Committee for Risk Assessment

RAC

Annex 1

Background document

to the Opinion proposing harmonised classification and labelling at EU level of

N-{2-[[1,1'-bi(cyclopropyl)]-2-yl]phenyl}-3-(difluoromethyl)-1-methyl-1*H*-pyrazole-4-carboxamide; sedaxane

EC Number: -
CAS Number: 874967-67-6

CLH-O-0000001412-86-280/F

The background document is a compilation of information considered relevant by the dossier submitter or by RAC for the proposed classification. It includes the proposal of the dossier submitter and the conclusion of RAC. It is based on the official CLH report submitted to public consultation. RAC has not changed the text of this CLH report but inserted text which is specifically marked as 'RAC evaluation'. Only the RAC text reflects the view of RAC.

Adopted

15 March 2019

CLH report

Proposal for Harmonised Classification and Labelling

Based on Regulation (EC) No 1272/2008 (CLP Regulation), Annex VI, Part 2

International Chemical Identification: Sedaxane

CAS Number: **874967-67-6**

Index Number: **616-RST- VW-Y**

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Version number: 3 **Date: May 2018**

Note on confidential information

Please be aware that this report is intended to be made publicly available. Therefore it should not contain any confidential information. Such information should be provided in a separate confidential Annex to this report, clearly marked as such.

ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON *N*-{2-[[1,1'-BI(CYCLOPROPYL)]-2-YL]PHENYL}-3-(DIFLUOROMETHYL)-1-METHYL-1*H*-PYRAZOLE-4-CARBOXAMIDE; SEDAXANE

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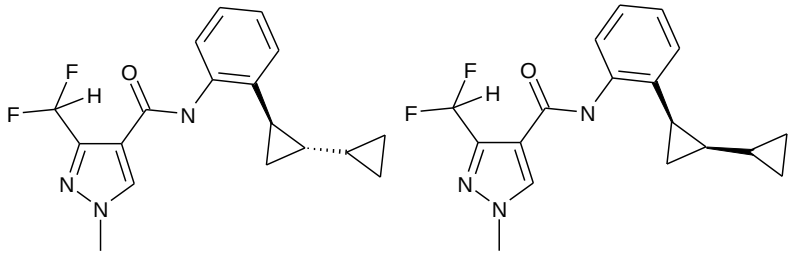
1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substance

Table 1: Substance identity and information related to molecular and structural formula of the substance

Name(s) in the IUPAC nomenclature or other international chemical name(s)	*N*-{2-[[1,1'-bi(cyclopropyl)]-2-yl]phenyl}-3-(difluoromethyl)-1-methyl-1*H*-pyrazole-4- carboxamide
Other names (usual name, trade name, abbreviation)	CA: 1H-pyrazole-4-carboxamide, N-[2-[1,1'-bicyclopropyl]-2-ylphenyl]-3-(difluoromethyl)-1-methyl-
Common name (if available and appropriate)	Sedaxane
EC number (if available and appropriate)	Not available
EC name (if available and appropriate)	3-difluoromethyl-1-methyl-1H-pyrazole-4-carboxylic acid (2-bicyclopropyl-2-yl-phenyl)-amide
CAS number (if available)	874967-67-6 (*trans* isomer: 599197-38-3 / *cis* isomer: 599194-51-1)
Other identity code (if available)	Syngenta code: SYN524464 Consists of two isomers with the following Syngenta codes: SYN508210 (*trans* isomer) SYN508211 (*cis* isomer)
Molecular formula	$C_{18}H_{19}F_2N_3O$
Structural formula	 *Trans* isomer (SYN508210) (racemate of enantiomers) *Cis* isomer (SYN508211) (racemate of enantiomers)
SMILES notation (if available)	FC(F)c4nn(C)cc4C(=O)Nc3ccccc3[C@@H]2C[C@@H]2C1CC1 FC(F)c4nn(C)cc4C(=O)Nc3ccccc3[C@H]2C[C@H]2C1CC1 FC(F)c4nn(C)cc4C(=O)Nc3ccccc3[C@H]2C[C@@H]2C1CC1 FC(F)c4nn(C)cc4C(=O)Nc3ccccc3[C@@H]2C[C@H]2C1CC1
Molecular weight or molecular weight range	331.4
Information on optical activity and typical ratio of (stereo) isomers (if applicable and appropriate)	sedaxane is a mixture of cis and trans isomers. Each cis and trans isomer constitute a racemate of enantiomers. Range 820-890 for the 2 trans isomers (SYN208210), range 100-150g/kg for the 2 cis isomers (SYN508211)

Description of the manufacturing process and identity of the source (for UVCB substances only)	Not relevant
Degree of purity (%) (if relevant for the entry in Annex VI)	≥960 g/kg for sedaxane (range 820-890 for the 2 trans isomers (SYN208210), range 100-150g/kg for the 2 cis isomers (SYN508211)

1.2 Composition of the substance

Table 2: Constituents (non-confidential information)

Constituent (Name and numerical identifier)	Concentration range (% w/w minimum and maximum in multi-constituent substances)	Current CLH in Annex VI Table 3.1 (CLP)	Current self-classification and labelling (CLP)
Sedaxane	≥960 g/kg (range 820-890 for the 2 trans isomers (SYN208210), range 100-150g/kg for the 2 cis isomers (SYN508211)	None	Aquatic Acute 1 H400 (M-factor: 1) Aquatic Chronic 2 H411

Table 3: Impurities (non-confidential information) if relevant for the classification of the substance

Impurity (Name and numerical identifier)	Concentration range (% w/w minimum and maximum)	Current CLH in Annex VI Table 3.1 (CLP)	Current self-classification and labelling (CLP)	The impurity contributes to the classification and labelling
Not relevant				

Table 4: Additives (non-confidential information) if relevant for the classification of the substance

Additive (Name and numerical identifier)	Function	Concentration range (% w/w minimum and maximum)	Current CLH in Annex VI Table 3.1 (CLP)	Current self-classification and labelling (CLP)	The additive contributes to the classification and labelling
Not relevant					

The purity of sedaxane tested in the studies ranged from 94.2 to 99.6%. Information on the actual purity is provided in the relevant tables of this report. The tested material in all cases is considered to be equivalent and representative of that specified above.

2 PROPOSED HARMONISED CLASSIFICATION AND LABELLING

2.1 Proposed harmonised classification and labelling according to the CLP criteria

Table 5:

	Index No	International Chemical Identification	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard statement Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry	Not included										
Dossier submitters proposal	None	N-{2-[[1,1'-bi(cyclopropyl)]-2-yl]phenyl}-3-(difluoromethyl)-1-methyl-1H-pyrazole-4-carboxamide; sedaxane	-	874967-67-6	Carc. 2	H351	GHS08 Wng	H351			
					Aquatic Acute 1 Aquatic Chronic 2	H400 H411	GHS09 Wng	H410 H411		M = 1	
Resulting Annex VI entry if agreed by RAC and COM											

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Table 6: Reason for not proposing harmonised classification and status under public consultation

Hazard class	Reason for no classification	Within the scope of public consultation
Explosives	conclusive but not sufficient for classification	Yes
Flammable gases (including chemically unstable gases)	not applicable	Yes
Oxidising gases	not applicable	Yes
Gases under pressure	not applicable	Yes
Flammable liquids	not applicable	Yes
Flammable solids	conclusive but not sufficient for classification	Yes
Self-reactive substances	conclusive but not sufficient for classification	Yes
Pyrophoric liquids	not applicable	Yes
Pyrophoric solids	conclusive but not sufficient for classification	Yes
Self-heating substances	conclusive but not sufficient for classification	Yes
Substances which in contact with water emit flammable gases	conclusive but not sufficient for classification	Yes
Oxidising liquids	not applicable	Yes
Oxidising solids	conclusive but not sufficient for classification	Yes
Organic peroxides	not applicable	Yes
Corrosive to metals	conclusive but not sufficient for classification	Yes
Acute toxicity via oral route	conclusive but not sufficient for classification	Yes
Acute toxicity via dermal route	conclusive but not sufficient for classification	Yes
Acute toxicity via inhalation route	conclusive but not sufficient for classification	Yes
Skin corrosion/irritation	conclusive but not sufficient for classification	Yes
Serious eye damage/eye irritation	conclusive but not sufficient for classification	Yes
Respiratory sensitisation	No data	Yes
Skin sensitisation	conclusive but not sufficient for classification	Yes
Germ cell mutagenicity	conclusive but not sufficient for classification	Yes
Carcinogenicity	**Carc. 2 – H351**	Yes
Reproductive toxicity	conclusive but not sufficient for classification	Yes
Specific target organ toxicity-single exposure	conclusive but not sufficient for classification	Yes
Specific target organ toxicity-repeated exposure	conclusive but not sufficient for classification	Yes
Aspiration hazard	not applicable	Yes

Hazard class	Reason for no classification	Within the scope of public consultation
Hazardous to the aquatic environment	Harmonised classification proposed: Aquatic Acute 1 H400 (M-factor: 1) Aquatic Chronic 2 H411	Yes
Hazardous to the ozone layer	conclusive but not sufficient for classification	Yes

3 HISTORY OF THE PREVIOUS CLASSIFICATION AND LABELLING

Sedaxane is a new active substance with no history of previous classification and labelling.

RAC general comment

The substance *N*-{2-[[1,1'-bi(cyclopropyl)]-2-yl]phenyl}-3-(difluoromethyl)-1-methyl-1*H*-pyrazole-4-carboxamide; sedaxane (Syngenta code CYN524464) is a new active substance (broad-spectrum seed treatment fungicide) in the meaning of Regulation EC 1107/2009 and has no history of previous classification and labelling. The substance is a mixture of the trans isomers (SYN508210) and cis isomer (SYN508211). Each isomer constitutes a racemate of enantiomers. The purity is 960 g/kg with a purity range in the studies from 94.2 to 99.6%.

The dossier submitter (DS) proposed a classification as Carc. 2 – H351, Aquatic Acute 1 – H400, and Aquatic Chronic 2 – H411. Formerly, no classification as regards to carcinogenicity was proposed in the conclusion of the peer review of the pesticide risk assessment of the active substance sedaxane (EFSA, 2012). In 2011, U.S. EPA classified sedaxane "Likely to be carcinogenic to humans" based on the presence of multiple site tumours in two species. Following a request of the European Commission for reconsideration and confirmation of the conclusion of the toxicological assessment, sedaxane was re-discussed at the Pesticide Peer Review Meeting in November 2012 with the conclusion that classification as Carc. 2 – H351 would be required (EFSA, 2013).

The applicant (Syngenta) then has generated numerous mechanistic studies and performed mode of action (MoA) analysis for liver, thyroid and uterine tumours according to the WHO/IPCS Framework for analysing the relevance of a cancer MoA for humans. The proposed MoAs are reported *in extenso* in Appendix 1-3 of the CLH report and have been evaluated by the dossier submitter. During the public consultation, additional data have been submitted by the applicant to be considered in the RAC opinion making process.

Sedaxane is a succinate dehydrogenase inhibitor (SDHI). During public consultation, the DS highlighted that a high concern regarding the use of as fungicides in agriculture has been recently been raised by researchers and clinicians from French institutes with respect to the carcinogenic potential linked to the SDH inhibition (Benit *et al*., 2018). ANSES set up an emergency expert group to analyse the alert issued, and to identify

whether immediate actions or additional risk management measures for the active substances and related products containing SDHI active substances should be taken.

4 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

As a substance that is an active substance in the meaning of Regulation EC 1107/2009, Sedaxane is subject to harmonised classification and labelling.

5 IDENTIFIED USES

Sedaxane is a broad-spectrum, seed treatment fungicide.

6 DATA SOURCES

The data source is the dossier supporting registration as an active substance in the meaning of Regulation EC 1107/2009.

7 PHYSICOCHEMICAL PROPERTIES

Table 7: Summary of physicochemical properties

Property	Value	Reference	Comment (e.g. measured or estimated)
Physical state at 20°C and 101,3 kPa	Pure substance: white powder Technical substance:grey-beige powder	Das (2008) Das (2009)	Visual assessment Pure substance 99.6% Tech. substance 97.5%
Melting/freezing point	121.4°C	Geoffroy (2008a)	OECD 102 Pure substance 99.6%
Boiling point	> 270°C	Geoffroy (2008a)	OECD 103 Pure substance 99.6%
Relative density	1.23×10^3 kg/m^3 at 20°C corresponding to a relative density of 1.23	Poux (2008)	OECD 109 Pure substance 99.6%
Vapour pressure	Mean vapour pressure of the 2 diastereosisomers at 20°C: 6.5×10^{-8} Pa mean vapour pressure of the 2 disatereoisomers at 25°C: 1.7×10^{-7} Pa	Geoffroy (2008b)	OECD 104 Pure substance 99.6%
Surface tension	48.6 mN/m at 20°C for a 90% saturated aqueous solution	Gasser (2009)	OECD 115 Tech. substance 97.5%
Water solubility	The solubility in pure water at 25°C was determined to be:14 mg/L	Khot S.B (2008)	OECD 105 Pure substance 99.6%

Property	**Value**	**Reference**	**Comment (e.g. measured or estimated)**
Partition coefficient n-octanol/water	The octanol/water partition coefficient (P_{ow}) and its logarithm to base 10 (log P_{ow}) at 25°C was determined to be: P_{ow} = 2100 (± 14) log P_{ow} = 3.3.	Hosmani (2009)	OECD 117 (shake flask method) Pure substance 99.6%
Flash point			Not applicable for a solid
Flammability	Not ignition occurred. Not classified in terms of its burning characteristics	Jackson (2009)	EU Test A.10 Tech. substance 97.5% (also acceptable with CLP criteria)
Explosive properties	Not an explosive substance. The substance did not explode when exposed to heat, mechanical shock or friction.	Jackson (2009)	EU Test A.14 Tech. substance 97.5% (acceptable with CLP criteria since the prelimerary tests are negative)
Self-ignition temperature	No ignition below the melting point	Jackson (2009)	EU Test A.16 Tech. substance 97.5% (also acceptable with CLP criteria)
Oxidising properties	Not an oxidizing substance according to DSD criteria (method EEC A17) The active substance contains oxygen and fluorine. However, these atoms are only bounded to carbon. According to CLP, no further test is needed. However, a test according to method O.1 was provided and confirm that the active substance is not classified.	Jackson (2009) Jackson (2017)	EEC A.17/ O.1 Tech. substance 97.5% (also acceptable with CLP criteria according to the chemical structure of sedaxane)
Granulometry			Not relevant for CLP
Stability in organic solvents and identity of relevant degradation products			No evidence of instability in organic solvents. Not required.
Dissociation constant	No pKa was found in the range of 1.0 to 12.0 by spectro-photometric titration of a solution of Sedaxane in water.	Martin (2008)	OECD 112 (spectrometric titration) Tech. substance 99.6%
Viscosity			Not applicable for a solid

Property	Value	Reference	Comment (e.g. measured or estimated)
Corrosion	No corrosion of different materials (tin plante, galvanized sheet metal, sheet steel and stainless steel) after immersion 7 days at 54°C in technical sedaxane.	Das (2009)	ASM G31-72 (equivalent to C.1 test described in Manua UN RTDG) Test. Substance 97.5%

8 EVALUATION OF PHYSICAL HAZARDS

Sedaxane has no physical properties warranting classification under CLP. It is not flammable, explosive or oxidising.

RAC evaluation of physical hazards

Summary of the Dossier Submitter's proposal

Sedaxane has no physical properties warranting classification under CLP. It is not flammable, explosive or oxidising.

Comments received during public consultation

No comments were received.

Assessment and comparison with the classification criteria

RAC supports the DS's proposal for **no classification of sedaxane regarding physical hazards**.

9 TOXICOKINETICS (ABSORPTION, METABOLISM, DISTRIBUTION AND ELIMINATION)

9.1 Short summary and overall relevance of the provided toxicokinetic information on the proposed classification(s)

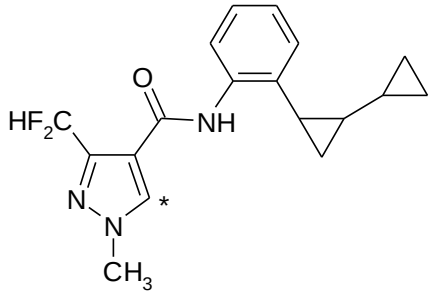
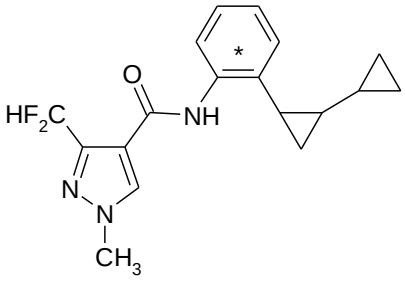
The mammalian metabolism of sedaxane (SYN524464) has been assessed in studies investigating the absorption, distribution, metabolism and excretion of sedaxane in rats (Anonymous, 2009 *Annex I. 2.1* to *Annex I. 2.7).*

In a biotransformation study, the nature of the metabolites formed was determined both qualitatively and quantitatively. The fate of sedaxane was investigated following both single and multiple doses.

Biliary elimination studies were conducted with ^{14}C-pyrazole and ^{14}C-phenyl SYN524464. As there was evidence of only very limited cleavage of the SYN524464 molecule during the biotransformation process, a single radiolabelled form of SYN524464 was used in the remaining metabolism studies. ADME studies used radiolabelled material comprising an approximate 6:1 mixture of the *trans* and *cis* isomers, SYN508210 and SYN508211.

The structure and position of the radiolabel in SYN524464 for the two radiolabelled forms used is shown below:

Table 8: Radiolabelled forms of SYN524464 used in ADME studies – Structure and position of the radiolabel.

Radiolabel	Structure and position of label
[Pyrazole-5-^{14}C]-SYN524464	
[Phenyl-U-^{14}C]-SYN524464	

Absorption

The absorption of SYN52464 was estimated following a single gavage administration at low and high dose levels (1 or 80 mg/kg) to bile duct cannulated male and female rats by measuring the radioactivity present in urine, bile, cage wash, faeces and carcass over 48 hours post dosing. Two different radiolabelled forms of SYN524464 were used; [pyrazole-5-^{14}C]-SYN524464 and [phenyl-U-^{14}C]-SYN524464. Absorption was similar in both sexes and at both dose levels and with both radiolabelled forms of SYN52464. Absorption at the low dose level was estimated to be 87.4 and 87.9% using ^{14}C-pyrazole radiolabel and 89.1 and 87.5% using ^{4}C-phenyl radiolabel in males and females respectively. At the high dose level, absorption was estimated to be 89.5 and 92.5% using ^{14}C-pyrazole radiolabel and 93.9 and 87.1% using ^{14}C-phenyl radiolabel in males and females respectively.

Distribution

Whole body autoradiography conducted in a preliminary study showed that the administered radioactivity was distributed widely throughout the internal organs after 5 hours. At 24 h post dose, levels of total radioactivity had decreased markedly in all tissues. This preliminary investigation also confirmed that no non-standard tissues needed to be collected during subsequent tissue distribution studies.

The excretion and tissue distribution studies, at both dose levels, showed that residues of radioactivity were very low in blood and tissues seven days after dosing and were only reliably measured in both sexes in the liver and kidney. Tissue distribution was generally similar in both sexes and at both dose levels, with the only difference being slightly higher residues in thyroid, spleen, lung and the gastrointestinal tract in male rats at the high dose level. In total, radioactive residues in tissues accounted for <0.1% of the dose at both dose levels. Radioactivity in the residual carcass accounted for <0.2% of the dose at both dose levels.

These very low tissue residues were consistent with the extensive excretion of the administered dose.

Pharmacokinetics

Following a single oral administration at a low or a high dose level to male and female rats, there was little apparent difference between the pharmacokinetic parameters calculated for blood and plasma. Maximum plasma concentrations of total radioactivity were achieved at 1-6 h post dose and declined with estimated terminal half-life values ranging from 22.6-28.8 h. Similar terminal half-life values of 20.7-39.9 hours were obtained for blood. At both dose levels systemic exposure was similar for males and females.

Tissue depletion following a single oral dose

Pharmacokinetic data were used to select appropriate time points for the tissue depletion study. At the low dose level the termination times were 1 (males only), 1.5 (females only), 8, 24, 48 and 96 hours after dosing and at the high dose level 5, 12, 24, 48 and 96 hours after dosing. Following administration, the absorbed radioactivity was widely and generally similarly distributed to the tissues in both sexes at both dose levels. For all dose groups, peak tissue concentrations were observed at the first time point measured with the highest concentrations of radioactivity present in the liver and kidney at the low dose level and liver and fat at the high dose level. Thereafter tissue concentrations progressively declined, with elimination half-lives for tissue depletion of between 0.1 and 3.2 days. The calculation of half-lives for some tissues was complicated by variable, but low tissue concentrations measured over the course of this study. However, most mean tissue concentrations were close to or below the limit of reliable measurement by 96 hours post dose, when mean total tissue and carcass residues accounted for less than 0.8% of the dose, irrespective of sex or dose level.

Accumulation and tissue depletion following repeated oral dosing

Following repeated daily oral administration of 1 mg [^{14}C]-SYN524464/kg to male rats, tissue distribution of radioactivity was extensive and most tissue concentrations appeared either to have attained or to be approaching steady state levels after 14 doses. During the period of dosing, tissue concentrations of radioactivity were highest in the liver followed by the kidney. Following the cessation of dosing, all tissue concentrations declined, with no evidence of any persistence. By the final sampling time (42 days post dose 14), concentrations of radioactivity were detectable only in the liver, kidney and spleen. The elimination half-lives for tissue depletion were variable and ranged from 2.3 days for plasma to 33.0 days for the spleen. The calculation of half-lives for some tissues was complicated by variable, but low tissue concentrations measured over the course of this study. However, with the exceptions of liver, kidney and spleen, tissue concentrations had declined to values close to or below the limit of reliable measurement by day 28 after the cessation of dosing.

Metabolism

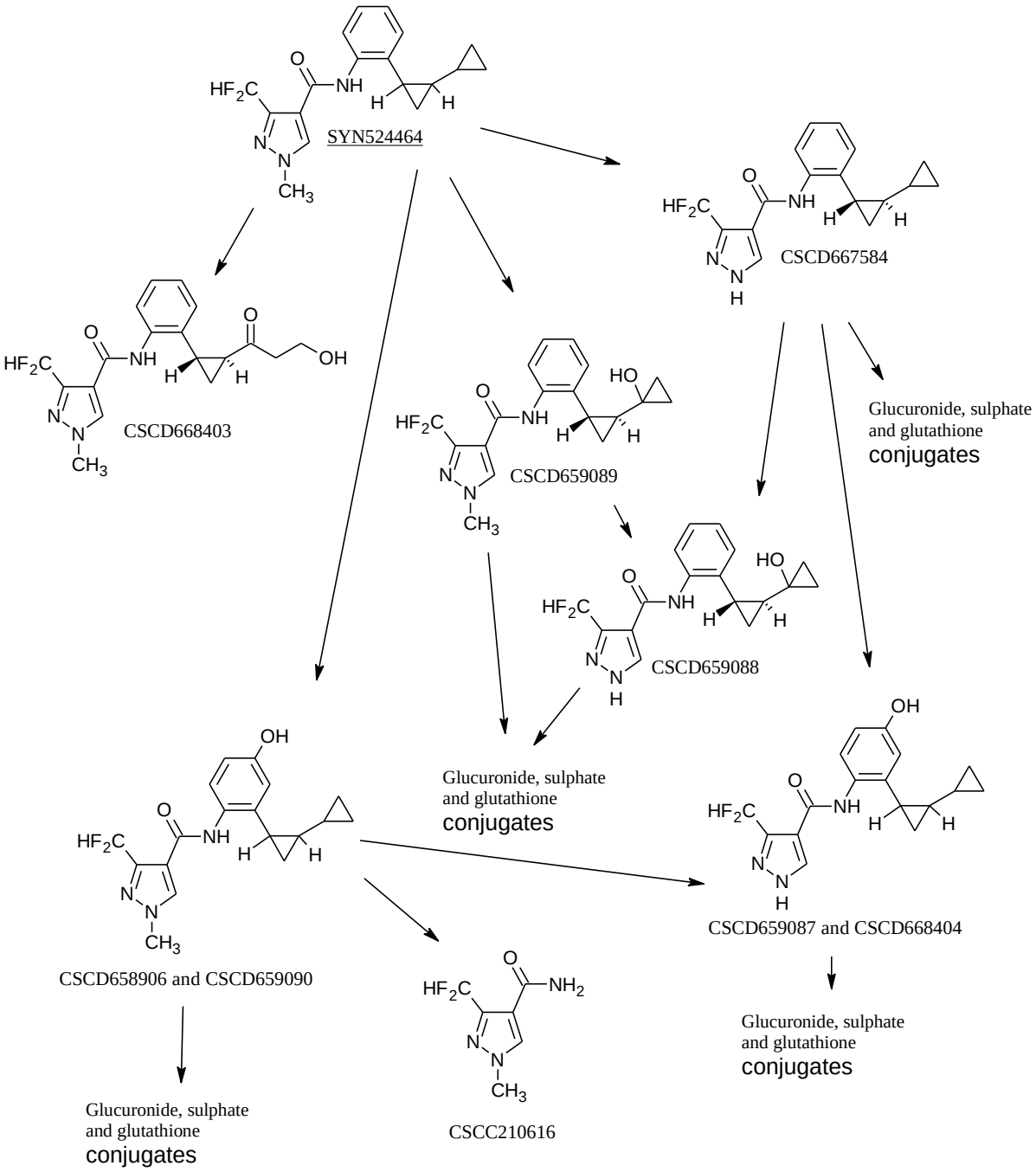
SYN524464 was extensively metabolised in the rat via demethylation, hydroxylation, oxidation and conjugation affording an array of hydroxylated metabolites and metabolites formed by cleavage of the terminal cyclopropyl moiety. An equivalent range of metabolites of desmethyl SYN524464 were also formed. The major metabolites were identified as the *trans* para phenol CSCD658906 and the desmethyl *trans* para phenol CSCD659087 which together with the equivalent *cis* para phenol isomers CSCD659090 and CSCD668404 accounted for approximately half the administered dose. There were no major sex or dose related differences apparent in the qualitative metabolite profile for SYN524464. Little evidence of any cleavage of the SYN524464 molecule between the phenyl and pyrazole moieties was seen with samples obtained from rats receiving ^{14}C-pyrazole or ^{14}C-phenyl SYN524464 affording similar metabolic profiles. A small amount (<1%) of CSCC210616 (pyrazole amide metabolite) was detected in bile samples. The phenolic and hydroxylated metabolites of SYN524464 and desmethyl SYN524464 were subject to glucuronic acid, sulphate and glutathione conjugation.

Excretion

The routes and rates of excretion were similar in male and female rats and at both dose levels. Over seven days after dosing, males and females excreted 97.2% and 96.3% respectively of a 1 mg/kg dose and 102.1% and 104.9% of an 80 mg/kg dose. The major route of excretion was via the faeces accounting for 88.4 and 79.4% of a low dose level in males and females respectively and 83.1 and 74.9% of a high dose level. Urinary excretion accounted for 11.8 and 19.6% of a low dose level in males and females and 11.9 and 17.6 % of a high dose level. In a preliminary study, no radioactivity was measured in expired air, consistent with the anticipated metabolically stable location of the radiolabel in the molecule and with the generally high recoveries of administered dose.

Biliary elimination was significant at both dose levels in both sexes, accounting over 48 hours after dosing for between 78.6 and 81.1% of the dose in males and females at the low dose level and between 81.0 and 85.3% of the dose in males and females at the high dose level in the studies conducted with ^{14}C-pyrazole and ^{14}C-phenyl SYN524464.

Figure 1: Biotransformation pathways based on identified metabolites of SYN524464 in the rat



10 EVALUATION OF HEALTH HAZARDS

Acute toxicity

10.1 Acute toxicity - oral route

Table 8: Summary table of animal studies on acute oral toxicity

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance,	Dose levels, duration of exposure	Value LD_{50}	Reference
Acute Oral Toxicity Study in the Rat (Up and Down Procedure) OECD 425 GLP	Rat HanRCC:WIST (SPF) 13 Females 7 – 5000 mg/kg 4 – 1750 mg/kg 1 – 550 mg/kg 1 – 175 mg/kg	SYN524464 Batch No.: SMU6LP006/ MILLED Purity: 95.3% Vehicle: 0.5% CMC/purified water	5000, 1750, 550 or 175 mg/kg Single dose followed by 14 day observation period.	Estimated LD_{50} is 5000 mg/kg bw - Females Approximate 95% profile-likelihood based confidence interval = 2513 to 9210 mg/kg body weight.	Anonymous (2008) *Annex I. 3.1.1.1*

Table 9: Summary table of human data on acute oral toxicity

Type of data/report	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
There are no reports of adverse effects following oral exposure to humans				

Table 10: Summary table of other studies relevant for acute oral toxicity

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
No relevant studies				

10.1.1 Short summary and overall relevance of the provided information on acute oral toxicity

The acute oral toxicity of sedaxane was assessed in a standard guideline study (UP and Down procedure) in female HanRcc:WIST rats (Anonymous, 2008 *Annex I. 3.1.1.1*). A limit test was initially conducted at 5000 mg sedaxane/kg (by gavage) in 1 female. This animal died shortly after dosing. Thereafter, following the AOT 425 Statistical program, a main test was conducted starting at a dose of 175 mg/kg (one animal) and using further animals treated at 550 mg/kg (1 animal), 1750 mg/kg (4 animals) and 5000 mg/kg (6 animals). Two animals, one treated at 175 and one at 550 mg/kg and the four animals treated at 1750 mg/kg, survived until the end of the study period. From the 7 animals treated at 5000 mg/kg, four animals were killed for ethical reasons after treatment at 5000 mg/kg 3 or 5 hours post-dosing on test day 1 and one female was found dead on test day 2; the remaining two animals survived.

Clinical observations noted at 5000 mg/kg included ruffled fur, hunched posture, sedation, poor co-ordination, ventral recumbency, deep respiration, rales, salivation and bradypnea. The surviving two animals showed signs of toxicity up to test day 8 and 10 respectively. At 1750 mg/kg, clinical signs included hunched posture and slight sedation in some animals up to day 5; poor coordination or ventral recumbency were seen in two animals on day 1. At 550 and 175 mg/kg, observations in the single animals dosed were limited to ruffled fur, hunched posture and slight sedation after dosing. These animals were symptom free from days 2 or 4, respectively.

In surviving animals, there were no effects on body weight and there were no significant macroscopic findings. A yellowish discoloured jejunum was recorded in the first 5000 mg/kg treated female terminated in extremis. In the last 5000 mg/kg treated female the lungs, whilst not collapsed, were discoloured, pale at necropsy.

The median lethal dose of sedaxane after single oral administration to female rats, observed over a period of 14 days, was estimated to be 5000 mg/kg body weight, with an approximate 95 % profile likelihood based (PL) confidence interval (of 2513 to 9210 mg/kg body weight).

See Annex I to the CLH report 3.1.1.

10.1.2 Comparison with the CLP criteria

As the MLD following an acute oral dose in rats was > 2000 mg/kg bw, the criteria for classification are not met.

10.1.3 Conclusion on classification and labelling for acute oral toxicity

Not classified (conclusive but not sufficient for classification)

10.2 Acute toxicity - dermal route

Table 11: Summary table of animal studies on acute dermal toxicity

Method, guideline, deviations if any	**Species, strain, sex, no/group**	**Test substance,**	**Dose levels duration of exposure**	**Value LD_{50}**	**Reference**
Acute dermal	Rat	SYN524664	5000 mg/kg	>5000 mg/kg bw	Anonymous

Method, guideline, deviations if any	**Species, strain, sex, no/group**	**Test substance,**	**Dose levels duration of exposure**	**Value LD_{50}**	**Reference**
(semi-occlusive) OECD 402 GLP	HanRcc:WIST (SPF) 5/sex/dose	Batch No.: SMU6LP006/ MILLED Purity: 95.3%	24 hour application followed by a 14 day observation period	Males/females	(2007) *Annex I. 3.2.1.1*

Table 12: Summary table of human data on acute dermal toxicity

Type of data/report	**Test substance,**	**Relevant information about the study (as applicable)**	**Observations**	**Reference**
There are no reports of adverse effects following oral exposure to humans				

Table 13: Summary table of other studies relevant for acute dermal toxicity

Type of study/data	**Test substance,**	**Relevant information about the study (as applicable)**	**Observations**	**Reference**
No relevant studies				

10.2.1 Short summary and overall relevance of the provided information on acute dermal toxicity

In an acute dermal toxicity study in the HanRcc:WIST rat (Anonymous, 2007 *Annex I. 3.1.2.1*), there were no mortalities following application of 5000 mg/kg bw. No clinical signs of systemic toxicity were observed during the study period. Signs of slight skin irritation (erythema) at the application site were observed in three males and five females on day 2 only. All animals gained weight during the study and there were no macroscopic abnormalities at necropsy. The acute dermal median lethal dose was >5000 mg/kg bw in male and female rats.

See Annex I to the CLH report 3.2.1.

10.2.2 Comparison with the CLP criteria

As the MLD following an acute dermal dose in rats was > 2000 mg/kg bw (5000 mg/kg in males and females), the criteria for classification are not met.

10.2.3 Conclusion on classification and labelling for acute dermal toxicity

Not classified (conclusive but not sufficient for classification)

10.3 Acute toxicity - inhalation route

Table 14: Summary table of animal studies on acute inhalation toxicity

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance, form and particle size (MMAD)	Dose levels, duration of exposure	Value LC_{50}	Reference
Acute inhalation (nose-only) OECD 403 GLP	Rat HanRcc:WIST(SPF) 5/sex/group	SYN524664 Batch No.: SMU6LP006/ MILLED Purity: 95.3% Aerosol (liquefied by warming) MMAD: 3.02, 2.97 µm	5.244 mg/L air 4 hour exposure, followed by 14 day observation period	>5.244 mg/L Males & Females	Anonymous (2008) *Annex I. 3.3.1.1*

Table 15: Summary table of human data on acute inhalation toxicity

Type of data/report	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
There are no reports of adverse effects following oral exposure to humans				

Table 16: Summary table of other studies relevant for acute inhalation toxicity

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
No relevant studies				

10.3.1 Short summary and overall relevance of the provided information on acute inhalation toxicity

In an acute, nose-only, inhalation toxicity study in HanRcc:WIST rats (Anonymous, 2008 *Annex I. 3.1.3.1*), a group of 5 males and 5 females were exposed to aerosolised sedaxane for 4 hours, at a mean gravimetric exposure concentration of 5.244 mg/L (s.d. $\pm$ 0.062). In order to facilitate the generation of a suitable aerosol, the test item was liquefied by warming it and an aerosol was generated from the liquefied test item by use of a nebuliser and pre-warmed air. Extra diluent air was not pre-warmed. Two gravimetric measurements of particle size distribution during the exposure produced mass median aerodynamic diameters (MMAD) of 3.02 and 2.97 µm and geometric standard deviations (GSD) of 2.84 and 2.87.

There were no deaths during the study. Clinical signs after exposure included: effects on breathing (bradypnea and rales), decreased spontaneous activity, hunched posture and ruffled fur in all animals. Bradypnea and hunched posture had cleared in all animals by the day after exposure (test day 2) and rales, decreased spontaneous activity, and ruffled fur by day 3. From test day 3 until termination of the study on test day 15, all animals remained free from clinical signs. Transient effects on body weight were seen in both sexes on days 1-3 and were followed by normal body weight gain in all

animals. There were no treatment related macroscopic findings at necropsy. The acute inhalation 4 hour LC_{50} value was determined to be greater than 5.244 mg/L in male and female rats.

See Annex I to the CLH report 3.3.1.

10.3.2 Comparison with the CLP criteria

The acute LC_{50} value for aerosolised sedaxane (5.244 mg/L) was > 5 mg/L for dust/mists and therefore does not meet the criteria for classification.

10.3.3 Conclusion on classification and labelling for acute inhalation toxicity

Not classified (conclusive but not sufficient for classification)

RAC evaluation of acute toxicity

Summary of the Dossier Submitter's proposal

Acute toxicity oral route

Sedaxane was tested for acute oral toxicity in female HanRcc:WIST rats according to Up and Down Procedure (OECD TG 425, GLP) after an initial limit test with 5000 mg/kg bw by gavage in one female. The main test was conducted with 1 animal at 175 mg/kg bw, 1 animal at 550 mg/kg bw, 4 animals at 1750 mg/kg bw and 6 animals at 5000 mg/kg bw. The LD_{50} after single oral administration to female rats was estimated to be 5000 mg/kg bw with an approximately 95% profile likelihood confidence interval of 2513 to 9210 mg/kg bw. Since the acute oral LD_{50} was > 2000 mg/kg bw no classification was proposed by the DS for acute oral toxicity.

Acute toxicity dermal route

Sedaxane was tested for acute dermal toxicity (semi-occlusive) in HanRcc:WIST rats (5/sex) according to OECD TG 403 (GLP) at a dose level of 5000 mg/kg bw. The LD_{50} was > 5000 mg/kg bw. Since the acute dermal LD_{50} was > 2000 mg/kg bw no classification was proposed for acute dermal toxicity.

Acute toxicity inhalation route

Aerosolised Sedaxane was tested for acute inhalation toxicity (nose-only) in HanRcc:WIST rats (5/sex) according to OECD TG 402 (semi-occlusive, GLP) with 5.244 mg/L mean gravimetric exposure concentration for 4 hours. Gravimetric measurements of particle size distribution yielded MMAD of 3.02 and 2.97 µm and standard deviations of 2.84 and 2.87. Since the acute inhalation LC_{50} of aerosolised sedaxane of 5.244 mg/L was > 5 mg/L for dust/mists no classification was proposed by the DS for acute inhalation toxicity.

Comments received during public consultation

No comments were received.

Assessment and comparison with the classification criteria

As described above, three guideline acute toxicity studies investigating the effects of a single dose of sedaxane via oral, dermal and inhalation routes are available. In addition there is a guideline acute oral (gavage) neurotoxicity test in HanRcc:WIST rats (10/sex/dose) according to OECD TG 424 (GLP) with gavage dosing of 0, 30, 250, and 2000 mg/kg bw available.

Table: *Overview of LD50/LC50 values or mortalities in acute toxicity studies with sedaxane.*

	Acute oral	**Acute dermal**	**Acute inhalation**
Rat	4/7 females dosed 5000 mg/kg bw killed in extremis day 1 and 1/7 females died day 2; no death (0/4) at 1750 mg/kg bw; Estimated LD_{50} of 5000 mg/kg bw (95% PL confidence interval = 2513-9210 mg/kg bw females)	No deaths; LD_{50} > 5000 mg/kg bw males and females	No deaths, LC_{50} > 5.244 mg/L males and females
Rat neurotoxicity	4/10 males and 3/10 females dosed 2000 mg/kg bw killed in extremis day 1		
Criteria Category 4	300-2000 mg/kg bw	1000-2000 mg/kg bw	1-5 mg/L (dusts and mists, 4 h)
	Not fulfilled	Not fulfilled	Not fulfilled

Taking into account the data on acute toxicity by the oral, dermal, and inhalation routes, and the acute neurotoxicity by oral route, and with reference to the numeric criteria of Annex I, 3.1.2.1, table 3.1.1 of CLP, RAC is of the opinion that sedaxane does not meet the criteria for classification for acute toxicity via the oral, dermal and inhalation route and **no classification is proposed for acute toxicity**.

10.4 Skin corrosion/irritation

Table 17: Summary table of animal studies on skin corrosion/irritation

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance,	Dose levels duration of exposure	Results -Observations and time point of onset -Mean scores/animal -Reversibility	Reference
Acute skin irritation OECD 404 GLP	Rabbit, New Zealand white 3/group	SYN524664 Batch No.: SMU6LP006/ MILLED Purity: 95.3%	0.5 g applied to shorn flank, moistened with water. 4 hour topical semi-occlusive application. Irritation response assessed at 1 hour, 1, 2 & 3 days after removal of dressings.	No adverse clinical signs. No skin reactions or staining in 3/3 animals. Mean scores at 24, 48 and 72 hours: Erythema: 0, 0, 0 Oedema: 0, 0, 0 Non –irritating to skin.	Anonymous (2007) *Annex I. 3.4.1.1*

Table 18: Summary table of human data on skin corrosion/irritation

Type of data/report	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
No evidence for skin corrosion/irritation in humans				

Table 19: Summary table of other studies relevant for skin corrosion/irritation

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
No relevant studies				

10.4.1 Short summary and overall relevance of the provided information on skin corrosion/irritation

The primary skin irritation potential of sedaxane was investigated in a standard guideline study in rabbits (Anonymous, 2008b *Annex I. 3.1.4.1*). No signs of systemic toxicity were observed in any of the 3 animals during the 72 hour observation period, there were no signs of skin irritation or corrosion and no test item staining of the treated skin. The mean erythema and oedema scores were zero at all time points. The primary irritation index was calculated by totalling the mean cumulative scores at 24, 48 and 72 hours and then dividing by the number of data points. The primary irritation index was 0.00. Sedaxane is, therefore, considered to be “not irritant” to rabbit skin.

See Annex I to the CLH report 3.4.1.

10.4.2 Comparison with the CLP criteria

As there was no evidence of skin irritation in any animal, classification as skin corrosive or skin irritant is not applicable.

10.4.3 Conclusion on classification and labelling for skin corrosion/irritation

Not classified (conclusive but not sufficient for classification)

RAC evaluation of skin corrosion/irritation

Summary of the Dossier Submitter's proposal

Sedaxane was tested for acute skin irritation in New Zealand White rabbits (3/group) according to OECD 404 (GLP) with 0.5 g topically applied for 4 hours semi-occlusive. There were no signs of skin irritation or corrosion and the DS concluded that classification was not applicable.

Comments received during public consultation

No comments were received.

Assessment and comparison with the classification criteria

In a guideline compliant *in vivo* skin irritation study according to OECD 404 in NZW rabbits no signs of irritation and corrosive effects were noted on the treated skin of any animal at any of the measuring intervals (1, 24, 48, 72 h) and no clinical signs were observed. The mean score for erythema and oedema was zero at all the time points. The primary irritation index was calculated by totalling the mean cumulative scores at 24, 48 and 72 hours and then dividing by the number of data points. The primary irritation index was 0.00. No evidence for skin corrosion/irritation in humans is available. Taking into account the above animal data with reference to the criteria of Annex I, 3.2.2.6 of CLP, RAC is of the opinion that sedaxane does not meet the criteria and **no classification is proposed for skin corrosion/irritation**.

10.5 Serious eye damage/eye irritation

Table 20: Summary table of animal studies on serious eye damage/eye irritation

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance,	Dose levels duration of exposure	Results - Observations and time point of onset - Mean scores/animal - Reversibility	Reference
Acute eye irritation OECD 405 GLP	Rabbit New Zealand White 3/group	SYN524664 Batch No.: SMU6LP006/ MILLED Purity: 95.3%	0.1 g (ground prior to instillation to left eye). Single exposure.	Mean scores/animal (24, 48 and 72 hours): Cornea: 0.00, 0.00, 0.00 Iris: 0.00, 0.00, 0.00 Conjunctivae (redness): 0.33, 0.67, 0.33 Conjunctivae (chemosis): 0.00, 0.00, 0.00 All signs had fully reversed by 72 hours after instillation.	Anonymous (2007) *Annex I. 3.5.1.1*

Table 21: Summary table of human data on serious eye damage/eye irritation

Type of data/report	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
No evidence of eye irritation in humans				

Table 22: Summary table of other studies relevant for serious eye damage/eye irritation

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
No relevant studies				

10.5.1 Short summary and overall relevance of the provided information on serious eye damage/eye irritation

The primary eye irritation potential of sedaxane was investigated in a standard regulatory study (Anonymous, 2007b *Annex I. 3.1.1.1*). Mild, early-onset and transient ocular changes, such as reddening of the conjunctivae and sclerae, discharge and chemosis were observed at 1 hour after instillation. These effects were reversible and were no longer evident 72 hours after treatment, the end of the observation period for all animals. No abnormal findings were observed in the cornea or iris of any animal at any of the examinations. No corrosion was observed at any of the measuring intervals. No staining of the treated eyes by the test item was observed and there were no clinical signs of systemic toxicity.

See Annex I to the CLH report 3.5.1.

10.5.2 Comparison with the CLP criteria

Substances are classified as irritating to eyes (Category 2) if, when applied to the eye of an animal, it produces:

– at least in 2 of 3 tested animals, a positive response of:

– corneal opacity ≥ 1 and/or

– iritis ≥ 1, and/or

– conjunctival redness ≥ 2 and/or

– conjunctival oedema (chemosis) ≥ 2

– calculated as the mean scores following grading at 24, 48 and 72 hours after installation of the test material, and which fully reverses within an observation period of 21 days.

In the study of (Anonymous, 2007 *Annex I. 3.5.1.1*), mean scores did not meet the criteria for classification and sedaxane is considered not to be an eye irritant.

10.5.3 Conclusion on classification and labelling for serious eye damage/eye irritation

Not classified (conclusive but not sufficient for classification)

RAC evaluation of serious eye damage/irritation

Summary of the Dossier Submitter's proposal

Sedaxane was tested for acute eye irritation in New Zealand White rabbits (3/group) according to OECD 405 (GLP) with 0.1 g single exposure to the rabbit eye. The instillation into the eye resulted in mild, early-onset and transient ocular changes, reversible within 72 hours after treatment. No classification was proposed by the dossier submitter.

Comments received during public consultation

No comments were received.

Assessment and comparison with the classification criteria

In a guideline compliant *in vivo* eye irritation study according to OECD 405 in NZW rabbits, only mild, early-onset and transient ocular changes reversible within 72 hours were recorded. No abnormal findings were observed in the treated eye of any animal 72

hours after treatment. The individual mean scores for all three animals were:

- corneal opacity and iris effects: 0, 0, 0,
- conjunctivae redness: 0.33, 0.67, 0.33,
- conjunctival chemosis: 0, 0, 0.

Taking into account the above animal data with reference to the criteria of Annex I, 3.3.2.6., table 3.3.1 and 3.3.2 of CLP, RAC is of the opinion that sedaxane does not meet the criteria and **no classification is proposed for eye damage/irritation.**

10.6 Respiratory sensitisation

Table 23: Summary table of animal studies on respiratory sensitisation

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance,	Dose levels, duration of exposure	Results	Reference
No relevant studies					

Table 24: Summary table of human data on respiratory sensitisation

Type of data/report	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
No evidence of respiratory sensitisation in humans				

Table 25: Summary table of other studies relevant for respiratory sensitisation

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
No relevant studies				

10.6.1 Short summary and overall relevance of the provided information on respiratory sensitisation

No formally recognised and validated animal tests currently exist for respiratory sensitisation. There was no evidence of respiratory irritation in single dose inhalation studies in rats and there was no indication of sensitisation. There is no reported evidence of respiratory sensitisation in humans.

10.6.2 Comparison with the CLP criteria

As there are no animal data and no evidence in humans that sedaxane exposure can lead to specific respiratory hypersensitivity, classification is not possible.

10.6.3 Conclusion on classification and labelling for respiratory sensitisation

Not classified (conclusive but not sufficient for classification)

RAC evaluation of respiratory sensitisation

Summary of the Dossier Submitter's proposal

No specific animal or human data was available on respiratory sensitisation. There was no evidence of respiratory irritation or indication of sensitisation observed in the single dose inhalation toxicity study. There is no reported evidence of respiratory sensitisation in humans available. The dossier submitter did not propose a classification of sedaxane as respiratory sensitiser.

Comments received during public consultation

No comments were received.

Assessment and comparison with the classification criteria

No data are available in both human and animals. In agreement with the dossier submitter, **RAC does not propose to classify sedaxane as a respiratory sensitiser due to lack of data**.

10.7 Skin sensitisation

Table 26: Summary table of animal studies on skin sensitisation

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance,	Dose levels duration of exposure	Results	Reference
Local Lymph Node Assay OECD 429 GLP	Mouse CBA/Ca Female 5/group	SYN524664 Batch No.: SMU6LP006/ MILLED Purity: 95.3% Vehicle: acetone/olive oil 4:1 Concurrent positive control study (15%	Concentrations in vehicle: 0, 10, 25 and 50% w/w. 50 µl (25µl/ear) was applied topically to the ears of mice in each group for 3 consecutive	Increase in Isotope incorporation: less than 3-fold at concentrations of 10, 25 and 50% w/w in acetone/olive oil 4:1. Stimulation Index: 10% - 1.12 25% - 0.96	Anonymous (2007). *Annex I. 3.7.1.1*

Method, guideline, deviations if any	Species, strain, sex, no/group	Test substance,	Dose levels duration of exposure	Results	Reference
		w/v hexylcinnamaldehyde in action/olive oil 4:1).	days. Day 6 – termination following intravenous injection of radiolabelled thymidine.	50% - 0.71 <u>Positive control</u> Positive responses. SI = 5.67 **Skin sensitising potential** : **non sensitising**.	

Table 27: Summary table of human data on skin sensitisation

Type of data/report	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
No evidence of skin sensitisation in humans				

Table 28: Summary table of other studies relevant for skin sensitisation

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
No relevant studies				

10.7.1 Short summary and overall relevance of the provided information on skin sensitisation

In a Local Lymph Node Assay in mice (Anonymous, 2007, *Annex I. 3.1.7.1*), the skin sentisation of sedaxane was assessed. The test substance was applied as 10, 25 or 50% w/w preparations in acetone/olive oil 4:1. The level of T lymphocyte proliferation in the lymph nodes draining the site of chemical application was determined by measuring the amount of radiolabelled thymidine incorporated into the dividing cells. The results are expressed as disintegrations per minute (dpm) value per lymph node for each animal. The activity of each test group was then divided by the activity of the vehicle control group to give a test: control ratio known as the stimulation index (SI), for each concentration. At concentrations of up to 50% w/w sedaxane, the increase in isotope incorporation was less than 3-fold at all concentrations. Consequently, sedaxane was considered to be a non-sensitiser under the conditions of the test.

In a concurrent positive control study, hexylcinnamaldehyde induced positive responses when applied as 15% w/v preparations in Acetone/olive oil 4:1, confirming the validity of the protocol used in this study.

See Annex I to the CLH report 3.7.1.

10.7.2 Comparison with the CLP criteria

As the stimulation index for sedaxane in a mouse local lymph node assay was < 3 it does not meet the requirements for classification and is considered not to be a skin sensitiser.

10.7.3 Conclusion on classification and labelling for skin sensitisation

Not classified (conclusive but not sufficient for classification)

RAC evaluation of skin sensitisation

Summary of the Dossier Submitter's proposal

The dossier does not contain any human data. Sedaxane was tested for skin sensitisation in the Local Lymph Node Assay (LLNA) in female CBA/Ca mice (5/group) according to OECD TG 429 (GLP) with 0, 10, 25 and 50% concentrations applied topically to the ear of mice in each group for 3 consecutive days. Based on the analysed level of T-lymphocyte proliferation in the lymph nodes draining the site of chemical application, the test material was considered to be a non-sensitiser under the conditions of the test. No classification was proposed by the dossier submitter.

Comments received during public consultation

No comments were received.

Assessment and comparison with the classification criteria

In a LLNA performed on CBA/Ca mice, sedaxane was negative with 10, 25 and 50% w/w in acetone/olive oil 4:1 resulting in an increase in isotope incorporation of less than 3-fold at all concentrations (stimulation index: 10% - 1.12; 25% - 0.96; 50% - 0.71). The validity of the protocol used was confirmed with a concurrent positive control (hexylcinnamaldehyde). Consequently, the test substance was not considered a skin sensitiser under the conditions of the study.

Taking into account the negative LLNA result with reference to the criteria of Annex I, 3.4.2.2. of CLP, **RAC does not propose classification for skin sensitisation.**

10.8 Germ cell mutagenicity

Table 29: Summary table of mutagenicity/genotoxicity tests *in vitro*

Method	Test substance,	Organisms/ strain	Concentrations tested	Result	Reference
Reverse mutation in bacteria OECD 471 (1997) GLP	SYN524464, batch SMU6LP006/Milled, purity 95.3 % (sum of 83.0 % SYN508210 and 12.3 % SYN508211) Solvent DMSO	*Salmonella typhimurium* strains TA1535, TA1537, TA98, and TA100, and *Escherichia coli* strains WP2 *uvrA* pKM101 and WP2 pKM101	Experiment I; 3, 10, 33; 100; 333; 1000; 2500; and 5000 µg/plate and Experiment II; 33; 100; 333; 1000; 2500; and 5000 µg/plate, with and without metabolic activation	Negative No toxic effects Positive controls included	Anonymous (2009) *Annex I. 3.8.1.1*
In vitro cytogenetics Chromosome aberration test OECD 473 (1997) GLP	SYN524464, batch SMU6LP006/Milled, purity 95.3 % (sum of 83.0 % SYN508210 and 12.3 % SYN508211) Solvent DMSO	Human lymphocytes	Without S9 mix: Experiment 1; 4h exposure 70.8, 123.9, 216.8 µg/mL Experiment 2; 22h exposure 23.1, 40.5, 70.8 µg/mL With S9 mix: Experiment 1; 4h exposure 70.8, 123.9, 216.8 µg/mL Experiment 2; 4h exposure 100.0, 150.0, 200.0 µg/mL	Negative Reduced mitotic index below 50 % of control could be observed at the highest evaluated concentration, Positive controls included	Anonymous (2009) *Annex I. 3.8.1.2*
Mammalian cell gene mutation (Mouse lymphoma assay) OECD 476 (1997)	SYN524464, batch SMU6LP006/Milled, purity 95.3 % (sum of 83.0 % SYN508210 and 12.3 % SYN508211) Solvent DMSO	Mouse lymphoma L5178Y cells	Without S9 mix: Experiment I; 6.9, 13.8, 27.5, 55.0 and 82.5 µg/mL and Experiment II; 20.0, 40.0, 60.0, 80.0 and 90.0	Negative Concentration range of the main experiments was limited by cytotoxicity Positive controls included	Anonymous (2009) *Annex I. 3.8.1.3*

Method	Test substance,	Organisms/ strain	Concentrations tested	Result	Reference
GLP			µg/mL With S9 mix Experiment I; 13.8, 27.5, 55.0, 82.5 and 110.0 µg/mL and Experiment II; 20.0, 40.0, 80.0, 90.0 and 100.0 µg/mL		

Table 30: Summary table of mutagenicity/genotoxicity tests in mammalian somatic or germ cells *in vivo*

Method	Test substance	Organisms/ strain	Concentrations tested	Result	Reference
Mouse bone marrow micronucleus test OECD 474 (1997) GLP	SYN524464, batch SMU6LP006/Milled, purity 95.3 % (sum of 83.0 % SYN508210 and 12.3 % SYN508211) Solvent 0.5% aqueous carboxy-methyl cellulose (CMC)	NMRI mouse 6 males/ group	24 hour preparation interval: 0, 500, 1000 and 2000 mg/kg bw 48 hour preparation interval: 2000 mg/kg b.w	Negative Highest dose was maximum recommended dose by the OECD guideline No proof of bone marrow exposure in the study (no systemic toxicity, PCE/(PCE +NCE) ratio not altered. However bone marrow exposure is assumed based on ADME data. Positive and negative control groups included	Anonymous (2010) Annex I. 3.8.2.1
Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells *in vivo'* OECD 486 (1997). GLP	SYN524464, batch SMU6LP006/Milled, purity 95.3 % (sum of 83.0 % SYN508210 and 12.3 % SYN508211) Solvent	Rat, Sprague-Dawley CD (Crl : CD® (SD) IGS BR) 4 males/ test group, and 5 males /control group	667, and 2000 mg/kg Experiment 1 the livers were perfused approximately 16 hours after dosing and, in Experiment 2; perfusion was performed approximately 2 hours after dosing	Equivocal, at 2000 mg/kg 16 hours harvest time point, the mutagenic parameters were numerically increased. While the increases were not statistically significant they slightly exceeded the historical control data.Highest dose was maximum recommended dose by the OECD guideline	Anonymous (2009) *Annex I. 3.8.2.2*

Method	Test substance	Organisms/ strain	Concentrations tested	Result	Reference
	0.5% aqueous carboxy-methyl cellulose (CMC)			Positive and negative control groups included	
Unscheduled DNA Synthesis (UDS) Test with Mammalian Liver Cells *in vivo* OECD 486 (1997). GLP	Sedaxane (95.3 % (w/w) content of SYN524464 comprised of, 83.0 % (w/w) SYN508210 (*trans* isomer), 12.3 % (w/w) SYN508211 (*cis* isomer), Solvent 0.5% aqueous carboxymethyl cellulose (CMC)	RatWistar 4 males per group/ time (Sedaxane). 2 males per group/ time (controls)	1000 and 2000 mg/kg Single oral treatment, post-treatment period of 4 or 16 hours	Negative Highest dose was maximum recommended dose by the OECD guideline Positive and negative control groups included	Anonymous (2011) *Annex I. 3.8.2.3*

Table 31: Summary table of human data relevant for germ cell mutagenicity

Type of data/report	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
There are no human relevant data				

10.8.1 Short summary and overall relevance of the provided information on germ cell mutagenicity

Sedaxane has been examined in a range of *in vitro* and *in vivo* genotoxicity assays, including endpoints of gene mutation, chromosomal damage and DNA repair. *In vitro*, sedaxane was negative for gene mutation in bacteria (Ames test, Anonymous (2009) *Annex I. 3.8.1.1*) and mammalian cells (L5178Y TK+/- mouse lymphoma, Anonymous (2009) *Annex I. 3.8.1.1*)). In the *in vitro* cytogenetic assay using primary human lymphocyte cultures, sedaxane did not induce chromosomal aberrations (Anonymous (2009) *Annex I. 3.8.1.2*).

In vivo, sedaxane was found to be non-clastogenic in the mouse bone marrow micronucleus assay (Anonymous (2009) *Annex I. 3.8.2.1*). Two rat liver UDS (unscheduled DNA synthesis) assays were performed, one gave equivocal results (Anonymous (2009) *Annex I. 3.8.2.2*) and the other was negative (Anonymous (2009) *Annex I. 3.8.2.3*) . All three *in vivo* studies were conducted up to the limit dose of 2000 mg/kg.

The overall picture indicates that sedaxane does not present a genotoxic potential.

See Annex I to the CLH report 3.8.

10.8.2 Comparison with the CLP criteria

The classification criteria for germ cell mutagenicity takes into account test results from mutagenicity or genotoxicity tests *in vitro* and from studies with mammalian somatic and germ cells *in vivo*. The overall body of toxicological data coming from a number of *in vitro* and *in vivo* assays indicates that there is no concern regarding genotoxic potential.

Based on the CLP criteria sedaxane does not require classification and labelling for germ cell mutagenicity.

10.8.3 Conclusion on classification and labelling for germ cell mutagenicity

Not classified (conclusive but not sufficient for classification)

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

Sedaxane has been tested in a series of *in vitro* and *in vivo* genotoxicity assays. *In vitro*, the substance was negative for gene mutations in bacteria (Ames test) and in mammalian cells (L5178Y TK+/- mouse lymphoma), as well as for chromosomal aberrations in human primary lymphocyte cultures. *In vivo,* up to the limit dose, sedaxane was not clastogenic in the mouse bone marrow micronucleus assay, and in two rat liver UDS assays the compound gave equivocal results in one and negative results in the other study. The DS did not propose classification for germ cell mutagenicity.

Comments received during public consultation

No comments were received during the public consultation.

Assessment and comparison with the classification criteria

In an OECD TG 471 and GLP -compliant Reverse Mutation Test using bacteria, sedaxane was tested in the *Salmonella typhimurium* strains TA1535, TA1537, TA98, and TA100 or in the *Escherichia coli* strains WP2 uvrA/pKM101 and WP2 uvrA/pKM101. No increase in the number of revertant colonies were observed in any of the six tested strains following treatment with sedaxane at dose levels up to the recommended maximum test concentration of 5 mg/plate in two independent experiments each in the presence or absence of metabolic activation (S9 mix). The validity of the protocol used was confirmed with a concurrent positive control. Sedaxane was also negative for mammalian gene mutations in the *in vitro* mouse lymphoma assay according to OECD TG 476 and GLP. No mutations were observed at the thymidine kinase locus using the cell line L5178Y in two independent experiments up to cytotoxic concentration range, each in the presence and absence of S9 metabolic activation system. The validity of the protocol used was confirmed with a concurrent positive control. In an OECD TG 473 and GLP -compliant Chromosome Aberration Test in Human Lymphocytes, sedaxane was negative for cytogenicity. No increase in structural chromosomal aberrations was observed in two independent experiments up to cytotoxic concentrations (50% reduction in mitotic index at the highest concentrations), each in the presence and absence of an exogenous metabolic activation system S9 mix. The validity of the protocol used was confirmed with a concurrent positive control.

In vivo, sedaxane was tested in the mammalian erythrocyte micronucleus assay in NMRI mice according to OECD TG 474, and GLP compliant. No increase in micronuclei was detected in mice treated with a single dose of sedaxane up to the recommended limit dose for 24 hours (500, 1000, 2000 mg/kg bw) and for 48 hours (2000 mg/kg bw). No toxicity to the bone marrow was evident based on the unchanged PCE ratio, thus it was not demonstrated that the target organ was reached. It is noted that the available toxicokinetic studies suggest bone marrow exposure, as there was a rapid oral absorption (at least 87% of the administered dose) and a wide distribution throughout the body (peak plasma concentrations at 1-5 hours for low and high dose level) with extensive metabolism via demethylation, hydroxylation, oxidation and conjugation. Rapid and extensive elimination was detected with the majority of the administered dose (>85%) excreted within 48 hours mainly via faeces. Also, the validity of the micronucleus assay protocol used was confirmed with a concurrent positive control. Thus, since the substance was tested at the recommended limit dose and no cytogenicity is expected based on the *in vitro* results, the result is considered acceptable and suggested that sedaxane is not genotoxic *in vivo* in the micronucleus assay. In addition, there are two available Unscheduled DNA Synthesis (UDS) tests with mammalian liver cells *in vivo* in rats according to OECD TG 486 and GLP compliant. The first test in Sprague Dawley rats was equivocal with all the parameters assessing mutagenicity (NNGC, N-C and % in repair) increased at the limit dose of 2000 mg/kg bw at the 16 hour harvest time point, exceeding the Historical Control Data (HCD) but not reaching statistical significance. The second study in Wistar rats was negative for DNA repair up

to the limit dose of 2000 mg/kg bw. The UDS assay is an indicator test only indirectly showing DNA lesions and there is not sufficient information to conclude on the induction of gene mutation by the substance. No germ cell data are available.

RAC concludes that sedaxane was negative for *in vitro* genotoxicity and is unlikely to be genotoxic *in vivo.* Thus the classification criteria of Annex I, 3.5.2 of CLP are not met, which would require a positive evidence obtained from *in vivo* somatic cell mutagenicity, or positive results from other *in vivo* genotoxicity assays supported by *in vitro* mutagenicity results, in order to classify in category 2. Therefore, RAC agrees with the DS that **classification for germ cell mutagenicity is not warranted**.

10.9 Carcinogenicity

Background: Formerly, taking into consideration historical control range (neoplastic findings in affected tissues were generally within historical control ranges), no classification with regard to carcinogenicity was proposed in the Conclusion on the peer review of the pesticide risk assessment of the active substance sedaxane (EFSA 2012).

In 2011, US-EPA classified sedaxane as "Likely to be Carcinogenic to Humans." This classification was based on the presence of tumours at multiple sites in two species: liver and thyroid tumours in male rats, uterine tumours in female rats, and liver tumours in male mice.

Following a request from the European Commission to re-consider the toxicological assessment and confirm the conclusions on sedaxane, carcinogenicity was re-discussed at the Pesticides Peer Review Meeting 98 in November 2012 and it was concluded that the overall pattern of tumours in rats and mice suggests that a 'Carc cat 2, H351, suspected of causing cancer' classification regarding carcinogenicity would be required for sedaxane (EFSA, 2013).

Since that time, the applicant has generated mechanistic studies and has proposed modes of action for liver, thyroid, and uterine tumours. Based on the new *in vivo* and *in vitro* exploratory toxicity studies as well as studies from the core dossier, the applicant has performed a MoA analysis according to the WHO/IPCS Framework for analysing the relevance of a cancer mode of action for humans. Those postulated MoA are reported in extenso in Appendix 1, Appendix 2 and Appendix 3 for uterine, liver and thyroid tumours respectively.

Table 32: Summary table of animal studies on carcinogenicity

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
2 year chronic toxicity/ carcinogenicity OECD 453 (2009),	SYN524464; batch SMU6LP006/ MILLED; purity 95.3% (83.0% trans	**Non-neoplastic findings** 3600 ppm (218 mg/ kg bw/day in males and 261 mg/kg bw/day in females) ↓ Body weight gain: 23.5% males week 104; 49.6%	Annonymous (2010) Amendment 1 Anonymous(2014)

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
OPPTS 870.4300 (1998), EU Directive 96/54/EEC B.33 (2001) GLP Rat: CrL:WI(Han) 52/sex/group for carcino-genicity 12/group/sex interim kill after 12 months	isomer (SYN508210) and 12.3% cis isomer (SYN508211). 0, 200, 1200 and 3600 ppm Continuous in the diet for 24 months	females week 104 ↓ Food consumption: 14.1% week 1 males, 13.3%, 14.1%, 15.8% and 12.4% females weeks 1, 13, 52 and 104 respectively ↓ Food utilisation: males 15.8% weeks 1-13, females 22.1% weeks 1-13 ↑ Liver weight: 33.7% males, 26.6% females adjusted values week 104 Liver -↑ hepatocyte hypertrophy, centrilobular: 16/52 males, 38/52 females (0/52 controls both sexes); ↑ eosinophilic cell focus: 25/52 males (8/52 controls), 14/52 females (2/52 control); ↑ hepatocyte pigment: 15/52 females (2/52 control) Thyroid - ↑ follicular cell hyperplasia 16/52 males (7/16 control); ↑ colloid basophilia 16/52 males (7/52 control), 17/52 females (3/52 control); ↑ desquamation, epithelial follicular: 14/52 females (2/52 control), ↓ diffuse C-cell hyperplasia 10/52 males (27/52 control), 5/52 females (29/52 control) Vagina- ↓ mucification (not statistically significant) Mammary gland- ↓ lobular hyperplasia <u>1200 ppm (67 mg/ kg bw/day in males and 86 mg/kg bw/day in females)</u> ↓ Body weight gain: maximum of 11% lower than control in females ↑ Liver weight: 11.7% males, 9.4% females adjusted values week 104.↑ hepatocyte hypertrophy in males at week 104. Thyroid - ↑ colloid basophilia 11/52 females (3/52 control); ↑ desquamation, epithelial follicular: 9/52 females (2/52 control) <u>200 ppm (11 mg/ kg bw/day in males and 14 mg/kg bw/day in females)</u> No treatment-related findings **The NOAEL for this study was 200 ppm for both sexes** (11 mg kg bw/day in males and 14 mg/kg bw/day in females).	*Annex I. 3.9.1.1*

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, dose levels duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr><td></td><td></td><td>

Neoplastic findings

Statistically significant increased incidence of uterine adenoma in females at 3600 ppm and reduction in mammary gland and anterior pituitary tumours.

<table>
<tr><th>Females</th><th colspan="4">Dietary Concentration of SYN524464 (ppm)</th></tr>
<tr><th>Tumour findings</th><th>0</th><th>200</th><th>1200</th><th>3600</th></tr>
<tr><td>Number examined</td><td>52</td><td>52</td><td>52</td><td>52</td></tr>
<tr><td>Uterine adenocarcinoma[a]$</td><td>0</td><td>3 (6%)</td><td>2 (4%)</td><td>9** (17%)</td></tr>
<tr><td>Uterine adenoma</td><td>0</td><td>0</td><td>1 (2%)</td><td>0</td></tr>
<tr><td>Mammary gland fibroadenoma</td><td>14 (27%)</td><td>9 (18%)</td><td>10 (20%)</td><td>0***</td></tr>
<tr><td>Pituitary adenoma anterior lobe</td><td>23 (44%)</td><td>28 (56%)</td><td>20 (38%)</td><td>16 (31%)</td></tr>
</table>

**p<0.01; *** p<0.001 , pairwise Fisher' Exact Test.

$ p<0.05, Positive trend by Peto Trend Test (Groups 1-4). P-value for linear trend including groups 1 to 4 = 0.002. P-value for linear trend including groups 1 to 3 = 0.22

[a]Historical control data from the testing laboratory including 5 prior or concurrent studies at CRL in 2002-2012 ranged from 0-19% mean 7% for uterine adenocarcinoma

Higher incidence of hepatocellular adenomas and thyroid follicular cell adenomas and thyroid adenoma/carcinoma combined in males at 3600 ppm above HCD.

<table>
<tr><th>MALES</th><th colspan="4">Dietary Concentration of SYN524464 (ppm)</th></tr>
<tr><th>Tumour findings</th><th>0</th><th>200</th><th>1200</th><th>3600</th></tr>
<tr><td>Number examined</td><td>52</td><td>52</td><td>52</td><td>52</td></tr>
<tr><td>Hepatocellular adenoma[a]</td><td>1 ^ (2%)</td><td>1 (2%)</td><td>1 (2%)</td><td>5 (10%)</td></tr>
<tr><td>Thyroid follicular cell adenoma[a]</td><td>3 ^ (6%)</td><td>3 (6%)</td><td>4 (8%)</td><td>8 (15%)</td></tr>
<tr><td>Thyroid follicular cell carcinoma[a]</td><td>0</td><td>0</td><td>2 (4%)</td><td>1 (2%)</td></tr>
<tr><td>Combined thyroid follicular cell adenoma and carcinoma</td><td>3 ^ (6%)</td><td>3 (6%)</td><td>6 (12%)</td><td>9 (17%)</td></tr>
</table>

No statistically significant differences from control group by Fisher's Exact Test (p<0.05)

^p < 0.05 Trend analysis (significance of trend denoted at control) by

</td><td></td></tr>
</table>

<table>
<tr><th>Method, guideline, deviations if any, species, strain, sex, no/group</th><th>Test substance, dose levels duration of exposure</th><th>Results</th><th>Reference</th></tr>
<tr><td></td><td></td><td>Exact Trend Test
a Historical control data from the testing laboratory including 5 prior or concurrent studies at CRL in 2002-2005 ranged from 0-3% for hepatocellular adenomas, 2-11% for follicular cell adenoma and 0-6% for follicular cell carcinoma

NOAEL for carcinogenicity: 1200 ppm (67 mg kg bw/day in males and 86 mg/kg bw/day in females)</td><td></td></tr>
<tr><td>OECD 451 (1981): OPPTS 870.4200 (1998): 87/302/EEC B.32 (1988):

GLP

Mouse: Crl:CD-1 (ICR)

50/sex/group</td><td>SYN524464; batch SMU6LP006/ MILLED; purity 95.3% (83.0% trans isomer (SYN508210) and 12.3% cis isomer (SYN508211).

0, 200, 1250 and 7000 ppm Continuous in the diet for at least 80 weeks</td><td><u>Non-neoplastic findings</u>

<u>7000 ppm (900 mg/kg bw/day in males and 1001 mg/kg bw/day in females)</u>

↓ Body weight: maximum 7% males; 9% females

↓ Food utilisation: 1.5 g/100g of diet consumed males weeks 9-13 [control 2.0]; 2.2 g/100g of diet consumed females weeks 1-4 [control 2.7];

↑ Adjusted liver weight: 16% males [covariate analysis]

<u>1250 ppm (157 mg kg bw/day in males and 185 mg/kg bw/day in females)</u>

No treatment-related findings

<u>200 ppm (25 mg/kg bw/day in males and 29 mg/kg bw/day in females)</u>

No treatment-related findings

The NOAEL for this study was 1250 ppm for both sexes, equating to achieved dose levels of 157 mg SYN524464/kg/day in males and 185 mg SYN524464/kg/day in females.

<u>Neoplastic findings</u>

At 7000ppm incidences of hepatocellular adenomas and adenomas/carcinomas combined were statistically significantly, higher than those of the control group by pair-wise comparison.

<table>
<tr><th>MALES</th><th colspan="4">Dietary Concentration of SYN524464 (ppm)</th></tr>
<tr><th>Finding incidence</th><th>0 ppm</th><th>200 ppm</th><th>1250 ppm</th><th>7000 ppm</th></tr>
<tr><td>Animals per group</td><td>48</td><td>45</td><td>45</td><td>48</td></tr>
</table>
</td><td>Anonymous (2010).

Annex I. 3.9.1.2</td></tr>
</table>

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference

excluding animals that died before week 49					
Hepatocellular adenoma[a]	7^ (14%)	9 (18%)	10 (20%)	15* (30%)	
Hepatocellular Carcinoma[a]	5^ (10%)	5 (10%)	3 (6%)	10 (20%)	
Adenoma/carcinoma combined	9^ (19%)	13 (29%)	12 (27%)	15* (40%)	

^ $p < 0.05$ Trend analysis : significance of trend denoted at control by Exact Test for trend
* $p < 0.05$ Pair-wise comparison : significance denoted at dose level by Fisher Exact Test
[a]Historical control data from the testing laboratory including 4 studies 10-28% for hepatocellular adenoma and 6-10% for hepatocellular carcinoma.

NOAEL for carcinogenicity: 1250 ppm (157 mg kg bw/day in males and 185 mg/kg bw/day in females)

Table 33: Summary table of human data on carcinogenicity

Type of data/report	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
No evidence of carcinogenicity in humans				

Table 34: Summary table of other studies relevant for carcinogenicity

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
Non-guideline investigative study Dose ranging study Non GLP Mouse CD-1. Crl:CD-1 (ICR) 5 males/dose	Sedaxane (SYN524464); batch SMU6LP006/ MILLED; purity 95.3%). Doses 7000, 10000 or 14000 ppm in diet 14 days	Aim: determine an appropriate high dose level for subsequent study investigating liver tumour mode of action.	14000 ppm (2155 mg/kg bw/day) ↑ Liver weights: 16%, Minimal hepatic centrilobular hypertrophy in 2/5 animals 10000 ppm (1389mg/kg/bw) No treatment-related effects 7000 ppm (970 mg/kg/bw) No treatment-related effects	Anonymous (2015) *Annex I. 3.9.4.1*

Type of study/data	**Test substance,**	**Relevant information about the study (as applicable)**	**Observations**	**Reference**
			No effect on transaminases levels at any dose levels. No excessive adverse effect anticipated at 14000 ppm	
Non-guideline investigative (supplemental to EPA Guideline 870.4200). Some elements GLP Mouse CD-1. Crl:CD-1 (ICR) 6 males/dose and time point	Sedaxane (SYN524464); batch SMU6LP006/ MILLED; purity 95.3%). Doses 1250, 7000 or 14000 ppm 1, 3, 7 and 21 days Vehicle Rat and Mouse (Modified) No. 1 Diet	Liver assessed for liver pathology and weight, Ki67, BrdU incorporation, up-regulation of hepatic mRNA levels, biochemical analysis, liver toxicogenomics. Positive control included: TCPOBOP	<u>14000 ppm (1792 mg/kg bw/day)</u> ↓ ALT and AST 39% and 55% day 22 ↑ Liver weights: 20%, 29% and 33% day 4, 8 and 22 respectively (adjusted values) ↑ Hepatocyte hypertrophy (centrilobular or diffuse): 2/6, 4/6, 6/6 and 6/6 days 2, 4, 8 and 22 respectively ↑ Ki67 and BrdU labelling indices: slight transient increase relative to controls on Day 8 ↑ Hepatic Cyp2b10 and Cyp2c65 mRNA levels all times ↑ Gadd45β mRNA levels on Days 2, 4 and 8 ↑ (PROD) activity; testosterone 6β-hydroxylase activity Findings from the biochemical analysis supported by the microarray toxicogenomics analysis. <u>7000 ppm (944 mg.kg bw/day</u> ↓ ALT and AST 41% and 49% day 22 ↑ Liver weights: 15% and 12% day 8 and 22 respectively (adjusted values) ↑ Hepatocyte hypertrophy (centrilobular or diffuse): centrilobular or diffuse): 6/6, and 5/6 days 8 and 22 respectively ↑ Ki67 and BrdU labelling indices: slight transient increase relative to controls on Day 8 ↑ Hepatic Cyp2b10 and Cyp2c65 mRNA levels ↑ Gadd45β mRNA levels on Days 2 and 4 ↑ (PROD) activity Findings from the biochemical	Anonymous (2016) *Annex I. 3.9.4.2*

Type of study/data	**Test substance,**	**Relevant information about the study (as applicable)**	**Observations**	**Reference**
			analysis supported by the microarray toxicogenomics analysis. 1250 ppm (170 mg/kg/bw) No treatment-related effects **Study characterises the effects of sedaxane on the mouse liver.**	
Non-guideline investigative study. GLP Rat Crl:WI(Han) 15 males/dose and time point	Sedaxane; batch SMU6LP006/ MILLED; purity 95.3% 0, 1200 and 3600 ppm in diet For 2, 4, 8, 15, and 29 days Recovery period 60 days 0, 3600 ppm Positive control sodium phenobarbital Vehicle powdered rodent diet, RM1 (E) FG SQC	Mechanistic study to evaluate effects of sedaxane on the liver and thyroid	3600 ppm (208.6 – 363.5 mg/kg bw/day) ↓ Body weight: 9.8% day 29 and BWG (34%) ↑ Triglycerides: approx. 2-fold day 8 and 15 ↑ Liver weight (adjusted): 21.6-37.6% days 4-29 ↑ Thyroid weight (adjusted): 28.6% day 29 ↑ Centrilobular hepatocyte hypertrophy: 10 to 14/15 (0/15 controls) day 4/8/15/29 ↑ BrdU labelling index: approx. 4-fold day 2 ↓ Total T3: 16.1-47.9% days 2/4/8/15 ↓ Total T4: 29.5% day 2 ↑ Thyroid follicular cell hypertrophy: 4/15 day 29 (0/15 controls) 1200 ppm (95.4 – 134.5 mg/kg bw/day) ↑ Liver weight (adjusted): 9.4-19.2% days 4-29 ↑ Thyroid weight (adjusted): 28.6% day 29 ↑ Centrilobular hepatocyte hypertrophy: 6 to 12/15 (0/15 controls) day 4/8/15/29 ↑ BrdU labelling index: approx. 3-fold day 2 ↓ Total T3: 19.3-45.5% days 4/8/15 **Conclusion: findings support the proposed MOA for liver and thyroid effects** Uncertainties: clear statistically significant changes in TSH were not discernible for the time points assessed in this study.	Anonymous (2015) *Annex I. 3.9.4.4*
Non-guideline	Sedaxane	Effect on hepatocellular	↑ PROD and BROD activities,	Anonymous

Type of study/data	**Test substance,**	**Relevant information about the study (as applicable)**	**Observations**	**Reference**
investigative study. Non-GLP Han Wistar rat hepatocyte cultures	(SYN524464); batch SMU6LP006/ MILLED; purity 95.3%). 1, 3, 10, 30, 65 and 100 µM	proliferation (measured as the change in replicative DNA synthesis [S-phase of the cell cycle]) and cytochrome P450 (CYP) enzyme activities	[mainly representative of CYP2B and CYP2B/3A induction] from 30 µM ↑ replicative DNA synthesis as determined by the S-phase labelling index from 1 **µM** **Treatment sedaxane caused a statistically-significant increase in replicative DNA synthesis in cultures of primary rat hepatocytes**.	(2016) *Annex I. 3.9.4.5*
Non-guideline investigative study. Non-GLP Cultured male human hepatocytes	Sedaxane (SYN524464); batch SMU6LP006/ MILLED; purity 95.3%). 1, 3, 10, 30, 65 and 100 µM	Effect on hepatocellular proliferation (measured as the change in replicative DNA synthesis [S-phase of the cell cycle]) and cytochrome P450 (CYP) enzyme activities	↑ BROD activities, [mainly representative of CYP2B induction] No effect on PROD representative CYP2B/3A induction No effect at any concentration on replicative DNA-synthesis, as determined by the S-phase labelling index **Treatment with sedaxane caused concentration-dependent increases in BROD activity, which is mainly representative of CYP2B/3A induction but had no effect on replicative DNA synthesis in cultures of primary human hepatocytes.**	Vardy (2016) *Annex I. 3.9.4.6*
Non-guideline investigative study. Non-GLP Mouse: CD1 5 males/group	SYN524464 Lot: SMU6LP006 95.3% pure (83% *trans* isomer, 12.3% *cis* isomer) Vehicle: diet 0, 1000, 7000 ppm (28 days duration) 0, 7000 ppm (90 days duration)	Analysis of liver samples from 28 and 90 day dietary studies with sedaxane in CD-1 mice for protein and cytochrome P450 (CYP) content and selected enzyme activities Positive control CYP inducers β-naphthoflavone, phenobarbital, dexamethasone and clofibric acid, which are known to induce CYP1A, CYP2B, CYP3A and CYP4A subfamily enzymes,	<u>28 Day 7000 ppm</u> ↑ Microsomal total CYP: 148% ↑ PROD activity: 1933% ↑ Testosterone 6β-hydroxylase activity: 135% [not statistically significant] No effect palmitoyl-CoA oxidation <u>28 Day 1000 ppm</u> ↑ PROD activity: 233 % <u>90 Day 7000 ppm</u> ↑ Microsomal total CYP: 140% ↑ PROD activity: 1400% ↑ Testosterone 6β-hydroxylase activity: 147% No effect palmitoyl-CoA oxidation **Conclusions: Increases in CYP2B-PROD activity; lesser increases in CYP3A-dependent testosterone 6β-**	Anonymous (2013) *Annex I. 3.9.4.7*

Type of study/data	**Test substance,**	**Relevant information about the study (as applicable)**	**Observations**	**Reference**
			hydroxylase activity.	
CAR3 Transactivation assay with mouse, rat and human CAR Non-Guideline Non-GLP	Sedaxane (SYN524464); batch SMU6LP006/ MILLED; purity 95.3%. 1, 3, 10, and 30 µM	CAR3 Transactivation assay with mouse, rat and human CAR	↑ Activation mouse CAR (from 3 µM) up to ~19-fold ↑ Activation rat CAR (from 10 µM) up to ~6-fold activation of rat ↑ Activation human CAR only statistically significant at 30 µM ~4-fold **Conclusion: Sedaxane is a direct activator of mouse, rat and human CAR. Under the conditions of this analysis, the activation of mouse CAR was stronger than the activation of rat or human CAR.**	Omiecinski (2014) *Annex I. 3.9.4.8*
Human PXR assay Non-Guideline Non-GLP	Sedaxane (SYN524464); batch SMU6LP006/ MILLED; purity 95.3%. 30,000, 10,000, 3,333, 1,111, 370, 123, 41, and 14 nM	Agonist activity directed against human, rat and mouse PXR. Potential to activate PXR, a nuclear receptor that transcriptionally regulates genes encoding transporters and drug-metabolizing enzymes primarily in the liver and intestine	Human PXR agonist activity in the Human PXR assay 3.33 µM to 30 µM, maximum 3.9-fold higher Rat PXR agonist activity in the rat PXR assay concentration 30 µM maximum activity 3.1-fold higher Mouse PXR No activity **Conclusion: Sedaxane has agonist activity on human and rat but not mouse PXR.**	Toyokawa and Sherf (2014) *Annex I. 3.9.4.9*
In vitro dopamine D2S receptor binding assay Non-Guideline Non-GLP Only one concentration tested D2S isoform, human recombinant, obtained from HEK-293 cells	SYN524464; batch SMU6LP006/ MILLED; purity 95.3% (83.0% trans isomer (SYN508210) and 12.3% cis isomer (SYN508211). 10 µM Vehicle DMSO	Potential of sedaxane to bind the dopamine D2S receptor *in vitro*, assessed by displacement of [3H]methyl-spiperone, a known binder of the dopamine receptor	Sedaxane was not considered to bind to the dopamine D2S receptor *in vitro*.	Jolas (2015) *Annex I. 3.9.4.10*
Non-guideline investigative study (supplemental to EPA Guideline 870.4300). Non-GLP except pathology	Sedaxane Samples from rats age 3, 12 or 24 months. [Anonymous 2015 *Annex I. 3.9.4.*1. and Anonymous 2009	Determine the cycle stage based on the microscopic examination of the vagina, uterus, and ovary of female rats exposed to sedaxane	Rats from 13 weeks to 52 weeks of age, the majority of animals were cycling and there were no clear differences between the SYN524464 treated groups and the control group In older rats (53 – 104 weeks) the majority of animals were at	Anonymous (2016) *Annex I. 3.9.4.11*

Type of study/data	**Test substance,**	**Relevant information about the study (as applicable)**	**Observations**	**Reference**
methodology and reporting. Rat Wistar	*Annex I. 3.12.1.2*] Dose levels, 0 and 4000 ppm 3 months, 0, 1200 and 3600 ppm for up to 24 months		senescent stages (either repetitive pseudopregnancy or persistent anoestrus) a numerically lower incidence of repetitive pseudopregnancy and vaginal mucification, and a numerically higher incidence of persistent anoestrus, was observed for the 3600 ppm animals compared to the controls. In the 1200 ppm animals, no differences from the concurrent group were observed in cycle stages or in descriptors in individual tissues.	
Non-guideline investigative study. Non-GLP Rat Wistar 8-10/females/group	Samples from control Wistar rats. Age 3, 12 or 24 months	Visualise and quantify dopaminergic neurons in the TIDA region of the hypothalamus from control female Wistar rats of different ages tyrosine hydroxylase (TH) immunohistochemistry and RNAscope™ in situ hybridisation	TH expression in the TIDA neurons of the hypothalamus of the 90-day, 12-month and 2-year control groups showed that the mRNA expression of TH in the cell bodies (ARC) and ARC+ axons (ME) decreased progressively with age. Conficting results between tyrosine hydroxylase (TH) immunohistochemistry and RNAscope™ in situ hybridisation were obtained at 12-month time point. While TH protein staining animals was statistically significantly lower at 2-year time point than at 1-year time point. No statistical difference was observed between 2-year time point and 90-d time point. observed. **The absence of correlation between mRNA staining and protein staining weakens the strength of this study to support age-related senescence of dopaminergic TIDA neurons**.	Anonymous (2015a) *Annex I. 3.9.4.12*
Non-guideline investigative study. Non-GLP Brain samples from female Han Wistar rats 12/group	Sedaxane Stored tissue from 2-year rat study (Anonymous 2015 *Annex I. 3.9.4.1*). 0, 200, 1200 and 3600 ppm	Brain samples examined for hypothalamic tyrosine hydroxylase via immunohistochemistry and in situ hybridization	<u>3600 ppm</u> ↑ TH protein in the dopaminergic neurons that control the release of prolactin from the anterior pituitary ↑ TH mRNA levels by ISH, <u>1200 ppm</u> ↑ TH protein in the dopaminergic neurons that control the release of prolactin	Anonymous (2015b) *Annex I. 3.9.4.13*

Type of study/data	**Test substance,**	**Relevant information about the study (as applicable)**	**Observations**	**Reference**
			from the anterior pituitary NB: ↑ TH protein not dose-related. **Conclusion: At 3600 ppm sedaxane increased TH expression in the TIDA region at 2 years.**	
Non-guideline investigative study. GLP in part only Serum samples from female Han Wistar rats 12/group	Sedaxane Stored tissue from 2-year rat study (Anonymous 2015 *Annex I. 3.9.4.1*) 0, 1200 and 3600 ppm	Frozen 1-year (52-week) serum samples from the interim sacrifice. Satisfactory stability of samples stored for extended periods at -20°C demonstrated before study samples analysed. Radioimmunoassay or enzyme-immunoassay methods with serum for analysis of prolactin, leptin and adiponectin	Differences in prolactin could not be demonstrated due to inherent level of variation in levels between individual animals No differences in adiponectin levels Mean leptin level at the high dose of 3600 ppm 15% lower than control value (not statistically significant) **Conclusion lower leptin levels at 3600 ppm correlated with the 13% lower body weights at 52 weeks. Prolactin and adinopectin levels not affected by treatment.**	Anonymous (2016) *Annex I. 3.9.4.14*
Uterotrophic assay OECD Guideline 440 (2007) GLP Deviation: only one dose level Rat Crl:WI(Han) 6 ovariectomized rats/group	Sedaxane; batch SMU6LP006/ MILLED; purity 95.3% (83.0% trans isomer (SYN508210) and 12.3% cis isomer (SYN508211) 375 mg/kg bw/day once daily for 3 consecutive days Vehicle 0.5% w/v carboxy-methylcellulose) positive control group 17α-ethynylestradiol	Gross examination of the uterus conducted; uterine weights (wet and blotted) were recorded	**Sedaxane was negative for oestrogenicity in the uterotrophic assay.**	Anonymous (2014) *Annex I. 3.9.4.15*
Effect on rat thyroid peroxidase activity *in vitro* Non-Guideline Non-GLP Rat:Wistar Han	SYN524464; batch SMU6LP006/ MILLED; purity 95.3% (83.0% trans isomer (SYN508210) and 12.3% cis isomer (SYN508211).	Samples assayed for thyroid peroxidase activity by determining the monoiodination of L-tyrosine	**Sedaxane is not an inhibitor of rat thyroid peroxidase activity *in vitro*.**	Anonymous (2014) *Annex I. 3.9.4.16*

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
5 males	Vehicle DMSO 0 (control), 0.01, 0.1, 1 and 10 µM Positive control 6-propyl-2-thiouracil			
Sedaxane - Mode of action and human relevance assessment of uterine tumors in female Han Wistar rats.				Peffer R and Yi K (2016) *Appendix 1*
Sedaxane - Mode of action and human relevance assessment of liver tumor incidences in rats and mice.				Peffer R & Minnema D (2016) *Appendix 2*
Mode of action and human relevance assessment of thyroid follicular cell tumors in male rats.				Peffer R & Cowie D (2015) *Appendix 3*

10.9.1 Short summary and overall relevance of the provided information on carcinogenicity

Two long-term toxicity/carcinogenicity studies in rodents were conducted with sedaxane. In the rat study (Anonymous, 2010 *Annex I. 3.9.1.1*), there was a marked effect on body weight in males and females in the 3600 ppm group which by the end of study represented a 23.5% and 49.6% decrease in body weight gain in males and females respectively. The magnitude of the body weight effect in the 3600 ppm dose greatly exceeds the maximum tolerated dose (MTD), conventionally defined by a 10% or greater decrease in body weight gain as compared to control. However, there was no evidence of overt toxicity at this dose level since the survival rate was not affected and no increase in clinical observations was observed. Therefore, the high dose level is not considered to be excessive to assess the potential carcinogenicity of sedaxane.

At this high dose of 3600 ppm, there was a statistically significant increased incidence of uterine adenocarcinomas in females (pair wise comparison) as well as a positive trend (Groups 1-4). At high dose level, there was also a numerically increased incidence of hepatocellular adenomas and thyroid follicular cell adenomas in the males.

In the mouse study (Anonymous, 2010 *Annex I. 3.9.1.2*), the incidences of hepatocellular adenomas and adenomas/carcinomas combined were statistically significantly, higher than those of the control group by pair-wise comparison. The incidences were slightly above the historical control range of the laboratory.

See Annex I to the CLH Report 3.9.1.1 and 3.9.1.2.

<u>Uterine tumours mode of action</u>

ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON *N*-{2-[[1,1'-BI(CYCLOPROPYL)]-2-YL]PHENYL}-3-(DIFLUOROMETHYL)-1-METHYL-1*H*-PYRAZOLE-4-CARBOXAMIDE; SEDAXANE

Syngenta has undertaken a series of investigative studies to determine the mode of action for sedaxane in the higher incidence of uterine adenocarcinomas and has performed the assessment of the putative MoA and its human relevance using the framework developed by the International Programme on Chemical Safety (IPCS) and the International Life Science Institute (ILSI) reported *in extenso* in Appendix 1 (Sedaxane - Mode of action and human relevance assessment of uterine tumors in female Han Wistar rats. Peffer R and Yi K , 2016).

Proposed mode of action by Sengenta:

Briefly, in response to sedaxane treatment at 3600 ppm a large, sustained deficit in body weight gain occurs which leads to lower amounts of adipose tissue associated with lower blood levels of leptin. Reductions in body weight gain and adipose tissue throughout the animals' lifetime causes a delay in the normal age-related loss of the tuberoinfundibular dopaminergic (TIDA) neurons of the hypothalamus. Retention of a greater number of functional TIDA neurons in aging sedaxane-treated rats results in continued production of dopamine, which suppresses prolactin release from the anterior pituitary. Thus, the age-related increase in circulating prolactin levels is delayed and/or diminished by sedaxane treatment, which leads to lower prolactin drive in the mammary gland (i.e. lower incidence of mammary gland fibroadenomas). The lack of a rise in circulating prolactin levels in blood results in a change in progression of sedaxane-treated rats into reproductive senescence and these rats continue to experience more regular oestrous cycles compared to control rats. Continued estrous cycles results in a greater cumulative exposure of the uterus to a higher oestrogen: progesterone ratio (i.e., reduced progesterone dominance of oestrogen) in aged female Wistar rats treated with sedaxane, which leads to a pro-proliferative oestrogenic stimulation of the uterine endometrial cells. Over time, the oestrogenic proliferative drive on the uterus leads to increased promotion of spontaneously initiated tumours (i.e., an increased incidence of uterine adenocarcinomas).

Key events:	**Associated events:**
Key event 1: Decreased BW gain and adipose tissue	**Associated event 1: Decreased leptin and signalling to the hypothalamus**
Key event 2: Suppression of age-related decreased in dopaminergic signalling	**Associated event 2: Decreased pituitary gland proliferative findings**
Key event 3: Suppression of age-related increase in prolactin	**Associative event 3: Decreased mammary proliferating findings**
Key event 4: Increased age of reproductive senescence	**Associative event 4: Decreased senescent mucification of the vagina and related changes observed at 2 years**
Key event 5: Increase in total number of estrous cycles and proliferation	
Final adverse outcome: Increase in uterine adenocarcinomas	

DS assessment of the postulated mode of action:

Key event 1: Significant treatment-related decreased body weight gain is supported by experimental data (50% decrease in high dose females at the end of the 2-year rat study). However, since adipose tissue was not measured in the 2-year rat study, there is no sedaxane-specific data regarding the supposed decrease in adipose tissue (Anonymous, 2010, *Annex I. 3.9.1.1*).

Furthermore, decreased body weight gain is not a specific molecular initiating event. It is a broad event, observed in many high dose groups of guidelined carcinogenicity studies. It is therefore questionable why uterine tumours are not observed with all chemical inducing significant body weight changes.

Associated event 1: In the analysis of the 1-year sacrificed rat females, the non-statistically significant decreased in mean leptin value observed in high dose females may indicate a decrease in adipose tissue, it is however not supported by mean values for adiponectin which were not affected by treatment (Anonymous, 2016, *Annex I. 3.9.4.14*).

Key event 2: In 2-year brain samples, both protein and mRNA staining support that in high dose group sedaxane treatment increased tyrosine hydroxylase expression in the TIDA region at 2 years. However, it does not automatically mean that a suppression of "age-related decrease in dopaminergic signalling" had occurred (Anonymous, 2015b, *Annex I. 3.9.4.13*). .

Key event 3: there is no direct experimental data to support a "suppression of age-related increase in prolactin" (i.e.: decreased prolactin level). Indeed the only measurements performed at 1-year time point did not show any treatment effect (Anonymous, 2016, *Annex I. 3.9.4.14*).

Associated events 2 and 3: experimental data support associative event 3 and to a lesser extent associative event 2. There was a tendency (not statistically significant) for decreased pituitary adenomas and a statistically significant decrease in the incidence of mammary fibroadenomas in the high dose rat females (Anonymous, 2010, *Annex I. 3.9.1.1*).

Key event 4: The blinded histopathology re-evaluation of the vagina, ovaries and uterus from existing histology slides from a 90-day rat study and the 2-year rat study did not support key event 4. Indeed, no differences in cyclicity measurements were observed in young animals (i.e., from 13 weeks up through the 52-week sacrifice). At 2-year time point, a similar high rate of senescence (repetitive pseudo pregnancy or persistent anoestrus) was noted in all groups (Anonymous, 2016, *Annex I. 3.9.4.11*).

Associated event 4: According to the blinded histopathology re-evaluation of the vaginas from existing histology slides from the 2-year rat study, the incidence of vaginal mucification was only slightly lower in 3600 ppm group compared to control group (21/51 vs 29/50) (Anonymous, 2016, *Annex I. 3.9.4.11*).

Key event 5: from the experimental data there is no supportive evidence of increased total number of estrous cycles and proliferation. There might have been differences in estrous cycles between 1 year and 2 year but this allegation is not substantiated by experimental data and the putative higher oestrogen: progesterone ratio has not been objectified. Furthermore, no histopathological findings indicative of overt estrogenic stimulation (as squamous metaplasia or endometrial hyperplasia) was observed at 1-year or 2-year sacrifice (Anonymous, 2016, *Annex I. 3.9.4.11*).

Based on the above listed deficiencies, DS is of the opinion that the experimental data do not provide enough evidence to support the postulated mode of action of rat uterine tumours induced by sedaxane.

Liver tumours mode of action

Syngenta has undertaken a series of investigative studies to determine the mode of action for sedaxane in the higher incidence of hepatocellular adenomas and carcinomas in rats and mice and has performed the assessment of the putative MoA and its human relevance using the framework developed by the International Programme on Chemical Safety (IPCS) and the International Life

Science Institute (ILSI) reported *in extenso* in Appendix 2 (Sedaxane - Mode of action and human relevance assessment of liver tumor incidences in rats and mice. Peffer R & Minnema D, 2016).

Proposed mode of action by Syngenta:

Briefly, sedaxane treatment results in the activation of the Constitutive Androstane Receptor (CAR) and/or Pregnane X Receptor (PXR) in the liver. This results in the altered expression of CAR-responsive genes that promote a pro-proliferative and anti-apoptotic environment in the liver and an early, transient, increase in hepatocellular proliferation. Increased hepatocellular foci as a result of clonal expansion of spontaneously mutated cells in the mouse and rat result in slight increases in liver tumour incidence compared to concurrent controls. This MoA is supported by a series of associative events including: increased expression of genes encoding cytochrome P450s (CYPs), particularly Cyp2b and (to a lesser extent) Cyp3a isoforms, increased microsomal (endoplasmic reticulum) proliferation and hepatocellular hypertrophy and increased liver weight.

Key events:	**Associated events:**
Key event 1: CAR/PXR activation	
Key event 2: Altered expression of CAR-responsive genes	**Associated event 1: Increased expression of genes encoding cytochrome P450s (CYPs), particularly Cyp2b and Cyp3a families**
Key event 3: Altered expression of pro-proliferative genes/anti-apoptotic genes	**Associated event 2: Hepatocellular hypertrophy**
Key event 4: Transient increased hepatocellular proliferation	**Associative event 3: Increased liver weight.**
Key event 5: Clonal expansion and development of altered hepatic foci	
Final adverse outcome: Increased incidence of liver adenomas	

DS assessment of the postulated mode of action:

Key event 1: The results from the *in vitro* CAR and PXR reporter assays support the fact that sedaxane activates CAR from rat, mouse and human (Omiecinski, 2014 *Annex I. 3.9.4.8)* and PXR from rat and human (Toyokawa and Sherf, 2014 *Annex I. 3.9.4.9).*

Associative event 1: In rat, the results from 28-day liver MOA (Anonymous, 2015 *Annex I. 3.9.4.4*) study as well as the 28-day study with various ratios of the isomers of sedaxane (Anonymous, 2010 *Annex I. 3.12.1.1*) support associative event 1 (increase in PROD activity from 500 ppm and in testosterone 16β-hydroxylase activity from 2000 ppm both markers of CYP 2b activity as well as increase in testosterone 6β-hydroxylase, a marker of CYP3a activity.

In mouse, combined data from RT-PCR and microarray analysis support Associative event 1 with increased expression of hepatic Cyp2b10 mRNA, Cyp2c65 mRNA, and PROD activity observed from at 1250ppm (Anonymous, 2016 *Annex I. 3.9.4.2*).

Key event 2 and Key event 3: The increase in Gadd45β mRNA from 7000 ppm and the increases in expression of xenobiotic metabolizing enzymes and other genes associated with CAR/PXR activation

from 7000 ppm in the microarray are consistent with Key event 2 and Key event 3 (Anonymous, 2016 *Annex I. 3.9.4.2*). No microarray assay is available in rat.

Associative event 2 and Associative event 3: increases in centrilobular hypertrophy and in liver weight were observed consistently in mechanistic data and regulatory studies in male rats. In male mice, while increased liver weight was observed throughout the toxicity studies, centrilobular hypertrophy was not noted in the 80-week mouse study.

Key event 4: In male rats, Sedaxane induced a transient increase in hepatocellular proliferation from 1200 ppm as measured by the BrdU labelling index (Anonymous, 2015 *Annex I. 3.9.4.4*). In male mice, as measured by Ki67 (statistically significant increase) and the BrdU label index (numerical increase), sedaxane induced a slight increase in hepatocellular proliferation from 7000 ppm at Day 8 (Anonymous, 2016 *Annex I. 3.9.4.2*).

Key event 5: In the 2-year rat study, sedaxane led to increase in eosinophilic cell foci from 1200 ppm (Anonymous, 2010 *Annex I. 3.9.1.1*). However, a minor inconsistency in the database is the lack of an increase in eosinophilic foci in the 80-week mouse study. It is plausible that an increase in altered foci in the mouse liver may have preceded the development of tumours, but it could not be observed because no interim sacrifice is made in a Guideline mouse carcinogenicity study.

Throughout the database, a good dose-concordance and a temporal concordance between the causal key events, associative events and the apical outcome, (liver tumours) were observed in both male rats and male mice.
The available data permitted to adequately rule out alternative MoAs (i.e., genotoxicity, peroxisome proliferation, AhR induction, cytotoxicity, estrogenic stimulation, statins, infections, iron/copper overload, and increased apoptosis).

In summary DS is of the opinion the available data provide enough evidence to support the postulated MoA (CAR activation) to be the underlying MoA of liver tumours observed in rodent males.

Similarly to phenobarbital (known CAR inducer), sedaxane did not induce DNA replication (prerequisite for tumour formation) in human hepatocytes following induction of human CAR, in contrast to rat. Due to this qualitative difference, the liver tumours as a result of CAR-activation by sedaxane are considered to be of little relevance to humans.

Thyroid tumours mode of action

Syngenta has undertaken a series of investigative studies to determine the mode of action for sedaxane in the higher incidence of thyroid follicular cell adenomas in male rats and has performed the assessment of the putative MoA and its human relevance using the framework developed by the International Programme on Chemical Safety (IPCS) and the International Life Science Institute (ILSI) reported *in extenso* in Appendix 3 (Mode of action and human relevance assessment of thyroid follicular cell tumors in male rats. Peffer R & Cowie D, 2015).

Proposed mode of action by Syngenta:

Briefly, activation of the CAR/PXR nuclear receptors by sedaxane leads to induction of hepatic UDP-glucuronosyltransferase (UDPGT), resulting in increased conjugation and excretion of

triiodothyronine (T3) and thyroxine (T4) and a decrease in serum T3 and T4 levels. A compensatory increase in thyroid stimulating hormone (TSH) levels secreted via the hypothalamus-pituitary-thyroid (HPT) axis results in the chronic proliferative stimulus of thyroid follicular cells by TSH prompting hypertrophy and hyperplasia, and eventually progress to form follicular cell adenomas and/or carcinomas.

Key events:	**Associated events:**
Key event 1: CAR/PXR activation	
Key event 2: Induction of hepatic UGT activity	**Associated event 1: Hepatocellular hypertrophy and increased liver weight**
Key event 3: Reduced circulating T3 and T4	
Key event 4: Increased circulating TSH	**Associated event 2: Hepatocellular hypertrophy**
Key event 5: Increased thyroid follicular cell proliferation (hyperplasia)	
Final adverse outcome: Increase in thyroid tumours incidence	

DS assessment of the postulated mode of action:

Key event 1: The results from the *in vitro* CAR and PXR reporter assays support the fact that sedaxane activates CAR from rat, mouse and human (Omiecinski, 2014 *Annex I. 3.9.4.8)* and PXR from rat and human (Toyokawa and Sherf, 2014 *Annex I. 3.9.4.9).*

Key event 2 : In the 28-day rat mechanistic study, sedaxane induced increased hepatic UGT activity at 1200 and 3600 ppm in line with Key event 2 (Anonymous, 2015 *Annex I. 3.9.4.4).*

Associated event 1: In the 28-day rat mechanistic study, sedaxane induced increased liver weight and hepatocellular hypertrophy at 1200 and 3600 ppm in line with associative event 1 (Anonymous, 2015 *Annex I. 3.9.4.4)*. Associative event 1 was consistently observed in mechanistic data and regulatory studies in male rats.

Key event 3: In the 28-day rat mechanistic study, total T3 showed a statistically significant decrease in one or both Sedaxane treatment groups on Days 2, 4, 8 and 15. However total T4 was statistically significantly decreased by treatment with Sedaxane only at Day 2 (Anonymous, 2015 *Annex I. 3.9.4.4).*

Key event 4 : In the 28-day rat mechanistic study, a clear increase of circulating TSH was not observed after sedaxane treatment (Anonymous, 2015 *Annex I. 3.9.4.4).*

Key events 3 and 4 are therefore weakly supported by experimental data. However, the shifts in thyroid hormone concentrations may be difficult to capture if the potency of effect is weak as it is the case for sedaxane (slight increased incidence of thyroid adenomas in male rats at high dose level)
Sedaxane seem to have a mild effect on thyroid hormone homeostasis, in a manner that is similar to the pattern seen with phenobarbital, but less severe.

Associative event 2: In the 28-day rat mechanistic study, sedaxane induced increased thyroid weight from 1200 ppm and thyroid follicular cell hypertrophy at 3600 ppm in line with associative event 2. Thyroid follicular cell hypertrophy was also observed at 4000 ppm in a 90-day rat study as well as in the 2-year rat study from 1200 ppm.

Key event 5: In the 2-year rat study, sedaxane induced thyroid follicular cell was also observed at 3600 ppm in line with Key event 5 (Anonymous, 2010 *Annex I. 3.9.1.1).*

Throughout the database, a good dose-concordance and a temporal concordance between the causal key events, associative events and the apical outcome (thyroid adenomas) were observed in male rats.

The available data permitted to rule out alternative MoAs: genotoxicity and inhibition of thyroid peroxidase (TPO) peroxisome proliferation (Anonymous 2014, *Annex I. 3.9.4.16).* Indeed sedaxane was negative according to genotoxicity package and was not an inhibitor of rat thyroid peroxidase activity *in vitro.*

In summary, DS is of the opinion the available data provide plausible evidence to support the postulated MoA (CAR-mediated induction of hepatic UGT activity) to be the underlying MoA of the slight increased incidence of thyroid adenomas observed in high dose male rats.

The thyroid tumours induced by sedaxane are caused by a CAR mediated MoA.
The increase in the activity of hepatic UDPG-transferase results in increased clearance of thyroid hormone levels (T4), resulting in thyroid stimulation. Such a mechanism/effect cannot be directly extrapolated to humans due to T4 binding protein that greatly reduces susceptibility to plasma T4 depletion.
The thyroid effects observed in rats are therefore considered of insufficient concern for classification (Guidance on the Application of the CLP Criteria, ECha 2017).

Table 35: Compilation of factors to be taken into consideration in the hazard assessment

Species and strain	Tumour type and background incidence	Multi-site responses	Progression of lesions to malignancy	Reduced tumour latency	Responses in single or both sexes	Confounding effect by excessive toxicity?	Route of exposure	MoA and relevance to humans
Rat: Han Wistar	Uterine adeno-carcinomas 17.3% in female rats at 3600 ppm (261 mg/kg bw/d) HCD [1] Range: 0-19% Mean: 7%	No	Yes, malignant tumour type	No	Females only	Yes, very marked decrease in body weight gain However no incidence on survival rate and clinical signs	Oral diet	Proposed MoA: Suppression of the age-related increases in prolactin levels in aging sedaxane-treated rats due to body weight deficit results in delayed reproduce tive senescence**. Not considered sufficiently supported by experimental data.**
Rat: Han Wistar	Hepato-cellular adenoma 10% in male rats at 3600 ppm (218 mg/kg bw/d) HCD [2] Range: 0-3%	Yes	No	No	Males only	Yes, marked decrease in body weight gain However no incidence on survival rate and clinical signs	Oral diet	Liver induction MoA is not relevant for humans due to the established qualitative differences in response to CAR/PXR activation between rodents (rats and mice) and humans
Rat: Han Wistar	Thyroid follicular cell adenoma 15% in male rats at 3600 ppm (218 mg/kg bw/d) CR [2] Range: 0-3%	Yes	No	No	Males only	Yes, marked decrease in body weight gain However no incidence on survival rate and clinical signs	Oral diet	MoA CAR-mediated hepatic UDP glucuronyl-transferase induction, not relevant to humans

Species and strain	Tumour type and background incidence	Multi-site responses	Progression of lesions to malignancy	Reduced tumour latency	Responses in single or both sexes	Confounding effect by excessive toxicity?	Route of exposure	MoA and relevance to humans
	Thyroid follicular cell adenomas/carcinomas combined17% in male rats at 3600 ppm (218 mg/kg bw/d) HCD [2] Range: 0-3%							
Mice: CD-1 (ICR)	Hepatocellular adenoma Males 30% in male mice at 7000 ppm (900 mg/kg bw/d) HCD [3] Range 10-28%	No	Yes	No	Males only	No	Oral diet	Liver induction MoA is not relevant for humans due to the established qualitative differences in response to CAR/PXR activation between rodents (rats and mice) and humans
	Hepatocellular carcinoma males 20% in male mice at 7000 ppm (900 mg/kg bw/d) HCD [3] Range 6-10%	No		No	Males only	No	Oral diet	

1 Lab historic data = 10 studies, all started between 2002 and 2012
2 Lab historic data = 5 prior or concurrent studies at CRL in 2002-2005
3 Lab historic data = 4 studies 2007-2010

10.9.2 Comparison with the CLP criteria

According to Regulation (EC) No 1272/2008 a substance is classified for carcinogenicity:

CATEGORY 1- Known or presumed human carcinogens on the basis of epidemiological and/or animal data.

- *Category 1A, known to have carcinogenic potential for humans, classification is largely based on human evidence, or*
- *Category 1B, presumed to have carcinogenic potential for humans, classification is largely based on animal evidence.*

CATEGORY 2: Suspected human carcinogens on the basis of evidence obtained from human and/or animal studies, but which is not sufficiently convincing to place the substance in Category 1A or 1B, based on strength of evidence together with additional considerations

As regard sedaxane, in the absence on human data category 1A is not triggered.

In order to assess the strength of evidence and to conclude whether sedaxane triggers cat.1B, cat.2 or no classification, the following factors have been taken into consideration:

- Genotoxicity:

Sedaxane is not genotoxic.

- Tumour type and background incidence:

Uterine tumours:

Statistically significant increased incidence in uterine adenocarcinomas in the rat at 3600 ppm- tumour incidence was within the range of historical control data from the laboratory. However, looking at the HCD distribution, the incidence of uterine adenocarcinoma in sedaxane high dose group is far above the HCD mean and is above the incidence of nine out of ten historical controls.

Liver tumours:

Increased incidence of hepatocellular adenomas, in male rats at 3600 ppm was not statistically significant by pairwise analysis but above HCD range.

Increased incidence in hepatocellular adenomas and hepatocellular carcinomas in the male mice at 7000 ppm was not statistically significant by pairwise analysis but above HCD range. Furthermore when excluding animals that died before week 49, statistical analysis showed that the incidences of hepatocellular adenoma and adenomas/carcinomas combined were statistically significantly higher than those of the control group by pair-wise comparison.

Thyroid tumours:

Increased incidence of thyroid follicular cell adenomas and thyroid follicular cell adenomas/carcinomas combined in male rats at 3600 ppm- not statistically significant by pairwise analysis but above HCD range.

- Multi-site responses :

In male rats increased incidences of both liver and thyroid adenoma were observed. While in female rats and male mice only one organ was affected, uterus and liver respectively.

- Progression of lesions to malignancy:

Uterine tumours:

Malignant tumours adenocarcinomas were increased at the top dose. There was no increase of adenoma incidence.

Liver tumours:

In male rats, liver tumour were limited to adenoma, there was no progression to malignancy while in male mice both hepatocellular adenomas and hepatocellular carcinomas incidences were increased at the top dose.

Thyroid tumours:

Increased incidence of thyroid follicular cell tumours was driven by the increased of adenomas (benign tumours) in male rats at 3600 ppm.

- Reduced tumour latency:

There was no evidence of reduced latency for any kinds of tumours.

- Single or both sexes:

Uterine tumours are obviously limited to females.

As regard **liver and thyroid tumours** they were only observed in males.

- Single species or several species:

Uterine tumours:

Only female rats were affected.

Liver tumours:

Both male rats and male mice were affected.

Thyroid tumours:

Only male rats were affected.

- Structural similarity to a substance(s) for which there is good evidence of carcinogenicity:

Among the fungicides inhibiting succinate dehydrogenase (SDHI) and belonging to the same chemical class (pyrazole-carboxamide) as sedaxane:

Isopyrazam also induced increased incidences of liver hepatocarcinomas and uterine adenocarcinoma in rats (EFSA Journal 2012;10(3):2600, no harmonised classification available).

Fluxapyroxad induced increased incidences of liver and thyroid tumours in rats, (EFSA Journal 2012; 10(1):2522, CLH report currently under public consultation, no classification for carcinogenicity by the DS).

Penthiopyrad induced thyroid follicular epithelial adenomas in male rats and hepatocellular adenomas and carcinomas in male mice (EFSA Journal 2013;11(2):3111, not classified for carcinogenicity CLP ATP10).

Penflufen induced increased incidences of several types of tumours including liver tumours in rats and mice (EFSA Journal 2012; 10(8):2860, CLH report recently under public consultation, proposal for carc.cat.2 classification by the DS).

- Routes of exposure:

Only experimental studies by oral route are available.

- Comparison of ADME between test animals and humans:

No human data are available.

- Possible confounding effect of excessive toxicity at test doses:

The increased incidences of tumours were only observed in top dose groups in rats and mice.

In the rat study, there was a marked effect on body weight in males and females in the 3600 ppm group which by the end of study represented a 23.5% and 49.6% decrease in body weight gain in males and females respectively. The magnitude of the body weight effect in the 3600 ppm dose greatly exceeds the maximum tolerated dose (MTD), conventionally defined by a 10% or greater decrease in body weight gain as compared to control. However, there was no evidence of overt toxicity at this dose level since the survival rate was not affected and no increase in clinical observations was observed.

In the mouse study there was no overt toxicity at the highest dose tested.

- Mode of action and its relevance for humans:

Uterine tumours: the experimental data do not provide enough evidence to support the postulated mode of action of rat uterine tumours induced by sedaxane. Indeed, several deficiencies were identified. Firstly, proposed key event 1 (decreased bodyweight gain and adipose tissue) is not a molecular initiating event. Decreased bodyweight gain is a broad event, observed in many high dose groups of guidelined carcinogenicity studies. It is therefore questionable why uterine tumours are not observed with all chemical inducing significant body weight changes. Furthermore, the downstream proposed key events 2 to 5 (Suppression of age-related decreased in dopaminergic signalling, Suppression of age-related increase in prolactin, increased age of reproductive senescence and Increase in total number of estrous cycles and proliferation) were not substantiated by experimental data.

Liver tumours: Based on the available data, DS considers that a CAR mediated MoA is sufficiently supported and that alternative MoA have been adequately ruled out. MoA gives rise to liver tumours in rodents, but there is evidence that the effects in human cells differ from rodent cells. The CAR mediated MoA is assumed to be of no relevance to humans. The CAR activation-dependent mode of action for the formation of liver tumours has been assessed for human relevance and "The data on species differences was considered [by the majority of the panel] to be sufficient to determine that this MOA would be qualitatively not plausible for humans. Thus compounds that cause rat or mouse liver tumours through this CAR-mediated MOA, similar to PB, would not be expected to increase the risk of liver tumour development in humans" (Elcombe et al 2014). "The MOA for phenobarbital (PB)-like P450 inducers was determined to be unlikely in humans after kinetic and dynamic factors were considered" (Holsapple et al, 2006).

Thyroid tumours: Based on the available data, DS considers that the CAR-mediated induction of hepatic UGT activity is sufficiently supported and that alternative MoA have been ruled out. This MoA might give rise to thyroid tumours in rodents. The relevance of such a MoA based on enhancement of the metabolism and excretion of thyroid hormone by the liver, largely through induction of UGT enzymes, is considered not to be relevant to humans. ECHA CLP Guidance (2017) lists "certain thyroid tumours in rodents mediated by UDP glucuronyltransferase (UGT) induction (IARC, 1999; EU Specialised Experts, 1999)" as not relevant to humans.

Overall weight of evidence analysis:

Based on the above listed considerations, DS considers that **liver tumours** observed in male rats and male mice at high dose levels as well **thyroid adenomas** observed in male rats at high dose levels do not trigger classification for carcinogenicity taking into account that an underlying CAR-mediated MoA is substantiated by the available data, MoA considered not relevant to humans.

As regard **uterine tumours**, in the absence of an established MoA, classification for carcinogenicity is warranted. While these tumours are malignant and also observed in one structurally similar compound, DS considers classification as Carc. 2 – H351 as appropriate since the uterine tumours were limited to a very high dose level in a single species.

In conclusion, DS is of the opinion that classification as Carc. 2 – H351 is warranted based on the increased incidence of uterine carcinomas observed in rat females.

10.9.3 Conclusion on classification and labelling for carcinogenicity

Carc. 2 – H351 Suspected of causing cancer

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

The DS proposed to classify sedaxane as Carc. 2. Oral administration of sedaxane to rats and mice for two years resulted in increased incidences of three types of tumours: malignant uterine adenocarcinoma, benign thyroid tumours and hepatocellular adenoma in rats, and hepatocellular adenomas and adenomas/carcinomas (combined) were reported in mice. The tumour findings indicated that sedaxane has a carcinogenic potential. Additional factors were also taken into account by the DS when assessing the overall level of concern and for making the decision on the category of classification.

Two carcinogenicity studies were included in the CLH report for sedaxane, one 2-year chronic/carcinogenicity study according to OECD TG 453 in rats and one OECD TG 451 - compliant study in mice. No classification with regard to carcinogenicity was proposed in

the Conclusion on the peer review of the pesticide risk assessment of the active substance sedaxane (EFSA, 2012). In 2011, US-EPA classified sedaxane as "Likely to be Carcinogenic to Humans" based on the presence of the three tumour types at multiple sites in two species observed in these two studies. Following a request from the European Commission to re-consider the toxicological assessment and confirm the conclusions on sedaxane, carcinogenicity was re-discussed at the Pesticides Peer Review Meeting 98 in November 2012 and it was concluded that the overall pattern of tumours in rats and mice suggests that classification of sedaxane as Carc. 2, H351 would be required (EFSA, 2013). Following this, the pesticide applicant has generated numerous mechanistic studies for the assessment of modes of action for liver, thyroid, and uterine tumours. The putative MoAs and their human relevance were assessed using the framework developed by the International Programme on Chemical Safety (IPCS) and the International Life Science Institute (ILSI).

In the rat study in the high dose of 3600 ppm (218/261 mg/kg bw/d males/females), a statistically significantly increased incidence in malignant uterine adenocarcinomas was observed. This incidence was within the historical control data range from the laboratory, however, the incidence was far above the HCD mean and above the incidence of nine out of ten historical controls. At this dose, there was also an increased incidence of hepatocellular adenomas and thyroid follicular cell adenomas in the males. The tumour incidences were accompanied by a marked effect on body weight in males and females, which by the end of study represented a 23.5% and 49.6% decrease in body weight gain in males and females, respectively. There was no other evidence of toxicity since the survival rate was not affected and there was no difference in clinical observations between the groups. The DS considered the effects at high dose relevant for assessing the carcinogenic potential of sedaxane.

In the mouse study, statistically significantly higher incidences of hepatocellular adenomas and adenomas/carcinomas (combined) were reported. These incidences were slightly above the historical control range of the laboratory.

The Pesticide applicant had performed a range of non-guideline MoA studies for sedaxane-induced hepatocellular adenoma/carcinoma and thyroid follicular cell adenomas, these included:

- Dose-range finding study in mice to investigate liver tumour mode of action,
- A mouse study to assess liver pathology (weight, Ki67, BrdU, mRNA levels, biochemical analysis, toxicogenomics),
- A rat study to evaluate effects of sedaxane on the liver and thyroid,
- A rat hepatocyte culture study to assess effects on hepatocellular proliferation (measured as the change in replicative DNA synthesis [S-phase of the cell cycle]) and cytochrome P450 enzyme activities),
- A study on cultured male human hepatocytes to assess effects on hepatocellular proliferation (measured as the change in replicative DNA synthesis [S-phase of

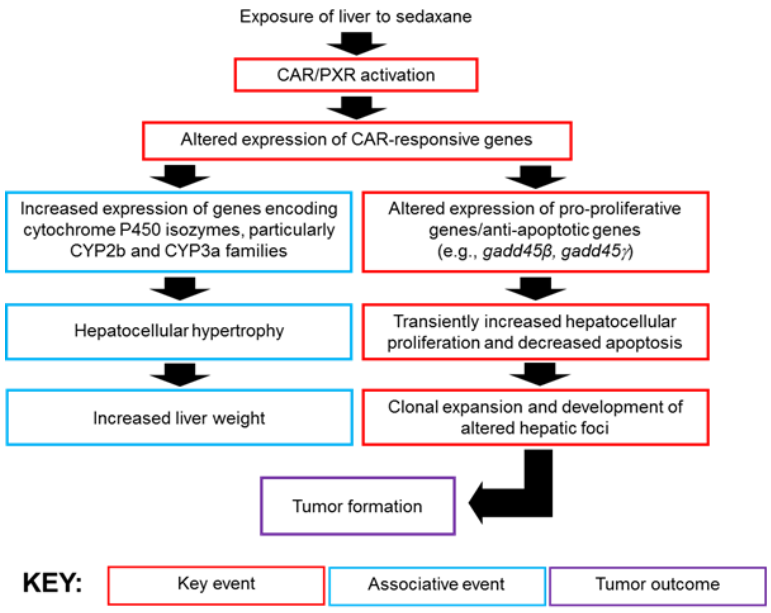
the cell cycle]) and cytochrome P450 enzyme activities,

- A mouse study to analyse liver samples from 28- and 90-day dietary studies with sedaxane for protein and cytochrome P450 content and selected enzyme activities,
- CAR3 Transactivation assay with mouse, rat and human CAR,
- Human PXR assay on agonist activity directed against human, rat and mouse PXR,
- *In vitro* study on effect on rat thyroid peroxidase activity.

Based on the results of the MoA studies, the applicant had proposed the following mode of action for the observed liver tumours in rats and mice that were considered not relevant to humans:

Sedaxane treatment resulted in the activation of the Constitutive Androstane Receptor (CAR) and/or Pregnane X Receptor (PXR) in the liver. This resulted in the altered expression of CAR-responsive genes that promoted a pro-proliferative and anti-apoptotic environment in the liver and an early transient increase in hepatocellular proliferation. Increased hepatocellular foci as a result of clonal expansion of spontaneously mutated cells in the mouse and rat resulted in slight increases in liver tumour incidences compared to concurrent controls. This MoA was supported by a series of associative events including: increased expression of genes encoding cytochrome P450s, increased microsomal (endoplasmic reticulum) proliferation and hepatocellular hypertrophy and increased liver weight. The MoA hypothesis as postulated by the pesticide applicant is represented below, with the identified causal key events and associative events:

Figure: *Proposed MoA for liver tumours in rats and mice*

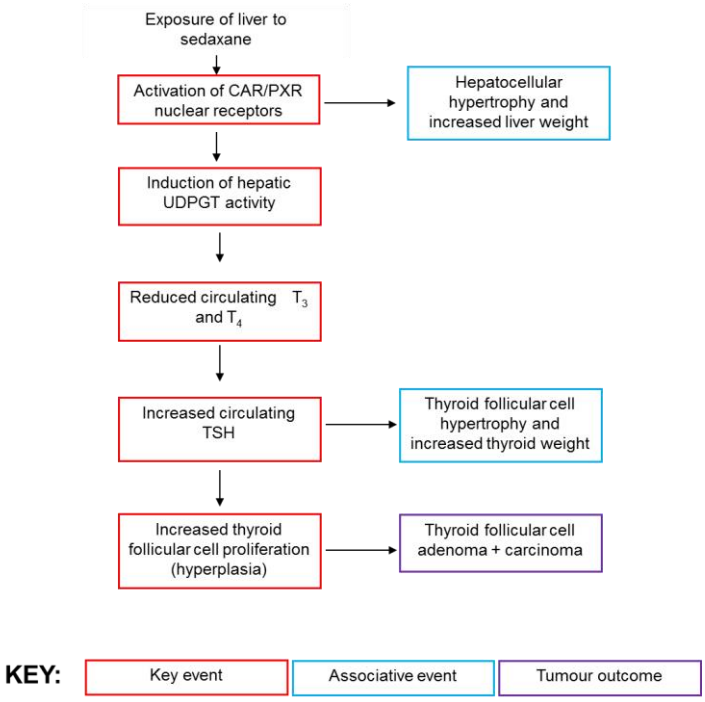


The DS concluded that the available data provided enough evidence to support the postulated MoA (CAR activation) for the liver tumours observed in rodent males. Similar to phenobarbital (known CAR inducer), sedaxane did not induce DNA replication (prerequisite for tumour formation) in human hepatocytes following induction of human CAR, in contrast to rat. Due to this qualitative difference, the liver tumours as a result of CAR-activation by sedaxane were considered to be of little relevance to humans.

The applicant had proposed the following MoA for the observed thyroid tumours in rats that were considered not relevant to humans:

The activation of the CAR/PXR nuclear receptors by sedaxane led to an induction of hepatic UDP-glucuronosyltransferase (UDPGT), resulting in an increased conjugation and excretion of triiodothyronine (T3) and thyroxine (T4) and in a decrease in serum T3 and T4 levels. A compensatory increase in thyroid stimulating hormone (TSH) levels secreted via the hypothalamus-pituitary-thyroid (HPT) axis resulted in a chronic proliferative stimulus of thyroid follicular cells by TSH prompting hypertrophy and hyperplasia, that eventually progressed to form follicular cell adenomas and/or carcinomas. This MoA hypothesis is represented below, with the identified causal key events and associative events:

Figure: *Proposed MoA for thyroid tumours in rats*



The DS concluded that the available data provided plausible evidence to support the postulated MoA (CAR-mediated induction of hepatic UGT activity for the slightly increased incidence of thyroid adenomas observed in high dose male rats. The increased activity of hepatic UDPG-transferase resulted in an increased clearance of thyroid hormone levels (T4), resulting in thyroid stimulation. Such a mechanism/effect cannot be

directly extrapolated to humans due to a T4 binding protein that greatly reduces susceptibility to plasma T4 depletion. The thyroid effects observed in rats were therefore considered of insufficient concern for classification (with a reference to the ECHA guidance on the application of the CLP Criteria).

The applicant had performed also a range of non-guideline mechanistic studies and an OECD TG 440 study for sedaxane-induced uterine adenocarcinomas:

- *In vitro* dopamine D2S receptor binding assay (assessed by a displacement of [3^H]methyl-spiperone, a known binder of the dopamine receptor),
- Determination of the oestrous cycle stage based on the microscopic examination of the vagina, uterus, and ovary of female rats exposed to sedaxane,
- Visualisation and quantification of dopaminergic neurons in the TIDA region of the hypothalamus from control female Wistar rats of different ages (Tyrosine hydroxylase (TH), immunohistochemistry and RNAscope™ *in situ* hybridisation),
- Examination of brain samples for hypothalamic TH expression via immunohistochemistry and *in situ* hybridization on stored tissue from the 2-year rat study,
- Radio- or enzyme-immunoassay for prolactin, leptin and adiponectin using frozen 1-year (52-week) serum samples,
- Uterotrophic assay according to OECD TG 440 with gross examination of the uterus and recording of uterine weights.

Based on the results the applicant had proposed a mode of action for uterine adenocarcinomas:

Sedaxane treatment induced a large and sustained reduction in body weight gain which was suggested by the applicant to lead to lower amounts of adipose tissue and consequently to lower blood levels of leptin. Reductions in body weight gain and in the following adipose tissue throughout the animals' lifetime were suggested to cause a delay in the normal age-related loss of the tuberoinfundibular dopaminergic (TIDA) neurons of the hypothalamus. The suggested retention of a greater number of functional TIDA neurons in aging sedaxane-treated rats would result in continued production of dopamine, which would suppress prolactin release from the anterior pituitary. The delayed and/or diminished prolactin drive in the mammary gland was considered to explain the lower incidence of mammary gland fibroadenomas. The suppressed circulating prolactin levels in blood resulted in a delay in reproductive senescence as the oestrous cycles were more regular in the aging sedaxane-treated rats as compared to the aging control rats. Thereby the cumulative exposure of the uterus was higher to oestrogen and lower to progesterone in the aged sedaxane-treated females as compared to the controls, which in turn lead to a pro-proliferative estrogenic stimulation of the uterine endometrial cells of the sedaxane-treated rats. Over the time, the estrogenic proliferative drive on the uterus was considered to lead to an increased spontaneous

incidence of uterine adenocarcinomas.

The DS however considered the experimental data did not provide sufficient evidence to support the postulated mode of action of rat uterine tumours induced by sedaxane, due to several deficiencies identified:

- *Key event 1:* A significant treatment-related decrease in body weight gain was supported by the experimental data (50% lower in high dose females as compared to controls at the end of the 2-year rat study). However, since adipose tissue was not measured in the 2-year rat study, there was no evidence for a sedaxane induced decrease. Furthermore, decreased body weight gain was considered not to be a specific initiating event for uterine tumours - such tumours were not systematically observed as a result of exposure to substances at high doses even if significant reductions in body weight gain were observed.
- *Associated event 1*: The statistically non-significant decrease in the mean leptin values observed in the high dose female rats sacrificed at 1 year may indicate a decrease in adipose tissue. However, this was not supported by the mean values for adiponectin, which were not affected by the treatment.
- *Key event 2*: In the 2-year brain samples, both protein and mRNA staining supported an increased TH expression in the TIDA region of the high dose group at 2 years. However according to the DS, it did not automatically mean an age-related decrease in dopaminergic signalling.
- *Key event 3*: There was no direct experimental data to support a "suppression of age-related increase in prolactin" (i.e.: decreased prolactin level) and the only measurements performed at 1-year time point did not show any treatment effect.
- *Associated events 2 and 3*: the DS considered that experimental data supported associative event 3 and to a lesser extent associative event 2. There was a tendency (not statistically significant) for decreased pituitary adenomas and a statistically significant decrease in the incidence of mammary fibroadenomas in the high dose rat females.
- *Key event 4*: The blinded histopathology re-evaluation of the vagina, ovaries and uterus from existing histology slides from a 90-day rat study and the 2-year rat study did not support key event 4, as no differences in oestrous cyclicity measurements were observed in young animals (i.e., from 13 weeks up through the 52-week sacrifice). At 2-year time point, a similar high rate of senescence (repetitive pseudopregnancy or persistent anoestrus) was recorded in all groups.
- *Associated event 4*: According to the blinded histopathology re-evaluation of the vaginas from existing histology slides from the 2-year rat study, the incidence of vaginal mucification was only slightly lower in 3600 ppm group compared to control group (21/51 vs 29/50).
- *Key event 5*: From the experimental data there was no supportive evidence of

increased total number of oestrous cycles and proliferation. There might have been differences in oestrous cycles between 1 year and 2 years, but this allegation was not substantiated by experimental data and the putative higher oestrogen:progesterone ratio has not been objectified. Furthermore, no histopathological findings indicative of overt estrogenic stimulation (as squamous metaplasia or endometrial hyperplasia) was observed at 1-year or 2-year sacrifice.

In the absence of an established MoA that could be considered not relevant to humans, the DS considered classification for carcinogenicity warranted. Overall, the DS considered classification as Carc. 2; H351 warranted, since the uterine tumours were limited to a very high dose level in a single species.

Comments received during public consultation

Five Member States Competent Authorities (MSCA) provided comments to carcinogenicity classification proposal.

Three MSCA supported the proposed classification as Carc. 2:

One MSCA considered the MoA for liver tumours (via the CAR/PXR pathway) and thyroid tumours (CAR-mediated hepatic UGT activation) sufficiently supported by the mechanistic data, but concluded that the human relevance of the uterine tumours observed cannot be excluded based on the uncertainty involved in the MoA. Therefore, the MSCA shared the opinion of the dossier submitter that classification of sedaxane as Carc. 2 is warranted based on the uterine tumours observed in female rats.

Another MSCA supported the classification as Carc. 2 based on a significant increase in incidences of uterine adenocarcinomas in female rats, liver adenomas in male rats, liver adenomas in male mice and liver carcinomas in male mice, and raised several issues to be critically discussed, in particular:

- The definition of "sufficient" evidence was partially met (CLP Annex I, 3.6.2.2.3), due to the 2-fold increase in liver carcinomas in mice over the concurrent control and HCD supported with the occurrence of liver adenomas in two species, mice and rats accordingly,
- Observed tumour may also occur in humans (uterus, liver, thyroid),
- Incidences of observed tumours are outside the HCD including follicular adenoma in male rats, liver adenoma in male rats, liver adenoma in male mice, liver carcinoma in male mice,
- The tumours are not spontaneous tumour types (liver tumours ware observed in Crl:CD-1(ICR), but not in B6C3F1 mice),
- Multiple site response in male rats was observed,
- Uterine tumours in female rats and liver tumours in male mice progressed to

malignancy,

- The postulated MoA for uterine tumours, in support of the position of DS, the submitted experimental data were inconclusive to substantiate the postulated MoA (for further details see RCOM).

The third MSCA concurring with the DS' conclusion provided further considerations in relation to the mechanistic data for uterine tumours, i.e. further uncertainties have been highlighted such as the lack of experimental evidence for decrease in adipose tissue or the role of leptin levels in dopaminergic signalling, also the fact that a role and causality for prolactin levels in uterine adenocarcinoma has not been proven.

Two MSCA considered classification for carcinogenicity not warranted:

In contrast to the DS, one MSCA considered that the increased incidence of uterine carcinoma in female rats provide weak and inconsistent evidence not sufficient to warrant carcinogenicity classification, because most importantly, the slightly statistically significant increased incidence was within the range of HCD from the test laboratory during the period (2002-2012) and uterine adenocarcinoma is a common finding in aging Wistar rats.

The other MSCA also highlighted that the incidence (17%) was within the range of historical control data (0-19%) and that RITA Wistar rat data (0-28%) can be used as evidence of high rate of spontaneous tumours. Moreover, a significant body weight decrease (50%) in animals at the top dose interferes with the interpretation of the study.

Industry provided also comments, the pesticide applicant and an industry trade association:

The trade association did not agree with the DS' assessment on the uterine tumours because the overall weight of evidence demonstrated that the observed uterine tumours are not relevant to human due to fundamental differences in physiological control of reproductive senescence between humans and rats. It was considered that the key events for the proposed MoA are well-described in the scientific literature, and the shift in tumour incidence was dependent on a marked and sustained deficit in body weight gain occurring in the female rats. The different tumour outcomes observed at 1200 ppm and 3600 ppm sedaxane indicated that the observed dose-response for the decrement in body weight gain translates into a dose response and threshold for the consequential shift in tumour incidence.

The pesticide applicant highlighted the non-relevance of the uterine tumours in rats being based on the fundamental physiological differences between humans and rats with regard to reproductive senescence as well as the role of prolactin during reproductive cycles. The uterine tumours observed in the 2-year carcinogenicity study at the high dose as a consequence of an increased duration of a persistent oestrous state would not be observed in humans (for further details see BD). To complete the overall assessment as presented in the CLH report and to address the data gaps noted by the DS, the applicant has recently completed additional investigations into the proposed MoA for the observed shift in tumour profile in rats treated with a structurally related SDHI,

isopyrazam. According to the CLH dossier, isopyrazam showed a similar uterine tumour profile in the 2-year carcinogenicity as sedaxane (i.e. increased uterine tumours with a concomitant decrease in mammary gland fibroadenomas and pituitary adenomas). The applicant considered the new isopyrazam data provide convincing evidence supporting the MoA for sedaxane. The additional data submitted consisted of the following:

- 18-month Investigative Dietary Study in the Female Han Wistar Rat on the structural analogue isopyrazam and one of its metabolites,
- OECD summary of the 18-Month Investigative Dietary Study in the Female Han Wistar Rat on isopyrazam,
- Detailed weight of evidence document describing the MoA and human non-relevance of uterine tumours,
- Short summary addressing the data gaps identified in the MoA by the DS in the CLH report.

In the view of the applicant, the new data confirmed the proposed MoA in rats and the overall database demonstrated that the observed shift in tumour profile, including the higher incidence of uterine tumours, has no relevance to human health. The applicant had slightly changed the initially proposed MoA (e.g.: initial key event: decreased food utilisation versus decreased bodyweight):

The DS considered the newly submitted mechanistic study with isopyrazam and acknowledged that the data substantiated some key events not previously observed in the data package with sedaxane (e.g.: decreased adipose tissue and statistical decreased plasma leptin and prolactin). The proposed initial key event however was still considered a broad event. It was further raised by the applicant that the described not typical pattern of response of sedaxane and isopyrazam with decreased food utilisation and body weight deficit sustained throughout the entire lifetime of the study, could be linked to their common fungicidal mode of action (SDH inhibitors) and inhibition of succinate dehydrogenase could be the molecular initiating event (however there is no specific supporting data). The dossier submitter highlighted still a range of remaining uncertainties in the data package (for details please see BD).

In conclusion, the dossier submitter was still of the opinion that the experimental data do not provide sufficient evidence to support the postulated mode of action of rat uterine tumours induced by sedaxane. It was further pointed out that an alternative potential mode of action through SDH inhibition and accumulation of succinate (considered as oncometabolite) could not be ruled out in respect to the alert recently raised by researchers and clinicians from French institutes (Benit, 2018), who established that SDH inhibitors readily inhibit the earthworm and the human enzyme, thereby raising a new concern because the loss of function, partial or total, of SDH activity caused by genetic variants causes severe human neurological diseases, or leads to the development of tumours and/or cancers. The proposal for classification therefore was maintained.

- Sedaxane: suppression of age-related decrease in hypothalamic signalling (higher

functionality of TIDA neurons): the evidence was weak with sedaxane (higher protein levels of tyrosine hydroxylase in 1200 ppm treated females than in 3600 ppm dosing group),

- Isopyrazam top dose: the mean dopamine concentrations in the median eminence of the hypothalamus were only statistically significantly higher at week 26 and were not affected later. The measure of dopamine turnover in the median eminence was unaffected by treatment. Furthermore, across the time points in this study, the concentration of dopamine (DA) and 3,4-Dihydroxyphenylacetic acid (DOPAC) in the median eminence remained fairly constant in the control animals from weeks 26 through 80,
- Isopyrazam: there was no difference in the amount of tyrosine hydroxylase staining in the arcuate nucleus by immunohistochemistry (for protein) or *in situ* hybridization (for RNA) between control and test substance-treated groups at week 52,
- Isopyrazam: there were no test substance-related differences in the number of tyrosine hydroxylase-positive (dopaminergic) neurons in the arcuate nucleus between control and treated groups by unbiased stereology at weeks 66 and 80.
- These results do not support a decreased of dopamine with time (up to 80 weeks) and a preservation of the dopaminergic activity with isopyrazam treatment as postulated,
- While there was no direct sedaxane data on differences in oestrous cycling between 1-year and 2 years, the 18-month isopyrazam MoA study suggested that high dose of isopyrazam can delay the time of onset of reproductive senescence. It is however noteworthy that in the GLP statement of the study report it is mentioned that the systems used for calculation and tabulation of oestrous cycle data were not validated,
- No histopathological findings indicative of overt estrogenic stimulation were observed in sedaxane data package and there were also no definitive adverse test substance-related histologic changes across all time points, and there were no apparent test substance-related effects on proliferative lesions in the uterus, cervix, and vagina in the 18-month isopyrazam MoA study.

Assessment and comparison with the classification criteria

Sedaxane was tested in two OECD guideline compliant chronic/carcinogenicity studies, one OECD TG 453 study in Crl:WI (Han) rats and one OECD TG 451 study in Crl:CD-1 (ICR) mice.

Rat

In rats, 0, 200 (11/14 mg/kg bw/d males/females), 1200 (67/86 mg/kg bw/d

males/females) or 3600 (218/261 mg/kg bw/d males/females) ppm sedaxane was administered to groups of 52 rats per sex via the diet for at least 104 consecutive weeks. In addition, four smaller groups of 12 animals per sex were included and dosed for 52 weeks. There were no statistically significant differences in mortality between the control and any other groups for males and no treatment related effect for females for both the 104-weeks carcinogenicity groups and the 52-weeks toxicity dosing groups. There were no increases in clinical observations, which could be attributed to test substance treatment.

Males and females treated at the high dose of 3600 ppm showed a consistent and lower body weight and weight gain compared to their respective controls throughout the treatment period. The reduced cumulative body weight gain throughout the study in the high dose represented a maximum of 23.5% decrease in males and 49.6% decrease in females at termination (reduction of terminal body weight in females by 37%). Lower values for food consumption (for females throughout the study, for males week 1-7) and reduced food utilisation (reported for week 1-13 in the CLH report) were noted in males and females at 3600 ppm. At 1200 ppm, body weight and body weight gain were also decreased in females but not in males (consistently lower weight gain than control from week 66 to the end of the study, terminal weight reduced by 8%).

Dose related higher adjusted liver weights were observed in males and females of the mid and high dose (both in the 104-weeks and 52-weeks groups), correlating with micropathology findings of centrilobular hepatocyte hypertrophy and hepatocyte pigmentation. Also selected changes in clinical chemistry parameters were observed, involving higher total protein, albumin and globulin levels in males and females considered to be treatment related and indicating adaptive changes in the liver (see table 3.9.1.1-9 of Annex I to CLH report), further changes included higher gamma glutamyl transferase (GGT) for the high dose males, higher cholesterol levels in high dose females, higher phosphate levels in high dose males, high glucose levels in mid and high dose males, and higher prothrombin for high dose males.

In the thyroid, follicular cell hypertrophy was observed in both sexes at 52 weeks with higher incidences of follicular cell hyperplasia after the 104 weeks in the 3600 ppm males, colloid basophilia and desquamation of the follicular epithelium at 1200 and 3600 ppm for both sexes. The incidence of diffuse C-cell hyperplasia was decreased in both sexes receiving 3600 ppm.

The incidence of mucification of the vagina and mammary gland lobular hyperplasia were decreased in females receiving 3600 ppm compared to controls. A blinded histopathology re-evaluation of the vagina, ovaries and uterus has been performed and according to this re-evaluation, the incidence of vaginal mucification was only slightly lower in 3600 ppm group compared to control group (21/51 vs 29/50).

Non-neoplastic findings

Table: *Non-neoplastic findings in the 2-year carcinogenicity study of sedaxane in rats*

	Males				**Females**			
Sedaxane (ppm)	**0**	**200**	**1200**	**3600**	**0**	**200**	**1200**	**3600**
Mortality	43/52 (83%)	40/52 (77%)	43/52 (83%)	44/52 (85%)	44/52 (85%)	35/52 (67%)	37/52 (71%)	44/52 (85%)
Body weight gain								
0-1	49.1	50.7	45.4**	33.3**	23.2	22.6	20.5**	12.4**
0-3	112.1	116.8	109.2	83.0**	54.0	55.1	50.6	35.4**
0-13	232.5	247.9*	228.0	187.3**	106.1	108.1	102.2	72.9**
0-52	370.3	387.3	360.9	300.7**	163.5	165.6	151.1**	108.1**
0-104	464.9	509.7*	447.9	355.7**	262.6	259.2	232.7*	132.4**
(week 104)	(-)	(+9.6%*)	(-3.6)	(-23.5%**)	(-)	(-1.3%)	(-11.4%*)	(-49.6%**)
Food intake (g/rat/day)								
-1	19.5	19.7	19.2	19.7	16.4	16.5	16.7	17.0*
1	22.7	23.0	21.6*	19.5**	16.6	16.7	16.2	14.4**
7	21.6	21.8	21.3	20.3*	17.1	17.5	16.8	15.2**
13	20.7	21.2	20.8	20.3	17.0	17.0	17.1	14.6**
28	21.0	21.7	21.9	21.6	16.6	17.5*	17.1	14.1**
52	22.3	22.4	21.5	21.6	18.4	18.2	17.3	15.5**
104	22.2	22.4	21.0	21.3	18.5	19.7	18.2	16.2*
Food utilisation (g/100 g diet)								
1-4	21.3	22.1	21.5	16.8**	13.7	13.6	12.8*	10.1**
5-8	8.4	8.6	7.7	9.3	4.8	5.3	5.4	5.4
9-13	5.4	6.1	5.9	4.1*	2.8	2.6	2.8	1.5**
1-13	11.4	12.0*	11.4	9.6**	6.8	6.8	6.6	5.3**
Liver								
Liver weights adjusted (g)	18.10	18.06	20.21** (11.7%)	24.20** (33.7%)	11.56	12.05	12.65** (9.4%)	14.64** (26.6%)
Hypertrophy	0/52	0/52	8/52**	16/52***	0/52	0/52	1/52	38/52***
Eosinic cell focus	8/52	7/52	15/52	25/52***	2/52	10/52*	12/52**	14/52**
Hepatocyte pigment	0	1	0	1	2	3	1	15
Thyroid gland								
Desquamation. epithelial follicular	7	8	11	16	2	5	9*	14**
Basophilia colloid	7	9	12	16+	3	6	11*	17***
Diffuse C-cell hyperplasia	27	27	24	10***	29	31	27	5***
Focal follicular cell hyperplasia	7	8	8	16+	0	4+	0	4+
Vagina and mammary gland								
Vagina mucification*					29/52 (15/52)	(22/52)	29/52 (16/52)	21/52 (3/52**)
Mammary gland lobular hyperplasia	7/43	1/43	1/45*	4/41	34/52	34/50	32/51	21/52*

Histology re-evaluation corrected the originally reported incidences (in brackets) with 29/52, 29/52, and 21/52 for control, mid and high dose, respectively (Annex I, 3.9.4.11).

*, ** and ***: Statistically-significantly different from control with p<0.05, p<0.01 and p<0.001, respectively. (Dunnett's test or Fisher's Exact Test).

Neoplastic findings

A statistically significant increased incidence (by pairwise test p<0.01) of uterine

adenocarcinoma in females at 3600 ppm (17.3%) and statistical significant reduction in mammary gland tumours (27% control vs 0% for the high dose) and a numerical, not significant, decrease in anterior pituitary tumours was observed for this high dose. For the mammary and pituitary tumours, these are toxicologically not significant based on the direction of change, but, based on the comparison with the control group, may reflect a result of treatment. Uterine adenocarcinomas were statistically significant also by trend test. There were no treatment-related effects on the uterus at the 52-week interim sacrifice, and no non-neoplastic micropathology changes to the uterus in the 104-week study groups. No increase in pre-neoplastic glandular endometrial hyperplasia was recorded. HCD for uterine adenocarcinoma for the testing laboratory have been submitted for the years in 2002-2012 (± 5 years from start of the sedaxane study) ranged from 0-19% with a mean of 7% for 10 studies. In addition, RITA historic control data have been provided as well, for studies of 22 to 25 months of duration, showing a range of 0-28%. RAC notes that the concurrent control is the most important control and that HCD can be used as supportive information for assessment of study results, in particular for assessing any limitations of the concurrent control group and assessing the range of normal for the endpoint. HCD should be from the same laboratory with comparable housing and feeding conditions, strain, animal supplier, and similar time periods (± 4-5 years). Therefore, the RITA data are not considered relevant for the assessment. Concerning the Charles River HCD, RAC acknowledges that the sedaxane related incidence of 17% is within the range of the HCD and that the concurrent control incidence of zero appears low. However, not only the range but also the distribution of the HCD is important. In agreement with the DS, it is noted that 2 of the 10 HCD studies had also a control incidence of zero, thus the concurrent control is considered reliable. Furthermore, the incidence of 17% exceeded the control incidence of 9 out of 10 HCD studies, thus the sedaxane treatment group does not appear to reflect the normal range of variation. It cannot be excluded that the single historical control incidence at the upper range was an outlier. In addition, the dossier submitter pointed out that another structurally related substance of the group of SDH-inhibitors isopyrazam also induced uterine adenocarcinoma at a comparable dose level, this concomitant with reduced mammary gland tumours, i.e. a similar pattern of effects. RAC notes that isopyrazam carcinogenicity data are not available to RAC and not subject for assessment in relation to the current CLH proposal. The brief information provided by the dossier submitter could indicate that the two substances may have a common MoA due to their similar chemical structure.

Based on the information available in the CLH report, RAC considers that the increased incidence of uterine adenocarcinoma is potentially a treatment related effect.

A higher incidence of hepatocellular adenomas (10% vs 2% in control), thyroid follicular cell adenomas (15% vs 6% in control) and thyroid adenoma/carcinoma combined (17% vs 6% in control) in males at 3600 ppm was observed. The incidences clearly exceeded the concurrent control incidences and also the HCD from the testing laboratory including 5 prior or concurrent studies at CRL in 2002-2005 with tumour incidences ranging from 0-3% for hepatocellular adenomas, 2-11% for follicular cell adenoma and 0-6% for follicular cell carcinoma.

In line with the dossier submitter, RAC considers the increased incidence hepatocellular adenoma, thyroid follicular cell adenoma and adenoma/carcinoma combined in the high dose males a treatment related effect (the combined thyroid tumour increase was driven by the increased incidence of adenomas, i.e. no increase in malignant tumours).

Table: *Neoplastic findings in the 2-year carcinogenicity study of sedaxane in rats*

Tumour findings	**Sedaxane (ppm)**			
	0	**200**	**1200**	**3600**
Females (# animals examined: 52)				
Uterine adenocarcinoma[a]$	0	3 (6%)	2 (4%)	**9** ** **(17%)**
Uterine adenoma	0	0	1 (2%)	0
Mammary gland fibroadenoma	14 (27%)	9 (18%)	10 (20%)	**0*** **
Pituitary adenoma anterior lobe	23 (44%)	28 (56%)	20 (38%)	**16 (31%)**
Males (# animals examined: 52)				
Hepatocellular adenoma[b]	1^ (2%)	1 (2%)	1 (2%)	**5 (10%)**
Thyroid follicular cell adenoma[b]	3^ (6%)	3 (6%)	4 (8%)	**8 (15%)**
Thyroid follicular cell carcinoma[b]	0	0	2 (4%)	1 (2%)
Combined thyroid follicular cell adenoma and carcinoma	3^ (6%)	3 (6%)	6 (12%)	**9 (17%)**

**p<0.01; *** p<0.001, pairwise Fisher' Exact Test.

$ p<0.05, Positive trend by Peto Trend Test (Groups 1-4). P-value for linear trend including groups 1 to 4 = 0.002. P-value for linear trend including groups 1 to 3 = 0.22

a Historical control data from the testing laboratory including 10 studies started in CRL between 2002-2012, ranged from 0-19% mean 7% for uterine adenocarcinoma

No statistically significant differences from control group by Fisher's Exact Test (p<0.05)

^p < 0.05 Trend analysis (significance of trend denoted at control) by Exact Trend Test

b Historical control data from the testing laboratory including 5 prior or concurrent studies at CRL in 2002-2005 ranged from 0-3% for hepatocellular adenomas, 2-11% for follicular cell adenoma and 0-6% for follicular cell carcinoma

Based on the findings reported, RAC notes that the high dose of 3600 ppm (equal to about 218/261 mg/kg bw/d males/females), where tumour incidences were increased, induced marked reduction in body weight and weight gain. According to the OECD TG 453, "*unless limited by the physical-chemical nature or biological effects of the test chemical, the highest dose level should be chosen to identify the principal target organs and toxic effects while avoiding suffering, severe toxicity, morbidity, or death*". In the view of RAC, this condition is fulfilled with the selection of dose levels, as there was no increase in mortality or clinical observations that would indicate suffering, morbidity or severe toxicity. Although the body weight gain reduction exceeded the 10%, which is commonly given as a convention for the Maximum Tolerable Dose (MTD), RAC considers the MTD not exceeded and the findings relevant for classification and labelling. No other signs of severe toxicity were apparent and there is no general (causal) association of body weight reduction with higher tumour incidences. There was no excessive toxicity and associated cell necrosis in the target tissues (or any other tissue) that would indicated that regenerative cell proliferation might have occurred, neither was hyperplasia recorded. However, RAC takes note of the MoA hypothesized by the pesticide applicant, which postulates marked body weight reduction / feed utilisation as initial event, and considers further assessment warranted.

Mouse

In the mouse study, groups of 50 male and 50 female CD-1 mice were fed diets containing 0, 200 ppm (25/29 mg/kg bw/d), 1250 ppm (157/185 mg/kg bw/d) and 7000 ppm (900/1001 mg/kg bw/d) of sedaxane for a period of at least 80 weeks.

Non-neoplastic findings

The treatment of sedaxane in mice results in mild body weight decrease with maximum of 7% in males and 9% in females and increased adjusted liver weight in males up to 16%. All non-neoplastic histology findings were considered background findings associated with this age and strain of mice, on this kind of study at Charles River, Edinburgh.

Neoplastic findings

In male mice at 7000 ppm, the incidence of hepatocellular adenomas was numerically higher than in control or other male treatment groups (14%, 18%, 20%, 30% for control, low, mid and high dose, respectively), but there were no statistically significant differences by the Peto trend test or a pairwise Fishers Exact test. A comparison to historic control data, and the RITA database, clearly shows that the incidence at 7000 ppm was above the range of normal variability for male hepatocellular adenomas in this laboratory and strain of mice.

Similarly, in male mice at 7000 ppm, the incidence of hepatocellular carcinomas was numerically higher than incidence in the control (10%, 10%, 6%, 20% for control, low, mid and high dose, respectively), but was not statistically significantly different from the concurrent control value by the Peto trend test or a pairwise Fishers Exact test (including animals dying before week 49). A comparison to the historic control data range of the study-performing laboratory shows that the incidence of the high dose exceeded the background variability. However, it is acknowledged that the liver tumours is a relatively common finding in male CD-1 mice. In addition, the RITA control data showed a range up to 22%, but these data are of less relevance as HCD should be from the same laboratory, strain and similar housing and feeding conditions and similar time (± 4-5 years).

The incidences of hepatocellular adenomas and carcinomas in female mice in the current study were extremely low in all control and treated groups (maximum 1/50).

Considering the background variability, the incidence of liver tumours in male mice are of minor concern. RAC notes however a clear dose-response for adenoma, which is indicative of a treatment-related effect. For carcinoma, the incidence in the high dose exceeded the concurrent and historical control data. In addition, the findings were not related to excessive or significant toxicity as the body weight data and clinical findings were not indicating exceedance of the MTD. Therefore, it is concluded that a treatment-related effect on carcinoma induction cannot be ruled out either.

Table: *Neoplastic findings in mice treated with sedaxane*

	Dietary concentration sedaxane (ppm)				**Historical Control Incidence**	
	0	**200**	**1250**	**7000**	**Lab (Range)** [a]	**RITA (Range)** [b]
Adenoma						
No. Animals	50	50	50	50	30/150	
Intercurrent	1	2	1	3		
Terminal kill	6	7	9	12		
Total	7 (14%)	9 (18%)	10 (20%)	15 (30%)	10-28%	0.0 – 13.6%
Carcinoma						
No. Animals	50	50	50	50	11/150	
Intercurrent	1	0	0	4		
Terminal kill	4	5	3	6		
Total	5 (10%)	5 (10%)	3 (6%)	10 (20%)	6-10%	4.0 – 22.0%

RAC assessment of the mode of action for liver tumours

The findings included higher incidences of hepatocellular adenomas in male rats and in male mice and hepatocellular carcinomas in male mice. In summary, the following incidences were reported:

Table: *Overview of liver tumours observed in rodent carcinogenicity studies with sedaxane*

	Dietary Concentration of sedaxane (ppm)			
MALES Rats	**0**	**200**	**1200**	**3600**
Number examined	52	52	52	52
Hepatocellular adenoma[a]	1 (2%)	1 (2%)	1(2%)	5 (10%)
MALES Mice	**0**	**200**	**1250**	**7000**
Number examined	48	45	45	48
Hepatocellular adenoma[a]	7 (14%)	9 (18%)	10 (20%)	15*(30%)
Hepatocellular carcinoma[a]	5 (10%)	5 (10%)	3 (6%)	10 (20%)
Adenoma/carcinoma combined	9 (19%)	13 (29%)	12 (27%)	19* (40%)[1]

* $p < 0.05$ Pair-wise comparison: significance denoted at dose level by Fisher Exact Test, excluding animals died before week 49.
[1] RAC noted that the CLH report Annex I, Table 3.9.1.2-11 reports an incidence of 15/48* (40%), which however equals 31%. As 6 animals are stated bearing adenoma and carcinoma, RAC concludes a combined incidence of 19/48* (40%).

A range of mechanistic studies was performed by the pesticide applicant to support the hypothesis of a CAR-mediated MoA for liver tumours. The data have been assessed by the dossier submitter. The DS concluded that the data convincingly demonstrated the CAR-PXR mechanism being the most plausible mechanism for liver tumour formation. The dose ranges in the mechanistic assays were in the same order of magnitude as the doses used in the long term studies in rats and mice.

RAC assessment for the specific key and associative events for the MoA

Key event 1: CAR/PXR activation

Sedaxane was evaluated in an *in vitro* CAR3 reporter assay for its ability to activate CAR from rat, mouse and human, by a method that has previously been shown to detect known species-specific activators of this nuclear receptor. In addition, sedaxane was evaluated in PXR reporter assays in the rat, mouse and human. In each assay, model compounds that are known to activate the specific CAR or PXR receptors were also tested to confirm the performance of the assays. The results from the *in vitro* CAR and PXR transactivation assays demonstrate that sedaxane activates CAR from rat, mouse and human and PXR from rat origin.

Associative event 1: Increased expression of genes encoding CYP2B/3A

In rat, the results from two 28-day studies with sedaxane and isomers support associative event 1. Increased PROD and testosterone 16β-hydroxylase activity are markers of CYP2B and cyp3A activity indicative of CAR and PXR activation. In mouse, data from RT-PCR and microarray analysis shows increased expression of hepatic CYP2B10 mRNA, CYP2Cc65 m RNA and PROD activity. Testosterone 6β-hydroxylase activity was noted.

Key event 2 and 3: Altered gene expression and altered expression of pro-proliferative genes/anti-apoptotic genes

Increase in Gadd45β mRNA and increase in xenobiotic metabolizing enzymes and other genes associated with associated with CAR/PXR activation were detected in the microarray assay in mice.

Associative event 2 and 3: Hepatocellular hypertrophy and increased liver weights

The 28-days and 90-day rat studies, and the 21-day liver MoA study in mouse together with the 90 days/80 weeks repeated dose toxicity study in mice confirm the findings in the carcinogenicity studies as increases in centrilobular hypertrophy (rats) and in liver weight (rats and mice) were observed. No histopathology including centrilobular hypertrophy or increased hepatic foci were noted in mice at any dose levels.

Key event 4: Transient increased hepatocellular proliferation and decreased apoptosis

In male rats, sedaxane induced a transient increase in hepatocellular proliferation measured by the BrdU labelling index. In male mice, sedaxane induced a slight increase in hepatocellular proliferation measured by Ki67 and BrdU labelling index.

Key event 5: Clonal expansion and development of altered hepatic foci

In the 2-year carcinogenicity study in rats, sedaxane led to increased eosinophilic cell foci.

Overall, RAC notes that in accordance to the available data, a good dose-concordance

between the causal key events, associative events and the apical outcome (liver tumours) were observed in both male rats and male mice.

Key events in the animal MoA plausible in humans

To explore the species differences in response to sedaxane, an *in vitro* investigative study using primary hepatocytes isolated from male Wistar rats was conducted to assess the effects of sedaxane on PROD (CAR-marker) and BROD (PXR/CAR-marker) activities and hepatocellular proliferation and a similar experiment was conducted with isolated male human hepatocytes from one donor.

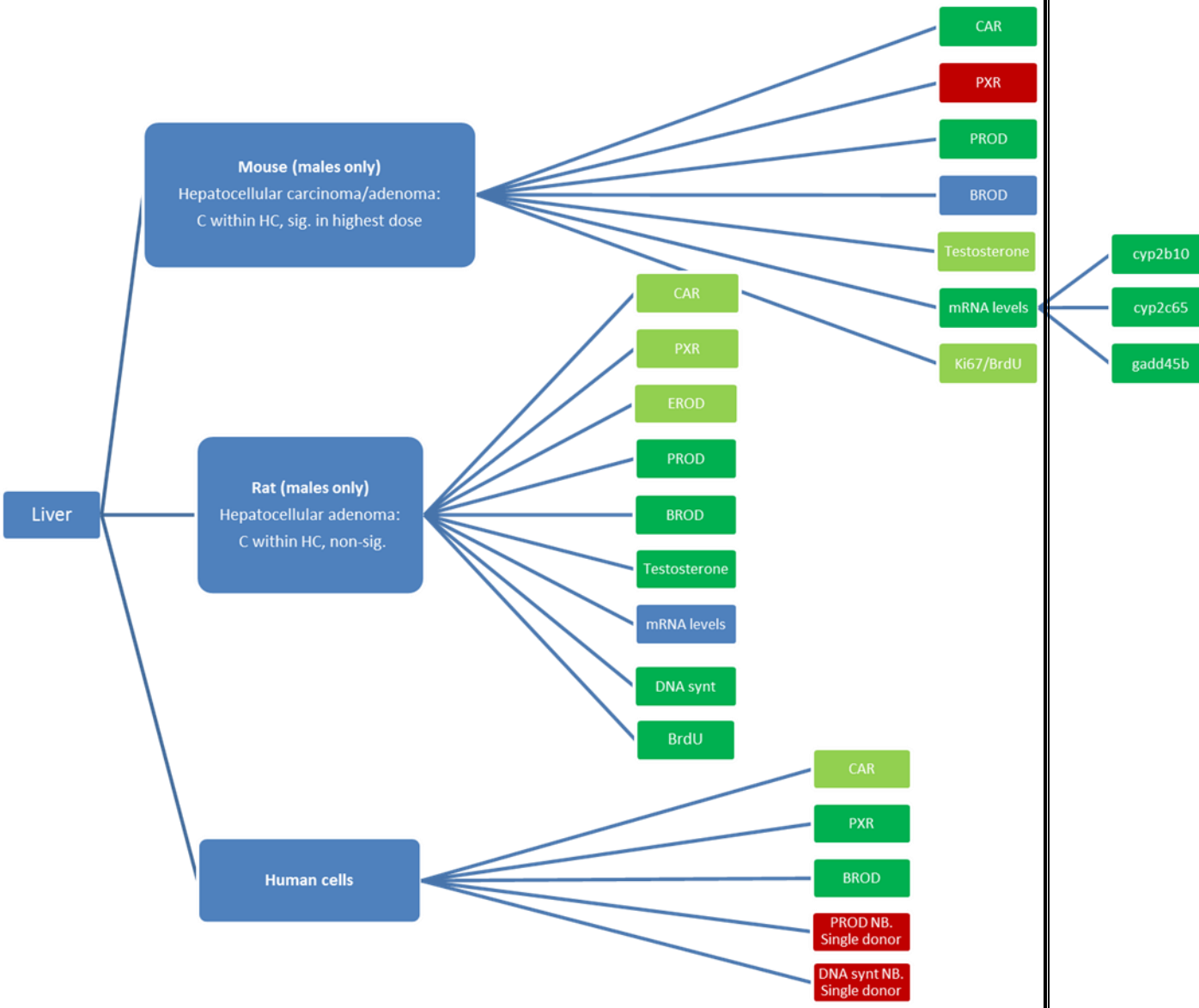
Table: *In vitro* comparison to the suggested MoA (CAR-PXR) with rat and human hepatocytes

Species	**CYP induction**	**Cell proliferation**
Rat Hepatocytes	↑ PROD activity and ↑ BROD activity	↑ S-phase labelling index
Human Hepatocytes **Only one male donor**	↑ BROD activity; no effect on PROD activity	None

The experiments showed a PXR/CAR activity (BROD activity) in human cells but negative activity for PROD activity (no CYP2B activity). Based on experimental data with one donor, the human hepatocytes have been shown to be non-responsive to sedaxane regarding the causal key event of cell proliferation. It was concluded by the pesticide applicant and supported by the DS that these data indicate that the tumourigenic MoA established for sedaxane in male rats and male mice, although likely operative in humans, may be expected to show qualitative differences between rodents and humans in their response to sedaxane, i.e. different in the critical key event cell proliferation that would ultimately lead to tumour formation. However as only one donor was tested, RAC acknowledges significant uncertainties remaining in the conclusion that the pattern of effects matches the known species differences that have been demonstrated for phenobarbital and other CAR activators as regards to a qualitative difference in the tumourigenic MoA for rodents (rats and mice) and humans. As an additional uncertainty it is noted, that no CAR-knock-out studies have been conducted.

The following figure presents a summary on the available evidence in support of a CAR-mediated MoA for liver tumour formation:

Figure: *Available data and strength of evidence for liver tumour MoA based on mechanistic studies in CLH report Appendix 2*



Colour coding and the relevant receptors, the enzymes and the gene expression:

Colour coding

Positive test
Slightly positive/less potent
Negative
Not tested

CAR	*PROD*	*CYP2B*
PXR/CAR	*BROD*	*CYP2B/3A*
AhR	*EROD*	*CYP1A*
PPAR		*CYP4A*

Overall, the available data for sedaxane support the proposed MoA to account for the higher incidences of liver tumours in male rats and male mice. The changes in the liver

seem attributable to activation of CAR (with a possible lesser activation of PXR in rats), which results in a series of well-documented downstream events, ultimately leading to a higher incidence of tumours vs. the concurrent controls. The available data provide an indication that the causal event cell proliferation, and thus related adverse outcome liver tumours, of this MoA seem of less relevance to humans. RAC however points out that the key experiments in the mechanistic data package in support of the hypothesis that the tumours are likely not relevant for humans, including studies with CAR-knock-mice and convincing data on cell proliferation of more than one human hepatocyte donor would have increased RAC´s confidence in the assessment. As human liver cell proliferation represents the key data to discount human relevance of the tumour findings observed in rodents, testing of only human donor is considered a significant uncertainty in the data package.

RAC assessment of alternative mechanisms for liver tumour formation

In addition to CAR/PXR activation, alternative MoA for induction of liver tumours in rodents or humans have been demonstrated with the data package. The DS did perform an assessment of alternative MoA in appendix 2 in the CLH report. Most important the exclusion of genotoxicity, peroxisome proliferation, AhR mediated and cytotoxicity MoA seem to be supported:

- Direct genotoxicity

It was considered that this MoA can be excluded as sedaxane has been demonstrated to be negative for genotoxicity in a comprehensive package of *in vitro* and *in vivo* assays. RAC agrees that a direct genotoxic MoA appears unlikely. Sedaxane has been tested negative in series of *in vitro* and *in vivo* genotoxicity assays and it is concluded that the substance is unlikely to be genotoxic (see section of germ cell mutagenicity).

RAC concludes that the data do not support a (direct) genotoxic MoA for liver tumours.

- Cytotoxicity / regenerative cell proliferation

Chronic cytotoxicity with subsequent regenerative cell proliferation is considered a mode of action by which tumour development can be enhanced. Following administration to rats and mice, sedaxane did not produce elevations in markers of hepatocyte damage nor was there any evidence of cytotoxicity or regenerative proliferation (the proliferation noted following treatment with sedaxane was transient and not sustained).

RAC concludes that the data do not support a cytotoxicity-mediated MoA.

- Peroxisome proliferator

Treatment with sedaxane did not increase male mouse hepatic peroxisomal fatty acid β-oxidation or lauric acid 12-hydroxylation activity (a marker of Cyp4a activity). Also, in a rat 28-day study, electron microscopy of the livers showed no evidence of peroxisome formation.

RAC concludes that the data indicate that peroxisome proliferator-MoA is unlikely.

- AhR-mediated

Treatment with sedaxane did not result in increased EROD activities in rats or in mice. In addition, no strong induction of CYP1A isoform expression of the magnitude seen with AhR activators was observed in mouse liver microarrays.

RAC concludes that the data indicate that AhR-mediate-MoA is unlikely.

In conclusion on the MoA for liver tumours*, RAC is of the opinion that the available data provide evidence to support the postulated MoA – CAR activation - to be the underlying MoA of liver tumours observed in rodent males. However, uncertainty remains especially for the hepatocellular carcinoma in male mice, as no studies with CAR-Knock-Out mice have been performed and only one single donor for the human hepatocytes assay was available.*

RAC assessment of the mode of action for thyroid tumours in rats

The findings included higher incidences of thyroid follicular cell adenoma and carcinoma in male rats. The following incidences were reported:

Table: *Overview of thyroid tumours observed in rodent carcinogenicity studies with sedaxane*

MALES Rats	**Dietary Concentration of sedaxane (ppm)**			
Tumour findings	**0**	**200**	**1200**	**3600**
Number examined	52	52	52	52
Thyroid follicular cell adenoma[a]	3 (6%)	3 (6%)	4 (8%)	8 (15%)
Thyroid follicular cell carcinoma[a]	0	0	2 (4%)	1 (2%)
Combined thyroid follicular cell adenoma and carcinoma	3 (6%)	3 (6%)	6 (12%)	9 (17%)

a Historical control data from the testing laboratory including 5 prior or concurrent studies at CRL in 2002-2005 ranged from 0-3% for hepatocellular adenomas, 2-11% for follicular cell adenoma and 0-6% for follicular cell carcinoma.

The pesticide applicant has undertaken a series of investigative studies to determine the mode of action for sedaxane in the higher incidence of thyroid follicular cell adenomas in male rats. The studies have been assessed by the dossier submitter and it was agreed that the postulated MoA involving activation of the CAR/PXR nuclear receptors by sedaxane and consequent induction of hepatic UDP-glucuronosyltransferase (UDPGT) is the most plausible mechanism. The dose ranges in the mechanistic assays were in the same order of magnitude as the doses used in the long term studies in rats and mice.

<u>RAC assessment for the specific key and associative events for the MoA</u>

Key event 1: CAR/PXR activation

The results from the *in vitro* CAR and PXR reporter assays support that sedaxane activates CAR from rat, muse and human and PXR from rat.

Associative event 1: Hepatocellular hypertrophy and increased liver weight

In the 28-day, 90-day and 2-year rat studies, sedaxane induced increased liver weight and hepatocellular hypertrophy.

Key event 2: Induction of hepatic UGT

In the 28-day rat mechanistic study, sedaxane induced increased hepatic UGT activity.

Associative event 2: Thyroid follicular cell hypertrophy and increased thyroid weight

In the 28-day, 90-day and 2-year rat studies, sedaxane induced increased thyroid weight and thyroid follicular cell hypertrophy.

Key event 3: Reduced circulating T3 and T4

In the 28-day rat mechanistic study, total T3 showed a statistically significant decrease in one or both sedaxane treatment groups on days 2, 4, 8 and 15. However, total T4 was statistically significantly decreased by treatment with sedaxane only at day 2.

This key event is only weakly supported by the experimental data.

Key event 4: Increased circulating TSH

In the 28-day rat mechanistic study, a clear increase of circulating TSH was not observed after sedaxane treatment. A marginal TSH increase could possibly be present after 14-28 days of sedaxane treatment, but definitive increases in TSH levels for sedaxane treated groups were not discernible for the time points that were assessed in the study. The individual animal data were quire variant, while the positive control phenobarbital behaved as expected with a clear effect on thyroid hormones and TSH.

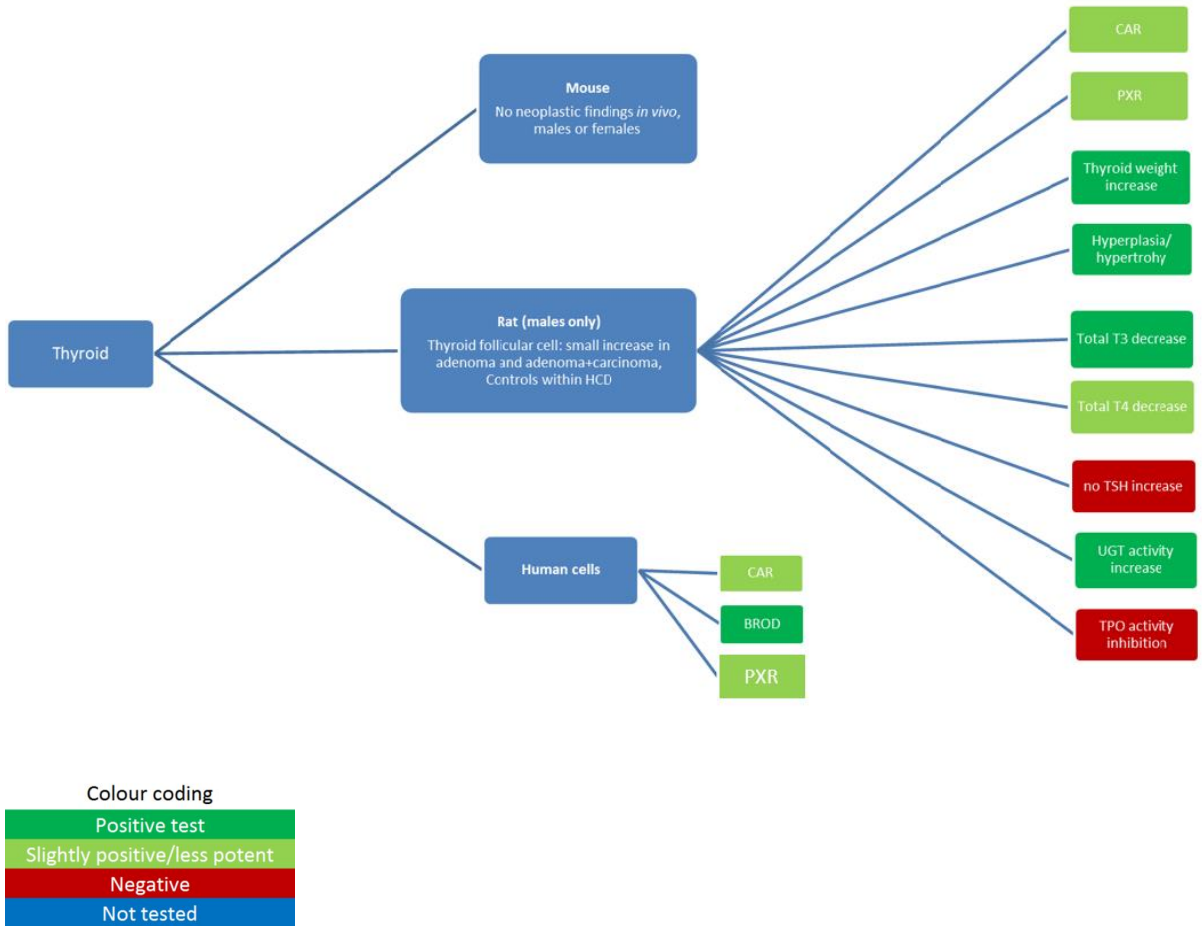
This key event is only weakly supported by experimental data.

Key event 5: Increased thyroid follicular cell proliferation

In the 2-year rat study, sedaxane induced thyroid follicular cell proliferation.

The following figure presents a summary on available data and strength of evidence in support of a UGT-mediated MoA for thyroid tumour formation:

Figure: *Available data and strength of evidence for thyroid tumour MoA based on mechanistic studies in CLH report Appendix 2*



RAC assessment of alternative mechanisms for thyroid tumour formation in rats

In addition to the MoA described, alternative modes of action for the induction of thyroid tumours exist, of which some can be assessed:

- Genotoxicity

One such alternative MoA is genotoxicity. This MoA can be excluded as sedaxane has been demonstrated to be negative for genotoxicity in a comprehensive package of *in vitro* and *in vivo* assays for genotoxicity (see previous section).

- Cytotoxicity

Generally, chronic cytotoxicity with subsequent regenerative cell proliferation is considered a MoA by which tumour development can be enhanced. Sedaxane and/or its metabolites reach the target organ as indicated by the toxicokinetic data (CLH report,

Annex I, section 2), however direct toxicity on the thyroid gland is unlikely the cause. Following administration to rats and mice, there was no evidence of cytotoxicity. The observed organ weight increase and hypertrophy is suggestive of a characteristic feedback-regulated increase in thyroid gland activity to meet higher demands of thyroid hormones.

RAC concludes the data do not suggest this cytotoxicity-mediated MoA.

- Direct inhibition of the thyroid hormone synthesis

A second alternative MoA is direct inhibition of the thyroid hormone synthesis. Organification of iodine via monoiodination of L-tyrosine is the first step in the synthesis of T3 and T4 and is catalysed by the enzyme thyroid peroxidase (TPO). Inhibition of TPO, in order to reduce circulating T3/T4, by compounds such as propylthiouracil (PTU) is exploited as a treatment for hyperthyroidism in humans, such as in Graves' disease. PTU has also been shown to induce thyroid follicular cell adenomas in rats (IARC, 2001). Sedaxane was evaluated for its potential to inhibit TPO *in vitro* across a concentration range up to 10 µM compared to the appropriate controls. Taken together with the evidence supporting the proposed MoA for thyroid tumours, these data provide compelling evidence that sedaxane lacks the intrinsic properties to interact with and inhibit TPO.

This MoA can be excluded for sedaxane as it was found not to be an inhibitor of male rat thyroid-derived TPO *in vitro*, whereas PTU was shown to be a potent inhibitor.

In conclusion on the MoA for thyroid tumours*, based on the assessment of the available data, RAC agrees with the dossier submitter that the CAR-mediated induction of hepatic UGT activity is the most plausible mechanism. This MoA might give rise to thyroid tumours in rodents. Such MoA based on enhancement of the metabolism and excretion of thyroid hormone (TH) by the liver, largely through induction of UGT enzymes, is considered less relevant to humans due to known differences in sensitivity. Two key events are only weakly supported by the data package, which leaves some uncertainty, but mild effects on thyroid hormone homeostasis have been noted. The available data permitted to rule out three alternative MoAs, i.e. genotoxicity, cytotoxicity, and inhibition of thyroid peroxidase (TPO). No other potentially alternative or additional MoA was identified but other operative pathways on thyroid hormone disruption, such as e.g. deiodinase inhibition or NIS inhibition, have not been investigated. The pattern of effects, hypertrophy and organ weight increase, is suggestive of a MoA involving disruption of TH homeostasis. Overall, some uncertainties on the underlying MoA are left.*

RAC assessment of the mode of action for uterine adenocarcinoma in rats

The DS assessed the MoA as postulated by the applicant. The dossier submitter considered the proposed MoA as not sufficiently demonstrated and thus did not analyse human relevance.

RAC assessment of the postulated mode of action

Initial key event "Decreased food utilisation", key event "Decreased adipose tissue" and associated "Decreased body weight"

The pesticide applicant considers the mechanism of secondary nature due to reduced food utilisation and body fat tissue. In the view of RAC, this is not sufficiently supported. A decrease in body weight and body weight gain throughout the 2-year carcinogenicity study was observed with 49.6% decrease in body weight gain in females at termination (reduction of terminal body weight in females by 37%). Food utilisation was measured only in the first 13 weeks of the study and was statistically significantly reduced. No direct evidence for decreased adipose tissue via measurement of whole body fat content is available for sedaxane.

Thus, RAC acknowledges that a marked reduction on body weight gain is reported for the carcinogenic dose level. However, RAC agrees with the dossier submitter and commenting MSCA that reduced body weight/food utilisation (leading to decrease in adipose tissue) is a very broad, commonly observed effect in chronic toxicity studies with the high doses, and usually not leading to increased tumour formation. It has been raised by RAC that the marked decrease in body weight gain might have even masked a more pronounced tumourigenic effect.

Further assessment of the initial key event, i.e. a potential secondary versus direct effect on the dopaminergic system, see below section "RAC assessment of Initiating Key Event".

Key event "Decreased plasma leptin and signalling to the hypothalamus"

Further downstream of the postulated MoA, plasma leptin levels and adiponectin levels were measured as weight reduction and caloric restriction are related to increase in adiponectin and decrease in leptin and the hormones provide neuroendocrine signalling to the arcuate nucleus in the hypothalamus. For the sedaxane one-year interim sacrifice of the 2-year rat study, leptin levels were reduced but not statistically significant by 15% for the 3600 ppm sedaxane group only (body weight reduction stated -13% at 1-year). The dossier submitter pointed out that also a decrease in adiponectin due to treatment with sedaxane would be expected, but was measured unchanged. The ratio of adiponectin:leptin remained fairly unchanged for sedaxane 3600 ppm (1.15-fold).

RAC concludes that leptin decrease in sedaxane-treated animals was weak with -15% and biological significance of this marginal change is questionable. In relation to the marked decrease in body weight gain, the leptin data are not plausible based on the known correlation of leptin with body fat content. It is further considered that the postulated decrease in leptin signalling to the hypothalamus is speculative. Leptin signalling to the hypothalamus has not been investigated, nor was a functional role proven.

Key event: Hypothalamus "Suppression of age-related decrease in hypothalamic signalling"; Associative event "Higher DOPAC levels in median eminence/increased dopaminergic signalling"

It was postulated that the decrease in adipose tissue and leptin-signalling to the hypothalamus would lead to a suppression of age-related increase in pituitary prolactin secretion caused by increased dopaminergic signalling due to maintained functional activity of tuberoinfundibular dopamine neurons (TIDA) neurons. TIDA function decreases with age in rats, and it was postulated by the pesticide applicant that age-related decrease in TIDA function and dopamine release by this pathway is supressed by sedaxane. In the 2-year rat sedaxane study, formalin-fixed paraffin-embedded brains from the 104-week sacrifice have been analysed for the relative abundance of tyrosine hydroxylase (TH), the enzyme hydroxylating tyrosine to DOPA (which is then converted to dopamine), in the TIDA region of the hypothalamus, in the arcuate nucleus (ARC) and medium eminence (ME) using in situ hybridisation (ISH) for mRNA expression and immunohistochemistry (IHC) to evaluate protein expression. In addition, age-related changes in these neurons as indicated by TH expression have been analysed in a control Wistar rat population after 90 days, 1 year, and 2 years.

For the latter experiment with a control population, the DS concluded that protein staining did not support age-related decrease in TIDA-TH (no difference 2y-vs-90d) in control rats and noted a lack of correlation between mRNA and protein staining. RAC agrees with the dossier submitter on this experiment, that age-related TIDA senescence has not been conclusively demonstrated. In the brain samples from the sedaxane 2-year study, 12 rats per group were quantified. TH mRNA was increased by approximately 2-fold for sedaxane 3600 ppm in the ARC and ARC+ME quantification (no increase for 1200 ppm). For the data as presented, in contrast the mid dose of 1200 ppm sedaxane increased protein levels in the ARC, ME and ARC+ME significantly and this to higher extend than the high dose of 3600 ppm, thus not dose-related (see Figure 6 of CLH report, section 3.2.4.1 on tyrosine hydroxylase mRNA and protein expression in brain samples from 2-year study). In the view of RAC, these results on sedaxane do not present a robust evidence. It is noted that protein expression by immunohistochemistry staining is a semi-quantitative methodology and quantified differences and statistical analysis need to be taken with caution. Furthermore, TIDA neuron activity is sensitive upon a variety of stimuli such as stress, hormones, i.e. oestrogens in females, and even endogenous daily activity (Ben-Jonathan *et al.*, 2001). Hypothalamic TH protein is constitutively active to meet the high dopamine demand and TH activity might be influenced by post-translational modifications, importantly the functional activity has not been proven to be increased by sedaxane. Regarding biological plausibility of the proposed MoA, considering an increase in TH protein in the mid dose, it is difficult to establish an association to increased uterine tumours and decreased mammary gland tumours, since the incidence were unchanged for the mid dose and the tumour shift observed only for the high dose group.

To summarise, 1) For aging control rats, decrease in dopamine with time, TH protein staining, did not support age-related TIDA senescence. 2) For sedaxane-treated rats (2-

yr-study), experiments on TH expression are inconclusive (no prove of dose-response in TH protein, no correlation in mRNA and protein levels for the mid dose, potentially related to methodological issues). 3) The functional TH activity has not been objectified and thus not proven to be increased by sedaxane. RAC agrees with the DS that this important key event in the postulated MoA, preservation of dopaminergic signalling, is not sufficiently supported.

Key event: pituitary "Suppression of age-related increase in pituitary prolactin secretion", Associated event "Lower plasma prolactin"

It has been postulated that maintenance of dopaminergic activity in the hypothalamus suppresses age-related increase in pituitary prolactin secretion into the blood and consequently decreases prolactin-mediated progesterone secretion from the corpora lutea from the ovaries. The lower levels of prolactin in sedaxane-treated aging rats should cause a change in transition into reproductive senescent. Prolactin is an established trophic drive for mammary tumour formation in rats. Indeed, a reduced incidence of mammary tumours was associated with sedaxane treatment: fibroadenoma 14/52 (27%), 9/50 (18%), 10/51 (20%), 0/52 (0%) for control, low, mid and high dose, respectively. Prolactin levels have been analysed in 1-year (frozen) serum samples from the 2-year carcinogenicity study with sedaxane. Due to the inherent level of variation in prolactin levels between individual animals at 52 weeks of age, it was not possible to determine any differences in prolactin concentrations between control and sedaxane-treated groups from the available serum samples. No further studies have been performed, such as prolactin measurements at later time points.

The dossier submitter correctly concluded that there is no direct experimental evidence for suppression of age-related prolactin suppression by sedaxane, and that the data for 52-weeks do not show a treatment-related effect. To summarise, prolactin alteration has not been proven for sedaxane.

Key event: Ovary "Delayed progression from persistent oestrus to persistent dioestrus"; Associated event: Vagina "Decreased mucification and related changes"

It has been postulated that the lack of rise in circulating prolactin levels in blood results in a delay in reproductive senescence of rats, which continue to experience more periodic oestrous cycles compared to control rats. The objective of the histology re-evaluation investigation (CLH report Annex I, 3.9.4.11) was to determine the cycle stage based on the microscopic examination of the vagina, uterus, and ovary of rats exposed to sedaxane in their diets for intervals ranging from 13 weeks in the available 90-day dietary study to 104 weeks in the 2-years carcinogenicity study. Based on the time period when necropsy occurred in the 90-day and 2-year studies, the following groups have been analysed for oestrous cyclicity and reproductive senescence: A = sacrificed at 13 weeks, B = died 0 - 52 weeks, C = sacrificed at 52 weeks, D = died 53 - 104 weeks, E = sacrificed at 104 weeks, for the mid (1200 ppm) and high dose (3000 ppm) as compared to the control group. Looking across all age groups, the results indicated that regardless of treatment group, virtually all of the females in the 13-week study as well as the 52-week interim sacrifice subgroup were cycling at the time of death/sacrifice. In

older rats (subgroups D and E combined) by 104 weeks, the vast majority of animals showed evidence of senescent stages (either in repetitive pseudopregnancy or persistent anoestrus) comparable for all treatment groups (44/50, 47/52, 47/51 for control, mid and high dose, respectively). The only difference was a lower incidence of repetitive pseudopregnancy (29/50 (control), 28/52 (1200 ppm), 19/51 (3600 ppm)) and a higher incidence of persistent anoestrus (15/50 (control), 18/52 (1200 ppm), 27/51 (3600 ppm)), the last stage of reproductive senescence, observed for the 3600 ppm animals compared to the control. Regardless of treatment group, persistent oestrus was virtually absent at all ages up through 104 weeks in the subchronic and chronic study with sedaxane. It needs however to be considered that oestrous cycling staging need to be performed in regular time intervals by vaginal lavage and therefore these data may not be sufficient to draw firm conclusions. No hormone levels (estrogen:progesterone) have been measured in any of the studies for sedaxane.

Therefore, RAC agrees with the conclusion of the dossier submitter, that a similar rate of reproductive senescence was noted in all dosing groups and a delay in reproductive senescence of rats by sedaxane cannot be concluded from these data. From these data, an increase in the total number of oestrous cycles and proliferation is not supported and a change in oestrogen:progesterone levels that would lead to a sustained stimulation of the uterine endometrium (key event, uterus, leading to higher incidence of uterine adenocarcinomas) has not been objectified. Overall, RAC concludes that the data do not evidence the postulated key event of a delayed progression into persistent oestrus and from there to persistent dioestrus. A higher estrogenic state with altered (=elevated) estrogen:progesterone levels for sedaxane remains hypothetical.

Key event: uterus "Sustained stimulation of uterine epithelium" (eventually leading to a higher incidence of uterine adenocarcinoma)

A sustained proliferative drive of the uterus epithelium has been postulated to result from an increased estrogenic state. It is noted that no data have been provided to substantiate this key event ultimately leading to uterine adenocarcinoma. For sedaxane, there were no treatment-related effects on the uterus indicative for sustained proliferation/hyperplasia of the endometrium at the 52-week interim sacrifice, and no non-neoplastic micropathology changes to the uterus in the 104-week carcinogenicity study.

RAC concludes that this key event, as assumed is plausible based on the literature (glandular endometrium hyperplasia is a pre-neoplastic lesion of uterine adenocarcinoma), but was not recorded in the data package.

RAC assessment of Initiating Key Event of the postulated MoA – further considerations

The applicant considered the mechanism of secondary nature due to reduced food utilisation and body fat tissue, this hypothesis should decrease the concern. In the view of RAC, this is not sufficiently supported:

It is noted, based on the toxicokinetic studies, that the substance (or metabolites) reach the target organ uterus (Annex 1 of the CLH report, table 2-18). Furthermore it should

be noted, that in the two-generation reproduction toxicity study (see next section) in the top dose females (1500 ppm) of the P and F1 generation, effects on the reproductive organs (ovary weight reduction, number of corpora lutea and number of antral follicles reduced, uterus weights reduction) were recorded, in absence of marked toxicity or body weight gain reduction.

Generally, it is not considered very plausible, as not all body weight reductions lead to increased tumour formation in carcinogenicity studies, not all chemically induced alterations in reproductive senescence lead to uterine tumour formation, and not all Wistar rat feed restriction studies show an increase in uterine adenocarcinoma. However, it is acknowledged that some studies have shown an association of diet / feeding status with uterine tumour incidences (Tucker *et al.,* 1979; Roe *et al.,* 1995), but the diet composition itself rather than caloric restriction could play a role. Also, an association of caloric/feeding status with (decrease) in mammary gland tumours is known. In the view of RAC it is true that reproduction and oestrous cycling is sensitive to feeding and weight loss (McShane and Wise, 1996; Frisch *et al.,* 1975; Tropp *et al.,* 2001). However, not all studies on caloric restriction that show a delay in reproductive senescence result in induction of uterine carcinoma (Keenan *et al.,* 1995). Then, in the literature it is not well demonstrated whether activity of TIDA neurons changes during different feeding states and inconsistent results have been reported. Recent findings in transgenic mice suggested that short-term fasting attenuated TIDA neuron activity and increased serum prolactin levels (Kubota *et al.,* 2018).

Ultimately, as the HPO-key events have not been robustly proven, the question of a primary or secondary effect on the hypothalamic dopamine system seem of less importance to RAC. Nevertheless, RAC notes that further data have been generated in order to exclude a direct effect on the dopamine system, such as those of the dopamine receptor agonist bromocriptine. The potential of sedaxane to bind to isolated dopamine D2S receptor was investigated:

- Dopamine receptor agonist activity

Sedaxane was tested in triplicate at a single concentration of 10 µM for its potential to bind the dopamine D2S receptor (Eurofins Cerep assay for binding potential to dopamine receptor D2S isoform, human recombinant, obtained from HEK-293 cells transfected at Eurofins Cerep with and stably expressing the human D2S gene). The assay evaluated binding by displacement of [3H]methyl-spiperone, a known binder of the dopamine receptor. When tested at a concentration of 10 µM, sedaxane did not trigger any significant reduction in control specific binding (<50%). A reference substance has been included as control in the assay and performed as expected. According to the CLH report, for the test substance, strict criteria for determination of a positive response for dopamine D2S receptor binding were not applied to the assay. However, a guideline value of $\geqslant$ 50% inhibition of control specific binding was used to indicate a positive response, in conjunction with other considerations, if applicable, such as increasing effect with increasing concentration. Under the conditions of the study control specific binding was > 93% and inhibition by sedaxane less than 7%. Therefore, sedaxane was not considered to bind to the dopamine D2S receptor *in vitro*. According to the CLH report,

the concentration of 10 µM has been selected based on pharmacokinetic considerations, the maximum µM concentration was calculated based upon the Cmax at day 14 of dietary administration, which would represent the maximum concentration at steady state. RAC notes uncertainties in the results interpretation, i.e. only the result of one single concentration has been presented and no log competition curve of ligand binding, which is usually set up in such type of studies. This is considered important, as the dopamine receptor is a neuro-endocrine receptor and as such operates rather in the nM range (high concentrations could, for instance, result in steric hindrance). Then, sedaxane is extensively metabolised *in vivo* (see Annex 1 of the CLH report, table 2-26) and RAC wonders whether metabolic conversion of the parent compound would not be required for full evaluation of receptor binding. Finally, the experiments have been conducted to exclude direct effects of sedaxane on the dopamine system and consequent prolactin alteration. Apart from RAC's observation, that no direct evidence for prolactin alteration by sedaxane is available, the presented experiment does not exclude the possibility of effects on one or several downstream components of the different dopamine signal transduction pathways. Other factors than hypothalamic dopamine, within the brain, pituitary gland, and peripheral organs have been shown to inhibit or stimulate prolactin secretion as well (Freeman *et al.,* 2000). In the view of RAC, further specific robust mechanistic evidence on prolactin regulation would be required. This is in particular true for investigating whether other effects than an indirect markedly reduced body weight gain would have contributed.

In conclusion, the result of this biochemical dopamine receptor-binding assay provides limited information in support of the postulated MoA.

RAC conclusion on the sedaxane mechanistic data

The uterus is an oestrogen-dependent organ and endometrial cells proliferate as a result of oestrogen stimulation and early neoplastic growth requires continuous presence of oestrogen, later becoming oestrogen-independent. Yoshida *et al.* (2015) described five mode of actions, among three the major pathways for uterine carcinogenesis considered relevant for humans and rodents: 1) oestrogens/chemicals with estrogenic activity, 2) continuous increase in E2:P4 ratio, and 3) modulation of oestrogen metabolism via CYP induction. For the other two MoA, i.e. 4) decreased E2 excretion/increased E2 levels in the blood or 5) increase *in situ* aromatase, little or no evidence so far in rodents has been reported. The authors also discussed other factors, i.e. the role of prolactin and dopamine modulating activity in uterine, pituitary and mammary carcinogenesis. This MoA however is less investigated and the key events are not sufficiently well described in the literature, so far.

Based on literature references, the applicant postulated the MoA involving sustained dopaminergic activity and prolactin-dependent alteration of reproductive senescence with an estrogenic state leading to sustained cell proliferation, the adverse outcome, typically observed with higher frequency in Wistar rats, an increase incidence in uterine adenocarcinoma and concomitant decrease in mammary gland and pituitary tumours. This pathway is discussed by Harleman *et al.* (2012). The authors point out that such a tumour shift is seen in dietary restriction studies in Wistar rats, but less frequent in SD

rats. The prolactin-mediated effect in rats on increased oestradiol:progesterone ratio and resulting uterine tumour formation is considered as not relevant for humans, since prolactin is not luteotrophic in humans. Yoshida *et al.* (2015) concluded that for such a MoA extrapolation to humans, more clear evidence is needed.

RAC acknowledges that a dopaminergic-prolactin, i.e. disruption of the HPO-axis dependent mechanism, is discussed, rather recently, in the available literature as one possible mechanism that could lead to endometrium carcinogenesis, and that relevance for humans is uncertain. In particular, however, the regulatory role of hypothalamic dopamine in prolactin release from the pituitary and the role of prolactin in mammary gland carcinogenesis is settled (O´Connor *et al.*, 2000; Ben-Jonathan and Hnasko, 2001).

It is further considered that this Adverse Outcome Pathway (AOP) in Wistar rats is so far not robustly developed with its molecular initiating and key events, and thus is still in an uncertain stage. A limited amount of publications investigating/discussing this MoA is available (in mice: Gunin *et al.* 2002; in rats: Klaunig *et al.*, 2016; Harleman *et al.,* 2012; Yoshida *et al.,* 2009, 2015) for a limited number of substances, such as dopamine agonists bromocriptine and antagonist sulpiride. Up to know, only a putative AOP on an *increased dopaminergic activity leading to endometrial adenocarcinomas (in Wistar rat)* is under development (see AOP Wiki, for comparison, molecular initiating (MIE) and key events (KE)*: MIE: Increase, dopaminergic activity; KE: Decreased prolactin; KE: Increased oestrogen receptor (ER) activity; KE: Decreased progesterone from corpus luteum; KE: Increase hyperplasia of glandular epithelial cells of endometrium; AO: Increase endometrial adenocarcinomas*).

For RAC however, it is of outmost importance that key events are robustly established and measured. The toxicology and mechanistic data package for the substance in question should demonstrate the association of key events with the adverse outcome. Moreover, a causality of key events with the adverse outcome should be valid. Unless a MoA has not been demonstrated with sufficient certainty, human relevance assessment is not warranted, as the standard assumption in toxicology is that effects observed in animals are relevant for humans. RAC further points out, that endometrial cancer is highly relevant in humans, and is both in humans and in rodents an oestrogen sensitive lesion.

RAC summarises the following deficiencies in the sedaxane data package. Key events in the disruption of the HPO-axis are not supported and/or only assumptions have been made*:*

- The dopaminergic activity has not been conclusively proven, this is considered a major deficiency as it represents a key event in the hypothesis,
- Prolactin secretion was unchanged for sedaxane, respectively has been insufficiently investigated (not measured after 52 weeks), this is considered a major deficiency as it represents a key event in the hypothesis (data on a structural analogue is not considered sufficient), and is the basis for the postulate that the sedaxane-associated uterine tumours are not relevant for humans, as

prolactin is not luteotrophic in humans,

- Important key events have not been measured/objectified with robust methodologies, in particular oestrous cycling and alteration of (cycle-sensitive) oestrogen:progesterone levels,
- Sustained proliferative stimulation is postulated, but endometrial hyperplasia due to an estrogenic-mediated stimulation was not apparent in the 2-year carcinogenicity study with sedaxane, nor in any other repeated dose studies of the available data package.

Therefore, an association of the key events with the adverse outcome has not been demonstrated.

To further substantiate some key events, additional supportive data have been generated with the structural analogue isopyrazam. This included key serial events as postulated (table 13 of the position paper submitted in the PC). However, isopyrazam carcinogenicity has not been assessed so far by RAC and is not subject to this CLH proposal. A read across to isopyrazam would require prior assessment of toxicokinetics and toxicity of this substance on its own based on the substance's data package, including conclusions on carcinogenicity and underlying MoA, furthermore a robust justification for read across from isopyrazam to sedaxane. In absence of such assessment, RAC has reservations to consider the proposed read across from isopyrazam and related conclusions as robust evidence for the sedaxane MoA. According to the EFSA Peer Review conclusion (EFSA, 2012), long-term exposure to isopyrazam produced liver hepatocellular adenomas and uterine endometrial adenocarcinomas in rats. Apart from the similar tumour profile, that may indeed by related to the structural similarity of the two compounds, RAC takes note of a distinct classification proposal in the EFSA conclusion for isopyrazam regarding human health compared to sedaxane (Carc. 2; H351, Repr. 2; H361d, Acute Tox. 4; H302, Skin Sens. 1; H317).

During public consultation, the pesticide applicant concluded on sedaxane mainly based on an 18-months MoA-study conducted with isopyrazam, which has been submitted in PC. RAC cannot consider these conclusions due to the aforementioned reasons. It is further noted that the dossier submitter considered these data as not sufficient to establish the underlying MoA for uterine carcinogenesis.

RAC assessment of alternative MoA for the induction of uterine tumours

Data in relation to alternative MoA have been submitted and assessed:

- Direct genotoxicity

It was considered that this MoA can be excluded as sedaxane has been demonstrated to be negative for genotoxicity in a comprehensive package of *in vitro* and *in vivo* assays. RAC agrees that a direct genotoxic MoA appears unlikely. Based on the toxicokinetic studies it is noted that the substance (or metabolites) reach the target organ uterus (Annex 1, table 2-18). However, sedaxane has been tested negative in series of *in vitro* and *in vivo* genotoxicity assays and it is concluded that the substance is unlikely to be

genotoxic (see section of germ cell mutagenicity).

RAC concludes that the data do not support a (direct) genotoxic MoA for uterine adenocarcinoma.

- Direct endocrine/estrogenic activity

Sedaxane was investigated in the uterotrophic assay according to OECD TG 440. The substance was administered orally, by gavage, to one group of six young adult female ovariectomized Crl:WI (Han) rats once daily for three consecutive days at a dose level of 375 mg/kg bw/d. A positive control group of 6 ovariectomized rats received the oestrogenic positive control agent (17α-ethynylestradiol) in corn oil at a dose level of 0.3 mg/kg bw/day and a control group of 6 ovariectomized rats received the vehicle on a comparable regimen. As a result, mean body weight in this group was slightly (4.0%) lower than the control group on study day 3. Mean wet and blotted uterus weights in the 375 mg/kg bw/day group were similar to the control group values. The absence of effects on uterus weights demonstrated a lack of oestrogenicity for the test substance at the dose level evaluated. For the positive control, increases in mean wet and blotted uterus weights (7.1 and 3.9-fold, respectively) were noted compared to the control group. In conclusion, sedaxane was considered to be negative for oestrogenicity in the uterotrophic assay. RAC agrees to this conclusion. Although no oestrogen-receptor-binding studies have been conducted, the uterotrophic assay is highly sensitive assay for detection of *in vivo* estrogenic activity of an oestrogen/chemical (either parent or metabolite). Sedaxane has been tested with 375 mg/kg bw/d, which exceeds the carcinogenic dose level of the 2-year study (3600 ppm: 218/261 mg/kg bw/d males/females). It is also noted by RAC, that for an oestrogenic mechanism, focal glandular endometrial hyperplasia is an obligatory pre-neoplastic precursor lesions. For sedaxane, no such hyperplasia is reported. Then, for sufficiently high treatment levels, leading to oestrus cycle disruption, vaginal cytology and morphology show persistent oestrus and cornification. No increase in cornification and no increase in persistent oestrous is reported for sedaxane in the 2-years study (notable insufficient data is available on oestrous cycling).

RAC concludes that direct oestrogen-activity as a MoA for uterine adenocarcinoma is not supported by the data.

- Modulation of oestrogen metabolism via induction of CYP and related oxidative stress

In the liver and other tissues, oestrogens are converted to 2- or 4-hydroxylestradiol (2-HE, 4-HE), catechol oestrogens, by oxidative drug metabolising enzymes such as CYP1A1 and CYP1B1. CYP induction therefore can modulate oestrogen metabolism and 4-HE has a suspected role in carcinogenesis. In a 28-day rat study (Annex I, 3.12.1), sedaxane has been demonstrated to increase liver weights and induce liver centrilobular hypertrophy (of adaptive type). However, liver hypertrophy is a commonly observed event and not specific for this MoA and as such not a sufficient indication. The substance also increased the metabolic capacity of the liver, being a potent CYP2B inducer based on

hepatic PROD activity and increased 16β hydroxylation of testosterone, immunoblotting showed increased levels of CYP2B and CYP3A, and 2- and 6β hydroxylation of testosterone also supported CYP3A activity. However, only a weakly increased EROD activity indicated that CYP1A/1B induction was unlikely. *In vitro*, treatment of isolated male Han Wistar rat hepatocyte cultures with sedaxane resulted in increases in PROD and BROD activities, again, mainly representative of CYP2B and CYP2B/3A induction (Annex I of the CLH report, 3.9.4.5). In isolated male human hepatocyte cultures from one donor, treatment with sedaxane BROD activity was induced, and PROD activity was unaffected by treatment (Annex I, 3.9.4.6). Analysis of increased 4-HE in the blood would be a direct evidence for this mechanism, but such data is not available.

RAC concludes that the data provide no evidence for CYP-induction mediated modulation of oestrogen metabolism and ROS formation.

RAC considers furthermore the following alternative mechanisms:

- Increase in oestrogen:progesterone ratio

Indirect senescent or chemically-induced imbalance in sex steroid hormones in the ovary leads to decrease of both oestrogen and progesterone and a status similar to a high oestrogen status may manifests by persistent oestrous in vaginal cytology, an atrophic ovary with cystic atretic follicles, lack/few corpora lutea, cornification of the cervix/vagina mucosa, and/or squamous metaplasia of endometrial epithelial cells. Atypical precancerous hyperplasia may be increased (Yoshida *et al.,* 2015; Cruz *et al.,* 2017). For sedaxane no oestrous cycle staging study with vaginal lavage at regular time intervals is available, only the 2-years study where for the animals dying between 52 and 104 weeks in the histopathology re-evaluation persistent oestrous was virtually absent (Annex I, 3.9.4.11). In this study, as compared to the control group, no increase in cornification, metaplasia, precancerous hyperplasia, was recorded, and no decrease in corpora lutea and no ovary atrophy was reported, but an increase in atretic follicles was apparent for the animals dying from 52-104 weeks (30/63, 49/63, 56/61 for control, 1200 ppm, 3600 ppm, respectively). In the 2-generation reproduction study, ovarian atrophy and decrease in corpora lutea was noted. Importantly for clarification, no oestrous-cycle-sensitive hormone measurements on oestrogen:progesterone ratio are available.

RAC considers that the data available do not allow a firm conclusion on the role of this MoA due to insufficient data.

- Modulation of oestrogen excretion

A decrease in oestrogen excretion and related increase in oestrogen blood levels can be related to test substance ADME (Yoshida *et al*., 2015; Sanders *et al.,* 2016; Mungenast *et al.,* 2016) and oestrogen metabolism and excretion might be modulated *in situ*. Sedaxane does reach the uterus as seen in the toxicokinetic studies, but no specific data are available, such as oestrogen levels and phase 2 enzyme induction and activity.

Therefore, no conclusions are possible on this MoA.

- Increased *in situ* aromatase

According to Yoshida *et al.* (2015), this is a mechanism with no evidence so far in rodents, but human relevance has been demonstrated with increased protein and mRNA expression for aromatase (and related *in situ* oestrogen production) in epithelial stromal cells in endometrial carcinomas (Watanabe *et al.* 1995). Aromatase is a key factor for mammary carcinogenesis.

Based on the tumour profile of sedaxane (tumour shift with decrease in mammary tumours) such pathway is unlikely.

- Cytotoxicity / regenerative cell proliferation

Chronic cytotoxicity with subsequent regenerative cell proliferation is considered a mode of action by which tumour development can be enhanced. For sedaxane no persistent inflammation/cytotoxicity and regenerative hyperplasia is evident in the repeated dose and carcinogenicity studies. Carcinogenesis due to excessive toxicity and necrosis that would trigger regenerative proliferation is unlikely as there is no histopathological hint for this mechanism. Sedaxane is not irritating and apart from body weight reduction, no clinical signs indicative of excess toxicity was evident in the 2-years study.

RAC concludes that the data do not support a cytotoxicity-mediated MoA.

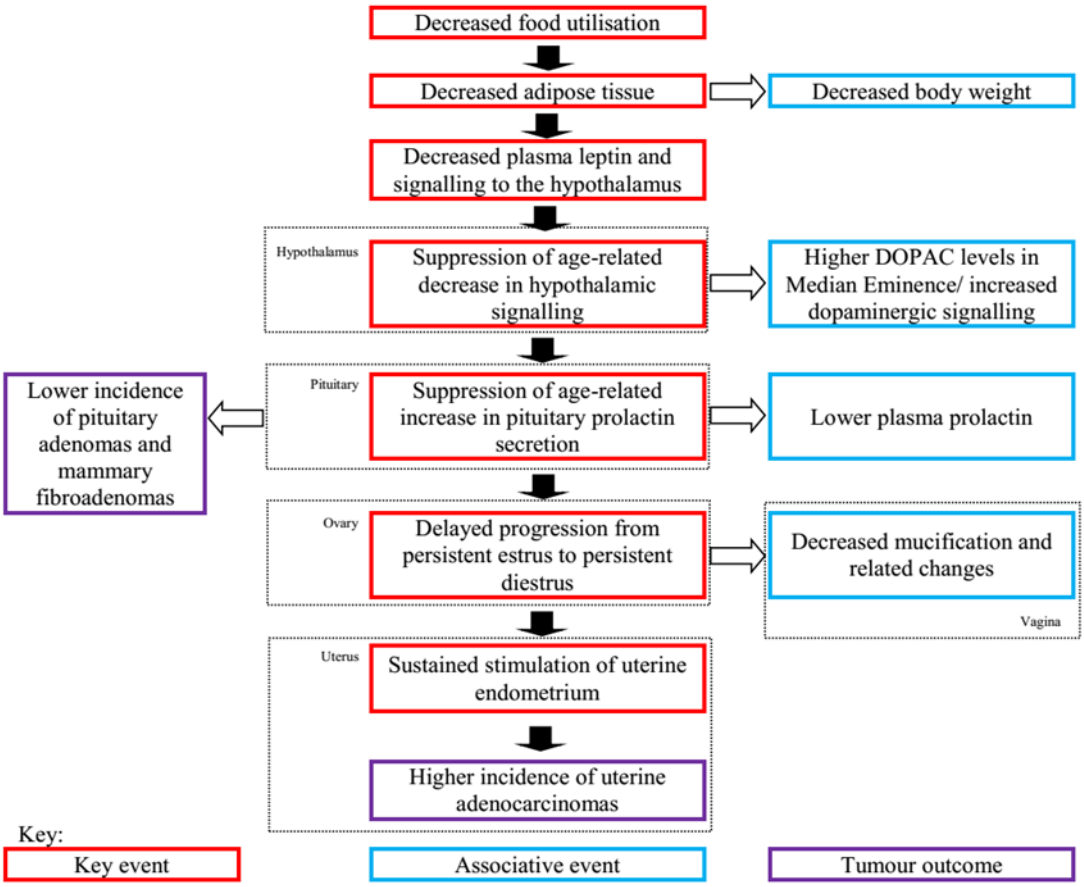
- Inhibition of Succinate Dehydrogenase Inhibition

Concerning alternative MoA, the dossier submitter raised the possibility that the molecular mechanism of tumour formation might be SDH inhibition. This idea was based on the observation, that the SDHI isopyrazam also induced uterine adenocarcinoma at a comparable dose level and this was also associated with significant loss of body weight. Recently, Bénit *et al.* (2018) published that SDHIs readily inhibited the human enzyme of respiratory chain complex II. This is seen as a concern because human germline mutations in one of the four genes encoding SDH subunits have been observed to result in different types of cancer, and epigenetic modifications due to long-term succinate accumulation rather than random mutations seem associated with this. RAC notes that the carcinogenicity study on isopyrazam is not subject of the current assessment, which hampers the conclusion on such association. RAC further notes that other SDHI have been assessed by RAC in the past. While apparently the liver and thyroid is a common target of these substances (in general liver is a frequent target of toxic compounds and the observation therefore might be of unspecific nature), uterine tumours as observed for sedaxane and isopyrazam are not a common effect. No specific data are available to analyse a relevance of succinate accumulation acting as an oncometabolite.

RAC concludes that SDHI as the relevant MoA cannot be assessed.

In summary, a prediction of an alternative MoA is not possible and the MoA remains undefined (see decision tree in Yoshida *et al.,* 2015).

Figure: *Proposed MoA for uterine tumours in rats*



In conclusion on the MoA for uterine adenocarcinoma*, RAC considers that the available data on the MoA of uterine adenocarcinoma are insufficient to support the by the applicant-postulated MoA. The remaining uncertainties are considerably high, such that no human relevance assessment seem warranted. This conclusion is in line with the dossier submitter. Regarding alternative MoA, a prediction is not possible and the MoA remains undefined.*

Comparison with the criteria

Carcinogenic potential of sedaxane has been observed with increased incidences of three types of tumours in two species: malignant uterine tumours and benign thyroid tumours in rats, higher incidences of hepatocellular adenomas in male rats and in male mice and hepatocellular carcinomas in male mice.

The dossier submitter considered that liver tumours observed in male rats and male mice at high dose levels as well thyroid adenomas observed in male rats at high dose levels do not trigger classification for carcinogenicity taking into account that an underlying CAR-mediated MoA is substantiated by the available data, the tumour outcome

considered of limited relevance to humans. As regards to uterine tumours, in the absence of an established MoA, classification for carcinogenicity is warranted. While these tumours are malignant and also observed in one structurally similar compound, the DS considered classification as Carc. 2; H351 as appropriate since the uterine tumours were limited to a very high dose level in a single species.

According to Regulation (EC) No 1272/2008 a substance is classified for carcinogenicity, *Category 1 - Known or presumed human carcinogens on the basis of epidemiological and/or animal data.*

- *Category 1A, known to have carcinogenic potential for humans, classification is largely based on human evidence, or*
- *Category 1B, presumed to have carcinogenic potential for humans, classification is largely based on animal evidence.*

Category 2 - Suspected human carcinogens on the basis of evidence obtained from human and/or animal studies, but which is not sufficiently convincing to place the substance in Category 1A or 1B, based on strength of evidence together with additional considerations.

RAC is of the opinion that classification in category **Carc. 1A is not warranted**. According to the CLP criteria for carcinogenicity Category 1A, *known to have carcinogenic potential for humans*, classification is largely based on human evidence. For sedaxane, no information on carcinogenicity in humans is available.

According to the CLP criteria (Annex 3.6.2.2.3) for Category 1B *"sufficient evidence of carcinogenicity", a causal relationship has been established between the agent and an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in (a) two or more species of animals or (b) two or more independent studies in one species carried out at different times or in different laboratories or under different protocols. An increased incidence of tumours in both sexes of a single species in a well conducted study, ideally conducted under Good Laboratory Practices, can also provide sufficient evidence […]".*

Placing of a substance in category 2 is done on the "*basis of evidence obtained from human and/or animal studies, but which is not sufficiently convincing to place the substance in Category 1A or 1B based on strength of evidence together with additional considerations. Such evidence may be derived either from limited evidence of carcinogenicity in human studies or from limited evidence of carcinogenicity in animal studies".*

Based solely on a combination of benign and malignant neoplasms in two species, a classification as Category 1B could be argued. However, there were several additional factors that were considered by RAC when assessing the overall level of concern:

- Background incidences

For malignant uterine tumours in rats, control incidences were zero and although the top dose tumour incidence with 17% was just inside the HCD range (0-19%), the distribution

of HCD (mean of 7%) indicate that the effects are treatment-related.

The higher incidences of hepatocellular adenomas (10% vs 2% in control), thyroid follicular cell adenomas (15% vs 6% in control) and thyroid adenoma/carcinoma combined (17% vs 6% in control) in males at the top dose in rats clearly exceeded the concurrent control incidences and also the HCD from the testing laboratory.

Considering the background variability, the incidence of liver tumours in male mice are of minor concern. But for carcinoma, the incidence in the high dose (20%) exceeded the concurrent (10%) and historical control data (6-10%).

- Multi-site responses

In male rats increased incidences of both liver and thyroid adenoma were observed. While in female rats and male mice only one organ was affected, uterus and liver respectively.

- Progression to malignancy

Malignant tumours, uterine adenocarcinomas, were increased at the top dose in rats. There was no increase of adenoma incidence or pre-neoplastic lesions. In male rats, liver tumour were limited to adenoma with no progression to malignancy, however in male mice both hepatocellular adenomas and hepatocellular carcinomas incidences were increased at the top dose. For the thyroid only benign adenoma were increased in male rats at the top dose.

- Single or both species

Uterine tumours were only observed in rats. No uterine lesions, including pre-neoplastic lesions or non-neoplastic lesion were observed in any other tested species in the repeated dose studies (mice, dogs), which decreases the concern. Liver tumours were observed in two species, rats and mice. For thyroid tumours only male rats were affected.

- Single or both sex

Tumour types were only observed in one sex. Uterine tumours are obviously limited to females. Liver and thyroid tumours were only observed in males.

- Confounding effects of excessive toxicity

The malignant uterine tumours, and thyroid adenoma, and liver adenoma in rats occurred at doses with markedly reduced body weight (gains). Animals treated at the tumorigenic high dose of 3600 ppm showed a consistent and lower body weight and weight gain compared to their respective controls throughout the treatment period. The reduced cumulative body weight gain throughout the study in the high dose represented a maximum of 23.5% decrease in males and 49.6% decrease in females at termination (reduction of terminal body weight in females by 37%). However, RAC considers the MTD not exceeded and the findings relevant for classification and labelling. No other signs of severe toxicity were apparent and there is no general (causal) association of

body weight reduction with higher tumour incidences. According to the CLH dossier, in addition it is considered that the structural analogue isopyrazam also induced increased incidences of uterine adenocarcinoma in rats (EFSA, 2012). This may indicate a specific effect.

For the liver adenoma and carcinoma in mice, no excessive toxicity was apparent.

- Reduced tumour latency

There was no evidence of reduced latency for any kinds of tumours. For uterine tumours, control incidence was zero.

- Mode of action and its relevance for humans

According to the EU specialised experts (1999), classification for thyroid tumours in rodents was not recommended for non-genotoxic substances causing thyroid tumours mediated by UDP glucuronyltransferase (UGT) induction. For thyroid tumours, RAC concludes that the sequence of events for the MoA proposed by the DS, i.e. an induction of the UDP glucuronyltransferase (UDPGT) leading to decrease in serum T4 and T3 levels and a compensatory increase in TSH that would in turn result in thyroid hyperplasia and tumours, is overall supported, however uncertainties remain as not all events were fully demonstrated. Some alternative MoAs have been ruled out, but not all. However, tumours were benign and only observed in rats in the high dose level, and RAC concludes that these thyroid adenomas do not warrant classification for carcinogenicity taking into account that a CAR-mediated MoA via UGT induction is likely based on the available data. Such MoA is considered less relevant for humans.

Concerning liver tumours in rats and mice, RAC concludes that the liver adenomas in rats and mice together with the carcinoma in mice most likely are caused by a CAR-mediated MoA. However, still significant uncertainties remain, as the data not sufficiently support this hypothesis, mainly as no assay in CAR-Knock-Out mice has been performed, and in the human hepatocyte assay on cell proliferation, only one human donor has been used in order to demonstrate that the adverse outcome would not be relevant for humans. The liver tumours therefore are considered in the overall weight-of-evidence assessment.

As regards to the uterine tumours, in the absence of an established MoA, RAC concludes that sedaxane warrants classification for carcinogenicity. These tumours are malignant and endometrial cancer is considered highly relevant for women, both in humans and in rodents this type of cancer is an oestrogen sensitive lesion. The mode of action of sedaxane has not been conclusively investigated.

RAC considers the uterine neoplastic lesions together with the remaining uncertainty related to the missing or insufficient mechanistic data package for the liver carcinoma in mice. Uterine adenocarcinoma were reported only in the top dose of one species, accompanied by marked decrease in body weight gain. In addition, sedaxane is unlikely to be genotoxic. RAC considers therefore that classification in Category 1B is not justified and overall pattern of effects justifies downgrading classification.

In a weight-of-evidence approach, RAC agrees with the dossier submitter’s proposal and

recommends **classification of sedaxane as Carc. 2; H351 (suspected of causing cancer).** As it has not been proven that no other routes of exposure cause the hazard, the route of exposure should not be stated.

10.10 Reproductive toxicity

10.10.1 Adverse effects on sexual function and fertility

Table 36: Summary table of animal studies on adverse effects on sexual function and fertility

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
Two generation reproduction (one litters) OECD 416 (2001) GLP Oral (continuous in diet) Rat, Han Wistar (HanRcc:WIST) 25/sex/group	Sedaxane (SYN524464, batch no. SMU6LP006/MILLED, purity 95.3%) 0, 200, 500 or 1500 ppm Vehicle: laboratory animal diet	***Parental toxicity*** 1500 ppm (120 mg/kg bw/day – F0 males pre-pairing) **P:** In females, mean body weight was significantly reduced (less than 10%) in the pre-pairing, gestation and lactation period. Decreased body weight gain during prepairing (days 1-70, 16%), during gestation (days 0-21, 16%) and lactation (↓day1-21, 12%); ↓ food consumption pre-pairing: males weeks 1-4 (days 15-16, 5%), females throughout (days 43-44, 10.5%); ↓ food consumption gestation days 0-11 (days 4-7, 10%) and throughout lactation (days 5-7, 13%); ↑ absolute thyroid weight males (~19%); ↑ liver weight adjusted for body weight (males 22%, females 30%); ↑ incidence and severity of centrilobular hypertrophy (males 20/25 severity 1.3 cf. controls 13/25 severity 1.1: females 10/25 severity 1.3 cf. controls 0/25); ↓ absolute ovary weight (~14%), ↓ number of corpora lutea (15%) and ↓ number of antral follicles (13%) ↑ females in lactational diestrus at termination (12/25 cf. 7/25 controls) **F1:** In females, mean BW was significantly reduced (less than 10%). ↓ body weight gain females pre-pairing (days 1-91, 6% not stat. sig.); ↓ food consumption pre-pairing females throughout (days 43-45, 6.5%); ↓ food consumption gestation (days 4-7, 14.5%) and lactation (days 7-9, 10%); ↑ liver weight adjusted for body weight (males 21%, females 42%); ↑ incidence and severity of centrilobular hypertrophy (males 22/25 severity 1.1 cf. controls 10/25 severity 1.0: females 17/25 severity 1.1 cf. controls 0/25); ↑ incidence and severity of thyroid follicular hypertrophy (males 9/25 severity	Anonymous (2010), final report amendment 2, Anonymous (2013) *Annex I. 3.10.1.1*

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
		1.2 cf. controls 5/25 severity 1.0); ↓ ovary weight (~ 19.5% absolute, ~ 15.5% adjusted), ↓ number of corpora lutea (12.3 cf. 17.2 controls), ↓ number of antral follicles (5.2 cf. 10.8 controls), ↓ uterus weight (absolute and adjusted) and ↑ females in lactational diestrus at termination (20/25 cf. 8/25 controls). Liver changes considered adaptive. 500 ppm (41mg/kg bw/day – F0 males pre-pairing) P: ↑ liver weight adjusted for body weight (males 9%) F1: ↑ liver weight adjusted for body weight (males 6%) Small magnitude of change and absence of associated histopathology indicate no adverse effect of treatment. 200 ppm (16 mg/kg bw/day – F0 males pre-pairing) P: No treatment-related effects F1: No treatment-related effects **NOAEL parental toxicity: 41 mg/kg bw/day** ***Reproductive toxicity*** 1500 ppm (120 mg/kg bw/day – F0 males pre-pairing) P and F1: Reproductive performance was not affected by treatment. **NOAEL reproductive toxicity: 120 mg/kg bw/day** ***Offspring toxicity*** 1500 ppm (120 mg/kg bw/day) F1: ↓ body weight from PND 14 (10% day 28); ↑ time to vaginal patency (34.2 days cf. 32.5 although no difference in body weight at time of patency); ↑ liver weight adjusted for body weight (14% males, 11% females) F2: ↑ anogenital distance females (8%) small magnitude ↓ body weight from PND 14 (9% day 21); ↑ liver weight adjusted for body weight (14% males, 13% females); 500 ppm (41 mg/kg bw/day) F1: No adverse effects F2: No adverse effects **NOAEL offspring toxicity: 41 mg/kg bw/day**	

Table 37: Summary table of human data on adverse effects on sexual function and fertility

Type of data/report	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
No evidence of adverse effects on sexual function or fertility in humans				

Table 38: Summary table of other studies relevant for toxicity on sexual function and fertility

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
No relevant studies				

10.10.2 Short summary and overall relevance of the provided information on adverse effects on sexual function and fertility

Sedaxane was evaluated for its potential to cause effects on sexual function and fertility in adults and on development of the offspring in a two-generation reproduction toxicity study in Wistar rat (Anonymous, 2010; final report amendment, Anonymous, 2013 *Annex I. 3.10.1.1*) conducted according to the current OECD test guideline (OECD 416, 2001) including sensitive endpoints to investigate endocrine disruption properties.

Parental systemic toxicity in the high-dose group (120mg/kg bw/d) consisted of reductions in food consumption, body weights in females in both generations and in the F1 males, increased liver weights and hepatocellular hypertrophy in both P and F1 sexes, increased thyroid weights and follicular hypertrophy in P and F1 males. At the same level, systemic toxicity observed in pups consisted of decreased body weights and increased liver weights suggesting that developing organism were not more susceptible.

At the high dose level, decreased ovarian weights, reductions in ovarian follicles, an increased number of females in lactational diestrus at sacrifice, delayed vaginal patency in F1 female offspring, slightly increased ano-genital distance in F2 female offspring were observed in the presence of general toxicity. Some of those effects could be secondary to decreased bodyweight. Ovarian weights, reductions in ovarian follicles could be consistent with the reduction in body weight seen in the 1500 ppm maternal animals and their pups during lactation, and a delay in returning to estrous cycling could result from the prolonged stimulus of nursing by the pups. The time until vaginal patency was reached was statistically significantly increased, but the weight at sexual maturation was similar to control. However, in the absence of any mechanistic studies potential endocrine properties of sedaxane cannot be totally ruled out.

Mating, fertility, gestation, survival indexes and sperm parameters were not impacted at any dose levels. Overall, fertility and reproductive performance were not affected by treatment even at the highest dose level that induced parental toxicity.

Systemic toxicity was observed in parents and offspring at the highest dose of 120 mg/kg bw/day with a NOAEL of 41 mg/kg bw/day. The NOAEL for reproductive toxicity was 120 mg/kg bw/day, the highest dose tested. The same conclusions were reached by the peer review of the pesticide risk assessment of the active substance sedaxane (EFSA 2013).

See Annex I to the CLH report 3.10.1.1.

10.10.3 Comparison with the CLP criteria

In the classification system, reproductive toxicity is subdivided under two main headings: Adverse effects on sexual function and fertility and Adverse effects on development of the offspring.

Adverse effects on sexual function and fertility

Any effect of substances that has the potential to interfere with sexual function and fertility. This includes, but is not limited to, alterations to the female and male reproductive system, adverse effects on onset of puberty, gamete production and transport, reproductive cycle normality, sexual behaviour, fertility, parturition, pregnancy outcomes, premature reproductive senescence, or modifications in other functions that are dependent on the integrity of the reproductive systems.

Fertility and reproduction remained unaffected by treatment even at the highest dose level that induced parental toxicity. The differences in ovarian follicle and corpora lutea counts may be an indirect consequence of high dose effects on pup and maternal body weights and the increased number of dams in lactational diestrus. The effects on female sexual maturation were considered to be equivocal since the changes from controls were marginal and concurrent body weight decrease was observed.

Overall, there was no adverse effect on sexual function and fertility or on development of the offspring in the rat to warrant classification of sedaxane.

This position is consistent with EFSA conclusion. No additional data on sedaxane have become available since the EFSA review was concluded.

10.10.4 Adverse effects on development

Table 39: Summary table of animal studies on adverse effects on development

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
Developmental toxicity OECD 414 (2001) GLP Oral (gavage) Rat, HanRcc:WIST 24 mated	Sedaxane (SYN524464, batch no. SMU6LP006/MILLED, purity 95.3%) Dose levels 0, 25, 100 and 200 mg/kg bw/day Dosing on gestation days 6-20 Vehicle: 0.5% CMC	***Maternal toxicity*** 200 mg/kg bw/day: ↓ body weight gain days 6-13 (44%), 6-21 (17%), adjusted days 6-21 (53%); ↓ food consumption days 9-12 (14%), days 18-21 (11%) 100 mg/kg bw/day: ↓ body weight gain days 6-13 (12.5%), 6-21 (4.5% not stat. sig.) adjusted days 6-21 (13% not stat. sig.); ↓ food consumption days 9-12 (7%) 25 mg/kg bw/day: No effects	Anonymous (2009) *Annex I. 3.10.1.2*

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
females/group		**Maternal NOAEL 25 mg/kg bw/day** ***Developmental toxicity*** 200 mg/kg bw/day: ↓ foetal body weight (4%) stat. sig. for females only and not for combined sexes 100 mg/kg bw/day: No effects **Developmental NOAEL 200 mg/kg bw/day**	
Developmental toxicity OECD 414 (2001) GLP Oral (gavage) Rabbit, New Zealand White (Hra:NZW) 25 mated females/group	Sedaxane (SYN524464, batch no. SMU6LP006/MILLED, purity 95.3%) Dose levels 0, 25, 100 and 200 mg/kg bw/day Dosing on gestation days 7-28 Vehicle: 0.5% CMC	***Maternal toxicity*** 200 mg/kg bw/day: ↓ defaecation (73 occurrences in 14 animals cf. 11 occurrences in 1 control animal), ↓ body weight gain days 7-13 (-33g cf. + 36g controls), 7-29 (+71g cf. +150g controls), day 29 body weight (-1.5%); ↓ food consumption days 10-13 (21%); ↑ liver weight, absolute (13%), adjusted for body weight (14%) 100 mg/kg bw/day: ↑ liver weight, absolute (11%), adjusted for body weight (9%) considered adaptive 25 mg/kg bw/day: No effects **Maternal NOAEL 100 mg/kg bw/day** ***Developmental toxicity*** 200 mg/kg bw/day: ↓ foetal body weight (male, female, and combined) 8.0% to 8.6% lower than the control group values, stat. sig. for females only . Increased incidence of 13th full rib(s). 100 mg/kg bw/day: No effects **Developmental NOAEL 100 mg/kg bw/day**	Anonymous (2010) *Annex I. 3.10.1.3*

Table 40: Summary table of human data on adverse effects on development

Type of data/report	**Test substance,**	**Relevant information about the study (as applicable)**	**Observations**	**Reference**
No evidence of adverse effects on development in humans				

Table 41: Summary table of other studies relevant for developmental toxicity

Type of study/data	**Test substance,**	**Relevant information about the study (as applicable)**	**Observations**	**Reference**
No relevant studies				

10.10.5 Short summary and overall relevance of the provided information on adverse effects on development

The prenatal developmental toxicity of sedaxane was investigated in the rat (Anonymous, 2009 *Annex I. 3.10.1.2*) and in the rabbit (Anonymous, 2010 *Annex I. 3.10.1.3*). Both studies were conducted according to the current OECD test guideline, (OECD 414, 2001). Overall, the results from the two studies showed no potential for sedaxane to induce teratogenic effects, and the foetal NOAEL values were equal to or higher than the maternal NOAEL values.

Maternal toxicity was observed in both species at the highest dose tested of 200 mg/kg bw/day. In the rat, the maternal NOAEL was 25 mg/kg bw/day and in the rabbit the maternal NOAEL was 100 mg/kg bw/day.

Treatment-related maternal effects included decreased bodyweight gain or body weight loss, and decreased food consumption in rat, body weight loss, decreased defecation and food consumption and increased liver weights (absolute and adjusted) in rabbit.

With respect to developmental toxicity, the NOAEL was 200 and 100 mg/kg bw/day in rat and rabbit respectively.

In the rat developmental toxicity, no developmental toxicity (including teratogenicity) was observed up to the highest dose tested. In the rabbit developmental toxicity study, decrease in fetal weights (-9%) and an increased incidence of 13th full ribs were observed in the presence of maternal toxicity.

See Annex I to the CLH report 3.10.1.2 and 3.10.1.3.

10.10.6 Comparison with the CLP criteria

In the classification system, reproductive toxicity is subdivided under two main headings: Adverse effects on sexual function and fertility and Adverse effects on development of the offspring.

Adverse effects on development of the offspring:

Any effect of substances that has the potential to interfere with sexual function and fertility. This includes, but is not limited to, alterations to the female and male reproductive system, adverse effects on onset of puberty, gamete production and transport, reproductive cycle normality, sexual behaviour, fertility, parturition, pregnancy outcomes, premature reproductive senescence, or modifications in other functions that are dependent on the integrity of the reproductive systems.

According to Annex I: 3.7.1.4 of CLP Regulation, the major manifestations of developmental toxicity include (1) death of the developing organism, (2) structural abnormality, (3) altered growth, and (4) functional deficiency.

In rat and rabbit prenatal developmental toxicity studies there was no evidence of foetal death (1) or teratogenicity (2).

In the rat developmental toxicity, no developmental toxicity was observed up to the highest dose tested.

In the rabbit, the only structural abnormality observed was an increased incidence of 13th full ribs (2) in high-dose foetuses. Supernumerary lumbar ribs are considered not to impact foetal survival or health (Solecki R et al., 2013)[1] and are therefore categorized as foetal variations. Supernumerary

[1] Solecki R et al., (2013) Harmonization of description and classification of fetal observations: Achievements and still standing problems. Report of the 7th Workshop on the Terminology in Developmental Toxicology Berlin, 4–6 May 2011 Reprod Toxicol in press

lumbar ribs may be induced by maternal stress (Moore, 2013)[2]. At the same dose level, a 9% decrease in foetal weights (3) was also noticed. Those two effects were observed in the presence of maternal toxicity and are therefore not considered to represent a higher foetal sensitivity.

In light of the nature of the effects observed in high-dose rabbit foetuses and the concurrent maternal toxicity, no classification for developmental toxicity is proposed.

10.10.7 Adverse effects on or via lactation

Table 42: Summary table of animal studies on effects on or via lactation

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, dose levels duration of exposure	Results	Reference
Two generation reproduction (one litters) OECD 416 (2001) GLP Oral (continuous in diet) Rat, Han Wistar (HanRcc:WIST) 25/sex/group	Sedaxane (SYN524464, batch no. SMU6LP006/MILLED, purity 95.3%) 0, 200, 500 or 1500 ppm Vehicle: laboratory animal diet	***Parental toxicity*** 1500 ppm (120 mg/kg bw/day – F0 males pre-pairing) **P:** In females, mean body weight was significantly reduced (less than 10%) in the pre-pairing, gestation and lactation period. Decreased body weight gain during prepairing (days 1-70, 16%), during gestation (days 0-21, 16%) and lactation (↓day1-21, 12%); ↓ food consumption pre-pairing: males weeks 1-4 (days 15-16, 5%), females throughout (days 43-44, 10.5%); ↓ food consumption gestation days 0-11 (days 4-7, 10%) and throughout lactation (days 5-7, 13%); ↑ absolute thyroid weight males (~19%); ↑ liver weight adjusted for body weight (males 22%, females 30%); ↑ incidence and severity of centrilobular hypertrophy (males 20/25 severity 1.3 cf. controls 13/25 severity 1.1: females 10/25 severity 1.3 cf. controls 0/25); ↓ absolute ovary weight (~14%), ↓ number of corpora lutea (15%) and ↓ number of antral follicles (13%) ↑ females in lactational diestrus at termination (12/25 cf. 7/25 controls) **F1:** In females, mean BW was significantly reduced (less than 10%). ↓ body weight gain females pre-pairing (days 1-91, 6% not stat. sig.); ↓ food consumption pre-pairing females throughout (days 43-45, 6.5%); ↓ food consumption gestation (days 4-7, 14.5%) and lactation (days 7-9, 10%); ↑ liver weight adjusted for body weight (males 21%, females 42%); ↑ incidence and severity of	Anonymous (2010), final report amendment 2, Anonymous (2013) *Annex I. 3.10.1.1*

[2] Moore et al; Guidance on classification for reproductive toxicity under the globally harmonized system of classification and labelling of chemicals (GHS).. Crit Rev Toxicol. 2013 Nov;43(10):850-91. doi: 10.3109/10408444.2013.854734. Review

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, dose levels duration of exposure**	**Results**	**Reference**
		centrilobular hypertrophy (males 22/25 severity 1.1 cf. controls 10/25 severity 1.0: females 17/25 severity 1.1 cf. controls 0/25); ↑ incidence and severity of thyroid follicular hypertrophy (males 9/25 severity 1.2 cf. controls 5/25 severity 1.0); ↓ ovary weight (~ 19.5% absolute, ~ 15.5% adjusted), ↓ number of corpora lutea (12.3 cf. 17.2 controls), ↓ number of antral follicles (5.2 cf. 10.8 controls), ↓ uterus weight (absolute and adjusted) and ↑ females in lactational diestrus at termination (20/25 cf. 8/25 controls). Liver changes considered adaptive. 500 ppm (41mg/kg bw/day – F0 males pre-pairing) P: ↑ liver weight adjusted for body weight (males 9%) F1: ↑ liver weight adjusted for body weight (males 6%) Small magnitude of change and absence of associated histopathology indicate no adverse effect of treatment. 200 ppm (16 mg/kg bw/day – F0 males pre-pairing) P: No treatment-related effects F1: No treatment-related effects **NOAEL parental toxicity: 41 mg/kg bw/day** ***Reproductive toxicity*** 1500 ppm (120 mg/kg bw/day – F0 males pre-pairing) P and F1: Reproductive performance was not affected by treatment. **NOAEL reproductive toxicity: 120 mg/kg bw/day** ***Offspring toxicity*** 1500 ppm (120 mg/kg bw/day) F1: ↓ body weight from PND 14 (10% day 28); ↑ time to vaginal patency (34.2 days cf. 32.5 although no difference in body weight at time of patency); ↑ liver weight adjusted for body weight (14% males, 11% females) F2: ↑ anogenital distance females (8%) small magnitude ↓ body weight from PND 14 (9% day 21); ↑ liver weight adjusted for body weight (14% males, 13% females); 500 ppm (41 mg/kg bw/day) F1: No adverse effects F2: No adverse effects **NOAEL offspring toxicity: 41 mg/kg bw/day**	

Table 43: Summary table of human data on effects on or via lactation

Type of data/report	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
No evidence of adverse effects on or via lactation in humans				

Table 44: Summary table of other studies relevant for effects on or via lactation

Type of study/data	Test substance,	Relevant information about the study (as applicable)	Observations	Reference
No relevant studies				

10.10.8 Short summary and overall relevance of the provided information on effects on or via lactation

The two generation study of sedaxane in rats (Anonymous, 2010 and amendement 2 Anonymous, 2013 *Annex I. 3.10.1.1*) has already been described. Systemic toxicity was observed in parents and offspring at the highest dose of 120 mg/kg bw/day with a NOAEL of approximately 41 mg/kg bw/day. The reduction in F1 & F2 pup body weight from post natal day 14 was considered to result from direct consumption of the diet and not to be maternally mediated. There was no indication of impaired nursing behaviour or decreased pup viability during lactation. The results of the study do not indicate any direct, adverse effect on the offspring due to transfer of the chemical via the milk or to the quality of the milk.

See Annex I to the CLH report 3.10.1.1.

10.10.9 Comparison with the CLP criteria

The classification is intended to indicate when a substance may cause harm due to its effects on or via lactation and is independent of consideration of the reproductive or developmental toxicity of the substance. There were no effects to warrant classification of sedaxane for effects on or via lactation.

10.10.10 Conclusion on classification and labelling for reproductive toxicity

Not classified (conclusive but not sufficient for classification)

RAC evaluation of reproductive toxicity

Summary of the Dossier Submitter's proposal

Sedaxane was tested in HanWistar rats in an oral two-generation reproductive toxicity study according to OECD TG 416, and GLP compliant, at 200, 500 or 1500 ppm, equal to

16, 41 or 120 mg/kg bw/d. The test included an investigation of endocrine disrupting properties. Fertility and reproductive performance were not affected by treatment up to the highest dose that induced parental toxicity. No adverse effects were observed that would warrant classification for fertility, and this position of the dossier submitter is in line with the EFSA conclusion. In the F1 and F2 offspring, body weights from PND 14 were reduced, which could reflect toxicity of the test substance by direct intake by the pups, and suggesting a similar sensitivity for the offspring and adults. Similar to parental animals, adjusted liver weights were increased in the F1 offspring at the high dose.

Two developmental toxicity studies according to OECD TG 414, and GLP compliant, are available, one in rats and one in rabbits, both at concentrations of 25, 100, and 200 mg/kg bw/d administered by oral gavage. The results showed no potential for sedaxane to induce foetal death or teratogenic effects. In rabbits, a 13th full rib, considered a foetal variation, and 9% decrease in foetal weights as compared to controls was observed in the presence of maternal toxicity. The dossier submitter did not propose classification for developmental toxicity due to the nature of the effects observed in high-dose rabbit foetuses and the concurrent maternal toxicity.

Comments received during public consultation

No comments were received during the public consultation.

Assessment and comparison with the classification criteria

Sexual function and fertility

In the two-generation reproduction toxicity study, the mid dose of 41 mg/kg bw/d gave the NOAEL for parental toxicity. The high dose of 120 mg/kg bw/d was the NOAEL for toxicity for sexual function and fertility and 41 mg/kg bw/d was the NOAEL for developmental toxicity.

The main effects observed in the parental generations in the high dose included reductions in body weights as compared to controls with changes of generally less than 10% and rarely statistically significant for males, but often significant for females of both generations throughout the study. As compared to controls, the body weight gain reduction in females during the pre-mating period was 15% in P generation, but less than 5% in F1 parental females. Reduced food consumption in females in both P and F1 generations and in P males, increased liver weights and centrilobular hypertrophy in P and F1 animals of both sexes, as well as increased thyroid weights and thyroid follicular hypertrophy in P and F1 males were apparent. In the mid dose, liver weights were only slightly increased by 9% and 6% for males in P and F1 generation, respectively, without histopathological correlates.

Reproductive performance was not affected by treatment at any dose level as parameters such as mating index, fertility indexes, number of pups at birth, or litter size were not different between the treated and control groups.

In the high dose females of the P and F1 generation, effects on the reproductive organs were observed. Absolute (P: -14%; F1: -19.5%) and adjusted ovary weights (F1, -15.5%) were reduced and the reduction was apparent at both ovary sites. The number of corpora lutea and the number of antral follicles were significantly reduced for P and F1 generations. Absolute and adjusted uterus weights were reduced (statistically significant for F1) and the number of females in the lactational dioestrus at the time of termination was increased in P and F1 generations.

Effects on the female reproductive organs may be due to a direct organ-toxic effect or due to primary or secondary – stress-related - endocrine disturbance. The latter could be a result of reduced food consumption and decreased body weight (Everds *et al.,* 2013). The DS suggested that the effects of sedaxane on ovary weights and the decreased number of ovarian follicles could be secondary to decreased body weights, and the delay in returning to oestrous cycling could be related to the prolonged nursing stimulus by the pups. To assess the hypothesis by the DS, a careful analysis of the individual animal data, which is not available to RAC, would be required. RAC notes, however, that the body weight changes were rather mild indicating only mild stress that was unlikely to be the cause of the reduction in uterus and ovary weights and of the decrease in the number of ovarian follicles and corpora lutea. For example, in female rats, a dietary restriction leading to a > 16% decrease in mean body weight compared to controls has been associated with persistent dioestrus, decreased number of corpora lutea, and decreased fertility (likely due to the decreased corpora lutea; Terry *et al.,* 2005). On the basis of minor changes seen in the oestrous cyclicity, decreases of 10 to 15% in body weight gain were considered not to cause adverse effects on sexual function and fertility in the female rat. Studies in rats evaluating the effects of feed restriction have also demonstrated that female body weight must be reduced to approximately 70% of that in controls before the ovary weights will decrease (Chapin *et al*., 1993; Seki *et al.,* 1997). The effects on female body weight observed in the available 2-generation study on sedaxane were not of similar magnitude. In the repeated dose toxicity studies, no findings were recorded that would suggest specific toxicity on reproductive organs. Moreover, the changes observed in the 2-generation study were rather moderate and not associated with compromises in sexual function and fertility.

The mean time to vaginal patency (VP) was increased from day 32.5 to day 34.2 in the F1 offspring at the top dose. In F2 female offspring the mean anogenital distance was statistically significantly increased by 8% as compared to control. No adverse effects on the offspring were observed at the lower doses. In general, a delay in puberty could be related to a general growth retardation as the VP, the primary sexual development landmark for females, can positively correlate with the body weight. However, in the absence of body weight changes, differences of 2.0 days or more is a general indicator for test substance-related toxicity. For sedaxane, the delay of less than 2 days was statistically significant in the F1 pups. The body weights of pups were reduced as compared to controls, but only between PND 14 to 21, and at the time of sexual maturation the body weights of pups were similar compared to control as stated in the CLH dossier (no body weight data at the time of vaginal opening is available in the CLH dossier). No data on VP were recorded for F2 pups, thus it cannot be judged whether

both generations would be consistently affected. In addition, only the high dose in F1 showed the delay in VP. Difference in the mean litter anogenital distance (AGD) of 5% or greater is an indicator of toxicity and the primary landmark for sexual development (the most frequent effects on AGD are observed in response to antiandrogenic agents or 5a-reductase inhibitors) in addition to the balanopreputial separation in males. The 8% sedaxane-associated increase in AGD in F2 female pups is of unclear biological significance. No data were recorded for F1 offspring, no effects were observed in F2 male pups, and the increase in F2 females is only slight.

Overall, it can be stated that the effects on the female sexual maturation and offspring development were only apparent at the top dose without any impact at lower doses where no general systemic toxicity or growth retardation in the offspring was observed. Reproductive performance was not affected at any of the tested dose level. However, RAC notes that in this study a top dose level of 1500 ppm (corresponding to 120 mg/kg bw/d) was administered, which induced only limited parental toxicity. According to OECD TG 416 the highest dose level should be chosen with the aim to induce toxicity but not death or severe suffering, taking into account any existing toxicity data. According to the CLH report, Annex I, 3.10.1, dietary concentrations were based on the results of a single generation study (not included in the CLH report) and long term feeding studies in rats. To compare, in the 2-year chronic study a dietary level of 3600 ppm was applied as the top dose, not inducing severe toxicity, suffering or death. Further, RAC takes note of the study summary of a single-generation dose-range finder reproductive toxicity study[3], with nominal dose levels of 500, 1500, and 3600 ppm for 10 weeks before pairing until weaning of the F1 generation. It was concluded that, based on the results of this single-generation range finder and the 2-year chronic study, the top dose chosen in the two-generation study is too low. This leaves uncertainties on the informative value of the chosen dosing regimen. With reference to the criteria set out in Annex I, 3.7.2.2 of CLP, RAC therefore concludes that **no classification is warranted for sexual function and fertility** based on inconclusive data.

Developmental toxicity

According to the CLH report, Annex I, 3.10.1.3, the dosing regimen of the rabbit developmental toxicity studies was selected based on a range finder tolerability study in non-pregnant (day 0 to 9; 250, 500, 750, 1000 mg/kg bw/d) and in pregnant rabbits (day 7 to 28; 100, 300, 500 mg/kg bw/d). Administration to pregnant rabbits resulted in moribund condition at 500 mg/kg bw/d, body weight loss and reduced food consumption at 300 and 500 mg/kg bw/d, and a trend for increased liver weights was noted at 100, 300, 500 mg/kg bw/d. There was no evidence of developmental toxicity at any dose level.

Based on the range finder results, dose levels of 25, 100, 200 mg/kg bw/d were selected for the main study.

[3] SEDAXANE 769–839 JMPR 2012, *who.int/pesticide-residues-jmpr-database/Document/58*

In rabbits, the NOAELs for maternal and developmental toxicity were the mid dose of 100 mg/kg bw/d. Maternal toxicity encompassed defaecation and increased (adjusted) liver weights. Body weights were not different between the groups, but the weight gain was statistically significantly reduced in the first week of treatment. In the offspring, foetal examination revealed 8-9% lower mean foetal weights at 200 mg/kg bw/d in males, females and combined sexes, being statistically significant in females, and considered treatment-related. On the other hand, such rather slight reductions < 10% in intrauterine growth in the presence of maternal toxicity are of minor concern and do not warrant classification. No effects on intrauterine growth and development were observed at 25 and 100 mg/kg bw/d. At 200 mg/kg bw/d, a decrease in the foetal incidence of unossified #5 and/or #6 sternebrae was within the historical control range and is considered by RAC a normal variation in this species. Statistically significant increase in the foetal incidence of skeletal variations included the 13th full ribs at 200 mg/kg bw/d. RAC considers that the increased incidence of full 13th ribs is a developmental variation and presents no toxicological or teratogenic concern that would warrant classification. In rabbits this variation is common. In the sedaxane-treated groups, there was no major or minor malformations, foetal deaths or functional impairment.

For rats, the dose levels were also selected based on a dose range-finding toxicity study in Han Wistar rats. A dose of 200 mg/kg bw/d resulted in decreased maternal body weight gain and food consumption and was therefore expected to produce some effects on maternal body weight and food consumption without excessive toxicity in the main study (CLH report, Annex I, 3.10.1.2).

Based on the range finder results, dose levels of 25, 100, 200 mg/kg bw/d were selected for the main study.

In the rat study, the NOAEL for maternal toxicity was the low dose of 25 mg/kg bw/d and the developmental NOAEL was the high dose of 200 mg/kg bw/d. Maternal toxicity at 100 mg/kg bw/d constituted of moderate reduction of weight gain (statistically significant only on days 6-13 with 12.5%) and food consumption. Foetal weights of the female offspring were reduced by 4% at the high dose. No developmental toxicity was overt up to the highest dose level.

With reference to the CLP criteria, RAC concludes that no adverse effects on the developing organism, including death, structural abnormalities, altered growth or functional deficiency were associated with the exposure to sedaxane during pregnancy or as a result of parental exposure in developing rats or rabbits that would warrant classification. Therefore, RAC agrees with the dossier submitter that **classification for developmental toxicity is not warranted.**

10.11 Specific target organ toxicity-single exposure

Table 45: Summary table of animal studies on STOT SE

Method, guideline, deviations if any, species, strain, sex, no/group	**Test substance, route of exposure, dose levels, duration of exposure**	**Results**	**Reference**
Acute Neurotoxicity OECD 424 GLP Rat HanRcc:WIST (SPF) 10/sex/dose	SYN524664 Batch No.: SMU6LP006 Purity: 95.3% 0, 30, 250 and 2000 mg/kg Single oral (gavage) dose followed by 16 day observation period	Mortalities: **2000 mg/kg** – 4/10 males and 3/10 females on day 1. Clinical signs: **2000 mg/kg** - Decreased activity /swaying gait (females), weak condition (males & females) – days 1-4. Prostration (1 female) – day 2, hunched posture (1 female) – day 5. **250 mg/kg** - Decreased activity /swaying gait (females), weak condition (females) – days 1-4 Food consumption: **2000 mg/kg -** Reduced in both sexes on day 1. **250 mg/kg** - Reduced in both sexes on day 1. Bodyweight: **2000 mg/kg** – statistically significantly lower on day 8 in both sexes. Bodyweight gain: **2000 mg/kg** –lower in males on day 8. **250 mg/kg** lower in males on day 8. No signs of neurotoxicity in the FOB/MLA assessments. No effects on brain weights and no neurohistopathological lesions.	Anonymous (2009a). *Annex I. 3.11.1.1*

Table 46: Summary table of human data on STOT SE

Type of data/report	**Test substance**	**Route of exposure** **Relevant information about the study (as applicable)**	**Observations**	**Reference**
No human data are available				

Table 47: Summary table of other studies relevant for STOT SE

Type of study/data	**Test substance**	**Relevant information about the study (as applicable)**	**Observations**	**Reference**
No relevant studies				

10.11.1 Short summary and overall relevance of the provided information on specific target organ toxicity – single exposure

In standard single dose oral and dermal toxicity studies there was no evidence of specific target organ toxicity. In the acute inhalation study at the limit concentration of 5.244 mg/l, minimal clinical signs consistent with inhalation of aerosols were seen (bradypnea 3 h from the start of exposure, and rales from 4 h) which had fully recovered by day 3 post exposure. There were no macroscopic findings in the lungs or lung weight changes at necropsy.

In an acute neurotoxicity study (Anonymous, 2009a *Annex I. 3.11.1.1*), rats were dosed orally (by gavage) with up to 2000 mg/kg sedaxane and observed for 16 days. At 2000 mg/kg, 4/10 males and 3/10 females were killed on day 1 in a moribund condition. The remaining animals at this dose level survived until scheduled termination.

Signs of transient general toxicity at the high dose (2000 mg/kg) and in some cases mid dose (250 mg/kg) included lower bodyweights and bodyweight gains, lower food consumption on day 1, weakness, and lower bodyweight gains in males, decreased activity and/or swaying gait, which had disappeared by day 7 post dosing. In addition, a single incidence of prostration and hunched posture was recorded in females at 2000 mg/kg on day 2 and 5, respectively. Clinical effects in the FOB (slightly reduced activity decreased rearing, lower body temperature, lower grip strength and decreased locomotor activity) were observed in animals at 2000 mg/kg on day 1 only and are considered to reflect the clinical condition of the animals at this time.

There were no specific signs of neurotoxicity observed, there were no treatment related effects on brain weights and no neurohistopathological lesions present at microscopic examination.

See Annex I to the CLH report 3.1-3.3 and 3.11.

10.11.2 Comparison with the CLP criteria

Specific target organ toxicity (single exposure) is defined as specific, non-lethal target organ toxicity arising from a single exposure to a substance. All significant health effects that can impair function, reversible and irreversible, immediate and/or delayed effects are considered.

STOT-SE categories 1 and 2 are assigned on the basis of clear evidence of significant or severe toxicity to a specific target organ arising from a single exposure to a substance. STOT-SE category 3 is assigned for the transient effects of respiratory tract irritation and narcotic effects.

There is no evidence from single or repeated dose studies (including acute neurotoxicity studies) of any clinical signs or other adverse effects indicative of specific target organ toxicity following single exposures to sedaxane at non-lethal doses meeting the classification criteria for specific target-organ toxicity category 1, 2 or 3 no classification is proposed.

10.11.3 Conclusion on classification and labelling for STOT SE

Not classified (conclusive but not sufficient for classification)

RAC evaluation of specific target organ toxicity – single exposure (STOT SE)

Summary of the Dossier Submitter's proposal

In the standard single dose oral and dermal acute toxicity studies there was no evidence of specific target organ toxicity. In the acute inhalation toxicity study, minimal clinical signs consistent with aerosol inhalation were observed at the limit concentration of 5.244 mg/L (bradypnea 3 h from the start of exposure, rales as of 4 h) which fully recovered by day 3. There were no macroscopic findings or weight changes of the lungs at necropsy.

Sedaxane was tested in an acute neurotoxicity study in HanRcc:WIST rats (10/sex/dose) according to OECD 424 (GLP) at dose levels of 0, 30, 250, and 2000 mg/kg bw by gavage. Transient clinical signs of generalised toxicity were noted at non-lethal doses and the treatment did not produce any evidence of neurotoxicity, effects on brain weights, and there were no treatment-related neurohistopathological findings.

The dossier submitter concluded that there is no evidence from single or repeated dose studies (including acute neurotoxicity studies) of any clinical signs or other adverse effects indicative of specific target organ toxicity following single exposures to sedaxane at non-lethal doses meeting the classification criteria for specific target-organ toxicity category 1, 2 or 3. Therefore, no classification was proposed for STOT SE.

Comments received during public consultation

No comments were received.

Assessment and comparison with the classification criteria

In the standard single dose studies, oral and dermal routes, there was no evidence of specific target organ toxicity. At non-lethal oral doses clinical observations included ruffled fur, hunched posture, slight sedation, poor coordination and ventral recumbency, resolving symptom free after few days. No clinical signs of systemic toxicity were noted after dermal dosing. After acute inhalation exposure of the limit dose of 5.244 mg/L there were no macroscopic pathology findings. Transient clinical signs comprised effects on breathing (bradypnea and rales), decreased spontaneous activity, hunched posture and ruffled fur, and transient, slight retardation in bodyweight gain or marginal to moderate bodyweight loss.

In the acute neurotoxicity study only slight clinical signs of general toxicity were observed. Treatment-related findings were noted at 250 and 2000 mg/kg bw, and included reduced activity, decreased rearing and a decreased body weight in males and females at 2000 mg/kg bw and a lower body weight gain in males at 250 and 2000

mg/kg bw, and a decreased food consumption in males and females at 250 and 2000 mg/kg bw. These findings were transient as there was no evidence of treatment related findings subsequent to day 8 of the study. Clinical signs observed in the FOB at 2000 mg/kg bw on day 1 seem to reflect the actual clinical condition of the animals. The treatment did not produce any evidence of neurotoxicity, effects on brain weights, and there were no treatment-related neurohistopathological findings.

Classification for STOT SE category 3 (respiratory tract irritation and narcotic effects) is primarily based on human data, if available, animal data can be included in the evaluation. No human data are available. Taking into account available acute toxicity (inhalation) animal data as described above, RAC concludes that the criteria for classifying in STOT SE 3 for transient target organ effects as provided in Annex I, 3.8.2.2.1 and 3.8.2.2.2 of CLP are not met.

According to the criteria, STOT SE Categories 1 and 2 are assigned on the basis of findings of "significant" or "severe" toxicity. "Significant" means changes which clearly indicate functional disturbance or morphological changes which are toxicologically relevant. "Severe" effects are generally more profound and serious and of considerable adverse nature with significant impact on health. Based on available animal studies as outlined above and the classification criteria according Annex I, 3.8.2.1. and applicable guidance value ranges for single exposure provided in table 3.8.2 of CLP, there is no evidence for signs of specific non-lethal target organ toxicity meeting the classification criteria. **RAC concludes that classification of sedaxane for STOT-SE is not warranted.**

10.12 Specific target organ toxicity-repeated exposure

Table 48: Summary table of animal studies on STOT RE

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
Oral Studies			
28-day comparative toxicity study Rat: HsdBrlHan: Wistar Not a guideline study Conducted to GLP standards,	SYN508210 (*trans* isomer) Lot: KI 7193/5 Purity: not confirmed SYN508211 (*cis* isomer) Lot: KI-7245/5 Purity not	**NOTE:** the chemical named SYN524464 in this study was a 1:1 mix of the *cis* and *trans* isomers. The active substance Sedaxane (SYN524464 is a mixture of a minimum 81% *trans* and a maximum 15% *cis* isomers). **SYN508211** **5000 ppm (equivalent to 438,2 and 435.8 mg/kg bw/day for males and females respectively):** *Body weights:* ↓ throughout the study; at termination, ↓ 17% for males and ↓14% females. *Food consumption:* ↓ for males and females, especially	Anonymous, 2010 *Annex I. 3.12.1.1*

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
except protocol, procedures and report not audited; liver biochemistry analyses were not performed in a GLP facility. 5/sex/group (a further 3/sex/group were included for toxicokinetics – not reported here).	confirmed SYN524464 (1:1 mixture of the *cis* and *trans* isomers) Vehicle: diet 0, 500, 2000, 5000 ppm 28 days duration	during week1. *Clinical chemistry:* ↑protein, cholesterol and triglycerides. Bilirubin ↑ females. ɤGT ↑ males and females. *Liver biochemistry:* Pentoxyresorufin (PROD) markedly ↑male (x46) and females (x9). Slight ↑EROD activity. Slight ↑ hydroxylation of 16 βhydroxytestosterone and ↓ 16 α and 2α testosterone hydroxylase activity in males, and ↑ in females. ↑ in 2β and 6β testosterone hydroxylase activity in females. *Liver weights*: absolute and relative weights ↑for males and females. *Uterus with cervix weight:* statistically significantly ↓ *Liver histopathology:* ↑centrilobular hypertrophy in males and females and increase in smooth endoplasmic reticulum and condensed cells. *Immunoblotting:* ↑CYP 2B and CYP 3A **2000 ppm (equivalent to 182.7 and 179.6 mg/kg bw/day for males and females respectively) :** *Body weights:* slightly ↓, statistically significant for males at termination (↓7%) *Food consumption:* slightly ↓, especially during week 1. *Clinical chemistry:* ↑ cholesterol and triglycerides. *Liver biochemistry:* Pentoxyresorufin (PROD) markedly ↑ males (x52) and females (x7). Slight ↑ EROD activity. Slight ↑ hydroxylation of 16 βhydroxytestosterone and ↓ 16 α and 2α testosterone hydroxylase activity in males, and ↑ in females. ↑ in 2β and 6β testosterone hydroxylase activity in females. *Liver weights*: absolute and relative weights ↑for males and females. *Immunoblotting:* ↑CYP 2B and CYP 3A **500 ppm (equivalent to 45.9 and 47.6 mg/kg bw/day for males and females respectively):** Clinical chemistry: triglycerides and cholesterol slightly ↑ Liver biochemistry: Pentoxyresorufin (PROD) ↑ Males (x5.50 and females (x2.3). Slight ↑ EROD activity. Slight ↑ hydroxylation of 16 βhydroxytestosterone and ↓ 16 α and 2α testosterone hydroxylase activity in males, and ↑ in females. ↑ in 2β and 6β testosterone hydroxylase activity in	

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
		females. *Immunoblotting:* ↑CYP 2B and CYP 3A	
		SYN508210 **5000 ppm (equivalent to 438.2 and 384.3 mg/kg be/day for males and females respectively):** *Body weights:* ↓ throughout the study; at termination, ↓ 15% for males and ↓17% females. *Food consumption:* ↓ for males and females, especially during week1. *Haematology*: Prothrombin time ↑males *Clinical chemistry:* ↑protein, cholesterol and triglycerides. Bilirubin ↑ males. ɤGT ↑ females. *Liver biochemistry:* Pentoxyresorufin (PROD) markedly ↑ in males (x118) and females (x12). Slight ↑ EROD activity in females. Slight ↑ hydroxylation of 16 βhydroxytestosterone and ↓ 16 α and 2α testosterone hydroxylase activity in males, and ↑ in females. ↑ in 2β and 6β testosterone hydroxylase activity in females. *Liver weights*: absolute and relative weights ↑for males and females. *Liver histopathology:* ↑centrilobular hypertrophy in males and females and increase in smooth endoplasmic reticulum and condensed cells. *Immunoblotting:* ↑CYP 2B and CYP 3A **2000 ppm (equivalent to 187.4 and 177.1 mg/kg bw/day for males and females respectively):** *Body weights:* slightly ↓ (not statistically significant). *Food consumption:* slightly ↓, especially during week 1. *Haematology*: Prothrombin time ↑males *Clinical chemistry:* ↑protein, cholesterol and triglyceride *Liver biochemistry:* Pentoxyresorufin (PROD) ↑ in males (x64) and females (x18). Slight ↑ hydroxylation of 16 βhydroxytestosterone and ↓ 16 α and 2α testosterone hydroxylase activity in males, and ↑ in females. ↑ in 2β and 6β testosterone hydroxylase activity in females. *Liver weights*: absolute and relative weights ↑for males and females. *Liver histopathology:* ↑centrilobular hypertrophy in males	

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
		Immunoblotting: ↑CYP 2B and CYP 3A **<u>500 ppm: (equivalent to 47 and 48.4 mg/kg be/day for males and females respectively):</u>** *Clinical chemistry:* triglycerides slightly ↑ *Liver biochemistry:* Pentoxyresorufin (PROD) ↑ males (x2). Slight ↑ hydroxylation of 16 βhydroxytestosterone and ↓ 16 α and 2α testosterone hydroxylase activity in males, and ↑ in females. ↑ in 2β and 6β testosterone hydroxylase activity in females. *Immunoblotting:* ↑CYP 2B and CYP 3A **<u>SYN524464</u>** **<u>5000 ppm (equivalent to 444.6 and 428.1 mg/kg bw/day for males and females respectively):</u>** *Body weights:* ↓ throughout the study; at termination, ↓ 16% for males and ↓12% females. *Food consumption:* ↓ for males and females, especially during week1. *Clinical chemistry:* ↑protein, cholesterol and triglyceride. Bilirubin ↑ males and females. ɤGT ↑ males and females. *Liver biochemistry:* Pentoxyresorufin (PROD) ↑ Males (x50 and females (x12). Slight ↑ EROD activity in females. Slight ↑ hydroxylation of 16 β hydroxytestosterone and ↓ 16 α and 2α testosterone hydroxylase activity in males, and ↑ in females. ↑ in 2β and 6β testosterone hydroxylase activity in females. *Liver weights*: absolute and relative weights ↑for males and females. *Liver histopathology:* ↑centrilobular hypertrophy in males and females and increase in smooth endoplasmic reticulum and condensed cells. *Immunoblotting:* ↑CYP 2B and CYP 3A **<u>2000 ppm (equivalent to181.2 and 181.1 mg/kg bw/day for males and females respectively):</u>** *Body weights:* slightly ↓ (not statistically significant). *Food consumption:* slightly ↓, especially during week 1. *Clinical chemistry:* ↑protein, cholesterol and triglyceride *Liver biochemistry:* Pentoxyresorufin (PROD) ↑ Males (x73 and females (x10). Slight ↑ EROD activity in females. Slight ↑ hydroxylation of 16 βhydroxytestosterone	

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
		and ↓ 16 α and 2α testosterone hydroxylase activity in males, and ↑ in females. ↑ in 2β and 6β testosterone hydroxylase activity in females. *Liver weights*: absolute and relative weights ↑for males and females. *Immunoblotting:* ↑CYP 2B and CYP 3A **500 ppm (equivalent to 47.5 and 46.7 mg/kg bw/day for males and females respectively):** *Clinical chemistry:* triglycerides slightly ↑ *Liver biochemistry:* Pentoxyresorufin (PROD) ↑ Males (x3.5 and females (x2). Slight ↑ hydroxylation of 16 β hydroxytestosterone and ↓ 16 α and 2α testosterone hydroxylase activity in males, and ↑ in females. ↑ in 2β and 6β testosterone hydroxylase activity in females. *Immunoblotting:* ↑CYP 2B and CYP 3A	
90-day oral Rat: Han Wistar (Crl:WI (Han)) OECD 408 (1998) 2001/59/EC B 26 GLP 10/sex/group	SYN524464 Lot: SMU6LP006 95.3% pure (83% trans isomer, 12.3% cis isomer) Vehicle: diet 0, 300, 2000, 4000 ppm 90 days duration	**4000 ppm (325.1 mg/kg bw/day):** *Bodyweights:* ↓ males from day 7 (9% day 28 and 11% day 91) and ↓ females (10% day 63 and 12% day 91). *Food consumption:* consistently ↓ throughout the study (e.g. ↓ approx. 14% for both sexes week 4 and ↓5% for males and ↓17% females at week 13. *Food utilisation:* ↓ weeks 5-8 for females. *FOB (week 12-13):* females showed ↑ hunched posture and piloerection. ↓ fore- and hind limb grip strength. *Haematology:* ↑prothrombin time in males (14%) and females (9%). A 5%↓ in RBC in males was considered not related to treatment. *Clinical chemistry:* ↑ in ɤ glutamyl transferase (34% males, 67% females); ↑cholesterol (50% females); ↑ triglycerides (59% males) and ↑ total protein (4% males). ↓AST (18% females). *Organ weights:* relative liver weights were ↑ (36% males and 50% females). Absolute liver weights were also ↑ (23% males and 29% females). *Histopathology:* centrilobular hepatocyte hypertrophy was evident in all animals, occasionally accompanied by hepatocyte was seen in 5/10 males and 1/10 females. **2000 ppm (168 mg/kg bw/day):** *Bodyweights:* ↓ females from day 70; ↓10% day 91.	Anonymous, 2009 *Annex I. 3.12.1.2*

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
		FOB (week 12-13): ↓ fore limb grip strength. *Organ weights:* relative liver weights were ↑ (15% males and 25% females). **<u>300 ppm (24.8 mg/kg bw/day):</u>** No adverse effects. **NOAEL: 300 ppm (24.8mg/kg bw/day and 28.3mg/kg bw/day) for males and females respectively.**	
90 Day Dietary Neurotoxicity Study. OECD 424 (1997): 67/548//EEC B.43 (2000): Rat: HanRcc: WIST (SPF) GLP 12/sex/group	SYN524464 Lot: SMU6LP006 95.3% pure (83% trans isomer, 12.3% cis isomer) Vehicle: diet 0, 300, 1000, 4000 ppm 92 days duration	**<u>4000 ppm (260 and 302.9 mg/kg bw/day for males and females respectively):</u>** *Body weights:* body weights were generally↓in males and females throughout the study: at the end of treatment body weight gains were ↓ 18.1% males and ↓24.8% females. *Food consumption:* Food consumption was ↓ throughout the study. During week 1 food consumption was ↓ 26.5% and 34.8%, respectively for males and females. *FOB*: ↓ Mean locomotor activity total distance values considered reflective of general toxicity (decreased bw and food consumption). *Histopathology:* neuronal organs and tissues revealed no treatment-related findings **<u>1000 ppm (66 and 79.7 mg/kg bw/day for males and females respectively)</u>** No adverse effects. **<u>300 ppm (19.7 and 24.3 mg/kg bw/day for males and females respectively)</u>** No adverse effects. **NOAEL: 1000 ppm (66mg/kg bw/day and 79.7mg/kg bw/day) for males and females respectively.**	Anonymous 2009b *Annex I. 3.12.1.3*
2-year rat carcinogenicity and chronic toxicity – interim kill at 12 months. OECD 453 (2009), OPPTS 870.4300 (1998), EU Directive 96/54/EEC	SYN524464 Lot: SMU6LP006 95.3% pure (83% trans isomer, 12.3% cis isomer) Vehicle: diet 0, 200, 1200, 3600 ppm Interim kill at	**<u>Note: dose received (in mg/kg bw/day) are based on data at 2 years.</u>** **<u>3600 ppm (218 and 261 mg/kg bw/day for males and females respectively);</u>** *Mortality*: 2 females were terminated on welfare grounds, considered not related to treatment. *Body weight:* body weight gain was reduced throughout the study and at week 52 was ↓ 19% males and ↓ 34% females. *Food consumption:* consistently ↓ for females by approximately 15%. Males showed reduced food intake	Anonymous, 2010 Amendment 1, Anonymous, 2014 *Annex I. 3.9.1.1*

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
B.33 (2001) GLP Rat: CrL:WI(Han) 52/sex/group for carcinogenicity 12/group/sex interim kill after 12 months	12 months.	during weeks 1-7. *Haematology:* Prothrombin time was ↑in males at weeks 14, 27, 52. *Clinical chemistry:* GGT was ↑ in males at most time points. Small, but statistically ↑ in total protein, albumin and globulin for males at most time points. Globulin ↑ females. *Organ weights*: Adjusted liver weights were ↑40% males and ↑ 29% females. *Histology:* Minimal to moderate centrilobular hepatocyte hypertrophy in the liver was seen in all females and most males. There was ↑ incidence of pigment in centrilobular or midzonal hepatocytes. Minimal to mild thyroid follicular cell hypertrophy in some animals of both sexes. **1200 ppm (67 and 86 mg/kg bw/day for males and females respectively);** *Mortality:* 1 female terminated on welfare grounds, considered not related to treatment. *Body weight:* body weight gain was reduced for males and females during week 1; at week 52 body weight gain was ↓ 7% females. *Organ weights:* adjusted liver weights were ↑ 18% for males. *Histology:* Minimal to mild thyroid follicular cell hypertrophy in some animals of both sexes, being statistically significant in males. **200 ppm (11 and 14 mg/kg bw/day for males and females respectively);** *Mortality:* 1 female terminated on welfare grounds, considered not related to treatment. No adverse effects **The NOAEL for this study was 200 ppm for both sexes (11 mg kg bw/day in males and 14 mg/kg bw/day in females).**	
90-day oral Mouse: Crl:CD-1(1CR) OECD 408 (1998)	SYN524464 Lot: SMU6LP006 95.3% pure (83% *trans* isomer, 12.3%	**7000 ppm (1167 and 1455 mg/kg bw/day for males and females, respectively):** *Body weight:* ↓ males throughout the study; overall, body weight gain ↓32%. *Food utilisation:* ↓ for males over the 13 weeks.	Anonymous, 2008 *Annex I. 3.12.1.5*

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
GLP 10/sex/group	*cis* isomer) Vehicle: diet 0, 500, 3500, 7000 ppm 90 days duration	*Clinical chemistry:* in males, AST ↓ (29%) and bilirubin ↓ (41%). *Organ weights:* ↑ relative liver weight (27%) ↑ absolute and relative testis weight **3500 ppm (567 and 810 mg/kg bw/day for males and females, respectively):** No adverse effect (a 24%↓ in male AST was considered not toxicologically significant). **500 ppm (80 and 112 mg/kg bw/day for males and females, respectively):** No adverse effect **NOAEL: 3500 ppm (567 mg/kg bw/day) for males and 7000 ppm (1455 mg/kg bw/day) for females.**	
OECD 451 (1981): OPPTS 870.4200 (1998): 87/302/EEC B.32 (1988): GLP Mouse: Crl:CD-1 (ICR) 50/sex/group	SYN524464; batch SMU6LP006/ MILLED; purity 95.3% (83.0% trans isomer (SYN508210) and 12.3% cis isomer (SYN508211). 0, 200, 1250 and 7000 ppm Continuous in the diet for at least 80 weeks	**Non-neoplastic findings** **7000 ppm (900 mg/kg bw/day in males and 1001 mg/kg bw/day in females)** *Body weight:* ↓ maximum 7% males; 9% females *Food utilisation:* ↓ 1.5 g/100g of diet consumed males weeks 9-13 [control 2.0]; 2.2 g/100g of diet consumed females weeks 1-4 [control 2.7]; ↑ Liver weight: 16% males [covariate analysis] **1250 ppm (157 mg kg bw/day in males and 185 mg/kg bw/day in females)** No treatment-related findings **200 ppm (25 mg/kg bw/day in males and 29 mg/kg bw/day in females)** No treatment-related findings **The NOAEL for this study was 1250 ppm for both sexes (157 mg/kg bw/day in males and 185 mg/kg bw /day in females)**.	Anonymous (2010). *Annex I. 3.9.1.2*
90 day oral Dog: Beagle OECD 409 (1998)	SYN524464 Lot: SMU6LP006 95.3% pure	**400 mg/kg bw/day:** *Body weight:* ↓ during week 1 and throughout the study for males and females. Body weight gain at day 85 ↓ 5% males and ↓18% females.	Anonymous, 2008 *Annex I. 3.12.1.6*

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
2001/59/EC B27 GLP 4/sex/group	(83% *trans* isomer, 12.3% *cis* isomer) Vehicle: capsule 0, 50, 150, 400 mg/kg bw/day 90 days duration	*Food consumption:* ↓ during week 1 and throughout the study for males and females. *Clinical chemistry:* cholesterol was ↓ in males from week 4, being ↓26% at week 13. *Haematology:* statistical differences were evident in white cells (e.g. total leucocytes ↓ 26% males and ↓ 33% females), decreased lymphocyte and monocyte counts in females and lymphocyte counts in males. *Organ weights:* decreased spleen weights in males and females. **150 mg/kg bw/day:** *Body weight:* ↓ during week 1 and throughout the study for females. Body weight gain at day 85 ↓19% females. *Food consumption:* ↓ during week 1. *Haematology:* decreased total leucocytes (lymphocyte and monocyte counts) in females **50 mg/kg bw/day:** No adverse effect. **NOAEL : 50 mg/kg bw/day**	
52-week oral Dog: Beagle OECD 452 (1981) 88/303/EC B30 (1988) GLP 4/sex/group	SYN524464 Lot: SMU6LP006 95.3% pure (83% *trans* isomer, 12.3% *cis* isomer) Vehicle: capsule 0, 15, 50, 200 mg/kg bw/day 52weeks duration	**200 mg/kg bw/day:** *Body weight:* 1/4 males and all females lost weight during week 1. Weights remained low throughout the study. Body weight gain at week 52 was ↓30% males and ↓66% females. *Food consumption:* During week 1, ↓19% for males and ↓41% females. Food consumption improved with a change in the feeding regime was lower than controls throughout the study (approximately ↓ 5-16% males and ↓ 13-38% females). *Clinical chemistry:* blood glucose levels were ↓ for males and females throughout the study (e.g. ↓13-14% at week 52). ALP was ↓ throughout the study; at week 52, males ↑ approx. 3x and approx. 2x for females. Cholesterol levels were ↓ for males by approx. 30% throughout the study. Phosphorous levels were ↓ in males at weeks 13 and 26. *Organ weights:* ↑ mean liver weights were recorded in males and females; adjusted liver weight for males (↑18%) and for females (↑21%).↓mean spleen weights in both sexes. ↓ mean testes weight (absolute and adjusted).	Anonymous, 2009 *Annex I. 3.12.1.7*

Method, guideline, deviations if any, species, strain, sex, no/group	Test substance, route of exposure, dose levels, duration of exposure	Results	Reference
		50mg/kg bw/day: No adverse effects. **15 mg/kg bw/day:** No adverse effects. **NOAEL : 50 mg/kg bw/day**	
Dermal studies			
28 day dermal Rat: HanRcc: WIST (SPF) OECD 410 (1981) EC 440/EC B9 (2008) GLP 10/sex/group	SYN524464 Lot: SMU6LP006 95.3% pure (83% *trans* isomer, 12.3% *cis* isomer) Vehicle: bi-distilled water 0, 100, 300,1000 mg/kg bw/day 6 hr/day, 5 days/week for 28 days. Applied to 25cm^2 dorsal area.	**1000 mg/kg bw/day:** No adverse effects. **300 mg/kg bw/day:** No adverse effects. **100 mg/kg bw/day:** No adverse effects. **NOAEL : 1000 mg/kg bw/day**	Anonymous, 2009c *Annex I. 3.12.1.8*

Table 49: Summary table of human data on STOT RE

Type of data/report	Test substance	Route of exposure Relevant information about the study (as applicable)	Observations	Reference
No relevant data.				

Table 50: Summary table of other studies relevant for STOT RE

Type of study/data	Test substance	Observations	Reference

Type of study/data	Test substance	Observations	Reference
Two generation reproduction (one litters) OECD 416 (2001) GLP Oral (continuous in diet) Rat, Han Wistar (HanRcc:WIST) 25/sex/group	Sedaxane (SYN524464, batch no. SMU6LP006/MILLED, purity 95.3%) 0, 200, 500 or 1500 ppm Vehicle: laboratory animal diet	***Parental toxicity*** 1500 ppm (120 mg/kg bw/day – F0 males pre-pairing) **P:** In females, mean body weight was significantly reduced (less than 10%) in the pre-pairing, gestation and lactation period. Decreased body weight gain during prepairing (days 1-70, 16%), during gestation (days 0-21, 16%) and lactation (↓day1-21, 12%); ↓ food consumption pre-pairing: males weeks 1-4 (days 15-16, 5%), females throughout (days 43-44, 10.5%); ↓ food consumption gestation days 0-11 (days 4-7, 10%) and throughout lactation (days 5-7, 13%); ↑ absolute thyroid weight males (~19%); ↑ liver weight adjusted for body weight (males 22%, females 30%); ↑ incidence and severity of centrilobular hypertrophy (males 20/25 severity 1.3 cf. controls 13/25 severity 1.1: females 10/25 severity 1.3 cf. controls 0/25); ↓ absolute ovary weight (~14%), ↓ number of corpora lutea (15%) and ↓ number of antral follicles (13%) ↑ females in lactational diestrus at termination (12/25 cf. 7/25 controls) **F1:** In females, mean BW was significantly reduced (less than 10%). ↓ body weight gain females pre-pairing (days 1-91, 6% not stat. sig.); ↓ food consumption pre-pairing females throughout (days 43-45, 6.5%); ↓ food consumption gestation (days 4-7, 14.5%) and lactation (days 7-9, 10%); ↑ liver weight adjusted for body weight (males 21%, females 42%); ↑ incidence and severity of centrilobular hypertrophy (males 22/25 severity 1.1 cf. controls 10/25 severity 1.0: females 17/25 severity 1.1 cf. controls 0/25); ↑ incidence and severity of thyroid follicular hypertrophy (males 9/25 severity 1.2 cf. controls 5/25 severity 1.0); ↓ ovary weight (~ 19.5% absolute, ~ 15.5% adjusted), ↓ number of corpora lutea (12.3 cf. 17.2 controls), ↓ number of antral follicles (5.2 cf. 10.8 controls), ↓ uterus weight (absolute and adjusted) and ↑ females in lactational diestrus at termination (20/25 cf. 8/25 controls). Liver changes considered adaptive. 500 ppm (41mg/kg bw/day – F0 males pre-pairing) P: ↑ liver weight adjusted for body weight (males 9%) F1: ↑ liver weight adjusted for body weight (males 6%) Small magnitude of change and absence of associated histopathology indicate no adverse effect of treatment. 200 ppm (16 mg/kg bw/day – F0 males pre-pairing) P: No treatment-related effects F1: No treatment-related effects **NOAEL parental toxicity: 41 mg/kg bw/day**	Anonymous (2010), final report amendment 2, Anonymous (2013) *Annex I. 3.10.1.1*

Type of study/data	Test substance	Observations	Reference
		Reproductive toxicity 1500 ppm (120 mg/kg bw/day – F0 males pre-pairing) P and F1: Reproductive performance was not affected by treatment. **NOAEL reproductive toxicity: 120 mg/kg bw/day** ***Offspring toxicity*** 1500 ppm (120 mg/kg bw/day) F1: ↓ body weight from PND 14 (10% day 28); ↑ time to vaginal patency (34.2 days cf. 32.5 although no difference in body weight at time of patency); ↑ liver weight adjusted for body weight (14% males, 11% females) F2: ↑ anogenital distance females (8%) small magnitude ↓ body weight from PND 14 (9% day 21); ↑ liver weight adjusted for body weight (14% males, 13% females) 500 ppm (41 mg/kg bw/day) F1: No adverse effects F2: No adverse effects **NOAEL offspring toxicity: 41 mg/kg bw/day**	
Developmental toxicity OECD 414 (2001) GLP Oral (gavage) Rat, HanRcc:WIST 24 mated females/group	Sedaxane (SYN524464, batch no. SMU6LP006/MILLED, purity 95.3%) Dose levels 0, 25, 100 and 200 mg/kg bw/day Dosing on gestation days 6-20 Vehicle: 0.5% CMC	***Maternal toxicity*** 200 mg/kg bw/day: ↓ body weight gain days 6-13 (44%), 6-21 (17%), adjusted days 6-21 (53%); ↓ food consumption days 9-12 (14%), days 18-21 (11%) 100 mg/kg bw/day: ↓ body weight gain days 6-13 (12.5%), 6-21 (4.5% not stat. sig.) adjusted days 6-21 (13% not stat. sig.); ↓ food consumption days 9-12 (7%) 25 mg/kg bw/day: No effects **Maternal NOAEL 25 mg/kg bw/day** ***Developmental toxicity*** 200 mg/kg bw/day: ↓ foetal body weight (4%) stat. sig. for females only and not for combined sexes 100 mg/kg bw/day: No effects **Developmental NOAEL 200 mg/kg bw/day**	Anonymous (2009) Annex I. 3.10.1.2
Developmental toxicity OECD 414 (2001) GLP	Sedaxane (SYN524464, batch no. SMU6LP006/MILLED, purity 95.3%) Dose levels 0,	***Maternal toxicity*** 200 mg/kg bw/day: ↓ defaecation (73 occurrences in 14 animals cf. 11 occurrences in 1 control animal), ↓ body weight gain days 7-13 (-33g cf. + 36g controls), 7-29 (+71g cf. +150g controls), day 29 body weight (-1.5%); ↓ food consumption days 10-13 (21%); ↑ liver weight, absolute (13%), adjusted for body weight (14%)	Anonymous (2010) Annex I. 3.10.1.3

Type of study/data	Test substance	Observations	Reference
Oral (gavage) Rabbit, New Zealand White (Hra:NZW) 25 mated females/group	25, 100 and 200 mg/kg bw/day Dosing on gestation days 7-28 Vehicle: 0.5% CMC	100 mg/kg bw/day: ↑ liver weight, absolute (11%), adjusted for body weight (9%) considered adaptive 25 mg/kg bw/day: No effects **Maternal NOAEL 100 mg/kg bw/day** ***Developmental toxicity*** 200 mg/kg bw/day: ↓ foetal body weight (male, female, and combined) 8.0% to 8.6% lower than the control group values, stat. sig. for females only . Increased incidence of 13th full rib(s). 100 mg/kg bw/day: No effects **Developmental NOAEL 100 mg/kg bw/day**	

10.12.1 Short summary and overall relevance of the provided information on specific target organ toxicity – repeated exposure

Oral

The toxicity of sedaxane following repeated exposure has been evaluated by the oral route of administration in rats, dogs and mice, including lifetime studies in rats and mice. In addition, dermal toxicity was evaluated in rats in a 28-day study (no adverse effects were seen).

Sedaxane is generally of a low order of toxicity in all species tested in repeat dose studies. The main evidence of systemic toxicity in all species was on body weight and food consumption.

The most consistent effect in all species was seen in the **liver** (with most marked effects seen in the rat), indicating that this is a target organ for sedaxane. In a 28day oral study in **rats** (Anonymous, 2010 *Annex I. 3.12.1.1*), statistically significant increased liver weight was seen at the high and mid doses (5000 and 2000ppm, respectively). Histopathologically, hepatocellular centrilobular hypertrophy was seen, together with hepatic enzyme induction (specifically CYP450 2B and 3A) as evidenced by immunoblotting and increased PROD activity (CYP450 2B). This was further confirmed in subsequent in vitro studies (Anonymous, 2016, *Annex I. 3.9.4.5* and Vardy, 2016 *Annex I. 3.9.4.6*) and investigative studies on stored tissues from longer term studies (Anonymous, 2013, *Annex I. 3.9.4.7*). Similar increases in liver weight and histopathological findings were seen in rats following 90 days exposure (Anonymous, 2009 *Annex I. 3.12.1.2*) at dietary inclusion levels of 325.1 and 168 mg/kg bw/day, and following exposure for up to 2 years (Anonymous, 2010, *Annex I. 3.9.1.1*) at 218/261 mg/kg bw/day. Increased liver weight and centrilobular hypertrophy were also seen in the two-generation reproductive study in rats at the highest dose of 120 mg/kg bw/day (Anonymous (2010), final report amendment 2, Anonymous, 2013 *Annex I. 3.10.1.1*).

In **mice**, treatment for 90 days resulted in increased liver weight only at the highest dose tested (7000ppm, Anonymous (2008) *Annex I. 3.12.1.5*) but there were no accompanying histopathological changes. In **dogs**, liver weights were higher than control values in male and female dogs after one year at 200 mg/kg/day (Anonymous (2009) *Annex I. 3.12.1.7*) in the absence of associated histopathological findings. In the 90-day dog study (Anonymous (2008) *Annex I. 3.12.1.6*), there were no effects on liver weights and no histopathology findings in the liver. In the developmental

toxicity study in **rabbits** (Anonymous, 2010 *Annex I. 3.10.1.3*) increased liver weight was also seen at 200 mg/kg bw/day.

There was some evidence for effects in the **thyroid** (rat only). In the carcinogenicity study in rats (Anonymous, 2010, *Annex I.3.9.1.1*) at the 52 week interim kill, minimal-mild follicular cell hypertrophy was seen at the highest dose (3600ppm: 218mg/kg (M), 261mg/kg (F)). Mode of action studies (see Section 10.9, Appendix 3) showed that the thyroid effects in rats were secondary to the activation of the CAR/PXR nuclear receptors in the liver and are not relevant to humans.

See Annex I to the CLH report 3.12 and 3.9.4.

Dermal

Dermal administration of sedaxane up to the limit dose of 1000 mg/kg bw/d for 28 days did not result in any adverse local or systemic effects in rats.

10.12.2 Comparison with the CLP criteria

Substances are classified as specific target organ toxicants following repeated exposure on the basis of "significant" or "severe" toxicity. In this context "significant" means changes which clearly indicate functional disturbance or morphological changes which are toxicologically relevant. "Severe" effects are generally more profound or serious than "significant" effects and are of a considerably adverse nature which significantly impact on health.

In accordance with the guidance on the application of the CLP criteria, the following effects might be indicative of significant or severe toxicity and thus merit classification for STOT-RE.

a) Morbidity or death resulting from repeated or long-term exposure.

b) Significant functional changes in the central or peripheral nervous systems or other organ systems

c) Any consistent and significant adverse change in clinical biochemistry, haematology or urinalysis parameters

d) Significant organ damage noted at necropsy and/or subsequently seen or confirmed at microscopic examination

e) Multi-focal or diffuse necrosis, fibrosis or granuloma formation in vital organs with regenerative capacity

f) Morphological changes that are potentially reversible but provide clear evidence of marked organ dysfunction (e.g., severe fatty change in liver)

The above mentioned effects were not features of exposure to sedaxane at doses below/equal to the guidance cut-off values for category 2 (see table below). The only target organ at such doses was the liver with changes not sufficiently severe or reproducible to warrant classification:

- in the 28-d rat study (with increased absolute and relative liver weights and hepatic enzyme induction CYP450 2B and CYP3A)
- in the rabbit developmental toxicity study (increased liver weight)

Study	**(Adjusted) guidance value category 1 / 2 (mg/kg bw/d)**	**Effects at doses below guidance cut-off values**
28-d rat study Anonymous, 2010	30/300	Category 1: Lowest dose = 45.9/47.6 mg/kg bw/d

Annex I. 3.12.1.1		Category 2: At 45.9/47.6 mg/kg bw/d : No adverse effects At 182.7 and 179.6 mg/kg bw/d: Body weights: slightly ↓(7%) in males Liver effects: ↑absolute and relative weights , ↑ cholesterol and triglycerides, Pentoxyresorufin (PROD) markedly ↑, slight ↑ EROD activity, ↑CYP 2B and CYP 3A
90-d rat study Anonymous, 2009 Annex I. 3.12.1.2	10 / 100	Category 1: Lowest dose = 24.8 mg/kg bw/d Category 2: No adverse effects at 24.8 mg/kg bw/d
90-d neurotoxicity study Anonymous, 2009b Annex I. 3.12.1.3	10 / 100	Category 1: Lowest dose = 19.7/24.3 mg/kg bw/d Category 2: No adverse effects at 19.7/24.3 mg/kg bw/d and 66/79.7 mg/kg bw/d
90-d mouse study Anonymous, 2008 Annex I. 3.12.1.5	10 / 100	Category 1: Lowest dose = 80/112 mg/kg bw/d Category 2: No adverse effects at 80/112 mg/kg bw/d
90-d dog study Anonymous, 2008 Annex I. 3.12.1.6	10 / 100	Category 1: Lowest dose = 50 mg/kg bw/d Category 2: No adverse effects at 50 mg/kg bw/d
1-year dog study Anonymous, 2009 Annex I. 3.12.1.7	2.5 / 25	Category 1: Lowest dose = 15 mg/kg bw/d Category 2: No adverse effects at 15 mg/kg bw/d and 50 mg/kg bw/d
2-year rat study Anonymous, 2010 Amendment 1, Anonymous, 2014 *Annex I. 3.9.1.1*	1.25 / 12.5	Category 1: Lowest dose = 11/14 mg/kg bw/d Category 2: No adverse effects at 11/14 mg/kg bw/d
18-month mouse study Anonymous (2010). *Annex I. 3.9.1.2*	1.7 / 17	Category 1: Lowest dose = 25 mg/kg bw/d Category 2: Lowest dose = 25 mg/kg bw/d
2-generation study Anonymous (2010), final report amendment 2, Anonymous (2013)	10/100*	Category 1: Lowest dose = 16 mg/kg bw/d Category 2: No adverse effects at 16 mg/kg bw/d and 41 mg/kg bw/d

Annex I. 3.10.1.1		
Rat developmental study Anonymous (2009) *Annex I. 3.10.1.2*	60/600	Category 1: No adverse effects at 25 mg/kg bw/d Category 2: At 100 mg/kg bw/d and 200 mg/kg bw/d Dams: ↓ body gain and food consumption, Foetuses: no adverse effects
Rabbit developmental study Anonymous (2010) *Annex I. 3.10.1.3*	45/450	Category 1: No adverse effects at 25 mg/kg bw/d Category 2: No adverse effects at 100 mg/kg bw/d At 200 mg/kg bw/d Dams: ↓ body gain and food consumption, ↓ defaecation ↑ liver weight Foetuses: ↓ foetal body weight, increased incidence of 13th full rib(s).

*: proposed cut-off value for 90-d study as a worst case.

At dose levels above cut-off values for category 2, more severe effects on liver and thyroid were observed. However mechanistic data were generated to explore the underlying modes of action and their relevance to human health.

Mode of action studies showed that the liver effects observed and the increased incidence of tumours in long term studies can be explained due to changes in the liver that are attributable to activation of CAR (with a possible lesser activation of PXR in rats), resulting in a series of well-documented downstream events, ultimately leading to a higher incidence of tumours vs. the concurrent controls. This MOA is not relevant for human hazard/risk assessment purposes due to qualitative differences in response to CAR/PXR activation between rodents (rats and mice) and humans (see section 10.9 and Appendix 2). Therefore, no classification is warranted.

Effects in the thyroid are considered to be due to the activation of the CAR/PXR nuclear receptors in the liver (Omiecinski, 2014, *Annex I. 3.9.4.8*; Toyokawa and Sherf, 2014 *Annex I. 3.9.4.9*), resulting in perturbation of the hypothalamus-pituitary-thyroid (HPT) axis as a secondary consequence of liver xenobiotic metabolising enzyme induction. This MOA is not relevant for humans and considered of insufficient concern for classification (see section 10.9, Appendix 3 and ECBI/22/98 Add 1, EU Commission Meeting of the Commission Working Group on C&L of dangerous substances ECBI/27/98 Rev.2)

Overall, the only target organ at doses below the guidance cut-off value for category 2 was the liver. Based on the nature of the effects and the doses at which these occurred, liver effects did not warrant classification.

10.12.3 Conclusion on classification and labelling for STOT RE

Not classified (conclusive but not sufficient for classification)

RAC evaluation of specific target organ toxicity– repeated exposure (STOT RE)

Summary of the Dossier Submitter's proposal

The toxicity of sedaxane following repeated exposure has been evaluated by the oral route of administration in rats, dogs and mice, including lifetime studies in rats and mice. In addition, dermal toxicity was evaluated in rats in a 28-day study (no adverse effects were seen). Sedaxane is generally of a low order of toxicity in all species tested in repeat dose studies. The main evidence of systemic toxicity in all species was on body weight and food consumption. The most consistent effect in all species was seen in the liver (with most marked effects seen in the rat), indicating that this is a target organ for sedaxane.

There was some evidence for effects in the thyroid (rats only). In the carcinogenicity study in rats (Anonymous, 2010) at the 52-week interim kill, minimal-mild follicular cell hypertrophy was seen at the highest dose (3600 ppm ~ 240 mg/kg bw/d).

On basis of the mode of action, the DS concluded the only target organ at doses below the guidance cut-off value for category 2 was the liver. Based on the nature of the effects and the doses at which these occurred, liver effects did not warrant classification.

Comments received during public consultation

No comments were received.

Additional key elements

An exploratory 28-day study in rats of the same strain is available: *cis*/*trans isomer comparative study.* This non-guideline study was conducted with the individual diastereomers of sedaxane (SYN508210 and SYN508211) plus a 1:1 mixture of the two. The study clearly demonstrated that each isomer was capable of producing similar qualitative and quantitative effects on body weight and liver micropathology. Hepatic cytochrome P450 induction was also determined in this study, and marked increases were seen in CYP2B activity in particular, along with other hepatic enzyme induction. Therefore, the liver effects in rats are considered to be reflective of induction of specific hepatic cytochrome P450 isoenzymes, and the overall types of effects across multiple studies represent a transient, adaptive response rather than evidence of hepatotoxicity. This exploratory 28-day rat study with sedaxane indicated that no changes in thyroid hormone levels (T3, T4, and TSH) were observed after 28 days of treatment at dose levels up to 5000 ppm in the diet.

Assessment and comparison with the classification criteria

In accordance with the guidance on the application of the CLP criteria, the effects for justifying classification for STOT RE were not features of exposure to sedaxane at doses below or equal to the guidance cut-off values for category 2. The only target organ at such doses was the liver with changes not sufficiently severe or reproducible to warrant classification. The observations related to the 28 days study (rats) where increased absolute and relative liver weights and hepatic enzyme CYP450 induction; CYP2B and CYP3A, increased cholesterol and triglycerides. In the rabbit developmental toxicity study increased liver weight was observed in the 200 mg/kg bw/d dose group.

Rat

In a 28 day oral study in rats (Anonymous, 2010), statistically significant increased liver weight was seen at the high and mid doses (5000 ppm (~ 437 mg/kg bw/d) and 2000 ppm (~ 180 mg/kg bw/d) respectively). Histopathologically, hepatocellular centrilobular hypertrophy was seen, together with hepatic enzyme induction (specifically CYP2B and CYP3A) as evidenced by immunoblotting and increased PROD activity. This was further confirmed in subsequent *in vitro* studies (Anonymous, 2016, Vardy, 2016) and investigative studies on stored tissues from longer-term studies (Anonymous, 2013).

Similar increases in liver weight and histopathological findings were seen in rats following 90 days exposure (Anonymous, 2009) at dietary inclusion levels of 325 and 168 mg/kg bw/d, and following exposure for up to 2 years (Anonymous, 2010) at 218/261 mg/kg bw/d. Increased liver weight and centrilobular hypertrophy were also seen in the two-generation reproductive study in rats at the highest dose of 120 mg/kg bw/d (Anonymous, 2010).

Dermal rat

Dermal administration of sedaxane up to the limit dose of 1000 mg/kg bw/d for 28 days did not result in any adverse local or systemic effects in rats.

Mouse

In mice, treatment for 90 days resulted in increased liver weight only at the highest dose tested at 7000 ppm (~ 1300 mg/kg bw/d) (Anonymous, 2008) but there were no accompanying histopathological changes.

Dog

In dogs, liver weights were higher than control values in male and female dogs after one year at 200 mg/kg bw/day (Anonymous, 2009) in the absence of associated histopathological findings. In the 90-day dog study (Anonymous, 2008) there were no effects on liver weights and no histopathology findings in the liver in doses up to 400 mg/kg bw/d.

Rabbit

In the developmental toxicity study in rabbits (Anonymous, 2010) increased liver weight was also seen at 200 mg/kg bw/d.

Mode of action studies showed that the liver and thyroid effects in rats were secondary to the activation of the CAR/PXR nuclear receptors in the liver and are less relevant to humans. This will be discussed in detail in the carcinogenicity section.

Table: *Summary of studies relevant for STOT RE assessment and the guidance values*

Study	**Cut off values Cat. 1/2**	**Effects at doses below guidance cut-off values**
28-d rat study Anonymous, 2010 Annex I. 3.12.1.1	30/300	Category 1: Lowest dose = 45.9/47.6 mg/kg bw/d Category 2: At 45.9/47.6 mg/kg bw/d : No adverse effects At 182.7 and 179.6 mg/kg bw/d: Body weights: slightly ↓(7%) in males Liver effects: ↑absolute and relative weights , ↑ cholesterol and triglycerides, Pentoxyresorufin (PROD) markedly ↑, slight ↑ EROD activity, ↑CYP 2B and CYP 3A
90-d rat study Anonymous, 2009 Annex I. 3.12.1.2	10 / 100	Category 1: Lowest dose = 24.8 mg/kg bw/d Category 2: No adverse effects at 24.8 mg/kg bw/d
90-d neurotoxicity study Anonymous, 2009b Annex I. 3.12.1.3	10 / 100	Category 1: Lowest dose = 19.7/24.3 mg/kg bw/d Category 2: No adverse effects at 19.7/24.3 mg/kg bw/d and 66/79.7 mg/kg bw/d
90-d mouse study Anonymous, 2008 Annex I. 3.12.1.5	10 / 100	Category 1: Lowest dose = 80/112 mg/kg bw/d Category 2: No adverse effects at 80/112 mg/kg bw/d
90-d dog study Anonymous, 2008 Annex I. 3.12.1.6	10 / 100	Category 1: Lowest dose = 50 mg/kg bw/d Category 2: No adverse effects at 50 mg/kg bw/d
1-year dog study Anonymous, 2009 Annex I. 3.12.1.7	2.5 / 25	Category 1: Lowest dose = 15 mg/kg bw/d Category 2: No adverse effects at 15 mg/kg bw/d and 50 mg/kg bw/d
2-year rat study Anonymous,	1.25 / 12.5	Category 1: Lowest dose = 11/14 mg/kg bw/d Category 2:

2010 Annex I. 3.9.1.1		No adverse effects at 11/14 mg/kg bw/d
18-month mouse study Anonymous, 2010 Annex I. 3.9.1.2	1.7 / 17	Category 1: Lowest dose = 25 mg/kg bw/d Category 2: Lowest dose = 25 mg/kg bw/d
2-generation study Anonymous (2010) Annex I. 3.10.1.1	10/100	Category 1: Lowest dose = 16 mg/kg bw/d Category 2: No adverse effects at 16 mg/kg bw/d and 41 mg/kg bw/d
Rat developmental study Anonymous (2009) Annex I. 3.10.1.2	60/600	Category 1: No adverse effects at 25 mg/kg bw/d Category 2: At 100 mg/kg bw/d and 200 mg/kg bw/d Dams: ↓ body gain and food consumption, Foetuses: no adverse effects
Rabbit developmental study Anonymous, (2010) Annex I. 3.10.1.3	45/450	Category 1: No adverse effects at 25 mg/kg bw/d Category 2: No adverse effects at 100 mg/kg bw/d At 200 mg/kg bw/d Dams: ↓ body gain and food consumption, ↓ defaecation ↑ liver weight Foetuses: ↓ foetal body weight, increased incidence of 13th full rib(s).

RAC concludes, in accordance with the proposal by the dossier submitter that **no classification is justified for STOT RE.**

10.13 Aspiration hazard

Table 52: Summary table of evidence for aspiration hazard

Type of study/data	Test substance	Relevant information about the study (as applicable)	Observations	Reference
No relevant studies				

10.13.1 Short summary and overall relevance of the provided information on aspiration hazard

Liquid substances and mixtures have to be classified which contain hydrocarbons to ≥ 10% and which show a kinematic viscosity of < 20.5 cSt (mm²/sec).

10.13.2 Comparison with the CLP criteria

Since sedaxane is a solid, the criteria for classification are not met.

10.13.3 Conclusion on classification and labelling for aspiration hazard

Not classified (conclusive but not sufficient for classification)

RAC evaluation of aspiration toxicity

Summary of the Dossier Submitter's proposal

Liquid substances and mixtures which contain hydrocarbons ≥ 10% and which show kinematic viscosity < 20.5 cSt (mm^2/s) should be classified. Sedaxane is a solid, therefore the classification criteria are not met.

Comments received during public consultation

No comments were received.

Assessment and comparison with the classification criteria

RAC agrees with the dossier submitter that sedaxane does not require classification as regards to aspiration toxicity.

11 EVALUATION OF ENVIRONMENTAL HAZARDS

Studies on the environmental properties of sedaxane have been previously evaluated in the framework of approval of sedaxane at EU level and therefore have been included in the DAR of sedaxane (June, 2012). They were all considered acceptable and are summarized below. No new studies were submitted.

Either indicated otherwise, the following ecotoxicological studies have been previously evaluated in the framework of approval of sedaxane at EU level and are considered fully reliable.

11.1 Rapid degradability of organic substances

Relevant studies on degradation of sedaxane are listed in the table below. These studies show that sedaxane is not readily biodegradable and is not considered to be rapidly degradable for the purposes of classification and labelling.

Table 53: Summary of relevant information on rapid degradability

Method	Results	Remarks	Reference

Method	Results	Remarks	Reference
Ready biodegradability 28 day, 22°C, pH 7.2-7.6 Test substance: Sedaxane (purity 95.3%) Test concentration = 101 mg/L OECD 301F GLP	No biodegradation in 28 days; Not readily biodegradable	43% degradation observed in the toxicity control, therefore no significant inhibitory effect	Seyfried (2007)
Hydrolysis, pH 4, 5, 7 and 9, 25°C, 30 days, dark. Test substance: [Phenyl-U-^{14}C]- -Sedaxane (Radiochemical purity 99.1%, chemical purity 99.6%) Nominal concentration = 0.0017 mg/mL OECD 111 EPA subdivision N-161-1 GLP	Prelim study at 50°C; <10% degradation at pH 4,5,7 and 9 after 5 days. After 30 days at 25°C, Sedaxane accounted for 95.9, 102.8 and 101.3% of Applied radioactivity at pH 5, 7 and 9, respectively. $DegT_{50}$ > 1 year		Nicollier (2007a)
Direct and indirect photolysis pH 7, up to 34 days, 25 ± 2°C, sterile and natural water, Test substance: ^{14}C-phenyl and ^{14}C-pyrazole sedaxane (purity >99%) Test concentration = *c.a.* 2.0 mg/L OECD draft guideline Aug 2000, JMAFF 12 Nousan no. 8147, 2001 EPA 540/9-82-021 GLP	Direct photolysis in sterile buffer; $DegT_{50}$ = 42, 52 and 71 days at 30, 40 and 50°N	Sedaxane level 57.3% AR after 34 days continuous irradiation (95.2% in dark controls) Total ^{14}C recoveries 98.1–101.4 % (phenyl) and 91.5-99.2% (pyrazole) Multiple degradates and minimal volatiles (max 1.8%)	Hand and Flemming (2007)
	Indirect photolysis in natural water; $DegT_{50}$ = 16.3, 16.5 and 17.1 days at 30, 40 and 50°N	Sedaxane level 23.9% AR after 28 days continuous irradiation (97.7% in dark controls) Total ^{14}C recoveries 94.5–107.6 % (phenyl) and 98.2-105.4% (pyrazole) Multiple degradates and minimal volatiles (max 11.1% (phenyl only))	
Water-sediment degradation, aerobic (179 days) and anaerobic (360 days), 20 ± 1°C, dark, pond & river systems Test substance: ^{14}C-phenyl	Total system $DegT_{50}$ Aerobic: Pond; >> 1 year River; >> 1 year	For aerobic and anaerobic systems; CO_2 evolution ≤2.0% AR Total mean recoveries	Stoll and Nicollier (2008)

Method	Results	Remarks	Reference
sedaxane (purity >96%) Nominal concentration = 0.03 µg/mL OECD 308 EPA subdivision N-162-3 GLP	Anaerobic: Pond; >> 1 year River; >> 1 year	93.9-105.2 %	
Soil adsorption/desorption, 6 soils Test substance: ^{14}C-phenyl sedaxane (purity >99%) OECD 106 EPA subdivision N-163-1 GLP	Mean $K_{FOC(ads)}$ = 534L/kg for all soils (range 262-666 L/kg) Mean $K_{FOC(des)}$ = 704L/kg for all soils (range 367-907 L/kg)	Total ^{14}C recovery 90-110% in all soils	Nicollier (2008)

11.1.1 Ready biodegradability

One study (Seyfried, 2007) showed no mineralisation after 28 days, hence sedaxane does not fulfil the criteria for ready biodegradability.

Study 1: Seyfried (2007)

The 28 day ready biodegradability of sedaxane (101 mg/L) in aerobic activated sludge (30mg/L) was determined using manometric methods. The test employed an inoculum control, procedure control (with sodium benzoate), abiotic control (with test material and mercury dichloride) and toxicity control (with test substance and sodium benzoate).

The test material sedaxane was found not to be readily biodegradable under the conditions of the test within 28 days. In the toxicity control 43% degradation of sodium benzoate was observed within 14 days of exposure, thus the test substance had no inhibitory effect on activated sludge microorganisms.

11.1.2 BOD_5/COD

No additional studies.

11.1.3 Hydrolysis

One study (Nicollier, 2007a) showed that sedaxane was hydrolytically stable at all pH values and temperatures tested. Predicted half-lives were > 1 year, therefore sedaxane is not considered to be rapidly degradable by hydrolysis.

Study 1: Nicollier (2007a)

The hydrolysis of sedaxane was studied at 50°C for up to 5 days in sterile aqueous solutions buffered at pH 4, pH 5, pH 7 and pH 9 in the dark (preliminary test). Subsequently hydrolysis was studied at 25°C for up to 30 days in sterile aqueous solutions buffered at pH 5, pH 7 and pH 9 in the dark (confirmatory test). The nominal concentration of sedaxane was 0.0017 mg/mL for all pH values tested.

In each test, duplicate samples were taken for analysis at 5 (preliminary test) or 3 intervals (confirmatory test) during incubation.

For the preliminary test at 50°C, the mean recoveries of radioactivity ranged from 96.1% to 110.9% of the applied radioactivity for all pH values. For the confirmatory test at 25°C, the corresponding range was 95.9% to 109.7% of the applied radioactivity.

Sedaxane was shown to be stable to hydrolysis at all four pH values. Less than 10% hydrolysis of sedaxane was observed for all four pH values after 5 days at 50°C (which equates to a $DegT_{50}$ >1 year at 25 C).

Two minor degradates were formed, not exceeding 5.4% and 2.4% of the applied radioactivity at any time point throughout the preliminary or confirmatory tests.

11.1.4 Other convincing scientific evidence

No additional studies.

11.1.4.1 Field investigations and monitoring data (if relevant for C&L)

No additional studies.

11.1.4.2 Inherent and enhanced ready biodegradability tests

No additional studies.

11.1.4.3 Water, water-sediment and soil degradation data (including simulation studies)

One relevant study on the degradation of ^{14}C sedaxane in aquatic water-sediment systems showed primary degradation half-lives >>1 year. CO_2 evolution was negligible (<2%).

Based on these results sedaxane is considered not to be rapidly biodegradable in natural water and water/ sediment systems.

Study 1: Stoll and Nicollier (2008)

The degradation of ^{14}C phenyl sedaxane, was investigated in two laboratory incubated aquatic sediment systems, using river and pond sediments, under aerobic and anaerobic conditions, in the dark. The flow through gases for the systems were air for the aerobic systems and oxygen-free nitrogen for the anaerobic systems. ^{14}C-Sedaxane was applied to the surface water in each vessel to give a nominal initial concentration of 0.03 μg / mL in the water phase, equivalent to a single surface overspray application of 148 g ai/ha, evenly distributed to a depth of 30 cm. The test systems were incubated in a flow-through system at 20±1ºC in the dark for up to 179 days for the aerobic system and 360 days for the anaerobic system. The mean mass balance from all aerobic water/sediment systems ranged from 93.9 to 104.0%. The mean mass balance from all anaerobic water/sediment systems ranged from 97.1 to 105.2%.

Under aerobic conditions in both systems, sedaxane dissipated rapidly from the water phase to the sediment; and slowly under anaerobic conditions, however degradation of the sedaxane in the whole water/sediment systems was very slow. The dissipation and degradation rates for the various systems were calculated using simple first-order kinetics (SFO) see Table 54.

Table 54: Dissipation and degradation rates of sedaxane in two water/sediment systems

Water/sediment	SFO					
	Rate of dissipation from water phase			Rate of degradation in whole system		
	DT_{50} [days]	DT_{90} [days]	χ^{2} *	$DegT_{50}$ [days]	$DegT_{90}$ [days]	χ^{2} *
Aerobic						
River	6.4	21.2	19.0	>>1 year	>> 1 year	1.5
Pond	5.5	18.4	18.9	>> 1 year	>> 1 year	2.1
Anaerobic						
River	43.4	144.0	14.7	>> 1 year	>> 1 year	1.0
Pond	31.3	104.0	15.9	>> 1 year	>> 1 year	1.1

* χ^2 was calculated using FOCUS_DEGKIN V2 (visual assessment and chi2-test for SFO kinetics)

In all systems the ratio of the isomer SYN508210 (*trans* isomer) and SYN508211 (*cis* isomer) remained unchanged during the study.

Degradation of sedaxane under aerobic conditions led to the production of at least 15 minor metabolites, all observed in the total systems at less than 1% of applied radioactivity. Volatile radioactivity in form of CO_2 was negligible at <2% and the unextracted radioactivity reached a maximum of 12.6% at the end of the study.

Degradation of sedaxane under anaerobic conditions led to the production of up to four minor metabolites, all observed at less than 1% of applied radioactivity. Volatile radioactivity in form of CO_2 was negligible at <1% and the unextracted radioactivity reached a maximum of 7.9% at the end of the study.

11.1.4.4 Photochemical degradation

One study (Hand and Fleming, 2007) shows that photolysis is a potentially significant degradation mechanism in natural waters. Since available information on photolysis is provided here as supporting information only. This information does not impact the classification and labelling of sedaxane.

Study 1: Hand and Flemming (2007)

The photolysis of sedaxane was investigated in both sterile pH 7 phosphate buffer (direct photolysis) and sterile natural water (indirect photolysis).

^{14}C-Sedaxane (both ^{14}C-phenyl and ^{14}C-pyrazole labelled) was applied, at rates equivalent to ca. 2 µg mL^{-1}, to the aqueous media in individual photolysis vessels. Aliquots (15 mL) were continuously irradiated using light from a Suntest xenon arc lamp, filtered to give a spectral distribution close to that of natural sunlight. The samples were maintained at 25±2°C and were irradiated for periods at least the equivalent of 30 days summer sunlight.

Direct photolysis of sedaxane followed first order kinetics. The DT_{50} was estimated as 42, 52 and 71 days summer sunlight at 30, 40 and 50°N, respectively. At the end of the irradiation period ^{14}C-sedaxane represented 57.3% of the applied radioactivity.

Indirect photolysis of sedaxane followed first order kinetics with an estimated DT_{50} of 371.6 hours (15.5 days) continuous irradiation. From this, the DT_{50} was estimated as 16.3, 16.5 and 17.1 days summer sunlight at 30, 40 and 50°N, respectively (using the calculation specified in the OECD test

guideline). At the end of the irradiation period ^{14}C-sedaxane represented 23.9% of the applied radioactivity.

In both direct and indirect studies several degradates were formed. The main degradates observed in both tests were metabolites CSAA798670, CSCC210616, CSCD668095 and CSCD668094. Increased levels of metabolites were observed in the indirect photolysis test with maximum levels of 25.7, 5.4, 15.8 and 14.8% for CSAA798670, CSCC210616, CSCD668095 and CSCD668094 respectively. No significant degradation was apparent in the 'dark controls' indicating that the degradation in irradiated samples was due to photodegradation only.

In summary, both direct and indirect photodegradation of sedaxane were shown to be extensive, with indirect photolysis being approximately 3 times faster than direct photolysis.

11.2 Environmental transformation of metals or inorganic metals compounds

Not applicable.

11.2.1 Summary of data/information on environmental transformation

Not applicable.

11.3 Environmental fate and other relevant information

Available information on adsorption and volatilisation are provided here as supporting information only. This information does not impact the classification and labelling of sedaxane.

Adsorption

One study (Nicollier, 2008) is available on the adsorption and desorption of sedaxane to a range of soil types. This study showed that sedaxane is moderately to strongly adsorbed to soil and is expected to have low to medium mobility.

Study 1: Nicollier G (2008)

The adsorption characteristics of ^{14}C-phenyl ring labelled sedaxane was investigated in six different soils: Gartenacker (loam), Marsillargues (silty clay), 18 Acres (sandy clay loam), Visalia (sandy loam), Champaign (silty clay) and Washington (sand) using a standard batch equilibrium method. The soil adsorption coefficients Kd and K_{OC}, together with the Freundlich adsorption constants K_F and K_{FOC}, were determined for each soil. The reversibility of the adsorption (desorption) was also determined.

The mass balance from all soils was between 90 and 110% of the applied radioactivity.

Sedaxane can be classified as having a "low" potential mobility in 18 Acres, Visalia, Washington, Champaign and Marsillargues soils and "medium" potential mobility in Gartenacker. The mean K_{FOC} from all soils was 534 mL/g and the slopes (1/n) of the adsorption and desorption isotherms ranged from 0.8 to 0.9. These data indicate that the Freundlich adsorption / desorption isotherms tended to follow the Freundlich distribution law with increasing sorption strength at decreasing equilibrium concentrations.

A summary of the key values is shown in Table 55.

The desorption constants of sedaxane were higher than the adsorption constants thus demonstrating that adsorption was not fully reversible.

Table 55: Soil adsorption constants for Sedaxane in 6 Soils

Parameter	Gartenacker (loam)	Marsillargues (silty clay)	18 Acres (sandy clay loam)	Visalia (sandy loam)	Champaign (silty clay)	Washington (sand)
pH (0.01M $CaCl_2$)	6.8	7.5	5.1	5.7	7.1	7.0
%OC	2.6	1.04	2.78	0.52	2.44	0.3
K_F	6.82	5.72	16.74	3.06	13.13	2.00
K_{FOC}	262	548	602	588	538	666
1/n	0.81	0.86	0.91	0.91	0.84	0.86
r^2	0.99	1.00	1.00	0.99	1.00	1.00
K_F (desorption)	9.55	7.39	19.90	3.94	18.75	2.72
K_{FOC} (desorption)	367	708	716	758	769	907
1/n	0.80	0.86	0.89	0.92	0.86	0.87
r^2	0.99	0.99	0.99	0.98	1.00	0.99

Volatilisation

Based on its physical-chemical properties (vapour pressure = 6.5×10^{-8} Pa at 20°C and 1.7×10^{-7} Pa at 25°C (Geoffroy, 2008b), Henry's constant H = 4.0×10^{-6} Pa m^3 mol^{-1} at 25°C (Stulz, 2009), sedaxane has a low potential for volatilization and is no considered as volatile (Focus AIR, 2008[4]).

11.4 Bioaccumulation

Table 56: Summary of relevant information on bioaccumulation

Substance	Species	Test guidelines	Endpoint	Value	Condition	Reference
^{14}C-sedaxane purity 95.2%, radiochem purity 99.1%	Rainbow trout (*Oncorhynchus mykiss*)	OECD 305	BCF (whole fish)	97	Flow-through, 14 day uptake and 14 day depuration 0.5 µg/L + Solvent (DMF) control. pH 7.24-7.69 15.1-15.3°C GLP	Authors of vertebrate study (2010) *Annex I. 4.2.1.1*

[4] FOCUS (2008) "Pesticides in Air: Considerations for Exposure Assessment". Report of the FOCUS Working Group on Pesticides in Air, EC Document Reference SANCO/10553/2006 Rev 2 June 2008. 327 pp.

11.4.1 Estimated bioaccumulation

The experimentally derived Log Kow of sedaxane is 3 (Section 7). For classification and labelling purposes a substance with Log Kow <4 may be considered unlikely to bioaccumulate in aquatic organisms. However, a measured BCF is available and is presented below.

11.4.2 Measured partition coefficient and bioaccumulation test data

For pesticide registration, a Log Kow >3 triggers the requirement for a bioconcentration study. Since the Log Kow of sedaxane is 3.3 a BCF study has been undertaken (Authors of vertebrate study, 2010). The reported BCF for parent sedaxane was 97 L/kg. According to CLP criteria, a measured BCF ≥ 500 indicates a potential for bioaccumulation. Since the BCF for sedaxane is < 500, sedaxane is considered not to be bioaccumulative for the purpose of classification and labelling.

Study 1: Authors of vertebrate study (2010) *Annex I. 4.2.1.1*

In a 28 day flow-through bio-concentration study, groups of 80 rainbow trout (*Oncorhynchus mykiss*) were exposed to a single nominal concentration of 0.5 µg/L ^{14}C-sedaxane and a solvent (0.050 ml/L dimethylformamide) control. One treatment tank was employed for each of the treatment and solvent control and the fish were exposed for a period of 14 days (uptake phase), followed by a period of 14 days in fresh water without test substance or solvent (depuration phase).

During the 28-day exposure period the mean measured concentration of ^{14}C-sedaxane in the treatment tank was 0.49 µg/L (98% of nominal), indicating that the test substance remained stable in solution throughout the duration of the test.

The plateau concentration of radioactivity in whole fish (µg ^{14}[C]-sedaxane equivalents/kg) was attained on day 1 of the exposure phase. On transfer to clean water, the depuration of accumulated residue from the whole body was rapid, with <95% of the steady state concentration remaining within 3 days.

The measured steady state BCF value (to 2 significant figures) obtained for whole fish tissues was 97. The uptake rate constant (k1) was 1934 day^{-1} and the depuration rate constants (k2) was 20.2 day^{-1}. The kinetic BCF (k1/k2) was 96.

Table 57: Uptake and depuration of ^{14}[C]-sedaxane in rainbow trout (*Oncorhynchus mykiss*)

Day		Mean concentration of ^{14}[C]-sedaxane (µg/kg)		
		Edible tissues	Non-edible tissues	Whole body
Uptake phase	1	90	158	48
	3*	14	234	40
	7*	16	200	54
	10*	16	287	51
	14*	15	235	43
Depuration phase	3	0.93	8.98	1.90
	7	0.58	4.05	1.20
	10	0.24	4.39	0.94
	14	0.30	3.85	0.80
Average steady state		15.25	239	47
Upper confidence limit (+ 20%)		18.3	287	56.4
Lower confidence limit (- 20%)		12.2	191	37.6
BCF_{SS}		n/a	n/a	97

* = steady state, n/a = not applicable.

According to EFSA journal 2012 (10(7):2823), this study was not considered as fully valid but was considered to be of sufficient quality for informative data (Please refer to the EU review of sedaxane 2012). For classification purpose, this study is sufficiently robust to support that sedaxane has a low bioaccumulation potential as indicated by its log K_{ow} value < 4.

11.5 Acute aquatic hazard

Table 58: Summary of relevant information on acute aquatic toxicity

Substance	Species	Test guidelines	Endpoint	Toxicity value (mg a.i./L)	Conditions	Reference
Fish						
Sedaxane (purity 95.3%)	*Cyprinus carpio* (Common carp)	OECD 203 OPPTS 850.1074	96 h LC_{50}	0.62 mg/L (mm)	96 h static test. Dilution water control. pH 8.3 – 8.5 21.5 – 22.1°C GLP	Authors of vertebrate study (2008a) ***SYN524464_11104*** *Annex I. 4.3.1.1*

Sedaxane (purity 95.3%)	*Oncorhynchus mykiss* (Rainbow trout)	OECD 203 OPPTS 850.1074	96 h LC_{50}	1.1 mg/L (mm)	96 h static test. Dilution water control. pH 8.2 – 8.4 13°C GLP	Author of vertebrate study (2008) ***SYN524464/0067*** *Annex I. 4.3.1.2*
Sedaxane (purity 98.2%)	*Pimephales promelas* (Fathead minnow)	OECD 203 OPPTS 850.1075	96 h LC_{50}	0.98 mg/L (mm)	96 h static test. Dilution water control. pH 7.59 – 8.29 24.1 – 24.5°C GLP	Author of vertebrate study (2006) ***SYN524464/0012*** *Annex I. 4.3.1.3*
Sedaxane (purity 95.3%)	*Cyprinodon variegatus* (Sheepshead minnow)	OPPTS 850.1075	96 h LC_{50}	4.2 mg/L (mm)	96 h static test. Dilution water control. pH 8.0 – 8.3 21.8 – 22.7°C GLP	Authors of vertebrate study (2008b) ***SYN524464/0062*** *Annex I. 4.3.1.4*
Aquatic Invertebrates						
Sedaxane (purity 95.3%)	*Americamysis bahia* (saltwater mysid)	OPPTS 850.1035	96 hour LC_{50}	1.5 mg/L (mm)	96 hour static test Dilution water control. pH 8.1 – 8.2 24.9– 25.4°C (start) 25.2 – 25.3°C (end) GLP	Gallagher, Kendall & Krueger. (2008c) ***SYN524464/0059***
Sedaxane (purity 98.2%)	*Daphnia magna* (Cladoceran)	OECD 202	48 h EC_{50}	6.10 mg/L (mm)	48 hour static test Dilution water control. pH 7.50–7.59 20.7 – 21.2°C GLP	Ricketts & Paddick (2006) ***SYN524464/0011***
Algae and aquatic plants						
Sedaxane (purity 95.3%)	*Pseudokirchneriella subcapitata* (freshwater green alga)	OECD 201	72-h E_bC_{50} 72-h E_rC_{50} 72-h E_yC_{50}	1.9 mg/L 2.8 mg/L 1.6 mg/L (mm)	96 hour static Culture medium control pH 8.2 (start) 8.1 - 9.2 (end) 22 - 23°C	Bätscher, (2007a) ***SYN524464/0037*** *****

			96-h E_bC_{50} 96-h E_rC_{50} 96-h E_yC_{50}	1.9 mg/L 3.0 mg/L 1.8 mg/L (mm)	GLP	
Sedaxane (purity 95.3%)	*Navicula pellicosa* (Freshwater diatom)	OECD 201	72-h E_bC_{50} 72-h E_rC_{50} 72-h E_yC_{50}	4.8 mg/L 8.7 mg/L 4.8 mg/L (mm)	96 hour static Culture medium & filtrate control. pH: 7.4 (start) 7.7 – 9.2 (end) 23ºC GLP	Büche, (2007a) ***SYN524464/0044****
			96-h E_bC_{50} 96-h E_rC_{50} 96-h E_yC_{50}	5.3 mg/L 10 mg/L 5.7 mg/L (mm)		
Sedaxane (purity 95.3%)	*Anabaena flos-aquae* (freshwater Cyanobacteria)	OECD 201	72-h E_bC_{50} 72-h E_rC_{50} 72-h E_yC_{50}	>6.5 mg/L >6.5 mg/L >6.5 mg/L (mm)	96 hour static Culture medium & filtrate control. pH: 8.5 (start) 9.0-9.1 (end) 22 - 23°C GLP	Büche (2007b) ***SYN524464/0045****
			96-h E_bC_{50} 96-h E_rC_{50} 96-h E_yC_{50}	>6.5 mg/L >6.5 mg/L >6.5 mg/L (mm)		
Sedaxane (purity 95.3%)	*Skeletonema costatum* (Marine diatom)	OECD 201	72-h E_bC_{50} 72-h E_rC_{50} 72-h E_yC_{50}	>6.0 mg/L >6.0 mg/L >6.0 mg/L (mm)	96 hour static Culture medium control. pH: 8.1-8.1 (start) 8.4-8.5 (end) 19.8 – 20.5ºC GLP	Minderhout, Kendall & Krueger (2007) ***SYN524464/0058****
			96-h E_bC_{50} 96-h E_rC_{50} 96-h E_yC_{50}	>6.0 mg/L >6.0 mg/L >6.0 mg/L		

				(mm)		
Sedaxane (purity 95.3%)	*Lemna gibba* (duckweed)	OECD 221	Frond No 7-day E_rC_{50} 7-day E_yC_{50}	6.5 mg/L 3.6 mg/L (mm)	7 day semi-static Dilution water control. pH: 7.3–7.4 (start) 8.6 - 9.0 (end) 23°C GLP	Bätscher (2007b) ***SYN524464/0039 ****
			Dry Weight 7-day E_rC_{50} 7-day E_yC_{50}	4.8 mg/L 2.7 mg/L (mm)		

Results are based on the mean measured concentrations (mm)
* The chronic endpoints of the studies are detailed in Table 65, below.

11.5.1 Acute (short-term) toxicity to fish

Four studies on sedaxane, with supporting specific analysis, showed short-term (96 hour) acute toxicity to fish across a range of species (see below). 96 hour LC_{50} values ranged from 0.62 to 4.2 mg a.i./L with the lowest LC_{50} value (LC_{50}=0.62 mg a.i./L) being for common carp (Gallagher *et al*, 2008). Common carp (a warm water species) is considered the most sensitive species relevant for classification of acute toxicity to fish.

Table 59: Summary of relevant acute endpoints for fish

Test type	Test substance	Test species	Endpoint	Value (mg a.i./L)	Reference
Acute Toxicity to Fish	Sedaxane technical (purity 95.3%)	*Cyprinus carpio* (common carp)	96 h LC_{50} (static)	0.62 mg a.i./L (mm)	Authors of vertebrate study (2008a) SYN524464_11104 *Annex I. 4.3.1.1*
	Sedaxane technical (purity 95.3%)	*Oncorhynchus mykiss* (rainbow trout)	96 h LC_{50} (static)	1.1 mg a.i./L (mm)	Author of vertebrate study (2008) SYN524464/0067 *Annex I. 4.3.1.2*
	Sedaxane technical (purity 98.2%)	*Pimephales promelas* (fathead minnow)	96 h LC_{50} (static)	0.98 mg a.i./L (mm)	Author of vertebrate study (2006) SYN524464/0012 *Annex I. 4.3.1.3*
	Sedaxane technical (purity 95.3%)	*Cyprinodon variegatus* ***(***sheepshead minnow***)***	96 h LC_{50} (static)	4.2 mg a.i./L (mm)	Authors of vertebrate study (2008b) SYN524464/0062 *Annex I. 4.3.1.4*

mm = mean measured

Study 1: Authors of vertebrate study (2008a; SYN524464_11104) *Annex I. 4.3.1.1*

The acute toxicity of sedaxane to common carp (*Cyprinus carpio)* was investigated in a 96-hour static test. Fish were exposed to nominal concentrations of 0.10, 0.20, 0.40, 0.80 and 1.6 mg a.i./L, alongside a dilution water control. Seven fish per tank were used for each concentration and for the

control treatment. Specific analysis showed mean measured concentrations of 0.12, 0.27, 0.37, 0.73 and 1.6 mg a.i./L. Measured concentrations at the start, at 48 hours and at the end of the test were in the range 93 to 99.4, 89.7 to 147% and 90.9 to 158%, respectively, of the nominal concentrations. Based on mean measured concentrations, the 96-hour LC_{50} for sedaxane to common carp (*Cyprinus carpio*) was determined to be 0.62 mg ai/L.

Table 60: Effects of Sedaxane on Common Carp (*Cyprinus carpio)*

Nominal Concentration (mg/L)	Mean Measured Concentration (mg/L)	Cumulative Mortality (n = 7)			
		24 hours	48 hours	72 hours	96 hours
Dilution water control	Dilution water control	0	0	0	0
0.10	0.12	0	0	0	0
0.20	0.27	0	0	0	0
0.40	0.37	0	0	0	0
0.80	0.73	1	5	5	5
1.6	1.6	6	7	7	7
LC_{50} mg/L		1.1	0.62	0.62	0.62
95% confidence interval		0.73 – 1.7	0.37 – 1.6	0.37 – 1.6	0.37 – 1.6

Study 2: Author of vertebrate study (2008; SYN524464/0067) *Annex I. 4.3.1.2*

The acute toxicity of technical sedaxane to rainbow trout (*Oncorhynchus mykiss)* was investigated in a 96-hour static test. Fish were exposed to dilutions 1:80, 1:40, 1:20, 1:10, 1:5 and 1:2.5 of a saturated solution , alongside a dilution water control. Corresponding mean measured test concentrations were 0.11, 0.23, 0.49, 1.0, 2.0 and 4.2 mg a.i./L. Seven fish per tank were used for each concentration and for the control treatment. At the start of the test, the measured concentrations of the test item in the dilutions 1:80, 1:40, 1:20, 1:10, 1:5 and 1:2.5 of the saturated solution were 0.12, 0.25, 0.53, 1.0, 2.1 and 4.2 mg/L, respectively. At the end of the test, 84 to 92% of the initially measured concentrations were found in the dilutions 1:80 to 1:10. The concentration measured in the dilution 1:5 of the filtrate after the test period of 24 hours was 98% of the initially measured concentration. Based on mean measured concentrations, the 96-hour LC_{50} for sedaxane to rainbow trout (*Oncorhynchus mykiss*) was 1.1 mg ai/L.

Study 3: Author of vertebrate study (2006; SYN524464/0012) *Annex I. 4.3.1.3*

The acute toxicity of technical sedaxane to fathead minnow (*Pimephales promelas)* was investigated in a 96-hour static test. Fish were exposed to nominal concentrations of 0.65, 1.3, 3.0, 5.0 and 10 mg a.i./L, alongside a dilution water control. Seven fish per tank were used for each concentration and for the control treatment. Specific analysis showed measured concentrations of sedaxane at the start, at 48 hours and at the end of the test were in the range 76 to 85%, 72 to 93% and 69 to 86%, respectively, of nominal. Mean measured concentrations were 0.48, 1.0, 2.5, 3.9 and 7.7 mg a.i./L. Based on mean measured concentrations, the 96-hour LC_{50} for sedaxane to fathead minnow (*Pimephales promelas*) was determined to be 0.98 mg a.i./L.

Study 4: Authors of vertebrate study (2008b; SYN524464/0062) *Annex I. 4.3.1.4*

ANNEX 1 - BACKGROUND DOCUMENT TO RAC OPINION ON *N*-{2-[[1,1'-BI(CYCLOPROPYL)]-2-YL]PHENYL}-3-(DIFLUOROMETHYL)-1-METHYL-1*H*-PYRAZOLE-4-CARBOXAMIDE; SEDAXANE

The study has not been submitted for the EU review of sedaxane. An extended summary is therefore presented for this study.

Report:	Authors of vertebrate study. 2008b, SYN524464 – A 96-Hour Static Acute Toxicity Test with the Sheepshead Minnow (Cyprinodon variegatus), Report Number 528A-163A. 8 April 2008. Wildlife International Ltd, Easton, MD, USA. .(Syngenta File No. SYN524464/0062)

GUIDELINES: OPPTS 850.1075 Ecological Effects Test Guidelines 'Fish Acute Toxicity Test, Freshwater and Marine'. Public draft April 1996.: U.S. Environmental Protection Agency. 1985. Standard Evaluation Procedure, *Acute Toxicity Test for Estuarine and Marine Organisms (Estuarine Fish 96-Hour Acute Toxicity Test)*. Lab of vertebrate study. 1996. Standard Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians. American Society for Testing and Materials.

GLP: Yes

Validity: Yes

EXECUTIVE SUMMARY

The acute toxicity of SYN524464 to sheepshead minnow (*Cyprinodon variegatus*) was determined under static conditions. Fish were exposed to a range of mean measured concentrations of 0.36, 0.67, 1.4, 3.0 and 5.8 mg a.i./L and a dilution water control. The 96 hour LC50 (based on mean measured concentrations of SYN524464) was 4.2 mg a.i./L.

MATERIALS AND METHODS

Materials:

Test Material	SYN524464
Lot/Batch #:	SMU6LP006/MILLED
Purity:	95.3% (sum of 83.0% SYN508210 and 12.3% SYN508211)
Description:	Off-white powder
Stability of test compound:	Stable under standard conditions
Reanalysis/expiry date:	January 2011
Treatments	
Test concentrations:	Dilution water control and nominal concentrations of 0.44, 0.88, 1.8, 3.5 and 7.0 mg a.i./L (mean measured 0.36, 0.67, 1.4, 3.0 and 5.8 mg a.i./L)
Dilution water:	Saltwater (0.45 μm filtered seawater)
Solvent:	None
Analysis of test concentrations:	Yes (0, 48 and 96 hours)
Test organisms	
Species:	Sheepshead minnow (*Cyprinodon variegatus*)
Source:	Aquatic BioSystems, Inc. of Fort Collins, Colorado USA

Acclimatisation period:	At least 14 days
Treatment for disease:	None
Weight and length of dilution water control fish at end of exposure period:	Mean length: 2.6 cm (range of 2.3 to 2.9 cm) Mean weight: 0.22 grams (range of 0.16 to 0.32 grams)
Feeding:	None during test
Test design	
Test vessels:	9 L Glass aquaria containing 7 L water. The depth of test water in a representative chamber was approximately 13 cm.
Test medium:	Natural seawater (0.45 μm filtered)
Replication:	None
No of fish per tank:	7
Exposure regime:	Static
Duration:	96 hours
Environmental conditions	
Test temperature:	21.8 – 22.7 °C
pH:	8.0 – 8.3 measured daily
Dissolved oxygen:	6.2 – 7.6 mg/L measured daily
Salinity of dilution water:	20‰ to 21‰ measured in the control replicate at test initiation and termination
Lighting:	16 hours fluorescent light and 8 hours dark with 30 minute dawn and dusk transition periods

Study Design and Methods
Experimental dates: Start 5 August End 1 September 2007.

Three stock solutions were prepared at a nominal concentration of 10 mg a.i./L, by mixing a calculated amount of SYN524464 into dilution water (filtered saltwater). The three stock solutions were stirred overnight with top-down electric mixers. Sonication was also added to two of the three solutions while they stirred overnight. At the termination of stirring, stock solutions were clear and colorless with particles of precipitate on the surface. The stocks were allowed to settle for approximately four hours after stirring. The three stocks were then analyzed to determine the highest concentration achieved with the mixing methods employed and to identify the stock solution to be used for preparation of lower concentrations. The analyses indicated that the highest stock concentration, using sonication and stirring, was approximately 7 mg a.i./L.

The 7.0 mg a.i./L stock solution was used as the highest concentration test solution. Aliquots of the 7.0 mg a.i./L stock solution were siphoned off, being careful to avoid any particulate matter, and proportionally diluted with saltwater to prepare 6 L of test solution at target concentrations of 0.44, 0.88, 1.8 and 3.5 mg a.i./L. The solutions were mixed by stirring. At test initiation and termination, all solutions appeared clear and colorless.

At the start of the test seven fish were indiscriminately allocated to each of the test concentrations and the dilution water control. Test chambers were indiscriminately positioned in an environmental chamber set to maintain the desired test temperature. Observations for mortalities and symptoms of toxicity were made at 3.5, 24, 48, 72 and 96 hours. The LC_{50} values were estimated from the data obtained.

Temperature was measured in each test chamber at test initiation and at approximately 24-hour intervals during the test using a liquid-in-glass thermometer. A continuous temperature recorder (Fulscope ER/C Recorder) was used to measure the temperature in the negative control test chamber throughout the test.
The concentrations of SYN524464 in the test solutions were measured at 0, 48 and 96 hours using high performance liquid chromatography (HPLC) using variable wavelength detection.

RESULTS AND DISCUSSION
The concentrations of SYN524464 were determined in the test. Mean measured concentrations calculated from the average of all samples ranged from 76 to 86% of nominal concentrations. Mean measured concentrations were used for the reporting of the results.

All sheepshead minnows in the negative control group and in the 0.36 and 0.67 mg a.i./L treatment groups appeared normal throughout the test. Percent mortality in the 1.4, 3.0 and 5.8 mg a.i./L treatment groups at test termination was 0, 0 and 100%, respectively. All fish in the 1.4 mg a.i./L treatment group appeared lethargic at test termination. All fish in the 3.0 mg a.i./L treatment group were also exhibiting signs of toxicity at test termination. The no-mortality concentration was 3.0 mg a.i./L. The NOEC was 0.67 mg a.i./L.

Table 61: Effects of SYN524464 on the survival of the Sheepshead Minnow (*Cyprinodon variegates*)

Measured concentration (mg a.i./L)	**Cumulative percentage mortality observed**				
	3 hour	**24 hours**	**48 hours**	**72 hours**	**96 hours**
Dilution water control	0	0	0	0	0
0.36	0	0	0	0	0
0.67	0	0	0	0	0
1.4	0	0	0	0	0
3.0	0	0	0	0	0
5.8	0	0	0	0	100
LC_{50} mg formulation/L	>5.8	>5.8	>5.8	>5.8	4.2
(95% confidence interval)	-	-	-	-	3.0 – 5.8

CONCLUSION: The 96-hour LC_{50} for SYN524464 to sheepshead minnow (*Cyprinodon variegatus*) was 4.2 mg a.i./L, based on the mean measured concentrations of SYN524464.

11.5.2 Acute (short-term) toxicity to aquatic invertebrates

Two studies on sedaxane, with supporting specific analysis, showed short-term (48 or 96 hour) acute toxicity to several aquatic invertebrates (see below). A 96 hour EC_{50} value in Eastern oyster was 3.5 mg a.i./L is also presented in the Environmental Risk Assessment for Registration of the new chemical Sedaxane from US-EPA. However, the study report and the study summary are not

available. This toxicity data has not been included in the dataset used for the sedaxane classification proposal.

The 48 hour EC_{50} value in daphnia was 6.10 mg a.i./L. The lowest EC_{50} of 1.5 mg a.i./L in the saltwater mysid shrimp (Gallagher, Kendall & Kreuger, 2008c) is considered appropriate to use for classification of acute toxicity to aquatic invertebrates.

Table 61: Summary of relevant acute endpoints for aquatic invertebrates

Test type	Test substance	Test species	Endpoint	Value (mg a.i./L)	Reference
Acute Toxicity to Aquatic Invertebrates	Sedaxane technical	*Americamysis bahia* Mysid shrimp	96 hour EC_{50} (static)	1.5 mg/L (mm)	Gallagher, Kendall & Krueger. (2008c)
		Daphnia magna	48 hour EC_{50} (static)	6.10 mg/L (mm)	Ricketts & Paddick (2006)

mm = mean measured

Study 1: Gallagher, Kendall & Krueger(2008c; SYN524464/0059)

The study has not been provided for the EU review of sedaxane. An extended summary is therefore presented for this study.

Report:	Gallagher, T. Kendall and H. Krueger. 2008c, SYN524464 – A 96-hour static acute toxicity test with the saltwater mysid (Americamysis bahia). Report Number 528A-162. 15 February 2008. Wildlife International Ltd, Easton, MD, USA. (Syngenta File No. SYN524464/0059)

GUIDELINES: U.S. Environmental Protection Agency. 1996. OPPTS Number 850.1035: *Mysid Acute Toxicity Test*. Series 850 – Ecological Effects Test Guidelines (*draft*).: U.S. Environmental Protection Agency. 1985. *Standard Evaluation Procedure, Acute Toxicity Test for Estuarine and Marine Organisms (Shrimp 96-Hour Toxicity Test)*. Hazard Evaluation Division. Office of Pesticide Programs. EPA-540/9-85-010. Washington, DC.: ASTM Standard E729-96. 1996. *Standard Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians*. American Society for Testing and Materials.

GLP: Yes

Validity: Yes

EXECUTIVE SUMMARY

The acute toxicity of SYN524464 to saltwater mysids (*Americamysis bahia*) was determined under static conditions. This study was run with mean measured concentrations of 0.57, 1.2, 2.3, 4.7, and 8.1 mg a.i./L together with a negative control.

The LC_{50} was 1.5 mg a.i./L based on mean measured concentrations.

MATERIALS AND METHODS

Materials:

Test Material SYN524464

Lot/Batch #:	SMU6LP006/MILLED
Purity:	95.3% (sum of 83.0% SYN508210 and 12.3% SYN508211)
Description:	Off-white powder
Stability of test compound:	Stable under standard conditions
Reanalysis/expiry date:	January 2011

Treatments

Test concentrations:	Dilution water control and nominal concentrations of 0.63, 1.3, 2.5, 1.3, 2.5 and 5.0 mg a.i./L (mean measured 0.57, 1.2, 2.3, 4.7 and 8.1 mg a.i./L)
Dilution water:	Saltwater (0.25 μm filtered seawater)
Solvent:	None
Analysis of test concentrations:	Yes (0 and 96 hours)

Test organisms

Species:	Saltwater mysid (*Americamysis bahia*)
Source:	Test facility
Acclimatisation period:	Adults acclimated 14 days before collection of juveniles
Treatment for disease:	None
Life stage of test organism:	Juvenile
Feeding:	Live brine shrimp (*Artemia* sp.) daily during test

Test design

Test vessels:	Test chambers were 2L glass beakers containing 1.5 L of test solution. The depth of the test water in a representative test chamber was approximately 11.8 cm.
Replication:	2 replicates, 10 mysids per replicate
Exposure regime:	Static
Duration:	96 hours

Environmental conditions

Test temperature:	24.9 to 25.4 °C at start and 25.2 to 25.3 °C at end, also monitored continuously in one negative control replicate
pH range:	8.1 to 8.2 measured daily
Dissolved oxygen:	6.5 to 7.1 mg/L measured daily
Salinity of dilution water:	20‰ at test start
Lighting:	16 hours fluorescent light and 8 hours dark daily, with 30 minute dawn and dusk transition periods. Light intensity ≈313 lux at water surface.

Study Design and Methods

Experimental dates: Start 8 August End 13 August 2007

The chambers were indiscriminately positioned by treatment group in an environmental chamber designed to maintain the desired test temperature throughout the test period. Two replicate tanks were prepared for the control and each test solution. Ten mysids were randomly allocated to each prepared test vessel.

Stock solutions were prepared at nominal concentrations of 5.0 or 10 mg a.i./L, the two highest test concentrations, by mixing a calculated amount of SYN524464 into dilution water (filtered saltwater). The stock solutions were sonicated for approximately 30 minutes and then stirred overnight. At the termination of stirring, stock solutions were clear and colorless with a few particles of precipitate on the surface. The 10 mg a.i./L stock solutions had more precipitate than the 5.0 mg a.i./L solutions. The 10 mg a.i./L stock solutions were used as the highest concentration test solution. Aliquots of the 5.0 mg a.i./L stock solution were proportionally diluted with saltwater to prepare 1500 mL of test solution at nominal concentrations of 0.63, 1.3 and 2.5 mg a.i./L. The solutions were mixed by stirring. All test solutions were adjusted to 100% active ingredient during preparation, based on the test substance purity (95.3%).

The concentrations of SYN524464 in the test solutions were measured at 0 and 96 hours using high performance liquid chromatography (HPLC) using variable wavelength detection.

Observations were made for mortality and clinical symptoms of toxicity at approximately 4.5, 24, 48, 72 and 96 hours.

RESULTS AND DISCUSSION

Mean measured concentrations for the study were 0.57, 1.2, 2.3, 4.7 and 8.1 mg a.i./L, representing 90, 92, 92, 94 and 81% of nominal concentrations, respectively. Mean measured concentrations were used for the reporting of the results.

All saltwater mysids in the negative control group and in the 0.57 mg a.i./L treatment group appeared normal throughout the test. Percent mortality in the 1.2, 2.3, 4.7 and 8.1 mg a.i./L treatment groups at test termination was 10, 100, 100 and 100%, respectively. Surviving mysids in the 1.2 mg a.i./L treatment group were normal in appearance at test termination. The no-mortality concentration and the NOEC were both 0.57 mg a.i./L.

Table 62: Effects of test material on the survival of saltwater mysids (Americamysis bahia) following exposure for 96 hours in a flow-through test

Mean measured concentration	Cumulative mortality observed, n = 20 [a]				
(mg a.i./L)	4.5 hour	24 hour	48 hour	72 hour	96 hour
Dilution water control	0	0	0	0	0
0.57	0	0	0	0	0
1.2	0	0	2	2	2
2.3	0	12	20	20	20
4.7	0	20	20	20	20
8.1	0	20	20	20	20
LC_{50} (mg ai/L)	-	2.1	1.5	1.5	1.5
95% confidence limits	-	1.2 – 4.7	1.2 – 2.3	1.2 – 2.3	1.2 – 2.3
Method	-	Nonlinear Interpolation			

a Ten mysids were exposed in each test vessel, two replicates per treatment.

CONCLUSION: The 96 hour LC_{50} for test material to the saltwater mysid (*Americamysis bahia*) was calculated to be 1.5 mg a.i./L, based on mean measured concentrations.

Study 2: Ricketts & Paddick (2006; SYN524464/0011)

In a 48 hour static toxicity study to *Daphnia magna*, 20 animals/ group were exposed to nominal concentrations of 0.5, 1.0, 2.0, 4.0 and 8.0 mg sedaxane/L and a dilution water control. Four replicate vessels (5 daphnia per vessel) were used for each concentration and for the control treatment. Specific analysis showed mean measured concentrations of 0.395, 0.810, 1.61, 3.79 and 6.14 mg a.i./L. Measured sedaxane concentrations ranged from 77 – 96% of nominal concentrations at the start of the test and 77 – 93% of nominal at the end of the test. Based on mean measured concentrations, the 48-hour EC_{50} for sedaxane to *Daphnia magna* was estimated to be 6.10 mg a.i./L (95 % confidence interval of 5.05-11.0 mg a.i./L).

11.5.3 Acute (short-term) toxicity to algae or other aquatic plants

Four studies showed short-term (72 and 96 hour) acute toxicity of sedaxane to algae across a range of species, summarised in the table below, together with one study on the effect of sedaxane on the aquatic plant *Lemna gibba* (duckweed). For consideration of acute toxicity the E_rC_{50} (growth rate) is preferred, whilst for consideration of chronic toxicity, the EC_x or NOEC values are used (see section 11.6.2).

The lowest EC_{50} value (72-h E_rC_{50}) in freshwater green algae (*Pseudokirchneriella subcapitata*) of 2.8 mg a.i./L (Bätscher, 2007a) is considered appropriate to use for classification of acute toxicity to algae and aquatic plants.

Table 63: Summary of relevant acute growth endpoints for algae and aquatic plants

Test type	Test substance	Test species	Endpoint	Value (mg a.i./L)	Reference
Acute Toxicity to Algae and Aquatic Plants	Sedaxane	*Pseudokirchneriella subcapitata* Freshwater Green Algae	72 hour E_rC_{50} 96 hour E_rC_{50} (static)	2.8 mg/L 3.0 mg/L (mm)	Bätscher, (2007a) *SYN524464/0037*
		Navicula pellicosa Freshwater Diatom	72 hour E_rC_{50} 96 hour E_rC_{50} (static)	8.7 mg/L 10 mg/L (mm)	Büche, (2007a) ***SYN524464/0044***
		Anabaena flos-aquae (freshwater Cyanobacteria)	72 hour E_rC_{50} 96 hour E_rC_{50} (static)	>6.5 mg/L (mm)	Büche (2007b) ***SYN524464/0045***
		Skeletonema costatum (Marine diatom)	72 hour E_rC_{50} 96 hour E_rC_{50} (static)	>6.0 mg/L (mm)	Minderhout, Kendall & Krueger (2007) ***SYN524464/0058***
		Lemna gibba (Duckweed)	7 day E_rC_{50} (frond) 7 day E_rC_{50} (dry weight) (semi-static)	6.5 mg/L 4.8 mg/L (mm)	Bätscher (2007b) ***SYN524464/0039***

mm = mean measured

Study 1: Bätscher (2007a; SYN524464/0037)

In a 96-hour static toxicity study to freshwater green algae (*Pseudokirchneriella subcapitata*), algae cells (starting density 10000 cells/mL) were exposed to sedaxane (purity 95.3%) at the following dilutions: 1:46, 1:22, 1:10, 1:4.6, 1:2.2 and the undiluted filtrate, and a culture medium control. There were three replicate cultures for each exposure concentration and six replicates for the control. Specific analysis at the start of the test showed that the measured concentrations of the 1:10, 1:4.6, 1:2.2 dilutions and the undiluted filtrate were 1.1, 2.2, 4.7 and 9.9 mg a.i./L, respectively. At the end of the test, the measured values ranged from 97 to 100% of the initial measured values. Based on mean measured sedaxane concentrations, the 72 hour E_bC_{50} and E_rC_{50} to the green alga *Pseudokirchneriella subcapitata* (formerly *Selenastrum capricornutum*) was 1.9 mg a.i./L and 2.8 mg a.i./L respectively. The 96-hour E_bC_{50} and E_rC_{50} was 1.9 mg a.i./L and 3.0 mg a.i./L respectively. The 72- and 96 hour NOEC (biomass, growth and yield) was 1.0 mg a.i./L.

Table 64: Effects of sedaxane on green algae *(Pseudokirchneriella subcapitata)*

Nominal concentration (mg/L)	Mean measured concentration (mg/L)	Mean cell density (cells/mL*10000)				Area under the curve		Growth rate	
		24 hours	48 hours	72 hours	96 hours	Mean inhibition (%) 0 - 72 hours	Mean inhibition (%) 0 - 96 hours	Mean inhibition (%) 0 - 72 hours	Mean inhibition (%) 0 - 96 hours
Control	n.a.	4.0	13.4	53.1	113.6	0	0	0	0
0.21	n.a.	4.6	14.8	64.3	129	-18.4	-16.9	-4.8	-2.7
0.45	n.a.	4.1	15.1	60.8	120.5	-13.7	-10.5	-3.4	-1.3
1.0	1.1	4.3	13.9	55.5	113	-4.8	-2.4	-1.1	0.1
2.2	2.2	3.8	6.0	11.3	30.7	68.7	73.4	39.0	27.7
4.6	4.7	2.6	1.9	2.1	2.4	92.8	96.6	82.0	81.6
9.8	9.9	3.0	2.0	2.4	2.5	91.0	95.9	79.0	80.7

n.a. = not analysed

- % inhibition: increase in growth relative to that of the solvent control

Study 2: Büche (2007a; SYN524464/0044)

In a 96-hour static toxicity study of sedaxane to freshwater diatom (*Navicula pelliculosa*), algal cells (starting density 10,000 cells/mL) were exposed to a saturated solution (undiluted filtrate) and dilutions 1:2, 1:4, 1:8, 1:16, and 1:32 of the filtrate and a culture medium control. There were four replicate vessels for each exposure concentration and six replicates for the control. HPLC analysis showed that mean measured concentrations of sedaxane were: 1.2, 2.4, 4.7 and 12 mg a.i./L for the 1:8, 1:4, 1:2 and the undiluted filtrate, respectively. The concentrations measured at the end of the test corresponded to between 74 and 123% of the initially measured values. The algal cell densities were measured at 24, 48, 72 and 96 hours and the mean biomass, growth rate and yield calculated. Based on mean measured concentrations, the 72-hour E_rC_{50} for sedaxane to *Navicula pelliculosa* was 8.7 mg a.i./L, the E_yC_{50} was 4.8 mg a.i./L and the E_bC_{50} was 4.8 mg a.i./L. The 72-hour E_rC_{10} was 4.3 mg/L, the E_yC_{10} was 3.0 mg/L and the E_bC_{10} was 3.0 mg/L. The 72-hour NOE_rC was 2.4 mg/L, the NOE_yC was 2.4 mg/L and the NOE_bC was 2.4 mg/L. The 96-hour E_rC_{50} for sedaxane was 10 mg a.i./L, the E_yC_{50} was 5.7 mg a.i./L and the E_bC_{50} was 5.3 mg a.i./L. The 96-hour E_rC_{10} was 5.3 mg/L, the E_yC_{10} was 3.0 mg/L and the E_bC_{10} was 2.9 mg/L. The 96-hour NOE_rC was 2.4 mg/L, the NOE_yC was 1.2 mg/L and the NOE_bC was 1.2 mg/L.

Study 3: Büche, (2007b; SYN524464/0045)

The effect of sedaxane on the growth of the freshwater cyanobacterium ("blue alga") *Anabaena flos-aquae* was investigated in a 96-hour static test. Algal cells (starting density 10,000 cells/mL) were exposed to a saturated solution (undiluted filtrate) and dilutions 1:2.2, 1:4.6, 1:10, 1:22 and 1:46 of the filtrate and a control. There were three replicate cultures for each exposure concentration and six replicates for the control. HPLC analysis showed that mean measured concentrations of sedaxane were 4.3 and 6.5 mg a.i./L for the 1:2.2 dilution and the undiluted filtrate, respectively. Concentrations at the start of the test were 4.55 and 10.2 mg a.i./L, respectively, for the 1:2.2 dilution and the undiluted filtrate (loading rate of 100 mg a.i./L); at the end of the test they were 4.06 and 4.12 mg a.i./L. The algal cell densities were measured at 24, 48, 72 and 96 hours and the mean biomass, growth rate and yield calculated. Based on mean measured concentrations, the 72- and 96-hour E_rC_{50},

E_bC_{50} and E_yC_{50} values for sedaxane to *Anabaena flos-aquae* were >6.5 mg a.i./L. The 72- and 96-hour NOEC (biomass, growth and yield) was 4.3 mg a.i./L.

Study 4: Minderhout, Kendall and Krueger (2007; SYN524464/0058)

The study has not been provided for the EU review of sedaxane. An extended summary of this study is therefore presented

Report:	Minderhout T, Kendall T and Krueger H, 2007, Report title SYN524464: a 96-hour toxicity test with the marine diatom (*Skeletonema costatum*), Report Number 528A-165, 24 January 2008. Wildlife International, Ltd., Easton, Maryland. (Syngenta File No. SYN524464/0058)

GUIDELINES: ISO 10253 Standard: *Water Quality – Marine Algal Growth Inhibition Test with Skeletonema costatum and Phaeodactylum tricornutum.* 2nd Edition, Technical Committee ISO/TC 147, Water Quality Subcommittee SC 5, Biological Methods (2006): OECD Guidelines for Testing of Chemicals, Section 2 - Effects on Biotic Systems, Method 201: Freshwater Alga and Cyanobacteria, Growth Inhibition Test (2006): Official Journal of the European Communities, Dir 92/69/EEC, O.J. L383A, Part C.3: Algal inhibition test (1992): US EPA Ecological Effects Test Guidelines, OPPTS 850.5400: Algal Toxicity, Tiers I and II, (1996): ASTM, *Standard Guide for Conducting Static 96-Hour Toxicity Tests with Microalgae* (1990)

GLP: Yes

Validity: Yes (One of the validity criteria of the OECD guideline is not met: the mean coefficient of variation for section-by-section specific growth rates in the control cultures is 72% but should not have exceeded 35%. The validity criteria of the guideline are not designed for the marine algae *S. costatum*. Given that no effect has been observed in the study, the exceedance of this validity criteria is considered to have no outcome on the study results. The study is considered less reliable but sufficiently informative for the purpose of classificiation.)

EXECUTIVE SUMMARY

The toxicity of SYN524464 to the marine diatom *Skeletonema costatum* was determined. Algae were exposed to nominal concentrations of 0.44, 0.88, 1.8, 3.5 and 7.0 mg ai/L alongside a culture medium control. Based on mean measured concentrations, the 72-hour E_bC_{50}, E_rC_{50} and E_yC_{50} were all >6.0 mg ai/L, the highest concentration tested. The 96-hour E_bC_{50}, E_rC_{50} and E_yC_{50} were also all >6.0 mg ai/L, the highest concentration tested.

MATERIALS AND METHODS

Materials:

Test Material	SYN524464
Lot/Batch #:	SMU6LP006/MILLED
Purity:	95.3% (sum of 83.0% SYN508210 and 12.3% SYN508211)
Description:	Solid
Stability of test compound:	Stable under standard conditions
Reanalysis/expiry date:	January 2011

Treatments

Test concentrations:	Culture medium control and nominal concentrations of 0.44, 0.88, 1.8, 3.5 and 7.0 mg ai/L	
Solvent:	None	
Positive control:	Not applicable	
Analysis of test concentrations:	Yes, analysis of SYN524464 at 0 and 96 hours	
Test organism		
Species:	*Skeletonema costatum*, Strain No. CCMP 1332	
Source:	Continuous laboratory cultures, originally obtained from Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP), West Booth Bay Harbor, Maine, USA.	
Test design		
Test vessels:	250 mL glass Erlenmeyer flasks containing 100 mL of media covered with glass dishes	
Test medium:	Saltwater algal medium	
Replication:	Three vessels for the control and three vessels for each test concentration	
Starting cell density:	7.7×10^4 cells/mL	
Exposure regime:	Static	
Aeration:	No	
Duration:	96 hours	
Environmental conditions		
Test temperature:	19.8 – 20.5°C	
pH:	test start:	8.1 to 8.1
	test end:	8.4 to 8.5
Lighting:	16 hours per day of cool-white fluorescent lighting at an intensity of 3,680 to 4,460 Lux, and 8 hours of darkness	

Study Design and Methods
Experimental dates: Start 2 November End 6 November, 2007.

A stock solution with a nominal concentration of 7.0 mg ai/L was prepared by dissolving 0.1049 g of the test item completely in 1,000 mL of algal medium. Appropriate volumes of the stock solution were diluted to give the test concentration series. The control consisted of culture medium only.

An aliquot of test solution was placed into each test vessel and the test was started by inoculation of 77,000 algal cells per mL of test medium. Test solutions were constantly shaken and were held in a temperature controlled incubator under continuous illumination.

Small volumes of all test concentrations and controls were taken from all test flasks after 24, 48, 72 and 96 hours of exposure. The algal cell densities in these samples were determined by counting using a hemacytometer and microscope. In addition, after 96 hours exposure, a sample was taken from the control and from each treatment group. The shape of the algal cells was examined microscopically in these samples.

The pH was measured at the start and at the end of the test. The water temperature was measured daily in a flask incubated under the same conditions as the test flasks and ranged from 19.8 to 20.5°C.

The test concentrations were verified by chemical analysis of SYN524464 at 0 and 96 hours, using high performance liquid chromatography.

RESULTS AND DISCUSSION

At the start of the test, the measured concentrations were in the range 99.0 to 101% of the nominal values and at the end of the test were in the range 69.1 to 89.9% (see table below). The limit of quantification in this study was 0.300 mg ai/L. Mean measured concentrations were used for the calculation and reporting of results.

Table 64-2: Analytical results

Nominal concentrations of ai (mg/L)	% of nominal measured at 0 hours	% of nominal measured at 96 hours	Mean measured concentration (mg ai/L)
Control (0.0)	<LOQ	<LOQ	--
0.44	99.5	85.8	93
0.88	99.5	88.3	94
1.8	99.0	87.0	94
3.5	99.8	89.9	94
7.0	101	69.1	86

The algal cell densities were measured at 24, 48, 72 and 96 hours and the mean biomass, growth rate and yield calculated. The 72-hour and 96-hour E_bC_{50}, E_yC_{50} and E_rC_{50} values (defined as the concentration resulting in 50% reduction of each parameter) were calculated using non-linear regression and linear interpolation analysis. For determination of the LOEC (Lowest Observed Effect Concentration) and NOEC (No Observed Effect Concentration) values, a Dunnett's test was used to identify significant differences in the calculated mean biomass, growth rate and yield of test item treatments compared to the control.

There were no abnormalities, observed microscopically, in the control or any of the treatment groups at 96 hours.

Cell Density*:* The cell density values for 0 to 72 hours and 0 to 96 hours were calculated for each replicate culture and the means are shown below, alongside the estimated EC_{50} values.

Table 64-3: Mean values at each concentration of SYN524464 for the cell density at 72 and 96 hours for *Skeletonema costatum* and relevant endpoints

Mean measured concentrations of ai (mg/L)	Mean cell density ($x 10^6$) 0 – 72 hrs *	Percentage inhibition	Mean cell density ($x 10^6$) 0 – 96 hrs *	Percentage inhibition
Control (0.0)	1.6	--	2.7	--
0.41	1.8	-9.0	2.7	1.2
0.83	2.0	-20	2.9	-6.1
1.7	1.3	23	2.2	20
3.3	2.1	-26	2.7	-1.0
6.0	2.2	-32	2.9	-6.9
EC_{50} mg ai/L (95% confidence limits)	>6.0 (Not Calculable)		>6.0 (Not Calculable)	
NOEC	6.0		6.0	
LOEC	>6.0		>6.0	

* = No statistically significant differences (p>0.05) from the negative control

Biomass (area under the growth curve)*:* The areas under the growth curve for 0 to 72 hours and 0 to 96 hours were calculated for each replicate culture and the means are shown below, alongside the estimated EC_{50} values.

Table 64-4: Mean values at each concentration of SYN524464 or the biomass integral (area under the growth curve) at 72 and 96 hours for *Skeletonema costatum* and relevant endpoints

Mean measured concentrations of ai (mg/L)	Mean biomass integral ($x 10^7$) 0 – 72 hrs *	Percentage inhibition	Mean biomass integral ($x 10^7$) 0 – 96 hrs *	Percentage inhibition
Control (0.0)	44,155,780	--	94,496,656	--
0.41	47,256,500	-7.0	98,972,780	-4.7
0.83	47,208,928	-6.9	103,549,592	-9.6
1.7	28,728,148	35	68,051,956	28
3.3	45,607,312	-3.3	101,367,644	-7.3
6.0	51,463,636	-17	110,353,608	-17
E_bC_{50} mg ai/L (95% confidence limits)	>6.0 (Not Calculable)		>6.0 (Not Calculable)	
NOEC	6.0		6.0	
LOEC	>6.0		>6.0	

* = No statistically significant differences (p>0.05) from the negative control

Growth rates: The growth rate 0 to 72 hours and 0 to 96 hours were calculated for each replicate culture and the means are shown below, alongside the estimated EC_{50} values.

Table 64-5: Mean values at each concentration of SYN524464 for the growth rate at 72 and 96 hours for Skeletonema costatum and relevant endpoints

Mean measured concentrations of ai (mg/L)	Mean growth rate 0 – 72 hrs *	Percentage inhibition	Mean growth rate 0 – 96 hrs *	Percentage inhibition
Control (0.0)	0.0425	--	0.0370	--
0.41	0.0436	-2.4	0.0369	0.23
0.83	0.0451	-6.0	0.0377	-1.8
1.7	0.0376	12	0.0345	6.6
3.3	0.0456	-7.2	0.0372	-0.39
6.0	0.0463	-8.8	0.0377	-1.9
E_rC_{50} mg ai/L (95% confidence limits)	>6.0 (Not Calculable)		>6.0 (Not Calculable)	
NOEC	6.0		6.0	
LOEC	>6.0		>6.0	

* = No statistically significant differences (p>0.05) from the negative control

Yield: The yield 0 to 72 hours and 0 to 96 hours were calculated for each replicate culture and the means are shown below, alongside the estimated EC_{50} values.

Table 64-6: Mean values at each concentration of SYN524464 for the yield at 72 and 96 hours for *Skeletonema costatum* and relevant endpoints

Mean measured concentrations of ai (mg/L)	Mean yield (x 10^6 cells/mL) 0 – 72 hrs *	Percentage inhibition	Mean yield (x 10^6 cells/mL) 0 – 96 hrs *	Percentage inhibition
Control (0.0)	1,571,175	--	2,623,898	--
0.41	1,718,768	-9.4	2,590,922	1.3
0.83	1,905,282	-21	2,789,773	-6.3
1.7	1,198,017	24	2,078,967	21
3.3	1,995,238	-27	2,651,456	-1.1
6.0	2,097,777	-34	2,809,721	-7.1
E_yC_{50} mg ai/L (95% confidence limits)	>6.0 (Not Calculable)		>6.0 (Not Calculable)	
NOEC	6.0		6.0	
LOEC	>6.0		>6.0	

* = No statistically significant differences (p>0.05) from the negative control

CONCLUSION: Based on mean measured concentrations, the 72-hour EC_{50}, E_bC50, E_rC50 and E_yC50 values for SYN524464 to *Skeletonema costatum* were all >6.0 mg ai/L. The 96-hour EC_{50}, E_bC50, E_rC50 and E_yC50 values for SYN524464 to *Skeletonema costatum* were all >6.0 mg ai/L.

The Lowest Observed Effect Concentration at 72 and 96 hours, based on cell density, biomass integral, growth rate and yield, was >6.0 mg ai/L, and the No Observed Effect Concentration was 6.0 mg ai/L.

One of the validity criteria of the OECD guideline is not met: the mean coefficient of variation for section-by-section specific growth rates in the control cultures is 72% but should not have exceeded 35%. The validity criteria of the guideline are not designed for the marine algae *S. costatum*. Given that no effect has been observed in the study, the exceedance of this validity criteria is considered to have no outcome on the study results. The study is considered less reliable but sufficiently informative for the purpose of classificiation.

Study 5: Bätscher (2007b; SYN524464/0039)

The study has not been provided for the EU review of sedaxane. An extended summary of this study is therefore presented.

Report:	Bätscher R 2007b, SYN524464 – Toxicity to the aquatic higher plant *Lemna gibba* in a 7-day semi-static growth inhibition test. Report Number B27898, 17 August 2007. RCC Ltd, CH-4452 Itingen, Switzerland. (Syngenta file no SYN524464/0039)

GUIDELINES: OECD 221 (2004), EPA OPPTS 850.4400 (1996)

GLP: Yes

Validity: Yes

EXECUTIVE SUMMARY

The toxicity of SYN524464 to the aquatic plant *Lemna gibba* was determined in a 7-day semi-static test. The test incorporated 6 concentrations (0.28, 0.59, 1,2, 2.4, 5.0 and 9.9 mg ai/L, based on mean measured concentrations) and a dilution water control.

For frond number, the 7-day EC_{50} for yield (EyC_{50}) and growth rate (ErC_{50}) for SYN524464 to *Lemna gibba* were 0.67 and 1.8 mg ai/L respectively, based on mean measured concentrations.

For dry weight, the 7-day EC_{50} for yield (EyC_{50}) and growth rate (ErC_{50}) for SYN524464 to *Lemna gibba* were 0.73 and 1.0 mg ai/L respectively, based on mean measured concentrations.

MATERIALS AND METHODS

Materials:

Test Material:	SYN524464
Description:	Off-white powder
Batch number:	SMU6LP006/MILLED
Purity:	95.3% SYN508210 (trans isomer): 83.0% SYN508211 (cis isomer): 12.3%
Stability:	Reanalysis date: January 2011
Treatments	
Test concentrations:	Dilution water control; mean measured concentration of 0.28, 0.59, 1,2, 2.4, 5.0 and 9.9 mg ai/L.
Vehicle and/or positive control:	n/a

Analysis of test concentrations:	Yes, days 0 (fresh solutions) and 7 (old solutions) (based on analysis of SYN524464)
Test organisms	
Species:	*Lemna gibba*
Source:	In-house cultures
Test design	
Test vessels:	250 mL glass dishes
Test medium:	
Replication:	Three vessels for the control and each test concentration
Initial frond number:	3 plants each consisting of 4 fronds, total 12 fronds
Exposure regime:	Semi-static (medium renewal on day 5)
Environmental conditions	
Temperature:	23°C
pH:	7.3 to 7.4 at test initiation; 8.6 to 9.0 at test termination
Lighting:	Continuous illumination, mean light intensity of 7500 Lux (range 6700 – 8200 Lux).

Study Design and Methods
Experimental dates: Start 15 May End 3 July 2006

Saturated stock solutions of SYN524464 were prepared by stirring 100 mg (test initiation) and 100.1 mg (medium renewal) of test item in 1000 mL nutrient medium for 3 hours. These were then filtered (0.45µm) and diluted 1:2, 1:4, 1:8, 1:16 and 1:32 to produce the test concentration range. Test solutions were transferred into 250 mL crystallizing dishes and inoculated with *Lemna* plants. Cultures were then transferred to a temperature-controlled room where they were maintained for 7 days under the conditions indicated above. Assessments of frond number were made on days 0, 3, 5 and 7. Fronds were harvested for measurement of dry weight after 7 days, and the initial dry weight was determined using 12 fronds from pre-test cultures collected at day 0. Temperature was measured daily while pH was recorded at 0 and 7 days. Light intensity was recorded at nine locations over the experimental area before the start of the test.

RESULTS AND DISCUSSION
Chemical analyses of fresh solutions (days 0 and 5) indicated that test concentrations were 0.31 and 0.26, 0.64 and 0.58, 1.3 and 1.2, 2.4 and 2.4, 5.0 and 5.1 and 9.7 and 10.3 mg ai/L respectively. At the end of the respective medium renewal periods 87 – 103% of the initially measured concentrations were found. Results were expressed in terms of mean measured concentrations. Mean frond numbers and dry weights are presented below:

Table 64-7: Effect of SYN524464 on frond number and dry weight of *Lemna gibba*

Mean measured concentrations (TWA) (mg ai/L)	Mean No. fronds/replicate (day 7)	Frond No. yield	Frond No. average specific growth rate	Mean dry weight (mg)/replicate (day 7)	Dry weight yield	Dry weight average specific growth rate
Control	142.3	130.3	0.353	16.1	14.4	0.321
0.28	130.7	118.7(*)	0.341	15.2	13.5	0.313
0.59	128.3	116.3(*)	0.338	15.3	13.6	0.314
1.2	120.7	108.7*	0.330*	14.4	12.8	0.306
2.4	102.7	90.7*	0.307*	9.6	7.9*	0.246*
5.0	64.7	52.7*	0.240*	4.9	3.2*	0.151*
9.9	23.3	11.3*	0.094*	2.0	1.2*	0.074*

Inoculum = 12 fronds/vessel (1.7 mg dry weight),
* = mean value statistically significantly lower than the control (p = 0.05)

Data for frond number and dry weight was used to fit growth curves from which average specific growth rates were calculated for the control and each exposure concentration. A probit model was then used to calculate the 7-day ErC_{50} and EyC_{50} and their respective 95% confidence intervals, based on percent inhibition relative to the control. Results are shown below:

Table 64-8: Summary of EC_{50} parameters and confidence limits for SYN524464 to *L. gibba* (mg ai/L)

Parameter	Frond numbers		Dry weight of plants	
	Growth rate (r)	Yield (y)	Growth rate (r)	Yield (y)
7-d EC_{50} (95% confidence interval)	6.5 (5.3 – 8.2)	3.6 (2.5 – 5.2)	4.8 (4.4 – 5.2)	2.7 (2.3 – 3.3)
7-d EC_{10} (95% confidence interval)	2.4 (1.2 – 3.3)	0.97 (0.29 – 1.6)	1.5 (1.2 – 1.7)	0.98 (0.61 – 1.3)
NOEC	0.59	0.59	1.2	1.2

CONCLUSION: For frond number, the 7-day ErC_{50} and EyC_{50} for SYN524464 to *Lemna gibba* are 6.5 and 3.6 mg ai/L respectively, based on mean measured concentrations.
For dry weight, the 7-day ErC_{50} and EyC_{50} for SYN524464 to *Lemna gibba* are 4.8 and 2.7 mg ai/L respectively, based on mean measured concentrations.
For frond number, the 7-day ErC_{10} and EyC_{10} for SYN524464 to *Lemna gibba* are 2.4 and 0.97 mg ai/L respectively, based on mean measured concentrations.
For dry weight, the 7-day ErC_{10} and EyC_{10} for SYN524464 to *Lemna gibba* are 1.5 and 0.98 mg ai/L respectively, based on mean measured concentrations.
For frond number, the 7-day NOEC was 0.59 mg ai/L, based on mean measured concentrations.
For dry weight, the 7-day NOEC was 1.2 mg ai/L, based on mean measured concentrations.

11.5.4 Acute (short-term) toxicity to other aquatic organisms

No additional studies.

11.6 Long-term aquatic hazard

Table 65: Summary of relevant information on chronic aquatic toxicity

Substance (purity)	Species	Test guidelines	Endpoint	Toxicity value	Conditions	Reference
Fish						
Sedaxane (95.3%)	*Pimephales promelas* (Fathead minnow)	OECD 210 OPPTS 850.1400	21 day LC_{50} NOEC	0.469 mg/L 0.165 mg/L (mm)	33 day flow-through (28 days post hatch) Dilution water and solvent control. pH 8.1 - 8.3 23.9 – 25.4°C GLP	Authors of vertebrate study (2008d). ***SYN524464/0065*** *Annex I. 4.4.1.1*
Aquatic Invertebrates						
Sedaxane (95.3%)	*Daphnia magna*	OECD 211	21 day EC_{50} (reproduction) 21 day NOEC (survival and reproduction)	1.5 mg/L 0.82 mg/L (nom)	21 day semi-static Culture medium and solvent control. pH 7.6 - 8.1 20°C GLP	Bätscher (2007c) SYN524464/0038
Algae and aquatic plants						
Sedaxane (purity 95.3%)	*Pseudokirchneriella subcapitata* (freshwater green alga)	OECD 201	72-h NOErC 96-h NOErC	1.0 mg/L (mm) 1.0 mg/L (mm)	96 hour static Culture medium control pH 8.2 (start) 8.1 - 9.2 (end) 22 - 23°C GLP	Bätscher, (2007a) ***SYN524464/0037***
Sedaxane (purity 95.3%)	*Navicula pellicosa* (Freshwater diatom)	OECD 201	72-h NOErC 72h ErC_{10} 96-h NOErC 96h ErC_{10}	2.4 mg/L 4.3 mg/L (mm) 2.4 mg/L 5.3 mg/L (mm)	96 hour static Culture medium & filtrate control. pH: 7.4 (start) 7.7 – 9.2 (end) 23ºC GLP	Büche, (2007a) ***SYN524464/0044***
Sedaxane (purity 95.3%)	*Anabaena flos-aquae* (freshwater	OECD 201	72-h NOErC	4.3 mg/L (mm)	96 hour static Culture medium & filtrate control.	Büche (2007b) ***SYN524464/0045***

	Cyanobacteria)		96-h NOErC	4.3 mg/L (mm)	pH: 8.5 (start) 9.0-9.1 (end) 22 - 23°C GLP	
Sedaxane (purity 95.3%)	*Skeletonema costatum* (Marine diatom)	OECD 201	72-h NOErC	6.0 mg/L (mm)	96 hour static Culture medium control. pH: 8.1-8.1 (start) 8.4-8.5 (end) 19.8 – 20.5ºC GLP	Minderhout, Kendall & Krueger (2007) ***SYN524464/0058***
			96-h NOErC	6.0 mg/L (mm)		
Sedaxane (purity 95.3%)	*Lemna gibba* (duckweed)	OECD 221	Frond No 7-d NOEC 7-d ErC_{10}	0.59 mg/L 2.4 mg/L (mm)	7 day semi-static Dilution water control. pH: 7.3–7.4 (start) 8.6 - 9.0 (end) 23°C GLP	Bätscher (2007b) ***SYN524464/0039***
			Dry Weight 7-d NOErC 7-d ErC_{10}	1.2 mg/L 1.5 mg/L (mm)		

Results are based on the mean measured or (mm) or nominal (nom) concentrations

11.6.1 Chronic toxicity to fish

One study is available on the long-term toxicity of sedaxane to fish, with supporting specific analysis. In a toxicity study to the early life-stages of fathead minnow (*Pimephales promelas*) the NOEC = 0.165 mg a.i./L. This NOEC is considered representative of fish species for chronic classification purposes.

Table 66: Summary of relevant chronic endpoints for fish

Test type	Test substance	Test species	Endpoint	Value (mg a.i./L)	Reference
Early life stage toxicity to fish	Sedaxane	Fathead minnow (*Pimephales promelas*	NOEC (flow-through)	0.165 mg/L (mm)	Authors of vertebrate study (2008d). *Annex I. 4.4.1.1*

mm = mean measured

Study 1: Authors of vertebrate study (2008d; SYN524464/0065) *Annex I. 4.4.1.1*

The toxicity of sedaxane (purity 95.3%) to early life-stages of fathead minnow (*Pimephales promelas*) was determined in a flow-through test. Fish were exposed to a range of nominal concentrations of 6.2, 19, 56, 170 and 500 µg a.i./L, a solvent control (dimethylformamide) and a dilution water control for 33 days (28 days post-hatch). There were four replicate tanks for each exposure concentration with 20 eggs per tank. Specific analysis showed mean measured test concentrations to be 94- 100 % of nominal and mean measured concentrations were 6.1, 18, 56, 165 and 469 µg a.i./L. Mean water temperatures were 23.9 to 25.4°C and mean pH was 8.1 to 8.3. Mean

water hardness ranged between 138 - 148 mg/L as $CaCO_3$ and the dissolved oxygen ranged from 6.5 – 8.2 mg/L.

There were no statistically significant treatment-related effects on hatching success at any of the concentrations tested. There were also no statistically significant treatment-related effects on survival or growth at the 6.1, 18, 56 and 165 µg a.i./L test concentrations. There was a statistically significant reduction in survival at the 469 µg a.i./L test concentration that resulted in 100% mortality for this treatment group. Based on mean measured concentrations, the 33-day NOEC for sedaxane to early life-stages of fathead minnow (*Pimephales promelas*) was 165 µg a.i./L, resulting from effects on fry survival.

Table 66-1: Effects of sedaxane on *Pimephales promelas*

Mean measured concentration (µg a.i./L)	Quantal responses		Non quantal responses		
	Hatching success (%)[1]	Fry survival to test end (%)[2]	Mean length (mm)±SD	Mean wet weight (mg)±SD	Mean dry weight (mg)±SD
Control	100	86	19.8 ± 0.59	53.1 ± 4.71	9.6 ± 0.89
Solvent control	96	83	20.5 ± 0.65	63.8 ± 7.74	11.1 ± 1.41
Pooled control	98	85	20.2 ± 0.70	58.4 ± 8.23	10.3 ± 1.34
6.1	96	91	19.8 ± 0.10	55.4 ± 2.65	9.7 ± 0.37
18	99	89	19.7 ± 0.40	55.9 ± 3.03	9.6 ± 0.64
56	98	94	20.0 ± 0.18	59.0 ± 2.79	10.3 ± 0.59
165	98	88	20.4 ± 0.32	65.5 ± 3.84	11.3 ± 0.89
469	100	0*	-	-	-
NOEC	469 µg/L	165 µg/L	165 µg/L	165 µg/L	165 µg/L

[1] The number of live larvae on the day they are transferred from the egg cups to the test vessel (day 5) expressed as a percentage of the number of eggs added at the start of the test (day 0).
[2] The number of surviving larvae at the end of the test (day 33) expressed as a percentage of the number of eggs added on day 0.
* Statistically different from the pooled control.

11.6.2 Chronic toxicity to aquatic invertebrates

One study is available on the long-term toxicity of sedaxane to aquatic invertebrates (*Daphnia magna*), with supporting specific analysis.

Table 67: Summary of relevant chronic endpoints for aquatic invertebrates

Test type	Test substance	Test species	Endpoint	Value (mg a.i./L)	Reference
Survival and reproduction	Sedaxane	Freshwater crustacean (*Daphnia magna)*	21 day EC_{50} (reproduction) (semi-static)	1.5 mg/L (nom)	Bätscher R (2007c)
			21 day NOEC (survival and reproduction) (semi-static)	0.82 mg/L (nom)	

nom = nominal

Study 1: Bätscher (2007c; SYN524464/0038)

In a 21 day semi-static toxicity study of sedaxane technical (95.3% purity) to the freshwater crustacean *Daphnia magna*, groups of ten P generation animals (1 × 10 replicates) were exposed to each of nominal test concentrations 0.025, 0.080, 0.26, 0.82, 2.6 and 8.4 mg a.i./L, plus a culture medium and solvent control. The measured concentrations of the test item in the old and new test media were between 90 and 103% of nominal, demonstrating stability of the test item in the test medium over the renewal periods of 48 and 72 hours. Nominal concentrations were used for the calculation and reporting of the results.Water temperature, pH and dissolved oxygen concentration were 20°C, 7.6 - 8.1 and 8.1 – 9.4 mg/L, respectively, over the test period.

Exposure to sedaxane concentrations up to and including 2.6 mg a.i./L did not have any significant effect on mortality but at 8.4 mg a.i./L all P generation daphnids were dead on day 5. Time to first brood was unaffected at concentrations up to and including 0.82 mg a.i./L but at 2.6 mg a.i./L no juveniles were produced.

The 21-day NOEC for adult mortality and reproduction was 0.82 mg a.i./L and the 21-day EC_{50} for reproduction was 1.5 mg a.i./L (geometric mean of 0.82 and 2.6 mg a.i./L).

Table 68: Effects of sedaxane on *Daphnia magna*

Nominal test concentration (mg a.i./L)	Number of surviving parent	Mean No. of juveniles per surviving parent	± SD	CV
Solvent control	9	86.3	6.7	7.8
Control	10	88.6	6.7	7.6
0.025	10	86.8	8.1	9.4
0.080	10	85.5	7.9	9.2
0.26	10	88.8	11.9	13.4
0.82	10	92.3	6.2	6.8
2.6	10	0*	0	n.a.
8.4	0	0*	0	n.a.
21-day NOEC	**2.6 mg a.i./L**	**0.82 mg a.i./L**	-	-

* Significant difference (p=0.05) from the control

11.6.3 Chronic toxicity to algae or other aquatic plants

The algae and aquatic plant studies are described in Section 11.5.3 and the relevant chronic endpoints are summarised in Table 65 above. The lowest NOErC is for duckweed *Lemna gibba*, (Bätscher, 2007b), with a 7-day NOEC value of 0.59 mg a.i./L for frond number. However, a reliable ErC_{10} of 2.4 [1.2 – 3.3] mg/L for growth rate based on frond number has been estimated and is considered more relevant for classification purpose. The lowest relevant endpoint for classification purpose for algae and other aquatic plants is the 72-h NOErC of 1.0 mg/L for *P. subcapitata*.

Table 69: Summary of relevant chronic endpoints for algae and aquatic plants

Test type	Test substance	Test species	Endpoint	Value (mg a.i./L)	Reference
Chronic toxicity to algae and aquatic plants	Sedaxane	*Pseudokirchneriella subcapitata* Freshwater Green Algae	72- and 96-h $NOEC_{b, r, y}$ (static)	1.0 mg/L (mm)	Bätscher (2007a)
	Sedaxane	*Navicula pellicosa* Freshwater Diatom	72-h $NOEC_{r, b, y}$ 96-h $NOEC_{r}$ 96-h $NOEC_{b, y}$ (static)	2.4 mg/L 2.4 mg/L 1.2 mg/L (mm)	Büche (2007a)
	Sedaxane	*Anabaena flos-aquae* (freshwater Cyanobacteria)	72- and 96-h $NOEC_{b, r, y}$ (static)	4.3 mg/L (mm)	Büche (2007b)
	Sedaxane	*Skeletonema costatum* (Marine diatom)	72- and 96-h $NOEC_{b, r, y}$ (static)	6.0 mg/L (mm)	Minderhout, Kendall & Krueger (2007)
	Sedaxane	*Lemna gibba* (Duckweed)	NOEC (frond number) NOEC (dry weight) (semi-static)	0.59 mg/L 1.2 mg/L (mm)	Bätscher (2007b)

Results are based on the mean measured or (mm) or nominal (nom) concentrations

11.6.4 Chronic toxicity to aquatic invertebrates

No additional studies.

11.7 Comparison with the CLP criteria

11.7.1 Acute aquatic hazard

Table 70: Acute endpoints relevant to classification of sedaxane

Species group	Species	Lowest representative L/EC_{50}	Reference
Fish	*Cyprinus carpio* (common carp)	0.62 mg a.i./L	Authors of vertebrate study (2008a)

			Annex I. 4.3.1.1
Aquatic invertebrates	*Americamysis bahia* (Mysid shrimp)	1.5 mg a.i./L	Gallagher, Kendall & Krueger. (2008c)
Algae and aquatic plants	*Pseudokirchneriella subcapitata* (Freshwater Green Algae)	2.8 mg a.i./L	Bätscher, (2007a)

Based on these results the lowest EC_{50} is for fish (LC_{50}= 0.62 mg a.i./L). On this basis, the following acute classification and labelling of sedaxane is proposed:

Aquatic Acute 1 H400 (Very toxic to aquatic life); as the lowest L(E)C_{50} is between 0.1 and 1.0 mg/L, the associated M-factor is 1.

11.7.2 Long-term aquatic hazard (including bioaccumulation potential and degradation)

Toxicity

Table 71: Chronic endpoints relevant to classification of sedaxane

Species group	Species	Lowest representative NOEC	Reference
Fish	Fathead minnow *(Pimephales promelas)*	0.165 mg a.i./L	Authors of vertebrate study (2008d). SYN524464/0065 *Annex I. 4.4.1.1*
Aquatic invertebrates	*Daphnia magna*	0.82 mg a.i./L	Bätscher R (2007c)
Algae and aquatic plants	Algae (*Pseudokirchneriella subcapitata)*	1.0 mg a.i./L	Bätscher R (2007a)

Based on these results the lowest NOEC for aquatic organisms is for *P. promelas* (NOEC = 0.165 mg a.i./L).

Bioaccumulation

For classification and labelling purposes, substance with a log K_{ow} < 4 are not considered to have a potential for bioaccumulation. This is the case for sedaxane (log K_{ow} = 3.3). This is also supported by the measured BCF value of 97 L/kg which is below the trigger value of 500 L/kg according to CLP criteria.

Degradation

Sedaxane is not readily biodegradable (Seyfried, 2007) and is hydrolytically stable (Nicollier, 2007a).

One relevant study on the degradation of sedaxane in aquatic water-sediment systems (Stoll and Nicollier, 2008) shows primary degradation half-lives >> 1year and negligible (<2%) CO_2 evolution.

Overall, based on the data available, sedaxane is considered not to be rapidly degradable for classification purposes.

On this basis, the following classification and labelling of sedaxane is proposed:

- **Aquatic Chronic 2 H411 (Toxic to aquatic life with long lasting effects)**

11.8 Conclusion on classification and labelling for environmental hazards

On the basis of the above information on chronic toxicity, bioaccumulation and rapid degradability, the following classification and labelling of sedaxane is proposed:

Hazard Class and Category code(s)	Hazard Statement Code
Aquatic Acute 1, H400	Very toxic to aquatic life (M-factor 1)
Aquatic Chronic 2 H411	Toxic to aquatic life with long lasting effects

Labelling

Pictogram	 Hazardous to the environment
Signal word	Warning
Precautionary statements	P273 Avoid release to the environment
	P391 Collect spillage
	P501 Dispose of contents/container in accordance with local/regional/national/international regulations

RAC evaluation of aquatic hazards (acute and chronic)

Summary of the Dossier Submitter's proposal

The DS proposal was to classify sedaxane as Aquatic Acute 1 – H400 (Very toxic to aquatic life) with M-factor = 1 and Aquatic Chronic 2 – H411 (Toxic to aquatic life with

long lasting effects).

Degradation

The DS's summary of relevant information on degradability:

Table: *Summary of studies on degradation*

Method	Results	Remarks	Reference
Ready biodegradability 28 days, 22°C, pH 7.2-7.6 Test substance: sedaxane (purity 95.3%) Test concentration = 101 mg/L OECD TG 301F GLP	No biodegradation in 28 days; Not readily biodegradable	43% degradation observed in the toxicity control, therefore no significant inhibitory effect	Seyfried, 2007
Hydrolysis, pH 4, 5, 7 and 9, 25°C, 30 days, dark. Test substance: [Phenyl-U-^{14}C] - sedaxane (Radiochemical purity 99.1%, chemical purity 99.6%) Nominal concentration = 0.0017 mg/mL OECD TG 111 EPA subdivision N-161-1 GLP	Prelim study at 50°C; < 10% degradation at pH 4, 5, 7 and 9 after 5 days. After 30 days at 25°C, sedaxane accounted for 95.9, 102.8 and 101.3% of applied radioactivity at pH 5, 7 and 9, respectively. $DegT_{50}$ > 1 year		Nicollier, 2007a
Direct and indirect photolysis pH 7, up to 34 days, 25 ± 2°C, sterile and natural water, Test substance: ^{14}C-phenyl and ^{14}C-pyrazole sedaxane (purity > 99%) Test concentration = *c.a.* 2.0 mg/L OECD draft guideline Aug 2000, JMAFF 12 Nousan no. 8147, 2001 EPA 540/9-82-021 GLP	Direct photolysis in sterile buffer; $DegT_{50}$ = 42, 52 and 71 days at 30, 40 and 50°N	Sedaxane level 57.3% AR after 34 days continuous irradiation (95.2% in dark controls) Total ^{14}C recoveries 98.1–101.4% (phenyl) and 91.5-99.2% (pyrazole) Multiple degradates and minimal volatiles (max 1.8%)	Hand and Flemming, 2007
	Indirect photolysis in natural water; $DegT_{50}$ = 16.3, 16.5 and 17.1 days at 30, 40 and 50°N	Sedaxane level 23.9% AR after 28 days continuous irradiation (97.7% in dark controls) Total ^{14}C recoveries 94.5–107.6% (phenyl) and 98.2-105.4% (pyrazole) Multiple degradates and minimal volatiles (max 11.1% (phenyl only))	
Water-sediment degradation, aerobic (179 days) and anaerobic (360 days), 20 ± 1°C, dark, pond & river systems Test substance: ^{14}C-phenyl sedaxane (purity > 96%) Nominal concentration = 0.03 µg/mL OECD TG 308 EPA subdivision N-162-3 GLP	Total system $DegT_{50}$ Aerobic: Pond; >> 1 year River; >> 1 year Anaerobic: Pond; >> 1 year River; >> 1 year	For aerobic and anaerobic systems; CO_2 evolution ≤ 2.0% AR Total mean recoveries 93.9-105.2%	Stoll and Nicollier, 2008
Soil adsorption/desorption, 6 soils	Mean $K_{FOC(ads)}$ = 534L/kg for all	Total ^{14}C recovery 90-	Nicollier,

Test substance: ^{14}C-phenyl sedaxane (purity > 99%) OECD TG 106 EPA subdivision N-163-1 GLP	soils (range 262-666 L/kg) Mean $K_{FOC(des)}$ = 704L/kg for all soils (range 367-907 L/kg)	110% in all soils	2008

Sedaxane is not readily biodegradable (Seyfried, 2007) and is hydrolytically stable (Nicollier, 2007a).

One relevant study on the degradation of sedaxane in aquatic water-sediment systems (Stoll and Nicollier, 2008) shows primary degradation half-lives >> 1 year and negligible (< 2%) CO_2 evolution.

Overall, based on the data available, sedaxane is considered not to be rapidly degradable for classification purposes.

Bioaccumulation

Table: *Summary of relevant information on bioaccumulation*

Substance	Species	Test guidelines	Endpoint	Value	Condition	Reference
^{14}C-sedaxane purity 95.2%, radiochemical purity 99.1%	Rainbow trout (*Oncorhynchus mykiss*)	OECD TG 305	BCF (whole fish)	97	Flow-through, 14 day uptake and 14 day depuration 0.5 µg/L + solvent (DMF) control. pH 7.24-7.69 15.1-15.3°C GLP	Anonymous, 2010 *Annex I. 4.2.1.1*

The experimentally derived Log Kow of sedaxane is 3.3. For classification and labelling purposes, a substance with Log Kow < 4 may be considered unlikely to bioaccumulate in aquatic organisms. This is the case for sedaxane (Log Kow = 3.3). This is also supported by the measured BCF value of 97 L/kg which is below the trigger value of 500 L/kg according to CLP criteria. A measured BCF ≥ 500 indicates a potential for bioaccumulation. Since the BCF for sedaxane is < 500, it is considered not to be bioaccumulative for the purpose of classification and labelling.

Acute aquatic hazard

The summary of acute aquatic toxicity, as presented by the DS is presented below.

Table: *summary of the acute aquatic toxicity studies*

Substance	Species	Test guidelines	Endpoint	Toxicity value (mg/L)	Conditions	Reference
Fish						

Sedaxane (purity 95.3%)	*Cyprinus carpio* (Common carp)	OECD TG 203 OPPTS 850.1074	96h LC_{50}	0.62 mg/L (mm)	96 h static test. Dilution water control. pH 8.3 – 8.5 21.5 – 22.1°C GLP	Anonymous, 2008a ***SYN524464_1 1104***
Sedaxane (purity 95.3%)	*Oncorhynchus mykiss* (Rainbow trout)	OECD TG 203 OPPTS 850.1074	96h LC_{50}	1.1 mg/L (mm)	96 h static test. Dilution water control. pH 8.2 – 8.4 13°C GLP	Anonymous, 2008 ***SYN524464/0 067***
Sedaxane (purity 98.2%)	*Pimephales promelas* (Fathead minnow)	OECD TG 203 OPPTS 850.1075	96h LC_{50}	0.98 mg/L (mm)	96 h static test. Dilution water control. pH 7.59 – 8.29 24.1 – 24.5°C GLP	Anonymous, 2006 ***SYN524464/0 012***
Sedaxane (purity 95.3%)	*Cyprinodon variegatus* (Sheepshead minnow)	OPPTS 850.1075	96h LC_{50}	4.2 mg/L (mm)	96 h static test. Dilution water control. pH 8.0 – 8.3 21.8 – 22.7°C GLP	Anonymous, 2008b ***SYN524464/0 062***
Aquatic Invertebrates						
Sedaxane (purity 95.3%)	*Americamysis bahia* (saltwater mysid)	OPPTS 850.1035	96h LC_{50}	1.5 mg/L (mm)	96 hour static test Dilution water control. pH 8.1 – 8.2 24.9– 25.4°C (start) 25.2 – 25.3°C (end) GLP	Gallagher *et al*., 2008c ***SYN524464/0 059***
Sedaxane (purity 98.2%)	*Daphnia magna* (Cladoceran)	OECD TG 202	48h EC_{50}	6.10 mg/L (mm)	48 hour static test Dilution water control. pH 7.50–7.59 20.7 – 21.2°C GLP	Ricketts and Paddick, 2006 ***SYN524464/0 011***
Algae and aquatic plants						
Sedaxane (purity 95.3%)	*Pseudokirchneriella subcapitata* (freshwater green alga)	OECD TG 201	72h E_bC_{50} 72h E_rC_{50} 72h E_yC_{50}	1.9 mg/L 2.8 mg/L 1.6 mg/L (mm)	96 hour static Culture medium control pH 8.2 (start) 8.1 - 9.2 (end) 22 - 23°C GLP	Bätscher, 2007a ***SYN524464/0 037***
			96h E_bC_{50} 96h E_rC_{50} 96h E_yC_{50}	1.9 mg/L 3.0 mg/L 1.8 mg/L (mm)		
Sedaxane (purity 95.3%)	*Navicula pellicola* (Freshwater diatom)	OECD TG 201	72h E_bC_{50} 72h E_rC_{50} 72h	4.8 mg/L 8.7 mg/L 4.8 mg/L (mm)	96 hour static Culture medium & filtrate control. pH: 7.4 (start) 7.7 – 9.2 (end)	Büche, 2007a ***SYN524464/0 044***

			E_yC_{50}		23ºC GLP	
			96h E_bC_{50} 96h E_rC_{50} 96h E_yC_{50}	5.3 mg/L 10 mg/L 5.7 mg/L (mm)		
Sedaxane (purity 95.3%)	*Anabaena flos-aquae* (freshwater Cyanobacteria)	OECD TG 201	72h E_bC_{50} 72h E_rC_{50} 72h E_yC_{50}	> 6.5 mg/L > 6.5 mg/L > 6.5 mg/L (mm)	96 hour static Culture medium & filtrate control. pH: 8.5 (start) 9.0-9.1 (end) 22 - 23°C GLP	Büche, 2007b ***SYN524464/0045***
			96h E_bC_{50} 96h E_rC_{50} 96h E_yC_{50}	> 6.5 mg/L > 6.5 mg/L > 6.5 mg/L (mm)		
Sedaxane (purity 95.3%)	*Skeletonema costatum* (Marine diatom)	OECD TG 201	72h E_bC_{50} 72h E_rC_{50} 72h E_yC_{50}	> 6.0 mg/L > 6.0 mg/L > 6.0 mg/L (mm)	96 hour static Culture medium control. pH: 8.1-8.1 (start) 8.4-8.5 (end) 19.8 – 20.5ºC GLP	Minderhout *et al.*, 2007 ***SYN524464/0058***
			96h E_bC_{50} 96h E_rC_{50} 96h E_yC_{50}	> 6.0 mg/L > 6.0 mg/L > 6.0 mg/L (mm)		
Sedaxane (purity 95.3%)	*Lemna gibba* (duckweed)	OECD TG 221	<u>Frond No</u> 7d E_rC_{50} 7d E_yC_{50}	6.5 mg/L 3.6 mg/L (mm)	7 day semi-static Dilution water control. pH: 7.3–7.4 (start) 8.6 - 9.0 (end) 23ºC GLP	Bätscher, 2007b ***SYN524464/0039 ****
			<u>Dry Weight</u> 7d E_rC_{50} 7d E_yC_{50}	4.8 mg/L 2.7 mg/L (mm)		

Based on the results from the four available and reliable experimental studies on fish, the lowest acute toxicity value has been derived for *Cyprinus carpio* (Common carp), with an LC_{50} value of 0.62 mg/L).

The 48 hour EC_{50} value in daphnia was 6.10 mg/L. The lowest EC_{50} of 1.5 mg/L in the saltwater mysid shrimp (Gallagher *et al.*, 2008c) is considered appropriate to use for classification of acute toxicity to aquatic invertebrates.

The lowest EC_{50} value (72h ErC_{50}) in freshwater green algae (*Pseudokirchneriella subcapitata*) of 2.8 mg/L (Bätscher, 2007a) is considered appropriate to use for classification of acute toxicity to algae and aquatic plants.

On this basis, the following acute classification and labelling of sedaxane is proposed by the dossier submitter:

Aquatic Acute 1 – H400 (Very toxic to aquatic life); as the lowest L(E)C_{50} (= 0.62 mg/L) is between 0.1 and 1.0 mg/L, the associated M-factor is 1.

Long-term aquatic hazard (including bioaccumulation potential and degradation

The endpoints for chronic aquatic toxicity endpoints relevant for classification of sedaxane are summarised in the table below:

Table

Substance (purity)	Species	Test guidelines	Endpoint	Toxicity value	Conditions	Reference
Fish						
Sedaxane (95.3%)	*Pimephales promelas* (Fathead minnow)	OECD TG 210 OPPTS 850.1400	21 days LC_{50} NOEC	0.469 mg/L 0.165 mg/L (mm)	33 days flow-through (28 days post hatch) dilution water and solvent control. pH 8.1 - 8.3 23.9 – 25.4°C GLP	Anonymous, 2008d ***SYN524464/0065*** *Annex I. 4.4.1.1*
Aquatic Invertebrates						
Sedaxane (95.3%)	*Daphnia magna*	OECD TG 211	21 days EC_{50} (reproduction) 21 days NOEC (survival and reproduction)	1.5 mg/L 0.82 mg/L (nom)	21 days semi-static culture medium and solvent control. pH 7.6 - 8.1 20°C GLP	Bätscher, 2007c SYN524464/0038
Algae and aquatic plants						
Sedaxane (purity 95.3%)	*Pseudokirchneriella subcapitata* (freshwater green alga)	OECD TG 201	72h NOE_rC 96h NOE_rC	1.0 mg/L (mm) 1.0 mg/L (mm)	96 hours static Culture medium control pH 8.2 (start) 8.1 - 9.2 (end) 22 - 23°C GLP	Bätscher, 2007a ***SYN524464/0037***
Sedaxane (purity 95.3%)	*Navicula pellicola* (Freshwater diatom)	OECD TG 201	72h NOE_rC 72h E_rC_{10} 96h NOE_rC 96h E_rC_{10}	2.4 mg/L 4.3 mg/L (mm) 2.4 mg/L 5.3 mg/L (mm)	96 hours static culture medium & filtrate control. pH: 7.4 (start) 7.7 – 9.2 (end) 23ºC GLP	Büche, 2007a ***SYN524464/0044***

Sedaxane (purity 95.3%)	*Anabaena flos-aquae* (freshwater Cyanobacteria)	OECD TG 201	72h NOE_rC	4.3 mg/L (mm)	96 hours static culture medium & filtrate control. pH: 8.5 (start) 9.0 - 9.1 (end) 22 - 23°C GLP	Büche, 2007b ***SYN524464/0045***
			96h NOE_rC	4.3 mg/L (mm)		
Sedaxane (purity 95.3%)	*Skeletonema costatum* (Marine diatom)	OECD TG 201	72h NOE_rC	6.0 mg/L (mm)	96 hours static culture medium control. pH: 8.1 - 8.1 (start) 8.4 - 8.5 (end) 19.8 – 20.5ºC GLP	Minderhout *et al.*, 2007 ***SYN524464/0058***
			96h NOE_rC	6.0 mg/L (mm)		
Sedaxane (purity 95.3%)	*Lemna gibba* (duckweed)	OECD TG 221	Frond No 7d NOEC	0.59 mg/L	7 days semi-static Dilution water control. pH: 7.3 – 7.4 (start) 8.6 - 9.0 (end) 23°C GLP	Bätscher, 2007b ***SYN524464/0039***
			7d E_rC_{10}	2.4 mg/L (mm)		
			Dry Weight 7d NOE_rC	1.2 mg/L		
			7d E_rC_{10}	1.5 mg/L (mm)		

One study is available on the long-term toxicity of sedaxane to fish referring to a toxicity study to the early life-stages of fathead minnow (*Pimephales promelas*), which derived a NOEC value of 0.165 mg/L. There were no statistically significant treatment-related effects on hatching success at any of the concentrations tested. There were also no statistically significant treatment-related effects on survival or growth at the 6.1, 18, 56 and 165 µg/L test concentrations. There was a statistically significant reduction in survival at the 469 µg/L test concentration that resulted in 100% mortality for this treatment group. Based on mean measured concentrations, the 33 days NOEC for sedaxane to early life-stages of fathead minnow (*Pimephales promelas*) was 165 µg/L, resulting from effects on fry survival.

Effects of sedaxane on Pimephales promelas:

Table

Mean measured concentration (µg/L)	Quantal responses		Non quantal responses		
	Hatching success (%)[1]	Fry survival to test end (%)[2]	Mean length (mm) ± SD	Mean wet weight (mg) ± SD	Mean dry weight (mg) ± SD
Control	100	86	19.8 ± 0.59	53.1 ± 4.71	9.6 ± 0.89
Solvent control	96	83	20.5 ± 0.65	63.8 ± 7.74	11.1 ± 1.41
Pooled control	98	85	20.2 ± 0.70	58.4 ± 8.23	10.3 ± 1.34
6.1	96	91	19.8 ± 0.10	55.4 ± 2.65	9.7 ± 0.37
18	99	89	19.7 ± 0.40	55.9 ± 3.03	9.6 ± 0.64
56	98	94	20.0 ± 0.18	59.0 ± 2.79	10.3 ± 0.59

165	98	88	20.4 ± 0.32	65.5 ± 3.84	11.3 ± 0.89
469	100	0*	-	-	-
NOEC	469 µg/L	165 µg/L	165 µg/L	165 µg/L	165 µg/L

Concerning aquatic invertebrates, a long-term study on *Daphnia magna* performed according to OECD TG 211 derived a 21 days NOEC value of 0.82 mg/L based on survival and reproduction.

Concerning algae and aquatic plants, the lowest relevant endpoint for classification purposes was considered to be the 72 hours NOE_rC value of 1.0 mg/L for *Pseudokirchneriella subcapitata*.

Overall, based on all long-term results, the lowest NOEC for aquatic organisms was that of *Pimephales promelas* (NOEC = 0.165 mg/L). On this basis, the following classification and labelling of sedaxane was proposed by the dossier submitter:

Aquatic Chronic 2 – H411 (Toxic to aquatic life with long lasting effects).

Comments received during public consultation

There was general support for the Aquatic Acute 1 – H400 classification from the commenting MSCAs.

Regarding the aquatic chronic classification, one MSCA pointed out that the key chronic toxicity test was not performed with the most sensitive species like as in the acute toxicity test: *Pimephales promelas* NOEC 0.165 mg/L (mm). This test species was not the most acutely sensitive, as the lowest 96h LC_{50} of 0.62 mg/L (mm) was for *Cyprinus carpio,* while the *Pimephales promelas* 96h LC_{50} was 0.98 mg/L (mm). According to the commenting MSCA, considering the surrogate approach using the lowest acute effects endpoint would result in Aquatic Chronic 1 (M-factor = 1) for a non-rapidly degradable substance. An additional argument for following this approach was the fact that, although both fish species exhibited acute endpoints in the 0.1 - 1.0 mg/L range, the chronic NOEC for fish is close to the regulatory threshold value of 0.1 mg/L.

On this basis, the MSCA wondered whether Aquatic Chronic 1 (M-factor 1) should be considered and commented that it might be useful to also consider acute:chronic ratios and if EC_{10} endpoints were available.

On their response, the DS stated that there is only slight difference in sensitivity between the two fish species from the acute tests and considered their sensitivity to sedaxane as similar. Furthermore, they considered the NOEC of 165 µg/L robust, as it corresponds to the highest tested concentration without significant effects while significant effects were observed at the highest tested concentration in the study. No reliable EC_{10} value could be derived from the reported results of the study.

Assessment and comparison with the classification criteria

The substance sedaxane is not readily biodegradable and is hydrolytically stable. The experimentally derived Log Kow of sedaxane is 3.3 and may be considered unlikely to bioaccumulate in aquatic organisms. However, sedaxane may have surface-active properties that introduce uncertainty to the results of the experimental bioconcentration study. The BCF value of 97 L/kg is below the trigger value of 500 L/kg in CLP, although the BCF has not been growth-corrected. This will not have any influence on the classification.

Based on the LC_{50} = 0.62 mg/L for *Cyprinus carpio,* RAC agrees with the proposal by the dossier submitter that the substance should be classified as **Aquatic Acute 1 – H400 (very toxic to aquatic life) with an M-factor of 1**.

RAC notes that the acute toxicity dataset for fish seems to indicate that *Cyprinus carpio* (LC_{50} = 0.62 mg/L) may be slightly more sensitive to sedaxane than *Pimephales promelas* (LC_{50} = 0.98 mg/L). However, both acute toxicity values are within the same order of magnitude, with this small difference probably falling within the test variability range.

Furthermore, RAC considers that the substance is not a data poor one, there is reliable chronic toxicity data for all three trophic levels and the chronic toxicity study for *Pimephales promelas* should not be discarded. As such, the aquatic chronic classification should be based on the *Pimephales promelas* chronic study that derived a NOEC value of 0.165 mg/L.

Thus, RAC agrees with the proposal by the dossier submitter that the substance should be classified as **Aquatic Chronic 2 – H411 (Toxic to aquatic life with log lasting effects)**.

12 EVALUATION OF ADDITIONAL HAZARDS

12.1 Hazardous to the ozone layer

12.1.1 Short summary and overall relevance of the provided information on ozone layer hazard

Transport of sedaxane in air is considered to be negligible due to its very low vapour pressure (6.5×10^{-8} Pa at 20°C and 1.7×10^{-7} Pa at 25°C) and Henry's constant (4.0×10^{-6} Pa m^3 mol^{-1} at 25°C). Furthermore, the photochemical oxidative degradation of sedaxane in air is expected to be rapid. The estimated half-life is 5.1 hours, calculated using Atkinson method (Hayes, 2010).Therefore long-range transport is not considered to be of relevance (Focus AIR, 2008).

12.1.2 Comparison with the CLP criteria

Sedaxane is not listed in Annex I to Regulation (EC) No 1005/2009.

12.1.3 Conclusion on classification and labelling for hazardous to the ozone layer

Due to the low volatility and rapid photochemical oxidative degradation in air of sedaxane; local and global effects are expected to be negligible.

No classification is warranted.

Hazardous to the ozone layer

Summary of the Dossier Submitter's proposal

Transport of sedaxane in air is considered to be negligible due to its very low vapour pressure (6.5 × 10-8 Pa at 20°C and 1.7 × 10-7 Pa at 25°C) and Henry's constant (4.0 × 10-6 Pa m3 mol-1 at 25°C). Furthermore, the photochemical oxidative degradation of sedaxane in air is expected to be rapid. The estimated half-life is 5.1 hours, calculated using Atkinson method. Therefore, long-range transport is not considered to be of relevance.

Comments received during public consultation

No comments were received during the public consultation.

Assessment and comparison with the classification criteria

Transport of sedaxane in air is considered to be negligible due to its very low vapour pressure and Henry's constant, whilst its photochemical oxidative degradation in air is expected to be rapid. Therefore, local and global effects are expected to be negligible.

Thus, RAC agrees with the DS' proposal that **no classification** is warranted for this hazard class.

13 ADDITIONAL LABELLING

No additional labels.

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Modes of action and human relevance of tumours observed in experimental studies.

Dossier Submitter:

Context: Formerly, taking into consideration historical control range (neoplastic findings in affected tissues were generally within historical control ranges), no classification with regard to carcinogenicity was proposed in the Conclusion on the peer review of the pesticide risk assessment of the active substance sedaxane (EFSA 2012).

However, in 2011, US-EPA classified sedaxane as "Likely to be Carcinogenic to Humans." This classification was based on the presence of tumours at multiple sites in two species: liver and thyroid tumours in male rats, uterine tumours in female rats, and liver tumours in male mice.

Following a request from the European Commission to re-consider the toxicological assessment and confirm the conclusions on sedaxane, carcinogenicity was re-discussed at the Pesticides Peer Review Meeting 98 in November 2012 and it was concluded that the overall pattern of tumours in rats and mice suggests that a 'Carc cat 2, H351, suspected of causing cancer' classification regarding carcinogenicity would be required for sedaxane (EFSA, 2013).

Since that time, the applicant has submitted new studies and has proposed modes of action for liver, thyroid, and uterine tumours. Based on the new *in vivo* and *in vitro* exploratory toxicity studies as well as studies from the core dossier, the applicant has performed a MoA analysis according to the WHO/IPCS Framework for analysing the relevance of a cancer mode of action for humans (see WHO Harmonization Project Document No. 4, parts 1 and 2, 2009) which is endorsed in the ECHA Guidance on the Application of the CLP Criteria, Version 4.1 - June 2015.

The same data have been submitted to US-EPA and the Cancer Assessment Review Committee (CARC) has re-evaluated the cancer classification of sedaxane.

The applicant postulated modes of action for uterine tumours, liver tumours and thyroid tumours which are directly reported in appendix 1, appendix 2 and appendix 3 respectively.

For better clarity and as requested by the rapporteurs all text sections prepared by Syngenta have been highlighted in yellow.

The DS comments are inserted in the text in bold and the final assessment at the end of each proposed mode of action.

15 APPENDIX 1: MODE OF ACTION AND HUMAN RELEVANCE ASSESSMENT OF UTERINE TUMOUR INCIDENCE IN FEMALE RATS

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1.0 EXECUTIVE SUMMARY

Sedaxane is a fungicidal succinate dehydrogenase inhibitor. Following dietary administration to Han Wistar rats for 2 years, a high dose (3600 ppm) of sedaxane resulted in a slightly higher incidence of uterine endometrial adenocarcinomas in female rats compared to concurrent controls. Syngenta, the performing laboratory, and the rapporteur member state (RMS), France, [are] *were*[5] of the view that the higher incidence of uterine tumours at 3600 ppm is within the range of the variable spontaneous tumour incidence of Han Wistar rats in the test laboratory and is not related to sedaxane treatment. However, the European Food Safety Authority (EFSA) concluded that the overall pattern of tumours in rats and mice suggests that a 'Carc cat 2, H351, suspected of causing cancer' classification regarding carcinogenicity would be required for sedaxane (EFSA, 2013). This opinion followed US EPA classification of sedaxane as "likely to be carcinogenic to humans," based in part on an increased uterine adenocarcinoma incidence at 3600 ppm that EPA considered a high-dose treatment-related effect (U.S. Environmental Protection Agency, 2011b). Based on that alternative view, a programme of work was initiated to assess a hypothesized mode of action (MOA) for the higher incidence of uterine tumours using the weight of evidence framework developed by the International Programme on Chemical Safety (IPCS) and the International Life Science Institute (ILSI). The weight of evidence for the proposed MOA in female rats is described in detail in this document, and the human relevance of the identified MOA is assessed via the IPCS/ILSI framework.

The available data for sedaxane support a proposed MOA in female Han Wistar rats involving the following differences vs. control rats that occur in 3600 ppm sedaxane-treated rats:

- A large, sustained deficit in body weight gain occurs as a consequence of lower amounts of adipose tissue and associated with lower blood levels of leptin
- Reductions in body weight gain and adipose tissue throughout the animals' lifetime causes a delay in the normal age-related loss of the tuberoinfundibular dopaminergic (TIDA) neurons of the hypothalamus
- Retention of a greater number of functional TIDA neurons in aging sedaxane-treated rats results in continued production of dopamine, which suppresses prolactin release from the anterior pituitary. Thus, the age-related increase in circulating prolactin levels is delayed and/or diminished by sedaxane treatment
- The lack of a rise in circulating prolactin levels in blood results in a change in progression of sedaxane-treated rats into reproductive senescence, and these rats continue to experience more periodic estrous cycles compared to control rats.
- Continued estrous cycles results in a greater cumulative exposure of the uterus to a higher estrogen: progesterone ratio (*i.e.,* reduced progesterone dominance of estrogen) in aged female Wistar rats treated with sedaxane, which leads to a pro-proliferative estrogenic stimulation of the uterine endometrial cells. Over time, the estrogenic proliferative drive on the uterus leads to increased promotion of spontaneously initiated tumours (*i.e.,* an increased incidence of uterine adenocarcinomas).

At the same time, the maintenance of higher dopamine levels released from the TIDA neurons in aging sedaxane-treated rats blocks proliferative changes in the pituitary, causing a lower pituitary adenoma incidence. In turn, the lower prolactin release by the pituitary in aging sedaxane-treated rats results in the absence of mammary fibroadenomas in the 3600 ppm sedaxane-treated rats. This MOA has been well characterized and described in Wistar rats (Harleman *et al.*, 2012), and rats tested in

[5] : Dossier submitter: this conclusion refers to Draft Assessment Report 2012 (see context)

lifetime dietary restriction studies (Roe *et al.*, 1995; Tucker, 1979), where the same pattern of changes as with 3600 ppm sedaxane treatment was observed (*i.e.*, lower body weight gain plus lower incidences of pituitary adenomas and mammary gland fibroadenomas, and higher incidences of uterine adenocarcinomas). Therefore, there is a well-established biological plausibility for this uterine tumour MOA

Clear thresholds exist for the key events in this MOA. Moreover, the control of the female reproductive cycle and the drivers for reproductive senescence in humans are fundamentally different than those in rats; therefore, this MOA for uterine tumours in Wistar rats is not relevant to humans.

2.0 INTRODUCTION

Although the incidence of uterine tumours in female Wistar rats was higher than concurrent controls at the top dose of 3600 ppm in a 2-year combined chronic/carcinogenicity study (Anonymous, 2010b), Syngenta maintains the view that it is within the range of variable spontaneous tumour incidences for the test species and is not related to sedaxane treatment. Syngenta does not consider that there is robust evidence that sedaxane has carcinogenic potential in rats at the doses tested, as the frequency of neoplastic findings in affected tissues were generally within historical control ranges, the doses at which neoplastic findings were identified exceeded the maximum tolerated dose, and no evidence of mutagenicity/genotoxicity were identified in the full suite of required genotoxicity studies.

In 2011, the Cancer Assessment Review Committee (CARC) of the US EPA evaluated the carcinogenic potential of sedaxane and concluded that sedaxane is "Likely to be Carcinogenic to Humans" with application of a linear low-dose extrapolation model (Q1*) for quantification of cancer risk to humans (U.S. Environmental Protection Agency, 2011b). Upon reconsideration of the toxicological assessment of sedaxane as requested by the European Commission, EFSA concluded that the overall pattern of tumours in rats (multiple sited) and mice (liver) suggests that a 'Carc cat 2, H351, suspected of causing cancer' classification regarding carcinogenicity would be required for sedaxane (EFSA, 2013). After these regulatory positions were published, Syngenta conducted a program of work to investigate the possible tumour incidences described by EPA and/or EFSA. Based on this work, uterine, thyroid, and liver tumour weight of evidence (WOE) assessments were prepared by Syngenta to evaluate the MOA and human relevance of each tumour type to support a Cancer Reclassification Decision by the US EPA for sedaxane. In combination with similar WOE documents that address the MOA and human relevance of liver and thyroid tumours, this uterine tumour WOE assessment is intended to support no cancer classification for sedaxane in the EU.

The purpose of this current document is to review the available data from a perspective that the higher uterine tumour incidence at 36000 ppm may be related to treatment with sedaxane. The overall Weight of Evidence (WOE) is evaluated according to the MoA framework developed by the IPCS and ILSI which considers the strength of evidence for establishing a mode of action in the animal model and the relevance of any proposed mode of action to humans. This evaluation is used as a basis for determining if any cancer classification according to the CLP criteria (European Parliament and the Council of the European Union, 2008) is justified for sedaxane based on the higher incidence of uterine tumours.

2.1 Overview of 2-year rat carcinogenicity data for sedaxane

In a combined chronic toxicity and carcinogenicity study, Han Wistar rats (strain designation CrL:WI(Han)) were treated for up to 2 years with sedaxane at dietary inclusion levels of 0, 200, 1200 and 3600 ppm, which corresponded to achieved dose levels of 0, 14, 86 and 261 mg/kg/day for female rats (Anonymous, 2010, *Annex I. 3.9.1.1*).The study authors noted that the incidence of endometrial adenocarcinomas at 3600 ppm in the uterus was statistically significantly higher than the control group by Fisher's Exact Test ($p<0.01$), and that a significant increasing trend was observed by the Peto trend test. No significant increase in uterine tumour incidence was observed at the mid-dose of 1200 ppm. A statistically significant decrease in the incidence of mammary fibroadenomas was also reported at 3600 ppm, but not at 1200 ppm. There was also a tendency for decreased pituitary adenomas in the high dose females (Table 1).

TABLE 1 Incidences of Uterine Endometrial Tumours, Mammary Gland Fibroadenomas, and Pituitary Gland Adenomas in Female Han Wistar Rats at the Conclusion of a 2-Year Carcinogenicity Study – Terminal Sacrifice + Decedents

Table 1A: Sedaxane 2-year study - Selected tumour incidences:

Tumour Type	Dietary inclusion level of Sedaxane in ppm (mg/kg/day)			
	0	**200 (14 mg/kg/day)**	**1200 (86 mg/kg/day)**	**3600 (261 mg/kg/day)**
Uterine				
Adenomas (%)	0/52 (0%)	0/52 (0%)	1/52 (2%)	0/52 (0%)
Adenocarcinomas (%)	0/52 (0%)	3/52 (6%)	2/52 (4%)	9/52** (17%)
Mammary Gland				
Fibroadenoma (%)	14/52 (27%)	9/50 (18%)	10/51 (20%)	0/52*** (0%)
Pituitary Gland				
Adenoma, Anterior lobe (%)	23/52 (44%)	29/52 (56%)	20/52 (38%)	16/52 (31%)

, * Statistically-significantly different from control with $p<0.01$, $p<0.001$ (Fisher's Exact Test) as reported in the original rat 2-year report [mammary gland and pituitary gland (Anonymous, 2010, *Annex I. 3.9.1.1*)].

Table 1B: Historic control data in Wistar rats (± 5 years from start of sedaxane study)

	Historic Control Data - Range	
Uterine tumours:	Lab (CRL)	RITA
Adenomas	0-6%	0-6%
Adenocarcinomas	0-19%	0-22%

Lab (CRL) data refers to 10 prior or concurrent studies at CRL in 2002-2012, ± 5 years from start of sedaxane rat chronic/carcinogenicity study (see Appendix A). RITA data refers to 22 studies conducted in the Wistar rat from 2002-2012, ± 5 years from start of sedaxane rat chronic/carcinogenicity study. Registry of Industrial Toxicology Animal Data (RITA), (see Appendix B) http://reni.item.frauhofer.de/reni.

Based on the historical control data in the table above, the study authors concluded that the incidence of uterine adenocarcinomas in the 3600 ppm group was within the normal range of biological variability and was not therefore an effect of treatment. In addition, there were no treatment-related effects on the uterus at the 52-week interim sacrifice, non-neoplastic micropathology changes to the uterus in the 104-week carcinogenicity study animals or changes to the uterus in two 90-day rat studies at doses up to 4000 ppm, which corresponded to 349.8 mg/kg/day for (Anonymous, 2009 *Annex I. 3.12.1.2*).

Within Table 1B, updated Historic Control Data (HCD) from both the performing laboratory (CRL) and RITA are summarized, to include all studies that were within five years of the current study, both before and after the study was conducted [*i.e.*, consistent with OECD (2009) recommendations regarding HCD]. The results from the individual studies that compromise these HCD are tabulated in Appendices 1 and 2.

Compared to the HCD ranges (Table 1), the incidence of uterine adenocarcinomas (17%) in female rats at 3600 ppm was within the range of values from the test laboratory and the RITA database. In addition, the incidence of uterine adenocarcinomas and combined uterine tumours in the concurrent control group of the sedaxane rat study (0%) was somewhat lower than a typical study in this strain of rat. Of the 10 studies where HCD values were available from the test laboratory, only two had an incidence of 0% for adenocarcinomas. Two studies in the RITA database out of 22 studies had 0% for adenocarcinomas. Therefore, the incidences of uterine adenocarcinomas in all treatment groups are within normal range of HCD for both the performing laboratory as well as the RITA database.

There was no evidence for a treatment-related effect on the incidence of uterine tumours in an 80 week study in CD-1 mice (Anonymous, 2010, *Annex I. 3.9.1.2*).
In this study, male and female CD-1 mice were treated with sedaxane at 25, 157 and 900 mg/kg/day for males, and 29, 185 and 1001 mg/kg/day for females, corresponding to dietary inclusion levels of 200, 1250 and 7000 ppm respectively for both sexes. The no observed adverse effect level (NOAEL) for this study was 1250 ppm, based on a decrease in body weight and body weight gain in males and females, and a decrease in food utilization during the early stages of the study.

Syngenta and the performing laboratorymaintain that the higher incidence of uterine tumours at 3600 ppm is within the range of the variable spontaneous tumour incidence of Han Wistar rats and is not related to sedaxane treatment. However, the European Food Safety Authority (EFSA) concluded that the uterine adenocarcinoma incidence at 3600 ppm was an effect of treatment, and that the overall pattern of tumours in rats and mice suggests that a 'Carc cat 2, H351, suspected of causing cancer' classification regarding carcinogenicity would be required for sedaxane (EFSA, 2013).

DS comment: Uterine adenocarcinoma is a common finding in aging Wistar rats, as demonstrated by historical control data from the laboratory (2002-2005). It is also acknowledged that uterine adenocarcinoma incidence in the concurrent control animals was low (0%). However, as shown in the Historic control data from Charles River (CRL) available (Appendix A), two other studies out of the ten during this period (2002-2012) had a control group with a 0.0% incidence.
As regard HCD from RITA database (Appendix B), they are not considered appropriate (not the same laboratory).
Furthermore, regarding structure-activity relationships, another SDHI fungicide similar to sedaxane, "isopyrazam" also induced uterine adenocarcinoma at a high dose level of 3000 ppm (233 mg/kg/day). Therefore, the statistically increased incidence of uterine tumours observed at

high dose level (3600 ppm), above the HCD mean could not be ruled out as unrelated to treatment.
See annex I to the CLH Report 3.9.1.1

3.0 WEIGHT OF EVIDENCE ASSESSMENT FOR A PROPOSED MODE OF ACTION FOR UTERINE TUMOURS

A framework for assessing and communicating the relevance of tumour findings in rodent studies to humans has been developed by the International Programme on Chemical Safety (IPCS) (Boobis *et al.*, 2006; Sonich-Mullin *et al.*, 2001) and the International Life Science Institute (ILSI) (Meek *et al.*, 2003). The framework aims to answer three questions: (i) Has a mode-of-action (MOA) been established in the test species?; (ii) based on qualitative assessment of the differences between species in terms of toxicokinetics and toxicodynamics, is that MOA plausible in humans?; and (iii) based on an assessment of the quantitative differences between species in terms of toxicokinetics and toxicodynamics, is the MOA plausible in humans?

First, a MOA is established in the rodent using an approach developed by the IPCS/ILSI (Boobis *et al.*, 2006; Meek *et al.*, 2003; Sonich-Mullin *et al.*, 2001), which begins with a postulated theory of cause and the series of requisite and measureable events that are necessary for the induction of the toxicity. A recent workshop on nuclear receptor induced liver tumour MOAs has defined a number of types of events that may be useful in describing the MOA (Andersen *et al.*, 2014). A causal key event is an empirically observable causal precursor step to the adverse outcome that is itself a necessary element of the MOA. Such key events are required for a MOA, but often are not sufficient to induce the adverse outcome in the absence of other key events. Associative events are measurable biological processes that are not themselves necessary causal key events for the MOA, but are reliable indicators or markers for key events. As such, associative events can often be used as surrogates for a causal key event in a MOA. Finally, modulatory factors are biological features or responses that are not necessary to induce an adverse event but could modulate the dose-response or probability of inducing one or more key events or the adverse outcome. A body of experimental evidence is then developed and assessed to support the association between these key events and the apical endpoint. This assessment is made using "tests for causation" proposed by Bradford Hill (1965) and involves answering a number of simple questions, namely:

- Are the dose and temporal relationships consistent with causality?
- Are the effects consistent and reproducible between studies?
- Could other causes have given rise to the key events?
- Are the effects biologically plausible given our current state of knowledge?

Only when a MOA has been established in an experimental species, can the human relevance assessment begin and, if, on the basis of experimental results, it can be shown that one or more of the necessary key events seen in the animal MOA is not plausible in humans (on either qualitative or quantitative grounds), then the adverse outcome in rodents is not appropriate for further consideration due to a lack of human relevance.

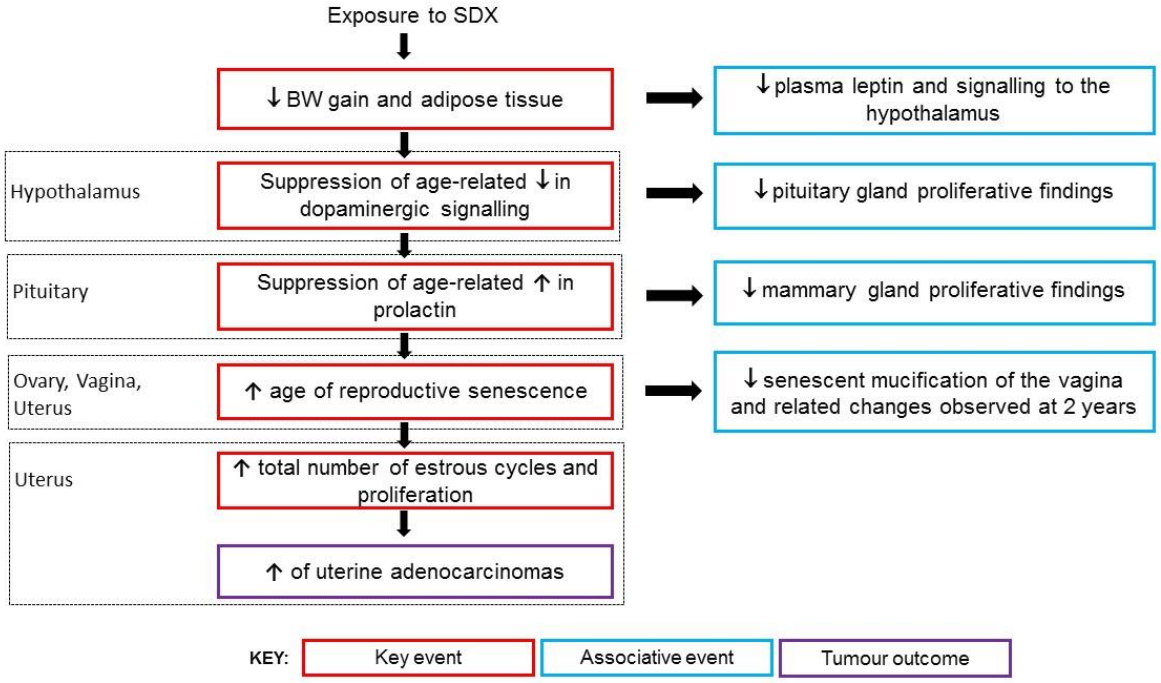
3.1 Mode of Action Hypothesis

Based on the published conclusions by by US EPA (2011b) and EFSA (2013) that the increase in uterine adenocarcinomas at 3600 ppm in the 2-year rat study may be an effect of treatment, Syngenta conducted a program of work to investigate the MOA for uterine tumours in the rat and assess their

potential human relevance The evaluation that was undertaken was influenced by the publication of Harleman et al (2012) who noted an association between increased uterine tumour incidence and a decrease in mammary tumour incidence and, to a lesser extent, pituitary tumour incidence. They also noted that the Han Wistar rat appeared particulary susceptible to this shift in tumour profile compared to other laboratory strains of rat.

Figure 1 shows the proposed mode of action for the uterine adenocarcinomas in female Wistar rats following 2 years of treatment with sedaxane. Treatment-related decreases in body weight gain and mean group body weight in aging Wistar rats would be associated with lower levels of circulating leptin and result in lower levels of prolactin secretion due to maintenance of dopaminergic activity in the hypothalamus, and consequently decreased prolactin-mediated progesterone secretion from the corpora lutea of the ovaries. This may also be described as a lack of the physiological increase in blood prolactin levels that normally occurs in aging rats as they enter reproductive senescence. Prolactin is a key factor that influences the onset of reproductive senescence in Wistar rats, and lower levels of prolactin release (*i.e.*, maintenance of the pattern seen in young rats) in aging sedaxane-treated rats would cause a change in their transition into senescence. The result is a greater cumulative exposure of the uterus to a higher estrogen: progesterone ratio (*i.e.*, reduced progesterone dominance of estrogen) in aged female sedaxane-treated rats, which would lead to a pro-proliferative estrogenic stimulation of the uterine endometrial cells. Over time, the estrogenic proliferative drive would lead to promotion of spontaneously initiated tumours (*i.e.*, increased incidence of uterine adenocarcinomas). At the same time, the lack of an age-related increase in prolactin signaling would lead to decreased proliferation of the anterior pituitary and mammary glands, which would in turn be associated with a reduction in the incidences of pituitary adenomas and prolactin-driven mammary gland fibroadenomas.

FIGURE 1 Mode of Action Hypothesis for Induction of Uterine Endometrial Adenocarcinomas in Female Han Wistar Rats by Sedaxane



3.2 Sedaxane-specific Data to Support the Proposed MOA

To assess the proposed MOA, data from a number of repeat dose dietary and *in vitro* studies were evaluated to build a weight-of-evidence supporting the key and associative events.

3.2.1 Lower body weight gain in the two-year chronic/carcinogenicity study for sedaxane

The initial key event in the proposed MOA for sedaxane uterine tumours is a large, sustained deficit in body weight gain. Data from the 104-week study are shown in Table 2 and Figure 2. In females at 3600 ppm, group mean body weight and cumulative body weight gain were statistically significantly lower than control from Week 1 to the end of the study, and the magnitude of the difference increased progressively throughout the study. As shown in both Table 2 and Figure 2, the divergence from the control group values were accelerated in the second year of the study, with a greater percentage difference from control at 104 weeks (-50%) than at 52 weeks (-34%) for cumulative body weight change. It can be argued that the top dose in this study is beyond the maximum tolerated dose (MTD), as indicated by the 50% decrease in body weight gain as compared to control, which equates to ~33% decrease in body weight at the end of the study.

Sedaxane at 1200 ppm also produced statistically significantly lower body weights (-8%) and body weight gain values (-11%) compared to the concurrent control group. The 200 ppm group displayed no treatment-related differences from the control group. Therefore, the mid-dose group may be considered near the MTD as defined by a 10% or greater retardation of body weight gain as compared to control.

Food consumption was statistically lower than the control group for females at 3600 ppm throughout the treatment period (data not shown). In contrast, food consumption profiles in females that received 200 or 1200 ppm closely resembled those displayed by the control group.

Food utilization (g/100 g of diet consumed) was statistically significantly lower for the 3600 ppm females during the intervals of Weeks 1-4, Weeks 9-13 and overall for Weeks 1-13 in comparison to the control group (Table 3). In contrast, there were no consistent differences in the 200 or 1200 ppm females compared to the controls.

In summary, these data from the 104-week study indicate that 3600 ppm sedaxane produced a very large and progressive deficit in body weight gain compared to the control females. Although a small proportion this deficit may be explained by lower food consumption, the lower food utilization values at 3600 ppm indicate that there was a lower efficiency in utilizing the consumed calories. Thus, the profound deficit in body weight gain in 3600 ppm animals relative to controls is similar to a caloric restricted state [*e.g.*, (Roe *et al.*, 1995); discussed in a later section regarding Biological Plausibility].

In the 104-week chronic/carcinogenicity study in rats, specific measurements that would reflect a decrease in the percentage of adipose tissue [*e.g.*, percentage (by weight) abdominal fat pads, omental fat] were not a routine part of the study design, and therefore, a direct measure of adipose tissue in the 3600 ppm female rats was not performed. However, based on known responses in rat studies to caloric restriction, and the increasing percentage of body weight in obese rats that is represented by fat at the end of a 2-year *ad libitum* feeding study, it can be inferred that 3600 ppm sedaxane-treated female rats that were greatly lower than controls in body weight (-33% at 104 weeks) had lower percentages of their body weight as adipose tissue than the controls. This proposed key event of reduced adipose tissue in 3600 ppm sedaxane-treated rats is discussed further in Section 4.1.6.

DS comment: <u>Key event 1: "decreased body weight gain/decreased adipose tissue</u>"

Significant treatment-related decreased body weight gain is supported by experimental data. However, since adipose tissue was not measured in the 2-year rat study, there is no sedaxane-specific data regarding the supposed decrease in adipose tissue.

Furthermore, decreased bodyweight gain is not a molecular event. It is a broad event, observed in many high dose groups of guidelined carcinogenicity studies. It is therefore questionable why uterine tumours are not observed with all chemical inducing significant body weight changes.

TABLE 2 Intergroup Comparison of Female Body Weights and Cumulative Bodyweight Change in the 104-Week Rat Study

Week	0 ppm	200 ppm (14 mg/kg/day)	1200 ppm (86 mg/kg/day)	3600 ppm (261 mg/kg/day)
Body Weight (g):				
13	235.5	238.0	232.2 (-1%)	205.4** (-13%)
26	259.0	259.0	249.0** (-4%)	219.4** (-15%)
52	292.9	295.5	281.1 (-4%)	240.2** (-18%)
104	392.5	389.9	362.2* (-8%)	264.1** (-33%)
Cumulative Bodyweight Change (g):				
0-13	106.1	108.1	102.2 (-4%)	72.9** (-31%)
0-52	163.5	165.6	151.1** (-8%)	108.1** (-34%)
0-104	262.6	259.2	232.7* (-11%)	132.4** (-50%)

Values are group means (g) for females at selected intervals (% difference vs. control values in parentheses)

* Statistically significant difference from control group mean, $p<0.05$ (Dunnett's test, 2-sided)

** Statistically significant difference from control group mean, $p<0.01$ (Dunnett's test, 2-sided)

Data are from Anonymous, 2010, *Annex I. 3.9.1.1.*

TABLE 3 Food Utilization in Females from the 104-Week Rat Study

Week	**0 ppm**	**200 ppm (14 mg/kg/day)**	**1200 ppm (86 mg/kg/day)**	**3600 ppm (261 mg/kg/day)**
1-4	13.7	13.6	12.8*	10.1**
5-8	4.8	5.3	5.4	5.4
9-13	2.8	2.6	2.8	1.5**
1-13	6.8	6.8	6.6	5.3**

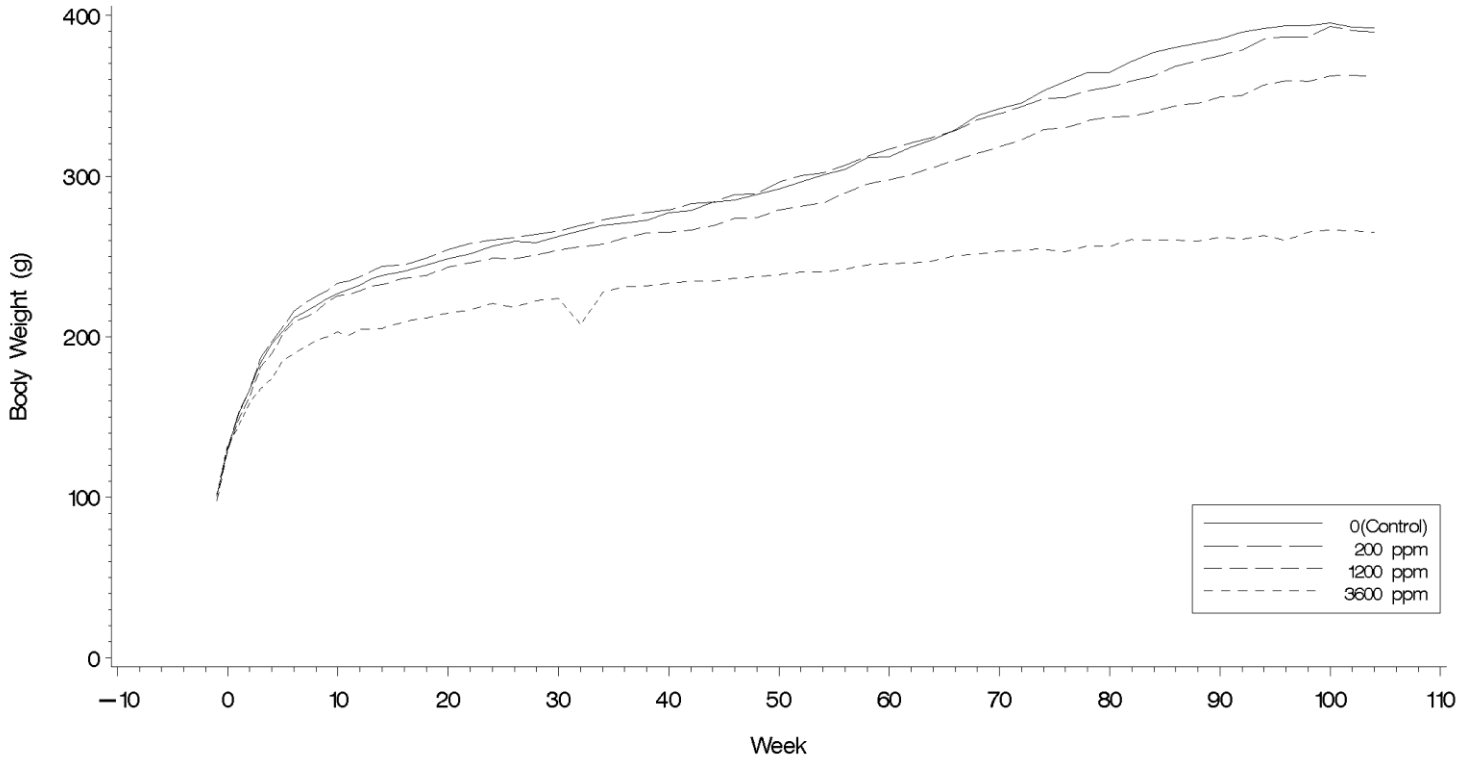
Values are group means (g per 100 g of diet consumed) for females during the indicated durations.

* Statistically significant difference from control group mean, $p<0.05$ (Dunnett's test, 2-sided)

** Statistically significant difference from control group mean, $p<0.01$ (Dunnett's test, 2-sided)

Data are from (Anonymous, 2010, Annex I. 3.9.1.1).

FIGURE 2 Group Mean Body Weights vs. Time in Female Rats during the Sedaxane 104-Week Chronic/Carcinogenicity Study



Data are from Anonymous, 2010, *Annex I. 3.9.1.1*.

A slight decrease in male and female group mean body weight was noted during Week 32 when compared to previous measurements in 3600 ppm animals; the deficit was made up by the following measurement. No explanation for this transient effect could be found (as described in the study report), and it was not considered to have affected the integrity of the study.

3.2.2 Lower Plasma Leptin Levels in rats treated with Sedaxane for 1-Year

Serum samples from female Wistar rats taken after 1 year in the carcinogenicity study with sedaxane (Anonymous, 2010, *Annex I. 3.9.1.1*) were evaluated for concentrations of prolactin, leptin and adiponectin in the 0, 1200 and 3600 ppm groups by radioimmunoassay or enzyme-immunoassay methods, and mean values were compared to determine any differences related to treatment (Anonymous, 2016 *Annex I. 3.9.4.14*). Due to the inherent level of variation in prolactin levels between individual animals at 52 weeks of age, it was not possible to determine any differences in prolactin concentrations between control and sedaxane-treated groups from the available serum samples.

Group means for leptin levels at 1 year were 4.46, 4.37 and 3.65 ng/mL for the 0, 1200 and 3600 ppm females, respectively (Table 4). The mean leptin value for the 3600 ppm females was 18% lower than the control group mean value, and this difference (although not statistically significant), matched the 13% lower body weights in the 3600 ppm females that were statistically significant. Mean values for adiponectin at 1 year showed no differences between groups (Table 5). Based on these comparisons, and an observed correlation of leptin levels with body weight in all groups, the lower leptin levels for the 3600 ppm group were considered to be a treatment-related effect.

DS comment: <u>Associative event 1: "Decreased plasma leptin and signalling to the hypothalamus"</u>
While the non-statistically significant decreased in mean leptin value observed in high dose females may indicate a decrease in adipose tissue, it is not supported by mean values for adiponectin which were not affected by treatment.
There is no evidence on decreased signalling to the hypothalamus.
(see Annex 1 to the CLH Report 3.9.4.14)

TABLE 4. Plasma Leptin Levels (ng/mL) in Rats Treated with Sedaxane for 1 Year.

	0 ppm	**1200 ppm**	**3600 ppm**
Mean	4.46	4.37	3.65
Std. Dev.	2.62	3.21	1.49
N	12	11	10
% Decrease vs. control		-2.0%	-18.0%

Not Statistically significant by ANOVA, Dunnett's ($P<0.05$)

TABLE 5. Plasma Adiponectin Levels (µg/mL) in Rats Treated with Sedaxane for 1 Year.

	0 ppm	**1200 ppm**	**3600 ppm**
Mean	29.93	24.76	28.70
Std. Dev.	6.83	11.11	6.75

N	11	9	9

Not Statistically significant by ANOVA, Dunnett's (P<0.05)

3.2.3 Shift in tumour profile with sedaxane treatment

Concominant to the uterine adenocarcinoma incidence, there is a decrease in the incidences of mammary gland fibroadenomas and anterior pituitary adenomas in the 3600 ppm females (Table 1). As a further exploration of these associations, both neoplastic and non-neoplastic pathology findings in the mammary gland and pituitary gland are summarized in Table 6.

Examining the mammary gland data, there is a statistically significant decrease in mammary gland fibroadenomas, and the overall data across all groups showed a statistically significant negative trend (Peto trend test; including decedents). In addition, the 3600 ppm females from the 2-year terminal sacrifice, and among the decedent animals, had a statistically significantly higher incidence of "no abnormality detected" in the mammary gland (Table 6). Remarkably, in the 3600 ppm group (2-year sacrifice and decedents), there was a zero incidence of mammary fibroadenoma. In addition, the non-neoplastic finding of "lobular hyperplasia with atypia" was statistically significantly lower in the 3600 ppm females at the terminal sacrifice. These incidences are considered an Associative Event that are reflective of the proposed lower levels of circulating prolactin, as mammary fibroadenomas in rats are considered to be responsive to this hormonal input over time (Greaves, 2007; Hargreaves and Harleman, 2011; Keenan *et al.*, 1996).

Examining the pituitary gland data, the 3600 ppm females from the 2-year terminal sacrifice had a statistically significantly higher incidence of "no abnormality detected" in the pituitary gland compared to the controls. Along with this observation, there was a numerically lower incidence of pituitary adenomas (anterior lobe) compared to the control group or the 200 and 1200 ppm groups, and the incidence of this finding in the decedent animals was also lower at 3600 ppm (2/8) than in the control group (4/8). A related non-neoplastic marker of proliferation, focal hyperplasia of the anterior pituitary, was also numerically lower in the 3600 ppm terminal sacrifice animals than in the other control and treated groups. Only the incidence of "no abnormality detected" achieved statistical significance, but a pattern of lower proliferative changes in the anterior pituitary at 3600 ppm was evident. These lower incidences of pituitary proliferative changes with high dose sedaxane treatment are an Associative Event in the MOA, as the age-related increase in pituitary adenomas in control rats is known to reflect loss of dopaminergic suppression of the pituitary lactotroph cells (Freeman *et al.*, 2000).

There were no differences from controls in findings in the mammary gland or the pituitary gland at the 1-year sacrifice (data not shown); therefore, the data indicate that these effects were late onset findings, *i.e.*, after 1 year of exposure.

TABLE 6 Incidence of Pathology Findings in Pituitary and Mammary Gland – Associative Events in the 2-Year Carcinogenicity Study (Female Rats)

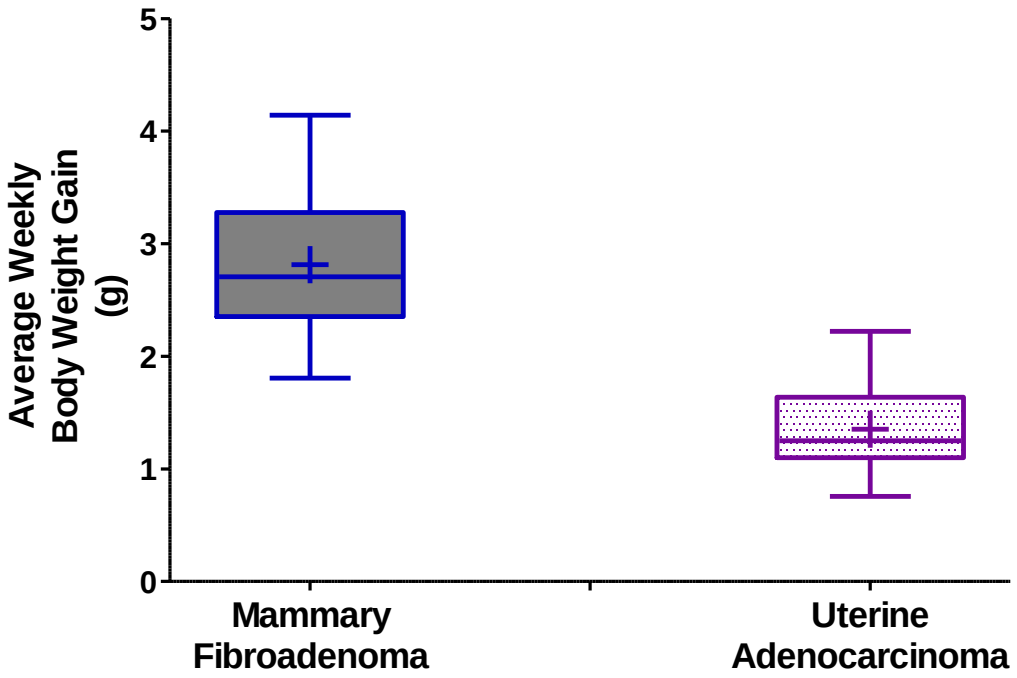
	Survivors (104 Weeks)				Decedents			
	Dose Groups							
Pathology findings	**0**	**200 ppm (14 mg/kg/day)**	**1200 ppm (86 mg/kg/day)**	**3600 ppm (261 mg/kg/day)**	**0**	**200 ppm (14 mg/kg/day)**	**1200 ppm (86 mg/kg/day)**	**3600 ppm (261 mg/kg/day)**
Pituitary Gland	(44)	(35)	(37)	(44)	(8)	(17)	(15)	(8)
No abnormality detected	7	9	8	19**	2	3	6	3
Focal hyperplasia, anterior lobe	14	11	17	9	1	2	0	3
Adenoma, anterior lobe [B]	19	16	15	14	4	13	5	2
Carcinoma, anterior lobe [M]	0	0	0	0	0	0	1	0
Adenoma, intermediate lobe [B]	1	0	0	0	1	0	0	0
Ganglioneuroma [B]	1	0	0	0	0	0	0	0
Mammary Gland	(44)	(34)	(36)	(44)	(8)	(16)	(15)	(8)
No abnormality detected	8	8	7	25***	0	2	5	6**
Fibroadenoma [B]	11	6	7	0***	3	3	3	0
Lobular hyperplasia, with atypia	13	11	7	1***	0	0	0	0
Adenoma [B]	1	0	0	0	1	0	0	0
Adenocarcinoma [M]	3	1	0	0	0	0	1	0

[B] – benign tumour; [M] – malignant tumour. Data from Anonymous, 2010, *Annex I. 3.9.1.1.*
*, **, *** statistically significant by pairwise Fisher's Exact Test ($p<0.05$, 0.01, 0.001)

By looking across all treatment groups in the 2-year rat study with sedaxane on an individual animal basis, certain other patterns and correlations are noteworthy:

- Notably, in animals with uterine adenocarcinomas (regardless of dose group), there is no co-incidence of mammary gland fibroadenomas.
- Considering the large deficits in body weight gain vs. control in the 3600 ppm females and (to a lesser extent) in the 1200 ppm females (Table 2), along with the patterns of tumour incidence shown in Tables 1 and 6, there is a clear relationship between average weekly body weight gain and the incidences of these tumour types.
    - Mammary gland fibroadenomas and pituitary adenomas occur in rats with higher body weight gains, and uterine adenocarcinomas occur in rats with lower body weight gains. This correlation is illustrate graphically in Figure 3.

FIGURE 3 Relationship of Average Weekly Body Weight Gain with Tumour Type in Sedaxane 104-Week Rat Study



The box extends from the 25th and 75th percentiles of average weekly body weight gain while the whiskers are the minimum and maximum average weekly body weight gain. The line within the box is the median while the plus sign is the mean weekly body weight gain. Data from all female rats in this study, regardless of treatment group, were combined in this analysis. Data from Anonymous, 2010, *Annex I. 3.9.1.1.*

In summary, lower incidences of proliferative responses in the pituitary (adenomas) and mammary gland (fibroadenomas) occur only in the 3600 ppm females, and these data provide strong evidence that prolactin is an integral component of the proposed MOA.

These data are consistent with the caloric restriction mediated changes in tumour profiles in Wistar rats (Harleman *et al.*, 2012; Roe *et al.*, 1995; Tucker, 1979), as discussed in Section 4.1.4. regarding Biological Plausibility of the proposed MOA.

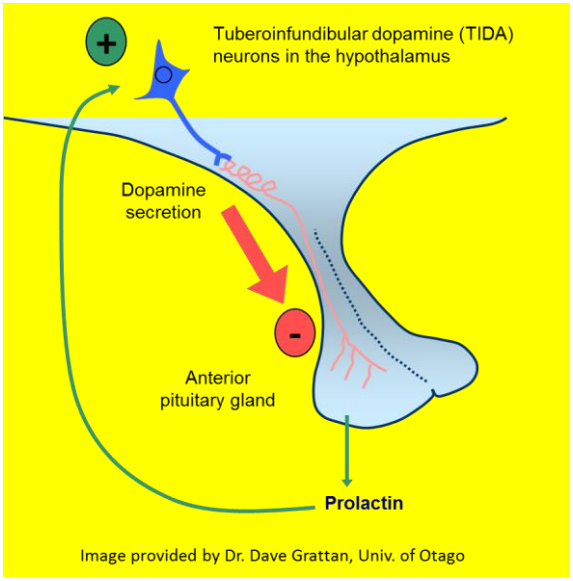
DS comment: <u>Associative event 2 "decreased pituitary gland findings" and associative event 3 "decreased mammary gland findings".</u>
It is agreed that experimental data support associative event 3 and to a lesser extent associative event 2.
However, contrary to the applicant statement, there is no strong evidence that "prolactin is an integral component of the proposed MOA". Indeed, no direct evidence supports decreased prolactin levels. Prolactin levels were only measured in one-year sacrificed females and no differences in prolactin concentrations between control and sedaxane-treated groups were observed.
(see Annex 1 to the CLH Report 3.9.4.14)

3.2.4 Suppression by Sedaxane Treatment of Age-Related Changes in Dopaminergic Neurons in the TIDA Region of the Hypothalamus

As outlined in Figure 1, the proposed MOA includes the preservation of dopaminergic signalling in the TIDA neurons of the hypothalamus as rats age, possibly via altered serum levels of leptin and other adipokine signals to the hypothalamus, which are directly proportional to body weight, nutritional status and adipose tissue content of the animals (Arner, 2003; Tena-Sempere, 2015; Woodside *et al.*, 1998). To help orient the reviewer of this Assessment to the underlying physiology, Figure 4 provides a schematic of the TIDA region and how it relays dopamine to the anterior pituitary to control the release of prolactin.

The TIDA neurons have their cell bodies in the arcuate nucleus (ARC) region of the hypothalamus, and their axons extend into the median eminence (ME) region. These TIDA neurons synthesize dopamine, which is released from the ME and travels via the primary plexus (a network of capillaries) into the anterior pituitary. In the pituitary, dopamine has an inhibitory effect on the lactotrophs that synthesize and release prolactin into the systemic circulation. Circulating prolactin also plays a role in down-regulating dopamine release by the TIDA neurons, completing a known negative feedback loop.

FIGURE 4 Anatomy of the TIDA Neurons and Control of Prolactin Release in the Anterior Pituitary



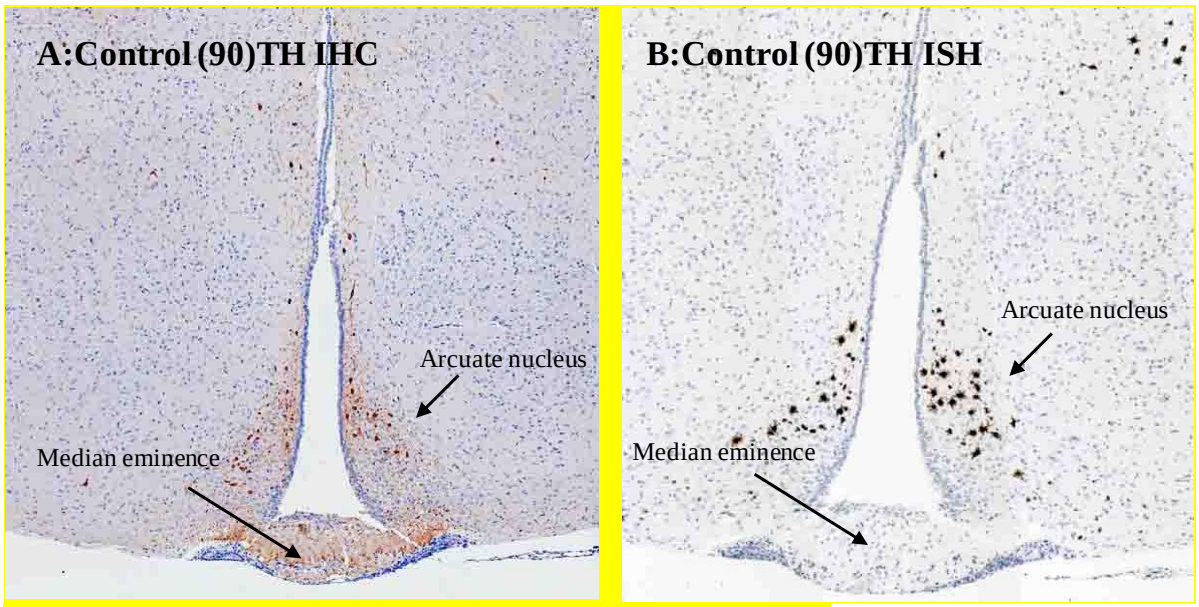
3.2.4.1 Age-related changes in dopaminergic neurons as indicated by tyrosine hydroxylase mRNA and protein expression

As female Wistar rats age and begin to enter reproductive senescence, a variety of stimuli trigger a decrease in the number and activity of dopaminergic neurons in the TIDA region. To assess the changes with age in female Wistar rats, a study with control rats that had been sacrificed at 90 days, 1 year or 2 years was performed, using formalin-fixed paraffin-embedded (FFPE) brains (just reference the studies) Tyrosine hydroxylase (TH) is the rate-limiting step in the synthesis of dopamine. The relative abundance of TH in the TIDA region was assessed in these rats by two methods:

- In-situ hybridization (ISH): a method that selectively stains mRNA for TH.
- Immunohistochemistry (IHC): a method that uses selective antibodies to selectively stain TH protein.

Detailed methods for the ISH and IHC techniques are provided in the study report (Anonymous, 2015a *Annex I. 3.9.4.12*). In short, to quantify the extent of staining in each slide, a grid was overlaid and the positively stained pixel area within regions of the arcuate nucleus or the median eminence were determined by image analysis. Similar approaches were followed for slides stained for tyrosine hydroxylase mRNA and protein. Landmarks in these sections were noted that indicated an appropriate brain section (Bregma 2.4) [as defined in an anatomical atlas of the rat brain (Paxinos and Watson, 2009)]. Representative images of the 90-day control rat TIDA region following IHC or ISH staining for TH are shown in Figure 5.

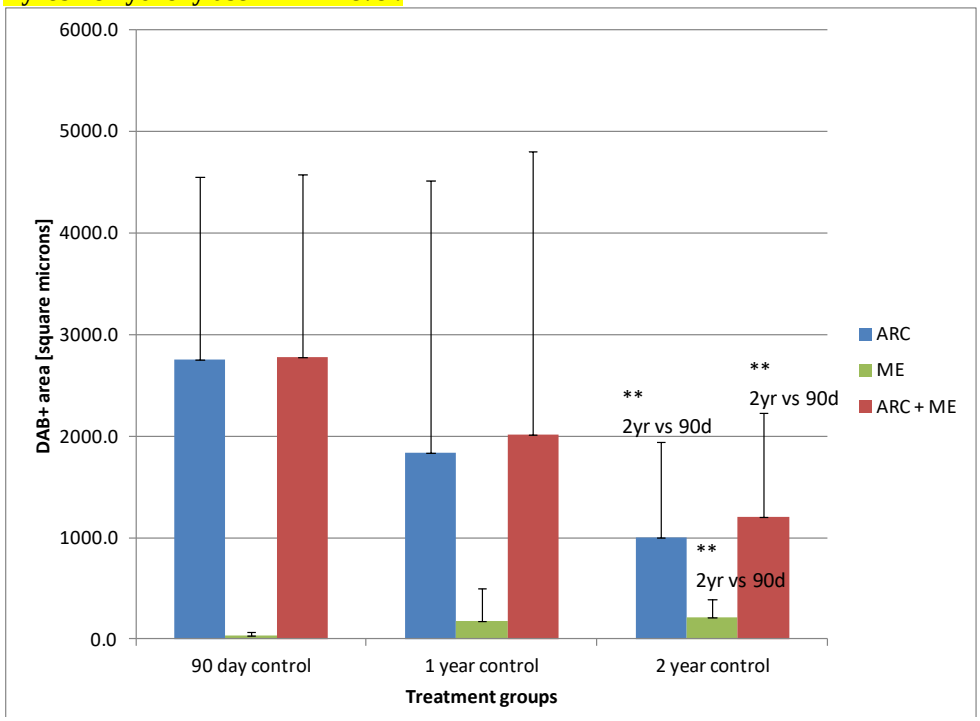
FIGURE 5 Representative Images of the TIDA Region of Control (90-Day) Rats Stained for Tyrosine Hydroxylase by IHC or ISH



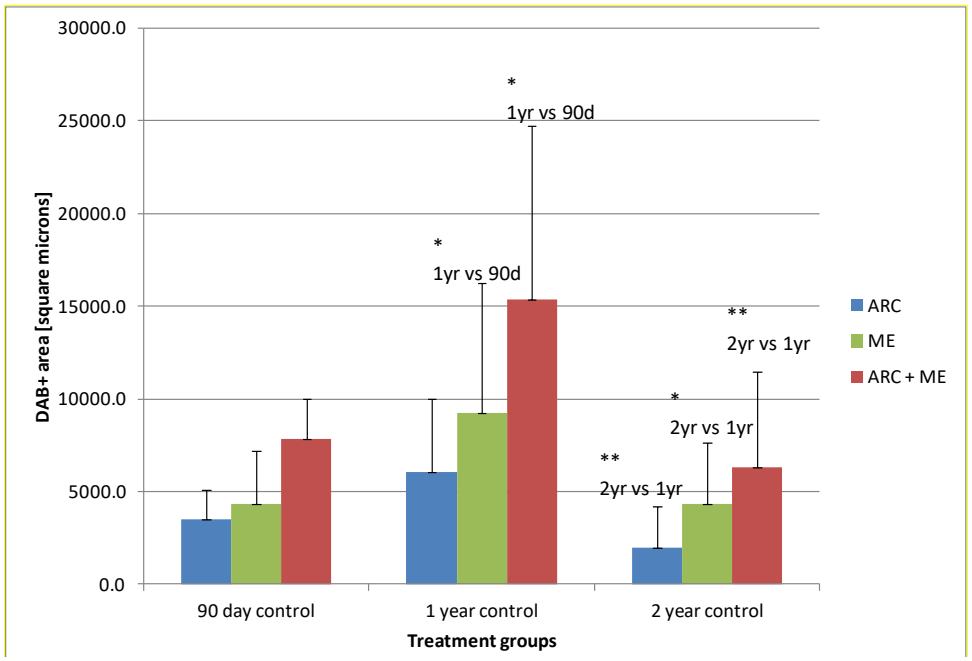
A: TIDA region of a 90-day control rat stained for tyrosine hydroxylase protein (IHC)
B: TIDA region of a 90-day control rat stained for tyrosine hydroxylase mRNA (ISH).
Brown color staining by diaminoazobenzene (DAB) is observed in the arcuate nucleus and median eminence. Sections were taken from the brain in the region of Bregma 2.4 mm. Images from Anonymous, 2015a *Annex I. 3.9.4.12*.

FIGURE 6 Quantitation of Tyrosine Hydroxylase in the TIDA Region in Control Groups of Increasing Age

Tyrosine hydroxylase mRNA level:



Tyrosine hydroxylase protein level:



Results are mean (positive stained area, μM^2)± SD, n=8-10. *, **, statistically significantly different P<0.05, P<0.01, respectively, by students T test 1 tailed type 2 . Data from Anonymous, 2015a Annex I. 3.9.4.12.

As shown in Figure 5, the mRNA for tyrosine hydroxylase was localized primarily in the arcuate nucleus, where the cell bodies were located, whereas the protein for tyrosine hydroxylase was located in both the median eminence and the arcuate nucleus. Quantification of the TH protein and mRNA in control female Han Wistar rats at 90 days, 1 year and 2 years are shown in Figure 6. The results demonstrate an age-related decrease in TIDA tyrosine hydroxylase-positive neurons. The TH mRNA levels at 2 years were statistically significantly lower than the values at 90 days. Also, there was a significantly lower amount of TH protein in all three regions (ARC, ME and ARC+ME) for the 2-year animals compared to the 1-year animals. While there was a slight but significant increase in TH protein in the ME and ARC+ME regions of the 1-year control animals compared to the 90-day control animals, it is most likely due to variation given that the standard deviations at 90 days and 1 year were large for both protein and mRNA staining. For both TH protein and mRNA, the mean values were consistently lower at 2 years, and the relative standard deviations were smaller at 2 years, giving confidence that a clear age-related decrease in tyrosine hydroxylase was observed in the TIDA neurons of control female rats.

In summary, the results of this analysis with control rats are consistent with literature showing a similar decline in the expression of TH protein in the ARC and ME regions of the hypothalamus in aging rats caused by senescence of domaminergic TIDA neurons (Sanchez *et al.*, 2003).

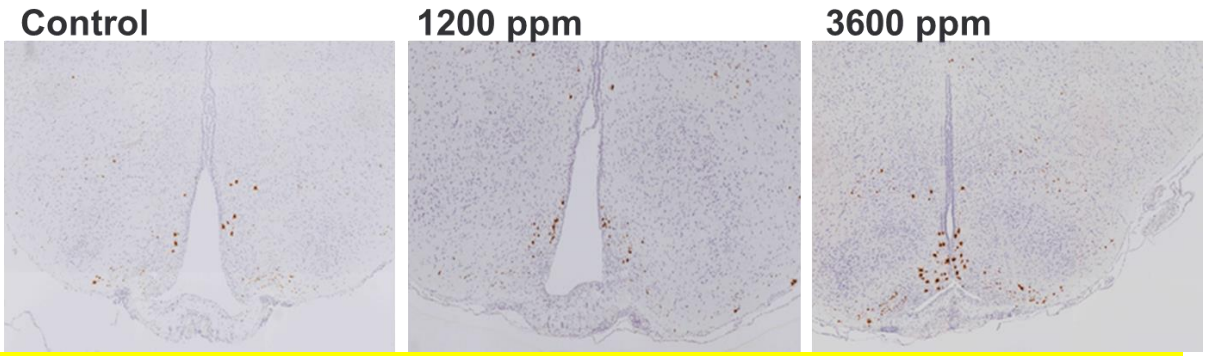
DS comment: In this study, while an age-related decrease in TIDA mRNA expression of TH was observed, it was not supported by TH protein staining (no difference between 2-year time point and 90-d time point).
The absence of correlation between mRNA staining and protein staining weakens the strength of this study to support age-related senescence of dopaminergic TIDA neurons.
(See Annex I to the CLH Report 3.9.4.12).

3.2.4.2 Changes in dopaminergic neurons as indicated by tyrosine hydroxylase mRNA and protein expression in sedaxane treatment groups

Given the age-related changes in TH protein and mRNA levels in control rats, FFPE brains from the 2-year female Wistar rats [control, 1200 ppm and 3600 ppm sedaxane groups (Anonymous, 2010, *Annex I. 3.9.1.1*)]were prepared and analyzed by the same methods, as detailed in Anonymous, 2015b Annex I. 3.9.4.13 for TH protein and mRNA.

In Figure 7, representative images for TH mRNA from control, 1200ppm and 3600 ppm sedaxane-treated brain sections are displayed. Out of all the available brain samples with proper topography and staining, 12 rats per group were quantified.

FIGURE 7 Representative Images for Quantitation of Tyrosine Hydroxylase mRNA in the TIDA Region of 2-Year Control vs. Sedaxane-Treated Rats



Brown color staining by diaminoazobenzene (DAB) is observed primarily in the arcuate nucleus. Images from Anonymous, 2015b Annex I. 3.9.4.13.

Quantification of tyrosine hydroxylase protein and mRNA in the TIDA region for control, 1200 ppm and 3600 ppm sedaxane-treated female Wistar rats at 2 years are shown in Figure 8.

The quantified amounts of tyrosine hydroxylase staining were similar to the pattern observed previously in control rats of various ages (Figures 5 and 6), with ISH producing staining of mRNA predominantly in the arcuate nucleus, and with IHC producing staining of TH protein to a similar extent in both the median eminence and the arcuate nucleus. There was a significantly higher tyrosine hydroxylase mRNA expression in the ARC and ARC+ME regions in the 3600 ppm group compared to the controls. Moreover, there were no statistically significant differences in tyrosine hydroxylase mRNA expression in the 1200 ppm treated group compared to the controls. There was a statistically significantly higher tyrosine hydroxylase protein level in both the 1200 ppm and 3600 ppm sedaxane groups compared to the control group.

In summary, these data indicated that compared to controls at 2 years of age, the 3600 ppm sedaxane group displayed preservation of the dopaminergic activity in the TIDA region of the hypothalamus as indicated by higher levels of tyrosine hydroxylase mRNA and protein. In the 1200 ppm sedaxane group, there was some evidence for the preservation of dopaminergic activity, with increases only in tyrosine hydroxylase protein but not mRNA levels compared to controls. Based on these results, it can be concluded that there is a higher capacity for dopamine production in the TIDA neurons in the 3600 ppm group compared to control rats after 2 years of treatment.

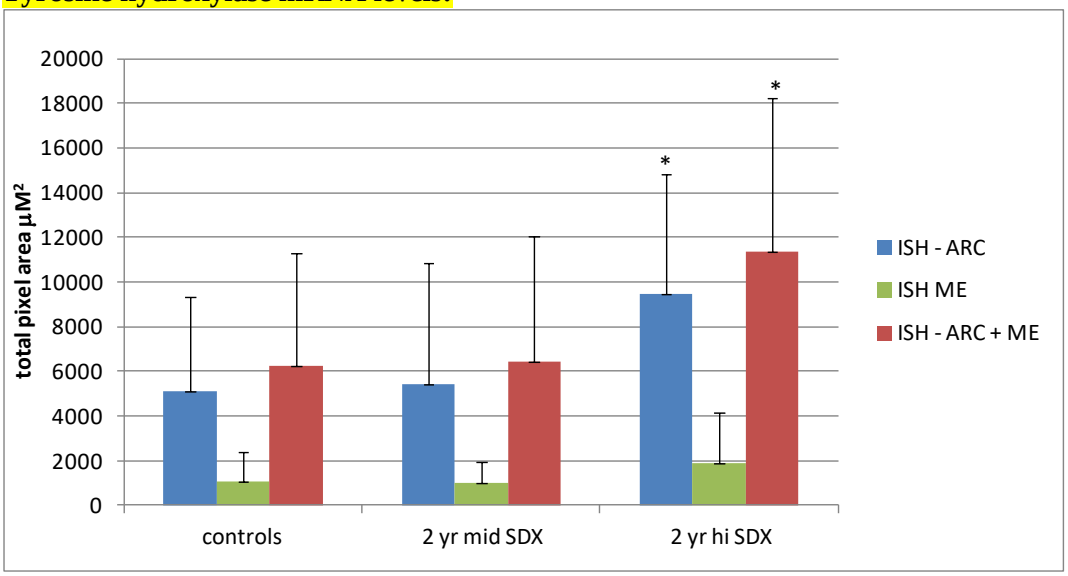
DS comment: <u>Key event 2 "suppression of age-related decrease in dopaminergic signalling" and key event 3 "suppretion of age-related increase in prolactin"</u>

In 2-year brain samples from 1200 ppm and 3600 ppm treated female rats, statistically significantly higher levels of TH protein were observed (compared to control animals) in the dopaminergic neurons. However it was not dose-related (higher TH protein level in 1200 ppm group compared to 3600 ppm group). At 3600 ppm there were also higher TH mRNA levels by ISH. While both protein and mRNA staining support that in high dose group sedaxane increased TH expression in the TIDA region at 2 years, this does not automatically mean that a suppression of "age-related decrease in dopaminergic signalling" (KE 2) had occurred. As regard KE 3 "suppression of age-related increase in prolactin" there is no direct experimental

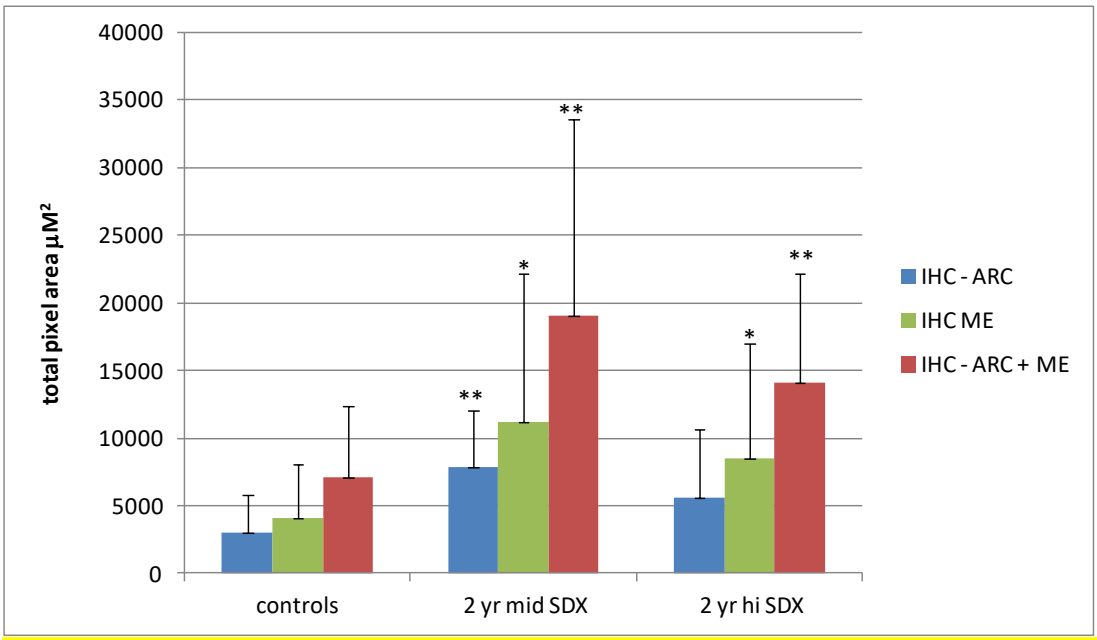
data to support it. Indeed the only measurements performed at 1-year time point did not show any treatment effect.
(See Annex I to the CLH Report 3.9.4.13).

FIGURE 8 Quantitation of Tyrosine Hydroxylase in the TIDA Region in Control and Sedaxane-Treated Groups (by ISH and IHC) for 2-Year Female Rats

Tyrosine hydroxylase mRNA levels:



Tyrosine hydroxylase protein levels:



Results are mean (positive stained area, μm^2)± SD, n=12. *, **, statistically significantly different P<0.05, P<0.01, respectively, by students T test 1 tailed type 2. Data from Anonymous, 2015b Annex I. 3.9.4.13

2yr mid SDX = 2-year rats, 1200 ppm sedaxane. 2yr hi SDX = 2-year rats, 3600 ppm sedaxane.

3.2.4.3 Reproductive Senescence in 3600 ppm Sedaxane –Treated Female Rats

In a long-term study with 104 control female Wistar rats, Mitchard and Klein (2016) performed estrous cycle determinations from 18 weeks to 112 weeks of age. Most animals showed normal 4-day estrous cycles up to 6 months of age. From that point up through 12 months of age, an increasing number showed irregular cycles; however, the majority of Wistar rats were still reproductively competent at 12 months of age. From 12-18 months of age, an increasing percentage of Wistar rats showed senescent stages characterized as repetitive pseudopregnancy (persistent diestrus with occasional proestrus) or persistent anestrus (continuous diestrus, with no evidence of cycling). Mating of 20 female Wistar rats at approximately 18 months of age with younger males showed that a high percentage of females mated, but none yielded viable litters. From 16-24 months of age, fewer surviving animals were available, but the profiles continued to show some rats with irregular cycles and higher percentages with senescent stages of repetitive pseudopregnancy or persistent anestrus (Mitchard and Klein, 2016).

Based on this long-term study and a prior investigation in 17-18 month old female Wistar rats (Kachi *et al.*, 2006; Mitchard and Klein, 2016), the incidence of rats in "persistent estrus" is quite small in aging Wistar rats compared to Sprague-Dawley rats (Eldridge *et al.*, 1999; Wetzel *et al.*, 1994). Similar to the Fischer 344 rat, Wistar rats have a relatively low spontaneous incidence of mammary adenomas and carcinomas compared to Sprague-Dawley rats, which is consistent with a change to repetitive pseudopregnancy where the estradiol : progesterone ratio would remain relatively low compared to aging Sprague-Dawley rats which experience long periods of constant estrogenic input.

Several lines of evidence are available regarding the effects of sedaxane on female Wistar rats related to aging and reproductive senescence. Mucification of the vagina is a trait that commonly occurs in repetitive pseudopregnancy (Westwood, 2008). It can also be observed to a lesser degree in other stages of the estrous cycle, particularly during irregular cycles prior to progression into reproductive senescence.

The pathology data from the 2-year rat study with sedaxane showed no difference between control and treated groups after 1 year for findings in the vagina (Anonymous, 2010, *Annex I. 3.9.1.1*). All animals appeared to be in some stage of estrous cycling, and a low incidence of vaginal mucification (0 – 2 per group; n=12 per group) was observed in all groups, consistent with the expectation that most Wistar rats of this age experience irregular estrous cycles, but they are still reproductively competent (Kachi *et al.*, 2006; Mitchard and Klein, 2016). However, at 2 years, there was a significantly lower incidence of vaginal mucification in the 3600 ppm treated females (Table 7). Based on these observations, the pathology data indicated that a lower number of rats in the 3600 ppm group had progressed into repetitive pseudopregnancy, which in turn suggests that the females in the 3600 ppm group continued to experience estrous cycling for longer than the controls, or enter reproductive senescence later in life.

TABLE 7 Incidence of Pathology Findings in the Vagina at 1 Year and 2 Years in the Carcinogenicity Study (Female Rats) as a Marker of Reproductive Senescence

Histopathology findings	2-year carcinogenicity study			
	0 ppm	200 ppm (14 mg/kg/day)	1200 ppm (86 mg/kg/day)	3600 ppm (261 mg/kg/day)
Vagina – 2-Years (survivors + decedents)	(52)	(52)	(52)	(52)
No abnormality detected	23	16	21	35*
Mucification	15	22	16	3**
Atrophy	13	11	14	11
Inflammation / inflammatory cell infiltration	0	5	0	0
Squamous cell hyperplasia +/- inflammation	0	0	0	1

Data from Anonymous, 2010, *Annex I. 3.9.1.1*, are pathology incidences in the original report from Charles River Laboratories, UK.
*, ** statistically significant by pairwise Fisher's Exact Test ($p<0.05$, 0.01)

To further investigate the possible age-related changes in organs related to estrous cycling, histology of the vagina, ovaries and uterus from existing histology slides from a 90-day study in Wistar rats (Anonymous, 2009 Annex I. 3.12.1.2) and the 2-year chronic/carcinogenicity study in Wistar rats (Anonymous, 2010, *Annex I. 3.9.1.1*) were re-evaluated by an independent pathologist (Anonymous, 2016, *Annex I. 3.9.4.11*). The objective of this work was to determine the cycle state from the microscopic examination of the vagina, uterus and ovary of female rats exposed to sedaxane in their diets for intervals ranging from 13 to 104 weeks. Slides from each rat were coded and divided into the following Subgroups (A – E) based on time of death or sacrifice, as follows:

Time Subgroup	Time Period / Fate
A	13 Weeks – Scheduled Sacrifice
B	0 – 52 Weeks – Animals Deceased
C	52 Weeks – Scheduled Sacrifice
D	53- 104 Weeks – Animals Deceased
E	104 Weeks – Scheduled Sacrifice

The pathologist was aware of the Subgroups for each animal but blind to the treatment groups. The histology as defined by Westwood (2008) in vagina, ovaries and uterus was used as a guide to facilitate making a determination of cycle stage or senescent stage for each animal. After an evaluation of the three tissues (where possible), an overall estimate of the cycle stage or the senescent stage was recorded under "vagina" for that animal. For cycling rats, if the ovary and uterus had changes that did not match the agreed cycle stage from the vagina, an additional descriptor of "asynchronous" was recorded. The incidences of each stage/finding by treatment and subgroup were tabulated; patterns and incidences compared to the control group were examined, but no formal statistical analysis was performed. A summary of findings for rats that lived beyond 52 weeks (*i.e.*, Subgroups D+E combined) is shown in Table 8.

TABLE 8 Summary Findings – Histology Re-evaluations of Control and Sedaxane-Treated Tissues Related to Cycle Stage: Combined 104-Week Sacrifice + Decedents from Weeks 53-104 (Groups D & E).

Subgroups D + E: Sacrifice at 104 week and decedents from weeks 53-104 (combined):

Time Groups D + E	Combined Incidence		
	0 ppm	**1200 ppm (86 mg/kg/day)**	**3600 ppm (261 mg/kg/day)**
Vagina Descriptive Findings (N[a])	(50)	(52)	(51)
Nothing Abnormal Discovered	4	4	1
Epithelium; Inactive	15	18	27
Mucification	29	29	21
Cornification	2	1	2
Vagina Estrous Cycle (N[a])	(50)	(52)	(51)
Repetitive Pseudopregnancy	29	28	19
Persistent Anestrus	15	18	27
Persistent Estrus	0	1	1
Total – Senescent Stages	44	47	47
Diestrus	2	2	1
Metestrus	1	2	0
Estrus	2	0	1
Proestrus	1	1	2
Total – Cycling Stages	6	5	4

For additional descriptive findings and results for other time intervals, see original report (Anonymous, 2016 *Annex I. 3.9.4.11*)

[a] For Subgroup D, N = 7 (0 ppm), 15 (1200 ppm), and 7 (3600 ppm). For Subgroup E, N = 44 (0 ppm), 37 (1200 ppm), and 44 (3600 ppm).

In younger animals (*i.e.*, from 13 weeks up through the 52-week sacrifice), the three tissues showed correlation with respect to cyclicity state, and the majority of rats in all groups were experiencing estrous cycles (Anonymous, 2016 *Annex I. 3.9.4.11*). In contrast, as the animals age there is a great deal of variability across the vagina, uterus, and ovaries with respect to reproductive senescence, as well as within individual animals; therefore, a lack of concordance observed across these three tissues was typical in animals from 53 – 104 weeks of age.

Despite this observed variability, the finding of a lower incidence of vaginal mucification and the corresponding cycle stage of "repetitive pseudopregnancy" in the 3600 ppm animals at 104 weeks was reproduced (Table 8). The total incidences of vaginal mucification in the control, 1200 and 3600 ppm groups were numerically higher in each group than those in the original pathology read (Table 7) given that the objective of the histology re-evaluation was to evaluate subtle changes related to reproductive cycle stages rather than finding adverse pathological changes as in the original study.

Most Wistar rats begin to cycle irregularly and enter various senescent stages somewhere between 53 and 80 weeks of age (Kachi *et al.*, 2006; Mitchard and Klein, 2016). Long-term studies in rats have demonstrated that some individuals experience irregular cycles followed by repetitive pseudopregnancy then persistent anestrus, while others proceed directly to persistent anestrus (vom Saal and Finch, 1988). Although a lower incidence of "repetitive pseudopregnancy" in the 3600 ppm animals at 104 weeks was observed, the total number of animals in senescent stages was unchanged

in response to sedaxane treatment at that timepoint (Table 8). Examination of a single section of tissue at a single point in time for animals at 104 weeks with physiological irregular/failing physiological processes cannot provide sufficient details to fully understand the continuum of changes and time to irregular cycling that leads to reproductive senescence in these groups of female rats.

While the specificity of progression into reproductive senescence is unknown in these rats, dopaminergic activity in the hypothalamus leads to suppression of the age-related decrease in prolactin signaling which is supported by the tumor profile observed in the two-year carcinogenicity study (i.e. significant decrease in mammary fibroadenomas in the 3600 ppm group). The blinded re-evaluation of the three tissues indicated differences in the cycle staging/ senescence for female rats treated at 3600 ppm sedaxane vs. controls and a lack of differences from controls at 1200 ppm. As such, these data provide supportive evidence of delayed senescence in Wistar rats with 3600 ppm sedaxane treatment compared to controls that occurred at some time during the 53 – 104-week period.

In summary, the following findings with 3600 ppm sedaxane treatment are considered Associative Events in the MOA, *i.e.*, they are markers for the Key Event of a delay in the age of reproductive senescence:

- A decreased incidence of vaginal mucification at 2 years.
- A decrease in animals at 2 years that were in repetitive pseudopregnancy.

These data are consistent with the caloric restriction mediated changes in reproductive senescence in Wistar rats, as discussed in Section 4.1.4. regarding Biological Plausibility of the proposed MOA. Taken together with the presence of a massive body weight deficit and associated neuroendocrine changes that have been demonstrated at 3600 ppm, it is reasonable to conclude that high dose sedaxane treatment altered the time course and the nature of these time-dependent changes in cycle stage. Thus, a higher incidence of 3600 ppm rats in persistent anestrus at 104 weeks and a lower incidence of vaginal mucification and rats in repetitive pseudopregnancy is a plausible indication of a difference in the transition into senescence over time in this treatment group.

DS comment: <u>Key event 4 "Increased age of reproductive senescence", key event 5 "increased total number of oestrous cycles and proliferation" and associative event 4 "decreased senescent mucification of the vagina and related changes observed at 2 years".</u>
The blinded re-evaluation of the data did not support key event 4 and associated event 4. Indeed, no differences in cyclicity measurements were observed in young animals (i.e., from 13 weeks up through the 52-week sacrifice). At 2-year time point, a similar high rate of senescence (repetitive pseudo pregnancy or persistent anoestrus) was noted in all groups and the incidence of vaginal mucification was only slightly lower in 3600 ppm group compared to control group (21/51 vs 29/50).
As regard key event 5, from the experimental data there is no supportive evidence of increased total number of oestrous cycles and proliferation. There might have been differences in estrous cycles between 1 year and 2 year but this allegation is not substantiated by experimental data and the putative higher oestrogen: progesterone ratio has not been objectified. Furthermore, no histopathological findings indicative of over estrogenic stimulation (as squamous metaplasia or endometrial hyperplasia) was observed at 1-year or 2-year sacrifice.
(See Annex I to the CLH Report 3.9.4.11).

3.2.5 13-Week study in Han Wistar rats

In a subchronic 90-day rat study, a limited number of parameters associated with the proposed MOA (vagina, ovary and uterus pathology; ovarian weights) were assessed (Anonymous, 2009 *Annex I. 3.12.1.2)* .There were no effects of sedaxane treatment on ovary weights, uterine weights and pathology findings in the ovaries, uterus and vagina.

4.0 IPCS/ILSI FRAMEWORK FOR THE EVALUATION OF THE HUMAN HEALTH RELEVANCE OF A HYPOTHESISED MODE OF ACTION

4.1 Has the Mode of Action Been Established in the Animal Model(s)?

The Hill (1965) criteria, as updated in a more recent description of the IPCS Framework for evaluation of a proposed MOA (Boobis *et al.*, 2006), require that, for the key events to be causally related to the formation of tumours, they must:

- Show dose-concordance of key events and dose levels that produce tumours
- Occur in a logical temporal sequence
- Be supported by data showing strength, consistency and specificity of association of key events and tumour response
- Be plausible and consistent with the current state of knowledge of the relevant biological processes, and coherent with what is known about the test substance specifically
- Demonstrate that alternative MOAs have been considered and are not operative
- Explore any uncertainties, inconsistencies and data gaps
- Assess the overall outcome of this analysis and the level of confidence in the postulated MOA

4.1.1 Dose-concordance of key events

With respect to the incidence of uterine adenomas and adenocarcinomas in female Han Wistar rats (Table 1), dose levels of 200 and 1200 ppm sedaxane (14 and 86 mg/kg/day, respectively) can be considered non-tumourigenic doses. At 3600 ppm (261 mg.kg.day) the incidence of uterine adenocarcinomas was statistically significantly higher than concurrent controls, but within the range of historic control values from the test laboratory and the RITA database, especially in light of the rarity of zero incidences of these tumours in the historical control databases.

Overall, there is good dose concordance of the proposed key events. The initial key event of decreased body weight was observed throughout the 2-year rat study at 3600 ppm and in a 13-week rat study at a similar high dose level (4000 ppm). As observed in both Table 2 and Figure 2, the divergence of the body weight and body weight gain was accelerated in the second year of the study for the 3600 ppm females compared to the control group. The final body weight gains at this dose relative to the control group was lower by approximately 50%, while in the 1200 ppm group it was approximately 11% lower. Plasma leptin levels tended to be lower than controls after 1 year of dosing at 3600 ppm with no differences seen in plasma adiponectin levels. Leptin and adiponectin are two factors that provide feedback signaling to the neuroendocrine control of appetite, energy usage and reproductive control centers in the brain (Tena-Sempere, 2015), and caloric restriction has been consistently shown to be preserve neuronal function in aging rodents (Lin *et al.*, 2015; Pani, 2015). Consistent with this feedback due to lower body weight gains and adipose tissue, the TIDA neurons in the hypothalamus retain function as indicated by higher levels of tyrosine hydroxylase in the 2 year 3600 ppm group compared to concurrent controls. Specifically, higher levels of TH mRNA and protein were observed only in the 3600 ppm sedaxane group (Figure 8).

TABLE 9 Summary of Dose-Concordance of Associative Events and (Causal) Key Events

Dietary inclusion level of Sedaxane (ppm) [a]	Decrease in body weight gain (Key Event)	Decreased plasma leptin levels, (Associative)	Hypothalamus: Increased DA activity in TIDA neurons after 2 years; ↑ TH mRNA levels (Key Event) [c]	Hypothalamus: Increased DA activity in TIDA neurons after 2 years; ↑ TH Protein levels (Key Event) [c]	Marker of ↑ Dopamine from TIDA: Decreased proliferation in anterior pituitary (Associative)	Marker of ↓ blood Prolactin levels: Decreased mammary gland hyperplasia and fibro-adenoma (Associative)	Decreased senescent mucification of the vagina, plus related changes observed at 2 years (Associative)	↑Age at Reproductive Senescence = ↑ Total number of estrus cycles + uterine endometrial proliferation (Key Event)	Significantly lower incidence of mammary fibroadenoma compared to concurrent controls (Associative)	Significantly higher incidence of uterine adenocarcinoma at 3600 ppm compared to concurrent controls (Outcome)
200	No	No data	No data	No data	No	No	No	No[d]	No	No
1200 (2000) [a]	Yes (slight)	No	No	Yes	No	No	No	No[d]	No	No
3600 (4000) [a]	Yes (large)	Yes[b]	Yes	Yes	Yes	Yes	Yes	Yes[d]	Yes	Yes

200 ppm = 14 mg/kg/day; 1200 ppm = 86 mg/kg./day, 2000 ppm = 186 mg/kg/day, 3600 ppm = 261 mg/kg/day, 4000 ppm = 350 mg/kg/day

[a] Values in parentheses are subchronic dose levels (that are similar to the chronic dose level).

[b]Not statistically significant, but similar in magnitude to the decrease in body weight and thus considered an effect of treatment (Anonymous, 2016, *Annex I. 3.9.4.14)*

[c]Tyrosine hydroxylase mRNA and protein = ↑ dopaminergic (DA) neurons in TIDA region = ↑ dopamine release.

[d]Captured from previously column, which is a Marker for these Key Events that occur progressively from 1-2 years.

Associative events present in the histology findings from the 2-year rat study that served as markers for two additional proposed key events are as follows (Table 6):

- In the hypothalamus, retention of a greater number of functional TIDA neurons in the 3600 ppm sedaxane group resulted in continued production of dopamine, which tonically inhibits prolactin release from the anterior pituitary. This Associative Event is indicated by a lower incidence of proliferative changes in the anterior pituitary and a significantly higher number of animals with no abnormalities in the pituitary. These decreased incidences of proliferative changes as well as pituitary adenomas were observed at 3600 ppm, but not at 200 or 1200 ppm sedaxane treatment.
- In the mammary gland, the resulting lower circulating prolactin levels (due to continued dopamine –mediated tonic inhibition of prolactin release by the pituitary) led to a complete absence of fibroadenomas (0/52) in the 3600 ppm treatment group; in contrast, incidences in the 200 and 1200 ppm groups were similar to control incidences.

Several analyses provided evidence for a change in the transition of aging Wistar rats into reproductive senescence at the 3600 ppm sedaxane dose level. Both the initial study report (Table 7) and a blinded, retrospective evaluation of the vagina, ovaries and uterus of rats in the 2-year study (Table 8) demonstrated some degree of estrous cycling in control and treated Wistar rats at 52 weeks. The majority of control rats transitioned to a state of either repetitive pseudopregnancy or persistent anestrus between 53 and 105 weeks (Anonymous,2016 *Annex I. 3.9.4.11*).This histology re-evaluation also confirmed that unlike Sprague-Dawley rats, very few Wistar rats enter persistent estrus as a reproductive senescent stage. For the 2-year time interval, the original pathology results (Table 7) and the histology re-evaluation (Table 8) observed a lower incidence of senescent mucification (repetitive pseudopregnancy) at the high dose of 3600 ppm. In contrast, there were no effects on any of these markers at 200 or 1200 ppm in the original pathology report (Table 7) or in the retrospective histology evaluation (Table 8).

By changing/delaying the transition of 3600 ppm sedaxane treated rats into reproductive senescence, it can be inferred that these animals were exposed to unopposed estradiol for a greater cumulative period (*i.e.*, a higher estrogen : progesterone ratio for more of their lifetime compared to controls). This could not be measured in the available samples from sedaxane studies, but is strongly inferred based on the Associative Events demonstrated in 3600 ppm group, particularly by significantly lower incidence of mammary fibroadenoma compared to concurrent controls (Table 9). For the Han Wistar rat, most control Wistar rats enter reproductive senescence via repetitive pseudopregnancy (with minimal to no persistent estrus), and the histology re-evaluation of samples from the 2-year sedaxane study confirmed this pattern (Mitchard and Klein , 2016; Anonymous, 2016 *Annex I. 3.9.4.11).*

Thus, the key events of prolonged exposure of the uterus to unopposed estrogen and increased proliferation of the uterine endometrium occurred at 3600 ppm, based on the associative events that are markers of these changes (Table 9). The final outcome in this MOA, an increased incidence of uterine endometrial adenocarcinomas, was only higher at 3600 ppm as compared to controls.

In summary, all of the key events and/or the associative events that served as markers for the key events were observed at the tumourigenic dose level of 3600 ppm. At 1200 ppm, a slight effect on body weight gain was observed (-11%), but this was insufficient to produce most of the downstream key events. At 200 ppm, the overall NOAEL in the 2-year rat study, none of the key events occurred.

4.1.2 Temporal-concordance of key events

The temporal-concordance is summarized in Table 10.

The observed steps occur in a logical sequence, with the key events preceding the time of occurrence of uterine adenocarcinomas. In particular:

- A decrease in body weight gain was observed in the first week of treatment at 3600 ppm, and continued to progress for the duration of the 2-year study. As shown in Figure 2, the divergence of body weight in the 3600 ppm group from the control group was accelerated in the second year of the study
- Plasma leptin levels were lower than the control group after 1 year of dosing at 3600 ppm
- Retention of dopamine release from the TIDA region (*i.e.*, as indicated by decreased pituitary proliferation) and the resulting lower circulating prolactin levels (*i.e.*, as indicated by decreased mammary gland hyperplasia and fibroadenoma) were clearly demonstrated in the 3600 ppm group compared to controls at the 104-week sacrifice. For the low numbers of animals that died between 53-104 weeks (data in Table 6), there is some numerical and statistically significant data that suggests these markers were also affected at 3600 ppm sedaxane between 53 and 104 weeks (*e.g.*, a statistically higher incidence of "no abnormality detected" in the mammary glands).
- There was a decreased incidence of vaginal mucification in the 3600 ppm rats at 104 weeks (Table 7), and this treatment-related difference (representing fewer animals in repetitive pseudopregnancy) was confirmed in a retrospective histology re-evaluation of the vagina, ovaries and uterus (Table 8).
- A higher incidence of uterine adenocarcinomas was observed after 104 weeks compared to concurrent controls. Of the 14 animals with this tumour type, 4 were observed in late decedent animals (after week 89) and the remainder at termination. Therefore, uterine adenocarcinomas appeared after 1.5 – 2 years.
- Notably, clear data on certain time-sensitive key events are available from groups of rats that were sacrificed after 13 weeks of treatment at dose levels up to 4000 ppm sedaxane (Table 9) and after 52 weeks of treatment (Tables 6 and 7). At both time intervals, the majority of rats were cycling. Only 0 or 1 rats per treatment group may have started to show evidence of repetitive pseudopregnancy after 52 weeks [data in Anonymous, 2016 *Annex I. 3.9.4.11*] In addition, there were no histopathology changes related to treatment in the vagina, ovaries or uterus, and no effects on relevant organ weights (*e.g.*, ovaries or uterus) at these time points as indicated in the original chronic report. Therefore, the lack of effects on these endpoints is consistent with a mode of action where:
    - Large deficits in body weight gain, altered cycle stage progression and/or effects of treatment on the transition into reproductive senescence occur primarily after 53 weeks, and
    - Any histological changes in the reproductive tissues (vagina, ovaries, uterus) related to the MOA only occur between 53 and 104 weeks of age.

In summation, the time course of key and associative events, as well as other supporting data, are consistent with the proposed MOA whereby measurable changes on the target tissue (uterus) only begin to appear between 53-104 weeks. Conversely, the lack of any earlier effects on the uterus or other endocrine-sensitive tissues precludes alternative MOAs involving short-term, direct effects on these systems by sedaxane.

TABLE 10 Summary of Temporal Concordance of Associative and (Causal) Key Events

Time	Decrease in body weight gain (Key Event)	Decreased plasma leptin levels, (Associative)	Hypothalamus: Increased DA activity in TIDA neurons after 2 years; ↑ TH mRNA and Protein levels (Key Event)	Marker of ↑ Dopamine from TIDA: Decreased proliferation in anterior pituitary (Associative)	Marker of ↓ blood Prolactin levels: Decreased mammary gland hyperplasia and fibro-adenoma (Associative)	Decreased senescent mucification of the vagina, plus related changes observed at 2 years (Associative)	↑Age at Reproductive Senescence = ↑ Total number of estrus cycles + uterine endometrial proliferation (Key Event)	Significantly lower incidence of mammary fibroadenoma compared to concurrent controls (Associative)	Numerically higher incidence of uterine adenocarcinoma at 3600 ppm compared to concurrent controls (Outcome)
1 – 13 weeks	Yes	Possibly[a]	No data	No data	No	No	No	No data	No
13 – 52 weeks	Yes	Likely[a]	No data	No data	No	No	No	No data	No
53 weeks	Yes	Yes[b]	No data	No data	No	No	No	No	No
53 – 104 weeks	Yes	Likely[a]	No data	Possibly[c]	Possibly[c]	Likely[d]	Likely[d]	No data	No
104 weeks	Yes	Likely[a]	Yes	Yes	Yes	Yes	Yes	Yes	Yes

[a]Not specifically measured for sedaxane at these time points.
[b]Not statistically significant, but similar in magnitude to the decrease in body weight and thus considered an effect of treatment(Anonymous, 2016 Annex I. 3.9.4.14)
[c]See Table 6 – incidences in decedent animals
[d]Histology re-evaluation of vagina, ovaries and uterus from rats that died from 53 – 104 weeks (Anonymous, 2016 Annex I. 3.9.4.11) provided insufficient power to assess whether a difference in senescent stages was apparent in this age range. However, changes in 3600 ppm (261 mg/kg/day) females at this time interval are considered highly likely based on clear differences at 104 weeks (Tables 7 and 8) and the known biology of reproductive senescence in Wistar rats.

4.1.3 Strength, consistency, and specificity of the key events and tumour response

As recommended in an IPCS Framework analysis (Boobis *et al.*, 2006), this section discusses the weight of evidence linking the key events, precursor lesions, and the tumour response. Consistent observations in a number of such studies with differing experimental designs increase support for a proposed MOA, since different designs may reduce unknown biases or confounding factors.

For sedaxane, there was no evidence for a treatment-related effect on the incidence of uterine tumours in an 80 week study in CD-1 mice (Anonymous, 2010, *Annex I. 3.9.1.2*). In this study, male and female CD-1 mice were treated with sedaxane at 25, 157 and 900 mg SYN524464/kg/day for males, and 29, 185 and 1001 mg SYN524464/kg/day for females, corresponding to dietary inclusion levels of 200, 1250 and 7000 ppm respectively for both sexes. A lack of uterine tumours in mice is consistent with the lack of an excessive effect of sedaxane on body weight gain, as the 7000 ppm group only experienced ~9% decrease in mean group body weight compared to controls after 80 weeks of treatment. In addition, the transition of CD-1 mice into reproductive senescence could differ from that in Wistar rats. A lack of uterine tumours in mice following lifetime exposure to a limit dose of sedaxane (~1000 mg/kg/day) gives strength to the proposed MOA, which is likely to only be operative in female Wistar rats that experience a very large lifetime deficit in body weight gain.

Where parameters were measured in multiple studies, there is a high degree of reproducibility between studies and consistency between key events. The first key casual event in the proposed MOA, a dose-responsive decrease in body weight gain at dose levels of 1200 – 4000 ppm, was observed throughout the 2-year rat study, a 13-week rat study with a similar high dose level, and in multiple other short-term rat studies with sedaxane. As shown in Figure 2, the divergence from the control group values accelerated in the second year of the study at the 3600 ppm dose level. This degree of effect on body weight was not attained in the non-tumourigenic 1200 ppm dose level.

Downstream key events also had multiple sets of data to support them. Retention of functional TIDA neurons in 2-year rats was demonstrated by higher levels of both TH mRNA and protein in the 3600 ppm group vs controls. Also, the associative event of decrease pituitary proliferative effects was an indirect marker of continued dopamine production by the TIDA neurons at 2 years in the 3600 ppm. As a product of continued dopamine release by the TIDA neurons, prolactin release by the pituitary remained low, which was supported by a very marked absence in mammary fibroadenomas (prolactin-driven) and decreases in other proliferative changes of the mammary gland at 3600 ppm.

Finally, results of the original histopathology evaluation of all tissues in the context of a 2-year cancer bioassay (Anonymous, 2010, *Annex I. 3.9.1.1*) were reconfirmed, and expanded, by a retrospective, blinded evaluation of the histology in vagina, ovaries and uterus to determine cycle stage and reproductive senescence status (Anonymous, 2016 *Annex I. 3.9.4.11*). The treatment-related decrease in vaginal mucification in 3600 ppm rats at 2 years (*i.e.*, indicating fewer rats in the senescent stage of repetitive pseudopregnancy) observed in the original pathology examination (Table 7) was confirmed in the retrospective, blinded evaluation (Table 8).

Data in these studies and in the literature (Kachi *et al.*, 2006; Mitchard and Klein, 2016) indicate that the critical period where Wistar rats transition from normal cycling to irregular cycling to ultimately reproductive senescence is likely somewhere between 53 – 104 weeks. As discussed in Section 3.2.3., the confirmation that 3600 ppm rats differed in their senescent staging at 2 years compared to the control and 1200 ppm groups by a retrospective, blinded evaluation is evidence of reproducible

data that suggests a change in the progression into reproductive senescence did occur in the 3600 ppm sedaxane-treated rats.

4.1.4 Biological Plausibility and Coherence

As recommended in an IPCS Framework analysis (Boobis *et al.*, 2006), this section considers whether the MOA is consistent with what is known about carcinogenesis in general (biological plausibility) and also in relation to what is known for the test substance specifically (coherence), including structure-activity relationships or similar treatments that produce tumours via the same mechanism.

The uterine tumour MOA for sedaxane is proposed to occur via an initiating event involving large decreases in body weight gain (-50%) over the full 2-year span of this study in female Wistar rats, which is supported by a body of literature on caloric restriction and tumour profiles. In addition, the proposed MOA appears to be critically dependent on the strain of rat, based on known differences in the timing and preferred state of transition into reproductive senescence for different strains of rat (*e.g.*, Sprague-Dawley rats prefer persistent estrus while the predominant stage for Fischer-344 rats is repetitive pseudopregnancy). The majority of Wistar rats had regular estrous cycles up to 6 months of age, after which there was a progressive increase in irregular cycles, repetitive pseudopregnancy and persistent anestrus (Mitchard and Klein, 2016).

4.1.4.1 Large body weight gain deficits, caloric restriction, carcinogenesis, and reproductive senescence

A retrospective examination of the effects of excessively high dose levels in chronic bioassays in rodents has demonstrated that large body weight gain deficits at high dose levels are frequently associated with lower incidences of total tumours, as well as increases in longevity [reviewed in Haseman *et al.* (1997)]. In addition, long-term studies of caloric restriction in rats and mice have demonstrated a host of biochemically or hormonally-mediated effects, many of them beneficial in terms of survival or tumour outcome, when compared to *ad libitum*-fed control animals that develop obesity and adverse age-related changes in long-term studies [reviewed in Frame *et al.* (1998)]. As a result of these realizations and consistent experimental data, international consortiums and various Regulatory Agencies have revised their guidance on dose selection in long-term bioassays, to help avoid confounding effects due to large body weight gain deficits (Foran, 1997; U.S. Environmental Protection Agency, 2005). Current guidance recommends that, in carcinogenicity testing, the highest dose should not cause overt toxicity and not be anticipated to shorten the test animal's life expectancy for reasons other than the development of tumours. Operationally, a desired MTD dose produces minimal signs of toxicity such as slight depression of body weight gain not to exceed 10% (ECHA, 2015; OECD, 2012).

In a large investigative effort known as the Biosure study (Roe *et al.*, 1995), various methods to achieve caloric or dietary restriction were investigated in male and female Wistar rats to assess their impact on longevity and tumour incidences in lifetime feeding studies. One form of caloric restriction (code = LMA/LMA) consisted of *ad libitum* feeding of a low nutrient maintenance diet from weaning, through 13 weeks of treatment and then throughout the rats' normal life span (30 months). These animals were compared to a control group that was fed a standard maintenance diet *ad libitum* (code = SBA/SMA). Food consumption in the LMA/LMA group females (Group 11) was higher than controls (Group 1) ($p<0.001$) throughout the study (115 – 126% of control), but food spilling was also higher than the control group and had to be assessed carefully to estimate actual

food consumption. Based on the nutrient content in the different diets, the authors calculated that despite the higher food consumption, the LMA/LMA female rats had mean daily energy intakes that were 10-20% lower than the control rats. This form of caloric restriction produced a sustained decrease in body weights, which was ≥14% of mean values for the control group throughout the study. In addition, the caloric restriction produced a shift in the tumour profile that is illustrated in Table 11.

TABLE 11 Tumour Profile in Calorie-Restricted Female Wistar Rats from Roe *et al.* (1995).

	Incidence (Females) and Statistical Significance[a]	
	Control Group (SBA/SMA) = Group 1	Restricted Calorie Group (LMA/LMA) = Group 11
Mammary Gland (N):	(50)	(50)
Fibroadenoma [a]	17	4***
Anterior Pituitary (N):	(50)	(50)
Adenoma [a]	33	23***
Uterus (N):	(50)	(50)
Adenocarcinoma [a,b]	0	7**
combined - "glandular tumours of uterine horn or body" [c]	6%	20.5%**

*, **, *** $p<0.05$, $p<0.01$, $p<0.001$

[a]Statistics in Roe *et al.* (1995) were based on overall tumour incidence in that tissue; values shown above are the major contributor to the tumour incidences in that tissue. Statistics for pituitary tumours were only conducted by the authors across both sexes; similar incidences were seen in males and in females ($p<0.001$).

[b] Incidence of adenocarcinoma in "protocoled tissues" is shown as reported; it was not statistically analysed. However, the statistical significance of the total glandular tumours (next row) has been assigned to this row (**) for presentation purposes.

[c] Text describes statistics conducted on "glandular tumours of uterine horn or body" (adenocarcinoma, anaplatic carcinoma, adenomatous polyp and papillary adenoma).

The overall incidence of benign or malignant tumours was reduced significantly ($p<0.001$) in the LMA/LMA females compared to the control group. As shown in Table 11, this incidence included a statistically significant decrease in both anterior pituitary adenomas and mammary gland fibroadenomas. However, uterine tumours (primarily adenocarcinomas) were significantly increased in the restricted calorie rats compared to the controls.

In a different study by Tucker (1979), Wistar-derived male and female rats were divided into two groups (50 males and 50 females per group), an *ad libitum* fed control group and a restricted calorie group whose diet availability was limited to achieve approximately 20% of the control group value (in g food/rat/day). At the end of 24 months, the survival in the restricted calorie group of females (44%) was numerically higher than the control females (34%), but the difference was not statistically significant. In the females, specifically, there was a statistically significant reduction in the total tumour burden in the restricted calorie group compared to control. The patterns of tumours observed in the female rats were:

- Significantly lower incidences of mammary gland tumours in the restricted calorie group (3) compared to the control group (17) ($p<0.001$)

- Significantly lower pituitary tumours in the restricted calorie group (19) compared to the control group (33) ($p<0.005$)
- Higher numeric incidences of uterine tumours in the restricted calorie group (3) compared to the control group (1). All of these tumours were carcinomas, likely endometrial adenocarcinomas based on the typical background tumours described for Wistar rats in later publications.

In a review paper published more recently, Harleman *et al.* (2012) examined the incidence and coincidence of uterine tumours and mammary tumours in two different strains of rats (Wistar and Sprague-Dawley). The RITA database represents company-sponsored database of historic tumour data from the control groups of guideline compliant toxicology studies. In their review, Harleman *et al.* (2012) reviewed the incidence and coincidence of mammary, pituitary and uterine tumours in a total of 5419 Wistar rats and 2158 Sprague-Dawley rats. The authors did not differentiate between mammary gland fibroadenomas (prolactin dependent) and adenomas /carcinomas (estrogen-dependent). The results of their analysis showed the following:

- Wistar rats had a markedly lower incidence of mammary tumours (24%) than Sprague-Dawley rats (58%)
- Conversely, Wistar rats had a higher incidence of uterine tumours (5%) than Sprague-Dawley rats (0.9%)
- For Wistar rats, there was strong evidence of an inverse relationship between mammary tumours and uterine tumour incidence (chi-square = 14.364; $p<0.001$). In contrast, although there was some evidence of an inverse relationship, a statistically significant correlation was not established for Sprague-Dawley rats, most likely because of the higher incidences of mammary gland adenocarcinomas often observed in Sprague-Dawley strain (see below for details).
- Wistar rats that did not have a mammary tumour were significantly more likely to have a uterine tumour, and vice versa. The factor driving this response in Wistar rats was concluded to be prolactin, which increases with age due to reduced dopamine production in the hypothalamus (Greaves, 2007; Hargreaves and Harleman, 2011; Keenan *et al.*, 1996).

Harleman *et al.* (2012) also reviewed the hormonal regulation of the reproductive system in rats, in particular as it relates to changes during reproductive senescence and the onset of age-related tumours. As discussed in Section 4.3 on human relevance, there are fundamental differences in rats vs. humans in the onset of reproductive senescence and the hormonal mechanisms that accompany this transition, particularly with respect to the role of prolactin.

Moreover, as mentioned above, there is a difference in mammary tumour types in Sprague-Dawley rats as compared to Wistars due to the different manner in which these two strains progress into reproductive senescence. An investigation of reproductive aging in female Wistar rats (Mitchard and Klein, 2016) revealed that the majority of rats had regular estrous cycles up to 6 months of age,after which there was a progressive increase in irregular cycles, repetitive pseudopregnancy and persistent anestrus . By 106-107 weeks, the majority of animals were acyclic and the few remaining cyclic animals had irregular cycles. A total of 65 animals (61.5%) showed adenomas and/or pituitary hyperplasia in the pituitary gland at necropsy. The pituitary tumours were likely to be prolactin secreting that give rise to pseudopregnancy and mammary tumours, demonstrated by the fact that 43/65 (66%) of the affected animals had histopathological signs of these conditions. While the sub-strain of Wistar rats in this study (Mitchard and Klein, 2016) was slightly different from those used in the 104-week study with sedaxane, the data provides a reasonable estimate of the normal progression of change in cycle state for aging Wistar female rats.

In addition, caloric restriction also leads to profound effects on reproductive fitness and senescence in rats. In female rats in which caloric restriction is initiated early in life (~ 45 days of age) and continued until 6 – 16 months of age, reproductive senescence was delayed in these rats compared to controls, as indicated by fertility at ages between 16 and 24 months (Osborne *et al.*, 1917). Keenan *et al.* (1996) also confirmed a markedly delayed reproductive senescence in Sprague-Dawley female rats on caloric restriction diets.

4.1.4.2 Hormonal changes during transition into reproductive senescence in the Wistar female rat

As a second topic that is important to the biological plausibility of the proposed MOA for sedaxane, the normal biology of reproductive cycles and the transition into senescence by the Wistar rat has been described in the literature, and results in this Weight of Evidence document for sedaxane are consistent with that known biology.

As with any species, the normally aging female Wistar rats undergo a series of changes with increasing age, which involves normal age related increases in body weight as well as progressive changes to the hypothalamic – pituitary – gonadal (HPG) axis which in turn affects the hormonal milieu. As Wistar rats age, plasma prolactin levels increase which corresponds to age-dependent higher incidences of pituitary hyperplasia and tumours (Greaves, 2007; Tucker, 1997). In particular, the tuberoinfundibular dopaminergic (TIDA) neurons show a decrease in the number of dopamine-producing neurons and a decrease in the amount of dopamine that is released into the portal capillaries, which carry dopamine to the anterior pituitary. Dopamine exerts tonic inhibitory effect on the release of prolactin from the anterior pituitary gland; therefore, the decreased levels of dopamine result in higher levels of circulating prolactin. Prolactin is luteotropic in rats, promoting production of progesterone in the corpora lutea after ovulation, and sustained higher levels of prolactin (*i.e.*, in pregnancy or in repetitive pseudopregnancy) maintains the corpora lutea for longer periods of time. During the luteal phase of the normal rat 4-day reproductive cycle, progesterone is produced in large quantities by the corpora lutea (CL), which antagonizes estrogenic stimulation of uterine growth (Gambrell *et al.*, 1983). As a normal female Wistar rat goes into reproductive senescence, the loss of the tonic inhibition of prolactin release leads to higher baseline blood levels of prolactin and a progesterone dominance (*i.e.*, estrogen/progesterone ratio is lower).

In addition, prolactin plays an important role in maintaining normal reproductive cycling in the rat (Freeman, 2006; Grattan and Le Tissier, 2015), and therefore, perturbed prolactin release (*i.e.*, loss of tonic inhibition via loss of dopaminergic activity) leads animals into reproductive senescence. In Wistar rats, where repetitive pseudopregnancy appears to be the predominant initial senescent state (Kachi *et al.*, 2006; Mitchard and Klein, 2016), the influence of prolactin on the CL and reproductive senescence favors higher levels of progesterone release by the CL, and thus a lower estrogen: progesterone ratio. These hormonal and physiological changes in aging Wistar rats produce the following histological changes:

- An increase in pituitary proliferative changes (including adenomas), due to the increased activity of pituitary lactotrophs caused by lower dopamine release from the TIDA neurons.
- An increase in mammary fibroadenomas, due to the increase in circulating prolactin.
- A protective effect on the uterine endometrium, due to diminished estradiol: progesterone ratios and resulting lower proliferative signalling.

Additional literature reviews provide good evidence for the biochemical changes that occur, and the hormonal changes that are specifically observed in Wistar rats. It is well understood that most rat pituitary hyperplasias and adenomas are prolactin positive (Kovacs *et al.*, 1977) and are functional prolactin-producing tumours. Rats with pituitary adenomas have high levels of circulating prolactin which correlates with the size of the pituitary tumour (Greaves, 2007). Because prolactin is the major promoter of mammary gland fibroadenomas due to its trophic function, there is a direct correlation between circulating prolactin and the incidence of mammary fibroadenomas (Tucker, 1997; Welsch *et al.*, 1970). Therefore, substances that stimulate the anterior pituitary and increase the levels of prolactin in blood are associated with an increased incidence of pituitary and mammary tumours (Gopinath *et al.*, 1987; Greaves, 2007). On the other hand, chemicals that reduce prolactin secretion by acting as dopamine agonist, such as bromocriptine, cause a reduction in the incidence of pituitary hyperplasia and adenomas as well as a significant reduction in the incidence of mammary fibroadenomas (Griffith, 1977; O'Connor *et al.*, 2000). Caloric restriction in Wistar rats has been shown to reduce pituitary hyperplasia with a reduced incidence of pituitary adenomas as well as a reduction in mammary tumours, which were linked to the decreased levels of circulating prolactin (Roe *et al.*, 1995).

As discussed in the previous section, a decrease in mammary fibroadenomas and anterior pituitary adenomas plus an increase in uterine adenocarcinomas have been reproducibly demonstrated in caloric restriction studies in Wistar rats. In contrast, this spectrum of changes in the presence of large bodyweight deficits does not appear to happen in other strains (*i.e.*, Sprague-Dawley rats do not demonstrate an increase in uterine tumours compared to controls) (Keenan *et al.*, 1996). The typical phases of reproductive senescence to which a particular strain of rats is preferentially exposed (*e.g.*, persistent estrus for Sprague-Dawley rats vs. repetitive pseudopregnancy for Wistar rats) correlate with the observed strain differences in uterine adenocarcinoma incidence and other tumour types in control vs. calorie-restricted rats. These changes in tumour profiles have been demonstrated to be related to prolonged retention of normal dopamine levels in specific regions of the hypothalamus in calorie-restricted rats, which prevents the age-related increase in circulating prolactin that is seen in *ad libitum*-fed control Wistar rats (Hargreaves & Harleman, 2011; Sanchez, 2003).

4.1.4.3 Conclusions – Biological plausibility and coherence

To summarize, in Wistar rats, multiple studies of caloric restriction or chemical – induced large body weight deficits in lifetime studies have been shown to produce a characteristic shift in the incidences of certain specific tumour types, including an increase in uterine adenocarcinomas (Harleman *et al.*, 2012; Roe *et al.*, 1995; Tucker, 1979; U.S. Environmental Protection Agency, 2011a). Therefore, a strong scientific precedence for the postulated Mode of Action with sedaxane has been established, and the proposed key events in this MOA are consistent with the known biology in Wistar rats.

4.1.5 Alternative mode of action hypotheses

In addition to the MOA described (see Section 3.1; Figure 1), alternative modes of action for the induction of uterine tumours exist. The plausible alternative MOAs for rat uterine tumours with sedaxane treatment of Wistar rats have been considered, and data which supports or refutes these alternative MOAs are described below.

4.1.5.1 Sedaxane is not genotoxic

This MOA can be excluded as sedaxane has been demonstrated to be negative for genotoxicity in a comprehensive package of *in vitro* and *in vivo* assays for genotoxicity (see Table 12).

TABLE 12 Summary of Genotoxicity Studies with Sedaxane

Study	Dose Levels	Result
***In vitro* studies**		
Bacterial reverse mutation Sokolowski, A (2009)	3-5000 µg/plate	Negative
In vitro cytogenetics Bohnenberger S (2009)	23.1 – 216.8 µg/mL	Negative
Mammalian cell gene mutation (mouse lymphoma) Wollny H-E (2009)	6.9-110 µg/mL	Negative
In vivo studies		
Mouse bone marrow micronucleus Reichenbach M (2010)	2000 mg/kg	Negative
Unscheduled DNA synthesis – rat liver Durward R (2009)	2000 mg/kg	Negative
Unscheduled DNA synthesis – rat liver Hall C (2011)	1000 – 2000 mg/kg	Negative

4.1.5.2 Sedaxane is not estrogenic

Compounds that can mimic the normal ligands that bind to and activate the estrogen receptors (primarily ERα and ERβ) are known to cause an increase in uterine endometrial tumours (Sherman, 2000; Yoshida *et al.*, 2012). This estrogenic effect can occur via binding of the parent xenobiotic molecule to the estrogen receptor, or after conversion to metabolite(s) that can bind and activate the receptors. To investigate the potential for sedaxane to operate via this MOA, an OECD Guideline 440 Uterotrophic Assay (US EPA Guideline OPPTS 890.1600) was conducted in ovariectomized (OVX) Wistar rats (strain designation Crl:WI(Han)) (Anonymous, 2014 *Annex I. 3.9.4.15*). In summary, sedaxane was not estrogenic in an *in vivo* uterotrophic assay in female Wistar rats. In combination with the wider database of mammalian toxicology studies, in which sedaxane did not show any histopathology or organ weight changes that would be expected from an estrogenic substance (or its metabolites), it can be concluded that sedaxane does not show the potential to produce uterine proliferative changes via an estrogenic MOA.

4.1.5.3 Sedaxane is not a dopamine agonist

Compounds that act as dopamine agonists, such as bromocriptine, can decrease prolactin levels in rats. With bromocriptine, a 100-week study in male and female rats (strain not specified) was conducted as part of the safety testing requirements for this human drug (Richardson *et al.*, 1984). In female rats, bromocriptine caused a statistically significant increase in uterine adenocarcinomas at 9.9 and 44.5 mg/kg/day and a decrease in mammary tumours (specific types not mentioned) in all treated groups. Thus, dopamine agonists have been shown to increase the incidence of uterine tumours in

rats by suppressing prolactin and altering the pattern of estrous cycling or the transition into reproductive senescence (Griffith, 1977; Klaunig *et al.*, 2015; Richardson *et al.*, 1984).

To investigate the potential for sedaxane to act as a dopamine agonist, it was tested in triplicate in a competitive binding assay at a concentration of 10 micromolar with a human dopamine receptor (type D2S). In summary, sedaxane was demonstrated to be inactive for binding to the dopamine receptor *in vitro* (Jolas, 2015 *Annex I. 3.9.4.10*). In addition, the wider toxicology database for sedaxane did not display the same types of short-term toxicity as seen in preclinical studies with bromocriptine that would suggest a short-term, pharmacological effect as a dopamine agonist [summarized in Richardson *et al.* (1984)]. Taken together with the evidence supporting the proposed MOA for uterine tumours, these data provide compelling evidence that sedaxane lacks the intrinsic properties to interact withthe dopamine receptor. Therefore, an alternative MOA for rat uterine tumours via activity as a dopamine agonist has been excluded for sedaxane.

4.1.5.4 Exclusion of other potential Modes of Action

More recently, several authors have indicated that additional MOAs may be operative for rat uterine adenocarcinomas, based on initial key events such as induction of specific CYP enzymes involved in estradiol metabolism or inhibition of enzymes related to estradiol conjugation and excretion (Wikoff *et al.*, 2016; Yoshida *et al.*, 2015). These MOAs involve short-term effects on metabolism or neuroendocrine systems that result in a higher net estrogenic stimulation of the uterus, and would be expected to produce changes in estrogen-sensitive tissues and/or related estrogen pathway endpoints after short-term as well as long-term administration. Since sedaxane did not produce any changes in the uterus, ovaries, vagina or other similar tissues in rats, mice, dogs and rabbits during *in vivo* studies of 3 days(Anonymous, 2014 *Annex I. 3.9.4.15*), through 1 year in duration these short-term MOAs are not operative for sedaxane.

4.1.6 Uncertainties, inconsistencies and data gaps

Based on the guidance regarding the IPCS Framework (Boobis *et al.*, 2006), identification of any uncertainties should include those related to both the biology of tumour development and those for the database on the compound of interest. Inconsistencies should be flagged and data gaps identified. For the identified data gaps, there should be some indication of whether they are critical as support for the postulated MOA.

The available data support the proposed hypothesized MOA for the higher incidence of rat uterine tumours with sedaxane (Section 3.1; Figure 1), while excluding the alternative MOAs described in Section 4.1.5. There is a strong database in the literature for caloric restriction where similar deficits in body weight of the magnitude seen with sedaxane have produced the same shift in tumour profile in Wistar rats. The datapoints for sedaxane, as summarized in the Dose-Concordance Table (Table 9) provide either direct evidence for the key event, or associative events that can serve as markers for each key event.

One potential data gap that exists is a lack of data specifically for sedaxane-treated female Wistar rats showing the proposed key event of decreased adipose tissue. In the 104-week chronic/carcinogenicity study in rats, specific measurements that would reflect a decrease in the percentage of adipose tissue (*e.g.*, abdominal fat pads, omental fat) were not a routine part of the study design. Therefore, a direct measure of decreased adipose tissue in the 3600 ppm female rats was not obtained. However, plasma leptin levels were shown to be lower after 1 year of dosing at

3600 ppm, and leptin levels were shown to be directly proportional to body weight (Anonymous, 2016 *Annex I. 3.9.4.14*). Based on known responses in rat studies to caloric restriction, and the increasing percentage of body weight in obese rats that is represented by fat at the end of a 2-year *ad libitum* feeding study, it can be presumed that 3600 ppm sedaxane-treated female rats that had significantly lower body weights than controls (-33% at 104 weeks) would also have lower adipose tissue as a percentage of total body weight compared to controls.

For example, Wolden-Hanson *et al.* (1999) studied the progression of adipose tissue deposition in male Brown Norway rats from age 3 to 29 months. In this study, the total body fat rose with age faster than the body weight of the *ad libitum* fed rats, such that the percentage of body weight as fat increased from 8.8% at 3 months of age to 19.9% at 29 months of age. While the weight of the visceral fat pads increased also with age, it progressively represented a lower % of total fat. The peripheral fat was proportionately a higher % of total fat with increasing age. Leptin levels in the blood progressively increased in proportion to the increase in percentage of body fat as the rats aged. Leptin levels in blood are known to be directly proportional to fat deposition levels, and they influence appetite and other energy-balance signals via receptors in the hypothalamus and other regions of the brain (Arner, 2003; Tena-Sempere, 2015; Woodside *et al.*, 1998). In addition to leptin and adiponectin, other signalling factors or metabolism products (*e.g.*, ketone bodies, sirtuins, cAMP responsive element binding (CREB)) have also been shown to change in diet restriction studies, and multiple studies have linked these changes during diet restriction to a neuroprotective effect (Lin *et al.*, 2015; Pani, 2015).

In a study of the effect of caloric restriction on tumour profiles in Wistar rats (Roe *et al.*, 1995), decreases in body weights compared to *ad libitum* fed controls (code = SBA/SMA) were observed in female rats on a high-fiber restricted calorie regimen for 30 months (code = LMA/LMA). At the end of the study, the LMA/LMA caloric-restricted female rats had body weights that were -14% different from the *ad libitum* fed controls. This regimen produced an increase in uterine adenocarcinomas, and decreases in mammary fibroadenomas and pituitary adenomas (as discussed in a previous section on Biological Plausibility). The authors did not quantify the weight or percentage of body fat in the different feeding regimens, but they noted in their discussion that restricted animals were not insulated by the thick layer of fat in the body wall (*i.e.*, peripheral fat), as opposed to *ad libitum* fed rats, which may have caused them to use more energy to maintain body temperature.

In summary, published experimental data in rats has established that increasing fat deposition occurs with age in *ad libitum* fed rats, and that caloric restriction studies that produced body weight deficits (-14%) smaller than those seen in the 3600 ppm sedaxane females (-33%) resulted in less deposition of fat than in the *ad libitum* fed control rats. Therefore, it is reasonable to conclude that the 3600 ppm sedaxane-treated female rats had lower percentages of body fat than the control rats. Therefore, the lack of actual data related to adipose tissue content in sedaxane-treated rats does not detract from the overall weight of evidence for the proposed MOA.

Another potential data gap for the sedaxane MOA is the key event regarding the increased age at reproductive senescence. The proposed key event is that the high dose group experiences more estrus cycles in its lifetime, which leads to a greater cumulative exposure of estrogens to the uterus that ultimately leads to uterine endometrial proliferation (Table 10). To definitively demonstrate this key event, it would be necessary to determine cycle stage by continuous vaginal lavages throughout the lifestage of control and sedaxane-treated Wistar female rats, particularlybetween 53 and 104 weeks of a 2-year chronic study.

Continuous vaginal lavages between 53 and 104 weeks would allow for determination of the timing as well as progression of changes in estrous cycling that eventually lead to repetitive pseudopregnancy and persistent anestrus. In a published long-term study in control Wistar rats (Mitchard and Klein, 2016), the majority of Wistar rats had regular estrous cycles up to 6 months of age, after which there was a progressive increase over time in irregular cycles, repetitive pseudopregnancy and persistent anestrus. By 106-107 weeks, the majority of animals were acyclic and the few remaining cyclic animals had irregular cycles (Mitchard and Klein, 2016). Some Wistar rats may experience irregular cycles, followed by repetitive pseudopregnancy then persistent anestrus, while others may proceed from irregular cycles directly to persistent anestrus (vom Saal and Finch, 1988). In addition, histopathology examination of the reproductive organs (*i.e.*, vagina, ovaries, uterus) of a sufficiently large number of rats across a series of interim sacrifice time intervals from 53 – 104 weeks may be useful to further confirm the cycle stage as indicated by the vaginal lavages.

However, as described Section 4.1.1 for dose concordance of the key events (Table 9), ample data is available with sedaxane for an Associative Event that provides a suitable marker for the reproductive senescence related key event. For the terminal sacrifice time point of 104 weeks, the original pathology results (Table 7) and a retrospective, blinded histology evaluation (Table 8) detected a lower incidence of senescent mucification (repetitive pseudopregnancy) at the high dose of 3600 ppm. In contrast, there were no effects on any of these markers at 200 or 1200 ppm. In summary, these datasets provide ample evidence that the age of reproductive senescence was also perturbed by treatment with 3600 ppm sedaxane at some point between 53 and 104 weeks. Therefore, this potential data gap is not critical to the weight of evidence for this MOA, and there is supporting literature evidence that caloric restriction has profound effects on reproductive senescence in rats (Keenan *et al.*, 1995; Osborne *et al.*, 1917).

In summation, the few data gaps or inconsistencies noted above do not diminish the strength of the evidence for the proposed MOA. No other uncertainties, inconsistencies or data gaps have been identified.

4.2 Assessment of the Postulated Mode of Action

The concordance analyses presented in Section 4.1 have established that the proposed key events resulting in a higher incidence of uterine tumours in female Wistar rats exhibit good dose- and temporal-concordance with the tumour endpoint. This MOA for the induction of uterine tumours with sedaxane has ample precedence in the literature in terms of the shift in tumour profiles that occurs in Wistar rats when body weights are sufficiently impaired due to caloric restriction (Harleman *et al.*, 2012; Roe *et al.*, 1995; Tucker, 1979; U.S. Environmental Protection Agency, 2011a), and the associated precursor key events in rats, and the parameters essential for describing the MOA have been presented for sedaxane. In addition, alternative MOAs for generating uterine tumours in rats have been examined and excluded for sedaxane based on further experimentation.

4.3 Are the Key Events in the Animal Mode of Action Plausible in Humans?

Following establishment of a plausible MOA for the induction of uterine tumours in rats, the next step is to assess the relevance to humans by assessing the qualitative and quantitative differences between the rat and human for each of the key events.

With regard to the hormonal control of the HPG axis, and the changes that occur in the transition from normal reproductive age into reproductive senescence, there are fundamental differences

between rats and humans that are critical to evaluating the potential relevance of the MOA that is established for sedaxane-treated rat uterine tumours.

The human reproductive cycle (menstrual cycles) has very different control mechanisms compared to rats (4-5 day estrous cycles) (Table 13). First, the surge of prolactin during proestrus in rats is not observed in human menstrual cycles. Second, the normal luteal phase of a rat estrous cycle is short (~1 day), and the new corpora lutea (CL) will regress, but if a high prolactin level is maintained (*e.g.*, a twice per day surge of prolactin occurs upon the stimulation of the cervix in mating), this higher prolactin will rescue the new CL and stimulate it to produce sustained higher levels of progesterone. In contrast, human CLs are initially maintained by LH (during a menstrual cycle), and later by chorionic gonadotropin from the placenta when pregnancy occurs. In rats, but not humans, luteolysis is also under partial control by prolactin, which is thought to be mediated by recruitment of macrophages in the ovary to degrade prior CLs (Freeman, 2006; Grattan and Le Tissier, 2015; Harleman *et al.*, 2012).

Similar to the fundamental differences between rats and humans in the control of normal cycling or the control of pregnancy, reproductive senescence processes are also fundamentally different (Table 14). In Wistar rats, onset of senescence is driven by a progressive decrease in the activity of dopaminergic neurons in the hypothalamus, particularly the TIDA neurons. In contrast, menopause and reproductive senescence in humans is driven by an eventual depletion of a limited number of primordial follicles in the ovaries with age. In Wistar rats, as a consequence of the loss of the dopamine-mediated tonic inhibition, prolactin levels in the blood are elevated which results in a luteotrophic effect on the CLs, and this in turn results in elevated progesterone and lower estrogen in the blood. This regulation does not occur in humans. Human female reproductive senescence occurs at the level of the ovaries, since the onset of menopause is driven by depletion of the limited number of available follicles within the ovaries (Table 14). In addition, it is well known that menopause in human females is associated with a marked decrease in circulating estrogens and progesterone.

The main stages of senescence in rats can be strain-dependent; in Wistar rats, the aging animals proceed mainly into irregular cycles, followed by repetitive pseudopregnancy and eventually persistent anestrus (Kachi *et al.*, 2006; Mitchard and Klein, 2016). In repetitive pseudopregnancy, a higher baseline level of progesterone production by the CLs of the ovary achieves a low estradiol: progesterone ratio.

Compared to control rats, the large body weight gain deficits that can be produced in rats by calorie restriction or by treatment with 3600 ppm sedaxane have been shown to preserve the TIDA neurons in aging Wistar rats, which leads to continued low prolactin levels, longer continued estrous cycling and periodic exposure to estradiol unopposed by progesterone (*i.e.*, a higher estradiol: progesterone ratio). The increased time of exposure to a higher estradiol: progesterone ratios leads to a higher incidence of uterine tumours in Wistar rats. This MOA is not relevant to humans, since the pathways leading to reproductive senescence and the control of the HPG axis in humans are not mediated by dopaminergic signaling.

TABLE 13 Regulation of Reproductive Cycles in Rats and Humans

	Rat	**Human**
Prolactin surge	Occurs in proestrus	No significant changes during the menstrual cycle.
Luteal phase length	Short (~1 day)	Long (14-16 days)

Role of Prolactin in Luteal phase	Luteotrophic which rescues new CLs and ↑ Progesterone synthesis	None – mediated by LH (initially) + chorionic gonadotropin (in pregnancy).
	Luteolytic which recruits macrophages to degrade prior CLs – prolactin surge in proestrus	None

References: Freeman (2006); Grattan and Le Tissier (2015); Harleman *et al*. (2012).

TABLE 14 Reproductive Senescence in Rats and Humans

Parameter	Wistar Rat	Women
Principal cause of senescence	Hypothalamic failure to control prolactin surges – loss of dopaminergic function	Depletion of ovarian follicle content –[hypothalamic function is maintained]
Predominant cycle pattern	Repetitive Pseudopregnancy	Menopause
Estrogen/progesterone ratio during this senescent stage	Reduced	Reduced
Prolactin secretion in reproductive senescence	Elevated	Reduced
Dopaminergic control	Yes	No
Prolactin dependence	Medium	None

References: Freeman (2006); Grattan and Le Tissier (2015); Harleman *et al*..(2012); vom Saal and Finch (1988); Neal-Perry and Santoro (2006).

Moreover, it was discussed in Section 4.1.4 that caloric restriction influences the progression into reproductive senescence in rats. However, in non-human primates, these changes are not observed with caloric restriction. In female rhesus monkeys, long-term caloric restriction had no effect on menstrual cycling or age-related decline in menstrual cycling (Lane *et al.*, 2001).

In summary, a wealth of information in the literature has demonstrated that the key events that lead to uterine tumours in Wistar rats after large, sustained decreases in body weight (*i.e.*, as seen with 3600 ppm sedaxane) would not be operative in humans, because of fundamental species differences in the control of reproductive cycles and the transition into reproductive senescence. Therefore, based on qualitative differences, the MOA established in rats with sedaxane is not relevant to humans.

5.0 CONCLUSIONS

The available data for sedaxane support the proposed MOA, which is described in Section 3.1 and Figure 1. The higher incidence of uterine tumours in female rats is attributable to a large deficit in body weight, which results in changes/delay in reproductive senescence by preserving the dopaminergic neurons of the hypothalamus. The continued high dopamine activity has a tonic inhibitory effect on prolactin release by the pituitary. Specifically for Wistar rats, this change (mediated via a state similar to caloric restriction) compared to normal aging control rats leads to a

lower incidence of tumours in the pituitary and mammary glands, and a higher incidence of uterine adenocarcinomas. This same pattern of changes in Wistar rats has been demonstrated to occur in rats maintained for their lifetimes on a restricted calorie diet. The suppression of the age-related increases in prolactin levels by sustained dopamine activity results in changes/delay in reproductive senescence and consequently greater cumulative exposure of the uterus to a higher estrogen:progesterone ratio (*i.e.*, reduced progesterone dominance of estrogen) in aged female rats, which would lead to a pro-proliferative estrogenic stimulation of the uterine endometrial cells. Over time, the estrogenic proliferative drive leads to promotion of spontaneously initiated uterine adenocarcinomas. At the same time, the decreased prolactin signalling leads to decreased proliferation of the anterior pituitary and mammary glands, which in turn leads to lower incidences of pituitary adenomas and prolactin-driven mammary gland fibroadenomas. The control of the female reproductive cycles and the drivers for reproductive senescence in humans are fundamentally different than that in rats, and therefore, this MOA for uterine tumours in rats is not relevant to human risk assessment due to qualitative differences between the species.

Clear thresholds exist for the key events in this MOA. The control of the female reproductive cycles and the drivers for reproductive senescence in humans are fundamentally different than those in rats; therefore, this MOA for uterine tumours in Wistar rats is not relevant to human risk assessment due to significant qualitative differences between the species.

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APPENDICES SECTION

APPENDIX A Historical control data from performing laboratory (CRL) for spontaneously occurring uterine adenoma and adenocarcinoma in female Wistar rats

			Uterine Adenoma		Uterine Adenocarcinoma		Total Uterine Tumours	
Study Identifier	Study Start	Number of Animals Examined	Incidence	Percent Incidence	Incidence	Percent Incidence	Incidence	Percent Incidence
811	2002	50	2	4	0	0	2	2
894	2003	100	2	2	9	9	11	11
993	2004	99	4	4	10	10	14	14
384	2005	50	0	0	7	14	7	14
666	2005	110	4	4	21	19	25	23
325	2007	52	3	6	4	8	7	13
930	2007	110	0	0	8	7	8	7
580	2009	52	0	0	0	0	0	0
072	2009	120	0	0	4	3	4	3
962	2012	64	0	0	2	3	2	3
			Mean	2	Mean	7	Mean	9
			SD	2	SD	6	SD	7
			Range	0-6	Range	0-19	Range	0-23
			N	10	N	10	N	10

Lab (CRL) data refers to 10 prior or concurrent studies at CRL in 2002-2012, ± 5 years from start of sedaxane rat chronic/carcinogenicity study .

APPENDIX B Historical control data from the RITA database for spontaneously occurring uterine adenoma and adenocarcinoma in Wistar rats

			Uterine Adenoma		Uterine Adenocarcinoma		Total Uterine Tumours	
Study Identifier	Study Start	Number of Animals Examined	Incidence	Percent Incidence	Incidence	Percent Incidence	Incidence	Percent Incidence
184	2002	50	3	6	11	22	14	28
187	2003	50	1	2	9	18	10	20
190	2002	51	3	6	6	12	9	18
191	2002	51	0	0	9	18	9	18
192	2003	59	1	2	2	3	3	5
196	2002	100	0	0	5	5	5	5
201	2004	50	1	2	3	6	4	8
204	2002	64	0	0	0	0	0	0
205	2002	64	0	0	5	8	5	8
207	2003	50	2	4	5	10	7	14
208	2003	50	0	0	3	6	3	6
209	2005	50	0	0	5	10	5	10
212	2005	60	0	0	0	0	0	0
216	2003	50	1	2	7	14	8	16
217	2005	50	0	0	1	2	1	2
220	2002	50	0	0	9	18	9	18
224	2004	60	1	2	2	3	3	5
231	2006	50	0	0	2	4	2	4
237	2007	50	0	0	8	16	8	16
265	2009	50	0	0	11	22	11	22
286	2010	60	0	0	5	8	5	8
287	2010	60	0	0	1	2	1	2
			Mean	1	Mean	9	Mean	11
			SD	2	SD	7	SD	8
			Range	0-6	Range	0-22	Range	0-28
			N	22	N	22	N	22

RITA data refers to 22 studies conducted in the Wistar rat from 2002 – 2012, ± 5 years from start of sedaxane rat chronic/carcinogenicity study . Registry of Industrial Toxicology Animal Data (RITA), http://reni.item.frauhofer.de/reni.

DS assessment of the postulated mode of action underlying sedaxane-induced rat uterine tumours:

Based on the regulatory studies and the mechanistic studies generated, the applicant has proposed the following mode of action for sedaxane-induced rat uterine tumours

Key events	Associative events
1 Decreased BW gain and adipose tissue	**1 Decreased leptin and signalling to the hypothalamus**
2 Suppression of age-related decreased in dopaminergic signaling	**2 Decreased piyuitary gland proliferative findings**
3 Suppression of age-related increase in prolactin	**3 Decreased mammary proliferating findings**
4 Increased age of reproductive senescence	**4 Decreased senescent mucification of the vagina and related changes observed at 2 years**
5 Increase in total number of estrus cycles and proliferation	
Final adeverse outcome: Increase in uterine adenocarcinomas	

Assessment of the postulated mode of action:

Key event 1: Significant treatment-related decreased body weight gain is supported by experimental data (50% decrease in high dose females at the end of the 2-year rat study). However, since adipose tissue was not measured in the 2-year rat study, there is no sedaxane-specific data regarding the supposed decrease in adipose tissue.

Furthermore, decreased body weight gain is not a specific molecular initiating event. It is a broad event, observed in many high dose groups of guidelined carcinogenicity studies. It is therefore questionable why uterine tumours are not observed with all chemical inducing significant body weight changes.

Associated event 1: In the analysis of the 1-year sacrificed rat females, the non-statistically significant decreased in mean leptin value observed in high dose females may indicate a decrease in adipose tissue, it is however not supported by mean values for adiponectin which were not affected by treatment.

Key event 2: In 2-year brain samples, both protein and mRNA staining support that in high dose group sedaxane treatment increased tyrosine hydroxylase expression in the TIDA region at 2 years. However, it does not automatically mean that a suppression of "age-related decrease in dopaminergic signalling" had occurred.

Key event 3: there is no direct experimental data to support a "suppression of age-related increase in prolactin" (i.e.: decreased prolactin level). Indeed the only measurements performed at 1-year time point did not show any treatment effect.

Associated events 2 and 3: experimental data support associative event 3 and to a lesser extent associative event 2. There was a tendency (not statistically significant) for decreased pituitary adenomas and a statistically significant decrease in the incidence of mammary fibroadenomas in the high dose rat females.

Key event 4: The blinded histopathology re-evaluation of the vagina, ovaries and uterus from existing histology slides from a 90-day rat study and the 2-year rat study did not support key event 4. Indeed, no differences in cyclicity measurements were observed in young animals (i.e., from 13 weeks up through the 52-week sacrifice). At 2-year time point, a similar high rate of senescence (repetitive pseudo pregnancy or persistent anoestrus) was noted in all groups.

Associated event 4: According to the blinded histopathology re-evaluation of the vaginas from existing histology slides from the 2-year rat study, the incidence of vaginal mucification was only slightly lower in 3600 ppm group compared to control group (21/51 vs 29/50).

Key event 5: from the experimental data there is no supportive evidence of increased total number of oestrous cycles and proliferation. There might have been differences in estrous cycles between 1 year and 2 year but this allegation is not substantiated by experimental data and the putative higher oestrogen: progesterone ratio has not been objectified. Furthermore, no histopathological findings indicative of over estrogenic stimulation (as squamous metaplasia or endometrial hyperplasia) was observed at 1-year or 2-year sacrifice.

Based on the above listed deficiencies, DS is of the opinion that the experimental data do not provide enough evidence to support the postulated mode of action of rat uterine tumours induced by sedaxane.

As the key events are considered not sufficiently supported by data, the assessment of dose and temporal concordance of key events as well as the strength, consistency and specificity of association of key events and tumour response is unwarranted.

As regard alternative modes of action hypotheses:

- **Genotoxicity: sedaxane was not genotoxic in a comprehensive package of in vitro and in vivo assays for genotoxicity**
- **Estrogenicity: sedaxane was not estrogenic in an uterotrophic assay in Wistar rat. Furthermore no effect indicative of estrogenic stimulation was observed in the available mammalian toxicology studies**
- **Dopamine agonism: sedaxane was inactive in a competitive binding assay with a human dopamine receptor**

16 APPENDIX 2: MODE OF ACTION AND HUMAN RELEVANCE ASSESSMENT OF LIVER TUMOUR INCIDENCES IN RATS AND MICE

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1.0 EXECUTIVE SUMMARY

Sedaxane is a fungicide of the succinate dehydrogenase inhibitor (SDHI) class that is registered for use globally as a seed treatment. In long-term studies, a high dose of sedaxane resulted in a numerically higher incidence of liver tumours in male Wistar rats (at 3600 ppm) and male CD-1 mice (at 7000 ppm) when compared to concurrent controls.

Syngenta and the rapporteur member state (RMS), France, [maintain] *were of* [6] the view that the higher incidence of tumours was within the range of variable spontaneous tumour incidences for the test species and was not related to sedaxane treatment. However, the European Food Safety Authority (EFSA) concluded that the overall pattern of tumours in rats and mice suggests that a 'Carc cat 2, H351, suspected of causing cancer' classification regarding carcinogenicity would be required for sedaxane (EFSA, 2013). This opinion followed US EPA classification of sedaxane as "likely to be carcinogenic to humans" citing increased liver tumour incidences as weak evidence of a high-dose treatment-related effect (U.S. Environmental Protection Agency, 2011c). Based on that alternative view, a mode of action programme was initiated to investigate a hypothesised mode of action (MOA) for the higher incidence of liver tumours using the framework developed by the International Programme on Chemical Safety (IPCS) and the International Life Science Institute (ILSI). The weight of evidence for the proposed MOA in rats and mice is described in detail in this document, and the human relevance of the identified MOA is assessed via the IPCS/ILSI framework.

The available data for sedaxane support a proposed MOA in male rats and male mice involving the following causal key events:

- Activation of the constitutive androstane receptor (CAR) and/or pregnane-X receptor (PXR),
- Altered expression of CAR-responsive genes that promote a pro-proliferative and anti-apoptotic environment in the liver,
- An early, transient, increase in hepatocellular proliferation,
- Increased hepatocellular foci as a result of clonal expansion of spontaneously mutated (initiated) cells, and
- Slight increases in liver tumour incidence compared to concurrent controls.

And the following associative events:

- Increased expression of genes encoding cytochrome P450s (CYPs), particularly Cyp2b and (to a lesser extent) Cyp3a isoforms,
- Increases in microsomal (endoplasmic reticulum) proliferation and hepatocellular hypertrophy, and
- Increased liver weight.

The available data also demonstrates that this MOA is not relevant for humans due to the established qualitative differences in response to CAR/PXR activation between rodents (rats and mice) and humans. Experimental data demonstrate that sedaxane does not produce the key event of cell proliferation in human liver cells *in vitro*. This pattern of effects matches the known species differences that have been demonstrated for other CAR activators, and the weight of evidence indicates that it represents a qualitative difference in the established MOA for sedaxane between

[6] Dossier submitter: this statement refers to Draft Assessment Report 2012 (see context)

rodents (rats and mice) and humans. In summary, the data support the conclusion that sedaxane does not pose a carcinogenic hazard to humans.

2.0 INTRODUCTION TO HUMAN RELEVANCE ASSESSMENT

A framework for assessing and communicating the relevance of tumour findings in rodent studies has been developed by the International Programme on Chemical Safety (IPCS) (Boobis *et al.*, 2006; Sonich-Mullin *et al.*, 2001) and the International Life Science Institute (ILSI) (Meek *et al.*, 2003). The framework aims to answer three questions:

(i) Has a mode-of-action (MOA) been established in the test species?

(ii) Based on qualitative assessment of the differences between species in terms of toxicokinetics and toxicodynamics, is that MOA plausible in humans?

(iii) Based on an assessment of the quantitative differences between species in terms of toxicokinetics and toxicodynamics, is the MOA plausible in humans?

Firstly, a MOA is established in the rodent using an approach developed by the IPCS/ILSI (Boobis *et al.*, 2006; Meek *et al.*, 2003; Sonich-Mullin *et al.*, 2001), which begins with a postulated theory of cause and the series of requisite and measureable events that are necessary for the induction of the toxicity. A recent workshop on nuclear receptor-induced liver tumour MOAs has defined a number of types of events that may be useful in describing the MOA (Andersen *et al.*, 2014). A causal key event is an empirically observable precursor step to the adverse outcome that is itself a necessary element of the MOA. Such key events are required for a MOA, but often are not sufficient to induce the adverse outcome in the absence of other key events. Associative events are measurable biological processes that are not themselves necessary causal key events for the MOA, but are reliable indicators or markers for key events. As such, associative events can often be used as surrogates for a causal key event in a MOA. Finally, modulatory factors are biological features or responses that are not necessary to induce an adverse event but could modulate the dose-response or probability of inducing one or more key events or the adverse outcome. A body of experimental evidence is then developed and assessed to support the association between these key events and the apical endpoint. This assessment is made using "tests for causation" proposed by Bradford Hill (1965) and involves answering a number of simple questions, namely:

- Are the dose and temporal relationships consistent with causality?
- Are the effects consistent and reproducible between studies?
- Could other causes have given rise to the key events?
- Are the effects biologically plausible given our current state of knowledge?

Only when a MOA has been established in an experimental species, can the human relevance assessment begin and, if, on the basis of experimental results, it can be shown that one or more of the necessary key events seen in the animal MOA is not plausible in humans (on either qualitative or quantitative grounds), then the adverse outcome in rodents is not appropriate for further consideration due to a lack of human relevance.

3.0 MODE OF ACTION HYPOTHESIS FOR LIVER TUMOURS IN RATS AND MICE

3.1 Overview of Liver Carcinogenicity Data for Sedaxane

3.1.1 Liver tumour incidence in rats

In a combined chronic toxicity and carcinogenicity study, Han Wistar rats [Crl:WI(Han)] were treated with sedaxane at dietary inclusion levels of 0, 200, 1200 and 3600 ppm (Anonymous, 2010, *Annex I. 3.9.1.1*). In the study report, the authors concluded that the marginally higher incidences of liver hepatocellular adenomas noted for the 3600 ppm males were not statistically significant and were within the range of Historic Control Data (RITA) that was available, and therefore did not reflect a treatment-related effect. Syngenta agrees with this conclusion. There were no hepatocellular carcinomas in male rats in this study (zero incidence).

Compared to the Historic Control Data (Table 1), the incidence in males at 3600 ppm was outside of the HCD ranges for the performing laboratory, but it was equivalent to the upper end of the adenoma range for the RITA database. The data indicate that incidence of liver tumours at 3600 ppm in male rats was marginally higher than in the concurrent control group.

TABLE 1 Incidence of Liver Tumours in Male Han Wistar Rats in a 2 Year Carcinogenicity Study and Historic Control Data

	Dietary Inclusion Level of Sedaxane (ppm)				**Peto Trend Test (p-value)**
	0	**200**	**1200**	**3600**	
Incidence of Hepatocellular Adenoma in Males (%)	1/52 (2%)	1/52 (2%)	1/52 (2%)	5/52 (10%)	0.0521

No statistically significant differences from the control group by Fisher's Exact Test ($p<0.05$)
There were no instances of hepatocellular carcinomas in male rats.

	Historic Control Data - Range	
	Lab (CRL)	RITA
Hepatocellular Adenoma, Males	0-3%	0-6%

Lab (CRL) data refers to 5 prior or concurrent studies at CRL in 2002-2005.
RITA data refers to 35 studies conducted in the Wistar rat from 1997 – 2009.

3.1.2 Liver tumour incidence in mice

In an 18-month carcinogenicity study, male and female CD-1 mice [strain designation Crl:CD-1(ICR)] were treated with sedaxane at dietary inclusion levels of 0, 200, 1250 and 7000 ppm (Anonymous, 2010, *Annex I. 3.9.1.2*). In the study report, the authors acknowledge a numerically higher incidence of hepatocellular adenomas and hepatocellular carcinomas in 7000 ppm males compared to the concurrent control group; however, there were no statistically significant differences by the Peto Trend Test or a pairwise Fishers Exact Test. Compared to the HCD (Table 2), the incidences in 7000 ppm male mice of hepatocellular adenomas alone (30%) or hepatocellular carcinomas alone (20%) were within one of the two HCD ranges (i.e. from the same laboratory and from RITA) or close to the top of a range. In addition, the authors cited comparisons to the control and treated groups from a parallel study that was completed in the same time frame with a different test item (Table 2), which showed that the incidences at 7000 ppm were within the range of normal

spontaneous variability for male hepatocellular adenomas or male hepatocellular carcinomas in this laboratory and strain of mice. Based on these comparisons, the authors concluded that the incidences of adenomas and the incidences of carcinomas in male mice at 7000 ppm reflected normal background variability in these relatively common histopathologic findings in older male CD-1 mice, and were not related to treatment. Syngenta agrees with this conclusion.

TABLE 2 Incidence of Liver Tumours in Male CD-1 Mice in an 18-Month Carcinogenicity Study and Historic Control Data

	Dietary inclusion level of Sedaxane (ppm)				Peto Trend Test (p-value)
	0	200	1250	7000	
Incidence of Hepatocellular Adenoma in Males (%)	7/50 (14%)	9/50 (18%)	10/50 (20%)	15/50 (30%)	0.0535
Incidence of Hepatocellular Carcinoma in Males (%)	5/50 (10%)	5/50 (10%)	3/50 (6%)	10/50 (20%)	0.2110

* Statistically-significantly different from control with p<0.05 (Fisher's Exact Test).

	Historical Control Incidence (%)	
	Lab (Range) [a]	RITA (range) [b]
Hepatocellular Adenoma, Male	(5 - 28%)	(0.0 – 13.6%)
Hepatocellular Carcinoma, Male	(3 - 6%)	(4.0 – 22.0%)

	Concurrent Study Incidences (%) [c]			
	0 ppm	Low	Mid	High
Hepatocellular adenoma, Male	14/50 (28%)	17/50 (34%)	17/50 (34%)	13/50 (26%)
Hepatocellular carcinoma, Male	3/50 (6%)	10/50 (20%)	3/50 (6%)	4/50 (8%)

[a] Lab historic control data = 3 studies, all started in 2007.

[b] RITA historic control data is shown for studies of 18 or 19 mo. Duration (9 studies).

[c] Concurrent Study Number 458346 in CD-1 mice conducted in parallel with SYN524464 (Sedaxane) study, but with different test item.

DS comment: In rat, although not statistically significant by pair-wise comparison there was a higher incidence of hepatocellular adenomas males at 3600 ppm with statistically significant trend and exceeding the HCD range from the same laboratory.
In mice, when excluding animals that died before week 49, statistical analysis showed that the incidences of hepatocellular adenoma and adenomas/carcinomas combined were statistically significantly, higher than those of the control group by pair-wise comparison (Table 3.9.1.2-11in Annex I to the CLH report 3.9.1.2). There was also a significant trend for adenoma, adenocarcinoma and adenomas/carcinomas combined (SEDAXANE 769–839 JMPR 2012). The incidences at 7000 ppm were also above the historic control data range of the same laboratory.

As regard HCD from RITA database they are considered appropriate (not the same laboratory).
Therefore, the increased incidence of liver tumours observed at high dose level both in rat males (3600 ppm) and mouse males (7000 ppm), could not be ruled out as unrelated to treatment.

See annex I to the CLH Report 3.9.1.1 and 3.9.1.2.

3.1.3 EFSA conclusions from the rat and mouse studies regarding liver carcinogenicity

Syngenta maintains the view that the higher incidences of tumours observed at 3600 ppm in rats and 7000 ppm in mice are within the range of variable spontaneous tumour incidences for the test species and are not related to sedaxane treatment. Syngenta does not consider that there is robust evidence that sedaxane has carcinogenic potential at the doses tested, as the frequency of neoplastic findings in affected tissues were generally within historical control ranges. Specifically in rats, the doses at which neoplastic findings were identified exceeded the maximum tolerated dose. No evidence of mutagenicity/genotoxicity were identified in the full suite of required genotoxicity studies.

In 2011, the Cancer Assessment Review Committee (CARC) of the US EPA evaluated the carcinogenic potential of sedaxane and concluded that sedaxane is "Likely to be Carcinogenic to Humans" with application of a linear low-dose extrapolation model (Q1*) for quantification of cancer risk to humans (U.S. Environmental Protection Agency, 2011c). Upon reconsideration of the toxicological assessment of sedaxane as requested by the European Commission, EFSA concluded that the overall pattern of tumours in rats (multiple sited) and mice (liver) suggests that a 'Carc cat 2, H351, suspected of causing cancer' classification regarding carcinogenicity would be required for sedaxane (EFSA, 2013). After these regulatory positions were published, Syngenta conducted a program of work to investigate the possible tumour incidences described by EPA and/or EFSA. Based on this work, uterine, thyroid, and liver tumour weight of evidence (WOE) assessments were prepared by Syngenta to evaluate the MOA and human relevance of each tumour type to support a Cancer Reclassification Decision by the US EPA for sedaxane. In combination with similar WOE documents that address the MOA and human relevance of uterine and thyroid tumours, this liver tumour WOE assessment is intended to support no cancer classification for sedaxane in the EU.

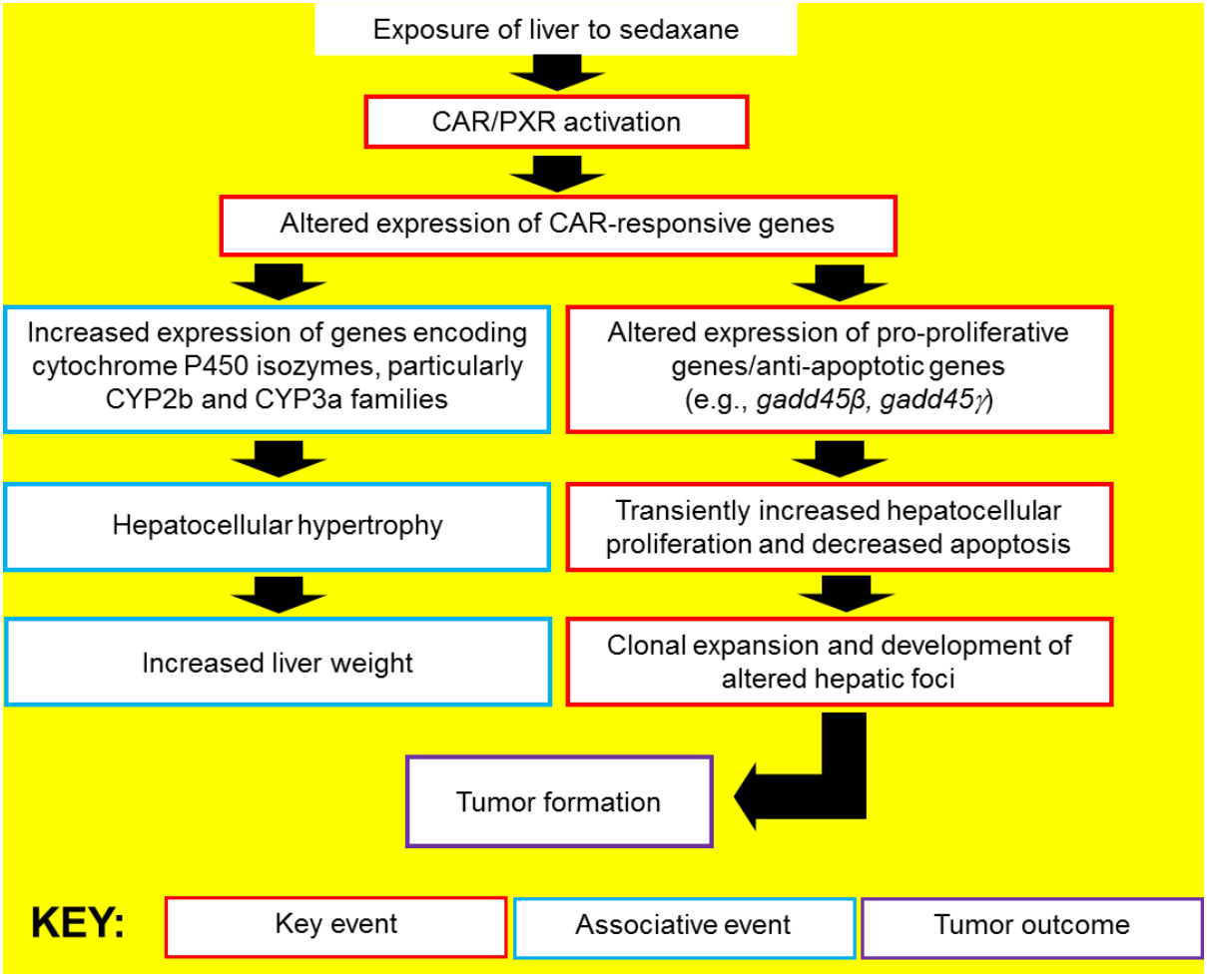
3.2 Statement of Mode of Action Hypothesis

A number of time- and dose-related key events have been identified that characterise the MOA for the higher incidences of liver tumours in male rats and male mice seen following sedaxane treatment. The proposed non-genotoxic MOA is initiated by activation of the constitutive androstane receptor (CAR) and/or pregnane X receptor (PXR). Activation of CAR/PXR in the liver results in altered expression of CAR-responsive and PXR-responsive genes, including induction of pro-proliferative/anti-apoptotic genes (e.g. *gadd45β*) and suppression of anti-proliferative genes/pro-apoptotic genes (e.g. *gadd45γ*) (Liebermann *et al.*, 2011; Ozawa *et al.*, 2011; Tojima *et al.*, 2012). CAR-mediated stimulation of cell proliferation (and associated replicative DNA synthesis) promotes an environment of increased cell replication that can result in a higher rate of spontaneous mutations. Suppression of apoptosis promotes an environment that would allow a spontaneously mutated cell to clonally expand before it could be removed by normal apoptotic control processes. Over time, transformed cells progress to pre-neoplastic foci, with clonal expansion eventually leading to the potential for development of liver tumours. In addition to the induction of pro-proliferative and anti-apoptotic genes, CAR/PXR activation in male mice and male rats also results in the induction of a

number of other genes, including some coding for members of specific cytochrome P450 families of isozymes, particularly those of CYP2b and, to a lesser extent, CYP3a. The activation of CAR/PXR and altered gene expression resulting in a pro-proliferative, anti-apoptosis environment are considered to be causal key effects, being necessary and directly resulting in the induction of liver tumours. In contrast, the effects on cytochrome P450s are considered to be associative events in that, while they are a characteristic hallmark of CAR/PXR activation, they are not central to the induction of liver tumours. A further associative event is liver hypertrophy, which is caused by proliferation of the smooth endoplasmic reticulum as a consequence of cytochrome P450 induction. This hypertrophy, in combination with the increased proliferation, results in an increase in liver weight.

The MOA hypothesis is represented diagrammatically and with more detail in Figure 1, with the causal key events and associative events identified.

FIGURE 2 Mode of Action Hypothesis for Induction of Liver Tumours in Rats and Mice



3.3 Relevant Data for Sedaxane

Data from several studies investigating the MOA hypothesis outlined in Figure 1 are presented below.

3.3.1 CAR and PXR activation assays

Sedaxane was evaluated in an *in vitro* CAR3 reporter assay for its ability to activate CAR from rat, mouse and human, by a method that has previously been shown to detect known species-specific activators of this nuclear receptor (Omiecinski *et al.*, 2011,). In addition, sedaxane was evaluated in PXR reporter assays in the rat, mouse and human. In each assay, model compounds that are known to activate the specific CAR or PXR receptors were also tested to confirm the performance of the assays. Results for sedaxane in CAR and PXR reporter assays (rat, mouse and human) are shown in Table 3.

TABLE 3 Results of Reporter Assays for CAR and PXR (Rat, Mouse and Human) with Sedaxane (data expressed as fold change vs. control)

Rat	**Rat CAR**		**Rat PXR**	
Concentration (µM)	Sedaxane	Model Activator [b]	Sedaxane	Model Activator [c]
1	1.1	---[a]	1.3	---
3	1.3	---	1.5*	---
10	2.7*	95.4*	1.7*	---
30	6.3*	---	3.1*	79.1*
Mouse	**Mouse CAR**		**Mouse PXR**	
Concentration (µM)	Sedaxane	Model Activator [b]	Sedaxane	Model Activator [c]
1	1.7	45.3*	1.2	---
3	5.2*	---	1.2	---
10	18.0*	---	1.2	---
30	19.3*	---	0.8	3.1*
Human	**Human CAR**		**Human PXR**	
Concentration (µM)	Sedaxane	Model Activator [b]	Sedaxane	Model Activator [c]
1	0.8	---	1.3	26.5*
3	0.8	10.3*	1.7*	---
10	2.1	---	3.0*	---
30	4.2*	---	3.9*	---

* $p<0.01$, Dunnett's test.

[a] --- not tested at this concentration, or data not shown for simplicity (rPXR)

[b] CAR receptor model activators = Clotrimazole (10 uM) for rat CAR, TCPOBOP (0.5 µM) for mouse CAR, CITCO (5 µM) for human CAR.

[c] PXR receptor model activators = Pregnenolone-16α-carbonitrile (20 uM) for rat PXR and mouse PXR, TO901317 (1 µM) for human PXR.

Data from Omiecinski (2014) *Annex I. 3.9.4.8* and Toyokawa and Sherf (2014) *Annex I. 3.9.4.9*

Sedaxane produced statistically significant increases in rat CAR activation at 10 and 30 µM, with a maximum increase of 6.3-fold *vs.* control at 30 µM. Sedaxane also was classified as a possible rat

PXR activator, with a statistically significant maximal increase of 3.1-fold *vs.* control only at the highest test concentration of 30 µM. The model activators clotrimazole (for rat CAR) and pregnenolone-16α-carbonitrile (for rat PXR) produced large fold-changes in activation of the respective reporter constructs, demonstrating that the assays responded as expected.

Sedaxane was also shown to activate mouse CAR (maximum 19-fold increase) and human CAR (maximum 4-fold increase) at concentrations up to 30 µM. Sedaxane did not activate mouse PXR, but it did activate human PXR (maximum 4-fold increase) at concentrations up to 30 µM. Model compounds for these species-specific assays produced large fold-change values, demonstrating appropriate responsiveness of the assays.

In summary, CAR and PXR reporter assays showed the following results:

- Sedaxane is an activator of both rat CAR and PXR
- Sedaxane is an activator of mouse CAR but not PXR
- Sedaxane is an activator of both human CAR and PXR

The CAR and PXR reporter gene assay data demonstrate that sedaxane has the intrinsic properties to interact with and activate the CAR receptor. The *in vivo* responses in mice and rats are concordant with CAR activation being the primary initiator of liver changes in those species, with markers of Cyp2b expression and activity being much greater than those of Cyp3a in sedaxane-treated mice and rats. Taken together, these data provide compelling evidence for the initial key event of CAR activation following sedaxane treatment.

DS comment: <u>Key event 1 "CAR/PXR activation":</u>
The results from the *in vitro* CAR and PXR reporter assays support the fact that sedaxane activates CAR from rat, mouse and human and PXR from rat and human.

See annex I to the CLH Report 3.9.4.8 and 3.9.4.9.

3.3.2 Short term (28-Day) dietary sedaxane liver mode-of-action study in rats

Male Han Wistar rats [strain designation Crl:WI(Han); 15 rats/group/time-point] were treated with sedaxane at dietary inclusion levels of 0, 1200, and 3600 ppm for 1, 3, 7 , 14 or 28 days before termination (Study Days 2, 4, 8, 15 and 29), and a number of liver- and thyroid-related parameters were measured (Anonymous, 2015 *Annex I. 3.9.4.4*). The 1200 ppm dose was the mid-dose in the 2 year carcinogenicity study, and the 3600 ppm dose was the highest dose in the carcinogenicity study and the only dose where the EPA concluded that a higher incidence of liver adenomas compared to controls was a treatment-related effect. The reversibility of sedaxane treatment-related effects on liver- and thyroid-related parameters was assessed by including additional groups of animals treated with either 0 ppm or 3600 ppm sedaxane for 28 days, followed by a 60 day recovery period prior to termination (on Study Day 89). The duration of the recovery period was based on 5 times the estimated liver half-life of 12 days, which was determined in a repeated-dose ^{14}C-sedaxane tissue depletion study in male Wistar rats (Shaw, 2009). In addition to treatment with sedaxane, further animals were treated with 1200 ppm phenobarbital sodium salt (NaPB) as a positive control, as NaPB is a known promoter of liver adenomas in the rat by a MOA similar to that proposed for sedaxane (Elcombe *et al.*, 2014). The study design was as follows:

Group Number	Treatment	Number of Male Rats					
		Termination Timepoints (Study Day)					
		2	4	8	15	29	89 [R]
1	Control: Basal (untreated) Diet	n=15	n=15	n=15	n=15	n=15	n=15
2	Sedaxane: 1200 ppm in Diet	n=15	n=15	n=15	n=15	n=15	-
3	Sedaxane: 3600 ppm in Diet	n=15	n=15	n=15	n=15	n=15	n=15
4	Positive Control: 1200 ppm sodium phenobarbital in Diet	n=15	n=15	n=15	n=15	n=15	-

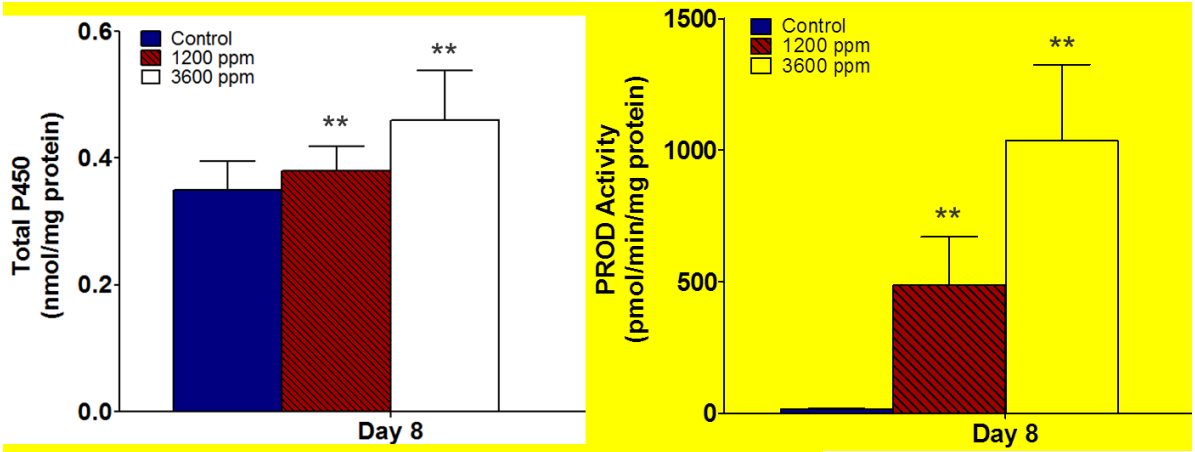
[R] Following 28 days of treatment, 15 animals from the control (Group 1) and 3600 ppm sedaxane (Group 3) groups were retained off-dose for a further 60 days to assess the reversibility of the effects of treatment.

3.3.3 Liver enzyme activity measurements in sedaxane treated rats

In the 28-day liver MOA study in male Wistar rats (Anonymous, 2015 *Annex I. 3.9.4.4*), the total CYP content in the liver and the PROD enzyme activity were measured in animals treated for 7 days. These results are displayed in Figure 2.

Sedaxane on Day 8 caused a small increase in total liver CYP protein that showed a clear dose response. The PROD activity (a marker of Cyp2b induction) was greatly increased, achieving a maximum of 69-fold increase at 3600 ppm. The positive control agent, sodium phenobarbital, produced similar increases in both of these liver endpoints (data not shown).

FIGURE 23 Effect of Treatment of Male Rats with Sedaxane for 7 Days on Hepatic Total Cytochrome P450 and PROD Activity



Results are presented as mean ± SD for groups of 10 animals. Data are from Anonymous, 2015 *Annex I. 3.9.4.4.* Values significantly different from control are: ***p*<0.01.

An earlier study was conducted with various ratios of the isomers of sedaxane, to investigate subchronic effects and liver enzyme profiles following 28-day treatment of Wistar rats (Anonymous, 2010 *Annex I. 3.12.1.1)* Groups of five male and five female rats (strain designation: HsdBrlHan:Wistar), from Harlan Labs UK were fed diets containing 0 (control), 500, 2000 or 5000 ppm SYN508210 (*trans* isomer), SYN508211 (*cis* isomer) or SYN524464 (1:1 mix of isomers) for 28 consecutive days. The key endpoints of interest to this liver tumour MOA are summarized in Table 6.

With all isomer ratios tested, PROD activity (a marker of Cyp2b activity) was greatly increased by treatment at 2000 ppm and 5000 ppm, whereas only a small increase in PROD activity was observed at 500 ppm. Testosterone 16β-hydroxylase activity (also a marker of Cyp2b activity) showed a similar pattern as PROD, with large increases at 2000 ppm and above.

EROD activity (ethoxyresorufin-O-dealkylase, a marker of Cyp1a activity) was virtually unaffected by each isomer, with small increases in activity following SYN508211 treatment that did not display a dose response relationship. Testosterone 6β-hydroxylase activity (a marker of Cyp3a activity) was increased somewhat at 2000 ppm and 5000 ppm with all isomers, although the response was variable for SYN508211 and SYN524464. The magnitude of this response indicated weak activity as a Cyp3a inducer for the isomers of sedaxane.

Corresponding to these liver enzyme activities, the liver weight adjusted for body weight was significantly increased at 2000 ppm and 5000 ppm, but not at 500 ppm. The only histopathology change observed in the livers was centrilobular hypertrophy. Other liver biochemical endpoints that were assessed in this study (e.g. immunoblots for different Cyp proteins, other testosterone hydroxylase activities) were either consistent with the patterns already described in Table 6, or were unaffected. The ratio of isomers in sedaxane technical, SYN508210 : SYN508211 (approximately 85:15 ratio), differs from the ratios tested in this study, but the results are informative of the major types of effects in the rat liver with sedaxane isomers.

TABLE 4 Summary of Liver Data from 28-Day Subchronic Rat Study with Sedaxane Isomers

	SYN508210 (trans isomer)			
	0 ppm	**500 ppm**	**2000 ppm**	**5000 ppm**
Liver Enzymes:				
PROD Activity (pmol/min/mg protein)	4.04	8.40**	258.81**	475.24**
EROD Activity (pmol/min/mg protein)	20.4	22.7	22.1	16.5
Testosterone 6β-hydroxylase Activity (nmol/10 min/mg protein)	5.098	5.720	6.885	9.627**
Testosterone 16β-hydroxylase Activity (nmol/10 min/mg protein)	0.293	0.647	7.086***	10.422***
Weights:				
Terminal Body Wt. (g)[a]	280.6	282.6	285.0	237.6**
Adjusted Liver Wt. (g)	9.4	10.0	11.9**	16.1**
Liver Histopathology (n):	(5)	(5)	(5)	(5)
Centrilobular hypertrophy	0	0	5**	5**
	SYN508211 (cis isomer)			
	0 ppm	**500 ppm**	**2000 ppm**	**5000 ppm**
Liver Enzymes:				
PROD Activity (pmol/min/mg protein)	4.04	22.21**	209.28**	184.77**
EROD Activity (pmol/min/mg protein)	20.4	39.9*	33.3*	28.4
Testosterone 6β-hydroxylase Activity (nmol/10 min/mg protein)	5.098	6.080	11.688***	8.464*
Testosterone 16β-hydroxylase Activity (nmol/10 min/mg protein)	0.293	1.411	5.838***	6.066***
Weights:				
Terminal Body Wt. (g)[a]	280.6	281.4	260.0*	231.8**
Adjusted Liver Wt. (g)	9.4	10.0	12.6**	15.6**
Liver Histopathology (n):	(5)	(5)	(5)	(5)
Centrilobular hypertrophy	0	0	0	5**
	SYN524464 (1:1 isomer ratio)			
	0 ppm	**500 ppm**	**2000 ppm**	**5000 ppm**
Liver Enzymes:				
PROD Activity (pmol/min/mg protein)	4.04	14.06**	294.53**	201.28**
EROD Activity (pmol/min/mg protein)	20.4	26.7	30.7	20.9
Testosterone 6β-hydroxylase (nmol/10 min/mg protein)	5.098	6.297	11.440***	6.986
Testosterone 16β-hydroxylase (nmol/10 min/mg protein)	0.293	1.274	6.835***	6.557***
Weights:				
Terminal Body Wt. (g)[a]	280.6	296.8	265.6	238.4**
Adjusted Liver Wt. (g)	9.4	9.8	12.6**	16.5**

Liver Histopathology (n):	(5)	(5)	(5)	(5)
Centrilobular hypertrophy	0	0	5**	5**

*, **, *** Statistically-significantly different from control with p<0.05, p<0.01 and p<0.001, respectively.
[a] Terminal body weight was statistically analysed after adjustment for initial Day 1 weight.
Data from Anonymous, 2010 *Annex I. 3.12.1.1*

DS comment:
Associative event 1 "Increased expression of genes encoding cytochrome P450s (CYPs), particularly Cyp2b and Cyp3a families":
In rat, the results from 28-day liver MOA study as well as the 28-day study with various ratios of the isomers of sedaxane support associative event 1 (increase in PROD activity from 500 ppm and in testosterone 16β-hydroxylase activity from 2000 ppm both markers of CYP 2b activity as well as increase in testosterone 6β-hydroxylase, a marker of CYP3a activity.

Associative event 2 "Hepatocellular hypertrophy" and Associative event 3 "Increased liver weights:
In the 28-day study, increases in centrilobular hypertrophy and in liver weight were also observed from 2000 ppm supporting Associative event 2 and Associative event 3.

See annex I to the CLH Report 3.9.4.4 and 3.12.1.1.

3.3.4 Short-term (21-Day) dietary sedaxane liver mode-of-action study in mice

Male CD-1 mice [strain designation Crl:CD-1 (ICR), 6 mice/group/time point] were treated with sedaxane at dietary inclusion levels of 0, 1250, 7000 and 14000 ppm for 1, 3, 7 or 21 days before termination (Study Days 2, 4, 8 and 22), and a number of liver-related parameters were measured (Anonymous, 2016, *Annex I. 3.9.4.2)*The 1250 ppm dose was the mid-dose in the 18-month carcinogenicity study, 7000 ppm was the highest dose in the carcinogenicity study and the only dose where a statistically significantly higher incidence of combined (adenoma + carcinoma) liver tumours was recorded , and 14000 ppm was used as a higher dose to explore dose-response effects. The higher dose of 14000 ppm sedaxane was shown to be well tolerated by mice in a preliminary 14-day dietary study at dose levels up to 14000 ppm (Anonymous, 2015, *Annex I. 3.9.4.1*).

The mouse CAR activator 1,4-bis [2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) was used as a positive control. TCPOBOP (3 mg/kg/dose, prepared in DMSO) was administered by intraperitoneal (ip) injection either once or twice. The 1st 6 mice in Group 5 were injected once with TCPOBOP on Day 1 then sacrificed 12 hours later (on Day 2). The 2nd 6 mice in Group 5 were injected twice with TCPOBOP, once on Day 1 and again on Day 3 (48 hr between doses), and then euthanized on Day 4 (12 hours after the 2nd dose). This positive control dosing regimen was similar to that used in a study of the whole mouse liver microarray effects on gene expression following acute dosing with TCPOBOP (Tojima *et al.*, 2012) and in a study of the liver tumour response and biochemical changes after longer-term TCPOBOP treatment (Huang *et al.*, 2005). The study design was as follows:

Group Number	Treatment	Number of Male Mice			
		Termination Timepoints (Study Day)			
		2	4	8	22
1	Control: Basal (untreated) Diet	n=6	n=6	n=6	n=6
2	Sedaxane: 1250 ppm in Diet	n=6	n=6	n=6	n=6
3	Sedaxane: 7000 ppm in Diet	n=6	n=6	n=6	n=6
4	Sedaxane: 14000 ppm in Diet	n=6	n=6	n=6	n=6
5	Positive Control: TCPOBOP in DMSO	n=6 [1]	n=6 [2]	-	-
6	DMSO Vehicle Control	n=6 [1]	n=6 [2]	-	-

[1] The first six mice in Groups 5 and 6 were euthanized approximately 12 hours after a single intraperitoneal (ip) 3 mg/kg dose of TCPOBOP (or vehicle).

[2] The last six mice in Groups 5 and 6 were dosed twice with TCPOBOP at 3 mg/kg/dose (or vehicle), appropriately 48 hours between doses, and euthanized approximately 12 hours after the second dose.

3.3.4.1 Altered gene expression in sedaxane treated mice: RT-PCR

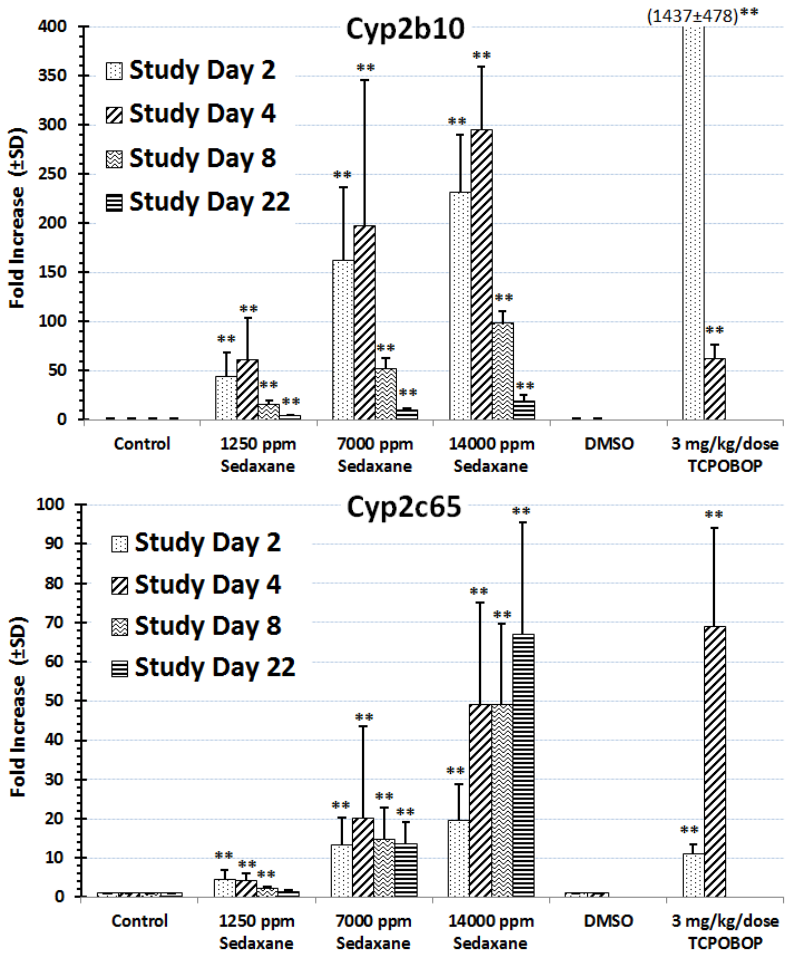
The effects of dietary sedaxane treatment on the expression of selected genes in the livers of mice were evaluated by RT-PCR. The following genes were selected for analysis by RT-PCR, based on their response in a previous study (Tojima *et al.*, 2012) in C57BL/6 mice given a single intraperitoneal 3 mg/kg dose of TCPOBOP at 12 hours prior to necropsy:

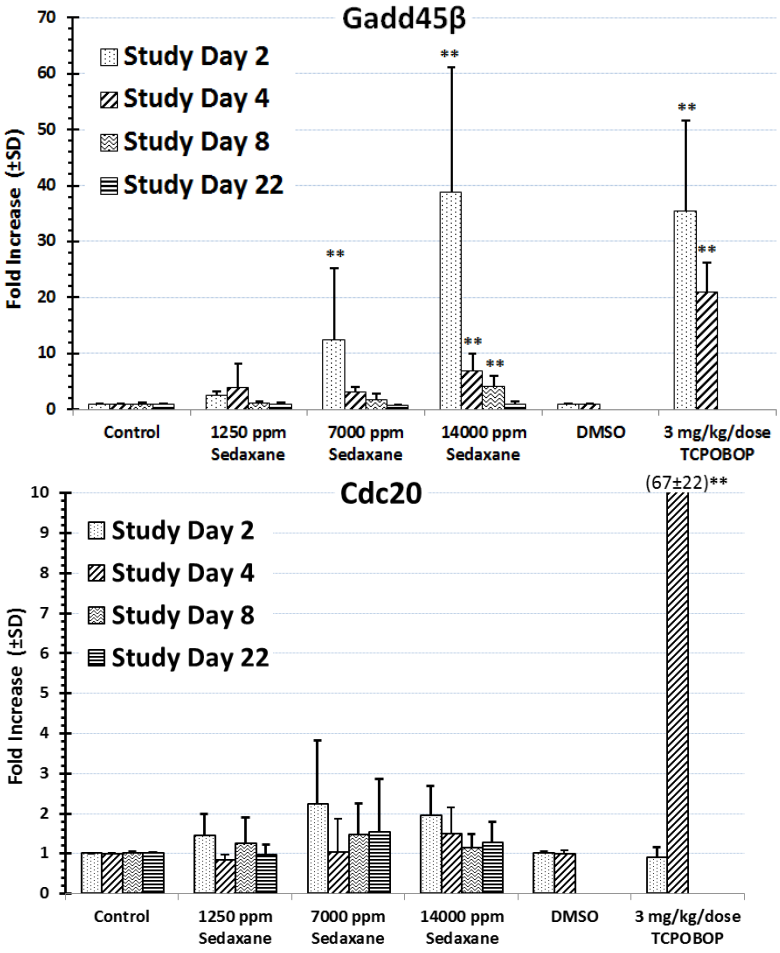
Gene Name	**Description**	**TCPOBOP-induced change in expression** (Tojima et al., 2012)
Cyp2b10	Cytochrome P450 isoform 2b10	increase
Cyp2c65	Cytochrome P450 isoform 2c65	increase
Gadd45β	growth arrest and DNA-damage-inducible 45 beta	increase
Cdc20	cell division cycle 20	increase
Fos	FBJ osteosarcoma oncogene	decrease

In the 21-day mouse study, treatment with either one or two 3 mg/kg doses of TCPOBOP resulted in a significant up-regulation of Cyp2b10, Cyp2c65, Gadd45β and Cdc20 mRNA levels, as shown in Figure 3. These responses are similar to those reported by Tojima *et al.* (2012) after one 3 mg/kg dose of TCOPOBOP, although the increase in Cdc20 in the current study was only observed after two doses of TCPOBOP. In contrast, no effects of TCPOBOP treatment on Fos expression were observed (data not shown), whereas Tojima *et al.* (2012) reported a down-regulation of this gene. Slight differences in the ability to decrease hepatic Fos mRNA levels or the timing of the increase in Cdc20 mRNA levels between these two studies could represent strain differences in the responsiveness to CAR activation for specific genes. Overall, the results of the investigations with TCPOBOP demonstrate the responsiveness of the batch of male CD-1 mice used in this study to a known CAR activator.

The treatment of male CD-1 mice with 1250, 7000 or 14000 ppm sedaxane in their diets for 1, 3, 7 and 21 days (Days 2, 4, 8 and 22) resulted in a significant dose-dependent up-regulation of Cyp2b10 mRNA levels at all dose levels and time-points, as shown in Figure 3. With the exception of the effect of treatment with 1250 ppm sedaxane at Day 22, a significant dose-dependent up-regulation of Cyp2c65 mRNA levels was also observed at all dose levels and time-points. The treatment of male mice with 14000 ppm sedaxane significantly up-regulated hepatic Gadd45β mRNA levels at Days 2, 4 and 8, and Gadd45β mRNA levels were also significantly up-regulated after treatment with 7000 ppm sedaxane at Day 2. The up-regulation of Gadd45β mRNA levels by sedaxane treatment was transient, with no significant differences in the fold-change at Day 22 of treatment. Sedaxane had no treatment-related effects on Cdc20 or Fos mRNA expression levels at any of the dose levels or time points.

FIGURE 4 Effect of Treatment of Male Mice with Sedaxane and TCPOBOP on Hepatic mRNA Levels (RT-PCR)





Values are mean ± st.dev. for fold change *versus* control. *p<0.05; **p<0.01, statistically significant by Dunnett's test.

3.3.4.2 Altered gene expression in sedaxane treated mice: Microarrays

As a further exploration of the mRNA expression patterns produced by sedaxane in male CD-1 mice, Agilent Whole Mouse Genome Oligo Microarray slides were run and analysed for liver samples from Day 2, 4 and 22 of treatment (in Anonymous, 2016 Annex I. 3.9.4.2). A set of differentially expressed genes (DEGs) was obtained by comparisons of treated and control microarray results, and the patterns of DEGs were evaluated with Genespring and Ingenuity Pathways Analysis™ (IPA™). The total numbers of DEGs at each dose level showed a clear dose response (Table 5), with very minimal differences from control at the low dose levels of 1250 ppm.

TABLE 5 Genespring Analysis: Total Number of Differentially Expressed Genes as a Function of Dose Level and Study Day

	Study Day 2	Study Day 4	Study Day 22
1250 ppm sedaxane	0	3	0
7000 ppm sedaxane	9	17	106
14000 ppm sedaxane	395	178	963

Details of the results from analysis of affected pathways or genes are contained in the study report, but a brief summary of the most striking observations can be summarized as follows:

- The top ten IPA pathways in mouse liver following treatment with model CAR activators (Oshida *et al.*, 2015) were virtually identical to those identified for sedaxane by IPA analysis.
- Large changes in characteristic genes involved in the CAR pathway were observed at 7000 ppm and 14000 ppm, including Cyp2b10 and Cyp3a11. In contrast, only a minimal response in the number of DEGs was observed in the 1250 ppm group.
- Focused analysis showed that changes consistent with a potential proliferative effect in the liver were seen at 14000 ppm (Days 2, 4 and 22) and possibly at 7000 ppm (Day 22), but not at 1250 ppm. These changes included up-regulation of Gadd45β at 14000 ppm and down-regulation of Gadd45gamma at 7000 ppm, both of which reflect a pro-proliferative, anti-apoptotic signal (Liebermann *et al.*, 2011; Ozawa *et al.*, 2011).
- A lack of Cyp4a induction coupled with minimal PPAR-α pathway gene induction suggests no significant involvement of peroxisome proliferator-activated receptor alpha (PPAR-α).
- In the absence of Cyp1a1 induction there is no strong evidence for Aryl hydrocarbon receptor (AhR) activation. Minor pathway changes related to AhR were relatively small in number and are not definitive of AhR activation due to the fact that they are also regulated by other nuclear hormone receptors.

3.3.4.3 Liver enzyme activity measurements in sedaxane treated mice

As a complement to the increased gene expression of specific Cyp isoforms in the liver, the activity of the following liver enzymes were measured in Day 8 samples from the mouse MOA study:

- 7-Pentoxyresorufin O-depentylase (PROD) – a marker of Cyp2b activity
- Testosterone 6β-hydroxylase – a marker of Cyp3a activity

Results of these assessments are shown in Table 6.

TABLE 6 Effect of Treatment of Male Mice with Sedaxane for 7 Days on Hepatic Microsomal Protein Content and 7-Pentoxyresorufin O-dependentylase and Testosterone 6β-hydroxylase Activities

Treatment[a]	Microsomal protein (mg/g liver)	7-Pentoxyresorufin O-dependentylase (pmol/min/mg protein)	Testosterone 6β-hydroxylase (nmol/min/mg protein)
Control	30.3 ± 2.25 [b] (100)	18 ± 6.2 (100)	0.98 ± 0.263 (100)
1250 ppm Sedaxane	32.6 ± 2.53 (108)	50 ± 12.3** (278)	0.96 ± 0.195 (98)
7000 ppm Sedaxane	32.1 ± 1.97 (106)	266 ± 45.5** (1478)	1.23 ± 0.158 (126)
14000 ppm Sedaxane	31.3 ± 2.22 (103)	439 ± 84.6** (2439)	2.55 ± 0.551** (260)

[a] Mice were fed control diet or diet containing sedaxane for 7 days. Data are from (Anonymous, 2016 Annex I. 3.9.4.2).

[b] Results are presented as mean ± SD for groups of 6 mice. Values in parentheses are percentage of control levels.

Values significantly different from control are: **p<0.01.

Previous studies have demonstrated that the 7-pentoxyresorufin O-dependentylase and testosterone 6β-hydroxylase activities are good markers for induction of Cyp2b and Cyp3a subfamily enzymes, respectively (Nims and Lubet, 1996; Parkinson, 2001). The treatment of male mice for 7 days with sedaxane resulted in a significant dose-dependent increase in 7-pentoxyresorufin O-dependentylase activity (indicating Cyp2b induction), and a lesser increase in testosterone 6β-hydroxylase activity (indicating Cyp3a induction). This pattern is consistent with the mouse CAR and mouse PXR activation assays (Table 3), which showed that sedaxane was a CAR activator but not a PXR activator for the mouse nuclear receptor. There is cross-talk between CAR and PXR and some overlap in the extent of activation of these Cyp isoforms, which could be responsible for the small increase in Cyp3a activity.

DS comment:

Associative event 1 "Increased expression of genes encoding cytochrome P450s (CYPs), particularly Cyp2b and Cyp3a families":

In mouse, combined data from RT-PCR and microarray analysis support Associative event 1 with increased expression of hepatic Cyp2b10 mRNA, Cyp2c65 mRNA, and PROD activity observed from at 1250ppm.

Key event 2 "Altered expression of CAR-responsive genes" and Key event 3 "Altered expression of pro-proliferative genes/anti-apoptotic genes":

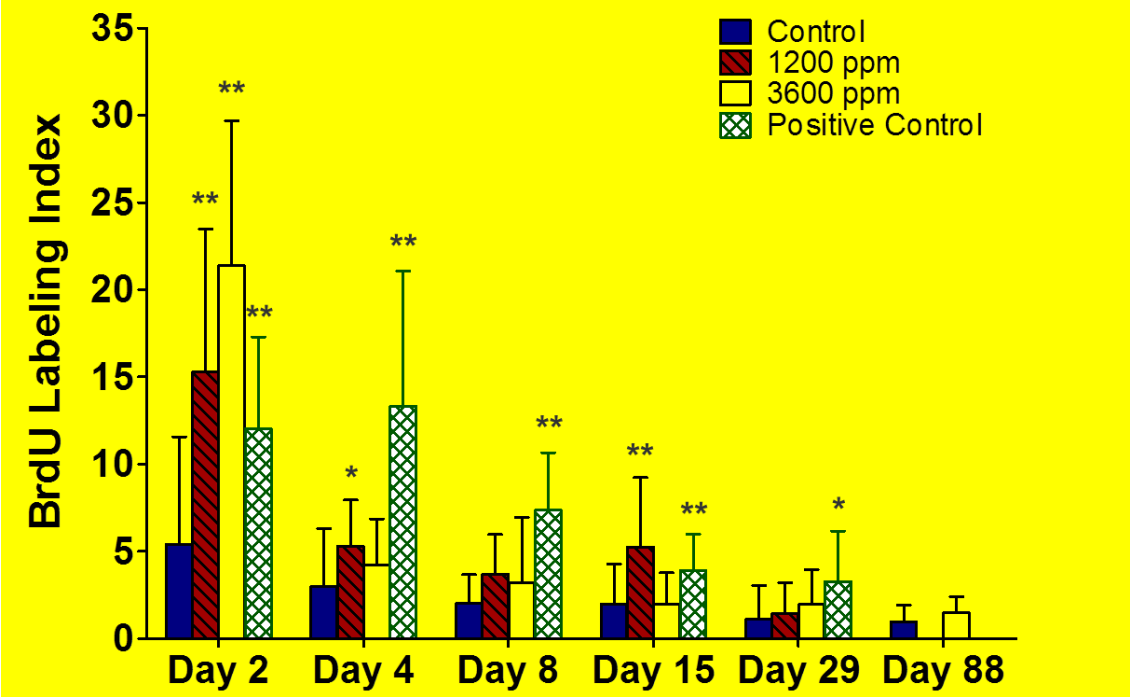
The increase in Gadd45β mRNA from 7000 ppm and the increases in expression of xenobiotic metabolizing enzymes and other genes associated with CAR/PXR activation from 7000 ppm in the microarray are consistent with Key event 2 and Key event 3.

See annex I to the CLH Report 3.9.4.2.

3.3.5 Cell proliferation – rats

In the 28-day MOA study in male Wistar rats (Anonymous, 2015 Annex I. 3.9.4.4), all animals received a subcutaneous injection of 5-bromo-2'-deoxyuridine (BrdU) in sterile 0.9% sodium chloride solution approximately 2 hours before termination. The formalin-fixed paraffin-embedded liver samples of all rats (along with a piece of duodenum as a positive control tissue) were processed by immunohistochemistry to detect and quantify the labelling index (%BrdU positive hepatocytes) as a measurement of S-phase or cell proliferation. Results of this BrdU labelling index determination are shown in Figure 4.

FIGURE 5 Cell Proliferation Results by BrdU Labeling Index in Rat Livers



*, ** Statistically-significantly different from control with $p<0.05$ and $p<0.01$, respectively.
Data from Anonymous, 2015 *Annex I. 3.9.4.4.*

A statistically significant transient increase in hepatocellular proliferation, as indicated by an elevated hepatic BrdU labelling index, was maximal on Day 2 of treatment with sedaxane at both 1200 and 3600 ppm. Proliferation, as measured by the labelling index, was less evident on Day 4 of treatment, with only the 1200 ppm sedaxane treatment achieving a statistically significant increase, and by Day 29 of treatment, the labelling index in all the sedaxane treatment groups returned to baseline levels. After a recovery period of 60 days (Day 89), the 3600 ppm sedaxane-treated animals showed no difference from control values for BrdU labelling index.

Labelling indices of the positive control (Sodium Phenobarbital) increased in a manner consistent with the known toxicity of the test item, with a transient profile that was maximal at Days 2 – 4. The response to sodium phenobarbital diminished with time, but was slightly elevated up though Day 28.

DS comment: <u>Key event 4 "Transient increased hepatocellular proliferation and decreased apoptosis":</u>
In male rats, as measured by the BrdU labelling index, sedaxane induced a transient increase in hepatocellular proliferation from 1200 ppm consistently with key event 4.

See annex I to the CLH Report 3.9.4.4.

3.3.6 Cell proliferation – mice

In the 21-day MOA study in male mice, cell proliferation was evaluated by the zonal distribution of Ki67 in the liver, as illustrated in Figure 5. Zone 1 corresponded to the periportal region (including the portal triad), zone 2 was the midzonal region and zone 3 corresponded to the centrilobular region (including the hepatic vein). Ki67 is a nuclear protein that is expressed during all phases of the proliferating cell cycle (G1, S and G2), but not during the quiescent G0 phase. In contrast, BrdU is incorporated into newly synthesized DNA only during S-phase of the cell cycle. Published work comparing the utility of Ki67 to other markers of cell proliferation such as BrdU have shown that Ki67 can give similar or greater sensitivity than BrdU for detecting proliferation in a particular tissue (Kee *et al.*, 2002; Muskhelishvili *et al.*, 2003; Wood *et al.*, 2015). In addition, zonal counts of hepatic Labeling Index by various methods have demonstrated that for some specific xenobiotics, a cytotoxic or a cell proliferation response may be detected more readily within an individual zone of the liver than by counting all zones together (Bahnemann and Mellert, 1997; Wood *et al.*, 2015). Results of the Ki67 analysis of male mouse livers are shown in Figure 6.

In addition to the Ki67 analysis of liver Labeling Index for the sedaxane-treated mouse livers, an initial evaluation of BrdU labelling of livers was conducted. Mice received a subcutaneous injection of BrdU approximately 2 hours prior to termination. Results of BrdU immunohistochemistry are shown in Figure 7.

FIGURE 5 Liver Zone Designations – Ki67 Analysis of Mouse Livers

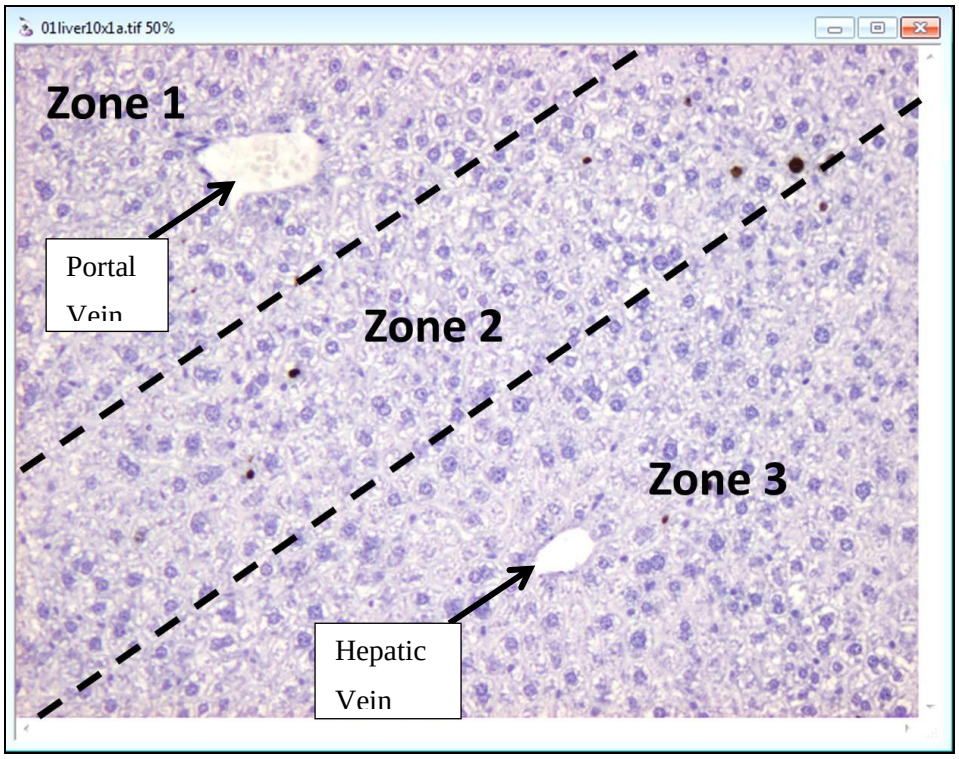
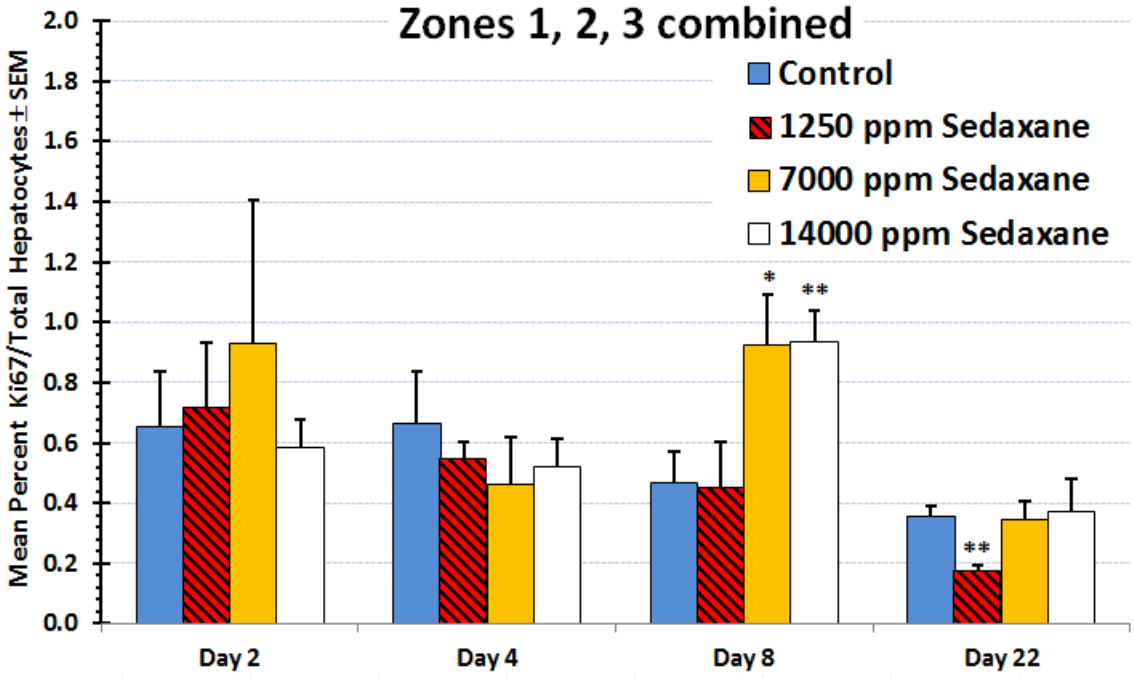
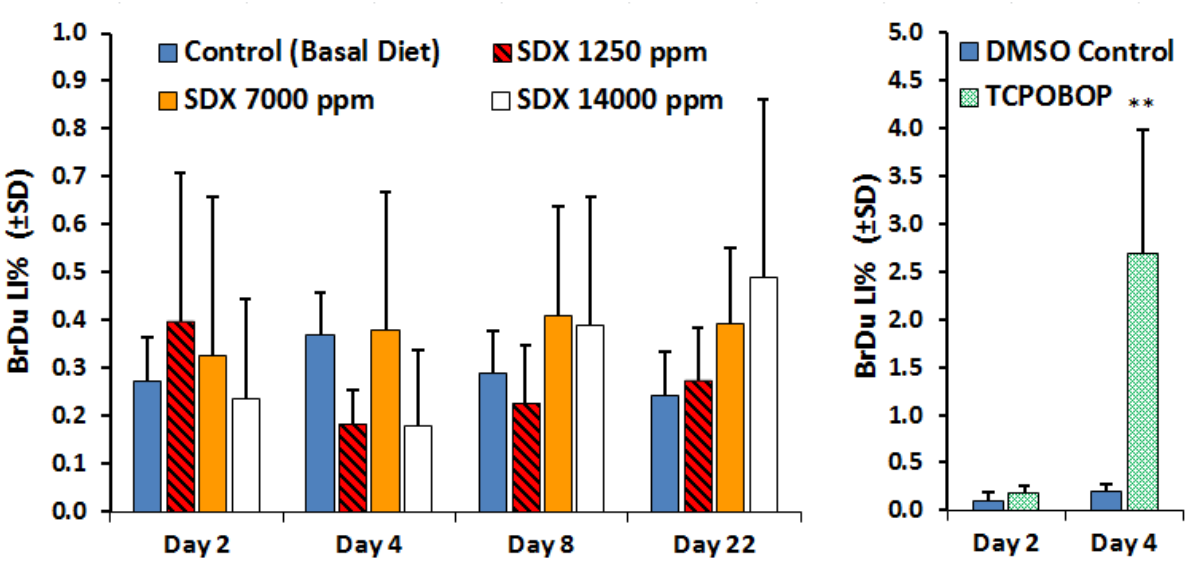


FIGURE 6 Results of Ki67 Cell Proliferation Analysis of Livers – Sedaxane-Treated Mice



*, ** Statistically-significantly different from control with $p<0.05$ and $p<0.01$, respectively.
Data from Anonymous, 2016 Annex I.3.9.4.2

FIGURE 7 CD-1 Mouse Liver BrdU Labelling Index Results



Results are means ±SD, n=6.
**p<0.01, Student's t-test
Data from Anonymous, 2016 Annex I. 3.9.4.2.

In the Ki67 labelling results (Figure 6), a slight treatment-related effect was observed at the Day 8 sacrifice, as indicated by statistically significant increases relative to controls in mean LI values for Zones 1, 2, and 3 combined in the 7000 ppm and 14000 ppm dose groups. The overall ANOVA analysis for all dose groups and combined zones at Day 8 was additionally significant at p = 0.0186. The labelling index on Day 8 was numerically higher than the control group values for each of the three separate zones (individually), and these differences were statistically significant in zone 1 (14000 ppm) and zone 3 (7000 and 14000 ppm) (data not shown). The 1250 ppm dose group was not significantly different from the control group at this time interval. At Day 22, a statistically significant decrease in Ki67 labeling index for the 1250 ppm group, in the absence of any effects at higher dose levels or other time intervals, is not considered toxicologically meaningful.

In the BrdU labelling index results (Figure 7), a trend toward numerically higher labelling index values at 7000 and 14000 ppm was observed at the Day 8 time interval, but this did not achieve statistical significance. For the TCPOBOP positive control groups, a large increase in BrdU labelling index was observed on Day 4, but not on Day 2. Because the expected response to the positive control was observed in the BrdU analysis, the livers of TCPOBOP-treated mice were not evaluated for Ki67 labelling.

In summation, the combined data from Ki67 and BrdU immunohistochemistry of the liver showed that a slight, treatment-related increase in cell proliferation was observed on Day 8 following treatment with 7000 and 14000 ppm sedaxane. This proliferative response was transient, with no effect on Ki67 labelling index observed on Day 22.

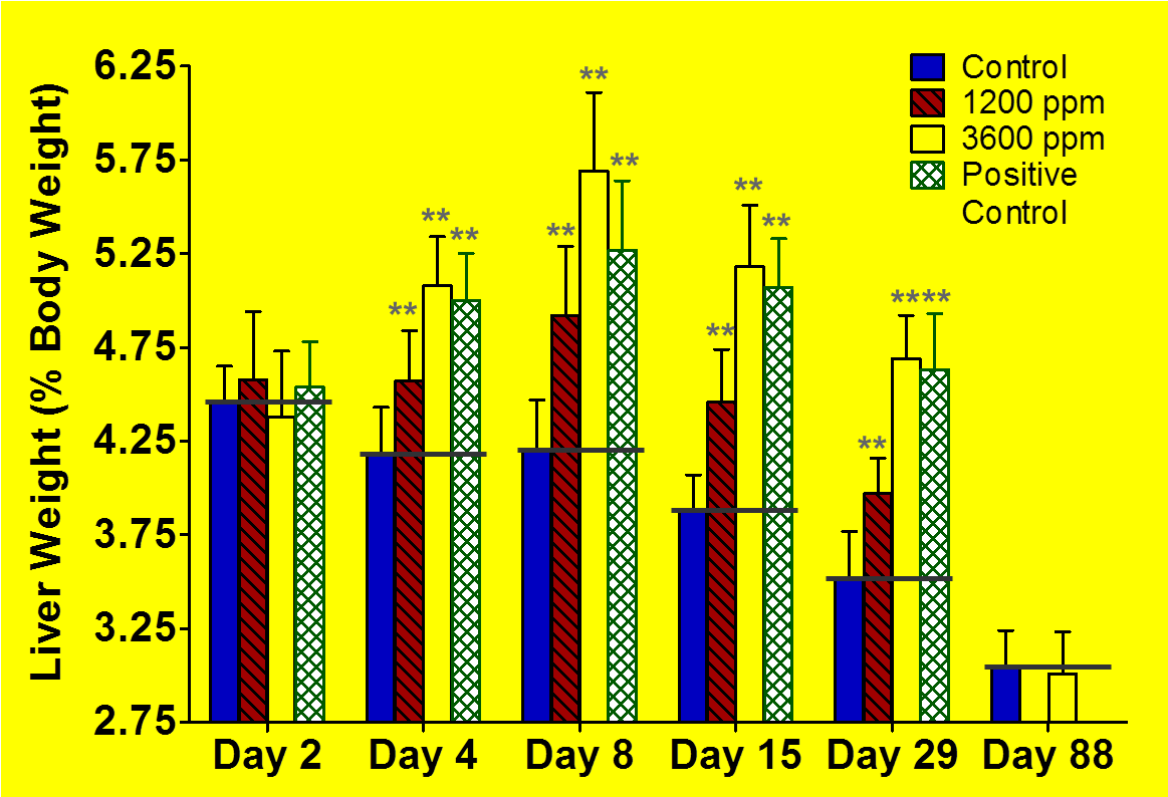
DS comment:
Key event 4 "Transient increased hepatocellular proliferation and decreased apoptosis":
In male mice, as measured by Ki67 (statistically significant increase) and the BrdU label index (numerical increase), sedaxane induced a slight increase in hepatocellular proliferation from 7000 ppm at Day 8, consistently with key event 4.

See annex I to the CLH Report 3.9.4.2.

3.3.7 Liver weight and histopathology – 28-day rat MOA study

The associative key events of liver weight and liver histopathology were also evaluated at each sacrifice interval in the 28-day liver MOA study in male Wistar rats. These results are summarized in Figure 8 and Table 7.

FIGURE 8 Liver Weights (Adjusted for Body Weight) – 28-Day MOA Study in Male Rats



*, ** Statistically-significantly different from control with p<0.05 and p<0.01, respectively.
Data from Anonymous, 2015 *Annex I. 3.9.4.4.*

TABLE 7 Liver Histopathology from 28-Day Rat MOA Study

	Sedaxane			NaPB
	0 ppm	**1200 ppm**	**3600 ppm**	**1200 ppm**
Liver: Centrilobular Hypertrophy (N)	(15)	(15)	(15)	(15)
Day 2	0	0	0	0
Day 4	0	7**	12**	15**
Day 8	0	6*	10**	13**
Day 15	0	11**	13**	15**
Day 29	0	12**	14**	15**
Day 29 (+60)	0	NA	0	NA

*, **Statistically significant difference from control group incidence by Fisher's Exact Test (p<0.05, p<0.01)
Values shown are total incidence (combining all severities). NA – Not applicable
Data from Anonymous, 2015 *Annex I. 3.9.4.4.*

The data shown in Figure 8 and Table 7 illustrate that treatment with sedaxane at 1200 and 3600 ppm produced a dose- and time-dependent increase in centrilobular hypertrophy in the liver, which

correlated with increases in liver weight adjusted for body weight. These changes were not observed on Day 2, but they were present beginning on Day 4 and continued throughout the dosing period. The incidence and severity of the hypertrophy of hepatocytes was greater at 3600 ppm (minimal to moderate severity) than at 1200 ppm sedaxane (minimal to slight severity). NaPB, the positive control agent, produced similar increases in liver weight and histopathology findings as sedaxane.

After the 60-day recovery period (Day 89), no differences from control were observed in the liver for animals that had previously been treated with 3600 ppm sedaxane.

3.3.8 Liver weight and histopathology – 21-day mouse MOA study

The liver weight and liver histopathology effects of treatment with sedaxane for 21 days, or with the positive control agent TCPOBOP, are shown in Tables 8 and 9. In this MOA study in male mice, no apparent effects on body weights were observed with any of the treatments and time intervals. Food consumption was slightly lower than control in the sedaxane-treated groups only during the first 3 days of the study.

TABLE 8 Liver Weights Adjusted for Body Weight from 21-Day Mouse MOA Study

	Sedaxane				TCPOBOP	
	0 ppm	**1250 ppm**	**7000 ppm**	**14000 ppm**	**0 mg/kg**	**3 mg/kg**
Day 2	1.98	2.06	1.87	1.91	2.15	2.29*
Day 4	2.18	2.20	2.34	2.61*	2.25	2.95**
Day 8	2.02	2.07	2.33*	2.60**	NA	NA
Day 22	1.78	1.85	1.99*	2.36**	NA	NA

NA – Not applicable
*, ** Statistically-significantly different from control with $p<0.05$ and $p<0.01$, respectively.
Data from Anonymous, 2016 Annex I. 3.9.4.2.

DS comment:
Associative event 2 "Hepatocellular hypertrophy" and Associative event 3 "Increased liver weights
In the 28-day liver MOA study in rat, increases in centrilobular hypertrophy and in liver weight were observed from 1200 ppm supporting Associative event 2 and Associative event 3.

See annex I to the CLH Report 3.9.4.4.

TABLE 9 Liver Histopathology from 21-Day Mouse MOA Study

	Sedaxane				TCPOBOP	
Liver, Hypertrophy	**0 ppm**	**1250 ppm**	**7000 ppm**	**14000 ppm**	**0 mg/kg**	**3 mg/kg**
Number Examined (at each interval)	(6)	(6)	(6)	(6)	(6)	(6)
Day 2						
centrilobular	0	0	0	2	0	4
Day 4						
centrilobular	0	0	0	3	0	4

diffuse	0	0	0	1	0	0
Day 8						
centrilobular	0	1	4	0	NA	NA
diffuse	0	0	2	6**	NA	NA
Day 22						
centrilobular	0	0	5*	6**	NA	NA
diffuse	0	0	0	0	NA	NA

NA – Not applicable

*, ** Statistically-significantly different from control with p<0.05 and p<0.01, respectively.

Data from Anonymous, 2016 Annex I. 3.9.4.2.

Statistically significant increases in mean adjusted liver weights were noted for the 14000 ppm sedaxane-treated mice terminated on Day 4, and for the 7000 and 14000 ppm sedaxane-treated mice terminated on Days 8 and 22, as compared to the mean liver weights of controls. No effects of treatment on liver weights were noted for the mice treated with 1250 ppm sedaxane. The increases in adjusted liver weights were a maximum of 33% higher than controls for 14000 ppm males and 31% higher than controls for 7000 ppm males.

For mice treated with the positive control agent, TCPOBOP, adjusted liver weights were statistically significantly higher than the corresponding control group on Day 2 (+7%) and on Day 4 (+31%).

Hepatocellular hypertrophy was the only treatment-related histopathology findings in the liver following sedaxane or TCPOBOP treatment. In sedaxane-treated groups, the severity was mild in the majority of animals, and it increased in incidence from Day 2 (14000 ppm group only) through Day 8 (present in all groups). There was also a shift in localization of the hypertrophy at 7000 and 14000 ppm from predominantly centrilobular (Day 2) to more diffuse (Day 8). Interestingly, at the Day 22 sacrifice, the hypertrophy had returned to entirely centrilobular in the 7000 and 14000 ppm sedaxane groups.

In the TCPOBOP positive control groups, centrilobular hypertrophy was observed in a majority of the mice on Day 2 and Day 4; there were no incidences of diffuse hypertrophy.

DS comment:

Associative event 2 "Hepatocellular hypertrophy" and Associative event 3 "Increased liver weights

In the 21-day liver MOA study in mouse, increases in centrilobular hypertrophy and in liver weight were observed from 7000 ppm supporting Associative event 2 and Associative event 3.

See annex I to the CLH Report 3.9.4.2.

3.3.9 Subchronic toxicity studies – rats

Two subchronic toxicity studies were conducted in rats with sedaxane. One of these studies (Anonymous, 2007 *Annex I. 3.12.1.1*) was conducted in a different strain from a different supplier than the carcinogenicity study, so it is only briefly mentioned here for completeness. In this study, Wistar rats (strain designation: HsdRccHan:WIST, from Harlan Lab, UK) were treated for 90 days with 0, 250, 1000 or 4000 ppm sedaxane in the diet. The dose level of 4000 ppm produced lower body weights, increased absolute and relative liver weights, and liver histopathology findings of centrilobular hypertrophy and increased pigmentation in males and females. The dose level of 1000 ppm produced minor increases in liver weight, but in the absence of any associated histopathology changes, this was not considered adverse. A smaller number of changes in clinical chemistry parameters were also observed at the 4000 ppm dose (including higher triglycerides, total protein), but there were no indicators of liver toxicity. The NOAEL in this study was 1000 ppm in males and females.

In a 90-day study in rats (strain designation CrL:WI(Han), from Charles River Labs, UK), groups of 10 male and 10 female Han Wistar rats were fed diets containing 0, 300, 2000 or 4000 ppm sedaxane for 90 days (Anonymous, 2009 *Annex I. 3.12.1.2*). In male and female rats treated at 4000 ppm, body weight, body weight gain and food consumption were significantly decreased. In female rats treated at 2000 ppm, body weight and body weight gain were also reduced. Sedaxane at 2000 ppm had no effect on male body weights, and 300 ppm was without effect on males or females.

Changes in clinical chemistry parameters that were considered treatment related in male rats at 4000 ppm included the following:

- Slightly higher gamma glutamyl transferase (GGT)
- Higher triglycerides
- Higher total protein

However, no clinical chemistry changes that would indicate liver toxicity were observed.

The findings in this study related to the proposed liver MOA are summarized in Table 10.

TABLE 10 Summary of Data from a 90 Day Study with Sedaxane – Liver-related Parameters from Male Rats Only

	0 ppm	**300 ppm**	**2000 ppm**	**4000 ppm**
Liver Wt. (adjusted for BW) (g)	15.38	15.71	17.73**	20.94**
Liver Histopathology: (N)	(10)	(10)	(10)	(10)
Centrilobular hypertrophy	0	0	0	10***
Hepatocyte pigment	0	1	0	4

*, ** and ***: Statistically-significantly different from control with $p<0.05$, $p<0.01$ and $p<0.001$, respectively.
Data from Anonymous, 2009 *Annex I. 3.12.1.2*

Liver weights adjusted for body weight were statistically significantly higher than control values for 2000 and 4000 ppm males. Histopathology findings in the liver including centrilobular hypertrophy and hepatocyte pigment were only increased in the 4000 ppm males.

DS comment:
Associative event 2 "Hepatocellular hypertrophy" and Associative event 3 "Increased liver weights
In the 90-day rat study, increases in centrilobular hypertrophy at 4000 ppm and in liver weight from 2000 ppm were observed consistently with Associative event 2 and Associative event 3.

See annex I to the CLH Report 3.12.1.2

3.3.10 Subchronic toxicity studies – mice

In CD-1 mice, subchronic toxicity studies were conducted as preliminary studies to facilitate dose level selection in the 18-month carcinogenicity study. Very few of the measured parameters showed statistically significant or treatment-related changes; therefore, only a brief summary of the overall results is provided in this Section.

In a 28-day study, CD-1 mice (strain designation: Crl:CD-1(ICR)) were treated with 0, 1000, 5000 and 7000 ppm sedaxane (Anonumou, 2008). This study showed:

- No effects on body weight, liver weight or liver histopathology in male or female mice.

In a 90-day study, CD-1 mice (strain designation: Crl:CD-1(ICR)) were treated with 0, 500, 3500 and 7000 ppm sedaxane (Anonymous, 2008 *Annex I. 3.12.1.5*). This study showed:

- A statistically significant lower body weights and a lower body weight gain in 7000 ppm males (-32%; $p<0.05$) during weeks 0 – 13 compared to the control males. A numerically lower body weight gain was observed in 500 and 3500 ppm males, but these were not considered treatment-related considering the lack of statistical significance and comparisons to historic control body weight ranges. There were no effects on body weight gain in female mice;
- No effects on liver histopathology;
- No effects on clinical chemistry parameters that would indicate liver toxicity; and
- Slightly higher, statistically significant liver weights in male mice were observed, but only after adjustment for body weight. The difference in adjusted liver weights in 7000 ppm males (+27%, $p<0.01$) was considered to be treatment-related, since the mean value was >25% higher than the control group mean, and histopathology changes were seen at 7000 ppm in the livers of mice treated for shorter durations (Table 9). However, the smaller differences in adjusted liver weights at 500 ppm (+12%, $p<0.05$) and 3500 ppm (+12%, $p<0.05$) were not considered effects of treatment, considering the lack of any histopathology changes, no effects on absolute weights, and lack of a dose-response.

After the 28-day and a 90-day mouse studies were completed, further investigations with frozen liver samples were performed to examine the liver enzyme induction profiles (Anonymous, 2013, *Annex I. 3.9.4.7*). At termination, after necessary sections of the liver were taken for histology processing (formalin fixation), 5 x 150 mg of liver was taken from the left lobe and stored in RNA-ase free tubes, snap frozen in liquid nitrogen and stored at -70°C. for future use in transcriptomics experiments. The remaining liver was wrapped in aluminium foil and stored deep frozen at -70°C. for future use in enzyme activity determinations (Anonymous, 2008)Samples of liver for future use were taken in a similar manner from the 90-day mouse study (Anonymous, 2008 *Annex I. 3.12.1.5)* and stored at -70°C. for future use.

From the 28-day mouse study and the 90-day mouse study, the snap-frozen samples in RNA-ase free tubes and the remaining liver sample in aluminium foil (both stored at -70°C.) were shipped on dry ice to LFR, Inc. and received in frozen condition (Anonymous, 2013, *Annex I. 3.9.4.7*) . The remaining liver samples wrapped in aluminium foil were used for enzyme activity determinations; the smaller subsamples in RNA-ase free tubes were not used and were continuously stored frozen at LFR Inc. The liver samples in aluminium foil were thawed and weighed. Whole liver homogenates were prepared, and via differential centrifugation they were separated into microsomal and cytosolic fractions. Aliquots of liver whole homogenate, microsomal and cytosolic fractions from each animal were stored at -70°C or below. Samples were analysed for the following activities:

- With whole liver homogenate: total protein and cyanide-insensitive palmitoyl-CoA oxidation activity
- With microsomes: total protein content, CYP content, PROD activity, EROD activity, testosterone 6β-hydroxylase activity and lauric acid 12-hydroxylase activity

Results of the enzyme activity determinations in liver fractions are shown in Table 11 for both the 28-day study and the 90-day study (Anonynous, 2013, *Annex I. 3.9.4.7*).

TABLE 11 Enzyme Activities in Frozen Liver Samples from 28-Day and 90-Day Studies in Male CD-1 Mice

	% of Control Value		
	28 Day		90 Day
	1000 ppm	7000 ppm	7000 ppm
Whole homogenate protein (mg/g liver)	106	103	99
Palmitoyl-CoA oxidation Activity (nmol/min/mg protein)	82	104	89
Microsomal protein (mg/g liver)	109	104	103
Cytochrome P450 content (nmol/mg protein)	100	148**	140**
EROD Activity (pmol/min/mg protein)	82	103	111
PROD Activity (pmol/min/mg protein)[b]	233**	1933**	1400**
Testosterone 6β-hydroxylase Activity (nmol/min/mg protein)	95	135	147**
Lauric acid 12-hydroxylase Activity (nmol/min/mg protein)	83	117	97

** Statistically-significantly different from control with p<0.01. Data from Anonymous, 2013, *Annex I.3.4.7*

Based on the pattern of results observed, sedaxane did not activate PPARα (peroxisome proliferator-activator receptor alpha). It did not produce any increases in activity for palmitoyl-CoA oxidation or lauric acid 12-hydroxylase (an indicator of microsomal CYP4A induction), two enzymes that are known markers of PPARα activation (Klaunig *et al.*, 2003; Lake, 2009). Sedaxane also did not

activate the aromatic hydrocarbon receptor (AhR). It did not produce any increases in EROD activity, which is a marker for Cyp1a and Cyp1b activities that are induced by AhR activation.

In contrast, sedaxane was a potent activator of CAR in mouse livers. PROD activity (a marker for Cyp2b activity) was increased in a dose-responsive manner, with maximal activity that was 1933% and 1400% of the control value at 28 days and 90 days, respectively. It also produced a lesser induction of testosterone 6β-hydroxylase activity (a marker of Cyp3a activity), but a treatment-related increase was observed only at 7000 ppm. This pattern of effects is consistent with the nuclear receptor reporter assays (Table 3), which indicated that sedaxane was a CAR activator but not a PXR activator for mice (Elcombe *et al.*, 2014; Lake, 2009).

In terms of generalized markers of liver induction, sedaxane at 7000 ppm produced an increase in total CYP content, but it had no effects on total hepatic protein in either the microsomal fraction or the liver homogenate.

DS comment:
Associative event 2 "Hepatocellular hypertrophy" and Associative event 3 "Increased liver weights
In the 90-day mouse study, increased liver weight was observed at 7000 ppm was observed consistently with Associative event 3. However no effects on liver histopathology were noted at any dose levels.

Associative event 1 "Increased expression of genes encoding cytochrome P450s (CYPs), particularly Cyp2b and Cyp3a families":
Determinations of enzyme activity in liver fractions showed that sedaxane treatment induced CAR activation (increase in CYP content, PROD activity, and testosterone 6β-hydroxylase activity) at 7000 ppm.

See annex I to the CLH Report 3.9.4.7 and 3.12.1.4.

3.3.11 Non-neoplastic findings in a combined chronic toxicity and carcinogenicity study in Han Wistar rats

In addition to the tumour incidence data described in Section 3.1.1, relevant toxicity data in the liver generated in the combined chronic toxicity and carcinogenicity study in rats is presented in Tables 12 and 13. Body weight and body weight gain were decreased throughout the study in male rats at 3600 ppm. Male rats at 3600 ppm also had lower food consumption during Weeks 1-7, but were comparable to controls thereafter (data not shown). After 52 weeks, dose-responsive increases in liver weights adjusted for body weight were observed at ≥1200 ppm, whereas the liver histopathology findings of centrilobular hypertrophy and hepatocyte pigment occurred only at 3600 ppm in male rats.

In the carcinogenicity phase of the study, terminal sacrifice animals at 104 weeks displayed increased liver weights, and the histopathology examination (including early decedents) revealed increased hepatocellular hypertrophy in males at ≥1200 ppm. In addition, eosinophilic foci were statistically significantly higher at 3600 ppm, and this was considered a treatment-related effect.

TABLE 12 Summary of Data from 52-Week Interim Sacrifice in 2-Year Rat Study with Sedaxane – Non-neoplastic Liver-related Parameters from Male Rats

		0 ppm	200 ppm	1200 ppm	3600 ppm
Weights:	**Week**				
Body weight gain (g) weeks 0 – 52	52	370.3	387.3	360.9	300.7** (-19%)
Liver wt. adjusted for body weight (g)	52	16.10	16.03	18.94**	22.63** (+41%)
Histopathology: (N)		(12)	(12)	(12)	(12)
Liver:					
Centrilobular hypertrophy	52	0	0	0	11***
Hepatocyte pigment	52	0	1	0	7**

Weights are mean and (percent of control).
*, ** and ***: Statistically-significantly different from control with p<0.05, p<0.01 and p<0.001, respectively. (Dunnett's test or Fisher's Exact Test).
Data from Anonymous 2015 *Annex I. 3.9.4.1*.

TABLE 13 Summary of Data from 104-Week Sacrifice + Decedents in 2-Year Rat Study with Sedaxane – Non-neoplastic Liver-related Parameters from Male Rats

		0 ppm	200 ppm	1200 ppm	3600 ppm
Weights:	**Week**				
Body weight gain (g) weeks 0 – 13	13	232.5	247.9*	228.0	187.3** (-19%)
Body weight gain (g) weeks 0 – 104	104	464.9	509.7*	447.9	355.7** (-23%)
Liver wt. adjusted for body weight (g)	104	18.10	18.06	20.21**	24.20** (+34%)
Histopathology: (N)		(52)	(52)	(52)	(52)
Liver:					
Centrilobular hypertrophy	104	0	0	8**	16***
Hepatocyte pigment	104	0	1	0	1
Eosinophilic cell focus	104	8	7	15	25***
Clear cell focus	104	33	39	37	19*
Angiectasis	104	0	1	6*	6*

Weights are mean and (percent of control).
*, ** and ***: Statistically-significantly different from control with p<0.05, p<0.01 and p<0.001, respectively. (Dunnett's test or Fisher's Exact Test).
Data from Anonymous 2015 *Annex I. 3.9.4.1*.

DS comment:
Associative event 2 "Hepatocellular hypertrophy" and Associative event 3 "Increased liver weights
Key event 5 "Clonal expansion and development of altered hepatic foci":
In the 2-year rat study, sedaxane led to hepatocellular hypertrophy and increased liver weight from 1200 ppm and increased eosinophilic cell foci from 3600 ppm supporting Associative events 2 & 3. And Key event 5.

See annex I to the CLH Report 3.9.1.1.

3.3.12 Non-neoplastic findings in a carcinogenicity study in CD-1 mice

In addition to the tumour incidence data described in Section 2.1.1, relevant toxicity data related to the liver that was generated in the 80-week carcinogenicity study in CD-1 male mice is presented in Table 14.

TABLE 14 Summary of Data from Terminal Sacrifice + Decedents in 80-Week Mouse Study with Sedaxane – Non-neoplastic Liver-related Parameters from Male Mice

		0 ppm	200 ppm	1250 ppm	7000 ppm
Weights:	**Week**				
Body weight gain (g) weeks 0 – 13	13	14.0	13.0	13.7	12.3 (-13%)
Body weight gain (g) weeks 0 – 80	80	27.5	25.7	25.8	24.7 (-10%)
Liver wt. adjusted for body weight (g)	80	3.04	3.13	3.15	3.53* (+16%)
Histopathology:					
Liver (N):		(50)	(50)	(50)	(50)
Centrilobular hypertrophy	80	0	1	2	1
Periportal hypertrophy	80	0	0	0	0
Hepatocyte vacuolation	80	4	6	12*	8
Necrosis	80	2	4	3	3
Eosinophilic cell focus	80	3	1	3	3
Basophilic cell focus	80	2	1	3	6
Clear cell focus	80	4	4	7	3
Haemopoiesis, extramedullary	80	0	0	1	4*
Erythrophagocytosis, hepatocyte	80	0	1	0	0
Inflammatory cell infiltrate	80	1	1	0	1
Degeneration, hepatocyte	80	0	0	0	0

Weights are mean and (percent of control).
*, ** and ***: Statistically-significantly different from control with $p<0.05$, $p<0.01$ and $p<0.001$, respectively. (Dunnett's test or Fisher's Exact Test).
Data from Anonymous 2015 *Annex I. 3.9.4.2*.

In the 80-week mouse study, lower body weights and body weight gains were observed for males (and females – data not shown) at 7000 ppm. The maximum difference from control for body weight was 7% in male mice at 7000 ppm. Food consumption was unaffected.

Liver weight adjusted for body weight was statistically significantly increased in males at 7000 ppm (+16%). However, the report concluded that no histopathology changes in the liver were attributed to treatment with sedaxane.

In Table 14, all of the non-neoplastic histopathology changes in the liver that were tabulated in the original report are included for the male mice, summing all severities to give a total incidence. Although a small number of statistically significant values are observed (Fisher's Exact Test), the

pattern of incidences and the relatively low numbers for each finding listed are consistent with the observation in the final report, that none of these non-neoplastic histopathology changes are considered treatment-related.

DS comment:
Associative event 3 "Increased liver weights
In the 80-week mouse study, sedaxane led to increased liver weight at 7000 ppm supporting Associative event 3.
However no centrilobular hypertrophy or increased hepatic foci were noted at any dose levels.

See annex I to the CLH Report 3.9.1.2.

4.0 IPCS/ILSI FRAMEWORK FOR THE EVALUATION OF THE HUMAN HEALTH RELEVANCE OF A HYPOTHESISED MODE OF ACTION

4.1 Is the Weight of Evidence Sufficient to Establish the Mode of Action (MOA) in Animals?

After the key events within a postulated MOA have been described, the Hill (1965) criteria and the framework outlined by IPCS (Boobis *et al.*, 2006) require that they be evaluated by a standardized weight of evidence evaluation. For the key events to be causally related to the formation of tumours, they must:

- Be supported by data showing strength, consistency and specificity of association of key events and tumour response,
- Show dose-concordance of key events and dose levels that produce tumours,
- Occur in a logical temporal sequence,
- Be reproducible,
- Demonstrate that alternative MOAs have been considered and are not operative, and
- Be plausible and consistent with the current state of knowledge of the relevant biological processes.

A weight of evidence analysis for the animal MOA with sedaxane is described in the following sections.

4.1.1 Dose-concordance of key events

Tables 15 summarizes the dose-concordance of the associative and causal key events for male rat liver tumours. Overall, there is good dose concordance of the proposed key events with tumour outcome. The key event of transient cell proliferation was demonstrated to occur at both 1200 ppm and 3600 ppm, but the longer-term key event of increased altered foci (observed at 2 years) was only observed at 3600 ppm. Effects at non-tumourigenic dose levels in the range of 500 – 1200 ppm included short-term increases in markers of liver Cyp2b induction (e.g. PROD activity), but at the lower dose of 500 ppm this did not translate into increased liver weight or liver hypertrophy after 28 days of treatment (Table 4). The associative key events of increases in liver hepatocellular hypertrophy and liver weight were observed in a dose-responsive manner consistent with the proposed MOA. No effects on these parameters were observed at the low dose of 300 ppm from a 90-day subchronic rat study or at the low dose of 200 ppm from the 2-year rat study.

Table 16 summarizes the dose-concordance of the key events for male mouse liver tumours. In this comparison, dose levels of 7000 ppm and 14000 ppm are considered tumourigenic, since the EPA concluded that an increased liver tumour incidence occurred at 7000 ppm in the 80-week study. At dose levels of 1000 – 1250 ppm evaluated in the 28-day subchronic study (Table 11) and the 21-day mouse MOA study (Tables 8-9), limited effects of small increases in PROD activity were observed, but no observable increases in liver weight or liver hypertrophy occurred. Further, the key event of transiently increase cell proliferation (by Ki67 staining) was only observed at the tumourigenic dose levels of 7000 and 14000 ppm, but not at the non-tumourigenic 1250 ppm dose (Figure 6). Overall, the pattern of effects in the mouse liver were relatively weak in magnitude, compared with the rat, but the dose levels where the earlier key events were uniformly observed matched with the tumourigenic dose level of ≥7000 ppm.

Toxicokinetic data that offers a likely explanation for the observed differences in sensitivity to liver effects between male rats and male mice are described in the following section (Section 4.1.2)

DS comment:
According to the available data, a good dose-concordance between the causal key events, associative events and the apical outcome, (liver tumours) was observed in both male rats and male mice.

TABLE 15 Summary of Dose-Concordance of Associative Events and (Causal) Key Events - Male Rat Liver Tumours

Dose of Sedaxane (ppm)[a]	CAR/PXR activation (Causal)	Induction of *Cyp* gene expression/ increased CYP activity[c] (Associative)	Increased hepatocellular hypertrophy (Associative)	Increased liver weight (Associative)	Induction of pro-proliferative and anti-apoptotic genes[d]	Transiently increased hepato-cellular proliferation (Causal)	Altered hepatic foci (Causal)	Numerically Higher Incidence of Liver Tumours Compared to Concurrent Controls
200 (300) [a]	No data	No data	No	No	No data[d]	No data	No	No
500	Yes[b]	Yes	No	No	No data	No data		
1200	Yes[b]	Yes	Yes	Yes	No data	Yes	No	No
2000	Yes[b]	Yes	Yes	Yes	No data	No data	No data	No data
3600 (4000) [a]	Yes[b]	Yes	Yes	Yes	No data	Yes	Yes	Yes
5000	Yes[b]	Yes	Yes	Yes	No data	No data	No data	No data

a Values in parentheses are subchronic dose levels (that are similar to the chronic dose levels).
b Confirmed *in vitro* (CAR, PXR transactivation) and *in vivo* (dose levels inferred from observed increases in *cyp* gene expression and/or increased CYP activity).
c Increased Cyp activity = primarily Cyp2b activity >> Cyp3a activity, based on PROD activity and Testosterone 16β-hydroxylase activity (Cyp2b markers) >> Testosterone 6β-hydroxylase activity (Cyp3a marker).
d Analysis of gene expression was not assessed for rats, but can be inferred from the large changes in 1) cell proliferation in rat hepatocytes, and 2) mouse RT-PCR and microarray data

TABLE 16 Summary of Dose-Concordance of Associative Events and (Causal) Key Events - Male Mouse Liver Tumours

Dose of Sedaxane (ppm)[a]	CAR activation (Causal)	Induction of *Cyp* gene expression/ increased CYP activity[c] (Associative)	Increased hepatocellular hypertrophy (Associative)	Increased liver weight (Associative)	Induction of pro-proliferative and anti-apoptotic genes [d] (Causal)	Transiently increased hepato-cellular proliferation (Causal)	Altered hepatic foci (Causal)	Numerically Higher Incidence of Liver Tumours Compared to Concurrent Controls
200 (500) [a]	No data	No data	No	No	No data	No data	No	No
1250 (1000) [a]	Yes[b]	Yes	No	No	No [d]	No	No	No
3500 (5000) [e]	No data	No data	No	No	No data	No data	No data	No data
7000	Yes[b]	Yes	Yes / No[f]	Yes / No[f]	Yes[d]	Yes	No	Yes
14000	Yes[b]	Yes	Yes	Yes	Yes[d]	Yes	No data	No data

a Values in parentheses are subchronic dose levels (some of which are similar to the chronic dose levels of 200, 1250, or 7000 ppm).
b Confirmed *in vitro* (CAR transactivation) and *in vivo* (dose levels inferred from observed increases in *cyp* gene expression and/or increased CYP activity).
c Increased Cyp activity = primarily Cyp2b activity >> Cyp3a activity, based on PROD activity (Cyp2b markers) >> Testosterone 6β-hydroxylase activity (Cyp3a marker).
d Gene expression changes in mouse liver that indicated a pro-proliferative, anti-apoptotic signal included increased Gadd45β expression , decreased Gadd45gamma expression, and further changes in the microarray KEGG pathway for cell cycle (Anonymous, 2016 Annex I. 3.9.4.2).
e Dose levels of 3500 ppm and 5000 ppm were tested in a 90-day study and a 28-day study, respectively.
f At 7000 ppm, increases in liver weight and hepatocellular hypertrophy were observed only at certain time intervals and studies (See Time Concordance Table).

4.1.2 Toxicokinetic differences that correlate with differences in liver effects in mice vs. rats

The CAR reporter assays with sedaxane showed that sedaxane was an activator of rat, mouse and human CAR (Table 3), and the relative fold-change at 30 μM in the different species indicated a greater response with mouse CAR (19-fold) than with rat CAR (6-fold) or human CAR (4-fold). Despite this apparent difference in the affinity for CAR receptor site between the species, sedaxane showed greater non-neoplastic liver responses at lower doses in male Wistar rats *in vivo* than in male CD-1 mice *in vivo*. Tables 15 and 16 provide an overview of the effects related to the liver at different doses (ppm in diet) for rats and for mice. The magnitude of responses in mice to sedaxane for liver related parameters was generally weak, and this included the lack of apparent hepatocellular hypertrophy or liver weight increases at 28 days, lack of any eosinophilic foci in the 80-week study and a marginal increase in the incidence of a relatively common tumour type (hepatocellular adenoma + carcinoma) despite lifetime dosing at a limit dose (7000 ppm or approximately 1000 mg/kg/day). In addition, mice tolerated a dose as high as 14000 ppm in diet (equivalent to 2155 mg/kg/day) for 14 days with only a slight decrease in body weight gain and a small increase in liver weight, whereas the maximum dose that could be given to rats for repeated durations was 3600 – 4000 ppm (chronic or 90-day rat study).

Blood toxicokinetic data available from 14-day dosing studies in rats and mice provide a strong suggestion that differences in clearance of the parent molecule (isomers of sedaxane) between mice and rats accounts for this apparent difference in sensitivity, as shown in Table 17. In the rat study, samples were taken every 4 hours starting at approximately 17.00 h, which was approximately 8 h after dietary feeding commenced. The rat Tmax (time of the maximum blood concentration (Cmax)) appeared in the morning, which is consistent with overnight feeding, so that a comparison to the blood samples from the 14-day mouse study at termination provides a reasonable comparison of approximate steady-state Cmax values. As shown in Table 17, the blood concentrations of the *Trans*-isomer and the *Cis*-isomer in male mice were less than the Limit of Quantitation (LOQ <10 ng/mL) in the majority of the mice, such that a reliable mean value could not be determined. This was the opposite of the pattern in male rats, where quantifiable Cmax concentrations were observed consistently that were roughly proportionate to the administered dose. In a rat ^{14}C-sedaxane metabolism study (Green, 2009), the major circulating moieties in blood at a dose of 80 mg/kg were parent sedaxane (isomers) and a demethylated metabolite (*Trans*- and *Cis*-isomers). The HPLC analysis of mouse blood in the 14-day study detected only trace amounts of the desmethyl sedaxane isomers or of sedaxane isomers in a small proportion of the mice (Anonymous, 2015 Annex I. 3.9.4.1). These data suggest that sedaxane is rapidly metabolized to downstream metabolites and thus cleared from the blood, at a greater rate than in the rat, and this quantitative difference translates into less generalized toxicity (e.g. body weight decrease) and less effects on the liver (e.g. liver weight and hypertrophy increases) in mice than in rats.

TABLE 17 Comparison of Blood Concentrations of Sedaxane Isomers – Short-term Studies in Male Rats and Male Mice

Dose Level	Administered Dose (mg/kg/day)	Day	Mean Cmax[a] Trans (SYN508210) (ng/mL)	Mean Cmax[a] Cis (SYN508211) (ng/mL)
Male Rats:	SYN524464 (50:50)			
500 ppm	48	1	112	28
		14	60	32
2000 ppm	181	1	1220	101
		14	153	---
5000 ppm	445	1	1610	120
		14	613	32
Male Mice:	SYN524464 (83.0 : 12.3)			
7000 ppm	970	1	---	---
		14	<10	<10
10000 ppm	1389	1	---	---
		14	<10	<10
14000 ppm	2155	1	---	---
		14	<10	<10

--- = no data available

a Values are mean Cmax from 28-day rat study (Anonymous, 2010 *Annex I. 3.12.1.1*) and from a rangefinding 14-day mouse dietary study (Anonymous, 2015 *Annex I. 3.9.4.1*). In the rat study, samples were taken from eight hours after the administration of test diets and at four hour intervals thereafter (approximately 17.00, 21.00, 01.00, 05.00, 09.00 and 13.00 hours) on Day 1/2 and Day 14/15 of the study. Blood was centrifuged and the plasma was taken for analysis. In the mouse study, samples were taken by cardiac puncture under CO_2 anesthesia into EDTA tubes at the time of sacrifice. Whole blood samples were diluted with an equal volume of water and frozen prior to analysis. Rat plasma and mouse blood samples were analysed by HPLC for determination of the *Trans* isomer (SYN508210) and the *Cis* isomer (SYN508211). Tmax in rats was (in general) between 12 – 20 hr into the sampling regime, which equated to the morning hours (5.00 – 13.00 h). Thus, the sampling at a single sacrifice time for mice (morning) is considered a reasonable approximation of a Cmax measurement.

4.1.3 Temporal-concordance of key events – male rats

The observed effects on parameters associated with the key events occur in a logical, time-dependent manner consistent with the proposed MOA. The temporal concordance for the rat tumour MOA is summarized in Table 18.

The observed steps occur in a logical sequence, with the key events preceding the time of occurrence of liver hepatocellular adenomas and carcinomas. In particular:

- Activation of CAR is inferred to occur after 1 day, based on a correlating increase in cell proliferation by BrdU labelling;
- This causal key event of hepatocellular proliferation was transiently affected, with significant increases at 1-3 days, but no measurable sustained increase above control at 7 days and longer;

- Induction of CYP activities (Cyp2b >> Cyp3a), increased hepatocellular hypertrophy, and increased liver weight occurred early (3-7 days), and remained consistently; affected over time. The CYP activities were assessed after 7 or 28 days of treatment (Figure 2 and Table 4), and they would be expected to stay elevated after longer durations in concordance with the increased liver weights;
- A long-term consequence of these early key events included the postulated formation of altered hepatic foci (eosinophilic foci), which were only observed beyond one year; and
- Potential for a higher incidence of liver tumours vs. the concurrent control group with sustained treatment for 1.5 – 2 years

While the activation of specific genes was not an endpoint measured in rat MOA studies, these data are available in the mouse MOA studies, and they can be inferred from the *in vitro* CAR/PXR transaction results, the CYP enzyme activity measurements and cell proliferation response in rat liver.

The temporal concordance for the mouse liver tumour MOA is summarized in Table 19. The observed steps occur in a logical sequence, with the key events preceding the time of occurrence of liver hepatocellular adenomas and carcinomas. In particular:

- Activation of CAR is inferred to occur after 1 day, based on a correlating increase in gene expression (*Cyp2b10*, *Cyp2c65*, *Gadd45β);*
- Induction of CYP activities (Cyp2b >> Cyp3a), increased hepatocellular hypertrophy, and increased liver weight occurred early (1-7 days), and remained consistently affected over time;
- Differential expression of genes that promote a pro-proliferative and anti-apoptotic environment in the liver (e.g. increased *Gadd45β,* decreased *Gadd45γ*) also occurred early (detected at 1-7 days by RT-PCR; at 1-21 days by microarrays). The gradually diminished response with time for *Gadd45β* induction as well as *Cyp2b10* induction by RT-PCR (Figure 2) is of interest. This pattern may be associated with a time-dependent greater metabolism of sedaxane by the induced CYP enzymes (discussed above);
- The diminished response for *Gadd45β* beyond 7 days is concordant with the transient effect on cell proliferation. The causal key event of hepatocellular proliferation was significantly increased after 7 days, but no measurable sustained increase was observed at 21 days (Figure 6);
- Despite the consistent increase in certain markers of CYP gene expression (*Cyp2c65*) or activity (PROD), the phenotypic endpoints of increased liver weight and liver hypertrophy were minimally affected or unaffected at 7000 ppm after longer time intervals; and
- Potential for a higher incidence of liver tumours vs. the concurrent control group with sustained treatment ror 80 weeks .

The temporal relationships in mice are consistent with the proposed MOA and the known biology of CAR activators (Elcombe *et al.*, 2014). In addition, they point to a gradually diminished response in the livers of mice beyond 21 days, that is consistent with the relatively small increase in incidences vs. the concurrent control group of a fairly common tumour type in male CD-1 mice.

DS comment:
Based on the available data, a temporal concordance between the causal key events, associative events and the apical outcome, (liver tumours) could be established in both male rats and male mice.

TABLE 18 Temporal Concordance of Associative Events and (Causal) Key Events in the Proposed MOA – Male Rat Liver Tumours

Time	CAR/PXR activation (Causal)	Induction of *cyp* gene expression/ increased CYP activity[b] (Associative)	Increased hepatocellular hypertrophy (Associative)	Increased liver weight (Associative)	Induction of pro-proliferative and anti-apoptotic genes [c]	Hepato-cellular proliferation (Causal)	Altered hepatic foci (Causal)	Numerically Higher Incidence of Liver Tumours Compared to Concurrent Controls
1 days	Yes[a]	No data	No	No	No data [c]	Yes	No	No
3 days	Yes[a]	No data	Yes	Yes	No data	Yes	No	No
7 days	Yes[a]	Yes	Yes	Yes	No data	No	No	No
28 days	Yes[a]	Yes	Yes	Yes	No data	No	No	No
90 days	No data	No data	Yes	Yes	No data	No[c]	No	No
1 year	No data	No data	Yes	Yes	No data	No data	No	No
1.5-2 years	No data	No data	Yes	Yes	No data	No data	Yes	Yes

a Confirmed *in vitro* (CAR, PXR transactivation) and *in vivo* (inferred from observed increases in cell proliferation, *cyp* gene expression and/or increased CYP activity).

b Increased Cyp activity = primarily Cyp2b activity >> Cyp3a activity, based on PROD activity and Testosterone 16β-hydroxylase activity (Cyp2b markers) >> Testosterone 6β-hydroxylase activity (Cyp3a marker).

c Analysis of gene expression was not assessed for rats, but can be inferred from the large changes in 1) cell proliferation in rat hepatocytes, and 2) mouse RT-PCR and microarray data

TABLE 19 Temporal Concordance of Associative Events and (Causal) Key Events at the Tumourigenic Dose Level and Higher – Male Mouse Liver Tumours

Time	CAR activation (Causal)	Induction of *cyp* gene expression/ increased CYP activity[b] (Associative)	Increased hepatocellular hypertrophy (Associative)	Increased liver weight (Associative)	Induction of pro-proliferative and anti-apoptotic genes [c] (Causal)	Hepato-cellular proliferation (Causal)	Altered hepatic foci (Causal)	Numerically Higher Incidence of Liver Tumours Compared to Concurrent Controls
1 days	Yes[a]	Yes	Yes	No	Yes[c]	No	No	No
3 days	Yes[a]	Yes	Yes	Yes	Yes[c]	No	No	No
7 days	Yes[a]	Yes	Yes	Yes	Yes[c]	Yes	No	No
21 days	Yes[a]	Yes	Yes	Yes	Yes[c]	No	No	No
28 days	Yes[a]	Yes	No	No	No data	No data	No	No
90 days	Yes[a]	Yes	No	Yes	No data	No data	No	No
14 – 80 weeks	No data	No data	No	Yes	No data	No data	No	Yes

a Confirmed *in vitro* (CAR, PXR transactivation assays) and *in vivo* (inferred from observed increases in cell proliferation, *cyp* gene expression and/or increased CYP activity).

b Increased Cyp activity = primarily Cyp2b activity >> Cyp3a activity, based on PROD activity (Cyp2b marker) >> Testosterone 6β-hydroxylase activity (Cyp3a marker).

c Gene expression changes in mouse liver that indicated a pro-proliferative, anti-apoptotic signal included increased *Gadd45β* expression , decreased *Gadd45γ* expression, and further changes in the microarray KEGG pathway for cell cycle (Anonymous, 2016 Annex I. 3.9.4.2).

4.1.4 Reproducibility and consistency

For both the rat and the mouse, where parameters were measured in multiple studies, there is a high degree of reproducibility between studies and consistency between key events. The first key event in the postulated MOA, activation of CAR (mice) or CAR and PXR (rat), was demonstrated *in vitro* in direct activation assays for CAR and for PXR, and it was evident in multiple *in vivo* studies based on increases in *Cyp2b* gene expression and Cyp2b enzymatic activity (both PROD and testosterone 16β-hydroxylase activities). The liver weight increases and associated hepatocellular hypertrophy were consistently observed in all of the rat *in vivo* studies; in mice, these phenotypic changes were much less affected by 7000 ppm sedaxane at later times. However, use of a dose (14000 ppm) higher than the tumourigenic dose produced stronger responses in the 21-day mechanistic study, giving greater confidence that the sequence of key events is well understood. The transient increase in hepatocellular proliferation was observed in both rats and in mice, and corresponding changes in gene expression in mice that indicate a pro-proliferative environment are further support for this key event. Finally, the matching key events and similar overall MOA pathways in Wistar rats and CD-1 mice provides a strong measure of reproducibility across similar rodent species.

The only minor inconsistency in the database is the lack of an increase in eosinophilic foci in the 80-week mouse study. This late key event was clearly observed at the tumourigenic 3600 ppm dose in male rats. However, it is plausible that an increase in altered foci in the mouse liver may have preceded the development of tumours, but it could not be observed because no interim sacrifice is made in a Guideline mouse carcinogenicity study. In addition, this minor difference between the species is consistent with the overall pattern of much milder non-neoplastic effects on the liver occurring in mice than in rats, likely due to differences in toxicokinetics (Table 17).

4.1.5 Biological plausibility

The liver is the most common target tissue affected in carcinogenicity studies in rodents (Gold *et al.*, 2001). This may be due to the fact that the liver is the major site of metabolic processing of xenobiotics, as well as being the first organ exposed following absorption from the gastrointestinal tract (if administered orally, as in the case of the carcinogenicity studies with sedaxane). The induction of liver tumours in male mice subsequent to the activation of CAR is a comprehensively studied and characterised MOA for a number of compounds, including the archetypal CAR activator phenobarbital (Elcombe *et al.*, 2014; Meek *et al.*, 2003; Whysner *et al.*, 1996) and the potent mouse CAR activator TCPOBOP (Huang *et al.*, 2005).

4.1.6 Alternative mode of action hypotheses

In addition to CAR/PXR activation, a number of alternative MOAs for induction of liver tumours in rodents and/or humans have been demonstrated (Cohen, 2010). These alternative MOAs, and the reasons why they can be excluded for sedaxane, are described below.

TABLE 20 Alternative Modes of Action for Induction of Liver Tumours in Rodents and Reason(s) for their Exclusion for Sedaxane

Alternative MOA	Reason for exclusion
Genotoxicity	Sedaxane has been tested in a wide variety of *in vitro* and *in vivo* assays for genotoxicity. There is no evidence that sedaxane is genotoxic (Table 21).

Peroxisome proliferator	Treatment with sedaxane did not increase male mouse hepatic peroxisomal fatty acid β-oxidation or lauric acid 12-hydroxylation activity (a marker of Cyp4a activity) (Anonymous, 2013, *Annex I. 3.9.4.7*). Also, in a rat 28-day study, electron microscopy of the livers showed no evidence of peroxisome formation (Anonymous, 2010 *Annex I. 3.12.1.1*).
Enzyme induction (aryl hydrocarbon receptor [AhR]-mediated)	Treatment with sedaxane did not result in increased EROD activities in rats or in mice (Anonymous, 2013, Annex I. 3.9.4.7 and (Anonymous, 2010 *Annex I. 3.12.1.1*). In addition, no strong induction of Cyp1a isoform expression of the magnitude seen with AhR activators was observed in mouse liver microarrays (Anonymous, 2016 Annex I. 3.9.4.2).
Estrogenic stimulation	In the large mammalian toxicological database available for sedaxane, including the studies summarised in this document, as well as studies of the effects of sedaxane on reproduction and development, there is no evidence for estrogenic stimulation. In addition, sedaxane showed no estrogenic activity in a rat uterotrophic assay (Anonymous, 2014 Annex I. 3.9.4.15).
Statins	Sedaxane was not designed to inhibit HMG-CoA reductase. Also, the toxicology study database shows that cholesterol levels are mildly increased in rats by sedaxane treatment (not decreased) and there were no effects of sedaxane on cholesterol levels in 28 and 90-day studies in mice.
Cytotoxicity	Following administration to rats and mice, sedaxane did not produce elevations in markers of hepatocyte damage nor was there any evidence of cytotoxicity or regenerative proliferation (the proliferation noted following treatment with sedaxane was transient and not sustained).
Infection	Following administration to rats and mice, sedaxane did not produce any signs of hepatic infection, cytotoxicity or regenerative proliferation. The proliferation noted following treatment with sedaxane was transient and not sustained.
Iron/copper overload	Following administration to rats and mice, sedaxane did not produce elevations in markers of hepatocyte damage, or specific staining of tissues reflective of iron deposition, nor was there any evidence of cytotoxicity or regenerative proliferation.
Increased apoptosis	There was no consistent evidence that administration of sedaxane increased hepatic apoptosis in rats or in mice.

TABLE 21 Summary of Genotoxicity Studies with Sedaxane

Study	Dose Levels	Result
***In vitro* studies**		
Bacterial reverse mutation (Sokolowski, 2009)	3 – 5000 µg/plate	Negative
In vitro cytogenetics (Bohnenberger, 2009)	23.1 – 216.8 µg/mL	Negative
Mammalian cell gene mutation (mouse lymphoma) (Wollny, 2009)	6.9 – 110 µg/mL	Negative
***In vivo* studies**		
Mouse bone marrow micronucleus (Reichenbach, 2010)	2000 mg/kg	Negative
Unscheduled DNA synthesis – rat liver (Durward, 2009)	2000 mg/kg	Negative
Unscheduled DNA synthesis – rat liver (Hall, 2011)	1000 – 2000 mg/kg	Negative

DS comment:

The available data permitted to adequately rule out alternative MoAs (i.e., genotoxicity, peroxisome proliferation, AhR induction, cytotoxicity, estrogenic stimulation, statins, infections, iron/copper overload, increased apoptosis).

See annex I to the CLH Report especially 3.8 (genotoxicity studies), 3.9.4.2 and 3.9.4.4 (investigative 21-day with microarray in mouse and investigative 28-day in rat), 3.9.4.15 (uterotrophic assay) as well as all the toxicological database.

4.1.7 Uncertainties, inconsistencies and data gaps

The available data strongly support the proposed MOA for induction of rat and mouse liver tumours by sedaxane (Figure 1) and exclude the alternative MOAs described in Table 20. No critical uncertainties or inconsistencies have been identified.

4.2 Assessment of the Postulated Mode of Action

The concordance analyses presented in Section 4.1 have established that the proposed key events resulting in the induction of liver tumours in male rats and male mice are reproducible across a number of studies and exhibit strong dose- and temporal-concordance with the tumour endpoint. This is a well described MOA for the induction of liver effects including liver tumours in rats and mice, and the parameters essential for describing this MOA have been demonstrated experimentally for sedaxane. Therefore, there is a high level of confidence that the hypothesised MOA (Figure 1) was responsible for the higher incidences of liver tumours in male rats following dietary exposure to 3600 ppm sedaxane, and in male mice following exposure to 7000 ppm sedaxane.

DS comment:
DS is of the opinion that based on the available data, there is strong evidence that the postulated MoA (CAR activation) is the underlying MoA of liver tumours observed in rodent males.

4.3 Are the Key Events in the Animal Mode of Action Plausible in Humans?

Following establishment of a plausible MOA for the induction of liver tumours in mice, the next step is to assess the relevance to humans by assessing the qualitative and quantitative differences between the mouse and human for each of the key events. As described in a recent extension of the IPCS Mode of action Framework (Boobis *et al.*, 2006), the questions to be asked are:

- Can human relevance of the MOA be reasonably excluded on the basis of fundamental, qualitative differences in key events between experimental animals and humans?, and
- Can human relevance of the MOA be reasonably excluded on the basis of quantitative differences in either kinetic or dynamic factors between experimental animals and humans?

4.3.1 Qualitative differences in key events

As described in section 3.3.1 and Table 3, sedaxane was shown to be a direct activator of mouse, rat and human CAR, and of rat and human PXR *in vitro*. Therefore it can be concluded that the human is not qualitatively different to the mouse and rat with respect to the initial causal key event of CAR and/or PXR activation following sedaxane treatment, although quantitative differences were observed. The magnitude of the response in the CAR and PXR transactivation assays indicated that CAR (6.3-fold) is more responsive than PXR (3.1-fold) to sedaxane in rats, and that in mice, only CAR activation was observed (19-fold). The *in vivo* liver responses in mice and rats are concordant with CAR activation being the primary initiator of liver changes in rats and mice, with markers of Cyp2b activity being much greater than Cyp3a markers of activity in sedaxane-treated mice and rats (Tables 5 and 6). On this basis, the following discussion will focus primarily on the human response to CAR activation.

A number of studies have shown that CAR can be activated by compounds such as phenobarbital in the mouse, rat, Syrian hamster, primates, and humans resulting in altered gene expression, hypertrophy, and CYP2B enzyme induction (Diwan *et al.*, 1986; Elcombe *et al.*, 2014; Huang *et al.*, 2005; Olsen *et al.*, 1989; Weaver *et al.*, 1994; Yamamoto *et al.*, 2004). In contrast, while phenobarbital enhances cell proliferation and decreases apoptosis in the mouse and rat, other species appear to be refractory to the proliferative and anti-apoptotic responses. For example, phenobarbital has been reported not to stimulate DNA synthesis and not to inhibit apoptosis in cultured Syrian hamster and guinea pig hepatocytes (James and Roberts, 1996). In keeping with the lack of effect of phenobarbital on cell proliferation (Parzefall *et al.*, 1991) in the Syrian hamster, chronic phenobarbital treatment does not produce liver tumours in this species when given in the drinking water at 500 ppm (Diwan *et al.*, 1986).

Although phenobarbital can increase liver size in both rodents and humans (Aiges *et al.*, 1980), significant species differences in the mitogenic and anti-apoptotic properties of phenobarbital and related compounds have been demonstrated. In contrast to effects in cultured rodent hepatocytes, phenobarbital does not induce replicative DNA synthesis and does not inhibit apoptosis in human hepatocytes (Hasmall and Roberts, 1999; Hirose *et al.*, 2009). While phenobarbital can act as a non-genotoxic carcinogen and tumour promoter in the rat and mouse, it does not appear to produce liver

tumours in humans. A number of epidemiological studies have demonstrated that in human subjects receiving phenobarbital for many years at doses producing plasma concentrations similar to those that are carcinogenic in rodents, there is no evidence of increased liver tumour risk (Friedman *et al.*, 2009; IARC, 2001; Olsen *et al.*, 1989; Olsen *et al.*, 1995; Whysner *et al.*, 1996).

To explore the species differences in response to sedaxane, an *in vitro* investigative study using primary hepatocytes isolated from male Wistar rats was conducted to assess the effects of sedaxane on PROD and BROD activities and hepatocellular proliferation (Anonymous, 2016, *Annex I. 3.9.4.5*) and a similar experiment was conducted with isolated male human hepatocytes (Vardy, 2016b, *Annex I. 3.9.4.6*).
In these experiments, hepatocytes were exposed for 96 h to 6 concentrations of sedaxane, up to a maximum of 100 µM. A value of 100 µM was estimated to be the highest concentration that could be tested based on cytotoxicity that had been observed in prior *in vitro* studies with sedaxane. Testing up to the highest tolerable concentration allows for a robust assessment of the intrinsic potential of sedaxane to induce CYP2B/3A activity and hepatocellular proliferation. These experiments also included appropriate controls: sodium phenobarbital as a known inducer of PROD/BROD activities in both species and an inducer of cell proliferation in rat, and epidermal growth factor (EGF) as a known inducer of cell proliferation in both species.

Table 22 provides a summary of the data from the two studies and shows that treatment with sedaxane caused induction of CYP2B/3A activity (observed as an increase in PROD and BROD activity) and a proliferative response in the rat hepatocytes. In contrast, sedaxane did not produce a cell proliferation response in the human hepatocytes. It did cause an increase in BROD activity. Both control compounds gave the expected responses for both species, indicating the test systems were responding as expected.

TABLE 22 Results of Primary Hepatocyte Mode of Action Studies with Sedaxane

Species	**CYP induction[a]**	**Cell proliferation**	**Reference**
Rat Hepatocytes	↑ PROD activity and ↑ BROD activity	↑ S-phase labelling index	Anonymous, 2016, *Annex I. 3.9.4.5*
Human Hepatocytes	↑ BROD activity; no effect on PROD activity	None	Vardy, 2016, *Annex I. 3.9.4.6*

[a] Abbreviations:
pentoxyresorufin-O-dependentylation (PROD) is a mainly a marker for CYP2B activity.
benzyloxyresorufin-O-debenzylation (BROD) is mainly a marker for CYP2B/3A activity

Therefore, based on experimental data, human hepatocytes have been shown to be non-responsive to sedaxane regarding the causal key event of cell proliferation. This pattern of effects matches the known species differences that have been demonstrated for phenobarbital and other CAR activators, and the weight of evidence indicates that it represents a qualitative difference in the established MOA for sedaxane between rodents (rats and mice) and humans (Elcombe *et al.*, 2014). Therefore, it can be concluded that the tumourigenic MOA established for sedaxane in male rats and male mice is not operative in humans based on qualitative differences between rodents and humans in their response to sedaxane.

Therefore, the answer to the question "Can human relevance of the MOA be reasonably excluded on the basis of fundamental, qualitative differences in key events between experimental animals and humans?", is clearly "Yes" for sedaxane.

Considering that a qualitative difference has been established, the question of quantitative differences in key events between experimental animals and humans does not need to be reviewed in this assessment.

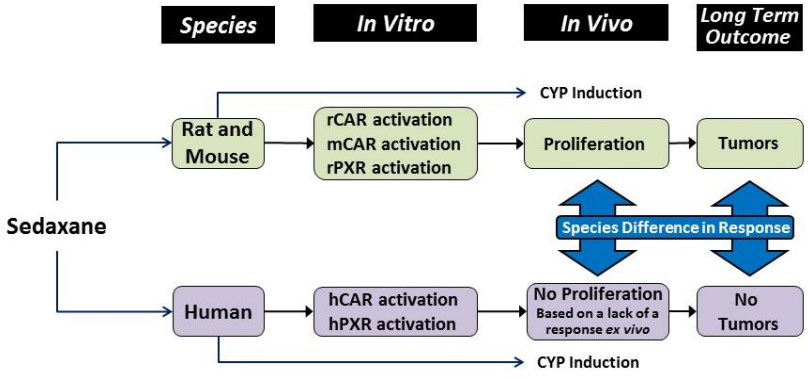
DS comment :
Similarly to phenobarbital (known CAR inducer), sedaxane did not induce DNA replication in human hepatocytes following induction of human CAR, in contrast to rat. Due to this qualitative difference, the liver tumours as a result of CAR-activation by sedaxane are considered to be of little relevance to humans.

5.0 CONCLUSIONS

The available data for sedaxane support the proposed MOA to account for the higher incidences of liver tumours in male rats (at 3600 ppm) and male mice (at 7000 ppm), The changes in the liver are attributable to activation of CAR (with a possible lesser activation of PXR in rats), which results in a series of well-documented downstream events, ultimately leading to a higher incidence of tumours vs. the concurrent controls, as described in section 3.2 and Figure 1. The available data also demonstrate that this MOA is not relevant to humans. Figure 9 summarises the interspecies differences in response to sedaxane.

Based on the overall weight of evidence including marginal increases in tumour incidence, observed tumour incidences within the range of what is known to occur spontaneously in the test strain and a demonstrated mode of action which has no relevance to humans, Syngenta considers that the marginally higher incidences of liver tumours in rats and mice have no relevance to hazard classification for sedaxane.

FIGURE 9 Summary of Interspecies Differences in Response to Sedaxane.



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DS assessment of the postulated mode of action underlying sedaxane-induced rodent liver tumours:

Based on the regulatory studies and the mechanistic studies generated, the applicant has proposed the following mode of action for sedaxane-induced rodent liver tumours:

Key events	Associative events
1-CAR/PXR activation	
2-Altered expression of CAR-responsive genes	
3-Altered expression of pro-proliferative genes/anti-apoptotic genes	**1-Increased expression of genes encoding cytochrome P450s (CYPs), particularly Cyp2b and Cyp3a families**
4-Transient increased hepatocellular proliferation and decreased apoptosis	**2-Hepatocellular hypertrophy**
5-Clonal expansion and development of altered hepatic foci	**3-Increased liver weight.**
Final adeverse outcome: liver tumour foramtion	

Assessment of the postulated mode of action:

Key event 1: The results from the *in vitro* CAR and PXR reporter assays support the fact that sedaxane activates CAR from rat, mouse and human and PXR from rat and human.

Associated event 1: In rat, the results from 28-day liver MOA study as well as the 28-day study with various ratios of the isomers of sedaxane support associative event 1 (increase in PROD activity from 500 ppm and in testosterone 16β-hydroxylase activity from 2000 ppm both markers of CYP 2b activity as well as increase in testosterone 6β-hydroxylase, a marker of CYP3a activity.
In mouse, combined data from RT-PCR and microarray analysis support Associative event 1 with increased expression of hepatic Cyp2b10 mRNA, Cyp2c65 mRNA, and PROD activity observed from at 1250ppm.

Key event 2 and Key event 3: The increase in Gadd45β mRNA from 7000 ppm and the increases in expression of xenobiotic metabolizing enzymes and other genes associated with CAR/PXR activation from 7000 ppm in the microarray are consistent with Key event 2 and Key event 3. No microarray assay is available in rat.

Associative event 2 and Associative event 3: increases in centrilobular hypertrophy and in liver weight were observed consistently in mechanistic data and regulatory studies in male rats. In male mice, while increased liver weight was observed throughout the toxicity studies, centrilobular hypertrophy was not noted in the 80-week mouse study.

<u>Key event 4:</u> In male rats, Sedaxane induced a transient increase in hepatocellular proliferation from 1200 ppm as measured by the BrdU labelling index. In male mice, as measured by Ki67 (statistically significant increase) and the BrdU label index (numerical increase), sedaxane induced a slight increase in hepatocellular proliferation from 7000 ppm at Day 8.

<u>Key event 5:</u> In the 2-year rat study, sedaxane led to increase in eosinophilic cell foci from 1200 ppm. However, a minor inconsistency in the database is the lack of an increase in eosinophilic foci in the 80-week mouse study. It is plausible that an increase in altered foci in the mouse liver may have preceded the development of tumours, but it could not be observed because no interim sacrifice is made in a Guideline mouse carcinogenicity study.

Throughout the database, a good dose-concordance and a temporal concordance between the causal key events, associative events and the apical outcome, (liver tumours) were observed in both male rats and male mice.
The available data permitted to adequately rule out alternative MoAs (i.e., genotoxicity, peroxisome proliferation, AhR induction, cytotoxicity, estrogenic stimulation, statins, infections, iron/copper overload, and increased apoptosis).

In summary DS is of the opinion the available data provide enough evidence to support the postulated MoA (CAR activation) to be the underlying MoA of liver tumours observed in rodent males.

Similarly to phenobarbital (known CAR inducer), sedaxane did not induce DNA replication (prerequisite for tumour formation) in human hepatocytes following induction of human CAR, in contrast to rat. Due to this qualitative difference, the liver tumours as a result of CAR-activation by sedaxane are considered to be of little relevance to humans.

17 APPENDIX 3: MODE OF ACTION AND HUMAN RELEVANCE ASSESSMENT OF THYROID FOLLICULAR CELL TUMOURS IN MALE RATS

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1.0 EXECUTIVE SUMMARY

Sedaxane is a fungicidal succinate dehydrogenase inhibitor. Following dietary administration to Han Wistar rats for two years, the high dose (3600 ppm) of sedaxane had a numerically higher incidence of thyroid follicular cell adenomas in male rats which did not achieve statistical significance.

Syngenta and the rapporteur member state (RMS), France,[maintain] *were of* [7] the view that the higher incidence of tumours is within the range of variable spontaneous tumour incidences for the test species and was not related to sedaxane treatment. However, the European Food Safety Authority (EFSA) concluded that the overall pattern of tumours in rats and mice suggests that a 'Carc cat 2, H351, suspected of causing cancer' classification regarding carcinogenicity would be required for sedaxane (EFSA, 2013). This opinion followed US EPA classification of sedaxane as "likely to be carcinogenic to humans" citing increased thyroid tumour incidences in male rats as weak evidence of a high-dose treatment-related effect (U.S. Environmental Protection Agency, 2011b). Based on that alternative view, a programme of work was initiated to investigate a hypothesised mode of action (MOA) using a weight of evidence framework developed by the International Programme on Chemical Safety (IPCS) and the International Life Science Institute (ILSI). The weight of evidence for the proposed MOA in rats is described in detail in this document, and the human relevance of the identified MOA is assessed via the IPCS/ILSI framework.

The available data for sedaxane provide a possible MOA for the higher incidence of thyroid follicular cell adenomas observed in male rats at 3600 ppm:

- Activation of hepatic CAR/PXR nuclear receptors and induction of hepatic UDP-glucuronosyltransferase (UDPGT), resulting in increased conjugation and excretion of triiodothyronine (T_3) and thyroxine (T_4)
- A decrease in serum T_3 and T_4 levels,
- A compensating increase in thyroid stimulating hormone (TSH) levels secreted via the hypothalamus-pituitary-thyroid (HPT) axis; and
- Under the chronic proliferative stimulus of TSH, thyroid follicular cells undergo hypertrophy and hyperplasia, and eventually progress to form follicular cell adenomas and/or carcinomas

The available data also demonstrate that a threshold exists for the induction of the key events and that this MOA is not relevant for humans due to the well-established qualitative and quantitative differences in response to UDPGT induction and increased T_3/T_4 clearance between rats and humans. In summary, the data support the conclusion that sedaxane does not pose a carcinogenic hazard to humans.

[7] Dossier submitter: this statement refers to Draft Assessment Report 2012 (see context)

2.0 INTRODUCTION TO HUMAN RELEVANCE ASSESSMENT

A framework for assessing and communicating the relevance of tumour findings in rodent studies to humans has been developed by the International Programme on Chemical Safety (IPCS) (Boobis *et al.*, 2006; Sonich-Mullin *et al.*, 2001) and the International Life Science Institute (ILSI) (Meek *et al.*, 2003). The framework aims to answer three questions:

(i) Has a mode-of-action (MOA) been established in the test species?
(ii) Based on qualitative assessment of the differences between species in terms of toxicokinetics and toxicodynamics, is that MOA plausible in humans?
(iii) Based on an assessment of the quantitative differences between species in terms of toxicokinetics and toxicodynamics, is the MOA plausible in humans?

Firstly, a MOA is established in the rodent using an approach developed by the IPCS/ILSI (Boobis *et al.*, 2006; Meek *et al.*, 2003; Sonich-Mullin *et al.*, 2001), which begins with a postulated theory of cause and the series of requisite and measureable events that are necessary for the induction of the toxicity. A recent workshop on nuclear receptor induced liver tumour MOAs has defined a number of types of events that may be useful in describing the MOA (Andersen *et al.*, 2014). A causal key event is an empirically observable precursor step to the adverse outcome that is itself a necessary element of the MOA. Such key events are required for a MOA, but often are not sufficient to induce the adverse outcome in the absence of other key events. Associative events are measurable biological processes that are not themselves necessary causal key events for the MOA, but are reliable indicators or markers for key events. As such, associative events can often be used as surrogates for a causal key event in a MOA. Finally, modulatory factors are biological features or responses that are not necessary to induce an adverse event but could modulate the dose-response or probability of inducing one or more key events or the adverse outcome. A body of experimental evidence is then developed and assessed to support the association between these key events and the apical endpoint. This assessment is made using "tests for causation" proposed by Bradford Hill (Hill, 1965) and involves answering a number of simple questions, namely:

- Are the dose and temporal relationships consistent with causality?
- Are the effects consistent and reproducible between studies?
- Could other causes have given rise to the key events?
- Are the effects biologically plausible given our current state of knowledge?

Only when a MOA has been established in an experimental species, can the human relevance assessment begin and, if, on the basis of experimental results, it can be shown that one or more of the necessary key events seen in the animal MOA is not plausible in humans (on either qualitative or quantitative grounds), then the adverse outcome in rodents is not appropriate for further consideration due to a lack of human relevance.

3.0 MODE OF ACTION HYPOTHESIS FOR THYROID TUMOURS IN MALE RATS

3.1 Proposed Mode of Action for Thyroid Tumours

3.1.1 Overview of thyroid carcinogenicity data for sedaxane

In a combined chronic toxicity and carcinogenicity study, Han Wistar rats (strain designation CrL:WI(Han)) were treated for up to 2 years with sedaxane at dietary inclusion levels of 0, 200, 1200 and 3600 ppm (Anonymous 2015 *Annex I. 3.9.4.1*) . The study authors noted that the incidences of thyroid follicular cell adenomas and carcinomas were not statistically significant and were within the wider range of Historic Control Data (HCD) from the RITA database. On this basis they concluded that there were no treatment-related increases in thyroid follicular cell tumours in this study. The study findings are summarised in Table 1.

The incidences in males at 200 ppm and 1200 ppm for thyroid follicular cell tumours were within the range of HCD from the performing laboratory and from RITA, and were therefore considered to reflect normal background variability. In males treated with 3600 ppm sedaxane, there was a higher incidence of thyroid follicular cell adenomas, compared to the control group, but these incidences were not statistically significant in a pairwise, 2-sided Fisher's Exact Test nor in the Peto Trend Test (2-sided), Compared to the HCD (Table 1), the incidence of follicular cell adenomas in male rats at 3600 ppm was just outside the range of values from the test laboratory, but within the range of the RITA database. There was no increase in the incidence of thyroid tumours in female rats.

Syngenta maintains the view that the higher incidence of tumours observed at 3600 ppm in rats is within the range of variable spontaneous tumour incidences for the test species and is not related to sedaxane treatment. Syngenta does not consider that there is robust evidence that sedaxane has carcinogenic potential at the doses tested, as the frequency of neoplastic findings in affected tissues were generally within historical control ranges, the doses at which neoplastic findings were identified exceeded the maximum tolerated dose, and no evidence of mutagenicity/genotoxicity were identified in the full suite of required genotoxicity studies.

In 2011, the Cancer Assessment Review Committee (CARC) of the US EPA evaluated the carcinogenic potential of sedaxane and concluded that sedaxane is "Likely to be Carcinogenic to Humans" with application of a linear low-dose extrapolation model (Q1*) for quantification of cancer risk to humans (U.S. Environmental Protection Agency, 2011b). Upon reconsideration of the toxicological assessment of sedaxane as requested by the European Commission, EFSA concluded that the overall pattern of tumours in rats (multiple sited) and mice (liver) suggests that a 'Carc cat 2, H351, suspected of causing cancer' classification regarding carcinogenicity would be required for sedaxane (EFSA, 2013). After these regulatory positions were published, Syngenta conducted a program of work to investigate the possible tumour incidences described by EPA and/or EFSA. Based on this work, uterine, thyroid, and liver tumour weight of evidence (WOE) assessments were prepared by Syngenta to evaluate the MOA and human relevance of each tumour type to support a Cancer Reclassification Decision by the US EPA for sedaxane. In combination with similar WOE documents that address the MOA and human relevance of uterine and liver tumours, this thyroid tumour WOE assessment is intended to support no cancer classification for sedaxane in the EU.

TABLE 1 Incidence of thyroid follicular cell adenoma and carcinoma in male Han Wistar rats at the conclusion of a 2 year carcinogenicity study and Historic Control Data

Thyroid follicular cells:	Dietary inclusion level of Sedaxane (ppm)			
	0	**200**	**1200**	**3600**
Adenoma	3/52 (6%)	3/52 (6%)	4/52 (8%)	8/52 (15%)
Carcinoma	0/52 (0%)	0/52 (0%)	2/52 (4%)	1/52 (2%)

No mean values were statistically-significantly different from control with $p<0.05$ (Fisher's Exact Test).

	Historic Control Data - Range	
	Lab (CRL)	RITA
Adenomas	2-11%	0-28%
Carcinomas	0-6%	0-6%

Lab (CRL) data refers to 5 prior or concurrent studies at CRL in 2002-2007.
RITA data refers to 41 studies conducted in the Wistar rat from 1997 – 2006. Registry of Industrial Toxicology Animal Data (RITA), http://reni.item.frauhofer.de/reni.

DS comment: In rat, although not statistically significant by pair-wise comparison there was a higher incidence of hepatocellular adenomas, thyroid follicular cell adenomas and thyroid follicular cell adenomas/carcinomas combined in males at 3600 ppm with statistically significant trend and exceeding the HCD range from the same laboratory.
The combined thyroid tumour increase was driven by the increased incidence of adenomas (no increase in malignant tumours).
As regard HCD from RITA database they are considered appropriate (not the same laboratory).
Therefore, the increased incidence of thyroid adenoma observed at high dose level both in rat males (3600 ppm), could not be ruled out as unrelated to treatment.

See annex I to the CLH Report 3.9.1.1.

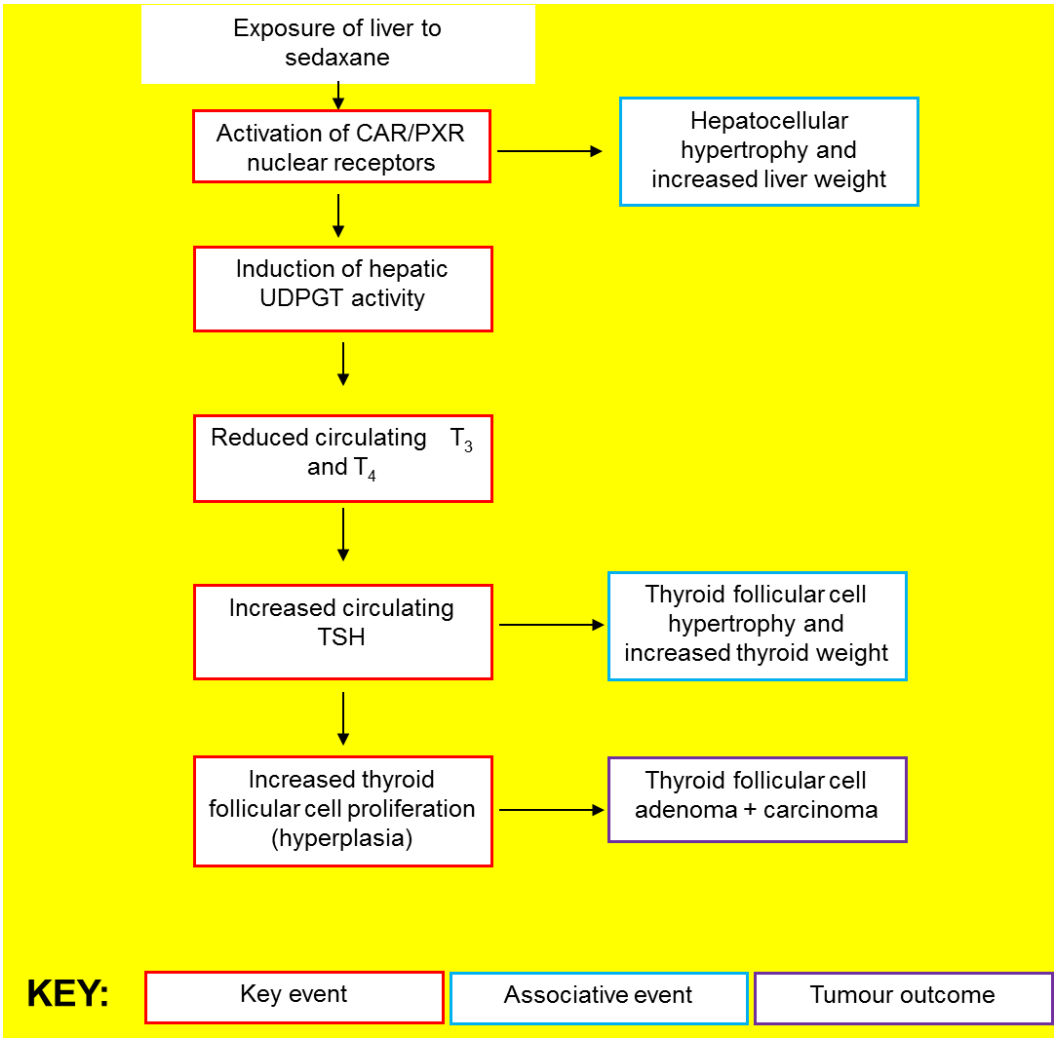
There was no evidence for a treatment-related effect on the incidence of thyroid tumours in an 80 week study in CD-1 mice (Anonymous 2015 Annex I. 3.9.4.2). In this study, male and female CD-1 mice were treated with sedaxane at dietary inclusion levels of 0, 200, 1250 and 7000 ppm. The no observed adverse effect level (NOAEL) for this study was 1250 ppm, based on a decrease in body weight and body weight gain in males and females, and a decrease in food utilization during the early stages of the study.

3.1.2 Statement of mode of action hypothesis

A proposed MOA for thyroid follicular cell tumours is described in Figure 1 and involves a number of key events and associative events. Treatment of male Han Wistar rats with sedaxane results in the activation of the constitutive androstane receptor (CAR) and pregnane X receptor (PXR) in the liver, which causes induction of hepatic UDP-glucuronosyltransferase (UDPGT). UDPGT is a phase II liver enzyme that catalyses the glucuronidation of endogenous circulating thyroxine (T_4). These glucuronide conjugates are readily excreted via the bile, and so induction of hepatic UDPGT results in lower circulating thyroxine (T_4) and/or triiodothreonine (T_3) due to their increased metabolic and

biliary clearance. The reduced concentration of circulating T_3/T_4 is detected in the hypothalamus. In order to maintain homeostasis, the secretion of thyrotropin-releasing hormone [TRH] (by the hypothalamus) is triggered and consequently increases the secretion of thyroid stimulating hormone (TSH) by the pituitary. Increased circulating TSH results in thyroid follicular cell hypertrophy and proliferation (resulting in increased thyroid weight) in order to increase the capacity for production of T_3/T_4 by the thyroid and thereby return circulating T_3 and T_4 to basal levels. Increased circulating TSH and a persistent proliferative stimulation of thyroid follicular cells leads to hyperplasia of follicular cells, and eventually results in the formation of thyroid follicular cell adenomas + carcinomas. This MOA can be described as a perturbation of the hypothalamus-pituitary-thyroid (HPT) axis as a secondary consequence of liver xenobiotic metabolising enzyme induction and has been well characterized and described for a number of compounds (Hurley, 1998; McClain, 1995).

FIGURE 10 Mode of action hypothesis for induction of thyroid follicular cell tumours in male Han Wistar rats by sedaxane



3.1.3 CAR and PXR activation assays

Sedaxane was evaluated in an *in vitro* CAR3 reporter assay for its ability to activate CAR from rat, mouse and human, by a method that has previously been shown to detect known species-specific activators of this nuclear receptor (Omiecinski *et al.*, 2011). In addition, sedaxane was evaluated in PXR reporter assays (rat, mouse and human). In each assay, model compounds that are known to activate the specific CAR or PXR receptors were also tested to confirm the performance of the assays.

Results for sedaxane in rat CAR and rat PXR reporter assays are shown in Table 2. Sedaxane produced statistically significant increases in rat CAR activation at 10 and 30 µM, with a maximum increase of 6.3-fold vs. control at 30 µM. Sedaxane also was classified as a possible rat PXR activator, with a statistically significant maximal increase of 3.1-fold vs. control at 30 µM. The model activators clotrimazole (for rat CAR) and pregnenolone-16α-carbonitrile (for rat PXR) produced large fold-changes in activation of the respective reporter constructs, demonstrating that the assays responded as expected.

Sedaxane was also shown to activate mouse CAR (maximum 19-fold increase) and human CAR (maximum 4-fold increase) at concentrations up to 30 µM. Sedaxane did not activate mouse PXR, but it did activate human PXR (maximum 4-fold increase) at concentrations up to 30 µM.

The CAR and PXR reporter gene assay data demonstrate that sedaxane has the intrinsic properties to interact with and activate the CAR receptor. The *in vivo* responses in mice and rats are concordant with CAR activation being the primary initiator of liver changes in those species, with markers of Cyp2b expression and activity being much greater than those of Cyp3a in sedaxane-treated mice and rats. Taken together, these data provide compelling evidence for the initial key event of CAR activation following sedaxane treatment.

TABLE 2 Results of transactivation assays for rat CAR and rat PXR with sedaxane (fold change vs. control)

	rCAR		rPXR	
Concentration (µM)	Sedaxane	Model Activator: Clotrimazole (10 µM)	Sedaxane	Model Activator: Pregnenolone-16α-carbonitrile (20 µM)
1	1.1	---	1.3	---
3	1.3	---	1.5*	---
10	2.7*	95.4*	1.7*	---
30	6.3*	---	3.1*	79.1*

*P<0.01, Dunnett's test.
--- not tested at this concentration, or data not shown for simplicity (rPXR)
Data from Omiecinski (2014) *Annex I. 3.9.4.8* and Toyokawa and Sherf (2014) *Annex I. 3.9.4.9*

DS comment: <u>Key event 1 "CAR/PXR activation"</u>
The results from the *in vitro* CAR and PXR reporter assays support the fact that sedaxane activates CAR from rat, mouse and human and PXR from rat and human.

See annex I to the CLH Report 3.9.4.8 and 3.9.4.9.

3.1.4 28 Day mode of action study in male Han Wistar rats

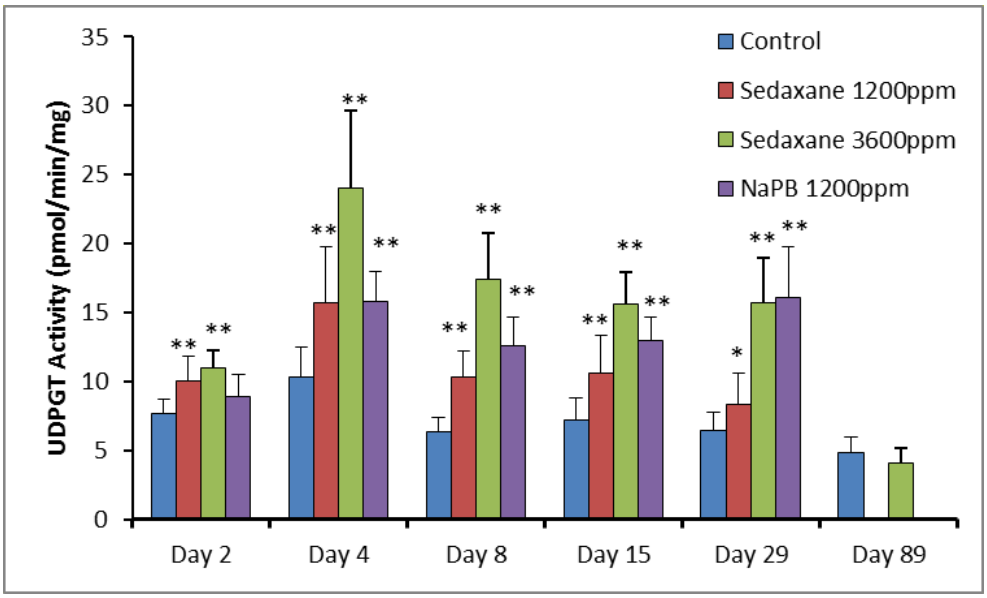
Male Han Wistar rats (strain designation CrL:WI(Han); 15/group/time point) were treated with sedaxane at dietary inclusion levels of 0 ppm (control), 1200 ppm (mid dose in the 2 year carcinogenicity study), and 3600 ppm (highest dose in the 2 year carcinogenicity study and the only dose where an increased incidence of thyroid follicular cell adenoma wasobserved). Rats were dosed for 1, 3, 7, 14 or 28 days before termination (Study Days 2, 4, 8, 15 and 29), and a number of liver- and thyroid-related parameters were measured (Anonymous, 2015 *Annex I. 3.9.4.4)*. The reversibility of sedaxane treatment-related effects was assessed by including additional groups of animals treated with either 0 ppm or 3600 ppm sedaxane for 28 days, followed by a 60 day recovery period prior to termination (on Study Day 89). The duration of the recovery period was based on 5 times the estimated liver half-life of 12 days, which was determined in a repeated-dose ^{14}C-sedaxane tissue depletion study in male Wistar rats (Shaw, 2009). In addition to treatment with sedaxane, further animals were treated with 1200 ppm phenobarbital sodium salt (NaPB). NaPB was included as a positive control as it is a known promotor of thyroid follicular cell adenomas in the rat by a MOA similar to that proposed for sedaxane (Meek *et al.*, 2003). The study design was as follows:

Group Number	Treatment	Number of Male Rats					
		Termination Timepoints (Study Day)					
		2	4	8	15	29	89 [R]
1	Control: Basal (untreated) Diet	n=15	n=15	n=15	n=15	n=15	n=15
2	Sedaxane: 1200 ppm in Diet	n=15	n=15	n=15	n=15	n=15	-
3	Sedaxane: 3600 ppm in Diet	n=15	n=15	n=15	n=15	n=15	n=15
4	Positive Control: 1200 ppm sodium phenobarbital in Diet	n=15	n=15	n=15	n=15	n=15	-

[R] Following 28 days of treatment, 15 animals from the control (Group 1) and 3600 ppm sedaxane (Group 3) groups were retained off-dose for a further 60 days to assess the reversibility of the effects of treatment.

The data from this study are summarised in Figure 2 (UDPGT activity in liver), Table 3 (liver-related parameters), Table 4 (thyroid-related parameters), Figure 3 (thyroid hormones), and Table 5 (TSH).

FIGURE 2 UDP-glucuronosyltransferase activity in the liver following sedaxane or NaPB treatment



UDPGT activity was assessed toward thyroxine (T4) as a substrate. Similar responses were observed when UDPGT activity was expressed per gram of liver, per total liver and per relative liver weight.
*, **: Statistically-significantly different from control with $p<0.05$ and $p<0.01$, respectively. Data from Anonymous, 2015 Annex I. 3.9.4.4.

TABLE 3 Summary of data from 28-day mode of action study with sedaxane – Liver-related parameters

	Sedaxane			NaPB
	0 ppm	**1200 ppm**	**3600 ppm**	**1200 ppm (0 ppm)[a]**
PROD Activity (pmol/min/mg protein)				
Day 8	15	486**	1035**	1569**
Liver wt. adjusted for body weight (g)				
Day 2	8.62	8.87	8.61	9.18 (8.97)
Day 4	9.62	10.52**	11.70**	11.98** (10.08)
Day 8	9.46	11.28**	13.02**	12.55** (10.01)
Day 15	10.43	12.06**	13.86**	14.02** (10.72)
Day 29	10.15	11.48**	13.53**	14.17** (10.86)
Day 29 (+60)	11.56	NA	11.49	NA
Liver: Centrilobular hypertrophy (N)	(15)	(15)	(15)	(15)
Day 2	0	0	0	0
Day 4	0	7** (minimal)	12** (minimal)	15** (minimal – slight)
Day 8	0	6* (minimal)	10** (minimal – slight)	13** (minimal – slight)
Day 15	0	11** (minimal – slight)	13** (minimal – slight)	15** (slight – moderate)
Day 29	0	12** (minimal – slight)	14** (minimal – moderate)	15** (minimal – moderate)
Day 29 (+60)	0	NA	0	NA

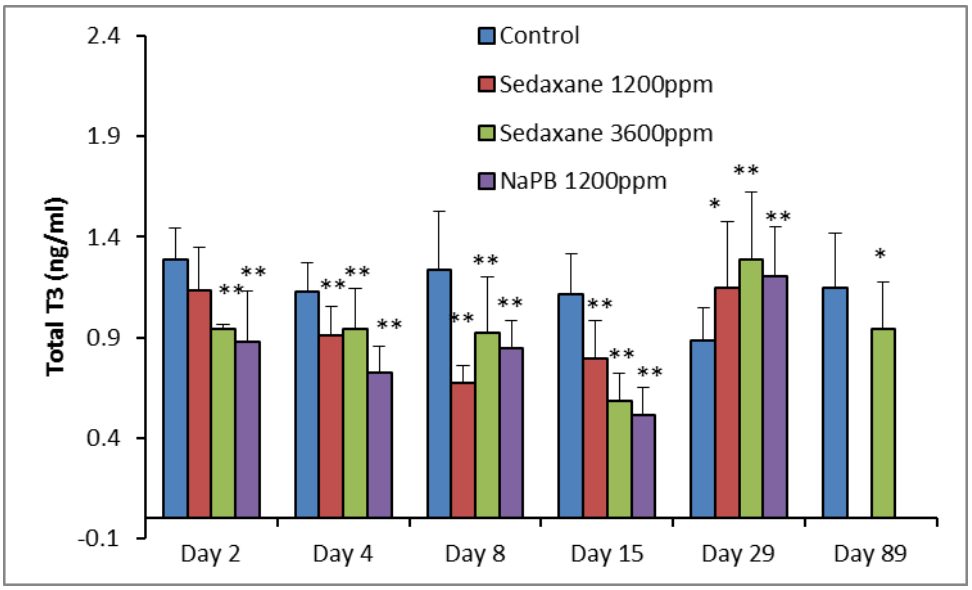
[a]For NaPB groups, adjustment of liver weights for body weight generated a different adjusted control group mean value (shown in parentheses).
[b]For liver centrilobular hypertrophy, range of severities observed are shown in parenthesis.
NA – Not applicable
*, ** Statistically-significantly different from control with $p<0.05$ and $p<0.01$, respectively.
Data from Anonymous, 2015 *Annex I. 3.9.4.4.*

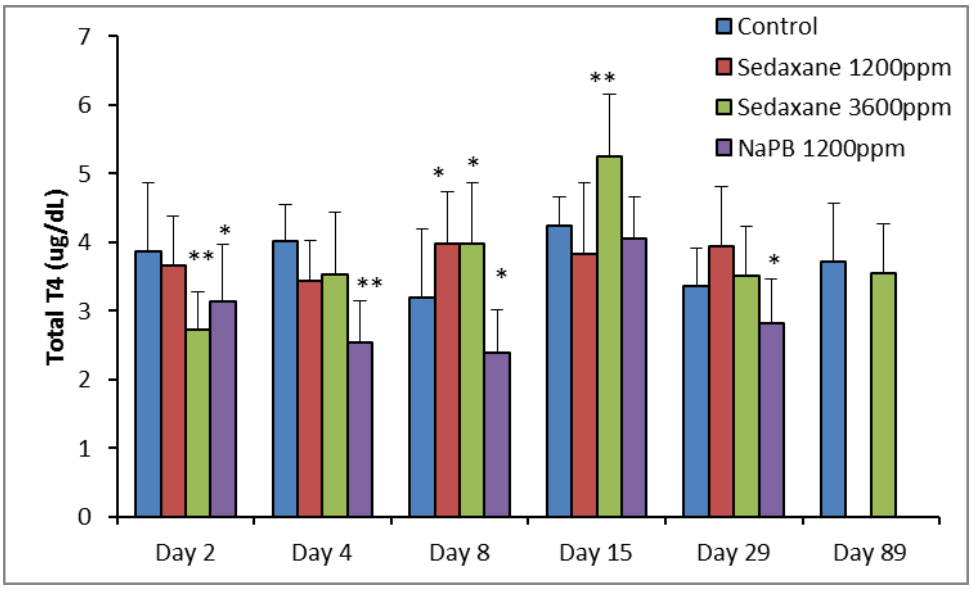
TABLE 4 Summary of data from 28-day mode of action study with sedaxane – Thyroid-related parameters

	Sedaxane			NaPB
	0 ppm	**1200 ppm**	**3600 ppm**	**1200 ppm (0 ppm)[a]**
Thyroid wt. adjusted for body weight (g)				
Day 2	0.014	0.014	0.015	0.016* (0.014)
Day 4	0.018	0.017	0.019	0.018 (0.018)
Day 8	0.015	0.014	0.015	0.016 (0.016)
Day 15	0.026	0.026	0.026	0.029** (0.026)
Day 29	0.014	0.018**	0.018**	0.025** (0.015)
Day 29 (+60)	0.021	NA	0.020	NA
Thyroid: Follicular cell hypertrophy (N) [b]	(15)	(15)	(15)	(15)
Day 2	0	0	0	0
Day 4	0	0	0	0
Day 8	0	0	0	0
Day 15	0	1 (minimal)	2 (minimal)	8** (minimal – slight)
Day 29	0	1 (minimal)	4 (minimal – slight)	14** (minimal – moderate)
Day 29 (+60)	0	NA	0	NA

[a]For NaPB groups, adjustment of thyroid weights for body weight generated a different adjusted control group mean value (shown in parentheses).
[b]For thyroid follicular cell hypertrophy, range of severities observed are shown in parenthesis. In the study report (Anonymous, 2015 *Annex I. 3.9.4.4*) , the terminology "epithelial hypertrophy" was used, which is synonymous with the term "follicular cell hypertrophy".
NA – Not applicable
*, ** Statistically-significantly different from control with $p<0.05$ and $p<0.01$ respectively. Data from Anonymous, 2015 *Annex I. 3.9.4.4*.

FIGURE 3 Summary of data from 28-day mode of action study with sedaxane – Thyroid Hormones (Total T_3 and Total T_4)





*, ** Statistically-significantly different from control with $p<0.05$ and $p<0.01$ respectively. Data from Anonymous, 2015 *Annex I. 3.9.4.4*.

TABLE 5 Time Course of Changes in TSH Levels and T3 and T4 Levels in Sedaxane-Treated or NaPB-Treated Rats (Percent of Control)

Sedaxane-Treated:

	Percent of Control Value					
Total T3:	Day 2	Day 4	Day 8	Day 15	Day 29	Day 89
Sedaxane 1200ppm	**88%**	**81%**	**54%**	**71%**	130%	
Sedaxane 3600ppm	**73%**	**84%**	**75%**	**52%**	146%	82%
Total T4:						
Sedaxane 1200ppm	**95%**	**85%**	125%	90%	117%	
Sedaxane 3600ppm	**71%**	**88%**	125%	124%	105%	96%
TSH:						
Sedaxane 1200ppm	148%[a]	89%	145%	**195%**	**123%**	
Sedaxane 3600ppm	111%	73%	102%	**126%**	**134%**	128%

NaPB-Treated:

	Percent of Control Value				
Total T3:	Day 2	Day 4	Day 8	Day 15	Day 29
NaPB 1200ppm	**68%**	**64%**	**69%**	**46%**	136%
Total T4:					
NaPB 1200ppm	**81%**	**63%**	**75%**	96%	84%
TSH:					
NaPB 1200ppm	112%	105%	**172%**	**314%**	**220%**

[a] TSH value statistically-significantly different from control with $p<0.05$ for the 1200 ppm sedaxane group on Day 2, but not considered a treatment effect due to lack of a dose response. Data from Anonymous, 2015 *Annex I. 3.9.4.4.*

Values in **Bold font** are considered treatment-related effects on T3, T4 and TSH, based on the difference from control, the dose-response pattern, and comparisons to the effects with NaPB (the positive control). TSH levels for NaPB were statistically significantly greater than control at Day 15 and Day 29. See Figure 3 for statistical evaluation of total T3 and total T4.

TSH values were via Radioimmunoassay (RIA) for TSH. Preliminary values for TSH obtained by ELISA were largely outside the range of the standard curve, and therefore were repeated (with remaining serum samples) by RIA to confirm the data.

The data from this MOA study show that treatment with sedaxane results in dose- and time-related effects on the proposed causal and associative key events that are consistent with the hypothesized MOA. Consistent with its activity as a CAR/PXR activator, sedaxane caused dose-dependent increases in pentoxyresorufin O-dependentylase (PROD) activity, liver weight and hepatocellular hypertrophy beginning on Day 4 of treatment. PROD was only measured on Day 8 samples, but would be assumed to have increased at each time point when liver weights were increased; PROD is a marker of Cyp2b enzyme activity, which is induced by CAR and PXR nuclear receptor activation. Related to effects on the thyroid, administration of sedaxane at concentrations of ≥1200 ppm resulted in increased hepatic microsomal UDP-glucuronosyltransferase activity towards thyroxine as substrate from Day 2 – Day 29, dose dependent increases in thyroid follicular cell hypertrophy from Day 15 – Day 29, and elevated thyroid weights on Day 29 of treatment.

Reductions in total T3 were observed on Days 2, 4, 8 and 15 of treatment, and decreased total T4 was observed on Days 2 and 4 of treatment (Figure 3). The mean values for total T4 were statistically significantly lower than the control group on Day 2 (3600 ppm sedaxane), and mean values on Day 4 were numerically lower than control (1200 and 3600 ppm) with some individual animal values noticeably lower than the range of control values. TSH levels were numerically higher than control on Days 15 and 29 of treatment, which matched the time points when the effect of NaPB on TSH was maximal. These differences with TSH in the sedaxane groups were not statistically significant at the time intervals that were assessed in this study. However, as shown in Table 5, the increase in TSH (as % of control values) was maximal for the 1200 ppm and 3600 ppm sedaxane groups at the same time (Day 15 – 29) that the total T3 values returned to control levels and/or were slightly higher than the control means. This time course of changes was also the same for NaPB, and indicates the known biological sequence of events where higher TSH levels produce a stimulation of the thyroid to achieve a normalization of T3 and T4 levels (Hurley, 1998; McClain, 1995).

The stimulation of the thyroid by TSH in sedaxane-treated groups also produced slight increases in thyroid weights and thyroid follicular hypertrophy beginning on Day 15 (Table 4). The magnitude of these responses in the sedaxane-treated groups are consistent with the marginal increase in thyroid tumour incidence and the temporal relationship of effects between the liver and the thyroid are consistent with the effects on the thyroid being secondary to and driven by the increase in liver metabolic capacity.

Sodium phenobarbital at 1200 ppm in the diet also increased hepatic microsomal UDP-glucuronosyltransferase activity towards thyroxine as substrate from Day 2 – Day 29 of treatment. In accord with this elevated T4 conjugation activity, reductions in total T3 and total T4 levels were observed at the majority of the time points assessed, and elevations in TSH were observed on Days 8, 15 and 29 of treatment. Sodium phenobarbital produced elevated thyroid weights on Days 15 and 29 of treatment and dose and time dependent increases in thyroid follicular cell hypertrophy at these same time intervals. The results with NaPB in this study are consistent with the expected set of responses and therefore demonstrate the sensitivity of the test system.

The spectrum of hepatic and thyroid effects observed with 3600 ppm sedaxane treatment was reversible upon treatment cessation, following a 60-day period where control diet was received (Day 89). Minor differences in a very limited set of parameters (e.g., lower total T3, with no difference in total T4 or TSH) are considered a reflection of normal variability in this measurement and not an effect of treatment at the end of the recovery period.

DS comment:
Key event 2 "Induction of hepatic UGT"
In the 28-day rat mechanistic study, sedaxane induced increased hepatic UGT activity at 1200 and 3600 ppm in line with Key event 2.

Associative event 1 "UGT Hepatocellular hypertrophy and increased liver weight"
In the 28-day rat mechanistic study, sedaxane induced increased liver weight and hepatocellular hypertrophy at 1200 and 3600 ppm in line with associative event 1.

Key event 3 "Reduced circulating T3 and T4":
In the 28-day rat mechanistic study, total T3 showed a statistically significant decrease in one or both Sedaxane treatment groups on Days 2, 4, 8 and 15. However total T4 was statistically significantly decreased by treatment with Sedaxane only at Day 2.

Key event 4 "Increased circulating TSH":
In the 28-day rat mechanistic study, a clear increase of circulating TSH was not observed after sedaxane treatment.

Key events 3 and 4 are therefore weakly supported by experimental data.

Associative event 2 "Thyroid follicular cell hypertrophy and increased thyroid weight":
In the 28-day rat mechanistic study, sedaxane induced increased thyroid weight from 1200 ppm and thyroid follicular cell hypertrophy at 3600 ppm in line with associative event 2.

See annex I to the CLH Report 3.9.4.4.

3.1.5 90 Day study in Han Wistar rats

Male and female Han Wistar rats (strain designation CrL:WI(Han)) were treated with sedaxane at dietary inclusion levels of 0, 300, 2000 and 4000 ppm following the OECD 408 test guideline (Anonymous, 2009 *Annex I. 3.12.1.2*). This study was conducted in the same laboratory and strain of rat as the 2-year rat study with sedaxane. Only a limited number of parameters associated with the proposed MOA (liver and thyroid weights and histopathology) were assessed in this study. These data for the male rats are summarised in Table 6.

Liver weights adjusted for body weight were statistically significantly higher than control values for 2000 and 4000 ppm males. Thyroid weights showed statistical significance at 300 ppm and 4000 ppm. However, the mean value at 2000 ppm was virtually identical to the control values. In the absence of a dose-related response and considering that all mean values were within the historical control range for both absolute weight and relative weight, these differences in thyroid weights reflect normal variability in a relatively small organ weight, and they do not represent a treatment related effect.

Histopathology findings in the liver including centrilobular hypertrophy and hepatocyte pigment were only increased in the 4000 ppm males. Thyroid histopathology findings consisted of follicular cell hypertrophy (minimal to mild) and were only observed at 4000 ppm.

TABLE 6 Summary of data from a 90 day study with sedaxane – Liver- and thyroid-related parameters from male rats only

	0 ppm	**300 ppm**	**2000 ppm**	**4000 ppm**
Organ Weights:				
Liver wt. adjusted for body weight (g)	15.38	15.71	17.73**	20.94**
Thyroid wt. adjusted for body weight (g)	0.0259	0.0177**	0.0261	0.0210**
Histopathology: (N)	(10)	(10)	(10)	(10)
Liver:				
Centrilobular hypertrophy (mild - moderate)	0	0	0	10***
Hepatocyte pigment (minimal - mild)	0	1	0	4
Thyroid:				
Follicular cell hypertrophy (minimal – moderate)	0	0	0	5*

*, ** and ***: Statistically-significantly different from control with $p<0.05$, $p<0.01$ and $p<0.001$, respectively.
Data from Anonymous, 2009 *Annex I. 3.12.1.2*

RMS comment: In the 90-day rat study, sedaxane induced increased liver weight from 2000 ppm, centrilobular hypertrophy at 4000 ppm, and follicular cell hypertrophy at 4000 ppm in line with associative events 1 & 2.

See annex I to the CLH Report 3.12.1.2.

3.1.6 Additional subchronic studies in a different strain of rat

A 28-day study and a 90-day study with sedaxane or its isomers were conducted in a different strain of Han Wistar rats (designation: HsdRccHan:WIST). These studies were also conducted in a different laboratory (Syngenta Central Toxicology Laboratory, UK) than the 2-year rat study with sedaxane. In both of these studies, liver weight increases were observed at dose levels of ≥1000 ppm in the diet, and microscopic findings of centrilobular hypertrophy and increased pigmentation in the liver were observed at dose levels of ≥2000 ppm in the diet in male rats. However, no treatment-related changes in thyroid weights or microscopic findings in the thyroid were observed in either of these studies. In addition, no effects on serum levels of thyroid hormones or TSH were observed at the end of the 28-day study (Anonymos, 2010 *Annex I. 3.12.1.1*).
Based on the results of these studies, the HsdRccHan:WIST rat strain supplied by Harlan Laboratories did not demonstrate the thyroid changes that were seen in the CrL:WI(Han) supplied by Charles River Laboratories. However, both strains showed similar responses in the liver after dietary treatment with sedaxane.

RMS comment: In a 28-day with sedaxane isomers, the trans isomer, the cis isomer and the 1:1 mix of the isomers induced increased liver weights from 2000 ppm and centrilobular

hypertrophy from 5000 ppm in line with associative events 1. However, no effects on thyroid weight, histopathology or thyroid hormones levels were observed.

See annex I to the CLH Report 3.12.1.1.

3.1.7 Non-neoplastic findings in a combined chronic toxicity and carcinogenicity study in Han Wistar rats

In addition to the tumour incidence data described in Section 2.1.1, relevant toxicity data in the liver and thyroid generated in the combined chronic toxicity and carcinogenicity study in rats is presented in Tables 7 and 8. After 52 weeks, dose-responsive increases in liver weights adjusted for body weight were observed at ≥1200 ppm, whereas the liver histopathology findings of centrilobular hypertrophy and hepatocyte pigment occurred only at 3600 ppm in male rats. Thyroid weights were not measured, but the thyroid histopathology finding of follicular cell hypertrophy was increased at ≥1200 ppm (similar to the findings in a 28-day and a 90-day study)

In the carcinogenicity phase of the study, terminal sacrifice animals at 104 weeks (plus early decedents for histopathology assessments) displayed increased liver weights and increased hepatocellular hypertrophy in males at ≥1200 ppm. In the thyroid, follicular cell hyperplasia was increased only at the high dose (3600 ppm) after 104 weeks; this response was considered an indicator of increased cell proliferation, and is a causal key event in the MOA. Colloid basophilia was also increased in the 3600 ppm males at 104 weeks. The other reported histopathology incidences in the male thyroid shown in Table 8 were either marginally different from controls at the higher dose levels, or represented a decrease in incidence at 3600 ppm and were thus of limited biological relevance.

TABLE 7 Summary of data from 52-week interim sacrifice in 2-year rat study with sedaxane – Non-neoplastic liver- and thyroid-related parameters from male rats only

		0 ppm	200 ppm	1200 ppm	3600 ppm
Organ Weights:	**Week**				
Liver wt. adjusted for body weight (g)	52	16.10	16.03	18.94**	22.63**
Histopathology: (N)		(12)	(12)	(12)	(12)
Liver:					
Centrilobular hypertrophy	52	0	0	0	11***
Hepatocyte pigment	52	0	1	0	7**
Thyroid:					
Follicular cell hypertrophy	52	0	0	5*, +	4+

*, ** and ***: Statistically-significantly different from control with p<0.05, p<0.01 and p<0.001, respectively. (Dunnett's test or Fisher's Exact Test).
+p<0.05, Mann-Whitney U-test. Data from Anonymous 2015 *Annex I. 3.9.4.1*.

TABLE 8 Summary of data from 104-week sacrifice + decedents in 2-year rat study with sedaxane – Non-neoplastic liver- and thyroid-related parameters from male rats only

		0 ppm	200 ppm	1200 ppm	3600 ppm
Organ Weights:	**Week**				
Liver wt. adjusted for body weight (g)	104	18.10	18.06	20.21**	24.20**
Histopathology: (N)		(52)	(52)	(52)	(52)
Liver:					
Centrilobular hypertrophy	104	0	0	8**	16***
Hepatocyte pigment	104	0	1	0	1
Thyroid:					
Desquamation, epithelial follicular	104	7	8	11	16
Basophilia, colloid	104	7	9	12	16+
Diffuse C-cell hyperplasia	104	27	27	24	10***
Focal follicular cell hyperplasia	104	7	8	8	16+

*, ** and ***: Statistically-significantly different from control with p<0.05, p<0.01 and p<0.001, respectively. (Dunnett's test or Fisher's Exact Test).
+p<0.05, Mann-Whitney U-test. Data from Anonymous, 2015 *Annex I. 3.9.4.1*.

DS comment:
In the 2-year rat study, sedaxane induced increased liver weight, liver and thyroid hypertrophy from 1200 ppm in line with associative events 1 & 2. Thyroid follicular cell was also observed at 3600 ppm in line with Key event 5.

See annex I to the CLH Report 3.12.1.2

4.0 IPCS/ILSI FRAMEWORK FOR THE EVALUATION OF THE HUMAN HEALTH RELEVANCE OF A HYPOTHESISED MODE OF ACTION

4.1 Has the Mode of Action Been Established in the Animal Model(s)?

The Hill (1965) criteria require that, for the key events to be causally related to the formation of tumours, they must:

- Be supported by data showing strength, consistency and specificity of association of key events and tumour response,
- Show dose-concordance of key events and dose levels that produce tumours,
- Occur in a logical temporal sequence,
- Be reproducible,
- Demonstrate that alternative MOAs have been considered and are not operative, and
- Be plausible and consistent with the current state of knowledge of the relevant biological processes.

4.1.1 Dose-concordance of key events

From consideration of the incidence of thyroid follicular cell adenomas and carcinomas in male Han Wistar rats (Table 1), 200 and 1200 ppm can be considered non-tumourigenic doses. At 3600 ppm the incidence of thyroid adenomas was numerically higher than concurrent controls but not statistically significant using Fisher's Exact Test and was within the range of spontaneous tumour incidence for the test strain as indicated by the RITA data base. In the studies described in this Assessment, data was generated at a number of different dose levels from 200 ppm to 4000 ppm. Table 9 describes the key events observed at each dose level.

TABLE 9 Summary of Dose-Concordance of Associative Events and (Causal) Key Events

Dietary inclusion level of Sedaxane (ppm) [a]	CAR/PXR activation (Causal)	Hepatic UDPGT induction (Causal)	Increased hepatocellular hypertrophy and/or liver weight (Associative)	Reduced circulating total T_3/T_4 (Causal)	Increased circulating TSH (Causal)	Increased thyroid follicular cell hypertrophy (Associative)	Increased thyroid weight (Associative)	Increased thyroid follicular cell proliferation and hyperplasia (Causal)	Higher Incidence of Combined Thyroid Adenomas (Outcome)
200 (300) [a]	No data	No data	No	No data	No data	No	No	No	No
1200	Yes[b]	Yes	Yes	Yes	Yes[c]	Yes	Yes (28 days)	No	No
(2000) [a]	Yes[d]	Yes[d]	Yes	Yes[d]	Yes[c,d]	Yes	No (90 days)	No data	No data
3600 (4000) [a]	Yes[b]	Yes	Yes	Yes	Yes[c]	Yes	Yes (28 days)	Yes	Yes

[a] Values in parentheses are subchronic dose levels (that are similar to the chronic dose levels, or in between the chronic dose levels).
[b]CAR/PXR activation was demonstrated based on *in vitro* studies, and by increased PROD activity (a marker of Cyp2b activity) in the livers of rats on Day 8 of the 28-day MOA study at the indicated dose levels (Anonymous, 2015 *Annex I. 3.9.4.4)*.
[c]TSH levels were numerically higher than the control values on Days 15 and 29, the same time points that NaPB produced maximal increases in TSH, and correlated with a return of T3 and T4 levels to the same as (or slightly above) control levels.
[d]Assumed response (not evaluated at 2000 ppm), based on responses at higher and lower dose levels.

Overall, there is good dose concordance of the proposed key events. The initial causal key event of CAR/PXR activation was assessed based on *in vitro* reporter assays and the surrogate measure of PROD activity in the liver. PROD activity is a marker of Cyp2b induction, an isoform that is characteristic of CAR and/or PXR activation, and this activity was increased in a dose-responsive manner at 1200 and 3600 ppm sedaxane. The large increase in PROD activity (e.g. 69-fold at 3600 ppm sedaxane) is very characteristic of CAR activators, including NaPB. Similarly, the causal key event of UDPGT induction (with thyroxine as substrate) was also increased in a dose-responsive manner at both 1200 and 3600 ppm (Figure 2). As a consequence of this UDPGT induction, increased clearance of T4 led to decreased T3 and T4 levels at 1200 and 3600 ppm at early time points. Later (Days 15-29), a marginal increase in TSH was observed at these same dose levels. While all of these early effects could be demonstrated in a dose-responsive manner at both 1200 ppm and 3600 ppm, the later key event of hyperplasia was only observed at 3600 ppm. The incidence of thyroid adenomas at 3600 ppm, although numerically higher, was not statistically significant using Fisher's Exact Test or the Peto Trend test.

For the associative events, the expected increases in liver centrilobular hypertrophy and weight were observed in a dose-responsive manner consistent with the proposed MOA. These liver parameters were affected consistently across multiple studies, and they occurred at dose levels at or below the tumourigenic dose level of 3600 ppm. Thyroid follicular cell hypertrophy was observed with low severity and partial incidence at the higher doses of 1200 and 3600 ppm only, but not at the low dose of 200 ppm (1-year and 2-year time points) or 300 ppm (90-day study). Increases in thyroid weight were observed at only selected early time intervals at 1200 and 3600 ppm (Day 29; Table 4), but not after 90 days of treatment (Table 6). Overall, the associative events in the liver were clearly and consistently observed across multiple studies, and the associative events in the thyroid after sedaxane treatment were affected in multiple studies (but to a lesser extent than the liver effects).

DS comment:
According to the available data, a good dose-concordance between the causal key events, associative events and the apical outcome (thyroid tumours) was observed in male rats.

4.1.2 Temporal-concordance of key events

The observed effects on parameters associated with the key events occur in a logical, time-dependent manner consistent with the proposed MOA. The temporal-concordance is summarised in Table 10.

The observed steps occur in a logical sequence, with the key events preceding the time of occurrence of thyroid follicular adenomas. In particular:

- CAR/PXR activation, induction of hepatic UDPGT, elevated total P450 and microsomal enzyme activities, increased liver hypertrophy and increased liver weight occurred early (within 1-7 days) and remained consistently affected over time
- Decreases in circulating T_3/T_4 occurred early (within 1-7 days), and these values had returned to control levels by the last time of measurement (28 days)
- TSH was unaffected after 1-7 days, but showed a marginal increase at 14-28 days, reflecting a response of the HPT axis to lower T_3/T_4 levels

- Thyroid follicular cell hypertrophy was not apparent at 1-7 days, but it was observed at 14 days up through 1 year. This was consistent with increased stimulation of the thyroid by increased TSH levels.
- After >1-2 years, increased thyroid follicular hyperplasia (as an indicator of increased thyroid follicular cell proliferation) was observed.
- A higher incidence of adenomas of the thyroid required >1-2 years before it was observed.

DS comment:
Based on the available data, a temporal concordance between the causal key events, associative events and the apical outcome (thyroid tumours) could be established in male rats.

TABLE 10 Summary of Temporal Concordance of Associative and (Causal) Key Events

Time[c]	CAR/PXR Activation and Hepatic UDPGT induction (Causal) [a]	Increased hepatocellular hypertrophy and liver weight (Associative)	Reduced circulating T_3/T_4 (Causal)	Increased circulating TSH (Causal)	Increased thyroid follicular cell hypertrophy (Associative)	Increased thyroid weight (Associative)	Increased thyroid follicular cell proliferation and hyperplasia (Causal)	Higher Incidence of Thyroid Adenomas (Outcome)
1-7 days	Yes	Yes	Yes	No	No	No	No	No
14 days	Yes	Yes	Yes	Yes[b]	Yes	No	No	No
28 days	Yes	Yes	No	Yes[b]	Yes	Yes	No	No
90 days	No data	Yes	No data	No data	Yes	Yes	No	No
1 year	No data	Yes	No data	No data	Yes	No data	No	No
>1 - 2 years	No data	Yes	No data	No data	No	No data	Yes	Yes

[a]CAR/PXR activation was demonstrated based on *in vitro* studies, and by increased PROD activity (a marker of Cyp2b activity) in the livers of rats on Day 8 of the 28-day MOA study at the indicated dose levels (Anonymous, 2015 *Annex I. 3.9.4.4).*

[b]In the mode of action study, TSH levels were numerically higher than the control values on Days 15 and 29, and correlated with a return of T3 and T4 levels to the same as (or slightly above) control levels

[c]Time of continuous treatment – as opposed to the Study Day (e.g. Day 2, Day 4, Day 8), which is 1 day longer by convention.

4.1.3 Reproducibility and consistency

Where parameters were measured in multiple studies, there is a high degree of reproducibility between studies and consistency between key events. The first key causal event in the proposed MOA, activation of CAR/PXR in the liver, was confirmed both by *in vitro* reporter assays and by increases in Cyp2b isoenzymes (PROD activity) in the liver *in vivo*. The causal key event of induction of hepatic UDPGT showed a high degree of consistency across multiple time points in the 28-day mechanistic study. Similarly the associative events of hepatomegaly and hepatocellular hypertrophy were observed in every study in the rat. Thyroid hormone measurements were only determined in this strain of rat in a single study so their reproducibility between studies cannot be assessed. Thyroid follicular cell hypertrophy was seen in the 28-day MOA study, the 90-day study and at the 1-year sacrifice of the chronic toxicity/carcinogenicity study in rats.

In studies involving 28 or 90 days treatment with sedaxane, the HsdRccHan:WIST rat strain supplied by Harlan Laboratories did not demonstrate the thyroid changes that were seen in the CrL:WI(Han) supplied by Charles River Laboratories. However, both strains showed similar responses in the liver after dietary treatment with sedaxane. These differences are considered a reflection of possible strain differences in sensitivity for this thyroid MOA, and the definitive data to investigate the key events were produced in studies with the CrL:WI(Han) strain of rat, the same strain that was used in the 2-year chronic/carcinogenicity study.

4.1.4 Biological plausibility

The induction of thyroid follicular cell adenomas in rats is a common finding in chronic toxicity and carcinogenicity studies (Finch *et al.*, 2006; Hurley, 1998; Wilson *et al.*, 1996). The proposed MOA for the thyroid effects seen with sedaxane (see Section 2.1.2; Figure 1), which can be described as a perturbation of the HPT axis secondary to induction of hepatic UDPGT, is well described for a number of compounds, including the archetypal UDPGT inducer, NaPB (Finch *et al.*, 2006) and a number of SDHI fungicides including benzovindiflupyr (U.S. Environmental Protection Agency, 2014a), fluopyram (U.S. Environmental Protection Agency, 2014b) and fluxapyroxad (U.S. Environmental Protection Agency, 2011a).

4.1.5 Alternative mode of action hypotheses

In addition to the MOA described (see Section 2.1.2; Figure 1), alternative modes of action for the induction of thyroid tumours exist. One such alternative MOA is genotoxicity. This MOA can be excluded as sedaxane has been demonstrated to be negative for genotoxicity in a comprehensive package of *in vitro* and *in vivo* assays for genotoxicity (see Table 11).

TABLE 11 Summary of genotoxicity studies with sedaxane

Study	Dose Levels	Result
***In vitro* studies**		
Bacterial reverse mutation (Sokolowski, 2009)	3-5000 µg/plate	Negative
In vitro cytogenetics (Bohnenberger, 2009)	23.1 – 216.8 µg/mL	Negative
Mammalian cell gene mutation (mouse lymphoma) (Wollny, 2009)	6.9-110 µg/mL	Negative
***In vivo* studies**		
Mouse bone marrow micronucleus (Reichenbach, 2010)	2000 mg/kg	Negative
Unscheduled DNA synthesis – rat liver (Durward, 2009)	2000 mg/kg	Negative
Unscheduled DNA synthesis – rat liver (Hall, 2011)	1000 – 2000 mg/kg	Negative

A second alternative MOA is direct inhibition of the thyroid hormone synthesis. Organification of iodine via monoiodination of L-tyrosine is the first step in the synthesis of T_3 and T_4 and is catalysed by the enzyme thyroid peroxidase (TPO). Inhibition of TPO, in order to reduce circulating T_3/T_4, by compounds such as propylthiouracil (PTU) is exploited as a treatment for hyperthyroidism in humans, such as in Graves' disease. PTU has also been shown to induce thyroid follicular cell adenomas in rats (IARC, 2001). Sedaxane was evaluated for its potential to inhibit TPO *in vitro* across a concentration range up to 10 micromolar compared to the appropriate controls. Taken together with the evidence supporting the proposed MOA for thyroid tumours, these data provide compelling evidence that sedaxane lacks the intrinsic properties to interact with and inhibit TPO. This MOA can be excluded for sedaxane as it was found not to be an inhibitor of male rat thyroid-derived TPO *in vitro*, whereas PTU was shown to be a potent inhibitor (Anonymous, 2014 *Annex I. 3.9.4.16*).

DS comment:
The available data permitted to rule out alternative MoAs: genotoxicity and inhibition of thyroid peroxidase (TPO) peroxisome proliferation. Indeed sedaxane was negative according to genotoxicity package and was not an inhibitor of rat thyroid peroxidase activity in vitro.

See annex I to the CLH Report 3.8 (genotoxicity studies) and 3.9.4.16.

4.1.6 Uncertainties, inconsistencies and data gaps

The available data support the proposed hypothesized MOA for the slightly increased incidence of rat thyroid tumours by sedaxane (Section 2.1.2; Figure 1), while excluding the alternative MOAs described in Section 3.1.5. Only minor uncertainties and no data gaps remain.

TSH levels were numerically higher than control on Days 15 and 29 of treatment, which matched the time points when the effect of NaPB on TSH was maximal, but these differences in the sedaxane groups

were not statistically significant at the time intervals that were assessed in this study (Table 5). Considering the weaker response for most key events with sedaxane treatment, relative to the responses with NaPB treatment, a marginal effect on TSH levels is not unexpected. The timing of the increases in TSH levels with compounds that produce thyroid effects via induction of UDGPT in the liver can vary by compound and rat strain, such that TSH increases following sedaxane treatment might have been maximal during a time window that was not among the time points selected for this sedaxane MOA study.

The hypothesised consequence of UDPGT induction, increased clearance of T_3/T_4 from the blood into the bile, has not been directly demonstrated; however, the consequence of this increased clearance, namely decreased T_3/T_4 in the serum was demonstrated and was associated with the increased hepatic UDPGT. It is therefore reasonable to infer that all of the intermediate key events are operating.

In a 90-day capsule study in Beagle dogs, sedaxane was administered to 4 male and 4 female dogs at 0, 50, 150 and 400 mg/kg/day. There was a higher incidence of minimal thyroid follicular cell hypertrophy in male treated groups (0, 1, 2, 1) and in females only at the high dose (0, 0, 0, 2) (Anonymous, 2008 *Annex I. 3.12.1.6*). However, there were no effects in a 1-year dog study at dose levels up to 200 mg/kg/day (Anonymous, 2009 *Annex I. 3.12.1.7*), and no effects on thyroid weights in either the 90-day or the 1-year dog study. Considering the lack of a dose response and the minimal severity, plus the lack of any effects with longer treatment, the thyroid changes in the 90-day study in dogs were not considered adverse. Pharmaceutical compounds that perturb thyroid hormone clearance via liver enzyme induction have been shown to also produce this type of effect in dogs (Muller *et al.*, 2000), so these minor changes with sedaxane treatment in dogs are consistent with its established MOA in rats.

No other uncertainties, inconsistencies or data gaps have been identified.

4.2 Assessment of the Postulated Mode of Action

The concordance analyses presented in section 3.1 have established that the proposed key events resulting in a higher incidence of thyroid tumours in male rats exhibit good dose- and temporal-concordance with the tumour endpoint. This is a well described MOA for the induction of thyroid tumours and associated precursor key events in rats, and the parameters essential for describing the MOA have been presented for sedaxane. Therefore, there is a high level of confidence that the hypothesised MOA (Figure 1) was responsible for the higher incidence of thyroid tumours in male rats following dietary exposure to 3600 ppm sedaxane.

DS comment:
DS is of the opinion the available data provide plausible evidence to support the postulated MoA (CAR-mediated induction of hepatic UGT activity) to be the underlying MoA of the slight increase incidence of thyroid adenomas observed in high dose male rats.

4.3 Are the Key Events in the Animal Mode of Action Plausible in Humans?

Following establishment of a plausible MOA for the induction of thyroid tumours in rats, the next step is to assess the relevance to humans by assessing the qualitative and quantitative differences between the rat and human for each of the key events.

In contrast to rats, serum TSH levels in humans are more stable following exposure to hepatic enzyme inducers (Dellarco *et al.*, 2006; Meek *et al.*, 2003). The human HPT axis is qualitatively very similar to that of rats and it has been demonstrated that human administration of pharmaceuticals that result in the induction of UDPGT, including phenobarbital, phenytoin and carbamazepine also result in reduced circulating T_3/T_4. However, despite the reduced T_3/T_4 levels, TSH levels in humans remain largely unaffected, whereas in the rat TSH levels increase in order to compensate (Curran and DeGroot, 1991). Therefore, although the HPT axis is responsible for homeostatic control of thyroid hormones in both species, there is a large difference in their sensitivity to perturbation, with the human being considerably less susceptible (Dellarco *et al.*, 2006).

In addition to differential sensitivity of the HPT axis, another factor resulting in lower sensitivity of humans as compared to rats is that the half-life of T_3 and T_4 in humans is considerably longer than that in rats, being 5-9 days in humans and 12 hours in rats for T_4 (Dohler *et al.*, 1979; U.S. Environmental Protection Agency, 1998). The substantially longer half-life in humans is a result of binding to a high-affinity thyroid-binding globulin, which binds T_4 (and T_3 to a lesser degree), and is not present in rats (Hill *et al.*, 1998; U.S. Environmental Protection Agency, 1998). These differences mean that rats have a higher rate of turnover of T_3/T_4. As a result of this higher turnover, rats have a much higher (approximately 25-fold) basal level of TSH when compared to humans (Dohler *et al.*, 1979). This means that the compensatory reaction in rats towards a T_3/T_4 deficiency is much more pronounced than in humans.

Finally, it has been suggested that interspecies differences in thyroid histology play a role in the differential sensitivity. In humans, the thyroid follicular cell epithelium is composed of short, cuboidal cells, indicative of their quiescent nature. In rats, however, the thyroid follicular cells are tall and cuboidal and appear to be continually active in synthesis. Therefore, it appears that the rodent thyroid gland is chronically stimulated by TSH levels to compensate for the increased clearance of thyroid hormones. It follows that increases in TSH levels above basal levels in rats more readily moves that gland towards increased growth and potential neoplastic change than in humans (Dellarco *et al.*, 2006; U.S. Environmental Protection Agency, 1998). Interestingly, adult male rats have higher serum TSH levels than female rats (Chen, 1984), and they are often more sensitive to stimulation of thyroid growth and carcinogenesis. Overall, the histological differences in thyroid follicular cells between rats and humans is related to a higher rate of production of T_4 in rats to maintain a consistent serum concentration, thus making the rat thyroid more "functionally active" than primates including humans (Dellarco *et al.*, 2006; McClain, 1995).

Even though certain agents can cause a reduction in T_3/T_4 levels in humans, there is no evidence that these agents can induce an increased susceptibility to thyroid cancer in humans (Dellarco *et al.*, 2006; Ron *et al.*, 1987). Epidemiology studies with phenobarbital have not shown any increased risk for thyroid cancer in humans (Olsen *et al.*, 1993). As a result, the only known human thyroid carcinogen is radiation, which is a mutagenic mode of action.

In summary, a wealth of information in the literature has established a lack of susceptibility of humans to thyroid hormone alterations, resulting changes in TSH, and thyroid tumour responses that are initiated in rats by induction of UDPGT and increased T_3/T_4 clearance. Therefore, based on qualitative differences (presence of high-affinity thyroid binding globulin in humans but not rodents) and quantitative differences (including lower responsiveness to fluctuations in thyroid hormone levels), the MOA established in rats with sedaxane is not relevant to humans.

DS comment :

There is strong evidence that the thyroid tumours induced by sedaxane are caused by a CAR mediated MoA.

The increase in the activity of hepatic UDPG-transferase results in increased clearance of thyroid hormone levels (T4), resulting in thyroid stimulation. Such a mechanism/effect cannot be directly extrapolated to humans due to T4 binding protein that greatly reduces susceptibility to plasma T4 depletion.

The thyroid effects observed in rats are therefore considered of insufficient concern for classification (Guidance on the Application of the CLP Criteria, ECha 2017).

5.0 CONCLUSIONS

The available data for sedaxane support the proposed MOA that the higher numerical incidence of thyroid tumours in male rats is attributable to activation of CAR/PXR and induction of hepatic UDPGT, which results in a series of downstream events, ultimately leading to a higher incidence of tumours vs. the concurrent controls as described in Section 2.1.2 and Figure 1. The available data also demonstrates that this threshold-based mode of action is not relevant to humans due to qualitative and quantitative differences in response to UDPGT induction and increased T_3/T_4 clearance between rats and humans. In summary, the data support the conclusion that sedaxane does not pose a carcinogenic hazard to humans.

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DS comments:

Based on the regulatory studies and the mechanistic studies generated, the applicant has proposed the following mode of action for sedaxane-induced thyroid tumours in male rats:

Key events	Associative events
1-CAR/PXR activation	**1- Hepatocellular hypertrophy and increased liver weight**
2- Induction of hepatic UGT activity	
3- Reduced circulating T3 and T4	
4- Increased circulating TSH	**2-Thyroid follicular cell hypertrophy and increased thyroid weight**
5-Increased thyroid follicular cell proliferation (hyperplasia)	
Final adverse outcome: Increase in thyroid tumours incidence	

Assessment of the postulated mode of action:

Key event 1: The results from the *in vitro* CAR and PXR reporter assays support the fact that sedaxane activates CAR from rat, mouse and human and PXR from rat and human.

Key event 2 "Induction of hepatic UGT"
In the 28-day rat mechanistic study, sedaxane induced increased hepatic UGT activity at 1200 and 3600 ppm in line with Key event 2

Associated event 1: In the 28-day rat mechanistic study, sedaxane induced increased liver weight and hepatocellular hypertrophy at 1200 and 3600 ppm in line with associative event 1.
Associative event 1 was consistently observed in mechanistic data and regulatory studies in male rats.

Key event 3 "Reduced circulating T3 and T4":
In the 28-day rat mechanistic study, total T3 showed a statistically significant decrease in one or both Sedaxane treatment groups on Days 2, 4, 8 and 15. However total T4 was statistically significantly decreased by treatment with Sedaxane only at Day 2.

Key event 4 "Increased circulating TSH":
In the 28-day rat mechanistic study, a clear increase of circulating TSH was not observed after sedaxane treatment

Key events 3 and 4 are therefore weakly supported by experimental data. However, the shifts in thyroid hormone concentrations may be difficult to capture if the potency of effect is weak as it is the case for sedaxane (slight increased incidence of thyroid adenomas in male rats at high dose level)
Sedaxane seem to have a mild effect on thyroid hormone homeostasis, in a manner that is similar to the pattern seen with phenobarbital, but less severe.

<u>Associative event 2 "Thyroid follicular cell hypertrophy and increased thyroid weight":</u>
In the 28-day rat mechanistic study, sedaxane induced increased thyroid weight from 1200 ppm and thyroid follicular cell hypertrophy at 3600 ppm in line with associative event 2.
Thyroid follicular cell hypertrophy was also observed at 4000 ppm in a 90-day rat study as well as in the 2-year rat study from 1200 ppm.

<u>Key event 5:</u> In the 2-year rat study, sedaxane induced thyroid follicular cell was also observed at 3600 ppm in line with Key event 5

Throughout the database, a good dose-concordance and a temporal concordance between the causal key events, associative events and the apical outcome (thyroid adenomas) were observed in male rats.

The available data permitted to rule out alternative MoAs: genotoxicity and inhibition of thyroid peroxidase (TPO) peroxisome proliferation. Indeed sedaxane was negative according to genotoxicity package and was not an inhibitor of rat thyroid peroxidase activity *in vitro*.

In summary, DS is of the opinion the available data provide plausible evidence to support the postulated MoA (CAR-mediated induction of hepatic UGT activity) to be the underlying MoA of the slight increased incidence of thyroid adenomas observed in high dose male rats.

The thyroid tumours induced by sedaxane are caused by a CAR mediated MoA.
The increase in the activity of hepatic UDPG-transferase results in increased clearance of thyroid hormone levels (T4), resulting in thyroid stimulation. Such a mechanism/effect cannot be directly extrapolated to humans due to T4 binding protein that greatly reduces susceptibility to plasma T4 depletion.
The thyroid effects observed in rats are therefore considered of insufficient concern for classification (Guidance on the Application of the CLP Criteria, ECha 2017).