Scientific Committee on Consumer Safety

SCCS

OPINION ON

HC Blue 16

COLIPA n° B119

The SCCS adopted this opinion at its 16th plenary meeting

of 18 September 2012

About the Scientific Committees

Three 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.
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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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This opinion has been subject to a commenting period of four weeks after its initial publication. Comments received during this time have been considered by the SCCS and discussed in the subsequent plenary meeting. Where appropriate, the text of the relevant sections of the opinion has been modified or explanations have been added. In the cases where the SCCS after consideration and discussion of the comments, has decided to maintain its initial views, the opinion (or the section concerned) has remained unchanged.

Revised opinions carry the date of revision.

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1. BACKGROUND

Submission I for the new hair dye substance HC Blue 16 with the chemical name N,N-dimethyl-3-{[4-(methylamino)-9,10-dioxo-9,10-dihydro-1-anthracenyl]amino}-N-propyl-1-propanaminium bromide (CAS 502453-61-4) was submitted in October 2008 by COLIPA[1].

According to the submission the intended use is as a semi-permanent hair dye substance with a final concentration on the scalp of up to max 3.0%.

2. TERMS OF REFERENCE

1. *Does SCCS consider N,N-dimethyl-3-{[4-(methylamino)-9,10-dioxo-9,10-dihydro-1-anthracenyl]amino}-N-propyl-1-propanaminium bromide safe for use as an non-oxidative hair dye with a concentration on-head of maximum 3.0 % taken into account the scientific data provided?*

2. *And/or does the SCCS have any further scientific concerns with regard to the use N,N-dimethyl-3-{[4-(methylamino)-9,10-dioxo-9,10-dihydro-1-anthracenyl]amino}-N-propyl-1-propanaminium bromide in non-oxidative hair dye formulations?*

[1] COLIPA - European Cosmetics Toiletry and Perfumery Association

3. OPINION

3.1. Chemical and Physical Specifications

3.1.1. Chemical identity

3.1.1.1. Primary name and/or INCI name

HC Blue 16 (INCI name)

3.1.1.2. Chemical names

- 1-Propanaminium, 3-[[9,10-dihydro-4-(methylamino)-9,10-dioxo-1-anthracenyl] amino]-N,N-dimethyl-N-propyl-, bromide (CA INDEX NAME, 9CI)
- N,N-dimethyl-3-{[4-(methylamino)-9,10-dioxo-9,10-dihydro-1-anthracenyl] amino}-N-propyl-1-propanaminium bromide (IUPAC)
- dimethyl-(3-(4-methylamino-9,10-dioxo-9,10-dihydro-anthracen-1-ylamino)propyl)propylammonium bromide

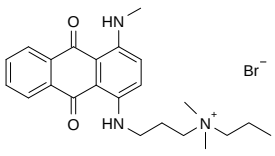
3.1.1.3. Trade names and abbreviations

BLUEQUAT-BROMID
A015035
COLIPA n° B119

3.1.1.4. CAS / EC number

CAS: 502453-61-4
EC: 481-170-7

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula: $C_{23}H_{30}N_3O_2Br$

3.1.2. Physical form

Dark blue powder

3.1.3. Molecular weight

Molecular weight: 460.42 g/mol

3.1.4. Purity, composition and substance codes

Chemical characterisation of HC Blue 16 was performed by NMR, MS, IR and elemental analysis.
Purity of HC Blue 16 in two batches used for toxicity testing and impurities in these batches are described in the table below.

Description of sample	Batch James Robinson lot PP 1-4	Batch James Robinson lot 8080 AB001
References of Analyses	2006/1445 GLP	2008/2831
NMR content , %, w/w (calculated as mono bromide)	97.9	98.1
HPLC* purity, area %		
210 nm	99.3	99.6
254 nm	99.4	99.5
635 nm	99.9	99.8
Bromide content, % w/w	16.94	17.21
Water content, % w/w	0.27	0.27
Residue on ignition, % w/w	0.02	0.03
N-Methyl-2-pyrrolidinone , ppm	176	305
1-Bromo-4-(methylamino)-9,10-anthraquinone, ppm	Not detectable (LOD 3 ppm)	About 5 ppm (near LOD)
1-((3-(dimethylamino)propyl)amino)-4-(methylamino)-9,10-anthraquinone ppm	147	119
Element screening (impurities), ppm	10 (Na), 69 (Si), 18 (P), 51 (Fe), 53 (Cu)	89 (Na), 26 (Fe), 16 (Cu)

*: Column Purosphere RP-C18e, 250/4-5; CH_3CN:0,005M KH_4PO_4 pH 3,0; 40:60, 1 mL/min, 40 °C
LOD: Limit of detection

3.1.5. Impurities / accompanying contaminants

See 3.1.4.
NDELA content less than 25 ppm (detection limit).

3.1.6. Solubility

Water solubility:	218 g/l (20°C, pH 5.6)	(EU - A.6)	reference 11
Water/acetone (1:1):	> 100 g/L (pH 5.3)		
DMSO:	> 50 g/L		

3.1.7. Partition coefficient (Log P_{ow})

Log P_{ow}: 2.44 (pH 6; room temperature) (EU – A.8)

3.1.8. Additional physical and chemical specifications

Particle size distribution:	> 250 µm (mean; > 66% of raw material)	(CIPAC MT59.1)
Melting point:	226.7 °C (decomposition)	(EU-A.1)
Boiling point:	/	
Vapour pressure:	5.3 10-13 hPa (20 °C, ex.-polated)	(EU-A.4)
Surface tension:	59.44 mN/m (19.7 °C, in water)	(EU-A.5)
Density:	1.445 g/ml	(EU-A.3)
pH:	6.1 (20%, w/w aqueous solution, 20 °C)	
pKa:	7.0 ± 0.2 (HL/H+L) [calculated] 2.86 ± 0.2 (H2L/H+HL) [calculated]	
Flammability:	not highly flammable	(EU-A.10)
Explosive properties:	not explosive	(EU-A.14)
Relative self-ignition:	> 394 °C	(EU-A.16)
Oxidising properties:	not oxidising	(EU-A.17)
UV_Vis abs. spectrum:	λmax 261 nm, 589 nm, 637 nm	

3.1.9. Stability

HC Blue 16 formulations at concentration 6 mg/g and 60 mg/g, used in 28 days oral toxicity study, were shown to be homogeneous (maximum variation 2%). These formulations were stable at room temperature for 4 hours study period (maximum variation 2%).

General comments on physico-chemical properties

- HC Blue 16 is a secondary amine, and therefore, it can be nitrosated to form nitrosamine.
- Nitrosamine content corresponding to HC Blue 16 was not reported.
- Stability of HC Blue 16 in typical hair dye formulations was not reported.

3.2. Function and uses

HC Blue 16 is used as a non-reactive hair colouring agent ("Direct Dye") in semi-permanent hair dye formulations at a maximum on-head concentration of 3%.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

No data submitted
ECHA inventory: Acute Tox 4; H302

3.3.1.2. Acute dermal toxicity

No data submitted

3.3.1.3. Acute inhalation toxicity

No data submitted

Comment
No acute oral toxicity study was performed for HC Blue 16 since the applicant considered that data on the maximum tolerated dose (MTD) after oral application (*in vivo* micronucleus assay in mice –and *in vivo* Comet assay in rats –) was available. The MTD value of HC Blue 16 was 2000 mg/kg bw in the study with mice, and 600 mg/kg bw in the study with rats, since all rats died at 2000 mg/kg bw. Therefore, the LD50 value is estimated to be between 600 and 2000 mg/kg bw in rats, and greater than 2000 mg/kg bw in mice.

3.3.2 Irritation and corrosivity

3.3.2.1. Skin irritation

Guideline: OECD 404 (1992)

Species/strain: Albino rabbit, New Zealand White, (SPF-quality)
No. of animals: 3 males
Test substance: BLUEQUAT-BROMID
Batch: James Robinson lot pp 1-4
Purity: 99.7 area% (HPLC, 254 nm)
Dose: 0.5 g moistened with water
GLP: in compliance
Study period: 6 – 16 August 2002

0.5g BLUEQUAT-BROMID, moistened with water, was applied on the clipped dorsal skin under a 2x3 cm semi-occlusive dressing for 4 hours. Observations were made 1, 24, 48 and 72 hours after exposure.
There was blue staining of the skin of all animals at all time-points; this staining "did not hamper" observations. No reactions were observed on any animal at any time point.

Ref.: 16

Comment
It is unknown whether the blue staining may have prevented observation of slight erythema.

3.3.2.2. Mucous membrane irritation

Guideline: OECD 405 (1987)
Species/strain: Albino rabbit, New Zealand White, (SPF-quality)
No. of animals: 3 males
Test substance: BLUEQUAT-BROMID
Batch: James Robinson lot pp 1-4
Purity: 99.7 area% (HPLC, 254 nm)
Dose: 33 mg (0.1 mL)
GLP: in compliance
Study period: 19 – 22 August 2002

33 mg (approximately 0.1ml) of BLUEQUAT-BROMID was instilled into one eye of each rabbit. Observations were made a 1, 24, 48 and 72 hours after exposure.
In all animals, blue staining of the lower lid at 1 hour prevented scoring.
Conjunctival redness, chemosis and discharge occurred in all animals but this had cleared at 24 hours in 1 animal, at 48 hours in the other 2 animals.
No iris or corneal changes were noted. At 24 hours, 2% fluorescein was instilled and this showed that no corneal epithelial damage occurred.

Ref.: 17

Comment
Blue staining prevented some observations. Neat BLUEQUAT-BROMID is an irritant to the rabbit eye.

Neutral Red Uptake assay (NRU) on Human Keratinocytes (HaCaT)

Guideline: /
Species/strain: human keratinocytes (HaCaT)
Test substance: BLUEQUAT-BROMID
Batch: James Robinson lot pp 1-4
Purity: 99.7 area% (HPLC, 254 nm)
Dose: 681.3, 1000, 1468, 2154, 3162, 4642, 6813, 10000 µg/mL
Reference item: sodium lauryl sulfate (SLS)
GLP: in compliance (no QAU checking)
Study period: 8 July – 21 August 2002

The aim of this study was to assess the eye irritation potential of the test item by measuring its cytotoxicity in the NRU assay. Monolayers of human keratinocytes (HaCaT) were exposed for 24 h in 96-well microtitre plates to concentrations of 681.3, 1000.0, 1468.0, 2154.0, 3162.0, 4642.0, 6813.0 and 10000.0 µg/ml to BLUEQUAT-BROMID.

After a 24 h exposure period, the treatment medium was replaced by maintenance medium containing 50 µg/ml neutral red. The plates were returned to the incubator for 3 h. Thereafter, the medium was removed, the cells were quickly washed with a fixative (1% formaldehyde / 1% calcium chloride solution) and then an aliquot of 100 µl of extraction solution (1% glacial acetic acid in 50% ethanol) was added to each well to extract the dye. After 20 minutes incubation at room temperature, cell viability was measured by the absorbance of neutral red at 540 nm with a microplate reader. The NRU-50 value in µg/ml, which is the test item concentration required to reduce the neutral red uptake to 50% of negative control, was determined from the graph by interpolation.

Classification of the test item based on the NRU-50 values (µg/ml):
NRU-50: ≥ 750 = non-irritant
NRU-50: 110-749 = not classifiable
NRU-50: < 110 = severe irritant

Results
In two independent experiments, NRU-50 values of 1514 µg/ml and 1673 µg/ml were obtained, resulting in a median NRU-50 value of 1594 µg/ml.

Conclusion
According to the NRU classification system used for the assessment of eye irritation potential, the obtained NRU-50 value of 1594 µg/ml (> 750 µg/ml) results in a classification of BLUEQUAT-BROMID as being non-irritant.

Ref.: 18

Comment
This is a non-guideline study.

3.3.3. Skin sensitisation

Local lymph node assay (LLNA)

Guideline:	OECD 429 (draft 2000)
Species:	mouse: CBA/J
Group:	5 female rats per group
Substance:	801736
Batch:	James Robinson lot pp 1-4
Purity:	99.7 area% (HPLC, 254 nm)
Concentration:	0, 1, 2.5, 5 and 10%
Dosage volume:	25 µL
Vehicle:	acetone/water (1:1) mixed with olive oil (3:1)
Positive control:	801571 (Bandrowski base *vide infra*) at 0.001, 0.01, 0.1 and 1.0% in the vehicle
GLP:	in compliance
Study period:	April 2002

A dose of 25 µl of 0 (vehicle only), 1.0, 2.5, 5.0 and 10.0% 801736 in a mixture of acetone/water (1:1), which was mixed with olive oil (3:1) was applied to the surface of the ear of five female mice per group for three consecutive days. The formulations were prepared daily and used within 5 h after preparation. The maximum solubility of the test item in the chosen vehicle determined the highest test concentration of 10%.

As a positive control, 801571 (*not otherwise defined in the report but stated to be Bandrowski base by the applicant*) at 0.001, 0.01, 0.10 and 1.0% in the vehicle was investigated under identical test conditions. As a negative control the vehicle alone was investigated under the same test conditions.
At day 5, the mice received an intravenous injection of 250 µl solution containing 21.1 µCi of [H3] methyl thymidine. Approximately 5 h later, the mice were killed, and the draining auricular lymph nodes were removed and collected in phosphate buffered saline. After preparing a single cell suspension for each mouse, cells were precipitated by 5% trichloroacetic acid and the radioactivity was determined (incorporation of [H3] methyl thymidine in the pellets) by means of liquid scintillation counting as disintegration per minute (dpm).

Results
The positive control induced a positive response, and elicited at least a 3-fold increase in isotope incorporation relative to the vehicle, with a calculated EC3 value of 0.33%.

801736 induced a negative response: it did not elicit an increase in isotope incorporation relative to the vehicle. The mean stimulation indices were 0.8, 0.6, 0.6 and 0.6 at the concentrations of 1.0, 2.5, 5.0 and 10.0%, respectively.

Conclusion
801736 is not a skin sensitizer under the defined experimental conditions.

Ref.: 19

Comment
801736 was found not to be a sensitizer in this LLNA. The highest dilution used was 10% as this was the maximum solubility in the vehicle. By reference to the batch number and cross referencing this to other studies, the identity of 801736 is BLUEQUAT-BROMID.

3.3.4. Dermal / percutaneous absorption

Guideline:	OECD 428 (2004)
Tissue:	pig skin (back and flanks), 3 donors (2 males and 1 female)
Membranes:	split thickness (approximately 1000 µm thick)
Skin integrity:	tritiated water
Method:	Diffusion Teflon-chambers (9.1 cm² surface)
Replicate cells:	2 independent experiments, 6 chambers each; 10 valid skin samples
Test substance:	BLUEQUAT-BROMID [carbonyls-^{14}C]-BLUEQUAT-BROMID, 5 mCi/mL
Batch:	James Robinson lot pp 1-4 CFQ14699 Batch 1 (labelled test substance)
Purity:	99.6 w% (by NMR) 99.7% (radiochemical purity)
Formulation:	colour cream formulation with 3% BLUEQUAT-BROMID
Dose applied:	100 mg/cm²
Receptor fluid:	physiological receptor fluid
Solubility receptor fluid:	80.3 mg/ml (pH 7.3)
Stability in receptor:	/
Analytical method:	liquid scintillation counting
GLP:	in compliance
Study period:	25 September – 10 October 2006

Two independent experiments were performed with 6 diffusion cells per experiment. For calculations, the mean value of all valid skin samples (n=10) in contact with 3.0% BLUEQUAT-BROMID in a typical non-oxidative hair dye formulation was used. The 2 skin

samples with integrity values above 2% were not taken into consideration for the calculation.

400 mg of the formulation (= 100 mg/cm^2), containing 3.0% BLUEQUAT-BROMID, was applied to the skin samples (= 3.0 mg of test item/cm^2) for 60 minutes and subsequently rinsed off with water and shampoo.

The determination of the amount of BLUEQUAT-BROMID in the washings (i.e. the amount dislodgeable from the skin surface) was determined by measuring the radioactivity by means of scintillation counter. At 16, 24, 40, 48, 64 and 72 h, the content of BLUEQUAT-BROMID was determined in the receptor fluid by the same method. At termination of the experiment, the skin was heat-treated and the "upper skin" (stratum corneum and upper stratum germinativum) was mechanically separated from the "lower skin" (lower stratum germinativum and upper dermis). Both skin compartments were extracted separately and the radioactivity was quantified by means of scintillation counting.

Results

Summary of both experimental series:

	Skin	Integrity-Test	1)		2)		3)		4)		1) + 2) + 3) + 4)	
	No (series)	^3H_2O Permeation (4 hours cumulative)	Receptor fluid (72 hours cumulative)		Lower skin (72 hours cumulative)		Upper skin (72 hours cumulative)		Rinsing solution (after 60 minutes)		Total***	
		[% Dose]	[µg/cm^2]	[% Dose]**	[µg/cm^2]	[% Dose]**	[µg/cm^2]	[% Dose]**	[µg/cm^2]	[% Dose]**	[µg/cm^2]	[% Dose]
Application of 100 mg 3 % 3-((9,10-Dihydro-9,10-dioxo-4-(methylamino)-1-anthracenyl)amino)-N,N-dimethyl-N-propyl-1-Propanaminium-bromid in a typical hair dye formulation* per 1 cm^2 skin	2 (1)	1.5	BLD**** (0.486)	0.016	0.128	0.005	4.448	0.157	2668.36	93.92	2831.95	99.68
	4 (1)	2.0	BLD**** (0.486)	0.016	0.269	0.009	4.077	0.143	2700.03	94.68	2852.56	100.03
	6 (1)	1.7	0.614	0.021	0.143	0.005	2.676	0.093	2753.40	96.08	2890.71	100.87
	8 (1)	1.2	BLD**** (0.486)	0.016	0.240	0.008	2.247	0.078	2830.46	98.33	2954.40	102.64
	10 (1)	1.3	BLD**** (0.486)	0.016	0.182	0.006	1.821	0.062	2937.09	99.95	3000.61	102.11
	12 (1)	1.5	BLD**** (0.486)	0.016	0.207	0.007	3.939	0.136	2787.90	96.20	2893.89	99.85
	*2 (2)*****	*2.4*	*BLD**** (0.486)*	*0.016*	*0.370*	*0.013*	*1.821*	*0.065*	*2576.22*	*91.91*	*2775.31*	*99.01*
	4 (2)	1.7	BLD**** (0.486)	0.016	0.218	0.008	2.516	0.088	2637.02	92.46	2787.70	97.74
	6 (2)	1.5	BLD**** (0.486)	0.016	0.148	0.005	2.258	0.078	2716.31	93.69	2819.36	97.24
	*8 (2)*****	*2.7*	*BLD**** (0.486)*	*0.016*	*0.195*	*0.007*	*1.645*	*0.057*	*2728.12*	*94.51*	*2843.23*	*98.49*
	10 (2)	1.8	BLD**** (0.486)	0.016	0.296	0.010	2.899	0.100	2790.39	95.90	2883.81	99.11
	12 (2)	1.5	BLD**** (0.486)	0.016	0.204	0.007	2.360	0.081	2774.75	95.13	2860.53	98.07
Mean		**1.7**	**0.499**	**0.017**	**0.204**	**0.007**	**2.924**	**0.102**	**2759.57**	**95.63**	**2877.55**	**99.73**
± S.D		0.4	0.041	0.002	0.055	0.002	0.904	0.032	86.48	2.22	63.23	1.79
(n)		(12)	(10)	(10)	(10)	(10)	(10)	(10)	(10)	(10)	(10)	(10)

*vehicle: (typical hair dye formulation as detailed in Annex III); **Corrected for individual applied dose; *** Total is corrected for losses on tips;****below the limit of detection, explanation on theoretically calculated amounts is given in the Annex VI); ***** Outlier; not considered for the calculation of the mean (with the exception for the integrity test)

The majority of the test substance was detected in the rinsing solutions (2759.57 ± 86.48 µg/cm^2). Small amounts of BLUEQUAT-BROMID were found in the upper skin (2.924 ± 0.904 µg/cm^2), in the lower skin (0.204 ± 0.055 µg/cm^2) and in the fractions of the receptor fluid collected over the period of 72 h (0.499 ± 0.041 µg/cm^2).

With respect to the receptor fluid, BLUEQUAT-BROMID was detectable only in a single fraction (fractions 0-16 h) of one skin sample. The absence of amounts of BLUEQUAT-BROMID found in the remaining receptor fluid fractions collected after 16 h indicate that BLUEQUAT-BROMID does not migrate to the receptor fluid over time. Therefore BLUEQUAT-BROMID is considered to not be available from a potential reservoir in the upper skin compartments. Consequently, a maximum amount of 0.703 + 0.069 µg/cm^2 of BLUEQUAT-BROMID is considered biologically available (n=10, three donors; receptor fluid + lower skin; 0.499 µg/cm^2 + 0.204 µg/cm^2).

Amount of BLUEQUAT-BROMID in:	µg/cm^2 (mean ± S.D, n=10)			%* (mean ± S.D, n=10)		
Receptor fluid (72 h)	0.499	±	0.041	0.017	±	0.002
Lower skin (72 h)	0.204	±	0.055	0.007	±	0.002
Upper skin (72 h)	2.924	±	0.904	0.102	±	0.032
Rinsing solution (after 60 min.)	2759.57	±	86.48	95.63	±	2.22
Total balance (recovery)**	2877.55	±	63.23	99.17	±	1.79

* Corrected for individual applied dose; ** Total is corrected for losses on tips

Ref.: 20

Comment
The dosing of the formulation was 100 mg/cm². After 72 hours, the amount of BLUEQUAT-BROMID considered biologically available (receptor + lower skin) from a cream formulation containing 3% BLUEQUAT-BROMID is 0.703 + 0.069 = 0.772 µg/cm^2 or 0.024 + 0.002 (= 0.026)% of the applied dose. The data indicated that any release from the upper dermis was below the limit of detection.

3.3.5. Repeated dose toxicity

3.3.5.1. Repeated Dose (28 days) oral / dermal / inhalation toxicity

Guideline:	OECD 407 (1995)
Species/strain:	rat: Wistar Crl:WI) BR (outbred, SPF-quality)
Group size:	5 males and 5 females
Test substance:	BLUEQUAT-BROMID
Batch:	James Robinson lot PP 1-4
Purity:	99.7 area% (HPLC, 254 nm)
Vehicle:	water (Milli-U)
Dose levels:	0, 30, 100 and 300 mg/kg bw/day
Dose volume:	5 ml/kg bw
Administration:	once daily for at least 28 days, 7 days per week
Route:	oral (gavage)
GLP:	in compliance
Study period:	6 August – 3 September 2002

In this 28 days OECD oral toxicity study, Wistar rats were exposed by gavage to BLUEQUAT-BROMID once per day, 7 days per week for 28 days at the doses of 0, 30, 100 and 300 mg/kg bw/day (5 males and 5 females per dose). The stability of the test substance over 4 hours was checked. Clinical signs were reported daily, body weight and food consumption weekly. Clinical pathology and macroscopic examinations were done at termination as well as organ weights and histopathology on selected tissues.

Results
A decrease in bilirubin level and increase in glucose and potassium values in female rats at the highest dose (300 mg/kg bw/day) was observed. Increased chloride values were observed in male and females from this group. In male rats a slight increase in relative liver weight was also observed at this dose (3.21 g versus 2.94 in the control group).
At the dose of 100 mg/kg bw/day, only increased chloride values were observed in females. No other effects were observed except a discolouration of the faeces and/or various part of the body among all high dose rats.

The authors considered the decrease in bilirubin and the slight increase in liver weights of no toxicological significance. They also considered that the increase in glucose and

potassium levels may be due to a lack of insulin in female at the dose of 300 mg/kg bw/day and the increased chloride value to an artefact in the analysis due to interference with bromide.

Conclusion
Only some biochemical blood parameters were modified at the two highest doses. A NOAEL of 100 mg/kg bw/day was derived.

Ref.: 31

Comment
Comparison of bilirubin and potassium between control and treated animals was unreliable as only 2 female rats were used in the control group.

3.3.5.2. Sub-chronic (90 days) oral / dermal / inhalation toxicity

Guideline:	OECD 408 (1998)
Species/strain:	rat, HanRcc:WIST (SPF)
Group size:	10 males and 10 females (testing) 5 males and 5 females (recovery: vehicle and highest dose group)
Test substance:	BLUEQUAT-BROMID
Batch:	PP 1-4
Purity:	99.7 area% (HPLC)
Vehicle:	bidistilled water
Dose levels:	0, 30, 100 and 300 mg/kg bw/day
Dose volume:	10 ml/kg bw
Administration:	once daily for 91/92 days
Route:	oral (gavage)
GLP:	in compliance
Study period:	26 June 2006 – 15 May 2008

In this 90 days OECD oral toxicity study, Wistar rats were exposed by gavage to BLUEQUAT-BROMID once per day, 7 days per week for 91/92 days at the doses of 0, 30, 100 and 300 mg/kg bw/day (10 males and 10 females per dose). 5 males and 5 females were also used for 28-day recovery assessment following exposure to the vehicle and the highest dose. Clinical signs were reported daily, body weight and food consumption weekly. Clinical pathology and macroscopic examinations were done at termination as well as organ weights and histopathology on selected tissues. Serum insulin analyses were also performed at the end of the exposure period to investigate the hypothesis made in the previous study concerning the increase level of glucose and potassium. The stability of the test substance over 7 days was checked

Results
2 females from the highest dose group (300 mg/kg bw/day) died before the end of the study (one on day 34 and the second on day 76). No findings could explain the cause of death.

No test item-related clinical signs of toxicological relevance were noted during daily or weekly observations.

Faeces and urine discoloration was observed in animals treated at 100 and 300 mg/kg bw/d.

Concerning the haematological parameters, the following observations were made after 13 weeks of treatment: at the dose of 300 mg/kg bw/day, males showed decreased mean corpuscular haemoglobin concentration, decreased haemoglobin distribution width, and elevated haematocrit values. Females showed only a decreased haemoglobin distribution width. At 100 mg/kg bw/day, elevated mean corpuscular volume in females was observed.

After 17 weeks (additional 4 weeks treatment-free recovery), male rats treated previously with 300 mg/kg bw/day showed elevated haematocrit and haemoglobin levels, as well as elevated platelet counts. Even though these findings are statistically significant, all parameters mentioned above were within the limits of historical data. Furthermore, some of the parameters were within the values of the controls from weeks 13 and 17. None of the differences correlated with any histopathological or biochemical changes, no indications of a disturbance in the haematopoiesis were observed. Therefore, these findings are considered by the authors as biological variations commonly seen in this strain of rats at this age.

Concerning the clinical biochemistry, at the dose of 300 mg/kg bw/day, changes were noted such as elevated sodium, potassium, chloride or calcium levels in male and female rats, elevated lactate dehydrogenase and creatinine kinase in females and reduced urea, alanine aminotransferase activity, triglyceride and phospholipid in males. At the end of the recovery period, the following modifications were reported: elevated alkaline phosphatase in females, decreased potassium in males and decreased calcium, protein, and globulin in females. At the dose of 100 mg/kg bw/day, changes in clinical biochemistry such as elevated chloride levels were observed in male and female rats, and elevated sodium in males and decreased triglyceride in males. At the dose of 30 mg/kg bw/day, changes in clinical biochemistry were still noted such as elevated chloride levels in male and female rats, and elevated potassium in females and reduced aspartate aminotransferase activity in males. Again, even though these findings are statistically significant, all parameters mentioned above were within the limits of historical data. Furthermore, some of the parameters were within the values of the controls from weeks 13 and 17. None of the differences correlated with any histopathological or biochemical changes, no indications of a disturbance in the haematopoiesis were observed. Therefore, these findings are considered by the authors as biological variations commonly seen in this strain of rats at this age.

No significant differences were noted between treated rats and control animals concerning serum insulin levels.

No test item-related changes in mean or absolute organ weights were noted at any dose level. No macroscopic or histopathological test item-related changes were noted at any dose levels tested.

Conclusion
A NOAEL above of 300 mg/kg bw/day and a NOEL of 30 mg/kg bw/day based on the excretory discoloration are proposed by the authors.

Ref.: 21

Comment
The clinical observations presented in the study report, as well as the other findings reported, are not considered to be treatment-related and do not give any hints regarding a possible explanation for the death of the female on day 76.
The SCCS agrees with the NOAEL of 300 mg/kg bw/day. This is also supported by the findings of the other toxicity studies.

3.3.5.3. Chronic (> 12 months) toxicity

No data submitted

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1. Mutagenicity / Genotoxicity *in vitro*

Bacterial reverse mutation test
Guideline: OECD 471 (1997)

Species/strain:	*Salmonella typhimurium* TA98, TA100, TA102, TA1535, TA1537	
Replicates:	triplicate cultures in 3 independent experiments	
Test substance:	Bluequat-Bromid	
Batch:	PP 1-4	
Purity:	99.6 area% (HPLC, 254 nm)	
Solvent:	DMSO	
Concentrations:	experiment 1:	0, 1, 10, 100, 1000 and 5000 µg/plate without and with S9-mix
	experiment 2:	0, 10, 30, 100, 300, 1000 and 3000 µg/plate without and with S9-mix
	experiment 3:	0, 300, 600, 1000, 2000 and 3000 µg/plate with S9-mix strain TA1537 only
Treatment:	direct plate incorporation with 48 h incubation without and with S9-mix	
GLP:	in compliance	
Study period:	11 March 2002 - 28 March 2002	

Bluequat-Bromid was investigated for the induction of gene mutations in *Salmonella typhimurium* (Ames test). Liver S9 fraction from Aroclor 1254-induced rats was used as exogenous metabolic activation system. Toxicity was evaluated on the basis of a reduction in the number of revertant colonies and a qualitative evaluation of the bacterial lawn. In the main test the bacteria were exposed up to the prescribed maximum concentration of 5000 µg/plate in the first experiment. The results were repeated in 2 other experiments with concentrations up to 3000 µg/plate. All experiments were performed with the direct plate incorporation method. Negative and positive controls were in accordance with the OECD guideline.

Results
Without S9-mix toxic effects were observed at concentrations ≥ 300 µg/plate with TA102, ≥ 1000 µg/plate with TA98 and TA100 and ≥ 3000 µg/plate with the other strains: with S9-mix at concentrations ≥ 1000 µg/plate with TA100 and TA102, ≥ 2000 µg/plate with TA1537 and ≥ 3000 µg/plate with the other strains.
Bluequat-Bromid treatment resulted in an increase in the number of revertant colonies in strain TA1537. As these effects did not show a clear and relevant concentration-response relationship over a range of concentrations and were observed near to toxic concentrations they are considered biologically not relevant.

Conclusion
Under the experimental conditions used Bluequat-Bromid was not mutagenic in this gene mutation tests in bacteria.

Ref.: 30

Bacterial reverse mutation test

Guideline:	OECD 471 (1997)	
Species/strain:	*Salmonella typhimurium* TA98, TA100, TA102, TA1535, TA1537	
Replicates:	triplicate cultures in 2 independent experiments	
Test substance:	Bluequat-Bromid	
Batch:	James Robinson lot PP 1-4	
Purity:	99.7 area% (HPLC)	
Solvent:	deionised water	
Concentrations:	experiment I:	3, 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate without and with S9-mix
	experiment II:	10, 33, 100, 333, 1000, 2500 and 5000 µg/plate without and with S9-mix
	experiment IIA:	10, 33, 100, 333, 1000, 2500 and 5000 µg/plate (TA1537 only) without and with S9-mix
Treatment:	experiment I:	direct plate incorporation method with 48 h incubation

		without and with S9-mix
	experiment II:	pre-incubation method with 60 minutes pre-incubation and 48 h incubation without and with S9-mix
GLP:	in compliance	
Study period:	19 December 2006 – 5 February 2007	

Bluequat-Bromid was investigated for the induction of gene mutations in *Salmonella typhimurium* (Ames test). Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. Test concentrations were based on the level of toxicity in a preliminary toxicity test with all Salmonella strains both without and with S9-mix. Toxicity was evaluated for 8 concentrations up to the prescribed maximum concentration of 5000 µg/plate on the basis of a reduction in the number of spontaneous revertant colonies and/or clearing of the bacterial background lawn. Since in this pre-experiment evaluable plates were obtained for five concentrations or more in the strains used, the pre-experiment is reported as experiment I. Experiment I was performed with the direct plate incorporation method, experiment II with the pre-incubation method with 60 min pre-incubation. Negative and positive controls were in accordance with the OECD guideline.

Results
Both without and with S9-mix, the plates incubated with Bluequat-Bromid showed normal background growth up to 5000 µg/plate. In experiment I both without and with S9-mix toxic effects, evident as a reduction in the number of revertants, were observed at the highest concentrations in TA98, TA100 and TA102, in experiment II without S9-mix in TA98, TA100, TA102 and TA1535 and with S9-mix in TA100, TA102 and TA1535.
In experiment II and IIA (performed to confirm the results from experiment II) Bluequat-Bromid treatment resulted in a biologically relevant and concentration dependent increase in the number of revertant colonies in strains TA 1537. A biologically relevant increase in the number of revertants was not seen in any of the other strains used, in both experiments both without and with S9-mix.

Conclusion
Under the experimental conditions used Bluequat-Bromid was mutagenic in this gene mutation tests in bacteria.

Ref.: 22

In vitro mammalian cell gene mutation assay (_tk_-locus)

Guideline:	OECD 476 (1997)	
Cells:	mouse lymphoma L5178Y $tk^{+/-}$ cells	
Replicates:	duplicate culture in 2 independent experiments	
Test substance:	Bluequat-Bromid	
Batch:	PP 1-4	
Purity:	99.7 area% (HPLC)	
Solvent:	cell culture medium	
Concentrations:	experiment 1:	0.5, 1, 5, 10, 50, 100, 500, 1000, 2500 and 5000 µg/ml, without and with S9-mix.
	experiment 2:	25, 50, 100, 250, 500, 750, 1250, 2500, 3500 and 5000 µg/ml with S9-mix.
	experiment 2:	0.005, 0.01, 0.51, 1.02, 5.10, 10.2, 51, 102, 510 and 1020 µg/ml without S9-mix.
Treatment:	experiment 1:	4 h treatment both without and with S9-mix; expression period 72 h and a selection period of 11-14 days
	experiment 2:	24 h treatment without S9-mix; expression period 48 h and a selection period of 11-14 days 4 h treatment with S9-mix; expression period 72 h and a selection period of 11-14 days

GLP: in compliance
Study period: 9 April – 20 August 2002

Bluequat-Bromid was assayed for gene mutations at the *tk* locus of mouse lymphoma cells both in the absence and presence of S9 metabolic activation. Liver S9 fraction from phenobarbital/β-naphthoflavone-induced rats was used as exogenous metabolic activation system. Test concentrations were based on the results of a pre-test for toxicity with 6 concentrations up to 5000 µg/ml, the prescribed maximum concentration according to the OECD guideline, in the absence of S9-mix measuring suspension growth relative to the concurrent vehicle control cell cultures. The pre-test was performed under the same experimental conditions as in the main test. In the main tests, cells were treated for 4 h (both without and with S9-mix) followed by an expression period of 72 h or for 24 h (without S9-mix only) followed by an expression period of 48 h, to fix the DNA damage into a stable *tk* mutation and a selection growth 11-14 days. Toxicity was measured in the main experiments as percentage suspension and relative total growth of the treated cultures relative to the concurrent vehicle control cell cultures. To discriminate between large (indicative for mutagenic effects) and small colonies (indicative for a clastogenic effect) colony sizing was performed. An increased occurrence of small colonies indicated by a low large/small colonies ratio (<4) was associated with clastogenic effects and/or chromosomal aberrations. Negative and positive controls were in accordance with the OECD guideline.

Results
In the pre-test the appropriate level of toxicity (about 10-20% survival after the highest concentration) was reached at the highest concentration tested. Therefore, 5000 µg/ml was chosen as the highest concentration. In the main experiment, although tested at this prescribed maximum concentration, the appropriate level of toxicity was not reached in the tests without S9-mix.
A concentration dependent and biologically relevant increase in the mutant frequency was only observed in experiment 2 after 24 h exposure to Bluequat-Bromid without metabolic activation. The results of colony sizing were inconclusive. A biologically relevant increase in the mutant frequency was not found after 4 h exposure both without and with metabolic activation.

Conclusion
Under the experimental conditions used, Bluequat-Bromid was mutagenic in this mouse lymphoma assay using the *tk* locus as reporter gene. The results of colony sizing did not allow a conclusion on a mutagenic or a more clastogenic effect.

Ref.: 23

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

Mammalian erythrocyte micronucleus test

Guideline: OECD 474 (1997)
Species/strain: mouse, NMRI
Group size: 5 males and 5 females per dose group
Test substance: Bluequat-Bromid
Batch: James Robinson lot PP 1-4
Purity: 99.7 area% (HPLC)
Vehicle: deiosined water
Dose levels: 0, 500, 1000, 2000 mg/kg bw/day
Route: twice orally at 24h intervals
Sacrifice times: 24 h after the last treatment
GLP: in compliance
Study period: 3 March 2003 – 1 April 2003

Bluequat-Bromid has been investigated for induction of micronuclei in bone marrow cells of mice. Test doses were based on the results of a preliminary study on acute toxicity performed under identical conditions as in the mutagenicity study. Male and female mice were treated orally twice 24 h apart with 200 and 2000 mg/kg bw and examined for acute toxic symptoms and/or mortality at 1, 2-4, 6 and 24 h after each treatment. In the main experiment male and female mice were exposed orally twice at 24 h intervals to 0, 500, 1000, 2000 mg/kg bw/day. The mice were examined for acute toxic symptoms and/or mortality at 1, 2-4, 6 and 24 h after each treatment. Bone marrow cells were collected 24 h after the last treatment. Toxicity and thus exposure of the target cells was determined by measuring the ratio between polychromatic and total erythrocytes (PCE/TE). Negative and positive controls were in accordance with the OECD guideline.

Results
In the pre-test all mice survived the dose levels up to 2000 mg/kg bw/day. Clinical observations observed included: reduction in spontaneous activity (after both treatments) and ruffled fur (after the first treatment only). In the micronucleus test, next to a reduction in spontaneous activity and ruffled fur in all doses both after the first and the second treatment also occasionally eyelid closure and abdominal position were found particularly in the highest dose. Both in the pre-test and in the micronucleus test mice treated with 1000 and 2000 mg/kg bw/day had blue to green coloured urine
Both in males and females a decrease in the PCE/TE ratio due to treatment with Bluequat-Bromid was not observed. However, the clinical signs reported, particularly the coloured urine, indicated systemic distribution and thus bioavailability of Bluequat-Bromid. A biologically relevant and dose dependent increase in the number of cells with micronuclei was not found at any dose level of Bluequat-Bromid.

Conclusions
Under the experimental conditions used Bluequat-Bromid did not induce an increase in the number of bone marrow cells with micronuclei and, consequently, Bluequat-Bromid is not genotoxic (clastogenic and/or aneugenic) in bone marrow cells of mice.

Ref.: 24

Alkaline Comet assay

Guideline:	The experiment and evaluation of the slides were performed according to an internationally accepted protocol (see ref. 25).
Species/strain:	rat, CRL:(WI) BR Wistar
Group size:	5 animals per dose group
Test substance:	Bluequat-Bromid
Batch:	PP 1-4
Purity:	99.6 area% (HPLC)
Vehicle:	deionised water
Concentrations:	0, 150, 300 and 600 mg/kg bw/day
Route:	twice orally at 24h intervals
Sampling:	23 h after the first treatment
GLP:	in compliance
Study period:	26 January – 29 April 2004

Bluequat-Bromid was evaluated for its potential to induce DNA damage in cells of the liver, urinary bladder and stomach epithelium after oral administration of Bluequat-Bromid to male rats. Test doses were selected on the results of 2 pilot experiments on acute toxicity performed under identical conditions as in the main study. Male rats were treated orally once with 2000 mg/kg bw and twice 24 h apart with 100, 200, 400 and 600 mg/kg bw/day and examined for acute toxic symptoms and/or mortality.
In the Comet assay, food was withdrawn about 4-5 h before the first treatment. Bluequat-Bromid was administered by oral gavage twice at a 24 h interval at 0, 150, 300 and 600 mg/kg bw/day. Animals received food again 1 h after the first treatment. Tissue sampling

occurred 23 h after the first treatment. The animals were killed by perfusion through the vena cava.
After perfusion the liver, stomach and urinary bladder were carefully removed and hepatocyte, stomach cell and bladder epithelium cell suspensions were prepared. At least two slides per cell suspension were exposed to alkali (pH>13) for 20 minutes, followed by electrophoresis for 40 minutes (stomach cell for 30 min) at 25 V and 300 mA, stained with ethidium bromide and scored for comets. Tail length, defined as distance between the middle of the head and the end of the tail, was used as assessment parameter and determined in 50 cells per each of two slides. Negative and positive controls were incorporated in the study.

Results
In the pilot tests the rats treated with 2000 mg/kg bw all died shortly after treatment. The clinical symptoms found were roughened fur and rapid breathing in all rats treated and additionally palmospasm and discoloured faeces in the rats of the two highest doses. Identical symptoms were observed in the Comet assay. When opening the rats for perfusion, stomach, small intestine and colon partly showed a clear blue colour for a few rats treated with Bluequat-Bromid. These findings demonstrate relevant systemic exposure of of males to Bluequat-Bromid.
Following oral administration of dose levels up to 600 mg/kg bw/day administered on 2 consecutive days, Bluequat-Bromid did not induce a biologically relevant increase in tail length values in hepatocytes, stomach cells or urinary bladder epithelial cells compared to those of the concurrent tissue control values.

Conclusion
Under the experimental conditions used Bluequat-Bromid did not induce DNA damage in cells from the liver, stomach or urinary bladder of treated rats and, consequently, Bluequat-Bromid was not genotoxic (clastogenic and/or mutagenic) in these tissues of rats.

Ref.: 26

Comment
In this study, only the tail length was evaluated and not the tail moment which is considered a more sensitive parameter.

3.3.7. Carcinogenicity

No data submitted

3.3.8. Reproductive toxicity

3.3.8.1. Two generation reproduction toxicity

No data submitted

3.3.8.2. Teratogenicity

Prenatal development toxicity study

Guideline:	OECD 414 (2001)
Species/strain:	rat, HanRcc:WIST (SPF quality)
Group size:	22 mated females per group
Test substance:	Bluequat-Bromid
Batch:	James Robinson lot PP 1-4
Purity:	99.7 area% (HPLC)
Vehicle:	highly purified water
Dose levels:	0, 100, 300, 1000 mg/kg bw/day

Dosage volume:	10 mL/kg bw
Route:	oral (gavage)
Administration:	once daily from day 6 through to day 20 *post-coitum*
GLP statement:	in compliance
Study period:	6 June – 23 November 2006

In this oral developmental toxicity study, Bluequat-Bromid was administered orally once daily to 22 presumed-pregnant rat HanRcc:WIST, from day 6 to day 20 of gestation at the doses of 0, 100, 300 and 1000 mg/kg bw/d in purified water. The dosage volume was 10 ml/kg bw. Dose levels were based on a dose range-finding study where the animal group administered with high dose of 1000 mg/kg bw did not show any toxic effect.

Viabilities, clinical observations, body weights and feed consumption values were recorded. All surviving rats were sacrificed on day 21. The gravid uterus was weighed and examined for gross external alterations and sex. Caesarean-sectioning and subsequent foetal observations were conducted.

Results
Maternal parameters:

All dams except two dams from the 1000 mg/kg bw/day dosage group survived to GD 21. The death of both animals was considered incidental due to misadministration.

No clinical symptoms were recorded in all tested rats. Black faeces due to coloration by the test compound were noted in all treated rats.

Mean body weights and food consumption in all dosed groups were comparable to the control group.

No significant differences were observed in the number of *corpora lutea* in all treated rats when comparing with the control rats. No significant differences in the number of implantations or resorptions were observed in the treated rats when comparing with the control rats.

Foetal parameters:

No significant differences in: body weight, litter sizes, number of live or resorbed foetuses or in foetal abnormalities were observed between treated and control animals.

Conclusion
Based on the results of this oral prenatal developmental toxicity study, a NOAEL of 1000 mg/kg bw/d for maternal and embryo-foetal toxicity was derived. This NOAEL corresponds to the highest tested dose in this study.

Ref.: 27

Comment
2 dams died at 1000 mg/kg bw/d, it cannot be excluded that it is treatment related. Therefore, the SCCS sets the NOAEL for maternal toxicity at 300 mg/kg bw/d.

3.3.9. Toxicokinetics

Guideline:	OECD 417 (1984), OECD 427 (2004)
Species/strain:	rat: Wistar Crl:WI
Group size:	4 females per dose level (groups 1–3) ((ADME) 6 females per dose level (groups 4-6) (Toxicokinetics)
Test substance:	Bluequat-Bromid

	[carbonyl-^{14}C]Bluequat-Bromid (radiolabelled)
Batch:	James Robinson lot PP 1-4
	CFQ14699 batch 1 (radiolabelled)
Purity:	99.7 area% (HPLC)
	99.7% (HPLC) (radiochemical purity)
Vehicle:	0.9% NaCl (i.v.)
	Milli-Q water (oral)
	Water/acetone 1:1 (dermal)
Dose levels:	1 mg/kg bw (i.v.)
	100 mg/kg bw (oral)
	125 mg/mL (dermal)
Dose volume:	2 mL/kg bw (i.v.)
	5 mL/kg bw (oral)
	0.1 mL per dosing area (dermal)
Administration:	single dose
GLP statement:	in compliance
Study period:	19 February – 23 March 2007

Absorption, distribution, metabolism and excretion of ^{14}C-Bluequat-Bromid were investigated in Wistar rats after a single oral, intravenous or dermal dose. Six groups were used: three groups for the mass balance study and three groups for toxicokinetics. The doses used were: 1 mg/kg bw intravenously, 100 mg/kg bw orally and 100 mg/kg bw dermally (125 mg/mL or 1.25 mg/cm^2). The vehicles were 0.9% saline intravenously, milli-Q water orally, acetone/water 1:1 dermally.

In the mass-balance groups (1-3) urine and faeces were collected in 0-8, 8-24, 24-48, 48-72 and 72-96 hr intervals. Total radioactivity in urine, faeces, tissues and organs was determined. Selected urine and faeces samples were pooled per group and the metabolite profile was investigated. In the toxicokinetic groups (4-6) blood was sampled alternatively from several rats per time point at 0.25, 0.5, 1, 2, 4, 8, 24 and 48 h after dosing.

Results
Mortality was observed in this study after dosing 10 mg/kg bw intravenously. One animal died immediately after *iv* dosing. Therefore, a dose level of 1 mg/kg bw was selected for further experiments.

All animals of the *iv* exposure groups displayed quick breathing just after dosing, and two rats showed partly blue colouring of the tail. After oral exposure, coloration of urine and faeces was observed. Three animals showed piloerection. After dermal dosing, all rats displayed red discharge from eye and/or nose, due to the collar around the neck.

The average total recovery of radioactivity in the intravenous and oral group was 88% and 90% of the applied dose respectively and 97% of the applied dose in the dermal group.

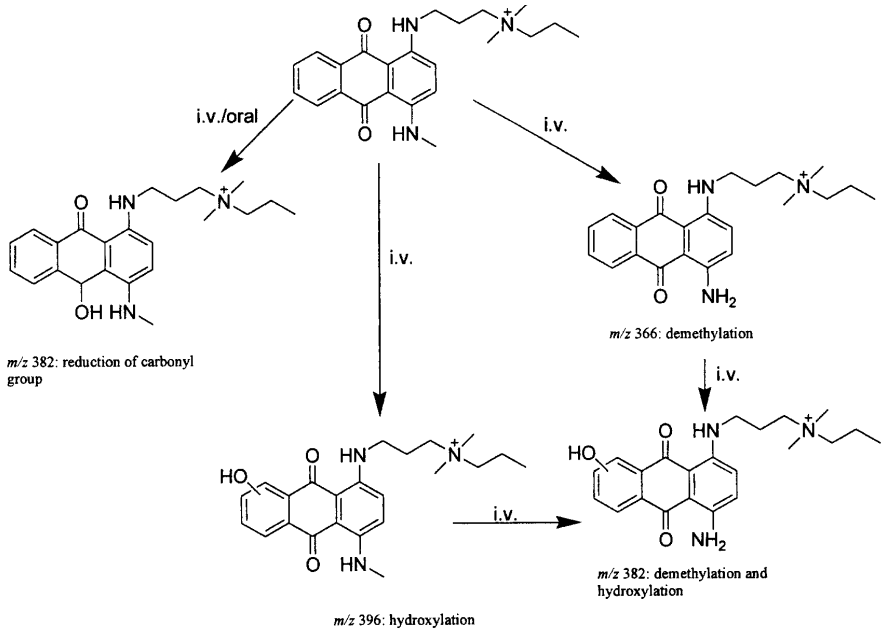
After iv injection, two rats in the group 4 showed plasma concentrations that were over 10-fold higher than the plasma concentrations of 3 other rats in the same group. No reason could be given by the authors to explain this high variability between animals. Urinary excretion accounted for 17% and faecal excretion for 67.3%. The terminal half-life was between 5.85 and 10.4 hours after iv injection

After oral exposure, the average oral absorption was low: 5.1% when calculated from the urine data and 0.8% and 10.9% when calculating from the plasma data (comparison with the iv group 4). The oral absorption was then 0.8% based upon all animals of group 4, and 10.9% based upon the parameters calculated for the two rats with the highest plasma concentrations. Urinary excretion accounted for 0.9% of the administered dose and faecal excretion accounted for 87%. T_{max} value was 0.25 h and C_{max} value was 0.309 mg/kg bw and AUC_{∞} value 5.66 h x mg/kg bw, The terminal half-life was 22.1 hours.

After dermal exposure, the absorption was low and plasma concentrations were below the limit of quantification. 0.2% absorption was calculated (0.0003 mg/cm^2) from excretion, cage-wash, carcass and unexposed skin and 0.25% (0.0004 mg/cm^2) when adding skin residue dose. Urinary excretion accounted for 0.01% of the administered dose and faecal excretion for 0.08%. T_{max}, C_{max} and AUC values were not determined due to the too low concentrations in the plasma.

In plasma sample at termination, no significant radioactivity could be detected and therefore, no metabolite analysis was performed on these samples. The urine samples contained only one peak corresponding to the parent compounds; samples were not subjected to metabolite screening further.

In the faeces, extracts of four possible metabolites have been detected after iv dosing, one after oral dosing and no metabolite after dermal dosing. Reduction of carbonyl group, demethylation and hydroxylation are the deduced metabolic pathways after iv exposure. The suggested metabolite profile is presented in the following figure:



Following dermal exposure, no metabolite could be detected.

Conclusion
Bluequat-Bromid administered orally showed only limited absorption; the absorbed fraction was distributed into all organs, metabolised to some extent and excreted mainly in the faeces. Following dermal exposure, no metabolite could be detected.

Dermal absorption of Bluequat-Bromid was low after 1 hour of exposure and when absorbed, excretion took also place mainly in the faeces.

Reduction of carbonyl group, demethylation and hydroxylation are the supposed metabolic pathway after iv exposure.

Ref.: 28

Comment
Only female rats and one dose per routes of exposure were used. A very low oral absorption of Bluequat-Bromid was observed. The very high variability observed in the toxicokinetic

results between rats dosed by the *iv* route make the whole results of this part of the study very approximative. The oral bioavailability should then be based on the ADME study. Based on the results of this study, a value of 5% will be applied to correct the oral NOAEL used to calculate the MoS.

3.3.10. Photo-induced toxicity

3.3.10.1. Phototoxicity / photoirritation and photosensitisation

No data submitted

3.3.10.2. Phototoxicity / photomutagenicity / photoclastogenicity

No data submitted

3.3.11. Human data

No data submitted

3.3.12. Special investigations

No data submitted

3.3.13. Safety evaluation (including calculation of the MoS)

CALCULATION OF THE MARGIN OF SAFETY

HC Blue 16

Absorption through the skin	**A**	**=**	**0.772 µg/cm²**
Skin Area surface	**SAS**	**=**	**580 cm²**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**0.45 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED) bw/d	**SAS x A x 0.001/60**	**=**	**0.0075 mg/kg**
No Observed Adverse Effect Level (90-day, oral, rat)	**NOAEL**	**=**	**300 mg/kg bw/d**
NOAEL adjusted for 5% bio-availability		**=**	**15 mg/kg bw/d**
MOS		**=**	**2000**

3.3.14. Discussion

Physico-chemical specifications
HC Blue 16 is used as hair colouring agent in semi-permanent hair dye formulations at a maximum on-head concentration of 3%.
HC Blue 16 is a secondary amine, and therefore, it can be nitrosated to form nitrosamine. Nitrosamine content corresponding to HC Blue 16 was not reported. It should not be used together with nitrosating agents. Nitrosamine content should be <50 ppb. Stability of HC Blue 16 in typical hair dye formulations was not reported.

General toxicity
In the 90-day study, the clinical observations presented in the study report, as well as the other findings reported, are not considered to be treatment-related and do not give any hints regarding a possible explanation for the death of the female on day 76. The SCCS considers the NOAEL to be 300 mg/kg bw/day.
In an oral prenatal developmental toxicity study, 2 dams died at 1000 mg/kg bw/d, it cannot be excluded that it is treatment related. Therefore, the SCCS sets the NOAEL for maternal toxicity at 300 mg/kg bw/day. The NOAEL for embryo-foetal toxicity is 1000 mg/kg bw/day.
Based on the results of an ADME study, a value of 5% will be applied to correct the oral NOAEL used to calculate the MoS.

Irritation, sensitisation
Neat HC Blue 16 is an irritant to the rabbit eye. It was not classified as an eye irritant in a non-guideline study (Neutral Red Uptake assay). Although blue staining may have masked slight erythema, no reactions on rabbit skin were noted from application of neat HC Blue 16.

HC Blue 16 was not a sensitizer in a LLNA. The highest test concentration of HC Blue 16 used was 10% in this study as this was the maximum solubility in the vehicle.

Dermal absorption
After 72 hours, the amount of HC Blue 16 considered biologically available (receptor + lower skin) from a cream formulation containing 3% HC Blue 16 is 0.703 + 0.069 (= 0.772) µg/cm^2 or 0.024 + 0.002 (= 0.026) % of the applied dose. The data indicated that any release from the upper dermis was below the limit of detection.

Mutagenicity
Overall, the genotoxicity of HC Blue 16 is sufficiently investigated in valid genotoxicity tests for the 3 endpoints of genotoxicity: gene mutations, chromosome aberrations and aneuploidy. HC Blue 16 induced gene mutations both in a gene mutation test in bacteria and in a mouse lymphoma assay in mammalian cells. An *in vitro* test for clastogenicity was not performed. However, in an *in vivo* micronucleus test in mice an increase in bone marrow cells with micronuclei was not found.
The positive findings from the *in vitro* tests for gene mutations were not confirmed in an *in vivo* tests. In a Comet assay, a test nowadays considered as indicative for both clastogenic (chromosome breaks) and mutagenic (gene mutations) effects, an increase in tail length in cells of the liver, stomach and urinary bladder was not observed.
Consequently, on the basis of these tests, HC Blue 16 can be considered to have no genotoxic potential and additional tests are unnecessary.

Carcinogenicity

No data submitted

4. CONCLUSION

The SCCS is of the opinion that the use of HC Blue 16 with a maximum on-head concentration of 3.0% in non-oxidative hair dye formulations does not pose a risk to the health of the consumer.

A sensitisation potential of HC Blue 16 cannot be excluded.

HC Blue 16 is a secondary amine, and therefore, it can be nitrosated to form nitrosamine . Nitrosamine content corresponding to HC Blue 16 was not reported.
It should not be used in combination with nitrosating substances. The nitrosamine content should be < 50 ppb.

5. MINORITY OPINION

Not applicable

6. REFERENCES

Submission I, 2008

1. Umbricht, G.; Comparison of different batches of HC Blue No. 16; COSMITAL SA; 2008
2. Umbricht, G.; Data concerning definition of structure of HC Blue No. 16; COSMITAL SA; 2008
3. Lange, J.; Partition coefficient (n-octanol / water) using HPLC-method; NOACK; 2007
4. Bodsch, J.; Sieve analysis and dust content; NOACK; 2008
5. Dougoud, P.; Solubility and pH in solution (Draft); COSMITAL SA; 2001
6. Bodsch, J.; Melting point / melting range; NOACK; 2008
7. Bodsch, J.; Statement on boiling point; NOACK; 2008
8. Bodsch, J.; Determination of the density; NOACK; 2008
9. Smeykal, H.; Vapour pressure A.4. (OECD 104); SIEMENS AG; 2008
10. Bodsch, J.; Determination of surface tension; NOACK; 2008
11. Lange, J.; Water solubility (Flask Method); NOACK; 2007
12. Schulz, M.; Flammability of solids; NOACK; 2007
13. Smeykal, H.; Explosive properties A.14.; SIEMENS AG; 2008
14. Smeykal, H.; Auto-flammability (Solids-determination of relative self-ignition temperature) A.16.; SIEMENS AG; 2008
15. Lange, J.; Oxidizing properties of solids; NOACK; 2008
16. van Otterdijk, F. M.; Primary skin irritation/corrosion study with Bluequat-Bromid / 801 736 in the rabbit (4-h-semi-occlussive application) Protocol (page 1-8); External protocol amendment (1 page); NOTOX; 2002
17. van Otterdijk, F. M.; Acute eye irritation/corrosion study with Bluequat-Bromid / 801736 in the rabbit; NOTOX; 2002
18. Faller, C.; Assessment of the eye irritation potential of WR 801736 by cytotoxicity measurement in the neutral red uptake assay (NRU) on human keratinocytes (HaCaT); COSMITAL SA; 2002
19. Christ, M.; Local Lymph Node Assay - 801736; MDS PHARMA SERVICES; 2002
20. Sieber, T. P.; Cutaneous absorption of 3% 3-((9,10-Dihydro-9,10-dioxo-4-(methylamino)-1-anthracenyl)amino)-N,N-dimethyl-N-propyl-1-Propanaminium-bromid (WR801736) in a typical hair dye formulation through pig skin in vitro; COSMITAL SA; 2006
21. Braun, W. H.; Brunke, A.; Flade, D.; Burri, R.; Heider, K.; Bluequet-Bromid (WR 801736): 13-week oral toxicity (gavage) study in Wistar Rats; RCC; 2008
22. Sokolowski, A.; Salmonella typhimurium reverse mutation assay with Bluequat-Bromid (WR 801736); RCC-CCR; 2007
23. Hamann, U.; In vitro mammalian cell gene mutation assay (Thymidine Kinase Locus/TK+/-) in mouse lymphoma L5178Y cells with WR801736; BIOSERVICE; 2002
24. Honarvar, N.; Micronucleus assay in bone marrow cells of the mouse with Bluequat-Bromid / 801736; RCC-CCR; 2003

25. Hartmann, A.; Agurell, E.; Beevers, C.; Brendler-Schwaab, S.; Burlinson, B.; Clay, P.; Collins, A.; Smith, A.; Speit, G.; Thybaud, V.; Tice, R. R.; Recommendations for conducting the in vivo alkaline comet assay; MUTAGENESIS; 18, 45-51; 2003
26. Brendler-Schwaab, S.; Bluequat-Bromid (WR 801736) Comet Assay in vivo in male rat liver, stomach and urinary bladder epithelium; BAYER AG; 2005
27. Pössnecker, A.; Flade, D.; Bluequat-Bromid (WR 801736): Prenatal developmental toxicity study in the Han Wistar rat; RCC; 2007
28. Wenker, M.A.M.; Absorption, distribution, metabolism and excretion of Bluequat-Bromid (WR 801736, lot PP 1-4) in the Wistar rat; NOTOX; 2008
29. König, P.; Data base search for references for 3-((9,10-Dihydro-9,10-dioxo-4-(methylamino)-1-anthracenyl)amino)- N,N-dimethyl-N-propyl-1-Propanaminium-bromid; WELLA SERVICE GMBH; 1; 2006

References provided upon request

30. Aeby, P.; Assessment of the potential mutagenicity of WR 801736 in the AMES reversion assay with Salmonella Typhimurium; COSMITAL SA; 2002
31. Otterdijk, F. M.; Subacute 28-day oral toxicity with Bluequat-Bromid / 801736 by daily gavage in the rat; NOTOX; 2004