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**SCCS OPINION ON Dimethylpiperazinium
Aminopyrazolopyridine HCl (A164) - SCCS/1584/17 –
Final Version**

Ulrike Bernauer, Laurent Bodin, Leonardo Celleno, Qasim Mohammad Chaudhry, Pieter-Jan Coenraads, Maria Dusinska, Janine Ezendam, Eric Gaffet, Lodovico Corrado Galli, Berit Granum, et al.

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Scientific Committee on Consumer Safety

SCCS

OPINION ON
Dimethylpiperazinium
Aminopyrazolopyridine HCl (A164)

The SCCS adopted this Opinion
on 6 June 2017

About the Scientific Committees

Two independent non-food Scientific Committees provide the Commission with the scientific advice it needs when preparing policy and proposals relating to consumer safety, public health and the environment. The Committees also draw the Commission's attention to the new or emerging problems which may pose an actual or potential threat.

They are: the Scientific Committee on Consumer Safety (SCCS) and the Scientific Committee on Health, Environmental and Emerging Risks (SCHEER) and are made up of scientists appointed in their personal capacity.

In addition, the Commission relies upon the work of the European Food Safety Authority (EFSA), the European Medicines Agency (EMA), the European Centre for Disease prevention and Control (ECDC) and the European Chemicals Agency (ECHA).

SCCS

The Committee shall provide Opinions on questions concerning all types of health and safety risks (notably chemical, biological, mechanical and other physical risks) of non-food consumer products (for example: cosmetic products and their ingredients, toys, textiles, clothing, personal care and household products such as detergents, etc.) and services (for example: tattooing, artificial sun tanning, etc.).

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http://ec.europa.eu/health/scientific_committees/consumer_safety/index_en.htm

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All Declarations of Working Group members are available on the following webpage:
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This opinion has been subject to a commenting period of 9 weeks (from 12 March 2017 to 14 May 2017) after its initial publication.
There were no comments received and the final version of the opinion remained unchanged compared to the preliminary one.

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1. BACKGROUND

Submission I on the hair dye Dimethylpiperazinium Aminopyrazolopyridine HCl (A164), with the chemical name 4-(3-aminopyrazolo[1,5-A]pyridin-2-yl)-1,1-dimethylpiperazin-1-ium chloride hydrochloride (CAS 1256553-33-9), was transmitted by Cosmetics Europe in March 2016.

The new ingredient Dimethylpiperazinium Aminopyrazolopyridine HCl (A164) is intended to be used in oxidative hair colouring products up to a maximum on-head concentration of 2 %.

2. TERMS OF REFERENCE

- (1) *In light of the data provided, does the SCCS consider Dimethylpiperazinium Aminopyrazolopyridine HCl (A164), safe when used in oxidative hair colouring products up to a maximum on-head concentration of 2 %?*

- (2) *Does the SCCS have any further scientific concerns with regard to the use of Dimethylpiperazinium Aminopyrazolopyridine HCl (A164) in cosmetic products?*

3. OPINION

3.1 Chemical and Physical Specifications

3.1.1 Chemical identity

3.1.1.1 Primary name and/or INCI name

Dimethylpiperazinium Aminopyrazolopyridine; chloride; hydrochloride

3.1.1.2 Chemical names

4-(3-aminopyrazolo[1,5-A]pyridin-2-yl)-1,1-dimethylpiperazin-1-ium chloride hydrochloride

3.1.1.3 Trade names and abbreviations

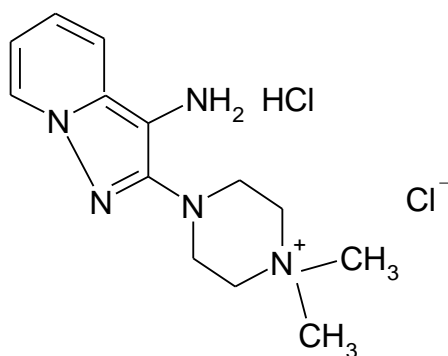
IMEXINE® OBM

3.1.1.4 CAS / EC number

CAS: 1256553-33-9

EC: /

3.1.1.5 Structural formula



3.1.1.6 Empirical formula

C₁₃H₂₁Cl₂N₅

3.1.2 Physical form

A164 is a light grey to dark blue/dark green (when it is oxidised) powder.

3.1.3 Molecular weight

Molecular weight: 318.25

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3.1.4 Purity, composition and substance codes

Chemical characterisation of A164 was performed by IR, ¹H NMR, UV-Visible and mass spectrometry using Electrospray Ionization (ESI) in positive ion mode and HPLC-PDA.

Table 1. Summary of the analytical characteristics of different batches of A164

| Analytical test | 007 L 001 | 002 L 002 | 001 P 001 | 001 P 002 |
|---|---|----------------------|-----------------------------|---|
| Identification | | | | |
| Appearance * | Blue grey powder | Light blue powder | Light blue powder | Light grey powder |
| Infra red spectrometry | In accordance with the proposed structure | Not performed | Not performed | In accordance with the proposed structure |
| UV spectrometry | Compatible with the proposed structure | Not performed | Not performed | Compatible with the proposed structure |
| ¹ H NMR spectrometry | In accordance with the proposed structure | | | |
| Mass spectrometry | Compatible with the proposed structure | | Not performed | Compatible with the proposed structure |
| Assakys | | | | |
| Titre (% w/w) | Not provided | | 99.6 % (NMR) | 98.08 % (HPLC) 98.42 % (NMR) |
| Purity (HPLC) (UV- Area %) | > 99 % | > 95 % | | |
| HPLC Profile | Two impurities (content above 0.1% - Relative purity, UV- Area%) were detected. One of them corresponds to the saturated ring compound (R0069603A). The other is eluted at 21.8 min. Other detected impurities were below 0.1% (Relative purity, UV- area % - Traces) | | | |
| Impurity content: R0069603A (Saturated ring compound) (% w/w) | 0.5% (NMR) | <i>Not performed</i> | 0.3% (HPLC) 0.26 % (NMR) | 0.87 % (HPLC) 0.79 % (NMR) |
| Impurity content Rt : 21.8 | 0.6% (Area) | <i>Not detected</i> | 0.4% (Area) | 0.2% (Area) |
| Solvent content: Isopropyl alcohol (%) | 0.15 % (GC/HS) | <i>Not performed</i> | < 0.1 % (NMR) | 1 % (GC) |

Opinion on Dimethylpiperazinium Aminopyrazolopyridine HCl (A164)

| | | | | |
|--|---------------------------|--------|--------|--------|
| w/w) | | | | |
| Chloride content (Theoretical value: 22.3% w/w) | 21.9 % (Potentiometry) | 21.8 % | 22.6 % | 21.4 % |

* The difference in colour is due to the slight oxidation of the compound. The compound is a light grey powder when not oxidised.

Elemental analysis (% w/w)

Batch 007 L001

| | C | H | N | Cl |
|---------------------|------|-----|------|------|
| Theoretical values | 49.1 | 6.7 | 22.0 | 22.3 |
| Experimental values | 49.0 | 6.7 | 22.0 | 21.9 |

Batch 002 L002

| | C | H | N | Cl | O | Zn |
|---------------------|------|-----|------|------|------|-----------|
| Theoretical values | 49.1 | 6.7 | 22.0 | 22.3 | - | - |
| Experimental values | 48.5 | 6.6 | 21.5 | 21.8 | 1.27 | 213 mg/kg |

Batch 001 P001

| | C | H | N | Cl | O |
|---------------------|------|-----|------|------|-----|
| Theoretical values | 49.1 | 6.7 | 22.0 | 22.3 | - |
| Experimental values | 49.7 | 6.8 | 22.1 | 22.6 | 0.6 |

Batch 001 P002

| | C | H | N | Cl | O |
|---------------------|------|-----|------|------|-----|
| Theoretical values | 49.1 | 6.7 | 22.0 | 22.3 | - |
| Experimental values | 48.4 | 6.8 | 21.5 | 21.4 | 1.9 |

Opinion on Dimethylpiperazinium Aminopyrazolopyridine HCl (A164)

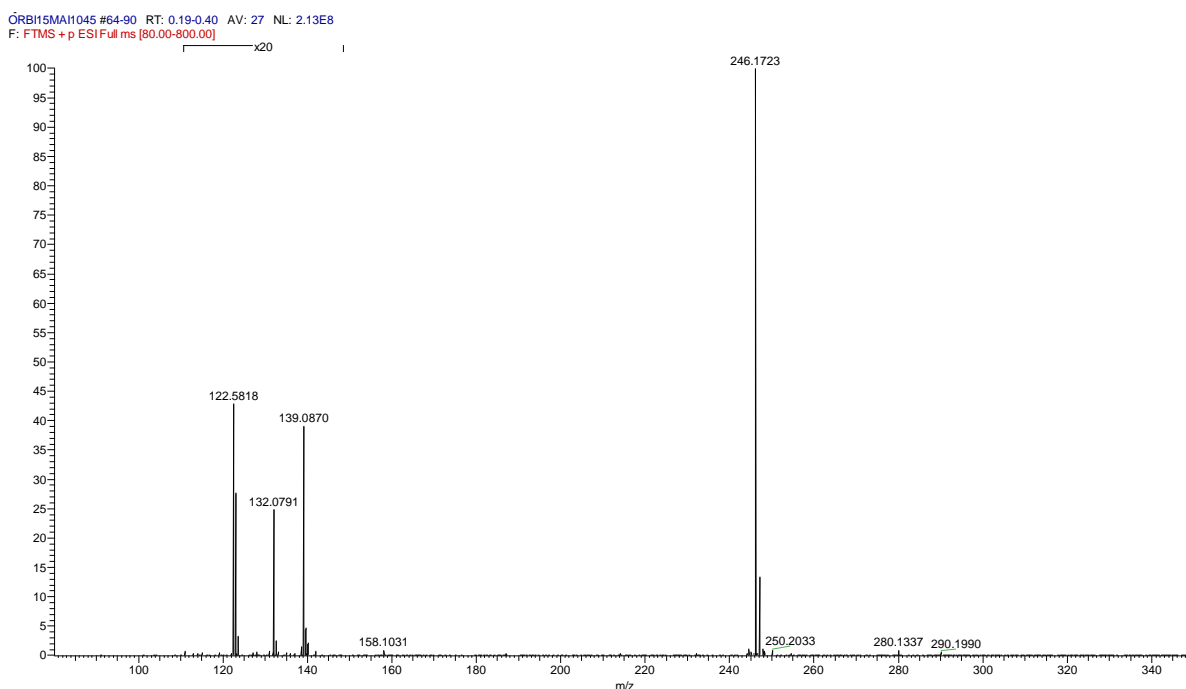


Fig. 1: Positive ion full scan mass spectrum of diluted solutions of A164 in the mass per charge range 50 to 500.

The ultra-violet/visible light absorption spectrum, in the range 200 to 800 nm of a 3.19×10^{-5} mol/L (0.001016 g/100 mL) solution of A164 batch 007 L001 in water, exhibited three maxima at 243nm, 309nm and 609nm.

- the absorbance at 243 nm was about 0.672
- the absorbance at 309 nm was about 0.155
- the absorbance at 609 nm was about 0.061 Path length of the cell = 1cm

Note: The weak absorbance at 609 nm is probably due to the slight oxidation of the sample.

SCCS comment

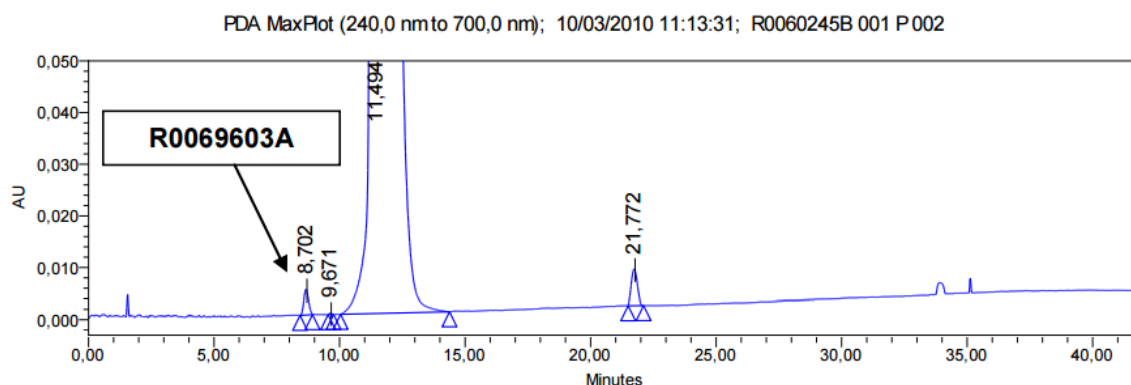
Quantitation of A164 (% w/w) for the batches 007 L001 and 002 L002 was not provided. Detection limit for the impurity at RT 21.8 min was not provided.

Isopropyl alcohol was not quantified in the batch 002 L 002.

3.1.5 Impurities / accompanying contaminants

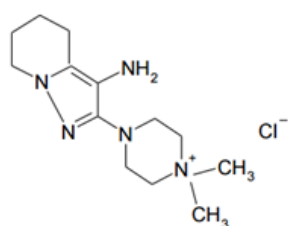
Potential impurities of A164 can originate from starting material, synthesis intermediates, by-products or residual solvents.

Opinion on Dimethylpiperazinium Aminopyrazolopyridine HCl (A164)

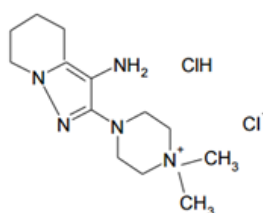


| | |
|--|--|
| <p>R0069603A</p> <p>(Saturated ring compound)</p> <p>Molecular formula: $C_{13}H_{24}N_5, Cl$</p> <p>Molecular weight: 285.82</p> | |
| <p>Molecular formula: $C_{12}H_{17}N_5$</p> <p>Molecular weight: 231.15</p> <p>Exact mass: 231.14839</p> <p>Eluted with Rt: 21.8 min.</p> | |
| <p>Isopropyl alcohol (Isopropanol)</p> <p>(Residual solvent)</p> <p>Molecular formula: C_3H_8O</p> <p>Molecular weight: 60.1</p> | |

Determination of R0069603A content was performed by HPLC using R0069603B as primary reference standard for external standard approach.



R0069603A: MW 285.82



R0069603B : MW 322.28

SCCS comment

Chemical characterisation of the impurity eluted at 9.67 min (<0.1%) should be provided. Chemical characterisation and quantification of the oxidation product(s) should be provided for a typical batch.

3.1.6 Solubility

Water solubility: > 500 g/L at 22°C at pH 1.3 (OECD TG 105, determined on batch 001 P003).

SCCS comment

A considerable decrease in pH value has been reported at high concentration of A164 in water, making the solution strongly acidic. This indicates the corrosive nature of the substance, which should be considered in regard to potential local effects.

3.1.7 Partition coefficient (Log P_{ow})

Log P_{o/w}: < -4.6 at 22°C

3.1.8 Additional physical and chemical specifications

Decomposition of A164 started at about 270°C before melting occurred.

Batch 002 L 002 and 003 L 001* – half wave oxidation potential: -85 mv/ECS (measured with Hg electrode at pH=9.9).

Ionization constant: experimental (potentiometry, GLpKa Sirius) (determination on batch 003 L 001*): pKa is 4.15 (25°C and ionic strength 0.15M) for an equilibrium NH⁺/N.

* Batch used for *in vitro* primary cutaneous tolerance Episkin

3.1.9 Homogeneity and Stability

According to the analytical certificate, A164 is considered to be stable when stored refrigerated (+4°C) under inert gas, away from light and shielded from humidity.

SCCS comment

Information concerning the stability of A164 at 2% concentration and under use conditions should be provided.

3.2 Function and uses

The ingredient A164 is used in oxidative hair colouring formulations at a maximum concentration of 4%, which after mixing typically in a ratio of 1:1 with hydrogen peroxide prior to use, corresponds to a concentration of 2% upon application (final, on-head concentration).

3.3 Toxicological evaluation

3.3.1 Acute toxicity

3.3.1.1 Acute oral toxicity

| | |
|-----------------|---|
| Guideline: | OECD guideline No. 420 (Fixed Dose Method), 17 th December 2004 |
| Species/strain: | female rat, Sprague-Dawley Rj: SD (IOPS Han). |
| Group size: | one female for the sighting test and one group of two females for the main test |
| Test substance: | R0060245B |
| Batch: | R0060245B 002 L002 |
| Purity: | 95% |
| Vehicle: | purified water |
| Dose levels: | 500 mg/kg bw |
| Administration: | oral (gavage), single |
| GLP: | in compliance |
| Study period: | 29 November 2007 - 13 March 2009 |

Fresh dosage forms were prepared (using degassed and inert-gas-stored vehicle) extemporaneously under inert atmosphere on the morning of administration. The test item dosage forms were kept under inert gas and administered within 1 hour after preparation.

No chemical analysis of the dosage forms was performed, but according to the authors of the study this was not considered to impact on the overall GLP status of the study.

The test item was administered to the animals under a volume of 10 mL/kg at the dose-level of 500 mg/kg to one female. As no mortality occurred at the dose-level of 500 mg/kg in the sighting test, the test item was administered at the dose-level of 500 mg/kg to one group of two females in the main test. The definitive test included the female of the sighting test and the two females of the main test.

The single administration was performed in the morning of day 1; it was followed by a 14-day observation period.

Results

No deaths and no clinical signs were noted during the study.

When compared to historical control animals, a slightly lower body weight gain was noted in one female between day 8 and day 15. The body weight gain of the other animals was not affected by treatment with the test item.

Macroscopic examination of the main organs of the animals revealed no apparent abnormalities.

Conclusion

Under the experimental conditions of this study, no deaths were observed after a single oral administration of the test item R0060245B (batch No. R0060245B 002 L002) at the dose-level of 500 mg/kg in rats.

Ref.: 11. Rokh N (2009a)

SCCS comment

It is not clear why the dose of 500 mg/kg was selected for the sighting study (requested by the Sponsor). According to OECD TG 420, the starting dose for the sighting study should be selected from the fixed dose levels of 5, 50, 300 and 2000 mg/kg. In the absence of such information, the starting dose should be 300 mg/kg. Based on the data, the SCCS considers this study as not having been performed adequately and therefore not useful for this evaluation.

3.3.1.2 Acute dermal toxicity**3.3.1.3 Acute inhalation toxicity****3.3.1.4 Acute intraperitoneal toxicity****3.3.2 Irritation and corrosivity****3.3.2.1 Skin irritation*****In vitro* EpiskinSM Skin Irritation Test**

| | |
|---------------------------------|--|
| Guideline: | <i>In vitro</i> Episkin SM Skin Irritation Test, ECVAM validated protocol (ESAC statement 2007) |
| Test system: | Reconstructed Human Epidermis Model Episkin SM (small model, 0.38 cm ²) |
| Replicates: | 3 tissues per condition |
| Test substance: | R0060245B |
| Batch: | R0060245B 001 P 002 |
| Purity: | 98 % (NMR) |
| Dose level: | 10 ± 2 mg |
| Treatment period: | 15 ± 0.5 minutes |
| Post-treatment incubation time: | 42 h ± 1 h |
| Positive control: | 10 µL of 50 mg/mL aqueous solution of Sodium Dodecyl Sulfate |
| Negative control: | 10 µL of PBS+ |
| Direct interaction with MTT: | negative |
| Colouring of tissue: | negative |
| Solubility in vehicle: | yes |
| GLP: | in compliance |
| Study period: | 10 April - 26 May 2014 |

The test item, positive and negative controls were tested in triplicate. 10 ± 2 mg of the test substance and 10 µL of the different controls were applied onto the epidermis. After a 15 ± 0.5-minute treatment period at room temperature, tissues were rinsed with 25 mL PBS+ and the epidermis were transferred in 2 mL/well of fresh maintenance medium for 42 ± 1 hours at 37°C, 5% CO₂ and 95% humidity. Maintenance culture media were kept frozen at -20°C for further IL-1α measurements. After the 42-hour incubation period, each epidermis unit was transferred to another 12-well plate containing 2 mL/well of dye solution (0.30 mg/mL MTT in assay medium). After a 3-hour ± 15-minute incubation period at 37.0°C, 5.0% CO₂ and 95% humidity, a biopsy of the entire epidermis was taken. The epidermis was separated from the collagen matrix and both were transferred into a tube containing 500 µL acidified isopropanol. Formazan crystals were extracted (5°C ± 3°C) and after homogenisation the optical density was measured at 570 nm versus acidified isopropanol as blank and the % cell viability was calculated.

IL-1α released in the culture medium was determined by a classic quantitative sandwich immunoassay technique. Monoclonal specific IL-1α antibodies were pre-coated onto microplates. 200 µL of standards or samples were added in the wells enabling IL-1α to bind to immobilised antibodies. After washing, an enzyme-linked polyclonal antibody specific to

IL-1 α was added to the wells. A substrate solution was added and the intensity of the colour developed was measured at 450 nm.

Results

The mean viability value for undiluted A164 was 92.4 ± 1.7 % and the mean IL-1 α release was 9.8 ± 5.7 pg/mL.

Conclusion

Under the conditions of this study, undiluted A164 is predicted to be non-irritant to the skin.

Ref.: 1. Ansellem C. (2014a)

SCCS comment

The SCCS notes a discrepancy between date of verification of the study plan by QA unit (April 10, 2014) and starting the tests (April 8, 2014). According to GLP guidelines for each study, a written plan should exist prior to the initiation of the study. However this minor deviation should have not compromised the validity or integrity of the study.

In vitro EpiskinSM Skin Irritation Test

| | |
|---------------------------------|--|
| Guideline: | <i>In vitro</i> Episkin SM Skin Irritation Test, ECVAM validated protocol (ESAC statement 2007) |
| Test system: | Reconstructed Human Epidermis Model Episkin SM (small model, 0.38 cm ²) |
| Replicates: | 3 different tissue batches |
| Test substance: | R0060245B |
| Batch: | R0060245B 001 P 002 |
| Purity: | 98 % (NMR) |
| Test item: | 2.0% in water |
| Dose level: | 10 μ L |
| Treatment period: | 15 \pm 0.5 minutes |
| Post-treatment incubation time: | 42 h \pm 1 h |
| Positive control: | 10 μ L of 50 mg/mL aqueous solution of Sodium Dodecyl Sulfate |
| Negative control: | 10 μ L of PBS+ |
| Solvent control: | 10 μ L of solvent used to dilute the raw material (water) |
| Direct interaction with MTT: | negative |
| Colouring of tissue: | negative |
| GLP: | in compliance |
| Study period: | 10 April - 26 May 2014 |

The test item, positive and negative controls were tested in triplicate. 10 μ L of the test substance at 2% in water and 10 μ L of the different controls were applied onto the epidermis. After a 15 \pm 0.5 minutes treatment period at room temperature, tissues were rinsed with 25 mL PBS+ and the epidermis were transferred in 2 mL/well of fresh maintenance medium for 42 \pm 1 hours at 37°C, 5% CO₂ and 95% humidity. Maintenance culture media were kept frozen at -20°C for further IL-1 α measurements. After the 42-hour incubation period, each epidermis unit was transferred to another 12-well plate containing 2 mL/well of dye solution (0.30 mg/mL MTT in assay medium). After a 3-hour \pm 15-minute incubation period at 37.0°C, 5.0% CO₂ and 95% humidity, a biopsy of the entire epidermis was taken. The epidermis was separated from the collagen matrix and both were transferred into a tube containing 500 μ L acidified isopropanol. Formazan crystals were extracted (5°C \pm 3°C) and after homogenisation, the optical density was measured at 570 nm versus acidified isopropanol as blank and the % cell viability was calculated.

IL-1 α released in the culture medium was determined by a classic quantitative sandwich immunoassay technique. Monoclonal specific IL-1 α antibodies were pre-coated onto microplates. 200 μ l of standards or samples were added in the wells enabling IL-1 α to bind to immobilised antibodies. After washing, an enzyme-linked polyclonal antibody specific to IL-1 α was added to the wells. A substrate solution was added and the intensity of the colour developed was measured at 450 nm.

Results

The mean viability value for a 2% solution of A164 in water was $93.7 \pm 2.2\%$ and the mean IL-1 α release was 5.5 ± 2.2 pg/mL.

Conclusion

Under the conditions of this study, A164 diluted at 2% in water is predicted to be non-irritant to the skin.

Ref.: 2. Ansellem C. (2014b)

SCCS comment

The SCCS notes a discrepancy between the date of verification of the study plan by QA unit (April 10, 2014) and starting the tests (April 8, 2014). According to GLP guidelines for each study, a written plan should exist prior to the initiation of the study. However this minor deviation should have not compromised the validity or integrity of the study.

3.3.2.2 Mucous membrane irritation / Eye irritation

Acute Eye Irritation in Rabbits

Guideline: OECD TG 405 (24th April 2002)
Species/strain: male New Zealand White rabbit
Group size: 3 rabbits
Test substance: R0060245B
Batch: R0060245B 002 L 002
Purity: 95%
Vehicle: water purified by reverse osmosis
Concentration: 10 % (w/w)
Positive control:
GLP: in compliance
Study period: 29 November – 13 March 2009

Before preparation, the vehicle was degassed by sonication for 15 minutes and then saturated with inert gas and kept under inert atmosphere for 15 minutes. Fresh dosage form preparations were prepared extemporaneously under inert atmosphere on the morning of administration. The test item dosage forms were kept under inert gas and administered within 1 hour after preparation. No chemical analysis of the dosage forms was performed in this study, but according to the authors of the study, this was not considered to impact on the overall GLP status of the study.

The dosage form (pH~4) was first administered to a single male rabbit. Since the dosage form was not severely irritating on this first animal, it was then evaluated simultaneously in two other animals. A single dose of 0.1 mL of the test item at the requested by the Sponsor concentration of 10% (w/w) in purified water was instilled into the left conjunctival sac. The right eye was not treated and served as control. The eyes were not rinsed after administration of the test item. Ocular reactions were observed approximately 1 hour, 24, 48 and 72 hours after the administration. The study was stopped on day 4 in the absence of

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persistent ocular reactions. The mean values of the scores for chemosis, redness of the conjunctiva, iris lesions and corneal opacity were calculated for each animal.

Results

Slight chemosis and redness of the conjunctiva were observed in 1 animal on day 1 only. Mean scores calculated for each animal over 24, 48 and 72 hours were 0. For chemosis, for redness of the conjunctiva, for iris lesions and for corneal opacity it was 0.

Conclusion

Under the experimental conditions of the study, A164 (batch No. R0060245B 002 L 002) at the concentration of 10% in purified water, was non-irritant when administered by ocular route to rabbits.

Ref: 12. Rokh N (2009b)

3.3.3 Skin sensitisation

| | |
|-------------------|---|
| Guideline: | OECD TG 429 "Skin Sensitization: Local Lymph Node Assay", 24 th April 2002 |
| Species/strain: | female CBA/J mice |
| Group size: | 4 mice per group, 20 animals per experiment, 2 independent experiments |
| Test substance: | R0060245B |
| Batch: | R0060245B 002 L 002 |
| Purity: | > 95% |
| Vehicle: | propylene glycol |
| Concentration: | 0.1, 1 and 10 % (I experiment); 5, 10 and 15 % (II experiment) |
| Positive control: | α -hexylcinnamaldehyde (HCA) in propylene glycol at the concentration of 25% (v/v) |
| GLP: | in compliance |
| Study period: | 13 June – 12 September 2008 |

At the request of the Sponsor, in the first experiment the test item was tested at the concentrations of 0.1, 1 and 10% and then in the second experiment at the concentrations of 5, 10 and 15%. The test item was not soluble in any of the recommended vehicles. However, a homogeneous suspension was obtained at the maximum tested concentrations of 10% and 15%, with propylene glycol, after sonication for 10 minutes. Therefore propylene glycol was selected as vehicle.

On days 1, 2 and 3 of each experiment, a dose-volume of 25 μ L of the control or dosage form preparations was applied to the dorsal surface of both ears. On day 6 of each experiment, all animals of all groups received a single intravenous injection of 20 μ Ci of 3H-TdR. Approximately 5 hours later, the animals were killed and the auricular lymph nodes were excised. The lymph nodes were pooled for each experimental group. The test item was considered as a skin sensitizer when the Stimulation Index (SI) for a dose group was ≥ 3 . A dose-response relationship was also assessed while considering the results.

Results

In the first experiment no mortality and no clinical signs were observed during the study. No cutaneous reactions and no noteworthy increase in ear thickness were observed in the animals of the treated groups. No noteworthy lymphoproliferation was noted at any of the tested concentrations (SI: 1.6, 0.97, 1.04 and 10.53, for R0060245B at 0.1%, 1%, 10% and HCA, respectively).

In the second experiment no mortality and no clinical signs were observed during the study. No cutaneous reactions and no noteworthy increase in ear thickness were observed in the animals of the treated groups. No noteworthy lymphoproliferation was noted at any of the

tested concentrations (SI: 1.21, 0.70, 1.47 and 4.69, for R0060245B at 5%, 10%, 15% and HCA, respectively).

Ref: 6. Pelcot C (2008).

SCCS comment

Based on this LLNA study in which a maximum concentration of 15% was used, A164 is considered not to have skin sensitising potential.

3.3.4 Dermal / percutaneous absorption

| | |
|-------------------------------|---|
| Guideline: | OECD TG 428 (2004) |
| Species/strain: | Frozen human dermatomed skin (400 µm) |
| Membrane integrity: | checked by electrical resistance, at least 10 kΩ |
| Replicates: | 12 intact skin samples (5 donors) |
| Test substance: | [¹⁴ C]-R0060245B |
| Batch and purity: | R0060245B 001 P 001 (>95% pure) and CFQ41090 of [¹⁴ C]-R0060245B (99.6% radiochemical pure) |
| Test item: | Hair dye formulation (pre-formulation P1120828, developer 178914) containing 2% w/w R0060245B |
| Membrane Integrity: | Membranes with a resistance of <10 kΩ excluded |
| Dose applied: | 20 mg/cm ² of the test formulation (400 µg/cm ² R0060245B) |
| Exposure area: | 2.54 cm ² |
| Exposure period: | 30 minutes |
| Sampling period: | 24 hours |
| Receptor fluid: | Degassed phosphate buffered saline (PBS) |
| Solubility in receptor fluid: | > 10 g/l (25°C) |
| Mass balance analysis: | Provided |
| Tape stripping: | Yes (20) |
| Method of Analysis: | Liquid scintillation counting (LSC) |
| GLP: | in compliance |
| Study period: | August 2011 – April 2012 |

Human skin samples were obtained from five different donors from a tissue bank. Each membrane was stored frozen, at approximately -20°C, on aluminium foil until required for use.

A typical oxidative hair dye formulation containing a nominal 4% A164 concentration was mixed with peroxide developer (1:1, w/w) resulting in a concentration of 2% A164. The dose was applied to the surface of 12 intact skin membranes (from 5 human donors) at a rate of 20 mg/cm², corresponding to 400 µg/cm² of A164. At the end of the 30-minute exposure period, the skin surface was washed with water (followed by 2% sodium dodecyl sulphate (SDS) in water. The *stratum corneum* was removed by a tape stripping process removing up to a maximum of 20 strips from each skin membrane. The flange skin was cut away from the dermis and the epidermis on the remaining skin disc was separated from the dermis using a heat separation technique.

The penetration process was monitored using [¹⁴C]-radiolabelled A164, which was incorporated into the formulation, prior to application. The distribution of A164 within the test system was measured and a 24-hour penetration profile was determined by collecting receptor fluid samples 0.5, 1, 2, 4, 8, 12, 16, 20 and 24 hours following application. The samples were analysed by liquid scintillation counting (LSC).

Results

LSC analysis of the dose preparations confirmed that the dose preparations were homogeneous both prior to and following dosing. The HPLC analysis of the hair dye formulation performed following the dosing procedure and 24 hours post application was

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96.1 and 95.8%, respectively, confirming that the formulation was stable for a 24-hour period.

None of the 12 dosed cells were rejected.

Mean recovery of the applied test material was very good at 98.5%, with individual cell values ranging from 95.1% to 106% (n=12).

Table 1 presents the distribution of A164 in the test system.

Table 1. Summary of A164 distribution in the Test System

| Test compartment N=12 | µg A164 /cm ² | | % of applied dose | |
|-------------------------------|--------------------------|--------------|-------------------|--------------|
| | Mean | SD | Mean | SD |
| Donor chamber | 0.320 | 0.450 | 0.079 | 0.111 |
| Skin wash at 30 minutes | 396 | 15.4 | 98.0 | 3.82 |
| Skin wash at 24 hours | 1.32 | 0.333 | 0.327 | 0.083 |
| <i>Stratum corneum</i> | 0.261 | 0.124 | 0.065 | 0.031 |
| Remaining epidermis | 0.129 | 0.205 | 0.032 | 0.051 |
| Dermis | 0.023 | 0.033 | 0.006 | 0.008 |
| Flange | 0.043 | 0.031 | 0.011 | 0.008 |
| Receptor fluid | 0.009 | 0.008 | 0.002 | 0.002 |
| Total non-absorbed | 398 | 15.4 | 98.5 | 3.81 |
| Systemically available | 0.158 | 0.227 | 0.039 | 0.056 |
| Total recovered | 398 | 15.5 | 98.5 | 3.84 |

Remaining epidermis = Epidermal tissue after tape stripping.

Stratum corneum = Amount in tape strips.

Total non-absorbed = Sum of donor chamber, skin wash, flange and *stratum corneum*.

Systemically available = Sum of remaining epidermis, dermis and receptor fluid.

Conclusion

The results obtained in this study indicate that A164 at 2% in a typical oxidative hair dye formulation penetrated through human dermatomed skin at a very slow rate. The extent of A164 penetration through human skin amounted to only 0.002% (0.009 ± 0.008 µg/cm²) of the applied dose, after 24 hours.

The mean total systemically available dose of A164 (remaining epidermis plus dermis and receptor fluid) was 0.039% of the applied dose (corresponding to 0.158 µg/cm²).

Ref.: 4. Davies D (2012)

SCCS comment

The SCCS considers that the mean + 2SD, i.e. 0.612 µg/cm² should be used for the MoS calculation because of high variability of the results.

3.3.5 Repeated dose toxicity**3.3.5.1 Repeated Dose (14 days) oral toxicity**

| | |
|-----------------|--|
| Guideline: | OECD TG 407 (3 October 2008) |
| Species/strain: | Rat / Wistar Hannover |
| Group size: | 5 animals/sex/group |
| Test substance: | R0060245B |
| Batch: | R0060245B 001 P 001 (grey very light powder) |
| Purity: | > 95% |
| Vehicle: | deionised water |
| Dose levels: | 0, 100, 250 and 500 mg/kg bw/day |
| Dose volume: | 10 mL/kg bw |
| Route: | oral |
| Administration: | by gavage |
| GLP: | in compliance |
| Study period: | 14 December 2009 – 29 May 2013 |

The test substance was administered by oral gavage to male and female rats (5 animals/sex/group) at dose levels of 100, 250, 500 mg/kg bw/day once daily for 14 days. The dosage levels were selected in agreement with the Sponsor based on the results of a previous acute oral (gavage) toxicity study in which administration of A164 at the highest dose tested 500 mg/kg/day did not modify the body weight or induce clinical signs or macroscopic changes.

Results

The administration of the test item R0060245B to male and female Wistar rats induced no mortality or clinical signs. There were no toxicologically significant effects on body weight, body weight gain or food consumption. No toxicologically significant changes were observed in hematological, coagulation and clinical chemistry parameters. The presence of proteins and occult blood in the urine was observed in males at 500 mg/kg/day. A lower volume of urine and the presence of protein, urobilinogen, bilirubin and occult blood in the urine were observed in females at 500 mg/kg/day. At 250 mg/kg/day, the presence of proteins in the urine was observed in males and the presence of occult blood in the urine was observed in females. In the absence of any microscopic treatment-related findings in kidneys, these effects were considered of minimal toxicological significance.

On the basis of the results obtained in this study, the authors suggested the following doses for the repeated dose 13-week oral toxicity study with R0060245B in Wistar rats: 60, 200 and 500 mg/kg/day.

Ref. 10. Rodríguez Gómez J. (2013)

3.3.5.2 Sub-chronic (90 days) toxicity (oral)

| | |
|-----------------|---|
| Guideline: | OECD TG 408 (21 st September 1998) |
| Species/strain: | Rat / Wistar Hannover |
| Group size: | 10 animals/sex/group |
| Test substance: | R0060245B |
| Batch: | R0060245B 001 P 001 (grey very light powder) |
| Purity: | > 95% |
| Vehicle: | deionised water |
| Dose levels: | 0, 60, 200 and 600 mg/kg bw/day |
| Dose volume: | 10 mL/kg |
| Route: | oral |
| Administration: | by gavage |

GLP: in compliance
Study period: 20 November 2009 – 20 September 2012

The test substance was administered by oral gavage to male and female rats (10/sex/group) at dose levels of 0, 60, 200, 600 mg/kg bw/day once daily for 90 days. The dosage levels were selected based on the results of a previous 14-day oral (gavage) toxicity (see 3.3.5.1).

Results

The concentration of the test item in samples of each control and test solutions prepared for use were determined on week 1, week 7 and week 13. All values were within the acceptable range (nominal value $\pm 10\%$). Test solutions were stable up to day 9 when stored between $+2^{\circ}\text{C}$ and $+8^{\circ}\text{C}$, under inert gas and protected from light.

No mortality due to the treatment was observed. The death of one female animal from the 600 mg/kg/day group (week 11 day 72) was attributed to a gavage error.

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

No differences in body weight and body weight gain were observed in male and female treated groups compared to the control group, except for a statistically significantly lower body weight gain for females at 200 mg/kg/day during week 3 which was not considered test-item related. In both sexes, negative values of body weight gain were observed around week 12 related to concurrent FOB analysis.

No differences in food consumption were observed in male or female treated rats during the study when compared to the control group.

In FOB testing, mean passing through the centre was lower in male animals exposed at 600 mg/kg/day compared to the control group (0.3 ± 0.5 vs. 1.1 ± 1.0 , respectively). This finding was considered to be without toxicological significance because no other parameters were affected by treatment during the open-field evaluation or in the FOB. No differences were found in treated female groups.

Mean red blood cell count (RBC), hemoglobin concentration (HGB) and hematocrit (HCT) were statistically lower in male rats exposed at 600 mg/kg/day compared to the control group. These effects were of minimal magnitude (-6.3% ; -5.5% and -6.8% , respectively) and not dose-related, thus considered not toxicologically significant. In female treated rats, there were no differences on hematological parameters compared to the control group except for the lowest percentage of monocytes (70 ± 46 vs. $136 \pm 80/\text{mm}^3$, respectively), which was isolated and thus considered as not toxicologically relevant.

Variations in Alanine Aminotransferase (ALT) were considered be within normal biological variation. Mean blood urea nitrogen (BUN) in females exposed at 200 mg/kg/day was statistically lower than the control (-14.4%). This difference was isolated, at mid dose, not dose-related and consequently considered as incidental.

Mean urinary pH values of males at 60 mg/kg/day (7.1 ± 0.3 vs. 6.8 ± 0.3) and females at 600 mg/kg/day (7.0 ± 0.5 vs. 6.2 ± 0.2) were statistically significantly higher compared to the control group. The effect in males was not dose related, and therefore considered as not related to the test item. However, in females, the effect could be considered as test-item related. The urine from males and females at 200 and 600 mg/kg/day was cloudier than control urine and the colour varied from dark yellow to green, brown or blue. This change in colour could be related to a metabolite of the test item and was considered not to be adverse. The presence of protein, urobilinogen and occult blood in the urine was higher in males and females at 200 mg/kg/day and 600 mg/kg/day. The presence of bilirubin and ketones in urine was higher in females at 600 mg/kg/day. These findings were considered to be treatment related.

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No differences in the absolute organ weights were observed in treated males and females compared to controls. In male rats exposed at 200 mg/kg/day, a statistically higher relative weight in both kidneys (right +13.9% and left +11.7%) was observed. Since this finding was only observed at the mid dose, it was considered incidental. Female rats exposed at 600 mg/kg/day showed a statistically higher relative right kidney weight (+8.7%). This effect was of very small magnitude (<10%) and thus was considered not toxicologically relevant.

No macroscopic lesions were found in either male or female treated groups.

The main microscopic lesions in treated male and female rats were observed in the kidneys. These findings consisted in initial stage of membranous glomerulonephritis in male (5/10 and 5/10 vs. 0/10) and female (4/10 and 4/10 vs. 0/10) rats exposed at 200 and 600 mg/kg/day. Slight tubular vacuolation was observed in male rats exposed at 600 mg/kg/day (4/10 vs. 0/10). Tubular-like proteinaceous material was found in male (4/10 vs. 0/10) and female (4/10 vs. 0/10) rats at 600 mg/kg/day. These findings were considered to be related to the treatment with the test item. The remaining lesions were considered incidental.

Conclusion

According to the applicant, the NOEL (No Observed Effect Level) of this 90-day oral toxicity study was considered to be 60 mg/kg bw/day for both male and females.

Ref.: 7. Rodríguez Gómez J (2012a)

SCCS comment

Daily administration of 60, 200 and 600 mg/kg bw/day A164 by oral gavage to male and female Wistar Hannover rats for 90 days resulted in a treatment-related pathological changes in kidneys of the animals exposed to the 2 highest doses (200 and 600 mg/kg bw). Therefore NOEL for this study can be established at 60 mg/kg bw.

3.3.5.3 Chronic (> 12 months) toxicity

3.3.6 Mutagenicity / Genotoxicity

3.3.6.1 Mutagenicity / Genotoxicity *in vitro*

Bacterial Reverse Mutation Test

Guideline: OECD TG 471 (1997)
Species/Strain: *Salmonella typhimurium* TA98, TA100, TA1535, TA1537 and TA102
Replicates: triplicates plates in two separate experiments
Test substance: R0060245B
Batch: R0060245B 001 P 001 (grey very light powder)
Purity: > 95% (UV detection)
Solvent: water (at concentrations up to at least 50 mg/mL)
Positive controls: -S9 MIX: 2-nitrofluorene, sodium azide, 9-aminoacridine, mitomycin C;
+S9 MIX: benzo[a]pyrene, 2-aminoanthracene
Concentrations: experiment I (range-finder and mutation): 0, 1.6, 8, 40, 200, 1000, 5000
µg/plate without and with S9-mix
experiment II: 0, 51.2, 128, 320, 800, 2000 and 5000 µg/plate without
and with S9-mix
Treatment: direct plate incorporation incubated for 3 days protected from light without
and with S9-mix
GLP: in compliance
Study period: 6 October 2009 – 21 January 2010

A164 was investigated for the induction of gene mutations in five strains of *Salmonella typhimurium* (Ames test). Liver S9-fraction from Aroclor 1254 induced rats was used as an exogenous metabolic activation system. Experiment 1 was performed with the direct plate incorporation method and experiment 2 with the pre-incubation step. Negative and positive controls were in accordance with the OECD guideline.

The test article was completely soluble in the aqueous assay system at all concentrations treated, in each of the experiments performed. It was noted that prior to treatment, all test article formulations were clear yellow liquids, but following plate incubation a blue colouration (attributed to test article treatment) was observed at 2000 and 5000 µg/plate.

Experiment 1 consisted of an initial toxicity range-finding carried out in the absence and in the presence of S-9 in strain TA100 only, using final concentrations of A164 at 1.6, 8, 40, 200, 1000 and 5000 µg/plate, plus negative (vehicle) and positive controls. Following these treatments, evidence of toxicity in the form of a reduction in revertant numbers was observed at 5000 µg/plate in the absence and presence of S-9 MIX. These data were considered to be acceptable for mutation assessment. Experiment 1 treatments of the remaining test strains were performed in the absence and in the presence of S-9 MIX and retained the same test concentrations as employed for the range-finder experiments. Following these treatments, evidence of toxicity ranging from a diminution of the background bacterial lawn (with a concomitant reduction in revertant numbers) to a complete killing of the test bacteria was observed at 5000 µg/plate in strains TA98, TA1537 and TA102 in the absence and presence of S-9 MIX. No evidence of toxicity was observed in strain TA1535.

Experiment 2 treatments included narrowed concentration intervals covering the range 51.2-5000 µg/plate, in order to examine more closely those concentrations of A164 approaching the maximum test concentration. In addition, all treatments in the presence of S-9 MIX were further modified by the inclusion of a pre-incubation step in order to increase the probability of detecting any mutagenic effect. Evidence of toxicity ranging from a slight thinning of the background bacterial lawn and/or a reduction in revertant numbers to a complete killing of the test bacteria was observed at 5000 µg/plate in strains TA98, TA1537 and TA102 in the absence and presence of S-9 MIX. No clear evidence of toxicity was observed in strains TA100 and TA1535. Negative and positive controls were in accordance with the OECD guideline.

Results

Following treatments of all the test strains in the absence and presence of S-9 MIX, only Experiment 1 treatments of strain TA102 in the presence of S-9 MIX at 1.6 µg/plate resulted in an increase in revertant numbers that was statistically significant when the data were analysed at the 1% level using Dunnett's test. The increase was small in magnitude and occurred solely at the lowest tested concentration with no indication of a concentration relationship. Accordingly, this increase was considered to have been due to normal biological variability and not evidence of mutagenic activity.

No other increases in revertant numbers were observed that were statistically significant when the data were analysed at the 1% level using Dunnett's test. This study was considered therefore to have provided no evidence of any A164 mutagenic activity in this assay system.

Conclusion

A164 did not induce gene mutants 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 in the absence and in the presence of a rat liver metabolic activation system (S-9 MIX).

Ref.: 3. Beevers C. (2010).

SCCS comment

In the highest concentrations of A164, used slight signs of bacteriotoxicity were noted. In both Experiment 1 and 2, small increases in revertant colony numbers were observed in TA102 strain after exposure with low concentrations of A164 in the presence of S-9 MIX. However, the values of all these increases were within historical negative (vehicle) control values for the TA102 strain, thus these observations were not considered biologically relevant. Considering all the data, the SCCS agrees that the study provided no evidence of mutagenic activity of A164 in this assay system.

***In vitro* Mammalian Cell Gene Mutation Test (Hprt-locus)**

| | |
|--------------------|---|
| Guideline: | OECD TG 476 (1997) |
| Cells: | L5178Y <i>tk</i> ⁺ / ₋ (3.7.2C) mouse lymphoma cells |
| Replicates: | duplicate cultures in two independent experiments |
| Test substance: | R0060245B |
| Batch: | R0060245B 001 P 001 (light grey powder) |
| Purity: | >95% (UV detector) |
| Solvent: | purified water |
| Positive controls: | -S9 MIX: 4-nitroquinoline 1-oxide; +S9 MIX: benzo[a]pyrene |
| Concentrations: | range-finding experiment: 0, 99.47, 198.9, 397.9, 795.8, 1592, 3183 µg/mL with and without S9-mix experiment I: 0, 200, 400, 600, 800, 1000, 1100, 1200, 1300, 1400, 1500, 1750 µg/mL without S-9 and 0, 100, 200, 400, 800, 1200, 1600, 2000, 2400, 2800, 3183 µg/mL with S-9; experiment II: 0, 150, 300, 600, 900, 1200, 1500, 1800, 2100, 2400, 2700, 3183 µg/mL without S-9 and 0, 200, 400, 600, 800, 1200, 1600, 2000, 2400, 2800, 3183 µg/mL with S-9 |
| Treatment: | experiment I: 3 h treatment both without and with S9-mix; expression period 7 days experiment II: 3 h treatment both without and with S9-mix; expression period 7 days |
| GLP: | in compliance |
| Study period: | 5 October 2009 – 24 February 2010 |

A164 was assayed for mutation at the hypoxanthine-guanine phosphoribosyl transferase (*hprt*) locus (6-thioguanine [6TG] resistance) in mouse lymphoma cells using a fluctuation protocol. The study consisted of a cytotoxicity range-finder experiment followed by two independent experiments, each conducted in the absence and presence of metabolic activation by an Aroclor 1254-induced rat liver post-mitochondrial fraction (S-9 MIX). Formulations of the test article were prepared in purified water. Negative and positive controls were in accordance with the OECD guideline.

Range-Finder Experiment. Six concentrations were tested in the absence and presence of S-9 MIX, ranging from 99.47 to 3183 µg/mL (equivalent to 10 mM at the highest concentration tested). Extreme toxicity (<10% relative survival - RS) was observed at 1592 µg/mL and above in the absence of S-9 MIX only. The highest concentration to provide >10% RS in the absence of S-9 MIX was 795.8 µg/mL, which gave 43% RS. The highest concentration tested in the presence of S-9 MIX, 3183 µg/mL, which gave 51% RS.

Experiment 1. Eleven concentrations were tested in the absence of S-9 MIX, ranging from 200 to 1750 µg/mL, and ten concentrations were tested in the presence of S-9 MIX, ranging from 100 to 3183 µg/mL. Seven days after treatment, concentrations of 600, 1000 and 1400 µg/mL in the absence of S-9 MIX and 100 and 2400 µg/mL in the presence of S-9 MIX were not selected to determine viability and 6TG resistance as there were sufficient concentrations to define an appropriate toxicity profile. All other concentrations were

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selected. The highest concentrations selected were 1750 µg/mL in the absence of S-9 MIX and 3183 µg/mL in the presence of S-9 MIX, which gave 39% and 75% RS, respectively. It may be noted that the highest concentration tested in the absence of S-9 MIX did not achieve the desired toxicity level of 10-20% RS and therefore a limit concentration was not achieved. However, as this experiment is clearly negative, this has not affected the integrity of the data and higher concentrations were tested in Experiment 2 in order to achieve an appropriate limiting concentration.

In **Experiment 2**, eleven concentrations were tested in the absence of S-9 MIX (100 - 3183 µg/mL), and ten concentrations were tested in the presence of S-9 MIX (200 - 3183 µg/mL). Seven days after treatment, concentrations of 150, 900, 1200, 1800 and 3183 µg/mL in the absence of S-9 and 200, 400, 800 and 1600 µg/mL in the presence of S-9 MIX were not selected to determine either viability or 6TG resistance because there were sufficient concentrations to define an appropriate toxicity profile or due to excessive toxicity (3183 µg/mL in the absence of S-9 MIX). All other concentrations were selected. The highest concentrations selected were 2700 µg/mL in the absence of S-9 and 3183 µg/mL in the presence of S-9 MIX, which gave 15% and 88% RS, respectively.

Results

No statistically significant increases in mutant frequency were observed following treatment with A164 at any concentration tested, in the absence or presence of S-9 MIX, in Experiment 1 or 2. Significant linear trends were observed in the absence of S-9 MIX in Experiments 1 and 2. However, the linear trend observed in Experiment 1 was thought to be due to a slightly lower than normal (although still acceptable) mutant frequency in the vehicle control: In Experiment 2, the linear trend was weak and might have been due to a slight drop in mutant frequency at the lowest concentration tested. Therefore, these observations were not considered biologically relevant.

Conclusion

It is concluded that A164 did not induce gene mutants at the Hprt locus of L5178Y mouse lymphoma cells when tested under the conditions employed in this study. These conditions included treatments up to or approaching the limit of toxicity in the absence of S-9 MIX (2700 µg/mL), or up to 3183 µg/mL (10 mM) in the presence of S-9 MIX, in two independent experiments in the absence and presence of a rat liver metabolic activation system.

Ref.: 13. Stone V (2010).

SCCS comment

In both experiments, a linear trend in mutant frequency was noted in the absence of S-9 MIX. The trends were weak, especially in Experiment 2, and most probably resulted from low mutant frequency in the control cells or cell exposed to the lowest A164 concentration, in Experiment 1 and Experiment 2, respectively. Furthermore, the mutant frequencies in cells exposed to all concentrations of A164 used in both experiments were within historical control ranges. Considering all the data, the SCCS agrees that A164 did not show a gene mutation potential in this study.

Micronucleus Test in Human Lymphocytes

| | |
|-----------------|--|
| Guideline: | OECD TG 487 (2008 Draft proposal) |
| Cells: | human lymphocytes (blood was pooled using equal volumes from each of two male donors prior to use) |
| Replicates: | duplicate cultures in 1 experiment |
| Test substance: | R0060245B |
| Batch: | R0060245B 001 P 001 (grey powder) |
| Purity: | >95% (UV detector) |
| Solvent: | Water for injection |

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Positive controls: -S9 MIX: mitomycin C and vinblastine; +S9 MIX: cyclophosphamide

Concentrations and treatment for the Micronucleus Experiment based on the results from the cytotoxicity Range-Finder Experiment:

- 3-h treatment + 21 recovery: 0 (vehicle), 3000, 4000, 5000 µg/mL without S9 MIX

- 3-h treatment + 21 recovery: 0 (vehicle), 2500, 3000, 4000 with S9 MIX

- 24-h treatment + 0 recovery: 0 (vehicle), 200, 400, 500, 600 without S9 MIX

Treatment: 3 + 21-hour treatment both with and without S9 MIX; test article added at 48 h following culture initiation (stimulation by PHA). Cytochalasin B was added at 51 h (3-h exposure to the chemical +/-S9) or at 48 h (24-h exposure without S9). Cultures were harvested 72 h after initiation.

GLP: in compliance

Study period: 6 October 2009 - 17 February 2010

A164 was tested in an *in vitro* micronucleus assay using duplicate human lymphocyte cultures prepared from the pooled blood of two male donors in a single experiment. Treatments covering a broad range of concentrations, separated by narrow intervals, were performed both in the absence and presence of metabolic activation (S-9 MIX) from Aroclor 1254 induced rats. The test article was formulated in water for injection (purified water) and the highest concentration used of 5000 µg/mL was determined following a preliminary cytotoxicity Range-Finder Experiment. Negative and positive controls were in accordance with the OECD guideline.

Results

3+21 hour treatment in the presence of S-9 MIX

Treatment of cells with A164 in the presence of S-9 MIX resulted in frequencies of MNBN cells which were similar to and not significantly ($p \leq 0.05$) higher from those observed in concurrent vehicle controls for all concentrations analysed. The MNBN cell frequency of all A164 treated cultures fell within historical vehicle control (normal) ranges.

3+21 and 24+0 hour treatments in the absence of S-9 MIX

Treatment of cells with A164 in the absence of S-9 MIX in both treatment regimens resulted in frequencies of MNBN cells that were similar to and not significantly higher than those observed in concurrent vehicle controls for the majority of concentrations analysed. Exceptions to this were at single concentrations as follows:

1) at the intermediate concentration analysed post 3+21 hour -S-9 MIX treatment (4000 µg/mL, inducing 9% cytotoxicity

2) at the highest concentration analysed post 24+0 hour -S-9 MIX treatment (600 µg/mL, inducing 63% cytotoxicity

In both instances, the increases observed were isolated and small with the MNBN cell frequencies of replicate cultures at these and all other A164 concentrations analysed fell within historical vehicle control (normal) ranges. The observed statistical increases were therefore not considered of biological importance.

Conclusion

A164 did not induce micronuclei in cultured human peripheral blood lymphocytes following treatment in the absence and presence of a rat liver metabolic activation system (S-9). Concentrations were analysed up to either 5000 µg/mL (3+21 hour -S-9 MIX) treatment or 4000 µg/mL (3+21 hour +S-9 MIX), limited by precipitation or to 600 µg/mL (24+0 hour treatment), a concentration inducing high cytotoxicity (63%).

Ref.: 14. Whitwell J (2010).

SCCS comment

In both treatment regimens without S-9 MIX, a statistically significant increase in micronucleus frequency was observed. After 3-h treatment only at the medium concentration of A164, a small but significant increase of MNBN frequency was observed. After a longer treatment (24-h), a small concentration dependent increase in micronucleus frequency was observed. Linear trend analysis was not performed. However, the values of MNBN frequency for all A164 concentrations tested were within historical vehicle control ranges. Considering all the data, the SCCS agrees that A164 did not show a mutation potential under the experimental condition studied.

3.3.6.2 Mutagenicity / Genotoxicity *in vivo***Micronucleus Test in Bone Marrow Cells of the Rat**

| | |
|--------------------|--|
| Guideline: | OECD TG 474 |
| Species/strain: | Rat (Wistar) |
| Group size: | 6 males and 6 females per test group |
| Test substance: | R0060245B |
| Batch: | R0060245B 007 L 001 (blue grey powder) |
| Purity: | 99% |
| Vehicle: | Deionised water |
| Dose levels: | 24-h preparation interval: 500, 1000 and 2000 mg/kg b.w. 48-h preparation interval: 2000 mg/kg b.w. |
| Dose volume: | 10 mL/kg |
| Route: | oral |
| Administration: | gavage |
| Positive controls: | cyclophosphamide at 20 mg/kg b.w. |
| GLP: | in compliance |
| Study period: | 07 January 2009 - 17 June 2010 |

A164 has been investigated for the induction of micronuclei in bone marrow cells of rats. Test concentrations were based on acute toxicity in a preliminary range-finding study. The animals received the test item, the vehicle or the positive control substance once. Twelve animals (6 males and 6 females) were treated per dose group and sampling time. The animals of all dose groups were examined for acute toxic symptoms at intervals of around 1 h, 2 - 4 h, 6 h and 24 h after administration of the test item. Because of mortality, the high-dose group for the 48-hour treatment interval using females was repeated with 6 female rats. Sampling of the bone marrow was done 24 and 48 hours after treatment. Six thousand polychromatic erythrocytes (PCE) were analysed per animal for micronuclei, 2000 PCE for the repeated female group of additional 6 animals. To describe a cytotoxic effect, the ratio between polychromatic and normochromatic erythrocytes (NCE) was determined in the same sample and expressed in PCE per 2000 erythrocytes. All surviving animals per test group were evaluated as described. Negative and positive controls were in accordance with the OECD guideline.

Results

On the day of the experiment, the test item was formulated in deionised water. Stability in solvent was not indicated by the sponsor.

As estimated by a pre-experiment, 2000 mg A164 per kg b.w. (the maximum guideline-recommended dose) was suitable. However, 1 female from the dose group of 2000 mg/kg b.w. (24 h preparation interval) and 2 females from the dose group of 2000 mg/kg b.w. (48h preparation interval) died.

The mean number of PCE was not decreased after treatment with the test item as compared to the mean value of PCEs of the vehicle control, indicating that A164 did not have any cytotoxic properties in the bone marrow. The urine of the treated animals was discoloured, indicating the systemic distribution of the test item and thus, its bioavailability.

For the 24-h sampling time, in comparison to the corresponding vehicle controls there was no biologically relevant or statistically significant enhancement in the frequency of the detected micronuclei after administration of the test item at any dose level used. Mean values and individual number of micronuclei were within the historical vehicle control data range.

For the 48-h sampling time, the mean value of micronucleated PCE per 2000 erythrocytes of the vehicle group was slightly above the historical vehicle control data range and above the mean value of the vehicle at the 24-h sampling time (MayGrunwald staining: 0.313% in 48 h vs. 0.233% in 24 h vehicle groups). However, the individual number of micronuclei per animal for the vehicle at 48 h was within the historical data range. Thus the highest treatment dose (48-h interval) was compared to the 24-h vehicle control group instead of the 48-h vehicle group. The mean values of PCE with micronuclei for the high-dose test item treated animals sampled at 48 h did not show any statistically significant increase and additionally were within the historical vehicle control data range. The repetition of the evaluation by usage of acridine orange stained slides did not confirm the difference observed previously with MayGrunwald staining between the vehicle group sampled at 24 h and the vehicle group sampled at 48 h. With the specific acridine orange dye, values were similar for both vehicle groups and all treated animals. This confirmed the data obtained for the test item indicating a non-mutagenic effect of the test item and demonstrating the validity of the study.

Conclusion

Under the experimental conditions reported, i.e. oral administration of up to 2000 mg/kg, the maximal recommended dose according to the regulatory guideline, the test item did not induce micronuclei as determined by the micronucleus test in the bone marrow cells of the rat.

Ref.: 5. Merker M (2010)

SCCS overall comment on mutagenicity

The genotoxicity of A164 was investigated in the three endpoints of genotoxicity: gene mutations, structural chromosome aberrations and aneuploidy.

It can be concluded that under the experimental conditions used, A164 did not induce gene mutations in either bacteria or in mammalian cells, and also did not induce an increase in micronucleated cells in human lymphocytes and, most importantly, not in rat bone marrow cells either. Thus, A164 can be considered to have no genotoxic potential.

3.3.7 Carcinogenicity

3.3.8 Reproductive toxicity

3.3.8.1 Two-generation reproduction toxicity

3.3.8.2 Other data on fertility and reproduction toxicity

3.3.8.3 Developmental Toxicity

Prenatal Developmental Toxicity Study in Rats

Guideline: OECD TG 414 (2001)
Species/strain: Rat, Wistar Hannover
Group size: 25 females/group
Test substance: R0060245B
Batch: R0060245B 001 P 001 (grey very light powder)

Opinion on Dimethylpiperazinium Aminopyrazolopyridine HCl (A164)

Purity: >95%
Vehicle: deionised water
Dose levels: 0, 60, 200 and 600 mg/kg bw/day
Dose volume: 10 mL/kg bw
Route: Oral
Administration: Gavage
GLP: In compliance
Study period: 19 November 2009- 15 July 2012

The dose levels were based on the findings from the 14-day dose-range finding study where the highest dose tested, 500 mg/kg/day, induced only slight maternal toxicity characterised by lower body weight gain from day 12 to day 15 and lower corrected body weight gain. In the present study, the test solutions (corresponding to 60, 200 and 600 mg/kg bw/day) or deionised water were administered to the mated female rats daily (25/group), by gavage, from implantation until one day prior to the scheduled caesarean section (i.e. from day 6 to day 19 of gestation). Animals that were not pregnant were excluded from calculations regarding mean maternal bodyweights, food consumption and gestational parameter (n=7, 4, 5 and 5, for 0, 60, 200 and 600 mg/kg bw/day, respectively).

Results

The stability of the substance was confirmed with the certificate of analysis provided by the sponsor. The concentration of the test item in samples of each control and test solutions prepared for use were determined at each new preparation. The acceptance criteria used for the analyses was the nominal value \pm 10% for the actual concentration. The results of the analyses met the acceptance criteria. The test solutions were prepared daily and administered within 2 hours after preparation.

No mortality and no clinical signs were observed in any group during the study. No statistically significant alterations on food consumption were observed in treated animals when compared to the control animals.

Although not statistically significant, lower body weight gains were observed at 60 mg/kg/day and 200 mg/kg/day from gestation day 6 to day 9 and at 600 mg/kg/day from gestation day 6 to day 15. According to the authors of the report, as these differences at 60 mg/kg/day and 200 mg/kg/day were observed only from day 6 to day 9, they were considered related to the treatment with the test item but did not represent adverse effects. On the contrary, at 600 mg/kg/day, this difference was maintained up to day 15 and was thus considered as adverse.

Lower corrected body weight gains were observed in treated dams compared to control group (-32.9%; -18.1% and -33% at 60, 200 and 600 mg/kg/day, respectively), statistically significant at 60 mg/kg/day and 600 mg/kg/day. According to the authors of the report, at 60 mg/kg/day, this difference could be explained by the higher number of fetuses in litters and higher fetus body weight and was considered not related to the treatment with the test item. On the contrary, at 600 mg/kg/day, this difference was considered related to the treatment with the test item.

More foetuses per litter were observed at 60 mg/kg/day when compared to the control group. This finding occurred only at the lowest dose level, thus was considered without relation to the test item. Statistically significantly lower numbers of resorption (early and late) and post implantation loss was observed at 600 mg/kg/day. According to the authors of the report, this effect was not clinically relevant and thus was considered not related to the treatment with the test item.

No significant effects were observed in maternal necropsy findings in any treated group when compared to the control group.

Opinion on Dimethylpiperazinium Aminopyrazolopyridine HCl (A164)

No changes in foetal or mean placental weights were observed when compared to control values.

External examinations of the fetuses did not reveal any variations or malformations in control or exposed dams.

No soft tissue malformations were observed in treated or control fetuses. No statistically significant difference existed between treated and control groups, in both foetal and litter incidence of soft tissue variations.

No foetal skeletal malformations were observed in treated or control fetuses.

Applicant's summary of foetal skeletal variations and retardations observed in the study is presented below:

Foetal skeletal variations:

- Statistically significant higher total skeletal variations were observed at 60 mg/kg/day (in a litter basis) and at 600 mg/kg/day (in foetal and litter basis) but without dose relation in litter basis and with small differences among the groups. These effects were thus considered not related to treatment with the test item.
- Higher foetal incidence of dumbbell-shaped thoracic vertebra was observed in all treated groups but only significant at 600 mg/kg/day. As this observation is a common finding in this kind of study, it was considered not to be related to the treatment with the test item.

Foetal skeletal retardations:

- There was a statistical difference in total skeletal retardations, on foetal incidence at 600 mg/kg/day.
- The foetal incidence of rudimentary sternbrae at 600 mg/kg/day was statistically significantly higher when compared to the control group. This effect was not dose-related and thus, was considered not related to the treatment with the test item.
- The litter and foetal incidences of frontal and parietal incomplete ossification were higher at 60 mg/kg/day (not statistically significant), at 200 mg/kg/day (statistically significant in a foetal basis) and at 600 mg/kg/day (statistically significant on a foetal and litter basis).
- The foetal and litter incidences of interparietal incomplete ossification were higher at 600 mg/kg/day (statistically significant in a foetal basis) and at 200 mg/kg/day (not statistically significant). These effects were dose-related and could be considered related to the treatment with the test item. This kind of effect could be considered of low concern in the absence of effect on foetal body weight and, as they were associated with a slight maternal toxicity observed at the initiation of treatment, they could be considered as not adverse.
- A statistically significant higher number of posterior phalanges ossification centres were observed at 60 mg/kg/day. Absence of dose trend effect and occurrence only in the lower dose excluded a possible test-item effect.

Conclusion

Based on the above results, the authors of the study report established the No Observed Adverse Effect Level (NOAEL) for maternal toxicity of R0060245B at 200 mg/kg/day and the NOAEL for foetal developmental toxicity at 600 mg/kg/day.

Ref.: 8. Rodríguez Gómez J (2012b).

SCCS comment

Concerning foetal skeletal variations, a higher foetal incidence of dumbbell-shaped thoracic vertebra was observed in all treated groups, but this was only significant at 600 mg/kg/day.

Opinion on Dimethylpiperazinium Aminopyrazolopyridine HCl (A164)

Concerning foetal skeletal retardations, there was a statistically significant increase in foetal incidences of total skeletal retardations at 600 mg/kg/day.

The SCCS agrees that the NOAEL value for maternal toxicity can be established at 200 mg/kg/day.

As the incidence of incomplete ossification was statistically higher in fetuses at 600 mg/kg bw which was not associated with any decrease in foetal body weight but was associated with a significant maternal toxicity, the SCCS cannot exclude that the effect might be due to the compound. Therefore, the SCCS considers that the NOAEL value for developmental toxicity of A164 should be established at 200 mg/kg/day.

3.3.9 Toxicokinetics

3.3.9.1 Toxicokinetics in laboratory animals

3.3.9.2 Toxicokinetics in humans

3.3.10 Photo-induced toxicity

3.3.10.1 Phototoxicity / photo-irritation and photosensitisation

3.3.10.2 Photomutagenicity / photoclastogenicity

3.3.11 Human data**3.3.12 Special investigations****3.3.13 Safety evaluation (including calculation of the MoS)****CALCULATION OF THE MARGIN OF SAFETY**

(oxidative conditions)
(4% formulation, on head concentration 2%)

| | | | |
|---|----------------------------|---|--------------------------------------|
| Absorption through the skin | A | = | 0.612 µg/cm² |
| Skin Area surface | SAS | = | 580 cm² |
| Dermal absorption per treatment | SAS x A x 0.001 | = | 0.35 mg |
| Typical body weight of human | | = | 60 kg |
| Systemic exposure dose (SED) | SAS x A x 0.001/... | = | 5.92x10⁻³ mg/kg bw |
| No observed adverse effect level (90-day, oral, rat) | NOAEL | = | 60 mg/kg bw/d |
| Bioavailability 50%* | | = | 30 mg/kg bw/d |

| | |
|-------------------------|----------------------------------|
| Margin of Safety | adjusted NOAEL/SED = 5068 |
|-------------------------|----------------------------------|

* standard procedure according to the SCCS's Notes of Guidance for the testing of cosmetic ingredients and their safety evaluation.

3.3.14 Discussion

Physicochemical properties

Quantitation of Dimethylpiperazinium Aminopyrazolopyridine HCl (% w/w) for the batches 007 L001 and 002 L002 was not provided.

Detection limit for the impurity at RT 21.8 min was not provided.

Toxicological Evaluation

Acute toxicity

An acute oral toxicity study with Dimethylpiperazinium Aminopyrazolopyridine HCl in 3 rats revealed no deaths after a single oral administration of the test item at the dose-level of 500 mg/kg. Based on the data submitted, the SCCS considers the study as not performed according to the OECD TG 420.

Irritation/sensitisation

The *in vitro* tests did not indicate skin irritancy. Dimethylpiperazinium Aminopyrazolopyridine HCl at the concentration of 10% in purified water was non-irritant when administered by ocular route to rabbits. The *in vivo* tests (LLNA) did not indicate a skin-sensitising potential.

Dermal absorption

The submitted documents indicate a very low rate of dermal penetration of Dimethylpiperazinium Aminopyrazolopyridine HCl. Because of the high variability of the results in the *in vitro* test system, the mean + 2 SD is used for the calculation of the MoS.

Repeated dose toxicity

Daily administration of 60, 200 and 600 mg/kg bw/day Dimethylpiperazinium Aminopyrazolopyridine HCl by oral gavage to male and female Wistar Hannover rats for 90 days resulted in treatment-related pathological changes in kidneys of the animals exposed to the 2 highest doses (200 and 600 mg/kg bw). Therefore the NOEL for this study can be established at 60 mg/kg bw.

Reproductive toxicity

No fertility study with Dimethylpiperazinium Aminopyrazolopyridine HCl has been provided. Based on a developmental toxicity study in rats, a NOAEL for maternal toxicity of Dimethylpiperazinium Aminopyrazolopyridine HCl can be established at 200 mg/kg/day. Dimethylpiperazinium Aminopyrazolopyridine HCl did not show any teratogenic potential. Foetal skeletal variations (retardations) were observed at all three doses tested (60, 200 and 600 mg/kg bw/d). As the incidence of incomplete ossification was statistically higher in fetuses at 600 mg/kg bw, which was not associated with any decrease in foetal body weight but was associated with a significant maternal toxicity, the SCCS cannot exclude that the effect might be due to the compound. Therefore, the SCCS considers that the NOAEL value for developmental toxicity of Dimethylpiperazinium Aminopyrazolopyridine HCl should be established at 200 mg/kg/day.

Mutagenicity / genotoxicity

The genotoxicity of Dimethylpiperazinium Aminopyrazolopyridine HCl was investigated in the three endpoints of genotoxicity: gene mutations, structural chromosome aberrations and aneuploidy. Dimethylpiperazinium Aminopyrazolopyridine HCl did not induce gene mutants in five strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537 and TA102) up to concentration of 5000 µg/plate in the presence or absence of a rat liver metabolic activation system (S-9 MIX), it did not induce gene mutations at the *Hprt* locus in L5178Y mouse lymphoma cells in the presence or absence of S-9 mix up to 3183 µg/mL, or micronuclei in cultured human peripheral blood lymphocytes in the presence or absence of S-9 mix. It also did not induce micronuclei in bone marrow cells of the rat. Thus,

Dimethylpiperazinium Aminopyrazolopyridine HCl can be considered to have no genotoxic potential and additional tests are not required.

4. CONCLUSION

1. In light of the data provided, does the SCCS consider Dimethylpiperazinium Aminopyrazolopyridine HCl (A164), safe when used in oxidative hair colouring products up to a maximum on-head concentration of 2%?

On the basis of data provided, the SCCS considers Dimethylpiperazinium Aminopyrazolopyridine HCl (A164) safe when used in oxidative hair colouring products up to a maximum on-head concentration of 2%.

2. Does the SCCS have any further scientific concerns with regard to the use of Dimethylpiperazinium Aminopyrazolopyridine HCl (A164) in cosmetic products?

Chemical characterisation and quantification of the oxidation product(s) detected in some batches have not been provided.

5. MINORITY OPINION

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6. REFERENCES

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