Document Title

Summary of the toxicological and metabolism studies for spiroxamine

Part 2

Data Requirement(s)

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Document MCA

Section 5: Toxicological and metabolism studies

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

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ERM

On behalf of Bayer AG
Crop Science Division

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Version history

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

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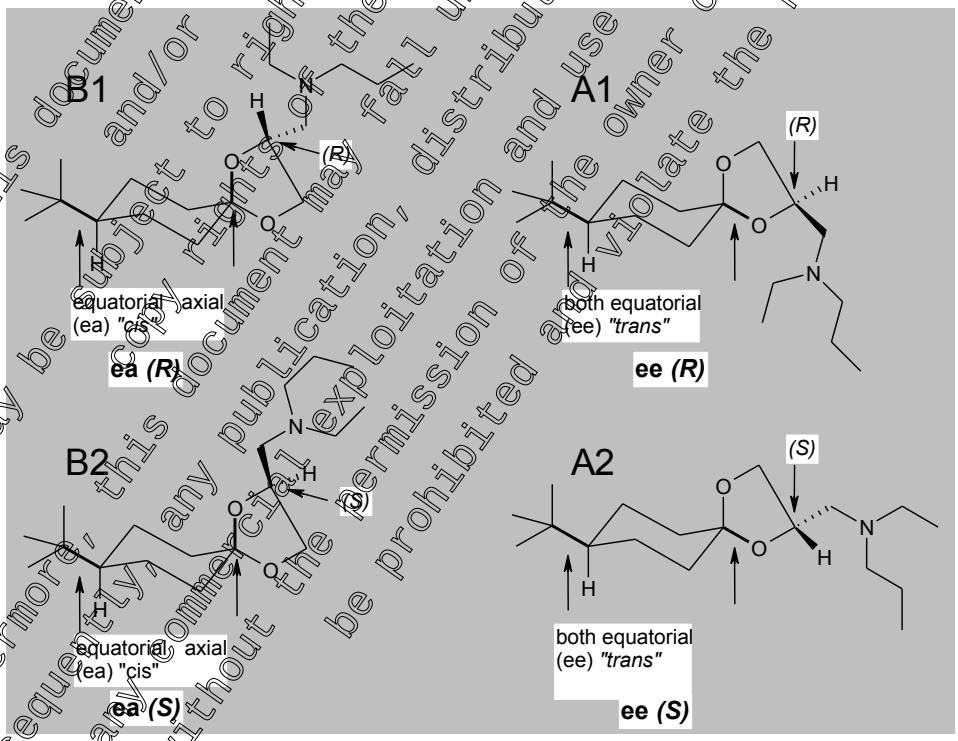
CA 5 TOXICOLOGICAL AND METABOLISM STUDIES ON THE ACTIVE SUBSTANCE

Spiroxamine was included in Annex I to Council Directive 91/414/EEC in 1999 (Directive 1999/73/EC, Entry into Force on 1 September 1999). This Supplementary Dossier contains data which were not submitted at the time of the Annex I inclusion of spiroxamine under Council Directive 91/414/EEC and which were therefore not evaluated during the first EU review. However, all studies submitted for the first approval and subsequent first renewal of spiroxamine have also been summarised according to current guidance and included in the dossier. Where studies meet relevant validity criteria, new robust study summaries have been provided in the appropriate dossier section. However, where studies do not meet relevant validity criteria and are not considered acceptable, less detailed summaries may have been provided alongside discussions of study deficiencies. All relied upon study reports are submitted in Document K for this second renewal of approval dossier or in Document K for the previous Annex I inclusion and first renewal submissions.

All data which were already submitted by Bayer AG (former Bayer CropScience) for the Annex I inclusion and first renewal under Council Directive 91/414/EEC are contained in the draft Re-Assessment Report (RAR) 2010 and its revised RAR 2017 and are included in the Baseline Dossier provided by Bayer AG..

Spiroxamine consists of four isomers (two diastereomers each with its corresponding two enantiomers which are in a 1:1 ratio) as shown in the schematic below. The isomer nomenclature presented in some historical documentation may differ with respect to the A/B and corresponding trans/cis notation as a result of a discrepancy in referencing, which is discussed in detail in position paper M-761468-01-1 (see CA 1.7/01). It is recommended that the stereo assignments depicted here, together with the A and B notation should be used exclusively going forward to ensure continuity of information throughout the dossier.



Relevant information for classification as detailed in the "*Combined Draft (Renewal) Assessment Report prepared according to Regulation (EC) N° 1107/2009 and Proposal for Harmonised Classification and*

Labelling (CLH Report) according to Regulation (EC) N° 1272/2008 – Volume 1, Level 2" is provided in Document N1, Sections 6.1.1 – 6.1.10 and 6.2, and highlighted in light grey.

CA 5.8 Other toxicological studies

CA 5.8.1 Toxicity studies on metabolites

A myriad of acute oral, genotoxicity and repeat dose toxicity studies have been undertaken on spiroxamine metabolites, with a summary provided.

Acute oral toxicity studies conducted with M03 and M28 LD_{50} trigger classification (Acute Tox. Cat. 2, H302) with LD_{50} values of 707 and >550 to <2000 mg/kg bw, respectively. A complete *in vitro* genotoxicity test battery (Ames, mammalian forward (V79 *hprt*$^{+/-}$ or L5178Y *tk*$^{+/-}$) gene mutation and mammalian (human peripheral blood lymphocyte micronucleus)) tested up to suitable maximum concentrations, confirmed both M03 and M28 to be devoid of both gene mutation and structural/numerical chromosomal effects.

In the 28-day rat oral dietary study conducted on M03, the NOAEL was deemed to be 150 ppm (equivalent to 12.9/13.2 mg/kg bw/day for males/females). This conclusion is based reduction in body weight gain (exceeding 10%), hyperkeratosis of the stomach and oesophagus with mild transitional cell hyperplasia in the urinary bladder. These effects are associated with the irritant nature of the test article.

In the 90-day rat oral dietary study conducted on M03, the NOAEL was deemed to be 125 ppm (equivalent to 8.8/9.7 mg/kg bw/day for males/females) based on reduction in body weight and body weight gain, hyperkeratosis of the stomach and oesophagus. Increases in liver enzyme induction were observed, without concurrent hepatic histopathology. An additional dose group were also treated with 625 ppm spiroxamine (equivalent to 48.7/52.7 mg/kg bw/day for males/females). Similar effects reported for spiroxamine N-oxide were also observed for spiroxamine.

For M13, both the acute oral and dermal studies indicated that the LD_{50} was >2000 mg/kg bw for both endpoints. A complete *in vitro* genotoxicity test battery (Ames, mammalian forward (L5178Y *tk*$^{+/-}$) gene mutation and mammalian (human peripheral blood lymphocyte micronucleus)) tested up to suitable maximum concentrations, confirmed M13 to be devoid of both gene mutation and structural/numerical chromosomal effects.

In the 28-day rat oral *via* gavage study with a 14 day recovery period conducted on M13, the NOAEL was deemed to be 50 mg/kg bw/day for males/females based on reductions in body weight and body weight gain (males) and moderate to severe clinical effects following dosing with a peak period approximately 15 minutes post dosing. Unlike the parent compound, spiroxamine and spiroxamine N-oxide, which are considered tertiary amines, spiroxamine cyclohexanol, which has lost the tertiary amine group consequently did not display histopathological lesions associated with tertiary amines (hyperkeratosis of the epithelium of the oesophagus and forestomach), with the NOAEL for spiroxamine following 28 days of dosing *via* oral gavage was deemed to to be 10 mg/kg bw/day.

In order to understand the metabolic fate of M13-acetate in mammalian systems it was incubated with rat plasma and the reaction products were identified. Under the conditions of this study, M13-acetate, is readily hydrolysed in plasma of male and female rats to M13.

In the rat developmental study conducted on M13-acetate, the NOAEL for maternal toxicity was considered to be 40 mg/kg bw/day based maternal mortality, body weight losses, reduced body weight gains and food consumption values at a dosage level that exceeded the MTD (640 mg/kg bw/day).

The developmental NOAEL was considered to be 160 mg/kg bw/day based on transient retardations in fetal development, with reduction in fetal bodyweight and associated significant increases in moderate dilation of the renal pelvis and delayed ossification of the caudal vertebrae, fore- and hind limb phalanges and metatarsals. These retardations occurred at a dosage level of 640 mg/kg bw/day, which exceeded the MTD for maternal animals.

When accounting for acetate to M13 conversion, NOAELs for maternal and developmental toxicity equate to 31.5 and 126.1 mg/kg bw/day, respectively.

It can be concluded that M13 is less toxic than the parent, spiroxamine in the rat with a *ca.* 9-fold, 2-fold and 8-fold increase in sub-acute, maternal and developmental NOAELs, respectively when compared to the spiroxamine equivalent studies.

In the 28-day rat oral dietary study conducted on M28, the NOAEL was deemed to be 400 ppm (equivalent to 28.4/31.4 mg/kg bw/day for males/females) based on no adverse effects when tested up to the maximum dose level. Unlike the cyclohexane dimethyl ethyl containing spiroxamine and spiroxamine N-oxide (M03), spiroxamine aminodiol (M28) does not contain this structure, with only the aminodiol group present. Consequently, the adverse histopathological lesions associated with the cyclohexane dimethyl ethyl containing tertiary amines (hyperkeratosis of the epithelium of the oesophagus and forestomach) were not observed here. Furthermore, no test article related histopathological lesions were observed. The presence of the two diol groups vastly reduces the overt pH and pKa values observed with both the parent and M03, and therefore the mucosal membrane containing tissues, which would be site of first contact are not targeted. The two diol groups present in M28 vastly increases the water solubility and with a smaller chemical structure (*i.e.* omission of the cyclohexane dimethyl ethyl group) results in rapid absorption, with a likely scenario of absorption between mucosal cells within the gastric environment, rather than crossing between membranes. Therefore the existence within such an environment is markedly reduced compared to parent and M03.

The NOAEL for spiroxamine in the rat 28-day oral dietary study was 30 ppm (equivalent to 3.4/3.8 mg/kg bw/day for males/females). Thus, M28 is less toxic than the parent, spiroxamine in the rat, with an order of magnitude of difference between established NOAELs.

The NOAEL for maternal toxicity was considered to be 150 mg/kg bw/day based maternal mortality, body weight losses, gaseous content of the GI tract and adverse clinical signs at a dosage level that exceeded the MTD (500 mg/kg bw/day). Gaseous content of the GI tract was also observed in a single dam at 150 mg/kg bw/day.

The developmental NOAEL was considered to be 30 mg/kg bw/day based on incomplete ossification.

It can be concluded that M28 is less toxic than the parent, spiroxamine in the rat with a *ca.* 15-fold, 9-fold and 2-fold increase in sub-acute, maternal and developmental NOAELs, respectively when compared to the spiroxamine equivalent studies.

Refer to Section 6 (CA 6.7.1.5/01 [M-472579-01-1]) for an overview of stereochemistry of M13 and M28.

Table CA 5.8.1-1: Comparison of Group B and C metabolite NOAELs

Parameters	Repeat dose toxicity NOAELs	MWT[a] (g/mol)	M-13 equivalent (NOAEL *x* (M13 MWT/M13-acetate MWT))	SPX-equivalent (NOAEL *x* (SPX MWT/metabolite MWT))	NOAEL fold difference (metabolite *vs.* SPX)
M13	28-d (gavage) NAOEL: 50 mg/kg bw/d	156.27	n/a	95.2 mg/kg bw/d (50 *x* (297.48/156.27)	*x*9.52 (9.52/10)
M13-acetate	Maternal NOAEL: 40 mg/kg bw/d	198.30	31.5 mg/kg bw/d (40 *x* (156.27/198.30))	60.0 mg/kg bw/d (31.5 *x* 297.48/156.27)	*x*2 (60.0/30)
	Developmental NOAEL: 160 NOAEL		126.1 mg/kg bw/d (160 *x* (156.27/198.30))	240.0 mg/kg bw/d (126.1 *x* (297.48/156.27)	*x*8 (240/30)
M28	28-d (dietary) NAOEL: 28.4 mg/kg bw/d	164.24	n/a	51.4 mg/kg bw/d (28.4 *x* (297.48/164.24)	*x*15.1 (51.4/3.4)
	Maternal NOAEL: 150 mg/kg bw/d		n/a	271.7 mg/kg bw/d (150 *x* (297.48/164.24)	*x*9.1 (571.7/30)
	Developmental NOAEL: 30 NOAEL		n/a	54.3 mg/kg bw/d (30 *x* (297.48/164.24)	*x*1.8 (54.3/30)
SPX:	28-d (gavage) NAOEL: 10 mg/kg bw/d	297.48	n/a	n/a	n/a
	28-d (dietary) NAOEL: 3.4 mg/kg bw/d		n/a	n/a	n/a
	Maternal NOAEL: 30 mg/kg bw/d		n/a	n/a	n/a
	Developmental NOAEL: 30 NOAEL		n/a	n/a	n/a

a MWT calculated from ACD/ChemSketch 2016 1.1

For M35 and M37, *in vitro* human peripheral blood lymphocyte micronucleus assays confirmed a lack of structural/numerical chromosomal effects for both metabolites when tested up to suitable maximum concentrations.

It is acknowledged that no repeat dose toxicity data are available for M35 or M37. Comparison of the chemical properties of M37 and M13 leads to the conclusion that both molecules are similar with respect to physicochemical properties, predicted chemical and biological activity and disposition, with the double bond in the benzene ring of M37 not impacting upon the assessment. Therefore it is appropriate to group both molecules together for the purposes of read-across of toxicity data.

For M36 discuss gastric acid hydrolysis study when data available.

Following *in silico* assessment using two methods showed there were no concerns for mutagenicity for any of the metabolites assessed. There was high confidence in the predictions for metabolites M05, M06, M07, M08 and M10 due to their close structural similarity to spiroxamine and M03 or, in the case of M06, being a major rat metabolite. For the sulfate conjugates M25, M26 and M27 confidence in the prediction was assigned as low, since the sulfate moiety was not assessed in any of the Leadscope sub-models therefore the prognosis was based on a single method. There was high confidence in the predictions for metabolites M13 and M14 due to the availability of experimental data for M13 and the structural similarity to M14. For metabolites M35, M36 and M37 there was high confidence in the prediction for chromosome damage due to the availability of experimental data for M35 and M37 plus the structural similarity between M35 and M36, though the overall confidence level for genotoxicity was medium, based on the mutagenicity endpoint, for which there was no experimental data. For metabolites M15, M16 and M17 there was medium confidence in the predictions because they were based on two *in silico* methods. There was high confidence in the predictions for Metabolite M28 due to the availability of experimental data, medium confidence in the predictions for M30 and M31 and a low confidence in the prediction for M29 because the chromosome damage component of the genotoxicity assessment was based on a single method.

Table CA 5.8.1-2: Summary of toxicity studies conducted on spiroxamine metabolites

Type of study	Species	Doses	mg/kg bw/day		Key effects	Annex CA Point/Reference
			NOAEL	LOAEL		
Toxicity data on spiroxamine N-oxide (Group A, M03[1]) (primary plant metabolite minor metabolite in fruits <5% TRR; 1 – 22% TRR in cereals)						
Acute oral	Rat	500 1000 mg/kg bw	n/a		LD_{50} 707 mg/kg bw (Acute Tox. Cat. 4, H302)	CA 5.8.1/01 [M-016338-01-1]
In vitro bacterial reverse (Ames) gene mutation	*S. typhimurium* strains	**Expt. 1: plate incorporation (±S9)**			+/-S9 negative	CA 5.8.1/02 [M-016297-01-1]
		TA98, TA1537, TA100, TA1535:	16 – 5000 µg/plate			
		Expt. 2: Pre incorporation (±S9)				
		All strains:	5 – 1581 µg/plate			
		TA100:	20.48 – 5000 µg/plate			
In vitro bacterial reverse (Ames) gene mutation	*S. typhimurium* strains	**Expt. 1: plate incorporation (±S9)**			+/-S9 negative	CA 5.8.1/20 [M-756858-02-1]
		All strains:	5 – 5000 µg/plate			
		Expt. 2: plate incorporation (-S9)				
		TA98, TA1535, TA1537, TA102:	8.912 – 2000 µg/plate			

Type of study	Species	Doses	mg/kg bw/day		Key effects	Annex CA Point/Reference
			NOAEL	LOAEL		
		TA100:	20.48 – 5000 µg/plate			
		Expt. 2: pre-incubation (+S9)				
		TA98, TA102:	8.912 – 2000 µg/plate			
		TA100, TA1535, TA1537	20.48 – 5000 µg/plate			
In vitro mammalian forward gene mutation	V79 (*hprt*) cells	5 h –S9:	0, 50, 200, 350, 500, 650 µg/mL		+/-S9 negative (sufficient maximum concentration not tested)	CA 5.8.1/03 [M-006500-01-1]
		5 h +S9:	0, 100, 200, 300, 400, 500, 600 µg/mL			
V79 chrom abs	V79 cells	4 h (+18 h recovery) – S9	0, 200, 300, 400 µg/mL		+/-S9 negative (sufficient maximum concentration not tested)	CA 5.8.1/04 [M-006495-01-1]
		4 h (+30 h recovery) – S9	0, 400 µg/mL			
		4 h (+18 h recovery) +S9	0, 300, 400, 500 µg/mL			
		4 h (+30 h recovery) +S9	0, 500 µg/mL			
In vitro mammalian micronucleus	Human peripheral blood lymphocytes	3 h (+21 h recovery) – S9	0, 150, 250, 300 µg/mL		+/-S9 negative	CA 5.8.1/21 [M-755221-02-1]
		3 h (+21 h recovery) +S9	0, 225, 275, 325 µg/mL			
		24 h (+24 h recovery) – S9	0, 20, 50, 90 µg/mL			
28-day	Rat	0, 30, 150, 1000 ppm	♂: 12.9 ♀: 13.2	♂: 114.6 ♀: 94.3	↓ bwt gain, stomach, oesophagus (hyperkeratosis) and urinary bladder (mild transitional cell hyperplasia) histopathology	CA 5.8.1/05 [redacted] [M-006504-02-1]
		Spiroxamine N-oxide				
90-day	Rat	0, 25, 125, 625 ppm	♂: 8.8 ♀: 9.7	♂: 45.0 ♀: 53.6	↓ bwt gain, stomach, oesophagus (hyperkeratosis) histopathology. ↑ liver enzymes, without concurrent hepatic histopathology	CA 5.8.1/06 [M-016585-01-2]
		Spiroxamine				

Type of study	Species	Doses	mg/kg bw/day		Key effects	Annex CA Point/Reference
			NOAEL	LOAEL		
		0, 625 ppm	♂: - ♀: -	♂: 48.7 ♀: 52.7	Similar effects reported for spiroxamine N-oxide were also observed for spiroxamine	
Toxicity data on spiroxamine cyclohexanol (Group B, M13) (not a primary plant metabolite only detected in fruit crops following exhaustive hydrolysis)						
Acute oral	Rat	200, 2500, 3200, 4000, 5000 mg/kg bw	n/a		LD_{50} 4200 mg/kg bw (CLP: insufficient)	CA 5.8.1/07 [M-471767-01-1]
Acute dermal	Rabbit	5000 mg/kg bw	n/a		LD_{50} >5000 mg/kg bw (CLP: insufficient)	
In vitro bacterial reverse (Ames) gene mutation	*S. typhimurium* strains	**Expt. 1: plate incorporation (±S9)** All strains: 0, 1.0, 3.16, 10.0, 31.6, 100, 316 µg/plate **Expt. 2: Pre incorporation (±S9)** All strains: 0, 1.0, 3.16, 10.0, 31.6, 100, 316 µg/plate			+/-S9 negative	CA 5.8.1/08 [M-471123-01-1]
In vitro bacterial reverse (Ames) gene mutation	*S. typhimurium* strains	**Expt. 1: plate incorporation (±S9)** All strains: 5 – 5000 µg/plate **Expt. 2: plate incorporation (-S9)** All strains: 7 – 1600 µg/plate **Expt. 2: pre-incubation (+S9)** All strains: 7 – 1600 µg/plate			+/-S9 negative	CA 5.8.1/22 [M-755223-02-1]
In vitro mammalian forward gene mutation	L5178Y (*tk*) cells	3 h, 24 h – S9; 3 h +S9:	15.63 – 250 µg/mL		+/-S9 negative	CA 5.8.1/09 [M-471125-01-1]
V79 chrom abs	V79 cells	3 h (+15 h recovery) – S9	10, 60, 100 µg/mL		+/-S9 negative (sufficient maximum concentration not tested)	CA 5.8.1/10 [M-471187-01-1]
		3 h (+15 h recovery) – S9	50, 250, 500 µg/mL			
		3 h (+25 h recovery) - S9	20, 100, 200 µg/mL			
		3 h (+25 h recovery) +S9	60 µg/mL			
			200 µg/mL			
In vitro mammalian micronucleus	Human peripheral blood lymphocytes	3 h (+21 h recovery) – S9	0, 100, 150, 210 µg/mL		+/-S9 negative	CA 5.8.1/23 [M-755227-02-1]
		3 h (+21 h recovery) +S9	0, 120, 180, 230 µg/mL			
			0, 40, 80, 95, 100 µg/mL			

Type of study	Species	Doses	mg/kg bw/day		Key effects	Annex CA Point/Reference
			NOAEL	LOAEL		
		24 h (+24 h recovery) – S9	0, 40, 80, 105 µg/mL			
28-day oral (*via* gavage)	Rat	0, 50, 150, 300 mg/kg bw/d	♂: 50 ♀: 50	♂: 150 ♀: 150	↓ bwt / bwt gain (♂), moderate to severe clinical effect following dosing with a peak period of effects 15 minutes post dosing	CA 5.8.1/11 [CA471x06-01-1]
Toxicity data on spiroxamine cyclohexyl acetate (Group B, M13-acetate) (Not a plant metabolite)						
Developmental, oral (gavage)	Rat	0, 40, 160, 640 mg/kg bw/day	**Maternal**		Mortality, ↓ bwt, bwt gain, food consumption, clinical signs	CA 5.8.1/12 [M-471532-01-2]
			40	640		
			Developmental		↓ bwt, ↓ dilation of renal pelvis, delayed ossification	
			160	640		
Bio-transformation in plasma	Rat	10 µL of M13-acetate stock incubated for 15, 60 min	n/a	n/a	M13-acetate readily hydrolysed in plasma of ♂ and ♀ rats to M13	CA 5.8.1/13 [M-472817-01-1]
Toxicity data on spiroxamine aminodiol (Group C, M28) (primary plant metabolite in fruit crops only (up to 38% TRR). Not formed in cereals						
Acute oral	Rat	550, 2000 mg/kg bw	n/a		LD_{50} >550 <2000 mg/kg bw (Acute Tox. Cat. 4, H302)	CA 5.8.1/14 [M-462551-02-1]
In vitro bacterial reverse (Ames) gene mutation	*S. typhimurium* strains	**Expt. 1: plate incorporation (±S9)** All strains: 3 – 5000 µg/plate **Expt. 2: Pre incorporation (±S9)** All strains: 3 - 5000 µg/plate			+/-S9 negative	CA 5.8.1/15 [M-463413-01-1]
In vitro mammalian forward gene mutation	V79 (*hprt*) cells	4 h –S9:	0, 101.9, 203.8, 407.5, 815, 1630 µg/mL		+/-S9 negative	CA 5.8.1/16 [M-465292-01-1]
		4 h +S9:	0, 101.9, 203.8, 407.5, 815, 1630 µg/mL			
		24 h -S9:	0, 101.9, 203.8, 407.5, 815, 1630 µg/mL			
		24 h +S9:	0, 203.8, 407.5, 815, 1222.5, 1630 µg/mL			
In vitro mammalian micronucleus	Human peripheral blood lymphocytes	4 h (+36 h recovery) – S9	0, 525.7, 920, 1610 µg/mL		+/-S9 negative	CA 5.8.1/17 [M-469334-01-1]
		4 h (+36 h recovery) +S9	0, 525.7, 920, 1610 µg/mL			
			0, 525.7, 920, 1610 µg/mL			

Type of study	Species	Doses	mg/kg bw/day		Key effects	Annex CA Point/Reference
			NOAEL	LOAEL		
		20 h (+20 h recovery) – S9	0, 525.7, 920, 1610 µg/mL			
In vitro mammalian micronucleus	Human peripheral blood lymphocytes	3 h (+21 h recovery) – S9	0, 200, 1000, 1613 µg/mL		+/-S9 negative	CA 5.8.1/24 [M-755220-02-1]
		3 h (+21 h recovery) +S9	0, 200, 1000, 1613 µg/mL			
		24 h (+24 h recovery) – S9	0, 200, 1000, 1613 µg/mL			
			0, 400, 1000, 1400 µg/mL			
28-day oral (*via* dietary)	Rat	0, 45, 135, 400 ppm	28.4 ♂ 31.4 ♀	- ♂ - ♀	No adverse effects observed when tested up to the highest dose level	CA 5.8.1/18 [M-471499-01-1]
Developmental, oral (gavage)	Rat	0, 30, 150, 500 mg/kg bw/day	**Maternal**		Mortality, ↓ bwt, clinical signs, gaseous content in GI tract	CA 5.8.1/19 [M-472720-01-1]
			150	500		
			Developmental		↑ incidence of incomplete ossification	
			30	150		
Toxicity data on spiroxamine docosanoic acid (Group B, M35) (primary plant metabolite in grapes only (14% TRR). Not formed in cereals						
In vitro mammalian micronucleus	Human peripheral blood lymphocytes	3 h (+21 h recovery) – S9	0, 25, 50, 75 µg/mL		+/-S9 negative	CA 5.8.1/25 [M-753775-01-1]
			0, 25, 50, 75 µg/mL			
		3 h (+21 h recovery) +S9	0, 10, 25, 50 µg/mL			
		24 h (+24 h recovery) – S9	0, 10, 25, 50 µg/mL			
Toxicity data on spiroxamine tetracosanoic acid ester (Group B, M36) (minor plant metabolite in grapes only (4% TRR). Not formed in cereals						
In vitro gastric acid stability	Awaiting data	Awaiting data	Awaiting data		Awaiting data	CA 5.8.1.5/01 Author (YYYY)
Toxicity data on spiroxamine cyclohexenol (Group B, M37) (Not a primary plant metabolite Formed in small quantities (3% TRR) only after exhaustive hydrolysis)						
In vitro mammalian micronucleus	Human peripheral blood lymphocytes	3 h (+21 h recovery) – S9	0, 220, 280, 300 µg/mL		+/-S9 negative	CA 5.8.1/26 [M-761547-01-1]
			0, 240, 280, 300, 310 µg/mL			
		3 h (+21 h recovery) +S9	0, 225, 270, 310 µg/mL			
			0, 100, 140, 170 µg/mL			

Type of study	Species	Doses	mg/kg bw/day		Key effects	Annex CA Point/Reference
			NOAEL	LOAEL		
		24 h (+24 h recovery) – S9	0, 75, 100, 140 µg/mL			
In silico toxicology testing	QSAR models used		Result			CA 5.8.1/27 [M-763154-01-1]
	ECOSAR v2.0, OECD Toolbox v4.4		It is concluded that M37 can be grouped with M13 for the purposed of read across of toxicity data			
***In silico* analysis on Group A, B and C metabolites**						
In silico toxicology testing	QSAR models used		Result			CA 5.8.1/28 [M-763152-01-1]
	Derek Nexus v6.0.1 [knowledge base expert system]; OECD (Q)SAR Toolbox v 4.4 [profiler and category formation tool] Leadscope Model Applier v3.0.1-1		Group A metabolites (M01, M02, M03, M04, M05, M06, M07, M08 M09, M10, M11, M12, M23, M26, M38): no concerns for genotoxicity for any of the metabolites assessed			
			Group B metabolites (M13, M14, M15, M16, M17, M35, M36, M37): no concerns for genotoxicity for any of the metabolites assessed			CA 5.8.1/29 [M-763153-01-1]
			Group C metabolites (M28, M29, M30, M31): no concerns for genotoxicity for any of the metabolites assessed			CA 5.8.1/30 [M-763154-01-1]

1. M03: this primary plant metabolite, once absorbed will undergo reduction to the parent (spiroxamine [SPX]). This reduction takes place both enzymatically and non-enzymatically in the stomach. Since the N-oxide is also formed by oxygenation of SPX in the same manner as N-oxides of other tertiary amines an equilibrium between SPX and its N-oxide is formed. The balance of this equilibrium is mainly on the side of the parent compound. However, it is prudent to emphasise that due to this equilibrium there is a low but permanent level of N-oxide maintained in the rat as long as SPX is administered (*i.e.* under chronic feeding conditions). This also means that the major part of the absorbed N-oxide originating from plant residues will not remain as such in the organism but rather be reduced to SPX and then metabolised. Therefore, the toxicity of the primary plant metabolite, M03 is addressed by the toxicity profile of the parent, SPX.

Spiroxamine-N-oxide (M03)

Data Point:	KCA 5.8.1/31
Report Author:	[redacted]
Report Year:	1997
Report Title:	Position paper on the metabolic and toxicological aspects of Spiroxamine-N-oxide
Report No:	M-008167-02-1
Document No:	M-008167-02-1
Guideline(s) followed in study:	not specified
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted DAR (2010)
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	Supportive only

Executive Summary

After application of the fungicide Spiroxamine to cereals the N-oxide of the parent compound was identified as one of the main constituents of the residue in grain. The metabolite spiroxamine-N oxide was not found as a terminal excretion product in urine or faeces of rats.

The aim of this position paper was to collectively assess additional studies was demonstrate that the N-oxide is formed *in vivo* by the rat, that its toxicological potential is not of concern, and its toxicity is lower as compared to spiroxamine.

Spiroxamine, orally administered at 100 mg/kg to rats were sacrificed at the maximum plasma level 4 hours post applicaton (CA 5.1.2/01 [M-006044-01-1]). The livers were sampled and extracted. After purification the extract was analysed chromatographically and the N-oxide identified by comparison with the authentic reference compound and by mass spectroscopy. The quantification yielded an amount of 0.11 % of the liver radioactivity which is equivalent to a concentration of 0.12 ppm in this organ.

These results clearly show that the N-oxide is formed in the rat upon administration of spiroxamine *in vivo* as an intermediary product. However, its absolute amount is very low. The reasons for this will be discussed in more detail below.

In further experiments the radioactively labelled N-oxide as well as the parent compound were incubated for 4 hours at 37°C with freshly prepared samples of gastric juice from the rat (CA 5.1.2/01 [M-006044-01-1]). The incubation solution was extracted and analysed. It was demonstrated that both compounds were hydrolysed under the prevailing acidic conditions by approximately 20% to t-butyl cyclohexanone.

Following absorption, the N-oxide will undergo reduction to the parent compound like other N-oxides of tertiary amines. This reduction takes place enzymatically and non-enzymatically[1,2]. Since the N-oxide is also formed by oxygenation of spiroxamine in the same manner as N-oxides of other tertiary amines[3] an equilibrium between spiroxamine and its N-oxide is formed. The balance of this equilibrium is mainly on the side of the parent compound. However, it has to be emphasised at this point, that due to this equilibrium there is a low but permanent level of N-oxide maintained in the rat as long as spiroxamine is administered, *e.g.* under chronic feeding conditions.

This also means that the major part of the absorbed N-oxide originating from plant residues will not remain as such in the organism but rather be reduced to spiroxamine and then metabolised as described in the metabolism reports and also shown on the degradation pathway (refer to Figure CA 5.1.1).

Additionally, the fraction which is neither reduced nor hydrolysed will be eliminated from the body even faster than the parent compound. This is due to the increased water solubility of the N-oxide as compared to spiroxamine as a consequence of the high polarity of the N-»0-bond[2]. These physical-chemical properties render an accumulation in the tissues highly unlikely.

The N-oxide was tested for its acute oral toxicity in female rats which was the more sensitive gender with Spiroxamine. The method was essentially the same as with the spiroxamine. It could be demonstrated that the N-oxide has a lower acute toxicity than the parent compound. The lowest lethal dose was in the same range.

1 Mitchard, M. (1971). Bioreduction of organic nitrogen. *Xenobiotica* **1**, pp 469
Bickel, M. (1971). Liver metabolic reactions. Tertiary amine N-dealkylation, tertiary smine N-oxidation, N-oxide reduction, and N-oxide N-dealkylation *Arch. Biochem. Biophys*. **148**. Pp 54-62
White, R.H., Sutanger, M., Mattocks, A.R., Bailey, E., Farmer, P.B.& Connors, T.A. (1989) Reduction of nitromin to nitrogen mustard: Unscheduled DNA synthesis in aerobic or anaerobic rat hepatocytes, JB1, BL8, and Walker carcinoma cell lines. *Carcinogenesis* **10**, pp 2113

2 Bickel, M. (1969). The pharmacology and biochemistry of N-oxides. *Pharmacol. Rev*. **21**., pp 325-355

3 Hamill, S. & Cooper, D.Y. (1984). The role of cytochrome P-450 in the dual pathways of N-demethylation of N.N'-dimethylaniline by hepatic microsomes. *Xenobiotica* **14**, pp 139-149
Ziegler, D.M. (1980). Microsomal flavin-containing monooxygenase: oxygenation of nucleophilic nitrogen and sulfur compounds. In: Enzymatic Basis of Detoxification, Vol. 1, pp. 201-227 (Academic Press).
Akesson, B., Vinge, E. & Skerfving, S. (1989) Pharmacokinetics of triethylamine and triethylamine-N-oxide in man. *Toxicol. Appl. Pharmacol*. **100**. Pp 529

The N-oxide was also tested in the *Salmonella*/microsome test for induction of point mutations. The test was performed with 5 *Salmonella* strains with and without metabolic activation. The test was negative which is in line with the knowledge of the reactivity of N-oxides of tertiary amines.

When assessing the toxicological potential of the N-oxide, the reactions as described have to be taken into account. An intake of the N-oxide resulting from residues will lead prior to absorption to a certain extent to a hydrolytic formation of t-butyl-cyclohexanone which is virtually non-toxic. Its NOAEL after acute oral and dermal application in rats is at least 5000 mg/kg bw. It also exhibits no reactive properties in the primary irritation and sensitization test[4]. This may be related to the easy and quick metabolisation leading to the corresponding alcohol and its conjugates[5]. The non hydrolized part which will be absorbed will be reduced to the parent compound and undergo the same metabolic pathway. Accordingly, the toxicity of the N-oxide is covered by the toxicity of the spiroxamine.

Thus, the available data for the N-oxide, including limited toxicity data and special studies for its hydrolysis in gastric juice, as well as existing knowledge on the reactions and metabolic behaviour of N-oxides from literature lead to the conclusion that the N-oxide is not of toxicological concern and is covered by the toxicity data of spiroxamine.

This information and the quantitative consideration as described in may be used to conclude that any derived acceptable exposure level taken from the spiroxamine data base may be representative also for the N-oxide.

Data Point:	KCA 5.8.1/04
Report Author:	[redacted]
Report Year:	1995
Report Title:	KWG 4168-N-Oxide: Pilot study for acute oral toxicity in female rats
Report No:	23716
Document No:	M-016338-01-1
Guideline(s) followed in study:	OECD 401; US-EPA Series 81-1
Deviations from current test guideline:	Yes Although the study was broadly comparable to the now deleted OECD 401 (1987) test guideline, a number of deficiencies were noted (refer to Results, Deficiencies section below).
Previous evaluation:	yes, evaluated and accepted DAR (2010)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

In an acute oral toxicity study, broadly comparable to OECD 401 test guideline, a group of female Han Wistar rats (5/group) were administered the test article, spiroxamine N-oxide as a single dose at 500 or 1000 mg/kg bw, suspended in 2% Cremophor, employing a dose volume of 10 mL/kg bw. The observation period was 14 days post-exposure.

Clinical signs of toxicity occurred shortly after administration, lasting until a maximum of either 5 or 3 days for animals dosed at 500 or 1000 mg/kg bw, respectively. Observations included signs which were reflective of CNS toxicity.

4 Opdyke, D.L.J. (1975). Fragrance raw materials monographs p-tert-butylcyclohexanone. *Food Cosmet. Toxicol*. **13**. Pp 729

5 Cheo, K.L., Elliott T.H. & Tao R.C.C. (1967). The metabolism of the isomeric tert.-butylcyclohexanones. *Biochem.J*. **104**, pp 198-204

A single animal from the 500 mg/kg bw and four animals from the 1000 mg/kg bw group died within 1 day of dosing. No gross necropsy was performed.

Under the conditions of this study the acute oral LD_{50} for spiroxamine N-oxide was 707 mg/kg bw in female rats. Therefore, according to Annex I for Regulation (EC) 1272/2008 spiroxamine N-oxide must be classified in Category 4. The signal word "Warning" and hazard statement H302 "Harmful if swallowed" are required.

Materials and Methods

A. Materials:

1. Test Material:	KWG 4168-N-oxide (alternative names: Spiroxamine N-oxide, [(8-tert-butyl-1,4-dioxaspiro[4.5]dec-2-yl)methyl](ethyl)(propyl)amine-N-oxide, M03)
Description:	Yellowish liquid
Lot/Batch No.:	940315ELB01
Purity:	90.0% (w/w) (correction for purity not undertaken)
CAS No.:	148044-85-3
Stability of test compound:	Confirmed stable for the duration of the study (expiry date: 7 November 1995)
2. Vehicle and/or positive control:	2% Cremophor / not relevant
3. Test animals:	
Species:	Rat
Strain:	Han Wistar
Age at dosing:	11-13 wks
Weight at dosing:	♀: 171 - 205g
Source:	[redacted]
Acclimation period:	7 days
Diet:	Altromin® 1324 Diet for rats and mice, *ad libitum* (withdrawn 17 and 2 h prior and post dosing, respectively)
Water:	Municipal water, *ad libitum*
Housing:	5 animals/sex/cage during acclimatisation. Individually housed during study period
4. Environmental conditions:	
Temperature:	21 ±1.5°C
Humidity:	40-70%
Air changes:	12-25 changes/h
Photoperiod:	12 h light/dark cycle

B. Test Performance:

1. In life dates:	9 November 1994 to 29 November 1994 (experimental dates)
2. Animal assignment and treatment:	Animals were selected before administration based on their body weight on the day of grouping. After overnight fasting, the test article was suspended in 2% Cremophor (on the day of dosing) and administered orally *via* gavage at a dose level of 500 and 1000 mg/kg bw, employing a dose volume of 10 mL/kg bw. Feeding was resumed 2h post dosing. The observation period was 14 days post-exposure.

3. Statistics:	LD_{50} was calculated according to Bliss[6] in the manner described by Rosiello *et al*[7] and Baird & Balster[8].

C. Methods:

1. Homogeneity and achieved concentration analysis of the dose:	Not conducted
2. Test article formulation preparation:	The test article was formulated in demineralised water (for *ca.* 1 hour) before administration with the aid of Cremophor EL (2% v/v). Homogeneity was achieved by drawing into a syringe and then pumping out several. No correction for purity was taken into account.
3. Observations:	Animals were observed several times on the day of dose administration (epochs to time not given), with daily observation up until day 14.
3. Body weights:	Weighed on the day before dosing and on days 4, 8 and 15.
4. Food consumption:	Not recorded.
5. Sacrifice and pathology:	All animals were killed at terminal sacrifice, but no gross necropsy was undertaken.

Results

A. Homogeneity and achieved concentration analysis:

Not undertaken. Analyses for achieved concentration, homogeneity or stability of test article formulations were not conducted as part of this study, as this is not a requirement of the regulatory test guidelines.

B. Observations:

1. Clinical signs of toxicity:	Clinical signs of toxicity occurred shortly after administration, lasting until a maximum of either 5 or 3 days for animals dosed at 500 or 1000 mg/kg bw, respectively. Observations included signs which were reflective of CNS toxicity, manifest as laboured breathing, decreased motility, spastic gait, piloerection, narrowed palpebral slits, salivation, red-encrusted nose, and in some cases bloody nasal discharge, periodic lying on side, cramped stretching of extremities, soft or no faeces. The report did not detail how these clinical signs were distributed between the dose groups, with a general onset time of signs (collectively) were observed only.
2. Mortality:	Refer to Table CA 5.8.1/01-1 A single animal from the 500 mg/kg bw and four animals from the 1000 mg/kg bw group died within 1 day of dosing.

C. Body weight and food consumption:

1. Body weight:	Slight body weight loss was observed in 1 ♀ (#1105) at day 3, with increased body weight gain occurring from day 7 onwards. This was an isolated incidence, with the transient decrease in body weight deemed not treatment-related. All other animals body weight increased from day 1 through to day 14 (refer to Table CA 5.2.1/01-1).
2. Food consumption:	Not applicable.

6 Bliss, C.I. (1935). The calculation of the dosage-mortality curve. *Ann. Appl. Biol.* **22**, pp 134
Bliss, C.I. (1938). The determination of the dosage-mortality curve from small numbers. *Q.J. Pharm. Pharmacol.* **11**, pp 192-216

7 Rosiello, A.P., Essigmann, J.M. & Wogan, G.N. (1977). Rapid and accuracte determination of the media lethal dose (LD_{50}) and its error with a small computer. *J. Toxicol. Environ. Health*, **3**, pp 797-809

8 Baird, J.B. & Balster, R.L. (1979). Analysis of nominal dose-effect data with an advanced programmable calculator. *Neurobehaviour. Tox.* **1**, pp 73-7 7

Table CA 5.8.1/01-1: Overview of acute oral toxicity study in rats treated with spiroxamine N-oxide: mortality and body weight

Parameter	♀ (mg/kg bw) 500			♀ (mg/kg bw) 1000		
Mortality[a]	1/5			4/5		
Day	1	8	15	1	8	15
Body weight (g) ±s.d.	202 ±2.7	219 ±5.4	---[b]	173 ±1.8	213 ±0.0	270 ±0.0
Net body weight gain (g)	17.8 ±6.7 (day 1 – 8)			73.9 ±8.5		
Acute oral LD_{50}	>2000 mg/kg bw					

a Mortality: no. of animals found dead / no. of animals treated
b no individual animal data reported

D. Necropsy:

Not undertaken.

E. Deficiencies:

Although the study was broadly comparable to the now deleted OECD 401 (1987) test guideline, the following deficiencies are noted when compared to this guideline.

- Dosing was limited to a single sex (females), therefore it is not known if males are markedly more sensitive or not to the test article.
- No gross histopathological analysis was undertaken
- Tabulation of clinical signs were not provided
- The LD_{50} value estimated was provided without confidence intervals.

Assessment and conclusion by applicant:

Assessment: Study meets the current guidance and the requirements in 283/2013.

Conclusion: Under the conditions of this study the acute oral LD_{50} of spiroxamine N-oxide was 707 mg/kg bw in female rats. Therefore, according to Annex I for Regulation (EC) 1272/2008 spiroxamine N-oxide must be classified in Category 4. The signal word "Warning" and hazard statement H302 "Harmful if swallowed" are required.

Data Point:	KCA 5.8.1/02
Report Author:	[redacted]
Report Year:	1995
Report Title:	KWG 4168-N-Oxid - Salmonella/microsome test plate incorporation and preincubation method
Report No:	24105
Document No:	M-016297-01-1
Guideline(s) followed in study:	Directive 92/69/EEC, Method B.14.; OECD 471; US-EPA PB 84-233295, HG-Gene Muta-S. typhimurium
Deviations from current test guideline:	Yes A number of deficiencies were noted (refer to Results, Deficiencies section below).
Previous evaluation:	yes, evaluated and accepted DAR (2010)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Supportive only

Executive Summary

In a reverse gene mutation assay in bacteria, *S. typhimurium* strains TA98, TA1537, TA100 and TA1535 were exposed to spiroxamine N-oxide (M03) formulated in DMSO using the both the plate incorporation and pre-incubation methodologies in the absence and presence of an Aroclor-1254-induced rat liver post-mitochondrial fraction (S9).

In the plate incorporation assay treatments of the tester strains were performed in the absence and presence of S9 using final concentrations of spiroxamine N-oxide at 16, 50, 158, 500, 1581, 5000 µg/plate (the maximum recommended concentration according to current regulatory guidelines). No evidence of precipitate was observed upon plating. Following these treatments, evidence of toxicity ranging from a complete killing of the test bacteria to a slight thinning of the background bacterial lawn, with or without a concurrent marked reduction in revertant numbers or reductions in titre levels was observed in all the tester strains in the absence and presence of S9 at 1581 and/or 5000 µg/plate.

For Experiment 2 treatments of the tester strains were performed in the absence and presence of S9 utilising the pre-incubation methodology with the maximum test concertation for each strain selected as an estimate of the lower limit of toxicity based on the observations in Experiment 1. The maximum concentrations were 1581 µg/plate for all strains ±S9.

Following these treatments, evidence of toxicity was again observed in all the tester strains in both the absence and presence of S9 at 500 µg/plate and extended down to 1581 µg/plate in each tester strain.

Vehicle and positive control treatments were included for all strains in both experiments. The mean numbers of revertant colonies were generally comparable with acceptable ranges for vehicle control treatments in both treatments. However it was noted that in the pre-incubation experiment, TA100 ±S9 vehicle control individual and mean revertant colonies exceeded the laboratory's historical control range, confirming the data were not representative of the strain.

The positive controls induced an acceptable increase in revertant colony numbers, thereby demonstrating the sensitivity and specificity of the test system. However, the sensitivity of the S9 was only confirmed with a single positive control, 2-aminoanthracene (2-AA). 2-AA can be activated by enzymes other than the microsomal cytochrome P450 family. Consequently, concerns over the specificity of the S9 to metabolise other known mutagens was not conclusively confirmed.

It was concluded that spiroxamine N-oxide did not induce mutation in five histidine-requiring strains (TA98, TA100, TA1535 and TA1537) of *Salmonella typhimurium* when tested under the conditions of this study. These conditions included treatments at concentrations up to 5000 µg/plate (the maximum recommended concentration according to current regulatory guidelines), in the absence and in the

presence of a rat liver metabolic activation system (S9) or up to concentration limited by toxicity using both the plate incorporation and pre-incubation methodologies.

The data generated under this study is considered supplementary with the bacterial reverse gene mutation endpoint sufficient addressed with a new, up to date test guideline compliant GLP study (refer to CA 5.8.1/20 [M-756858-02-1]).

Materials and Methods

A. Materials:

1. Test Material: KWG 4168-N-oxid
(alternative names: Spiroxamine N-oxide, [(8-tert-butyl-1,4-dioxaspiro[4.5]dec-2-yl) methyl](ethyl)(propyl)amine-N-oxide, M03)

Description:	Viscous, colourless mass
Lot/Batch No.:	940315ELB01
Purity:	90.0% (w/w) (correction for purity not undertaken)
CAS No.:	148044-85-3
Stability of test compound:	Confirmed stable for the duration of the study (expiry date: 7 November 1995)

2. Control materials:

Negative:

Solvent/final concentration: DMSO (dimethyl sulphoxide)/0.1 mL/plate

Positive: -S9

Strain	*Mutagen*	*Conc. (µg/plate)*
TA98, TA1537	4-nitro-1,2-phenylene diamine (NPDA)	0.5, 10
TA100	2-nitrofluorene (2-NF)	0.2
TA1535	Sodium azide (NaN_3)	10

Positive: +S9

Strain	*Mutagen*	*Conc. (µg/plate)*
TA98, TA100, TA1535, TA1537	2-aminoanthracene[9] (2-AA)	3

3. Activation: S9 was prepared in house from ♂ Sprague Dawley rats treated with Aroclor 1254 (protein content 26.3 mg/mL). Each batch of S9 was checked for metabolising capacity using reference mutagens. These mutagens however were not specified.

The composition of the 10% S9 reaction mix was: S9 (10%), $MgCl_2$ PBS (2.3 mg/mL), KCl (3.5 mg/mL), glucose-6-phosphate (2.6 mg/mL), β-NADP (4.5 mg/mL), PBS (1.4 mg/mL).

4. Test organisms: *S. typhimurium* strains TA98, TA100, TA1535, TA1537
All test organisms were properly maintained and were checked for appropriate genetic markers (*S. typhimurium*: histidine and biotin requirement, *rfa* mutation, *uvr*B sensitivity, ampicillin-resistance) regularly.

5. Test Concentrations:

a) Mutation assay 1: *Plate incorporation: +/-S9 all strains*:
0, 16, 50, 158, 500, 1581, 5000 µg/plate

b) Mutation assay 2: *Pre-incubation +/-S9*:

9 Of note, some researchers have suggested that 2-AA should not be used as the only positive control to evaluate S9-mix activity as it has been shown that the chemical may be activated by enzymes other than the microsomal cytochrome P450 family. Although no other +S9 positive control was used in this study, the report confirmed that each batch of S9 was checked for metabolising capacity using reference mutagens. These mutagens however were not specified. Therefore, concerns over the specificity of the S9 to metabolise other known mutagens was not conclusively confirmed.

0, 5, 16, 50, 158, 500, 1581 µg/plate

B. Test Performance:

1. In life dates:	19 April 1995 to 2 May 1995 (experimental dates)
2. Experiment 1:	*Plate incorporation assay:* The following sequence of additions of 2 mL of supplemented molten top agar, test article solution/vehicle/positive control (0.1 mL), either buffer (not specified) (0.5 mL) or S9 mix (0.5 mL) for treatments in the absence and in the presence of metabolic activation respectively and bacterial suspension (0.1 mL) were mixed and poured on to Vogel-Bonner E agar plates (minimal glucose agar plate). When set, plates were inverted and incubated at 37°C, protected from light for 2 days.
3. Experiment 2:	*Pre-incubation assay:* The test article solution or vehicle/positive control solution (0.1 mL), bacteria (0.1 mL) and S9 mix (0.5 mL) were mixed in a small test tube and incubated for 20 minutes at 37°C. After additional of 2 mL of top agar solution the mixture was poured onto a minimal glucose agar plate and allowed to solidify. All plates were incubated for 2 days at 37°C. In this way, it was hoped to increase the range of mutagenic chemicals that could be detected using this assay system. For each experiment, quadruplicate plating was undertaken for each dose level.
4. Statistics:	None applied.
5. Acceptance criteria:	The assay was considered valid if the following criteria were met: 1. The vehicle controls fell within the expected range as defined by published data and/or laboratory's historical control ranges 2. The positive control chemicals had to show sufficient effects as defined by the laboratory's experience; 3. Titer determinations had to demonstrate sufficient bacterial density in the suspension.
5. Evaluation criteria:	The test article was considered mutagenic in this assay if: 1. A concentration related increase in revertant numbers was ≥2-fold (TA98, TA100, TA1535), ≥3-fold (TA1537) above the concurrent vehicle control values 2. Any observed response was reproducible under the same treatment conditions. The test article was considered positive in this assay if all the above criteria were met The test article was considered negative in this assay if none of the above criteria were met.

C. Methods:

1. Homogeneity and achieved concentration analysis of the dose:	Not undertaken
2. Test article formulation preparation:	No details provided. However, from the available data, it can be confirmed that the test article, spiroxamine N-oxide was soluble in DMSO at concentrations up to at least 30 mg/mL. Thereby, confirming a maximum concentration of 5000 µg/plate was achievable.
3. Toxicity Assessment:	The background lawns of the plates were examined for signs of toxicity. Revertant plate count data were also assessed, as a marked reduction in revertants compared to the concurrent vehicle controls were also considered as evidence of toxicity. In addition, the titre levels were determined with total bacterial counts taken on 2 plates/dose level studied with S9.
4. Scoring:	The number of revertant colonies were counted with the unaided eye or a colony counter (details not provided). Individual plate counts were recorded

separately and the mean and standard deviation of the plate counts for each treatment were determined. Control counts (vehicle/positive) were compared with the laboratory's historical control range. The purpose of the vehicle historical control range was to assess strain characteristics, and not to interpret or justify a negative or positive result[3].

Results and Discussion

A. Analytical determinations:

Not undertaken. Analyses for achieved concentration, homogeneity or stability of test article formulations were not conducted as part of this study, as this is not a requirement of the regulatory test guidelines.

B. Mutation experiment 1:

In the plate incorporation assay treatments of the tester strains were performed in the absence and presence of S9 using final concentrations of spiroxamine N-oxide at 16, 50, 158, 500, 1581, 5000 µg/plate (the maximum recommended concentration according to current regulatory guidelines). No evidence of precipitate was observed upon plating. Following these treatments, evidence of toxicity ranging from a complete killing of the test bacteria to a slight thinning of the background bacterial lawn, with or without a concurrent marked reduction in revertant numbers or reductions in titre levels was observed in all the tester strains in the absence and presence of S9 at 1581 and or 5000 µg/plate.

Following spiroxamine N-oxide treatments of all the test strains in the absence and in the presence of S9, no increases in revertant numbers were observed that were ≥2-fold (TA98, TA100, TA1537); ≥3-fold (TA1537) above the concurrent vehicle control values.

Vehicle and positive control treatments were included for all strains. The mean numbers of revertant colonies were comparable with acceptable ranges for vehicle control treatments. The positive controls induced an acceptable increase in revertant colony numbers, thereby demonstrating the sensitivity and specificity of the test system.

Table CA 5.8.1/02-1: Spiroxamine N-oxide: bacterial reverse gene mutation data (mean revertant colonies): Mutation experiment 1 (plate incorporation)

Type of mutation	Frame-shift				Base-pair substitution			
Conc. (µg/plate)	TA98		TA1537		TA100		TA1535	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0	18 ±3	30 ±4	8 ±2	7 ±1	78 ±3	91 ±14	10 ±2	10 ±3
16	20 ±4	29 ±2	8 ±1	7 ±1	74 ±7	81 ±12	5 ±2	10 ±3
50	19 ±2	23 ±2	6 ±2	9 ±9	84 ±10	91 ±8	7 ±2	6 ±3
158	22 ±7	25 ±7	8 ±2	7 ±1	68 ±±9	91 ±8	6 ±3	9 ±1
500	18 ±1	24 ±2	6 ±2	7 ±2	65 ±9	82 ±8	7 ±2	8 ±3
1581	7 ±3[B]	13 ±4[B,T]	5 ±2[B]	4 ±1[B]	38 ±2	91 ±10[T]	4 ±1	8 ±4[T]
5000	[B]	[B,T]	0 ±0	0 ±0[B]	[B]	50 ±17[B,T]	[B]	[B,T]
Positive control	153 ±5	1010 ±93	129 ±21	109 ±27	229 ±20	789 ±44	743 ±40	105 ±17

B: diminution of background lawn

T: bacteriotoxic effect evident from marked reduction in titre levels

Positive controls:
-S9: strains:
TA98; TA1537: NPDA
TA100: 2-NF
TA1535: NaN_3

+S9: strains:
TA98; TA1537; TA100; TA1535: 2-AA

B. Mutation experiment 2:

For Experiment 2, treatments of the tester strains were performed in the absence and presence of S9 utilising the pre-incubation methodology with the maximum test concertation for each strain selected as

an estimate of the lower limit of toxicity based on the observations in Experiment 1. The maximum concentrations were 1581 µg/plate for all strains ±S9.

Following these treatments, evidence of toxicity was again observed in all the tester strains in both the absence and presence of S9 at 500 µg/plate and extended down to 1581 µg/plate in each tester strain.

Following spiroxamine N-oxide treatments of all the test strains in the absence and in the presence of S9, no increases in revertant numbers were observed that were ≥2-fold (TA98, TA100, TA1537); ≥3-fold (TA1537) above the concurrent vehicle control values.

Vehicle and positive control treatments were included for all strains. The mean numbers of revertant colonies were generally comparable with acceptable ranges for vehicle control treatments, however it was noted that TA100 ±S9, vehicle control individual and mean revertant colonies exceeded the laboratory's historical control range, without an impact assessment made. The concern from the data presented is that the data were not representative of the strain, raising the concern of genetic drift. The positive controls induced an acceptable increase in revertant colony numbers, thereby demonstrating the sensitivity and specificity of the test system.

Table CA 5.8.1/02-2: Spiroxamine N-oxide: bacterial reverse gene mutation data (mean revertant colonies): Mutation experiment 2

Type of mutation	Frame-shift				Base-pair substitution			
Conc. (µg/plate)	TA98		TA1537		TA100		TA1535	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0	28 ±3	37 ±8	14 ±1	16 ±3	153 ±9	201 ±16	13 ±2	15 ±4
5	30 ±4	44 ±5	10 ±4	11 ±3	153 ±6	192 ±10	14 ±2	14 ±3
16	25 ±2	37 ±4	13 ±4	14 ±1	162 ±19	211 ±8	13 ±3	15 ±3
50	23 ±5	40 ±3	16 ±1	13 ±4	124 ±9	209 ±5	11 ±2	13 ±3
158	33 ±4	45 ±6	8 ±3	13 ±3	123 ±28	167 ±17	12 ±1	15 ±3
500	26 ±6[B]	31 ±5	13 ±2[B]	8 ±3	53 ±12	126 ±15	7 ±2[B]	9 ±1[T]
1581	9 ±3[B]	18 ±5[B,T]	[B]	4 ±1[B,T]	0 ±0[B]	45 ±18[B,T]	[B]	6 ±2[B,T]
Positive control	167 ±15	1320 ±34	130 ±14	322 ±23	320 ±25	1422 ±49	712 ±33	215 ±32

B: diminution of background lawn

T: bacteriotoxic effect evident from marked reduction in titre levels

Positive controls:
-S9: strains:
TA98; TA1537: NPDA
TA100: 2-NF
TA1535: NaN_3

+S9: strains:
TA98; TA1537; TA100; TA1535: 2-AA

C. Discussion:

In two independent experiments and in all strains, in the absence and presence of a rat liver metabolic activation system (S9) no increases in revertant numbers were observed that were ≥1≥2-fold (TA98, TA100, TA1537); ≥3-fold (TA1537) above the concurrent vehicle control values. This study was therefore considered to have provided no evidence of any spiroxamine N-oxide mutagenic activity in this assay system.

The positive controls induced an acceptable increase in revertant colony numbers, thereby demonstrating the sensitivity and specificity of the test system.

Table CA 5.8.1/02-3: Bacterial reverse gene mutation data (mean revertant colonies): historical control ranges – plate incorporation

Type of mutation	Frame-shift				Base-pair substitution			
Parameter	TA98		TA1537		TA100		TA1535	
	-S9	+S9 (10%)	-S9	+S9 (10%)	-S9	+S9 (10%)	-S9	+S9 (10%)
Vehicle control								
Date range	Jan 1994 – Dec 1994		Jan 1994 – Dec 1994		Jan 1994 – Dec 1994		Jan 1994 – Dec 1994	
Median	21	27	9	8	82	105	9	11
Semi Q range	4	5	2	2	9	15	2	3
Positive control								
Date range	Jan 1994 – Dec 1994		Jan 1994 – Dec 1994		Jan 1994 – Dec 1994		Jan 1994 – Dec 1994	
Median	138	1301	105	103	263	1110	706	171
Semi Q range	23	208	35	33	33	330	98	49

The report provided historical control ranges over a multitude of dates (Jul – Dec 1991; Jan – Jul 1992; Jul – Dec 1992; Jan – Jun 1993; Jul – Dec 1993), including numerous vehicles and S9 concentrations. The data presented above pertain to the ranges relevant for this study.
Positive controls:
-S9: strains:
TA98; TA1537: NPDA
TA100: 2-NF
TA1535: NaN_3

+S9: strains:
TA98; TA1537; TA100; TA1535: 2-AA

Table CA 5.8.1/02-4: Bacterial reverse gene mutation data (mean revertant colonies): historical control ranges – pre-incubation

Type of mutation	Frame-shift				Base-pair substitution			
Parameter	TA98		TA1537		TA100		TA1535	
	-S9	+S9 (10%)	-S9	+S9 (10%)	-S9	+S9 (10%)	-S9	+S9 (10%)
Vehicle control								
Date range	Jan 1994 – Dec 1994		Jan 1994 – Dec 1994		Jan 1994 – Dec 1994		Jan 1994 – Dec 1994	
Median	22	29	8	8	99	115	10	11
Semi Q range	5	6	2	2	10	13	2	3
Positive control								
Date range	Jan 1994 – Dec 1994		Jan 1994 – Dec 1994		Jan 1994 – Dec 1994		Jan 1994 – Dec 1994	
Median	137	1268	98	178	402	1357	185	787
Semi Q range	26	208	33	55	41	166	35	116

The report provided historical control ranges over a multitude of dates (Jul – Dec 1992; Jan – Jun 1993; Jul – Dec 1993), including numerous vehicles and S9 concentrations. The data presented above pertain to the ranges relevant for this study.
Positive controls:
-S9: strains:
TA98; TA1537: NPDA
TA100: 2-NF
TA1535: NaN_3

+S9: strains:
TA98; TA1537; TA100; TA1535: 2-AA

D. Deficiencies:

When the study methodology is compared to current test guideline requirements (OECD 471, 1997) the following deficiencies are noted:

- The absence of a 5th strain, *S. typhimurium* TA102 or *E. coli* WP2*uvr*A/WP2*urv*A (pKM101).

- In the absence of S9 certificate of analysis presented in the report, the sensitivity of the exogenous rat liver post-mitochondrial fraction (S9) was only confirmed with a single positive control, 2-aminoanthracene (2-AA). 2-AA can be activated by enzymes other than the microsomal cytochrome P450 family. Consequently, concerns over the specificity of the S9 to metabolise other known mutagens was not conclusively confirmed.
- A collective acceptance of validity criteria was adopted to confirm acceptable vehicle control data, as defined by published data/and or laboratory's historical control ranges. No data for the former were presented. For the bacterial (Ames) reverse mutation assay the laboratory's historical control range should be established with at the very least a 99% reference range (at least 300 data points needed for this) applied. The inclusion to accepted published data is inappropriate and creates confusion, with data generated from numerous laboratories, undertaking the Ames assay slightly differently to one another, using different consumables etc. which are known to have effects upon revertant colonies numbers.
- From the data presented in the report, vehicle control revertant colony counts for TA100 ±S9, pre-incubation exceeded the relevant historical control ranges, which confirm that the data are not characteristic of the strain, with genetic drift of likely concern.
- Due to the low spontaneous revertant colony frequency of TA1535, ≥2-fold increase above the concurrent vehicle control values is inappropriate, with the current test guideline recommending ≥3-fold.

In conclusion, the data generated under this study are considered supplementary with the bacterial reverse gene mutation endpoint sufficient addressed with a new, up to date test guideline compliant GLP study (refer to CA 5.8.1/20 [M-756858-02-1]).

Assessment and conclusion by applicant:

Assessment: This study is deemed supplementary as a number of deficiencies are identified when assessed against current test guideline requirements.

Conclusion: It was concluded that spiroxamine N-oxide did not induce mutation in four histidine-requiring strains (TA98, TA100, TA1535 and TA1537) of *Salmonella typhimurium* when tested under the conditions of this study. These conditions included treatments at concentrations up to 5000 µg/plate (the maximum recommended concentration according to current regulatory guidelines), in the absence and in the presence of a rat liver metabolic activation system (S9) or up to concentration limited by toxicity using both the plate incorporation and pre-incubation methodologies.

Data Point:	KCA 5.8.1/20
Report Author:	[redacted]
Report Year:	2020
Report Title:	Amendment no. 01: Spiroxamine N-oxide: Bacterial reverse mutation assay
Report No:	8406980
Document No:	M-756858-02-1
Guideline(s) followed in study:	OECD Guideline 471 (OECD, 1997)
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

In a reverse gene mutation assay in bacteria, *S. typhimurium* strains TA98, TA1537, TA100, TA1535 and TA102 were exposed to spiroxamine N-oxide (M03) formulated in DMSO using the both the plate incorporation and pre-incubation methodologies in the absence and presence of an Aroclor-1254-induced rat liver post-mitochondrial fraction (S9).

Mutation Experiment 1 treatments of all the tester strains were performed in the absence and in the presence of S9, using final concentrations of spiroxamine N-oxide at 0, 5, 16, 50, 160, 500, 1600 and 5000 µg/plate. Following these treatments, evidence of toxicity manifest as thinning of the background lawn and/or complete killing of the bacterial background lawn was observed at 1600 and/or 5000 µg/plate, respectively in all the tester strains in both the absence and presence of S9.

Mutation Experiment 2 treatments of all the tester strains were performed in the absence (plate incorporation methodology) and in the presence of S9 (pre-incubation methodology). The maximum test concentration for each strain was selected as an estimate of the lower limit of toxicity based on the observations in Experiment 1, and these were 5000 µg/plate for strain TA100 in the absence and presence of S9 and strains TA1535 and TA1537 in the presence of S9, and 2000 µg/plate for all other strain treatments. Narrowed concentration intervals were employed covering the range 8.192 – 2000 µg/plate or 20.48 – 5000 µg/plate, in order to examine more closely those concentrations of spiroxamine N-oxide approaching the maximum test concentration and considered therefore most likely to provide evidence of any mutagenic activity. Following these treatments, evidence of toxicity was again observed in all the tester strains in both the absence and presence of S9, and extended down to either 800, 2000 or 5000 µg/plate.

Following spiroxamine N-oxide treatments of all the test strains in the absence and in the presence of S9, no increases in revertant numbers were observed that were ≥1.5-fold (TA102); ≥2-fold (TA98, TA100); ≥3-fold (TA1535, TA1537) above the concurrent vehicle control in either experiment.

Vehicle and positive control treatments were included for all strains in both experiments. The mean numbers of revertant colonies were comparable with acceptable ranges for vehicle control treatments. The positive controls induced an acceptable increase in revertant colony numbers, thereby demonstrating the sensitivity and specificity of the test system.

It was concluded that spiroxamine N-oxide did not induce mutation in four histidine-requiring strains (TA98, TA100, TA1535, TA1537 and TA102) of *Salmonella typhimurium* when tested under the conditions of this study. These conditions included treatments at concentrations up to 5000 µg/plate (the maximum recommended concentration according to current regulatory guidelines), in the absence and in the presence of a rat liver metabolic activation system (S9) or up to concentration limited by toxicity using both the plate incorporation and pre-incubation methodologies.

Materials and Methods

A. Materials:

1. Test Material: Spiroxamine N-oxide
(alternative name: [(8-tert-butyl-1,4-dioxaspiro[4.5]dec-2-yl) methyl](ethyl)(propyl)amine-N-oxide, M03)

Description:	Light yellow liquid
Lot/Batch No.:	AE 1344305 00 1C74 0001
Purity:	72.9% (w/w) (correction factor 1.37 applied)
CAS No.:	148044-85-3
Stability of test compound:	Confirmed stable for the duration of the study (expiry date: 28 November 2022)

2. Control materials:

Negative:

Solvent/final concentration: DMSO (dimethyl sulphoxide) 0.1 or 0.05 mL/plate (plate incorporation or pre-incubation, respectively)

Positive: -S9

Strain	*Mutagen*	*Conc. (µg/plate)*
TA98	2-nitrofluorene (2-NF)	5
TA100, TA1535	Sodium azide (NaN_3)	2
TA1537	9-aminoacridine (9-AAC)	50
TA102	Mitomycin C (MMC)	0.2

Positive: +S9

Strain	*Mutagen*	*Conc. (µg/plate)*
TA98	Benzo[a]pyrene (B[a]P)	5
TA100, TA1535, TA1537	2-aminoanthracene[10] (2-AA)	5
TA102		20

3. Activation: S9 was purchased from a commercial source. ♂ Sprague Dawley rats were treated with Aroclor 1254 (supplied by [redacted] lot no.: 4029, protein content 3.7 mg/mL). The composition of the 10% S9 reaction mix was: 100 µL S9, Na PBS (100 µM), glucose-6-phosphate (5 µM), β-NADP (4 µM), $MgCl_2$ (8 µM), KCl (33 µM), water (to volume).

4. Test organisms: *S. typhimurium* strains: TA98, TA100, TA1535, TA1537, TA102
All test organisms were properly maintained and were checked for appropriate genetic markers (*S. typhimurium*: histidine and biotin requirement, *rfa* mutation, *uvr*B sensitivity, ampicillin-resistance) regularly.

5. Test Concentrations:

a) Mutation assay 1: *Plate incorporation, +/-S9 all strains*:
0, 5, 16, 50, 160, 500, 1600, 5000 µg/plate

b) Mutation assay 2: *Plate incorporation, -S9*:
TA98, TA1535, TA1537, TA102: 0, 8.192, 20.48, 51.2, 128, 320, 800, 2000 µg/plate
TA100: 0, 20.48, 20.48, 128, 320, 800, 2000, 5000 µg/plate
Pre-incubation +S9:
TA98, TA102: 0, 8.192, 20.48, 51.2, 128, 320, 800, 2000 µg/plate
TA100, TA1535, TA1537: 0, 20.48, 20.48, 128, 320, 800, 2000, 5000 µg/plate

10 Of note, some researchers have suggested that 2-AA should not be used as the only positive control to evaluate S9-mix activity as it has been shown that the chemical may be activated by enzymes other than the microsomal cytochrome P450 family. In this study, S9 activity was evaluated with both 2-AA and B[a]P. In addition, each batch was checked by the manufacturer of S9 for sterility, protein content, ability to convert ethidium bromide, cyclophosphamide, B[a]P and AAN to bacterial mutagens, and cytochrome P-450-catalysed enzyme activities (alkoxyresorufin-*O*-dealkylase activities). Therefore, there is no concern over S9 activity.

B. Test Performance:

1. In life dates:	25 April 2019 to 8 May 2019 (experimental dates)
2. Experiment 1:	*Plate incorporation assay:* The following sequence of additions of 2 mL of supplemented molten top agar, test article solution/vehicle control (0.1 mL) or positive control solution (0.05 mL), either 0.1 M Na phosphate buffer (0.5 mL pH 7.4) or S9 mix (0.5 mL) for treatments in the absence and in the presence of metabolic activation, respectively and bacterial suspension (0.1 mL) were mixed and poured on to Vogel-Bonner E agar plates (minimal glucose agar plate). When set, plates were inverted and incubated at 37°C protected from light for 3 days.
3. Experiment 2:	*Plate incorporation assay:* Undertaken for bacterial strains treated in the absence of S9 (refer above). *Pre-incubation assay:* The test article solution or vehicle/positive control solution (0.05 mL), bacteria (0.1 mL) and S9 mix (0.5 mL) were mixed in a small test tube and incubated for 20 minutes at 37°C. After additional of 2 mL of top agar solution the mixture was poured onto a minimal glucose agar plate and allowed to solidify. All plates were incubated for 3 days at 37°C. In this way, it was hoped to increase the range of mutagenic chemicals that could be detected using this assay system. For each experiment triplicate plating was undertaken for each dose level.
4. Statistics:	None applied.
5. Acceptance criteria:	The assay was considered valid if the following criteria were met: 1. The vehicle controls fell within the laboratory's historical control ranges 2. The positive control chemicals induced increases in revertant numbers of ≥1.5-fold (TA102); ≥2-fold (TA98, TA100); ≥3-fold (TA1535, TA1537) above the concurrent vehicle control confirming discrimination between difference strains and an active S9 preparation.
5. Evaluation criteria:	The test article was considered mutagenic in this assay if: 1. A concentration related increase in revertant numbers was ≥1.5-fold (TA102); ≥2-fold (TA98, TA100); ≥3-fold (TA1535, TA1537) above the concurrent vehicle control values 2. Any observed response was reproducible under the same treatment conditions. The test article was considered positive in this assay if all the above criteria were met The test article was considered negative in this assay if none of the above criteria were met.

C. Methods:

1. Homogeneity and achieved concentration analysis of the dose:	Not undertaken.
2. Test article formulation preparation:	To correct for purity, a correction factor of 1.37 was applied. A preliminary solubility test confirmed spiroxamine N-oxide was soluble in DMSO at concentrations equivalent to 116 mg/mL. Thereby, confirming a maximum concentration of 50 mg/mL could be prepared and dosed into the test system at a maximum concentration of 5000 µg/plate. Test article stock solutions were prepared by formulating spiroxamine N-oxide under subdued lighting in DMSO with the aid of vortex mixing and warming at 37°C, to give the maximum required treatment solution concentration. Subsequent dilutions were made using DMSO. The test article solutions were protected from light and used within approximately 4.5 h of initial formulation.
3. Toxicity Assessment:	The background lawns of the plates were examined for signs of toxicity. Revertant plate count data were also assessed, as a marked reduction in

revertants compared to the concurrent vehicle controls were also considered as evidence of toxicity.

4. Scoring: The number of revertant colonies were counted with the unaided eye or a colony counter (Ames Scorer, Perceptive Instruments). Manual scoring with the unaided eye was used when confounding factors such as bubbles or splits in the agar affected the accuracy of the automated counter.
Individual plate counts were recorded separately and the mean and standard deviation of the plate counts for each treatment were determined. Control counts (vehicle/positive) were compared with the laboratory's historical control range. The purpose of the vehicle historical control range was to assess strain characteristics, and not to interpret or justify a negative or positive result[3].

Results and Discussion

A. Analytical determinations:

Not undertaken. Analyses for achieved concentration, homogeneity or stability of test article formulations were not conducted as part of this study, as this is not a requirement of the regulatory test guidelines.

B. Mutation experiment 1:

In the plate incorporation assay treatments of the tester strains were performed in the absence and presence of S9 using final concentrations of spiroxamine N-oxide at 5, 16, 50, 160, 500, 1600 and 5000 µg/plate (the maximum recommended concentration according to current regulatory guidelines). No evidence of precipitate was observed upon plating. Following these treatments, evidence of toxicity ranging from a complete killing of the test bacteria to a slight thinning of the background bacterial lawn, with or without a concurrent marked reduction in revertant numbers was observed in all the tester strains in the absence and presence of S9 at 1600 and/or 5000 µg/plate. Following spiroxamine N-oxide treatments of all the test strains in the absence and in the presence of S9, no increases in revertant numbers were observed that were ≥1.5-fold (TA102); ≥2-fold (TA98, TA100); ≥3-fold (TA1535, TA1537) above the concurrent vehicle control.

Table CA 5.8.1/20-1: Spiroxamine N-oxide: bacterial reverse gene mutation data (mean revertant colonies): Mutation experiment 1 (plate incorporation)

Type of mutation	Frame-shift				Base-pair substitution					
Conc. (µg/plate)	TA98		TA1537		TA100		TA1535		TA102	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0	30.7 ±7.5	50.7 ±2.1	14.3 ±2.3[MB]	20.0 ±4.6	102.0 ±1.6	123.0 ±3.0	10.7 ±4.9	13.0 ±4.4	236.7 ±18.5	337.7 ±15.7
5	40.7 ±4.5	47.0 ±6.6	14.0 ±1.0	21.7 ±10.4	102.0 ±4.6	138.0 ±15.1	11.3 ±1.5	16.0 ±3.0	247.7 ±14.2	348.3 ±5.5
16	37.7 ±1.5	56.3 ±10.8	16.7 ±2.9	24.3 ±4.9	116.0 ±0.8	125.0 ±1.7	9.0 ±2.6	15.7 ±4.6	256.7 ±3.8	342.3 ±15.6
50	41.0 ±13.9	61.0 ±10.	12.3 ±2.3	28.0 ±2.0	108.7 ±5.1	123.3 ±11.0	9.7 ±2.3	12.3 ±6.0	280.7 ±9.0	334.7 ±14.2
160	39.7 ±10.1	61.5 ±2.1	13.3 ±2.1	29.0 ±3.0	109.3 ±4.2	125.3 ±17.2	12.7 ±4.2	13.7 ±4.0	270.7 ±6.4	370.0 ±34.4
500	33.3 ±8.6	64.0 ±6.1	16.0 ±3.6	28.3 ±10.4	115.7 ±12.5	115.3 ±11.5	14.3 ±3.8	12.3 ±2.3	220.7 ±16.0	309.0 ±20.0
1600	19.0 ±3.6	27.0 ±4.4	3.0 2.1[S]	18.0 ±3.5	98.3 ±7.5	106.3 ±16.3	9.0 ±4.6[S]	12.3 ±5.0	27.3 ±12.7[S]	26.0 ±7.5[S]
5000	14.3[S] ±2.3	19.7 ±1.5[S]	T	T	30.0 ±7.9[S]	9.7 ±5.7[S]	T	T	T	T
Positive control	1393.7 ±67.8	319.0 ±21.1	793.0 ±151.9	359.0 ±31.4	1198.0 ±42.6	3478.0 ±53.7	721.3 ±28.0	252.3 ±13.5	914.7 ±134.0	2179.3 ±299.2

Type of mutation	Frame-shift				Base-pair substitution					
Conc.	TA98		TA1537		TA100		TA1535		TA102	
(µg/plate)	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9

B: bubbles in agar
C: based on 2 replicates only. 1 replicate contaminated
M: manual counting
Positive controls:
-S9: strains:
TA98: 2-NF
TA1537: 9-AAC
TA100; TA1535: NaN_3
TA102: MMC

S: slight thinning of background lawn
T: Toxic, no revertant colonies

+S9: strains:
TA98; B[a]P
TA1537; TA100; TA1535; TA102: 2-AA

B. Mutation experiment 2:

For Experiment 2, treatments of the tester strains were performed in the absence (utilising the plate incorporation methodology) and presence of S9 (pre-incubation) with the maximum test concentration for each strain selected as an estimate of the lower limit of toxicity based on the observations in Experiment 1. The maximum concentrations were 5000 µg/plate for strain TA100 ±S9, TA1535 and TA1537 +S9; for all other strains and treatments this was 2000 µg/plate. Concentration intervals were narrowed covering the range 8.192 – 2000 µg/plate or 20.48 – 5000 µg/plate in order to examine more closely those concentrations of spiroxamine N-oxide approaching the maximum test concentration and considered therefore most likely to provide evidence of any mutagenic activity.

Following these treatments, evidence of toxicity was again observed in all the tester strains in both the absence and presence of S9, and extended down to either 800, 2000 or 5000 µg/plate in each tester strain.

Following spiroxamine N-oxide treatments of all the test strains in the absence and in the presence of S9, no increases in revertant numbers were observed that were ≥1.5-fold (TA102); ≥2-fold (TA98, TA100); ≥3-fold (TA1535, TA1537) above the concurrent vehicle control.

Vehicle and positive control treatments were included for all strains in both experiments. The mean numbers of revertant colonies were comparable with acceptable ranges for vehicle control treatments. The positive controls induced an acceptable increase in revertant colony numbers, thereby demonstrating the sensitivity and specificity of the test system.

Table CA 5.8.1/20-2: Spiroxamine N-oxide: bacterial reverse gene mutation data (mean revertant colonies): Mutation experiment 2

Type of mutation	Frame-shift				Base-pair substitution					
Conc.	TA98		TA1537		TA100		TA1535		TA102	
(µg/plate)	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0	35.3 ±7.5	41.0 ±9.5	6.0 ±1.7	13.7 ±2.3	105.7 ±7.2	139.7 ±19.1	11.0 ±6.0	12.7 ±6.4	265.7 ±21.8	302.0 ±29.5
8.192	37.3 ±7.5	39.3 ±1.5	6.7 ±1.2	-	-	-	16.7 ±3.1	-	281.0 ±9.8	343.3 ±17.6
20.48	33.3 ±3.8	37.3 ±3.2	7.0 ±0.0	15.7 ±0.6	97.3 ±13.3	117.7 ±17.8	6.7 ±1.5	11.0 ±4.0	267.3 ±8.1	319.0 ±26.1
51.2	40.7 ±2.9	46.7 ±8.3	8.7 ±4.7	13.7 ±0.6	104.3 ±8.1	131.3 ±7.0	9.0 ±2.0	14.3 ±2.3	267.0 ±6.6	366.3 ±4.2
128	32.3 ±1.5	42.3 ±5.8	6.0 ±2.0	18.3 ±4.0	101.7 ±10.0	130.3 ±9.6	8.3 ±1.5	9.7 ±1.5	257.3 ±8.5	335.3 ±33.0
320	38.0 ±3.0	51.0 ±13.1	7.7 ±1.2	14.7 ±2.1	94.3 ±8.7	124.3 ±5.7	11.0 ±4.4	12.7 ±4.2	233.0 ±5.2	316.3 ±27.5
800	27.3 ±6.4	26.7 ±7.1^{S}	7.7 ±1.2	8.7 ±4.0^{S}	93.3 ±6.8	121.7 ±18.0	5.0 ±0.0	12.0 ±1.7	87.0 ±7.0^{S}	132.0 29.5^{S}

Type of mutation	Frame-shift				Base-pair substitution					
Conc.	TA98		TA1537		TA100		TA1535		TA102	
(µg/plate)	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
2000	6.3 ±3.1[S]	[T]	3.7[S] ±1.5	[T]	95.3 ±2.1	89.3 ±10.7[S]	2.0 ±1.0[S]	[T]	[T]	[T]
5000	-	-	-	[T]	20.0 ±12.5[S]	[T]	-	[T]	-	[T]
Positive control	1415.3 ±24.5	368.0 ±15.6	429.7 ±45.1	442.3 ±76.7	1054.7 ±24.2	3149.3 ±117.8	727.0 ±22.6	159.7 ±34.0	824.7 ±64.6	927.3 ±94.5

S: slight thinning of background lawn
T: Toxic, no revertant colonies
Positive controls:
-S9: strains (plate incorporation):
TA98: 2-NF
TA1537: 9-AAC
TA100; TA1535: NaN_3
TA102: MMC

T: Toxic, no revertant colonies
- dose level not treated

+S9: strains (pre-incubation):
TA98: B[a]P
TA1537; TA100; TA1535; TA102: 2-AA

C. Discussion:

In two independent experiments and in all strains in the absence and presence of a rat liver metabolic activation system (S9), no increases in revertant numbers were observed that were ≥1.5-fold (in strain TA102), ≥2-fold (in strains TA98 and TA100) or ≥3-fold (in strains TA1535 and TA1537) the concurrent vehicle control. This study was therefore considered to have provided no evidence of any spiroxamine N-oxide mutagenic activity in this assay system.

The positive controls induced an acceptable increase in revertant colony numbers, thereby demonstrating the sensitivity and specificity of the test system.

Table CA 5.8.1/20-3: Bacterial reverse gene mutation data (mean revertant colonies): historical control ranges

Type of mutation	Frame-shift				Base-pair substitution					
Parameter	TA98		TA1537		TA100		TA1535		TA102	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Vehicle control										
Date range	Jan 19 – Mar 19		Oct 17 – Feb 18		Oct 17 – Feb 18		Jun 18 – Sep 18		Oct 17 – Feb 18	
n (studies)	77	75	76	76	103	100	78	76	72	72
n (plates)	300	291	299	292	377	370	287	281	266	274
Mean	23.1	36.3	10.0	13.9	101.8	108.7	19.6	18.8	290.4	315.7
99% L.R.R	10	20	1	5	56	72	7	5	220	193
99% U.R.R	46	64	22	29	168	168	35	37	403	411
Positive control										
Date range	Jan 19 – Mar 19		Oct 17 – Feb 18		Oct 17 – Feb 18		Jun 18 – Sep 18		Oct 17 – Feb 18	
n (studies)	77	75	75	76	102	98	78	76	72	71
n (plates)	285	275	294	276	372	351	287	278	264	255
Mean	1170.8	350.8	303.3	286.3	650.2	1524.3	668.1	190.2	936.9	1559.8
99% L.R.R	328	203	84	41	431	455	234	37	454	368
99% U.R.R	3312	711	885	550	1470	2884	927	614	2148	3566

Positive controls:
-S9: strains:
TA98: 2-NF
TA1537: 9-AAC
TA100; TA1535: NaN_3
TA102: MMC

+S9: strains:
TA98: B[a]P
TA1537; TA100; TA1535: TA102: 2-AA
Upper / lower reference range

D. Deficiencies:

It is noted that OECD TG 471 has been recently updated (29 June 2020). However, the updated test guideline has only included a correction to a CAS number of an example positive control –S9 for E.coli strain WP2uvrA. Therefore, it is reasonable to conclude that this study, whilst conducted in accordance with the test guideline issued in 1997, is also in accordance with the updated test guideline.

Assessment and conclusion by applicant:

Assessment: Study meets the current guidance and the requirements in 283/2013.

Conclusion: It was concluded that spiroxamine N-oxide did not induce mutation in five histidine-requiring strains (TA98, TA100, TA1535, TA1537 and TA102) of *Salmonella typhimurium* when tested under the conditions of this study. These conditions included treatments at concentrations up to 5000 µg/plate (the maximum recommended concentration according to current regulatory guidelines), in the absence and in the presence of a rat liver metabolic activation system (S9) or up to concentration limited by toxicity using both the plate incorporation and pre-incubation methodologies.

Data Point:	KCA 5.8.1.03
Report Author:	[redacted]
Report Year:	1998
Report Title:	KWG4168-N-Oxid - V79-HPRT test in vitro for the detection of induced forward mutations
Report No:	28143
Document No:	M-006500-01-2
Guideline(s) followed in study:	Directive 88/302/EEC; OECD 476; US-EPA 712-C-98-221; OPPTS 870.5300
Deviations from current test guideline:	Yes A number of deficiencies were noted (refer to Results, Deficiencies section below).
Previous evaluation:	yes, evaluated and accepted DAR (2010)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Supportive only

Executive Summary

Spiroxamine N-oxide was assayed for the ability to induce mutation at the *hprt* locus (6-thioganine (6-TG) resistance) in Chinese hamster V79 lung cells. The study consisted of a preliminary cytotoxicity assay followed by a Mutation Experiment, each conducted in the absence and presence of metabolic activation by an Aroclor-1254 induced rat liver post-mitochondrial fraction (S9).

The test article was formulated in deionised water and dosed at 2% v/v.

A 3-hour treatment incubation period was used in the presence of S9. In the absence of S9, treatments were performed using 3 and 24-hour treatment incubation periods.

In the preliminary cytotoxicity assay, nine concentrations were tested in the absence (5 hours) and presence (5 hours) of S9 ranging from 19.5 to 5000 µg/mL (a concentration deemed to be the maximum recommended concentration in accordance with the *in vitro* genotoxicity test guidelines followed at the time of the study. However, when assessed against current test guideline requirements, the maximum concentration tested should be 10 mM, or 2000 µg/mL, whichever is the lowest. In the case of spiroxamine N-oxide, the maximum concentration tested was equivalent to 16 mM in the preliminary test). No precipitate (observed by eye at the end of treatment) was observed at any concentration tested in the either the absence or presence of S9. Exposure to spiroxamine N-oxide at concentrations from

19.5 to 5000 µg/mL in the absence and presence of S9 resulted in RCE values from 172.8 to 33.2% and 144.2 to 46.7%, respectively. It is noted that overtly high RCE values >100% were observed in the absence of precipitate. It is highly likely that this was attributed to dilution errors when plating for cloning efficiency assessment.

No marked changes in osmolality or pH were observed at the highest concentration tested compared to the concurrent vehicle controls.

In a single gene mutation assay, following exposure in the absence and presence of a rat liver metabolic activation system (S9) no increases mutant frequency were observed that exceeded >2-fold the respective concurrent vehicle control values. This study was therefore considered to have provided no evidence of any spiroxamine N-oxide mutagenic activity in this assay system. However it is recognised that a sufficient level of toxicity was not achieved in either treatment condition, with concerns raised over the assay sensitivity with overtly low spontaneous mutant frequency values observed in the vehicle controls.

The positive controls induced an acceptable increase in mutant frequency, thereby demonstrating the sensitivity and specificity of the test system when assessed against the laboratory's evaluation criteria. Yet, as discussed concerns are raised when evaluated against current test guideline requirements.

It is concluded that spiroxamine N-oxide did not show any increases in the mutant frequency of V79 cells at the *hprt* locus. These conditions included treatments that were neither limited by toxicity (*i.e.* reduction in RCE 10-20%) or precipitate, in the absence (5 hours) and presence (5 hours at 2% (v/v) final concentration of S9 fraction) of rat liver metabolic activation system.

For the reasons identified in the deficiencies section this study is deemed supplementary.

Materials and Methods

A. Materials:

1. Test Material:	KWG 4168-N-oxid (alternative names: Spiroxamine N-oxide, (8-tert-butyl-1,4-dioxaspiro[4.5]dec-2-yl)methyl(ethyl)(propyl)amine-N-oxide, M03)	
Description:	Clear viscous liquid	
Lot/Batch No.:	M001190	
Purity:	90.8% (correction for purity not undertaken) (molecular weight: 313.48 g/mol)	
CAS No.:	148044-85-3	
Stability of test compound:	Confirmed stable for the duration of the study (expiry date: 26 August 1998)	
2. Control materials:		
Vehicle / final concentration:	Deionised water / 2% (v/v)	
Positive: -S9	Ethyl methanesulphonate (EMS, 5 h: 900 µg/mL)	
Positive: +S9	Dimethyl benzanthracene (DMBA, 5 h: 20 µg/mL)	
3. Activation:	S9 was purchased from a commercial source. ♂ Sprague Dawley rats were treated with Aroclor 1254 (supplied by [redacted] lot no.: 85681, protein content 39.0 mg/mL). The composition of the S9 reaction mix was: 40% S9, $MgCl_2$ *x* H_2O (8 mM), KCl (33 mM), glucose-6-phosphate (5 mM), NADP (1 mM), buffer (60%).	
4. Test cells:	V79 cells derived from the lung of ♂ Chinese hamster were stored as frozen stocks in liquid nitrogen. Each batch was purged of *hprt*- mutants, checked for spontaneous mutant frequency and confirmed to be free of mycoplasma. For each experiment the cells were diluted in MEM (see culture medium below) and incubated in a humidified atmosphere of 5% (v/v) CO_2 in air.	

5. Culture medium:	During growth, and post treatment: Eagle's minimal essential medium (MEM) supplied with L-glutamine (2 mM), MEM-vitamins $NaHCO_3$, penicillin (100 units/mL), streptomycin (100 µg/mL), heat-inactivated fetal calf serum (10%). During treatment, serum concentration was reduced to 2%. For selection of mutants, MEM as supplemented above, with selective agent 6-thioganine (6-TG, 10 µg/mL).
6. Source of cells:	V79 cell line, originally derived from the lung of a Chinese hamster obtained from [redacted] (University of Ulm, Germany). These cells were recloned to maintain karyotypic stability. A modal chromosome number of 22 and a doubling time of 10-14 h.
7. Locus examined:	*hprt* (hypoxanthine-guanine phosphoribosyltransferase) locus. The selection agent was 6-thioguanine (6-TG).
8. Test article Concentrations:	
a) Preliminary cytotoxicity assay:	5 h +/-S9: 0, 19.5, 39.1, 78.1, 156.3, 312.5, 625, 1250, 2500, 5000 µg/mL (a dose in excess of the current guideline requirements of 10 mM, or 2000 µg/mL, whichever is the lowest)
b) Mutation assays:	Experiment 1: 5 h +/-S9: 0, 100, 200, 300, 400, 500, 600, 700 µg/mL (contamination noted 2 days after treatment. Although cultures were plated for cloning efficiency and mutant frequency (MF) assessment and reported, these data have not been considered further) Experiment 2: 5 h -S9: 0, 50, 200, 350, 500, 650, 800, 950 µg/mL 5 h +S9: 0, 100, 200, 300, 400, 500, 600, 700 µg/mL (concentrations underlined were assessed for MF)

B. Study design:

1. In life dates:	4 May 1998 to 23 July 1998 (experimental dates)
2. Vehicle selection:	Spiroxamine N-oxide was soluble at 250 mg/mL in deionised water gave a final concentration of 5000 µg/mL when dosed at 2% v/v (a concentration deemed to be the maximum recommended concentration in accordance with the *in vitro* genotoxicity test guidelines followed at the time of the study. However, when assessed against current test guideline requirements, the maximum concentration tested should be 10 mM, or 2000 µg/mL, whichever is the lowest. In the case of spiroxamine N-oxide, the maximum concentration tested was equivalent to 16 mM in the preliminary test). Osmolality and pH assessments of the test article in cell culture medium were undertaken for the preliminary cytotoxicity study.
3. Statistics:	Dunnett's test
4. Acceptance criteria:	*For test article*: The highest concentration tested was one that allowed the maximum exposure up to 5000 µg/mL for freely soluble compounds, or the limit of toxicity (*i.e.* cloning efficiency reduced to 10% of the concurrent vehicle control) or the limit of solubility. For the calculation of MF, at least 5 dishes/culture had to be available. For a toxic substance, at least 4 analysable concentrations should have been achieved which ideally spanned the toxicity range of 100 - 10% RTG. *For vehicle controls*: The mean vehicle control value for cloning efficiency was ≥50%. The mean MF was <25 $x10^{-6}$ mutants *per* 10^6 viable cells. No heterogeneity between cultures, as assessed by MF <5 mutants *per* 10^6 viable cells for each culture.

For positive controls: Positive controls showed an absolute increase in mean total MF at least 3-fold above that of the mean concurrent vehicle control. There was an absence of confounding technical problems such as contamination, excessive numbers of outliers and excessive toxicity.

5. Evaluation criteria:

The test article was considered mutagenic if:

- A concentration-related increase in mutant frequency (MF) was observed which was at least 2 times that of the vehicle control, with reproducibility in a second experiment.
- Biological relevance was only considered if in addition to the above if no significant change in osmolality compared to the vehicle control was observed.

Equivocal results were concluded if no concentration-related increase in MF, but one or more concentrations induced a biologically relevance increase in MF.

A result was considered negative if no reproducible increase in MF was observed.

C. Methods:

1. Homogeneity and achieved concentration analysis of the dose:

Stability analysis was undertaken at nominal concentrations of 0.02 and 250 mg/mL (room temperature) for 24 h.

Homogeneity and achieved concentration of the dose solution were not undertaken.

2. Cell treatment:

Preliminary cytotoxicity assay: Following cell attachment (16-24 h post culture establishment), cells (4 *x* 10^6 cells/flask) were exposed to test article formulations or vehicle control (added at 2% v/v[11]) for either 5 hours in the absence of S9 or 5 hours in the presence of S9 (final concentration of S9, 2%, v/v). The cell culture media containing 2% FCS was used during test article exposure. At the end of treatment cultures were washed, cells trypsinised and replaced in cell culture media containing 10% FCS at a cell density of 200 cells/Petri dish (3 dishes/culture). These dishes were incubated for 6-8 days to allow colony establishment.

Following colony establishment, colonies were fixed with 95% methanol, stained with Giemsa and counted automatically. Cytotoxicity was expressed by comparison of colonies in treated cultures *vs.* vehicle control cultures (relative cloning efficiency, %RCE). Single cultures were used throughout. Culture volume was 20 mL.

Mutation assays: cells were treated as indicated above, with the exception that duplicate cultures were used. At the end of treatment cultures were washed, cells trypsinised and split for toxicity and mutagenicity assessment:

- Toxicity assessment, as indicated above for the preliminary cytotoxicity assay to allow assessment of toxicity after treatment.
- Mutagenicity assessment: 1 *x* 10^6 cells/culture treated were incubated to express and fix the phenotype. These cells were sub-cultured on day 4, reseeding 1 x 10^5 cells. At the end of the expression period (*ca*. 7 days) cultures were reseeded at 3 x 10^6 cells/dish (8 dishes/culture) in culture medium containing the selective again, 6-TG. A further 200 cells/dish (3 dishes/culture) were seeded in culture medium to determine the absolute CE for each concentration. After 6-7 days of incubation, colonies were fixed, stained with Giemsa and counted to determine the number of 6-TG resistant colonies in the mutation assay dishes and the number of colonies in the CE dishes.

Results

11 It should be noted that the report stated that the final concentration of S9 in the cell culture was 5% v/v. This does not equate, when a 1 mL S9 fraction containing 40% S9 was prepared and this was added to provide a total treatment volume of 20 mL *i.e.* a 5% volume addition of a 40% S9 mix equates to a final S9 concentration of 2% v/v

A. Analytical determinations:

Homogeneity and achieved concentration of the dose solution were not undertaken. Stability analysis in the vehicle (deionised water) confirmed stability at nominal concentration of 0.02 and 250 mg/mL over a 24 hour period, when stored at room temperature. Stability analysis were within 10% of nominal values.

B. Preliminary cytotoxicity assay:

No marked changes in osmolality or pH were observed at the highest concentration tested compared to the concurrent vehicle controls.

In the range-finding experiment, no precipitate (observed by eye at the end of treatment) was observed at any concentration tested in the either the absence or presence of S9.

Exposure to spiroxamine N-oxide at concentrations from 19.5 to 5000 µg/mL in the absence and presence of S9 resulted in RCE values from 172.8 to 33.2% and 144.2 to 46.7%, respectively. It is noted that overtly high RCE values >100% were observed in the absence of precipitate. It is highly likely that this was attributed to dilution errors when plating for cloning efficiency assessment.

Table CA 5.8.1/03-1: Spiroxamine N-oxide mutant frequency data from V79 *hprt*$^-$ cells: short term treatment

Concentration (µg/mL)	5 h -S9		5 h +S9	
	ACE (%)	RCE (%)	ACE (%)	RCE (%)
0[a]	50.2	100.0	58.8	100.0
19.5	58.0	115.6	84.8	144.2
39.1	57.3	114.1	45.8	77.9
78.1	64.5	128.6	63.2	107.4
156.3	73.2	145.8	47.7	81.0
312.5	69.3	138.2	48.0	81.6
625	56.8	113.1	67.7	115.0
1250	76.3	152.2	63.5	107.9
2500	86.7	172.8	29.3	49.9
5000	16.7	33.2	27.5	46.7

RCE: Relative cloning efficiency
ACE: Absolute cloning efficiency
[a] deionised water (1% v/v)

C. Mutation assay:

1. Experiment 1: Cultures were exposure to spiroxamine N-oxide at concentrations from 100 - 5000 µg/mL. Contamination was noted two days after treatment, but the cultures were still carried forward to cloning efficiency and MF assessment. For this reason, these data have not been considered further in this submission.

2. Experiment 2: -S9: Cultures were exposed to spiroxamine N-oxide at concentrations from 50 – 950 µg/mL. Precipitate (assessed by eye at the end of treatment) was not observed at any concentration. Cultures exposed to spiroxamine N-oxide at concentrations from 50 – 650 µg/mL were assessed for determination of MF. Mean RCE values from 103 - 32% were obtained relative to the vehicle control. There were no increases in the mean MF of any of the test concentrations assessed that exceeded the sum of the mean concurrent vehicle control MF and the GEF, within acceptable levels of toxicity.

+S9: Cultures were exposed to spiroxamine N-oxide at concentrations from 20 – 420 µg/mL. Precipitate (assessed by eye at the end of treatment) was observed at concentrations of 260 µg/mL and above. Cultures exposed to spiroxamine at concentrations from 20 – 180 µg/mL were assessed for determination of MF (were plated for determination of MF). RTG values from 91 - 20% were obtained relative to the vehicle control. There were no increases in the mean MF of any of the test concentrations assessed that exceeded the

sum of the mean concurrent vehicle control MF and the GEF, within acceptable levels of toxicity.

3. Positive controls: Vehicle and positive control treatments were included in the Mutation assays in the absence and presence of S9. MF in vehicle control cultures fell within laboratory's observed ranges, and clear increases in mutation were induced by the positive control chemicals EMS (without S9) and DMBA (with S9). Therefore the study was accepted as valid under the laboratory's acceptance criteria.

Table CA 5.8.1/03-2: Spiroxamine N-oxide mutant frequency data from V79 *hprt*$^{+/-}$ cells: short term treatment

Conc. (µg/mL)		5 h -S9			Conc. (µg/mL)		5 h +S9		
		RCE (%)[a]	Mutant plates				RCE (%)[a]	Mutant plates	
			ACE (%)[a]	MF[a]				ACE (%)[a]	MF[a]
0[a]	A	-	72.2	0	0[a]	A	-	81.0	0.5
	B	-	51.8	1.6		B	-	51.5	2.8
Mean		100	62.0	0.8	Mean		100	66.3	1.7
50	A	113.5	94.7	0.4	100	A	146.6	87.3	0.7
	B	63.1	51.5	1.6		B	47.0	52.0	2.7
Mean		88.3	73.1	1.0	Mean		96.8	55	1.7
200	A	131.8	69.3	1.2	200	A	90	99.7	0.4
	B	70.9	96.3	0.4		B	69.4	54.8	0.9
Mean		101	82.8	0.8	Mean		79.7	77.3	0.7
350	A	124.0	65.0	0.6	300	A	133.5	103.3	0.8
	B	81.2	80.3	1.0		B	29.0	55.0	0.7
Mean		102.6	72.7	0.8	Mean		81.2	79.2	1.3
500	A	60.7	86.5	0.6	400	A	115.7	58.3	0.7
	B	57.7	51.0	1.9		B	27.1	84.3	0.6
Mean		59.1	68.8	1.2	Mean		71.4	71.3	0.7
650	A	144	102.0	0.5	500	A	39.8	93.5	1.5
	B	49.7	66.0	0.7		B	35.5	51.5	1.4
Mean		32.1	84.0	0.6	Mean		37.7	7.5	1.5
EMS	A	67.3	59.3	120.1	600	A	75.5	87.3	1.0
	B	89.8	62.0	73.7		B	14.3	62.3	0.8
Mean		78.55	60.7	96.9*	Mean		44.9	74.8	0.9
					DMBA	A	57.2	100.3	62.3
						B	42.2	68.0	68.0
					Mean		49.7	84.2	51.8*

* $p \leq 0.05$
RCE: Relative cloning efficiency
ACE: Absolute cloning efficiency
MF: Mutant Frequency (mutants *per* 10^6 viable cells)

a data presented as individual values in report. Mean values calculated and presented
b deionised water (2% v/v)
Positive control:
-S9: Ethyl methanesulphonate (900 µg/mL)
+S9: Dimethyl benzanthracene (20 µg/mL)

Table CA 5.8.1/03-3: Spiroxamine N-oxide mutant frequency data from V79 *hprt*$^{+/-}$ cells: historical control data

Parameter	Laboratory historical control range (April 1995 to August 1996 based on 20 experiments)			
	Vehicle control		Positive control	
	-S9[a]	+S9[a]	-S9[a]	+S9[a]
MF ±sd	4.2 ±5.0	2.9 ±2.9	650.6 ±172.9	51.9 ±25.1
Observed range	0.0 – 23.4	0.3 – 13.8	227.1 – 992.9	14.6 – 110.7

a Treatment duration which the laboratory historical control data is based upon is not provided
MF: Mutant Frequency (mutants *per* 10^6 viable cells)

Positive control:
-S9: Ethyl methanesulphonate
+S9: Dimethyl benzanthracene

D. Discussion:

In a single gene mutation assay, following exposure in the absence and presence of a rat liver metabolic activation system (S9) no increases mutant frequency were observed that exceeded ≥2-fold the respective concurrent vehicle control values. This study was therefore considered to have provided no evidence of any spiroxamine N-oxide mutagenic activity in this assay system. However it is recognised that a sufficient level of toxicity was not achieved in either treatment condition, with concerns raised over the assay sensitivity with overtly low spontaneous mutant frequency values observed in the vehicle controls.

The positive controls induced an acceptable increase in mutant frequency, thereby demonstrating the sensitivity and specificity of the test system when assessed against the laboratory's evaluation criteria. Yet, as discussed concerns are raised when evaluated against current test guideline requirements.

E. Deficiencies:

Although the study was conducted according to test guideline OECD 476 (1984), this test guideline has since been updated twice in the intervening periods (1997, 2016). When assessed against current test guideline requirements the following deficiencies are noted:

- An insufficient number of cells were treated in the mutation assay (1×10^6 cells/culture) compared to the test guideline requirements (20×10^6 cells/culture). Consequently, a very low spontaneous mutant frequency rate was obtained for the vehicle controls (0.8 – 1.7 mutants *per* 10^6 viable cells) compared to current test guideline recommendations (5 – 20 mutants *per* 10^6 viable cells). This raises the concern over assay sensitivity to detect gene mutations.
- The acceptable range that the laboratory used for acceptance criteria were observed ranges, without a confidence interval applied (ideally 95%). Consequently, the mean mutant frequency values presented are wide and varying (as evidence by a standard deviation value which is either comparable to or exceeds the mean value). This further raises concerns over the laboratory's test methodology adopted to be deemed sufficiently sensitivity to detect gene mutation potential.
- A sufficient level of toxicity was not achieved in either the absence or presence of S9 following a 5 hour treatment exposure with RCE reduced to 32% and 45% of the concurrent vehicle control, respectively.
- Concerns are raised over the heterogeneity between replicated cultures

For the reasons listed above, this study is deemed supplementary. Spiroxamine N-oxide is deemed a fruit metabolite. Under the '*Guidance on the establishment of the residue definition for dietary risk assessment*' (EFSA Journal 2016; 14(12):4549) an *in vitro* mammalian gene mutation assay is not deemed necessary to evaluable the genotoxic potential, with spiroxamine N-oxide deemed devoid of genotoxic potential with a robust, GLP up to date *in vitro* genotoxicity package generated (CA 5.8.1/20, CA 5.8.1/21).

Assessment and conclusion by applicant:

Assessment: For the reasons identified in the deficiencies section this study is deemed supplementary.

Conclusion: It is concluded that spiroxamine N-oxide did not show any increases in the mutant frequency of V79 cells at the *hprt* locus. These conditions included treatments that were neither limited by toxicity (*i.e.* reduction in RCE 10-20%) or precipitate, in the absence (5 hours) and presence (5 hours at 2% (v/v) final concentration of S9 fraction) of rat liver metabolic activation system.

Data Point:	KCA 5.8.1/04
Report Author:	[redacted]
Report Year:	1998
Report Title:	KWG 4168-N-Oxid - In vitro chromosome aberration test with chinese hamster V79 cells
Report No:	27715
Document No:	M-006495-01-1
Guideline(s) followed in study:	Directive 92/69/EEC, Method B.10.; OECD 473; US-EPA 712-C-96-223; OPPTS 870.5375
Deviations from current test guideline:	Yes A number of deficiencies were noted (refer to Results, Deficiencies section below).
Previous evaluation:	yes, evaluated and accepted DAR (2010)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Supportive only

Executive Summary

In a mammalian cell chromosome aberration assay, cultured Chinese hamster V79 lung cells were exposed to spiroxamine N-oxide. The study consisted of a preliminary cytotoxicity assay followed by a two independent chromosome aberration experiment, each conducted in the absence and presence of metabolic activation, by an Aroclor-1254 induced rat liver post-mitochondrial fraction (S9).

The test article was formulated in deionised water and dosed at 2% v/v.

A 5-hour treatment incubation period was used in the absence and presence of S9, with recovery periods of 18 hours (preliminary cytotoxicity and chromosome aberration tests) and a 30 hour (chromosome aberration test only).

Following treatment, no precipitate (observed by eye at the end of treatment) was observed at any concentration tested. Exposure to spiroxamine N-oxide at concentrations of 5 to 5000 µg/mL caused complete toxicity at the highest concentration tested in both the absence and presence of S9. At the next highest concentration, 1800 µg/mL, a reduction in mitotic index of 75% and 38.2% was observed in the absence and presence of S9, respectively. In a further experiment, spiroxamine N-oxide was exposed to cultures at 250 and 500 µg/mL, with a reduction in MI to 79.5% and 35.1% in the absence and presence of S9, respectively.

In the chromosomal aberration assay cultures exposed to spiroxamine N-oxide exhibited insufficient reductions in relative mitotic index (40-50%) in both treatment conditions and recovery periods.

The positive controls, MMC (-S9) and CPA (+S9) induced a statistically significant increase in chromosomal aberrations (excluding gaps), thus confirming the test systems ability to detect potential clastogenic effects in the 18 hour recovery treatments. No positive controls were included in the extended recovery periods.

It is recognised that a sufficient level of toxicity was not achieved in either treatment condition, with concerns raised over the assay sensitivity with overtly low chromosome aberrant frequency observed in the vehicle controls.

It is concluded that spiroxamine N-oxide did not induce biologically relevant increases in the incidence of chromosome aberrations in Chinese hamster V79 cells. These conditions included treatments that were neither limited by toxicity (*i.e.* reduction in % relative MI 45±5%) or precipitate in the absence (4 hours) and presence (4 hours at 2% (v/v) final concentration of S9 fraction) of rat liver metabolic activation system with recovery periods of 18 and 30 hours in both treatment conditions.

Materials and Methods

A. Materials:

1. Test Material:	KWG 4168-N-oxid (alternative names: Spiroxamine N-oxide, [(8-tert-butyl-1,4-dioxaspiro[4.5]dec-2-yl) methyl](ethyl)(propyl)amine-N-oxide, M03)
Description:	Clear viscous liquid
Lot/Batch No.:	M00190
Purity:	90.8% (correction for purity not undertaken) (molecular weight: 313.48 g/mol)
CAS No.:	148044-85-3
Stability of test compound:	Confirmed stable for the duration of the study (expiry date: 26 August 1998)
2. Control materials:	
Vehicle / final concentration:	Deionised water / 2% (v/v)
Positive: -S9	Mitomycin C (MMC, 5 h: 0.1 µg/mL)
Positive: +S9	Cyclophosphamide (CPA, 5 h: 2 µg/mL)
3. Activation:	S9 was prepared in house from Sprague Dawley rats treated with Aroclor 1254 (protein content 40.3 mg/mL). The composition of the S9 reaction mix was: 40% S9, $MgCl_2.6H_2O$ (13.3 mM), KCl (33 mM), glucose-6-phosphate (10 mM), NADP (1.65 mM).
4. Test cells:	V79 cells derived from the lung of Chinese hamster were obtained from Dr. Ulesch, Merk AG, Darmstadt and stored as frozen stocks in liquid nitrogen. For each experiment the cells were diluted in MEM (see culture medium below) and incubated in a humidified atmosphere of 5% (v/v) CO_2 in air. A modal chromosome number of 22 and a doubling time of 12 h. Cells were confirmed mycoplasma free.
5. Culture medium:	During growth, and post treatment: Eagle's minimal essential medium (MEM) supplied with L-glutamine (2 mM), MEM-vitamins $NaHCO_3$, penicillin (50 units/mL), streptomycin (50 µg/mL), heat-inactivated fetal calf serum (10%). During treatment, serum concentration was reduced to 2%
6. Test article Concentrations:	
a) Preliminary cytotoxicity assay:	1st preliminary test: 4 h +/-S9: 0, 5, 10, 50, 100, 500, 1000, 5000 µg/mL (a dose in excess of the current guideline requirements of 10 mM, or 2000 µg/mL, whichever is the lowest) 2nd preliminary test: 4 h +/-S9: 0, 250, 500, 750, 1000, 2000, 3000 µg/mL (concentrations underlined were assessed for MI)
b) Chromosomal aberration assay:	Experiment 1: 4 h (+14 h recovery) -S9: 0, 100, 200, 300, 400, 500 µg/mL 4 h (+14 h) +S9: 0, 200, 300, 400, 500, 600 µg/mL Experiment 2: 4 h (+26 h) -S9: 0, 300, 400, 500 µg/mL 4 h (+26 h) +S9: 0, 400, 500, 600 µg/mL (concentrations underlined were scored for chromosome aberrations)

B. Study design:

1. In life dates:	23 March 1998 to 14 May 1998 (experimental dates)
2. Vehicle selection:	Spiroxamine N-oxide was soluble at 333 mg/mL in deionised water which gave a final concentration of 5000 µg/mL when dosed at 2% v/v (a concentration

deemed to be the maximum recommended concentration in accordance with the *in vitro* genotoxicity test guidelines followed at the time of the study. However, when assessed against current test guideline requirements, the maximum concentration tested should be 10 mM, or 2000 µg/mL, whichever is the lowest. In the case of spiroxamine N-oxide, the maximum concentration tested was equivalent to 16 mM in the preliminary test).
pH and osmolality of the test culture medium was assessed at 5000 µg/mL. No fluctuations in pH of the medium of more than 1 pH unit or osmolality of more than 50 mOsm/kg were observed when compared with the vehicle control. The maximum final concentration tested in the preliminary toxicity test was 5000 µ/mL.

3. Statistics: Fisher's Exact test was used to determine increases in the incidence of chromosome aberrations (5% level).

4. Acceptance criteria: *For vehicle controls:* The mean vehicle control was within the laboratory's historical control range.
For positive controls: Positive controls induced a response that were compatible with the laboratories historical positive control range.

5. Evaluation criteria: The criteria for determining a positive result a relevant and statistically significant increase in aberration rate was obtained.
A negative result was concluded if there were statistical significant value which were within the historical controls.

C. Methods:

1. Homogeneity and achieved concentration analysis of the dose: Stability analysis was undertaken at nominal concentrations of 0.02 and 250 mg/mL (room temperature) for 24 h.
Homogeneity and achieved concentration of the dose solution were not undertaken.

2. Preliminary cytotoxicity assay: Following establishment of cultures (1×10^6 cells), S9 mix or KCl was added as appropriate for treatments in the presence or absence of S9, respectively. Cells were exposed to the test article for 4 hours in the absence or presence of S9 (2% v/v). These cultures were sampled at 18 hours after the beginning of treatment.
At the end of treatment, the test article was removed by washing the monolayer cultures, cells removed by trypsinisation. Cytotoxicity was determined by both MI (a total of 1000 cells assessed for metaphase, refer below) and cell survival (haemocytometer, without fixation).
Single cultures were used for the vehicle control and each test article concentration.

Harvesting and fixation: Two hours before the cells were harvested, mitotic activity was arrested by addition of Colcemid® to each culture at a final concentration of 0.4 µg/mL to inhibit cytokinesis. After 2 hours incubation, each cell suspension was trypsinised, transferred to a centrifuge tube and centrifuged for 5 minutes at 700 rpm. The cell pellets were treated with a hypotonic solution (0.56% KCl). The suspensions were centrifuged the cell pellets fixed by addition of ice-cold fixative (ethanol: acetic acid (3:1 v/v)). Following further centrifugation the supernatant was removed and replaced with fixative; this was repeated until the fixative was clear.

Slide preparation: The fixed pellets were re-suspended, then centrifuged and re-suspended in a small volume of fixative. A few drops of the cell suspensions were dropped onto pre-cleaned microscope slides and allowed to air dry. Two slides were prepared per culture. The slides were then stained in 5% Giemsa. After rinsing in water and once in acetone and then kept in xylene for *ca*. 30 minutes.

Cytotoxicity: The MI, which indicates the ratio of cells in metaphase divided by the total no. of cells observed in a population of cells, was determined using the formula below:

$$MI\ (\%) = \frac{No.\ of\ mitotic\ cells}{Total\ no.\ of\ cells\ scored} \times 100$$

Relative MI (expressed in terms of percentage) for each treated culture was calculated as follows:

$$\text{Relative MI (\%)} = \frac{\text{MI of treated cultures}}{\text{MI of vehicle control}} \times 100$$

Cytotoxicity (%) was expressed as 100 – Relative MI.
A minimum of 1000 cells/culture were examined for cytotoxicity.

3. Chromosomal aberration assay:

Cell treatment: Cells were exposed to the test article formulation, vehicle or positive control as described above, with the exception of duplicate cultures used for the vehicle, positive controls and each test article concentration. Two independent experiment were undertaken, with treatment conditions of 4 hours for both experiments. Recovery times of 18 and 30 hours from the start of treatment were employed for Experiment 1 and 2, respectively in both the absence and presence of S9. As spiroxamine N-oxide is not known to cause cell cycle delay, the extended recovery period was neither justified nor warranted. Untreated controls were included in the 18 hour recovery treatment. However is unclear why these were included as the vehicle used was deionised water. For this reason, the untreated control data has not been included.
Duplicate slides were prepared for each culture. The MI was determined for each culture, expect where there was clear evidence of overt toxicity or no indications of cytotoxicity.

4. Slide scoring: Concentrations were selected for analysis of chromosome aberrations with modal chromosome number of 22. Toxicity was not deemed a limiting factor. Current test guideline requirements consider toxicity a limiting factor, with a reduction in mitotic index of 45±5%. Slides were coded prior to scoring.
The following were considered to be classes of aberrations
Chromatid type:
Chromatid gap: achromatic lesion with a chromatid arm, without obvious dislocation of the chromatid ends. Whilst these should not be counted in the final totals of aberrations, as they may not all be true breaks, the laboratory included them.
Isochromatid gap: same as chromatid gap but at the same locus in both sister chromatids.
Chromatid exchange: exchange of chromatid-parts between different chromosomes
Chromatid break (an achromatic region in one chromatid, larger than the width of a chromatid. The associated fragment may be partially or completely displaced).
Chromosome-type:
Break ("Chromosome gap"). Same as chromatid gap but at the same locus in both sister chromatids. If the gap is large or chromosome fragment displaced, the break is included with chromosome breaks).
Multiple aberrations: when 5 or more structural changes (excluding gaps occur) within one metaphase.
100 metaphase figures from each slide of each culture were examined. The incidence of polyploidy cells (but not endoreduplicated) were also recorded as a percentage of the 100 metaphases analysed.

Results and Discussion

A. Analytical determinations:

Not undertaken. Analyses for achieved concentration or homogeneity of test article formulations were not conducted as part of this study, as this is not a requirement of the regulatory test guidelines.

Stability analysis in the vehicle (deionised water) confirmed stability at nominal concentration of 0.02 and 250 mg/mL over a 24 hour period, when stored at room temperature. Stability analysis were within 10% of nominal values.

B. Preliminary cytotoxicity assay:

1. **Absence of S9:** Following treatment, no precipitate (observed by eye at the end of treatment) was observed at any concentration tested. Exposure to spiroxamine N-oxide at concentrations of 5 to 5000 µg/mL caused complete toxicity at the highest concentration tested. At the next highest concentration, 1000 µg/mL, a reduction in mitotic index of 7.5% was observed. In a further experiment, spiroxamine N-oxide was exposed to cultures at 250 and 500 µg/mL, with a reduction in MI to 79.5%.
2. **Presence of S9:** Following treatment, no precipitate (observed by eye at the end of treatment) was observed at any concentration tested. Exposure to spiroxamine N-oxide at concentrations of 5 to 5000 µg/mL caused complete toxicity at the highest concentration tested. At the next highest concentration, 1000 µg/mL, a reduction in mitotic index of 38.2% was observed. In a further experiment, spiroxamine N-oxide was exposed to cultures at 250 and 500 µg/mL, with a reduction in MI to 35.1%.

   Whilst %survival index was calculated for the preliminary cytotoxicity treatments, this data has not been considered further as it is not considered a reliable assessment of toxicity for this assay type.

   In all cases, overt toxicity (reduction in %MI) was observed, without a clear indication of the toxicity response curve to allow reliable selection of dose concentrations for the chromosomal aberration experiment.

Table CA 5.8.1/04-1: Spiroxamine N-oxide overview of preliminary cytotoxicity data in V79 cells

Dose level (µg/mL)	4 h exposure (+ 18 h recovery) –S9		4 h exposure (+ 18 h recovery) –S9	
	Relative MI (%)	Cytotoxicity (%)	Relative MI (%)	Cytotoxicity (%)
Preliminary cytotoxicity experiment 1				
0	100	0	100	0
5	112.4	-12.4	109.3	-9.3
10	89.1	10.9	114.2	-14.2
50	99.5	0.5	111.1	-11.1
100	109.5	-9.5	102.7	-2.7
500	73.1	26.9	91.6	8.4
1000	92.5	7.5	61.8	38.2
5000	0	100	0	100
Preliminary cytotoxicity experiment 2				
0	100	0	100	0
250	104.1	-4.1	64.9	35.1
500	20.5	79.5	79.7	20.3

C. Chromosome aberration assay:

1. **Short-term treatment in the absence of S9:** 4 h + 18 h recovery:

   Following treatment, no precipitate (observed by eye at the end of treatment) was observed at any concentration tested. Exposure to spiroxamine N-oxide at concentrations of 100 to 500 µg/mL caused reductions in %MI to 79.6%. . The concentrations selected for metaphase analysis were 100, 200 and 300 µg/mL, with % reductions in MI of no cytotoxicity, 8.1% and 33.8%, respectively. No increase in the incidence of cells with structural aberrations or polyploidy cells was observed in any analysed treatment concentrations. However, a sufficient level of cytotoxicity (reduction in %MI 40-50%) was not achieved.

- The positive control, MMC induced a statistically significant increase in chromosomal aberrations (excluding gaps), thus confirming the test systems ability to detect potential clastogenic effects.

4 h + 30 h recovery:

- Following treatment, no precipitate (observed by eye at the end of treatment) was observed at any concentration tested. Exposure to spiroxamine N-oxide at concentrations of 300 to 500 µg/mL caused reductions in %MI to 82.2%. The following concentration was selected for metaphase analysis: 400 µg/mL, with % reduction in MI 9.8%. No increase in the incidence of cells with structural aberrations was observed. An increased incidence of cells with polyploidy was observed (9.0% vs. 2.5% in the vehicle). The biological relevance of this is not understood as no historical control data for polyploidy was presented, and this assay is not specifically designed to assess numerical aberrations, with assessment limited to a qualitative assessment only. A sufficient level of cytotoxicity (reduction in %MI 40-50%) was not achieved, with metaphase analysis limited to a single dose level.
- No positive control was included, therefore the laboratory's ability to detect clastogenic damage following an extended recovery was not adequately demonstrated.

Refer to Table CA 5.8.1/04-2.

Table CA 5.8.1/04-2: Spiroxamine N-oxide overview of chromosomal aberration data in V79 cells in the absence of S9

Dose level (µg/mL)	4 h exposure (+ 18 h recovery) –S9				4 h exposure (+ 30 h recovery) –S9			
	Relative MI (%)	Cyto-toxicity (%)	Aberrant cells excl. gaps (%)	Numerical aberrant cells (%)	Relative MI (%)	Cyto-toxicity (%)	Aberrant cells excl. gaps (%)	Numerical aberrant cells (%)
0	100	0	0.5	4.0	100	0	0.5	2.5
100	101.7	-1.7	0.5	5.5	---[b]	---[b]	---[b]	---[b]
200	91.9	8.1	0.5	9.5	---[b]	---[b]	---[b]	---[b]
300	66.2	33.8	1.5	7.5	96.3	3.7	---[a]	---[a]
400	80.3	19.7	---[a]	---[a]	90.2	9.8	1.0	9.0
500	23.1	76.9	---[a]	---[a]	17.8	82.2	---[a]	---[a]
MMC	100.9	-0.9	27.5**	12.5**	---[c]	---[c]	---[c]	---[c]
Laboratory historical control ranges								
Vehicle control (water) 4 h +18 h recovery (*n* = 4)					**Vehicle control (water) 4 h +30 h recovery (*n* = 4)**			
Year	1994	1995	1996	1997	1994	1995	1996	1997
Range:	---[d]	---[d]	---[d]	---[d]	---[d]	---[d]	---[d]	---[d]
Median:	3.0	0.0	0.0	1.0	1.5	1.0	0.0	1.0
Positive control (MMC) 4 h +18 h recovery (*n* = 4)					**Positive control (MMC) 4 h +30 h recovery (*n* = 4)**			
Range:	35.0 – 57.0	18.0 – 43.5	29.5 – 50.5	15.5 – 44.5	---[c]	---[c]	---[c]	---[c]
Median:	43.5	34.5	37.5	39.5				

** $p \leq 0.01$ Positive control: Mitomycin C [0.1 µg/mL]

a metaphases not assessed for chromosome aberrant incidence
b concentration not treated for this treatment condition
c no positive control data generated for this treatment condition
d no observed range presented

2. Short-term treatment in the presence of S9:

4 h + 18 h recovery:

- Following treatment, no precipitate (observed by eye at the end of treatment) was observed at any concentration tested. Exposure to spiroxamine N-oxide at concentrations of 200 to 600 µg/mL caused reductions in %MI to 80.9%. The concentrations selected for metaphase analysis were 300, 400 and 500 µg/mL, with % reductions in MI of no cytotoxicity at the low and mid dose groups, with complete toxicity (80.9%) at the high dose group. No increase in the incidence of cells with structural aberrations or polyploidy cells was observed in any analysed treatment concentrations. However, a sufficient level of cytotoxicity (reduction in %MI 40-50%) was not achieved.
- The positive control, CPA induced a statistically significant increase in chromosomal aberrations (excluding gaps), thus confirming the test systems ability to detect potential clastogenic effects.

4 h + 30 h recovery:

- Following treatment, no precipitate (observed by eye at the end of treatment) was observed at any concentration tested. Exposure to spiroxamine N-oxide at concentrations of 400 to 600 µg/mL caused reductions in %MI to 88%. The following concentration was selected for metaphase analysis, 500 µg/mL, with % reduction in MI confirming no toxicity. No increase in the incidence of cells with structural aberrations was observed. An increased incidence of cells with polyploidy was observed (5.5% vs. 3.0% in the vehicle). The biological relevance of this is not understood as no historical control data for polyploidy was presented, and this assay is not specifically designed to assess numerical aberrations with assessment limited to a qualitative assessment only. . A sufficient level of cytotoxicity (reduction in %MI 40-50%) was not achieved, with metaphase analysis limited to a single dose level.
- No positive control was included, therefore the laboratory's ability to detect clastogenic damage following an extended recovery was not adequately demonstrated.

Refer to Table CA 5.8.1/04-3.

Table CA 5.8.1/04-3: Spiroxamine N-oxide overview of chromosomal aberration data in V79 cells in the presence of S9

Dose level (µg/mL)	4 h exposure (+ 18 h recovery) +S9				4 h exposure (+ 30 h recovery) +S9			
	Relative MI (%)	Cyto-toxicity (%)	Aberrant cells excl. gaps (%)	Numerical aberrant cells (%)	Relative MI (%)	Cyto-toxicity (%)	Aberrant cells incl. gaps (%)	Numerical aberrant cells (%)
0	100	0	3.0	6.0	100	0	1.0	3.0
200	136.2	-36.2	---[a]	---[a]	---[b]	---[b]	---[b]	---[b]
300	163.8	-63.8	3.0	2.5	---[b]	---[b]	---[b]	---[b]
400	145.4	-45.4	4.0	5.5	124	-24	---[a]	---[a]
500	98.6	1.4	3.0	6.5	119.7	-19.7	2.5	5.5
600	19.1	80.9	---[a]	---[a]	12	88	---[a]	---[a]
Positive control	117	-17	32.0**	6.0	---[c]	---[c]	---[c]	---[c]
Laboratory historical control ranges								
Vehicle control (water) 4 h +18 h recovery (*n* = 4)					**Vehicle control (water) 4 h +30 h recovery (*n* = 4)**			
Year	1994	1995	1996	1997	1994	1995	1996	1997
Range	---[d]	---[d]	---[d]	---[d]	---[d]	---[d]	---[d]	---[d]
Median:	7.0	2.0	2.0	1.5	4.0	1.5	1.0	1.0

Positive control (MMC) 4 h +18 h recovery (n = 4)					Positive control (MMC) 4 h +30 h recovery (n = 4)			
Range:	14.5 – 48.5	9.0 – 35.0	13.5 – 38.0	25.0 – 42.5	---[c]	---[c]	---[c]	---[c]
Median:	31.0	15.0	21.8	32.5				

** $p \leq 0.01$ Positive control: Cyclophosphamide [2 µg/mL]

a metaphases not assessed for chromosome aberrant incidence
b concentration not treated for this treatment condition
c no positive control data generated for this treatment condition
d no observed range presented

D. Deficiencies:

Although the study was conducted according to test guideline OECD 473 (1984), this test guideline has since been updated twice in the intervening periods (1997, 2016). When assessed against current test guideline requirements the following deficiencies are noted:

- It is unclear why the recovery period of 30 hours was selected for a follow up confirmatory experiment as there is no evidence that spiroxamine N-oxide causes cell cycle delay.
- No assessment of exposure in the absence of S9 following an extended exposure was undertaken.
- Concerns are raised over the sensitivity and specificity of the assay, with only 100 metaphases scored/culture, compared with test guideline requirements of 150/culture, 300/concentration. This is further impacted with the overly low background spontaneous chromosome aberrant frequency observed in the vehicle controls
- Toxicity was the limiting factor in both the absence and presence of S9. In both cases, a sufficient level of toxicity was not achieved and therefore a complete assessment of spiroxamine N-oxide's potential to induce chromosome aberrations has not been sufficiently investigated.
- For the extended recovery period undertaken, no concurrent positive controls were included. It is therefore unclear if the extended recovery methodology employed by the laboratory was sufficiently sensitive to demonstrate clastogenic damage.
- Limited historical control data were presented, with observed ranges only for the positive control data and median values for both the vehicle and positive controls. As the data are limited (4 studies over 4 years), there are insufficient data to undertake statistical analysis.
- The laboratory's criteria for assay acceptance and evaluation criteria are somewhat lacking when compared to current test guideline requirements. Toxicity is was not deemed a limiting factor, with a positive result concluded based on the summation aberrant cells including and excluding gaps. Gaps should be excluded from the final totals of aberrations, as they may not all be true breaks.
- Although not a deficiency of the test guideline, numerical aberrations are only qualitatively assessed in this assay.

For the reasons listed above, this study is deemed supplementary. Both the clastogenic and aneugenic endpoints have been adequately addressed with a robust, GLP up to date *in vitro* human peripheral blood lymphocyte micronucleus study (CA 5.8.1/21 [M-755221-02-1]).

Assessment and conclusion by applicant:

Assessment: For the reasons identified in the deficiencies section this study is deemed supplementary.

Conclusion: It is concluded that spiroxamine N-oxide did not induce biologically relevant increases in the incidence of chromosome aberrations in Chinese hamster V79 cells. These conditions included treatments that were neither limited by toxicity (*i.e.* reduction in % relative MI 45±5%) or precipitate in the absence (4 hours) and presence (4 hours at 2% (v/v) final concentration of S9 fraction) of rat liver metabolic activation system with recovery periods of 18 and 30 hours in both treatment conditions.

Data Point:	KCA 5.8.1/21
Report Author:	[redacted]
Report Year:	2020
Report Title:	Spiroxamine N-oxide: In vitro human lymphocyte micronucleus assay
Report No:	8406983
Document No:	M-755221-02-1
Guideline(s) followed in study:	OECD 487 (2016)
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

Spiroxamine N-oxide was tested in an *in vitro* micronucleus assay using duplicate human lymphocyte cultures prepared from the pooled blood of two female donors in a single experiment. Treatments covering a broad range of concentrations, separated by narrow intervals, were performed both in the absence and presence of metabolic activation (S9) from Aroclor 1254-induced rats. The test article was formulated in anhydrous analytical grade dimethyl sulphoxide (DMSO) and the highest concentration tested in the micronucleus experiment, 500 µg/mL (limited by toxicity), was determined following a preliminary cytotoxicity range-finder experiment. All test article concentrations, formulated in DMSO were dosed into the test system at 1% v/v.

Following establishment of cultures, concentrations ranging from 100 to 500 µg/mL in the absence (3 hours + 21 hour recovery) and presence of S9 (3 h + 21 h), and from 1 to 90 µg/mL in the extended treatment in the absence of S9 (24 h + 24 h). The test article concentrations for micronucleus analysis were selected by evaluating the effect of spiroxamine N-oxide on the replication index (RI). Micronuclei were analysed at three concentrations and a summary of the data are presented below.

The frequency of MNBN cells in vehicle controls fell within the normal ranges. The positive control chemicals induced statistically significant increases in the proportion of MNBN cells. Both replicate cultures at the positive control concentration analysed under each treatment condition demonstrated MNBN cell frequencies that clearly exceeded the normal range, thereby demonstrating the sensitivity and specificity of the assay. A minimum of 50% of cells had gone through at least one cell division (as measured by binucleate + multinucleate cell counts) in vehicle control cultures at the time of harvest. The maximum concentration analysed under each treatment condition met the criteria. The assay data were therefore considered valid and acceptable.

Treatment of cells with Spiroxamine N-oxide in the absence and presence of S9 resulted in MNBN cells that were similar to and not significantly ($p \leq 0.05$) higher than those observed in concurrent vehicle control cultures for all concentrations analysed. With the exception of a single culture (B) at 50 µg/mL following 24+24 S9 treatment, the MNBN cell frequency of all test article treated cultures for all three treatment conditions fell within the 95th percentile of the current historical vehicle control (normal) ranges. The isolated increase was small, and was not reproduced in the replicate culture, with the mean MNBN cell frequency (0.55%) falling within the normal range and therefore considered of no biological relevance.

It is concluded that spiroxamine N-oxide did not induce micronuclei in human peripheral blood lymphocytes following treatment in the absence or presence of an Aroclor-induced rat liver metabolic

activation system (S9). Maximum concentrations analysed were limited by cytotoxicity, in line with recommendations in the current regulatory guidelines for the *in vitro* micronucleus assay.

Materials and Methods

A. Materials:

1. Test Material:	Spiroxamine N-oxide (alternative name: [(8-tert-butyl-1,4-dioxaspiro[4.5]dec-2-yl) methyl](ethyl)(propyl)amine-N-oxide, M03)
Description:	Light yellow liquid
Lot/Batch No.:	AE 1344305 00 1C74 0001
Purity:	72.9% (w/w) (correction factor 1.37 applied) (molecular weight: 313.48 g/mol)
CAS No.:	148044-85-3
Stability of test compound:	Confirmed stable for the duration of the study (expiry date: 28 November 2022)
2. Control materials:	
Negative:	
Solvent/final concentration:	DMSO (dimethyl sulphoxide)/1% (v/v)
Positive: -S9	Mitomycin C (MMC, 3 h: 0.3 µg/mL) [clastogenic control] Vinblastine (VIN, 24 h: 0.04 µg/mL) [aneugenic control]
Positive: +S9	Cyclophosphamide (CPA, 3 h: 7 µg/mL)
3. Activation:	S9[12] was purchased from a commercial source. ♂ Sprague Dawley rats were treated with Aroclor 1254 (supplied by [redacted] lot no. 4030, protein content 3.3 mg/mL). The composition of the 10% S9 reaction mix was: 100 µL S9, Na PBS (100 µM), glucose-6-phosphate (5 µM), β-NADP (4 µM), $MgCl_2$ (8 µM), KCl (33 µM), water (to volume).
4. Test organisms:	Human peripheral blood lymphocytes were collected from 2 healthy, non-smoking adult donors aged between 23 and 30 years, pooled and diluted with HML media. Cells were stimulated to divide by the addition of phytohaemagglutinin (PHA).
5. Culture medium:	HEPES buffered RPMI supplemented with 10% (v/v) inactivated fetal calf serum, 0.52% penicillin-streptomycin cell culture.
6. Test article Concentrations:	
a) Preliminary cytotoxicity test:	3 h (+21 h recovery) -/+S9; 24 h (+24 h) –S9: 0, 7.256, 12.09, 20.16, 33.59, 55.99, 93.31, 155.5, 259.2, 432.0, 720, 1200, 2000 µg/mL (maximum recommended concentration)
b) Micronucleus assay:	3 h (+21 h recovery) -S9: 0, 100, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 500 µg/mL 3 h (+21 h recovery) +S9: 0, 100, 150, 175, 200, 225, 250, 275, 300, 325, 350, 375, 400, 500 µg/mL 24 h (+24 h) –S9: 0, 1, 5, 10, 15, 20, 30, 40, 45, 50, 55, 60, 70, 80, 90, 100 µg/mL (concentrations underlined scored for micronucleus frequency)

B. Test Performance:

1. In life dates:	7 May 2019 to 10 June 2019 (experimental dates)
2. Vehicle selection:	For the micronucleus experiments to correct for purity, a correction factor of 1.37 was applied.

12 In this study, S9 activity was evaluated with CPA. In addition, each batch was checked by the manufacturer of S9 for sterility, protein content, ability to convert ethidium bromide, cyclophosphamide, B[a]P and AAN to bacterial mutagens, and cytochrome P-450-catalysed enzyme activities (alkoxyresorufin-*O*-dealkylase activities). Therefore, there is no concern over S9 activity.

A preliminary solubility test confirmed spiroxamine N-oxide was soluble in DMSO up to at least 158.9 mg/mL (not corrected for purity).
The solubility limit in culture medium was in the range of 794.5 to 1589 µg/mL (correction factor not applied), as indicated by precipitation at the higher concentration which persisted for at least 24 h after test article addition. A maximum concentration of 2000 µg/mL was selected for the cytotoxicity Range-Finder Experiment, in order that treatments were performed up to the maximum recommended concentration according to current regulatory guidelines. Concentrations for the micronucleus experiment were selected based on the results of the cytotoxicity range-finder experiment.
Test article stock solutions were prepared by formulating spiroxamine N-oxide under subdued lighting in DMSO with the aid of vortex mixing and warming at 37°C, to give the maximum required treatment solution concentration. Subsequent dilutions were made using DMSO. The test article solutions were protected from light and used within approximately 3.5 h of initial formulation.

3. Statistics: The proportion of MNBN cells for each treatment condition were compared with the proportion in vehicle controls by using Fisher's exact test. A Cochran-Armitage trend test was applied to each treatment condition. Probability values of $p \leq 0.05$ were accepted as significant.

4. Acceptance criteria: The following acceptance criteria had to be met for assay acceptability:

1. The binomial dispersion test demonstrated acceptable heterogeneity (in terms of MNBN cell frequency) between replicate cultures, particularly where no positive responses were seen;
2. The frequency of MNBN cells in vehicle controls fell within the current 95th percentile of the observed historical vehicle control (normal) ranges;
3. The positive control chemicals induced statistically significant increases in the proportion of cells with micronuclei. Both replicate cultures at the positive control concentration analysed under each treatment condition demonstrated MNBN cell frequencies that clearly exceeded the normal range;
4. A minimum of 50% of cells had gone through at least one cell division (as measured by binucleate + multinucleate cell counts) in vehicle control cultures at the time of harvest;
5. The maximum concentration analysed under each treatment condition met the specified criteria (*i.e.* the highest concentration selected for micronucleus analysis following all treatment conditions was one at which 50-60% cytotoxicity was achieved).

5. Evaluation criteria: For valid data, the test article was considered to induce clastogenic and/or aneugenic events if:

1. A statistically significant increase in the frequency of MNBN cells at one or more concentrations was observed;
2. An incidence of MNBN cells at such a concentration that exceeded the normal range in both replicates was observed;
3. A concentration-related increase in the proportion of MNBN cells was observed (positive trend test).

The test article was considered positive in this assay if all of the above criteria were met.
The test article was considered negative in this assay if none of the above criteria were met.

C. Methods:

1. Homogeneity and achieved concentration analysis of the dose: Not undertaken.

2. Preliminary cytotoxicity assay: Whole blood cultures were established by placing 0.4 mL of pooled heparinised blood into 8.5 mL pre-warmed HEPES-buffered RPMI medium

containing 10% (v/v) heat inactivated fetal calf serum and 0.52% penicillin/streptomycin, so that the final volume following addition of S9 mix/KCl and the test article in its chosen vehicle was 10 mL. The mitogen, phytohaemagglutinin (PHA, reagent grade) was included in the culture medium at a concentration of approximately 2% of culture to stimulate the lymphocytes to divide. Blood cultures were incubated at 37±1°C for approximately 48 hours and rocked continuously. S9 mix or KCl (1 mL/culture) was added appropriately. Cultures were treated with the test article or vehicle (0.1 mL/culture). Positive control treatments were not included. Duplicate cultures were used for the vehicle control and single cultures were used for each test article treated concentration.

For removal of the test article, cells were pelleted (approximately 300 g, 10 minutes, washed twice with sterile saline and resuspended in fresh pre-warmed medium containing fetal calf serum and penicillin/streptomycin. Cyto-B (formulated in DMSO) was added to post wash-off culture medium to give a final concentration of 6 µg/mL/culture to inhibit cytokinesis, resulting in binucleate cells (without effecting karyokinesis), thereby arresting cells in interphase.

Changes in osmolality of more than 50 mOsm/kg and fluctuations in pH of more than one unit may be responsible for an increase in chromosome aberrations. Osmolality and pH measurements on post-treatment incubation medium were taken in the cytotoxicity range-finder experiment.

Cytotoxicity was determined by examination of the proportion of mono, bi and multinucleate cells to a minimum of 500 cells/concentration. From this, the replication index (RI) was calculated for each concentration (see Cytotoxicity section below).

3. Micronucleus assay:

Cell treatment: Cells were exposed to the test article formulation, vehicle or positive control as described above, with the exception of quadruplicate cultures were used for the vehicle control and duplicate controls used for the positive controls and each test article concentration. Concentrations selected for micronucleus analysis were assessed for cytotoxicity uncoded and for the proportions of mono-, bi- and multinucleate cells to a minimum of 200 or 500 cells/culture (preliminary cytotoxicity range finder or micronucleus experiment, respectively)./

Spindle inhibitor: Cyto-B was added post-wash to cultures to inhibit cytokinesis.

Slide preparation: Slides were prepared by spreading the fixed cultures on clean slides. The slides were stained with acridine orange (12.5 µg/mL) dropped on to slides, coverslipped and scored prior to analysis.

Cytotoxicity: The replication index (RI), which indicates the relative number of nuclei compared to controls, was determined using the formula below:

$$RI = \frac{no.\ of\ binucleate\ cells + 2(no.\ of\ multinucleate\ cells)}{total\ no.\ of\ cells\ in\ treated\ cultures}$$

Relative RI (expressed in terms of percentage) for each treated culture was calculated as follows:

$$Relative\ RI\ (\%) = \frac{RI\ of\ treated\ cultures}{RI\ of\ vehicle\ control} \times 100$$

Cytotoxicity (%) was expressed as (100 – Relative RI).

Micronucleus assessment: Slides from the highest selected concentration and two lower concentrations were taken for microscopic analysis.

Immediately prior to analysis, 1-2 drops of PBS were added to the slides before mounting with glass coverslips. One thousand binucleate cells from each culture (total of 2000 binucleate cells/concentration) were analysed for micronuclei. The number of cells containing micronuclei and the number of micronuclei/cell on each slide were noted.

Nucleoplasmic bridges (NPBs) between nuclei in binucleate cells were recorded during micronucleus analysis to provide an indication of chromosome rearrangement. Various mechanisms may lead to NPB formation following DNA misrepair of strand breaks in DNA in this assay, binucleate cells with NPBs were recorded as part of the micronucleus analysis.

Results

A. Analytical determinations:

Not undertaken. Analyses for achieved concentrations, homogeneity or stability of test article formulations were not conducted as part of this study, as this is not a requirement of the regulatory test guidelines.

B. Preliminary cytotoxicity assay:

Test article precipitate was observed at concentrations of 432 µg/mL and above in both short term treatments, with no scorable cells present at concentrations of 720 µg/mL and above. In the long term treatment no test article precipitate was observed, with no scorable cells present at concentrations of 290 µg/mL and above.

No marked changes in osmolality or pH were observed at the highest three concentrations tested as compared to the concurrent vehicle controls (individual data not reported).

The results of the cytotoxicity preliminary cytotoxicity range-finder experiment were used to select suitable maximum concentrations for the micronucleus experiment.

Table CA 5.8.1/21-1: Spiroxamine N-oxide: human lymphocyte preliminary cytotoxicity range finder experiment

Conc. (µg/mL)	3 h (+ 21 h recovery) –S9		3 h (+ 21h recovery) +S9		24 h (+ 24 h recovery) –S9	
	Replication index (RI)	Cytotoxicity based on RI (%)	Replication index (RI)	Cytotoxicity based on RI (%)	Replication index (RI)	Cytotoxicity based on RI (%)
0	0.67	-	0.73	-	0.87	-
7.256	0.65	2	0.82	0	0.76	13
12.09	0.73	0	0.68	7	0.64	27
20.16	0.71	0	0.63	13	0.47	47
33.59	0.70	0	0.80	0	0.50	43
55.99	0.63	5	0.70	4	0.32	64
93.31	0.57	15	0.65	11	0.12	87
155.5	0.61	9	0.61	17	0.04	95
259.2	0.52	23	0.65	11	NE	-
432.0	0.04[ppt]	95	0.05[ppt]	94	NE	-
720.0	NE, ppt, H-ppt	-	NE, ppt, H-ppt	-	NE, ppt, E-ppt,	-
1200	NE, ppt, E-ppt, H-ppt	-	NE, ppt, E-ppt, H-ppt	-	NE, ppt, E-ppt, H-ppt	-
2000	NE, ppt, E-ppt, H-ppt	-	NE, ppt, E-ppt, H-ppt	-	NE, ppt, E-ppt, H-ppt	-

NE: not evaluated due to no scorable cells

ppt: precipitate observed at treatment
E-ppt: precipitate observed at end of treatment
H-ppt: precipitate observed at harvest

C. Micronucleus assay:

The frequency of MNBN cells in vehicle controls fell within the normal ranges. The positive control chemicals induced statistically significant increases in the proportion of MNBN cells. Both replicate cultures at the positive control concentration analysed under each treatment condition demonstrated MNBN cell frequencies that clearly exceeded the normal range, thereby demonstrating the sensitivity and specificity of the assay. A minimum of 50% of cells had gone through at least one cell division (as measured by binucleate + multinucleate cell counts) in vehicle control cultures at the time of harvest.

The maximum concentration analysed under each treatment condition met the criteria. The assay data were therefore considered valid and acceptable.

Treatment of cells with Spiroxamine N-oxide in the absence and presence of S9 resulted in MNBN cells that were similar to and not significantly ($p \leq 0.05$) higher than those observed in concurrent vehicle control cultures for all concentrations analysed. With the exception of a single culture (B) at 50 µg/mL following 24+24 –S9 treatment, the MNBN cell frequency of all test article treated cultures for all three treatment conditions fell within the 95th percentile of the current historical vehicle control (normal) ranges. The isolated increase was small such that it was not reproduced in their replicate culture with the mean MNBN cell frequency (0.55%) falling within the normal range and therefore considered of no biological relevance.

Table CA 5.8.1/21-2: Spiroxamine N-oxide: human lymphocyte micronuclei assay: 3 h (+ 21 h recovery) –S9 treatment and laboratory historical control data

Conc. (µg/mL)		3 h (+ 21 h recovery) –S9				Vehicle historical control ranges (♀ donors)	
		Total BN	Total MN-BN	Frequency of MN-BN (%)	Cyto. (%)	Feb 17 – Feb 18	% MNBN
Vehicle	A	1000	5	0.50		No. of expts	15
	B	1000	3	0.30		Number of cultures	40
Total		2000	8	Mean: 0.40	-	Mean ±SD	0.41 ±0.28
150	A	1000	3	0.30		min. – max.	0.00 – 1.30
	B	1000	7	0.70		95% reference range	0.00 – 1.01
Total		2000	10	Mean: 0.50	Mean: 15		
250	A	1000	5	0.50			
	B	1000	1	0.10		Positive historical control ranges (♀ donors)	
Total		2000	6	Mean: 0.30	Mean: 33	MMC (0.3 µg/mL)	
300	A	1000	2	0.20		Aug 15 – Dec 17	% MNBN
	B	1000	3	0.30		No. of expts	19
Total		2000	5	Mean: 0.25	Mean: 60	Number of cultures	40
Linear trend: p 0.8603						Mean ±SD	5.68 ±1.66
MMC (0.3)	A	1000	70	7.00		min. – max.	2.80 – 9.20
	B	1000	79	7.90		95% reference range	3.39 – 8.81
Total		2000	149	Mean: 7.45***	Mean: 56		

*** $p<0.001$

No test article related increases in cells with NPBs were observed (data not reported)

Table CA 5.8.1/21-3: Spiroxamine N-oxide: human lymphocyte micronuclei assay: 3 h (+ 21 h recovery) +S9 treatment and laboratory historical control data

Conc. (µg/mL)		3 h (+ 21 h recovery) +S9				Vehicle historical control ranges (♀ donors)	
		Total BN	Total MN-BN	Frequency of MN-BN (%)	Cyto. (%)	Feb 17 – Feb 18	% MNBN
Vehicle	A	1000	4	0.40		No. of expts	16
	B	1000	2	0.20		Number of cultures	40
Total		2000	6	Mean: 0.30	-	Mean ±SD	0.55 ±0.30
225	A	1000	2	0.20		min. – max.	0.00 – 1.20
	B	1000	2	0.20		95% reference range	0.10 – 1.20
Total		2000	4	Mean: 0.20	Mean: 8		
275	A	1000	5	0.50			
	B	1000	2	0.20		Positive historical control ranges (♀ donors)	
Total		2000	7	Mean: 0.35	Mean: 28		
300	A	1000	4	0.40		CPA (3 µg/mL)[1]	
	B	1000	3	0.30		Feb 16 – Feb 18	% MNBN
Total		2000	7	Mean: 0.35	Mean: 51	No. of expts	21
Linear trend: p 0.2917						Number of cultures	41

CPA (7.0)	A	1000	24	2.40		Mean ±SD	2.60 ±1.07
	B	1000	18	1.80		min. – max.	0.80 – 5.00
Total		2000	42	Mean: 2.10***	Mean: 33	95% reference range	1.00 – 4.70

*** $p<0.001$
There is currently no historical control range for CPA 7 µg/mL, the concentration analysed in this study, therefore the range for the highest CPA concentration normally analysed (3 µg/mL) has been included for comparative purposes
No test article related increases in cells with NPBs were observed (data not reported)

Table CA 5.8.1/21-4: Spiroxamine N-oxide: human lymphocyte micronuclei assay: 24 h (+ 24 h recovery) -S9 treatment and laboratory historical control data

Conc. (µg/mL)		24 h (+ 24 h recovery) –S9				Vehicle historical control ranges (♀ donors)	
		Total BN	Total MN-BN	Frequency of MN-BN (%)	Cyto. (%)	Feb 17 – Feb 18	% MNBN
Vehicle	A	1000	6	0.60		No. of expts	14
	B	1000	4	0.40		Number of cultures	40
Total		2000	10	Mean: 0.50	-	Mean ±SD	0.34 ±0.21
20	A	1000	4	0.40		min. – max.	0.00 – 0.90
	B	1000	4	0.40		95% reference range	0.10 – 0.80
Total		2000	8	Mean: 0.40	Mean: 12		
50	A	1000	4	0.40			
	B	1000	4	0.40		**Positive historical control ranges (♀ donors)**	
Total		2000	8	Mean: 0.40	Mean: 55		
90	A	1000	1	0.10		**VIN (0.04 µg/mL)**	
	B	1000	10	1.10>HC		Feb 17 – Jan 18	% MNBN
Total		2000	11	Mean: 0.55	Mean: 32	Number of cultures	20
Linear trend: p 0.5875						Mean:	40
VIN (0.04)	A	1000	60	6.00		Mean ±SD	5.59 ±2.05
	B	1000	53	5.30		min. – max.	2.50 – 10.10
Total		2000	113	Mean: 5.65***	Mean: 26	95% reference range	2.50 – 8.93

*** $p<0.001$
>HC: exceeds historical control
No test article related increases in cells with NPBs were observed (data not reported)

D. Deficiencies:

Whilst not deemed a deficiency, it is considered prudent to detail the rationale for undertaking an extended treatment which included a recovery period.

Following revision of the OECD 487 TG in 2014 the cell treatment and harvest times have been somewhat simplified with emphasis placed on performing the extended treatment without recovery, with sampling times or recovery times extended by up to a further 1.5-2.0 normal cell cycles where justified.

Given the purpose for the *in vitro* micronucleus assay is to determine both clastogenic and aneugenic potential, the assay is compromised if an extended treatment with recovery is not included for sensitive detection of certain chemical classes.

Average generation times and replication index data demonstrated that the human peripheral blood lymphocytes were actively dividing beyond 96 hours, indicating that the extended treatment with recovery within the assay system did not compromise the results. This is directly in contrast to the statement in the OECD 487 test guideline, which includes the statement 'for lymphocytes exponential growth may be declining at 96 hours following stimulation and monolayer cultures of cells may become confluent'. This statement in the test guideline is not supported by any published data, but rather a throw away statement and may provide the 'get out clause' why testing laboratories did not use this approach as they may have had reduction in growth at 96 hours.

The extended treatment with recovery allows adequate detection of chemicals that either act on a narrow portion of the cell cycle (e.g. aneugenic compounds, G2/metaphase) and/or that induce cell cycle delay

or require two rounds of DNA replication. The extended treatment without recovery in the micronucleus assay may be seen as somewhat compromised. It is widely known that direct acting clastogens such as azidothymidine and cytosine arabinoside are negative in the *in vitro* micronucleus assay with the 24 + 0 h approach, but positive with the with 24+24 h protocol. Therefore, the extended treatment with recovery is deemed more sensitive and in accordance with the latest published data (refer to Whitwell et al., 2019[8]), with the OECD test guideline to be updated to reflect this change.

Assessment and conclusion by applicant:

Assessment: Study meets the current guidance and the requirements in 283/2013.

Conclusion: It is concluded that spiroxamine N-oxide did not induce micronuclei in human peripheral blood lymphocytes following treatment in the absence or presence of an Aroclor-induced rat liver metabolic activation system (S9). Maximum concentrations analysed were limited by cytotoxicity, in line with recommendations in the current regulatory guidelines for the *in vitro* micronucleus.

Data Point:	KCA 5.8.1/03
Report Author:	[redacted]
Report Year:	2000
Report Title:	KWG 4168 N-Oxide - Study for subacute oral toxicology in rats (feeding study over 4 weeks) - 1st revised version of report no. 28161 from Nov 17, 1998 -
Report No:	28161A
Document No:	M-006504-02-1
Guideline(s) followed in study:	OECD 407; Directive 67/548/EEC, Method B.7.
Deviations from current test guideline:	Yes A number of deficiencies were noted (refer to Results, Deficiencies section below).
Previous evaluation:	yes, evaluated and accepted DAR (2010)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

In this study, spiroxamine N-oxide was administered continuously *via* the diet for 28 days to Wistar rats. Animals (5/sex/group) were administered test diet at concentrations of 0, 30, 150, 1000 ppm (equivalent to males/females: 0/0, 2.6/2.7, 12.9/13.2, 114.6/94.3 mg/kg bw/day). Animals were subjected to body weight, food consumption which were measured at regular intervals. Clinical pathology evaluations (haematology, clinical chemistry and urinalysis) were performed with all surviving animals subjected to complete gross necropsy and full histopathology.

No treatment-related effects were seen on survival, clinical signs, food consumption, urinalysis or serum chemistry/ haematology parameters. High dose effects were limited to slightly decreased cholesterol levels in both males and females. This was deemed to be a treatment related effect, but likely slight (without correlating liver histopathology) rather than adverse.

At 1000 ppm, males showed a reduction in body weight, with a -7% reduction by the end of treatment when compared to the concurrent control, with statistically significant reductions in body weight from day 7 through to day 21. Body weight gain in this group was markedly reduced, with 16% reduction. In females at 1000 ppm body weights were comparable to the concurrent control group. Whilst statistically significant increased in body weight were observed at 30 and 150 ppm dose groups, this was not dose

related. When body weight gain at the end of the treatment period was examined, a 19% reduction compared to the concurrent control group was observed.

In high group animals, hyperkeratosis of the epithelium of the oesophagus and forestomach were observed with mild transitional cell hyperplasia in the urinary bladder. As with the parent compound, spiroxamine, spiroxamine N-oxide is a tertiary amine, and like all tertiary amines has a high pKa, and a high pH with comparable histopathological effects reported. As with irritant compounds, tissues containing mucosal membrane is deemed to be a target organ critical effect. This is acknowledged in the literature, with optimal pH ranges for substances administered orally *via* gavage not to exceed pH 9, with administration of substances outside of the recommended pH ranges resulting in tissue necrosis, pH partitioning *etc.* This should not be dismissed.

Hyaline droplet nephropathy was observed in the kidneys of two high dose group males. This lesion is due the accumulation of α2μ-globulin which is known to be a lesion specific to male rats with no relevance to humans, with Azan specific staining used to confirm α2μ-globulin presence.

Under the conditions of this study the NOAEL following 28 days oral is deemed to be 150 ppm (equivalent to 12.9/13.2 mg/kg bw/day for males/females) based on reduction in body weight gain (exceeding 10%), hyperkeratosis of the stomach and oesophagus with mild transitional cell hyperplasia in the urinary bladder. These effects are associated with the irritant nature of the test article.

Materials and Methods

A. Materials:

1. Test Material:	KWG 4168-N-oxide (alternative names: Spiroxamine N-oxide, [(8-tert-butyl-1,4-dioxaspiro[4.5]dec-2-yl) methyl](ethyl)(propyl)amine-N-oxide; M03)
Description:	Colourless liquid
Lot/Batch No.:	M00190
Purity:	90.8% (w/w) (correction for purity not undertaken)
CAS No.:	148044-85-3
Stability of test compound:	Confirmed stable for the duration of the study (expiry date: 26 August 1998)
2. Vehicle and/or positive control:	Basal diet, not relevant
3. Test animals:	
Species:	Rat
Strain:	Wistar
Age at dosing:	4-5 wks
Weight at dosing:	♂: 129-143g; ♀: 120-138g
Source:	[redacted]
Acclimation period:	Animals were acclimatised upon arrival, but the duration of this period was not detailed
Diet:	Altromin® 1321 fixed-formula standard diet (Altromin GmbH, Lage, Germany) *ad libitum*
Water:	Municipal water, *ad libitum*
Housing:	Individually housed
4. Environmental conditions:	
Temperature:	22 ±2°C
Humidity:	55 ± 5%
Air changes:	*ca.* 10/h
Photoperiod:	12 hour light/dark

B. Study Design:

1. In life dates: 8 December 1997 to 6 January 1998 (experimental dates)

2. Animal assignment and treatment: After an acclimatisation period rats were allocated to groups by computer-based stratified random sampling. Dose levels selected based on the results from the sub-chronic study undertaken on the parent compound, spiroxamine, in which animals were dosed at 0, 25, 125, 625 ppm were dosed.

The test article, spiroxamine N-oxide was administered continuously *via* the diet to groups of rats for a period of 28 days. Animals (5/sex/gp), were administered test diet at concentrations of 0, 30, 150, 1000 ppm (equivalent to ♂/♀: 0/0, 2.6/2.7, 12.9/13.2, 114.6/94.3 mg/kg bw/day). Following 28 d of treatment 5 animals/sex were subjected to complete necropsy. Body weight, water and food consumption were measured at regular intervals. Clinical pathology evaluations (haematology, clinical chemistry and urinalysis) were performed with all surviving animals subjected to complete gross necropsy and full histopathology.

3. Statistics: Dunnett's test: body weight, feed and water consumption, organ weight data.

Table CA 5.8.1/05-1: Overview of sub-acute toxicity study in rats treated orally (*via* diet) with spiroxamine N-oxide: study design and dose received

Parameters	(ppm)				(ppm)			
	0	**30**	**150**	**1000**	**0**	**30**	**150**	**1000**
Dose/animal (mg/kg bw/day)	0	2.6	12.9	114.6	0	2.7	13.2	94.3
Animals assigned/sex	5	5	5	5	5	5	5	5

C. Methods:

1. Test diet preparation and analysis: The spiroxamine N-oxide was prepared at dietary concentrations of 0, 30, 150 and 1000 ppm. 1% peanut oil was added to minimize dust formation. The prepared test diets were prepared once and stored frozen (approximately −15°C) until before use.

Stability and homogeneity of the diet preparation containing the test article were determined by the analysis of two samples of the diet from 10 and 2000 ppm (refer to Doc MCA Section 4 [M-020615-01-1] for method validation).

2. Observations: Animals were inspected twice daily for signs of toxicity and mortality.

3. Body weights: Animals were weighed prior to study start and then on Days 0, 7, 14, 21, and 28.

4. Food consumption: Determined by weighing food supplied and food that remained on days 7, 14, 21, and 28.

From the food consumption data, compound consumption was calculated using the following equation:

$$\text{Cpd consumption (mg/kg bw/d)} = \frac{\text{Food consumption (g/rat/d)} \times \text{test article conc. (ppm)}}{\text{Body weight (g)}}$$

Food efficiency was not calculated.

5. Water consumption: Whilst detailed in the statistical analysis that this was undertaken, no data are report. Therefore it is assumed that water consumption was not conducted.

6. Ophthalmological examination: Not conducted.

7. Neurological / functional examinations: Not conducted.

8. Haematology and clinical chemistry: Conducted on day 29. Animals were not fasted prior to blood sampling.

Haematology: red blood cell parameters (haematocrit (commonly termed PCV), haemoglobin concentration (Hb), mean haemoglobin concentration

(MHC), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV), erythrocyte count, platelet count, reticulocyte count), white blood cell parameters (total and differential (neutrophils, lymphocytes, eosinophils, basophils, monocytes) leukocyte count), coagulation parameters (prothrombin time (PT) – termed Hepato-Quick Test).
Clinical chemistry: electrolytes (sodium, potassium, calcium, chloride, inorganic phosphorus), kidney function test (creatinine, urea), glucose, liver function tests (albumin, alkaline phosphatase (AlP), alanine aminotransferase (ALT [commonly referred to as glutamic pyruvic transaminase (GPT)]), aspartate aminotransferase (AST [commonly referred to as glutamic oxaloacetic transaminase (GOT)]), total bilirubin (TBili), total protein (TP), lipid profile (total cholesterol).

9. Urinalysis: Conducted on days 28. The following urinary parameters were measured: specific gravity, pH, total volume, protein, glucose, ketones, bilirubin, blood, urobilinogen, sediment

10. Organ weights: Adrenal glands, brain, epididymides, heart, kidney, liver, lung, ovary, spleen, testis.

11. Sacrifice and pathology: Conducted on day 30. Gross pathological examination was performed on all animals and included examination of the external surface, all orifices and associated tissues.
The following tissues were preserved in 10% neutral buffered formalin for subsequent histopathological examination (with the exception of urinary bladder and lungs (+2nd liver lobe) which were fixed with Davidson's solution and 4% formaldehyde solution, respectively) and performed on control and high dose group animals. The kidneys were stained with azan methodology (chromotrope-aniline blue stain) and analysed for all ♂ for the presence of α2µ-globulin[13]:
Accessory sex glands (♂:epididymides, prostate, seminal vesicle, testes; ♀: ovary, oviduct, uterus, vagina), cardiovascular/haematological system (aorta, bone (sternum, tibial or femur for marrow), heart, lymph nodes (mandibular, mesenteric), spleen, thymus), gastrointestinal tract (oesophagus, tongue, stomach, intestine (caecum, colon duodenum, ileum, jejunum, rectum), liver, pancreas), neurological (brain, eyes (+optic nerve, Harderian gland), sciatic nerve, spinal cord), respiratory system (nose, trachea, lung), urogenital system (kidneys, urinary bladder), other (skeletal muscle, bone (femur, sternum), skin, all gross lesions and masses);
Other endocrine producing/sensitive glands (adrenals, mammary gland, pituitary, thyroid (+parathyroid)).
In liver tissue aminopyrine-N-demethylase, *p*-nitroanisole-*O*-demethylase and cytochrome P450 activity was determined in all animals.

12. Neurohisto-pathology: No specific neurohistopathology with specific fixatives were performed in addition to the standard histopathology undertaken on neuronal tissues.

Results and discussion

A. Test diet analysis:

Spiroxamine N-oxide was homogenously distributed and chemically stable for at least 6 weeks within the concentration range of 10 to 2000 ppm. The analytical data verify that during the treatment period concentrations of the test article in the diet preparations ranged from -26.5% to -2.5% of nominal concentrations 30, 150 and 1000 ppm, which were within acceptable limits.

B. Observations:

13 De Rijk, E.P.C.T., Ravesloot, W.T.M., Wijnands, Y. & van Esch, E. (2003). A fast histochemical staining method to identify hyaline droplets in the rat kidney. *Toxicological Pathology*, **31** pp 462-464. Available online: https://journals.sagepub.com/doi/pdf/10.1080/01926230390213775

1. Clinical signs of toxicity:	No treatment related effects observed. A single ♀ in the 1000 ppm dose group exhibited piloerection on day 10 to 12.
2. Mortality:	All animals survived until the scheduled necropsy.
3. Ophthalmoscopic examination:	Not conducted.
4. Neurological functional examinations	Not conducted.

C. Body weight and body weight gain:

At 1000 ppm, males showed a reduction in body weight, with a -7% reduction by the end of treatment when compared to the concurrent control, with statistically significant reductions in body weight from day 7 through to day 21. Body weight gain in this group was markedly reduced, with 16% reduction. In females at 1000 ppm body weights were comparable to the concurrent control group. Whilst statistically significant increases in body weight were observed at 30 and 150 ppm dose groups, this was not dose related. When body weight gain at the end of the treatment period was examined, a 19% reduction compared to the concurrent control group was observed.

Table CA 5.8.1/05-2: Overview of sub-acute toxicity study in rats treated orally (*via* diet) with spiroxamine N-oxide: body weight effects

Parameters		♂ (ppm)				♀ (ppm)			
		0	**30**	**150**	**1000**	**0**	**30**	**150**	**1000**
Dose/animal (mg/kg bw/d)		0	2.6	12.9	114.6	0	2.7	13.2	94.3
Body wt (g)	Day 0	135 ±4.2	135 ±6.0	133 ±1.8	137 ±5.4	124 ±4.1	128 ±6.0	131 ±6.0	128 ±1.6
	Day 7	181 ±6.5	184 ±8.4	181 ±5.4	164 ±5.2**	141 ±4.5	152 ±5.8*	151 ±8.4	143 ±2.6
	Day 14	225 ±6.4	231 ±7.4	219 ±12.6	202 ±5.0**	157 ±4.4	170 ±3.1**	168 ±6.1**	156 ±2.6
	Day 21	261 ±10.4	270 ±11.3	254 ±22.3	236 ±5.2*	166 ±4.3	181 ±6.5**	180 ±6.6**	168 ±4.3
	Day 28	263 ±9.7	281 ±12.3	258 ±22.3	244 ±5.6	166 ±3.5	178 ±5.3*	177 ±11.1	162 ±3.3
Body wt gain (g)	Day 0-28	128	146 (↑14%)	121 (↓6%)	107 (↓16%)	42	50 (↑20%)	46 (↑10%)	34 (↓19%)

* $p \leq 0.05$; ** $p \leq 0.01$

D. Food consumption, food efficiency and water consumption:

1. Food consumption:	Initially an increase in feed consumed was observed in ♂ during the first 2 week of treatment when expressed as g/kg/day and then reduced from day 21 to day 28. All other treatment groups, both ♂ and ♀ showed a reduction in feed consumed throughout the dosing period.
2. Food efficiency:	Not conducted.
3. Water consumption:	No effects observed.

Table CA 5.8.1/05-3: Overview of sub-acute toxicity study in rats treated orally (*via* diet) with spiroxamine N-oxide: food consumption

Parameters		♂ (ppm)				♀ (ppm)			
		0	30	150	1000	0	30	150	1000
Dose/animal (mg/kg bw/d)		0	2.6	12.9	114.6	0	2.7	13.2	94.3
Food consum. (g/kg/d)	Day 7	97 ±3.7	105 ±10.9	101 ±5.8	153 ±13.8*	99 ±10.5	105 ±12.2	100 ±5.4	109 ±22.3
	Day 14	89 ±5.8	90 ±2.2	92 ±4.9	124 ±17.6*	109 ±23.9	96 ±9.0	93 ±2.2	103 ±9.5
	Day 21	79 ±5.6	84 ±5.0	81 ±2.8	100 ±12.4	89 ±12.2	87 ±6.2	85 ±4.3	90 ±8.8
	Day 28	68 ±5.8	73 ±3.7	71 ±2.9	81 ±9.8	78 ±10.2	78 ±5.4	73 ±3.4	76 ±2.9

* $p \leq 0.05$

E. Blood and urinalysis:

1. Haematological findings:

The following changes were observed in ♂ at 1000 ppm, clotting time (PT) was extended and eosinophils increased at dose levels of 150 ppm and above. In ♀ Hb concentration and haematocrit (PCV) values were decreased at 1000 ppm, which would be deemed evidence of anaemia. However without effects on RBC, MCV, MHC, MCHC values or a compensatory increase in reticulocytes or increases in spleen weights, these effects are deemed serendipitous, isolated to a single sex and within the laboratory's historical control range. The increases in eosinophil counts were isolated, without effect on total leukocyte counts.

Table CA 5.8.1/05-4: Overview of sub-acute toxicity study in rats treated orally (*via* diet) with spiroxamine N-oxide: selected haematological parameters

Parameters	♂ (ppm)				♀ (ppm)			
	0	30	150	1000	0	30	150	1000
Dose/animal (mg/kg bw/d)	0	2.6	12.9	114.6	0	2.7	13.2	94.3
PT (sec)	26.7 ±0.74	26.5 ±0.76	27.4 ±0.89	29.4 ±1.96*	25.7 ±0.78	25.7 ±0.72	25.7 ±0.87	26.7 ±0.78
Eosino. (10^9/L)	0.02 ±0.011	0.03 ±0.008	0.05 ±0.013**	0.05 ±0.013**	0.04 ±0.015	0.05 ±0.015	0.06 ±0.027	0.05 ±0.022
Leuko. (10^9/L)	5.19 ±2.327	6.43 ±0.918	7.07 ±0.993	5.53 ±1.498	5.19 ±0.666	6.34 ±0.791	6.41 ±1.075	6.49 ±1.667
RBC (10^{12}/L)	7.97 ±0.336	7.95 ±0.272	8.16 ±0.414	7.90 ±0.134	8.23 ±0.291	8.24 ±0.184	7.96 ±0.386	7.89 ±0.310
Ret. (%)	25 ±5.1	26 ±4.6	24 ±3.9	32 ±6.2*	20 ±4.4	14 ±4.9	17 ±3.6	20 ±2.9
Hb (g/L)	152 ±6.0	155 ±5.7	155 ±6.4	147 ±2.5	152 ±4.5	150 ±3.6	150 ±5.4	144 ±6.2*
PCV (L/L)	0.483 ±0.0202	0.492 ±0.0212	0.503 ±0.0139	0.473 ±0.0100	0.473 ±0.0137	0.461 ±0.0085	0.459 ±0.0212	0.438 ±0.0236
MCV (fL)	60.6 ±1.24	62.0 ±3.47	61.7 ±1.93	59.8 ±1.93	57.5 ±1.37	56.0 ±0.96	57.7 ±1.12	55.5 ±1.70
MHC (ρg)	19.1 ±0.55	19.3 ±0.89	19.0 ±0.68	18.6 ±0.46	18.5 ±0.37	18.2 ±0.29	18.8 ±0.30	18.2 ±0.46
MCHC (g/L RBC)	314 ±4.2	311 ±3.2	308 ±4.8	311 ±4.5	322 ±6.6	325 ±6.3	326 ±4.3	328 ±4.1
Laboratory historical control data (rat, Wistar 8 -11 wks of age)								
PT (sec)	Date range:		1994 - 1995		-		-	
	n:		65					
	Mean ±SD:		28.1 ±1.46					
	Range ±2SD:		25.2 – 31.0					
Eosino. (10^9/L)	Date range:		1997		-		-	
	n:		30					

Parameters	♂ (ppm)				♀ (ppm)			
	0	30	150	1000	0	30	150	1000
	Mean ±SD: 0.04 ±0.025 Range ±2SD: 0 – 0.09							
Hb (g/L)	-		-		Date range: 1994 - 1995 *n:* 53 Mean ±SD: 137 ±7.7 Range ±2SD: 121 - 152			
PCV (L/L)	-		-		Date range: 1994 - 1995 *n:* 53 Mean ±SD: 0.442 ±0.0267 Range ±2SD: 0.388 – 0.495			

*$p \leq 0.05$
PT: prothrombin time
Eosino.: eosinophils
Leuko.: leukocytes
RBC: red blood cell
Reti. reticulocytes
Hb: haemoglobin
PCV: packed corpuscular volume (haematocrit)
MCV: mean corpuscular volume
MHC: Mean haemoglobin concentration
MCHC: mean corpuscular haemoglobin concentration

2. Clinical chemistry findings: High dose effects were limited to slightly decreased cholesterol levels in both ♂ and ♀. For ♂ this was below the historical control range, lacking statistical significance. For ♀ this was within the historical control range. This was deemed to be a treatment related effect, but likely slight (without correlating liver histopathology) rather than adverse.
For other clinical chemistry parameters analysed (electrolytes, kidney and liver functional tests) no treatment related effects were observed.

3. Urinalysis: There were no treatment related effects. ♂ at 150 ppm and above showed increased incidence pf urine reacting positive for blood. This qualitative assessment was considered incidental as it was limited to a single sex, the incidence were consistent with the historical control range, did not correlate with any associate adverse histopathology.

Table CA 5.8.1/05-5: Overview of sub-acute toxicity study in rats treated orally (*via* diet) with spiroxamine N-oxide: selected clinical chemistry parameters

Parameters	♂ (ppm)				♀ (ppm)			
	0	30	150	1000	0	30	150	1000
Dose/animal (mg/kg bw/d)	0	2.6	12.9	114.6	0	2.7	13.2	94.3
T.chol (mmol/L)	1.55 ±0.138	1.82 ±0.248	1.72 ±0.280	1.29 ±0.205	1.85 ±0.157	1.86 ±0.187	1.69 ±0.237	1.52 ±0.097*
Laboratory historical control data (rat, Wistar 8 -11 wks of age)								
T.chol (mmol/L)	Date range: 1994 - 1995 *n:* 64 Mean ±SD: 2.04 ±0.290 Range ±2SD: 1.46 – 2.61				Date range: 1994 - 1995 *n:* 56 Mean ±SD: 2.08 ±0.362 Range ±2SD: 1.35 – 2.80			

* $p \leq 0.05$
T.chol.: total cholesterol

F. Sacrifice and pathology:

1. Organ weight: At 1000 ppm decreased absolute heart and liver weights were observed in ♂, reflective of reductions in terminal body weight. When expressed as relative organ weights, organ weights were comparable to the concurrent control (including endocrine producing and endocrine sensitive organs, where weighed).

Table CA 5.8.1/05-6: Overview of sub-acute toxicity study in rats treated orally (*via* diet) with spiroxamine N-oxide: selected organ weights

Parameters		♂ (ppm)				♀ (ppm)			
		0	**30**	**150**	**1000**	**0**	**30**	**150**	**1000**
Dose/animal (mg/kg bw/d)		0	2.6	12.9	114.6	0	2.7	13.2	94.3
Terminal bwt (g)		283 ±9.4	305 ±12.6 (↑8%)	278 ±24.8 (↓2%)	260 ±6.2 (↓8%)	197 ±42.9	191 ±4.9 (↑8%)	189 ±6.6 (↑7%)	176 ±4.7 (↓1%)
Liver	Abs (g)	12.9 ±0.6	14.8 ±0.8 (↑15%)	12.4 ±1.3 (↓4%)	11.3 ±0.3* (↓12%)	6.8 ±0.7	7.1 ±0.7 (↑4%)	7.2 ±0.5 (↑6%)	6.9 ±0.3 (↑1%)
	Rel. (g%)	4.6 ±0.2	4.9 ±0.1 (↑7%)	4.5 ±0.3 (↓2%)	4.3 ±0.2 (↓7%)	3.9 ±0.3	3.7 ±0.3 (↓5%)	3.8 ±0.3 (↓3%)	3.9 ±0.2 (-)
Heart	Abs (g)	0.94 ±1.0	0.99 ±0.6 (↑5%)	0.86 ±0.7 (↓9%)	0.81 ±1.7 (↓14%)	0.65 ±0.05	0.71 ±0.07 (↑9%)	0.67 ±0.08 (↑3%)	0.63 ±0.06 (↓3%)
	Rel. (g%)	0.33 ±0.03	0.33 ±0.01 (-)	0.31 ±0.02 (↓6%)	0.31 ±0.002 (↓6%)	0.37 ±0.03	0.38 ±0.03 (↑3%)	0.35 ±0.04 (↓5%)	0.36 ±0.03 (↓3%)
Spleen	Abs (g)	0.51 ±0.06	0.63 ±0.06 (↑24%)	0.51 ±0.03 (-)	0.56 ±0.09 (↑10%)	0.38 ±0.02	0.41 ±0.05 (↑8%)	0.49 ±0.1 (↑29%)	0.41 ±0.07 (↑8%)
	Rel. (g%)	0.18 ±0.02	0.21 ±0.02 (↑17%)	0.18 ±0.02 (-)	0.20 ±0.03 (↑11%)	0.21 ±0.04	0.21 ±0.03 (-)	0.26 ±0.06 (↑24%)	0.23 ±0.04 (↑10%)
Adrenals	Abs (mg)	44 ±1.5	48 ±3.2 (↑9%)	36 ±5.5 (↓18%)	38 ±5.8 (↓14%)	53 ±6.5	57 ±9.3 (↑8%)	60 ±9.2 (↑13%)	53 ±6.0 (-)
	Rel. (mg%)	16 ±0.3	16 ±1.6 (-)	13 ±1.6 (↓19%)	15 ±1.9 (↓6%)	30 ±3.0	30 ±5.5 (-)	32 ±4.6 (↑7%)	30 ±3.6 (-)
Testes	Abs (g)	3.1 ±0.3	3.4 ±0.2 (↑10%)	2.5 ±0.6 (↓20%)	3.2 ±0.4 (↑6%)	-	-	-	-
	Rel. (g%)	1.1 ±0.06	1.1 ±0.05 (-)	0.91 ±0.2 (↓17%)	1.3 ±0.2 (↑18%)	-	-	-	-
Epidid.	Abs (g)	1.3 ±0.2	1.2 ±0.2 (↓8%)	1.1 ±0.9 (↓15%)	1.2 ±0.2 (↓8%)	-	-	-	-
	Rel. (g%)	0.45 ±0.07	0.40 ±0.06 (↓11%)	0.38 ±0.06 (↓16%)	0.46 ±0.08 (↑2%)	-	-	-	-
Ovary	Abs (g)					109 ±29.2	114 ±25.3 (↑5%)	110 ±20.0 (↑1%)	93 ±12.6 (↓15%)
	Rel. (g%)	-	-	-	-	62 ±17.9	60 ±13.6 (↓3%)	58 ±9.0 (↓6%)	53 ±6.8 (↓15%)

* $p \leq 0.05$
Abs.: absolute
Rel.: relative to body weight

Epidid: epididymides

2. Gross pathology: Macroscopic findings were limited to the liver and consisted of evident lobular or discoloration in the high dose ♂ and ♀

3. Histopathology: In high group animals, hyperkeratosis of the epithelium of the oesophagus and forestomach were observed with mild transitional cell hyperplasia in the urinary bladder. As with the parent compound, spiroxamine, spiroxamine N-oxide is a tertiary amine, and like all tertiary amines has a high pKa and a high pH with comparable histopathological effects reported. As with irritant compounds, tissues containing mucosal membrane is deemed to be a target organ critical effect. This is acknowledged in the literature, with optimal pH ranges for substances administered orally *via* gavage not to exceed pH 9, with administration of substances outside of the recommended pH ranges resulting in tissue necrosis, pH partitioning *etc.* (Gad *et al.*[18], Turner *et al.*[19]) This should not be dismissed.

Hyaline droplet nephropathy was observed in the kidneys of two high dose group males. This lesion is due to the accumulation of α2μ-globulin which is known to be a lesion specific to male rats with no relevance to humans, with Azan specific staining used to confirm α2μ-globulin presence.

Table CA 5.8.1/05-7: Overview of sub-acute toxicity study in rats treated orally (*via* diet) with spiroxamine N-oxide: selected histopathology observations

Parameters	♂ (ppm)				♀ (ppm)			
	0	**30**	**150**	**1000**	**0**	**30**	**150**	**1000**
Dose/animal (mg/kg bw/day)	0	2.6	12.9	114.6	0	2.7	13.2	94.3
Histopathology: [incidence / total no. examined [minimal, slight, moderate]]								
Kidney - Hyaline droplet nep.	0/5 [0,0,0]	0/5 [0,0,0]	0/5 [0,0,0]	2/5 [0,2,0]	-		-	-
Urinary bladder - Hyperpl. mild trans.	0/5 [0,0,0]	0/5 [0,0,0]	0/5 [0,0,0]	2/5 [1,1,0]	0/5 [0,0,0]	0/5 [0,0,0]	0/5 [0,0,0]	5/5 [3,2,0]
Stomach - Hyperkeratosis	0/5 [0,0,0]	0/5 [0,0,0]	0/5 [0,0,0]	5/5 [0,4,1]	0/5 [0,0,0]	0/5 [0,0,0]	0/5 [0,0,0]	4/5 [2,2,0]
Oesophagus - Hyperkeratosis	0/5 [0,0,0]	0/5 [0,0,0]	0/5 [0,0,0]	5/5 [1,4,0]	0/5 [0,0,0]	0/5 [0,0,0]	0/5 [0,0,0]	5/5 [0,4,1]

* $p \leq 0.05$; ** $p \leq 0.01$

Hyaline droplet nep.: hyaline droplet nephropathy Hyperpl. mild trans.: hyperplasia, mild transitional cell

4. Liver tissue enzyme analysis: Investigations of the liver tissue showed no treatment related effects at 150 ppm and below. In ♂ aminopyrine-N-demethylase activity was increased at 150 and 1000 ppm achieving statistical significance at the high dose group. In both ♂ and ♀ in the high dose group, cytochrome P450 activity was increased. These changes were deemed treatment related, but deemed adaptive and not adverse as no concurrent liver histopathological findings were evident, with other liver enzymes which were analysed (ALP, ALT, AST) remaining unaffected.

Table CA 5.8.1/05-8: Overview of sub-acute toxicity study in rats treated orally (*via* diet) with spiroxamine N-oxide: liver tissue enzyme analysis

Parameters	♂ (ppm)				♀ (ppm)			
	0	30	150	1000	0	30	150	1000
Dose/animal (mg/kg bw/day)	0	2.6	12.9	114.6	0	2.7	13.2	94.3
N-DEM (mU/g)	118.3 ±19.61	127.8 ±21.29	149.6 ±20.43	151.4 ±11.52*	83.9 ±5.48	78.8 ±11.03	67.7 ±6.44	88.0 ±12.98
O-DEM (mU/g)	10.4 ±0.66	10.3 ±1.42	9.1 ±1.30	10.5 ±0.98	7.3 ±1.43	7.8 ±0.48	6.7 ±0.66	8.0 ±1.29
Cyto-P450 (nmol/g)	37.1 ±2.11	36.8 ±2.83	39.5 ±3.09	46.7 ±2.12**	34.4 ±1.36	36.4 ±1.74	33.3 ±4.46	41.9 ±2.52**

** $p \leq 0.01$
N-DEM: aminopyrine-N-demethylase
O-DEM: *p*-nitroanisole-*O*-demethylase
Cyto-P450: cytochrome P450 activity

G. Discussion:

No treatment-related effects were seen on survival, clinical signs, food consumption, urinalysis or serum chemistry / haematology parameters. High dose effects were limited to slightly decreased cholesterol levels in both males and females. This was deemed to be a treatment related effect but likely slight (without correlating liver histopathology) rather than adverse.

At 1000 ppm, males showed a reduction in body weight, with a -7% reduction by the end of treatment when compared to the concurrent control, with statistically significant reductions in body weight from day 7 through to day 21. Body weight gain in this group was markedly reduced, with 16% reduction. In females at 1000 ppm body weights were comparable to the concurrent control group. Whilst statistically significant increases in body weight were observed at 30 and 150 ppm dose groups, this was not dose related. When body weight gain at the end of the treatment period was examined, a 19% reduction compared to the concurrent control group was observed.

In high group animals, hyperkeratosis of the epithelium of the oesophagus and forestomach were observed with mild transitional cell hyperplasia in the urinary bladder. As with the parent compound, spiroxamine, spiroxamine N-oxide is a tertiary amine, and like all tertiary amines has a high pKa, and a high pH with comparable histopathological effects reported. As with irritant compounds, tissues containing mucosal membrane is deemed to be a target organ critical effect. This is acknowledged in the literature, with optimal pH ranges for substances administered orally *via* gavage not to exceed pH 9, with administration of substances outside of the recommended pH ranges resulting in tissue necrosis, pH partitioning *etc*. This should not be dismissed.

Hyaline droplet nephropathy was observed in the kidneys of two high dose group males. This lesion is due the accumulation of α2μ-globulin which is known to be a lesion specific to male rats with no relevance to humans, with Azan specific staining used to confirm α2μ-globulin presence.

H. Deficiencies:

Although the study was conducted according to test guideline OECD 407 (1995), this test guideline has since been updated in the intervening period (2008). When assessed against current test guideline requirements the following deficiencies are noted:

- Whilst not a requirement, the test guideline makes reference to determination of serum thyroid hormones (T3, T4, TSH). These were not analysed. However, the gold standard to assess thyroid effects is histopathological analysis. This was undertaken on all animals and revealed no histopathological findings. Consequently the lack of serum thyroid hormone analysis is not deemed critical, with potential thyroid effects adequately addressed with histopathological analysis.
-

Assessment and conclusions by applicant:

Assessment: Study meets the current guidance and the requirements in 283/2013.

Conclusion: Under the conditions of this study the NOAEL following 28 days oral is deemed to be 150 ppm (equivalent to 12.9/13.2 mg/kg bw/day for males/females) based on reduction in body weight gain (exceeding 10%), hyperkeratosis of the stomach and oesophagus with mild transitional cell hyperplasia in the urinary bladder. These effects are associated with the irritant nature of the test article.

Data Point:	KCA 5.8.1/06
Report Author:	[redacted]
Report Year:	1998
Report Title:	KWG 4168 N-Oxide - Study for subchronic oral toxicity in rats (feeding study over 13 weeks)
Report No:	27475
Document No:	M-016585-01-2
Guideline(s) followed in study:	OECD 408; US-EPA §82-1; Directive 87/302/EEC, Part B
Deviations from current test guideline:	Yes Although the study was conducted according to test guideline OECD 408 (1981), this test guideline has since been updated in the intervening period (1998, 2008). When assessed against current test guideline requirements the following deficiencies are noted: Serum thyroid hormones (T3, T4, TSH) and clinical chemistry parameters sensitive to thyroid pathway perturbations (LDL, HDL) were not analysed. However, the gold standard to assess thyroid effects is histopathological analysis. This was undertaken on all animals and revealed no histopathological findings. Consequently the lack of serum thyroid hormone analysis is not deemed critical, with potential thyroid effects adequately addressed with histopathological analysis.
Previous evaluation:	yes, evaluated and accepted DAR (2010)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

In this study, spiroxamine N-oxide was administered continuously *via* the diet for 90 days to Wistar rats. Animals (10/sex/group) were administered test diet at concentrations of 0, 25, 125, 625 ppm (equivalent to males/females/♀: 0/0, 1.7/1.9, 8.8/9.7, 45.0/53.6 mg/kg bw/day). An additional group (10/sex) was included which were fed with spiroxamine at 625 ppm (equivalent to 48.7/52.7 mg/kg bw/da for males/females). Animals were subjected to body weight, food and water consumption which were measured at regular intervals. Clinical pathology evaluations (haematology, clinical chemistry and urinalysis) were performed with all surviving animals subjected to complete gross necropsy and full histopathology.

No treatment-related effects were seen on survival, clinical signs, food consumption, urinalysis or serum chemistry / haematology parameters. High dose effects were limited to slightly decreased cholesterol levels in both males and females. This was deemed to be a treatment related effect, but likely slight (without correlating liver histopathology) rather than adverse.

For animals treated with spiroxamine N-oxide, at 625 ppm, males showed a reduction in body weight from week 1 onwards, with statistically significant reductions in body weight from the second week through to week 9 with body weights being approximately 9-10% lower than the concurrent control. At termination of treatment, body weight gain in high dose group males was 9% lower. For females dosed

with spiroxamine N-oxide at 625 ppm, whilst body weight was lower throughout the dosing period from the first week onwards, but not no greater than 5% lower than the concurrent control group. At termination of treatment, body weight gain in high dose group females was 1% lower.

For animals treated with spiroxamine, at 625 ppm, males showed a statistically significant reduction in body weight from week 1 onwards, with body weights being approximately 10-17% lower than the concurrent control. At termination of treatment, body weight gain in this dose group was 14% lower. For females dosed with spiroxamine at 625 ppm females showed a statistically significant reduction in body weight from week 1 onwards, with body weights being approximately 12% lower than the concurrent control. At termination of treatment, body weight gain in this dose group was 19% lower.

Throughout the dosing period, food consumption was marginally greater in animals of the 625 ppm dose group for both test articles when expressed as g/kg bw. Whilst not measured, a qualitative assessment from increased food consumption of high dose group males and reduction in body weight implies that food efficiency dropped. Water intake at 625 ppm dose group for both test articles were marginally reduced in both sexes when expressed as g/animal. When corrected for body weight this effect was not replicated and deemed a result of a reduction in body weight.

No treatment related haematological effects were reported with either test article, however clotting time (PT) was significantly extended at termination in high dose group males receiving spiroxamine N-oxide.

For animals receiving spiroxamine, alterations in red blood cell parameters were evident, suggestive of anaemia, with reductions in haematocrit, MCV, MHC, MCHC, reticulocyte counts and hypochromasia (*i.e.* RBC pale in colour, suggestive of reduced Hb concentration) were increased. Total and differential WBC parameters were affected in both sexes, with leucocytes, neutrophils, basophils, lymphocytes were increased and eosinophils and atypical leukocytes were decreased. Clotting time was significantly extended in both sexes at both sample times. Extended PT was likely a secondary effect resultant from decreases in both serum albumin and total protein.

In high group animals, hyperkeratosis of the epithelium of the oesophagus and forestomach were observed in both males and females dosed with spiroxamine N-oxide. Similar effects were observed in animals receiving spiroxamine, however the incidence and severity were greater in both instances. In addition, hyperkeratosis of the ear was observed in spiroxamine treated animals. Mild transitional cell hyperplasia in the urinary bladder was observed in females dosed with spiroxamine. As with the parent compound, spiroxamine, spiroxamine N-oxide is a tertiary amine, and like all tertiary amines has a high pKa, and a high pH with comparable histopathological effects reported. As with irritant compounds, tissues containing mucosal membrane is deemed to be a target organ critical effect. This is acknowledged in the literature, with optimal pH ranges for substances administered orally *via* gavage not to exceed pH 9, with administration of substances outside of the recommended pH ranges resulting in tissue necrosis, pH partitioning *etc.* This should not be dismissed.

Hyaline droplet nephropathy was observed in the kidneys of all treated males. This lesion is due the accumulation of α2μ-globulin which is known to be a lesion specific to male rats with no relevance to humans, with Azan specific staining used to confirm α2μ-globulin presence.

Under the conditions of this study the NOAEL following 90 days oral administration is deemed to be 150 ppm (equivalent to 12.9/13.2 mg/kg bw/day for males/females) based on reduction in body weight gain (exceeding 10%), hyperkeratosis of the stomach and oesophagus with mild transitional cell hyperplasia in the urinary bladder. These effects are associated with the irritant nature of the test article.

Materials and Methods

A. Materials:

1. Test Material:	KWG 4168-N-oxide (alternative names: Spiroxamine N-oxide, [(8-tert-butyl-1,4-dioxaspiro[4.5]dec-2-yl) methyl](ethyl)(propyl)amine-N-oxide, M03)
Description:	Colourless liquid

Lot/Batch No.:	0153560
Purity:	90.8% (w/w) (correction for purity not undertaken)
CAS No.:	148044-85-3
Stability of test compound:	Confirmed stable for the duration of the study (expiry date: 26 August 1998)
1. Test Material (continued):	Spiroxamine (alternative name: [8-(1,1-dimethylethyl)-N-ethyl-N-propyl-1,4-dioxaspiro[4,5]decane-2-methanamine; KWG 4168)
Description:	Clear light brown oil
Lot/Batch No.:	202740026
Purity:	96.5% (w/w) (correction for purity not undertaken)
CAS No.:	118134-30-8
Stability of test compound:	Confirmed stable for the duration of the study (expiry date: 3 September 1998)
2. Vehicle and/or positive control:	Basal diet / not relevant
3. Test animals:	
Species:	Rat
Strain:	Wistar
Age at dosing:	5-6 wks
Weight at dosing:	♂: 125-152g; ♀: 105-133g
Source:	[redacted]
Acclimation period:	At least 7 days
Diet:	Altromin® 1231 fixed-formula standard diet (Altromin GmbH, Lage, Germany) *ad libitum*
Water:	Municipal water, *ad libitum*
Housing:	Individually housed
4. Environmental conditions:	
Temperature:	22 ± 2°C
Humidity:	55 ± 5%
Air changes:	*ca.* 15-20h
Photoperiod:	12 hour light/dark

B. Study Design:

1. In life dates:	5 January 1998 to 15 April 1998 (experimental dates)
2. Animal assignment and treatment:	After an acclimatisation period rats were allocated to groups by computer-based stratified random sampling. Dose levels selected based on the results from the sub-chronic study undertaken on the parent compound, spiroxamine, in which animals were dosed at 0, 25, 125, 625 ppm were dosed. The test article, spiroxamine N-oxide was administered continuously *via* the diet to groups of rats for a period of 90 days. Animals (10/sex/gp), were administered test diet at concentrations of 0, 25, 125, 625 ppm (equivalent to ♂/♀: 0/0, 1.7/1.9, 8.8/9.7, 45.0/53.6 mg/kg bw/day). An additional group (10/sex) was included which were fed with spiroxamine at 625 ppm (equivalent to 48.7/52.7 mg/kg bw/day for ♂/♀). Following 90 days of treatment 10 animals/sex were subjected to complete necropsy. Body weight, water and food consumption were measured at regular intervals. Clinical pathology evaluations (haematology, clinical chemistry and urinalysis) were performed with all surviving animals subjected to complete gross necropsy and full histopathology.
3. Statistics:	Dunnett's test: body weight, feed and water consumption, organ weight data.

Adjusted Welch test for heterogeneous error
The non-parametric Kruskal-Wallis Test was used. If the *p* value indicated a nominal significance, pairwise treatment control comparisons were performed with the Mann-Whitney-Wilcoxon test.

Table CA 5.8.1/06-1: Overview of sub-chronic toxicity study in rats treated orally (*via* diet) with spiroxamine N-oxide: study design and dose received

Parameters	♂ (ppm)					♀ (ppm)				
	SPX N-oxide				SPX	SPX N-oxide				SPX
	0	25	125	625	625	0	25	125	625	625
Dose/animal (mg/kg bw/day)	0	1.7	8.8	45.0	48.7	0	1.9	9.7	53.6	52.7
Animals assigned/sex	10	10	10	10	10	10	10	10	10	10

C. Methods:

1. Test diet preparation and analysis: Spiroxamine N-oxide was prepared at dietary concentrations of 0, 25, 125 and 625 ppm. 1% peanut oil was added to minimize dust formation. The prepared test diets were prepared once and stored frozen (approximately −15°C) until before use.
Stability and homogeneity of the diet preparation containing the test article were three times throughout the study (refer to Doc MCA Section 4 [M-020915-01-1] for method validation).

2. Observations: Animals were inspected twice daily for signs of toxicity and mortality.

3. Body weights: Animals were weighed prior to study start and then weekly thereafter.

4. Food consumption: Determined by weighing food supplied and food that remained from day 3 onwards and thereafter at twice weekly intervals.
From the food consumption data, compound consumption was calculated using the following equation:

$$\text{Cpd consumption (mg/kg bw/d)} = \frac{\text{Food consumption (g/rat/d)} \times \text{test article conc. (ppm)}}{\text{Body weight (g)}}$$

Food efficiency was not calculated.

5. Water consumption: Calculated for each animal according to measurement of body weight from day 7 onwards and thereafter at weekly intervals.

6. Ophthalmological examination: Performed before the start of the study and on day 84 for all control and high dose animals.

7. Neurological functional examinations: Not conducted.

8. Haematology and clinical chemistry: Conducted on day 28/29 and 91/92. Animals were not fasted prior to blood sampling.
Haematology: red blood cell parameters (haematocrit (commonly termed PCV), haemoglobin concentration (Hb), mean haemoglobin concentration (MHC), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV), erythrocyte count, platelet count, reticulocyte count), white blood cell parameters (total and differential (neutrophils, lymphocytes, eosinophils, basophils, monocytes) leukocyte count), coagulation parameters (prothrombin time (PT) – termed Hepato-Quick Test).
Clinical chemistry: electrolytes (sodium, potassium, calcium, chloride, inorganic phosphorus), kidney function test (creatinine, urea), glucose, liver function tests (albumin, alkaline phosphatase (ALP), alanine aminotransferase (ALT [commonly referred to as glutamic pyruvic transaminase (GPT)]), aspartate aminotransferase (AST [commonly referred

to as glutamic oxaloacetic transaminase (GOT)), total bilirubin (T.Bili), total protein (TP), lipid profile (total cholesterol).

In addition, on day 24 blood was collected for glucose determination, with animals having fasted overnight.

9. Urinalysis: On day 84/85 of the study, all animals were given an oral dose of 5 mL water/animal and urine was collected for a subsequent 16 hours. During this time food was withdrawn. The following urinary parameters were measured: specific gravity, pH, total volume, protein, glucose, ketones, bilirubin, blood, urobilinogen, sediment

10. Organ weights: Adrenal glands, brain, epididymides, heart, kidney, liver, lung, ovary, spleen, testis

11. Sacrifice and pathology: Conducted on day 91. Gross pathological examination was performed on all animals and included examination of the external surface, all orifices and associated tissues.

The following tissues were preserved in 10% neutral buffered formalin for subsequent histopathological examination (with the exception of urinary bladder and lungs (+2nd liver lobe) which were fixed with Davidson's solution and 4% formaldehyde solution, respectively) and performed on control and high dose group animals. Tissues in bold: complete histopathological analysis of all groups. The kidneys were stained with azan methodology (chromotrope-aniline blue stain) and analysed for all ♂ for the presence of α2μ-globulin[13].

Accessory sex glands (♂:epididymides, prostate, seminal vesicle, seminal vesicles (+coagulating gland), testes; ♀: ovary, oviduct, uterus (+cervix), vagina),

cardiovascular/haematological system (aorta, bone (sternum, tibial or femur for marrow), heart, lymph nodes (mandibular, mesenteric), spleen, thymus), gastrointestinal tract (**oesophagus**, **tongue**, **stomach** (fore-, glandular stomach), intestine (caecum, colon duodenum, ileum, jejunum, rectum), **liver**, pancreas), salivary glands; neurological (brain (cerebrum, cerebellum, pons/medulla), eyes (+optic nerve, Harderian gland, extraorbital lacrimal glands), sciatic nerve, spinal cord (cervical, thoracic, lumbar), respiratory system (nose, trachea, **lung**), urogenital system (**kidneys** (ureters, urethra), **urinary bladder**), other (skeletal muscle (thigh), bone (femur, sternum), ear (**pinna**, Zymbal gland), **skin**, **tail**, **all gross lesions and masses**)

Other endocrine producing/sensitive glands (adrenals, mammary gland, pituitary, **thyroid (+parathyroid)**).

In liver tissue aminopyrine-N-demethylase, *p*-nitroanisole-*O*-demethylase and cytochrome P450 activity was determined in all animals.

12. Neurohisto-pathology: No specific neurohistopathology with specific fixatives were performed in addition to the standard histopathology undertaken on neuronal tissues.

Results and discussion

A. Test diet analysis:

Spiroxamine N-oxide was homogenously distributed and chemically stable for at least 6 weeks within the concentration range of 25 to 625 ppm. The analytical data verify that the during the treatment period concentrations of the test article in the diet preparations ranged from -17.7% to +15% of nominal concentrations 25, 125 and 625 ppm, which were within acceptable limits.

B. Observations:

1. Clinical signs of toxicity: No treatment related effects observed with either test article.

2. Mortality: All animals survived until the scheduled necropsy.

3. Ophthalmoscopic examination: There were no treatment related effects were observed in animals treated with either spiroxamine N-oxide or spiroxamine.

4. Neurological functional examinations	Not conducted.

C. Body weight and body weight gain:

1. Spiroxamine N-oxide:	At 625 ppm, ♂ showed a reduction in body weight from week 1 onwards, with statistically significant reductions in body weight from the second week through to week 9 with body weights being approximately 9-10% lower than the concurrent control. At termination of treatment, body weight gain in high dose group ♂ was 9% lower. In ♀ at 625 ppm spiroxamine N-oxide, whilst body weight was lower throughout the dosing period from the first week onwards, but not no greater than 5% lower than the concurrent control group. At termination of treatment, body weight gain in high dose group ♀ was 1% lower.
2. Spiroxamine:	At 625 ppm, ♂ showed a statistically significant reduction in body weight from week 1 onwards, with body weights being approximately 10-17% lower than the concurrent control. At termination of treatment, body weight gain in this dose group was 14% lower. At 625 ppm, ♀ showed a statistically significant reduction in body weight from week 1 onwards, with body weights being approximately 12% lower than the concurrent control. At termination of treatment, body weight gain in this dose group was 19% lower.

Table CA 5.8.1/06-2: Overview of sub-chronic toxicity study in rats treated orally (*via* diet) with spiroxamine N-oxide: food consumption

Parameters		♂ (ppm)					♂ (ppm)				
		SPX N-oxide				SPX	SPX N-oxide				SPX
		0	25	125	625	625	0	25	125	625	625
Dose/animal (mg/kg bw/d)		0	1.7	8.8	45.0	48.7	0	1.9	9.7	53.9	52.7
Body wt (g)	Day 0	140 ±7.4	137 ±7.8	141 ±6.6	141 ±9.4	141 ±10.1	123 ±6.3	117 ±6.1*	117 ±6.3*	121 ±3.1	118 ±5.9
	Day 7	191 ±13.5	185 ±9.4	190 ±10.8	182 ±9.1	172 ±13.6**	146 ±8.3	139 ±8.6	138 ±7.6	141 ±6.8	132 ±7.8**
	Day 14	234 ±15.7	224 ±12.5	236 ±14.1	217 ±12.2*	203 ±18.7**	163 ±7.5	153 ±10.1	152 ±9.5*	155 ±5.9	144 ±8.4**
	Day 21	275 ±19.7	260 ±16.3	278 ±18.6	250 ±16.7*	232 ±25.2**	178 ±9.2	170 ±12.2	168 ±10.1	169±6.4	156 ±10.3**
	Day 28	295 ±20.3	277 ±17.5	297 ±21.5	269 ±16.5*	246 ±39.9**	185 ±8.9	177 ±12.0	174 ±14.1*	178 ±7.5	164 ±11.3**
	Day 35	319 ±21.1	298 ±20.2	324 ±24.3	289 ±22.4*	264 ±32.9**	193 ±10.4	186 ±10	184 ±14.1	189 ±9.1	170 ±12.2**
	Day 42	345 ±23.2	320 ±21.5	351 ±25.7	314 ±23.3*	287 ±36.6**	204 ±9.7	195 ±12.9	193 ±13.7	199 ±9.6	179 ±161**
	Day 49	361 ±23.3	337 ±23.0	368 ±29.9	332 ±26.1*	308 ±39.4**	208 ±9.5	201 ±15.4	198 ±14.9	204 ±10.2	184 ±13.4**
	Day 56	378 ±25.4	354 ±24.9	391 ±31.9	347 ±28.7*	327 ±41.4**	215 ±10.8	208 ±15.2	205 ±15.9	211 ±108	189 ±14.5**
	Day 63	393 ±25.5	369 ±25.6	405 ±34.5	361 ±26.1*	342 ±42.7**	220 ±11.8	213 ±15.0	208 ±16.3	219 ±13.5	195 ±14.6**
	Day 91	428 ±30.3	412 ±30.2	450 ±36.6	403 ±24.1	388 ±44.4*	233 ±13.2	226 ±15.6	222 ±18.9	232 ±13.6	207 ±15.8**
Body wt gain (g)	Day 0-91	288	275 (↓5%)	309 (↑7%)	262 (↓9%)	247 (↓14%)	110	108 (↓2%)	105 (↓5%)	109 (↓1%)	89 (↓19%)

* $p \leq 0.05$; ** $p \leq 0.01$

D. Food consumption, food efficiency and water consumption:

1. Food consumption:	Throughout the dosing period, food consumption was marginally greater in animals of the 625 ppm dose group for both test articles when expressed as a g/kg bw.
2. Food efficiency:	Whilst not measured, a qualitative assessment from increased food consumption of high dose group ♂ and reduction in body weight implies that food efficiency dropped.
3. Water consumption:	Water intake at 625 ppm dose group for both test articles were marginally reduced in both sexes when expressed as g/animal. When corrected for body weight this effect was not replicated and deemed a result of a reduction in body weight.

Table CA 5.8.1/06-3: Overview of sub-chronic toxicity study in rats treated orally (*via* diet) with spiroxamine N-oxide: food consumption

Parameters		♂ (ppm)					♂ (ppm)				
		SPX N-oxide				SPX	SPX N-oxide				SPX
		0	25	125	625	625	0	25	125	625	625
Dose/animal (mg/kg bw/d)		0	1.7	8.8	45.0	48.7	0	1.9	9.7	53.6	52.7
Food consum. (g/kg/d)	Day 7	107 ±4.0	108 ±3.2	114 ±11.1	114 ±10.6	109 ±11.6	104 ±3.0	106 ±9.0	104 ±4.0	128 ±21.4**	97 ±9.4*
	Day 14	93 ±6.2	93 ±5.3	97 ±5.8	101 ±12.9	118 ±29.9**	102 ±13.4	100 ±8.7	100 ±7.2	129 ±31.1	123 ±39.1
	Day 21	83 ±5.9	81 ±4.5	84 ±3.2	88 ±9.7	94 ±10.1**	97 ±10.2	90 ±6.4	91 ±6.1	102 ±13.9	105 ±27.4
	Day 28	79 ±6.1	11 ±5.1	82 ±4.0	86 ±3.9	93 ±14.3*	95 ±7.5	90 ±4.9	89 ±11.0	87 ±17.0	103 ±17.5
	Day 49	56 ±3.7	57 ±5.3	61 ±5.2*	63 ±3.2**	70 ±7.3**	72 ±4.2	71 ±7.6	72 ±6.7	81 ±19.8	92 ±17.6
	Day 66	53 ±3.7	54 ±3.7	54 ±4.6	60 ±3.5**	68 ±9.4	68 ±7.2	68 ±4.9	66 ±11.5	70 ±7.6	72 ±11.1
	Day 70	52 ±4.2	55 ±5.4	53 ±3.4	58 ±3.8*	63 ±7.1**	68 ±7.2	66 ±4.6	71 ±10.3	70 ±13.3	69 ±6.9
	Day 73	49 ±3.8	52 ±5.3	55 ±4.3	57 ±4.4**	60 ±6.6**	60 ±5.9	66 ±6.3	67 ±9.4	66 ±17.3	69 ±6.5**
	Day 77	46 ±4.1	50 ±6.1	51 ±4.5	54 ±3.9*	57 ±5.3**	62 ±4.9	62 ±6.1	64 ±5.2	65 ±6.1	65 ±7.9
	Day 80	51 ±3.9	50 ±6.4	50 ±4.4	53 ±2.9	58 ±6.4*	70 ±13.9	62 ±6.6	62 ±7.3	66 ±4.5	66 ±7.8
	Day 84	45 ±3.9	46 ±5.9	53 ±20.8	52 ±3.2*	55 ±4.6**	61 ±6.1	64 ±24.4	62±6.9	64 ±6.3	64 ±5.5
	Day 87	38 ±3.7	43 ±5.0	39 ±3.5	41 ±3.0	44 ±4.5**	44 ±4.5	48 ±5.4	51 ±3.3*	47 ±4.5	47 ±5.1
	Day 91	47 ±3.0	49 ±5.6	48 ±2.9	52 ±4.1*	55 ±4.9**	64 ±4.2	63 ±5.9	65 ±4.4	63 ±4.0	65 ±5.1

* $p \leq 0.05$

E. Blood and urinalysis:

1. Haematological findings:

Spiroxamine N-oxide:

In high dose group ♀ whilst reticulocyte counts were statistically significantly increased at the interim time point (day 29), all individual values were in the historical control range and were not present at the day 91. As other RBC parameters were unchanged (*i.e.* Hb concentration and haematocrit (PCV), RBC, MCV, MHC, MCHC values) and spleen weights were not affected, the reticulocyte change was deemed serendipitous.

In ♂, although thrombocyte count was unaffected, clotting time (PT) was significantly extended on day 91/92. This was also seen in the 28 day study in males dosed 1000 ppm (114 mg/kg bw/day).

Spiroxamine:

In ♂ and ♀ at the interim sample point and/or in ♀ at day 91/92 alterations in red blood cell parameters were evident, suggestive of anaemia, Reductions in haematocrit (♀, interim), MCV (♂♀ interim; ♀ day 91/92), MHC (♂ interim). MCHC, reticulocyte counts (♀ interim) and ♂ showing hypochromasia (*i.e.* RBC pale in colour, suggestive of reduced Hb concentration) were increased. Total and differential WBC parameters were affected in both sexes, with leucocytes, neutrophils, basophils (♂, interim), lymphocytes (♀ day 91/92) were increased and eosinophils and atypical leukocytes (♂, day 91/92) were decreased. Clotting time was significantly extended in both sexes at both sample times.

Extended PT was likely a secondary effect resultant from decreases in both serum albumin and total protein

Table CA 5.8.1/06-4: Overview of sub-chronic toxicity study in rats treated orally (*via* diet) with spiroxamine N-oxide: selected haematological RBC parameters

Parameters		**♂ (ppm)**					**♀ (ppm)**				
		SPX N-oxide			**SPX**		**SPX N-oxide**			**SPX**	
	day	**0**	**25**	**125**	**625**	**625**	**0**	**25**	**125**	**625**	**625**
Dose/animal (mg/kg bw/d)		0	1.7	8.8	45.0	48.7	0	1.9	9.7	53.6	52.7
RBC (10^{12}/L)	28	7.81 ±0.280	7.82 ±0.429	7.92 ±0.350	7.77 ±0.473	8.05 ±0.459	8.05 ±0.239	7.96 ±0.341	8.03 ±0.458	8.00 ±0.268	8.13 ±0.449
	91	8.55 ±0.180	8.54 ±0.502	8.63 ±0.413	8.33 ±0.305	8.55 ±0.180	7.92 ±0.183	7.84 ±0.284	7.89 ±0.359	8.02 ±0.232	7.99 ±0.256
Hb (g/L)	28	150 ±4.3	148 ±4.9	150 ±6.8	150 ±6.8	148 ±6.3	151 ±2.7	152 ±5.4	150 ±5.6	149 ±3.9	150 ±5.1
	91	145 ±5.2	144 ±5.1	147 ±4.2	146 ±5.1	142 ±6.7	140 ±2.9	142 ±4.0	141 ±5.8	141 ±7.0	138 ±3.9
PCV (L/L)	28	0.486 ±0.0577	0.464 ±0.0177	0.464 ±0.0202	0.464 ±0.0202	0.460 ±0.0160	0.457 ±0.0111	0.458 ±0.0140	0.455 ±0.0162	0.448 ±0.0106	0.444 ±0.0162[*]
	91	0.445 ±0.0166	0.441 ±0.0121	0.448 ±0.0131	0.449 ±0.0139	0.437 ±0.0237	0.421 ±0.0089	0.430 ±0.0102	0.426 ±0.0200	0.426 ±0.0169	0.414 ±0.0110
MCV (fL)	28	60.0 ±1.90	59.3 ±0.456	59.8 ±2.27	59.8 ±2.27	57.3 ±2.20[**]	56.8 ±1.33	57.6 ±1.64	56.7 ±2.10	56.0 ±1.73	54.6 ±1.76[**]
	91	52.1 ±1.98	51.7 ±2.88	52.0 ±1.46	53.9 ±1.64	51.3 ±1.88	53.2 ±1.33	54.9 ±1.62	53.8 ±2.45	53.2 ±1.85	51.8 ±1.59[*]
MHC (ρg)	28	19.2 ±0.45	18.9 ±01.21	19.3 ±0.62	19.3 ±2.27	18.4 ±0.73[*]	18.8 ±0.36	19.1 ±0.51	18.7 ±0.66	18.6 ±0.65	18.5 ±0.63
	91	16.9 ±0.57	16.9 ±1.14	17.1 ±0.73	17.6 ±0.61	16.7 ±0.61	17.7 ±0.37	18.2 ±0.33	17.8 ±0.73	17.6 ±0.74	17.3 ±0.64
MCHC (g/L RBC)	28	320 ±5.5	319 ±07.1	323 ±6.2	323 ±2.9	321 ±6.0	330 ±5.1	332 ±3.5	330 ±3.4	332 ±3.2	338 ±5.6[**]
	91	325 ±10.5	327 ±7.1	329 ±7.6	326 ±9.7	325 ±5.5	333 ±6.2	331 ±6.6	331 ±4.4	331 ±7.3	334 ±3.6
Ret. (%)	28	27 ±3.5	26 ±4.8	27 ±3.2	29 ±4.1	29 ±5.0	21 ±3.7	22 ±4.5	21 ±4.7	25 ±3.2	26 ±6.4[*]
	91	23 ±3.6	24 ±4.2	21 ±3.5	25 ±4.4	25 ±3.2	24 ±3.4	26 ±3.3	26 ±5.4	25 ±4.9	25 ±4.2
Thro ($10^{9/L}$)	28	1169 ±124.9	1178 ±83.0	1190 ±83.7	1111 ±81.5	1061 ±116.6	1095 ±42.2	1081 ±44.2	1081 ±102.4	1045 ±131.5	989 ±173.0
	91	1026 ±169.9	1017 ±135.7	1042 ±104.9	978 ±159.0	1054 ±141.3	985 ±119.3	971 ±115.9	960 ±109.5	1006 ±83.0	974 ±127.0
PT (sec)	28	32.1 ±0.60	31.9 ±1.63	32.2 ±1.43	32.9 ±2.29	34.0 ±1.60[*]	28.9 ±1.34	29.8 ±0.99	29.9 ±2.16	30.0 ±1.03	30.8 ±1.98[*]
	91	28.9 ±0.81	29.6 ±1.18	29.1 ±1.11	30.6 ±1.02	30.7 ±1.02[**]	27.9 ±1.01	27.7 ±0.88	27.5 ±1.20	27.8 ±1.11	28.9 ±1.24[*]
Hypochrom	28	0 ±0.3	1 ±0.7	1 ±0.7	0 ±0.3	1 ±0.9	0 ±0.0	0 ±0.0	0 ±0.0	0 ±0.0	0 ±0.0
	91	0 ±0.7	0 ±0.6	0 ±0.3	0 ±0.6	0 ±0.3	0 ±0.0	0 ±0.0	0 ±0.0	0 ±0.0	0 ±0.0
Laboratory historical control data (rat, Wistar, 1994-1995)											
PCV (L/L)		Age of animals: *n:* Mean ±SD: Range ±2SD:	- - - -				Age of animals: *n:* Mean ±SD: Range ±2SD:	8 – 11 wks 330 0.440 ±0.0253 0.389 – 0.491			
MCV (fL)		Age of animals: *n:*	8 – 11 wks 299				Age of animals: *n:*	8 – 11 wks 329		12 - 25 wks 257	

Parameters		♂ (ppm)					♀ (ppm)				
		SPX N-oxide			SPX		SPX N-oxide			SPX	
	day	0	25	125	625	625	0	25	125	625	625
		Mean ±SD: Range ±2SD:	57.0 ±2.40 52.2 – 61.8				Mean ±SD: Range ±2SD:	55.6 ±2.09 51.4 – 59.8		52.7 ±2.49 47.7 – 57.6	
MHC (ρg)		Age of animals: *n:* Mean ±SD: Range ±2SD:	8 – 11 wks 304 18.2 ±0.82 16.6 – 19.9				Age of animals: *n:* Mean ±SD: Range ±2SD:	-			
MCHC (g/L RBC)		Age of animals: *n:* Mean ±SD: Range ±2SD:	- - - -				Age of animals: *n:* Mean ±SD: Range ±2SD:	8 – 11 wks 324 324 ±8.2 307 - 340			
Ret. (%)		Age of animals: *n:* Mean ±SD: Range ±2SD:		- - - -			Age of animals: *n:* Mean ±SD: Range ±2SD:		8 – 11 wks 209 22 ±7.3 12 - 37		
PT (sec)		Age of animals: *n:* Mean ±SD: Range ±2SD:	8 – 11 wks 304 27.3 ±1.41 24.5 – 30.1		12 – 25 wks 249 27.3 ±1.42 24.4 – 30.1		Age of animals: *n:* Mean ±SD: Range ±2SD:	8 – 11 wks 334 26.0 ±1.53 22.9 – 29.0		12 – 25 wks 250 25.7 ±1.19 23.3 – 28.0	

**p* ≤0.05; ***p* ≤0.01
RBC: red blood cell
Hb: haemoglobin
PCV: packed corpuscular volume (haematocrit)
MCV: mean corpuscular volume
MHC: Mean haemoglobin concentration
MCHC: mean corpuscular haemoglobin concentration
Ret.: Reticulocytes
Thro.: Thrombocytes
PT: prothrombin time
Hypochrom.: hypochromasia

Table CA 5.8.1/06-5: Overview of sub-chronic toxicity study in rats treated orally (*via* diet) with spiroxamine N-oxide: selected haematological WBC parameters

Parameters		♂ (ppm)					♀ (ppm)				
		SPX N-oxide				SPX	SPX N-oxide				SPX
	day	0	25	125	625	625	0	25	125	625	625
Dose/animal (mg/kg bw/d)		0	1.7	8.8	45.0	48.7	0	1.9	9.7	53.6	52.7
Leuko. (10^9/L)	28	9.74 ±2.105	10.31 ±2.051	9.43 ±2.122	9.39 ±1.609	12.12 ±2.814*	8.06 ±1.243	7.65 ±1.076	8.08 ±2.176	8.99 ±1.178	9.77 ±2.388
	91	6.82 ±0.708	6.51 ±1.504	7.04 ±1.542	6.31 ±1.261	6.91 ±1.499	3.16 ±0.692	3.29 ±1.035	3.75 ±1.455	4.18 ±1.127	3.77 ±0.847

Parameters		♂ (ppm)					♀ (ppm)				
		SPX N-oxide				SPX	SPX N-oxide				SPX
	day	0	25	125	625	625	0	25	125	625	625
Neutro. (10^9/L)	28	0.80 ±0.3116	1.02 ±0.592	0.82 ±0.678	0.92 ±0.324	1.94 ±2.064*	0.79 ±0.271	0.67 ±0.343	0.71 ±0.301	0.79 ±0.514	0.91 ±0.427
	91	0.56 ±0.206	0.28 ±0.191	0.61 ±0.160	0.56 ±0.235	0.68 ±0.218	0.37 ±0.181	0.33 ±0.148	0.36 ±0.146	0.32 ±0.106	0.31 ±0.105
Lymph. (10^9/L)	28	8.33 ±1.821	8.66 ±1.692	8.08 ±1.408	7.94 ±1.529	9.44 ±1.122	6.74 ±1.076	6.56 ±0.778	6.93 ±1.958	7.76 ±1.304	8.37 ±2.182
	91	5.86 ±0.531	5.57 ±1.461	6.04 ±1.448	5.44 ±1.114	5.93 ±1.230	2.65 ±0.582	2.82 ±0.913	3.23 ±1.335	3.05 ±0.019	3.30 ±0.725
Eosino. (10^9/L)	28	0.12 ±0.063	0.10 ±0.035	0.08 ±0.026	0.09 ±0.047	0.11 ±0.031	0.11 ±0.042	0.08 ±0.042	0.08 ±0.035	0.09 ±0.035	0.09 ±0.033
	91	0.15 ±0.046	0.16 ±0.071	0.014 ±0.070	0.11 ±0.051	0.10 ±0.026	0.06 ±0.019	0.07 ±0.044	0.05 ±0.013	0.05 ±0.018	0.08 ±0.064
Baso. (10^9/L)	28	0.03 ±0.013	0.03 ±0.016	0.03 ±0.013	0.03 ±0.013	0.05 ±0.016*	0.03 ±0.009	0.03 ±0.011	0.03 ±0.009	0.03 ±0.012	0.04 ±0.024
	91	0.02 ±0.005	0.02 ±0.010	0.02 ±0.007	0.02 ±0.013	0.02 ±0.007	0.00 ±0.004	0.00 ±0.004	0.00 ±0.005	0.01 ±0.005	0.00 ±0.005
Atyp. leuko. (10^9/L)	28	0.16 ±0.051	0.20 ±0.059	0.016 ±0.071	0.15 ±0.042	0.21 ±0.085	0.14 ±0.038	0.11 ±0.034	0.11 ±0.046	0.12 ±0.037	0.12 ±0.049
	91	0.09 ±0.021	0.07 ±0.030	0.08 ±0.029	0.06 ±0.015*	0.06 ±0.018*	0.02 ±0.006	0.02 ±0.012	0.03 ±0.008	0.03 ±0.013	0.02 ±0.012
Laboratory historical control data (rat, Wistar, 1997)											
Atyp. leuko. (10^9/L)		Age of animals: 12 – 25 wks *n:* 80 Mean ±SD: 0.13 ±0.078 Range ±2SD: 0 – 0.28					Age of animals: - *n:* Mean ±SD: Range ±2SD:				
Baso. (10^9/L)		Age of animals: 8 – 11 wks *n:* 80 Mean ±SD: 0.04 ±0.019 Range ±2SD: 0 – 0.07					Age of animals: - *n:* Mean ±SD: Range ±2SD:				
Lymph. (10^9/L)		Age of animals: - *n:* Mean ±SD: Range ±2SD:					Age of animals: 12 – 25 wks *n:* 77 Mean ±SD: 6.13 ±1.182 Range ±2SD: 2.50 – 9.75				
Eosino. (10^9/L)		Age of animals: 12 – 25 wks *n:* 80 Mean ±SD: 0.16 ±0.092 Range ±2SD: 0 – 0.34					Age of animals: - *n:* Mean ±SD: Range ±2SD:				
Neutro. (10^9/L)		Age of animals: 8 – 11 wks *n:* 80 Mean ±SD: 0.87 ±0.433 Range ±2SD: 0.01 – 1.74					Age of animals: - *n:* Mean ±SD: Range ±2SD:				

*$p \leq 0.05$
Leuko.: leukocytes Eosino.: eosinophils

Parameters		♂ (ppm)					♀ (ppm)				
		SPX N-oxide				SPX	SPX N-oxide				SPX
	day	0	25	125	625	625	0	25	125	625	625

Neutro.: neurophils
Lymph.: lymphocytes
Baso.: basophiles
Atyp.leujo.: atypical lymphocytes

2. Clinical chemistry findings:	*Spiroxamine N-oxide:* In the high dose group, increased liver enzyme activities (ALT, ALP) were observed, with decreased total cholesterol at both the interim and terminal sample time points. Total protein and albumin were decreased at the interim time point in both sexes and at day 91/92 in ♀. *Spiroxamine*: Animals treated at 625 ppm, increased ALT (♂/♀, interim and terminal time points) and AST (♂ terminal time point) were observed, with ALP (♀ terminal time point) and total cholesterol (♂♀, interim and terminal time points) decreased. Total protein, albumin (♂, interim; ♀, interim and terminal time points) were decreased. Whilst creatinine, glucose, total bilirubin and electrolytes showed significance, these were considered incidental and not adverse as not correlating histopathology was present.
3. Urinalysis:	There were no treatment related effects were observed in animals treated with either spiroxamine N-oxide or spiroxamine.

Table CA 5.8.1/06-6: Overview of sub-chronic toxicity study in rats treated orally (*via* diet) with spiroxamine N-oxide: selected clinical chemistry parameters

Parameters		♂ (ppm)					♀ (ppm)				
		SPX N-oxide			SPX		SPX N-oxide			SPX	
	Day	0	25	125	625	625	0	25	125	625	625
Dose/animal (mg/kg bw/day)		0	1.7	8.8	45.0	48.7	0	1.9	9.7	53.6	52.7
ALT (U/L)	28	49.7 ±6.50	49.2 ±5.82	52.5 ±7.99	59.9 ±8.90	71.4 ±9.97**	47.9 ±5.31	46.9 ±6.84	45.1 ±4.30	55.5 ±6.89*	59.7 ±7.94**
	91	44.5 ±7.69	46.2 ±6.14	45.8 ±4.70	50.4 ±5.18	54.9 ±7.23**	42.1 ±40.1	49.3 ±6.80	47.4 ±4.23	50.6 ±6.18	53.1 ±9.22*
ALP (U/L)	28	454 ±48.9	462 ±55.6	447 ±67.0	553 ±103.5	448 ±79.6	265 ±46.1	279 ±48.3	289 ±39.8	402 ±132.9*	292 ±30.2
	91	256 ±29.7	267 ±22.8	267 ±33.3	347 ±63.4	272 ±33.5	174 ±33.9	202 ±32.9	237 ±22.3**	272 ±53.0**	219 ±33.4**
AST (U/L)	28	35.6 ±4.27	37.8 ±3.82	35.6 ±1.81	38.2 ±4.12	41.6 ±4.91**	39.0 ±3.01	38.4 ±4.44	36.4 ±3.20	41.2 ±2.65	43.8 ±4.39*
	91	42.1 ±5.57	39.4 ±8.64	35.4 ±5.85	36.4 ±3.94*	36.9 ±4.53*	34.5 ±6.10	39.8 ±6.40	37.4 ±3.81	41.8 ±8.55	41.6 ±5.54*
T.prot.	28	65.9 ±1.64	64.8 ±1.50	64.8 ±1.29	61.4 ±3.07**	59.0 ±2.70**	65.7 ±2.30	63.2 ±1.76*	64.0 ±2.70	62.2 ±1.67**	59.2 ±2.60**
	91	70.0 ±1.80	69.7 ±2.07	70.2 ±2.90	68.9 ±3.28	68.4 ±1.92	71.0 ±2.50	70.1 ±2.76	70.3 ±3.49	67.6 ±2.16*	67.0 ±2.97**
T.chol (mmol/L)	28	2.44 ±0.187	2.39 ±0.347	2.40 ±0.276	1.86 ±0.205*	1.66 ±0.331**	2.10 ±0.268	2.29 ±0.285	2.14 ±0.166	1.89 ±0.175	1.59 ±0.284**
	91	2.46 ±0.207	2.46 ±0.477	2.51 ±0.315	2.14 ±0.263	2.19 ±0.258*	2.23 ±0.322	2.36 ±0.400	2.23 ±0.193	2.04 ±0.235	1.77 ±0.318**
Alb. (g/L)	28	32.1 ±0.81	32.3 ±0.47	32.0 ±1.29	30.6 ±1.20**	29.8 ±2.24**	34.2 ±1.44	33.2 ±1.45	34.0 ±1.44	33.0 ±1.50	30.7 ±1.12**
	91	32.9 ±1.45	33.6 ±0.62	33.1 ±1.01	33.7 ±1.63	33.1 ±0.97	37.9 ±1.84	38.0 ±2.57	37.2 ±1.47	35.7 ±1.27*	35.9 ±1.47*
Laboratory historical control data (rat, Wistar 1994 – 1995)											
ALT (U/L)		Age of animals:	8 – 11 wks		12 – 25 wks		Age of animals:	8 – 11 wks		12 – 25 wks	
		n:	311		251		*n:*	336		25	
		Mean ±SD:	45.8 ±8.19		40.8 ±7.10		Mean ±SD:	40.9 ±7.08		40.5 ±8.61	
		Range ±2SD:	29.4 – 62.2		26.6 – 55.1		Range ±2SD:	26.8 – 55.1		23.3 – 57.7	
ALP (U/L)		Age of animals:	8 – 11 wks		12 – 25 wks		Age of animals:	8 – 11 wks		12 – 25 wks	
		n:	310		250		*n:*	332		257	
		Mean ±SD:	468 ±98.4		241 ±50.3		Mean ±SD:	314 ±64.4		177 ±36.5	
		Range ±2SD:	271 – 665		140 - 342		Range ±2SD:	185 - 443		104 - 250	
AST (U/L)		Age of animals:	8 – 11 wks		12 – 25 wks		Age of animals:	8 – 11 wks		12 – 25 wks	
		n:	306		249		*n:*	327		255	
		Mean ±SD:	41.0 ±7.26		36.7 ±7.76		Mean ±SD:	40.0 ±6.91		38.8 ±8.15	
		Range ±2SD:	26.5 – 55.5		21.2 – 52.2		Range ±2SD:	26.2 – 53.8		22.5 – 55.1	
T. prot.		Age of animals:	8 – 11 wks				Age of animals:	8 – 11 wks		12 – 25 wks	
		n:	301				*n:*	330		247	
		Mean ±SD:	63.4 ±2.81				Mean ±SD:	63.6 ±3.31		67.9 ±3.66	
		Range ±2SD:	57.8 – 69.1				Range ±2SD:	57.0 – 70.2		60.5 – 75.2	

Parameters	Day	♂ (ppm)					♀ (ppm)				
		SPX N-oxide			SPX		SPX N-oxide			SPX	
		0	25	125	625	625	0	25	125	625	625
T.chol (mmol/L)		Age of animals:		8 – 11 wks		12 – 25 wks	Age of animals:		8 – 11 wks		12 – 25 wks
		n:		301		246	*n:*		331		247
		Mean ±SD:		2.10 ±0.344		2.36 ±0.471	Mean ±SD:		1.98 ±0.338		2.08 ±0.376
		Range ±2SD:		1.41 – 2.78		1.41 – 3.30	Range ±2SD:		1.30 – 2.65		1.33 – 2.84
Alb. (g/L)		Age of animals:		8 – 11 wks			Age of animals:		8 – 11 wks		12 – 25 wks
		n:		253			*n:*		275		244
		Mean ±SD:		32.5 ±1.74			Mean ±SD:		34.3 ±2.15		36.1 ±2.31
		Range ±2SD:		29.0 – 36.0			Range ±2SD:		30.0 – 38.6		31.4 – 40.7

* $p \leq 0.05$; ** $p \leq 0.01$
ALT: alanine aminotransferase
ALP: alkaline phosphatase
AST: aspartate aminotransferase
T.prot: total protein
T.chol.: total cholesterol
Alb.: albumin

F. Sacrifice and pathology:

1. Organ weight:

For the majority of organs, absolute organ weights were reduced for animals in the 625 ppm dose group for both test articles. However when expressed as relative organ to body weight, organ weights were comparable to the concurrent control (including endocrine producing and endocrine sensitive organs, where weighed).

Table CA 5.8.1/06-7: Overview of sub-chronic toxicity study in rats treated orally (*via* diet) with spiroxamine N-oxide: selected organ weights

Parameters		♂ (ppm)					♀ (ppm)				
		SPX N-oxide				SPX	SPX N-oxide				SPX
		0	25	125	625	625	0	25	125	625	625
Dose/animal (mg/kg bw/day)		0	1.7	8.8	45.0	48.7	0	1.9	9.7	53.6	52.7
Terminal bwt (g)		432 ±30.4	415 ±30.0 (↓4%)	454 ±38.9 (↑5%)	408 ±26.9 (↓6%)	390 ±45.5** (↓10%)	234 ±14.5	227 ±6.8 (↓3%)	223 ±18.2 (↓5%)	231 ±13.7 (↓1%)	207 ±16.5** (↓12%)
Liver	Abs (g)	14.1 ±0.15	13.7 ±0.18 (↓3%)	15.1 ±0.1 (↑7%)	13.3 ±0.17 (↓6%)	13.0 ±2.1 (↓8%)	7.5 ±0.69	7.0 ±0.92 (↓7%)	7.0 ±1.1 (↓7%)	7.0 ±0.53 (↓7%)	6.6 ±0.72 (↓12%)
	Rel. (g%)	3.3 ±0.18	3.3 ±0.22 (-)	3.3 ±0.18 (-)	3.3 ±0.22 (-)	3.3 ±0.18 (-)	3.2 ±0.24	3.1 ±0.30 (↓3%)	3.1 ±0.26 (↓3%)	3.0 ±0.18 (↓6%)	3.3 ±0.24 (↑3%)
Spleen	Abs (mg)	695 ±58.5	698 ±171.8 (-)	718 ±88.8 (↑3%)	682 ±100.1 (↓2%)	660 ±0.10 (↓5%)	415 ±63.0	391 ±67.4 (↓6%)	398 ±78.9 (↓4%)	442 ±50.4 (↑7%)	430 ±78.7 (↑4%)
	Rel. (mg%)	161 ±13.9	167 ±29.7 (↑4%)	158 ±13.3 (↓2%)	167 ±21.4 (↑4%)	169 ±18.5 (↑5%)	177 ±23.3	172 ±22.7 (-)	177 ±24.4 (-)	192 ±18.5 (↑8%)	177 ±23.3 (-)
Adrenals	Abs (mg)	45 ±8.9	43 ±9.7 (↓4%)	49 ±6.7 (↑9%)	40 ±5.7 (↓11%)	43 ±5.1 (↓4%)	59 ±8.8	57 ±11.8 (↓3%)	57 ±8.4 (↓3%)	60 ±11.7 (↑2%)	58 ±6.4 (↓2%)
	Rel. (mg%)	10 ±2.1	10 ±2.1 (-)	11 ±1.3 (↑10%)	10 ±1.6 (-)	11 ±1.7 (↑10%)	25 ±3.0	25 ±5.0 (-)	26 ±3.4 (↑4%)	26 ±5.3 (↑4%)	28 ±4.2 (↑12%)
Testes	Abs (g)	3.5 ±0.23	3.7 ±0.11 (↑6%)	3.6 ±0.30 (↑3%)	3.6 ±0.23 (↑3%)	3.4 ±0.58 (↓3%)	-	-	-	-	-
	Rel. (g%)	0.81 ±0.08	0.93 ±0.25	0.79 ±0.07	0.89 ±0.07	0.86 ±0.08	-	-	-	-	-
Ovary	Abs (mg)	-	-	-	-	-	138 ±28.5	132 ±28.9 (↓4%)	134 ±27.5 (↓3%)	136 ±27.1 (↓1%)	128 ±29.0 (↓7%)
	Rel. (mg%)	-	-	-	-	-	59 ±12.8	59 ±14.4 (-)	60 ±9.7 (↑2%)	60 ±15.5 (↑2%)	61 ±9.6 (↑3%)

** $p \leq 0.01$
Abs.: absolute
Rel.: relative to body weight

2. Gross pathology: There were no treatment related effects were observed in animals treated with either spiroxamine N-oxide or spiroxamine.

3. Histopathology: In high group animals, hyperkeratosis of the epithelium of the oesophagus and forestomach were observed in both ♂ and ♀ animals dosed with spiroxamine N-oxide. Similar effects were observed in animals receiving spiroxamine, however the incidence and severity were greater in both instances. In addition, hyperkeratosis of the ear was observed in spiroxamine treated animals. Mild transitional cell hyperplasia in the urinary bladder was observed in ♂ dosed with spiroxamine. As with the parent compound, spiroxamine, spiroxamine N-oxide is a tertiary amine, and like all tertiary amines has a high pKa, and a high pH with comparable histopathological effects reported. As with irritant compounds, tissues containing mucosal membrane is deemed to be a target organ critical effect. This is acknowledged in the literature, with optimal pH ranges for substances administered orally *via* gavage not to exceed pH 9, with administration of substances outside of the recommended pH ranges resulting in tissue necrosis, pH partitioning *etc.* (Gad *et al*[18]; Turner *et al*[19]). This should not be dismissed.
Hyaline droplet nephropathy was observed in the kidneys of all treated ♂. This lesion is due the accumulation of α2u-globulin which is known to be a lesion specific to rats with no relevance to humans.

Table CA 5.8.1/06-8: Overview of sub-chronic toxicity study in rats treated orally (*via* diet) with spiroxamine N-oxide: selected histopathology observations

Parameters	(ppm)					(ppm)				
	SPX N-oxide				SPX	SPX N-oxide				SPX
	0	25	125	625	625	0	25	125	625	625
Dose/animal (mg/kg bw/day)	0	1.7	8.8	45.0	48.7	0	1.9	9.7	53.6	52.7
Histopathology: [incidence / total no. examined [minimal, slight, moderate]										
Kidney - Hyaline droplet nep.	10/10 [1,7,2]	10/10 [0,9,1]	10/10 [0,7,3]	10/10 [0,10,0]	10/10 [0,10,0]	-	-	-	-	-
Urinary bladder - Hyperpl. mild trans.	- -	-	-	-	-	0/10 -	0/10 -	0/10 -	0/10 -	2/10 [2,0,0]
Stomach - Hyperkeratosis	0/10 -	0/10 -	0/10 -	3/10 [0,2,1]	6/10 [0,2,4]	0/10 -	0/10 -	0/10 -	1/10 [0,1,0]	5/10 [0,2,3]
Oesophagus - Hyperkeratosis	0/10 -	0/10 -	0/10 -	6/10 [3,2,1]	9/10 [1,5,3]	0/10 -	0/10 -	0/10 -	8/10 [8,0,0]	8/10 [0,5,3]
Pinna - Hyperkeratosis	0/10 -	1/10 [1,0,0]	0/10 -	2/10 [2,0,0]	2/10 [2,0,0]	2/10 [1,1,0]	0/10 -	0/10 -	5/10 [3,2,0]	

* $p \leq 0.05$; ** $p \leq 0.01$
Hyaline droplet nep.: hyaline droplet nephropathy; Hyperpl. mild trans.: hyperplasia, mild transitional cell

4. Liver tissue enzyme analysis: Investigations of the liver tissue showed no treatment related effects at 125 ppm and below. In ♂ at 625 ppm and ♀ at 125 and 625 ppm, aminopyrine-N-demethylase activity was increased, achieving statistical significance at the high dose group ♀. Similar effects were also observed in animals dosed with spiroxamine. In both ♂ and ♀ in the high dose group, cytochrome P450 activity was increased in animals dosed with both spiroxamine N-oxide and spiroxamine. These changes were deemed treatment related, but adaptive and not adverse as no concurrent liver histopathological findings were evident.

Table CA 5.8.1/06-9: Overview of sub-chronic toxicity study in rats treated orally (*via* diet) with spiroxamine N-oxide: liver tissue enzyme analysis

Parameters	♂ (ppm)					♀ (ppm)				
	SPX N-oxide				SPX	SPX N-oxide				SPX
	0	25	125	625	625	0	25	125	625	625
Dose/animal (mg/kg bw/day)	0	1.7	8.8	45.0	48.7	0	1.9	9.7	53.6	52.1
N-DEM (mU/g)	101.4 ±19.21	101.9 ±20.17	101.0 ±13.64	107.1 ±12.88	112.3 ±12.05	56.2 ±4.06	54.5 ±9.47	60.9 ±10.24	63.7 ±6.83*	63.0 ±7.24
O-DEM (mU/g)	10.0 ±1.57	9.7 ±1.94	10.4 ±1.41	10.1 ±1.38	9.3 ±1.10	9.1 ±1.37	9.3 ±1.14	10.2 ±1.35	10.1 ±0.87	10.1 ±1.09
Cyto-P450 (nmol/g)	47.3 ±4.46	48.6 ±4.68	46.0 ±4.65	56.7 ±4.10**	57.6 ±4.30**	38.7 ±1.87	37.6 ±3.50	39.4 ±2.95	43.7 ±4.10*	42.7 ±4.26*
Laboratory historical control data (rat, Wistar, 1994 - 1995)										
N-DEM (mU/g)	Age of animals:		12 – 25 wks			Age of animals:		12 – 25 wks		
	n:		75			*n*:		75		
	Mean ±SD:		127.4 ±31.12			Mean ±SD:		68.6 +14.77		
	Range ±2SD:		65.2 – 189.6			Range ±2SD:		39.0 – 98.1		
O-DEM (mU/g)	Age of animals:		12 – 25 wks			Age of animals:		12 – 25 wks		
	n:		75			*n*:		75		
	Mean ±SD:		9.9 ±2.49			Mean ±SD:		9.3 ±1.84		
	Range ±2SD:		5.0 – 14.9			Range ±2SD:		5.6 – 12.9		
Cyto-P450 (nmol/g)	Age of animals:		12 – 25 wks			Age of animals:		12 – 25 wks		
	n:		75			*n*:		75		
	Mean ±SD:		38.9 ±5.34			Mean ±SD:		34.2 ±5.10		
	Range ±2SD:		28.2 – 49.6			Range ±2SD:		24.0 – 44.4		

* $p \leq 0.05$; ** $p \leq 0.01$
N-DEM: aminopyrine-*N*-demethylase Cyto-P450: cytochrome P450 content
O-DEM: *p*-nitroanisole-*O*-demethylase

G. Discussion:

No treatment-related effects were seen on survival, clinical signs, food consumption, urinalysis or serum chemistry / haematology parameters. High dose effects were limited to slightly decreased cholesterol levels in both males and females. This was deemed to be a treatment related effect, but likely slight (without correlating liver histopathology) rather than adverse.

For animals treated with spiroxamine N-oxide, at 625 ppm, males showed a reduction in body weight from week 1 onwards, with statistically significant reductions in body weight from the second week through to week 9 with body weights being approximately 9-10% lower than the concurrent control. At termination of treatment, body weight gain in high dose group males was 9% lower. For females dosed with spiroxamine N-oxide at 625 ppm, whilst body weight was lower throughout the dosing period from the first week onwards, but not no greater than 5% lower than the concurrent control group. At termination of treatment, body weight gain in high dose group females was 1% lower.

For animals treated with spiroxamine, at 625 ppm, males showed a statistically significant reduction in body weight from week 1 onwards, with body weights being approximately 10-17% lower than the concurrent control. At termination of treatment, body weight gain in this dose group was 14% lower. For females dosed with spiroxamine at 625 ppm females showed a statistically significant reduction in body weight from week 1 onwards, with body weights being approximately 12% lower than the concurrent control. At termination of treatment, body weight gain in this dose group was 19% lower.

Throughout the dosing period, food consumption was marginally greater in animals of the 625 ppm dose group for both test articles when expressed as g/kg bw. Whilst not measured, a qualitative assessment from increased food consumption of high dose group males and reduction in body weight implies that food efficiency dropped. Water intake at 625 ppm dose group for both test articles were marginally reduced in both sexes when expressed as g/animal. When corrected for body weight this effect was not replicated and deemed a result of a reduction in body weight.

No treatment related haematological effects were reported with either test article, however clotting time (PT) was significantly extended at termination in high dose group males receiving spiroxamine N-oxide.

For animals receiving spiroxamine, alterations in red blood cell parameters were evident, suggestive of anaemia, with reductions in haematocrit, MCV, MHC, MCHC, reticulocyte counts and hypochromasia (*i.e.* RBC pale in colour, suggestive of reduced Hb concentration) were increased. Total and differential WBC parameters were affected in both sexes, with leucocytes, neutrophils, basophils, lymphocytes were increased and eosinophils and atypical leukocytes were decreased. Clotting time was significantly extended in both sexes at both sample times. Extended PT was likely a secondary effect resultant from decreases in both serum albumin and total protein.

In high group animals, hyperkeratosis of the epithelium of the oesophagus and forestomach were observed in both males and females dosed with spiroxamine N-oxide. Similar effects were observed in animals receiving spiroxamine, however the incidence and severity were greater in both instances. In addition, hyperkeratosis of the ear was observed in spiroxamine treated animals. Mild transitional cell hyperplasia in the urinary bladder was observed in females dosed with spiroxamine. As with the parent compound, spiroxamine, spiroxamine N-oxide is a tertiary amine and like all tertiary amines has a high pKa, and a high pH with comparable histopathological effects reported. As with irritant compounds, tissues containing mucosal membrane is deemed to be a target organ critical effect. This is acknowledged in the literature, with optimal pH ranges for substances administered orally *via* gavage not to exceed pH 9, with administration of substances outside of the recommended pH ranges resulting in tissue necrosis, pH partitioning *etc.* This should not be dismissed.

Hyaline droplet nephropathy was observed in the kidneys of all treated males. This lesion is due the accumulation of α2μ-globulin which is known to be a lesion specific to male rats with no relevance to humans, with Azan specific staining used to confirm α2μ-globulin presence.

H. Deficiencies:

Although the study was conducted according to test guideline OECD 408 (1981), this test guideline has since been updated in the intervening period (1998, 2008). When assessed against current test guideline requirements the following deficiencies are noted:

- Serum thyroid hormones (T3, T4, TSH) and clinical chemistry parameters sensitive to thyroid pathway perturbations (LDL, HDL) were not analysed. However, the gold standard to assess thyroid effects is histopathological analysis. This was undertaken on all animals and revealed no histopathological findings. Consequently the lack of serum thyroid hormone analysis is not deemed critical, with potential thyroid effects adequately addressed with histopathological analysis.

Assessment and conclusions by applicant:

Assessment: Study meets the current guidance and the requirements in 283/2013.

Conclusion: Under the conditions of this study the NOAEL for spiroxamine N-oxide following 90 days oral (*via* diet) treatment is deemed to be 125 ppm (equivalent to 8.8/9.7 mg/kg bw/day for males/females) based on reduction in body weight and body weight gain, hyperkeratosis of the stomach and oesophagus. Increases in liver enzyme induction were observed, without concurrent hepatic histopathology.

Similar effects reported for spiroxamine N-oxide were also observed for spiroxamine.

Spiroxamine-cyclohexanol (M13)

Data Point:	KCA 5.8.1/07
Report Author:	[redacted]
Report Year:	1973
Report Title:	p-t-Butyl cyclohexanol - Acute oral toxicity rat (rat LD50) - Dermal toxicity (rabbit)
Report No:	M-471767-01-1
Document No:	M-471767-01-1
Guideline(s) followed in study:	not specified
Deviations from current test guideline:	Yes A number of deficiencies were noted (refer to Results, Deficiencies section below).
Previous evaluation:	yes, evaluated and accepted RAR (2010)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

In an acute oral toxicity study, broadly comparable to the OECD 401 test guideline, groups of rats (10/group) were administered the test article, spiroxamine cyclohexanol as a single dose at 200, 2500, 3200, 4000 or 5000 mg/kg bw. In the same study, rabbits (6/sex) received a single dermal application of test article at 5000 mg/kg bw. The observation period was 14 days post-exposure.

Clinical signs of toxicity were limited to rats, with immediate stimulation followed by ataxia for animals dosed at 3200 mg/kg bw and above. Clinical signs resolved by day 3.

Mortality was observed in rats at dose levels of 2500 mg/kg bw and above.

Under the conditions of this study the acute oral LD_{50} of spiroxamine cyclohexanol was 4200 mg/kg bw. The acute dermal LD_{50} in rabbits was >5000 mg/kg bw. Therefore, according to Annex I for Regulation (EC) 1272/2008 the test article has no obligatory labelling requirement for acute oral or dermal toxicity and is unclassified.

Materials and Methods

A. Materials:

1. **Test Material:** Spiroxamine cyclohexanol
(alternative name: 1(4-tert-butylcyclohexanol, M13)

Description:	Not reported
Lot/Batch No.:	72-86 (bl 2006)
Purity:	Not reported
CAS No.:	98-52-2
Stability of test compound:	Not reported

2. **Vehicle and/or positive control:** Not reported / not relevant

3. **Test animals:**

Species:	Rat (oral) / rabbit (dermal)
Strain:	Not reported
Age at dosing:	Not reported
Weight at dosing:	Not reported
Source:	Not reported
Acclimation period:	Not reported

Diet:	Not reported
Water:	Not reported
Housing:	Not reported
4. Environmental conditions:	
Temperature:	Not reported
Humidity:	Not reported
Air changes:	Not reported
Photoperiod:	Not reported

B. Test Performance:

1. In life dates:	Not reported
2. Animal assignment and treatment:	Limited information was provided in the report. For the acute oral phase of the study, rats (10/sex/group) received a single oral gavage dose of spiroxamine cyclohexanol at dose levels of 2000, 2500, 3200, 4000 and 5000 mg/kg bw. For the acute dermal phase of the study, rabbits (6/group) received a single dermal application of spiroxamine cyclohexanol at 5000 mg/kg bw. It was not reported if a non-occlusive, semi-occlusive or occlusive dressing was applied following application. Rats Animals were selected before administration based on their body weight The observation period was 14 days post-exposure.
3. Statistics:	A 95% confidence limit was applied to the oral LD_{50}, details of this calculation are not reported.

C. Methods:

1. Homogeneity and achieved concentration analysis of the dose:	Not conducted
2. Test article formulation preparation:	Not reported
3. Observations:	From the limited data presented, animals were observed once daily for 14 days.
3. Body weights:	Not reported
4. Food consumption:	Not reported
5. Sacrifice and pathology:	All animals were killed at terminal sacrifice, but no gross necropsy was undertaken.

Results

A. Homogeneity and achieved concentration analysis:

Not undertaken. Analyses for achieved concentration, homogeneity or stability of test article formulations were not conducted as part of this study. This is not a requirement of the current regulatory test guidelines for this study type.

B. Observations:

1. Clinical signs of toxicity:	Acute oral: Clinical signs of toxicity reported for rats were immediate stimulation followed by ataxia for animals dosed at 3200 mg/kg bw and above. Clinical signs resolved by day 3. Acute dermal: No clinical signs of toxicity reported for rabbits.
2. Mortality:	Refer to Table CA 5.8.1/07-1

Acute oral:
Mortality was observed at dose levels of 2500 mg/kg bw and above.
Acute dermal:
All animals survived until scheduled termination.

C. Body weight and food consumption:

1. **Body weight:** Not reported
2. **Food consumption:** Not reported

Table CA 5.8.1/07-1: Overview of acute oral toxicity study in rats treated with spiroxamine cyclohexanol: mortality and body weight

Parameter	Rat oral (mg/kg bw)					Rabbit dermal (mg/kg bw)
	2000	2500	3200	4000	5000	5000
Mortality[a]	0/10	1/10	2/10	4/10	8/10	0/6
Acute LD_{50}	4200 mg/kg bw (95% confidence limit: 3620 – 4870 mg/kg bw)					>5000 mg/kg bw

a Mortality: no. of animals found dead / no. of animals treated
b no individual animal data reported

D. Necropsy:

Not undertaken.

E. Deficiencies:

Although the study was broadly comparable to the now deleted OECD 401 (1987) test guideline for the oral exposure and OECD 402 (1981) for the dermal exposure with the limited data reported, the following deficiencies are noted when compared with the respective guidelines:

- No gross histopathological analysis was undertaken.
- The tabulation of data presented it is unclear when mortality occurred.
- Body weights were not recorded.
- Whilst group sizes are detailed, it is unclear if a single sex or groups were equally split between sexes. Furthermore, the strain(s) of animal used is not reported.
- Details regarding dermal application are unclear.

Whilst there are recognized deficiencies, it is considered ethically unjustifiable to perform new acute oral and acute dermal toxicity studies to estimate acute toxicity. The data in this report provide a reliable estimation of acute toxicity from the oral and dermal routes.

Assessment and conclusion by applicant:

Assessment: Whilst there are recognized deficiencies, it is considered ethically unjustifiable to perform new acute oral and acute dermal toxicity studies to estimate acute toxicity. The data in this report provides a reliable estimation of acute toxicity from the oral and dermal routes.

Conclusion: Under the conditions of this study the acute oral LD_{50} of spiroxamine cyclohexanol was 4200 mg/kg bw. The acute dermal LD_{50} in rabbits was >5000 mg/kg bw. Therefore, according to Annex I for Regulation (EC) 1272/2008 the test article has no obligatory labelling requirement for acute oral or dermal toxicity and is unclassified.

Data Point:	KCA 5.8.1/08
Report Author:	[redacted]
Report Year:	2012
Report Title:	Mutagenicity study of 4-tert-butylcyclohexanol (PTBCOL) in the Salmonella typhimurium reverse mutation assay (in vitro)
Report No:	2012-0124-DGM
Document No:	M-471123-01-1
Guideline(s) followed in study:	Regulation (EC) No. 440/2008, part B, B.13/14; OECD 471; US-EPA OPPTS 870.5100
Deviations from current test guideline:	Yes A number of deficiencies were noted (refer to Results, Deficiencies section below).
Previous evaluation:	yes, evaluated and accepted RAR (2010)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

In a reverse gene mutation assay in bacteria, *S. typhimurium* strains TA98, TA1537, TA100, TA1535 and TA102 were exposed to spiroxamine cyclohexanol (M13) formulated in DMSO using the both the plate incorporation and pre-incubation methodologies in the absence and presence of an Aroclor-1254-induced rat liver post-mitochondrial fraction (S9).

For the preliminary cytotoxicity test, TA100 in the absence and presence of S9 at concentration ranging from 0.316 to 5000 µg/plate. Following these treatments, evidence of toxicity ranging from a complete killing of the test bacteria at 1000 µg/plate to a thinning of the background bacterial lawn and/or a marked reduction in revertants at 316 µg/plate.

In two independent experiments using the plate incorporation and pre-incubation methodologies and in all strains in the absence and presence of S9 no increases in revertant numbers were observed that were ≥2-fold (in strains TA98, TA100 and TA102) or ≥3-fold (in strains TA1535 and TA1537) the concurrent vehicle control. This study was therefore considered to have provided no evidence of any spiroxamine cyclohexanol mutagenic activity in this assay system.

The positive controls induced an acceptable increase in revertant colony numbers, thereby demonstrating the sensitivity and specificity of the test system.

In conclusion, the data generated under this study are considered supplementary with the bacterial reverse gene mutation endpoint sufficient addressed with a new, up to date test guideline compliant GLP study (refer to CA 5.8.1/22 [M-755223-02-1]).

It was concluded that spiroxamine cyclohexanol did not induce mutation in five histidine-requiring strains (TA98, TA100, TA1535 and TA1537) of *Salmonella typhimurium* when tested under the conditions of this study. These conditions included treatments at concentrations up to 5000 µg/plate (the maximum recommended concentration according to current regulatory guidelines), in the absence and in the presence of a rat liver metabolic activation system (S9) or up to concentration limited by toxicity using both the plate incorporation and pre-incubation methodologies.

Materials and Methods

A. Materials:

1. Test Material: Spiroxamine cyclohexanol
(alternative name: [(4-tert-butylcyclohexanol, M13)

Description: Solid, viscid, melt

Lot/Batch No.: 80322

Purity:	98.6% (correction factor of 1.014 applied)
CAS No.:	98-52-2
Stability of test compound:	Confirmed stable for the duration of the study (expiry date: February 2013)

2. Control materials:

Negative:

Solvent/final concentration: DMSO (dimethyl sulphoxide)/0.1 or 0.05 mL/plate (plate incorporation or pre-incubation, respectively)

Positive: -S9

Strain	*Mutagen*	*Conc. (µg/plate)*
TA98	2-nitrofluorene (2-NF)	10
TA100, TA1535	Sodium azide (NaN_3)	10
TA1537	9-aminoacridine (9-AAC)	100
TA102	Methyl methanesulphonate (MMS)	1300

Positive: +S9

Strain	*Mutagen*	*Conc. (µg/plate)*
TA100, TA1535	Cyclophosphamide (CPA)	1500
TA98, TA102, TA1537	2-aminoanthracene[14] (2-AA)	2

3. Activation: S9 was prepared in house from rats treated with Aroclor 1254 (protein content 33.1 mg/mL).

The composition of the S9 reaction mix was: S9 (5%), $MgCl_2$ (8 mM); KCl (33 mM); glucose-6-phosphate (5.4 mM), NADP (3.89 mg/mL), PBS (0.1 M).

4. Test organisms: *S. typhimurium* strains: TA98, TA100, TA1535, TA1537, TA102
All test organisms were properly maintained and were checked for appropriate genetic markers (*S. typhimurium*: histidine and biotin requirement, *rfa* mutation, *uvr*B sensitivity, ampicillin-resistance) regularly.

5. Test Concentrations:

a) Preliminary cytotoxicity test: *Plate incorporation: +/-S9 TA100*:
0, 0.316, 1.0, 3.16, 10.0, 31.6, 100, 316, 1000, 360, 5000 µg/plate

b) Mutation assay 1: *Plate incorporation: +/-S9 all strains*:
0, 1.0, 3.16, 10.0, 31.6, 100, 316 µg/plate

c) Mutation assay 2: *Pre-incubation +/-S9 all strains*:
0, 1.0, 3.16, 10.0, 31.6, 100, 316 µg/plate

B. Test Performance:

1. In life dates: 23 April 2012 to 24 May 2012 (experimental dates)

2. Preliminary cytotoxicity test: *Plate incorporation assay:*
The following sequence of additions of 2 mL of supplemented molten top agar, test article solution/vehicle control (0.1 mL), either 0.1 M Na phosphate buffer (0.5 mL pH 7.4) or S9 mix (0.5 mL) for treatments in the absence and in the presence of metabolic activation, respectively and bacterial suspension (0.1 mL) were mixed and poured on to Vogel-Bonner E agar plates (minimal glucose agar plate). When set, plates were inverted and incubated at 37°C, protected from light for 3 days. Duplicate plates were used, with only a single strain, TA100 tested in the absence and presence of S9 without a positive controls

2. Experiment 1: *Plate incorporation assay:*

14 Of note, some researchers have suggested that 2-AA should not be used as the only positive control to evaluate S9-mix activity as it has been shown that the chemical may be activated by enzymes other than the microsomal cytochrome P450 family. In this study, S9 activity was evaluated with both 2-AA and CPA. Therefore, there is no concern over S9 activity.

	Refer above. The exception was that 0.1 mL of positive control was also added to each strain.
3. Experiment 2:	*Pre-incubation assay:* The test article solution or vehicle/positive control solution (0.5 mL), bacteria (0.1 mL), either 0.1 M Na phosphate buffer (0.5 mL pH 7.4) or S9 mix (0.5 mL) for treatments in the absence and in the presence of metabolic activation, respectively were mixed in a small test tube and incubated for 20 minutes at 37°C. After additional of 2 mL of top agar solution the mixture was poured onto a minimal glucose agar plate and allowed to solidify. All plates were incubated for 3 days at 37°C. In this way, it was hoped to increase the range of mutagenic chemicals that could be detected using this assay system. For each experiment triplicate plating was undertaken for each dose level.
4. Statistics:	None applied
5. Acceptance criteria:	Assay acceptance criteria was not provided.
5. Evaluation criteria:	The test article was considered mutagenic in this assay if: 1. A concentration related increase in revertant numbers was ≥2-fold (TA98, TA100, TA102), ≥3-fold (TA1535, TA1537) above the concurrent vehicle control values; 2. Any observed response was reproducible under the same treatment conditions. The test article was considered negative in this assay if none of the above criteria were met.

C. Methods:

1. Homogeneity and achieved concentration analysis of the dose:	Not undertaken.
2. Test article formulation preparation:	Correction for purity was made. A preliminary solubility test confirmed spiroxamine cyclohexanol was soluble in DMSO at concentrations equivalent to 50 mg/mL. Thereby confirming a maximum concentration of 50 mg/mL could be prepared and dosed into the test system at a maximum concentration of 5000 µg/plate. Test article stock solutions were prepared by formulating spiroxamine cyclohexanol in DMSO on the day of dosing into the test system.
3. Toxicity Assessment:	The background lawns of the plates were examined for signs of toxicity. Cytotoxicity was evidenced by a reduction in the number of spontaneous revertants, a clear diminution of the background lawn or by the degree of survival of the treated cultures.
4. Scoring:	It is unclear how the number of revertant colonies were counted *i.e.* unaided eye or a colony counter. Individual plate counts were recorded separately and the mean and standard deviation of the plate counts for each treatment were determined. Control counts (vehicle/positive) were compared with the laboratory's historical control range. The purpose of the vehicle historical control range was to assess strain characteristics, and not to interpret or justify a negative or positive result[3].

Results and Discussion

A. Analytical determinations:

Not undertaken. Analyses for achieved concentration, homogeneity or stability of test article formulations were not conducted as part of this study, as this is not a requirement of the regulatory test guidelines.

B. Preliminary cytotoxicity test:

For the preliminary cytotoxicity test, TA100 in the absence and presence of S9 at concentration ranging from 0.316 to 5000 µg/plate. Following these treatments, evidence of toxicity ranging from a complete killing of the test bacteria at 1000 µg/plate to a thinning of the background bacterial lawn and/or a marked reduction in revertants at 316 µg/plate.

Table CA 5.8.1/08-1: Spiroxamine cyclohexanol: bacterial reverse gene mutation data (mean revertant colonies): Preliminary cytotoxicity experiment (plate incorporation)

Type of mutation	Frame-shift	
Conc. (µg/plate)	TA100	
	-S9	+S9
0	130 ±3.5	151 ±7.1
0.316	135 ±1.4	113 ±2.7
1.0	137 ±4.9	132 ±4.2
3.16	138 ±1.4	129 ±10.6
10.0	127 ±5.7	132 ±12.7
31.6	105 ±0.0	108 ±2.1
100	115 ±1.4	122 ±2.1
316	55 ±0.7 S	46 ±7.8 S
1000	0 S,T	0 S,T
3160	0 S,T	0 S,T
5000	0 S,T	0 S,T

T: Toxic, no revertant colonies S: thinning of background lawn

C. Mutation experiment 1:

In the plate incorporation assay, treatments of the tester strains were performed in the absence and presence of S9 using final concentrations of spiroxamine cyclohexanol at 1.0, 3.16, 10.0, 31.6, 100 and 316 µg/plate (a concentration limited by cytotoxicity). No precipitation of test article was observed. Following these treatments, evidence of toxicity manifest as a thinning of the background bacterial lawn and a marked reduction in revertants was observed at 316 µg/plate in all strains in the absence and in the presence of S9.

Following spiroxamine cyclohexanol treatments of all the test strains in the absence and in the presence of S9, no increases in revertant numbers were observed that were ≥2-fold (TA98, TA100, TA102); ≥3-fold (TA1535, TA1537) above the concurrent vehicle control.

Table CA 5.8.1/08-3: Spiroxamine cyclohexanol: bacterial reverse gene mutation data (mean revertant colonies): Mutation experiment 1 (plate incorporation)

Type of mutation	Frame-shift				Base-pair substitution					
Conc. (µg/plate)	TA98		TA1537		TA100		TA1535		TA102	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0	31.0 ±10.4	28.0 ±2.0	3.3 ±2.3	2.0 ±1.0	173.6 ±1.5	125.0 ±15.5	18.7 ±0.6	16.0 ±5.6	274.3 ±9.1	261.3 ±2.9
1.0	22.0 ±0.0	31.3 ±6.4	4.7 ±0.6	3.0 ±1.7	168.0 ±1.7	137.7 ±7.4	20.0 ±1.7	17.3 ±6.1	272.7 ±5.5	263.7 ±11.7
3.16	25.3 ±2.1	29.0 ±5.2	7.7 ±2.5	7.3 ±2.3	168.7 ±5.9	137.0 ±4.4	18.3 ±2.5	17.3 ±3.2	247.0 ±5.0	267.0 ±3.6
10	22.7 ±3.1	29.3 ±3.8	3.3 ±0.6	5.7 ±4.5	158.0 ±8.5	128.3 ±5.5	14.3 ±3.5	15.3 ±3.5	276.7 ±30.9	265.3 ±3.1
31.6	25.3 ±7.6	27.7 ±9.9	3.7 ±0.6	4.3 ±4.9	123.3 ±27.8	133.3 ±5.1	13.3 ±1.5	14.7 ±4.2	260.0 ±2.6	293.7 ±19.9
100	24.7 ±1.5	20.7 ±1.2	7.0 ±1.0	4.0 ±3.5	110.0 ±6.9	119.3 ±7.6	19.7 ±4.9	13.0 ±4.4	264.0 ±3.6	267.3 ±1.5

Type of mutation	Frame-shift				Base-pair substitution					
Conc.	TA98		TA1537		TA100		TA1535		TA102	
(µg/plate)	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
316	16.0 ±1.0[S]	16.0 ±1.0[S]	1.0 ±0.0[s]	1.3 ±0.6[S]	83.7 ±4.5[S]	90.3 ±4.9[S]	6.7 ±1.5[S]	7.0 ±1.7[s]	240.0 ±1.0[S]	222.0 ±16.1[S]
Positive control	239.0 ±3.6	336.7 ±5.5	200.3 ±67.3	142.3 ±20.6	1024.0 ±18.7	1030.3 ±25.7	212.7 ±10.8	217.0 ±10.8	1039.7 ±31.5	1047.3 ±16.5

S: thinning of background lawn

Positive controls:
-S9: strains:
TA98: 2-NF
TA1537: 9-AAC
TA100; TA1535: NaN_3
TA102: MMS

+S9: strains:
TA100, TA1537 CPA
TA98; TA102; TA1537 2-AA

D. Mutation experiment 2:

In the pre-incubation assay treatments of the tester strains were performed in the absence and presence of S9 using final concentrations of spiroxamine cyclohexanol at 1.0, 3.16, 10.0, 31.6, 100 and 316 µg/plate (a concentration limited by cytotoxicity). No precipitation of test article was observed. Following these treatments, evidence of toxicity manifest as a thinning of the background bacterial lawn and a marked reduction in revertants was observed at 316 µg/plate in all strains in the absence and in the presence of S9.

Following spiroxamine cyclohexanol treatments of all the test strains in the absence and in the presence of S9, no increases in revertant numbers were observed that were ≥2-fold (TA98, TA100, TA102); ≥3-fold (TA1535, TA1537) above the concurrent vehicle control.

Vehicle and positive control treatments were included for all strains in both experiments. The mean numbers of revertant colonies were comparable with acceptable ranges for vehicle control treatments. The positive controls induced an acceptable increase in revertant colony numbers, thereby demonstrating the sensitivity and specificity of the test system.

Table CA 5.8.1/08-3: Spiroxamine cyclohexanol: bacterial reverse gene mutation data (mean revertant colonies) - Mutation experiment 2 (pre-incubation)

Type of mutation	Frame-shift				Base-pair substitution					
Conc.	TA98		TA1537		TA100		TA1535		TA102	
(µg/plate)	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0	38.3 ±9.7	30.0 ±4.4	6.7 ±0.6	7.3 ±1.2	131.0 ±15.6	132.3 ±7.6	14.7 ±3.2	25.3 ±3.5	290.0 ±23.4	281.0 ±10.4
1.0	34.3 ±3.8	23.3 ±1.2	3.3 ±2.1	5.3 ±1.5	111.3 ±12.1	131.0 ±2.6	24.0 ±1.0	15.0 ±3.0	273.7 ±11.5	270.3 ±3.2
3.16	33.3 ±6.5	35.0 ±10.4	5.0 ±2.6	6.0 ±1.0	104.3 ±3.8	129.3 ±3.8	16.7 ±3.2	14.7 ±0.6	287.3 ±28.0	289.7 ±9.1
10	29.0 ±13.0	30.3 ±4.2	4.7 ±2.3	5.3 ±0.6	124.3 ±5.9	125.7 ±17.5	19.7 ±8.1	18.3 ±5.5	268.7 ±6.7	271.3 ±4.7
31.6	28.0 ±1.7	27.0 ±1.0	2.7 ±2.1	5.0 ±2.0	107.7 ±10.8	111.3 ±8.0	18.0 ±7.0	23.7 ±5.1	270.0 ±40.7	274.3 ±3.1
100	29.7 ±3.5	28.3 ±3.8	6.3 ±0.6	4.7 ±0.6	117.7 ±11.6	127.0 ±27.2	17.7 ±2.9	19.3 ±7.6	289.0 ±4.0	264.3 ±22.5
316	14.0 ±2.6[S]	11.0 ±1.0[S]	1.0 ±0.0[S]	1.0 ±0.0[S]	82.7 ±5.0[S]	85.7 ±10.7[S]	5.3 ±0.6[S]	5.0 ±1.0[S]	107.7 ±5.8[S]	197.0 ±3.5[S]
Positive control	275.0 ±8.9	267.7 ±2.5	128.3 ±2.1	134.0 ±10.0	973.3 ±12.1	1017.3 ±7.8	196.0 ±5.3	132.0 ±7.9	975.0 ±24.5	132.0 ±7.9

S: thinning of background lawn

Type of mutation	Frame-shift				Base-pair substitution					
Conc.	TA98		TA1537		TA100		TA1535		TA102	
(µg/plate)	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9

Positive controls:
-S9: strains:
TA98: 2-NF
TA1537: 9-AAC
TA100; TA1535: NaN_3
TA102: MMS

+S9: strains:
TA100, TA1537: CPA
TA98; TA102; TA1537: 2-AA

E. Discussion:

In two independent experiments and in all strains in the absence and presence of a rat liver metabolic activation system (S9) no increases in revertant numbers were observed that were ≥2-fold (in strains TA98, TA100 and TA102) or ≥3-fold (in strains TA1535 and TA1537) the concurrent vehicle control. This study was therefore considered to have provided no evidence of any spiroxamine cyclohexanol mutagenic activity in this assay system.

The positive controls induced an acceptable increase in revertant colony numbers, thereby demonstrating the sensitivity and specificity of the test system.

Table CA 5.8.1/08-4: Bacterial reverse gene mutation data (mean revertant colonies): historical control ranges

Type of mutation	Frame-shift				Base-pair substitution					
Parameter	TA98		TA1537		TA100		TA1535		TA102	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Vehicle control (2011)										
n (studies)	77	77	77	77	77	77	77	77	77	77
Mean	32.5 ±6.9	34.1 ±7.4	7.6 ±2.0	7.8 ±1.9	146.6 ±19.4	148.1 ±23.7	18.8 ±4.2	19.5 ±4.6	277.9 ±15.7	278.1 ±17.5
Min	10	17	1	0	102	101	10	10	233	213
Max	54	60	13	13	220	236	36	48	31.9	328
Positive control (2011)										
n (studies)	77	77	77	77	77	77	77	77	77	77
Mean	371.3 ±150.6	364.1 ±147.2	237.7 ±78.0	237.8 ±88.9	909.3 ±79.7	906.0 ±74.4	262.6 ±93.6	259.6 ±86.0	1062.0 ±66.6	1068.2 ±74.4
Min	186	132	62	77	705	695	102	93	874	854
Max	784	890	577	843	1131	1165	648	599	1586	1563

Positive controls:
-S9: strains:
TA98: 2-NF
TA1537: 9-AAC
TA100; TA1535: NaN_3
TA102: MMC

+S9: strains:
TA98: B[a]P
TA1537; TA100; TA1535: TA102: 2-AA
Upper / lower reference range

F. Deficiencies:

When the study methodology is compared to current test guideline requirements (OECD 471, 1997) the following deficiencies are noted:

- No acceptance criteria for the assay were provided. In the absence of this criteria, only mean data and observed ranges were presented, without a confidence interval applied (ideally 95%). Consequently, with observed ranges presented, genetic drift in bacterial strains cannot be detected, with the data assay data presented it is unclear if the revertant colonies are characteristic of the strain.
- The historical control data presented in the report were not subjected to QA assessment.

It is prudent to discuss the deviations listed in the previous evaluation:

- *'Stock solutions and serial dilutions of the test substance or positive control substances were not analysed to verify concentrations, homogeneity or stability*'. Analyses for achieved concentration, homogeneity or stability of test article formulations were not conducted as part of this study, as this is not a requirement of the regulatory test guidelines. Furthermore, there is no requirement to verify positive control concentrations. The purpose of the positive control is to show specificity and sensitivity of the test system, manifest as increases in revertant colonies. The data presented in this report concluded the suitability of the test system.

It is noted that OECD TG 471 has been recently updated (29 June 2020). However, the updated test guideline has only included a correction to a CAS number of an example positive control – S9 for *E.coli* strain WP2*uvr*A.

In conclusion, the data generated under this study are considered supplementary with the bacterial reverse gene mutation endpoint sufficient addressed with a new, up to date test guideline compliant GLP study (refer to CA 5.8.1/22 [M-755223-02-1]).

Assessment and conclusion by applicant:

Assessment: This study is deemed supplementary as a number of deficiencies are identified when assessed against current test guideline requirements.

Conclusion: It was concluded that spiroxamine cyclohexanol did not induce mutation in five histidine-requiring strains (TA98, TA100, TA1535 and TA1537) of *Salmonella typhimurium* when tested under the conditions of this study. These conditions included treatments at concentrations up to 5000 µg/plate (the maximum recommended concentration according to current regulatory guidelines), in the absence and in the presence of a rat liver metabolic activation system (S9) or up to concentration limited by toxicity using both the plate incorporation and pre-incubation methodologies.

Data Point:	KCA 5.8.1/22
Report Author:	[redacted]
Report Year:	2020
Report Title:	Spiroxamine cyclohexanol: Bacterial reverse mutation assay
Report No:	8406981
Document No:	M-755223-02-1
Guideline(s) followed in study:	OECD 471 (1997)
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

In a reverse gene mutation assay in bacteria, *S. typhimurium* strains TA98, TA1537, TA100, TA1535 and TA102 were exposed to spiroxamine cyclohexanol (M13) formulated in DMSO using the both the plate incorporation and pre-incubation methodologies in the absence and presence of an Aroclor-1254-induced rat liver post-mitochondrial fraction (S9).

Mutation Experiment 1 treatments of all the tester strains were performed in the absence and in the presence of S9, using final concentrations of spiroxamine cyclohexanol at 0, 5, 16, 50, 160, 500, 1600 and 5000 µg/plate. Precipitation of test article was observed on all the test plates treated at 5000 µg/plate.

Following these treatments, evidence of toxicity ranging from a complete killing of the test bacteria to a slight thinning of the background bacterial lawn, in some cases with a marked reduction in revertants was observed at 1600 µg/plate and above in all strains in the absence and in the presence of S9 and also on several plates treated at 500 µg/plate. It was noted that there was variable toxicity observed between the replicate plates treated at 1600 µg/plate in strain TA100 in the presence of S9. This was considered to have been indicative that these treatments were on a relatively steep part of the toxicity curve in this strain, meaning that possible small differences in plate volumes (for example, top agar volume) may have caused sufficient variation in the test article concentration to cause these differing toxic effects.

Mutation Experiment 2 treatments of all the tester strains were performed in the absence (plate incorporation methodology) and in the presence of S9 (pre-incubation methodology). For all strains, the maximum test concentration was reduced to 1600 µg/plate, this being an estimate of the lower limit of toxicity based on the toxicity observed in Experiment 1. Narrowed concentration intervals were employed covering the range 7-1600 µg/plate, in order to examine more closely those concentrations of spiroxamine cyclohexanol approaching the toxicity limit, and considered therefore most likely to provide evidence of any mutagenic activity. In addition, all treatments in the presence of S9 were further modified by the inclusion of a pre-incubation step. In this way, it was hoped to increase the range of mutagenic chemicals that could be detected using this assay system. Following these treatments, evidence of toxicity ranging from a complete killing of the test bacteria to a slight thinning of the background bacterial lawn and/or a marked reduction in revertants was observed on all plates treated at 1600 µg/plate, and also at 640 µg/plate in strain TA102 in the absence of S9 and on all plates treated at 250 or 640 µg/plate in the presence of S9.

No precipitation was observed on any of the test plates following incubation (which was performed using a maximum treatment concentration of 1600 µg/plate).

Following spiroxamine cyclohexanol treatments of all the test strains in the absence and in the presence of S9, no increases in revertant numbers were observed that were ≥1.5-fold (TA102); ≥2-fold (TA98, TA100); ≥3-fold (TA1535, TA1537) above the concurrent vehicle control in either experiment.

Vehicle and positive control treatments were included for all strains in both experiments. The mean numbers of revertant colonies were comparable with acceptable ranges for vehicle control treatments. The positive controls induced an acceptable increase in revertant colony numbers, thereby demonstrating the sensitivity and specificity of the test system.

It was concluded that spiroxamine cyclohexanol did not induce mutation in five histidine-requiring strains (TA98, TA100, TA1535, TA1537 and TA102) of *Salmonella typhimurium* when tested under the conditions of this study. These conditions included treatments at concentrations up to 16000 µg/plate (a toxic concentration), in the absence and in the presence of a rat liver metabolic activation system (S9) using both the plate incorporation and pre-incubation methodologies.

Materials and Methods

A. Materials:

1. Test Material: Spiroxamine cyclohexanol
(alternative name: 4-tert-butylcyclohexanol, M13)

Description:	White powder
Lot/Batch No.:	AE 1247868_PU-01
Purity:	98.2% (no correction factor applied)
CAS No.:	98-52-2
Stability of test compound:	Confirmed stable for the duration of the study (expiry date: 20 January 2021)

2. Control materials:

Negative:

Solvent/final concentration:	DMSO (dimethyl sulphoxide)/0.1 or 0.05 mL/plate (plate incorporation or pre-incubation, respectively)

Positive: -S9	*Strain*	*Mutagen*	*Conc. (µg/plate)*
	TA98	2-nitrofluorene (2-NF)	5
	TA100, TA1535	Sodium azide (NaN_3)	2
	TA1537	9-aminoacridine (9-AAC)	50
	TA102	Mitomycin C (MMC)	0.2
Positive: +S9	*Strain*	*Mutagen*	*Conc. (µg/plate)*
	TA98	Benzo[a]pyrene (B[a]P)	5
	TA100, TA1535, TA1537	2-aminoanthracene[15] (2-AA)	5
	TA102		20

3. Activation: S9 was purchased from a commercial source: Sprague Dawley rats were treated with Aroclor 1254 (supplied by [redacted] lot no. 4029, protein content 3.7 mg/mL). The composition of the 10% S9 reaction mix was: 100 µL S9, Na PBS (100 µM), glucose-6-phosphate (5 µM), β-NADP (4 µM), $MgCl_2$ (8 µM), KCl (33 µM), water (to volume).

4. Test organisms: *S. typhimurium* strains: TA98, TA100, TA1535, TA1537, TA102.
All test organisms were properly maintained and were checked for appropriate genetic markers (*S. typhimurium*: histidine and biotin requirement, *rfa* mutation, *uvrB* sensitivity, ampicillin resistance) regularly.

5. Test Concentrations:

a) Mutation assay 1: *Plate incorporation: +/-S9 all strains*:
0, 5, 16, 50, 160, 500, 1600, 5000 µg/plate

b) Mutation assay 2: *Plate incorporation S9*:
0, 7, 15, 40, 100, 250, 640, 1600 µg/plate
Pre-incubation +S9:
0, 7, 15, 40, 100, 250, 640, 1600 µg/plate

B. Test Performances:

1. In life dates: 25 April 2019 to 8 May 2019 (experimental dates)

2. Experiment 1: *Plate incorporation assay*:
The following sequence of additions of 2 mL of supplemented molten top agar, test article solution/vehicle control (0.1 mL) or positive control solution (0.05 mL), either 0.1 M Na phosphate buffer (0.5 mL pH 7.4) or S9 mix (0.5 mL) for treatments in the absence and in the presence of metabolic activation, respectively and bacterial suspension (0.1 mL) were mixed and poured on to Vogel-Bonner E agar plates (minimal glucose agar plate). When set, plates were inverted and incubated at 37°C, protected from light for 3 days.

3. Experiment 2: *Plate incorporation assay*:
Undertaken for bacterial strains treated in the absence of S9 (refer above).
Pre-incubation assay:
The test article solution or vehicle/positive control solution (0.05 mL), bacteria (0.1 mL) and S9 mix (0.5 mL) were mixed in a small test tube and incubated for 20 minutes at 37°C. After additional of 2 mL of top agar solution the mixture was poured onto a minimal glucose agar plate and allowed to solidify. All plates were incubated for 3 days at 37°C. In this way, it was hoped to increase the range of mutagenic chemicals that could be detected using this assay system.

15 Of note, some researchers have suggested that 2-AA should not be used as the only positive control to evaluate S9-mix activity as it has been shown that the chemical may be activated by enzymes other than the microsomal cytochrome P450 family. In this study, S9 activity was evaluated with both 2-AA and B[a]P. In addition, each batch was checked by the manufacturer of S9 for sterility, protein content, ability to convert ethidium bromide, cyclophosphamide, B[a]P and AAN to bacterial mutagens, and cytochrome P-450-catalysed enzyme activities (alkoxyresorufin-*O*-dealkylase activities). Therefore, there is no concern over S9 activity.

For each experiment triplicate plating was undertaken for each dose level.

4. Statistics: None applied

5. Acceptance criteria: The assay was considered valid if the following criteria were met:

4. The vehicle controls fell within the laboratory's historical control ranges
5. The positive control chemicals induced increases in revertant numbers of ≥1.5-fold (TA102); ≥2-fold (TA98, TA100); ≥3-fold (TA1535, TA1537) above the concurrent vehicle control confirming discrimination between difference strains and an active S9 preparation

5. Evaluation criteria: The test article was considered mutagenic in this assay if:

1. A concentration related increase in revertant numbers was ≥1.5-fold (TA102); ≥2-fold (TA98, TA100); ≥3-fold (TA1535, TA1537) above the concurrent vehicle control values
2. Any observed response was reproducible under the same treatment conditions.

The test article was considered positive in this assay if all the above criteria were met

The test article was considered negative in this assay if none of the above criteria were met.

C. Methods:

1. Homogeneity and achieved concentration analysis of the dose: Not undertaken.

2. Test article formulation preparation: No correction for purity was made, nor was it considered necessary. A preliminary solubility test confirmed spiroxamine cyclohexanol was soluble in DMSO at concentrations equivalent to 102.34 mg/mL. Thereby, confirming a maximum concentration of 50 mg/mL could be prepared and dosed into the test system at a maximum concentration of 5000 µg/plate. Test article stock solutions were prepared by formulating spiroxamine cyclohexanol under subdued lighting in DMSO with the aid of vortex mixing and warming at 37°C, to give the maximum required treatment solution concentration. Subsequent dilutions were made using DMSO. The test article solutions were protected from light and used within approximately 4 h of initial formulation.

3. Toxicity Assessment: The background lawns of the plates were examined for signs of toxicity. Revertant plate count data were also assessed, as a marked reduction in revertants compared to the concurrent vehicle controls were also considered as evidence of toxicity.

4. Scoring: The number of revertant colonies were counted with the unaided eye or a colony counter (Ames Scorer, Perceptive Instruments). Manual scoring with the unaided eye was used when confounding factors such as bubbles or splits in the agar affected the accuracy of the automated counter.

Individual plate counts were recorded separately and the mean and standard deviation of the plate counts for each treatment were determined. Control counts (vehicle/positive) were compared with the laboratory's historical control range. The purpose of the vehicle historical control range was to assess strain characteristics, and not to interpret or justify a negative or positive result[3].

Results and Discussion

A. Analytical determinations:

Not undertaken. Analyses for achieved concentration, homogeneity or stability of test article formulations were not conducted as part of this study, as this is not a requirement of the regulatory test guidelines.

B. Mutation experiment 1:

In the plate incorporation assay treatments of the tester strains were performed in the absence and presence of S9 using final concentrations of spiroxamine cyclohexanol at 5, 16, 50, 160, 500, 1600 and 5000 µg/plate (the maximum recommended concentration according to current regulatory guidelines). Precipitation of test article was observed on all the test plates treated at 5000 µg/plate. Following these treatments, evidence of toxicity ranging from a complete killing of the test bacteria to a slight thinning of the background bacterial lawn, in some cases with a marked reduction in revertants was observed at 1600 µg/plate and above in all strains in the absence and in the presence of S9 and also on several plates treated at 500 µg/plate. It was noted that there was variable toxicity observed between the replicate plates treated at 1600 µg/plate in strain TA100 in the presence of S9. This was considered to have been indicative that these treatments were on a relatively steep part of the toxicity curve in this strain, meaning that possible small differences in plate volumes (for example, top agar volume) may have caused sufficient variation in the test article concentration to cause these differing toxic effects.

Following spiroxamine cyclohexanol treatments of all the test strains in the absence and in the presence of S9, no increases in revertant numbers were observed that were ≥1.5-fold (TA102); ≥2-fold (TA98, TA100); ≥3-fold (TA1535, TA1537) above the concurrent vehicle control.

Table CA 5.8.1/22-1: Spiroxamine cyclohexanol: bacterial reverse gene mutation data (mean revertant colonies): Mutation experiment 1 (plate incorporation)

Type of mutation	Frame-shift				Base-pair substitution					
Conc. (µg/plate)	TA98		TA1537		TA100		TA1535		TA102	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0	36.0 ±8.7	54.3 ±7.0	17.3 ±2.1	23.0 ±6.1	108.0 ±6.6	115.3 ±6.4	8.0 ±1.0	20.0 ±13.6	293.7 ±3.8	354.7 ±21.1
5	45.3 ±5.5	47.7 ±1.2	15.7 ±5.7	23.0 ±5.3	107.0 ±1.0	131.7 ±16.0	12.7 ±2.9	17.3 ±13.3	302.7 ±6.4	332.3 ±25.4
16	40.0 ±6.2	57.0 ±2.0	13.3 ±3.1	25.3 ±4.0	102.0 ±6.0	121.0 ±10.6	9.7 ±1.2	19.0 ±12.0	291.7 ±14.5	368.3 ±15.0
50	43.0 ±6.2	49.0 ±2.0	9.3 ±4.0	28.0 ±5.0	105.7 ±16.6	118.7 ±16.3	8.0 ±2.0	14.3 ±5.5	296.7 ±13.4	317.7 ±6.0
160	41.7 ±9.1	52.7 ±2.5	11.0 ±3.0	24.7 ±4.2	116.0 ±3.0	123.7 ±3.5	10.7 ±6.0	16.0 ±1.0	297.7 ±15.0	323.7 ±6.0
500	35.3 ±8.1 S	41.0 ±5.6 S	15.3 ±1.5	23.0 ±1.7 S	96.7 ±3.8 S	133.7 ±12.7 S	11.0 ±2.0	11.0 ±5.6	215.0 ±21.7 S	267.0 ±11.4 S
1600	T	T	T	T	T	70.3 ±4.9 S,T	T	T	T	T
5000	T,P	T,P	T,P	T,P	T,P	T,P	T,P	T,P	T,P	T,P
Positive control	1485.0 ±66.5	397.7 ±4.7	546.7 ±389.5	320.7 ±41.6	1168.3 ±49.5	3205.7 ±139.5	817.3 ±16.0	192.0 ±14.5	1190.0 ±79.1	2571.0 ±243.2

P: precipitate on plate
T: Toxic: no revertant colonies
S: slight thinning of background lawn
Positive controls:
-S9: strains:
TA98: 2-NF
TA1537: 9-AAC
TA100; TA1535: NaN_3
TA102: MMC

+S9: strains:
TA98: B[a]P
TA1537; TA100; TA1535: TA102: 2-AA

B. Mutation experiment 2:

For Experiment 2 treatments of all the tester strains were performed in the absence (plate incorporation methodology) and in the presence of S9 (pre-incubation methodology). For all strains, the maximum test concentration was reduced to 1600 µg/plate, this being an estimate of the lower limit of toxicity based on the toxicity observed in Experiment 1. Narrowed concentration intervals were employed covering the range 7-1600 µg/plate, in order to examine more closely those concentrations of spiroxamine cyclohexanol approaching the toxicity limit, and considered therefore most likely to provide evidence of any mutagenic activity. In addition, all treatments in the presence of S9 were further

modified by the inclusion of a pre-incubation step. In this way, it was hoped to increase the range of mutagenic chemicals that could be detected using this assay system. Following these treatments, evidence of toxicity ranging from a complete killing of the test bacteria to a slight thinning of the background bacterial lawn and/or a marked reduction in revertants, was observed on all plates treated at 1600 µg/plate, and also at 640 µg/plate in strain TA102 in the absence of S9, and on all plates treated at 250 or 640 µg/plate in the presence of S9.

No precipitation was observed on any of the test plates following incubation (which was performed using a maximum treatment concentration of 1600 µg/plate).

Following spiroxamine cyclohexanol treatments of all the test strains in the absence and in the presence of S9, no increases in revertant numbers were observed that were ≥1.5-fold (TA102); ≥2-fold (TA98, TA100); ≥3-fold (TA1535, TA1537) above the concurrent vehicle control.

Vehicle and positive control treatments were included for all strains in both experiments. The mean numbers of revertant colonies were comparable with acceptable ranges for vehicle control treatments. The positive controls induced an acceptable increase in revertant colony numbers, thereby demonstrating the sensitivity and specificity of the test system.

Table CA 5.8.1/22-2: Spiroxamine cyclohexanol: bacterial reverse gene mutation data (mean revertant colonies): Mutation experiment 2

Type of mutation	Frame-shift				Base-pair substitution					
Conc. (µg/plate)	TA98		TA1537		TA100		TA1535		TA102	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0	31.3 ±2.5	46.6 ±11.9	5.3 ±0.6	11.3 ±1.5	99.3 ±8.1	140.0 ±3.6	8.7 ±2.5	15.3 ±1.2	266.3 ±10.0	338.3 ±14.4
7	24.3 ±3.5	50.3 ±3.2	7.0 ±3.6	9.0 ±1.0	103.0 ±10.4	127.3 ±18.1	8.0 ±2.6	13.0 ±7.0	284.0 ±7.0	357.3 ±34.0
15	35.7 ±4.0	45.0 ±5.3	6.7 ±1.2	14.7 ±4.0	90.7 ±24.1	122.0 ±14.2	12.0 ±4.4	13.3 ±4.5	284.3 ±12.7	325.3 ±19.4
40	32.0 ±3.6	47.0 ±6.1	5.3 ±3.5	21.0 ±5.0	108.7 ±12.3	128.0 ±14.8	11.3 ±2.9	13.3 ±2.5	292.3 ±10.8	351.0 ±11.5
100	34.0 ±5.0	36.0 ±7.9	4.0 ±1.0	11.0 ±3.0	97.3 ±3.1	121.0 ±5.3	5.0 ±3.6	12.0 ±3.6	269.3 ±19.7	324.7 ±39.6
250	32.3 ±6.1	18.7 ±3.1	6.0 ±1.0	12.7 ±8.4[S]	96.3 ±21.2	85.3 ±7.8	8.7 ±4.5	10.3 ±1.2[S]	245.7 ±21.0	240.3 ±45.5[S]
640	20.7 ±6.1	[T]	6.3 ±2.5	[T]	86.7 ±13.4	[T]	7.3 ±3.8	[T]	164.7 ±8.4	[T]
1600	[T]	[T]	[T]		[T]	[T]	[T]	[T]	[T]	[T]
Positive control	1151.7 ±119.3	361.7	819 ±223.3	309.3 ±28.5	992.0 ±6.2	2801.0 ±455.7	745.3 ±37.1	211.3 ±37.6	847.3 ±3.2	2105.7 ±102.1

S: slight thinning of background lawn
T: Toxic, no revertant colonies
Positive controls:
-S9: strains (plate incorporation):
TA98: 2-NF
TA1537: 9-AAC
TA100; TA1535: NaN_3
TA102: MMC

T: Toxic, no revertant colonies
- dose level not treated

+S9: strains (pre-incubation):
TA98: B[a]P
TA1537; TA100; TA1535: TA102: 2-AA

C. Discussion:

In two independent experiments and in all strains in the absence and presence of a rat liver metabolic activation system (S9) no increases in revertant numbers were observed that were ≥1.5-fold (in strain TA102), ≥2-fold (in strains TA98 and TA100) or ≥3-fold (in strains TA1535 and TA1537) the concurrent vehicle control. This study was therefore considered to have provided no evidence of any spiroxamine cyclohexanol mutagenic activity in this assay system.

The positive controls induced an acceptable increase in revertant colony numbers, thereby demonstrating the sensitivity and specificity of the test system.

Table CA 5.8.1/22-3: Bacterial reverse gene mutation data (mean revertant colonies): historical control ranges

Type of mutation	Frame-shift				Base-pair substitution					
Parameter	TA98		TA1537		TA100		TA1535		TA102	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Vehicle control										
Date range	Jan 19 – Mar 19		Oct 17 – Feb 18		Oct 17 – Feb 18		Jun 18 – Sep 18		Oct 17 – Feb 18	
n (studies)	77	75	76	76	103	100	78	76	72	72
n (plates)	300	291	299	292	377	370	287	281	266	274
Mean	23.1	36.3	10.0	13.9	101.8	108.7	19.6	18.8	290.4	315.7
99% L.R.R	10	20	1	5	56	72	7	5	220	193
99% U.R.R	46	64	22	29	168	168	35	37	403	411
Positive control										
Date range	Jan 19 – Mar 19		Oct 17 – Feb 18		Oct 17 – Feb 18		Jun 18 – Sep 18		Oct 17 – Feb 18	
n (studies)	77	75	75	76	102	98	78	76	72	71
n (plates)	285	275	294	276	372	351	287	278	264	255
Mean	1170.8	350.8	303.3	286.3	650.2	1524.3	668.1	190.2	936.9	1559.8
99% L.R.R	328	203	84	41	431	455	234	37	454	368
99% U.R.R	3312	711	885	550	1470	2884	925	614	2148	3566

Positive controls:
-S9: strains:
TA98: 2-NF
TA1537: 9-AAC
TA100; TA1535: NaN3
TA102: MMC
+S9: strains:
TA98: B[a]P
TA1537; TA100; TA1535: TA102: 2-AA
Upper / lower reference range

D. Deficiencies:

It is noted that OECD TG 471 has been recently updated (29 June 2020). However, the updated test guideline has only included a correction to a CAS number of an example positive control –S9 for E.coli strain WP2uvrA. Therefore, it is reasonable to conclude that this study, whilst conducted in accordance with the test guideline issued in 1997, is also in accordance with the updated test guideline.

Assessment and conclusion by applicant:

Assessment: Study meets the current guidance and the requirements in 283/2013.

Conclusion: It was concluded that spiroxamine cyclohexanol did not induce mutation in five histidine-requiring strains (TA98, TA100, TA1535, TA1537 and TA102) of *Salmonella typhimurium* when tested under the conditions of this study. These conditions included treatments at concentrations up to 16000 µg/plate (a toxic concentration), in the absence and in the presence of a rat liver metabolic activation system (S9) using both the plate incorporation and pre-incubation methodologies.

Data Point:	KCA 5.8.1/09
Report Author:	[redacted]
Report Year:	2012
Report Title:	Mutagenicity study of 4-tert-butylcyclohexanol (PTBCOL) in the mouse lymphoma forward mutation assay -in vitro -
Report No:	2012-0126-DGM
Document No:	M-471125-01-1
Guideline(s) followed in study:	Regulation 2000/32/EC, Method B.17; OECD 476; US-EPA 712-C96-22; OPPTS 870.5300
Deviations from current test guideline:	Yes A number of deficiencies were noted (refer to Results, Deficiencies section below).
Previous evaluation:	yes, evaluated and accepted RAR (2010)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

Spiroxamine cylohexanol was assayed for the ability to induce mutation at the *tk* locus (5-trifluorothymidine [TFT] resistance) in mouse lymphoma L5178Y cells using a fluctuation protocol. The study consisted of a preliminary cytotoxicity assay followed by two independent Mutation Experiments, each conducted in the absence and presence of metabolic activation by an Aroclor 1254-induced rat liver post-mitochondrial fraction (S9).

The test article was formulated in anhydrous analytical grade dimethyl sulphoxide (DMSO) and dosed at 1% v/v.

In a two independent gene mutation experiments following exposure in the absence (3 and 24 hours) and presence (3 hours) of a rat liver metabolic activation system (S9) no increases mutant frequency (MF) were observed that exceeded the sum of the MF of any of the test concentrations assessed that exceeded the sum of the mean concurrent vehicle control MF and the GEF (126 *x* 10^6). This study was therefore considered to have provided no evidence of any spiroxamine cyclohexanol mutagenic activity in this assay system. However it is recognised that in all treatment conditions on overt level of toxicity (RTG <10%) was observed.

Vehicle and positive control treatments were included in the Mutation assays in the absence and presence of S9. MF in vehicle control cultures fell within acceptable ranges, and clear increases in mutation were induced by the positive control chemicals MMS (without S9) and 3MC (with S9). Therefore the study was accepted as valid.

It is concluded that spiroxamine cyclohexanol did not show any increases in the mutant frequency of L5178Y cells at the *tk* locus employing the fluctuation protocol. These conditions included treatments up to 250 µg/mL (a concentration deemed overtly toxic i.e. RTG <10%, in accordance with current *in vitro* mouse lymphoma genotoxicity regulatory test guideline) in the absence (3 and 24 hours) or presence (3 hours) of rat liver metabolic activation system.

Materials and Methods

A. Materials:

1. Test Material: Spiroxamine cyclohexanol
(alternative name: [(4-tert-butylcyclohexanol, M13)

Description: Solid, viscid, melt
Lot/Batch No.: 80322
Purity: 98.6% (correction factor of 1.014 applied) (molecular weight 156.27 g/mol)
CAS No.: 98-52-2

Stability of test compound:	Confirmed stable for the duration of the study (expiry date: February 2013)
2. Control materials:	
Vehicle / final concentration:	DMSO (dimethyl sulphoxide) / 1% (v/v)
Positive: -S9	Methyl methanesulphonate (MMS, 3/24 h: 10, 15 µg/mL)
Positive: +S9	3-methlcholanthrene (3MC, 3 h: 2.5, 4.0 µg/mL)
3. Activation:	S9 was prepared in house from rats treated with Aroclor 1254 (protein content 33.1 mg/mL). The composition of the S9 reaction mix was: S9 (5%), KCl (30 mM), glucose-6-phosphate (138 mM), NADP (6.7 mM);
4. Test cells:	L5178Y *tk*$^{+/-}$ mouse lymphoma cells were stored as frozen stocks in liquid nitrogen. Each batch was purged of TK- mutants, checked for spontaneous mutant frequency and that it was mycoplasma free. Cultures were used within 8 days of recovery from frozen stock. For each experiment the cells were diluted in R10p and incubated in a humidified atmosphere of 5% (v/v) CO_2 in air.
5. Culture medium:	For growth maintenance of the cell line: R10p: RPMI 1640 medium supplemented with heat inactivated horse serum (10% v/v) for growing and 0.05% Pluronic F68, 2 mM L-glutamine, 300 mM Na pyruvate, 100 µg/mL gentamycin, 2.5 µg/mL Fungizone. During treatment: R5p: RPMI 1640 medium supplemented with heat inactivated horse serum (5% v/v) 0.05% Pluronic F68, 2 mM L-glutamine; Mutant selective medium: R10p with TFT (3 µg/mL)
6. Locus examined:	*tk* (thymidine kinase) locus. The selection agent was TFT (5-trifluorothymidine)
7. Test article Concentrations:	
a) Preliminary cytotoxicity assay:	0, 25, 100, 250, 1000, 2500, 5000 µg/mL
b) Mutation assays:	3 h +/-S9: 0, 15.63, 31.3, 62.5, 250 µg/mL 24 h –S9: 0, 15.63, 31.3, 62.5, 250 µg/mL

B. Test Performance:

1. In life dates:	23 April 2012 to 14 August 2012 (experimental dates)
2. Vehicle selection:	Spiroxamine cyclohexanol was soluble at 500 mg/mL in deionised water gave a final concentration of 5000 µg/mL when dosed at 1% v/v (a concentration deemed to be the maximum recommended concentration in accordance with the *in vitro* genotoxicity test guidelines followed at the time of the study. However, when assessed against current test guideline requirements, the maximum concentration tested should be 10 mM, or 2000 µg/mL, whichever is the lowest. In the case of spiroxamine cyclohexanol, the maximum concentration tested was equivalent to 32 mM in the preliminary test). Osmolality and pH assessments of the test article in cell culture medium were undertaken for the preliminary cytotoxicity study.
3. Statistics:	None performed.
4. Acceptance criteria:	<u>*For test article*</u>: The highest concentration tested was one that allowed the maximum exposure up to 5000 µg/mL or 10 mM for freely soluble compounds, or the limit of toxicity (*i.e.* relative total growth (RTG) reduced to ~10 - 20% of the concurrent vehicle control) or the limit of solubility. For a toxic substance,

at least 4 analysable concentrations should have been achieved which ideally spanned the toxicity range of 100 - 10% RTG.

For vehicle controls: The mean vehicle control value for mutant frequency was between 50 – 170 mutants *per* 10^6 viable cells.

The mean cloning efficiency was between 65 - 120%.

The mean suspension growth was between 8 - 32 on Day 2 following 3 h treatments and between 32 - 180 on Day 2 following a 24 h treatment.

For positive controls: Positive controls showed an absolute increase in mean total MF above the mean concurrent vehicle control MF of at least 300 mutants *per* 10^6 mutants. At least 40% of this was due to the number of small mutant colonies.

Mean RTGs for the positive controls were greater than 10%.

5. Evaluation criteria: The test article was considered mutagenic if a concentration-related increase in mutant frequency (MF) was observed.

The global evaluation factor (GEF) devised by Moore and colleagues[1] has been used to evaluate the data retrospectively. For a test material to be considered positive the mutant frequency of any test concentrations must exceed the sum of the mean concurrent solvent control mutant frequency + the GEF (126 mutants *per* 10^6 viable cells) and was accompanied by a positive linear trend.

C. Methods:

1. Homogeneity and achieved concentration analysis of the dose: Not conducted.

2. Cell treatment: *Preliminary cytotoxicity assay*: Cells were exposed to test article formulations, or vehicle controls for either 3 hours in the absence of S9 or 3 hours in presence the of S9. At the end of treatment cultures were washed, cell cultures were adjusted to 8 cells/mL and for each concentration 0.2 mL was plated into 32 microtitre wells (1.6 cells/well). Plates were incubated at 37°C for 7 days. Wells containing viable clones were counted, with cloning efficiency used as an assessment for toxicity.

Mutation assays: Cells (12.75 x 10^6 cells, diluted to 5*x* 10^5 cells/mL) were exposed to test article formulations, solvent or positive controls for either 3 hours in the absence of S9 or 3 hours in presence the of S9. At the end of treatment cultures were washed, cell cultures were adjusted to 8 cells/mL for cloning efficiency, with 0.2 mL (~1.6 cells/well) plated into two 96 well plates. Plates were incubated for 7 days. After this period the number of wells without growth of cells was counted.

Mutation frequency was determined by plating ~2000 cells/well in cell culture medium containing 2 µg/ TFT/mL. Plates were incubated for 11-14 days. After this period the number of well without growth was counted to provide CE in TFT. Wells with growth in indicated evidence of TFT-resistance mutants. Colony sizing was performed on negative and positive controls.

Duplicate vehicle and single cultures for test article concentrations and positive controls were used throughout. The mutation assay was repeated with cells exposed to test article formulations, solvent or positive controls for either 24 hours in the absence of S9 or 3 hours in presence the of S9.

Results and Discussion

A. Analytical determinations:

Not undertaken. Analyses for achieved concentration, homogeneity or stability of test article formulations were not conducted as part of this study, as this is not a requirement of the regulatory test guidelines.

B. Preliminary cytotoxicity assay:

In the range-finding experiment, precipitate was observed at concentrations ≥1000µg/mL both in the absence and presence of S9 following a 3 hour of treatment.

Exposure to spiroxamine cyclohexanol at concentrations from 25 to 250 µg/mL in the absence and presence of S9 mix (3 hour exposure) resulted in relative survival (RS) values from 109 to 9% and from 91 to 19%, respectively.

Table CA 5.8.1/09-01: Spiroxamine cyclohexanol mutant frequency data from L5178Y *tk*$^{+/-}$ 3.7.2C cells: preliminary cytotoxicity test

Conc. (µg/mL)	3 h -S9		3 h +S9	
	CE (%)	RS (%)	CE (%)	RS (%)
0[a]	0.6674	100	0.4332	100
25	0.6674	100	0.3953	91
100	0.7270	109	0.4332	100
250	0.0615	9	0.0831	19
1000	0.0615	9[ppt]	0.0198	5[ppt]
2500	0.0000	0[ppt]	0.0000	0[ppt]
5000	0.0198	2[ppt]	0.0198	5[ppt]

CE – cloning efficiency
RS: relative survival
[a] DMSO
ppt precipitate observed at end of treatment

C. Mutation assay:

1. Experiment 1:

3 hour -S9: Cultures were exposed to spiroxamine cyclohexanol at concentrations from 15.63 – 250 µg/mL. No precipitate (assessed by eye at the end of treatment) was observed. All concentrations were assessed for determination of MF. RTG values from 136 - 5% were obtained relative to the vehicle control. There were no increases in the MF of any of the test concentrations assessed that exceeded the sum of the mean concurrent vehicle control MF and the GEF (126 x 10^6). It is observed that the highest concentration plated for MF assessment was overtly toxic (*i.e.* RTG <10%).

3 hour +S9: Cultures were exposed to spiroxamine cyclohexanol at concentrations from 15.63 – 250 µg/mL. No precipitate (assessed by eye at the end of treatment) was observed. All concentrations were assessed for determination of MF. RTG values from 75 - 5% were obtained relative to the vehicle control. There were no increases in the mean MF of any of the test concentrations assessed that exceeded the sum of the mean concurrent vehicle control MF and the GEF. It is observed that the highest concentration plated for MF assessment was overtly toxic (*i.e.* RTG <10%).

2. Experiment 2:

3 hour +S9: Cultures were exposed to spiroxamine cyclohexanol at concentrations from 15.63 – 250 µg/mL. No precipitate (assessed by eye at the end of treatment) was observed. All concentrations were assessed for determination of MF. RTG values from 80 - 5% were obtained relative to the vehicle control. There were no increases in the mean MF of any of the test concentrations assessed that exceeded the sum of the mean concurrent vehicle control MF and the GEF. It is observed that the highest concentration plated for MF assessment was overtly toxic (*i.e.* RTG <10%).

24 hour -S9: Cultures were exposed to spiroxamine cyclohexanol at concentrations from 15.63 – 250 µg/mL. No precipitate (assessed by eye at the end of treatment) was observed. All concentrations were assessed for determination of MF. RTG values from 116 - 1% were obtained relative to the vehicle control. There were no increases in the mean MF of any of the test concentrations assessed that exceeded the sum of the mean concurrent vehicle control MF and the GEF. It is observed that the highest concentration plated for MF assessment was overtly toxic (*i.e.* RTG <10%).

3. Positive controls:

Vehicle and positive control treatments were included in the Mutation assays in the absence and presence of S9. MF in vehicle control cultures fell within acceptable ranges, and clear increases in mutation were induced by the positive

control chemicals MMS (without S9) and 3MC (with S9). Therefore the study was accepted as valid.

Table CA 5.8.1/09-02: Spiroxamine cyclohexanol mutant frequency data from L5178Y *tk*$^{+/-}$ 3.7.2C cells: experiment 1

Conc. (µg/mL)	3 h -S9					3 h +S9				
	RTG (%)	SG	CE (%)	MF (x10^6)	SC MF (%)	RTG (%)	SG	CE (%)	MF (x10^6)	SC MF (%)
0[a]	100	23.5	70.15	99.42	45.0	100	21.7	98.83	69.06	54.1
15.63	98	21.6	74.81	74.49	51.2	67	20.1	71.67	80.83	52.4
31.3	136	26.3	85.36	79.96	51.0	72	18.4	84.10	60.27	48.6
62.5	89	17.7	82.86	77.00	52.2	75	20.0	80.46	86.68	48.0
125	74	18.8	64.87	91.56	53.5	73	22.4	69.65	62.44	52.5
250	5	5.8	12.98	60.57	50.0	5	6.3	16.69	67.97	25.0
MMS 10	23	15.2	24.37	1016.17	68.7	22	18.2	25.94	902.92	45.4
MMS 15	18	14.6	20.64	1360.45	67.3	3	22.3	32.03	956.98	54.0

RTG: Relative Total Growth
SG: suspension growth
CE: Cloning Efficiency
MF: Mutant Frequency (mutants *per* 10^6 viable cells)
SC MF: small colony mutant frequency

[a] dimethyl sulphoxide (DMSO)
Positive control:
-S9: Methyl methanesulphonate (10 µg/mL)
+S9: Benzo[a]pyrene (1.5 µg/mL)

Table CA 5.8.1/09-03: Spiroxamine cyclohexanol mutant frequency data from L5178Y *tk*$^{+/-}$ 3.7.2C cells: experiment 2

Conc. (µg/mL)	3 h +S9					24 h -S9				
	RTG (%)	SG[b]	CE (%)	MF (x10^6)	SC MF (x10^6)	RTG (%)	SG	CE (%)	MF (x10^6)	SC MF (x10^6)
0[a]	100	26.8	74.28	82.92	51.7	100	39.0	88.62	77.02	51.0
15.63	55	16.5	65.79	83.63	40.0	93	48.4	66.73	95.61	47.8
31.3	53	13.6	77.00	73.34	41.5	76	34.1	77.00	86.71	54.2
62.5	59	13.6	86.64	61.83	53.8	113	49.3	79.27	65.76	50.0
125	80	23.4	68.67	67.47	47.1	116	51.9	77.00	65.82	54.1
250	5	4.1	24.37	87.39	37.5	1	1.0	28.34	75.15	50.0
Positive control	11[b]	9.8	23.42	1258.17	52.6	16[d]	32.4	17.55	1830.35	72.5
	11[c]	8.8	25.34	1367.69	43.8	22[d]	27.4	28.34	1376.55	79.3

RTG: Relative Total Growth
SG: suspension growth
CE: Cloning Efficiency
MF: Mutant Frequency (mutants *per* 10^6 viable cells)
SC MF: small colony mutant frequency

[a] dimethyl sulphoxide (DMSO)
Positive control:
+S9: 3-methylcholanthrene: 2.5[b], 4.0[c] µg/mL)
-S9: Methyl methanesulphonate: 10[d], 15[e] µg/mL

Table CA 5.8.1/09-03: Spiroxamine cyclohexanol mutant frequency data from L5178Y *tk*$^{+/-}$ 3.7.2C cells laboratory historical control mutant frequency data 2009 - 2011

Parameter	Vehicle control (n = 14)			Positive control (n = 14)		
	3 h –S9	3 h +S9	24 h –S9	3 h –S9	3 h +S9	24 h –S9
Mutant frequency (mutants *per* 10^6 viable cells)						
Mean ±sd	93.0 ±27.1	97.0 ±26.7	96.6 ±24.4	2646.7 ±1890.8	2441.6 ±2068.3	3245.1 ±2560.0
Range	43.1 – 130.4	55.1 – 145.6	61.6 – 157.6	315 – 8987.4	351.6 – 8437.8	799.4 – 9589.9
Small colony : Large colony ratio						
Mean ±sd	0.77 ±0.34	0.85 ±0.38	0.79 ±0.27	2.19 ±0.76	0.93 ±0.50	1.99 ±0.56
Range	0.29 – 1.50	0.18 – 0.150	0.42 – 1.43	1.34 – 5.00	0.22 – 2.33	1.05 – 3.09

D. Discussion:

In a two independent gene mutation experiments following exposure in the absence (3 and 24 hours) and presence (3 hours) of a rat liver metabolic activation system (S9) no increases in mutant frequency (MF) were observed that exceeded the sum of the mean concurrent vehicle control MF and the GEF (126×10^6). This study was therefore considered to have provided no evidence of any spiroxamine cyclohexanol mutagenic activity in this assay system. However it is recognised that in all treatment conditions on overt level of toxicity (RTG <10%) was observed.

Vehicle and positive control treatments were included in the Mutation assays in the absence and presence of S9. MF in vehicle control cultures fell within acceptable ranges, and clear increases in mutation were induced by the positive control chemicals MMS (without S9) and 3MC (with S9). Therefore the study was accepted as valid.

E. Deficiencies:

Although the study was conducted according to test guideline OECD 476 (1984), this test guideline has since been updated twice in the intervening periods (1997 and 2016 with the publication of OECD 490 for the *tk* version of the assay). When assessed against current test guideline requirements the following deficiencies are noted:

- An insufficient number of cells were treated in the mutation assay (12.75×10^6 cells) compared to the test guideline requirements (20×10^6 cells/culture), however the spontaneous mutant frequency rate observed in the vehicle control along with small colony recovery was acceptable.
- At the time of study conduct the Moore evaluation criteria, whilst not formally adopted in the OECD test guideline, were universally accepted by laboratories. Consequently, the Moore evaluation criteria were followed and used to interpret the data. Whilst deemed a deficiency from the test guideline at the time of study conduct, the approach adopted was scientifically robust and acceptable, and therefore did not impact upon the data interpretation
- Although historical control ranges have been provided, these are not used to determine the data, but rather confirms the expertise of the laboratory to conduct the assay along with confirming the stability of the cell line used.

Assessment and conclusions by applicant:

Assessment: Study meets the current guidance and the requirements in 283/2013.

Conclusion: It is concluded that spiroxamine cyclohexanol did not show any increases in the mutant frequency of L5178Y cells at the *tk* locus employing the fluctuation protocol. These conditions included treatments up to 250 µg/mL (a concentration deemed overtly toxic *i.e.* RTG <10%, in accordance with current *in vitro* mouse lymphoma genotoxicity regulatory test guidelines) in the absence (3 and 24 hours) or presence (3 hours) of rat liver metabolic activation system.

Data Point:	KCA 5.8.1/10
Report Author:	[redacted]
Report Year:	1997
Report Title:	In vitro chromosomal abberation assay with P-tert. Butylcyclohexanol
Report No:	97-0366-DGM
Document No:	M-471187-01-1
Guideline(s) followed in study:	OECD 473; Guideline 97/548/EWG, Part B, B.10; EEC Guidence Note, Annex V, Method B10
Deviations from current test guideline:	Yes A number of deficiencies were noted (refer to Results, Deficiencies section below).
Previous evaluation:	yes, evaluated and accepted RAR (2005)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Supportive only

Executive Summary

In a mammalian cell chromosome aberration assay, cultured Chinese hamster V79 lung cells were exposed to spiroxamine cyclohexanol. The study consisted of a three independent chromosome aberration experiments, each conducted in the absence and presence of metabolic activation, by an Aroclor-1254 induced rat liver post-mitochondrial fraction (S9).

The test article was formulated in ethanol, which was assumed to be dosed at 1% v/v.

Experiment 2 was in part a direct repeat of experiment 1, with cultures exposure to spiroxamine cyclohexanol for 3 hours, followed by a 15 hour recovery period. Single cultures (with the exception of duplicate cultures for the vehicle control) were exposed to the test article, with concentrations of 10, 60 and 100 µg/mL in experiment 1 and 2 in the absence of S9 selected for metaphase analysis. In the presence of S9, concentrations of 50, 250 and 500 µg/mL and 20, 100 and 200 µg/mL for experiments 1 and 2, respectively were selected for metaphase analysis. In each treatment condition across both experiments resulted in no increase in the incidence of cells with structural aberrations in any analysed treatment concentration. However, a sufficient level of cytotoxicity (reduction in %MI 40-50%) was not achieved.

In experiment 3, cultures were exposed to a single test article concentration of spiroxamine cyclohexanol in the absence and presence of S9, with a recovery period of 25 hours post the end of exposure. In the absence of S9, a concentration of 60 µg/mL resulted in a reduction in MU of 11%. Whilst no increase in the incidence of cells with structural aberrations was observed, a sufficient level of cytotoxicity (reduction in %MI 40-50%) was not achieved. In the presence of S9, a concentration of 200 µg/mL resulted in a reduction in MU of 44%. Whilst an acceptable level of cytotoxicity was achieved, only a single concentration was treated, no assessment of a dose response available.

The positive controls, MMC (-S9) and CPA (+S9) induced a statistically significant increase in chromosomal aberrations (excluding gaps) in all experiments. However, these increases were observed in the presence of overt cytotoxicity (*i.e.* ≥50%). Therefore the ability for the assay to assess direct acting clastogens was not sufficiently demonstrated, with overt cytotoxicity a contributing factor.

It is concluded that spiroxamine cyclohexanol did not induce biologically relevant increases in the incidence of chromosome aberrations in Chinese hamster V79 cells. These conditions included treatments that were neither limited by toxicity (*i.e.* reduction in % relative MI 45±5%) or precipitate in the absence (3 hours) and presence (3 hours) of rat liver metabolic activation system with recovery periods of 15 and 25 hours in both treatment conditions.

Materials and Methods

A. Materials:

1. Test Material:	Spiroxamine cyclohexanol (alternative name: [(4-tert-butylcyclohexanol, M13)
Description:	White crystals or flakes, with a faint odour of camphor
Lot/Batch No.:	96/189V
Purity:	98.7% (no correction for purity) (molecular weight 156.27 g/mol)
CAS No.:	98-52-2
Stability of test compound:	Confirmed stable for the duration of the study (expiry date: >1 year stated)
2. Control materials:	
Vehicle / final concentration:	Ethanol / 1% (v/v)
Positive: -S9	Mitomycin C (MMC, 3 h: 0.03, 0.04 µg/mL)
Positive: +S9	Cyclophosphamide (CPA, 3 h: 3, 4 µg/mL)
3. Activation:	S9 was purchased from a commercial source. Sprague Dawley rats were treated with Aroclor 1254 (supplied by [redacted] lot no.: 85675, 85679, protein content not reported). The composition of the 15% S9 reaction mix was: 1 % parts S9, glucose-6-phosphate (5 mM), NADP (4 mM), $MgCl_2$ (10 mM), KCl (30 mM), Na_2HPO_4/NaH_2PO_4 (50 mM).
4. Test cells:	V79 cells derived from the lung of Chinese hamster were obtained from Dr. Engelhardt, BASF, Ludwigshafen and stored as frozen stocks in liquid nitrogen. For each experiment the cells were diluted in MEM4 (see culture medium below) and incubated in a humidified atmosphere of 5% (v/v) CO_2 in air. A modal chromosome number of 22 and a doubling time of 16.5 h. Mycoplasma status was not reported.
5. Culture medium:	MEM4: Eagle's minimal essential medium (MEM) supplied with L-glutamine (2 mM), penicillin (100 units/mL), streptomycin (100 µg/mL), heat-inactivated fetal calf serum (4%). MEM0: as MEM4, but without fetal calf serum. Used as a negative control for +S9 treatment.
6. Test article Concentrations:	
c) Chromosomal aberration assay:	Experiment 1 3 h (+15 h recovery) -S9: 0, 2.5, 5, 10, 20, 40, 60, 100, 180, 300, 500 µg/mL 3 h (+15 h recovery) +S9: 0, 10, 25, 50, 75, 150, 250, 500, 750, 1200, 2000 µg/mL Experiment 2 3 h (+15 h recovery) -S9: 0, 10, 60, 100 µg/mL 3 h (+15 h recovery) +S9: 0, 20, 100, 200 µg/mL Experiment 3 3 h (+25 h recovery) -S9: 0, 60 µg/mL 3 h (+25 h recovery) +S9: 0, 200 µg/mL (concentrations underlined were scored for chromosome aberrations)

B. Study design:

1. In life dates:	Not reported
2. Vehicle selection:	Spiroxamine N-oxide was soluble at 200 mg/mL in ethanol which gave a final concentration of 2000 µg/mL when dosed at 1% v/v v (a concentration deemed to be the maximum recommended concentration in accordance with the *in vitro* genotoxicity test guidelines followed at the time of the study. However, when assessed against current test guideline requirements, the maximum concentration tested should be 10 mM, or 2000 µg/mL, whichever is the

lowest. In the case of spiroxamine cyclohexanol, the maximum concentration tested was equivalent to 12.8 mM in the preliminary test).
pH and osmolality of the test culture medium was not assessed. The maximum final concentration tested in the preliminary toxicity test was 2000 µ/mL.

3. Statistics: Chi-square test was used to determine increases in the incidence of chromosome aberrations. (5% level).

4. Acceptance criteria:

For vehicle controls:

- The mean vehicle control for chromosomal aberration frequency (excluding gaps) was within the laboratory's historical control range.
- The percentage of polyploidy and endoreduplicated cells was <10%.
- 200 cells/treatment group were analysable

For positive controls:

- Positive controls induced a response that was >5%.
- At least 100 cells for each positive control were available for analysis.
- There was acceptable homogeneity between replicate cultures as demonstrated by binomial dispersion test.

5. Evaluation criteria: The criteria for determining a positive result a relevant and statistically significant increase in aberration rate was obtained that exceeded the laboratory's historical control range. Increases in chromosomal aberrant frequency had to be reproducible.
A negative result did not meet the above criteria.

C. Methods:

1. Homogeneity and achieved concentration analysis of the dose: Not conducted.

2. Chromosomal aberration assay, experiment 1: Following establishment of cultures (24 hours), 1×10^7 cells were seeded, where S9 mix was required this was added as appropriate. Cells were exposed to the test article for 3 hours in the absence or presence of S9. These cultures were sampled at 15 hours after the end of treatment (*i.e.* 18 hours after the start of treatment).
At the end of treatment (3 hours) it was assumed that the test article was removed by washing the monolayer cultures, cells removed by trypsinisation. Cytotoxicity was determined by MI (a total of 1000 cells assessed for metaphase).
Duplicate cultures were used for the vehicle, positive controls and each test article concentration. Three test article treated concentrations for each treatment condition were analysed.

2. Chromosomal aberration assay, experiment 2: For experiment 2, a repeat of the above procedure was undertaken, with a three test article treated concentrations analysed in the presence of S9.
A further treatment was undertaken in the absence of S9 (5×10^4 cells) with an extended recovery period of 25 hours (*i.e.* 28 hours after the start of treatment). A single test article treated concentration was analysed.

3. Chromosomal aberration assay, experiment 3: For experiment 3, a 3 hour treatment in the presence of S9 was undertaken, with an extended recovery period of 25 hours employed, as detailed above. A single test article treated concentration was analysed.

4. Harvesting, fixation and slide preparation:

Harvesting and fixation: Two hours before the cells were harvested, mitotic activity was arrested by addition of Colcemid® to each culture at a final concentration of 0.2 µg/mL to inhibit cytokinesis. After 2 hours incubation, each cell suspension was trypsinised, cells were fixed by addition of ice-cold fixative (methanol: acetic acid (3:1 v/v)) for 5 minutes followed by centrifugation (800 rpm). This step was repeated.

Slide preparation: The fixed pellets were re-suspended, then centrifuged and re-suspended in a small volume of fixative. A few drops of the cell suspensions were dropped onto microscope slides and allowed to air dry. Two slides were prepared per culture. The slides were then stained in Giemsa.

Cytotoxicity: The MI, which indicates the ratio of cells in metaphase divided by the total no. of cells observed in a population of cells, was determined using the formula below:

$$MI\ (\%) = \frac{No.\ of\ mitotic\ cells}{Total\ no.\ of\ cells\ scored} \times 100$$

Relative MI (expressed in terms of percentage) for each treated culture was calculated as follows:

$$Relative\ MI\ (\%) = \frac{MI\ of\ treated\ cultures}{MI\ of\ vehicle\ control} \times 100$$

Cytotoxicity (%) was expressed as (100 – Relative MI.
A minimum of 1000 cells/culture were examined for cytotoxicity.

5. Slide scoring: Concentrations were selected for analysis of chromosome aberrations with modal chromosome number of 22. Toxicity was not deemed a limiting factor. Current test guideline requirements consider toxicity a limiting factor, with a reduction in mitotic index of 45±5%. Slides were coded prior to scoring.
Definitions of chromosomal aberrations were taken from ISCN[6].
Chromatid type:
cte = Chromatid exchange
ctb = Chromatid break (an achromatic region in one chromatid, larger than the width of a chromatid. The associated fragment may be partially or completely displaced)
Chromatid gap (an achromatic (unstained) region in one chromatid, the size of which is equal to or smaller than the width of a chromatid. These are noted but not usually included in final totals of aberrations as they may not all be true breaks)
Chromosome-type:
Isochromatid gap (("Chromosome gap"). Same as chromatid gap but at the same locus in both sister chromatids. If the gap is large or chromosome fragment displaced, the break is included with chromosome breaks)
Acentric Fragment (Two parallel chromatids with no evident 'centromere. The fragment can be of any size greater than the width of a chromatid)
Chromosome exchange: dicentric (dicentric with fragment), ring (a chromosome which forms a circle containing a centromere. This is often associated with an acentric fragment in which case it is classed as ring fragment)
csb = Chromosome break (Chromosome has a clear break, forming an abnormal (deleted) chromosome with an acentric fragment that is dislocated but apparently related)

Results and Discussion

A. Analytical determinations:

Not undertaken. Analyses for achieved concentration or homogeneity or stability of test article formulations were not conducted as part of this study, as this is not a requirement of the regulatory test guidelines.

B. Chromosome aberration assay:

1. Chromosomal aberration assay, experiment 1:

3 h + 13 h recovery – S9:

- Following treatment, no precipitate (observed by eye at the end of treatment) was observed at any concentration tested. Exposure to spiroxamine cyclohexanol at concentrations of 10 to 500 µg/mL caused reductions in

%MI to 52% at 100 µg/mL, with no cells in metaphase at concentrations >100 µg/mL. The concentrations selected for metaphase analysis were 10, 60 and 100 µg/mL, with % reductions in MI of 27%, 63% and 52%, respectively. No increase in the incidence of cells with structural aberrations was observed in any analysed treatment concentrations. However, a sufficient level of cytotoxicity (reduction in %MI 40-50%) was not achieved.

- The positive control, MMC induced a statistically significant increase in chromosomal aberrations (excluding gaps), however this was at a level of cytotoxicity that exceeded current test guideline reduction in %MI 40-50%).

3 h + 13 h recovery +S9:

- Following treatment, no precipitate (observed by eye at the end of treatment) was observed at any concentration tested. Exposure to spiroxamine cyclohexanol at concentrations of 10 to 2000 µg/mL caused reductions in %MI to 93% at 500 µg/mL, with no cells in metaphase at concentrations >500 µg/mL. The concentrations selected for metaphase analysis were 50, 250 and 500 µg/mL, with % reductions in MI of 7%, no cytotoxicity and 7%, respectively. No increase in the incidence of cells with structural aberrations was observed in any analysed treatment concentrations. However, a sufficient level of cytotoxicity (reduction in %MI 40-50%) was not achieved.
- The positive control, CPA induced a statistically significant increase in chromosomal aberrations (excluding gaps), however this was at a level of cytotoxicity that exceeded current test guideline reduction in %MI 40-50%).

Table CA 5.8.1/10-1: Spiroxamine cyclohexanol overview of chromosomal aberration data in V79 cells: experiment 1

3 h exposure (+ 13 h recovery) –S9					3 h exposure (+ 13 h recovery) –S9				
Dose level (µg/mL)	**Relative MI (%)**	**Cyto-toxicity (%)**	**Aberrant cells excl. gaps (%)**	**Num. aberrant cells (%)**	**Dose level (µg/mL)**	**Relative MI (%)**	**Cyto-toxicity (%)**	**Aberrant cells excl. gaps (%)**	**Num. aberrant cells (%)**
0[a]	100	-	0.5	---[b]	0[a]	100	-	1.0	---[b]
10	73	27	2.0	---[b]	50	93	7	3.5	---[b]
60	37	63	1.5	---[b]	250	111	-	3.0	---[b]
100	48	52	1.8	---[b]	500	93	7	3.0	---[b]
MMC 0.04	46	54	25.4*	---[b]	CPA 3	47	53	27.5*	---[b]

Laboratory historical control ranges				
Vehicle control 3 h +15 h recovery –S9 (*n* = 19)			**Vehicle control 3 h +15 h recovery +S9 (*n* = 18)**	
	Aberrant cells excl. gaps (%)	**% MI**	**Aberrant cells excl. gaps (%)**	**% MI**
Year	Date range not reported		Date range not reported	
Range: Mean:	0.00 – 7.00 0.97 ±1.12	- 8.38	0.00 – 9.00 1.25 ±1.26	- 6.82
Positive control (MMC) 3 h +15 h recovery –S9 (*n* = 8)			**Positive control (CPA) 3 h +15 h recovery +S9 (*n* = 18)**	
	Aberrant cells excl. gaps (%)	**% MI**	**Aberrant cells excl. gaps (%)**	**% MI**
Year	Date range not reported		Date range not reported	
Range: Mean:	7.50 – 21.50 14.06 ±4.27	- 5.30	12.50 – 38.50 25.41 ±6.60	- 2.94

* $p \leq 0.05$
a ethanol
b data not reported

2. Chromosomal aberration assay, experiment 2:

3 h + 13 h recovery – S9:

- Following treatment, no precipitate (observed by eye at the end of treatment) was observed at any concentration tested. Exposure to spiroxamine cyclohexanol at concentrations of 10, 60 and 100 µg/mL caused reductions in %MI of 19%, 21% and 39%, respectively with all concentrations assessed for metaphase analysis. No increase in the incidence of cells with structural aberrations was observed in any analysed treatment concentrations. However, a sufficient level of cytotoxicity (reduction in %MI 40-50%) was not achieved.
- The positive control, MMC induced a statistically significant increase in chromosomal aberrations (excluding gaps), however this was at a level of cytotoxicity that exceeded current test guideline reduction in %MI 40-50%).

3 h + 13 h recovery +S9:

- Following treatment, no precipitate (observed by eye at the end of treatment) was observed at any concentration tested. Exposure to spiroxamine cyclohexanol at concentrations of 20, 100 and 200 µg/mL caused no cytotoxicity at any of the concentrations assessed, with all concentrations assessed for metaphase analysis. No increase in the incidence of cells with structural aberrations was observed in any analysed treatment concentrations. However, a sufficient level of cytotoxicity (reduction in %MI 40-50%) was not achieved.
- The positive control, CPA induced a statistically significant increase in chromosomal aberrations (excluding gaps), however this was at a level of cytotoxicity that exceeded current test guideline reduction in %MI 40-50%).

Table CA 5.8.1/10-2: Spiroxamine cyclohexanol overview of chromosomal aberration data in V79 cells: experiment 2

3 h exposure (+ 13 h recovery) –S9					3 h exposure (+ 13 h recovery) –S9				
Dose level (µg/mL)	**Relative MI (%)**	**Cyto-toxicity (%)**	**Aberrant cells excl. gaps (%)**	**Num. aberrant cells (%)**	**Dose level (µg/mL)**	**Relative MI (%)**	**Cyto-toxicity (%)**	**Aberrant cells excl. gaps (%)**	**Num. aberrant cells (%)**
0[a]	100	-	0.5	---[b]	0	100	-	1.5	---[b]
10	81	19	0.5	---[b]	20	113	-	2.0	---[b]
60	79	21	1.0	---[b]	100	134	-	0.5	---[b]
100	61	39	0.0	---[b]	200	131	-	0.0	---[b]
MMC 0.03	29	71	19.5	---[b]	CPA 3	39	61	31.5	---[b]
MMC 0.04	40	60	29.5	---[b]	-	-	-	-	-

* $p \leq 0.05$

a ethanol

b data not reported

3. Chromosomal aberration assay, experiment 3:

3 h + 25 h recovery – S9:

- Following treatment, no precipitate (observed by eye at the end of treatment) was observed at the single concentration of 60 µg/mL tested. Exposure to spiroxamine cyclohexanol at this concentrations reduced %MI to 11%, with this single test article treated concentration assessed for metaphase analysis. No increase in the incidence of cells with structural aberrations was observed. However, a sufficient level of cytotoxicity (reduction in %MI 40-50%) was not achieved.
- The positive control, MMC induced a statistically significant increase in chromosomal aberrations (excluding gaps), however this was at a level of cytotoxicity that exceeded current test guideline reduction in %MI 40-50%).

3 h + 25 h recovery +S9:

- Following treatment, no precipitate (observed by eye at the end of treatment) was observed at the single concentration of 200 µg/mL tested. Exposure to spiroxamine cyclohexanol at this concentrations reduced %MI to 44%, with this single test article treated concentration assessed for metaphase analysis. No increase in the incidence of cells with structural aberrations was observed. Whilst an acceptable level of cytotoxicity was achieved, only a single concentration was treated, no assessment of a dose response available.
- The positive control, CPA induced a statistically significant increase in chromosomal aberrations (excluding gaps), however this was at a level of cytotoxicity that exceeded current test guideline reduction in %MI 40-50%.

Table CA 5.8.1/10-3: Spiroxamine cyclohexanol overview of chromosomal aberration data in V79 cells: experiment 3

3 h exposure (+ 25 h recovery) –S9					**3 h exposure (+ 25 h recovery) –S9**				
Dose level (µg/mL)	**Relative MI (%)**	**Cyto-toxicity (%)**	**Aberrant cells excl. gaps (%)**	**Num. aberrant cells (%)**	**Dose level (µg/mL)**	**Relative MI (%)**	**Cyto-toxicity (%)**	**Aberrant cells excl. gaps (%)**	**Num. aberrant cells (%)**
0[a]	100	-	3.5	---[b]	0	100		0.0	---[b]
60	89	11	2.0	---[b]	200	56	44	3.8	---[b]
MMC 0.03	25	75	39.0	---[b]	CPA 3	80	20	14.0	---[b]
Laboratory historical control ranges									
Vehicle control 3 h +25 h recovery –S9 (*n* = 10)					**Vehicle control 3 h +25 h recovery +S9 (*n* = 9)**				
	Aberrant cells excl. gaps (%)		**% MI**			**Aberrant cells excl. gaps (%)**		**% MI**	
Year	Date range not reported				Date range not reported				
Range:	0.00 – 7.00				0.00 – 3.00			-	
Mean:	2.10 ±1.97		7.13		0.56 ±0.97			6.99	
Positive control (MMC) 3 h +25 h recovery –S9 (*n* = 8)					**Positive control (CPA) 3 h +25 h recovery +S9 (*n* = 9)**				
	Aberrant cells excl. gaps (%)		**% MI**			**Aberrant cells excl. gaps (%)**		**% MI**	
Year	Date range not reported				Date range not reported				
Range:	19.50 – 45.50				13.00 – 62.50			-	
Mean:	32.00 ±7.91		4.31		28.33 ±16.82			5.63	

* $p \leq 0.05$

a ethanol b data not reported

D. Deficiencies:

Although the study was conducted according to test guideline OECD 473 (1984), this test guideline has since been updated twice in the intervening periods (1997, 2016). When assessed against current test guideline requirements the following deficiencies are noted:

- Whilst stated in the report that pH and osmolality data following test article formulation additions to culture medium would be used to aid interpretation of the data (*i.e.* to exclude confound factors), no impact statement was provided to conclude on the result. Therefore, it is unclear if these measurement were performed.
  It is unclear why the recovery period of 25 hours was selected for a follow up confirmatory experiment as there is no evidence that spiroxamine cyclohexanol causes cell cycle delay.
- Based on the doubling time of 16.5 hours reported by the laboratory for V79 cells, a recovery period of 15 hours (*i.e.* 18 hours post start of treatment) is an insufficient time period to arrest cells in metaphase following the treatment exposure (*i.e.* 1.5*x* doubling time required, equivalent to 22 hour recovery or 25 hours from the start of treatment). Where an acceptable recovery

period was used, only a single concentration was assessed, without either establishment of a dose response or a sufficient level of cytotoxicity induced.
- No assessment of exposure in the absence of S9 following an extended exposure was undertaken.
- Concerns are raised over the sensitivity and specificity of the assay, with only 100 metaphases scored/culture, compared with test guideline requirements of 150/culture, 300/concentration. This is further impacted with the overtly low background spontaneous chromosome aberrant frequency observed in the vehicle controls
- Toxicity was the limiting factor in both the absence and presence of S9. In both cases a sufficient level of toxicity was not achieved and therefore a complete assessment of spiroxamine cyclohexanol's potential to induce chromosome aberrations has not been sufficiently investigated.
- Concerns over the laboratory's ability to conduct the assay are raised as overt cytotoxicity (*i.e.* ≥50%) was observed for each positive control tested. Therefore the ability for the assay to assess direct acting clastogens was not sufficiently demonstrated, with overt cytotoxicity a contributing factor.
- Limited historical control data were presented, with mean and observed ranges only for the vehicle and positive control data without a confidence interval applied (ideally 95%). No date range is provided for the historical control data presented, therefore it is not understood the applicability of the data to aid in the test article clastogenic potential and assay acceptability.
- Although not a deficiency of the test guideline, numerical aberrations were only qualitatively assessed in this assay, however no data were presented.

For the reasons listed above, this study is deemed supplementary. Both the clastogenic and aneugenic endpoints have been adequately addressed with a robust, GLP up to date *in vitro* human peripheral blood lymphocyte micronucleus study (CA 5.8.1/23 [M-755227-03-1]).

Assessment and conclusion by applicant:

Assessment: For the reasons identified in the deficiencies section this study is deemed supplementary.

Conclusion: It is concluded that spiroxamine cyclohexanol did not induce biologically relevant increases in the incidence of chromosome aberrations in Chinese hamster V79 cells. These conditions included treatments that were neither limited by toxicity (*i.e.* reduction in % relative MI 45±5%) or precipitate in the absence (3 hours) and presence (3 hours) of rat liver metabolic activation system with recovery periods of 15 and 25 hours in both treatment conditions.

Data Point:	KCA 5.8.1/23
Report Author:	[redacted]
Report Year:	2020
Report Title:	Spiroxamine cyclohexanol: In vitro human lymphocyte micronucleus assay
Report No:	8406984
Document No:	M-755227-02-1
Guideline(s) followed in study:	OECD 487 (2016)
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

Spiroxamine cyclohexanol was tested in an *in vitro* micronucleus assay using duplicate human lymphocyte cultures prepared from the pooled blood of two male donors in two experiments. Treatments covering a broad range of concentrations, separated by narrow intervals, were performed both in the absence and presence of metabolic activation (S9) from Aroclor 1254-induced rats. The test article was formulated in anhydrous analytical grade dimethyl sulphoxide (DMSO) and the highest concentration tested in the micronucleus experiment, 230 µg/mL (limited by toxicity), was determined following a preliminary cytotoxicity range-finder experiment. All test article concentrations, formulated in DMSO were dosed into the test system at 1% v/v.

Following establishment of cultures, concentrations ranging from 120 to 320 µg/mL in the absence (3 hours + 21 hour recovery), 100 to 280 µg/mL in the presence of S9 (3 h + 21 h) and 10 to 200 µg/mL in the extended treatment in the absence of S9 (24 h + 24 h). The test article concentrations for micronucleus analysis were selected by evaluating the effect of spiroxamine cyclohexanol on the replication index (RI). Micronuclei were analysed at three concentrations and a summary of the data are presented below.

The frequency of MNBN cells in vehicle controls fell within the normal ranges. The positive control chemicals induced statistically significant increases in the proportion of MNBN cells. Both replicate cultures at the positive control concentration analysed under each treatment condition demonstrated MNBN cell frequencies that clearly exceeded the normal range, thereby demonstrating the sensitivity and specificity of the assay. A minimum of 50% of cells had gone through at least one cell division (as measured by binucleate + multinucleate cell counts) in vehicle control cultures at the time of harvest. The maximum concentration analysed under each treatment condition met the criteria. The assay data were therefore considered valid and acceptable.

Treatment of cells with spiroxamine cyclohexanol for 3 hours (+21 hour recovery) in the absence and presence of S9 resulted in MNBN cells that were similar to and not significantly ($p \leq 0.05$) higher than those observed in concurrent vehicle control cultures for all concentrations analysed.

Treatment of cells with spiroxamine cyclohexanol for 24 hours (+24 hour recovery) in the absence of S9 resulted in frequencies of MNBN cells that were significantly ($p \leq 0.001$) higher than those observed in concurrent vehicle control cultures for the highest concentration analysed (100 µg/mL, inducing 53% cytotoxicity. The MNBN cell frequency of both test article treated cultures marginally (0.95%, 1.05%) exceeded the normal range (0.00 – 0.90%), with statistically significant linear trend test, indicating evidence of a weak linear trend.

A confirmatory experiment was performed in order to confirm reproducibility and biological relevance of the small increases in MN-BN cells observed only at the highest concentration analysed following 24 hour (+24 hour recovery) treatment in the absence of S9.

In the confirmatory 24 hours (+24 hour recovery) in the absence of S9, cells treated with spiroxamine cyclohexanol in frequencies of MN-BN cells that were similar to and not significantly ($p \leq 0.05$) higher than those observed in concurrent vehicle control cultures for the majority of concentrations analysed. A single exception to this was the intermediate concentration (80 µg/mL, inducing 35% cytotoxicity) where a weak but statistically significant ($p \leq 0.05$) MN-BN cell frequency was observed. However, this increase was small such that, the MN-BN cell frequency of both test article treated cultures at this concentration (80 µg/mL) and the remaining cultures at the lowest and the highest concentrations analysed fell within the normal range. Furthermore, there was no evidence of any concentration-related effect (non-significant linear trend test). Therefore, the small statistical increase observed at the intermediate concentration was not considered of any biological relevance.

The marginal effect seen at 100 µg/mL (inducing 53% cytotoxicity) in Experiment 1 was not reproduced in Experiment 2 at 105 µg/mL (inducing 51% cytotoxicity). According to the current data interpretation strategy, weak non-reproducible increases may be considered of little to no genotoxicity concern. Therefore, it can conclude that the response observed in Experiment 1 was non-reproducible and not biologically relevant

It was concluded that spiroxamine cyclohexanol did not induce biologically relevant increases in the frequency of micronuclei in human peripheral blood lymphocytes following treatment in the absence or presence of an Aroclor-induced rat liver metabolic activation system (S9). Maximum concentrations analysed were limited by cytotoxicity, consistent with recommendations in the current regulatory test guidelines for the *in vitro* micronucleus assay.

Materials and Methods

A. Materials:

1. Test Material:	Spiroxamine cyclohexanol (alternative name: [(4-tert-butylcyclohexanol, M13)
Description:	White powder
Lot/Batch No.:	AE 1247868-PU-01
Purity:	98.2% (no correction factor applied)
CAS No.:	98-52-2
Stability of test compound:	Confirmed stable for the duration of the study (expiry date: 20 January 2021)
2. Control materials:	
Negative:	
Solvent/final concentration:	DMSO (dimethyl sulphoxide)/1% (v/v)
Positive: -S9	Mitomycin C (MMC, 3 h: 0.3; 24 h : 0.20 µg/mL) [clastogenic control] Vinblastine (VIN, 24 h: 0.04 µg/mL) [aneugenic control]
Positive: +S9	Cyclophosphamide (CPA, 3 h: 7 µg/mL)
3. Activation:	S9[16] was purchased from a commercial source. ♂ Sprague Dawley rats were treated with Aroclor 1254 (supplied by [redacted] lot no.: 4030, protein content 3.3 mg/mL). The composition of the 10% S9 reaction mix was: 100 µL S9, Na PBS (100 µM), glucose-6-phosphate (5 µM), β-NADP (4 µM), $MgCl_2$ (8 µM), KCl (33 µM), water (to volume).
4. Test organisms:	Human peripheral blood lymphocytes were collected from 2 healthy, non-smoking adult donors aged between 23 and 30 years, pooled and diluted with HML media. Cells were stimulated to divide by the addition of phytohaemagglutinin (PHA).

16 In this study, S9 activity was evaluated with CPA. In addition, each batch was checked by the manufacturer of S9 for sterility, protein content, ability to convert ethidium bromide, cyclophosphamide, B[a]P and AAN to bacterial mutagens, and cytochrome P-450-catalysed enzyme activities (alkoxyresorufin-*O*-dealkylase activities). Therefore, there is no concern over S9 activity.

5. Culture medium: HEPES buffered RPMI supplemented with 10% (v/v) inactivated fetal calf serum, 0.52% penicillin-streptomycin cell culture.

6. Test article Concentrations:

a) Preliminary cytotoxicity test: 3 h (+21 h recovery) -/+S9; 24 h (+24 h) –S9: 0, 5.671, 9.451, 15.75, 26.25, 43.75, 72.92, 121.5, 202.6, 337.6, 562.7, 937.8, 1563 µg/mL (maximum recommended concentration, equivalent to 10 mM)

b) Micronucleus assay: 3 h (+21 h recovery) -S9: 0, 100, 120, 140, 160, 180, 190, 200, 210, 220, 240, 260, 280, 300, 320 µg/mL

3 h (+21 h recovery) +S9: 0, 100, 120, 140, 160, 180, 190, 200, 210, 220, 230, 240, 250, 260, 280 µg/mL

24 h (+24 h) –S9: 0, 10, 20, 40, 60, 70, 75, 80, 85, 90, 95, 100, 110, 125, 150, 175 200 µg/mL

Confirmatory 24 h (+24 h) –S9: 0, 20, 40, 60, 80, 90, 95, 100, 105, 110, 115, 120, 130, 140, 150 µg/mL (concentrations underlined scored for micronucleus frequency)

B. Test Performance:

1. In life dates: 3 June 2019 to 20 September 2019 (experimental dates)

2. Vehicle selection: A preliminary solubility test confirmed spiroxamine cyclohexanol was soluble in DMSO up to at least 102.34 mg/mL (not corrected for purity).

The solubility limit in culture medium was in the range of 255.85 to 511.7 µg/mL, as indicated by precipitation at the higher concentration which persisted for at least 24 hours after test article addition. A maximum concentration of 1563 µg/mL was selected for the cytotoxicity Range-Finder Experiment, in order that treatments were performed up to the maximum recommended concentration according to current regulatory guidelines (*i.e.* a concentration equivalent to 10 mM). Concentrations for the micronucleus experiment were selected based on the results of the cytotoxicity range-finder experiment.

Test article stock solutions were prepared by formulating spiroxamine cyclohexanol under subdued lighting in DMSO with the aid of vortex mixing and warming at 37°C, to give the maximum required treatment solution concentration. Subsequent dilutions were made using DMSO. The test article solutions were protected from light and used within approximately 4 h of initial formulation.

3. Statistics: The proportion of MNBN cells for each treatment condition were compared with the proportion in vehicle controls by using Fisher's exact test. A Cochran-Armitage trend test was applied to each treatment condition. Probability values of $p < 0.05$ were accepted as significant.

4. Acceptance criteria: The following acceptance criteria had to be met for assay acceptability:

1. The binomial dispersion test demonstrated acceptable heterogeneity (in terms of MNBN cell frequency) between replicate cultures, particularly where no positive responses were seen;
2. The frequency of MNBN cells in vehicle controls fell within the current 95th percentile of the observed historical vehicle control (normal) ranges;
3. The positive control chemicals induced statistically significant increases in the proportion of cells with micronuclei. Both replicate cultures at the positive control concentration analysed under each treatment condition demonstrated MNBN cell frequencies that clearly exceeded the normal range;
4. A minimum of 50% of cells had gone through at least one cell division (as measured by binucleate + multinucleate cell counts) in vehicle control cultures at the time of harvest;
5. The maximum concentration analysed under each treatment condition met the specified criteria (*i.e.* the highest concentration selected for

micronucleus analysis following all treatment conditions was one at which 50-60% cytotoxicity was achieved).

5. Evaluation criteria:

For valid data, the test article was considered to induce clastogenic and/or aneugenic events if:

1. A statistically significant increase in the frequency of MNBN cells at one or more concentrations was observed;
2. An incidence of MNBN cells at such a concentration that exceeded the normal range in both replicates was observed;
3. A concentration-related increase in the proportion of MNBN cells was observed (positive trend test).

The test article was considered positive in this assay if all of the above criteria were met.

The test article was considered negative in this assay if none of the above criteria were met.

C. Methods:

1. Homogeneity and achieved concentration analysis of the dose:

Not undertaken

2. Preliminary cytotoxicity assay:

Whole blood cultures were established by placing 0.4 mL of pooled heparinised blood into 8.5 mL pre-warmed HEPES-buffered RPMI medium containing 10% (v/v) heat inactivated fetal calf serum and 0.52% penicillin/streptomycin, so that the final volume following addition of S9 mix/KCl and the test article in its chosen vehicle was 10 mL. The mitogen, phytohaemagglutinin (PHA, reagent grade) was included in the culture medium at a concentration of approximately 2% of culture to stimulate the lymphocytes to divide. Blood cultures were incubated at 37±1°C for approximately 48 hours and rocked continuously. S9 mix or KCl (1 mL/culture) was added appropriately. Cultures were treated with the test article or vehicle (0.1 mL/culture). Positive control treatments were not included. Duplicate cultures were used for the vehicle control and single cultures were used for each test article treated concentration.

For removal of the test article, cells were pelleted (approximately 300 g, 10 minutes, washed twice with sterile saline and resuspended in fresh pre-warmed medium containing fetal calf serum and penicillin/streptomycin. Cyto-B (formulated in DMSO) was added to post wash-off culture medium to give a final concentration of 6 µg/mL/culture to inhibit cytokinesis, resulting in binucleate cells (without effecting karyokinesis), thereby arresting cells in interphase.

Changes in osmolality of more than 50 mOsm/kg and fluctuations in pH of more than one unit may be responsible for an increase in chromosome aberrations. Osmolality and pH measurements on post-treatment incubation medium were taken in the cytotoxicity range-finder experiment.

Cytotoxicity was determined by examination of the proportion of mono, bi and multinucleate cells to a minimum of 500 cells/concentration. From this, the replication index (RI) was calculated for each concentration (see Cytotoxicity section below).

3. Micronucleus assay:

Cell treatment: Cells were exposed to the test article formulation, vehicle or positive control as described above, with the exception of quadruplicate cultures were used for the vehicle control and duplicate controls used for the positive controls and each test article concentration. Concentrations selected for micronucleus analysis were assessed for cytotoxicity uncoded and for the proportions of mono-, bi- and multinucleate cells to a minimum of 200 or 500 cells/culture (preliminary cytotoxicity range finder or micronucleus experiment, respectively)./

Spindle inhibitor: Cyto-B was added post-wash to cultures to inhibit cytokinesis.

Slide preparation: Slides were prepared by spreading the fixed cultures on clean slides. The slides were stained with acridine orange (12.5 µg/mL) dropped on to slides, coverslipped and scored prior to analysis.

Cytotoxicity: The replication index (RI), which indicates the relative number of nuclei compared to controls, was determined using the formula below:

$$RI = \frac{\textit{no. of binucleate cells + 2(no. of multinucleate cells)}}{\textit{total no. of cells in treated cultures}}$$

Relative RI (expressed in terms of percentage) for each treated culture was calculated as follows:

$$\textit{Relative RI (\%)} \frac{\textit{RI of treated cultures}}{\textit{RI of vehicle control}} \times 100$$

Cytotoxicity (%) was expressed as (100 – Relative RI).

Micronucleus assessment: Slides from the highest selected concentration and two lower concentrations were taken for microscopic analysis.

Immediately prior to analysis, 1-2 drops of PBS were added to the slides before mounting with glass coverslips. One thousand binucleate cells from each culture (total of 2000 binucleate cells/concentration) were analysed for micronuclei. The number of cells containing micronuclei and the number of micronuclei/cell on each slide were noted.

For the 24 hour (+24 hour recovery) treatment condition in the absence of S9 in Micronucleus Experiment 1, it was subsequently deemed necessary to analyse the two remaining vehicle control cultures, to aid data interpretation.

Nucleoplasmic bridges (NPBs) between nuclei in binucleate cells were recorded during micronucleus analysis to provide an indication of chromosome rearrangement. Various mechanisms may lead to NPB formation following DNA misrepair of strand breaks in DNA in this assay, binucleate cells with NPBs were recorded as part of the micronucleus analysis.

Results

A. Analytical determinations:

Not undertaken.

B. Preliminary cytotoxicity assay:

Test article precipitate was observed at concentrations of 202.6 µg/mL and above in both short term treatments, with no scorable cells present at concentrations of 202.6 and 337.6 µg/mL and above in the extended and short term treatments, respectively.

No marked changes in osmolality or pH were observed at the highest three concentrations tested as compared to the concurrent vehicle controls (individual data not reported).

The results of the cytotoxicity preliminary cytotoxicity range-finder experiment were used to select suitable maximum concentrations for the micronucleus experiment.

Table CA 5.8.1/23-1: Spiroxamine cyclohexanol: human lymphocyte preliminary cytotoxicity range finder experiment

Conc. (µg/mL)	3 h (+ 21 h recovery) –S9		3 h (+ 21h recovery) +S9		24 h (+ 24 h recovery) –S9	
	Replication index (RI)	Cytotoxicity based on RI (%)	Replication index (RI)	Cytotoxicity based on RI (%)	Replication index (RI)	Cytotoxicity based on RI (%)
0	0.69	-	0.76	-	1.06	-
5.67	0.75	0	0.69	9	1.07	0
9.451	0.73	0	0.77	0	1.14	0
15.75	0.74	0	0.75	1	1.10	0
26.25	0.74	0	0.65	14	0.97	8

Conc. (µg/mL)	3 h (+ 21 h recovery) –S9		3 h (+ 21h recovery) +S9		24 h (+ 24 h recovery) –S9	
	Replication index (RI)	Cytotoxicity based on RI (%)	Replication index (RI)	Cytotoxicity based on RI (%)	Replication index (RI)	Cytotoxicity based on RI (%)
43.75	0.70	0	0.81	0	0.93	12
72.92	0.74	0	0.71	6	0.74	30
121.5	0.63	9	0.71	5	0.20	82
202.6	0.26 P-ppt	62	0.30 P-ppt	60	NE, P-ppt	-
337.6	P-ppt	-	P-ppt	-	E-ppt, H-ppt, P-pptt	-
562.7	E-ppt, P-ppt	-	E-ppt, P-ppt	-	E-ppt, H-ppt, P-pptt	-
937.8	E-ppt, P-ppt	-	E-ppt, P-ppt	-	E-ppt, H-ppt, P-pptt	-
1563	E-ppt, H-ppt, P-pptt	-	E-ppt, H-ppt, P-pptt	-	E-ppt, H-ppt, P-pptt	-

NE: not evaluated due to no scorable cells

ppt: precipitate observed at treatment
E-ppt: precipitate observed at end of treatment
H-ppt: precipitate observed at harvest

C. Micronucleus assay:

1. Assay acceptability: The frequency of MNBN cells in vehicle controls fell within the normal ranges. The positive control chemicals induced statistically significant increases in the proportion of MNBN cells. Both replicate cultures at the positive control concentration analysed under each treatment condition demonstrated MNBN cell frequencies that clearly exceeded the normal range, thereby demonstrating the sensitivity and specificity of the assay. A minimum of 50% of cells had gone through at least one cell division (as measured by binucleate + multinucleate cell counts) in vehicle control cultures at the time of harvest. The maximum concentration analysed under each treatment condition met the criteria. The assay data were therefore considered valid and acceptable.

2. Short term treatment in the absence and presence of S9: Treatment of cells with spiroxamine cyclohexanol for 3 hours (+21 hour recovery) in the absence and presence of S9 resulted in MNBN cells that were similar to and not significantly ($p \leq 0.05$) higher than those observed in concurrent vehicle control cultures for all concentrations analysed.

Table CA 5.8.1.23-2: Spiroxamine cyclohexanol: human lymphocyte micronuclei assay: 3 h (+ 21 h recovery) –S9 treatment and laboratory historical control data

Conc. (µg/mL)		3 h (+ 21 h recovery) –S9				Vehicle historical control ranges (♂ donor)	
		Total BN	Total MN-BN	Frequency of MN-BN (%)	Cyto. (%)	Feb 17 – Feb 18	% MNBN
Vehicle	A	1000	4	0.40		No. of expts	17
	B	1000	5	0.50		Number of cultures	40
Total		2000	9	Mean: 0.45	-	Mean ±SD	0.40 ±0.35
100	A	1000	4	0.40		min. – max.	0.00 – 0.80
	B	1000	7	0.70		95% reference range	0.00 – 0.70
Total		2000	11	Mean: 0.55	Mean: 15		
150	A	1000	5	0.50			
	B	1000	3	0.30			
Total		2000	8	Mean: 0.40	Mean: 34		
210	A	1000	5	0.50			
	B	1000	3	0.30			
Total		2000	8	Mean: 0.40	Mean: 43		
Linear trend: p 0.6730							
MMC (0.2)	A	1000	58	5.80			
	B	1000	42	4.20			
Total		2000	100	Mean: 5.00***	Mean: 33		

Positive historical control ranges (♂ donor)	
MMC (0.3 µg/mL)	
Feb 17 – Nov 17	% MNBN
No. of expts	22
Number of cultures	40
Mean ±SD	5.57 ±1.74
min. – max.	1.50 – 9.20
95% reference range	2.57 – 8.52

*** $p<0.001$
No test article related increases in cells with NPBs were observed (data not reported)

Table CA 5.8.1/23-3: Spiroxamine cyclohexanol: human lymphocyte micronuclei assay: 3 h (+ 21 h recovery) +S9 treatment and laboratory historical control data

Conc. (µg/mL)		3 h (+ 21 h recovery) +S9			
		Total BN	Total MN-BN	Frequency of MN-BN (%)	Cyto. (%)
Vehicle	A	1000	5	0.50	
	B	1000	5	0.50	
Total		2000	10	Mean: 0.50	-
120	A	1000	5	0.50	
	B	1000	0	0.00	
Total		2000	5	Mean 0.25:	Mean: 10
180	A	1000	4	0.40	
	B	1000	2	0.20	
Total		2000	6	Mean: 0.30	Mean: 27
230	A	1000	5	0.50	
	B	1000	5	0.50	
Total		2000	10	Mean: 0.50	Mean: 53[ppt]
Linear trend: p 0.4679					
CPA (7.0)	A	1000	19	1.90	
	B	1000	21	2.10	
Total		2000	40	Mean: 2.00[***]	Mean: 44

Vehicle historical control ranges (♂ donor)	
Feb 17 – Feb 18	% MNBN
No. of expts	16
Number of cultures	40
Mean ±SD	0.46 ±0.36
min. – max.	0.00 – 1.00
95% reference range	0.10 – 0.90

Positive historical control ranges (♂ donor)	
CPA (3 µg/mL)[1]	
Feb 17 – Dec 17	% MNBN
No. of expts	22
Mean:	40
Mean ±SD	2.21 ±0.85
min. – max.	1.00 – 4.70
95% reference range	1.00 – 3.63

*** $p<0.001$
ppt precipitate observed at the end of treatment
There is currently no historical control range for CPA 2 µg/mL, the concentration analysed in this study, therefore the range for the highest CPA concentration normally analysed (3 µg/mL) has been included for comparative purposes
No test article related increases in cells with NPBs were observed (data not reported)

3. Extended treatment in the absence of S9:

Treatment of cells with spiroxamine cyclohexanol for 24 hours (+24 hour recovery) in the absence of S9 resulted in frequencies of MNBN cells that were significantly ($p\leq0.001$) higher than those observed in concurrent vehicle control cultures for the highest concentration analysed (100 µg/mL, inducing 53% cytotoxicity. The MNBN cell frequency of both test article treated cultures marginally (0.95%, 1.05%) exceeded the normal range (0.00 – 0.90%), with statistically significant linear trend test, indicating evidence of a weak linear trend.

A confirmatory experiment was performed in order to confirm reproducibility and biological relevance of the small increases in MNBN cells observed only at the highest concentration analysed following 24 hour (+24 hour recovery) treatment in the absence of S9.

Table CA 5.8.1/23-3: Spiroxamine cyclohexanol: human lymphocyte micronuclei assay: 24 h (+ 24 h recovery) -S9 treatment and laboratory historical control data

Conc. (µg/mL)		24 h (+ 24 h recovery) –S9			
		Total BN	Total MN-BN	Frequency of MN-BN (%)	Cyto. (%)
Vehicle	A	1000	12	0.60	
	B	1000	5	0.25	
	C	1000	4	0.40	
	D	1000	5	0.50	
Total		4000	26	Mean: 0.43	-
40	A	2000	12	0.60	
	B	2000	11	0.55	
Total		4000	23	Mean: 0.58	Mean: 7

Vehicle historical control ranges (♂ donor)	
Jul 17 – Jan 18	% MNBN
No. of expts	16
Number of cultures	40
Mean ±SD	0.34 ±0.23
min. – max.	0.00 – 0.90
95% reference range	0.00 – 0.80

80	A	2000	7	0.35	
	B	2000	5	0.25	
Total		4000	23	Mean: 0.30	Mean: 29
95	A	2000	12	0.60	
	B	2000	11	0.55	
Total		4000	40	Mean: 0.58	Mean: 41
100	A	2000	19	0.95$^{>HC}$	
	B	2000	21	1.05$^{>HC}$	
Total		4000	23	Mean: 1.00	Mean: 53
Linear trend: p 0.0010					
VIN (0.04)	A	2000	80	8.00	
	B	2000	83	83.0	
Total		4000	163	Mean: 8.15	Mean: 74

Positive historical control ranges (♂ donor)	
VIN (0.04 µg/mL)	
Feb 17 – Jan 18	% MNBN
No. of expts	20
Mean:	4.1
Mean ±SD	6.43 ±2.38
min. – max.	2.50 – 13.60
95% reference range	2.80 – 13.50

*** $p<0.001$
>HC: exceeds historical control
No test article related increases in cells with NPBs were observed (data not reported)

4. Extended treatment in the absence of S9, confirmatory experiment:

Treatment of cells for 24 hours (+24 hour recovery) in the absence of S9 resulted in frequencies of MNBN cells that were similar to and not significantly ($p\leq0.05$) higher than those observed in concurrent vehicle control cultures for the majority of concentrations analysed. A single exception to this was the intermediate concentration (80 µg/mL; inducing 33% cytotoxicity) where a weak but statistically significant ($p\leq0.05$) MNBN cell frequency was observed. However, this increase was small such that, the MNBN cell frequency of both test article treated cultures at this concentration (80 µg/mL) and the remaining cultures at the lowest and the highest concentrations analysed fell within the normal range. Furthermore, there was no evidence of any concentration-related effect (non-significant linear trend test). Therefore, the small statistical increase observed at the intermediate concentration was not considered of any biological relevance.

The marginal effect seen at 100 µg/mL (inducing 53% cytotoxicity) in Experiment 1was not reproduced in Experiment 2 at 105 µg/mL (inducing 51% cytotoxicity). According to the current data interpretation strategy (Thybaud *et al.*[17]), weak non-reproducible increases may be considered of little to no genotoxicity concern. Therefore, it can conclude that the response observed in Experiment 1 was non-reproducible and not biologically relevant.

Table CA 5.8.1/23-4: Spiroxamine cyclohexanol: human lymphocyte micronuclei assay: 24 h (+ 24 h recovery) –S9 treatment, micronucleus experiment 2 and laboratory historical control data

Conc. (µg/mL)		24 h (+ 24 h recovery) –S9				Vehicle historical control ranges (♂ donor)	
		Total BN	Total MN-BN	Frequency of MN-BN (%)	Cyto. (%)	Jul 17 – Jan 18	% MNBN
Vehicle	A	1000	2	0.20		No. of expts	16
	B	1000	2	0.20		Number of cultures	40
Total		2000	4	Mean: 0.20	-	Mean ±SD	0.34 ±0.23
40	A	1000	7	0.70		min. – max.	0.00 – 0.90
	B	1000	4	0.40		95% reference range	0.00 – 0.80
Total		2000	11	Mean: 0.55	Mean: 6	Positive historical control ranges (♂ donor)	
80	A	1000	4	0.40		MMC (0.2 µg/mL)	
	B	1000	8	0.80			

17 Thybaud V, Aardema M, Clements J, Dearfield K, Galloway S, Hayashi M, Jacobson-Kram D, Kirkland D, MacGregor J T, Marzin D, Ohyama W, Schuler M, Suzuki H and Zeiger E (2007). Strategy for genotoxicity testing: Hazard identification and risk assessment in relation to *in vitro* testing. *Mutation Research* **627**, pp 41-58

Total		2000	12	Mean: 0.60	Mean: 35	**Dec 14 – Jan 18**	**% MNBN**
105	A	1000	4	0.40		No. of expts	16
	B	1000	4	0.40		Number of cultures	29
Total		2000	8	Mean: 0.40	Mean: 51	Mean ±SD	31.18 ±10.58
Linear trend: p 0.1623						min. – max.	16.35 – 52.50
MMC	A	1000	385	38.50		95% reference range	n/a
(0.20)	B	1000	397	39.70		**VIN (0.04 µg/mL)**	
Total		2000	781	Mean: 39.10***	Mean: 42	**Feb 17 – Jan 18**	**% MNBN**
VIN	A	1000	39	3.90		No. of expts	20
(0.04)	B	1000	48	4.80		Number of cultures	41
Total		2000	87	Mean: 4.35***	Mean: 48	Mean ±SD	6.43 ±2.38
						min. – max.	2.50 – 13.60
						95% reference range	2.80 – 13.50

*** p<0.001
>HC: exceeds historical control
For VIN historical control range, refer to Table CA 5.8.1/22-5

D. Deficiencies:

Whilst not deemed a deficiency, it is considered prudent to detail the rationale for undertaking an extended treatment which included a recovery period.

Following revision of the OECD 487 TG in 2014 the cell treatment and harvest times have been somewhat simplified with emphasis placed on performing the extended treatment without recovery, with sampling times or recovery times extended by up to a further 1.5-2.0 normal cell cycles where justified.

Given the purpose for the *in vitro* micronucleus assay is to determine both clastogenic and aneugenic potential, the assay is compromised if an extended treatment with recovery is not included for sensitive detection of certain chemical classes.

Average generation times and replication index data demonstrated that the human peripheral blood lymphocytes were actively dividing beyond 96 hours, indicating that the extended treatment with recovery within the assay system did not compromise the results. This is directly in contrast to the statement in the OECD 487 test guideline, which includes the statement '*for lymphocytes exponential growth may be declining at 96 hours following stimulation and monolayer cultures of cells may become confluent*'. This statement in the test guideline is not supported by any published data, but rather a throw away statement and may provide the 'get out clause' why testing laboratory's did not use this approach as they may have had reduction in growth at 96 hours.

The extended treatment with recovery allows adequate detection of chemicals that either act on a narrow portion of the cell cycle (e.g. aneugenic compounds, G2/metaphase) and/or that induce cell cycle delay or require two rounds of DNA replication. The extended treatment without recovery in the micronucleus assay may be seen as somewhat compromised. It is widely known that direct acting clastogens such as azidothymidine and cytosine arabinoside are negative in the *in vitro* micronucleus assay with the 24 + 0 h approach, but positive with the with 24+24 h protocol. Therefore, the extended treatment with recovery is deemed more sensitive and in accordance with the latest published data (refer to Whitwell *et al.*, 2019[8]), with the OECD test guideline to be updated to reflect this change.

Assessment and conclusion by applicant:

Assessment: Study meets the current guidance and the requirements in 283/2013.

Conclusion: It was concluded that spiroxamine cyclohexanol did not induce biologically relevant increases in the frequency of micronuclei in human peripheral blood lymphocytes following treatment in the absence or presence of an Aroclor-induced rat liver metabolic activation system (S9). Maximum concentrations analysed were limited by cytotoxicity, consistent with recommendations in the current regulatory test guidelines for the *in vitro* micronucleus assay.

Data Point:	KCA 5.8.1/11
Report Author:	[redacted]
Report Year:	1999
Report Title:	4-tert.-Butylcyclohexanol - A 28-day subacute oral toxicity study (gavage) in the rat
Report No:	SA-98/0210
Document No:	M-471106-01-1
Guideline(s) followed in study:	Commission Directive 96/54/EEC, Part B, Method B.7; OECD 407
Deviations from current test guideline:	Yes Although the study was conducted according to test guideline OECD 407 (1995), this test guideline has since been updated in the intervening period (2008). When assessed against current test guideline requirements the following deficiencies are noted: Whilst not a requirement, the test guideline makes reference to determination of serum thyroid hormones (T3, T4, TSH). These were not analysed. However, the gold standard to assess thyroid effects is histopathological analysis. This was undertaken on all animals and revealed no histopathological findings. Consequently the lack of serum thyroid hormone analysis is not deemed critical, with potential thyroid effects adequately addressed with histopathological analysis.
Previous evaluation:	yes, evaluated and accepted RAR (2010)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

In this study, spiroxamine cyclohexanol was administered orally *via* gavage to groups of rats for a period of 28 days. Animals (5/sex/group), were administered test article concentrations at 0, 50, 150, 300 mg/kg bw/day once daily, employing a dose volume of 5 ml/kg bw. Additional control and high dose groups were included, dosed for 28 days, with a 14-day recovery to assess for reversible effects post dosing.

Following 28 days of treatment (or 14 days recovery) 5 animals/sex were subjected to complete necropsy. Body weight, water and food consumption were measured at regular intervals, with FOB and motor activity included. Clinical pathology evaluations (haematology, clinical chemistry and urinalysis) were performed with all surviving animals subjected to complete gross necropsy and full histopathology.

Treatment-related effects were seen in respective of clinical signs in both the mid and high dose groups with moderate to severe effects observed (manifest as prone and squatting positon, straub tail and vocalisation). This was in part attributed to the difficulty in dosing the test article, with defensive clinical signs observed. These effects were apparent *ca.*15 minutes post dosing. These effects were not apparent in the recovery phase. Consequently the functional observational battery, which was conducted in the after, following morning dosing revealed clinical abnormalities in the high dose group animals. These abnormalities included severe defence against application of test article prior to dosing, aggressiveness against touching during weekly open field observations. The stress connection to dosing is further supported by statistically significant increases in relative adrenal glands weights, without associated histopathology. These increased organ weights are suggestive of increased adrenal activity due to the stress of dosing, rather than a direct endocrine related effect, which again is further evidenced by the unscheduled deaths of two high dose group males due to a dosing error.

No test article related histopathological lesions, relevant to humans were observed. High dose group males showed an increase in eosinophilic hyaline droplets in the epithelial cell cytoplasm of the proximal

tubules. Hyaline droplet nephropathy is due to the accumulation of α2μ-globulin which is known to be a lesion specific to ♂ rats with no relevance to humans. It however is acknowledge that Azan specific staining used to confirm α2μ-globulin presence was not undertaken.

Under the conditions of this study, the NOAEL following 28 days of dosing *via* oral gavage is deemed to be 50 mg/kg bw/day for males/females based on reductions in body weight and body weight gain (males) and moderate to severe clinical effects following dosing with a peak period approximately 15 minutes post dosing.

Materials and Methods

A. Materials:

1. Test Material:	Spiroxamine cyclohexanol (alternative name: 4-tert-butylcyclohexanol, M28)
Description:	Solid substance with weak camphor smell
Lot/Batch No.:	M00190
Purity:	99.0% (w/w) (correction for purity not undertaken)
CAS No.:	98-52-2
Stability of test compound:	Confirmed stable for the duration of the study (expiry date: March 1998)
2. Vehicle and/or positive control:	Corn oil / not relevant
3. Test animals:	
Species:	Rat
Strain:	Wistar
Age at dosing:	6-8 wks
Weight at dosing:	♂: 129-145g ♀: 120-138g
Source:	[redacted]
Acclimation period:	At least 5 days
Diet:	Ssniff R 10 diet pellet form (Ssniff Spezialfutter GmbH, Germany) *ad libitum*
Water:	Municipal water, *ad libitum*
Housing:	Housed 5/sex/cage
4. Environmental conditions:	
Temperature:	22 ±3°C
Humidity:	55 ±15%
Air changes:	*ca* 10/h
Photoperiod:	12-hour light/dark

B. Study Design:

1. In life dates:	6 April 1998 to 26 May 1998 (experimental dates)
2. Animal assignment and treatment:	After an acclimatisation period rats were allocated to groups by random permutation table approach. Dose levels selected based on the results from a range-finder study which was reported separately and not available for review. The test article, spiroxamine cyclohexanol was administered orally *via* gavage to groups of rats for a period of 28 days. Animals (5/sex/gp), were administered test article concentrations at 0, 50, 150, 300 mg/kg bw/day once daily, employing a dose volume of 5 mL/kg bw. Additional control and high dose groups were included, dosed for 28 days, with a 14-day recovery to assess for reversible effects post dosing. Following 28 days of treatment (or 14 days recovery) 5 animals/sex were subjected to complete necropsy. Body weight, water and food consumption were

measured at regular intervals, with FOB and motor activity included. Clinical pathology evaluations (haematology, clinical chemistry and urinalysis) were performed with all surviving animals subjected to complete gross necropsy and full histopathology.

3. Statistics: FOB, motor activity, absolute body weights / body weight changes, organ weights, differential blood counts and urine analysis: non-parametric analysis (Kruskal Wallis test). In case of significance, a pair wise comparison between the control and dose groups was undertaken (Wilcoxon, Mann and Whitney U-test).

Haematological data, serum clinical chemistry data: ANOVA incorporating Bartlett's test for homogeneity of variance. If ANOVA was significant, group means were compared by Scheffe test.

Table CA 5.8.1/11-1: Overview of sub-acute toxicity study in rats treated orally (*via* gavage) with spiroxamine cyclohexanol: study design and dose received

Parameters	♂ (mg/kg bw/d)				♀ (mg/kg bw/d)			
	0	50	150	300	0	50	150	300
Animals assigned/sex	5	5	5	5	5	5	5	5
No. of daily doses	28	28	28	28	28	28	28	28
Animals assigned/sex	5	-	-	5	5	-	-	5
No. of daily doses +recovery period	28+14			28+14	28+14			28+14

C. Methods:

1. Test article preparation and analysis: During the range-finder experiment, doses were prepared weekly, but analytical examination confirmed instability of the dosing formulation. Therefore, for the sub-acute study dosing formulations were prepared daily. The test article was transferred to a beaker, melted and the corresponding weight of corn oil added and stirred for 15 minutes.

Homogeneity and concentration of the test article were determined for all preparations prepared at all dose levels.

2. Observations: Animals were inspected twice daily for signs of toxicity and mortality.

3. Body weights: Animals were weighed prior to study start and then on Days 0, 7, 14, 28, and the day of necropsy.

4. Food consumption: Measured in weekly intervals throughout the study. Mean food intake/rat (g/rat/week) was calculated from the amount of food consumed in each cage and the number of rats in each cage.

Food efficiency was calculated using the following formula:

$$\text{Food efficiency} = \frac{\text{Mean food consumption/week}}{\text{Mean body weight gain/week}} \times 100$$

5. Water consumption: Water intake was observed daily for each cage group by visual inspection

6. Ophthalmological examination: Not conducted.

7. Neurological functional examinations: *Functional observation battery:*

Detailed functional observation of each animal was conducted in their home cage and in an open field arena once each week.

Home cage observations: posture, co-ordination or movement/gait, tremor and conclusion, abnormal behaviour.

Open field arena observations: behaviour, autonomic functions, fur, skin and external appearance, muscle tone, posture, co-ordination or movement/gait, respiration, activity/attention, tremor and conclusion, abnormal behaviour, eyes/palpepral closure, faeces, urine

In week 4, in addition to the home cage and open field arena observations the high dose group (without recovery period) underwent additional observations

including rearing, landing foot-splay and grip strength within the home cage. Additional open field observations included vision test, pupillary reflex, winking reflex, pinna-reflex, auditory startle response, olfactory test, examination of catalepsy, right response, tail pinch.

Motor activity:

Performed in week 4. Following 2 minutes of habituation, motor activity of each animal was measured in special cages with infrared photobeam pairs. Each time a photobeam was interrupted an activity count was registered. The number of activity counts/movement in 20 intervals a 3 minute period were determined.

8. Haematology and clinical chemistry:

At the end of the treatment and recovery period blood was collected. Animals were not fasted prior to blood sampling.

Haematology: red blood cell parameters (haematocrit (commonly termed PCV), haemoglobin concentration (Hb), mean haemoglobin concentration (MHC), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV), platelet count), white blood cell parameters (total and differential (neutrophils, lymphocytes, eosinophils, basophils, monocytes) leukocyte count), coagulation parameters (prothrombin time (PT) – termed Hepato-Quick Test).

Clinical chemistry: electrolytes (sodium, potassium, calcium), kidney function test (creatinine, urea), glucose, liver function tests (albumin, alkaline phosphatase (ALP), alanine aminotransferase (ALT [commonly referred to as glutamic pyruvic transaminase (GPT)]), aspartate aminotransferase (AST [commonly referred to as glutamic oxaloacetic transaminase (GOT)), total bilirubin (T.Bili), total protein (TP), lipid profile (total cholesterol, triglycerides).

9. Urinalysis:

At the end of the treatment and recovery period, animals were placed in metabolism cages for 6 hours. The following urinary parameters were measured: specific gravity, pH, total volume, protein, glucose, ketones, bilirubin, blood, urobilinogen, sediment, colour, leukocytes, erythrocytes, bacteria, epithelial cells (squamous, renal), oxalate crystals, phosphate crystals, carbonate, granular cylinder, urate crystals.

10. Organ weights:

Adrenal glands, brain, epididymides, heart, kidney, liver, spleen, testis, thymus.

11. Sacrifice and pathology:

At the end of the treatment and recovery period gross pathological examination was performed on all animals and included examination of the external surface, all orifices and associated tissues.

The following tissues were preserved in 10% neutral buffered formalin for subsequent histopathological undertaken on all control and high dose group animals from the treatment and recovery periods:

Accessory sex glands (♂:epididymides, prostate, seminal vesicle, testes; ♀: ovary, uterus, vagina), cardiovascular/haematological system (aorta [thoracic], heart, lymph nodes (skin, cervical, mesenteric), spleen, thymus), gastrointestinal tract (oesophagus, tongue, stomach, intestine (caecum, colon duodenum, ileum, jejunum, rectum (+anus)), liver, pancreas, salivary glands), neurological (brain, eyes (+exorbital lacrimal glands), sciatic nerve, spinal cord), respiratory system (trachea, lung), urogenital system (kidneys, urinary bladder), other (skeletal muscle, bone (sternum), skin, all gross lesions and masses);

Other endocrine producing/sensitive glands (adrenals, mammary gland, pituitary, thyroid (+parathyroid)).

12. Neurohisto-pathology:

No specific neurohistopathology with specific fixatives were performed in addition to the standard histopathology undertaken on neuronal tissues.

Results and discussion

A. Test diet analysis:

Spiroxamine cyclohexanol was homogenously distributed and within the concentration range of 10 to 60 mg/L. The analytical data verify that the during the treatment period concentrations of the test article in the corn oil preparations ranged from 98 – 104% of nominal concentrations, which were within acceptable limits.

B. Observations:

1. Clinical signs of toxicity:	Animals in the mid and high dose group showed moderate to severe clinical signs of toxicity manifest as animals in a prone and squatting positon, straub tail and vocalisation. This was in part attributed to the difficulty in dosing the test article, with defensive clinical signs observed. These effects were apparent *ca.*15 minutes post dosing. Convulsions disappeared after a while and animals began to recover. Individual animals still showed mild clinical symptoms in the afternoon. During the recovery period, no clinical signs of toxicity were observed.
2. Mortality:	No test article related effects upon mortality were observed. Two high dose group ♂ died on day 20 and 26 due to a dosing error.
3. Ophthalmoscopic examination:	No test article related effects observed
4. Neurological functional examinations	*Functional observation battery:* - Home cage and open field arena observations: in the low, mid and high dose groups observations included ataxia, fasciculations, paddling movements, defence against touching, aggressiveness, hunchback/squatting position, reduced respiration, hyperactivity, straub tail, piloerection and slight convulsions. - Additional open field observations: high dose group animals showed minimal or high sensitivity to pain. One ♂ showed positive response when examined for catalepsy. - Rearing, landing foot-splay and grip strength: high dose group ♂ showed statistically significant increase of group mean values for landing foot-splay and rearing and decrease of group mean values for grip strength when compared to the recovery control group. The described differences in the recovery group were minor, with no clear pattern evidence and therefore considered to be of minor toxicological significance. - Recovery period home cage and open field arena observations: no significant dose-related clinical signs were observed in either ♂ or ♀. *Motor activity:* - Group mean values of activity counts of all intervals showed no statistically significant increase in any of the treatment groups, or the ♂ recovery group. An increase of group mean motor activity values (not achieving statistical significance) was observed.

C. Body weight and body weight gain:

Of the groups assigned to the treatment period, a slight reduction in body weight and body weight gains were observed in high dose group males at weeks 1 – 3.

For males assigned to the recovery group, in the final weeks of treatment a statistically significant reduction of group mean weekly body weight gain was observed. This correlates with the statistically significant reduction of group mean body weight in the male high dose recovery group at the end of the treatment period. At the end of the recovery period, no statistically significant differences were evident.

The female high dose recovery group showed at the beginning of the study a statistically significant increase of group mean weekly body weight change. At the end of the recovery period, no statistically significant differences were evident.

Table CA 5.8.1/11-2: Overview of sub-acute toxicity study in rats treated orally (*via* gavage) with spiroxamine cyclohexanol: body weight effects

Parameters		♂ (mg/kg bw/d)				♀ (mg/kg bw/d)			
		0	50	150	300	0	50	150	300
28 day treatment period									
Body wt (g)	Day 0	189 ±8.3	183 ±6.9	187 ±8.3	185 ±16.5	144 ±5.9	144 ±4.3	150 ±6.5	146 ±6.6
	Day 7	227 ±8.7	220 ±10.6	226 ±8.4	217 ±19.4	159 ±7.8	162 ±4.4	165 ±3.6	165 ±9.3
	Day 14	254 ±11.3	247 ±10.1	257 ±12.9	242 ±22.0	170 ±10.1	177 ±7.3	180 ±8.5	185 ±13.8
	Day 21	276 ±11.6	269 ±11.9	281 ±12.7	261 ±22.2	183 ±10.9	189 ±8.1	193 ±8.1	204 ±16.0
	Day 28	269 ±14.4	290 ±13.1	305 ±13.3	278 ±30.9	191 ±11.4	195 ±8.6	205 ±9.1	212 ±21.6
Body wt gain (g)	Day 0-28	80	107 (↑34%)	118 (↑48%)	93 (↑16%)	47	51 (↑9%)	55 (↑17%)	66 (↑40%)
28 day treatment period + 14 day recovery									
Body wt (g)	Day 0	183 ±7.8	-	-	189 ±7.9	150 ±6.7	-	-	147 ±5.7
	Day 7	224 ±5.7	-	-	221 ±5.4	168 ±8.0	-	-	160 ±9.0
	Day 14	254 ±8.8	-	-	247 ±9.6	181 ±8.7	-	-	187 ±8.5
	Day 21	285 ±9.2	-	-	266 ±8.6	192 ±7.5	-	-	205 ±11.7
	Day 28	310 ±11.0	-	-	278 ±12.4*	204 ±9.9	-	-	214 ±16.6
	Day 35	325 ±12.3	-	-	303 ±13.4	214 ±9.5	-	-	221 ±18.3
	Day 42	350 ±13.1	-	-	331 ±16.9	220 ±13.4	-	-	231 ±16.7
Body wt gain (g)	Day 0-28	127	-	-	89 (↓30%)	54	-	-	67 (↑24%)
	Day 28-42	40	-	-	53 (↑33%)	16	-	-	17 (↑6%)
	Day 0-42	167	-	-	142 (↑15%)	70	-	-	84 (↑20%)

* $p \leq 0.05$

D. Food consumption, food efficiency and water consumption:

1. Food consumption and food efficiency:

During the 28 treatment period, a slight reduction in food consumption was observed in treated ♂ when compared to the concurrent control group. Conversely, for treated ♀, food consumption was increased. Food efficiency for treated ♀ was decreased, and for ♂ was increased.

For the recovery phase, food consumption was increased for ♂ and ♀ high dose groups during this period. Food efficiency rate of treated ♂ and ♀ at the end of the treated period was still increased and reduced, respectively when compared with the concurrent control.

Without a clear pattern in these changes across the sexes, these differences were considered of little toxicological relevance.

2. Water consumption: No effects observed.

E. Blood and urinalysis:

1. Haematological findings:

The following changes were observed during the 28-day treatment period total WBC count for both high dose ♂ and ♀ were increased (achieving statistical significance of ♂ only). However, differential WBC counts for both genders showed no significant changes.

At the end of the recovery period, high dose group ♂ showed a slight decrease in total WBC. In addition, statistically significant decreases of RBC, Hb and platelet count were observed in ♂.

These changes were deemed of little toxicological concern with effects observed within the normal laboratory range, confined to a single sex with reversibility observed during the recovery period.

Table CA 5.8.1/11-3: Overview of sub-acute toxicity study in rats treated orally (*via* gavage) with spiroxamine cyclohexanol: selected haematological parameters

Parameters	♂ (mg/kg bw/d)				♀ (mg/kg bw/d)			
	0	50	150	300	0	50	150	300
28 day treatment period								
Platelets (10^9/L)	1025 ±111.2	944 ±117.2	988 ±97.0	1006 ±122.5	1055 ±97.5	993 ±91.5	977 ±50.6	976 ±108.3
RBC (10^{12}/L)	7.77 ±0.470	7.84 ±0.595	8.24 ±0.616	8.10 ±0.356	7.63 ±0.272	7.58 ±0.587	7.28 ±0.628	7.55 ±0.456
Hb (mmol/L)	9.43 ±0.646	9.70 ±0.541	10.06 ±0.566	9.70 ±0.680	8.99 ±0.468	9.06 ±0.542	8.96 ±0.679	9.47 ±0.270
Total WBC (10^9/L)	10.10 ±2.469	14.30 ±3.553	13.10 ±1.505	16.39 ±1.700*	8.65 ±0.943	7.98 ±1.697	7.94 ±1.033	11.51 ±1.531
28 day treatment period + 14 day recovery								
Platelets (10^9/L)	1046 ±102.8	-	-	796 ±109.0	940 ±105.8	-	-	1007 ±101.2
RBC (10^{12}/L)	8.18 ±0.211	-	-	7.39 ±0.211**	7.37 ±1.140	-	-	7.79 ±1.036
Hb (mmol/L)	9.76 ±0.314	-	-	8.91 ±0.234	8.86 ±0.282	-	-	9.27 ±0.772
Total WBC (10^9/L)	13.88 ±1.647	-	-	11.43 ±0.488	6.74 ±0.725	-	-	9.05 ±2.191
Laboratory historical control data (rat, Wistar 7-13 wks of age)								
Platelets (10^9/L)	Date range: *n:* Mean ±SD:	Not stated 105 1017.9 ±166.3			Date range: *n:* Mean ±SD:	Not stated 102 1052.3 ±146.1		
RBC (10^{12}/L)	Date range: *n:* Mean ±SD:	Not stated 105 7.615 ±0.816			Date range: *n:* Mean ±SD:	Not stated 102 7.322 ±0.990		
Hb (mmol/L)	Date range: *n:* Mean ±SD:	Not stated 105 9.248 ±0.684			Date range: *n:* Mean ±SD:	Not stated 102 8.833 ±1.347		
Total WBC (10^9/L)	Date range: *n:* Mean ±SD:	Not stated 105 12.403 ±3.015			Date range: *n:* Mean ±SD:	Not stated 112 8.272 ±3.232		

*$p \leq 0.05$
RBC: red blood cell
Hb: haemoglobin
Total WBC: total white blood cell count

2. Clinical chemistry findings:

No toxicological relevant changes were observed during the 28-day treatment period in treated ♂. In mid and high dose group ♂ a statistically significant decrease in glucose was observed. A similar pattern was observed in high dose group ♀, but not achieving statistical significance.

Increases in creatinine were observed in all treated ♂, achieving statistical significance in the mid dose group. Conversely, ♀ showed decreases in creatinine at all doses, but did not achieve statistical significance. For other clinical chemistry parameters analysed (electrolytes, kidney functional tests) no treatment related effects were observed.

AST was statistically significant increase in high dose group ♂ when compared to the concurrent control. Conversely, ♀ showed a marginal decrease in AST activity, without statistical significance. In all cases these changes were within the historical control range.

At the end of the recovery period, no toxicological relevant changes were observed in ♂.

Glucose values of treated high dose group ♂ and ♀ were increased (without statistical significance) when compared to the concurrent control group. A

treatment related effect cannot be discounted, with a likely rebound effect following the withdrawal of treatment with decreased glucose levels observed during the treatment period.
In high dose group ♀ a statistically significant decrease in cholesterol, triglycerides, total bilirubin and ALT were observed compared to the concurrent control group. These changes however were within the laboratory historical control range, and therefore deemed or little toxicological concern.
Whilst contradictory results were observed between the genders for creatinine and AST, a treatment related effect cannot be dismissed, but likely slight (without correlating liver histopathology) rather than adverse.
For other clinical chemistry parameters analysed (electrolytes, kidney and liver functional tests) no treatment related effects were observed.

Table CA 5.8.1/11-4: Overview of sub-acute toxicity study in rats treated orally (*via* gavage) with spiroxamine cyclohexanol: selected clinical chemistry parameters

Parameters	♂ (mg/kg bw/d)				♀ (mg/kg bw/d)			
	0	50	150	300	0	50	150	300
28 day treatment period								
Gluc. (mmol/L)	9.83 ±0.448	9.92 ±0.273	8.45 ±0.475*	8.40 ±0.897*	8.58 ±1.062	8.84 ±0.530	8.55 ±0.263	7.42 ±0.267
T.chol (mmol/L)	2.26 ±0.324	2.17 ±0.370	2.24 ±0.308	2.29 ±0.091	1.79 ±0.249	1.71 ±0.203	1.91 ±0.110	1.70 ±0.184
Trig (mmol/L)	2.24 ±1.039	1.19 ±0.200	1.19 ±0.277	3.41 ±1.825	1.42 ±0.671	0.80 ±0.273	0.95 ±0.173	1.02 ±0.183
T.bili (µmol/L)	1.92 ±0.116	1.85 ±0.298	1.79 ±0.192	1.62 ±0.262	1.90 ±0.317	1.91 ±0.307	1.79 ±0.219	1.67 ±0.222
Creat. (µmol/L)	41 ±3.6	50 ±6.8	54 ±4.8*	48 ±4.6	47 ±3.2	41 ±2.9	44 ±4.6	43 ±1.7
AST (U/L)	44.0 ±2.28	51.0 ±4.98	47.0 ±3.55	64.6 ±7.85**	52.2 ±8.11	49.8 ±3.13	51.0 ±10.09	50.9 ±10.95
ALT (U/L)	22.1 ±2.86	22.7 ±1.79	20.6 ±1.28	22.6 ±3.18	27.4 ±4.97	26.0 ±5.35	26.7 ±3.83	23.0 ±7.02
28 day treatment period + 14 day recovery								
Gluc. (mmol/L)	9.15 ±1.005	-	-	9.61 ±0.850	8.30 ±0.802	-	-	8.62 ±0.406
T.chol (mmol/L)	2.29 ±0.313	-	-	2.22 ±0.316	2.20 ±0.195	-	-	1.77 ±0.213*
Trig (mmol/L)	2.00 ±0.433	-	-	1.81 ±0.382	1.59 ±0.203	-	-	1.13 ±0.100**
T.bili (µmol/L)	2.20 ±0.309	-	-	2.12 ±0.124	2.44 ±0.315	-	-	1.96 ±0.113*
Creat. (µmol/L)	47 ±4.6	-	-	51 ±1.2	48 ±3.6	-	-	49 ±7.3
AST (U/L)	47.9 ±4.13	-	-	52.2 ±11.33	46.8 ±3.50	-	-	47.7 ±8.21
ALT (U/L)	24.6 ±1.52	-	-	25.7 ±0.17	26.3 ±1.25	-	-	22.4 ±1.50**
Laboratory historical control data (rat, Wistar 8 -11 wks of age)								
Gluc. (mmol/L)	Date range: *n:* Mean ±SD:		Not stated 100 9.19 ±1.519		Date range: *n:* Mean ±SD:		Not stated 97 9.06 ±1.208	
T.chol (mmol/L)	Date range: *n:* Mean ±SD:		Not stated 115 2.27 ±0.371		Date range: *n:* Mean ±SD:		Not stated 112 1.96 ±0.325	
Trig (mmol/L)	Date range: *n:* Mean ±SD:		Not stated 115 1.86 ±0.663		Date range: *n:* Mean ±SD:		Not stated 102 1.35 ±0.548	

Parameters	♂ (mg/kg bw/d)				♀ (mg/kg bw/d)			
	0	50	150	300	0	50	150	300
T.bili (µmol/L)	Date range: *n:* Mean ±SD:		Not stated 114 3.15 ±1.543		Date range: *n:* Mean ±SD:		Not stated 112 3.02 ±0.371	
Creat. (µmol/L)	Date range: *n:* Mean ±SD:		Not stated 115 40 ±7.0		Date range: *n:* Mean ±SD:		Not stated 112 44 ±7.1	
AST (U/L)	Date range: *n:* Mean ±SD:		Not stated 115 55.8 ±15.97		Date range: *n:* Mean ±SD:		Not stated 112 57.7 ±18.04	
ALT (U/L)	Date range: *n:* Mean ±SD:		Not stated 115 27.8 ±9.37		Date range: *n:* Mean ±SD:		Not stated 112 26.8 ±11.12	

* $p \leq 0.05$; ** $p \leq 0.01$
Gluc.: glucose
T.chol.: total cholesterol
Trig.: triglycerides
T.bili.: total bilirubin
Creat.: creatinine
AST: aspartate aminotransferase
ALT: alanine aminotransferase

3. Urinalysis:

There were no toxicologically relevant treatment related effects observed. Specific gravity was significantly decreased in mid dose group ♀, with no effects observed in ♂ following 28 days of treatment.

At the end of the recovery period high dose group ♀ showed a statistically significant increase in specific gravity and a statistically significant decrease in pH. These effects were not replicated in ♂. These semi-quantitate / qualitative assessment was considered incidental as it was limited to a single sex, the incidence were consistent with the historical control range, did not correlate with any associate adverse histopathology and therefore deemed not toxicologically relevant.

F. Sacrifice and pathology:

1. Organ weight:

In the treatment phase, a statistically significant increase in relative adrenal weights in high dose group ♂ were observed. It is prudent however to acknowledge that relative adrenal weights for all test article treated ♂ and mid and high dose group ♀ were elevated above that of the respective concurrent control groups. These increases were not replicated in the recovery group, and whilst no concurrent organ histopathology was evident, the increased adrenal weights during the dosing phase correlate with the difficulty in dosing along with aggressiveness against touching in the weekly open field tests. Therefore, these increased organ weights are suggestive of increased adrenal activity due to the stress of dosing, rather than a direct endocrine related effect.

At the end of the recovery period for high dose group ♂, relative epididymides weights were statistically significantly increased for high dose group ♂. These findings did not correlate with any associated adverse histopathology and therefore deemed not toxicologically relevant.

Table CA 5.8.1/11-5: Overview of sub-acute toxicity study in rats treated orally (*via* gavage) with spiroxamine cyclohexanol: selected organ weights

Parameters		♂ (mg/kg bw/d)				♀ (mg/kg bw/d)			
		0	50	150	300	0	50	150	300
28 day treatment period									
Terminal bwt (g)		284 ±10.5	274 ±11.3	289 ±11.7	265 ±27.4	184 ±12.9	188 ±7.22	197 ±8.6	206 ±23.6
Liver	Abs (g)	9.159 ±0.7109	8.683 ±0.3137 (↓5%)	9.154 ±0.7043 (↓1%)	8.649 ±1.5178 (↓6%)	5.907 ±0.4526	5.798 ±0.3332 (↓2%)	6.510 ±0.5147 (↑10%)	6.482 ±0.4431 (↑10%)
	Rel. (g%)	3.228 ±0.1903	3.172 ±0.0973 (↓2%)	3.166 ±0.1361 (↓2%)	3.246 ±0.2381 (↑1%)	3.223 ±0.2551	3.078 ±0.1153 (↓4%)	3.310 ±0.2351 (↑3%)	3.174 ±0.2106 (↓2%)
Adrenals	Abs (g)	0.045 ±0.0054	0.046 ±0.0069 (↑2%)	0.053 ±0.0045 (↑18%)	0.057 ±0.0115 (↑27%)	0.055 ±0.0084	0.056 ±0.0039 (↑2%)	0.064 ±0.0040 (↑16%)	0.073 ±0.0131 (↑33%)
	Rel. (g%)	0.016 ±0.0018	0.017 ±0.0023 (↑6%)	0.018 ±0.0015 (↑13%)	0.022 ±0.0026* (↑38%)	0.030 ±0.0051	0.030 ±0.0028 (-)	0.032 ±0.019 (↑7%)	0.035 ±0.0046 (↑17%)
Testes	Abs (g)	3.173 ±0.2174	2.690 ±0.8045 (↓15%)	3.290 ±0.2756 (↑4%)	2.850 ±0.1548 (↓10%)	-	-	-	-
	Rel. (g%)	1.119 ±0.00759	0.979 ±0.2582 (↓13%)	1.137 ±0.0529 (↑2%)	1.066 ±0.1043 (↓5%)	-	-	-	-
Epidid.	Abs (g)	0.981 ±0.0546	0.908 ±0.2240 (↓7%)	1.052 ±0.4079 (↑7%)	0.949 ±0.0780 (↓4%)	-	-	-	-
	Rel. (g%)	0.347 ±0.0256	0.333 ±0.0877 (↓4%)	0.363 ±0.0383 (↑5%)	0.359 ±0.0323 (↑3%)	-	-	-	-
28 day treatment period + 14 day recovery									
Terminal bwt (g)		334 ±13.8	-	-	312 ±15.0 (↓7%)	208 ±110.2	-	-	217 ±16.1 (↑4%)
Liver	Abs (g)	10.262 ±0.5306	-	-	9.260 ±0.6878 (↓10%)	6.221 ±0.3754	-	-	6.683 ±0.7043 (↑7%)
	Rel. (g%)	3.077 ±0.1349	-	-	2.966 ±0.1040 (↓4%)	3.007 ±0.3032	-	-	3.077 ±0.2441 (-)
Adrenals	Abs (g)	0.047 ±0.0029	-	-	0.050 ±0.0042 (↑6%)	0.055 ±0.0038	-	-	0.064 ±0.0054 (↑16%)
	Rel. (g%)	0.014 ±0.0011	-	-	0.016 ±0.0005 (↑14%)	0.027 ±0.0023	-	-	0.029 ±0.0017 (↑7%)
Testes	Abs (g)	3.129 ±0.3861	-	-	2.966 ±0.1068 (↓5%)	-	-	-	-

Parameters		♂ (mg/kg bw/d)				♀ (mg/kg bw/d)			
		0	50	150	300	0	50	150	300
	Rel. (g%)	0.942 ±0.1382	-	-	0.954 ±0.0768 (↑1%)	-	-	-	-
Epidid.	Abs (g)	1.089 ±0.0991	-	-	1.283 ±0.0817 (↓18%)	-	-	-	-
	Rel. (g%)	0.326 ±0.0282	-	-	0.411 ±0.0136* (↑26%)	-	-	-	-

* $p \leq 0.05$
Abs.: absolute
Rel.: relative to body weight

Epidid: epididymides

2. Gross pathology: No test article related macroscopic findings were evident. Two high dose group ♂ (#68 and #69) were confirmed to have had a perforated oesophagus, resultant from a dosing error, with test article formulation apparent in the thoracic cavity.

3. Histopathology: No test article related histopathological lesions, relevant to humans were observed. Where histopathological changes were observed (hydrometra [distended uterus], sperm granuloma [lump of extravasated sperm that appears along the vasa deferentia], minimal focal inflammatory cell foci in the liver) in the high dose group, these were isolated cases, comparable to the concurrent control incidence and therefore considered normal background variation.
Although inflammatory foci in the lungs were characterised by multi-focal interstitial lymphocyte infiltrates, these lesions were not dose related and likely caused by a viral infection.
The kidney of 5 high dose group ♂ and 1 control ♂ animal showed an increase in eosinophilic hyaline droplets in the epithelial cell cytoplasm of the proximal tubules. Hyaline droplet nephropathy is due to the accumulation of α2μ-globulin which is known to be a lesion specific to ♂ rats with no relevance to humans. It however is acknowledge that Azan specific staining used to confirm α2μ-globulin presence was not undertaken.
Both the parent compound, spiroxamine and spiroxamine N-oxide are considered tertiary amines. As all tertiary amines have a high pKa, and a high pH with comparable histopathological effects reported. As with irritant compounds, tissues containing mucosal membrane are deemed to be a target organ critical effect. Spiroxamine cyclohexanol, which has lost the tertiary amine group consequently did not display histopathological lesions associated with tertiary amines (hyperkeratosis of the epithelium of the oesophagus and forestomach).
No adverse histopathology was reported in the recovery groups.

Table CA 5.8.1/11-6: Overview of sub-acute toxicity study in rats treated orally (*via* gavage) with spiroxamine cyclohexanol: selected histopathology observations

Parameters	♂ (mg/kg bw/d)				♀ (mg/kg bw/d)			
	0	50	150	300	0	50	150	300
28 day treatment period								
Histopathology: [incidence. Note, lesions were not graded]								
Epididymis - Sperm granuloma	0/5	-	-	1/5	-	-	-	-
Kidney - Hyaline droplet nep.	1/5	-	-	0/5	0/5	-	-	0/5
Liver - Inflam. cell foci	0/5	-	-	1/5	1/5	-	-	1/5
Lung - Inflam. cell foci	2/5	-	-	3/5	2/5	-	-	0/5
Uterus - Hydrometra	-	-	-	-	1/5	-	-	1/5

Hyaline droplet nep.: hyaline droplet nephropathy

G. Discussion:

Treatment-related effects were seen in respective of clinical signs in both the mid and high dose groups with moderate to severe effects observed (manifest as prone and squatting positon, straub tail and vocalisation). This was in part attributed to the difficulty in dosing the test article, with defensive clinical signs observed. These effects were apparent ca.15 minutes post dosing. These effects were not apparent in the recovery phase. Consequently the functional observational battery, which was conducted in the

after, following morning dosing revealed clinical abnormalities in the high dose group animals. These abnormalities included severe defence against application of test article prior to dosing, aggressiveness against touching during weekly open field observations. The stress connection to dosing is further supported by statistically significant increases in relative adrenal glands weights, without associated histopathology. These increased organ weights are suggestive of increased adrenal activity due to the stress of dosing, rather than a direct endocrine related effect, which again is further evidenced by the unscheduled deaths of two high dose group males due to a dosing error.

No test article related histopathological lesions, relevant to humans were observed. High dose group males showed an increase in eosinophilic hyaline droplets in the epithelial cell cytoplasm of the proximal tubules. Hyaline droplet nephropathy is due to the accumulation of α2μ-globulin which is known to be a lesion specific to ♂ rats with no relevance to humans. It however is acknowledge that Azan specific staining used to confirm α2μ-globulin presence was not undertaken.

H. Deficiencies:

Although the study was conducted according to test guideline OECD 407 (1995), this test guideline has since been updated in the intervening period (2008). When assessed against current test guideline requirements the following deficiencies are noted:

- Whilst not a requirement, the test guideline makes reference to determination of serum thyroid hormones (T3, T4, TSH). These were not analysed. However, the gold standard to assess thyroid effects is histopathological analysis. This was undertaken on all animals and revealed no histopathological findings. Consequently the lack of serum thyroid hormone analysis is not deemed critical, with potential thyroid effects adequately addressed with histopathological analysis.

Assessment and conclusions by applicant:

Assessment: Study meets the current guidance and the requirements in 283/2013.

Conclusion: Under the conditions of this study, the NOAEL following 28 days of dosing *via* oral gavage is deemed to be 50 mg/kg bw/day for males/females based on reductions in body weight and body weight gain (males) and moderate to severe clinical effects following dosing with a peak period approximately 15 minutes post dosing.

Data Point:	KCA 5.8.1/12
Report Author:	[redacted]
Report Year:	2007
Report Title:	Oral (gavage) developmental toxicity study of 4-tert butylxcyclohexyl acetate (4-tBCHA) in rats
Report No:	52639
Document No:	M-471532-01-1
Guideline(s) followed in study:	US-FDA Guideline for Industry: detection of toxicity to reproduction for medicinal products, (ICH) S5A
Deviations from current test guideline:	Yes Methods: SANCO/3029/99 rev. 4 Accuracy n = 4 Toxicology: The test guideline OECD 414 was updated 2018, the following deficiencies were noted: -Thyroid gland weights and histopathological assessment of every dam treated dam not performed. -Thyroid hormone measurements of dams not undertaken. -Anogenital distance of fetuses not performed.
Previous evaluation:	yes, evaluated and accepted RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

In an embryo-fetal developmental toxicity study, spiroxamine cyclohexyl was administered to female Sprague Dawley rats (25/group) by oral gavage once daily at dose levels of 0 (corn oil), 40, 160 or 640 mg/kg bw, from days 7 through 20 of gestation, employing a dose volume of 10 mL/kg bw. Surviving dams were sacrificed on GD 21. The following parameters and end points were evaluated in this study: clinical signs, body weights, body weight changes, food consumption, ovarian and uterine examinations, gravid uterine weights, fetal examinations, gross necropsy findings and histopathology.

Based on the results of this study, spiroxamine cyclohexyl administered to rats at a dose of 640 mg/kg bw/day caused maternal mortality, body weight losses (up to 9.2% reduction), reduced body weight gains (32%) and food consumption values at a dosage level that exceeded the MTD. The incidence of excessive salivation at dose level of 160 and 640 mg/kg bw/day (moderate and severe, respectively) was evident compared to the concurrent control group.

Pregnancy occurred in 24, 24, 25 and 25 rats in the 0, 40, 160 and 640 mg/kg bw/day dosage groups, respectively. The litter averages for *corpora lutea*, implantations, pre-implantation loss, early resorptions, and post-implantation loss were comparable among the three dose groups when compared with the concurrent control. There were no embryonic death in the control or test article treated groups. All placentae appeared normal.

Statistically significant reductions in total fetal weights (both presented as combined sex and individual sex) were observed in the 640 mg/kg bw/day dose group, which were considered test-article related. No test article related or statistically significant difference were observed in sex ratio between the vehicle control and test article treated groups.

Visceral examinations were based on 161, 170, 147 and 166 live, GD 21 caesarean-delivered fetuses in 24, 24, 25 and 24 litters in dose groups 0, 40, 160 and 640 mg/kg bw/day dosage groups, respectively. Slight or moderate dilation of the pelvis of one or both kidneys was observed. This variation is considered to be reversible, typically common in rat strains. This variation occurred in 1, 2, 0 and 8 fetuses from 1, 2, 0 and 2 litters from each respective group. It is noted that the fetal incidence of

moderate enlargement of the pelvis of both kidneys was significantly increased ($p \leq 0.01$) in the high dose group, compared to the concurrent vehicle control, however when assessed as litter incidence (the more relevant parameter), was not significantly increased. No other alterations occurred in these fetuses. The incidence observed in this study exceeded the laboratory' historical control range for fetal incidence, with litter incidence exceeding the concurrent vehicle control.

No treatment related skeletal alterations were observed.

Skeletal examinations were based on 175, 187, 188 and 177 live, GD 21 caesarean-delivered fetuses in 24, 24, 25, and 24 litters in dose groups 0, 40, 160 and 640 mg/kg bw/day dosage groups, respectively. In total skeletal variations were limited to vertebrae and ribs, with no malformations. Reductions in the average number of ossified caudal vertebrae, forelimb phalanges and hind limb metatarsals and phalanges were significantly reduction in the 640 mg/kg bw/day dosage groups, compared to the concurrent control. There were no other statistically significant or biologically relevant difference among the four dosage groups in the average number of ossification sites/fetus for the hyoid, vertebrae (cervical, thoracic, lumbar, sacral), ribs sternum (manubrium, sternal centers, xiphoid) forelimbs (carpals, metacarpals) or hind limbs (tarsals).

Under the conditions of this study, the NOAEL for maternal toxicity was considered to be 160 mg/kg bw/day based maternal mortality, body weight losses, reduced body weight gains and food consumption values at a dosage level that exceeded the MTD (640 mg/kg bw/day).

The developmental NOAEL was considered to be 40 mg/kg bw/day based on transient retardations in fetal development, with reduction in fetal bodyweight and associated significant increases in moderate dilation of the renal pelvis and delayed ossification of the caudal vertebrae, fore- and hind limb phalanges and metatarsals. These retardations occurred at a dosage level of 640 mg/kg bw/day, which exceeded the MTD for maternal animals.

Materials and Methods

A. Materials:

1. Test Material:	Spiroxamine cyclohexyl acetate (alternative name: 4-tert-butylcyclohexyl acetate, 4-tBCHA, M13 acetate)	
Description:	Colourless liquid	
Lot/Batch No.:	1003915	
Purity:	99.3% (w/w) (correction for purity not undertaken)	
CAS No.:	Not assigned	
Stability of test compound:	Confirmed stable for the duration of the study (expiry date: April 2007)	
2. Vehicle and/or positive control:	Corn oil / not relevant	
3. Test animals:		
Species:	Rat	
Strain:	Sprague Dawley	
Age at dosing:	9 weeks	
Weight at dosing:	221-257g	
Source:	[redacted]	
Acclimation period:	7 days	
Diet:	Certified Rodent Diet® #5002 (PMI® Nutrition International, USA) *ad libitum*	
Water:	Municipal water, *ad libitum*	
Housing:	Individually housed	

4. Environmental conditions:

Temperature:	22 ±4°C
Humidity:	55 ± 15%
Air changes:	*ca.* 10/h
Photoperiod:	12 hour light/dark

B. Study Design:

1. In life dates: 26 June 2006 to 14 July 2006 (experimental dates)

2. Animal assignment and treatment: Upon arrival, rats were assigned to individual housing on the basis of computer-generated randomisation. After an acclimatisation period of 7 days, virgin ♀ rats were paired 1:1 with breeder rats of the same source for up to 5 days. The day which spermatozoa observed in a smear of the vaginal contents and/or a copulatory plug observed *in situ* were considered to be day 0 of gestation. Each group consisted of 25 ♀/group.
The dose levels were selected based on the results from a range-finding study in which spiroxamine cyclohexyl acetate was dosed, orally *via* gavage at 375, 50, 150, 300 mg/kg bw/d to pregnant rats from GD 7 to 20. No mortality occurred up to the highest dose tested. Whilst body weight and food consumption reductions were observed at 300 mg/kg bw/day, caesarean sectioning, litter or fetal gross external evaluations were not adversely affected. In the absence of an MTD being established, the main study was conducted with an increased higher dose level.
Based on these data, dosages of 0, 40, 160, 640 mg/kg bw/day of spiroxamine cyclohexanol were employed for the developmental toxicity study in rats, using a dose volume of 10 mL/kg bw. Surviving dams were sacrificed on GD 21.

3. Statistics: Continuous data:
Parametric:
- Barlett's Test
- If not significant:
  - ANOVA. If not significant no further statistical analysis
  - If ANOVA significant, Dunnett test
- If significant, non-parametric approach

Non-parametric:
- Kruskal-Wallis test.
- If not significant no further statistical analysis
- If significant, Dunn test

For proportional data, variance test for homogeneity of the binomial distribution.
Indices: All appropriate indices were calculated from caesarean section records of animals in the study.

$$\% \text{ pre-implantation loss} = \frac{\text{No. of } \textit{corpora lutea} - \text{No. of implantations}}{\text{number of } \textit{corpora lutea}} \times 100$$

$$\% \text{ post-implantation loss} = \frac{\text{No. of implantation no. of live fetuses}}{\text{No. of implantations}} \times 100$$

$$\text{Sex ratio} = \frac{\text{No. of ♂ fetuses / No. of ♀ fetuses}}{\text{Total no. of fetuses}} \times 100$$

C. Methods:

1. Test article formulation preparation and analysis:	The test article formulations were prepared on weekly basis at concentrations of 0, 4, 16, 64 mg/mL. Spiroxamine cyclohexyl acetate was weighed into a glass beaker, with vehicle added. The mixture was homogenized using a magnetic stirrer during dosing. Verification of concentration and homogeneity of the test article were determined on one occasion from the initial dose preparation. Fourteen day stability data bracketing the dose range used in this study were confirmed (refer to Doc MCA Section 4 [M-471532-01-1] for method validation).
2. Observations:	*Maternal observations*: The animals were checked for mortality twice daily. The rats were observed for general appearance twice during the acclimation period, on GD 0, daily before administration and 1-2 hours post dosing, and once daily during the post-dose period. *Fetal observations*: examined for sex and external abnormalities. Dead fetuses and late resorptions were examined for sex and external abnormalities to the extent possible.
3. Body weights:	*Maternal body weights:* Recorded from GD 0, and then from 7 through to 21. *Fetal body weights*: body weight of each fetus was recorded. Fetuses were individually identified with litter number and uterine distribution
4. Food consumption:	Recorded for the following periods GD 0, 7-10, 12-15, 18-20 and 21. From the food consumption data, compound consumption was calculated using the following equation: $\text{Mean daily food consumption} = \frac{\text{Food consumption (g rat } per \text{ period)}}{\text{Days } per \text{ period}}$
5. Water consumption:	Not conducted
6. Ophthalmological examination:	Not conducted
7. Mating performance:	Evaluated daily during the co-habitation period. Dams were sacrificed on day 21 of gestation.
8. Haematology and clinical chemistry:	Not conducted
9. Urinalysis:	Not conducted
10. Organ weights:	Not undertaken.
11. Maternal sacrifice and pathology:	*Ovarian and uterine examinations:* the uterus was opened and the contents were examined. The fetuses were removed from the uterus and placed in individual containers (or a tray). The ovaries and uterus were examined for number and distribution of *corpora lutea*, implantation sites, placentae (size, color or shape), live and dead fetuses, and early and late resorptions. An early resorption was defined as one in which organogenesis was not grossly evident. A late resorption was defined as one in which the occurrence of organogenesis was grossly evident. A live fetus was defined as a term fetus that responded to stimuli. Non-responding term fetuses were considered to be dead. Dead fetuses and late resorptions were differentiated by the degree of autolysis present; marked to extreme autolysis indicated that the fetus was a late resorption. Uteri of apparently non-pregnant dams were examined to confirm absence of implantation sites. *Necropsy*: rats were subjected to a gross necropsy examination, which included an evaluation of the thoracic, abdominal, and pelvic cavities with their associated organs and tissues. Gross lesions were collected for all animals. Representative samples of the tissues (cervix, collected with uterus: including non-pregnant animals; gravid uterus, all animals; gross lesions, all animals; liver, all animals; ovaries, including all non-pregnant animals; uterus, including all non-pregnant animals) were collected and preserved in 10% neutral buffered formalin.

Histopathology: the lungs, trachea, oesophagus, heart, liver, stomach and spleen of dams of the 640 mg/kg bw/day dosage group were perfused in 10% NBF and retained for possible histopathology examination.

12. Fetal sacrifice and pathology: Fetuses were euthanized by *ip* injection of sodium pentobarbital. Approximately one-half of the fetuses in each litter were examined for visceral abnormalities by using a modification of the micro-dissection technique of Wilson (1965). Each fetus was fixed in a Bouin's solution and then preserved in a solution of ethyl alcohol. Examination of the viscera and brain were performed. The remaining fetuses (approximately one-half of the fetuses in each litter) were examined for skeletal abnormalities after staining with alizarin red S. Following examination, skeletal preparations were preserved in glycerin with thymol.

Results and discussion

A. Test article formulation analysis:

Spiroxamine cyclohexyl acetate was homogenously distributed and chemically stable for at least 14 days and within the concentration range of 4 to 64 mg/mL. The analytical data verify that the during the treatment period concentrations of the test article in the formulation preparations ranged from -15% to +15% of nominal concentrations which were within acceptable limits.

B. Maternal toxicity:

1. Clinical signs of toxicity: At dose levels of 160 and 640 mg/kg bw/day the incidence of excessive salivation (moderate and severe, respectively) was evident compared to the concurrent control group. In addition to the clinical signs already discussed, at 640 mg/kg bw/day the incidences of red perioral substance and sparse hair coat on the limbs. The increased salivation occurred *ca.* 1-2 hours following dosing. In the single animal (#19476) in the 640 mg/kg bw/day killed *in extremis*, in addition to the clinical signs already reported, scant faeces, decreased motor activity, urine stained abdominal fur, ptosis, coldness to touch and apparent dehydration were evident.
All other clinical signs reported were considered unrelated to treatment.

2. Mortality: One dam (#19476) in the 640mg/kg bw/day dosage group was killed *in extremis* on GD20 due to adverse clinical signs of toxicity (discussed above) attributed to the test article.

3. Ophthalmoscopic examination: Not conducted.

4. Body weight: Body weight effects were limited to the high dose group, 640 mg/kg bw/day. Significantly reduced average maternal body weights on GD 9 through to 21 (exception to this was GD 11). These values reflected a significant reduction in maternal body weight gain for the entire treatment period (GD 7 – 21), with a 32% body weight loss compared to the concurrent control group. Significant body weight losses occurred on GD 7, 8 and 8-9 after the first two administrations of spiroxamine cyclohexyl acetate, with significant reductions in body weight gains occurring in GD 15-18 and 18-21. Maternal body weight gains on GD 7-10 and for the entire gestation period were also significantly reduced.

Table CA 5.8.1/12-1: Overview of developmental toxicity study in rats treated orally (*via* gavage) with spiroxamine cyclohexyl acetate: body weight effects

Parameters	♀ (mg/kg bw/d)			
	0	40	160	640
No. of animals treated	25	25	25	25

Parameters		♀ (mg/kg bw/d)			
		0	40	160	640
Body wt (g)	Day 0	238 ±9.3	239 ±9.7 (↑0.4%)	239 ±9.5 (-)	239 ±9.3 (↑0.4%)
	7	275 ±8.8	273 ±1.7 (↓0.7%)	274 ±13.8 (↓0.4%)	279 ±12.9 (↑1.5%)
	8	276 ±9.0	276 ±13.0 (-)	275 ±13.3 (↓0.4%)	273 ±14.1 (↓1.1%)
	9	280 ±9.1	279 ±12.4 (↓0.4%)	279 ±14.2 (↓0.4%)	270 ±15.8* (↓3.6%)
	10	285 ±8.5	284 ±13.7 (↓0.4%)	285 ±14.4 (-)	273 ±15.5** (↓4.2%)
	15	313 ±10.3	313 ±14.6 (-)	315 ±18.8 (↑0.6%)	302 ±21.5** (↓3.5%)
	16	326 ±13.6	326 ±14.4 (-)	325 ±19.7 (↓0.3%)	310 ±22.7** (↓4.9%)
	17	339 ±14.4	340 ±16.7 (↑0.3%)	338 ±21.6 (↓0.3%)	321 ±26.1** (↓5.3%)
	18	355 ±15.7	358 ±18.5 (↑0.8%)	355 ±23.9 (-)	333 ±26.6** (↓6.2%)
	19	370 ±17.3	373 ±16.5 (↑0.8%)	371 ±25.5 (↑0.3%)	345 ±29.9** (↓6.8%)
	20	385 ±18.2	389 ±18.9 (↑1%)	387 ±28.5 (↑0.5%)	353 ±34.9** (↓8.3%)
	21	402 ±18.7	402 ±21.9 (-)	403 ±30.4 (↑0.2%)	365 ±33.7** (↓9.2%)
Body weight gain (g)	Day 7-21	127 ±17.9	130 ±15.2 (↑2.4%)	129 ±21.7 (↑1.6%)	86 ±30.9** (↓32%)
	0-21	164 ±20.0	164 ±17.7 (-)	165 ±25.2 (↑0.6%)	126 ±31.2** (↓23%)

* $p \leq 0.05$; ** $p \leq 0.01$

5. Food consumption: Absolute and mean food consumption during the treatment period was significantly reduced ($p \leq 0.01$) during the entire dosing period for high dose group dams.

Food consumption was unaffected in the low and mid dose groups.

Table CA 5.8.1/12-2: Overview of developmental toxicity study in rats treated orally (*via* gavage) with spiroxamine cyclohexyl acetate: absolute and relative food consumption data

Parameters	♀ (mg/kg bw/d)							
	0	40	160	640	0	40	160	640
	Absolute feed consumption (g/day) [% change]				Relative feed consumption (g/kd bw/day) [% change]			
Day								
0-7	23.1 ±1.6	22.5 ±2.0 [↓2.6]	23.0 ±2.4 [↓0.4]	23.8 ±2.2 [↑3.0]	90.0 ±6.6	87.8 ±5.9 [↓2.4]	89.4 ±6.9 [↓0.7]	91.8 ±6.3 [↑2.0]
7-10	19.1 ±2.2	19.5 ±3.0 [↑2.1]	19.9 ±2.9 [↑4.2]	12.5 ±3.8** [↓35]	68.6 ±7.7	70.1 ±9.0 [↑2.2]	71.3 ±9.2 [↑3.9]	45.5 ±12.6** [↓33.7]
10-12	19.7 ±1.9	19.8 ±2.8 [↑0.5]	20.8 ±2.9 [↑5.6]	13.3 ±2.8** [↓33]	68.1 ±6.3	68.5 ±7.6 [↑0.6]	71.3 ±8.2 [↑4.7]	47.6 ±8.4** [↓30.1]
12-15	19.9 ±2.0	19.9 ±2.1 [-]	20.4 ±3.0 [↑2.5]	14.5 ±3.0** [↓27]	65.4 ±6.2	65.5 ±5.7 [↑0.2]	66.8 ±8.4 [↑2.1]	50.1 ±7.8** [↓23.4]
15-18	21.4 ±3.1	22.0 ±2.6 [↑2.8]	21.5 ±2.8 [↑0.5]	15.6 ±3.5** [↓27]	64.0 ±8.4	65.9 ±6.4 [↓3.0]	64.5 ±51.0 [↑0.8]	49.0 ±9.0** [↓23.4]
18-21	19.2 ±3.1	18.4 ±3.9 [↓4.2]	19.3 ±3.1 [↑0.5]	14.2 ±3.5** [↓26]	50.9 ±8.3	48.2 ±9.5 [↓4.9]	51.0 ±7.6 [↑0.2]	40.6 ±8.9** [↓20.2]
7-21	19.9 ±1.9	20.0 ±2.0 [↑0.5]	20.4 ±2.4 [↑2.5]	14.1 ±3.2** [↓29]	62.1 ±5.6	62.2 ±4.7 [↑0.2]	63.6 ±6.2 [↑2.4]	46.0 ±5.0** [↓25.9]
0-21	20.9 ±1.6	20.8 ±1.9 [↓0.5]	21.2 ±2.1 [↑1.4]	17.4 ±1.9** [↓17]	66.4 ±4.9	66.0 ±4.1 [↓0.6]	67.3 ±4.7 [↑1.4]	57.6 ±3.3** [↓12.8]

* $p \leq 0.05$; ** $p \leq 0.01$

6. Organ weight	Not conducted
7. Gross pathology:	No gross pathology changes were observed.
8. Histopathology:	Not conducted
9. Caesarean section data:	Pregnancy occurred in 24, 24, 25 and 25 rats in the 0, 40, 160 and 640 mg/kg bw/day dosage groups, respectively. The litter averages for *corpora lutea*, implantations, pre-implantation loss, early resorptions, and post-implantation loss were comparable among the three dose groups when compared with the concurrent control. There were no embryonic death in the control or test article treated groups. All placentae appeared normal.

Table CA 5.8.1/12-3: Overview of developmental toxicity study in rats treated orally (*via* gavage) with spiroxamine cyclohexyl acetate; selected caesarean section data

Parameters	(mg/kg bw/d)							
	0		40		160		640	
No. of animals mated	25		25		25		25	
Animals pregnant and caesarean section on GD 21	24		24		25		24	
Unscheduled deaths	0		0		0		1	
Corpora lutea [/dam]	378 [15.8 ±2.8]		377 [15.7 ±1.4]		396 [15.7 ±2.0]		395 [16.4 ±3.1]	
Implantation sites [/dam]	352 [14.7 ±2.5]		366 [15.2 ±1.5]		383 [15.3 ±1.8]		363 [15.1 ±3.2]	
Total no. of litters	25		24		25		24	
Total live fetus [/dam]	336 [14.0 ±2.4]		357 [14.9 ±1.8]		362 [14.5 ±2.9]		344 [14.3 ±3.5]	
Total live ♂ / ♀	159/177		174/183		182/180		161/183	
Total dead fetus	0		0		0		0	
Early/late resorptions [/dam]	16 / 0 [0.7 ±0.9 / 0.0 ±0.0]		9 / 0 [0.4 ±0.9 / 0.0 ±0.0]		21 / 0 [0.8 ±1.8 / 0.0 ±0.0]		18 / 2 [0.8 ±1.5 / 0.0 ±0.2]	
No. of dams with resorptions [%]	11 [45.8]		6 [25.0]		11 [44.0]		11 [45.8]	
	♂	♀	♂	♀	♂	♀	♂	♀
Fetal wt (g)	5.45 ±0.31	5.19 ±0.35	5.40 ±0.31	5.17 ±0.27	5.42 ±0.31	5.12 ±0.34	4.85 ±0.49**	4.58 ±0.44**
Mean fetal wt (g)	5.31 ±0.33		5.26 ±0.29 (↓1%)		5.27 ±0.30 (↓0.8%)		4.70 ±0.46** (↓11%)	
Sex ratio (%♂)	46.6 ±13.1		49.0 ±11.8		49.5 ±11.9		47.2 ±11.4	
% resorbed conceptuses/litter	4.3 ±5.9		2.5 ±5.8		6.1 ±14.9		5.3 ±11.1	

** $p < 0.01$

C. Developmental toxicity:

1. Body weights:	Statistically significant reductions in total fetal weights (both presented as combined sex and individual sex) were observed in the 640 mg/kg bw/day dose group, which were considered test-article related.
2. Sex ratio:	No test article related or statistically significant difference were observed in sex ratio between the vehicle control and test article treated groups.
3. External examination:	Fetal gross external alterations were limited to a single occurrence of an absent tail in the mid dose group. Skeletal examination of this fetus confirmed the gross absence of the tail which was evident as the presence of fewer than

normal ossified lumbar, sacral and caudal vertebrae (5, 1 and 0 respectively, compared to normal values of 6, 3 and 7).

Table CA 5.8.1/12-4: Overview of developmental toxicity study in rats treated orally (*via* gavage) with spiroxamine cyclohexyl acetate: overview of fetal abnormalities (fetus/litter incidence)

Parameters	♀ (mg/kg bw/d)			
	0	40	160	640
Total external, visceral and skeletal examinations (fetus/litter)				
Fetus/litters examined	336/24	357/24	362/25	344/24
Fetus/litter with any alteration (%)	8 (33.3)/9 (2.7)	11 (45.8)/12 (3.4)	6 (24.0)/7 (1.9)	7 (29.3)/18 (5.2)
% Fetus with any alteration/litter (%)	2.6 ±3.9	3.2 ±3.8	1.9 ±3.6	4.7 ±10.2
External examinations (fetus/litter)				
Fetus/litters examined	336/24	357/24	362/25	344/24
Abnormal findings	0/0	0/0	1/1	0/0
Visceral examinations (fetus/litter)				
Fetus/litters examined	161/24	170/24	174/25	166/24
Abnormal findings	7/7	6/6	1/1	9/3
Skeletal examination (fetus/litter)				
Litters examined	24/175	24/187	24/188	24/178
Abnormal findings	3/2	6/5	6/5	13/10

4. Visceral and skeletal observations:

Fetal alterations were defined as:

- malformations (irreversible changes that occur at low incidences in this species and strain)
- variations (common findings in this species and strain and reversible delays or accelerations in development).

Visceral examinations: Fetal evaluations were based on 161, 170, 147 and 166 live, GD 21 caesarean-delivered fetuses in 24, 24, 25, and 24 litters in dose groups 0, 40, 160 and 640 mg/kg bw/day dosage groups, respectively. Each of these fetuses were examined for visceral alterations, using the Wilson technique. In total soft tissue variations were limited to 3 tissues, with no malformations:

Eyes: one fetus in each of the 40 and 160 mg/kg bw/day dosage groups had a folded retina in the right eye. In the absence of a dose related response, and considering that this finding occurs as a result of artefact of fixation/sectioning, this was not deemed test article related. In the absence of a dose related response, this finding was not considered test article-related. No other alterations occurred in these fetuses.

Vessels: the umbilical artery descended to the left of the urinary bladder in 3, 1 and 1 fetus from separate litters in the 0, 40 and 160 mg/kg bw/day dosage groups, respectively. No other alterations occurred in these fetuses.

Kidneys: slight or moderate dilation of the pelvis of one or both kidneys was observed. This variation is considered to be reversible, typically common in rat strains. This variation occurred in 1, 2, 0 and 8 fetuses from 1, 2, 0 and 2 litters from each respective group. It is noted that the fetal incidence of moderate enlargement of the pelvis of both kidneys was significantly increased ($p \leq 0.01$) in the high dose group, compared to the concurrent vehicle control, however when assessed as litter incidence (the more relevant parameter), was not significantly increased. No other alterations occurred in these fetuses. The incidence observed in this study exceeded the laboratory' historical control range for fetal incidence, with litter incidence exceeding the concurrent vehicle control.

When examining the laboratory historical control data, of the 50 studies available, 10 litters and 11 fetuses (0.9% and 0.14%, respectively) had slight

dilation of the pelvis of one or both, with a maximum of 3 (13.6%) litters and 4 fetuses (2.6%)/study, with 3 litters (0.27%) and 3 fetuses (0.04%) having moderate dilation of the pelvis of one or both kidneys, with a maximum of 1 (4.5%) litters and 1 fetus (0.7%)/study.

Table CA 5.8.1/12-4: Overview of developmental toxicity study in rats treated orally (*via* gavage) with spiroxamine cyclohexyl acetate: overview of visceral variations (fetus/litter incidence)

Parameters	♀ (mg/kg bw/d)			
	0	40	160	640
Fetus/litters examined	161/24	170/24	174/25	166/24
Eyes : retina folded (%)	0/0 (0.0/0.0)	1/1 (4.2/0.6)	0/0 (0.0/0.0)	1/1 (4.2/0.6)
Vessels: umb. art. ↓ - L UB (%)	333 (12.5/1.9)	1/1 (4.2/0.6)	1/1 (4.0/0.6)	0/0 (0.0/0.0)
Kidneys: pelvis, sli. dilat. (%)	0/0 (0.0/0.0)	2/2 (8.3/1.2)	0/0 (0.0/0.0)	1/1 (4.2/0.6)
Kidneys: pelvis, mod. dilat. (%)	1/1 (4.2/0.6)	0/0 (0.0/0.0)	0/0 (0.0/0.0)	1/1 (4.2/4.2**)
Genitalia: testis, malpos. (%)	3/3 (12.5/1.9)	2/2 (8.3/1.2)	0/0 (0.0/0.0)	0/0 (0.0/0.0)
Laboratory historical control data (rat, SD June 2004 – June 2006)				
Kidneys: pelvis, sli. dilat.	*n*: 50 studies Fetus/litter evaluated: 7805/1112 Fetal incidence [*n*] (%): 11 (0.14%) [max. study incidence 4 (2.6%)] Litter incidence [*n*] (%): 10 (0.9%) [max. study incidence 3 (13.6%)]			
Kidneys: pelvis, mod. dilat.	*n*: 50 studies Fetus/litter evaluated: 7805/1112 Fetal incidence [*n*] (%): 3 (0.04%) [max. study incidence 1 (0.7%)] Litter incidence [*n*] (%): 3 (0.27%) [max. study incidence 1 (4.5%)]			

** p <0.01
umb. art. ↓ - L UB: umbilical artery descends to left of urinary bladder
pelvis, sli. dilat.: pelvis, slight dilation
pelvis, mod. dilat.: pelvis, moderate dilation
testis, malpos.: testis, malpositioned:

4. Visceral and skeletal observations (continued):

Skeletal examinations: Fetal evaluations were based on 175, 187, 188 and 177 live, GD 21 caesarean-delivered fetuses in 24, 24, 25, and 24 litters in dose groups 0, 40, 160 and 640 mg/kg bw/day dosage groups, respectively. Each of these fetuses were examined for skeletal alterations, after staining with alizarin red S. In total skeletal variations were limited to vertebrae and ribs, with no malformations:

Vertebrae: one of both arches in the 6th cervical vertebra had the appearance of an arch in the 7th cervical vertebra in 1, 1, 1 and 4 fetus from 1, 1, 1 and 2 fetuses in the 0, 40, 160 and 320 mg/kg bw/day, respectively. No other alterations occurred in these fetuses.

A bifid centrum in the 11th, 12th or 13th thoracic vertebra occurred in 0, 2, 2 and 3 fetuses all from separate litters in the respective 4 dosage groups. No other alterations occurred in these fetuses.

Ribs: presence of a cervical rib at the 7th cervical vertebra were observed in 1, 2, 2 from separate litters in the 0, 40 and 160 mg/kg bw/day dosage groups, respectively. This variation in common in this strain of rat. No other alterations occurred in these fetuses.

A single fetus (#19417/14) in the vehicle control group and two fetuses from the same litter (19486/1 and /8) in the 640 mg/kg bw/day dosage group had a short 13th rib. The vehicle control fetus, discussed above (#19417/14) also had a rib present on the 7th cervical vertebra. No other alterations occurred in the fetus in the 640 mg/kg bw/day dosage group.

No other skeletal variations occurred.

Table CA 5.8.1/12-5: Overview of developmental toxicity study in rats treated orally (*via* gavage) with spiroxamine cyclohexyl acetate: overview of skeletal variations (fetus/litter incidence)

Parameters	♀ (mg/kg bw/d)			
	0	40	160	640
Fetus/litters examined	175/24	187/24	188/25	178/24
Vertebrae:				
Cervi: Arch, 6th app. like 7th (%)	1/1 (4.2/0.6)	1/1 (4.2/0.5)	1/1 (4.0/0.5)	2/4 (8.3/2.2)
Cervi: cervi. rib at 7th cervi. vertebrae (%)	1/1 (4.2/0.6)	2/2 (8.3/1.1)	2/2 (8.0/1.1)	0/0 (0.0/0.0)
Thoracic: centrum, bifid (%)	0/0 (0.0/0.0)	2/2 (8.3/1.1)	2/2 (8.0/1.1)	3/3 (12.5/1.7)
Lumbar: 5 present (%)	0/0 (0.0/0.0)	0/0 (0.0/0.0)	1/1 (4.0/0.5)	0/0 (0.0/0.0)
Sacral: 1 present (%)	0/0 (0.0/0.0)	0/0 (0.0/0.0)	1/1 (4.0/0.5)	0/0 (0.0/0.0)
Caudal: 0 present (%)	0/0 (0.0/0.0)	0/0 (0.0/0.0)	1/1 (4.0/0.5)	0/0 (0.0/0.0)
Ribcage				
Ribs: short (%)	1/1 (4.2/0.6)	0/0 (0.0/0.0)	0/0 (0.0/0.0)	1/2 (4.2/1.1)
Manubrium: duplicate (%)	0/0 (0.0/0.0)	1/1 (4.2/0.5)	0/0 (0.0/0.0)	0/0 (0.0/0.0)
Sternal centra: duplicate (%)	0/0 (0.0/0.0)	1/1 (4.2/0.5)	0/0 (0.0/0.0)	0/0 (0.0/0.0)

Cervi: Arch, 6th app. like 7th: cervical vertebrae: arch, 6th has appearance like the 7th

Cervi: cervi. rib at 7th cervical vertebrae: cervical rib present at 7th cervical vertebrae

5. Fetal ossification: Reductions in the average number of ossified caudal vertebrae, forelimb phalanges and hind limb metatarsals and phalanges were significantly reduction in the 640 mg/kg bw/day dosage groups, compared to the concurrent control.

Forelimb phalanges: the average number of ossified sites, when expressed per fetus per litter (7.76) was within the historical control range of the testing facility.

Hind limb metatarsals: litter averages (4.48) below historical control range
Caudal vertebrae: litter averages (6.58) below historical control range
Hind limb phalanges: litter averages (5.40) below historical control range

There were no other statistically significant or biologically relevant difference among the four dosage groups in the average number of ossification sites/fetus for the hyoid, vertebrae (cervical, thoracic, lumbar, sacral), ribs sternum (manubrium, sternal centers, xiphoid) forelimbs (carpals, metacarpals) or hind limbs (tarsals).

Table CA 5.8.1/12-6: Overview of developmental toxicity study in rats treated orally (*via* gavage) with spiroxamine cyclohexyl acetate: overview of ossification site (/fetus/litter)

Parameters	♀ (mg/kg bw/d)			
	0	40	160	640
Fetus/litters examined	175/24	187/24	188/25	178/24
Hyoid	1.00 ±0.00	1.00 ±0.00	1.00 ±0.00	1.00 ±0.02
Vertebrae - Cervical - Thoracic	 7.00 ±0.00 13.07 ±0.10	 7.00 ±0.00 13.09 ±0.15	 7.00 ±0.00 13.08 ±0.15	 7.00 ±0.00 13.15 ±0.14

Parameters	♀ (mg/kg bw/d)			
	0	40	160	640
- Lumbar	5.92 ±0.11	5.90 ±0.15	5.91 ±0.15	5.85 ±0.14
- Sacral	3.00 ±0.00	3.00 ±0.00	3.00 ±0.00	3.00 ±0.00
- Caudal	7.64 ±0.63	7.59 ±0.62	7.64 ±0.70	6.58 ±0.69**
Ribs (pairs)	13.06 ±0.08	13.07 ±0.11	13.06 ±0.12	13.14 ±0.13
Sternum				
- Manubrium	1.00 ±0.00	1.00 ±0.00	1.00 ±0.00	1.00 ±0.00
- Sternal centers	3.99 ±0.04	3.99 ±0.03	4.00 ±0.00	3.99 ±0.04
- Xiphoid	1.00 ±0.00	1.00 ±0.00	1.00 ±0.00	1.00 ±0.00
Forelimb				
- Carpals	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00
- Metacarpals	3.99 ±0.04	4.00 ±0.00	4.00 ±0.00	4.00 ±0.00
- Digits	5.00 ±0.00	5.00 ±0.00	5.00 ±0.00	5.00 ±0.00
- Phalanges	8.20 ±0.62	8.39 ±0.43	8.28 ±0.59	7.76 ±0.84
Hind limb				
- Tarsals	0.04 ±0.11	0.02 ±0.06	0.04 ±0.11	0.00 ±0.00
- Metatarsals	4.86 ±0.18	4.84 ±0.17	4.79 ±0.23	4.48 ±0.38*
- Digits	5.00 ±0.00	5.00 ±0.00	5.00 ±0.00	5.00 ±0.00
- Phalanges	6.04 ±0.93	6.15 ±0.90	6.10 ±1.03	5.40 ±0.59*
Laboratory historical control data (rat, SD June 2004 – June 2006)				
Vertebrae - Caudal	*n:* Fetus/litter evaluated: Fetal incidence [*n*]:	50 studies 8312/1113 7.47 [study incidence 6.69 – 8.23]		
Hind limb - Metatarsals	*n:* Fetus/litter evaluated: Fetal incidence [*n*]:	50 studies 8312/1113 4.84 [study incidence 4.67 – 5.00]		
Hind limb - Phalanges	*n:* Fetus/litter evaluated: Fetal incidence [*n*]:	50 studies 8312/1113 6.23 [study incidence 5.54 – 7.75]		

* $p \leq 0.05$; ** $p < 0.01$

D. Deficiencies:

The test guideline OECD 414 was updated 2018, the following deficiencies were noted:

- Thyroid gland weights and histopathological assessment of every dam treated dam not performed.
- Thyroid hormone measurements of dams not undertaken.
- Anogenital distance of fetuses not performed.

E. Discussion:

Based on the results of this study, spiroxamine cyclohexyl administered to rats at a dose of 640 mg/kg bw/day caused maternal mortality, body weight losses (up to 9.2% reduction), reduced body weight gains (32%) and food consumption values at a dosage level that exceeded the MTD. The incidence of excessive salivation at dose level of 160 and 640 mg/kg bw/day (moderate and severe, respectively) was evident compared to the concurrent control group.

Pregnancy occurred in 24, 24, 25 and 25 rats in the 0, 40, 160 and 640 mg/kg bw/day dosage groups, respectively. The litter averages for *corpora lutea*, implantations, pre-implantation loss, early resorptions, and post-implantation loss were comparable among the three dose groups when compared with the concurrent control. There were no embryonic death in the control or test article treated groups. All placentae appeared normal.

Statistically significant reductions in total fetal weights (both presented as combined sex and individual sex) were observed in the 640 mg/kg bw/day dose group, which were considered test-article related. No test article related or statistically significant difference were observed in sex ratio between the vehicle control and test article treated groups.

Visceral examinations were based on 161, 170, 147 and 166 live, GD 21 caesarean-delivered fetuses in 24, 24, 25, and 24 litters in dose groups 0, 40, 160 and 640 mg/kg bw/day dosage groups, respectively. Slight or moderate dilation of the pelvis of one or both kidneys was observed. This variation is considered to be reversible, typically common in rat strains. This variation occurred in 1, 2, 0 and 8 fetuses from 1, 2, 0 and 2 litters from each respective group. It is noted that the fetal incidence of moderate enlargement of the pelvis of both kidneys was significantly increased ($p \leq 0.01$) in the high dose group, compared to the concurrent vehicle control, however when assessed as litter incidence (the more relevant parameter), was not significantly increased. No other alterations occurred in these fetuses. The incidence observed in this study exceeded the laboratory' historical control range for fetal incidence, with litter incidence exceeding the concurrent vehicle control.

No treatment related skeletal alterations were observed.

Skeletal examinations were based on 175, 187, 188 and 177 live, GD 21 caesarean-delivered fetuses in 24, 24, 25, and 24 litters in dose groups 0, 40, 160 and 640 mg/kg bw/day dosage groups, respectively. In total skeletal variations were limited to vertebrae and ribs, with no malformations. Reductions in the average number of ossified caudal vertebrae, forelimb phalanges and hind limb metatarsals and phalanges were significantly reduction in the 640 mg/kg bw/day dosage groups, compared to the concurrent control. There were no other statistically significant or biologically relevant difference among the four dosage groups in the average number of ossification sites/fetus for the hyoid, vertebrae (cervical, thoracic, lumbar, sacral), ribs, sternum (manubrium, sternal centers, xiphoid) forelimbs (carpals, metacarpals) or hind limbs (tarsals).

Assessment and conclusions by applicant:

Assessment: Study meets the current guidance and the requirements in 283/2013.

Conclusion: Under the conditions of this study, the NOAEL for maternal toxicity was considered to be 40 mg/kg bw/day based maternal mortality, body weight losses, reduced body weight gains and food consumption values at a dosage level that exceeded the MTD (640 mg/kg bw/day).

The developmental NOAEL was considered to be 160 mg/kg bw/day based on transient retardations in fetal development, with reduction in fetal bodyweight and associated significant increases in moderate dilation of the renal pelvis and delayed ossification of the caudal vertebrae, fore- and hind limb phalanges and metatarsals. These retardations occurred at a dosage level of 640 mg/kg bw/day, which exceeded the MTD for maternal animals.

Data Point:	KCA 5.8.1/13
Report Author:	[redacted]; [redacted]
Report Year:	2013
Report Title:	4-tert-butylcyclohexyl acetate: Biotransformation in plasma from male and female rats
Report No:	EnSa-13-1118
Document No:	M-472817-01-1
Guideline(s) followed in study:	Regulation (EC) No 1107/2009 (Europe) amended by the Commission Regulation (EU) No 283/2013 (Europe) US EPA OCSPP not applicable
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated, not accepted RAR (2017) Some data not reported: validity of analytical methods, animal source, number; duration; concentrations of solutions
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Supportive only

Executive Summary

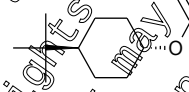
In order to understand the metabolic fate of 4-tert-butylcyclohexyl acetate in mammalian systems it was incubated with rat plasma and the reaction products were identified.

Under the conditions of this study, 4-tertbutylcyclohexylacetate, is readily hydrolysed in plasma of male and female rats to 4-tertbutylcyclohexanol.

Materials and Methods

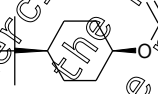
A. Materials:

1. **Test Material (non-labelled):** Trans-4-tert-butylcyclohexyl acetate

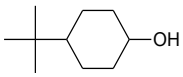


Description: Colourless liquid
Lot/Batch No.: BCS-CX98424-01-01
Purity: 99.6% w/w
CAS No.: Not available
Stability of test compound: Confirmed stable for the duration of the study (expiry date: 2014-05-07)

2. **Test Material (non-labelled):** Cis-4-tert-butylcyclohexyl acetate



Description: Colourless liquid
Lot/Batch No.: BCS-AH21306-01-01
Purity: 99.8% w/w
CAS No.: Not available
Stability of test compound: Confirmed stable for the duration of the study (expiry date: 2014-05-27)

3. Reference Compound (non-labelled):	4-Tert-butylcyclohexanol (M-13) 
Description:	White solid
Lot/Batch No.:	BCS-AC12532-01-01
Purity:	99.3% w/w
CAS No.:	Not available
Stability of test compound:	Confirmed stable for the duration of the study (expiry date: 14 March 2014)
3. Vehicle and/or positive control	None /nor relevant
4. Test system:	
Species:	Rat
Strain:	Wistar
Plasma	A single ♂ and ♀ rat were anaesthetised then sacrificed by transection of the cervical vessels and exsanguination. Blood was collected in heparinised tubes and separated into plasma and red blood cells by centrifugation. Plasma was stored frozen prior to use on the study.
6. Preparation of dosing solutions:	Stock solutions of both isomers of test compound were prepared in acetonitrile.

B. Study Design and Methods:

1. Experimental dates:	18 December 2013 to 20 December 2013 (experimental dates)
2. Incubation:	Incubations were performed by adding *ca* 10 µL of the respective stock solution to *ca* 2 mL plasma from male and female rats and the incubation vials were gently shaken and placed in a water bath at 37°C. Aliquots were removed immediately and at 15 and 60 minutes after the start of the experiment. The reaction was stopped by the addition of acetonitrile and formic acid to the removed aliquots and the samples centrifuged. To the supernatant was added dichloromethane:water (5:6 v/v) and following phase separation an aliquot of the organic phase was directly analysed by GC-MS.
3. Mass spectrometry (GC-MS):	Electron impact (EI) mass spectra were acquired on a TSQ Quantum GC spectrometer interfaced to a Trace GC Ultra. The GC retention time and mass spectra obtained from the test and reference items were compared to those obtained from samples of the incubate.

Results and Discussion

A. Biotransformation data:

The incubations of cis and trans 4-tertbutylcyclohexylacetate with plasma from male and female rats clearly showed that both compounds were significantly transformed to 4-tertbutylcyclohexanol, with evidence that the transformation started immediately after contact with plasma.

B. Deficiencies:

None.

Assessment and conclusions by applicant:

Assessment: This study is deemed acceptable and meets the requirements in 283/2013.

Conclusion: Under the conditions of this study, 4-tertbutylcyclohexylacetate, is readily hydrolysed in plasma of male and female rats to 4-tertbutylcyclohexanol.

Spiroxamine-aminodiol (M28)

Data Point:	KCA 5.8.1/14
Report Author:	[redacted]
Report Year:	2013
Report Title:	Amendment 1 to the final report - KWG 4168-aminodiol - Acute oral toxicity study in rats (up and down procedure)
Report No:	13/165-001P
Document No:	M-462551-02-1
Guideline(s) followed in study:	OECD 425; EEC Directive 440/2008, B.1.tris; US-EPA 712-C-98-190, OPPTS 870.1100
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted RAR (2010)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The method used to investigate the acute oral toxicity of spiroxamine aminodiol was the up and down procedure (OECD 425). The test article was formulated in deionised water and administered to a single female Han Wistar rat, orally *via* gavage at 550 mg/kg bw (employing a dose volume of 10 mL/kg bw). As no death was observed, a further female rat was dosed as previously described at a dose level of 2000 mg/kg bw. This proceed repeated until in total 3 and 4 female rats were dosed at 550 and 2000 mg/kg bw, respectively. The observation period was 14 days post exposure.

Clinical signs of toxicity were limited to animals dosed at 2000 mg/kg bw. Observations included signs which were reflective of CNS toxicity, manifest as decreased activity, hunched back, red discharge from eye, piloerection and death.

Three of the four animals dosed at 2000 mg/kg bw died prior to scheduled sacrifice. Two animals were found dead 1 day post dosing, with the remaining animal found dead 6 hours post dosing.

Macroscopic findings were limited to animals dosed at 2000 mg/kg bw. Diffused red discolouration of the glandular stomach and/or duodenum, jejunum, ileum, caecum or colon in 2/3 animals found dead. Other reported sign were considered related to test article administration (*i.e.* test article / diet in gastrointestinal tract).

Clinical signs of toxicity occurred shortly after administration, lasting until a maximum of either 5 or 3 days for animals dosed at 550 or 1000 mg/kg bw, respectively. Observations included signs which were reflective of CNS toxicity.

A single animal from the 550 mg/kg bw and four animals from the 1000 mg/kg bw group died within 1 day of dosing. No gross necropsy was performed.

Under the conditions of this study the acute oral LD_{50} for spiroxamine N-oxide was 707 mg/kg bw in female rats. Therefore, according to Annex I for Regulation (EC) 1272/2008 spiroxamine N-oxide must be classified in Category 4. The signal word "Warning" and hazard statement H302 "Harmful if swallowed" are required.

Materials and Methods

A. Materials:

1. Test Material:	KWG 4168-aminodiol (alternative name: Spiroxamine aminodiol, [3-ethyl(propyl)amino]propane-1,2-diol, M28)
Description:	Colourless liquid
Lot/Batch No.:	4310612/0780313
Purity:	98.9% (w/w) (correction not applied)
CAS No.:	Not assigned
Stability of test compound:	Confirmed stable for the duration of the study (expiry date: 8 November 2013)
2. Vehicle and/or positive control:	Distilled water / not relevant
3. Test animals:	
Species:	Rat
Strain:	Han Wistar
Age at dosing:	13 wks
Weight at dosing:	♀: 190 – 239g
Source:	[redacted]
Acclimation period:	At least 27 days
Diet:	ssniff® SM R/M 'Autoclavable complete diet for rats and mice – breeding and maintenance', *ad libitum* (fasted overnight prior to dosing)
Water:	Municipal water, *ad libitum*
Housing:	Group housed during acclimatisation. Individually housed during study period
4. Environmental conditions:	
Temperature:	21 ±3°C
Humidity:	30-70%
Air changes:	12-20 changes/h
Photoperiod:	12 h light/dark cycle

B. Test Performance:

1. In life dates:	11 June 2013 to 10 July 2013 (experimental dates)
2. Animal assignment and treatment:	Based on information provided by the client, a single, fasted rat received the test article formulated in distilled water at a dose level of 550 mg/kg bw, and administered orally *via* gavage employing a dose volume of 10 mL/kg bw. Further doses of 2000 and 550 mg/kg bw were employed using the up and down dosing procedure.
3. Statistics:	Data was evaluated using the acute oral toxicity (OECD 425) statistical Programme (AOT 425 Stat Pgm).

C. Methods:

1. Homogeneity and achieved concentration analysis of the dose:	Not conducted
2. Test article formulation preparation:	The test article was formulated in deionised water on the day of dose administration at 200 and 55 mg/mL. Homogeneity was achieved by the dose preparation being stirred with magnetic stirrer during the dosing window. No correction for purity was taken into account.
3. Observations:	Animals were observed several times on the day of dose administration (30 minutes, 1, 2, 3, 4 hours post dosing), with daily observation up until day 14.

3. Body weights:	Weighed on the day before dosing, the day of dosing and then weekly thereafter.
4. Food consumption:	Not recorded.
5. Sacrifice and pathology:	All animals were killed at terminal sacrifice, with gross necropsy performed (cranial, thoracic, abdominal cavities opened and organs inspected)

Results

A. Homogeneity and achieved concentration analysis:

Not conducted.

B. Observations:

1. Clinical signs of toxicity:	Clinical signs of toxicity were limited to animals dosed at 2000 mg/kg bw. Observations included signs which were reflective of CNS toxicity, manifest as decreased activity, hunched back, red discharge from eye, piloerection and death. No clinical signs of toxicity were observed in animals dosed at 550 mg/kg bw.
2. Mortality:	Refer to Table CA 5.8.1/14-1. Three of the four animals dosed at 2000 mg/kg bw died prior to scheduled sacrifice. Two animals were found dead 1 day post dosing, with the remaining animal found dead 6 hours post dosing.

C. Body weight and food consumption:

1. Body weight:	No indication of effects on body weight were observed.
2. Food consumption:	Not applicable.

Table CA 5.8.1/14-1: Overview of acute oral toxicity study in rats treated with spiroxamine aminodiol: mortality and body weight

Parameter	♀ (mg/kg bw) 550			♀ (mg/kg bw) 2000		
Mortality[a]	0/3			3/4		
Day	1	7	15	0	7	14
Body weight (g) ±s.d.	199.7 ±8.4	222.7 ±13.5	229.3 ±17.6	227.0 ±13.8	226.0	242.0
Net body weight gain (g)	30.7 ±11.9			15.0		
Acute oral LD_{50}	>550 <2000 mg/kg bw (95% confidence interval 614 – 5110 mg/kg bw)					

a Mortality: no. of animals found dead / no. of animals treated

D. Necropsy:

Macroscopic findings were limited to animals dosed at 2000 mg/kg bw. Diffused red discolouration of the glandular stomach and/or duodenum, jejunum, ileum, caecum or colon in 2/3 animals found dead. Other reported sign were considered related to test article administration (*i.e.* test article / diet in gastrointestinal tract).

E. Deficiencies:

None.

Assessment and conclusion by applicant:

Assessment: Study meets the current guidance and the requirements in 283/2013.

Conclusion: Under the conditions of this study the acute oral LD_{50} of spiroxamine aminodiol was >550, but <2000 mg kg/bw in female rats (95% confidence interval 614.6 to 5110 mg/kg bw). Therefore, according to Annex I for Regulation (EC) 1272/2008 spiroxamine aminodiol must be

classified in Category 4. The signal word "Warning" and hazard statement H302 "Harmful if swallowed" are required.

Data Point:	KCA 5.8.1/15
Report Author:	[redacted]
Report Year:	2013
Report Title:	KWG 4168-aminodiol: Salmonella typhimurium reverse mutation assay
Report No:	1558901
Document No:	M-463413-01-1
Guideline(s) followed in study:	OECD 471; Commission Regulation (EC) No. 440/2008, B13/14; US-EPA 712-C-98-247, OPPTS 870.5100
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted RAR (2010)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

In a reverse gene mutation assay in bacteria, *S. typhimurium* strains TA98, TA1537, TA100, TA1535 and TA102 were exposed to spiroxamine aminodiol (M28) formulated in deionized water using the both the plate incorporation and pre-incubation methodologies in the absence and presence of an phenobarbital/β-naphthoflavone-induced rat liver post-mitochondrial fraction (S9).

In the plate incorporation assay treatments of the tester strains were performed in the absence and presence of S9 using final concentrations of spiroxamine aminodiol at 3, 10, 33, 100, 333, 100, 2500 and 5000 µg/plate (the maximum recommended concentration according to current regulatory guidelines). No evidence of precipitate was observed upon plating. Following these treatments, no evidence of toxicity (*i.e.* slight thinning of the background bacterial lawn or reduction in revertant numbers) was observed in all the tester strains in the absence and presence of S9. Following spiroxamine aminodiol treatments of all the test strains in the absence and in the presence of S9, no increases in revertant numbers were observed that were ≥2-fold (TA98, TA100, TA102); ≥3-fold (TA1535, TA1537) above the concurrent vehicle control.

In the pre-incubation assay treatments of the tester strains were performed in the absence and presence of S9 using final concentrations of spiroxamine aminodiol at 33, 100, 333, 100, 2500 and 5000 µg/plate (the maximum recommended concentration according to current regulatory guidelines). No evidence of precipitate was observed upon plating. Following these treatments, no evidence of toxicity (*i.e.* slight thinning of the background bacterial lawn or reduction in revertant numbers) was observed in all the tester strains in the absence and presence of S9. Following spiroxamine aminodiol treatments of all the test strains in the absence and in the presence of S9, no increases in revertant numbers were observed that were ≥2-fold (TA98, TA100, TA102); ≥3-fold (TA1535, TA1537) above the concurrent vehicle control.

Following spiroxamine aminodiol treatments of all the test strains in the absence and in the presence of S9, no increases in revertant numbers were observed that were ≥2-fold (TA98, TA10, TA102); ≥3-fold (TA1535, TA1537) above the concurrent vehicle control in either experiment.

Vehicle and positive control treatments were included for all strains in both experiments. The mean numbers of revertant colonies were comparable with acceptable ranges for vehicle control treatments. The positive controls induced an acceptable increase in revertant colony numbers, thereby demonstrating the sensitivity and specificity of the test system.

It was concluded that spiroxamine aminodiol did not induce mutation in five histidine-requiring strains (TA98, TA100, TA1535, TA1537 and TA102) of *Salmonella typhimurium* when tested under the conditions of this study. These conditions included treatments at concentrations up to 5000 µg/plate (the maximum recommended concentration according to current regulatory guidelines), in the absence and in the presence of a rat liver metabolic activation system (S9) or up to concentration limited by toxicity using both the plate incorporation and pre-incubation methodologies.

Materials and Methods

A. Materials:

1. Test Material: KWG 4158-aminodiol
(alternative name: Spiroxamine aminodiol, [3-ethyl(propyl)amino]propane-1,2-diol, M28)

Description: Colourless liquid
Lot/Batch No.: AE 1344304-01-01
Purity: 98.9% (w/w) (correction not applied)
CAS No.: Not assigned
Stability of test compound: Confirmed stable for the duration of the study (expiry date: 8 November 2013)

2. Control materials:

Negative:
Solvent/final concentration: Deionised water/0.1 or 0.05 mL

Positive: -S9

Strain	*Mutagen*	*Conc. (µg/plate)*
TA98, TA1537	4-nitro-*o*-phenylene-diamine (4-NOPD)	10, 50
TA100, TA1535	Sodium azide (NaN_3)	10
TA102	Methyl methane sulphonate (MMS)	2

Positive: +S9

Strain	*Mutagen*	*Conc. (µg/plate)*
TA98	Benzo[a]pyrene (B[a]P)	5
TA98, TA100, TA1535, TA1537 TA102	2-aminoanthracene[18] (2-AA)	2.5, 10 (TA102)

3. Activation: S9 was prepared in house from Wistar rats treated with phenobarbital/β-naphthoflavone (protein content 44.9 mg/mL). Each batch of S9 was checked for metabolising capacity using reference mutagens.

The composition of the 10% S9 reaction mix was: S9 (10%), $MgCl_2$ 8 mg/mL); KCl (33 mM), glucose-6-phosphate (5 mM), NADP (4 mM).

4. Test organisms: *S. typhimurium* strains: TA98, TA100, TA1535, TA1537, TA102
All test organisms were properly maintained and were checked for appropriate genetic markers (*S. typhimurium*: histidine and biotin requirement, *rfa* mutation, *uvrB* sensitivity, ampicillin-resistance) regularly.

5. Test Concentrations:

c) Mutation assay 1: *Plate incorporation +/-S9 all strains*:
0, 3, 10, 33, 100, 333, 1000, 2500, 5000 µg/plate

d) Mutation assay 2: *Pre-incubation +/-S9 all strains*

18 Of note, some researchers have suggested that 2-AA should not be used as the only positive control to evaluate S9-mix activity as it has been shown that the chemical may be activated by enzymes other than the microsomal cytochrome P450 family. In this study, S9 activity was evaluated with 2-AA. In addition, each batch was checked for sterility, protein content, ability to convert B[a]P to reactive mutation. Therefore, there is no concern over S9 activity.

0, 33, 100, 333, 1000, 2500, 5000 µg/plate

B. Test Performance:

1. In life dates:	25 April 2019 to 8 May 2019 (experimental dates)
2. Experiment 1:	*Plate incorporation assay:* The following sequence of additions of 2 mL of supplemented molten top agar, test article solution/vehicle or positive control (0.1 mL), either 0.1 M Na phosphate buffer (0.5 mL pH 7.4) or S9 mix (0.5 mL) for treatments in the absence and in the presence of metabolic activation, respectively, and bacterial suspension (0.1 mL) were mixed and poured on to Vogel-Bonner E agar plates (minimal glucose agar plate). When set, plates were inverted and incubated at 37°C, protected from light, for 2 days.
3. Experiment 2:	*Pre-incubation assay:* The test article solution or vehicle/positive control solution (0.1 mL), bacteria (0.1 mL) and S9 mix (0.5 mL) were mixed in a small test tube and incubated for 60 minutes at 37°C. After additional of 2 mL of top agar solution the mixture was poured onto a minimal glucose agar plate and allowed to solidify. All plates were incubated for 2 days at 37°C. In this way, it was hoped to increase the range of mutagenic chemicals that could be detected using this assay system. For each experiment triplicate plating was undertaken for each dose level.
4. Statistics:	None applied.
5. Acceptance criteria:	The assay was considered valid if the following criteria were met: 6. The vehicle controls fell within the laboratory's historical control ranges 7. The positive control chemicals induced a significant increase in revertant numbers 8. A minimum of 5 analysable dose concentration were available.
5. Evaluation criteria:	The test article was considered mutagenic in this assay if: 1. A concentration related increase in revertant numbers was ≥2-fold (TA98, TA100, TA102), ≥3-fold (TA1535, TA1537) above the concurrent vehicle control value. 2. Any observed response was reproducible under the same treatment conditions. The test article was considered positive in this assay if all the above criteria were met The test article was considered negative in this assay if none of the above criteria were met.

C. Methods:

1. Homogeneity and achieved concentration analysis of the dose:	Not undertaken.
2. Test article formulation preparation:	A preliminary solubility test confirmed spiroxamine aminodiol was soluble in deionised water at concentrations equivalent to 50 mg/mL. Thereby, confirming a maximum concentration of 50 mg/mL could be prepared and dosed into the test system at a maximum concentration of 5000 µg/plate. Test article stock solutions were prepared by formulating spiroxamine cyclohexanol in deionised water and neutralised with HCl 2N. Subsequent dilutions were made using deionised water. The test article solutions were used within 2 h of initial formulation.
3. Toxicity Assessment:	The background lawns of the plates were examined for signs of toxicity Revertant plate count data were also assessed, as a marked reduction in revertants compared to the concurrent vehicle controls were also considered as evidence of toxicity with a reduction in the number of revertants below the indication factor of 0.5.

4. Scoring: The number of revertant colonies were counted with a colony counter (Petri Viewer Mk2, Perceptive Instruments). Individual plate counts were recorded separately and the mean and standard deviation of the plate counts for each treatment were determined. Control counts (vehicle/positive) were compared with the laboratory's historical control range. The purpose of the vehicle historical control range was to assess strain characteristics, and not to interpret or justify a negative or positive result[3].

Results and Discussion

A. Analytical determinations:

Not undertaken. Analyses for achieved concentration, homogeneity or stability of test article formulations were not conducted as part of this study, as this is not a requirement of the regulatory test guidelines.

B. Mutation experiment 1:

In the plate incorporation assay treatments of the tester strains were performed in the absence and presence of S9 using final concentrations of spiroxamine aminodiol at 3, 10, 33, 100, 333, 100, 2500 and 5000 µg/plate (the maximum recommended concentration according to current regulatory guidelines). No evidence of precipitate was observed upon plating. Following these treatments, no evidence of toxicity (*i.e.* slight thinning of the background bacterial lawn or reduction in revertant numbers) was observed in all the tester strains in the absence and presence of S9. Following spiroxamine aminodiol treatments of all the test strains in the absence and in the presence of S9, no increases in revertant numbers were observed that were ≥2-fold (TA98, TA100, TA102); ≥3-fold (TA1535, TA1537) above the concurrent vehicle control.

Table CA 5.8.1/15-1: Spiroxamine aminodiol: bacterial reverse gene mutation data (mean revertant colonies): Mutation experiment 1 (plate incorporation)

Type of mutation	Frame-shift				Base-pair substitution					
Conc. (µg/plate)	TA98		TA1537		TA100		TA1535		TA102	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0	30 ±1	48 ±5	10 ±3	19 ±5	112 ±17	131 ±10	15 ±1	17 ±3	343 ±12	553 ±86
3	28 ±4	48 ±4	10 ±3	21 ±3	116 ±8	123 ±3	16 ±2	15 ±5	320 ±8	481 ±45
10	32 ±9	46 ±4	8 ±1	23 ±9	123 ±19	137 ±8	12 ±2	17 ±6	351 ±4	515 ±30
33	25 ±3	39 ±5	12 ±4	18 ±3	117 ±10	132 ±9	14 ±2	18 ±2	360 ±13	555 ±13
100	31 ±8	49 ±11	10 ±4	17 ±5	119 ±16	138 ±4	14 ±2	18 ±2	345 ±6	555 ±41
333	35 ±3	56 ±8	10 ±3	23 ±1	123 ±9	128 ±8	15 ±5	21 ±7	301 ±19	451 ±5
1000	38 ±11	49 ±9	11 ±3	22 ±7	110 ±5	144 ±18	13 ±7	19 ±3	342 ±17	513 ±20
2500	28 ±6	48 ±5	10 ±4	18 ±4	112 ±9	150 ±12	13 ±3	15 ±3	342 ±5	526 ±43
5000	31 ±3	52 ±7	7 ±3	21 ±1	112 ±20	145 ±8	10 ±3	20 ±4	330 ±15	518 ±25
Positive control	265 ±34	2732 ±346	163 ±2	426 ±38	2301 ±47	3353 ±159	1805 ±2	433 ±33	5100 ±422	2693 ±336

Untreated control data not included as the vehicle used is common to the Ames assay
Positive controls:
-S9: strains:
TA98, TA1537: 4-NOPD
TA100, TA1535: NaN_3
TA102: MMS

S: slight thinning of background lawn
T: Toxic, no revertant colonies

+S9: strains:
All strains: 2-AA

B. Mutation experiment 2:

In the preincubation assay, treatments of the tester strains were performed in the absence and presence of S9 using final concentrations of spiroxamine aminodiol at 33, 100, 333, 100, 2500 and 5000 µg/plate (the maximum recommended concentration according to current regulatory guidelines). No evidence of precipitate was observed upon plating. Following these treatments, no evidence of toxicity (*i.e.* slight thinning of the background bacterial lawn or reduction in revertant numbers) was observed in all the

tester strains in the absence and presence of S9. Following spiroxamine aminodiol treatments of all the test strains in the absence and in the presence of S9, no increases in revertant numbers were observed that were ≥2-fold (TA98, TA100, TA102); ≥3-fold (TA1535, TA1537) above the concurrent vehicle control.

Following spiroxamine aminodiol treatments of all the test strains in the absence and in the presence of S9, no increases in revertant numbers were observed that were ≥2-fold (TA98, TA100, TA102); ≥3-fold (TA1535, TA1537) above the concurrent vehicle control.

Vehicle and positive control treatments were included for all strains in both experiments. The mean numbers of revertant colonies were comparable with acceptable ranges for vehicle control treatments. The positive controls induced an acceptable increase in revertant colony numbers, thereby demonstrating the sensitivity and specificity of the test system.

Table CA 5.8.1/15-2: Spiroxamine N-oxide: bacterial reverse gene mutation data (mean revertant colonies): Mutation experiment 2 (pre-incubation)

Type of mutation	Frame-shift				Base-pair substitution					
Conc. (µg/plate)	TA98		TA1537		TA100		TA1535		TA102	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0	24 ±6	43 ±12	10 ±3	25 ±3	108 ±10	149 ±6	14 ±2	18 ±2	405 ±21	523 ±70
33	27 ±1	49 ±12	11 ±3	28 ±6	119 ±11	142 ±6	15 ±7	19 ±3	380 ±22	531 ±42
100	28 ±6	43 ±10	10 ±2	22 ±2	117 ±9	149 ±7	14 ±4	17 ±4	405 ±13	592 ±17
333	26 ±5	52 ±4	8 ±2	30 ±7	115 ±28	144 ±4	13 ±2	20 ±1	405 ±34	595 ±50
1000	27 ±7	39 ±5	11 ±2	22 ±2	111 ±4	143 ±12	14 ±1	19 ±0	348 ±18	521 ±8
2500	25 ±7	50 ±7	9 ±2	27 ±1	106 ±7	139 ±4	14 ±4	18 ±4	373 ±12	563 ±21
5000	31 ±2	47 ±4	9 ±1	25 ±2	104 ±9	133 ±10	13 ±4	19 ±3	354 ±22	597 ±24
Positive control	365 ±18	2996 ±276	68 ±7	464 ±19	2081 ±109	3989 ±129	1970 ±74	381 ±71	3780 ±117	3255 ±264

Untreated control data not included as the vehicle used is common to the Ames assay

Positive controls:
-S9: strains:
TA98, TA1537: 4-NOPD
TA100, TA1535: NaN_3
TA102: MMS

S: slight thinning of background lawn
T: Toxic, no revertant colonies

+S9: strains
All strains: 2-AA

C. Discussion:

In two independent experiments and in all strains in the absence and presence of a rat liver metabolic activation system (S9) no increases in revertant numbers were observed that were ≥2-fold (in strains TA98, TA100 and TA102) or ≥3-fold (in strains TA1535 and TA1537) the concurrent vehicle control. This study was therefore considered to have provided no evidence of any spiroxamine aminodiol mutagenic activity in this assay system.

The positive controls induced an acceptable increase in revertant colony numbers, thereby demonstrating the sensitivity and specificity of the test system.

Table CA 5.8.1/15-3: Bacterial reverse gene mutation data (mean revertant colonies): historical control ranges

Type of mutation	Frame-shift				Base-pair substitution					
Parameter	TA98		TA1537		TA100		TA1535		TA102	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Vehicle control										
Mean ±SD	30 ±5.60	40 ±6.08	12 ±3.31	16 ±4.34	142 ±29.42	156 ±29.4	14 ±2.37	20 ±3.75	380 ±43.64	502 ±89.8
Min	17	21	5	7	86	99	9	11	305	321
Max	47	58	26	30	243	249	23	35	510	677

Type of mutation	Frame-shift				Base-pair substitution					
Parameter	TA98		TA1537		TA100		TA1535		TA102	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
Positive control										
Mean ±SD	372 ±78.05	2167 ±717.60	88 ±38.92	342 ±144.31	1741 ±488.75	2642 ±796.59	1751 ±226.44	362 ±93.12	502 ±89.88	2329 ±598.00
Min	158	249	61	77	569	825	710	126	321	1091
Max	595	4089	448	809	3082	4503	2385	703	677	3972

Historical control data generated from January 2011 to December 2011. Based on ca. 550 experiments (for TA102 ca. 200 experiments)
Positive controls:
-S9: strains:
TA98, TA1537: 4-NOPD
TA100, TA1535: NaN_3
TA102: MMS
+S9: strains:
All strains: 2-AA

D. Deficiencies:

It is noted that OECD TG 471 has been recently updated (29 June 2020). However, the updated test guideline has only included a correction to a CAS number of an example positive control –S9 for *E.coli* strain WP2*uvr*A. Therefore, it is reasonable to conclude that this study, whilst conducted in accordance with the test guideline issued in 1997, is also in accordance with the updated test guideline.

Assessment and conclusion by applicant:

Assessment: Study meets the current guidance and the requirements in 283/2013.

Conclusion: It was concluded that spiroxamine aminodiol did not induce mutation in five histidine-requiring strains (TA98, TA100, TA1535, TA1537 and TA102) of *Salmonella typhimurium* when tested under the conditions of this study. These conditions included treatments at concentrations up to 5000 µg/plate (the maximum recommended concentration according to current regulatory guidelines), in the absence and in the presence of a rat liver metabolic activation system (S9) or up to concentration limited by toxicity using both the plate incorporation and pre-incubation methodologies.

Data Point:	KCA 5.8.1/16
Report Author:	[redacted]
Report Year:	2013
Report Title:	KWG4168-aminodiol: Gene mutation assay in Chinese hamster V79 cells in vitro (V79/HPRT)
Report No:	1558902
Document No:	M-465292-01-1
Guideline(s) followed in study:	OECD 476; Commission Regulation (EC) No. 440/2008, B17; US-EPA 712-C-98-221, OPPTS 870.5300; JEPA Kanpoan No. 287; JMHW Eisei No. 127; JMITI Heisei 09/10/31 Kikyoku No. 2; JMAFF Notification No. 12 Nousan-8147
Deviations from current test guideline:	Yes Although the study was conducted according to test guideline OECD 476 (1997), this test guideline has since been updated in the intervening period (2016). When assessed against current test guideline requirements, a number of deficiencies are noted (refer to Results, Deficiencies section below)
Previous evaluation:	yes, evaluated and accepted RAR (2010)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive summary

In a mammalian cell gene mutation assay, V79 Chinese hamster cells were exposed to spiroxamine aminodiol formulated in deionised water. Forward mutation at the hypoxanthine-guanine-phosphoribosyl-transferase (*hprt*) gene locus was measured. The study consisted of a preliminary cytotoxicity assay followed by a Mutation Experiment each conducted in the absence (4 and 24 hour) and presence (4 hour) of metabolic activation by a phenobarbital / β-naphthoflavone-induced rat liver post-mitochondrial fraction (S9).

The test article was formulated in deionised water and dosed at 10% v/v.

Cultures were exposed to spiroxamine aminodiol at concentrations from 50.9 - 1630 µg/mL. No precipitate (assessed by eye at the end of treatment) was observed in any of the treatment conditions. Concentrations of 101.9 – 1630 µg/ml were assessed for determination of MF in both the short term treatments in the absence and presence of S9 with RCE values from 99.4 – 96.6% and 101.0 – 84.6%, respectively were obtained relative to the vehicle control. In the extended treatment in the absence of S9, concentrations of 203.9 – 1630 µg/mL were assessed for determination of MF. RCE values from 101.6 – 72.5% were obtained relative to the vehicle control. There were no increases in the MF of any of the test concentrations assessed that exceeded 3-fold the concurrent vehicle control MF that were observed in any of the treatment conditions.

DMBA and EMS were used as positive controls and showed distinct and biologically relevant effects in mutation frequency, thus demonstrating the sensitivity and specificity of the test system.

It is concluded that spiroxamine aminodiol did not show any increases in the mutant frequency of Chinese Hamster V79 cells at the *hprt* locus when tested up to a concentration of 1603 µg/L (maximum recommended concentration equivalent to 10 mM in accordance with the current regulatory test guideline for the *in vitro* gene mutation assay) in the absence and presence of a rat liver metabolic activation system (S9).

Materials and Methods

A. Materials:

1. Test Material: Spiroxamine aminodiol
(alternative name: KWG 4158-aminodiol, [3-ethyl(propyl)amino]propane-1,2-diol, AE 134430, technical, M28)

Description:	Colourless liquid
Lot/Batch #:	AE 1344304-01-01
Purity:	98.9% (w/w) (correction not applied) (molecular weight: 161.24 g/mol)
CAS #:	Not assigned
Stability of test compound:	Confirmed stable for the duration of the study (expiry date: 8 November 2013)

2. Control materials -

Vehicle / final concentration:		Deionised water / 10% (v/v)
Positive:	**-S9**	Ethyl methane sulphonate (EMS, 150 µg/mL)
	+S9	7,12-dimethylbenz(*a*)anthracene (DMBA; 1.1 µg/mL)

3. Activation: S9[19] was purchased from a commercial source. Sprague Dawley rats were treated with phenobarbital / β-naphthoflavone, prepared in house (lot no.: 080313, 220313, protein content 31.4, 38.4 mg/mL respectively. The composition of the S9 reaction mix was: glucose-6-phosphate (G6P: 5 mM), β-Nicotinamide adenine dinucleotide phosphate (NADP: 4 mM), potassium chloride (KCl: 33 mM), magnesium chloride ($MgCl_2$: 8 mM). The final protein concentration in the S9 cofactor solution was 0.75 mg/mL. The final concentration of rat liver S-9 in the test system was 2.25% (v/v).

4. Test cells: Chinese hamster V79 cells were stored as frozen stocks in liquid nitrogen. Each batch was purged of *hprt*- mutants, checked for spontaneous mutant frequency and that it was mycoplasma free. Doubling time 12-16 hours.

5. Culture medium: Complete culture medium: MEM supplemented with fetal bovine serum (10% v/v), 100 U/100 µg/mL penicillin/streptomycin, 2 mM L-glutamine, 25 mM HEPES, 2.5 µg/mL amphotericin B. Used for seeding and post treatment.

Treatment medium: for short term exposure identical to complete culture medium but no fetal bovine serum. For long term exposure identical to complete culture medium.

Selective medium: complete culture medium supplemented with 6-TG (11 µg/mL)

6. Locus examined Chinese hamster V79 *hprt* (hypoxanthine-guanine-phosphoribosyl-transferase) locus cells were obtained from Laboratory for Mutagenicity Testing, Technical University, Germany. The selection agent was 6-thioguanine (6-TG).

7. Test article concentrations:

a)	**Preliminary cytotoxicity assay:**	4 h -/+S9; 24 h –S9: 12.7, 25.5, 50.9, 101.9, 203.8, 407.5, 815, 1630 µg/mL (maximum recommended concentration, equivalent to 10 mM)
b)	**Mutation assays:**	Experiment 1: 4h -/+S9: 0, 50.9, 101.9, 203.8, 407.5, 815, 1630 µg/mL 24 h -S9: 0, 50.9, 101.9, 203.8, 407.5, 815, 1222.5, 1630 µg/mL Experiment 2: 4 h+S9: 0, 50.9, 101.9, 203.8, 407.5, 815, 1630 µg/mL (concentrations underlined were scored)

B. Test Performance:

1. In life dates:	11 June 2013 to 15 August 2013 (experimental dates)
2. Vehicle selection:	Spiroxamine aminodiol was soluble at 16.3 mg/mL in deionised water. When dosed at 10% v/v, a suitable volume:volume addition for aqueous vehicles, a maximum concentration of 1630 µg/mL (maximum recommended

19 In this study, S9 activity was evaluated with CPA. In addition, each batch was checked by the laboratory with known positive controls requiring metabolic activation, B[a]P and 2-AA with TA98. Therefore, there is no concern over S9 activity.

concentration, equivalent to 10 mM) was selected for the initial cytotoxicity Range-Finder Experiment in order that treatments were performed up to a suitable maximum concentration.
Osmolality and pH assessments of the test article in cell culture medium were undertaken for the preliminary cytotoxicity study.

3. Statistics: Linear regression was performed to assess a possible dose dependent increase in MF ($p \leq 0.05$).

4. Acceptance criteria: *For test article*: The highest concentration tested was one that allowed the maximum exposure up to 2000 µg/mL or 10 mM for freely soluble compounds, or the limit of toxicity (*i.e.* relative cloning efficiency (RCE) reduced to ~10 - 20% of the concurrent vehicle control) or the limit of solubility. For a toxic substance, at least 4 analysable concentrations should have been achieved which ideally spanned the toxicity range of 100 - 10% RCE.
For vehicle controls: The mean vehicle control value for mutant frequency (MF) fell within the laboratory's historical control range.
The mean cloning efficiency was ~50%.
For positive controls: Positive controls showed a statistically significant increase in mean total MF above the mean concurrent vehicle control MF, and remained within the laboratory's historical control range.

5. Evaluation criteria: The criteria for determining a positive result:
- a reproducible three times higher MF than the solvent control for at least one of the concentrations;
- a concentration related increase of the MF; such an evaluation may be considered also in the case that a three-fold increase of the MF was not observed;
- if there is by chance a low spontaneous mutation rate in the corresponding negative and solvent controls a concentration related increase of the mutations within their range has to be discussed.

C. Methods:

1. Homogeneity and achieved concentration analysis of the dose: Not conducted.

2. Cell treatment: *Preliminary cytotoxicity assay*: 1.5 *x* 10^6 cells/culture were establish and grown for 24 hours, and then were exposed to test article or solvent control for 4 h (in the absence and presence of S9) and 24 h (absence of S9) thereby *ca.* 6.0 x 10^6 cells/culture. After 4 or 20 h the treatment medium containing the test article was removed and the cells were washed twice with PBS. Subsequently complete medium (MEM supplemented with 10% FBS) was added. During the following expression period the cells of the logarithmic growing culture were sub-cultured 48 to 72 h after treatment. For toxicity criteria the cell density of every concentration tested was measured with a cell counter and adjusted to 1 x 10^6 cells/mL.
Mutation assay: The procedure for preparing the cell suspension was the same as for the preliminary toxicity test, with 5 *x* 10^2 cells/culture establish and grown for 24 hours, and then were exposed to test article, solvent or positive controls for 4 h (in the absence and presence of S9) and 24 h (absence of S9) thereby *ca.* 20 *x* 10^2 cells/culture. At the end of the expression period for selection the mutants, about 4 *x* 10^5 cells from each treatment group, were seeded in cell culture Petri dishes (diameter 90 mm) with selection medium containing 11 µg/mL thioguanine (TG) for further incubation (about one week). At the end of the selection period, colonies were fixed and stained for counting.
The cloning efficiencies (CE) were determined in parallel to the selection of mutants. For each treatment group two 25 cm^2 flasks were seeded with approx. 200 cells to determine cloning efficiencies. After incubation for an appropriate time colonies were fixed with methanol, stained with Giemsa and counted

Results and Discussion

A. Analytical determinations:

Not undertaken. Analyses for achieved concentration, homogeneity or stability of test article formulations were not conducted as part of this study, as this is not a requirement of the regulatory test guidelines.

B. Preliminary cytotoxicity assay:

No precipitate, observed by eye was observed at the end of treatment at concentrations up to 1630 µg/mL in any of the treatments undertaken. Exposure to spiroxamine aminodiol at concentrations from 12.7 to 1630 µg/mL resulted in relative cloning efficiency (RCE) values from 96.3 to 86.4%, 100 to 82.6% and 96.9 to 12.7% for the 4 hour –S9, 4 hour +S9 and 24 hour –S9 treatments, respectively.

No marked changes in osmolality or pH were observed at the highest three concentrations tested as compared to the concurrent vehicle controls (individual data not reported).

The results of the cytotoxicity preliminary cytotoxicity range-finder experiment were used to select suitable maximum concentrations for the mutation experiment.

Table CA 5.8.1/16-1: Spiroxamine aminodiol: Chinese hamster V79 mammalian cell preliminary cytotoxicity range experiment

Dose level (µg/mL)	4 h –S9 RCE (%)	4 h +S9 RCE (%)	24 h –S9 RCE (%)
0	100	100	100
12.7	95.2	100.8	96.9
25.5	96.3	99.7	96.4
50.9	95.7	98.7	96.6
101.9	91.5	92.4	92.4
203.8	88.6	94.7	96.2
407.5	87.6	91.5	91.8
815	88.7	84.4	57.1
1630	86.4	82.6	12.6

C. Mutation assay:

1. **Short term treatments in the absence and presence of S9:** Cultures were exposed to spiroxamine aminodiol at concentrations from 50.9 - 1630 µg/mL. No precipitate (assessed by eye at the end of treatment) was observed. Concentrations of 101.9 – 1630 µg/mL were assessed for determination of MF. RCE values from 99.4 – 96.6% and 101.0 – 84.6 were obtained relative to the vehicle control for the –S9 and +S9 treatments, respectively. There were no increases in the MF of any of the test concentrations assessed that exceeded 3-fold the concurrent vehicle control MF.
2. **24 hour -S9:** Cultures were exposed to spiroxamine aminodiol at concentrations from 50.9 - 1630 µg/mL. No precipitate (assessed by eye at the end of treatment) was observed. Concentrations of 203.9 – 1630 µg/mL were assessed for determination of MF. RCE values from 101.6 – 72.5% were obtained relative to the vehicle control. There were no increases in the MF of any of the test concentrations assessed that exceeded 3-fold the concurrent vehicle control MF.
3. **Positive controls:** Vehicle and positive control treatments were included in the Mutation assays in the absence and presence of S9. MF in vehicle control cultures fell within acceptable ranges, and clear increases in mutation were induced by the positive control chemicals MMS (without S9) and DMBA (with S9). Therefore, the study was accepted as valid.

Table CA 5.8.1/16-2: Spiroxamine aminodiol: Chinese hamster V79 mammalian cell mutation test: toxicity and mutation data (group mean values)

Dose level (µg/mL)	Expt 1 4h –S9		Expt 1 4h +S9	
	RCE (%)	MF	RCE (%)	MF
0	100	19.8	100	17.6
101.9	99.4	8.2	93.4	16.8
203.8	97.8	12.9	100.0	8.4
407.5	97.8	24.3	95.0	20.8
815	96.6	18.0	101.0	8.8
1630	97.9	6.25	98.4	17.5
Positive control	98.7	129.5	84.6	983.3

Dose level (µg/mL)	Expt 2 24h –S9		Expt 2 4h +S9	
	RCE (%)	MF	RCE (%)	MF
0	100	13.3	100	7.4
101.9	-	-	101.4	5.6
203.8	91.4	9.8	94.0	10.0
407.5	91.1	6.4	101.6	12.5
815	87.9	12.5	99.5	13.4
1222.5	95.6	11.4	-	-
1630	86.0	9.15	72.5	12.8
Positive control	88.1	353.1	98.9	284.8

Mutant frequency: mutants/10^6 viable cells

Positive control: -S9: EMS, +S9: DMBA

Table CA 5.8.1.3/3-3: Spiroxamine aminodiol: Chinese hamster V79 mammalian cell mutation test: laboratory historical control range (2011 – 2012)

Parameter	4 hour –S9		4 hour +S9		24 h –S9	
	Vehicle	EMS 150 µg/mL	Vehicle	DMBA 1.1 µg/mL	Vehicle	EMS 150 µg/mL
No. of studies	75	75	75	75	60	60
Mean ±sd	17.2 ±8.3	131.0 ±75.5	15.5 ±6.9	723.8 ±342.9	16.0 ±8.4	357.6 ±275.0
Obs. range	2.6 – 43.5	54.8 – 889.0	3.4 – 44.2	91.4 – 2666.3	2.4 – 40.5	124.0 – 2746.9

D. Deficiencies:

Although the study was conducted according to test guideline OECD 476 (1997), this test guideline has since been updated in the intervening period (2016). When assessed against current test guideline requirements the following deficiencies are noted:

- An insufficient number of cells were treated in the mutation assay (20 *x*10^2 cells/culture) compared to the test guideline requirements (20 *x* 10^6 cells/culture), however spontaneous mutant frequency rate obtained for the vehicle controls (7.4 – 19.8 mutants *per* 10^6 viable cells) was comparable to current test guideline recommendations (5 – 20 mutants *per* 10^6 viable cells).
- The acceptable range that the laboratory used for acceptance criteria were observed ranges, without a confidence interval applied (ideally 95%). Consequently, the observed range mutant frequency values presented are wide and varying. This raises concerns over the laboratory's ability to detect genetic drift in the cell line.

In conclusion, the data generated under this study are still considered valid.

Assessment and conclusions by applicant:

Assessment: Study meets the current guidance and the requirements in 283/2013.

Conclusion: It is concluded that spiroxamine aminodiol did not show any increases in the mutant frequency of Chinese Hamster V79 cells at the *hprt* locus when tested up to a concentration of 1603 µg/L (maximum recommended concentration, equivalent to 10 mM in accordance with the current regulatory test guideline for the *in vitro* gene mutation assay) in the absence and presence of a rat liver metabolic activation system (S9).

Data Point:	KCA 5.8.1/17
Report Author:	[redacted]
Report Year:	2013
Report Title:	KWG 4168-aminodiol: Micronucleus test in human lymphocytes in vitro
Report No:	1558903
Document No:	M-469334-01-1
Guideline(s) followed in study:	OECD 487 (2010); Commission Regulation (EU) No 640/2012, B49 (2012)
Deviations from current test guideline:	Yes Although the study was conducted according to test guideline OECD 487 (2010), this test guideline has since been updated in the intervening period (2016). When assessed against current test guideline requirements, a number of deficiencies are noted (refer to Results, Deficiencies section below).
Previous evaluation:	yes, evaluated and accepted RAR (2010)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Supportive only

Executive summary

Spiroxamine aminodiol was tested in an *in vitro* micronucleus assay using duplicate human lymphocyte cultures prepared from the pooled blood of a single female donor. Treatments covering a broad range of concentrations, separated by narrow intervals, were performed both in the absence and presence of metabolic activation (S9) from phenobarbital/β-naphthoflavone -induced rats. The test article was formulated in deionised water and the highest concentration tested in the micronucleus experiment, 1610 µg/mL (maximum recommended concentration, equivalent to 10 mM), was determined following a preliminary cytotoxicity range-finder experiment. All test article concentrations, formulated in deionised water were dosed into the test system at 10% v/v.

Treatment of cells with spiroxamine aminodiol for 4 hours (+36 hour recovery) in the absence and presence of S9 and 20 hours (+20 hour recovery) in the absence of S9 resulted in MNBN cells that were similar to and not significantly ($p \leq 0.05$) higher than those observed in concurrent vehicle control cultures for all concentrations analysed.

The positive controls MMC (-S9), DMC (-S9) and CPA (+S9) induced a statistically significant increase in %MNBN in all treatment conditions thereby demonstrating the sensitivity and specificity of the assay under these treatment conditions.

It is concluded that spiroxamine aminodiol did not induce biologically relevant increases in the frequency of micronuclei in human peripheral blood lymphocytes when tested up to 1610 µg/mL (equivalent to 10 mM) in the absence (4 hours (+36 hour recovery) and 20 hours(+20 hour)) and presence (4 hours (+36 hour)) of rat liver metabolic activation system.

Materials and Methods

A. Materials:

1. **Test Material:** Spiroxamine aminodiol
(alternative name: KWG 4158-aminodiol, [3-ethyl(propyl)amino]propane-1,2-diol, AE 134430, technical, M28)

Description:	Colourless liquid
Lot/Batch #:	NLL 9095-4-3
Purity:	98.9% (w/w) (correction not applied) (molecular weight: 161.24 g/mol)
CAS #:	Not assigned
Stability of test compound:	Confirmed stable for the duration of the study (expiry date: 8 November 2013)

2. Control materials -

Vehicle / final concentration:		Deionised water / 10% (v/v)
Positive:	**-S9**	Mitomycin C (MMC, 2 µg/mL) Demecolcin (DMC, 0.175 µg/mL)
	+S9	Cyclophosphamide (CPA, 12.5, 15 µg/mL)

3. Activation: S9[20] was purchased from a commercial source. ♂ Sprague Dawley rats were treated with phenobarbital/β-naphthoflavone, prepared in house (lot no.: 080313, 220313, protein content 31.4, 38.4 mg/mL, respectively). The composition of the S9 reaction mix was: glucose-6-phosphate (G6P: 5 mM); β-Nicotinamide adenine dinucleotide phosphate (NADP: 4 mM), potassium chloride (KCl: 33 mM), magnesium chloride ($MgCl_2$: 8 mM). The final protein concentration in the S9 cofactor solution was 0.75 mg/mL. The final concentration of rat liver S9 in the test system was 5% (v/v).

4. Test organisms: Human peripheral blood lymphocytes were collected from a single healthy, non-smoking ♀ donor aged 29 years, pooled and diluted with HML media. Cells were stimulated to divide by the addition of phytohaemagglutinin (PHA).

5. Culture medium: Dulbecco's modified Eagles medium/Ham's F12 (DMEM/F12 1:1) supplemented with 200 mM GlutaMAX™, 10% (v/v) inactivated fetal bovine serum, 0.52% penicillin/streptomycin (100 U/mL/100 µg/mL), PHA (3 µg/mL), heparin (125 USP-U/mL)

6. Test article Concentrations:

a)	**Preliminary cytotoxicity test:**	4 h (+36h recovery) -/+S9; 20 h (+20 h) –S9: 0, 10.5, 18.3, 32, 56.1, 98.1, 171.7, 300.4, 525.7, 920, 1610 µg/mL (maximum recommended concentration, equivalent to 10 mM)
b)	**Micronucleus assay:**	Experiment 1: 4 h (+26h recovery) -/+S9: 0, 10.5, 18.3, 32, 56.1, 98.1, 171.7, 300.4, 525.7, 920, 1610 µg/mL Experiment 2: 4 h (+26h recovery) +S9; 20 h (+20 h) –S9: 0, 10.5, 18.3, 32, 56.1, 98.1, 171.7, 300.4, 525.7, 920, 1610 µg/mL (concentrations underlined scored for micronucleus frequency)

B. Test Performance:

1. In life dates: Not stated

2. Vehicle selection: Spiroxamine aminodiol was soluble at 16.1 mg/mL in deionised water. When dosed at 10% v/v, a suitable volume:volume addition for aqueous vehicles, a maximum concentration of 1610 µg/mL (maximum recommended concentration, equivalent to 10 mM) was selected for the initial cytotoxicity Range-Finder Experiment in order that treatments were performed up to a suitable maximum concentration.

Changes in osmolality of more than 50 mOsm/kg and fluctuations in pH of more than one unit may be responsible for an increase in chromosome aberrations. Osmolality and pH measurements on post-treatment incubation medium were taken in all experiments.

3. Statistics: The proportion of MNBN cells for each treatment condition were compared with the proportion in vehicle controls by using Chi-square analysis. Linear trend test applied to assess for dose response were not detailed.

4. Acceptance criteria: The following acceptance criteria had to be met for assay acceptability:

20 In this study, S9 activity was evaluated with CPA. In addition, each batch was checked by the laboratory with known positive controls requiring metabolic activation, B[a]P and 2-AA with TA98. Therefore, there is no concern over S9 activity.

1. The binomial dispersion test demonstrated acceptable heterogeneity (in terms of MNBN cell frequency) between replicate cultures, particularly where no positive responses were seen;
2. The frequency of MNBN cells in vehicle controls fell within the current 95th percentile of the observed historical vehicle control (normal) ranges;
3. The positive control chemicals induced statistically significant increases in the proportion of cells with micronuclei. Both replicate cultures at the positive control concentration analysed under each treatment condition demonstrated MNBN cell frequencies that clearly exceeded the normal range;
4. A minimum of 50% of cells had gone through at least one cell division (as measured by binucleate + multinucleate cell counts) in vehicle control cultures at the time of harvest;
5. The maximum concentration analysed under each treatment condition met the specified criteria (*i.e.* the highest concentration selected for micronucleus analysis following all treatment conditions was one at which 50-60% cytotoxicity was achieved).

5. Evaluation criteria:

For valid data the test article was considered to induce clastogenic and/or aneugenic events if:

1. A statistically significant increase in the frequency of MNBN cells at one or more concentrations was observed;
2. An incidence of MNBN cells at such a concentration that exceeded the normal range in both replicates was observed;
3. A concentration-related increase in the proportion of MNBN cells was observed (positive trend test).

The test article was considered positive in this assay if all of the above criteria were met.

The test article was considered negative in this assay if none of the above criteria were met.

C. Methods:

1. Homogeneity and achieved concentration analysis of the dose:

Not undertaken.

2. Preliminary cytotoxicity assay:

Whole blood cultures were established by placing 11% heparinised blood into DMEM/F12 containing 10% (v/v) heat inactivated fetal bovine serum and penicillin/streptomycin, so that the final volume following addition of S9 mix/KCl and the test article in its chosen vehicle was 10 mL. The mitogen, phytohaemagglutinin (PHA, reagent grade) was included in the culture medium at a concentration of approximately 3% of culture to stimulate the lymphocytes to divide. Blood cultures were incubated at 37±1°C for approximately 48 hours and rocked continuously. S9 mix or KCl (1 mL/culture) was added appropriately. Cultures were treated with the test article or vehicle (1 mL/culture) in serum free media. Positive control treatments were not included. Duplicate cultures were used for the vehicle control for each test article treated concentration.

For removal of the test article, cells were pelleted and resuspended in fresh pre-warmed medium containing fetal calf serum and penicillin/streptomycin and cultured for 16 hours. After this period Cytochalasin-B was added and the cells cultured for a further 20 hours to give a final concentration of 4 µg/mL/culture to inhibit cytokinesis, resulting in binucleate cells (without effecting karyokinesis), thereby arresting cells in interphase (equivalent to 2.5*x* the doubling time)

Cytotoxicity was determined by examination of the proportion of mono, bi and multinucleate cells to a minimum of 500 cells/concentration. From this, the

cytokinesis block proliferation index (CPBI) was calculated for each concentration (see Cytotoxicity section below).

3. Micronucleus assay:

Cell treatment: Cells were exposed to the test article formulation, vehicle or positive control as described above, with the exception that a continuous treatment in the absence of S9 for 20 hours was also undertaken, with cyto-B added to the cultures at the end of the exposure period with cultures undergoing a recovery period of 20 hours. Concentrations selected for micronucleus analysis were assessed for cytotoxicity uncoded and for the proportions of mono-, bi- and multinucleate cells to a minimum of 500 cells/culture.

Spindle inhibitor: Cyto-B was added at either 16 hours post end of treatment or immediately post-wash to cultures to inhibit cytokinesis for the short term or continuous treatments, respectively.

Slide preparation: Slides were prepared by spreading the fixed cultures on clean slides. The slides were stained with Giemsa dropped on to slides, coverslipped and scored prior to analysis.

Cytotoxicity: The replication index (RI), which indicates the relative number of nuclei compared to controls, was determined using the formula below:

$$CBPI = \frac{(\text{no. of mononucleate cells}) + (2 \times \text{no. of binucleate cells}) + (3 \times \text{no. of multinucleate cells})}{\text{total no. of cells in treated cultures}}$$

$$\%\text{cytostasis} = 100 - 100 \{(CBPI_T - 1) / (CBPI_C - 1)\}$$

Micronucleus assessment: Slides from the highest selected concentration and two lower concentrations were taken for microscopic analysis.

One thousand binucleate cells from each culture (total of 2000 binucleate cells/concentration) were analysed for micronuclei. The number of cells containing micronuclei and the number of micronuclei/cell on each slide were noted.

Nucleoplasmic bridges (NPBs) between nuclei in binucleate cells which provide an indication of chromosome rearrangement resulting from various mechanisms which may lead to NPB formation following DNA misrepair of strand breaks in DNA in this assay were not recorded.

Results and Discussion

A. Analytical determinations:

Not undertaken. Analyses for achieved concentration, homogeneity or stability of test article formulations were not conducted as part of this study, as this is not a requirement of the regulatory test guidelines.

B. Preliminary cytotoxicity assay:

Data were not reported.

C. Micronucleus assay:

1. Assay acceptability: The frequency of MNBN cells in vehicle controls fell within the normal ranges. The positive control chemicals induced statistically significant increases in the proportion of MNBN cells. Both replicate cultures at the positive control concentration analysed under each treatment condition demonstrated MNBN cell frequencies that clearly exceeded the normal range, thereby demonstrating the sensitivity and specificity of the assay. A minimum of 50% of cells had gone through at least one cell division (as measured by mononucleate + binucleate + multinucleate cell counts) in vehicle control cultures at the time of harvest. The maximum concentration analysed under each treatment condition met the criteria. The assay data were therefore considered valid and acceptable.

2. Short term treatment in the absence and presence of S9:

Treatment of cells with spiroxamine aminodiol for 4 hours (+36 hour recovery) in the absence and presence of S9 resulted in MNBN cells that were similar to and not significantly ($p \leq 0.05$) higher than those observed in concurrent vehicle control cultures for all concentrations analysed.

Osmolality and pH measurements were taken, with pH adjustment undertaken with 1N HCl at concentrations of 525.7 µg/mL and above to achieve a pH of between 7.4-7.6. However, it was not detailed what the pH measurements were prior to correction or when the measurements were undertaken.

Table CA 5.8.1/17-1: Spiroxamine aminodiol: human lymphocyte micronuclei assay: experiment 1, 4 h (+ 36 h recovery) –S9 and historical control ranges

Conc. (µg/mL)	4 h (+ 36 h recovery) –S9				
	Total BN	Total MN-BN	Frequency of MN-BN (%)	CBPI	Cytotoxicity based on CBPI (%)
0	2000	10	0.50	2.12	-
525.7	2000	8	0.40	2.13	-
920	2000	9	0.45	2.14	-
160	2000	5	0.25	1.56	50.1
MMC	2000	667	33.35*	1.30	73.6
Laboratory historical control data (2009 – 2012)					
	Vehicle %MNBN ranges		**Positive (MMC) %MNBN ranges**		
No. of expts	35		26		
Mean ±SD	0.38 ±0.28		11.27 ±5.67		
min. – max.	0.15 – 1.40		3.60 – 25.10		

* $p<0.05$

Table CA 5.8.1/17-2: Spiroxamine aminodiol: human lymphocyte micronuclei assay: experiment 1, 4 h (+36 h recovery) +S9 and historical control ranges

Conc. (µg/mL)	4 h (+ 36 h recovery) –S9				
	Total BN	Total MN-BN	Frequency of MN-BN (%)	CBPI	Cytotoxicity based on CBPI (%)
0	2000	6	0.30	1.71	-
525.7	2000	1	0.05	1.73	-
920	2000	7	0.35	1.59	16.5
160	2000	2	0.10	1.62	12.4
MMC	2000	67	3.75*	1.31	56.4
Laboratory historical control data (2009 – 2012)					
	Vehicle %MNBN ranges		**Positive (CPA) %MNBN ranges**		
No. of expts	70		47		
Mean ±SD	0.66 ±0.35		5.19 ±2.22		
min. – max.	0.15 – 1.70		2.20 – 11.05		

* $p<0.05$

3. Short term treatment in the presence of S9 and continuous treatment in the absence of S9:

Treatment of cells with spiroxamine aminodiol for 4 hours (+36 hour recovery) in the presence of S9 and 20 hours (+20 hour recovery) in the absence of S9 resulted in MNBN cells that were similar to and not significantly ($p \leq 0.05$) higher than those observed in concurrent vehicle control cultures for all concentrations analysed.

Osmolality and pH measurements were taken, with pH adjustment undertaken with 1N HCl at concentrations of 525.7 µg/mL and above to achieve a pH of between 7.4-7.6. However, it was not detailed what the pH measurements were prior to correction or when the measurements were undertaken.

Table CA 5.8.1/17-3: Spiroxamine aminodiol: human lymphocyte micronuclei assay: experiment 2, short term and continuous treatments

Conc. (µg/mL)	4 h (+ 36 h recovery) +S9			20 h (+ 20 h recovery) –S9		
	CBPI	Cytotoxicity based on CBPI (%)	Frequency of MN-BN (%)	CBPI	Cytotoxicity based on CBPI (%)	Frequency of MN-BN (%)
0	1.79	-	0.50	2.15	-	0.65
525.7	1.75	3.9	0.95	2.10	4.6	1.15
920	1.88	-	0.50	2.11	3.3	0.75
1610	1.83	-	0.80	2.15	-	0.50
Positive control	1.42	46.9	8.75	1.64	44.6	10.30

Laboratory historical control data (2009 – 2012)		
	Vehicle %MNBN ranges	Positive (DMC) %MNBN ranges
No. of expts	34	28
Mean ±SD	0.39 ±0.27	3.12 ±1.22
min. – max.	0.05 – 1.45	1.40 – 6.105

* $p<0.05$

D. Deficiencies:

Although the study was conducted according to test guideline OECD 487 (2010), this test guideline has since been updated in the intervening period (2016). When assessed against current test guideline requirements the following deficiencies are noted:

- It is unclear why a total recovery period of 36 hours was selected for the short term treatments. With a typical doubling time of 14 hours for human lymphocytes, the recovery period of 4 hours post exposure is insufficient to allow an asynchronous cell population to go through 1.5 times cell cycle. With the prolonged recovery time, lymphocytes would have gone through 2.5 times cell cycling thereby missing cells in interphase.
- Concerns are raised over the sensitivity and specificity of the short term treatment in the absence of S9, with increases in micronuclei binucleate cells only observed with the positive control MMC at overtly high levels of cytotoxicity. Consequently, it is unclear if the assay is able to detect true clastogenicity/aneugenicity compared with structural chromosomal events secondary to cytotoxicity.
- Limited historical control data were presented, with mean and observed ranges only for the vehicle and positive control data without a confidence interval applied (ideally 95%).

For the reasons listed above, this study is deemed supplementary. Both the clastogenic and aneugenic endpoints have been adequately addressed with a robust, GLP up to date *in vitro* human peripheral blood lymphocyte micronucleus study (*CA* 5.8.1/24 [M-755220-02-1]).

Assessment and conclusion by applicant:

Assessment: For the reasons identified in the deficiencies section this study is deemed supplementary.

Conclusion: It is concluded that spiroxamine aminodiol did not induce biologically relevant increases in the frequency of micronuclei in human peripheral blood lymphocytes when tested up to 1610 µg/mL (equivalent to 10 mM) in the absence (4 hours (+36 hour recovery) and 20 hours(+20 hour)) and presence (4 hours (+36 hour)) of rat liver metabolic activation system.

Data Point:	KCA 5.8.1/24
Report Author:	[redacted]
Report Year:	2020
Report Title:	Spiroxamine aminodiol: In vitro human lymphocyte micronucleus assay
Report No:	8408577
Document No:	M-755220-02-1
Guideline(s) followed in study:	OECD (2016)
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

Spiroxamine aminodiol was tested in an *in vitro* micronucleus assay using duplicate human lymphocyte cultures prepared from the pooled blood of two male donors in two experiments. Treatments covering a broad range of concentrations, separated by narrow intervals, were performed both in the absence and presence of metabolic activation (S9) from Aroclor 1254-induced rats. The test article was formulated in water and the highest concentration tested in the micronucleus experiment, 1613 µg/mL (equivalent to 10 mM), was determined following a preliminary cytotoxicity range-finder experiment. . All test article concentrations, formulated in water were dosed into the test system at 10% *v/v*.

Following establishment of cultures, concentrations ranging from 200 to 1613 µg/mL were tested in the absence (3 hours + 21 hour recovery) and presence of S9 (3 h + 21 h), and following extended treatment in the absence of S9 (24 h + 24 h). The test article concentrations for micronucleus analysis were selected by evaluating the effect of spiroxamine aminodiol on the replication index (RI). Micronuclei were analysed at three concentrations and a summary of the data are presented below.

The frequency of MNBN cells in vehicle controls fell within the normal ranges. The positive control chemicals induced statistically significant increases in the proportion of MNBN cells. Both replicate cultures at the positive control concentration analysed under each treatment condition demonstrated MNBN cell frequencies that clearly exceeded the normal range, thereby demonstrating the sensitivity and specificity of the assay. A minimum of 50% of cells had gone through at least one cell division (as measured by binucleate + multinucleate cell counts) in vehicle control cultures at the time of harvest. The maximum concentration analysed under each treatment condition met the criteria. The assay data were therefore considered valid and acceptable.

Treatment of cells with spiroxamine aminodiol for 3 hours (+21 hour recovery) in the absence and presence of S9 resulted in MNBN cells that were similar to and not significantly ($p \leq 0.05$) higher than those observed in concurrent vehicle control cultures for all concentrations analysed.

Treatment of cells with spiroxamine aminodiol for 24 hours (+24 hour recovery) in the absence of S9 resulted in frequencies of MNBN cells that were significantly ($p \leq 0.05$) higher than those observed in concurrent vehicle control cultures for the highest two concentrations analysed (1000 and 1613 µg/mL, inducing 14% and 36% cytotoxicity. These increases were small such that the MNBN cell frequency of only a single replicate at 1613 µg/mL exhibited an MNBN cell value (0.9%) that exceeded the normal range (0 to 0.8%). However, evidence of a concentration-related increase (positive linear trend test) was apparent and as such, these small statistical increases may be considered of biological relevance and therefore the result is considered equivocal.

In the confirmatory 24 hours (+24 hour recovery) in the absence of S9, cells treated with spiroxamine aminodiol resulted in frequencies of MNBN cells that were similar to and not significantly ($p \leq 0.01$) higher than those observed in concurrent vehicle control cultures for the majority of concentrations

analysed (two of three concentrations analysed). The MNBN cell frequency of the majority of the test article treated cultures fell within the normal range. A single exception to this was observed at the intermediate concentration, 1000 µg/mL (inducing 21% cytotoxicity), where the MNBN cell value of one of the two replicate cultures marginally exceeded the normal range. However, this increase was small (0.9% versus normal range of 0 to 0.8%) and was not observed in the replicate culture or at the higher and lower concentrations analysed. Furthermore, the mean MNBN cell value at 1000 µg/mL (0.80%) fell within the normal range with no evidence of any concentration-related effect. As such, this small isolated increase was therefore not considered of biological relevance.

It is concluded that spiroxamine aminodiol induced small increases in the frequency of micronuclei in human peripheral blood lymphocytes when tested up to 1613 µg/mL (equivalent to 10 mM) for 24 hour (+24 hour recovery) treatment in the absence of an Aroclor-induced rat liver metabolic activation system (S9). However, these increases were not reproducible and according to current data interpretation strategies, may be considered of very low or no toxicological concern. Therefore these increases were considered of no biological relevance under the experimental conditions described. In the same test system, spiroxamine aminodiol did not induce increase in the frequency of micronuclei when tested up to 1613 µg/mL for 3 hour (+21 hour recovery) in the absence and presence of S9.

Materials and Methods

A. Materials:

1. Test Material:	Spiroxamine aminodiol (alternative name: [3-ethyl(propyl)amino]propane-1,2-diol, M28)
Description:	Clear amber oily liquid
Lot/Batch No.:	AE 1344304-PU-01
Purity:	96.6% (w/w) (correction not applied) (molecular weight: 161.24 g/mol)
CAS No.:	Not assigned
Stability of test compound:	Confirmed stable for the duration of the study (expiry date: 12 January 2023)
2. Control materials:	
Negative:	
Solvent/final concentration:	Water/10% (v/v)
Positive: -S9	Mitomycin C (MMC, 3 h: 0.3; 24 h : 0.20 µg/mL) [clastogenic control] Vinblastine (VIN, 24 h: 0.04 µg/mL) [aneugenic control]
Positive: +S9	Cyclophosphamide (CPA, 3 h: 7 µg/mL)
3. Activation:	S9[21] was purchased from a commercial source. ♂ Sprague Dawley rats were treated with Aroclor 1254 (supplied by [redacted] lot no.: 4029, protein content 3.7 mg/mL). The composition of the 10% S9 reaction mix was: 100 µL S9, Na PBS (100 µM), glucose-6-phosphate (5 µM), β-NADP (4 µM), $MgCl_2$ (8 µM), KCl (33 µM), water (to volume).
4. Test organisms:	Human peripheral blood lymphocytes were collected from 2 healthy, non-smoking adult donors aged between 23 and 30 years, pooled and diluted with RPMI media. Cells were stimulated to divide by the addition of phytohaemagglutinin (PHA).
5. Culture medium:	HEPES buffered RPMI supplemented with 10% (v/v) inactivated fetal calf serum, 0.52% penicillin-streptomycin cell culture.
6. Test article Concentrations:	

21 In this study, S9 activity was evaluated with CPA. In addition, each batch was checked by the manufacturer of S9 for sterility, protein content, ability to convert ethidium bromide, cyclophosphamide, B[a]P and AAN to bacterial mutagens, and cytochrome P-450-catalysed enzyme activities (alkoxyresorufin-*O*-dealkylase activities). Therefore, there is no concern over S9 activity.

a)	**Preliminary cytotoxicity test:**	3 h (+21 h recovery) -/+S9; 24 h (+24 h) –S9: 0, 5.852, 9.753, 16.26, 27.09, 45.15, 75.26, 125.4, 209.0, 348.4, 580.7, 967.8, 1613 µg/mL (maximum recommended concentration, equivalent to 10 mM)
b)	**Micronucleus assay:**	3 h (+21 h recovery) +/-S9: 0, 200, 400, 600, 800, 1000, 1200, 1400, 1613 µg/mL 24 h (+24 h) –S9: 0, 200, 400, 600, 800, 1000, 1200, 1400, 1613 µg/mL Confirmatory 24 h (+24 h): 0, 200, 400, 600, 800, 1000, 1200, 1400, 1613 µg/mL (concentrations underlined scored for micronucleus frequency)

B. Test Performance:

1. In life dates: 1 May 2019 to 18 July 2019 (experimental dates)

2. Vehicle selection: A preliminary solubility test confirmed spiroxamine aminodiol was soluble in water up to at least 17.15 mg/mL (not corrected for purity).
The solubility limit in culture medium was in excess of 1715 µg/mL, as indicated by a lack of precipitation at this concentration *ca.* 24 h after test article addition. A maximum concentration of 1613 µg/mL was selected for the cytotoxicity Range-Finder Experiment, in order that treatments were performed up to the maximum recommended concentration according to current regulatory guidelines (*i.e.* a concentration equivalent to 10 mM).
Concentrations for the micronucleus experiment were selected based on the results of the cytotoxicity range-finder experiment.
Test article stock solutions were prepared by formulating spiroxamine aminodiol under subdued lighting in water with the aid of vortex mixing and warming at 37°C, to give the maximum required treatment solution concentration. Subsequent dilutions were made using water. The test article solutions were protected from light and used within approximately 4 h of initial formulation.

3. Statistics: The proportion of MNBN cells for each treatment condition were compared with the proportion in vehicle controls by using Fisher's exact test. A Cochran-Armitage trend test was applied to each treatment condition. Probability values of $p \leq 0.05$ were accepted as significant.

4. Acceptance criteria: The following acceptance criteria had to be met for assay acceptability:

1. The binomial dispersion test demonstrated acceptable heterogeneity (in terms of MNBN cell frequency) between replicate cultures, particularly where no positive responses were seen;
2. The frequency of MNBN cells in vehicle controls fell within the current 95th percentile of the observed historical vehicle control (normal) ranges;
3. The positive control chemicals induced statistically significant increases in the proportion of cells with micronuclei. Both replicate cultures at the positive control concentration analysed under each treatment condition demonstrated MNBN cell frequencies that clearly exceeded the normal range;
4. A minimum of 50% of cells had gone through at least one cell division (as measured by binucleate + multinucleate cell counts) in vehicle control cultures at the time of harvest;
5. The maximum concentration analysed under each treatment condition met the specified criteria (*i.e.* the highest concentration selected for micronucleus analysis following all treatment conditions was one at which 50-60% cytotoxicity was achieved).

5. Evaluation criteria: For valid data, the test article was considered to induce clastogenic and/or aneugenic events if:

1. A statistically significant increase in the frequency of MNBN cells at one or more concentrations was observed;
2. An incidence of MNBN cells at such a concentration that exceeded the normal range in both replicates was observed;
3. A concentration-related increase in the proportion of MNBN cells was observed (positive trend test).

The test article was considered positive in this assay if all of the above criteria were met.
The test article was considered negative in this assay if none of the above criteria were met.

C. Methods:

1. Homogeneity and achieved concentration analysis of the dose:

Not undertaken.

2. Preliminary cytotoxicity assay:

Whole blood cultures were established by placing 0.4 mL of pooled heparinised blood into 8.5 mL pre-warmed HEPES-buffered RPMI medium containing 10% (v/v) heat inactivated fetal calf serum and 0.52% penicillin/streptomycin so that the final volume following addition of S9 mix/KCl and the test article in its chosen vehicle was 10 mL. The mitogen, phytohaemagglutinin (PHA, reagent grade) was included in the culture medium at a concentration of approximately 2% of culture to stimulate the lymphocytes to divide. Blood cultures were incubated at 37±1°C for approximately 48 hours and rocked continuously. S9-mix or KCl (1 mL/culture) was added appropriately. Cultures were treated with the test article or vehicle (0.1 mL/culture). Positive control treatments were not included. Duplicate cultures were used for the vehicle control and single cultures were used for each test article treated concentration.
For removal of the test article, cells were pelleted (approximately 300 g, 10 minutes), washed twice with sterile saline and resuspended in fresh pre-warmed medium containing fetal calf serum and penicillin/streptomycin. Cyto-B (formulated in DMSO) was added to post wash-off culture medium to give a final concentration of 6 µg/mL/culture to inhibit cytokinesis, resulting in binucleate cells (without effecting karyokinesis), thereby arresting cells in interphase.
Changes in osmolality of more than 50 mOsm/kg and fluctuations in pH of more than one unit may be responsible for an increase in chromosome aberrations. Osmolality and pH measurements on post-treatment incubation medium were taken in the cytotoxicity range-finder experiment.
Cytotoxicity was determined by examination of the proportion of mono, bi and multinucleate cells to a minimum of 500 cells/concentration. From this, the replication index (RI) was calculated for each concentration (see Cytotoxicity section below).

3. Micronucleus assay:

Cell treatment: Cells were exposed to the test article formulation, vehicle or positive control as described above, with the exception of quadruplicate cultures were used for the vehicle control and duplicate controls used for the positive controls and each test article concentration. Concentrations selected for micronucleus analysis were assessed for cytotoxicity uncoded and for the proportions of mono-, bi- and multinucleate cells to a minimum of 200 or 500 cells/culture (preliminary cytotoxicity range-finder or micronucleus experiment, respectively)./

Spindle inhibitor: Cyto-B was added post-wash to cultures to inhibit cytokinesis.

Slide preparation: Slides were prepared by spreading the fixed cultures on clean slides. The slides were stained with acridine orange (12.5 µg/mL) dropped on to slides, coverslipped and scored prior to analysis.

Cytotoxicity: The replication index (RI), which indicates the relative number of nuclei compared to controls, was determined using the formula below:

$$RI = \frac{\textit{no. of binucleate cells} + 2(\textit{no. of multinucleate cells})}{\textit{total no. of cells in treated cultures}}$$

Relative RI (expressed in terms of percentage) for each treated culture was calculated as follows:

$$\text{Relative RI (\%)} = \frac{\text{RI of treated cultures}}{\text{RI of vehicle control}} \times 100$$

Cytotoxicity (%) was expressed as (100 – Relative RI).

Micronucleus assessment:

Slides from the highest selected concentration and two lower concentrations were taken for microscopic analysis.

Immediately prior to analysis, 1-2 drops of PBS were added to the slides before mounting with glass coverslips. One thousand binucleate cells from each culture (total of 2000 binucleate cells/concentration) were analysed for micronuclei. The number of cells containing micronuclei and the number of micronuclei/cell on each slide were noted.

For the 24 hour (+24 hour recovery) treatment condition in the absence of S9 in Micronucleus Experiment 1, it was subsequently deemed necessary to analyse the two remaining vehicle control cultures, to aid data interpretation.

Nucleoplasmic bridges (NPBs) between nuclei in binucleate cells were recorded during micronucleus analysis to provide an indication of chromosome rearrangement. Various mechanisms may lead to NPB formation following DNA misrepair of strand breaks in DNA in this assay. Binucleate cells with NPBs were recorded as part of the micronucleus analysis.

Results

A. Analytical determinations:

Not undertaken. Analyses for achieved concentration, homogeneity or stability of test article formulations were not conducted as part of this study, as this is not a requirement of the regulatory test guidelines.

B. Preliminary cytotoxicity assay:

No test article precipitate was observed at any concentration tested. No marked changes in osmolality or pH were observed at the highest three concentrations tested as compared to the concurrent vehicle controls (individual data not reported).

The results of the cytotoxicity preliminary cytotoxicity range-finder experiment were used to select suitable maximum concentrations for the micronucleus experiment.

Table CA 5.8.1/24-1: Spiroxamine aminodiol: human lymphocyte preliminary cytotoxicity range finder experiment

Conc. (µg/mL)	3 h (+ 21 h recovery) –S9		3 h (+ 21h recovery) +S9		24 h (+ 24 h recovery) –S9	
	Replication index (RI)	Cytotoxicity based on RI (%)	Replication index (RI)	Cytotoxicity based on RI (%)	Replication index (RI)	Cytotoxicity based on RI (%)
0	0.93	-	0.91	-	0.96	-
5.852	0.89	5	0.92	3	0.99	0
9.753	0.91	2	0.88	6	0.91	5
16.26	0.84	9	0.86	4	1.04	0
27.09	0.80	14	0.87	2	1.01	0
45.15	0.91	2	0.89	0	0.98	0
75.26	0.72	23	0.91	0	0.97	0
125.4	0.89	4	0.92	0	1.02	0
209.0	0.88	6	0.93	0	1.07	0
348.4	0.89	4	0.99	0	0.95	1
580.7	0.90	3	0.92	0	0.97	0
967.8	0.93	0	0.90	1	0.74	24
1613	0.84	9	0.84	8	0.63	35

C. Micronucleus assay:

1. Assay acceptability: The frequency of MNBN cells in vehicle controls fell within the normal ranges. The positive control chemicals induced statistically significant increases in the proportion of MNBN cells. Both replicate cultures at the positive control concentration analysed under each treatment condition demonstrated MNBN cell frequencies that clearly exceeded the normal range, thereby demonstrating the sensitivity and specificity of the assay. A minimum of 50% of cells had gone through at least one cell division (as measured by binucleate + multinucleate cell counts) in vehicle control cultures at the time of harvest. The maximum concentration analysed under each treatment condition met the criteria. The assay data were therefore considered valid and acceptable.

2. Short term treatment in the absence and presence of S9: Treatment of cells with spiroxamine aminodiol for 3 hours (+ 21 hour recovery) in the absence and presence of S9 resulted in MNBN cells that were similar to and not significantly ($p \leq 0.05$) higher than those observed in concurrent vehicle control cultures for all concentrations analysed.

Table CA 5.8.1/24-2: Spiroxamine aminodiol: human lymphocyte micronuclei assay: 3 h (+ 21 h recovery) –S9 treatment and laboratory historical control data

Conc. (µg/mL)		3 h (+ 21 h recovery) – S9				Vehicle historical control ranges (♂ donors)	
		Total BN	Total MN-BN	Frequency of MN-BN (%)	Cyto. (%)	Feb 17 – Feb 18	% MNBN
Vehicle	A	1000	3	0.30		No. of expts	17
	B	1000	6	0.60		Number of cultures	40
Total		2000	9	Mean: 0.45		Mean ±SD	0.40 ±0.35
200	A	1000	6	0.60		min. – max.	0.00 – 0.80
	B	1000	5	0.50		95% reference range	0.00 – 0.70
Total		2000	11	Mean: 0.55	Mean: 0		
1000	A	1000	4	0.40			
	B	1000	5	0.50			
Total		2000	9	Mean: 0.45	Mean: 0		
1613	A	1000	5	0.50			
	B	1000	5	0.50			
Total		2000	10	Mean: 0.50	Mean: 0		
Linear trend: p 0.4714							
MMC (0.3)	A	1000	52	5.20			
	B	1000	49	4.90			
Total		2000	104	Mean: 5.20***	Mean: 3.7		

Positive historical control ranges (♂ donors)	
MMC (0.3 µg/mL)	
Feb 17 – Feb 18	% MNBN
No. of expts	21
Number of cultures	40
Mean ±SD	5.57 ±1.74
min. – max.	1.50 – 9.20
95% reference range	2.57 – 8.52

*** $p<0.001$

No test article related increases in cells with NPBs were observed (data not reported)

Table CA 5.8.1/24-3: Spiroxamine aminodiol: human lymphocyte micronuclei assay: 3 h (+ 21 h recovery) +S9 treatment and laboratory historical control data

Conc. (µg/mL)		3 h (+ 21 h recovery) +S9				Vehicle historical control ranges (♂ donors)	
		Total BN	Total MN-BN	Frequency of MN-BN (%)	Cyto. (%)	Feb 17 – Feb 18	% MNBN
Vehicle	A	1000	4	0.40		No. of expts	16
	B	1000	2	0.20		Number of cultures	40
Total		2000	6	Mean: 0.30	-	Mean ±SD	0.40 ±0.36
200	A	1000	4	0.40		min. – max.	0.00 – 1.00
	B	1000	5	0.50		95% reference range	0.10 – 0.90
Total		2000	9	Mean: 0.45	Mean: 0		
1000	A	1000	3	0.30			
	B	1000	5	0.50			

Total		2000	8	Mean: 0.40	Mean: 0	**Positive historical control ranges (♂ donors)**	
1613	A	1000	6	0.60		**CPA (3 µg/mL)[1]**	
	B	1000	5	0.50		**Feb 17 – Dec 17**	**% MNBN**
Total		2000	11	Mean: 0.55	Mean: 0	No. of expts:	22
Linear trend: p 0.1410						Mean:	40
CPA	A	1000	21	2.10		Mean ±SD	2.21 ±0.85
(7.0)	B	1000	21	2.10		min. – max.	1.00 – 4.50
Total		2000	42	Mean: 2.10***	Mean: 28	95% reference range	1.00 – 3.63

*** $p<0.001$
There is currently no historical control range for CPA 7 µg/mL, the concentration analysed in this study, therefore the range for the highest CPA concentration normally analysed (3 µg/mL) has been included for comparative purposes.
No test article related increases in cells with NPBs were observed (data not reported)

3. Extended treatment in the absence of S9: Treatment of cells with spiroxamine aminodiol for 24 hours (+24 hour recovery) in the absence of S9 resulted in frequencies of MNBN cells that were significantly ($p\leq0.05$) higher than those observed in concurrent vehicle control cultures for the highest two concentrations analysed (1000 and 1613 µg/mL), inducing 14% and 36% cytotoxicity. These increases were small such that the MNBN cell frequency of only a single replicate at 1613 µg/mL exhibited an MNBN cell value (0.9%) that exceeded the normal range (0 to 0.8%). However, evidence of a concentration-related increase (positive linear trend test) was apparent and as such, these small statistical increases may be considered of biological relevance and therefore, the result is considered equivocal.

Table CA 5.8.1/24-4: Spiroxamine aminodiol: human lymphocyte micronuclei assay: 24 h (+ 24 h recovery) -S9 treatment and laboratory historical control data

Conc. (µg/mL)		**24 h (+ 24 h recovery) –S9**				**Vehicle historical control ranges (♂ donors)**	
		Total BN	**Total MN-BN**	**Frequency of MN-BN (%)**	**Cyto. (%)**	**Jul 17 – Jan 18**	**% MNBN**
Vehicle	A	1000	2	0.20		No. of expts	16
	B	1000	4	0.40		Number of cultures	40
	C	1000	2	0.20		Mean ±SD	0.34 ±0.23
	D	1000	1	0.10		min. – max.	0.00 – 0.90
Total		4000	9	Mean: 0.23	-	95% reference range	0.00 – 0.80
200	A	2000	14	0.70			
	B	2000	2	0.10			
Total		4000	16	Mean: 0.40	Mean: 5		
1000	A	2000	6	0.30			
	B	2000	16	0.80		**Positive historical control ranges (♂ donors)**	
Total		4000	22	Mean: 0.55	Mean: 14		
1613	A	2000	15	0.75		**VIN (0.04 µg/mL)**	
	B	2000	18	0.90>HC		**Feb 17 – Jan 18**	**% MNBN**
Total		4000	33	Mean: 0.83	Mean: 36	No. of expts	20
Linear trend: p <0.0001						Mean:	41
VIN	A	2000	30	3.00		Mean ±SD	6.43 ±2.38
(0.04)	B	2000	20	2.00		min. – max.	2.50 – 13.60
Total		4000	50	Mean: 2.50***	Mean: 37	95% reference range	2.80 – 13.50

*** $p<0.001$
>HC: exceeds historical control
No test article related increases in cells with NPBs were observed (data not reported)

4. Extended treatment in the absence of S9, confirmatory experiment:	Treatment of cells for 24 hours (+24 hour recovery) in the absence of S9 resulted in frequencies of MNBN cells that were similar to and not significantly (p≤0.01) higher than those observed in concurrent vehicle control cultures for the majority of concentrations analysed (two of three concentrations analysed). The MNBN cell frequency of the majority of the test article treated cultures fell within the normal range. A single exception to this was observed at the intermediate concentration, 1000 µg/mL (inducing 21% cytotoxicity), where the MNBN cell value of one of the two replicate cultures marginally exceeded the normal range. However, this increase was small (0.9% versus normal range of 0 to 0.8%) and was not observed in the replicate culture or at the higher and lower concentrations analysed. Furthermore, the mean MNBN cell value at 1000 µg/mL (0.80%) fell within the normal range, with no evidence of any concentration-related effect. As such, this small isolated increase was therefore not considered of biological relevance (Thybaud *et al*[7]).

Table CA 5.8.1/24-5: Spiroxamine aminodiol: human lymphocyte micronuclei assay: 24 h (+ 24 h recovery) -S9 treatment, micronucleus experiment 2 and laboratory historical control data

Conc. (µg/mL)		24 h (+ 24 h recovery) –S9				Vehicle historical control ranges (♂ donors)	
		Total BN	Total MN-BN	Frequency of MN-BN (%)	Cyto. (%)	Jul 17 – Jan 18	% MNBN
Vehicle	A	1000	2	0.20		No. of expts	16
	B	1000	1	0.10		Number of cultures	40
Total		2000	3	Mean: 0.15	-	Mean ±SD	0.34 ±0.23
400	A	1000	4	0.40		min. – max.	0.00 – 0.90
	B	1000	2	0.20		95% reference range	0.00 – 0.80
Total		2000	6	Mean: 0.30	Mean: 1	**Positive historical control ranges (♂ donors)**	
1000	A	1000	9	0.90$^{>HC}$		**MMC (0.2 µg/mL)**	
	B	1000	7	0.70		**Dec 14 – Jan 18**	**% MNBN**
Total		2000	16	Mean: 0.80	Mean: 21	No. of expts	16
1400	A	1000	2	0.20		Number of cultures	29
	B	1000	4	0.40		Mean ±SD	31.18 ±10.58
Total		2000	6	Mean: 0.30	Mean: 47	min. – max.	16.35 to 52.50
Linear trend: *p* 0.0631						95% reference range	n/a
MMC (0.20)	A	1000	564	56.40		**VIN (0.04 µg/mL)**	
	B	1000	544	54.40		**Feb 17 – Jan 18**	**% MNBN**
Total		2000	1108	Mean: 55.40***	Mean: 52	No. of expts	20
VIN (0.04)	A	1000	68	6.80		Number of cultures	41
	B	1000	79	7.90		Mean ±SD	6.43 ±2.38
Total		2000	147	Mean: 7.35***	Mean: 53	min. – max.	2.50 to 13.60
						95% reference range	2.80 to 13.50

*** $p<0.001$
>HC: exceeds historical control
No test article related increases in cells with NPBs were observed (data not reported)
For VIN historical control range, refer to Table CA 5.8.1/09-5

D. Deficiencies:

Whilst not deemed a deficiency, it is considered prudent to detail the rationale for undertaking an extended treatment which included a recovery period.

Following revision of the OECD 487 TG in 2014 the cell treatment and harvest times have been somewhat simplified with emphasis placed on performing the extended treatment without recovery, with sampling times or recovery times extended by up to a further 1.5-2.0 normal cell cycles where justified.

Given the purpose for the *in vitro* micronucleus assay is to determine both clastogenic and aneugenic potential, the assay is compromised if an extended treatment with recovery is not included for sensitive detection of certain chemical classes.

Average generation times and replication index data demonstrated that the human peripheral blood lymphocytes were actively dividing beyond 96 hours, indicating that the extended treatment with recovery within the assay system did not compromise the results. This is directly in contrast to the statement in the OECD 487 test guideline, which includes the statement '*for lymphocytes exponential growth may be declining at 96 hours following stimulation and monolayer cultures of cells may become confluent'*. This statement in the test guideline is not supported by any published data, but rather a throw away statement and may provide the 'get out clause' why testing laboratory's did not use this approach as they may have had reduction in growth at 96 hours.

The extended treatment with recovery allows adequate detection of chemicals that either act on a narrow portion of the cell cycle (*e.g.* aneugenic compounds, G2/metaphase) and/or that induce cell cycle delay or require two rounds of DNA replication. The extended treatment without recovery in the micronucleus assay may be seen as somewhat compromised. It is widely known that direct acting clastogens such as azidothymidine and cytosine arabinoside are negative in the *in vitro* micronucleus assay with the 24 + 0 h approach, but positive with the with 24+24 h protocol. Therefore, the extended treatment with recovery is deemed more sensitive and in accordance with the latest published data (refer to Whitwell *et al.*, 2019[8]), with the OECD test guideline to be updated to reflect this change.

Assessment and conclusion by applicant:

Assessment: Study meets the current guidance and the requirements in 283/2013.

Conclusion: It is concluded that spiroxamine aminodiol induced small increases in the frequency of micronuclei in human peripheral blood lymphocytes when tested up to 1613 µg/mL (equivalent to 10 mM) for 24 hour (+24 hour recovery) treatment in the absence of an Aroclor-induced rat liver metabolic activation system (S9). However, these increases were not reproducible and according to current data interpretation strategies, may be considered of very low or no toxicological concern. Therefore these increases were considered of no biological relevance under the experimental conditions described. In the same test system, spiroxamine aminodiol did not induce increase in the frequency of micronuclei when tested up to 1613 µg/mL for 3 hour (+21 hour recovery) in the absence and presence of S9.

Data Point:	KCA 5.8.1/18
Report Author:	[redacted]
Report Year:	2013
Report Title:	KWG4168-aminodiol - Exploratory 28-day toxicity study in the rat by dietary administration
Report No:	SA 13129
Document No:	M-471499-01-1
Guideline(s) followed in study:	OECD 407; EEC Directive 96/54/EC, Method B.7
Deviations from current test guideline:	Yes Methods: SANCO/3029/99 rev. 4 No linearity data, no chromatograms and method only briefly described. Toxicology: A number of deficiencies are noted (refer to 'Deficiencies' section below)
Previous evaluation:	yes, evaluated and accepted RAR (2017)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

In this study, spiroxamine aminodiol was administered continuously *via* the diet for 28 days to Wistar rats. Animals (10/sex/group) were administered test diet at concentrations of 0, 45, 135, 400 ppm (equivalent to males/females: 0/0, 3.18/3.51, 9.42/10.81, 28.4/31.4 mg/kg bw/day). Animals were subjected to body weight, food consumption were measured at regular intervals, with FOB and motor activity included. Clinical pathology evaluations (haematology, clinical chemistry and urinalysis) were performed with all surviving animals subjected to complete gross necropsy and full histopathology.

Unlike the cyclohexane dimethyl ethyl containing spiroxamine and spiroxamine N-oxide (M03), spiroxamine aminodiol (M28) does not contain this structure, with only the aminodiol group present. Consequently, the adverse histopathological lesions associated with the cyclohexane dimethyl ethyl containing tertiary amines (hyperkeratosis of the epithelium of the oesophagus and forestomach) were not observed here. Furthermore, no test article related histopathological lesions were observed. The presence of the two diol groups vastly reduces the overt pH and pKa values observed with both the parent and M03, and therefore the mucosal membrane containing tissues, which would be site of first contact are not targeted. The two diol groups present in M28 vastly increases the water solubility, and with a smaller chemical structure (*i.e.* omission of the cyclohexane dimethyl ethyl group) results in rapid absorption, with a likely scenario of absorption between mucosal cells within the gastric environment, rather than crossing between membranes. Therefore the existence within such an environment is markedly reduced compared to parent and M03.

No treatment-related effects were seen for any of the parameters assessed, up to and including 400 ppm, the highest dose tested.

Under the conditions of this study the NOAEL for spiroxamine aminodiol following 28 days oral (*via* diet) treatment is deemed to be 400 ppm (equivalent to 28.4/31.4 mg/kg bw/day for males/females) based on adverse effects when tested up to the maximum dose level.

Materials and Methods

A. Materials:

1. Test Material: Spiroxamine aminodiol
(alternative name: KWG 4158-aminodiol, 3-ethyl(propyl)amino]propane-1,2-diol, AE 134430, technical, M28)

Description: Colourless liquid

Lot/Batch No.: NLL 9095-1

Purity:	98.14% (w/w) (correction for purity not undertaken)
CAS No.:	Not assigned
Stability of test compound:	Confirmed stable for the duration of the study (expiry date: 7 December 2013)
2. Vehicle and/or positive control:	Basal diet / not relevant
3. Test animals:	
Species:	Rat
Strain:	Wistar
Age at dosing:	6-7 wks
Weight at dosing:	♂: 286-329g; ♀: 177-216g
Source:	[redacted]
Acclimation period:	11 days
Diet:	A04CP1-10 powdered diet form (Scientific Animal Food and Engineering, France) *ad libitum*
Water:	Municipal water, *ad libitum*
Housing:	Individually housed
4. Environmental conditions:	
Temperature:	22±2°C
Humidity:	55 ± 15%
Air changes:	10-15/h
Photoperiod:	12-hour light/dark

B. Study Design:

1. In life dates:	8 July 2013 to 15 October 2013 (experimental dates)
2. Animal assignment and treatment:	After an acclimatisation period rats were allocated to groups by randomisation. Dose levels selected to enable comparison with the parent compound, spiroxamine. The test article, spiroxamine aminodiol was administered continuously *via* the diet to groups of rats for a period of 28 days. Animals (10/sex/gp), were administered test diet at concentrations of 0, 45, 135, 400 ppm (equivalent to ♂/♀: 0/0, 3.18/3.51, 9.42/10.81, 28.4/31.4 mg/kg bw/day). Following 28 days of treatment 10 animals/sex were subjected to complete necropsy. Body weight, food consumption were measured at regular intervals, with FOB and motor activity included. Clinical pathology evaluations (haematology, clinical chemistry and urinalysis) were performed with all surviving animals subjected to complete gross necropsy and full histopathology.
3. Statistics:	Body weight change parameters, terminal body weight, organ weights, haematology parameters (PT, Hb, haematocrit, MCV, MHC, MCHC, %neutrophils, %lymphocytes, %reticulocytes), clinical chemistry parameters, urinalysis (volume, refractive index), FOB assessments: - Bartlett Test. - If not significant: - ANOVA. If not significant no further statistical analysis - If ANOVA significant, Dunnett test - If significant: - Kruskal-Wallis test. If not significant no further statistical analysis - If Kruskal-Wallis test significant, Dunn test Body weight parameters, haematology parameters (RBC, platelet count, WBC, neutrophil, lymphocyte and reticulocyte counts), total cytochrome P-450 and liver enzyme activities:

- Barlett Test.
  - If not significant:
    - ANOVA. If not significant no further statistical analysis
    - If ANOVA significant, Dunnett test
  - If significant:
    - Barlett Test on transformed data
    - If Bartlett test on transformed data not significant, ANOVA on transformed data.
      - ANOVA on transformed data. If not significant no further statistical analysis
      - If ANOVA on transformed data, significant, Dunnett test on transformed data
    - If Bartlett test on transformed data significant Kruskal-Wallis test.
      - If Kruskal-Wallis test not significant no further statistical analysis
    - If Kruskal-Wallis test significant, Dunn test

Urinalysis pH parameter:

- Kruskal-Wallis. If not significant no further statistical analysis
  - If significant, Dunn test

Table CA 5.8.1/18-1: Overview of sub-acute toxicity study in rats treated orally (*via* diet) with spiroxamine aminodiol: study design and dose received

Parameters	♂ (ppm)				♀ (ppm)			
	0	**45**	**135**	**400**	**0**	**45**	**135**	**400**
Dose/animal (mg/kg bw/day)	0	3.18	9.42	28.4	0	3.51	10.81	31.4
Animals assigned/sex	10	10	10	10	10	10	10	10

C. Methods:

1. Test article preparation and analysis: The spiroxamine aminodiol was prepared at dietary concentrations of 0, 45, 135 and 400 ppm. The prepared test diets were prepared once and stored frozen (approximately −18°C) until before use.

Stability of the test article incorporated into the diet was confirmed at 45 and 400 ppm for a time, which covered the period of usage. Homogeneity was verified at the lowest and highest concentration, with the mean homogeneity vale taken to confirm measured concentration (refer to Doc MCA Section 4 [M-671499-01-1] for method validation).

2. Observations: Animals were inspected twice daily for signs of toxicity and mortality.

3. Body weights: Animals were weighed prior to study start and then on Days 0, 7, 14, 28, and the day of necropsy.

4. Food consumption: Determined by weighing food supplied and food that remained from day 5 and then at twice weekly intervals.

From the food consumption data, compound consumption was calculated using the following equation:

$$\text{Cpd consumption (mg/kg bw/d)} = \frac{\text{Food consumption (g/rat/d)} \times \text{test article conc. (ppm)}}{\text{Body weight (g)}}$$

Food efficiency was not calculated.

5. Water consumption: Not conducted.

6. Ophthalmological examination: Conducted prior to the start of dosing and during week 4.

7. Neurological functional examinations: *Functional observation battery:*

Detailed functional observation of each animal was conducted in their home cage and in an open field arena during week 3.

Home cage observations: posture, co-ordination or movement/gait, tremor and conclusion, abnormal behaviour.
During handling: ease to remove from cage, reaction to being handled, muscle tone, eyelid lacrimation, salivation, nasal discharge, staining or other signs (*e.g.* alopecia, emaciation, temperature upon touching)
Open field arena observations: piloerection, respiration, arousal, gait abnormalities, posture, involuntary motor movements, stereotypic movements, vocalisation, number of rearings, urine, faecal output.
Addition to the home cage and open field arena observations the high dose group (without recovery period) underwent additional observations including rearing, landing foot-splay and grip strength within the home cage. Additional open field observations included vision test, pupillary reflex, winking reflex, pinna-reflex, auditory startle response, olfactory test, examination of catalepsy, right response, tail pinch.

Motor activity:

Spontaneous motor activity was recorded during the 1st 60 minutes, with data collected at regular intervals throughout the session.

8. Haematology and clinical chemistry:

At the end of the treatment period blood was collected. Animals were fasted prior to blood sampling.

Haematology: red blood cell parameters (haematocrit (commonly termed PCV), haemoglobin concentration (Hb), mean haemoglobin concentration (MHC), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV), platelet count), white blood cell parameters (total and differential (neutrophils, lymphocytes, eosinophils, basophils, monocytes) leukocyte count), coagulation parameters (prothrombin time (PT)). A blood smear was prepared and stained using May-Grünwold-Giemsa and examined if haematological parameters were abnormal.
Clinical chemistry: electrolytes (sodium, potassium, calcium, chloride, phosphorus), kidney function test (creatinine, urea), glucose, liver function tests (albumin, globulin [A:G], alkaline phosphatase (ALP), alanine aminotransferase (ALT [commonly referred to as glutamic pyruvic transaminase (GPT)]), aspartate aminotransferase (AST [commonly referred to as glutamic oxaloacetic transaminase (GOT), γ-glutamyltransferase (γ-GT), total bilirubin (T.Bil), total protein (TP), lipid profile (total cholesterol, triglycerides).

9. Urinalysis:

At the end of the treatment period, animals were placed in metabolism cages overnight and fasted. The following urinary parameters were measured: specific gravity, pH, refractory index, total volume, protein, glucose, ketones, bilirubin, blood, urobilinogen, sediment, colour, leukocytes, erythrocytes, bacteria, epithelial cells, casts, crystals

10. Organ weights:

Adrenal glands, brain, epididymides, heart, kidney, liver, ovary, pituitary gland, prostate gland, spleen, testis, thymus, thyroid gland (+parathyroid), uterus (+cervix)

11. Sacrifice and pathology:

At the end of the treatment period gross pathological examination was performed on all animals and included examination of the external surface, all orifices and associated tissues. In addition, kidney, liver, lung, thyroid gland were examined in the intermediate dose groups.

The following tissues were preserved in 10% neutral buffered formalin for subsequent histopathological undertaken on all control and high dose group animals. Davidson's fixative was used for the eye (+optic nerve), Harderian gland, epididymis, testis:

Accessory sex glands (♂:epididymides, prostate, seminal vesicle, testes; ♀: ovary, uterus (+cervix), vagina), cardiovascular/haematological system (aorta, heart, lymph nodes (mesenteric, submaxillary), spleen, thymus), gastrointestinal tract (oesophagus, tongue, stomach, intestine (caecum, colon duodenum, ileum, jejunum, rectum), liver, pancreas, salivary glands (submaxillary)), neurological (brain, eyes (+exorbital lacrimal glands and optic nerve), sciatic nerve, spinal cord (cervical, thoracic, lumbar)),

	respiratory system (nasal cavities, larynx/pharynx ,trachea, lung), urogenital system (kidneys, urinary bladder), other (skeletal muscle, bone (sternum + marrow and femorotibial joint), skin, all gross lesions and masses); Other endocrine producing/sensitive glands (adrenals, mammary gland, pituitary, thyroid (+parathyroid)). Samples of liver tissue were collected for total cytochrome P450 and subsets of P450 responsible for Phase I metabolism (1A1, 1A2 (EROD), 2B1, 2B2, 2E (PROD) and 3A1, 3A2 (BROD) activity) were determined in all animals. Phase II enzymatic activities were determined by measuring UDPGT (UDP-glucuronosyltransferase) with 4-nitrophenol as a substrate.
12. Neurohisto-pathology:	No specific neurohistopathology with specific fixatives were performed in addition to the standard histopathology undertaken on neuronal tissues.

Results and discussion

A. Test diet analysis:

Spiroxamine aminodiol was homogenously distributed and chemically stable for at least 4 weeks and within the concentration range of 45 to 400 ppm. The analytical data verify that the during the treatment period concentrations of the test article in the diet preparations ranged from -15% to +18% of nominal concentrations which were within acceptable limits.

B. Observations:

1. Clinical signs of toxicity:	No test article related effects observed
2. Mortality:	No test article related effects upon mortality were observed, with all animals surviving until scheduled sacrifice.
3. Ophthalmoscopic examination:	No test article related effects observed
4. Neurological functional examinations	*Functional observation battery:* - Home cage, open field arena observations and additional open field observations: no test article related effects were observed. This pattern also was observed in regarding to grip strength, landing foot splay and rectal temperature. *Motor activity:* - No treatment related change in group mean values of activity counts of all intervals were observed.

C. Body weight and body weight gain:

No treatment related effects on body weight were observed, with all animals gaining weight over the treatment phase.

Table CA 5.8.1/18-2: Overview of sub-acute toxicity study in rats treated orally (*via* diet) with spiroxamine aminodiol: body weight effects

Parameters		♂ (ppm)				♀ (ppm)			
		0	45	135	400	0	45	135	400
Dose/animal (mg/kg bw/day)		0	3.18	9.42	28.4	0	3.51	10.81	31.4
Body wt (g)	Day 1	305 ±10.6	305 ±12.2	305 ±9.8	306 ±12.0	191 ±10.4	193 ±9.8	197 ±9.0	195 ±4.0
	Day 8	350 ±17.1	353 ±21.7	351 ±16.0	353 ±21.2	215 ±11.0	213 ±8.9	216 ±15.4	211 ±7.9
	Day 15	390 ±16.7	395 ±24.4	391 ±21.8	397 ±27.7	237 ±10.3	231 ±11.6	237 ±15.1	230 ±12.9
	Day 22	418 ±19.7	425 ±27.7	420 ±23.3	429 ±26.7	248 ±14.7	244 ±12.9	249 ±16.5	245 ±13.7
	Day 29	437 ±20.4	446 ±31.6	438 ±28.5	452 ±32.9	257 ±18.6	247 ±14.3	256 ±20.0	251 ±13.1
Body wt gain (g)	Day 0-29	132	141 (↑7%)	133 (↑1%)	146 (↑11%)	66	54 (↓18%)	59 (↓11%)	56 (↓15%)

D. Food consumption, food efficiency and water consumption:

1. Food consumption:	No treatment-related effects on food consumption were observed. Whilst it is noted that a slight reduction in food consumption was observed between study days 22 and 26 this was due to fasting for urine collection.
2. Food efficiency:	Whilst not measured, a qualitative assessment from food consumption data and body weight fain is suggestive that food efficiency was not affected.
2. Water consumption:	Not conducted

E. Blood and urinalysis:

1. Haematological findings:	No toxicological relevant changes were observed following the 28-day treatment period.
2. Clinical chemistry findings:	No toxicological relevant changes were observed following the 28-day treatment period.
3. Urinalysis:	No toxicological relevant changes were observed following the 28-day treatment period.

F. Sacrifice and pathology:

1. Organ weight:	No toxicological relevant changes were observed in absolute or relative organ weights following the 28-day treatment period, this included endocrine sensitive and producing organs.

Table CA 5.8.1/18-3: Overview of sub-acute toxicity study in rats treated orally (*via* diet) with spiroxamine aminodiol: selected organ weights

Parameters		♂ (ppm)				♀ (ppm)			
		0	45	135	400	0	45	135	400
Dose/animal (mg/kg bw/d)		0	3.18	9.42	28.4	0	3.51	10.81	31.4
Terminal bwt (g)		415 ±22.7	425 ±33.2 (↑2%)	417 ±25.9 (-)	429 ±29.9 (↑3%)	243 ±14.3	236 ±13.5 (↓2%)	241 ±18.5 (-)	239 ±12.5 (↓1%)
Liver	Abs (g)	10.293 ±0.847	10.770 ±1.017 (↑5%)	10.750 ±1.018 (↑4%)	10.479 ±1.14 (↑2%)	6.077 ±0.553	5.753 ±0.376 (↓5%)	6.121 ±0.581 (↑1%)	6.083 ±0.881 (-)
	Rel. (g%)	2.478 ±0.148	2.539 ±0.142 (↑2%)	2.576 ±0.163 (↑4%)	2.501 ±0.146 (↑1%)	2.511 ±0.195	2.443 ±0.0956 (↓3%)	2.539 ±0.122 (↑1%)	2.539 ±0.221 (↑1%)
Adrenals	Abs (g)	0.0570 ±0.013	0.0592 ±0.011 (↑4%)	0.0634 ±0.0045 (↑11%)	0.0599 ±0.0046 (↑5%)	0.0734 ±0.0083	0.0704 ±0.0072 (↓4%)	0.0790 ±0.0099 (↑8%)	0.0709 ±0.0096 (↓3%)
	Rel. (g%)	0.0137 ±0.0032	0.0139 ±0.0023 (↑1%)	0.0153 ±0.0016 (↑10%)	0.0140 ±0.0015 (↑2%)	0.0304 ±0.0037	0.0299 ±0.0030 (↓2%)	0.0328 ±0.0037 (↑8%)	0.0297 ±0.0040 (↓2%)
Testes	Abs (g)	3.418 ±0.311	3.256 ±0.912 (↓5%)	3.671 ±0.702 (↑7%)	3.561 ±0.320 (↑4%)	-	-	-	-
	Rel. (g%)	0.825 ±0.0884	0.764 ±0.205 (↓7%)	0.879 ±0.157 (↑7%)	0.831 ±0.058 (↑1%)	-	-	-	-
Epidid.	Abs (g)	1.174 ±0.119	1.094 ±0.233 (↓7%)	1.138 ±0.147 (↓3%)	1.152 ±0.0703 (↓2%)	-	-	-	-
	Rel. (g%)	0.284 ±0.0339	0.258 ±0.0547 (↓9%)	0.273 ±0.0338 (↓4%)	0.269 ±0.0268 (↓5%)	-	-	-	-
Prostate	Abs (g)	0.469 ±0.114	0.444 ±0.122 (↓5%)	0.434 ±0.136 (↓7%)	0.496 ±0.107 (-)	-	-	-	-
	Rel. (g%)	0.113 ±0.028	0.105 ±0.031 (↓7%)	0.104 ±0.0315 (↓8%)	0.116 ±0.024 (↑3%)	-	-	-	-
Ovary	Abs (g)	-	-	-	-	0.0933 ±0.0124	0.0824 ±0.0140 (↓12%)	0.0892 ±0.0208 (↓4%)	0.0857 ±0.0099 (↓8%)
	Rel. (g%)	-	-	-	-	0.0384 ±0.0037	0.0350 ±0.0059 (↓9%)	0.0370 ±0.0078 (↓4%)	0.0359 ±0.0042 (↓7%)
Uterus	Abs (g)	-	-	-	-	0.561 ±0.244	0.541 ±0.257 (↓4%)	0.588 ±0.273 (↑5%)	0.553 ±0.225 (↓1%)
	Rel. (g%)	-	-	-	-	0.232 ±0.0991	0.230 ±0.106 (↓1%)	0.243 ±0.110 (↑5%)	0.232 ±0.095 (-)

Parameters		♂ (ppm)				♀ (ppm)			
		0	45	135	400	0	45	135	400
Pit. gland	Abs (g)	0.0114 ±0.0007	0.0119 ±0.0020 (↑4%)	0.0111 ±0.0013 (↓3%)	0.0112 ±0.0013 (↓2%)	0.0123 ±0.0017	0.0121 ±0.0025 (↓2%)	0.0121 ±0.0024 (↓2%)	0.0116 ±0.0015 (↓6%)
	Rel. (g%)	0.0027 ±0.0002	0.0028 ±0.0004 (↑4%)	0.0027 ±0.0004 (-)	0.0026 ±0.0003 (↓4%)	0.0051 ±0.0007	0.051 ±0.0009 (-)	0.049 ±0.00097 (↓4%)	0.0049 ±0.0006 (↓4%)
Thyroid	Abs (g)	0.0169 ±0.0028	0.0168 ±0.0024 (↓1%)	0.0173 ±0.0024 (↑2%)	0.0179 ±0.0023 (↑6%)	0.0141 ±0.0020	0.0124 ±0.0016 (↓12%)	0.0142 ±0.0025 (↑1%)	0.0136 ±0.0024 (↓4%)
	Rel. (g%)	0.0041 ±0.0007	0.0040 ±0.0007 (↓2%)	0.0041 ±0.0004 (-)	0.0042 ±0.0006 (↑2%)	0.0058 ±0.0008	0.0053 ±0.0008 (↓9%)	0.0060 ±0.0012 (↑3%)	0.0057 ±0.0010 (↓2%)

Abs.: absolute
Rel.: relative to body weight

Epidid. epididymides
Pit. gland: pituitary gland

2. Gross pathology:	No test article related macroscopic findings were evident.
3. Histopathology:	Unlike the cyclohexane dimethyl ethyl containing spiroxamine and spiroxamine N-oxide (M03), spiroxamine aminodiol (M28) does not contain this structure, with only the aminodiol group present. Consequently, the adverse histopathological lesions associated with the cyclohexane dimethyl ethyl containing tertiary amines (hyperkeratosis of the epithelium of the oesophagus and forestomach) were not observed here. Furthermore, no test article related histopathological lesions were observed. The presence of the two diol groups vastly reduces the overt pH and pKa values observed with both the parent and M03, and therefore the mucosal membrane containing tissues, which would be site of first contact are not targeted. The two diol groups present in M28 vastly increases the water solubility, and with a smaller chemical structure (*i.e.* omission of the cyclohexane dimethyl ethyl group) results in rapid absorption, with a likely scenario of absorption between mucosal cells within the gastric environment, rather than crossing between membranes. Therefore the existence within such an environment is markedly reduced compared to parent and M03.
4. Liver tissue enzyme analysis:	Investigations of the liver tissue showed no treatment related effects at on either total cytochrome P450, Phase I (EROD, PROD, BROD) or Phase II (UDP-GT) enzyme activities.

G. Deficiencies:

Although the study was conducted according to test guideline OECD 407 (2008), the following minor deficiencies is noted:

- Whilst not a requirement, the test guideline makes reference to determination of serum thyroid hormones (T3, T4, TSH). These were not analysed. However, the gold standard to assess thyroid effects is histopathological analysis. This was undertaken on all animals and revealed no histopathological findings. Consequently the lack of serum thyroid hormone analysis is not deemed critical, with potential thyroid effects adequately addressed with histopathological analysis.
- Whilst it can be argued that a maximum tolerated dose was not achieved, the objective of this study was to enable a direct toxicological comparison back to the parent compound, spiroxamine when treated at comparable dose levels.

These deficiencies are considered minor and do not impact upon the scientific integrity of the study data.

Assessment and conclusions by applicant:

Assessment: Study meets the current guidance and the requirements in 283/2013.

Conclusion: Under the conditions of this study the NOAEL for spiroxamine aminodiol following 28 days of oral (*via* diet) treatment is deemed to be 400 ppm (equivalent to 28.4/31.4 mg/kg bw/day for males/females) based on adverse effects when tested up to the maximum dose level.

Data Point:	KCA 5.8.1/19
Report Author:	[redacted]
Report Year:	2013
Report Title:	KWG 4168-aminodiol: Oral (gavage) prenatal developmental toxicity study in the wistar rat
Report No:	10939
Document No:	M-472720-01-1
Guideline(s) followed in study:	US-EPAOPPTS 870.3700; OECD 414; Health Canada PMRA DACO 4.5.2; JMAFF 12 Nousan No. 8147; EU 88/302/EEC
Deviations from current test guideline:	Yes Methods: SANCO/3029/99 rev.4 Accuracy n = 4 No calibration data Toxicology: Yes The test guideline OECD 414 was updated 2018, the following deficiencies were noted: -Thyroid gland weights and histopathological assessment of every dam treated dam not performed. -Thyroid hormone measurements of dams not undertaken. -Anogenital distance of fetuses not performed.
Previous evaluation:	yes, evaluated and accepted RAR (2017)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

In an embryo-fetal developmental toxicity study, spiroxamine aminodiol was administered to female Han Wistar rats (27/group) by oral gavage once daily at dose levels of 0 (deionised water), 30, 150 or 500 mg/kg bw from days 7 through 19 of gestation, employing a dose volume of 10 mL/kg bw. Surviving dams were sacrificed on GD 20. The following parameters and end points were evaluated in this study: clinical signs, body weights, body weight changes, food consumption, ovarian and uterine examinations, gravid uterine weights, fetal examinations, gross necropsy findings and histopathology.

Based on the results of this study, spiroxamine aminodiol administered to rats at a dose of 500 mg/kg bw/day caused maternal mortality and adversity in clinical signs of toxicity (rales, gasping) at a dosage level that exceeded the MTD. Body weight effects were limited to the high dose group, 500 mg/kg bw/day. Overall group mean data were unaffected, but decrease body weight gain and/or body weight loss was observed in some individual high dose dams. These animals were terminated early without fetal examinations being undertaken. Gross pathology of these animals confirmed that either died or were killed *in extremis* included gaseous contents or gas-filled regions of the gastrointestinal tract. A single mid dose group dam was noted with gaseous content of the intestines. Based on gross pathology observations in both the range finder (at 540 and 800 mg/kg bw/day) and high dose group animals, this single incidence was deemed treatment related.

At dose levels of 30 and 150 mg/kg bw/day no adversity in clinical signs of toxicity, body weight or food consumption effects were evident compared to the concurrent control group.

Pregnancy occurred in 24, 25 and 24 rats in the 0, 3 and 150 mg/kg bw/day dosage groups, respectively. The litter averages for *corpora lutea*, implantations, pre-implantation loss, early resorptions, and post-implantation loss were comparable among the two dose groups when compared with the concurrent control. There were no embryonic deaths in the test article treated groups. All placentae appeared normal. Whilst an increase in absolute gravid uterine weight was observed for low mid dose group animals (increased by 14% and 29%, respectively), this was attributed to slightly increased litter sizes, which was considered within normal variation.

Total fetal weights (both presented as combined sex and individual sex) were comparable to the concurrent control in the 40 and 150 mg/kg bw/day dose groups. No test article related or statistically significant difference were observed in sex ratio between the vehicle control and test article treated groups.

Visceral examinations were based on 108, 121 and 128 live, GD 20 caesarean-delivered fetuses in 24, 25, and 24 litters in dose groups 0, 30 and 150 mg/kg bw/day dosage groups, respectively. In total soft tissue variations were limited to 3 tissues, with a single incidence of malformation (eyes). A single incidence of retinal folding was observed in a control fetus. This finding can occur as a result of artefact of fixation/sectioning, with a single incidence occurring, this was the likely outcome here. The umbilical artery descended to the left in 10, 15 and 18 fetus at a litter incidence of 7, 11 and 10 in the 0, 30 and 150 mg/kg bw/day dosage groups, respectively.

Skeletal evaluations were based on 116, 138 and 145 live, GD 20 caesarean-delivered fetuses in 24, 25, and 24 litters in dose groups 0, 30 and 150 mg/kg bw/day dosage groups, respectively. Incidental findings primarily included incomplete ossification (variation) of various bones, occurring across all groups (including controls) as well as variations in the ribs (way/curved, ossification centre, rudimentary, small and extra), also occurring across all groups. All incidences were within the laboratory historical control range. No treatment related skeletal alterations were observed.

Under the conditions of this study, the NOAEL for maternal toxicity was considered to be 150 mg/kg bw/day based maternal mortality, body weight losses gaseous content of the GI tract, and adverse clinical signs at a dosage level that exceeded the MTD (500 mg/kg bw/day).

The developmental NOAEL was considered to be 30 mg/kg bw/day based on incomplete ossification.

Materials and Methods

A. Materials:

1. **Test Material:** KWG 4168-aminodiol
(alternative name: Spiroxamine aminodiol, 3-ethyl[propyl)amino]propane-1,2-diol, M28)

Description:	Colourless liqui
Lot/Batch No.:	Nct. 9095-21
Purity:	98.1% (w/w) (correction for purity undertaken, with a correction factor of 1.019)
CAS No.:	Not assigned
Stability of test compound:	Confirmed stable for the duration of the study (expiry date: 7 December 2013)

2. **Vehicle and/or positive control:** Deionised water / not relevant

3. **Test animals:**

Species:	Rat
Strain:	Wistar Han
Age at dosing:	10 weeks
Weight at dosing:	164-265g
Source:	[redacted]
Acclimation period:	7 days
Diet:	Certified Rodent Diet® #5002 (PMI® Nutrition International, USA) *ad libitum*
Water:	Municipal water, *ad libitum*
Housing:	Individually housed, except during cohabitation period with 1♂ paired with 2♀ overnight with sexually mature ♂.

4. Environmental conditions:

Temperature:	22 ±4°C
Humidity:	55 ± 15%
Air changes:	*ca.* 15/h
Photoperiod:	12 hour light/dark

B. Study Design:

1. In life dates: 23 September 2013 to 25 October 2013 (experimental dates)

2. Animal assignment and treatment: Upon arrival, rats were assigned to individual housing on the basis of computer-generated randomisation. After an acclimatisation period (not stated), virgin ♀ rats were paired 2:1 with breeder rats of the same source. The day on which spermatozoa observed in a smear of the vaginal contents and/or a copulatory plug observed *in situ* were considered to be day 0 of gestation. Group assignment was designated when dams were confirmed sperm positive. Each group consisted of 27 ♀/group.

The dose levels were selected based on the results from a range-finding study in which spiroxamine aminodiol was dosed orally *via* gavage at 0, 30, 120, 480, 800 mg/kg bw/day to 8 pregnant rats/group from GD 6-19. At 800 mg/kg bw/day a single animal showed resistance to dosing, which contributed to mis-dosing/perforation of the oesophagus in 3/8 rats. Two animals were found dead on GD 7, with a third animal killed *in extremis* on GD 8. Due to increased mortality, the 800 mg/kg bw/day dose group was terminated early. Findings in the 480 mg/kg bw/day dose group were limited to a reductions in body weight compared to the concurrent control (16%). Consequently, to ensure that an MTD was identified, an additional 5 time mated animals were dosed from GD 6 to 14 only at 540 mg/kg bw/day. No clear effect on body weight was observed, however 1/5 animals was noted with rales prior to necropsy, with gas-filled caecum and colon at gross necropsy. It was concluded that the 540 mg/kg bw/day dose group also exceeded the MTD, and was therefore not suitable for the main study.

Based on these data, dosages of 0, 30, 150 and 500 mg/kg bw/day of spiroxamine aminodiol were employed for the developmental toxicity study in rats, using a dose volume of 10 mL/kg bw. Surviving dams were sacrificed on GD 20.

3. Statistics: Continuous data:

Parametric (*e.g.* dam body weight, food consumption):
- ANOVA
- If not significant no further statistical analysis
- If ANOVA significant, Dunnett test

Non-parametric (*e.g.* litter size, number of *corpora lutea*):
- Kruskal-Wallis test
- If not significant no further statistical analysis
- If significant, Dunn test

Non-parametric dichotomous data (*e.g.* number normal/abnormal):
- Chi-square test.
- If not significant no further statistical analysis
- If significant, Fisher's Exact test, with Bonferroni adjustment

Indices: All appropriate indices were calculated from caesarean section records of animals in the study:

$$\text{\% pre-implantation loss} = \frac{\text{No. of } corpora\ lutea - \text{No. of implantations}}{\text{number of } corpora\ lutea} \times 100$$

$$\frac{\text{No. of implantation no. of live fetuses}}{} \times 100$$

$$\% \text{ post-implantation loss} = \frac{\text{No. of implantations}}{}$$

$$\text{Sex ratio} = \frac{\text{No. of ♂ fetuses / No. of ♀ fetuses}}{\text{Total no. of fetuses}} \times 100$$

C. Methods:

1. Test article formulation preparation and analysis: The test article formulations were prepared on weekly basis (1-3 days) at concentrations of 0, 30, 15 50 mg/mL. Spiroxamine aminodiol was weighed into a glass beaker, with vehicle added. Following preparation the stock formulations were refrigerated (2-9°C). The mixture was homogenized using a magnetic stirrer during dosing. Verification of concentration and homogeneity of the test article were determined as part of the method validation during the course of the dose range-finding study. One day stability at room temperature and 7 days refrigerated over a concentration rang of 0.080 – 120 mg/mL were confirmed (refer to Doc MCA Section 4 [M-472720-01-1] for method validation). Concentration of test article in the dose formulation was analytically verified for four of the batches prepared.

2. Observations: *Maternal observations*: The animals were checked for mortality twice daily. The rats were observed for general appearance twice during the acclimation period, on GD 0 and GD 5, GD 6 (prior to and 1 hour post dosing) and then daily thereafter.
Fetal observations: examined for sex and external abnormalities. Dead fetuses and late resorptions were examined for sex and external abnormalities to the extent possible.

3. Body weights: *Maternal body weights:* Recorded daily from GD 0 through to GD 19 and prior to necropsy on GD 20.
Fetal body weights: body weight of each fetus was recorded. Fetuses were individually identified with litter number and uterine distribution

4. Food consumption: Recorded for the following periods GD 0-6, 6-9, 9-12, 12-15, 15-18 and 18-20. From the food consumption data, compound consumption was calculated using the following equation:

$$\text{Mean daily food consumption} = \frac{\text{Food consumption (g rat } \textit{per} \text{ period)}}{\text{Days } \textit{per} \text{ period}}$$

5. Water consumption: Not conducted

6. Ophthalmological examination: Not conducted

7. Mating performance: Evaluated daily during the co-habitation period. Dams were sacrificed on day 20 of gestation.

8. Haematology and clinical chemistry: Not conducted

9. Urinalysis: Not conducted

10. Organ weights: Restricted to maternal animals, with gravid uterus, placenta, liver and kidney

11. Maternal sacrifice and pathology: *Ovarian and uterine examinations*: the uterus was opened and the contents were examined. The fetuses were removed from the uterus and placed in individual containers (or a tray). The ovaries and uterus were examined for number and distribution of *corpora lutea*, implantation sites, placentae (size, color or shape), live and dead fetuses, and early and late resorptions. An early resorption was defined as one in which organogenesis was not grossly evident. A late resorption was defined as one in which the occurrence of organogenesis was grossly evident. A live fetus was defined as a term fetus that responded to stimuli. Non-responding term fetuses were considered to be dead. Dead fetuses and late resorptions were differentiated by the degree of autolysis present; marked to extreme autolysis indicated that the fetus was a late resorption. Uteri

of apparently non-pregnant dams were examined to confirm absence of implantation sites.

Necropsy: rats were subjected to a gross necropsy examination, which included an evaluation of the thoracic, abdominal, and pelvic cavities with their associated organs and tissues. Gross lesions were collected for all animals. Representative samples of the tissues (cervix, collected with uterus: including non-pregnant animals; gravid uterus, all animals; gross lesions, all animals; liver, all animals; ovaries, including all non-pregnant animals; uterus, including all non-pregnant animals) were collected and preserved in 10% neutral buffered formalin.

Histopathology: the liver, kidney and gross lesions were of dams were perfused in 10% NBF and retained for possible histopathology examination

12. Fetal sacrifice and pathology: Fetuses were euthanized by *ip* injection of sodium pentobarbital. Approximately one-half of the fetuses in each litter were examined for visceral abnormalities by using a modification of the micro-dissection technique of Wilson (1965). Each fetus was fixed in a Bouin's solution and then preserved in a solution of ethyl alcohol. The remaining fetuses (approximately one-half of the fetuses in each litter) were examined for skeletal abnormalities after staining with alizarin red S and alcian blue. Fetal cranial examination of fetuses were undertaken, with sectioning of the head performed according to the method of Wilson.

Results and discussion

A. Test article formulation analysis:

Spiroxamine aminodiol was homogenously distributed and chemically stable for at least 7 days (refrigerated) and within the concentration range of 9 to 50 mg/mL. The analytical data verify that the during the treatment period concentrations of the test article in the formulation preparations ranged from +1% to +5% of nominal concentrations which were within acceptable limits.

B. Maternal toxicity:

1. Clinical signs of toxicity: Clinical signs of toxicity were limited to the 500 mg/kg bw/day dosage group. Rales were evident from GD 8-20 in 10/27 dams. Of the dams that were found dead / killed *in extremis* two dams (#906, #93) exhibited gasping on GD 14 or 15.

Due to the severity of clinical signs of toxicity, unscheduled deaths and animals killed *in extremis*, collectively the MTD was deemed to have been exceeded. These animals were terminated early without fetal examinations being undertaken.

All other clinical signs reported were considered unrelated to treatment.

2. Mortality: Two dams (#14, #363) in the 500 mg/kg bw/day dosage group were found dead on GD 16 and 19, respectively. Two further dams (#93, #906) were killed *in extremis* on GD 14 and 15.

3. Ophthalmoscopic examination: Not conducted

4. Body weight: Body weight effects were limited to the high dose group, 500 mg/kg bw/day. Overall group mean data were unaffected, but decrease body weight gain and/or body weight loss was observed in some individual high dose dams.

Table CA 5.8.1/19-1: Overview of developmental toxicity study in rats treated orally (*via* gavage) with spiroxamine aminodiol: body weight effects

Parameters	♀ (mg/kg bw/d)			
	0	30	150	500
No. of animals treated	27	27	27	27

Parameters		♀ (mg/kg bw/d)			
		0	30	150	500
Body wt (g)	Day 0	204 ±2.5	204 ±2.8 (-)	208 ±2.8 (↑2%)	210 ±3.8 (↑3%)
	6	224 ±3.7	224 ±2.8 (-)	230 ±2.7 (↑3%)	231 ±3.8 (↑3%)
	10	238 ±3.7	236 ±2.9 (↓0.8%)	243 ±2.9 (↑2%)	236 ±5.6 ↓0.8%)
	16	268 ±3.5	266 ±3.2 (↓0.7%)	271 ±3.7 (↑1%)	263 ±7.0 (↓2%)
	19	297 ±3.8	296 ±3.7 (↓0.3%)	303 ±5.2 (↑2%)	293 ±7.7 (↓1%)
	20	308 ±4.3	308 ±4.1 (-)	318 ±5.7 (↑3%)	306 ±8.6 (↓0.6%)
Gravid uterine wt (g)		48.5 ±3.63	55.2 ±2.44 (↑14%)	62.4 ±2.49** (↑29%)	60.6 ±2.3 (↑25%)
Corrected body wt (g)	Day 20[a]	260 ±3.4	253 ±3.8 (↓3%)	259 ±3.5 (↓0.4%)	239 ±7.0 (↓8%)
Body weight gain (g)	Day 6-20	104 ±4.2	108 ±2.6 (↑4%)	110 ±4.7 (↑6%)	97 ±25.4 (↓7%)
	0-20	83.9 ±2.73	84.8 ±2.46 (↑2%)	87.5 ±4.58 (↑5%)	74.2 ±25.93 (↓12%)
Corrected body wt gain (g)	Day 20[a]	55.5 ±2.62	49.7 ±2.02 (↓10%)	50.9 ±1.66 (↓8%)	39.6 ±4.6 (↓29%)

* $p \leq 0.05$; ** $p \leq 0.01$

a gestational body weight – gravid uterine weight (note rounding errors may result)

5. Food consumption: Absolute and mean food consumption during the treatment period was not significantly affected in any of the dose groups.

6. Organ weight Organ weights were limited to gravid uterus weight, placenta, liver and kidney from low and mid dose groups. Whilst an increase in absolute gravid uterine weight was observed for low mid dose group animals (increased by 14% and 29%, respectively), this was attributed to slightly increased litter sizes, which was considered within normal variation.

For placenta, liver and kidney weights taken, no increase in either absolute or relative weights were observed for low or mid dose group animals.

Organ weights for high dose group animals were not taken.

7. Gross pathology: For high dose group animals that either died or were killed *in extremis*, gross observations included gaseous contents or gas-filled regions of the gastrointestinal tract was evident in all four animals. In one animal surviving until GD 20, similar observation were also observed.

A single mid dose group dam (#298) was noted with gaseous content of the intestines. Based on gross pathology observations in both the range finder (at 540 and 800 mg/kg bw/day) and high dose group animals, this single incidence was deemed treatment related.

8. Histopathology: Not conducted.

9. Caesarean section data: Pregnancy occurred in 24, 25 and 24 rats in the 0, 30 and 150 mg/kg bw/day dosage groups, respectively. The litter averages for *corpora lutea*, implantations, pre-implantation loss, early resorptions, and post-implantation loss were comparable among the two dose groups when compared with the concurrent control. Litter sizes for the 30 and 150 mg/kg bw/da dosage groups were marginally greater than that of the concurrent control (10.4 and 11.4 *vs* 9.4, respectively), which would account for the increased weights of the gravid uterus, with litter size consistent with the laboratory's historical control range (9.8 – 11.8). There were no embryonic death in the test article treated groups, with 2 fetuses found dead in 1 single control dam. All placentae appeared normal.

Table CA 5.8.1/19-2: Overview of developmental toxicity study in rats treated orally (*via* gavage) with spiroxamine aminodiol: selected caesarean section data

Parameters	♀(mg/kg bw/d)			
	0	30	150	500
No. of animals mated	27	27	27	27
Animals pregnant and caesarean section on GD 21	24	25	24	-
Animals with non-viable fetuses	1	0	0	-
Unscheduled deaths	0	0	0	4
Corpora lutea [/dam]	299 [12.5 ±0.41]	300 [12.0 ±0.31]	316 [13.2 ±0.35]	-
Implantation sites [/dam]	245 [10.2 ±0.75]	278 [11.1±0.51]	298 [12.4 ±0.39]	-
Total no. of litters	24	25	24	-
Mean litter size/dam	9.4 ±0.73	10.4 ±0.49	11.4 ±0.50	-
Total live fetus [/dam]	224 [9.3 ±0.75]	259 [10.4 ±0.49]	273 [11.4 ±0.50]	-
Total live ♂ / ♀	113/111	123/136	139/134	-
Total dead fetus	2	0	0	-
Early/late resorptions [/dam]	19 / 0 [0.8 ±0.13 / 0.0 ±0.0]	19 / 0 [0.8 ±0.28 / 0.0 ±0.0]	25 / 0 [1.0 ±0.46 / 0.0 ±0.0]	-
	♂ ♀	♂ ♀	♂ ♀	♂ ♀
Fetal wt (g)	3.6 ±0.05 3.4 ±0.05	3.7 ±0.07 3.4 ±0.03	3.8 ±0.06 3.6 ±0.05	- -
Mean fetal wt (g)	3.6 ±0.05	3.6 ±0.07	3.7 ±0.05	-
Sex ratio (% ♂)	50.4	47.5	50.9	-
% resorbed conceptuses/litter	4.3 ±5.9	2.5 ±5.8	6.1 ±14.9	-
Total pre-/post-implantation loss (mean pre-/post-)	54 / 21 (2.3 ±0.51 / 0.9 ±0.14)	22 / 19 [0.9 ±0.30 / 0.8 ±0.28]	18 / 25 [0.8 ±0.16 / 1.0 ±0.46*]	-

C. Developmental toxicity:

1. Body weights: Fetal body weights in the 30 and 150 mg/kg bw/day dosage groups were comparable to that of the concurrent control.

2. Sex ratio: No test article related or statistically significant difference were observed in sex ratio between the vehicle control and test article treated groups.

3. External examination: No fetal gross external alterations were observed in either the control, 30 or 150 mg/kg bw/day.

Table CA 5.8.1/19-3: Overview of developmental toxicity study in rats treated orally (*via* gavage) with spiroxamine aminodiol: overview of fetal abnormalities (fetus/litter incidence)

Parameters	♀ (mg/kg bw/d)			
	0	30	150	500
Total external, visceral and skeletal examinations (fetus/litter)				
Fetus/litters examined	224/24	259/25	273/24	-

Parameters	♀ (mg/kg bw/d)			
	0	30	150	500
Fetus/litter with any alteration (%)	3/1 (1.3/4.2)	0/0 (0.0/0.0)	0/0 (0.0/0.0)	-
% Fetus with any alteration/litter (%)	2.6 ±3.9	3.2 ±3.8	1.9 ±3.6	
External examinations (fetus/litter)				
Fetus/litters examined	224/24	259/25	273/24	-
Abnormal findings	0/0	0/0	0/0	-
Visceral examinations (fetus/litter)				
Fetus/litters examined	108/24	121/25	128/24	-
Malformations (%)	1/1 (0.9/4.2)	0/0 (0.0/0.0)	0/0 (0.0/0.0)	-
Variations (%)	12/8 (11.1/33.3)	15/11 (12.4/44)	20/12 (15.6/50.0)	-
Skeletal examination (fetus/litter)				
Litters examined	116/24	138/25	145/24	-
Malformations (%)	0/0 (0.0/0.0)	0/0 (0.0/0.0)	0/0 (0.0/0.0)	-
Variations (%)	116/24 (100/100)	138/25 (100/100)	145/24 (100/100)	-

4. Visceral and skeletal observations:

Fetal alterations were defined as:
- malformations (irreversible changes that occur at low incidences in this species and strain)
- variations (common findings in this species and strain and reversible delays or accelerations in development).

Visceral examinations: Fetal evaluations were based on 108, 121 and 128 live, GD 21 caesarean-delivered fetuses in 24, 25, and 24 litters in dose groups 0, 30 and 150 mg/kg bw/day dosage groups, respectively. Each of these fetuses were examined for visceral alterations, using the Wilson technique. In total soft tissue variations were limited to 3 tissues, with a single incidence of malformation (eyes).

Eyes: a single incidence of retinal folding was observed in a control fetus, deemed to be malformation. This finding can occur as a result of artefact of fixation/sectioning; with a single incidence occurring, this was the likely outcome here.

Vessels: the umbilical artery descended to the left in 10, 15 and 18 fetus at a litter incidence of 7, 11 and 10 in the 0, 30 and 150 mg/kg bw/day dosage groups, respectively.

Liver: mottled liver appearance in a single control fetus.

Kidney: pale kidney appearance in two control and two mid dose fetuses (from separate litters).

Table CA 5.8.1/19-3: Overview of developmental toxicity study in rats treated orally (*via* gavage) with spiroxamine aminodiol: overview of visceral findings (fetus/litter incidence)

Parameters	♀ (mg/kg bw/d)			
	0	30	150	500
Fetus/litters examined	108/24	121/25	128/24	-
Malformations				
Retinal folding (%)	1/1 (0.9/4.2)	0/0 (0.0/0.0)	0/0 (0.0/0.0)	-
Variations				
Vessels: umb. art., L (%)	10/7 (9.3/29.2)	15/11 (12.4/44.0)	18/10 (14.1/41.7)	-

Parameters	♀ (mg/kg bw/d)			
	0	30	150	500
Kidney: pale (%)	2/2 (1.9/8.3)	0/0 (0.0/0.0)	0/0 (0.0/0.0)	-

umb. art. L: umbilical artery, leftside

4. Visceral and skeletal observations (continued):

Skeletal examinations: Fetal evaluations were based on 116, 138 and 145 live, GD 20 caesarean-delivered fetuses in 24, 25, and 24 litters in dose groups 0, 30 and 150 mg/kg bw/day dosage groups, respectively. Each of these fetuses were examined for skeletal alterations, after staining with alizarin red S and alcian blue. In total skeletal variations were limited to ribs, with no malformations:

Ribs: wavy/curved ribs were observed in 14, 8 and 7 fetuses across 9, 5 and 7 litters in the 0, 40 and 150 mg/kg bw/day dosage groups, respectively. This variation in common in this strain of rat.

Rudimentary ribs were observed in 15, 11 and 11 fetuses across 8, 9 and 9 litters in the 0, 40 and 150 mg/kg bw/day dosage groups, respectively.

Small ribs were observed in in a single fetus in both the 30 and 150 mg/kg bw/day dosage groups, with no incidence observed in the control group.

Extra ribs were observed in 2, 1 and 0 fetuses in the 0, 30 and 150 mg/kg bw/day dosage groups, respectively.

In each case, incidence was consistent with the historical control range.

No other skeletal variations occurred.

Table CA 5.8.1/19-4: Overview of developmental toxicity study in rats treated orally (*via* gavage) with spiroxamine aminodiol: overview of skeletal variations (fetus/litter incidence)

Parameters	♀ (mg/kg bw/d)			
	0	30	150	500
Fetus/litters examined	116/24	138/25	145/24	-
Ribcage				
Ribs: wavy/curved (%)	14/6 (12.1/37.5)	8/5 (5.8/20.0)	7/5 (4.8/20.8)	-
Ribs: rudimentary (%)	15/8 (12.9/33.3)	11/9 (8.0/36.0)	11/9 (7.6/37.5)	-
Ribs: small (%)	0/0 (0.0/0.0)	1/1 (0.7/4.0)	1/1 (0.4/4.2)	-
Ribs: extra (%)	2/2 (1.7/8.3)	1/1 (0.7/4.0)	0/0 (0.0/0.0)	-

5. Fetal ossification:

Incidentals findings primarily included incomplete ossification of various bones, occurring across all groups (including the controls) were observed without dose-response, with all incidences within the historical control.

There were no other statistically significant or biologically relevant difference among the two dosage groups in the average number of ossification sites/fetus for the hyoid, vertebrae (cervical, thoracic, lumbar, sacral), ribs sternum (manubrium, sternal centers, xiphoid) forelimbs (carpals, metacarpals) or hind limbs (tarsals).

D. Deficiencies:

The test guideline OECD 414 was updated 2018, the following deficiencies were noted:

- Thyroid gland weights and histopathological assessment of every dam treated dam not performed.
- Thyroid hormone measurements of dams not undertaken.
- Anogenital distance of fetuses not performed.

E. Discussion:

Based on the results of this study, spiroxamine aminodiol administered to rats at a dose of 500 mg/kg bw/day caused maternal mortality and adversity in clinical signs of toxicity (rales, gasping) at a dosage level that exceeded the MTD. Body weight effects were limited to the high dose group, 500 mg/kg bw/day. Overall group mean data were unaffected, but decrease body weight gain and/or body weight loss was observed in some individual high dose dams. These animals were terminated early without fetal examinations being undertaken. Gross pathology of these animals confirmed that either died or were killed *in extremis* included gaseous contents or gas-filled regions of the gastrointestinal tract. A single mid dose group dam was noted with gaseous content of the intestines. Based on gross pathology observations in both the range finder (at 640 and 800 mg/kg bw/day) and high dose group animals, this single incidence was deemed treatment related.

At dose levels of 30 and 150 mg/kg bw/day no adversity in clinical signs of toxicity, body weight or food consumption effects were evident compared to the concurrent control group.

Pregnancy occurred in 24, 25 and 24 rats in the 0, 30 and 150 mg/kg bw/day dosage groups, respectively. The litter averages for *corpora lutea*, implantations, pre-implantation loss, early resorptions, and post-implantation loss were comparable among the two dose groups when compared with the concurrent control. There were no embryonic deaths in the test article treated groups. All placentae appeared normal. Whilst an increase in absolute gravid uterine weight was observed for low mid dose group animals (increased by 14% and 29%, respectively), this was attributed to slightly increased litter sizes, which was considered within normal variation.

Total fetal weights (both presented as combined sex and individual sex) were comparable to the concurrent control in the 30 and 150 mg/kg bw/day dose groups. No test article related or statistically significant difference were observed in sex ratio between the vehicle control and test article treated groups.

Visceral examinations were based on 108, 121 and 128 live, GD 20 caesarean-delivered fetuses in 24, 25, and 24 litters in dose groups 0, 30 and 150 mg/kg bw/day dosage groups, respectively. In total soft tissue variations were limited to 3 tissues, with a single incidence of malformation (eyes). A single incidence of retinal folding was observed in a control fetus. This finding can occur as a result of artefact of fixation sectioning, with a single incidence occurring, this was the likely outcome here. The umbilical artery descended to the left in 10, 15 and 18 fetus at a litter incidence of 7, 11 and 10 in the 0, 30 and 150 mg/kg bw/day dosage groups, respectively.

Skeletal evaluations were based on 116, 138 and 145 live, GD 20 caesarean-delivered fetuses in 24, 25, and 24 litters in dose groups 0, 30 and 150 mg/kg bw/day dosage groups, respectively. Incidental findings primarily included incomplete ossification (variation) of various bones, occurring across all groups (including controls) as well as variations in the ribs (way/curved, ossification centre, rudimentary, small and extra), also occurring across all groups. All incidences were within the laboratory historical control range. No treatment related skeletal alterations were observed.

Assessment and conclusions by applicant:

Assessment: Study meets the current guidance and the requirements in 283/2013.

Conclusion: Under the conditions of this study, the NOAEL for maternal toxicity was considered to be 150 mg/kg bw/day based maternal mortality, body weight losses gaseous content of the GI tract, and adverse clinical signs at a dosage level that exceeded the MTD (500 mg/kg bw/day).

The developmental NOAEL was considered to be 30 mg/kg bw/day based on incomplete ossification.

Spiroxamine-docosanoic acid ester (M35)

Data Point:	KCA 5.8.1/25
Report Author:	[redacted]
Report Year:	2020
Report Title:	4-tert-butylcyclohexyl docosanoate: In vitro human lymphocyte micronucleus assay
Report No:	8425089
Document No:	M-753775-01-1
Guideline(s) followed in study:	OECD guideline 487 (OECD, 2016)
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

Spiroxamine docosanoic acid ester was tested in an *in vitro* micronucleus assay using duplicate human lymphocyte cultures prepared from the pooled blood of two female donors in two experiments. Treatments covering a broad range of concentrations, separated by narrow intervals, were performed both in the absence and presence of metabolic activation (S9) from Aroclor 1254-induced rats. The test article was formulated in tetrahydrofuran (THF) and the highest concentration tested in the micronucleus experiment, 50 µg/mL was limited by precipitate observed by eye at the end of treatment. All test article concentrations, formulated in THF were dosed into the test system at 0.25% v/v.

Following establishment of cultures, concentrations ranging from 5 to 200 µg/mL were used in the absence (3 hours + 21 hour recovery), presence of S9 (3 h + 21 h), and for extended treatment in the absence of S9 (24 h + 24 h). The test article concentrations for micronucleus analysis were selected by evaluating the effect of spiroxamine docosanoic acid ester on the replication index (RI). Micronuclei were analysed at three concentrations and a summary of the data are presented below.

The frequency of MNBN cells in vehicle controls fell within the normal ranges. The positive control chemicals induced statistically significant increases in the proportion of MNBN cells. Both replicate cultures at the positive control concentration analysed under each treatment condition demonstrated MNBN cell frequencies that clearly exceeded the normal range, thereby demonstrating the sensitivity and specificity of the assay. A minimum of 50% of cells had gone through at least one cell division (as measured by binucleate + multinucleate cell counts) in vehicle control cultures at the time of harvest. The maximum concentration analysed under each treatment condition met the criteria. The assay data were therefore considered valid and acceptable.

Treatment of cells with spiroxamine docosanoic acid ester for 3 hours (+21 hour recovery) in the absence and presence of S9 resulted in MNBN cells that were similar to and not significantly ($p \leq 0.05$) higher than those observed in concurrent vehicle control cultures for all concentrations analysed. It was however noted that one replicate from each test article concentration had a %MN-BN frequency that exceeded both the observed and the 95% percentile reference ranges. To understand the biological relevance of these increases, an additional short term treatment in the absence of S9 was undertaken.

Treatment of cells with spiroxamine docosanoic acid ester for 24 hours (+24 hour recovery) in the absence and presence of S9 resulted in frequencies of MNBN cells that were similar to and not significantly ($p \leq 0.05$) higher than those observed in concurrent vehicle control cultures for all concentrations analysed.

In the confirmatory 3 hours (+21 hour recovery) in the absence of S9, cells treated with spiroxamine docosanoic acid ester resulted in frequencies of MNBN cells that were similar to and not significantly ($p \leq 0.05$) higher that those observed in the concurrent vehicle control cultures for all concentrations analysed. The MNBN cell frequency of the majority of test article treated cultures for all concentrations analysed fell within the normal 95% reference range. The exception to this were single replicate cultures, each at the intermediate and the highest concentrations analysed (25 and 50 µg/mL, inducing 7% and 11% cytotoxicity, respectively) exhibiting MNBN cell values (0.90% and 1.10%, respectively) that marginally exceeded the normal 95% reference range (0.1 -0.80%). However, these small increases were not reproduced in their replicate cultures with the mean MNBN cell frequency at 25 µg/mL (0.55%) falling within the normal 95% reference range and the mean MNBN cell frequency at 50 µg/mL (0.85%) marginally exceeding the normal 95% reference range but falling within the observed range (0 -0.90%). As such, this small isolated increase was therefore not considered of biological relevance.

It is concluded that spiroxamine docosanoic acid ester did not induce biologically relevance increases in the frequency of micronuclei in human peripheral blood lymphocytes when tested up to 50 µg/mL (a concentration limited by precipitate observed at the end of treatment) in the absence (3 hour (+21 hour recovery) and 24 hour (+24 hour)) and presence (3 hour (+21 hour) of an Aroclor induced rat liver metabolic activation system (S9, final concentration 1% v/v).

Materials and Methods

A. Materials:

1. Test Material:	Spiroxamine docosanoic acid ester (alternative name: 4-tert-butlcyclohexyl docosanoate, M35)
Description:	White crystalline powder
Lot/Batch No.:	BCS-DH86302
Purity:	96.2% (w/w) (correction not applied) (molecular weight: 478.83 g/mol)
CAS No.:	Not assigned
Stability of test compound:	Confirmed stable for the duration of the study (expiry date: 25 February 2022)
2. Control materials:	
Solvent/final concentration:	Tetrahydrofuran (THF)/0.2% (v/v)
Positive: -S9	Mitomycin C (MMC, 3 h: 0.3, 24 h : 0.20 µg/mL) [clastogenic control] Vinblastine (VIN, 24 h: 0.04 µg/mL) [aneugenic control]
Positive: +S9	Cyclophosphamide (CPA, 3 h: 7 µg/mL)
3. Activation:	S9[22] was purchased from a commercial source. ♂ Sprague Dawley rats were treated with Aroclor 1254 (supplied by [redacted], lot no.: 4179, protein content 3.3 mg/mL). The composition of the 10% S9 reaction mix was: 100 µL S9, Na PBS (100 µM), glucose-6-phosphate (5 µM), β-NADP (4 µM), $MgCl_2$ (8 µM), KCl (33 µM), water (to volume).
4. Test organisms:	Human peripheral blood lymphocytes were collected from 2 healthy, non-smoking adult donors aged between 23 and 30 years, pooled and diluted with HML media. Cells were stimulated to divide by the addition of phytohaemagglutinin (PHA).
5. Culture medium:	HEPES buffered RPMI supplemented with 10% (v/v) inactivated fetal calf serum, 0.52% penicillin-streptomycin cell culture.

22 In this study, S9 activity was evaluated with CPA. In addition, each batch was checked by the manufacturer of S9 for sterility, protein content, ability to convert ethidium bromide, cyclophosphamide, B[a]P and AAN to bacterial mutagens, and cytochrome P-450-catalysed enzyme activities (alkoxyresorufin-*O*-dealkylase activities). Therefore, there is no concern over S9 activity.

6. Test article Concentrations:

a)	**Preliminary cytotoxicity test:**	3 h (+21 h recovery) -/+S9; 24 h (+24 h) –S9: 0, UTC, 1.814, 3.023, 5.039, 8.398, 14.0, 23.33, 38.88, 64.8, 108, 300, 500 µg/mL (maximum limited by solubility in the test system)
b)	**Micronucleus assay:**	3 h (+21 h recovery) -/+S9; 24 h (+24 h) –S9: 0, UTC, 5, 10, 25, 50, 75, 100, 200 µg/mL 3 h (+21 h recovery) +S9; 24 h (+24 h) –S9: 0, UTC, 5, 10, 25, 50, 75, 100, 200 µg/mL Confirmatory 3 h (+21 h) -S9: 0, UTC, 5, 10, 25, 50, 75, 100, 200 µg/mL (concentrations underlined scored for micronucleus frequency)

B. Test Performance:

1. In life dates: 6 April 2020 to 17 June 2020 (experimental dates)

2. Vehicle selection: A preliminary solubility test confirmed spiroxamine docosanoic acid ester was soluble in tetrahydrofuran (THF) up to at least 720.22 mg/mL.
The solubility limit in culture medium was below 28.13 µg/mL, as indicated by a lack of precipitation at this concentration *ca.* 24 h after test article addition. A maximum concentration of 500 µg/mL was selected for the cytotoxicity Range-Finder Experiment, in order that treatments were performed up to a precipitating concentration. Concentrations for the micronucleus experiment were selected based on the results of the cytotoxicity range-finder experiment.
Test article stock solutions were prepared by formulating spiroxamine dosanoic acid ester under subdued lighting in THF with the aid of vortex mixing and warming at 37°C, to give the maximum required treatment solution concentration. Subsequent dilutions were made using THF. The test article solutions were protected from light and used within approximately 3 hours of initial formulation.

3. Statistics: The proportion of MNBN cells for each treatment condition were compared with the proportion in vehicle controls by using Fisher's exact test. A Cochran-Armitage trend test was applied to each treatment condition. Probability values of $p \leq 0.05$ were accepted as significant.

4. Acceptance criteria: The following acceptance criteria had to be met for assay acceptability:

1. The binomial dispersion test demonstrated acceptable heterogeneity (in terms of MNBN cell frequency) between replicate cultures, particularly where no positive responses were seen;
2. The frequency of MNBN cells in vehicle controls fell within the current 95th percentile of the observed historical vehicle control (normal) ranges;
3. The positive control chemicals induced statistically significant increases in the proportion of cells with micronuclei. Both replicate cultures at the positive control concentration analysed under each treatment condition demonstrated MNBN cell frequencies that clearly exceeded the normal range;
4. A minimum of 50% of cells had gone through at least one cell division (as measured by binucleate + multinucleate cell counts) in vehicle control cultures at the time of harvest;
5. The maximum concentration analysed under each treatment condition met the specified criteria (*i.e.* the highest concentration selected for micronucleus analysis following all treatment conditions was one at which 50-60% cytotoxicity was achieved).

5. Evaluation criteria: For valid data, the test article was considered to induce clastogenic and/or aneugenic events if:

1. A statistically significant increase in the frequency of MNBN cells at one or more concentrations was observed;

2. An incidence of MNBN cells at such a concentration that exceeded the normal range in both replicates was observed;
3. A concentration-related increase in the proportion of MNBN cells was observed (positive trend test).

The test article was considered positive in this assay if all of the above criteria were met.
The test article was considered negative in this assay if none of the above criteria were met.

C. Methods:

1. Homogeneity and achieved concentration analysis of the dose: Not undertaken.

2. Preliminary cytotoxicity assay: Whole blood cultures were established by placing 0.4 mL of pooled heparinised blood into 8.5 mL pre-warmed HEPES-buffered RPMI medium containing 10% (v/v) heat inactivated fetal calf serum and 0.52% penicillin/streptomycin, so that the final volume following addition of S9 mix/KCl and the test article in its chosen vehicle was 10 mL. The mitogen, phytohaemagglutinin (PHA, reagent grade) was included in the culture medium at a concentration of approximately 2% of culture to stimulate the lymphocytes to divide. Blood cultures were incubated at 37±1°C for approximately 48 hours and rocked continuously. S9 mix or KCl (1 mL/culture) was added appropriately. Cultures were treated with the test article or vehicle (0.1 mL/culture). An untreated control was also included as THF is not a typical vehicle for this study type. Positive control treatments were not included. Duplicate cultures were used for the vehicle control and single cultures were used for each test article treated concentration.
For removal of the test article, cells were pelleted (approximately 300 g, 10 minutes, washed twice with sterile saline and resuspended in fresh pre-warmed medium containing fetal calf serum and penicillin/streptomycin. Cyto-B (formulated in DMSO) was added to post wash-off culture medium to give a final concentration of 6 µg/mL culture to inhibit cytokinesis, resulting in binucleate cells (without effecting karyokinesis), thereby arresting cells in interphase.
Changes in osmolality of more than 50 mOsm/kg and fluctuations in pH of more than one unit may be responsible for an increase in chromosome aberrations. Osmolality and pH measurements on post-treatment incubation medium were taken in the cytotoxicity range-finder experiment.
Cytotoxicity was determined by examination of the proportion of mono, bi and multinucleate cells to a minimum of 500 cells/concentration. From this, the replication index (RI) was calculated for each concentration (see Cytotoxicity section below).

3. Micronucleus assay:

Cell treatment: Cells were exposed to the test article formulation, vehicle or positive control as described above, with the exception of quadruplicate cultures were used for the vehicle control and duplicate controls used for the positive controls and each test article concentration. Concentrations selected for micronucleus analysis were assessed for cytotoxicity uncoded and for the proportions of mono-, bi- and multinucleate cells to a minimum of 200 or 500 cells/culture (preliminary cytotoxicity range finder or micronucleus experiment, respectively)./

Spindle inhibitor: Cyto-B was added post-wash to cultures to inhibit cytokinesis.

Slide preparation: Slides were prepared by spreading the fixed cultures on clean slides. The slides were stained with acridine orange (12.5 µg/mL) dropped on to slides, coverslipped and scored prior to analysis.

Cytotoxicity: The replication index (RI), which indicates the relative number of nuclei compared to controls, was determined using the formula below:

$$RI = \frac{\textit{no. of binucleate cells + 2(no. of multinucleate cells)}}{\textit{total no. of cells in treated cultures}}$$

Relative RI (expressed in terms of percentage) for each treated culture was calculated as follows:

$$\textit{Relative RI (\%)} = \frac{\textit{RI of treated cultures}}{\textit{RI of vehicle control}} \times 100$$

Cytotoxicity (%) was expressed as (100 – Relative RI).

Micronucleus assessment: Slides from the highest selected concentration and two lower concentrations were taken for microscopic analysis.
Immediately prior to analysis, 1-2 drops of PBS were added to the slides before mounting with glass coverslips. One thousand binucleate cells from each culture (total of 2000 binucleate cells/concentration) were analysed for micronuclei. The number of cells containing micronuclei and the number of micronuclei/cell on each slide were noted.
For the 24 hour (+24 hour recovery) treatment condition in the absence of S9 in Micronucleus Experiment 1, it was subsequently deemed necessary to analyse the two remaining vehicle control cultures, to aid data interpretation.
Nucleoplasmic bridges (NPBs) between nuclei in binuclear cells were recorded during micronucleus analysis to provide an indication of chromosome rearrangement. Various mechanisms may lead to NPB formation following DNA misrepair of strand breaks in DNA in this assay, binucleate cells with NPBs were recorded as part of the micronucleus analysis.

Results

A. Analytical determinations:

Not undertaken. Analyses for achieved concentration, homogeneity or stability of test article formulations were not conducted as part of this study, as this is not a requirement of the regulatory test guidelines.

B. Preliminary cytotoxicity assay:

Test article precipitate was observed at concentrations of 64.80 µg/mL and above in both short term and long term treatments, with no overt toxicity observed.

No marked changes in osmolality or pH were observed at the highest three concentrations tested as compared to the concurrent vehicle controls (individual data not reported).

The results of the cytotoxicity preliminary cytotoxicity range-finder experiment were used to select suitable maximum concentrations for the micronucleus experiment, with the maximum concentration tested limited by precipitate observed at the end of treatment.

Table CA 5.8.1/25-1: Spiroxamine docosanoic acid ester: human lymphocyte preliminary cytotoxicity range finder experiment

Conc. (µg/mL)	3 h (+ 21 h recovery) –S9		3 h (+ 21h recovery) +S9		24 h (+ 24 h recovery) –S9	
	Replication index (RI)	Cytotoxicity based on RI (%)	Replication index (RI)	Cytotoxicity based on RI (%)	Replication index (RI)	Cytotoxicity based on RI (%)
0	0.90	-	0.90	-	0.88	-
Untreated	0.87	-	0.95	-	0.87	-
1.814	0.86	4	0.84	6	0.92	0
3.023	0.82	8	0.88	3	1.02	0
5.039	0.96	0	0.91	0	0.98	0

Conc. (µg/mL)	3 h (+ 21 h recovery) –S9		3 h (+ 21h recovery) +S9		24 h (+ 24 h recovery) –S9	
	Replication index (RI)	Cytotoxicity based on RI (%)	Replication index (RI)	Cytotoxicity based on RI (%)	Replication index (RI)	Cytotoxicity based on RI (%)
8.398	0.93	0	1.00	0	1.07	0
14.00	0.87	3	0.92	0	0.96	0
23.33	0.91	0	0.98	0	1.13	0
38.88	0.93	0	0.94	0	1.01	0
64.80	0.91 E-ppt	0	0.95 E-ppt	0	1.12 E-ppt	0
108	0.95 ppt, E-ppt	0	0.90 ppt, E-ppt	0	1.21 ppt, E-ppt	0
180	0.88 ppt, E-ppt	2	0.94 ppt, E-ppt	0	1.17 ppt, E-ppt	0
300	0.93 ppt, E-ppt, H-ppt	0	0.95 ppt, E-ppt, H-ppt	0	1.17 ppt, E-ppt, H-ppt	0
500	0.90 ppt, E-ppt, H-ppt	0	0.90 ppt, E-ppt, H-ppt	12	1.10 ppt, E-ppt, H-ppt	0

ppt: precipitate observed at treatment
H-ppt: precipitate observed at harvest
E-ppt: precipitate observed at end of treatment

C. Micronucleus assay:

1. Assay acceptability: The frequency of MNBN cells in vehicle controls fell within the normal ranges. The positive control chemicals induced statistically significant increases in the proportion of MNBN cells. Both replicate cultures at the positive control concentration analysed under each treatment condition demonstrated MNBN cell frequencies that clearly exceeded the normal range, thereby demonstrating the sensitivity and specificity of the assay. A minimum of 50% of cells had gone through at least one cell division (as measured by binucleate + multinucleate cell counts) in vehicle control cultures at the time of harvest. The maximum concentration analysed under each treatment condition met the criteria. The assay data were therefore considered valid and acceptable.

2. Short term treatment in the absence and presence of S9: Treatment of cells with spiroxamine docosanoic acid ester for 3 hours (+21 hour recovery) in the absence and presence of S9 resulted in the mean MNBN cell frequency that were similar to and not significantly ($p \leq 0.05$) higher than those observed in concurrent vehicle control cultures for all concentrations analysed. It was however noted that one replicate from each test article concentration had a %MN-BN frequency that exceeded both the observed and the 95% percentile reference ranges. To understand the biological relevance of these increases, an additional short term treatment in the absence of S9 was undertaken.

Table CA 5.8.1/25-2: Spiroxamine docosanoic acid ester micronuclei assay: 3 h (+ 21 h recovery) –S9 treatment and laboratory historical control data

Conc. (µg/mL)		3 h (+ 21 h recovery) –S9				Vehicle historical control ranges (♀ donors)	
		Total BN	Total MN-BN	Frequency of MN-BN (%)	Cyto. (%)	Feb 17 – Feb 18	% MNBN
Vehicle	A	1000	10	1.00		No. of expts	15
	B	1000	4	0.40		Number of cultures	40
Total		2000	14	Mean: 0.70	-	Mean ±SD	0.40 ±0.28
Untreated	A	---[a]		---[a]		min. – max.	0.00 – 1.30
	B	---[a]		---[a]		95% reference range	0.00 – 1.01
Total		---[a]		---[a]	Mean: 0		
25	A	1000	13	1.30 >HC			
	B	1000	7	0.70			
Total		2000	20	Mean: 1.00	Mean: 0		
50	A	1000	11	1.10 >HC			

						Positive historical control ranges (♀ donors)	
	B	1000	5	0.50			
Total		2000	16	Mean: 0.80	Mean: 0		
75[E-ppt]	A	1000	15	1.50[>HC]		MMC (0.3 µg/mL)	
	B	1000	8	0.80		Aug 15 – Dec 17	% MNBN
Total		2000	23	Mean: 1.15	Mean: 0	No. of expts	19
Linear trend: p 0.0655 NS						Number of cultures	40
MMC	A	1000	60	6.00		Mean ±SD	5.68 ±1.66
(0.3)	B	1000	78	7.80		min. – max.	2.80 – 9.20
Total		2000	138	Mean: 6.90***	Mean: 33	95% reference range	3.39 – 8.81

*** p<0.001
>HC: exceeds historical control
a as vehicle controls were considered acceptable, %MN-BN frequency not scored
E-ppt: precipitate observed at end of treatment
No test article related increases in cells with NPBs were observed (data not reported)

Table CA 5.8.1/25-3: Spiroxamine docosanoic acid ester: human lymphocyte micronuclei assay: 3 h (+ 21 h recovery) +S9 treatment and laboratory historical control data

Conc. (µg/mL)		3 h (+ 21 h recovery) +S9				Vehicle historical control ranges (♀ donors)	
		Total BN	Total MN-BN	Frequency of MN-BN (%)	Cyto. (%)	Apr 17 – Feb 18	% MNBN
Vehicle	A	1000	5	0.50		No. of expts	16
	B	1000	6	0.60		Number of cultures	40
Total		2000	11	Mean: 0.55	-	Mean ±SD	0.40 ±0.55
Untreated	A	---[a]	---[a]	---[a]		min. – max.	0.00 – 1.20
	B	---[a]	---[a]	---[a]		95% reference range	0.10 – 1.20
Total		---	---[a]	Mean: ---[a]	Mean: 0		
10	A	1000	5	0.50			
	B	1000	9	0.90			
Total		2000	14	Mean: 0.70	Mean: 13		
25	A	1000	5	0.50			
	B	1000	3	0.30		Positive historical control ranges (♀ donors)	
Total		2000	8	Mean: 0.40	Mean: 2		
50[E-ppt]	A	1000	3	0.30		CPA (3 µg/mL)[1]	
	B	1000	7	0.70		Feb 16 – Feb 18	% MNBN
Total		2000	14	Mean: 0.70	Mean: 1	No. of expts	21
Linear trend: p 0.7826 NS						Mean:	41
CPA	A	1000	35	3.50		Mean ±SD	2.60 ±1.07
(7.0)	B	1000	35	3.50		min. – max.	0.80 – 5.00
Total		2000	70	Mean: 3.50**	Mean: 54	95% reference range	1.00 – 4.70

*** p<0.001
a as vehicle controls were considered acceptable, %MN-BN frequency not scored
E-ppt: precipitate observed at end of treatment
No test article related increases in cells with NPBs were observed (data not reported)
1 There is currently no historical control range for CPA 7 µg/mL, the concentration analysed in this study, therefore the range for the highest CPA concentration normally analysed (3 µg/mL) has been included for comparative purposes

3. Extended treatment in the absence of S9: Treatment of cells with spiroxamine docosanoic acid ester for 24 hours (+24 hour recovery) in the absence of S9 resulted in frequencies of MNBN cells that were significantly (p≤0.05) higher that those observed in the concurrent vehicle control cultures for all concentrations analysed. The MN-BN cell frequency of the majority of test article treated cultures for all concentrations analysed fell within the normal 95% reference range. The exception to this were single replicate cultures, each at the intermediate and the highest concentrations analysed (25 and 50 µg/mL, inducing 7% and 11% cytotoxicity, respectively)

exhibiting MN-BN cell values (0.90% and 1.10%, respectively) that marginally exceeded the normal 95% reference range (0.1 -0.80%). However, these small increases were not reproduced in their replicate cultures with the mean MNBN cell frequency at 25 µg/mL (0.55%) falling within the normal 95% reference range and the mean MN-BN cell frequency at 50 µg/mL (0.85%) marginally exceeding the normal 95% reference range but falling within the observed range (0 - 0.90%). Therefore, in the absence of any statistically significant increases for all concentrations analysed and no evidence of concentration-related increase, the two isolated increases were considered of no biological relevance.

Table CA 5.8.1/25-4: Spiroxamine docosanoic acid ester: human lymphocyte micronuclei assay - 24 h (+ 24 h recovery) -S9 treatment and laboratory historical control data

Conc. (µg/mL)		24 h (+ 24 h recovery) –S9				Vehicle historical control ranges (♀ donors)	
		Total BN	Total MN-BN	Frequency of MN-BN (%)	Cyto. (%)	Apr 17 – Jan 18	% MNBN
Vehicle	A	1000	6	0.60		No. of expts	14
	B	1000	6	0.60		Number of cultures	40
Total		2000	12	Mean: 0.60	Mean: -	Mean ±SD	0.34 ±0.21
Untreated	A	---[a]	---[a]			min. – max.	0.00 – 0.90
	B	---[a]	---[a]			95% reference range	0.10 – 0.80
Total		---[a]	---[a]	Mean: ---[a]	Mean:-		
10	A	1000	8	0.80			
	B	1000	6	0.60			
Total		2000	14	Mean: 0.70	Mean: 11		
25	A	1000	9	0.90[>HC]			
	B	1000	2	0.20			
Total		2000	11	Mean: 0.55	Mean: 7		
50[E-ppt]	A	1000	6	0.60		Positive historical control ranges (♀ donors)	
	B	1000	11	1.10[>HC]		VIN (0.04 µg/mL)	
Total		2000	17	Mean: 0.85	Mean: 11	Feb 17 – Jan 18	% MNBN
Linear trend, p 0.1812 NS						No. of expts	20
						Mean:	40
VIN (0.04)	A	1000	54	5.40		Mean ±SD	5.59 ±2.05
	B	1000	31	3.10		min. – max.	2.50 – 10.10
Total		2000	85	Mean: 4.25***	Mean: 47	95% reference range	2.50 – 8.93

*** p<0.001
a as vehicle controls were considered acceptable, %MN-BN frequency not scored
E-ppt: precipitate observed at end of treatment
>HC: exceeds historical control
No test article related increases in cells with NPBs were observed (data not reported)

4. Short-term treatment in the absence of S9, confirmatory experiment:

Treatment of cells with spiroxamine docosanoic acid ester for 3 hours (+21 hour recovery) in the absence of S9 resulted in frequencies of MNBN cells that were similar to and not significantly ($p \leq 0.05$) higher than those observed in concurrent vehicle control cultures for all three concentrations analysed. With the exception of a single replicate culture (A) at the intermediate concentration (5 µg/mL, inducing 0% cytotoxicity), the MNBN cell frequency of all remaining test article treated cultures for all concentrations analysed fell within the normal 95% reference range. The group mean MNBN cell frequency of all concentrations analysed also fell within the normal 95% reference range. As such, this isolated and marginal increase observed only at a single culture was considered of no biological relevance. According to the current data interpretation strategy (Thybaud *et al*[17]), weak non-reproducible increases may

be considered of little to no genotoxicity concern. Therefore, it can conclude that the response observed in Experiment 1 was non-reproducible and not biologically relevant.

Table CA 5.8.1/25-5: Spiroxamine docosanoic acid ester: human lymphocyte micronuclei assay: 3 h (+ 21 h recovery) -S9 treatment, micronucleus experiment 2 and laboratory historical control data

Conc. (µg/mL)		3 h (+ 21 h recovery) –S9			
		Total BN	Total MN-BN	Frequency of MN-BN (%)	Cyto. (%)
Vehicle	A	1000	7	0.70	
	B	1000	4	0.40	
Total		2000	11	Mean: 0.55	-
Untreated	A	---[a]	---[a]	---[a]	
	B	---[a]	---[a]	---[a]	
Total		---[a]	---[a]	Mean: ---[a]	Mean: 0
10	A	1000	5	0.50	
	B	1000	3	0.30	
Total		2000	8	Mean: 0.40	Mean: 0
25	A	1000	11	1.10[>HC]	
	B	1000	5	0.70	
Total		2000	16	Mean: 0.90	Mean: 0
50[E-ppt]	A	1000	15	1.50	
	B	1000	8	0.70	
Total		2000	23	Mean: 1.10	Mean: 0
Linear trend: p 0.6345 NS					
MMC (0.3)	A	1000	60	3.50	
	B	1000	78	5.50	
Total		2000	138	Mean: 4.50***	Mean: 33

Vehicle historical control ranges (♀ donors)	
Feb 17 – Feb 18	% MNBN
No. of expts	15
Number of cultures	40
Mean ±SD	0.40 ±0.28
min. – max.	0.00 – 1.30
95% reference range	0.00 – 1.01

Positive historical control ranges (♀ donors)	
MMC (0.3 µg/mL)	
Aug 15 – Dec 17	% MNBN
No. of expts	19
Number of cultures	40
Mean ±SD	5.68 ±1.66
min. – max.	2.80 – 9.20
95% reference range	3.39 – 8.81

*** p<0.001
a as vehicle controls were considered acceptable, %MN-BN frequency not scored
>HC: exceeds historical control
E-ppt: precipitate observed at end of treatment
No test article related increases in cells with NPBs were observed (data not reported)

D. Deficiencies:

Whilst not deemed a deficiency, it is considered prudent to detail the rationale for undertaking an extended treatment which included a recovery period.

Following revision of the OECD 487 TG in 2014 the cell treatment and harvest times have been somewhat simplified with emphasis placed on performing the extended treatment without recovery, with sampling times or recovery times extended by up to a further 1.5-2.0 normal cell cycles where justified.

Given the purpose for the *in vitro* micronucleus assay is to determine both clastogenic and aneugenic potential, the assay is compromised if an extended treatment with recovery is not included for sensitive detection of certain chemical classes.

Average generation times and replication index data demonstrated that the human peripheral blood lymphocytes were actively dividing beyond 96 hours, indicating that the extended treatment with recovery within the assay system did not compromise the results. This is directly in contrast to the statement in the OECD 487 test guideline, which includes the statement '*for lymphocytes exponential growth may be declining at 96 hours following stimulation and monolayer cultures of cells may become confluent'*. This statement in the test guideline is not supported by any published data, but rather a throw away statement and may provide the 'get out clause' why testing laboratory's did not use this approach as they may have had reduction in growth at 96 hours.

The extended treatment with recovery allows adequate detection of chemicals that either act on a narrow portion of the cell cycle (*e.g.* aneugenic compounds, G2/metaphase) and/or that induce cell cycle delay or require two rounds of DNA replication. The extended treatment without recovery in the micronucleus assay may be seen as somewhat compromised. It is widely known that direct acting clastogens such as azidothymidine and cytosine arabinoside are negative in the *in vitro* micronucleus assay with the 24 + 0 h approach, but positive with the with 24+24 h protocol. Therefore, the extended treatment with recovery is deemed more sensitive and in accordance with the latest published data (refer to Whitwell et al., 2019[8]), with the OECD test guideline to be updated to reflect this change.

Assessment and conclusion by applicant:

Assessment: Study meets the current guidance and the requirements in 283/2013.

Conclusion: It is concluded that spiroxamine docosanoic acid ester did not induce biologically relevance increases in the frequency of micronuclei in human peripheral blood lymphocytes when tested up to 50 µg/mL (a concentration limited by precipitate observed at the end of treatment) in the absence (3 hour (+21 hour recovery) and 24 hour (+24 hour)) and presence (3 hour (+21 hour) of an Aroclor induced rat liver metabolic activation system (S9, final concentration 1% v/v).

Spiroxamine-tetracosanoic acid ester (M36)

Dossier node	Draft title	Study ID	Planned submission
CA 5.8.1.5/01	Spiroxamine docosanoic acid ester (M35): stability in stimulated gastric fluids	8420315	Final: September 2021

Spiroxamine-cyclohexenol (M37)

Data Point:	KCA 5.8.1/26
Report Author:	[redacted]
Report Year:	2020
Report Title:	Spiroxamine cyclohexenol (M37): In Vitro human lymphocyte micronucleus assay
Report No:	8415892
Document No:	M-761547-01-1
Guideline(s) followed in study:	OECD 487 (2016)
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

Spiroxamine cyclohexenol was tested in an *in vitro* micronucleus assay using duplicate human lymphocyte cultures prepared from the pooled blood of two male donors in a two experiments. Treatments covering a broad range of concentrations, separated by narrow intervals, were performed, both in the absence and presence of metabolic activation (S9) from Aroclor 1254-induced rats. The test article was formulated in dimethyl sulphoxide (DMSO) and the highest concentration tested in the micronucleus experiment was limited cytotoxicity. All test article concentrations, formulated in DMSO were dosed into the test system at 1% v/v.

Following establishment of cultures, concentrations ranging from 200 to 340 µg/mL were tested in the absence (3 hours + 21 hour recovery), 150 to 350 µg/mL in the presence of S9 (3 h + 21 h) and 40 to 190 µg/mL in the extended treatment in the absence of S9 (24 h + 24 h). The test article concentrations for micronucleus analysis were selected by evaluating the effect of spiroxamine cyclohexenol on the replication index (RI). Micronuclei were analysed at three concentrations and a summary of the data are presented below.

The frequency of MNBN cells in vehicle controls fell within the normal ranges. The positive control chemicals induced statistically significant increases in the proportion of MNBN cells. Both replicate cultures at the positive control concentration analysed under each treatment condition demonstrated MNBN cell frequencies that clearly exceeded the normal range, thereby demonstrating the sensitivity and specificity of the assay. A minimum of 50% of cells had gone through at least one cell division (as measured by binucleate + multinucleate cell counts) in vehicle control cultures at the time of harvest. The maximum concentration analysed under each treatment condition met the criteria. The assay data were therefore considered valid and acceptable.

Treatment of cells with spiroxamine cyclohexenol for 3 hours (+21 hour recovery) in the absence of S9 resulted in MNBN cells that were similar to and not significantly ($p \leq 0.05$) higher than those observed in concurrent vehicle control cultures for all concentrations analysed. The MNBN cell frequency of the majority of test article treated cultures for all concentrations analysed, fell within the normal range. A single exception to this was a replicate culture (A) at 290 µg/mL which exhibited a MNBN cell value that marginally exceeded the normal range, but the mean MNBN cell frequency at this concentration fell within the normal 95% reference range. A weak but statistically significant linear trend was observed, however the majority of individual (and all group mean) MNBN cell values fell within the normal range. Therefore, these observations were considered of no biological relevance. It is noted that, one of the vehicle control cultures (B) exhibited a MNBN cell value that marginally exceeded the normal 95% reference range but fell within the observed range. The vehicle control group mean MNBN cell frequency also fell within the normal range.

Treatment of cells with spiroxamine cyclohexenol for 3 hours (+21 hour recovery) in the presence of S9 resulted in frequencies of MNBN cells that were similar to and not significantly ($p \leq 0.05$) higher that those observed in concurrent vehicle control cultures for all concentrations analysed. However, single cultures of the highest three concentrations analysed (280, 300 and 310 µg/mL, inducing 29%, 47% and 64% cytotoxicity, respectively) exhibited MNBN cell values that marginally exceeded the normal range. These increases were not reproduced in their replicate cultures and all remaining test article treated cultures fell within the normal range. Therefore, these sporadic increases were considered of questionable biological relevance.

Treatment of cells with spiroxamine cyclohexenol for 24 hours (+24 hour recovery) in the absence of S9 resulted in frequencies of MNBN cells that were significantly ($p \leq 0.05$) higher that those observed in the concurrent vehicle control cultures for the highest concentration analysed (170 µg/mL, inducing 59% cytotoxicity) with a statistically significant linear trend. The MNBN cell frequency of both test article treated cultures at this concentration exceeded the normal 95% reference range. It is noted that, one of the vehicle control cultures (A) exhibited a MNBN cell value that marginally exceeded the normal 95% reference range, but fell within the observed range. The vehicle control group mean MNBN cell frequency also fell within the normal 95% reference range. Overall, the MN response observed in the

test article treated cultures was present only at a high concentration (170 µg/mL), inducing a high cytotoxicity of 59% and as such these increases were considered of questionable biological relevance.

A second experiment was conducted for both the short term treatment in the presence of S9 and in the extended treatment in the absence of S9 in order to assess reproducibility and biological relevance of the increases observed

In the confirmatory 3 hours (+21 hour recovery) in the presence of S9 and the 24 hours (+24 hour recovery) in the absence of S9, cells treated with spiroxamine cyclohexenol resulted in frequencies of MNBN cells that were similar to and not significantly ($p \geq 0.05$) higher than those observed in concurrent vehicle control cultures for all three concentrations analysed for both treatment conditions. The sporadic marginal increases observed in Experiment 1 were not reproduced in the confirmatory Experiment 2, where similar concentrations inducing broadly similar cytotoxicity levels were analysed across the two independent experiments. As such, this small isolated increases were therefore not considered of biological relevance.

It is concluded that spiroxamine cyclohexenol did not induce biologically relevance increases in the frequency of micronuclei in human peripheral blood lymphocytes when tested up to a concentration limited by cytotoxicity in the absence (3 hour (+21 hour recovery) and 24 hour (+24 hour)) and presence (3 hour (+21 hour) of an Aroclor induced rat liver metabolic activation system (S9, final concentration 1% v/v).

Materials and Methods

A. Materials:

1. Test Material:	Spiroxamine cyclohexenol (alternative name: 4-tert-butlcyclohex-2-en1-ol, M37)
Description:	White crystalline powder
Lot/Batch No.:	BCS-AB45695-PU-01
Purity:	97.2% (w/w) (correction not applied) (molecular weight: 154.25 g/mol)
CAS No.:	Not assigned
Stability of test compound:	Confirmed stable for the duration of the study (expiry date: 3 January 2022)
2. Control materials:	
Negative:	
Solvent/final concentration:	Dimethyl sulphoxide (DMSO)/1.0% (v/v)
Positive: -S9	Mitomycin C (MMC, 3 h: 0.3, 24 h : 0.20 µg/mL) [clastogenic control] Vinblastine (VIN, 24 h: 0.04 µg/mL) [aneugenic control]
Positive: +S9	Cyclophosphamide (CPA, 3 h: 7 µg/mL)
3. Activation:	S9[23] was purchased from a commercial source. ♂ Sprague Dawley rats were treated with Aroclor 1254 (supplied by [redacted] lot no.: 4157, 4158, 4197 protein content 4.1, 4.2, 3.3 mg/mL, respectively). The composition of the 10% S9 reaction mix was: 100 µL S9, Na PBS (100 µM), glucose-6-phosphate (5 µM), β-NADP (4 µM), $MgCl_2$ (8 µM), KCl (33 µM), water (to volume).
4. Test organisms:	Human peripheral blood lymphocytes were collected from 2 healthy, non-smoking adult 2 ♂ donors aged between 30 and 34 years, pooled and diluted with HML media. Cells were stimulated to divide by the addition of phytohaemagglutinin (PHA).

23 In this study, S9 activity was evaluated with CPA. In addition, each batch was checked by the manufacturer of S9 for sterility, protein content, ability to convert ethidium bromide, cyclophosphamide, B[a]P and AAN to bacterial mutagens, and cytochrome P-450-catalysed enzyme activities (alkoxyresorufin-*O*-dealkylase activities). Therefore, there is no concern over S9 activity.

5. Culture medium:	HEPES buffered RPMI supplemented with 10% (v/v) inactivated fetal calf serum, 0.52% penicillin-streptomycin cell culture.
6. Test article Concentrations:	
a) Preliminary cytotoxicity test:	3 h (+21 h recovery) -/+S9; 24 h (+24 h) –S9: 0, 4.716, 7.861, 13.10, 21.84, 36.39, 60.65, 101.1, 168.5, 280.8, 468, 780, 1300 µg/mL (maximum limited by solubility in the test system)
b) Micronucleus assay:	3 h (+21 h recovery) -S9: 0, 200, 220, 240, 260, 280, 290, 300, 305, 310, 315, 320, 330, 340 µg/mL 3 h (+21 h recovery) +S9: 0, 150, 200, 200, 240, 260, 280, 290, 300, 310, 320, 330, 340, 350 µg/mL 24 h (+24 h) –S9: 0, 40, 60, 80, 100, 120, 130, 140, 150, 160, 170, 180, 190 µg/mL Confirmatory Experiments: 3 h (+21 h recovery) +S9: 0, 200, 225, 250, 270, 290, 295, 300, 305, 310, 315, 320 ,330 µg/mL 24 h (+24 h) –S9: 0, 50, 75, 100, 120, 140, 150, 160, 165, 170, 175, 180, 190 µg/mL (concentrations underlined scored for micronucleus frequency)

B. Test Performance:

1. In life dates:	4 March 2020 to 22 May 2020 (experimental dates)
2. Vehicle selection:	A preliminary solubility test confirmed spiroxamine cyclohexenol was soluble in dimethyl sulphoxide (DMSO) up to at least 130 mg/mL. The solubility limit in culture medium was in the range 650 to 1300 µg/mL, as indicated by precipitation at the higher concentration *c.a.* 24 h after test article addition. A maximum concentration of 1300 µg/mL was selected for the cytotoxicity Range-Finder Experiment, in order that treatments were performed up to a precipitating concentration. Concentrations for the micronucleus experiment were selected based on the results of the cytotoxicity range-finder experiment. Test article stock solutions were prepared by formulating spiroxamine cyclohexenol under subdued lighting in DMSO with the aid of vortex mixing and warming at 37°C, to give the maximum required treatment solution concentration. Subsequent dilutions were made using DMSO. The test article solutions were protected from light and used within approximately 3 hours of initial formulation.
3. Statistics:	The proportion of MNBN cells for each treatment condition were compared with the proportion in vehicle controls by using Fisher's exact test. A Cochran-Armitage trend test was applied to each treatment condition. Probability values of $p \leq 0.05$ were accepted as significant.
4. Acceptance criteria:	The following acceptance criteria had to be met for assay acceptability: 1. The binomial dispersion test demonstrated acceptable heterogeneity (in terms of MNBN cell frequency) between replicate cultures, particularly where no positive responses were seen; 2. The frequency of MNBN cells in vehicle controls fell within the current 95th percentile of the observed historical vehicle control (normal) ranges; 3. The positive control chemicals induced statistically significant increases in the proportion of cells with micronuclei. Both replicate cultures at the positive control concentration analysed under each treatment condition demonstrated MNBN cell frequencies that clearly exceeded the normal range; 4. A minimum of 50% of cells had gone through at least one cell division (as measured by binucleate + multinucleate cell counts) in vehicle control cultures at the time of harvest;

5. The maximum concentration analysed under each treatment condition met the specified criteria (*i.e.* the highest concentration selected for micronucleus analysis following all treatment conditions was one at which 50-60% cytotoxicity was achieved).

5. Evaluation criteria:

For valid data, the test article was considered to induce clastogenic and/or aneugenic events if:

1. A statistically significant increase in the frequency of MNBN cells at one or more concentrations was observed;
2. An incidence of MNBN cells at such a concentration that exceeded the normal range in both replicates was observed;
3. A concentration-related increase in the proportion of MNBN cells was observed (positive trend test).

The test article was considered positive in this assay if all of the above criteria were met.

The test article was considered negative in this assay if none of the above criteria were met.

C. Methods:

1. Homogeneity and achieved concentration analysis of the dose:

Not undertaken.

2. Preliminary cytotoxicity assay:

Whole blood cultures were established by placing 0.4 mL of pooled heparinised blood into 8.5 mL pre-warmed HEPES-buffered RPMI medium containing 10% (v/v) heat inactivated fetal calf serum and 0.52% penicillin/streptomycin, so that the final volume following addition of S9 mix/KCl and the test article in its chosen vehicle was 10 mL. The mitogen, phytohaemagglutinin (PHA, reagent grade) was included in the culture medium at a concentration of approximately 2% of culture to stimulate the lymphocytes to divide. Blood cultures were incubated at 37±1°C for approximately 48 hours and rocked continuously. S9 mix or KCl (1 mL/culture) was added appropriately. Cultures were treated with the test article or vehicle (0.1 mL/culture). An untreated control was also included as THF is not a typical vehicle for this study type. Positive control treatments were not included. Duplicate cultures were used for the vehicle control and single cultures were used for each test article treated concentration.

For removal of the test article, cells were pelleted (approximately 300 g, 10 minutes, washed twice with sterile saline and resuspended in fresh pre-warmed medium containing fetal calf serum and penicillin/streptomycin. Cyto-B (formulated in DMSO) was added to post wash-off culture medium to give a final concentration of 6 µg/mL/culture to inhibit cytokinesis, resulting in binucleate cells (without effecting karyokinesis), thereby arresting cells in interphase.

Changes in osmolality of more than 50 mOsm/kg and fluctuations in pH of more than one unit may be responsible for an increase in chromosome aberrations. Osmolality and pH measurements on post-treatment incubation medium were taken in the cytotoxicity range-finder experiment.

Cytotoxicity was determined by examination of the proportion of mono, bi and multinucleate cells to a minimum of 500 cells/concentration. From this, the replication index (RI) was calculated for each concentration (see Cytotoxicity section below).

3. Micronucleus assay:

Cell treatment:

Cells were exposed to the test article formulation, vehicle or positive control as described above, with the exception of quadruplicate cultures were used for the vehicle control and duplicate controls used for the positive controls and each test article concentration. Concentrations selected for micronucleus analysis were

assessed for cytotoxicity uncoded and for the proportions of mono-, bi- and multinucleate cells to a minimum of 200 or 500 cells/culture (preliminary cytotoxicity range finder or micronucleus experiment, respectively)./

Spindle inhibitor: Cyto-B was added post-wash to cultures to inhibit cytokinesis.

Slide preparation: Slides were prepared by spreading the fixed cultures on clean slides. The slides were stained with acridine orange (12.5 µg/mL) dropped on to slides, coverslipped and scored prior to analysis.

Cytotoxicity: The replication index (RI), which indicates the relative number of nuclei compared to controls, was determined using the formula below:

$$RI = \frac{no.\ of\ binucleate\ cells + 2(no.\ of\ multinucleate\ cells)}{total\ no.\ of\ cells\ in\ treated\ cultures}$$

Relative RI (expressed in terms of percentage) for each treated culture was calculated as follows:

$$Relative\ RI\ (\%) = \frac{RI\ of\ treated\ cultures}{RI\ of\ vehicle\ control} \times 100$$

Cytotoxicity (%) was expressed as (100 – Relative RI).

Micronucleus assessment: Slides from the highest selected concentration and two lower concentrations were taken for microscopic analysis.

Immediately prior to analysis, 1-2 drops of PBS were added to the slides before mounting with glass coverslips. One thousand binucleate cells from each culture (total of 2000 binucleate cells/concentration) were analysed for micronuclei. The number of cells containing micronuclei and the number of micronuclei/cell on each slide were noted.

For the 24 hour (+24 hour recovery) treatment condition in the absence of S9 in Micronucleus Experiment 1, it was subsequently deemed necessary to analyse the two remaining vehicle control cultures, to aid data interpretation.

Nucleoplasmic bridges (NPBs) between nuclei in binucleate cells were recorded during micronucleus analysis to provide an indication of chromosome rearrangement. Various mechanisms may lead to NPB formation following DNA misrepair of strand breaks in DNA in this assay, binucleate cells with NPBs were recorded as part of the micronucleus analysis.

Results

A. Analytical determinations:

Not undertaken. Analyses for achieved concentration, homogeneity or stability of test article formulations were not conducted as part of this study, as this is not a requirement of the regulatory test guidelines.

B. Preliminary cytotoxicity assay:

Test article precipitate was observed at concentrations of 280 µg/mL and above in both short term and long term treatments, overt toxicity observed.

No marked changes in osmolality or pH were observed at the highest three concentrations tested as compared to the concurrent vehicle controls (individual data not reported).

The results of the cytotoxicity preliminary cytotoxicity range-finder experiment were used to select suitable maximum concentrations for the micronucleus experiment, with the maximum concentration tested limited by precipitate observed at the end of treatment.

Table CA 5.8.1/26-1: Spiroxamine cyclohexenol: human lymphocyte preliminary cytotoxicity range finder experiment

Conc. (µg/mL)	3 h (+ 21 h recovery) –S9		3 h (+ 21h recovery) +S9		24 h (+ 24 h recovery) –S9	
	Replication index (RI)	Cytotoxicity based on RI (%)	Replication index (RI)	Cytotoxicity based on RI (%)	Replication index (RI)	Cytotoxicity based on RI (%)
0	0.88	-	0.89	-	1.14	-
4.716	0.82	6	0.95	0	1.15	0
7.861	0.91	0	0.91	0	1.14	0
13.10	0.95	0	0.88	1	1.06	7
21.84	0.91	0	0.88	1	1.20	0
36.39	0.88	0	0.86	3	1.13	1
60.65	0.83	6	0.88	1	1.05	8
101.1	0.90	0	0.91	0	0.78	31
168.5	0.78	11	0.81	9	0.40	65
280.8	0.28[ppt]	68	0.51[ppt]	43	0.07[ppt]	94
468.0	NE, ppt	-	NE, ppt, H-ppt	-	NE, ppt, E-ppt, H-ppt	-
780.0	NE, ppt, E-ppt, H-ppt	-	NE, ppt, E-ppt, H-ppt	-	NE, ppt, E-ppt, H-ppt	-
1300	NE, ppt, E-ppt, H-ppt	-	NE, ppt, E-ppt, H-ppt	-	NE, ppt, E-ppt, H-ppt	-

NE: not evaluated due to no scorable cells

ppt: precipitate observed at treatment
E-ppt: precipitate observed at end of treatment
H-ppt: precipitate observed at harvest

C. Micronucleus assay:

1. Assay acceptability: The frequency of MNBN cells in vehicle controls fell within the normal ranges. The positive control chemicals induced statistically significant increases in the proportion of MNBN cells. Both replicate cultures at the positive control concentration analysed under each treatment condition demonstrated MNBN cell frequencies that clearly exceeded the normal range, thereby demonstrating the sensitivity and specificity of the assay. A minimum of 50% of cells had gone through at least one cell division (as measured by binucleate + multinucleate cell counts) in vehicle control cultures at the time of harvest. The maximum concentration analysed under each treatment condition met the criteria. The assay data were therefore considered valid and acceptable.

2. Short term treatment in the absence of S9: Treatment of cells with spiroxamine cyclohexenol for 3 hours (+21 hour recovery) in the absence of S9 resulted in frequencies of MNBN cells that were similar to and not significantly ($p \leq 0.05$) higher that those observed in the concurrent vehicle control cultures for all concentration analysed. The MNBN cell frequency of the majority of test article treated cultures for all concentrations analysed, fell within the normal range. A single exception to this was a replicate culture (A) at 290 µg/mL which exhibited a MNBN cell value (0.80%) that marginally exceeded the normal range, but the mean MNBN cell frequency at this concentration (0.68%) fell within the normal 95% reference range (0 to 0.70%). A weak but statistically significant linear trend ($p \leq 0.05$) was observed, however the majority of individual (and all group mean) MNBN cell values fell within the normal range. Therefore, these observations were considered of no biological relevance. It is noted that, one of the vehicle control cultures (B) exhibited a MNBN cell value (0.80%) that marginally exceeded the normal 95% reference range (0 to 0.70%) but fell within the observed range (0 to 0.80%). The vehicle control group mean MNBN cell frequency (0.40%) also fell within the normal range (0 to 0.70%).

Table CA 5.8.1/26-2: Spiroxamine cyclohexenol: micronuclei assay: 3 h (+ 21 h recovery) –S9 treatment and laboratory historical control data

Conc. (µg/mL)		3 h (+ 21 h recovery) –S9				Vehicle historical control ranges (♂ donors)	
		Total BN	Total MN-BN	Frequency of MN-BN (%)	Cyto. (%)	Feb 17 – Dec 17	% MNBN
Vehicle	A	1000	2	0.20		No. of expts	17
	B	1000	8	0.80[>HC]		Number of cultures	40
	C	1000	1	0.10		Mean ±SD	0.35 ±0.1
	D	1000	5	0.50		min. – max.	0.00 – 0.80
Total		4000	16	Mean: 0.40	-	95% reference range	0.00 – 0.70
220	A	2000	7	0.35			
	B	2000	9	0.45			
Total		4000	16	Mean: 0.40	Mean: 12		
260	A	2000	10	0.50		**Positive historical control ranges (♂ donors)**	
	B	2000	7	0.35			
Total		4000	17	Mean: 0.43	Mean: 43	**MMC (0.3 µg/mL)**	
290[ppt]	A	2000	16	0.80[>HC]		**Feb 16 – Nov 17**	**% MNBN**
	B	2000	11	0.55		No. of expts	21
Total		4000	27	Mean: 0.68	Mean: 61	Number of cultures	40
Linear trend: p 0.0420						Mean ±SD	5.57 ±1.74
MMC (0.3)	A	1000	56	5.60		min. – max.	1.50 – 9.20
	B	1000	61	6.10		95% reference range	2.57 – 8.52
Total		2000	117	Mean: 5.85***	Mean: 48		

*** $p<0.001$
>HC: exceeds historical control
ppt: precipitate observed at treatment
No test article related increases in cells with NPBs were observed (data not reported)

3. Short term treatment in the presence of S9:

Treatment of cells with spiroxamine cyclohexenol for 3 hours (+21 hour recovery) in the presence of S9 resulted in frequencies of MNBN cells that were similar to and not significantly ($p \leq 0.05$) higher that those observed in concurrent vehicle control cultures for all concentrations analysed. However, single cultures of the highest three concentrations analysed (280, 300 and 310 µg/mL; inducing 29%, 47% and 64% cytotoxicity, respectively) exhibited MNBN cell values (1% or 1.1% in each case) that marginally exceeded the normal range (0.10 to 0.90%). These increases were not reproduced in their replicate cultures and all remaining test article treated cultures fell within the normal range. Therefore, these sporadic increases were considered of questionable biological relevance.

A second experiment was conducted, in order to assess reproducibility and biological relevance of the increases observed

Table CA 5.8.1/26-3: Spiroxamine cyclohexenol: human lymphocyte micronuclei assay: 3 h (+ 21 h recovery) +S9 treatment and laboratory historical control data

Conc. (µg/mL)		3 h (+ 21 h recovery) +S9				Vehicle historical control ranges (♂ donors)	
		Total BN	Total MN-BN	Frequency of MN-BN (%)	Cyto. (%)	Feb 17 – Dec 17	% MNBN
Vehicle	A	1000	5	0.50		No. of expts	18
	B	1000	8	0.80		Number of cultures	40
Total		2000	13	Mean: 0.65	-	Mean ±SD	0.36 ±0.20
240	A	1000	6	0.60		min. – max.	0.00 – 1.00
	B	1000	8	0.80		95% reference range	0.10 – 0.90
Total		2000	14	Mean: 0.70	Mean: 17		

280[ppt]	A	1000	9	0.90	
	B	1000	11	1.10[>HC]	
Total		2000	20	Mean: 1.00[>HC]	Mean: 29
300[ppt]	A	1000	10	1.00[>HC]	
	B	1000	7	0.70	
Total		2000	17	Mean: 0.85	Mean: 47
310[ppt]	A	1000	3	0.30	
	B	1000	10	1.00[>HC]	
Total		2000	13	Mean: 0.65	Mean: 64
Linear trend: p 0.4041 NS					
CPA (5.0)	A	1000	49	4.90	
	B	1000	47	4.90	
Total		2000	96	Mean: 4.80[***]	Mean: 38

*** $p<0.001$
>HC: exceeds historical control
ppt: precipitate observed at treatment

Positive historical control ranges (♂ donors)	
CPA (3 µg/mL)	
Feb 16 – Feb 18	% MNBN
No. of expts	22
Number of cultures	40
Mean ±SD	2.21 ±0.85
min. – max.	1.00 – 4.70
95% reference range	1.00 – 3.63

No test article related increases in cells with NPBs were observed (data not reported)
There is currently no historical control range for CPA (5 µg/mL), the concentration analysed in this study, therefore the range for the highest CPA concentration normally analysed (3 µg/ml) has been included for comparative purposes.

4. Extended treatment in the absence of S9:

Treatment of cells with spiroxamine cyclohexenol for 24 hours (+24 hour recovery) in the absence of S9 resulted in frequencies of MNBN cells that were significantly ($p\leq0.05$) higher that those observed in the concurrent vehicle control cultures for the highest concentration analysed (170 µg/mL, inducing 59% cytotoxicity) with a statistically significant linear trend ($p\leq0.05$). The MNBN cell frequency of both test article treated cultures at this concentration exceeded the normal 95% reference range. It is noted that, one of the vehicle control cultures (A) exhibited a MNBN cell value (0.85%) that marginally exceeded the normal 95% reference range (0 to 0.80%), but fell within the observed range (0 to 0.90%). The vehicle control group mean MNBN cell frequency (0.66%) also fell within the normal 95% reference range (0 to 0.80%). Overall, the MN response observed in the test article treated cultures was present only at a high concentration (170 µg/mL), inducing a high cytotoxicity of 59% and as such these increases were considered of questionable biological relevance.

A second experiment was conducted, in order to assess reproducibility and biological relevance of the increases observed

Table CA 5.8.1/26-4: Spiroxamine cyclohexenol: human lymphocyte micronuclei assay: 24 h (+ 24 h recovery) -S9 treatment and laboratory historical control data

Conc. (µg/mL)		24 h (+ 24 h recovery) –S9				Vehicle historical control ranges (♂ donors)	
		Total BN	Total MN-BN	Frequency of MN-BN (%)	Cyto. (%)	Jul 17 – Jan 18	% MNBN
						No. of expts	16
Vehicle	A	2000		0.85[>HC]		Number of cultures	40
	B	2000		0.55		Mean ±SD	0.34 ±0.23
	C	2000		0.55		min. – max.	0.00 – 0.90
	D	2000		0.60		95% reference range	0.10 – 0.80
Total		8000		Mean: 0.66	Mean: -		
100	A	1000	5	0.50			
	B	1000	7	0.70			
Total		2000	12	Mean: 0.60	Mean: 16		
140	A	1000	7	0.70			
	B	1000	3	0.30			

						Positive historical control ranges (♂ donors)	
Total		2000	10	Mean: 0.50	Mean: 34		
170[ppt]	A	1000	17	1.70[>HC]		VIN (0.04 µg/mL)	
	B	1000	12	1.20[>HC]		Feb 17 – Jan 18	% MNBN
Total		2000	29	Mean: 1.45	Mean: 59	No. of expts	20
Linear trend: p 0.6234 NS						Number of cultures	41
VIN (0.04)	A	1000	50	5.00		Mean ±SD	6.43 ±2.38
	B	1000	61	6.10		min. – max.	2.50 – 13.60
Total		2000	111	Mean: 5.55***	Mean: 65	95% reference range	2.80 – 13.50

*** p<0.001
a as vehicle controls were considered acceptable, %MN-BN frequency not scored
E-ppt: precipitate observed at end of treatment
>HC: exceeds historical control

No test article related increases in cells with NPBs were observed (data not reported)

5. Short-term treatment in the presence of S9, confirmatory experiment:

Treatment of cells with spiroxamine cyclohexenol for 3 hours (+21 hour recovery) in the presence of S9 resulted in frequencies of MNBN cells that were similar to and not significantly ($p \leq 0.05$) higher than those observed in concurrent vehicle control cultures for all three concentrations analysed. The sporadic marginal increases observed following 3 hours (+21 hour recovery) in the presence of S-9 in Experiment 1 were not reproduced in the confirmatory experiment 2, where similar concentrations inducing broadly similar cytotoxicity levels were analysed across the two independent experiments. As such, this isolated and marginal increase observed in a single experiment was considered of no biological relevance.

According to the current data interpretation strategy (Thybaud *et al*[17]), weak non-reproducible increases may be considered of little to no genotoxicity concern. Therefore, it can conclude that the response observed in Experiment 1 was non-reproducible and not biologically relevant.

6. Extended treatment in the absence of S9, confirmatory experiment:

Treatment of cells with spiroxamine cyclohexenol for 24 hours (+24 hour recovery) in the absence of S9 resulted in frequencies of MNBN cells that were similar to and not significantly ($p \leq 0.05$) higher than those observed in concurrent vehicle control cultures for all three concentrations analysed. The sporadic marginal increases observed following 24 hours (+24 hour recovery) in the absence of S-9 in Experiment 1 were not reproduced in the confirmatory experiment 2, where a lower cytotoxicity level was induced (54%).

According to the current data interpretation strategy (Thybaud *et al*[17]), weak non-reproducible increases may be considered of little to no genotoxicity concern. Therefore, it can conclude that the response observed in Experiment 1 was non-reproducible and not biologically relevant.

Table CA 5.8.1/26-5: Spiroxamine cyclohexenol: human lymphocyte micronuclei assay: 3 h (+ 21 h recovery) +S9 and 24 h (+ 24 h recovery) -S9 treatments, micronucleus experiment 2

Conc. (µg/mL)		3 h (+ 21 h recovery) +S9				24 h (+ 24 h recovery) –S9			
		Total BN	Total MN-BN	Frequency of MN-BN (%)	Cyto. (%)	Total BN	Total MN-BN	Frequency of MN-BN (%)	Cyto. (%)
Vehicle	A	1000	7	0.70		1000	4	0.40	
	B	1000	4	0.40		1000	5	0.50	
Total		2000	11	Mean: 0.55	-	2000	9	Mean: 0.45	-
75	A	---[a]	---[a]	---[a]		1000	5	0.50	
	B	---[a]	---[a]	---[a]		1000	6	0.60	
Total		2000	---[a]	---[a]	---[a]	---[a]	11	Mean: 0.55	Mean: 18
100	A	---[a]	---[a]	---[a]		1000	2	0.20	
	B	---[a]	---[a]	---[a]		1000	7	0.70	

Total		2000	---[a]	---[a]	---[a]	---[a]	9	Mean: 0.45	Mean: 28
140	A	---[a]	---[a]	---[a]		1000	8	0.80	
	B	---[a]	---[a]	---[a]		1000	6	0.60	
Total		2000	---[a]	---[a]	---[a]	---[a]	14	Mean: 0.70	Mean: 55
225	A	1000	4	0.40		---[a]	---[a]	---[a]	
	B	1000	6	0.60		---[a]	---[a]	---[a]	
Total		2000	10	Mean: 0.50	Mean: 13		---[a]	---[a]	---[a]
270[ppt]	A	1000	5	0.50		---[a]	---[a]	---[a]	
	B	1000	7	0.70		---[a]	---[a]	---[a]	
Total		2000	12	Mean: 0.60	Mean: 30		---[a]	---[a]	---[a]
305[ppt]	A	1000	2	0.20		---[a]	---[a]	---[a]	
	B	1000	3	0.30		---[a]	---[a]	---[a]	
Total		2000	5	Mean: 0.25	Mean: 54	---[a]	---[a]	---[a]	---[a]
Linear trend: p 0.8777 NS						Linear trend: p 0.5119 NS			
Positive control	A	1000	34	3.40		1000	69	6.90	
	B	1000	39	5.90		1000	63	6.30	
Total		2000	73	Mean: 4.65***	Mean: 33	2000	132	Mean: 6.60***	Mean: 47

*** p<0.001
>HC: exceeds historical control
E-ppt: precipitate observed at end of treatment
a not treated in this treatment condition
No test article related increases in cells with NPBs were observed (data not reported)

D. Deficiencies:

Whilst not deemed a deficiency, it is considered prudent to detail the rationale for undertaking an extended treatment which included a recovery period.

Following revision of the OECD 487 TG in 2014 the cell treatment and harvest times have been somewhat simplified with emphasis placed on performing the extended treatment without recovery, with sampling times or recovery times extended by up to a further 1.5-2.0 normal cell cycles where justified.

Given the purpose for the *in vitro* micronucleus assay is to determine both clastogenic and aneugenic potential, the assay is compromised if an extended treatment with recovery is not included for sensitive detection of certain chemical classes.

Average generation times and replication index data demonstrated that the human peripheral blood lymphocytes were actively dividing beyond 96 hours, indicating that the extended treatment with recovery within the assay system did not compromise the results. This is directly in contrast to the statement in the OECD 487 test guideline, which includes the statement '*for lymphocytes exponential growth may be declining at 96 hours following stimulation and monolayer cultures of cells may become confluent'*. This statement in the test guideline is not supported by any published data, but rather a throw away statement and may provide the 'get out clause' why testing laboratory's did not use this approach as they may have had reduction in growth at 96 hours.

The extended treatment with recovery allows adequate detection of chemicals that either act on a narrow portion of the cell cycle (*e.g.* aneugenic compounds, G2/metaphase) and/or that induce cell cycle delay or require two rounds of DNA replication. The extended treatment without recovery in the micronucleus assay may be seen as somewhat compromised. It is widely known that direct acting clastogens such as azidothymidine and cytosine arabinoside are negative in the *in vitro* micronucleus assay with the 24 + 0 h approach, but positive with the with 24+24 h protocol. Therefore, the extended treatment with recovery is deemed more sensitive and in accordance with the latest published data (refer to Whitwell *et al.*, 2019[8]), with the OECD test guideline to be updated to reflect this change.

Assessment and conclusion by applicant:

Assessment: Study meets the current guidance and the requirements in 283/2013.

Conclusion: It is concluded that spiroxamine cyclohexenol did not induce biologically relevance increases in the frequency of micronuclei in human peripheral blood lymphocytes when tested up to a concentration limited by cytotoxicity in the absence (3 hour (+21 hour recovery) and 24 hour (+24 hour)) and presence (3 hour (+21 hour) of an Aroclor induced rat liver metabolic activation system (S9, final concentration 1% v/v).

Data Point:	KCA 5.8.1/27
Report Author:	[redacted]
Report Year:	2020
Report Title:	Justification for toxicological read-across from spiroxamine metabolite M13 to M37
Report No:	0471836-TOX2
Document No:	M-761554-01-1
Guideline(s) followed in study:	ECHA RAAF (2017)
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	Yes

Executive Summary

The suitability of grouping spiroxamine plant metabolites M13 and M37 with a view to read-across of toxicology endpoints from M13 to M37 was evaluated, following the principles outlined in the ECHA read-across assessment framework as far as practicable.

ECHA RAAF Scenario 2 was considered an appropriate hypothesis to assess the suitability of grouping. Following evaluation according to this scenario it was concluded that the two metabolites are similar in respect of structure, molecular weight, physicochemical properties, chemical reactivity profiles and predicted ADME. Therefore grouping and read-across is justified.

Comparison of the chemical properties of M37 and M13 leads to the conclusion that both molecules are similar with respect to physicochemical properties, predicted chemical and biological activity and disposition. Therefore it is appropriate to group both molecules together for the purposes of read-across of toxicity data.

Results

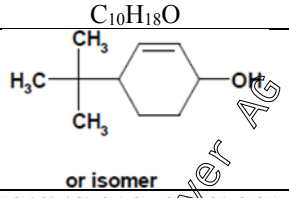
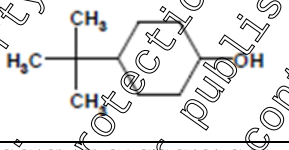
A. Substance identification and comparison of physicochemical properties:

The structure and physicochemical properties of M37 (target) and M13 (source) are provided in below.

Properties were predicted using ECOSAR v2.0 and similarity was measured using the Pubchem similarity calculator embedded in OECD Toolbox V4.4.

The physical properties of a molecule such as size, aqueous and lipid solubility contribute to the potential for absorption, distribution, metabolism and excretion. Both compounds have similar structure, size and solubilities. Both compounds contain the same major functional groups.

Table CA 5.8.1/27-1: Structure and physico-chemical properties of M37 and M13

Property	Spiroxamine cyclohexenol (M37)	Spiroxamine cyclohexanol (M13)
Chemical name	4-tert-butylcyclohex-2-en-1-ol	4-tert-butylcyclohexan-1-ol
CAS	not available	98-52-2
Molecular formula	$C_{10}H_{18}O$	$C_{10}H_{20}O$
Structure	 or isomer	
Smiles code	CC(C)(C)C1C=CC(O)CC1	CC(C)(C)C1CCC(O)CC1
Molecular weight	154.25	156.27
Log Kow (mg/L)	3.2 (estimated)	3.4 (estimated) 3.09 (measured)
Water solubility (mg/L)	433 (estimated)	326 (estimated) 100 (measured)
Similarity	80%	-
Impurities	Not relevant, compound is a plant metabolite	Not relevant, compound is a plant metabolite

B. Comparison of chemical and biological reactivity profiles:

The OECD Toolbox v4.4 was used to create a comparative profile of both compounds using the following profilers embedded in OECD Toolbox v4.4 under the following headings: general mechanistic, endpoint specific (human health), empiric and toxicological. The results of this profiling are presented below. It is noted that the double bond present in M37 is not part of a conjugated system and therefore not expected to be susceptible to Michael addition. This is supported by the results of the comparative profiling which showed that there were no notable differences between M13 and M37 in the profiles examined.

The presence of the double bond in M37 is likely to slightly limit the freedom of the cyclohexyl ring to assume different conformations but not completely remove it. Since the substituents on the ring are the same in M37 and M13 the most favourable conformation will also be the same in both molecules.

Table CA 5.8.1/27-2: Comparison of chemical and biological reactivity profiles (OECD Toolbox V4.4)

Profilers	Spiroxamine cyclohexenol (M37)	Spiroxamine cyclohexanol (M13)
General Mechanistic		
Protein binding by OECD	No alert found	No alert found
Toxic hazard classification by Cramer	Intermediate (Class II)	Low (Class I)
Uncouplers (MITOTOX)	Undefined	Non concern for uncoupling of OxPhos (pKa ranges)
DNA binding by OECD	No alert found	No alert found
Toxic hazard classification by Cramer (extended)	Intermediate (Class II)	Low (Class I)
Protein binding by OASIS	No alert found	No alert found
DNA binding by OASIS	No alert found	No alert found
Protein binding potency Cys (DPRA 13%)	DPRA less than 9% (DPRA 13%) DPRA less than 9% (DPRA 13%) >> Alcohols	DPRA less than 9% (DPRA 13%) DPRA less than 9% (DPRA 13%) >> Alcohols
Estrogen Receptor Binding	Weak binder, OH group	Weak binder, OH group
Protein binding potency Lys (DPRA 13%)	DPRA less than 9% (DPRA 13%)	DPRA less than 9% (DPRA 13%)

Profilers	Spiroxamine cyclohexenol (M37)	Spiroxamine cyclohexanol (M13)
	DPRA less than 9% (DPRA 13%) >> Alcohols	DPRA less than 9% (DPRA 13%) >> Alcohols
Protein binding potency GSH	Not possible to classify according to these rules (GSH)	Not possible to classify according to these rules (GSH)
Endpoint Specific		
Skin irritation/corrosion Exclusion rules by BfR	Undefined	Undefined
Oncologic Primary Classification	Not classified	Not classified
Protein binding alerts for skin sensitization according to GHS	No alert found	No alert found
Protein binding alerts for skin sensitization by OASIS	No alert found	No alert found
Eye irritation/corrosion Exclusion rules by BfR	Undefined	Undefined
Bioaccumulation - metabolism half-lives	Fast	Fast
Acute Oral Toxicity	Not categorized	Basic toxicity
Protein Binding Potency h-CLAT	No alert found	No alert found
rtER Expert System - USEPA	No alert found	Alkylcyclohexanols
Keratinocyte gene expression	Not possible to classify according to these rules	Not possible to classify according to these rules
DART scheme	Not known precedent reproductive and developmental toxic potential	Known precedent reproductive and developmental toxic potential Piperazine-, dioxane-, morpholine-, tetrahydrothiopyran-like derivatives and cyclohexanamine (17c)
Skin irritation/corrosion Inclusion rules by BfR	Inclusion rules not met	Inclusion rules not met
in vitro mutagenicity (Ames test) alerts by ISS	No alert found	No alert found
Carcinogenicity (genotox and nongenotox) alerts by ISS	No alert found	No alert found
Respiratory sensitisation	No alert found	No alert found
Retinoic Acid Receptor Binding	Not possible to classify according to these rules	Not possible to classify according to these rules
Protein binding alerts for Chromosomal aberration by OASIS	No alert found	No alert found
in vivo mutagenicity (Micronucleus) alerts by ISS	No alert found	No alert found
DNA alerts for AMES, CA and MNT by OASIS	No alert found	No alert found
Bioaccumulation - metabolism alerts	Aliphatic alcohol [-OH] -C=CH [alkenyl hydrogen] Carbon with 4 single bonds & no hydrogens -CH - [cyclic] -CH2- [cyclic] Methyl [-CH3]	Aliphatic alcohol [-OH] Carbon with 4 single bonds & no hydrogens -CH - [cyclic] -CH2- [cyclic] Methyl [-CH3]
Eye irritation/corrosion Inclusion rules by BfR	Inclusion rules not met	Inclusion rules not met

Profilers	Spiroxamine cyclohexenol (M37)	Spiroxamine cyclohexanol (M13)
Toxicological		
Repeated dose (HESS)	Not categorized	Not categorized

C. Comparison of ADME properties:

Human exposure is *via* consumption of plants and plant commodities therefore only the oral route is considered relevant. As demonstrated in Section 2, both compounds have similar structure, size and solubilities and both compounds contain the same major functional groups. Therefore the oral absorption, distribution and excretion of both molecules is expected to be similar. Biotransformation of both molecules is also expected to be similar, with hydroxylation at one or more positions being the major likely step possibly followed by conjugation of the hydroxy function with glucuronic acid.

D. Consideration of potential bias and/or other factors:

The ECHA RAAF framework requires evaluation of potential for bias in the selection of source molecules for read-across and the potential for exposure to other compounds than those linked to the prediction (e.g. manufacturing impurities). It is considered that these factors are not relevant for the justification of grouping of plant metabolites. Selection of lead/or source molecules comes from a very limited group of identified plant metabolites, rather than a whole chemical database. Likely human exposure is *via* consumption of metabolites present in plants and plant commodities and not from manufactured sources.

Table CA 5.8.1/27-3: Summary of available toxicity data

Spiroxamine cyclohexanol (M13)		
Study	**Endpoint**	**Reference**
Acute oral toxicity (rat) Acute dermal toxicity (rabbit)	LD_{50}: 4200 mg/kg bw LD_{50} > 5000 mg/kg bw	CA 5.8.1/07 [redacted] (1973)
In vitro bacterial reverse (Ames) gene mutation	+/-S9 negative	CA 5.8.1/22 [redacted] (2020)
In vitro mammalian L5178Y forward ($tk^{+/-}$) gene mutation	+/-S9 negative	CA 5.8.1/09 [redacted] (2012)
In vitro human peripheral blood lymphocytes micronucleus	+/-S9 negative	CA 5.8.1/23 [redacted] (2020)
Rat sub-acute toxicity (28 day dietary route)	NOAEL: 50 mg/kg bw/d	CA 5.8.1/11 [redacted] (1999)
Rat developmental toxicity study (conducted using M13 acetate ester)	Maternal NOAEL: 40 mg/kg bw/d Developmental NOAEL: 160 mg/kg bw/d	CA 5.8.1/12 [redacted] (2007)
Spiroxamine cyclohexenol (M37)		
Study	**Endpoint**	**Reference**
In vitro human peripheral blood lymphocytes micronucleus	+/-S9 negative	CA 5.8.1/26 [redacted] (2020)

Assessment and conclusion by applicant:

Assessment: Study meets the current guidance and the requirements in 283/2013.

Conclusion: Comparison of the chemical properties of M37 and M13 leads to the conclusion that both molecules are similar with respect to physicochemical properties, predicted chemical and biological activity and disposition. Therefore it is appropriate to group both molecules together for the purposes of read-across of toxicity data.

QSAR data on metabolites

Following expert review, *in silico* assessment using two methods showed there were no concerns for mutagenicity for any of the metabolites assessed. There was high confidence in the predictions for metabolites M05, M06, M07, M08 and M10 due to their close structural similarity to spiroxamine and M03 or, in the case of M06, being a major rat metabolite. For the sulfate conjugates M25, M26 and M27 confidence in the prediction was assigned as low, since the sulfate moiety was not assessed in any of the Leadscope sub-models therefore the prognosis was based on a single method. There was high confidence in the predictions for metabolites M13 and M14 due to the availability of experimental data for M13 and its structural similarity to M14. For metabolites M35, M36 and M37 there was high confidence in the prediction for chromosome damage due to the availability of experimental data for M35 and M37 plus the structural similarity between M35 and M36, but the overall confidence level for genotoxicity was medium, based on the mutagenicity endpoint for which there was no experimental data. For metabolites M15, M16 and M17 there was medium confidence in the predictions because they were based on two *in silico* methods. There was high confidence in the predictions for Metabolite M28 due to the availability of experimental data, medium confidence in the predictions for M30 and M31 and a low confidence in the prediction for M29 because the chromosome damage component of the genotoxicity assessment was based on a single method.

The QSAR analysis of spiroxamine metabolites for genotoxicity endpoints was split into three reports to keep document sizes manageable. Since the methods used were identical all three reports are summarised as one here.

Data Point:	KCA 5.8.1/28
Report Author:	[redacted]
Report Year:	2021
Report Title:	In silico prognosis of the genotoxic potential of spiroxamine group A metabolites (M01-M12, M25-M27, M38 & M41)
Report No:	0566398-TOX1
Document No:	M-763152-01-1
Guideline(s) followed in study:	ECHA Guidance on information requirements and chemical safety assessment Chapter R.6: QSARs and grouping of chemicals. May 2008. Guidance for the implementation of REACH
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	Yes

Data Point:	KCA 5.8.1/29
Report Author:	[redacted]; [redacted]
Report Year:	2021
Report Title:	In silico prognosis of the genotoxic potential of spiroxamine broup B metabolites (M13-M17 & M35-M37)
Report No:	0566398-TOX2
Document No:	M-763153-01-1
Guideline(s) followed in study:	ECHA Guidance on information requirements and chemical safety assessment Chapter R.6: QSARs and grouping of chemicals. May 2008. Guidance for the implementation of REACH
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	Yes

Data Point:	KCA 5.8.1/30
Report Author:	[redacted]; [redacted]
Report Year:	2021
Report Title:	In silico prognosis of the genotoxic potential of spiroxamine group C metabolites (M28-M31)
Report No:	0566398-TOX3
Document No:	M-763154-01-1
Guideline(s) followed in study:	ECHA Guidance on information requirements and chemical safety assessment Chapter R.6: QSARs and grouping of chemicals. May 2008. Guidance for the implementation of REACH
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	Yes

Executive Summary

In order to aid human health assessment, an *in silico* prognosis has been made of the genotoxicity potential of seventeen, eight and four plant and livestock metabolites, M01-M12, M25-M27, M38 & M41 (also known as Group A metabolites), M13-M17 & M35-M37 (also known as Group B metabolites) and M28-M31 (also known as Group C metabolites), respectively. The prognosis was made using Derek Nexus and Leadscope Model Applier software which represents two different methods, rule based and statistical, respectively. In addition, the OECD (Q)SAR Toolbox was used to create a profile for each structure, focusing on chemical and biological reactivity endpoints relevant for a genotoxicity prognosis.

The *in silico* results for each structure were subject to expert review taking into account available appropriate experimental data to give an overall assessment and an indication of confidence in the assessment. A summary of predictions following expert review taking into account available experimental data, which are considered as the final results of the in silico investigations, is presented in Tables CA 5.8.1/28-1 to CA 5.8.1/28-3.

Table CA 5.8.1/28-1: Summary of expert review of in silico modelling outputs for Group A metabolites

Endpoint	Mutagenicity				Chromosome damage				Overall expert call
Compound	Derek/ Toolbox	L/scope	Data	Expert call	Derek/ Toolbox	L/scope	Data	Expert call	
Spiroxamine				High				High	High
M01/M02/ M04/M09/ M11/ M12/M38/M41			-	Med			-	Med	Med
M03				High				High	High
M05/M06/M07 /M08/M10			-	High			-	High	High
M25/M7			-	Low			-	Low	Low
M26			-	Med			-	Low	Low

Table CA 5.8.1/28-2: Summary of expert review of in silico modelling outputs for Group B metabolites

Endpoint	Mutagenicity				Chromosome damage				Overall expert call
Compound	Derek/ Toolbox	L/scope	Data	Expert call	Derek/ Toolbox	L/scope	Data	Expert call	
Spiroxamine				High				High	High
M13				High				High	High
M14				High				High	High
M15/M16/ M17			-	Med			-	Med	Med
M35/M37				Med				High	Med
M36			-	Med			-	High	Med

Table CA 5.8.1/28-3: Summary of expert review of *in silico* modelling outputs for Group C metabolites

Endpoint	Mutagenicity				Chromosome damage				Overall expert call
Compound	Derek/ Toolbox	L/scope	Data	Expert call	Derek/ Toolbox	L/scope	Data	Expert call	
Spiroxamine				High				High	High
M28				High				High	High
M29				Med			-	Low	Low
M30/M31			-	Med			-	Med	Med

Key

Indicator	Expert review
	Positive/confidence
	Negative/confidence
	Cannot call

Following expert review, *in silico* assessment using two methods showed there were no concerns for mutagenicity for any of the metabolites assessed. There was high confidence in the predictions for metabolites M05, M06, M07, M08 and M10 due to their close structural similarity to spiroxamine and M02 or, in the case of M06, being a major rat metabolite. For the sulfate conjugates M25, M26 and M27 confidence in the prediction was assigned as low, since the sulfate moiety was not assessed in any of the Leadscope sub-models therefore the prognosis was based on a single method.

For metabolites M35, M36 and M37 there was high confidence in the prediction for chromosome damage due to the availability of experimental data for M35 and M37 plus the structural similarity between M35 and M36, though the overall confidence level for genotoxicity was medium, based on the mutagenicity endpoint, for which there was no experimental data. For metabolites M15, M16 and M17 there was medium confidence in the predictions because they were based on two *in silico* methods.

There was high confidence in the predictions for Metabolite M28 due to the availability of experimental data, medium confidence in the predictions for M30 and M31 and a low confidence in the prediction for M29 because the chromosome damage component of the genotoxicity assessment was based on a single method. There was high confidence in the predictions for metabolites M13 and M14 due to the availability of experimental data for M13 and the structural similarity to M14.

A. Materials and Methods

1. Substance identity: Refer to results for individual metabolite identity details.

2. Information on QSAR models:

Derek Nexus:

Parameter	Details
Prediction endpoint: Mutagenicity *in vitro* in bacterium (Ames test)	
QMRF protocol:	Q19-761-0004 (updated May 2020)
Algorithm of the model	Expert-derived structural alerts for mutagenicity, physicochemical properties and associated reasoning (2D SAR). Following alert evaluation, Derek evaluates whether non-alerting query compounds contain any features that are either (i) also present in non-alerting mutagens in a large Ames test reference set (misclassified features) or (ii) not present in a large Ames test reference set (unclassified features).
Statistics	i) Internal: not available given that it is a knowledge-based system. ii) External: no data available
Domains	The compounds in the dataset are primarily small and medium-sized chemicals and so are representative of the structures used to build the model. The Ames test reference set contains 5780 mutagens and 5994 non-mutagens (v6.1) The scopes of the structure-activity relationships describing the mutagenicity endpoint are defined by the developer to be the applicability domain for the model. Therefore, if a chemical activates an alert describing a structure-activity for mutagenicity it can be considered to be within the applicability domain. If a compound does not activate an alert or reasoning rule then Derek makes a negative prediction. The applicability of the negative prediction to the query compounds can be determined by an expert, if required, by investigating the presence (or absence) of misclassified and/or unclassified features. Misclassified features in the molecule are found in non-alerting mutagens in the Lhasa reference set. The prediction remains negative and the misclassified features are highlighted to enable the negative prediction to be verified by expert assessment. Inactive, contains unclassified features, some features in the molecule have not been found in the Lhasa reference set. The prediction remains negative and the unclassified features are highlighted to enable the negative prediction to be verified by expert assessment. Mechanistic

	information is detailed in the comments associated with an alert and can include information on both the mechanism of action and biological target
Analogues	Non-proprietary elements of the training set are available through the references, and illustrated by the examples, within Derek Nexus.
Prediction endpoint: Chromosomal damage	
QMRF protocol	Q19-762-0007 (updated May 2020)
Algorithm of the model	Expert derived structural alerts for chromosome damage (2D SARs), physicochemical properties and associated reasoning
Statistics	i). Internal: not available given that it is a knowledge-based system. ii). External: no data available
Domains	Data derived from *in vitro* and *in vivo* chromosome damage derived from several sources of data. The scopes of the structure-activity relationships describing the chromosome damage endpoint are defined by the developer to be the applicability domain for the model. Therefore, if a chemical activates an alert describing a structure-activity for chromosome damage it can be considered to be within the applicability domain. If a compound does not activate an alert or reasoning rule in Derek, a result of 'nothing to report' is presented to the user. This can be interpreted as a negative prediction or that the query compound is outside the domain of the model. Which of these is more appropriate may depend on the endpoint of interest. All alerts describing structure-activity relationships for the chromosome damage endpoint have a mechanistic basis wherever possible. Mechanistic information is detailed in the comments associated with an alert and can include information on both the mechanism of action and biological target.
Analogues	Non-proprietary elements of the training set are available through the references, and illustrated by the examples, within Derek Nexus.

Derek Nexus, which assigns various levels of non-numeric likelihood to its predictions as follows:

Certain: There is proof that the proposition is true

Probable: There is at least one strong argument that the proposition is true and there are no arguments against it

Plausible: The weight of evidence supports the proposition

Equivocal: There is an equal weight of evidence for and against the proposition

Doubted: The weight of evidence opposes the proposition

Improbable: There is at least one strong argument that the proposition is false and there are no arguments that it is true

Impossible: There is proof that the proposition is false

Open: There is no evidence that supports or opposes the proposition

Contradicted: There is proof that the proposition is both true and false

In addition to the above, Derek Nexus contains expert-derived functionality to provide negative predictions for bacterial *in vitro* mutagenicity. The query compound is compared to a Lhasa reference set of Ames test data and the

software determines whether there are structures that have been misclassified by Derek Nexus or is unknown. This provides a high confidence in negative predictions for this endpoint

Leadscope Model Applier Genetox Suite:

Parameter	Details
Prediction endpoint: Gene mutation: mammalian in vitro	
MODEL	**HGPRT Mut v1**
QMRF protocol	JRC reference number not available (protocol date October 2014)
Comment on endpoint	Combination of results from all in vitro Chinese hamster ovary (CHO) and Chinese hamster lung (CHL) gene mutation tests using the hgprt locus.
Algorithm of the model	Partial logistic regression. The descriptors are generated using the Leadscope software. Two QSAR models are generated with a balance of positive and negative compounds and the results are calculated as an average of two models.
Statistics	i). Internal: no data available. ii). External: no data available
Domains	Leadscope uses two parameters to guide the applicability of the model domain: 1) having at least one structural feature defined in the model in addition to all the property descriptors 2) having at least one chemical in a training neighbourhood with at least 30% global similarity to the test structure. The model uses 277 descriptors for 643 chemicals.
Analogues	Presented in model output.
MODEL	**Mouse Lymphoma Activated v2**
QMRF protocol	JRC reference number not available (protocol date April 2016)
Comment on endpoint	Re-scored results of the mouse lymphoma mutation assays at the thymidine kinase (tk) locus using L5178Y cells in culture
Algorithm of the model	Partial logistic regression. The descriptors are generated using the Leadscope software. Two QSAR models are generated with a balance of positive and negative compounds and the results are calculated as an average of two models.
Statistics	i). Internal: no data available. ii). External: no data available
Domains	Leadscope uses two parameters to guide the applicability of the model domain: 1) having at least one structural feature defined in the model in addition to all the property descriptors 2) having at least one chemical in a training neighbourhood with at least 30% global similarity to the test structure. The model uses 234 descriptors for 674 chemicals.
Analogues	Presented in model output
MODEL	**Mouse Lymphoma Unactivated v2**
QMRF protocol	JRC reference number not available (protocol date April 2016)
Comment on endpoint	Re-scored results of the mouse lymphoma mutation assays at the thymidine kinase (tk) locus using L5178Y cells in culture

Algorithm of the model	Partial logistic regression. The descriptors are generated using the Leadscope software. Two QSAR models are generated with a balance of positive and negative compounds and the results are calculated as an average of two models.
Statistics	i). Internal: no data available. ii). External: no data available
Domains	Leadscope uses two parameters to guide the applicability of the model domain: 1) having at least one structural feature defined in the model in addition to all the property descriptors 2) having at least one chemical in a training neighbourhood with at least 30% global similarity to the test structure. The model uses 245 descriptors for 750 chemicals.
Analogues	Presented in model output
Prediction endpoint: Gene mutation: microbial in vitro	
MODEL	**Bacteria Mutation v2**
QMRF protocol	JRC reference number not available (protocol date April 2019)
Comment on endpoint	Combination of results from the S. typhimurium histidine reversion gene mutation using tester strains TA97, TA97a, TA1537, TA98, TA 100, TA1535, TA102, E.coli (any variant)
Algorithm of the model	Partial logistic regression. The descriptors are generated using the Leadscope software or coded from the literature as substructure queries.
Statistics	i). Internal: no data available. ii). External: no data available
Domains	Leadscope uses two parameters to guide the applicability of the model domain: 1) having at least one structural feature defined in the model in addition to all the property descriptors 2) having at least one chemical in a training neighbourhood with at least 30% global similarity to the test structure. The model uses 477 descriptors for 9254 chemicals.
Analogues	Presented in model output
MODEL	**E. Coli Sal 102 A-T Mut v2**
QMRF protocol	JRC reference number not available (protocol date April 2019)
Comment on endpoint	Combination of results from the E. coli WP2 uvrA, E.coli WP2 uvrA (pKM101), and s.typhimurium TA102
Algorithm of the model	Partial logistic regression. The descriptors are generated using the Leadscope software or coded from the literature as substructure queries.
Statistics	i). Internal: no data available. ii). External: no data available
Domains	Leadscope uses two parameters to guide the applicability of the model domain: 1) having at least one structural feature defined in the model in addition to all the property descriptors 2) having at least one chemical in a training neighbourhood with at least 30% global similarity to the test structure. The model uses 333 descriptors for 1198 chemicals.
Analogues	Presented in model output

MODEL	Salmonella v4
QMRF protocol	JRC reference number not available (protocol date April 2019)
Comment on endpoint	Combination of results from the S typhimurium histidine reversion gene mutation test using tester strains TA97, TA98, TA100, TA 1535, TA1536, TA1537 and TA1538
Algorithm of the model	Partial logistic regression. The descriptors are generated using the Leadscope software or coded from the literature as substructure queries.
Statistics	i). Internal: no data available. ii). External: no data available
Domains	Leadscope uses two parameters to guide the applicability of the model domain: 1) having at least one structural feature defined in the model in addition to all the property descriptors 2) having at least one chemical in a training neighbourhood with at least 30% global similarity to the test structure. The model uses 438 descriptors for 4326 chemicals.
Analogues	Presented in model output
Prediction endpoint: Clastogenicity in vitro	
MODEL	**In vitro chrom ab CHL (v2)**
QMRF protocol	JRC reference number not available (protocol date April 2015)
Comment on endpoint	In vitro chromosome aberration test using Chinese hamster lung cells
Algorithm of the model	Partial logistic regression. The descriptors are generated using the Leadscope software. Two QSAR models are generated with a balance of positive and negative compounds and the results are calculated as an average of the two models.
Statistics	i). Internal: no data available. ii). External: no data available
Domains	Leadscope uses two parameters to guide the applicability of the model domain: 1) having at least one structural feature defined in the model in addition to all the property descriptors 2) having at least one chemical in a training neighbourhood with at least 30% global similarity to the test structure. The model uses 250 descriptors for 874 chemicals.
Analogues	Presented in model output
MODEL	**In vitro chrom ab CHO (v2)**
QMRF protocol	JRC reference number not available (protocol date April 2015)
Comment on endpoint	In vitro chromosome aberration test using Chinese hamster ovary cells
Algorithm of the model	Partial logistic regression. The descriptors are generated using the Leadscope software. Two QSAR models are generated with a balance of positive and negative compounds and the results are calculated as an average of the two models.
Statistics	i). Internal: no data available. ii). External: no data available

Domains	Leadscope uses two parameters to guide the applicability of the model domain: 1) having at least one structural feature defined in the model in addition to all the property descriptors 2) having at least one chemical in a training neighbourhood with at least 30% global similarity to the test structure. The model uses 274 descriptors for 819 chemicals.
Analogues	Presented in model output
Prediction endpoint: Clastogenicity in vivo	
MODEL	**Chrom ab comp (v1)**
QMRF protocol	JRC reference number not available (protocol date July 2012)
Comment on endpoint	Chromosome aberrations in vivo using rats, mice and other species that are not defined in the EPA GENE-TOX database
Algorithm of the model	Partial logistic regression. The descriptors are generated using the Leadscope software. Two QSAR models are generated with a balance of positive and negative compounds and the results are calculated as an average of the two models.
Statistics	i). Internal: no data available. ii). External: no data available
Domains	Leadscope uses two parameters to guide the applicability of the model domain: 1) having at least one structural feature defined in the model in addition to all the property descriptors 2) having at least one chemical in a training neighbourhood with at least 30% global similarity to the test structure. The model uses 207 descriptors for 285 chemicals.
Analogues	Presented in model output
MODEL	**Chrom ab other rodent(v1)**
QMRF protocol	JRC reference number not available (protocol date July 2012)
Comment on endpoint	Chromosome aberrations in vivo using rats and mice
Algorithm of the model	Partial logistic regression. The descriptors are generated using the Leadscope software. Two QSAR models are generated with a balance of positive and negative compounds and the results are calculated as an average of the two models.
Statistics	i). Internal: no data available. ii). External: no data available
Domains	Leadscope uses two parameters to guide the applicability of the model domain: 1) having at least one structural feature defined in the model in addition to all the property descriptors 2) having at least one chemical in a training neighbourhood with at least 30% global similarity to the test structure. The model uses 132 descriptors for 153 chemicals.
Analogues	Presented in model output
MODEL	**Chrom ab rat(v1)**
QMRF protocol	JRC reference number not available (protocol date July 2012)

Comment on endpoint	Chromosome aberrations in vivo using rats
Algorithm of the model	Partial logistic regression. The descriptors are generated using the Leadscope software. Two QSAR models are generated with a balance of positive and negative compounds and the results are calculated as an average of the two models.
Statistics	i). Internal: no data available. ii). External: no data available
Domains	Leadscope uses two parameters to guide the applicability of the model domain: 1) having at least one structural feature defined in the model in addition to all the property descriptors 2) having at least one chemical in a training neighbourhood with at least 30% global similarity to the test structure. The model uses 182 descriptors for 110 chemicals.
Analogues	Presented in model output
MODEL	**In vivo micronucleus mouse**
QMRF protocol	JRC reference number not available (protocol date July 2012)
Comment on endpoint	In vivo micronucleus in mice
Algorithm of the model	Partial logistic regression. The descriptors are generated using the Leadscope software. Two QSAR models are generated with a balance of positive and negative compounds and the results are calculated as an average of the two models.
Statistics	i). Internal: no data available. ii). External: no data available
Domains	Leadscope uses two parameters to guide the applicability of the model domain: 1) having at least one structural feature defined in the model in addition to all the property descriptors 2) having at least one chemical in a training neighbourhood with at least 30% global similarity to the test structure. The model uses 207 descriptors for 624 chemicals.
Analogues	Presented in model output

Leadscope Model Applier predicts toxicity by comparing the entered structure with empirical data then assigns a probability value based on likelihood of occurrence. Leadscope takes into account both toxifying and detoxifying structures. The prediction results for each model are presented as the "prediction" and the "positive prediction probability". The prediction can be "Positive", "Negative", and "Not-In-Domain". The higher the probability, the greater chance of the test chemical being toxic in a particular endpoint. For most models, a test chemical is evaluated as active for a set of models if the average probability is ≥0.5 and inactive if the average probability is <0.5. The exceptions are the Genetox Bacterial, Salmonella and E. coli AT models where the accepted cutoffs are inactive <0.4 and active ≥0.6.

3. Method: Spiroxamine and each metabolite was processed using Derek Nexus and Leadscope Model Applier using the constraints described in Table CA 5. The endpoint specific and general mechanistic profilers used in the OECD (Q)SAR Toolbox for the assessment of genotoxicity are also shown below. The results obtained from *in silico* modelling were subject to expert review conducted

according to the principles described in Myatt *et al*., 2018[24].. In addition to the automatic domain applicability evaluation embedded in Leadscope Model Applier, the query structure features contributing to the prediction were checked against the total structure and any exceptions were noted. Where exceptions were significant the prediction was assessed as out of domain. For Leadscope endpoints, which are made up of a number of sub-models for each endpoint, the overall outcome was based on the majority call. Spiroxamine and metabolite M03 were used as reference compounds as appropriate due to the availability of recent, GLP, experimental genotoxicity data for these compounds. A confidence level (high, medium or low) was assigned for each endpoint following expert review as follows:

High:
- *In silico* prediction matches recently conducted experimental data
- *In silico* prediction matches recently conducted experimental data for compound with close structural similarity

Medium:
- Matching *in silico* predictions using two different methods, following expert review

Low:
- *In silico* prediction available using one method only, following expert review

4. Available experimental data:

Spiroxamine has been previously evaluated by EFSA, with the data package updated and the relevant results of the evaluation with respect to genotoxicity are summarised in in Table CA 5.8.1/28-6. Experimental genotoxicity data are also available for metabolite M03 and the results are also summarised in Table CA 5.8.1/28-4. Reference has also been made to the rat metabolism data available for spiroxamine (EFSA DAR, 2017) with the data updated for the impending renewal of approval.

Table CA 5.8.1/28-4: Derek Nexus and Leadscope Model Applier processing constraints

Endpoint	Endpoint specific	Alert/model
Derek Nexus v6.0.1[A]		
Genotoxicity	Mutagenicity	Mutagenicity *in vitro*
		Mutagenicity *in vivo*
		Photomutagenicity *in vitro*
	Chromosome damage	Chromosome damage *in vitro*
		Chromosome damage *in vivo*
		Photo-induced chromosome damage *in vitro*
	Non-specific genotoxicity	Non-specific genotoxicity *in vitro*
		Non-specific genotoxicity *in vivo*
		Photo-induced non-specific genotoxicity *in vitro*
		Photo-induced non-specific genotoxicity *in vivo*
Leadscope Model Applier v3.0.1 Genotox Statistical Suite[BC]		
Gene mutation	Mammalian *in vitro*	HGPRT Mut v1
		Mouse Lymphoma Act v2

[24] Myatt, G.J., Ahlberg, E., Akahori, Y., Allen, D., Amberg, A., Anger, L.T., Aptula, A., Auerbach, S., Beilke, L., Bellion, P., Benigni, R., Bercu, J., Booth, E.D., Bower, D., Brigo, A., Burden, N., Cammerer, Z., Cronin, M.T.D., Cross, K.P., Custer, L., Detweiler, M., Dobo, K., Ford, K.A., Fortin, M.C., Gad-McDonald, S.E., Gellatly, N., Gervais, V., Glover, K.P., Glowienke, S., Van Gompel, J., Gutsell, S., Hardy, B., Harvey, J.S. Hillegass, J. Honma, M., Hsieh, J-H., Hsu, C-W., Hughes, K., Johnson, C., Jolly, R., Jones, D., Kemper, R., Kenyon, M.O., Kim, M.T., Kruhlak, N.L., Kulkarni, S.A., Kümmerer, K., Leavitt, P., Majer, B., Masten, S., Miller, S., Moser, J., Mumtaz, M., Muster, W., Neilson, L., Oprea, T.I., Patlewicz, G., Paulino, P., Piparo, E., Powley, M., Quigley, D.P., Reddy, M.V. Richarz A-N., Ruiz, P., Schilter, B., Serafimova, R., Simpson, W., Stavitskaya, L., Stidl, R., Suarez-Rodriguez, D., Szabo, D.T., Teasdale, A., Trejo-Martin, A., Valentin J-P., Vuorinen, A., Wall, B.A., Watts, P., White, A.T., Wichard, J., Witt, K.L., Woolley, A., Woolley, D., Zwickl, C. & Hasselgren, C. (2018). *In silico* toxicology protocols. *Regulatory Toxicology and Pharmacology*, **96**, pp 1-17

Endpoint	Endpoint specific	Alert/model
		Mouse Lymphoma Unact v2
	Microbial *in vitro*	Bacterial Mutation v2
		E Coli - Sal 102 A-T Mut v2
		Salmonella Mut v4
Clastogenicity	Chromosome aberrations *in vitro*	*In Vitro* Chrom Ab CHL v2
		In Vitro Chrom Ab CHO v2
	Chromosome aberrations *in vivo*	*In Vivo* Chrom Ab Comp v2
		In Vivo Chrom Ab Other v1
		In Vivo Chrom Ab Rat v1
	Micronucleus *in vivo*	*In Vivo* Micronuc Mouse v2

[A] All available species (Bacteria: *Escherichia coli* and *Salmonella typhimurium*. Mammal: dog, monkey and human (primate); rabbit; and rat, hamster, mouse and guinea pig (rodent)) and option to perceive tautomers were selected. Only predictions at the EQUIVOCAL level or above are reported in the text.

[B] The following endpoint specific models are available in this suite but are considered not appropriate for this evaluation. The results from these models are presented in the relevant Annexes for completeness but are not considered in the report text
Gene mutation- *In Vivo* Rodent DL Mut v1 & *In Vivo* Rodent Mut v1
Sister Chromatid Exchange- *in vitro* SCE CHO v1, *in vitro* SCE Comp v1 & *in vitro* SCE Other v1

Table CA 5.8.1/28-5: Derek Nexus and Leadscope Model Applier processing constraints

Profiler type	Endpoint associated	Profiler alert
Endpoint specific	Mutagenicity	DNA alerts for AMES, CA and MNT by OASIS
		In vitro mutagenicity (Ames test) alerts by ISS
		In vivo mutagenicity (Micronucleus) alerts by ISS
	Chromosome damage	Protein binding alerts for chromosomal aberration by OASIS
General mechanistic	Mutagenicity	DNA binding by OASIS
		DNA binding by OECD
	Chromosome damage	Protein binding by OASIS
		Protein binding OECD

Table CA 5.8.1/28-6: Relevant toxicity endpoints for spiroxamine and M03

Substance	Endpoint		Reference
	Mutagenicity (*In vitro* bacterial reverse (Ames) gene mutation)	**Chromosome damage (*In vitro* mammalian micronucleus)**	
Spiroxamine	No genotoxic potential		EFSA LoEP, 2017 CA 5.4.1/05 [M-756857-02-1]; CA 5.4.1/06 [M-687035-02-1]; CA 5.4.1/07 [M-755219-02-1];
M03	Negative	Negative	CA 5.8.1/20 [M-756858-02-1]; CA 5.8.1/21 [M-755221-02-1]
M13	Negative	Negative	CA 5.8.1/22 [M-755223-02-1] CA 5.8.1.2/03

Substance	Endpoint		Reference
	Mutagenicity (*In vitro* bacterial reverse (Ames) gene mutation)	Chromosome damage (*In vitro* mammalian micronucleus)	Reference
			[M-471125-01-1] CA 5.8.1/23 [M-775227-02-1]
M35	-	Negative	CA 5.8.1/25 [M-753775-01-1]
M37	-	Negative	CA 5.8.1/26 [M-761547-01-1]
M28	Negative	Negative	CA 5.8.1/15 [M-463412-01-1] CA 5.8.1.3/03 [M-465292-02-1]; CA 5.8.1.3/04 [M-469334-01-1]

Results

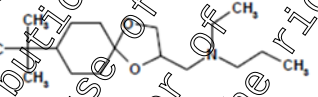
A. QSAR predictions:

1. Spiroxamine [reference compound]:

Chemical (IUPAC): name: 8-(1,1-dimethylethyl)-N-ethyl-N-propyl-1,4-dioxaspiro[4.5]decane-2-methanamine

CAS No.: 118134-30-8

Chemical structure:



SMILES: CCN(CC1COC2(CCC(C(C)(C)C)CC2)O1)CCC

- **Results and discussion:**

Derek Nexus: predicted to be inactive for bacterial mutagenicity with no misclassified or unclassified features. There were no alerts for mammalian mutagenicity or chromosome damage. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applier:

- Mutagenicity: no alerts for the bacterial or mammalian mutagenicity endpoints (refer to Table CA 5.8.1/28-5), with the *in silico* bacterial and mammalian predictions in agreement with the experimental data
- Chromosome damage: no alerts for any of the chromosome damage specific endpoints (refer to Table CA 5.8.1/28-5), with the *in silico* prediction in agreement with the experimental data.

OECD(Q)SAR Toolbox:

- Mutagenicity: profiled as potential for DNA binding by OECD SN1 mechanism and *in vitro* mutagenicity (Ames) by ISS with the H-acceptor-path3-H-acceptor alert. The predictive performance of this alert is poor, with 163 substances examined, with only 55 substances returning true positive results (34%). This alert is based on broad structural features that describe the planarity of the compound, which is associated with the potential to intercalate with DNA. The query compound does not contain a core structure associated with intercalation and therefore this profiler should be regarded as low concern. However, a valid robust *in vitro* bacterial mutagenicity study and *in vitro* mammalian forward gene mutation studies are available, which concludes that spiroxamine does not increase bacterial revertant colony or mammalian gene mutation frequency, respectively. It is

concluded therefore that these alerts are not realized experimentally, and deemed not biologically relevant (refer to Table CA 5.8.1/28-6)..

- Chromosome damage: no alerts identified.

Expert evaluation of in silico prognosis for mutagenicity

Experimental data show that spiroxamine is not mutagenic. *In silico* predictions using two methods are in agreement with the experimental result. The OECD (Q)SAR Toolbox (Toolbox) alerts are considered not relevant taking into account the experimental data. The 'H-acceptor-path3-H-acceptor' alert in Toolbox is considered not relevant.

Expert evaluation of in silico prognosis for chromosome damage

Experimental data show that spiroxamine does not cause chromosomal damage. *In silico* predictions using two methods are in agreement with the experimental result.

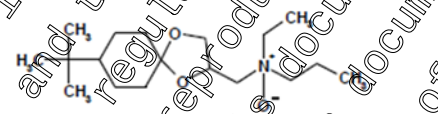
Conclusion

Experimental data show that spiroxamine is not genotoxic and the *in silico* analysis using two methods corresponds with this finding.

2. Spiroxamine-N-oxide (M03) [reference compound]:

Chemical name (IUPAC): N-[(8-tert-butyl-1,4-dioxaspiro[4.5]decan-2-yl)methyl]-N-ethylpropan-1-amine N-oxide

Chemical structure:



SMILES: CC(C)(C)C1CCC2(CC1)OCC(C[N+]([O-])(CC)CCC)O2

- Results and discussion:

M03 differs from spiroxamine with the oxidation of the N-ethyl group.

Derek Nexus: predicted to be inactive for bacterial mutagenicity with no misclassified or unclassified features. There were no alerts for mammalian mutagenicity or chromosome damage. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applier:

- Mutagenicity: no alerts for the bacterial or mammalian mutagenicity endpoints (refer to Table CA 5.8.1/28-5).
- Chromosome damage: no alerts for any of the chromosome damage specific endpoints (refer to Table CA 5.8.1/28-5).

OECD (Q)SAR Toolbox:

- Mutagenicity: profiled as potential for *in vitro* mutagenicity (Ames test) by ISS with the H-acceptor-path3-H-acceptor alert (refer above to 1. Spiroxamine regarding predictive performance of this alert). However, a valid robust *in vitro* bacterial mutagenicity study is available, which concludes that M03 does not increase bacterial revertant colony frequency,. It is concluded therefore that this alert is not realized experimentally, and deemed not biologically relevant.
- Chromosome damage: no alerts identified.

Expert evaluation of in silico prognosis for mutagenicity

Experimental data show that M03 is not mutagenic. *In silico* predictions using two methods are in agreement with the experimental result. The Toolbox alert is triggered by the same substructure as for spiroxamine and given the available experimental data is considered not relevant. The 'H-acceptor-path3-H-acceptor' alert in Toolbox is considered not relevant.

Expert evaluation of in silico prognosis for chromosome damage

Experimental data show that M03 does not cause chromosome damage. *In silico* predictions using two methods are in agreement with the experimental result although all the Leadscope sub-models for chromosome damage are considered out of domain because the N-oxide is not considered as a feature.

Conclusion

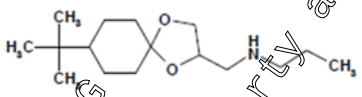
M03 is not genotoxic and the rule based *in silico* prognoses correspond with this finding.

3. Spiroxamine-desethyl (M01):

Chemical name (IUPAC): N-[(8-tert-butyl-1,4-dioxaspiro[4.5]decan-2-yl)methyl]propan-1-amine

CAS No.: Not available

Chemical structure:



SMILES: CC(C)(C)C1CCC2(CC1)OCC(CNCCC)O2

M01 differs from spiroxamine by the loss of the N-ethyl group giving rise to a secondary amine function.

Derek Nexus: no alerts activated for bacterial mammalian mutagenicity or chromosome damage specific endpoint with no misclassified or unclassified features. There were no alerts for mammalian mutagenicity. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applier:

- Mutagenicity: predicted to be inactive for bacterial mutagenicity with no misclassified or unclassified features. There were no alerts for mammalian mutagenicity (refer to Table CA 5.8.1/28-5).
- Chromosome damage: no alerts for any of the chromosome damage specific endpoints (refer to Table CA 5.8.1/28-5).

OECD (Q)SAR Toolbox:

- Mutagenicity: profiled as potential for *in vitro* mutagenicity (Ames test) by ISS with the H-acceptor-path3-H-acceptor alert (refer above to 1. Spiroxamine regarding predictive performance of this alert).
- Chromosome damage: no alerts identified.

Expert evaluation of in silico prognosis for mutagenicity

M01 is predicted not to be mutagenic by Derek Nexus and five of the six mutagenicity sub-models in Leadscope, it was out of domain for the remaining sub-model. The Toolbox alert is considered not relevant since it is also present in a similar chemical space to spiroxamine. Furthermore, a secondary amine is considered to have a low probability of increasing genotoxic potential (Benigni *et al*[25]).

Expert evaluation of in silico prognosis for chromosome damage

M01 gave no alerts for chromosome damage in Derek Nexus or Toolbox and returned negative predictions two of the six models in Leadscope. It is out of domain for remaining four Leadscope models.

Conclusion

In silico analysis by two methods predicts M01 is neither mutagenic nor causes chromosomal damage thus there is medium confidence in this prediction.

[25] Benigni, R., Battistelli, C.L., Bossa, C., Giuliani, A., Fioravanzo, E., Bassan, A., Gatnik, M.F., Rathman, J., Yang, C., Tcheremenskaia, O. (2019) Evaluation of the applicability of existing (Q)SAR models for predicting the genotoxicity of pesticides and similarity analysis related with genotoxicity of pesticides for facilitating of grouping and read across. EFSA Supporting publication 2019:EN-1598

Table CA 5.8.1/28-7: Leadscope Model Applier mutagenicity and chromosome damage predictions: spiroxamine, M03, M01

Mutagenicity predictions										
Effect	Sub-model	Spiroxamine			M03			M01		
		QSAR prediction	Positive probability	Expert review	QSAR prediction	Positive probability	Expert review	QSAR prediction	Positive probability	Expert review
Mammalian *in vitro*	HGPRT Mut v1	NID	0.178	Accepted	Negative[A]	0.178	OoD	NID	0.178	Accepted
	Mouse Lymphoma Act v2	NID	0.285	Accepted	Negative	0.451	Accepted	Negative	0.180	Accepted
	Mouse Lymphoma Unact v2	NID	0.295	Accepted	Negative[A]	0.295	OoD	Negative[A]	0.343	Accepted
Microbial *in vitro*	Bacterial Mutation v2	Negative	0.078	Accepted	Negative	0.108	Accepted	Negative	0.039	Accepted
	E Coli - Sal 102 A-T Mut v2	NID	0.083	Accepted	Negative	0.255	Accepted	Negative	0.090	Accepted
	Salmonella Mut v4	Negative	0.030	Accepted	Negative	0.088	Accepted	Negative	0.020	Accepted
Chromosome damage predictions										
CA *in vitro*	*In vitro* Chrom Ab CHL v2	NID	0.299	Accepted	NID	0.350	Accepted	NID	0.356	Accepted
	In vitro Chrom Ab CHO v2	NID	0.121	Accepted	Negative[A]	0.119	OoD	Negative[B]	0.231	Accepted
										Accepted
CA i*n vivo*	*In vivo* Chrom Ab Comp v2	NID	0.038	Accepted	Negative[A]	0.041	OoD	NID	0.056	Accepted
	In vivo Chrom Ab Other v1	NID	0.373	Accepted	Negative[A]	0.360	OoD	NID	0.265	Accepted
	In vivo Chrom Ab Rat v1	NID	0.160	Accepted	NID	0.155	Accepted	NID	0.101	Accepted
Micronucleus *in vivo*	*In vivo* Micronuc Mouse v2	Negative[A]	0.425	Accepted	Negative[A,B]	0.434	OoD	Negative[B]	0.307	Accepted

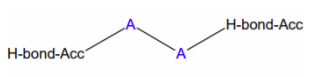
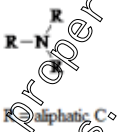
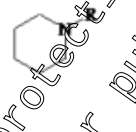
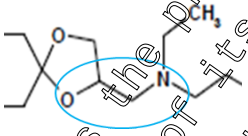
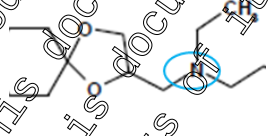
CA: chromosome aberrations
NID: not in domain
OoD: out of domain

Domain exceptions:
[A] Cyclohexane

Domain exceptions:
[A] N-oxide
[B] Cyclohexane

Domain exceptions:
[A] Dioxolane ring
[B] Cyclohexane

Table CA 5.8.1/28-8: QECD (Q)SAR Toolbox structural alerts

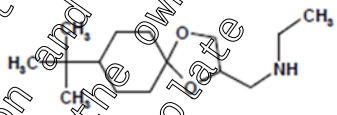
Alert	
H-acceptor-path3-H-acceptor (*In vitro* mutagenicity (Ames) / *in vivo* mutagenicity (micronucleus) alerts by ISS)  A = any atom, except Hydrogen H-bond Acc = any atom that is a potential Hydrogen bond acceptor	SN1 (DNA binding by OECD)   R = aliphatic C The cyclic aliphatic ring system can be any size above $n = 3$ (*i.e.* not aziridine). The ring system cannot be heterocyclic
Compounds where alert is activated: spiroxamine, M03, M01, M02, M04, M05, M06, M07, M08, M09, M10, M11, M12, M25, M26, M27, M38, M41  	*Compounds where alert is activated*: spiroxamine, M04, M05, M06, M07, M08, M25, M38 

4. Spiroxamine-despropyl (M02):

Chemical name (IUPAC): N-[(8-tert-butyl-1,4-dioxaspiro[4.5]decan-2-yl)methyl]ethanamine

CAS No.: Not available

Chemical structure:



SMILES: CC(C)(C)C1CCC2(CC1)OCC(CNCC)O2

- Results and discussion:

M02 differs from spiroxamine by the loss of the N-propyl group giving rise to a secondary amine function.

Derek Nexus: predicted to be inactive for bacterial mutagenicity with no misclassified or unclassified features. There were no alerts for mammalian mutagenicity or chromosome damage. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applier:

- Mutagenicity: no alerts for the bacterial or mammalian mutagenicity endpoints (refer to Table CA 5.8.1/28-7).
- Chromosome damage: no alerts for any of the chromosome damage specific endpoints (refer to Table CA 5.8.1/28-7).

OECD (Q)SAR Toolbox:

- Mutagenicity: profiled as potential for *in vitro* mutagenicity (Micronucleus test) by ISS with the H-acceptor-path3-H-acceptor alert (refer above to 1. Spiroxamine regarding predictive performance of this alert).
- Chromosome damage: no alerts identified.

Expert evaluation of in silico prognosis for mutagenicity

M02 is predicted not to be mutagenic by Derek Nexus and three of the six mutagenicity sub-models in Leadscope, it was out of domain for the remaining sub-models. The Toolbox alert is considered not relevant since it is also present in a similar chemical milieu to spiroxamine. Furthermore a secondary amine is

considered to have a low probability of increasing genotoxic potential (Benigni *et al.*)[2].

Expert evaluation of in silico prognosis for chromosome damage

M02 gave no alerts for chromosome damage in Derek Nexus or Toolbox and returned negative predictions one of the six models in Leadscope. It is out of domain for remaining five Leadscope models.

Conclusion

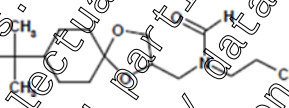
In silico analysis by two methods predicts M02 is neither mutagenic nor causes chromosomal damage thus there is medium confidence in this prediction.

5. Spiroxamine-N-formyl-desethyl (M04):

Chemical name (IUPAC): N-[(8-tert-butyl-1,4-dioxaspiro[4.5]decan-2-yl)methyl]-N-propylformamide

CAS No.: Not available

Chemical structure:



SMILES: CC(C)(C)C1CCC2(CC1)OCC(CN(C=O)CCC)O2

- Results and discussion:

M04 differs from spiroxamine by oxidation at the N-ethyl group giving rise to a tertiary amide function.

Derek Nexus: predicted to be inactive for bacterial mutagenicity with no misclassified or unclassified features. There were no alerts for mammalian mutagenicity or chromosome damage. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applier:

- Mutagenicity: no alerts for the bacterial or mammalian mutagenicity endpoints (refer to Table CA 5.8.1/28-7).
- Chromosome damage: no alerts for any of the chromosome damage specific endpoints (refer to Table CA 5.8.1/28-7).

OECD (Q)SAR Toolbox:

- Mutagenicity: profiled as potential for DNA binding by OECD SN1 mechanism and *in vitro* mutagenicity (micronucleus) by ISS with the H-acceptor-path3-H-acceptor alert (refer above to 1. Spiroxamine regarding predictive performance of this alert).
- Chromosome damage: no alerts identified.

Expert evaluation of in silico prognosis for mutagenicity

M04 is predicted not to be mutagenic by Derek Nexus and all three of the bacterial mutagenicity sub models in Leadscope, it is out of domain for the Leadscope mammalian mutagenicity sub-models. The Toolbox alerts are considered not relevant since it is also present in a similar chemical space to spiroxamine.

Expert evaluation of in silico prognosis for chromosome damage

M04 gives no alerts for chromosome damage in Derek Nexus or Toolbox and two of the six sub-models in Leadscope give negative predictions, it is out of domain of the remaining four sub-models, though it is noted that one of the out of domain predictions gives a positive probability of >0.5. The positive prediction is based on a single feature, the alkyl formamide moiety which is also identified as a deactivating feature in the model output and the probability score is considered not relevant. Therefore, *in silico* analysis by two methods predicts M04 does not cause chromosome damage with medium confidence in the prediction.

Conclusion

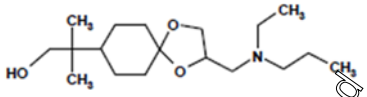
In silico analysis by two methods predicts M04 is neither mutagenic nor causes chromosomal damage thus there is medium confidence in this prediction.

6. Spiroxamine-hydroxyl (M05):

Chemical name (IUPAC): 2-(2-{[ethyl(propyl)amino]methyl}-1,4-dioxaspiro[4.5]decan-8-yl)-2-methylpropan-1-ol

CAS No.: Not available

Chemical structure:



SMILES: CC(C)(CO)C1CCC2(CC1)OCC(CN(CC)CCC)O2

- **Results and discussion:**

M05 differs from spiroxamine by oxidation at the t-butyl group giving a hydroxy metabolite.

Derek Nexus: predicted to be inactive for bacterial mutagenicity with no misclassified or unclassified features. There were no alerts for mammalian mutagenicity or chromosome damage. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applier:

- Mutagenicity: no alerts for the bacterial or mammalian mutagenicity endpoints (refer to Table CA 5.8.1/28-7).
- Chromosome damage: no alerts for any of the chromosome damage specific endpoints (refer to Table CA 5.8.1/28-7).

OECD (Q)SAR Toolbox:

- Mutagenicity: profiled as potential for DNA binding by OECD SN1 mechanism and *in vitro* mutagenicity (Ames) by ISS with the H-acceptor-path3-H-acceptor alert (refer above to 1. Spiroxamine regarding predictive performance of this alert).
- Chromosome damage: no alerts identified.

Expert evaluation of in silico prognosis for mutagenicity

M04 is predicted not to be mutagenic by Derek Nexus and five of the six of the mutagenicity sub-models in Leadscope, it was out of domain the remaining Leadscope model. The Toolbox alerts are considered not relevant since they are also present in a similar chemical space to spiroxamine. Since the structure of M05 is similar to spiroxamine, with the only structural difference between the two being the hydroxyl group on the t-butyl moiety and hydroxy functions are considered to have a low probability of increasing genotoxicity (Benigni *et al.* [5]) the negative outcome of recently conducted, GLP mutagenicity tests for spiroxamine can be used to support the negative in silico prediction for M05.

Expert evaluation of in silico prognosis for chromosome damage

M05 does not give any alerts for chromosome damage in Derek Nexus or Toolbox and gives negative predictions in four of the six Leadscope sub-models, it is out of domain in the remaining models. Furthermore, since the structure of M05 is similar to spiroxamine, with the only structural difference between the two being the hydroxyl group on the t-butyl moiety which gives no alerts in Derek Nexus or Toolbox, the negative outcome of recently conducted, GLP chromosome aberration tests for spiroxamine can be used to support the negative in silico prediction for M05.

Conclusion

In silico analysis by two methods predicts M05 is neither mutagenic nor causes chromosomal damage and comparison with spiroxamine supports high confidence in this prediction.

Table CA 5.8.1/28-9: Leadscope Model Applier mutagenicity and chromosome damage predictions: M02, M04, M05

Mutagenicity predictions										
Effect	Sub-model	M02			M04			M05		
		QSAR prediction	Positive probability	Expert review	QSAR prediction	Positive probability	Expert review	QSAR prediction	Positive probability	Expert review
Mammalian *in vitro*	HGPRT Mut v1	NID	0.186	Accepted	NID	0.176	Accepted	Negative	0.174	Accepted
	Mouse Lymphoma Act v2	NID	0.184	Accepted	NID	0.383	Accepted	NID	0.293	Accepted
	Mouse Lymphoma Unact v2	Negative[A]	0.370	Accepted	NID	0.356	Accepted	Negative[A]	0.351	Accepted
Microbial *in vitro*	Bacterial Mutation v2	Negative	0.050	Accepted	Negative	0.043	Accepted	Negative	0.207	Accepted
	E Coli - Sal 102 A-T Mut v2	Negative[B]	0.099	OoD	Negative	0.021	OoD	Negative	0.091	Accepted
	Salmonella Mut v4	Negative	0.023	Accepted	Negative	0.018	Accepted	Negative	0.081	Accepted
Chromosome damage predictions										
CA *in vitro*	*In vitro* Chrom Ab CHL v2	NID	0.475	Accepted	NID	0.677	Accepted	Negative[A]	0.301	Accepted
	In vitro Chrom Ab CHO v2	NID	0.285	Accepted	Negative	0.131	Accepted	Negative[B]	0.121	Accepted
				Accepted			Accepted			
CA i*n vivo*	*In vivo* Chrom Ab Comp v2	NID	0.063	Accepted	Negative	0.030	Accepted	Negative	0.038	Accepted
	In vivo Chrom Ab Other v1	NID	0.279	Accepted	Negative[C]	0.377	OoD	NID	0.364	Accepted
	In vivo Chrom Ab Rat v1	NID	0.104	Accepted	NID	0.115	Accepted	Negative [B,C,D]	0.149	OoD
Micronucleus *in vivo*	*In vivo* Micronuc Mouse v2	Negative[A]	0.313	Accepted	NID	0.397	Accepted	Negative[B,D]	0.419	Accepted

CA: chromosome aberrations
NID: not in domain
OoD: out of domain

Domain exceptions:
[A] Dioxolane ring
[B] Secondary amide
[C] Cyclohexane

Domain exceptions:
[A] Dioxolane ring
[B] Secondary amide
[C] Alkyl formamide

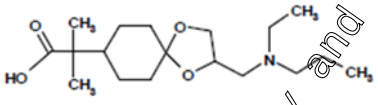
Domain exceptions:
[A] Dioxolane ring
[B] T-butyl OH
[C] Tertiary amide
[D] Cyclohexane ring

7. Spiroxamine-acid (M06):

Chemical name (IUPAC): 2-(2-{[ethyl(propyl)amino]methyl}-1,4-dioxaspiro[4.5]decan-8-yl)-2-methylpropanoic acid

CAS No.: Not available

Chemical structure:



SMILES: O=C(O)C(C)(C)C1CCC2(CC1)OCC(CN(CC)CCC)O2

- Results and discussion:

M06 differs from spiroxamine by oxidation at the t-butyl group giving an acid metabolite. M06 was the major metabolite found in the rat biotransformation study and formation of the acid is accounts for a major pathway of the biotransformation of spiroxamine.

Derek Nexus: predicted to be inactive for bacterial mutagenicity with no misclassified or unclassified features. There were no alerts for mammalian mutagenicity or chromosome damage. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applier:

- Mutagenicity: no alerts for the bacterial or mammalian mutagenicity endpoints (refer to Table CA 5.8.1/28-8).
- Chromosome damage: no alerts for any of the chromosome damage specific endpoints (refer to Table CA 5.8.1/28-8).

OECD (Q)SAR Toolbox:

- Mutagenicity: profiled as potential for DNA binding by OECD SN1 mechanism and *in vitro* mutagenicity (Ames) by ISS with the H-acceptor-path3-H-acceptor alert (refer above to 1. Spiroxamine regarding predictive performance of this alert).
- Chromosome damage: no alerts identified.

Expert evaluation of in silico prognosis for mutagenicity

M04 is predicted not to be mutagenic by Derek Nexus and five of the six of the mutagenicity sub-models in Leadscope. It was out of domain the remaining Leadscope model. The Toolbox alerts are considered not relevant since they are also present in a similar chemical space to spiroxamine. Since the structure of M05 is similar to spiroxamine, with the only structural difference between the two being the hydroxyl group on the t-butyl moiety and hydroxy functions are considered to have a low probability of increasing genotoxicity (Benigni *et al.*[25]) the negative outcome of recently conducted, GLP mutagenicity tests for spiroxamine can be used to support the negative in silico prediction for M05.

Expert evaluation of in silico prognosis for chromosome damage

M05 does not give any alerts for chromosome damage in Derek Nexus or Toolbox and gives negative predictions in four of the six Leadscope sub-models, it is out of domain in the remaining models. Furthermore, since the structure of M05 is similar to spiroxamine, with the only structural difference between the two being the hydroxyl group on the t-butyl moiety which gives no alerts in Derek Nexus or Toolbox, the negative outcome of recently conducted, GLP chromosome aberration tests for spiroxamine can be used to support the negative in silico prediction for M05.

Conclusion

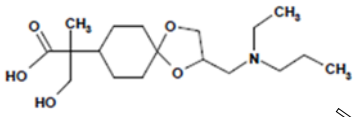
In silico analysis by two methods predicts M05 is neither mutagenic nor causes chromosomal damage and comparison with spiroxamine supports high confidence in this prediction.

8. Spiroxamine-hydroxy acid (M07):

Chemical name (IUPAC): 2-(2-{[ethyl(propyl)amino]methyl}-1,4-dioxaspiro[4.5]decan-8-yl)-3-hydroxy-2-methylpropanoic acid

CAS No.: Not avavailable

Chemical structure:



SMILES: O=C(O)C(C)(CO)C1CCC2(CC1)OCC(CN(CC)CC C)O2

- **Results and discussion:**

M07 differs from spiroxamine by oxidation in two positions at the t-butyl group giving a hydroxylated acid metabolite

Derek Nexus: predicted to be inactive for bacterial mutagenicity with no misclassified or unclassified features. There were no alerts for mammalian mutagenicity or chromosome damage. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applier:

- Mutagenicity: no alerts for the bacterial or mammalian mutagenicity endpoints (refer to Table CA 5.8.1/28-8).
- Chromosome damage: no alerts for any of the chromosome damage specific endpoints (refer to Table CA 5.8.1/28-8).

OECD (Q)SAR Toolbox:

- Mutagenicity: profiled as potential for DNA binding by OECD SN1 mechanism and *in vitro* mutagenicity (Ames) by ISS with the H-acceptor-path-3-H-acceptor alert (refer above to 1. Spiroxamine regarding predictive performance of this alert).
- Chromosome damage: no alerts identified.

Expert evaluation of in silico prognosis for mutagenicity

M07 is predicted not to be mutagenic by Derek Nexus and two of the six of the mutagenicity sub-models in Leadscope, it is out of domain in the remaining Leadscope sub-models. The Toolbox alerts are considered not relevant since they are also present in a similar chemical space in spiroxamine. It is noted that individually, the hydroxy and acid metabolites (M05 and M06, respectively) are predicted not to be mutagenic and the relative position of each of these functional groups in M07 is unlikely to give extra cause for concern *via* creation of additional centres of reactivity.

Expert evaluation of in silico prognosis for chromosome damage

M07 does not give any alerts for chromosome damage in Derek Nexus or Toolbox. It was in domain for two of the Leadscope models, where negative predictions were returned and out of domain for the remaining models. It is noted that individually, the hydroxy and acid metabolites (M05 and M06, respectively) are predicted not to cause chromosome damage and the relative position of each of these functional groups in M07 is unlikely to give extra cause for concern *via* creation of additional centres of reactivity.

Conclusion

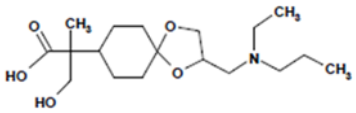
In silico analysis by two methods predicts M07 is neither mutagenic nor causes chromosomal damage, and by reference to M05 and M06 there is high confidence in the prediction.

9. Spiroxamine-8-hydroxyacid (M08):

Chemical name (IUPAC): 2-(2-{[ethyl(propyl)amino]methyl}-8-hydroxy-1,4-dioxaspiro[4.5]decan-8-yl)-2-methylpropanoic acid

CAS No.: Not available

Chemical structure:



SMILES: O=C(O)C(C)(CO)C1CCC2(CC1)OCC(CN(CC)CC C)O2

- **Results and discussion:**

M08 differs from spiroxamine by oxidation on the t-butyl group and the cyclohexyl ring giving a hydroxylated acid metabolite.

Derek Nexus: predicted to be inactive for bacterial mutagenicity with no misclassified or unclassified features. There were no alerts for mammalian

mutagenicity or chromosome damage. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applier:

- Mutagenicity: no alerts for the bacterial or mammalian mutagenicity endpoints (refer to Table CA 5.8.1/28-8).
- Chromosome damage: no alerts for any of the chromosome damage specific endpoints (refer to Table CA 5.8.1/28-8).

OECD (Q)SAR Toolbox:

- Mutagenicity: profiled as potential for DNA binding by OECD SN1 mechanism and *in vitro* mutagenicity (Ames) by ISS with the H-acceptor-path3-H-acceptor alert (refer above to 1. Spiroxamine regarding predictive performance of this alert).
- Chromosome damage: no alerts identified.

Expert evaluation of in silico prognosis for mutagenicity

M08 is predicted not to be mutagenic by Derek Nexus and five of the six mutagenicity models in Leadscope. It is out of domain in the remaining Leadscope model. The Toolbox alerts are considered not relevant since they is also present in a similar chemical space in spiroxamine. It is noted that individually the acid metabolite (M06) is predicted not to be mutagenic and the relative position of the acid and hydroxy functional groups in M08 is unlikely to give extra cause for concern *via* creation of additional centres of reactivity

Expert evaluation of in silico prognosis for chromosome damage

M08 does not give any alerts for chromosome damage in Derek Nexus or Toolbox. It was in domain for one of the Leadscope models where a negative prediction was returned. t is noted that individually the acid metabolite (M06) and a similarly placed hydroxy metabolite (M05) are also predicted not to cause chromosome damage, the relative position of each of these functional groups in M08 is unlikely to give extra cause for concern *via* creation of additional reactive centres of reactivity.

Conclusion

In silico analysis by two methods predicts M08 is neither mutagenic nor causes chromosomal damage., and by reference to M05 and M06 there is high confidence in the prediction.

Table CA 5.8.1/28-10: Leadscope Model Applier mutagenicity and chromosome damage predictions: M06, M07, M08

Mutagenicity predictions									
Effect	**Sub-model**	**M06**			**M07**			**M08**	
		QSAR prediction	**Positive probability**	**Expert review**	**QSAR prediction**	**Positive probability**	**Expert review**	**QSAR prediction**	**Positive probability**	**Expert review**
Mammalian *in vitro*	HGPRT Mut v1	Negative	0.174	Accepted	NID	0.058	Accepted	Negative	0.051	Accepted
	Mouse Lymphoma Act v2	NID	0.293	Accepted	NID	0.284	Accepted	Negative	0.233	Accepted
	Mouse Lymphoma Unact v2	Negative[A]	0.361	Accepted	NID	0.483	Accepted	Negative[A]	0.432	Accepted
Microbial *in vitro*	Bacterial Mutation v2	Negative	0.207	Accepted	Negative	0.108	Accepted	Negative	0.048	Accepted
	E Coli - Sal 102 A-T Mut v2	Negative	0.091	Accepted	NID	0.014	Accepted	NID	0.014	Accepted
	Salmonella Mut v4	Negative	0.081	Accepted	Negative	0.052	Accepted	Negative	0.023	Accepted
Chromosome damage predictions										
CA *in vitro*	*In vitro* Chrom Ab CHL v2	Negative[A]	0.301	Accepted	Negative[A]	0.096	Accepted	NID	0.132	Accepted
	In vitro Chrom Ab CHO v2	Negative[B]	0.121	Accepted	NID	0.127	Accepted	NID	0.129	Accepted
CA i*n vivo*	*In vivo* Chrom Ab Comp v2	Negative	0.038	Accepted	NID	0.035	Accepted	NID	0.036	Accepted
	In vivo Chrom Ab Other v1	NID	0.364	Accepted	NID	0.304	Accepted	NID	0.307	Accepted
	In vivo Chrom Ab Rat v1	Negative[B,C,D]	0.149	OoD	NID	0.312	Accepted	NID	0.312	Accepted
Micronucleus *in vivo*	*In vivo* Micronuc Mouse v2	Negative[B,D]	0.419	Accepted	Negative[B]	0.260	Accepted	Negative[B]	0.317	Accepted

CA: chromosome aberrations
NID: not in domain
OoD: out of domain

Domain exceptions:
[A] Dioxolane ring
[B] T-butyl OH
[C] Tertiary amide
[D] Cyclohexane ring

Domain exceptions:
[A] Cyclohexane dioxolane
[B] Cyclohexane

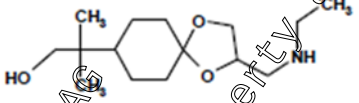
Domain exceptions:
[A] Dioxolane ring
[B] Cyclohexanol

10. Spiroxamine-hydroxy-despropyl (M09):

Chemical name (IUPAC): 2-{2-[(ethylamino)methyl]-1,4-dioxaspiro[4.5]decan-8-yl}-2-methylpropan-1-ol

CAS No.: Not available

Chemical structure:



SMILES: CC(C)(CO)C1CCC2(CC1)OCC(CNCC)O2

- Results and discussion:

M09 differs from spiroxamine by oxidation on the t-butyl and N-dealkylation giving a hydroxy N-despropyl metabolite.

Derek Nexus: predicted to be inactive for bacterial mutagenicity with no misclassified or unclassified features. There were no alerts for mammalian mutagenicity or chromosome damage. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applier:

- Mutagenicity: no alerts for the bacterial or mammalian mutagenicity endpoints (refer to Table CA 5.8.1/28-9).
- Chromosome damage: no alerts for any of the chromosome damage specific endpoints (refer to Table CA 5.8.1/28-9).

OECD (Q)SAR Toolbox:

- Mutagenicity: profiled as potential for *in vitro* mutagenicity (Ames) by ISS with the H-acceptor-path3-H-acceptor alert (refer above to 1. Spiroxamine regarding predictive performance of this alert).
- Chromosome damage: no alerts identified.

Expert evaluation of in silico prognosis for mutagenicity

M09 is predicted not to be mutagenic by Derek Nexus and four sub-models in Leadscope, deemed out of domain for the remaining two sub-models. The Toolbox alert is considered not relevant since it is also present in a similar chemical space in spiroxamine. It is noted that individually, the despropyl and hydroxy metabolites (M02 and M05, respectively) are also predicted not to cause mutagenicity. The relative position of each of these functional groups in M09 is unlikely to give extra cause for concern *via* creation of additional reactive centres of reactivity

Expert evaluation of in silico prognosis for chromosome damage

M09 does not give any alerts for chromosome damage in Derek Nexus or Toolbox. Negative predictions were returned for all of the models within Leadscope. It is noted that individually, the despropyl and hydroxy metabolites (M02 and M05, respectively) are also predicted not to cause chromosome damage and the relative position of each of these functional groups in M09 is unlikely to give extra cause for concern *via* creation of additional reactive centres of reactivity.

Conclusion

In silico analysis by two methods predicts M09 is neither mutagenic nor causes chromosomal damage , there is medium confidence in the prediction.

M09 differs from spiroxamine by oxidation on the t-butyl and N-dealkylation giving a hydroxy N-despropyl metabolite.

Derek Nexus: predicted to be inactive for bacterial mutagenicity with no misclassified or unclassified features. There were no alerts for mammalian mutagenicity or chromosome damage. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applier:

- Mutagenicity: no alerts for the bacterial or mammalian mutagenicity endpoints (refer to Table CA 5.8.1/28-9).

- Chromosome damage: no alerts for any of the chromosome damage specific endpoints (refer to Table CA 5.8.1/28-9).

OECD (Q)SAR Toolbox:

- Mutagenicity: profiled as potential for *in vitro* mutagenicity (Ames) by ISS with the H-acceptor-path3-H-acceptor alert (refer above to 1. Spiroxamine regarding predictive performance of this alert).
- Chromosome damage: no alerts identified.

Expert evaluation of in silico prognosis for mutagenicity

M09 is predicted not to be mutagenic by Derek Nexus and four sub-models in Leadscope, deemed out of domain for the remaining two sub-models. The Toolbox alert is considered not relevant since it is also present in a similar chemical space in spiroxamine. It is noted that individually, the despropyl and hydroxy metabolites (M02 and M05, respectively) are also predicted not to cause mutagenicity. The relative position of each of these functional groups in M09 is unlikely to give extra cause for concern *via* creation of additional reactive centres of reactivity

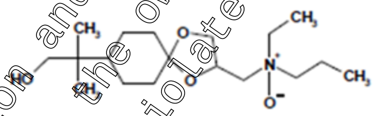
Expert evaluation of in silico prognosis for chromosome damage

M09 does not give any alerts for chromosome damage in Derek Nexus or Toolbox. Negative predictions were returned for all of the models within Leadscope. It is noted that individually, the despropyl and hydroxy metabolites (M02 and M05, respectively) are also predicted not to cause chromosome damage and the relative position of each of these functional groups in M09 is unlikely to give extra cause for concern *via* creation of additional reactive centres of reactivity.

Conclusion

In silico analysis by two methods predicts M09 is neither mutagenic nor causes chromosomal damage, there is medium confidence in the prediction.

11. Spiroxamine – hydroxy-N-oxide (M10):

Chemical name (IUPAC):	N-ethyl-N-{[8-(1-hydroxy-2-methylpropan-2-yl)-1,4-dioxaspiro[4.5]decan-2-yl]methyl}propan-1-amine N-oxide
CAS No.:	Not available
Chemical structure:	
SMILES:	CC(C)(CO)C1CCC2(CC1)OCC(C[N+]([O-])(CC)CCC)O2

- Results and discussion:

M10 differs from M03 by oxidation on the t-butyl moiety giving rise to a hydroxylated spiroxamine N-oxide.

Derek Nexus: predicted to be inactive for bacterial mutagenicity with no misclassified or unclassified features. There were no alerts for mammalian mutagenicity or chromosome damage. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applier:

- Mutagenicity: no alerts for the bacterial or mammalian mutagenicity endpoints (refer to Table CA 5.8.1/28-9).
- Chromosome damage: no alerts for any of the chromosome damage specific endpoints (refer to Table CA 5.8.1/28-9).

OECD (Q)SAR Toolbox:

- Mutagenicity: profiled as potential for *in vitro* mutagenicity (Ames) by ISS with the H-acceptor-path3-H-acceptor alert (refer above to 1. Spiroxamine regarding predictive performance of this alert).
- Chromosome damage: no alerts identified.

Expert evaluation of in silico prognosis for mutagenicity

M10 is predicted not to be mutagenic by Derek Nexus and all four mutagenicity sub-models in Leadscope, it was considered out of domain for the remaining two.

The Toolbox alert is considered not relevant since it is also present in a similar chemical space in M03. It is noted that individually, the N-oxide and hydroxy metabolites (M03 and M05, respectively) are also predicted not to cause mutagenicity and experimental data shows that M03 is not mutagenic. The relative position of each of these functional groups in M10 is unlikely to give extra cause for concern *via* creation of an additional reactive centre

Expert evaluation of in silico prognosis for chromosome damage

M10 does not give any alerts for chromosome damage in Derek Nexus and Toolbox. Following expert review the predictions were considered out of domain for all of the models within Leadscope, due to lack of representation of the N-oxide. It is noted that individually, the N-oxide and hydroxy metabolites (M03 and M05, respectively) are shown experimentally chromosome damage or are predicted with high confidence not to cause chromosome damage, respectively. The relative position of each of these two moieties is considered not to give rise to additional concerns *via* creation of an additional reactive centre.

Conclusion

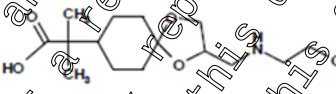
In silico analysis by two methods predicts M10 is neither mutagenic nor causes chromosomal damage, and by reference to M03 and M05 there is high confidence in the prediction.

12. Spiroxamine – desethyl acid (M11):

Chemical name (IUPAC): 2-methyl-2-{2-[(propylamino)methyl]-1,4-dioxaspiro[4.5]decan-8-yl}propanoic acid

CAS No.: Not available

Chemical structure:



SMILES: O=C(O)C(C)(C)C1CCC2(CC1)OCC(CNCCC)O2

- Results and discussion:

M11 differs from spiroxamine by N-dealkylation and oxidation at the t-butyl giving N-desethyl spiroxamine acid.

Derek Nexus: predicted to be inactive for bacterial mutagenicity with no misclassified or unclassified features. There were no alerts for mammalian mutagenicity or chromosome damage. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applier:

- Mutagenicity: no alerts for the bacterial or mammalian mutagenicity endpoints (refer to Table CA 5.8.1/28-9).
- Chromosome damage: no alerts for any of the chromosome damage specific endpoints (refer to Table CA 5.8.1/28-9).

OECD (Q)SAR Toolbox:

- Mutagenicity: profiled as potential for *in vitro* mutagenicity (Ames) by ISS with the H-acceptor-path3-H-acceptor alert (refer above to 1. Spiroxamine regarding predictive performance of this alert).
- Chromosome damage: no alerts identified.

Expert evaluation of in silico prognosis for mutagenicity

M11 is predicted not to be mutagenic by Derek Nexus and by two of the six mutagenicity sub-models in Leadscope, it is out of domain for the other Leadscope sub-models. The Toolbox alert is considered not relevant since it is also present in a similar chemical space to spiroxamine. It is noted that individually, the desethyl and acid metabolites (M01 and M06) are predicted not to cause mutagenicity and the prediction for M06 is supported by experimental weight of evidence. The relative position of each of these functional groups in M11 is unlikely to give extra cause for concern *via* creation of an extra centre of reactivity

Expert evaluation of in silico prognosis for chromosome damage

M11 does not give any alerts for chromosome damage in Derek Nexus or Toolbox. Negative predictions were returned for one of the models within Leadscope and was out of domain for the other five models. It is noted that

individually, the desethyl and acid metabolites (M01 and M06, respectively) are also predicted not to cause chromosome damage and that and the prediction for M06 is supported by experimental weight of evidence. The relative position of each of these two moieties is considered not to give rise to additional concerns *via* creation of an additional reactive centre.

Conclusion

In silico analysis by two methods predicts M11 is neither mutagenic nor causes chromosomal damage , there is medium confidence in the prediction.

Table CA 5.8.1/28-11: Leadscope Model Applier mutagenicity and chromosome damage predictions: M09 M10, M11

Effect	Sub-model	M09			M10			M11		
		QSAR prediction	Positive probability	Expert review	QSAR prediction	Positive probability	Expert review	QSAR prediction	Positive probability	Expert review
Mutagenicity predictions										
Mammalian *in vitro*	HGPRT Mut v1	Negative	0.183	Accepted	Negative[A]	0.174	OoD	NID	0.060	Accepted
	Mouse Lymphoma Act v2	Negative[A,B]	0.187	OoD	Negative[B]	0.462	Accepted	NID	0.173	Accepted
	Mouse Lymphoma Unact v2	Negative[C]	0.432	Accepted	Negative[A]	0.354	OoD	NID	0.483	Accepted
Microbial *in vitro*	Bacterial Mutation v2	Negative	0.139	Accepted	Negative	0.270	Accepted	Negative	0.047	Accepted
	E Coli - Sal 102 A-T Mut v2	Negative[A,B]	0.109	OoD	Negative[B]	0.207	Accepted	NID	0.036	Accepted
	Salmonella Mut v4	Negative	0.065	Accepted	Negative	0.220	Accepted	Negative	0.025	Accepted
Chromosome damage predictions										
CA *in vitro*	*In vitro* Chrom Ab CHL v2	Negative[D,B]	0.477	Accepted	Negative[D,B]	0.352	OoD	NID	0.339	Accepted
	In vitro Chrom Ab CHOc2	Negative[B]	0.285	Accepted	Negative[D,E]	0.119	OoD	NID	0.240	Accepted
CA i*n vivo*	*In vivo* Chrom Ab Comp v2	Negative	0.063	Accepted	Negative	0.042	OoD	NID	0.052	Accepted
	In vivo Chrom Ab Other v1	Negative[B]	0.270	Accepted	Negative[D,E]	0.350	OoD	NID	0.217	Accepted
	In vivo Chrom Ab Rat v1	Negative[B,E]	0.096	Accepted	Negative[D,B]	0.145	OoD	NID	0.225	Accepted
Micronucleus *in vivo*	*In vivo* Micronuc Mouse v2	Negative[B,E]	0.297	Accepted	Negative[D,B]	0.431	OoD	Negative[A]	0.228	Accepted

CA: chromosome aberrations
NID: not in domain
OoD: out of domain

Domain exceptions:
[A] Secondary amide
[B] Hydroxy
[C] Dioxolane ring
[D] Cyclohexane-dioxolane
[E] Cyclohexane

Domain exceptions:
[A] N-oxide
[B] Hydroxy
[D] Dioxolane
[D] Cyclohexane-dioxolane
[E] Cyclohexane

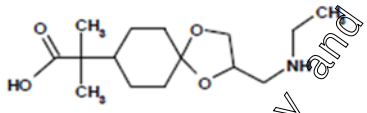
Domain exceptions:
[A] Cyclohexane

13. Spiroxamine - despropyl acid (M12):

Chemical name (IUPAC): 2-{2-[(ethylamino)methyl]-1,4-dioxaspiro[4.5]decan-8-yl}-2-methylpropanoic acid

CAS No.: Not available

Chemical structure:



SMILES: O=C(O)C(C)(C)C1CCC2(CC1)OCC(CNCC)O2

- Results and discussion:

M12 differs from spiroxamine by N-dealkylation and oxidation at the t-butyl giving N-despropyl spiroxamine acid.

Derek Nexus: predicted to be inactive for bacterial mutagenicity with no misclassified or unclassified features. There were no alerts for mammalian mutagenicity or chromosome damage. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applier:

- Mutagenicity: no alerts for the bacterial or mammalian mutagenicity endpoints (refer to Table CA 5.8.1/28-10).
- Chromosome damage: no alerts for any of the chromosome damage specific endpoints (refer to Table CA 5.8.1/28-10).

OECD (Q)SAR Toolbox:

- Mutagenicity: profiled as potential for in vitro mutagenicity (Ames) by ISS with the H-acceptor-path3-H-acceptor alert (refer above to 1. Spiroxamine regarding predictive performance of this alert).
- Chromosome damage: no alerts identified.

Expert evaluation of *in silico* prognosis for mutagenicity

M12 is predicted not to be mutagenic by Derek Nexus and by two of the six mutagenicity models in Leadscope and is out of domain for the other Leadscope models. It is noted that M12 returns a weakly positive result in the out of domain unactivated mouse lymphoma model. The Toolbox alert is considered not relevant because it is present in a similar chemical space in spiroxamine. Examination of the contributing features for out of domain but weakly positive prediction indicates this is due to the carbonyl group in the acid moiety, and the same moiety is present in the acid M06. It is noted that individually, the despropyl and acid metabolites (M02 and M06) are predicted not to cause mutagenicity and that and the prediction for M06 is supported by experimental weight of evidence. The relative position of each of these functional groups in M12 is unlikely to give extra cause for concern *via* creation of an extra reactive centre.

Expert evaluation of *in silico* prognosis for chromosome damage

M12 does not give any alerts for chromosome damage in Derek Nexus or Toolbox. Negative predictions were returned for one of the models within Leadscope and was out of domain for the other five models. It is noted that individually, the despropyl and acid metabolites (M02 and M06, respectively) are also predicted not to cause chromosome damage and the prediction for M06 is supported by experimental weight of evidence. The relative position of each of these two moieties is considered not to give rise to additional concerns *via* creation of an extra reactive centre.

Conclusion

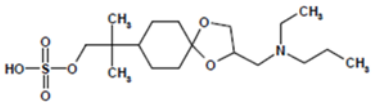
In silico analysis by two methods predicts M12 is neither mutagenic nor causes chromosomal damage , there is medium confidence in the prediction.

14. Spiroxamine – sulfate (M25):

Chemical name (IUPAC): 2-(2-{[ethyl(propyl)amino]methyl}-1,4-dioxaspiro[4.5]decan-8-yl)-2-methylpropyl hydrogen sulfate

CAS No.: Not available

Chemical structure:



SMILES: O=S(=O)(O)OCC(C)(C)C1CCC2(CC1)OCC(CN(CC)CCC)O2

- **Results and discussion:**

M25 differs from spiroxamine by oxidation and conjugation at the t-butyl moiety giving hydroxy spiroxamine sulfate.

Derek Nexus: predicted to be inactive for bacterial mutagenicity with no misclassified or unclassified features. There were no alerts for mammalian mutagenicity or chromosome damage. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applier:

- Mutagenicity: no alerts for the bacterial or mammalian mutagenicity endpoints (refer to Table CA 5.8.1/28-10).
- Chromosome damage: no alerts for any of the chromosome damage specific endpoints (refer to Table CA 5.8.1/28-10).

OECD (Q)SAR Toolbox:

- Mutagenicity: profiled as potential for DNA binding by OECD SN1 mechanism and *in vitro* mutagenicity (Ames) by ISS with the H-acceptor-path3-H-acceptor alert (refer above to 1. Spiroxamine regarding predictive performance of this alert).
- Chromosome damage: no alerts identified.

Expert evaluation of in silico prognosis for mutagenicity

M25 is predicted not to be mutagenic by Derek Nexus and it is out of domain for the Leadscope models. The Toolbox alerts are considered not relevant since they are also present in a similar chemical space in spiroxamine.

Expert evaluation of in silico prognosis for chromosome damage

M25 does not give any alerts for chromosome damage in Derek Nexus or Toolbox. It was not possible to obtain a prediction of chromosome damage using Leadscope since M25 was out of domain for all six models.

Conclusion

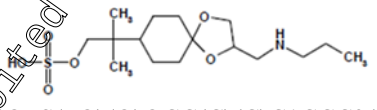
In silico analysis a single method predicts M25 is neither mutagenic nor causes chromosomal damage , there is low confidence in the prediction.

15. Spiroxamine – desethyl-sulfate (M26):

Chemical name (IUPAC): 2-methyl-2-{2-[(propylamino)methyl]-1,4-dioxaspiro[4.5]decan-8-yl}propyl hydrogen sulfate

CAS No: Not available

Chemical structure:



SMILES: O=S(=O)(O)OCC(C)(C)C1CCC2(CC1)OCC(CNCCC)O2

- **Results and discussion:**

M26 differs from spiroxamine by N-dealkylation and oxidation and conjugation at the t-butyl moiety giving hydroxy desethyl spiroxamine sulfate.

Derek Nexus: predicted to be inactive for bacterial mutagenicity with no misclassified or unclassified features. There were no alerts for mammalian mutagenicity or chromosome damage. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applier:

- Mutagenicity: no alerts for the bacterial or mammalian mutagenicity endpoints (refer to Table CA 5.8.1/28-10).
- Chromosome damage: no alerts for any of the chromosome damage specific endpoints (refer to Table CA 5.8.1/28-10).

OECD (Q)SAR Toolbox:

- Mutagenicity: profiled as potential for *in vitro* mutagenicity (Ames) by ISS with the H-acceptor-path3-H-acceptor alert (refer above to 1. Spiroxamine regarding predictive performance of this alert).
- Chromosome damage: no alerts identified.

Expert evaluation of in silico prognosis for mutagenicity

M26 is predicted not to be mutagenic by Derek Nexus and one of the sub-models in Leadscope, it is out of domain for the Leadscope models. The Toolbox alert is considered not relevant since it is also present in a similar chemical space in spiroxamine.

Expert evaluation of in silico prognosis for chromosome damage

M26 does not give any alerts for chromosome damage in Derek Nexus or Toolbox, it was out of domain for the Leadscope sub-models.

Conclusion

In silico analysis predicts M26 is not genotoxic. There is medium confidence in this prediction with respect to mutagenicity but low confidence with respect to chromosome damage, and the lowest confidence level is taken for the combined endpoints.

Table CA 5.8.1/28-12: Leadscope Model Applier mutagenicity and chromosome damage predictions: M12 M25, M11

Mutagenicity predictions										
Effect	**Sub-model**	**M12**			**M25**			**M11**		
		QSAR prediction	**Positive probability**	**Expert review**	**QSAR prediction**	**Positive probability**	**Expert review**	**QSAR prediction**	**Positive probability**	**Expert review**
Mammalian *in vitro*	HGPRT Mut v1	NID	0.064	Accepted	NID	0.170	Accepted	NID	0.170	Accepted
	Mouse Lymphoma Act v2	NID	0.174	Accepted	NID	0.210	Accepted	NID	0.128	Accepted
	Mouse Lymphoma Unact v2	NID	0.512	Accepted	NID	0.272	Accepted	Negative[A]	0.319	Accepted
Microbial *in vitro*	Bacterial Mutation v2	Negative	0.060	Accepted	Negative[A]	0.183	OoD	NegativeA[B]	0.097	Accepted
	E Coli - Sal 102 A-T Mut v2	NID	0.040	Accepted	NID	0.193	Accepted	NID	0.208	Accepted
	Salmonella Mut v4	Negative	0.029	Accepted	Negative[A]	0.062	OoD	Negative[B]	0.043	Accepted
Chromosome damage predictions										
CA *in vitro*	*In vitro* Chrom Ab CHL v2	NID	0.456	Accepted	NID	0.256	Accepted	NID	0.308	Accepted
	In vitro Chrom Ab CHOc2	NID	0.296	Accepted	NID	0.106	Accepted	NID	0.206	Accepted
CA i*n vivo*	*In vivo* Chrom Ab Comp v2	NID	0.058	Accepted	NID	0.016	Accepted	NID	0.024	Accepted
	In vivo Chrom Ab Other v1	NID	0.229	Accepted	NID	0.184	Accepted	NID	0.120	Accepted
	In vivo Chrom Ab Rat v1	NID	0.230	Accepted	NID	0.140	Accepted	NID	0.087	Accepted
Micronucleus *in vivo*	*In vivo* Micronuc Mouse v2	Negative[A]	0.234	Accepted	NID	0.431	Accepted	Negative[B,C]	0.318	OoD

CA: chromosome aberrations

NID: not in domain
OoD: out of domain

Domain exceptions:
[A] Cyclohexane

Domain exceptions:
None

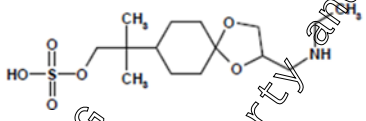
Domain exceptions:
[A] Dioxolane ring
[B] Sulfate
[C] Cyclohexane

16. Spiroxamine – despropyl-sulfate (M27):

Chemical name (IUPAC): 2-{2-[(ethylamino)methyl]-1,4-dioxaspiro[4.5]decan-8-yl}-2-methylpropyl hydrogen sulfate

CAS No.: Not available

Chemical structure:



SMILES: O=S(=O)(O)OCC(C)(C)C1CCC2(CC1)OCC(CNCC)O2

- **Results and discussion:**

M27 differs from spiroxamine by N-dealkylation and oxidation and conjugation at the t-butyl moiety giving hydroxy despropyl spiroxamine sulfate.

Derek Nexus: predicted to be inactive for bacterial mutagenicity with no misclassified or unclassified features. There were no alerts for mammalian mutagenicity or chromosome damage. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applier:

- Mutagenicity: no alerts for the bacterial or mammalian mutagenicity endpoints (refer to Table CA 5.8.1/28-11).
- Chromosome damage: no alerts for any of the chromosome damage specific endpoints (refer to Table CA 5.8.1/28-11).

OECD (Q)SAR Toolbox:

- Mutagenicity: profiled as potential for *in vitro* mutagenicity (micronucleus) by ISS with the H-acceptor-path3-H-acceptor alert (refer above to 1. Spiroxamine regarding predictive performance of this alert).
- Chromosome damage: no alerts identified.

Expert evaluation of in silico prognosis for mutagenicity

M27 is predicted not to be mutagenic by Derek Nexus and it is out of domain for the Leadscope models. The Toolbox alert is considered not relevant since it is also present in a similar chemical space in spiroxamine.

Expert evaluation of in silico prognosis for chromosome damage

M27 does not give any alerts for chromosome damage in Derek Nexus or Toolbox and was out of domain for the Leadscope models.

Conclusion

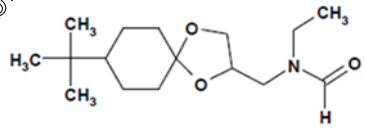
In silico analysis a single method predicts M27 is neither mutagenic nor causes chromosomal damage , there is low confidence in the prediction.

17. Spiroxamine – N-formyl-despropyl (M38):

Chemical name (IUPAC): N-[(8-tert-butyl-1,4-dioxaspiro[4.5]decan-2-yl)methyl]-N-ethylformamide

CAS No.: Not available

Chemical structure:



SMILES: CC(C)(C)C1CCC2(CC1)OCC(CN(C=O)CC)O2

- **Results and discussion:**

M38 differs from spiroxamine by oxidation at the N-ethyl group giving rise to a tertiary amide function.

Derek Nexus: predicted to be inactive for bacterial mutagenicity with no misclassified or unclassified features. There were no alerts for mammalian mutagenicity or chromosome damage. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applier:

- Mutagenicity: no alerts for the bacterial or mammalian mutagenicity endpoints (refer to Table CA 5.8.1/28-11).

- Chromosome damage: no alerts for any of the chromosome damage specific endpoints (refer to Table CA 5.8.1/28-11).

OECD (Q)SAR Toolbox:

- Mutagenicity: profiled as potential for DNA binding by OECD SN1 mechanism and *in vitro* mutagenicity (Ames) by ISS with the H-acceptor-path3-H-acceptor alert (refer above to 1. Spiroxamine regarding predictive performance of this alert).
- Chromosome damage: no alerts identified.

Expert evaluation of in silico prognosis for mutagenicity

M38 is predicted not to be mutagenic by Derek Nexus and all three of the bacterial sub-models in Leadscope, it is out of domain for the mammalian Leadscope models. The Toolbox alerts are considered not relevant since they are also present in a similar chemical space in spiroxamine.

Expert evaluation of in silico prognosis for chromosome damage

M38 gives no alerts for chromosome damage in Derek Nexus or Toolbox and two of the six sub-models in Leadscope give negative predictions, it is out of domain of the remaining four sub-models. It is noted that one of the out of domain predictions gives a positive probability of ~0.5. The positive prediction is based on a single feature, the alkyl formamide moiety which is also identified.

Conclusion

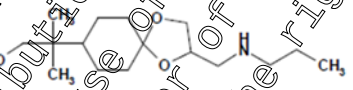
In silico analysis by two methods predicts M38 is neither mutagenic nor causes chromosomal damage , and there is medium confidence in the prediction.

18. Spiroxamine – hydroxyl-desethyl (M41):

Chemical name (IUPAC): 2-methyl-2-{2-[(propylamino)methyl]-1,4-dioxaspiro[4.5]decan-8-yl}propan-1-ol

CAS No.: Not available

Chemical structure:



SMILES: CC(C)(CO)C1CCC2(CC1)OCC(CNCCC)O2

- **Results and discussion:**

M41 differs from spiroxamine by the loss of the N-ethyl group giving rise to a secondary amine function and by oxidation at the t-butyl group giving a hydroxyl function.

Derek Nexus: predicted to be inactive for bacterial mutagenicity with no misclassified or unclassified features. There were no alerts for mammalian mutagenicity or chromosome damage. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applier:

- Mutagenicity: no alerts for the bacterial or mammalian mutagenicity endpoints (refer to Table CA 5.8.1/28-11).
- Chromosome damage: no alerts for any of the chromosome damage specific endpoints (refer to Table CA 5.8.1/28-11).

OECD (Q)SAR Toolbox:

- Mutagenicity: profiled as potential for DNA binding by OECD SN1 mechanism and *in vitro* mutagenicity (Ames) by ISS with the H-acceptor-path3-H-acceptor alert (refer above to 1. Spiroxamine regarding predictive performance of this alert).
- Chromosome damage: no alerts identified.

Expert evaluation of in silico prognosis for mutagenicity

M41 is predicted not to be mutagenic by Derek Nexus and six mutagenicity models in Leadscope. The Toolbox alert is considered not relevant since they is also present in a similar chemical space in spiroxamine.

Expert evaluation of in silico prognosis for chromosome damage

M41 gave no alerts for chromosome damage in Derek Nexus and Leadscope returned a negative prediction for chromosome damage in all six models. There were no alerts for chromosome damage in Toolbox.

Conclusion

In silico analysis by two methods predicts M41 is neither mutagenic nor causes chromosomal damage, and there is medium confidence in the prediction.

Table CA 5.8.1/28-13: Leadscope Model Applier mutagenicity and chromosome damage predictions: M27 M38, M41

Mutagenicity predictions										
Effect	**Sub-model**	**M27**			**M38**			**M41**		
		QSAR prediction	**Positive probability**	**Expert review**	**QSAR prediction**	**Positive probability**	**Expert review**	**QSAR prediction**	**Positive probability**	**Expert review**
Mammalian *in vitro*	HGPRT Mut v1	NID	0.178	Accepted	NID	0.183	Accepted	Negative	0.174	Accepted
	Mouse Lymphoma Act v2	NID	0.129	Accepted	NID	0.334	Accepted	Negative	0.186	Accepted
	Mouse Lymphoma Unact v2	NID	0.345	Accepted	NID	0.383	Accepted	Negative[A]	0.404	Accepted
Microbial *in vitro*	Bacterial Mutation v2	Negative[A]	0.112	OoD	Negative	0.055	Accepted	Negative	0.111	Accepted
	E Coli - Sal 102 A-T Mut v2	NID	0.226	Accepted	NID	0.023	Accepted	Negative[B]	0.099	Accepted
	Salmonella Mut v4	Negative[A]	0.050	OoD	Negative	0.021	Accepted	Negative	0.056	None
Chromosome damage predictions										
CA *in vitro*	*In vitro* Chrom Ab CHL v2	NID	0.422	Accepted	NID	0.774	Accepted	Negative[C]	0.357	Accepted
	In vitro Chrom Ab CHO v2	NID	0.257	Accepted	Negative	0.270	Accepted	Negative[B]	0.231	Accepted
CA i*n vivo*	*In vivo* Chrom Ab Comp v2	NID	0.027	Accepted	Negative	0.034	Accepted	Negative	0.057	Accepted
	In vivo Chrom Ab Other v1	NID	0.138	Accepted	Negative	0.393	OoD	Negative[B]	0.257	OoD
	In vivo Chrom Ab Rat v1	NID	0.090	Accepted	NID	0.118	Accepted	Negative[D]	0.094	Accepted
Micronucleus *in vivo*	*In vivo* Micronuc Mouse v2	Negative[A]	0.324	OoD	NID	0.403	Accepted	Negative[D]	0.291	Accepted

CA: chromosome aberrations
NID: not in domain
OoD: out of domain

Domain exceptions:
[A] Sulfate

Domain exceptions:
[A] N-alkyl formamide

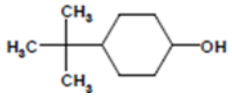
Domain exceptions:
[A] Dioxolane
[B] Hydroxy
[C] Dioxolane-cyclohexane
[D] Cylcohexane

19. Spiroxamine – cyclohexanol (M13) Used as a reference:

Chemical name (IUPAC): 4-tert-butylcyclohexan-1-ol

CAS No.: Not available

Chemical structure:



SMILES: CC(C)(C)C1CCC(O)CC1

- Results and discussion:

Derek Nexus: predicted to be inactive for bacterial mutagenicity with no misclassified or unclassified features. There were no alerts for mammalian mutagenicity or chromosome damage. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applier:

- Mutagenicity: no alerts for the bacterial mutagenicity and a single alert for mammalian mutagenicity endpoints (refer to Table CA 5.8.1/28-12), with the *in silico* bacterial prediction in agreement with the experimental data.
- Chromosome damage: no alerts for any of the chromosome damage specific endpoints (refer to Table CA 5.8.1/28-12), with the *in silico* prediction in agreement with the experimental data.

OECD (Q)SAR Toolbox:

- Mutagenicity: no alerts identified.
- Chromosome damage: no alerts identified.

Expert evaluation of in silico prognosis for mutagenicity

M13 is not mutagenic. *In silico* predictions using two methods are in agreement with the experimental result.

Expert evaluation of in silico prognosis for chromosome damage

M13 does not cause chromosome damage. *In silico* predictions using two methods are in agreement with the experimental result.

Conclusion

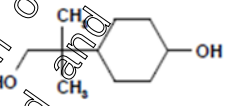
Experimental data show that M13 does not cause bacterial mutagenicity or chromosome damage. *In silico* predictions using two methods are in agreement with the experimental result.

20. Spiroxamine – diol (M14):

Chemical name (IUPAC): 4-(1-hydroxy-2-methylpropan-2-yl)cyclohexan-1-ol

CAS No.: Not available

Chemical structure:



SMILES: OCC1CCC(CC1)C(C)(C)CO

- Results and discussion:

M14 differs from M13 by the addition of a hydroxy function on the t-butyl group.

Derek Nexus: no alerts activated for bacterial, mammalian mutagenicity or chromosome damage specific endpoint with no misclassified or unclassified features. There were no alerts for mammalian mutagenicity. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applier:

- Mutagenicity: no alerts for the bacterial mutagenicity and one alerts for mammalian mutagenicity endpoints (refer to Table 5.8.1.7/01-12).
- Chromosome damage: a single alerts for *in vitro* chrom Ab CHL v2 was returned (refer to Table 5.8.1.7/01-12).

OECD (Q)SAR Toolbox:

- Mutagenicity: no alerts identified.
- ***Expert evaluation of in silico prognosis***.
- Chromosome damage: no alerts identified.

Expert evaluation of in silico prognosis for mutagenicity

M14 is predicted not to be mutagenic in Derek, in four of the Leadscope sub-models in Leadscope and there were no alerts in Toolbox. It was deemed out of domain for two of the Leadscope sub-models. M14 is structurally similar to M13 which is not mutagenic (according to a recently conducted, GLP Ames test). Furthermore a hydroxy group is considered to have a low probability of increasing genotoxic potential (Benigni *et al*, 2019).

Expert evaluation of in silico prognosis for chromosome damage

M14 gave no alerts for chromosome damage in Derek Nexus or Toolbox and was predicted to be negative in one Leadscope sub-models and deemed out of domain for the other five sub-models. M14 is structurally similar to M13 which does not cause chromosome damage (according to a recently conducted, GLP *in vitro* micronucleus assay) and this result can be used to support the negative *in silico* prediction for M14.

Conclusion

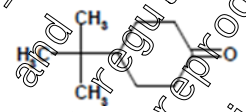
M14 is predicted not to be genotoxic by two methods and by reference to M13 there is high confidence in this prediction.

21. Spiroxamine – ketone (M15):

Chemical name (IUPAC): 4-tert-butylcyclohexan-1-one

CAS No.: Not available

Chemical structure:



SMILES: O=C1CCC(CC1)C(C)(C)C

Results and discussion:

M15 differs from spiroxamine in that the dioxolane ring and amide functions have been lost. M15 differs from M13 by having a ketone rather than a hydroxy group on the cyclohexane ring.

Derek Nexus: predicted to be inactive for bacterial mutagenicity with no misclassified or unclassified features. There were no alerts for mammalian mutagenicity or chromosome damage. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applier:

- Mutagenicity: no alerts for the bacterial or mammalian mutagenicity endpoints (refer to Table 5.8.1.7/01-12).
- Chromosome damage: no alerts for any of the chromosome damage specific endpoints (refer to Table 5.8.1.7/01-12).

OECD (QSAR Toolbox):

- Mutagenicity: no alerts identified.
- Chromosome damage: no alerts identified.

Expert evaluation of in silico prognosis for mutagenicity

M15 is predicted not to be mutagenic in Derek, Leadscope and there were no alerts for mutagenicity in Toolbox. The structural features of M15 were represented as contributing features and training set analogues in Leadscope.

Expert evaluation of in silico prognosis for chromosome damage

M15 gave no alerts for chromosome damage in Derek Nexus or Toolbox and was predicted to be negative in four of the Leadscope sub-models, being out of domain for the remaining sub-models.

Conclusion

In silico analysis by two methods predicts M15 is neither mutagenic nor causes chromosomal damage, there is medium confidence in this prediction.

Table CA 5.8.1/28-14: Leadscope Model Applier mutagenicity and chromosome damage predictions: spiroxamine, M13, M14 and M15

Effect	Sub-model	M13			M14			M15		
		QSAR prediction	Positive probability	Expert review	QSAR prediction	Positive probability	Expert review	QSAR prediction	Positive probability	Expert review
Mutagenicity predictions										
Mammalian *in vitro*	HGPRT Mut v1	Negative	0.174	Accepted	Negative	0.171	Accepted	Negative	0.215	Accepted
	Mouse Lymphoma Act v2	Negative	0.270	Accepted	Negative[A]	0.278	OoD	Negative	0.277	Accepted
	Mouse Lymphoma Unact v2	Negative	0.324	Accepted	Negative	0.383	Accepted	Negative	0.303	Accepted
Microbial *in vitro*	Bacterial Mutation v2	Negative	0.056	Accepted	Negative	0.154	Accepted	Negative	0.104	Accepted
	E Coli - Sal 102 A-T Mut v2	Negative[A]	0.110	OoD	Negative[A,B]	0.121	OoD	Negative	0.086	Accepted
	Salmonella Mut v4	Negative	0.045	Accepted	Negative	0.120	Accepted	Negative	0.070	Accepted
Chromosome damage predictions										
CA *in vitro*	*In vitro* Chrom Ab CHL v2	Negative	0.218	Accepted	Negative[A]	0.219	OoD	Negative	0.450	Accepted
	In vitro Chrom Ab CHO v2	Negative[A]	0.379	OoD	Negative[A,B]	0.379	OoD	Negative[A]	0.377	OoD
CA i*n vivo*	*In vivo* Chrom Ab Comp v2	Negative	0.045	Accepted	Negative	0.045	Accepted	Negative	0.059	Accepted
	In vivo Chrom Ab Other v1	Negative[A]	0.302	OoD	Negative[A,B]	0.293	OoD	Negative[A]	0.304	OoD
	In vivo Chrom Ab Rat v1	Negative[B]	0.125	OoD	Negative[A,C]	0.116	OoD	Negative	0.075	Accepted
Micronucleus *in vivo*	*In vivo* Micronuc Mouse v2	Negative	0.430	OoD	Negative[A,C]	0.426	OoD	Negative	0.337	Accepted

CA: chromosome aberrations
NID: not in domain
OoD: out of domain

Domain exceptions:
[A] Hydroxyl
[B] Cyclohexanol

Domain exceptions:
[A] Hydroxy (t-butyl)
[B] Hydroxy (cyclohexane)
[C] Cyclohexanol

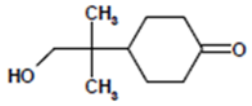
Domain exceptions:
[A] Ketone

22. Spiroxamine – hydroxy-ketone (M16):

Chemical name (IUPAC): 4-(1-hydroxy-2-methylpropan-2-yl)cyclohexan-1-one

CAS No.: Not available

Chemical structure:



SMILES: OCC(C)(C)C1CCC(=O)CC1

- **Results and discussion:**

M16 differs from spiroxamine in that the dioxolane ring and amide functions have been lost and the t-butyl group is hydroxylated. M16 differs from M13 by having a ketone rather than a hydroxy group on the cyclohexane ring and the t-butyl group is hydroxylated.

Derek Nexus: predicted to be inactive for bacterial mutagenicity with no misclassified or unclassified features. There were no alerts for mammalian mutagenicity or chromosome damage. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applier:

- Mutagenicity: no alerts for the bacterial or mammalian mutagenicity endpoints (refer to Table CA 5.8.1/28-13).
- Chromosome damage: no alerts for any of the chromosome damage specific endpoints (refer to Table CA 5.8.1/28-13).

OECD (Q)SAR Toolbox:

- Mutagenicity: no alerts identified.
- Chromosome damage: no alerts identified.

Expert evaluation of in silico prognosis for mutagenicity

M16 is predicted not to be mutagenic in Derek, in four Leadscope sub-models and there were no alerts for mutagenicity in Toolbox. M16 was deemed out of domain for the remaining Leadscope sub-models.

Expert evaluation of in silico prognosis for chromosome damage

M16 gave no alerts for chromosome damage in Derek Nexus or Toolbox and was predicted to be negative one Leadscope sub-model. It was deemed out of domain for the remaining models.

Conclusion

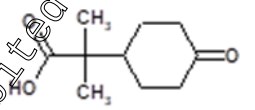
In silico analysis by two methods predicts 161 is neither mutagenic nor causes chromosomal damage, there is medium confidence in this prediction.

23. Spiroxamine – ketone-acid (M17):

Chemical name (IUPAC): 2-methyl-2-(4-oxocyclohexyl)propanoic acid

CAS No.: Not available

Chemical structure:



SMILES: O=C1CCC(CC1)C(C)(C)C(=O)O

- **Results and discussion:**

M17 differs from M13 by having a ketone rather than a hydroxy group on the cyclohexane ring and the t-butyl group has an acid group.

Derek Nexus: predicted to be inactive for bacterial mutagenicity with no misclassified or unclassified features. There were no alerts for mammalian mutagenicity or chromosome damage. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applier:

- Mutagenicity: no alerts for the bacterial or mammalian mutagenicity endpoints (refer to Table CA 5.8.1/28-13).
- Chromosome damage: no alerts for any of the chromosome damage specific endpoints (refer to Table CA 5.8.1/28-13).

OECD (Q)SAR Toolbox:

- Mutagenicity: no alerts identified.
- Chromosome damage: no alerts identified.

Expert evaluation of in silico prognosis for mutagenicity

M17 is predicted not to be mutagenic in Derek, Leadscope and there were no alerts for mutagenicity in Toolbox.

Expert evaluation of in silico prognosis for chromosome damage

M17 gave no alerts for chromosome damage in Derek Nexus or Toolbox and was predicted to be negative in four Leadscope sub-models and out of domain in the remaining two.

Conclusion

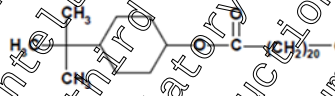
In silico analysis by two methods predicts M17 is neither mutagenic nor causes chromosomal damage, there is medium confidence in this prediction.

24. Spiroxamine – docosanoic acid ester (M35):

Chemical name (IUPAC): 4-tert-butylcyclohexyl docosanoate

CAS No.: Not available

Chemical structure:



SMILES: O=C(OC1CCC(CC1)C(C)(C)C)CCCCCCCCCCCCCCCCCCCCC

- Results and discussion:

Derek Nexus: predicted to be inactive for bacterial mutagenicity with no misclassified or unclassified features. There were no alerts for mammalian mutagenicity or chromosome damage. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applier:

- Mutagenicity: no alerts for the bacterial or mammalian mutagenicity endpoints (refer to Table CA 5.8.1/28-13).
- Chromosome damage: no alerts for any of the chromosome damage specific endpoints (refer to Table CA 5.8.1/28-13) with the *in silico* prediction in agreement with the experimental data.

OECD (Q)SAR Toolbox:

- Mutagenicity: no alerts identified.
- Chromosome damage: no alerts identified.

Expert evaluation of in silico prognosis for mutagenicity

M35 is predicted not to be mutagenic in Derek, Leadscope and there were no alerts for mutagenicity in Toolbox.

Expert evaluation of in silico prognosis for chromosome damage

Experimental data shows that M35 does not cause chromosome damage. M35 gave no alerts for chromosome damage in Derek Nexus or Toolbox and was predicted to be negative in five Leadscope sub-models and out of domain in the remaining Leadscope sub-model.

Conclusion

M35 is predicted not to be genotoxic and there is medium confidence in the prediction for mutagenicity which is based on two methods, and high confidence in the prediction for chromosome damage since it is supported by experimental data.

Table CA 5.8.1/28-15: Leadscope Model Applier mutagenicity and chromosome damage predictions: M16, M17 and M35

Mutagenicity predictions										
Effect	Sub-model	M16			M17			M35		
		QSAR prediction	Positive probability	Expert review	QSAR prediction	Positive probability	Expert review	QSAR prediction	Positive probability	Expert review
Mammalian *in vitro*	HGPRT Mut v1	Negative	0.194	Accepted	Negative	0.088	Accepted	Negative	0.029	Accepted
	Mouse Lymphoma Act v2	Negative[A]	0.285	OoD	Negative	0.267	Accepted	Negative	0.004	Accepted
	Mouse Lymphoma Unact v2	Negative	0.360	Accepted	Negative	0.436	Accepted	Negative	0.004	Accepted
Microbial *in vitro*	Bacterial Mutation v2	Negative	0.262	Accepted	Negative	0.123	Accepted	Negative	0.019	Accepted
	E Coli - Sal 102 A-T Mut v2	Negative[A]	0.094	OoD	Negative	0.034	Accepted	Negative	0.008	Accepted
	Salmonella Mut v4	Negative	0.180	Accepted	Negative	0.086	Accepted	Negative	0.013	Accepted
Chromosome damage predictions										
CA *in vitro*	*In vitro* Chrom Ab CHL v2	Negative[A]	0.451	OoD	Negative	0.431	Accepted	Negative	0.028	Accepted
	In vitro Chrom Ab CHO v2	Negative[A,B]	0.377	OoD	Negative[A]	0.390	OoD	Negative[B]	0.236	Accepted
CA i*n vivo*	*In vivo* Chrom Ab Comp v2	Negative	0.047	Accepted	Negative	0.042	Accepted	Negative	0.000	Accepted
	In vivo Chrom Ab Other v1	Negative[A,B]	0.295	OoD	NID	0.250	Accepted	Negative	0.011	Accepted
	In vivo Chrom Ab Rat v1	Negative[A]	0.069	OoD	Negative	0.173	Accepted	Not in Domain	0.100	Accepted
Micronucleus *in vivo*	*In vivo* Micronuc Mouse v2	Negative[A]	0.334	OoD	Negative	0.244	Accepted	Negative	0.477	Accepted

CA: chromosome aberrations
NID: not in domain
OoD: out of domain

Domain exceptions:
[A] Hydroxy (t-butyl)
[B] Ketone

Domain exceptions:
[A] Ketone

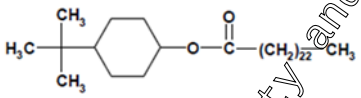
Domain exceptions:
[A] Cyclohexane
[B] T-butyl

25. Spiroxamine – tetracosanoic acid ester (M36):

Chemical name (IUPAC): 4-tert-butylcyclohexyl tetracosanoate

CAS No.: Not available

Chemical structure:



SMILES: O=C(OC1CCC(CC1)C(C)(C)C)CCCCCCCCCCCCCCCCCCCCCCC

- **Results and discussion:**

M36 differs from M35 in the chain length in the acid-ester function (C_{22} compared to C_{20}.

Derek Nexus: predicted to be inactive for bacterial mutagenicity with no misclassified or unclassified features. There were no alerts for mammalian mutagenicity or chromosome damage. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applier:

- Mutagenicity: no alerts for the bacterial or mammalian mutagenicity endpoints (refer to Table CA 5.8.1/28-14).
- Chromosome damage: no alerts for any of the chromosome damage specific endpoints (refer to Table CA 5.8.1/28-14).

OECD (Q)SAR Toolbox:

- Mutagenicity: no alerts identified.
- Chromosome damage: no alerts identified.

Expert evaluation of in silico prognosis for mutagenicity

M36 is predicted not to be mutagenic in Derek, Leadscope and there were no alerts for mutagenicity in Toolbox.

Expert evaluation of in silico prognosis for chromosome damage

M36 gave no alerts for chromosome damage in Derek Nexus or Toolbox and was predicted to be negative in five Leadscope sub-models and out of domain in one sub-model. Furthermore, M36 is structurally very similar to M35 which has been shown in a recently conducted GLP study not to cause chromosome damage and this result can be used to support the negative *in silico* prediction for M36.

Conclusion

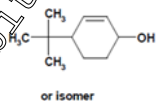
M36 is predicted not to be genotoxic. There is medium confidence in the prediction for mutagenicity which is based on two methods, and high confidence in the prediction for chromosome damage by reference to M35. The lowest confidence value is taken for the overall genotoxicity prognosis.

26. Spiroxamine – hexenol (M37):

Chemical name (IUPAC): 4-tert-butylcyclohex-2-en-1-ol

CAS No.: Not available

Chemical structure:



or isomer

SMILES: CC(C)(C)C1C=CC(O)CC1

- **Results and discussion:**

M37 differs from M13 only in the fact that there is a double bond in the cyclohexane ring.

Derek Nexus: predicted to be inactive for bacterial mutagenicity with no misclassified or unclassified features. There were no alerts for mammalian mutagenicity or chromosome damage. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applier:

- Mutagenicity: no alerts for the bacterial or mammalian mutagenicity endpoints (refer to Table CA 5.8.1/28-14).

- Chromosome damage: no alerts for any of the chromosome damage specific endpoints (refer to Table CA 5.8.1/28-14) with the *in silico* prediction in agreement with the experimental data.

OECD (Q)SAR Toolbox:

- Mutagenicity: no alerts identified.
- Chromosome damage: no alerts identified.

Expert evaluation of in silico prognosis for mutagenicity

M37 is predicted not to be mutagenic in Derek, one of the sub-models in Leadscope and there were no alerts for mutagenicity in Toolbox. Expert review deemed M37 out of domain for the remaining Leadscope sub-models.

Expert evaluation of in silico prognosis for chromosome damage

Data from recently conducted a GLP study shows that M37 does not cause chromosome damage. M37 gave no alerts for chromosome damage in Derek Nexus or Toolbox, was predicted to be negative in two Leadscope sub-models and out of domain in the remaining Leadscope sub-models.. Therefore, experimental data show that M37 does not cause chromosomal damage. *In silico* predictions using two methods are in agreement with the experimental result.

Conclusion

M37 is predicted not to be genotoxic. There is medium confidence in the prediction for mutagenicity which is based on two methods, and high confidence in the prediction for chromosome damage because of the availability of experimental data. The lowest confidence value is taken for the overall genotoxicity prognosis.

Table CA 5.8.1/28-16: Leadscope Model Applier mutagenicity and chromosome damage predictions: M36, M37, M28

Effect	Sub-model	M36			M37			M28		
		QSAR prediction	Positive probability	Expert review	QSAR prediction	Positive probability	Expert review	QSAR prediction	Positive probability	Expert review
Mutagenicity predictions										
Mammalian *in vitro*	HGPRT Mut v1	Negative	0.028	Accepted	Negative	0.234	Accepted	Negative	0.165	OoD
	Mouse Lymphoma Act v2	Negative	0.003	Accepted	Negative[A]	0.391	OoD	Positive	0.827	Refuted
	Mouse Lymphoma Unact v2	Negative	0.004	Accepted	Negative[A,B]	0.487	OoD	Negative	0.487	Accepted
Microbial *in vitro*	Bacterial Mutation v2	Negative[A]	0.019	Accepted	Negative[A]	0.044	OoD	Negative	0.078	Accepted
	E Coli - Sal 102 A-T Mut v2	Negative	0.007	Accepted	Negative[A,B]	0.218	OoD	Negative	0.083	Accepted
	Salmonella Mut v4	Negative[A]	0.013	Accepted	Negative[A]	0.026	OoD	Negative	0.030	Accepted
Chromosome damage predictions										
CA *in vitro*	*In vitro* Chrom Ab CHL v2	Negative	0.024	Accepted	Negative	0.295	Accepted	Negative	0.307	Accepted
	In vitro Chrom Ab CHO v2	Negative[B]	0.191	Accepted	Negative[A]	0.112	OoD	Negative	0.018	Accepted
CA i*n vivo*	*In vivo* Chrom Ab Comp v2	Negative	0.060	Accepted	Negative	0.035	Accepted	Negative[A]	0.224	OoD
	In vivo Chrom Ab Other v1	Negative	0.009	Accepted	NID	0.305	Accepted	Not in	0.393	Accepted
	In vivo Chrom Ab Rat v1	NID	0.094	Accepted	Negative[A]	0.101	OoD	Domain	0.131	OoD
								Negative[A]		
Micronucleus *in vivo*	*In vivo* Micronuc Mouse v2	Negative	0.475	Accepted	Negative[D]	0.433	OoD	Negative[A]	0.427	OoD

CA: chromosome aberrations
NID: not in domain
OoD: out of domain

Domain exceptions:
[A] Cyclohexane
[B] T-butyl

Domain exceptions:
[A] Hydroxyl
[B] Cyclohexene ring
[C] Double bond
[D] Cyclohexenol

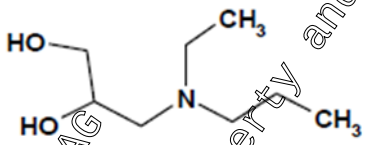
Domain exceptions:
[A] Hydroxy

27. Spiroxamine – aminodiol (M28) [reference compound]:

Chemical name (IUPAC): 3-[ethyl(propyl)amino]propane-1,2-diol

CAS No.: Not available

Chemical structure:



SMILES: CCCN(CC(O)CO)CC

- Results and discussion:

Derek Nexus: predicted to be inactive for bacterial mutagenicity with no misclassified or unclassified features. There were no alerts for mammalian mutagenicity or chromosome damage. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applier:

- Mutagenicity: no alerts for the bacterial mutagenicity and a single alert for mammalian mutagenicity endpoints (refer to Table CA 5.8.1/28-14), with the *in silico* bacterial prediction in agreement with the experimental data.
- Chromosome damage: no alerts for any of the chromosome damage specific endpoints (refer to Table CA 5.8.1/28-14), with the *in silico* prediction in agreement with the experimental data.

OECD (Q)SAR Toolbox:

- Mutagenicity: profiled as potential for DNA binding by OECD SN1 mechanism and *in vitro* mutagenicity (Ames) by ISS with the H-acceptor-path3-H-acceptor alert (refer above to 1. Spiroxamine regarding predictive performance of this alert).
- Chromosome damage: no alerts identified.

Expert evaluation of in silico prognosis for mutagenicity

Experimental data show that M28 is not mutagenic in microbial and mammalian systems. *In silico* predictions using two methods are in agreement with the experimental result with the exception of the Mouse Lymphoma Act v.2 sub-model which gave a positive prediction. The features contributing to the positive prediction at a level of >10% partial property are: 1,2-diol (22.57%); scaffold 140 (15.15%) and scaffold 478 (11.48%). Given the negative experimental mutagenicity result obtained for M28 this prediction is refuted. The Toolbox alerts are triggered by the same substructures as for spiroxamine and given the available experimental data is considered not relevant.

Expert evaluation of in silico prognosis for chromosome damage

Experimental data show that metabolite M28 does not cause chromosome damage. *In silico* predictions using two methods are in agreement with the experimental result.

Conclusion

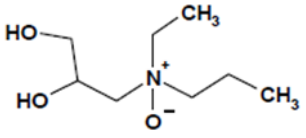
Experimental data show that M28 does not cause chromosome damage. *In silico* predictions using two methods are in agreement with the experimental result.

28. Spiroxamine – aminodiol-N-oxide (M29):

Chemical name (IUPAC): N-ethyl-2,3-dihydroxy-N-propylpropan-1-amine N-oxide

CAS No.: Not available

Chemical structure:



SMILES: CCC[N+]([O-])(CC(O)CO)CC

- **Results and discussion:**

The only difference between M29 and M28 is the presence of an N-oxide moiety.

Derek Nexus: no alerts activated for bacterial, mammalian mutagenicity or chromosome damage specific endpoint with no misclassified or unclassified features. There were no alerts for mammalian mutagenicity. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applier:

- Mutagenicity: no alerts for the bacterial mutagenicity and one alerts for mammalian mutagenicity endpoints (refer to Table CA 5.8.1/28-15).
- Chromosome damage: a single alerts for *in vitro* chrom Ab CHL v2 was returned (refer to Table CA 5.8.1.7/03-5 and Table CA 5.8.1/28-15).

OECD (Q)SAR Toolbox:

- Mutagenicity: profiled as potential for *in vitro* mutagenicity (Ames) by ISS with the H-acceptor-path2-H-acceptor alert (refer above to 1. Spiroxamine regarding predictive performance of this alert).
- Chromosome damage: no alerts identified.

Expert evaluation of in silico prognosis for mutagenicity

Metabolite M29 is predicted not to be mutagenic by Derek Nexus and three of the six mutagenicity sub-models in Leadscope. A positive prediction was returned from the Mouse Lymphoma Act v2 sub-model and the other two sub-models were deemed out of domain. The Toolbox alert was triggered by the same sub-structure as in spiroxamine and M28 and is therefore considered not relevant. Investigation of the positive prediction in the Leadscope Mouse Lymphoma Act v2 sub-model showed features contributing to the positive prediction at a level of >10% partial property are: scaffold 245 (22.47%) 1,2-diol (16.82 %) and scaffold 140 (11.29%). Scaffold 478 (8.549%) is also a contributing feature. These features with the exception of scaffold 245 were also the major contributors to the positive prediction for M28 . Since M28 is shown experimentally not to be mutagenic the positive prediction for M29 is refuted.

Expert evaluation of in silico prognosis for chromosome damage

Metabolite M29 gave no alerts for chromosome damage in Derek Nexus or Toolbox was considered out of domain for all Leadscope sub-models.

It is noted that the In Vitro Chrom Ab CHL v2 sub-model, returned a positive call. Even though expert review deemed M29 to be out of the model domain the call was investigated. Inspection of the model output files showed a training set analogue, LS-199129, which is similar to M29 since it contains an N-oxide and an aliphatic alcohol and is of comparable molecular size. As the extract of the model output below shows the call for this compound was "not in domain" with a probability score of 0.627, indicating a positive prediction. Since the experimental result for this compound was negative it is clear that this sub-model does not predict well for structures in this chemical space.

Conclusion

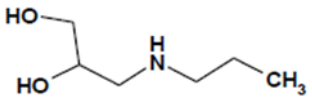
M29 is predicted not to be genotoxic. There is medium confidence in this prediction for mutagenicity and low confidence for chromosome damage. The lowest confidence level is taken for the overall prediction.

29. Spiroxamine – desethylaminodiol (M30):

Chemical name (IUPAC): 3-(propylamino)propane-1,2-diol

CAS No.: 2137135-67-0

Chemical structure:



SMILES: CCCNCC(O)CO

- **Results and discussion:**

The only difference between M30 and M28 is the loss of N-ethyl giving rise to a secondary amine.

Derek Nexus: predicted to be inactive for bacterial mutagenicity with no misclassified or unclassified features. There were no alerts for mammalian mutagenicity or chromosome damage. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applier:

- Mutagenicity: no alerts for the bacterial mutagenicity and two alerts for mammalian mutagenicity endpoints (refer to Table CA 5.8.1/28-15).
- Chromosome damage: no alerts for any of the chromosome damage specific endpoints (refer to Table CA 5.8.1/28-15).

OECD (Q)SAR Toolbox:

- Mutagenicity: profiled as potential for *in vitro* mutagenicity (Ames) by DNA with the H-acceptor-path3-H-acceptor alert (refer above to 1. Spiroxamine regarding predictive performance of this alert).
- Chromosome damage: no alerts identified.

Expert evaluation of in silico prognosis for mutagenicity

M30 is predicted not to be mutagenic by Derek Nexus and four of the six mutagenicity sub-models in Leadscope, with the remaining two models giving a positive prediction. The major feature contributing to the positive prediction in the Mouse Lymphoma Act v.2 sub-model was 1,2-diol (41.44%). This feature was also present as contributing to the positive prediction for M28, which was shown experimentally not to be mutagenic. The next feature contributing to the positive result is the property descriptor ALogP (12.6%), rather than a chemical feature and this indicates that the model prediction is not robust in this chemical space. Similarly, the positive alert for Mouse Lymphoma Unact v2 sub-model is mainly dependent on property descriptors ALogP (21.39%) and hydrogen bond donors (12.91%) and is therefore similarly refuted. Furthermore, the hydroxy and secondary amine functions are considered to have a low probability of increasing genotoxic potential (Benigni *et al*, 2019). The Toolbox alert was triggered by the same sub-structure as in spiroxamine and M28 and is therefore considered not relevant.

Expert evaluation of in silico prognosis for chromosome damage

Metabolite M30 gave no alerts for chromosome damage in Derek Nexus or Toolbox and returned negative predictions for four of the Leadscope sub-models and was deemed out of domain for the remaining sub-models (due to the lack of consideration of any hydroxyl groups in the structure).

Conclusion

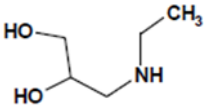
In silico analysis by two methods predicts M30 is neither mutagenic nor causes chromosomal damage, there is medium confidence in this prediction.

30. Spiroxamine – despropyl-aminidiol (M31):

Chemical name (IUPAC): 3-(ethylamino)propane-1,2-diol

CAS No.: 1218657-33-0

Chemical structure:



SMILES: CCNCC(O)CO

- **Results and discussion:**

The only difference between M29 and M28 is the loss of N-propyl giving rise to a secondary amine.

Derek Nexus: predicted to be inactive for bacterial mutagenicity with no misclassified or unclassified features. There were no alerts for mammalian mutagenicity or chromosome damage. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applier:

- Mutagenicity: no alerts for the bacterial mutagenicity and two alerts for mammalian mutagenicity endpoints (refer to Table CA 5.8.1/28-15).

- Chromosome damage: no alerts for any of the chromosome damage specific endpoints (refer to Table CA 5.8.1.7/03-8).

OECD (Q)SAR Toolbox:

- Mutagenicity: profiled as potential for *in vitro* mutagenicity (Ames) by ISS with the H-acceptor-path3-H-acceptor alert (refer above to 1. Spiroxamine regarding predictive performance of this alert).
- Chromosome damage: no alerts identified.

Expert evaluation of in silico prognosis for mutagenicity

M31 is predicted not to be mutagenic by Derek Nexus and four of the six mutagenicity sub-models in Leadscope, with the remaining two models giving a positive prediction. The major feature contributing to the positive prediction in the Mouse Lymphoma Act v.2 sub-model was 1,2-diol (29.68%). This feature was also present as contributing to the positive prediction for M28, which was shown experimentally not to be mutagenic. The next feature contributing to the positive result is the property descriptor ALogP (13.29%), rather than a chemical feature and this indicates that the model prediction is not robust in this chemical space. Similarly, the positive alert for Mouse Lymphoma Unact v2 sub-model is mainly dependent on property descriptors ALogP (15.81%) and hydrogen bond donors (8.91%) and is therefore similarly refuted. Furthermore, the hydroxy and secondary amine functions are considered to have a low probability of increasing genotoxic potential (Benigni *et al*, 2019). The Toolbox alert was triggered by the same sub-structure as in spiroxamine and M28 and is therefore considered not relevant (refer to Table CA 5.8.1/28-15).

Expert evaluation of in silico prognosis for chromosome damage

M31 gave no alerts for chromosome damage in Derek Nexus or Toolbox and returned negative predictions for three Leadscope sub-models and was deemed out of domain for the remaining three models (due to the lack of consideration of any hydroxy function).

Conclusion

In silico analysis by two methods predicts M31 is neither mutagenic nor causes chromosomal damage, there is medium confidence in this prediction.

Table CA 5.8.1/28-17: Leadscope Model Applier mutagenicity and chromosome damage predictions: spiroxamine, M29, M30, M31

Mutagenicity predictions										
Effect	**Sub-model**	**M29**			**M30**			**M31**		
		QSAR prediction	**Positive probability**	**Expert review**	**QSAR prediction**	**Positive probability**	**Expert review**	**QSAR prediction**	**Positive probability**	**Expert review**
Mammalian *in vitro*	HGPRT Mut v1	Negative[A,B]	0.275	OoD	Negative[A]	0.165	Accepted	Negative[A]	0.177	Accepted
	Mouse Lymphoma Act v2	Positive	0.908	Refuted	Positive	0.724	Refuted	Positive	0.742	Refuted
	Mouse Lymphoma Unact v2	Negative[A]	0.490	OoD	Positive	0.543	Refuted	Positive	0.709	Refuted
Microbial *in vitro*	Bacterial Mutation v2	Negative	0.249	Accepted	Negative	0.078	Accepted	Negative	0.099	Accepted
	E Coli - Sal 102 A-T Mut v2	Negative	0.155	Accepted	Negative	0.144	Accepted	Negative	0.148	Accepted
	Salmonella Mut v4	Negative	0.325	Accepted	Negative	0.077	Accepted	Negative	0.089	Accepted
Chromosome damage predictions										
CA *in vitro*	*In vitro* Chrom Ab CHL v2	Positive[A]	0.673	OoD	Negative	0.364	Accepted	Negative	0.108	Accepted
	In vitro Chrom Ab CHO v2	Negative[A]	0.018	OoD	Negative	0.102	Accepted	Negative	0.193	Accepted
CA i*n vivo*	*In vivo* Chrom Ab Comp v2	Negative[A,C]	0.240	OoD	Negative[B]	0.303	OoD	Negative[B]	0.329	OoD
	In vivo Chrom Ab Other v1	NID	0.379	Accepted	Negative[B]	0.282	Accepted	Negative[B]	0.296	OoD
	In vivo Chrom Ab Rat v1	Negative[A,C]	0.115	OoD	Negative[B]	0.082	Accepted	Negative[A]	0.084	OoD
Micronucleus *in vivo*	*In vivo* Micronuc Mouse v2	Negative[A,C]	0.438	OoD	Negative[B]	0.298	OoD	Negative[B]	0.303	OoD

CA: chromosome aberrations
NID: not in domain
OoD: out of domain

Domain exceptions:
[A] N-oxide
[B] Hydroxy
[C] Dihydroxy

Domain exceptions:
[A] Hydroxy
[B] Dihydroxy

Domain exceptions:
[A] Hydroxy
[B] Dihydroxy

B. Deficiencies:

None

Assessment and conclusion by applicant:

Assessment: Study meets the current guidance and the requirements in 283/2013.

Conclusion: Following expert review, *in silico* assessment using two methods showed there were no concerns for mutagenicity for any of the metabolites assessed. There was high confidence in the predictions for metabolites M05, M06, M07, M08 and M10 due to their close structural similarity to spiroxamine and M03 or, in the case of M06, being a major rat metabolite. For the sulfate conjugates M25, M26 and M27 confidence in the prediction was assigned as low, since the sulfate moiety was not assessed in any of the Leadscope sub-models therefore the prognosis was based on a single method. There was high confidence in the predictions for metabolites M13 and M14 due to the availability of experimental data for M13 and its structural similarity to M14. For metabolites M35, M36 and M37 there was high confidence in the prediction for chromosome damage due to the availability of experimental data for M35 and M37 plus the structural similarity between M35 and M36, but the overall confidence level for genotoxicity was medium, based on the mutagenicity endpoint for which there was no experimental data. For metabolites M15, M16 and M17 there was medium confidence in the predictions because they were based on two *in silico* methods. There was high confidence in the predictions for Metabolite M28 due to the availability of experimental data, medium confidence in the predictions for M30 and M31 and a low confidence in the prediction for M29 because the chromosome damage component of the genotoxicity assessment was based on a single method.

CA 5.8.2 Supplementary studies on the active substance

Three supplementary studies on spiroxamine have been conducted. An acute intraperitoneal study confirmed LD_{50} values of 114 mg/kg bw in male rats and 150 mg/kg bw in female rats. Two sensory irritation studies conducted in mice and rats confirmed RD_{50} values of 0.713 mg/L (equivalent to 46.03 mg/kg bw) and >1.584 mg/L (equivalent to 71.28 mg/kg bw), respectively. In mice respirable aerosolised spiroxamine had a sensory irritant potential, with respiratory changes (tidal volume, respiratory rate and minute volume lowered) evident and deemed related to irritation to the peripheral region of the lung. Based on the most sensitive parameter, the minute volume, 0.016 mg/L (equivalent to 1.03 mg/kg bw) is considered to be the non-irritant threshold concentration. In rats, 0.450 mg/L (equivalent to 20.25 mg/kg bw) is considered to be tolerated without any toxicologically relevant changes in breathing.

Table CA 5.8.2-1: Summary of supplementary studies on the active substance toxicity, primary irritation and sensitisation studies

Type of study	Species	Doses	mg/kg bw/day		Key effects	Annex CA Point/Reference
			NOAEL	LOAEL		
Acute intraperitoneal route	Rat	♂: 10, 100, 112, 125 mg/kg bw ♀: 10, 100, 125, 140, 180 mg/kg bw	n/a		LD_{50} ♂: 114 mg/kg bw LD_{50} ♀: 150 mg/kg bw	CA 5.8.2/01 [M-007996-01-1]

Type of study	Species	Doses	mg/kg bw/day		Key effects	Annex CA Point/Reference
			NOAEL	LOAEL		
Sensory irritant potential (inhalation)	Mouse	0.063, 0.142, 0.407, 0.811, 1.367 mg/L	n/a		Sensory irritant RD_{50}: 0.713 mg/L (46.03 mg/kg bw) Non irritant RD_0: 0.016 mg/L (1.03 mg/kg bw)	CA 5.8.2/02 [M-007789-01-1]
Sensory irritant potential (inhalation)	Rat	0.450, 0.858, 1.584 mg/L	n/a		Sensory irritant RD_{50}: 1.584 mg/L (71.28 mg/kg bw) Tolerated RD_0: 0.450 mg/L (20.25 mg/kg bw)	CA 5.8.2/03 [M-007784-01-1]

Data Point:	KCA 5.8.2/01
Report Author:	[redacted]
Report Year:	1991
Report Title:	KWG 4168 - Study for acute intraperitoneal toxicity in rats
Report No:	20419
Document No:	M-007996-01-1
Guideline(s) followed in study:	complied with OECD 401; US-EPA Series 81-1
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted DAR (2010)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The acute intraperitoneal toxicity of spiroxamine was investigated in a study in rats performed to GLP. Groups of Wistar rats (5/sex/group) received a single intraperitoneal injection, employing a dose volume of 10 mL/kg bw, for the following doses: males: 10, 100, 112 and 125 mg/kg bw; females: 10, 100, 125, 140 and 180 mg/kg bw. The animals were then observed for a period of 21 days, the exception to this was females dosed at 180 mg/kg bw, which was for 15 days. Signs of toxicity were observed at dose levels of 100 mg/kg bw and above which were reflective of CNS type effects (including but not limited to piloerection, apathy, decreased motility, staggering gait, increased salivation, temporal convulsions). A transient decrease in body weight was observed on day 4 in animals dosed at ≥100 mg/kg bw.

There was no evidence of test-article related gross lesions in animals sacrificed at the end of the study. In decedent rats there were observations of hepatocytes depleted with glycogen, inflammatory changes in the intestine, with the latter a result of a perforated jejunum resulting from dosing.

Under the conditions of this study the acute intraperitoneal LD_{50} for spiroxamine is 114 mg/kg bw in male rats and 150 mg/kg bw in female rats.

Materials and Methods

A. Materials:

1. Test Material: Spiroxamine
(alternative name: [8-(1,1-dimethylethyl)-N-ethyl-N-propyl-1,4-dioxaspiro[4,5]decane-2-methanamine; KWG 4168)

Description:	Dark brown liquid
Lot/Batch No.:	17002/90
Purity:	93.60%
CAS No.:	118134-30-8
Stability of test compound:	Confirmed stable for the duration of the study (expiry date: 1 April 1991)
2. Vehicle and/or positive control:	2% v/v Cremophor® EL/not applicable
3. Test animals:	
Species:	Rat
Strain:	Wistar
Age at dosing:	♂: 7 - 8 wks; ♀: 10 wks
Weight at dosing:	♂: 165 - 188 g; ♀: 176 - 187 g
Source:	[redacted]
Acclimation period:	6-7 days
Diet:	Altromin® 1324 diet for rats and mice, *ad libitum* (except for 17 h before and 2 h after dosing)
Water:	Municipal water, *ad libitum*
Housing:	Group housed (5/sex/cage)
4. Environmental conditions:	
Temperature:	22 ±2°C
Humidity:	*ca.* 50%
Air changes:	At least 10/h
Photoperiod:	*ca.* 12 h light/dark

B. Study Design:

1. In life dates:	October 1990 to November 1990 (experimental dates)
2. Animal assignment and treatment:	After an acclimatisation period of *ca.* 7 days, rats were pre-arranged based on weight classes and allocated to groups by computer-based stratified random sampling. After being fasted (duration not stated), rats (5/sex/gp) were administered the test article by single intraperitoneal injection, employing a dose volume of 10 mL/kg bw, for the following doses: ♂: 10, 100, 112 and 125 mg/kg bw; ♀: 10, 100, 125, 140 and 180 mg/kg bw. The animals were then observed for a period of 21 days, the exception to this was ♀ dosed at 180 mg/kg bw, which was for 15 days.
3. Statistics:	Not undertaken. For body weight, the mean value and standard deviation were calculated.

C. Methods:

1. Homogeneity and achieved concentration analysis of the dose:	Whilst no details are provided in the report, stability and homogeneity data are presented in the Appendix of the report. Formulations dosed within 1 hour of preparation.
2. Observations:	Appearance and behaviour was recorded several times on the day of treatment, and at least once a day thereafter for up to 21 days.
3. Body weights:	Recorded on study day 1 (prior to dosing), 4, 8, 15 and 22 post dosing.
4. Food consumption:	Not recorded.
5. Sacrifice and pathology:	Organs/tissues were examined macroscopically. No histopathological analysis was undertaken

Results

A. Homogeneity and achieved concentration analysis:

Homogeneity and stability data was confirmed at 0.01% solution and a 50% emulsion for 24 hours. The conditions to which this was applicable were not detailed. Analyses for achieved concentration and homogeneity of test article formulations were not conducted as part of this study, as this is not a requirement of the regulatory test guidelines.

B. Observations:

1. Clinical signs of toxicity:

10 mg/kg bw: no clinical signs of toxicity observed for either sex

100 mg/kg bw: both sexes showed signs of CNS toxicity (including but not limited to piloerection, apathy, decreased motility, staggering gait, increased salivation, temporal convulsions). These effects were immediate in their onset, lasting until day 2. These effects abated on day 6.

112 mg/kg bw: with only ♀ treated, clinical signs of toxicity were comparable to those reported at 100 mg/kg bw. These effects were immediate in their onset, lasting until the animals were found dead between *ca* 34 - 45 minutes post dosing, or lasting until day 22 post dosing in the animals that survived.

125 mg/kg bw: both sexes showed clinical signs of toxicity comparable to those reported at 100 mg/kg bw. These effects were immediate in their onset, lasting until day 4 for ♂♂. For ♀, onset was immediate, lasting until the animals were found dead between *ca* 36 minutes to 7 days post dosing, or in the surviving animal lasting until day 7.

140 mg/kg bw: with only ♀ treated, clinical signs of toxicity were comparable to those reported at 100 mg/kg bw. These effects were immediate in their onset, lasting until day 6.

180 mg/kg bw: with only ♀ treated, signs of CNS toxicity comparable to those already observed. These effects were immediate in their onset, lasting until the animals were found dead between *ca* 22 minutes to 1 day post dosing, or lasting until day 4 post dosing in the single animal that survived.

2. Mortality: Mortality was observed at doses of 112 and 125 mg/kg bw and above for ♂ and ♀, respectively.

Refer to Table CA 5.8.2/01-1 and Table CA 5.8.2/01-2.

Table CA 5.8.2/01-1-: Overview of acute intraperitoneal toxicity study in male rats treated with spiroxamine: mortality and body weight

Parameter	Dose level (mg/kg bw)																			
	10					100					112					125				
Overall mortality[a]	0/5					0/5					2/5					4/5				
Day	1	4	8	15	22	1	4	8	15	22	1	4	8	15	22	1	4	8	15	22
Mortality[a]	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	2/5	0/3	0/3	0/3	0/3	3/5	1/2	0/1	0/1	0/1
Body weight (g) ±s.d	173 ±2.3	189 ±2.8	207 ±2.6	228 ±2.0	247 ±3.3	185 ±3.1	178 ±3.1	187 ±4.8	208 ±2.6	228 ±2.5	178 ±5.6	163 ±11.5	180 ±9.6	209 ±12.7	230 ±9.6	175 ±6.0	163 ±4.9	182 ±0.0	215 ±0.0	250 ±0.0
Net body weight gain (g)	74.6 ±2.97					43.5 ±4.73					-39.3 ±34.19					72.0				
Acute intraperitoneal LD_{50}	114 mg/kg bw																			

a Mortality: no. of animals found dead / no. of animals treated

Table CA 5.8.2/01-2-: Overview of acute intraperitoneal toxicity study in female rats treated with spiroxamine: mortality and body weight

Parameter	Dose level (mg/kg bw)																							
	10					100					125					140					180			
Overall mortality[a]	0/5					0/5					1/5					5/5					4/5			
Day	1	4	8	15	22	1	4	8	15	22	1	4	8	15	22	1	4	8	16	22	1	4	8	15
Mortality[a]	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	1/5	0/4	0/4	0/4	0/4	0/5	0/5	0/5	0/5	0/5	4/5	0/1	0/1	0/1
Body weight (g) ±s.d	180 ±4.0	181 ±6.3	183 ±6.3	185 ±6.8	185 ±7.8	184 ±2.6	178 ±3.8	181 ±2.8	185 ±4.8	188 ±4.5	180 ±1.8	180 ±4.1	182 ±2.8	187 ±3.2	193 ±4.4	182 ±4.0	180 ±5.2	188 ±2.6	194 ±4.8	195 ±3.6	182 ±3.6	173 ±0.0	173 ±0.0	173 ±0.0
Net body weight gain (g)	4.6 ±4.93					4.0 ±5.83					1.4 ±3.71					13.2 ±4.87					10 ±0.0			
Acute intraperitoneal LD_{50}	150 mg/kg bw																							

a Mortality: no. of animals found dead / no. of animals treated

C. Body weight and food consumption:

1. Body weight:	A transient decrease in body weight was observed on day 4 in animals dosed at ≥100 mg/kg bw. Overall body weight gain was affected during the post-treatment observation period in both sexes (refer to Table CA 5.8.1/01-1 and Table CA 5.8.1/01-2). The exception to this statement however applied to ♂ dosed at 125 mg/kg bw and ♀ dosed at 180 mg/kg bw, which only 1 animal survived to day 22 or day 15, respectively. ♀ dosed at 140 mg/kg bw whilst showing a transient decrease in body weight on day 4, net body weight gain was *ca.* 3-fold higher compared to the 10 mg/kg bw dosage group.
2. Food consumption:	Not measured

D. Necropsy:

There was no evidence of test-article related gross lesions in animals sacrificed at the end of the study. In decedent rats there were observations of hepatocytes depleted with glycogen, inflammatory changes in the intestine, with the latter a result of a perforated jejunum resulting from dosing.

E. Deficiencies:

None, no valid test guideline.

Assessment and conclusions by applicant:

Assessment: This study is deemed acceptable and meets the requirements in 283/2013.

Conclusion: Under the conditions of this study the acute intraperitoneal LD_{50} for spiroxamine is 114 mg/kg bw in male rats and 150 mg/kg bw in female rats.

Data Point:	KCA 5.8.2/02
Report Author:	[redacted]
Report Year:	1991
Report Title:	KWG 4168 - Study for sensory irritant potential in the mouse (RD50 determination)
Report No:	20370
Document No:	M-002780-01-1
Guideline(s) followed in study:	ASTM E981-84; OECD 403; FIFRA §81-3; Directive 84/449/EC B.2.
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted DAR (2010)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The sensory irritant potential of spiroxamine was investigated in a study in mice performed to GLP. Groups of CF1 mice (4/females/group) were exposed nose only for a single 1 hour period to an aerosol atmosphere to a mean achieved aerosolised concentrations of 63, 142, 407, 811 and 1367 mg/m^3 [0.063, 0.142, 0.407, 0.811, 1.367 mg/L], with MMAD ±GSD ranging from 1.12 ±1.48 to 1.48 ±1.48 obtained for the aerosol size distribution, with >95% of the inhalable fraction <3 µm. The observation period was 7 days post-exposure.

Prior to test article exposure and following the 1 hour exposure, lung function tests were conducted examining peak expiratory flow, tidal volume, respiration rate, minute volume, inspiration time, expiration time, quotient. The purpose of these data were to determine a sensory irritant potential in order to establish a threshold concentration for the irritant potential.

Clinical signs were observed at concentrations of 811 and 1367 mg/m³ and included slightly to moderately slower breathing, reduced motility, with animals returning to normal 1 day post treatment. No test article related effects on body weight, body weight gain or mortality were observed.

A concentration related change in specific respiratory parameters were observed with tidal volume, respiratory rate and minute volume lowered in animals receiving concentrations ≥811 mg/m³. These animals exhibited characteristic pauses between breaths. The sporadic increase in the tidal volume and the respiratory rate, which was evident in these groups, is related to pulmonary irritation.

Spiroxamine when administered as an aerosol had a low sensory irritant potential in the mouse following a 1 hour nose only exposure. Signs of an irritant effect on the lung periphery were also observed.

Based on the most sensitive parameter, the minute volume 16 mg/m³ is considered to be the non-irritant threshold concentration, equivalent to 1.03 mg/kg bw.

Animals sacrificed at the end of the observation period had no evidence of concentration related changes in the lungs or other organs.

Under the conditions of this study the sensory irritant potential of spiroxamine in mice following a 1 hour nose only exposure RD_{50} is 0.713 mg/L (equivalent to 46.03 mg/kg bw). Respirable aerosolised spiroxamine had a sensory irritant potential, with respiratory changes (tidal volume, respiratory rate and minute volume lowered) evident and deemed related to irritation to the peripheral region of the lung. Based on the most sensitive parameter, the minute volume 0.016 mg/L (equivalent to 1.03 mg/kg bw) is considered to be the non-irritant threshold concentration.

Materials and Methods

A. Materials:

1. **Test Material:** Spiroxamine
   (alternative name: [8-(1,1-dimethylethyl)-N-ethyl-N-propyl-1,4-dioxaspiro[4,5]decane-2-methanamine; KWG 4168)
   Description: Brown liquid (pH 10.4)
   Lot/Batch No.: 17002/90
   Purity: 94.9%
   CAS No.: 118134-30-8
   Stability of test compound: Assumed stable for the duration of the study (expiry date not given)
2. **Vehicle and/or positive control:** For low concentrations polyethylene glycol 400 - ethanol / not relevant
3. **Test animals:**
   Species: Mouse
   Strain: OF1
   Age at dosing: ♀ 5-7 wks
   Weight at dosing: ♀: 23.0 – 28.0 g
   Source: [redacted]
   Acclimation period: 7 days
   Diet: Altromin® 1324 diet for rats and mice, *ad libitum*
   Water: Municipal water, *ad libitum*
   Housing: Group housed (4/cage)

4. Environmental conditions:	
Temperature:	22 ±2°C
Humidity:	*ca.*50%
Air changes:	At least 10/h
Photoperiod:	*ca.* 12 h light/dark

B. Study Design:

1. In life dates:	13 November 1990 to 23 November 1990 (experimental dates)
2. Animal assignment and treatment:	Following acclimatisation mice were randomly assigned to the test groups. Groups of rats (4/sex) were exposed (nose only) for 1 hours to atmospheres containing spiroxamine (aerosol) at measured concentrations of 0 (air control and vehicle control [polyethylene glycol 400 – ethanol]), 63, 142, 407, 811 or 1367 mg/m^3 in air. The observation period was 7 days post-exposure.
3. Generation of the test atmosphere/chamber description:	During the 1 hour exposure period, rats were housed individually in plexiglass exposure tubes (following a period of acclimatisation prior to dosing). Spiroxamine at concentrations of 0, 300, 1000, 3000, 10000 and 13300 mg/m^3 was automatically injected into a baffle with compressed air (air that has had water, dust and oil removed). This mixture was then pumped into the inhalation chamber (volume: *ca.* 20 L). The baffle increased the efficiency of aerosol generation, whilst also removing larger particles. The air flows (10 L/minute, ensuring at least *ca.* 30 exchanges of the inhalation chamber air volumes/hour) were continuously monitored with rotameters and re-adjusted to the nominal settings where necessary. Air flows were monitored continuously Determination of the concentration of spiroxamine in the test atmosphere was performed using gas chromatography (FI detector). Temperature and air humidity in the exposure chamber were measured once during the exposure period. Particle size distribution analysis were taken from the immediate vicinity of the breathing zone and analysis performed by means of a Berner cascade impactor. The impactor media were gravimetrically evaluated.
4. Statistics:	Mean values and simple standard deviations were calculated for the body weights. more frequent findings for the respiratory tract were evaluated using Fisher's Pairwise Test with a preceding RxC chi square test

C. Methods:

1. Observations:	Observed several times on the day of the exposure, then twice daily (morning and evening). They were also assessed at weekends. The animals were only assessed while they were in the tubes if there were clear signs occurring such as spasms, abnormal movements, and severe dyspnoea. An assessment of their reflexes was also undertaken. After exposure animals were assessed with particular regard to the following signs: - Gross appearance of the mucus membranes of the eyes, respiratory tract - General state of muzzle skin, pinnae, state of fur, grooming activity, respiration - Cardiovascular parameters, - Somato-motor system and behaviour pattern (including tremors, convulsions, hypersalivation, dyspnoea, diarrhoea, lethargy, sedation, coma) - Central nervous and autonomic signs
2. Body weights:	The body weights of the rats were recorded manually before exposure, and on days 1, 3 and 7 of the post-treatment observation period.
3. Food consumption:	Not recorded.
4. Lung function test:	Prior to test article exposure animals were adapted to the inhalation chamber and the relevant basal lung function parameters were measured for *ca.* 15

minutes. Following the 1 hour exposure, a 30 to 40 minute recovery period was included. Lung function test and calculation of the relevant parameters were performed under isothermic conditions. Air flows were measured from the pressure difference across 4 *x* 400-mesh wire gauzes using a differential pressure transducer mounted on the chamber wall. The following lung function parameters were determined:

Peak expiratory flow, tidal volume, respiration rate, minute volume, inspiration time, expiration time, quotient.

5. Sacrifice and pathology: All animals were sacrificed post-treatment and subjected to a gross necropsy.

Results and Discussion

A. Atmospheric data:

Findings indicate that particles were well within the respirable range.

Table CA 5.8.2/02-1: Overview of acute sensory irritant potential in mice treated with spiroxamine: exposure parameters

Parameter		Value				
Dose group (nominal mg/m³)		300	1000	3000	10000	13300
Mean achieved atmosphere concentration (mg/m³)		63	142	407	811	1367
Mean achieved atmosphere concentration (mg/L)		0.063	0.142	0.407	0.811	1.367
Dose group (internal dose mg/kg bw/d)[a]		4.07	9.2	26.3	52.4	88.3
Chamber flow rate (L/min)		5.85	5.85	5.85	5.85	5.85
Particle size (MMAD ± GSD)		1.41 ± 1.45	1.42 ± 1.46	1.12 ± 1.48	1.48 ± 1.48	1.39 ±1.49
Aerosol mass <3 µm (%)		98	98	100	97	98
Chamber air temperature (°C)	During exposure	22				
Relative humidity (%)	During exposure	32 ±5				
Air changes (/h)	During exposure	30				
O_2 conc. (%)	During exposure	Not detailed				
CO_2 conc. (%)	During exposure	Not detailed				

a Internal dose (mg/kg bw) = inhalation dose (mg/L) *x* 64.56 L/kg bw/h (rat respiration rate)[26] *x* 1 h (daily inhalation exposure) x 1 (default respiratory absorption: 100%). No further correction considered necessary [adapted from SANCO 7531-rev.10]

B. Observations:

1. Clinical signs: The single animal in the air control group, which died on day 4 exhibited reduced motility and piloerection on day 3. All other animals in the air control, vehicle control, 63, 142 and 407 mg/m³ dose groups exhibited no signs of toxicity.

811 and 1367 mg/m³ (52.4 and 88.3 mg/kg bw): clinical signs of toxicity included slightly to moderate slower breathing, reduced motility. All animals returned to normal from day 1 post treatment.

2. Mortality: Deaths were limited to a single animal in the air control group on day 4. Refer to Table CA 5.8.2/02-1 and Table CA 5.8.2/02-2.

26 R.W. Bide, S.J. Armour & E. Yee (1997). Estimation of human toxicity from animal inhalation toxicity data: 1. Minute volume-body weight relationships between animals and man. DRES (Defence Research Establishment Suffield). https://apps.dtic.mil/dtic/tr/fulltext/u2/a336351.pdf
Body weight of mouse: 0.025 kg; Respiratory rate: 0.0269 L/minute. Conversion to L/kg bw/h = (0.0269 *x* 60) / 0.025 = 64.56 L/kg bw/h

Table CA 5.8.2/02-2-: Overview of acute sensory irritant potential in mice treated with spiroxamine: mortality and body weight

Parameter	♀ (Actual concentration (mg/m³) [nominal mg/m³])															
	Air control				Vehicle control				63 [300]				142 [1000]			
Overall mortality[a]	2/4				0/4				0/4				0/4			
Day	0	1	3	7	0	1	3	7	0	1	3	7	0	1	3	7
Mortality[a]	0/4	0/4	0/4	1/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
Body weight (g) ±s.d	25.0 ±1.8	23.3 ±1.9	23.3 ±4.1	25.7 ±2.9	25.3 ±1.5	24.0 ±1.8	25.0 ±1.8	25.0 ±2.2	25.5 ±2.4	24.3 ±2.6	25.5 ±2.4	25.0 ±3.2	25.5 ±1.0	24.0 ±0.8	25.5 ±1.0	25.8 ±1.0
Net body weight gain (g)	1.0 ±1.00				-0.3 ±0.96				-0.5 ±1.73				0.3 ±0.50			
Parameter	**♀ (Actual concentration (mg/m³) [nominal mg/m³])**															
	407 [3000]				**811 [10000]**				**1367 [13300]**							
Overall mortality[a]	0/4				0/4				0/4							
Day	**0**	**1**	**3**	**7**	**0**	**1**	**3**	**7**	**0**	**1**	**3**	**7**				
Mortality[a]	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4				
Body weight (g) ±s.d	24.5 ±2.5	23.5 ±2.1	24.8 ±2.2	25.8 ±3.6	24.0 ±0.8	22.8 ±0.5	23.3 ±1.0	25.3 ±0.5	24.3 ±1.5	21.8 ±1.5	22.0 ±1.4	24.3 ±2.2				
Net body weight gain (g)	1.3 ±1.71				-1.3 ±0.50				0.0 ±0.82							

C. Body weight and food consumption:

1. Body weight:	No test article related effects on body weight or body weight gain were observed.
2. Food consumption:	Not measured

D. Lung function and sensory irritant potential:

1. Lung function test:	A concentration related change in specific respiratory parameters was observed, with tidal volume, respiratory rate and minute volume lowered. The RD_{50} value was calculated on the basis of the minute volume, as this was proved to be the most sensitive parameter. RD_{50} (minute volume) = 713 mg/m³ (equivalent to 0.713 mg/L; 46.03 mg/kg bw) RD_0 (extrapolated from the regression curve) = 16 mg/m³ (equivalent to 0.016 mg/L; 1.03 mg/kg bw) Confidence level (95%) = 594 – 886 mg/m³ (equivalent to 0.594 – 0.886 mg/L; 38.35 – 57.20 mg/kg bw) Animals in the 811 and 1367 mg/m³ groups exhibited characteristic pauses between breaths. The sporadic increase in the tidal volume and the respiratory rate, which was evident in these groups is related to pulmonary irritation.
2. Sensory irritant potential:	Spiroxamine when administered as an aerosol had a low sensory irritant potential in the mouse following a 1 hour nose only exposure. Signs of an irritant effect on the lung periphery were also observed. Based on the most sensitive parameter, the minute volume 16 mg/m³ is considered to be the non-irritant threshold concentration, equivalent to 1.03 mg/kg bw.

Table CA 5.8.2/02-3: Overview of acute sensory irritant potential in mice treated with spiroxamine: respiratory decrease parameters

Parameter	(Actual concentration (mg/m³) [nominal mg/m³])				
	63 [300]	**142 [1000]**	**407 [3000]**	**811 [10000]**	**1367 [33000]**
Respiratory decrease (%)	18	30	42	50	60
RD_{50}	713 mg/m³ [(0.713 mg/L), equivalent to 46.03 mg/kg bw] (based on minute volume)				
Non-irritant threshold	16 mg/m³ (0.016 mg/L), equivalent to 1.03 mg/kg bw] (based on minute volume)				

D. Necropsy:

Animals sacrificed at the end of the observation period had no evidence of concentration related changes in the lungs or other organs.

E. Deficiencies:

None, no valid test guideline.

Assessment and conclusions by applicant:

Assessment: This study is deemed acceptable and meets the requirements in 283/2013.

Conclusion: Under the conditions of this study the sensory irritant potential of spiroxamine in mice following a 1 hour nose only exposure RD_{50} is 0.713 mg/L (equivalent to 46.03 mg/kg bw). Respirable aerosolised spiroxamine had a sensory irritant potential, with respiratory changes (tidal volume, respiratory rate and minute volume lowered) evident and deemed related to irritation to the peripheral region of the lung. Based on the most sensitive parameter, the minute volume,

0.016 mg/L (equivalent to 1.03 mg/kg bw) is considered to be the non-irritant threshold concentration.

Data Point:	KCA 5.8.2/03
Report Author:	[redacted]
Report Year:	1991
Report Title:	KWG 4168 - Studies of sensory irritation potential in rats (determination of the median irritation dose ID50)
Report No:	20375
Document No:	M-007784-01-1
Guideline(s) followed in study:	ASTME981-84; OECD 403; EHRA88I-3
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted DAR (2010)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The sensory irritant potential of spiroxamine was investigated in a study in rats performed to GLP. Groups of Wistar rats (4/females/group) were exposed nose only for a single 1 hour period to mean achieved aerosolised concentrations of 450, 858 and 1584 mg/m^3 [0.450, 0.858, 1.584 mg/L], with MMAD ±GSD ranging from 1.02 ±1.3 to 1.17 ±1.42 obtained for the aerosol size distribution, with 100% of the inhalable fraction <3 µm. The observation period was 7 days post-exposure.

Prior to test article exposure and following the 1 hour exposure, lung function tests were conducted examining peak expiratory flow, tidal volume, respiration rate, minute volume, inspiration time, expiration time, quotient. The purpose of these data were to determine a sensory irritant potential in order to establish a threshold concentration for the irritant potential.

Animals in the 1584 mg/m^3 dosage group mildly slowed down and laboured breathing, piloerection, reduced motility, sniffing noises. All animals returned to normal from day 1 post treatment. No test article related effects on body weight, body weight gain or mortality were observed.

No clear concentration related change in respiratory parameters were observed, with respiratory rate and minute volume unchanged. A mild decrease in the tidal volume from ≥858 mg/m^3, with a toxicologically meaningful influence on the inspiration time/expiration time (IT/ET) ratio observed in animals in the 1584 mg/m^3 dosage group.

No specific irritant potential were observed in rats exposed to spiroxamine as an aerosol. However, in rats exposed to 1584 mg/m^3 exhibited certain alterations in breathing (*i.e.* increase in time between IT and ET), with influence on the lung periphery only detected after high exposure concentrations.

Based on these results 450 mg/m^3 is considered to be tolerated without any toxicologically relevant changes in breathing.

Animals sacrificed at the end of the observation period had no evidence of concentration related changes in the lungs or other organs.

Under the conditions of this study no sensory irritant potential of spiroxamine in rats following a 1 hour nose only resulted with an RD_{50} value >1.584 mg/L (equivalent to 71.28 mg/kg bw). Although these

animals exhibited certain alterations in their breathing pattern, these were deemed casually connected with action on the lung periphery with a pause between breaths detected in this group of animals.

Based on these results 0.450 mg/L (equivalent to 20.25 mg/kg bw) is considered to be tolerated without any toxicologically relevant changes in breathing.

Materials and Methods

A. Materials:

1. Test Material:	Spiroxamine (alternative name: [8-(1,1-dimethylethyl)-N-ethyl-N-propyl-1,4-dioxaspiro[4,5]decane-2-methanamine; KWG 4168)
Description:	Brown liquid (pH 10.4)
Lot/Batch No.:	17002/90
Purity:	94.9%
CAS No.:	118134-30-8
Stability of test compound:	Assumed stable for the duration of the study (expiry date not given)
2. Vehicle and/or positive control:	None / not relevant
3. Test animals:	
Species:	Rat
Strain:	Bor: WISW (SPF-Cpb)
Age at dosing:	*ca.* 2 – 3 months
Weight at dosing:	*ca.* 170-203g
Source:	[redacted]
Acclimation period:	7 days
Diet:	Altromin® 1324 diet for rats and mice, *ad libitum*
Water:	Municipal water, *ad libitum*
Housing:	Group housed (4/cage)
4. Environmental conditions:	
Temperature:	22 ± 2°C
Humidity:	*ca.* 50%
Air changes:	At least 10/h
Photoperiod:	*ca.* 12 h light/dark

B. Study Design:

1. In life dates:	22 October 1990 to 31 October (experimental dates)
2. Animal assignment and treatment:	Following acclimatisation rats were randomly assigned to the test groups. Groups of rats (4/sex) were exposed (nose only) for 1 hours to atmospheres containing spiroxamine (aerosol) at measured concentrations of 0 (air control), 450, 858 or 1584 mg/m^3 in air. The observation period was 7 days post-exposure.
3. Generation of the test atmosphere/chamber description:	During the -hour exposure period, rats were housed individually in plexiglass exposure tubes (following a period of acclimatisation prior to dosing). Spiroxamine at concentrations of 0, 3000, 10000 and 13333 mg/m^3 was automatically injected into a baffle with compressed air (air that has had water, dust and oil removed). This mixture was then pumped into the inhalation chamber (volume: *ca.* 20 L). The baffle increased the efficiency of aerosol generation, whilst also removing larger particles. The air flows (10 – 15 L/minute) were continuously monitored with rotameters and re-adjusted to

the nominal settings where necessary. Air samples were taken on three occasions; at the start, halfway through and towards the end. Determination of the concentration of spiroxamine in the test atmosphere was performed using gas chromatography (FI detector). Temperature and air humidity in the exposure chamber were measured over 10 minute intervals. Particle size distribution analysis were taken from the immediate vicinity of the breathing zone and analysis performed by means of a Berner cascade impactor. The impactor media were gravimetrically evaluated.

4. Statistics: Mean values and simple standard deviations were calculated for the body weights. more frequent findings for the respiratory tract were evaluated using Fisher's Pairwise Test with a preceding RxC chi square test.

C. Methods:

1. Observations: Observed several times on the day of the exposure, then twice daily (morning and evening). They were also assessed at weekends. The animals were only assessed while they were in the tubes if there were clear signs occurring such as spasms, abnormal movements, and severe dyspnoea. An assessment of their reflexes was also undertaken.

After exposure animals were assessed with particular regard to the following signs:

- Gross appearance of the mucus membranes of the eyes, respiratory tract
- General state of muzzle skin, pinnae, state of fur, grooming activity, respiration
- Circulation
- Somato-motor system and behaviour pattern (including tremors, convulsions, hypersalivation, dyspnoea, diarrhoea, lethargy, sedation, coma)
- Central nervous and autonomic signs

2. Body weights: The body weights of the rats were recorded manually before exposure, and on days 1, 3 and 7 of the post-treatment observation period.

3. Food consumption: Not recorded.

4. Lung function test: Prior to test article exposure animals were adapted to the inhalation chamber and the relevant basal lung function parameters were measured for *ca.* 15 minutes. Following the 1 hour exposure, a 30 to 60 minute recovery period was included. Lung function test and calculation of the relevant parameters were performed under isothermic conditions. Air flows were measured from the pressure difference across 4 x 400-mesh wire gauzes using a differential pressure transducer mounted on the chamber wall. The following lung function parameters were determined:

Peak expiratory flow, tidal volume, respiration rate, minute volume, inspiration time, expiration time, quotient.

5. Sacrifice and pathology: All animals were sacrificed post-treatment and subjected to a gross necropsy.

Results and Discussion

A. Atmospheric data:

Findings indicate that particles were well within the respirable range.

Table CA 5.8.2/03-1: Overview of acute sensory irritant potential in rats treated with spiroxamine: exposure parameters

Parameter	Value		
Dose group (nominal mg/m³)	3000	10000	13333
Mean achieved atmosphere concentration (mg/L)	450	858	1584
Mean achieved atmosphere concentration (mg/L)	0.450	0.858	1.584

Parameter		Value		
Dose group (internal dose mg/kg bw/d)[a]		20.25	38.61	71.28
Chamber flow rate (L/min)		25	10	15
Particle size (MMAD ± GSD)		1.02 ± 1.38	1.11 ±1.38	1.17 ±1.42
Aerosol mass <3 µm (%)		100	100	100
Chamber air temperature (°C)	During exposure	22		
Relative humidity (%)	During exposure	30-40		
Air changes (/h)	During exposure	20		
O_2 conc. (%)	During exposure	Not detailed		
CO_2 conc. (%)	During exposure	Not detailed		

a Internal dose (mg/kg bw) = inhalation dose (mg/L) *x* 45 L/kg bw/h (rat respiration rate) *x* 4 h (daily inhalation exposure) *x* 1 (default respiratory absorption: 100%). No further correction considered necessary [taken from SANCO 7531-rev.10]

B. Observations:

1. Clinical signs: All animals in the air control, 450 and 858 mg/m³ dose groups exhibited no signs of toxicity.
Animals in the 1584 mg/m³ dosage group mildly slowed down and laboured breathing, piloerection, reduced motility, sniffing noises. All animals returned to normal from day 1 post treatment.

2. Mortality Deaths were limited to a single animal in the air control group on day 4.
Refer to Table CA 5.8.2/03-1.

Table CA 5.8.2/03-1-: Overview of acute sensory irritant potential in rats treated with spiroxamine: mortality and body weight

Parameter	♀ (Actual concentration (mg/m³) [nominal mg/m³])															
	Air control				450 [3000]				858 [10000]				1584 [13333]			
Overall mortality[a]	0/4				0/4				0/4				0/4			
Day	**0**	**1**	**3**	**7**	**0**	**1**	**3**	**7**	**0**	**1**	**3**	**7**	**0**	**1**	**3**	**7**
Body weight (g) ±s.d	184 ±6.4	182 ±7.6	184 ±6.6	189 ±7.1	191 ±3.9	192 ±4.8	193 ±3.3	197 ±3.4	199 ±3.7	196 ±2.7	195 ±7.0	198 ±5.7	195 ±6.3	190 ±6.8	191 ±6.7	197 ±5.1
Net body weight gain (g)	2.5 ±3.42				6.0 ±1.83				-0.3 ±5.91				1.5 ±1.29			

a Mortality: no. of animals found dead / no. of animals treated

C. Body weight and food consumption:

1. Body weight:	No test article related effects on body weight or body weight gain were observed.
2. Food consumption:	Not measured

D. Lung function and sensory irritant potential:

1. Lung function test:	No clear concentration related change in respiratory parameters were observed with respiratory rate and minute volume unchanged. A mild decrease in the tidal volume from ≥858 mg/m^3, with a toxicologically meaningful influence on the inspiration time/expiration time (IT/ET) ratio observed in animals in the 1584 mg/m^3 dosage group. RD_{50} ≥1584 mg/m^3 (equivalent to 1.584 mg/L; 71.28 mg/kg bw) ID_0 450 mg/m^3 (equivalent to 0.450 mg/L; 20.25 mg/kg bw) Animals at ≥858 mg/m^3 group exhibited characteristic pauses between breaths, suggestive of pulmonary irritation.
2. Sensory irritant potential:	No specific irritant potential were observed in rats exposed to spiroxamine as an aerosol. However, in rats exposed to 1584 mg/m^3 exhibited certain alterations in breathing (i.e. increase in time between IT and ET), with influence on the lung periphery only detected after high exposure concentrations. Based on these results 450 mg/m^3 is considered to be tolerated without any toxicologically relevant changes in breathing.

Table CA 5.8.2/03-3: Overview of acute sensory irritant potential in rats treated with spiroxamine: respiratory parameters (data relative to control period [5])

Parameter	♀ (Actual concentration (mg/m³) [nominal mg/m³])																							
	0 [air control]						450 [3000]						858 [10000]						1584 [13333]					
	PEF	TV	R8	MV	IT	ET	PEF	TV	R8	MV	IT	ET	PEF	TV	R8	MV	IT	ET	PEF	TV	R8	MV	IT	ET
Min	66.3	81.0	80.4	67.5	91.1	83.9	88.6	79.8	86.7	85.4	77.3	86.3	72.5	67.2	89.0	77.4	78.3	74.1	61.1	62.4	67.2	44.8	79.5	72.3
Max	122.7	103.1	108.9	107.2	137.8	130.3	123.9	108.6	117.6	114.9	111.0	119.5	122.9	106.7	139.9	109.9	122.5	104.3	132.9	102.0	114.4	108.6	243.9	154.9
RD_{50}	>1584 mg/m³ [(1.584 mg/L), equivalent to 71.28 mg/kg bw]																							
Non-irritant threshold	450 mg/m³ [(0.450 mg/L), equivalent to 20.25 mg/kg bw]																							

PEV
TV: tidal volume
R8: rate
MV: minute volume
IT: inspiration time
ET: expiration time

D. Necropsy:

Animals sacrificed at the end of the observation period had no evidence of concentration related changes in the lungs or other organs.

E. Deficiencies:

None, no valid test guideline.

Assessment and conclusions by applicant:

Assessment: This study is deemed acceptable and meets the requirements in 283/2013.

Conclusion: Under the conditions of this study m.sensory irritant potential of spiroxamine in rats following a 1 hour nose only resulted with an RD_{50} value >1.584 mg/L (equivalent to 71.28 mg/kg bw). Although these animals exhibited certain alterations in their breathing pattern, these were deemed casually connected with action on the lung periphery with a pause between breaths detected in this group of animals.

Based on these results 0.450 mg/L (equivalent to 20.25 mg/kg bw) is considered to be tolerated without any toxicologically relevant changes in breathing.

For procedural reasons studies listed in the Table 5.8.2-2 below are included in the current dossier as available data or information previously submitted but not necessarily evaluated. However, these reports have been fully superseded by newer studies. Consequently, no summaries of the reports have been included in the dossier.

Table CA 5.8.2-2: Studies previously submitted and not relied upon for the risk assessment

Data Point	Document No.	Date	Title
KCA 5.8.2/04	M-008149-02-1	1997	KWG 4168 - Position paper on the toxicological no-observed effect level which is relevant for the calculation of a systemic AOEL

Literature review manuscripts relevant to mammalian toxicity

Spiroxamine is known to effect fungal sterol biosynthesis by inhibiting either δ14 reductase and δ7, δ 8 isomerase or both; however, data reported in this paper suggest that spiroxamine has a higher affinity to inhibit DHCR7 in human cells. Indeed spiroxamine inhibited the human homologs to δ 14 reductase or δ7, δ8 isomerase, it would be expect decreases in 7-DHC and 8-DHC, not increases. The lack of significant change in cholesterol in the human neuroblastoma SK-N-SH cell line in response to spiroxamine could be due to a biological adaptation of these cells to store cholesterol during acute exposure to small molecule inhibitors of cholesterol biosynthesis. The distinct sterolomic profiles across cell lines that match closely with their tissues of origin have the potential to provide additional insights. Even though our *in vitro* data suggested that the lead-hits were most potent in neural cells, the results also demonstrated that the lead-hits could impair cholesterol biosynthesis in other tissues as well. When extrapolating this study's findings to an actual human, it would be important to consider the impact of the exposure beyond the brain, because liver and lung sterol profiles will likely be disrupted as well.

This data in the context of the apical mammalian toxicity data on spiroxamine support the findings of perturbations in cholesterol homeostasis observed across species, which do not impact upon the steriodogenesis pathway, *in vivo*. Whilst the paper makes reference to characterize the potential to inhibit cholesterol biosynthesis in developing human neurons, from the available apical mammalian data on spiroxamine, there is no evidence to support this. Data generated from two individual two generation

studies (CA 5.6.1/01 [M-016566-01-1]; CA 5.6.1/02 [M-304231-01-1]) confirm no such effects. Furthermore, data from both the acute and sub-chronic neurotoxicity studies (CA 5.7.1/01 [M-016556-01-1]; CA 5.7.1/02 [M-006914-01-1]) confirm spiroxamine is not a neurotoxicant, nor does it show structural relationship to known neurotoxicants (*e.g.*, organophosphates, carbamates). With the thyroid gland being fulcrum to neurodevelopmental effects, the extensive *in vivo* mammalian toxicity data pacakage confirms that spiroxamine is devoid of thyroid gland effects. Therefore, the need to investigate DNT effects are null and void in the respect of spiroxamine.

Data Point:	KCA 5.8.2/21
Report Author:	[redacted]
Report Year:	2020
Report Title:	Screening toxcast for chemicals that affect cholesterol biosynthesis: studies in cell culture and human induced pluripotent stem cell-derived neuroprogenitors
Report No:	M-689223-01-1
Document No:	M-689223-01-1
Guideline(s) followed in study:	not applicable
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Supportive only

Overview

Changes in cholesterol metabolism are common hallmarks of neurodevelopmental pathologies. A diverse array of genetic disorders of cholesterol metabolism support this claim as do multiple lines of research that demonstrate chemical inhibition of cholesterol biosynthesis compromises neurodevelopment. Recent work has revealed that a number of commonly used pharmaceuticals induce changes in cholesterol metabolism that are similar to changes induced by genetic disorders with devastating neurodevelopmental deficiencies.

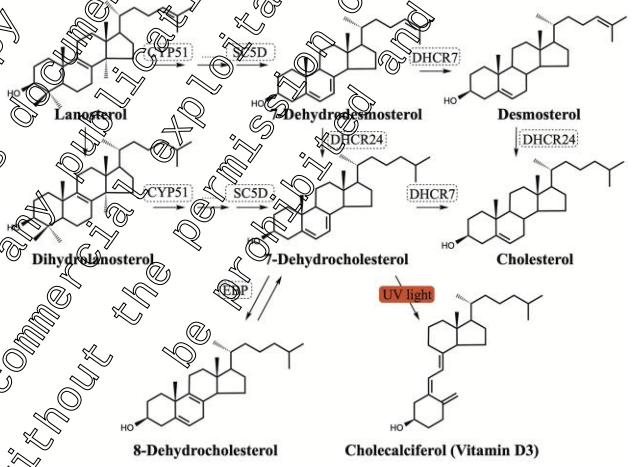


Figure CA 5.8.2/21-1: schematic of cholesterol biosynthesis

Hypocholesterolaemia is a common metabolic feature of many neurodevelopmental disorders and neurodegenerative diseases, including Huntington's disease and Alzheimer's disease. Cholesterol is an indispensable lipid in the central nervous system, with more than 25% of the total cholesterol content of

a human body residing in the brain. In addition to cholesterol serving as an important structural component for cellular membranes and myelin sheathes, a number of cholesterol metabolites, including neurosteroids, serve as critical signaling molecules. To maintain this indispensable pool of cholesterol, the anatomy of the central nervous system requires an active yet independent cholesterol biosynthetic pathway. This requirement is because plasma cholesterol from either diet or hepatosynthesis does not freely cross the blood–brain barrier. During the 1960s' drug-discovery efforts to develop efficacious cholesterol-lowering pharmaceuticals, numerous lead-hit compounds failed in commercial development due to toxicity. AY-9944 was designed to inhibit DHCR7 but the compound never passed preclinical phases due to its potent teratogenicity. Given the history with the small molecule AY-9944, the question arises: are there environmental chemicals that affect human neural development by disrupting cholesterol metabolism? Some environmental sterol biosynthesis disruptors have already been identified by cell culture experiments and the use of *in silico* predictive modeling (*e.g.* benzalkonium chloride which inhibits DHCR7). Another class of environmental sterol metabolism disruptors are the conazole fungicides. These agricultural pesticides are known to inhibit CYP51 by coordinating with the heme group, which halts substrate binding with a resulting increase of lanosterol in cell culture. The most common drug class to treat hypercholesterolaemia, statins, is contraindicated for pregnancy due to an increased risk of spontaneous pregnancy loss yet it is controversial whether this loss is due to teratogenicity.

The objective of this work was to test the hypothesis that common environmental toxicants may also impair cholesterol metabolism and thereby possibly contribute to neurodevelopmental toxicity.

Results

A. Lead-hit validation and *in vitro* dosing:

Using high-through put screening with a targeted lipidomic analysis and the mouse neuroblastoma cell line, Neuro-2a,theToxCast™ chemical library (1851 chemicals) was screened for compounds that impact sterol metabolism. Validation of chemical effects was conducted by assessing cholesterol biosynthesis in human induced pluripotent stem cell (hiPSC)-derived neuroprogenitors using an isotopically labeled cholesterol precursor and by monitoring product formation with UPLC-MS/MS.

Twenty-nine compounds were identified as validated lead-hits, and four were prioritized for further study (endosulfan, sulfate, tributyltin chloride, fenpropimorph,and spiroxamine). All four compounds were validated to cause hypocholesterolemia in Neuro-2a cells. The morpholine-like fungicides, fenpropimorph and spiroxamine, mirrored their Neuro-2a activity in four immortalized human cell lines and in a human neuroprogenitor model derived from hiPSCs, but endosulfan, sulfate and tributyltinchloride did not. Further details of the data generated on spiroxamine are discussed.

Lead-hit determination of ToxCast™ Chemical Library for environmental cholesterol biosynthesis disruptors. The workflow of high-throughput screen from the entire library identified four lead-hit compounds (tributyltin chloride, endosulfan, sulfate, fenpropi morph, spiroxamine). Lead-hit compounds were identified through the results of two independent screens of the ToxCast™ library using Neuro-2a cells as an *in vitro* model at a screening exposure of 1 µM for24 h. Of the compounds screened, those determined as lead-hits for elevating 7-dehydrocholesterol included spiroxamine and fenpropimorph, with a focus on the former.

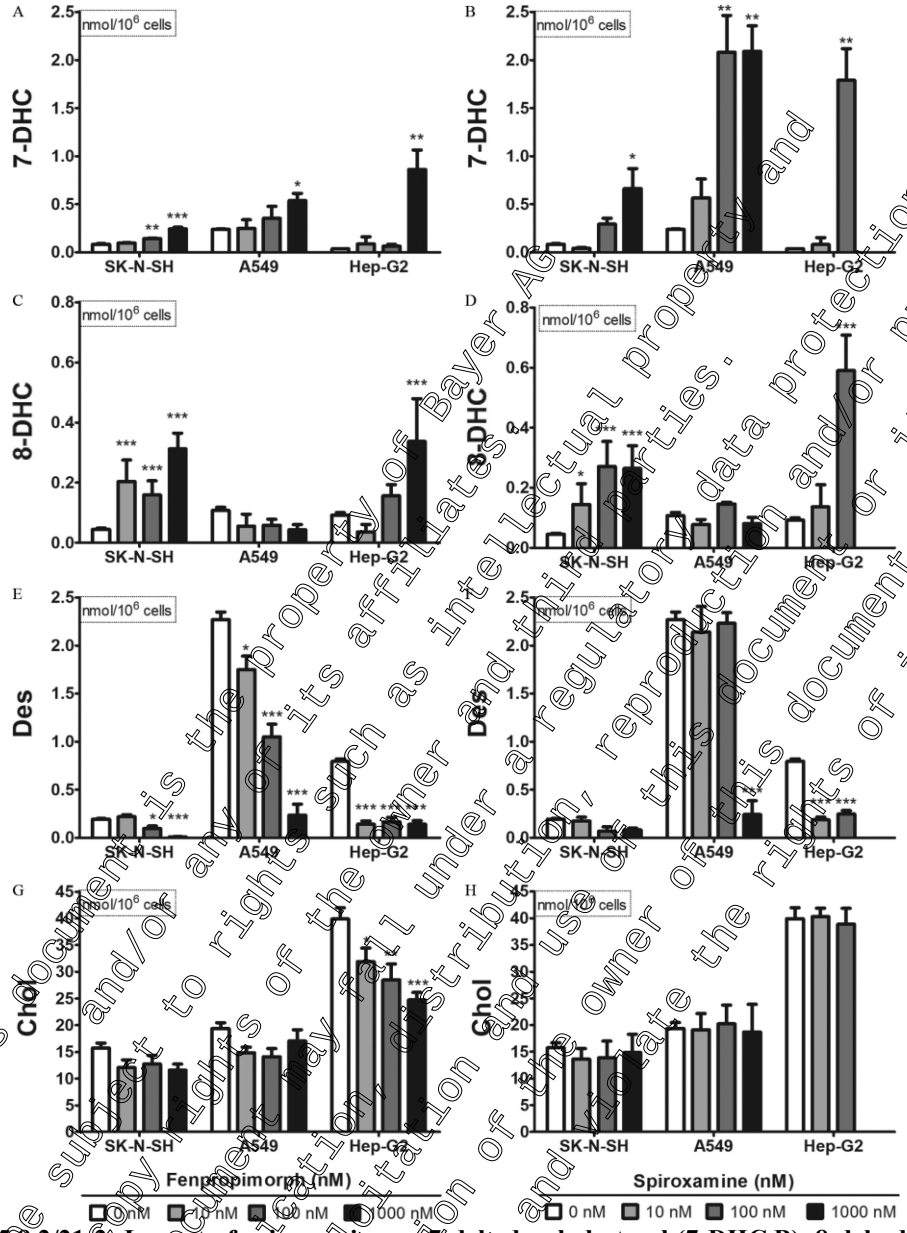


Figure CA 5.8.2/21-2: Impact of spiroxamine on 7-dehydrocholesterol (7-DHC,B), 8-dehydrocholesterol(8-DHC; D),desmosterol (Des; F),and cholesterol levels (Chol;H) in three different human-derived cell lines: (from left to right)SK-N-SH, A549 and Hep-G2. Cells were exposed to compound (0, 10, 100, 1,000nM) for 24h. Omitted bars reflect significant toxicity as determined by release of lactate dehydrogenase. Data presented as nmolsterol/million cells(n= 4,±SEM). *p<0:05, **p<0:01, ***p<0:001 as determined by post hoc Dunnett's test following one-way ANOVA, using vehicle as the control comparison.

B. Temporal changes in neuroprogenitor sterol levels exposed to 7-DHC elevating pesticides:

The accumulated observations for the effect of spiroxamine, suggested inhibition of human DHCR7. To model the cell type most likely affected by a developmental neurotoxicant such as a DHCR7 inhibitor, hiPSCs were differentiated into neuroprogenitors of the cortical glutamatergic lineage and then exposed to chemicals. To account for differentiation-induced changes in cholesterol biosynthesis, each de-identified donor cell line was used to produce three separate sets of hiPSC-derived neuroprogenitors for experiments, and the results were then averaged derived from three distinct differentiations. In addition to spiroxamine, the most potent 7-DHC elevating lead-hit from the original screen and validation, haloperidol, was used as a reference positive control. Haloperidol at 10 and 1000 nM concentration

significantly elevated 7-DHC levels from 0:81 ±0:17nmol/10^6 cells to 1:66 ±0:19 and 2.2 ±0:30 nmol 7-DHC/10^6 cells, respectively, after 8 h of exposure in hiPSC neuroprogenitors. The maximum effect of haloperidol was observed with a 1000 nM, 24-h exposure, plateauing at 5.37 ±0:28 nmol 7-DHC/10^6 cells.

Spiroxamine at 1000 nM led to elevated 7-DHC levels and reduced desmosterol levels in the hiPSC-derived neuroprogenitors in comparison to the time-matched vehicle control. Exposure to spiroxamine at 10 nM did not significantly change sterol levels in the cells. However, beginning at 4 h, exposure to both pesticides at 1000 nM altered hiPSC sterol profiles as indicated by an increase in 7-DHC levels and decrease in desmosterol levels with spiroxamine. After 24 h, similar absolute levels of 7-DHC were observed (spiroxamine, 1:54 nmol 7-DHC/10^6 cells).

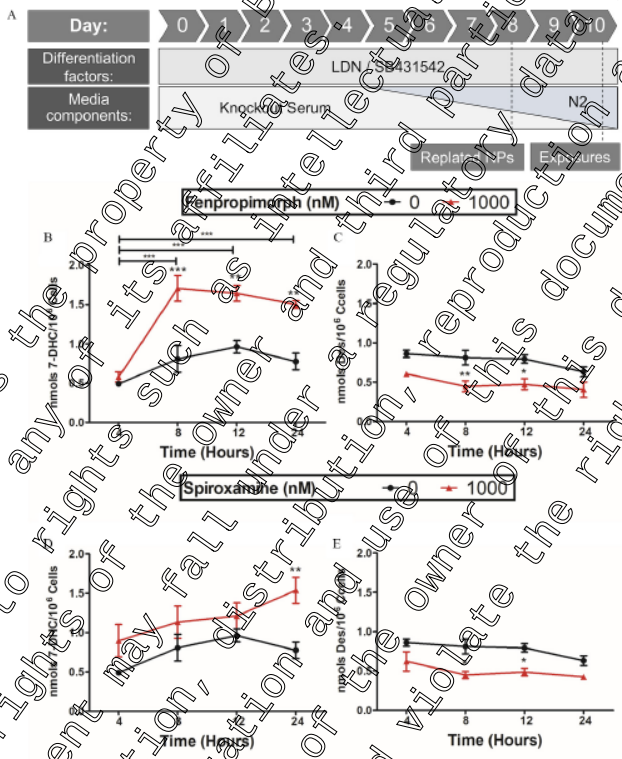


Figure CA 5.8.2/21-3: Human induced hiPSCs were differentiated towards a neuroectoderm lineage as shown (B).These hiPSC-derived neuroprogenitor cells were exposed on day 10 of differentiation to spiroxamine (D,E) at 1000 nM (red triangle) for 4, 8, 12 and 24 h. Neuroprogenitor cells were then analyzed for 7-dehydrocholesterol (D) or desmosterol (E) and compared with vehicle control (black circle; 0.01% DMSO). Three distinct differentiations were conducted for each donor and averaged; shown is the average of the three donors ±SEM (n=3). *p<0:05, **p<0:01 as determined by Bonferroni post tests following two-way ANOVA

C. *De novo* cholesterol Synthesis in hiPSC cells treated with spiroxamine:

An isotopically labeled sterol precursor provides a highly sensitive means to track the enzymatic turnover of post-lanosterol metabolites during a chemical exposure. Thus, hiPSCs were incubated with a synthetic lanosterol labeled with three ^{13}C atoms and the isotopically labeled sterol products of this precursor 7-DHC,8-DHC, desmosterol and cholesterol were analyzed by LC-MS/MS. A gradual increase in $^{13}C_3$-cholesterol levels was observed in the neuroprogenitor cells with a doubling of the absolute amount occurring between 12 and 24h. Between those same time points, a significant decrease of $^{13}C_3$-7-DHC and an increase in $^{13}C_3$-desmosterol were also observed.

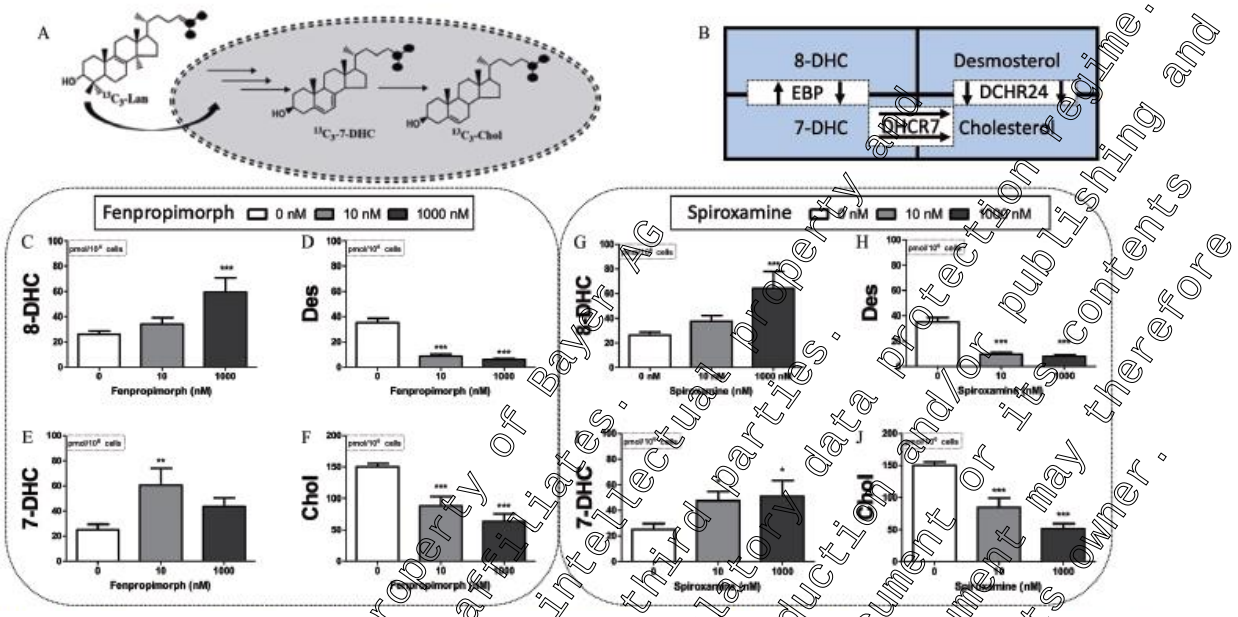


Figure CA 5.8.2/21-4: ***De novo*** **synthesis of cholesterol and sterol precursors was accomplished by incubating hiPSC-derived neuroprogenitor cells with $^{13}C_3$ -lanosterol for 24 h and monitoring for $^{13}C_3$ -sterols including $^{13}C_3$ -7-dehydrocholesterol and $^{13}C_3$ -cholesterol (A).Simplified cholesterol biosynthetic pathway is shown (B). Neuroprogenitor cells were exposed to spiroxamine (G–J) at10 nM and 1000 nM for the same duration as the $^{13}C_3$ -lanosterol incubation. Absolute values of $^{13}C_3$ -8-dehydrocholesterol (G), $^{13}C_3$-desmosterol (H), $^{13}C_3$ -7-dehydrocholesterol (I) and $^{13}C_3$ -cholesterol (J) were detected, quantified and normalized to cell number. Three distinct differentiations were conducted for each donor and averaged;shown is theaverage of the three donor s±SEM (n=3). *p<0:05, **p<0:01, ***p<0:001 as determined by post hoc Dunnett's test following one-way ANOVA**

D. Concentration-dependent effects of environmental cholesterol biosynthesis disruptors:

The capacity of spiroxamine to impact 7-DHC levels was assessed by exposing Neuro-2a cells across a range of concentrations (0–4 µM). IC_{50} value for spiroxamine was 0.55 µM.

The IMR-32 cell line was particularly sensitive to spiroxamine, with concentrations as low as 10nM inducing significant cytotoxicity as determined by lactate dehydrogenase detected in the media after a 24-hexposure. It is noteworthy that the 1000 nM exposure of spiroxamine in the Hep-G2 cells was cytotoxic.

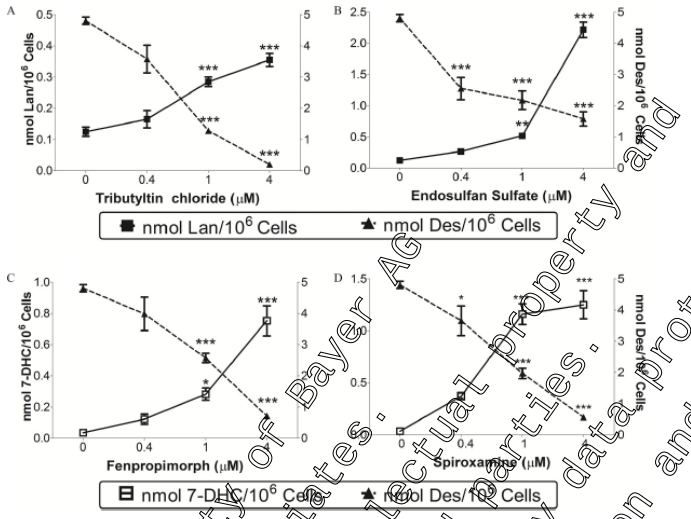


Figure CA 5.8.2/21-5: Concentration-dependent response of lead-hit compounds on intracellular levels of 7-dehydrocholesterol (D, open squares) and desmosterol (solidtriangles) in Neuro-2a cells. Cells were exposed to (C) spiroxamine at the indicated concentration for 24 h. Data presented as nmolsterol/million cells (*n*=4, ±SEM). *p<0:05, ***p<0:001 as deter mined by post hoc Dunnett's test following one-way ANOVA, using vehicle as the control comparison.

Further experimentation with spiroxamine was undertaken to evaluate levels of other 7-DHC relevant metabolites. In addition to 7-DHC serving as a precursor to both cholesterol and vitamin D, this sterol is also isomerized biosynthetically to 8-dehydrocholesterol (8-DHC,) by the enzyme Emopamil Binding Protein (EBP,3-b-hydroxysteroid-D8, D7-isomerase). Thus, if DHCR7 is selectively inhibited, and EBP is unaffected by a compound, then 8-DHC would be elevated. All concentrations tested in the neural SK-N-SH cells for spiroxamine demonstrated a significant elevation of 8-DHC levels. Because a concomitant increase in 7-DHC and 8-DHC levels along with decreases in the DHCR7 metabolites desmosterol and cholesterol is observed following exposure to spiroxamine, it leads to the conclusion that spiroxamine inhibits DHCR7, ultimately leading to a reduction in circulating cholesterol levels. The data from the high throughput screening of spiroxamine also supports the perturbations observed in the apical mammalian toxicity studies.

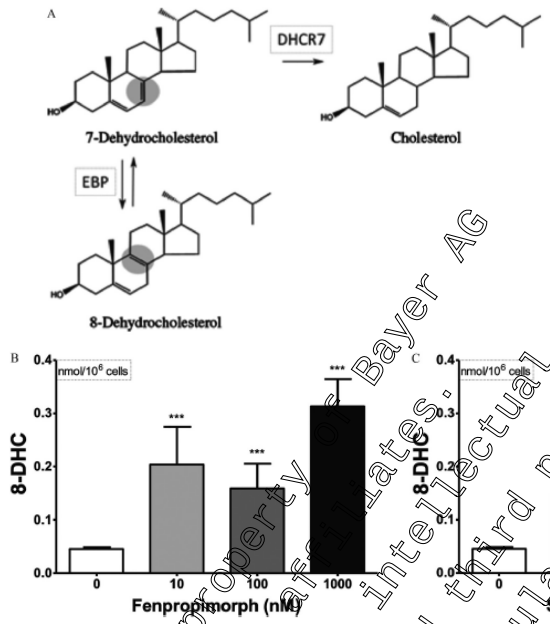


Figure CA 5.8.2/21-6: Biological synthesis of 8-dehydrocholesterol *via* isomerization of 7-dehydrocholesterol by the enzyme EBP is shown (A). SK-N-SH cells were exposed to spiroxamine (C) at indicated concentrations for 24 h. Data presented as nmol 8-dehydrocholesterol/million cells (n=4, ±SEM). *p<0:05, ***p<0:001 as determined by post hoc Dunnett's test following one-way ANOVA , using vehicle as the control comparison

E. *De novo* cholesterol synthesis in hiPSC cells treated with spiroxamine:

Spiroxamine affects *de novo* cholesterol biosynthesis. Exposure at 10 nM for 24 h increased $^{13}C_3$-7-DHC levels and at a higher dose of 1000 nM, $^{13}C_3$-8-DHC levels were also significantly elevated. Of importance is that both products of the enzyme DHCR7, $^{13}C_3$-desmosterol and $^{13}C_3$-cholesterol were significantly decreased in all exposures tested. The enzymatic capacity of DHCR7 to convert 7-DHC to cholesterol can be assessed by the ratio of [$^{13}C_3$-Chol]:[$^{13}C_3$-Chol+$^{13}C_3$-7-DHC], also known as the RCS. Ideally, the RCS is a value of 1 in normal, functional tissues. The hiPSC-derived neuroprogenitors exposed to the vehicle control (0.01% DMSO) provided an RCS value of 0:96±0:01, where as the reference positive control of haloperidol at 10 nM and 1000 nM for 24h led to an RCS of 0:50±0:10 and 0:28±0:14, respectively. Spiroxamine reduced DHCR7 activity at 1000 nM with an observed RCS of 0:50±0:10.

Conclusions

Spiroxamine is known to effect fungal sterol biosynthesis by inhibiting either δ14 reductase and δ7, δ 8 isomerase or both; however, data reported in this paper suggest that spiroxamine has a higher affinity to inhibit DHCR7 in human cells. Indeed spiroxamine inhibited the human homologs to δ 14 reductase or δ7, δ8 isomerase, it would be expect decreases in 7-DHC and 8-DHC, not increases. The lack of significant change in cholesterol in the human neuroblastoma SK-N-SH cell line in response to spiroxamine could be due to a biological adaptation of these cells to store cholesterol during acute exposure to small molecule inhibitors of cholesterol biosynthesis. The distinct sterolomic profiles across cell lines that match closely with their tissues of origin have the potential to provide additional insights. Even though our *in vitro* data suggested that the lead-hits were most potent in neural cells, the results also demonstrated that the lead-hits could impair cholesterol biosynthesis in other tissues as well. When extrapolating this study's findings to an actual human, it would be important to consider the impact of the exposure beyond the brain, because liver and lung sterol profiles will likely be disrupted as well.

This data in the context of the apical mammalian toxicity data on spiroxamine support the findings of perturbations in cholesterol homeostasis observed across species, which do not impact upon the

steriodogenesis pathway, *in vivo*. Whilst the paper makes reference to characterize the potential to inhibit cholesterol biosynthesis in developing human neurons, from the available apical mammalian data on spiroxamine, there is no evidence to support this. Data generated from two individual two generation studies (CA 5.6.1/01 [M-016566-01-1]; CA 5.6.1/02 [M-304231-01-1]) confirm no such effects. Furthermore, data from both the acute and sub-chronic neurotoxicity studies (CA 5.7.1/01 [M-016536-01-1]; CA 5.7.1/02 [M-006914-01-1]) confirm spiroxamine is not a neurotoxicant, nor does it show structural relationship to known neurotoxicants (*e.g.*, organophosphates, carbamates). With the thyroid gland being fulcrum to neurodevelopmental effects, the extensive *in vivo* mammalian toxicity data pacakage confirms that spiroxamine is devoid of thyroid gland effects. Therefore, the need to investigate DNT effects are null and void in the respect of spiroxamine.

Toxicity studies on impurities

Refer to Doc J for assessment on impurities in the context of mammalian toxicology.

CA 5.8.3 Endocrine disrupting properties

A complete in vitro and in vivo test battery of endocrine disruption studies have been conducted on spiroxamine, with a summary an overview provided below. Spiroxmaine is not considered a endocrine drisruptor from the available mammalian data.

The potential of spiroxamine to interact with endocrine systems in mammals has been reviewed based on results from in vitro and mammalian toxicology studies. A WoE assessment for human health based on the 'Guidance for the identification of endocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009' published 2018 has been conducted.

Based on the available mammalian evidence, EATS-modalities were considered sufficiently investigated and, on the WoE, no adversity was consistently observed in the relevant studies. Therefore, spiroxamine does not meet the ED criteria for the T- and EAS-modalities, according to Scenario 1a and 1b, respectively.

T:	No indication that there are any adverse effects on the T-modality based upon an endocrine mode of action for spiroxamine that occur in the absence of overt toxicity. In addition to the apical studies, results from Tox21 EDSP TR indicate that spiroxamine is negative for T activity.
EAS:	Although sporadic effects were observed in *in vitro* mechanistic (decreased testosterone, progesterone, estradiol) and *in vivo* (delays in developmental milestones for males and females) studies, these effects were not deemed evidence of ED mediated effects. Where both male and female pup developmental milestone delays occurred, these were not driven by A- or E-modality involvement respectively, but rather secondary to maternal toxicity. In addition to the apical studies, results from ToxCast ER and AR indicate that spiroxamine is negative for E- and A- activity, with further Level 2/3 A-modality studies confirming a lack of A-modality involvement. *In vitro* data confirm that aromatase, the terminal enzyme in the steroidogenesis pathway is not inhibited. The reductions in steroid hormone production observed in the steroidogeneses data is attributed to upstream effects related to reduction in serum cholesterol levels, which impact upon the availability for this principal sterol feeding into the steroidogenesis pathway. Reductions in these steroid hormones observed *in vitro* are not manifest in the available, comprehensive apical *in vivo* mammalian toxicity data. Collectively, adrenal weights were unaffected in the apical toxicity studies, with no test article related effects observed upon adrenal gland histopathological analysis. Because steroidogenesis is among the functional roles of the adrenal gland, the absence of effects on this organ argues against spiroxamine having a potential steroidogenesis pathway interaction, *in vivo*. It is therefore concluded that spiroxamine is devoid effects on the EAS-modalities, with further Level 5 *in vivo* studies to address the *in vitro* steroidogenesis data not required.

Table CA 5.8.3-1: Summary of endocrine disruption studies conducted on spiroxamine

Type of study	Species	Doses	Key effects	Annex CA Point / Reference
E-modality				
US EPA ToxCast	ToxCast bioactivity ER model	n/a	No evidence of a direct interaction with oestrogenic or anti-oestrogenic pathway	CA 5.8.3/03 [M-762777-01-1]
A-modality				
US EPA ToxCast	ToxCast bioactivity AR model	n/a	No evidence of a direct interaction with androgenic or anti-androgenic pathway	CA 5.8.3/04 [M-762779-01-1]
In vitro androgen receptor and antagonist activity androgen receptor transcriptional activation assay	CHO-K1 cell line stably transfected with human AR expression vector	**AR agonist assay:**		CA 5.8.3/05 [M-761548-01-1]
		0.01, 0.1 1, 10, 100 nM, 1, 3.16 µM	No evidence of androgen receptor agonist activity	
		AR antagonist assay:		
		0.1, 1, 10, 100 nM, 1, 3.16 µM	No evidence of androgen receptor antagonist activity	
In vivo Hershberger DRF study	Young mature rats	0, 125, 250, 500 mg/kg bw/day	125 mg/kg bw/day deemed to be the MTD for the *in vivo* Hershberger assay (CA 5.8.3/07), with additional dose levels of 31.25 and 62.5 mg/kg bw/day selected	CA 5.8.3/06 [M-761549-01-1]
In vivo Hershberger study	Castrated young mature rats	0, 31.25, 62.5, 125 mg/kg w/day	No potency to exhibit androgenic, anti-androgenic or 5α-reductase inhibitory properties	CA 5.8.3/07 [M-764008-01-1]
T-modality				
US EPA ToxCast	HEK293T cells	n/a	No evidence of a direct interaction with thyroid pathway	CA 5.8.3/08 [M-762778-01-1]
S-modality				
US EPA ToxCast	H295 cells	n/a	Inhibition of mineralocorticoids (11-deoxycorticosterone, progesterone); glucocorticoids (11-deoxycortisol, 17α-hydroxyprogesterone) androgens (androstenedione) and testosterone	CA 5.8.3/09 [M-762780-01-1]
In vitro inhibition assay with human recombinant aromatase	Human recombinant microsomes containing CYP19 and P450 reductase	0.01, 0.1, 1, 10, 100 µM	Spiroxamine is deemed to not inhibit aromatase activity	CA 5.8.3/01 [M-301971-01-1]
In vitro steroidogenesis assay	*Ex vivo* in rat testis homogenate	1, 100 µM	Spiroxamine is deemed to not inhibit steroidogenesis when examining testosterone production	CA 5.8.3/02 [M-303122-01-1]

Type of study	Species	Doses	Key effects	Annex CA Point / Reference
In vitro steroidogenesis assay	H295R human adrenocortical carcinoma cells	Expt 1: : 0, 0.0001, 0.001, 0.01, 0.1, 1, 10, 31.6 µM Exp 2 & 3: 0, 0.001, 0.01, 0.1, 0.316, 1, 3.16, 10 µM	New steroidogenesis assay	CA 5.8.3/10; [M-764156-01-1]

E-modality

Data Point:	KCA 5.8.3/03
Report Author:	[redacted]
Report Year:	2021
Report Title:	Spiroxamine: US EPA ToxCast data retrieval for the E-modality
Report No:	0471836-TOX5
Document No:	M-762777-01-1
Guideline(s) followed in study:	None
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	Yes

Executive Summary

Using the computational network model to determine a composite value that takes all oestrogen receptor (ER)-related assays into account to assess the *in vitro* oestrogenicity of a chemical, this model evaluated that spiroxamine was not predicted to act as an oestrogen or anti-oestrogen. The data that underpins the prediction for spiroxamine are results from 17 ER ToxCast high-throughput screening (HTS) assays that discriminate bioactivity from assay-specific interference and cytotoxicity.

The ToxCast model scores range from 0 (no activity) to 1 (bioactivity of 17β-estradiol). ToxCast ER model performance was evaluated for reference chemicals, as well as results of EDSP Tier 1 screening assays in current practice. The ToxCast ER model accuracy was 86% to 93% when compared to reference chemicals and predicted results of EDSP Tier 1 guideline and other uterotrophic studies with 84% to 100% accuracy. The performance of high-throughput assays and ToxCast ER model predictions demonstrates that these methods correctly identify active and inactive reference chemicals, provide a measure of relative ER bioactivity, and rapidly identify chemicals with potential endocrine bioactivities for additional screening and testing. EPA is accepting ToxCast ER model data for 1812 chemicals as alternatives for EDSP Tier 1 ER binding, ER transactivation, and uterotrophic assays.

Of the HTS assay, only OT_ER_ERaERa_1440 a protein stabilisation assay examining receptor function and kinetics of the human oestrogen receptor 1 in a kidney cell line (HEK293T) following 24 hours exposure showed activity with an AC50 value of 36.05 µM obtained. However, the biological

relevance of this is low as activity was observed only at a concentration that occurred in the presence of cytotoxic, which was observed at 15.32 µM.

In conclusion, negative results in all ER-associated assays and negative results in the ER network model provides a strong mechanistic argument against a direct interaction of spiroxamine with oestrogenic or anti-oestrogenic pathways. In accordance with the ECHA guidance on endocrine disruption, the ToxCast bioactivity ER model detailing an absence of oestrogenic effects is deemed sufficient to address the E-modality.

Results

Table CA 5.8.3/03-1: Overview of ToxCast models and data output for the E-modality

ToxCast model	Assay description	E / A-E[a]	Chosen model and outputs	Figure
Data retrieved from US EPA Endocrine: EDSP universe of chemicals website (https://comptox.epa.gov/dashboard/dsstoxdb/results?abbreviation=EDSPUOC&search=DTXSID1034232#details) 12 August 2020				
ToxCast bioactivity ER model	The publications associated with the ToxCast Pathway Models are: ESTROGEN: As described in Browne, *et al* (2015) DOI: 10.1021/acs.est.5b02641 and presented to December 2014 FIFRA SAP under EPA-HQ-OPP-2014-0614.	E/A-E	No oestrogen agonist activity No oestrogen antagonist activity	CA 5.8.3/03-1
ACEA_ER_80hr	Cell proliferation assay that measures time-dependent cell growth using impedance using the ER-responsive T-47D breast cancer cells.	E	Constant model. AIC: 125.77; RMSE: 11.27; AC_{50}: n/a Cytotoxicity: 15.32 µM	CA 5.8.3/03-2
ATG_ERa_TRANS_up	Protein stabilisation assays, examining a mRNA reporter sequence unique to the transfected trans-acting reporter gene and exogenous transcription factor GAL4-ERα induction in HepG2 cells	E	Hill model. AIC: -4.97; RMSE: 0.1; Top: 0.68; AC_{50} 8.09 µM; Cytotoxicity: 15.32 µM	CA 5.8.3/03-3
ATG_ERE_CIS_up	protein stabilisation assays, examining a mRNA reporter sequence unique to the transfected cis-acting reporter gene and exogenous transcription factor GAL4-ERα induction in HepG2 cells	E	Gain-loss model: AIC: -12; RMSE: 0.06; Top: 0.40; AC_{50} 0.5 µM; Cytotoxicity: 15.32 µM	CA 5.8.3/03-4
NVS_NR_bER	Receptor binding assay in bovine uterine membranes examining receptor-ligand binding of the key ligand [[^{3}H]-oestradiol] to cattle oestrogen receptor 1	E/A-E	Constant model. AIC: 62.37; RMSE: 10.1; AC_{50}: n/a Cytotoxicity: 15.32 µM	CA 5.8.3/03-5
NVS_NR_hER	Human extracted gene proteins from MCF7 breast cancer cell line receptor-ligand binding of the key ligand [[^{3}H]-oestradiol] to human oestrogen receptor 1 (ER-α)	E/A-E	Constant model. AIC: 103.94; RMSE: 7.3; AC_{50}: n/a Cytotoxicity: 15.32 µM	CA 5.8.3/03-6
OT_ER_ERaERa_0480	Protein stabilisation assay conducted in HEK293T kidney cell line following 8 h exposure examining receptor function and kinetics for the human oestrogen receptor 1 and 2.	E/A-E	Constant model. AIC: 89.29; RMSE: 3.2; AC_{50}: n/a Cytotoxicity: 15.32 µM	CA 5.8.3/03-7
OT_ER_ERaERa_1440	Protein stabilisation assay conducted in HEK293T kidney cell line following 24 h exposure examining receptor function and kinetics for the human oestrogen receptor 1 and 2.	E/A-E	Hill model: AIC: 59.43; RMSE: 1.08; Top: 2.23; AC_{50} 36.05 µM; Cytotoxicity: 15.32 µM	CA 5.8.3/03-8
OT_ER_ERaERb_0480	Protein stabilisation assay conducted in HEK293T kidney cell line following 8 h exposure examining receptor function and kinetics for the human oestrogen receptor 1 and oestrogen receptor 2 (ER-β)	E/A-E	Constant model. AIC: 115.62; RMSE: 5.58; AC_{50}: n/a Cytotoxicity: 15.32 µM	CA 5.8.3/03-9

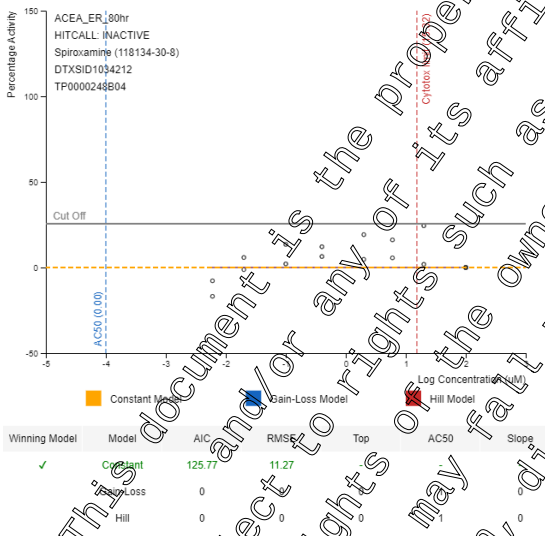
ToxCast model	Assay description	E / A-E[a]	Chosen model and outputs	Figure
OT_ER_ERaERb_1440	Protein stabilisation assay conducted in HEK293T kidney cell line following 24 h exposure examining receptor function and kinetics for the human oestrogen receptor 1 and oestrogen receptor 2 (ER-β)	E/A-E	Hill model. AIC: 87.65; RMSE: 2.92; Top: 33.48; AC_{50} 32.84 µM; Cytotoxicity: 15.32 µM	CA 5.8.3/03-10
OT_ER_ERbERb_0480	Protein stabilisation assay conducted in HEK293T kidney cell line following 8 h exposure examining receptor function and kinetics for the human oestrogen receptor 2 (ER-β)	E/A-E	Constant model. AIC: 89.09; RMSE: 2.84; AC_{50}: n/a Cytotoxicity: 15.32 µM	CA 5.8.3/03-11
OT_ER_ERbERb_1440	Protein stabilisation assay conducted in HEK293T kidney cell line following 24 h exposure examining receptor function and kinetics for the human oestrogen receptor 2 (ER-β)	E/A-E	Gain-loss model. AIC: 74.05; RMSE: 1.42; Top: 8.3; AC_{50} 29.97 µM; Cytotoxicity: 15.32 µM	CA 5.8.3/03-12
OT_ERa_EREGFP_0120	Gene expression assay in human cervix cell line. 2 h exposure	E/A-E	Gain-loss model. AIC: 65.09; RMSE: 1.07; Top: 7.19; AC_{50} 0.19; Cytotoxicity: 15.32 µM	CA 5.8.3/03-13
OT_ERa_EREGFP_0480	Gene expression assay in human cervix cell line. 8 h exposure	E/A-E	Gain-loss model. AIC: 47.95; RMSE: 0.65; Top: 4.29; AC_{50} 0.77; Cytotoxicity: 15.32 µM	CA 5.8.3/03-14
Tox21_ERa_BLA_Agonist_ratio	Inducible ERα transcription factor activity detected by ß-lactamase oestrogen response element reporter in agonist mode using HEK293T, a human kidney cell line. 24 h exposure	E/A-E	Constant model. AIC: 151.85; RMSE: 1.23; AC_{50}: n/a Cytotoxicity: 15.32 µM	CA 5.8.3/03-15
Tox21_ERa_BLA_Antagonist_ratio	Inducible ERα transcription factor activity detected by ß-lactamase oestrogen response element reporter in antagonist mode using HEK293T, a human kidney cell line. 24 h exposure	E/A-E	Constant model. AIC: 275.52; RMSE: 5.2; AC_{50}: n/a Cytotoxicity: 15.32 µM	CA 5.8.3/03-16
TOX21_ERa_LUC_VM7_Agonist	Inducible ERα transcription factor activity detected by luciferase-coupled ATP quantitation in agonist mode using VM7, a human breast tissue cell line. 48 h exposure	E/A-E	Constant model. AIC: 231.79; RMSE: 3.26; AC_{50}: n/a Cytotoxicity: 15.32 µM	CA 5.8.3/03-17
TOX21_ERa_LUC_VM7_Antagonist_0.5nM_E2	Inducible ERα transcription factor activity detected by luciferase-coupled ATP quantitation in antagonist mode using VM7, a human breast tissue cell line. 48 h exposure	E/A-E	Constant model. AIC: 262.92; RMSE: 4.24; AC_{50}: n/a Cytotoxicity: 15.32 µM	CA 5.8.3/03-18

a: oestrogen (E) or anti-oestrogen (A-E) signal detection
AIC: Akaike Information criteria: appropriate model set on the lowest AIC value, essential model that best fits the data
RMSE: median root mean-squared error across all winning models. RMSE is lower for higher performance models

Figure CA 5.8.3/03-1:
ToxCast model

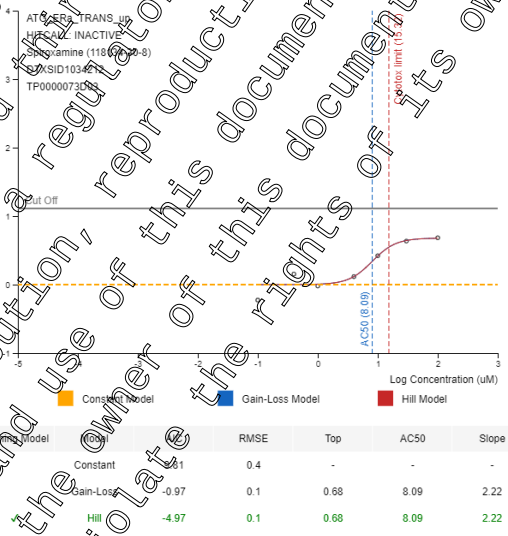
Model	Receptor	Agonist	Antagonist	Binding
ToxCast Pathway Model (AUC)	Androgen	0.00	0.00	-
ToxCast Pathway Model (AUC)	Estrogen	0.00	0.00	-
COMPARA (Consensus)	Androgen	Inactive	Inactive	Inactive
CERAPP Potency Level (From Literature)	Estrogen	Inactive (Inactive)	Inactive (Inactive)	Inactive (Inactive)
CERAPP Potency Level (Consensus)	Estrogen	Inactive (Inactive)	Inactive (Inactive)	Inactive (Inactive)

Figure CA 5.8.3/03-2:
ACEA_ER_80hr



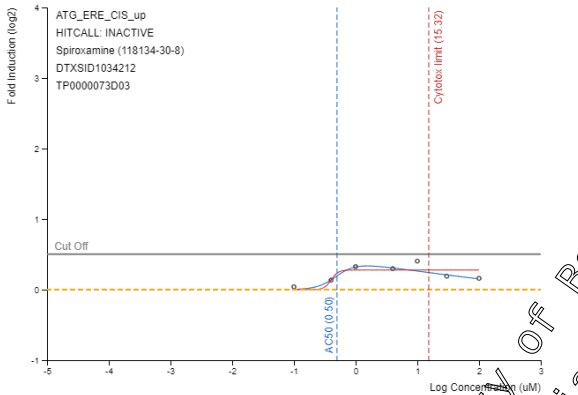
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	125.77	11.27	-	-	-
	Gain-Loss	0	0	0	0	0
	Hill	0	0	0	0	0

Figure CA 5.8.3/03-3:
ATG_ERa_TRANS_up



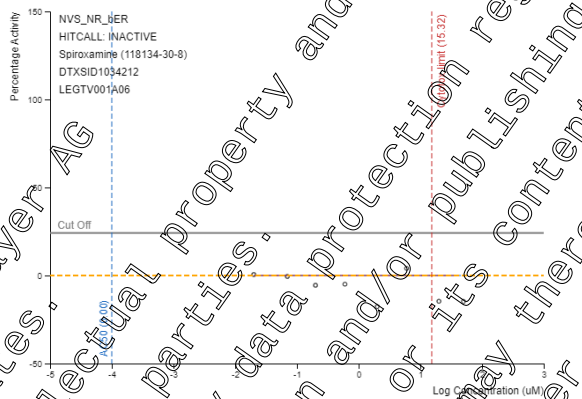
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
	Constant	3.31	0.4	-	-	-
	Gain-Loss	-0.97	0.1	0.68	8.09	2.22
✓	Hill	-4.97	0.1	0.68	8.09	2.22

Figure CA 5.8.3/03-4: ATG_ERE_CIS_up



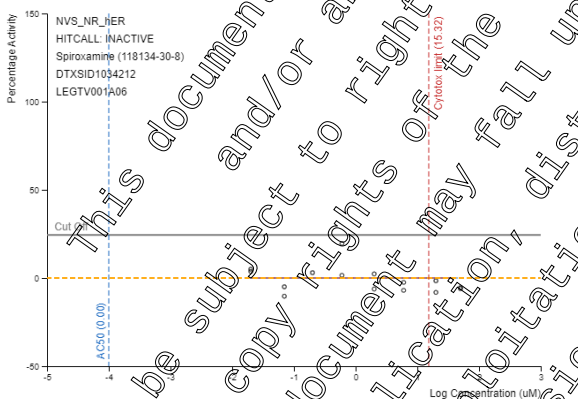
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
	Constant	3.61	0.25	-	-	-
✓	Gain-Loss	-12	0.06	0.49	0.5	2.84
	Hill	-7.17	0.08	0.28	0.4	8

Figure CA 5.8.3/03-5: NVS_NR_bER



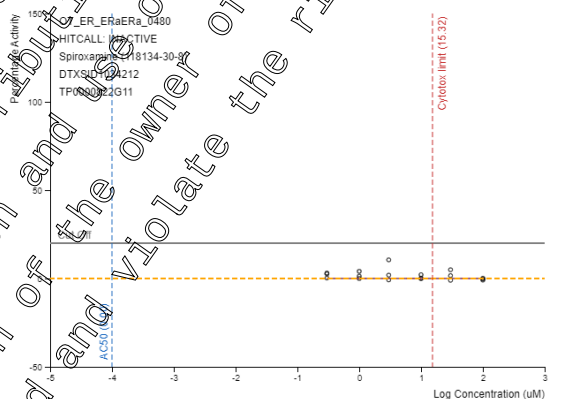
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	62.37	10.1	-	-	-
	Gain-Loss	72.3	10	0	0.45	7.76
	Hill	74.3	10	0	0.55	7.77

Figure CA 5.8.3/03-6: NVS_NR_hER



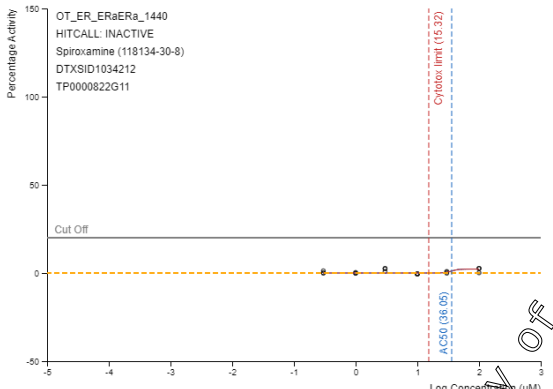
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	103.94	7.3	-	-	-
	Gain-Loss	0	0	0	1	0
	Hill	0	0	0	1	0

Figure CA 5.8.3/03-7: OT_ER_ERaERa_0480



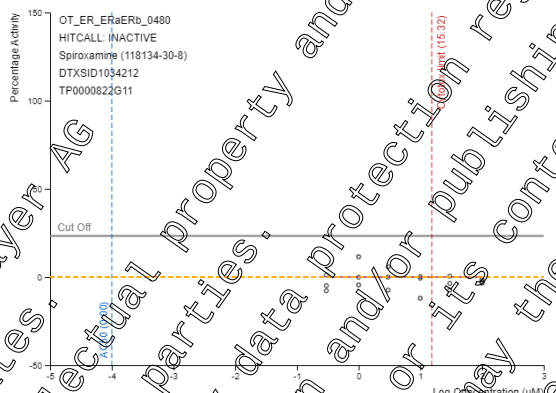
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	89.29	3.2	-	-	-
	Gain-Loss	0	0	0	1	0
	Hill	0	0	0	1	0

Figure CA 5.8.3/03-8:
OT_ER_ERaERa_1440



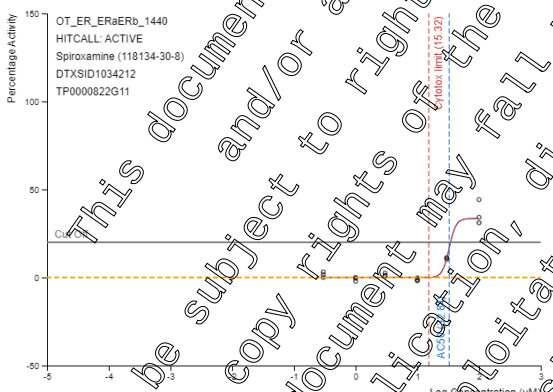
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
	Constant	61.36	1.3	-	-	-
	Gain-Loss	63.43	1.08	2.23	36.06	8
✓	Hill	59.43	1.08	2.23	36.05	8

Figure CA 5.8.3/03-9:
OT_ER_ERaERb_0480



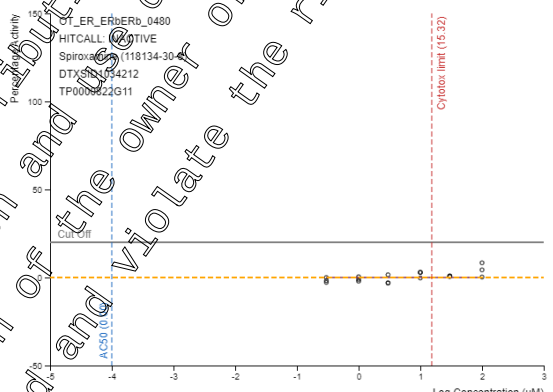
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	115.62	5.58	-	-	-
	Gain-Loss	0	0	0	1	0
	Hill	0	0	0	1	0

Figure CA 5.8.3/03-10:
OT_ER_ERaERb_1440



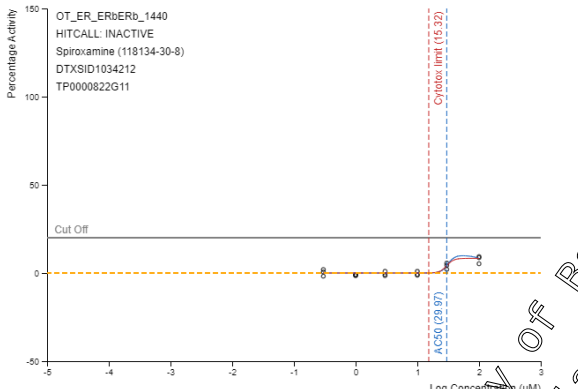
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
	Constant	145.65	15.78	-	-	-
	Gain-Loss	91.65	2.84	93.5	32.84	8
✓	Hill	87.65	2.84	93.5	32.84	8

Figure CA 5.8.3/03-11:
OT_ER_ERbERb_0480



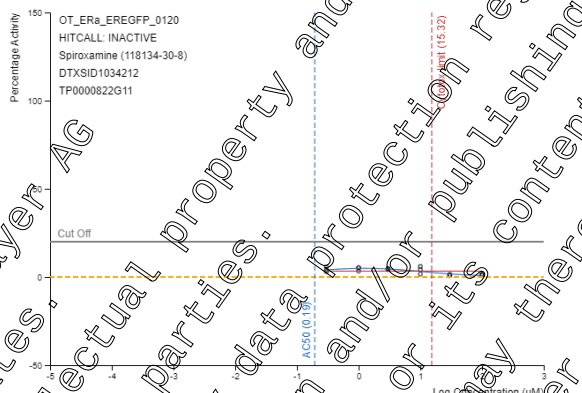
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	89.09	2.84	-	-	-
	Gain-Loss	0	0	0	1	0
	Hill	0	0	0	1	0

Figure CA 5.8.3/03-12:
OT_ER_ERbERb_1440



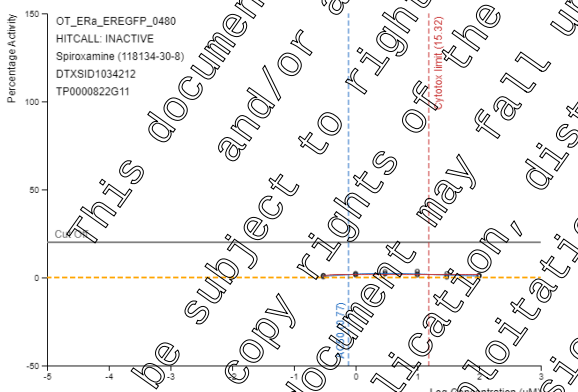
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
	Constant	99.67	3.83	-	-	-
	Gain-Loss	78.05	1.42	10.14	31.36	8
✓	Hill	74.05	1.42	8.3	29.97	8

Figure CA 5.8.3/03-13:
OT_ERa_EREGFP_0120



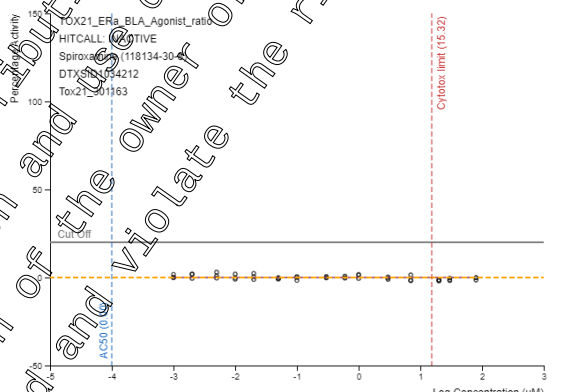
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
	Constant	103.4	3.73	-	-	-
✓	Gain-Loss	65.33	1.07	7.15	0.19	0.82
	Hill	62.05	1.04	7.15	0.19	4.09

Figure CA 5.8.3/03-14:
OT_ERa_EREGFP_0480



Winning Model	Model	AIC	RMSE	Top	AC50	Slope
	Constant	77.65	1.84	-	-	-
	Gain-Loss	47.93	0.8	6.29	0.7	1.3
	Hill	52.68			0.3	

Figure CA 5.8.3/03-15:
Tox21_ERa_BLA_Agonist_ratio



Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	151.85	1.23	-	-	-
	Gain-Loss	0	0	0	1	0
	Hill	0	0	0	1	0

Figure CA 5.8.3/03-16:
Tox21_ERa_BLA_Antagonist_ratio

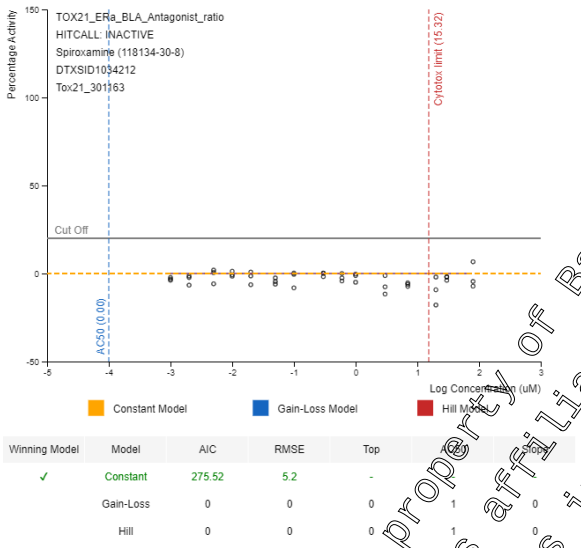


Figure CA 5.8.3/03-17:
TOX21_ERa_LUC_VM7_Agonist

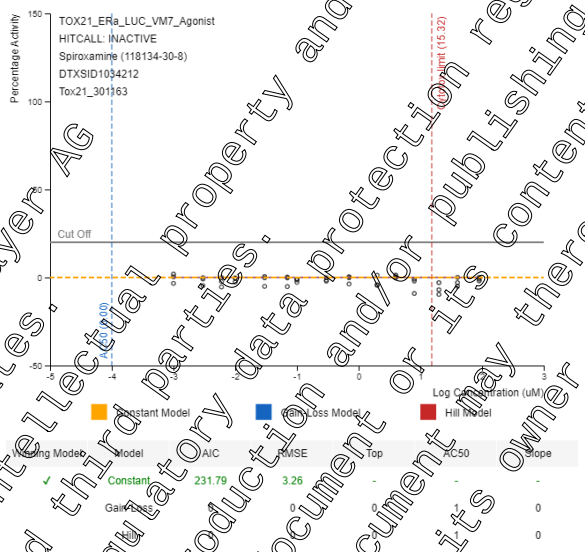
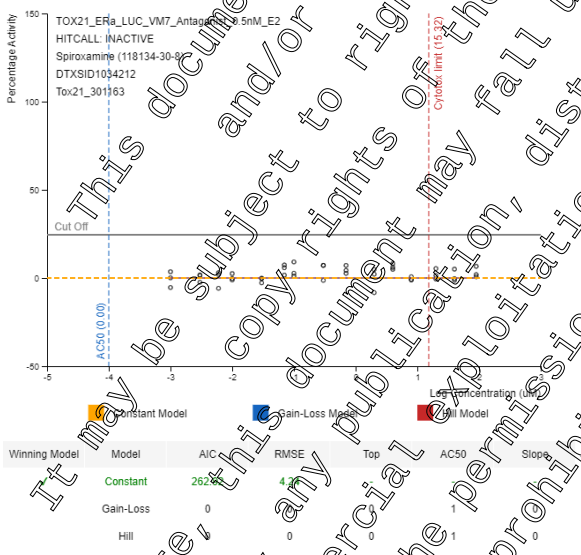


Figure CA 5.8.3/03-18:
TOX21_ERa_LUC_VM7_
Antagonist_0.5nM_E2



Of the HTS assays, only OT_ER_ERaERa_1440, a protein stabilisation assay examining receptor function and kinetics of the human oestrogen receptor 1 in a kidney cell line (HEK293T) following 24 hours exposure, showed activity with an AC50 value of 36.05 µM obtained. However, the biological relevance of this is low as activity was observed only at a concentration that occurred in the presence of cytotoxic, which was observed at 15.32 µM.

Assessment and conclusions by applicant:

Assessment: Study meets the current guidance and the requirements in 283/2013.

Conclusion: In conclusion, negative results in all ER-associated assays and negative results in the ER network model provides a strong mechanistic argument against a direct interaction of spiroxamine with oestrogenic or anti-oestrogenic pathways. In accordance with the ECHA guidance on endocrine disruption, the ToxCast bioactivity ER model detailing an absence of oestrogenic effects is deemed sufficient to address the E-modality

A-modality

Data Point:	KCA 5.8.3/04
Report Author:	[redacted]
Report Year:	2021
Report Title:	Spiroxamine: US EPA ToxCast data retrieval for the A-modality
Report No:	0471836-TOX6
Document No:	M-762779-01-1
Guideline(s) followed in study:	None
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted -
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	Supportive only

Executive Summary

Using the computational network model to determine a composite value that takes all androgen receptor (AR)-related assays into account to assess the *in vitro* androgenicity of a chemical, this model evaluated that spiroxamine was not predicted to act as an androgen or anti-androgen. The data that underpins the prediction for spiroxamine are results from 10 AR ToxCast high-throughput screening (HTS) assays that discriminate bioactivity from assay-specific interference and cytotoxicity.

The ToxCast model scores range from 0 (no activity) to 1 (bioactivity of testosterone propionate). ToxCast ER model performance was evaluated for reference chemicals, as well as results of EDSP Tier 1 screening assays in current practice. The ToxCast ER model accuracy was 95.2% for agonist and 97.5% for antagonist reference chemicals. Out of 1855 chemicals screened in the AR pathway model, 220 chemicals demonstrated AR agonist or antagonist activity and an additional 174 chemicals were predicted to have potential weak AR pathway activity.

Of the HTS assay, only OT_AR_ARSRC1_0960 a luciferase protein-fragment complementation assay measuring AR binding to cofactor SRC1 detected by microscopy in HeLA cells following 24 hours exposure showed activity with an AC50 value of 48.74.05 µM obtained. However, the biological relevance of this is low as activity was observed only at a concentration that occurred in the presence of cytotoxic, which was observed at 15.32 µM.

In conclusion, negative results in all AR-associated assays and negative results in the AR network model provides a strong mechanistic argument against a direct interaction of spiroxamine with androgenic or anti-androgenic pathways. In accordance with the ECHA guidance on endocrine disruption, the ToxCast bioactivity AR model detailing an absence of androgen effects is not deemed sufficient to address the A-modality. Consequently both Level 2 and Level 3 studies have been conducted to conclusively address this endpoint.

Results

Table CA 5.8.3/04-1: Overview of ToxCast models and data output for the A-modality

ToxCast model	Assay description	A / A-A[a]	Chosen model and outputs	Figure
Data retrieved from US EPA Endocrine: EDSP universe of chemicals website (https://comptox.epa.gov/dashboard/dsstoxdb/results?abbreviation=EDSPUOC&search=DTXSID1034212#details) 12 August 2020				
ToxCast bioactivity AR model	The publications associated with the ToxCast AR Pathway Model is described in Kleinstreuer et al (2017) 10.1021/acs.chemrestox.6b00347 and presented to November 2017 FIFRA SAP under EPA-HQ-OPP-2017-0214	A/A-A	No androgen agonist activity No androgen antagonist activity	CA 5.8.3/04-1
NVS_NR_hAR	Receptor binding assay conducted in human extracted gene-proteins from LNCaP human prostate adenocarcinoma cell line receptor-ligand binding of the key ligand [[^{3}H]-methyltrienolone] to the human androgen receptor	A/A-A	Constant model. AIC: 51.79; RMSE: 5.08; AC_{50}: n/a Cytotoxicity: 15.32 µM	CA 5.8.3/04-2
OT_AR_ARELUC_AG_1440	Luciferase protein-fragment complementation assay measuring AR binding to the androgen response element detected in CHO-K1 cells by microscopy following a 24 h exposure	A/A-A	Constant model. AIC: 126.63; RMSE: 7.24; AC_{50}: n/a Cytotoxicity: 15.32 µM	CA 5.8.3/04-3
OT_AR_ARSRC1_0480	Luciferase protein-fragment complementation assay measuring AR binding to cofactor SRC1 detected by microscopy in HeLA cells following a 8 h exposure	A/A-A	Constant model. AIC: 94.02; RMSE: 2.98; AC_{50}: n/a Cytotoxicity: 15.32 µM	CA 5.8.3/04-4
OT_AR_ARSRC1_0960	Luciferase protein-fragment complementation assay measuring AR binding to cofactor SRC1 detected by microscopy in HeLA cells following a 16 h exposure	A/A-A	Hill model: AIC: 110; RMSE: 20.27; Top: 2.23; AC_{50} 36.05 µM; Cytotoxicity: 15.32 µM	CA 5.8.3/04-5
Tox21_AR_BLA_Agonist_ratio	Inducible AR transcription factor activity detected by ß-lactamase androgen response element reporter in agonist mode using HEK293T, a human kidney cell line following a 24 h exposure	A	Constant model. AIC: 251.68; RMSE: 4.05; AC_{50}: n/a; Cytotoxicity: 15.32 µM	CA 5.8.3/04-6
Tox21_AR_BLA_Antagonist_ratio	Inducible AR transcription factor activity detected by ß-lactamase androgen response element reporter in antagonist mode using HEK293T, a human kidney cell line following a 24 h exposure	A	Constant model. AIC: 302.79; RMSE: 7.35; AC_{50}: n/a Cytotoxicity: 15.32 µM	CA 5.8.3/04-7
Tox21_AR_LUC_MDAKB2_Agonist	Inducible AR transcription factor activity detected by luciferase-androgen response element report in agonist mode using MDA cells following a 24 h exposure	A	Constant model. AIC: 140.55; RMSE: 1.16; AC_{50}: n/a Cytotoxicity: 15.32 µM	CA 5.8.3/04-8
Tox21_AR_LUC_MDAKB2_Antagonist	Inducible AR transcription factor activity detected by luciferase-androgen response element report in antagonist mode using MDA cells following a 24 h exposure	A	Constant model. AIC: 293.65; RMSE: 6.7; AC_{50}: n/a Cytotoxicity: 15.32 µM	CA 5.8.3/04-9

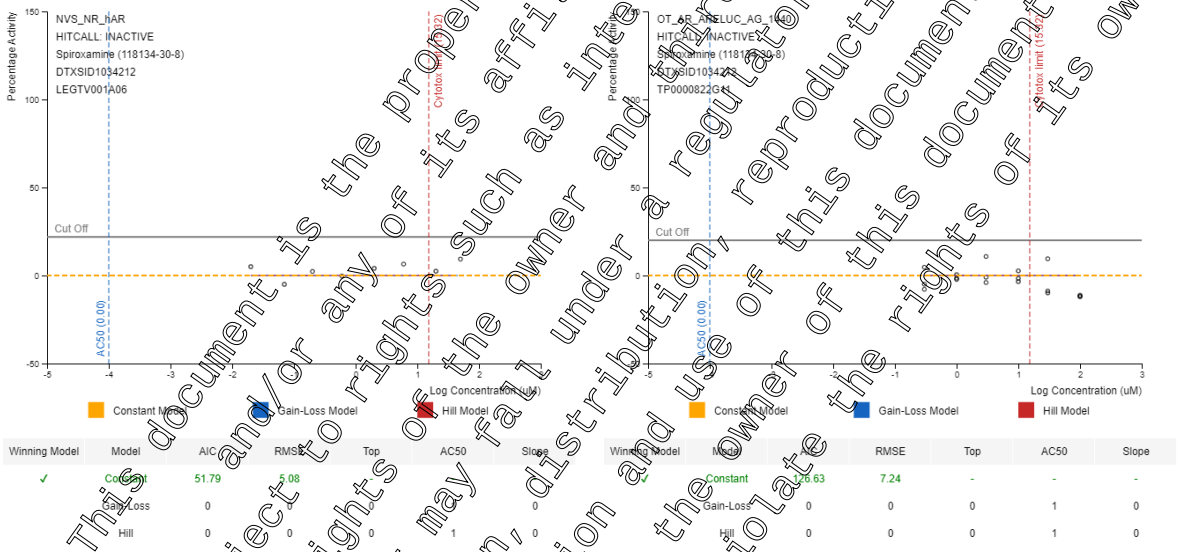
ToxCast model	Assay description	A / A-A[a]	Chosen model and outputs	Figure
UPITT_HCI_U2OS_AR_TIF2_Nucleoli_Agonist	Receptor binding assay conducted in U2OS, a human osteosarcoma cell line, expressing a human androgen receptor fused to red fluorescent protein and the coactivator TIF2 fused to green fluorescent protein in agonist mode. Cells exposure for 3 h.	A	Constant model. AIC: 139.28; RMSE: 7.01; AC_{50}: n/a Cytotoxicity: 15.32 µM	CA 5.8.3/04-10
UPITT_HCI_U2OS_AR_TIF2_Nucleoli_Antagonist	Receptor binding assay conducted in U2OS, a human osteosarcoma cell line, expressing a human androgen receptor fused to red fluorescent protein and the coactivator TIF2 fused to green fluorescent protein in antagonist mode. Cells exposure for 3 h.	A	Constant model. AIC: 151.15; RMSE: 10.55; AC_{50}: n/a Cytotoxicity: 15.32 µM	CA 5.8.3/04-11

a: androgen (A) or anti-androgen (A-A) signal detection
AIC: Akaike Information criteria: appropriate model set on the lowest AIC value, essential model that best fits the data
RMSE: median root mean squared error across all winning models. RMSE is lower for higher performance models

Figure CA 5.8.3/03-1:
ToxCast model

Model	Receptor	Agonist	Antagonist	Binding
ToxCast Pathway Model (AUC)	Androgen	0.00	0.00	-
ToxCast Pathway Model (AUC)	Estrogen	0.00	0.00	-
COMPARA (Consensus)	Androgen	Inactive	Inactive	Inactive
CERAPP Potency Level (From Literature)	Estrogen	Inactive (Inactive)	Inactive (Inactive)	Inactive (Inactive)
CERAPP Potency Level (Consensus)	Estrogen	Inactive (Inactive)	Inactive (Inactive)	Inactive (Inactive)

Figure CA 5.8.3/04-2:
NVS_NR_hAR

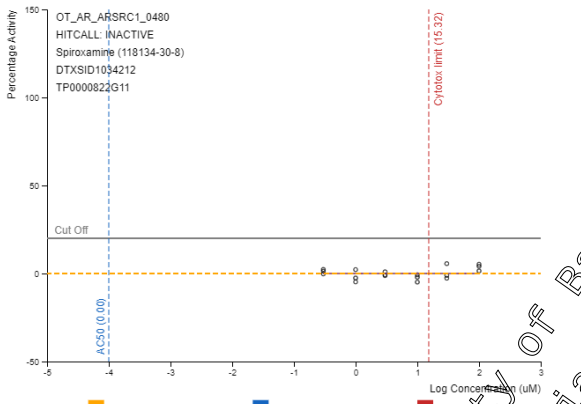


Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	51.79	5.08	-	-	-
	Gain-Loss	0	0	0	0	0
	Hill	0	0	0	1	0

Figure CA 5.8.3/04-3:
OT_AR_ARELUC_AG_1440

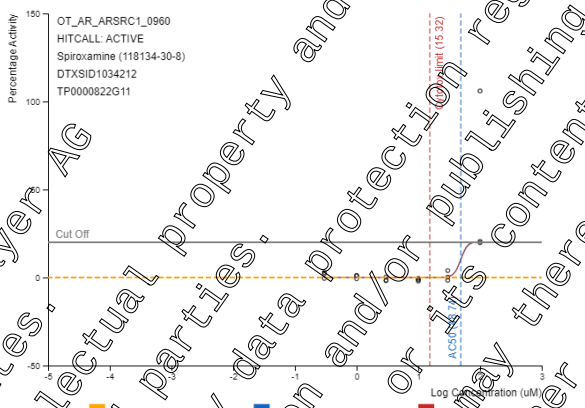
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	178.63	7.24	-	-	-
	Gain-Loss	0	0	0	1	0
	Hill	0	0	0	1	0

Figure CA 5.8.3/04-4:
OT_AR_ARSRC1_0480



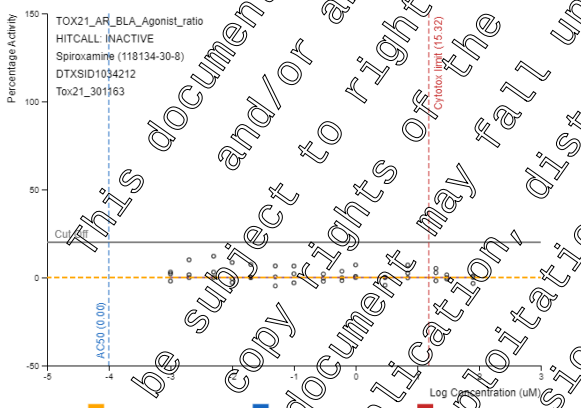
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	94.02	2.98	-	-	-
	Gain-Loss	0	0	0	1	0
	Hill	0	0	0	1	0

Figure CA 5.8.3/04-5:
OT_AR_ARSRC1_0960



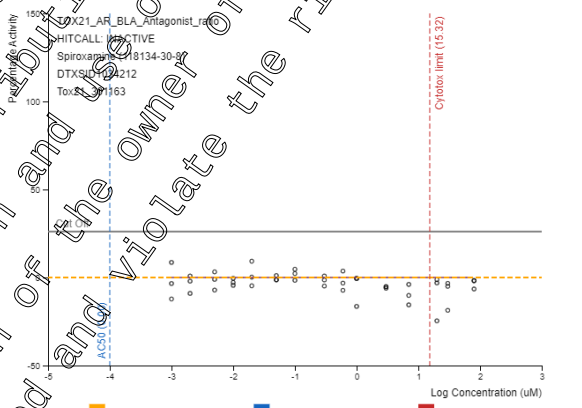
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
	Constant	136.14	25.9	-	-	-
	Gain-Loss	1[illegible]	20.[illegible]	21.[illegible]	49	8
✓	[illegible]	[illegible]	[illegible]	[illegible]	49.7	8

Figure CA 5.8.3/04-6:
Tox21_AR_BLA_Agonist_ratio



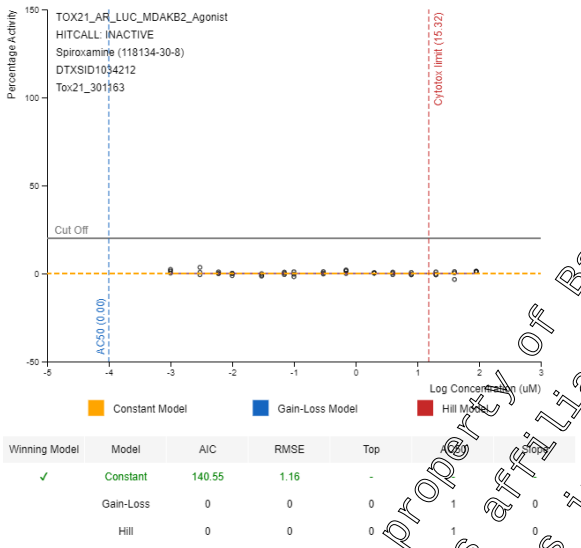
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	251.68	4.05	-	-	-
	Gain-Loss	0	0	0	1	0
	Hill	0	0	0	1	0

Figure CA 5.8.3/04-7:
Tox21_AR_BLA_Antagonist_ratio



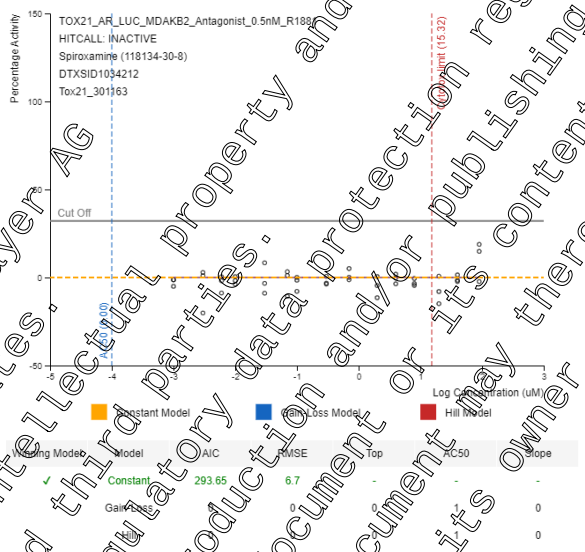
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	302.79	7.35	-	-	-
	Gain-Loss	0	0	0	1	0
	Hill	0	0	0	1	0

Figure CA 5.8.3/04-8:
Tox21_AR_LUC_MDAKB2_Agonist



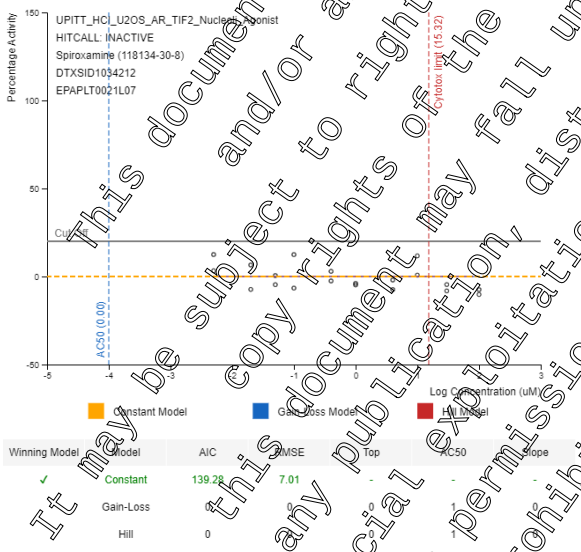
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	140.55	1.16	-	-	-
	Gain-Loss	0	0	0	1	0
	Hill	0	0	0	1	0

Figure CA 5.8.3/04-9:
Tox21_AR_LUC_MDAKB2_Antagonist



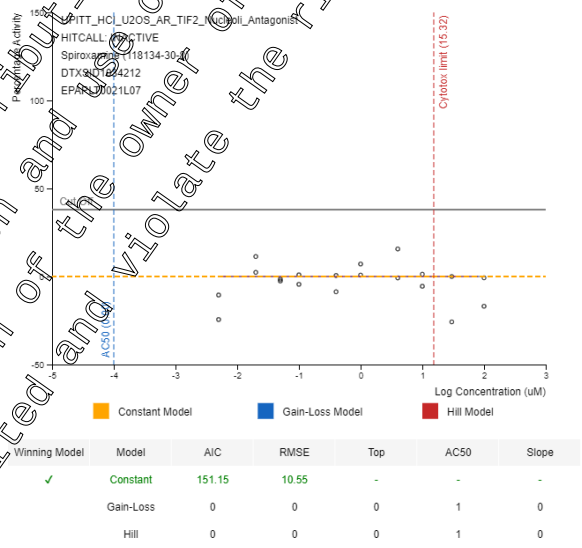
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	293.65	6.7	-	-	-
	Gain-Loss	0	0	0	1	0
	Hill	0	0	0	1	0

Figure CA 5.8.3/03-10:
UPITT_HCI_U2OS_AR_TIF2_Nucleoli_Agonist



Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	139.28	7.01	-	-	-
	Gain-Loss	0	0	0	1	0
	Hill	0	0	0	1	0

Figure CA 5.8.3/03-11:
UPITT_HCI_U2OS_AR_TIF2_Nucleoli_Antagonist



Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	151.15	10.55	-	-	-
	Gain-Loss	0	0	0	1	0
	Hill	0	0	0	1	0

Of the HTS assays, only OT_AR_ARSRC1_0960 a luciferase protein-fragment complementation assay measuring AR binding to cofactor SRC1 detected by microscopy in HeLA cells following 24 hours exposure showed activity with an AC_{50} value of 36.05 µM obtained. However, the biological relevance of this is low as activity was observed only at a concentration that occurred in the presence of cytotoxic, which was observed at 15.32 µM.

Assessment and conclusions by applicant:

Assessment: This study is considered supplemental only.

Conclusion: In conclusion, negative results in all AR-associated assays and negative results in the AR network model provides a strong mechanistic argument against a direct interaction of spiroxamine with androgenic or anti-androgenic pathways. In accordance with the ECHA guidance on endocrine disruption, the ToxCast bioactivity AR model detailing an absence of androgen effects is not deemed sufficient to address the A-modality. Consequently both Level 2 and Level 3 studies have been conducted to conclusively address this endpoint.

Data Point:	KCA 5.8.3/05
Report Author:	[redacted]
Report Year:	2021
Report Title:	Evaluation of the androgen receptor agonist and antagonist activity of spiroxamine using the stably transfected human androgen receptor transcriptional activation assay (AR-EcoScreen™)
Report No:	20249038
Document No:	M-761548-01-1
Guideline(s) followed in study:	OECD 458 (2020)
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

In an *in vitro* stably transfected human androgen receptor transcriptional activation assay examining both AR agonist and antagonist activity. The study consisted of a solubility test followed by two independent AR agonist and AR antagonist experiments.

The test article was formulated in dimethyl sulphoxide (DMSO) and dosed at 0.1% v/v, with a maximum exposure concentration of 3.16 µM limited by solubility in the test system. Cytotoxicity was evaluated by determining the *Renilla* luciferase activity in the AR antagonist assay experiments, with no cytotoxicity observed up to 3.16 µM.

In the two valid AR agonist assay experiments spiroxamine was tested at seven concentrations ranging from 10 pM to 3.16 together with vehicle controls, positive controls and complete concentration-response curves of the control items 5α-dihydrotestosterone (DHT), mestanolone and di(2-ethylhexyl)phthalate (DEHP).

All assay acceptability criteria were met with exception of the log PC_{10} and log PC_{50} values in the first valid experiment and the log PC_{50} value in second valid experiment obtained for mestanolone. Since these values were lower than the acceptance criteria (PC_{10} and PC_{50} responses at lower concentrations) this indicated sufficient responsiveness and therefore these data were accepted. As the control items were correctly classified as positive (DHT and mestanolone) or negative (DEHP), both AR agonist assay experiments were considered valid.

The maximum level of response induced by spiroxmaine compared to the response induced by 10 nM DHT (the RPC_{max}) was 0.0% and -0.6% in each valid experiment. Since the RPC_{max} values were below 10% in both independent experiments, spiroxamine was considered to be devoid of AR agonist activity, *in vitro*.

In the two valid AR antagonist assay experiments spiroxamine was tested at seven concentrations ranging from 100 pM to 3.16 together with vehicle controls, positive controls and complete concentration-response curves of the control items hydroxyflutamide (HF), bisphenol A (BPA) and DEHP. All assay acceptability parameters were met and the control items were correctly classified as positive (HF and BPA) or negative (DEHP). Therefore, both AR antagonist assay experiments were considered valid.

In both valid experiments, no log IC_{30} could be determined for spiroxamine. As such, spiroxamine was concluded to be devoid of AR antagonist activity, *in vitro*.

It is concluded that spiroxamine did no show evidence of either androgen receptor agonist or antagonist activity in the Stably Transfected Human Androgen Receptor Transcriptional Activation Assay (AR-EcoScreen™) following two independent experiments. Maximum concentrations analysed were limited by solubility, in line with current regulatory guidelines for this assay.

Materials and Methods

A. Materials:

1. Test Material:	Spiroxamine (alternative name: [8-(1,1-dimethylethyl)-N-ethyl-N-propyl-1,4-dioxaspiro[4,5]decane-2-methanamine; KWG 4168)
Description:	Clear light yellow liquid
Lot/Batch No.:	EDTH0114499
Purity:	97.0% (w/w) (correction for purity not undertaken)
CAS No.:	118134-30-8
Stability of test compound:	Confirmed stable for the duration of the study (expiry date, 4 June 2021)
2. Vehicle and/or positive control:	Vehicle control: DMSO, Negative control: AR agonist and antagonist assay: bis-(2-ethylhexyl)-phthalate (DEHP, 10, 100 (pM), 1, 10, 100 (nM), 1, 10 (µM)) Positive controls: AR agonist assay: - 5α-dihydrotestosterone (DHT, 100 fM, 1, 10, 100 pM, 1, 10, 100 nM); - 17α-methylandrostan-17-β-ol-3-one (mestanolone, 100 *f*M, 1, 10, 100 pM, 1, 10, 100 nM); AR antagonist assay: - hydroxyflutamide (HF, 100 pM, 1, 10, 100 nM, 1, 10 µM), - bisphenol A (BPA, 100 pM, 1, 10, 100 nM, 1, 10 µM)
3. Test system:	AR-EcoScreen™ cell line, derived from CHO-K1 cell line stably transfected with human AR expression vector and a firefly luciferase reporter vector bearing four tandem repeats of androgen responsive element (ARE) from prostate C3 gene-responsive element driven by a minimal heat shock protein promoter. In addition, a *Renilla* luciferase reporter construct under the SV40 promotor, stably and non-inducibly expressed, is transfected as to distinguish pure antagonism from a cytotoxicity-related decrease of luciferase activity. The cell line was sourced from Health Science Research Resources Bank (HSRRB), Osaka Japan and stored frozen. Cells were regularly checked for mycoplasma contamination.
4. Cell culture conditions:	Dulbecco's Modified Eagle Medium/Ham's F12 nutrient mix (DMEM/F12) without phenol red, supplemented with 5% fetal bovine serum (FBS), 100 U/mL Penicillin, 100 µg/mL Streptomycin, 100 µg/mL Hygromycin B and 200 µg/mL Zeocin.

For seeding into a 96-well plate, DMEM/F12 without phenol red, supplemented with 5% charcoal stripped FBS, 100 U/mL Penicillin and 100 µg/mL Streptomycin.

For exposure, DMEM/F12 without phenol red was used.

6. Test concentrations: *AR agonist assay*: 0, 10, 100 (pM), 1, 10, 100 (nM), 1, 3.16 µM

AR antagonist assay: 0, 100 pM, 1, 10, 100 (nM), 1, 3.16 µM

B. Test Performance:

1. In life dates: 18 August 2020 to 16 October 2020 (experimental dates)

2. Vehicle selection: The test article was soluble in DMSO at a concentration of 1 M (inspection under the microscope). However, a 10-fold dilution of this 1 M solution and 316 mM, 100 mM, 31.6 mM and 10 mM in exposure medium resulted in precipitation of the test article. A 10-fold dilution of a 3.16 mM solution of the test article in exposure medium resulted in a clear solution. This concentration was used as the highest test article spiking concentration in the first main experiments (final concentration in the well: 3.16 µM) based on 0.1% additions into the test system.

3. Statistics: No statistics performed.

4. Acceptance criteria: *AR agonist assay:*

Considered acceptable if the results met the following criteria:

a) The mean luciferase activity of the positive control (10 nM DHT) was ≥6.4-fold that of the mean vehicle control on each plate.

b) The fold induction corresponding to the PC_{10} value of the concurrent positive control ($PC_{A@}$ 10 nM DHT) was greater than 1+2SD of the fold induction value of the concurrent vehicle control (which is set at 1).

c) The % co-efficient of variation (CV) of the raw data of triplicate wells (*i.e.* luminescence intensity data) was <20%.

d) The shape of the concentration-response curve of positive control items was sigmoidal.

e) The results of the three control items were within the acceptable range and classified in the correct class *i.e.*, negative or positive for androgenic activity.

Control Item	Log PC_{10} (M)	Log PC_{50} (M)	Purpose of Control Item
DHT	-12.08 ~ -9.87	-11.03 ~ -9.00	Positive
Mestanolone	-10.92 ~ -10.41	-10.15 ~ -9.26	Positive
DEHP		-	Negative

f) The results obtained were reproducible in at least one independent repeated experiment.

AR antagonist assay:

Considered acceptable if the results meet the following criteria:

a) In the antagonist assay the fold induction of AG ref should be ≥5.0.

b) The RTA of IC_{ATG} (1 µM HF) was ≤46%.

c) The % CV of the raw data of triplicate wells (*i.e.*, luminescence intensity data) was <20%.

d) The shape of the concentration-response curve of positive control items was sigmoidal.

e) The results of the three control items was within the acceptable range and classified in the correct class *i.e.*, negative or positive for anti-androgenic activity

Control Item	Log PC_{10} (M)	Log PC_{50} (M)	Purpose of Control Item
HF	-8.37 ~ -6.41	-7.80 ~ -6.17	Positive

BPA	-7.52 ~ -4.48	-7.05 ~ -4.29	Positive
DEHP	-	-	Negative

f) The results obtained were reproducible in at least one independent repeated experiment.

5. Evaluation criteria:

AR agonist assay:

- A test article was considered positive if the maximum response induced by the test article (RPC_{max}) was equal to or exceeded 10% of the response of the positive control (10 nM DHT) in at least two out of two or two out of three independent experiments.
- A test article was considered negative if the maximum response induced by the test article (RPC_{max}) failed to achieve at least 10% of the response of the positive control (10 nM DHT) in at least two out of two or two out of three independent experiments.

AR antagonist assay:

- A test article was considered positive if a log IC_{30} could be calculated in two out of two or two out of three experiments.
- A test article was considered negative if no log IC_{30} can be calculated in two out of two or two out of three experiments.

C. Methods:

1. Homogeneity and achieved concentration analysis of the dose:

Not undertaken.

2. AR agonist assay:

AR-EcoScreen™ cells were seeded (at 0.9×10^4 cells/90 µL/well) into a 96-well plate and cultured for 24 hours prior to treatment. Subsequently cells exposed for 24 hours to negative (DMSO, DEHP), positive controls for AR agonism (DHT, mestanolone), the positive control for cytotoxicity (cycloheximide), or seven test article concentrations all included on each plate.

The luciferase activity was determined using the Steady-Glo Luciferase assay system to evaluate AR-mediated transcriptional activation. After exposure, 40 µL of Steady-Glo reagent was added to each well of the 96-well plate, The plate was shaken and incubated for at least 5 minutes at room temperature in the dark. The luminescence intensity was measured using a luminometer (Infinite® M200 Pro, TECAN, Austria). Luminescence data was presented as Relative Light Units (RLU).

3. AR antagonist assay:

AR-EcoScreen™ cells were seeded (at 0.9×10^4 cells/90 µL/well) into a 96-well plate and cultured for 24 hours prior to treatment. Subsequently cells exposed for 24 hours to negative (DMSO, DEHP), positive controls for AR antagonism (BPA, HF) and AR agonist (DHT), the positive control for cytotoxicity (cycloheximide), or six test article concentrations all included on each plate.

The luciferase activity was determined using the Dual-Glo luciferase assay to evaluate AR-mediated transcriptional inhibition and cytotoxicity simultaneously.

After incubation, 40 µL of Dual-Glo reagent was added to each well of the 96 well plate. The plate was shaken, and after an incubation for at least 10 minutes at room temperature, the firefly luminescence intensity was measured using a luminometer. After the measurements, 60 µL of the medium was removed and 40 µL of Stop-Glo luciferase reagent was added to each well and the plate was shaken and incubated at room temperature for at least 10 minutes after which *Renilla* luminescence intensity was measured. Luminescence data will be presented as RLU

a) cytotoxicity assay:

Cytotoxicity was determined by reduction of the *Renilla* luciferase activity (in the AR antagonist assay). The cytotoxicity was calculated using the following formula:

$$\text{Cytotoxicity (\%)} = 100 - \frac{(\text{RLU of each well} - \text{Mean RLU of PC}_{CT})}{(\text{Mean RLU of AG ref} - \text{Mean RLU of PC}_{CT})} * 100$$

All test article doses that exhibit a reduction in *Renilla* luciferase activity of >20% were excluded from further evaluation.

4. Data analysis:

a) AR agonist assay:

The mean values (expressed RLU – relative light units) of the vehicle controls were calculated for each plate. All RLU values from a plate were normalised by subtracting the mean RLU of the vehicle control of the specific plate from the data from each well of the same plate. The mean (normalized) RLU values for the positive control (PC_{AGO}) were calculated. For each well of the plate, the normalized RLU value were divided by the mean value of the normalized positive control (PC_{AGO}) to calculate the relative transcriptional activity for that well compared to the positive control response (PC) = 100%). For each concentration group of the test article or control item, the mean value of transcriptional activity was calculated.

Fold-induction of PC_{AGO} were be calculated using the following equation:

$$\text{Fold induction of PC}_{AGO} = \frac{\text{Mean RLU of PC}_{AGO}\ (10\text{ nM DHT})}{\text{Mean RLU of VC}}$$

For each test and control item, the maximum level of response induced by the test item, expressed as a percentage against the response by PC_{AGO} on the same plate (RPC_{max}) was calculated. If applicable, the concentrations that induce an effect corresponding to that of a 10% effect of the positive control (log PC_{10}) and to 50% effect for the positive control (log PC_{50}) was determined.

b) AR antagonist assay:

The mean RLU values of the vehicle controls was calculated for each plate. All RLU values from a plate were normalised by subtracting the mean RLU of the vehicle control of the specific plate from the RLU data from each well of the same plate. The mean (normalized) RLU values for the spike-in control (500 pM DHT) was calculated (=normalized AG ref). For each well of the plate, the normalized value was divided by the mean value of the normalized AG ref (AG ref is set to 100%). The mean RTA was calculated for each concentration group of control or proficiency test item.

The fold induction of AG ref was calculated by the following equation:

$$\text{Fold induction of AG ref} = \frac{\text{Mean RLU of AG ref (500 pM DHT)}}{\text{Mean RLU of VC}}$$

The RTA of PC_{ATG} (%) was calculated by the following equation:

$$\text{RTA of PC}_{ATG}\ (\%) = \left(\frac{\text{Mean RLU of PC}_{ATG} - \text{Mean RLU of VC}}{\text{Mean RLU of AG ref} - \text{Mean RLU of VC}}\right) * 100$$

The concentrations of 20% inhibition of transcriptional activity induced by 500 pM DHT (log IC_{30}) and, if appropriate, to 50% inhibition of activity of 500 pM DHT (log IC_{50}) was calculated for each positive control and test item. Each log IC_x value was calculated using the following equation:

$$lin\ ICx = \frac{a - (b - (100 - x)) \times (a - c)}{(b - d)}$$

Results and Discussion

A. Analytical determinations:

Not undertaken. Analyses for achieved concentration, homogeneity or stability of test article formulations were not conducted as part of this study, as this is not a requirement of the regulatory test guidelines.

B. Androgen agonist assay:

1. Experiment 1:

Cell from passage 9 were used. The mean (normalized) RLU values for the DHT 10 nM, the positive control (PC_{AGO}), PC_{10} and vehicle control +2 standard

deviations were 8.96, 1.80 and 1.04 for the reference plate and 9.03, 1.80 and 1.02, respectively. The fold induction of DHT corresponding to the PC_{10} value was higher than the fold induction of vehicle control +2SD and therefore these acceptance criteria were met.
The log PC_{10} and log PC_{50} values obtained for mestanolone were outside the acceptance criteria, however, since these values were lower than the acceptance criteria (PC_{10} and PC_{50} responses at lower concentrations) this indicated sufficient responsiveness and therefore these data were accepted. All other acceptability criteria were met. The control items were correctly classified as positive (DHT and mestanolone) or negative (DEHP). Since all acceptance criteria were met, the androgen receptor (AR) agonist experiment was considered valid, with both sensitivity and specify for androgen agonist activity demonstrated.
The maximum level of response induced by spiroxamine compared to the response induced by 10 nM DHT (the RPC_{max}) was 0.0%. Since the RPC_{max} values were below 10% spiroxamine was concluded negative in the AR agonist assay.

2. Experiment 2: Data rejected due to a possible pipetting error in the mestanolone standard curve. Therefore, this data were not used in interpret the androgen agonist potential of spiroxamine.

3. Experiment 3: Cell from passage 18 were used. The mean (normalized) RLU values for the DHT 10 nM, the positive control (PC_{AGO}), PC_{10} and vehicle control +2 standard deviations were 8.93, 1.79 and 1.05 for the reference plate and 8.85, 1.78 and 1.05, respectively. The fold induction of DHT corresponding to the PC_{10} value was higher than the fold induction of vehicle control +2SD and therefore these acceptance criteria were met.
The log PC_{50} values obtained for mestanolone was outside the acceptance criteria, however, since these values were lower than the acceptance criteria (PC_{50} responses at lower concentrations) this indicated sufficient responsiveness and therefore these data were accepted. All other acceptability criteria were met. The control items were correctly classified as positive (DHT and mestanolone) or negative (DEHP). Since all acceptance criteria were met, the AR agonist experiment was considered valid, with both sensitivity and specify for androgen agonist activity demonstrated.
The maximum level of response induced by spiroxamine compared to the response induced by 10 nM DHT (the RPC_{max}) was -0.6%. Since the RPC_{max} values were below 10% spiroxamine was concluded negative in the AR agonist assay.
Collectively, in two independent agonist experiments, spiroxamine did not show any evidence of androgen receptor agonist activity and therefore concluded to be devoid of AR agonist activity *in vitro*.

Table CA 5.8.3/05-1: Androgen receptor and antagonist activity of spiroxamine: agonist assay – Experiment 1

Positive control data				Negative control data		Test article data	
DHT		Mestanolone		DEHP		Spiroxamine	
Log conc. (M)	Mean ±SD	Log conc. (M)	Mean ±SD	Log conc. (M)	Mean ±SD	Log conc. (M)	Mean ±SD
10^{-7}	107.5 ±2.4	10^{-7}	99.9 ±2.4	10^{-5}	1.0 ±0.2	$10^{-5.5}$	-1.4 ±0.5
10^{-8}	101.1 ±0.8	10^{-8}	99.3 ±3.5	10^{-6}	0.7 ±0.2	$10^{-6.0}$	-0.1 ±0.3
10^{-9}	97.5 ±0.6	10^{-9}	89.2 ±0.8	10^{-7}	0.2 ±0.1	$10^{-7.0}$	-0.7 ±0.1
10^{-10}	55.1 ±1.3	10^{-10}	67.9 ±1.4	10^{-8}	0.8 ±0.3	$10^{-8.0}$	-0.3 ±0.4
10^{-11}	1.7 ±0.4	10^{-11}	5.3 ±0.8	10^{-9}	-0.1 ±0.3	10^{-9}	0.0 ±0.3
10^{-12}	-0.4 ±0.1	10^{-12}	-0.4 ±0.2	10^{-10}	0.6 ±0.2	$10^{-10.0}$	-0.8 ±0.1
10^{-13}	-0.3 ±0.4	10^{-13}	0.1 ±0.2	10^{-11}	-0.4 ±0.3	$10^{-11.0}$	-0.4 ±0.3
RPC_{max}	107.5	RPC_{max}	99.9	RPC_{max}	1.0	RPC_{max}	0.0
PC_{50} (M)	8.02E-11	PC_{50} (M)	5.18E-11	PC_{50} (M)	-	PC_{50} (M)	-
PC_{10} (M)	1.43E-11	PC_{10} (M)	1.19E-11	PC_{10} (M)	-	PC_{10} (M)	-
Log PC_{50} (M)	-10.10	Log PC_{50} (M)	-10.29	Log PC_{50} (M)	-	Log PC_{50} (M)	-
Log PC_{10} (M)	-10.84	Log PC_{10} (M)	-10.92	Log PC_{10} (M)	-	Log PC_{10} (M)	-
Judgement	Positive	Judgement	Positive	Judgement	Negative	Judgement	Negative

Fold Induction of the positive control, DHT obtained in AR agonist experiment 1

	Passage	PC_{AGO} (10 nM DHT)	PC_{10} (10 nM DHT)	Vehicle control (DMSO) +2SD
Reference plate	P9	8.96	1.80	1.04
Test article plate	P9	9.03	1.80	1.02

DHT: 5α-dihydrotestosterone
DEHP: bis-(2-ethylhexyl)-phthalate

RPC_{max}: Maximum level of response induced by a test item, expressed as a percentage to the response induced by PC_{AGO} (10 nM DHT)
PC_{AGO}: Positive AR agonist control, wells treated with 10 nM DHT

PC_{10}: conc. of SPX with a response of 10% of the response induced by the positive control (10 nM DHT)
PC_{50}: conc. of SPX with a response of 50% of the response induced by the positive control (10 nM DHT)

Table CA 5.8.3/05-2: Androgen receptor and antagonist activity of spiroxamine: agonist assay – Experiment 3

Positive control data				Negative control data		Test article data	
DHT		Mestanolone		DEHP		Spiroxamine	
Log conc. (M)	Mean ±SD	Log conc. (M)	Mean ±SD	Log conc. (M)	Mean ±SD	Log conc. (M)	Mean ±SD
10^{-5}	7.0 ±0.4	10^{-5}	14.2 ±0.1	10^{-5}	111.9 ±1.1	$10^{-5.5}$	84.5 ±0.9
10^{-6}	2.3 ±0.4	10^{-6}	77.8 ±2.5	10^{-6}	104.2 ±0.7	$10^{-6.0}$	87.5 ±1.8
10^{-7}	17.5 ±0.9	10^{-7}	95.7 ±2.0	10^{-7}	98.1 ±2.2	$10^{-7.0}$	98.3 ±1.9
10^{-8}	65.2 ±2.2	10^{-8}	99.0 ±1.3	10^{-8}	103.4 ±8.9	$10^{-8.0}$	99.0 ±2.1
10^{-9}	90.6 ±0.7	10^{-9}	98.5 ±0.6	10^{-9}	101.2 ±0.6	$10^{-9.0}$	98.8 ±1.1
10^{-10}	93.0 ±1.3	10^{-10}	96.4 ±1.0	10^{-10}	100.6 ±1.3	10^{-10}	101.0 ±3.5
RPC_{max}	93.0	RPC_{max}	99.0	RPC_{max}	111.9	RTA_{max}	101
PC_{50} (M)	2.09E-08	PC_{50} (M)	2.74E-08	PC_{50} (M)	-	PC_{50} (M)	-
PC_{10} (M)	6.49E-09	PC_{10} (M)	1.33E-06	PC_{10} (M)	-	PC_{10} (M)	-
Log PC_{50} (M)	-7.68	Log PC_{50} (M)	-7.56	Log PC_{50} (M)	-	Log PC_{50} (M)	-
Log PC_{10} (M)	-8.19	Log PC_{10} (M)	-5.88	Log PC_{10} (M)	-	Log PC_{10} (M)	-
Judgement	Positive	Judgement	Positive	Judgement	Negative	Judgement	Negative

Fold Induction of the positive control, DHT obtained in AR agonist experiment 2				
	Passage	PC_{AGO} (10 nM DHT)	PC_{10} (10 nM DHT)	Vehicle control (DMSO) +2SD
Reference plate	P18	8.93	1.79	1.05
Test article plate	P18	8.85	1.78	1.05

DHT: 5α-dihydrotestosterone
DEHP: bis-(2-ethylhexyl)-phthalate

RPC_{max}: Maximum level of response induced by a test item, expressed as a percentage to the response induced by PC_{AGO} (10 nM DHT)
PC_{AGO}: Positive AR agonist control, wells treated with 10 nM DHT

PC_{10}: conc. of SPX with a response of 10% of the response induced by the positive control (10 nM DHT)
PC_{50}: conc. of SPX with a response of 50% of the response induced by the positive control (10 nM DHT)

C. Androgen antagonist assay:

1. Experiment 1:

Cell from passage 9 were used. The RTA value for the positive control, HF was <46% and the fold-induction of the AG agonist reference (AG ref) was ≥5.0, therefore these acceptance criteria were met.
The log IC_{30} and log IC_{50} values for HF, BPA and DEHP were within the acceptability criteria and the control items were correctly classified as positive (HF and BPA) or negative (DEHP) in the AR antagonist assay. Since all acceptance criteria were met, the AR antagonist experiment was considered valid, with both sensitivity and specify for androgen antagonist activity demonstrated.
Since no log IC_{30} and no log IC_{50} could be determined for spiroxamine, spiroxamine was concluded negative in the AR antagonist experiment.

2. Experiment 2:

Cell from passage 14 were used. The RTA value for the positive control, HF was <46% and the fold-induction of the AG agonist reference (AG ref) was ≥5.0, therefore these acceptance criteria were met.
The log IC_{30} and log IC_{50} values for HF, BPA and DEHP were within the acceptability criteria and the control items were correctly classified as positive (HF and BPA) or negative (DEHP) in the AR antagonist assay. Since all acceptance criteria were met, the AR antagonist experiment was considered valid, with both sensitivity and specify for androgen antagonist activity demonstrated.
Since no log IC_{30} and no log IC_{50} could be determined for spiroxamine, spiroxamine was concluded negative in the AR antagonist experiment.
Collectively, in two independent antagonist experiments, spiroxamine did not show any evidence of AR antagonist activity and therefore concluded to be devoid of AR activity *in vitro*.

3. Cytotoxicity:

Cytotoxicity was evaluated by determining the *Renilla* luciferase activity in the two AR antagonist experiments. With exception to the 100 pM HF and DEHP in experiment 2, no cytotoxicity (≥20% reduction of the *Renilla* luciferase activity) was observed for any of the control items. In addition, the observed cytotoxicity at these two instances where considered assay variation, and not actual cytotoxicity, due to the fact that it was observed at the lowest concentration tested, and the effect was not dose dependent.
No cytotoxicity (< 20% reduction of the *Renilla* luciferase activity) was observed for any of the test item concentrations tested, with exception to the 100 pM concentration in experiment 2. However, since this was the lowest tested concentration and due to the fact that the effect was not dose dependent, it was evaluated that this decrease in cell viability was assay variation.

Table CA 5.8.3/05-3: Androgen receptor and antagonist activity of spiroxamine: antagonist assay Experiment 1

Positive control data				Negative control data		Test article data	
HF		Bisphenol A		DEHP		Spiroxamine	
Log conc. (M)	Mean ±SD	Log conc. (M)	Mean ±SD	Log conc. (M)	Mean ±SD	Log conc. (M)	Mean ±SD
10^{-5}	4.7 ±0.3	10^{-5}	11.7 ±1.3	10^{-5}	108.6 ±4.1	$10^{-5.5}$	87.7 ±2.5
10^{-6}	3.3 ±0.6	10^{-6}	84.4 ±1.9	10^{-6}	101.9 ±1.9	$10^{-6.0}$	94.0 ±1.5
10^{-7}	48.5 ±1.1	10^{-7}	103.0 ±0.9	10^{-7}	106.5 ±2.0	$10^{-7.0}$	101.9 ±0.6
10^{-8}	91.6 ±1.4	10^{-8}	105.3 ±3.7	10^{-8}	104.8 ±n.a	$10^{-8.0}$	98.1 ±2.6
10^{-9}	98.4 ±3.1	10^{-9}	101.4 ±1.4	10^{-9}	103.2 ±1.6	$10^{-9.0}$	98.4 ±1.6
10^{-10}	97.7 ±1.5	10^{-10}	105.7 ±1.4	10^{-10}	97.4 ±2.6	$10^{-10.0}$	98.9 ±1.0
RTA_{max}	98.4	RTA_{max}	105.7	RTA_{max}	108.6	RTA_{max}	101.9
IC_{50} (M)	9.21E-08	IC_{50} (M)	2.97E-06	IC_{50} (M)	-	IC_{50} (M)	-
IC_{30} (M)	3.17E-08	IC_{30} (M)	1.58E-06	IC_{30} (M)	-	IC_{30} (M)	-
Log IC_{50} (M)	-7.04	Log IC_{50} (M)	-5.53	Log IC_{50} (M)	-	Log IC_{50} (M)	-
Log IC_{30} (M)	-7.50	Log IC_{30} (M)	-5.80	Log IC_{30} (M)	-	Log IC_{30} (M)	-
Judgement	Positive	Judgement	Positive	Judgement	Negative	Judgement	Negative

Fold Induction of the positive control, HF obtained in AR antagonist experiment 1			
	Passage	RTA (% of PC_{ATG} (1µM HF)	Fold induction AG ref
Reference plate	P9	1.5	7.6
Test article plate	P9	2.0	7.8

HF: hydroxyflutamide
DEHP: bis-(2-ethylhexyl)-phthalate
RTA: Relative transctiption activity
PC_{ATG}: Positive AR antagonist control, wells treated with 500 pM DHT and 1 µM of HF)
AG ref: Agonist reference (500 pM of DHT) in the antagonist assay
IC_{30}: Inhibition at 30% of max activity
IC_{50}:Inhibition at 50% of max activity

Table CA 5.8.3/05-4: Androgen receptor and antagonist activity of spiroxamine: agonist assay – Experiment 2

Passage				RTA (%) of PC_{ATG} (1 µM HF)		Fold induction AG ref	
DHT		Mestanolone		DEHP		Spiroxamine	
Log conc. (M)	Mean ±SD	Log conc. (M)	Mean ±SD	Log conc. (M)	Mean ±SD	Log conc. (M)	Mean ±SD
10^{-5}	7.0 ±0.4	10^{-5}	14.2 ±0.1	10^{-5}	111.9 ±1.1	$10^{-5.5}$	84.5 ±0.9
10^{-6}	2.3 ±0.4	10^{-6}	77.8 ±2.5	10^{-6}	104.2 ±0.7	$10^{-6.0}$	87.5 ±1.8
10^{-7}	17.5 ±0.9	10^{-7}	95.7 ±2.0	10^{-7}	98.1 ±2.2	$10^{-7.0}$	98.3 ±1.9
10^{-8}	65.2 ±2.2	10^{-8}	99.0 ±1.3	10^{-8}	102.4 ±8.9	$10^{-8.0}$	99.0 ±2.1
10^{-9}	90.6 ±0.7	10^{-9}	98.5 ±0.6	10^{-9}	101.2 ±0.6	$10^{-9.0}$	98.8 ±1.1
10^{-10}	93.0 ±1.3	10^{-10}	96.4 ±1.0	10^{-10}	100.6 ±1.3	10^{-10}	101.0 ±3.5
RTA_{max}	93.0	RTA_{max}	99.0	RTA_{max}	111.9	RTA_{max}	101
IC_{50} (M)	2.09E-08	IC_{50} (M)	2.74E-06	IC_{50} (M)	-	IC_{50} (M)	-
IC_{30} (M)	6.49E-09	IC_{30} (M)	1.33E-06	IC_{30} (M)	-	IC_{30} (M)	-
Log IC_{50} (M)	-7.68	Log IC_{50} (M)	-5.56	Log IC_{50} (M)	-	Log IC_{50} (M)	-
Log IC_{30} (M)	-8.19	Log IC_{50} (M)	-5.88	Log IC_{30} (M)	-	Log IC_{30} (M)	-
Judgement	Positive	Judgement	Positive	Judgement	Negative	Judgement	Negative

Fold Induction of the positive control, DHT obtained in AR agonist experiment 2

	Passage	PC_{AGO} [a] (10 nM DHT)	PC 0 (10 nM DHT)	VC +2SD
Reference plate	P18	8.93	1.79	1.05
Test article plate	P18	8.85	1.78	1.05

HF: hydroxyflutamide
DEHP: bis-(2-ethylhexyl)-phthalate
RTA: Relative transcription activity
PC_{ATG}: Positive AR antagonist control, wells treated with 500 pM DHT and 0 µM of HF)
AG ref: Agonist reference (500 pM of DHT) in the antagonist assay
IC_{30}: Inhibition at 30% of max activity
IC_{50}:Inhibition at 50% of max activity

C. Deficiencies:

None.

Assessment and conclusions by applicant:

Assessment: Study meets the current guidance and the requirements in 283/2013

Conclusion: It is concluded that spiroxamine did no show evidence of either androgen receptor agonist or antagonist activity in the Stably Transfected Human Androgen Receptor Transcriptional Activation Assay (AR-EcoScreen™) following two independent experiments. Maximum concentrations analysed were limited by solubility, in line with current regulatory guidelines for this assay.

Data Point:	KCA 5.8.3/06
Report Author:	[redacted]
Report Year:	2020
Report Title:	A dose range-finding oral (gavage) toxicity study of spiroxamine in young adult male rats
Report No:	00543028
Document No:	M-761549-01-1
Guideline(s) followed in study:	None
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

In a dose range finding study to select dose levels for the Hershberger assay (CA 5.8.3/07 [M-764008-01-1]), young adult male Sprague Dawley rats (5 animals/group) were administered spiroxamine suspended in 1% methylcellulose orally *via* gavage for 7 consecutive days at dose levels of 0 (1% MC), 125, 250 and 500 mg/kg bw/day, employing a dose volume of 5 mL/kg bw.

Spiroxamine at dose levels of ≥ 250 mg/kg bw/day were not tolerated in young adult male rats when administered once daily via oral gavage, as evidenced by the effects on body weights, body weight gains, food consumption, and clinical observations leading to the death or euthanasia of all animals in these groups during days 2–4 of dosing. At 125 mg/kg bw/day, lower mean body weight gains with corresponding lower mean food consumption were generally noted throughout the dosing period, resulting in a slightly lower mean absolute body weight on day 7 compared to the concurrent control group. Based on these data, dose levels of 31.25, 62.5, and 125 mg/kg/day (deemed to be a maximum tolerated dose) were selected for a Hershberger assay administered orally by gavage to peripubertal, orchidoepididymectomized Sprague Dawley rats (CA 5.8.3/07 [M-764008-01-1]).

Under the conditions of this study dose levels ≥250 mg/kg bw/day were not tolerated by young adult male rats when administered once daily via oral gavage. Based on these data, dose levels of 31.25, 62.5, and 125 mg/kg/day (deemed to be a maximum tolerated dose) were selected for a Hershberger assay administered orally by gavage to peripubertal, orchidoepididymectomized Sprague Dawley rats (CA 5.8.3/07 [M-764008-01-1]).

Materials and Methods

A. Materials:

1. Test Material:	Spiroxamine (alternative name: [8-(1,1-dimethylethyl)-N-ethyl-N-propyl-1,4-dioxaspiro[4,5]decane-2-methanamine; KWG 4168)
Description:	Brown fluid
Lot/Batch No.:	AE 1344293-01-07
Purity:	97.0% (w/w) (correction for purity not undertaken)
CAS No.:	118134-30-8
Stability of test compound:	Confirmed stable for the duration of the study (expiry date: 4 June 2021)
2. Vehicle and/or positive control:	1% methylcellulose; not relevant
3. Test animals:	
Species:	Rat
Strain:	Sprague Dawley
Age at dosing:	7 weeks
Weight at dosing:	♂: 229 – 283 g
Source:	[redacted]
Acclimation period:	At least 13 days
Diet:	Altromin® 1324 diet for rats and mice, *ad libitum* (except during prior to dosing with animals fasted overnight [*ca.* 15 h])
Water:	Municipal water, *ad libitum*
Housing:	Group housed (2-3/cage)
4. Environmental conditions:	
Temperature:	22.5 ±2.5°C
Humidity:	55 ±15%
Air changes:	12±15/h
Photoperiod:	12 hour light/dark

B. Study Design:

1. In life dates:	27 May 2020 to 3 June 2020 (experimental dates)
2. Animal assignment and treatment:	After an acclimatisation period rats were allocated to groups by randomisation to achieve similar group mean body weights (*i.e.* body weight within ±20% of group mean body weight for each group). Dose level selection was based on existing information available on spiroxamine confirming moderate acute rat oral toxicity (LD_{50} *ca.* 500 mg/kg bw [CA 5.2.1/01, M-007791-01-1]) the target organ and critical effects observed by spiroxamine are the liver and irritant effects on the mucosal epithelium of the oesophagus and forestomach. No specific neurotoxic effects were found in acute and repeated-dose neurotoxicity studies, but an acute NOAEL of 10 mg/kg bw was obtained from the acute neurotoxicity study (CA 5.7.1/01, M-016556-01-1]. The test article, spiroxamine was administered orally *via* gavage once daily at doses of 0 (1% MC), 125, 250 and 500 mg/kg bw/day, employing a dose volume of 5 mL/kg bw, for seven consecutive days to 5 ♂/group.
3. Statistics:	Body weights and body weight changes were subjected to a parametric one-way ANOVA to determine intergroup differences. If the ANOVA revealed significant intergroup variance, Dunnett's test was used to compare the test article-treated groups to the control group.

C. Methods:

1. Test article formulation preparation and analysis:	Dose formulations were prepared daily, with formulations stirred for a minimum of 30 minutes before dosing and continuously during dosing. Dose formulations were administered to animals within 2 hours of dosing. No stability, homogeneity or concentration analysis on the dose formulations were undertaken.
2. Observations:	Cage side observations, including mortality, clinical signs of toxicity were conducted twice daily, with post dose observations conducted at 3 hours. Detailed clinical observations were recorded daily for all animals.
3. Body weights:	Recorded daily during dosing.
4. Food consumption:	Recorded daily during dosing.
5. Water consumption:	Not conducted.
6. Ophthalmological examination:	Not conducted.
7. Preputial separation:	Not conducted.
8. Haematology and clinical chemistry:	Not conducted.
9. Urinalysis:	Not conducted.
10. Organ weights:	Not conducted.
11. Sacrifice and pathology::	All animals were subjected to a complete gross necropsy, which included evaluation of the external surface, all orifices, the cranial cavity, external surface of the brain, and the thoracic, abdominal, and pelvic cavities, including viscera. Gross lesions, if any were collected and preserved in 10% NBF.

Results and discussion

A. Test article formulation analysis:

Test article formulation analysis was not undertaken for this dose range finder study.

B. Observations:

1. Clinical signs of toxicity:	Clinical signs of toxicity were limited to the 250 and 500 mg/kg bw/day dosage groups. These clinical signs were evident of general toxicity, consisting of red material around the right eye, a prostrate body, piloerection, cool extremities, and dilated pupils (500 mg/kg bw/day). At 250 mg/kg bw/day brown material around the anogenital area at the daily examinations on the day of death/euthanasia. A single ♂ that was found dead was also noted with a cool body. Several ♂ were also noted to have hunched posture, yellow/orange diarrhoea, prostate body and or decreased activity during study days 2-4.
2. Mortality:	All ♂ in the 250 and 500 mg/kg/day groups were found dead or euthanised *in extremis* during days 2 - 4.
3. Ophthalmoscopic examination:	Not conducted.

C. Body weight and body weight gain:

Statistically significantly lower mean body weight gains or mean body weight losses were noted in the 250 and 500 mg/kg bw/day dosage groups beginning on day 1 and continuing through to death or early euthanasia (on day 4 or 3, respectively) compared to the control group. As a result, mean absolute body weights were 6.3% to 13.7% lower at 250 mg/kg bw/day on days 3 and 4 and 5.8% and 13.5% lower at 500 mg/kg bw/day on days 2 and 3 compared to the control group; the differences were statistically significant on day 4 and 3, respectively. Further evaluation of body weight data at 250 and 500 mg/kg bw/day was precluded due to the death or euthanasia of all animals on or before days 4 and 3, respectively.

In the 125 mg/kg bw/day mean absolute body weights were 5.7% lower than the control group on day 7. Mean body weight gain were generally lower than the control group for the remained of the dosing period (days 1-7), achieving statistical significance on days 4-5, 6-7 and when the entire treatment period was evaluated (days 0-7).

Table CA 5.8.3/06-1: Overview of dose range finding study in young adult male rats treated orally (*via* gavage) with spiroxamine: body weight effects

Parameters		♂ (mg/kg bw/d)			
		0	125	250	500
Body wt (g)	Day				
	-1	252 ±11.07	253 ±14.88 (↑0.3%)	254 ±14.69 (↑1%)	253 ±18.82 (↑0.2%)
	0	263 ±14.26	259 ±9.37 (↓2%)	261 ±15.98 (↓1%)	261 ±20.11 (↓1%)
	1	269 ±12.56	267 ±14.36 (↓1%)	270 ±17.07 (↑0.3%)	269 ±18.41 (↑0.2%)
	2	280 ±13.35	274 ±16.61 (↓2%)	271 ±17.74 (↓3%)	264 ±14.82 (↓6%)
	3	287 ±12.29	283 ±15.96 (↓1%)	269** ±16.10 (↓6%)	248 ±8.35** (↓14%)
	4	295 ±14.07	289 ±16.49 (↓2%)	255 ±23.64**	-
	5	306 ±16.80	295 ±16.30 (↓4%)	↓14%)	-
	6	309 ±18.26	296 ±17.63 (↓4%)	--	-
	7	322 ±18.99	304 ±16.58 (↓6%)		

** $p \leq 0.01$ - all animals dead, no available data

D. Food consumption, food efficiency and water consumption:

1. **Food consumption:** Mean food consumption in the 250 and 500 mg/kg bw/day groups was lower than the control group during days 1–4 and 1–3, respectively. The lower mean food consumption corresponded with the lower mean body weight gains or mean body weight losses noted in these group during these same periods. Further evaluation of food consumption data at 250 and 500 mg/kg bw/day was precluded due to the death or euthanasia of all animals on or before days 4 and 3, respectively.
2. **Food efficiency:** Not conducted.
3. **Water consumption:** Not conducted.

E. Blood and urinalysis:

1. **Haematological findings:** Not conducted
2. **Clinical chemistry findings:** Not conducted
3. **Urinalysis:** Not conducted

F. Sacrifice and pathology:

1. **Organ weight:** Not conducted.
2. **Gross pathology:** No gross pathological findings were evident in animals that survived until the end of the treatment period or in animals that were found dead or were killed *in extremis*.
3. **Histopathology:** Not conducted.

G. Deficiencies:

None.

H. Discussion:

Spiroxamine at dose levels of ≥ 250 mg/kg bw/day were not tolerated in young adult male rats when administered once daily via oral gavage, as evidenced by the effects on body weights, body weight gains, food consumption, and clinical observations leading to the death or euthanasia of all animals in these groups during days 2–4 of dosing. At 125 mg/kg bw/day, lower mean body weight gains with

corresponding lower mean food consumption were generally noted throughout the dosing period, resulting in a slightly lower mean absolute body weight on day 7 compared to the concurrent control group. Based on these data, dose levels of 31.25, 62.5, and 125 mg/kg/day (deemed to be a maximum tolerated dose) were selected for a Hershberger assay administered orally by gavage to peripubertal, orchidoepididymectomized Sprague Dawley rats (CA 5.8.3/07 [M-764008-01-1]).

Assessment and conclusions by applicant:

Assessment: Study meets the current guidance and the requirements in 283/2013

Conclusion: Under the conditions of this study dose levels ≥250 mg/kg bw/day were not tolerated by young adult male rats when administered once daily via oral gavage. Based on these data, dose levels of 31.25, 62.5, and 125 mg/kg/day (deemed to be a maximum tolerated dose) were selected for a Hershberger assay administered orally by gavage to peripubertal, orchidoepididymectomized Sprague Dawley rats (CA 5.8.3/07 [M-764008-01-1]).

Data Point:	KCA 5.8.3/07
Report Author:	[redacted]
Report Year:	2021
Report Title:	A hershberger assay of spiroxamine administered orally in peripubertal orchidoepididymectomized rats
Report No:	00543029
Document No:	M-764008-01-1
Guideline(s) followed in study:	US EPA OPPTS 890.1400 (Oct 2009) OECD Test Guideline 441 (Sep 2009)
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The androgenic, anti-androgenic or 5α-reductase inhibitory effects of spiroxamine were evaluated using the Hershberger Assay. Animals (castrated by the supplier) selected for the study were allocated to each group by stratified randomization method based on the body weight of each animal using computer program into four groups of 6 animals so that the mean body weight would be nearly equal among all groups prior to dosing on the day of administration. The study was split into two phases:

- Phase I: androgenic activity assessment
- Phase II: anti-androgenic activity assessment

The test article was formulated in 1% methyl cellulose, with all animals receiving a single oral gavage dose at 0, 31.25, 62.5, 125 mg/kg bw/day for 10 consecutive days from PND 60, using a dose volume of 5 mL/kg. Additional positive control groups were included in each phase of the study:

- Phase I: Testosterone propionate administered once daily for 10 consecutive days *via* subcutaneous injection at 0.4 mg/kg bw/day, employing a dose volume of 0.5 mg/kg bw
- Phase II: Flutamide administered once daily for 10 consecutive days *via* oral gavage at 3 mg/kg bw/day, employing a dose volume of 5 mg/kg bw.
- In addition, all animals in this phase of the study also were received testosterone propionate administered once daily for 10 consecutive days *via* subcutaneous injection at 0.4 mg/kg bw/d,

employing a dose volume of 0.5 mg/kg bw to screen for potential anti-androgenic effects and 5α-reductase inhibitory effects.

All animals were euthanized and underwent necropsy with selected organs weighed (including five androgen-dependent tissues: levator ani-bulbocavernosus (LABC), seminal vesicles with coagulating glands and fluid (SVCGs), Cowper's glands (COW), ventral prostate (VP) and glans penis (GP)) 24 hours post the final dose.

No test article related clinical signs of toxicity were observed. Mean body weight gains in the 125 mg/kg/day groups (administered alone [Phase I] or in conjunction with TP [Phase II]) were generally lower than the respective control group throughout the treatment period and when the overall treatment period (Study Days 0-10) was evaluated; differences for the overall treatment period were statistically significant from the respective control group.

There were no significant increases or decreases in the mean weights of the five androgen-dependent tissues (LABC, SVCGs, COW, VP and GP) in the three spiroxamine dose groups from either Phase I (androgenic effects) or Phase II (anti- androgenic effects).

In the respective positive control groups, either statistically significant increases in the mean weights of the five androgen-dependent tissues were observed (testosterone propionate) or statistically significant reductions in the mean weights of these tissues were observed, thus showing sensitivity and specificity of the test system to detect androgenic and anti-androgenic effects.

Under the conditions of this study, spiroxamine did not cause any treatment related changes in reproductive/endocrine organ weights in the Hershberger assay when administered to castrated male rats up to a maximum dose of 125 mg/kg bw/day (deemed to be a maximum tolerated dose). Therefore, it is concluded that spiroxamine did not exhibit androgenic, anti-androgenic or 5α-reductase inhibitory properties under the conditions of this study.

Materials and Methods

A. Materials:

1. Test Material:	Spiroxamine (alternative name: [8-(1,1-dimethylethyl)-N-ethyl-N-propyl-1,4-dioxaspiro[4.5]decane-2-methanamine; KWG 4168)
Description:	Brown fluid
Lot/Batch No.:	AE 1344293-01-07
Purity:	97.0% (w/w) (correction for purity not undertaken)
CAS No.:	118134-30-8
Stability of test compound:	Confirmed stable for the duration of the study (expiry date: 4 June 2021)
2. Vehicle and/or positive control:	1% methylcellulose/ testosterone propionate (TP, 0.2 mg/kg bw/day), flutamide (3 mg/kg bw/day)
3. Test animals:	
Species:	Rat
Strain:	Sprague Dawley
Age at dosing:	7 weeks
Weight at dosing:	229 – 283 g
Source:	[redacted]
Acclimation period:	At least x days
Diet:	Altromin® 1324 diet for rats and mice, *ad libitum* (except during prior to dosing with animals fasted overnight [*ca.* 15 h])
Water:	Municipal water, *ad libitum*
Housing:	Group housed (2-3/cage)

4. Environmental conditions:

Temperature:	22.5 ±2.5°C
Humidity:	55 ±15%
Air changes:	12-15/h
Photoperiod:	12 hour light/dark

B. Study Design:

1. In life dates: 26 June 2020 to 7 July 2020 (experimental dates)

2. Animal assignment and treatment: Animals (castrated by the supplier) selected for the study were allocated to each group by stratified randomization method based on the body weight of each animal using computer program into four groups of 6 animals so that the mean body weight would be nearly equal among all groups prior to dosing on the day of administration. The study was split into two phases:

- Phase I: androgenic activity assessment
- Phase II: anti-androgenic activity assessment

The test article was formulated in 1% MC, with all animals receiving a single oral gavage dose at 0, 31.25, 62.5, 125 mg/kg bw/d for 10 consecutive days from PND 60, using a dose volume of 5 mL/kg bw. Additional positive control groups were included in each phase of the study:

- Phase I: Testosterone propionate administered once daily for 10 consecutive days *via* subcutaneous injection at 0.2 mg/kg bw/d, employing a dose volume of 0.5 mg/kg bw
- Phase II: Flutamide administered once daily for 10 consecutive days *via* oral gavage at 3 mg/kg bw/d, employing a dose volume of 5mL/kg bw.
- In addition, all animals in this phase of the study also were received testosterone propionate administered once daily for 10 consecutive days *via* subcutaneous injection at 0.2 mg/kg bw/d, employing a dose volume of 0.5 mL/kg bw to screen for potential anti-androgenic effects and 5α-reductase inhibitory effects

All animals were euthanized and underwent necropsy with selected organs weighed 24 h post the final dose.

3. Statistics: Clinical observations and other proportional data were analysed using the Variance Test for Homogeneity of the Binomial Distribution.

Continuous data (*e.g.*, body weights, body weight changes, and feed consumption values,) were analysed using Bartlett's Test of Homogeneity of Variances and the ANOVA, when appropriate [*i.e.* Bartlett's Test was not significant (p >0.001)]. If the ANOVA was significant (p ≥0.05), Dunnett's Test was used to identify the statistical significance of the individual groups. If the ANOVA was not appropriate [*i.e.*, Bartlett's Test was significant (p≤0.001)], the Kruskal-Wallis Test was use. In cases where the Kruskal-Wallis Test was statistically significant (p ≤0.05), Dunn's Method of Multiple Comparisons was used to identify the statistical significance of the individual groups. Wet and blotted uterine weights and liver weights were analysed by Analysis of Covariance (ANCOVA), using terminal body weight as the covariate.

Mean daily body weight values were reported to the nearest 0.1 g. Food consumption values were measured/nesting box/day, and the results were expressed in grams/rat/day.

Phase I: all comparisons for the spiroxamine dose groups and for the testosterone propionate positive control were made relative to the vehicle control. A statistically significant increase (p ≤0.05) in any 2 or more of the 5 required androgen-dependent tissue weights was considered a positive androgen agonist result.

For Phase II: all comparisons for the spiroxamine dose groups were made relative to the vehicle control. A statistically significant reduction ($p \leq 0.05$) in any 2 or more of the 5 required androgen-dependent tissue weights was considered a positive androgen antagonist result.
Coefficients of variation (CVs) for the vehicle control and the spiroxamine high-dose groups (were compared to the CV values in the US EPA OPPTTS test guideline [refer below]).

4. Acceptance criteria:

The Hershberger assay performance criteria do not include absolute organ weights for positive and negative controls. The CV for a tissue has an inverse relationship with statistical power, therefore performance criteria are based on maximum CV value for each tissue weighed. The maximum allowable CV determined are as follows:

Tissue	Anti-Androgenic Effects	Androgenic Effects
Seminal vesicles	40%	40%
Ventral prostate	40%	45%
LABC muscle group	20%	30%
Bulbourethral gland	35%	55%
Glans penis	17%	22%

Where negative outcomes were concluded, CVs from the control group and high dose group are determined if the maximum CV performance criteria had been exceeded.
Where 3 or more of the 10 possible individual CVS in the control and high dose groups exceed the maximum designated agonist and antagonist studies and at least 2 target tissues were marginally insignificant (*i.e.* p 0.05 to 0.10) then a study repeat was undertaken.

5. Evaluation criteria:

Evidence of androgen agonist activity:
- A statistically significant increase ($p \leq 0.05$) in any two or more of the five androgen-dependent tissue weights (VP, LABC, GP, CG, SVCG) with some degree of increased growth combined evaluation of all accessory sex organs.

Evidence of androgen antagonism:
- A statistically significant reduction ($p \leq 0.05$) in any two or more of the five androgen-dependent tissue weights (VP, LABC, GP, CG, SVCG) relative to TP treatment alone with some degree of reduced growth.

Table CA 5.8.3/07-1: Overview of the Hershberger study in young castrated adult male rats treated orally (*via* gavage) with spiroxamine: doses received

Parameters	(mg/kg bw/day)				
	0 (1% MC)	31.25	62.5	125	Positive control
Phase I – androgenic activity					
Animals assigned/sex	6	6	6	6	6
Dose volume (mL/kg bw)	5	5	5	5	TP: 0.2
Phase II – anti-androgenic activity					
Animals assigned/sex	6	6	6	6	6
Dose volume (mL/kg bw)	5^{TP}	5^{TP}	5^{TP}	5^{TP}	Flutamide/TP: 3/0.2

TP: testosterone propionate 0.4 mg/kg bw/d administered *via* subcutaneous injection once daily from study days 1 to 10
Flutamide: 3 mg/kg bw/d administered *via* oral gavage once daily from study days 1 to 10

C. Methods:

1. Test article formulation preparation and analysis:	Dose formulations were prepared daily, with formulations stirred for a minimum of 30 minutes before dosing and continuously during dosing. Dose formulations were administered to animals within 2 hours of dosing. Test article formulations have been previously shown to be stable and homogeneous at concentrations of 5 and 100 mg/mL for at least 8 days in a refrigerator set to maintain a target of 5°C (refer to Doc MCA Section). Therefore, stability and resuspension homogeneity of test article formulations will not be assessed on this study.
2. Observations:	Cage side observations, including mortality, clinical signs of toxicity were conducted twice daily, with post dose observations conducted at 5 hours. Detailed clinical observations were recorded daily for all animals.
3. Body weights:	Recorded daily during dosing.
4. Food consumption:	Recorded daily during dosing.
5. Water consumption:	Not conducted.
6. Ophthalmological examination:	Not conducted.
7. Haematology and clinical chemistry:	Blood was collected at termination, with serum separated and frozen for further analysis if required.
8. Urinalysis:	Not conducted.
9. Organ weights:	Brain, kidney, liver, prostate (ventral), seminal vesicles (including coagulating glands), levator ani plus bulbocavernosus muscle complex (LABC), Cowper's gland (bulbourethral gland), glans penis
10. Sacrifice and pathology:	Conducted on PND 71. Gross pathological examination was performed on all animals and included examination of the thoracic abdominal and pelvic viscera. The following tissues were preserved in 10% neutral buffered formalin for subsequent histopathological examination: Kidney, liver, prostate (ventral), seminal vesicles (including coagulating glands), Cowper's gland, gross lesions. For animals that died prior to scheduled sacrifice (including all vehicle control animals) the following additional tissue were sampled: oesophagus, heart, lung, spleen stomach, trachea

Results and discussion

A. Test article formulation analysis:

Spiroxamine was homogenously distributed and within the concentration range of 6.25 to 25 mg/mL. The analytical data verify that the during the treatment period concentrations of the test article in the formulation preparations ranged from 98.5% to 111% of nominal concentrations, with %RSD values of 1.5 to 2.6% which were within acceptable limits.

B. Observations:

1. Clinical signs of toxicity:	*Phase I & II:* No test article-related effects were observed.
2. Mortality:	*Phase I & II:* All animals survived until the scheduled necropsy
3. Ophthalmoscopic examination:	Not conducted.

C. Body weight and body weight gain:

Mean body weight gains in the 125 mg/kg/day groups (administered alone [Phase I] or in conjunction with TP [Phase II]) were generally lower than the respective control group throughout the treatment period and when the overall treatment period (Study Days 0-10) was evaluated; differences for the overall treatment period were statistically significant from the respective control group. As a result, mean body weights were lower in the 125 mg/kg/day group [Phase I, 6.69% to 10.48% during Study

Day 7-10) and in the 125 mg/kg/day group co-administered TP group [Phase II, 5.50% to 7.17% during Study Days 8-10) when compared to the shared concurrent vehicle control group or TP positive control group, respectively; differences from the respective control groups were not statistically significant.

No test article-related effects on mean body weights or body weight changes were noted in the 31.25 and 62.5 mg/kg bw/day groups [Phase I] when compared to the vehicle control group. Likewise, in the 31.25 and 62.5 mg/kg bw/day groups co-administered TP [Phase II] mean body weights and body weight changes were similar to the TP positive control group.

Mean body weights and body weight changes in the TP positive control group were comparable to the shared concurrent vehicle control group throughout the study. In addition, mean body weights and body weight changes in the flutamide/TP positive control group were comparable to the TP positive control group throughout the study.

Table CA 5.8.3/07-2: Overview of the Hershberger study in young castrated adult male rats treated orally (*via* gavage) with spiroxamine: Phase I, androgenic activity, body weight effects

Parameters		♂ (mg/kg bw/d)				
		0	31.25	62.5	125	TP (0.2)
Body wt (g)	Day					
[% change in bwt]	-1	208 ±18.94 (9.10)	204 ±26.30 [↓2.00%] (12.90)	206 ±19.37 [↓0.75%] (9.58)	208 ±19.94 [↓0.26%] (9.61)	207 ±21.94 [↓0.38%] (10.61)
(CV)	0	214 ±17.24 (8.05)	210 ±27.74 [↓1.89%] (13.20)	214 ±19.24 [↓0.05%] (8.99)	213 ±20.88 [↓0.78%] (9.83)	212 ±25.83 [↓1.07%] (12.19)
	1	221 ±18.56 (8.39)	216 ±28.67 [↓2.64%] (13.30)	222 ±21.14 [↑0.11%] (9.54)	219 ±19.64 [↓1.09%] (8.97)	220 ±24.63 [↓0.68%] (11.21)
	2	229 ±17.66 (7.72)	223 ±30.01 [↓2.68%] (13.49)	229 ±21.62 [↑0.21%] (9.44)	225 ±18.44 [↓1.68%] (8.20)	227 ±23.48 [↓0.65%] (10.33)
	3	233 ±17.56 (7.53)	228 ±28.48 [↓2.29%] (12.49)	234 ±23.20 [↑0.32%] (9.91)	229 ±18.87 [↓1.81%] (8.24)	234 ±21.92 [↑0.07%] (9.39)
	4	242 ±18.36 (7.60)	236 ±28.55 [↓2.39%] (12.11)	241 ±22.15 [↓0.24%] (9.19)	233 ±17.34 [↓3.40%] (7.44)	242 ±21.03 [↑0.04%] (8.70)
	5	247 ±16.66 (6.76)	242 ±27.90 [↓1.96%] (11.54)	245 ±21.78 [↓0.66%] (8.89)	237 ±16.49 [↓3.98%] (6.96)	249 ±19.69 [↑1.12%] (7.90)
	6	254 ±16.56 (6.53)	247 ±26.61 [↓2.46%] (10.76)	253 ±21.82 [↓0.41%] (8.64)	242 ±16.29 [↓4.69%] (6.74)	255 ±19.44 [↓0.40%] (7.64)
	7	262 ±16.05 (6.13)	255 ±26.42 [↓2.40%] (10.35)	258 ±22.24 [↓1.44%] (8.63)	248 ±14.11 [↓5.69%] (5.70)	264 ±18.98 [↑1.06%] (7.17)
	8	267 ±16.24 (6.08)	262 ±25.79 [↓2.12%] (9.86)	264 ±20.30 [↓1.14%] (7.69)	247 ±13.06 [↓7.57%] (5.29)	274 ±20.80 [↑2.63%] (7.59)
	9	271 ±14.00 (5.17)	267 ±24.49 [↓1.51%] (9.18)	265 ±21.80 [↓2.33%] (8.24)	248 ±10.41 [↓8.68%] (4.21)	278 ±18.22 [↑2.66%] (6.55)
	10	278 ±15.84 (5.70)	271 ±26.11 [↓2.37%] (9.62)	273 ±22.35 [↓1.83%] (8.19)	249 ±12.69 [↓10.48%] (5.10)	283 ±20.05 [↑1.73%] (7.09)
Body wt gain (g) [% change in bwt][a] (CV)	0-10	63.9 ±8.28 (12.96)	61.3 ±11.04 [↓4.07%] (18.01)	58.9 ±5.97 [↓7.82%] (10.14)	36.4 ±19.73 [↓43.04%] (54.21)*	71.0 ±6.28 [↑11.11%] (8.85)

* $p \leq 0.05$ relative to the concurrent vehicle control group

[a] % change in bwt relative to the concurrent vehicle control group

Table CA 5.8.3/07-3: Overview of the Hershberger study in young castrated adult male rats treated orally (*via* gavage) with spiroxamine: Phase II, anti-androgenic activity, body weight effects

Parameters		♂ (mg/kg bw/d)				
		0	31.25/TP (0.2)	62.5/TP (0.2)	125/TP (0.2)	Flutamide/TP (3/0.2)
Body wt (g)	Day					
[% change in bwt]	-1	208 ±18.94 (9.10)	209 ±18.09 [↑0.91%] (8.65)	207 ±24.86 [↑1.01%] (12.02)	208 ±19.46 [↑0.46%] (9.35)	212 ±26.00 [↑2.21%] (11.91)
(CV)	0	214 ±17.24 (8.05)	213 ±22.55 [↑0.54%] (10.59)	214 ±25.05 [↑1.60%] (11.71)	216 ±19.02 [↑1.94%] (8.81)	218 ±26.00 [↑3.08%] (11.91)
	1	221 ±18.56 (8.39)	223 ±19.93 [↑1.36%] (8.94)	223 ±26.35 [↑0.24%] (11.80)	223 ±20.13 [↑1.61%] (9.01)	225 ±27.20 [↑2.43%] (12.08)
	2	229 ±17.66 (7.72)	230 ±19.34 [↑1.35%] (8.40)	228 ±25.64 [↑0.89%] (11.26)	228 ±20.21 [↑0.22%] (8.87)	233 ±26.93 [↑2.31%] (11.59)
	3	233 ±17.56 (7.53)	239 ±19.64 [↑2.46%] (8.21)	236 ±25.87 [↑1.07%] (10.98)	234 ±19.09 [↑0.16%] (8.16)	242 ±26.37 [↑3.48%] (10.91)
	4	242 ±18.36 (7.60)	247 ±20.71 [↑2.09%] (8.40)	244 ±28.03 [↑0.94%] (11.48)	240 ±19.86 [↓0.57%] (8.27)	247 ±26.93 [↑2.20%] (10.91)
	5	247 ±16.66 (6.76)	254 ±19.60 [↑1.989%] (7.71)	252 ±27.21 [↑2.4%] (10.81)	245 ±18.17 [↓1.72%] (7.41)	258 ±27.82 [↑3.38%] (10.79)
	6	254 ±16.56 (6.53)	261 ±23.16 [↑2.70%] (8.86)	261 ±28.07 [↑1.40%] (10.77)	249 ±18.61 [↓2.23] (7.48)	263 ±26.98 [↑3.16%] (10.27)
	7	262 ±16.05 (6.13)	269 ±21.07 [↑1.83%] (7.83)	268 ±29.27 [↓0.65%] (10.92)	255 ±18.27 [↓3.66%] (7.18)	272 ±28.32 [↑2.86%] (10.42)

Parameters		♂ (mg/kg bw/d)				
		0	31.25/TP (0.2)	62.5/TP (0.2)	125/TP (0.2)	Flutamide/TP (3/0.2)
	8	267 ±16.24 (6.08)	278 ±21.44 [↑1.34%] (7.72)	272 ±29.73 [↑0.40%] (10.91)	259 ±16.39 [↓5.50%] (6.33)	278 ±26.67 [↑1.27%] (9.60)
	9	271 ±14.00 (5.17)	282 ±19.89 [↑1.21%] (7.06)	279 ±29.96 [↑0.40%] (10.73)	258 ±16.31 [↓7.17%] (6.31)	280 ±23.60 [↑0.79%] (8.42)
	10	278 ±15.84 (5.70)	289 ±23.87 [↑2.22%] (8.26)	284 ±29.92 [↑0.52%] (10.53)	265 ±15.01 [↓6.27%] (5.66)	286 ±27.07 [↑1.24%] (9.45)
Body wt gain (g) [% change in bwt][a] (CV)	0-10	61.3 ±11.04 (18.01)	76.1 ±8.89 [↑7.18%] (11.68)	70.3 ±9.99 [↓1.41%] (14.21)	49.2 ±9.27 [↓30.70%] (18.87)*	68.0 ±5.60 [↓4.23%] (8.23)

* $p \leq 0.05$ relative to the TP control group

[a] % change in bwt relative to the TP control group [Phase I]

D. Food consumption, food efficiency and water consumption:

1. Food consumption:	No effect on food consumption.
2. Food efficiency:	Not conducted.
3. Water consumption:	Not conducted.

E. Blood and urinalysis:

1. Haematological findings:	Not conducted.
2. Clinical chemistry findings:	Not conducted.
3. Urinalysis:	Not conducted.

F. Sacrifice and pathology:

1. Assay performance criteria: The %CV for all weights from the vehicle control, TP positive control, flutamide/TP positive control and 125 mg/kg/day (administered alone or in conjunction with TP) groups were within the performance criteria range for androgenic or anti-androgenic effects, except for the following:

- The %CV for the weight of the glans penis in all groups ranged from 22.66% to 41.47% and exceeded the maximum values in the performance criteria for both androgenic (22%) and anti-androgenic (17%) effects.
- The %CV for the weight of the ventral prostate in the vehicle control group (51.73%) exceeded the maximum value in the performance criteria.
- The assay however was still considered acceptable for the following reasons:
- All other %CV values for androgenic or anti-androgenic effects in the vehicle control, TP positive control, flutamide/TP positive control and 125 mg/kg/day (administered alone or in conjunction with TP) groups were within the performance criteria range.
- The %CV for the ventral prostate in the vehicle control group was within the historical control range of %CV values for the conducting laboratory (11.2% to 60.2%).

2. Organ weight:

Phase1, androgenic activity:

- Mean weights of the five androgen-dependent tissues (levator ani-bulbocavernosus (LABC), seminal vesicles with coagulating glands and fluid (SVCGs), Cowper's glands (COW), ventral prostate (VP) and glans penis (GP) in the three spiroxamine dose groups did not differ statistically from the respective vehicle control group values.
- The mean weights of the adrenal gland in the three spiroxamine dose groups did not differ statistically from the respective vehicle control group values, confirming no involvement in the organ fulcrum to steroidogenesis were observed.
- When covariate analysis was conducted using the body weights recorded on DS 1 of study as the covariate, the mean tissue weight values did not differ statistically from the vehicle control group values.
- Coefficients of variation (CVs) for the five androgen-dependent tissues in the vehicle control and high-dose groups did not exceed the maximum allowable CVs specified in OPPTS 890.1400, with the following exceptions:
  - the GP in both the vehicle control group (39.39%), test article treated groups and the positive control group (range 25.58 – 41.47%);
  - ventral prostate in the vehicle control group (51.73%), which exceeded the maximum allowable CV (45%) by 6.73%. for the reasons discussed above, these data did not hinder study interpretation.

Positive control group:

- Increases or statistically significant increases in the mean weights of the five androgen-dependent tissues were observed in the testosterone propionate positive control group as compared with the vehicle control group values.

Phase II, anti-androgenic activity:

- There were no test article-related differences observed in the five androgen-dependent tissues in the 31.25, 62.5 and 125 mg/kg bw/day dose groups (compared with the positive control, testosterone propionate).
- The mean weights of the adrenal gland in the three spiroxamine dose groups did not differ statistically from the respective vehicle control group values, confirming no involvement in the organ fulcrum to steroidogenesis were observed.
- When covariate analysis was conducted using the body weights recorded on DS 1 of study as the covariate, the mean tissue weight value did not differ statistically from the vehicle control group value.
- CV for the five androgen-dependent tissues in the vehicle control and high-dose groups did not exceed the maximum allowable CVs specified in OPPTS 890.1400, with the following exceptions:
  - the GP in both the vehicle control group (39.39%), test article treated groups and the positive control group (range 25.58 – 41.47%);
  - ventral prostate in the vehicle control group (51.73%), which exceeded the maximum allowable CV (40%) by 11.73%. for the reasons discussed above, these data did not hinder study interpretation.

Positive control group:

- Reductions or statistically significant reductions in the mean weights of the five androgen-dependent tissues were observed (compared with the positive control group, testosterone propionate value).
- Statistically significantly higher mean kidney weights were noted in the positive control group compared to the TP positive control group; however, the value was within the laboratory historical control range (mean ± 3 S.D.).

Table CA 5.8.3/07-3: Overview of the Hershberger study in young castrated adult male rats treated orally (*via* gavage) with spiroxamine: Phase I, androgenic activity organ weights

Parameters		(mg/kg bw/day)				
		0	31.25	62.5	125	TP
No. of animals		6	6	6	6	6
Terminal bwt (g)		278 ±15.84	271 ±26.11 [↓2.37%]	273 ±22.35 [↓1.83%]	249 ±12.69 [↓10.48%]	283 ±20.05 [↑1.73%]
Adrenals	Abs (mg)	51.4 ±6.95	53.4 ±6.95 [↑3.86%]	47.0 ±4.54 [↓8.62%]	58.8 ±7.51 [↑14.33%]	52.6 ±4.58 [↓1.49%]
	C.V	13.52	13.01	9.66	12.78	8.71
Kidney	Abs (mg)	1812.7 ±140.03	1716.7 ±192.12 [↓5.30%]	1800.6 ±195.29 [↓0.67%]	1727.2 ±110.85 [↓4.71%]	1933.3 ±91.38 [↑6.65%]
	C.V	7.73	11.19	10.85	6.42	4.73
Liver	Abs (g)	10.9 ±0.67	10.2 ±1.71 [↓6.42%]	10.5 ±0.77 [↓3.86%]	9.3 ±0.77 [↓15.84%]	10.9 ±0.81 [↓0.52%]
	C.V	6.11	16.67	7.35	8.27	7.45
LABC	Abs (mg)	166.6 ±15.61	164.0 ±19.22 [↓1.58%]	151.8 ±29.16 [↓8.91%]	127.6 ±21.13 [↓23.41%]	371.8 ±123.13* [↑123.13%]
	C.V	9.37	11.72	19.21	16.56	19.98
SVCGs	Abs (mg)	70.1 ±11.72	79.4 ±23.59 [↑13.37%]	74.7 ±8.80 [↑6.64%]	74.7 ±16.97 [↑6.66%]	327.2 ±48.13* [↑366.98%]
	C.V	6.11	29.70	25.10	22.70	14.71
COW	Abs (mg)	6.5 ±1.78	5.9 ±3.31 [↓9.44%]	5.8 ±1.84 [↓11.99%]	5.5 ±1.46 [↓15.82%]	28.6 ±6.79* [↑123.13%]
	C.V	27.22	56.00	31.93	26.50	23.79
VP	Abs (mg)	20.0 ±20.32	18.6 ±13013 [↓6.93%]	13.8 ±4.82 [↓30.83%]	74.7 ±16.97 [↓12.70%]	112.0 ±27.62* [↑461.40%]
	C.V	**51.73**	70.77	25.16	22.70	24.66
GP	Abs (mg)	108.4 ±42.69	81.8 ±24.49 [↓24.52%]	96.0 ±24.57 [↓11.42%]	86.1 ±28.73 [↓20.57%]	153.8 ±63.79 [↑41.90%]
	C.V	**39.39**	**29.94**	**25.58**	**33.37**	**41.47**

* p <0.05 increase to concurrent vehicle control group
a. shared control with androgen antagonist assay
% difference of test article groups and positive control relative to the concurrent vehicle control
Values in bold exceeded maximum allowable CV for respective organ
TP: testosterone propionate 0.2 mg/kg bw/d administered *via* subcutaneous injection once daily from study days 1 to 10
Abs.: absolute
C.V.: coefficient of variation
LABC: levator ani-bulbocavernosus
SVCGs: seminal vesicles with fluids and coagulation gland
COW: Cowper's gland
VP: ventral prostate
GP: glans penis

Table CA 5.8.3/07-4: Overview of the Hershberger study in young castrated adult male rats treated orally (*via* gavage) with spiroxamine: Phase II anti-androgenic activity organ weights

Parameters		♂ (mg/kg bw/day)				
		0[a]	31.25	62.5	125	Flutamide/TP
No. of animals		6	6	6	6	6
Terminal bwt (g)		278 ±15.84	289 ±23.87 [↑2.22%]	284 ±29.92 [↑0.52%]	265 ±15.01 [↓6.27%]	286 ±27.07 [↑1.24%]
Adrenals	Abs (mg)	51.4 ±6.95	47.0 ±5.35 [↓10.64%]	47.8 ±4.47 [↓9.15%]	52.4 ±7.38 [↑0.44%]	51.8 ±9.07 [↓1.49%]
	C.V	13.52	11.35	992	14.09	17.50
Kidney	Abs (mg)	1812.7 ±140.03	1925.8 ±142.42 [↓0.39%]	1896.0 ±220.41 [↓1.93%]	1829.3 ±145.14 [↓5.38%]	1831.6 ±90.68* [↓5.26%]
	C.V	7.73	7.40	11.63	7.94	90.68
Liver	Abs (g)	10.9 ±0.67	10.9 ±1.33 [↑0.28%]	11.1 ±1.69 [↑2.27%]	9.8 ±0.85 [↓10.09%]	11.0 ±0.82 [↑1.50%]
	C.V	6.11	12.17	15.17	8.65	7.42
LABC	Abs (mg)	166.6 ±15.61	398.5 ±48.68 [↑7.18%]	388.5 ±84.54 [↑4.49%]	346.7 ±24.15 [↓6.77%]	232.2 ±29.56* [↓37.55%]
	C.V	9.37	12.22	21.76	6.97	12.73
SVCGs	Abs (mg)	70.1 ±11.72	368.4 ±109.15 [↑12.58%]	342.8 ±55.09 [↑4.78%]	319.8 ±46.44 [↓2.27%]	94.4 ±14.09* [↓71.14%]
	C.V	6.11	29.63	16.07	14.52	14.92
COW	Abs (mg)	9.5 ±1.78	33.1 ±10.06 [↑16.05%]	27.8 ±3.68 [↓2.57%]	32.2 ±4.27 [↑12.84%]	9.5 ±1.83* [↓66.61%]
	C.V	27..22	30.36	13.23	13.27	19.18
VP	Abs (mg)	20.0 ±20.32	121.3 ±44.72 [↑8.32%]	138.4 ±8.35 [↑23.60%]	147.3 ±22.07 [↑31.47%]	31.8 ±12.49* [↓71.65%]
	C.V	**51.73**	36.86	8.35	14.99	39.35
GP	Abs (mg)	108.4 ±42.69	175.3 ±59.43 [↑13.96%]	136.6 ±41.52 [↑11.18%]	165.2 ±42.62 [↑7.32%]	123.6 ±28.01 [↓19.64%]
	C.V	**39.39**	**33.90**	**30.39**	**25.81**	**22.66**

* p <0.05 increase to TP control group
a. shared control with androgen agonist assay
% difference of test article groups and positive control, flutamide/TP relative to the TP positive control
Flutamide: 3 mg/kg bw/d administered *via* oral gavage once daily from study days 1 to 10
Abs.: absolute
Rel.: relative (to body weight)
C.V.: coefficient of variation
LABC: levator ani-bulbocavernosus
SVCGs: seminal vesicles with fluids and coagulation gland
COW: Cowper's gland
VP: ventral prostate
GP: glans penis

2. Gross pathology:	No test article related effects were observed in animals from either phase of the study.
3. Histopathology:	Not conducted.

G. Deficiencies:

None.

H. Discussion:

The androgenic, anti-androgenic or 5α-reductase inhibitory effects of spiroxamine were evaluated using the Hershberger Assay. Animals (castrated by the supplier) selected for the study were allocated to each group by stratified randomization method based on the body weight. The study was split into two phases:

- Phase I: androgenic activity assessment
- Phase II: anti-androgenic activity assessment

The test article was formulated in 1% methyl cellulose, with all animals receiving a single oral gavage dose at 0, 31.25, 62.5, 125 mg/kg bw/day for 10 consecutive days from PND 60, using a dose volume of 5 mL/kg. Additional positive control groups were included in each phase of the study:

- Phase I: Testosterone propionate administered once daily for 10 consecutive days *via* subcutaneous injection at 0.4 mg/kg bw/day employing a dose volume of 0.5 mg/kg bw
- Phase II: Flutamide administered once daily for 10 consecutive days *via* oral gavage at 3 mg/kg bw/day, employing a dose volume of 5 mg/kg bw.

There were no significant increases or decreases in the mean weights of the five androgen-dependent tissues (LABC, SVCGs, COW, VP and LGP) in the three spiroxamine dose groups from either Phase I (androgenic effects) or Phase II (anti-androgenic effects).

Assessment and conclusions by applicant:

Assessment: Study meets the current guidance and the requirements in 283/2013.

Conclusion: Under the conditions of this study, spiroxamine did not cause any treatment related changes in reproductive/endocrine organ weights in the Hershberger assay when administered to castrated male rats up to a maximum dose of 125 mg/kg bw/day (deemed to be a maximum tolerated dose). Therefore, it is concluded that spiroxamine did not exhibit androgenic, anti-androgenic or 5α-reductase inhibitory properties under the conditions of this study.

T-modality

Data Point:	KCA 5.8.3/08
Report Author:	[redacted]
Report Year:	2021
Report Title:	Spiroxamine: US EPA ToxCast data retrieval for the T-modality
Report No:	0471836-TOX7
Document No:	M-762778-01-1
Guideline(s) followed in study:	None
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted -
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	Supportive only

Executive Summary

Whilst it is recognised that the ToxCast/Tox21 screening assays for the major molecular initiating events involved in thyroid perturbation (Na-I symporter modulation, TPO release, transport or thyroid hormones, T4 to T3 conversion by deiodinases, increased systemic clearance) are not covered in the high throughput screening assays, the ToxCast/Tox21 data includes coverage of direct thyroid receptor mediated, thyroid hormone-related hepatic catabolism are available. The available data is considered as supplementary data only.

Of the HTS assays, two models confirmed thyroid activity which require further discussion. The LTEA_HepaRG_THRSP_dn, a metabolically competent HepaRG cell culture mode examining alterations in transcription activity with an AC_{50} value of 0.33 µM obtained. The Tox21_TR_LUC_GH3_Antagonist, an inducible endogenous thyroid hormone receptor transcription factor activity detected by luciferase-fusion response element in rat pituitary gland GH3 cell line as regulated by the human THR-α and THR-β in antagonist mode confirmed activity with an AC50 value of 66.56 µM. However, the biological relevance of these results are low as activity was observed only at a concentration that occurred in the presence of cytotoxic, which was observed at 15.32 µM.

A computational network model based on ToxCast/Tox21 data has not been developed for the thyroid.

In conclusion, negative results in all HTS TR-associated assays provides a strong mechanistic argument against a direct interaction of spiroxamine with thyroid or anti-thyroid pathways. In accordance with the ECHA guidance on endocrine disruption, the Toxcast HTS TR-associated assays detailing an absence of thyroid effects is not deemed sufficient to address the T-modality. However, the apical mammalian toxicity studies undertaken confirm that the thyroid gland is not a target organ, with no adverse histopathology reported in these studies. Therefore it is concluded that spiroxamine is devoid of T-modality perturbations.

Results

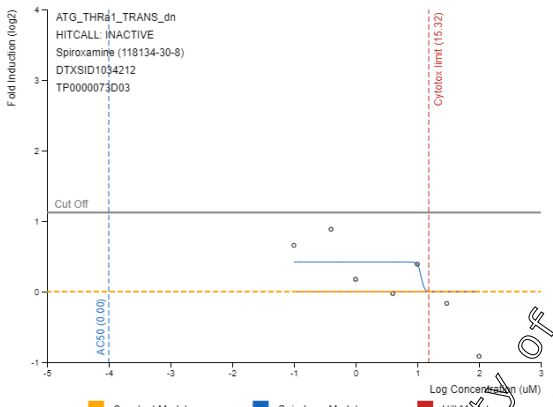
Table CA 5.8.3/08-1: Overview of ToxCast models and data output for the T-modality

ToxCast model	Assay description	T / A-T[a]	Chosen model and outputs	Figure
Data retrieved from US EPA Endocrine: EDSP universe of chemicals website (https://comptox.epa.gov/dashboard/dsstoxdb/results?abbreviation=EDSPUOC&search=DTXSID1034232#details) 12 August 2020				
ATG_THRa1_TRANS_dn	Inducible TR transcription activity detected with fluorescence intensity signals by RT-PCR of mRNA reporter sequence unique to the transfected trans-acting reporter gene and exogenous transcription factor GAL4-THRα [known as human thyroid hormone receptor, α]) in HepG2 liver cells exposed for 24 h.	T	Constant model. AIC: 14.75; RMSE: 0.75; AC_{50}: n/a; Cytotoxicity: 15.32 µM	CA 5.8.3/08-1
ATG_THRa1_TRANS_up		T	Constant model. AIC: 14.75; RMSE: 0.75; AC_{50}: n/a; Cytotoxicity: 15.32 µM	CA 5.8.3/08-2
LTEA_HepaRG_THRSP_dn	Metabolically-competent HepaRG cell cultures were exposed for 48 h before cytotoxicity (LDH) and alterations in transcription were assessed.	T	Gain-loss model: AIC: 20.79; RMSE: 0.31; AC_{50}: 0.33 µM; Cytotoxicity: 15.32 µM	CA 5.8.3/08-3
LTEA_HepaRG_THRSP_up		T	Constant model. AIC: 43.19; RMSE: 0.86; AC_{50}: n/a; Cytotoxicity: 15.32 µM	CA 5.8.3/08-4
Tox21_TR_LUC_GH3_Agonist	Inducible endogenous thyroid hormone receptor transcription factor activity detected by luciferase-fusion response element in rat pituitary gland GH3 cell line as regulated by the human THR-α and THR-β in agonist mode for 28 h	T	Hill model: AIC: 125.81; RMSE: 0.94; AC_{50}: 0 uM; Cytotoxicity: 15.32 µM	CA 5.8.3/08-5
Tox21_TR_LUC_GH3_Antagonist	Inducible endogenous thyroid hormone receptor transcription factor activity detected by luciferase-fusion response element in rat pituitary gland GH3 cell line as regulated by the human THR-α and THR-β in antagonist mode for 28 h	A-T	Hill model: AIC: 297.15; RMSE: 16.67; AC_{50}: 66.56 uM; Cytotoxicity: 15.32 µM	CA 5.8.3/08-6
TOX21_TSHR_Agonist_ratio	Inducible reporter gene examining mapping to the TSHR gene in HEK293T (human kidney cell line) through increased cAMP generation in agonist mode for 0.5 h	T	Constant model. AIC: 307.8; RMSE: 8.15; AC_{50}: n/a; Cytotoxicity: 15.32 µM	CA 5.8.3/08-7
TOX21_TSHR_Anagonist_ratio	Inducible reporter gene examining mapping to the TSHR gene in HEK293T (human kidney cell line) through increased cAMP generation in antagonist mode for 0.5 h	A-T	Constant model. AIC: 191.51; RMSE: 2.15; AC_{50}: n/a; Cytotoxicity: 15.32 µM	CA 5.8.3/08-8
TOX21_TSHR_wt_ratio	Inducible TR transcription activity detected with fluorescence intensity signals by targeting background cAMP generation in cells lacking TSHR in HEK293T (human kidney cell line) for 0.5 h	A-T	Constant model. AIC: 265.03; RMSE: 4.47; AC_{50}: n/a; Cytotoxicity: 15.32 µM	CA 5.8.3/08-9

a: thyroid (T) or anti-thyroid (A-T) signal detection

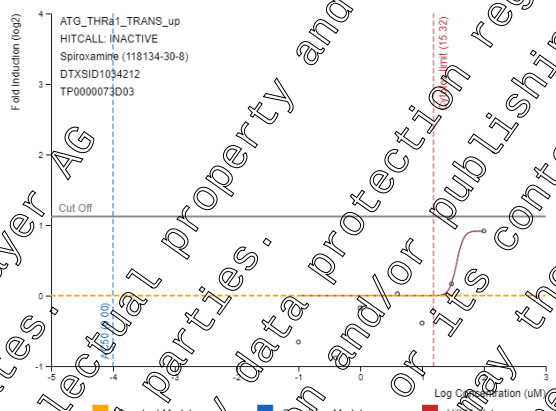
AIC: Akaike Information criteria: appropriate model set on the lowest AIC value, essential model that best fits the data
RMSE: median root mean squared error across all winning models. RMSE is lower for higher performance models

Figure CA 5.8.3/08-1:
ATG_THRa1_TRANS_dn



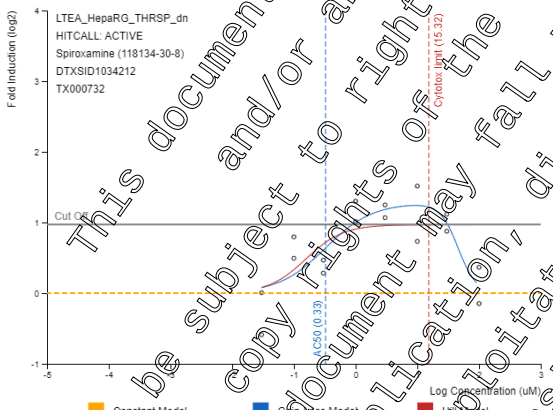
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	14.75	0.57	-	-	-
	Gain-Loss	20.92	0.45	0.42	0.02	7.34
	Hill	20.75	0.57	0	114.4	0.93

Figure CA 5.8.3/08-2:
ATG_THRa1_TRANS_up



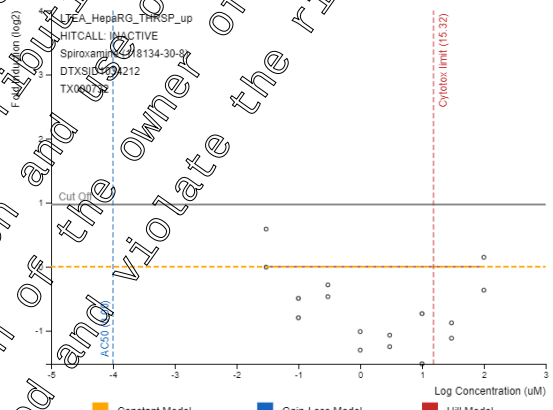
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	14.75	0.57	-	-	-
	Gain-Loss	20.92	0.45	0.42	36.19	8
	Hill	20.75	0.57	0	36.19	8

Figure CA 5.8.3/08-3:
LTEA_HepaRG_THRSP_dn



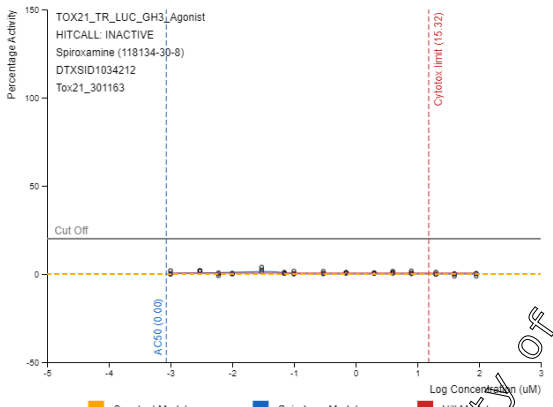
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
	Constant	45.19	0.86	-	-	-
✓	Gain-Loss	20.92	0.31	1.28	0.33	1.14
	Hill	27.92	0.31	1.28	0.11	1.14

Figure CA 5.8.3/08-4:
LTEA_HepaRG_THRSP_up



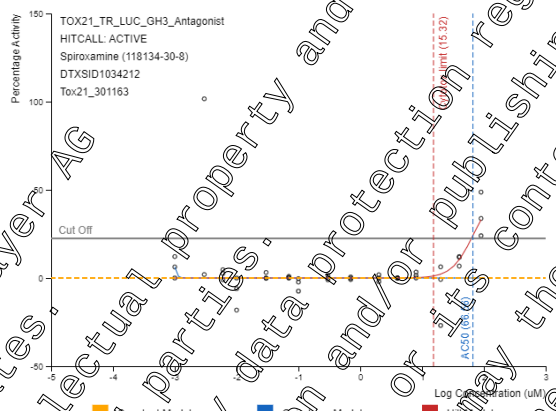
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	45.19	0.86	-	-	-
	Gain-Loss	55.19	0.86	0	0.69	1.14
	Hill	51.19	0.86	0	0.43	1.14

Figure CA 5.8.3/08-5:
Tox21_TR_LUC_GH3_Agonist



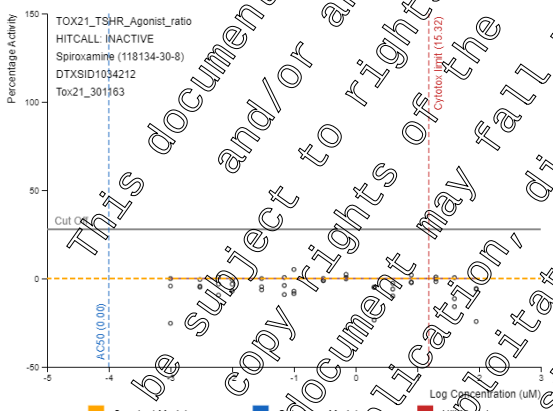
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
	Constant	137.2	1.13	-	-	-
	Gain-Loss	134.25	0.92	2.95	0.04	0.56
✓	Hill	125.81	0.94	0.53	0	8

Figure CA 5.8.3/08-6:
Tox21_TR_LUC_GH3_Antgonist



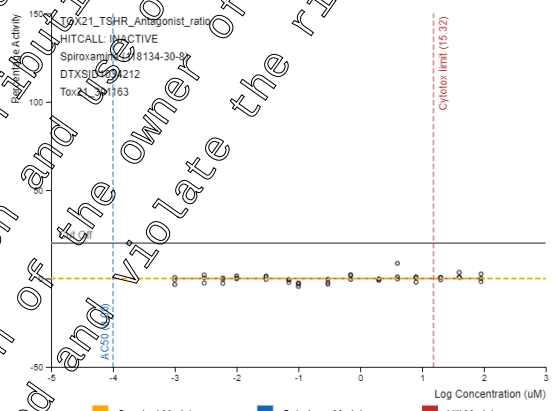
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
	Constant	334.92	19.18	-	-	-
	Gain-Loss	348.09	19.18	49.95	0	0.35
✓	Hill	283.4	15.67	49.95	66.58	2.54

Figure CA 5.8.3/08-7:
TOX21_TSHR_Agonist_ratio



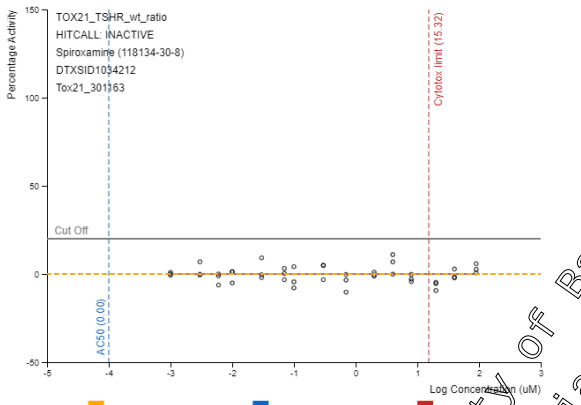
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	307.8	8.15	-	-	-
	Gain-Loss	0	0	0	1	0
	Hill	0	0	0	1	0

Figure CA 5.8.3/08-8:
TOX21_TSHR_Antagonist_ratio



Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	191.51	2.15	-	-	-
	Gain-Loss	0	0	0	1	0
	Hill	0	0	0	1	0

Figure CA 5.8.3/08-9:
TOX21_TSHR_wt_ratio



Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	265.03	4.47	-		
	Gain-Loss	0	0	0	1	0
	Hill	0	0	0	1	0

Of the HTS assays, two models confirmed thyroid activity, which require further discussion. The LTEA_HepaRG_THRSP_dn, a metabolically competent HepaRG cell culture mode examining alterations in transcription activity with an AC50 value of 0.334µM obtained. The Tox21_TR_LUC_GH3_Antagonist, an inducible endogenous thyroid hormone receptor transcription factor activity detected by luciferase-fusion response element in rat pituitary gland GH3 cell line as regulated by the human THR-α and THR-β in antagonist mode confirmed activity with an AC50 value of 66.56 µM. However, the biological relevance of these results are low as activity was observed only at a concentration that occurred in the presence of cytotoxic, which was observed at 15.32 µM.

A computational network model based on ToxCast/Tox21 data has not been developed for the thyroid.

Assessment and conclusions by applicant:

Assessment: This study is considered supplemental only.

Conclusion: In conclusion, negative results in all HTS TR-associated assays provides a strong mechanistic argument against a direct interaction of spiroxamine with thyroid or anti-thyroid pathways. In accordance with the ECHA guidance on endocrine disruption, the ToxCast HTS TR-associated assays detailing an absence of thyroid effects is not deemed sufficient to address the T-modality. However, the apical mammalian toxicity studies undertaken confirm that the thyroid gland is not a target organ, with no adverse histopathology reported in these studies. Therefore it is concluded that spiroxamine is devoid of T-modality perturbations.

S-modality

Data Point:	KCA 5.8.3/09
Report Author:	[redacted]
Report Year:	2021
Report Title:	Spiroxamine: US EPA ToxCast data retrieval for the S-modality
Report No:	0471836-TOX8
Document No:	M-762780-01-1
Guideline(s) followed in study:	None
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	Supportive only

Executive Summary

From the US EPA ToxCast data, of the HTS assays, 6 models confirmed steroidogenesis perturbations, which require further discussion. Of the mineralocorticoids, two assays CEETOX_H295R_DOC_dn (CA 5.8.3/09-2) and CEETOX_H295R_PROG_dn (Figure CA 5.8.3/09-4) confirmed AC_{50} values of 0.77 µM and 0.37 µM, respectively. Both models utilise H295 cells, with the former model examining the ability to inhibit 11-deoxycorticosterone and the latter model examining the ability to inhibit progesterone.

Of the glucocorticoids, CEETOX_H295R_11DCORT_dn (Figure CA 5.8.3/09-8) and CEETOX_H295R_OHPROG_dn (Figure CA 5.8.3/09-12) confirmed AC_{50} values of 0.18 µM and 0.15 µM, respectively. Both models utilise H295 cells, with CEETOX_H295R_11DCORT_dn examining the inhibition of 11-deoxycortisol and CEETOX_H295R_OHPROG_dn examining the inhibition of inhibit 17α-hydroxyprogesterone.

The androgen model, CEETOX_H295R_ANDR_dn, examining the inhibit androstenedione in H295 cells, with an AC_{50} value of 0.21 µM. CEETOX_H295R_TESTO_dn, examining the inhibit testosterone in H295 cells confirmed an AC_{50} of 0.88 µM. In all six cases, the AC_{50} values obtained were in the absence of overt cytotoxicity (cut off 15.32 µM).

A computational network model based on ToxCast/Tox21 data has not been developed for the steroidogenesis pathway.

In conclusion, the US EPA ToxCast data suggest that spiroxamine disrupts steroidogenesis in HTS *in vitro* assays, however no direct interference of androgen or oestrogen receptor activity was observed in toerh ED studies.

Results

Of the HTS assays, 6 models confirmed steriodgenesis perturbations, which require further discussion. Of the mineralocorticoids, two assays CEETOX_H295R_DOC_dn (Figure CA 5.8.3/09-2) and CEETOX_H295R_PROG_dn (Figure CA 5.8.3/09-4) confirmed AC_{50} values of 0.77 µM and 0.37 µM, respectively. Both models utilise H295 cells, with the former model examing the ability to inhibit 11-deoxycorticosterone and the latter model examining the ability to inhibit progesterone.

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A computational network model based on ToxCast/Tox21 data has not been developed for the steroidogenesis pathway.

Table CA 5.8.3/09-1: Overview of ToxCast models and data output for the S-modality

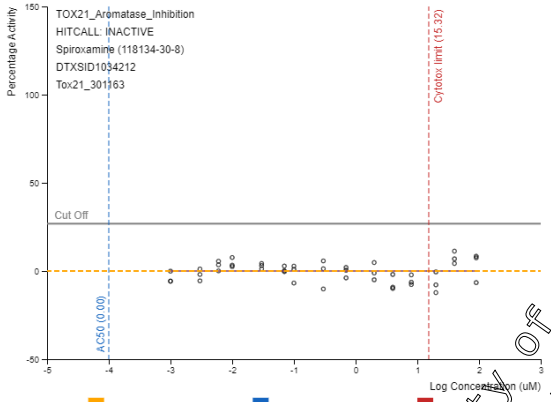
ToxCast model	Assay description	Response effect	Chosen model and outputs	Figure
Data retrieved from US EPA Endocrine: EDSP universe of chemicals website (https://comptox.epa.gov/dashboard/dsstoxdb/results?abbreviation=EDSPUOC&search=DTXSID1034212#details) 12 August 2020				
TOX21_Aromatase_Inhibition	Assay for inhibition of aromatase using MCF-7, a human breast cell line aro ERE cells stably-transfected with a testosterone receptor-responsive luciferase reporter following 24 h	-ve	Constant model. AIC: 288.28; RMSE: 5.55; AC_{50}: n/a; Cytotoxicity: 15.32 µM	CA 5.8.3/09-1
Mineralocorticoids				
CEETOX_H295R_DOC_dn	Assessment of potential to inhibit 11-deoxycorticosterone in H295 cells	+ve	Hill model. AIC: -2.99; RMSE: 0.14; AC_{50}: 0.77 uM; Cytotoxicity: 15.32 µM	CA 5.8.3/09-2
CEETOX_H295R_DOC_up	Assessment of potential to stimulate 11-deoxycorticosterone in H295 cells	-ve	Constant model. AIC: 42.61; RMSE: 1.18; AC_{50}: n/a; Cytotoxicity: 15.32 µM	CA 5.8.3/09-3
CEETOX_H295R_PROG_dn	Assessment of potential to inhibit progesterone in H295 cells	+ve	Gain-loss model: AIC: -103.43; RMSE: 0.04; AC_{50}: 0.37 µM; Cytotoxicity: 15.32 µM	CA 5.8.3/09-4
CEETOX_H295R_PROG_up	Assessment of potential to stimulate progesterone in H295 cells	-ve	Constant model. AIC: 6832.47; RMSE: 1.11; AC_{50}: n/a; Cytotoxicity: 15.32 µM	CA 5.8.3/09-5
CEETOX_H295R_CORTIC_dn	Assessment of potential to inhibit corticosterone in H295 cells	-ve	Constant model. AIC: 6729.69; RMSE: 0.46; AC_{50}: n/a; Cytotoxicity: 15.32 µM	CA 5.8.3/09-6
CEETOX_H295R_CORTIC_up	Assessment of potential to inhibit corticosterone in H295 cells	-ve	Constant model. AIC: 6729.69; RMSE: 0.46; AC_{50}: n/a; Cytotoxicity: 15.32 µM	CA 5.8.3/09-7
Glucorticoids				
CEETOX_H295R_11DCORT_dn	Assessment of potential to inhibit 11-deoxycortisol in H295 cells	+ve	Hill model. AIC: -34.92; RMSE: 0.04; AC_{50}: 0.18 µM; Cytotoxicity: 15.32 µM	CA 5.8.3/09-8

ToxCast model	Assay description	Response effect	Chosen model and outputs	Figure
CEETOX_H295R_11DCORT_up	Assessment of potential to stimulate 11-deoxycortisol in H295 cells	-ve	Constant model. AIC: 42.87; RMSE: 1.18; AC_{50}: n/a; Cytotoxicity: 15.32 µM	CA 5.8.3/09-9
CEETOX_H295R_OHPREG_dn	Assessment of potential to inhibit 17α-hydroxypregnenolone in H295 cells	-ve	Constant model. AIC: 6839.01; RMSE: 1.13; AC_{50}: n/a; Cytotoxicity: 15.32 µM	CA 5.8.3/09-10
CEETOX_H295R_OHPREG_up	Assessment of potential to stimulate 17α-hydroxypregnenolone in H295 cells	-ve	Constant model. AIC: 6839.01; RMSE: 1.13; AC_{50}: n/a; Cytotoxicity: 15.32 µM	CA 5.8.3/09-11
CEETOX_H295R_OHPROG_dn	Assessment of potential to inhibit 17α-hydroxyprogesterone in H295 cells	+ve	Hill model. AIC: -19.84; RMSE: 0.08; AC_{50}: 0.15 µM; Cytotoxicity: 15.32 µM	CA 5.8.3/09-12
CEETOX_H295R_OHPROG_up	Assessment of potential to stimulate 17α-hydroxyprogesterone in H295 cells	-ve	Constant model. AIC: 59.07; RMSE: 2.32; AC_{50}: n/a; Cytotoxicity: 15.32 µM	CA 5.8.3/09-13
CEETOX_H295R_CORTISOL_dn	Assessment of potential to inhibit cortisol in H295 cells	-ve	Hill model. AIC: -12.98; RMSE: 0.11; AC_{50}: 0.23 µM; Cytotoxicity: 15.32 µM	CA 5.8.3/09-14
CEETOX_H295R_CORTISOL_up	Assessment of potential to stimulate cortisol in H295 cells	-ve	Constant model. AIC: 22.71; RMSE: 0.52; AC_{50}: n/a; Cytotoxicity: 15.32 µM	CA 5.8.3/09-15
Androgens				
CEETOX_H295R_ANDR_dn	Assessment of potential to inhibit androstenedione in H295 cells	+ve	Hill model. AIC: -10.25; RMSE: 0.12; AC_{50}: 0.21 µM; Cytotoxicity: 15.32 µM	CA 5.8.3/09-16
CEETOX_H295R_ANDR_up	Assessment of potential to stimulate androstenedione in H295 cells	-ve	Constant model. AIC: 46.22; RMSE: 1.28; AC_{50}: n/a; Cytotoxicity: 15.32 µM	CA 5.8.3/09-17
CEETOX_H295R_TESTO_dn	Assessment of potential to inhibit testosterone in H295 cells	+ve	Hill model. AIC: -13.79; RMSE: 0.1; AC_{50}: 0.88 µM; Cytotoxicity: 15.32 µM	CA 5.8.3/09-18
CEETOX_H295R_TESTO_up	Assessment of potential to stimulate testosterone in H295 cells	-ve	Constant model. AIC: 47.45; RMSE: 1.44; AC_{50}: n/a; Cytotoxicity: 15.32 µM	CA 5.8.3/09-19

ToxCast model	Assay description	Response effect	Chosen model and outputs	Figure
Oestrogens				
CEETOX_H295R_ESTRADIOL_dn	Assessment of potential to inhibit oestradiol in H295 cells	-ve	Constant model. AIC: 21.02; RMSE: 0.53; AC_{50}: n/a; Cytotoxicity: 15.32 µM	CA 5.8.3/09-20
CEETOX_H295R_ESTRADIOL_up	Assessment of potential to stimulate oestradiol in H295 cells	-ve	Hill model. AIC: -6.21; RMSE: 0.13; AC_{50}: 21.75 µM; Cytotoxicity: 15.32 µM	CA 5.8.3/09-21
CEETOX_H295R_ESTRONE_dn	Assessment of potential to inhibit oestrone in H295 cells	-ve	Constant model. AIC: 15.4; RMSE: 0.4; AC_{50}: n/a; Cytotoxicity: 15.32 µM	CA 5.8.3/09-22
CEETOX_H295R_ESTRONE_up	Assessment of potential to stimulate oestrone in H295 cells	-ve	Hill model. AIC: 2.03; RMSE: 0.15; AC_{50}: 16.24 µM; Cytotoxicity: 15.32 µM	CA 5.8.3/09-23

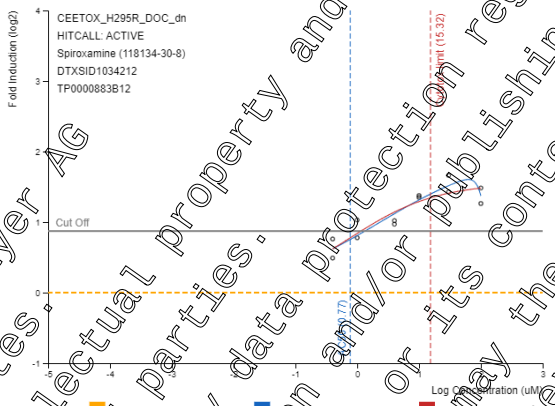
AIC: Akaike Information criteria: appropriate model set on the lowest AIC value, essential model that best fits the data
RMSE: median root mean squared error across all winning models. RMSE is lower for higher performance models

Figure CA 5.8.3/09-1:
TOX21_Aromatase_Inhibition



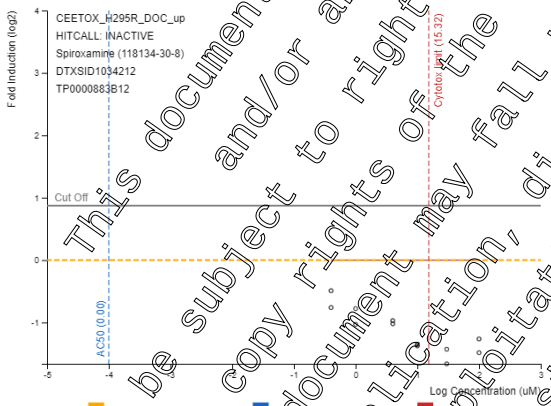
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	288.28	5.55	-	-	-
	Gain-Loss	0	0	0	1	0
	Hill	0	0	0	1	0

Figure CA 5.8.3/09-2:
CEETOX_H295R_DOC_dn



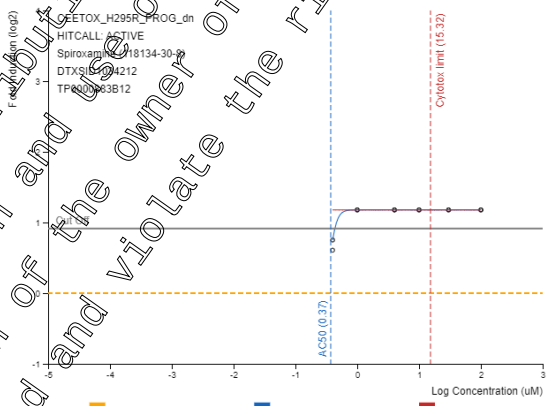
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
	Constant	42.61	1.18	-	-	-
	Gain-Loss	-2.51	0.12	1.95	2.33	0.45
✓	Hill	-4.51	0.11	1.95	0.71	0.62

Figure CA 5.8.3/09-3:
CEETOX_H295R_DOC_up



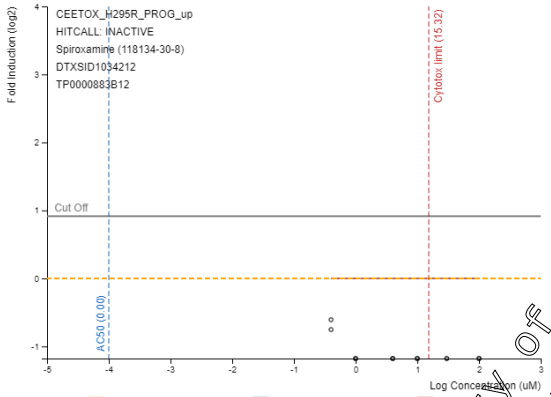
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	42.61	1.18	-	-	-
	Gain-Loss	1	1	1	1	1
	Hill	0	1	1	1	1

Figure CA 5.8.3/09-4:
CEETOX_H295R_PROG_dn



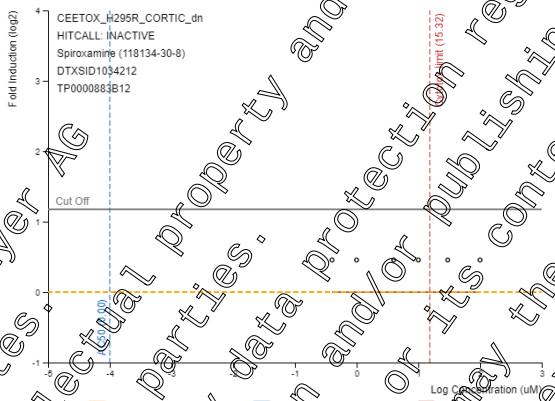
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
	Constant	6832.47	1.11	-	-	-
✓	Gain-Loss	-103.43	0.04	1.18	0.37	8
	Hill	-96.96	0.21	1.18	0.04	8

Figure CA 5.8.3/09-5:
CEETOX_H295R_PROG_up



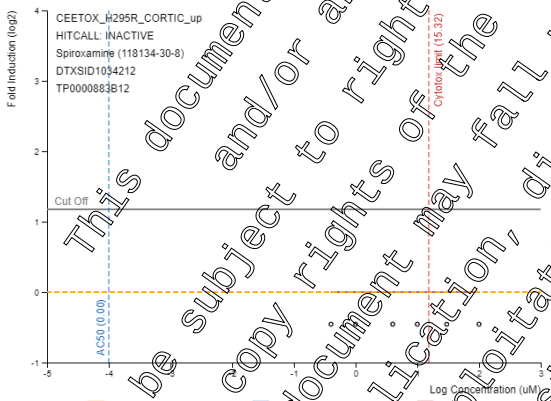
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	6832.47	1.11	-	-	-
	Gain-Loss	0	0	0	1	0
	Hill	0	0	0	1	0

Figure CA 5.8.3/09-6:
CEETOX_H295R_CORTIC_dn



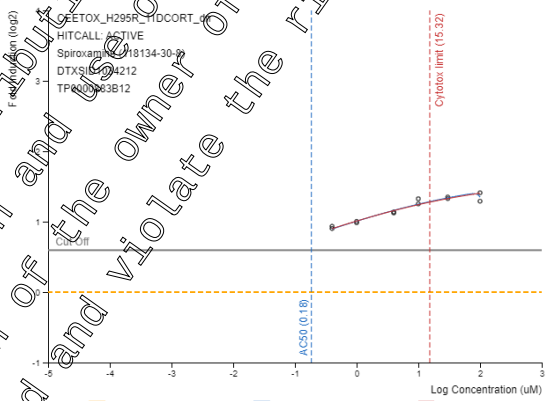
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	6729.69	0.46	-	-	-
	Gain-Loss	0	0	0	1	0
	Hill	0	0	0	1	0

Figure CA 5.8.3/09-7:
CEETOX_H295R_CORTIC_up



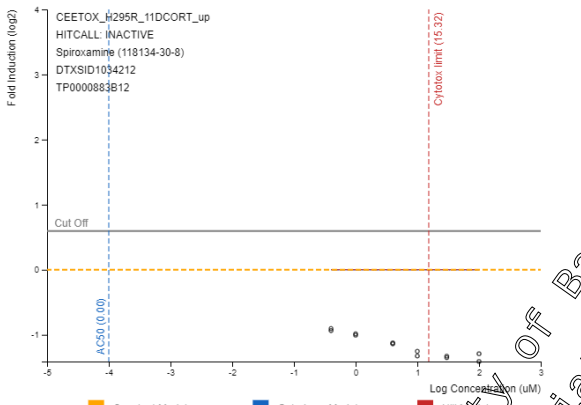
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	6729.69	0.46	-	-	-
	Gain-Loss	0	0	0	1	0
	Hill	0	0	0	1	0

Figure CA 5.8.3/09-8:
CEETOX_H295R_11DCORT_dn



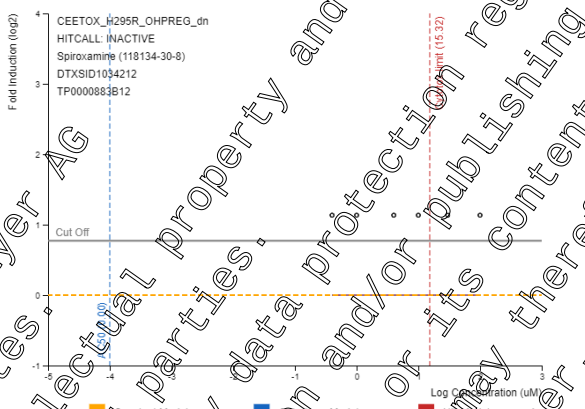
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
	Constant	42.87	1.18	-	-	-
	Gain-Loss	-32.01	0.04	1.64	0.21	0.3
✓	Hill	-34.92	0.04	1.62	0.18	0.3

Figure CA 5.8.3/09-9:
CEETOX_H295R_11DCORT_up



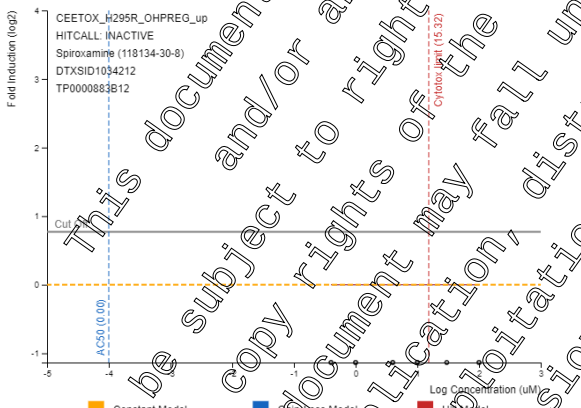
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	42.87	1.18	-	-	-
	Gain-Loss	0	0	0	1	0
	Hill	0	0	0	1	0

Figure CA 5.8.3/09-10:
CEETOX_H295R_OHPREG_dn



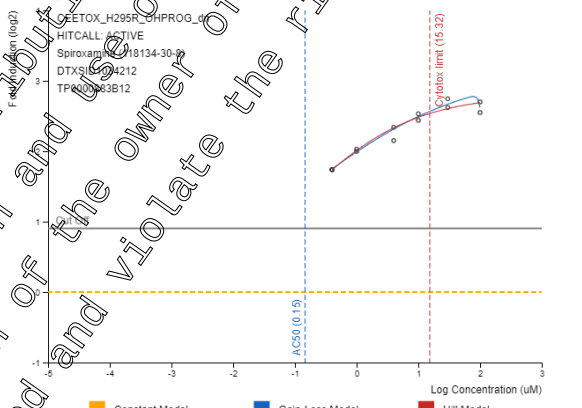
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	6839.01	1.13	-	-	-
	Gain-Loss	0	0	0	1	0
	Hill	0	0	0	1	0

Figure CA 5.8.3/09-11:
CEETOX_H295R_OHPREG_up



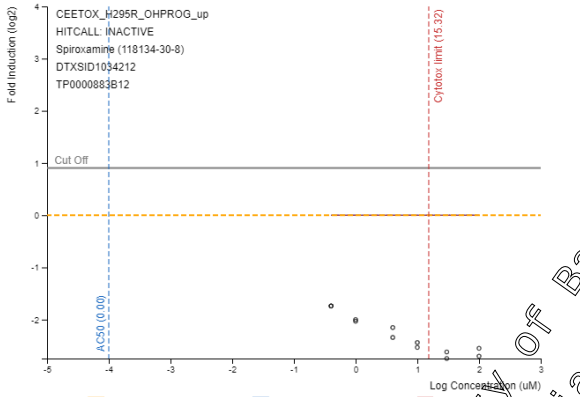
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	6839.01	1.13	-	-	-
	Gain-Loss	0	0	0	1	0
	Hill	0	0	0	1	0

Figure CA 5.8.3/09-12:
CEETOX_H295R_OHPROG_dn



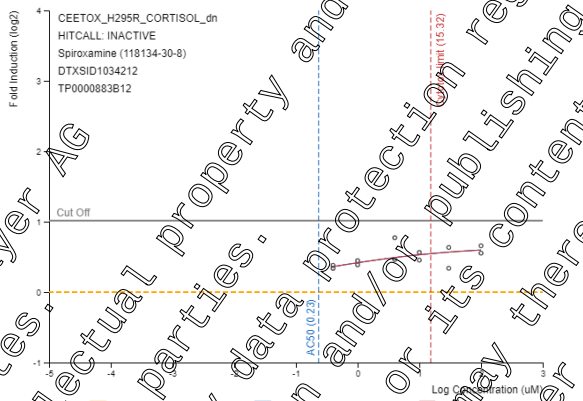
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
	Constant	59.07	2.32	-	-	-
	Gain-Loss	-18.8	0.07	3.14	0.21	0.35
✓	Hill	-19.84	0.08	2.8	0.15	0.48

Figure CA 5.8.3/09-13: CEETOX_H295R_OHPROG_up



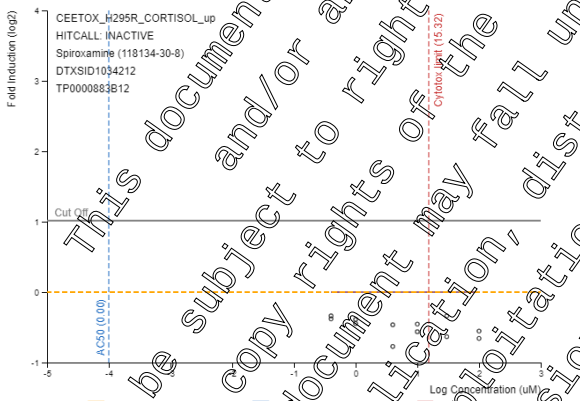
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	59.07	2.32	-	-	-
	Gain-Loss	0	0	0	1	0
	Hill	0	0	0	1	0

Figure CA 5.8.3/09-14: CEETOX_H295R_CORTISOL_dn



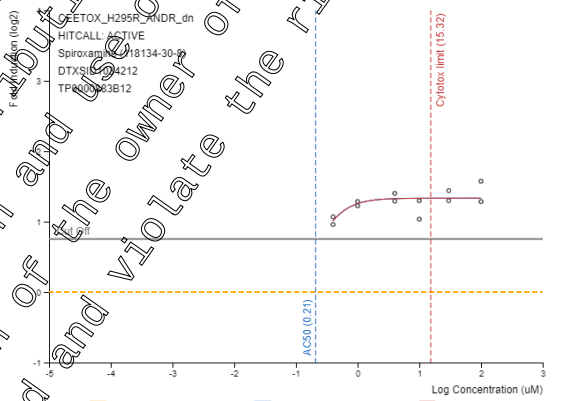
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
	Constant	22.71	0.52	-	-	-
	Gain-Loss	-8.86	0.14	0.66	0.23	0.38
✓	Hill	-10.86	0.12	0.66	0.23	0.38

Figure CA 5.8.3/09-15: CEETOX_H295R_CORTISOL_up



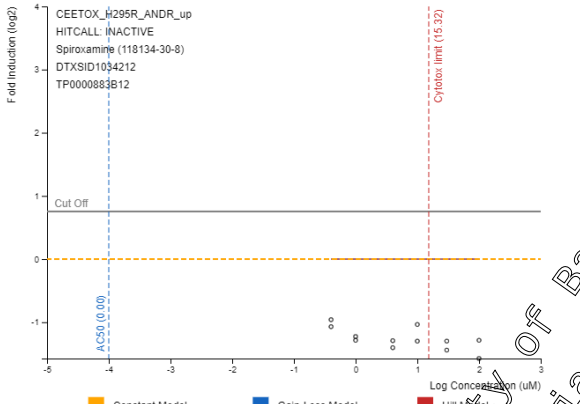
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	22.71	0.52	-	-	-
	Gain-Loss	0	0	0	1	0
	Hill	0	0	0	1	0

Figure CA 5.8.3/09-16: CEETOX_H295R_ANDR_dn



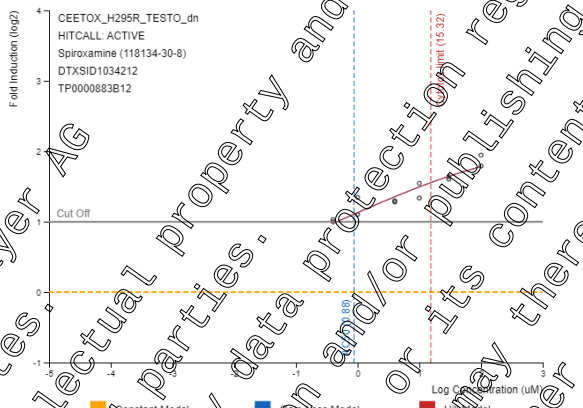
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
	Constant	46.22	1.28	-	-	-
	Gain-Loss	-6.25	0.12	1.33	0.21	1.78
✓	Hill	-10.25	0.12	1.33	0.21	1.78

Figure CA 5.8.3/09-17: CEETOX_H295R_ANDR_up



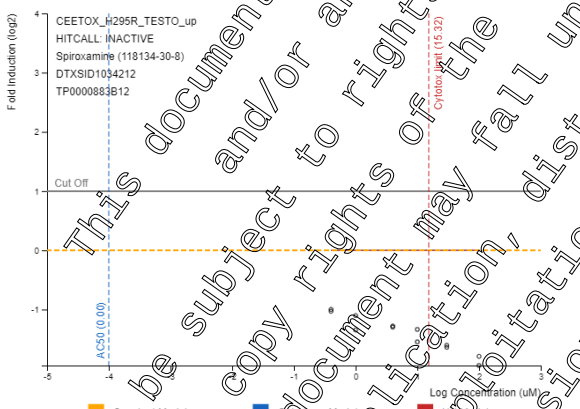
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	46.22	1.28	-	-	-
	Gain-Loss	0	0	0	1	0
	Hill	0	0	0	1	0

Figure CA 5.8.3/09-18: CEETOX_H295R_TESTO_dn



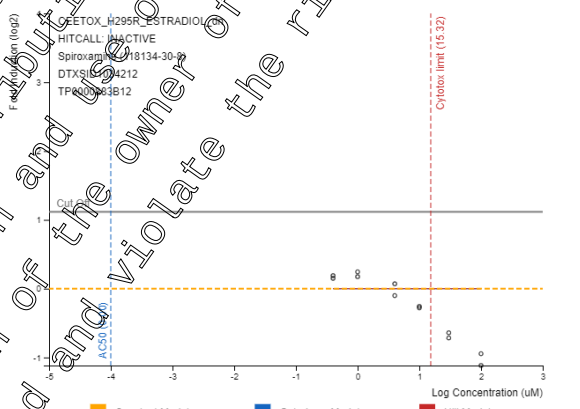
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
	Constant	47.45	1.44	-	-	-
	Gain-Loss	9.36	0.1	2.26	0.88	0.3
✓	Hill	11.36	0.1	2	0.88	0.3

Figure CA 5.8.3/09-19: CEETOX_H295R_TESTO_up



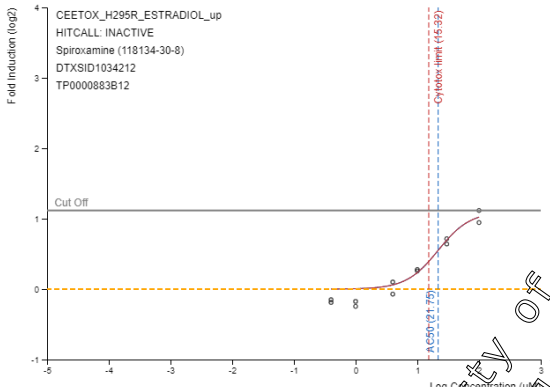
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	47.45	1.44	-	-	-
	Gain-Loss	0	0	0	1	0
	Hill	0	0	0	1	0

Figure CA 5.8.3/09-20: CEETOX_H295R_ESTRADIOL_dn



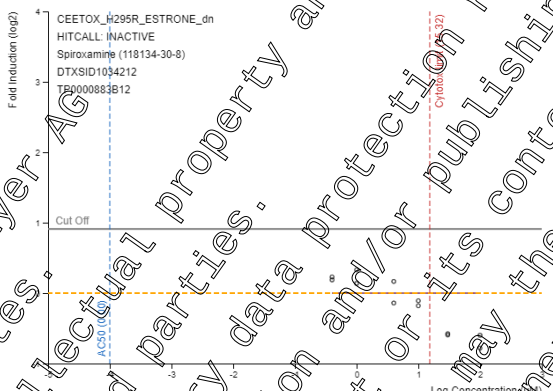
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	21.02	0.53	-	-	-
	Gain-Loss	31.02	0.53	0	51.73	1.32
	Hill	27.02	0.53	0	45.49	1.29

Figure CA 5.8.3/09-21:
CEETOX_H295R_ESTRADIOL_up



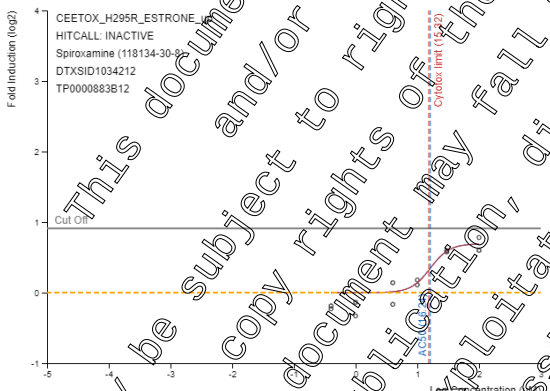
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
	Constant	21.02	0.53	-	-	-
	Gain-Loss	-2.21	0.13	1.1	21.75	1.69
✓	Hill	-6.21	0.13	1.1	21.75	2.1

Figure CA 5.8.3/09-22:
CEETOX_H295R_ESTRONE_dn



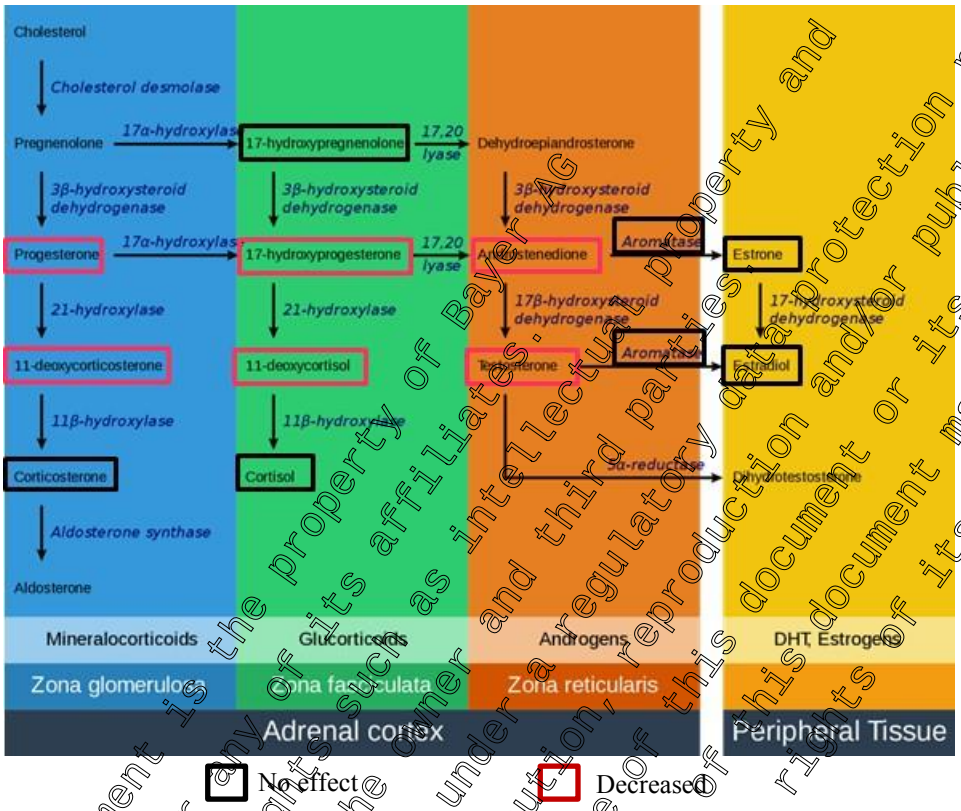
Winning Model	Model	AIC	RMSE	Top	AC50	Slope
✓	Constant	19.4	0.4	-	-	-
	Gain-Loss	23.4	0.4	0	33.46	1.67
	Hill	21.4	0.4	0	66.09	0.75

Figure CA 5.8.3/09-23:
CEETOX_H295R_ESTRONE_up



Winning Model	Model	AIC	RMSE	Top	AC50	Slope
	Constant	15.4	0.2	-	-	-
	Gain-Loss	1.97	0.15	0.69	16.24	2.81
✓	Hill	1.03	0.15	0.69	16.24	2.81

Figure CA 5.8.3/09-24:
Overview of steroidogenesis pathway, with indicated perturbations of spiroxamine according to US EAP ToxCast data



Collectively these data suggest that spiroxamine disrupts steroidogenesis in HTS *in vitro* assays. However, spiroxamine did not show any aromatase inhibition in human recombinant CYP19 cells when treated up to 100 µM (CA 5.8.3/01 [M-301972-01-1]). Spiroxamine was negative in rat testicular homogenate steroidogenesis assay *in vitro* up to 100 µM (CA 5.8.3/09 [M-303122-01-1]). Whilst it is recognised that only the terminal hormone in the androgen pathway was examined (testosterone), this assay did not examine upstream effects reported in the ToxCast data or potential effects on estradiol. Consequently, a new *in vitro* steroidogenesis assay was conducted (CA 5.8.3/10 [M-764156-01-1]) examining testosterone, estradiol and the upstream mineralocorticoid, progesterone. The recently conducted OECD CF Level 2 and Level 3 tests, *in vitro* androgen receptor transcriptional activation (CA 5.3.2/02 [M-760548-01-1]) and the *in vivo* Hershberger assay (CA 5.8.3/07 [M-764008-01-1]), respectively confirmed that spiroxamine was devoid of androgenic and anti-androgenic modalities, thereby addressing the CEETOX_H295R_ANDR_dn and CEETOX_H295R_TESTO_dn ToxCast data which reported inhibition of androstenedione and testosterone, respectively.

Whilst it is recognised that the reported effects on glucocorticoids (17-hydroxyprogesterone and 11-deoxycortisol) and the mineralocorticoid, 11-deoxycorticosterone have not directly been investigated, as these hormones are fulcrum to the steroidogenesis pathway, biologically relevant inhibition occurring would be reflective downstream and manifest in the hormones analysed.

Assessment and conclusions by applicant:

Assessment: This study is considered supplemental only.

Conclusion: In conclusion, the US EPA ToxCast data suggest that spiroxamine disrupts steroidogenesis in HTS *in vitro* assays, however no direct interference of androgen or oestrogen receptor activity was observed in toerh ED studies.

Data Point:	KCA 5.8.3/01
Report Author:	[redacted]
Report Year:	2008
Report Title:	Spiroxamine - Investigation on potential in vitro aromatase (CYP19) inhibition
Report No:	AT04594
Document No:	M-301971-01-1
Guideline(s) followed in study:	not applicable; special study
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted DAR (2010)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Yes

Executive Summary

The objective of this study was to evaluate the ability of spiroxamine to act as an inhibitor of aromatase activity using human CYP19 (aromatase) and P450 reductase by means of a radiometric assay. The substrate for the assay was [^{3}H]-androstenedione (ASDN), which is converted by aromatase to estrone

Final concentrations of spiroxamine tested in the aromatase assay ranged from 0.01 to 100 µM in a single assay. The positive controls, letrozole and ketoconazole, were included to demonstrate specificity and sensitivity of the assay.

The mean aromatase activity was determined to be 110.6% of control at the highest spiroxamine test concentration of 100 µM. Based on the data interpretation criteria established for the assay, spiroxamine did not inhibit human recombinant aromatase, and is therefore concluded to be devoid of aromatase inhibiting potential.

The positive controls, letrozole and ketoconazole both concentration-dependently inhibited aromatase activity with IC_{50} values of *ca.* 6 nM and *ca.* 21 nM, respectively thereby confirming the sensitivity and specificity of the assay.

Based on the data interpretation criteria established for the assay, spiroxamine did not inhibit human recombinant aromatase, and therefore concluded to be devoid of aromatase inhibiting potential.

Under the conditions of the study, spiroxamine did not inhibit human recombinant aromatase, and is therefore concluded to be devoid of aromatase inhibiting potential in a single experiment.

Materials and Methods

A. Materials:

1. **Test Material:** Spiroxamine
   (alternative name: [8-(1,1-dimethylethyl)-N-ethyl-N-propyl-1,4-dioxaspiro[4.5]decane-2-methanamine; KWG 4168)

   Description: Light brown oil
   Lot/Batch No.: EDTH004650
   Purity: 97.0% (w/w) (correction for purity not undertaken)
   CAS No.: 118134-30-8
   Stability of test compound: Confirmed stable for the duration of the study (expiry date: 2 August 2009)

2. Vehicle and/or positive control:	DMSO / positive control: Ketoconazole (12.5, 25, 50, 100 µM), Letrozole (0.0005, 0.0015, 0.005, 0.015 µM)
3. Substrate:	A mixture of non-radiolabelled androstenedione (ASDN) and radiolabelled [1β-^{3}H(n)]-ASDN was used to measure the release of tritiated water during the conversion of ASDN to oestrone as a direct assessment of aromatase activity. The final substrate solution had a concentration of 0.42 µM of ASDN (non-radiolabelled and radiolabelled combined).
4. Test system:	Human recombinant microsomes containing CYP19 and P450 reductase were selected as the test system based on the recommendation from the EPA test guideline. Determination of protein concentration of microsomes was determined daily. Aromatase activity of human recombinant microsomes was conducted to confirm that the microsomes had sufficient activity for each assay. The aromatase activity in microsomes was 5.2 pmole product
5. Dose preparation and analysis:	A stock solution of the test article was prepared in DMSO on the day of treatment. Subsequent dilutions of the stock solution were prepared in DMSO: 0.01, 0.1, 1, 10, 100 µM
6. Radiochemical analysis:	The amount of [^{3}H]-ASDN (measured in disintegrations per minute, DPM) in each standard was determined by subjecting a diluted (up to 1 mL with acetonitrile) 5 µL aliquot of the standard to radio-analysis by liquid scintillation counting (LSC).

B. Study Design:

1. In life dates:	15 October 2007 to 16 October 2007 (experimental dates)
2. Cell treatment:	The ability of the test article to inhibit human recombinant microsomal cytochrome P$_{450}$ (CYP19) aromatase activity using a mixture of ASDN and [^{3}H]-ASDN as the substrate was evaluated according to a test methodology similar to the EPA test guideline. The experiment was performed by pre-incubation of human CYP19 Aromatase (1 µL), NADPH (240 µmol/L), NADPH for 5 minutes in at 0 (DMSO), test article or positive control (ketoconazole, letrozole) for 5 minutes at 37 °C. Vehicle, test article and positive controls were dosed into the test system at 2% v/v. Incubations containing test article were performed in duplicate, for vehicle and positive controls five replicates were performed. The reaction was started with the addition of 420 nmol/L (16.3 KBq/mL) [1ß-^{3}H(n)]-ASDN and incubation for 20 minutes. Reactions were terminated by the addition of 0.25 mL trichloroacetic acid (5 %).
3. Evaluation criteria:	Not stated in the report, but assumed to follow the US EPA test guideline, as detailed below: <u>Classification of receptor binding affinity</u>: The data interpretation criteria were as follows: Aromatase activity at the highest concentration was ≤50%. Inhibitor Aromatase activity at the highest concentration was between 50-75%: Equivocal Aromatase activity at the highest concentration was ≥75%. Non-inhibitor.
4. Statistics:	None performed.

C. Methods:

1. Homogeneity and achieved concentration analysis of the dose:	Not undertaken.

2. Determination of aromatase activity:

Tritiated water was separated from the labelled steroid by solid phase extraction, subsequently [^{3}H]-activity in the eluents were measured by liquid scintillation counting. Samples were counted for maximally 15 minutes or until a 2σ% value of ≤1 was achieved. Decays per minute (DPM) were calculated from counts per minute using a calibration curve established by a set of differentially quenched ^{3}H standards.

Results and Discussion

A. Analytical determinations:

Not undertaken.

B. Aromatase assay:

A single experiment was undertaken to evaluate aromatase activity in the presence of spiroxamine. Solubility/precipitation of spiroxamine in the assay buffer was not assessed in the assay. [^{3}H]-ASDN was used as a substrate to assess aromatase activity. The mean aromatase activity was determined to be 110.6% of control at the highest spiroxamine test concentration of 100 µM.

Based on the data interpretation criteria established for the assay, spiroxamine did not inhibit human recombinant aromatase, and tis herefore concluded to be devoid of aromatase inhibiting potential.

The positive controls, letrozole and ketoconazole both concentration-dependently inhibited aromatase activity with IC_{50} values of *ca.* 6 nM and *ca.* 21 nM, respectively thereby confirming the sensitivity and specificity of the assay.

Table CA 5.8.3/01-1: Spiroxamine: *in vitro* human recombinant aromatase assay: aromatase activity results

Test article (µM)	^3H_2O released [dpm]	% Activity
0	125658 ±1338	
0.01	126731 ±880	100.9 ±0.7
0.1	128368 ±2093	102.2 ±1.6
1	126928 ±129	101.0 ±0.1
10	133178 ±327	106.0 ±0.2
100	138924 ±824	110.6 ±0.6
Positive controls		
Letrozole (µM)		
- 0.0005	113931 ±184	90.7 ±0.2
- 0.0015	86669 ±1051	69 ±1.2
- 0.005	6251 ±1973	51.9 ±3.0
- 0.015	40359 ±336	32.1 ±0.8
Ketoconazole (µM)		
- 12.5	86600 ±1003	68.9 ±1.2
- 25	58503 ±9463	46.6 ±16.2
- 50	44095 ±3263	35.1 ±7.4
- 100	22280 ±1422	17.7 ±6.4

C. Deficiencies:

When the study methodology is compared to current test guideline requirements (US EPA OPPTS 890.1200-2009) the following deficiencies are noted:

- A solubility assessment of spiroxamine in DMSO added to the test system was not performed
- The approved positive control, 4-OH ASDN was not included, with letrozole and ketoconazole used instead, however these strongly and weakly inhibiting compounds of aromatase activity, respectively which demonstrated the sensitivity and specificity of the test system.

- No acceptance or evaluation criteria were defined, however aromatase activity in the presence of spiroxamine was comparable with the concurrent control
- A response curved was not established, with model fitting using a non-linear regression program not undertaken, however spiroxamine did not show evidence of aromatase inhibition.

Whilst the following deficiencies are detailed, the results produced in the aromatase assay are still valid.

Assessment and conclusions by applicant:

Assessment: Study meets the current guidance and the requirements in 283/2013

Conclusion: Under the conditions of the study, spiroxamine did not inhibit human recombinant aromatase, and therefore concluded to be devoid of aromatase inhibiting potential in a single experiment.

Data Point:	KCA 5.8.3/02
Report Author:	[redacted]
Report Year:	2008
Report Title:	Spiroxamine - Investigation on potential in vitro steroidogenesis inhibition
Report No:	AT04646
Document No:	M-303122-01-1
Guideline(s) followed in study:	not applicable; mechanistic study
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and accepted DAR (2010)
GLP/Officially recognised testing facilities:	No, not conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Supportive only

Executive Summary

The objective of this study was to evaluate the ability of spiroxamine to affect the steroidogenic pathway beginning with the sequence of reactions occurring after the gonadotropin hormone receptors through the production of testosterone using rat testis.

Final concentrations of spiroxamine tested in the steroidogenesis assay were 1 and 100 µM in a single assay. The positive controls, ketoconazole, an inhibitor of steroidogenesis production was included to demonstrate specificity and sensitivity of the assay.

The mean testosterone concentration was determined to be 116% of control at the highest spiroxamine test concentration of 100 µM in the medium.

The positive control, ketoconazole (an inhibitor of steroidogenesis) significantly decreased testosterone concentration in the medium by 67% and by 83% in testicular fragments thereby confirming the sensitivity and specificity of the assay.

Under the conditions of the study, spiroxamine did not inhibit steroidogenesis ex vivo in rat testis homogenate and therefore concluded to be devoid of steroidogenesis inhibiting potential in a single experiment when examining testosterone production.

Materials and Methods

A. Materials:

1. Test Material:	Spiroxamine (alternative name: [8-(1,1-dimethylethyl)-N-ethyl-N-propyl-1,4-dioxaspiro[4,5]decane-2-methanamine; KWG 4168)
Description:	Light brown oil
Lot/Batch No.:	EDTH004650
Purity:	97.0% (w/w) (correction for purity not undertaken)
CAS No.:	118134-30-8
Stability of test compound:	Confirmed stable for the duration of the study (expiry date: 2 August 2009)
2. Vehicle and/or positive control:	DMSO / positive control: Ketoconazole (0.5, 20 µM)
3. Test animals:	
Species:	Rat
Strain:	Wistar
Age at sacrifice:	♂: 12-14 wks
Source:	[redacted]
4. Primary cell line:	Testicular fragments isolated from a single rat.
5. Cell culture conditions:	Minimum essential medium Eagle, (MEME) modified supplemented with $NaHCO_3$, L-glutamine (5 mM), Na pyruvate (1 mM), hCG (1 IU hCG [human chorion gonadotropin]/mL) adjusted to pH 7.4 – 7.5
6. Test concentrations:	1, 100 µM

B. Study Design:

1. In life dates:	12 October 2007 to 30 October 2007 (experimental dates)
2. Cell fragment preparation:	Following sacrifice of a single rat, one testis was removed. Testicular capsular was discarded and the testis cut into small pieces. Fragments were assigned to control and treatment groups, ensuring that size distribution of each group was comparable. Each group comprised of at least 9 fragments and cultured in MEME supplemented with hCG.
3. Evaluation criteria:	Not stated in the report, but assumed to follow the US EPA test guideline, as detailed below: The results of the hormone analyses were normalised to the mean solvent control value and then expressed as changes relative to the solvent control in each exposure plate. All doses that exhibited cytotoxicity greater than 20% or exceeded the limits of solubility (precipitation) were omitted from further evaluation. The data were evaluated for evidence of a dose response. The test article was judged to potentially affect steroidogenesis if the fold induction or inhibition was statistically different from the solvent control at concentrations falling within the increasing or decreasing portion of the dose-response curve.
4. Statistics:	One way analysis of variance eventually followed by pairwise comparisons versus the control group using Dunnett's method. If heterogeneity was observed, Kruskal-Wallis one way analysis of variance of ranks was performed, followed by pairwise comparisons versus the control group using Dunn's method.

C. Methods:

1. Homogeneity and achieved	Not undertaken.

concentration analysis of the dose:	
2. Cell treatment:	Testicular fragments cultured in MEME supplemented with hCG containing either vehicle (DMSO), test article or positive control were placed into a dynamic organ culture incubator for 6 hours at 35°C. Vials were rotated 2 times/minute, with a flow rate of carbogen of 3.4 L/minute. For the vehicle control 12 replicates were used, for all other groups 9 replicates were used. Six fragments were directly deep-frozen without incubation and served as a control to demonstrate effective stimulation of steroidogenesis by hCG. At the end of the incubation period, 500µL medium were collected from each incubation and stored at -20°C until analysis. Testicular fragments were recovered, weighed and stored deep frozen until processed further.
3. Analysis:	Medium was directly analysed after thawing. Fragments were thawed on ice in 500 µL MEME without hCG. Following centrifugation (7.5 minutes, 1300 x g) the supernatant was analysed for testosterone. Duplicate determinations were performed. Testosterone in medium was assayed directly, testosterone in fragments was measured after work-up by RIA kit.

Results and Discussion

A. Analytical determinations:

Not undertaken.

B. Solubility assay:

A solubility assay was not undertaken, with the maximum concentration limited to 100 µM. The highest concentration recommended for testing in the absence of solubility is 10^{-3} M.

C. Steroidogenesis assay:

A single experiment was undertaken to evaluate steroidogenesis in the presence of spiroxamine. Solubility/precipitation of spiroxamine in the assay buffer was not assessed in the assay, nor was cytotoxicity evaluated. The mean testosterone concentration was determined to be 116% of control at the highest spiroxamine test concentration of 100 µM in the medium.

Based on the data interpretation criteria established for the assay, spiroxamine did not inhibit human recombinant aromatase, and therefore concluded to be devoid of aromatase inhibiting potential.

The positive control, ketoconazole (an inhibitor of steroidogenesis) significantly decreased testosterone concentration in the medium by 67% and by 83% in testicular fragments, thereby confirming the sensitivity and specificity of the assay.

Table CA 5.8.3/02-1: Spiroxamine: *in vitro* rat steroidogenesis assay: testosterone concentrations

Test article (µM)		Testosterone in medium		Testosterone in tissue	
	n	ng/mg tissue	% reduction	ng/mg tissue	% reduction
No incubation	6	-	-	0.44 ±0.10	-
0	12	0.91 ±0.30	-	2.26 ±0.82	-
1	9	1.22 ±0.53	-	3.37 ±1.51	-
100	9	1.06 ±0.46	-	3.55 ±2.25	-
Positive controls					
Ketoconazole (µM)					
0.5	9	0.61 ±0.22*	33	1.57 ±0.73	31
20	9	0.31 ±0.07*	66	0.39 ±0.19*	83

D. Deficiencies:

When the study methodology is compared to current test guideline requirements (OECD 456, 2011) the following deficiencies are noted:

- The H295 cell line, as recommended by the test guideline was not used
- The assay set utilised only examined interference of testosterone production and not conversions of testosterone into 17β-estradiol.
- No assessment of spiroxamine solubility within the test system was undertaken. Consequently, there is no justification as to the maximum concentration test (100 µM), which 10x fold lower than the recommended concentration (10 mM).
- Concurrent measure of cytotoxicity was not undertaken
- Only 2 concentration of test article concentration were treated. Test guideline requirements are 7 concentrations.
- Known inducers (forskolin) and inhibitors (prochloraz) of testosterone and 17β-estradiol synthesis were not used

In conclusion, the data generated under this study are considered supplementary with the steroidogenesis endpoint sufficiently addressed with a new, up to date test guideline compliant GLP study (refer to CA 5.8.3/10 [M-764156-01-1]).

Assessment and conclusions by applicant:

Assessment: This study is deemed supplementary as a number of deficiencies are identified when assessed against current test guideline requirements.

Conclusion: Under the conditions of the study, spiroxamine did not inhibit steroidogenesis *ex vivo* in rat testis homogenate, and therefore concluded to be devoid of steroidogenesis inhibiting potential in a single experiment when examining testosterone production.

Data Point:	KCA 5.8.3/10
Report Author:	[redacted]
Report Year:	2021
Report Title:	Screening spiroxamine for modulation of steroidogenesis using the human H295R adreno-carcinoma cell line
Report No:	20249039
Document No:	M-764156-01-1
Guideline(s) followed in study:	OECD Test Guideline No. 456 (July 2011)
Deviations from current test guideline:	--
Previous evaluation:	
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	

Executive Summary

The objective of this study was to evaluate the ability of spiroxamine to affect the steroidogenic pathway from the precursor cholesterol leading to the formation of progesterone, testosterone and estradiol using the human H295R cell line.

Three valid steroidogenesis assay experiments were performed whereby the test article was tested at seven concentrations together with the positive control inducer forskolin and positive control inhibitor prochloraz.

Dimethylsulfoxide (DMSO) was used as vehicle and the concentration of vehicle in the incubations was kept constant at 0.1% (v/v).

H295R cells were exposed for 48 hours to the vehicle, the test article and positive controls. After exposure, the viability of the cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. The concentration of estradiol, testosterone and progesterone in the exposure medium was determined using a commercially available Enzyme-Linked Immuno Sorbent Assay (ELISA) (estradiol) or by UPLC-MS/MS (testosterone and progesterone).

Experiment 1:

- A statistically significant decrease in testosterone production was observed at 0.1, 1 and 10 µM spiroxamine. Overall the result was concluded to show evidence of a decrease in testosterone synthesis.
- A statistically significant decrease in estradiol production was observed at 0.0001, 0.001, 0.01, 0.1 and 1 µM spiroxamine. Overall the result was concluded to show evidence of a decrease in estradiol synthesis.
- A statistically significant decrease in progesterone production was observed at 0.1, 1 and 10 µM spiroxamine. Overall the result was concluded to show evidence of a decrease in progesterone synthesis.

Experiment 2:

- A statistically significant decrease in testosterone production was observed at 0.1, 0.316, 1, 3.16 and 10 µM spiroxamine. Overall the result was concluded to show evidence of a decrease in testosterone synthesis.
- A statistically significant decrease in estradiol production was observed at 0.1 µM and a statistically significant increase in estradiol production was observed at 10 µM spiroxamine. Overall the result was concluded equivocal.
- A statistically significant decrease in progesterone production was observed at 0.01, 0.1, 0.316, 1, 3.16 and 10 µM spiroxamine. Overall the result was concluded to show evidence of a decrease in progesterone synthesis.

Experiment 3:

- A statistically significant decrease in testosterone production was observed at 0.001. 0.01, 0.1, 0.316, 1, 3.16 and 10 µM spiroxamine. Overall the result was concluded to show evidence of a decrease in testosterone synthesis.
- A statistically significant decrease in estradiol production was observed at 0.001, 0.01, 0.1, 0.316 µM and a statistically significant increase in estradiol production was observed at 10 µM spiroxamine. Overall the result was concluded to show evidence of a decrease in estradiol synthesis.
- A statistically significant decrease in progesterone production was observed at 0.001, 0.01, 0.1, 0.316, 10 µM.

Cytotoxicity (>20%) was observed at the highest test article concentration (31.6 µM) in Experiment 1 only. The samples from this concentration were therefore excluded from hormone analysis. No cytotoxicity was observed at any of the other spiroxamine concentrations tested over Experiments 2 and 3.

The positive control inducer forskolin and positive control inhibitor prochloraz induced acceptable fold changes to the solvent control for testosterone and estradiol plates when assessed against the assay performance criteria, with all other parameters passing the performance criteria, thereby confirming the sensitivity and specificity of the assay test system over all three experiments..

Spiroxamine was judged as positive in the H295R steroidogenesis assay. The Lowest Observed Effect Concentration (LOEC) was 100 pM, 100 nM and 10 nM for estradiol, testosterone and progesterone, respectively. The maximum strength of response was 1.45-fold for estradiol, 0.63-fold for testosterone and 0.28-fold for progesterone, which was observed upon exposure of 10 µM (experiment 3), 10 µM (experiment 1) and 10 µM (experiment) test article, respectively

It is concluded that spiroxamine showed evidence of steroidogenesis in human adrenocortical carcinoma cell line H295R following three independent experiments. Statistically significant decreases in oestradiol, testosterone and progesterone were observed.

Materials and Methods

A. Materials:

1. Test Material:	Spiroxamine (alternative name: [8-(1,1-dimethylethyl)-N-ethyl-N-propyl-1,4-dioxaspiro[4,5]decane-2-methanamine; KWG 4168)
Description:	Clear light yellow liquid
Lot/Batch No.:	EDTH0114499
Purity:	97.0% (w/w) (correction for purity not undertaken)
CAS No.:	118134-30-8
Stability of test compound:	Confirmed stable for the duration of the study (expiry date: 4 June 2021)
2. Vehicle and/or positive control:	Vehicle control: DMSO, Positive controls: Steroid metabolism inducer: - forskolin (1, 10 µM) Steroid metabolism inhibitor: - prochloraz (0.1, 1 µM) Cytotoxicity: - methanol (70%)
3. Test system:	Human adrenocortical carcinoma cell line H295R obtained from adult adrenal cortex were sourced from American Type Culture Collection (ATCC), with cells passaged at least 5 times from the frozen stock.
4. Cell culture conditions:	Dulbecco's Modified Eagle Medium/Ham's F12 nutrient mix (DMEM/F12), supplemented with 2.5% Nu serum, 100 U/mL Penicillin, 100 µg/mL Streptomycin, 15 mM HEPES, 6.25 µg/mL insulin, 6.25 µg/mL transferrin, 6.25 ng/mL selenium, 1.25 mg/mL bovine serum albumin.
6. Test concentrations:	*Experiment 1:* 0, 0.0001, 0.001, 0.01, 0.1, 1, 10, 31.6 µM *Experiment 2, 3:* 0, 0.001, 0.01, 0.1, 0.316, 1, 3.16, 10 µM

B. Test Performance:

1. In life dates: 29 September 2020 to 7 December 2020 (experimental dates)

2. Vehicle selection: The test article was soluble in DMSO at a concentration of 1 M (inspection under the microscope). However, a 1000-fold dilution of this 1 M solution and 316 mM, 100 mM, 31.6 mM and 10 mM in exposure medium resulted in precipitation of the test article. A 1000-fold dilution of a 3.16 mM solution of the test article in exposure medium resulted in a clear solution. This concentration was used as the highest test article spiking concentration in the first main experiments (final concentration in the well: 3.16 µM) based on 0.1% additions into the test system.

3. Statistics: Normality was evaluated using a Shapiro-Wilk´s test. If data were not normally distributed, the data were transformed to approximate a normal distribution. If the data were normally distributed or approximate normal distribution, differences between test article treatments and solvent controls were analyzed using the Dunnett's Test.

If the data were not normally distributed, the non-parametric Kruskal Wallis test was performed. A Levene's test was performed to test for variance homogeneity. If variance was non-homogenous another appropriate test was performed. Differences were considered significant at $p \leq 0.05$. Statistical evaluation was performed based on average values for each well that represented independent replicate data points.

ToxRat Professional software (ToxRat Solutions® GmbH, Germany) was used for statistical evaluation.

4. Acceptance criteria: *Steroidogenesis assay:*

Considered acceptable if the results met the following criteria:

	Comparison between	Testosterone	Estradiol
Basal production of hormone in the SC	Fold-greater than LOQ	≥ 5-fold	≥ 2.5-fold
Exposure experiments – Within plate CV for SCs (replicate wells)	Absolute concentrations	≤ 30%	≤ 30%
Exposure experiments – Between plate CV for SCs (replicate experiments)	Absolute concentrations	≤ 30%	≤ 30%
Hormone measurement system – Sensitivity	Detectable fold-change relative to SC	≥ 5-fold	≥ 2.5-fold
Hormone measurement system – Replicate measure CV for SCs	Absolute concentrations	≤ 25%	≤ 25%
Induction (10 µM forskolin)	Fold-change compared to SC	≥ 1.5-fold	≥ 7.5 -fold
Inhibition (1 µM prochloraz)	Fold-change compared to SC	≤ 0.5-fold	≤ 0.5-fold

Accuracy and repeatability:

The analytical method was considered applicable for the quantitative analysis of testosterone and progesterone if the mean accuracy was in the range 70–130%.

5. Evaluation criteria: *Steroidogenesis interference:*

A test article was considered positive if the fold induction was statistically different ($p \leq 0.05$) from the solvent control at two adjacent concentrations in at least two independent runs.

Devoid of steroidogenesis interference:

A test article was considered negative following two independent negative runs, or in three runs, comprising two negative runs and one equivocal or positive run. If the data generated in three independent experiments does not meet the decision criteria listed in the table below, the experimental results are not interpretable:

Run 1	Run 2	Run 3	Decision

Scenario	Decision	Scenario	Decision	Scenario	
-ve	Confirm[a]	-ve	Stop		-ve
-ve	Confirm[a]	+ve	Refine[b]	-ve	-ve
Equivocal[c]	Refine[b]	-ve	Confirm[a]	-ve	-ve
Equivocal[c]	Refine[b]	-ve	Confirm[a]	+ve	+ve
Equivocal[c]	Refine[b]	+ve	Stop		+ve
+ve	Refine[b]	-ve	Confirm[a]	+ve	+ve
-ve	Confirm[a]	+ve	Refine[b]	+ve	+ve
+ve	Refine[b]	+ve	Stop		+ve

a Confirm previous run with the same experimental design
b Re-run assay at ½-log concentrations spacing (bracketing the concentration that tested significantly different in the preceding experiment)
c Fold-change at one concentration is statistically significantly different from the SC

C. Methods:

1. Homogeneity and achieved concentration analysis of the dose:

Not undertaken.

2. Steroidogenesis assay:

H295 cells were plated into 24 well plates (3.0×10^5 cells/mL) and grown for at least 5 passages from frozen, reaching approximately 85-90% confluency, before passage. The 24-well plates were pre-incubated for at least 24±1 h prior to exposure.

After 24 h of incubation, well plates were removed from the incubator. The media was removed and replaced by exposure medium. The exposure medium consisted of cell culture medium supplemented with:

- medium only (untreated control, in triplicate), or
- DMSO (solvent control, in triplicate), or
- forskolin (inducer, in triplicate at 1 µM and 10 µM for QC plate), or
- prochloraz (inhibitor, in triplicate at 0.1 µM and 1 µM for QC plate), or
- test article (7 concentrations in triplicate)

Following exposure for 48 h, the 24-well plates were removed from the incubator and every well checked microscopically for cell condition (attachment, morphology, degree of confluence) and signs of cytotoxicity and potential test article precipitation. The medium from each well was transferred to tube and stored in the freezer (≤ -75°C) until further processing.

3. Cell viability assay:

Cell viability was determined by the MTT assay. Medium from each well was replaced by 300 µL fresh medium. Subsequently, only the medium from wells A4-6 and B4-6 of the QC plate was removed and 300 µL methanol (70%) added to these wells. The plates were incubated at room temperature for 30 minutes.

After incubation, methanol was removed from wells A4-6 and B4-6 and these wells were rinsed carefully with medium (three times). After rinsing, 500 µL medium was added to these wells.

To all wells, 75 µL MTT (5 mg/mL in PBS) was added. The plates were incubated at 37°C and 5.0% CO_2 for 2 -3 h.

After incubation, the medium in each well was replaced with 500 µL DMSO. The plates were shaken on an orbital shaker for 5 minutes. Formation of the blue formazan in each well was measured at 560 nm using a spectrophotometer. In addition, the absorption was measured at 690 nm to correct for background absorption.

All wells that exhibit cytotoxicity >20% were omitted from further evaluation.

4. Chemical hormone assay:

Spiroxamine was tested for potential interference with estradiol, testosterone and progesterone hormone analysis. For this purpose, chemical spiked medium was prepared as described below.

Hormone stocks	Preparation	

Primary hormone stocks E2, T and P	1 mg testosterone dissolved in 1 mL ethanol 1 mg estradiol dissolved in 1 mL ethanol 1 mg progesterone dissolved in 1 mL ethanol
Secondary hormone stocks	10 µL of primary hormone stock diluted in 990 µL solution of 75% supplemented medium, 25% ethanol
Tertiary hormone stocks	10 µL of secondary hormone stock diluted in 990 µL supplemented medium
Final hormone stock	100 µL of tertiary hormone stock diluted in 900 µL supplemented medium

T: testosterone; E2: 17β-estradiol.

The tubes were vortexed and the hormone concentrations determined by ELISA (for estradiol) or UPLC-MS/MS (for testosterone and progesterone).

If interference occurred that was ≥30% of basal hormone production for E2 and/or T, the chemical hormone assay interference test was performed with additional test article spiking solutions to determine the threshold dose at which substantial interference occurred.

If interference exceeded 30%, the data were invalid and the data at these concentrations discarded.

5. Hormone analysis and recovery:

Hormone analysis was not be performed in medium obtained from wells that showed viability < 80%.

In addition, medium samples spiked with 100 pg/mL, 150 pg/mL and 250 pg/mL estradiol, 500 pg/mL, 2500 pg/mL and 5000 pg/mL testosterone or 50 pg/mL, 200 pg/mL and 400 pg/mL progesterone was included and analyzed with ELISA (for estradiol) or UPLC-MS/MS (testosterone and progesterone) to determine the recovery.

The measured hormone concentrations in spiked medium should not deviate more than 30% from the nominal concentrations.

6. Data analysis:

b) Cell viability:

The cell viability was expressed relative to the average response in the solvent controls, which is considered 100% viable cells, and was calculated as follows:

$$\%\ \text{viable cells} = \frac{(\text{response in well} - \text{average response in MeOH}_{\text{treated}}\ \text{well})}{(\text{response in SC wells} - \text{average response in MeOH}_{\text{treated}}\ \text{well})} \times 100$$

All doses that exhibit cytotoxicity >20% were omitted from further evaluation.

b) Testosterone and progesterone analysis:

Response (R) = Peak area of the analyte × (IS Conc. / IS peak area) [units]

Calibration curve: Regression analysis was performed using the least squares method.

Analyzed concentration (C_A): Analyzed concentration of the samples

QC prepared in supplemented medium

$$\text{Accuracy} = \frac{C_A - C_B}{C_N} \times 100\ [\%]$$

where C_B = analyzed concentration in supplemented medium blank sample

c) Calculation of estradiol, testosterone and progesterone concentrations:

Estradiol concentrations were calculated using the Excel-sheet delivered with the ELISA kits. Estradiol, testosterone and progesterone concentrations were normalized to the mean solvent control value for each assay. Results were expressed as changes relative to the solvent control in each exposure plate and calculated as follows:

$$\text{Relative change} = \frac{\text{hormone concentration in each well}}{\text{Mean solvent hormone concentration}}$$

Results and Discussion

A. Analytical determinations:

Not undertaken. Analyses for achieved concentration, homogeneity or stability of test article formulations were not conducted as part of this study, as this is not a requirement of the regulatory test guidelines.

B. Steroidogenesis assay – Experiment 1:

1. QC plates: QC plates for the test article plates, estradiol and testosterone confirmed that the acceptability criteria were met.

2. Testosterone analysis: The average basal testosterone production in the DMSO solvent control wells on the test article plate was 1742 pg/mL. The average testosterone concentrations in the medium from the H295R cells exposed to the test article ranged from 1103 pg/mL to 1772 pg/mL. The average relative change of the test article exposed wells to the solvent treated control wells ranged from 0.63 to 1.02. A statistically significant decrease in testosterone production was observed at 0.1, 1 and 10 µM spiroxamine. Overall the result was concluded to show evidence of a decrease in testosterone synthesis.

3. Estradiol analysis: The average basal estradiol production in the DMSO solvent control wells on the test article plate was 120 pg/mL. The average estradiol concentrations in medium from H295R cells exposed to the test article ranged from 83 pg/mL to 129 pg/mL. The average relative change of the test article exposed wells compared to the solvent treated control wells ranged from 0.69 to 1.07. A statistically significant decrease in estradiol production was observed at 0.0001, 0.001, 0.01, 0.1 and 1 µM spiroxamine. Overall the result was concluded to show evidence of a decrease in estradiol synthesis.

4. Progesterone analysis: The average basal progesterone production in the DMSO solvent control wells on the test article plate was 313 pg/mL. The average progesterone concentrations in the medium from the H295R cells exposed to the test article ranged from 115 pg/mL to 308 pg/mL. The average relative change of the test article exposed wells to the solvent treated control wells ranged from 0.37 to 0.98. A statistically significant decrease in progesterone production was observed at 0.1, 1 and 10 µM spiroxamine. Overall the result was concluded to show evidence of a decrease in progesterone synthesis.

5. Cell viability: Cytotoxicity (>20%) was observed at the highest test article concentration (31.6 µM). The samples from this concentration were therefore excluded from hormone analysis. No cytotoxicity was observed at any of the other spiroxamine concentrations tested.

6. Positive controls: The positive control inducer forskolin and positive control inhibitor prochloraz induced acceptable fold changes to the solvent control for testosterone and estradiol plates when assessed against the assay performance criteria, with all other parameters passing the performance criteria, thereby confirming the sensitivity and specificity of the assay test system.

It however is prudent to note that no performance criteria are available for progesterone, therefore, whilst a statistically significant decrease in progesterone was observed the biological relevance is unclear. Within H295R cells, androstenedione and 11-deoxycortisol are the most abundant, while steroids upstream and downstream show lower levels. Steroid levels (absolute and relative) can vary since they are highly dependent on culture conditions (basal levels of steroids in batch of Nu-serum used is very important) and the method of steroid analysis used. Xing *et al*[27] report that basal levels of progesterone are overtly low (0.6%).

27 Xing,Y., Edwards, M.A., Ahlem, C., Kennedy, M., Cohen, A., Gomez-Sanchez, C.E. & Rainey, W.E. (2011). The effect of ACTH on steroid metabolomics profiles in human adrenal cells. *J. Endocrin.* **209**, pp 327-335

Table CA 5.8.3/10-1: Steroidogenesis activity of spiroxamine: Experiment 1

MTT (%viability [%cytotoxicity])				Testosterone (relative change)				Estradiol (relative change)				Progesterone (relative change)			
QC plate		Spiroxamine plate		QC plate		Spiroxamine plate		QC plate		Spiroxamine plate		QC plate		Spiroxamine plate	
Blank	99.9 [0.1] 100	-	-	Blank	1.07 ±0.08	-	-	Blank	1.08 ±0.06	-	-	Blank	1.60 ±0.02	-	-
0[a]	100.0 [0.0]	DMSO	100.0 [0.0]	0[a]	1.00 ±0.01	0[a]	1.00 ±0.06	0[a]	1.00 ±0.11	0[a]	1.00 ±0.05	0[a]	1.00 ±0.04	0[a]	1.00 ±0.04
For: 1 uM	108.4 [-8.4]	0.0001 µM	101.3 [-1.3]	For: 1 uM	1.83 ±0.09	0.0001 µM	1.02 ±0.03	For: 1 uM	15.27 ±0.82	0.0001 µM	0.79 ±0.12	For: 1 uM	2.25 ±0.12	0.0001 µM	0.98 ±0.01
For: 10 uM	107.3 [-7.3]	0.001 µM	94.0 [6.0]	For: 10 uM	1.85 ±0.13	0.001 µM	0.93 ±0.02	For: 10 uM	37.03 ±2.36	0.001 µM	0.69 ±0.13*	For: 10 uM	12.42 ±0.25	0.001 µM	0.97 ±0.05
Pro: 0.1 uM	99.1 [0.9]	0.01 µM	99.3 [0.7]	Pro: 0.1 uM	0.28 ±0.01	0.01 µM	0.93 ±0.04	Pro: 0.1 uM	0.63 ±0.04	0.01 µM	0.80 ±0.03*	Pro: 0.1 uM	4.26 ±0.03	0.01 µM	0.96 ±0.02
Pro: 1 uM	99.1 [0.9]	0.1 µM	104.6 [-4.1]	Pro: 1 uM	0.08 ±0.01	0.1 µM	0.76 ±0.02*	Pro: 1 uM	0.26 ±0.00	0.1 µM	0.78 ±0.03*	Pro: 1 uM	23.0 ±0.62	0.1 µM	0.62 ±0.03*
		1 µM	100.2 [-0.2]			1 µM	0.72 ±0.07*			1 µM	0.78 ±0.08*			1 µM	0.54 ±0.02*
		10 µM	91.21 [8.8]			10 µM	0.63 ±0.01*			10 µM	1.07 ±0.04			10 µM	0.37 ±0.01*
		31.6 µM	77.1 [22.9]			31.6 µM	-			31.6 µM	-			31.6 µM	-

* $p \leq 0.05$
For: forskolin
Pro: prochloraz

Table CA 5.8.3/10-2: Steroidogenesis activity of spiroxamine: Experiment 1, performance criteria

		Testosterone			Estradiol		
	Comparison between	**Performance criteria**	**QC plate**	**Spiroxamine plate**	**Performance criteria**	**QC plate**	**Spiroxamine plate**
LOQ	NA	NA	50 pg/mL	50 pg/mL	NA	31.3 pg/mL	31.3 pg/mL
Basal production of hormone in the SCs	Absolute concentration	NA	1591 pg/mL	1742 pg/mL	NA	118 pg/mL	120 pg/mL
	Fold-greater than LOQ	≥ 5-fold	32-fold	35-fold	≥ 2.5-fold	3.8-fold	3.8-fold
Within plate CV for SCs (replicate wells)	Absolute concentrations	≤ 30%	1.2%	5.6%	≤ 30%	11%	4.9%
Induction (10 µM forskolin)	Fold-change compared to SC	≥ 1.5-fold	1.9-fold	-	≥ 7.5-fold	37-fold	-
Inhibition (1 µM prochloraz)	Fold-change compared to SC	≤ 0.5-fold	0.077-fold	-	≤ 0.5-fold	≤ 0.26-fold [a)]	-
Hormone measurement system: Sensitivity	Detectable fold-change relative to SC	≥ 5-fold	32-fold	35-fold	≥ 2.5-fold	3.8-fold	3.8-fold
Hormone measurement system: Replicate measure CV for SCs	Absolute concentrations	-	-	-	≤ 25%	≤113%	≤18%

C. Steroidogenesis assay – Experiment 2:

1. QC plates: QC plates for the test article plates, estradiol and testosterone confirmed that the acceptability criteria were met. Since progesterone is not an endpoint included in OECD guideline 456, there are no acceptance criteria available.

2. Testosterone analysis: The average basal testosterone production in the DMSO solvent control wells on the test article plate was 1965 pg/mL. The average testosterone concentrations in the medium from the H295R cells exposed to the test article ranged from 1237 pg/mL to 2011 pg/mL. The average relative change of the test article exposed wells to the solvent treated control wells ranged from 0.63 to 1.02. A statistically significant decrease in testosterone production was observed at 0.1, 0.316, 1, 3.16 and 10 µM spiroxamine. Overall the result was concluded to show evidence of a decrease in testosterone synthesis.

3. Estradiol analysis: The average basal estradiol production in the DMSO solvent control wells on the test article plate was 111 pg/mL. The average estradiol concentrations in medium from H295R cells exposed to the test article ranged from 72 pg/mL to 160 pg/mL. The average relative change of the test article exposed wells compared to the solvent treated control wells ranged from 0.64 to 1.44. A statistically significant decrease in estradiol production was observed at 0.1 µM and a statistically significant increase in estradiol production was observed at 10 µM spiroxamine. Overall the result was concluded equivocal.

4. Progesterone analysis: The average basal progesterone production in the DMSO solvent control wells on the test article plate was 269 pg/mL. The average progesterone concentrations in the medium from the H295R cells exposed to the test article ranged from 76 pg/mL to 264 pg/mL. The average relative change of the test article exposed wells to the solvent treated control wells ranged from 0.28 to 0.98. A statistically significant decrease in progesterone production was observed at 0.01, 0.1, 0.316, 1, 3.16 and 10 µM spiroxamine. Overall the result was concluded to show evidence of a decrease in progesterone synthesis.

5. Cell viability: Cytotoxicity (>20%) was not observed at any concentration.

6. Positive controls: The positive control inducer forskolin and positive control inhibitor prochloraz induced acceptable fold changes to the solvent control for testosterone and estradiol plates when assessed against the assay performance criteria, with all other parameters passing the performance criteria, thereby confirming the sensitivity and specificity of the assay test system.

Table CA 5.8.3/10-3: Steroidogenesis activity of spiroxamine: Experiment 2

MTT (%viability [%cytotoxicity])				Testosterone (relative change)				Estradiol (relative change)				Progesterone (relative change)			
QC plate		Spiroxamine plate		QC plate		Spiroxamine plate		QC plate		Spiroxamine plate		QC plate		Spiroxamine plate	
Blank	103.4 [-3.4]	-	-	Blank	1.03 ±0.04	-	-	Blank	1.01 ±0.10	-	-	Blank	1.71 ±0.07	-	-
0[a]	100.0 [0.0]	DMSO	100.0 [0.0]	0[a]	1.00 ±0.02	0[a]	1.00 ±0.01	0[a]	1.00 ±0.03	0[a]	1.00 ±0.20	0[a]	1.00 ±0.07	0[a]	1.00 ±0.03
For: 1 uM	105.4 [-5.4]	0.001 µM	102.8 [-2.8]	For: 1 uM	1.85 ±0.08	0.001 µM	1.02 ±0.05	For: 1 uM	18.14 ±1.17	0.001 µM	0.84 ±0.04	For: 1 uM	2.60 ±0.17	0.001 µM	0.98 ±0.02
For: 10 uM	116.5 [-16.5]	0.01 µM	96.7 [3.3]	For: 10 uM	1.82 ±0.08	0.01 µM	0.95 ±0.03	For: 10 uM	68.51 ±14.50	0.01 µM	0.76 ±0.13	For: 10 uM	2.78 ±0.07	0.01 µM	0.90 ±0.02*
Pro: 0.1 uM	99.5 [0.5]	0.1 µM	111.6 [-11.6]	Pro: 0.1 uM	0.27 ±0.01	0.1 µM	0.20 ±0.03*	Pro: 0.1 uM	0.62 ±0.01	0.1 µM	0.64 ±0.15*	Pro: 0.1 uM	3.82 ±0.14	0.1 µM	0.53 ±0.02*
Pro: 1 uM	106.3 [-6.3]	0.316 µM	103.8 [-3.8]	Pro: 1 uM	0.07 ±0.00	0.316 µM	0.64 ±0.04*	Pro: 1 uM	0.21 ±0.02	0.316 µM	0.92 ±0.05	Pro: 1 uM	21.5 ±0.55	0.316 µM	0.52 ±0.04*
		1 µM	101.5 [-1.5]			1 µM	0.63 ±0.00*			1 µM	0.86 ±0.03			1 µM	0.47 ±0.04*
		3.16 µM	95.1 [4.9]			3.16 µM	0.64 ±0.02*			3.16 µM	0.88 ±0.13			3.16 µM	0.39 ±0.01*
		10 µM	88.8 [11.2]			10 µM	0.63 ±0.01*			10 µM	1.44 ±0.03*			10 µM	0.28 ±0.01*

* $p \leq 0.05$
For: forskolin
Pro: prochloraz

Table CA 5.8.3/10-4: Steroidogenesis activity of spiroxamine: Experiment 2, performance criteria

		Testosterone			Estradiol		
	Comparison between	**Performance criteria**	**QC plate**	**Spiroxamine plate**	**Performance criteria**	**QC plate**	**Spiroxamine plate**
LOQ	NA	NA	50 pg/mL	50 pg/mL	NA	31.3 pg/mL	31.3 pg/mL
Basal production of hormone in the SCs	Absolute concentration	NA	1681 pg/mL	1961 pg/mL	NA	162 pg/mL	111 pg/mL
	Fold-greater than LOQ	≥ 5-fold	34-fold	39-fold	≥ 2.5-fold	5.2-fold	3.6-fold
Within plate CV for SCs (replicate wells)	Absolute concentrations	≤ 30%	2.4%	2.2%	≤ 30%	3.1%	20%
Induction (10 µM forskolin)	Fold-change compared to SC	≥ 1.5-fold	1.8-fold	-	≥ 7.5-fold	69-fold	-
Inhibition (1 µM prochloraz)	Fold-change compared to SC	≤ 0.5-fold	0.07-fold	-	≤ 0.5-fold	0.21-fold	-
Hormone measurement system: Sensitivity	Detectable fold-change relative to SC	≥ 5-fold	34-fold	39-fold	≥ 2.5-fold	5.2-fold	3.6-fold
Hormone measurement system: Replicate measure CV for SCs	Absolute concentrations	-	-	-	≤ 25%	≤16.3%	≤26%

C. Steroidogenesis assay – Experiment 3:

1. QC plates:	QC plates for the test article plates, estradiol and testosterone confirmed that the acceptability criteria were met. Since progesterone is not an endpoint included in OECD guideline 456, there are no acceptance criteria available.
2. Testosterone analysis:	The average basal testosterone production in the DMSO solvent control wells on the test article plate was 1589 pg/mL. The average testosterone concentrations in the medium from the H295R cells exposed to the test article ranged from 1069 pg/mL to 1693 pg/mL. The average relative change of the test article exposed wells to the solvent treated control wells ranged from 0.67 to 1.07. A statistically significant decrease in testosterone production was observed at 0.001, 0.01, 0.1, 0.316, 1, 3.16 and 10 µM spiroxamine. Overall the result was concluded to show evidence of a decrease in testosterone synthesis.
3. Estradiol analysis:	The average basal estradiol production in the DMSO solvent control wells on the test article plate was 90 pg/mL. The average estradiol concentrations in medium from H295R cells exposed to the test article ranged from 48 pg/mL to 131 pg/mL. The average relative change of the test article exposed wells compared to the solvent treated control wells ranged from 0.53 to 1.45. A statistically significant decrease in estradiol production was observed at 0.001, 0.01, 0.1, 0.316 µM and a statistically significant increase in estradiol production was observed at 10 µM spiroxamine. Overall the result was concluded to show evidence of a decrease in estradiol synthesis.
4. Progesterone analysis:	The average basal progesterone production in the DMSO solvent control wells on the test article plate was 319 pg/mL. The average progesterone concentrations in the medium from the H295R cells exposed to the test article ranged from 101 pg/mL to 311 pg/mL. The average relative change of the test article exposed wells to the solvent treated control wells ranged from 0.32 to 1.00. A statistically significant decrease in progesterone production was observed at 0.001, 0.01, 0.1, 0.316, 10 µM. Overall the result was concluded to show evidence of a decrease in progesterone synthesis.
5. Cell viability:	Cytotoxicity (>20%) was not observed at any concentration.
6. Positive controls:	The positive control inducer forskolin and positive control inhibitor prochloraz induced acceptable fold changes to the solvent control for testosterone and estradiol plates when assessed against the assay performance criteria, with all other parameters passing the performance criteria, thereby confirming the sensitivity and specificity of the assay test system.

Table CA 5.8.3/10-5: Steroidogenesis activity of spiroxamine: Experiment 3

MTT (%viability [%cytotoxicity])				Testosterone (relative change)				Estradiol (relative change)				Progesterone (relative change)			
QC plate		Spiroxamine plate		QC plate		Spiroxamine plate		QC plate		Spiroxamine plate		QC plate		Spiroxamine plate	
Blank	98.2 [1.8]	-	-	Blank	1.04 ±0.00	-	-	Blank	1.13 ±.08	-	-	Blank	1.51 ±0.03	-	-
0[a]	100.0 [0.0]	DMSO	100.0 [0.0]	0[a]	1.84 ±0.01	0[a]	1.00 ±0.02	0[a]	1.00 ±0.05	0[a]	1.00 ±0.10	0[a]	1.00 ±0.02	0[a]	1.00 ±0.01
For: 1 uM	105.1 [-5.1]	0.001 µM	100.3 [-0.3]	For: 1 uM	1.84 ±0.01	0.001 µM	1.07 ±0.01*	For: 1 uM	28.87 ±1.11	0.001 µM	0.72 ±0.10*	For: 1 uM	2.42 ±0.02	0.001 µM	1.00 ±0.02
For: 10 uM	109.9 [-9.9]	0.01 µM	100.2 [-0.2]	For: 10 uM	2.18 ±0.02*	0.01 µM	0.98 ±0.01	For: 10 uM	65.21 ±2.35*	0.01 µM	0.62 ±0.07*	For: 10 uM	2.57 ±0.08	0.01 µM	0.89 ±0.01*
Pro: 0.1 uM	97.9 [2.1]	0.1 µM	106.9 [-6.9]	Pro: 0.1 uM	0.29 ±0.01	0.1 µM	0.78 ±0.01*	Pro: 0.1 uM	0.78 ±0.03	0.1 µM	0.53 ±0.16*	Pro: 0.1 uM	3.68 ±0.12	0.1 µM	0.56 ±0.03*
Pro: 1 uM	103.7 [-3.7]	0.316 µM	101.3 [-1.3]	Pro: 1 uM	0.08 ±0.00	0.316 µM	0.67 ±0.01*	Pro: 1 uM	≤0.35 n/a	0.316 µM	0.68 ±0.07	Pro: 1 uM	16.89 ±0.51	0.316 µM	0.56 ±0.03*
		1 µM	101.3 [-1.3]			1 µM	0.68 ±0.02*			1 µM	0.76 ±0.02			1 µM	0.46 ±0.00*
		3.16 µM	101.4 [-1.4]			3.16 µM	0.67 ±0.01*			3.16 µM	0.82 ±.17			3.16 µM	0.38 ±0.02*
		10 µM	89.3 [10.7]			10 µM	0.68 ±0.02*			10 µM	1.45 ±0.06*			10 µM	0.32 ±0.02*

* $p \leq 0.05$
For: forskolin
Pro: prochloraz

Table CA 5.8.3/10-6: Steroidogenesis activity of spiroxamine: Experiment 3, performance criteria

		Testosterone			Estradiol		
	Comparison between	**Performance criteria**	**QC plate**	**Spiroxamine plate**	**Performance criteria**	**QC plate**	**Spiroxamine plate**
LOQ	NA	NA	50 pg/mL	50 pg/mL	NA	31.3 pg/mL	31.3 pg/mL
Basal production of hormone in the SCs	Absolute concentration	NA	1474 pg/mL	1589 pg/mL	NA	90 pg/mL	90 pg/mL
	Fold-greater than LOQ	≥ 5-fold	29-fold	32-fold	≥ 2.5-fold	2.9-fold	2.9-fold
Within plate CV for SCs (replicate wells)	Absolute concentrations	≤ 30%	3.6%	1.7%	≤ 30%	5.4%	9.6%
Induction (10 µM forskolin)	Fold-change compared to SC	≥ 1.5-fold	2.2-fold	-	≥ 7.5-fold	65-fold	-
Inhibition (1 µM prochloraz)	Fold-change compared to SC	≤ 0.5-fold	0.079-fold	-	≤ 0.5-fold	0.35-fold	-
Hormone measurement system: Sensitivity	Detectable fold-change relative to SC	≥ 5-fold	29-fold	32-fold	≥ 2.5-fold	2.9-fold	2.9-fold
Hormone measurement system: Replicate measure CV for SCs	Absolute concentrations	-	-	-	≤ 25%	≤14%	≤16%

C. Deficiencies:

None.

Assessment and conclusions by applicant:

Assessment: Study meets the current guidance and the requirements in 283/2013.

Conclusion: It is concluded that spiroxamine showed evidence of steroidogenesis in human adrenocortical carcinoma cell line H295R following three independent experiments. Statistically significant decreases in oestradiol, testosterone and progesterone were observed.

Data Point:	KCA 5.8.3/11
Report Author:	[redacted]
Report Year:	2021
Report Title:	Spiroxamine: Proposed mode of action for cholesterol perturbations and the knock on consequences
Report No:	0471836-Tox3
Document No:	M-763156-01-2
Guideline(s) followed in study:	None
Deviations from current test guideline:	None
Previous evaluation:	No, not previously submitted
GLP/Officially recognised testing facilities:	not applicable
Acceptability/Reliability:	Supportive only

Executive Summary

Spiroxamine (8-tert-butyl-1,4-dioxaspiro[4.5]decan-2-ylmethyl(ethyl)(propyl)amine), is a tertiary amine fungicide of the chemical class of spiroketalamines acting through sterol biosynthesis inhibition. It is used solely as a fungicide belonging to the amine group, FRAC code 5 fungal control agents, which inhibit δ14-reductase, a member of the HMG (3-hydroxy-3-methylglutaryl)-CoA reductase family. Inhibition of this enzyme is considered related to the adverse toxicity observed in the apical mammalian toxicity data set. The activity of spiroxamine in the *in vitro* steroidogenesis assay is also likely to be due to inhibition of this enzyme, however it is noted that this activity is not associated with any *in vivo* adversity related to the EAS modalities.

This position paper provides an understanding of the *in vivo* consequences of inhibition of this enzyme, which do not drive any endocrine mode of action pathway in the comprehensive *in vivo* mammalian data package.

Although sporadic effects were observed in in vitro mechanistic (decreased testosterone, progesterone, estradiol) and in vivo (delays in developmental milestones for males and females) studies, these effects were not deemed evidence of ED mediated effects. Where both male and female pup developmental milestone delays occurred, these were not driven by A- or E-modality involvement respectively, but rather secondary to maternal toxicity. In addition to the apical studies, results from ToxCast ER and AR indicate that spiroxamine is negative for E- and A- activity, with further Level 2/3 A-modality studies confirming a lack of A-modality involvement. In vitro data confirm that aromatase, the terminal enzyme in the steroidogenesis pathway is not inhibited. The reductions in steroid hormone production observed in the in vitro H295R assay are attributed to upstream effects related to reduction in serum cholesterol levels, which impact upon the availability for this principal sterol feeding into the steroidogenesis pathway in vitro. Reductions in these steroid hormones observed in vitro are not manifest in the

available, comprehensive apical in vivo mammalian toxicity data. Collectively, adrenal weights were unaffected in the apical toxicity studies, with no test article related effects observed upon adrenal gland histopathological analysis. Because steroidogenesis is among the functional roles of the adrenal gland, the absence of effects on this organ argues against spiroxamine having a potential steroidogenesis pathway interaction, in vivo. It is therefore concluded that spiroxamine is devoid effects on the EAS-modalities, with further Level 5 in vivo studies to address the in vitro steroidogenesis data not required.

The available *in vivo* data for spiroxamine support the proposed mode of action that a reduction in cholesterol synthesis across all mammalian species is attributed to HMG-CoA reductase inhibition (in part with influence from the inflammatory cascade due to the inherent irritancy profile of spiroxamine), resulting in interference in cholesterol synthesis, manifest as increased incidence of cataract formation in the apical data package. Due to the high cholesterol demands of the eye, along with the corneal epithelial cell population which account for essentially all of the *de novo* cholesterol synthesis, this makes the eye overtly sensitive to perturbations in cholesterol synthesis.

Attempting to provide a causative link to the known mode of action of spiroxamine (HMG-CoA reductase inhibition) and the *in vitro* steroidogenesis data is not appropriate or scientifically justifiable as *in vivo* adversity is not observed across a comprehensive *in vivo* mammalian data package, with no biological plausible link evident between the *in vitro-in vivo* data.

The *in vitro* steroidogensis data should be viewed with caution since it only considers a portion of the pathway, does not consider the upstream effects from the available serum cholesterol (the primary sterol) entering the steriodogenesis pathway. Furthermore, the assay is conducted in the absence of an exogenous metabolic liver fraction (S9), whereas *in vivo* ADME studies, confirm that spiroxamine undergoes extensive metabolism. From the in vivo studies there were no findings suggesting that HMG-CoA reductase inhibition resulted in steriodogenesis effect in the sub-acute, sub-chronic, chronic, Hershberger or reproductive toxicity studies. Adrenal weights and adrenal histopathology were unaffected across a myriad of studies. Because steroidogenesis is among the functional roles of the adrenal glands, the absence of effects on this organ argues against spiroxamine having a potential steroidogenesis pathway interaction *in vivo*. There is cross over between different species (rat, dog, mice), with the effects (perturbations in serum cholesterol, triglycerides, eye effects) observed in the *in vivo* studies.

Collectively, the reductions in sterol hormone production observed in the *in vitro* steriodogenesis data do not warrant further *in vivo* testing, with no pre-neoplastic lesions or tumour production in EAS sensitive/producing organs (in two separate rodent models), no HPG axis involvement observed across rodent/non-rodent species; up to date two-generation study which confirms no adveristy in sperm parameters, angogenital distance, with developmental delays attributed to prolonged maternal stress. Finally, Level 2/3 data confirm no E, A-receptor or aromatase interference. It is therefore concluded that spiroxamine is devoid of effects on the EAS-modalities, with further Level 5 *in vivo* studies to address the *in vitro* steroidogenesis data not required.

CA 5.9 Medical data

Occupational health surveillance did not reveal any health effects. No reports on epidemiological studies were submitted. Clinical cases and poisoning incidences were summarised by the notifier. However, the correlation between spiroxamine and the observed symptoms is not sure, besides findings of skin and eye irritation from splashes with spiroxamine containing products. No specific therapy upon ingestion is available. The usual first aid measures and symptomatic treatment apply. Special care should be taken for possible lesions due to irritation properties along the route of exposure and for liver and eye lesions upon systemic exposure.

CA 5.9.1 Medical surveillance on manufacturing plant personnel and monitoring studies

There were no reported incidences involving spiroxamine during its manufacture at any of the monitored sites. Monitoring of workers involved in the production of spiroxamine revealed no unusual findings and no ill effects were reported to medical professionals. The summary of the data can be found in the confidential Document JCA (CA 5.9.1 [M-762352-02-1]).

CA 5.9.2 Data collected on humans

The irritation and sensitisation potential of spiroxamine was investigated in human subject using the an abbreviated version of the Shelanski & Shelanski Repeated Insult Patch Test (RIPT) conducted under double blind conditions with doses of ranging from 0.027 to 1.02%. Dermal occlusive patches containing 0.15 mL/patch was applied to the upper arms. The study was split into the following phases: i) nitial exposure (induction) phase was 4 weeks with repeated daily application for 4 days/week; ii) iIntermediate phase (rest period) allowed normalisation of the skin following any adverse effects. This allowed the opportunity for the patching of subjects who may not have completed the patch application phase; iii) Challenge (elicitation) phase, 4 consecutive days.

Whilst a concentration of 1.02% spiroxamine produced a visible dermal reaction, up to 1.02% did not cause persistent irritant effects or sensitisation reaction that remained. A concentration of 0.2% was the highest concetration that did not elicit a dermal reaction in human volunteers using the abbreviated version of Shelanski & Shelanski Repeated Insult Patch Test (RIPT).

Table CA 5.9.2-1: Summary of acute toxicity, primary irritation and sensitisation studies

Type of study	Species	Results	Classification (Annex I for Regulation (EC) 1272/2008)	Annex CA Point / Reference
Skin irritation	Human	0.2% did not elicit reveal any skin irritating or skin sensitising properties (abbreviated version of the Shelanski & Shelanski RIPT)	Study not suitable for classification	CA 5.9.2/01 [redacted] (2001)
Skin irritation	Human	1.02% did not elicit reveal any skin irritating or skin sensitising properties (abbreviated version of the Shelanski & Shelanski RIPT)	Study not suitable for classification	CA 5.9.202 [redacted] (2000)

Data Point:	KCA 5.9.2/01
Report Author:	[redacted]
Report Year:	2001
Report Title:	A patch test procedure to facilitate the expression and detection of the irritating and sensitizing propensities of KWG 4168
Report No:	107791
Document No:	M-086474-02-1
Guideline(s) followed in study:	not applicable
Deviations from current test guideline:	None
Previous evaluation:	yes, evaluated and classified DAR (2010)
GLP/Officially recognised testing facilities:	Yes, conducted under GLP/Officially recognised testing facilities
Acceptability/Reliability:	Supportive only

Main report

Executive Summary

An intensified version of the Shelanski & Shelanski Repeated Insult Patch Test (RIPT) was conducted under double blind conditions with the intention of identifying irritation and sensitisation responses. Group sizes of 45 males and 166 females were used. The study was conducted in two stages on two panels of subjects. The effects of nominal doses of 0.02%, 0.066%, and 0.20% solutions of spiroxamine (as solution in 0.2% Cremophor® EL in physiological saline) were studied on the subjects in both stages. Volumes of 0.15 mL of each solution were used to load the patching devices. This corresponds to doses of 7.5 µg/cm², 25.0 µg/cm² and 75.0 µg/cm² spiroxamine, available on the 2 cm *x* 2 cm contact area. Dose selection was based on preliminary investigations.

Dermal, occlusive patches containing 0.15 mL/patch was applied to the upper arms. The study was split into the following phases: i) Initial exposure (induction) phase was 4 weeks with repeated daily application for 4 days/week; ii) Intermediate phase (rest period) allowed normalisation of the skin following any adverse effects. This allowed the opportunity for the patching of subjects who may not have completed the patch application phase; iii) Challenge (elicitation) phase: 4 consecutive days.

During the challenge phase an absence of perceptible gross changes of the skin indicated that non-irritating solutions of 0.02, 0.066 and 0.20% spiroxamine have no skin sensitising properties in humans.

Under the conditions of this study, spiroxamine up to 0.2% did not reveal any skin irritating or skin sensitising properties in human volunteers using the Intensified Shelanski Repeated Insult Patch Test (RIPT).

Materials and Methods

A. Materials:

1. **Test Material:** Spiroxamine
   (alternative name: [8-(1,1-dimethylethyl)-N-ethyl-N-propyl-1,4-dioxaspiro[4,5]decane-2-methanamine; KWG 4168)

Description:	Viscous yellow liquid
Lot/Batch No.:	17002/90
Purity:	95.5%
CAS No.:	118134-30-8
Stability of test compound:	Assumed stable for the duration of the exposure

2. Vehicle and/or positive control: 0.2% Cremophor® EL in saline / not relevant

3. Test animals:

Species:	Human
Strain:	Not relevant
Age at dosing:	♂: 19 – 84 years; ♀: 20 – 84 years
Weight at dosing:	Not relevant
Source:	Not relevant
Acclimation period:	Not relevant
Diet:	Not relevant
Water:	Not relevant
Housing:	Not relevant

4. Environmental conditions:

Temperature:	Not relevant
Humidity:	Not relevant
Air changes:	Not relevant
Photoperiod:	Not relevant

B. Study Design:

1. In life dates: 23 February 1998 to 30 July 1998 (experimental dates)

2. Study design: An intensified version of the Shelanski & Shelanski Repeated Insult Patch Test (RIPT) was conducted under double blind conditions with the intention of identifying irritation and sensitisation responses. Group sizes of 45 ♂ and 166 ♀ were used. The study was conducted in two stages on two panels of subjects. The effects of nominal doses of 0.02%, 0.066%, and 0.20% solutions of spiroxamine (as solution in 0.2% Cremophor® EL in physiological saline) were studied on the subjects in both stages. Volumes of 0.15 mL of each solution were used to load the patching devices. This corresponds to doses of 7.5 µg/cm², 25.0 µg/cm² and 75.0 µg/cm² spiroxamine, available on the 2 cm *x* 2 cm contact area. Dose selection was based on preliminary investigations (CA 5.2.4/02).

Dermal, occlusive patches containing 0.15 mL/patch was applied to the upper arms. The study was split into the following phases:

- Initial exposure (induction) phase was 4 weeks with repeated daily application for 4 days/week.
- Intermediate phase (rest period) allowed normalisation of the skin following any adverse effects. This allowed the opportunity for the patching of subjects who may not have completed the patch application phase.
- Challenge (elicitation) phase: 4 consecutive days.

3. Evaluation criteria:

Morphological change	Visible change	Grade
Absent	None	0
Vascular dilatation	Redness: faint to moderate, with distinct border	1
	Redness: moderate, with distinct border	2
	Redness: intense, with distinct border	3
Infiltration	Redness, plus oedema or papules	4
	Redness, plus vesicles, blisters or bullae	5
	Redness, plus extension beyond border	6

4. Statistical analysis: Not undertaken

Table CA 5.9.2/01-1: Summary of human repeated insult patch test scheme: overview treatment regimen

Group size	45 ♂			166 ♀	
Treatment day	**Monday**	**Tuesday**	**Wednesday**	**Thursday**	**Friday**
Activation/induction phase					
Wk 1	B/A	R/E/A	R/E/A	R/E/A	R/E
Wk 2	E/A	R/E/A	R/E/A	R/E/A	R/E
Wk 3	E/A	R/E/A	R/E/A	R/E/A	R/E
Wk 4	E/H	(E)H	(E)H	(E)H	(E)
Challenge phase					
Wk 5	B/A	R/E/A	R/E/A	R/E/A	R/E
Wk 6	E/D				
Dose concentration (%) applied to each subject					
Band L1: 0.2% SPX	Band L2: 0.066% SPX	Band L3: 0.02% SPX		Band L5: 0.2% Cremophor® EL	Band L5: physiological saline

B: baseline examination
R: patch removed under supervision
D: subject discharged
A: patch applied
E: site examined and grade recorded
H: hiatus (rest period) or application to make up for any missed during induction phase

C. Methods:

1. Homogeneity and achieved concentration analysis of the dose: Not undertaken.

2. Observations: The application sites were observed daily, 5/days a week. The occuled patching device (consisiting of 4 x 4 cm square impermeable plastic film with adhesive coating on one side and a 2 x 2 cm webril pad centred on the adhesive coated surface to which the test article was applied), was removed, discarded, the site graded and a freshly prepared device was applied on the same site

Results and Discussion

A. Homogeneity and achieved concentration analysis:

Not undertaken. Analyses for achieved concentration, homogeneity or stability of test article formulations were not conducted as part of this study, as this is not a requirement of the regulatory test guidelines.

B. Preliminary investigations:

Preliminary investigations confirmed that 0.20% was the highest spiroxamine concentration which was tolerated without any visible irritation after repeated dermal application for up to 4 days. Higher concentrations of 0.30% up to 1.02% induced gross skin changes.

C. Observations:

1. Initial phase: There were no gross changes of the skin perceptible at the application sites after repeated dermal application of solutions containing 0.02, 0.066 and 0.20% spiroxamine.

2. Challenge phase: The absence of perceptible gross changes of the skin during the challenge phase indicated that non-irritating solutions of 0.02, 0.066 and 0.20% spiroxamine have no skin sensitising properties in humans.

3. Follow up phase: No skin findings at the application sites were reported from any of the human volunteers during the 2 weeks of the follow-up phase.

Table CA 5.9.2/01-2: Summary of human repeated insult patch test scheme: maximum assigned grade/individual participant

Grade	Induction phase (%)					Challenge phase				
	0.20	0.066	0.02	Crem[a]	Saline	0.20	0.066	0.02	Crem[a]	Saline
0	210	210	210	208	206	204	204	204	204	204
1	0	0	0	1	3	0	0	0	0	0
2	0	0	0	0	0	0	0	0	0	0
3	0	0	0	0	0	0	0	0	0	0
4	0	0	0	0	0	0	0	0	0	0
No. providing data	210	210	210	209	209	204	204	204	204	204
No. not providing data	1	1	1	1	1	7	7	7	7	7
No. of responders	0	0	0	1	3	0	0	0	0	0

a 2% Cremophor® EL

D. Deficiencies:

None.

Assessment and conclusions by applicant:

Assessment: This study is deemed supplementary.

Conclusion: Under the conditions of this study, spiroxamine up to 0.2% did not reveal any skin irritating or skin sensitizing properties in human volunteers using the Intensified Shelanski & Shelanski Repeated Insult Patch Test (RIPT).

Supplement to M-086474-02-1

Executive Summary

The irritation and sensitisation potential of spiroxamine was investigated in human subjects. An abbreviated version of the Shelanski & Shelanski Repeated Insult Patch Test (RIPT) was conducted under double blind conditions. The objectives were 2-fold. To determine the maximum concentration of spiroxamine that elicited gross dermal effects during or following a 24 hour exposure period under occluded conditions, along with the course of 4 consecutive applications of 24 hours in duration to provide substantial enough data to confirm the maximum not irritating concentration that did not cause irritation or sensitisaiton. A total of 21 males were used. The study was conducted in two stages. The effects of nominal doses of 0.027, 0.04, 0.06, 0.09 and 0.135% solutions of spiroxamine (as solution in 0.2% Cremophor® EL in physiological saline) were studied on 11 subjects, applied for up to 5 days. Since the first series of solutions did not show any evidence of irritancy, a second series of solutions were prepared and dosed at 0.2, 0.3, 0.45, 0.68 and 1.02% to group of 10 further males were treated.

Dermal, occlusive patches containing 0.15 mL/patch was applied to the upper arms. The study was split into the following phases: i) Initial exposure (induction) phase was 3 weeks with repeated daily application for 4 days/week. ii) Intermediate phase (rest period) allowed normalisation of the skin following any adverse effects. This allowed the opportunity for the patching of subjects who may not have completed the patch application phase; iii) Challenge (elicitation) phase: 4 consecutive days

The solution containing 0.2% spiroxamine was the highest concentration which did not elicit any gross skin changes. The solution containing 1.02% spiroxamine was the highest concentration which did not elicit gross changes that remained unabated over the weekend.

Under the conditions of this study, whilst a concentration of 1.02% spiroxamine produced a visible dermal reaction, up to 1.02% did not cause persistent irritant effects or sensitisation reaction that remained. A concentration of 0.2% was the highest concetration that did not elicit a dermal reaction in human volunteers using the abbreviated version of Shelanski & Shelanski Repeated Insult Patch Test (RIPT).

Materials and Methods

A. Materials:

1.	**Test Material:**	Spiroxamine (alternative name: [8-(1,1-dimethylethyl)-N-ethyl-N-propyl-1,4-dioxaspiro[4,5]decane-2-methanamine; KWG 4168)
	Description:	Viscous yellow liquid
	Lot/Batch No.:	Not provided
	Purity:	Not provided
	CAS No.:	118134-30-8
	Stability of test compound:	Assumed stable for the duration of the exposure
2.	**Vehicle and/or positive control:**	0.2% Cremophor® EL in saline / not relevant
3.	**Test animals:**	
	Species:	Human
	Strain:	Not relevant
	Age at dosing:	♂: age not stated
	Weight at dosing:	Not relevant
	Source:	Not relevant
	Acclimation period:	Not relevant
	Diet:	Not relevant
	Water:	Not relevant
	Housing:	Not relevant
4.	**Environmental conditions:**	
	Temperature:	Not relevant
	Humidity:	Not relevant
	Air changes:	Not relevant
	Photoperiod:	Not relevant

B. Study Design:

1.	**In life dates:**	23 February 1998 to 30 July 1998 (experimental dates)
2.	**Study design:**	An abbreviated version of the Shelanski & Shelanski Repeated Insult Patch Test (RIPT) was conducted under double blind conditions. The objectives were 2-fold: To determine the maximum concentration of spiroxamine that elicited gross dermal effects during or following a 24 hour exposure period under occluded conditions; along with the course of 4 consecutive applications of 24 hours in duration to provide substantial enough data to confim the maximum not irritating concentration that did not cause irritation or sensitisaiton. A total of 21 ♂ were used. The study was conducted in two stages. The effects of nominal doses of 0.027, 0.04, 0.06, 0.09 and 0.135% solutions of spiroxamine (as solution in 0.2% Cremophor® EL in physiological saline) were studied on 11 subjects, applied for up to 5 days. Since the first series of solutions did not show any evidence of irritancy, a second series of solutions were prepared and dosed at 0.2, 0.3, 0.45, 0.68 and 1.02% to group of 10 further ♂ were treated.

Dermal, occlusive patches containing 0.15 mL/patch was applied to the upper arms. The study was split into the following phases:

- Initial exposure (induction) phase was 4 weeks with repeated daily application for 4 days/week.
- Intermediate phase (rest period) allowed normalisation of the skin following any adverse effects. This allowed the opportunity for the patching of subjects who may not have completed the patch application phase.
- Challenge (elicitation) phase: 4 consecutive days.

3. Evaluation criteria:

Morphological change	Visible change	Grade
Absent	None	0
Vascular dilatation	Redness: faint to moderate, with distinct border	1
	Redness: moderate, with distinct border	2
	Redness: intense, with distinct border	3
Infiltration	Redness, plus oedema or papules	4
	Redness, plus vesicles, blisters or bullae	5
	Redness, plus extension beyond border	6

4. Statistical analysis: Not undertaken

Table CA 5.9.2/02-1: Summary of human repeated insult patch test scheme: overview treatment regimen

Group size	21				
Treatment day	**Monday**	**Tuesday**	**Wednesday**	**Thursday**	**Friday**
Activation/induction phase					
Wk 1	B/P	R/E/P	R/E/P	R/E/P	R/E
Wk 2	E/A	R/E/P	R/E/P	R/E/P	R/E
Wk 3	E/P	R/E/P	R/E/P	R/E/P	R/E
Wk 4	H(M)	H(M)	H(M)	H(M)	H(E)
Challenge phase					
Wk 5	E/B/P	R/E/P	R/E/P	R/E/P	R/E
Wk 6	E/D				
Dose concentration (%) applied to each subject – Group 1					
Band L1: 0.027% SPX	Band L2: 0.04% SPX	Band L3: 0.06% SPX	Band L5: 0.09% SPX	Band L5: 0.135% SPX	
Dose concentration (%) applied to each subject – Group 2					
Band L1: 0.2% SPX	Band L2: 0.3% SPX	Band L3: 0.45% SPX	Band L5: 0.68% SPX	Band L5: 1.02% SPX	

B: baseline examination
P: patch applied
R: patch removed under supervision
E: site examined and grade recorded
H: rest period
M: make up applications, *i.e.* missed during induction phase
D: subject discharged

C. Methods:

1. Homogeneity and achieved concentration analysis of the dose: Not undertaken.

2. Observations: The application sites were observed daily, 5/days a week. The occuled patching device (consisiting of 4 *x* 4 cm square impermeable plastic film with adhesive coating on one side and a 2 *x* 2 cm webril pad centred on the adhesive coated surface to which the test article was applied) was removed, discarded, the site graded and a freshly prepared device was applied on the same site

3. **Body weights:** Not applicable

Results and Discussion

A. Homogeneity and achieved concentration analysis:

Not undertaken. Analyses for achieved concentration, homogeneity or stability of test article formulations were not conducted as part of this study.

C. Observations:

1. **Group 1:** There were no gross changes of the skin perceptible at the application sites after repeated dermal application of solutions containing 0.04, 0.06, 0.09 and 0.135% spiroxamine.
2. **Group 2:** The solution containing 0.2% spiroxamine was the highest concentration which did not elicit any gross skin changes.
   The solution containing 1.02% spiroxamine was the highest concentration which did not elicit gross changes that remained (i.e. not reversible) over the weekend.

Table CA 5.9.2/02-2: Summary of human repeated insult patch test scheme: maximum assigned grade/individual participant

Grade	Group 1 (%)					Group 2 (%)				
	0.027	0.04	0.06	0.09	0.135	0.20	0.30	0.45	0.68	1.02
No. of patients eliciting a response (no. of patients eliciting a response on both arms/total no. of patients treated										
No. providing data	10	10	10	10	10	9	9	9	9	9
No. not providing data	1	1	1	1	1	1	1	1	1	1
No. of responders	0/10	0/10	0/10	0/10	0/10	4/9	4/9	7/9	7(2)/9	9(4)/9

D. Deficiencies:

None

Assessment and conclusions by applicant:

Assessment: This study is deemed supplementary.

Conclusion: Under the conditions of this study, whilst a concentration of 1.02% spiroxamine produced a visible dermal reaction, up to 1.02% did not cause persistent irritant effects or sensitisation reaction that remained. A concentration of 0.2% was the highest concetration that did not elicit a dermal reaction in human volunteers using the abbreviated version of Shelanski & Shelanski Repeated Insult Patch Test (RIPT).

CA 5.9.3 Direct observations

See CA 5.9.1 and CA 5.9.2

CA 5.9.4 Epidemiological studies

No epidemiological studies have been conducted on spiroxamine.

CA 5.9.5 Diagnosis of poisoning (determination of active

substance, metabolites), specific signs of poisoning, clinical tests

Test article-specific poisoning signs in humans after oral ingestion other than irritation are not expected. The analytical demonstration of parent compound, spiroxamine or metabolites in blood, urine or gastrointestinal contents is required for an exact diagnosis of poisoning.

CA 5.9.6 Proposed treatment: first aid measures, antidotes, medical treatment

The table presented below has been taken from the medical surveillance report presented in the Doc I (CA 5.9.1/02 [M-762352-02-1]).

First Aid::

Inhalation	-	Remove patient from exposure/terminate exposure
Skin Contact	-	Thorough skin decontamination with copious amounts of water and soap, if available with polyethylene glycol 300 followed by water. Note: Most formulations with this active ingredient can be decontaminated with water (and soap), so for formulations polyethylene glykol 300 is not required
Eye contact	-	Flushing of the eyes with lukewarm water for 15 minutes
Ingestion	-	Induction of vomiting can be considered if a large amount has been swallowed, if the ingestion was less than one hour ago, and if the patient is fully conscious. Induced vomiting can remove maximum 50% of the ingested substance. **Note**: Induction of vomiting is prohibited, if a formulation containing organic solvents has been ingested!

Treatment:

Ingestion	-	Gastric lavage can be considered in cases of significant ingestions within the first (2) hour(s).
	-	The application of activated charcoal and sodium sulphate (or other carthartic) should be considered in significant ingestions.
	-	As there is no antidote, treatment has to be symptomatic and supportive

CA 5.9.7 Expected effects of poisoning

The effects in men following oral uptake of toxic doses of spiroxamine are not known. Irritation-induced effects on the gastrointestinal mucosa can be expected from the studies in experimental animal. Liver and eye were target organs in experimental animals.