





OWNERSHIP STATEMENT

This document, the data contained in it and copyright therein are owned by Bayer AG and/or affiliated entities. No part of the document or any information contained therein may be disclosed to any third party without the prior written authorisation of Bayer AC and/or aftinated entities.

The summaries and evaluations contained in this document are based on unpublished proprietary data submitted for the purpose of the assessment undertaken by the regulatory authority of their registration authorities should not grant amount of the control of the con e from other applicants once the speriod of data protection has expired. registration authorities should not grant, amend, or renew a registration on the basis of the summaries and evaluation of unpublished propeletary data contained in this document puless they



Version history

| | Version history | Øj° 🗞 |
|----------------------------------|--|--|
| Date [yyyy-mm-dd] | Data points containing amendments or additions ¹ and brief description | Document identifier and Sersion number |
| <u> </u> | brief description | Sersion number |
| | | |
| | | |
| It is suggested the SANCO/10180/ | Data points containing amendments or additions¹ and brief description at applicants adopt a similar approach to showing revisions 2013 Chapter 4, 'How to revise an Assessment Report'. | and version history as outlined in |

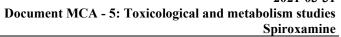




Table of Contents

| CA 5 | TOXICOLOGICAL AND METABOLISM STUDIES ON THE ACTIVE SUBSTANCE. Other toxicological studies. Supplementary studies on the active substance. 279 Endocrine disrupting properties. 303 Medical data. 389 Medical surveillance on manufacturing plant personnel and monitoring studies. 390 Data collected on humans. 290 Direct observations. Epidemiological studies. 397 Diagnosis of poisoning (determination offactive substance, metabolites), specific signs of poisoning (diagnostic studies). 397 Proposed treatment: first aid measures antidotes, medical dreatment. 398 Expected effects of poisoning. 398 |
|--|---|
| CA 5.8 | Other toxicological studies |
| CA 5.8.1 | Toxicity studies on metabolites |
| CA 5 8 2 | Supplementary studies on the active substance |
| CA 5 8 3 | Endocrine disrupting properties |
| CA 5.9 | Medical data |
| CA 5.9.1 | Medical surveillance on manufacturing plant personnel and monitoring studies |
| CA 5 9 2 | Data collected on humans |
| CA 5.9.3 | Direct observations |
| CA 5.9.4 | Epidemiological studies |
| CA 5.9.5 | Diagnosis of poisoning (determination of active substance, metabolitis). |
| | specific signs of poisoning clinical tests 397 |
| CA 5.9.6 | Proposed treatment: fire aid measure antidotes, nedical treatment |
| CA 5.9.7 | Expected effects of possoning 398 |
| | |
| | |
| | |
| | |
| | |
| | |
| | |
| | |
| | |
| | |
| | |
| ₩ | |
| | |
| | |
| « ¥ | |
| | |
| | |
| | |
| . Q | |
| | |
| | |
| × 1 | |
| Ay" | |
| | |
| A. A | |
| | |
| | |
| ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ | |
| | |
| | |



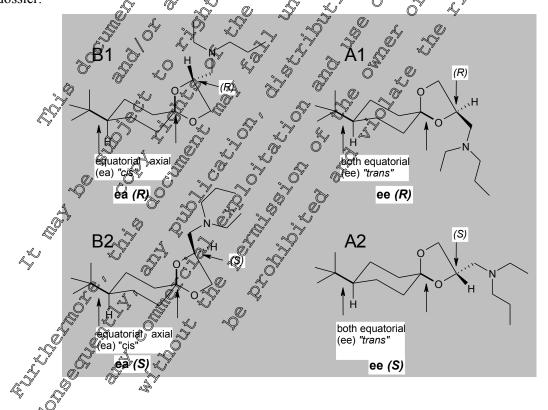


CA 5 TOXICOLOGICAL AND METABOLISM STUDIES ON THE **ACTIVE SUBSTANCE**

Spiroxamine was included in Annex I to Council Directive 91/414/EEC in 1999 (Directive 1999) 3/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 cuteria new robust study summaries have been provided in the appropriate dossier section. However, where studies to not, or meet relevant validity criteria and are not considered acceptable, less detailed summaries may have been provided alongside discussions of study deficiences. All relied upon study coports are submitted in Document K for this second renewal of approval dossier or in Document K for the previous Amoex 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 Possier 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 AP and corresponding trans/es 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 indertaken on spiroxamine metabolites, with a summary provided.

Acute oral toxicity studies conducted with M03 and M28 LD₅₀ trigger classification (Acute Pox. Cat. 2, H302) with LD₅₀ values of 707 and >550 to <2000 mg/kg bw, respectively. A complete in vitros genotoxicity test battery (Ames, mammalian forward (V79 hprt⁺ or L5178Y th⁻) gene mutation and mammalian (human peripheral blood lymphocode micronucleus)) to set allow maximum concentrations, confirmed both M03 and M28 to be devoid of both cone mutation and structural/numerical chromosomal effects.

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

In the 90-day rat oral dietary study conducted on 1003, the NOACL 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 historathology. 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 agrice oral and termal studies indicated that the LD3 was 2000 mg/kg bw for both endpoints. A complete *in stro* genetoxicity test batter (Ames, manipalian Corward (L5178Y $tk^{+/-}$) gene mutation and manmalian (human peripheral blood, lymphocyte micronycleus)) tested up to suitable maximum consentrations, confirmed M13 to be devoid of both seen 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 noales/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 Noxide, which are considered terriary unines spiroxamine oclohexanol, which has lost the tertiary amine group consequently did not display histopathological lesions associated with tertiary amines (hyperkeratosis of the epithelium of the ocsophagus 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 fechale rats to M13.

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

The developmental NOAFX 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. 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 comethyl ethy containing spiroxanine and spiroxamine N-oxide (M03), spiroxamine aminodiol (M28) does not contain this spructure with only the aminodiol group present. Consequently, the adverse histopathological lesions associated with the cyclohexane dimethyl ethyl containing tertiary amines (hyperCeratosis of the epithelium) of the oesophagus and forestomach) were not observed here. Furtherwore, no test article related histopathological lesions were observed. The presence of the two diot groups vasity 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 25 day and distary 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 NOALLs.

The NOAEL for maternal oxicity was considered to be 150 mg/kg bw/day based maternal mortality, body weight losses, gaseous content of the of track and werse clinical signout 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 har/day,

The developmental NQAEL 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, 9fold and 24 fold increase in sub-acute material and developmental NOAELs, respectively when compared to the spiroxamine equivalent studies.

Refer to Section 6 (CA 6.7 1.5 /01 (M-472 //9-01.31)) for an overview of stereochemistry of M13 and M28. fold and 25 fold increase in sub-acute maternal and developmental NOAELs, respectively when



a

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

| | MWT ^a | M-13 equivalent √ © × | SEX-equivalent | MAEL fold |
|---|---|---|---|--|
| Repeat dose toxicity NOAFI s | (g/mol) | | (NOAIO x (SPX MWT/metabolite | difference |
| Repeat dose toxicity NOALLS | | acetate MWT)) | MWT)) C V | (metabolite vs. |
| | | 0,00 | | SPX) |
| 28-d (gavage) NAOEL: 50 mg/kg bw/d | 156.27 | n/a | 95.2 mg/kg bw/d © | $x_{9.52}$ |
| | | | (50 x (297.48/15\$.27) 20 20 20 20 20 20 20 20 20 20 20 20 20 | 9 .52/10) |
| Maternal NOAEL: 40 mg/kg bw/d | | 31 5cmg/kg bw/d | 60.0 mg/kg bw/d | x2 |
| | 100 20 | (40x ×156.27/198.30)) | 31.5 x 6297.48/156.27) | (60.0/30) |
| Developmental NOAEL: 160 NOAEL | 198.30 | 3 126.1 mg/kg bw/d | 240.0 mg/kg bw/d | <i>x</i> 8 |
| _ | R. W. | (160 x (356.27/19830)) | (29 P.48/156.21) | (240/30) |
| 28-d (dietary) NAOEL: 28.4 mg/kg bw/d 💃 | S. | n/a O | 51 Amg/kg bw | x15.1 |
| × 1 | | | (28 4x (297,48/164.24) C | (51.4/3.4) |
| Maternal NOAEL: 150 mg/kg by | ai 21 | S n/a S | 271.7 kg bw/d\" | x9.1 |
| | - SP | | (150,32,297.48/264.24) | (571.7/30) |
| Developmental NOAEI | | n/a | 54.3 mg/kg/bw/d | <i>x</i> 1.8 |
| | | | $30 \times (30 \times (297.48/164.24))$ | (54.3/30) |
| 28-d (gavage) NAOEL: 10 mg/kg bw/d 🔾 | <u> </u> | na l | n/a | n/a |
| 28-d (dietary) NAOEL: 3.4 mg/kg.bw/d | 207 40 | n/a | n/a | n/a |
| Maternal NOAEL: 30 mg/kg w/d × | \$297.48 ; | No national | n/a | n/a |
| Developmental NOAEL NOAEL | | n/a | n/a | n/a |
| | Maternal NOAEL: 40 mg/kg bw/d Developmental NOAEL: 160 NOAEL 28-d (dietary) NAOEL: 28.4 mg/kg bw/d Maternal NOAEL: 150 mg/kg bw/d Developmental NOAEL: 10 mg/kg bw/d 28-d (gavage) NAOEL: 10 mg/kg bw/d Maternal NOAEL: 3.4 mg/kg bw/d Maternal NOAEL: 30 mg/kg bw/d | Repeat dose toxicity NOAELs 28-d (gavage) NAOEL: 50 mg/kg bw/d Maternal NOAEL: 40 mg/kg bw/d Developmental NOAEL: 160 NOAEL 28-d (dietary) NAOEL: 28.4 mg/kg bw/d Maternal NOAEL: 150 mg/kg bw/d Developmental NOAEL: 10 mg/kg bw/d 28-d (gavage) NAOEL: 10 mg/kg bw/d Maternal NOAEL: 3.4 mg/kg bw/d Maternal NOAEL: 30 mg/kg bw/d Maternal NOAEL: 30 mg/kg bw/d Maternal NOAEL: 30 mg/kg bw/d | Repeat dose toxicity NOAELs 28-d (gavage) NAOEL: 50 mg/kg bw/d Maternal NOAEL: 40 mg/kg bw/d Developmental NOAEL: 160 NOAEL 28-d (dietary) NAOEL: 28.4 mg/kg bw/d Maternal NOAEL: 150 mg/kg bw/d 28-d (gavage) NAOEL: 10 mg/kg bw/d 28-d (dietary) NAOEL: 3.4 mg/kg bw/d Maternal NOAEL: 30 mg/kg bw/d | Companies Comp |

Developmental NOAEL 30 NOAEL MAN ACD/ChemSkee 2016 1. kg. The MWT calculated from ACD/



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 appropriated 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 mutagenioty 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 piroxamine and M03 or, in the case of M06, being a major rat metabolite. For the suffate conjugates M25 M26 and M27 confidence in the prediction was assigned as low, since the sulfate morely was not assessed in any of the Leadscope submodels 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 M33 and M37 plus the structural similarity between M35 and M36, though the overall confidence level for genotoxicity was medium, based on the mutagenicity endfoint, 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 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 of a single method.

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

| Type of study | Species S | Desses nig/kg bw/day New LOACL | Key effects | Annex CA Point/Refer ence | | | | |
|--|--|--|--|------------------------------------|--|--|--|--|
| | Voxicity data on spiroxamine N-oxide (Group A, M031) (promary plant metabolite minor metabolite in ruits <5% TRR; 1 22% TRR in cereals | | | | | | | |
| Acute oral | Rat | 5000 1000 mg/kg n/ac | LD ₅₀ 707 mg/kg bw (Acute Tox. Cat. 4, H302) | CA 5.8.1/01 [M-016338- 01-1] | | | | |
| In vite bacterial reverse (Ames) gene mutation | typhim Fium strains | TA98, 16 5000 μg/plate TA1537 TA1535: Expt. 2: Por incorporation (±S9) All strains 5 – 1581 μg/plate TA100: 20.48 – 5000 μg/plate | +/-S9 negative | CA 5.8.1/02 [M-016297- 01-1] | | | | |
| In the backerial reverse (Arnes) gene mutation | typhimurium strains | Expt. 1: plate incorporation (±S9) All strains: 5 – 5000 μg/plate Expt. 2: plate incorporation (-S9) TA98, 8.912 – 2000 μg/plate TA1535, TA1537, TA102: | +/-S9 negative | CA 5.8.1/20 [M-756858- 02-1] | | | | |



| Type of study | Species | Doses | es mg/kg bw/day | | Key effects | Annex CA |
|--|--|-------------------------------|----------------------------|----------------------------|-----------------------------------|---------------------------|
| | | | NOAEL | LOAEL | | Point/Refer ence@ |
| | | TA100: | 20.48 - 50 | 000 μg/plate | | |
| | | Expt. 2: p | re-incubatio | on (+S9) | Ď | |
| | | TA98, TA102: | 8.912 – 20 | 000 μg/plate | F | |
| | | TA102. | 20.48 - 50 | 000 μg/plate | | |
| | | TA1535, | | Ö | | |
| | | TA1537 5 h –S9: | 0 50 200 | 0, 350, 500, | OF/-S9 negative | |
| In vitro mammalian | V79 (hprt) | 3 H 37. | | ug/mL | (sufficient © | CA 5:8.1/03@ |
| forward gene | cells | 5 h +S9: | 0, 100, 200 | | maximuno voncentration not | [M-006500 |
| mutation | | | 500, 600 μι | g/mL 🎻 🎉 | tested) | |
| | | 4 h (+18 h | Q, 200, \$00 | , 400 μg/mJ | 8 8 4 | A |
| | | recovery) – 1 | | | A | |
| | | 4 h (+36)h | 0,400 | μg/mJL Ó | +/-Sonegatove | |
| | | recovery) – ¿ | | | (sufficient | CA 5.8.1/04 |
| V79 chrom abs | V79 cells | 4® (+18 h | 0,300,400 | 500 kg/mL | Oraximum S | <u> [M-006495-</u> |
| | | recovery) | | | concentration flot tested) | <u>[<u>W1-1</u>]</u> |
| | 4 | 4 % (+30 hc) | \$0,500 | <u>~V @√V</u> bug/mL √V | | |
| | ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ | recovery | | Greans , | | |
| | | +S9 × | 00 158 | - 200 μg/m/L | | |
| | | recovery) | 90, 13 0 /230 | , ayo μg/μπ. | | |
| | Juman Juman | \$\sqrt{9}\sqrt{9} | | | | G |
| In vitro mammalian | penguciai | (421 h / recovery), @ | 0, 225, 2 75 | , 325 μg/m () | +/-\$9 negative | CA 5.8.1/21 [M-755221- |
| micronucleus | blood © lymphocytes | +S9 | | | © | 02-1] |
| l Ön | | 29 h (+24 h | 3 , 20, 50 , | 90 µg/mL | ∀ | |
| ĘĠ" | | S ⁹ | | | | |
| | 9 4 | 0.30, 150 0.000 ppp | | , 3 | ↓ bwt gain, | |
| (| | | | | stomach, oesophagus | G + 5 0 1 /05 |
| | | 0.30 150 | ₹ 0 3≈12 9 ° | ♂: 114.6 | (hyperkeratosis) | CA 5.8.1/05 |
| 28-day | Rat 💝 | 000 ppp | #: 13.2° | Ç: 94.3 | and urinary bladder (mild | [M-006504- |
| | | | | | transitional cell | <u>02-1</u>] |
| ₹ 1 | JÝ A | | | | hyperplasia) | |
| | | | Spirov | amine N-oxid | histopathology | |
| | , | | Spirox | amme 11-0alu | ↓ bwt gain, | |
| | | | | | stomach, | |
| | | | 10- | 4 - | oesophagus (hyperkeratosis) | CA 5.8.1/06 |
| 90-day 🔊 | Rat 🔊 | 0, 25, <u>125,</u> 625 ppm | ♂: 8.8 ♀: 9.7 | ♂: 45.0 ♀: 53.6 | histopathology. ↑ | [M-016585- |
| | | 023 ppiii | + • • • • • • | +. 55.0 | liver enzymes, without concurrent | <u>01-2</u>] |
| | S | | | | hepatic | |
| 90-day 7 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 6 | | | | | histopathology | |
| | | | Sp | iroxamine | | |



| Type of study | Species | Doses | Doses mg/kg bw/day | | Key effects | Annex CA |
|---|---|---|--|--|--|---|
| | | | NOAEL | LOAEL | | Point/Refer ence@ |
| | | 0, 625 ppm | ⊘: - 9: - | ♂: 48.7 ♀: 52.7 | Similar effects reported for spiroxamine N- oxide were also observed for spiroxamine | |
| | Toxicity | data on spirox | | | oup B, M13) | |
| | only det | <u>not</u> a pr) ected in fruit c | imary plant rops followi | | oup B, M13) | |
| Acute oral | Rat | 200, 2500, 3200, 4000, 5000 mg/kg bw | n/aQ | | LP20 4200 mg/kg / bw (CLP2 msufficent) | © 0 © 0 © 0 © 0 © 0 0 0 0 0 0 0 0 0 0 0 |
| Acute dermal | Rabbit | 5000 mg/kg bw | | | LID >5000 mg/kg/ bw CLP: Psufficient) | 01-1 |
| In vitro bacterial reverse (Ames) gene mutation | S. typhimurium strains | │ _{ॣॣ} Expt,ॣ <i>ॠ</i> ?₽r | 0, 1,0,3.16 100, 3.16 e incorpora 0, 1.0,9.16 | 5 10.0, 3106, 6 μg/plate | OH/-S9@cgative | GA 5.8.1/08 SM-471123- 01-1] |
| In vitro bacterial reverse (Ames) gene mutation | S. typuimurjum | AON strains. | te incorpora 5 - 2000 te incorpora 7 - 1660 cre incubation | ition (±S9)) µg/plate vijon (-SD)) µg/plate | O-S9 negative | CA 5.8.1/22 [M-755223- 02-1] |
| In vitro mammalian forward sene mutation | L51784/ (tk) enls | 3h, 24 h – \$9; 3 h 89: | 5.63 | 50 μg/mL × | +/-S9 negative | CA 5.8.1/09 [M-471125- 01-1] |
| V79 chrom abs | V. V. V. Cells | 3 h (+15 h ecovery) - 3 h (+15 h ecovery) - 3 h (+25 h ecovery) - 3 h (+25 h ecovery) - yecovery) - | 20, 250 20, 250 20, 100, 2 | 00 μg/mL 500 μg/mL 200 μg/mL ug/mL | +/-S9 negative (sufficient maximum concentration not tested) | CA 5.8.1/10 [<u>M-471187-</u> 01-1] |
| In sitro mammasian micronucleus | fuman peripheral blood lymphocytes | 3 h (+21 h recovery) – S9 3 h (+21 h recovery) | 0, 120, 180 | 210 μg/mL 2, 230 μg/mL 80, 95, μg/mL | +/-S9 negative | CA 5.8.1/23 [M-755227- 02-1] |



| Type of study | Species | Doses | mg/kg bw/day | | Key effects | Annex CA |
|------------------------------|--|--|---------------------------|---------------------------------|--|-----------------------------|
| | | | NOAEL | LOAEL | | Point/Refer |
| | | 24 h (+24 h | 0 40 80 | 105 μg/mL | | ence |
| | | recovery) – | 0, 10, 00, | 100 pg mil | | |
| | | S9 | | | | |
| | | | | | ↓ bwt / bowt gain (♂), booderate to | |
| | | 0.50.150 | | | severe clinical | |
| 28-day oral (via | Rat | 0, <u>50</u> , 150, 300 mg/kg | ♂: 50 | % : 150 | effect following | CA 5/8.1/14/ |
| gavage) | Kut | bw/d | ♀: 50 | \$\times \tau: 150 | Rosing with a peak period of effects | 291-1] Q |
| | | | | Q. | 15 minutes post | |
| | | | | ~ | dosing Q , O | |
|] | Toxicity data | on spiroxamine (Not | cyclohexyl a płant met | acetate Grou | ip/B, M43-acetate) | |
| | | (1101 | Mat | | Mortality, Powt, & | |
| | | | Winter Contract | eggiai Q | bwt gair food | |
| Davidanmentel | | 0, 40, 169, | 40 ° | / 16 40 6 | Consumption Colinical signs | CA 5 8 /1/12 |
| Developmental, oral (gavage) | Rat | 640 mQ/kg | | poprental O | ↓ byt, ↓ dotation | [<u>M-471532-</u> |
| (881) | | bw day | / | porental | conal pelvis, | |
| | | | ©160 © | | Odelay (0) O ossification \(\) | ~ |
| | ************************************** | 10 μL of «C | n/a | n/a | M23-acetate | |
| Bio- | Ö | M13 acetate ″ | | v ~ | readily bydrolysed | CA 5.8.1/13 |
| transformation | Rat 🔊 | stock© Aincubated | F F | | in plasma of and ♀ rats to Mas | [<u>M-472817-</u> |
| in plasma | | for 15, 60 | | | | <u>01-1</u>] |
| | | Şmin 🖑 | | | 0 4 | |
| (nrima) | Nankmeta | tý∧ďata orkspiro Krajte in fruit či | erns only (u | inodio I (Grøy n to 38% I ki | rp C, W2 8) R). <u>Not</u> formed in cer | reals |
| (P. III) | | | рэ ожу <u>(а</u> | D 2 | $L_{D_{50}} > 550$ | CA 5.8.1/14 |
| Acute oral & | Rat | 2 © 000 mg2kg | | y O i/a ** | ₹2000 mg/kg bw | [M-462551- |
| | | by by | | | (Acute Tox. Cat. 4, H302) | <u>02-1</u>] |
| | 5 0 4 | Expt. 1: pla | te incorpora | tion (± \$9) | 11302) | |
| In vitro bacterial | | All strains: | 3 - 500 | | | CA 5.8.1/15 |
| reverse (Ames) gene mutation | Typhimarium strains | Expr 2: Pr | e incorporat | tion (±S9) | +/-S9 negative | [<u>M-463413-</u> 01-1] |
| gene mutation | | All strains: | 30-5000 | μg/plate | | <u>01-1</u> j |
| 4 | 8 | n −SP | | 03.8, 407.5, | | |
| In vitro | , Q | 2 4 h +SO: \$ | | 30 μg/mL 03.8, 407.5, | | |
| mammalian | V 9 (hpr#) | 4 h +S9: | \$15, 16. | 05.8, 407.5, 30 μg/mL | . / 00 | CA 5.8.1/16 |
| forward gene | cells | | 7, 101.9, 2 | 03.8, 407.5, | +/-S9 negative | [<u>M-465292-</u> 01-1] |
| mutation | | | | 30 μg/mL | | <u>01 1</u> j |
| | | ⁷ 24 √ -S9: [∞] | | 407.5, 815, 630 μg/mL | | |
| | | 4 h (+36 h | | 920, 1610 | | |
| | | recovery) – | μg | /mL | | |
| In vitte 0 | Haiman J | S9 4 h (+36 h | 0 525 7 | 920, 1610 | | CA 5.8.1/17 |
| mammalian | blood | recovery) | | /mL | +/-S9 negative | [<u>M-469334-</u> |
| mičronuđeus | lymphocytes | | | | | <u>01-1</u>] |
| | | | | 920, 1610 | | |
| | | | μg | /mL | | |



| Type of study | Species | Doses | mg/kg bw/day | | | | |
|--|----------------------|--------------------------------|-----------------------------------|-----------------------------|--|-------------------------------|--|
| | | | NOAEL | LOAEL | | Point/Refer ence@ | |
| | | 20 h (+20 h | 0, 525.7, | 920, 1610 | | | |
| | | recovery) – S9 | μg | /mL | ð | | |
| | | 3 h (+21 h | 0, 200 | , 1000, | O O | | |
| | | recovery) – | | μg/mL | | 5° 5° ,¢ | |
| | Human | S9 3 h (+21 h | 0.200 | 3 00, | | | |
| <i>In vitro</i> mammalian | peripheral | recovery) | | μg/mL | %-S9 negative | 69 5.8.1/24 24 -755020- 4 | |
| micronucleus | blood lymphocytes | +S9 24 h (+24 h | 0300 | , 1000, | | 02-1 | |
| | Tymphocytes | recovery) – | | μg/mL 🦠 | | | |
| | | S9 | 0,400 | b, 1000. μg/ xn) | | | |
| | | | * 144,00 | μg/tanje 🕎 | Nordverse effects | | |
| 28-day oral (via | Rat | 0, 45, 135 | ∂; 28 .4 ^ | √ <u>1: -</u> 4 | observed when | CAOS.8.1/18 [M-471499- | |
| dietary) | | 400 ppm | 31.4 | / <u> </u> | tested up to the highest dose level | 01-1 | |
| | | - P | Mat | echal T | Mortality bwt, | 8 | |
| | | | , , | , , | Onical signs, Si | | |
| Developmental, | Rat ≈ | 0, 30, 150, \$500 mg/kg | \$\tilde{\pi}\)150 \$\tilde{\pi}\ | \$000 A | GI tract | ČA 5.8.1/19 [M-472720- | |
| oral (gavage) | Kat | bw/day | Develo | pmentał√ | incidence of | <u>01-1</u>] | |
| | | | 30 \$ | <u></u> | Incomplete ossification | | |
| | Tokicity: | ata on spiroxa | | 0 | - O | | |
| (p 1 | rimæry plant i | metabolite in gi | rapeSonly (| 4% TRR). <u>N</u> | of formed in cereals | | |
| į | Š ,õ | % h (+24) h % recovery) - ^ | 0, 25, 90, | 75 pg/mL 4 | | | |
| | | | | | \\\\\'\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\ | | |
| In vitro 🖔 | H o man | Ö 3 | 25, 56C | 775 μg/PnL 🐰 | S . | CA 5.8.1/25 | |
| mammalian | peripheral | h (+2) h recovery) | 0, 10, 25, | 50 kg/mL | +/-S9 negative | [M-753775- | |
| micronacteus | lymphocytes | Secovery) | | | | <u>01-1</u>] | |
| | \$. | 24 h (+24 h Secover√) – | 3 , 10, 3 , | 50 μg/mL | | | |
| a a a a a a a a a a a a a a a a a a a | | Secovery) - | * 6 | Ţ | | | |
| ~ | Toxicity data | on spiroxamin | e tetracosar | oic acid ester | (Group B, M36) t formed in cereals | | |
| | | netabolite to gr | appes omygya | 76 IKK). <u>No</u> | Tormed in cereais | | |
| | | | | | | CA 5.0.1.5/01 | |
| In vitro gastric acid stability | data | Awaitin@ data | Awaiti | ing data | Awaiting data | 5.8.1.5/01 Author | |
| | | | \$ | | | (YYYY) | |
| Toxicity data on sporoxamine cyclohexenol (Group B, M37) | | | | | | | |
| (<u>Not</u> a primary p | lant me@boli | te Formêd in s | mall quantit | ies (3% TRR |) only after exhaustiv | ve hydrolysis) | |
| | | 3 h (+21 h recovery) – | | , 300 μg/mL | | | |
| In vitiro | Human. | S9 | | 280, 300, ug/mL | | CA 5.8.1/26 | |
| matnmalian | peripheral blood | 3 h (+21 h | | , 310 μg/mL | +/-S9 negative | [M-761547- | |
| micropuPéus | lymphocytes | recovery) +S9 | | | | <u>01-1</u>] | |
| | | 167 | 0, 100, 140 | , 170 μg/mL | | | |
| | | | | | | | |



| Type of study | Species | Doses | mg/kg bw/day | | Key effects | Annex CA |
|---------------|--------------------------|--------------------------|--|-------------------------|--|----------------------------|
| | | | NOAEL | LOAEL | | Point/Refer |
| | | 24 h (+24 h | 0, 75, 100, | 140 μg/mL | | ence |
| | | recovery) – S9 | | | Ö | |
| In silico | QSAR m | odels used | | Resul | lt Ö | CA 5.8 1/27 |
| toxicology | ECOSAR v2 | | It is conclu | ded that M37 | can be prouped with | PM-7@554-« |
| testing | OECD Tooll | oox v4.4 | M13 for the purposed of read across of toxicity date | | | |
| | In | silico analysis o | n Group A., | B and C meta | H oolites | |
| | QSAR m | SAR models used Result . | | | ít | CA 5:8.1/28@ |
| | Derek Nexus | e v 6 0 1 | M05, M06, | "M07, № 08 M | 01 M02, M03, M05, (09, M10 M11 no conserns for | [M-763152] Q[2] |
| In silico | [knowledge system]; | | genotoxicit assessed | y f ôr any of th | ne medabolite® | |
| toxicology | OECD (Q)S. | AR Toolbox ∜ ´ | Group Ban | etabol it s (M) | (3, M14, M15, W16, | ÇÃ 5.8√29 |
| testing | | and category | | | no concerns for | M-763153- |
| | formation to Leadscope M | ol] | | y for any of th | ne metabolites V | 01-1 |
| | v3.0.1-1 | | Gooup Côn | etabolites (M) | M29 M30, C notox vity for any & | °♥A 5.8.1/30 [M-763154- |
| | A | | | bolites assesse | | 01-1] |

1. M03: this primary plant metabolite, once absorbed will indergo reduction to the parent (sproxamine [SPX]). This reduction takes place both eazymatically 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 main 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: Report Author Report Year: Report Title: Position paper on the netabolic and toxicological aspects of Spiroxamine-Noxide Report No: M-008167-02-1 Document No: Guideline(s) followed in study: Deviations from current test guideline: Previous valuation: Previous valuation: GLP Officially recognised aesting facilities Acceptability/Reliability: Supportive only | | |
|---|----------------------------|---|
| Report Year: Report Title: Position paper on the metabolic and toxicological aspects of Spiroxamine-Noxide Report No: Document No: Guideline(s) followed in study: Deviations from current test guideline: Previous valuation: GLPOfficially recognised testing facilities | Data Point: | KCAS 8.1/2 |
| Report Title: Position paper on the metabolic and toxicological aspects of Spiroxamine-Noxide Report No: Nt-00816 - 02-1 Document No: Guideline(s) followed in study: Deviations from current test guideline: Previous valuation: yes valuated and accepted DAR (2010) GLP Officially recognised testing facilities | | |
| Report No: M-008165-02-1 Document No: | Report Year: Q | |
| Report No: Document No: Guideline(s) followed in study: Deviations from current test guideline: Previous valuation: GLPOfficial recognised testing facilities | Report Title; | Position paper on the metabolic and toxicological aspects of Spiroxamine-N- |
| Document No: Guideline(s) followed in study: Deviations from current test guideline: Previous valuation: Out-008 67-02 Not specified study: Deviations from current test guideline: Previous valuation: Out-008 67-02 Not specified study: Out-008 67-02 Out-008 67-02 | (%) | oxide S |
| Guideline(s) followed in not specified study: Deviations from current test guideline: Previous valuation: Quarticle of the property of the | | |
| Study: Deviations from current test guideline: Previous valuation: Previous valuation: GLPOfficial value and accepted DAR (2010) GLPOfficial value and accepted not applicable recognised testing facilities. | Document No: | <u>7√1-008</u> <u>167-02</u> |
| Deviations from current test guideline: Previous valuation: yes valuated and accepted DAR (2010) GLP officially not applicable recognised testing facilities. | Guideline(s) followed in | not specified. |
| Deviations from current test guideline: Previous valuation: yes valuated and accepted DAR (2010) GLP officially not applicable recognised testing facilities. | study: | |
| Previous valuation: yes valuated and accepted DAR (2010) GLP Official value of applicable recognised testing facilities. | Deviations from current | Mone W , |
| GLP/Officially hot applicable recognised testing facilities. | test guideliane. | D) · · · · · · · · · · · · · · · · · · · |
| GLP Officially hot applicable recognised testing facilities. | Previous valuation: © | |
| | 77 5 4 | |
| | GLP Officially S | not applicable |
| | recognised testing | |
| Acceptability/Reliability: Supportive only | | |
| | 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 New 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 evel 4 hours post application (CA 5.1.2/01 [M-006044-01-1]). The livers were sampled and extracted. After purification the extract was analysed chromatographically and the Noxide identified by compatison 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 Noxide as well as the parent compound were incubated for 4 hours at 37°C with freshly prepared samples of gastric juice from the rat (CA5-1.2/04 [M-06044-01-1]). 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-buth cyclonexanone.

Following absorption, the N-oxide will undergo reduction to the parent compound like other N-oxides of tertiary amines. This reduction takes place enzymentically 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 an equilibrium between spitoxamine 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 Noxida maintained in the rat as long as spiroxamine is administered, e.g. under chronic reeding conditions.

This also means that the major part of the absorbed N-oxide originating from plant residues will not remain as such in the meanism but rather be reduced to spiroxandine 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 factor 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-w0-bond². 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.

Mitchard, M. (1971). Bioreduction of organic nitrogen. *Xenobiotica* 1, pp 469

Bickel, M. (1971). Diver metabolic reactions: Certiary amine N-dealkylation, tertiary smine N-oxidation, N-oxide reduction, and N. (1981). Biochem. *Biochem. Biochem. Biophys.* 148. Pp 54-62

White, C.H., Suzanger, M., Mattacks, A.R., Bailey, E., Farmer, P.B.& Connors, T.A. (1989). Reduction of nitromin to nitrogen mustach. Unscheduled DNA synthesis in aerobic or anaerobic rat benatocytes. IBL BL8, and Walker carcinometers.

nitrogen mustaid: Unscheduled DNA synthesis in aerobic or anaerobic rat hepatocytes, JB1, BL8, and Walker carcinoma cell lines. *Cyclinogenesis* **10**, pp 2113

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

Hamill S. & Cooper, D.Y. (1984). The role of cytochrome P-450 in the dual pathways of N-demethylation of N.N'-

Mamill 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 test⁴. This may be related to the easy and quick metabolisation leading to the corresponding alcohol and its conjugates. The non-hydrolized paint 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 spiroxaroine.

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 oxicological 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 takes from the spiroxamine data has greatly be representative also for the N-oxide.

| Data Point: | KCAG5.8.1/04J V S S JV O S S |
|---|---|
| Report Author: | |
| Report Year: | |
| Report Title: | KWG 4168-No xide Pilot study for acute stral toxicity in ternale rats |
| Report No: | 237, 0 0 0 |
| Document No: | Mart 6338-01-1 |
| Guideline(s) followed in | OECD 1; US PA Series 813 |
| Guideline(s) followed in study: Deviations from current test guideline | OECD 401; US TEPA Series 8 Est |
| Deviations from current | Yes & & X |
| test guideline | Annough The strict was proadly comparable to the now deleted OECD 401 |
| | (1987) gest guideline a number of deficiencies were noted (refer to Results, |
| | Deficiencies ection Selow). |
| Previous evaluation: | yes evaluated and accepted of the second of |
| *** | DAR (2010) 5 4 4 |
| GLP/Officially | No, not conducted under GLP officially recognised testing facilities |
| recognised testing | |
| GLP/Officially recognised testing facilities: | Deficiencies section below). yes evaluated and accepted DAR (2010) No, not conducted under GLP officially recognised testing facilities |
| Acceptability Reliability: | |

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 artimals dosed at 300 of 1000 mg/kg bw, respectively. Observations included signs which were reflective of CNS toxicity.

⁴ Opdyle, 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 LD₅₀ for spiroxamine N-oxide was 707 mg/kg w in female rats. Therefore, according to Annex I for Regulation (EC) 1272/2008 spiroxamine N-oxide musto be classified in Category 4. The signal word "Warning" and hazard statement H302 "Harmful of swallowed" are required.

Materials and Methods

A. Materials:

1. Test Material: KWG 4168-N-oxide

(alternative names: Spirovamine N-oxide, \$48-text-butyl-

dioxaspiro[4.5]dec-2-yh methyl](ethyl)(propyl) mine-18-ox

Description: Yellowish liquid Lot/Batch No.: 940315ELB01

90.0% (w/w) (correction for purity not undertaken **Purity:**

148044-85-3 CAS No.:

Confirmed Stable for the duration of the Stability of test

compound:

2. Vehicle and/or positive

control:

3. Test animals:

Species: Han≟Wistar Strain: Age at dosing:

Weight at dosing

Source: Acclimation periodo

7 days 7 and 2 h prior Diet:

and post dosing, respectively

Water. Manicipal water, ad libitum

during acclimatisation. Individually housed during study Housing:

4. Environmenta conditions: @/

> Temperature: Humidity:

Air Thanges: Photoperiod:

B. Test Performance:

1. In life dates: [overeber 1994 to 29 November 1994 (experimental dates)

Animals were selected before administration based on their body weight on the 2. Animal assignment day of grouping. After overnight fasting, the test article was suspended in 2% Cemophor (on the day of dosing) and administered orally via gavage at a dose Yevel 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 postexposure.



LD₅₀ was calculated according to Bliss⁶ in the manner described by Rosiello et 3. Statistics:

al⁷ and Baird & Balster 8.

Not conducted

C. Methods:

1. Homogeneity and

achieved

concentration analysis

of the dose:

2. Test article formulation preparation: The test article was formulated in demineralised water (for ca. khour) before administration with the aid of Comophor EL (2% v/v). Homoscheity was

achieved by drawing into a syringe and then Omnping out several. No

correction for purity was taken into account

Animals were observed several times on the day of dos odministration (ep 3. Observations:

to time not given), with aily observation up until day 14.

Weighed on the day before dosing and on days 4, sand 15 3. Body weights:

4. Food consumption: Not recorded.

5. Sacrifice and All animals were

pathology: undertaken

Results

A. Homogeneity and achieved concentration analy

Not undertaken. Analyses for a drieved concentration homogeneit for stability of lest 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:

Omical signs of toxicits occurred shortly after administration, lasting until a maxing of Other 5 of 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, Moerection, narrowed alpebra slits, alivation, red-encrusted nose, and in some cases bloody nasal disorarge, periodic lying on side, cramped stretching of extremition, soft or no faeces. The report did not detail how these clinical sign's were distributed between the dose of ours, with a general onset time of signs (collectivery) were∕observed only.

2. Mortality: Referito Table/CA 5%.1/01-©

> A strigle againal from the 500 mgdeg bw and four animals from the 1000 mg/kg by group died wothin 1 Day of dosing.

C. Body weight and food consumption

1. Body weight:

Sheht body weight loss was observed in 1 \(\text{ (#1105)}\) at day 3, with increased body weight gain occurring from day 7 onwards. This was an isolated incidence, with the transpent decrease in body weight deemed not treatment-related. All other animals body weight increased from day 1 through to day 14 (refer to Table GA 5.2.4€1-1). Q

2. Food consum

Not applicable

Bliss (O. (1935). The calculation of the dosage-mortality curve. *Ann. Appl. Biol.* **22**, pp 134

Bliss Q.I. (1938). The determination of the dosage-mortality curve from small numbers. O.J. Pharm. Pharmacol. 11, pp 192-216

Rosiello, A.P., Essigmann, J.M. & Wogan, G.N. (1977). Rapid and accuracte determination of the media lethal dose (LD50) and its error with a small computer. J. Toxicol. Environ. Health, 3, pp 797-809

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

0



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

| Danier of ou | | ♀ (mg/kg bw) | | | ♀ (mg/kg bw) | |
|-----------------------------|---------------|------------------------------|------------------|-------------------------|--------------|--------------------|
| Parameter | | 500 | | | 1000 | |
| Mortality ^a | | 1/5 | | | 2 75 | |
| Day | 1 | 8 | 15 | 1 | 8 | → 15, ¬¬ |
| Body weight (g) | 202 ± 2.7 | 219 ±5.4 | b | 173 ±1.8 | ₹ 213 ±0.0 | \$270± 0 .0 |
| ±s.d. | | | | * | | P . Ø′ 🗸 |
| Net body weight | 17 | 1.8 ± 6.7 (day $1 - 3$) | 8) 💍 | 4 | 73.9 ±8.5© | |
| gain (g) | | | | Q | | \$' \" |
| Acute oral LD ₅₀ | | · | > 1 000 m | ıg/kg bw¦© [™] | W. | |

Mortality: no. of animals found dead / no. of animals treated

D. Necropsy:

Not undertaken.

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

Necropsy:

It undertaken.

Deficiencies:

though the study was broadly comparable to the new delered ODCD 407 (1987) test guideline, the llowing 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 Although the study was broadly comparable to the new deleted ODCD following deficiencies are noted when compared to this guideline.

- Dosing was limited to a single sex (females)

- No gross histopathological@nalysis/was_undertaken
- Tabulation of clinical signs were not provided
- The LD50 value estimated was provided without confidence intervals

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

gridance and the real as of this study the acute or. Therefore, according to Arinex to be classified in Category & The six and if a wallowed are required. Conclusion: Under the conditions of this study the acute oral LO50 of spiroxamine N-oxide was 707 mg/kg box in female rats. Therefore, according to Annex For Regulation (EC) 1272/2008 spiroxamine N-oxide must be classified in Calegory & The signal word "Warning" and hazard



| | 1 |
|----------------------------|---|
| Data Point: | KCA 5.8.1/02 |
| Report Author: | |
| Report Year: | 1995 |
| Report Title: | KWG 4168-N-Oxid - Salmonella/microsome test plate incorporation and |
| | preincubation method |
| Report No: | 24105 |
| Document No: | <u>M-016297-01-1</u> |
| Guideline(s) followed in | Directive 92/69/EEC, Method B.14.; OECD 471; USEPA PB 84-28 295, 56- |
| study: | Gene Muta-S. typhimurium |
| Deviations from current | Yes V V V V |
| test guideline: | A number of deficiencies were noted (refer to posults, Deficiencies section |
| | below). |
| Previous evaluation: | yes, evaluated and accepted & & & & & & & & & & & & & & & & & & & |
| | DAR (2010) |
| GLP/Officially | Yes, conducted under GLP/Officially (Cognised testing facilities |
| recognised testing | |
| facilities: | |
| Acceptability/Reliability: | Supportive only A A A A |

Executive Summary

In a reverse gene mutation assay in bacteria 3. typhimurium strain TA98, TA1537, TA100 and TA1535 were exposed to spiroxamine N-oxode (M03) formulated in DMSO using the both the plate-incorporation and pre-incubation methodologies in the absonce and presence of an Anoclor 1254-induced rat liver post-mitochondrial fraction (\$9).

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 μ g/plate (the maximum recommended oncentration 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 oblight finning of the background bacterial lawn, with or without a concurrent marked reduction in a vertain numbers of reductions in titre levels was observed in all the tester strains in the absolute and presence of S0 at 1581 and/or 5000 μ g/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 381 tig/plate for all strains ±S9.

Following these treatments, expense of toxicity was again observed in all the tester strains in both the absence and presence of S9 at 500 µg/plate and expended 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 control with acceptable ranges for vehicle control treatments in both treatments. However it was noted that in the pre-incubation experiment, $TA100 \pm S9$ vehicle control individual and mean reversant colonies exceeded the laboratory's historical control range, confirming the data were not representative of the strain.

The positive controls induced in 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 minoanthracene (2-AA). 2-AA can be activated by enzymes other than the microsomal cytochrome C450 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

Conc.



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: Spiroxapone N-oxide, [48-tert-butyl-1,4

dioxaspiro[4.5]dec-2-yl) methyl](ethyl)(propyl)amine-N-bxide, 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 Confirmed stable for the duration of the study expired ate: November 1995

compound:

2. Control materials:

Negative:

Solvent/final DMSO (dirfiethyl sulphoxide)/0.1 AL/plate concentration:

Spitro 2-phenylene diamine 0.5, 10

O' S(NPDA)

TA100 2-prirofluorene $(2_{T}NF)$ 0.2 TA1535 Sodium ande $(N_{0}N_{3})$ 10

(µg/plate)

TA98, TA190 2-aminoa@hracene (2-AA) 3

3. Activation:

So 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% SP reaction mix was: S9 (10%), MgCl₂ PBS (2.3 mg/mL), KCl₃ SP mg/mL), glucose-6-phosphate (2.6 mg/mL), β-NADP (4Q mg/mL), PBS (1.4 mg/mL).

4. Test organisms:

🕏 typhynurium strains 🛪 A98, TA100, TA1535, TA1537

All test organisms were properly maintained and were checked for appropriate genetic markers (*Styphimurium*: histidine and biotin requirement, *rfa* putation wrB sensitivity, ampicillin-resistance) regularly.

5. Test Concentrations:

a) Modation assay 1. Place incorporation: +/-S9 all strains:

%16, 50, 158, 500, 1581, 5000 μg/plate

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

Of new, 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 agadetest 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 (nominal glucose agar plate). When set plates were in order and incubated at 7°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 3 °C. After additional of 2 mL of top again solution the mixture was poured onto minimal glucose agar plate and allowed to shidify All plates were incubated for 2 days at 37°C. The this way, it was hoped to increase the range of mutagonic 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 whild if the following criteria were met.

The vehicle controls fell within the expected range as defined by published data and of aboratory's historical control ranges.

2. The positive control chemicals had to show sufficient effects as defined by the laboratory experience:

Tites determinations had to demonstrate sufficient bacterial density in the suspension.

The test article was considered mutagenic in this assay if:

1. A concentration related increase in Svertant humbers was ≥2-fold (TA98, TA 100, TA 1535) above the concurrent vehicle control values A

2 Any observed response was reproducible under the same treatment of conditions.

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

The test arcicle was considered negative in this assay if none of the above outeria-were med.

C. Methods:

5. Evaluation criteria

1. Homogeneity and achieved concentration analysis of the dose.

concentration analysis of the dose:

2. Test article

formulation: preparation: details provided. However, from the available data, it can be confirmed that

The test article piroxamine N-oxide was soluble in DMSO at concentrations up to at least 0 mg/mL. Thereby, confirming a maximum concentration of

5000 μg/plate was achievable.

3. Toxicity Assessment:

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 result³.

Results and Discussion

A. Analytical determinations:

Not undertaken. Analyses for achieved concentration homogeneits or stability formulations were not conducted as part of this study, as this is not a equirement of the 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 gardelines). No evidence of precipitate was observed upon playing. Following these treatments, evidence of toxicity ranging from a complete killing of the test bacteria to reslight thinning of the background bacterial lawn, with or without a concurrent marked education in a vertant numbers or reductions in intre levels was observed in all the tester strains in the absorce and presence of \$\sqrt{3}\) at 1581 and or 5000 \(\mu\gamma\) at 1581.

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 ≥ fold (TA98, TA100, TA1537); ≥3fold (TA1537) above the concurrent vehicle control values.

Vehicle and positive control treatments were well ded for all strains. The mean bumbers 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 text system.

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

| | | | . 4 | | | L j | | |
|------------|---------------------|------------------------|-----------------------------|----------------------|----------|-------------------|-------------|-------------------|
| 1 ypc or | ? | | e-shift | ~ 'O | | ∰ase-pair s | ubstitution | |
| mutation " | <i>a</i> | | | \bigcirc v | √ n° (°) | | | |
| Conc. | T | 98 🔊 , | | 1537 | ŽΥTA | 100 | TA1 | 1535 |
| (µg/plate) | -S9\$\(\text{S}\) " | ≯ S9 ≪ | » - S9 | +S9 | -\$9 | +89 | -S9 | +89 |
| 0 | 180#3 | △30 ±4 | 8 ±2 ∗ | 7 ±1 | Ø8 ±3 | 91 ±14 | 10 ±2 | 10 ±3 |
| 16 | 20 ±4 & | ²⁹ ±29 ±28° | ©8 ±1 🔊 | 7 4 1 | ≈74 ±7 | 81 ±12 | 5 ±2 | 10 ±3 |
| 50 | ~019 ±2℃ | 23 ±2 | % 6 ±2% | *9±9 ~ | 84 ±10 | 91 ±8 | 7 ±2 | 6 ±3 |
| 158 | 22 ±7 | ©5 ±7~0 | 8,4 | ~7±1@ | 68 ±±9 | 91 ±8 | 6 ±3 | 9 ±1 |
| 500 | " 18 ±1 | ⊘ 24 ±2° | ® ±2 ° € | / / <u>\</u> | 65 ±9 | 82 ±8 | 7 ±2 | 8 ±3 |
| 1581 | 7 ±3 ^B | 13 ±4 ^{18/T} | $\sim 5 \pm 2^{\mathrm{B}}$ | 4 (∌1 B | 38 ±2 | 91 ± 10^{T} | 4 ±1 | 8 ±4 ^T |
| 500,0€ | В | |) 0 ±00 | $\pm 0^{\mathrm{B}}$ | В | $50 \pm 17^{B,T}$ | В | B,T |
| Positive | 153 ±5 | 10 ±93 | 129 ¥21 | 0109 ±27 | 229 ±20 | 789 ±44 | 743 ±40 | 105 ± 17 |
| control | @1 [\] | | | 7 | | | | |

B: diminution of background laws

T: bacteriotoxic effect evident from marked reduction in titre levels

Positive contro

TA98: 131537

+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 μ g/plate for all strains \pm 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, TA1337); ≥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 generic drift. The positive controls induced an acceptable increase in revertant colony humbers, 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 & periment 2

| | | | | \checkmark | . ~ . " | Ĉ, | | |
|------------------|----------------|--|----------------------|-------------------|--------------------------------------|--|----------------------|---------------------|
| Type of mutation | | Fram | e-shift | | | Base-pair | abstitution TAI | Ş |
| Conc. | TA | .98 _{@.} | ♥ √©TA1 | 597 | | ************************************** | [_() JA | 535 |
| (µg/plate) | -S9 | +89 | ~ 5 9 € | +890 | √- S9 √ 153 4 9 | +S9 | © -S9 [®] √ | +S9 |
| 0 | 28 ±3 | 37≝∕8 | √ 14 ±1 √ | 16,±3 | <u>153</u> 49 | 201 ±16% | | 15 ±4 |
| 5 | 30 ±4 | 4 3 ±5 € | 10 ±2 13 ±4 | ⊉ 1 ±3 | 153 ± 6 | \$\text{92 \pm 10} | 19 ±2 | 14 ±3 |
| 16 | 25 ±2 | ₹7 ±4,∢ | 13 2 4 ∠ | 14 ±47 | 162 ±19 * | 211⁄∠±8 | ≈3 ±3 | 15 ±3 |
| 50 | 23 ±5 × | √ 40 ±3,** | @6±1 € | 1334 | , 124 ± 9 √ | 209 ±5 % | 11 ±2 | 13 ±3 |
| 158 | 33 ±4 🔊 | 45 ⊈6 ∝ | 8 ±3@ | 13 ±3 ≤ | √123 ±28 | Ø67 ±17√ | 12 ±1 | 15 ±3 |
| 500 | 26 ± | \$1 ±5 € |) 13,≇2 [®] | 8 ±3 | 5 3 ⊈12 ∫ | 126 + 5 | 7 ±2 ^B | 9 ±1 ^T |
| 1581 | 9 = 3 B | \\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\ | <i>B</i> ^ | 74 ±1387 | 20 ±0B € | 45 ≇ ℂ8 ^{B,T} | В | 6 ±2 ^{B,T} |
| Positive | 10 ±15 |)1320±34 | 30 ±140° | 32 2 ¥23 g | 320 ±25 | 1422 ±49 | 712 ±33 | 215 ±32 |
| control | | 4 | | | | .0 | | |

B: diminution of background lawn

Description of the state of the

Positive controls:

-S9: strains:

TA98; TA1537: NPD

TA100: 2-NF TA1535: NaN₃ +\$9% strains

A98; 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 (TA1557) 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

| | | 8 | • | • | | | | <i>a</i> , ` | |
|------------------|------------|----------|------------|---------------|------------------------|-------------------|---------------------|---------------|--|
| Type of mutation | | Fram | e-shift | | Base-pair substitution | | | | |
| Parameter | TA | 98 | TA1 | 1537 | TA | 100 | TA1535 | | |
| | -S9 | +89 | -S9 | +89 | -S9 | +S9 💍 | -S9 | ∀+S9 ₹ | |
| | | (10%) | | (10%) | | (10%) | d | (10%) | |
| | | | Ve | ehicle contro | 1 | Z) | ~ | | |
| Date range | Jan 1994 - | Dec 1994 | Jan 1994 – | Dec 1994 | 🕼 an 1994 – | - Dec 1994 | Jan 1 99 4 - | – Dec 1994 🤊 | |
| Median | 21 | 27 | 9 | 8 | § 82 | © 105 | | 9 11v | |
| Semi Q | 4 | 5 | 2 | 2 🐇 | 9 | © [♥] 15 | № 2 £ | 25 | |
| range | | | | , | | . 0 | 10 (| | |
| | | | | sitiy@ontro | ol 😞 🔻 | | | | |
| Date range | Jan 1994 - | Dec 1994 | Jan 1994 – | - Dev 1994 | Jan 👍 🦻 94 – | Dec 1994 | Jan 1994 | ∡Dec 1994 | |
| Median | 138 | 1301 | 105 | 103 | _^2€3 ¸₹ | 11,10 | 706 | 174 | |
| Semi Q | 23 | 208 | 35 | 73 | ِيُّ 33 گُرِ آھ | 33 0 | O' 98.C | 49 | |
| range | | | <u> </u> | . 0 ~ | V Q | | , Oʻ | | |

The report provided historical control ranges over a multitude of dates (July Dec 1994; Jan Jul 1992; Jul – Dec 1992)

Jan – Jun 1993; Jul – Dec 1993), including numerous related 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: NaN₃ +SQ: strains;

TA98; TA0337; TA100; TA935: 2

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

| | | 4 4 | | | <u> </u> | / · | |
|--------------|------------------------------------|------------|--|------------------------------|--------------|-------------|----------|
| Type of | Fram | e-shift @ | \$ X | | Base-pair | ubstitution | |
| mutation | | | ~Ô | TO | | | |
| Parameter | | TAI | 837 | TA | 100 | TA1 | 535 |
| | -S9 +S9 +S9 (4 b%) | S9.0 | # \$ 9 | -59 | ¥ S 9 | -S9 | +89 |
| | | , | 6. KIU /U/K | | (20%) | | (10%) |
| Ò | | · ¬ | hicle contro | 1 0 | V | | |
| Date range | Jan 1994 Dec 1994 | Jan 1994 – | Dec 19 94 | ~ © an 19 9 4√ | Dec 1994 | Jan 1994 – | Dec 1994 |
| Median | 22 🔎 🔩 | 8.00 | , % · ' | U 99 | 115 | 10 | 11 |
| Semi Q | 50 46 8 | 20 | √ 2 √ y | 440" | 13 | 2 | 3 |
| range | 59 46 8 | | Ø O | * | | | |
| | -Q, | | sitive@ontro | KŽ | | | |
| Date range | √a n 199 ₽ Dec©994 ° | Jan 1994 – | - De [©] 1994 <i>"</i> | [©] Jan 1994 – | Dec 1994 | Jan 1994 – | Dec 1994 |
| Median | 137 268 | , 98°, | \$\\\@\^178\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\ | 402 | 1357 | 185 | 787 |
| Semi Q range | 26 208 | 6,83 °≈ | \$\int 178 \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ | 41 | 166 | 35 | 116 |
| range | | | | | | | |

The report provided his orical 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

1A100: 2-N

+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 WP2uvrA/WP2urvA (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 cytochome 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 formers were presented. For the bacterial (Ames) reverse mutation assay the laboratory's historical control range should be established with at the very least a \$9\% reference range (at least 300 data points needed for this) applied. The inclusion to accepted published data is inappropriate and greates 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 ±89, preincubation exceeded the relevant historical control ranges, which confirm that the data are not characteristic of the strain, with generic drift of likely concern.
- Due to the low spontaneous revertant colony frequency of TA1535 22-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 [\$\frac{1}{2}\cdot 7568 \frac{1}{2}\cdot -02\cdot 7\cdot 7\

Assessment and conclusion by applicant

Assessment: This gudy is deemed supplementary as a number of deficiencies are identified when assessed against corrent lest guideline requirements.

Conclusion: It was concluded that spiroxamine Noxide did not induce mutation in four histidine-requiring strains (TA98, TA100. TA1535 and TA1537 of Sationella typhimurium when tested under the conditions of this study. These conditions fielded 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 negabolic activation system (S9) or up to concentration infinited by toxicity using both the plate incorporation and pre-incubation methodologies.



| Data Point: | KCA 5.8.1/20 |
|----------------------------|---|
| Report Author: | . 0 |
| Report Year: | 2020 |
| Report Title: | Amendment no. 01: Spiroxamine N-oxide: Bacterial reverse mutation assay |
| Report No: | 8406980 |
| Document No: | <u>M-756858-02-1</u> |
| Guideline(s) followed in | OECD Guideline 471 (OECD, 1997) |
| study: | |
| Deviations from current | None & X X X |
| test guideline: | |
| Previous evaluation: | No, not previously submitted (|
| | |
| GLP/Officially | Yes, conducted under GLP Officially recognised testing facilities |
| recognised testing | |
| facilities: | |
| Acceptability/Reliability: | Yes O O O O O |

Executive Summary

In a reverse gene mutation assay in bacteria, Styphiaurium strains TA98 TA1577, TA100, TA1535 and TA102 were exposed to spiroxamine Noxide (A03) formulated in DMSO sing 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 trains were performed in the absence and in the presence of S9, using final concentrations of spiroxamine N-oxide at 0.5, 5, 16, 50, 160, 500, 1600 and $5000 \, \mu g/plate$. Following these treatments, evidence of toxicity manifest as uninning of the background lawn and/or complete killing of the background lawn was observed at 1600 and/or $5000 \, \mu g/plate$, respectively in all the rester strains in both the absence and presence of S9.

Mutation Experiment 2 treatments of all the tester strain were performed in the absence (plate incorporation methodology) and in the presence of \$9 (pre-incultation 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 \$000 μg/plate for strain TA100 in the absence and presence of \$9 and strains TA1535 and VA1537 in the presence of \$9, 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 ovidence of any mutagenic activity. Following these treatments, evidence of toxicity was again observed in all the tester strains or both the absence and presence of \$9, and extended down to either 800, 2000 or 50000 g/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, TA190, TA7335, PA1537 and TA102) of *Salmonella typhimurium* when tested under the conditions of this study. These conditions included treatments at concentrations up to $5000 \, \mu \text{g/plate}$ (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 AE 1344305 00 1C74 0001 Lot/Batch No.:

Purity: 72.9% (w/w) (correction factor 1.37 applied)

CAS No.: 148044-85-3

Confirmed stable for the duration of the stud Stability of test

compound: 2022)

2. Control materials:

Negative:

plate incorporation or DMSO (dimethyl subhoxide)0 Solvent/final

concentration: incubation, respectively

Positive: -S9 Strain Mutagen **TA98** TA100, TA153@ 9-aminoacridine (9 TA 100/2 Anomycia C (MAYC Strain Positive: +S9

(μg/plate)

S9 was purchased from a commercial source. Sprague Dawley rats were treated with Arocoor 1254 (supplied by lot no.: 4029, protein Content 3.7 mg/mL). The composition of the 10% S9 reaction mix was: 100 µL S9, Na PBS (400 μl/h), glucose-6-phosphate 5 μM), β-NADP (4 μM), MgCl₂

(8,0M), K, (33 μM), water (to vorume)

Styphimurium Trains TA98, TA100 TA1535, TA1537, TA102 4. Test organism

All test organisms were properly maintained and were checked for appropriate generic markers (Skuyphimurium: Justidine and biotin requirement, rfa

mutation graph sensitivity, ampigulin-resistance) regularly.

5. Test Concentrations

3. Activation:

Mutation assay +/-S&all strains:

0, 5, 16, 50, 160, 500, 1600, 5000 μg/plate

Rlate incorporation.

TA98, TA1535, TA537, TA102: 0, 8.192, 20.48, 51.2, 128, 320, 800,

A100χ0, 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

\$\times_100\$, TA1535, TA1537: 0, 20.48, 20.48, 128, 320, 800, 2000, 5000 μg/plate

¹⁰ Of notes 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-450catalysed 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 pm 7.4) or S9 mix (0.5 mL) for treatments in the absence and in the presence of metabolic activation, respectively and bacterial suspension (10 mL) were mixed and poured on to Vogel-Bonner E agai plates (minimal glucose agai plate) when set, plates were inverted and incubated at 37° protected from light to 3 days.

3. Experiment 2: Plate incorporation assay:

Undertaken for bacterial strains treated in the absence of \$9 (refet above).

Pre-incubation assay:

The test article solution or vehicle/positive control solution (0.5 mL), bacteria (0.1 mL) and S9 mix (0.5 mL) were mixed in a small test the 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 solidiff. All plates were incubated for y days at 37°C. In this way, it was hoped to increase the range of mutagonic chemicals that could be decided 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 raboratory's historical control ranges

2. The positive control chemicals induced increases in revertant numbers of \$\times 1.5\$-fold (TA\$\frac{10}{2}); \$\geq 2\$ fold (TA\$\frac{9}{2}\$, TA\$\frac{100}{2}\$); \$\frac{100}{2}\$ fold (TA\$\frac{9}{2}\$). TA\$\frac{100}{2}\$ above the concurrence control confirming discrimination between difference contains and an active S9 preparation.

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

1. A concentration related increase in revertant numbers was ≥1.5-fold (TA002); 22 fold (TA98, TA100); 23-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

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:

Nøfundertaken

2. Test article of formulation preparation:

To correct for purity, a correction factor of 1.37 was applied. A preliminary colubility test confirmed spiroxamine N-oxide was soluble in DMSO at concentration 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 draximum 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 spins 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 frain characteristics, and not to interpret or justify degative or positive result³.

Results and Discussion

A. Analytical determinations:

Not undertaken. Analyses for achieved concentration, homogeneity of 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 corrent regulatory guidelines). No evidence of precipitate was observed upon plating. Following these treatments, evidence of toxicity ranging from a complete killing of the test occertation a shight thinning of the background bacterial lawn, with or without a concurrent marked reduction in revertant numbers was observed it wall the tester strains in the absence and presence of S9 at 1600 and/ox5000 ng/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 (PA102) ≥2-fold (TA98, TA100); ≥3-fold (TA1535, TA1537) above the concertent which controls

Table CA 5.8. 20-1: Spiroxamine N-oxide: bacterial reverse gene mutation data (mean revertant colonies): Mutation experiment 1 (plate incorporation)

| | Q | √ , | Li a | | , • | 01 - | <i>~</i> | | | | | |
|------------------|----------------------------|-------------------|----------------------------|---------------------|------------------------|-------------------|-------------------|-------|--------------------|-------------------|--|--|
| Type of mutation | 2 | | eShift | | Base-pair substitution | | | | | | | |
| Conc. | ŢØ | 98 🎸 | €TA1 | .53 ⁹ × | 🏏 🏗 🖈 100 🕮 🗀 | | TA1 | 1535 | TA | 102 | | |
| (µg/plate) | -S2 | ± § 9 | ₽Š9 ≰ | J"+ S9 // | ′ -S9 | ∂ \$9 | -S9 | +89 | -S9 | + S9 | | |
| 0 | 30.7 | \$0 .7 | \$ 14.3° | 20:0 | 102.0 | 23.0 | 10.7 | 13.0 | 236.7 | 337.7 | | |
| | <i>\$</i> 7.5 € | \$\text{2.1}C | ±2.5 MB | ≈± \$4.6 | %√±1.0 _∞ | ±3.0 | ±4.9 | ±4.4 | ±18.5 | ±15.7 | | |
| 5 2 | 40.7 | 47 | 2.0 | Q21.7 | 2 102 P | 138.0 | 11.3 | 16.0 | 247.7 | 348.3 | | |
| 3 2 | > ±4.5 | ±6.6 | ≫¥1.0 <i>%</i> | ±10A | ±4.6 | ± 15.1 | ±1.5 | ±3.0 | ±14.2 | ±5.5 | | |
| 16 | 37.7 | %56.3 ° | 🎖 16 <u>.</u> 7 ° | 248 | X1 6.0 | 125.0 | 9.0 | 15.7 | 256.7 | 342.3 | | |
| 10 | ±1.5 ~ | €±10. | ±2,9 | ₽ ¥.9 ° | ≥±0.8 | ±1.7 | ±2.6 | ±4.6 | ±3.8 | ±15.6 | | |
| 50 | 41.0 | 61.0 | 212 .3 | Q28.0 × | 108.7 | 123.3 | 9.7 | 12.3 | 280.7 | 334.7 | | |
| 30 | ±13.9 | ±10. | £2.3 ⊘ | ±2.0 | ±5.1 | ±11.0 | ±2.3 | ±6.0 | ±9.0 | ±14.2 | | |
| 160 | 39 .7 | ₄ 61.5 Q | 13.5 | 29 9 0 | 109.3 | 125.3 | 12.7 | 13.7 | 270.7 | 370.0 | | |
| 100 | l @10 1≪° | | ±2.1 | <u></u> #3.0 | ± 4.2 | ± 17.2 | ±4.2 | ±4.0 | ±6.4 | ±34.4 | | |
| 500 | 33.3 | 6400 | \$46.0 *\(\pm\ \pm 3.6 \) | \mathfrak{P} 28.3 | 115.7 | 115.3 | 14.3 | 12.3 | 220.7 | 309.0 | | |
| 300 g' | 33.3 ±86 | £6.1 | Õ±3.6 | ±10.4 | ±12.5 | ±11.5 | ±3.8 | ±2.3 | ±16.0 | ±20.0 | | |
| 1600 | ANG 0 | 4 ,27.0,% | | 18.0 | 98.3 | 106.3 | 9.0 | 12.3 | 27.3 | 26.0 | | |
| 1000 | ©±3.6 14.3 ^S | ±4,4 | 2.1 ^s | ±3.5 | ± 7.5 | ± 16.3 | ±4.6 ^S | ±5.0 | ±12.7 ^S | ±7.5 ^S | | |
| 1600 | 14.3 ^S | | Т | Т | 30.0 | 9.7 | T | T | Т | T | | |
| 2000 200 | ±2.3 | ±1.5 ^S | | | ±7.98 | ±5.7 ^s | | | | | | |
| Positive | 1393.7 | 319.0 | 793.0 | 359.0 | 1198.0 | 3478.0 | 721.3 | 252.3 | 914.7 | 2179.3 | | |
| control | ±67.8 | ±21.1 | ±151.9 | ±31.4 | ±42.6 | ±53.7 | ±28.0 | ±13.5 | ±134.0 | ±299.2 | | |



Document MCA - 5: Toxicological and metabolism studies **Spiroxamine**

| Type of mutation | | Fram | e-shift | | | В | ase-pair s | ubstitutio | n | |
|------------------|-------------|------|--|-----|--------------------|-----|------------|------------|-----|------|
| Conc. | TA98 TA1537 | | | | TA100 TA1535 TA102 | | | | | |
| (μg/plate) | -S9 | +89 | -S9 | +89 | -S9 | +89 | -S9 | +89 | -S9 | +\$9 |
| B: bubbles in | agar | | S: slight thinning of background by wn | | | | | | | |

C: based on 2 replicates only. 1 replicate contaminated

M: manual counting

Positive controls:

-S9: strains: TA98: 2-NF

TA1537: 9-AAC TA100; TA1535: NaN₃

TA102: MMC

+S9: strains: TA98 (B)[a]P

TA1537; TA100

T: Toxic, no revertant colonies

B. Mutation experiment 2:

TA102: 2-AD For Experiment 2, treatments of the tester strains were performed in the absence (utilising the plate incorporation methodology) and presence of \$9 (projection) 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 5000 µg/plate for strain 3A100 ±S9, TA1535 and TA1537 +S9; for all other strains and preatments this was 2000 µg plate. Concentration intervals were narrowed covering the range 8.192 -2000 ug plate or 2048 - 5000 µg plate in order to examine more closely those concentrations of spiroxamino N-oxide approaching the maximum test concentration and considered therefore most likely to provide evidence obany mitagenic activoty.

Following these treatments, exprence 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 (TX1535(TA155)) above the concurrent vekicle control.

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

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

| Type of | <u> </u> | ØFrom. | Shift O | | | D. | oco poir c | ubstitutio | ``` | | | |
|------------|--------------------|---------------------|--------------|-------------------|------------------------|------------|------------|------------|-------------------|-------------------|--|--|
| mutation | w . | | Shift J | , O" | Base-pair substitution | | | | | | | |
| Conc. | | | ~ / | 1597 A | | 100 | TA1 | 535 | TA | 102 | | |
| (μg/plate) | (μg/plate) -S9 +S9 | | S-S9 +S9 | | -89 | +89 | -S9 | +S9 | -S9 | +89 | | |
| 0 | 35.3 | ° √ 41.0 ′ | ₹ 6,Q . | 13.7 | ≈ 105.7 | 139.7 | 11.0 | 12.7 | 265.7 | 302.0 | | |
| 4 1 | ±7.5 🔏 | ¥9. 5 ♣, | ±1/27 | £ 2.3 | ≫±7.2 | ±19.1 | ±6.0 | ±6.4 | ±21.8 | ±29.5 | | |
| 8.192 | 37.3 | 393 | 26 .7 | \$ - \} | " - | - | 16.7 | - | 281.0 | 343.3 | | |
| 0.192 | ±7,5 | ±1.5 | €±1.2 @ | | | | ±3.1 | | ±9.8 | ± 17.6 | | |
| 20.48 | \$3. 3 | <u>3</u> 37.3 🔏 | 7.05 | 15.9 | 97.3 | 117.7 | 6.7 | 11.0 | 267.3 | 319.0 | | |
| 20.46 | £3.8 | ±3.2 | ±0.0 | ₩ 0.6 | ±13.3 | ± 17.8 | ±1.5 | ±4.0 | ±8.1 | ± 26.1 | | |
| 51.2 | 40,7 | 407 | 8.7 | ♥13.7 | 104.3 | 131.3 | 9.0 | 14.3 | 267.0 | 366.3 | | |
| 51.2 | ± X | ±8.3 | Ʊ4.7 | ±0.6 | ±8.1 | ±7.0 | ±2.0 | ±2.3 | ±6.6 | ±4.2 | | |
| 120 | \$ 2.3 | 4 2.3, § | 6.0 | 18.3 | 101.7 | 130.3 | 8.3 | 9.7 | 257.3 | 335.3 | | |
| 120 | ©±1.5 | ±5,8 | ±2.0 | ±4.0 | ±10.0 | ±9.6 | ±1.5 | ±1.5 | ±8.5 | ±33.0 | | |
| 128 | 38.0 | 54.0 | 7.7 | 14.7 | 94.3 | 124.3 | 11.0 | 12.7 | 233.0 | 316.3 | | |
| 320 p | ±3.0 | ±13.1 | ±1.2 | ±2.1 | ±8.7 | ±5.7 | ±4.4 | ±4.2 | ±5.2 | ±27.5 | | |
| 800 | 27.3 | 26.7 | 7.7 | 8.7 | 93.3 | 121.7 | 5.0 | 12.0 | 87.0 | 132.0 | | |
| 800 | ±6.4 | ±7.1 ^s | ±1.2 | ±4.0 ^S | ±6.8 | ±18.0 | ±0.0 | ±1.7 | ±7.0 ^S | 29.5 ^s | | |



| Type of mutation | Frame-shift Base-pair substitution | | | | | | | | | |
|-------------------------------------|------------------------------------|------------|------------------|--------------|-------------------|--------------------|--|------------|----------------|-----------------|
| Conc. | TA98 TA1537 | | | TA | 100 | TA1535 | | TA | 102 | |
| (µg/plate) | -S9 | +89 | -S9 | +89 | -S9 | +89 | -S9 | +89 | -S9 | +\$9 |
| 2000 | 6.3 | T | 3.7 ^S | T | 95.3 | 89.3 | 2.0 | T | T | O)i |
| 2000 | ±3.1 ^S | | ±1.5 | | ± 2.1 | $\pm 10.7^{S}$ | ±1.0 ^S | | | |
| 5000 | - | - | - | T | 20.0 | T | - | ØŤ | | 227 |
| 5000 | | | | | $\pm 12.5^{S}$ | | 4 | 1 | | ~ ~ . Q |
| Positive | 1415.3 | 368.0 | 429.7 | 442.3 | 1054.7 | 3149.3 | 727.0 | 159.7 | 824.7 | 27.3 |
| control | ±24.5 | ±15.6 | ±45.1 | ± 76.7 | ±24.2 & | ¥117.8 | ±2206 | ± 34.0 | 64.6 | 727.3 7±94.2 |
| S: slight thin | ning of bac | kground la | wn | | T: To | xic, no rev | ertant color | nies | W S | |
| T: Toxic, no | | olonies | | | - Ass | e level not t | treated | | / Q | |
| Positive cont | | | | | 4 | 4 | Q' & ° | 4 | 2 | |
| -S9: strains (| | oration): | | | %+S9: | strains (pre | -incubation | ı): 🦞 | | d i |
| TA98: 2-NF | | | | | TA98 | 8: B[a]P | ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ | m > | | * .7\$ |
| TA1537: 9-A | | | | <u>&</u> | , T ¢ ojl: | 537; TA 100 |); F Av1535; | TA 102: 🏖 | ÄA 💝 | *** |
| TA100; TA1 | | | | | | | | 7 | al a | 4 |
| TA102: MM | C | | | 1 | ~~ | | | ~ | O _x | O" 4 |
| C. Discussion (in two indeposition) | on: | | | | | | | | | |
| n two inde | nendent e | experime | nts and a | all strai | ns in €the | absence | and presi | ence P a | rat liver | metabolic |
| _4:4: | (O(| | | ¥ | t say Saale | Z 6 | | | 5 f.10 | h(in stusin |

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 \$5.5-fold (in strain TA102), ≥2-fold (in strains TA98 and TA109) 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 mutagente activity in this assay system.

The positive controls induced an acceptable increase in revertant colors numbers, thereby demonstrating the sensitivity and specificity of the test systems

Table CA 5.8.1/20-3: Bacterial reverse gene mutation data (mean revertant colonies): historical @ontrolyanges) A.

| | | | 0 // | | % Y | Q | ~ | | | |
|------------------------|-----------------------------------|---------------|-------------------------------|---------------------------------------|------------------|---------------------|--------------|------------|----------|-------------|
| Type of | () | Fram | e-shift((| | | ~ B | ase-pair's | ubstitutio | n | |
| mutation | (A) | , O | O ^v | K O' | |) Z | - (1) n | | | |
| Parameter _® | TOX | 98 | € TA1537 € | | A100 | | ₹ TA1 | .535 | TA | 102 |
| | -S9 | ₹ \$9 | ∜-S9 🧳 | +S90° | -S9 [©] | _ & S9 ∝ | ҈°-S9 | +89 | -S9 | + S9 |
| į Ģ | | | | \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ | cle contro | | 7 | | | |
| Date range | | Mar 🗚 🕅 | Oct_17 - | - Feb 18_ | **Oct 1:7 - | - Feb 48 | Jun 18 - | - Sep 18 | Oct 17 - | - Feb 18 |
| n (studies) | 77 | .75 | 3 76 | 🥎 76 🦓 | 103 | <u>100</u> | 78 | 76 | 72 | 72 |
| n (plates) | 360 | 29 1 . | § 299 🌡 | 292 | 377 | 3 70 | 287 | 281 | 266 | 274 |
| Mean | 2 3.1 | 3 6.3 | 10.0 | 13.9 | 3 01.8 4 | ⁷ 108.7 | 19.6 | 18.8 | 290.4 | 315.7 |
| 99% L.R.R * | © 10 C | 200 | | ₹ 5 | [≫] 56≫ | 72 | 7 | 5 | 220 | 193 |
| 99% | 46 | 64 | \$\text{22} \(\tilde{\pi} \) | 29 0 | 168 | 168 | 35 | 37 | 403 | 411 |
| U.R.R | e e | | y | 200 | (N) | | | | | |
| | ~ | Y | | Posiţ | i contro | 1 | | | | |
| Date range | Jan 19 | Mar 👣 | Q Q 17 - | eb 186 | Oct 17 - | - Feb 18 | Jun 18 - | - Sep 18 | Oct 17 - | - Feb 18 |
| n (studies) | 77ू | ® 5 | ్రీ 75 | > 76° | 102 | 98 | 78 | 76 | 72 | 71 |
| n (plates) | " © 8Š | 275 a | 294 P | 2₫8 | 372 | 351 | 287 | 278 | 264 | 255 |
| Mean | Ĵ [™] 170,8 ² | | 30 % /3 | 286.3 | 650.2 | 1524.3 | 668.1 | 190.2 | 936.9 | 1559.8 |
| 99% L.R.R. | ₹ 32 & √ | 263 | _≪ 84 ≈ | 5 41 | 431 | 455 | 234 | 37 | 454 | 368 |
| 99% | 33 2 | Ö 11 | ॐ 885 | 550 | 1470 | 2884 | 927 | 614 | 2148 | 3566 |
| U.R.R | | | 9 | | | | | | | |

Positive controls?

-S9: Strains:

TA98: 2-M

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 N-oxide aid not induce mutation in five histidine-requiring strains (TA98, TA100, TA1535, TA1537 and TA102) of Salmonella typinmurity when tested under the conditions of this study. These conditions included treatments at concentration up to 5000 μ g/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 μ 0 to concentration limited by toxicity using both the plate incorporation and μ 1 re-incorporation methodologies.

| Data Point: | KCA 5.8.1/8 47 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 5 |
|---|--|
| Report Author: | |
| Report Year: | |
| Report Title: | KW@4168-NOxid OV79-HORT tell in vitro for the detection of anduced |
| | forward mutations |
| Report No: | 28143 |
| Document No: | <u>[%1-006560-018</u>] |
| Guideline(s) followed in | Dife yve 8 302/EBE, Or 20 4/0003-EGA /12-C-98-22 OFF 13 8/0.3300 |
| study: | |
| study: Deviations from current test guideline: | Yes A number of deficiencies were noted or feer to Result. Deficiencies section below. |
| test guideline: | A number of deficiencies were noted orefer to Results, Deficiencies section |
| | below). |
| Previous evaluation: | yes, evaluated and accepted |
| | *DAR (2010) |
| GLP/Officially | Yes. Conducted under GLP/Officiall@recognosed testing facilities |
| 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 hanster (39) lung cells The study consisted of a preliminary cytotoxicity assay followed by a Motation Experiment, each conducted in the absence and presence of metabolic activation by an Arocor-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 seed in the presence of S9. In the absence of S9, treatments were performed using 3 and 24-hour treatment incubation periods.

In the prefining cytotoxicity assay, hine concentrations were tested in the absence (5 hours) and presence (5 hours) of \$9 ranging from 19.5 to 5000 μ g/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 μ g/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 \$9. Exposure to spiroxamine N-oxide at concentrations from



19.5 to $5000~\mu g/mL$ 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 fiver metabolic activation system (S9) no increases mutant frequency were observed that exceeded sylval 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's recognised that a sufficient level of toxicity was not achieved in either treatment condition, with concerns raised over the assay sensitivity with overtly low spontantous mutant frequency values observed in the velocile controls.

The positive controls induced an acceptable increase in national 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 evalvated against current test guideline requirements.

It is concluded that spiroxamine N-orded did not show any increases in the mulant frequency of V79 cells at the hprt locus. These conditions included reatments that were reithed limited by toxicity (i.e. reduction in RCE 10-20%) or precipitate, in the absence (5 lours) and presence (5 hours at 2% (v/v) final concentration of S9 fraction) of ratifiver metabolic activation system.

For the reasons identified in the deficiencies

Materials and Methods

A. Materials:

√KWGŒ) 68-NSoxid

(alternative names: Spiroxamine Noxide, 1/8-ter Outyl-1,4-1. Test Material;

dioxaspiro 4.5]de 2-yl methyl (ethyl) (propyl) amine-N-oxide, M03)

Description: Clear yiscous liquid %

Lot/Batch No .: M0@490

968% (correction for puory not undertaken) (molecular weight: 313.48 g/mol) Purity:

CAS No.:

Stability of tes Continued stable for the duration of the study (expiry date: 26 August 1998)

compound:

2. Control materials

Vehicle/ final

concentration:

Ethyl methanes Phona (EMS, 5 h: 900 μg/mL) Positive: -S9

Dimethyl berganthracene (DMBA, 5 h: 20 µg/mL) **Positive:** +S9

Sylwas pu@hased&from a commercial source. Sprague Dawley rats were 3. Activation: 4. Test cells theated with Aroctor 1254 (supplied by lot no.: 85681,

protein conten 39.0 mg/mL). The composition of the S9 reaction mix was: 40% \$9, MgCl₂ x H₂O (8 mM), KCl (33 mM), glucose-6-phosphate (5 mM),

NODP (1 mM), buffer (60%).

 $\sqrt[6]{79}$ cells derived from the lung of $\sqrt[3]{79}$ 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) CO₂ in air.



5. Culture medium: During growth, and post treatment: Eagle's minimal essential medium (MEM)

supplied with L-glutamine (2 mM), MEM-vitamins NaHCO₃, 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, or

thioganine (6-TG, $10 \mu g/mL$).

6. Source of cells: V79 cell line, originally derived from the lung of Chinese hamser obtained

from (University of Ulm, Gernary). These cells were ocloned to maintain karyotypic stability. A modal chromosome number of 23 and a

doubling time of 10-14 h. « @

7. Locus examined: hprt (hypoxanthine-guarone phosphoribosyltrans ferase) Ocus. The selection

agent was 6-thioguanine (6-TG).

8. Test article Concentrations:

a) Preliminary cytotoxicity assay:

5 h +/-S9: 0, 10.5, 39:1, 78.1; 156.3, 202.5, 605, 1250 2500 45000 μg/mL (10.5) dose in excess of the current guideline requirements of 10 m/M, or 2000 μg/mL

whichever the towest)

b) Mutation assays: Experiment 1:

5 h +/-\$9: 0, \$\tilde{Q}\$00, 20\$\tilde{Q}\$300, \$\tilde{Q}\$0, 500\$\tilde{G}\$00, 700 μg/\$\tilde{Q}\$

(confamination noted 2 days after treatment. Although cultures were plated for croning efficiency and mutant frequency (MF) assessment and reported, these

data have not been considered further)

Experiment 🗐

5 μ-89: 0.250, 2000350, 260, 650 800, 950 μg/mL 50 +89 9, 100 200, 300, 400 500, 600, 700 μg/mL (concentrations underlined were assessed for MF)

B. Study design:

1. In life dates:

May 1998 to 23 July 9998 (Experimental dates)

2. Vehicle selection:

Spiroxaming N-oxide was soluble at 250 mg/mL in deionised water gave a final concentration of 5000 µg/mL when losed at 2% v/v (a concentration deemed to be the maximum recommended concentration in accordance with the *invitro* ganotoxicity test stridelines followed at the time of the study. However, when assessed against corrent test guideline requirements, the maximum concentration ested 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 µM in the preliminary test).

Osmodality and planssessments of the test article in cell culture medium were undertaken for the preliminary cytotoxicity study.

3. Statistics:

√y ADunnerr's t

4. Acceptance criteria:

For action tested was one that allowed the measurement with the property of the concurrent which control of the limit of actions of the limit of actions of 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 $<25 \times 10^{-6}$ mutants per 10^6 viable cells.

No heterogeneity between cultures, as assessed by MF <5 mutants $per\ 10^6$ viable cells for each culture.



<u>For positive controls</u>: 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 is a second experiment.
- Biological relevance was only considered if in addition to the above if no significant change in osmolative compared to the vehicle control was observed.

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

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

C. Methods:

1. Homogeneity and achieved concentration analysis of the dose:

2. Cell treatment:

Stability analysis was undertaken at somina concentrations of 0.02 and 250 mg/mg coor temperature) for 24 h

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

Preliminary evitors/leity assay: Following cell attachment 16-24th post culture establishment), cells (4 x 106 cells/flask) were exposed to test article formulations or vehicle control added at 2% of 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% PCS 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 columns were used throughout. Culture cloning efficiency, %RCF 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.

- Mutage wicity assessment. 1 x 10° cells/culture treated were incubated to express and fix the planotype. These cells were sub-cultured on day 4, reseeding 1 10° cells. At the end of the expression period (ca. 7 days) cultures were reseeded at 3 x 10° 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 desistant 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 cominal values.

B. Preliminary cytotoxicity assay:

No marked changes in osmolality or pH were observed at the highest concentration tested compared 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 of presence of 9.

Exposure to spiroxamine N-oxide at concentrations from 193 to \$000 µg/mL in the absence and presence of S9 resulted in RCE values from 172,8 to 322% and 144,2 to 465%, respectively. It is noted that overtly high RCE values >100% were observed in the absence of presipitate it is highly likely that this was attributed to dilution errors when plating for clothing efficiency assessment.

Table CA 5.8.1/03-1: Spiroxamine Noxide mutant frequency data from X79 hpm? cells short form treatment

| Concentration | | +89 % |
|---------------|-----------------------------|----------------|
| $(\mu g/mL)$ | ACE (%) ROE (%) ACE (%) | © RCE (%) |
| 0^{a} | 50.2 7 100.0 7 58.8 | 0 100.0 |
| 19.5 | 58.0 \$ 0 1156 0 \$ 84.8 \$ | ô 144.2 |
| 39.1 | \$5.7.3 | \$ 77.9 |
| 78.1 | 64.5 64.5 64.5 | 107.4 |
| 156.3 | 73.20° 2 1450° 2 0 47° 2° | 81.0 |
| 312.5 | 69,3 67 138.2 48.0 | 81.6 |
| 625 | 96.8 Y13.1 Y | 115.0 |
| 1250 | 76.3 63.5 | 107.9 |
| 2500 | 17 867 . 1 × 1798 & 0 243 | 49.9 |
| 500Q | 216.7 2 23.2 2 27.5 | 46.7 |

RCE: Relative cloning efficiency ACE: Absolute cloning afficiency

a deignised water (1% v/v)

C. Mutation assay

1. Experiment 1

2. Experiment 2:

Cultures were exposure to spiro amine N-oxide at concentrations from 100 - 000 ug/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.

Cultures were expected to spiroxamine N-oxide at concentrations from 50 – 550 μcmL. 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. Tean 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.

20 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 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 DDBA (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

| C | | | 5 h -S9 | | Conc. | | 5(P+S9 | |
|----------------|----|----------------------|----------------------|----------------------------------|-------------------------------|----------------------|-------------------|---------------------|
| Con | | RCE (%) ^a | Mutan | t plates | | RČE (%) ^a | Q Mutan | t plates 🗳 |
| (μg/m | L) | | ACE (%) ^a | MF ^a | (μg/mL) | | ACE(%)a | ₩F |
| O ^a | Α | - | 72.2 | 0 🖔 | 0^{a} | | 8 0×0 | ≫ 0.5°° |
| | В | - | 51.8 | 1.6 | $\mathbb{Z}^{\mathbb{Z}}$ | Ø - P | © 1.5 🎸 | 28 |
| Mean | | 100 | 62.0 | .048 | OMean © | \$ 100 | € 66.3 °C | 91.7 ° |
| 50 | Α | 113.5 | 94.7 | €0.4 ~ | 100 A | 1,46.6 | O 8743 | 0.7 |
| | В | 63.1 | 51.5 | @ 1.6g | 100 % | Q47.0 <u></u> _ | \$2 .0 | × 25 |
| Mean | | 88.3 | 73.1 | 190 | Mean 🦠 | € 96.8° | \$55 Q | |
| 200 | Α | 131.8 | 69.3 96.3 | <u> 10,2</u> | 200 A | 907 | گر 99. ∑ ا | 1.7 \$\infty 0.4 |
| | В | 70.9 | | . % | | 69.4 | | ≫ 0.9 |
| Mean | | 101 | 82 ® « | 0.8 | Mean T | 279.7 ^O | 7 7.3 ≤ | 0.7 |
| 350 | Α | 124.0 | 67.0 | ″ QQ | 300 A | 133.90 | 103.3 | 0.8 |
| | В | 81.2 | 80.3 | ∑¥.0 | У 0° В | ″ 20 0 ° | 55. © | 0.7 |
| Mean | | 102.6 | × 12.1 | © 0.8 S | Mean 🔊 | *8/1.2 V | 79.2 | 1.3 |
| 500 | Α | 60.7 | 86,3 | 0.8 | 200 OA | £ 115,7 | \$ 058.3 | 0.7 |
| | В | 57.7 | ® ₹.0 🖔 | 19 | 48 2.° I B | © 27 × | 84.3 | 0.6 |
| Mean | | 59,1 [©] | 68.8 | ~(M.2 | Mean Q | | 71.3 | 0.7 |
| 650 | Α | 1,40,4 | O″ 102.0°″ | 0.5 | 500 A B | @ 3 9.8 | 93.5 | 1.5 |
| | В | ₽ 9.7 ∂ | 66.0 | 0.7 | B | 35.5 | 51.5 | 1.4 |
| Mean | | 32.1 | √84.0 [©] | 9 .6 Q | Mean | 37.7 | 7.5 | 1.5 |
| EMS | Αd | ·)) | 59.30 | ₹120.1× | 600 A | 43 .5 | 87.3 | 1.0 |
| | ₿ÿ | چ 89.8 | y 62 p | © [®] 73.7 [©] | a Pa | 14.3 | 62.3 | 0.8 |
| Mean & | | 78.55 @ | 60 %.7 | 96.9* | Mean V | 44.9 | 74.8 | 0.9 |
| | • | Ő | | . 0 4 | DMBA A | 57.2 | 100.3 | 62.3 |
| | | | | ~~ · · · · | \mathbb{O}^{r} \mathbb{B} | 42.2 | 68.0 | 68.0 |
| | | <u> </u> | * \$\$ | | Mean ✓ | 49.7 | 84.2 | 51.8* |

* *p*≤0.05

RCE: Relative Coning efficience

ACE: Absolute cloning efficiency

MF: Mutant Frequency (na) tants per 106 Wable cell

a data presented as individual values in report. Mean

values calculated and presented 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 hist | orical control range (A) experim | | 6 based on 20 |
|---------------------------------------|-----------------|-------------------------------------|-------------------|---------------|
| ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ | A Achicle | control | Positive | control |
| | ON SZSOa W | +S9 ^a | -S9 ^a | $+S9^a$ |
| MF⊋sd Ø | ©4.2 ±5.0 | 2.9 ±2.9 | 650.6 ± 172.9 | 51.9 ±25.1 |
| Observed ? | 0.0 - 23.4 | 0.3 - 13.8 | 227.1 – 992.9 | 14.6 - 110.7 |
| range 💍 | | | | |

a Treatment duration which the laboratory historical control data is based upon is not provided

MF: Mutant Frequency (mutants per 10⁶ 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 chicle 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 evaluation of terial 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 (1995, 2016). When assessed against corrent test guideline requirements the following defreiencies are noted:

- An insufficient number of colls were treated in the mutation assay of x10% cells/culture) compared to the test guideline requirements (20 x 10% cells/culture). Consequently, a very low spontaneous mutant frequency rate was obtained for the vehicle controls (0.85 1.7 mutants per 10% viable cells) compared to current test guideline recommendations (5 20 mutants per 10% viable cells). This raise the concern over as any sentivity of 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 first her raises concerns were the laboratory's test methodologo 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 how 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 bove 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 applicants

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

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



| | T |
|----------------------------|--|
| Data Point: | KCA 5.8.1/04 |
| Report Author: | |
| Report Year: | 1998 |
| Report Title: | KWG 4168-N-Oxid - In vitro chromosome aberration test with chinese hamster |
| | V79 cells |
| Report No: | 27715 |
| Document No: | <u>M-006495-01-1</u> |
| Guideline(s) followed in | Directive 92/69/EEC, Method B.10.; OECD 473; US=EPA 712-C-9@223; |
| study: | OPPTS 870.5375 |
| Deviations from current | Yes V V V |
| test guideline: | A number of deficiencies were noted (refer to besults, Deficiencies section |
| | below). |
| Previous evaluation: | yes, evaluated and accepted DAR (2010) |
| | DAR (2010) |
| GLP/Officially | Yes, conducted under GLP/Officially recognised testing facilities |
| recognised testing | |
| facilities: | |
| Acceptability/Reliability: | Supportive only Supportive onl |

Executive Summary

In a mammalian cell chromosome aberration assay, cultured Chinese hamster V7 lung cells were exposed to spiroxamine N-oxide. The study consisted of a prefiminary cytotoxicity assay followed by a two independent chromosome aberration experiment, each conducted in the absence and presence of metabolic activation, by an Aroclor-1254 included rat liver post-phrochandrial fraction (\$9).

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

A 5-hour treatment incubation period was used in the absence and presence of S95 with recovery periods of 18 hours (preliminary cytoloxicity and chromosome aberration test only).

Following treatment, no precipitate (observed by eve at the end of treatment) was observed at any concentration tested. Exposure to sproxanine Nexide at concentrations of 5 to 5000 μ g/mL caused complete toxicity at the highest concentration tested in both the absence and presence of S9. At the next highest concentration, 1000 μ g/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 μ g/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 clative mitotic index (40-50%) in both treatment conditions and recovery periods.

The positive controls, MMC \$59) and CPA (+\$9) induced a statistically significant increase in chromosomal aberrations (excluding gaps) thus confirming the test systems ability to detect potential clastogenic effects for the 18 hour recovery treatments. No positive controls were included in the extended recovery periods

It is recognised that a sufficient level of too reity 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 aberiations 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:

KWG 4168-N-oxid

1. Test Material: (alternative names: Spiroxamine N-oxide, [(8-tert-butyl-1,4-

dioxaspiro[4.5]dec-2-yl) methyl](ethyl)(propyl)amine-N₇oxide, M03)

Description: Clear viscous liquid

Lot/Batch No.: M00190

Purity: 90.8% (correction for purity not undertaken) (molecular weight: 3.19.48 g/mol)

CAS No.: 148044-85-3

Stability of test compound:

Confirmed stable for the duration of the study expiry date: 2@August 1998)

2. Control materials:

Vehicle / final concentration:

Deionised water / 2% (Vv)

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 Dam'ey rats treated with Aroclor 1254

(protein content 40% mg/m²/). The composition of the S9 feaction mix was: 40% S9 MgCl₂ H₂O (43.3 mM), KCl 55 mM), glucose-6-phosphate

(10 mM), NADP (1.65 mM).

4. Test cells: V79 cells derived from the jung of Chines Chamster were obtained from Dr. U25ch, Merk At Darmstadt and stored as frozen stocks in liquid nitrogen. For

each experiment the course were diluted in MEW (see culture medium below) and incubated in a hamidified atmosphere of 5% (y/y) CQ in air. A modal chronosome number of 22 and a dividing time of 12 h. Calls were confirmed

mycoplasina free.

5. Culture medium

During growth, and post treatment: Lagle's minimal essential medium (MEM) supplied with L-glutamine (2 mM) MEM vitamine NaHCO₃, penicillin (50 units mL), streptomeoin (50 ug/mL) heat-inactivated fetal calf serum

During treatment, serum concentration was reduced to 2%

6. Test article Concentrations:

a) Preliminary

lst preliminary test.

 $4\sqrt{7} + \sqrt{-500}, 5, 10, 50, 100, 5000, 5000 \mu g/mL$ (a dose in excess of the

ourrent guideline requirements of 10 mM, or 2000 μg/mL, whichever is the

2nd prelimitary test

4 h²+/-89; <u>0</u>, <u>250, 500</u>, \$30, 1000, 2000, 3000 μg/mL (concentrations underlined were assessed for MI)

b) Chromosomal aberration assay

Experiment 1:

46 (+14 (recove γ) -S9: 0, 100, 200, 300, 400, 500 μg/mL

Fh (+1 4h) +S9; 0, 200, 300, 400, 500, 600 μg/mL

*Experiment®

4 h (+26 h) -S9: 0, 300, 400, 500 μg/mL (+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 $\mu g/mL$, whichever 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 μ g/mL. No fluctuations in pH of the medium of more than 1 pH unit or osmolatry 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 facidence of chromosome aberrations. We level).

4. Acceptance criteria:

For vehicle controls: The mean vehicle control was within the laborators historical control range.

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 in yease in aberration rate was obtained.

A negative result was concluded if there were statistical senificant value which were within the historical controls.

C. Methods:

1. Homogeneity and achieved concentration analysis, of the dose:

2. Preliminary cytotoxicity assay

Stability analysis was undertaken at nonward consentrations of 002 and 250 mg/sp.L (rown temperature) for 24 h.

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

Following establishment of cultures (1.010° cells), S9 mix or KCl was added as appropriate for treatments in the presence of absence of S9, respectively. Cells were exposed to the test article for 4 hours in the absence or presence of S9 (2% v(x)). 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. Cototoxicity was determined by both Mica total of 1000 cells assessed for metaphase, refer below) and cell survival that mocytometes, without fixation).

(haemocytometer, without fixation).
Single cultures were jised for the vehicle control and each test article concentration.

Two hours before the cells were parvested, mitotic activity was arrested by addition of Colemid to each culture at a final concentration of 0.4 μg/mL to inhibit cytologiesis. After 2 lours 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: acord acid (3:1 v/v)). Following further centrifugation the suspensions were the controlled with fixative; this was repeated until the fixative was clear.

The fixed pell of swere re-suspended, then centrifuged and re-suspended in a small volume of fixative. A few drops of the cell suspensions were dropped only 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.

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:

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

Harvesting and fixation:

Slide preparation:

Cytotoxicity:



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

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

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 rest 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 spirotamine W-oxide is not known to cause cell cycle delay, the extended recovery period was neither justified not warranted. Untreated controls were included in the 18 hour recovery treatment. If however is unclear why these were included as the vehicle used was deionised water for this reason, the untreated control data has not been juctuated. The MI was determined for

Duplicate slides were prepared for each culture. The II was determined for each culture expect where there was clear vidence of over toxicity or no indications of cytotoxicity.

4. Slide scoring:

Concentrations were selected for analysis of chromosome aborations with modal chromosome comber 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:

Chromatic gap: achromatic lesion with a chromatic arm, without obvious Chromatic for the chromatid ends. Whits 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 breat an aethomatic 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 included with chromosome breaks).

Multiple aberrations: when 5 or more structural changes (excluding gaps occur) withit 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 Noxide at concentrations of 5 to 5000 µg/mL caused complete toxicity at the Aghest concentration tested. At the next highest concentration, 1000 µg/m²L, a reduction in mitotic index of 7.5% was observed. In a further experiment, spiroxamine N-oxide was exposed to culture at 250 and 500 fig/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 pg/mL caused complete toxicity at the highest concentration tested At the liext highest concentration, 1000 µg/mL, a reduction in mitotic index of 38.2% was observed. In a further experiment, spiroxamine Noxide was exposed to sultures at 250 and 500 µg/mL, with a reduction in 1 to 35.1%.

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

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

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

| Dose level | 4 h exposure | (+ 18 h recovery) -89 | O4 h exposure (4 1 | 8 h recovery) –S9 |
|---------------|--------------------|--|--------------------|-------------------|
| (μg/mL) | Relative Min | %) Cytotoxicity (%) | Relative MI (%) | Cytotoxicity (%) |
| (μg/mL) | Ÿ \O' }`∀Pro | eliminary cytotoxicity expe | IIIICIMA' N | |
| 0 | 100 | | 169 | 0 |
| 5 | \$ JP12.4 C |) \(\sigma\) \(\sigma\) \(\sigma\) \(\sigma\) \(\sigma\) | 160 | -9.3 |
| 10 | 89.10 | △ 10.9° | 14.2 | -14.2 |
| 50/ | 99.5 | | 111.1 | -11.1 |
| (1) 00 | 109.5 | | 102.7 | -2.7 |
| 500 | √73.1 ~ | 26.9 K | △ 91.6 | 8.4 |
| 1000 5000 | 92.5 | √, 7.5°° ≥ | 61.8 | 38.2 |
| 5000 | | 0 109 Q | 0 | 100 |
| | Pr | eliminary cytotoxicity expe | riment 2 | |
| 0 | 100 | | 100 | 0 |
| 250 | 10437 | -4.1 | 64.9 | 35.1 |
| £60 | 20,5 | 79/5 | 79.7 | 20.3 |

C. Chromosome aberration assay

1. Short-term treatment in the absence of

Following treatment, no precipitate (observed by eye at the end of treatment) was observed at any concentration tested. Exposure to spirous at concentrations and the end of treatment) 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 Noxide 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% & 2.5% in the Whicle) The biological relevance of the is not understood as no historical control data for polyploidy was presented, and this assay is not specifically designed to assess numerical about tions, with assessment limited to a qualitative assessment only. A sufficient level of cytotoxicity preduction in MI 40, 50%) was not achieved, with metaphase analysis Himited to a single dose
- 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 C. 8.1/942.

Refer to Table CA 5.8.1/642.

Spiroxamine N-62 ide overview of chromosomal abercation data in V79 cells in Table CA 5.8.1/04-2: the absence of S9

| | 4 | × | 7 | · \$ 60° | <u></u> | 0 | |
|------------|--------------------------------|--|---------------------------------------|-----------------------|----------------|---------------|---------------|
| Dose level | 4 h exposure | (+ 18/h recove | ry) 🔊 🦠 🤊 🧳 | ∌ ² ≇∕h e∑ | | 0 hæcovery | y) –S9 |
| (μg/mL) | Relative Cyt | o- Abôrran | Numeric | Relative | Cyto- | Aberran | Numeric |
| | MI (%) toxic | ity st cells | | MI (%) | toxicity | t cells | al |
| | ~ (%) |) 🔊 exci. | agerrang | | (%) (%) | excl. | aberrant |
| | | gaps (%) | cells (%) | , , , | 0 ~ | gaps (%) | cells (%) |
| 0 | 160 0 | ×0.5 | 4.09 | Ø100 & | , & | 0.5 | 2.5 |
| 100 | 10 1.7 1 .5 -1.5 | 1 05 A | y 55,5 | >>b | Ş-b | b | b |
| 200 | %91.9. | | 9.5 |) -4) | b | ^b | ^b |
| 300 | 66.20 33. | | 7.5 | 96.3 🐇 | 3.7 | a | a |
| 400 | 80.3 📞 19. | ZV Sza | Oa | 290.2 | 9.8 | 1.0 | 9.0 |
| 500 | 23.1 76 | | S ^a | 17.80 | 82.2 | a | a |
| MMČ | 100.9 | | ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ | , *** | c | c | c |
| | | Laboratory | bistorica co | ontrol ranges | S | | |
| Vehicle c | ontrol (water) 4 h | 18 h recovery | (4n = 4) | Vehicle con | trol (water) | 4 h +30 h r | ecovery (n |
| | | | | Õ, | = 4 | | - ' |
| Year ⋄ | Ф94 Ф 19 95 | 1996 | 1997 | 1994 | 1995 | 1996 | 1997 |
| Range: | d <u>Oʻ</u> d | %4 | 1997 2 d 1 0 | d | d | d | d |
| Median 💇 | 3.0 | | y 1 ₂ 0 | 1.5 | 1.0 | 0.0 | 1.0 |
| : 🗬 | | V A I Y | &()) · | | | | |
| Positive c | ontrok(MMC) Th | +180h recovery | (n¥4) | Positive con | trol (MMC |) 4 h +30 h r | ecovery (n |
| "\ | | | .0 | | = 4 | 4) | |
| Range: | 35. O. 18.0 – | [v] 2\$\text{\$\text{\$2.5}} - \text{\$\tau\$} | 15.5 − | ^c | ^c | ^c | ^c |
| Median | 5 7.0 43.5 | ₹50.5° | 44.5 | | | | |
| : [8 | ¥3.5 × 34.5 | 37.5 | 39.5 | | | | |

 $p \leq 0.01$

Positive control: Mitomycin C [0.1 µg/mL]

- p≤0.01 y y metaphases no assessed for chomosome aberrant
- incidence of this treatment condition of the treatment condition
- c no positive control data generated for this treatment condition
- d no bserved 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%. The concentrations selected for metaphase analysis were 300, 400 and 500 μg/mL, with % reductions in MI of no cytotoxicity at the lowend 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 treatmen Concentration Thowever, a sufficient level of cytotoxicity (reduction in MI 40-50%) was not
- The positive control, CPA induced a statistically significant increase in chromosomal aberrations (excluding gaps), wus confirming the test systems ability to detect potential clastogenic effects.

4 h + 30 h recovery

- Following treatment, as precipitate (observed by eye at the odd of treatment). was observed at any concentration tested. Exposure to spiroxamine W-oxide at concentrations of 400 to 600 µc/mL caused reductions in %ML 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 polyploids was observed 5.5% @ 3.0% in the whicle) The biological relevance of this is not understood as no historical control data for polygloidy was presented, and this assay is not specifically des@ned to assess rumerical aberrations, with assessment mited to a qualitative assessment only. A conflicient level of cytotoxicity (reduction in MI 4050%) Was not schieved, with metaphase analysis limited to a single Odose Jevel. 👩
- Noopsitive control was included. Grerefore the laboratory's ability to detect glastogenic damage following an extended recovery was not adequately demonstrated ~

Refer to Table CA 5.8.004-3.

Spiroxamine Noxide overview of choomosomal aberration data in V79 cells in the presence of S9

| Dose level | 49 76 | exposure (± | 78 h recove | ry +S9 | 4 h e | xposure (+ 3 | 0 h recovery | y) +S9 |
|------------------|--------------------------------|----------------------------|------------------|--------------------|--------------|--------------|---------------|--------------|
| (μg/mL) | Relative | | Aberran | Numeric | Relative | Cyto- | Aberran | Numeric |
| | M I (%) | toxicity | . Ot cello | ″ "Oål ′ | 🎖 МІ (%) | toxicity | t cells | al |
| • | Q U | Q 0/0) | excl. | aberrant | | (%) | incl. | aberrant |
| 4 | | 0" 3 | / gaps (%) | očells (%) | | | gaps (%) | cells (%) |
| 200 | 100 。 | \$ 90° | ©3.0 g | ×600 | 100 | 0 | 1.0 | 3.0 |
| 200 | 136.2 | ,736.2 | ~ ³ Ç | aaaa | ^b | ^b | ^b | ^b |
| ₂ 390 | 163% | ₹ 63.8 _× | 7 3.6 | 2.5 | b | b | b | b |
| 400 | 145.4 | © -45,40 | 4.0 | $_{r}\Phi^{r}$ 5.5 | 124 | -24 | a | a |
| 500 | . Ø8.6 | 1,2 | 3.0 | 6.5 | 119.7 | -19.7 | 2.5 | 5.5 |
| 600 | [™] 19,1 [™] | 80 .9 | 4a | a | 12 | 88 | a | a |
| Positive A | 140 | \$ -17 € | , 3200** | 6.0 | c | c | c | c |
| control | | ک ک | » | | | | | |
| | 110 | | Laboratory | historical co | ntrol range | S | | |
| Vehicle o | ontrol (wa | iter) 4∕h +1 | 8 h recovery | (n=4) | Vehicle con | ntrol (water |) 4 h +30 h r | ecovery (n |
| | | | | | | = | 4) | |
| Year Y | 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 |



| Positi | ve control (N | MMC) 4 h +1 | 18 h recover | Positive co | ntrol (MMC |) 4 h +30 h ı | ecovery (n | |
|---------|---|-------------|--------------|-------------|------------|---------------|------------|---|
| | • | | | | | = | 4) | 0 |
| Range: | 14.5 - 48.5 | 9.0 - 35.0 | 13.5 - 38.0 | 25.0 - 42.5 | c | c | c | |
| Median: | 31.0 | 15.0 | 21.8 | 32.5 | | | | |

** p < 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 47% (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 splotament N-oxide causes cell cycle delay.
- No assessment of exposure in the absence of So following an extended exposure was undertaken
- Concerns are raised over the ensitt ity and specificity of the assay, with only 100 metaphases scored/culture, compared with test guideline requirements of 150/continue. 300/concentration. This is further impacted with the overfly low background spontaneous chromosome aberrant frequency observed in the vehicle controls.
- Toxicity was the limiting factor of 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.
- sufficiently sensitive to demonstrate elastogenic damage.

 Limited distorical 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 eriteria for assay acceptance and evaluation criteria are somewhat lacking when compared to corrent out guideline requirements. Voxicity is was not deemed a limiting factor, with a positive result concluded based on the summation aberrant cells including and excluding gaps. Gap should be evaluded from the fina Potals of aberrations, as they may not all be true breaks.
- Although not a deficiency of the lest guideline, numerical aberrations are only qualitatively assessed in this assay.

For the reasons listed above, this study is defined 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 5.81/21 (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 $45\pm5\%$) 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: | |
| Report Year: | 2020 |
| Report Title: | Spiroxamine N-oxide: In vitro human lymphocyte micronucleus assay |
| Report No: | 8406983 |
| Document No: | <u>M-755221-02-1</u> |
| Guideline(s) followed in | OECD 487 (2016) |
| study: | |
| Deviations from current | None Q Q Q Q |
| test guideline: | A Q o Q Q |
| Previous evaluation: | No, not previously submitted \sim \sim \sim \sim \sim |
| | |
| GLP/Officially | Yes, conducted under GLP/Officially recognised testing faculties |
| recognised testing | |
| facilities: | |
| Acceptability/Reliability: | Yes Y Y Y Y Y Y Y Y Y Y Y Y Y Y Y Y Y Y |

Executive Summary

Spiroxamine N-oxide was tested in an *in vitro* micronucleus as ay using duplicate human lymphocyte cultures prepared from the pooled blood of two female deflors 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 proclor 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 me/mL dimited by toxicity), was determined following a preliminary cytotoxicity range finder experiment. All test article concentrations, formulated in DMSO were dosed into the less system at 1% v/v

Following establishment of cultures, concentrations ranging from 00 to 500 µg/mL in the absence (3 hours + 21 hour recovery) and presence of 89 (3 h + 21 h), and from 1 to 90 µg/mL in the extended treatment in the absence of 89 (24 h + 24 h). The test which concentrations for micronucleus analysis were selected by evaluating the effect of spiroxamine Noxide 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 agnificant increases of 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 freatment condition met the criteria. The assay data were therefore considered valid and acceptable.

Treatment of cells with Spiroxantine N-oxide in the absence and presence of S9 resulted in MNBN cells that were similar to and not significantly $Q \le 0.05$) higher than those observed in concurrent vehicle control cultures for all concentrations analysed. With the exception of a single culture (B) at 50 μ g/mL following 24+24 59 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 (molecular weight: 313.48 g/mol) **Purity:**

CAS No.:

Stability of test Confirmed stable for the dur

compound: 2022)

2. Control materials:

Negative:

Solvent/final

concentration:

Positive: -S9

Vinblastine (VIN, 24 h: 0.04 ug/ml/ Paneugenic control) C

Crophosphamide (CPA, 9 h: 7 fg/mL) Positive: +S9

was purchased from a commercial source. Sprague Dayley rats were 3. Activation:

> Tot no 4030, protein treated with Aroclor 254 (supplied by content 3.2 mg/mI_Q. The composition of the 10% S9 reaction mix was: 100 μL SO, Na PSS (100 μM), gracose -6 phospQate (5 μM), β₂ NADP (4 μM), MgCl₂

(8 μM) KCl (3 μM), water (6 volume).

4. Test organisms

Human peripheral blood lymphocytes were collected from 2 healthy, nonsmoking adult dopors aged between 23 and 30 years, pooled and diluted with HML media. Cells were stimulated to divide by the addition of

phytokaemagaluting PHA)@

HEPES buffered RPMI supplemented with/10% (v/v) inactivated fetal calf 5. Culture medium:

6 penicillin-streptomycin cell culture.

6. Test article Concentrations

> Preliminary cytotoxicity test:

(+21 (+25) (j 5.5, 259.2, 43≥0, 720, 1200, 2000 μg/mL (maximum

recommended concentration

+21 h recovery -S9: 0, 100, 150, 175, 200, 225, 250, 275, 300, 325, 350,

21 h recovery \$9: <u>0</u>, 100, 150, 175, 200, <u>225</u>, 250, <u>275</u>, 300, <u>325</u>, 350,

400, 500 μg/m/L

26 h (+2**-**0 n) –**S9** <u>0</u>, 1, 5, 10, 15, <u>20,</u> 30, 40, 45, <u>50,</u> 55, 60, 70, 80, <u>90,</u>

100 µg/mL (concentrations underlined scored for micronucleus frequency)

May 2019 to 10 June 2019 (experimental dates)

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-450catalysed 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 $\mu g/p \mathcal{U}$ (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 wer 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 cytotocicity range-finder experiment Test article stock solutions were prepared by formulating spin xamine N-oxide under subdued lighting in DNSO with the aid of vortex mixing and warming at 37°C, to give the maximum required treatment solution concentration. Subsequent dilutions were made using DMSQ. The test article Solutions were @ protected from light and used within approximately 36 h of initial formulation. The proportion of MNBN cents for each treatment condition were compared with the proportion in vehicle controls by using Wher's exact test. A Cochran-Armitage trend test was applied to each treatment condition. Probability value of p≤0.05 were accepted as significant."

3. Statistics:

4. Acceptance criteria:

of p≤0.05 were accepted as significant!

The following acceptance exteria had to be met for assay acceptability:

- 1. The binomial dispersion test demonstrated acceptable heterogeneits (in tem's of MNBN cell frequency) between eplicate cultures, particularly yhére popositivé responses were seen
- The frequency of MNBN cells in vehicle controls fell within the current 95th percentage of the observed historical vehicle control (normal) ranges;
- 3. The positive control chemicals induced statistically significant increases in the proportion of cells with misronuclei. Both replicate cultures at the positive control concentration analysed under each treatment condition demonstrated MNISN cell frequencies that clearly exceeded the normal raunge; 🦴
 - 4. A minimum of 70% of cells have goned frought least one cell division (as measured by binucleare + multinucleare cell counts) in vehicle control cultures at the timeof harvest;
 - 5. The maximum concentration analysed under each treatment condition met The specified criteria (i.e. the trighest concentration selected for micronucleus analysis following all treatment conditions was one at which 50% cootoxicity was achieved).

5. Evaluation criteria:

For while data, the test article was considered to induce clastogenic and/or anengenic events if.

- A statistically significant increase in the frequency of MNBN cells at one or more concentrations was observed;
 - An inextence of MNRO cells at such a concentration that exceeded the normal rangem both replicates was observed;
- 3. A Soncentration-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

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

1. Homogeneity and achieved congentration analysis

> Whole blood cultures were established by placing 0.4 mL of pooled heparinised blood into 8.5 mL pre-warmed HEPES-buffered RPMI medium

'Not undertaken.

of the dose:

2. Preliminary cytotoxicity assay:



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 medicin at a concentration of approximately 2% of culture to stimulate the lymphosytes 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. Dyplicate 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 perfeted (approximately 300 g, 16) minutes, washed twice with sterile saline and resuspended in fresh pre-warmed medium containing fetal calf serum and penicillar/streptomycin Cyto B (formulated in DMSO) was added to nost wash off calture medium to give a final concentration of 6 µg/pt2/culture to inhibit cytokinesis resulting in binucleate cells (without effecting taryokaresis) 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. Osmulality and pH recasurements or post-treatment on 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 \$6.00 cells/concentration. From this, the replication index (RI) was calculated for each concentration (see Cytotoxicity section below).

3. Micronucleus assay: > Cell treatment: <

Cols were exposed to the test article formulation, vehicle of 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. Concentration selected for micronucleus analysis were assessed for cytoloxicity discoded and for the proportions of mono-, bi- and multinucleate cells to a minimum of 200 or 500 cells/culture (preliminary cytotoxicity range funder or micronucleus experiment, respectively)./

Spinele inhibitor:

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

Shows were prepared by spreading the fixed cultures on clean slides. The slides were started with acridine orange (12.2 mg/mL) dropped on to slides, covershipped and scored prior of analysis.

Cytotoxicity

The replication index (RIC which indicates the relative number of nuclei compared to compols, was determined using the formula below:

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

$$\frac{\text{RI of treated cultures}}{\text{RI of vehicle control}} \times 100$$

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

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 concentration homogeneits or stability of test articles formulations were not conducted as part of this study, as this is not a equirement of the regulators test guidelines.

B. Preliminary cytotoxicity assay:

Test article precipitate was observed at concentrations of 430 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 A were observed at the highest three concentrations tested as compared to the concurrent vehicle controls undividual data not reported.

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

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

| Conc. | 3 h (+ 21/h | | 3 h (+2) h | recovery) 489 (| 24 h (💬 24 h | recovery) -S9 |
|---------------|-----------------------|---------------------|-------------------------------------|-----------------|-----------------|---------------|
| (μg/mL) | Replication | Cytotoxicity | Replication | Cytotoxicity 0 | Replication | Cytotoxicity |
| | index (KI) | √basędon RI | index (RI) | based on RI | index (RI) | based on RI |
| | | (%) | | ~~(%) Ø | Z, | (%) |
| 0 | 0.67 | - 🖔 | , ® ⁷ 0.73, ** | <u>-</u> G | ♥ 0.87 | - |
| 7.256 | 0.65 | | [∞] √ Q. & 2) / | O S | 0.76 | 13 |
| 12.09 | b 0.73 , | , (h) A | % 88 € | 7 7 | 0.64 | 27 |
| 20.16 | 0.71 | | 0.63 | ~ 13° × | 0.47 | 47 |
| 33.59 | 0.70 | | % 0.80° | | 0.50 | 43 |
| 55.99 | 0.63) | \$ 5° . (| D" 0 <i>zj</i> ď 🧐 | Z Z | 0.32 | 64 |
| 93.31 | 0.57 a | | ® 65 [©] | ≫ 11 | 0.12 | 87 |
| 155.5 | 0.61 | 7 5 9 | ~0.61 C | ₽ 17 | 0.04 | 95 |
| 259.2 | @0.52° | © 23 _~ , | 0.65 | 11 | NE | - |
| 432.0 | 0.04ppt | | 0.05 ppt | ÿ 94 | NE | - |
| 720.0 1200 | NE, ppt, H-ppt | <u> </u> | NE, ppt, H-ppt | - | NE, ppt, E-ppt, | - |
| 1200 | NE, ppt, E-ppt, | l 💜 | ppt, E-ppt, | - | NE, ppt, E-ppt, | - |
| | H-pp | 4. | H _s ppty | | H-ppt | |
| 2000 | NE, ppt, Eppt, | | NE APOT E-ppt, | - | NE, ppt, E-ppt, | - |
| , | H _z ppt "(| | Al-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. Micromucleus assay

The frequency of MNBN cells in vehicle controls fell within the normal ranges. The positive control changes in the proportion of MNBN cells. Both replicate cultures of 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 ($p \le 0.05$) higher than those observed in concurrent chicle control cultures for all concentrations analysed. With the exception of a single culture (B) at $90 \mu g/m$ 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 micronicolei assay: 3 h + 21 h recovery) –S9 treatment and laboratory historical control data

| | | | | Cr. | • | | |
|-----------|------------|----------------|----------------------|----------------------------|----------------|------------------------|-----------------|
| Conc. | | | 3 h (+ 2 | 21 h recovery≯∕S | 89 Q 2 | Vehicle historical c | ontrol ranges |
| (μg/mL |) | Total | Total | Frequency of | CytoC (%) | (♀ T ono | rela 🛋 a |
| | | BN | MN- | MN-BN (%) _∞ | (%) | Felp-1,7 – Felp 18 , , | 0/ WINDN |
| | | | BN | | ſ ∕v° .C | | % MINBAG |
| Vehicle | Α | 1000 | 5 | Ø.50 & Y | | No. okexpts | 150 |
| | В | 1000 | 3 | 0.30€ ° | | Number of cultures | V 40 |
| Total | | 2000 | 8 | Mean: 0.40 | × _~ . | Moean ±SD 🗳 | 0.40±±0.28 |
| 150 | Α | 1000 | 3 | ♥ 0 030 © | | gnin. wax. | 0.00 - 1.30 |
| | В | 1000 | 7. | 0.70 | | 95% reference range | 80.00 - 1.01 |
| Total | | 2000 | 10 | Mean: \$30 | Mean: 15 | | 0 |
| 250 | Α | 1000 | 5 | 9 0.50 <i>0</i> | y 0 | | |
| | В | 1000 | 1 , | ©.10 S | | Positive historical c | ontrol ranges |
| Total | | 2000≼ | , 6 | Mgean: 000 | Mean: 93 | (Pa)ono | rs) |
| 300 | Α | 1000 | D | L 💝 0.2 4 0 🔏 | | ΜΜ (C) (0.3 μ | g/mL) |
| | В | 1000 | £3 (|) je j 30 | ()(a | / Aug 15 – Dec 17 | % MNBN |
| Total | | 20 00 v | | Mean: 0,25/ | Mean: 60 | No. Ocexpts | 19 |
| | ۸ (| | | p 0.8603 " 🔊 🛴 . | | Number of cultures | 40 |
| MMC | ĄĈ | | _{&} 7,0 | 7.00 0 | | Mean ±SD | 5.68 ± 1.66 |
| (0.3) | ∂ B | 1000 | , 79 J | 7.00 2.90 Mean: 7.45 | o ^y | min. – max. | 2.80 - 9.20 |
| Total | , } | 2000 @ | 149 | Mean: 7.45*** | Mean: | 95% reference range | 3.39 - 8.81 |
| *** p<000 | | ·_@ | | | | | |

No test article related jacreases in cells with NPB3 were observed (data not) reported)

Table CA 5.8.1/213: Spiroxamine Noxide: human lymphocyte micronuclei assay: 3 h (+ 21 h requery) \$59 treatment and laborator historical control data

| Conc. (µg/mL) | Total Total Frequency of Cyto. | | | | Vehicle historical control ranges (♀ donors) | | |
|---------------|--------------------------------|---------------|-------------------------|----------|--|------------------|--|
| 4 | BN | MNQ ÆN | MN-BN %) | (%) | Feb 17 – Feb 18 | % MNBN | |
| Vehicle A | 1000 | \$\frac{4}{2} | Q 40 \$ | | No. of expts | 16 | |
| В | @ ₀ 1000 | 2, | @ 0.20 & | | Number of cultures | 40 | |
| Total | 200 <u>@</u> \ | "W" | Mean: 030 | - | Mean ±SD | 0.55 ± 0.30 | |
| 225 | 1000 | 2 | 0 20 | | min. – max. | 0.00 - 1.20 | |
| B | 1000 | .° 2 .∜ | 0.20 | | 95% reference range | 0.10 - 1.20 | |
| Total | © 2000 ' | 40 | Mean: 0.20 | Mean: 8 | | | |
| 275 AS | 1000 | Z \$7 | 0.50 | | | | |
| 275 A | 10000 | ~~ 2 | 0.20 | | Positive historical c | ontrol ranges | |
| Totak | 2000 | 7 | Mean: 0.35 | Mean: 28 | (♀ dono | rs) | |
| 300≥ A | 1000 | 4 | 0.40 | | СРА (3 µg/ | mL) ¹ | |
| В | 1000 | 3 | 0.30 | | Feb 16 – Feb 18 | % MNBN | |
| Total | 2000 | 7 | Mean: 0.35 | Mean: 51 | No. of expts | 21 | |
| | Lir | near trend: | p 0.2917 | · | Number of cultures | 41 | |



| CPA | Α | 1000 | 24 | 2.40 | Mean ±SD | | 2.60 ± 1.07 |
|-------|---|------|----|---------------|----------|---------------------|-----------------|
| (7.0) | В | 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 highest CPA concentration powerful and the concentration analysed in this study. 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)

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

| | | 24 h (+ | 24 h recovery) – | S9 , " | Vehicle historical e | ontroDranges |
|---|---|--|---|---|--|--|
| | Total | Total | Frequency of | Æyto. | (\text{pono} | rs) 💝 🔎 📜 |
| | BN | MN- | MN-BN (%) | ♣ (%) | Fal 19 Eal 0 | O MNDN |
| | | BN | L | | · . · · · · · · · · · · · · · · · · · · | Ö % MNBN Ö |
| Α | 1000 | 6 | 0.60 🐇 | | No. of expts | 14 |
| В | 1000 | 4 | 0.40 | Ů, | Number of cultures | 40 |
| | 2000 | 10 | Mean: 0.50 | (7)≥ (7) | Mean ±SD | ⊙ [¥] 0.34⁄ 5 0.21 √ |
| Α | 1000 | 4 | 0×40° × | | min. – max. | 0.00 - 0.90 |
| В | 1000 | 4 | 6 40 | | 95% reference range | Q 10 - 0 3 0 |
| | 2000 | 8 | M@an: 0.49 | Mean; 12 | | |
| A | 1000 | 4 | 0°0.40° | J' J | | |
| В | 1000 | 4 | \mathbb{Q}^{r} 0.40 | | Positive historical c | ontrol ranges |
| | 2000 | 8 @1 | Mean: 0.40 | Mean: 55 | | |
| Α | 1000 | 1,5 | °>> 0.10 _{>>} | \rangle \text{\partial} | | g(mL) |
| В | 1000 | | | Y 00 | Feb √7 – Jan 18 | % MNBN |
| | 2000 | | ○ Mean: 0.55 | Mean: 32 | Number of cultures | 20 |
| | Lir | near trend: | p 0.5875 | | Mean: | 40 |
| Α | 1000 | 605 | ₹ 1 0.00 | | o″ Mean ±SDy″ | 5.59 ± 2.05 |
| В | 1000 | 53 | S 5 ,9 0 S | | mɨn. – max. | 2.50 - 10.10 |
| | 2000 | <u></u> | Mean, 5.65*** | Mean: 26 | 95% reference range | 2.50 - 8.93 |
| | A A B B A A A A A A A A A A A A A A A A | BN A 1000 B 1000 A 1000 B 1000 C 2000 C Lin A 1000 B 1000 C 2000 C Lin A 1000 C 2000 C Lin C 2000 C 2000 C Lin C 2000 C 20 | Total BN MN-BN A 1000 6 B 1000 4 2000 10 A 1000 4 B 1000 10 B 1000 10 C 1000 10 | Total BN MN-BN (%) BN MN-BN (%) A 1000 6 0.60 B 1000 4 0.40 2000 10 Mean: 0.50 A 1000 4 0.40 B 1000 4 0.40 A 1000 4 0.40 B 1000 4 0.40 A 1000 4 0.40 B 1000 4 0.40 B 1000 4 0.40 B 1000 10 0.10 B 1000 10 0.55 Linear trend: p 0.5875 A 1000 60 60 6.00 B 1000 53 5 50 2000 113 Mean: 5.65**** | Total Total Frequency of MN-BN (%) (%) | Total Total Frequency of MN-BN (%) Feb 1/- Feb 18 A 1000 6 0.60 No. of expts B 1000 4 0.40 No. of expts B 1000 5 No. of expts D Mean ±SD No. of expts D No. of expts No. of expts No |

No test article related increases in

D. Deficiencies:

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

Following revision of the QDCD 457 TG in 2014 the Fell treatment and harvest times have been somewhat simplified with emphasis placed on performing the extended treatment without recovery, with sampling times or recovered times extended by up to a curther 1.5-2.0 normal cell cycles where justified.

Given the purpose for the in tro micronuseus as ay 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 ordex data demonstrated that the human peripheral blood lymphocytes overe 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 statemen@n the@ECD 487 test guideline, which includes the statement 'for lymphocytes exponential growth may be declining at 6 hours following stimulation and monolayer cultures of cells may become conflicent'. 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 pay 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

>HC: exceeds historical control



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 4 + 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., 20198), 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 equirements is 283/2013

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

| Data Point: | KCA 5.8.1/05 |
|--------------------------------|---|
| Report Author: | |
| Report Year: | |
| Report Title: | KWG_4168 N-Oxide Study for subscrate or toxicology in ats (feeding study |
| | over weeks) - 1st revised version of report no. 28161 from Nov 77, 1998 - |
| Report No: | 28461A6 2 4 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 |
| Document No: | <u>Ø1-006.04-02</u> |
| Guideline(s) followed in | OECD 407; Directive 67/548/EEC, Wethod B.7. |
| study: | |
| Deviations from current | Yes A number of deficiencies were noted feefer to Results, Deficiencies section below). |
| test guideline: | A number of deficiencies were noted fefer to Results, Deficiencies section |
| | below). |
| Previous evaluation: | yes, evaluated and accepted and accepted |
| O' Ş | \mathbb{Z} AR (2010) \mathbb{Z} \mathbb{Z} \mathbb{Z} |
| GLP/Officially | No, no conducted under GLP Officially recognised testing facilities |
| recognised testing | |
| recognised testing facilities. | |
| Acceptability/Reliability: | Kes Z O J & A |

Executive Summary

In this study sorroxanine Noxide 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, 12.9/16.2, 1146/94.3 mg/kg bw/day). Animals were subjected to body weight, Good consumption which were measured at regular intervals. Clinical pathology evaluations (haeptatology, clinical chemistry and urifallysis) were performed with all surviving animals subjected to complete gross necrops and fall histopathology.

No treatment-related effects were seen on stervival, clinical signs, food consumption, urinalysis or serum chemistry/ haematology parameters. High dose effects were limited to slightly decreased cholesterol levels in both makes and Gemales. 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 amine has a high pla, and a high pH with comparable histopathological effects reported. As with irrelant 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 and gavage not to exceed pH with administration of substances outside of the recommended pH ranges resulting to tissue necrosis, pH partitioning etc. This should not be dismissed.

Hyaline droplet nephropathy was observed in the kidneys of two logh dose group males. This desion is due the accumulation of $\alpha 2\mu$ -globulin which is known to be a lesion specific to wale rates with no relevance to humans, with Azan specific staining used to confirm $\alpha 2\mu$ globulin presence.

Under the conditions of this study the NOAEL following 28 days or is defined to be 450 ppm (equivalent to 12.9/13.2 mg/kg bw/day for male females) based on reduction in body weight gain (exceeding 10%), hyperkeratosis of the stomach and oesophagus with mild transitional cell hyperblasia in the urinary bladder. These effects are associated with the uritant pature of the sest article.

Materials and Methods

A. Materials:

1. Test Material: KWG 4168-N-oxde

(alternative names: Spiroxamifie N-oxide, [(& tert-buty1-1,4-dioxaspiro[4.5]dec-

2-yl) methyl@ethyl)@ropyl)@mine_N_oxide,\M03),°

Description: Coourles Aiquid

Lot/Batch No.: M00190

Purity: \$\int 90.82 \text{(w/w) correction for Qurity of undertaken)} \text{\$\int 90.82 \text{(w/w) correction for Qurity of undertaken)} \text{\$\int 90.82 \text{(w/w)} \text{correction for Qurity of undertaken)} \text{\$\int 90.82 \text{(w/w)} \te

CAS No.: 0 148044-85-3

Stability of test Confirmed stable for the duration of the study (expiry date: 26 August 1998)

compound:

2. Vehicle and/or positive Basel diet Fot relevant

control:

3. Test animals:

Species: Rational Wistar Age at dosing: 4-5 years

Source:

Acclimation period: Animals were acclimatised upon arrival, but the duration of this period was not

detailed 0,

Diet: Stromin 1321 Exed-formula standard diet (Altromin GmbH, Lage, Germany)

ad libitum 🔟

Water Musicipal water, ad libitum

Housing: ______ Individually housed

4. Environmental

Temperature: $22 \pm 2^{\circ}\text{C}$ **Humidity:** $55 \pm 5\%$ **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 continuous 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 ppin (equivalent to 1/2. 1/2. 2.6/2.7, 12.9/13.2, 114.6/94.3 mg/kg bw/day) Collowing 28 of treatment 54 animals/sex were subjected to complete negropsy. Body weight, water and food consumption were measured at regular intervals. Clinical pathology evaluations (haematology, clinical charmstry 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.

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

| Danamatana | | (ppm) | | | O Pop | m) | |
|----------------|---|---------------|------------------|----------|-------------|----------------|--------|
| Parameters | 0 | 3.0 750 | ~ 10 60 ° | ~ 0 | · · · · · · | ₹50 √ | 7 1000 |
| Dose/animal | 0 | % 12.9 | 134.6 | | | 〕 13.2~√ | 94.3 |
| (mg/kg bw/day) | | | | | | & , | |
| Animals | 5 | 5 7 55 | 5 | | 5 | Q, | 5 |
| assigned/sex | | ¥ &, ĉ,³ | | * ° | | r Ta | |

C. Methods:

1. Test diet preparation and analysis:

The spirovamine N-oxide was prepared at dietary concentrations of 0, 30, 150 and 1000 ppm % pearlut oil was added to minimize dust formation. The prepared test diets were prepared once and stored frozen (approximately -15%C) uotil before use. ✓

Cability@nd horrogeneity of the diet preparation containing the test article were determined by the analysis of two samples of the diet from 10 and 200@ppm (refer to Doc MCA Section 4 [M0020615-01-1] for method validation).

Animals were inspected wice daily for signs of toxicity and mortality.

Animas were weighed prior of study start and then on Days 0, 7, 14, 21, and

4. Food consumption:

Determined by weigh mg food supplied and food that remained on days $\sqrt[3]{7}$, 14 $\sqrt[3]{2}$ 1, and $\sqrt[3]{2}$ 8.

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

Cpd consumption (g/rat/d) x test article conc. (ppm) Body weight (g)

5. Water consumption

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

6. Ophthalmological examination:

Not conducted.

7. Newrolog wal **functional** exam@ations:

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



9. Urinalysis:

10. Organ weights:

11. Sacrifice and pathology:

(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 (AP), alanine aminotransferase (ALT [commonly referred to as glutamic pyrtuvic transaminase (GPT)]), aspartate aminotransferase (AST [commonly referred to as glutamic oxaloacetic transaminase (GOT)), total bilitubin (TBili), total protein (TP), lipid profile (total cholested).

Conducted on days 28 The following usinary parameters were measured: specific gravity, pH, total volume, protein, glucose, kerones priirubiin, blood, urobilinogen, sediment

Adrenal glands, brain, epiteldymides, heart, kidney, liver, lung, wary, spiten, testis.

Conducted or day 30. Gross pathological examination was performed on all animals and included examination of the external surface all orifices and associated dissues.

The following tissues were preserved in 10% neutral bufferest 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% formalder yet solution respectively) and performed on control and high dose group animals. The kidneys were stained with azarmethodology (chromotrope aniline blue stain) and analysed for all β for the presence of α2μ-globulin 13 β.

Accessory sex glands (cepitalymides, prostate, sertinal vesicle, testes; covary oviduct, uterus, vagina), cardrovascular/haematological system (aorta, bone (sternum, tibial or tenur for marrow), heart lymph nodes (mandibular, mesenteric), spheen, thy hous), gastroint stinal tract (oesophagus, tongue, stomach, intestine (caccum, colon da denum ileum, jejunum, rectum), liver, pancreas), neurological (brain, eyes (+optionerve, Harderian gland), sciatic nerve, spiral cord), respiratory, stem (aose, trachea, lung), urogenital system (kidneys; urinary bladder), other (skeletal muscle, bone (femur, sternum), skin, all gross lesions and masses);

Other endorine producing sensitive glands (adrenals, mammary gland, putuitary hyroid + parathyroid)

In liver dissue Ominop Pine-N-Gemethylase, p-nitroanisole-O-demethylase and cytochrome P450 activity was determined in all animals.

No specific heurohistopathology with specific fixatives were performed in addition to the standard histopathology undertaken on neuronal tissues.

12. Neurohistopathology:

Results and discussion

A. Test diet analysis:

Spiroxamine oxide was komogoously 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 4000 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: 3. Ophthalmoscopic All animals survived until the scheduled necropsy.

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 06% reduction. In females at 1000 ppm body weights were comparable to the concarrent control group. Whilst statistically significant increases in body weight were observed at 30 and 150 from dose groups, this was not dose related. When body weight gain at the end of the treatment period was examined, \$19% reduction compared to the concurrent control group was observed.

Overview of sub-acute toxicity study in rats treated orally wia diety with Table CA 5.8.1/05-2: spiroxamine N-oxide: body weight effects

| | | | <u> </u> | 9 | | ~ () | <i>~</i> , | | |
|----------|----------|--------------|---------------------------------|-----------------|--|--------------------|-------------------|--------------|-----------|
| Danas | neters | | ~~~ (p | 29 m) 🖔 | | | O(p | pr) 🔊 | |
| Farai | neters | 0 | @ 30 📡 | 150 | № 000 € | 1 1 | ○30 % | 150 | 1000 |
| Dose/ani | mal | 0 & | 2,6 | 2.9 | 114.6° | | 2.7 | 1 9.2 | 94.3 |
| (mg/kg b | w/d) | æ. | | | ' | | | Ò | |
| Body | Day 0 | 135 | 135 G |) 13 7 © | 4137 | , | <u></u> | 💇 131 | 128 |
| wt (g) | | ±4.2 | ≠6.0° | ±458 4 | ©±5.4 Õ | # 4,1 | ±6.0 | ±6.0 | ± 1.6 |
| | Day 7 | J81 @ | 7 184J | 181 _C | 164 | O 41 § | √ 15 2 °√″ | 151 | 143 |
| | , | @±6.5 | ≠8 24 ∞ | €±5.4 🍣 | ±5,2** | © ±4.5 | ±5.8* | ±8.4 | ± 2.6 |
| | Day 14 | × 225× | % 231 ≪ | j" 21Q, | ~ 2 02 [| o 157√ | 2 470 | 168 | 156 |
| | | ±8.4 | */±7.4€ | ±12.6 | ∡,¥5.0** [©] | ★ 4 | £3.1** | ±6.1** | ± 2.6 |
| | Day 🏖 🗎 | 261 € | 27 © ° | & 2 54 | J 2366y | ≥ 366 | 181 | 180 | 168 |
| | ₩ | ®±10.4♥ | ±11.3 | ±22.3 | ±5,2* | ©±4.36 | ±6.5** | ±6.6** | ± 4.3 |
| | √Day 28 | 263 | 281 | ® 25®° | . 244 @ | 166° | 178 | 177 | 162 |
| × | y | ±9 .7 | \$\pi\tau\tau\tau=12.2\$\pi\tau | ±22.3 | \$\psi \pm | ±3 ² .5 | ±5.3* | ±11.1 | ±3.3 |
| Body 📎 | Day | ×128 × | 1,46 | \$121 × | 107 | ~ 42 | 50 | 46 | 34 |
| wt gain | 0-28 | 9 4 | (1Q4%) _% | Ç(↓6%) | (48%) | | (†20%) | (10%) | (19%) |
| (g) | | | | | | · | | | |

D. Food consumption, food efficiency and water consumption:

1. Food consumption:

Initially analycrease 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 \exists and \supseteq showed a reduction in feed consumed throughout the dosing period.

2. Food efficiency?

Not conducted.

3. Water consumption: Net conducted. So effects observed.

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

| | | | | | | | | | (7/ n | |
|----------|------------|--------------|----------------|--------------|------------------|----------------|----------------|-----------|--------------------------|--|
| Дама | Parameters | | ∂ (p | pm) | | ් (ppm) | | | | |
| Гага | | | 30 | 150 | 1000 | 0 | 30 | 150 | 1000 | |
| Dose/ani | imal | 0 | 2.6 | 12.9 | 114.6 | 0 | 2.7 | 13.2 | © 94.3 | |
| (mg/kg b | ow/d) | | | | | | | | 4 . Ç | |
| Food | Day 7 | 97 ± 3.7 | 105 ± 10.9 | 101 ±5.8 | 153 ±13.8* | 99 ± 10.5 | 105(±12.2 | 100 ±5. | 109 \$22.3 | |
| consum. | Day 14 | 89 ± 5.8 | 90 ± 2.2 | 92 ± 4.9 | 124 ±17.6* | 109 ± 23.9 | 26 ±9.0 | 93 \pm2.2 | 109(\$22.3) 193 ±9.5(| |
| (g/kg/d) | Day 21 | 79 ± 5.6 | 84 ± 5.0 | 81 ± 2.8 | 100 ±1294 | 89 ±12.2 | 87 ± 6.2 | 85<≠4.3 4 | 90 ±8.8 | |
| | Day 28 | 68 ± 5.8 | 73 ± 3.7 | 71 ± 2.9 | 81 ± % .8 | 78 ± 10.20 | 78 ± 5.4 | #3.4° |) 76 ⊭ 2.9 | |

^{*} *p* ≤0.05

E. Blood and urinalysis:

1. Haematological findings:

The following changes were observed on 3 at 1000 ppm, clothing time (PT) was extended and easinophil increased at dose levels of 150 ppm and above. In \$\times\$ Hb concentration and haematicrit (POV) values were decreased at 1000 ppm, which would be deemed evidence of anaema. However without effects on RBC MCV MHC MCHC alues 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 distorical control range. The increases in cosinophil cours were solated without effect on total reukosyte counts.

Table CA 5.8.1/05-4: Overseew of sub-acute toxicity study in rate treated orally (via thet) with spiroxamine N-oxide: selected paematological parameters

| | - 9 | | | -f. | | ~\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\ | <u>(</u> | |
|------------------------------|----------------------|-----------------------------|--------------------|--------------------|------------------|--|--------------|--------------|
| Parameters | * * | | pm) | Ů Š | | | pm) | |
| | 40 | Ş" 30, Q | |) 100 0 | W & | 30 ∼√ | 150 | 1000 |
| Dose/animal | | 200 | @12.9 \$ | 114.6 | 0 0 | 2.5 | 13.2 | 94.3 |
| (mg/kg bw/d) | | | | | | () | | |
| PT (sec) | 216.7 | L 26.5 | 27,Å | يُّكُ 9.4£ | 2 <i>\$</i> \$# | , \$25.7 | 25.7 | 26.7 |
| 20 | 2 0.74 | ±0.76 | &£00.89 ≤ | ±1. 06 * | 30 .78 | ≈ ±0.72 | ± 0.87 | ± 0.78 |
| Eosino. (10 ⁹ /L) | ® 0.02√J | 0.03 | 0.05 ±0.00 | 0.05 | 00.04 | 0.05 | 0.06 | 0.05 |
| , Q | ±0.011 | ±0.008 | ±0.0 | ±0.013** | ±0.035 | ± 0.015 | ± 0.027 | ± 0.022 |
| Leuko. (10°/L) | 5019 | \$ 6.43 | 7,07 | \$ 5.5 3 \$ | 5 19 240.666 | 6.34 | 6.41 | 6.49 |
| K√y . | 2.327 | ± 0.918 | £0.993 | ±1.498 | ° ≥ 0.666 | ± 0.791 | ±1.075 | ±1.667 |
| RBC (10 ¹² /L) | <i>© "</i> 7.97√√" | Z 95 _% | © 8.1 <i>6</i> €″ | 2 :90 | 8.23 | 8.24 | 7.96 | 7.89 |
| | ±0,336 | ₽ 0.272≪ | ±0,4,94 | ±0.134 | ±0.291 | ± 0.184 | ± 0.386 | ± 0.310 |
| Ret. (%) | 23Q±5.1 _& | \$26 ± 1≥6 | 2 4 ± 3.9 ≥ | \$32 ±6\$ | 20 ± 4.4 | 14 ± 4.9 | 17 ± 3.6 | 20 ± 2.9 |
| Hb (g/L) | ر 152 ر [©] | 135 | 155 | 147 | 152 | 150 ± 3.6 | 150 | 144 |
| 4 | ±6.0 | 3.7 <i>₹</i> | Q ±6,40 | 2 2.5 | ±4.5 | | ±5.4 | ±6.2* |
| PCV (L/L | 0,483 | <i>≈</i> ₩0.492 <i>@</i> 'n | 0.503 | ≪ 0.473 | 0.473 | 0.461 | 0.459 | 0.438 |
| | ±0,0202 ° | Q±0.Q212 | ±0.0139€ | ¥±0.0100 | ±0.0137 | ± 0.0085 | ± 0.0212 | ± 0.0236 |
| MCV(fL) | 60.6 | 62,0 | © 61.7°√ | 59.8 | 57.5 | 56.0 | 57.7 | 55.5 |
| Y | ±1,7 | <u></u> \$3.47 [₹] | ₽ ±1, 93 ° | ±1.93 | ±1.37 | ±0.96 | ±1.12 | ±1.70 |
| MHC (ρg) | 19.1 | € 19.3© | 19.0 | 18.6 | 18.5 | 18.2 | 18.8 | 18.2 |
| A A | <u></u> ±0.55 & | ±0,39 | ₹0.68 | ± 0.46 | ±0.37 | ±0.29 | ± 0.30 | ± 0.46 |
| MCHC (g/L | 314 | 311 | 2 308 | 311 | 322 | 325 ±6.3 | 326 | 328 |
| | ±4.0° | ±3.2 [∞] | ±4.8 | ±4.5 | ±6.6 | | ±4.3 | ±4.1 |
| PT (see) | Laborato | 👸 historica | al control d | ata (rat, W | 'istar 8 -11 | wks of age |) | |
| PT (see) | Date | range: | | - 1995 | | | - | - |
| | r in | | 6 | 5 | | | | |
| | Mean | ±SD: | 28.1 | ±1.46 | | | | |
| ⊳ O _ν | Range | ±2SD: | 25.2 - | - 31.0 | | | | |
| Eosino. (10 ⁹ /L) | Date | range: | 19 | 97 | | - | - | - |
| | r | ı: | 3 | 0 | | | | |



| Damamatana | | ∂ (1 | opm) | | ♀ (ppm) | | | | |
|------------|-------|------------------------------|--------|----------------|----------------|------------------|--------------|-------------------|--|
| Parameters | 0 | 30 | 150 | 1000 | 0 | 30 | 150 | 1000 _° | |
| | | n ±SD: | 0.04 = | ±0.025 | | | | | |
| | Range | Range $\pm 2SD$: $0 - 0.09$ | | | | | | | |
| Hb (g/L) | | - | | = | Date | range: 🤝 | 1994 | - 1995 | |
| | | | | | | n: Ş | 5 | | |
| | | | | | Mea | n ±SD:"0" | 137 | ±7.7% | |
| | | | | | Rang | e ±2 S D: | j j | - 152 | |
| PCV (L/L) | | - | | - _Č | Date | range: | 79 94 | - 11995 N | |
| | | | | - V | | W | | | |
| | | | | .r | Mea | ¥SD: | © 0.442 | £0.02 67 / | |
| | | | | .Õ | Rang | e ±2SD: | 0.388 | - 0, 49 5 | |

 $*p \le \overline{0.05}$

PT: prothrombin time Eosino.: eosinophils Leuko.: leukocytes RBC: red blood cell Reti. reticulocytes Hb: haemoglobin

PCV: packed corpus dalar volume (haematocrity

MCV: mean corpriscular volume

MHC: Mean harmogloby concernation &

MCHO: mean Corpuscular haemoglobin Concentration

2. Clinical chemistry findings:

High dose affects were limited to slightly decreased cholesterol levels in both and a for a finis was below the historical control range, lasting statistical significance. For a this was within the historical control range. This was deemed to be a treatment related effect, but likely light (without correlating light historiathology) rather than adverse.

For other clinical chemistry parameters analyses (electrolytes, kidney and liver Founctional tests) no treatment related effects were observed.

3. Urinalysis:

There were no treatment related effects. A at 150 ppm and above showed increased incidence of usine reacting positive for blood. This qualitative assessment was considered incidental as it was limited to a single sex, the incidence were consistent with the lastorical control range, did not correlate with any associate diverse instopathology.

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

| Parameters | | W A.W | ppm)\ C | | , O * | ○ | | | | |
|--------------------------|-------------------------|-----------------|------------------------|-------------|-------------|-------------|-----------------|---------------|--|--|
| | \bigcirc 0 \bigcirc | 30 | 150 | 1000 | ∆ | 30 | 150 | 1000 | | |
| Dose/animal (mg/kg bw/d) | | ©2.6 2 \$ 50 | 12.90 | P14.6 | 0 | 2.7 | 13.2 | 94.3 | | |
| T.chol (mmol/D) | <u></u> 9.55 | 1.82 | Q.72. | 1.29 | 1.85 | 1.86 | 1.69 | 1.52 | | |
| | ±0.138 | ±0,248 | ±0.280) | ±0205 | ± 0.157 | ± 0.187 | ±0.237 | $\pm 0.097^*$ | | |
| | Laborator | vistoric | al control da | uta (rat, W | istar 8 -11 | wks of age |) | | | |
| T.chol (mol/L) | Date range | | 1994 - 199 | § | Date range | e: | 1994 - 199 | 95 | | |
| | | | 64 2 | | n: | | 56 | | | |
| | Mean⊕SD: | | Q2.04 ₩0 .29 | 0 | Mean ±SI |) : | 2.08 ± 0.36 | 52 | | |
| # 10.05 | Range ±2S | ĸ | 1.46 [©] 2.61 | | Range ±25 | SD: | 1.35 - 2.8 | 0 | | |

* *p* ≤0.05

T.chol.: total cholesterol

F. Sacrifice and pathology:

1. Organ weight

1000 ppm decreased absolute heart and liver weights were observed in 3, 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).



| | | | | | | | | DO. | 1 TOP 3 |
|--|------------------------|-----------------------|---|---|--|-------------------|---|---------------------------------|---------------------------------|
| | | | | | | p.Ġ | 4 | r C | ATUR OFFICE |
| able CA 5 | 5.8.1/05-6: | Overview of sub- | -acute toxicity stud | ly in rats treated o | orally (<i>via</i> diet) wit | h spiroxamine N-o | oxide: selected org | an weights | ALINE ALLE |
| Ромо | meters | | ♂ (p | pm) | 4 | 3 ⁶⁰⁻⁷ | ♀ (ppm) ♪ ↓ | | |
| | | 0 | 30 | 150 | 1000 | 0 < | □″ 20 | © 150 D | S 1000 |
| Dose/anin (mg/kg bw | | 0 | 2.6 | 12.9 | 114.6 | | \$ 30 \$ 2.7 \$ \$ 0 | ppm) > 150 | r W |
| Terminal l | bwt (g) | 283 ±9.4 | 305 ±12.6 (†8%) | 278 ±24.8 (\12%) | \$6.2.(\%\%) | 7±42.9 | | ¥89 ±6.€(₹7%) | ©6±4.7 (↓1% |
| Liver | Abs (g) | 12.9 ±0.6 | 14.8 ±0.8 (↑15%) | 12.4 ±1.3 (↓4%) | \$\tag{\pmu}.3\pm\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\ | .6√8⊈0.7 .1 | 7.1 ±0.7% 4%) | 72±0.5 (100) | 6.9 ±0.3 (†1% |
| | Rel. (g%) | 4.6 ±0.2 | 4.9 ±0.1 (↑7%) | 4.5 ±0.3 (12%) | 4.3 🕸 🗘 (17%) | 3.9 ±0.3 | 37 ±0.3 (15%) | 3 € 10.3 (↓3%) | 3.9 ±0.2 (-) |
| Heart | Abs (g) | 0.94 ± 1.0 | 0.99 ±0.6 (†5%) | | 0.81 ±1.7 (\14%) | 0.37 0.03 | \$\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\ | 0.67 ±608 | 0.63 ±0.06 (\dagger*3%) |
| | Rel. (g%) | 0.33 ± 0.03 | 0.33 ±0 0 (-) | (16%) ±0.02 \$\) | 0.3€£0.002 (↓6%) | | 0.38 ±0.03 | (↓5%) (↓5%) | 0.36 ± 0.03 (\$\dagger\$3%) |
| Spleen | Abs (g) | 0.51 ±0.06 | 0.63 ±0.04 0 ° (↑240°) | 0.5 D 0.03 (-) | 0.56 @0.09 10%), O | 0.38 ±0.02 | (18%) (18%) | 0.49 ± 0.1 (†29%) | 0.41 ±0.07 (†8%) |
| | Rel. (g%) | 0.18 ±0.02 | (†240) 0.21±0.02 (†17%). | 0.18 \$0.02 (-) | 0.20 ±0.03 (101%) | 0.21 ±0,040 | 0.2 ±0.03 (-) | 0.26 ± 0.06 (†24%) | 0.23 ± 0.04 (†10%) |
| Adrenals | Abs (mg) Rel. (mg%) | 44 ±1.5 16 ±0.3 | 48 ±33 (19%) 16 ±1.6 (=) | (36 ±5.5 (↓18%) 13 ±4 6 (↓19%) | 38±5.8 (\1426) 15±12 (\16%) | 59±6.5 30±3.0 | 57 ±9.3 (↑8%) 30 ±5.5 (-) | 60 ±9.2 (†13%) 32 ±4.6 (†7%) | 53 ±6.0 (-) 30 ±3.6 (-) |
| Testes | Abs (g) Rel. (g%) | 3.1 ±0.3 1.1 ±0.06 | 3.4 ±0.2 (\$10%) 1.1 ±0.05 (-) | 2.5\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\ | 3.3 ±0.4 (↑6%) 1.3 ±0.2 (↑18%) | | - | - | |
| Epidid. | Abs (g) Rel. (g%) | 0.45 ±0.07 | 1.2 ±0.2 (1.8%) 0.00 ±0.06 (111%) | 0.38±0.06 0.16%) | 1.2 ±0.2 (18%) 0.46 ±0.98 (12%) | - | 1 1 | - | |
| Ovary | Abs (g) | | | FF 155 2 | Office | 109 ± 29.2 | 114 ±25.3 (↑5%) | 110 ±20.0 (↑1%) | 93 ±12.6 (\(\psi 15\%) |
| | Rel. (g%) | | | Start - i in | - | 62 ±17.9 | 60 ±13.6 (\13%) | 58 ±9.0 (\(\d\)6%) | 53 ±6.8 (\15% |
| * <i>p</i> ≤0.05 Abs.: absol Rel.: relativ | ute ve to body were | erro | aner the b | | Epidid: epid | lidymides | | | |
| | | 0.45 ±0.07 | podit pe | | | | | | |



2. Gross pathology:

Macroscopic findings were limited to the liver and consisted of evident lobule@ or discoloration in the high dose \emptyset and \mathbb{Q}

3. Histopathology:

In high group animals, hyperkeratosis of the epithelium of the oesophaguond forestomach were observed with mild transitional cell hyperplasia in the urinary bladder. As with the parent compound, spiroxamine, spiroxamine N-2 oxide is a tertiary amine, and like all tertiary amines has a high pKa and a tigh pH with comparable histopathological effects reported. As with invitant % 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 of 9, with administration of substances outside of the ecommended of ranges resulting in tissue necrosis, pH parotioning etc. (Gad et al.), Turner et al.) This should not be dismissed.

Hyaline droplet nephropathy was observed in the kitcheys of two high dose group males. This lesion is due to the accomulation of α200 globylin which, is known to be a lesson specific to male rats with no relevance to humans with Azan specific staining used to confirm 2 µ-globulin presence

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

| Danamatans | , | a, & K | pm) | | | | pm) _C | |
|---------------------------|-----------------------|-----------|-------------------|-------------------|--------------------|--------------------|------------------|---------|
| Parameters | 0 🖔 | ₹ 30∀ | 150 | © 100€√ | | 30 ♥ | 150 | 1000 |
| Dose/animal | 0 💝 | Q,6 | © 12.9√ | 114.6 | | ,200 | 13.2 | 94.3 |
| (mg/kg bw/day) | Ò | 0' & | | | | ~(S) × | 1 / 1 | |
| Histopathology: [incident | lence∜ tota <u>l</u> | no. exami | ned [minim | af slight of | noderate] | W S | 7 | |
| Kidney | № 0/5 № | , , , O/S | 90/5 | 275/ | o [™] - « | , ' | - | - |
| - Hyaline droplet | [0,0,0] | [0,0,0] | @[0,0, 0 | [0,2,0] | | 4 | | |
| nep. | * | | V | | | _@j | | |
| Urinary bladder | <u></u> | 0/5 | ~0/5 ₄ | Ŷ 2/5 <i>\</i> | <i>@5</i> | % 0/5 | 0/5 | 5/5 |
| - Hyperpl. mild© | 90,0,0 | [0,0,0] | (20,0,0) | [1;4,0] | (0,0,0] | $^{\circ}$ [0,0,0] | [0,0,0] | [3,2,0] |
| trans. |) W | <u> </u> | Y Q | Ş | | | | |
| Stomach , Q | 20/5 | J 0/5 🐴 | (65 ⁷ | ©5/5 _@ | 0/5 | 0/5 | 0/5 | 4/5 |
| - Hyperkeratosis | (0,0,0] | 7 0/3 T | [0,0,0] & | > [0,4;¶ | [0,0,0] | [0,0,0] | [0,0,0] | [2,2,0] |
| Oesophagus ? | © 0/5 _~ | 0/5 | © 0/5° | 5/5 | ° 0/5 | 0/5 | 0/5 | 5/5 |
| - Hyperkeratosis | [0,0,0] | [0,0,0] | [0,0 x0] | [4,4,0] | 2 [0,0,0] | [0,0,0] | [0,0,0] | [0,4,1] |

* *p* ≤0.05; ** *p* ≤0.03

Hyaline droplet nep.

pers. mild trans.: hyperplasia, mild transitional cell

In the high dose group, cytochrome P450 activity was increased. These changes were deemed treatment related, but deemed adaptivation and not accept the high dose group, cytochrome P450 activity was increased. These changes were deemed treatment related, but deemed adaptivation and not accept the high dose group, cytochrome P450 activity was increased. These changes were deemed treatment related, but deemed adaptivation and not accept the high dose group, cytochrome P450 activity was increased. These changes were deemed treatment related, but deemed adaptivation and not accept the high dose group, cytochrome P450 activity was increased. These changes were deemed treatment related, but deemed adaptivation and not accept the high dose group, cytochrome P450 activity was increased. These changes were deemed treatment related, but deemed adaptivation and not accept the high dose group, cytochrome P450 activity was increased. These changes were deemed treatment related, but deemed adaptivation and not accept the high dose group, cytochrome P450 activity was increased. These changes were deemed treatment related, but deemed adaptivation and not accept the high dose group, cytochrome P450 activity was increased. These changes were deemed treatment related, but deemed adaptivation and not accept the high dose group, cytochrome P450 activity was increased. The high dose group and not accept the high dose group, cytochrome P450 activity was increased. The high dose group and not accept the high dose group a Investigations of the liver tusue showed no treatment related effects at 150 ppm and below. In Jaminopyrine-N-demethylase activity was increased at 150 and 1000 ppm, whieving statistical significance at the high dose group. ingreased. These changes were deemed treatment related, but deemed adaptive and not adverse as no concurrent liver histopathological findings were evident,



| 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 | | | | | | | | | |

| Danamatana | | ♂ (p | pm) | | ♀ (ppm) | | | | | |
|--------------------|--------|--------|-------------|-----------------|------------|------------------|------------------|---|--|--|
| Parameters | 0 | 30 | 150 | 1000 | 0 | 30 | 150 | 1000 | | |
| Dose/animal | 0 | 2.6 | 12.9 | 114.6 | 0 | 2.70 | 13.2 | Ø 94.3 | | |
| (mg/kg bw/day) | | | | | | | <i>*</i> | Y\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\ | | |
| N-DEM (mU/g) | 118.3 | 127.8 | 149.6 | 151.4 | 83.9 | ₹78.8 | 67.7\$ | 88 .0 | | |
| | ±19.61 | ±21.29 | ± 20.43 | ±11.52* | ± 5.48 | √ ∓ 11.03 | ±6,44 | <u>,</u> ₽ 12.98√ | | |
| O-DEM (mU/g) | 10.4 | 10.3 | 9.1 | 10.5 | 7.3 | § 7.8 | <i>\$</i> 6.7 ∧ | 8.0 | | |
| | ±0.66 | ±1.42 | ±1.30 | ±0.98 | ±1.430 | ±0.48 | <i>@</i> ¥0.66\$ | ±1,29 | | |
| Cyto-P450 (nmol/g) | 37.1 | 36.8 | 39.5 | 4 6.7 | 34,AD* | 36.4 | ₩ 33 .2 | ₽ .9 ♀ | | |
| | ±2.11 | ±2.83 | ±3.09 | <u>4</u> 2.12** | ±036 | ء±1.74 | ±4.46 | \$\frac{1}{2}\displays \text{.9} \text{\text{\$\circ}}{2}\displays \text{.52**\text{\$\circ}} | | |

** *p* ≤0.01

N-DEM: aminopyrine-N-demethylase O-DEM: *p*-nitroanisole-*O*-demethylase

Cyto-P450; sytochrome P450 activity

G. Discussion:

No treatment-related effects were seen on survival, clinical signs, food consumption urinalysis or corum chemistry / haematology parameters. High dose effects were limited to slightly decreased chotesterol levels in both males and females. This was deemed to be a treatment to lated effect. Out likely slight (without correlating liver histopathology) fother 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 gair in this group was markedly reduced, with 16% reduction. In females at 1000 ppm body weight 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 with a true 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 hadder. As with the parent compound, spiroxamine, spiroxamine woxide 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 elserved in the kidneys of two high dose group males. This lesion is due the accumulation of $\alpha 2\mu$ -globuling which is known to be a lesion specific to male rats with no relevance to humans, with Azan specific staining used to confirm $\alpha 2\mu$ -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:

Whist not a requirement, the test guideline makes reference to determination of serum thyroid hormone (T3, T4, TSI). 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 defined critical, with potential thyroid effects adequately addressed with histopathological malysis.



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.

| | Y Q Q Y Y |
|----------------------------|--|
| Data Point: | KCA 5.8.1/06 |
| Report Author: | |
| Report Year: | 1998 Q [*] |
| Report Title: | KWG 4168 N-Oxide - Study for subchronic oral toxicity in rets (feeding study) |
| | over 13 weeks) |
| Report No: | 27475 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 |
| Document No: | M-016585-01-2, A |
| Guideline(s) followed in | OECD 408; US-EPA \$\$2-1; Directive \$7/302(EEC, Part B |
| study: | |
| Deviations from current | |
| test guideline: | Although the study was conducted according to est guideline (ECD 408 (1981), |
| | this test guideline has since been updated in the interconing period (1998, 2008). |
| | Whereassessed against current test guideline requirements be following |
| | deficiencies are noted: |
| | Serum thyroid hormones (T3, T% TSH) and clinical chemistry parameters |
| % | Sensitive to the foid pathway perturbations (IDL, HDC) were not analysed. |
| 4 n | However, the gold standard assess thyroid effects is historathological analysis. |
| | This was undertaken on all animals and revealed too histopathological findings. |
| | Consequently the lack of serum thyroid hormone analysis is not deemed critical, |
| | with potential thyroid effects adequately additessed with histopathological |
| | analysis. |
| Previous evaluation: | yes, evaluated and accepted |
| CLD/OCC (A) | DAR (2010) |
| GLP/Officially | Yes, conducted under LP/Officially recognised testing facilities |
| recognised testing | |
| lacilities. | Ves 2 0 V 4 A |
| Acceptability/Reliability: | Yes V V V |

Executive Summary

In this study, spiroxamine Noxide was administered continuously *via* the diet for 90 days to Wistar rats. Animals (10/sex/group) were administered test first at concentrations of 0, 25, 125, 625 ppm (equivalent to males/emales/2: 0/6, 1.7/39, 8.8/37, 45 3/33.6 mg/kg bw/day). An additional group (10/sex) was included which were fied with spiroxamine at 625 ppm (equivalent to 48.7/52.7 mg/kg bw/da for males/females). Arimals 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. havinatology 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 instopathology) 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 60-17% lower than the concurrent control. At termination of treatment, body weight gain in this dose group was 14% hower. 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 the dose group was 19% lower.

Throughout the dosing period, food consumption was marginally greater in animals of the \$25 pph dose group for both test articles when expressed as g/kg. w. 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 priroxemine boxide.

For animals receiving spiroxamine, afterations in red blood cell parameters were evident, suggestive of anaemia, with reductions in haematocrit, MCV MHC MCHC retic docyte 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, neutrophilis, basophils, lonphocytes were increased and eosinophils and attrical 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 spiror mines worde. Similar effects were observed in animals receiving spiror amine, however the incidence and severtly were greater in both instances. In addition, hyperkeratosis of the ear was observed in spiror amine treated animals. Mild transitional cell hyperplasia in the utinary bradder was observed in females dosed with spiroxamine. As with the parent compound, spiroxamine spiroxamine of oxide is a tertiary artine and like all tertiary amines has a high pKa, and a high pH with comparable histopathological effects oported. As with irritant compounds, tissues containing mucosal membrane of deepred 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 necroses, pH partitioning are. This should not be dismissed.

Hyaline draplet nephropathy was observed in the kidneys of all treated males. This lesion is due the accumulation of $\alpha 2\mu$ -globulin which is known to be a lesion specific to male rats with no relevance to humans, with Azan specific staining used to confirm $\alpha 2\mu$ -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 urmary badder. These effects are associated with the irritant nature of the test article.

Material and Methods

A. Materials©

Execution 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



0153560 Lot/Batch No.:

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

(alternative name: [8-(1,1-dimethylethyl)-N-ethyl-Mpropyl-1,4 (continued):

dioxaspiro[4,5]decane-2-methanamine; KWG 41689

Clear light brown oil **Description:**

Lot/Batch No.: 202740026

purity not und taken). **Purity:** 96.5% (w/w) (correction for

CAS No.: 118134-30-8

Stability of test compound:

Confirmed stable for the duration of the study (expiredate).

Basal diet / not relevant

2. Vehicle and/or positive

control:

Basal diet / not rele

3. Test animals:

Species: Strain: Age at dosing:

Weight at dosing:

Source:

Acclimation period: Animat least 7 days

ast 7 days

1231 fixed-formula standard diet (Altromin SmbH, Lage, Germany)

water, and libitum

y housed

/dark Diet:

Water: umigipal water, ad

Housing:

4. Environmental conditions:

> Temperature: **Humidity:** Air changes: **Photoperiod**:

B. Study Design:

1. In life dates:

to 19 April 1998 (experimental dates)

2. Animal assignment and treatment:

sub-bronic study undertaken on the parent compound. After an acclimatisation period rats were allocated to groups by computer-based animals were dosed at 0, 25, 125, 625 ppm were dosed.

We test article, spiroxamine N-oxide was administered continuously via the diet to groups@f rats for a period of 90 days. Animals (10/sex/gp), were adminustered test diet at concentrations of 0, 25, 125, 625 ppm (equivalent to (36): 0/0, 1.7/1.9, 8.8/9.7, 45.0/53.6 mg/kg bw/day). An additional group (\$\text{\$\pi\$0/sex}\$) was included which were fed with spiroxamine at 625 ppm (equivalent to 48.7/52.7 mg/kg bw/day for \Im/\Im). 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

| | | | ∂ (ppm) | | | | ♀ (ppm) _% | | |
|----------------|----|-------|---------|------|--------------|---------|----------------------|--------------|------------------|
| Parameters | | SPX N | l-oxide | | SP® | SPX N | N-oxide 🔊 | | SPX |
| | 0 | 25 | 125 | 625 | 625 | 0 25 | 125 | 625 | 625 |
| Dose/animal | 0 | 1.7 | 8.8 | 45.0 | 4 8.7 | 0 ° 1.9 | 94 | 53 .6 | \$\$2.7 ♀ |
| (mg/kg bw/day) | | | | _4 | | | | * (| ``J |
| Animals | 10 | 10 | 10 | 100 | 10 | | Q"10, 0" | 10 | 10 |
| assigned/sex | | | | . ~ | , | | | , W | |

C. Methods:

1. Test diet preparation and analysis:

Spiroxamine N-oxide was prepared at dietary concentrations of 0, 25, 25 and 625 ppm. 1% beauty 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 attricle were three times throughout the study referred Doc MCA Section 4 M-02065-01 for method worldation.

2. Observations:

Animals were inspected twice daily for signs of foxicity and mortality.

3. Body weights:

Animats were weighed prior to study start and then weekly thereafter.

Determined by weighing food supplied and food that remained from day 3

4. Food consumption:

orwards and thereafter at wice weekly intervals.

From the rood @nsumption data, compound consumption was calculated using

the following equation:

Food consumption

Food consumption (g/raf g) x test article conc. (ppm)

Cpd consumption Food consumption (g/rafty) x test article conc. (ppm) Body weight (g)

Food Officiency was not calculated

5. Water consumption:

Calculated for each animal according to measurement of body weight from day Shwards 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 mimaks.

7. Neurological functional examinations:

Not conducted

8. Haematology and clinical chemistry:

Conducted and 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 (MCHC), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCV), erythrocyte count, platelet count, reticulocyte count), which blood cell parameters (total and differential (neutrophils, lymphocytes, eosinophils, basophils, monocytes) leukocyte count), coagulation parameters (prothrombin time (PT) – termed Hepato-Quick Test).

<u>Clinical chemistry</u>: 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 close of 5 mL

water/animal and urine was collected for a subsequent behours. During this time food was withdrawn. The following urinary parameters were measured specific gravity, pH, total volume, protein, glucose betones, bilirular, bloods

urobilinogen, sediment

10. Organ weights: Adrenal glands, brain, epididymodes, heart, kiddey, liver, lung, ovary pricen,

testis

11. Sacrifice and pathology:

Conducted on day 91. Gros pathological examination was performed on the external surface all orifices and associated tissues.

The following tissues were preserved in 10% neutral buffer of forman for subsequent histopathological examination with the exception of urinary bladder and lungs (+2nd byer lobe) which were fixed with Davidson's solution and 4% formal derives solution, respectively) and performed on control and high dose group animals. Tissues in hold: complete histopathological analysis of all groups. The kidneys were stained with azarmethod logy (phromotropeaniline blue stain) and analysed for all. For the presence of α20 globulan¹³.

Accessory sex glands (depididymides, prostate, serinal vesicle, seminal vesicles (+coagulating gland), testes, Q: ovary, widuct, werus (+cervix), wigina),

cardievascular haematological system (aorta hone (ternum, tibial or femur for marrow) heart symph nodes (mandibular, mesenteric), spleen, thymus), gastrointestinal tract (oesophagus, tongue, stornach (fore-, glandular stornach) intestine (cacoum, colon duadenum, ileum; ie num, rectum), liver pancress), salivary glands; neurological (brain (cerebrum, cerebellum, pons medulla), eyes (+optionerve, flarderian gland, extraorbital lacrimal glands), sciatic nerve, spinal cord (cervical, thouseic, lumbar), respiratory system (nose, tochea lumg), urogenital system (kidneys (ureters, urethra), urinary bladder), other (ske tetal moscle (though), bone (femur, sternum), ear (pina, Zymbal gland), skin, tail all gross lesions and masses) other endocrine producing/sensurve glands (adrenals, mammary gland, pituitary, thypoid (+parathyroid)).

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

No specific neuronistopathology with specific fixatives were performed in addition to the standard histopathology undertaken on neuronal tissues.

12. Neurohistopathology:

Results and discussion

A. Test@iet 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 arocle in the det preparations ranged from -17.7% to +15% of nominal concentrations 25, 123 and 625 ppm, which were within acceptable limits.

B. Observations:

1. Clirical signs of treatment related effects observed with either test article.

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

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



wed a reduction in body weight from the search of the control. At termingian of treatment, body weight set to a control. At termingian of treatment, body weight weight set to another than the originary of treatment, body weight weight to another than the originary of treatment, body weight weight to another than the originary of the control for unpart terminating for it as yield gain in highly acting them to taggitate and the concurrent option and the concurrent option of the concurrent option. A fermination of control for the concurrent option of the concurrent option. A fermination of control for the concurrent option of the concurrent option. A fermination of the concurrent option of the concurrent option of the concurrent option of the concurrent option of the concurrent option. A fermination of the concurrent option of the concurrent option.



| _ | | | ♂ (ppm) | | | | | C. | ∂ (ppm) | ion Coji | |
|------------------------------------|------------------|----------------------------------|----------------------------------|------------------------|---------------------------------------|--|---|----------------------------------|-------------------------|---------------------------------------|------------|
| Parameters | | SPX N-oxide 0 25 125 625 | | | SPX © | 0 | ch spiroxamine N-oxide: food consumption (ppm) SPX N-oxide 0 25 0 9.7 53.6 | | |) | |
| Dose/animal (mg/kg bw/d |) | 0 | 1.7 | 8.8 | 45.0 | 48.7 | 0 0 ©123 ±6.2 | 25 © 25 | 9.7 | | .0, |
| Body wt (g) | Day 0 | 140 ±7.4 | 137 ±7.8 | 141 ±6.6 | 141 - 4 | 314NL¥10.1 a | L ((2)[23 ±6 &) | 117±64* | 152 39.5* | \bigcirc 121 \perp 361 \bigcirc | 110 |
| | Day 7 | 191 ±13.5 | 185 ±9.4 | 190 ± 10.8 | 189±9.1 | 172 ±13 6 5 203 ± 18.7** | 146 ± 83 \$\pm\$ ±7.5 | 330 ±8.6 | 138 ±7.65 | 141-66.8 | 132 |
| | Day 14 | 234 ± 15.7 | 224 ± 12.5 260 ± 16.3 | 236 ±14.1 | \$17 ±12.25 | 203 + 18.7 | 178 + 0.3° | 153 ±10.20 | 152 \$9.5 | 185 ±5.9 169±6.4 | 144 156 |
| | Day 21 Day 28 | 275 ± 19.7 295 ± 20.3 | 260 ± 16.3 277 ± 17.5 | 278 ±186 297 ±21.5 | 250 ±6.7* 269 ±16.5* | 2320±25.2 % \$246 ±29.9 | 178 ±9.35 185, \(\frac{1}{2}\)8.9 | 170 £2.2 177 ±12.0 | 168 ±10.1 174 ±11 1* | 169 ± 6.4 178 ± 7.5 | 164 |
| | Day 28 Day 35 | 293 ± 20.3 319 ± 21.1 | 277 ± 17.3 298 ± 20.2 | 324 ±24.3 | 289 ±32.4* | 264 \$2.9** | 193 ±10,4 | 186 ±19 | 184 \$14.1 | $1/8 \pm 7.3$ 189 ± 9.1 | 170 |
| | Day 42 | 345 +23 2 | 320 +21 5 ₩ | 251 +25 7° | 289 ±32 4* 314 £23.3* | 289 + 36 6* | D) 2014 +Q(D) " | 186 ±19 195@12.9 201 ±15.4 | 93 ±13.70 | 199 ± 9.6 | 179 |
| | Day 49 | 361 ± 23.3 | 337 ±200 | 368 29.9 | 392 ±26.1*© | 308 ±39.4** | 204 ±9.5 208 ±9.5 | 201 ±15.4 | 198 ± 19.9 | 204 ± 10.2 | 184 |
| | Day 56 | 378 ± 25.4 | 337 ±200 3543 24.9 | 391 ±31,90° | 347 ±24.7* 361 ±26.1* 403 ±24.7 | 327 \$\overline{4}\)1.4** | 200 ±9.5 215 ±10.80 | 208 ±45.2 | 20\$\psi\psi15.9 | 211 ±108 | 189 |
| | Day 63 | 393 ±25.5 | Q69 ±25.6 [©] | 405 ±3 .5 | 361 ±26.1* ≥ | 342 ±42.7** | | 283°±15.0 | 208 ± 16.3 | 219 ± 13.5 | 195 |
| | Day 91 | 428 ±303 | 412 + 30.2 | 450 ¥36.6 _¥ | 403 ±24. | 388 +44.4* | 23 3 ±13.2 | \$ 226 ±15 6 | 222 ±18.9 | 232 ± 13.6 | 207 |
| Body wt | Day | 288 | 275 | 309 | 262 | 247 | 110 | 108 | 105 | 109 | 4 1 |
| $gain (g)$ * $p \le 0.05$; ** p | 0-91 | <i>y</i> | (13%) | | (19%) · \ | D (114%) <u></u> | | (12%) | (↓5%) | (\1%) | (\ |
| | | ore this | docume docume docume | exploite | rion and | 308 ± 39.4** 327 ± 41.4** 342 ± 42.7** 388 ± 44.4* 247 () 14%) | * Chi | | | | |



onsumption:

.ented, fixed consumption was marginally greater in a dose group for both test articles when expressed as a sign dose group of an detection in body weight implied that desped.

at 025 pm dose group for both test attricles when expressed as a desped.

at 025 pm dose group for both test stricles were marginals, onthe sexes when expressed as graining? When complete for yeary as effect was not replicated and deeped a result of articles for the deeped and deeped a result of articles for the deeped and de The state of the s



| Day 7 1 Day 14 9 Day 21 1 Day 28 1 Day 49 1 Day 66 1 | 0 0 07 ±4.0 93 ±6.2 83 ±5.9 79 ±6.1 56 ±3.7 | SPX N 25 1.7 108 ±3.2 93 ±5.3 81 ±4.5 | -oxide 125 8.8 114 ±11.1 97 ±5.8 | 625 45.0 | 625× | with spinoxam | «()) ~ ~ = | -oxide 125 9.7 0 9.7 | 2. 2625 € \$ |) i |
|--|---|--|---|--|--|--|--|--|------------------------|------------------|
| Day 14 Day 21 Day 28 Day 49 Day 66 | 0 07 ±4.0 93 ±6.2 83 ±5.9 79 ±6.1 | 1.7 108 ±3.2 93 ±5.3 | 8.8 114 ±11.1 | 45.0 | 48.7 | | \$ 25 | 9.7 | 536 | 5 |
| Day 14 Day 21 Day 28 Day 49 Day 66 | 93 ±6.2 83 ±5.9 79 ±6.1 | 93 ±5.3 | | 114 +10 | | ₩ . • | | | | |
| Day 14 Day 21 Day 28 Day 49 Day 66 | 93 ±6.2 83 ±5.9 79 ±6.1 | 93 ±5.3 | | | 1000118 | | | 1000 | 0128 ±21640 | <u>⊘</u> 1 97 |
| Day 21 Day 28 Day 49 Day 66 | 83 ±5.9 79 ±6.1 | | | 101 # 92.9 | 109 \(\frac{1}{2} \) 0 0**\(\) | \$64 ±3.0 | 106 ±900 100 ±8.7 | 100 ±4.0 0100 ±7.25 91 ±6.17 80 ±11.0 | 129 ±31.1 | 123 |
| Day 28 Day 49 Day 66 | 79 ±6.1 | 01 ±4.3 | 97 ± 3.8 84 ± 3.2 | 101 ± 12.9 8 ± 9.7 @ | 0.4 ± 10.8 | 102 ±13Q 97 210.2 | | 01 kg | 129 ±31.1 100 ±13.9 | 105 |
| Day 49 2 | | 11 ±5.1 | 82 ±4.0, | 86 ±3.95 | 94 ±10 10 | 9% ₹10.2 \$95 +7 5 \$ | 90 ±0.4 % | 89 ±11.0 | 87 ±17.0 | 103 |
| Day 66 | .)() エ | 57 ± 5.3 | 61 ±5 2*** | 63 \$3.2** | 109 ±1.6 109 ±1.6 109 ±1.6 109 ±2.9.** 109 ±1.3** 109 ±1.3** 109 ±1.3** 109 ±1.3** | 72 # | 7) ± 7 6 | 72 ±6.75 | Q1 ±10 Q | 92 |
| | 53 ±3.7 | 54 ± 3.7 | 61 ±5.2*** 54 ±4.6 | 60 ±3.5** | 68 ±9.4 | -68 ±7.2 | © 68 ±4.90 | 66 ± 1.5 | 70 ±7.6 | 72 |
| ay / 0 . | 52 ±4.2 | 55 ±5.4 | ≈53 ±3.4 ¶ | 58 ±3.8 | 63 ² 7.1** . | 968 ±7,20° | 66 4.6 | ₩ ±10.3 | 70 ± 13.3 | 69 |
| You 73 | 40 ±2 € | 52 ⊥5 2 <i>@</i> | 55 1 A CE | _57 [©] 4.4** _ | © 60 ±6.6** € | 68 ±7.2 68 ±7.2 60 \$.9 60 \$.9 | 66 ±6.3 | 97 ±10.3 67 ±90 | 66 ± 17.3 | 69 |
| Day 77 | 46 ±4.1 | 50 ±631 (1) (50 ±6.4 (1) | 55 \$405 51 ±4.5 | 54 ±3,9***** | 57 ₂ ±5.9** | \$\frac{1}{2}\frac{1}{2}\frac{1}{2}.9 | 90 ±6.4 90 ±0.9 71 ±7.6 68 ±4.9 66 ±6.3 62 ±6.3 62 ±6.3 | 6 4 ±5.2 | 65 ± 6.1 | 65 |
| Day 80 | 51 ± 3.9 | 3 0±6.4 | 050 ±4.40 | 53 ±2.9 | 8±6.4* | 70 ±13.0 | 62€6.6 | 62 ± 7.3 | 66 ± 4.5 | 66 |
| Day 84 | 45 ±3.9 | 46 ±5.9 | 53 ±20.8 | ±3.2* | 55 ±4.6 | 61, 46.1 | \$64 ±24.4\$ | 62±6.9 | | 64 |
| 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 65 |
| L'E TO | | one the | nent ior lication exponis | cation and | Tajjoja, | | | | | |
| | ay 84 ay 87 ay 91 | ay 84 ay 87 ay 91 45 ±3.9 38 ±3.7 47 ±3.0 AT ENTROPE OF ENTR | ay 84 ay 87 ay 91 45 ± 3.9 46 ± 5.9 43 ± 9.0 47 ± 3.0 40 ± 5.6 49 ± 5.6 49 ± 5.6 40 40 40 40 40 40 40 40 40 40 40 40 40 | ay 84 ay 87 ay 91 45 ±3.9 46 ±5.9 39 ±3.5 48 ±2.9 48 ±2.9 And | ay 77 ay 80 ay 84 ay 87 ay 87 ay 91 47 ± 320 48 ± 2.90 48 ± 2.90 41 ± 3.0 53 ± 2.9 41 ± 3.0 53 ± 4.1* 39 ± 3.5 41 ± 3.0 53 ± 4.1* 54 ± 5.6 55 ± 4.45 56 ± 4.45 57 ± 5.6 58 ± 4.45 58 ± 3.9 59 ± 3.5 59 ± 3.5 50 ± 4.45 50 ± 4.1* 50 ± 4. | ay 73 | ay 84 ay 87 ay 87 ay 97 38 ± 3.7 38 ± 3.7 43 ± 5.6 49 ± 5.6 49 ± 5.6 48 ± 2.9 39 ± 3.5 41 ± 3.0 42 ± 3.2 43 ± 3.0 44 ± 4.5 43 ± 4.1 44 ± 4.5 44 ± 4.5 45 ± 4.9 47 ± 3.0 48 ± 2.9 48 ± 2 | ay 77 | 30 | ay 84 |



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 20), all indicate its content of the country of historical control range and were not present at the day. 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 ⊖, armough thrombocyte could was unaffected, clotting time (PT) was significantly extended on day 91/92. This was also seen in the 28 day study to males dosed 1000 ppm (114 mg/kg bw/day).

Spiroxamine:

In ∂ and ♀ at the interior sample point and/or 200 and In \circlearrowleft , although thrombocyte could was unaffected, clotting time (PT) was

. C valu.
deemed s.
accyte copif w
ed on day 91/92.
. ppm (114/mg/kg bw)

.t the interior were evident sug.
crit (2, inferim), MCV (2/2) interior.
.bl. margine is evident sugress accuration were very evident sugress.
.centration were very evident sugress accuration of were very evident sugress.
.centration of were very evident sugress and bosts leukocytes (2, day 91/92) were funct asset (1, day 91/92) we was also seen in the 28 day study to the contract of the cont concentration) were increased. Total and differential WBC parameters were affected in both sexes, with eucocytes, neutrophils, basoning (25day 91/85)..... leukocytes (3, day 91/92) were decreased. Clotting time was significantly

extended in both sexes at both sample times Extended Pf was likely a secondar effect resultant from secreases in both



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

| | _ | | | ♂ (ppm) | | | SPX N-grade SPX | | | | |
|--|---------|--|------------------|-------------------------|---|---------------------|-------------------------|---------------------------|----------------------------|-----------------|----------------------|
| Parameters | | SP | X N-oxide | | SPX | | SP | X N-oxide | | SPX | |
| | day | 0 | 25 | 125 | 625 | 625 | . 0 | \$ 25 | | °.4025 ≥ | \$ 625 |
| Dose/animal | | 0 | 1.7 | 8.8 | 45.0 | 48.7 | | \$ 25 | 2 9.7 2 9.7 | 53.6 | 52.7 |
| (mg/kg bw/d) |) | | | | | | | | 0 P | | r O |
| RBC | 28 | 7.81 ± 0.280 | 7.82 ± 0.429 | 7.92 ± 0.350 | 7.77 ±0.473 | | $8.02/\pm0.239$ | 37.96 ±0.3 € } | 8.03 ⊕0.458 | 800 ±0.2680 | |
| $(10^{12}/L)$ | 91 | 8.55 ± 0.180 | 8.54 ± 0.502 | 8.63 ± 0.413 | 8.33 ±0.305 | 8 .55 ±0.180 | 7.92 ±Q.183° | 7.8 4 @ .284 | ₩9±0.35% | | 7.99 ± 0.256 |
| Hb (g/L) | 28 | 150 ±4.3 | 148 ±4.9 | 150 ±6.8 | | ©~148 ±635°° | 1.54€±2.7 | 1152 ±5.4 ℃ | | 149°±3.9 | 150 ± 5.1 |
| | 91 | 145 ± 5.2 | 144 ± 5.1 | 147 ±4.2 🔏 | \$\frac{1}{2}\frac{1}{46} \pm \frac{1}{2}\frac{1}{2}\frac{1}{2} | 142 ±6.7 | \$\frac{1}{40} \pm 2.90 | 142 | ₩ ±5.8 | √141 ±7.0 | 138 ± 3.9 |
| PCV (L/L) | 28 | 0.486 | 0.464 | 0.464 | ©0.464 | 0.460 ±0.0100 | 0.450 | 0.458 ±0.0140 | 0.455 | 0.448 | 0.444 |
| | | ±0.0577 | ±0.0177 | ±0.0202 | 0.0202 | ±0.0160 | # 0 :0111 @ | > ±0.014 ₽ | . ∩ ∩ ill./≥5 | 6 0.0106 | ±0.0162* |
| | 91 | 0.445 | 0.441 | 0.448 ±0.012 | 0.449 | 0.437 | 0.4210 | 0.430 | ±0.0162 0.426 ±0.020 | 0.426 | 0.414 |
| | | ±0.0166 | ±0.0121 | ±0.01% | ±0.0139 | ©±0.0237 | ±0.6089 | | | ±0.0169 | ±0.0110 |
| MCV (fL) | 28 | 60.0 ± 1.90 | 59.3 ±0356 | 598±2.27 | √\$9.8 ±2.2 5 | | 56.8 ±1.33 | 57.6±64 | 56€€2.10 | 56.0 ± 1.73 | $54.6 \pm 1.76^{**}$ |
| | 91 | 52.1 ± 1.98 | 51.0±2.88 | 52.0 ±1,46 | 53,9@1.64 | 61.3 ±1.88 | 1 53.2 41 33 | 54.9 ¹ €1.62 € | 53.8 ±2.45 | 53.2 ± 1.85 | $51.8 \pm 1.59^*$ |
| MHC (ρg) | 28 | 19.2 ±0.45 % | \$8.9 ±01 | 19.3 ±0.62 | | 18.4 ±0.73* | 18.8 ±0.36 | \$19.1 ±0.50 | 18.7 ± 0.66 | 18.6 ± 0.65 | 18.5 ± 0.63 |
| | 91 | 16.9 ±0.5 | 16.9 ±1.14 | 10.73° | | 16.2€0.61 | ⊙17.7 ±0.3℃ | 18.2,±0.33 | 17.8 ± 0.73 | 17.6 ± 0.74 | 17.3 ± 0.64 |
| MCHC | 28 | 320 ± 5.5 | 319 ±07.1 | | 32, 9±2.9 | ≥321 ±6,0€ | 330 \$5.1 | 382 ±3.5 | 330 ±3.4 | 332 ±3.2 | $338 \pm 5.6^{**}$ |
| (g/L RBC) | 91 | 325 ± 10.5 | 327 ±₹₽ | 3 ₹9¥7.6 | 326 ±93° | | √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 | Ø9 ±5.0 | 21 ±3@ | 22 ±4.5 | 21 ±4.7 | 25 ±3.2 | 26 ±6.4* |
| | 91 | 23 ±3.6 | 24 ±4.2 | 21 📆 .5 | 25 ±4.4 | 25 ±30 | 2443.4 | 26 ±3.3 | 26 ± 5.4 | 25 ±4.9 | 25 ±4.2 |
| Thro (10 ^{9/L}) | 28 | 1169 ±1249 | 1178 | | 1111± 6 1.5 | 106@±116.6 s | \$095 ±42.2 | 1081 ±44.2 | 1081 ± 102.4 | 1045 ±131.5 | 989 ± 173.0 |
| | 91 | 1026 169.9 | 1017 ±135.7° | A *// NO | 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 ± | 32,2 ^{C±} 1.43 | \$2.9 ±2.29 | | 28.9 ± 1.34 | 29.8 ± 0.99 | 29.9 ± 2.16 | 30.0 ± 1.03 | $30.8 \pm 1.98^*$ |
| | 91 🖠 | 28.9 ± 0.81 | | ©29.1 ±1.1√ | 30.6⊕1.02 | 30.7 ±1.02** | 27.9 ± 1.01 | 27.7 ± 0.88 | 27.5 ± 1.20 | 27.8 ± 1.11 | $28.9 \pm 1.24^*$ |
| Hypochrom | 28 | 0 ±0.3 | 1 ±0.7° | 1 ±0.7 | 10.3 ±0.3 | 1 ±0.9 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | $0.0\pm$ | 0 ± 0.0 |
| | 91 | 0 ± 0.7 | 0,49,6 | 0±0.3 % | 0 ±0.06 | 0 ±0.3 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 | 0.0 ± 0.0 |
| | | 0 ±0.7 | 9 m 1 6 | Laborátory | historical con | trol data (rat, ` | <u>Wistar, 1994-1</u> | | | | |
| PCV (L/L) | | Age of animal | ls: | Laboratory | D. D. | | Age of anima | | 11 wks | | |
| | n.e | h: | - 100 | | " | | n: | 330 | | | |
| | | Mean ±SD: | | | | | Mean ±SD: | | 40 ± 0.0253 | | |
| Range Contraction of the Contrac | | Age of animal n: Mean ±SD: Mange ±2SD: Age of animal n: | <u> </u> | S. F. | | | Range ±2SD: | | 39 – 0.491 | | |
| MCV (fL) | 40DB | Age of animal | | 9 wks | | | Age of anima | | 11 wks | 12 - 25 v | vks |
| (| 4 (Q) » | n: 💆 | ري | | | | n: | 329 | | 257 | |



| | | | | | | | | | · · |
|-------------------|-----|-----------------|--------------|---------------------|--|----------|--------------------------|---|-----------------|
| | | | | ♂ (ppm) | | | ŗĜ | Ç (ppm) | |
| Parameters | | SPX | N-oxide | | SPX | | ŠPX | N-oxide | «SPX O |
| | day | 0 | 25 | 125 | 625 | 625 | _{si} ©ð | 25 2 125 | 625 625 |
| | | Mean ±SD: | 57.0 | 0 ± 2.40 | | | Mean ±SD: | 55.6 ±2.09 € | 520 ¥2.49 |
| | | Range ±2SD: | 52.2 | 2 – 61.8 | | | Range ±2SD: | 51.4 - 59.8 C | ~ ~ ~ 57% |
| MHC (ρg) | | Age of animals: | | 11 wks | | Q. | Age of animals | | |
| | | n: | 304 | | | | n: | | e court of e |
| | | Mean ±SD: | | 2 ±0.82 | | 2 | Mean 4SD: | · Para Maria | |
| | | Range ±2SD: | | 5 – 19.9 | OF | | Mean ±SD: Range ±2SD. | | |
| MCHC | | Age of animals: | - | | | | Age of animals | 2 0 *********************************** | E Cher |
| (g/L RBC) | | n: | - | ** | | | - 12" | 324 | ₹ [™] |
| | | Mean ±SD: | _ | , Ġ | | | Mean ±SD: Range ±2SD: | 2 32 T = 0.2 | |
| Dot (0/) | | Range ±2SD: | - | | | | Range ±2SD. | 307-340 | 1 10 % |
| Ret. (%) | | Age of animals: | | | SUCILIA DE LA COMPANSION DE LA COMPANSIO | | Age of animals | | 1 turks |
| | | Mean ±SD: | 120° | O | | | Mora®±SD: Ĉ | : 200 | +7.3 |
| | | Range ±2SD: | | 10 ³ - 3 | | ~ 0.S | Range ±2SD: | 200 3 20 22 = 12 - | 37 |
| PT (sec) | | Age of animals | 80 | Wks & | ₩2 - 25 w | WO " OLD | Age of animals | :\$ 8 11 wks | 12 – 25 wks |
| , | | n: | 3 04 | ~0 É | | | | 334 | 250 |
| | | Mean ±SD. | 27,3 | 3 ± 1.41 | 273 ±1.4 | | Mean ★SD. | 26.0 ±1.53 | 25.7 ± 1.19 |
| at0 0 5 at at | | Range ±2SD: | | 5 − 30.1 \$ | <u>\$24.4 – 30</u> | . P | Rango £2SD: % | <u> </u> | 23.3 - 28.0 |

*p \leq 0.05; **p \leq 0.01
RBC: red blood cell
Hb: haemoglobin
PCV: packed corpuscular volume (haematocrit)
MCV: mean corpuscular volume.
MCV: mean haemoglobin concentration

Table CA 5.8.1/06-5:

Overview of sub-chronic loxicity study in rats treated mally (via diet) with spiroxamine N-oxide: selected haematological WBC parameters

| | • | ** | , a | P(ppm) @ | | | Ç (ppm) | | | | | | |
|-------------------|---------|--------------|---------------------|------------------|--------------------|-------------|------------------|------------------|------------------|------------------|------------------|--|--|
| Parameters | | ~ € 1 | SPX | oxide | , * C | SPX | | SPX N | -oxide | | SPX | | |
| | day | | 25 C | | © [™] 625 | 625 | 0 | 25 | 125 | 625 | 625 | | |
| Dose/animal | w.E | | Q.7 | 8.8 N | 45.0 | 48.7 | 0 | 1.9 | 9.7 | 53.6 | 52.7 | | |
| (mg/kg bw/d) | | . 0.10 | | | | | | | | | | | |
| Leuko. | 28 | Ø.74 ±2.10\$ | 10.31 \$\dag{2.051} | 9.43 ±2.122 | 9.39 ± 1.609 | 12.12 | 8.06 ± 1.243 | 7.65 ± 1.076 | 8.08 ± 2.176 | 8.99 ± 1.178 | 9.77 ±2.388 | | |
| $(10^{9}/L)$ | _ &@ | | | | | ±2.814* | | | | | | | |
| | 1 Oje 1 | 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 | | |



| | = | | | | | | | | a de | . art | Se , |
|-------------------------------|------------|---|--|----------------------|--|----------------------|---|--------------------------|-------------------------------|------------------|------------------|
| | | | | ♂ (ppm) | | | PĠ. | | Ç ©ppm) | | |
| Parameters | | | | -oxide | | SPX | Al Al | D1 11 P | -oxide | | ØŠPX |
| | day | 0 | 25 | 125 | 625 | 625 | I O O | 25 | | 625 € 625 | 625 |
| Neutro. | 28 | 0.80 ± 0.3116 | 1.02 ± 0.592 | 0.82 ± 0.678 | 0.92 ± 0.324 | 1.94 ±2.064* | ©.79 ±0.271 | 0.67 ⊕0.343 | 0.71 ±0.301 | 0.79 ±80\$14 | 0.91 ± 0.427 |
| $(10^9/L)$ | 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 - 146 | 0.32 ±0.106 | 0.31 ± 0.105 |
| Lymph. | 28 | 8.33 ± 1.821 | 8.66 ± 1.692 | 8.08 ± 1.408 | 7.94 ± 1.529 | 9.44 ±1.122 | 26.74 ±1.076 | 6.56 | | 7.76 ±1204 | 8.37 ± 2.182 |
| $(10^{9}/L)$ | 91 | 5.86 ± 0.531 | 5.57 ± 1.461 | 6.04 ± 1.448 | 5.44 ±1.114 | \$5.93 ±12 50 | 2.65 (3.582 | £82 ±0.913 ° | 23.23 ±1.335 | 0.05 0.019 | CS:30 ±0.725 |
| Eosino. | 28 | 0.12 ±0.063 | 0.10 ± 0.035 | 0.08 ± 0.026 | 0.09 ±0.00 | 0.11 ±0.031. | 0.14 ±0.0420 | 0.08 ±0042 | 0.0890.035 | 0.09 ±0.035 | 0.09 ± 0.033 |
| $(10^9/L)$ | 91 | 0.15 ± 0.046 | 0.16 ± 0.071 | 0.014 ± 0.070 | 0.11 9.051 | Ø10±0.026© | 0.06 #0.019 | 0.00±0.044 | 0.05 ±0.043 | 0.05 40.018 | 0.08 ± 0.064 |
| Baso. | 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 | $0.03 \pm 0.011^{\circ}$ | 0.03 ± 0.009 | 0.03 ± 0.012 | 0.04 ± 0.024 |
| $(10^9/L)$ | 91 | 0.02 ± 0.005 | 0.02 ± 0.010 | 0.02 ±0.00% | 0.02 ±0.013 | 0.02 ± 0.007 | (0.00 ±0.00) | 0.00 ±0.004 | 000±0.005 | 0.01 ± 0.005 | 0.00 ± 0.005 |
| Atyp. leuko. | 28 | 0.16 ± 0.051 | 0.20 ± 0.059 | 0.016± © 071 | 0.042 | 9.21 ±0.085 | 0.14 \(0.038 | P.M ±0.034 | | 0.12 ± 0.037 | 0.12 ± 0.049 |
| $(10^9/L)$ | 91 | 0.09 ± 0.021 | 0.07 ± 0.030 | 0.08 ± 0.029 | 9.06 ± 0.00 | | | 0.02 ±0 012 | 0.03 ±0.008 | 0.03 ± 0.013 | 0.02 ± 0.012 |
| | | | | Labocato | ry historical c | opti ol data (r | t, Wistar (199' | 7) | |)) } | |
| Atyp. leuko. (| $(10^9/L)$ | Age of anima | ls: 12 35 w | ks | ry historical c | | Age Fanima | | | | |
| | | n: | 28 0 0 | 10, "9 | | 26 ⁷ | n: S | 90 | | | |
| | | Mean ±SD: | 0.13 ± 0.0 | 78 _V | | | n: S Mean SD: Range ±2SD | | | | |
| | | Range ±2SD | 0-0028 | | | | Range ±2SD | | | | |
| Baso. (10 ⁹ /L) | | Age of anima | 0 - 628 ls: 8 - 11 wk 80 0 04 €0.0 | s O' | | and one | Range ±2SQ Age of anima n: Mean ±SD: Range QSD: | ls: - | | | |
| | | n: | 80 C | | | | | | | | |
| | | Mean ±SD: | 0.07 | | 1 338 | - A | Mean ±SD: | , * | | | |
| I | τ.\ | Range ±2SD: | 1-50 - 0.0 /c | The Aller | Ope (| | Age of anima | 1 1/ | 25 - 1 - | | |
| Lymph. (10 ⁹ / | L) | Age of anima | is: - | | | <i>@</i> . | Age%i anima | .is: 1. 7' | 2-25 wks | | |
| | | Marrison: | COF | | | * D | Mean ±SD: | 6 | .13 ±1.182 | | |
| | | Range +2SD: | 30 ^C | , Çõ | *,0 | * O } | Range +2SD: | 2 | $.13 \pm 1.182$.50 - 9.75 | | |
| Eosino. (10 ⁹ /I | () < | Age of anima | Is: @12 - 25 w | k 0 0 0 | » 0° | option data (18) | Age of anima | ls: - | .50 7.75 | | |
| 2051110. (10 /1 | L) >> | n: | 80 00 N | | # Ojr. | | n: | | | | |
| | | Mean ±SD: [™] | 0.16 ± 0.0 | 92 ^{et} . ¢ | \$ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ | » | Mean ±SD: | | | | |
| | | Range 2SD: | 0.34 | | | | Range $\pm 2SD$: | | | | |
| Neutro. (10 ⁹ /I | L) | A of anima | ls: 8 – 17 wk | s e | | | Age of anima | ls: - | | | |
| ` | | Ch: | · | | | | n: | | | | |
| | A DE | Age of anima n: Mean ±SD: Range ±2SD: Age of anima n: Mean ±SD: Range ±2SD: Age of anima n: Mean ±SD: Range ±2SD: Age of anima n: Mean ±SD: Range ±SD: Range ±SD: Range ±SD: Age of anima | 0.9 ±0.9 | | | | Mean ±SD: | | | | |
| | | Range ±2SD | 0.01 - 1.7 | 4 9" | | | Range ±2SD: | | | | |
| *p ≤0.05 | | Mean ±SD: Range ±2SD: Age of anima n: Meant \$SD: Range ±2SD: Age of anima n: Mean ±SD: Range 2SD: Age of anima n: Meant \$SD: Range 2SD: Age of anima n: Meant \$SD: Range \$Range \$SD: Range \$Range \$SD: Range \$Range \$SD: Range \$Range \$SD: Range \$Range \$SD: Range \$SD: Range \$SD: Range \$SD: Range \$SD: Range \$Range \$Ran | ~ 000 T |) — — | | | | | | | |
| * $p \le 0.05$ Leukoc: leukoc | ytes [] by | Org. " # | The state of the s | • | | Eosino.: eo | sinophils | | | | |
| (| ي _ | | - | | | | | | | | |
| | | * | | | | | | | | | |



| | | | | | | | | | Spi | roxamine |
|--|---|-------|---------|------|-----|---------------------------|---------|-----------------|----------------------|----------|
| | | | | | | | | 29 | | , |
| | | | ♂ (ppm) | | | Ĝ | | Ç (ppm) | O Dille | ~Q |
| Parameters | | SPX N | N-oxide | | SPX | | SPX N-0 | | 200 L | SPX |
| day | 0 | 25 | 125 | 625 | 625 | _{a1} ©ð | 25 | 125 | 625 · 6 | 625 |
| Neutro.: neurophils Lymph.: lymphocytes | | | | 700° | | partes atypical lympho | CAPE OF | orecri | iontents contents | |

Furthernore, this document may distribution use of this document of any commercial exploitation and use and use of this document or its owner.

The commercial exploitation of the violate the richts of and violate the prohibited and violate. and use of this document or its and use of this document or its and use of this document copy rights of the owner and third par reproducting distribution, reproducting distribution, reproductive distribution, and publication, and p the permission of the owner the rights of the prohibited and wholate the prohibited



group, incremed liver enzyme activities (
decreased total cholesterol at both the ingrin,
points. Total protein and albumin were deglesse.

a both sexes and at day 1919. 20 m.

a treated at 625 ppm, increased AL1 (%%-mierim and tag)

a) and AST (6 terminal brigs point) were greeved, with \$40 P (
aintal time point) and total foliosiested (52% metrim and legifunist),
orns) decreased.

Total protein, albumin (26 metrin), incline and terminal time points) of decreased. Whits creating the total protein and green specific at the control of the co

point) were observed, with ALP (point) and total cholesterol () interim and terminal time points) decreased.

Total protein, albumin (interim; interim and terminal time points) were decreased. Whilst creatione, glucose total bilination and electrolytes showed significance, these were considered incidental and not advise correlating histopathology was present there were no treatment. notal protein, albumin (Anterim; \$\square\$, interim and terminal time points) were decreased. Whilst creatione, glucose, total bilination and electrolytes showed significance, these were considered incidental and not adverse as not correlating histopathology was present.

There were no treatment related and interior spirores.



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

| | | | | ♂ (ppm) | | | JE" | | ♀ (ppm) * | | | |
|-------------------|-------------|------------------------------------|-----------------|--|---|-----------------------------|-----------------------|-------------------------|---------------------------------------|-------------------|-------------------|--|
| Parameters | | SP | X N-oxide | | SPX | * | SI | X N-øxide | 18/2 | | | |
| | Day | 0 | 25 | 125 | 625 | 625 | 0 « | 25 | ,125 [©] | 625 | 625 | |
| Dose/animal | | 0 | 1.7 | 8.8 | 45.0 | 48.7 | | 106 | ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ | 53.6 | 52.7 | |
| (mg/kg bw/d | ay) | | | | | | | | | 1 10 V | r O | |
| ALT (U/L) | 28 | 49.7 ± 6.50 | 49.2 ± 5.82 | 52.5 ± 7.99 | 59.9 ±8.90 | | 4789±5.31 | 46.9 ±6.8 | 45 ₁ 1 ₄ .30 | \$5.5 ±6.89*○ | | |
| | 91 | 44.5 ± 7.69 | 46.2 ± 6.14 | 45.8 ± 4.70 | 50.4 ± 5.98 | 54.9. <u>₹</u> 7.23** | \$42.1 ±40.℃ | 49.3.26.80 | ₩ ±4.23\$ | 50.6 ≱€28 | 53.1 ±9.22 | |
| ALP (U/L) | 28 | 454 ±48.9 | 462 ± 55.6 | 447 ± 67.0 | 553°¥103.5 | 6448 ±796 | 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 × | \$47 ±63.4\$ | 272 ±33.5 | | 0 11 11 . | 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 ₹.12 | 41.8±4.91** | 39.0±201 | 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. | | 345£6.10 ₂ | 39.8 ±6.40 | 37.4 ±3.81 | #1.8 ±8.55 | 41.6 ± 5.54 | |
| T.prot. | 28 | 65.9 ± 1.64 | 64.8 ± 1.50 | 64.8 ±1.29J | | 59.0 ±2.70** | 63.7 ±2,30° | | | 62.2 ±1.67** | $59.2 \pm 2.60^*$ | |
| | 91 | 70.0 ± 1.80 | 69.7 ±2.07 | ₽70.2 ±1 39 0°° | 68.9 ±3.28 | €8.4±1,92 | 71.0 2.50 | 20.4 ±2.76 | 70.3 ±3.49°° | $67.6 \pm 2.16^*$ | $67.0 \pm 2.97^*$ | |
| T.chol | 28 | 2.44 ± 0.187 | 2.39 ±0347 | 2.46(±0.276 | 1.86 ±0.205 € | 1.66 ±0.331** | 2.10 ±0.268 | 2.29 ±9385 | 2,14,€0.166 | 1.89 ± 0.175 | 1.59 ± 0.284 | |
| (mmol/L) | 91 | 2.46 ± 0.207 | 2.40 0.477 | 2.51 ±0.315 | 2.14 + 20.263 | 2 ±0.258 | 12.23 ±0.322 | 2.36 0.400 _C | 2.23 ±0.193 | 2.04 ± 0.235 | 1.77 ±0.318 | |
| Alb. (g/L) | 28 | 32.1 ±0.81 % | \$2.3 ±0.47 | 32.0 ±1.29 | 300 ±1.20** | 29.8 ±2.24 | 34.2 1.44 | \$3.2 ±1.40 | 34.0 ±1.44 | 33.0 ± 1.50 | $30.7 \pm 1.12^*$ | |
| , | 91 | 32.9 ±1 | 33.6 ± 0.62 | 33.9 ±1.01 | ≥ 33.7 ±1.63 | 33 , 3 ,₹0.97 | 37.9 ±1.84 | 38.0 ± 2.57 | 37.2 ±1.47 | $35.7 \pm 1.27^*$ | $35.9 \pm 1.47^*$ | |
| Laboratory h | istorical | control data (ra | t, Wistar 1994 | ~1995) A | 6 B | | Age of anima | | | | | |
| ALT (U/L) | | Age of anima | ls: 311 | 11 wks | 12 - 35 40.8 ±7. | wks . | Arge of anima | ils: 8 – | 11 wks | 12 - 25 v | wks | |
| | | n: | 311 | 11 11 11 11 11 11 11 11 11 11 11 11 11 | 251 | | Chi. | 336 | | 25 | | |
| | | Mean ±SD: | 45. | ₹±8.19% | 40.8 ± 7.5 | O'IL | on: Mean ♣SD: | 40.9 | 9 ± 7.08 | 40.5 ± 8.0 | 61 | |
| | | Range ±250. | 29.4 | 4 <u>-</u> 62 ¹ 2 , c | 26,65°£55 | 5.1 | Range ±2SD | 26.8 | 3 - 55.1 | 23.3 - 57 | 7.7 | |
| ALP (U/L) | | Age of anima | ls: Ç 8 | ₩ks 🌾 📜 | ≈ ∫2 – 25 v | wks a o | Age of anima | als: 8 – | 11 wks | 12 - 25 v | wks | |
| | | n: 1000 | 30 10 | , C ^o - , | 250 K | , | n: | 332 | | 257 | | |
| | 4 | €Mean ±SD: | 468 | ±98.4 0 | ²⁴¹ ¥50. | .3, 4 | Mean \pm SD: | 314 | ± 64.4 | 177 ± 36 | .5 | |
| | > | Range ±2SD: | 270 | ¥665 , 0 × 1 | <u> </u> | 20- | Range ±2SD | : 185 | - 443 | 104 - 250 | 0 | |
| AST (U/L) | | Age of anima | ls." . 8- | 11 w | ,\$ 12 <u>,</u> - 2 \$ | wks | Age of anima | | 11 wks | 12 - 25 v | wks | |
| | | n: | 306 | | ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ | | n: | 327 | | 255 | | |
| | | Mean ±SD: | AY.0 | 5±7.26 | 36.7 ± 7.7 | 76 | Mean \pm SD: | 40.0 | 0 ± 6.91 | $38.8 \pm 8.$ | 15 | |
| | | Range ±2SD | 26.5 | 5 - 595 🐪 | 21.2 - 52 | 2.2 | Range ±2SD | | 2 - 53.8 | 22.5 - 55 | | |
| T. prot. | " 10 E | Age of anima Nean ±SD: Range 2SD: | ls: 0110 8 10 | ₩ wks ~ O W | 12 - 25 v 40.8 ±7. 26.6 55: 27 - 25 v 241 ±50. 241 ±50. 240 - 34. 12 - 25 v 36.7 ±7. 21.2 - 52 | | Age of anima | | 11 wks | 12 - 25 v | wks | |
| - 4 | 120 | night of | ,° , 301 | S. | | | n: | 330 | | 247 | | |
| E. | J». | n: Mean ±SD: Range 22SD: | 63.4 | 1 2.81 3 – 69.1 | | | Mean \pm SD: | 63.0 | 6 ± 3.31 | 67.9 ± 3.0 | 66 | |
| | -10.S | Range 2SD: | ے 57.8 فیل | 3 [™] – 69.1 | | | Range ±2SD | 57.0 | 0 - 70.2 | 60.5 - 75 | 5.2 | |



| | | | | | | | | | <u> </u> | 100° | |
|-------------------------------------|---------------------|------------------|-----------|--|------------------|------------|----------------|---------------------------|------------------------|---|-----|
| | | | | ♂ (ppm) | | | Ġ | | <u>, ♀(ppm)</u> | | |
| Parameters _ | | SPX | N-oxide | | SPX | | ŠP | X N-oxide | | ≪SPX 🌣 | |
| | Day | 0 | 25 | 125 | 625 | 625 | a Co | 25 8 - 11 331 | 125 | SPX 625 wks | 625 |
| T.chol (mmol/ | L) | Age of animals | 8 – | 11 wks | 12 - 25 | wks 🤻 | Rege of animal | ls: ൃ©്⊭ 8 – 11 | wks . | 12\25 wks | |
| | | n: | 301 | | 246 | E, | n: | 2 331 | ا کی ا | ~ \$247 , \$ | |
| | | Mean ±SD: | 2.10 | 0 ± 0.344 | 2.36 ± 0.6 | 471 ° | Moan ±SD; | ≥ | 0388 | 2.08 9 376 | |
| | | Range ±2SD: | 1.41 | -2.78 | 1.41 - 3 | 300 | ∕Range,≠23D: | 1.30 | 2.65 | 035-2.84 | |
| Alb. (g/L) | | Age of animals | 8 – | 11 wks | € | | Ago of anima | kg [™] &_− 111 | wks > | 23 - 2.84 12 - 25 ks 244 36.1 ±2.31 31.4 - 40.7 | |
| | | n: | 253 | | COE | | M. D. | 275 | 31 | S 244 | |
| | | Mean ±SD: | 32.5 | 5 ± 1.74 | \$ P | | Mean ⊕SD: | 343 | 2.15 | Ø6.1 ±2.31 | |
| | | Range ±2SD: | 29.0 | <u>) – 36.0</u> | | | Range ±2SD | action 30.0- | 38.6 | 31.4 – 40.7 | |
| * <i>p</i> ≤0.05; ** <i>p</i> ≤0.05 | 0.01 | | | | | | | | | J | |
| ALI: alanine an | ninotrar bosphat | isterase | | | | I.prot Oot | al protein | | I I III | | |
| AST: aspartate a | minotr: | asc ansferase | | ~~ J | | Alh alhur | al Chytesicion | | | | |
| 1101. uspartate t | 41111110111 | | - TO | | , Ş ⁻ | | | 20° .100° | Ö | da. | |
| | | | | | | % .Os | | | , * \$ | | |
| | | | 30 2 | | | ye, | | 90 | D | | |
| | | 9 | \$ _ D | | E. K. | | E The | | | | |
| | | | . Os | *\0 | | | | , \$ | | | |
| | | , 11, 11 | ~* | | C Or | | - & | | | | |
| | | | , e, c | | | | 0" | 39" | | | |
| | | | | 7 9 J | | - à | e ^y | | | | |
| | | | \$ J. 1 | The Alle | Ope | | | | | | |
| | | ~C | -04 | 6.D | | @. | . 0, | | | | |
| | | 4 1 | COF . | | | | | | | | |
| | | | ~ ^ C' | | . D | * 0 | | | | | |
| | R | * | | | | JŽ | | | | | |
| | 1 | , " | | | . O.D. | å. | | | | | |
| | | ₩" | The Sa | | _6 ⁾ | | | | | | |
| | | @ 1 | | | \$. ° ° | | | | | | |
| | | | | The State of the s | | | | | | | |
| | | and and | | | 1 0 m | | | | | | |
| | me | | | | 7 | | | | | | |
| | | | Office Ry | | | | | | | | |
| | 3. E | 200 41 | | e F | | | | | | | |
| y | 10.B | | | Õ | | | | | | | |
| Ĉ | | | | | | | | | | | |
| | • | 40° | | | | | | 25 8 - 11 331 1.98 ± 1.30 | | | |
| | | | | | | | | | | | |
| | | | | | | | | | | | |
| | | | | | | | | | | | |



F. Sacrifice and pathology:

1. Organ weight:

The state of the s or an pressed to the nine sension to the nine The state of the s



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

| | | | ♂ (ppm) | | | 41e | | ♀ (ppm) ¸ | | |
|--|-----------------------------------|--|-------------------------------------|---|--|-----------------------|---|--|---|--|
| Parameters | | SPX N | | | SPX 📎 | % } | "⊘ŠPX N | | | SPX |
| | 0 | 25 | 125 | 625 | 625 | 0 | P 25 | 125 | 625 | 625 |
| Dose/animal (mg/kg bw/day) | 0 | 1.7 | 8.8 | 45.0 | 48.7 | | \$ 25 | 9.7 | 53.6 | 52.7 |
| Terminal bwt (g) | 432 ±30.4 | 415 ±30.0 (\dagger*4%) | 454 ±38.9 (↑5%) | 408 ±26 8 C | 390 ±45.5** | 284 ±14.5 | 227 ±6% (3%) | 2230 78.2 (\) (\) 5%), \(\) | (↓1°© | 207 ±16.5** (\12%) |
| Liver Abs (g) | 14.1 ±0.15 | 13.7 ±0.18 (\(\pm\)3%) | 15.1 ±0.1 (↑7%) | $ \begin{array}{c} (13.3 \pm 0.17) \\ (16.4) \\ (16.4) \\ 3.3 \pm 0.22 \\ (-) \end{array} $ | 13.0 ±2.4 | 7.5 \$0.69 | 7.0 ±0.92 © | 7.0 ± 1 1 | 7. 6 ±0.53 (\pm\7%) | 6.6 ±0.72 (\pm\12%) |
| Rel. (g%) | 3.3 ±0.18 | 3.3 ±0.22 (-) | 3.3 ±0.18 (-) | ar do | 3.9 ±0.18 (-) | 3.2 ±9.24 | 3.1×0.30 (\13%) | 3.1 ±0.26 (13%) | 3.0 ±0.18 (\$6%) | 3.3 ±0.24 (↑3%) |
| Spleen Abs (mg) | 695 ±58.5 | 698 ±171.8 (-) | (†3%) (**) | 682 ±109.1 | 660°£0.10 © (15%) | &()) V | 391 % 7.4 | \$\frac{14\%}{14\%} | 442 ±50.4 (†7%) | 430 ±78.7 (↑4%) |
| Rel. (mg%) | 161 ±13.9 | 1670 ⁷⁷ ± 29 ©(†4%) | 158 ±13.3 (\)\(\)\(\)(\)\(\)\(\) | (14%) | 169±18.5 | ₹23.3 \$ | 172 ±223 | 177 4.4 (-) | 192 ±18.5 (†8%) | 177 ±23.3 (-) |
| Adrenals Abs (mg) | 45 ±8.9 10 ±2.1 | 43 ±9 7 ○ (↓4%) | " 49 ±% 6.7″ " | ±5.7 | 43 ±5 (34%) | 5 .0 ≥ 8.8 ° | \$57 ±11.9 | 57 ±8.4 (\dagger*3%) | 60 ±11.7 (†2%) | 58 ±6.4 (↓2%) |
| Rel. (mg%) | | 10 ±2.1 (-) | 11 ±1.30 (11.6%) | (\11\%) 10\#\0.6 (-) | ↑10%) (↑10%) | 25 \$3.0 | 35 ± 5.0 (-) | 26 ±3.4 (†4%) | 26 ±5.3 (†4%) | 28 ±4.2 (†12%) |
| Testes Abs (g) Rel. (g%) | 3.5 ± 0.23 0.81 ± 0.08 | 37±0.11 (†6%) 4 0.93±0.25 | (†3%) (*) 0.7940.07 | 3.6 ±0.23 (\$\infty\$) 0.89 ±0.27 | 3.4±0.58 (\\\\)3%\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\ | | - | - | - | - |
| Ovary Abs (mg) Rel. (mg%) | | | | Carrie of | 0.86 ±0.08 | 138 ±28.5 59 ±12.8 | 132 ±28.9 (\(\pm4\%\)) 59 ±14.4 (-) | 134 ±27.5 (↓3%) 60 ±9.7 (↑2%) | 136 ±27.1 (↓1%) 60 ±15.5 (↑2%) | 128 ±29.0 (\(\frac{7}{9}\)) 61 ±9.6 (\(\frac{3}{9}\)) |
| Testes Abs (g) Rel. (g%) Ovary Abs (mg) Rel. (mg%) *** p ≤0.01 Abs.: absolute | onently, | hout the | explis | | Rel.: relative | e to body weight | | (270) | (270) | (1370) |



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 \triangleleft and \triangleleft 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 freated animals, Mild transitional cell hyperplasia in the urinary bladder was observed in dose with spiroxamine. As with the parent compound, spiroxamine, spiroxamine, spiroxamine, spiroxamine Noxide is a tertiary amine, and like all tertiary amines has a high Ka, and a high pH with comparable histopathological effects ported. As with irritant compounds, tissues containing mucosal metobrane is deemed to be starged organ critical effect. This is acknowledged in the literature, with optimal pH ranges for substances administered orally via govage not to exceed pHO, with administration of substances outside of the recommended plananges resulting in tissue necrosis, partitioning etc. (Gad et al 18 Turner et al 19). This should not be dismissed.

Hyaline droplet hophropathy was observed in the kidneys of all treated. The lesion is due the accumulation of α2μ globully which is known to be a lesion specific to frats with no refevance to humans.

Overview of sub-chronic toxicity study in rats theated orally (win diet) with Table CA 5.8.1/06-8: spiroxamine N-oxide selected histograthology observations

| | , Ç | 🧗 (ppm) | 100 | 4 | , O | <u>.</u> | ♀ (ppm)(|) | |
|-------------------------------|-----------------------------|------------------------|-----------------|----------------------|----------------|--------------|--------------|---------|---------|
| Parameters | | PXN-oxide | \$\ @\ | SPX | 4 | SPX S | oxide | | SPX |
| | $\mathbf{Q}^{\mathbb{Q}}$ 2 | 25 | 625 | 625 | | 25 | 125 | 625 | 625 |
| Dose/animal | | .7 8.8 | \$°45,Q@ | ″ 48 <i>₫</i> √″ | Q_{μ}^{0} | 1.9 | 3 9.7 | 53.6 | 52.7 |
| (mg/kg bw/day) | | | | | O ^v | | y | | |
| Histopathology: [incident | lence / total n | @ examined [| minimal, | | | 0 % | , | | |
| Kidney - Hyaline dropletonep. | 1060 [10 | /10 40 //10 / | 10/10 | 910/1 % | | Ž | - | - | - |
| - Hyaline droplet | [41,7,2] [6,9 | 9,1]([0,7,3] | [0,10,0] | [0,10,0] | | W. | | | |
| | \sim 0 | | | ð | | Ð. | | | |
| Urinary bladder | r - ♥ | \$ _3-` | ~~ - | \$ - | O/10 | 0/10 | 0/10 | 0/10 | 2/10 |
| - Hyperplanild | | | | w w | ~-0 | - | - | - | [2,0,0] |
| trans. | | | S, | | 0 | | | | |
| Stomach | 0/10 0/ | 1.0, 0/10 | 3/1/0 | _{(,,,} 6/10 | 20 /10 | 0/10 | 0/10 | 1/10 | 5/10 |
| - Hyperkeratosis | _~ | | (2,1] | [0,2,4] | 7 | - | - | [0,1,0] | [0,2,3] |
| - Hyperkeratosis Oesophagus | 0410 | 10 % 0/10 × | 6/10 | 9/100 | 0/10 | 0/10 | 0/10 | 8/10 | 8/10 |
| - Hyperkeratosis | ~~~~~~. | <u>- 0 - 6"</u> | [3,25] | [1,5,3] | - | - | - | [8,0,0] | [0,5,3] |
| Pinna 🔊 🤇 | 0/10 1/ | 10% | 0 710 | <u>2</u> /10 | 2/10 | 2/10 | 0/10 | 0/10 | 5/10 |
| - Hyperkeratosis | <u>-0</u> [10 | 9,0] 🖇 | & - (| [2,0,0] | [2,0,0] | [1,1,0] | - | - | [3,2,0] |

* $p \le 0.05 p$ ** $p \le 0.01$

* $p \le 0.05$ %* $p \le 0.01$ Whyaline Groplet nep.: hyatine droplet nephropathy

Hyperpl. mild trans.: hyperplasia, mild transitional cell

4. Liver tissue @zyme analysis:

Investigations of the liver tissue showed no treatment related effects at \$\frac{1}{2}\$5 pp\(\text{p}\) and below. In $\[\cap \cap \]$ at 625 ppm and $\[\ \ \ \]$ at 125 and 625 ppm, aminopyrine-N-demethylase activity was increased, achieving statistical significance at the high dose group \mathcal{L} . Similar effects were also observed in animals dosed with spectromagnetic spectrum in both \circlearrowleft and \circlearrowleft 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

| | | | ♂ (ppm) |) | | | | ♀ (ppm) |) | |
|--------------------|-------------|-----------|-----------------|---------------------|-----------------|-------------------------------|--------------------|-----------------------------|-------------------|-----------------|
| Parameters | | SPX N | -oxide | | SPX | | SPX N | l-oxide | | SPX 4 |
| | 0 | 25 | 125 | 625 | 625 | 0 | 25 | 2 125 | 625 @ | 625 |
| Dose/animal | 0 | 1.7 | 8.8 | 45.0 | 48.7 | 0 | 1.9 | 9.7 | 53.6 | 5297 |
| (mg/kg bw/day) | | | | | | | 4 | | Ş | |
| N-DEM (mU/g) | 101.4 | 101.9 | 101.0 | 107.1 | 112.3 | 56.2 | 54.5 | 60.9 | × 63.7 × | Ø63.0≰ |
| | ± 19.21 | ±20.17 | ±13.64 | ± 12.88 | ±1 23 05 | ± 4.06 | ₽ 9.47 | ±10.24 | V±6.83*√ | ±7.2 |
| O-DEM (mU/g) | 10.0 | 9.7 | 10.4 | 10.1 | % .3 | 9.1 | 9.3 | 10.3 | 109 | 101 |
| | ±1.57 | ±1.94 | ±1.41 | ±1.38 | ⊈ ±1.10 | ±1.370 | *±1.14 | ±14.36 | ±0.87 | ₽ ₹.09 @ |
| Cyto-P450 (nmol/g) | 47.3 | 48.6 | 46.0 | 56.7 <u>.</u> 4 | 57.6 | 3807 | 37.6 | 39.4 | 43.7 (| J 42.7,© |
| | ±4.46 | ±4.68 | ±4.65 | ±4_100*** | ±4.30** | ±1.87 | #3.50 · | Q±2.95© | $\pm 4.10^{*}$ | ±4.26* |
| | Labora | atory his | torical co | ontrol da | ta (rat, \ | Mistar 🎉 |)94 - 1 <i>9</i> 9 | 5) | , W | |
| N-DEM (mU/g) | Age of | animals: | 12 | 2 5 wks | | Agerof | animals: | 12 | -23 wks | |
| | n: | | 7,5 | | | n:0° | 8 | 75 | | } |
| | Mean ± | SD: | J412' | 7.4.± 9 0°.1 | 2 | Mean ± | SD: « | > 68 | 86 +14 9 7 | ~~~ |
| | Range = | ±2SD: | 65. | 2 ∼ ,189.€ | | Range | SD: 0 | ×3 9. | .0 – 98.1 | S |
| O-DEM (mU/g) | Age of | animals: | 2 12 | _25 wks | | Age©f | animals: | 7 12 | -25 wks | |
| | n: | Ċ | 5 7 5) | * . Ç | | $n_{\widetilde{\mathcal{O}}}$ | | J 75 | Q' | |
| | Mean ± | | 10 .9 | | , | Mean € | |)" <i>9</i> 3 | 5±1.84 | |
| | Range = | ±2SD:∜ | | <i>-</i> ∅34.9 | <u>Ö</u> | Range | ±2SD© | \$25.6 | 5 - Î Z.9 | |
| Cyto-P450 (nmol/g) | Age of | an@nals: | 2 12 | ©25 wks | y | Agoof | animals: | | % 25 wks | 3 |
| | n: 😸 | | | ſ | ~ | $\eta^{\mathscr{Q}}$ | | 75 [©] | | |
| | Mean ± | | , 9 8. | 9 +5 /34 | ~ | Mean | v **/ | Ø & 4. | $.2 \pm 5.10$ | |
| | Range = | ±2SD. | Ø 28. | 2 ♥ 49.6, | (, ~ | Nange | ±2SÞÇ″ | . \$ ∕24. | 0 - 44.4 | |

^{*} *p* ≤0.05; ** *p* ≤0.01

N-DEM: aminopyrine-N-demethylase

O-DEM: p-nitroanisole demethylase

Cyto P450: cytochronic P450 content

G. Discussion:

No treatment related effects were seen on survival clinical signs food consumption, urinalysis or serum chemistry / haematology parameters. High dose effects were united 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 ody 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 bydy 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, a 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 cosed 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 inco ased 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. Clothing 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 esophagus 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 instance. In addition, hyperkeratosis of the ear was observed in spiroxamine treated animals. Mild transitional cell hyperplasia in the urinary bladder was observed in families dosed with spiroxamine. As with the parent compound, spiroxamine, spiroxamine N-oxide is a fertiary amine and like all tertiary amines has a high pKa, and a high pH with comparable historiathological 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 dispussed.

Hyaline droplet nephropathy was observed in the ladneys of all peated males. This resion is due the accumulation of $\alpha 2\mu$ -globulin which is known to be a lesion specific to male as with no relevance to humans, with Azan specific staining used to confirm $\alpha 2\mu$ -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 thyroic hormones (T3, T4, TSH) and clinical chamistry 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 the histopathological bridings. Consequently the lack of a rum thyroid hormone analysis is not deemed critical, with potential dryroid 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 25 ppm (equivalent to 8.8/9.7 mg/kg bw/day for males/females) based on Eduction in bedy weight and body weight gain, hyperkeratosis of the stomach and oesophagus. Increases in liver Pazyme induction were observed, without concurrent hepatic histopathology.

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

Spiroxamine-cyclohexanol (M13)



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

Executive Summary

In an acute oral toxicity study, broadly comparable to the OECD 400 test enideline, groups of rats (10/group) were administered the test article, spiroxamine cyclohexanol as 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 raits, 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 rate at dose levels of 2500 mg/kg bw and above.

Under the conditions of this study the acute oral LDG of sproxamine 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 (EQ 12722008 the test article has no obligatory labelling requirement for acute oral or dermal toxicity and α unclassified.

Materials and Methods

A. Materials:

1. Test Material Spicoxamine cyclohexanol

(alternative name) (4-ter butyles clohexanol, M13)

Description:
Lot/Batch No.:
Purity:
No.:
Stability of test
compound:

2. Vehicle and or positive of reported / not relevan

3. Test animals:

Species: Rat (oral) / rabbit (dermal)

Age dosing:

Weight at dosing:

Not reported



Diet:Not reportedWater:Not reportedHousing: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 Limited info and treatment: study, rats (1

Limited information was provided in the report. For the acute oral phase of the study, rats (10/sex/group) received a single oral gavage dos of spiroxamine cyclohexanol at dose levels of 2000, 2500 3200, 3000 and 5000 mg/kg two. For the acute definal phase of the study, rabbits (6/group) received a single dermal application of spiroxamine cyclohexanol at \$000 mg/kg bw. It was not reported if a non-occlusive semi-occlusive or occlusive dessing was applied

following pplication.

Rats Ammals were selected before administration based on their body weight

The observation period was A days post-exposure

3. Statistics: A % confidence limit was applied to the oral LD50, details of this calculation

re not reported

C. Methods:

1. Homogeneity and A Nor conducted achieved

concentration analysis of the dose:

2. Test article of formulation of reported

preparation:

3. Observations: From the binited data presented, animals were observed once daily for 14 days.

3. Body weights: Not reported
4. Food consumption: Not reported

5. Sacrifice and Alkanimal were killed at terminal sacrifice, but no gross necropsy was pathology: Alkanimal were killed at terminal sacrifice, but no gross necropsy was

Results

A. Homogeneity and achieved concentration analysis:

Not undertaken. Avalyses 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 tope.

B. Observations

1. Climical signs of A Zacute oral

xicity: 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:

C. Body weight and food consumption:

Table CA 5.8.1/07-1: cyclohexanol: mortality and body weight

| Mortality was observed at dose levels of 2500 mg/kg bw and above. | | | | | | | | | | | |
|---|--------------------------------------|-------------------------|---------------|-------------------|-------------|---------------------------------------|--------------------|----------|--|--|--|
| | | Acute de | rmal: | | | | | | | | |
| | | All anim | als surviv | ed until scl | neduled ter | rmination. | | | | | |
| C. Body weigh | t and food | consump | otion: | | | | | | | | |
| 1. Body weight: | ; | Not repor | rted | | | 4 | 10 | | | | |
| 2. Food consum | ption: | Not repor | rted | | | | 'n % | | | | |
| Table CA 5.8.1/0 | | verview of xanol: mo | | | | ats treated wi | | ning V | | | |
| Parameter | | Rat or | al (mg/kg | g bw) 🚔 | | ຶ່∀ Raabbi | t dermal (m | g/kg bw) | | | |
| 1 ai ailletei | 2000 | 2500 | 3200 | 4000 | 5000 ^ | y "©ʻ | 5000 | | | | |
| Mortality ^a | 0/10 | 1/10 | 2/10 | & 4/10 E | ° 8/10 | | <i>(0</i> ° (006°) | | | | |
| Acute LD ₅₀ | | 420 | 0 mg/kg t | (V) | | ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ | >5000 mg/kg | bw 4 | | | |
| | (95% cor | nfidence lin | nit: 3620 | – 487 0 mg | /kg/gw) | | | | | | |
| a Mortality b no individual anim | y: no. of animal nal data reporte | ls found dead ed | / no. of anim | nals treated | | | | | | | |

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 QECD 401 (1987) test guideline for the oral exposure and OECD 402 (D81) for the Germal exposure with the Timited 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 prortality occurred.
- Body weights were not recorded.
- Whilst grown size are detailed by is unclear if a single sex or growns were equally split between sexes. Furthermore, the strain(s) of animal used is not reported.
- Details@garding dermal approcation are unclear.

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

Assessment and conclusion by applicant

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

Conclusion: Under the conditions of this study the acute oral LD₅₀ of spiroxamine cyclohexanol was 4200 mg/kg bw. The acute dermal LD & An rabbits was >5000 mg/kg bw. Therefore, according to Annex I for Regulation (EC) 1272/2008 the test article has no obligatory labelling requirement mal toxicity for acute or or definal to ricity and is unclassified.



| Data Point: | KCA 5.8.1/08 |
|----------------------------|---|
| Report Author: | 10113.0.1100 |
| 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 | Regulation (EC) No. 440/2008, part B, B.13/14; OEQD 471; US-EPD OPP S |
| study: | 870.5100 |
| Deviations from current | Yes V V V |
| test guideline: | A number of deficiencies were noted (refer to results, Deficiencies section |
| | below). |
| Previous evaluation: | yes, evaluated and accepted RAR (2010) |
| | |
| GLP/Officially | Yes, conducted under GLP/Officially (Cogniced testing facilities |
| recognised testing | |
| facilities: | |
| Acceptability/Reliability: | Yes A A A |

Executive Summary

In a reverse gene mutation assay in Pacteria, S. typhimutium strains TA98, TA 1537 TA 100, TA 1535 and TA 102 were exposed to spirocamine cyclohexanol (M13) Tormutated 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 \$9 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 tomning of the background pacterial lawn and/or a marked reduction in severtants at \$100 µg/plate.

In two independent experiments using the plate incorporation and pre-incubation methodologies and in all strains in the obsence and presence of S9 no increases in reverant numbers were observed that were ≥2-fold (in strains TAP8, TAP00 and TA162) or ≥3-fold in strains TAP535 and TA1537) the concurrent vehicle control. This study was therefore considered to have provided no evidence of any spiroxamine cyclohexanol mutagenic activity in the 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 general attitude and point sufficient addressed with a new, up to date test guideline compliant GLP study (refer to CA 5.8.1/20 [M-75/2234Q-1])

It was concluded that spiroxamine cyclohexanol did not induce mutation in five histidine-requiring strains (TA98, TA100, TA1535 and TA7537) of Salmonella typhimurium when tested under the conditions of this study. These conditions included treatments at concentrations up to 5000 μ g/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.

Material and Methods

A. Materials

1. Test Material: Spiroxamine cyclohexanol

(alternative name: [(4-tert-butylcyclohexanol, M13)

Description: Solid, viscid, melt

Lot/Batch No.: 80322



98.6% (correction factor of 1.014 applied) **Purity:**

98-52-2 CAS No.:

Stability of test compound:

Confirmed stable for the duration of the study (expiry date: February 201)

2. Control materials:

Negative:

DMSO (dimethyl sulphoxide)/0.1 or 0.05 mL/plate incorporation Solvent/final concentration:

incubation, respectively)

Positive: -S9 Strain

> **TA98** TA100, TA1535 TA1537 aminoacriône (9) TA102 Acthyl methanes Uphonati

Strain Positive: +S9

TA1537

S9 was prepared in house from rats to ated (protent 3. Activation:

33, 1@ng/mLY.

The composition of the 89 reaction mix was: S9 5%), **M**gCl₂ (8 mM); KCl (33 mM); glucose-6-pleosphate (5.4 mM), N/QPP (3,89 mg/m/2), PBS (0.1 M).

S. tylhimurium strans: TAOS, TADO, TA1535, TAI537, TA102 4. Test organisms:

Affilest organisms were properly maintained and were execked for appropriate genetic marker & S. typhimurium. histidine and biotin fequirement, rfa

mutation, uvily sensitivity, applicilling resistance) regularly.

5. Test Concentrations

Prelimerary ∡lflate incorporation: +®\$9 TA€0: cytotoxicity test:

0, 0, 3, 96, 1.0, \$1.16, \$6, 0, 31.6, 100, 3, 16, 100, 0, 360, 5000 μg/plate

Place incorporation: +/-SQ all strains: Metation assay

Mutation assay

B. Test Performance

1. In life dates: (o 24 %) (experimental dates)

Plate incorporation assay 2. Preliminary

cytotoxicity test The following sequence of additions of 2 mL of supplemented molten top agar, test article solution/repicle control (0.1 mL), either 0.1 M Na phosphate buffer

(0.50mL pH 7.4) or \$9 mix (0.5 mL) for treatments in the absence and in the presence of metabolic activation, respectively and bacterial suspension (minimal were mixed and poured on to Vogel-Bonner E agar plates (minimal glucose agasolate). When set, plates were inverted and incubated at 37°C, projected 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 ≪øŏntrols

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), bacterial (0.1 mL), either 0.1 M Na phosphate buffer (0.5 mL pH 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 solvery. 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 triplies te plating was undertaken for each dose level

4. Statistics:

None applied

5. Acceptance criteria:5. Evaluation criteria:

Assay acceptance criteria was not provided

The test article was Considered mutagenic of this assay if O

- 1. A concentration related increase in revertant numbers was 2-fold TA98 TA100, TA102), 3-fold TA1575, TA1537) above the concurrent vehicle control values;
- 2. Any observed response was reproducible under the same treament conditions.

The test article was considered negative in the assay of none of the above critera were net.

C. Methods:

1. Homogeneity and achieved concentration analysis of the dose:

Not undertak@n

2. Test article formulation preparation

Correction for purify was made. A preliminary solubility test confirmed spiroxamine cyclobexanol was soluble in MSC at concentrations equivalent to 50 mg/mL. Thereby confirming a new image concentration of 50 mg/mL could be prepared and closed and to the test system at a maximum concentration of 5000 µg/plate. Test article stock colutions were prepared by formulating sproxamine cyclobexanol 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. Cytoroxicity was evidenced by a reduction in the number of spontaneous reverants, a clear diminution of the background lawn or by the degree of survival of the treated courses.

4. Scoring

Of is unclear how the number of revertant colonies were counted *i.e.* unaided eye of 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 result³.

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 μ g/plate. Following these treatments, evidence of toxicity ranging from a complete killing of the test bacteria at 1000 μ g/plate to a thinning of the background bacterial lawn and a marked reduction in revertants at 316 μ g/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 Frame-shift |
|------------------|--|
| Conc. | TAPOO Q Q X |
| (µg/plate) | -s9 +s9 \$ \$ |
| 0 | 130 ±3.5 |
| 0.316 | 135 ±1.4 |
| 1.0 | 137 ±4.9 |
| 3.16 | 138 ± 1.4 |
| 10.0 | 127 ±5.7 |
| 31.6 | 105 ±0.0 108 ±2.1 22 ±2.1 115 ±1.4 22 ±2.2 22 ±2.2 24 24 25 ±2.2 ±2.2 ±2.2 ±2.2 ±2.2 ±2.2 ±2.2 ± |
| 100 | 115 ±1.4 |
| 316 | 55 ±0.7 ^S |
| 1000 | 0 S.T. V V V V V V V V V V V V V V V V V V |
| 3160 | |
| 5000 | |

T: Toxic, no revertant colonies

B. thinging of background lawn

C. Mutation experiment &

In the plate incorporation assay, treatments of the rester Grains were performed in the absence and presence of S9 using final concentrations of spirosamine cyclohexanol at $1.0 \, \mathring{3}.16$, $10.0 \, 31.6$, $10.0 \, 31$

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

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

| Type of | | Form | e-shift | | | В | ase-pair s | ubstitutio | on | | | |
|-----------------|----------------|------------------|----------------|----------------------|--------------------|------------|------------|------------|-------|-------|--|--|
| mutation |) TE 4 | | TA1 | | s.**V | | | | | | | |
| Conc | | ~ T . | Y AA | 537 | TA100 | | TA | 1535 | TA102 | | | |
| (µg/plate) | -S9 🦿 | ** + S9 = | -89 | ₽ \$9 | '≫"-S9 | +S9 | -S9 | +89 | -S9 | +S9 | | |
| * 0 | 31.0 | 28 | 3 7.3 | \$2.0 \tilde{\infty} | [*] 173.6 | 125.0 | 18.7 | 16.0 | 274.3 | 261.3 | | |
| | ±1,0,4 | ±2.0 | €±2.3 © | | ±1.5 | ± 15.5 | ±0.6 | ±5.6 | ±9.1 | ±2.9 | | |
| 1.0 | \$2 .0 | 31.3 ±6. | 4.7 | 3.0 | 168.0 | 137.7 | 20.0 | 17.3 | 272.7 | 263.7 | | |
| | 40.0 | √"±6, € | ±0.6 | ₩ 1.7 | ±1.7 | ±7.4 | ±1.7 | ±6.1 | ±5.5 | ±11.7 | | |
| 3.16 | 25.2 | 2,9 <u>0</u> 0 | <i>≈</i> 7.7 ' | [₩] 7.3 | 168.7 | 137.0 | 18.3 | 17.3 | 247.0 | 267.0 | | |
| 3.10 | ± 2 | 2 ±5.2 € | ○±2.5 | ±2.3 | ±5.9 | ±4.4 | ±2.5 | ±3.2 | ±5.0 | ±3.6 | | |
| 186 | ₿ 2.7 . | 29.3 | 3.3 | 5.7 | 158.0 | 128.3 | 14.3 | 15.3 | 276.7 | 265.3 | | |
| | ©±3.1∂ | ±3.8 | ±0.6 | ±4.5 | ±8.5 | ±5.5 | ±3.5 | ±3.5 | ±30.9 | ±3.1 | | |
| \$1.6 | 25.3 | 27.7 | 3.7 | 4.3 | 123.3 | 133.3 | 13.3 | 14.7 | 260.0 | 293.7 | | |
| ⊳© _A | ±7.6 | ±9.9 | ±0.6 | ±4.9 | ±27.8 | ±5.1 | ±1.5 | ±4.2 | ±2.6 | ±19.9 | | |
| 100 | 24.7 | 20.7 | 7.0 | 4.0 | 110.0 | 119.3 | 19.7 | 13.0 | 264.0 | 267.3 | | |
| | ±1.5 | ±1.2 | ±1.0 | ±3.5 | ±6.9 | ±7.6 | ±4.9 | ±4.4 | ±3.6 | ±1.5 | | |



| Type of mutation | | Fram | e-shift | | | | | | | | |
|------------------|-------------------|-------------------|---|-------------------|----------------------|-------------------|-------------------|-----------|------------------------------------|----------------|-----|
| Conc. | TA | 98 | TA1 | 1537 | TA | TA100 | | TA1535 | | TA102 🔎 | |
| (μg/plate) | -S9 | +89 | -S9 | +89 | -S9 | +\$9 | -S9 | +\$9 | -S9 | + \$9 | T. |
| 316 | 16.0 | 16.0 | 1.0 | 1.3 | 83.7 | 90.3 | 6.7 | 7. | 240.0 | \$2.0 a | 1.0 |
| | ±1.0 ^S | ±1.0 ^S | ±0.0s | ±0.6 ^S | ±4.5 ^S | ±4.9 ^S | ±1.5 ^S | ±107s | ±1.0 ^S _{&} | DZ16.1₽ | |
| Positive | 239.0 | 336.7 | 200.3 | 142.3 | 1024.0 | 1030.3 | 212.7 | 297.0 | 1039,7 | 1047,3 | |
| control | ±3.6 | ±5.5 | ± 5.5 ± 67.3 ± 20.6 ± 18.7 ± 25.7 ± 10.8 ± 10.8 ± 3 | | | | | | | | |
| S: thinning o | f backgrou | nd lawn | | | | Ö | Ž | J. * | | | |
| Positive cont | rols: | | | | <i>*</i> | V. | | | | | |
| -S9: strains: | | | | | +\$0: | strains: | OA | × | | | &O' |
| TA98: 2-NF | | | | | AX 10 | 00, TA1537 | PA . | | e 😽 | | Ů |
| TA1537: 9-A | | | | | ₩A98 | 3; TA102; T | ÎX153703- | AA 👸 | ~~ × | | 1 |
| TA100; TA1 | | | | | Q) | | ~~ | * | | | |
| TA102: MM | S | | | <u>k</u> | , b° | | | | | | |
| D. Mutation | n experi | ment 2: | | (O | | | * & | | | A 4 | 0 |
| In the pre-in | ncubation | assay tro | eatments | of the tes | s te r strain | were po | erformed | in the ab | sence and | i presenc | ee |

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 carclohexanol at 1.0, 3.16, 40.0, 34.6, 190 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 thirming of the background backerial lawn and a marked reduction in revertants was observed at \$16 \mugget at 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 (TX98, TX100, TA102); ≥3fold (TA1535, TA1537) above the concurrent vehicle control.

Vehicle and positive control reatments were included for all strains or 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 \$ 1/08-3: Spiroxamine cyclohexanol: bacterial reverse gene mutation data (mean revertant colonies): Mutation experiment 2 (pre-incubation)

| Type of mutation | ~ | Fram | e-shift | | | B | ase-pair s | ubstitutio | n | |
|--|----------------------|----------------|----------------------|-------------------|-------------------|--------------------|-------------------|-------------------|-------------------|-------------------|
| Conc. | TA98 A TAY | | ≶ 37 √ ″ | , TA | 1000 | TA1 | 1535 | TA102 | | |
| (μg/plate) | <u>~</u> \$9 | SS9 É | ි් -S9උ [©] | +\$9 | | | -S9 | +S9 | -S9 | +S9 |
| 0 | | ∑30.0√ | 6.7% | ^5 /.3 | 31.0 | 132.3 | 14.7 | 25.3 | 290.0 | 281.0 |
| . 1 | ±9.7 | ±40 | ₩ 0.6 | <u></u> \$41.2 ₺ | ±1500 | ±7.6 | ±3.2 | ±3.5 | ±23.4 | ±10.4 |
| 1.0 | 34.3 | 23 .3 | ~ × 2 | √ 5. 3 ≫ | 114.3 | 131.0 | 24.0 | 15.0 | 273.7 | 270.3 |
| | ±3.8 | *±1.2_ " | 2 3.3 (v) +2.1 | ±17.5 | ∞ 12.1 | ±2.6 | ±1.0 | ± 3.0 | ±11.5 | ±3.2 |
| 3,46 | 33.3 🔏 | > 35. 0 | 570) | 6 .0 | ≫ 104.3 | 129.3 | 16.7 | 14.7 | 287.3 | 289.7 |
| | ±6.5 | ±1/0.4 | £2.6 | Q±1.00 | ±3.8 | ± 3.8 | ± 3.2 | ±0.6 | ±28.0 | ±9.1 |
| 10 | 2 9 ,0° | 30.3 | | 1 | 124.3 | 125.7 | 19.7 | 18.3 | 268.7 | 271.3 |
| | ₹ ¥3.0 ¿ | _3_£4.2≪ | ±2,3 | ±0.6 | ±5.9 | ±17.5 | ± 8.1 | ±5.5 | ±6.7 | ±4.7 |
| 31.6 | 28.0 | ∕″27 . | 1 27 | . <i>₩</i> . 5 ∩ | 107.7 | 111.3 | 18.0 | 23.7 | 270.0 | 274.3 |
| | ? ±1,0% | ₽ 0 | <u></u> \$2.1 ' | ©±2.0 | ±10.8 | ±8.0 | ±7.0 | ±5.1 | ±40.7 | ±3.1 |
| 100 | 2 , | 28.3 | O 6.3 | 4.7 | 117.7 | 127.0 | 17.7 | 19.3 | 289.0 | 264.3 |
| * *********************************** | \$\$3.5 _€ | ±3.8 | ±0.6 | ±0.6 | ±11.6 | ± 27.2 | ± 2.9 | ±7.6 | ± 4.0 | ±22.5 |
| 3\$6 | © 14.00 | 11.70 | 1.0 | 1.0 | 82.7 | 85.7 | 5.3 | 5.0 | 107.7 | 197.0 |
| 356 | $\pm 2.6^{S}$ | ±1:0s | ±0.0 ^S | ±0.0 ^S | ±5.0 ^S | ±10.7 ^S | ±0.6 ^S | ±1.0 ^S | ±5.8 ^S | ±3.5 ^S |
| Positive | 275.0 | 267.7 | 128.3 | 134.0 | 973.3 | 1017.3 | 196.0 | 132.0 | 975.0 | 132.0 |
| control | ±8.9 | ±2.5 | ±2.1 | ±10.0 | ±12.1 | ±7.8 | ±5.3 | ±7.9 | ±24.5 | ±7.9 |

S: thinning of background lawn



| Type of mutation | | Fram | e-shift | | Base-pair substitution | | | | | | | |
|------------------|-------------|------|---------|-----|------------------------|-----|-----|------|-----|-------|--|--|
| Conc. | TA98 TA1537 | | | | TA | 100 | TA | 1535 | TA | 102 🔎 | | |
| (µg/plate) | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 | -S9 | +\$9 | | |

Positive controls:

-S9: strains:

TA98: 2-NF TA1537: 9-AAC TA100; TA1535: NaN₃

TA102: MMS

+S9: strains:

TA100, TA1537: CPA

TA98; TA102; TA1537: 2-A

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 ≥ 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 spiror amine 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 gove mutation data (mean revertant colonies) historical control ranges

| | | | 04 .a. × | | | D _A | * | ¥ 0 | - /- | | | |
|---|--------------|------------------------|---|---------------------------------|-------------------|---------------------|--|--------------------|------------|------------|--|--|
| Type of | | Fram | eshift 🤿 | | W. | B | ase-pair s | ubstitatio | n 🖔 | | | |
| mutation | | K | i la " | | | | | | | | | |
| Parameter | TA | 98 🔊 | ⊕TA1 | 537 | Ž TĀ | 100 ° | ~©TA1 | 5 35 | 7 TA102 | | | |
| | -S9 | 198 ¥ \$ ∮ | ∢ -S9 | 🗣 +S9🖫 | '- S \$ | ÆS9 | ₹ S9 € | + S9 | -S9 | +S9 | | |
| | | &1 A | \$\ \bar{\bar{\bar{\bar{\bar{\bar{\bar{ | Veh@le | control (2 | QQ) | | ~ (N) | | | | |
| n (studies) 77 \$\frac{1}{27} \frac{1}{27} \frac{1}{27} \frac{1}{27} \frac{1}{27} \frac{1}{27} \frac{1}{27} \frac{1}{27} \frac{1}{27} \frac{1}{27} \frac{1}{27} \frac{1}{27} \frac{1}{27} \frac{1}{27} \frac{1}{27} \frac{1}{27} \frac{1}{27} \frac{1}{27} \frac{1}{27} | | | | | | | | | | | | |
| Mean | 32.5 ±6.9 | 34(1 | , Ø | ,°\$7.8 ' | 146 | 148/1 | .18.8 | _{».} 19.5 | 277.9 | 278.1 | | |
| | ±6.9 | √±Ø.4 | <u>*</u> ŽŽ.0 * | 5 ±1,97 | ±19,4 | .≠23.7 | ∂,\±4.2√ | ±4.6 | ± 15.7 | ± 17.5 | | |
| Min | . 80 | № 17 | 1 🖔 | De y | √202 _∧ | 101 | 7 10 [©] | 10 | 233 | 213 | | |
| Max | 5 4 & | × 60,0 | 13 | ¥3 | چ 220 چ | 236 | 3 6 | 48 | 31.9 | 328 | | |
| Ò | " D | , | | Positive. | control 2 | 011). | | | | | | |
| n (studies) | 77 | <i>2</i> ,₹7 ≈ | 决 77 🏖 | 7 77 [©] | 27 | ~~17 ^ | 77 | 77 | 77 | 77 | | |
| n (studies) | 371.3 🗞 | Ø364.1 O | ž 237.7° | 237.8 | . 9 09.3 § | &906 ₂ Q | 262.6 | 259.6 | 1062.0 | 1068.2 | | |
| , | ±150,6 | ±147.2 | ± % .0 | 237\8 ∰88.9 _{&} | Ĵ±79.� | ±744 | ±93.6 | ±86.0 | ±66.6 | ±74.4 | | |
| Min | 186 | 132 | ©62 _√ | 🔊 77, O | 70 © | % 695 | 102 | 93 | 874 | 854 | | |
| Max | 784 | 890 | § 577 _, © | 843 | JH31 | A 165 | 648 | 599 | 1586 | 1563 | | |

Positive control

-S9: strains: \(\sigma\)
TA98: 2-NF

TA1537: 96AAC

TA100; A1535: NaN₃

TA102; MMC

+\$\(\frac{1}{2}\) strains: T\(\frac{1}{2}\)98: B[a]P

🕍 A1537; TA100; TA1535: TA102: 2-AA

Upper / lower reference range

F. Déficiencies:

When the study methodology is compared to current test guideline requirements (OECD 471, 1997) the following descriptors are moted;

- 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 specificary 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 to guideline has only included a correction to a CAS number of an example positive control —So for E. College Strain WP2uvrA.

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 @LP 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 cyclobexand did not induce mutation in five histidine-requiring strains (TA98, TA100, TA1535 and TA1537) of Salmorella typhimum when tested under the conditions of this study. These conditions included treatments at concentrations up to 5000 μ g/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: | KCÃ5.8.1422 |
|---------------------------|--|
| Report Author | |
| Report Year: | 2020 🛕 💢 👸 |
| Report Title: | Spiroxamin@yclohexanol; Bacterial reverse mutation assay |
| Report 0: | 8406981 |
| Document No: | <u>M-7552-3-02-10</u> |
| Guideline(s) followed in | OECD 471 (1997) 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 |
| study: | |
| Deviations from current | None O O O |
| test guideline. | |
| Previous aluation: | No, not previously sufficient of |
| | |
| GLP/Officially | Yes, conducted under GD/Officially recognised testing facilities |
| 1 ' 👽 | S S Q S |
| facilities: | |
| Acceptability/R@nability; | Y |

Executive Summary

In a reverse gene mutation assay in bacteria, *S. typhimurium* strains TA98, TA1537, TA100, TA1535 and TA 102 were exposed to piroxamine 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 ractiver 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 column) 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 concentrations 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 16θ0 μg/plate).

Following spiroxamine cyclohexanol treatments of all the test strains in the absence and in the presence of S9, no increases in revertable numbers were observed that were ≥ 1.5 -fold (TA102); ≥ 2 -fold (TA98, TA100); ≥ 3 -fold (TA5535, TA1550) above the concurrent vehicle control in other 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 colonie numbers, thereby demonstrating the sensitivity and specificity of the test system.

It was concluded that spiroxomine cyclohexanol did not induce mutation in five histidine-requiring strains (TA98, TA160, TA1535, KA1537 and TX102) 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 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 Oclohevanol

(alternative name: 4-tert-butylcyclohexanol, M13)

Description: White powder Lot/Batch No.: AE 1247868 PU-01

Purity, 98.2% (no correction factor applied)

CASNo.: 5 4 2552-2

Stability of test of Confirmed stable for the duration of the study (expiry date: 20 January 2021)

eompo@nd:

2. Control materials:

Negative:

Solvent/final DMSO (dimethyl sulphoxide)/0.1 or 0.05 mL/plate (plate incorporation or pre-

concentration: incubation, respectively)

Conc.



| Positive: -S9 | Strain | Mutagen | Conc. (µg/plate) 。 |
|------------------------------------|--|--|----------------------------------|
| | TA98 | 2-nitrofluorene (2-NF) | $(\mu g/piute)$ 5 |
| | TA100, TA1535 | Sodium azide (NaN ₃) | |
| | TA1537 | 9-aminoacridine (9-AAC) | 50 |
| | TA102 | Mitomycin C (MMC) | 02 \$ 8 |
| | | • • | 0.2 |
| Positive: +S9 | Strain | Mutagen | Confe. |
| | TA98 | Benzo Ppyrene (B[a]P) | Core. (ug/plate/) 5 |
| | TA100, TA1535, | 2-aminoanthracene (2-AA) | T. 9 4 |
| | TA1537 | z-animoantinacene (z-AA) | |
| | TA102 | | 20 ° C C |
| 3 Activations | | Dominion of Carrows Dowl | |
| 3. Activation: | treated with Aroclor 25 | commercial source. Sprague Dawl | 29, protein |
| | content 3.7 mg/mI OTh | e composition of the 10% \$9 reaction r | 29, pixotem ♥ niv was: 100 uI |
| | S9 Na PBS (100% IM) g | Aprose-Cophosphate (5, μM), β-NADP (| ΦuM) MoCh |
| | (8 μM), KCl (35 μM), w | | (Paris) Care Care |
| | | TA98, TA100, TA1935, TA7537, 7A1 | 02 🛴 |
| 4. Test organisms: | | properly Waintained and were checked | |
| | genetic byarkers 95. tvph | | |
| | mutation, uvr sensition | ty, appicilling esistance) regularly | |
| 5. Test Concentrations: | | | 4 |
| | | | 0 |
| a) Mutation assay 1: | Plate incorporation: +f | imurium: histidine and biotin requirem ty, ampicilling esistance) regularly (89 all mains: 600,5000 µg/plate (10, 1600 µg/plate) |) |
| °>> | , 0, 5, 16, 50, 000, 500 C10 | 500,0000 µg/plate | |
| b) Mutation assay 2: | Plate incorporation \$59: 001, 15, 40, 100, 250, 64 | | |
| | Pre-incubation +S9: | to, roug μg/prate ον | |
| | 0, 7, 1/5, 40, 100, 250, 64 | 10°1800 ug@late | |
| | 0, 1, 13, 40, 100, 230, 0 | A A A A A A A A A A A A A A A A A A A | |
| B. Test Performance | | | |
| 1. In life dages: | 25 April 2012 to 8 N | 2019 (experimental dates) | |
| 1. In life dates: 2. Experiment 1: | Place incorporation asso | | |
| | The following sequence | of additions of mL of supplemented | molten top agar |
| ~ ~ · | Stest article solution/version | cle control (0.1 mL) or positive control | |
| | (0.0 CmL), either 0.1 M | Na phosphate buffer (0.5 mL pH 7.4) of | or S9 mix |
| a. Q* | (Q. James) Just the difference of | in the absonce and in the presence of m | netabolic |
| | activation, respectively | and bacterial suspension (0.1 mL) were | mixed and |
| <u> </u> | | iner Kagar plates (minimal glucose aga | |
| | | and incubated at 37°C, protected from | light for 3 days. |
| 3. Experiment 2: | Plate incorporation asso | | (C1) |
| | | strains treated in the absence of S9 (re | ier above). |
| | Pre-incubation assay | or vehicle/positive control solution (0.0 | 05 mI) haataria |
| L, 4 \ | | 5 mL) were mixed in a small test tube | |
| | | After additional of 2 mL of top agar so | |
| | | o a minimal glucose agar plate and allo | |
| | | ed for 3 days at 37°C. In this way, it wa | |

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.

assay system.

Altoplates were incubated for 3 days at 37°C. In this way, it was hoped to the rease the range of mutagenic chemicals that could be detected using this



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

4. Statistics: None applied

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 overtant 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:

5. Acceptance criteria:

The test article was considered not agenic in this assay if:

- 1. A concentration related increase in revertant numbers was 1.5-10d (TA102); ≥2-fold (TA9%, TA100); ≥3-fold (TA1535, TA1537) above to 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 mone of the above criteria were met.

C. Methods:

1. Homogeneity and achieved concentration analysis of the dose:

2. Test article formulation preparation:

Not undertaken.

No correction for purity was made, nor was it considered necessary. A preliminary solubility test confirmed piroxamine yelohexanol was soluble in DMSO at 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 spift amine 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 acticle solutions were protected from light and used within approximately. In of initial formulation.

The background lawns of the plates were examined for signs of toxicity.

Revertant plate out data were also assessed, as a marked reduction in reversants compared to the concurrent vehicle controls were also considered as evidence of axicity.

The number of revertant Colonics were counted with the unaided eye or a Colony Counter (Ames Scoret 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/postave) 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 result³.

3. Toxicity Assessment

4. Scoring:

Results and Discussion

A. Analytical determinations:

Not indertaken. Onalyses 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 bactoria to a slight minning 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 \$9 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 aused sufficient variation in the test article concentration to cause these differing toxic offects.

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 \$\times \times 5-fold (TA192); \(\times 2 \times fold (TA192); \(\times 2 \times fold (TA192); \(\times 2 \times 6 TA100); ≥3-fold (TA1535, TA1537) above the concurrent vehicles control

Spiroxamine cyclonexanol: bacterial recerse gene mutation data (mean revertant Table CA 5.8.1/22-1: colonies): Mutation & perintent 1 (plate incorporation)

| | | | 4 |) /.\/ | × V | ~~~ | | | @\\\ | • | | |
|------------|---------------|---|-----------------------|---------------------|-------------------------------|----------------------|--------------------|----------------|-------------------------|--------------------|--|--|
| Type of | | Fram | e-shift 🔎 | | Base-pair substitution TA102 | | | | | | | |
| mutation | | | Ď. ^y | | | | | | | | | |
| Conc. | TA | TA98 | | TA1537 | | | | 1 | | 102 | | |
| (µg/plate) | -S9 | +S9 a | ⊘-S9 🦠 | ≥ + S9 | - \$9 | √+S9 | % -S9 _∞ | +S9° | 8 9 | +89 | | |
| 0 | 36.0 | 54.3 | J 1703 | 28,0 26.1 | A08.0 | , 115.3 [©] | % -S9 8≉Q, | 26 0.0 | 293.7 | 354.7 | | |
| | ±8.7 | ±7.00 | ±ØĨ | № 6.1 | © ±6.6 | ±6.4 | 3 7.0 | Ž¥Ì3.6√ | ₽ ±3.8 | ±21.1 | | |
| 5 | 45.3 | 47.7 | <u>_</u> 15.7 | 23.0 | 107.9 | 1 9 7.7 | ¥2.7 ≰ |) 17. 3 | 302.7 | 332.3 | | |
| | ±5.5 | ≈ 1.2 | \$¥5.7 <i>,</i> \$ | ±5.® | 1 .0 | √¥16.0 [©] | √ ±2Ø | ±13.3 | ±6.4 | ± 25.4 | | |
| 16 | 40.0 | \$57.0 °C | 13.3 | 25 .3 | \$02.0 ₺ | ₽ 121.U | 90 | 49 .0 | 291.7 | 368.3 | | |
| | ±6.2 | ±2.0 | _± 3)1 | °¥4.0 | ±6.00 | ±1, % 6 | ±1.2 | $_{7}$ ± 12.0 | ±14.5 | ± 15.0 | | |
| 50 | 43.0 | 49.0 | £9.3 | \sim 28.Q \sim | 10 <i>5</i> /7 | . 1 √8.7 | ® 8.0 % | 14.3 | 296.7 | 317.7 | | |
| | ±6.2 | <u></u> 2.0 € | ±4.0 | ±5709 ²⁷ | ≠ Ĵ6.6 ⁄ | ±16.3 | ±2.0 | ±5.5 | ±13.4 | ±6.0 | | |
| 160 | Q1.7 | \$ 52.7 ₆ | 11.0 | Ž¥.7 。 | \$16.0 _C | ±16.3 | 1 % 7 | 16.0 | 297.7 | 323.7 | | |
| | b ±9.1 € | ±2.5 | <i>£</i> 3 0.0 | △±4.2 % | ≯ ±3.00° | ± 3.5 | ≱ 6.0 | ±1.0 | ± 15.0 | ±6.0 | | |
| 500 | 35.3 | 4) .0 | 3 5.3 | 23.0 °C | 9,6√7 | 33.7 | √ 11.0 | 11.0 | 215.0 | 267.0 | | |
| | =0.1 | <u></u> \$5.6 ₹ |) ±1.5 ° ° | ±1,7% | ° 8.€ | ¥12.7 SC | ±2.0 | ±5.6 | $\pm 21.7^{\mathrm{S}}$ | ±11.4 ^S | | |
| 1600 | T «C | T | | , O, | J T 📞 | 70 Å | T | T | T | T | | |
| | T,PQ | A | | | , O. | ±4.9 ^{S,T} | | | | | | |
| 5000 | T, F Q | ÓT P | T,P O | T.P | Z.P | ₹,P | T,P | T,P | T,P | T,P | | |
| Positive | 1485.0 | \$\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\ | 546 | 32697 | . 1968 3 | ©3205 7 | 817.3 | 192.0 | 1190.0 | 2571.0 | | |
| control | ±66.5 | ±4,70 | ±389.5 | 4 1.6 € | ±49.50 | ±139.5 | ±16.0 | ± 14.5 | ±79.1 | ± 243.2 | | |

P: precipitate on plate

T: Toxic of revertant colonges

Positive controls:

-S9; strains: TA98: 2-NF

TA1537: 9-AAC

TA100; TA1535

TA102: MMCC

Sslight thinning of background lawn

+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 it 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 of 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 we ated 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 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 \$\sum_5^5\$-fold (TA\f\tau_2); \sum_6^5\text{fold} (FA98) 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 wehicle control treatments. The positive controls induced an acceptable increase in revertant colony numbers. Ithereby demonstrating the sensitivity and specificity of the test system.

Spiroxamine cyconexamol: hacterial reverse gene mutation data incan Table CA 5.8.1/22-2: revertant colonies Mutation experiment 2 m

| | 1 | | | | | | | | | Q | |
|--------------------------|----------------|---|------------------------------------|-------------------------------|------------------------------|-----------------------------|----------------|---------------------|--------------------|--------------------|--|
| Type of | | Fram | e-shift) | | | B | ase pair s | ubstitutic | | | |
| mutation | | | _ <i></i> , ``````` | | | | \$ 2 | | • | | |
| Conc. | TA | .98 - | TAN | 537 | ÕŤА | | TA1 | 535 🛇 | TA | 102 | |
| (µg/plate) | -S9 | +S9 [≪] | ຶ່-S Q ຼ | +59 | | ⇒ +S9√ | -89 | <i>₿</i> \$9 | -S9 | +89 | |
| 0 | 31.3 | 46.3 | 5©" | Ĵĭ.3 _∠ | © 99.3 ° | 140,0 | \$.7 ±2.5 & | \$15.3 \(\) | [©] 266.3 | 338.3 | |
| | ±2.5 | ±117.9 | _3±0.6 | ±1.5 | ±871 | \$ 7.6 | ¥2.5 ≰ | √ ±1: 2 ♡ | ±10.0 | ±14.4 | |
| 7 | 24.3 | ≈ 50.3 € | (1.0 °) | 9. © | 103.0 | × 27.3 | √ 8.0 √ 8.0 | 13.0 | 284.0 | 357.3 | |
| | ±3.5 | \$\frac{1}{2}\pm 3.2 | ±3.6 | ₩ .0 | ₽ 10.4 × | ñ18.1 | ± <i>L</i> .xo | ♣ ¶.0 | ±7.0 | ±34.0 | |
| 15 | 35.7 | 45 4 | ~ () | 79 4.7 | 90,7 | 12,00 | 12.0 | _{7/1} 13.3 | 284.3 | 325.3 | |
| | ±4.00 | \$ 5.3 | <u>₹</u> 1.2 | ±4,0 > | ±2,4×1 | ₹14.2 | ©±4.4≈© | ±4.5 | ±12.7 | ±19.4 | |
| 40 | 30.0 23.6 | ⊘ 47.0 | 5.3 | 21⁄69 ⁷ ±₹.0 、 | . 10 8.7 € | 128.0 | 11.3 | 13.3 | 292.3 | 351.0 | |
| | ©3.6 g | \$\pm\delta \delta \delt | ±3.5 | ±₹.0 ¸ | ≇ 12.3 | ±148 | ± 2 /9 | ±2.5 | ±10.8 | ±11.5 | |
| 100 0 | 🤰 34.0 | 36.0 | J I 2 2 2 2 3 | 4 11.0% | ₹ 97. 3 © | 121.0 | 3.0 | 12.0 | 269.3 | 324.7 | |
| | ±5.0 | ₽ 7.9 4 | € ±1.0 € | 9"±3.0 | ±2>1 | ~@ * >3. <i>5</i> ′′ | →±3.6 | ±3.6 | ±19.7 | ±39.6 | |
| 250 | 32.3 | | 🔊 6.0 🦠 | 12,7 8.4s | _∞ 9 6.3 | ₩85.3 ±7.88 | 8.7 | 10.3 | 245.7 | 240.3 | |
| | ±6.1 | 18.7 | ± 1 ,0 | 、 € 8.4 ^S γ | ¥21.24 | ±7.88″ | ±4.5 | ±1.2 ^s | ±21.0 | ±45.5 ^S | |
| 640 | 20.7 | T | 6 3.3 | J T | ° 86.₽ | T | 7.3 | T | 164.7 | T | |
| | ±6.1 | | \$\disp\delta \text{2.5} \text{\@} | b 6. ~ | ±43.4 | .0 | ±3.8 | | ±8.4 | | |
| 1600 | Ø ^T | O TO | | | O T | "О" Т | T | T | T | T | |
| Positive | 7151.7 | 361 | 819 | £309.3 € | 5 ⁷ 992. © | 2801.0 | 745.3 | 211.3 | 847.3 | 2105.7 | |
| control | ±119.3 | , O | 2 23.3 | ₹¥28.5© | ±6.2 | ± 455.7 | ±37.1 | ± 37.6 | ±3.2 | ±102.1 | |
| S: slight thin | | kgrøund læ | Qn (C) | | °∕yT: To | xic, no rev | ertant color | nies | | | |
| T: Toxic, no | revertant e | olonies 🔏 | | | 🔊 - dos | e level not t | treated | | | | |
| Positive cont | rols: 🔏 | | ~~ | Ø. « | | | | | | | |
| -S9: strains (| plate incorp | orat@ň): | Ö' | | +S9: | strains (pre | e-incubation | 1): | | | |
| TA98: 2-NF | .∧ď®` | , \ <i>(i</i> | | , O.Y | TA98 | S: B[a]P |). TA1525. | TA 102. 2 | | | |
| TA100: TA1 | 1600 | | | | | | | | | | |
| TA100, TA1 | Po. Nains | | & n 6 | | | | | | | | |
| 771102. WIN | | Õ | Ĭ | A | | | | | | | |
| C. Diseussi | on: | A ~ |) | | | | | | | | |
| In the second size of se | | \$.J | سدم مسطنہ | . all atmai | | a h aan aa | | | not livron | matabal: | |

S: slight thinning of background lawn

In two independent experiments and in all strains in the absence and presence of a rat liver metabolic activation system (\$9) no increases in revertant numbers were observed that were ≥1.5-fold (in strain TA102) \bigcirc 2-fold (in strains TA98 and TA100) or \ge 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.

T: Toxic, no revertant colonies



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 ð

| | | | | | | | | ~~~ | | |
|------------------|-------------|-------------|----------|--------------|------------------------|----------------------|---------------------|--------------|----------------------|------------------|
| Type of mutation | Frame-shift | | | | Base-pair substitution | | | | | |
| Parameter | TA98 | | TA1537 | | TA100 | | TAJ535 | | ° TA102 | |
| | -S9 | +89 | -S9 | +\$9 | -S9 | 🕅+S9 | -S9 🗸 | +89 | ≈ \$9 ∧ | +S9 |
| | | • | | Vehi | cle contro | Ý | Q, | (| 7) \$ | |
| Date range | Jan 19 – | - Mar 19 | Oct 17 - | - Feb 18 | Oct \$\sqrt{7} - | - Feb 18 | Jun 18 - | - Sep 18 | Oct 0 7 - | - F eb 18 |
| n (studies) | 77 | 75 | 76 | 76 | 103 | 100 . | Q* 78 <u> </u> | 7 . 6 | ر _ي 72 ° | ن 72 © |
| n (plates) | 300 | 291 | 299 | 292 | <i>_</i> @r17 | 370 | 287 | 2Qi, | ©266 ∂ | 274 ₀ |
| Mean | 23.1 | 36.3 | 10.0 | 13.9 | 101.8 。 | 10807 | ,f9/.6 | @18.8 € | [№] 290.26° | 31,57 |
| 99% L.R.R | 10 | 20 | 1 | 5 🖔 | 5 6 | √72 [™] | £ 7 | 9 5 Q | 220 | 193 |
| 99% | 46 | 64 | 22 | 29 | 168 | ©168 _ | ð 35 💍 | 370 | 403 | 411 |
| U.R.R | | | | | | | .4 | | | #411 <i>(</i> |
| | | | | ∠ Posît | ive contro | | | | | |
| Date range | Jan 19 – | - Mar 19 | Oct 17 | ŬFeb &§≫ | Oct 77 - | - FeB 18 . | , 9un 18≪ | Sep 🔊 | O. 17 - | - F© b 18 |
| n (studies) | 77 | 75 | 75,6% | 76 | °. ₹02 | \$ 98 _{. ©} | 78 | | ₽12 _{&} | _a 71 |
| n (plates) | 285 | 275 | 29* | 2976 | 372 ~ | 351 | 287 | Ž78 . | Š~264 <i>∜</i> | 255 |
| Mean | 1170.8 | 350.8 | 303.3 | ©286.3 © | 6500 | 1504.3 | √668.1 _≫ | ©190.2° | 936.9 | 1559.8 |
| 99% L.R.R | 328 | 203 😹 | 🌷 84 % | 41 '° | 431 | £455 | Q"234 | 3 | 45 4 | 368 |
| 99% | 3312 | 711 ≪ | 885 | 5 \$0 | 1470 | 2884 | 92 | 6 14 | 2148 | 3566 |
| U.R.R | | L_ <i>`</i> | | | | , , | | | þ | |

Positive controls:

TA98: 2-NF
TA1537: 9-AAC
TA100; TA1535: Na
TA102: MMC

D. Deficiencia

TAN 37; TA 100; TA 935: TA 702: 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 spiroxanine exclohexanol did not induce mutation in five histidine-requiring strains (TA98, FA109, TA1595, TA1537 and TA102) of Salmonella typhilmurium when tested under the conditions of this study. These conditions included treatments at concentrations up to \$\infty6000 \text{ fg/plate}\$ (a toxic concentration), in the absence and in the presence of a rat liver metabolic activation setem (\$9) using both the plate incorporation and pre--yogie F



| Data Point: | KCA 5.8.1/09 |
|----------------------------|--|
| Report Author: | o |
| Report Year: | 2012 |
| Report Title: | Mutagenicity study of 4-tert-butylcyclohexanol (PTBCOL) in the mouse |
| Donart No. | lymphoma forward mutation assay -in vitro - |
| Report No: | 2012-0126-DGM |
| Document No: | <u>M-471125-01-1</u> |
| Guideline(s) followed in | Regulation 2000/32/EC, Method B.17; OECD 476; FS-EPA 712-CO6-22 |
| study: | OPPTS 870.5300 |
| Deviations from current | Yes v v v v |
| test guideline: | A number of deficiencies were noted (refer to Results, Deficiencies section |
| | below). |
| Previous evaluation: | yes, evaluated and accepted of the second of |
| | yes, evaluated and accepted RAR (2010) |
| GLP/Officially | Yes, conducted under GLP/Officially recognised testing facilities |
| recognised testing | |
| facilities: | |
| Acceptability/Reliability: | Yes O Y A A S |

Executive Summary

Spiroxamine cylohexanol was assayed for the ability to induce mutation of the tk locus (5-trifluorothymidine [TFT] resistance) in mouse lymphoma 1778 yells using a ductuation protocol. The study consisted of a pretiminary cytoloxicity 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 grace 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 (39) 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 (126×10^6). 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 10^6) was observed.

Vehicle and positive control treatments were included in the Mutation assays in the absence and presence of S9. MF in vehicle control cultives 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 spitoxamine cyclonexanor did not show any increases in the mutant frequency of L5178Y cells at the procuse employing the fluctuation protocol. These conditions included treatments up to 250 µg/mL (a concentration deemed overly 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. Materals:

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:

Positive: -S9

Positive: +S9

Vehicle / final concentration:

DMSO (dimethyl sulphoxide) / 1% (v/v)

Methyl methanesulphonate (MMS, 3/24 h: 10, 15 μg/m²) 3-methlcholanthrene (3MC, 3 h: 2.5, 4.0 μg/mL)

roclor 1254 (protein content 3. Activation: S9 was prepared in house from rest treated with

33.1 mg/mL).

The composition of the S9 reaction mix was

6-phosphate (138 mM), NADP (6.7 mM), Q

L5178Y $tk^{+/-}$ mouse lymphoma cells were stored as frozen stocks in liquid 4. Test cells:

nitrogen. Each batch was purged of TK-mutants, checked for spontaneous mutant frequency and that I was procoplasma free Culturos were used within 8. days of recovery from frozen stock. For each experiment the ceOs were conducted. in R10p and incubated in a humidified atmosphere of 6% (v/v) CO2 in air.

For growth maintenance of the cell line: 5. Culture medium:

> R10p: RPMI 1640 medium supplemented with theat inactivated biorse serum (10% Q) for growing and 0.05% Plyronic Fox, 2 mA/L-glyromine 300 mM

Na pyruvate 100 μφ/nL gentamycin, 2.5 μg/mL Fingizon@

During treatment

R5p: RPMI 1640 med@m supplemented with theat in a fivate Chorse serum (5%

0.05% Pharonic 58, 2 fpM L-glutamine,

RĬ0p (Ath TFI)/3 μg/aði

6. Locus examined (thymidine kinase) lo

fluorothymi@ine)

7. Test article Concentrations:

Preliminary

Mutation assays

B. Test Performance:

1. In life dates: august 2012 (experimental dates)

Spiroxamine cyclohexanol was soluble at 500 mg/mL in deionised water gave a 2. Vehicle selection:

final concentration of \$000 μg/mL when dosed at 1% v/v (a concentration deepned to be the maximum recommended concentration in accordance with the in vitragenotoricity 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). Smolality and pH assessments of the test article in cell culture medium were

Yundertaken for the preliminary cytotoxicity study.

None performed.

4. Acceptance criteria: For test article: 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 \sim 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.

<u>For vehicle controls</u>: The mean vehicle control value for mutant frequency we 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 30 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 invitants per 106 mutants. At least 40% of his was due to the number of small payant colonies

Mean RTGs for the positive ontrols were exeater 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 (GFF) devised by Moore and colleagues has been

The global evaluation factor (GEF) devised by Moore and colleagues has been used to evaluate the data redospectively. For a testinaterial 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 viable cells) and was accompanied by a positive linear trends

C. Methods:

1. Homogeneity and achieved concentration analysis of the dose:

Not commucted

2. Cell treatment:

Preliminary enotoxically assay: Cells were exposed to test atticle formulations, or vehicle controls for either 3 hours in the absence of S907 3 hours in presence the of S9. At the end of treatment cultures were washed, cell cultures, were adjusted to 8 cells in Land for each concentration 0.2 mL was plated into 32 microtites 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 to foxicity.

Mutation assays: Cells (12.75) 10° cells, diluted to 5x 10° cells/mL) were expressed to the article formulations solven for 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 cittures 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. Other this period the number of wells without growth of cells was counted.

growth of tells was counted.

Mutation frequency was determined by plating ~2000 cells/well in cell culture medium containing 3 mg/ TFF/mL. Plates were incubated for 11-14 days. After this period the number of well without growth was counted to provide CE in TFF. Wells with growth in indicated evidence of TFT-resistance mutants. Colony string 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 glosed to test article formulations, solvent or positive controls for either 4 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 to preliminary cytotoxicity test

| Conc. | 3 h -S9 | ₹ | 3 h +S9 | |
|----------------|---------|------------|----------------------------------|---------------------------------------|
| (µg/mL) | CE (%) | RS (%) | CE (%) ₽O♥ | RS (%) 5 |
| O ^a | 0.6674 | 100 (C) | # 332 ° | |
| 25 | 0.6674 | 100 | Ø.395 3 | 91 |
| 100 | 0.7270 | 109♥ | 0.4332 | FOR S |
| 250 | 0.0615 | Quest Q | √° 0,0831 √° | ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ |
| 1000 | 0.0615 | SPP V | Ø.0198 | O & 5ppt A |
| 2500 | 0.0000 | 1 Oppt O C | ⁷ \Q0.0000 \(\infty\) | O. Obba, A |
| 5000 | 0.0198 | 2001 N | 0.0 19 8 °° | ∠ 5ppt √ |

CE – cloning efficiency RS: relative survival

C. Mutation assay:

1. Experiment 1:

3 hour -S9: Cultures were sposed to spiro amine Cyclohes anol at concentrations from 15.63 – 250 µg/mL. Wo precipitate (assessed by eye at the end of the atment was precional concentrations were assessed for Odetermination of MF KTG values from 136-3% were obtained relative to the vehicle control. There were no increases in the MF of any of the test concentrations assessed that excended the sum of the mean concurrent vehicle control MF and the GEF (126 × 10°). It is observed that the highest concern ation plated for MF assessment was overthous vici (i.e. RTG < 10%). 3 hour +\$9: Cultures were exposed to spic xamine cyclohexanol at concentrations from 15 63 - 250 µg/mL. No precipitate (assessed by eye at the and of treatment) was observed. All concentrations were assessed for determination of Mi RTG values from 75 5% were obtained relative to the vehicle control. There were no intereases in the mean MF of any of the test concentrations assessed that exceeded the sum of the mean concurrent vehicle

control MF and the OEF. It is observed that the highest concentration plated for MF sessment was Evertly toxic (2). RTG <10%).

3 bour +SP. Cultures were exposed to spiroxamine cyclohexanol at Foncentrations from 15.63 – 250 μ g/mL. No precipitate (assessed by eye at the end of freatment) was observed. All concentrations were assessed for determination of ME. RTG values from 80 - 5% were obtained relative to the verscle 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 SEF. It is observed that the highest concentration plated for MF assessment was overtly toxic (i.e. RTG <10%).

24 hour \$9: Cultures were exposed to spiroxamine cyclohexanol at Soncentrations from $15.63 - 250 \,\mu\text{g/mL}$. No precipitate (assessed by eye at the end & treatment) was observed. All concentrations were assessed for determination of MF. RTG values from 116 - 1% were obtained relative to the whicle 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%).

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

2. Experimen

3. Positive controls:



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+/- 3.7.20 experiment 1

| | | | 3 h -S9 | | | | | 3 +S9 | 4 | <u> </u> |
|------------------|------------|------|-----------|------------------------|-----------------------|---------------------------------|-----------------------|----------------|---------------------------------|--------------------|
| Conc. (μg/mL) | RTG (%) | SG | CE (%) | MF (x10 ⁶) | SC MF | RTG (%) | SG | CE (%) | ME (x 106) | ŠĆ ŠMF « |
| | , | | , , | , , | (%) | Ġ` | A. | , | *\'\ | (%)\$ [®] |
| O ^a | 100 | 23.5 | 70.15 | 99.42 | 45.0 | § 100 | 2,5,7 | 98.83 | ⁽² 69.0 ((2) | 54.1 |
| 15.63 | 98 | 21.6 | 74.81 | 74.49 | 51.25 | 67 | _@0.1 | 71.67 | 80,83 | \$2.4 |
| 31.3 | 136 | 26.3 | 85.36 | 79.96 | 51.0 | 72 , | o [™] 18.4 。 | 84,10 | 60.27 | &48.6 _€ |
| 62.5 | 89 | 17.7 | 82.86 | 77.00 | 52 .2 | 75_ | 20 | 8Q,46 | 86.68 | 48 |
| 125 | 74 | 18.8 | 64.87 | 91.56 | [∞] 53.5 ° | 73 ₆₀ " | 22.4 | 69.65 | 62.44 | 62,5 |
| 250 | 5 | 5.8 | 12.98 | 60.57 | 2 50 ,0 0 | J. P. | €6.3 | | 67.97 | 25.0 |
| MMS 10 | 23 | 15.2 | 24.37 | 1016.19 | 7 50 © 68,7 | | 18.2 | 25. ® 4 | 902.92 | 45.4 ° |
| MMS 15 | 18 | 14.6 | 20.64 | 1360,45 | ° 7.3 × | \mathbb{Z}_{2} 3 \mathbb{Q} | 22.3 | 22 .03 | 956.98 | 5400 |

RTG: Relative Total Growth

SG: suspension growth CE: Cloning Efficiency

MF: Mutant Frequency (mutants per 10⁶ yiable

SC MF: small colony mutant frequency.

S9: Wethylanethanesilphonate

Spiroxamine cyclohexanol motant frequence Table CA 5.8.1/09-03: */- 3.7.2C cells: experiment/2

| | | | 3 h ¥S9 | Ď L | Ž L | | 4) ¼ | 24 h -\$9 | | |
|----------------|------------------------------|-----------------------|--------------------|---------------------|----------------|----------------|-----------------|------------|-----------|-------------|
| Conc. | RTG | SG ⁶ | CE O | ME | "SO" | ® TG ⟨⟨ | SG [≫] | CAL | MF | SC |
| (μg/mL) | (%) | | \$ (%) | (x10 ⁶) | MF | ⊘ (%)⊘' | | <u>(%)</u> | $(x10^6)$ | MF |
| | 6 | | | | ≈(x10°) | ľ | 0 | ~ | | $(x10^{6})$ |
| O ^a | 100 | 26.8 | 74.28 | ×82.92 | 51.9 | 150 0 | √ 39.0 √ | 88.62 | 77.02 | 51.0 |
| 15.63 | 5 <i>5</i> 0 | 16.5 | 85.79 ₄ | 83.63/ | 40.0 | ≈93 | 9 48. 4 | 66.73 | 95.61 | 47.8 |
| 31.3 | 733 | ₩3.6 C |) 77.0 0 ° | 72,34 | 4 1.5 |) 76 🔊 | 34.1 | 77.00 | 86.71 | 54.2 |
| 62.5 | ₂ 59 ⁴ | 13.6 | 86,64 | 461.83°≽ | 53.8 | 113 | 4 9.3 | 79.27 | 65.76 | 50.0 |
| 125 % | § 80 | 238.A | 68 .67 (| ₹67.47© | ♠ · | 2 46 ~ | © 51.9 | 77.00 | 65.82 | 54.1 |
| 250 | 5 | 4 .1 | 24.37 | 87.39 | <i>3</i> ₹.5 ∘ | J 1. O | 1.0 | 28.34 | 75.15 | 50.0 |
| Positive | 11 ^b | ^{ال} م 9.8 ك | 23:42 | 12\$8.17 | °∕52.6€ | 16 | 32.4 | 17.55 | 1830.35 | 72.5 |
| control | 11° | 8.8 | 25×34 | 1367.69 | 43.85 | 22ª | 27.4 | 28.34 | 1376.55 | 79.3 |

RTG: Relative Total Growth

SG: suspension growth CE: Cloning Efficiency

MF: Mutant Frequency (mutants per 10 viable @

SC MF: small colony mutant frequency

dimethyl sulphoxide (DMSO)

Positive control:

3-methylcholanthrene: 2.5^b, 4.0^c µg/mL) Methyl methanesulphonate: 10^d, 15^e µg/mL

Spiroxamine cyclobexanol mutant frequency data from L5178Y tk+/- 3.7.2C cells Taboratory historical control of utant frequency data 2009 - 2011

| Parameter | | icle control (n # | | Positive control $(n = 14)$ | | | | | |
|---|-----------------|-------------------|-----------------|-----------------------------|-----------------|-----------------|--|--|--|
| rarameter | 3 A _S9 | 3/h/+S9 | 24 n –89 | 3 h –S9 | 3 h +S9 | 24 h –S9 | | | |
| Mutant frequency (mutants per 10 ⁶ viable cells) | | | | | | | | | |
| Mean ±\$d | \$3.0 ±25.1 | \$7.0 ±26.7 | 96.6 ± 24.4 | 2646.7 | 2441.6 | 3245.1 | | | |
| | | ٥ | | ±1890.8 | ± 2068.3 | ± 2560.0 | | | |
| Range O | 43.4 | 55.1 – 145.6 | 61.6 - 157.6 | 315 - 8987.4 | 351.6 - | 799.4 – | | | |
| | 0' '\ | | | | 8437.8 | 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 EF (126×10^6). This study was therefore considered to have provided no evidence of any spirox mine cyclohexanol mutagenic activity in this assay system. However it is recognised that in all preatment 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 4/6 (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 nutation assay (12.75) 106 cells) compared to the test guideline requirements (20 x 100 cells culture), however the spontaneous mutant frequency rate observed in the vehicle control along with small colony accords was acceptable.
- At the time of study conduct the Moore evaluation enterial 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 preets the current guidance and the requirements in 283/2013.

Conclusion: It is concluded that spiroxamin reveloperate and did not show any increases in the mutant frequency of L5178Y cells and the historia comploying 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 vivo mouse lymphoma genotoxicity regulatory test guideline) 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: | |
| Report Year: | 1997 |
| Report Title: | In vitro chromosomal abberation assay with P-tert. Butylcyclohexanol |
| Report No: | 97-0366-DGM |
| Document No: | <u>M-471187-01-1</u> |
| Guideline(s) followed in | OECD 473; Guideline 97/548/EWG, Part B, B.10; EEC Guidence Note, Annex |
| study: | V, Method B10 |
| Deviations from current | Yes A A A A |
| test guideline: | Yes A number of deficiencies were round (refer to Results, Deficiencies section |
| | below). |
| Previous evaluation: | yes, evaluated and accepted of the second se |
| | RAR (2005) |
| GLP/Officially | Yes, conducted under GOP/Officially recognise desting facilities |
| recognised testing | |
| facilities: | |
| Acceptability/Reliability: | Supportive only |

Executive Summary

In a mammalian cell chromosome aberration assay cultured Chinese namster V79 Jung 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 (59).

The test article was formulated in ethanol, which was assumed to be dosed at 3% v/v.

Experiment 2 was in part a direct repeat of experiment 1, with oultures exposure to spiroxamine cyclohexanol for 3 hours, followed by a 15 hour receivery 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 each with structural aberrations in any analysed treatment concentration. However, a sufficient evel of cytotoxicity and action in %MI 40-50%) was not achieved.

In experiment 3, cultities were exposed to single test article concentration of spiroxamine cyclohexanol in the absence and presence of \$9, with a recovery period of 25 hours post the end of exposure. In the absence of \$9, a concentration of 60 mg/mL resulted in a reduction in MU of 11%. Whilst no increase in the incidence of calls with structural aberrations was observed, a sufficient level of cytotoxicity (reduction in MI 40-50%) was not achieved in the presence of \$9, 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, MAC (-S9) and CPA (+S9) induced a statistically significant increase in chromosomal aberration (excluding gaps) in all experiments. However, these increases were observed in the presence overt cytotoxicity (1.2. ≥50%). Therefore the ability for the assay to assess direct acting clastogens was not attricipally demonstrated, with overt cytotoxicity a contributing factor.

It is concluded that spiroxamine cyclonexanol did not induce biologically relevant increases in the incidence of of fromosome aperrations 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 5 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 156.2 g/mol)

CAS No.: 98-52-2

Stability of test compound:

Confirmed stable for the duration of the study (expire date: >1 yea@tated)

2. Control materials:

Vehicle / final concentration:

Ethanol / 1% (v/v)

Positive: -S9
Positive: +S9

Mitomycin C (MMC, 30): 0.03, 0.04 µcmL) Cyclophosphamide (CPA, 3 b) 3, 4 µcmL)

3. Activation:

S9 was purchased from a commercial source. Sprague Dawley rats were treated with Arcelor 1254 (supplied by 1254) lot no.: 85675, 85679, protein content not reported). The composition of the \$15% S9 reaction mix was: 1 Sparts 59, glucose-6-phosphate 5 m/W NADP (4 m/M), Mg(0) (10 mM), C1 (30 mM), Sa2HPO NAHAD (4 50 mM).

4. Test cells:

V79 ccus derived from the lung of Chinese hamster were obtained from Dr. Engthardt, BASF, Ludwigstaffen and stored as frozen stock 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) CO₂ in air. A modal chromosome pumber of 22 and a doubling time of 16.5 h. Mycoplasma status was not reported.

5. Culture medium:

MDEM4: Fagle's minimal essential medium (MEM) supplied with L-glutamine (2 mM) peniculin (100 units mL), steeptomycin (100 µg/mL), heat-inactivated fetal calf serum (4%).

MEM0: as MEM0; but without fetal call serum. Used as a negative control for \$1.50 treatment.

6. Test article Concentrations:

c) Chromosoma aberration assay

Experiment 1:

- 3 h Ø5 h revovery Ø59: <u>0</u>, 2.5, 5<u>30</u>, 20, 40, <u>60, 100</u>, 180, 300, 500 μg/mL
- 3, 5 (+15 k secovery) +S9 (2), 10, 28, 50, 75, 150, 250, 500, 750, 1200,

⊉000 μg√ymL

Experiment 2

3 h 15 h σ covery -S9; 6 10, 60, 100 μg/mL 3 h +15 h recovery +S9; 0, 20, 100, 200 μg/mL

Experiment 3

- 3 h (+25 h recovery) +\$9: 0, 60 μg/mL 3 h (+25 h recovery) +\$9: 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



3. Statistics:

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 maximup final concentration tested in the preliminary toxicity test was 2000 μ/mL.

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

For vehicle controls: 4. Acceptance criteria:

For positive controls:

At least 100 cells for each positive control were available for analysis.

There was acceptable homogeneity between replicate cultures as demonstrated to binomial dispersion for centrols in aberration rate was obtained that every's historical control range increase in aberration rate was obtained that even replicate cultures as demonstrated to binomial dispersion for central range in aberration rate was obtained that even replicate cultures as demonstrated to be reproducible attice resulted in the resulted of the resulted in the resulte The criteria for determining a positive result a relevant and statistically significant increase in abstraction retained to the significant in abstraction retained to the significant in abstraction retained to the significant significant increase in aberration rate was obtained that exceeded the laboratory's historical control range Increases in chromosostal aberrant frequency and to be reproducible

A negative resulted in order the above criteria.

Not conducted.

5. Evaluation criteria:

C. Methods:

1. Homogeneity and achieved concentration analysis% of the dose:

2. Chromosomal aberration assay 🎉 experiment 1

Following establishment of cultures (24 Diours) 4 x 10 cells were seeded, where mix was required this was added as appropriate. Cells were exposed to the test article for 3 hours in the disence or presence of S9. These cultures west sampled at 15 hours ofter the end of treatment (i.e. 18 hours after the start of treatmont). 🐒

At the end of treatment (3 hours) it was assumed that the test article was removed by washing the monolaye culture, cells removed by trypsinisation. Contoxicaty was determined by MM (a total of 1000 cells assessed for nætaphase).

Duplicate cultures were used for the vehicle, positive controls and each test article concentration. Three test article treated concentrations for each treatment condition were maly soft.

For experiment 2, a repeat of the above procedure was undertaken, with a three test apricle treated concentrations analysed in the presence of S9.

A further the atment was undertaken in the absence of S9 (5 x 10⁴ cells) with an extended recovery period of 25 hours (i.e. 28 hours after the start of treatment). A single test atticle treated concentration was analysed.

For experiment 3, a hour treatment in the presence of S9 was undertaken, with an extended secovery period of 25 hours employed, as detailed above. A Single test article treated concentration was analysed.

2. Chromosomal aberration assay, experiment 2:

3. Chromosomal aberration assay, experimen#3:

4. Harvesting, fixation and slide preparation Harvesting and

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:

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

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

Cytotoxicity (%) was expressed as 100 Relatio MI

A minimum of 1000 cells/culture were examined for swotoxicity.

Concentrations were selected for analysis of phromosome aberrations with 5. Slide scoring:

modal chromosome number of 22 Voxicity was not deemed a linguing factor. Current test guideline requirements consider toxicity a limiting factor, with a reduction in mitotic index of 45±5%. Stides were coded prior to scoring.

Definitions of chromosomal soerrations were taken from 180 No

Chromatid type:

cte = Ckromatid exchange

Octb = Chromatia break an achromatic region in one chromatid, larger than the width of a chromatic. The associated fragment may be partially or completely displaced)

Chromand gap can achromatic funstained) region in one chromatid, the size of which equal to or smaller than the width of a chromatid. These are noted but not usually included in fund totals of aborations as they may not all be true breaks)

(Chromosome-type:

locus in both sister chromatids. If the gap is large or chromosome fragment displaced, the break is included with chromosome breaks)

Acento Fragment (Two parallel chromatids with no evident 'centromere. The fraggeent can be of any size greater than the width of a chromatid)

Chromosome exchange: (Scentric dicentric with fragment), ring (a chromosome which forms a circle containing a centromere. This is often associated with an acentic fragment in which case it is classed as ring fragment)

csb Chromosome break Chromosome has a clear break, forming an abnormal (deteted) chromosome with an acentric fragment that is dislocated but apparently

Results and Discussion®

A. Analytical determinations:

Not undertaken. Analyse 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.

Thromosome 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 μ g/mL, with % reductions in MI of 27%, 63% and 52%, respectively. No increase in the incidence of cells with structural aberrations sufficient level of cytotoxicity (reduction in %MI 40,50%) was not achieved. was observed in any analysed treatment concentrations. However, a

cytotoxicity that exceeded current test guideline reduction in MI 40/50% 1+13 h recovery +S9: - The positive control, MMC induced a statistically significant increase in

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 congentrations of 10 to 200 @ig/mL caused couctions 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 achneved. W
- The positive Control, CPA induced a statistically significant increase in chromosomal abertations (excluding gaps), however this was at a level of Stotoxicity that exceeded current test equideline reduction in MII 40-50%).

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

| 3 | h exposure 413 h | covery) –S | 59 0 0 | | h exposur | e (+ 13 A) | recovery) –S | 9 |
|------------------|--------------------------|-------------------|----------------------------|-------------|-------------------------|-----------------------|------------------------|---------|
| Dose | Relativ Cyto- | Aberra | Num | Dose | Relativ | | Aberra | Num. |
| level | e MI 🛇 toxicity | on cells | | ≪Qevel ∂ | e Mî | toxicity | nt cells | aberran |
| (μg/mL | (%) | exel. | f cells / | μg/m1 | | (%) | excl. | t cells |
|) | | gaŷš | (%) × | <i>*</i> | | | gaps | (%) |
| | - 0° × | (%) | | - G | 0 4 | | (%) | , |
| 0 ^a § | © 100 🛫 | & *0.5 | yO* | 0° _@ | A - 0 | - | 1.0 | b |
| 10 | 73 <u>©</u> 27 | 2.0 | b | \$ 50 | 3 | 7 | 3.5 | b |
| 60 | 37 63 | <u>4,5</u> | \$b | 250 | № 111 | - | 3.0 | b |
| 100 | 48 9 52 4 46 6 44 | \$1.8 % | | 300 | 93 | 7 | 3.0 | b |
| MMC | 46 0 44 | 25.4 | | ČPA 3 | 47 | 53 | 27.5* | b |
| 0.04 | | <u> </u> | Lö ^y Ö | A 03 | _ | | | |
| | | Labora | tory histor | ical contro | | | | 80.4 |
| Vehi | cle control 3 h + C5 h r | ecovery 4 | 89 (n 5 19) | ֻּעֶּׁ Ve | | | | |
| | | | 000 | * | 18) | | | |
| | Aberrant colls excl. | - W | %MI | Ab | errant cell | | % N | 11 |
| Year | gaps (%) | 0" | | | gaps (%) | | | |
| | Dangerrang | e not repor | steg ⊘, | | | | ot reported | |
| Range: | Ø.00 − 7.00 | | و وي | | 0.00 - 9.0 | | - (9' | , |
| Mean: | 70.77 | | 8.38 The CO (*** | - 0) D | 1.25 ±1.2 | | 6.82 | |
| Positive | control (MMC) 39i +1 | z n recove | sry –39 (n = | - o) Po | ositive cont | roi (CPA) +S9 (n : |) 3 h +15 h r = 18) | ecovery |
| | Aberrant cells exc | | % MI | Λh | errant cell | | - 10) % N | 11 |
| | gaps (%) | | / U 1711 | Au | gaps (% | | /0 IV | 11 |
| Year | | e not repor | ted | | Date range not reported | | | |
| Range: | 7.50 – 21.50 | 100 10001 | - | | 12.50 - 38 | | - | |
| Means O | 14.06 ± 4.27 | | 5.30 | | 25.41 ± 6.6 | | 2.94 | 4 |

* p≤0.05

a ethanol



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/m 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 aberration (excluding gaps), however this was at a level of cytotoxicity that exceeded current test guideline reduction in MMI 40-50%

3 h + 13 h recovery + S

- Following treatment, no precipitate (observed by eye at the end of treatment) was observed at any concentration tested. Exposite to prioxamine cyclohexanol at concentrations of 20,000 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 fevel of cytotoxicity (reduction in %MQ40-50%) was not achieved.
- The positive contest, CPA induced a start stically ignificant 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 abertation data in V79 cells: experiment 2

| 3 | W. A. | V(+ 13 h re | | | CAPOSUTE (FIG. IT TECOVERY) S | | | | |
|--------|-----------------|--------------------|-------------------|--|-------------------------------|------------------|-----------------|----------|---------|
| Dose | Relativ | Ç yl o- | Xberra | Num. | Dose | Relativ | Eyto- | Aberra | Num. |
| level | e Ma (%) | toxicity | nt cells | aberran | ∀ level | €MI § | toxicity | nt cells | aberran |
| (μg/mL | (%) | \$ (%) | excl. | '≯t cells⊘ | (µganL | (%) _@ | (%) | excl. | t cells |
|) | & |), . | _ ©g aps ∡ | (%) | (M) | | | gaps | (%) |
| \$2 | y | 2. | 0.5 | | ~ | | | (%) | |
| 0a | 100 | , Ø - , Ò | | ~>b, C | | ° Q00 | - | 1.5 | b |
| 10 | 81 | 19 | @ W.J | O | £ 20 | 🕉 🌂 113 | ı | 2.0 | b |
| 60 | 79 🔊 | 21 " | 1.0 | *40° | Ol'00 👟 | 134 | ı | 0.5 | b |
| 100 | 61 [©] | (3)9 (5) | S 0.0 | <u>~</u> b | > 200 | 131 | ı | 0.0 | b |
| MMC | 3 | _°71 €~ | 19 ^C 3 | \\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\ | ° CPÆ3́ | 39 | 61 | 31.5 | b |
| 0.03 | 2 | | | | ð | | | | |
| MMC | 4 0 | 60 | \$29.5₽ | , <u>.</u> | , Ø - | - | - | - | - |
| 0.04 |) | | | | ~ \$ | | | | |

* p≤0.05[®]

a ethanol

b data not reported

3. Chromosomal aberration assay experiment 3

\$\hat{h} + 25\hat{h} \text{ recovery} − S9:

- Following reatment, 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 as %ME00-50%.

Table CA 5.8.1/10-3: Spiroxamine cyclohexanol overview of chromosomal abertation data in √79 cells: experiment 3

| | | | | . v | | <u> </u> | <i>~</i> | | |
|----------------|---|---------------------------------------|---------------------|----------------------|-------------|----------------|--------------|--------------------|------|
| 3 | h exposur | e (+ 25 h re | covery) –S | @ " | | 1 (%) | (C) | recovery)×S9 | |
| Dose | Relativ | Cyto- | Aberra | Num. | J Dose | Relativ | Cyto- | Aberra Anum | le ° |
| level | e MI | toxicity | nt cells | aberran C | lever | e⁄MI ₄ | toxicity | nt cells aber | an |
| (μg/mL | (%) | (%) | excl. | , t cells, | (ng/mL | (%) <i>(</i> | | excl. t cell | İs |
| () | | , , | gaps_@ | (%)) | /®) 💉 | ¥ `_^^ | | gaps %) |) |
| , | | | (%) [©] | & Z 4 | | | | | |
| O ^a | 100 | - | 25 | 0°b ^> | <i>™</i> | 100 6 | | \$0.0 ×b | |
| 60 | 89 | 11 | % .0 0 |) b | 2 00 | 56° | <u>_</u> | © 3.8 →b | |
| MMC | 25 | 75 | © 39.Q [©] | _08 | ©€PA 3© | 80 | ©20 ? | 1 kgb | |
| 0.03 | | ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ | , 'Y | | | | | | |
| | Laboratory historical control ranges & | | | | | | | | |
| Vehi | Vehicle control 3 h +25 h recovery 489 (n = 10) Vehicle control 3 h +25 h recovery +89 (n = | | | | | | | | |
| | | | - A | | | ' &, . | . % | | |
| | Aberran | t cells excl. | | % MI S | / Al | oeri@nt cell | s/excl. | % MI | |
| | gap | % (%) | 23 4 | | | agaps (% |) '*\ | | |
| Year | Š | Date rang | è pot repor | ted 🦴 | | ∑ .≸Da | te range no | ot reported | |
| Range: | Q. 00 | - ₹ 0 0 ′ | 4 6 | | Ç" [~ | 0.00 – 3.0 | HO 2. | = | |
| Mean: | 2 .10 | £¥.97 _ O | 0" | Ø.13 | | 6±0.9 | 7 | 6.99 | |
| Positive | control (M | -7 00 +7.97 O MC) 3 17 +2 | 25 h recove | ery – S9 (ň = | = 8) P | ositive cont | trol (CPA) | 3 h +25 h recovery | 7 |
| | `^ .\ | % <i>I</i> | | <i>'</i> | W. | | +S9 (n | = 9) | |
| | 🌂 Aberran | t cells excl | y' 🖓 | | S XI | perrant cell | | % MI | |
| * % | gap | 50/01 27 | % <i> </i> | | <i>Q</i> | gaps (% | | | |
| Year | S | Date rang | e not repor | ed 31 0 | | | | ot reported | |
| Range: | 19 % 0 | - 43.50 0 5.91 | | | " I 🔊 | 713.00 - 62 | | - | |
| Mean: | @32.00 |) ± 7%91 ≥ | | <u>#3</u> 1 6 | D. D. | 28.33 ± 16 | .82 | 5.63 | |
| * n<0.05 | ~O | | . "> | | | | | | |

n n c 👼 .

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

data not reported

- 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 inclear 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.* 1.5x 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 proximine 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 over 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 over cytotoxicity a contributing factor
- 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 inderstood the applicability of the data to aid in the test article clastogenic poential 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 germed supplementary. Both the chastogenic and aneugenic endpoints have been adequately addressed with Frobust, GLP up to date in vitro human peripheral blood lymphocyte micronucleus study (CA 5.8.1/23 M-755227-021).

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 oclohesanol did not induce biologically relevant increases in the incidence of chromosome aborations 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: | |
| Report Year: | 2020 |
| Report Title: | Spiroxamine cyclohexanol: In vitro human lymphocyte micronucleus assay |
| Report No: | 8406984 |
| Document No: | <u>M-755227-02-1</u> |
| Guideline(s) followed in | OECD 487 (2016) |
| study: | |
| Deviations from current | None & S |
| test guideline: | |
| Previous evaluation: | No, not previously submitted Ves. conducted under GLP/@ffcially recognized testing for this submitted |
| GLP/Officially | 1 i es, conducted under OLI / Minerally recognised testing radiaties " > 0 |
| recognised testing | |
| facilities: | |
| Acceptability/Reliability: | Yes & & & Z Z Z Z Z Z Z Z Z Z Z Z Z Z Z Z |

Executive Summary

Spiroxamine cyclohexanol was tested in an in vitro micronucleus assay using duplicate homan lymphocyte cultures prepared from the profed blood of two male domors 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 Afoclor 254 induced rats. The test article was formulated in anhydrous analytical grade dimethyl suphoxide (DMSO) and the highest concentration tested in the micronucleus experiment, 230 µg/mL timited 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 % v/v

Following establishment of cultures, concernation ranging from 120 to 320 pg/mL in the absence (3 hours + 21 hour recovery) 100 to 280 µg/mL in the presence of S9 (3 h + 2 h 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 spirox mine cyclohexanol on the replication index (RI). Nicronuclei 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 have gone through at least one cell division (as measured by binuclears + 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 spiroximine cyclobexanot 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 continues for all concentrations analysed.

Treatment of cells with spiror amine cyclonexanol for 24 hours (+24 hour recovery) in the absence of S9 resulted in frequencies of MNBN cells that were significantly ($p \le 0.001$) 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 format range (0.00 – 0.90%), with statistically significant linear trend test, indicating evidence of wealthnear 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 ($p \le 0.05$) 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 ($p \le 0.05$) 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 elated 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 $100 \,\mu\text{g/mL}$ (inducing 53% cytotoxicity) in Experiment was not reproduced in Experiment 2 at $105 \,\mu\text{g/mL}$ (inducing 51% cytotoxicity). According to the convent 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 (89). 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: Spiraxamine cyclobexanol@ Atternative name: (4-terP **Description:** Lot/Batch No stable for the duration of the Stabilit of test study (expiry date: 20 January 2021) compound: 2. Control material **Negative:** Solvent/final concentration: ③ h: 0 24 h: 0.20 μg/mL) [clastogenic control] Vin Plastin (VIN, 24 h: 0.64 μg/mL) [aneugenic control] včlopkospham(de (CPA, 3 h: 7 μg/mL) 3. Activation: was purchased from a commercial source. Sprague Dawley rats were treated with Aroclo 1254 (supplied by lot no.: 4030, protein content 3 mg/mQ). The composition of the 10% S9 reaction mix was: 100 μL S9, Na PBS (1900 μM), glucose-6-phosphate (5 μM), β-NADP (4 μM), MgCl₂ (8 μΜ), KClQ33 μM), water (to volume). Homan peripheral blood lymphocytes were collected from 2 healthy, non-

phytohaemagglutinin (PHA).

Smoking adult donors aged between 23 and 30 years, pooled and diluted with

HML media. Cells were stimulated to divide by the addition of

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 1, 15.75, 26, 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: <u>0</u>, <u>100</u>, <u>120</u>, 140, 160, <u>180</u>, 190, 200, <u>210</u>, 220, <u>248</u>, 260, 280, 300, 320 µg/mL

3 h (+21 h recovery) +S9: 0, 100, 120, 140, 166, 180, 190, 200, 210, 230, 230, 240, 250, 260, 280 µg/mL

24 h (+24 h) –S9: <u>0</u>, 10, 20, 40, 60, 70, 75; <u>80</u>, 85, 90, <u>95, 400</u>, 110, 125, 150, 175 200 μg/mL

Confirmatory 24 h (+24 h) -89:0, 20, 40, 60, 80, 90, 95, 100 005, 110, 115, 120, 130, 140, 150 µg/mb (concentrations underlined sensed for inferonucleus frequency)

B. Test Performance:

1. In life dates:

2. Vehicle selection:

3 June 2010 to 20 September 2012 (experimental dates)

A preliminary solubility test confirmed spiroxed in exception was soluble in DMSD up to at least 102.34 mg/mg (not corrected for purity).

The solubility limit for culture medium was in the range of \$25.85 to 51.77 μg/mλ, as indicated by precipitation at the higher concentration which persisted for at least 24 hours after test article addition. A maximum concentration of 1562 μg/mL was selected to the extrovociety 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 fr/m). Concentrations for the micronucleus experiment were selected based on the results of the cytotoxicity range-finder experiment.

Test article stock colutions were prepared by formulating spiroxamine cyclohexanol under subdued lighting DMSO with the aid of vortex mixing and warming at 37% 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.

solutions were protected from light and used within approximately 4 h of initial formulation.

The froportion of MNBN cells for each treatment condition were compared with the proportion in vehicle controls by using Fisher's exact test. A Cochran-

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:

3. Statistics:

The following acceptance witeria had to be met for assay acceptability:

The binomial dispersion test demonstrated acceptable heterogeneity (in terms of MOBN cell frequency) between replicate cultures, particularly where no positive responses were seen;

- The fequency 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 arone 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

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:

2. Preliminary cytotoxicity assay:

Not undertaken.

Whole blood cultures were established by placing 0.4 pt. of pooled heparitised blood into 8.5 mt pre-warmed HPPES-buffered RPMI medium containing 10% (v/% heat inactivated fetal calf serum and 52% pencillin/streptomycin, so that the final following addition of S9 mix/KCL and the test article in its chosen vehicle was 10 mL. The mitogen, phytohaemags lutining 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±4°C for approximately 48 hours and rocked continuously. S9 mix or KCl (1 ml culture) was added appropriately cultures were freated with the test article or vehicle (0.1 mL culture). Positive control and single cultures were used for each test article treated concentration.

For removal of the less article, cells were polleted (approximately 300 g, 10 minutes, washed twice with steril caline and resuspended in fresh pre-warmed medium containing fetal calf serum and penicillin/streptomycin. Cyto-B formulated in PMSO was added to post wash-off culture medium to give a final concentration of 6 µg/ml/culture to inhibit cytokinesis, resulting in binarcleate cells (without affecting karyokinesis), thereby arresting cells in interphase.

Changes in camolality of more than 50 mOsm/kg and fluctuations in pH of more than the 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.

Cytoroxicity 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. Micronûcleus assay Coll treatment:

Cols were exposed to the test article formulation, vehicle or positive control as excribed 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 = no. of binucleate cells + 2(no. of mylinucleate cells)

total no. of cells in treated cultures

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

Cytotoxicity (%) was expressed as (100 - Rolative RO.

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

Immediately prior to analysis, 12 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 containing micronucles and the number of micronucles. The number of cells containing micronucles and the number of micronucles on each slide were noted.

For the 24 hour (+24 hour recovery) the atment condition in the absence of S9 in Micronucleus Experiment 15 it was subsequently deemed accessive to analyse the two remaining vehicle control cultures, to aid data interpretation.

Nucleoplasmic bridge (NPBs) between nucleon binucleate cells were recorded during micronucleus analysis to provide an indication of chromosome represent. Various mechanisms may lead to NPB formation following INA misrepair of strand breaks in DNA in this assay to mucleate cells with NPBs were recorded as part of the moronucleus analysis.

Results

A. Analytical determinations:

Micronucleus assessment:

Not undertaken.

B. Preliminary cytotoxicity assay:

Test article precipitate was observed at concentrations of 202.6 μ g/mL and above in both short term treatments, with no scorable consequences at concentrations of 202.6 and 337.6 μ g/mL and above in the extended and contraction respectively.

No marked changes in osmolator or provided 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 microwiceus experiment.

Table CA 5.8.103-1: Spir Camine cyclohexanol: human lymphocyte preliminary cytotoxicity range funder experiment

| Conc. | 3 1 2 + 21 16 | recovery) -S9 | 3 h (+ 21h | recovery) +S9 | 24 h (+ 24 h recovery) –S9 | | |
|-----------|----------------------|---------------------|--|---------------|----------------------------|--------------|--|
| (μg/m³IQ) | Replication Contoxic | | tion Cytotoxicity Replication Cytotoxicity | | Replication | Cytotoxicity | |
| | index (RI) | ∜based on RI | index (RI) | based on RI | index (RI) | based on RI | |
| \$ 0 \$ | | (%) | 0.76 | (%) | 1.06 | (%) | |
| 5.670 | 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. | 3 h (+ 21 h | 3 h (+ 21 h recovery) -S9 3 h (+ 21h recovery) +S9 | | | 24 h (+ 24 h recovery) –S9 | | |
|---------|--------------------------|--|---------------------|--------------|----------------------------|----------------|--|
| (μg/mL) | Replication Cytotoxicity | | Replication | Cytotoxicity | Replication | Cytotoxicity 。 | |
| | index (RI) | based on RI | index (RI) | based on RI | index (RI) | based on RV | |
| | | (%) | | (%) | | (%)% | |
| 43.75 | 0.70 | 0 | 0.81 | 0 | 0.93 | 120 | |
| 72.92 | 0.74 | 0 | 0.71 | 6 | 0. Z . | 3 0 .0. | |
| 121.5 | 0.63 | 9 | 0.71 | 5 | 0920 | 82 | |
| 202.6 | 0.26 ^{P-ppt} | 62 | 0.30 P-ppt | 60 | NE, P-ppt | Š - Š | |
| 337.6 | P-ppt | - | P-ppt | <i>≿</i> ₄ - | 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 | - 8 | E-ppt, H-ppt, P-pp | Ž - Ž | |
| 1563 | E-ppt, H-ppt, P-pptt | - | E-ppt, H-ppt, P-pps | - 5 | E-ppt, H-ppt, Poptt | Q - ,0° | |

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 an arves

C. Micronucleus assay:

1. Assay acceptability:

The positive control chemicals induced statistically significant increases in the proportion of MNBN cells. Both replicate controls of the positive control concentration analysed under each freatment 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 preasured by binucleate + milimucleate cell counts) in vehicle control cultures at the time of harvest. The maximum concentration analysed under each treatment condition met the criteria. The assay dark were therefore considered valid and acceptable.

2. Short term treatment in the absence and presence of S9:

Treatment of cells with spin xamine cyclohexanol for 3 hours (+21 hour recovery) in the absence and presence of \$9 resulted in MNBN cells that were similar to and not significantly (\$\psi = 0.05\$) higher than those observed in concurrent vehicle control ordines for all concentrations analysed.

Table CA 5.8.123-2: Spiroxamine cyclobexanol human lymphocyte micronuclei assay: 3 h (+ 21 h

| <i>`</i> | rgcovery -3) treatment and about a tory mistorical control data | | | | | | | | | |
|----------------|---|-------------------------|-------------------|-----------------------|-----------------|--|--|--|--|--|
| Cong. | ≥ 3 h | €21 h recovery) – | S9 _~ ~ | Vehicle historical co | ontrol ranges | | | | | |
| $(\mu g/m^2)$ | | | | (donor) | | | | | | |
| , v | Total Tot | al Frequency of | Cyto. | Feb 17 – Feb 18 | % MNBN | | | | | |
| | BN MN | I- 🧳 MŊ-BN (%) | (Mar) & | | | | | | | |
| | Ø ∌ BN | | | | | | | | | |
| Vehicle A@ | , 10000 A | J 0.40 . | 0, 0, | No. of expts | 17 | | | | | |
| °B? | 1000 | \$ \$30 £ | * % | Number of cultures | 40 | | | | | |
| Total | 2000 9 | Mean: 0,450 | | Mean ±SD | 0.40 ± 0.35 | | | | | |
| 100 A | 1000 4 | 0.40 | | min. – max. | 0.00 - 0.80 | | | | | |
| B | 1000 47 | y 0.70° | P' | 95% reference range | 0.00 - 0.70 | | | | | |
| ∡ Total | 20% 20% | Mean. 0.55 | Mean: 15 | | | | | | | |
| 150 A | 1000 5 | | | | | | | | | |
| B | ©1000 \ 3 | 0.300 | | Positive historical c | ontrol ranges | | | | | |
| Total O | 2000 | [™] Meap; 0.40 | Mean: 34 | (♂ dono | r) | | | | | |
| 210 | 100 00 5 5 | ≪ Q .50 | | MMC (0.3 μ | g/mL) | | | | | |
| | ©r000 ♥ 3 | 0.30 | | Feb 17 – Nov 17 | % MNBN | | | | | |
| Total T | ₾ 20000 🚜 | Mean: 0.40 | Mean: 43 | No. of expts | 22 | | | | | |
| \$ 0° | | end: p 0.6730 | | Number of cultures | 40 | | | | | |
| ANTMC PA | 1000 🔌 58 | 5.80 | | Mean ±SD | 5.57 ± 1.74 | | | | | |
| (0.2) B | 1000 42 | | | min. – max. | 1.50 - 9.20 | | | | | |
| Total | 2000 100 | Mean: 5.00*** | Mean: 33 | 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. | | | 3 h (+ 2 | 21 h recovery) +S | 9 | Vehicle historical c | ontrol ranges |
| (μg/mL | <i>a</i>) | Total BN | Total MN- | Frequency of MN-BN (%) | Cyto. | (å dono | or) |
| | | DIN | BN | MIN-DIV (%) | (%) | Feb 17 – Feb 8 | % MNBN |
| Vehicle | Α | 1000 | 5 | 0.50 | | No. of expts | <u></u> |
| | В | 1000 | 5 | 0.50 | | Number of cultures | ~ Q 40 Q s |
| Total | | 2000 | 10 | Mean: 0.50 | - 8 | Mean ±SD | <20.40 ±0.36 € |
| 120 | Α | 1000 | 5 | 0.50 | V | min. – max. | 0.00-1.00 |
| | В | 1000 | 0 | 0.00 | 4 | 95% Peference range | QTO - 0 PB |
| Total | | 2000 | 5 | Mean 0.25: | Mean: 10 | | . 0 . |
| 180 | Α | 1000 | 4 | 0.40 | * | | |
| | В | 1000 | 2 | 0.20 | | Positive historical | |
| Total | | 2000 | 6 | Mean: 0.30 | Mean: 24 | (de dono | or) / |
| 230 | A | 1000 | 5 | 0.50 | | © CPA (3 µg/ | $(\mathfrak{o}L)^1$ |
| | В | 1000 | 5 | 0; \$0 ⁷ | | Feb 17 – Dec 17 | %MNBN |
| Total | | 2000 | 10 | M. 2n: 0.50 | Mean; 53ppt | No. of expts | 220 |
| | | Lir | ear trend: | p 9 9679 2 | | Mean: | Q 200 |
| CPA | Α | 1000 | 19 | Q 1.90 | | Mean + D | 2.21 ±0.85 |
| (7.0) | В | 1000 | 21 🕡 | £2.10 % | | min. max. | |
| Total | | 2000 | 4 9 \$ | Mean: 2,00*** | Mean: 44 | % reference range | 01.00 - 3.63 |
| | | | | | | | |

*** p<0.001

ppt precipitate observed at the and of tratment.

There is currently no historical control range for CPA [Mg/mL] the concentration analysed in this study, therefore the range for the highest CPA concentration normally analysed [Mg/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 spiroxamino cyclobexanol for 24 hours (+24 hour recovery) in the absence of S9 resulted in frequencies of MNBN cells that were significantly ($p \ge 0.001$) higher than those observed in concurrent vehicle control cultures for the highest concentration analysed (100 µg/mL, inducing 53% cytotoxicity. The MNBN cells frequency of both test article treated cultures marginally (0.95%, 1.05%) exceeded the normal range (0.00 – 0.90%), with stanstically significant linear trend test, indicating evidence of a weak linear trend.

A confirmatory experiment was performed in order to confirm reproducibility and biological refevance 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 \$9.

Table CA 5.8.1/23-3, Spiroxamine cyclobexamely human lymphocyte micronuclei assay: 24 h (+ 24 h recovery) -S9 weatment and laboratory historical control data

| Conc. | 6 | (| 24 h (+ | 24 L recovery) - | S9 | Vehicle historical control ranges | | | |
|----------|-----|---------------|-------------|------------------|-----------|-----------------------------------|-----------------|--|--|
| (µg/mL) | 0.0 | Total, | Total | Frequency of | Cyto. | (♂ donor) | | | |
| A On | | BN | BN.S | MN-8N (%) | (%) | Jul 17 – Jan 18 | % MNBN | | |
| Vehic≰e≫ | A | \$\text{1000} |]2O | 0.60 | | No. of expts | 16 | | |
| | BO | 1000 | ~ <i>\$</i> | 0.25 | | Number of cultures | 40 | | |
| | | 10000 | 3 4 | 0.40 | | Mean ±SD | 0.34 ± 0.23 | | |
| W Q | Ď | 1000 | 5 | 0.50 | | min. – max. | 0.00 - 0.90 | | |
| Total | | 4000 | 26 | Mean: 0.43 | - | 95% reference range | 0.00 - 0.80 | | |
| 40 | Α | 2000 | 12 | 0.60 | | | | | |
| | В | 2000 | 11 | 0.55 | | | | | |
| Total | | 4000 | 23 | Mean: 0.58 | Mean: 7 | | | | |



| _ | | | | | | |
|------------------------------------|-----------------|------------------------|-------------|------|---|--------|
| | | 0.35 | 7 | 2000 | Α | 80 |
| ۰ | | 0.25 | 5 | 2000 | В | |
| | Mean: 29 | Mean: 0.30 | 23 | 4000 | | Total |
| | | 0.60 | 12 | 2000 | Α | 95 |
| Positive historical control ranges | | 0.55 | 11 | 2000 | В | |
| (Sdonor) L | Mean: 41 | Mean: 0.58 | 40 | 4000 | | Total |
| VIN (0.04 μg/mL) | | 0.95 ^{>HC} | 19 | 2000 | Α | 100 |
| Feb 17 🗐 an 18 💮 MNBN 💃 | | 1.05 ^{>HC} | 21 | 2000 | В | |
| No. Let expts 20% 20% | Mean: 53 | Mean: 1.00 | 23 | 4000 | | Total |
| Mean: V 41 | | p 0.0010 | near trend: | Lin | | |
| (Nean ±SD) 6 3 ±2.38 | A, | 8.00 | 80 | 2000 | Α | VIN |
| min. – max. O 2.30 – 19.60 | 4W ^T | 83.0 | 83 | 2000 | В | (0.04) |
| 95% reference range \$2.80 - 13.50 | Mean: 74 | Mean: 8.15 | 163 | 4000 | | Total |

^{***} p<0.001

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 Dequencies of MNBN cells that were similar to and not significantly ($p \le 0.05$) ingher than those observed in concurrent vehicle control cultures for the majority of concentrations analysed. A single exception to this was the intermediate concentration (80 μ g/ μ g), indusing 35% cytotoxicity) where a weak but statistically significant ($p \le 0.05$) MNBN cell frequency was observed. However, this increase was small such that, the MNBN cell frequency of both lest article treated cultures at this concentration (80 μ g/ μ mL) and the remaining cultures at the lowest and the highest concentrations analysed fell within the notifical range. Furthermore, there was no evidence of any concentration-related effect (μ g) significant the article test). Therefore, the small statistical increase observed at the intermediate concentration was not considered of any biological relevance.

The marginal effect seep 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 (Thybaud et al.) 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: Spiroxomine cyclohexanol: human tymphocyte micronuclei assay: 24 h (+ 24 h recovery) 59 treatment micronucleus experiment 2 and laboratory historical control data

| Conco (µg/mL) | Total Total | 24 hæcovery) – S Frequency of | Vehicle historical control ranges (♂ donor) | | | |
|------------------|------------------|----------------------------------|--|------------------------------------|-----------------|--|
| 4 | BN BN | MN-120 (%) | (%) | Jul 17 – Jan 18 | % MNBN | |
| Vehicle A | @1000 24 | © 0.20 % | | No. of expts | 16 | |
| B | / 100 <u>0</u> | 0.20 | | Number of cultures | 40 | |
| Total 🝣 | 2000 24 | Mea 0.20 | - | Mean ±SD | 0.34 ± 0.23 | |
| 40 A | 2000 P 7 | 0.70 | | min. – max. | 0.00 - 0.90 | |
| B | © 1000 40 | 0.40 | | 95% reference range | 0.00 - 0.80 | |
| Total 6 | 2000 | Mean: 0.55 | Mean: 6 | Positive historical control ranges | | |
| S B | 10000 \$74 | 0.40 | | (donor) | | |
| B S | 1000 8 | 0.80 | | MMC (0.2 μg/mL) | | |

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

>HC: exceeds historical control



| Total | | 2000 | 12 | Mean: 0.60 | Mean: 35 | Dec 14 – Jan 18 | % MNBN |
|--------|---|------|------------|---------------|------------------|---------------------|----------------------|
| 105 | Α | 1000 | 4 | 0.40 | | No. of expts | 16 。 |
| | В | 1000 | 4 | 0.40 | | Number of cultures | 29 🌷 |
| Total | | 2000 | 8 | Mean: 0.40 | Mean: 51 | Mean ±SD | 31.18 ±10.58 |
| | | Lin | ear trend: | p 0.1623 | | min. – max. | 16.35 - \$2.50 , |
| MMC | Α | 1000 | 385 | 38.50 | | 95% reference ange | n a |
| (0.20) | В | 1000 | 397 | 39.70 | | VIN (0.04 μ | g/mL) |
| Total | | 2000 | 781 | Mean: | Mean: 42 | Feb 17 🕰 an 18 | MNBN |
| | | | | 39.10*** | Ĉ | | |
| VIN | Α | 1000 | 39 | 3.90 | | No@of expts(| |
| (0.04) | В | 1000 | 48 | 4.80 | ٠, ١ | Number of cultures | 3 41 3 |
| Total | | 2000 | 87 | Mean: 4.35*** | M ean: 48 | Mean ±SD O | 643 ±298 |
| | | • | • | | | ™ min⊘ max | \$2.50 - 13.60 \$ |
| | | | | D | n n | 95% reference range | 2.86 13.50 |

^{***} p<0.001

D. Deficiencies:

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

Following revision of the OECD 487 FG in 2014 the cell treatment and har ast times have been somewhat simplified with emphasis placed on performing the extended treatment without recovery, with sampling times or recovery times extended by up a further 1.52.0 normal cell cycles where justified.

Given the purpose for the *in vitro* micronucles assay is to determine both clastogenic and aneugenic potential, the assay is compromised it an extended beatment 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 and not compromise the results. This is directly in contrast to the statement in the OECD 487 test quideline, which includes the statement 'for lymphocytes exponential growth may be declining at 96 hours following stimulation and morphayer 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, C2/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 happroach, 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., 20198), with the OECID 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.

>HC: exceeds historical control



| | · · · · · · · · · · · · · · · · · · · |
|--------------------------------|---|
| Data Point: | KCA 5.8.1/11 |
| Report Author: | |
| Report Year: | 1999 |
| Report Title: | 4-tertButylcyclohexanol - A 28-day subacute oral toxicity study (gayage) in the |
| | rat |
| Report No: | SA-98/0210 |
| Document No: | M-471106-01-1 |
| Guideline(s) followed in | Commission Directive 96/54/EEC, Part B, Method B.7; OECD2407 |
| study: | |
| Deviations from current | Yes Q Q Q |
| test guideline: | Although the study was enducted according to cost guide ine OBCD 407 (1995) |
| | this test guideline has since been updated in the intervening period (2008). When |
| | assessed against current test grideline requirements the following deficiencies are |
| | noted: |
| | Whilst not a requirement the test guideline makes reference to determination of |
| | serum thyroid tormores (T3, T4, TSHO) These were not analysed. However, the |
| | gold standard to assess thyroid effects is histopathological malysis. This was |
| | undertaken on all animals and revealed no histopathological findings. |
| | Consequently the Cack of serum thyroid hormone analysis is not deemed critical, |
| | with potential chyroid offects adequately addressed with histopathological |
| D : 1 :: | analy&is. |
| Previous evaluation: | yes evaluated and accepted |
| CLD/OCC : II | RAR (2010) |
| GLP/Officially | Yes, conducted under GLP/Officially recognised testing facilities |
| recognised testing facilities: | |
| | |
| Acceptability/Reliabaty: | Yes |

Executive Summary

In this study, peroxagene cyclohexarol was administered orally via gayage 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 theatment (or 14 days recovery) Canimals/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 inpart attributed to the difficulty in dosing the test article, with defensive clinical signs observed. These effects were apparent on 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 osing revealed clinical abnormalities in the high dose group animals. These abnormalities included overe defence against application of test article prior to dosing, aggressiveness against fourhing during weekly open field observations. The stress connection to dosing is further supported by statistically significant increases in relative adrenal glands weights, without associated historathology. These increased organ weights are suggestive of increased adrenal activity due to the stress of sosing, 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 $\alpha 2\mu$ -globulin which is known to be a lesion specific to δ rats with no relevance to humans. It however is acknowledge that Azan specific staining used to confirm $\alpha 2\mu$ -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 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 cyclohexang

(alternative name: 4-text butylcyclohexanol.

Solid substance with weak comphor smell **Description:**

Lot/Batch No.: M00190

99.0% (w/w) (correction for purely not undertaken **Purity:**

CAS No.: 98-52-2

Confirmed stable for the duration of Stability of test

compound:

2. Vehicle and/or positive control:

3. Test animals:

Species: Strain:

Age at dosing:

Weight at dosing

Source:

Acclimation period.

At least days Some Saniff Spezial futter GmbH, Germany) ad libitum

Municipal outer, ad libitum

Housed 5/sex/cage Diet:

Water

Housing:

4. Environmental conditions:

Temperature:

Humidit Air changes:

Photoperiod:

B. Study Design:

1. In life dates: @/

6 April 1998 to 26 May 1998 (experimental dates)

2. Animal assignment 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 ar@ 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)

Haematological data, serum cl <u>@ata:</u> ANOVA. Incorporating Bartlett's test for homogeneity of variance. If ANOVA was significant, group means were compared by Sereffe test.

Overview of sub-acute toxicity study in rays treated orally (via gavage) with Table CA 5.8.1/11-1: spiroxamine cyclohexanol: study design and fose received @

| Damamatana | | ♂ (mg/k | g bw/d) | | _07 | (mg@k | g bw(d) | 4 |
|--------------------|-----------|---------|-------------------|----------------|--------------|-------------|----------|--------------|
| Parameters | 0 | 50 | <i>,</i> ₄450 ¸,⁴ | 300 | Q 0 4 | 5 0⊘ | 950 g | 30 0% |
| Animals | | | | | | , O' | | |
| assigned/sex | 5 | 5 Q | , * \$\%' | _\@_5 _\ | / 5° | 5 | 7 5° | 6 5 |
| No. of daily doses | 28 | 28 | <u>&</u> 28 | 28 | 28 | © 28 © | _28 | 28 |
| Animals | | 2 | © ′ * | ¥′ % | | | | 8 |
| assigned/sex | 5 | Q, a |) - () | 5 | S 5,0° | | Ö - ~ | 5 |
| No. of daily doses | 28+14 | @ ,*\ | ~ O' | 2 8+14@ | 28+04 | |) | 28+14 |
| +recovery period | \$ \$1 | Ğ ~ | |]*U 😽 | , O | b . | 0" | |

C. Methods:

1. Test article preparation and analysis:

Duting the range-fander experiment, doses were prepared veekly, but analytical examination confirmed instability of the dosing formulation. Therefore, for the sub-acute study dosing formulations were prepared daily. The test article was transforred to a beaker, melted and the corresponding weight of corn oil added and stirred for 15 minutes.

Momogenetity and concentration of the sest article were determined for all preparations prepared at all dose levels.

2. Observations:

Animals were inspected twice dails for signs of toxicity and mortality.

3. Body weights:

And mals we're weighed poor to study star and then on Days 0, 7, 14, 28, and the day of necropsy. 🎺 🧷

4. Food consumption

Measured in Weekly interval throughout the study. Mean food intake/rat (g/sat/week) was satculated from the amount of food consumed in each cage and the number of rats in each cage.

Food efficiency was colculated using the following formula:

Food efficiency

Nean food consumption/week

5. Water consumption:

6. Ophthalmological examination 🕼

7. Neurological functiona

Water make was observed daily for each cage group by visual inspection Note onducted.

Euncti&vål observation battery:

Detailed functional observation of each animal was conducted in their home case and in an open field arena once each week.

Some 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 mimals were not fasted prior to blood sampling.

Haematology: red blood well parameters (naematocrit commonly termed PCV), haemoglobin concentration (Hb), mean naemoglobin concentration (MHC), mean corpuscular haemoglobin concentration (MCHC), mean corpuscular volume (MCW), platelet count), white blood well parameters (total and differential (neutrophils, lymphocytes) eosinophils, basophils, monocytes) leukocyte count), coagulation parameters (prothrombin time (PC) – termed Hepato-Quick Test).

Clinical chemistry: electrolytes (Sodium potassium, calcium), kulney function test (creatinine urea), plucosodiver function tests (albumin alkaline phospiratase (ALP), alanine aminotrarisferase (ALT) commonly referred to as glutamic pyruvic transaminase (GPT)]), aspartate aminotrarisferase (AST Leommonly referred to as glutamic oxaloacetic transaminase (GPT)), total bilirubin (T.Bil), total protein (TP), lipid proffe (total cholesterol, trigly cerides)

At the end of the treatment and recovery period, arithals were placed in metabolism cages for 6 hours. The following urinary parameters were measured, specific gravity, pHxtotal volume, protein, glucose, ketones, biliruloid, blood, urobilinoger, sediment, colour, leukocytes, erythrocytes, bacteria, epithelial colls (squamous renal) exalate rystals, phosphate crystals, carbonate granular cylinder, urate crystals,

Adrenal glands, brain, spididy ordes, to art, kidney, liver, spleen, testis, thymus. At the end of the treatment and recovery period gross pathological examination was performed on all animals and included examination of the external surface, afforifices and associated tissues.

The following Ossues were preserved in 10% neutral buffered formalin for subsequent histopathological undertaken on all control and high dose group and rate in the speatment and recovery periods:

Accessory sex glands (:epididymides, prostate, seminal vesicle, testes; ♀: ovary, uterus, vagina), cardiovascular/haematological system (aorta [thoracic) heart Jomph nodes (skin, cervical, mesenteric), spleen, thymus), sastrointestinal fract (ocophagus, tongue, stomach, intestine (caecum, colon duoderum, 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, orinary bladded, other (skeletal muscle, bone (sternum), skin, all gross lesions and masses);

Other endorine producing/sensitive glands (adrenals, mammary gland, pruitary, thyroid (+parathyroid)).

specific neurohistopathology with specific fixatives were performed in addition to the standard histopathology undertaken on neuronal tissues.

9. Urinalysis:

10. Organ weights: 5
11. Sacrific and pathology:

12. Newrohisto pathology

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 chinical signs of toxicity manifest as animals in a prone and squatting position, strate 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 are animals began to recover. Individual animals still showed mild clipical symptoms in the afternoon.

During the recovery period, no clinical rights of toxicity were observed. No test article related effects apon mortality were observed wo high dose group 3 died on day 20 and 26 due to a doring epor.

No test article related effects observed

2. Mortality:

3. Ophthalmoscopic examination:

4. Neurological functional examinations

Functional Deservation battery:

- Home cage and open field arena observations in the low, mid and high dose groups observations included ataxia, rasciculations paddling movements, defence against touching aggressiveness nunchback/squarting position, reduced respiration, hyperactivity, straub tail, piloerection and slight convulsions.
- Convulsions.

 Additional open field observations: high dose group animals showed minimal of high sensitivity to pain. One showed positive response when examined for cataleps.
- Rearing, landing foot splay and grip strength high dose group of showed statistically significant increase of group mean values for landing foot-splay and rearing and recrease of group mean values for grip strength when compared to the recovery control group. The doscribed differences in the recovery group were minor with no slear pattern evidence and therefore considered to be of minor poxicological significance.
- Recovery period home cage and open field arena observations: no significant dose-related clinical signs of ere observed in either of or \(\text{?} \). Motor sativity
- 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 bean 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

| | | | | | | | | | a, č |
|----------|--------|----------------|----------------|----------------|--------------------------|--------------------------------|----------------------|--|-----------------|
| Paramet | OMG | | ∂ (mg/k | kg bw/d) | | | ♀ (mg/k | kg bw/d) | . Ø |
| raramet | ers | 0 | 50 | 150 | 300 | 0 | 50 | 150 | 300 |
| | | | | 28 day trea | tment perio | d | Ö | | |
| Body | Day 0 | 189 ±8.3 | 183 ±6.9 | 187 ±8.3 | 185 ± 16.5 | 144 ± 5.9 | 144 #3.3 | 150 ±6.5 ° | 146 <u>±6.6</u> |
| wt (g) | Day 7 | 227 ± 8.7 | 220 ± 10.6 | 226 ± 8.4 | 217 ± 19.4 | 159 ± 7.8 | 162 ± 4.4 | 165 ±3.6° | |
| | Day 14 | 254 ± 11.3 | 247 ± 10.1 | 257 ± 12.9 | 242 ± 22.0 | 170 ± 10.1 | 1/17/×±7.3 | 180 ± 8.5 | 183 ±13.8 |
| | Day 21 | 276 ± 11.6 | 269 ± 11.9 | 281 ± 12.7 | 261 ±2002 | 183 ± 10.9 | √489 ±8.1 | 19 % #8.1 / | 204 ±1650 |
| | Day 28 | 269 ± 14.4 | 290 ± 13.1 | 305 ± 13.3 | 278 ± 30.9 | 191 ±11,4 | 195 ±8.6 | 203 ±9.10 | |
| Body wt | Day | 80 | 107 | 118 | Q 3 | 47 ₆ 0 ⁹ | 51 | (55 ₀ | 26 6 (|
| gain (g) | 0-28 | | (†34%) | (†48%) | (\$\display \text{16%}) | | (†9%) ₍ C | (†1 <i>7%</i>) | (340%)@ |
| | | | 28 day tr | eatment per | 🍻 d + 14 da | y recovery | | - Ô' | |
| Body | Day 0 | 183 ± 7.8 | - | - 😽 | 189 ± 7.9 | \$50 ±6.7 | | ~\~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ | 147 \$ 5.7 |
| wt (g) | Day 7 | 224 ± 5.7 | - | -& | @ \ | 0168 ± 8 .0 | \$\ \frac{1}{2}\ | 9 - × | 160±9.0 |
| | Day 14 | 254 ± 8.8 | - | 0" | 247 ±96 | 18128.7 | \$ - \$ | Æ, | £87 ±8,5° |
| | Day 21 | 285 ± 9.2 | - | 4 6 | 266 ± & 6 | 198/±1.3 | <u>-</u> ~ | 0- | Q205 ±13\.7 |
| | Day 28 | 310 ± 11.0 | - | | 278±12.4* | 204 ±9, 9 ₹ | | w ~ | 214,416.6 |
| | Day 35 | 325 ± 12.3 | - @ | | 303/±13,4 | √214 ± 9 .5 | ~~ | 7 - 4 | 224 18.3 |
| | Day 42 | 350 ± 13.1 | - & | 4-4 | 33/1 ±16.9 | 220 43.4 | <u>0</u> - 8 | Ď | 231 ±16.7 |
| Body wt | Day | 127 | - / | 0√2 - × | ₹ 8 9 €* | ~√54 ~~ | | | 9 67 |
| gain (g) | 0-28 | | Q' | | (\$9%) | | | 8 - °N | (†24%) |
| | Day | 40 | _@ - 、≪ | - <u>-</u> | \$53 @ | 16 |) | \$ | 17 |
| | 28-42 | .// | | ~~ | (†33%) √ | , Ö ^r | <u> </u> | O [®] | (†6%) |
| | Day | 167 ~ | | D" - "S | 1422 | \$70 \hat{5} | ~_Q | l & - | 84 |
| | 0-42 | , Ö | Q' | ~ - ~ | (\$15%) | | | Ŭ - | (†20%) |

^{*} $p \le 0.05$

D. Food consumption, food officiency and water consumption.

food efficiency

1. Food consumption and During the 28 treatment period, a slight reduction in food consumption was observed in treated when compared to the concurrent control group. Conversely, for the ated & food consumption was increased. Food efficiency For treated ♀ was degreased, 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 β and φ at the end of the treated period was still increased and reduced, respectively when compared

with the concurrent courtrol. Without a clear pattern in these changes across the sexes, these differences were considered of little exicological relevance. No effects observed.

2. Water consimption

E. Blood and urinal

1. Haematological findings:

The following changes were observed during the 28-day treatment period total WBC (wint for both high dose of and \$\varphi\$ were increased (achieving statistical significance of of one). However, differential WBC (achieving statistical ways) showed no significant changes.

the end of the recovery period, high dose group of showed a slight decrease in total WBC in addition, statistically significant decreases of RBC, Hb and platelet count were observed in 3.

These changes were deemed of little toxicological concern with effects Cobserved 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

| Damamatana | | ♂ (mg/l | kg bw/d) | | | ♀ (mg/l | kg bw/d) | . Ø |
|--------------------------------|------------|-------------|----------------------|-------------------|-----------------|---------------|-----------------|---|
| Parameters | 0 | 50 | 150 | 300 | 0 | 50 | 150 | 300 |
| | | | 28 day trea | tment perio | od | Ď | • | |
| Platelets (10 ⁹ /L) | 1025 | 944 | 988 | 1006 | 1055 | 993 | 977 " | 9.76 |
| | ±111.2 | ±117.2 | ±97.0 | ±122.5 | ±97.5 | ±91.5 | ±50.00 | ±108.3 |
| RBC $(10^{12}/L)$ | 7.77 | 7.84 | 8.24 | 8.10 | 7.63 | ₹ 7.58 | 7:28 | \$\alpha \gamma \qu |
| | ±0.470 | ± 0.595 | ±0.616 | ±0.356 | ±0.272 | ±0.587 | ±6.628 / | ±0.456 |
| Hb (mmol/L) | 9.43 | 9.70 | 10.06 | 9.70 | 8.99 | 9.06 | 8.96 | 9.47 |
| | ±0.646 | ± 0.541 | ±0.566 | ±€,680 | ±0.46® | ±0.542 | ()±0.679 | ± 6 270 ¢ |
| Total WBC | 10.10 | 14.30 | 13.10 | ₫ 6.39 | 8.63 | 7.98 و ،7 | 7,94 | Q1.51, @ |
| $(10^9/L)$ | ±2.469 | ± 3.553 | | ∂°¥1.700* | ±0.943 | ±1.69Q" | ±6.033 g | ±1.53 |
| | | 28 day tr | eatment per | iod + 14 da | ıy⁄o¢covery∕ | | | |
| Platelets (10 ⁹ /L) | 1046 | - | - % | \$996 £ | 940 | | , - ° | ı 1007 |
| | ±102.8 | | | ≼£109.0℃ | ±1/05.8 | | <u> </u> | ≠ 101.2 ∘ |
| RBC $(10^{12}/L)$ | 8.18 | - | - ~ | 729 | 7.37 s | , <u>°</u> | J - & | 7.79 |
| | ±0.211 | | | ±0.2/11** | ©±1.14 0 | | | ±1,036 |
| Hb (mmol/L) | 9.76 | - @ | | 8.91 | 8.86 | 🎺 - 🧷 | | 3 .27 |
| | ±0.314 | | W. | ©±0.23 4 | ±6,282 | | | ± 0.772 |
| Total WBC | 13.88 | Ď | 0 | 11.43 | 6.74 | | [,5] - , L | 9.05 |
| $(10^9/L)$ | ±1.647 | ** J | | ±0488 | 5±0.729 | | | ±2.191 |
| | | ((V | al control d | | | | | |
| Platelets (10 ⁹ /L) | Date rang | ej. (| Not stated | , , | Date rang | | Not stated | |
| | n: | O´ | 505 | , "O | n: | ` ` ~ | 2 002 | |
| 10 | Mean ≠SI | | @1017 .9 ,°±1 | 66 B | Mean⊌SI | | 1052.3 ± 1 | |
| RBC $(10^{12}/L)$ | Date rang | | Norstated | % ~°°. | Date rang | e: S | Not stated | |
| | ncy | | 105 | | $n^{O'}$ | | 102 | |
| TT (1/T) | |): | 7.615 ±0.8 | A (/ 1) A | Mean ±S1 |): | 7.322 ± 0.9 | |
| Hb (mmol/L) | Date Tang | | Not stated | | Daterang | e: | Not stated | |
| | n: | | 1057 | do 4 m | n: | ₩ . | 102 | 2.47 |
| T-4-1 WDC | Mean ±SD |): Oʻ | 9.248 ±0 | (/\) | Mean ±SI | | 8.833 ±1 | |
| Total WBC | Date range | e: Ø | Not stated | | Date rang | e: | Not stated | l |
| $(10^{9}/L)$ | n: 🔑 | | 105 | • • | n. | ٦. | 112 | 222 |
| * <0.05 | Mean ±SI | <u> </u> | 12.403 ±3 | 3 y 113 ≪√ | Menn ±SI | J. | 8.272 ± 3.2 | 232 |

* $p \le 0.05^{\circ}$

RBC: red blood cell Hb: haemoglobin Total WBC: total white blood cell count

2. Clinical chemistry findings:

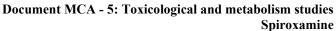
No toxicological relevant clanges were observed during the 28-day treatment period in the ated. In mid and high dose group of a statistically significant decrease in glucose was observed. A similar pattern was observed in high dose group of, but not achieving statistical significance.

Increases in creating were observed in all treated ♂, achieving statistical significant in the mid dose group. Conversely, ♀ showed decreases in featining 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 \triangle .

Glucose values of treated high dose group \lozenge and \lozenge 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 \mathcal{L} a statistically significant decrease in cholesterol, triglycerides, total bilirubin and ALT were observed compared to the historical control range, and therefore deemed or little exicological concern. Whilst contradictory results were observed between the genders for without correlating liver histopathology) rather than adverse.

For other clinical chemistry parameters analysed (electrolytes kidnes and liver functional tests) no treatment related effects were observed.

erview of sub-acute toxicity study in rate trees.

Overview of sub-acute toxicity study in rats treated orally (via gavage) with Table CA 5.8.1/11-4: spiroxamine cyclohexanol: selected clinical chemistry parameters

| | - | 71 / | | | | | * | |
|--|--------------------------|--|---|-----------------------------|----------------------|------------------|-----------------|-----------------|
| Parameters | | ∂ (mg/k | | | | | g bw(d) | |
| | 0 | 50 | 150 | 300 | ~ % 0 _4 | 50> | Q50 | ©″30 @ ∀ |
| | 1 | | 28 day trea | tment perio | | **O, | | |
| Gluc. (mmol/L) | 9.83 | 9.92 | 8,45/ | &.40 × | ⁄ 8.5®″ | 8.84 | 8. 55 | 3 .42 |
| | ± 0.448 | ±0.273 | ±0.475* | Ç¥0.89 7 \$″ | ±1 062 | ©±0.53@ | ±00263 | ± 0.267 |
| T.chol (mmol/L) | 2.26 | 2.3 | 2 .24 ^ | 2.29 | 1.79 6 | 1.7 | 3 7.91 | 1.70 |
| | ±0.324 | ±0.\$70_0 | ±0.30% | ±0091 | ±0.249 | ± <u>0</u> 203 | ©±0.110√ | ±0.184 |
| Trig (mmol/L) | 2.24 | <i>©</i> 1.19,∜ | 1.19 | 3.41 Q | 1,402 | 90.80 | 095 | 1.02 |
| | ±1.039 🐇 | \$\frac{1}{2}\display \display | ±0×277 | ±1.825 | ±0.671 | ≥±0.273 | ±@173 | ±0.183 |
| T.bili (µmol/L) | 1.92 | 1,85 | \$\tilde{1}.79 | 1.602 | 1.90 | 1.99 | <i>₯</i> 1.79 | 1.67 |
| | ±0.116 | ±0.298 @ |) ±0.1 .02 | # 0.262 _€ | , \$±0.34 3 ° | ±0 :307 | €±0.219 | ±0.222 |
| Creat. (µmol/L) | 41 ± 3.6 | ₹30 ±6,8 | 54 4.8* | Ø/8 ±4.5 | 47 <u>/</u> ±3.2 | 41 ±2.8 | 44 ±4.6 | 43 ± 1.7 |
| AST (U/L) | 4.0 | 51:40 | 47.0 | 64,67 | ©Ž.2 🖔 | # 49.8% ±3.13 | 51.0 | 50.9 |
| | ₩±2.28 | ±298 | ©±3.55 | ±7385** | @ ±8.11 [©] | | ±10.09 | ±10.95 |
| ALT (U/L) | 22 0 *2.86 | °≈22.7 ≪ | 20.6 | ×22.6 | 27/≱ | 2 6.0 | 26.7 | 23.0 |
| O | ★2 \86 | \$\frac{1.79}{\pmu}\$ | ±1\28 | £3.18 | ±4,97 | €¥±5.35 | ±3.83 | ±7.02 |
| ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ | <i>_</i> ~~_0 | 28 Qay tr | eatment per | 10d + 🕅 da | | | | |
| Gluc. (mmol/L) | © 9.15 [©] | \\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\ | → -57 | % 61 | 8.36 | - | - | 8.62 |
| | ±1,005 | | y O' | ±0.850@ | | | | ±0.406 |
| T.chol (mmol/L) | 3.29 E | - | ~~ . (| 2.22 | ° ©20 | - | - | 1.77 |
| · 'y | °€0.313°° | * | \$\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\ | ±0.316 | <u>\$</u> 20.195 | | | ±0.213* |
| Trig (mmol/L) | 2,00 | | | 9.81 | 1.59 | _ | _ | 1.13 |
| Q | ±0433 | | | ©±0.382 | ±0.203 | _ | _ | $\pm 0.100^*$ |
| <u>Q</u> | O ^v è | y Ç | , O, ^ (| | | | | * |
| T.bili (µmol⁄¶🎾 | © 2.200° | | 7 - 7 7 - 7 | 212 | 2.44 | - | - | 1.96 |
| 4 | ±0.399 | 9 4 | | 40 .124 | ±0.315 | | | ±0.113* |
| Creat. (µmol/L) | <i>47</i> 0 ≠ 4.6 | o - W | | 31 ± 1.2 | 48 ± 3.6 | - | - | 49 ± 7.3 |
| AST (U/L) | 4 7.9 | | \$ - \$ | 52.2 | 46.8 | - | - | 47.7 |
| | ±4.13 | ~ O' | | ±11.33 | ±3.50 | | | ±8.21 |
| ALT (U/L) | 24 % | Ö - | | 25.7 | 26.3 | - | - | 22.4 |
| v` | ±1.52 | | .Ó.Y | ±0.17 | ±1.25 | | | ±1.50** |
| | | | | | istar 8 -11 | wks of age |) | |
| Gluc. (mm L) « | Date singe | e: 🎣 🗼 🧠 | Not stated | | Date range | e: | Not stated | |
| Ĭ Î | n: 💍 | J' | 100 | | n: | | 97 | |
| | Mean ±SI |) | 9.19 ± 1.5 | 19 | Mean ±SI |): | 9.06 ± 1.20 | |
| T.chgl (mma/PL) | Date rang | e: | Not stated | | Date range | e: | Not stated | |
| | n: | | 115 | | n: | | 112 | |
| ~~ & | Mean ±SI |) : | 2.27 ± 0.3 | 71 | Mean ±SI | | 1.96 ± 0.32 | 25 |
| Trig (mmol/L) | Date range | e: | Not stated | [| Date range | e: | Not stated | |
| | n: | | 115 | | n: | | 102 | |
| | Mean ±SI |): | 1.86 ± 0.66 | 53 | Mean ±SI |) : | 1.35 ± 0.54 | 18 |



| Danamatana | | ♂ (mg | /kg bw/d) | • | | \bigcirc (mg/kg bw/d) | | | |
|-----------------|------------|-------|-----------------|-------|------------|-------------------------|-----------------|--------------|--|
| Parameters | 0 | 50 | 150 | 300 | 0 | 50 | 150 | 300 ° | |
| T.bili (μmol/L) | Date range | : | Not stated | | Date range | e: | Not stated | | |
| | n: | | 114 | | n: | | 112 | | |
| | Mean ±SD | : | 3.15 ± 1.54 | 13 | Mean ±SE |): 🌭 | 3.02 ± 0.37 | | |
| Creat. (µmol/L) | Date range | : | Not stated | | Date range | e: 👼 | Not stated | | |
| | n: | | 115 | | n: | <i>\O</i> | 112 | <i>`</i> | |
| | Mean ±SD | : | 40 ± 7.0 | | Mean ±SD |): 🕰 | 44 ±75° | | |
| AST (U/L) | Date range | : | Not stated | ĈA | Date range | | Not stated | | |
| | n: | | 115 | Ö | n: | | 100 | | |
| | Mean ±SD | : | 55.8 ± 15.9 | 7 , " | Mean ≠\$₹ |) : | _\$7.7 ±18.0 | 04 | |
| ALT (U/L) | Date range | : | Not stated | ,Ñ | Datestange | e: | Not stated | | |
| | n: | | 115 | 4 | n: 🌳 | o A | 1125/ | | |
| | Mean ±SD | : | 27.8 ±9.35 | Ur | Mean ±SQ | y ⁵. ~~ | 2698 ±1191 | 2 | |

* $p \le 0.05$; ** $p \le 0.01$

Gluc.: glucose T.chol.: total cholesterol Trig.: triglycerides T.bili.: total bilirubin

reat.: creatinine

AST: aspartate aminotrosferase O

3. Urinalysis:

There were no toxicologically retevant treatment related effects observed. Specific gravity was significantly descreased in mid to se group \(\times \), with no effe@s observed in @following 28@ays of treatment.

At the end of the recovery period high dose group \(\text{Spowed a statistically}\) significant increase in specific gravity and a stanstically significant decrease in PH. These effects were not replicated in S. Wese seri-quantitate / qualitative assessment was considered incidental as it was limited to single sex, the incidence were consistent with the historical control range, did not correlate with any associate adverse histopathology and therefore deemed not toxico egically relevant.

F. Sacrifice and pathoogy:

In the greatment phase a statistically significant increase in relative adrenal weights in high dose group of were observed. It is prudent however to and nowledge that relative adrenal weights for all test article treated 3 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 whitst no concurrent organ histopathology was evident, the increased adrenal At the end of the recovery period for high dos weights were that structure with any associated a therefore deemed not toxicologically relevant. 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 3, relative epididymides weights were satistically significantly increased for high dose group of. These findings did not correlate with any associated adverse histopathology and



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

| | | T | | | | | | - · · · · | <u> </u> |
|------------|------------|---------------------|--------------------|---|----------------------------------|--------------------------------|-------------------------------|----------------------|--|
| Para | meters | | ` ` ` | kg bw/d) | T | | | kg bw/d | - The state of the |
| Tara | incters | 0 | 50 | 150 | 300 | 0 0 | € 50 | | 300 |
| | | | | 28 da | y treatment period | | , (| | * \$ |
| Terminal b | bwt (g) | 284 ± 10.5 | 274 ±11.3 | 289 ± 11.7 | 265 ₁ ±27.4 | ≥\$ 184±1 ₽\$ | €88 ±7.220° | 1977±8.6 | 206 ±23.6 |
| Liver | Abs (g) | 9.159 ± 0.7109 | 8.683 ± 0.3137 | 9.154 ± 0.7043 | 8,649±1.5178 | 5.90 × ±0.4526 | >5.798 ±6@332 | 6.510 ± 0.5147 | 6,4 € 2 ±0.4431 |
| | | | (↓5%) | (↓1%) | OS (TENTO | | ≈ (2 2%) . \ C | (†1 0 %) | (†10%) |
| | Rel. (g%) | 3.228 ± 0.1903 | 3.172 ± 0.0973 | 3.166 ±0.1361 | 3.246 ±0.2381 | 3.223 ±0.2551 | \$6078 ±0,1053 | 3,340 ±0.2351© | 3.174 ± 0.2106 |
| | | | (↓2%) | (↓2%)♥* | % (↑1%) | i of | (1426) | (†3%)© [©] | (\12%) |
| Adrenals | Abs (g) | 0.045 ± 0.0054 | 0.046 ± 0.0069 | 0.0 5 @£0.0045_@ | ึ® 0.057 +0์≫า ี่ 15 | 0.055 ±0.0084 0.030 ±0.0051 | 00\$6 ±0.003\$ | 0.064 💇 .0040 | 0.073 ± 0.0131 |
| | | | (†2%) | (18%) | 27%) | | (\\2%) 0.030\(\phi\)0.0028 | 16%) 0.032 ±0.019 | (†33%) |
| | Rel. (g%) | 0.016 ± 0.0018 | 0.017 ± 0.0023 | °0.018 ±0 .0015 | √ 0.022 ±0 .0026 * | 0.030 ±0.00510 | 0.030 90.0028 | ©0.032 ±0.019 | 0.035 ± 0.0046 |
| | | | (↑6%),♥ | (13%) | (\$38%) | | 200 (-) 20 V | 0.032 = 0.019 | (†17%) |
| Testes | Abs (g) | 3.173 ± 0.2174 | 2.690 8045 | 2.290 ±0.2786 | 2 S0 ±0.1548 (\10%) | 168 yc | 0.039 0.0028 | 0.4 | - |
| | | | (15%) | | (10%) | | | 1 | |
| | Rel. (g%) | 1.119 ± 0.00759 | 9.979 ±0.2582 | 1.037 ±0.0529 | 1.06620.1043 | | | - | - |
| - · · · · | | \$ \$ | | (†289) (************************************ | 15% (15%) OTE | | | | |
| Epidid. | Abs (g) | 0.981 ±000346 | 0.968 ± 0.2240 | 1.052 ± 0.4079 | 0.949 \$0.0780 | | - ÷ | - | - |
| | D 1 (0/) | 0.247 : 0.0256 | (\7%) | | (4%) | | | | |
| | Rel. (g%) | 0.347 ± 0.0256 | 0.33540.0877 | | 0.359 ±0.0023 | of the | - | - | - |
| | | | | 5%) | (3%) | | | | |
| Tr ' 11 | 1 (() | 224 + 1220 | | * 28 day treatme | ent period + 14 day | recovery | T | | 217 +16 1 |
| Terminal b | bwt (g) | 334 ±138 | 96 j - 46 jr | | 312±15.0 | 208 ±110.2 | - | - | 217 ± 16.1 |
| Time | A la a (a) | 10262 ±0.5306 | | | (0.70) | (221 +0 2754 | | | (†4%) |
| Liver | Abs (g) | 16 202 ±0.5300 | 900 1 j.C. | | 9.260 ±0.68 78 (\10%) | 6.221 ± 0.3754 | - | - | 6.683 ±0.7043 (↑7%) |
| | Rel. (g%) | 3.077 ±0.1349 | | | 2 66 ±0.1040 | 3.007 ± 0.3032 | | | 3.077 ± 0.2441 |
| | Kei. (g/ø) | 3.077 ±0.1349 | | | (14%) | 3.007 ±0.3032 | _ | _ | (-) |
| Adrenals | Abs (g) | 0.047 +0.0029 | | Plojivation Plojivation Plojivation | 0.050 ± 0.0042 | 0.055 ± 0.0038 | _ | _ | 0.064 ± 0.0054 |
| Autonals | Aus (g) | 0.047 ±0.0029 | | | (†6%) | 0.000 ±0.0000 | _ | _ | (†16%) |
| | Rel (9%) | ©.014 ±0.0011 | | | 0.016 ± 0.0005 | 0.027 ± 0.0023 | _ | _ | 0.029 ± 0.0017 |
| | | P ~ >> " , | C. A. | | (†14%) | 0.027 =0.0023 | | | (†7%) |
| Testes | Abs (g) | 3@29 ±0.386 | | | 2.966 ± 0.1068 | _ | _ | _ | - |
| - | | | | | (\15%) | | | | |
| | v: . On | 1 * A | 191 10 0010 | | · \▼ / | • | • | | |



| | | | | | | | d | | |
|---------|------------|--------------------|---------|----------|--|--------------------|----------|-------|-------------|
| Вомо | · ma atawa | | ♂ (mg/l | kg bw/d) | | ♀ (mgkg bw/d) | | | <i>></i> |
| Рага | ameters | 0 | 50 | 150 | 300 | | 50 | 150 | |
| | Rel. (g%) | 0.942 ± 0.1382 | = | - | 0.954 ± 0.0768 | .10 ^y - | e y | - (2) | |
| | | | | | (↑1%) ⊘ | ∂ -1 | , O P | | |
| Epidid. | Abs (g) | 1.089 ± 0.0991 | - | - | 1.283 ±0.0817 | @ | - | | |
| | | | | | (18%) | s\$° | | | |
| | Rel. (g%) | 0.326 ± 0.0282 | - | - | 0.4*(*********************************** | | -05 | | Į. |
| | | | | | (†26%) | | | | |

* $p \le 0.05$ Abs.: absolute

Rel.: relative to body weight

The property of the continuent and use of this document may the there of this document may the there of this owner. Furthermore, this document may fail under a regulatory dere Furthermore, this document have a regulatory data production and have of this document or it comesquently, any connectial exploitation and has any connectial exploitation and has any connectial exploitation. AGREENTHY COMMERCIAL ESTRIBETOR OF THE OWNER THE ARTHUR THE PERMISE OF THE PROPERTY OF THE PRO



2. Gross pathology:

No test article related macroscopic findings were evident. Two high dose group of (#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 (hydrongera [distended uterus], sperm granuloma [lump of extrawasated 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 for in the lungs were characterised by multi-focal interstitial lymphocyte inflirates, these lesions were not lose related and likely caused by a viral infection.

The kidney of 5 high dose group α and 1 control α mimals nowed an increase in eosinophilic hyaline droplets in the epithelial cell cytophasm of the proximal tubules. Hyaline droplet hephropathy is one to the accumulation of α 20 globulin which is known to be a lesion specific to α rats with no relevance to humans. It however is acknowledge that A2an specific standing used to confirm α 2 μ -globulin presence was not undertaken.

Both the parent compound, spiroxamine and spiroxamine N-oxide are considered tetriary argines. As all terriary argines have a high pKa, and a high pH with comparable history thological effects reported. As with irritant compounds, tissues containing microsal membrage are deemed to be a target organ critical effect. Spiroxamine cyclohexanol, which has lost the tertiary amine group consequently did not display historial regions associated with tertiary amines (hypercerators) of the epithelium of the oesophagus and forestonach).

No adverse histopathology was reported in the recovery groups.

Table CA 5.8.1/106: Overview of sub-acute toxicity study in rats treated orally (via gavage) with spiroxamme cyclohexator: selected himpathology observations

| | p | ^ | ٥, ٧ | | | | | |
|---------------------------------------|-------------|-------------------|--------------|------------------|-----|---------|---------|-----|
| Parameters | J., . | √% (m g *) | kg bw∰d) | | | ♀ (mg/k | g bw/d) | |
| | 0°0 % | 500 | 150 | 3000 | | 50 | 150 | 300 |
| , , , , , , , , , , , , , , , , , , , | | . * 2 | 28 day treat | ment period | | | | |
| Histopathology: [in@ | dence.∕Note | , lesions w | ere not grac | led ∦ √ ⁴ | 7 | | | |
| Epididymis - Sperm granuloma | B | | 4 J | | | | | |
| - Sperm granuloma | Q0/5 \$ | | *> - & | 7 1/5 | - | - | - | - |
| Kidney 💍 🖰 | (()) | `%\ | | | | | | |
| - Hyaline droplet | | S - R | | ©0/5 | 0/5 | - | - | 0/5 |
| nep. | | | | V . | | | | |
| | [~]`````\ | . . | | * | | | | |
| - Inflam. cell foci | 0/54 | | ₹n″ - ~~~~ | 1/5 | 1/5 | - | - | 1/5 |
| Lung | | , | | | | | | |
| - Inflam. cell foci | 2/5 (| J -@ | ~~- | 3/5 | 2/5 | - | - | 0/5 |
| Uterus | | Š | Q. | | | | | |
| - Hydrometr | | - @ | <u> </u> | - | 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 assignated histopathology. These increased organ weights are suggestive of increased adroral activity due to the stress of dosing, rather than a direct endocrine related effect, which again is bother 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 dreplets in the estithelial cell cytoplasm of the proximal tubules. Hyaline droplet nephropathy is due to the accumplation of α2μ-globalin which is ο known to be a lesion specific to of rats with no relevance to humans. It however a 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 OE 05 407 (1995) whis 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 hyroid hormones (T3, T4, TSH). These were not analysed. However, the sold standard to assess thyroid effects is histopath logical analysis. This was undertaken or all animals and revealed no histopathological findings. Consequently the lack of serum thy and hormone analysis is not deemed critical, with potential thyroid effects adequately addressed with histopathological analysis.

Assessment: Study meets the current guidance and the requirements to 283/2913.

and ance and the required of this study? the NOAEL follog bw/flay formales lemaks base es) and moderate to severe clinical effects without sport downing. Conclusion: Under the conditions of this study, the MOAEL following 28 days of dosing via oral gavage is deemed to be 0 mg/kg bw/day for males remales based on reductions in body weight and body weight gain (males) and moderate to severe clinical effects following dosing with a peak



| Data Point: | KCA 5.8.1/12 |
|----------------------------|--|
| Report Author: | 0 |
| Report Year: | 2007 |
| Report Title: | Oral (gavage) developmental toxicity study of 4-tert butylxcyclohexyl acetaty (4- |
| | tBCHA) in rats |
| Report No: | 52639 |
| Document No: | <u>M-471532-01-1</u> |
| Guideline(s) followed in | US-FDA Guideline for Industry: detection of toxicity to reproduction for |
| study: | medicinal & |
| | products, (ICH) S5A |
| Deviations from current | Yes , O |
| test guideline: | Methods: SANCO/3029/99 (6). 4 |
| | Accuracy n = 4 |
| | Toxicology: The test guardline OECD 414 was tridated 2018, the following |
| | deficiencies were noted: -Thyroid gland weights and distopathological assessment of every dam treated |
| | - I hyroid gland weights and distopatiological assessment of every dam treated |
| | dam not performedThyroid hormone measurements of dams not undertaken. |
| | -Anogenital distance of fetuses not performed |
| Previous evaluation: | yes, evaluated and accepted yes, evaluated and accepted |
| Trevious evaluation. | RAR (2010) |
| GLP/Officially | Yes, conducted under GLP/Officially recognised testing facilities |
| recognised testing | 1 cs, confidence dilaci of 70 arctary recognized testing racing set |
| facilities: | |
| Acceptability/Reliability: | Yes a second |

Executive Summary

In an embryo-fetal developmental toxicity study, spiroxamine cyclohexyl was administered to female Sprague Dawley rats (25/group) by oral cavage 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, gravio uterine weights, fetal examinations, gross necropsy findings and histopathology.

Based on the results of this study spiroxamine cyclobexyl 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 compol group.

Pregnancy occurred in 24 Q4, 25 and 25 rats in the 0 40, 160 and 640 mg/kg bw/day dosage groups, respectively. The litter averages for corpora lunea, 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 fefal weights (both presented as combined sex and individual sex) were observed in the 650 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 which the sex ratio between the vehicle control and test which the sex ratio between the vehicle control and test which the sex ratio between the vehicle control and test which is the sex ratio between the vehicle control and test which is the sex ratio between the vehicle control and test which is the sex ratio between the vehicle control and test which is the sex ratio between the vehicle control and test which is the sex ratio between the vehicle control and test which is the sex ratio between the vehicle control and test which is the sex ratio between the vehicle control and test which is the sex ratio between the vehicle control and test which is the sex ratio between the vehicle control and test which is the sex ratio between the vehicle control and test which is the sex ratio between the vehicle control and test which is the sex ratio between the vehicle control and test which is the sex ratio between the vehicle control and test which is the sex ratio between the vehicle control and test which is the sex ratio between the vehicle control and test which is the sex ratio.

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 ($p \le 0.01$) 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 carear-delixered fetases 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 coalformations Reductions in the average number of ossified caudal vertebrae, forelynb phalanges and hind lynb meatars as 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 boologically recevant difference among the four dosage groups in the average number of ossification sites/fector for the hyord, vertebrae (cervical, thoracic, lumbar, sacral), ribs sternum (manuscium, sternal renters) xiphoid) forelimbs (carpals, metacarpals) or hind limbs (tarsals).

Under the conditions of this study, the NOAEL for maternal exicity 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 bodoweight and associated significant picreases in moderate dilation of the renal pelvis and delayed ossification of the caudal vortebrae, fore and hind limb phalanges and metatarsals. These retardations of ourred at a dosage level of 640 mg/kg bw/day, which exceeded the MTD for maternal animals

Materials and Methods

A. Materials:

Spiroxantine cycloriexyl acetate 1. Test Material

Olternative name. 4-tert-butylocclohexed acetate, 4-tBCHA, M13 acetate)

Description: Coloreless liquid Lot/Batch No.

(w/w) (correction for purity nor undertaken) Purity:

assigned CAS No.:

for assigned to the duration of the study (expiry date: April 2007) Stability of tes

compound

2. Vehicle and/or positi

control:

3. Test animals:

Species:

Strain: Age at dosing

Weight at dosing:

Source:

Acclimation period: **Adays**

Certified Rodent Diet® #5002 (PMI® Nutrition International, USA) ad libitum

Municipal water, ad libitum Høusing: Individually housed



4. Environmental conditions:

22 ±4°C **Temperature: Humidity:** $55 \pm 15\%$ 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 individua Dhousing on the basis of computer-generated random sation. After an acclimatisation period of 7 days, virgin \circ rats were paired 1.1 with breeder rate of \circ virgin 2 rats were paired. I with breeder rats of the same source for up to 5 days. The day which spormatozoa observed in a smear of the vaginal contents and/or a copulatory filing observed in Situ were considered to be day to of

gestation. Each group consisted of 25 \(\sigma\)/group. The dose levels were selected based on the results from a range finding study () in which spiroxamine cyclohexyl acetate was dosed, Wally vig gavage at 37,0 50, 150, 300 mg/kg bw/d to regnant rats from GD. T to 20 No mortality occurred up to the highest dose tested. Whilst body weight and food consumption reductions were observed at 300 mg/kg bwdday, cresarean sectioning, litter or fetal gross external evaluations were not adversely affected. In the absence of an MTD being established the main studowas conducted with an increased higher dose level.

Based on these data, dosages of 0, 40, 160, 640 mg/kg ow/day of spiroxamine ocyclohexanol were employed for the developmental toxicity study in rats, using a dose volume of 10 ml/kg bw. Surviving dams work sacrificed on GD

3. Statistics:

- Barlett's Test.
- O- If not sign theant:
 - ANOVA. If not significant no further statistical analysis
 - 🍑 If ANOVA significant, Duppett test 🥬
- S If significant, non-parametric approach

Von-parametrio

- Karaskal-Wallis test.
 - If not rignificant no further statistical analysis
 - If significator, Dun Prest O

For proportional data arian test for homogeneity of the binomial

<u>Indices</u>: All appropriate indices were calculated from caesarean section records of animals in the study

For proportional de distribution | Imalices: All approprior of animals in the strain loss | Post-implantation loss | Sev pre-No. of corpora lutea – No. of implantations number of corpora lutea

No. of implantation no. of live fetuses x 100 No. of implantations

Sex ratio = No. of \lozenge fetuses / No. of \lozenge fetuses x = 100Total no. of fetuses



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 homogeneous 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 [MO471532-01-1] for method validation).

2. Observations:

Maternal observations: The animals were checked for mortality twice daily The rats were observed for general appearance wice 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 abnortinalities. 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 work individually identified with litter number and atterine distribution Recorded for the following periods GD 0, 7 10, 12, 15, 18 20 and 21

4. Food consumption:

From the food consumption data compound consumption was conculated using the following equation:

Mean daily food consumption Foo Oconsumption (a rat per period)

5. Water consumption: 6. Ophthalmological

examination:

Not conducted Not conducted

7. Mating performance:

-habitation period, Dams were sacrificed on day Examulated daily during the co-29 of gestation

8. Haematology and clinical chemistry

10. Organ weights:

11. Maternal sacrifice and pathology:

Not conducted ∜Ñot undertaken

√Not conducted

Overvian and interincexaminations. 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. I live grus 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

Recrops 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 n-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.



<u>Histopathology</u>: 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 abnormanties after staining with alizarial red S. Following examination, skeletal preparations were preceived in glycerial with thymol.

Results and discussion

A. Test article formulation analysis:

Spiroxamine cyclohexyl acetate was homogenously distributed and chonically stable for at least 14 days and within the concentration range of 4 to 64 mg/mL. The analytical days 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 level 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 increased salivation occurred car 1-2 hours following dosing. In the single animal (#1966) in the 640 mg/kg/bw/day land in extremis, in addition to the clinical signs already reported, exant faces, decreased motor activity uring stained abdominal fur atosis, coldness to touch and apparent deliveration were evident.

2. Mortality:

All other chinical signs reported were considered unrelated to treatment.

One dam (#19476) in the 640 mg/kg bolday dosage group was killed in extraords on OD20 that 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. Overview of developmental toxicity study in rats treated orally (via gavage) with spiroxamine cyclohexyl acetate: body weight effects

| Parameters | | ♀ (mg/k | | |
|-----------------|-------|---------|-----|-----|
| | O' LO | 40 | 160 | 640 |
| No. of artimals | 25 | 25 | 25 | 25 |
| treated | | | | |



| Paran | neters | ្ (mg/kg bw/d) | | | |
|---|-----------------|----------------------------------|---|---|---|
| Parameters | | 0 | 40 | 160 | 640 。 |
| Body | Day | | | | 239 ±9.3 (†0 4%) 279 ±12.9 (†0 5%) |
| wt (g) | 0 | 238 ± 9.3 | $239 \pm 9.7 (\uparrow 0.4\%)$ | 239 ±9.5 (-) | 239 ±9.3 (↑0°4%) |
| | 7 | 275 ± 8.8 | $273 \pm 1.7 (\downarrow 0.7\%)$ | 274 ±13.8 (\\ 0.4\%) | 2/9 ±12.9 (14)5%) |
| | 8 | 276 ± 9.0 | $276 \pm 13.0 (-)$ | $275 \pm 13.3 (\downarrow 0.4\%)$ | 273 ±14.1 (1.1%) |
| | 9 10 | 280 ± 9.1 | $279 \pm 12.4 (\downarrow 0.4\%)$ | 279 ±14.2 (\\ \(\)0.4\%) | $270 \pm 15.8^{*}(\downarrow 3.6\%)$ $273 \pm 15.5^{**}(\downarrow 3.2\%)$ |
| | 15 | 285 ± 8.5 313 ± 10.3 | $284 \pm 13.7 (\downarrow 0.4\%)$ $313 \pm 14.6 (-)$ | 285 ±14.4 (2) 315 ±18.8 (30.6%) | 273 ±15.5** (\$2%) 302. £21.5* (\$3.5%) |
| | | | l `´ ((<i>)</i> ? | • | |
| | 17 | 320 ± 13.0 339 ± 14.4 | $326 \pm 14.4 (-)$ $340 \pm 16.7 (\uparrow 0.3\%)$ | 338 ± 21 ($\downarrow 0.3\%$) | $321 \pm 26.17 (15.3\%)$ |
| | 18 | 355 ± 15.7 | $358 \pm 18.5 (\uparrow 0.5\%)$ | 355(#23.9 (-) | 333 ±2Q6** (16.2%) |
| | 19 | 370 ± 17.3 | 373 ±16.5 (10.8%) | 371 \$25.5 (\$0.3%) \$ | 345,±29.9** (\$6.8%) |
| | 20 | 385 ± 18.2 | 389 ±18 (1%) | 387,±28.5@10.5%) | 35®±34.9₺ (↓8.3₺) |
| | 21 | 402 ±18.7 | 402 ±21.9 (-) ° | 40/3 ±30°.2√(↑0.2%) | 365 ±33.7** (\\9.2%) |
| Body | Day | 10- 1 | | | \$ 0 c 10 c 2** 1 |
| weight | 7-21 | 127 ± 17.9 | 130 ± 15.2 (\$\infty\$.4%) \(\) | 129°°21.7 (DF.6%) °° | 86 \$30.9 \$32%) |
| gain | 0-21 | 164 ± 20.0 | T 64 ± 1, (-) | 165 [±] 25. <u>4</u> (↑0.6 ⁴ 0) | 126 ±31 2 (\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\ |
| (g) * n<0.05 | · ** n/0 | <u> </u> | | | |
| <i>p</i> ≥0.03 | $p, p \leq 0.0$ | 01 | | | |
| | | 4 | | | |
| 5. Food | consum | ption: Absolute a | nd@nean food con imp | tion during the treatmen | t beriod was |
| significantly reduced (p<0,p\$\)) during the entire dosing period for kigh dose | | | | | |
| grup dams. | | | | | |
| 16 326 ±13.6 326 ±14.4 (-) 325 ±19.7∉(3.3%) 318.22.7; (14.9%) 318 15.5 ±15.7 358 ±18.5 (10.8%) 358.23.9 (-) 353.22.6 (-) (15.2%) 49.5 ±17.3 373 ±16.5 (10.8%) 358.23.9 (-) 373.2€6.0 (16.2%) 389.±18.2 20 385 ±18.2 389.±18.9 (11%) 387.±28.5 (0.3%) 435.22.9 (-) 385.±18.2 389.±18.9 (11%) 387.±28.5 (0.3%) 435.22.9 (-) 385.±18.2 389.±18.9 (11%) 387.±28.5 (0.3%) 435.22.9 (-) 402.±18.7 402.±219 (-) 403.±10.2 (10.3%) 265.±3.3 (-) (13.3%) 265.2 (-) (10.8%) 12.2 ±17.9 ±17.9 ±10.4 ±17.9 (-) 5.5 ±25.2 (-) (10.6%) 12.6 ±12.2 (-) (12.3%) 2.9 ±10.05; ** p≤0.01 5. Food consumption: Absolute anglanean good consumption/during the reforment ferriod was singificantly reduced (p≤0.07) during the reduc | | | | | |
| | | ~ A | | | Ğ. |
| | | | | |) |
| | | | | 0, 4, | |
| | | | | | |
| | | | | 9° 2° 2° | |
| | 20 | | | | |
| | . | | | | |
| | | | | | |
| Ž. | Ş" | | | , O _A | |
| 4 | ¥ | | | Ž | |
| | | J , J | | > | |
| | | | | Y | |
| | . 2 | | | | |
| | ,~ | | | | |
| | | | | | |
| Æ | | _~~~ Q~ _~ " | | | |
| . & 1 | | | | | |
| Ŋ | | | Q 0 | | |
| | (C | | | | |
| | Z. | ´,A`,&`.S | | | |
| | | | | | |
| | | | * | | |
| × | | | | | |
| | | | | | |
| | | | | | |
| ~~ | | 7 | | | |
| Ĉ' | Ĭ | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| | | | | | |



Overview of developmental toxicity study in rats treated orally (via gavage) with spiroxamine cyclohexyl acetate: absolute and relative Table CA 5.8.1/12-2: food consumption data

| | | | | ♀ (mg | /kg b | War - | | | N. S. |
|--------------------|------------------------|--|--|--|--|--|----------------------|--|--|
| Parameters | 0 | 40 | 160 | 640 | | 0 | <u>40</u> | 160 × 3 | \$ 640 |
| | Ab | osolute feed consumpt | tion (g/day) [% chans | ge] |) | ⊜° Rela | ive feed consumption | 1 (g/kd bw/dha) 1% ch | 2020 |
| Day | | | | | | , w , do | | | |
| 0-7 | 23.1 ± 1.6 | 22.5 \pm 2.0 [\downarrow 2.6] | $23.0 \pm 2.4 [\downarrow 0.4]$ | 23.8 +2.2 [↑3.0] | | 90.0-6.6 | ₩7.8 ±5.9 [↓2.¥] | 89.4 ±6.9 [16] | 91.8 ±6.3 [↑2.0] |
| 7-10 | 19.1 ± 2.2 | $19.5 \pm 3.0 [\uparrow 2.1]$ | $19.9 \pm 2.9 [\uparrow 4.2]$ | 125 3.8** [135] | <i>"</i> | 68 6 ±7.7 | 70.1 ± 09[↑2.2] | 71.3 ±9.2 [43.9] | 91 & £6.3 [↑2.0] \$5.3 ±12.6** [↓33.7] 47.6 ±8.4** [↓30.1] |
| 10-12 12-15 | 19. / ±1.9 | $19.8 \pm 2.8 [\uparrow 0.5]$ | $\begin{bmatrix} 20.8 \pm 2.9 \ [\uparrow 5.6] \\ 20.4 \pm 2.0 \ [\uparrow 5.6] \end{bmatrix}$ | $\begin{bmatrix} 14.5 \pm 2.8 \\ 14.5 \pm 2.8 \\ 14.5 \end{bmatrix}$ | JK,€ | 65.4 4 6 6 7 8 6 7 | 0800×±1.6[↑0.60] | 1.5 ±8.2 [] 4.7 kg | $47.6 \pm 8.4^{**} [\downarrow 30.1]$ $50.1 \pm 7.8^{**} [\downarrow 23.4]$ |
| 12-15 15-18 | 19.9 ±2.0 21 4 +3 1 | 19.9 ±2.1 [-] 22.0 ±2.6 [+2.8] | 20.4 ±3.0 [2.3 21.5 +2.8 [MM | 14.3 ±3.0 [12/] | | 03.94 = 0.2 1640 0 + 8.4 = 1 | 65 9 ±60 [13 N] | 1 64 5 +51 (P) R1 | $50.1 \pm 7.8 $ [\$\frac{1}{2}3.4\$] $49.0 \pm 9.0^{**} $ [\$\frac{1}{2}3.4\$] |
| 18-21 | 19.2 ± 3.1 | 18.4 ±3.9 [14.2] | $19.3 \pm 3.1 [10.5]$ | 14.2 ±3.5** (\$\mathre{D}_{2}61 | | 50.9 ±8.2 | 48.4 ±9.5 [14.9] | 51.Q\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\ | $49.0 \pm 9.0 $ [\$\pm\$23.4] $40.6 \pm 8.9^{**}$ [\$\pm\$20.2] |
| 7-21 | 19.9 ±1.9 | 20.0 ±2.0 [↑0.5] | 20.4 2.4 [12.5] | 14.1 * 2.2* [129] | 10° | 62.1 \$ 2.6 | \$2.2 ±4.7 \$09.21 | 63.6 ±6.2 [\$2.4] | $46.0 \pm 5.0^{**} [\downarrow 25.9]$ |
| 0-21 | 20.9 ± 1.6 | $20.8 \pm 1.9 [\downarrow 0.5]$ | 21.2 ±2.1 [21.4] | 17 C+1.9** []170 | | 669 ±4.9 | 66.0 (0.6] | 67.3 ±4.7 [1.4] | $57.6 \pm 3.3^{**} [\downarrow 12.8]$ |
| * p < 0.05; ** p < | <u>≤</u> 0.01 | | The Sun of | \$ <u> </u> | 7 | | | O NO | _ |
| | | | ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ | | ~ | 4. C.A. | | , Ġ | |
| | | 22.5 ±2.0 [\2.6] 19.5 ±3.0 [\2.1] 19.8 ±2.8 [\70.5] 19.9 ±2.1 [-] 22.0 ±2.6 [\72.8] 18.4 ±3.9 [\4.2] 20.0 ±2.0 [\70.5] 20.8 ±1.9 [\10.5] | 10 " WILL | e. 0 * 2 e,\$ | | , , , | 20 , | 160 (v/kd bw/day) % ch 71.3 ±9.2 [73.9] 71.3 ±8.2 [74.7] 60.8 ±8.4 [3.9] 64.5 ±51.0 [70.8] 51.9 ± [.6 [70.2] 6.6 ±6.2 [3.4] 67.3 ±4.2 [1.4] | |
| | | | », « ₂₂ »« | | , O.D | | | | |
| | À | | ,0 | | The state of the s | | The Solution | | |
| | M. | P. P. | V 0 V | | _ | 0" "" | | | |
| | | , act | | .o. "2, | , S | 07 | 1 9 h | | |
| | | NÖ. | ar ar | |)b.r. | | 77. | | |
| | | | The Was | Dr. Cor | 27E | to the | | | |
| | | | n. 1 | a∕\ | OAn | K. H. | | | |
| | | P _02 . | Wer I Om | # 0 _{Jr} , W6 | <u>_</u> 8 | K.C. | | | |
| | al | | r. INC . I | | 20 | ≫ ⁻ | | | |
| | " Willow " | 200 | A LOU LEO | | ,0 ″ | | | | |
| | | , \$.~ | J ~ _ ~ _ ~ _ ~ _ ~ _ ~ _ ~ _ ~ _ ~ | | | | | | |
| | 4 | | | | | | | | |
| | it, yeldreut, | | \ \\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\ | -O. | | | | | |
| | <u> </u> | 1 OTTAL | The state of the s | . _ | | | | | |
| | | - 11 · · · · · · · · · · · · · · · · · · | | ~ | | | | | |
| | | m ver | » Y MİL | | | | | | |
| | | | | | | | | | |
| A95 | | Ç .*, | P" | | | | | | |
| The way | i ge al | | J ^E | | | | | | |
| | 40 Kr. | " K.D. | • | | | | | | |
| (| √ ~ | " Lin | | | | | | | |
| | | • | | | | | | | |
| | | | | | | | | | |
| | | | | | | | | | |
| | | | | | | | | | |

^{*} p < 0.05; ** p < 0.01



6.Organ weight Not conducted

7. Gross pathology: No gross pathology changes were observed.

8. Histopathology: Not conducted

9. Caesarean section Pregnancy occurred in 24, 24, 25 and 25 rats in the 0, 40, 160 and 640 tog/kg bw/day dosage groups, respectively. The litter average 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 sembryonic doth in the control or test articles.

treated groups. All placentae appeared normal

Table CA 5.8.1/12-3: Overview of developmental toxicity study in rats treated smally (sia gavage) with spiroxamine cyclohexyl acetate selected caesarean section data

| | | | <i>10</i> 2 | <i>@</i> | | | | |
|----------------------|---|--|-------------------|---|-----------------|---------------|--|--|
| Parameters | 0 40 160 640 0 25 25 25 25 25 25 25 25 25 25 25 25 25 2 | | | | | | | |
| | 0 | 40 0 | © 10 | 60 00000 | S 64 | • 40 | | |
| No. of animals | 25 | 25 | ° √ 2 | 5 5 | | | | |
| mated | 4 | | | · 0. | V , · | Ş | | |
| Animals pregnant | 24 | 24, S | / O`2 | 5,\$\tag{\tag{\tag{\tag{\tag{\tag{\tag{ | 2 | 4 0 | | |
| and caesarean | ĺ Š | | . ~ | | | ð | | |
| section on GD 21 | \$ | <i>⊗ ∨ ∨</i> | N | 500 | \$ \v | * | | |
| Unscheduled | 0 ~ | | |) _0 (| O ~ 1 | | | |
| deaths | Q ,* | | Q.Y | 0 8 | & | | | |
| Corpora lutea | 378 | 377 | 30 | 7 6 | © 39 | | | |
| [/dam] | [15.8 ±2.8] | 377 [15]/±1.4]0 | " [15 <i>7</i>] | ±2.0] | . 9 - | ±3.1] | | |
| Implantation sites | 352 | \$\text{366} \text{\$\lambda\$} | ↓° | 83, \$ | ₩ 36 | 53 | | |
| [/dam] | [14.7 4 2.5] | [15.2×4.5] O | ₡ [15.3 | ±1.8] 💍 | [15.1 | ±3.2] | | |
| Total no. of litters | | 24 4 Y | 0' 2 | 8 12 | 2 | 4 | | |
| Total live fetus | 336 | 3 57. 2 | | 62 | 34 | 14 | | |
| [/dam] | [Q4.0 ±2%] * | (^[]/4.9 ±[]8] | J 4.5 | ±2.9] | [14.3 | ±3.5] | | |
| Total live 3 / 9 | > 159/Î♥7 | 174/483 | 182 | 480 | 161/ | /183 | | |
| Total dead fet@r | \$ \text{O} \text{O} \text{O} \text{O} \text{O} \text{O} \qua | | |) | (|) | | |
| Early/late @ | 16/0\& <u>2</u> | 0.4 ±0.9 / 0,0 ±0.0 | \$21 | / 0 | 18 | / 2 | | |
| resorptions | [0.7±0.9/0.0±0.00 | [0.4 \(\frac{1}{2} \) 0.9 \(\frac{1}{2} \) 0.9 \(\frac{1}{2} \) 0.9 \(\frac{1}{2} \) | [0.8.₽1.8] | $/0.0\pm0.0$] | $[0.8 \pm 1.5]$ | $(0.0\pm0.2]$ | | |
| [/dam] | | | | | | | | |
| No. of dams with | 14745.8 | 6 25.0 | 3 11 [4 | 44.0] | 11 [4 | 15.8] | | |
| resorptions [%] | | | | | | | | |
| resorptions [%] | | | 3 | 2 | <i>₹</i> | 9 | | |
| Fetal wt (g) | \$\tag{65.45} \text{5.16} | 5.1°0 5.1°0 ±0.31 ±0.27 | 5.42 | 5.12 | 4.85 | 4.58 | | |
| Q | ©±0.310 ±0,35 | ±0.31 ±0,27 | ±0.31 | ±0.34 | ±0.49** | ±0.44** | | |
| Mean fetal wt (g) | 5.31 ±0.93 | 5,2 © ±0.29 © 1%) | 5.27 ± 0.3 | 0 (\\$0.8%) | 4.70 ± | 0.46** | | |
| Ø ' | | | | | (↓1 | 1%) | | |
| Sex ratio (%3) | 46.6 ±13.1 | 49,00-11.8 | 49.5 | ±11.9 | 47.2 = | ±11.4 | | |
| % resorbed | \$\frac{1}{2}\psi_1 \frac{1}{2}\psi_2 \frac{1}{2} | 5 ±5.8 | 6.1 = | =14.9 | 5.3 ± | :11.1 | | |
| conceptuses/litter | | Y O' | | | | | | |
| ** n < 0.01 | ~ ~ | ě¥ | | | | | | |

C. Developmental tøxicité

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.

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/k | kg bw/d) | | | | | |
|------------------------------------|---|-----------------------|------------------|-------------------------------------|--|--|--|--|
| Parameters | 0 | 40 | 160∜ | \$640 × | | | | |
| То | Total external, visceral and skeletal examinations (fetus/litter) | | | | | | | |
| Fetus/litters examined | 336/24 | 357/24© | 362/25 | 344/24 | | | | |
| Fetus/litter with any | 8 (33.3)/9 (2.7) | 11 (45.8)/12 (3.4) | 6 (29,0)/7 (1.9) | 0,7 (29 3) /18 (52) | | | | |
| alteration (%) | | \$ | 10° × | | | | | |
| % Fetus with any | 2.6 ± 3.9 | 3,2 ±3.8 | 2 1.9 ±3.6 | 4.7 ± 10.2 °C | | | | |
| alteration/litter (%) | | ~ ~ | | | | | | |
| | External e | examinations (fetus | | | | | | |
| Fetus/litters examined | 336/24 | 35#24 | 36225 | 344/24 | | | | |
| Abnormal findings | 0/0 | 40 /0 C | 0 DH 0 | \$ 0\text{0}\$ | | | | |
| | Visceral e | xaminations (fetus/li | itter) 🐧 🏻 🛇 | | | | | |
| Fetus/litters examined | 161/24 | . ~ 1700224 | √ 174/25 × | 166/24\$ ³ | | | | |
| Abnormal findings | 7/7 | & 46/6 N | | <i>\$\$</i> 9/3♥ | | | | |
| Seletal examination (ferus/litter) | | | | | | | | |
| Litters examined | 24/jb)*5 | 24/187 | 24/1880 <u>,</u> | <i>2</i> ≠/178 | | | | |
| Abnormal findings | 3/2 | | & 6/5 O | 13/10 | | | | |

4. Visceral and skeletal observations:

Fetal alterations were defined as

malformations (interversible changes that occur at you incidences in this species and strain)

Ovariations (common tondings in this species and strain and reversible delays or accelerations in development).

Output

Description of the common tondings 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-defivered fetuses in 24, 24, 25, and 24 litters in dose groups 0,40, 160 and 600 mg/kg bw/day dosage groups, respectively. Each of these betuses were examined for visceral alterations, using the Wilson technique. To total soft tissue variations were limited to 3 tissues, with no molformations:

Eyes one fems in each of the 40 and 160 mg/kg bw/day dosage groups had a folded retire in the light 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.

Notice the approximation occurred in these fetuses.

Yessels: The unbifical affery descended to the left of the urinary bladder in 3, I and I fetus from separate litters in the 0, 40 and 160 mg/kg bw/day dosage grounds, respectively. No other alterations occurred in these fetuses.

Kidneys: slight of moderate dilution of the control of the co

Kidneys: slight or moderate dilation of the pelvis of one or both kidneys was abserved. 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 ucreased ($p \le 0.01$) 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 (4.5%) litters and 1 fetus (0.7%)/study.

Table CA 5.8.1/12-4: Overview of developmental toxicity study in rats treated or ally (via gayage) with spiroxamine cyclohexyl acetate: overview of visceral variations (fetus/litter incidence)

| D | | | | | | | | | |
|--------------------------|---|---|-------------------------|---------------------------------|--|--|--|--|--|
| Parameters | 0 | 40 💍 | 460 | \$ 640 S | | | | | |
| Fetus/litters examined | 161/24 | 170/24 | 3 74/25 | 0 196/24 V | | | | | |
| Eyes : retina folded (%) | 0/0 | 14 | 0/0 × | Ž 1/1 Š | | | | | |
| | (0.0/0.0) | (4.270.6) | $Q^{*}(0.0/0.0)$ | (4.2/006) | | | | | |
| Vessels: umb. art. ↓ - L | 333 | | , ' ₀ 9/1 Q' | | | | | | |
| UB (%) | (12.5/1.9) | (4.2/0.6) | * \$4.0/0.60 s | (0.0/0.0) | | | | | |
| Kidneys: pelvis, sli. | 0/0 | | 0/6 | 1/1 | | | | | |
| dilat. (%) | (0.0/0.0) | (8.3/1.2) | (0.20 (0.0) O' | (4.2/0.6) | | | | | |
| Kidneys: pelvis, mod. | 1/1 | 0/0 | S 0/0 € | | | | | | |
| dilat. (%) | (4.2/0.6) | \sim $(0.0/0)$ | (70.0/0.09° × | <i>y</i> (4.2/4.2** 3 \$ | | | | | |
| Genitalia: testis, | 3/3 | (2 ² 2 ³ | 0 0x0 0x | 0/00 | | | | | |
| malpos. (%) | (12.5/1.5) | × (853/1.2) \$ | (0.000.0) | V (0.0/0.0) | | | | | |
| Labo | ratory historical con | trol data (rat, SD Jú | re 2001 – June 2006 | | | | | | |
| Kidneys: pelvis, sli. | n: 👋 🗞 | 50 Dadies | |) . *\forall \tag{\tau} | | | | | |
| dilat. | Fetus/Meter exaluated | d: [©] 7 80 5/11 1 © ″ | | 4 | | | | | |
| | Fetal incidence [n] | %): 11 (0.14%) [ŋ | rax. stud@incidence 4 | (2.6%)] | | | | | |
| | Litter incidence [45] | (%):@¥10 (0. 9 %) [ma | ax. study incidence 3 | (2 53.6%)] | | | | | |
| Kidneys: pelvis, mod. | | 50 studies | | | | | | | |
| dilat. | Fetus/fitter exaluated | 1 5 7 805/1102 | | | | | | | |
| | Fetal incidence [n] (Litter incidence [n]) | %): (0.04%) [ma | S. study incidence 1 | (0.7%)] | | | | | |
| <u> V</u> | Litter incidence [4] | (%):🍑 3 (QQ7%) [## | ax. study incidence 1 | (4.5%)] | | | | | |

** p < 0.01

umb. art. 1 - L UB ambilical artery descends to left of urinary bladder

pelvis, sli. dilat. pelvis light dilation

pervis, mod. dilat belvis proderate dilation cestis, malpos: lestis, malpositioned:

4. Visceral and skeleral observations (continued):

Skeletal examinations: Fotal evaluations were based on 175, 187, 188 and 477 live GD 21 caesarean-delivered fetuses in 24, 24, 25, and 24 litters in dose froups 9, 40, 166 and 640 mg/kg bw/day dosage groups, respectively. Each of these fetuses were examined for skeletal alterations, after staining with alizarin red 5. In total skeletal variations were limited to vertebrae and ribs, with no malformations:

Vertebraezone 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 feruses in the 0, 40, 160 and 320 mg/kg bw/day, respectively. No other alterations occurred in these fetuses.

A bifid contrum is the 11th, 12th or 13th thoracic vertebra occurred in 0, 2, 2, and 3 setuses 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 io 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)

| Dawamatana | | ♀ (mg/kg bw/d) | | | | | | |
|--------------------------------------|---------------------------------|---------------------|--|--------------------------|--|--|--|--|
| Parameters | 0 | 40 | 160 | 640 🚀 | | | | |
| Fetus/litters examined | 175/24 | 187/24 | 188/25 | \$78/24\$ ⁹ | | | | |
| Vertebrae: | | | , L | | | | | |
| Cervi: Arch, 6 th app. | 1/1 | 1/1 | <i>@</i> 1/1 | 24 of | | | | |
| like 7 th (%) | (4.2/0.6) | $(4.2/0.5)^{7}$ | Q .0/0.5) | (\$3 /2.2) | | | | |
| Cervi: cervi. rib at 7 th | 1/1 | 2/\$ | 2/2 | ₹ | | | | |
| cervi. vertebrae (%) | (4.2/0.6) | (8371.1) | Q (8.0/1.1) L | (0.0/000) © | | | | |
| Thoracic: centrum, | 0/0 | ©2/2 × | 2/2 Q | © 3/3 © | | | | |
| bifid (%) | (0.0/0.0) | (8.3/1.1) | ************************************** | (12.5/1.7)\$ | | | | |
| Lumbar: 5 present (%) | 0/0 | \$ 99 Z | 142 5 | 0/0 | | | | |
| | (0.0/0.0) | (86/0.0) | (4.000.5) | (0.0 /0 /0) ° | | | | |
| Sacral: 1 present (%) | 0/0 | 0/0 | → <u> </u> | 6 00 0 7 | | | | |
| | (0.0/0.0) | (0.000) | (4.0/0,5) | (0.0/0.03) | | | | |
| Caudal: 0 present (%) | 0/0 | & 200 × | | 0/00 | | | | |
| | (0.0/0.0) | (Q,0/0.0) Q | © (4.0 0.5) © | (0.0/0.0) | | | | |
| Ribcage | | | | <u> J</u> | | | | |
| Ribs: short (%) | 1/Y © | | 0.400 | 1/2 | | | | |
| | 2/0.6 | (00,0.0) | Q' (0.0/0.0) | (4.2/1.1) | | | | |
| Manubrium: duplicate | √ 0/Q ° ≈ | 1/1 " | (~ % % % | 0/0 | | | | |
| (%) | (0.000) | ©(4.2/0.5) | ~(10.0/0.0) ⁴ | (0.0/0.0) | | | | |
| Sternal centra: | °√ , 0/0 © | | 0.00 × × | 0/0 | | | | |
| duplicate (%) | (\$\sqrt{0}\)\(\rangle 0/0.02\) | O (\$\\\\2/0.5\) O' | (0.0/0.0) | (0.0/0.0) | | | | |

Cervi: Arch, 6th app. like h: cervioal vericulae: arch, has appearance like the

Cervi, cervi. riv 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 metabrisals and phalanges were significantly reduction in the 640 mg/kg-bw/day dosage groups, compared to the consurrent control.

Foreling phalanges: the average number of ossified sites, when expressed per fetus per litter (7/6) was within the historical control range of the testing facility.

Caudal vertebrae: 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 feture for the hyoid, vertebrae (cervical, thoracic, lumbar, sacral), ribs sternum (manubrium, sternal centers, xiphoid) forelimbs
(cappals, apetacarpals) or hind limbs (tarsals).

Table CA 58.1/12-67 Overview of developmental toxicity study in rats treated orally (via gavage) with spiro-vamine cyclohexyl acetate: overview of ossification site (/fetus/litter)

| Parameters S | ♀ (mg/kg bw/d) | | | | | | |
|------------------------|------------------|------------------|------------------|------------------|--|--|--|
| | 3 | 40 | 160 | 640 | | | |
| Febus/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 | 7.00 ± 0.00 | 7.00 ± 0.00 | 7.00 ± 0.00 | 7.00 ± 0.00 | | | |
| - Thoracic | 13.07 ± 0.10 | 13.09 ± 0.15 | 13.08 ± 0.15 | 13.15 ± 0.14 | | | |



| Parameters | | ♀ (mg/kg bw/d) | | | | | | |
|----------------------------|-------------------------|---|--|--------------------------|--|--|--|--|
| rarameters | 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 ±0,00** | | | | |
| Ribs (pairs) | 13.06 ± 0.08 | 13.07 ± 0.11 | 13.06 ± 0.12 | 13.11 = 0.13 | | | | |
| Sternum | | | 10 | | | | | |
| - Manubrium | 1.00 ± 0.00 | 1.00 ± 0.00 | 1.00 ±€ 00 | (£000 ±0000 € | | | | |
| - 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 | 3.99 ±0.04 1.00 ±0.00 | | | | |
| Forelimb | | | 8 4 | , 20 ALV A | | | | |
| - Carpals | 0.00 ± 0.00 | 0.00 - 0.00 | $\sqrt[8]{0.00 \pm 0.00}$ | 6900 ±0.000 % | | | | |
| - Metacarpals | 3.99 ± 0.04 | 4.00 ± 0.00 | \$\frac{4.00}{2} \dot 0.00 \$\frac{4}{2}\$ | 4.00 ±0.00 | | | | |
| - Digits | 5.00 ± 0.00 | Ø0 ±0.00 △ | 4.00 ±0.00 5.00 ±0.00 | © 5.9 © ±0.00 © | | | | |
| - Phalanges | 8.20 ± 0.62 | 8.39 ±0:43 | 8,28±0.69 | 7.76 ± 0.84 | | | | |
| Hind limb | | | | | | | | |
| - Tarsals | 0.04 ± 0.11 | 0.02 ± 0.06 | U.U4₩±U.II | | | | | |
| - Metatarsals | 4.86 ±0.18 | 24,84 ±0.√7 | 4.79 ±0.25 | 4.48 0.38 | | | | |
| - Digits | 5.00 ±0.00 | 5.00 0.00 | \$5.00 ± 0 ,00 € | 5.00 ±0.00° | | | | |
| - Phalanges | | 5.00 ±0.00 5.00 ±0.00 6.14 ±0.90 2004 Sune 2006) | 6.10 4.03 | \$.40 ±0\$9* | | | | |
| Laboratory historical cont | rol data (rat, SD June | 2004 – Yune 2006) | cidence 4.67 5.00 | | | | | |
| Vertebrae | n: | 50 studies | | | | | | |
| - Caudal | Fetus/litter evaluated | d: 8342/11130 | |) | | | | |
| | retat macidence [n]. | 7 ⊘ 47 [study in | cidence 6.69 – 8.20] | ~ | | | | |
| Hind limb | n: V 👢 💍 | 50 studies d: \$\tilde{\mathcal{U}} 8312/1113 | | | | | | |
| - Metatarsals | Festus/litter evaluated | d: 💇 8312/1113 ् | | | | | | |
| | *Fetal incidence [n]: | 4 8 studevin | cidence 4.67 5.00 | ý | | | | |
| Hind limb | n: 🐬 🐧 | © So studies | cidence 4.67 5.000 | | | | | |
| - Phalanges | Fetus/litter evaluated | d: \$8312/41/13 | U Ö 4' | | | | | |
| - Finalanges | Retal incodence [4]: | 6.23 Study On | cidence 5.54 – 7.75] | | | | | |

The test guideline OECD 414 was updated 2018, the following deficiencies were noted:

- The foid gland weights and histopathological assessment of every dam treated dam not performed.
- Thyroid hormone measurements of cams not undertaken.

E. Discussion

Based on the results of this study spirox mine cyclohexyl administered to rats at a dose of 640 mg/kg bw/day caused ma@rnal 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 salvation at doc 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 werages for corpora lutea, implantations, pre-implantation loss, early resorptions, and post-inculantation 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) we're 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, 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, Orand 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 ($p \le 0.01$) 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 vetuses. 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.

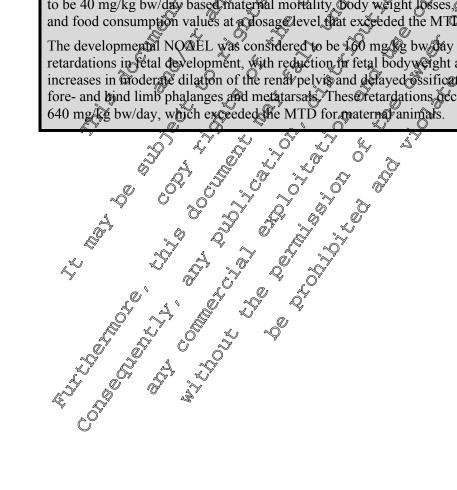
Skeletal examinations were based on 175, 187, 188 and 177 live, GD 22 caesarean-derivered fetus. In 24, 24, 25, and 24 litters in dose groups 0, 40, 160 and 640 mg/kg bw/day dosage groups, respectively. In total skeletal variations were limited to vertebrae and ribs with no maltormations. Reductions in the average number of ossified caudal vertebrae, forelimb phalanges and hind limb netators als and phalanges were significantly reduction in the 640 mg/kg by/day dosage froups, compared to the concurrent control. There were no other statistically significant of biologically relevant difference among the four dosage groups in the werage number of ossification sites fetus for the fivoid, vertebrae (cervical, thoracic, lumbar, sacral, ribs sternum (manubrium, sternal centers, xphoid) 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 MOARD for maternal toxicity was considered to be 40 mg/kg bw/day based material mortality body weight losses areduced body weight gains and food consumption values at cosag@level. That exceeded the MTD (640 mg/kg bw/day).

The developmental NOOEL was considered to be 160 mg/kg bw/day based on transient retardations in fetal development, with reduction in fetal body weight and associated significant increases in proderate dilation of the renal pelvis and delayed ssification of the caudal vertebrae, fore- and hand limb phalanges and metatarsals. These retardations occurred at a dosage level of





| Data Point: | KCA 5.8.1/13 |
|----------------------------|---|
| Report Author: | , |
| Report Year: | 2013 |
| Report Title: | 4-tert-butylcyclohexyl acetate: Biotransformation in plasma from male and |
| | female rats |
| Report No: | EnSa-13-1118 |
| Document No: | <u>M-472817-01-1</u> |
| Guideline(s) followed in | Regulation (EC) No 1107/2009 (Europe) |
| study: | amended by the Commission Regulation (EU) No (283/2013 (Europe) |
| | US EPA OCSPP not applicable 😿 🔑 💆 🗸 💯 |
| Deviations from current | None & S |
| test guideline: | |
| Previous evaluation: | yes, evaluated, not accepted RAR (2017) |
| | RAR (2017) |
| | Some data not reported: validity of analytical methods animal source number |
| | duration; concentrations of solutions |
| GLP/Officially | No, not conducted under PP/Officially recognised testing facilities |
| recognised testing | |
| facilities: | |
| Acceptability/Reliability: | Supportive only & & & & & & & & & & & & & & & & & & & |

Executive Summary

In order to understand the metabolic fate of 4-tori-but Ccyclobexyl apetate in manualian systems it was incubated with rat plasma and the reaction products were identified.

is reachly hydrolysee in plasma of male Under the conditions of this study tertboyley whexy and female rats to 4-tertbutylcyclohexarrol

Materials and Methods

A. Materials:

1. Test Material (non labelled):

Description: Lot/Batch No. **Purity:** CAS No Not axaîlable

Confirmed stable for the dustion of the study (expiry date: 2014-05-07) Stability of test compound:

2. Test Material (no yl acetate labelled):

Colourless liquid B🕉-AH21306-01-01

₹99.8% w/w Not available

Stability of test Confirmed stable for the duration of the study (expiry date: 2014-05-27)

compound:



3. Reference

4-Tert-butylcyclohexanol (M-13)

Compound (non-

labelled):

Description:

Lot/Batch No.:

Purity: CAS No.:

Stability of test

compound:

3. Vehicle and/or positive

control

4. Test system:

Species: Strain:

Plasma

None /nor relevant

None /nor relevant

At

Vistar

single of and are rat were areas thetised them same disparated into place.

stored frozen prior to use on the study;

6. Preparation of dosing

solutions:

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 @ 10 µ of the respective stock solution to cq 12 mL pasma from male and female rais and the incubation vials were gently shaken and placed in a water bath of 37°C. Aliquots were removed immediately and at 15 and 60 millutes after the start of the experiment.

The reaction was stopped by the addition of acctonitrile and formic acid to the removed alignots and the samples contrifugod. To the supernatant was added dicoloromethane: water (50 v/v) and following phase separation an aliquot of the organic phase was directly analysed by GC-MS.

3. Mass spectrometr (GC-MS):

Electron impact (EI) mass spectra were acquired on a TSQ Quantum GC spectrometer interfaced to a Trace OC Ultra. The GC retention time and mass spectra obtained from the vest and reference items were compared to those obtained from somples of the incubate.

Results and Discussion

The incubations of cis and trans 4-tertoutylex to hexylacetate with plasma from male and female rats clearly showed that both compounds were significantly transformed to 4-tertbutyleyclohexanol, with evidence that the transformation started immediately after contact with plasma.

None.

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: | |
| Report Year: | 2013 |
| Report Title: | Amendment 1 to the final report - KWG 4168-aminodiol - Acute or al toxicity |
| | study in rats (up and down procedure) |
| Report No: | 13/165-001P |
| Document No: | M-462551-02-1 |
| Guideline(s) followed in | OECD 425; EEC Directive 440/2008, B.1.ths; US-EPA 742-C-98-190, OPPTS |
| study: | 870.1100 |
| Deviations from current | None & G° J L J J |
| test guideline: | |
| Previous evaluation: | yes, evaluated and accepted RAR (2010) |
| | RAR (2010) |
| GLP/Officially | Yes, conducted under OLP/Otificially recognised testing facilities |
| recognised testing | |
| facilities: | |
| Acceptability/Reliability: | Yes of the second secon |

Executive Summary

The method used to investigate the acute of all 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 (simploying 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 fintil 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 rbg/kg bw. Observations included signs which were reflective of CNS toxicity, manifest as decreased activity, manched back, red discharge from eye, piloerection and death.

Three of the four animals dosed at 2000 mg/kg bworled 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, je unum rieum 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 1,000 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 tars. 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.

Materia 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:

2. Vehicle and/or positive

control:

3. Test animals:

Species: Strain: Age at dosing: Weight at dosing:

Source:

Acclimation period:

Diet:

Not assigned
Confirmed stable for the duration of the study (expiry date: 8 November 2013)

Distilled water / not relevant

Rat
Han Wistar
13 wks

2: 190 – 239e

At least 27 days

ssniff SM R/M 'Autolavable compute diet for rat and mice – breeding and maintenance, ad libitium (firsted overnight prior to dosing)

maintenance, ad librum (fired overnight prior to dosing)

Water: Municipal water Ad libitum

matisation. Individually housed during study period Housing:

4. Environmental conditions:

> Temperature: **Humidity:**

Air changes:

B. Test Performance:

1. In life dates:

L June 2013 to 00 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.

Data was exaluated sing the acute oral toxicity (OECD 425) statistical 3. Statistic nmme (AQP 425 Stat Pgm).

C. Methods:

1. Homogeneity@and achieved

2. Test article formulation

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.

All animals were killed at terminal sacrifice, with gross necropsy performed 5. Sacrifice and pathology:

(cranial, thoracic, abdominal cavities opened and organs impected)

Results

A. Homogeneity and achieved concentration analysis:

Not conducted.

B. Observations:

Clinical signs of toxicity were limited to animals dosed at 2000 ptg/kg bw 1. Clinical signs of toxicity:

Observations included signs which were reflective of CNS toxicity, manifest as decreased activity hunched back, to discharge from eye priocerection and death. No clinical signs of toxicity were observed in appinals dosed at a

550 mg/kg bw.

2. Mortality: Refer to Table \$4.5.8

Three of the four animals doced at 2000 mg/g bw died prioc to scheduled

sacrifice. Two animals were found dead 1 day post dosin

remaining animal found dead 6 hours post dosing

C. Body weight and food consumption

No indication of effects on body weight 1. Body weight:

Not applicable 2. Food consumption:

Overview of agute oral toxicity Table CA 5.8.1/14-1: treated with spiroxamine aminodiol: mortality and body weight

| | (/) | | | | <i>V</i> | |
|-----------------------------|------------|----------------|------------------|--|------------|-------|
| Parameter 2 | | mg/kg bw) | | | (mg/kg bw) | |
| rarameter | | € 550 ∼ | | | 2000 | |
| Mortality ^a | | 0/3 60 | | | 3/4 | |
| Day | | 7, | · 15 C | \bigcirc $\mathbb{Q}_{p}^{\mathbb{Z}}$ | 7 | 14 |
| Body weight (g) | 199.7 ±8.4 | 222.7 3.5 | \$229.3 £17.6 @ | _{227.0} 13.8 € | 226.0 | 242.0 |
| ±s.d. | | | \$.79 | | | |
| Net body weight | | 30.7 ±110° | | | 15.0 | |
| gain (g) | | | &' × | | | |
| Acute oral LD ₅₀ | | | © >550 <20@ | rg/kg bw | | |
| · · | (Q' Ş' | ~~(95%°cor | nfidence interva | ıl 614 – 5110 m | g/kg bw) | |

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 rejunum, 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. Deficienç

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 LD₅₀ 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

Ô



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: | |
| Report Year: | 2013 |
| Report Title: | KWG 4168-aminodiol: Salmonella typhimurium everse mutation assay |
| Report No: | 1558901 |
| Document No: | <u>M-463413-01-1</u> |
| Guideline(s) followed in | OECD 471; Commission Regulation (EC) No. 449/2008, \$13/14, US-EP \$2712- |
| study: | C-98-247, OPPTS 870.5190 |
| Deviations from current | None & S Y Y Y Y |
| test guideline: | |
| Previous evaluation: | yes, evaluated and accepted of the second of |
| | RAR (2010) |
| GLP/Officially | Yes, conducted under GLP/Officially recognized testing facilities |
| recognised testing | |
| facilities: | |
| Acceptability/Reliability: | Yes of The State of the State o |

Executive Summary

In a reverse gene mutation assay in bacteria, S. typhimurium strains T.598, T. 1537, TA100, TA1535 and TA102 were exposed to spirocamine 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 I ver post-mitochondial fraction (\$9).

In the plate incorporation assay treatments of the tester strains were performed in the absence and presence of S9 using final concentrations of spiroxamine animodis 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 trains in the absence and presence of S9. Following spiroxamine aminodral treatments of all the test strains in the absence and in the presence of S9, no increases in revertant numbers were observed that were 22-fold (TA98, TA100, TA102); ≥3-fold (TA1535, TA1537) above the concurrent whicle control

In the pre-incubation as ay treatments of the tester strains were performed in the absence and presence of S9 using final concentrations of spiroxamine amino old 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 upox plating. Following these treatments, no evidence of toxicity (*i.e.* slight thinning of the back round bacterial lawn or reduction in revertant numbers) was observed in all the tester strains in the absence and presence of S9 Following spiroxamine aminodial treatments of all the test strains in the absence and in the presence of S9, no increases in revertant numbers were observed that were \geq 2-fold (TA98, TA100, A102), \geq 3-fold (TA1535, TA1537) above the concurrent vehicle control.

Following spiros mine aminotial 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 loxicity using both the plate incorporation and pre-incubation methodologies.

Materials and Methods

A. Materials:

1. Test Material: KWG 4158-aminodiol

(alternative name: Spiroxarane aminodiol 3-ethyl(propyPamino)propage-1,2-

diol, M28)

Description: Colourless liquid AE 1344304-01-01

Purity: 98.9% (w/w) (correction for applied)

CAS No.: Not assigned *

Stability of test Confirmed stable for the duration of the study (expir) date (8) November 2013)

compound:

2. Control materials:

Negative: Solvent/final Dewnised vater/0.1 or 0.09 ml

concentration:

Positive: -S9 Strain Mayagen Conc. (µg/plate)

To 8, To 537 A-nitro phenolene-domine 4 10, 50

A NORD)

TA 100, TA 1535 Softum azode (NgN3) 10
TA 102 Methyl methane sulphroate (MMS) 2

Strain Mutagen Conc.

μg/plate)

Benzo[appyrene B[a]P)

5

TA98, TA100, 2-aminoanthracene¹⁸ (2-AA) 2.5, 10 (TA152), TA107 (TA102)

3. Activation: Sowas prepared in house from Wistar rats treated with phenobarbital/β-gaphthonavore 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%), MgCl₂ 8 mg/mL); KCl (33 mM), Queose Ophosphate (5 mM), NADP (4 mM).

4. Test organisms: S. typiumurium strains: TA98, TA100, TA1535, TA1537, TA102

All test organisms were properly maintained and were checked for appropriate godetic markers (2, typhimurium: histidine and biotin requirement, rfa mutation, uvrB sensitivity, ampicillin-resistance) regularly.

5. Test Concentrations:

c) Mutation assay 1: Late incorporation +/-S9 all strains:

 $\checkmark 0^{7}, 3, 10, 33, 100, 333, 1000, 2500, 5000 \,\mu\text{g/plate}$

Mulation assay 22 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 again 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 or to Vogel-Bonnor E again plates (minimal glucose agar plate). When set, plates were inverted and incubated at 2.720 metabolic activated from light 2.2 days

37°C, protected from light \$\infty\$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 3 °C. After additional of 2 mL of top agar solution the mixture was poured onto a minimal glucose agar plate and allowed to saidify All plates were incubated for 2 days (3.7°C. In this day, 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 whild if the following criteria were met.

The vehicle controls fell within the laboratory's historical control ranges

7. The positive control chemicals induced a significant increase in revertant

8 A minimum of analysable Ose concentration wer of vailable.

The test article was considered mutagenic in this assay if:

1. Aconceptration related increase in revertant numbers was ≥2-fold (TA98, YA100, TA102), ≥3-fold (TA4535, TA1537) above the concurrent vehicle control value.

2. Any observed response was reproducible onder the same treatment conditions.

The rest article was considered positive in this assay if all the above criteria

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

C. Methods:

1. Homogeneity and achieved concentration analysis of the dose:

5. Evaluation criteria

2. Test article formulation preparation

Not undertaken

A pretiminary solubility test confirmed spiroxamine aminodiol was soluble in detonised 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.

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.

3. Coxicity Assessment:



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 result³.

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 9. 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, VA100, TA102); ≥0 fold (TA1535, TA1537) above the concurrent vehicle control.

Table CA 5.8.1/15-1: Spiroxamine aminodist: bacterial roerse gene mutation data (mean revertant colonies): Nutation experiment (plate incorporation)

| Type of mutation | | Fram | e-shill | | | Va. | ase-pair s | ubstitutio 7 | n | | |
|------------------|-------------------|-------------------------------------|-----------|--------------------|------------------|-----------------|---------------------------|-----------------|--------------|--------------|--|
| Conc. | "⊜ TA | .98 | TM | 1537.0 | TA | 100 +SØ | ŤX1 | 535 | TA | 102 | |
| (μg/plate) | ©\$9 | \$\frac{1}{2} + \frac{1}{2} \tag{1} | -S9 | +89 . | Ø-S9 € | +SØ | ₋ - S 9 | +89 | -S9 | +S9 | |
| | ∂ 30 ±1 | 48,±5 | | △19 ±5⊘ | 112 ±107 | 13 <u>1</u> ±10 | @\$3 ±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 ⋄ | Ø46 ±4_ | 8 ±1 | 23≠9 | \$1 9 ±19 | √137 ±8 | 12 ±2 | 17 ±6 | 351 ±4 | 515 ± 30 | |
| 33 | 25 ±3 | | 12,₹4 | ° 6 ±3 € | St 1 / ± 1 € | 132≅99 | 14 ±2 | 18 ±2 | 360 ± 13 | 555 ± 13 | |
| 100 | 31 +8 | 49±11 | _@″±4 % | نِ*17 ± 5 إ | 119 ± 16 | 138 ±4 | 14 ±2 | 18 ±2 | 345 ±6 | 555 ±41 | |
| 333 | 35 ± 3 | | \$10 ±3.0 | 23 🖎 | 1 2 \$7±9 | \$28 ±8 | 15 ±5 | 21 ±7 | 301 ± 19 | 451 ±5 | |
| 1000 | _2 8 ±11 @ | ≥49 ±9° | 11343 | 22°±7 | 10 ±5 | 944 ±18 | 13 ±7 | 19 ±3 | 342 ± 17 | 513 ±20 | |
| 2500 | 28 ±6 | 48 | 10±4 | Q8 ±4, | 112 ± P | 150 ± 12 | 13 ±3 | 15 ±3 | 342 ±5 | 526 ±43 | |
| 5000 | ≥ 31 ±3 | 52 ±7 | ₹±3 € | 721 ±1 | 1124 20 | 145 ±8 | 10 ±3 | 20 ±4 | 330 ± 15 | 518 ± 25 | |
| Positive | 265 ±34 | | ©63 ±2 ° | 426 38 | 2301 | 3353 | 1805 ± 2 | 433 ±33 | 5100 | 2693 | |
| control | ~ | \$\disp\\\\\\\ | | | ±47 | ±159 | | | ±422 | ±336 | |

Unfreated control data not included as the vehicle used

S: slight thinning of background lawn

T: Toxic, no revertant colonies

common to the Ames assay Positive controls

-S9: strains: TA98, TA1567: 4-NOPD

TA100, TA1535: NON TA102: MMS +S9: strains: All strains: 2-AA

B. Mutation experiment 2

In the predicubation 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 μ g/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 aminodial 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 S9, no increases in revertant numbers were observed that were ≥2-fold (TA98, TA100, 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 reatmen The positive controls induced an acceptable increase in vevertant 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)

| | | | | <u> </u> | | | ∞ ′ <u>~</u> `∪ | | | |
|------------------|--------------|-------------------|---|--------------------|---------------------------------|---------------------|-----------------------------|-----------------|------------------|--------------|
| Type of mutation | | Fram | e-shift | | | ∅ 4 § | Á | ubstitutio | ono [®] | |
| Conc. | TA | .98 | TA1 | 53 7 ~ | , WA | 1004 | TA | 3 35 | Z TA | 102 |
| (µg/plate) | -S9 | +S9 | -S9 🍣 | , + S 9% | ₇ \$9 | ₹ S9 € | (S9 € | +500 | <i>-</i> \$9 | +S9 |
| 0 | 24 ±6 | 43 ±12 | 10 #3 | 25 ₀ ±3 | 108 ±10° | √149 ±6,″ | 14 | 180±2 | | \$523 ±70 |
| 33 | 27 ±1 | 49 ±12 | 11 Q 3 | 28 ±6 | 119 ± | 142,46 | 1⊚±7 | €±9 ±3 | ,380 ±22 | 531 ±42 |
| 100 | 28 ±6 | 43 ±10 | ∂ 0 ±2 ≈ | び22 ±2の | ″117 . ⊊9 | 149 47 | ∫1⁄4 ±4 Ĉ |) 17 ± ₽ | 405 ±13 | 592 ±17 |
| 333 | 26 ±5 | 52 ±4 🔏 | \$\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\ | ′ 30,⊭7 | 115 ⁴ 28 | 144 ±4.0 | √13 ± 2 | 20 ≝ | 405°±34 | 595 ± 50 |
| 1000 | 27 ±7 | 39 ±5 ° | 11 🞉 | 2© ₩2 | | ⊮143 ±1 ° 2∕ | 14*¥Ĭ | \$190±0 | 348 ±18 | 521 ±8 |
| 2500 | 25 ±7 | 50.±🔊 | 9 \mathbb{Q}_2 | 27 ±1 € | [∞] 106 ‡ 7 | 139 | .43¥±4 ≈ | (√18 ±4√ | 373 ±12 | 563 ± 21 |
| 5000 | 31 ±2 | 47 ≚ 4 | ∠9 ±1 ≥ | 25 ±2 | 1040±9 | 133 ±10 | | 19 🍣 | 354 ± 22 | 597 ± 24 |
| Positive | 365 ± 18 | J2996 j | \$68 ±7 | 464 ±19 | 29 81 | °∕3989⊙ | [₹] 19 ₹ 0⁄ | 381 ≠71 | 3780 | 3255 |
| control | (| *±276 | | 0 | Ĵ≟109.~ | ±129 | ±94 | * | ±117 | ±264 |

Untreated control data not included as the vehicle used is common to the Ames assay

P. Dight the ning of background lawn Proxication revertant colonies

Positive controls

-S9: strains: TA98, TA1537: 4-NOPD

TA100, TAN 335: NaN3

C. Discussion:

In two independent experiments and in all strains in the absence and presence of a rat liver metabolic activation system (S90 no increases n revertant no mber o were observed that were ≥2-fold (in strains TA98, TA10 Pand TA102 Par ≥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 systems

Table CA 5.8. 15-3: Bacterial reverse gene mutation data (mean revertant colonies): historical

| Type of mutation | | Fram | shift | * | |] | Base-pair s | ubstitutior | 1 | |
|------------------|----------|--------------|----------|----------|-------------|------------|-------------|-------------|-------------|------------|
| Parameter | | 808 <u>S</u> | TA | 1537 | TA | 100 | TA1 | 1535 | TA | 102 |
| | -S9® | <u></u> ±\$9 | -S9 | +89 | -S9 | +89 | -S9 | +S9 | -S9 | +89 |
| | | | | Veh | icle contro | 1 | | | | |
| Mean #S₽ | 30 ±5.60 | 40 ±6.08 | 12 ±3.31 | 16 ±4.34 | 142 | 156 | 14 ±2.37 | 20 ±3.75 | 380 | 502 |
| | | | | | ± 29.42 | ± 29.4 | | | ± 43.64 | ± 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 | e-shift | | | J | Base-pair s | ubstitution | 1 | |
|------------------|-------------|---------|-------------|--------------|--------------|--------------|-------------|------------------|----------|--------------|
| Parameter | TA | 198 | TA | 1537 | TA | 100 | TA1 | .535 | TA | .102 Ø |
| | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 | -S9 | +S9 | -S9 | +59 |
| | | | | Pos | itive contro | 1 | | | | |
| Mean ±SD | 372 | 2167 | 88 | 342 | 1741 | 2642 | 1751 | 360 | 502 | _@2329 & |
| | ± 78.05 | ±717.60 | ± 38.92 | ± 144.31 | ± 488.75 | ± 796.59 | ±226.44 | ±93×12 | ±89.88 " | /±598.00°° |
| Min | 158 | 249 | 61 | 77 | 569 | 825 | 710 | , 126 | 321 | 109Y |
| Max | 595 | 4089 | 448 | 809 | 3082 | 4503 | 2385 | 7 703 | 670° | 3 772 |

D. Deficiencies:

It is noted that OECD TG 471 has been recently applated (29 June 2020). However, the undated lest guideline has only included a correction to a CAS number of aprexample positive control—89 for *scoli* strain WP2uvrA. Therefore, it is reasonable to conclude that this study, whilst conducted in accordance with the updated test guideline issued in 1997 is also in accordance with the updated test guideline.

Assessment: Study meets the current guidance.

Conclusion: It was concluded.

quiren
...modiol did no
...35 FA1537 and
...ms of this study. These
...me maximum recommended
...e absence and in the presence of
...mon limited by toxicity using both the 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-



| D . D | WOA 5 0 1/16 |
|----------------------------|--|
| Data Point: | KCA 5.8.1/16 |
| Report Author: | 0 |
| 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 | OECD 476; Commission Regulation (EC) No. 440/2008, B17; UŞ-DA 712 C- |
| study: | 98-221, OPPTS 870.5300; JEPA Kanpoan No. 287; JMHW Eisei No. 127; JMITE |
| | Heisei 09/10/31 Kikyoku No. 2; MAFF Notification No. 12 Nowsan-& 7 |
| Deviations from current | Yes S. S. S. S. S. S. S. S. S. S. S. S. S. |
| test guideline: | 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 ost guideline requirements, a number of deficiencies are |
| | noted (refer to Results, Deficiencies section below). |
| Previous evaluation: | yes, evaluated and accepted of the second se |
| | RAR (2010) 4 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 2 |
| GLP/Officially | Yes, conducted ander CAP/Officially recognised testing facilities |
| recognised testing | |
| facilities: | |
| Acceptability/Reliability: | Yes O N N N N N N N N N N N N N N N N N N |

Executive summary

In a mammalian cell gene mutation assay V79 Chinese hamster cells were exposed to spiroxamine aminodiol formulated in deiorised 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 phenotrarbital β-maphthoff avone-induced rat liver post-mitochondrial fraction (S9).

The test article was formulated in dejonised water and dosed at 10% v/v, s

Cultures were exposed to prioxamine aninodial at concentrations from $50.9 - 1630 \,\mu\text{g/mL}$. No precipitate (assessed by eye at the end of treatment) was observed in any of the treatment conditions. Concentrations of $101.9 - 1630 \,\mu\text{g/mL}$ were assessed for determination of MF in both the short term treatments in the absence and presence of S9 with CCE 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 $203.9 - 1630 \,\mu\text{g/mL}$ 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 acpositive 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 od not show any increases in the mutant frequency of Chinese Hamster V79 cells at the hpre-locus when tested up to a concentration of 1603 μ g/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.Tes 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,Q.1 μg/mL)

3. Activation: S919 was purchased from a commercial source. Sprague Dawley rate were

treated with phenobarbital / β-naphthodavone, prepared in house (but no.: «080313, 220313, profein content 3 ¼ 4, 38.4 kmg/mL respectively... The

composition of the S9 reaction may was; stucose G-phosphate (GP: 5 mM), β-(Nicotinamide adenine dinucleotide phosphate (NADP) mM), potassiom chloride (KCL) 3 mM), magnesium chloride (MgCL) 8 mM). The final protein

concentration in the 39 concentration was 0.75 mg/mI The from concentration of the liver \$29 in the test system was 2.25% (v/v)

4. Test cells: Chinese hamster V79 gells were stored as frozen stocks in liquid nitrogen. Each

batcowas purged of hiprt-matants, whecked for sportaneous mutant frequency

and that it was moveoplasma free. Doubling time 2-16 hours.

5. Culture medium: Complete culture medium: MEM supplemented with Jetal boyine serum (10%

v/v) 100 U/100 μg/mL penjollin/streptomycin, 2 mM L-stramine, 25 mM HEPES, 29 μg/mD amphorericin B. Used for seeding and post treatment.

Treatment medium: for hort term exposure identical to complete culture medium but no fetal boving serum. For long term exposure identical to

complete culture in dium

Selectivemedism: commettee culture meanium supplemented with 6-TG

/11 μ**g**/mL)

6. Locus examinedChivese hanster V79 hpp (hypoxonthine guanine-phosphoribosyl-transferase)
Locus examined
Chivese hanster V79 hpp (hypoxonthine guanine-phosphoribosyl-transferase)
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Locus examined
Loc

University, Germany The selection agent was 6-thioguanine (6-TG).

7. Test article concentrations:

a) Preliminary cytotoxicity assay:

4 h → 89; 240 n − S9 212.7, 25.5, 50.9, 101.9, 203.8, 407.5, 815, 1630 μg/mL (maximum fecommended concentration, equivalent to 10 mM)

b) Mutation assays Experiment 1:

4h -/+S9. 0, 500, 101. 22203.8, 407.5, 815, 1630 μg/mL

⁶24 h 89: 0, 50.9, 1 9, 203.8, 407.5, 815, 1222.5, 1630 μg/mL

Experiment/2:

An+S9; 0, 50.9, 101.9, 203.8, 407.5, 815, 1630 μg/mL

(concentrations/underlined were scored)

B. Test Performance:

1. In the dates?

June 2013 to 15 August 2013 (experimental dates)

2. Yehicle collection? 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.



3. Statistics:

4. Acceptance criteria:

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.

Linear regression was performed to assess a possible dose dependent increase in MF ($p \le 0.05$).

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 finit of solubility. For atoxic 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 daboratory's historical control range.

The mean cloning dicience was \$50%%

For positive controls: Positive controls showed a statistically significant increase in mean total NF above the mean concurrent which control NF, 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 and evaluation may be considered also in the case that a three-told 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:

2. Cell preatment:

Not conducted

Preliminary cytotoxicity asay: \$\frac{1}{2}x\$ 10° cells/culture were establish and grown for 24 hours, and then were exposed to cest article or solvent control for 4 h (in the absence and presence of \$\frac{9}{2}\$) and 24 h (absence of \$9) thereby \$ca\$.

6.0 10° 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 (MFM supplemented with 10% FBS) was added. During the following expression period the cells of the logarithmic growing culture were subscultured 48 to \$\frac{1}{2}\$ h after treatment. For toxicity criteria the cell density of every concentration tester was measured with a cell counter and adjusted to 1 x \$\frac{1}{2}\$ of cells/mL.

Mutation assay: The procedure for preparing the cell suspension was the same as for the celliminary toxicity test, with 5×10^2 cells/culture establish and grown the 24 hours, and then were exposed to test article, solvent or positive controls for h (in the absoluce and presence of S9) and 24 h (absence of S9) thereby ca. 20×10^2 cells culture. At the end of the expression period for selection the motants, about 4×10^5 cells from each treatment group, were seeded in cell culture Petri dishes (diameter 90 mm) with selection medium containing 11 μ g/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 cm² 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 satisfies 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 ond of treatment at concentration's up to $1630~\mu g/mL$ in any of the treatments undertaken. Exposure to spiroxamine aminodio at concentrations from 12.7 to $1630~\mu g/mL$ resulted in relative cloning efficiency (RGE) values from $96.3~\omega$ 86.4%, 1000 to 82.6% and 96.9 to 12.7% for the 4 hour -S9, 4 hour +S9 and 2400 our -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-forder experiment west used to select suitable maximum concentrations for the contraction experiment.

Table CA 5.8.1/16-1: Spiroxamine aminodrol: Chinese hamster V79 mammalian cell preliminary cytotoxicity range experiment

| Dose | 4 h –S9 |
|---------|---|
| level | RCE (%) RCE (%) |
| (μg/mL) | |
| 0 | 100 7 100 100 |
| 12.7 | 95.2 |
| 25.5 | 96.3 |
| 50.9 | \$\infty 95.7@\ \infty \$\infty 98.7\ \O \infty 96.6\ |
| 101.9 | 91.5 |
| 203.8 | |
| 407.5 | 87.6 91.5 91.8 |
| 815 | |
| 1630 | 86.4 |

C. Mutation assay:

1. Short term treatments in the absence and presence of S9 Cultures were posed to spiroxamino aminodiol at concentrations from 50.9 - 1630 mg/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

3. Positive controls:

Wehicle 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 | Expt 1 | 4h –S9 | Expt 1 | 4h +S9 | Dose | Expt 2 | 24h –S9 | Expt 2 | 4h +\$9 |
|----------|--------|--------|--------|--------|-----------------------|--------------|---------------|---------------------|---|
| level | RCE | MF | RCE | MF | level | RCE | MĘ | RCE | MIF |
| (μg/mL) | (%) | | (%) | | (μg/mL) | (%) | Ö | (%) | |
| 0 | 100 | 19.8 | 100 | 17.6 | 0 | 100 | 133 | 100 ^ | 7. 4 |
| 101.9 | 99.4 | 8.2 | 93.4 | 16.8 | 101.9 | - | 4 - | 101 | 5 6 |
| 203.8 | 97.8 | 12.9 | 100.0 | 8.4 | 203.8 | 91.4 | € 9.8 | 949 | \$ 0.0 x |
| 407.5 | 97.8 | 24.3 | 95.0 | 20.8 | 4073 | 91.1 | 6.4 | 4.0 1.6 ∧ | J 12.5 |
| 815 | 96.6 | 18.0 | 101.0 | 8.8 | 818 | 87.90 | 12.5 | ₇ √99.5∑ | 133.4 |
| 1630 | 97.9 | 6.25 | 98.4 | 17.5 | 4 ♥222.5 | 95®* | 11.4 🖔 | | \$\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\ |
| Positive | 98.7 | 129.5 | 84.6 | 983.3 | ⊴ [©] 1630 | 8 6.0 | 。9.1 5 | 72.5 | ©12.8 ₂ © |
| control | | | | | [®] Positive | 88.1 | 353QÍ | Ø8.9 _Ø | 284 |
| | | | | i Q | | | · 6 2 | | .~\$ |

Mutant frequency: mutants/10⁶ viable cells

Positive control: \$9: EMS: +S9: DMBA

Table CA 5.8.1.3/3-3: Spiroxamine aminodiol: Chinese hamster V79 mammatian cell mutation tests laboratory historical control range (2011 – 2012)

| | 4 hou | | | ns.4S9 😂 🤇 | 24 % | |
|------------|------------|-------------------------|-------------|---------------|-------------|-------------------|
| Parameter | Vehicle | EMS 0 | 🔻 Vehicle 🗸 | DMBA | | * EMS |
| | | 150Qug/mL | | LT μg/mΩL | | %¥50 μg/mL |
| No. of | 75 | _@ 75 £\$ | ₩ 75-Ş | 0 75 V | O 60 (| 60 |
| studies | | | | Y ÖY O | | |
| Mean ±sd | 17.2 ±8.3 | [™] 131‰±75.5© | 15.5 ±6.90 | 723.8 ±342.9 | \$160.0 ±84 | 357.6 ± 275.0 |
| Obs. range | 2.6 – 43.5 | 54.8-88 9 9 | 3A - 44.2 | 91.4 - 2666.3 | ×2.4 – ×0.5 | 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 $(20 \times 10^2 \text{ cells/culture})$ compared to the test guideline requirements $(20 \times 10^6 \text{ cells/culture})$, however spontaneous motiant frequency rate obtained for the vehicle controls $(74-19.8 \text{ mutants } per 10^6 \text{ viable cells})$. This is comparable to current test guideline recommendations $(5-20 \text{ mutants } per 10^6 \text{ viable cells})$.
- The acceptable range that the laboratory used for acceptance criteria were observed ranges, without a confidence interval applies ideally 95%. Consequently, the observed range mutant frequency valoes presented are wide and varying of his raises concerns over the laboratory's ability to detect genetic drift in the confidence.

In conclusion, the data generated und this study are still considered valid.

Assessment and conclusions by applicant:

Assessment: Study meets the current guidence 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 ccOs at the *hprt* locus when tested up to a concentration of 1603 µC/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 that live metabolic activation system (S9).



| Data Point: | KCA 5.8.1/17 |
|----------------------------|--|
| Report Author: | |
| Report Year: | 2013 |
| Report Title: | KWG 4168-aminodiol: Micronucleus test in human lymphocytes in vitro |
| Report No: | 1558903 |
| Document No: | <u>M-469334-01-1</u> |
| Guideline(s) followed in | OECD 487 (2010); Commission Regulation (EU) No 640/2012, B49 (2012) |
| study: | |
| Deviations from current | Yes A A |
| test guideline: | Although the study was conducted according to lest guideline QDCD 487 (2010), |
| | this test guideline has since been updated in the intervening period (2016). When |
| | assessed against current test wideline requirements, a number of deficiencies are |
| | noted (refer to Results, Deficiencies section below) |
| Previous evaluation: | yes, evaluated and accepted |
| | RAR (2010) |
| GLP/Officially | Yes, conducted und@GLP/@fficially/recognised teging facilities |
| recognised testing | |
| facilities: | |
| Acceptability/Reliability: | Supportive only of the state of |

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 harrow intervals, were performed both in the absence and presence of metabolic activation (S9) from phenobal bital/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.

Treatment of cells with spirosomine aminociol for 4 hours (+36 hour recovery) in the absence and presence of S9 and 20 hour recovery) in the absence of S9 and 20 hour recovery) in the absence of S9 resulted in MNBN cells that were similar to and not regnificantly $(p \le 0.05)$ higher than those observed in concurrent vehicle control cultures for all concentrations analyses.

The positive controls MMC (-S9), DMC (-S9) and CPA (+S9) induced a statistically significant increase in %MNBO in all treatment conditions thereby demonstrating the sensitivity and specificity of the assay under these treatment conditions.

It is concluded that spiroxamine aminotol diction induce biologically relevant increases in the frequency of micronuclei in human peripheral blood lymphocytes when tested up to 1610 μ g/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 Materal: Spiroxamine againodiol

(alternative name: KWG 4158-aminodiol, [3-ethyl(propyl)amino]propane-1,2-

die, AE 134430, technical, M28)

Description Colourless liquid Lot/Baten #: VNLL 9095-4-3

Purity 98.9% (w/w) (correction not applied) (molecular weight: 161.24 g/mol)

CAS#: Not assigned

Stability of test Confirmed stable for the duration of the study (expiry date: 8 November 2013)

compound:



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:

S920 was purchased from a commercial source. Sprague Dawley ats were treated with phenobarbital/β-naphthoflavone, propared in house dot no 080313, 220313, protein content 31.4, 38.4 mg/mL, respectively. The composition of the S9 reaction mix was: glucose-6-phosphate (G6P) mM Nicotinamide adenine dinu deotide phosphare (NADP: 4 ppm), potassium chloride (KCl: 33 mM), magnesium chloride (MCCl₂: 8 mM). The final protein concentration in the S9 cofactor solution was Q 5 mg/mL. The final

concentration of rat giver S9 in the test system was 5% (v/v)

4. Test organisms:

Human peripheral blood kymphocotes were collected from a single health non-smoking and 29 years, pooled and diluted with HML media. Cells were stimulated to divide by the addition of phytohaemagglutinin (PHA). Dulbecco's prodified Eagles medium/Ham's F12 (NMEM/F) 2 1:10

5. Culture medium:

supplemented with 200 nM GlutaMAXII, 10% (v/v) in setivated fetal bovine serum, \$\sigmu 52\% pericillin/streptomycin (100 U/m) \(\bar{\chi}/100\) g/mL) \(\bar{\chi}/\text{HA (\chi)}\) \(\mug/\text{mL}),

heparin (125 SP-U/DL)

6. Test article **Concentrations:**

> **Preliminary** cytotoxicity test:

300.4, 525,7, 920, 1910 µg/mL (maximum, recommended soncentration, eginivalent to 10 mM)

Micronucleus assav:

\$9: 0, 10⁹, 18.3, \$2, 56.1, 98.1, 171.7, Experiment 1: 4h (+26b recover

Experiment 2

10.5, 18.3, 32, 56.1, 98.1, 171.7, 300.4,525.7,920, 1610 µg/ml (concentrations underlined scored for microniucleus frequent

B. Test Performance:

1. In life dates:

Not stated

2. Vehicle selection:

Special aminodiol was soluble at 16.1 mg/mL in deionised water. When dosed at 10% y/Q a suitable volume: volume addition for aqueous vehicles, a maximum comentration 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 comolalitize 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 riedium were takén in all experiments.

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.

otance 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) rances;
- 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 on cell division as measured by binucleate multinucleate cell counts) in vehicle control cultures at the time of arvest;
- 5. The maximum concentration analysed under each treatment condition med 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)

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 MNB scells 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.
- 3CA concentration-related increase in the proportion of MNB Scells was observed (positive trend test).

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

The test affecte was considered negative by this assay if mone of the above criteria were mone.

5. Evaluation criteria:

C. Methods:

1. Homogeneits and achieved concentration analysis of the dose:

2. Preliminary cytotoxicity assay

Mot undertaken

Whole blood cultures were established by placing 11% heparinised blood into DMEWF12 containing 10% (v) heat inactivated fetal bovine serum and pencifilin/streptomorin, so that the ornal volume following addition of S9 mb/KCl and the fest article in its chosen vehicle was 10 mL. The mitogen, bytohacmag luminin (RHA, 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 89 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 accelet treated concentration.

For removal of the test article, cells were pelleted and resuspended in fresh prewarmed medium containing fetal calf serum and penicillin/streptomycin and cutfured for 16 hours. After this period Cytochalasin-B was added and the cells of turned for a further 20 hours to give a final concentration of 4 μ g/mL/culture to inhibit cytokinesis, resulting in binucleate cells (without effecting karyokinesis), thereby arresting cells in interphase (equivalent to 2.5x 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 absorbe 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 valture.

Spindle inhibitor: Cyto-B was added at either 16 hours post entire treatment or immediately post-

wash to cultures to inhibit wookinesis for the short term or Continuous

treatments, respectively.

Slide preparation: Slides were prepared by preading the fixed cultures on clean slides. The slides

were stained with Gigmsa dropped on so slides covershipped and scored prior to

analysis.

Cytotoxicity: The replication index (RD), which indicates the relative number of nucleon

compared to controls was determined using the formal below:

CBPU= ((no. of Wonoucleate cells) + (2 x no. of binucleate cells) + (2 x no. of binucleate cells)

total no. of dells in reated cultures

%cybostasis ≥ 100 – 900 {6 BPI_L – 9) / (6 PI_C-1)

Micronucleus assessment:

Slides from the highest selected concentration and two lower concentrations

were taken for micros pic analysis.

One thousand binucteate 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

Nucleoplasmic bridges (NPPs) between nactei in binucleate cells which provide an indication of thromosome regarding ment resulting from various mechanisms which may lead to NPB formation following DNA misrepair of strangereaks 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 onducted as part of his story, 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 conference 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 ($p \le 0.05$) 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 andertaken.

Table CA 5.8.1/17-1: Spiroxamine aminodiol: human lymphocyte micronuclei assay: experiment 1, The (+ 36 h recovery) –S9 and historical control ranges

| 0 2000 10 0.50 2.12 2.12 525.7 2000 8 0.40 2.3 - 4 920 2000 9 0.4 2.14 - 2 160 2000 5 0.25 1.56 50.1 MMC 2000 66 33.35 1.30 33.6 Laboratory historical control data (200) - 2012/y | Conc. | 4 h (+ 36 | recovery) – 89 |
|--|--------------|-----------------------|---|
| O 2000 10 0.50 2.12 | (μg/mL) | Total BN Total | quency of SPI Q Cytotoxicity base |
| 525.7 2000 8 0.40 2.14 - 920 2000 9 0.40 2.14 - 160 2000 5 0.25 1.56 50.1 MMC 2000 66 3.35 1.30 3.6 Laborator historical control data (2009 - 2012) | | MN-BN MN-BN | N-BN (%) on CBO (%) |
| 920 2000 9 0.48 2.14 - 2.14 160 2000 5 0.48 2.35 1.56 50.1 2000 MMC 2000 66 2 3.35 1.30 3.6 1.30 3.6 1.30 1.30 1.30 1.30 1.30 1.30 1.30 1.30 | 0 | 2000 10 | 30 .50 2 2 .1 2 2 |
| 160 2000 5 4 9.25 1.56 50.1 MMC 2000 66 2 33.35* 1.30 33.6 C | 525.7 | 2000 8 | 0.400 289 0 4 - 4 |
| MMC 2000 660 33.35* 0 1.30 33.6 0 Laboratory historical control data (2009 – 2012) | 920 | 2000 9 🗐 | 0.4\$ 4 2.14 0 - |
| Laboratory historical control data (2009 – 2012) | 160 | 2000 5 | 1.56 ♥ 50.1 ♥ |
| Laboratory historical control data (2009 – 2012) | MMC | 2000 660 4 | \$\\\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ |
| VI 1: 1 0/ MENDAL (S) VI AND V | | | |
| Venicle % MNBN ranges Vositive (MML) % MSBN ranges | | Vehicle % WNBN ranges | Positive (MMC) %MSBN ranges |
| No. of expts | No. of expts | ₹35 % % | |
| Mean ±SD | | | ¥ 6 41.27 € .67 % |
| min. – max. 3.60 – 25.10 3.60 – | min. – max. | € 0.15 – 1.40 ° ° | y 3.60 − 25.10 ° |

* p<0.05

Table CA 5.8.1/17-2: Spiroxamine aminodiof: huntan lymphocyte micronuclei absay: experiment 1, 4 h (+ 56 h recovery) \$\forall \text{59}\$ and historical control ranges

| Conc. | | , O , S 4,h | (+ 36 korecovery) | -S2. @/ | |
|------------|-----------------------|---|----------------------------|--------------------|--------------------|
| (μg/mL) | Total BN | Total 🛴 | Frequency of | CBQ | Cytotoxicity based |
| | | MY-BN. ® | №N-BN (%) . | | on CBPI (%) |
| 0 | © 20 0 | 6 👸 | | @1.71 | = |
| 525.7 | © 2000 | | © .05 _{@1} | 1.73 | = |
| 920 | گ 2000 ک ^س | | © 0.35© | 1.59 | 16.5 |
| 160 | 2000 | 2 | 0.10 | 1.62 | 12.4 |
| MMC | 2,0000° 4,7° | 67, O | √″ % 75* △° | 1.31 | 56.4 |
| | La | aboratory/historic | i control data (200 | 19 – 2012) | |
| | w eni | che %MIVBN range | es 🖓 🧳 📑 | Positive (CPA) % | 6MNBN ranges |
| No. of exp | ots P | , ×70 × | · 0 | 4 | 7 |
| Mean ±SI | | 0.66 ±0.30 | | 5.19 = | ±2.22 |
| min. – mā | X . | $\sim 0^{\circ}.13 - \sigma_{\bullet} / 0 \sim$ | | 2.20 - | 11.05 |
| * p<0.05 | **** | Q S | | | |

3. Short term treatment of in the presence of S9 and continuous treatment in the

Treatment of cells with spiroxamine aminodiol for 4 hours (+36 hour recovery) in the presence of \$9 and 20 hours (+20 hour recovery) in the absence of \$9 and 20 hours (+20 hour recovery) in the absence of \$9 assulted in MNBN cells that were similar to and not significantly ($p \le 0.05$) higher than those observed in concurrent vehicle control cultures for all concentrations analysed.

Quite 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. | | 4 h (+ 36 h recovery) +S9 | | | 20 h (+ 20 h recov | rery) –S9 |
| $(\mu g/mL)$ | CBPI | Cytotoxicity | Frequency of | CBPI | Cytotoxicity | Frequency of |
| | | based on CBPI | MN-BN (%) | | based on CBPD | MN-BN(%) |
| | | (%) | | | (%) | |
| 0 | 1.79 | - | 0.50 | 2.15 | - 4 | 2 9.65 2 √ |
| 525.7 | 1.75 | 3.9 | 0.95 | 2.10 | 4.6 > | 1.150 |
| 920 | 1.88 | - | 0.50 | 2 .11 | \$.3 | . √ 0.₹\$ Q |
| 1610 | 1.83 | - | 0.80 | $\sqrt[8]{2.15}$ | <i>~</i> - | P 2950 V |
| Positive | 1.42 | 46.9 | 8.75 | 1.64 | °O [™] 44.6 | € 0.30 € |
| control | | | , | | | |
| | | Laboratory | historical control | data (20 | 09 – 2012) | |
| | | Vehicle %MN | BN ranges 🖤 | | Positive (DMC) % M | INBN ranges |
| No. of exp | pts | 34 | | | 28, | |
| Mean ±S | D | 0.39 ± 0 | | Õ | 3.12 £ 07.2 | 2 & 4 |
| min. – ma | ax. | 0.05 - 1 | 1.45 🐴 🐧 🔏 | (Q) (| 2 1.40 6.1 | 050 |

^{*} *p*<0.05

D. Deficiencies:

Although the study was conducted according to test guideline QECD 487 (2019), 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.

- It is unclear why a total recovery period of 36 hours was selected for the short term treatments. With a typical doubling time of Lanours for human lymphocytes, the recovery period of 4 hours post exposure is insufficient to allow an asymphocytes would have gone 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 cens only observed with the positive control MMC at overily high levels of cytotoxicity. Consequently, it is unclear if the assay is able to detect true obstogenicity/aneugenicity compared with structural chromosomal events secondary to cytotoxicity.
- As mitted historical control data were presented, with mean and observed ranges only for the vehicle and rositive control data without a confidence interval applied (ideally 95%).

For the reasons listed above, the study is deeped supplementary. Both the clastogenic and aneugenic endpoints have been adequately addressed with a robust, GP up to date *in vitro* human peripheral blood lymphocyte uncronucleus study (CA 5.84) 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: We's concluded that sput oxamine aminodiol did not induce biologically relevant increases in the frequency of micronuclei in human peripheral blood lymphocytes when tested up to 1610 µc mL (equivalent to 100 mM) on the absence (4 hours (+36 hour recovery) and 20 hours (+20 hour) and bresence (4 hours (+36 hour)) of rat liver metabolic activation system.



| Data Point: | KCA 5.8.1/24 |
|----------------------------|---|
| Report Author: | . 0 |
| Report Year: | 2020 |
| Report Title: | Spiroxamine aminodiol: In vitro human lymphocyte micronucleus assay |
| Report No: | 8408577 |
| Document No: | <u>M-755220-02-1</u> |
| Guideline(s) followed in | OECD (2016) |
| study: | |
| Deviations from current | None & X X X |
| test guideline: | |
| Previous evaluation: | No, not previously submitted (|
| | |
| GLP/Officially | Yes, conducted under GLP officially recognised testing facilities |
| recognised testing | |
| facilities: | |
| Acceptability/Reliability: | Yes O O O O O |

Executive Summary

Spiroxamine aminodiol was tested in an in vitro microvicleus assay using diplicate 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 (\$9) from Aroslor 1254-induced rats. The jest 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% y/w.

Following establishment of cultures, concentrations ranging from 200 to 1613 μ g/mL were tested in the absence (3 hours + 21 hour recovery) and presence of S9 (3 h + 25 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 symmatry 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 binar leate.) multinucleate cell counts) in vehicle control cultures at the time of harvest. The maximum concentration malysed under each treatment condition met the criteria. The assay data were therefore considered called and acceptable.

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

Treatment of cells with spiroxamine aminodial for 24 hours (+24 hour recovery) in the absence of S9 resulted in frequencies of M&BN cells that were significantly ($p \le 0.05$) higher than those observed in concurrent vehicle control collures for the highest two concentrations analysed (1000 and 1613 µg/mL, inducing 14% and 36% colotoxicity. 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 & infirmatory 24 hours (+24 hour recovery) in the absence of S9, cells treated with spiroxamine aminodial resulted in frequencies of MNBN cells that were similar to and not significantly ($p \le 0.01$) 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 at the higher and lower concentrations analysed. Furthermore, the mean MNBN central value at 1000 uson. (0.80%) fell within the normal range with no evidence of any concentration related effect so 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 micronucle in human peripheral blood lymphocytes when tested up to 1613 µg/mL requivalent to 10 mM for 24 hour © (+24 hour recovery) treatment in the absence of an Argelor-induced at 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 exicological 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 \$9

Materials and Methods

A. Materials:

Spiroxamine aminodial 1. Test Material:

(alternative name: [3 ethyl/pro

Description: Clear amber oil Miquid Lot/Batch No.:

Purity:

CAS No.: Vot assigned

Confirmed stable for the duration of the study expiry date: 12 January 2023) Stability of tes

compound:

2. Control materials

Negative:

Solvent/final concentration:

Positive: -S9

3 h. 0.3; 24 h : 0.20 μg/mL) [clastogenic control]

Vinblastine (VIN, 24): 0.04 (mL) [aneugenic control]

Cyclophosphamide (CPA, 3 h: 7 pg/mL)

was purchased from a commercial source. Sprague Dawley rats were lot no.: 4029, protein

treated with Apoclor 254 (supplied by content 3.7 mg/mL. The composition of the 10% S9 reaction mix was: 100 μL S⁹, Na PBS (100 M), gincose-6-phosphate (5 μM), β-NADP (4 μM), MgCl₂

18 μM) KCl (% μM), water (to volume).

4. Tést organisms

Human peripheral Mood lymphocytes were collected from 2 healthy, nonsmoking adult donors aged between 23 and 30 years, pooled and diluted with

MML media. Cells were stimulated to divide by the addition of phytohaemaga utinin (PHA).

HERES buffered RPMI supplemented with 10% (v/v) inactivated fetal calf serum, 0.52% penicillin-streptomycin cell culture.

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-450catalysed 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)

Micronucleus assay:

3 h (+21 h recovery) +/-S9: 0, 200, 400, 600, 800, 1000, 1200, 1400, 1613 μ₂ 24 h (+24 h) –S9: <u>0</u>, <u>200</u>, 400, 600, 800, <u>1000</u>, 1200, 140**0** <u>1613</u> μg/mL Confirmatory 24 h (+24 h): 0, 200, 400, 600, 800, 1000, 200, 1400, 1618 µg (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:

water up to at least 17.15 mg/mL (not corrected for purity) The solubility limit in capture medium was in excess of \$\sqrt{15} \mu \text{gmL}, as indicated by a lack of precipitation at On's concentration.ca. Wh after test article addition. A maximum concentration of 1613 vg/mL was selected for the cytotoxicity Range-Finder Experiment, in order that treatments were performed o up to the maximum recommended concentration according to current regulatory guidelines (i.e. a concentration equivalent of 10 mM). Concentrations for the microfucleus experiment were selected based on the results of the cytotoxicity range forder experiment!

A preliminary solubility test confirmed spiro amine amino fol was coluble

Test article stock solutions were prepared by formulating spiro amine aminodiol under subdied lighting in water with the od of vortex mixing and warming at T°C, to give the maximum required treatment solution concentration. Subsequent dilutions were made asing water. The est article solutions were protected from light and used within approximately 4 h of initial gformulation.

3. Statistics:

The proportion of MNBN cells for each treatment condition were compared with the proportion in vehicle controls by using Pisher'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 oriteria had to be met for assay acceptability:

- 9. The binomial dispersion test demonstrated acceptable heterogeneity (in terms of MNBN cell frequency) between replicate cultures, particularly where positive responses were seen
 - The frequency of MOBN calls in vehicle controls fell within the current 95 percentile of the observed historical vehicle control (normal) ranges;
 - The positive control chanicals induced statistically significant increases in The proportion of cells with micronuclei. Both replicate cultures at the positive compol concentration analysed under each treatment condition demonstrated MNBN cell frequencies that clearly exceeded the normal
 - A minimum of 50% of cells had gone through at least one cell division (as measured by binuc Pate + multinucleate cell counts) in vehicle control cultures of the time of harvest;
 - 5. The maximum concentration analysed under each treatment condition met the specified Fiteria (i.e. the highest concentration selected for micronucleus analysis following all treatment conditions was one at which 50-60% Sytotoxicity was achieved).

For alid data, the test article was considered to induce clastogenic and/or an eugenic 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 ploing 0.4 mL of pooled heparinised blood into 2.5 and the particle of the property of the property of the property of the particle of the property of the particle of the property of the particle of the property of the particle of the property of the particle o heparinised blood into 8.5 pt pre-warmed REPES-buffer RPMI medium containing 10% (v/v) heat mactivated fetal ralf of the containing 10% (v/v) heat mactivated fetal calf sorum and 0.52% penicillin/streptomycin so that the final volume following addition of \$9 mix/KCl and the test article in its chosen verticle was 10 mL The mitogen, phytohaemagglutino (PHACreager grade) was included in the culture medium at a concentration of approximately 2% of culture to stimulate the lymphocytess 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 of vehicle (0.1 mL/culture). Positive control treatments were not included. Duraticate control were used for the vehicle control and single cultures were used for each test article treated concentration

article treated concentration For the test article, cells were believed (approximately 300 g, 10 minutes, washed wice with sterile saline and resuspended in fresh pre-warmed medium containing feld calf serum and penjethin/streptomych. Cyto-B (formulated in DMSO) was added to post wash-off culture medium to give a final concentration of 6 permL/culture to inhibit cytokines, resulting in bunucleate cells without effecting karyokinesis, thereby arresting cells in interphase. 🔍

Changes in osmolality of more than 30 mosm/kg and fluctuations in pH of more than one unit may be responsible for an increase in chromosome aberrations. Osmolality and pH heasurements on post-treatment incubation medium were taken in the cylotoxicity range finder experiment.

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

3. Micronucleus assavz 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 displicate 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 multipucleat cells to minimum of 200 or 500 cells/culture (preliminary cytoxicity range ander or micronucleus experiment, respectively)./

(a) to-B was added post-wash to cultures to inhibit cytokinesis.

Slides were propared 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.

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

RI = 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:

Spindle inhibitor: Slide preparation:



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

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 \$9 in Micronucleus Experiment, it was subsequently deemed necessary to analyse the two remaining vehicle control cultures, to aid data interpretation.

Nucleoplasmic bridges (NPRs) between nuclei in binacleate cells were recorded during micronucleus analysis to provide an indication of chromosome rearrangement. Various mechanisms make ad to NPB formation following DNA misrepair of strains breaks in DNA in this assay binucleate cells with

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.

NPBs were recorded as part of the reconnecteus analysis

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 chotoxicity preliminary cytoroxicity range finder experiment were used to select suitable maximum concentrations for the microgracleur experiment.

Table (A 5.8.1/24-1: Spirovamine aminotiol: human lymphocyte preliminary cytotoxicity range finder speriment

| Conc. | 3 h2+21 h recovery) -S9 | | ҈ 3√h (+ 21h | recovery) +S9 | 24 h (+ 24 h recovery) –S9 | |
|------------------|-------------------------|----------------|---|---------------|----------------------------|--------------|
| (μg/mL) | Replication | Cortoxicity | Replication | Eytotoxicity | Replication | Cytotoxicity |
| | ndex (RA) | (b) ased on RI | yindex (RI) | 📡 based on RI | index (RI) | based on RI |
| _4 | , | | r bi . W | (%) | | (%) |
| 0 | ® 0.93 🗞 | , | 3 0.91 | = | 0.96 | - |
| 5.852 | 0.89 | 4 5~ | 0.91 | 3 | 0.99 | 0 |
| 9,7\S\3 | 0.941 | | 2 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 | 6 | 0.91 | 5 |
| 16.26 | 0.84 | | _ ® .86 | 4 | 1.04 | 0 |
| 27.09 | @ 0.80 | 14 | ∂ 0.87 | 2 | 1.01 | 0 |
| 45.15 | 0.914 | 25 | 0.89 | 0 | 0.98 | 0 |
| 75.26 | © 0.72° / | 23 | 0.91 | 0 | 0.97 | 0 |
| 125.4 | ′ ∮ \$89 ∂ | ⊅ 4 | 0.92 | 0 | 1.02 | 0 |
| 209.6 348.4 | .~√0.88 ∢ | 6 4 | 0.93 | 0 | 1.07 | 0 |
| 348,4 | O7 0.89 | ∜ 4 | 0.99 | 0 | 0.95 | 1 |
| <i>5</i> 800.7 ∂ | 0.990 á | 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 assav:



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 demonstration 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 aminodiation 3 hours (+2 hour ecovery) in the absence and presence of S9 resulted in MNBN cells that were similar to and not significantly ($p \le 0.05$) higher than those observed in concurrent vehicle control cultures for all concentrations analysed.

observed (data not reported)

Table CA 5.8.1/24-2: Spiroxamine aminodiol human lymphocyte hoicromaclei assay: 3 h (+ 21 h recovery) –S9 treatment and laboratory historical control data

| Conc. | | | 3 h (+ 2 | 21 h recovery) S | × 2 | Veficle historical c | ontrol ranges |
|----------------------|-------------|--|-----------------------------------|--|------------------|--|---|
| (μg/mL) | | | | | | O C donoi | rs) 🍣 💍 🥌 |
| | | | Total | Frequency of | Cyte | Feb 17 Feb 18 | ®% MNBN |
| | | | MN- | MN-BM (%) | (%) [*] | | |
| | | | BN | R' & & | 8 | | |
| Vehicle | A | 1000 | 3 @ | | | No. of expts | % , 17 |
| | В | 1000 | 6,5 | ⁷ 0.60 | '0' ¥ | @wmb@of cultures | © ^y 40 |
| Total | | 2000 | 9 | Mean 0.45 🔏 | y 0 | [™] Mean ±SP | 0.40 ± 0.35 |
| 200 | A | 1000 | ~~~6 | O \$60 \$ | | Anin Orax. 🗸 | 0.00 - 0.80 |
| | В | 1000 | 5 | 0.50 Nean: 0.55 | | 95% reference range | 0.00 - 0.70 |
| Total | | 2000 | 140 | √Mean: 0 55 | Mean 0 | | |
| | | 2009(8) | -4/7/P | 10un. 0.55 | y 11100y11.00 | | |
| 1000 | A | 1000 | 4 | S 040 S | | | |
| 1000 | A B | | 5 | ©.50 ~ | | Positive distorical c | ontrol ranges |
| 1000 Total | | 1000 | 4 5 9 | S 040 S | Mean: 0 | Positive distorical c | |
| | | 1000 1000 2000 1000 | . 6 | 0.50 × | | Positive distorical c | rs) |
| Total 1613 | В | 1000 1000 2000 | © *5 & | 0.50 Mean: 0.50 0.50 0.50 | Mean: 0 | Positive distorical c (3 donor MMC (0.3 µ Feb 17 – Feb 18 | rs) |
| Total 1613 | B A | 1000 1000 2000 1000 | . 6 | 0.50 Mean: 0.45 0.50 | | Positive distorical c (3 donor MMC (0.3 µ Feb 17 – Feb 18 | rs) g/mL) |
| Total 1613 Total | B A | 1000 2000 1000 1000 2000 | 5 & 10 \(\sqrt{2} \) tear trend: | 0.50 Mean: 0.50 0.50 0.50 | Mean: 0 | Positive distorical c (β donor MMC (0.3 μ | rs) g/mL) % MNBN |
| Total 1613 | B A | 1000 2000 1000 1000 2000 | 5 10 vear trend: | 0.50 Mean: 0.55 0.50 0.50 Mean: 0.50 | Mean: 0 | Positive bistorical c (β donor MMC (0.3 μ Feb 17 – Feb 18 No. of expts | rs) g/mL) % MNBN 21 |
| Total 1613 Total | B A | 1000 2000 1000 2000 1000 2000 1000 1000 | 5 10 ear trend: 58 | 0.50 Mean: 0.45 0.50 0.50 Mean: 0.50 Moan: 0.50 0.50 4.90 | Mean: 0 | Positive distorical c C donor MMC (0.3 µ Ftb 17 – Feb 18 No. of expts Oumber of cultures | rs) g/mL) % MNBN 21 40 |
| Total 1613 Totat MMC | B B B A B B | 1000 2000 1000 2000 1000 1000 | 5 10 vear trend: | 0.50 Mean: 0.45 0.50 0.50 Mean: 0.50 Mean: 0.50 Mean: 5.50 Mean: 5.20 | Mean: 0 | Positive distorical c (donor MMC (0.3 µ Feb 17 – Feb 18 No. of expts Oumber of cultures Mean ±SD | rs) g/mL) % MNBN 21 40 5.57 ±1.74 1.50 - 9.20 2.57 - 8.52 |

Table CA 5.8.1/24-3: Spiroxamine aninodiol: human lymphocyte micronuclei assay: 3 h (+ 21 h recovery) +89 treatment and laboratory historical control data

| «Conc. (μg/mL) |) | _ ^ | 3 h (+√ | h recovery | Vehicle historical control ranges (♂ donors) | | |
|-------------------|----|------------------|----------------|-------------------------|---|---------------------|-----------------|
| d | | Total BN | MN- BN « | To equency of MN-BN (%) | Cyto. (%) | Feb 17 – Feb 18 | % MNBN |
| Vehicle 🖔 | A | @¥000 | U 4,2 | 0.40 | | No. of expts | 16 |
| 47 | B |) 100 <u>0</u> | | 0.20 | | Number of cultures | 40 |
| Aptal | | ″ 20 0 0″ | \$ 6 | Mean: 0.30 | 1 | Mean ±SD | 0.40 ± 0.36 |
| 200 0 | ØΑ | 1000 | <u>گ</u> 4 | 0.40 | | min. – max. | 0.00 - 1.00 |
| , Ç | В | 1000 | 5 | 0.50 | | 95% reference range | 0.10 - 0.90 |
| Total | | 2000 | 9 | Mean: 0.45 | Mean: 0 | | |
| 1000 | Α | 1000 | 3 | 0.30 | | | |
| | В | 1000 | 5 | 0.50 | | | |



| Total | | 2000 | 8 | Mean: 0.40 | Mean: 0 | Positive historical control ranges (3 donors) | |
|-------|------------------------|------|----|---------------|----------|---|------------|
| 1613 | Α | 1000 | 6 | 0.60 | | CPA (3 μg/mL) ¹ | |
| | В | 1000 | 5 | 0.50 | | Feb 17 – Dec 17 | % MNB |
| Total | | 2000 | 11 | Mean: 0.55 | Mean: 0 | No. of expts | 220) |
| | Linear trend: p 0.1410 | | | | | Mean: | 40 2 |
| CPA | Α | 1000 | 21 | 2.10 | | Mean ±S® | 2.21 ±0.85 |
| (7.0) | В | 1000 | 21 | 2.10 | | min. 🗝 nax. | 600-450 |
| 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 μ g/mL, the concentration analysed in this study, therefore the range for the highest CPA concentration normally analysed (3 μ g/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 \$\times 24 hour recovery) in the absence of \$\times 9\$ resulted in frequencies of \$\times 100 \text{NBN cells that were significantly } (p \le 0.05) higher than those observed in concurrent chicle control cultures for the highest two concentrations analysed (\$\times 000 \text{ and } 1613 \text{ fight.} \)

inducing 14% and 36% cytomxicity. These precesses were small such that the MNBN cell trequency of only a single replicate at 1613 \text{ fight.} \)

MNBN cell trequency of only a single replicate at 1613 \text{ fight.} \)

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 ammodiot human lymphocyte micronuclei assay: 24 h (+ 24 h recevery) - 59 treatment and laboratory historical control data

| inges | |
|----------------------|--|
| | |
| Jul 17 Jan 18 % MNBN | |
| NBN | |
| | |
| | |
| 6 | |
| 0 | |
| ±0.23 | |
| - 0.90 | |
| - 0.80 | |
| | |
| | |
| | |
| | |
| inges | |
| | |
| | |
| NBN | |
| 0 | |
| 1 | |
| ±2.38 | |
| 13.60 | |
| 13.00 | |
| | |

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 \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 2% 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 colated increase was therefore not considered of biological relevance Thyband et 4%).

Table CA 5.8.1/24-5: Spiroxamine aminodiol: human lyophocyte micronuclei assay: A h (+24 h recovery) -S9 treatment, micronucleus experiment 2 and laboratory historical control data

| | 1 | | | V | | |
|-----------|-------------------|--------------------|---------------------|----------------------|-----------------------|-------------------|
| Conc. | | 24 h (+ | 24 h recovery) – S | | Vehicle historical co | patrol ranges |
| (μg/mL) | | | A (Q . | Y ~ | l 🤊 🤝 don Asi | *s) _ &_' |
| | Total | Total | Frequency of | © to. & | Jul 17 - Lan 18 | % ∕MNBN |
| | BN | MN_© | MN BN (%) | \$(%) _. © | | & |
| | | MN-© BN | | | | 0' |
| Vehicle A | 1000 | 2 | 0,29 | ¥ | No. of expts 🖔 | 16 |
| В | 1000 | × 1 | Mean: 0 5 | | Number of Cultures | 40 |
| Total | 2000 | 3 | Mean: 0 🗗 | Ž - Ö | Mean ±SD | 0.34 ± 0.23 |
| 400 A | 1000 | 46 | ∜ 0.40 × | | O mm/ – max. | 0.00 - 0.90 |
| В | 1000 | £2 | S 020 2 | | , 95% reference range | 0.00 - 0.80 |
| Total | 20 00 , | 0 6 | Mean: 0:30 | Mean: D | Positive historical c | ontrol ranges |
| | | | viean. 0.30 | Wiean. | 💸 🗸 (ð donor | rs) |
| 1000 A | 7 100 % | <i>"</i> © | 0.960 0 | | Ø MMC (0.2 μ | g/mL) |
| ∂B | 1000 | 7 . | 0 .70 🔊 | | Dec 14 – Jan 18 | % MNBN |
| Total | 2000 | ~ 16~~ | 0.70 Mean: 0.80 | Mean: 2 | No. of expts | 16 |
| 1400 A | 1000 | <u>,</u> 26) | , T | | wumber of cultures | 29 |
| B | 1000 | | | | Mean ±SD | 31.18 ± 10.58 |
| Total | 20 00 | , ⁸ 6 🔊 | Mean: 0.300 | M@an: 47 | min. – max. | 16.35 to 52.50 |
| | Q Lja | ear trend: | p 0,0631 | 2 | 95% reference range | n/a |
| MMC A@ | 1000 [©] | 56A | 56.40° , | O' O' | VIN (0.04 μ | g/mL) |
| (0.20) B | 1000 | 9 44 ^ | y 5 4 ,40 ⊗ | | Feb 17 – Jan 18 | % MNBN |
| Total | 2000 | 1108 | Mean; 955.40 | Mean: 52 | No. of expts | 20 |
| | | | 35.40 | | * | |
| VIN A | 1000 | 26 8 | ~ 6 .8 9 ~ | 7 | Number of cultures | 41 |
| (0.04) B | 1000 | \$779 °s | © 20,90 <i>≈</i> ©′ | | Mean ±SD | 6.43 ±2.38 |
| Total | 2000 | 147 °C | Mean: 7.36** | Mean: 53 | min. – max. | 2.50 to 13.60 |
| | | | , Q' | | 95% reference range | 2.80 to 13.50 |

^{***} p<0.001

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

D. Deficiencies:

Whilst to deemed a deficiency, it is considered prudent to detail the rationale for undertaking an extended treatment which included a recovery period.

>HC: exceed historical control

For VINOastorica, control range, refer to Table CA 5.8.1/09-5



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 an extended 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 perpheral 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 lymphocyte's 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. Out 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 defection of chemicals that either act of 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 arabidoside are negative in the invitro pocronucleus assay with the 24 + 0 h approach, but positive with the with 24+34 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., 20198), with the OECID test guideline to be updated to reflect this change.

Assessment and conclusion by applicant:

Assessment: Study meets the current guidance and the requirements in 283/2913.

Conclusion: It is concluded that spirovamine aminotifol 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 activations stem (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 micronucle; 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: | _ 0 |
| Report Year: | 2013 |
| Report Title: | KWG4168-aminodiol - Exploratory 28-day toxicity study in the rat by dietary |
| | administration & |
| Report No: | SA 13129 |
| Document No: | <u>M-471499-01-1</u> |
| Guideline(s) followed in | OECD 407; EEC Directive 96/54/EC, Method B.7 |
| study: | |
| Deviations from current | Yes V V |
| test guideline: | Methods: SANCO/3029/99 rey; 4 |
| | No linearity data, no chroma@grams and method only briefto described. |
| | Toxicology: A number of deficiencies are noted (toffer to Deficiencies' section & |
| | below) |
| Previous evaluation: | yes, evaluated and accepted of the second of |
| | RAR (2017) O' W ZY ZY ZY ZY |
| GLP/Officially | No, not conducted under GPP/Officially recognised testing facilities |
| recognised testing | |
| facilities: | |
| Acceptability/Reliability: | Yes O V V V V V V V V |

Executive Summary

In this study, spiroxamine aminodiol was administered commoust, 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: 0/6, 3.18/551, 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 grownecropsy and full histopathology.

Unlike the cycloboxane timethol ethal 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 arines (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 vasily reduces the overly H and pKa values observed with both the parent and M03, and therefore the mucosal menthrane 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. amission of the cycloboxane 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 NOAFL 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.

Material and Methods

A. Materials

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



98.14% (w/w) (correction for purity not undertaken) **Purity:**

CAS No.: Not assigned

Confirmed stable for the duration of the study (expiry date: 7 December 2013) Stability of test compound:

2. Vehicle and/or positive

control:

Basal diet / not relevant

3. Test animals:

Species: Rat Strain: Wistar 6-7 wks Age at dosing:

Weight at dosing: ♂: 286-329g; ♀: 177-21\$

Source:

Acclimation period: 11 days

A04CP1-10 powdered diet form Diet:

France) ad libitum

Municipal water, ad libitum Water: Housing: Individual housed

4. Environmental conditions:

> **Temperature: Humidity:** Air changes:

12 hour light/dark **Photoperiod:**

B. Study Design:

1. In life dates:

2. Animal assignment and treatment:

July 2013 to 15 October 2013 (experimental dates)

rm Scientific Animal Food and Engineering, After an acclimatisation period rats were allocated to groups by randomisation. Dose levels selected toonable Simparison with the parent compound, spiro@amine_1

The test article, spiroxamine aminodiol was administered continuously via the do to groups of rats for period of 28 days. Animals (10/sex/gp), were administered test diet af concentrations of 0, 45, 135, 400 ppm (equivalent to 3/2, 3/8/3.51, 42/10, treament in animals/sex were subjected to complete necropsy. Body weight, food consumption were measured at regular intervals, with FOB and motor Octivity included. Clinical pathology evaluations (haematology, clinical cherrystry and urinarysis) were performed with all surviving animals subjected to complete gross decropsy and full histopathology.

Body weight change parameters, terminal body weight, organ weights, Traematology Farameters (PT, Hb, haematocrit, MCV, MHC, MCHC, %neutrophils, %lymphocytes, %reticulocytes), clinical chemistry parameters, utinalysis (volume, refractive index), FOB assessments:

Barlett Test

∡If not si@nificant:

- ANOVA. If not significant no further statistical analysis
- If ANOVA significant, Dunnett test
- 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 of transformed data
- If Bartlett test on mansformed data significant Kanskal-Wallis test.
- If Kruskal-Walks test not significant no further statistical analysis
- If Kruskal-Wallis test significant, Dunn test

Urinalysis pH parameter:

- Kruskal-Walls. If not significant no farther statistical analysis
 - If significant, Dunn test

Table CA 5.8.1/18-1: Overview of 60b-acute toxicity study in rate treated oralle via diet) with spiroxamine amprodiol: study design and dose received

| Danamatana | <i>@</i> , | m) 🔗 🦃 🛭 | | ppm) 🖟 | |
|----------------|------------|--------------------------|--|--------------------|----|
| Parameters | 0 3 45 4 | 400 × | 20 6 45 | 135 40 | 0 |
| Dose/animal | 0 348 | ©9.42 € 28 04 | 3,50 | <u>6</u> 10.81 31. | .4 |
| (mg/kg bw/day) | | | | | |
| Animals | 10 10 | 10 10 | / _10 \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ | 10 10 |) |
| assigned/sex | | | | " | |

C. Methods:

1. Test article preparation and analysis:

2. Observations;

3. Body weights:

The spirogamine an inodical was prepared at dietary concentrations of 0, 45, 135 and 600 ppm. The prepared test diets were prepared once and stored frozen capproximately -18°C until bore 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 wrifted at the lowest and highest concentration, with the mean homogeneity vale taken to confirm measured concentration (refer to Doc MCA Section 4 [Mc/1499/01-1] for method validation).

Animals were inspected wice daily for signs of toxicity and mortality.

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 collowing equation:

 $\text{Cpd-consumption}_{\text{(five/g}/kg | byv/d)} = \frac{\text{Food consumption (g/rat/d) } x \text{ test article conc. (ppm)}}{\text{Body weight (g)}}$

Food efficiency was not calculated.

5. Water consumption:

Not conducted.

6. Ophthalmological

Conducted prior to the start of dosing and during week 4.

examination:
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.



<u>Home cage observations</u>: posture, co-ordination or movement/gait, tremor and conclusion, abnormal behaviour.

<u>During handling:</u> 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, offictory test, examination of catalepsy, right response, tail pinch.

Motor activity:

Spontaneous motor activity was recorded turing the 1st 60 minutes, with data collected at regular intervals throughout the session.

At the end of the freatment period blood was collected Animals were lasted prior to blood sampling.

rior to blood sampling.

Haematotogy: red blood cell parameters (haematocrit (Gmmost) termed PCV), haemoglobin concentration (Hby, mean raemoglobin concentration (MHO), mean corpuscular baemoglopin concentration (MCHC), mean corpuscular volume (MCV), plate of courts, while blood cell parameters (total and differential (newtrophils, lymphocytes, eosinophils, hasophils, monocytes) leakocyte count) coagulation parameters (prothrombin time (PT) A blood smeat was prepared and standed using May-Grünwold-Giemsa and examined if baematotogical parameters were abnormal. Chnical Chemister: electrolytes (sodium potassium, calcium, chloride, Thosphorus), kidney function test (creatining urea), klucose, liver function test Calbumin, globulin [A-5], alk dine phosphatase (ALP), alanine ammotransferase ALT formmonly referred to a glutamic pyruvic transaminase (GPT)]), aspartate aming transferase (AST [commonly referred to as glutamic oxalogoetic transaminase (GQT), γ-glutamyltransferase (γ-GTØ, total bilirubin (T.Billo, total protein (VP), lipid profile (total cholester@, triglycerides).

An the end of the treatment period, animals were placed in metabolism cages overnight and Osted. The following usinary parameters were measured: specific grayity, pH, refractory index, total volume, protein, glucose, ketones, billiabin, bood, urobilingsen, sectiment, colour, leukocytes, erythrocytes, lacteria, chithelia cells Casts, crystals

Adrenar glands, brain epididonides, heart, kidney, liver, ovary, pituitary gland, prostate gland, spleen, testis, thymus, thyroid gland (+parathyroid), uterus

At the end of the treatment period gross pathological examination was performed or all anithals and included examination of the external surface, all orithes and associated tissues. In addition, kidney, liver, lung, thyroid gland were examined with intermediate dose groups.

The following sissues 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 and, epididymis, testis:

Accessory sex glands (3:epididymides, prostate, seminal vesicle, testes; \$\varphi\$: 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)),

8. Haematology and clinical chemistry:

9. Urinalysis:

10. Organ weights:

11. Sacrifice and pathology:



<u>respiratory system</u> (nasal cavities, larynx/pharynx ,trachea, lung), <u>urogenital system</u> (kidneys, urinary bladder), <u>other</u> (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 cytochrope 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 annuals. Phase II enzymatic activities were determined by measuring UDP T (UDP-glucuronosyltransferase) with 4 patrophenol as Substrate.

12. Neurohisto-pathology:

No specific neurohistopathology with specific fixatives were performed in addition to the standard historathology undertaken on neuronal tissues.

Results and discussion

A. Test diet analysis:

Spiroxamine aminodiol was homogenously distributed and chendrally cable 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 +15% of naminal concentrations which were within acceptable limits

B. Observations:

1. Clinical signs of toxicity:

No tost articlo related effects observed

2. Mortality:

No test article related effects upon mortality were observed, with all animals surviving until schedoled sagrifice.

No test article related effects observed

3. Ophthalmoscopic examination:

4. Neurological functional examinations

Functional observation batters

- Home cage, open field arona 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

So treatment related change it group mean values of activity counts of all intervals were observed.

C. Body weight and body weight gains

No treatment placed effects in body weight were observed, with all animals gaining weight over the treatment phase.

Table C. S.8.1/18-2: Overview of sub-acute toxicity study in rats treated orally (via diet) with spiroxamine aminodiol, body weight effects

| | | W | <i>y</i> | | | | | | | |
|----------|-----------------|--|----------------|----------------|----------------|----------------|----------------|----------------|----------------|--|
| D 4 | | The state of the s | (ppm) | | | | ♀ (ppm) | | | |
| Гага | Parameters 0 | | √ 45 . © | 138 | 400 | 0 | 45 | 135 | 400 | |
| Dose | /animat | | 3.18 | 9.42 | 28.4 | 0 | 3.51 | 10.81 | 31.4 | |
| (mg/kg | bw/day) | | ,,,,,, | <u></u> | | | | | | |
| Body | 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 | |
| wt (g) ≈ | Day & | $[350] \pm 17.1$ | | | 353 ± 21.2 | 215 ± 11.0 | 213 ± 8.9 | 216 ± 15.4 | 211 ±7.9 | |
| × | √Day ∱ ≸ | 390, ₹16.7€ | 395 ±24.4 | 391 ±21.8 | 397 ±27.7 | 237 ± 10.3 | 231 ± 11.6 | 237 ± 15.1 | 230 ± 12.9 | |
| | Da 22 | 40/8 ±19.% | 425 ±27.7 | 420 ± 23.3 | 429 ± 26.7 | 248 ± 14.7 | 244 ± 12.9 | 249 ± 16.5 | 245 ±13.7 | |
| | D ay 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 | © Day | 132 | 141 | 133 | 146 | 66 | 54 | 59 | 56 | |
| wt gain | 0-29 | | (†7%) | (†1%) | (†11%) | | (18%) | (\11%) | (\15%) | |
| (g) | | | | | | | | | | |



sumption:

as on food consumption was obs.
as due to fasting for unite collection.

J. a qualitative assessment from foodyfon.
as suggestive that food efficiency wasprot aft.

Joegical relevant changes were observed following 159-23-48.
Are period.
Are period.
Activological relevant shanges were observed following 159-23-48.
Attending the collection of



Table CA 5.8.1/18-3: Overview of sub-acute toxicity study in rats treated orally (via diet) with spiro amine aminodiol; selected organ weighted in the contraction of

| | | | | • | , | | | | |
|------------------------|-----------------|---------------------|--------------------------------------|----------------------------------|------------------------------------|---------------------|---------------------------------------|--|--|
| Parameter | re. | ♂ (ppm) | | | | (ppm) | | | |
| Farameter | 13 | 0 | 45 | 135 | 400 | 0 | √O [©] 45 | 135 A | 400 |
| Dose/anim (mg/kg bw | | 0 | 3.18 | 9.42 | 28.4 | | 3.51 | 10.81 | \$ 31.4 |
| Terminal b | owt (g) | 415 ±22.7 | 425 ±33.2 (↑2%) | 417 ±25.9 (-) | 129±29.9 0 (†3%) | 24X+14.3 | 236 ±10.5 | 241 ±18.5 | 289 ±12.5 (\)1%) |
| Liver | Abs (g) Rel. | 10.293 ±0.847 | 10.770 ±1.017 (↑5%) | 10.750 ±1.018 (†4%) | 10.4%9±1.14 (†2%) | 6.077 ±0\$53 | \$753 ±0.306 (15%) | 6.431±0.581© | 6.083 ±0.881 (-) |
| | (g%) | 2.478 ± 0.148 | 2.539 ± 0.142 ($\uparrow 2\%$) | 2.5%0\(\frac{1}{4}\%\) | 2.501 ±0.146 | 2.511 ±0 ±0 ±0.5 | 2 43 ±0.0956 (\)\(\)\(\)\(\) | 2.539¥0.122 (↑1%) | 2.539 ± 0.221 (1%) |
| Adrenals | Abs (g) | 0.0570 ± 0.013 | 0.0592 ±0.011 | 0.0634 0.0045 | 0.0599 ±0.0046 | 0.0734 ±0.0083 | 0.070400.0072 | 0.0790 +0.0099 | 0.0709 ± 0.0096 |
| | Rel. (g%) | 0.0137 ± 0.0032 | 0.0139 0.0023 | (111%) (153 ±0.0016 (130%) | (\$5%) 0.0040 ±0.0015 (\$2%) | 0.030€€0.0037 | 0.0299 0.0030 | 08%) 00328 ±0.0037 (↑8%) | $ \begin{array}{c} (\downarrow 3\%) \\ 0.0297 \pm 0.0040 \\ (\downarrow 2\%) \end{array} $ |
| Testes | Abs (g) | 3.418 ±0.311 | 3.256 ±0.912 | 3.671 ±0.702 (↑7%) | 3.56 E 0.320 (†4%) O | | | - | - |
| | Rel. (g%) | 0.825 ±0,0884 | 0.764 ±0.2050 (\17%) | 0.879 ±0.157 (17%) | 0.831 40.058 | | L S | | |
| Epidid. | Abs (g) | 1.174 ± 0.119 | 1.094±0.233 × (17%) (17%) | 1.138 ±0.147 | 1.152 ±0.0\(\frac{1}{2}\)%) | | - - | - | - |
| | Rel. (g%) | 0.284 ±0.0339\$ | 0.258 ±0.0547 | 0.273 ±0.0938 | 0 ±0.0268 (15%) | er we | | | |
| Prostate | Abs (g) | 0.469 ±0.114 | \$0.444 ±0022 | 0 434 ±0.1360° | 0,4% ±0.107 | - - - | - | - | - |
| | Rel. (g%) | 0.113 ±0.028 | 0005 ±0.033 | | 0.116 4 0.024 | | | | |
| Ovary | Abs (g) | - 15 102 | a P- ef | | OTTO | 0.0933 ± 0.0124 | 0.0824 ± 0.0140 | 0.0892 ± 0.0208 | 0.0857 ± 0.0099 |
| | Rel. (g%) | | | | - | 0.0384 ± 0.0037 | (\12%) 0.0350 \pm 0.0059 (\19%) | $ \begin{array}{c} (\downarrow 4\%) \\ 0.0370 \pm 0.0078 \\ (\downarrow 4\%) \end{array} $ | $ \begin{array}{c} (\downarrow 8\%) \\ 0.0359 \pm 0.0042 \\ (\downarrow 7\%) \end{array} $ |
| Uterus | | | | | - | 0.561 ±0.244 | 0.541 ±0.257 | 0.588 ± 0.273 | 0.553 ± 0.225 |
| « | Abs (Q) | | 7 - 7 P | <u>-</u> | - | 0.232 ±0.0991 | (\\d\%) 0.230\pm 0.106 | $(\uparrow 5\%)$ 0.243 ±0.110 | (\psi 1%) 0.232 \pm 0.095 |
| | (g%)(\$) | | \forall | | | | (\1%) | (†5%) | (-) |



| | | | | | | | | , O | , ale ° |
|-------------------|---------|---------------------|---------------------|---------------------|--|---------------------|---|-----------------------|---------------------|
| D | | | ∂ (p | pm) | | a Ġ | | pm) | |
| Parameters | | 0 | 45 | 135 | 400 | 0 % | 45 | 135 | 400 0 |
| Pit. gland | Abs (g) | 0.0114 ± 0.0007 | 0.0119 ± 0.0020 | 0.0111 ± 0.0013 | 0.0112 ± 0.0013 | 0.0023 ±0.0017 | 0.012 ±0.0025 | 0.0121 \$0.0024 | 6 16 ±0.0015 |
| _ | | | (†4%) | (\13%) | (↓2%) | 9-7 | \bigcirc \bigcirc \bigcirc \bigcirc \bigcirc \bigcirc \bigcirc \bigcirc \bigcirc \bigcirc | 2%) | (↓6%) |
| | Rel. | 0.0027 ± 0.0002 | 0.0028 ± 0.0004 | 0.0027 ± 0.0004 | $0.0026 \pm 0.0003^{\circ}$ | 0.0051 ±0.0007© | 0.051 ± 0.0009 | ©049 ±0.00097 | 0.0949 ± 0.0006 |
| | (g%) | | (†4%) | (-) | (\J4%P * | sė° av ' | & ∘(-) | (1\P)\b) a | (↓4%) |
| Thyroid | Abs (g) | 0.0169 ± 0.0028 | 0.0168 ± 0.0024 | 0.0173 ± 0.0024 | 0.0179±0.0027 | 0.0141 (20.0020) | 0.0124 ±0.0016 | 0.6042 ±0.0025 | 0.0136 ± 0.0024 |
| | | | (↓1%) | (†2%) | (16%) D | | (412%) | \$\(\frac{120}{\pi}\) | (↓4%) |
| | Rel. | 0.0041 ± 0.0007 | 0.0040 ± 0.0007 | 0.0041 ±0.00045 | \$0.004 2 30 .0006 € | 0.0058 ±0.0008 | 0.0003 ±0.0008 | | 0.0057 ± 0.0010 |
| | (g%) | | (↓2%) | (-) | (************************************* | | (\J9}\P | \ \(\(^{3\%}\) | (↓2%) |
| Aba : abaaluta | | | | @ V | . Enidad oni | المستعمدة المستعمدة | O | | |

The may be good of the production of the product of the production of the product and use of this document may the owner.

| A commercial exploitation and violate the right permission and violate the prohibited Furthermore, any commercial exploitation and use of this document. any string the permise ion of the owner of this and the commercial exploitation of the owner of the commercial exploitation of the commercial c the permission of the prohibited and wiolate the prohibited



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 dimethylethyl 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 he 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 solithility, and with a smaller chemical structure (162 omission of the cyclohexane dimethyl ethyl group) results in rapid absorption, with a fixely scenario of absorption between mucosal cells within the pastric divironment, 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 ressue showed to treatment related effects at on either total cytochrome P450, Phase I (EROD BROD BROD) or Phase II (UDP-CT) 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 sest guideling makes reference to determination of serum thyroid hormones (T3, T4, T9H). These were not analysed. However, the gold standard to assess thyroid effects is hostopathological analysis. This was undertaken on all animals and revealed no histopathological findings. Consequently the lack of serom thyroid hormone analysis is not deeme Ocritical, with potential theroid effects adequately addressed with histopathological analysis.
 Whilst it can be argued that a maximum tolerated dose was not achieved, the objective of this
- Whast it can be argued that a maximum tolerated dose was not achieved, the objective of this soudy was to enable a direct to cological 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 exidence and the requirements in 283/2013.

Conclusion: Under the conditions of this suitly the NOAEL for spiroxamine aminodiol following 28 days of oral (via Met) treatment is deepned to be 400 ppm (equivalent to 28.4/31.4 mg/kg bw. day for males) based on adverse effects when tested up to the maximum dose level.



| Data Point: | KCA 5.8.1/19 |
|----------------------------|--|
| Report Author: | |
| Report Year: | 2013 |
| Report Title: | KWG 4168-aminodiol: Oral (gavage) prenatal developmental toxicity study in |
| _ | the wistar rat |
| Report No: | 10939 |
| Document No: | <u>M-472720-01-1</u> |
| Guideline(s) followed in | US-EPAOPPTS 870.3700; OECD 414; Health Canada PMRA DACO 4.52; |
| study: | JMAFF 12 Nousan No. 8147; Et 8/302/EEC 0 0 0 |
| Deviations from current | Yes |
| test guideline: | Methods: SANCO/3029/99 10 4 |
| | Accuracy n = 4 |
| | No calibration data |
| | Toxicology: Yes |
| | Accuracy n = 4 No calibration data Toxicology: Yes The test guideline OBCD 416 was undated 2018, the following deficiencies were noted: |
| | noted: |
| | - I hyroid gland weights and histopathological assessment of every dams reated |
| | noted: -Thyroid gland weights and histopathological assessment of every dan reated dam not performedThyroid hormone measurements of dams not undertaken. |
| | -Thyroid hormone measurements of dams not undertaken. |
| Previous evaluation: | yes, evaluated and accepted |
| Flevious evaluation. | RAR (2017) |
| GLP/Officially | Yes conducted under GLP officially recognised testing facilities |
| recognised testing | Tex conducted under OLI fornicially recognised testing facilities |
| facilities: | |
| Acceptability/Reliability: | Yes |
| receptuolity/iteliaolity. | |

Executive Summary

In an embryo-fetal developmental toxicity study, spiroxamine aminodiol was administered to female Han Wistar rats (2)/group) by oral gavage once daily at dose levels of Odeionised 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: dinical signs, body weights body weight changes, food consumption, ovarian and uterine examinations, gravid uferine wights retal examinations, gross necropsy findings and histopathology.

Based on the results of this study, spiroxamine aminodial administered to rats at a dose of 500 mg/kg bw/day caused material 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 defined treatment related.

At dose level of 30 and 150 mg/kg bw day no adversity in clinical signs of toxicity, body weight or food consumption effects were wident 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 related 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 arteract of fixation/sectioning, with a single incidence occurring, this was the beely outcome fere. 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/cursed, 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 as considered to be 50 mg/kg bw/day based maternal mortality, body weight losses gaseous content of the of tract, and adverse clinical signs at a dosage level that exceeded the MTD (500 mg/kg) w/day).

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

Materials and Methods²

A. Materials:

1. Test Material: KWGA168-aminodial

diol, M28)

Description: Colongless liqui Lot/Barch No.: NI 9095 PI

Purity: 981% (w/w) (correction for purity undertaken, with a correction factor of

J.019)

CAS No.: Not signed

compound

2. Vehicle and/or positive Deionsed water / not relevant control

3. Test, animals:

Source:

Acclimation period: Jays

Diet: Certified Rodent Diet® #5002 (PMI® Nutrition International, USA) ad libitum

Water: Municipal water, ad libitum

Housing: Individually housed, except during cohabitation period with $1 \circlearrowleft$ paired with $2 \circlearrowleft$

overnight with sexually mature ♂.



4. Environmental conditions:

> **Temperature: Humidity:** Air changes:

Photoperiod:

B. Study Design:

1. In life dates:

2. Animal assignment and treatment:

23 September 2013 to 25 October 2013 (experimental dates)
Upon arrival, rats were assigned to individual housing on the basis of computer-generated randomisation. After an acclimatisation period (not stated) virgin ♀ rats were paire 2:1 with breeder rats of the same source. The which spermatozoa observed in a smear of the vaginal content copulatory plug observed in situ were considered to be brought was designated when dame ach group consisted of 27 ♀ group he dose levels were select which sputoxame 0, 800.

480, 800 mg/kg/pw/day/to 8 pregnant rats/group from 60 6-19 At 800 mg/kg bw/day single animal showed resistance to do sing which contributed to mis-dosing/perforation of the oesophagus if 3/8 rats. Two drimals were found dead on GD/7, with a third animal killed in extremis on GD 8. Doe 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 MCD was dientified, an additional 5 time mated animals were dosed from GD 64o 14 only at 540 mg/kg bw/dwy. No clear effoct on blody weight was obserced, 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 by day dose group also exceeded the MTD, and was therefore not suitable for the main stody. 🛴

Based on these data Josages of 0, 30, 150 and 500 mg/kg bw/day of spicoxamine aminodiol were employed for the developmental toxicity study in ras, using a dose volume of 10 mL/kg by. Surviving dams were sacrificed on (GD 20℃

3. Statistics:

Containous data:

Posametric (e.g. dom bod weight food consumption):

If not Sprificant no further statistical analysis

If ANOVA significant, Dunnett test

Non-parametric (e.g. litter size, number of corpora lutea):

Kraskal-Walis test

If not significant no further statistical analysis

If significant Dunn test

on-pakametric 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 <u>Modices:</u> All appropriate indices were calculated from caesarean section records of animals in the study:

$$\frac{\text{% pre-}}{\text{implantation loss}} = \frac{\text{No. of } corpora \ lutea}{\text{number of } corpora \ lutea} x \ 100$$

No. of implantation no. of live fetuses



| | % post- | No. of implantations | |
|--|--------------------------------|--|-----------------------------|
| | implantation loss = | | |
| | | | |
| | Sex ratio = | No. of A fetuses / No. of Seruses | 200 A |
| | | Total no. of fetuse | |
| | | Total no. of fetuses | |
| C. Methods: | | No. of ♂ fetuses / No. of ♀ fetuses Total no. of fetuses tions were prepared on weekly basis (1 o da 15 50 mg/mL. Spirovamine aminodic was | |
| 1. Test article | The test article formulat | tions were prepared on Weekly basis (1 🕏 da | vsari "Ö "O |
| formulation | concentrations of 0, 30, | 15 50 mg/mL. Spiroxamine aminodio was | wenghed , O |
| preparation and | into a glass beaker, with | i velugere added. I onewing preparation the s | TWOIR SO OF |
| analysis: | formulations were refrig | gerated (2-9°C). The mixture was homogen | zed using a |
| | magnetic stirrer during | hosing. Verification of Concentration and ho etermined as part of the method validation | megeneity |
| | course of the dose range | e-finding stady. One day stability as room to | emperature |
| | | over a concentration range f 0.080 – 120 m | |
| | confirmed (refer to Doc | MCA Section 4 [M-472720-051] for meth | nod® Q" |
| | validation). Concentrati | on of test article in the dose formulation wa | s P |
| | | forth of the vatches prepared. | , |
| 2. Observations: | Maternal Observations: | The animals were checked for mortality w | ice caily. |
| | neriod on GIVO and GI | for general appearance (wice during the acc 0 5, 60 6 (p.f.) r to and 1 how post dosing) | and then |
| | daily thereafter. | The state of the s | , |
| | | nined for sex and external abnormalities. D | ead fetuses |
| | and lateresorptions were | e examined for sex and external abnormalit | |
| | extent possible. | | |
| 3. Body weights: | Moternal Body weights: | Recorded daily from GD 0 through to GD | 19 and prior |
| ĮŽ. | to necropsy on ©D 20. | Jacobson Company | |
| Ď, | | y weight of such fetus was recorded. Fetuse vith hitter number and uterfine distribution | es were |
| 4. Food consumption: | | ing periods GD \$26, 6-9, 9-12, 12-15, 15-18 | 8 and 18-20 |
| Tool consumption | | tion data, compound consumption was calc | |
| , Q | the following equation: | | <i>B</i> |
| | Mean daily food c | consumption = Food consumption (g rat p) | er period) |
| | | Days per period | |
| 5. Water consumption: | Not conducted & | | |
| 6. Ophthalmological | Not conducted \(\) | A A | |
| examination O | | | |
| 7. Mating performance: | Evaluated daily during t | he comabitation period. Dams were sacrific | ed on day |
| 0 H | 20 gg-gestanon. | Ž. | |
| 8. Haen@tology and clinical chemistry. | Not conducted | \mathbb{Q}^{y} | |
| 9. Upinalysis: | Not conducted S | <i>!</i> | |
| 10. Organ weights: | '() () · | nimals, with gravid uterus, placenta, liver a | nd kidnev |
| 11. Maternal sacrifice | | aminations: the uterus was opened and the o | |
| and pathology: | | uses were removed from the uterus and place | |
| | individual containers (o | r a tray). The ovaries and uterus were exam | ined for |
| | | of corpora lutea, implantation sites, placer | |
| | ©olor or shape), live and | dead fetuses, and early and late resorptions | s. An early |
| and pathology: | A late resortion was defined a | as one in which organogenesis was not grossefined as one in which the occurrence of org | siy evident. Zanogenesis |
| ¥ _~ | 11 late resorption was de | rinion as one in winon the occurrence of org | , |

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.

<u>Necropsy</u>: 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% reutral baffered formalin

<u>Histopathology</u>: the liver, kiddrey and gross tesions were of dams were perfored in 10% NBF and retained for possible histopathology examination

12. Fetal sacrifice and pathology:

Fetuses were euthanized by *ip* injection of sodium pentovarbital.

Approximately one-half of the fetuses in each litter were examined for viscoral abnormalities by using a modification of the micro-dissection technique of Wilson (1965). Each fetus was fixed in a fouring solution and then preserved in a solution of ethan alcohol. The remaining fetuses (approximately one half of the fetuses in each litter) were examined for skeletal abnormalities after staining with alizacin red Sand alcohol 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 cherocally stable for at least 7 days (refrigerated) and within the concentration range of to 50 mg/ral. 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 nonunal concentrations which were within acceptable limits.

B. Maternal toxicity:

1. Clinical signs of toxicity:

Clinical signs of toxicity were united to the 500 mg/kg bw/day dosage group. Rales were exident from GD \$20 in 10/27 dams. Of the dams that were found dead, kille to extremis two dams \$\pm\text{906} \pm\text{93}\$) exhibited gasping on GD 14 or

Due to the seventy of clinical signs of soxicity, 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 indertaken.

All other clinical signs reported were considered unrelated to treatment.

Two dams (#14, #363) in the 500 mg/kg bw/day dosage group were found dead or GD 16 and 19 respectively. Two further dams (#93, #906) were killed in extremis on GD 14 and 15.

3. Ophthalmoscopic examination:

Not ennducted,

4. Body weight:

2. Mortality

Weight effects were limited to the high dose group, 500 mg/kg bw/day. Everall group mean data were unaffected, but decrease body weight gain and by body weight loss was observed in some individual high dose dams.

Table (2.5.8.19-1: Overview of developmental toxicity study in rats treated orally (via gavage) with piroximine aminodiol: body weight effects

| Parameters | \mathcal{L} (mg/kg bw/d) | | | | | | |
|------------------------|----------------------------|----|-----|-----|--|--|--|
| Parameters | 0 | 30 | 150 | 500 | | | |
| No. of animals treated | 27 | 27 | 27 | 27 | | | |



| D. | 4 | | ♀ (mg/k | kg bw/d) | 0 |
|------------|----------|-----------------|----------------------------------|-----------------------|--|
| Parame | ters | 0 | 30 | 150 | 500 |
| Body wt | Day | | | | |
| (g) | 0 | 204 ± 2.5 | 204 ±2.8 (-) | 208 ±2.8 (†2%) > | 210 ±3.8 (\$\partial) \(\partial \) |
| | 6 | 224 ± 3.7 | 224 ±2.8 (-) | 230 ±2.7 (†3%) | 231 ±3,8 (†3%) |
| | 10 | 238 ± 3.7 | $236 \pm 2.9 (\downarrow 0.8\%)$ | 243 ±2.9 (†2%) | $236 \pm 5.6^{\circ} \downarrow 0.8\%$ |
| | 16 | 268 ± 3.5 | $266 \pm 3.2 (\downarrow 0.7\%)$ | 271 ±3.7 (14%) | 263 7.0 () |
| | 19 | 297 ± 3.8 | $296 \pm 3.7 (\downarrow 0.3\%)$ | 303 ±5.2 (\$\psi^2\%) | 299 ±7.7(11%) |
| | 20 | 308 ± 4.3 | 308 ±4.1 (-) | 318 ±5 Ø (↑3%) | 306 ±8.6 (10.6%) |
| Gravid ute | rine wt | 48.5 ± 3.63 | 55.2 ±2.44 (↑14%) | 62.4 ±2.49** (†29%) | |
| (g) | | | | | |
| Corrected | Day | 260 ± 3.4 | 253 ±3.8 (13%) | 259±3.56,0.4%) | 2 6 9 ±7.0 (78%) |
| body wt | 20^{a} | | | N OF R | |
| (g) | | | W 200 | | |
| Body | Day | | | | 7 4 |
| weight | 6-20 | 104 ± 4.2 | 108 ±2,6 (14%) | ±4.7Q↑6%) | 97/±25.47(*7%)(,° |
| gain (g) | 0-20 | 83.9 ± 2.73 | \$4.8 ±2,46 (↑2%) | 87.5 ±4.58 (↑5%) | 74.2 ±25\$3 (\12%) |
| Corrected | Day | 55.5 ±2.62 | √49.7±¥.02 (↓10%) | 50.9 41.66 (48%) | 39.6 ₂ ±4.6 (\20%) |
| body wt | 20^{a} | | | | |
| gain (g) | | ر ا | | | |

^{*} p \le 0.05; ** p \le 0.01

5. Food consumption:

6. Organ weight

7. Gross pathology:

8. Histopathology:

9. Caesarean section data:

Absolute and mean food consumption during the treatment period was not significantly affected in any of the cose groups.

Organ weights were limited to gravid uterus weight, placeta, liver and kidney from low and mid dose groups. Whilst an increase in absolute gravid uterine weight was observed for low wild 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 dodney relights 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.

For high dose group animals that either dred or were killed in extremis, gross subservations included gaseous contends 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.

Asingle 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 w/day and high dose group animals, this single incidence was deemed treatment related.

Not conducted

Pregrancy 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, precimplantation 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* 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.

a gestational body Weight Gravid attrine weight (note Crounding errors may result)



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

| _ | | \bigcirc (mg/kg bw/d) | | | | | | |
|--|-----------------------------------|--|--------------------------------------|----------------|--|--|--|--|
| Parameters | 0 | 30 | 150 | 50,00 | | | | |
| No. of animals mated | 27 | 27 | 27 | 27 . 7 | | | | |
| Animals pregnant and caesarean section on GD 21 | 24 | 25 V | 24 🗸 | | | | | |
| Animals with non-viable fetuses | 1 | | | | | | | |
| Unscheduled deaths | 0 | | | | | | | |
| Corpora lutea [/dam] | 299 [12.5 ±0.41] | 300 0 | 316 318 13.2±0.35] | | | | | |
| Implantation sites [/dam] | 245 [10.2 ±0.75] | 278 278 7 2131.1±05\$9 | [| | | | | |
| Total no. of litters | 24 | 25¢ 5¢ | 24 ^Q | | | | | |
| Mean litter | 9.4 ±0.73 | € 10.4 € 0.49 | 11.40=0.50 | | | | | |
| size/dam | 224@# | | | | | | | |
| Total live fetus [/dam] | 224 Ø (**) [9.3 *0 :75] | 259 210.4 ±0.49] | 273 ° 273 ° 6 | ~ - | | | | |
| Total live $\sqrt[3]{}$ / \mathbb{P} | 113/111 | 125/136 | 139/134 | <u> </u> | | | | |
| Total dead fetus | 2 | | | 2 - | | | | |
| Early/late resorptions [/dam] | 19 (0) [0.8 ±0.13 (0.0 ±0.0] | 19 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 | (25, / 0 (5)) [1.0±0.46 (0.0±0.0] | - | | | | |
| | | | @ 3. °° | 3 9 | | | | |
| Fetal wt (g) | 3.0° 3.4 * ±0.05 ±0.05 | 3.7 \$3.4 ±0.07 \$±0.03 | 3.6 ±0.06 \(\psi\)±0.05 | | | | | |
| Mean fetal wtog) | \$\times 3.6\times 0.05 \times | 3.6 0.07 | 3.70 0.05 | = | | | | |
| Sex ratio (%) | 50.4 | <u>√</u> 47.5 % | \$5 0.9 | - | | | | |
| % resorbed/conceptuses/litter | 50.4 Q 4.3 ±5 V | 2.5 ±5.8 | 6.1 ±14.9 | - | | | | |
| Total pre-/post- implantation loss (mean pre-/post-) | 54 721 (2.3 ±0.56) | 22,7 19 (c) 7 [009 ±0.30) & | △ × 18 / 25 | - | | | | |

C. Develor mental 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 behicle control and test article treated groups.

3. External examination: To fetal gross external alterations were observed in either the control, 30 or 150 mg/kg booday.

Table CN 5.8.1/19-3: A Overview of developmental toxicity study in rats treated orally (via gavage) with spiroxamine aminodiol: overview of fetal abnormalities (fetus/litter incidence)

| Parameters | Ç (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 | - | | | | | |



| Da a a 4 a a | | ♀ (mg/k | g bw/d) | o |
|------------------------|--------------------|------------------------------|---|------|
| Parameters - | 0 | 30 | 150 | 500 |
| Fetus/litter with any | 3/1 | 0/0 | 0/0 | - 🔊 |
| alteration (%) | (1.3/4.2) | (0.0/0.0) | (0.0/0.0) | |
| % Fetus with any | 2.6 ± 3.9 | 3.2 ±3.8 | 1.9 ±3.6 💸 | |
| alteration/litter (%) | | | Ø. | |
| | External | examinations (fetus/l | itter) | |
| Fetus/litters examined | 224/24 | 259/25 | 278/24 | |
| Abnormal findings | 0/0 | 0/0 | Ø 0/0 | |
| | Visceral | examinations (fetus/li | itter) 👸 🦼 | |
| Fetus/litters examined | 108/24 | 1 20 /25 | 128/24 | |
| Malformations (%) | 1/1 | ~ 0/0 | | - Z |
| | (0.9/4.2) | Q0.0/0.0) | y ~(0.0/0.0) | |
| Variations (%) | 12/8 | (15 <i>6</i>) 15. | | |
| | (11.1/33.3) | 0" (1 <i>2</i> 4 /44) | (15.6 /5 0.0) | a. A |
| | Skeleta <u>l</u> 4 | examination (letus/li | tt@r) | |
| Litters examined | 116/24 | ×138/25 % | A5/240 × | , |
| Malformations (%) | 0/0 | (%) 000° . ~ | 0,00,00 | |
| | (0.0/0.0) | (9.0/0.0) | (0.000.0) | |
| Variations (%) | 116/ 2 4 @ | ₹ °≥¥38/25° × | \$\frac{1}{24} \times \frac{1}{24} \times \frac | |
| · | (100 % 00) | (100/ 19 0) | (T00/1967) | |

4. Visceral and skeletal observations:

Fetal alterations were defined as:

matormations (irrefersible changes that occur at low incidences in this species and strains

- Variations (common findings in this species and strain and reversible delays or accelerations in development).

Viscefal examinations: Fetal evaluations were based on 108, 121 and 128 live, GDC1 caesarean delivered retuses in 24. 25, and 24 litters in dose groups 0, 30 and 150 mg/kg boolday dosage groups, respectively. Each of these fetuses were examined for visceral alterations, using the Woson technique. In total soft tissue variations were limited to 3 tissues, with a single incidence of mathematical (eyes).

Eves: a single incidence of retinal folding was observed in a control fetus, deepned to be malfornation. This finding can occur as a result of artefact of fixation/sectioning with a single incidence occurring, this was the likely succomposer.

Wessels: the uppoilical artery descended to the left in 10, 15 and 18 fetus at a little incidence of 72,11 and 00 in the 0, 30 and 150 mg/kg bw/day dosage groups, respectively.

Qver: mottled wer appearance in a single control fetus.

Kidney pale Adney appearance in two control and two mid dose fetuses (from separate litters).

Table CA 5.8.1419-3: Overview of developmental toxicity study in rats treated orally (via gavage) with spiroxagaine aminodion overview of visceral findings (fetus/litter incidence)

| Palameters | | ♀ (mg/k | kg bw/d) | |
|-----------------------|-----------------|-------------|-------------|-----|
| ragameters | 0 | 30 | 150 | 500 |
| Fetus Litters samined | \$\sqrt{108/24} | 121/25 | 128/24 | - |
| Madformations "O" | | | | |
| Retinal fording (%) | 1/1 | 0/0 | 0/0 | - |
| | (0.9/4.2) | (0.0/0.0) | (0.0/0.0) | |
| Variations | | | | |
| Vessels: umb. art., L | 10/7 | 15/11 | 18/10 | = |
| (%) | (9.3/29.2) | (12.4/44.0) | (14.1/41.7) | |



| Parameters | Ç (mg/kg bw/d) | | | | | |
|------------------|----------------|-----------|-----------|------------------|--|--|
| rarameters | 0 | 30 | 150 | 500 <i>&</i> | | |
| Kidney: pale (%) | 2/2 | 0/0 | 0/0 | - 🏋 | | |
| | (1.9/8.3) | (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 fetises were examined for skeletal alterations, after staining with alizarity and alciany blue. In total skeletal variations were limited to ribs, with an malformations:

Ribs: wavy/curved ribs were observed in 14, & and 7 Quises goross 9, 5 and 6 litters in the 0, 40 and 50 mg/kg by day do age groups, respectively. This variation in common in this strain of rat.

Rudimentary ribs were observed in 15. If and I fetuses across, 8, 9 and 9 litters in the 0.40 and 050 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, and 0 fotuses in the 0.50 and 00 mg/kg bw/day/dosag@groups, respectively.

In each case incidence was consistent with the historical control range.

No other skertetal variations occurred.

Table CA 5.8.1/19-4: Overview of developmental toxicity study in rate treated orally (via gavage) with spiroxamine aminodial: overview of skeletal variations (ferus/litter incidence)

| Parameters & | | ♥ ∰mg/k | g bw/dk | |
|------------------------|---|---------------------|----------------------------|-----|
| U " | | 30.~ | U 150 ≪ | 500 |
| Fetus/litters examined | 14,6/24 | 138025 | √ 145/ 2 4 | - |
| Ribcage & | \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ | | | |
| Ribs: wavy/curred (%) | 0 14/© | 9 × 8/5 6 | 7/5 | - |
| | (12.1/37.5) 4 | (5.8/200) | (4.8/20.8) | |
| Ribs: rudimentary (%) | £ \$\\\\\$\\\\\$\\\\\\$\\\\\\\\\\\\\\\\\\\\ | l ∅° 11″9 <i>@ı</i> | 11/9 | - |
| | (2.9/33 S) | (89/36.0) | $\bigcirc^{\%}$ (7.6/37.5) | |
| I Ribs: small (%) | | 1/1 | ¥ 1/1 | - |
| | (0,00.0) | (0.74.0) | (0.4/4.2) | |
| Ribs: extra (%) | 2/2 | 1/1 0 | 0/0 | - |
| | 1.7/8 3 | | (0.0/0.0) | |

5. Fetal estification:

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 twoid, 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:

- The oid 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 gastrointest and tract. A single mid dose group dam was noted with gaseous contents 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, and 100 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 lest article treated groups. All placentae appeared normal. Whilst an increase in absolute gravid uterine weight was observed for loss mid-dose group animals (increased by 14% and 29%, respectively), this was our butted to slightly increased litter sizes, which was considered within a final 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 by day dose groups. No test article related or statistically significant difference were observed in sex atto 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 fimited to 3 bissues 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 of a littor incidence of 7, 11 and 10 in the 0, 30 and 150 mg/kg bw/day dosage groups respectively.

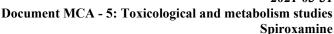
Skeletal evaluations were based in 116, 138 and 145 live, (2) 20 caesarean-delivered fetuses in 24, 25, and 24 litters in dose groups 0, 30 and 150 mg/kg bw/kay dosage groups, respectively. Incidental findings primarily included incomplete oscification (variation) of various bones, occurring across all 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 NOAE 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: | |
| Report Year: | 2020 |
| Report Title: | 4-tert-butylcyclohexyl docosanoate: In vitro human tymphocyte micronuçles |
| | assay (5) |
| Report No: | 8425089 |
| Document No: | M-753775-01-1 |
| Guideline(s) followed in | OECD guideline 487 (OECD, 2016) |
| study: | |
| Deviations from current | None V V V V |
| test guideline: | None & S S S S S S S S S S S S S S S S S S |
| Previous evaluation: | No, not previously submitted & & & & & & & & & & & & & & & & & & & |
| | |
| GLP/Officially | Yes, conducted inder of P/Officially recognised testing facilities |
| recognised testing | |
| facilities: | |
| Acceptability/Reliability: | Yes A A A A A A A A A A A A A A A A A A A |

Executive Summary

Spiroxamine docosanoic acid ester was tested in an in vitro micronucleus assay using applicate human lymphocyte cultures prepared from the pooled blood of two female donors in to two experiments. Treatments covering a broad range of concentrations, separated by narrow intervals, were performed both in the absence and presence of metabolic activation (SO) from Aroclor 1250 induced rats. The test article was formulated in tetral ydroforan (THF) and the highest concentration tested in the micronucleus experiment, 50 µg/m²L was limited by prompitate 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 oultures, concentrations ranging from 5 to 200 µg/mL were used in the absence (3 hours + 21 hour recovery), presence of S933 h + 21 h), and for extended treatment in the absence of \$9 (24 h + 24 h). The test article concentrations for micronucleus analysis were selected by evaluating the effect of spirosamine docosanoic and ester on the replication index (RI). Micronuclei were analysed at three concentrations and 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 pereases 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. Aminimum 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 analysed under each treatment condition met the criteria. The assay data were therefore considered validand acceptable.

Treatment of colls with spiro amine docosanoic acid ester for 3 hours (+21 hour recovery) in the absence and presence of S9 resulted in MNBN sells that were similar to and not significantly (p≤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 by 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≤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≤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 \(\mu g/mL \), 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 @.80%). However, these small increases were not reproduced in their replicate cultures with the mean MNBN cell frequency at 3 µg/m² (0.55%) falling within the normal 95% reference range and the rhean MNBN cell frequency at 50 μg/mL (\$185%). 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 esterolid not induce biologically relevance increases in the frequency of micronuclei in human peripheral brood lymphogytes when tested up to 50 µg/mL (a concentration limited by precipitate observed at the end of treatment) in the absence (I hour #21 bour recovery) and 24 hour (+24 hour)) and presence (3 hour (+2) hour) of an Aroctor induced rather metabolic activation system (S9, final concentration 1%

Materials and Methods

A. Materials:

1. Test Material:

White crystalline powder **Description:**

Lot/Batch No.:

96.2% (W/w) (Correction not applied) (molecular weight: 478.83 g/mol) **Purity:**

Not assigned CAS No.:

(expiry date: 25 February 2022) Stability of test

compound:

2. Control material

Solvent/final concentration

H: 0 2/24 h: 0.20 μg/mL) [clastogenic control] Positive: -S9

Vindastine (VIN, 24 h: 0.04 μg/mQ) [aneugenic control]

Cycloph Cynamide (CPA) 3 h: 70 mg/mL)

was purchased from a commercial source. A Sprague Dawley rats were

treated with Aroclar 1254 (supplied by , lot no.: 4179, protein content 3.3 mg/nd.). The composition of the 10% S9 reaction mix was: 100 µL \$9, Na PBS (100 μM), glucose-6-phosphate (5 μM), β-NADP (4 μM), MgCl₂

(8 μΜ), KCl-Q3 μΜΩ water (to volume).

Human perpheral blood lymphocytes were collected from 2 healthy, nonsmoking adult dowors aged between 23 and 30 years, pooled and diluted with

FIML media. Cells were stimulated to divide by the addition of

phytonaemagglutinin (PHA).

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-450catalysed 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 (maxumum limited by solubility in the test system)

Micronucleus assay:

3 h (+21 h recovery) -/+S9; 24 h (+24 h) -S9: 0, UT@, 5, 10, 25. $200 \,\mu g/mL$

3 h (+21 h recovery) +S9; 24 h $200 \,\mu g/mL$

Confirmatory 3 h (+21 h) -S%, UTC, 5, 10() (concentrations underlined scored for microgucleus frequency

B. Test Performance:

1. In life dates:

2. Vehicle selection:

6 April 2020 to 17 Jone 2020 (experimental dates)

A preliminary solubility set confirmed spiroxamme docosanoi cicid ester was soluble in tetrathy drofutan (THP) up to at least 20.22 mg/ml.

The solubility fimit in culture medium was below 2813 µg anL, as widicated by a lack of precipitation at this concentration ca. 24th after lest article addition. A maximum concentration of 500 ug/mL was selected for the cytotoxicit Range Dinder Experiment, in order that treatments were performed up to a precipitating concempation. Concempations for the microp@leus.experiment were selected based on the esults of the extotoxicity range-finder experiment.

Test article stock solutions were prepared by formulating spiroxamine dosanoic cacid ester under subduced 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 THT. The test article solutions were protected from hight and used within approximately 3 hours of ∕initial®rmulation. The propertion of MNBN cells for each treatment sondition were compared

3. Statistics:

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 ps0.05 were accepted as significant.

The following acceptance Fiteria had to be met for assay acceptability: The binoman dispersion test demonstrated acceptable heterogeneity (in terms of MNBN cell frequency) between replicate cultures, particularly

where he positive responses were seen;

The frequency of MNBN colls in vehicle controls fell within the current 95 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 depronstrated MNBN cell frequencies that clearly exceeded the normal

fange; A minomum of 50% of cells had gone through at least one cell division (as measured by binucleate + multinucleate cell counts) in vehicle control cultures whe 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).

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 assault none of the bove criteria were met.

C. Methods:

1. Homogeneity and achieved concentration analysis of the dose:

2. Preliminary cytotoxicity assay:

Not undertaken.

Whole blood cultures were established by pracing 0.4 mL of pooled heparinised blood into 8.5 mL pro warmed HEPES-buffered RPMI medium containing 10% (v/v) freat inactivated fetal cath 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, phytohaemaggluthin (PIIA, reagent grade) was included in the culture medium at a concentration of approximately 2% of culture to simulate the lymphocytes to divide. Blood cultures were incubated at 7±1° (for approximately 48 hours and rocked continuously. If mix is KCl of mL/culture) was added appropriately. Cultures were treated with the test article or vehicle (0.1 mL/culture). An untreated control was also included at THE is not a typical vehicle for this study type. Positive control treatments were not included. Dioplicate cultures were used for the vehicle control and shigle cultures were used for each test article freated concentration.

For repoval of the test article cells were pelleted (approximately 300 g, 10 minutes, washed twice with sterile saline and responded in fresh pre-warmed medium containing fetal call serum and conicillin/streptomycin. Cyto-B formulated in DMSQ) was added to post waste-off culture medium to give a final concentration of pug/me/culture to infibit cytokinesis, resulting in binus leate cells (without effecting caryokinesis), thereby arresting cells in interphase.

Change 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 about ation. Osmolality and pH measurements on post-treatment incubation and in the cytotoxicity range-finder experiment.

Cytotoxicity was determined by examination of the proportion of mono, bi and multipuclear cells (a minimum of 500 cells/concentration. From this, the replication index (R1) 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 ehicle 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)./

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

Slides were prepared by spreading the fixed cultures on clean slides. The slides were stained with acridine orange (12.5 $\mu g/mL$) dropped on to slides, coverslipped and scored prior to analysis.

Spin de inhibitor:



Cytotoxicity:

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

$$RI =$$
 no. of binucleate cells + 2(no. of multinucleate cells) total no. of cells in treated cours

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

Relative RI (%)=
$$RI$$
 of treated cultures RI of vehicle Qontrol RI of vehicle RI

Micronucleus assessment:

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

Slides from the highest effected 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 bidicleate cells from each culture (total of 2000 bindcleate cells/concentration) were analysed for micronuclei. The number of cells containing parcronuclei and the number of micronuclei feell on each slide were noted.

For the 2 hour (*24 hour recovery) treatment condition in the absence of S9 in Microngoleus Experiment 1, it was subsequently deemed necessary to analyse the two remaining vehicle control cultures, to aid data interpretation.

Nucleoplasmic bridges (NPBs) between naclei in binucleue cells were recorded during pricronucleus 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 incronucleus 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. It is not a requirement of the regulatory test guidelines.

B. Prehminary cytotoxicity assay:

Test article precipitate was observed at concentrations of $64.80~\mu g/mL$ and above in both short term and long term treatments, with no overt toxicity observed.

No marked changes on 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 cytoroxicity preliminary cytotoxicity range-finder experiment were used to select suitable maximum concentrations for the inicronucleus experiment, with the maximum concentration tested limited by precipitate observed at the end of treatment.

Table CA 5.8.1.75-1: Spir Vamine docosanoic acid ester: human lymphocyte preliminary cytotoxicity range finder experiment

| Conc. Of | Conc. 3 16 + 21 (crecovery) -S9 | | | recovery) +S9 | 24 h (+ 24 h recovery) –S9 | | |
|-----------|---------------------------------|---------------------------------|------------------------|------------------------------------|----------------------------|------------------------------------|--|
| (μg/mjL) | Replication Gridex (RT) | Cototoxicity Spased 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. | 3 h (+ 21 h | recovery) –S9 | 3 h (+ 21h | recovery) +S9 | 24 h (+ 24 h | recovery) –S9 。 |
|---------|-----------------------------|---------------|----------------------------|-----------------------------|----------------------------|-----------------------------------|
| (μg/mL) | Replication | Cytotoxicity | Replication | olication Cytotoxicity | | Cytotoxicit |
| | index (RI) | based on RI | index (RI) | based on RI | index (RI) | based on RI |
| | | (%) | | (%) | ~ | (%)D |
| 8.398 | 0.93 | 0 | 1.00 | 0 | 1.07 | |
| 14.00 | 0.87 | 3 | 0.92 | 0 | 096 | |
| 23.33 | 0.91 | 0 | 0.98 | 0 | 1.13 | \$ 0 \$\frac{1}{2}\$ |
| 38.88 | 0.93 | 0 | 0.94 | <i>⊳</i> _{&} 0 | 1.01 | |
| 64.80 | 0.91 ^{E-ppt} | 0 | 0.95 ^{E-ppt} | 0 | 1.12 ^{E-ppt} (| |
| 108 | 0.95 ^{ppt, E-ppt} | 0 | 0.90 ^{ppt, E-ppt} | 0 | 1.21 ^{ppt, E-pp} | ~ ~ ~ ~ |
| 180 | 0.88 ppt, E-ppt | 2 | 0.94 ^{ppt, E-ppt} | 0 | 1.17 ^{ppt, E} Opt | |
| 300 | 0.93 ^{ppt, E-ppt,} | 0 | 0.95 ^{ppt, E-ppt} | 0 💝 | ∂l°.17 ^{ppt} , | $\sqrt{2}$ 0° $\sqrt{2}$ |
| | H-ppt | | H-pp | ~ @ | Y H-pay | P' |
| 500 | 0.90 ^{ppt, E-ppt,} | 0 | 0.90 ppt, E-ppt, | ° 592 57 | 1 Oppt, E-p | ~ 0 V |
| | H-ppt | | O ^{Popt} O | | H-ppt | e 4 |

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 chicle control cell within the formal 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 ceatment condition depenstrated MNBN cell frequencies that clearly exceeded the difference, 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 bingcleate + multinucleate cell counts) in vehicle control cultures at the time of harvest. The maximum concentration aralysed under each treatment condition met the criteria. The as by data were therefore considered valid and acceptable.

in the absence and presence of \$9:

2. Short term treatment of cells with spiro amine socosahoic acidester for 3 hours (+21 horterecovery) in the absence and presence of S9 resulted in the mean MNBN cell frequency that were similar and that significantly ($p \le 0.05$) higher than Athose observed in concerrent Chicle Control of tures for all concentrations analysed. It was however noted that one reparcate from each test article concentration had a %MNSBN 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 underaken 🗸

Spiroxamine docosanoic acid ester micronuclei assay: 3 h (+ 21 h recovery) –S9 treatment and aboratory historical control data

| Conc (µg/mL) | | 4 | | | Vehicle historical control ranges (♀ donors) | | |
|-----------------|--------------------|-------------------|------------------------|--------------|---|-------------|--|
| (μg/mL) | Totail BN | ®MN-© | Frequency of MN-BN (%) | Cyto. (%) | Feb 17 – Feb 18 | % MNBN | |
| Vehicle 4 | 1000 | 4 2 | 1,00 | | No. of expts Number of cultures | 15 40 | |
| Total | 2000 | 14.5 | Mean: 0.70 | = | Mean ±SD | 0.40 ±0.28 | |
| Untreate A | 5 ^a ∢ | | a | | min. – max. | 0.00 - 1.30 | |
| d FO | Y (\$\frac{1}{2}\) | √√ ^y a | a | | 95% reference range | 0.00 - 1.01 | |
| A otal | <u>O</u> a | a | Mean: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} | | | | |



| | В | 1000 | 5 | 0.50 | | Positive historical c | control ranges 。 |
|---------------------|---|-------|-------------|------------------------|---------|-----------------------|-------------------|
| Total | | 2000 | 16 | Mean: 0.80 | Mean: 0 | (♀ dono | rs) |
| 75 ^{E-ppt} | Α | 1000 | 15 | 1.50 ^{>HC} | | MMC (0.3 μ | ıg/mL) |
| | В | 1000 | 8 | 0.80 | | Aug 15 – Dec 👭 | % MyBN |
| Total | | 2000 | 23 | Mean: 1.15 | Mean: 0 | No. of expts | 19 S |
| | | Linea | ar trend: p | 0.0655 NS | | Number of cultures | 40 |
| MMC | Α | 1000 | 60 | 6.00 | | Mean₄⊈SD | ©68 ±106 |
| (0.3) | В | 1000 | 78 | 7.80 | Ĉ | min — max. | 2.80 9 .20 |
| Total | | 2000 | 138 | Mean: 6.90*** | Mean 33 | 95% reference range | O 3.39 8.81, O' |

*** p<0.001

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

as vehicle controls were considered acceptable, %MN-BN frequency not scored

E-ppt: precipitate observed at end of treatment

3: Spiroxamine docosanoic acid ester: tuman tomphosyte micronuclei assay 3 h (+ ° 21 h recovery) +S9 treatment and laboratory historical control data **Table CA 5.8.1/25-3:**

| ` | | | 3 h (+ 2 | 21 h recovery) S | | Vehicle historical c | ontrol ranges |
|-----------------------|------------------|-------------------|--------------|--|------------------|------------------------------|------------------|
| (μg/mL |) | | | | Cyto, | C (Q Conor | rs) Ç |
| | | Total | Total | Frequency of MN-BN (%) | Cyto | Apr. 17 – Feb. 18 | % MONBN |
| | | BN | MN- | @MN-BN (%) | | | |
| | | | BN a | | | | - 10 |
| Vehicle | Α | 1000 | 5~~ | °>√0.50 | | No. of expts O | 16 |
| | В | 1000 | € | (4, 0.60) A | , V @ | Number of cutpures | 40 |
| Total | | 2000 | Ø 11 | \bigcirc Megn > 0.55 \bigcirc | , , | Mean #SD & | 0.40 ± 0.55 |
| Untreate | Α | ^a | ≫a <u></u> | a | | , min. ≪max. 🌣 | 0.00 - 1.20 |
| d | В | a ≪ | | √Qa O | | 95% reference range | 0.10 - 1.20 |
| Total | | - | | Mean@a | Mean/. 0 | 0, 4, | |
| 10 | Α | 1,600 | ₹5 ° | D 20,350 ° | | , L, Q) | |
| | В | <u>`</u> \$000, \ | 9 🛴 | 0.90 | | | |
| Total | | O 2000 O | ` 14 | Mean 070 | Mean: 13 | | |
| 25 | $A^{\mathbb{C}}$ | 1000 | `` \} | 0.50 | J Wiede 213 | J. Q. | |
| ♦ ^ | ©B | 1000 | ∠, 3 √) | 30 | - (<i>(//)</i> | Positive historical c | ontrol ranges |
| Total | 7 | 2000 | 85 | Mean: 0.40 | Mean: 2 | (♀ dono | rs) |
| 50 ^E -ppty | Α | 1000 |) }} | √, 0. 70 °° % | | | mL) ¹ |
| | В | 1000 | \$7 | ~Q90 & | | [№] Feb 16 – Feb 18 | % MNBN |
| Total | | 2 000 2 | 14 🍣 | | Mean: | No. of expts | 21 |
| | | Lipe | r trendr p | Mean: 0. 70 0.78 2 6 NS | 5 5 | Mean: | 41 |
| CPA | A O | 1000 | 3 | 3:500 | | Mean ±SD | 2.60 ± 1.07 |
| (7.0) | \mathbf{B}' | 1000 | ©35 <i>√</i> | R 50 R | | min. – max. | 0.80 - 5.00 |
| Total | 7 | 2000 @ | 70 | M@n: 3.50*** | √Mean: 54 | 95% reference range | 1.00 - 4.70 |

^{***} p<0.001

E-ppt: precipitate observed at end of Ceatment

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

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 ($p \le 0.05$) 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)

as yehicle controls were considered acceptable MN-BN frequency not scored



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 exidence of concentration. related increase, the two isolated increases were considered of pobiological relevance.

Table CA 5.8.1/25-4: Spiroxamine docosanoic acid ester: human lymphocyte micronuclei assay 24 24 h recovery) -S9 treatment an Plaboratory historical control data O

| | | | | | ¥ | | |
|---------------------|--------------|----------------|---------------------|----------------------------|-----------------------|--------------------------------|--------------------|
| Conc. | | | 24 h (+ | 24 h recovery – | S9 & Cyto | Vehicle historica Cc | ontrol ranges |
| (μg/mL) |) | | | 0′ | | Ç (Ç dono: | rs) |
| | | Total | Total | Frequency of | © Cyt@ | QApr 17 – Jan 18 | O" % MONBN & |
| | | BN | MN- | MN-BN (%) 🖔 | | | |
| | | | BN | | 1 0 i da | | |
| Vehicle | Α | 1000 | 6 | Q0.60, V | Ş Z | No. of expts | 140 |
| | В | 1000 | 6 | 0.66 | | Number of cultures & | © Q0 0.34 ±0.21 |
| Total | | 2000 | 12 | @ Mean: 0.60 | Mean: - | 🎢 🐧 Tean 🚓 D | |
| Untreate | Α | a | ^a @1 | | | min. Onax. | |
| d | В | ^a | G | | ~ ~ | % reference range | 0.10 - 0.80 |
| Total | | ^a | | Mean Mean | √ Meaga:- | | 1 |
| 10 | Α | 1000 | ~ Ø8 | | A | | |
| | В | 1000 | · 6 🕰 | Ž 0.60 Ž | | | |
| Total | | 2000 | 145 | √Mean: 0 70 | Mean [®] . I | % reference range | |
| 25 | Α | 1000 | 9 | © 0.90 A | | 0 4 | |
| | В | 1000 | [∞] 2 × | 0.20 | | Positive Bistorical c | ontrol ranges |
| Total | | 2 000 | 11 🕏 | Mean: 0.55 | 《Mean: 🎾 | (♀ dono | rs) |
| 50 ^{E-ppt} | A | 9 100 0 | 6 | 0.60 | | VIN (0.04 μ | g/mL) |
| | $^{\circ}$ B | 10000 | 4 1 6 | 1.10×HC | Mean: 10 | F c b 17 – Jan 18 | % MNBN |
| Totał⊳ | S S | 2000 | 17 <a>♥ | Mean: 0.85 | Mean: 10 | No. of expts | 20 |
| | | Line | r trene p | 0.18 NS \ | | Mean: | 40 |
| VIN | Α | 1000 | <u>`</u> 54 | √_ 5 A Ø <u>`</u> ` | | Mean ±SD | 5.59 ± 2.05 |
| (0.04) | В | 1000 | 31 | 3 3 10 N | 0 . | min. – max. | 2.50 - 10.10 |
| Total | | 2 000 g | ⁴ √ 85 € | Mean: 4.25 | Mean: 49 | 95% reference range | 2.50 - 8.93 |
| *** p<0.001 | | | | | No tesparticl | e related increases in cells v | with NPBs were |

^{*} p<0.001

as vehicle controls were copsidered acceptable %MN-BN frequency not fored

E-ppt: precipitate observed at end of treatment

>HC: exceeds historical control

4. Short-term treatment in the absence of S9, confirmatory

Treatment of cells with spiroxamine docosanoic acid ester for 3 hours (+21 were similar to and not significantly (p≤0.05) higher than those observed in concurrent vehicle control cultures for all there Hour recovery) in the absence of S9 resulted in frequencies of MNBN cells that concurrent vehicle control cultures for all three concentrations analysed. With the exception of a single replicate culture (A) at the intermediate concentration μg/mL, inducing 0% cytotoxicity), the MNBN cell frequency of all Temaining 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 al17), weak non-reproducible increases may

observed (data not reported)



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.

Spiroxamine docosanoic acid ester: human lymphocyte morronuclei assay **Table CA 5.8.1/25-5:** 21 h recovery) -S9 treatment, micronucleus experiment 2 an@laboratory historical control data

| Conc. | | | 3 h (+ 2 | 21 h recovery) –S | 89 🖔 | Vehicle historical c | ottrol ranges |
|---------------------|-----|--------------|------------------|---|---|-------------------------------|---------------------------|
| (μg/mL) |) | Total | Total | Frequency of | Cyto. | (♀ dong | |
| | | BN | MN- | MN-BN (%) | (%) | FC 17 F.L. 10 | O MANDE |
| | | | BN | , , , | a W | Feb 17 – Feb 18 | % MNBN |
| Vehicle | Α | 1000 | 7 | 0.70 | ~ | No of expto | 15 |
| | В | 1000 | 4 | 0.40 | | Number of cultures | 15 40 |
| Total | | 2000 | 11 | Mean: 0.5 % √ | \$ - \D | Mean⊯SD € | 0×40 ±0.28° |
| Untreate | Α | ^a | a | a O | | min max. V | ₹,0.00 ₹1.30 € |
| d | В | ^a | a | 24 | ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ | % reference range | 0.000°1.01 ₀ ° |
| Total | | ^a | a | Meana | Mean: 0 | | |
| 10 | Α | 1000 | 5 | Ø .50 , ~ | | | |
| | В | 1000 | 3 | ≈0.30¢ | | | |
| Total | | 2000 | 8 | Mean: 0.40 | Mean 0 | | |
| 25 | Α | 1000 | 11 | \$1. 139 >HC & | | To the same | |
| | В | 1000 | 5 | .×0.70 |] \$\forall \text{\$\exititt{\$\text{\$\text{\$\text{\$\text{\$\text{\$\text{\$\text{\$\text{\$\text{\$\text{\$\text{\$\text{\$\text{\$\text{\$\text{\$\text{\$\text{\$\exititt{\$\text{\$}\$\text{\$\text{\$\text{\$\text{\$\text{\$\text{\$\text{\$\tex{\$\text{\$\text{\$\exititt{\$\text{\$\text{\$\text{\$\text{\$\text{\$\text{\$\text{\$\text{\$\text{\$\text{\$\text{\$\text{\$\text{\$\text{\$\tex{\$\text{\$\text{\$\text{\$\text{\$\text{\$\exititint{\$\text{\$\exititt{\$\text{\$\text{\$\texititt{\$\text{\$\text{\$\text{\$\text{\$\text{\$\te | P ositive Historical c | oftrol ranges |
| Total | | 2000 | 16 | Mean: ©90 | , Mean: 0 | 🎺 🎺 🗯 dono: | r 9 |
| 50 ^{E-ppt} | Α | 1000 | 15 | 0. 5 0 | 7 0 | MMC (0.3@ | g/mL) |
| | В | 1000 | | Q .70 \$\sqrt{\sq}}}}}}}}}}} \end{\sqrt{\sqrt{\sqrt{\sqrt{\sqrt{\sqrt{\sqrt{\sqrt{\sqrt{\sqrt{\sqrt{\sqrt{\sqrt{\sqrt{\sqrt{\sqrt{\sqrt{\sqrt{\sq}}}}}}}}} \end{\sqrt{\sq}}}}}}}}} \end{\sqrt{\sq}}}}}}}}}} \end{\sqrt{\sqrt{\sqrt{\sqrt{\sqrt{\sqrt{\sqrt{\sqrt{\sqrt{\sqrt{\sqrt{\sqrt{\sqrt{\sqrt{\sqrt{\sqrt{\sqrt{\sqrt{\sqrt{\sq}}}}}}}}}} \end{\sqrt{\sqrt{\sqrt{\sq}}}}}}}} \end{\sqrt{\sq}}}}}} } \end{\sqrt{\sqrt{\sq}}}}}}} \end{\sqrt{\sqrt{\sqrt{\sq} | | Aug 15, Dec 17 | % MNBN |
| Total | | 2000 🛫 | 23 | Mean: 060 | Mean: 0 | No of expts | 19 |
| | | Line | ar trendr. p | 0,6345 NS | \$ \J | Number of cultures | 40 |
| MMC | Α | 1000 | £60 | \$ 3 C\$10 ~ | | √ Mean ±SĎ | 5.68 ± 1.66 |
| (0.3) | В | , 1000 v | ©″78 <u>,</u> °> | \$.50 \S | | min max. | 2.80 - 9.20 |
| Total | | 2000 | 138 | Mean: 450*** | Mean: 33 | ₹5% ref€rence range | 3.39 - 8.81 |
| *** p<0.001 | l 🖔 | | χ | 0 4 | No test articl | Felated increases in cells v | with NPBs were |

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

bserved (data not reported)

Following revision of the OPOD 480 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 to the OECD 487 test guideline, which includes the statement 'for lymphocytes exponential growth with 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.

as vehicle controls were considered acceptable, %MN∘BN frequency no≰scored ≰

>HC: exceeds historical control

E-ppt: precipitate observed at end of treatment



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 day or require two rounds of DNA replication. The extended treatment without recovery in the micropycleus. assay may be seen as somewhat compromised. It is widely known that direct acting clastogen, such as azidothymidine and cytosine arabinoside are negative in the *in vitro* micronucleus assay with the 244 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., 20198), 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 283/26

Conclusion: It is concluded that spiroxamine docosanoic acidester and not induce biologically relevance increases in the frequency of micropuclei in human peripheral blood lymphocytes when tested up to 50 μg/mL (a concentration limited by precipitate observed at the end of greatment) in 4 the absence (3 hour (+21 hour recovery) and 24 hour) and presence (3 hour (+21 hour) of an Aroclor induced rat liver metabolic activation system (\$9, final concentration 1\%\sqrt{v}).

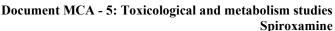
Spiroxamine-tetracosanoic acid ester

| Spiroxamine-tetracosanoic acid ester (M36) | | | |
|--|---|--|--|
| Dossier node | Draft title Study ID Planfed submission | | |
| CA 5.8.1.5/01 | Spiroxanime docusanoic acid ester \$4203 ft | | |

Spiroxamine-cyclohexenol (V137)

| Data Point: Report Author: Report Year: Report Title: Report No: Document No: Guideline(s) followed in study: Deviations from current test guideline Previous evaluation: No, not previously submitted | | | | |
|--|--|--|--|--|
| Report Year: Report Year: Report Title: Report No: Report No: Spiroxamine cyclohexerol (M37): In Vitro human lymphocyte micronucleus assay Report No: Buildine(s) followed in study: Deviations from current test guideline Previous evaluation: No, not previously submitted | Data Point: | KCA 58.1/26 0 | | |
| Report Title: Report No: Report No: Spirovamine cyclohexenol (M37): In Vitro human lymphocyte micronucleus assay Report No: Spirovamine cyclohexenol (M37): In Vitro human lymphocyte micronucleus assay Report No: Spirovamine cyclohexenol (M37): In Vitro human lymphocyte micronucleus assay Report No: Spirovamine cyclohexenol (M37): In Vitro human lymphocyte micronucleus assay Report No: Spirovamine cyclohexenol (M37): In Vitro human lymphocyte micronucleus assay Report No: Spirovamine cyclohexenol (M37): In Vitro human lymphocyte micronucleus assay Report No: Spirovamine cyclohexenol (M37): In Vitro human lymphocyte micronucleus assay Report No: Spirovamine cyclohexenol (M37): In Vitro human lymphocyte micronucleus assay Report No: Spirovamine cyclohexenol (M37): In Vitro human lymphocyte micronucleus assay Report No: Spirovamine cyclohexenol (M37): In Vitro human lymphocyte micronucleus assay Report No: Spirovamine cyclohexenol (M37): In Vitro human lymphocyte micronucleus assay Report No: Spirovamine cyclohexenol (M37): In Vitro human lymphocyte micronucleus assay Report No: Spirovamine cyclohexenol (M37): In Vitro human lymphocyte micronucleus assay Spirovamine cyclohexenol (M37): In Vitro human lymphocyte micronucleus assay Spirovamine cyclohexenol (M37): In Vitro human lymphocyte micronucleus assay Spirovamine cyclohexenol (M37): In Vitro human lymphocyte micronucleus assay Spirovamine cyclohexenol (M37): In Vitro human lymphocyte micronucleus assay Spirovamine cyclohexenol (M37): In Vitro human lymphocyte micronucleus assay Spirovamine cyclohexenol (M37): In Vitro human lymphocyte micronucleus assay Spirovamine cyclohexenol (M37): In Vitro human lymphocyte micronucleus assay Spirovamine cyclohexenol (M37): In Vitro human lymphocyte micronucleus assay Spirovamine cyclohexenol (M37): In Vitro human lymphocyte micronucleus assay Spirovamine cyclohexenol (M37): In Vitro human lymphocyte micronucleus assay Spirovamine cyclohexenol (M37): In Vitro human lymphocyte micronucleu | | | | |
| Report No. 8415892 Document No: M-76154-01-1 Guideline(s) followed in study: Deviations from current test guideline Previous evaluation: No, not previously submitted | Report Year: Report Year: | | | |
| Report No. 841 892 Document No: M-76154 01-1 Guideline(s) followed in study: Deviations from current test guideline Previous evaluation: No, not previously submitted | Report Title: Q | Opiroxanine cyclohexenol (M37): In Vitro human lymphocyte micronucleus | | |
| Document No: Guideline(s) followed in study: Deviations fron current test guideline Previous evaluation: No, not previously submitted | 4 | assa 🗘 🚑 👸 💇 | | |
| Guidefine(s) followed in study: Deviations from current test guideline Previous evaluation: No, not previously submitted | | 8415892 | | |
| Deviations from current test guideline No, not previously submitted | | | | |
| Deviations from current Note test guideline. Previous evaluation: No, not previously submitted | Guidefine(s) followed in OECD 487 (20 6) | | | |
| Previous evaluation: No, not previously submitted | J | | | |
| Previous evaluation: No, not previously submitted | Deviations from current, None | | | |
| | | | | |
| | Previous evaluation: | No, not previously submitted | | |
| | | | | |
| GLP/Oricially recognised testing facilities | GLP/Officially | Yes, conducted under GLP/Officially recognised testing facilities | | |
| Lecosuised (exams 7 a. 1 a | recognised testing | | | |
| identities. | TUSTATUCS. | Y | | |
| Acceptability/Reliability: Yes | 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 125 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 μ g/mL were tested in the absence (3 hours + 21 hour recovery), 150 to 350 μ g/mL in the presence of S9 (3, μ + 21 b) and 40 to 190 μ g/mL in the extended treatment in the absence of S9 (24 h + 24 h). The test orticle concentrations for micronucleus analysis were selected by evaluating the effect of spitoxamine 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 hormal 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 bast 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 effective.

Treatment of cells with spiroxamine cyclonexenol for 3 hours (+21 hour recovery) in the absence of S9 resulted in MNBN cells that were similar to and not significantly (p≤0.0%) 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. 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 reprecate culture (A) at 990 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 stanistically 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 Gological 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 exclohexenol 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 μ g/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 sproxamine cyclohexenol for 24 hours (+24 hour recovery) in the absence of S9 resulted in frequencies of MNBN cells that were significantly ($p \le 0.05$) higher that those observed in the concurrent vehicle control cultures for the highest concentration analysed (170 µg/mL, inducing 59% sylotoxicity) 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 prological 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 cyclohext nol resulted in frequencies of MNBN cells that were similar to and not significantly ($p \le 0.05$) higher than those observed in concurrent vehicle control cultures for all three concentrations analysed for both reatment concentrations in the sporadic marginal increases observed in Experiment 1 were not reproduced in the confirmatory experiment 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 do not induce bologically relevance increases in the frequency of micronuclei in human peripheral blood symphocytes when tested up to a concentration limited by cytotoxicity in the absence (32 hour (+21 hour) and presence (3 hour (+21 hour) of an Aroclor induced rat liver metabolic activation system 89, final concentration 1% v/v).

Materials and Methods

A. Materials:

1. Test Material: (alternative name: 4-tert-butleyclobex White rystalling powder **Description:** Lot/Batch No 97.2% (w/w) (correction not applied) (molecular weight: 154.25 g/mol) **Purity:** ot assioned € Configured stable for the duration of the study (expiry date: 3 January 2022) Stability of test compound: 2. Control material **Negative:** sulphoxide (DMSO)/1.0 Solvent/final@ concentration: Mitory in CAMMC 3 h: 0.3 24 h: 0.20 µg/mL) [clastogenic control] Positive: \$9 Vint Pastine AVIN, 24th: 0.0 Jug/mL) [aneugenic control] Positive: +S9 Colophosphamide (CPA 3 h: 7 μg/mL) \$923 was purchased from a commercial source. Sprague Dawley rats were 3. Activation: treated with Apoclor 1254 (supplied by lot no.: 4157, 4158, 4197 protein composition of the 10% So reaction mix was: 100 μL S9, Na PBS (100 μM), glucose-6-phosphate $(5 \mu M)$, β -NA(DP) (4 μ M), MgCl₂ (8 μ M), KCl (33 μ M), water (to volume). Human peripheral blood lymphocytes were collected from 2 healthy, nonspoking 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, 2.861, 13.10, 21 36.39, 60.65, 101.1, 168.5, 280.8, 468, 780, 1300 μg/mL (maximum thrittee solubility in the test system)

Micronucleus assay:

3 h (+21 h recovery) -S9: 0, 200 320, 330, 340 μg/mL

3 h (+21 h recovery) +S9: 0 $330, 340, 350 \,\mu g/mL$

24 h (+24 h) –S9: 0, 40 60, 80.

190 μg/mL

Confirmatory Experiments:

3 h (+21 h recovery) +S9

320 ,330 μg/mL/

24 h (+24 h) \$89:

 $190 \, \mu g/mL_{\odot}$

(concentrations underlined scored for micfonucleus fre

B. Test Performance:

1. In life dates:

2. Vehicle selection:

4 March 2020 to 22 May 2020 (experimental dates)

A preliminary solubility test confirmed spiroxamine cyclohexenol was soluble In dimethyl solphoxide (DMSO) up to at least 130 mg/mL.

The solubility limit in cultibe mednim was in the range 650 to 1300 μg/mL, as indicated by precipitation at the higher Oncentration.ca 24 h after test article addition. A maximum Sincentration 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 sesults of the cytotoxicity range-finder experiment.

Test article stock solutions were propared by formulating spiroxamine cy Pohexer under subdard lighting in PMSO with the aid of vortex mixing and warming at \$7°C, to give the maximum required treatment solution concentration Subsequent difficions were made using DMSO. The test article solutions were protected from light and used within approximately 3 hours of intral formulation

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 toond test was applied to each treatment condition. Probability values of \$≤0.05 were accepted as significant.

- The following acceptance criteria had to be met for assay acceptability: 1. The binomial dispersion test demonstrated acceptable heterogeneity (in frems of MNBN cell frequency) between replicate cultures, particularly where no positive responses were seen;
 - The frequency of MNBN cells in vehicle controls fell within the current 5th percentile of the observed historical vehicle control (normal) ranges; 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;

3. Statistics

4. Acceptance criteria



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 astogenic and aneugenic events if:

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

The test article was considered positive in this assay in an or were met.

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

C. Methods:

1. Homogeneity and achieved concentration analysis of the dose:

2. Preliminary cytotoxicity assay: Not underta

Whole blood cultures were established be placing 0.4 mL of pooled heparingsed blood into \$5 mL gre-warmed HEPRES-buffered BPMI medium Ocontaining 10% (v/v) beat inactivated fetal call serum and 052% penicillin/streptomycin, sochat the Final volume for Nowing addition of S9 mon KCl and the test artife in its chosen vehicle was 10 ml. The mitogen, phytologymaggletinin (BPIA, reagent grade) was included in the culture medium vat a concentration of approximately to of culture to stimulate the lymphocytes to divide Blood colliures were incubated 257±1. For approximately 48 hours and rocked continuously \$9 mix or KC\$1 mL/culture) was added appropriately. Cultures were treated with the test article or vehicle (0.1 mL/culture). An uniterated control was also pacluded as THF is not a typical velocile for his study type Positive control treatments were not included. Diplicate cultures were used for the vehicle control and single cultures were Lused for each test article treated concentration.

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

Changes in conolality of more than 50 mOsm/kg and fluctuations in pH of more than one unit may be responsible for an increase in chromosome aborrations. Osm@ality 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).

Ç∰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.

Slides were prepared by spreading the fixed cultures on coan slides. The slide Slide preparation:

were stained with acridine orange (12.5 μg/mL) dropped on to slides,

coverslipped and scored prior to analysis.

The replication index (RI), which indicates the relative number of nuclei **Cytotoxicity:**

compared to controls, was determined using the formula below

RI = no. of binycleate cells + Ono. of multinygleate \mathcal{R} is

notal no. of costs in treated cultures

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

Cytotoxicito (%) was expressed as \$100 - Belative

Micronucleus assessment:

Slides from the highest selected concentration and two fower concentrations were taken for microscopic analysis.

Immediately prior to analysis, 1-2 drops of RBS were added to the slides before mounting with glass cover Tips. Obe thousand binucleate cells from each culture (total of 2000 binucleate cells/concentration) were analysed for micronoclei. The number of cells containing pricroniclei and the number of micronuclei/cell on wach slifte were worted.

For the 24 hour (+94 hour ecovery) treatment condition for the absence of S9 in Micronnecieus Experiment 1, it was subsequently deemed necessary to analyse the two remaining vehicle control cultures, to aid data interpretation. Nucleoplasmic bridges (NPPs) between notici in Smucleate cells were

recorded during preronucleus analysis to provide an indication of chromosome rearrangement. Various mechanisms may lead to NPB formation following DNA Gnisrepair of sprand breaks in DNA in this assay, binucleate cells with were recorded as part of the micromycleus analysis.

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 guidelines₄

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 osnablality 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 cytotoxicito preliminary cytotoxicity range-finder experiment were used to select suitable maximum concentrations for the micronucleus experiment, with the maximum concentration tested imited by precipitate observed at the end of treatment.



Table CA 5.8.1/26-1: Spiroxamine cyclohexenol: human lymphocyte preliminary cytotoxicity range finder experiment

| | | | | | | . 10 |
|---------|-----------------------|---------------|------------------------|------------------------|------------------------|-------------------|
| Conc. | 3 h (+ 21 h | recovery) -S9 | 3 h (+ 21h | recovery) +S9 | 24 h (+ 24 h | recovery) 39 |
| (μg/mL) | Replication | Cytotoxicity | Replication | Cytotoxicity | Replication | Cytot@icity |
| | index (RI) | based on RI | index (RI) | based on RI | index (RI) | based√on RAÇ |
| | | (%) | | (%) | 4 | (%) |
| 0 | 0.88 | - | 0.89 | - | 1.14 | , O' - Ö' 🖟 |
| 4.716 | 0.82 | 6 | 0.95 | & 0 | ∜ 1.15 | |
| 7.861 | 0.91 | 0 | 0.91 | | 1.14 | / <u>~</u> 00 ~ |
| 13.10 | 0.95 | 0 | 0.88 | , 1 , O ^Y | 1.06 | 7 7 0 |
| 21.84 | 0.91 | 0 | 0.88 | 1 🔊 | . 1.20 [©] | |
| 36.39 | 0.88 | 0 | 0.86 | 3 | 9 1 AQ | |
| 60.65 | 0.83 | 6 | 0.88 | | 1.05 | √8 √3 · |
| 101.1 | 0.90 | 0 | 9 491 Q | | ₹ 0.78 ₹ | ≫ 31 [∞] |
| 168.5 | 0.78 | 11 | 9.81 | Č 9 %' | > 0.400° | & 6 ∮ , € |
| 280.8 | 0.28 ^{ppt} | 68 | , △, 0.51 P® | √© 43 [©] √ 4 | 0.07^{ppt} | |
| 468.0 | NE, ppt | - 2 | NE, ppt, M-ppt 🔈 | | NE, ppOE-ppt, Happt | / 200 |
| 780.0 | NE, ppt, E-ppt, H-ppt | - 0 | NE, ppr, F-ppt, H-pp | | NE, ppt, E-ppt 117ppt | |
| 1300 | NE, ppt, E-ppt, H-ppt | - 8 | NIC ppt, E-ppt, Hisppt | ~ - ~ · | (J), ppt, E-100, H-ppt | Q - |

NE: not evaluated due to no scorable cells

ppt: precipitate observed at treatment & & E-ppt: precipitate observed at end of treatment

Popt: progripitate bserve Ont 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 rignificant increases in the proportion of MNBN cells. Both replicate cultures at the positive control concentration analysed order each treatment condition demonstrated MNBN cell frequencies that clearly exceeded the normal range, thereby demonstrating the sensitivity and specificity of the assay of minimum of 50% of cells had gone through at least one sell 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.

To atment of cells with source and the cyclohexenol for 3 hours (+21 hour recovery) in the absence of S0 resulted in frequencies of MNBN cells that were similar to and not significantly ($p \le 0.05$) higher that those observed in the concurrent yehicle control cultures for all concentration analysed. The MNBN cell frequency of the material within the normal range. A single exception to this

cell frequency of the majority of these article treated cultures for all concentrations analysed, fell within the normal range. A single exception to this was 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 (7 to 0.70%). A weak but statistically significant linear trend (p≤0.05) was observed, however the majority of individual (and all group mean) MNBN cell value cell 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 (0.00.80%). The vehicle control group mean MNBN cell frequency (0.40%) also fell within the normal range (0 to 0.70%).

2. Show term treatment in the absence of \$92



Table CA 5.8.1/26-2: Spiroxamine cyclohexenol: micronuclei assay: 3 h (+ 21 h recovery) –S9 treatment and laboratory historical control data

| Conc. | | | 3 h (+ 2 | 21 h recovery) –S | 9 | Vehicle historical c | |
|--------------------|---|-------------|--------------|------------------------|---|------------------------|--------------------------|
| (μg/mL) |) | Total BN | Total MN- | Frequency of MN-BN (%) | Cyto. (%) | (A Conors) | |
| | | D1V | BN | 1VII (/0) | (70) | Feb 17 – Dee 17 | % MNBN |
| Vehicle | Α | 1000 | 2 | 0.20 | | No. of expts | , Oʻ 1,7 🗳 🦼 |
| | В | 1000 | 8 | 0.80 ^{>HC} | Ö | Number of cultures | ~ 40° ~ |
| | С | 1000 | 1 | 0.10 | \$ | Mean ±SD | 039±0.1 |
| | D | 1000 | 5 | 0.50 | 4 | 🛍 max. 🗸 | 0.00 - 0.80 |
| Total | | 4000 | 16 | Mean: 0.40 | , W - | 95% reference range | 0.00 -0.70 |
| 220 | Α | 2000 | 7 | 0.35 | | | |
| | В | 2000 | 9 | 0.45 | | | |
| Total | | 4000 | 16 | Mean: 0.4 🖔 | M © an: 12∾ | | |
| 260 | Α | 2000 | 10 | 0.50 | | Positive historical c | ontrol ranges |
| | В | 2000 | 7 | 0.34 | | 🌳 🦿 🚱 dono | rs) & V |
| Total | | 4000 | 17 | Mean, 0.43 | Mean: 43 |) MIMPC (0,354) | ıg/mL) 💮 |
| 290 ^{ppt} | Α | 2000 | 16 | 080>HC | | Feb 16 KNov 17 | % MNBN |
| | В | 2000 | 11 | 0.5 5 | \$. \$. \$. \$. \$. \$. \$. \$. \$. \$. | No. of expts | & ' 21 |
| Total | | 4000 | 27 | Mean: © .68 | Mean: 61 | Number of caltures | |
| | | Lin | ear trend: | | | Mean SD C | 5.37 ±1.74 |
| MMC | Α | 1000 | 56 🍭 | 5.60 | | Q'min. – max. | √1.50 − 9.20 |
| (0.3) | В | 1000 | 61 | 6.100 | , | 195% reference range | $^{\odot}$ 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 been did not reported

3. Short term treatment in the presence of \$9:

Treatment of cells with spiroxamine cyclonexemb for 3 hours (+21 hour covery) in the presence of \$9 \text{ estulted in frequencies of MNBN cells that were similar to and not significantly (p\u20120.054 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 \u20140g/mL\u2014\u2014\u20140 and 64\u2014

A second & periment was conducted, in order to assess reproducibility and biological relevance of the increases observed

Table CA 5.8.1/26-3: Spiroxamine cyclohex fol: human lymphocyte micronuclei assay: 3 h (+ 21 h recovery) + 99 treatment and laboratory historical control data

| Conc. | Q | | 3 h (+ 2 | Vehicle historical control ranges | | | | |
|----------|----|-------|-----------------|-----------------------------------|----------|---------------------|-----------------|--|
| Conc. | | Total | ÖŤotal U MN | Frequency of | Cyto. | (d donors) | | |
| | | ∑, ₹ | BN | MN-BN (%) | (%) | Feb 17 – Dec 17 | % MNBN | |
| Velricle | | 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 | Α | 1000 | 6 | 0.60 | | min. – max. | 0.00 - 1.00 | |
| | В | 1000 | 8 | 0.80 | | 95% reference range | 0.10 - 0.90 | |
| Total | | 2000 | 14 | Mean: 0.70 | Mean: 17 | | | |



| c | | 0.90 | 9 | 1000 | Α | 280 ^{ppt} |
|---|----------|------------------------------|-------------|-------|---|--------------------|
| Į. | | 1.10 ^{>HC} | 11 | 1000 | В | |
| | Mean: 29 | Mean: 1.00 ^{>HC} | 20 | 2000 | | Total |
| | | 1.00 ^{>HC} | 10 | 1000 | Α | 300 ^{ppt} |
| Positive historical control ranges | | 0.70 | 7 | 1000 | В | |
| (Odonors) | Mean: 47 | Mean: 0.85 | 17 | 2000 | | Total |
| EPA (3 μg/mLiĎ | | 0.30 | 3 | 1000 | Α | 310 ^{ppt} |
| Feb 16 Feb 18 / MNBN (| Ĉs | 1.00 ^{>HC} | 10 | 1000 | В | |
| No of expts \mathcal{O} \mathcal{O} \mathcal{O} | Mean 64 | Mean: 0.65 | 13 | 2000 | | Total |
| Number of cultures 40 & | ۳. | 0.4041 NS | ar trend: p | Linea | | |
| Mean ±SD © 2.21 ±9.85 | 4 W | 4.90 | 49 | 1000 | Α | CPA |
| min@- max \$\frac{1.00}{2} = 4.70 \$\frac{4}{3}\$ | | 4.90 | 47 | 1000 | В | (5.0) |
| 95% reference range 1.00 - 3.63 | Mean: 38 | Mean: 4.80*** | 96 | 2000 | | Total |

*** p<0.001

>HC: exceeds historical control ppt: precipitate observed at treatment Notest article related increases in cell with NPBs were

There is currently no historical control range for PA (5 µg/ml), the concentration analysed in this study therefore the range for the highest CPA concentration normally andlysed Jug/mJOhas been included for comparative

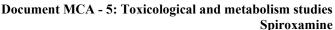
4. Extended treatment in the absence of S9:

Treatment of cells with spirsk amin cyclobexenol or 24 hours (#24 hours recovery) in the absence of S9 resolted in frequencies of MNBN cells that were significantly (p.0.05) ligher that those observed in the concurrent vehicle Control cultures for the highest concentration analysed (170 ug/mL, inducing 59% Cytotoxicity) with a stanstically significant linear treat (p≤0.05). 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 MNL cell value (\$285%) that marginally exocoded the normal 95% reference range to 0,85%), but fell within the observed range (Oto 0.90%). The vehicle control group mean MNBN cell trequency (0.66%) also Tell within the Dormal 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 (1/10 μg/mL), inducing a high contoxicity of 59% and as such these increases were considered of questionable bollogical relevance.

A second experiment was conducted, in order to assess reproducibility and biological relevance of the increases observed

Spiroxamine cyclohexenel: human lymphocyte micronuclei assay: 24 h (+ 24 h recovery) -So treatment and laboratory historical control data

| Conc. (µg/mL) | Ž, | 24 h (+ 24 h recovery) S | | | Vehicle historical control ranges (♂ donors) | | |
|------------------|--------------|--------------------------|-------------|----------|---|-----------------|--|
| ** | Total | Total T | Frequency@f | Cyto. | Jul 17 – Jan 18 | % MNBN | |
| e | Ø BN | MN-/ BN | MN-BN(%) | (%) | No. of expts | 16 | |
| Vehicle A | 2000 | | 0.83>HC | | Number of cultures | 40 | |
| ∂ B | Q 000 | | 0.55 | | Mean ±SD | 0.34 ± 0.23 | |
| Total | 2000 | | 0.55 | | min. – max. | 0.00 - 0.90 | |
| | 2000 | | 0.60 | | 95% reference range | 0.10 - 0.80 | |
| z Total | 80000 | | Mean: 0.66 | Mean: - | | | |
| 900 S A | 1000 | 5 | 0.50 | | | | |
| Ĉ B | 1000 | 7 | 0.70 | | | | |
| Total | 2000 | 12 | Mean: 0.60 | Mean: 16 | | | |
| 140 A | 1000 | 7 | 0.70 | | | | |
| В | 1000 | 3 | 0.30 | | | | |



No test article related increases in cells with News v



| Total | | 2000 | 10 | Mean: 0.50 | Mean: 34 | Positive historical control ranges (3 donors) | |
|--------------------|---|-------|-------------|------------------------|--------------------|---|-----------------|
| 170 ^{ppt} | Α | 1000 | 17 | 1.70 ^{>HC} | | VIN (0.04 μ | g/mL) |
| | В | 1000 | 12 | 1.20>HC | | Feb 17 – Jan 48 | % MNBN |
| Total | | 2000 | 29 | Mean: 1.45 | Mean: 59 | No. of expts | 20 0 |
| | | Linea | ar trend: p | 0.6234 NS | | Number of cultures | ~41 × |
| VIN | Α | 1000 | 50 | 5.00 | | Mean₄SD | 643 ±238 |
| (0.04) | В | 1000 | 61 | 6.10 | Ĉ _{&} | min — max. | ∑2.50 – 43.60 £ |
| Total | | 2000 | 111 | Mean: 5.55*** | Mean 65 | 95% reference range | َ 2.80 \ 13.50° |

^{***} p<0.001

E-ppt: precipitate observed at end of treatment

5. Short-term treatment in the presence of S9, confirmatory experiment: Treatment of cells with Piroxamine cyclohexenol for Shours (+21 hour recovery) in the presence of Syresulted in frequencies of MNBN cells that were similar to and not significantly, (p \le 0.05) higher than those observed to concurrent which 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 cyclotoxicity 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.

observed (data not reported)

According to the correct data interpretation strategy (Thybaud et al¹⁷), weak mon-reproducible increases may be considered of little to no genotoxicity concein. 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 sproxamine cyclonexend for 24 hours (+24 hour covery) in the bisence of S9 (esulted in frequencies of MNBN cells that were similar to and not significantly (p≤0.05) higher than those observed in concurrent reflicle central cultures for all three concentrations analysed. The sproadic 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 sytotoxicity 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

Table CA 5.8.1/26-5: Spiroxamine cyclohexenol human lymphocyte micronuclei assay: 3 h (+ 21 h

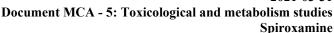
was non-reproducible and not biologically relevant.

| Conc. | | 3 h (+ 21 | h recovery) #\$ | Ď | | 24 h (+ 24 | h recovery) –S | 19 |
|-----------|----------------|-----------|-----------------|-------|--------------|------------|----------------|-----------|
| (μg/mL) | Tota | Notati | Frequency | Cyto. | Total | Total | Frequency | Cyto. |
| | DI I BN | MN- | of MN BN | (%) | BN | MN-BN | of MN-BN | (%) |
| | | BN 3 | (%) | | | | (%) | |
| Vehicle A | 3 000 | U 7 8 | 0.70 | | 1000 | 4 | 0.40 | |
| % /i L | ⊉ 1000₫ | | 0.40 | | 1000 | 5 | 0.50 | |
| Asotal @ | | | Mean: 0.55 | - | 2000 | 9 | Mean: 0.45 | - |
| \$75 PA | a | aa | a | | 1000 | 5 | 0.50 | |
| l , , , E | 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 | |
| E | a | a | a | | 1000 | 7 | 0.70 | |

a as vehicle controls were considered acceptable,

[%]MN-BN frequency not scored

>HC: exceeds historical control





| _ | | | | | | | | | |
|--------------------|---|--------------|--------------|----------------------|------------|-------|---------------------|--|---|
| Total | | 2000 | a | ^a | a | a | 9 | Mean: 0.45 | Mean: 28 o |
| 140 | Α | a | a | a | | 1000 | 8 | 0.80 | Q |
| | В | ^a | a | a | | 1000 | 6 | 0.60 | |
| Total | | 2000 | ^a | a | a | a | 14 | Mean: 0.70 | Mean: 55, |
| 225 | Α | 1000 | 4 | 0.40 | | a | a | ⟨¬a | |
| | В | 1000 | 6 | 0.60 | | a | a | ″0″a | |
| Total | | 2000 | 10 | Mean: 0.50 | Mean: | | a 🔑 | ^a . Ô | |
| | | | | | 13 | Ĉ. | | | |
| 270 ^{ppt} | Α | 1000 | 5 | 0.50 | | a | W | ^a 💍 | |
| | В | 1000 | 7 | 0.70 | ٦. | a | 8 | -7 <u>-</u> 0 % | |
| Total | | 2000 | 12 | Mean: 0.60 | Mean | | \$a | | |
| | | | | | 30 | | Q &° | | |
| 305 ^{ppt} | Α | 1000 | 2 | 0.20 | <i>Q</i> | a ^ | y <u></u> -W | ~{° | |
| | В | 1000 | 3 | 0.30 | | \$ | \$ _ | ~, ^ | |
| Total | | 2000 | 5 | Mean: 0.25 © |) Mea@ | | ¥a | \$\frac{1}{2}a \tag{\tag{\tag{\tag{\tag{\tag{\tag{ | 4a |
| | | | | . 1 | 5 ¥ | | | | |
| | | Linear | trend: p 0. | 8777 NS 🔊 | | y 🔊 | Li nd ar tre | p 0.5119 NS | |
| Positive | Α | 1000 | 34 | 3.40/ % | | 1000 | 689 ° | y 66 9 0 K | |
| control | В | 1000 | 39 | 50 90 (% | | 1000 | ₹ 63 € | Ø .30 | 0 |
| Total | | 2000 | 73 | Mean: | Mean: | Z000~ | 132 | Mean | Nean: 47 |
| | | | | 4.65*** [©] | ž 33 × | Ć, | | © 6.6 9 | *************************************** |

^{***} p<0.001

E-ppt: precipitate observed at end of treatment

No test article related increases in cells with NPBs were

D. Deficiencies:

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

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 by the contract times are placed by the placed on performing the extended by the contract times are placed by the contract times are placed by the contract times are placed by the contract times have been somewhat simplified with emphasis placed on performing the extended by the contract times have been somewhat simplified with emphasis placed on performing the extended by the contract times have been somewhat simplified with emphasis placed on performing the extended by the contract times have been somewhat simplified with emphasis placed on performing the extended by the contract times have been somewhat simplified with emphasis placed on performing the extended by the contract times have been somewhat simplified with emphasis placed on performing the extended by the contract times have been somewhat simplified with emphasis placed on performing the contract times are placed by th

Given the purpose for the *in our o* meronucleus as ay 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 ordex data depronstrated 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 on 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 confinent'. 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 Pause' 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 of g. an ugenic compounds, G2/metaphase) and/or that induce cell cycle delay or require two rounds of DNA eplication. 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 azido hymicine 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., 20198), with the OECD test guideline to be updated to reflect this change.

>HC: exceeds historical control

a not treated in this treatment condition



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 lympholytes 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: | KCA 5.8.1/2/ |
| Report Year: | 2020 |
| Report Title: | Justification for toxicological read-across from spiroxamine metabolite M13 to M37 |
| | M37 N N N N N N N N N N N N N N N N N N N |
| Report No: | 0471836-T@X2 |
| Document No: | |
| Guideline(s) followed in | ECHA (2017) |
| study: | |
| Deviations from current | None of the second seco |
| test guideline: | |
| Previous evaluation: | (No, not Oreviously suburitted |
| • | |
| GLP/Officially recognised testing facilities: | not applicable of the second o |
| recognised testing | |
| facilities: | |
| Acceptability/Rehability | Yes X Y Y Y Y |

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 projecular weight, physicochemical properties, chemical reactivity profiles and predicted ADME. Therefore grouping and read across is justified.

Comparison of the chemical properties of May 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.



| Property | Spiroxamine cyclohexenol (M37) | Spiroxamine cyclohexanol (Ma |
|-------------------|---|---|
| Chemical name | 4-tert-butylcyclohex-2-en-1-ol | 4-tert-buylcyclohexan- |
| CAS | not available | \$98-52-2 |
| Molecular formula | $C_{10}H_{18}O$ | C ₁₀ H ₂₀ O |
| Structure | CH ₃ CH ₃ Or isomer | CH ₃ CH ₃ CH ₃ CH ₃ CH ₄ CH ₅ |
| Smiles code | CC(C)(C)C1C=C(O)CC1 | \$\\ \(\\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \ |
| Molecular weight | 154.20 | 156,27 |
| Log Kow | 3.2 (estimated) 3.2 | 2 3.4 (Stimated) V |
| (mg/L) | 3.2 (estimated) | 3.09 (measured) |
| Water solubility | 433 (estimated) | 326 (estimated) |
| (mg/L) | 433 (estimated) | (tacasured) |
| Similarity | \$Q% Q" . 4 | |
| Impurities | Not red vant compound is a plant | Notatelevant compound is a plan |

Table CA 5.8.1/27-1: Structure and physico-chemical properties of M37 and M13

B. Comparison of chemical and biological reactivity profites:

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 fikely to slightly limb 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 through the 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)

| | | T = . |
|---------------------------------------|--------------------------|-------------------------------|
| Profilers A A | Spiroxamine cyclohexenol | Spiroxamine cyclohexanol |
| | (M37) | (M13) |
| General Mechanistic 🧳 🛴 🤝 | | |
| Protein binding by OECD | alert wund | No alert found |
| | Intermodiate (Class II) | Low (Class I) |
| Uncoupers (MITOTOX) | Underined O | Non concern for uncoupling of |
| DNA binding by QECD | | OxPhos (pKa ranges) |
| DNA binding by QECD O | No ale found | No alert found |
| Toxic hazard classification by Framer | Integnediate (Class II) | Low (Class I) |
| (extended) | V | |
| Protein binding by OASIS & | So alert found | No alert found |
| DNA binding by ASIS | No alert found | No alert found |
| Protein binding potency Cys (OPRA | DPRA less than 9% (DPRA | DPRA less than 9% (DPRA |
| 13% | 13%) | 13%) |
| 13% | DPRA less than 9% (DPRA | DPRA less than 9% (DPRA |
| | 13%) >> Alcohols | 13%) >> Alcohols |
| Estrogen Receptor Binding | Weak binder, OH group | Weak binder, OH group |
| Protein binding potency Lys (DPRA | DPRA less than 9% (DPRA | DPRA less than 9% (DPRA |
| 13%) | 13%) | 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 (GST) |
| Endpoint Specific | | |
| Skin irritation/corrosion Exclusion | Undefined | Indefined Indefined |
| rules by BfR | *** | |
| Oncologic Primary Classification | Not classified | Not classified Q |
| Protein binding alerts for skin | No alert found | No alert found |
| sensitization according to GHS | | W |
| Protein binding alerts for skin | No alert found | No altert found |
| sensitization by OASIS Eye irritation/corrosion Exclusion | Undefined & | Ordefined |
| rules by BfR | Undefined V | |
| Bioaccumulation - metabolism half- lives | | Fast 5 |
| Acute Oral Toxicity | Not categorized \$ 0 | Basic to city |
| Protein Binding Potency h-CLAT | No alert found | No alor found , V |
| rtER Expert System - USEPA | No alert found | Altorcyclonexanols |
| Keratinocyte gene expression | Not possible to classify | Not possible to dassify |
| 2 | | according to these rules |
| DART scheme | Not known precedent \ \ \ \ reproductive and developmental | Known presedent reproductive and developmental toxic |
| Skin irritation/corrosion Inclusion | toxic potential | Spotential |
| | | Piperážíne-, dioxane-, mo@holine-, |
| | | tetrahydrothiopyran-like |
| | | derivatives and |
| | | cyclohexanamine (17c) |
| rules by B#R _ O | Anclusion rules not met | Inclusion rules not met |
| in vitro mutagenicity Ames test) alerts by ISS | Noodert found | No alert found |
| Carcinogenicity (genotox and nongenotox) alerts by IS | No aler found | No alert found |
| Respiratory sensitisation | No alert found | No alert found |
| Retinoic Acid Receptor Bioling | Ot possible to Passify | Not possible to classify |
| | according to these rules | according to these rules |
| Protein binding alerts for Chronosomal aberration by OASISO | No bert found | No alert found |
| in vivo mutagenicity (Micronucleus) | No ale@found | No alert found |
| alerts by ISS | | N. 1 . C . 1 |
| DNA alerts for AMES, CA and MNT by OASIS | No alert found | No alert found |
| Bioaccum Nation metabolism alorts | Aliphatic alcohol [-OH] | Aliphatic alcohol [-OH] |
| | -C=CH [alkenyl hydrogen] | Carbon with 4 single bonds & |
| | Carbon with 4 single bonds & | no hydrogens |
| | no hydrogens -CH - [cyclic] | -CH - [cyclic] -CH2- [cyclic] |
| × 50, | -CH2- [cyclic] | Methyl [-CH3] |
| $igcup_{}$ | Methyl [-CH3] | |
| Eye irritation/corrosion Inclusion rules | Inclusion rules not met | Inclusion rules not met |
| by BfR | | |



| Profilers | Spiroxamine cyclohexenol (M37) | Spiroxamine cycle (M13) | ohexanol |
|----------------------|--------------------------------|-------------------------|----------|
| Toxicological | | | Z |
| 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 to 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, 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 retevant for the justification of grouping of plant metabolites. Selection of the production of grouping of 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: Suggmary of available to city data

| Spi | roxamine cyclonexanol (M13) | ? |
|--|--------------------------------------|-------------|
| Study | Frydpoint & S | Reference |
| Acute oral toxicity ((at) | LØ40: 4200 mg/kg bw | CA 5.8.1/07 |
| Acute dermal toxicity (rabbit) | (L)D 50 ≥ 5000 mg (kg bw) | (1973) |
| In vitro bacterial reverse Ames gene | +/-S9 negative | CA 5.8.1/22 |
| mutation \circ \circ \circ | | (2020) |
| In vitro mammalian \$5178 forward | +/-\$9 negative \$ | CA 5.8.1/09 |
| (tk ^{+/-}) gene mutation & | | (2012) |
| In vitra Duman peripheral blood | +/-S9 negative | CA 5.8.1/23 |
| lymphocytes microniecleus | | (2020) |
| Rat sub-acute toxicity (28 day dietary | NOAEL 50 mg/kg bw/d | CA 5.8.1/11 |
| route) | | (1999) |
| Rat developmental toxisty study | Maternal NOAEL: 30 mg/kg bw/d | CA 5.8.1/12 |
| (conducted sing Mi) acetate ester | Developinental NOAEL: 160 mg/kg bw/d | (2007) |
| ∆ Spi | roxamine cyclonexenol (M37) | |
| Study & Study | Endpoint | Reference |
| In vitro human peripheral blood ~ | +/-\$9 negative | CA 5.8.1/26 |
| lymphocytes microturcleus | | (2020) |
| V | - X | |

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 M30 and M31 and a low confidence in the prediction for M30 and M31 and a low confidence in the prediction for M30 and M31 and a low confidence in the prediction for M30 and M31 and a low confidence in the prediction for M30 and M31 and a low confidence in the prediction for M30 and M31 and a low confidence in the prediction for M30 and M31 and a low confidence in the prediction for M30 and M31 and a low confidence in the prediction for M30 and M31 and a low confidence in the prediction for M30 and M31 and a low confidence in the prediction for M30 and M31 and a low confidence in the prediction for M30 and M31 and a low confidence in the prediction for M30 and M31 and a low confidence in the prediction for M30 and M31 and a low confidence in the prediction for M30

The QSAR analysis of spiroxamine metabolites for genetoxicity 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: | KEA 5.8 1/28 & 4 4 5 6 |
|---------------------------------|--|
| Report Author: | THE TOTAL OF THE T |
| Report Year: | |
| Report Title: | In spico prognosis of the conotoxic potential of spiroxamine group A metabolites |
| Report Title: | 1 (N#O)1_NA@\$2 NAO\$2/NAOO7 ASM23 & Xr 3NAO/A 1) |
| | 9566398 TOX |
| Document No: | M-765152-01-1 |
| Guideline(s) followed in study: | ECHA Guidance, on information requirements and chemical safety assessment |
| study: | ECHA Guidance of information requirements and chemical safety assessment Chapter R.6: QSARs and grounding of Chemicals. May 2008. Guidance for the implementation of REACH |
| | implementation of REACH None |
| Deviations from current | Note S S S |
| test guideline: | None Solon Notice |
| Previous evaluation " | No, not previously subspitted > |
| GLP/Officially | not applicable |
| recognised testing | not applicable. |
| A countability Paliability: | |
| Acceptability/Renability. | |
| Ø, , | |
| | |
| | |
| <i>y</i> | |
| | |
| | |
| | |
| | |
| | |
| | |
| | |
| | Note |
| \bigcirc | |



| Data Point: | KCA 5.8.1/29 |
|----------------------------|--|
| Report Author: | ; |
| 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 | ECHA Guidance on information requirements and chemical safety assessment |
| study: | Chapter R.6: QSARs and grouping of chemicals May 2008. Guidance for the |
| | implementation of REACH |
| Deviations from current | None S S S S S S S S S S S S S S S S S S S |
| test guideline: | A Q 6° A A Q |
| Previous evaluation: | No, not previously subnorted |
| GLP/Officially | not applicable |
| recognised testing | not applicable |
| facilities: | |
| Acceptability/Reliability: | Yes A A A |
| | |
| Data Point: | KCA 5.8 ₄ /30 |
| Report Author: | |
| Report Year: | 2021 _Q & & & & & & & & & & & & & & & & & & & |
| Report Title: | In surco prognosis of the genotoxic potential of spiroxamine group C metabolites |
| | (M28-M21) O S S S S S S S S S S S S S S S S S S |
| Report No: | <u>M-763154-01-1</u> |
| Document No: | <u>M-763154-01-1</u> |
| Guideline(s) followed in | ECNA Guidance on information requirements and chemical safety assessment |
| study: | Chapter 1.6: QSARs and grouping of chemical May 2008. Guidance for the |
| | implementation of REACH O O |
| Deviations from current | None, S. S. S. S. S. S. S. S. S. S. S. S. S. |
| test guideline: | |
| Previous evaluation: | No, not previously submitted S |
| GLP/Officially | not applicable of the control of the |
| recognised testing | |

facilities.

Executive Summary

In order to an human health assessment an in silico prognosis has been made of the genotoxicity potential of seventeen, eight and four plant and live tock metabolites, M01-M12, M25-M27, M38 & M41 (also known as Group A metabolities), MF3-M17 & M35-M37 (also known as Group B metabolites) and M28-W31 (also known as Group 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 chomical and biological reactivity endpoints relevant for a genotoxicity orognosis.

The in silve 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. 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.



Document MCA - 5: Toxicological and metabolism studies
Spiroxamine

Table CA 5.8.1/28-1: Summary of expert review of in silico modelling outputs for Group A metabolites

| Endpoint | | Mutage | nicity | | (| Chromosome | damage | | Overall expert |
|---|-------------------|---------|--------|-------------|-------------------|------------|--------|-------------|-------------------|
| Compound | Derek/ Toolbox | L/scope | Data | Expert call | Derek/ Toolbox | L/scope | Data | Expert call | ca0 |
| Spiroxamine | | | | High | | | S. | High 🖋 | High A |
| M01/M02/ M04/M09/ M11/ M12/M38/M41 | | | - | Med | Ö | | _ | Med & | Land Chr. |
| M03 | | | | High | A., | ۰۵۷ | 4 | High N | High |
| M05/M06/M07 /M08/M10 | | | - | High | | | - 0 | High | High S |
| M25/M7 | | | - | Low | 02 | | - 4 | Low 4 | Low |
| M26 | | | - | Med | | V 4 | | Low | Low |

Table CA 5.8.1/28-2: Summary of expert review of in vilico modelling outputs for Group B metabolites

| Endpoint | | Mutage | nicity / | | | Chromosome | damage | | Overall |
|-------------|---------|---------|--|--------------|----------------|------------|-------------------|---------------|---------|
| Compound | Derek/ | L/scope | Pata 🕝 | Expert | Derek/ ~ | L/scope | Data | Expert | expert |
| | Toolbox | Ç |)" , | call | Toolbox | | <i>>></i> − | Çall 📈 | call |
| Spiroxamine | | | / | J ugh | | 4 8 | · • • | High | High |
| M13 | | | ************************************** | , High 💇 | 7 | | Ö' | High | High |
| M14 | | Ž, Š | | Hig⊮ | | * | | High | High |
| M15/M16/ | ٥. | | - B | Med | ? | Z . | ¥ 4. | Med | Med |
| M17 | 7 | × 4 | | Med | y Q | | | | |
| M35/M37 | 2 | | | Med | | X & | | High | Med |
| M36 | | | y- | | | 0' | <u>-</u> Y | High | Med |

Table CA 5.8. 28-3: Summary of in suco modelling outputs for Group C metabolites

| Endpoint 🖔 | | Mutage | | | | Chromosome | damage | | Overall |
|-------------|--------|---------|--------------|--------------|---------------------|------------|--------|-------------|----------------|
| Compound | Derek/ | L/scope | Parta | Experit Coll | Derek/ Toolbox % | Î/scope | Data | Expert call | expert call |
| Spiroxamine | \$ ° | 4 2 | | High | | | | High | High |
| M28 | 6 A | | | High | | | | High | High |
| M29 | | | | ″ Med 🎏 | Ö | | - | Low | Low |
| M30/M31 < | | | Y- > | Med | ð | | - | Med | Med |

| 7 | |
|---|----|
| ĸ | AT |
| | |

| Indreator | Expert review |
|-----------|---------------------|
| | Positive/confidence |
| | Negative/confidence |
| | Carmot cally |
| | 6 0 0 0 |



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 metabolities 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 of the confidence in the predictions for Metabolite M28 due to the availability of experimental of the confidence in the predictions for Metabolite M28 due to the availability of experimental of the confidence in the predictions for Metabolite M28 due to the availability of experimental of the confidence in the predictions for Metabolite M28 due to the availability of experimental of the confidence in the predictions for Metabolite M28 due to the availability of experimental of the confidence in the predictions for Metabolite M28 due to the confidence in the confidenc 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 M13 due to the availability of experimental data for M13 and the structural circilate to M14. availability of experimental data for M13 and the structural similarity to M14.

A. Materials and Methods

| Madae de capermientai | data for W13 and the sintetural similarity to W14. |
|--|--|
| A. Materials and Methods | |
| 1. Substance identity: | Refer to results for adividual metabolite identity details. |
| 2. Information on QSAR models: | Refer to results for Odividual metabolite identity details. |
| Derek Nexus: | Parameter Détails & S & S & S & S |
| | Prediction endpoint: Mutagenicity in vitro in bacterium (Ames test) |
| | QMRP Q19761-0004 (up atted May 2020) |
| | Expert-derived structural alerts of for mutagenicity, physichemical properties and associated reasoning (2D |
| | Algorithm SAN. Following Nert evaluation. Derect evaluates whether non-alerting query compounds contain any features that are model. Walso present in non-alerting mutagens in a large Ames |
| | test reference set (musclassified features) or (ii) not present in |
| ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ | Internal: not available given that it is a knowledge-based |
| | Statistics system. ii) External: no data available The compounds in the dataset are primarily small and medium- |
| | ii) External: no data available The compounds in the dataset are primarily small and medium- |
| | The compounds in the dataset are primarily small and medium- sized chemicals and so are representative of the structures used to boild 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 |
| | to boild the model. The Ames test reference set contains 5780 nauragens and 5994 non-mutagens (v6.1) The scopes of the |
| | 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 gructure 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 attent describing a structure-activity for mutagenicity it can be considered to be within the applicability domain. If a |
| | 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 |
| | 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 Derok makes a negative prediction. The applicability of the regative 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 |
| | Compound does not activate an alert or reasoning rule then |
| | Derek makes a negative prediction. The applicability of the |
| 4 4 | pomains pregative 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 |
| J D A | 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 |
| Ö [*] | features in the molecule have not been found in the Lhasa |
| - | ı |
| | unclassified features are highlighted to enable the negative prediction to be verified by expert assessment. Mechanistic |
| | prediction to be verified by expert assessment, internalistic |



| | | 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 er | ndpoint: Chromosomal damage 🔊 🔊 🗳 |
| | QMRF protocol | Q19-762-0007 (apdated May 20)20) |
| | Algorithm of the model | Expert derived structural agerts for chromosome damage (2D SARs) physicochemical properties and associated reasoning |
| | Statistics | i). Byternal not available given that it is a knowledge-based system. ii). External: no data available 1 |
| | Domains Androgues Data derived from the vitro and in two 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 apphicability domain for the model. Therefore, if a chemical activates an afert describing a structure activity for promosome damage it can be considered to be within the applicability domain. If a compound does not activate an alert or casoning rule in Derek, a result of mothing to report is presented to the user. This can be interpreted as a negative prediction or that the query compounds 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 classifications and 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. Non-stroprietary elements of the training set are available through the references, and illustrated by the examples, within Derek Nexus. |
| 4 | predictions as | |
| | Certain: 💇 | There is proof that the proposition is true |

There Pat least one strong argument that the proposition is Probable:

true and there are no arguments against it

The weight of evidence supports the proposition Plaûsible:

Eguivoeal. 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

yImpossible: There is proof that the proposition is false

Open: There is no evidence that supports or opposes the proposition

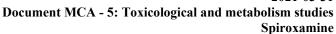
Contradicted: There is proof that the proposition is both true and false In addition to the above, Derek Nexus contains expert-derived functionality to provide negative predictions for bacterial in vitro mutagenicity. The query compound is compared to a Lhasa reference set of Ames test data and the



software determines whether there are structures that have been misclassified by Derek Nexus or is unknown. This provides a high confidence in negative productions for this provides a high confidence in negative.

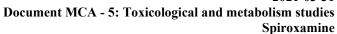
Leadscope Model **Applier Genetox Suite:**

| | predictions for | |
|--|---|---|
| Leadscope Model Applier Genetox | Parameter | Details |
| Applier Genetox Suite: | Prediction en | ndpoint: Gene mutation: mammalian 🕅 vitro |
| | MODEL | HGPRT Mut v1 |
| | QMRF protocol | JRC reference number not available (protocol date cotober 2014) |
| | Comment on endpoint | Combination of results from all in vitro Chinese handster of CHO) and Chinese hamster lung (CHL) gene foutation tests using the hyprt locus. |
| | Algorithm of the model | Partial logistic regression. The descriptors are generated using the Leads ope software I wo SAR models are generated with a bolance of positive and negative compounds and the results are calculated as an average of wo models. |
| | Statistics | i). Internal ho data available. |
| S. S. S. S. S. S. S. S. S. S. S. S. S. S | 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) paving at least one chemical in a training neighbourhood with at least 30% global similarity to the text structure. |
| % | 4 9 | The model uses 27 descriptors for 643 chemicals. |
| | Analogues MODEL | Presented in model output. Mouse Lymphoma Activated v2 |
| ĮŠ (Õ | MODILL | |
| | OMRF (| JRC reforence number not available (protocol date April 2016) |
| | Comment some contend on the contend of the contend | Re-cored results of the mouse lymphoma mutation assays at the thymidine kinase (tk) ocus 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. |
| | Domains | Leadscore 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 a least 30% global similarity to the test structure. |
| | | The model uses 234 descriptors for 674 chemicals. |
| | Amologues | 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: I having at least one structural feature defined in the model in addition to all the proper descriptors having at least one chemical in a training perghborohood with at least 300 global similarity to the test structure. The model uses 245 descriptor for 750 chemicals. |
| | Analogues | Presented in model output & & & & |
| | Prediction en | ndpoint: Gene mysation: Microbin in vitro |
| | MODEL 3 | Bacteria Mutation v2 A |
| | QMRF protocol | JRC reference number not available (protected date April 2019) |
| | Comment on Condpoint | Combination of results from the Sytyphich rium histidine reversion gene must non using tester strains TA97, TA97a, TA1537, TA98, TA 100, TA1535, TA102, E.coli (any variant) |
| | Algorithm of the model | Partial registic regression. The descriptors are generated using the Leadscope software of coded from the literature as substructure queries. |
| | Statistics | j). Internal: no data available. vi). External: no data@vailable |
| | Dongains A | Leadscope uses two parameters to guide the applicability of the model domain 1) having at least one structural feature defined in the model on 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. |
| ŽŶ 4 | Analogues, | Presented in model output |
| | MODEL | ExColi Sa 102 T Mut v2 |
| | QMRF protocol | JRC reference number not available (protocol date April 2019) |
| | Omment on endpoint | Combination of results from the E. coli WP2 uvrA, E.coli WP2 vvrA (pRM101), and s.typhimurium TA102 |
| | Algorithm Que Chodel | Partial logistic regression. The descriptors are generated using the Leadscope software or coded from the literature as substructure queries. |
| | Statistics 9 | 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 |
|------------------------------|--|
| | Samonena v4 |
| QMRF protocol | JRC reference number not available (protocol date April 2019) |
| Comment on endpoint | Combination of results from the S conhimurium his indine reversion gene mutation test using tester strains TA97, TA98, TA100, TA 1535, TA1536, TA153A 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: Qno data available. ii). External: no data available Q Q Q Q |
| 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 applications for 4326 chemicals. |
| Analogyes | Presented in model output |
| 4(5) | hdpojar: Clastogenicity in vitro |
| MODEL | In vitro chrom ab CHLQv2) |
| QMR6 protocol | JRC reference number not available (protocol date April 2015) |
| Comment on endpoint | In vitro Chromosome aberration test using Chinese hamster dung cells |
| Algorithm of the model | Partial logistic regression. The descriptors are generated using the Leadscope software I wo 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 5 | i). Internat no data available. |
| Domain | Leadscope uses two parameters to guide the applicability of the model domain: 1) Leaving 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 |
| MÕDEL Q | In varo chrom ab CHO (v2) |
| SMRF Sprotogol | RC reference number not available (protocol date April 2015) |
| Convenent of 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 |



| | | Leadscope uses two parameters to guide the applicability of the |
|----|------------------------|---|
| | | model domain: 1) having at least one structural feature defined |
| | Domains | in the model in addition to all the property descriptors having at least one chemical in a training peighbourhood with |
| | | at least 30% global similarity to the test structure. |
| | | The model uses 274 descriptors for 819 chemicals. |
| | Analogues | Presented in model output |
| | Prediction en | ndpoint: Clastogenicity in vivo 🗸 🧳 💆 |
| | MODEL | Chrom ab comp (v1) |
| | QMRF protocol | JRC reference number not available (projocol date July 2012) |
| | Comment on endpoint | Chromosomo aberrations in vivo using rats, mice and other species that are not defined in the PPA 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 another results are calculated as an average of the two plodels. |
| | Statistics & | i). Internal: A po Odata available. |
| ٨ | | Leadscope uses two parameters to guide the appleability of the phodel formain (P) having at least one spuctural feature defined |
| | Domains 🔊 | in the model in addition to all the property descriptors 2) having at least one chemical in a training reighbourhood with |
| Ž. | | at least 30% global similarity to the test structure. |
| | \$ 3 | The model uses 207 descriptors for 283 chemicals. |
| | Amalogues | Presented in model output |
| | MODE | Chrom ab other roden (v1) |
| | QM&F protocol | JR Geference number not available (protocol date July 2012) |
| | Comment on emploint | Chromosome aberrations in vivo using rats and mice |
| | Of the | Partial logistic regression. The descriptors are generated using the Leadscope of tware. Two QSAR models are generated with a balance of positive and negative compounds and the |
| | model | results are calculated as an average of the two models. |
| | Statistics | internal: no data available. |
| | | Leadscope uses two parameters to guide the applicability of the model domain: 1) having at least one structural feature defined |
| | Dománis S | 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. |
| | Änalogues | 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 varilable. 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 detyred 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 187 descriptors 60 110 chemicals. |
| | Analogues , | Presented in model output |
| | MODEL ® | In vivo miconucleus mouse V & V |
| | QMRF protocol | C reference number not available (protocol date July 2012) |
| | Comment Comment | In vivo migronucleus in mixe |
| | Algorithm of the model | Partial begistic 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 3 | no data available. ii) External no data available. |
| | | Madscope 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) |
| | Domains (| |
| | | The model uses 207 descriptors for 624 chemicals. |
| | Acalogues | Presented in model output |
| | Ceadscope Mo | data then assigns a probability value based on likelihood of |
| 4 | occurrence. | adscore take into account both toxifying and detoxifying |
| W W | | production results for each model are presented as the |

Evadscope Mode Applier predicts toxicity by comparing the entered structure with expirical data then assigns a probability value based on likelihood of occurrence deadscope take 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", "regative" 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.

Sproxamine and each metabolite was processed using Derek Nexus and Veadscope 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

3. Method:



according to the principles described in Myatt et al., 2018²⁴. 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, GLD experimental genotoristy data for these compounds. A confidence level thigh, medium or low was assigned for each endpoint following expert review as follows:

High:

- In since prediction matches recently conducted experimental data
- (In silice) prediction matches recently conducted experimental data for compound with close structural similarity

Medium:

Matching in silico predictions on two different methods, following expert review

Low

In stico prediction available using one method only following expert seviews

4. Available experimental data:

Spiroxamine has been previously evaluated by EFSA with the data mackage 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 nat metabolism data available for spirosamine (FSA DAR, 2017) with the data updated for the impending renewal of approval.

Table CA 5.8.1/28 Derek Nexus and Leadscope Model Applier spocessing constraints

| Endpoint > | Endpoint specific | Alert/model & |
|--------------------------------|--------------------------------|--|
| Derek Nexus 6.0.1 Genotoxicity | | |
| | Mutagenicity A | Mytagenicity in vitro |
| | | Mutage Heity in Vivo |
| <u> </u> | | Photophutage Dicity in vitro |
| | Chromosome damage | Chromosome damage in vitro |
| | Caromosone damage | Oromosome damage in vivo |
| Q (A | | Photo induced chromosome damage in vitro |
| l | Nor specific genotoxicity C | Non Specific genotoxicity in vitro |
| ₩ | | Non-specific genotoxicity in vivo |
| 4 | | Proto-induced non-specific genotoxicity in vitro |
| | * * | Photo-induced non-specific genotoxicity in vivo |
| Leadscope Model Appl | ier v3.0.1 Genotox statistical | Suite ^{BC} |
| Gene mutation | Mammalian in viro | HGPRT Mut v1 |
| | | Mouse Lymphoma Act v2 |
| (I) | | |

Myatt, G.D., Ahlberg, E., Dahori, V., Allen, D., Amberg, A., Anger, L.T., Aptula, A., Auerbach, S., Beilke, L., Bellion, P., Benghi, R., Bercu, J., Booth, E.D., Bower, D., Brigo, A., Burden, N., Cammerer, Z., Cronin, M.T.D., Cross, K.P., Custer, L., Detwiler, M., Doboth, Ford, K.A., Fortin, M.C., Gad-McDonald, S.E., Gellatly, N., Gervais, V., Glover, K.P., Glowrenke, V., Van Compel, J., Gutsell, S., Hardy, B., Harvey, J.S. Hillegass, J. Honma, M., Hsieh, J-H., Hsu, C-W., Hughes, B., Johnson, C., Jolly, R., Jones, D., Kemper, R., Kenyon, M.O., Kim, M.T., Kruhlak, N.L., Kulkarni, S.A., Kümmeter, 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., Seralimova, 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

Spiroxamine



| 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 Chrom Ab CHL v2 |
| | in vitro | In Vitro Chrom Ab CHO 2 |
| | Chromosome aberrations | In Vivo Chrom Ab Comp v2 |
| | in vivo | In Visco Chrom Ab Other v1 |
| | | In Vivo Chrom Aborat v1 |
| | Micronucleus in vivo | Mouse v2 |

All available species (Bacteria: Escherichia coli and Salmanolla typhimurium. Mananal: dog monke and human (primate); rabbit; and rat, hamster, mouse and guinea pignodent)) and option to perceive tautomers were selected. Only predictions at the EOUIVOCAL level or above are reported in the ext.

Only predictions at the EQUIVOCAL level or above are reported in the ext.

B The following endpoint specific models are available in this syste 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 Landscope Model Applier processing constraints

| Profiler type | Endpoint associated | Rrofile Plert |
|---------------------|---------------------|--|
| | | DNA alerts for AMES CA and MNT by OASIS |
| | Mutagenicity 5 | In Aro mutagenicity (Ames test) alerts by ISS |
| Endpoint specific | | |
| | Chromosomedamage | Protein binding alerts for chromosofinal aberration by OASIS |
| General mechanistic | | DNA binding by OASIS |
| | Mutagenicity & | DNA binding by OE D |
| General mechanistic | | Protein binging by OASIS |
| | Chromosope damage | Protein Dinding OECD |

Table CA 5.8.1/28-6 Relevant toxicity endpoints for spiroxamine and M03

| Substance Substance | Endpoint Mutagenicity (In vitro bacterial reverse (Ames) gene protation | Chromosome damage (Incarro mammalian meronucleus) | Reference |
|---|--|---|--|
| Spiroxamine M03 | | | 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 A A A A A A A A A A A A A A A A A A A | Negative 3 | 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 [<u>M-755223-02-1</u>] CA 5.8.1.2/03 |



| | Endpoint | Q)° | |
|-----------|--|---|---|
| Substance | Mutagenicity (In vitro bacterial reverse (Ames) gene mutation) | Chromosome damage (In vitro mammalian micronucleus) | Reference |
| | | | [M-471125-01-3] CA 5 8 1/23 [M-755227-02-1] |
| M35 | - | Negative S | (\$\frac{1}{2}\) 5.8.4\tilde{9}\(\frac{1}{2}\) \[\frac{1}{2}\] |
| M37 | - | Gegative S | CA 5.8.1/26 [M-761547-01~6] |
| M28 | Negative | Regative Nogative A A A A A A A A A A A A A A A A A A A | CA 5.8.1/15 [M\$463413701-1]; CA 5.8.8703 \$\frac{M}{4}-465292-0227]; CA \$\frac{8}{2}.1.3/00 [M\$469334_01-1] |

Results

- Results and discussion:

A. QSAR predictions:

1. Spiroxamine [reference compound]: (IUPA)

piame 8-(1,1-dimethylethyl)-

CCN(SC1CQ2(CCC(C(C)(C)C)CC2)O1)CCC

Derek Nexus: predicted to be inactive for bacterial mutagenicity with no misclassified or undassified features. There were no alerts for mammalian mittagenicaty or chromosome damage. The compound is considered to be within the applicability domain of the model.

- Leadscope Model Apprier:

 Mutagetifeity: no alerts for the Kacterial or mammalian mutagenicity endpoints (refer to Table CA 8.1/28-5), with the in silico bacterial and manimalian predictions in agreement with the control of the silico bacterial and manimalian predictions in agreement with the experimental data
- Chomosome 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 DECDAO)SAR Toolbox: agreement with the experimental data.

path 163 substances examined, with only 55 cm¹ @escribe 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 mutagence. *In silico* predictions using two methods are in agreement with the experimental result. The OECD (Q)SAR Toolbox (Toolbox) alerts are considered not relevant aking 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 fromosome dandage

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 genotopic and the *in silico* analysis using two methods corresponds with this finding.

2. Spiroxamine-N-oxide (M03) [reference compound]:

Chemical name N-[(8) (ert-b)tyl-1,4/dioxaspi0 [4.5] decan-2 (IUPAC): yl)methyl N-ethypropan-y-amine N-oxide

Chemical structure

SMILE'S

- Results and discussion:

MOS differs from sproxantine with the oxidation of the Northyl group.

<u>Detek Nexus</u>: predicted to be mactive for bacterial mutagenicity with no misclassified of 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.

Cadscoo Model Applier

- Mutagenicity: no aborts for the bacterial of mammalian mutagenicity endpoints refer to Table CA 5 \$21/28-550
- A hromosome damage no alexts for any of the chromosome damage specific endpoints (refer to Pable CA 5.8.1/28-5).

OECD OSAR Toolbox:

- Mutagenicity: profiled as potential for *in vitro* mutagenicity (Ames test) by SS with the Hyacceptor path Hyacceptor alert (refer above to 1.
- Spiroxamine regarding predictive performance of this alert). However, a valid robust in vitor bacterial mutagenicity study is available, which concludes that 2003 does not increase bacterial revertant colony frequency. It is concluded therefore that this alert is not realized experimentally, and deemed not cologically relevant.
- Chromosome da age: 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.

$^{ar{y}}$ 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 N-[(8-tert-butyl-1,4-dioxaspiro[4.5]decan-2name

(IUPAC): yl)methyl]propan-1-amine

CAS No.: Not available

Chemical structure:

CC(CNE)C1CCC2(C@X)OCC(CNC@ SMILES:

M01 differs from spiroxamine by the loss of the N-ethyl group giving rise to secondary amine function.

Derek Nexus: no alerts drivated for bacterial mammalian matagenicity chromosome damage specific endpoint with no misclassified or unclassified features. There were no alerts for manmalism mutagenicity. The compound is considered to be within the applicability danain of the model.

Leadscope Model Applies

- Mutagenicity predicted to be inactive for bacterial nutagenicity with no misclassified or maclassified features. There were no alects for mammalish mutager City (refer to Table CA 3.8.1 28-5).

- Chromosomo amage no alers for any of the chromosome damage specific end wints (refer to Table (5.8.1/28-5)

OEGD (Q)SAR Toobox:

Mutagemeity profiled as potential for it vitros mutagemeity (Immes test) by ISS with the P-acceptor-path H-acceptor abort (refer above to 1.

Spiroxamine regarding predictive performance of this aleut).

- Aromosome damage: Walert dentified.

Expert evaluation of invitico prognosifor mutagenifity

M01 is predicted not to be obutagenic by Derek Nexus and five of the six mutagenicity sub-models in beadscope, it was out of domain for the remaining sub-model. The Toolbox alert is considered not relevant since it is also present io a similar chemical space to oproxamine. Furthermore, a secondary amine is considered to have a low probability of increasing genotoxic potential (Benigni

Exfert evaluation of in Afico prognosis For chromosome damage

M01 gave no derts 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.

On silico analysis by two methods predicts M01 is neither mutagenic nor causes chronosomal damage thus Giere is medium confidence in this prediction.

ni, R., Battistellimenskaia, Cles and 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



Leadscope Model Applier mutagenicity and chromosome damage predictions: spiroxamine, M03. M01 **Table CA 5.8.1/28-7:**

| Effect Sub-model OSAR prediction probability review prediction probability probabil | | | | | Mutagenicity | predictions | , | TOP | | | |
|--|---|--|--|--------------------------------------|-------------------|---------------------------|-------------------------|---------------|-----------------------|-------------------------|----------------------------------|
| Mammalian in vitro Microbial in vitro E Coli - Sal 102 A-T Mut v2 NID Negative Salmonella Mut v4 Nid Nid Nid Nid Nid Nid Nid Nid Nid Nid | | | | Spiroxamine | 4 | o ^{\$} | · M03 | - Ĝ° | | MOLES | |
| in vitro Mouse Lymphoma Act v2 NID 0.285 Accepted Negative 0.451 Accepted Negative 0.343 Accepted Negative 0.295 Accepted Negative 0.343 Accepted Negative 0.295 Accepted Negative 0.343 Accepted Negative 0.295 Accepted Negative 0.343 Accepted Negati | Effect | Sub-model | | | review | QSAR prediction | Positive probability | Expert review | QSAR Dediction | Positive Probability | Expert review |
| Microbial in vitro Bacterial Mutation v2 Negative 0.078 Accepted Negative 0.108 Accepted Negative 0.039 Accepted Negative 0.255 Accepted Negative 0.090 Accepted Negative 0.090 Accepted Negative 0.020 Ac | | Mouse Lymphoma Act v2 | NID | | Accepted Accepted | Megative . | 0.451 | A aconto | Né≫ative ≪ | © 0.180 | Accepted Accepted Accepted |
| CA in vitro In vitro Chrom Ab CHL v2 NID Accepted NID Accepted NID Accepted NID 0.356 Acc | Microbial in vitro | E Coli - Sal 102 A-T Mut v2 | NID Negative | 0.0830 | Accepted & | Negative Negative | 0.25 0.008 | Accepted | Negative | 0.090 | Accepted Accepted Accepted |
| CA in vitro In vitro Chrom Ab CHL v2 NID Accepted NID Accepted NID O.356 Accepted NID O.3 | | | COUNTRY TO | O. P. Colo | romosome dan | nage prediction | *C. A. 9'C. | | , \$ | | |
| CA in vivo In vivo Chrom Ab Comp v2 In vivo Chrom Ab Other v1 In vivo Chrom Ab Other v1 In vivo Chrom Ab Other v1 In vivo Chrom Ab Rat v1 NID 0.373 Accepted NID 0.366 Accepted NID 0.366 Accepted NID 0.101 Accepted NID 0.10 | | | NAD O | 000 | & Accepted & | NID 1 | \$350 0.119 \ S | Accepted Oob | | | Accepted Accepted Accepted |
| Micronucleus in vivo Accepted Negative A. Donain exceptions: A Dioxolane ring B. Cyclohexane A Dioxolane ring B. Cyclohexane A Dioxolane ring B. Cyclohexane Accepted Negative A. Donain exceptions: A Dioxolane ring B. Cyclohexane A Dioxolane ri | CA in vivo | In vivo Chrom Ab Comp v2 In vivo Chrom Ab Other v1 In vivo Chrom Ab Rat v1 | NID OF | 0.038 0.373 0.360 | Accepted Accepted | Negative ^A NID | 0.041 | OoD OoD | NID | 0.265 | Accepted Accepted Accepted |
| CA: chromosome aberrations Domain exceptions: Domain exceptions: Domain exceptions: A Dioxolane ring Dioxolane rin | 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: chromoson NID: not in don OoD: out of dor | ne aberrations nain nain nain nain nain nain nain na | Domain Sceptic School S | APLOLIBBIC APLOLIBBIC ACOLIDIA | ed and | Domain except | ions: | | A Dioxolane rir | ıg | |

^A Dioxolane ring

^B Cyclohexane



Table CA 5.8.1/28-8: QECD (Q)SAR Toolbox structural alerts Alert H-acceptor-path3-H-acceptor (DNA binding by OECD) (In vitro mutagenicity (Ames) / in vivo mutagenicity (micronucleus) alerts by ISS) H-bond-Acc R = aliphatic CA = any atom, except Hydrogen The cyclic aliphatic ring system can be any size above H-bond Acc = any atom that is a potential Hydrogen @ = 3 (se. not aririding). The right system cannot be bond acceptor Compounds where alert is activated: sproxamille. Compounds where alert is activated: spirgstamine M03, M01, M02, M04, M05, M06, M07, M08, M09 M10, M11, M12, M25, M26, M27. 2038,

4. Spiroxaminedespropyl (M02) Chemical name (IUPAC):

N-[(S) tert-butyl-1,4-droxaspiro[4.5]decan-2yl) wethyl tethanaryjne

0

A NICA

Not available

@ ----- @ ----- & -

- Results and discussion:

CC(C)(C)C1CCC2(CC1)OCC(CNCC)O2

M02 differs from spiroxamine by the loss of the N-propyl group giving rise to a secondary awine function.

Derek Nexus: predicted to be mactive for bacterial mutagenicity with no misclassified of unclassified features. There were no alerts for mammalian mutagenicity of chromosome damage. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applier

- Mu@genicity. no alerts for the bacterial or mammalian mutagenicity enopoints (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)SAN Toolbox:

- Soutagenicity: profiled as potential for *in vitro* mutagenicity (Micronucleus est) 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 readscope. It is out of domain for remaining five Leadscope models.

Conclusion

In silico analysis by two methods predicts M02 is neither mutageric nor sauses chromosomal damage thus there is medium confidence in this prediction.

5. Spiroxamine-Nformyl-desethyl (M04): Chemical name N-[(8-tert-butyl-1,4-proxaspiro[4.5] decan-(IUPAC): y) whethyl]-N-proxylformamide

ot available

CAS No.:

Chemical structure: \(\)

- Results and discussion:

SMILES:

CC(C)(C)CECC2(CE1)QQQ(CN(G=0)CCQ)O2,Q

M04 differs from spinoxamine by oxidation at the N-ethyl group giving rice to a tertiary applies function.

Derek Nexus: predicted to be inactive for pacterial mutagenicity with no misclassified or unclassified features. There were no aletts for mammalian mutagenicity or chromosopic damage. The compound is considered to be within the applicability to main of the model.

Leadscore Model Applier:

- Mutagenicity: no aterts for the bacterial of manifoldian mutagenicity endpoints (refer of Table CA 5 1/2847).

Thromosome damage no alerts for any of the chromosome damage specific endpoints (refer to Table Ca 5.8.1/28-7).

OEÇD (Q)SAR Toolbox: 🛣

- Mutagonicity: profiled as potential for DNA binding by OECD SN1

mechanism and in varo mutagenicity micronucleus) by ISS with the Hacceptor-path3-H-acceptor alert (refer above to 1. Spiroxamine regarding
predictive performance of this alert).

Ochromosome damage Ono alerts identified.

Expert evaluation of in silico prognosis for mutagenicity

MO40's 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 manufacture mutagenicity sub-models. The Toolbox alerts are considered not relevant since it is also present in a similar chemical space to spiro amine.

Expert evaluation of in silico prognosis for chromosome damage

M04 gives no derts 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 will 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 2-(2-{[ethyl(propyl)amino]methyl}-1,4-(IUPAC): dioxaspiro[4.5]decan-8-yl)-2-methylpropan-1-ol



CAS No.:

Not available

Chemical structure:

- Results and

discussion:

SMILES: CC(C)(CO)C1CCC2(CC1)OCC(CN(CC)CCC)O2

M05 differs from spiroxamine by oxidation at the t-butyl group giving a hydroxy metabolite.

Derek Nexus: predicted to be mactive for betterial mutagenicity with no misclassified or unclassified features. There were no alerts for manmalian 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 QA 5.8, DZ8-7).
- Chromosome damage no alerte for an of the fromosome damage specific endpoints (refer to Table CA \$.8.1/28\$).

OECD (Q)SAR Toolkox:

- Mutagen City: provided as Gotential for DOA birding by DECD N1 mechanism and in vitro mutagenicity (Ames) by ISS with the A-acceptorpath3 H-acceptor aler (refer above to 1. Spinoxamine regarding predictive performance of this alert)
- Gromosome damage: po alerts dentified.

Expert evaluation of in silico prognosis for mutagenicity

M04 is predicted not to be mutagenic by Derck Nexus and five of the six of the mutagenicity Sub-models in Leadscope, it was out of domain the remaining Leadscope model The Tootbox, a letts are considered not be levant 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-but moiets and hydroxy functions are considered to have a low probability of increasing genotoxicity (Benigni et al. 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 progrosis for chromosome damage

Mos does not give any alerts for chronosome damage in Derek Nexus or Toolbox and gives negative predictions in four of the six Leadscope sub-models, it is and of domain in the remaining models. Furthermore, since the structure of MOS is similar to spiroxamine, with the only structural difference between the two being the horizoxylogioup on the t-butyl moiety which gives no alerts in Derek Nexus of Toolbox, the negative outcome of recently conducted, GLP chromosome averration test for spiroxamine can be used to support the negative in spico prediction for MOS!

n silico analysis by two metl chromosomal damage and conconfidence in this prediction. In silico analysis by two methods predicts M05 is neither mutagenic nor causes chroniosomal damage and comparison with spiroxamine supports high



Leadscope Model Applier mutagenicity and chromosome damage predictions: M02, M04, M05 **Table CA 5.8.1/28-9:**

| | | | | Mutagenicity | predictions | | KOB . | | | |
|--|---|--|-------------------------|-------------------|---|--------------------------|------------------------------|--|--------------------------|----------------------------------|
| | | | M02 | , | 0) | · MOA | Ġ° | | M05, | |
| Effect | Sub-model | QSAR prediction | Positive probability | Experit reform | OS AR Operation | Positive probability | Expero regiew | QSAR Drediction | Positive Oprobability | Expert review |
| Mammalian <i>in</i> vitro | HGPRT Mut v1 Mouse Lymphoma Act v2 Mouse Lymphoma Unact v2 | NID NID Negative ^A | 0.186 0.184 0.300 | Accepted Accepted | NID NID | 0.176 0.3834 0.006 | Accepted Accepted Secepted | Negative NID Negative ^A | 0.174 0.293 0.351 | Accepted Accepted Accepted |
| Microbial in vitro | Bacterial Mutation v2 E Coli - Sal 102 A-T Mut v2 Salmonella Mut v4 | Negative S Negative ^B Negative | 0.099° 0.023 | Accepted OoD | Negative Negative Negative | 0.043 | Accepted Accepted Accepted | Negative Negative © Negative | 0.207 0.091 0.081 | Accepted Accepted Accepted |
| | 6 | | Or Chr | omosome dam | age predictions | (C) O'_ | | , \$ | | |
| CA in vitro | In vitro Chrom Ab CHL v2 In vitro Chrom Ab CHO v2 | ONID O | 0.285 | Accepted | ND Negative | 0.131 | A coontrol | Negative ^A Negative ^B | 0.301 0.121 | Accepted Accepted |
| CA in vivo | In vivo Chrom Ab Comp v2 | ∂.¥ID . | \$ 0.063 & ® | Accepted Accepted | N@ative | 0.030 0.377 0.115 | Accepted OoD Accepted | Negative NID Negative ^{B,C,D} | 0.038 0.364 0.149 | Accepted Accepted OoD |
| Micronucleus in vivo | In vivo Micronuc Mouse 2 | J Negative C | 0.313 | Accepted | ONID | 0.397 | Accepted | Negative ^{B,D} | 0.419 | Accepted |
| CA: chromosome NID: not in doma OoD: out of doma | | omain Reeption Oxolane ring Secondary amide Cyclon Rane | | | Domain@xceptic Doxolane ring Secondary ami Alkyl formami | g ide | | Domain exception Domain exception Dioxolane ring Tobutyl OH Tertiary amide Dioxolane ring Dioxo | | |

^A Dioxolane ring

^B T-butyl OH

^C Tertiary amide

D Cyclohexane ring



7. Spiroxamine-acid (M06):

Chemical 2-(2-{[ethyl(propyl)amino]methyl}-1,4name

dioxaspiro[4.5]decan-8-yl)-2-methylpropanoic acid (IUPAC):

Not available CAS No.:

Chemical structure:

O=C(O)C(C)(C)C1CCC2(OC1)OCC(CNQ SMILES:

)02

- Results and discussion:

M06 differs from spiroxamine by oxidation at the t-butyl group giving an action metabolite. M06 was the major metabolite wind 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 the misclassified or unclassified features. There were no alors for mammatian mutagenicity or chromosome damage. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applier:

- Mutagenieny: no alerts for the harterial of mammalian pritagenicity
- endpoints (refer to Table CA 508.1/28.8).

 Chromosome damage pocific endpoints (gefer to Table (5.8.188-8)

OE (Q) SAR Toolbox:

Mutagenicity Profiled as potential for DNA funding by OECO SN1 mechanism and in vitro mutagenicity (Ames) by ISS with the H-acceptorpath3-H-acceptor Vert (refer above to 1. Spiroxa vine regarding predictive performance of this aler.

Thromosome damage no alerts identified.

Expendevaluation of in silica prognasis for mutagenicity

MOA is predicted not to be sputagenic by Derek Newus and five of the six of the mutagenicity submodels in Leadscope at was out of domain the remaining Cadscope model. The Toolbo Calerts are considered not relevant since they are also present in a similar cherical space to spiroxamine. Since the structure of MOS is simpler to spiroxamine, with the curry 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 progrative outcome of ocently conducted, GLP mutagenicity tests for spin xamine can be used to support the negative in silico prediction for M05.

Expert e duation of in Slico prognosis for chromosome damage

OM05 closes 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 spirotamine, with the only structural difference between the Two being the hydroxy group on the t-butyl moiety which gives no alerts in Derek Nexus or Toolbox, the negative outcome of recently conducted, GLP chromosoffe aberiation tests for spiroxamine can be used to support the negative in silice prediction for M05.

Conclusion

In stico analysis by two methods predicts M05 is neither mutagenic nor causes chromosomal damage and comparison with spiroxamine supports high **≪**∞onfidence in this prediction.

8. Spiroxamine-hydroxy acid (14907): (IUPAC):

*Chemical name 2-(2-{[ethyl(propyl)amino]methyl}-1,4dioxaspiro[4.5]decan-8-yl)-3-hydroxy-2-

methylpropanoic acid

CAS No.: Not avavailable



Chemical structure:

SMILES: O=C(O)C(C)(CO)C1CCC2(CCO)OCC(CN(CCO)

C)O2

- Results and discussion:

M07 differs from spiroxamine by oxidation in two positions at the toutyl group giving a hydroxylated acid metabolite

<u>Derek Nexus</u>: predicted to be inactive for batterial mutagenicity with no misclassified or unclassified features. There were no alerts for mammatian mutagenicity or chromosome damage. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applico

- Mutagenicity: no alerts for the bacterial or mammalian mutagenicity endpoints (refer to Table & 5.8 728-8).
- Chromosome damage: no alerts for an of the hromosome damage specific endpoints (refer to Table CA 5.8.1/28-8).

OECD (Q)SAR Toolbox:

- Mutagementy: profiled as potential for DNA bineing by ECDCSN1 mechanism and in vitro mutagenicity (Ames) by ISS with the H-acceptor-path H-acceptor alert (refer above to 1. Spiroxamme regarding predictive performance of this alert).
- nromosome damage: no alerts identified.

Expert evaluation of insilico prognosis for mutagenicity

M07 i 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 sprilar chemical space in spiroxamine. It is noted that individually, the hydroxy and acid metabolites (M05 and M06, respectively) are predicted not to be mutagened 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.

Experdevaluation of in silicoprognosis for chromosome damage

Mot 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 drydrox 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 M00 is unlikely to give extra cause for concern via creation of additional centres of reactivity.

Conclusion

In olico 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.

Chemical name (PAC)

2-(2-{[ethyl(propyl)amino]methyl}-8-hydroxy-1,4-dioxaspiro[4.5]decan-8-yl)-2-methylpropanoic acid

EAS No.: Not available Chemical structure:

SMILES:

O=C(O)C(C)(CO)C1CCC2(CC1)OCC(CN(CC)CC C)O2

- Results and discussion:

9. Spiroxamine-8-

hydroxyacid M08

M08 differs from spiroxamine by oxidation on the t-butyl group and the cyclohexyl ring giving a hydroxylated acid metabolite.

<u>Derek Nexus</u>: 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.

<u>Leadscope Model Applier:</u>

- Mutagenicity: no alerts for the bacterial or mammaliar mutagenicity
- Chromosome damage: no alerts for any of the chromosome damage specific endpoints (refer to Table CA 5.8.1/28-8).

 DECD (Q)SAR Toolbox:

OECD (Q)SAR Toolbox:

- Mutagenicity: profiled as potential for DNA sinding by OECD SNE mechanism and in vitro mutagenicity (Amos) by ISS with the H-acceptors path3-H-acceptor alert (per above to 1 Spiroxamine rearding predictive performance of this alent.
- Chromosome damage no alerts identified

Expert evaluation of in silico prognosis for mutagenicity

Aut and fly adequate in the selected for relevant si.

In spirotampic. It is 71 is precised not give mulgen across functional groups in Miss is up a creation of admittional centres of reactivities programs for throntosome illumage and admitted for chromosome illumage and admitted for chromosome damage in Berek Nex and dogston forces governed to cap and the continuous and the continuous and the continuous and the continuous across for chromosome damage, the relative position of each of these functional groups affoliately dogstone findels, where a neg a ferupada it is noted that independently content of each of these functional groups affoliately placed in the financial content of each of these functional groups affoliately dogstone forces are and of the functional groups affoliately placed in the financial content of each of these functional groups affoliately placed in the financial content of each of these functional groups afforced in the functional groups afforced in the financial content of each of these functional groups afforced in the financial content of each of these functional groups afforced in the financial content of each of these functional groups afforced in the functional groups afforced in the financial content of each of the functional groups afforced in the f M08 is predicted not to be mutagenic by Dereck Nexus and five of the six .



| | /28-10: Leadscope Model | | | Mutagenicity | predictions | | COP CONTRACTOR | reckion | regime. | |
|---|--|--|--------------------------|-------------------|-----------------------------|--|----------------------------------|--|---------------------------------|----------------------------------|
| Effect | Sub-model | | M06 | | | | Ġ° | | Mex | |
| | | QSAR prediction | Positive probability | Expert revow | QSAR prediction | Positive probability | Expero regiew | QSAR Prediction | Positive © probability | Expert review |
| Mammalian in vitro | HGPRT Mut v1 Mouse Lymphoma Act v2 Mouse Lymphoma Unact v2 | Negative NID Negative ^A | 0.174 0.293 0.300 | 0 .// 2 | NID NO | 0.058 0.284 0.483 | Accepted Accepted | Nogative Negative Negative | 0.233 0.432 | Accepted Accepted Accepted |
| Microbial in vitro | Bacterial Mutation v2 E Coli - Sal 102 A-T Mut v2 Salmonella Mut v4 | Negative Negative Negative | 0.207 0.0910 0.081 | Accepted Accepted | Negative NID Negative | 0.014 | Accepted Accepted | Negative Negative | 0.048 0.014 0.023 | Accepted Accepted Accepted |
| | | | | hromosome dar | nage@rediction | | | , \$ | | |
| CA in vitro | In vitro Chrom Ab CHL v2 In vitro Chrom Ab CHO | Negative ^B | ©301 © 0.121 × S | Accepted Accepted | Negatiwe ^A | 0.096 0.127 S | Accepted Accepted | NID NID | 0.132 0.129 | Accepted Accepted |
| CA in vivo | In vivo Chrom Ab Comp v2 In vivo Chrom Ab Other v1 In vivo Chrom Ab Rat v1 | Negative ND egative B,C,D | 0.364 0.149 | A | NIDO NIDO | W. Co | Accepted Accepted Accepted | NID NID NID | 0.036 0.307 0.312 | Accepted Accepted Accepted |
| Micronucleus in vivo | In vivo Micronuc Mouse v2 | Negative ^{B,D} | 0.419 | Accepted | ©egative ^B | 0.260 | Accepted | Negative ^B | 0.317 | Accepted |
| CA: chromoson NID: not in don OoD: out of dor | | Donate Except Dioxolane of T-but 1 Ori | tions. | of and | Don A Cy B Cy | nain exceptions: yclohexane dioxo yclohexane | olane | Domain e ^A Dioxola ^B Cyclohe | xceptions: ne ring exanol | |

^A Dioxolane ring

^B Cyclohexanol





10. Spiroxaminehydroxy-despropyl (M09):

Chemical name 2-{2-[(ethylamino)methyl]-1,4-

dioxaspiro[4.5]decan-8-yl}-2-methylpropan-1-ol (IUPAC):

CAS No.: Not available

Chemical structure:

SMILES: CC(C)(CO)C1CCC2(CC1)OCC(CNCC)O2

M09 differs from spiroxamine by oxidation on the t-buty and N-Cealky ation - Results and giving a hydroxy N-desproyl metabolite.

Derek Nexus: predicted to be inactive for Cacterial mutagenicity with misclassified or unclassified features. There were no alege 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 of mammalian mutagenicity endpoints (refer to Table CA 5 8 1/28-9)
- 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 vitro mutagenicity (Ames) by ISS with the H-acceptor fath 3-H-acceptor alert refer above to 1. Spiroxamine regarding predictive performance of this alert).
- Chromosome danage: no alerts dentified.

Expert exaluation of in Pico prognosis for mutageniçity

M09 is predicted not to be motagenic by Derek Nexus and four sub-models in Leadscope, deemed out of domain for the remarking 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 Laydroxy metabolites (M02 and M05, respectively) are also predicted not to cause mutagenicity. The rotative position of each of these functional groups in M09 is unfixely to give extra cause for confern via creation of additional reactive centres of reactivity

Expert evaluation of in silica prognosis for chromosome damage

Mos does not give any alerts for chromosome damage in Derek Nexus or Toolbox Regative predictions were returned for all of the models within Leadscope. It is noted that individually, the despropyl and hydroxy metabolites (MQ2 and MQ5, respectively) are also predicted not to cause chromosome damage and the relative position of each of these functional groups in M09 is untikely to give extra cause for concern via creation of additional reactive centres of reactivity.

🎤 Conctusion 🍳

Instilico agalysis by two methods predicts M09 is neither mutagenic nor causes Stromosomal dange, 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.

<u>Deek Nexus:</u> predicted to be inactive for bacterial mutagenicity with no inisclassified 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.

<u>Leadscope Model Applier:</u>

- Mutagenicity: no alerts for the bacterial or mammalian mutagenicity endpoints (refer to Table CA 5.8.1/28-9).

discussion:



- 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 SS 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 Dereck Nexus and four sub-models in Leadscope, deemed out of domain for the emaining two sub-models. The Toolbox alert is considered not relevant since it is also present in a smilar chemical space in spiroxymine. 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 sunctional groups in M09 is unlikely to give extra cause for contern via creation of additional reactive centres of reactivity

Expert evaluation of in silico prognosis for chromosome damage

M09 does not give any alous for onromosome damage in Derekt Nexus or Toolbox. Regative predictions were returned for all of the models within Leadscope. It is noted that individually the despropyland 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 feactivity.

Conclusion

In stlico analysis by two methods predicts M09 is wither wutagenic nor causes chromosofial danage, there is medium confidence in the prediction.

Chemical Uname N-ethyl-N-{[8-(1-hydroxy-2-methylpropan-2-yl)-1,4-diccaspiroff.5]degan-2-ylmethyl}propan-1-amine N-

AS Non W

Not avonlable.

Solution

Chemical structure:

\$MILES! O CC(C)(CO)(\$1CCC2(CC1)OCC(C[N+]([O-

Results and My differs from M03 or oxidation on the t-butyl moiety giving rise to a discussion: What differs from M03 or oxidation on the t-butyl moiety giving rise to a discussion:

Derek Nexus predicted to be inactive for bacterial mutagenicity with no misclassified or unclassified features. There were no alerts for mammalian mutagenicity or coromosome damage. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applier:

- Mutagonicity: 16 alerts for the bacterial or mammalian mutagenicity Cendpoints (refer to Table CA 5.8.1/28-9).
- Chromosome damage: no alerts for any of the chromosome damage specific empoints 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.

11. Spiroxamine – (hydroxy-N-oxide) (M10):





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 hydrogen metabolites (M03 and M05, respectively) are also predicted not to case mutagenicity and experimental data shows that M03 is not mutagenic. The relative position of each of these functional groups in 10 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 that mage 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 Noxide. It is noted that individually, the N-oxide and hydroxy metabolites (M03 and M05, respectively) and shown experimentally chromosome damage or are predicted with high confidence not to cause chromosome damage, respectivel The relative position of each of these for o more ties is considered not to give the to additional concerns via creation of an additional teactive centre.

Conclusion

In silico analysis, by two methods predicts M1Q is neither mutagenic nor cause chromosomal damage and by reference to M03 and M05 thore is high confidence in the prediction,

12. Spiroxamine – desethyl acid (M11):

name 2-methy 1-2-{2-propy amino methy 1,4dioxaspiro[45]decar@-yl}propanojoacid

Not available

Chemical structure

O≠Ç(O)C(Ç)(C)C1ČCC2(ÇC1)QCC(CNCCC)O2

MN differs from piroxamine by N-deptkylation and oxidation at the t-butyl gwing N-desethyl spirovamine 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: 0

- Mutagenicity: no alerts for the bacterial or mammalian mutagenicity Ondpoints (refer to TaOe CA 5/8.1/289).
- Chromoson@damage: no alerts for any of the chromosome damage specific empoints (refer to Table @A 5.8.1/28-9).

DEED (QISAR Toolbox:

- Mutagenicity profiled as potential for in vitro mutagenicity (Ames) by ISS with the Haccepto path 3 n-acceptor alert (refer above to 1. Spiroxamine regarding predictive performance of this alert).
- Chromosome mage 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 Loadscope 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





Abyl and acid metabolites (M01 and M06, respe.
o cause chromosome damage and that and the preco.
a by experimental weight of evidence. The relative post, we moneties its considered not to give rises; additional color, at an additional exactive centre.

n analysis by two methods predicts M11 is neighter mutageness or experimental damage, there is regular confidences in the prediction.

The state of the s

Spiroxamine



Table CA 5.8.1/28-11: Leadscope Model Applier mutagenicity and chromosome damage predictions: M09 M10, M10,

| | | | | Mutagenicity | predictions | | COR | | | | | | | |
|---|---|---|--|---------------|--|----------------------------------|--------------------|-----------------------------|-------------------------|----------------------------------|--|--|--|--|
| Effect | Sub-model | | M09 | 4T | O, P | · MIO | Ġ° (| | WHA | | | | | |
| | | QSAR prediction | Positive probability | Expert C | QSAR prediction | Positive probability | Expero regiew | QSAR Drediction | Positive probability | Expert review | | | | |
| in vitro | HGPRT Mut v1 Mouse Lymphoma Act v2 Mouse Lymphoma Unact v2 | Negative Negative ^{A,B} Negative ^C | 0.187 0.430 | Accepted | Negative ^A Negative ^B Negative ^A | 0.462 0.304 | OoD O | NUD NID NID | 0.060 0.173 0.483 | Accepted Accepted Accepted | | | | |
| vitro | Bacterial Mutation v2 E Coli - Sal 102 A-T Mut v2 Salmonella Mut v4 | Negative | © 0.139 0.109 0.465 | Accepted ODD | Negative Negative | 0.270 0.270 0.270 0.220 | Accepted Accepted | Negative NID Negative | 0.047 0.036 0.025 | Accepted Accepted Accepted | | | | |
| | | Negative | | hromosome dan | nageprediction | 16 9.0 | | , \$ | | | | | | |
| CA in vitro Chrom Ab CHL v2 Negative ^{D,B} Accepted Negative ^{D,B} OoD NID 0.339 Accepted In vitro Chrom Ab CHO 2 Negative ^B Accepted Negative ^{D,E} 0.119 OOD NID 0.240 Accepted | | | | | | | | | | | | | | |
| | In vivo Chrom Ab Other v1 In vivo Chrom Ab Other v1 In vivo Chrom Ab Rat v1 | Negative ^B | 0063 | Accepted N | Negative ^{D,E} | 0.042 0.350 0.145 | OoD OoD OoD | NID NID NID | 0.052 0.217 0.225 | Accepted Accepted Accepted | | | | |
| Micronucleus in vivo | In vivo Micronuc Mouse v | Negative ^{B,E} | 0.297 | | | | OoD | Negative ^A | 0.228 | Accepted | | | | |
| CA: chromosome NID: not in doma OoD: out of dom | ain 🥰 💍 | Bomain exception Secondary ame Bay Odroxy (Control of the Control | ons: Or of the control of the contro | i of the | Domain except A N-ox de B HOdroxy Dioxolane D Cyclohexane E Cyclohexane | -dioxolane | | Domain except A Cyclohexane | | | | | | |



13. Spiroxamine despropyl acid (M12): Chemical name 2-{2-[(ethylamino)methyl]-1,4-dioxaspiro[4.5]decan-

(IUPAC): 8-yl}-2-methylpropanoic acid

Not available CAS No.:

Chemical structure:

O=C(O)C(C)(C)C1CCC2/CC1)OCC(CN/C)O SMILES:

M12 differs from spiroxamine by N-dealkylation and oxidation at the t-buty - Results and discussion: giving N-despropyl spiroxamine acid.

Derek Nexus: predicted to be inactive to bacterial mutagenicity with no misclassified or unclassified features. There were no sterts for manufalian 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 Pacterial or material mutagenicity, endpoints (refer to Table CA 5.8.1/28-10).
- Chromosome damage: no aferts for any of the chromosome damage specific endpoints (refer to Table CA 5.8 1/28-10).

OECD (COSAR Twolbox:

- Mutagenicity: profiled as potential for in viero mutagenicity (Ames) by ISS with the H-acceptor-path H-acceptor alest (refer above to 1. Spiroxamine regarding predictive performance of this alert).
- Chromosome damage; no alerts identified. &

Exper Cevaluation of the silico prognosis for mutagenicity.

M12 is predicted not to be mutagenic by Derek Nexus and by two of the six magenicary models in Leadscope and is out of domain for the other Leadscope models. It is noted that \$12 returns a weakly positive result in the out of domain unactionted mouse lymphorna model. The Toolbox alert is considered not relevant because it is present in a similar chegoral space in spiroxamine. Examination of the contributing features for out of domain but weakly positive Apprediction indicates this is due to the corbony croup in the acid moiety, and the same moiet is present in the acid M06 It is noted that individually, the despropyland acid metabolites (102 and M06) are predicted not to cause nonagenicity 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 unlikely to goe extracause for concern via creation of an extra reactive

Expert evaluation of *in Bilico* prognosis for chromosome damage

M12 does not give any alegos for chromosome damage in Derek Nexus or Toolbox. Negative Gredictions 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) Tare 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 mojeries is considered not to give rise to additional concerns via creation of an extra reactive centre.

Conglusion

In silico analysis by two methods predicts M12 is neither mutagenic nor causes altromosomal damage, there is medium confidence in the prediction.

€hemical name 2-(2-{[ethyl(propyl)amino]methyl}-1,4-

(IUPAC): dioxaspiro[4.5]decan-8-yl)-2-methylpropyl hydrogen

CAS No.: Not available



Chemical structure:

SMILES: O=S(=O)(O)OCC(C)(C)C1CCO(CC1)OCC(G)CCC

CCC)O2

- Results and discussion:

M25 differs from spiroxamine by oxidation and conjugation at the t-botyl more giving hydroxy spiroxamine sulfate.

<u>Derek Nexus</u>: predicted to be mactive for bacterial mutagenicity with no misclassified or unclassified features. There were no alerts for manimalian mutagenicity or chromosome damage. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applier

- Mutagenicity: no alers for the bacterial or mammalian mutagenicity endpoints (refer to Table & 5.8, \$228-10)
- Chromosome damage: no alerte for any of the feromosome damage specific endpoints (refer to Table CA \$8.8.1/28 0).

OECD (Q)SAR Toolkox:

- Mutagenicity: profiled as potential for DNA birding by SECD SN1 mechanism and in vitro mutagenicity (Ames) by ISS with the M-acceptor-path 11-acceptor alert (refer above to 1. Spinoxamine regarding predictive performance of this alert).
- Gromosome damage: no alerts dentified.

Expert evaluation of in silico prognosis for mutagenicity

M25 is predicted not to mutagenic by Deret Nexus and it is out of domain for the Leadscope models. The Loolbox alerts are considered not relevant since they are also present in a similar chemical space in spiroxamina.

Expert evaluation of in Alico prognosis for chromosome damage

M25 does not give any alegs for coromosome 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 siller analysis a single method predicts \$125 is neither mutagenic nor causes chromosoped damage, there is low confreence in the prediction.

Chemica

name Q-meth 1-2-12

ĬUPĄCY: O 🏈 [(propylamino)methyl]-1,4-

dioxaspira[4.5]decan-8-yl}propyl

hydrogen sulfate

Not available

Chemidal structure

∠SMILES

O=S(=O)(O)OCC(C)(C)C1CCC2(CC 1)OCC(CNCCC)O2

- Results and discussion:

15. Spiroxamine –

(M26):

desethyl-sulfate

M26 differs from spiroxamine by N-dealkylation and oxidation and conjugation when the tability giving hydroxy desethyl spiroxamine sulfate.

Derek Nexus predicted to be inactive for bacterial mutagenicity with no miss assified or unclassified features. There were no alerts for mammalian magenicity or chromosome damage. The compound is considered to be within the applicability domain of the model.

- 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:



aicd as potential for in vitro mutage, anor-path-14-acceptor alert (refer above, acive performance of this alert); e damage in a letts identified attentified attentified attention of in illustration of in illustration for the Lastoner photoles The IQ erect not refervant since see also present it is an attention of the control of the c



Document MCA - 5: Toxicological and metabolism studies **Spiroxamine**

| | 1/28-12: Leadscope Model | | | Mutagenicity | predictions | | | Keck joi | TESTINE O | |
|---|--|--|-------------------------------|----------------------|------------------------------------|-------------------------|----------------------------|--|-------------------------|-------------------------------|
| Effect | Sub-model | | M12 | લ | | · M25 | - Ĝ° | | ~~\\.\\.\\\.\\\ | |
| | | QSAR prediction | Positive probability | Expert revow | QSAR prediction | | Expero Frequency | QSAR Drediction | Positive probability | Exper review |
| Mammalian in vitro | HGPRT Mut v1 Mouse Lymphoma Act v2 Mouse Lymphoma Unact v2 | NID NID NID | 0.064 0.174 0.502 | Accepted Accepted | NID NID | 0.170 0.210 0.002 | Accepted Accepted Accepted | NID Negative | 0.170 0.128 0.319 | Accepte Accepte Accepte |
| Microbial in vitro | Bacterial Mutation v2 E Coli - Sal 102 A-T Mut v2 Salmonella Mut v4 | Negative NID Negative | 0.060 \$ 0.040 \$ 0.029 | Accepted Accepted | Negative ^A NID Negative | 0.183 0.183 0.062 | Oot) Oot) OoD | NegativeA ^B NID Negative ^B | 0.097 0.208 0.043 | Accepte Accepte Accepte |
| | _ | - 7.T.C. | O. S. S. S. | hromosome dan | nage@rediction | 2 20 | | , \$ | | |
| CA in vitro | In vitro Chrom Ab CHL v2 In vitro Chrom Ab CHO | NO NO | \$ 0.296 K | Accepted Accepted | NADA WID | 0.106 0.106 | Accepted Accepted | NID NID | 0.308 0.206 | Accepte Accepte |
| CA in vivo | In vivo Chrom Ab Omp v2 In vivo Chrom Ab Other v1 In vivo Chrom Ab Rat v1 | NID NID | ©58 0.229 \$ 0.230 | Accepted Accepted | NIDO SAID NID A. | 0.016 0.184 0.140 | Accepted Accepted Accepted | NID NID NID | 0.024 0.120 0.087 | Accepte Accepte Accepte |
| Micronucleus in vivo | In vivo Micronuc Mouse v | Negative ^A | 0.234 | Accepted | OND . | ©.431 | Accepted | Negative ^{B,C} | 0.318 | OoD |
| CA: chromoson NID: not in don OoD: out of don | In vitro Chrom Ab CHL v2 In vitro Chrom Ab CHO 2 In vivo Chrom Ab Other v1 In vivo Chrom Ab Other v1 In vivo Chrom Ab Rat v1 In vivo Micronuc Mouse v2 me aberrations main main main mai | Domain Reption Control of the Contro | onstion that | iot en | Domain Excepti | ions: | | Domain except A Dioxolane rin B Sulfate C Cyclohexane | | |

^A Dioxolane ring

^B Sulfate

^C Cyclohexane



16. Spiroxamine – despropyl-sulfate (M27):

2-{2-[(ethylamino)methyl]-1,4-dioxaspiro[4.5]decan-Chemical name

(IUPAC): 8-yl}-2-methylpropyl hydrogen sulfate

CAS No.: Not available

Chemical structure:

S(=\$)(O)OCC(C)(\$)C1CCC2(C\$)O SMILES:

- Results and discussion:

M27 differs from spiroxamine by N-dealky fortion and oxiders on and conjugation at the t-butyl moiety giving hydroxydespropyl spiroxamine sulfate.

Derek Nexus: predicted to be inactive for bacterful mutagenicity with no misclassified or unclassified features. There were no afferts for mammalian mutagenicity or chromosome dansage. The compound is considered to be within , the applicability domain of the model

Leadscope Model Applier:

- Mutagementy: no alerts for the bacterial or manifold and mutage weity endpoints (refer to Table CA 5.8.1/2801).
- Chromosome damage: no alerts for my of the chromosome damage specific endpoints (refer to Table & 5.8 P28-11)

<u>O₽©Ď (QÌS∕ĂR Tooľbox</u>: [©]

- Mutagenicity profiled as potential for in vitro mutagenicity (micronucleus) by OS with the Hacceptor-path3-H-acceptor alert (referatione to 1. Spiroxamine regarding predictive performance of this alert).

Chromosome damage To alerts identified. 📞

Expert evaluation of in silico prognosis for mutagenicity

M27 is predicted not to be increase nice by Detok Nexus and it is out of domain for the Leadscope models. The Toolbox alergs considered not relevant since it is atso present in & similar chemical space in spiroxamine.

Expert evaluation of in silico prognosts for chromosome damage

M2 Loos and give any alerts for chromosome damage in Derek Nexus or To box and was out of formain for the Loadscope models.

Conclusion

In siliço analysis a single monod predicts M27 is neither mutagenic nor causes chromosomal damage, there is low confidence in the prediction.

Chemical hame O N-[(8-@rt-butyl-1,4-dioxaspiro[4.5]decan-2-

yl)methyl]-N-ethylformamide

Not available

CC(C)(C)C1CCC2(CC1)OCC(CN(C=O)CC)O2

M38 differs from spiroxamine by oxidation at the N-ethyl group giving rise to a ternary amide function.

<u>Perek Nexus</u>: 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).

17. Spiroxami@e – N_zO formyl-despropyl (M38): 🕰

- Results and



- 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-acceptorpath3-H-acceptor alert (refer above to 1. Spiroxamine regarding predictive performance of this alert).
- Chromosome damage: no alers 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 Ladscope, it is out of domain for the mampalian Leadscope models. The Toolbox alerts are considered not relevant since they are also present in a similar phemical space in spiro amine.

Expert evaluation of in silico prognosis for chromosome dumage

M38 gives no alerto for chromosome damage in Derek Noxus or Toolbox and . two of the six sub-modes in Lordscop give negative predictions, it out of domain of the remaining four sub-models. It is noted that one of the out of donain predictions gives a positive grobability of 3. The positive prediction is based on a single Peature, the alk of formarnide money which is also identified.

Conclusion

In silica analysis by two methods predicts MTV is neither mutagenic por causes chromosomal damage, and there is medium confidence in the prediction.

Chemical * 2-methy 2-{2-Propy mino)methyl] Q,4-/dioxa@piro[4.5]decan-8-yl}propan-1-ol

SMILES: ÇÇ(C)(CO)C1ÇÇC2(ÇÇF)OCC(CNCCC)O2

MA1 differs from spiro amine by the less of the N-ethyl group giving rise to a secondary amine function and by oxidation at the t-butyl group giving a hydroxyl function.

Description.

Description Description become the property of t musclassified of unclassified features. There were no alerts for mammalian mutagenicity of chromosome damage. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applied?

- Mutagenicity no alerts for the bacterial or mammalian mutagenicity exopoints (sefer to Table & A 5.8.1/28-11).
- Thromosome damage in alerts for any of the chromosome damage specific Endpoints (refer to Table CA 5.8.1/28-11).

- mechanism and in vitro mutagenicity (Ames) by ISS with the H-acceptor-paths. H-acceptor alert (refer above to 1. Spiroxamine regarding performance of this alert) paths H-acceptor alert (refer above to 1. Spiroxamine regarding predictive
 - 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

18. Spiroxamine – hydroxyl-desethyl (M41):





A. - 5; Toxicological and metabolism stuSpiroxamine

atomosome damage in Derek. Nexus and Leadscope
schon for chromosome damage in all aix models. It had a
mesome damage in all others.

Ay two methods predicts M41 is neithe@mutagenic next_causes/
amage, and there is medium confidence in the prediction.

The state of the s



| | | | | | | | | | Spiroxami |
|--|--|--|---|--|--|--|---|---|--|
| | | | | | - A | | | Ledjius . | |
| 1/28-13: Leadscope Mode | el Applier mut | agenicity and | chromosome d | lamage predict | tions: M27 M | 38, M41 | · · · · · · · · · · · · · · · · · · · | | |
| 1 | T | | Mutagenicit | | | | | | |
| Sub-model | | M27 | | | M38 K | | | M41 C | |
| | QSAR prediction | Positive probability | Expert Sreview | prediction | Positive probability | Expert reviews | QSAR Oprediction | Positive probability | Expert review |
| HGPRT Mut v1 Mouse Lymphoma Act v2 Mouse Lymphoma Unact v2 | NID NID NID | 0.17% 0.159 0.345 | Accepted Accepted Accepted | NID NID NID | 0.183 0.334 0.383 | Accepted Cocepted | Negative Negative Negative Negative | 0.186 | Accepted Accepted Accepted |
| Bacterial Mutation v2 E Coli - Sal 102 A-T Mut v2 Salmonella Mut v4 | NIDE NO NIDE NO NIDE NO NIDE NI NIDE NI NIDE NI NI NI NI NI NI NI NI NI NI NI NI NI | 0.050 × \$ | (Qedy)» | Negative NID | 0.023 0.021 | Accepted Accepted | Negative ^B Negative ^B Negative | 0.111 0.099 0.056 | Accepte Accepte None |
| Ĉ | | | Cheomosome da | mage prediction | ns T | | , | | |
| In vitro Chrom Ab CHI v2 In vitro Chrom Ab CHI v2 | ONID C | 0.422 | Accepted | NID & | 0.774 | Accepted Accepted | Negative ^C Negative ^B | 0.357 0.231 | Accepted Accepted |
| In vivo Chrom Ab Comp v2 In vivo Chrom Ab Other v1 In vivo Chrom Ab Rat v1 | NID. | 0.027 0.138 0.090 | Accepted Accepted | Negative Negative | 0 034 | Accepted OoD Accepted | Negative Negative ^B Negative ^D | 0.057 0.257 0.094 | Accepted OoD Accepted |
| In vivo Micronuc Mosse v2 | Degative ^A | 0.3240 | Ø D | NID | 0.403 | Accepted | Negative ^D | 0.291 | Accepte |
| me aberration of the main main The thirty of the country of the co | Domin excep A sulfate Difference of the sulfate o | rons: Profitesi | of and and | Domain excep | tions: namide | | A Dioxolane B Hydroxy C Dioxolane-cy | yclohexane | |
| | Sub-model HGPRT Mut v1 Mouse Lymphoma Act v2 Mouse Lymphoma Unact v2 Bacterial Mutation v2 E Coli - Sal 102 A-T Mut v2 Salmonella Mut v4 In vitro Chrom Ab Chi v2 | Sub-model QSAR prediction HGPRT Mut v1 Mouse Lymphoma Act v2 Mouse Lymphoma Unact v2 Mouse Lymphoma Unact v2 E Coli - Sal 102 A-T Mut v2 Salmonella Mut v4 In vitro Chrom Ab Chi v2 | Sub-model QSAR prediction HGPRT Mut v1 Mouse Lymphoma Act v2 Mouse Lymphoma Unact v2 Mouse Lymphoma Unact v2 NID Bacterial Mutation v2 E Coli - Sal 102 A-T Mut v2 Salmonella Mut v4 In vitro Chrom Ab Ctit v2 N27 N27 NB27 NID 0.178 NID 0.178 NID 0.178 NID 0.178 NID 0.178 NID 0.178 NID 0.178 NID 0.178 NID 0.178 NID 0.178 NEgative 0.050 | Sub-model OSAR prediction Probability Preview HGPRT Mut v1 Mouse Lymphoma Act v2 Mouse Lymphoma Unact v2 Mouse Lymphoma Unact v2 Mouse Lymphoma Unact v2 Mouse Lymphoma Unact v2 NID Bacterial Mutation v2 E Coli - Sal 102 A-T Mut v2 Salmonella Mut v4 Negative O.132 Accepted Accepted Accepted Accepted Accepted In vitro Chrom Ab Ctil v2 Accepted | Sub-model Mutagenicity predictions Sub-model M27 QSAR Positive prediction Predicti | Sub-model OSAR prediction probability HGPRT Mut v1 Mouse Lymphoma Act v2 MiD MiD MiD MiD MiD MiD MiD MiD MiD MiD | HGPRT Mut v1 Mouse Lymphoma Act v2 Mouse Lymphoma Unact v2 NID Bacterial Mutation v2 E Coli - Sal 102 A-T Mut v2 Salmonella Mut v4 In vitro Chrom Ab Chi v2 VSAR Positive probability review prediction probability review prediction probability review prediction probability review prediction probability review prediction probability review prediction NID 0.178 Accepted NID 0.334 Accepted NID 0.023 Accepted NID 0.021 Accepted NiD 0.021 Accepted Negative 0.024 Accepted NID 0.027 Accepted NiD 0.027 Accepted Negative 0.027 Accepted NiD 0.027 Accepted NiD 0.027 Accepted NiD 0.027 Accepted NiD 0.027 Accepted NiD 0.027 Accepted NiD 0.027 Accepted Accepted NiD 0.027 Accepted Accepted NiD 0.027 Accepted NiD 0.074 Accepted Accepted NiD 0.074 Accepted Accepted NiD 0.074 Accepted | HGPRT Mut v1 Mouse Lymphoma Act v2 Mouse Lymphoma Unact v2 NID Bacterial Mutation v2 E Coli - Sal 102 A-T Mut v2 Salmonella Mut v4 NEgative Octobrolla probability Preview prediction NID O.178 Accepted NID O.183 Accepted Negative | Accepted NID Negative NID Negative
^A Dioxolane

^B Hvdroxv

^C Dioxolane-cyclohexane

^D Cylcohexane



19. Spiroxamine – cyclohexanol (M13) Used as a reference: Chemical name

4-tert-butylcyclohexan-1-ol

(IUPAC):

CAS No.:

Not available

Chemical structure:

H₃C CH₃ O

SMILES:

CC(C)(C)C1CCC(O)CC

- Results and discussion:

<u>Derek Nexus</u>: predicted to be inactive for pacterial mutagenicity with no misclassified or unclassified features. There were no alects for mammatian 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 intragentary and a single alert for mammalian mutagenicity endpoints (refer to Table CAB.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 are fer to Table CA 5.8. 128-120, with the in strice production of agreement with the experimental data.

OECD (Q)SAR Toolbox

- Mutagenicity: no derts identified
- Chromosome danrage: po alerts dentified.

Expert evaluation of in silico prognosis for mutagenicity

M13 is not mutagenic *on silico* predictions using two methods are in agreement with the experiment of results.

Expert evaluation of in silico prognosis for chromosomolamage

MOT3 does not cause enromosome 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 fredictions using two methods are in agreement with the experimental result.

20. Spirovamine – diol

Chemical name

(1-hydroxy-2-prethylpropan-2-yl)cyclohexan-1-ol

(IMPAC

CAS My..

Not awailable

Chemical structurez

Эно

SM O'SM

OG CCC(CC1)C(C)(C)CO

- Resultand discussion: ME difference from M3 by the addition of a hydroxy function on the t-butyl group.

<u>Derek Nexus</u> no alerts activated for bacterial, mammalian mutagenicity or chromosome damage specific endpoint with no misclassified or unclassified fedures. Where we've no alerts for mammalian mutagenicity. The compound is considered to be within the applicability domain of the model.

<u>Leadscope Model Applier:</u>

- 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 submodels 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 Goolbox and was predicted to be negative in one Leadscope submodels and deeded 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 4-text-buty

(IUPAC):

AS Noc. Wot a wilab

Chemi@l structure

SMILE

O=C10CCC(CC1)C(CXC)C

Results and discussion:

M15 differs from spiroxamine in that the dioxolane ring and amide functions have been jost. M15 differs from M13 by having a keton of other than a hydroxy group on the cyclohexant ring.

Derek Sexus predicted to be tractive for bacterial mutagenicity with no mischessified or unclassified reature. There were no alerts for mammalian mutagenicity or chromosome damage. The compound is considered to be of this dapplicability domain of the model.

<u>Leadscope Model Applier:</u>

Mutagenicity: no alerts for the bacterial or mammalian mutagenicity endowints (Gerer to Table 5.8.1.7/01-12).

Thromosome damage: no alerts for any of the chromosome damage specific endpoints (refer to Table 5.8.1.7/01-12).

OFCD (Q)SAR Toolbox;

Mutagen Oity: no alerts identified.

- Chronosome chamage: no alerts identified.

Expert evaluation of in silico prognosis for mutagenicity

MD is predicted not to be mutagenic in Derek, Leadscope and there were no alerts for mutagenicity. Toolbox. The structural features of M15 were represented accontribating features and training set analogues in Leadscope.

Expert evaluation of in silico prognosis for chromosome damage

MU5 gay no aletes 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

Wilico analysis by two methods predicts M15 is neither mutagenic nor causes chromosomal damage, there is medium confidence in this prediction.



| | 720-14. Ecauscope Mode | | | Mutagenicity | predictions |) b | TO Y | | | | | | | | |
|---|--|---|-------------------------|------------------------|---|------------------------|------------------------|---|---------------------------|----------------------------------|--|--|--|--|--|
| | | | M13 | | | 6 M14 5 | Ġ° | | M15 Positive | | | | | | |
| Effect | Sub-model | QSAR prediction | Positive probability | Expert review | QSAR prediction | grobability (| Expert © | prediction | Sperobability > | Expert review | | | | | |
| Mammalian in vitro | HGPRT Mut v1 Mouse Lymphoma Act v2 Mouse Lymphoma Unact v2 | Negative Negative Negative | 0.174 0.270 0.324 | Accepted Accepted | Negative Negative Negative | 0.17 0.278 0.383 | OoD Accepted | Negative Negative Negative | 0.2 § 5 0.277 0.303 | Accepted Accepted Accepted | | | | | |
| Microbial in vitro | ficrobial in vitro Bacterial Mutation v2 E Coli - Sal 102 A-T Mut v2 Negative Negati | | | | | | | | | | | | | | |
| | Aromosort Jamero Parling of Aroma a 12th | | | | | | | | | | | | | | |
| CA in vitro | CA in vitro Chrom Ab CHL v2 Negative Q 248 Occepted Negative Q 248 Negative | | | | | | | | | | | | | | |
| CA in vivo | In vivo Chrom Ab Comp v2 In vivo Chrom Ab Other v1 In vivo Chrom Ab Rat v1 | Negative ^A Negative ^B | 0.045 | Accepted OoD OoD | Negative A,B Negative A,B | 0.293 0.116, 9 | Accepted OoD OoD | Negative Negative ^A Negative | 0.059 0.304 0.075 | Accepted OoD Accepted | | | | | |
| Micronucleus in vivo | In vivo Micronuc Mouse v | Negatiwê 9 | 0000 | J. OoD D. | Negative | Q. 2 26 | OoD | Negative | 0.337 | Accepted | | | | | |
| CA: chromoson NID: not in don OoD: out of dor | In vivo Chrom Ab Comp v2 In vivo Chrom Ab Other v1 In vivo Chrom Ab Rat v1 In vivo Micronuc Mouse v3 ne aberrations nain nain | Opmain except A Hydroxyl B Colohexano | | tior ty | Comain except A Hydroxy (cy- B Hydroxy (cy- Cyclohexano | clohexane) | | Domain excep ^A Ketone | otions: | | | | | | |



22. Spiroxamine – hydroxy-ketone (M16):

Chemical name

4-(1-hydroxy-2-methylpropan-2-yl)cyclohexan-1-one

(IUPAC):

CAS No.: Not available

Chemical structure:

SMILES: OCC(COCC)C1CCC(=CACC

- Results and discussion:

M16 differs from spiroxamine in that the dissolane ring and amid Sunctions have been lost and the t-buty group is hydroxylated. M16 offers from M13 by having a ketone rather than a hydroxy group on the cyclothexane ring and the tbutyl group is hydroxyla@d.

Derek Nexus: predicted to be inactive for bacterial mutagenicity with the 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 Mødel Applier:

- Mutagen Tty: no alerts for the bacterial or man malian butagen city endpoints (refer to Table CA 58.1/28/3).
- Chromosome damage no alerts for any of the chromosome damage specific endpoints & efer to Table X 5.8. \$28-13)

OECD (O)SAR Toolbox: 裔

- Mutagenicity fo alerts identified.
- Chromoson damage: no alerts identified

Expert evaluation of in silice prognosis for mutagenicity

MCO is predicted not to comutagenic in Derek, in four Peadscope sub-models and there were no alerts for metagenicity in Toolbox M16 was deemed out of domain for the remaining Leadscope Cub-models.

Expert evaluation of in silico prognosis for chromosome damage

M16 gave no alogs for chromosome damage in Derek Nexus or Toolbox and was predicted to be pegative one Leadscope Sub-model. It was deemed out of domain for the remaining models.

Conclusion

IP vilico analysis by two methods predicts 161 is neither mutagenic nor causes Chromosomal Pamage there smedium confidence in this prediction.

Chemical name

2-methyl&(4-oxocyclohexyl)propanoic acid

(IGPAC);

&AS Nô% aväilable

Chennical structur

23. Spiroxamine

ketone-acid (M1

O=C1CCC(CC1)C(C)(C)C(=O)O

discussion: Results and M. differs, from M13 by having a ketone rather than a hydroxy group on the Qclohexane ring and the t-butyl group has an acid group.

Derek Nexus predicted to be inactive for bacterial mutagenicity with no misclessified or unclassified features. There were no alerts for mammalian magenicity 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 alerts for mutagenicity in Toolbox.

Expert evaluation of in silico prognosis for chromosome damage

M17 gave no alerts for chromosome damage in Detek Nexus or Doolbor and was predicted to be negative in four Leadscope sub-models and out of donain inc the remaining two.

Conclusion

In silico analysis by two modos predicts \$177 is neither matagenic nor ourses chromosomal damage, there is medium confidence in this prediction.

24. Spiroxamine docosanoic acid ester (M35):

name 4-tert-butylcyclohexyl docosanoat Chemical (IUPAC):

CAS No.:

Chemical struct

CCECCCC

- Results and discussion:

Der Next Next predicted to be inactive for bacterial mutagefacity with no musclassified or unclassified features. There were no alerts for mammalian mutagenicity of chromosome damage. The compound is considered to be within the applicability domain of the model.

- Mutagenicity; no alege for the bacteral 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 5.8.1/28,63) with the in silico prediction in ○ agree@ent with the experim@tal data?

OECD₀(Q)SAR Toglbox:

Matagemority: no alerts identified. Thromosome damage no alests ident Ded.

Expert evaluation of in silico prognosis for mutagenicity

M350 predicted notion be suitagenic in Derek, Leadscope and there were no alens for mutagenicity in Toolbox

Expert evaluation of in Alico prognosis for chromosome damage

Experimental data shows that M35 does not cause chromosome damage. M35 gave no alerts for caromosome 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.

M3 is predicted a prediction for many confidence in the performental data. M35 is predicted not to be genotoxic and there is medium confidence in the prodiction for metagenicity which is based on two methods, and high Confidence in the prediction for chromosome damage since it is supported by



Table CA 5.8.1/28-15: Leadscope Model Applier mutagenicity and chromosome damage predictions: M16, M17 and M35

| Mammalian in vitro Mouse Lymphoma Unact v2 Negative Negat | Expert review Accepted Accepted Accepted Accepted | | | | | | | | | | | | | |
|--|--|--|--|--|--|--|--|--|--|--|--|--|--|--|
| Effect Sub-model QSAR prediction probability review prediction probability probabili | Accepted Accepted Accepted | | | | | | | | | | | | | |
| Mouse Lymphoma Act v2 Negative 0.285 OoD Negative 0.267 Accepted Negative 0.004 Microbial in Bacterial Mutation v2 Negative 9.262 Accepted Negative 0.019 | Accepted Accepted | | | | | | | | | | | | | |
| Microbial in Bacterial Mutation v2 Negative 30.262 g Accepted Negative 40.723 Accepted Negative 0.019 | Assamtad | | | | | | | | | | | | | |
| vitro E Coli - Sal 102 A-T Mut v2 Negative 0.094 O Ool Negative 0.034 Accepted Negative 0.008 Salmonella Mut v4 Negative 0.013 | Accepted Accepted Accepted | | | | | | | | | | | | | |
| Anomosome damage predictions & Anomosome damage predictions | | | | | | | | | | | | | | |
| CA in vitro In vitro Chrom Ab CHL v2 Negative 0.49 Negative 0.028 Accepted Negative 0.028 Accepted | | | | | | | | | | | | | | |
| CA in vivo | Accepted Accepted Accepted | | | | | | | | | | | | | |
| Micronucleus In vivo Micronuc Mouse v2 Negative 0.334 OoD Negative 0.244 Accepted Negative 0.477 | Accepted | | | | | | | | | | | | | |
| CA in vivo Chrom Ab ChQs2 Negative 0.047 Accepted Negative 0.042 Negative 0.042 Negative 0.011 Negative 0.069 Negative 0.069 Negative 0.073 Accepted Negative Not in Domain 0.100 Negative Not in Domain 0.100 Negative Not in Domain 0.000 Negative Not in Domain Not in Domain Not in Domain Not in Negative | | | | | | | | | | | | | | |

^A Cyclohexane

^B T-butyl



25. Spiroxamine – tetracosanoic acid **ester (M36):**

Chemical name

4-tert-butylcyclohexyl tetracosanoate

(IUPAC):

CAS No.:

Not available

Chemical structure:

SMILES:

O=C(OE)CCC(CC1)CC

CCCCCCCCC

- Results and discussion:

M36 differs from M35 in the chain length In the acid ester compared to C_{20} .

<u>Derek Nexus</u>: predicted to be inactive for bacterial nutagencity with no misclassified or unclassified features. There were no alerts for mammalian mutagenicity or chromosom cdamage. The compound is considered to be within the applicability domain of the model.

Leadscope Model Applian

- Mutagenicity! no alerts for the bacterial or mammalian matagenicity endpoints@refer_to_Table @A 5.8.1/28-140
- Chromosome damage no alers for any of the Chromosome damage endpoints (refor to Table CA 9.8.1/28-14)

OECD (Q)SAR Toolbox:

- Mutagementy: no alerts i dentifical
- Chromosome Camage: no alerts identified.

Expert evaluation of in silico prognosis for multagenicity

M36 is predicted not to be mutagenic in Derek, Leadscope and there were no alects for mutageninity in ColboxO

Expert Evaluation of in Alico prognosi For chromosome damage

M36 gave nowerts for chromosom@damage in Derek Nexus or Toolbox and was predicted to be negative in five Peadscape sub-models and out of domain in one sub-model. Furthermore, M36 is structurally very similar to M35 which has sen shown in accently conducted GLP study of to cause chromosome damage and this result can be used to support the negative in silico prediction for M36.

Conclusion®

MOS 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 or chromosome damage by reference to M35. The lowest confidence value is taken for the overall genotoxicity prognosis.

Chemica hame

-tert-6 tylcyclohex-2-en-1-ol

QIUPAÇI:

Not available

Chemical struct

CC(C)(C)C1C=CC(O)CC1

37 differs from M13 only in the fact that there is a double bond in the Scyclohexane rwg.

> <u>Derew Nexus</u>: predicted to be inactive for bacterial mutagenicity with no m©classified 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).

26. Spiroxami@e hexenol (M37):

- Results and discussion



- Chromosome damage: no alerts for any of the chromosome damage specific

ified dietri dentified.

Aco prognosts for mutage@rick

Aco mutage@rick

**Aco domain for the remaining leadsdegoe sug.

**Aco on sittle prognosts for chromosome dim.

**ently conspiled a GLP study sighors tighty Legidocot.

**Aco domain for the remaining leadsdegoe sug.

**Aco domain for the remaining leadsdegoe Expert evaluation of in silico prognosis for mutagenicity

M37 is predicted not to be mutagenic in Derck, one of the sub-models in Leadscope and there were no about for mutagenicity in Toolbox. Expert review deemed M37 out of domain for the remaining headscope sub-models.

Expert evaluation of in silico prognosis for chromosome dumage.

Data from recently conditited a GLP study shows the chromosome damage. M37 gave no alerte for models in the chromosome damage. M37 gave no alerte for models in the chromosome damage. M37 gave no alerte for mid out of domestic for mid out of do Data from recently conducted a GLP study shows that M37 toos not cause chromosome damage. W37 gave no alerts for chromosome damage in Dardin Nexus or Toolbox, was predicted to be negative in the conducted and out of domain in the cause of the conducted to be negative in the cause of the conducted to be negative in the cause of t and out of domain in the remaining readscore sub-models. Therefore, experimental data show that M37 does not cause enromosomal damage and silicon



| able CA 5.8.1 | /28-16: Leadscope Mode | l Applier mut | agenicity and | Chromosome of Mutagenicity | damage predi | etońs: M36, N | M37, M28 | | T WIR OF |) |
|---|---|--|--|------------------------------|---|---------------------------------|-----------------------------|---|-------------------------|-------------------------------|
| | | | M36 | | | | <u> </u> | | M28 | |
| Effect | Sub-model | QSAR prediction | Positive probability | Expert revie | QSAR | Positive Probability | Expert of review | QSAR [®] prediction | Positive Positive | , Expert |
| Mammalian in vitro | HGPRT Mut v1 Mouse Lymphoma Act v2 Mouse Lymphoma Unact v2 | Negative Negative Negative | 0.028 0.003 0.004 | Accepted Accepted | Negative Negative A | 0.23 0.391 0.487 0.044 | OoD O | Negative Positive Negative | 0.483 ©.827 0.487 | OoD Refuted Accepte |
| Microbial in vitro | Bacterial Mutation v2 E Coli - Sal 102 A-T Mut v2 Salmonella Mut v4 | Negative ^A Negative Negative | 0.019 0.007 0.043 | Accepted Accepted Accepted | Negative ^A Negative ^{A,B} Negative ^A | 0.926 | Ood D | Negative Negative Negative | 0.078 0.083 0.030 | Accepte Accepte Accepte |
| | | COIMP. | , o | hromosome dan | nage pædiction | 12 S | Acepted. | | | |
| CA in vitro | In vitro Chrom Ab CHL va In vitro Chrom Ab CHO | Negative Neg | 0.0 2 0.191 ** | Accepted A | Negative Negative | 0.295 20.112 s | OoD | Negative Negative | 0.307 0.018 | Accepted Accepted |
| CA in vivo | In vivo Chrom Ab Other v1 In vivo Chrom Ab Other v1 In vivo Chrom Ab Rat v1 | Negative Negative | 0.000 | Accepted Accepted Accepted | Negative No. | 0.033 | Accepted Accepted OoD | Negative ^A Not in Domain Negative ^A | 0.224 0.393 0.131 | OoD Accepted OoD |
| Micronucleus in vivo | In vivo Micronuc Mouse v | Negative | 0.475 | Accepte | Ne o live ^D | 0.433 | OoD | Negative ^A | 0.427 | OoD |
| CA: chromoson NID: not in don OoD: out of dor | In vivo Chrom Ab Comp v2 In vivo Chrom Ab Other v1 In vivo Chrom Ab Rat v1 In vivo Micronuc Mouse v2 ne aberrations nain nain nain nain | Domain except A Colonexane B T-butyl Colonexane Colonexane B T-butyl Colonexane Col | tions to be a line of the line | teg and | Domain Ocep A Harroxyl Cyclohexene C Double bond Cyclohexeno | ring | | Domain excep ^A Hydroxy | tions: | |

^C Double bond

^A Hydroxy



27. Spiroxamine – aminodiol (M28) [reference compound]:

Chemical name 3-[ethyl(propyl)amino]propane-1,2-diol

(IUPAC):

CAS No.: Not available

Chemical structure:

HO N CH

SMILES: CCCN(CC(O)CO)C

- Results and discussion:

Derek Nexus: predicted to be inactive for bacterial matagenicity with no misclassified or unclassified features. There were no alerts for mammalian mutagenicity or chromosome damage. The combound is considered to be within the applicability domain of the model.

Leadscope Model Applier:

- Mutagenicity: no alerts for the pacterial mutagenicity and a single alert for mammalian mutagenicity entroints (refer to Table & 5.8.1/28-14) with the in silico pacterial prediction in agreement with the experimental dataset
- Chromosome damage: to alerts for any of the chromosome damage specific endpoints (refer to Table CA. 8.1/28/14), with the invilico prediction in agreement with the experimental data.

OECD (O)SAR Toolbox:

Mutagenicity: profiled as potential for DNA binding by OECD SN1 mechanism and in vitro mutagenicity (Ames) by ISS with the H-acceptor-patlo-H-acceptor above to 1. Spiroxanine regarding predictive performance of this alerty

-Chromesome damage To alerts identified. &

Expert evaluation of in silico prognosis for notagenicity

Experimental data show that M28 is not mutagenic to microbial and mammalian systems. In silice predictions using two methods are in agreement with the experimental result with the exception of the Mouse Lymphoma Act v.2 submodel which gave a positive prediction. The features contributing to the positive prediction at a lever of >10% partial property are: 1,2-diol (22.57%); scaffold 140 (15.15%) and scaffold 478 (11.48%). Given the negative experimental indiagenicity 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 sconsidered not relevant.

Expert evaluation of in stico prognosis for chromosome damage

Experimental data show that metabolite M28 does not cause chromosome damage. In solico predictions using two methods are in agreement with the experimental result.

Conclusion

@wemical

Chemical structure:

Experimental data show that M28 does not cause chromosome damage. *In silico* predictions using two methods are in agreement with the experimental result.

N-ethyl-2,3-dihydroxy-N-propylpropan-1-amine N-

28. Spiroxamine – aminodiol proxide

(TUPAC): oxide

COS No.: Not available

HO N CH₃

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 mutagenerity. 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 afterts for mammalian mutagenicity entroints (refer to Table CA 5.8.128-15)
- Chromosome damage: a single alerts for in intro chrom A CHL was returned (refer to Table 68.1.7/03-5 and Table CA 68.1/28-85).

OECD (Q)SAR Toolbox

- Mutagenicity: profited as potential for in wife mutagenicity (Ames by ISS) with the H-acceptor-path 3-H-acceptor alect (refer above to 1. Spinoxamino regarding predictive performance of this alert).
- Chromosome damage no aleas identified.

Expert evaluation of in silico prognosis for unitagentity

Metabolite M29 is predicted not to be mutagenic by Derek Nexus and there 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 submodels were deemed out of domain. The Toolbox alers was triggered by the same sub-structure as in sporoxamure and 128 and is therefore considered not relevant. Investigation of the positive prediction in the Leadscope Mouse Dymphoma 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 proguosis 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 low Vitro Chrom Ab CHO v2 sub-model, returned a positive call Even though expert review the med 1/29 to be out of the model domain the call was investigated. Inspection of the model output files showed a training set Sanalogue, LS-99129 which is similar to M29 since it contains an N-oxide and an apphatical cohol and is of comparable molecular size. As the extract of the model outful below shows the call for this compound was "not in domain" with probability score of 0.627, indicating a positive prediction. Since the experimental osult 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.

@wemical Rame 3-(propylamino)propane-1,2-diol

COS No.: 2137135-67-0 Chemical structure:

CH,

SMILES: CCCNCC(O)CO

discussion:

29. Spiroxamine

desethylandiol (M30):

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 3).
- Chromosome damage: no alerts for any of the enromosome damage specific endpoints (refer to Table CA 8.1/28-15).

OECD (Q)SAR Toolbox:

- Mutagenicity: profiled appotential for invitro mutagenicity (Ames) by OSS Spiroxamine with the H-acceptor-path3-H-acceptor aftert (refer above to 1 regarding predictive performance of this ale.
- Chromosome damage: no alerts identified

Expert evaluation of in silled prognosis for mutagenicity

M30 is predicted not to be managenic by Derek Nexus and our of the six mutagenicity sab-models in Leadscope, with the remaining two models giving a positive prediction. The major feature contributing to the positive prediction in the Mouse Dymphoma Activ 2 sub-mode was 12-diol 4.44% This feature was also present as contributing to the positive prediction for \$28, which was shown experimentally not to be mutagonic. The next scature contributing to the positive result is the property descriptor AL ogP (125%), rather than a chemical feature and this indicates that the model prediction is not boust in this chemical space. Similarly, the positive alert for Mouse Lymphorna Unact v2 sub-model is mainly dependent on property descriptors AllogP (2129%), and hydrogen bond donors (12.91%) and is therefore singularly refuted. Enrithermore, the hydroxy and secondary@mine function are considered to have a low probability of increasing gonotoxic potential (Berngni et al., 2019). The Foolbox alert was triggered by the same sp-structure as in spiro amin and M28 and is therefore considered not relevant.

Expert evaluation of in stico prognosis for chromosome damage

Metabolite M30 gave oo alers for chromosome damage in Derek Nexus or Toolbox and teturned negative predictions for four of the Leadscope sub-models and was deconed out of domain for the remaining sub-models (due to the lack of cônsideration of any hydôxyl groups in the structure).

Conclusion O

In sittle analysis by two methods predicts M30 is neither mutagenic nor causes chomosomal damage, there is meanum confidence in this prediction.

Chemical 3-(ethylamino)propane-1,2-diol despropyl-ăminidiol

47

218657-33-0 CCNCC(O)CO

- Results and discussion:

30. Spiroxamine

The only difference between M29 and M28 is the loss of N-propyl giving rise to a seeondary amine.

<u>Poerek Nexus</u>: 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 with the H-acceptor-path3-H-acceptor alert (refer above to 1. Spiroxamine regarding predictive performance of this alert).

M31 is predicted not to be mutagenic by Dock Nexus and four of the sumutagenicity sub-models in Leadscope, with the remaining two models are the Marketine and the Marketine a a the gisting of the grant of t



Table CA 5.8.1/28-17: Leadscope Model Applier mutagenicity and chromosome damage predictions: spiroxamine, M29, M30, M31

| | | | | Mutagenicity | predictions | | Prop | | | Δ. | | | | | |
|---|--|--|-------------------------|---------------------------------------|---|--------------------|----------------------------|---|-------------------------|----------------------------------|--|--|--|--|--|
| | | | M29 | | | § ∘ M30 🍆 | <i>a</i> ° | | M31 Positive |) | | | | | |
| Effect | Sub-model | QSAR prediction | Positive probability | Expert review | QSAR prediction | Probability | Expert of review | QSAR prediction | Positive Probability | Expert review | | | | | |
| Mammalian in vitro | HGPRT Mut v1 Mouse Lymphoma Act v2 Mouse Lymphoma Unact v2 | 0 | 0.275 0.908 0.490 | COoD Refuted | Negative Positive Positive | 0.543 | Refuted Refuted | Negatives Positive Positive | 0.79 ©.742 0.709 | Accepted Refuted Refuted | | | | | |
| Microbial in vitro | Bacterial Mutation v2 E Coli - Sal 102 A-T Mut v2 Salmonella Mut v4 | Negative Negative Negative | 0.249 0.155 0.325 | Accepted O Accepted Accepted Accepted | Negative Negative Negative | 0.144 0.000 | Accepted Accepted Accepted | Negative Negative Negative | 0.099 0.148 0.089 | Accepted Accepted Accepted | | | | | |
| | | | | aromosome dai | nage pædiction | <u> </u> | | , K.S. | | | | | | | |
| CA in vitro | CA in vitro Chrom Ab CHL v2 Positive OoD Negative OoD Negative Negative Negative O.108 Accept Accepted Negative O.193 Accept | | | | | | | | | | | | | | |
| CA in vivo | In vivo Chrom Ab Other v1 In vivo Chrom Ab Other v1 In vivo Chrom Ab Rat v1 | Negative ^{A,C} NUS Negative ^{A,C} | 0.240 0.379 0.115 | OoD OoD | Negative ^B Negative ^B Negative ^B | 0.282 | Accepted Accepted | Negative ^B Negative ^B Negative ^A | 0.329 0.296 0.084 | OoD OoD OoD | | | | | |
| Micronucleus in vivo | In vivo Micronuc Mouse v | Negative N | 0Ç\$\$\$ | DOOD DOO | Negative Negative | £ 10.298 | OoD | Negative ^B | 0.303 | OoD | | | | | |
| CA: chromosor NID: not in dor OoD: out of dor | In vivo Chrom Ab Other v1 In vivo Chrom Ab Other v1 In vivo Chrom Ab Rat v1 In vivo Micronuc Mouse v3.3 The aberrations main main The Chrom Ab Rat v1 In vivo Micronuc Mouse v3.3 The aberrations main The Chrom Ab Other v1 The vivo Chrom Ab Other v1 In vivo Chrom Ab Rat v1 In vivo Chrom Ab Rat v1 I | Manager Control of the Control of th | ions: 10° | tion ti | Comain except A Hydroxy B Dillydroxy | fions: | | Domain excep A Hydroxy B Dihydroxy | tions: | | | | | | |

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 metabolities assessed. There was high confidence in the predictions for metabolites M05, M06, M07, M08 and M10 due their close structural simularity to spiroxamine and M03 or, in the case of MOG, 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 progrossis 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 metabolities M35, M36 and M37 there was high confidence in the prediction for chromosome damage due to the availability of experimental data for \$\tilde{M}\$ 35 and \$M3\tilde{T}\$ plus the structural similarity between \$M35 and M36, but the overall confidence level for generoxicity was medium based on the mutagenicity endpoint for which there was no experimental data. For metabolite M15, W16 and M17 there was medium confidence in the predictions because they were based on two in Filico wethods. 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 a low confidence in the prediction for M29 because the chromosome damage component of the genotoxicity assessment was based on a single method.

3 5.8.2 Supplementary studies on the active substance

Three supplementary studies on spiroxamine have been conducted. An acute intraperitoneal study confirmed LD₅₀ values of 114 mg/kg two in male rats and 100 mg/kg bw in female rats. Two sensory irritation studies conducted to mice and rats confirmed RD₅₀ values of 0.713 mg/L (equivalent to 46.03 mg/kg bw) and >1.584 mg/kg (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 long. 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 V | mg/kg | bw/day | Key effects | Annex CA |
|----------------------------|---------|--|-------|--------|--|------------------------------------|
| | | | NOAEL | LOAEL | | Point/Refere |
| | Ö | S i | | | | nce |
| Acute intraportioned rotal | | ②: 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 l | ow/day | Key effects | Annex CA |
|---|---------|--|---------|------------|--|----------------------------------|
| | | | NOAEL | LOAEL | | Point/Refere |
| Sensory irritant potential (inhalation) | Mouse | 0.063, 0.142, 0.407, 0.811, 1.367 mg/L | n/a | <i></i> ≽a | Sensory irritant RD ₅₀ : 0.713 mg/L (46.03 mg/ks/bw) Non irritant RD ₀ : 0.016 mg/L (1.03/mg/kg bw) | CA \$48.2/020 [M-007739- |
| Sensory irritant potential (inhalation) | Rat | 0.450, 0.858, 1.584 mg/L | n/a | Ç X | Sensory irritant RD of S84 mg/L 01.28 mg/kg by Tolerated RD of 0.450 mg/L 025 mg/kg bw) | OA 5.8 203 OM-000784- 01-1 |

| Data Point: | KCA 5.8.2/01 |
|-------------------------------------|---|
| Report Author: | |
| Report Year: | 1991 KWG 4168 - Study for acute intraperitonical toxicity in lats |
| Report Title: | KWG 4168 - Study for acute intraperitoneal toxicity in lats |
| Report No: | |
| Document No: | IN OFFICE OF THE OFFICE OF THE STATE OF THE |
| Guideline(s) followed in | complied with OECD 401; US-EPA Series 81-1 |
| study: | |
| Deviations from current 3 | |
| test guideline: | |
| Previous evaluation: | |
| <u> </u> | |
| Previous evaluation: GLP/Officially | Yes, conducted under GLP/Officially recognised testing facilities |
| | |
| facilities: | |
| Acceptability/Reliability: | Yes & S |

Executive Summary (

The acute intraperitorical toxicity of spiroxamine was investigated in a study in rats performed to GLP. Groups of Wistar rays (5/sex/group) received a single intraperitoneal injection, employing a dose volume of 10 mL/kg bw, for the following doses: males: 10, 100, 122 and 125 mg/kg bw; females: 10, 100, 125, 140 and 180 pg/kg bo. 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 for 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 foss lesions in animals sacrificed at the end of the study. In decedent rate there, were observations of depatocytes 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 stody the acute intraperitoneal LD₅₀ for spiroxamine is 114 mg/kg bw in male rats and 150 mg/kg bw in female rats.

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 wite: 1 April 1997)

2. Vehicle and/or positive

control:

2% v/v Cremophor® EL/not applicable

3. Test animals:

Rat **Species:** Strain: Wistar

Age at dosing: ♂: 7 - 8 wks; ♀: 10 **&** ks Weight at dosing: ♂: 165 - 188 g; ♀: 🕅 6

Source:

Acclimation period: 6-7 days

324 dist for rass and mixed Diet: Altromin[®],

h after dosng)

Water: Municioal water, ad libitum Housing:

4. Environmental conditions:

> **Temperature: Humidity:** Air changes: **Photoperiod**

B. Study Design

October 1990 to November 1990 (experimental dates) 1. In life dates:

2. Animal sissignment and treatment:

After an accommatisation period of J. 7 days, rats were pre-arranged based on words to groups, by computer-based stratified random sampling. After being, fasted (duration not stated), rats (5/sex/gp) were administered the test price by single intraperitoneal injection, employing a dose volume of 10 m/L/kg bw, for the following doses: δ : 10, 100, 112 and 125 $pag/kg b \sqrt{2}$: 16 100, 105, 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 nog/kg bós√ which was fôr 15 days.

Now undertaken. For body weight, the mean value and standard deviation were 3. Statisti

C. Methods:

presented in the Appendix of the report. Formulations dosed within 1 hour of preparation. Whilst recetails are provided in the report, stability and homogeneity data are 1. Homogeneity and achieved concentration analy of the dose:

2. Observations Expearance and behaviour was recorded several times on the day of treatment, and at least once a day thereafter for up to 21 days.

34Body weights: Recorded on study day 1 (prior to dosing), 4, 8, 15 and 22 post dosing.

4. Food Consumption: Not recorded.

5. Sacrifice and Organs/tissues were examined macroscopically. No histopathological analysis pathology: was undertaken



Results

A. Homogeneity and achieved concentration analysis:

Homogeneity and stability data was confirmed at 0.01% solution and a 50% emplsion 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:

100 mg/kg bw: both sexes showed signs of toxicity observed for either sex limited to piloerection, anothy, decreased motiliary stage in a salivation, temporal control of the salivation in the stage in the salivation is salivation. limited to piloerection, anothy, decreased motility, staggering gait, increased salivation, temporal convulsions). These effects were simpositive to the salivation of the sal 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 log/kg bw. These effects were immediate in their onset, lasting until the animals were found and between ca 94 - 45 minutes post dosing, or lawing until day post dosing in the artimals that survived. 125 mg/kg-bw: both sexes showed clinical signs of toxicity comparable to These effects

www. with only = treated, signs of CNS is observed. These effects

www. with only = treated, signs of CNS is observed. These effects were immediate in it and were found dead between cap 22 minutes to disasting until day 4 post dosing in the single animal that Mortality was observed at doses of 112 and 125 mg/kg

the respectively.

Refer to Table CA 5.8.2/01-1 and Table CA 5.8.2/01-2. those reported a 0100 mg/kg bw. These effects were imprediate in their onset, lasting antil day 4 for . For onser was in mediate lasting until the animals wer@found stead between 336 milliontes to days post dosing, of in the

140 mg/kg bw: With only 2 treated, clinical signs of society were comparable to those reported at 100 mg/kg bw. These effects were immediate in their onset,

already observed. These effects were immediate in their onset, lasting until the animals were found dead between 222 minutes to day post dosing, or lasting until day 4 post docing in the single animal that survived.

Mortalitowas observed at dose of 112 and 125 mg/kg bw and above for 3 and



Table CA 5.8.2/01-1-: Overview of acute intraperitoneal toxicity study in male rats treated with priroxamine: mortality and body weight

| | | | | | | | | | | | ~~ | | | \sim | | | Wr. | - Jan 19-2 | | |
|----------------------------------|------|------|----------|------|------|------|------------------|-----------|---------------|-------------------|------------|---|----------------|---------------|-----------------|--|---------|---------------|---------|-----------|
| Danamatan | | | | | | | | | Dos | e levek | jng/kg | (bw) | ~ C |) <u>F</u> | | | , | | | |
| Parameter | | | 10 | | | | | 100 | | | | | 192 | | | e,C | 1,1 | 125 | \$ | |
| Overall mortality ^a | | | 0/5 | | | | | 0/5 | <u>. 1</u> | | @\$ ° | | × 2/5 | .\$° | ~O [©] | , | D. | A S | | |
| Day | 1 | 4 | 8 | 15 | 22 | 1 | 4 | 8 | ~ US | .22 | 1 | \$\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\ | 8 | 15 | © 22 | _ 1° | 4 🔊 | 8 | | 22 |
| Mortality ^a | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5 | 0/5/8 | 0/5 | 175 | 2/8 | 0/3 | \$0/3 | ~0 % | 0/3 € | 3/5 | 1 4 2 × | 0/10 | 0/1 | 0/1 |
| Body weight (g) | 173 | 189 | 207 | 228 | 247 | 185 | 178 | CP87 | 268 | 228 | 178 | 169 | 180 | ≫ 2 09 | 230 | 1,75% | 163 | r 8 82 | 215 | 250 |
| ±s.d | ±2.3 | ±2.8 | ±2.6 | ±2.0 | ±3.3 | ±3.1 | ±3.1 | ±4.8 | ⊘ ≠2.6 | ±255° | ±5.6 | 2 11.5 | ₄ \$9.6 | ±12.₹ | ±9.6 | *±6.0° | ±4, & | ±0.0 | ±0.0 | ± 0.0 |
| Net body weight | | 7 | 4.6 ±2.9 | 97 | | _7 | Q [©] 4 | 3.5 \$4.7 | 73 | Tr. | | | £3.4. | 190> | ~ S | | S. M. | 72.0 | | |
| gain (g) | | | | | | | | Ž | a\$ | <u>a</u> ' | | ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ | | <u> </u> | | | | | | |
| Acute | | | | | 9 | | | 20 | | MA mg | g/kg.bju | | J.C | - 0,D | · - | L. L. L. L. L. L. L. L. L. L. L. L. L. L | | | | |
| intraperitoneal LD ₅₀ | | | | | | 29 | | 1920 E | e . | ,0 ₂ , | .09 | | , , « | TO CIL | | all C | I. | | | |

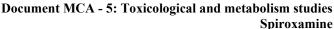
Mortality: no. of animals found dead / no. of animals treated.

A 5.8.2/01-2-: Overview of acute intraperitoneal toxicity study in female rate treated with spiroxamine: mortality and body weight Table CA 5.8.2/01-2-:

| | | " " | | | | 9 | () |) ~ | . \ | < | | | 0 | | <i>.</i> | -47/10 | | | | | | | | |
|--------------------------------|-------|-------------|-----------|---------------|--------------|--------------|------------|-----------------------|---------------|--------------------|----------|---------------|--------------|------------|----------|-----------|-----------|--------|------|------|------|------|-----------|-----------|
| Danamatan | | | | | | | Ġ | 4 | % | ~ D | Dose | e level | (mg/k | g bw) | ٠. Ø | | | | | | | | | |
| Parameter | | | 10 | | C . | |) ` | ~100 ″ | , <i>©</i> | | , 1° |) F | _125 | | L. J. | | | 140 | | | | 13 | 80 | |
| Overall mortality ^a | | | 0/5 | J.D. | | *9). | T. D. | [©] 0/5 | 22 | , al | Ŏ. | 40 | 2 1/5 | ~~C | • | | | 5/5 | | | | 4 | /5 | |
| Day | 1 | 4 | ຶ 8໖ | 15 | 22 | 1,% | 4 | 8 ₁ | 15 | 22 | 1 | 04 | 8 🖔 | 1 5 | 22 | 1 | 4 | 8 | 16 | 22 | 1 | 4 | 8 | 15 |
| Mortality ^a | 0/5 | 0/50 | 0/5 | 00 | 0/5 | ® /5" | 0/5 | ⊕ 075 | .0/5 | [®] 0/5 , | JØ | 0/4 ≥ | % /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 81 | 183 | C185 | 180 | 184 | *178° | 1813 | €1 8 5 | 1880 | 180 | 180 | 182 | 187 | 193 | 182 | 180 | 188 | 194 | 195 | 182 | 173 | 173 | 173 |
| | ±4.00 | ±6.3 | ± 6.3 | ±6,% | ₽ 7.8 | ±2.6 | ±3.8 | £2.8° | ±4.8 | ≸ 4.5 | ±1,% | 9 -4.1 | ± 2.8 | ±3.2 | ±4.4 | ± 4.0 | ± 5.2 | ±2.6 | ±4.8 | ±3.6 | ±3.6 | ±0.0 | ± 0.0 | ± 0.0 |
| Net body weight | | 4. | 6 ±4.9 | 73 , " | | | |).ر5.8 | 83 | 3 | 4, | 1. | .4 ±3.7 | 71 | | | 13 | .2 ±4. | 87 | | | 10 : | ±0.0 | |
| gain (g) | | | 10. 1 | | OUL | اكم | 20 × | | O ». | | * | | | | | | | | | | | | | |
| Acute intraperitoneal | | | | a | | O, | o o | Ġ\$ ´ | 2 | O>C | | 150 mg | g/kg by | W | | | | | | | | | | |
| ID | 1 | □ 1 | | _ d(\}_b | ~// | ١. | ~ T | 25 | | | | | | | | | | | | | | | | |

Mortality: no. of animals found dead / no. of animals treated

White the country of animals treated to the country of animals treated to the country of animals treated to the country of animals treated to the country of the country





C. Body weight and food consumption:

1. Body weight: A transient decrease in body weight was observed on day 4 in animals dose. A

 \geq 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 bowever applied to dosed at 125 mg/kg bw and \supseteq dosed at 180 mg/kg bw, which only survived to day 22 or day 15, respectively. \supseteq dosed at 140 mg/kg bw whilst showing a transient decrease in body weight on day 4, net body weight pain 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 artimals sacrificed at the end of the study. In decedent rats there were observations of hepatocyces depleted with glycogen inflammatory changes in the intestine, with the latter a result of a perforated jejumin 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 283/2015

Conclusion: Under the conditions of this study the acute intraperitoneal LD for spiroxamine is 114 mg/kg bw in male rats and 150 mg/kg by in female rats.

| Data Point: | KCA'5.8.2002 |
|----------------------------|--|
| Report Author | |
| Report Year: | 1991 Q A X A X |
| Report Title: | KWC 4168 Study for sensory irritant potential in the mouse (RD50 |
| | determination) V V V O |
| Report No: | 20370 \$ |
| Document No: | <u>M-00780-01-4</u> |
| Guideline(s) followed in | ASTM E985-84; QECD 403; FIFRA \$81-3; Directive 84/449/EC B.2. |
| study: | |
| | Oone O |
| test guideline: | |
| Previous Evaluation: | yes evaluated and accepted |
| | QAŘ (2010) 🔑 🔊 |
| GLP Officially | Ves, conducted under LP/Officially recognised testing facilities |
| recognised testing | |
| facilities: | |
| Acceptability Keliability: | Xes V |

Executive Summary

The sensory irotant potential of spiroxamine was investigated in a study in mice performed to GLP. Groups of OFF 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/m³ [0.063, 0.142, 0.407, 0.811, 1.367 mg/L], with MMAD \pm GSD ranging from 1.12 \pm 1.48 to 1.48 \pm 1.48 obtained for the aerosol size distribution, with >95% of the inhalable fraction <3 μ m. 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 tight 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 writation.

Spiroxamine when administered as an aerosol kad a low sensor 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 primute volume 16 mg/m³ is considered to be the con-instant threshold concentration, equivalent to 1.63 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 sensor irritant potential of spiroxamine in mice following a 1 hour nose only exposure RD is 0.743 mg/L (equivalent to 46.05 mg/kg bw). Respiratory rate and spiroxamine had a sensory irritant potential with respiratory changes (tidal volume, respiratory rate and minute volume lowered) evident and defined to attend 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 McGnods

A. Materials

1. Test Material: Spirogamine

(alternative pame: [8 (1,1-dimethylethyl) Vethyl-N-propyl-1,4-

distaspiro 4,5]decane-2 nivethanamine; KWG 4168)

Description: Brown Figuid (OH 10.4)

Lot/Batch No. 2 170(2/90 × Purity: 94.5% 2 18134-20-8

Stability of test Assumed stable for the duration of the study (expiry date not given)

compound:

2. Vehicle and/or positive For low concentrations polyethylene glycol 400 - ethanol / not relevant control:

3. Test animals

Species: Nouse Strain: OF 1/2

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:

> 22 ± 2 °C **Temperature:** ca.50% **Humidity:** 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 three were randomly assigned to the test groups. Groups of rats (4/sex) were exposed (nose only) for 1 hours to atmospheres containing spiroxamine vaerosol) at measured concentrations of 0 (air control) and vehicle control (polyethy)ene glacol 4000 ethacol]), 63 242, 407, 811 of 1367 mg/m³ 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 individual in plenglass exposure tubes (following a period of acclimatisation Prior to dosing). Spiroxamine at concentrations of 0, 300, 1000, 3000, 10000 and 1,300 mg/m³ was automatically injected into a buffle with compressed air (air that has had water, dust and of removed). This mixture was then puriped into the inhalation chamber (volume: ca, 20 L). The battle increased the efficiency of acrosol generation, whilst also removing larger particles. The air flows (10 L/minute, ensuring at Yeast eq. 30 exchanges of the Wahalation chamber air columes/hour) were continuously montored with rotal eters and re-algusted to the nominal Setting where necessary. Air flows were monitored continuously

Determination of the concentration of spiroxamine in the test atmosphere was performed using gas chromatography (FL detector). Temperature and air humidation 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.

Mean values and simple standard deviations were calculated for the body weights. more frequent findings for the responatory tract were evaluated using Fisher's Prowise Test with a preceding RXC chi square test

4. Statistics:

C. Methods:

1. Observations: 2

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 ssessed while they were in the tubes if there were clear signs occurring such as spassor, abnormal movements, and severe dyspnoea. An assessment of their reflexes was also indertaken.

After exposure animals were assessed with particular regard to the following

- Gross appearance of the mucus membranes of the eyes, respiratory tract
- General state Amuzzle skin, pinnae, state of fur, grooming activity, respiration
- Cardiovase flar parameters,
- Somato-motor system and behaviour pattern (including tremors, Convulsions, hypersalivation, dyspnoea, diarrhoea, lethargy, sedation, coma)
- Central nervous and autonomic signs

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-reatment and objected to a gross necropsy.

Results and Discussion

A. Atmospheric data:

Findings indicate that particles were well within the repirable range

Table CA 5.8.2/02-1: Overview of acute sensory fritant potential in mice treated with spiroramine exposure parameters

| <i></i> | | <i>0</i> .~Y | 0 4, | | |
|--|-------------|-------------------|-----------------------------|-----------------|-----------------|
| Parameter Q | | | √ Value | | |
| Dose group (nominal mg/m ³) | 300 | L ≪1000~° | 3000 | 10000 | ,©13300 |
| Mean achieved atmosphere concentration | 63 | 142 | 407 | 8 71 % | 1367 |
| (mg/m^3) | | | | 20° 4 | |
| Mean achieved atmosphere concentration | 0.063 | Ø√142 Ø | [∞] 0. 4 07 | 0.81 | 1.367 |
| (mg/L) | | ~ 4 | | | |
| Dose group (internal dose nig/kg bwd) ^a | 4.07 | 9.2 | 26.3 | √5 2.4 | 88.3 |
| Chamber flow rate (L/min) | 85 ® | 5 85 ₁ | 5.8 5 | \$75.85 | 5.85 |
| Particle size (MMAD #SD) | 1.41 ± 1.95 | 1,42 ± 1.46 | 1.12🗱 1.48° | 1.48 ± 1.48 | 1.39 ± 1.49 |
| \perp Aerosol mass $<3 \mu m (9\%)$ | 9.85 | 98 | 900 | 97 | 98 |
| Chamber air Buring exposure | * ** | 9 <i>b</i> | ∠ 22 © | | |
| temperature (°C)C | | | Ø' 25 | | |
| Relative humidly (%) During expoure | | 6 | | | |
| Air changes (/h) | | | √ 30 | | |
| O ₂ conc. (%) During exposure | 7 O | | ® ot detailed | | |
| CO ₂ copc (%) During exposure | | 4 F 0 | Not detailed | | |

a Internal dose mg/kg mg/kg inhalation dose mg/kg mg/k

B. Observations:

1. Clinical signs

The single anythal in the air control group, which died on day 4 exhibited reduced motivity and piloetection on day 3. All other animals in the air control, vehicle control, 63, 142 and 407 mg/m³ dose groups exhibited no signs of doxicity.

811 and 1367 ng/m \$2.4 and 88.3 mg/kg bw): clinical signs of toxicity included slightly to moderate slower breathing, reduced motility. All animals reduced \$\text{N}\$ normal from day 1 post treatment.

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 Bade, 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



Overview of acute sensory irritant potential in mice treated with spiroxamine: mortality and body weight Table CA 5.8.2/02-2-:

| Overall mortality ^a 2/4 0/4 | Parameter | (Actual concentration (mg/m³) [nominal mg/m³]) | | | |
|--|--------------------------------|--|---|---|---------------------|
| Body weight (g) | | Air control Vehicle control | | 63 300] | 142 [1000] |
| Body weight (g) 25.0 23.3 23.3 25.7 25.3 24.0 25.0 25.5 24.3 25.5 24.0 25. | Overall mortality ^a | 2/4 | 0/4 | | 6 44 |
| Body weight (g) 25.0 23.3 23.3 25.7 25.3 24.0 25.0 25.5 24.3 25.5 24.0 25. | | | 7 0 1 3 7 7 | | 30 30 7 |
| Body weight (g) | | 0/4 0/4 0/4 | 1/4 0/4 0/4 0/4 | 0/4 | 0/4 0/4 \$ 0/4 0/4 |
| Parameter | | 25.0 23.3 23.3 | 25.7 25.3 24.0 25.0 25.0 | 25.5 24.3 25.5 250° | 25.5 24.0 25.5 25.8 |
| Parameter | | ±1.8 ±1.9 ±4.1 | ±2.9 ±1.5 ±1.8 ±0.8 ±2.2 | ±2.4 ±2.4 ±3.2 | <u> </u> |
| Parameter 407 3000 314 10000 3257 13300 Day | , , | 1.0 ± 1.00 | -0.250.96 | Ø.5 ±1.73 ° | 0.3 ±0.50 |
| Parameter 407 3000 814 10000 92367 13300 Doy | gain (g) | | | | |
| Note | Parameter | | Actual conceOration (mg/m3) [neorinal | mg/no ¹) | |
| Overall mortality* Overall mortality* | | 407 [3000] | 814 (10000) | (C) (C) (C) (C) (C) (C) (C) (C) (C) (C) | |
| Day Mortality* O/4 | Overall mortality ^a | 0/4 | 0/4 | | |
| Mortality* 0.4 0.4 0.04 0.04 0.04 0.04 0.04 0.04 | Day | | | 1 30 7 7 X | |
| Body weight (g) | Mortality ^a | 0/4 0/4 00/4 | 0/4 0/4 0/4 0/4 | 0/4 0/4 0/4 | _ |
| He body weight gain (g) 1.3 ±1.71 Ret body weight gain (g) 1.3 ±1.71 Ret body weight gain (g) 1.3 ±1.71 Ret body weight gain (g) 1.3 ±1.71 Ret body weight gain (g) 1.3 ±1.71 Ret body weight gain (g) 1.3 ±1.71 Ret body weight gain (g) 1.3 ±1.71 Ret body weight gain (g) 1.3 ±1.71 Ret body weight gain (g) 1.3 ±1.71 Ret body weight gain (g) 1.3 ±1.71 Ret body weight gain (g) 1.3 ±1.71 Ret body weight gain (g) 1.3 ±1.71 Ret body weight gain (g) 1.3 ±1.71 Ret body weight gain (g) 1.3 ±1.71 Ret body weight gain (g) 1.4 ±2.2 Ret body weight gain (g) 1.5 ±1.4 Ret body weight g | Body weight (g) | 24.5 23.30 24.80 | 25.8 24.0 22.8 25.3 | 24.3 21.8 22.0 2 24.3 | |
| Net body weight gain (g) 1.3 ±1.71 Extract the copy the copy that the distribution the copy that t | ±s.d | ±2.5 \(\sqrt{2.1} \) ±2.2 | ± 3.6 ± 0.5 ± 1.0 ± 0.5 | <u>¥1.5</u> ₹1.5 ±1.4 ±2.2 | _ |
| gam (g) The glub of the triangle of triangle | Net body weight | 1.3 ±1.71 | | 0.059.82 | |
| Entry of any commercial permissive and connective perohibited | 5 2 | c way be cold | inention, of the or | | |
| | EUT'T' | County Front County | de bituitojteg | | |



C. Body weight and food consumption:

No test article related effects on body weight or body weight gain were 1. Body weight:

observed.

2. Food consumption: Not measured

D. Lung function and sensory irritant potential:

1. Lung function test:

observed, with tidal volume, respiratory rate and minute volume lowefed. The RD50 value was calculated on the basis of the winute volume.

proved to be the most sensitive parameter

 RD_{50} (minute volume) = $\sqrt{13}$ mg/m³ (equivalent $\sqrt{20}$ 0.71 $\sqrt{20}$ mg/I

(equivalent to RD₀ (extrapolated from the regression curve

0.016 mg/L; 1.03 mg/kg bw)

Confidence level 95% \$594 886 mg/sn3 (equivalence to 0.594)

38.35 – 57.20 mg/kg/bw)

Animals in the 81 kand 136 mg/m groups exhibited characteristic pauses between breaths. The spotdic increase in the tidal volume and the respiratory

rate, which was evident in these groups is related to put honary irritation. Spiroxamine when administered as an aeroso had a low sensory irritant

2. Sensory irritant potential:

potential in the mouse following a Phour wase only exposure. Signs of an

intitant effect on the lung periphery were also observed.

Based on the most sensitive parameter, the minute volume 162 ng/m³ is considered to be the fron-irritant threshold concentration, early valent to

1.03 mg/kg bw.

Table CA 5.8.2/02-3 Overview of acute sensory irritant potential in mice treated with spiroxamine: Fespiratory decrease parameters 🛣

| Parameter | | (Actual concer | ntra t ion (mgan ³) [1 | nominal mg/m³]) | |
|------------------|--------------|-----------------------------|---|----------------------|--------------|
| Paramete()* | 63 (300) | 142 [1000] | 300 0] , | 2 811 [10000] | 1367 [33000] |
| Respiratory | <u></u> 18 🗳 | 300 | | 50 | 60 |
| decrease (%) | | | | | |
| RD ₅₀ | × × 7 | 13 mg/m² [(0:7)3 | mg/L), equivalent t | to 46.03 mg/kg bw | v] |
| 6 | | (bas | sed on minute volui | ne) | |
| Non-irritant | | 16 mg/m ³ (0.016 | mg/L), equivalent t | o 1.03 mg/kg bw] | |
| threshold | | | šed or minute volu | me) | |

D. Necropsv:

bservation period had no evidence of concentration related changes Animals sacrificed at the end of in the lungs or other organs

E. Deficiencies:

None, no valid est guideline

Assessment and conclusions by applicant:

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 race following a 1 housanse only exposure RD₅₀ 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.

| concentration. | |
|---|---|
| | |
| Data Point: | KCA 5.8.2/03 |
| Report Author: | |
| 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: | <u>M-007784-01-1</u> |
| Guideline(s) followed in study: | M-007784-01-1 ASTME981-84; OECO 403; BFRASSI-3 |
| Deviations from current test guideline: | None A A A A A |
| Previous evaluation: | |
| GLP/Officially | Yes, conducted under GENOfficially recognised testing Pacilities |
| recognised testing facilities: | |
| Acceptability/Reliability: | Yes v |

Executive Summary

The sensory irritant potential of spiroxamine was givestigated in a study in rats performed to GLP. Groups of Wistar rats (4/females/group) were exposed ruse only for a single 1 hour period to mean achieved aerosolise concentrations of 450, 858 and 584 mg/m³ [0.450, 0.858, 1.584 mg/L], with MMAD \pm GSD ranging from 1.02 \pm 1.5 to 1.17 \pm 1.42 obtained for the aerosol size distribution, with 100% of the inharable fraction 3 μ m. The observation period was 7 days post-exposure.

Prior to test article positive and following the hour exposure, lung function tests were conducted examining beak 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/m³ docage group mifely slowed down and laboured breathing, piloerection, reduced motility, sniffing noises. All animals returned to formal from day 1 post treatment. No test article related effects on body weight, body weight gain of 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/m³, with a toxicologically meaningful influence on the inspiration time/expiration time (IT/ET) ratio observed in animals in the 1584 mg/m³ dosage group.

No specific irritant potential were observed in rats exposed to spiroxamine as an aerosol. However, in rats exposed to 1584 mg/m 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 ng/m³ 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 RD₅₀ 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 toxical acidal and the second of th any toxicologically relevant changes in breathing.

Materials and Methods

A. Materials:

1. Test Material:

(alternative name: [8-(1,1-dimethylethyl)-Nethyl-N-propy 4,4 dioxaspiro[4,5]decane-2-methanamine KMV2 4160

Brown liquid (pH 10.4%) **Description:**

Lot/Batch No.: 17002/90 **Purity:** 94.9% CAS No.: 118134-30-8

Assumed stable for the dur Stability of test

2. Vehicle and/or positive None / not control:

3. Test animals:

compound:

Species:

Strain:

ca. Д∕— 3 months Age at dosing:

Weight at dosing: Ã70-2**0**₹2

Source:

Acclimation perio

Altromin 1324 opet for rats and mice, addibitum funicipal water, ad libaum from housed (4/cage)

2 ±0/C

1 least 10/h Diet:

Water:

Housin@

4. Environmental conditions:

Temperature

Humidity:

Air changes:

10h light Park Photoperiod:

B. Study Design:

1. In life dates:

22 October 1990 to 34 October (experimental dates)

2. Animal assignment and treatment:

Following acclimatisation rats were randomly assigned to the test groups. Groups of ats (40ex) were exposed (nose only) for 1 hours to atmospheres Sontairing spiroxamine (aerosol) at measured concentrations of 0 (air control), 450,858 or 3584 mg/m³ in air. The observation period was 7 days postexpôsure.

3. Generation of the test atmosphere/chamber description:

Quring 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/m³ 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 waluated using Fisher's Pairwise Test with a preceding RxQ 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 yeekends. The armals were only assessed while they were in the tables if there were clear signs occurring such as spasms, abnormal movements; and severe dysphoea. 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 stain, pingae, state of fur grooming activity,
- Circolation
- Somato-motor system and behaviour pattern (including tremors, convulsions, hypersalivation, dyspinoea, diarrhoea, lethargy, sedation, coma)
- Central nervous and alionomic signs

The body weights of the rats were recorded manually before exposure, and on days is and of the post-treatment observation period.

3. Food consumption:

2. Body weights:

4. Lung function tests

Not recorded.

Dior to est 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 70 to 60 minute recovery period was included. Sung function test and calculation of the relevant parameters were performed under sothermic conditions. Air flows were measured from the pressure difference across 4 5 400-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 the expiration time, quotient.

5. Sacrifice and pathology:

All animal were acrificed post-treatment and subjected to a gross necropsy.

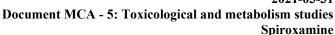
Results and Discussion

A. Atmospheri@data:

Findings indicate that particles were well within the respirable range.

Table CA 5.8.2/19-1: Over Dew of acute sensory irritant potential in rats treated with spiroxamine: exposure parameters

| Q Parameter | | Value | |
|---|-------|-------|-------|
| Dose growp (nominal mg/m ³) | 3000 | 10000 | 13333 |
| Mean whieved atmosphere concentration (mg/L) | 450 | 858 | 1584 |
| Mean achieved atmosphere concentration (mg/L) | 0.450 | 0.858 | 1.584 |





| BAYER E R | | Document MCA | - 5: Toxicological and | Page 291 of 39 2021-03-3 metabolism studio Spiroxamin |
|--|--|--|--|--|
| Parame | ter | | Value | |
| Dose group (internal dos | | 20.25 | 38.61 | 71.28 |
| Chamber flow rate (L/m | | 25 | 10 | 15 |
| | , | | | |
| Particle size (MMAD ± | | 1.02 ± 1.38 | 1.11 ±1.38 | 1.17 ±1.02 |
| Aerosol mass <3 μm (% | | 100 | 100 | 1.00 |
| Chamber air | During exposure | | 22 🚜 | |
| temperature (°C) | | | | $\overline{}$ |
| Relative humidity (%) | During exposure | Ĉ _A | 30-40 | |
| Air changes (/h) | During exposure | | 3 0 | |
| O ₂ conc. (%) | During exposure | P P | Not detailed | |
| CO ₂ conc. (%) | During exposure | | Not detailed O | # Q . O |
| a Internal dose (malka b | w) = inhalation dose (n | 1 | t respiration gate) x 4 ft (da clion considered necessary | uily abhalation |
| 7531-rev.10] B. Observations: 1. Clinical signs: | All animals in signs of toxicity Animals in the | he air control, 450 and | 1858 mg/m³ dose group coup mildly flowed www. | es exhibited no |
| 2. Mortality | breathing Piloe to normal from Deaths were in Refer to Table | rection, reduced notiled day 1 post treatment. Inited to a single animal CA 5.8.2/031. | ity Eniffine noises All in the air control ground | animals required |
| O ₂ conc. (%) CO ₂ conc. (%) a Internal dose (mg/kg beexposure) x 1 (default rows) 7531-rev.10] B. Observations: 1. Clinical signs: | | | | |
| | | | | |

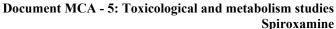


Table CA 5.8.2/03-1-: Overview of acute sensory irritant potential in rats treated with spiroxamore: mortality and body weight

| | | | | | | | | (2a) lb | ~'\ |) | , v | | |
|--------------------------------|------|-------------|-------|------|------------|------------------|-------------|------------|---------------|--------------------|---------------|----------------|------|
| Donomoton | | | | | | ♀ (Actual concer | ntration | mg/m³) [| nominal mg/m | ^{[3}]) | | | |
| Parameter | | Air control | | | 450 [3000] | O.K | a 0 | 858 j¥0000 | 1 % | , e ^o . | 1584 [133,38] | | |
| Overall mortality ^a | | 0 | /4 | | | 0/4 | | (C) | , 0/4 of | | | 2 2 4 2 | |
| Day | 0 | 1 | 3 | 7 | 0 | 1 | 1 20° | <u> </u> | 1 3 | \@\mathfrak{7} | ~ 0× | 3C | 7 |
| Body weight (g) | 184 | 182 | 184 | 189 | 191 | 192 993 | 197 | 199 | 196 19 | 5,0° 1981 | O 195 | C 190 € 991 | 197 |
| ±s.d | ±6.4 | ±7.6 | ±6.6 | ±7.1 | ±3.9 | ±4% ±3% | ±3.4 @ | €3.7 × | ¥2.7 ∂ | 2.0 | ±6.3 | ±6.7 | ±5.1 |
| Net body weight | | 2.5 = | ±3.42 | | *** | 6.0 ±1.830 | 1 W | * 2C | -0.3 ±5.91 | Or In | 1 | 1.5 ±1.29 | |
| gain (g) | | | | | ~ 101 | , K.S | The same of | M | ,, O , , c | | | | |

a Mortality: no. of animals found dead / no. of animals treated

copy rights of the owner and regulation, this docume this document has publication, and use of this and use of this document ton, and use of this and use of this and use of this and use of the commercial exploitation and use of this and u any commercial permission and violate the rights of the prohibited and violated the prohibited and violated the prohibited and violated the prohibited and violated the prohibited and violated the prohibited and violated the prohibited and violated the prohibited and violated the prohibited and violated the prohibited and violated the prohibited and violated the prohibited and violated the prohibited the prohibited and violated the prohibited and violated the prohibited and violated the prohibited and violated the prohibited the prohib any publication of the owner of this document and legin and use of this document





C. Body weight and food consumption:

1. Body weight:

weight gain were weight gain were with respiratory rate and minute volume unchanged. A mild decrease with tidal volume from ≥ 858 mg/m³, with a toxic logically meaningful influence the inspiration time/expiration time (IT/ET) ratio observed and ~ 1584 mg/m³ dosage group ~ 1584 mg/m³ (equivalent to ~ 150) ~ 150 0 Animals at ≥858 mg/m³ group exhibited characteristic pages between breaths, o suggestive of pulmonary diritation.

cluted eligible in respirately pound in ministrative pound in mini



PĠ

Overview of acute sensory irritant potential in rats treated with spiroxamine: respiratory parameters (data relative to control period [5]) Table CA 5.8.2/03-3:

| | PEF TV R8 MV IT ET | ♀ (Actual concer | itration (næfin |) [nominal mg/ng) | 1384 [13333] |
|------------------|--|------------------------------|------------------|----------------------------|---|
| Parameter | 0 [air control] | 450 [3000] | | 858 [10000] | 1384 [13333] |
| | PEF TV R8 MV IT ET | PEF TV R8 MV I | CALET PER | TV N MV SIT E | PEF R8 PV IT ET |
| Min | 66.3 81.0 80.4 67.5 91.1 83.9 | 88.6 79.8 86.7 85.4 | 86.3 ¥2.5 | 67:2 89.0 77 A 78.3 | F.1 61.1 62.4 67 2 44.8 39.5 72.3 |
| Max | 122.7 103.1 108.9 107.2 137.8 130.3 | 123.9 108.6 117.6 114 \$ 111 | 1.0, 149.5 122.9 | 206.7 139 9 109.9 122.5 10 | 4.3 632.9 102 914.4 108 243.9 154.9 |
| RD ₅₀ | >1584 mg/m ³ [(1.584 mg/L), equivalent | ent to 71.28 mg/kg/bw] | | | 4.5/052.9/102/07/14.4/2008/243.9/134.9/ |
| Non-irritant | 450 mg/m ³ [(0.450 mg/L), equivalent | to 20.25 mg/kg bw] | | | |
| threshold | | | | | |
| PEV | | | MV: minute vo | olume. The second | |
| TV: tidal volume | | | IT: in piration | thate and | |
| R8: rate | | in all all | ET. expiration | time O | |
| | | | | | O v |
| | 66.3 81.0 80.4 67.5 91.1 83.9 122.7 103.1 108.9 107.2 137.8 130.3 >1584 mg/m³ [(1.584 mg/L), equivalent 450 mg/m³ [(0.450 mg/L), equivalen | The may dietri | 0.S - 3 | | , to |
| | | | | | <i>y</i> |
| | | | | | |
| | Line. | | | | |
| | . O | | r "S | | |
| | | | | \$ " · | |
| | | | | | |
| | | | 04, | | |
| | | | de late | | |
| | | | | | |
| | | | J 2 | | |
| | | | | | |
| | | ett si | | | |
| | | | | | |
| | | | | | |
| | | | | | |
| ~ | De De Marie | | | | |
| | | S. P. | | | |
| | | • | | | |
| 409 | | | | | |
| C | | | | | |
| | ~ | | | | |
| | | | | | |
| | | | | | |



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 2832013

Conclusion: Under the conditions of this study not sensory irritant potential of spiror amine in rats following a 1 hour nose only resulted with an RD₅₀ value >1 \$84 mg/L (equivalent to 71.28 mg/L) bw). Although these animals exhibited certain alterations in their breathing pattern, these were deemed casually connected with action on the lung periphery with a partie between breaths detected in this group of animals.

Based on these results 0.450 mg/L (equivalent to 2023 mg/kg bw/is considered to be tolerated without any toxicologically relevant changes in breathing.

For procedural reasons studies insted in the Table 58.2-2 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: Stories previously submitted and not refled upon for the risk assessment

| Data | Document | Date | Tirle | | | . | | |
|----------|-------------|------------|----------|---------------------------|----------|-----------|---------------------------------------|--|
| Point | No. | \O' | | 0. | | Ş | | |
| KCA | M-O | 71997 | KWG 41 | 68 _C -Position | paper | on the to | twicological no-observed effect level | |
| 5.8.2/04 | 0081490 | 4 5 | which is | relevant for t | he calle | ulation | of a systemic AOEL | |
| | <u>02-1</u> | % _ a | | } '&' | ~°0° | | | |

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 chotesterol in the human neuroblastoma SK-N-SH cell line in response to spiroxamine could be due to a piological 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 thoughour in vitro that suggested that the lead-hits were most potent in neural cells, the results also demonstrate that the lead-hits could impair cholesterol biosynthesis in other tissues as well. When extrapolating this study's fractings to an actual human, it would be important to consider the impact of the exposure beyong the brain, because liver and lung sterol profiles will likely be disrupted as well.

This data on 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-01656-01-1]; CA 5.7.1/02 [M-006914-01-1]) confirm spiroxamine is not a neurotoxicant, nor does is how 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: | |
| | |
| Report Year: | 2020 |
| Report Title: | Screening toxcast for chemicals that affect cholesteral biosynthesis: studies to cell |
| | culture and human induced duripotent steps cell-derived neuroprogenitors |
| Report No: | M-689223-01-1 A & Q Q Q O O O O |
| Document No: | M-689223-01-10 |
| Guideline(s) followed in | not applicable of the control of the |
| study: | |
| Deviations from current | not applicable None |
| test guideline: | |
| Previous evaluation: | No, not previously submitted of the subm |
| | |
| GLP/Officially | No, not conducted under GLP/Officially recognised testing facilities |
| recognised testing | |
| facilities: | |
| Acceptability/Reliability | Supportive only Supportive onl |

Overview

Changes in chotestero 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.

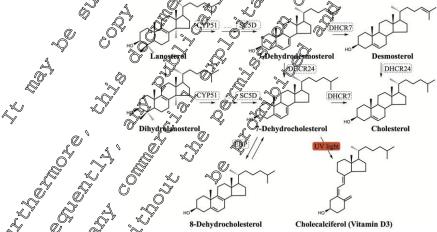


Figure CA 5.8.2/21-1: schematic of cholesterol biosynthesis

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 bepatosynthesis does not freely cross the blood-brain barrier. During the 1960s' drug-discovery efforts to develop effications. cholesterol-lowering pharmaceuticals, numerous lead-hit compounds failed in commercial development due to toxicity. AY-9944 was designed to inhibit DHCR 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 discipling cholesterol metabolism? Some environmental sterol biosynthesis digruptors have a fready been identified by cell culture experiments and the use of in silico predictive modeling (e.g. penzalkonium chloride which inhibits DHCR7). Another class of environmental stercometabolism 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, status, is contraindicated for pregnancy due to an increased risk of spontaneous pregnancy loss yet it is controversial whether this loss is due to teratogenicity.

teratogenicity.

The objective of this work was to jest the hypothesis that common covironmental toxicants may also impair cholesterol metabolism and thereby possibly contribute to neurodes dopmental toxicity.

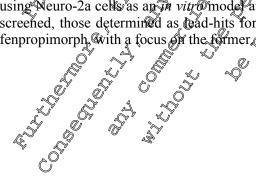
Results

A. Lead-hit validation and in vitto dosing:

Using high-through put screening with a targeted lipidomic analysis and the more neuroblastoma cell line, Neuro-2a, the Tox cast which mical library (1851 chemical) was screened for compounds that impact sterol metabolism. Validation of chemical effects was conducted by assessing cholesterol biosynthesis in laman and duced pluripotent stem cell (hipSC) derived neuroprogenitors using an isotopically labeled cholesterol precursor and by promitoring 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 chroride, fenpropimorph, and spiroxamine). All four compounds were validated to cause hypocholesterolemia in Neuro-2a activity in four immortalized human cell lines and in a human neuroprogentor 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 FoxCast^M Chemical Library for environmental cholesterol biosynthesis disruptors. The workflow of high-throughput screen from the entire library identified four lead-hit compounds (tributyltin chloride, endosultan, sulfate, fenpropi morph, spiroxamine). Lead-hit compounds were identified through the results of two independent screens of the ToxCastTM library using Neuro-2a cells as an in vitro mode at a screening exposure of 1 μ M for 24 h. Of the compounds screened, those determined as tead-hits for evating 7-dehydrocholesterol included spiroxamine and fenpropimorph with a focus of the former.





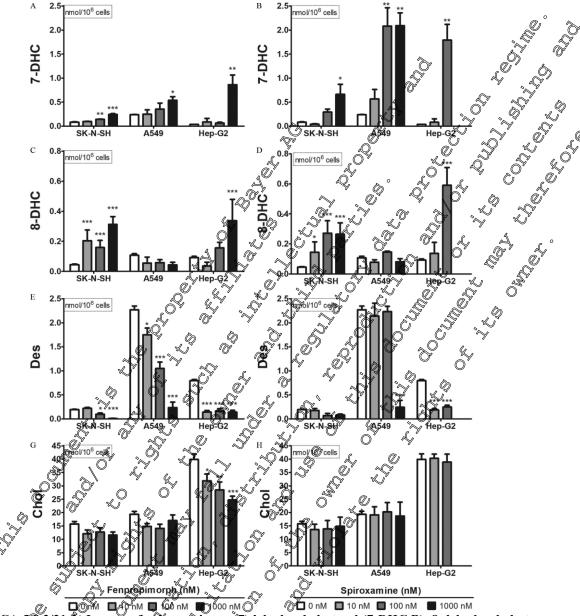


Figure CA 5.3.2/21-2. Impact of approxamine on 7-dehydrocholesterol (7-DHC,B), 8-dehydrocholesterol (8-DHC; D), desmosterol (Des; D), and cholesterol levels (Chol; H) in three different human-derived cell lines: (from left to right) SK-NSH, AS49 and Hep-G2. Cells were exposed to compound (0, 10, 100, 1,000nN) for 24h. Omitted bars reflect significant toxicity as determined by release of lactate dehydrogenase. Data presented as amolstorol/million cells (n=4,±SEM). *p<0:05, **p<0:01, ***p<0:001 as determined by post noc Dunnett's test following one-way ANOVA, using vehicle as the control comparison.

B. Temporalchanges in nearoprogenitor 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 observe the for differentiation-induced changes in cholesterol biosynthesis, each deidentified 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 $0.81 \pm 0.17 \text{nmol}/10^6$ 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 elect 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 hip SC-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 4h, exposure to both pesticides at 1000 nM altered hiPSC sterol profiles as indicated to an increase in 7-DMC levels and decrease in desmosterol levels with spiroxamine. After 24 h, similar absolute levels of DHC were observed (spiroxamine, 1:54 nmol 7-DHC/106 cells).

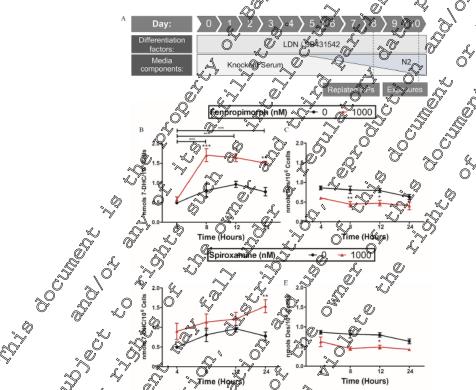


Figure CA 5.8.2/21-3: Human induced hiPSCs were differentiated towards a neuroectoderm lineage as shown (B). These hiPSCs derived neuroprogenitor cells were exposed on day 10 of differentiation to spiroxamine (D,E) at 1000 nM (red triangle) for 4, 8, 12 and 24 h. Neuroprogenitor cells were then analyzed for 7-dehydrocholesterol (D) for 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 \$\frac{1}{2}\text{EM} (\frac{1}{2}\text{3})\text{.**} \text{p<0:05, **p<0:01} as determined by Bonferroni post tests following two-way ANOVA

C. De novo chofesterol Synthesis in hiPSQ cells treated with spiroxamine:

An isotopically labeled serol 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 ¹³C atoms and the isotopically labeled sterol products of this precursor 7-PHC, desmosterol and cholesterol were analyzed by LC-MS/MS. A gradual increase in ¹³C₃-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 ¹³C₃-DHC and an increase in ¹³C₃-desmosterol were also observed.



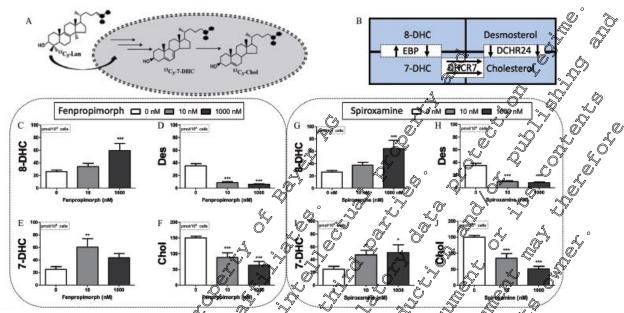


Figure CA 5.8.2/21-4: De novo sonthesis of cholesterol and sterol precursors was accomplished by incubating hiPSC-derived neuroprogenitor cells with C3-landsterol for 24-h and monitoring for ¹³C3-sterols including ¹³C3-7-dehydrocholesterol and ¹³C3-cholesterol (5). Simplified cholesterol biosynthetic pathway is shown (B). Neuroprogenitor cells were exposed to spiroxamine (G-1) at 10 nM and 1000 nM for the same duration as the ¹³C3-lanosterol incontion. Absolute values of ¹³C3-8-tehydrocholesterol (G), ¹³C3-desmosterol (H), ¹³C3-7-dehydrocholesterol (I) and ¹³C3-cholesterol (J) were detected, quantified and normalized to cell number. Three distinct differentiations were conducted for each donor and averaged; shown as thea wrage of the three donor s±8EM (n-3). *p<0:05, **p<0:01, ***p<0:001 as determined by post foc 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 and alue 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 lacfate delivergenase detected in the media after a 24-hexposure. It is noteworth that the 1000 nm exposure of spiroxamine in the Hep-G2 cells was cytotoxic.



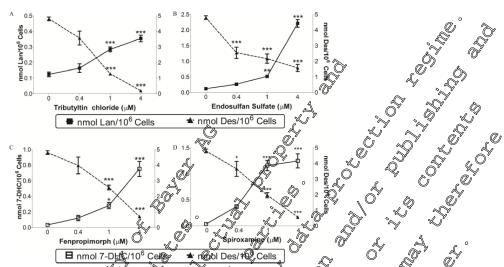


Figure CA 5.8.2/21-5: Concentration-dependent response of lead-hit compounds on intracellular levels of 7-dehydrocholesterol (D, open squares) and desmosferol (sofidtriangles) in Neuro-2a cells. Cells were exposed to (C) spiroxamine at the indicated concentration for 24 h Data presented as nuclisterol/million cells (n=4, ±SEM). *p<0:05, ****p<0:001 as determined by post hoc Dunnett y test following one-way ANOVA, using vehicle as the control comparison.

Further experimentation with spiroxamine was undertaken to evaluate levels of other 3-DHC relevant metabolites. In addition to 7-DHC serving as a precursor to both cholesterol and vitamin D, this sterol is also isomerized biosynthetically of 8-dehydrocholesterol (8-DHC) by the enzyme Emopamil Binding Protein (EBP,3-b-hydroxysteroid-D8, D7-isomeriase). Thus, if DHCR7 is solectively 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 5-DHC and 8-DHC levels along with decreases on the DHCR7 metabolites desmosterol and pholesterol is observed following exposure to spiroxamine, it leads to the conclusion that spiroxamine inhibits DHCR7, ultimately leading to acceleration in girculating cholesterol levels. The data from the high throughput screening of spiroxamine also supports the perturbations observed in the apical manimalian toxicity studies.



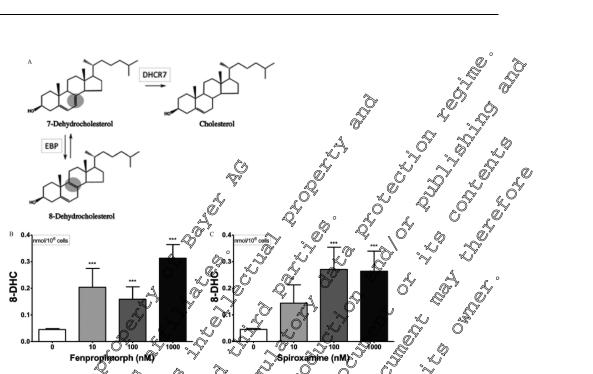


Figure CA 5.8.2/21-6: Biological synthesis of 8-dehydrocholesterol via isomerization of 7-dehydrocholesterol by the enzyme EBP is shown (A) SK-N-SH cells were exposed to spiroxamine (C) at indicated concentrations for 24 k. Data presented as amol 8-dehydrocholesterol/million cells (n=4, \pm SEM). *p<0:05, ***p<0:091 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 reated with spiroxomine:

Spiroxamine affects de novo cholesterol biosynthesis. Exposure at 10 nM for 24 h increased \$^{13}\text{C}_3\$-7-DHC levels and at a higher dose of 4,000 nM, \$^{13}\text{C}_3\$-DHC levels were also significantly elevated. Of importance is that both products of the enzyme DHCR, \$^{13}\text{C}_3\$-desmosterol and \$^{13}\text{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}\text{C}_3\$-Chol}, [^{13}\text{C}_3\$-chol+^{12}\text{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 half periods at 10 pM 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 $\delta 14$ reductase and $\delta 7$, $\delta 8$ 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 $\delta 14$ reductase or $\delta 7$, $\delta 8$ 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 line that match closely with their tissues of origin have the potential to provide additional insights. Even thoughour *in ritro* 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 extrapologing 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 date 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-01656-01-1]); CA 5.7.1/02 [M-006914-01-1]) confirm spiroxamine is not a neurotoxicant, nor close it show structural relationship to known neurotoxicants (*e.g.*, organophosphates, carbamates). With the phyroid 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 hvestigate 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 textcology

CA 5.8.3 Endocrine disrupting properties

A complete in vitro and in vivo test battery of endorine disruption studies have been conducted on spiroxamine, with a summary an overview provided below Spirox mainers 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 Work assessment for human health based on the 'Guidance for the identification of indocrine disruptors in the context of Regulations (EU) No 528/2012 and (EC) No 1107/2009 published 2018 has been conducted.

Based on the available maximalian evidence EATS modulities were considered sufficiently investigated and, on the WoE no adversity was consistently observed in the relevant studies. Therefore, spiroxamine does not meet the Electric 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 spirexamine that occur in the absence of wert toxicity. In addition to the apical studies, results from Tox21 EDSP TR indicate that spiroxamine is negative for Tactivity.
- EAS: Although sporator effects were observed in in infitro 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 As 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 spiroxyme 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 parties is not intribited. The reductions in steroid hormone production observed in the steroid geneses data is attributed to apstream effects related to reduction in serum cholesterol evels, which impact apon 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 agical in pivo manimalion toxicity data. Collectively, adrenal weights were unaffected in the appeal toxicity studies, with no test article related effects observed upon adrenal gland histopathological applysis 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 Sathway interaction, in vivo. It is therefore concluded that spiroxamine is devoid effects on the EASmodal thes, 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 A Point / Reference |
|--|---|---|---|--------------------------------------|
| | l | E-modalit | y S | 4 .4 |
| US EPA ToxCast | ToxCast bioactivity ER model | n/a | No evidence of a direct interaction with oestrogenic or interaction with oestrogenic fathway | ©A 5.83/03 [M-762777~ 01-17 |
| | | A-modalit | | |
| US EPA ToxCast | ToxCast bioactivity AR model | n/a | No evidence of a direct interaction with androgene or anti-androgenic pathway | EA 5.83/04 (1M-702779-0) 01-1 |
| In vitro androgen receptor and antagonist activity androgen receptor | CHO-K1 cell line stably transfected with human AR | | AR agonist assay: No evidence of androgen receptor agonist assay: R antagonist assay: | CA 5.8.3/05° [A-7615@- 01-11.8 |
| transcriptional activation assay | expression vector | 0.1¾, 10, © μ00 nM, ↓ 2.16 μΜ. | No evidence of androgen are receptor antagon st activity | |
| <i>In vivo</i> Hershberger DRF study | Young Mature Frats | 0, 125, 250 | 125 mg/kg hw/day reemed to be the MTD for the invivo hershberger assay (CA 5.8.3/07), with additional dose levels of 31.25 and 62.5 mg/kg by 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/cay | No potency to exhibit androgenic, and androgenic, and androgenic or 5a-galuctase inhibitory properties. | CA 5.8.3/07 [M-764008- 01-1] |
| | | T-modalit | | T |
| US EPA T®Cast | HEK2937 cells | n/a v | No evidence of a direct interaction with thyroid pathway | CA 5.8.3/08 [M-762778- 01-1] |
| | | S-mødalit | | |
| US EPA Tox Cast | H29 Ceells | | Inhibition of mineralocorticoids (1) 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 nficrosomes containing CV 19 and 2450 recontain | 0,0 ² , 0.1, 1, Φ, 100 μΜ | Spiroxamine is deemed to not inhibit aromatase activity | CA 5.8.3/01 [M-301971- 01-1] |
| In vitro | Ex viço in rat testis homogenate | 1, 100 μΜ | 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 C 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, | New steroidogerosis assay | CA 58/3/10, P [M-764156] 90/1 |

E-modality

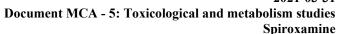
| Data Point: | KCA 5.8.3/00 |
|--|--|
| Report Author: | KCA 5.8.3/00 |
| Report Year: | |
| Report Title: | Spiroxamine US EPA ToxCast data retrieval for the E-machity |
| Report No: | 047\\$36-TOX5 |
| Document No: | M-7627\$Q-01-1 D |
| Guideline(s) followed in | Mone |
| study. | |
| Deviations from current | I Nume 4.5 |
| test guideline: | |
| Previous evaluation | No, not previously submitted |
| ر الله الله الله الله الله الله الله الل | |
| GLP/Officially recognised testing | not applicable of the state of |
| recognised testing | nay application of the state of |
| facilities: 🙎 | |
| Acceptability/Reliability | Yes S S S |

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 access an oestrogen or anti-oestrogen. The data that underpins the prediction for spiroxamine are results from 17 pr. Toxo ast high-throughput screening (HTS) assays that discriminate bioactivity from assay-specific interference and cytotoxicity.

The ToxCast model scores range from 0 tho 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 alternative for EDSP Tier 1 ER binding, ER transactivation, and uterotrophic assays.

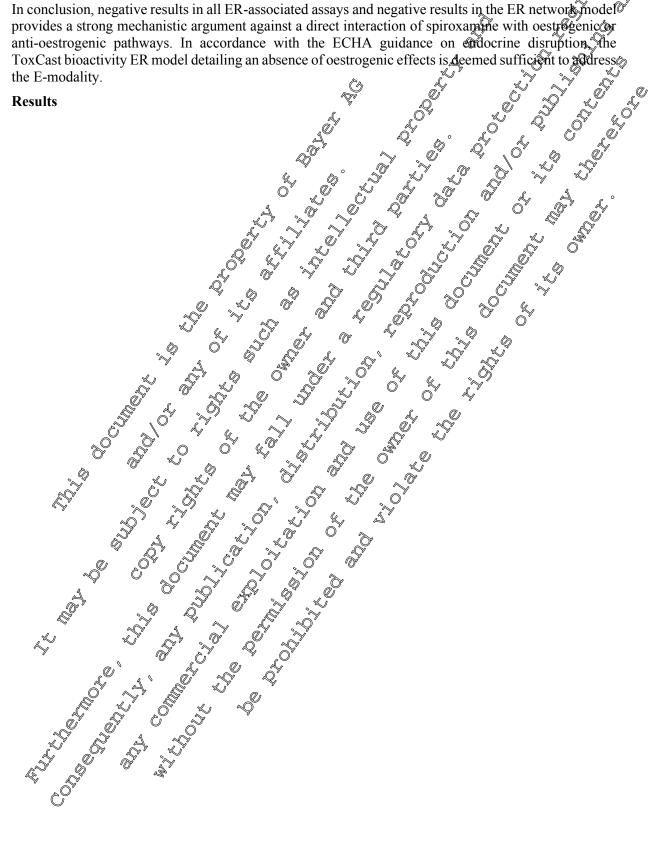
Of the PTS assay, only OT_ER_ERaERa_1440 a protein stabilisation assay examining receptor function and kinetics of the human oestrogen receptor 1 in a kidney cell line (HEK293T) following 24 hours exposure showed activity with an AC50 value of 36.05 µM obtained. However, the biological





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 critical and according to the extraction of critical and the extraction anti-oestrogenic pathways. In accordance with the ECHA guidance on codocrine disruption, the





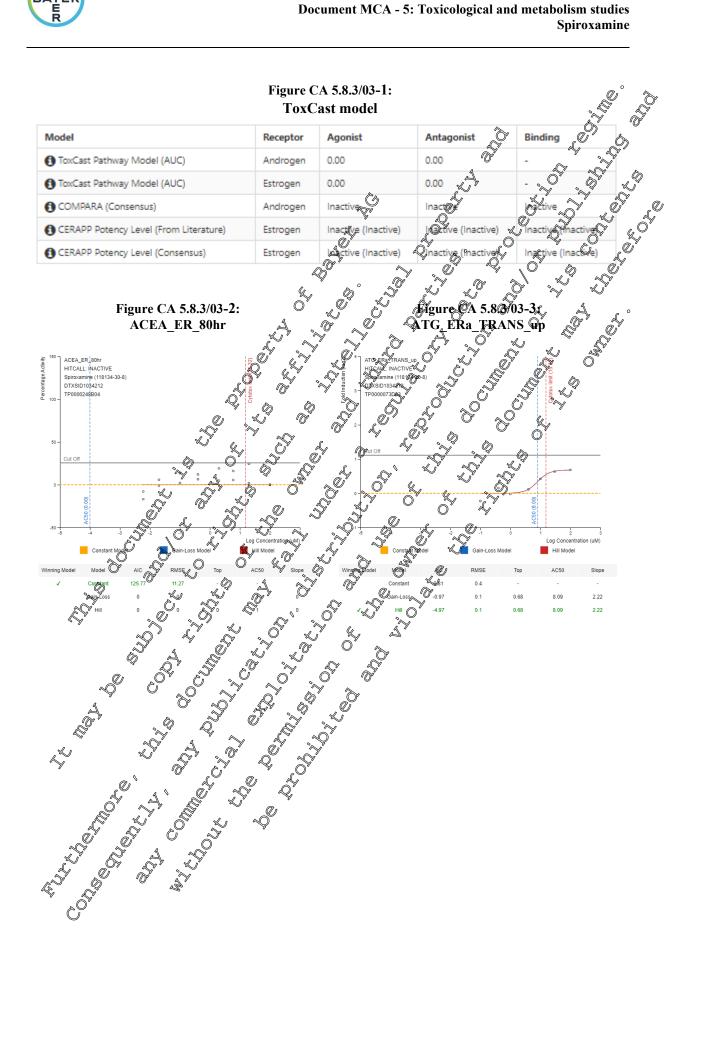
| | | | | Spiroxamine |
|---|---|-----------------------|---|---------------|
| | | P | - G | egind and |
| Table CA 5.8.3/03-1: Ov | verview of ToxCast models and data output for the E-modality | ale, | | |
| ToxCast model | Assay description | ©E / A-E ^a | Masson model and authorite a | Figure |
| | PA Endocrine: EDSP universe of chemicals website | ۵۰ | | e figure |
| | ashboard/dsstoxdb/results?abbreviation=EDSPUOC&scarch=I | T&SID10343 | Ø2#details № 2 August 2020 | Chr. |
| 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 | ¥É/A-E | No pestrogen agenist activity | CA 5-823/03-1 |
| | DOI: 10.1021/acs.est.5b02641 and presented to December 2014 FIFRA SAP under EPA-HQ-OPP-2014-06 . | | the grant of the next | |
| ACEA_ER_80hr | Cell proliferation assay that measures time-dependent cell growth using impedance using the ER-responsive T-4715 breast cancer cells. | EXT. | Constant model. AIC 125.77; RMSE: 11.27 AC ₅₀ : n/a Cytotoxicity: 15.32 | CA 5.8.3/03-2 |
| ATG_ERa_TRANS_up | and exogenous transcription factor GALA-ERa indiction in HepG2 cell | | Will model AIC: -407, RMSF 0.1; Top 9.68; AC, 08.09 μM; Ο Cytotoxicit 15.32 μM | CA 5.8.3/03-3 |
| ATG_ERE_CIS_up | protein cabilisation assays, examining and NA reporter sequence unique to the transfected cis-acting reporter general exogenous transcription factor GAL4-ERa induction in PlepG2 cells | | Guireloss model: AIC: -12; RMSE: 0.06; Tope 0.40; AC ₅₀ 0.5 μ M; Cytotoxicity: 15.32 μ M | CA 5.8.3/03-4 |
| NVS_NR_bER | Receptor binding assay in povine warme membranes examining receptor-thand binding of the key ligand [[41]-oestration] to cattle cestrogen acceptor 1 | EA-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 | Inne receptor-ligand finding of the key ligand [[3H]-oestradiol] of the human oestrogen receptor 1 (ER_o). | 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_0\$\ddot\delta\del | Protein stabilisation assay conducted in HER 293T kidney cell line following 8 hexposure examining seceptor function and kinetics for the human cestrogen 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 IEE 293T kidney cell line following 244 exposure examining receptor function and kinetics for the human estrogen 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_ERAER6_0480 | Protein subilisation assay conflucted in HEK293T kidney cell line following to hexposure examining receptor function and the first for the human destrogen 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 |



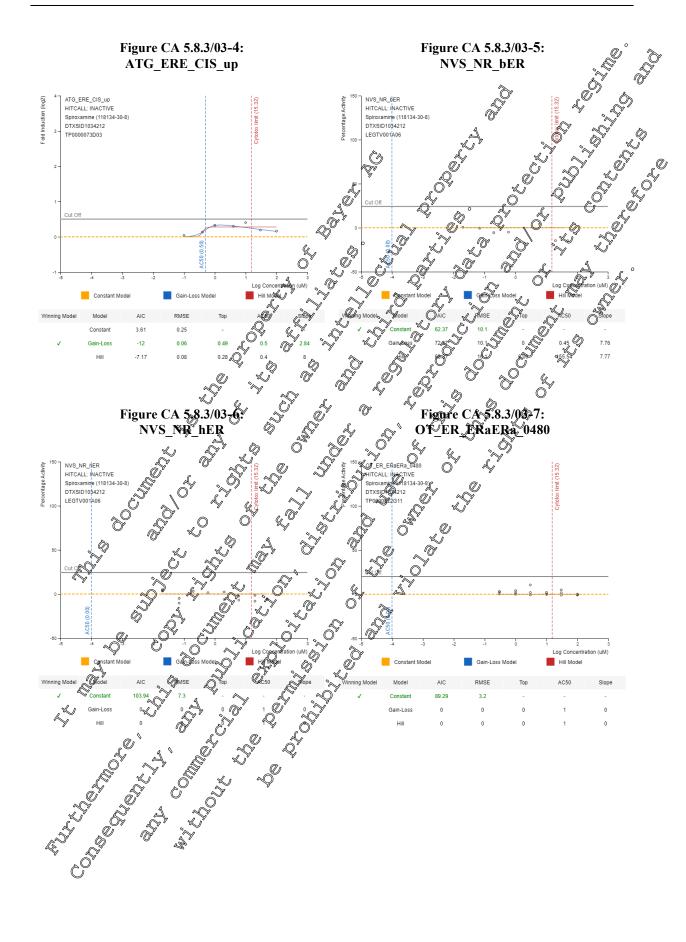
| | | | Ġ Dir | |
|--|---|-----------------------------|--|-------------------|
| ToxCast model | Assay description | E / A-Ea | Chosen model and outputs | Figure |
| OT_ER_ERaERb_1440 | Protein stabilisation assay conducted in HEK293T kidney cell | E/AIR | Hill model: (30°C: 87.65; RMSE: 20°2; | CA\$8.3/03-10 |
| | line following 24 h exposure examining receptor function and | OG J | Top: 33 (\$\$\text{\$\ext{\$\text{\$\text{\$\text{\$\text{\$\ext{\$\ext{\$\text{\$\text{\$\text{\$\text{\$\$\ext{\$\ext{\$\ext{\$\ext{\$\ext{\$\ext{\$\ext{\$\ext{\$\$\ext{\$\ext{\$\ext{\$\ext{\$\ext{\$\ext{\$\ext{\$\ext{\$\ext{\$\ext{\$\ext{\$\ext{\$\ext{\$\ext{\$\ext{\$\ext{\$\ext{\$\$\ext{\$\ext{\$\ext{\$\ext{\$\ext{\$\ext{\$\exititt{\$\ext{\$\ext{\$\ext{\$\ext{\$\ext{\$\ext{\$\exititt{\$\exititt{\$\exititt{\$\ext{\$\exititt{\$\ext{\$\exititt{\$\ext{\$\exitit{\$\ext{\$\exititt{\$\exititt{\$\exititt{\$\exititt{\$\exititt{\$\exititt{\$\exititt{\$\exititt{\$\exititt{\$\exititt{\$\exititt{\$\exititt{\$\exititt{\$\exititt{\$\exititt{\$\exitititt{\$\exititt{\$\exitititt{\$\ | O'D' |
| | kinetics for the human oestrogen receptor 1 and oestrogen | <i>y</i> | Cytenoxicity: 15.32 μM | , \$ |
| | receptor 2 (ER-β) | <u> </u> | | |
| OT_ER_ERbERb_0480 | Protein stabilisation assay conducted in HEK293T kidner cell | EA-E | Constant model. MC: 89.09; BMSE: | CA 5.8.3/03-11 |
| | line following 8 h exposure examining receptor function and | CTA-E | 2.84 AC50: n/a Cytotoxic(ty: 15.32 M | 1 c 0 2 c |
| OT ED EDIEDI 1440 | kinetics for the human oestrogen receptor 2 (ER) | | | 2 5 0 2 /02 12 |
| OT_ER_ERbERb_1440 | Protein stabilisation assay conducted in HE 293T kidney cell | Œ/A-E | Gain-loss model OTC: 74.05 RMSE | CA 5.8.3/03-12 |
| | line following 24 h exposure examining ecceptor function and | | 1.41; Top: 8.3; AC ₅₀ 29.97 μM; | |
| OT ED EDECED 0120 | kinetics for the human oestrogen receptor 2 (EKB) | E/A ES O | Cytotoxi Qy: 15.32 QM | CA 5.8.3/03-13 |
| OT_ERa_EREGFP_0120 | Gene expression assay in human cervix cell line. 2 h exposure | E/A-E | Gain-loss model AIC: 6509; RMSE:. | CA 5.8.3/03-13 |
| | an al ence | 200) - 40 | Cytotoxicity: 15.32 uM | |
| OT ERa EREGFP 0480 | Gene expression assay in human cervix call line. 8 hexposure | E/A-FC | Gan loss model. AIC: 47.95; RMSE: | CA 5.8.3/03-14 |
| | Gene expression as a in human cervix cell line. 8 the xposure | | 0.65; Top 4.29; AC 50.77; | |
| | | | Cytotoxicity: 15.32 μM | |
| Tox21_ERa_BLA_Agonis | Inducible ERα transcription factor activity detected by β- | E/A-E | Constant model. AIC: 151.85; RMSE: | CA 5.8.3/03-15 |
| t_ratio | | | 1.23; ACS: n/a Cytotoxicity: 15.32 μM | |
| | using HEK293T, a himnan kidney cell line 24 h exposure Inducible ERg manscription activity detected by B. | | J. O. | |
| Tox21_ERa_BLA_Antago | | E/A-E | Constant model. AIC: 275.52; RMSE: | CA 5.8.3/03-16 |
| nist_ratio | lactamase actrogen response element reporter in antagorist | / \\ \ \(\rangle \rangle \) | 5.2; AC ₅₀ : n/a Cytotoxicity: 15.32 μM | |
| | mode using HEK2937, a human kidney cell line. 24 dexposure | | | G + 5 0 0 /00 4 5 |
| TOX21_ERa_LUC_VM7_ | Induible ERe panscription factor activity detected by | E/&-E | Constant model. AIC: 231.79; RMSE: | CA 5.8.3/03-17 |
| Agonist | Auciferase-Coupled ATD quantitation in agenist mode tonig | 0 ≻ | 3.26; AC ₅₀ : n/a Cytotoxicity: 15.32 μM | |
| The state of the s | VM7, a human breast tissue cell line. 48 ff exposure | | G | G |
| TOX21_ERa_LUC_VM7_ | Inducible EBa transcript on factor activity detected by | E/A-E | Constant model. AIC: 262.92; RMSE: | CA 5.8.3/03-18 |
| Antagonist_0.5nM_É2 | luciferase coupled APP quantitation in an agonist mode using | | 4.24; AC ₅₀ : n/a Cytotoxicity: 15.32 μM | |
| | VM7, a human breast tissuccell line & h exposure | | | |

a: oestrogen (E) or anti-oestrogen (A-E) signal direction
AIC: Akaike Information criterial appropriate model server the lowest AIC value, essential model that best fits the data RMSE: median root mean squared error icross all winning models. RMSE as lower for higher performance models

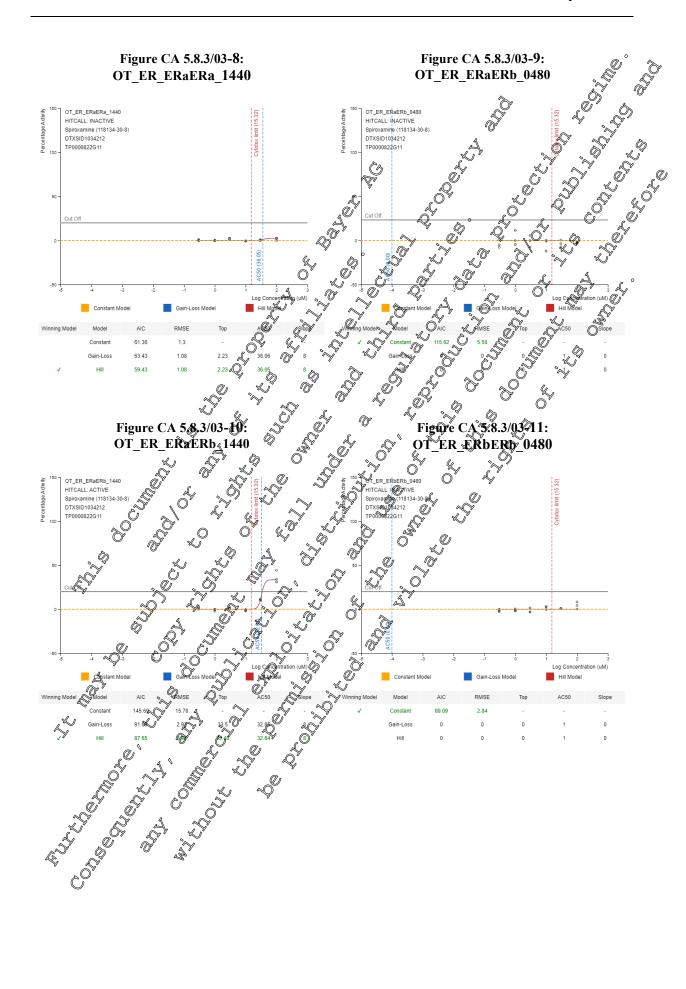




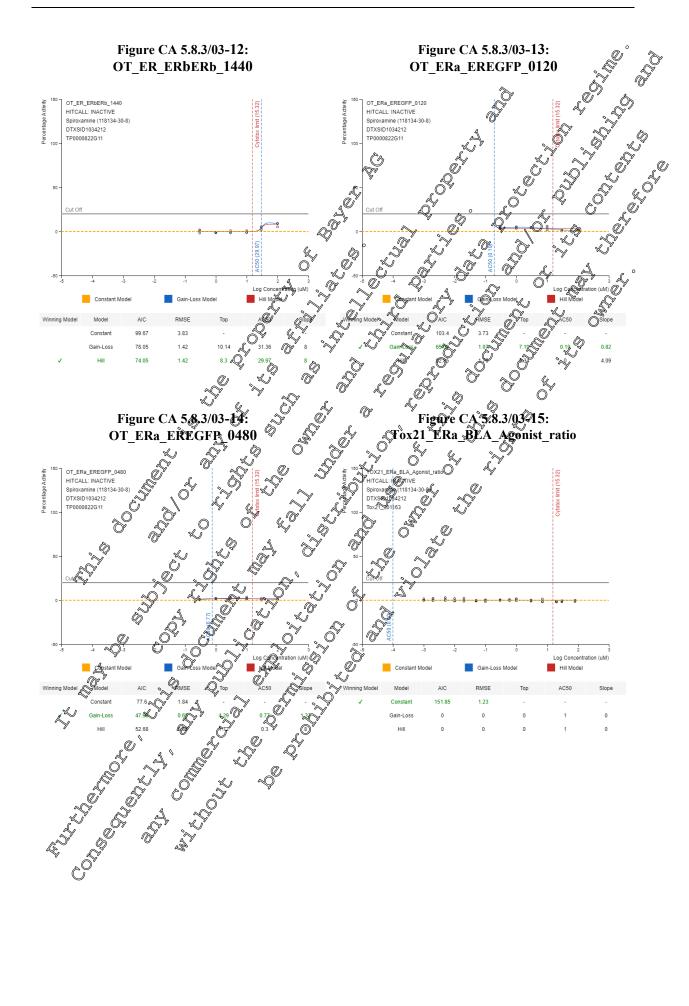




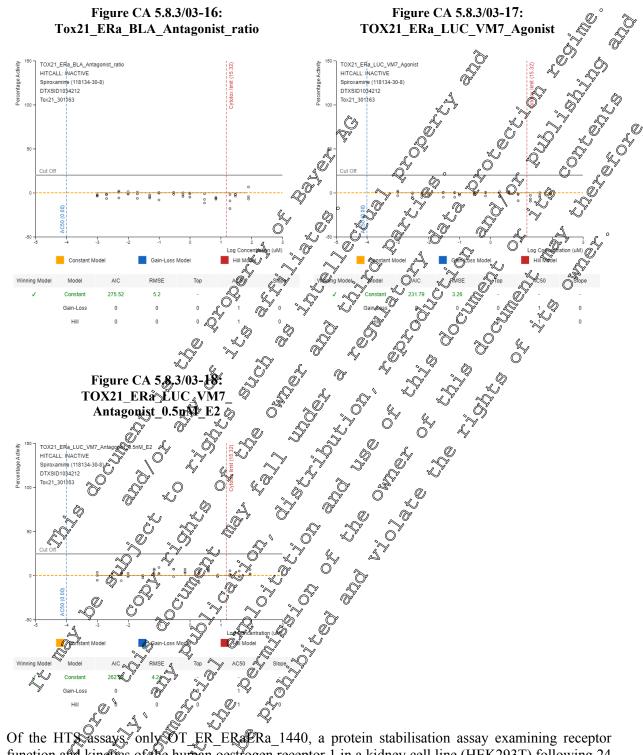












Of the HTS assays only OT_ER_ER PRa_1440, a protein stabilisation assay examining receptor function and kinetics of the human oestrogen receptor 1 in a kidney cell line (HEK293T) following 24 hours of showed activity with an AC50 value of 36.05 μM obtained. However, the biological relevance of this is low as activity was observed only at a concentration that occurred in the presence of cytotxic, 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

| guidance on endocrine o oestrogenic effects is de | disruption, the ToxCast bioactivity ER model detailing an absence of the model sufficient to address the E-modality |
|--|---|
| A-modality | KCA 5.8.3/04 |
| Data Point: | KCA 5.8.3/04 |
| Report Author: | |
| Report Year: | 2021 |
| Report Title: | Spiroxamine: US EPA ToxCast data retrieval for the A-modality. |
| Report No: | 0471836-TOX6, A S S S S S S S S S S S S S S S S S S |
| Document No: | M-762779-01-C |
| Guideline(s) followed in study: | None None |
| Deviations from current test guideline: | |
| Previous evaluation: | No set prexiously submitted 2 |
| GLP/Officially | not applicable |
| recognised testing | |
| facilities: | |
| Acceptability/Reliability: | Supportive only |

Executive Summary

Using the computational network model to determine a composite value that takes all androgen receptor (AR)-related assays the account to assess the in thro and open city 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 spiroxamme are results from 10 AR ToxCoxt high throughput screening (HTS) assays that discriminate bioactivity from assay-specific interference and cytotoxicity.

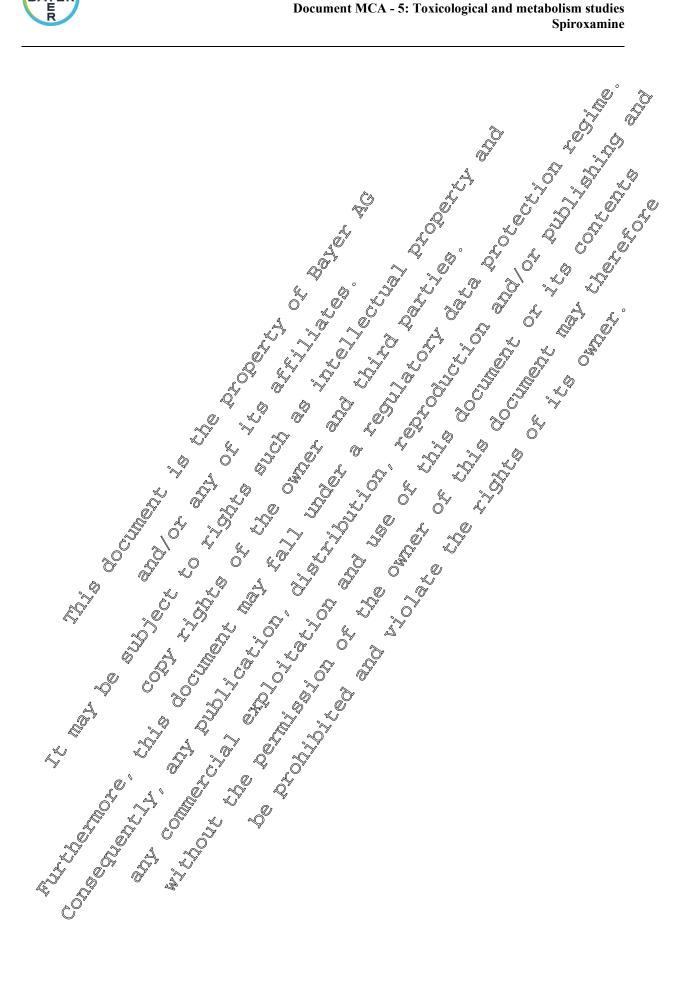
The ToxCast model scores range from 0 (no activity) to 1 (bioactivity of testosterone propionate). ToxCast ER model performance was evaluated for reference chemicals, as well as results of EDSP Tier 1 screening assays in current practice. The Tox ast ER model accuracy was 95.2% for agonist and 97.5% for antagonist reference chemicals. Out of 1856 chemicals screened in the AR pathway model, 220 chemicals demonstrated AR agonist or a tagonist activity and an additional 174 chemicals were predicted to have potential weak AR pathway activity.

Of the HTS assay, only OT AR ORSROW 0960 a luciferase protein-fragment complementation assay measuring AR binding to cofactor SRC1 delected by microscopy in HeLA cells following 24 hours exposure showed activity with an C50 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 15.320M.

In conclosion, regative result on 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 bioactivit. AR model detailing an absence of androgen effects is not deemed sufficient to address the A-modarity. Consequently both Level 2 and Level 3 studies have been conducted to conclusively address this endpoint.

Results







| | | | | Spiroxamine |
|----------------------------|--|--|--|---------------------|
| | | ************************************** | - G G | ind and |
| Table CA 5.8.3/04-1: Ov | verview of ToxCast models and data output for the A-modality | aie, | | |
| ToxCast model | Assay description | ♥A / A-Aa | Chosen model and outputs 🧠 🖒 | L' Figure |
| Data retrieved from US El | PA Endocrine: EDSP universe of chemicals website ashboard/dsstoxdb/results?abbreviation=EDSPUOC&scarch=I The publications associated with the ToxCast AR Pathway 200 | & ° | | e figure |
| (https://comptox.epa.gov/d | lashboard/dsstoxdb/results?abbreviation=EDSPUOC&scarch=I |)T&SID1034 | <u> 22#detail</u> 912 Augн | Chr. |
| ToxCast bioactivity AR | The publications associated with the ToxCast AR Pathway | A/A-A- | No and the control of the state | 1 C A 5 0222 /O A 1 |
| model | Model is described in Kleinstreuer et al | | So androgen antagonis Octivity C | CA 3-825/04-1 |
| | (2017) 10.1021/acs.chemrestox.6b00347 and firesente to the November 2017 FIEP A SAP under EPA | e ⁾ a. ` | | |
| | November 2017 FIFRA SAP under EPA-HS-OPP-26 7-0214 | 1,50 | | |
| NVS_NR_hAR | Receptor binding assay conducted in thuman extracted gene- proteins from LNCaP human prostate adenocarcinoma cell line receptor-ligand binding of the key ligand \(\frac{1}{3}\text{H}\)- | AXXXXXX | Constant model. AIC \$1.79; RMSE: 5.08; AC ₅₀ : n/a Cytotoxicity \$15.32 μM | CA 5.8.3/04-2 |
| | proteins from LNCaP human prostate adenocarcinoma cell line | ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ | 5.08; A© ₅₀ : n/a Cytotoxicity 15.32 μM | |
| | receptor-ligand binding of the key ligand [4]- | | | |
| | methyltrienolone] to the fuman and fogen receptor | | | |
| OT_AR_ARELUC_AG_1 | Luciferase protein-fragment complementation assay measuring | A/A-A | Constant model (NTC: 126.63; RMSE: | CA 5.8.3/04-3 |
| 440 | AR binding to the androgen response doment detected in CHO- | 201 | 7.24; AC ₅₀ : ^(a) a Cytotoxicity: 15.32 μM | |
| | K1 cells by apperoscopy following \$24 h exposure | | | |
| OT_AR_ARSRC1_0480 | Luciferase protein-tragment complementation assay measuring O | A/A-A | Constant model AIC: 94.02; RMSE: | CA 5.8.3/04-4 |
| | AR building to cofactor SROI detected by microscopy in HOLA | | Σ98; AC n/a Cytotoxicity: 15.32 μM | |
| | cells following a 8 h exposure | A/A-A | | |
| OT_AR_ARSRC1_0960 | Luciferase protein fragment complementation assay measuring | A/A-A | Hill model: AIC: 110; RMSE: 20.27; | CA 5.8.3/04-5 |
| | AR binding to coffactor SOC1 detected by microscopy in HeLA | e. | Top: 2.23; AC ₅₀ 36.05 μM; | |
| | cells following a 16 thexposure | | Cytotoxicity: 15.32 μM | |
| Tox21_AR_BLA_Agonist | Inducable AR transcription factor activity detected by B- | A | Constant model. AIC: 251.68; RIVISE: | CA 5.8.3/04-6 |
| _ratio | lactamase androgen response element reporter in agonis (mode | | 4.05; AC ₅₀ : n/a; Cytotoxicity: 15.32 | |
| | ersing HEK293T, a furman kidney cell line following a 24 h | | μΜ | |
| | exposure | | | |
| Tox21_AR_BLA_Artagon | Inducible AR transcription factor activity detected by B | A | Constant model. AIC: 302.79; RMSE: | CA 5.8.3/04-7 |
| ist_ratio | lactamase androgen response tement reporter in anagonist | | 7.35; AC ₅₀ : n/a Cytotoxicity: 15.32 μM | |
| | mode using HEK293T, a human kidney cell line following a 24 | | | |
| | Rexposure & Direction of the Control | | | |
| Tox21_AR_LUC_MD | Inductible AR transcription actor activate detected by luciferase- | Α | Constant model. AIC: 140.55; RMSE: | CA 5.8.3/04-8 |
| B2_Agonist | androgen response element report in agonist mode using MDA cells following a 24th exposure. | | 1.16; AC ₅₀ : n/a Cytotoxicity: 15.32 μM | |
| Tox21_AR_OUC_MDOR | Inducible AR transcription factor activity detected by luciferase- | A | Constant model. AIC: 293.65; RMSE: | CA 5.8.3/04-9 |
| B2_Antagonist | androgen reports in antagonist mode using | | 6.7; AC ₅₀ : n/a Cytotoxicity: 15.32 μM | |
| | MDA cells following a 24 h exposure | | | |

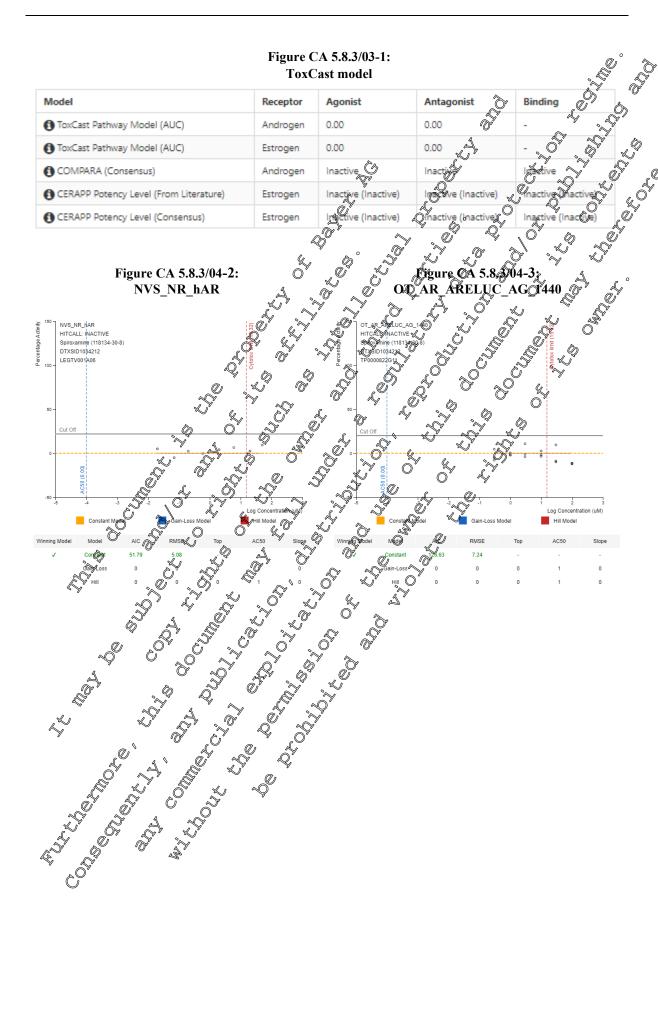


| | | | e and |
|--------------------------|---|----------|--|
| ToxCast model | Assay description | A/A-Aª | Chosen model and outputs Figure |
| UPITT_HCI_U2OS_AR_ | Receptor binding assay conducted in U2OS, a human | A | Constant model. AIC: 139.28; RMSE: CAS8.3/04-10 |
| TIF2_Nucleoli_Agonist | osteosarcoma cell line, expressing a human androgen receptor | A JEY | 7.01; AGO n/a Cytotoxicity: λ5.32 μM |
| | fused to red fluorescent protein and the coactivator TIF2 fused | | |
| | to green fluorescent protein in agonist mode. Cells exposure or | | |
| | 3 h. | | or is a contraction of the contr |
| UPITT_HCI_U2OS_AR_ | Receptor binding assay conducted in U2OS, a human | A CO | Constant model. AIC: 15 C 15; RMSD: CA 58.3/04-11 |
| TIF2_Nucleoli_Antagonist | osteosarcoma cell line, expressing a human and gen receptor | 1 2 2 | 10.55; ACS. n/a Cytotoxicity: |
| | fused to red fluorescent protein and the coachivator TIP2 fused | e" | 15.32 PM 30 30 30 30 30 30 30 30 30 30 30 30 30 |
| | to green fluorescent protein in antagonia mode. Cells exposure | | 15.32 4471 |
| | for 3 h. | K. T. H. | |

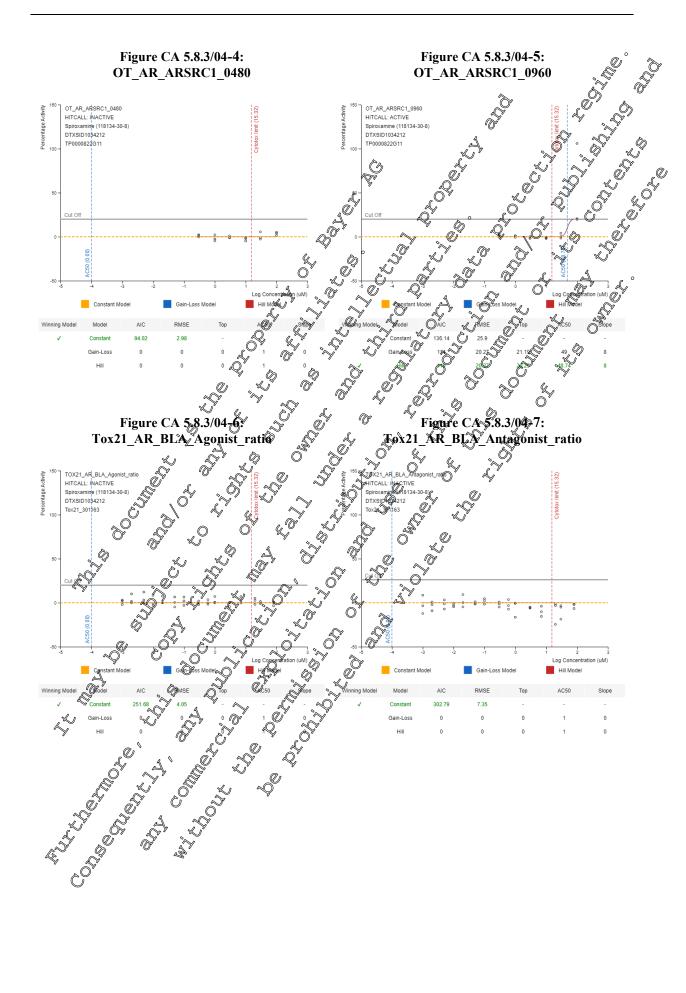
any commercial exploitation and violate the rights of its owner be prohibited and violate the prohibited any publication of the owner of this document and use of this document and use of this document and use of the owner of th the permission of the owner the rights of its owner.

be prohibited and violate the prohibited and violate. a: androgen (A) or anti-androgen (A-A) signal detection
AIC: Akaike Information criteria: appropriate model set on the lowest AIC value, essential prodel that he share RMSE: median root mean squared error across all winning models. RMSE is lower far bright performance models.

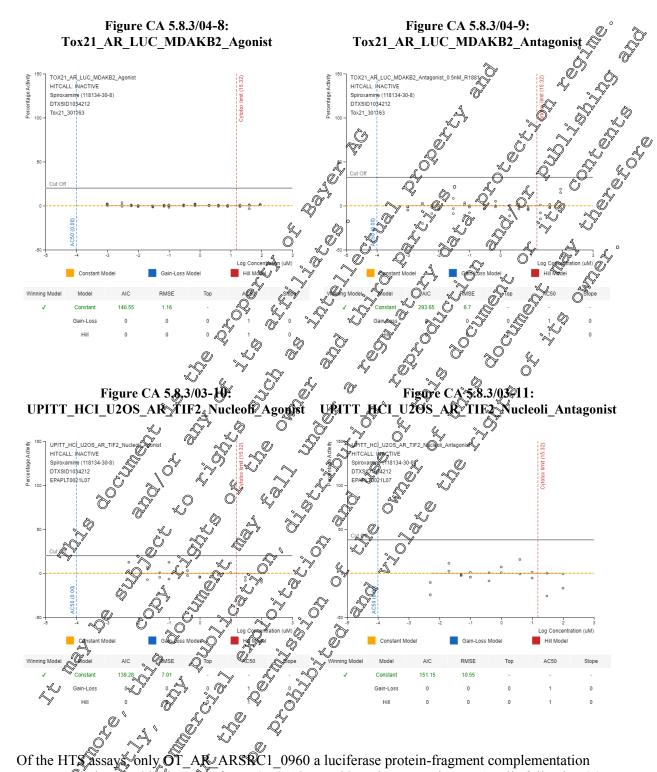












Of the HTS assays only OT_AR_ARSRC1_0960 a luciferase protein-fragment complementation assay measuring AR binding to cofactor SRC1 detected by microscopy in HeLA cells following 24 hours showed activity with an AC_{50} value of 36.05 μ M obtained. However, the biological relevance of this is low as activity was observed only at a concentration that occurred in the presence of cytotoxic, which was observed at 15.32 μ M.

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 absorbe 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: | |
| Report Year: | |
| Report Title: | Evaluation of the androgen receptor agonor and antagonist activity of |
| | spiroxamine using the stably transfected human androgon receptor transcriptional |
| | detivation assay (The Leosergen) |
| Report No: | 20249038 Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q Q |
| Document No: | M-761548-01-1 |
| Guideline(s) followed in | LOECD (C) (2020) |
| study: | |
| Deviations from current | None of the second of the seco |
| test guideline: | |
| Previous evaluation: | (No, not Previously subunitted |
| | |
| GLP/Officially | Yes conducted under GLOOfficially recognised testing facilities |
| recognised testing | |
| GLP/Officially recognised testing facilities: | |
| Acceptability/Rehability\ | Yes, The second |

Executive Summary

In an *in viffo* 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 agenist array experiments spiroxamine was tested at seven concentrations ranging from 10 pM to 3.16 logether with vehicle controls, positive controls and complete concentration-response curves of the control items 50 dihydrotestosterone (DHT), mestanolone and di(2-ethylhexyl)phthalate (DETIP).

All assay acceptability critera 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 difficient 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 IC₃₀ could be determined for spiroxamine. As such, spiroxamine concluded to be devoid of AR antagonist activity, in vitro

It is concluded that spiroxamine did no show evidence of either and ogen receptor agonistor antagonist activity in the Stably Transfected Human Androgen Receptor Transcriptional Activation Assay (APP EcoScreenTM) following two independent experiments. Maximum concentrations analysed were limited by solubility, in line with current regulatory guidelines for this assa

Materials and Methods

A. Materials:

1. Test Material: Spiroxamine

dioxaspiro[4,5]decane-2-methanamine: K

Description:

Lot/Batch No.:

97.0% (w/w) (correction for purity not undertain **Purity:**

CAS No.:

Stability of test compound:

2. Vehicle and/or pos control:

Negative control

AR agomst and antagorist lhexyl)-phthalate (DEHP, 10, 100 (pM), 1, 10, 100 (nM)

Positive compols:

5α-dihydrotestösteröne (DHT, 100 M, 1, 10, 100 pM, 1, 10, 100 nM);

7g-methykandrostan-17-2501-3-one (mestanolone, 100 fM, 1, 10, 100 pM, 1,

200 μg/mL Zeocin.

O hydroxyflutarnide (μ) , 100 μM, 1, 10, 100 nM, 1, 10 μM),

bisphenol (BPA) 100 pM, 1, 10, 100 nM, 1, 10 μM)

ARECOScreen Cell king, derived from CHO-K1 cell line stably transfected with homan Alexer expression vector and a firefly luciferase reporter vector bearing four tandem epeats of androgen responsive element (ARE) from prostate Cogene-responsive element driven by a minimal heat shock protein Momotor In addition, a Renilla luciferase reporter construct under the SV40 promotor, states and non-inducibly expressed, is transfected as to distinguish pure antagonism from a cytotoxicity-related decrease of luciferase activity. The cet line was sourced from Health Science Research Resources Bank (HSRRB), 🌠 saka Japan and stored frozen.

Cells were regularly checked for mycoplasma contamination.

Dulbecco's Modified Eagle Medium/Ham's F12 nutrient mix (DMEM/F12) conditions: without phenol red, supplemented with 5% fetal bovine serum (FBS), 100 U/mL Penicillin, 100 µg/mL Streptomycin, 100 µg/mL Hygromycin B and



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.

AR agonist assay: 0, 10, 100 (pM), 1, 10, 100 (nM), 1, 2.16 μM 6. Test concentrations:

AR antagonist assay: 0, 100 pM, 1, 10, 100 (nM)

B. Test Performance:

1. In life dates:

2. Vehicle selection:

The test article was soluble in DMSO at a concentration of 1 M (inspection under the microscope) Mowever, a 10-fold dilument of 1 M (inspection under the microscope) Mowever. under the microscope). However, a 10-fold dilugion of this 1 Molution and @ 316 mM, 100 mM, 31.6 mM and 10 mM in exposure medium resulted in precipitation of the test article. A 10-rold dilution of a 3.160mM 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 man experiments (final concentration in the well: 176 µMP baseck 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:

The mean luciferase activity of the positive control (P nM PHT) was ≥64-fold that of the mean yehicle control or each colate.

b) The fold induction corresponding to the RC10 value of the concurrent positive control PCAGO 10 nM DHT) was greater that 1+2SD of the fold mduction value of the concurrent vehicle control (which is set at 1).

(i.e. The co-officient 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 sigmoidad."

(e) The results of the three control items were within the acceptable range and elassified in the correct class i.e., negative or positive for androgenic

| | A 9 | | | |
|---|----------------|---|-----------------------------|----------------------------|
| , | Control Item | Log PC ₁₀ | Log PC ₅₀ (M) | Purpose of Control Item |
| | DHO, K | ©-12.08 [©] -9.87 _© | -11.03 ~ - 9.00 | Positive |
| | Mestanoloffe > | -10 9 2~-10 9 1 | -10.15 ~ -9.26 | Positive |
| C | Фенру 🦠 | <u>-</u> ~ | - | Negative |

f) The results obtained were reproducible in at least one independent repeated

ÄŘ^vant**ag**onist akšáy: 🎺

Considered acceptable if the results meet the following criteria:

a) In the antagoni@assay the fold induction of AG ref should be ≥ 5.0 .

The RAA of C_{ATG} (1 μ M HF) was \leq 46%.

c) The CV of the raw data of triplicate wells (i.e., luminescence intensity data) was∜20%.

The shape of the concentration-response curve of positive control items was sigmoidal.

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 | Log PC ₅₀ | Purpose of Control |
|--------------|-------------------------------|-------------------------------|--------------------|
| | (M) | (M) | Item |
| HF | - 8.37 ∼ - 6.41 | - 7.80 ∼ - 6.17 | Positive |



| BPA | -7.52 ~ -4.48 | -7.05 ~ -4.29 | Positive |
|------|---------------|---------------|------------|
| DEHP | - | - | Negative Q |

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 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 out of three independent experiments.
- A test article was considered negative if the maximum response induced by the test article (RPC ax) failed to achieve at least 10% of the response of the positive control (30 nM DHT) in at least two out of two or two out o three independent experiments.

AR antagonist assay.

- A test article was considered positive a log 100 30 color be calculated in two out of two or two out of three experiments.
- A test article was considered negative if no log IQ0 can be calculated in two out of two out of three experiments.

C. Methods:

1. Homogeneity and achieved concentration analysis of the dose:

2. AR agonist assay:

Not undertaken

AR-EcoScreta Mccoll were seeded (at 0.9×404 cells 90 µL well) into a 96-well plate and cultured for 24 boars prior to treatment. Subsequently cells exposed for 24 hours to negative OMSQ, DEHIQ positive controls for AR agonism (DHT prestantione), the positive control for cytotoxicity (cycloheximide), or seventest article concentrations all included on each plate.

The uciferase activity was determined using the Steady-Glo Luciferase assay Ostem to evaluate AR mediate otranscriptional activation. After exposure, 40 μLoof Steady-Glo roagent was added to each well of the 96-well plate, The plate was shaken and incubated for the least 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).

AR EcoScreen™ cells were seed of (at 0.9×10⁴ cells/90 µL/well) into a 96-well plate and cultured for 24 pours poor to treatment. Subsequently cells exposed of 24 hours to regative (DMSO, DEHP), positive controls for AR antagonism (BPAOHF) AR Ogonist (DHT), the positive control for cytotoxicity (cyclohexiloride), or six test article concentrations all included on each plate. The luciferase activity was determined using the Dual-Glo luciferase assay to avaluate AR-prediated transcriptional inhibition and cytotoxicity

simultaneously.

After incuration, #0 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 lumprometer. After the measurements, 60 µL of the medium was removed and 40 L 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

Cytotoxicity was determined by reduction of the Renilla luciferase activity (in the AR antagonist assay). The cytotoxicity was calculated using the following formula:

3. AR antagonis@as

a) cototoxicity assay:



Cytotoxicity (%) =
$$100 - \frac{(RLU \text{ of each well } - \text{Mean RLU of PC}_{CT})}{(Mean RLU \text{ of AG ref} - \text{Mean RLU of PC}_{CT})} * 100$$

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 control 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 of control item the mean value of transcriptional activity was calculated.

Fold-induction of PCAGO were be calculated using the following equation:

For each test and control term, the maximum level of response induced by the test item expressed as a percentage against the response by PC $_{\rm GO}$ on the same plate (RPC $_{\rm max}$) was calculated. If applicable, the concentrations that induce an effect corresponding to that of a 10% effect of the positive control (log PC $_{\rm 10}$) and to 50% effect for the positive control (log PC $_{\rm 30}$) was determined.

b) AR antagonist assay:

The mean RLU values of the vehicle controls was carefulated 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) RLO values for the spike-in control (500 pM DB) 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 practiciency test item.

The fold induction of AG reforms calculated by the following equation:

The RCA of RCATG (%) was alculated by the following equation:

The concentrations of 30% inhibition of transcriptional activity induced by 500 pM DHT (log IC₃₀) and, if appropriate, to 50% inhibition of activity of 500 pM DHT (log IC₅₀) was calculated for each positive control and test item. Fach log IC_x value was calculated using the following equation:

$$\lim_{x \to a} ICx = \frac{a - (b - (100 - x)) \times (a - c)}{(b - d)}$$

Results and Discussion

A. Analytical determinations

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

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}), PC₁₀ 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 PC₁₀ value was higher than the fold induction of vehicle control +2SD and therefore these acceptance criteria were met.

acceptance criteria, however, since these values were lower than the acceptance criteria (PC_{10} and PC_{50} responses at lower concentration with the acceptance criteria (PC_{10} and PC_{50} responses at lower concentration with the acceptance criteria (PC_{10} and PC_{50} responses at lower concentration with the acceptance criteria (PC_{10} and PC_{50} responses at lower concentration with the acceptance criteria (PC_{10} and PC_{50} responses at lower concentration with the acceptance criteria (PC_{10} and PC_{50} responses at lower concentration with the acceptance criteria (PC_{10} and PC_{50} responses at lower concentration with the acceptance criteria (PC_{10} and PC_{50} responses at lower concentration with the acceptance criteria (PC_{10} and PC_{50} responses at lower concentration with the acceptance criteria (PC_{10} and PC_{50} responses at lower concentration with the acceptance criteria (PC_{10} and PC_{50} responses at lower concentration with the acceptance criteria (PC_{10} and PC_{50} responses at lower concentration with the acceptance criteria (PC_{10} and PC_{50} responses at lower concentration with the acceptance criteria (PC_{10} and PC_{20} responses at lower concentration with the acceptance criteria (PC_{10} and PC_{20} responses at lower concentration with the acceptance criteria (PC_{10} and PC_{20} responses at lower concentration with the acceptance criteria (PC_{10} and PC_{20} responses at lower concentration with the acceptance criteria (PC_{10} and PC_{20} responses at lower concentration with the acceptance criteria (PC_{10} responses at lower concentration with the acceptance criteria (PC_{10} responses at lower concentration with the acceptance criteria (PC_{10} responses at lower concentration with the acceptance criteria (PC_{10} responses at lower concentration with the acceptance criteria (PC_{10} responses at lower concentration with the acceptance criteria (PC_{10} responses at l sufficient responsiveness and therefore these data were accepted. All other acceptability criteria were met acceptability criteria were met. The control items were correctly classified as positive (DHT and mestanolone) or negative (DEHP). Since a 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 PHT (the RPC nax) was 6.0%. Since the RPC nax values were below 10% spiroxamure was concluded negative in the AR amonist,

Data rejected due to a possible pipetting error in the mestanologie standard curve. Therefore, this data were not used in interpret the androgen gonist potentia Dof spiroxamine.

Spiroxamine? Cell from passage 18 were used. The mean (normalized) RLU values for the DHT 10 nM, the positive control (PC)GO), RC 10 and Sehicle control +2 standard deviations were 8.93, 1.79 and 1.05 for the reference plate and 8.85, 1.78 and 1405, respective of The fold induction of DHT corresponding to the PC₁₀ value was higher than the fold induction of vehicle control 25D and therefore these acceptance chaeria were met

The log PO00 values obtained for prestant one was outside the acceptance coteria however, since trese values were lower than the acceptance criteria (PC₅₀ (Sponses at lower concentrations) this indicated sufficient responsiveness and therefore these data well 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 Ale agonist experiment was considered whid, with both sensitivity and

ed vand, with demonstrated.

...sponses induced by spiroxan py 10 nM DHT (the RPC max) was -(
...cre below 16% spiroxamine was concluded n

...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses induced by spiroxan
...sponses indu The maximum level of response unduced by spiroxamine compared to the response induced by 10 nM DHT (the RPC_{max}) was -0.6%. Since the RPC_{max} value were below 10% spiroxamine was concluded negative in the AR agonist

Collectively, in two independent gonist experiments, spiroxamine did not Thoward evidence of androgen receptor agonist activity and therefore

2. Experiment 2:

3. Experiment 3:



| | | | | | | | Spiroxamine |
|--|--|----------------------------|--|--------------------------|--|---------------------------------------|-------------------------|
| Гable CA 5.8.3/05- | 1: Androgen receptor | and antagonist a | ctivity of spiroxamine: | agonist assay – P | eperiment 1 ve control data DEHP Alean ±SUP | A OTO | arthek data |
| | Positive con | trol data | | Negati | ve control date | l % Test | article data |
| | DHT | | estanolone | - Seguti | DEHP. | 0 1650 | · · · · · · · |
| Log conc. (M) | Mean ±SD | Log conc. (M) | Mean ±SD | Log conc | DEHP Mean ±SD | اء مار ا | iroxamine S Wean ±SD |
| 10-7 | 107.5 ±2.4 | 10-7 | 99.9 ±2.4 | 2 2 10-5 | Ø ±0.2 × Ø | , \Q\d{0}-5.5 \C | \$-Q4 ±0.5 |
| 10-8 | 101.1 ±0.8 | 10-8 | 99.3 ±3 5 | 10-6 | © 0.7 ±0 | 10-6.0 | -0.1 ±0.3 |
| 10-9 | 97.5 ±0.6 | 10-9 | 89.2 ±0.8 © | 2.402 | 0.21±0.1 | 102.0 | -0.7 ±0.1 |
| 10-10 | 55.1 ±1.3 | 10-10 | \$7.9 ±1.4 | 10-8 | \$\infty\$ | 10-8.0 | -0.3 ±0.4 |
| 10-11 | 1.7 ±0.4 | 10-11 | \$ 5.3 ±0.8 | 1959 | ~ 0.1,±0.3 × | 10-25 | 0.0 ± 0.3 |
| 10 ⁻¹² | -0.4 ±0.1 | 10 ⁻¹² | -04±0.2 | 20 -10 | +0.2 C | 10-10.0 | -0.8 ±0.1 |
| 10-13 | -0.3 ±0.4 | 10-13 | 0.1 ±0,20° | 10 ⁻¹¹ | 0-0.4 ±0.3 | 10-11.0 | -0.4 ±0.3 |
| RPC_{max} | 107.5 | RPCmax | 89.9° | RPC _{max} | er go | RPCmax | 0.0 |
| $PC_{50}(M)$ | 8.02E-11 | $\mathbb{P}C_{50}(M)$ | 305.48E-11 ○ | $PC_{50}(M)$ | | § *P C ₅₀ (M) | - |
| $PC_{10}\left(M\right)$ | 1.43E-11 | PC ₁₀ (M) | 1.19E-B1 | PC ₁₀ (M) | | $^{\flat\prime}$ PC ₁₀ (M) | - |
| $Log PC_{50}(M)$ | -10.10 | Logge E ₅₀ (M) | -10.29 | Log PC ₅₀ (M) | | $Log PC_{50}(M)$ | - |
| $Log PC_{10}(M)$ | -10.84 | Log PC ₁₀ (M) | <u>~~~10.93</u> ~~. | Oog PC ₁₀ (M) | - 15 | $Log PC_{10}(M)$ | - |
| Judgement | Positive | Judgerrent | S Positive | Judg@nent | Negative | Judgement | Negative |
| |] | old Induction of | the positive control, DI | IT obtained in Al | R agonist experiment 1 | 1 | |
| | Passage | | REAGO (16) pM DHT | | PC@(10 nM DHT) | Vehicle co | ontrol (DMSO) +2SD |
| Reference plate | P9 💆 | 4 | 8.96 | O | 1.80 | | 1.04 |
| Test article plate | 1 999 | | 9.030 | | 1.80 | 1077 | 1.02 |
| DHT: 5α-dihydrotest DEHP: bis-(2-ethylh | Positive Passage Positive Passage Positive Positive | expressed (10 m) De Ago: P | da a percentage to the response of the respons | wells treated with 10 | m, PC10: conc. of S induced by the PC50: conc. of S on M DHT induced by the | positive control (10: | of 50% of the response |
| | | | | | | | |



Androgen receptor and antagonist activity of spiroxamine: agonist assay – Experiment 3 Table CA 5.8.3/05-2:

| | Positive con | trol data | | Nogali | ve control data | . Tost article data |
|-----------------------|----------------|--|--|--------------------------|-------------------------------|--|
| 1 | DHT | | estanolone | Tregați | DEHP CONTOCULAR OF | Spirogamine |
| Log conc. (M) | Mean ±SD | Log conc. | Mean ±SD | Løg conc. | <i>6</i> 0 <i>f</i> | Log Conc (M) Mean +SD |
| Log cone: (ivi) | Witah LSD | (M) | Wieun 20D | Log conc. | Mean ±SD | |
| 10-5 | 7.0 ±0.4 | 10 ⁻⁵ | 14.2 ±0.1 | 100 | 111.9 \$1.1 | 19-5.5 84.5-40.9 |
| 10-6 | 2.3 ±0.4 | 10-6 | 77.8 ±2.5 | 10-6 | 100 ±0.7× 0 | \$\Q\delta^{6.0} \cdot\delta^{\text{\tint{\text{\tin}\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\text{\tex{\tex |
| 10-7 | 17.5 ±0.9 | 10-7 | 95.7 ±2.00 ° | 10-7 | | 10 ^{-7.0} 98.3 ±1.9 |
| 10-8 | 65.2 ±2.2 | 10-8 | 99,0 ±1.3 | · 40° ° | 102#±8.9 | 10 ^{8.0} 99.0 ±2.1 |
| 10-9 | 90.6 ±0.7 | 10-9 | 98.5 ±0.6 | 10-9 | ₩ 001.2 ±0.60 × | 98.8 ±1.1 |
| 10-10 | 93.0 ±1.3 | 10-10 | 96.4±1.0 | 10210 | - " | 101.0 ±3.5 |
| RPCmax | 93.0 | RPC _{max} | D 99.0 | RPC _{max} | 100.6%1.3 141.9 241.9 | RTA _{max} 101 |
| $PC_{50}(M)$ | 2.09E-08 | PC ₅₀ (MO) | 2.74E- 96 | PC50 (MI) | ~00 - ~211111 | PC50 (M) - |
| $PC_{10}(M)$ | 6.49E-09 | DC (NA) | 1 22E 06 | DC (NI) | 6.2 × 20 14.10 | $PC_{10}(M)$ - |
| $Log PC_{50}(M)$ | -7.68 | Log PC50 (M) | 30 ¥5.56 O ° ° | Log PC ₅₀ (M) | , & - 30 ^C | Lig PC ₅₀ (M) - |
| $Log PC_{10}(M)$ | -8.19 | Log PCMM) | -5,8 % | Log PC ₀ (M) | | $\operatorname{Log} \operatorname{PC}_{10}(M)$ - |
| Judgement | Positive | Judgement | Positive Office | Judgement | Negative O | Judgement Negative |
| | The I | old Induction of | f the positive control, DH | Tôbtained 🗑 🗚 | R agomist experiment 2 | |
| | Passage | | , & PCAGO (10 nM DH1) | | PC10 (10 nM DHT) | Vehicle control (DMSO) +2SD |
| Reference plate | P18 | | 8.93 | | 1.79 | 1.05 |
| Test article plate | P18 | | 885 | | 0.78 | 1.05 |
| DHT: 5α-dihydrotesto | sterone | RPC _{max} : | Maximum level of response | nduced a test ite | PC ₁₀ : conc. of S | PX with a response of 10% of the response |
| DEHP: bis-(2-ethylhe: | xyl)-phthalate | expresse | d as a percentage to the respo | ns@nduced by PCA | AGO induced by the p | PX with a response of 50% of the response |
| | | PCACO: I | Province AR and nist control v | vells treated with 10 | nM DHT induced by the r | positive control (10 nM DHT) |
| | The Third | O T CAGO. | Stative Area appliest control, v | vensured with re | mudeed by the p | ositive control (10 livi D111) |
| | I | | 10 30 3 | , | | |
| | | | | | | |
| | - 1 | al "a " | | | | |
| | ~ (°) | The state of the s | TO THE STATE OF TH | | | |
| | | ~ C > ~ (C) | | | | |
| | | | | | | |
| , e | | | | | | |
| | | | | | | |
| F | | | | | | |
| AC | | | | | | |
| | | | | | | |
| | | | Positive The positive control, DH PCAGO (10 nM DH) 8.93 8.83 Waximum leyel of response at as a percentage to the response | | | |
| | | | | | | |
| | | | | | | |
| | | | | | | |



C. Androgen antagonist assay:

1. Experiment 1:

Cell from passage 9 were used. The RTA value for the positive control, HF 346% and the fold-induction of the AG agonist and <46% and the fold-induction of the AG agonist reference ($\searrow G$ ref) was ≥ 5 therefore these acceptance criteria were met.

The log IC₃₀ and log IC₅₀ values for HF, BPA and DEHP were within the acceptability criteria and the control items were correctly classified possible (HF and BPA) or negative (DEHP) in the AR antagonist assay. Since all acceptance criteria were met, the AR antagonist@xperiment was considered valid, with both sensitivity and specify for and specify activity demonstrated.

Since no log IC₃₀ and no log IC₅₀ could be determined for spiroximine, 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 net.

The log IC30 and log IC30 values for IJE, BPA and DESP were within the acceptability criteria and the control tiems were correctly classified as positive (HF and BPA) or negative (DEHP) in the AR antagonist ssay. Since all acceptance criteria were niet, the AR antigonist experiment was considered valid, with both sensitivity and specification and ogen antagonist activity

demonstrated Since no log IC₃₀ and no log IC₅₀ could be determined for spirovarnine, spiroxagnine was concluded negative in the AR antagogist experiment. ©Collectively, in two independent antagonist experiments, spiroxamine did not show any evidence of AR anyagonist activity and therefore concluded to be

...g the Renilla fuciferase
...g the Renilla fuciferase
...g (20% reflection of the 100 pM H
...g any of the control items. In addition, the
...g two instances whose considered assay variatio.
...acity, the to the fact that it was observed at the lowest
...acity, the to the fact that it was observed at the lowest
...acity, the to the fact that it was observed at the lowest
...acity, the to the fact that it was observed at the lowest
...acity, the to the fact that it was observed at the lowest
...acity, the to the fact that it was observed at the lowest
...acity, the to the fact that it was observed at the lowest
...acity, the to the fact that it was observed at the lowest
...acity, the to the fact that it was observed at the lowest
...acity, the to the fact that it was observed at the lowest
...acity, the to the fact that it was observed at the lowest
...acity, the to the fact that it was observed at the lowest
...acity, the to the fact that it was observed at the lowest
...acity, the to the fact that it was observed at the lowest
...acity, the to the fact that it was observed at the lowest
...acity, the to the fact that it was observed at the lowest
...acity, the to the fact that it was observed at the lowest
...acity, the to the fact that it was observed at the lowest
...acity, the to the fact that it was observed at the lowest
...acity, the constitution to the fact that it was observed at the lowest
...acity, the to the fact that it was observed at the lowest
...acity, the constitution the fact that it was observed at the lowest
...acity, the constitution the fact that it was observed at the lowest
...acity, the constitution the fact that it was observed at the lowest
...acity, the constitution the fact that it was observed at the lowest
...acity, the constitution the fact that it was observed at the lowest
...acity the constitution the fact that it was observed at the lowest
...acity the constitution the fact that it was observed at the lowest
...acity the constitution the lowest constitution the lowest con Cytoto Courty was evaluated by determining the Renilla Juciferase activity in the two Are antagonist experiments. With exception to the 100 pM HF and DEHP in experiment 2, no cytotoxicity (>20% refluction of the Renilla luciferase activity) was observed for any of the control items. In addition, the observed soytotoxicity at these two instances whose considered assay variation, and not

Sobserved for any of the lest item concentrations tested, with exception to the 100 m concentration in experiment 2. However, since this was the lowest tested concentration and doe to the fact that the effect was not dose dependent,



Androgen receptor and antagonist activity of spiroxamine: antagonist assay Experiment 1 Table CA 5.8.3/05-3:

| Positive control data | | | Negati | ive control data | Test | article data | |
|--|------------------------------|--------------------------|--|--|----------------------------------|--------------------------|---------------------------|
| | HF Risphenal A DEHP & Shiriy | | iroxamine S | | | | |
| Log conc. (M) | Mean ±SD | Log conc. | Mean ±SD | Log con (M) | Mean ±SD | A Gordon Control | Mean ±SD |
| | | (M) | Z. | (M) | | | |
| 10-5 | 4.7 ± 0.3 | 10-5 | 11.7 ±1.3 84.4 ± 10 | \$\\\^10^{-5} \(\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\ | 108.6 ±4.1× 0 | Q0-5.5 | 80.7 ±2.5 |
| 10-6 | 3.3 ± 0.6 | 10-6 | 84.4 ±1.9 | 10-6 | 301.9 ± 30° | | 94.0 ±1.5 |
| 10-7 | 48.5 ± 1.1 | 10-7 | 103.0 ₹0.9 🎺 | 0 ./F. | 1065 ±2.0 | 100 | 101.9 ±0.6 |
| 10-8 | 91.6 ± 1.4 | 10-8 | € 105.3 ±3.7 € | 10-8 | 004.8 ±n/x0 | 0 10-8.0 | 98.1 ±2.6 |
| 10-9 | 98.4 ± 3.1 | 10-9 | 1014±1.4 | 1009 | 0 103.2€1.6 | 10-20 | 98.4 ±1.6 |
| 10-10 | 97.7 ±1.5 | 10-10 | 105.7±1.4 | 10 -10 | 103.4±1.0 108.6-1 | 10-10.0 | 98.9 ±1.0 |
| RTA _{max} | 98.4 | RTA | 105.7 | RTA _{nants} | 108.60 | RTA | 101.9 |
| IC ₅₀ (M) | 9.21E-08 | IC (M) | 2.93E-06 | IC _® (M) | | $IC_{50}(M)$ | - |
| IC ₃₀ (M) | 3.17E-08 | C30 (M) | | $IC_{30}(M)$ | , 6 - 20° | ≥ © (M) | - |
| Log IC ₅₀ (M) | -7.04 | Log IC (M) | -5.50 | Log IC (M) | Wagative S | $\log IC_{50}(M)$ | - |
| $Log IC_{30} (M)$ | -7.50 | Logo E ₃₀ (M) | <u>-\$.80</u> | Log L ₃₀ (M) | | Log IC ₃₀ (M) | |
| Judgement | Positive | Judgement | Positive > | Judgemen * | ₩ Negative \$ | Judgement | Negative |
| | <u>F</u> | old Indaction of | the positive control, His | obtained in AR a | aptagonist experiment 1 | E 11: 1 // | 1 C C |
| D C 1 . | <u> Passage</u> | | RTA (%) OF PC | ATG (DMM HEX | | Fold induction | AG ref |
| Reference plate | P9 | | | | | 7.6 | |
| Test article plate | P9 F | D.T.A.O. | <u> </u> |) O ₃ | A.C. or C. A. or or in | 7.8 | CDIT) in the contract of |
| HF: hydroxyflutamide DEHP: bis-(2-ethylhe: | yyl) nhthalata a'i | RIACKE | lative transcription activity | Wyalls traced with | AG ref: Agonis a 500 pM assay | st reference (500 pM | of DHT) in the antagonist |
| DEIII . 015-(2-cillyllic. | xyr)-pinnalate | DHT and | Orange Air antagonist contro | gowens acada with | IC300 pivi assay | at 30% of max activ | itv |
| | | | | J. D. | IC ₅₀ : Inhibition | at 50% of max activi | |
| | | | | > | | | |
| | The Market | 4 P 6. | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | ine ine | | | | | |
| | Ch. Wer. Collins | | | | | | |
| | | N NO I | , | | | | |
| V | |) | | | | | |
| Ç | | | | | | | |
| | Ma | | | | | | |
| | | | Positive positive control, HE RTA (%) of PC Lative transcription or rivity ositive AR antegorist control µM of HE | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |

Spiroxamine



Androgen receptor and antagonist activity of spiroxamine: agonist assay – Experiment 2 Table CA 5.8.3/05-4:

| DIT | | Passa | σe | | RTA (%) | PC _{ATG} (1 μM HP) | Fold induction AC ref |
|---|-----------------------|----------------------|-----------------------|--|------------------------------------|--|---------------------------------------|
| $ \begin{array}{ c c c c c c c c c c c c c c c c c c c$ | 1 | | | | KIA | | Spirozomino |
| $\begin{array}{c ccccccccccccccccccccccccccccccccccc$ | | | | | Leg conc. | | Log Ponc (M) Mean +SD |
| $\begin{array}{c ccccccccccccccccccccccccccccccccccc$ | 208 001101 (112) | 11 10411 = 22 | | 1/1 / 411 = 52 | (M). | | |
| $\begin{array}{c ccccccccccccccccccccccccccccccccccc$ | 10-5 | 7.0 ± 0.4 | | 14.2 ±0.1 | 100 | 111.9 21.1 | 10-5.5 84.5 90.9 |
| $\begin{array}{c ccccccccccccccccccccccccccccccccccc$ | 10-6 | | 10-6 | | 1 2 210-0 | 1004.2 ±0.7× 0 | \$\Q\0'-6.0 \Q\0'-6.0 \\\$\Q\0'-6.1.8 |
| $\begin{array}{c ccccccccccccccccccccccccccccccccccc$ | 10-7 | 17.5 ±0.9 | 10-7 | 95.7 ±2.0°° (| 10-7 | 98.1 ±2.2 | 0 10-7.0 |
| $\begin{array}{c ccccccccccccccccccccccccccccccccccc$ | 10-8 | 65.2 ± 2.2 | 10-8 | 99,0 ₹.3 | · 40° ° | 10,201 -0.7 | 10 ^{28.0} 99.0 ±2.1 |
| RTA _{max} 93.0 RTA _{max} 99.0 RTA _{max} 101 IC ₅₀ (M) 2.09E-08 IC ₅₀ (M) 1.33E-06 IC ₅₀ (M) IC ₃₀ (M) - Log IC ₅₀ (M) 7.68 IC ₅₀ (M) 1.33E-06 IC ₅₀ (M) - Log IC ₅₀ (M) 7.68 IC ₅₀ (M) 1.33E-06 IC ₅₀ | | 90.6 ±0.7 | | \$\tag{98.5 \pm 0.6} | | | |
| RTA _{max} 93.0 RTA _{max} 99.0 SPA _{max} 11.9 RTA _{max} 101 IC ₅₀ (M) 2.09E-08 IC ₅₀ (M) 2.74E-90 IC ₅₀ (M) - IC ₅₀ (M) - Log IC ₅₀ (M) -7.68 Log IC ₅₀ (M) -5.88 Log IC ₅₀ (M) - IC ₅₀ (M) - Log IC ₅₀ (M) -7.68 Log IC ₅₀ (M) -5.88 Log IC ₅₀ (M) - IC ₅₀ (M) - Log IC ₅₀ (M) -8.19 Log IC ₅₀ (M) - IC ₅₀ (M) - Judgement Positive Judgement Positive Judgement Negative Judgement Negative Reference plate P18 Reference plate Refere | 10-10 | 93.0 ± 1.3 | 10-10 | 96.4±1.0 | | ~ 6 100.6 € i.3 ~ € | 10^{-100} 101.0 ± 3.5 |
| ICso (M) | RTA_{max} | 93.0 | RTA _{max} | D 9.0 A | A A A A | DE 2011.9 2011 | RTA _{max} 101 |
| Control Cont | IC ₅₀ (M) | 2.09E-08 | IC ₅₀ (MO) | | | | - IC ₅₀ (M) - |
| Log IC ₃₀ (M) -7.68 | $IC_{30}(M)$ | | | | | | IC ₃₀ (M) - |
| Log IC ₃₀ (M) -8.19 | $Log IC_{50}(M)$ | -7.68 | L62 1C50 (M) | \$.56 © ™ | L og IC ₅₀ (M) ✓ | , s - 20 ^C | Dog 1C50 (M) |
| Passage Positive Judgement Negative Judgement Negative | $Log IC_{30}(M)$ | -8.19 | OLog ICO(M) | -5.80 | Log IC ₀ (M) | | |
| Passage Pass | Judgement | Positive | Judgement | Positive V | Judgement | Negative 0" | Judgement Negative |
| Reference plate P18 S23 T.79 1.05 Test article plate P18 S.85 T.78 T.05 HF: hydroxyflutamide DEHP: bis-(2-ethylhexyl)-phthalate A DEHP: bis-(2-ethylhexyl)-ph | | M. Pr. I | old Induction of | the positive control, DH | Tobtained in A | R agonist experiment 2 | |
| Reference plate P18 | | Passage | | CAGO a) (10 nM DHY) | | PCOS ^D (10 ACM DHT) | VC +2SD |
| Test article plate HF: hydroxyflutamide DEHP: bis-(2-ethylhexyl)-phthalate DEHP: description of the plate | Reference plate | P18 | | 100° × 833° × | | 1.79 | |
| HF: hydroxyflutamide DEHP: bis-(2-ethylhexyl)-phthalate DEHP: bis-(2-ethylhexyl)-phthalate The phase of the physical physical and the physical phys | Test article plate | P18 💝 | | (8.85 @ ¹ | | L 1.78 | |
| | DEHP: bis-(2-ethylhe: | xyl)-phthalate 1 | RIAGE PATE: POHT, and | lative transcription activity ositive AR antagonist control of His | Wells treated with | AG ref: Agonist assay IC ₃₀ : Inhibition IC ₅₀ :Inhibition a | at 30% 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 Tuman Androgen Receptor Transcriptional Activation Assay (AR-EcoScreen™) following two independent experiments. Maximum concentrations analysed were limited by solubility in line with current regulators guidelines for this assay.

| Data Point: | |
|--------------------------------|---|
| Report Author: | RCA 5.8.3/06 |
| Report Year: | |
| Report Title: | A dose range-finding oral gavager toxicity study of spiroxamine in young adult |
| | male rats |
| Report No: | 00543028 2 |
| Document No: | M-70/1549-04-1 |
| Guideline(s) followed in | None & D & W & S |
| study: | |
| | Nones Company of the |
| test guideline: | |
| Previous evaluation: | No, not previously submitted |
| | |
| GLP/Officially | No, not conducted under GLP/Officially recognised testing facilities |
| recognised testing | |
| recognised testing facilities: | |
| Acceptability/Reliability: | Yes Q A S O Q |

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 adolf made Spregue Dawley rats (5 animals/group) were administered spiroxamine suspended in 1% methy cellulose orally via gavage for 7 consecutive days at dose levels of 0 (1% MC), 125, 250 and 500 mg/kg by 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 date 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, orchidocordidy becomized 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 mate 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-provyl-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 curation of the study

2. Vehicle and/or positive

control:

1% methylcellulose no

3. Test animals:

Species: Rat

Strain: Sprague 💯 Age at dosing:

Weight at dosing:

Source:

At least 1/3 days **Acclimation period:**

At least 1/3 days

Altromin® 1324 diet for rats and miss, ad libitum (except during prior to dosing with animals fasted overwight [ca 05 h]) Diet:

with animals fasted overright [ca.05 h])(4)

Water: Municipal water, ad librum Housing:

4. Environmental conditions

> Temperature: Humidity:

Air changes: **Photoperiod**:

B. Study Design

1. In life dates

2. Animal assignment and treatment:

June 2020 (experimental dates)

After an acclustatisation period rats were allocated to groups by randomisation to achieve Cimilar Droup mean body weights (i.e. body weight within ±20% of existing information available on spiroxamine confirming moderate acute rat oral pixicity (2D₅₀ ca. 500 mg/kg by ICA 5.3 1/01 M 2007 oral (ED₅₀ © 500 mg/kg bw [CA 5.2.1/01, M-007791-01-1]) the target ordan and Critical offects observed by spiroxamine are the liver and irritant Hects on the mucosal epithelium of the oesophagus and forestomach. No Specific neurowxic effects were found in acute and repeated-dose neurotoxicity studies, but an acute NOAEL of 10 mg/kg bw was obtained from the acute neorotoxicity 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 $\frac{3}{2}$ group.

Body weights and body weight changes were subjected to a parametric oneway 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. Description No stability, homogeneity or concentration analysis on the dose formulations were undertaken. formulations were administered to animals within 2 hour of dosing.

2. Observations:

3. Body weights: 4. Food consumption:

5. Water consumption: 6. Ophthalmological examination:

7. Preputial separation: 8. Haematology and clinical chemistry:

9. Urinalysis: 10. Organ weights:

Not conducted.

Not constituted.

Not constituted.

All animals were subjected to a complete gross necropsy, which included evaluation of the external surface of the brain, and the thoracic, abdominal, and pelvic cavities, inclusives lesions, if anywere collected and preserved any 10% NPT

nativities.

Years not undertaken for the all signs. 11. Sacrifice and pathology::

Results and discussion

A. Test article formulation analysis:

Test article formulation analysis was not undertaken for

B. Observations:

1. Clinical signs of toxicity:

Clinical signs of toxicity were limited to the 250 and 500 mg/kg bw/day dosage gonips. These clinical signs were evident of general toxicity, consisting of red material around the right eye a prostrate body, piloerection, cool extremities, and divated pupils (500 mg/kg bw/day). At 250 mg/kg bw/day brown material around the mogenital area at the daily examinations on the day of death/euthanasiaOA singOe of that was found dead was also noted with a cool Gody: Several were also noted to have hunched posture, yellow/orange diar bea, prostate body and or decreased activity during study days 2-4.

Alog in the 250 and 500 mg/kg/day groups were found dead or euthanised in 2. Mortality: extremis during days 2 4.

Not conducted 3. Ophthalmoscopic examination:

C. Body weight and body weight gain:

Statistically significantly Dower mean body weight gains or mean body weight losses were noted in the 250 and 500 mg/kg bw/day do age groups beginning on day 1 and continuing through to death or early euthanasia (or day 40); 3, respectively) compared to the control group. As a result, mean absolute body weights wer 6.3% 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 dowing 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

| | | | 7 (() | | |
|-------------|------|-----------------|----------------------------------|---------------------------------|---|
| Paramete | arc | | | kg bw/d) | |
| 1 ai ailiet | C1 S | 0 | 125 | 250 | V 300 2 |
| Body wt | Day | | | | |
| (g) | -1 | 252 ± 11.07 | 253 ±14.88 (10.3%) | 254£14.69 (†1%){ | 253(±18.82 0.2%) |
| | 0 | 263 ± 14.26 | 259 ±957 (\12%) | 261 ±15Ø8 (↓1%) | 201 ±200 1 (↓196) |
| | 1 | 269 ± 12.56 | 267 ±14.36 (11%) | 2 70 ±1,7×07 (↑00%) | 269 ±18.41 (†0.2%) |
| | 2 | 280 ± 13.35 | 274 16.61 22%) | [* 271£]7.74[** 3%) | 264 ±14.82 (16%) |
| | 3 | 287 ± 12.29 | 283 ±15.96 (\1%) | 26 9 ±16. 10 (↓6%) | ²⁴⁸ ≠8.35***↓14%)° |
| | 4 | 295 ± 14.07 | $289 \pm 16.49 (\downarrow 2\%)$ | 255 ±23.64** | |
| | 5 | 306 ± 16.80 | £,295 £\6.30 (↑ \%) | (14%) O | |
| | 6 | 309 ± 18.26 | 296 \$17.63 (44%) | | 7 ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ |
| | 7 | 322 ± 18.99 | 304 ±16.53 (\16%) | | |

** p < 0.01

all animals dead, no wailable data

D. Food consumption, food efficiency and water consumption:

1. Food consumption: Moan food cons

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 josses wired in these group during these same periods. For ther evaluation of food consumption data at 250 and 300 mg/kg bw/day was precluded due to the death or cuthanasia of all animals on or before days 4 and

3, respectively.

2. Food efficiency:

Not conducted.

3. Water consumption

"Pot conducted.

E. Blood and urinalysis:

1. Haematological findings:

Not conducted

2. Clinical chemistry findings:

Not Conducted

3. Urinalysis:

Not computed

F. Sacrifice and nathology:

1. Organ weight:

Not conducted.

2. Gross pathology:

No gross path-hogical findings were evident in animals that survived until the end of the treatmen period or in animals that were found dead or were killed *in*

3. Histopathology:

And conducted

G. Deficiencies:

None

H. Discussion:

Spiroxarome 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-764008-012]).

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 olerated by young adult male rats when administered once dolly 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 periodbertal, orchidoepididymecromized Sprague Dawley rats (CA 5.8.3/07 [M-76400%-01-1])

| Data Point: | KCA 5.8.3/60 |
|--------------------------------------|---|
| Report Author: | KCA 5.8.3/00 |
| Report Year: | |
| Report Title: | A hershberger assay of spirosamine administered of the peripular tal |
| | orchidoepididymeetomized rats |
| Report No: | 00543029 |
| Document No: | <u>94-764098-01</u> |
| Guideline(s) followed in | US EPA OPPTS 890 1400 (Oct 2009) |
| study: | OFCD Test Guideline 44 (Sep 2009) |
| Deviations from current | None State of the |
| test guideline: Previous evaluation: | |
| Previous evaluation: | No, Not previously submitted & & |
| GLP/Officially recognised testing | Yes, conducted ander GLD/Officially recognised testing facilities |
| recognised testing | |
| facilities: | |
| Acceptability/Reliability | Y |

Executive Summary

The androgenic, anti-anchogenic or 50 reductase inhibitory effects of spiroxamine were evaluated using the Hershberger, Assay Animals (cast ated by the supplied 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 proof 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 10 methol cellulose, with all animals receiving a single oral gavage dose at 0, 3105, 625, 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 prepionate 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 D: 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 congulating glands and fluid (SVCGs), Cowper's glands (COW), ventral prostate (VP) and glans penis (OP)) 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 dese 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 tratistically significant reductions in the mean weights of these tissues were observed thus showing constitution and specificity of the test system to detect androgenic and anti-androgenic effects.

Under the conditions of this study spiroxamine and 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/sg bw/day (deemed to be a maximum tolerated dose). Therefore, it is concluded that spiroxamine did not exhibit androgenic, auti-androgenic or 50 reductase inhibitory properties under the conditions of this study.

Materials and Methods

A. Materials:

1. Test Material: Spiroxamine

(alternative name: [8,1,1-diffethylethyl)-Nethyl-N-propyl-1,4-

dioxaspiro (5,5]decane-2-methanaonne; KWG 4168)

Description: Brown fluid

Lot/Batch No.: AE 134429341-07

Purity: 97.6% (w/w) (correction for purity not undertaken)

Stability of test Confirmed stable for the duration of the study (expiry date: 4 June 2021)

compound:

2. Vehicle and/or positive 1 methylcellulose/ testosterone propionate (TP, 0.2 mg/kg bw/day), flutamide

control: 🧳

3. Test animals:

Species: A Left Strain: Sprague Dawle

Age af dosing 7 weeks

Weight at Abssing: 4 229 – 283 g

Source:

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: Humidity:** Air changes:

Photoperiod:

B. Study Design:

1. In life dates:

2. Animal assignment and treatment:

26 June 2020 to 7 July 2020 (experimental does)

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 emean body weight would be rearly equal among all groups the day of administration. The study was splitting.

Phase I: and openic agriculty assess.

The test articles.

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 Dw. Additional sositive control groups were included in each phase of the study:

- oups were included in each phase of the study:

 Phase I: Lestostewne provionat Cadministered once daily for 10 consecutive Pays via subcutaneous injection at 0.20 mg/kg/gw/d, employin@a dose volume of 0. Dimg/kg bw 10°
- Q- Phase II: Fintamide administered once daily for to consecutive days via oral gavage at 3 mg/kg bw/dzemplosing a dose volume of sml/kg bw.
 - In addition, all animal on this phase of the study also were received testosterone propionate administered once Quily for 10 consecutive days via subcataneous injection at 0.2 mg/kg bw/d, employing a dose volume of ILS mL/kg bw to screen for potential and and and senic effects and 5α-

reductase inhibitory effects with animals were euthanized and underwent peropsy with selected organs weighed 24 h post the final dose.

Cipical observations and other proportional data were analysed using the Wariance Test for Homogeneity of the Binomial Distribution.

Continuous data (e.g. body weights, body weight changes, and feed concernption values, were analysed using Bartlett's Test of Homogeneity of Variances and the ANOVA, when appropriate [i.e. Bartlett's Test was not Fignificant $(p \ge 0.001)$ of the ANOVA was significant $(p \ge 0.05)$, Dunnett's Test was used to identify the statistical significance of the individual groups. If the NOV was not appropriate [i.e., Bartlett's Test was significant (p=0.001)], the Karskal-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 Malysis of Covagance (ANCOVA), using terminal body weight as the

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 pressed 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 ($p \le 0.05$) 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 ($p \le 0.05$) 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 V for a tissue has an inverse relationship with statistical power, therefore performance criteria are based on maximum CV value for each rissue weighed. The maximum allowable CV determined are as follows:

| Tissue 🔎 | Anti-Androgenic Effects | Androgenic Feets |
|---------------------|---|------------------|
| Seminal vesicles | &° 540% & 4 | 0 40% V |
| Ventral prostate | © \$\frac{1}{2} 40\hat{3}\frac{1}{2} \text{\$\infty} | 45% |
| LABC muscle group | 20-00 | 030% Q 7 A |
| Bulboureth al gland | 35% | 55% |
| Glan@penis ~ | , O ~ \$17%, O & S | 22 |

Where negative outcomes were concluded. CVs from the control group and high dost 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. 0, 0.05 to 0.10) then a study topeat was undertaken

5. Evaluation criteria:

Evitence of androgen agoldst activity: &

A statistically significant increase (p=0.05) if any two or more of the five and gen-dependent issue weights (VP, LABC, GP, CG, SVCG) with some degree of increased growth combined explication of all accessory sex organs. Evidence of and agent and agent and agent approximation of the combined explication of the combined explication of the combined explication.

A statistically significant reduction \$\int 0.05\text{yin any two or more of the five and open-dependent tissue weights (VP, KABC, GP, CG, SVCG) relative to

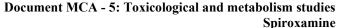
The treatment alone with some degree of reduced growth.

Table CA 5.8.3/07-1: Overview of the Horshberger study in young castrated adult male rats treated orally (via garage) with spirogramin@ doses received

| <i></i> | | | | | |
|-------------------------|---|-----------------|------------------|-----------------|------------------|
| @. | | | ♂ (mg/kg bw/day) | ı | |
| Parameters 0 | | 31.25 | 62.5 | 125 | Positive control |
| Phase I — androgeni | c activity | | Ĵ | | |
| Animak | ~ 6 ~ ~ | | 6 | 6 | 6 |
| assigned/sex | | | | | |
| Dose volume | \$\frac{1}{2}\frac{1}{2 | Q 5 👸 | 5 | 5 | TP: |
| (mL/kg bw) _₪ | | O Z | | | 0.2 |
| Phase II – anti-andro | ogenic activity | ¥ 4 | | | |
| | | & 6 | 6 | 6 | 6 |
| assigned/sex | | | | | |
| Dose volume | 5 ^{TP} | 5 ^{TP} | 5 ^{TP} | 5 ^{TP} | Flutamide/TP: |
| (mL/kg/bw) | | | | | 3/0.2 |

TP: textoster to proprovate 0.4 mg/kg bw/d administered *via* subcutaneous injection once daily from study days 1 to 10 Fluramide; 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 Do MCA Section). Therefore, stability and resuspension homogeneity at test article formulation will not be assessed on this study.

2. Observations:

3. Body weights:

Cage side observations, including mortality, cliffical signs of toxicity were conducted twice daily, with post dose observations conducted at 5 hours. Detailed clinical observations were recorded daily for all anomals.

4. Food consumption: Recorded daily during sing
5. Water consumption: Not conducted.
6. Ophthalmological
Not conducted.

examination:
7. Haematology and

7. Haematology and clinical chemistry: 8. Urinalysis:

9. Organ weights:

10. Sacrifice and pathology:

Not conducted.

Blood was collected attermination, with servin separated and frozen for further

analysis if required.

Recorded daily during dosing.

Brain, Jodney, liver, prostate (ventral) semina vesicles (including coagulating glands), levator ani plus bulbocaversosus muscle complex (CABC), Cowper's gland (bulbourethral gland) glans penis

gland (bulbourethral gland) glans penis

Conducted on PND 71. Gross pathological examination was performed on all canimals and included Camination of the thorse is abdominal and pelvic viscera.

The following tissues were preserved in 10% neutral buffered formalin for subsequent histopathological examination.

Kidney over, postate wentrally seminal vesicos (including coagulating glands) Cowper's gland, gross lesions.

For animals that died prior to scheduled satisfice (including all vehicle control animals) the following additional tissue were sampled: oesophagus, heart, lung, spleen stomach traches

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 1.19% of nominal concentrations, with %RSD values of 1.5 to 2.6% which were within acceptable limits.

B. Observations:

1. Clinical signs of Rhase & II

toxicity: No test article related effects were observed.

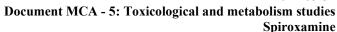
2. Mortality: Physe I & M:

animals survived until the scheduled necropsy

3. Ophthalooscopic 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 comprehable 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. shared concurrent vehicle control group throughout the study. In addition, mean body weight changes in the flutamide/TP positive control group were comparable to the group throughout the study. The state of the s



Overview of the Hershberger study in young castrated adult male rats treated orally (via gavage) with spiroxamine: Phase I Table CA 5.8.3/07-2: activity, body weight effects

| | | | | | 4 // | . (2) |
|--------------------------------|------|---------------------------|---------------------------------------|--|---|--|
| Parameters | | | | ∰g/kg bw/d) | | |
| rarameters | | 0 | 31.25 | 62. 5 \$ ° | \$ ° 125 | (P (0.2) |
| Body wt (g) | Day | | | | | |
| [% change in bwt] | -1 | $208 \pm 18.94 (9.10)$ | 204 ±26.30 [\\dig 2.00\%] (12.90) | 206 ±19.77 (\0.75%) (9.58) | 208 ±19.94 [\0.26%] (961) | 200°±21.94 [40°28%] (10.61) |
| (CV) | 0 | $214 \pm 17.24 (8.05)$ | 210 ±27.74 [↓1.89%] (13 🐠 | 214 19.24 [10.05%] (8.99) | 213 ±26.88 [↓0.78%] (9.83) | 212 ±25 83 [1.07%] (12.19) |
| | 1 | $221 \pm 18.56 (8.39)$ | 216 ±28.67 [\\2.64%]\(\sqrt{\\3.30}\) | 22 ±21.14 (20.11%) (2.54) | 210 19.64 [1409%] (8,95) | 220 ±34.63 [↓0.68%] (11.21) |
| | 2 | $229 \pm 17.66 (7.72)$ | | ©229 ±216221↑0.21%1€9.44) | 225 ±18.44 [\$1.68%] (8.20) | 223.48 [↓0.65%] (10.33) |
| | 3 | $233 \pm 17.56 (7.53)$ | 228 ±28.48 [4 22 9%] (1249) | 234 ±23.20 [↑0.30%] (9.91) | Ž29,± 15 87 [↓1.81%] (8.24) | 234 ±21.92 [↑0.07%] (9.39) |
| | 4 | $242 \pm 18.36 (7.60)$ | 236 ±28,55 [2.39%] (Î2.11) | 247 ±22.15 [\0.24%] (939) | 233, 217.34 [13,40%] (7.42) | 242 ±21.03 [↑0.04%] (8.70) |
| | 5 | $247 \pm 16.66 (6.76)$ | 242 ±27.90 [↓1.96%] (11.54) | , 245 ±21(38 [\0.66%] \8.89) , | 237 ±16.49 (\$3.98%] (696) | 24 9°±19.69 [↑1.12%] (7.90) |
| | 6 | $254 \pm 16.56 (6.53)$ | 247±26.61 [\2.46%] (10.76) | 253 ±21.82 [↓04P%] (8.64) (| 242 ±16.29 [↓4.69%] (6.74) | $255 \pm 19.44 \ [\downarrow 0.40\%] \ (7.64)$ |
| | 7 | 262 ±16.05 (6.13) | 255 ±26,42 2.40%] (\$0.35) | 258 ±22.24 [\$44%] (8.63) | 24££14.11 [J6£9%] (5.78) | 264 ±18.98 [↑1.06%] (7.17) |
| | 8 | 267 ±16.24 (6.08) | 262 ±25.79 [↓2,1258] (9.86) (| 264 ±20 3 9 [\1.14% \(\mathbb{E} \) \(\mathbb{E} \).69) | 247 ±13.060 7.57%] (5.29) | 274 ±20.80 [†2.63%] (7.59) |
| | 9 | 271 ±14.00 (5.17) | <u>1</u> 267 ±24.49 [3031 %] (9.18) | 265 £21.80 [↓2.33%] (8.24) | | $278 \pm 18.22 \ [\uparrow 2.66\%] \ (6.55)$ |
| | 10 | 278 ±15.84 (5. ©) | 271 ±26 1 (2.37%) (3.62) | ±22.35 [1.83%] (% [9) | 249 \(\frac{1}{2}\).69 [\(\frac{1}{2}\)10.48%] (5.10) | 283 ±20.05 [↑1.73%] (7.09) |
| Body wt gain (g) | 0-10 | 63.9 ±8.28 (12.96) | 61.3 ±11.04 [\4.0%] (18.01) | 58.9 ±3 9 [7.82%] (40.14) | 36.4 ±19.73 Q43.04%] (54.21)* | 71.0 ±6.28 [↑11.11%] (8.85) |
| [% change in bwt] ^a | | | | | r jg | |
| (CV) | | | | | | |

^{*} $p \le 0.05$ relative to the concurrent vehicle control group.

Overview of the Hershberger study in young castrated adult male rate treated of ally (via gavage) with spiroxamine: Phase II, anti-androgenic vity, body weight effects Table CA 5.8.3/07-3: activity, body weight effects

| Danamatana | | | C | | ি (ক্মুঞ্ছিkg bw/d) | | | | | |
|-------------------|----------|--------------------------|---------------------|--|--|--|---|--|--|--|
| Parameters | | BUILDE | 0 % | 3C25/TP (0.20) | 62.STP (0.2) | 125/TP (0.2) | Flutamide/TP (3/0.2) | | | |
| Body wt (g) | Day(| | . 6 | | | | | | | |
| [% change in bwt] | -1 | 208 ±1 | 8.94 (9.10) | 209 ±18.09 (30) (80) | 200±24.86 [†1.01%] (12.02) | 208 ±19.46 [†0.46%] (9.35) | 212 ±26.00 [↑2.21%] (11.91) | | | |
| (CV) | 0 | 214 ± 1 | 7.24(8.05) | | ②214 ±25.05 [↑1.60%] (11.71) | $216 \pm 19.02 [\uparrow 1.94\%] (8.81)$ | $218 \pm 26.00 \ [\uparrow 3.08\%] \ (11.91)$ | | | |
| | 1 | 221 ±1 | 8.56 (8.39) | 223 ±19.93 [↑\$36%] (8.94) | 223 ±26.35 [↑0.24%] (11.80) | $223 \pm 20.13 \ [\uparrow 1.61\%] \ (9.01)$ | 225 ±27.20 [†2.43%] (12.08) | | | |
| | 2 | 22941 | 7.66 (7. 7@) | 200 ±19.34 [\$1.35%] (8.46) | 228 ±25.64 [↑0.89%] (11.26) | $228 \pm 20.21 \ [\uparrow 0.22\%] \ (8.87)$ | 233 ±26.93 [†2.31%] (11.59) | | | |
| | 3 | 233 ±1 | 7,56 (7.53) | . C 239 ±19.64 [↑2.46%] (8.21) | 236 ±25.87 [↑1.07%] (10.98) | $234 \pm 19.09 \ [\uparrow 0.16\%] \ (8.16)$ | 242 ±26.37 [†3.48%] (10.91) | | | |
| | 4 @ | ∑\$ 242 ⇒ 1 | \$.36 (7.60) € | 247 ±20.71 [12.09%] (8.40) | 244 ±28.03 [↑0.94%] (11.48) | $240 \pm 19.86 \ [\downarrow 0.57\%] \ (8.27)$ | 247 ±26.93 [†2.20%] (10.91) | | | |
| | 1 3 D | 24₹ ±1 | 6.66 (60%) | \$34 ±19.60 [\P.989%] (7.71) | $252 \pm 27.21 \ [\uparrow 2.4\%] \ (10.81)$ | $245 \pm 18.17 \ [\downarrow 1.72\%] \ (7.41)$ | 258 ±27.82 [†3.38%] (10.79) | | | |
| | 6 | 254 ±1 | 6.56 (6.53) | 261 ±23\(\text{26} [\frac{1}{2}.70\(\text{70} \)] (8.86) | 261 ±28.07 [†1.40%] (10.77) | $249 \pm 18.61 [\downarrow 2.23] (7.48)$ | 263 ±26.98 [†3.16%] (10.27) | | | |
| | 7 . @ | >/ [□] > 262 ±1 | 6.05 (6.13) | 269 €21.07 [↑1.83%] (7.83) | $268 \pm 29.27 [\downarrow 0.65\%] (10.92)$ | $255 \pm 18.27 [\downarrow 3.66\%] (7.18)$ | $272 \pm 28.32 \uparrow 2.86\% (10.42)$ | | | |



| | | | | | | . I |
|--------------------------------|------|--------------------------|--|------------------------------|---------------------------------------|----------------------------|
| | | | | | ā and | ajule. |
| Danian et ana | | | | ් (mg/kg bw/d)ූ | | |
| Parameters | | 0 | 31.25/TP (0.2) | 62.5/TP (0.2) | 1255 (10.2) | Flutamide (DP (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 (\$\) [\[\] 5.50%] (6.33) | 278 ±26.67 [1.27%] (9.60) |
| | 9 | $271 \pm 14.00 (5.17)$ | $282 \pm 19.89 [\uparrow 1.21\%] (7.06)$ | 279 ±29.96 (10.73) | 258 46.31 [17.17%] (6.31) | 280 ±23.60 [↑0,79%] (8.42) |
| | 10 | $278 \pm 15.84 (5.70)$ | 289 ±23.87 [†2.22%] (8.26) | 284 ±29.9©(*0.52%) (10.53) | 265 ±15.01 [\dd(1.27\%] \(5,66) | 286 ±27.07 [1.24%] (9.45) |
| Body wt gain (g) | 0-10 | $61.3 \pm 11.04 (18.01)$ | 76.1 ±8.89 [↑7.18%] (11.68) | 70,3 49.99 [\1.41,6] (14.21) | 49.2 ±9&F [↓30.70%P18.87)* | 68.0 ±5.60 [\\ \] (8.23) |
| [% change in bwt] ^a | | | | | l . E ~ . F | |
| (CV) | | | | | | |

The probability of the probabili and violate the rights of the problem that the problem to the prob



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:

P positive control, winistered alone or nee criteria range form The %CV for all weights from the ehicle ontrol TP positive control, flutamide/TP positive control and 125 mg/kg/day (administered alone on in conjunction with TP) groups were within the performance criteria range for androgenic Canti-androgenic effects, except for the following:

- The %Co for the weight of the glans points in all groups range from 22.66% to 41.47% and exceeded the maximum values in the performance criteria for both androgenic (22%) and anti-androgenic (17%) offects
- The %CV for the weight of the wintral prostate in the varicle control group 131.73%) exceeded the maximum value in the operformance crueria.
 - The assay however was still considered acceptable for the collowing reasons: All other Volves for androgenic or anti-androgenic effects in the versicle control, it positive conool, flotamide/TP positive control and \$25 mc/kg/day (administered alone of contraction with TP) groups were within the performance criteria range.
- The CV for the ventral prostate on the ventral group was within the historical control range of %CV values for the conducting laboratory (11.2%

- Mean weights of the five androgen-dependent tissues (levator ani-Dulbocavernosus (LABO), servinal vesucles with coagulating glands and fluid Y(SV@Gs), Cowper's Wands (COW) Wentral prostate (VP) and glans penis (GF) in the three sproxamone dose groups did not differ statistically from the respective vehicle control group values.
- The mean weights of the adressal 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 with the body weights recorded on which weights recorded on DS Tof study as the covariate, the mean tissue weight values did not differ statistically from the vehicle control grows at statistically from the vehicle control group values.
 - Soefficients 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).
- three s, we white the control of the The mean weights of the adrenal gland in the three sproxamine dose groups did not differ statistically from the respective vehicle control group values



Overview of the Hershberger study in young castrated adult male rats treated orally (via gavage) with spiroxamine: Phase I, **Table CA 5.8.3/07-3:** activity organ weights

| | | | % 2 | | |
|----------|--|--|--|---|---------------------------------------|
| | | | (mg/kg bw/day) | | |
| ameters | 0 | 31.25 | 62.50 | \$ ° 125 € | (C) (C) TP |
| ls | 6 | 6 | | 2 0 2 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 | 6.0 |
| (g) | 278 ±15.84 | 271 ±26.11 [↓2.37%] €** | 278 22.35 [1283%] | \$\\^249 \pm\12\69 \[\]\10.48\%] | <u>~</u> 283 ±20 ⊙ 3 [↑1.73%] |
| Abs (mg) | 51.4 ±6.95 | 53.4 ±6.95 [↑3æ%] | \$\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\ | 588 ±7.51 [104!33%] & | 52,6% 4.58 [\1.49%] |
| C.V | 13.52 | 13.01 | ×9.66, ~ 0 | 1278 | 8.71 |
| Abs (mg) | 1812.7 ± 140.03 | 1716 ±192.12\$ | ¥800.6 ±19₹.29 | 1727.2 10.85 [18.71%] | ♥₹933.3 ±91.38 [↑6.65%] |
| C.V | 7.73 | \[\\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ | \$ [10.87%] | 6,42 | 4.73 |
| | | 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 | 10.85 | | ac ° |
| Abs (g) | 10.9 ±0.67 € | 10.2 ∉1.71 [↓6.43%] | 10.5 ±0.775 \$2.86%] | 9,2,90.77 [115,84%] | 10.9 ±0.81 [\dot0.52%] |
| C.V | 6.11 | 20 16.67 ° | 7.35 | 830° ON | 7.45 |
| Abs (mg) | | [164.0 ±18,22 [\ 1.58%] [| 151.8 429.16 [4.8.91%] | O 127.6 ±€1.13 [↓23.\$1%] | $371.8 \pm 123.13^*$ |
| C.V | 930 | 11.72 | 19,21 | 16.56 | [†123.13%] |
| | · · · · · · · · · · · · · · · · · · · | | OFF KILL | \$ 6 F | 19.98 |
| Abs (mg) | 70.1 ±11.72 | 79.4 ± 2 3.59 [↑13.37%] | \[\ \frac{747}{2} ±8.80 \[\frac{1}{2} 6.64 \] \] | 74.7±16.97 [†6.66%] | 327.2 ±48.13* [↑366.98%] |
| C.V | 6.11 | O 29.70 | 25.10 | 22.70 | 14.71 |
| Abs (mg) | 6.5 ±1.78 C | 3.9 ±3.31 49.44% | 5.8 \$1.84 [\11. 99 %] | 5.5 ±1.46 [↓15.82%] | $28.6 \pm 6.79^* [\uparrow 123.13\%]$ |
| C.V | 27,32) | 56.00 | 31,93 | 26.50 | 23.79 |
| Abs (mg) | 2000 ±20.32 | [_18.6 ±13013 [₽6.93%] _~ | 13.8 ±6.82 [↓30.85%] | 74.7 ±16.97 [\pm12.70%] | 112.0 ±27.62* [†461.40%] |
| C.V | 51.73 | 50!77 € | 25.16 | 22.70 | 24.66 |
| Abs (mg) | 108.4.042.69 | [81.8°±24.49 [J249.32%] , | \$\times 6.0 ±24.5\times [\left\11.42\times] | 86.1 ±28.73 [\\dig 20.57\%] | 153.8 ±63.79 [↑41.90%] |
| _ C.V | 39.39 | 29-94 | 25.58 | 33.37 | 41.47 |
| | Abs (mg) C.V Abs (mg) C.V Abs (g) C.V Abs (mg) | Abs (mg) C.V | S | S 6 6 6 6 6 6 6 6 6 | S |

^{*} p <0.05 increase to concurrent vehicle control group a. shared control with and open antagonist assay.

SVCGs: seminal vesicles with fluids and coagulation gland

a. shared control with androgen antagonist assay.

% difference of test article groups and positive control relative to the obscurrent vehicle control. SVCGs: seminal vesicles with fluids a Values in bold exceeded maximum allowable CV for respective organ.

TP: testosterone propionate 0.2 mg/kg/bw/d administered via subcutaneous injection one daily from study days 1 to 10

Abs.: absolute

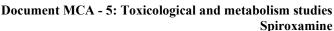
C.V.: coefficient of variation.

COW: Cowper's gland VP: ventral prostate GP: glans penis



Overview of the Hershberger study in young castrated adult male rats treated orany (via gavage) with spiroxamine: Phase II anti-androgenic Table CA 5.8.3/07-4: activity organ weights

| | | | | 7 (0 0 1 / 1 | | | |
|----------------|--|------------------------------|------------------------------------|--|---|---|--|
| Parameters | | 0.2 | 21.05 | (mg/kg bw/day) | | | |
| | | O ^a | 31.25 | 62.5 | 125 | Flutarnade/TP | |
| No. of anima | | 6 | 6 | | 60000 | 6 | |
| Terminal bw | \ U / | 278 ±15.84 | 289 ±23.87 [†2.22%] | 284-29.92 [1052%] | 265 ±15®1 [↓6.27%] ✓ | 286 ±27.07 (2) 1.24%] | |
| Adrenals | Abs (mg) | 51.4 ± 6.95 | 47.0 ±5.35 [↓10.6⊕%] | \$\$7.8 ±4.47 €9.15% } | 52:40±7.38.[\n0\044%] | © 51.8 € 9.07 [↓1.49%] | |
| | C.V | 13.52 | 11.35 | \$ \\ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ | 3°° 1409' | 17.50 | |
| Kidney | Abs (mg) | 1812.7 ± 140.03 | 1925.8 ±142.42 [\dot0.39\circ] | 1896 D±220.41 JJ 1.93% J | 1829.3 ± [45.14 [↓5. 3 8%] | 1.6 ±90.68* [\5.26%] | |
| | C.V | 7.73 | × 7.40, × 9 | 10.63 OV | 7.94 | 90.68 | |
| Liver | Abs (g) | 10.9 ± 0.67 | 40.9 ±1.33 [†0.28%] | 11 3 ±1.69 [1 2. 27 %] | 9.8 ±0.85 [\10.09% | $11.0 \pm 0.82 [\uparrow 1.50\%]$ | |
| | C.V | 0.11 | (1)1 / | 2 15 10 20° | 8.65 | 7.42 | |
| LABC | Abs (mg) | 166.6 ±15.61 | 398 (±48.68 [3 D. 18%] | 388.5 ±84.54 [↑4,49%] | 336.7 ±24 35 (16.77%) | 232.2 ±29.56* [\J37.55%] | |
| | C.V | 9.37 | 12,22 | 21.762 | O .97 O | 12.73 | |
| SVCGs | Abs (mg) | 70.1 ± 11 © 2 | 368.4 \$109.15 [1258%] | 342.8 ±55.09 [↑4.78%] | 318 ©±46.44 × 2.27%] | $94.4 \pm 14.09^* [\downarrow 71.14\%]$ | |
| | C.V | (OF) 3,1 | 29.6E | 16.07 | L - (C17.32 | 14.92 | |
| COW | Abs (mg) | €.5 ±1.78 | 33.1 ±10.86 [†16.05%] | 27.8 ±3.68 [\$2.57%] | 32.2 🗗 .27 [†12.84%] | 9.5 ±1.83* [\\displays 66.61\%] | |
| | C.V | 2722 | 30.36 | 3.23 K | \$ 13.27 | 19.18 | |
| VP | Abs (mg) | 20.0 ±20.32 51.73 | \$21.3 ±44 (20) (8.32%) | 138.0±8.35 [12\$60%] | \$\frac{1}{47.3} ±22.07 [↑31.47%] | $31.8 \pm 12.49^* [\downarrow 71.65\%]$ | |
| | C.V | 51.73 | 36.86 | \$35 \$ 35 | 14.99 | 39.35 | |
| GP | Abs (mg) | 108.432.69 | 175@£59.43 [\$\displaysing 196%]_(| D 136.6 ±4€.52 [↑116.8%] | 165.2 ±42.62 [↑7.32%] | $123.6 \pm 28.01 $ [$\downarrow 19.64\%$] | |
| | C.V | 39.39 | 33.90 | 30.39 | 25.81 | 22.66 | |
| * p <0.05 incr | rease to TP control gro | oup of open and of | | LABC LABC ator ani-bulbocave | | | |
| % difference | VP | | | | | | |
| control | control Supposed positive control and positive control and positive to provide a suppositive control and coagulation grand | | | | | | |
| Flutamide: 3 1 | mg/kg bw/d administe | red via oral ga@ge once 🐠 fr | om stad days 1 to 00 a | VP: ventral prostate | | | |
| Abs.: absolute | | | | GP: glans penis | | | |
| Rel.: relative | (to body weight) | | | | | | |
| C.V.: coeffici | C.V.: coefficient of variation | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | | | | | | |
| | | ~ | | | | | |
| | | | | | | | |





2. Gross pathology: No test article related effects were observed in animals from either phase of the

Not conducted. 3. Histopathology:

G. Deficiencies:

None.

H. Discussion:

The androgenic, anti-androgenic or 5α-reductase inhibitory effects of sorroxamine 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 artimals beceiving a single of 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.

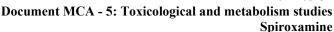
- Phase I: Testosterone propionate administered once daily for 10 consecutive days was subcutaneous injection at 0.4 mg/kg bw/day employing a dose volume of 0.5 mg/kg bw
- Phase II: Flutamide administered once daily for 00 consecutive days 0ia or gavage at 3 mg/kg bw/day, employing a dose folume of 5 mg/kg bw.

There were no significant increases or decreases in the mean weights of the live androgen-dependent tissues (LABC, SVCGs, COW, VP and CP) in the three spiroxaming dose groups from either Phase I (androgenic effects) or Phase II (anti-androgenic effects). Ô

Assessment and conclusions by applicant:

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

and the ania study spiroxa organ weights in the communities of 125 mg/kg to the constitution properties under the Conclusion: Inder the conditions of this study spirox amine 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 tose of \$\text{925 mg/kg b} day (deemed to be a maximum) tolerated dose). Therefore, it is concluded that spiroxamine did not exhibit androgenic, antiandrogenic or 5α-reductase inhibitory properties under the conditions of this study.





T-modality

| Data Point: | KCA 5.8.3/08 |
|----------------------------|--|
| Report Author: | |
| Report Year: | 2021 |
| Report Title: | Spiroxamine: US EPA ToxCast data retrieval for the T-modality |
| Report No: | 0471836-TOX7 |
| Document No: | <u>M-762778-01-1</u> |
| Guideline(s) followed in | None Q Q Q |
| study: | |
| Deviations from current | None A Q 6° A Q Q |
| test guideline: | |
| Previous evaluation: | No, not previously submitted of the subm |
| | |
| GLP/Officially | not applicable |
| recognised testing | |
| facilities: | |
| Acceptability/Reliability: | Supportive only & & & O |

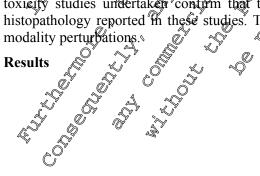
Executive Summary

Whilst it is recognised that the Tox Cast/Tox21 creening assays for the major molecular initiating events involved in thyroid perturbation (Na-I symported modulation TPO release, transport or thyroid hormones, T4 to T3 conversion by deiodoases, 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

Of the HTS assays two models confirmed thyroid activity which require further discussion. The LTEA HepaRG PHRSP dn, a metabolically competent HepaRG coll culture mode examining alterations in transcription activity with an AC_{50} yave of 0.33 μM obtained. Tox21 TR LUC GAS Artagonist, an inducible endogenous hyroid hormone receptor transcription factor activity detected by luciferase-fitsion response element in rat pituitary gland GH3 cell line as regulated by the human THR-wand TMR-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 cytojoxic, which was observed at 15.32 μM.

A computational network model based on ToxCast/Fox21 data has not been developed for the thyroid.

In conclusion negative results in all HTS VR-associated assays provides a strong mechanistic argument against a direct interaction of spitoxamine with thyroid or anti-thyroid pathways. In accordance with the ECHA guidance on endocrine disruption, the Tox Cast 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 Tmodality perturbations.





| | | | | Spiroxamine |
|--------------------------------|---|------------|---|---------------|
| | | | - G | Sajius . |
| Cable CA 5.8.3/08-1: Ov | erview of ToxCast models and data output for the T-modality | | | |
| ToxCast model | Assay description | T/A-Ta | Chosen model and outputs . § | Figure |
| Data retrieved from US EF | PA Endocrine: EDSP universe of chemicals website ashboard/dsstoxdb/results?abbreviation=EDSPUOC&search=D | T&\$D10343 | Aldetails 612 Augus 7020 | |
| ATG THRa1 TRANS dn | Inducible TR transcription activity detected with fluorescences | T AU | Country and delight C. 14.75 BMCE. | CA 5:83/08-1 |
| o_1111.u1_11011.10_u11 | intensity signals by RT-PCR of mRNA reporter sequence | | 25; AC ₅ Pa; Cytotoxicity: 15.62 | CA 3,825/08-1 |
| ATG_THRa1_TRANS_up | exogenous transcription factor GAL4-THRW [known@s human thyroid hormone receptor, a]) in HepQL liver cells exposed for | T | Constant model AIC: 14.75, RMSF. O.75; AC. 31/a; Cytos xicity: 15.32 | CA 5.8.3/08-2 |
| | | A (Ob) | μM | |
| LTEA_HepaRG_THRSP_ dn | Metabolically-competent HeparG cell outures were exposed for 48 h before cytotoxicity (LDH) and alterations in | T | Gom-loss model: AIC: 20:79; RMSC: 0:31; ACS 0:33 µM Cytotoxicity: | CA 5.8.3/08-3 |
| LTEA_HepaRG_THRSP_ up | transcription were assessed. and the sounder assessed and of the sounder assessed and the sounder assessed as the sounder assessed. | T | Constant model. AIC: 49.19; RMSE: 0.86; AO ₃₀ : n/a; Cylotoxicity: | CA 5.8.3/08-4 |
| Tox21 TR LUC GH3 A | In the ble endogenous there id hormone recentor transcription | т 🖟 | 15. 3 μM | CA 5.8.3/08-5 |
| gonist | factor activity detected by luciferase-fusion response alternent in | FF of | AC ₅ O uM; Cytotoxicity: 15.32 μM | CA 3.8.3/08-3 |
| | rat pituitary gland SH3 cell fine as regulated by the ruman THR-α and THR-β in agons to mode for 28 h. | | Ţ ¹⁹ | |
| Tox21_TR_LUC_GH3_A | Inducible and ogenous thyroid hormone receptor transcription | A-T | Hill model: AIC: 297.15; RMSE: | CA 5.8.3/08-6 |
| ntagonist | factor activity detected by the iterase-tosion response element in rat pituitary and GH3 well line as regulated by the human |)Č | 16.67; AC ₅₀ : 66.56 uM; Cytotoxicity: 15.32 μM | |
| TOWAL TOUR A | FHR-α and THR-β (in antagon) mode for 28 h | T | C | CA 5.0.2/00.7 |
| TOX21_TSHR_Agonist_r | Inducible reporter gene examining mapping to the TSHR gore in HEK293 P (human kidney cell line) through increased cAMP | 1 | Constant model. AIC: 307.8; RMSE: 8.15; AC ₅₀ : n/a; Cytotoxicity: | CA 5.8.3/08-7 |
| TOYAL TOUR | generation in agonist mode rows.5 h | A . T. | 15.32 μΜ | G 4 |
| TOX21_TSHR_Anagonist _ratio | Inducible reporter gene examining mapping to the TSHR gene in HEK2937 (human cidney call line) through increased cAMP generation in autagonist mode for 0.500 | 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_@no | Inducible TR franscription activity detected with fluorescence | A-T | Constant model. AIC: 265.03; RMSE: | CA 5.8.3/08-9 |
| Entry. Sedie | intensity signals by targeting background cAMP generation in cells lacking TSHR in HEK293T (human kidney cell line) for | | 4.47; AC ₅₀ : n/a; Cytotoxicity: 15.32 μM | |

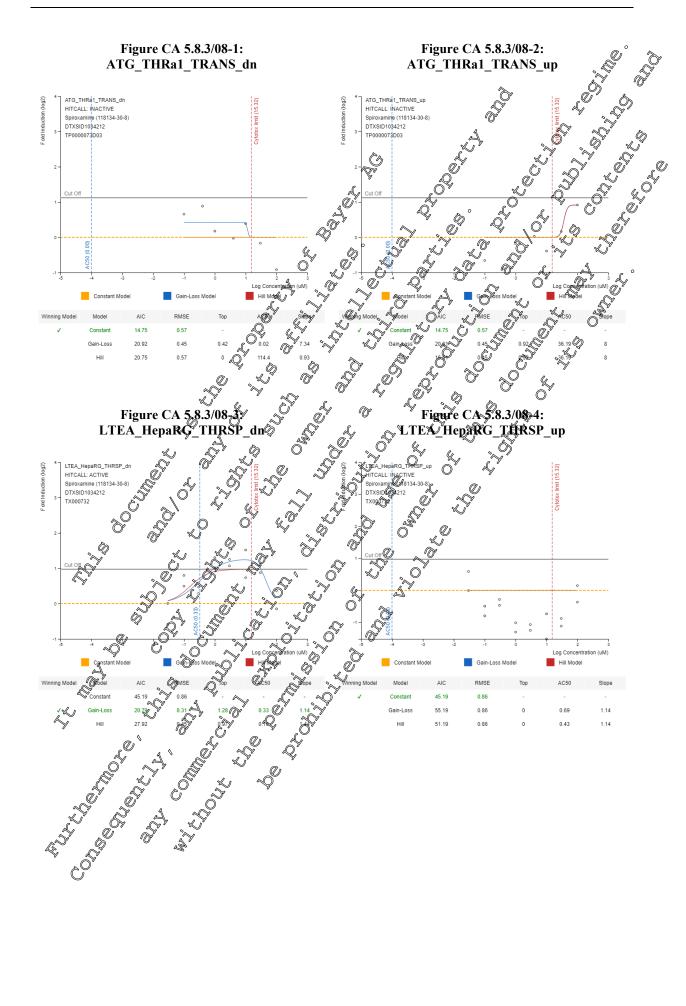
a: thyroid (T) or and thyroid (A-T) signal detection



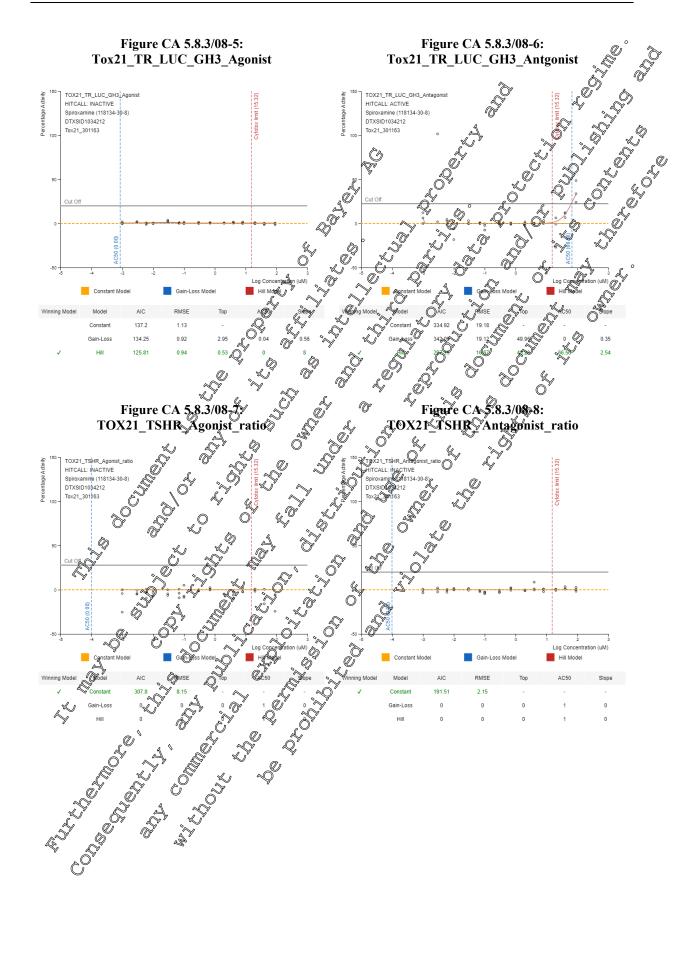
and third parties and third parties and or min laning and third parties and or min laning and cory and or min laning and third parties and or min laning and third parties and or min laning and third parties and or min laning and third parties and third parties and third parties and third parties and third parties and the parties and third parties and third parties and third parties and third parties and third parties and third parties and third parties and third parties and third parties and third parties and third parties and third parties and third parties and third parties and third parties and third parties and the parties and and or any of its such as intellectual property and third parties.

and to rights owner and third parties. Furthermore, this document is a regulated by the state of this document of the state of the stat 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. and withhout the prohibited and violate the rights of an and violate the rights.

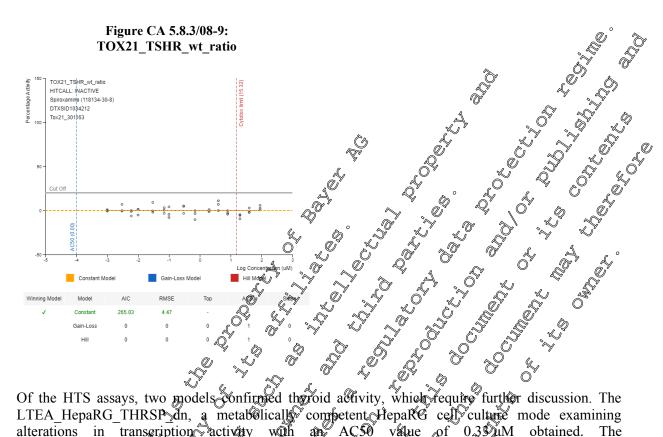












LTEA_HepaRG_THRSP_dn, a metabolically competent HepaRG cell culture mode examining alterations in transcription activity with an ACD value of 0.3 DiM obtained. The Tox21_TR_LUC_GHP_Antagonist an inducible endogenous thyroid hormone receptor transcription factor activity detected by Jucifethse-fusion response lement in rat pituitary gland GH3 cell line as regulated by the human PHR-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 Tox Cast/Tox 21 data has not been developed for the thyroid.

Assessment and conclusions by applicant:

Assessment: This study is copsidered supplemental only.

Conclusion: In conclusion regative results in all HTS OR-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 of endocrine disruption, the ToxCast HTS TRassociated assays detailing an absence of the roid effects is not deemed sufficient to address the Tno adverse has no adverse has devoid of modality. However the apical mainmalian 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

| | o was a second of the second o |
|----------------------------|--|
| Data Point: | KCA 5.8.3/09 |
| Report Author: | |
| Report Year: | 2021 |
| Report Title: | Spiroxamine: US EPA ToxCast data retrieval for the s-modality |
| Report No: | 0471836-TOX8 |
| Document No: | <u>M-762780-01-1</u> |
| Guideline(s) followed in | None Q Q Q |
| study: | |
| Deviations from current | None A Q 6° A A C |
| test guideline: | |
| Previous evaluation: | No, not previously submitted or the subm |
| GLP/Officially | not applicable |
| recognised testing | |
| facilities: | |
| Acceptability/Reliability: | Supportive on the supportive on the supportive on the supportive on the supportive on the supportive on the supportive on the supportive on the supportive on the supportive on the supportion of the support of the sup |

Executive Summary

From the US EPA ToxCast data, of the HTS assays, 6 models confirmed steroidogenesis perturbations, which require further discussion. Of the mineralocorricoids two assays Σ EET 0X H295R DOC dn (CA 5.8.3/09-2) and CEETOX H295R PROG dn Figure CA 5.8.3/09-4) confirmed AC₅₀ values of 0.77 μ M and 0.37 μ M, 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 glucocorpeoids, CEFTOX H295R 11DCORT dn (Figure CA 5.8.3/09-8) and CEETOX_H295R OHPROG_dn (Figure CA 5.8.3/09-12), confirmed AC50 values of 0.18 μ M and 0.15 μ M, respectively. Both models utilise H295 cells with CEETOX_H295R_11DCORT_dn examining the onhibition of 11-de-exycortisol and CEETOX_H295R_OHPROG_dn examining the inhibition of inhibit $\sqrt[3]{\alpha}$ -hydroxyprogesterone.

The androgen model, CFETOX_H293R_ANDR_dn, examining the inhibit androstenedione in H295 cells, with an AC_{50} value of 0.2 μ M. CEETOX_H295R_TESTO dn, examining the inhibit testosterone in H295 cells confirmed an AC_{50} of 0.88 μ M. An all six cases, the AC_{50} values obtained were in the absence of overt cytotoxicity (call off 15.32 μ M).

A computational network model based on Toxo ast/Tox21 data has not been developed for the steroidogeness pathway.

In conclusion, the US EPA Toxicast data suggest that spiroxamine disrupts steroidogenesis in HTS in vitro assays, however no direct interference of anatogen 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 AC₅₀ values of 0.77 μ M and 0.37 μ M, respectively. Both models unlike H295 cells, with the former model examing the ability to inhibit 11-deoxycors costerone and the latter model examining the ability to inhibit progesterone.

Of the glucocorticolds, CEETOX_H295R_11DCORT_dn (Figure CA 5.8.3/09-8) and CEETOX_H295R_OHPROG_dn (Figure CA 5.8.3/09-12) confirmed AC50 values of 0.18 μ M and 0.15 μ M, respectively. Both models utilise H295 cells, with CEETOX_H295R_11DCORT_dn examining the inhibition of 11-deoxycortisol and CEETOX_H295R_OHPROG_dn examining the inhibit 17 α -hydroxyprogesterone.



with an AC₅₀ value of 0.21 µM. CEETOX. H295R. TESTO, die, examining the inhibit testosteroig in H295 cells confirmed an AC₅₀ of 0.88 µM. In all six cases, the AC₅₀ values obtained were in the givence 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. The androgen model, CEETOX H295R ANDR dn, examing the inhibit androstenedione in H295 cells, H295 cells confirmed an AC₅₀ of 0.88 μ M. In all six cases, the AC₅0 values obtained were in the absence of overt cytotoxicity (cut off 15.32 μ M). with an AC₅₀ value of 0.21 μM. CEETOX H295R TESTO dn, examining the inhibit testosterous in The state of the s

The state of the s



| | | | | Spiroxami |
|----------------------------------|---|--|---|---------------|
| able CA 5.8.3/09-1: Overview o | f ToxCast models and data output for the S-modality | ale ^c | erti | |
| ToxCast model | Assay description | Response | Chosen model and ontputs | Figure |
| Data retrieved from US EPA Endo | crine: EDSP universe of chemicals website | @\$ | | |
| https://comptox.epa.gov/dashboar | d/dsstoxdb/results?abbreviation=EDSPUOC&search | XXSID1834 | 212#de (221s) 12 A Qust 2020 🖁 💮 | |
| FOX21_Aromatase_Inhibition | Assay for inhibition of aromatase using WCF-7, a human breast cell line aro ERE cells stably-transfected with a testosterone receptor-responsive lucilorase reporter following 24 h | -vo e | Constant model. AIC: 288.28; RMSE: 5.55; ACC: n/a; Cyloroxicity: 15.32 μΜ | (A)5.8.3/09-1 |
| Mineralocorticoids | | 1 Krist State of the State of t | | |
| CEETOX_H295R_DOC_dn | Assessment of potential to inhibit 11-deoxycortices terone in 11295 cells | +ve of | Hill model: AR: -2.99; RMSE: 0 14; ° AC ₅₀ : .0 10 uM; Cytotoxicity; 15.32 uM | CA 5.8.3/09-2 |
| CEETOX_H295R_DOC_up | Assessment of potential to stimulate 11 deoxycorticosterone in 1295 cells | -ve T | Constant model. AIG 42.61; RMSE: 1.18; AO ₃₀ : n/a; Cylotoxicity: 15.32 µM | CA 5.8.3/09-3 |
| CEETOX_H295R_PROG_dn | Assessment of potential to rahibit progesterone in H295 cells | | Cain-lossenodel: AIC: -103.43; RMSC 0.04; AC ₅₀ : 0.37 μM; Couroxicity: 15.32 μΜ | CA 5.8.3/09-4 |
| CEETOX_H295R_PROG_up | Assessment of potential to timulate progesterone in | | 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 Sotential to mhibit correcosterons in H295 cells | . ¥e | 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 | -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_11DQORT_dn | Assessment of poontial to inabit 11-deoxycortisol in | +ve | Hill model. AIC: -34.92; RMSE: 0.04; AC ₅₀ : 0.18 μM; Cytotoxicity: 15.32 μM | CA 5.8.3/09-8 |
| CEETOX_H295R_11DCORT_dn-1 | Assessment of potential to inhibit sorticosterone in H295 cells Assessment of potential to inhabit 11-deoxycortisol in H295 cells | | | |



| | | 40 | Ġ j Dir | |
|--|--|------------------|---|-------------------|
| ToxCast model | Assay description | Response | Chosen model and outputs | Figurre |
| | | effect | | 309 |
| CEETOX_H295R_11DCORT_up | Assessment of potential to stimulate 11-deoxycortisol | CO. | Constant Model. AIC: 42.87; AVISE: | ĈÃ 5.8.3/09-9 |
| | in H295 cells | | 1.180AC ₅₀ : n/a; Cytotoxicov. | , \$ |
| | | (// v | 15.32 μM | |
| CEETOX_H295R_OHPREG_dn | Assessment of potential to inhibit | (-#e | Constant model AC. 6839.00, RMSE 1.13, AC ₅₀ : 11/4; Cytotoxicity: | CA 5.83/09-10 |
| | 17α-hydroxypregnenolone in H295 cells | ~ e ^C | 1.13 $\stackrel{\circ}{A}C_{50}$: n/a; Cytotoxicity: | 1 6 0 T |
| | Assessment of potential to inhibit 17α-hydroxypregnenolone in H295 cells Assessment of potential to stimulate 17α-hydroxypregnenolone in H295 cells | | DP3.32 HIM ~ 21 | |
| CEETOX_H295R_OHPREG_up | Assessment of potential to stimulate 17α-hydroxypregnenolone in 1295 cells | ©ve 🄝 | Constant model MC: 683901; RMSE, | CA 5.8.3/09-11 |
| | 17α-hydroxypregnenolone in IC295 cells | | 1/13; AC ₅₀ : n/a; Cytotoxicity: | |
| | | | 95.32 pm | |
| CEETOX_H295R_OHPROG_dn | Assessment of potentia to inhibat 17α- | +ve 10 | Hillmodel. AK 19.84; AMSE: 0.08; | CA 5.8.3/09-12 |
| | hydroxyprogesterone in H295 Pells | | 300 50: 0.15 Wi, Cytotoxicity: | |
| | | | 15.32 mM | |
| CEETOX_H295R_OHPROG_up | Assessment of potential to stimulate 17α- | -ve CP | Constant model. AIC: 59.07; RMSE: | CA 5.8.3/09-13 |
| | hydroxyprogestem in H200 cells | | 2.32; AC n/a; Cytolexicity: | |
| | | | 15.32 μΜ | |
| CEETOX_H295R_CORTISOL_dn | Assessment of potential to inhibit cortisol in H295 cells | -ve | Hill model. APC: -12.98; RMSE: 0.11; | CA 5.8.3/09-14 |
| | | | AC ₅₀ : 029 μM; Cytotoxicity: | |
| GERMAN HARSE GODERGOL | | O.D | 15.23 uM | G + 5 0 2 /00 1 5 |
| CEETOX_H295R_CORTISOL_up | Assessment of parchial to stimulate cognisol in H293 | -vec | Constant model. AIC: 22.71; RMSE: | CA 5.8.3/09-15 |
| | cells , Ly, West grand | | 0.52; AC ₅₀ : n/a; Cytotoxicity: | |
| | | | 15.32 μΜ | |
| Androgens OF | | | TELL TO TO SE DIVERS OF TO | G + 5 0 2 /00 1 5 |
| CEETOX_H295R_ANDR_dal | Assessment of potential to inhibit androstene dione in | ⊅+ve | Hill model. AIC: -10.25; RMSE: 0.12; | CA 5.8.3/09-16 |
| The William | H295 & QUE TO TO TO THE TOP OF TH | | AC ₅₀ : 0.21 μM; Cytotoxicity: | |
| CEPTON HOOSE TO THE | | | 15.32 μΜ | G + 5 0 2 /00 15 |
| CEETOX_H295R_ANDR_up | Assessment of potential to stimulate androsteriedione | -ve | Constant model. AIC: 46.22; RMSE: | CA 5.8.3/09-17 |
| | in H295 cells | | 1.28; AC ₅₀ : n/a; Cytotoxicity: | |
| CELEBON HOOSE TRATE | | | 15.32 μM | G + 5 0 0 100 10 |
| CEETOX_H295R_TEST@ In | Assessment of potential to inhibit testosterone in H295 cells | +ve | Hill model. AIC: -13.79; RMSE: 0.1; | CA 5.8.3/09-18 |
| | cells * * * * * * * * * * * * * * * * * * | | AC ₅₀ : 0.88 μM; Cytotoxicity: | |
| CEPTON HOSE TRANSPORT | Mary Mary Constitution of the Constitution of | | 15.32 μΜ | G + 5 0 2 /00 1 0 |
| CEETOX H295R_TESTO_up | Assessment of potential to stimulate testosterone in | -ve | Constant model. AIC: 47.45; RMSE: | CA 5.8.3/09-19 |
| | H295 cells | | 1.44; AC ₅₀ : n/a; Cytotoxicity: | |
| The Street of the street of th | | | 15.32 μΜ | |



| | | | Ġ Du | |
|--------------------------|---|----------|--|----------------|
| ToxCast model | Assay description | Response | Chosen model and outputs | Figurre |
| | | effect | | |
| Oestrogens | | 20°7 | | |
| CEETOX_H295R_ESTRADIOL_d | Assessment of potential to inhibit oestradiol in H295 | -ve | Constant model. AIC: 21,02; RMSE. | CA 58.3/09-20 |
| n | cells | as ° | 0.53; AC ₅₆ ; n/a; Cytotoxicity: | |
| | | KO. MO | 15.32 11 15 | |
| CEETOX_H295R_ESTRADIOL_u | Assessment of potential to stimulate oestraction in H295 | -ve O | Hill model. AIC: 6.21; RMSE: 0.16 | CA \$8.3/09-21 |
| p | cells of the control | | ρΑC ₅₀ : 21, ΣμΜ; Cytotoxicity; | |
| | | e" | 15.32 PM 30 30 30 30 30 30 30 30 30 30 30 30 30 | ,\$C |
| CEETOX_H295R_ESTRONE_dn | Assessment of potential to inhibit oestrone in H295 | -ve | Constant model. AIC: 15.4; RMSE | CA 5.8.3/09-22 |
| | cells | R. T. E. | 9.4; AC ₀ 9n/a; CytoΦxicity: 15.32 μM | |
| CEETOX_H295R_ESTRONE_up | Assessment of potential to stimulate oestrone in H295 | · -ve | Hillomodel. A.K. 2.03; R.M. 2. 0.15; . | CA 5.8.3/09-23 |
| | cells | | A.C. 16.24 GM; Cytotoxicity: | |
| | | 50, 20 | 15.32 pM | |

and use of this document its of the rights o and connercial parmice in and armice in and armice in an armice in and armice in an armice in an armice in an armice in a marmice in a AIC: Akaike Information criteria: appropriate model sexual the lowest AIC value essential model that bear fits the deal RMSE: median root mean squared error across all numbers. RMSE services for higher performance models.

RMSE: median root mean squared error across all numbers. RMSE services for higher performance models.

RMSE: median root mean squared error across all numbers. RMSE services for higher performance models.

RMSE: median root mean squared error across all numbers. RMSE services for higher performance models.

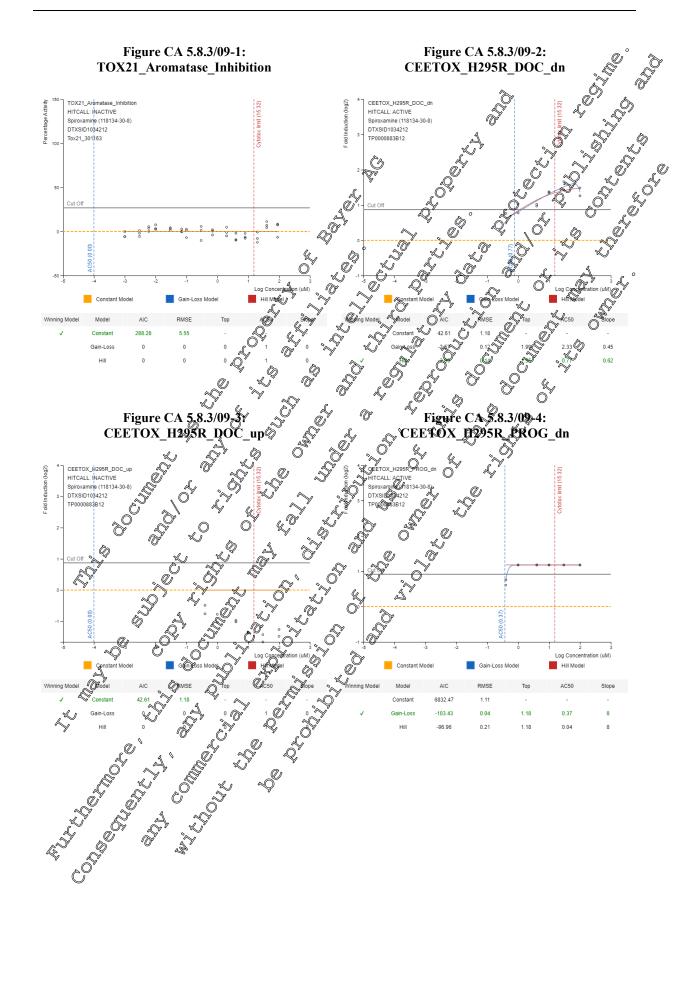
RMSE: median root mean squared error across all numbers. RMSE services for higher performance models.

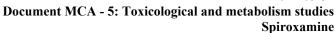
RMSE: median root mean squared error across all numbers. RMSE services for higher performance models.

RMSE: median root mean squared error across all numbers. RMSE services for higher performance models.

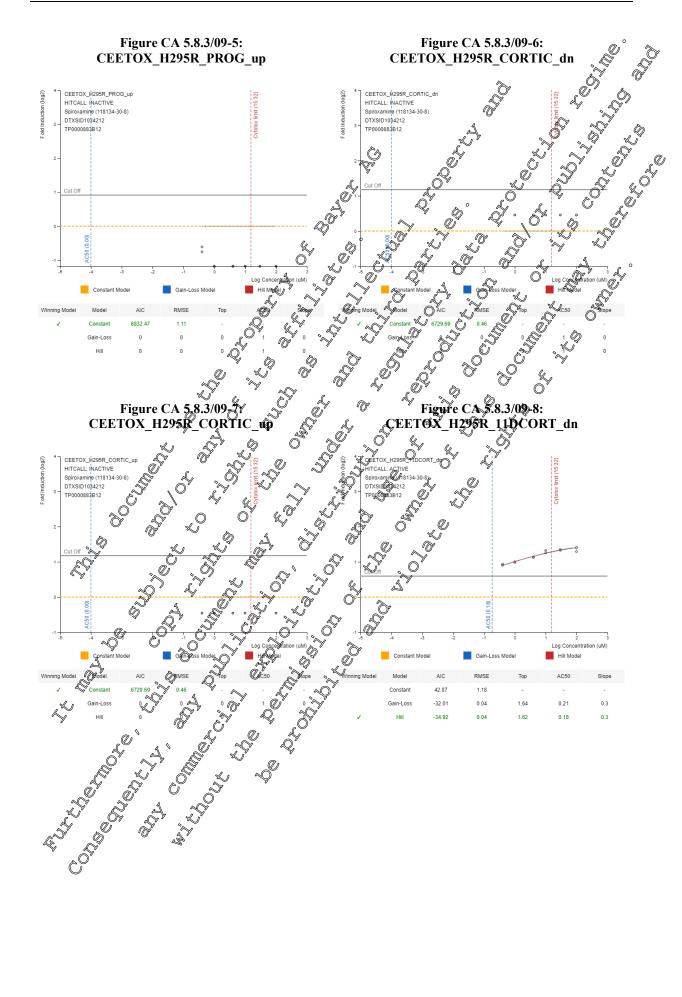
RMSE: median root mean squared error across all numbers. RMSE services for higher performance models.



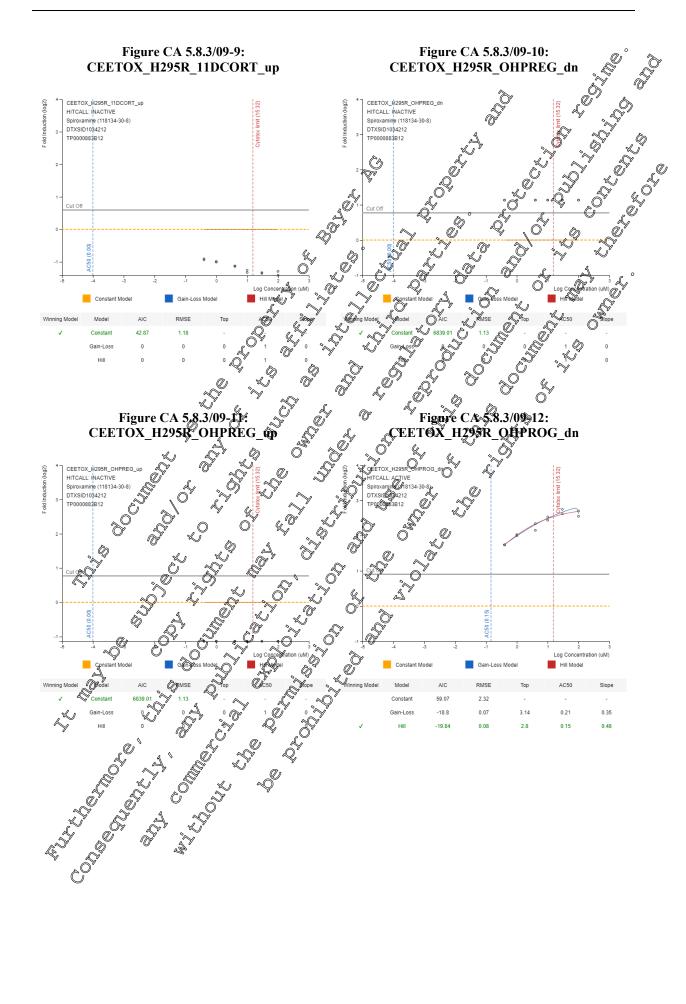


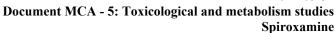




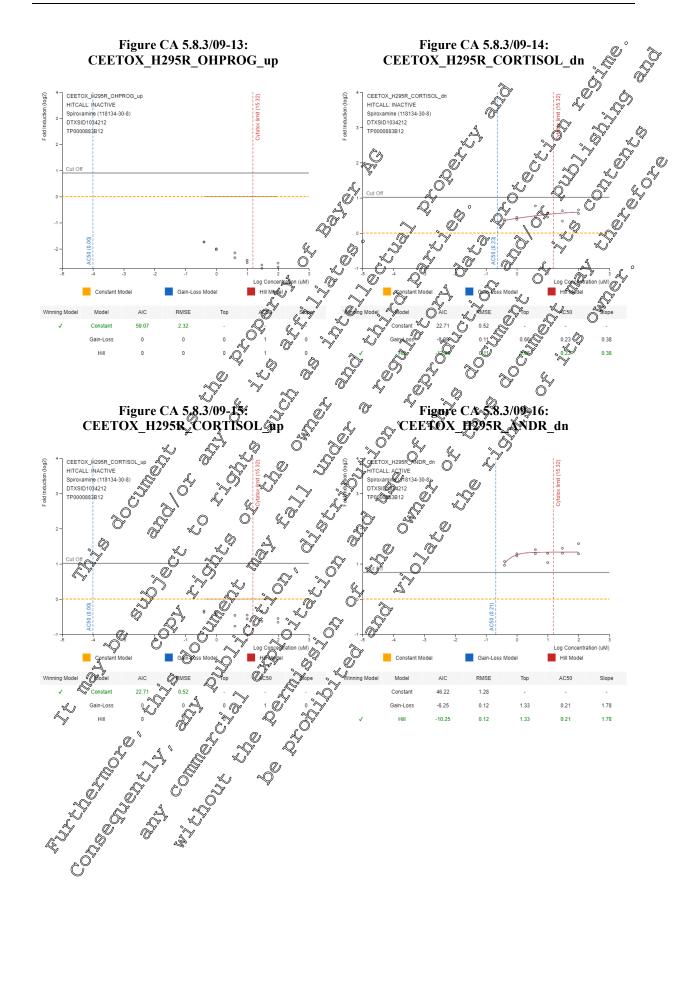




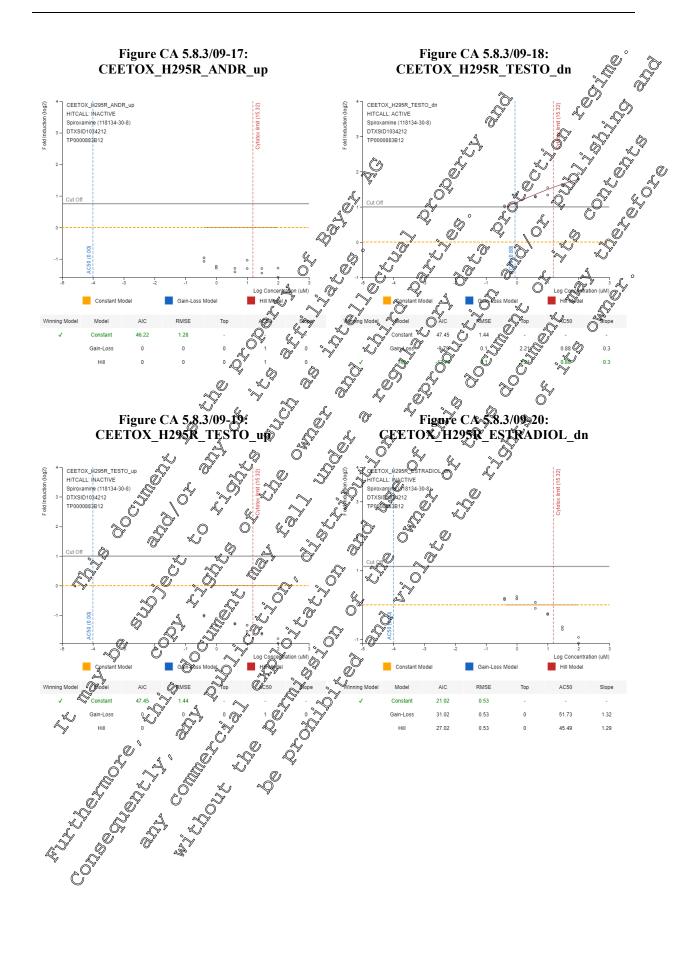


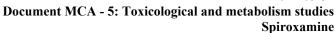




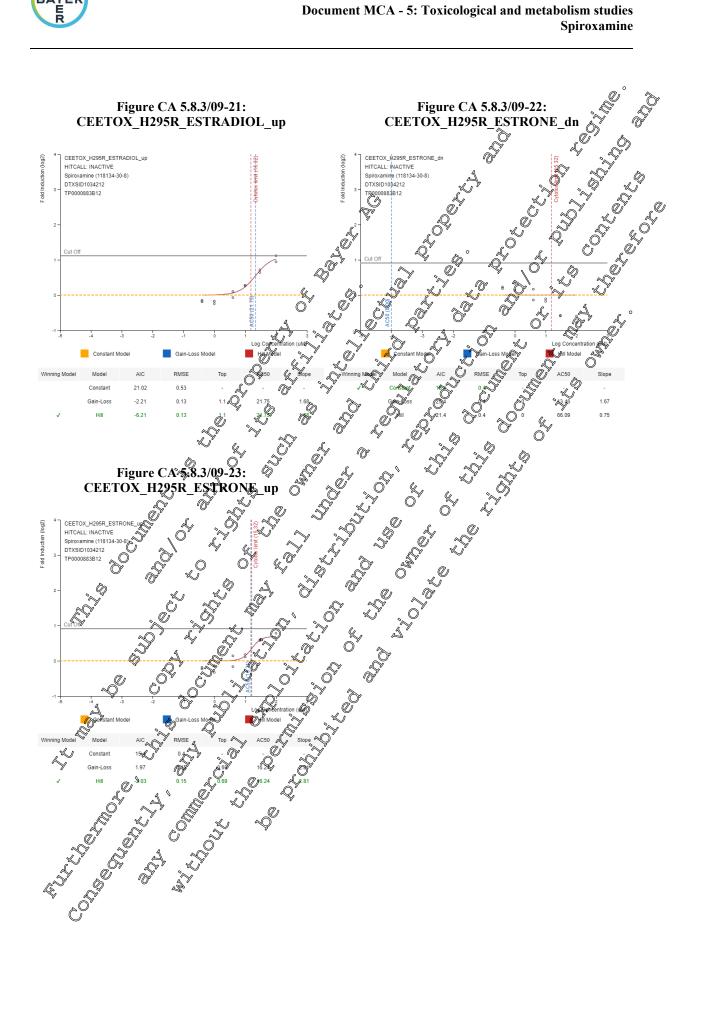




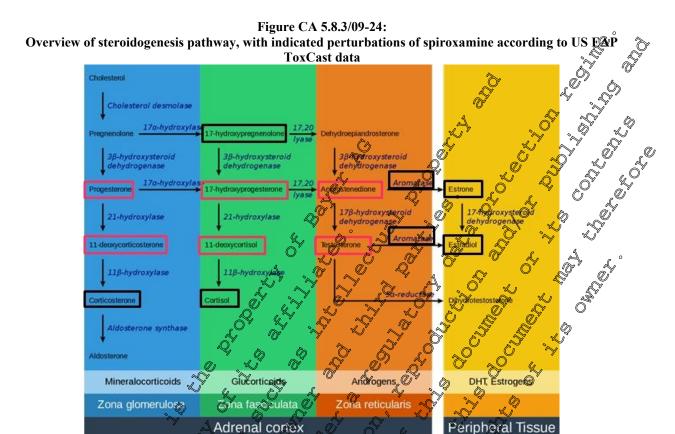












Collectively these data suggest that spiroxamine disrupts steroidogenesis in HTS *in vitro* assays. However, spiroxamine did not show any argunatase inhibition in human recombinant CYP19 cells when treated up to 100 µM (CA 5.8.3/01 [M-30197001-1]). Spiroxamine was negative in rat testicular homogenate steroidogenesis assay *in vitro* up of 100 µM (CA 5.8.3/09 [M-303122-01-1]). Whilst it is recognised that only the terminal horomone 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 apstream 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-70548-0-1]) 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 H297R ANDR dir and CEETOX H295R TESTO dn ToxCast data which reported inhibition of androstenedicine and Pestosterone, respectively.

No effect @

Decrease (D

Whilst it is recognised that the reported effects on glucocorticoids (17-hydroxyprogesterone and 11-deoxycortisol) and the mineral corticoid, 14-deoxycorticosterone have not directly been investigated, as these hormores are fulcrus to the steroidogenesis pathway, biologically relevant inhibition occurring would be reflective downstream and matters 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 steroeogenesis 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: | |
| Report Year: | 2008 |
| Report Title: | Spiroxamine - Investigation on potential in vitro aromatise (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 State of the |
| Previous evaluation: | yes, evaluated and accepted DAR (2010) |
| GLP/Officially | No, not conducted under GLP/Officially recognised testing facilities \ |
| recognised testing facilities: | |
| Acceptability/Reliability: | Yes V V V V |

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 \$2450 coluctase by poans of a radiometric assay. The substrate for the assay was [3H] candrostenedione (ASDN), which is converted by aromatase to estrone

Final concentrations of spiroxamine tested in the aromatase assay ranged from 0.01 to $100 \,\mu\text{M}$ in a single assay. The positive control eletrozole 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 inghest spiroxamine test concentration of 100 µM. Based on the data interpretation criteria established for the assay, spiroxamine did not inhibit harman 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 IC₅₀ values of ca, bind and ca. 21 nM, respectively thereby confirming the sensitivity and specifically 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 devoud of aromatase inhibiting potential.

Under the conditions of the study, spirox amine and not whibit human recombinant aromatase, and is therefore concluded to be sevoid of aromatase whibiting potential in a single experiment.

Material and Methods

A. Materials:

1. Test Material: Spiroxamine

(a)ternative name [8-(1,1-dimethylethyl)-N-ethyl-N-propyl-1,4-

alioxaspiro[4,5]decane-2-methanamine; KWG 4168)

Description: Light brown oil

Lot Batch No.: FOTH004650

Parity: 57.0% (w/w) (correction for purity not undertaken)

AS No.: " 118134-30-8

Stability of test Confirmed stable for the duration of the study (expiry date: 2 August 2009)

compound:



2. Vehicle and/or positive

control:
3. Substrate:

DMSO / positive control: Ketoconazole (12.5, 25, 50, 100 μ M), Letrozole (0.0005, 0.0015, 0.005, 0.015 μ M)

A mixture of non-radiolabelled androstenedione (ASDN) and radiolabell [1β-

³H(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 (\$\text{P}\$19 and P450 reductive were

selected as the test system based on the recommendation from the FA test guideline. Determination of protein concentration of microsomes was determined daily. Aromatase activity of human recombinant microsomes was

conducted to confirm that the microsoppes had sufficient activity for each assa

The aromatase activity in migrosomes was \$2 pmote product

5. Dose preparation and

analysis:

A stock solution of the test article was propared in DMSO on the day of treatment. Subsequent driutions of the stock solution were prepared in DMSO

0.01, 0.1, 1, 10, 100 µM

6. Radiochemical analysis:

The amount of [VI]-ASDN (measured in disintegrations per minute, DPM) in each standard was determined by subjecting addited up to 1 and with acetomerile) 5 µL aliquot of the standard to radio-analysis by liquid scintillation counting (ISC).

B. Study Design:

1. In life dates:

2. Cell treatment:

15 October 2007 to 6 October 2007 (experimental dates)

The ability of the lest article to inhibit human recombinant microsomal cytoclareme P4. (CYP19) aromatase activity using a mixture of ASDN and [3H]-ASDN as the substrate was evaluated according to a test methodology similar to the EPA test guideline. The experiment was performed by preincubation of human CYT19 Aromatase (1 µL), NADPH (240 µmol/L), NADPH for 5 minutes in at 0. PMSQ) test acticle or positive control (ketoconazole, letrozole) for minutes at 3 T.C. Vehicle, test article and positive controls were dosed into the test system at 2% v/v. Incubations containing test article were performed induplicate, for vehicle and positive controls five replicates, were performed.

The reaction was storted with the addition of 420 nmol/L (16.3 KBq/mL) [18-3H0n]-ASDN and incubation for 0 minutes. Reactions were terminated by the raddition of 0.25 on L triconoroactic acid (5 %).

3. Evaluation criteria:

Not started in the report, but assumed to follow the US EPA test guideline, as detailed below:

Classification of Sceptor binding affinity:

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%:

Kquivocal

Aromatase acovity at the highest concentration was ≥75%. Non-inhibitor.

Statistics: None performed.

C. Methods:

1. Homogeneity and

[≯]Not undertaken.

achieved

concentration analysis

of the dose:



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 $2\sigma\%$ value of \leq 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. [3H]-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 od not inhibit numan recombinant aromatase, and tis herefore concluded to be pevoid of aromatase inhibiting potential.

The positive controls, letrozole and ketoconazole both concentration dependently inhibited aromatase activity with IC₅₀ values of *ca*. and and *ca*. 20 nM, respectively thereby confirming the sensitivity and specificity of the assay.

Table CA 5.8.3/01-1: Spiroxamme: incitro homan recombinant aromatase assay aromatase activity results

| Test article (QM) | 311-11-0 = 0 = 0 = 0 | |
|--|--|-----------------|
| Test article (MM) | Tranzo reseaseu qupin | Activity |
| 0 | 125658 ±1358 | |
| | 1 2 6731 48 80 3 | 100.9 ±0.7 |
| | 12608 ±2093 12608 ±129 | 102.2 ±1.6 |
| | | _@ 101.0 ±0.1 |
| S 10 | | 106.0 ± 0.2 |
| 100 | 138924~*824~~ | 110.6 ± 0.6 |
| | Positive ontrols . | |
| | 103931 ±984 86669@1051 625[\$\frac{1}{2}\$1973 | |
| - 0.0005 - 0.0015 | ∫ JØ931 ±984 % | 90.7 ±0.2 |
| - 0.0015 | \$6669. ₩ 1051.₹ | 69 ± 1.2 |
| Ø.005 ° ° | © 625 1 1973 ° C | 51.9 ±3.0 |
| Letrozole (MI) - 0.0005 - 0.0015 - 0.0015 - 0.0015 | 40359 ±306 | 32.1 ±0.8 |
| Ketoconazole (μM) | 26600×1003 | |
| - 12.5 | \$6600 ≠1003 | 68.9 ± 1.2 |
| 25 200 4 | $1 \sim 50509 \pm 0.062$ | 46.6 ± 16.2 |
| - 25 - 50 - 100 - 100 | 44095 ±3263 | 35.1 ±7.4 |
| - 50° - 100° - 5° | 2280 ±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 use Dinstead, 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 prhibition.

Whilst the following deficiencies are detailed, the results produced in the argumatase assay are still valid

Assessment and conclusions by applicant:

Assessment: Study meets the current guidance and the requirement on 283/2013.

Conclusion: Under the conditions of the study, spic xamine did not inhibit human recombinant aromatase, and therefore concluded to be devoid fraromatase inhibiting potential in a single experiment.

| Data Point: | KCA 5.8.3/02/ |
|--------------------------------|---|
| Report Author: | |
| Report Year: | 2008 |
| Report Title: | Spiroxate ine - Investigation of potental in vito stero-tigenesis inhibition |
| Report No: | AT0#646 & |
| Document No: | |
| Guideline(s) followed in | not applicable; nuchanistic study |
| study: | |
| Deviations from current | None State of the |
| test guideline: | |
| Previous evaluation: | yes, evaluated and accepted |
| | (DAR(2010) & , , , , , , , , , , , , , , , , , , |
| GLP/Officially | No, not conducted under GLP/Officially recognised testing facilities |
| recognised testing facilities: | |
| | |
| Acceptabil Acceptability: | , Supportive of O |

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 that testost.

Final concentrations of spiroxamine tested in the sterodogenesis assay were 1 and 100 μ M in a single assay. The positive controls, keroconazole, 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 ketocorazole (in 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 August 2009)

2. Vehicle and/or positive

control:

DMSO / positive control. Ketoconazole (0.5, 20 µM)

3. Test animals:

Species:RatStrain:WistarAge at sacrifice:♂: 12-14Source:

4. Primary cell line:

Testicular fragments solated from Fingle Frat.

5. Cell culture conditions:

Minimum essential medium Eagle, (MEME) medified supplemented with NaHCO3, L-ghaming 5 mM, Na pyruvate (1 mM), NCG (12U hCG [human chorion goridotrophi]/mL) adjusted to pH 4 – 7.5

6. Test concentrations:

900 uM

B. Study Design:

1. In life dates:

2. Cell fragment preparation: 12 October 2007 of 30 October 2007 (experimental dates)

Following sacrifice of singles 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 wated in the report, but assumed to follow the US EPA test guideline, as defailed boow:

The results of the hormone analyses were normalised to the mean solvent control value and there expressed as changes relative to the solvent control in each exposite plate. All doses that exhibited cytotoxicity greater than 20% or exceeded the limits of solvibility (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 inhubition was statistically different from the solvent control at concentrations followed by main the increasing or decreasing portion of the dose-response curve. One way analysis of variance eventually followed by pairwise comparisons

versus the control group using Dunnett's method. If heterogeneity was observed, Kriskal-Wallis one way analysis of variance of ranks was performed, followed pairwise comparisons versus the control group using Dunn's method.

4. Statistics;

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 dynamic organ culture incubator for 6 hours at 35°C. Vias were rotated. times/minute, with a flow rate of carbogen of 3.4 L/minute. For the yehicle control 12 replicates were used, for all other groups preplicates 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 -200 until analysis. Testicular fragments were

recovered, weighed and stored deep frozen until processed further.

3. Analysis: Medium was directly analysed after that ring. Flagments were thawed on ice if

500 μL MEME without hCG. Following centrifugation (7.5 minutes, 1300 x g) the supernatant was malysed for testosterose. Dumicate determinations were performed. Testosterone in medium was assayed irectly, 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 underween to evaluate storoidogenesis in the presence of spiroxamine. Solubility/precipitation of spiroxamine in the assay buffer was oot assessed in the assay, nor was cytotoxicity exaluated. The mean testoster one 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 ketoconazof (an inhibitor of steroidogenesis) significantly decreased testosterone concentration in the medium by 67% and by 83% in testicular fragments, thereby confirming the sensitivity and specificate of the assau

Spiroxamine: in vitro voi steroldogenesis assay: testosterone concentrations

| Test acricle (μM) | Testoster | one in medium | Testostero | ne in tissue |
|--|-------------------|-------------------|-------------------|--------------|
| \sqrt{n} | ang/mg tissue | | ng/mg tissue | % reduction |
| No incubation 6 | | } | 0.44 ± 0.10 | - |
| 0 0 | 0.0 ±0.30 | | 2.26 ± 0.82 | - |
| 1 49 | \$22 ±0.53 | ₹ - | 3.37 ± 1.51 | = |
| 100 | 1.06 ±0.46 | V - | 3.55 ± 2.25 | - |
| | | Positive controls | | |
| Ketoconazole | | | | |
| (μM) | 7 G | | | |
| | $0.61 \pm 0.22^*$ | 33 | 1.57 ± 0.73 | 31 |
| 0.5 \$\frac{1}{26}\$ \$\tilde{\text{0}}\$ \$\text | $0.31 \pm 0.07^*$ | 66 | $0.39 \pm 0.19^*$ | 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 conversion 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 $(100 \, \mu M)$, which $(100 \, \mu M)$ than the recommended concentration $(10 \, mM)$
- Concurrent measure of cytotoxicity was no condertaken
- Only 2 concentration of test article concentration were treated. Test galideline requirements are 7 concentrations.
- Known inducers (forskolin) and inhibitors (prochloraz) of testosterone and 1712-estradiol synthesis were not used

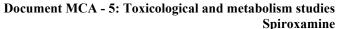
In conclusion, the data generated under this study are considered supplementary with the steroidogenesis endpoint sufficiently addressed with a new, up to date test guideline compliant GLP study (refer to CA 5.8.3/10 [M-764156-91-1])

Assessment and conclusions by applicant:

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

Conclusion: Under the conditions of the study spiroxamine did not inhibit steroidogenesis ex vivo in rat testis homogenate and therefore concluded to devoid of steroidogenesis inhibiting potential in a single experiment when examining testosterone production.

| Data Point: | KCA 5.8.3/10 |
|---------------------------|--|
| Report Author: 🐒 🏻 💆 | |
| Report Author: | 202 |
| Report Title: | Societing spiroxatrine for modulation of steroidogenesis using the human |
| | H295R adreno carcino ma cell line |
| | 20249639 |
| | M-204156-001-1 ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ ~ |
| Guideline(s) followed in | QECD Test Guideline No. 456 (July 2011) |
| study: | |
| Déviations from current | |
| test guideline: 🕡 🔪 | |
| Previous evaluation: | |
| | |
| GLP/Officially | Yes conducted under GLP/Officially recognised testing facilities |
| recognised testing | |
| facilities: | |
| Acceptabili /Reliability: | 9 |
| | · |





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 estradio 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 inducer prochloraz.

Dimethylsulfoxide (DMSO) was used as vehicle and the concentration of vehicle in the incurations was kept constant at 0.1% (v/v).

H295R cells were exposed for 48 hours to the whicle, the test article and positive controls. After exposure, the viability of the cells was determined using the 3-47,5-dimethylahiazol-2-yl]-27,5-dimenyl tetrazolium bromide (MTT) assay. The concentration of estradiol, testosterone and progesterone in the exposure medium was determined using a commercially available Engine-Linked Jamung Sorbent Assay (ELISA) (estradiol) or by UPLC-MS/MS (testosterone and progesterone).

Experiment 1:

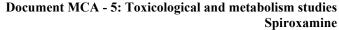
- A statistically significant decrease in restosterone production was observed at Ω, 1 and 10 μM spiroxamine. Overall the result was concluded to show evidence of decrease in restosterone synthesis.
- A statistically significant decrease in Stradiol production was observed at 0.0001, 0.001, 0.01, 0.1 and 1 μ M 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. Gerall 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 u.W. 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 progesterons 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 propesterone 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 und spiroxamine. Overall the result was concluded to show evidence of a decrease in testosterone synthesis.
- A stanstically significant occrease in estradiol production was observed at 0.001, 0.01, 0.1, 0.316 μ M and a stanstically significant increase in estradiol production was observed at 10 μ M spiroxamine. Everally 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, 0.916, 10 μ M.





Cytotoxicity (>20%) was observed at the highest test article concentration (31.6 µM) in Experiment_o1 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 and

The positive control inducer forskolin and positive control inhibitor prochlora 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 Officet Concentration (LOEC) was 100 pM, 100 nM and 10 nM for estration, 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), Jo μM (experiment 1) and 10 μM (experiment) test article, respectively

It is concluded that spiroxamine showed evidence of steroglogenesis in human adregiocortical 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: proxamine

(alternative name: [8-6] 1-dim@hylethyl)-N-elbyl-N-g

dioxaspiro[465]decar@2-methanamine; KWG 41689

Description:

Lot/Batch No.

97.0% (w/w) correction for ourity got undertaken) **Purity:**

CAS No.:

the tudy (expiry date: 4 June 2021)

compound:

2. Vehicle and/or positive control:

ositive controls

Steroid metabolism inducer forskoling(1, 10 µM)

∡methano¥(

Human adren cortical varcinoma 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.

Dulbecoo's Modified Eagle Medium/Ham's F12 nutrient mix (DMEM/F12), 4. Cell culture

supplemente@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,

625 ng/mL selenium, 1.25 mg/mL bovine serum albumin.

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:

2. Vehicle selection:

29 September 2020 to 7 December 2020 (experimental dates)

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 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: $3.16~\mu M$) based on

0.1% additions into the test system.

3. Statistics: Normality was evaluated using a Shapiro-Wife's test. If data were normally distributed, the data were transformed to approximate a normal distribution if

distributed, the data were transformed to approximate a normal distribution.

differences between tear article treatments and colvent controls were analyzed using the Dunnett's Test.

If the data were not formally distributed, the non-parametric Kruskal Wallis test was performed. A Levene's sest was performed to test for variance homogeneity. It variance was non-homogeneits another appropriate test was performed. Differences were considered significant at $p \le 0.05$. Statistical evaluation was performed based in average values for each well-that

represented independent replicate data points.

ToxRat Professional software (ToxRat Solutions® GrubH, Germany) was used for statistical evaluation.

4. Acceptance criteria:

Steroidogenesis assay:

Considered acceptable of the results med the following criteria

| , Ø | | Comparison between | Testostorone | Estradiol |
|--------------|--|----------------------------|-----------------|------------------|
| *\f\dagger\} | Basal production of an armonem the SO | Fold reater than LOQ | -fold | \geq 2.5-fold |
| | Exposure experiments S Within plate V for SCs (replicate wells) | Absolut@oncentrations | € 30% | ≤ 30% |
| | Exposure experiments – & Between plate V for Ses (replicate experiments) | Absolute concentrations | ≤ 30% | ≤ 30% |
| | Hormone measurement Stem - Consitivity | Detectable foldschange | \geq 5-fold | \geq 2.5-fold |
| | Phormone measurement S system - Repocate measure CV for SCS | Absolute Concentrations | ≤ 25% | ≤ 25% |
| | Muction 10 μM Porskol@) | Fold change compared to SC | ≥ 1.5-fold | \geq 7.5 -fold |
| | Inhibition (1 pm) | Fold-change compared to SC | \leq 0.5-fold | \leq 0.5-fold |
| A | 1 2 21 2 1 11. | • | • | • |

Aççılracy @d repentability!

The analytical method was considered applicable for the quantitative analysis of test steroid and progesterone if the mean accuracy was in the range 70–130%.

5. Evaluation cotteria:

Steroidogenesis jäverference:

A **test** article was considered positive if the fold induction was statistically different $p \le 0.05$) from the solvent control at two adjacent concentrations at least two independent runs.

Evoid 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:

| D 1 | D 2 | D 2 | Decision |
|---------|---------|-------|----------|
| I Run I | I Run 2 | Run 3 | Decision |



| Scenario | Decision | Scenario | Decision | Scenario | |
|------------------------|---------------------|----------|------------------------|----------|----------|
| -ve | Confirma | -ve | Stop | | -ve @ |
| -ve | Confirma | +ve | Refineb | -ve | -ve 🗬 |
| Equivocal ^c | Refineb | -ve | Confirma | ∝-ve | -v& |
| Equivocal ^c | Refine ^b | -ve | Confirma | Øve | #e (|
| Equivocal ^c | Refineb | +ve | Stop | | *¥ve 。.♀ |
| +ve | Refine ^b | -ve | Confirm ^a « | +ve | ~+ve~ |
| -ve | Confirma | +ve | Refine ^b | +ve | 0 +v |
| +ve | Refine ^b | +r\ | Stop | × 1 | +væ |

- Confirm previous run with the same experimental design
- Re-run assay at ½-log concentrations spacing Gracketing the concentration that tested significantly different in the preceding experiment)
- Fold-change at one concentration is statistically significantly different from the SC

C. Methods:

- 1. Homogeneity and achieved concentration analysis of the dose:
- 2. Steroidogenesis assay:

Not undertaken.

H295 cells overe plated into 24 well plates (3.0 x 10 cells 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

to exposure. After 24 liver 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 (pritreated control in triplicate) or
- ♥ĎMSØ (solvent contool, in troplicate);/or &
 - for Skolin (Orducer In triplicate at 1 μM and 10 μM for QC plate), or
- prochloraz (inhibitor, in Oriplicase at 0.1 μM and 1 μM for QC plate), or
- Litest article (7 soncentrations in triplicate)

Following exposure for 48 h, the 24-well plates were removed from the incubator and every well checked microscopically for cell condition (attechment Thorphology, 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.

Cell wability was defermined by the MTT assay. Medium from each well was replaced by 500 μk fresh medium Subsequently, only the medium from wells A 6 and β4-6 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, in than of was removed from wells A4-6 and B4-6 and these wens were rinsed carefully with medium (three times). After rinsing, 500 μL medium was added to these wells.

To all wells, 55 µL ATT (5 mg/mL in PBS) was added. The plates were incubated at 37°C and 5.0% CO₂ for 2 -3 h.

If the includation, the medium in each well was replaced with 500 µL DMSO. The plates we 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 Forrect for background absorption.

All wells that exhibit cytotoxicity >20% were omitted from further evaluation. 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

3. Cell viability as



| of |
|------|
| / |
| . \$ |
| |
| |
| |
| |

T: testosterone; E2: 17β-estradiol.

The tubes were vortexed and the hormone concentrations determined by ELISA (for estradiol) or UNC-MS/MS (for testosterone and progesterone). If interference occurred that was ≥30% of basal formone production for E2 and/or T, the chemical formone 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 the concentrations discarded.

5. Hormone analysis and recovery:

Hormone and ysis was not be performed in medium obtained from wells that showed viability \$0%.

In addition, medium samples spiked with 100 pg/mL 30 pg/mL and 250 pg/mL estratiol, 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-MSMS (testosterone and progesterone) to determine the recovery.

The measured hormore concentrations in spiked medium should not deviate more than 30% from the command oncentrations.

6. Data analysis:

b) Cell viability

The cell viability was expressed relative to the average response in the solvent controls, which is considered 100% riable cells, and was calculated as follows:

response in well – average response in MeOH_{treated} well) x 100 (response in SC well) – average response in MeOH_{treated}

b) Testosterone and progesterone analysis:

All coses that exhibit cytotoxicity 20% were omitted from further evaluation.

Response (R) = Peak area of the analyte × (IS Conc. / IS peak area) [units] rlibration curve Regression analysis was performed using the least squares method.

Analyzed Stalyzed Oncentration of the samples

OC Prepared in supplemented medium

$$Accuracy = \frac{\epsilon_A - C_B}{C_N} \times 100 \, [\%]$$

 C_B = analyzed concentration in supplemented medium blank sample

c) Calculation of estradio, testosterone and progesterone concentrations;

Total 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 adculated as follows:

Relative change= hormone concentration in each well

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 sest guidelines.

B. Steroidogenesis assay – Experiment 1:

QC plates for the test article plates, estradiol and testosterone confirmed that the 1. QC plates:

acceptability criteria were met.

The average basal testosterone production in the DMSO solvent control wells on 2. Testosterone analysis:

the test article plate was 1742 pernL. 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 and cle exposed wells to the solvent treated control wells ranged from 0.62 to 1.02. A statistically significant decrease in testosterone production was observed at Q1, 1 and 10 μW spiroxamine. Overall the result was coocluded to show evidence of a decrease in

testosterone synthesis/

The average basal estradio production in the DMSO solvent control wells on the ." 3. Estradiol analysis:

test article plate was 120 pg/mb. The average extradiol 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 wears ranged from 0.69 to 1.07. Estatistically significant decrease in estraction production was observed a 00.0001 0.001, 0.001, 0.001 μM spiroxamine. Overall the result was concluded to show evidence of a decrease in

estradiol synthesis.

4. Progesterone analysis:

The average basa progesterone production in the DMSQ solvent control wells on the test article plate was 313 pg/mL. The average progesteron concentrations in the medium from the H295R cells exposed to the test article ranged from 11 pg/ml/sto 308 pg/ml The average relative change of the test article exposed wells to the solvent treated control wells Panged from 0.37 to 0.98. A statistically significant degrease 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 progesterine synthesis.

Cytotoxicity (20%) was observed of the bighest test article concentration 5. Cell viability (31.600M). The samples from this concentration were therefore excluded from horizone arratysis. No cytotoxicity was observed at any of the other spiroxamine

concentrations tested.

The positive control inducer forskolus and positive control inhibitor prochloraz induced acceptable fold changes to the solvent control for testosterone and estadiol plates when assessed against the assay performance criteria, with all other parameters passing the performance criteria, thereby confirming the sensitivity and

Opecificity of the assay test system.

It however prudent to note that no performance criteria are available for progesterone, therefore, whilst a statistically significant decrease in progesterone was observed. The biplogical relevance is unclear. Within H295R cells, androstenedione and 1-deoxycortisol are the most abundant, while steroids upstream and down fream show lower levels. Steroid levels (absolute and relative) vary since they are highly dependent on culture conditions (basal levels of seroids in batch of Nu-serum used is very important) and the method of steroid analysis used King et al²⁷ report that basal levels of progesterone are overtly low

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



| | | | | | | | | | | | | | | Spi | roxamine |
|------------------|----------------------|----------------|--|-------------------|---------------------------------------|-----------------------|-----------------|----------------|-------------------|-------------|-------------------------|-----------------|----------------|----------------|----------------|
| | | a : | | | | | | | | pĠ | (ge) xamine ate\$ | A ard | Ç | | |
| Table CA | 5.8.3/10-1 | : Stero | oidogenesis | activity of | spiroxami | ine: Exper | riment 1 | | al [©] " | | | | | | |
| MTT | (%viability | y [%cytoto | oxicity]) | Testo | osterone (r | elative ch | ange) | Es | tradiol (re | lative char | (ge) | Prog | esterone (1 | elative cha | inge) |
| QC | plate | Spiro | xamine | QC | plate | Spiro | xamine | QE | plate 。 | Spire | xamine | * QC | plate | Spirox | |
| | | p] | late | | Т | pl | ate | 4 | * C 2 | p] | ate\$ | رo ^۳ | OUL . | , O Pla | ite |
| Blank | 99.9 [0.1] 100 | - | - | Blank | 1.07 ±0.08 | - | 1000 C | | \$0.06 | 90% | | Blank | 1.60 ±0002 | | - |
| O ^a | 100.0 | DMSO | 100.0 | 0 ^a | 1.00 | 0a 7 | 1.00% | 0a | 1.000 | Oa j | 1.00 | 0a 🎾 | 1.00 | O ^a | 1.00 |
| | [0.0] | | [0.0] | | ±0.01 | | ×± © .06 | -A | ¥0.11 | * O3/ 1 | ₫0.05 | 0\$ | ±0.04 | | ±0.04 |
| For: | 108.4 | 0.0001 | 101.3 | For: | 1.83 ±0.09 | 0.0001 | | For: | 15.27 | \$ 0.000 L | 100 | For: 2 | 2.25 ±0.42° | 0.0001 | 0.98 |
| 1 uM For: | [-8.4] 107.3 | μM 0.001 | [-1.3] 94.0 | 1 uM For: | ±0.0 9 | µ № ∡10.001 | ±0.03 | For: | ±0.82 × 0.03 | 0.001 | ±0.5% ±0.13 | Tulve' | 2.42 | μM 0.001 | ±0.01 0.97 |
| 10 uM | [-7.3] | μM | [6.0] | 10 uM | ±0.13 | μΜ. | ±0.02 | 10 uM | ±2.36© | uM \cap | ±0.13*0 | 10 uM© | ±0.25 | μM | ±0.05 |
| Dro | 00.1 | 0.01 mM | 00.3 | DATA ALL | $a \cap g$ | 0.0% | 0.300 | Pro: | 0% | 0.01 µM | | ું ઋજિ: | 4.26 | 0.01 μΜ | 0.96 |
| 0.1 uM | [0.9] | | [0.7] | 0.1 uM≥ | \\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\ | Qr. | ±0.04 | © .1 uM | 1±0.04 | | ©±0.03* | 🕅 uM | ±0.03 | | ± 0.02 |
| Pro: | 99.1 | 0.1 μΜ | 104\$ | Pro | 0.08 | 0.1 pg// | 0.76 | Pro:O' | 0.26 | 0.1 | 0.78 | Pro: | 23.0 | 0.1 μΜ | 0.62 |
| 1 uM | [0.9] | 1 uM | 100.2 | I uM | ₩ #9.01 | 01 uM @ | 0.02 | O JUNI | 1 0 .00 C | 1 uM% | 90.03* 0.78 | 1 uM | ±0.62 | 1 μM | ±0.03* 0.54 |
| | | 1 μινι | [-0.2] | . <i>o</i> Č | , TE | Ιμινίου | ±0.72 | ' , , | , O ₎ | , 1 μΜ.Υ | ±0.08* | | | Ι μινι | ±0.02* |
| | | 10 μΜ | 91.21 | | | ÖνμΜ | \$ 0.63 | à . | ~ex | . 10 μM | 1.07 | | | 10 μΜ | 0.37 |
| | | | [8.8]\$ | | | | ±0.01 | | | | ±0.04 | | | | ±0.01* |
| | | 31.6 μM | √ 2.1 | 06 ₂ 1 | Wells. | 34% µM | | ~C | * © | 31.6 μM | - | | | 31.6 μΜ | - |
| * <i>p</i> ≤0.05 | | | [[22.9] | | | | | | Let Fill | | | | | | |
| For: forsko | lin | | | 90 | ~ 1 CO. | | OF | | | | | | | | |
| Pro: prochle | oraz | | | \$ | | | D 3 | , | | | | | | | |
| | | | E. J. T. | . 1 P | e f | | a Olym | | | | | | | | |
| | | e (| 2, ¹ | | | | 2 [©] | | | | | | | | |
| | | | - | | | | | | | | | | | | |
| | 4.0 | C. C. Line | | | 2, °V | | | | | | | | | | |
| | | , 6 <u>7</u> 2 | | | |) ~ | | | | | | | | | |
| | E DI | | a1 . | | -C | | | | | | | | | | |
| | |) O | |) = // | Ų | | | | | | | | | | |
| | CO. | | | | | | | | | | | | | | |
| | | | [0.7] 1044 100.2 [-0.2] 91.21 [8.8] [22.9] | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | |



Table CA 5.8.3/10-2: Steroidogenesis activity of spiroxamine: Experiment 1, performance criteria

| | | | Testosterone | al [©] | | Estradiol > | |
|---------------------------|-------------------------|--------------|--------------------|-----------------|----------------------|-------------------------------|--------------------------|
| | Comparison between | Performance | QC plate 🧣 | Spiroxamine _ | Performance | QC plate | Spiroxamine |
| | Comparison between | criteria | | plate 🦿 🦠 | criteria 🤟 | | پرچ plate |
| LOQ | NA | NA | 50 pg/mL | © 50 pg/m@b≥ > | NA ZO | 31 3 pg/mL | 31.3 pg/mL |
| Basal production of | Absolute concentration | NA | 1591 pg/ml | 1742\pg/mL_% | NAQ NA | 』 1¥8 pg/m√ | 120 /pg/mL |
| hormone in the SCs | Fold-greater than LOQ | ≥ 5-fold | © 32-folk | √ Ø35-fold Ø | <u>≥</u> 2Ø-fold \ € | [™] 3.8- © id | €0 ⁸ 3.8-fold |
| Within plate CV for SCs | Absolute concentrations | ≤30% | £152% @ | 5,6% | 30% 5 30% | , \$11% ~ C | 4.9% |
| (replicate wells) | | | | 20° A | Or Br | | |
| Induction | Fold-change | ≥ %5-fold, × | 1.9-fold | - 0 · 0 | \$ 69.5-fold | 37-fold | - |
| (10 µM forskolin) | compared to SC | | 0° 2 | | (, ⁾ | | |
| Inhibition | Fold-change | × ≤ 0.55fold | \$\times 0.077-fg@ | | ≤@3-fold | ≤ 0.26 fold a | - |
| (1 µM prochloraz) | compared to SC | |) | | | ant De | |
| Hormone measurement | Detectable fold-change | © ≥ 5-føld | 32-fold | 3.Pfold 20 | ≥ 2.5 and | ©3.8-fold | 3.8-fold |
| system: Sensitivity | relative to SC | | | 3 000 | | ð | |
| Hormone measurement | Absolute oncentrations | ~ | | | © ≤ 25% × | ≤113% | ≤18% |
| system: Replicate measure | | | 272 1 2 On | | | | |
| CV for SCs | CO S | | | | , Ŝ | | |

The may be subject that the right and the right the right that the right the right that the right the right that the right the right that the right 

C. Steroidogenesis assay – Experiment 2:

QC plates for the test article plates, estradiol and testosterone confirmed that the 1. QC plates:

acceptability criteria were met. Since progesterone is not an endpoint included in

OECD guideline 456, there are no acceptance criteria avai@ble.

The average basal testosterone production in the DMS@solvent control wells on 2. Testosterone analysis:

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 Gerage relative Change of the test article exposed wells to the solvent treated control wells ranged from 0.63 to \$2. A spatistically significant decrease in testosterone production was observed at 0.1, 0516, 15.16 and 10 μM spiroxamine. Overall the resultowas concluded to show evidence of a @

decrease in testosterone sonthesis.

The average basal estraction production in the DMSO solvent control wells on the 3. Estradiol analysis:

test article plate was 1/1 pg/fal. The average estracted 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 1.40 A statistically significant decrease in @stradion production was observed at 0.1 up and statistically



2021-03-31 Document MCA - 5: Toxicological and metabolism studies

Spiroxamine Spiroxamine

Steroidogenesis activity of spiroxamine: Experiment 2 Table CA 5.8.3/10-3:

| M/TPT (0/ *. | .1.114 [0/ . 4.4. | *:4.1 | Tr | 44 | | | . | | 1.4 1 8 | <u> </u> | | Opr | | |
|----------------|--|----------------|---------------------------------------|-----------------|--------------------|----------------------|-------------|--|---------------------------------------|--|---------------|----------------------|--------------|----------------|
| | ability [%cytotox | • •/ | | tosterone (r | | nge) | % oc | stradiol (re | lative chang | | | | elative char | |
| QC plate | | mine plate | | plate | | nine plate | Q | plate | Spiroxai | nine plate | © QC | plate | Spirøxan | nine plate |
| Blank 103 | | - | Blank | 1.03 ± 0.04 | - | - % | Biank ⊗ | plate 1.00 ±0.10 1.00 ±0.03 | D " | \$ °- | Blank | 1 =0.07 | | - |
| 0a 100 | | 100.0 | O ^a | 1.00 ±0.02 | O ^a | 1.0000.01 | | 1.00 0.03 | O O O O O O O O O O O O O O O O O O O | 1 00-+0 90 | -0% | 1.00 0.07 | E OF | 1.00 |
| [0. | | [0.01 | | | | 1.000 | | 1.00.3 | | 1.00 ±0.20 | NO P | 1.00000.07 | EO, | ±0.03 |
| For: 105 | | 102.8 | For: 1 µM | 1 85 ±0 08 | 0.001 km | 1 02 ±0 05 | For: 1ы1 | 18 14 ±1217 | 7 0.001 uND | 0 84 ±0.404 | For: 1 18M | 2.60 ±0.10 | 0.001 μM | 0.98 |
| 1 uM [-5 | | [-2.8] | 1 01. 1 01.1 | 1.00 -0.00 | 0.001 kg/ | 1.02 O.W | 3 10 | \$ 7C 3 | المام المام | 0.0. Pri | 101. | | 0.001 pa.1 | ±0.02 |
| For: 116 | | 96.7 | For: 10 uM | 1.82 ±0.08 | 0.01 μΜ | 0,95 ±0.03 | For: 10 uN | 18.14 ± 17.7 18.14 ± 17.7 18 | @.01 μMs | ©76 ±0.13 | ₽or: 10 uM | 2.78 ±0.07 | 0.01 μM | 0.90 |
| 10 uM [-16 | 6.51 | [3.3] | | , Å |) · 3 | D. F | | ±14,5% | | - */ | |) } | • | ±0.02* |
| Pro: 0.1 99 | 0.1 μΜ | 111.6 | Pro: 0.1 uM | 0.27 0.01 | 0 <u></u> MM | 0.700±0.03* | Pro: Q.1 ul | M 0.623±0.01 | Q JAM | 0.64-20.15* | Pro: 0.1 uM | 3. 82 \$±0.14 | 0.1 μΜ | 0.53 |
| uM [0. | .5] | [-11.6] | a | | | D. C. | . 0 | r e d | 6 0° | M. C. L. | | 8N >> | | ±0.02 |
| Pro: 1 106 | 6.3 0.316 μM | 103.8 | Pro: 1 | 0.07 +0.00 | $0.316 \mu M$ | 0.64 ±0. 04 * | Pro: 1 uN | 0.21 ±0.02 | 0.316 M | 0.92 ±0005 | Pro: 1 100111 | 21.5 ± 0.55 | 0.316 μΜ | 0.52 |
| uM [-6 | [.3] | [-3.8] | | | | ON Pr | - Os | | 0,- | | × S | | | ±0.04* |
| | I μM | 101.5 | | 0. | P ^{ol} μM | 0.83 ±0.00 | | . 1 | DIμM (| 0.92 ±0.03 0.86 ±0.03 0.88 ±0.13 | | | 1 μM | 0.47 ±0.04* |
| | 2 16 uM | [-1,5] | | | 2 1A41M | 0.600002* | \$ Ob' | L. R. Mar. | 2. 10 VIV | 0.000012 | | | 3.16 µM | 0.39 |
| | 3.10 μΙνΙ | [4 0] | OF | *O | β.10 μΙνΙ | 0.04 \$20.02 | | | μνι γ | , Ø.00±0.13 | | | 3.10 μΙνΙ | ±0.01 |
| | 10 µM | 88.8 | | | 10.61/60> | 0.63 +000 | | - & | 10 m | 0.88 20.13 0.88 20.13 | | | 10 μΜ | 0.28 |
| | 10 μινι | [11.2] | \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ | ~K\$ | 10 301 | 0.05 70.01 | 47.5° | 0" | | 1.11 =0.03 | | | 10 μινι | ±0.01 |
| <i>p</i> ≤0.05 | • | 1 | | | 2 2 2 2 | | | e* . | | -I | <u>.</u> | | | l. |
| or: forskolin | | g ^V | L. D. | |), O., | | | | ? | | | | | |
| ro: prochloraz | | | Á | | 101 | -4O> | 0, | | | | | | | |
| | .1 | y | | | | 0, 70 | | | | | | | | |
| | | | | | | | | | | | | | | |
| | | | 90 4 | | | O | | | | | | | | |
| | | 16 | | | | | * | | | | | | | |
| | | | Q 0. | AFF. | | all. | | | | | | | | |
| | | 1 | J a | | | 3 | | | | | | | | |
| | -4.C | Or I | , | | , K. | 9 | | | | | | | | |
| | | . al 1 | | *O& * | · 102 | | | | | | | | | |
| | | | | | | | | | | | | | | |
| - N | | | | | | | | | | | | | | |
| | | . 0 | , K. | P . | | | | | | | | | | |
| | | |)~ ~0(| | | | | | | | | | | |
| ۵۵ | The Office | | , | | | | | | | | | | | |
| C | | | | | | | | | | | | | | |
| | | 4. | | | | | | | | | | | | |
| | | | | | | | | | | | | | | |
| | 0.5 0.1 μM 5.5 0.1 μM 6.3 0.316 μM 3.16 μM 10 μM | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | |



2021-03-31 Document MCA - 5: Toxicological and metabolism studies

| | | | | Docum | icht MCA - 5. 10. | Alcological and inc | |
|-----------------------------|--------------------------------|------------------|---------------------|----------------|---------------------|-------------------------------------|-------------------------|
| | | | | | | à | Spiroxamine |
| Table CA 5.8.3/10-4: Stere | oidogenesis activity of spirox | amine: Experimen | at 2, performance | criteria 🎉 | arti ar | , (°) | |
| | | | Testosterone | 10 | | Egradiol % | |
| | Comparison between | Performance | QC plate 🔊 | Spiroxamine | Performance | CC plate | Spiroxamine |
| i | • | criteria | Or | plate 💆 | criteria 🔪 🍳 | | 🎺 plate |
| LOQ | NA | NA | 50[pg/mL _ @ | 50 pg@mil 🛴 | O NA O | 3103 pg/mL | [≫] 31.3 pg/mL |
| Basal production of hormone | Absolute concentration | NA | √1681 pg/ma | 1964 pg/mL | NQ V | r ¥62 pg/mi∑ | ∡Ø1 pg/mL |
| in the SCs | Fold-greater than LOQ | ≥ 5-fold | 34-fold * | @ 39-fold | 2 .5-fold\ ○ | 5.2Gold | © 3.6-fold |
| Within plate CV for SCs | Absolute concentrations | ≤30% | \$2.4% | 2.2% | 3000° · . | \$ 3.1% C | 20% |
| (replicate wells) | | ~~ ~ ~ ~ ~ ~ ~ ~ | | | 20 0 1 12F | | |
| Induction | Fold-change | ×3.5-fold € | 1.8-föld | * <u>*</u> O** | | _{€¶} 69ٌ - fold | - |
| (10 µM forskolin) | compared to SC | | | 1 0 2K | | | |
| Inhibition | Fold-change | ≤ ੴfold | > 0.07 fo rd | 40° - 40° | 50.5-fold | 0.21% fold | = |
| (1 μM prochloraz) | compared to SC | al al | | | TIME STATE | ant Char | |
| Hormone measurement | Detectable fold-change | ≥ 5 fold | 34-fold | gg-fold | ≥ 2 Mold | © 5.2-fold | 3.6-fold |
| system: Sensitivity | relative to SC | | -4 | 20° | | | |
| Hormone measurement | Absolute concentrations | -0° | 30°- | | < 25%≫ | ≤16.3% | ≤26% |
| system: Replicate measure | | | The Source | | Op | | |
| CV for SCs | CO S | | | | ß | | |

and violate bernission and violate be prohibited and violate



C. Steroidogenesis assay – Experiment 3:

QC plates for the test article plates, estradiol and testosterone confirmed that the 1. QC plates:

acceptability criteria were met. Since progesterone is not an endpoint included in

OECD guideline 456, there are no acceptance criteria available.

The average basal testosterone production in the DMSQ solvent control wells on 2. Testosterone analysis:

the test article plate was 1589 pg/mL. The average test osterone concentrations in the medium from the H295R cells exposed to the test article manged 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 1.67. A stanstical of significant decrease in testosterone production was observed & 0.00000.01, 11, 0.316, 1, 3.16 and 10 μM spanoxamine. Overall the result was concluded to show

evidence of a decrease in testosterone synthesis.

The average basal estration production in the DMSO solvent control wells on the 3. Estradiol analysis:

> test article plate was 90 pg/mJ. The average ostradio concederations in medium from H295R cells exposed with test article ranged from 45 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 0.5340 1.45 A statistically significant decrease in estradiol production was observed at 0.001, 0.01, 0.16 μM and a statistically significant increase in estradiol production was observed at 10 μM

whe Dysko solvent control agenage progesterine conferental expectation of a decrease relative change of the fest article expectation was observed at 0.001, 0.01, verall the result was confeduded to show evidence of a decrease sometime.

In progesterine production was observed at 0.001, 0.01, verall the result was confeduded to show evidence of a decrease sometime.

In progestering production and positive control inhibitor prochlors induced acceptable fold charges to the sylvent control for testosterone and estração plates when a sessessed agaings the assay performance criteria, with all other pagameters ghassing the performance criteria whereby confirming the sensitivity and specificity of the assay test system.



Document MCA - 5: Toxicological and metabolism studies **Spiroxamine**

Steroidogenesis activity of spiroxamine: Experiment 3 Table CA 5.8.3/10-5:

| 3 57000 | (0/ 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 | FO / / : | • • • • | - | | | | | 0 3 | | <u> </u> | F. \$ | (O) (O) (O) | | |
|-----------------|---|----------|------------------|--|--------------------------|------------------|--------------|----------------|------------------|----------------------|--|------------------------------|-------------|--|----------------|
| | | | [%cytotoxicity]) | | | elative cha | | | stradiol (re | ative chang | (e)° | Progesterone velative change | | | |
| | olate | Spiroxan | iine plate | QC ₁ | | Spiroxan | nine plate | QC J | plate | Spiroxar | nine plate | | plate 🔑 | Spirøxan | ine plate |
| Blank | 98.2 [1.8] | - | - | Blank | 1.04 ± 0.00 | - | - .eV | Blank | 1.00 ±0.05 | 9. J. | ₹ ~0 [§] | Blank (| plate ±0.03 | S | - |
| 0 ^a | 100.0 [0.0] | DMSO | 100.0 [0.0] | O ^a | 1.84 ± 0.01 | O ^a | 1.00 0.02 | | . > | O O O | \$00 € \$\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\\ | 100 | 1.00 ⊕0.02 | a final contraction of the contr | 1.00 ±0.01 |
| For: 1 uM | 105.1 | 0.001 μΜ | 100.3 | For: 1 uM | 1.84 ± 0.01 | 0.001 | 1.07 ±0.61 | For: 1 July | 28.8 % | 0.001 μΜΦ | 0.72 ±0.10 | For: 1 ut | 2.42 ±0.62 | 0.001 μΜ | 1.00 ±0.02 |
| For: | 109.9 | 0.01 μΜ | 100.2 | For: 10 uM | 2.18 ±0.02× | 0.01 μM | 0.98 ±0.01 | For: 10 uM | 65.21 | Φ.01 μM _s | 0.62 | ⊕ or: 10 uM | 2.57 ±0.08 | 0.01 μΜ | 0.89 |
| 10 uM | [-9.9] | | [-0.2] | | | () | ∂ , ₹ | | ±2,35@ | | ±0.07* | | , | | ±0.01* |
| Pro: 0.1 uM | 97.9 [2.1] | 0.1 μΜ | 106.9 [-6.9] | Pro: 0.1 uM | 0.29° ≱ 0.01 ∜ | 00 MM | 0.78€¥0.01* | Pro ON uM | 0.78° ±0.03 | 0.78¥0.1 DµM | ₩ 33 1 2±0.16* | Pro: 0. uM | | | 0.56 ±0.03* |
| Pro: 1 | 103.7 [-3.7] | 0.316 μΜ | 101.3 | Pro: 1 | 0.08 ±0.00 | 0.316 μM | 0.67 ±0.001 | Pro: 1 uM | ≤0.35 | 0.316 M | 0.690 | Pro: 1 🐠 | 16.89 ±0.51 | 0.316 μΜ | 0.56 ±0.03* |
| WITT . | [3.,] | 1 μΜ | 101.3 | PC 31 | | ¹ μM | 0.68 ±0.02* | | Č _N 1 | \$ 1 μM | 0.76 | | | 1 μΜ | 0.46 ±0.00* |
| | | 3.16 μΜ | 10P.4 | all of the second | ,0 | 3.18 µM | 0.67 0.01* | | | Z ÎΣμΜ | 0.824.17 | | • | 3.16 μΜ | 0.38 |
| | | 10 μM | 89.3 | | | 10 (a) NO | 0.68 #0.92 |)> (_@ | | 1,0 ps/12 | 1.45 | | | 10 μΜ | ±0.02* 0.32 |
| <i>p</i> ≤0.05 | | | [10.7] | | | | | J.S | »\$ | 4 3 3 S | ±0.06* | | | | ±0.02* |
| or: forskol | in | | ĠŪ. | | | 9 | | | | | | | | | |
| o: prochlo | oraz | * | PC .(| 98. J | ST. | OD | 0 <u>1</u> 2 | e × | . C | | | | | | |
| | | Too's | C` | | | | | | | | | | | | |
| | Z.* | | \ <u>\</u> | | | | | J ² | | | | | | | |
| | v | | # Dig. | al Politic | e#P | | OTDOS | | | | | | | | |
| | | | | | | |) | | | | | | | | |
| | | | J ' | | Son of | | | | | | | | | | |
| | | | | | OLO _I | P 2 | | | | | | | | | |
| \(\frac{1}{2}\) | | | C | | 2 °F | | | | | | | | | | |
| | COURS | OIL I | | > | | | | | | | | | | | |
| | | 4 | | | | | | | | | | | | | |
| | | | | For: 10 uM Pro: 0.1 uM Pro: 1.00 Pro | | | | | | | | | | | |
| | | | | | | | | | | | | | | | |
| | | | | | | | | | | | | | | | |



Document MCA - 5: Toxicological and metabolism studies Spiroxamine

Table CA 5.8.3/10-6: Steroidogenesis activity of spiroxamine: Experiment 3, performance criteria

| | | | | | -4/2 | ~ " | \bigcirc |
|-----------------------------|-------------------------|---------------|------------------------|--------------------|--|--|----------------|
| | | | Testosterone @ | 4 | ~0 [©] ″ | Egradiol | (Car |
| | Comparison between | Performance | QC plate 💖 | Spiroxamine | Performance | CC plate | Spiroxamine |
| | Comparison between | criteria | | plate 🖁 | criteria 📈 🏽 | | 🎺 💆 plate |
| LOQ | NA | NA | 50[pg/mL _ @ | , 50 pg@miL , | O NA O | 3103 pg/mL | 31.3 pg/mL |
| Basal production of hormone | Absolute concentration | NA | ~(1¥74 pg/m a > | 1 589 pg/mL | N N | ≥ 90 pg/r0L | № pg/mL |
| in the SCs | Fold-greater than LOQ | \geq 5-fold | 29-fold _ | 32-fold | 1 | 2. Grold 🙊 | © У 2.9-fold |
| Within plate CV for SCs | Absolute concentrations | ≤30% | £ \$3.6% e | 1.7% | \$ | \$ 5.4% ~ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ \ | 9.6% |
| (replicate wells) | | | | 20° A | Org. | | |
| Induction | Fold-change | * 34.5-fold | 2.2-főld 🧳 | D O. | . ⊙≥7.5-fold > | 65-fold | - |
| (10 μM forskolin) | compared to SC | | | | | ~8 ³ | |
| Inhibition | Fold-change | ≤ ੴfold | > 0.079 -16 1d | - 200 - | (1).5-fold | 0.35⊈old | - |
| (1 μM prochloraz) | compared to SC | al all | ~. | | | «MDD | |
| Hormone measurement | Detectable fold-change | ≥ 5 fold | 29-fold " | 92-fold | ≥2,5 Yold | © 2.9-fold | 2.9-fold |
| system: Sensitivity | relative to SCO | | -2 | \$ 0° | | | |
| Hormone measurement | Absolute concentrations | | 36 1 | | © ≤25% | ≤14% | ≤16% |
| system: Replicate measure | | | | | O | | |
| CV for SCs | | £ . 1 | | | l Ġ | | |

and the state of t



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 steroit genesis in human adrenocortical carcinoma cell line H295R following three independent experiments. Statistically significant decreases in oestradiol, testosterone and projecterone were observed.

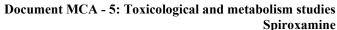
| Data Point: | KCA 5.8.3/11 & & & & & & & & & & & & & & & & & & |
|--------------------------------|--|
| Report Author: | |
| Report Year: | 2021 |
| Report Title: | Spiroxamine: Proposed mode of action for chalesterol perturbations and the |
| | I KHOCK OH COHSECUCIACES ./. " " " " " " " " " " " " " " " " " " |
| Report No: | knock on consequences 0 4 5 0 4 1 0 4 1 1 1 1 1 1 1 1 1 1 1 1 1 1 1 |
| Document No: | M-763156-01-2 0 |
| Guideline(s) followed in | None * & & & & & O & Y |
| study: | |
| Deviations from current | Nene & S & S & S & S & S & S & S & S & S & |
| test guideline: | |
| Previous evaluation: | No, not previously suppritted \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ \\ |
| GLP/Officially | not applicable |
| recognised testing facilities: | |
| facilities: | |
| Acceptability/Reliability: (| Supportive of the support of the sup |

Executive Sunonary

Spiroxamine (8-tert-butyl-1,4-dioxaspiro[4.5]decan-2 ylmethyl(ethyl)(propyl)amine), is a tertiary amine funcicide of the chemical class of spiroketalamines acting through sterol biosynthesis inhibition. It is used solely as a funcicide belonging to the amine group, FRAC code 5 fungal control agents, which inhibit δ 14-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 spirodimine in the wirro 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 modulities.

This position paper provides an understanding of the *in vivo* consequences of inhibition of this enzyme, which do not drive my 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 decreased 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 dexicity. In addition to the apical studies, results from ToxCast ER and AR indicate that proxamine 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 storoidogenesis pathway is not inhibited. The reductions in steroid hormone production observed in the movitro 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 deven 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 on 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* steroid ogenesis data as not appropriate or scient fically distifiable as *in vivo* adversity is not observed across a comprehensive in vivo mamma and data package, with no biological plausible link evident between the *invitro-fit 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 upsteam 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 (\$9), whereas *in vivo* ADMir 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 dexicity studies. Addenal weights and adrenal histopathology were unaffected across a moriad of studies. Because steroidogenesis is among the functional roles of the adrenal glands, the obsence 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, trigly crides, 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 pro-neoplastic bosons or tumour production in EAS sensitive producing organs (in two separate rodent models), no MPG 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 2/4, A-receptor architecture, with further Level 5 *in vivo* studies to address the *in vitro* steroidogenesis data not required.

CA 5.9 Medical data

Occupational health streetlance 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 and 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 symmic 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 indings 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 mathematical dependence of the Shelanski & Shelanski Repeated Insult Patch Test RIPT conducted under double blind conditions with doses of ranging from 0.027 to 1.02%. Dermat occlusive patches containing 0.15 mL/patch was applied to the upper agains. The study was split into the following phases: i) nitial exposure (induction) phase was 4 weeks with repeated daily application for 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 sensitivation feaction 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 | Species | Results Comments of the Commen | Classification | Annex CA |
|--------------|----------|--|--|-------------|
| study | | | Annex I for Regulation | Point / |
| · | Species | | Annex I for Regulation (EG) 12722008) | Reference |
| Skin | Human | I U 2970 CHQ/HOLEHCH/TEVEASLAHV | Studyasot sustable for | CA 5.9.2/01 |
| irritation 🍖 | | ©kin irr@ating or skin sensitising properties (abbreviated version of the | classification | |
| Č | o' « | sensitising properties, | | (2001) |
| | ₩ | (abbreviated version of the | | |
| | Human | Špělanskí & Shelanski KPT) 🦨 | | |
| Skin ** | Human | 1.02% did not elicit reveal any | Study not suitable for classification | CA 5.9.202 |
| irritation | , ° | skin writating or skin | classification | |
| | | sensitising properties | | (2000) |
| (| 7) Ô | Cobbreviated version of the | | |
| ~ | O A | Shelanski & Shelanski RIPI) | | |
| 4 | O' | | | |
| Ø" | , Ø | | | |
| | | | | |
| | | | | |
| Y | o' | | | |
| J | | | | |
| Ő | | | | |
| | | | | |
| Ű | | | | |
| | d e | | | |
| | | J [*] | | |
| | | | | |
| , O | | spelanski & Shelanski Kir I) y 1.02% did not sficit reveal any skin firitating or skij sensitising properties (abbreviated version of the Shelanski & Shelanski RIPT) | | |
| Õ | | | | |
| | | | | |



| Data Dainte | W.C.A. 5.0.2/01 |
|----------------------------|--|
| Data Point: | KCA 5.9.2/01 |
| Report Author: | |
| 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: | <u>M-086474-02-1</u> |
| Guideline(s) followed in | not applicable |
| study: | |
| Deviations from current | None V Q Q Q X |
| test guideline: | |
| Previous evaluation: | yes, evaluated and classified of the control of the |
| | DAR (2010) |
| GLP/Officially | Yes, conducted under GP/Officially repognised testing facilities |
| recognised testing | |
| facilities: | |
| Acceptability/Reliability: | Supportive only A |

Main report

Executive Summary

An intensified version of the Shekaski & Shelanski Repeated Insult atch Test (RPT) was conducted under double blind conditions with the intention of dentifying 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 lead 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 lased on preliminary investigations

Dermal, occlusive patches containing 0.15 mt/patch was applied to the upper arms. The study was split into the following plases: i) Initial exposure (finduction) phase was 4 weeks with repeated daily application for 4 days/week; ii) Intermediate phase (fest period) allowed normalisation of the skin following any adverse effects. This allowed the opportunit 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, 0.06 and 0.20% sproxamine have no skin sensitising properties in humans.

Under the conditions of this study spiroxonine of to 02% did not reveal any skin irritating or skin sensitising properties in human volunteers using the lotensified Shelanski Repeated Insult Patch Test (RIPT).

Materials and Methods

A. Materials:

1. Test Material. Souroxamme

Talternative name: [8-(1,1-dimethylethyl)-N-ethyl-N-propyl-1,4-

dioxaspiro[4©]decane-2-methanamine; KWG 4168)

Description Viscous yellow liquid

Lot/BatcloNo.: \$\ \pi\002/90 \\
\text{Durity:} \ \text{CAS.No.:} \ 118134-30-8

Stability of test Assumed stable for the duration of the exposure

compound:



2. Vehicle and/or positive 0.2% Cremophor® EL in saline / not relevant

control:

3. Test animals:

Species: Strain:

Age at dosing:

Weight at dosing: Source: **Acclimation period:** Diet: Water: Housing:

4. Environmental conditions:

> **Temperature: Humidity:** Air changes: **Photoperiod:**

B. Study Design:

1. In life dates:

2. Study design:

ant
velevant
Not relevant
Not relevant
Not relevant
Not relevant

1 in inchsified Version of the Shelanski & Shelanski Repeated

RIPT) was conducted unter doubte blind conditions with
ontifying irritation and Sensitisation responses. Gronwere Osed. Tile studywas conducted in two starself-ects of nominal doses of 10 22%, 0.066°
wamine (as solution in 0.2% Cremon'
studied on the subjects in both st
in were used to load the parclim, 23.0 up to 1.00 and in the parclim, 24.0 up to 1.00 and in the parclim, 25.0 up to

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 bjects who may not have completed the patch application

Challenge (elicitation) phase: 4 consecutive days.

ria: (

| 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 | Fricay |
| | | Activation/ind | uction phase | | |
| Wk 1 | B/A | R/E/A | R/E/A | R/19A | R/E |
| Wk 2 | E/A | R/E/A | R/E/A | E/A | R/E |
| Wk 3 | E/A | R/E/A | B/E/A | R/E/A | KE S |
| Wk 4 | E/H | (E)H | ♥(E)H | (E)H | $\mathcal{S}(E)$ |
| | | Challenge | e S hase | 0' 5 | |
| Wk 5 | B/A | R/E/A | R/E/A Q | °R/E/ A € | RÔE C |
| Wk 6 | E/D | | | 03 Q' \ | |
| | Dose conc | entration (%) : | applied to @ach s | yddject 🔗 🛚 🦠 | |
| Band L1: 0.2% | Band L2: 0.066% | 6 Band L3: | \$62% Bar | id L5: 0.2% | Band L5: |
| SPX | SPX | SR | | mop ho r® EL 💇 p | ohysiological salipe° |

- B: baseline examination
- R: patch removed under supervision
- D: subject discharged
- A: patch applied

E: sife examined and grade recorded

H: Matus (Test period) or application to make up for any with missed during induction phase

C. Methods:

1. Homogeneity and achieved concentration analysis of the dose:

Not@ndertak@i

2. Observations:

The application sites were observed daily, 5/days a week. The occuled patching device (consisiting of 4.4 cm square impermeable plastic film with adhesive coating on one of de and 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. Preliminar investigations

Preliminary investigation confunded that 0.20% was the highest spiroxamine concentration which was tolerated without any visible writation after repeated dermal application for up to 4 days. Higher concentrations of 0.30% up to 1.02% induced gress skin changes.

C. Øbservations:

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% piroxamine have no skin sensitising properties in humans.

3 rollow 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 |
|----------------------|--|
| gra | de/individual participant |

| Condi | | Induc | tion phas | se (%) | | | Cha | allenge pl | hase | |
|------------------------------|------|-------|-----------|--------|--------------|-------|----------------|-------------|-------------------|----------------|
| Grade | 0.20 | 0.066 | 0.02 | Crema | Saline | 0.20 | 0.066 | 0.02 | Crem ^a | S aline |
| 0 | 210 | 210 | 210 | 208 | 206 | 204 | 204 | 2 04 | 204 🎝 | 204 |
| 1 | 0 | 0 | 0 | 1 | 3 | 0 | 0 4 | 0 | Q:> | |
| 2 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | ~ 9° | \$ 0 K |
| 3 | 0 | 0 | 0 | 0 | 0 0 | 0 | Ø, | 0 | № ′0 🦠 | |
| 4 | 0 | 0 | 0 | 0 | Õ & | 0 | \mathbb{Q}^0 | 0 0 | | (A) |
| No. providing data | 210 | 210 | 210 | 209 | 1 000 | 204 A | 204 | 20 | 29 4 | 204 ° |
| No. not providing data | 1 | 1 | 1 | 1 & | | | | 77 7 | 7 0 | |
| No. of responders | 0 | 0 | 0 | | 3 (V | 0.0 | | \$ | | |

a 2% Cremophor® EL

D. Deficiencies:

None.

Assessment and conclusions by applicants

Assessment: This study is deemed supplementary

Conclusion: Under the conditions of this study, sproxamine up to 0.2% did not reveal any skin irritating or skin sensitizing properties in human colunteers using the latensified Shelanski & Shelanski Repeate Insult Patch Fest (RFPT).

Supplement to M-686474-92-1

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 elected cross dermal effects during of following a 24 hour exposure period under occluded conditions, along with the course of a consecutive applications of 24 hours in duration to provide substantial enough data to confin 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 aline) 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 1.2, 0.3, 0.45, 0.68 and 1.02% to group of 10 further males were treated.

Dermal, occlusive patches containing 0.05 mL/patch was applied to the upper arms. The study was split into the collowing phases: i) initial exposure (induction) phase was 3 weeks with repeated daily application for 4 days/weeks 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 concertation that did not elicit a dermal reaction in human volunteers using the abbreviated version of Shelanski & Shelanski Repeated Insult Park Test (RIPT).

Materials and Methods

A. Materials:

1. Test Material: Spiroxamine

(alternative name: [8-(1,1-dimethylethyl)-N-enyl-N-propyl-1,4-

dioxaspiro[4,5]decane-2-m@nanamine; KW 4168

Description: Viscous yellow liquid

Lot/Batch No.:Not providedPurity:Not providedCAS No.:118134-30-8

Stability of test Assumed stable for the duration of the exposure

compound:

2. Vehicle and/or positive 0.2% Crept phore EL in saline

control:

3. Test animals:

Species: Human Not relevant Strain: Not relevant Strain: Age at dosing: Strain: Not relevant Strain Not relevant Strain Not relevant Strain Not relevant Strain Not relevant Strain Not relevant Strain Not relevant Strain Not relevant Not relevant Strain Not relevant

Weight at dosing: Not relevant Source: Not relevant

Acclimation period: Not servent

Diet: Not releant
Water: Not releant
Housing: Not releant

4. Environmental conditions:

Temperature: Not recevan

Air changes:

Photoperiod:

Note elevant

No

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

Cold: 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 \circ 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 \circ 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
- nonowing 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 rphological

3. Evaluation criteria:

| | · 477 | Ø) * | <u>ر</u> آ | |
|------------------------------|------------------|------------------|------------------------|------------|
| Morphological | Visible hange | Q | W . | Grade |
| change | a.y | | | |
| Absent | None | Q' &° | | 00 |
| Vascular dilatation | Redness: faint t | ⊗ mode®ate, wi | th distince | |
| Q, | border 5 | ' LY L | ′ (\$) [*] `~ | |
| $\mathbb{O}_{_{\mathbb{A}}}$ | Re@ness: mødei | | | 24 |
| | Redness Intens | with distinct | border O | Or " |
| Infiltration () | Redness, plu | edemá or papu | Pes ≼ | 4 |
| | Redfess, plus v | esicæs, blisters | or ballae 🧳 | 5 0 |
| | Redness, plus e | xaension beyon | d Forder V | <i>6</i> 9 |

4. Statistical analysis:

Table CA 5.9.2/02-1: regimen

| Group size | | | 21 8 | | | , | |
|--|-------------------|---------------------|--------------|--|----------|-----------------|--|
| Treatment day | Monday | Tuesday | Wedne: | | Thursday | Friday | |
| | | ✓ Activation/in | duction/pha | so 🖔 | | | |
| | B /P | √ √ E/P √ | S R/E | P | R/E/P | R/E | |
| Wk 2 | OE/A | [∞] R/E/P/ | R P | W/// | R/E/P | R/E | |
| Wk 3 | | RAF P | R/E/ | P. S | R/E/P | R/E | |
| Wk 4 O | HOM) | H(M). | | | H(M) | H(E) | |
| , Q | Chollenge phase Q | | | | | | |
| Who 5 | ĈE/B/P\$ | R/E/P | R E/ | P | R/E/P | R/E | |
| ₩k 6 | E/D | | | ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ | | | |
| * | | tration (%) app | lied to each | subject – G | roup 1 | | |
| Band L1: 0.027% | Band L2: 9.04 | 4%,≪√ Roand L | 3: 0.06% | Band L5: | 0.09% | Band L5: 0.135% | |
| SPX | Q SPX | | X S | SPX | | SPX | |
| Dose concentration (%) applied to each subject – Group 2 | | | | | | | |
| Band L1; 0.2% | Bord L2:0.3 | 8% 🔏 Bander L | 3: 9,45% | Band L5: | | Band L5: 1.02% | |
| SP | SPX | S S | P\$V | SPX | ζ. | SPX | |

- B: basetine 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

1. Homogeneity and achiev@d concentration analysis

. Not windertaken

of the dose; 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 arriver 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 of the skin perceptible at the ski

after repeated dermal application of solution of solution of solutions on the solution of solutions of the solution of solutions of the soluti

0.135% spiroxamine.

2. Group 2: The solution containing 2 spiroxamine was the highest concentration which

did not elicit any gross kin changes.

The solution containing 1.07% spirs aming was the highest concentration which did not elicit gross changes that remained (L. not feversible) over the weekend.

Table CA 5.9.2/02-2: Summary of human repeated insult patch test scheme: maximum assigned grade/individual participant

| | | | ـــالم | O | * | / // | | ~ .~ | ~ ~ % | |
|---|-------------|-------------------|----------------|---------------------------|----------------|--------------|---------------|----------|-------------------|--------|
| Grade | Group 1 (%) | | | | , | S Geoup 27%) | | | | |
| | 0.027 | 0.04 | , 0.06 | 0.09 | Q. 13 5 | 90.20 | v 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 | | * | & , | 8 | | 4 | | | | |
| No. providing | 10 | Ø 10 | O'10 & |) 10. | 10 | ۷9 « | , § 9 , % | > 94) | 9 | 9 |
| data | | » | Q | | | | | | | |
| No. not | 1 🖔 | 1 | , © | \mathbb{O}_{l} | 🔊 1 % | 1 | <u>&1</u> | | 1 | 1 |
| providing data | | 10 | | | Ž | | 0 | ~ | | |
| No. of | 040 | Ø/10 _s | ©0/10° | 0/10 | 2000 | Ø1/9 | 4/9 | 7/9 | 7(2)/9 | 9(4)/9 |
| responders | | | , ~ | | | Š Û | | | · | |

D. Deficiencies

None

Assessment and conclusions by applicant

Assessment: This study is decored supplementary.

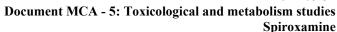
Conclusion: Under the conditions of this study, whilst a concentration of 1.02% spiroxamine produced a visible derma Deaction, up to 1.02% did not cause persistent irritant effects or sensitisation reaction that remained. A concentration of 0.2% was the highest concentration that did not elique a dermal reaction in human volunteers using the abbreviated version of Shelanski & Shelanski Repeated insult Patch Test (RIPT).

A 5.9.3 Direct observations

Epidemiological studies

No epident ological 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 metabolities in blood, wrine or gastrointestinal contents is required for an exact diagnosis of poisoning.

CA 5.9.6 Proposed treatment: first and measures antidotes, medical treatment

The table presented below has been taken from the medical surveillance report presented in the Doc (CA 5.9.1/02 [M-762352-02-1]).

First Aid::

<u>Inhalation</u> Skin Contact - Remove patient from expositive/terminate exposure

- Thorough skin decontamination with copious amounts of water and soap, if available with polyethylene glycol 300 followed by water Note: Nost formulations with this active ingredient can be decontaminated with water (and soap), so for formulations polyethylene glykol 300 is not required

Eye contact Ingestion - Flushing of the eyes with luke warm water for 15 minutes

- Induction of comiting 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 comiting can remove maximum 50% of the ingested substance.

Note: Induction of vomiting a profibited of a formulation containing organic

Solvents has been ingested D

Treatment:

Ingestion

- Gastric lawage can be considered in cases of significant ingestions within the Carst (20 hour (9).
- The application of activated chargoal and sodium sulphate (or other carthartic) should be considered in significant investions.
- -. Os there is no antidote, treatment has to be symptomatic and supportive

(X 5.95) 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 phocosa can be expected from the studies in experimental animals. Liver and eye were target organs in experimental animals.