Scientific Committee on Consumer Products

SCCP

OPINION ON

Basic Orange 69

The SCCP adopted this opinion at its 17th plenary of 30 September 2008

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.

They are: the Scientific Committee on Consumer Products (SCCP), the Scientific Committee on Health and Environmental Risks (SCHER) and the Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR) and are made up of external experts.

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

SCCP
Questions concerning the safety of consumer products (non-food products intended for the consumer).
In particular, the Committee addresses questions related to the safety and allergenic properties of cosmetic products and ingredients with respect to their impact on consumer health, toys, textiles, clothing, personal care products, domestic products such as detergents and consumer services such as tattooing.

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1. BACKGROUND

Submission I for the hair dye Basic Orange 69 with the chemical name 3-[(4-amino-2,5-dimethoxyphenyl)azo]-N,N,N-trimethyl benzenaminium chloride was submitted in May 2006 by EFfCI [1].

Basic Orange 69 is a new hair dye substance and a new chemical as well without an ELINCS number.

According to this submission, the substance is used in semi-permanent hair dye formulation at a maximum concentration of 2.0%. It can also be used in an oxidative hair dye formulation at a maximum final concentration, after mixing 1:1 with hydrogen peroxide, on the scalp of 1.0%.

2. TERMS OF REFERENCE

1. *Does SCCP consider Basic Orange 69 safe for consumers when used as a hair dye substance in semi-permanent hair dye formulations with a maximum 2.0% in the finished cosmetic product taken into account the scientific data provided.*

2. *Does SCCP consider Basic Orange 69 safe for consumers when used as a hair dye in oxidative hair dye formulations with a concentration of maximum 1.0% on the scalp taken into account the scientific data provided?*

[1] EFfCI – The European Federation for Cosmetics Ingredients

3. OPINION

3.1. Chemical and Physical Specifications

Some of the toxicological studies reported in this dossier refer to a zinc chloride salt of the molecule intended to be marketed (referred to as Zn Basic Orange 69). However, some of these studies were renewed with the current pure substance (referred to as Basic Orange 69) devoid of zinc chloride in order to assess the potential influence of zinc chloride in the initial studies. It is not clear, if physicochemical properties and impurities differ for the pure substance and the zinc chloride salt.

3.1.1. Chemical identity

3.1.1.1. Primary name and/or INCI name

Basic Orange 69

3.1.1.2. Chemical names

Benzenaminium, 3-[(4-amino-2,5-dimethoxyphenyl)azo]-N,N,N-trimethyl, chloride

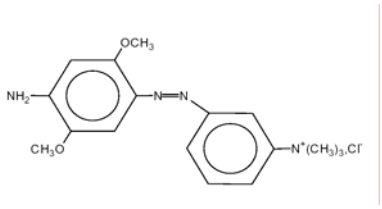
3.1.1.3. Trade names and abbreviations

306007 Arianor Orange
CI 112605

3.1.1.4. CAS / EINECS number

CAS: 226940-14-3
EINECS/ELINCS: /

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula: C17 H23N4O2, Cl

3.1.2. Physical form

Orange red to orange brown powder

3.1.3. Molecular weight

Molecular weight: 315.4 g/mol as cation
350.90 g/mol as chloride

3.1.4. Purity, composition and substance codes

Purity (HPLC):	95.0 % min (peak area)
Dye content:	85.0 % min as chloride salt
	76.4% min as cation
Volatile matter (water):	10 % max
Free chloride:	24 % max
Free sulfate:	0.05 % max
Water insoluble matter:	< 0.1%
Standardising agent:	none

3.1.5. Impurities / accompanying contaminants

Organic impurities (maximum):

Subsidiary dye content:	< 0.5 % calculated as the principal dye
3-aminophenyl trimethyl ammonium chloride:	0.01%
2,5 dimethoxyaniline:	not detected

Inorganic impurities (maximum):

Pb:	10 ppm
As:	3 ppm
Hg:	1 ppm
Fe:	50 ppm
Cd:	1 ppm
Zn:	20 ppm
Cu:	5 ppm
Cr:	5 ppm

3.1.6. Solubility

Water: > 240 g/L

3.1.7. Partition coefficient (Log P_{ow})

Log Pow: -3.0 (EC Testing method A8)

3.1.8. Additional physical and chemical specifications

Melting point:	95 °C
Boiling point:	/
Flash point:	/
Vapour pressure:	/
Density:	/
Viscosity:	/
pKa:	/
Refractive index:	/
pH (1% in water):	6.9 ± 1.0
UV_Vis spectrum (200-800 nm)	/
Decomposition temperature:	170 °C
Relative self ignition temperature (EC Testing method A16):	370 °C
Explosive properties (EC Testing method A14):	
BAM fallhammer:	negative
BAM friction:	negative

	Koenen: negative
Flammability of solid (EC Testing method A10):	not highly flammable
Relative density ($D4_{20}$ pycnometer):	0.75-0.80
Odour:	slight organic

3.1.9. Homogeneity and Stability

No data provided

General Comments to physico-chemical characterisation

- No analytical file was submitted.
- No data on homogeneity and stability of the test substance were provided.
- Purity and impurities for the batches investigated in the different toxicity test is mostly not given.
- It is not clear, if physicochemical properties and impurities differ for the pure substance Basic Orange 69 and the zinc-chloride salt Zinc Basic Orange 69.

3.2. Function and uses

Basic Orange 69 will be incorporated in semi-permanent hair dye formulations at a maximum concentration of 2.0%. The commercial product has to be applied without prior dilution with a maximum pause time of 30 minutes, and then rinsed. The volume of application intended to be used is in the range of 30 ml and 50 ml. In this case, the hair dye formulation may be used weekly.

It can also be used in oxidative hair dye formulations at a maximum final concentration (after dilution) of 1.0% with a pause of 30 to 45 minutes, rinsed, then followed with a neutralizing shampoo then rinsed again. In this case, the hair dye formulation may be used every 4 to 6 weeks.

3.3. Toxicological Evaluation

Part of the toxicological studies reported in this dossier refers to a zinc chloride salt of the molecule intended to be marketed (referred to as Zn Basic Orange 69). However, some of these studies were renewed with the current pure substance (referred to as Basic Orange 69) devoid of zinc chloride in order to assess the potential incidence of zinc chloride in the initial studies. Such studies were performed with the molecule Basic Orange 69 referring to a mentioned global 82.9% purity.

According to the conditions of use of the substance, 50 ml of final product containing 2% of Basic Orange 69 and considering 10% as amount of zinc chloride in hair dye formulation, the exposure to zinc chloride was estimated by the authors to be at most 17 µg/kg bw (50ml x 2% x 10% x 10% (partition coefficient) x 10% (retention coefficient) / 60 kg). Considering 60 mg/kg bw as lethal dose by intravenous route in the rat (Ref. 1, but Study not submitted), it appeared to the authors that the presence of zinc chloride associated with Basic Orange 69 should not have any impact on the results of these toxicological studies.

Overview of the test substance used in the different studies:

Study	Test substance	
Acute oral toxicity, study 1	Basic Orange 69	
Acute oral toxicity, study 2		Zn Basic Orange 69
Acute oral toxicity, study 3	Basic Orange 69	
Acute dermal toxicity	Basic Orange 69	
Skin irritation		Zn Basic Orange 69
Mucous irritation		Zn Basic Orange 69
Skin sensitisation (Guinea Pig)		Zn Basic Orange 69
Skin sensitisation (HRIPT)	Basic Orange 69	
Dermal absorption		Zn Basic Orange 69
Repeated dose oral toxicity	No data submitted	
Sub-chronic oral toxicity	No data submitted	
Bacterial gene mutation assay, study 1		Zn Basic Orange 69
Bacterial gene mutation assay, study 2	Basic Orange 69	
Mammalian chromosome aberration test		Zn Basic Orange 69
Bone marrow micronucleus test		Zn Basic Orange 69
Teratogenicity	Basic Orange 69	
Phototoxicity, study 1	Basic Orange 69	
Phototoxicity, study 2	Basic Orange 69	

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Study 1

Guideline: OECD 423 (2001)
Species/strain: NMRI mice
Group size: 3/6 females
Test substance: Basic Orange 69
Batch: RD 110/009
Purity: /
Dose: 50, 300 mg/kg bw at 20 ml/kg bw by gavage
Observ. Period: 14 days
GLP: in compliance

NMRI mice (body weight 20.6 – 21.6 g) were administered 50 mg (3 females) or 300 mg (6 females) Basic Orange 29 per kg body weight dissolved in distilled water at a volume of 20 ml/kg bw as single dose by gavage. Animals were observed for mortality and toxic effects

during the 30 minutes following gavage, 1h, 2h, 3h and 4h after administration and then at least once a day for a total of 14 days. In the low dose group body weight was recorded on days 1, 4, 8, and 15. All mice were examined macroscopically and necropsied when they had died or at the end of the observation period.

Results
At 300 mg/kg bw piloerection, decrease in respiratory and decrease in motor activity was reported for all animals up to 4h after administration of the test substance. On day 2 all animals were found dead. At necropsy congestive liver, spleen and kidneys were observed in all animals. At 50 mg/kg bw slight piloerection was reported after treatment. No further clinical signs were observed at any observation time or at necropsy. The LD50 in the mouse was reported to be in the range of 50 – 300 mg/kg bw.

Ref.: 2

Study 2

Guideline:	OECD 420 (1992)
Species/strain:	Wistar rats
Group size:	5 females, 5males
Test substance:	Zn Basic Orange 69
Batch:	KLS 2/147/1
Purity:	/
Dose:	50 mg/kg bw at 10 ml/kg bw by gavage
Observ. Period:	14 days
GLP:	in compliance

Wistar rats (body weight 133 – 169 g) were administered 50 mg Zn Basic Orange 69 per kg body weight dissolved in sterile water at a volume of 10 ml/kg bw as single dose by gavage. Dose was selected on the base of a pilot study were one female and one male given 2000 mg/kg bw and one female given 500 mg/kg bw of the test substance were found dead after dosing, while one female given 50 mg/kg bw showed no signs of toxicity. Animals were observed for mortality and toxic effects 1h, 3h and 6h after administration and then once a day for a total of 14 days. Body weight was recorded on days 1, 8, and 15. All animals were subjected to gross necropsy examination at the end of the observation period.

Results
In the pilot study the female who received 2000 mg/kg bw showed no evidence of toxicity in the day of dosing (day 1), but was found dead on day 2. Post-mortem inspection revealed dark almost black urine. The test substance was still present in the ventricle. The male who received 2000 mg/kg bw and the female who received 500 mg/kg bw showed subdued behaviour 1 h after dosing. The female was found dead 2 h and the male was found dead 6 h after dosing. Post-mortem inspection revealed bleeding in the ventricle of the male, while no abnormalities were reported for the female. In the main study no deaths occurred and no signs of evident toxicity were reported. Post-mortem inspection revealed no abnormalities. It was concluded that the minimal lethal dose was in the range of 50 – 500 mg/kg bw.

Ref.: 3

Study 3

Guideline:	OECD 423 (2001)
Species/strain:	Sprague-Dawley albino rats
Group size:	3 females/group
Test substance:	Basic Orange 69
Batch:	RD 110/009

Purity: /
Dose: 5, 50, 300 mg/kg bw at 5 ml/kg bw by gavage
Observ. Period: 14 days
GLP: in compliance

Female Sprague-Dawley albino rats (body weight 206.5 – 238.1 g) were administered 5, 50 mg or 300 mg Basic Orange 69 per kg body weight dissolved in distilled water at a volume of 5 ml/kg bw as single dose by gavage. Regarding the results, exposure to 5 and 50 mg/kg bw was repeated under the same conditions according to OECD guideline. Animals were observed for mortality and toxic effects during the 30 minutes following gavage, 1h, 2h, 3h and 4h after administration and then at least once a day for a total of 14 days. All rats were examined macroscopically and necropsied when they had died or at the end of the observation period.

Results
The test substance induced death in all 3 animals at 300 mg/kg bw. Deaths occurred on day 2 after treatment. Significant toxic effects appeared 30 minutes after the treatment and included piloerection and decrease in motor activity, hollow flanks and abdominal spasms. Test substance administered at 50 mg/kg bw induced death in 2 animals in the repeated test only. Toxic effects like piloerection, decrease in motor activity were reported. The post-mortem examination did not reveal tissue or organ lesions but organs were stained orange at 300 and 50 mg/kg bw. In the 5 mg/kg bw group no deaths occurred and no toxic effects were observed, however, slight piloerection was observed in all animals during 4 hours following administration. It was concluded that the LD_{50} is in the range of 5 to 50 mg/kg bw in the rat.

Ref.: 4

3.3.1.2. Acute dermal toxicity

Guideline: OECD 402 (1987)
Species/strain: Sprague-Dawley albino rats
Group size: 5 females, 5 males
Test substance: Basic Orange 69, 2% solution
Batch: 07 08 58101
Purity: /
Dose: 2000 mg/kg bw
Observ. Period: 14 days
GLP: in compliance

Female (body weight 214.6 – 225.2 g) and male (body weight 224.4 – 245.6 g) Sprague-Dawley albino rats were dermally administered 2000 mg Basic Orange 69 per kg body weight. The test substance was applied uniformly to a healthy, intact and clipped skin surface over an area which was not less than 10% of the total body surface (about 45 cm^2). The experimental area was then covered with a porous gauze dressing held in place with a non-irritating tape for 24 ± 1 h. At the end of the exposure period test element was removed. Animals were observed for clinical signs during the 30 minutes following application, 1h, 2h, 3h, 4h, 5h and 6h after administration and then at least once a day for a total of 14 days. All animals were necropsied at the end of the observation period.

Results
No deaths occurred. Animals were slightly excited after treatment. Effects reported were porphyrine round muzzle and orange coloured skin in both sexes. In females losses of weight occurred in two cases and body weight gain seemed to be low compared to males. However, no data for control animals are submitted. It was concluded that the LD50 is > 2000 mg/kg bw.

Ref.: 17

3.3.1.3. Acute inhalation toxicity

No data submitted

3.3.2 Irritation and corrosivity

3.3.2.1. Skin irritation

Guideline: OECD 404, 1992
Species/strain: Mol:Russian albino rabbits
Group size: 3 females
Test substance: Zn Basic Orange 69
Batch: KLS 2/147/1
Purity: /
Dose: 0.5 g under occlusive conditions
GLP: in compliance

Three rabbits were exposed to Zn Basic Orange 69 at two clipped skin sites on the back. To each test field (2.5 x 2.5 cm) 0.5 g of the test substance moistened with 0.5 ml sterile water was applied. Gauze patches were placed on each test field. After 4 hours of exposure, the test substance was removed, the skin was washed with lukewarm water and soap and the skin was examined 1, 24, 48 and 72 hours after termination of exposure.

Results
In one animal the short hairs at both test sites were coloured by the test article. Therefore, it was impossible to determine if no erythema or very slight erythema was present from 1 to 72 hours after termination of exposure. No erythema and no oedema were observed in any of the other animals at any of the observations.

Conclusion
Zn Basic Orange 69 was not irritant under the experimental conditions described.

Ref.: 5

3.3.2.2. Mucous membrane irritation

Guideline: OECD 405, 1987
Species: Mol:Russian albino rabbit
Group size: 1 female
Test substance: Zn Basic Orange 69
Batch: KLS 2/147/1
Purity: /
Dose: 0.1 g (but only 1/3 of the test substance applied stayed in the eye)
GLP: in compliance

A volume of 0.1 g of the test substance was placed in the left eye of the rabbit by gently pulling the lower lid away from the eyeball to form a cup into which the article was placed. Only about 1/3 of the test substance applied stayed in the eye. The right eye remained untreated and served as control. The eyes were examined and the changes were graded according to a numerical scale 1, 24, 48 and 72 hours as well as 7 and 14 days after dosing.

Results
Marked signs of irritation of cornea, iris and conjunctiva (chemosis, redness and discharge) were observed from 1 hour to 7 days after termination of exposure. On day 15 no abnormalities were observed in the eye.

Conclusion
Zn Basic Orange 69 was considered to be an eye irritant.

Ref.: 6

3.3.3. Skin sensitisation

Guinea Pig Maximisation Test

Guideline:	OECD 406, 1992	
Species/strain:	Hartley Crl:(HA)BR guinea pigs,	
Group size:	5 females (control group) 10 females (treated group)	
Test substance:	Zn Basic Orange 69	
Batch:	9923500306	
Purity:	/	
Concentration:	Induction:	1% test substance (w/w) in 0.9% NaCl and 1% test substance (w/w) in FCA/0.9% NaCl 50/50 (v/v) (day 1, intradermal injections, treated groups) 50% test substance (w/w) in 0.9% NaCl (day 8, topical application, treated groups)
	Challenge: substance	50% test substance (w/w) (left flank) and 1% test (w/w) (right flank) in 0.9% NaCl (day 22, topical application for 24 hours, all groups)
GLP:	in compliance	

On day 1 animals of the treated group received three intradermal injections of 0.1 ml into each side of an abraded interscapular region: Freund's complete adjuvants in 0.9% NaCl, test substance at 1% (w/w) in 0.9% NaCl and test substance at 1 % (w/w) in a mixture of Freund's complete adjuvants and 0.9% NaCl 50/50 (v/v). Animals of the control group received the vehicle under the same experimental conditions. On day 7 animals were treated with 0.5 ml of sodium lauryl sulfate at a concentration of 10% (w/w) in Vaseline in order to induce local irritation. On day 8, a pad of filter paper (approximately 8 cm^2) was loaded with the test substance at a concentration of 50% (w/w) and was then applied to the back region of the animals of the treated group for 48 hours under occlusion. The controls received an application of the vehicle alone under the same experimental conditions. On day 22 all groups were challenged by a topical application of the test substance to the posterior left (50% test substance) and right (1% test substance) flank for 24 hours under occlusion. At the end of the study animals were killed without examination of internal organs. Histological examination was performed on all preserved skin samples.

Results
No clinical signs and no deaths were noted during the study. On the right flank, given the test substance at a concentration of 1%, an orange coloration of the skin which could have masked a discrete or moderate erythema was noted at the 24-hour reading in all animals. At the 48-hour reading no cutaneous reactions were noted, however, a slight orange coloration of the skin persisted in all animals. Dryness of the skin was observed in two treated animals and crusts in one treated animal. On the left flank, given test substance at a concentration of 50%, an orange coloration of the skin, which could have masked a discrete to severe erythema was noted in most animals at the 24- and 48-hour readings. Dryness of the skin was observed in 7/10 animals and crusts in several treated animal. Microscopic examination revealed signs of irritation by Zn Basic Orange 69. Additionally the skin of 3/10 females showed microscopic findings for which skin sensitization could not be excluded.

Conclusion

Under experimental conditions chosen, the study authors concluded that Zn Basic Orange 69 did not induce delayed contact hypersensitivity in guinea pigs at a concentration of 1% (w/w). At the concentration of 50% (w/w), sensitisation cannot be definitely excluded.

Ref.: 7

Comment
A conclusion concerning sensitisation cannot be drawn. Coloration of the skin and crust at challenge at 50% were more frequent in test than in control animals, indicating sensitisation. Concentrations between 1 and 50% should have been tested.

Human data

A HRIPT study with 50 female and 6 male volunteers was performed. Test material (Basic Orange 69 – Pj 070) was applied as 1 % aqueous solution under a semi-occlusive path to the upper back and was allowed to remain in direct skin contact for 48 hours. Exposure was repeated for a total of 9 applications during the induction period (on Monday, Wednesday and Friday, respectively). The test sites were graded for dermal irritation and sensitisation 24 to 48 hours after removal of the patches. Following a 2 week rest period challenge patches were applied to the previously treated test sites in the back and additionally to newly defined sites, previously unexposed. After 48 hours the patches were removed and test sites were evaluated for dermal reactions. The test sites were re-evaluated at 72 and 96 hours. Two volunteers discontinued study participation. For the other volunteers no scores were noted for induction neither for the challenge period. It was concluded that Basic Orange 69 did not demonstrate a potential for eliciting dermal irritation or sensitisation.

Ref.: 8

Comment
The SCCP considers HRIPT-studies as unethical.

3.3.4. Dermal / percutaneous absorption

Guideline:	/
Species:	human, 3 donors, 4 skin samples/donor
Tissue:	skin preparations comprising stratum corneum and epidermis
Groups:	4 replicates/donor
Test substance:	Zn Basic Orange 69
Batch:	WRJ 85/7
Purity:	/
Dose levels:	1% (w/w) in shampoo formulation (FMc2/103/2, batch 174A), 100 µl/cm² corresponding to approximately 100 mg/cm² or 993 µg Zn Basic Orange 69/cm²
Exposure time:	20 minutes
Receptor fluid:	phosphate buffered saline (pH 7.4)
Analytical method:	HPLC, detection limit: 100 ng/ml
GLP:	in compliance

Human female abdominal and breast skin from three donors, obtained following cosmetic surgery was used for investigation of percutaneous absorption of the test substance in a shampoo formulation. Formulation was applied to the surface of skin samples (0.99 to 1.33 cm^2) mounted in diffusion cells at a dose level of 100 µl/cm^2 corresponding to a dose of 993 µg/cm^2 Zn Basic Orange 69. The authors considered a mean application of 99.3 µg/cm² of Zn Basic Orange 69. However, based on the quantity of formulation applied (100 µl/cm²) and considering that the formulation had a concentration of 1% Zn Basic Orange 69, the amount of substance applied was in reality 993 µg/cm² (d = 0.993). 20 minutes after application the donor chambers were rinsed with distilled water. 200 µl samples were taken from the receptor media at 2, 8, 24 and 48 hours. The receptor chambers were refilled with

receptor medium subsequently. After 48 hours diffusion cells were dismantled. Entire skin samples were used for extraction of test substance by 95/5 methanol/water containing 0.1% (v/v) phosphoric acid. Zn Basic Orange 69 from receptor fluid and skin extraction was measured by HPLC.

Results
Test substance was found to be below the detection limit of the analytical method (100 ng/ml corresponding to 0.2 µg/cm²) in the receptor fluid of 11 skin samples. In one sample Zn Basic Orange 69 was detected in the receptor fluid at 8, 24 and 48 hours (0.33 – 0.35 µg/cm²). It was concluded that these results were due to compromised skin membrane. Zn Basic Orange 69 in the skin samples was in the range of 1.3 ± 0.23 µg/cm^2.

Ref.: 9

Comment
A maximum skin absorption rate of approximately 1.5 µg/cm² could be derived from this study. However, the test was not performed under use conditions. In oxidative hair dyes, Basic Orange 69 is used as a 1% formulation applied for up to 45 minutes (corresponding to 200 µg dye/cm²); in semi-permanent hair dye formulation up to 2% of the substance is applied for 30 minutes (corresponding to 400 µg dye/cm²). The amount of formulation applied was excessive at 100 mg/cm². According to the SCCP Notes of guidance 20 mg formulation per cm² should have been tested. No mass balance was given. Too few chambers were used. No data were provided on the dermal absorption of Basic Orange 69 under oxidative conditions.
The study is considered inadequate for calculation of the Margin of Safety.

3.3.5. Repeated dose toxicity

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

No data submitted

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

No data submitted

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 gene mutation assay

Study 1

Guidelines: OECD 471 (1997)
Species/Strain: *Salmonella typhimurium* TA 98, TA 100, TA 102, TA 1535, TA 1537
Replicates: Triplicates, two independent tests
Test substance: Zn Basic Orange 69
Solvent: Distilled water
Batch: 9923500306
Purity: /

Concentrations:	50, 160, 500, 1600, 5000 µg/plate, with and without metabolic activation
Treatment:	Plate incorporation method and pre-incubation test with 48 to 72 h incubation time
GLP:	in compliance
Date:	June 2000

The Ames-test was performed with the bacterial tester strains *Salmonella typhimurium* TA 98, TA 100, TA 102, TA 1535 and TA 1537 with and without S9-mix. Liver S9 fraction from Aroclor-induced rats was used as exogenous metabolic activation system. The test substance was tested at five concentrations in the range of 50 to 5000 µg/plate. Tests were performed in triplicates in two independent tests. Sodium azide, 2-nitrofluorene and cumene hydroperoxide (for tests without S9-mix) as well as 2-aminoanthracene (for tests with S9-mix) served as positive controls, solvent as negative control. Toxicity was tested as change in the ratio: numbers of revertants after treatment with the test substance of strain TA 100 / number of revertants of vehicle controls.

Results
No evidence of toxicity was observed. Significant increases in the number of mutant colonies were observed at doses of 160 – 5000 µg/ml in the strain TA 98 with metabolic activation. These increases were reproducible between replicate plates and were observed in both tests. Weak mutagenic effects were evident in TA 98 in the absence of S9-mix and in TA 1537 both, in the presence and in the absence of S9-mix.

Conclusion
Under the experimental conditions used Zn Basic Orange 69 was mutagenic in the gene mutation tests in bacteria both in the absence and the presence of metabolic activation.

Ref.: 10

Study 2

Guidelines:	OECD 471 (1997)
Species/Strain:	*Salmonella typhimurium* TA 98, TA 100, TA 1535, TA 1537 *E. coli* WP2, *E. coli* WP2 uvrA
Replicates:	Triplicates, two independent tests
Test substance:	Basic Orange 69
Solvent:	/
Batch:	LOT/RD 110/009/1-2
Purity:	
Concentrations:	50, 150, 500, 1500, 5000 µg/plate, with and without metabolic activation
Treatment:	plate incorporation and preincubation method, 48 h incubation time
GLP:	in compliance
Date:	June – July 2003

The Ames-test was performed with the bacterial tester strains *Salmonella typhimurium* TA 98, TA 100, TA 1535, TA 1537 and *E. coli* WP2, *E. coli* WP2 uvrA with and without S9-mix. Liver S9 fraction from Aroclor-induced rats was used as exogenous metabolic activation system. The test substance was tested at five concentrations in the range of 50 to 5000 µg/plate. Tests were performed in triplicates in two independent tests. Sodium azide, 2-nitrofluorene and cumene hydroperoxide (for tests without S9-mix) as well as 2-aminoanthracene (for tests with S9-mix)) served as positive controls, solvent as negative control. Toxicity was tested as change in the ratio: numbers of revertants after treatment with the test substance of strain TA 100 / number of revertants of vehicle controls.

Results

The number of revertants decreased in the highest dose groups in several strains tested. Significant increases in the number of mutant colonies were observed at doses of 150 – 5000 µg/ml in the strains TA 98 with metabolic activation. These increases were reproducible between replicate plates and were observed in both tests performed. In the tester strain TA 100 the test substance was positive when tested with S9-mix in the pre-incubation assay. Weak mutagenic effects were also evident in TA 1537 in the presence of S9-mix.

Conclusion
The test substance induced mutations in *Salmonella typhimurium* strains TA 98 and TA 100 with metabolic activation.

Ref.: 11

In vitro Mammalian chromosome aberration test

Guideline:	OECD 473 (1997)	
Species/strain:	human lymphocytes	
Replicates:	Duplicates	
Test substance:	Zn Basic Orange 69	
Solvent:	culture medium	
Batch:	9923500306	
Purity:	/	
Concentrations:	Experiment 1:	25, 50, 100 µg/ml without S9-mix
		100, 200, 400 µg/ml with S9-mix
	Experiment 2:	12.5, 25, 50 µg/ml without S9-mix
		50, 100, 200 µg/ml with S9-mix
Treatment:	Experiment 1:	3 hours
	Experiment 2:	3 hours (with S9-mix)
		20 hours (without S9-mix)
GLP:	in compliance	
Date:	Before October 1999	

The test was performed with primary human lymphocytes obtained from two healthy male volunteers. Lymphocytes were cultured at 37 °C for 48 hours before treatment. Cell cycles times were determined to be 15.7 and 13.9 hours for the two donors, respectively. For metabolic activation S9-mix from Aroclor 1254 induced rat liver was used. A preliminary toxicity test and two main tests were performed. In the preliminary toxicity test and in experiment 1 all cultures were treated for 3 hours and harvested 20 hours after the start of treatment. In experiment 2 cultures were treated for 20 hours in the absence and for 3 hours in the presence of S9-mix. All cultures were harvested 20 hours after the start of treatment. Demecolchicin (0.1 µg/ml) was added to each culture for the last 2 hours before harvest. Cells were fixed onto slides and air dried. For scoring chromosomal aberrations slides were stained in 3% Giemsa. Where possible, 100 metaphases were scored for each dose. Selection of doses for metaphase analysis was based on mean mitotic indices which were in the range of 33 – 54 % for the highest concentrations. Danunomycin (0.015 µg/ml) in the absence and cyclophosphamide (6 µg/ml) in the presence of S9-mix served as positive controls.

Results
The test substance caused dose-related reductions in mitotic index both in the absence and presence of S9-mix. Treatment with Zn Basic Orange 69 did not affect the frequency of polyploid or endoreduplicated metaphases in either main test. No biologically or statistically significant increases in the frequency of metaphases with aberrant chromosomes were observed in cultures treated with Zn Basic Orange 69 in either main test, either in the absence or presence of S9-mix. The frequency of metaphases with chromosome aberrations

was within the normal range in negative control cultures. The positive control treatments produced statistically significant increases of aberrant metaphases.

Conclusion
Under the experimental conditions used Zn Basic Orange 69 was not clastogenic in this in vitro mammalian cytogenetic test.

Ref: 12

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

Bone marrow micronucleus test in mice

Guideline:	OECD 474, 1997
Species:	NMRI mice
Group sizes:	5 males/group, pretest: 2 females and 2 males/group
Test substance:	Zn Basic Orange 69
Batch:	9923500306
Purity:	/
Dose levels:	pretest 50, 100, 200 mg/kg bw 12.5, 25, 50 mg/kg bw
Route:	oral gavage, 10 ml/kg bw
Vehicle:	0.9% NaCl
Sacrifice times:	24 and 48 h (highest dose only) after treatment
GLP:	in compliance
Date:	Before May 2000

Pre-experiment
Two animals per sex were treated by oral gavage with solutions of Zn Basic Orange 69 in saline solution (0.9 % NaCl) at dose levels of 50, 100, and 200 mg/kg body weight. Both males, one female of the 100 mg/kg bw group and one male and one female of the 200 mg/kg bw group were found dead 6 to 8 hours after application of the test substance. The surviving animals were underactive with low laboured breathing and prone or hunched posture or shaking with fast respiration. They were killed for humane reasons. The maximum tolerated dose was found to be 50 mg/kg bw and this dose was selected as the highest dose level for the main test. As there was no evidence of a difference in toxicity between the sexes, main test was performed with males only.

Main experiment
In the main test male mice were treated with solutions of the test substance in saline solution at dose levels of 12.5, 25, and 50 mg/kg bw by single oral gavage at a dose volume of 10 ml/kg bw. A negative control group received the vehicle alone and the positive control group was dosed with cyclophosphamide at 20 mg/kg bw. Twenty-four and 48 hours (for the highest dose group and the controls only) after administration bone marrow cells of five male mice were collected for micronuclei analysis. At least 2000 polychromatic erythrocytes (PCEs) were scored per animal.

Results
No signs of toxicity were observed in mice. The urine of treated animals was red-coloured from two hours after dosing, demonstrating systemic distribution of the test substance. No reduction in the frequency of polychromatic erythrocytes among total erythrocytes was observed after treatment with Zn Basic Orange 69, indicating that there was no toxic effect on the bone marrow. No biologically or statistically significant increases in the frequency of micronucleated polychromatic erythrocytes were seen in mice treated with the test substance, while statistically significant increases were recorded for the positive control.

Conclusion

It was concluded that Zn Basic Orange 69 showed no evidence of a clastogenic or aneugenic activity in the mouse micronucleus test after administration by oral gavage.

Ref.:13

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

Guideline:	OECD 414, 2001
Species/strain:	Sprague-Dawley rats
Group size:	25 females/group
Test substance:	Basic Orange 69 in distilled water
Batch number:	RD 110/009
Purity:	/
Dose levels:	0, 3.5, 10, 25 mg/kg bw/day by gavage
Treatment period:	days 5 – 19 of gestation
GLP:	in compliance

Three groups of mated female Sprague-Dawley rats received the test substance by oral gavage at 3.5, 10, 25 mg/kg bw daily from day 5 to 19 of gestation at a volume of 5 ml/kg bw. Dose levels were chosen according to the results of a range finding test. In addition one group was given the vehicle alone and acted as control group. Clinical signs and mortality were checked daily. Food consumption and body weight were recorded at designated intervals during pregnancy. On day 20 of pregnancy, females were sacrificed. Foetuses were removed and females were examined macroscopically. Litter parameters as number of corpora lutea, implantation sites, early and late resorptions, or dead and live foetuses were recorded. Foetuses were weighed, sexed and submitted to an external examination as well as to examination of visceral and skeletal changes.

Results
Mortality was observed in the 25 mg/kg bw/day group only. Two females were found dead after 5 and 10 days of treatment respectively. Clinical signs reported from day 7 of treatment on include piloerection, thin body and chromodacryorrhea in the highest dose group. Administration of Basic Orange 69 affected the mean body weights significantly and dose-dependently in groups receiving 10 or 25 mg/kg bw/day from day 11 or day 8 on. Food consumption was also reduced in these treatment groups. The mean uterus weights were reduced in all treated groups compared to controls and changes were reported to be significant in the highest dose group. There were no statistically significant differences in the number of corpora lutea, implantation sites and live foetuses among the treated and control groups. The 25 mg/kg bw/day dosage was associated with early (3 dams) or late (1 dam) total resorptions and significant reduced foetal body weight per litter. No anomalies were observed at foetal examination. Incomplete ossification was observed in the highest dose group and was related to the lower mean foetal weight. A NOAEL for maternal toxicity and embryotoxicity of 10 mg/kg bw/day was derived.

Ref.: 14

Comment

The authors considered the increase in maternal body weight in the 10 mg/kg bw/day group not to be related to the test substance as mean body weight at the beginning of treatment in this group was lower than in untreated controls. The body weight gain in this treatment group, however, was also lower than in the controls. The SCCP therefore sets the NOAEL for maternal toxicity at 3.5 mg/kg bw.

3.3.9. Toxicokinetics

No data submitted

3.3.10. Photo-induced toxicity

3.3.10.1. Phototoxicity / photoirritation and photosensitisation

Study 1

Guideline:	OECD 432 (2003)
Species/strain:	Balb 3T3 mouse fibroblasts, clone A31
Test substance:	Basic Orange 69
Batch:	RD 110/009
Purity:	/
Controls:	solvent, chlorpromazine
Concentration:	0.001 %, 0.0025 %, 0.005 %, 0.01 %, 0.025 %, 0.05 %, 0.1 % in PBS (v/v)
GLP:	in compliance

The phototoxicity and cytotoxicity potential of Basic Orange 69 was evaluated in a 3T3 Neutral Red Uptake (NRU) Phototoxicity Assay with normal Balb/c 3T3 mouse fibroblasts. Duplicate 96 well mono-layers of 3T3 fibroblast were exposed to dilutions (8 wells per concentration) of the test substance for one hour. One plate was exposed to 5 J/cm^2 UVA irradiation for 50 minutes (phototoxicity), the other plate was kept in the dark (cytotoxicity). The treatment medium was then replaced by culture medium and cells were incubated for 18-22 hours. The number of viable cells was determined by Neutral Red Uptake. The IC_{50} concentration was determined for both the phototoxicity and cytotoxicity plates. The Photo-Irritancy Factor (PIF) was calculated as IC_{50} (-UV) / IC_{50} (+UV).

Results
The IC 50 was determined at 0.0304 % without and at 0.023 with UV radiation. The PIF was 1.3, indicating, that Basic Orange 69 has no phototoxic properties. The PIF for the positive control was 25.

Ref.: 15

Study 2

Guideline:	/
Species/strain:	Hartley albino guinea-pigs
Group size:	2 females for pretest 12 females for treatment group 10 females for positive control (10 % Bithionol in acetone/olive oil) 5 females for negative control
Test substance:	Basic Orange 69
Batch:	RD 110/009
Purity:	/
Concentration:	5% (w/v) in distilled water
GLP:	in compliance

Phototoxicity and photoallergic potential was investigated in a combined test with guinea pigs. Animals were anaesthetised before each treatment. In a pretest the maximal concentration inducing no cutaneous reaction by topical application was determined. Two animals received single topical applications of 0.5 ml test substance diluted at 5 % and 2.5 % with distilled water. Each application was performed to a skin surface at the posterior lumbar level of approximately 15 cm^2. For phototoxicity testing ten animals received a single cutaneous application of Basic Orange 69 diluted at 5 % with distilled water to the right abraded skin surface area of approximately 15 cm^2 while the left skin surface was not treated. 30 minutes after application of the test substance animals were exposed to UV radiation (14 J/cm^2 of UVA and 0.2 J/cm^2 of UVB). Phototoxic potential was assessed 24 and 48 hours after application. After a rest period of 10 days animals received 4 intradermal injections of 0.1 ml of a 1:1 mixture of Freund's Complete Adjuvant and distilled water. On days 2, 4, 9 and 11 right areas were treated with 0.5 ml of the test substance for induction and exposed to UV radiation (14 J/cm^2 of UVA and 0.2 J/cm^2 of UVB). Animals of the negative control group were not exposed to UV radiation. For challenge on day 23 0.5 ml of the test substance was applied to the right area while the left flank was not treated. UV radiation was performed after 30 minutes (7.2 J/cm^2 of UVA and 0.2 J/cm^2 of UVB). Negative control animals were not exposed to UV radiation. On days 3, 5, 10, and 12 during the induction phase and 24, 48 and 72 hours after the end of UV exposure during the challenge test local reactions were scored.

Results
One animal from the treatment group was found dead during the test on photoallergy. The post-mortem examination revealed no tissue or organic lesions attributable to the treatment. No phototoxic or photoallergic effects were observed after treatment with Basic Orange 69 while slight or well defined oedema were reported for 10 % of positive control animals.

Ref. 16

3.3.10.2. Phototoxicity / photomutagenicity / photoclastogenicity

No data submitted

3.3.11. Human data

See 3.3.3

3.3.12. Special investigations

No data submitted

3.3.13. Safety evaluation (including calculation of the MoS)

Not applicable

3.3.14. Discussion

Physico-chemical properties
No analytical file was submitted. No data on homogeneity and stability of the test substance were provided. Purity for the batches investigated in the different toxicity test is mostly not given. It is not clear if physicochemical properties and impurities differ for the pure substance and the zinc chloride salt.

General toxicity

Acute toxicity was investigated in rats and mice. LD_{50} for rats is in the range of 50 to 500 mg/kg bw Zn Basic Orange 69 or 5 to 50 mg/kg bw Basic Orange 69 after oral administration and >2000 mg/kg bw Basic Orange 69 after dermal application. In mice LD_{50} after oral administration was reported to be in the range of 50 to 300 mg/kg bw Basic Orange 69. No data on repeated dose toxicity or on chronic toxicity was submitted. From a study on teratogenicity with Basic Orange 69 a NOAEL of 3.5 mg/kg bw could be derived for maternal toxicity and a NOAEL of 10 mg/kg bw for embryotoxicity.

Irritation / Sensitisation
Zn Basic Orange 69 was not irritant in the rabbit. However, the substance was irritant to the rabbit eye. Zn Basic Orange 69 did not induce delayed contact hypersensitivity in guinea pigs at a concentration of 1 %. At the challenge concentration of 50 %, sensitisation could not be excluded. In the opinion of the SCCP, a conclusion concerning sensitisation cannot be drawn. Coloration of the skin and crust at challenge at 50% were more frequent in test than in control animals, indicating sensitisation. Concentrations between 1 and 50% should have been tested.
Tests on photoirritation and photosensitization were negative for Basic Orange 69.

Dermal absorption
A maximum skin absorption rate of approximately 1.5 µg/cm^2 Zn Basic Orange 69 (<0.2 µg/cm^2 in the receptor fluid and 1.3 µg/cm^2 in the skin) could be derived from a study with human skin samples. The amount of formulation applied was excessive at 100mg/cm^2. According to the SCCP Notes of guidance 20 mg formulation per cm^2 should have been tested. No mass balance was given. Too few chambers were used. No data were provided on the dermal absorption of Basic Orange 69 under oxidative conditions. The study is considered inadequate for calculation of the Margin of Safety.

Mutagenicity / Genotoxicity
Zn Basic Orange 69 and Basic Orange 69 were mutagenic in the gene mutation tests in bacteria both in the absence and the presence of metabolic activation. Zn Basic Orange 69 was not clastogenic in the *in vitro* mammalian chromosome aberration test. When investigated *in vivo* in the bone marrow micronucleus test in mice Zn Basic Orange showed no evidence of clastogenic or aneugenic activity. No *in vitro* mammalian cell gene mutation assay was performed to exclude the potential to induce gene mutations.

Carcinogenicity
No data submitted

4. CONCLUSION

The SCCP is of the opinion that the information submitted is insufficient to allow a final risk assessment to be carried out.

Before any further consideration, the following information must be submitted:

- complete physico-chemical characterisation of Basic Orange 69 and its Zinc-Chloride salt;
- a repeated dose oral toxicity study;
- an *in vitro* dermal absorption study in accordance with the SCCP Notes of Guidance;
- an *in vitro* mammalian cell gene mutation assay to exclude the potential to induce gene mutations.

The sensitising potential of Basic Orange 69 could not be excluded.

5. MINORITY OPINION

Not applicable

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