EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate C - Public Health and Risk Assessment
C7 - Risk assessment

SCIENTIFIC COMMITTEE ON CONSUMER PRODUCTS

SCCP

Opinion on

2-Amino-6-chloro-4-nitrophenol

COLIPA N° B99

Adopted by the SCCP
during the 7th plenary meeting of 28 March 2006

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1. BACKGROUND

Submission I of Chlororange, 2-Amino-6-chloro-4-nitrophenol (B 99) was submitted by COLIPA (European Cosmetics Toiletry and Perfumery Association) in March 1992. On 25 June 1993 the Scientific Committee on Cosmetics (SCC) classified the substance as a substance A (SPC/1080/93 rev. 06/93).

2-Amino-6-chloro-4-nitrophenol is listed in Annex III, part 2, no 56 of the Cosmetics Directive (76/768/EEC).

Submission II presents updated scientific data on the above mentioned substance in line with the second step of the strategy for the evaluation of hair dyes (http://europa.eu.int/comm/enterprise/cosmetics/doc/hairdyestrategyinternet.pdf) within the framework of the Cosmetics Directive 76/768/EEC.

2. TERMS OF REFERENCE

1. Is 2-Amino-6-chloro-4-nitrophenol safe for use in hair dye formulations taken into account the data provided?

2. Does the SCCP recommend any further restrictions with regard to the use 2-Amino-6-chloro-4-nitrophenol in hair dye formulations?

3. OPINION

3.1. Chemical and Physical Specifications

3.1.1.	Chemical identity

3.1.1.1.	Primary name and/or INCI name

2-amino-6-chloro-4-nitrophenol (INCI)

3.1.1.2.	Chemical names

Phenol, 2-amino-6-chloro-4-nitro- (CA Index name, 9CI)

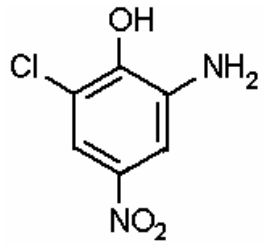
3.1.1.3.	Trade names and abbreviations

COLIPA: B99
Trade names: RODOL 9R (Lowenstein), Chlororange Base (Wella name)
Colour Index: /

3.1.1.4.	CAS / EINECS number

CAS: 6358-09-4
EINECS: 228-762-1

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula: $C_6H_5ClN_2O_3$

3.1.2. Physical form

Yellow-green moist powder

3.1.3. Molecular weight

Molecular weight: 188.57

3.1.4. Purity, composition and substance codes

Substance Code: A000119
Batches used: GST 009-01/30-07, Cos 198 (C8 15), AR 947, L-19503

Purity: 80.5-99.4% (w/w by NMR on a wet weight basis)
Purity: > 98.7% (w/w by NMR on a dry weight basis)
HPLC purity: > 99.8% (area% at 210 nm, 254 nm and 378 nm)
Water content: 0.7-18.2% (w/w)
Ash: < 1%

Description of sample	**GST 009-01/30-07**	**Cos 198 (CS 15)*****	**AR 947**	**R00057594**
References of Analyses	**A 2003/374-001**	**A 2003/374-002**	**A 2003/374-003**	**G 2002/001 A2003/374-004**
NMR content / weight %	99.4	94.9	94.8	80.5
HPLC purity / area %**				
210 nm	99.98	100	100	100
254 nm	99.97	100	100	100
378 nm	99.96	100	100	100
HPLC content weight %	97.2	92.0	92.0	81.4
Content of 2-Chlorophenol / weight %	< 25 ppm*	< 25 ppm*	< 25 ppm*	< 25 ppm*

Description of sample	**GST 009-01/30-07**	**Cos 198 (CS 15)*** **	**AR 947**	**R00057594**
References of Analyses	**A 2003/374-001**	**A 2003/374-002**	**A 2003/374-003**	**G 2002/001 A2003/374-004**
Content of 6-Chloro-2,4-dinitrophenol / weight %	90 ppm	< 25 ppm*	< 25 ppm*	< 25 ppm*
Water content / weight %	0.7	4..8	4.0	18.2

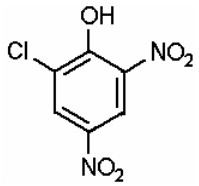
* Below detection limit. The indicated values show detection limits.

** HPLC conditions: Purospher RP18e 5µm 250 x 4 mm with precolumn; Eluent: 30% acetonitrile /70% 0.005M KH_2PO_4 buffer at pH 3.5; Flow: 1 ml/min

*** Batch Cos 198 is the test item code that was used for several studies. All samples were sent out in the same time period and were most likely part of the same batch. From one of these studies, a sample was retained with the name Cos 198 (CS 15), which most likely represents the quality used in all studies that have been performed with the substance code Cos 198.

3.1.5. Impurities / accompanying contaminants

Possible intermediates of synthetic route



6-Chloro-2,4-dinitrophenol: < 0.03% (<300ppm)

Residual Solvents (i.e. methanol, ethanol, isopropanol, n-propanol, acetone, ethyl acetate, cyclohexane, methyl ethyl ketone and monochlorobenzene) were not detected (<100ppm).

3.1.6. Solubility

Water solubility: 0.045% (450 mg/ml) at 25 °C (EU - A.5)

Ref. 8

Soluble in

Acetone/water 1:1:	8.7% (w/w)
DMSO:	> 10% (w/w)
Ethanol:	> 10% (w/w)

Ref. 1

3.1.7. Partition coefficient (Log P_{ow})

Log P_{ow}: 1.80 (P_{ow} = 63.10)

Ref. 2

3.1.8.	Additional physical and chemical specifications

Organoleptic properties

Melting point:	159-163 °C	(EU A.1)	Ref. 4
Boiling point:	Not detectable, decomposition at 170 °C		Ref. 4
Flash point:	/		
Relative self-ignition temp:	>380 °C	(EU A.16)	Ref. 11
Vapour pressure:	< 1.44 mPa (1 x 10^{-5} mmHg) at 20 °C	(EU A.4)	Ref. 6
Density:	1.488 g/ml at 20 °C	(EU A.3)	Ref. 5
Surface tension in water:	48.5 mN/m² at 20 °C	(EU A.5)	Ref. 7
Viscosity:	/		
pKa:	/		
Refractive index:	/		

3.1.9.	Stability

The substance on storage in dryness and darkness is considered (no data) to be stable for more than 5 years.
Stability in solution over a period of 7 days was:

Solution 0.05% (w/v) in water:	100.0-103.2%
Solution 10% (w/v) in DMSO:	99.1-100.0%
Solution 7% (w/v) in acetone / water, 1:1:	100.0-101.5%

General comment on analytical and physico-chemical characterisation

***** Data on characterization of the substance (eg IR, NMR, MS) were not provided.
* Stability in the marketed products was not reported.

3.2. Function and uses

The 2-amino-6-chloro-4-nitrophenol is intended for use at a maximum on-head concentration of 2% in semi-permanent hair dyes formulations as a direct dye, or as a non-reactive colorant in oxidative hair-dyes formulations. In the latter case, it has been found to be stable under the conditions during oxidative hair-dyeing, with the dye and developer (hydrogen peroxide) components mixed at a ratio between 1:1 and 1:3.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Guideline:	OECD 401 (limit test)
Species/strain:	Wistar rats, strain Crl: Wi/br (SPF)
Group size:	5 per sex
Test substance:	2-amino-6-chloro-4-nitrophenol
Batch:	COS 198
Purity:	100 area% (HPLC at 254 nm)
Dose:	2000 mg/kg bw in deionised water
GLP:	in compliance

The test compound was given once by stomach tube to Wistar rats (5 per sex). The animals were observed for 14 days for clinical signs and mortality.

Results
Red-orange-stained urine was observed in all animals up to 5 days. No clinical signs of toxicity and no mortalities were observed. No abnormalities were found during necropsy.

Ref.: 15

3.3.1.2. Acute dermal toxicity

No data submitted.

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
Species/strain:	white New Zealand rabbits
Group size:	6 animals (sex not specified)
Test substance:	Chlororange
Batch:	Cos 198
Purity:	not stated in report
Dose:	0.5 ml of a 0.5% dilution in propylene glycol (pH 6.0)
GLP:	in compliance

The aim of the study was to evaluate the irritating effect of chlororange to the shorn intact and scarified skin of WNZ rabbits. The exposure time of the test sample on the shorn skin (scarified and intact) was 4 hours. Reactions e.g. erythema and oedema were noticed during 72 h p.a. (Draize scheme).

Results
According to the EEC- guidelines 83/467/EEC, the test substance is considered as not irritant to the skin.

Ref.: 16

3.3.2.2. Mucous membrane irritation

Guideline:	OECD 405
Species/strain:	white New Zealand (WNZ) rabbits
Group size:	6 animals (sex not specified)
Test substance:	Chlororange
Batch:	Cos 198
Purity:	not stated in report
Dose:	0.1 ml of a 2 % dilution in propylene glycol
GLP:	in compliance

To assess the above-mentioned substance in mucous membrane irritation 6 WNZ rabbits were used. Every eye reactions were observed 1, 24, 48 and 72 h p.a. by Draize scheme. Prior to the start of the study and both, 24 h and 72 h p.a. the eyes were examined for ocular lesion by means of fluorescein and UV-light. All other observations were performed without fluorescein.

Results
The test sample chlororange was evaluated as not irritating.

Ref.: 17

3.3.3. Skin sensitisation

Local Lymph Node Assay (LLNA)

Guideline:	OECD 429 (2002)
Species/strain:	Mice CBA/J
Group size:	5 females per group
Test substance:	2-amino-6-chloro-4-nitrophenol
Batch:	L-19503
Purity:	99.9 % (HPLC at 254 nm)
Concentrations:	0.5, 1.5, 5.0 and 10.0 % (w/v) in DMSO and in aqua/acetone (1:1) mixed with olive oil (4:1)
GLP:	In compliance

The skin sensitising potential of 2-amino-6-chloro-4-nitrophenol was investigated in CBA/J mice by measuring the cell proliferation in the draining lymph nodes after topical application on the ear. Twenty five µl of 0 (vehicles only), 0.5, 1.5, 5 and 10 % of 2-amino-6-chloro-4-nitrophenol in DMSO or in a mixture of aqua/acetone (1:1) with olive oil (4:1) (equal to the maximum solubility) were applied to the surface of the ear of five female CBA/J mice per group for three consecutive days. After application, the ears were dried by means of a hair dryer for about 5 minutes. p-Phenylenediamine (PPD) at 1 % in DMSO was used as the positive control in parallel under identical test conditions.

At day 5, the mice received an intravenous injection of 250 µl phosphate buffered saline containing 23.3 µCi of [H^3] methyl thymidine. Approximately five hours later, the mice were killed by CO_2-inhalation and the draining auricular lymph nodes were removed and weighed. After preparing a single cell suspension for each mouse, cells were precipitated by TCA and the radioactivity was determined (incorporation of [H^3] methyl thymidine in the pellets) by means of liquid scintillation counting as disintegration per minute (dpm).

The mean dpm per treated group was determined and the stimulation index (test item compared to the concurrent vehicle control) was calculated.

Results
The mean stimulation indices were affected in a dose-dependent manner by the treatment with 2-amino-6-chloro-4-nitrophenol. With the test item dissolved in DMSO, mean stimulation indices of 1.2, 1.2, 2.0 and 4.7 were obtained for the 4 test concentrations of 0.5, 1.5, 5 and 10 %, respectively. An EC3 value (equal to the concentration inducing a stimulation index of 3) of 6.85 % was calculated.

In the second vehicle (aqua/acetone/olive oil), the indices were 2.8, 3.9, 3.7 and 5.2 for the 4 test concentrations of 0.5, 1.5, 5 and 10 %, respectively. An EC3 value of 0.68 % was calculated from those findings. However, the concurrent vehicle control showed an unusually low value possibly indicating that the value of 0.68 may overestimate the sensitising potency of 2-amino-6-chloro-4-nitrophenol*;* there was also a lack of a clear dose-response for the concentrations below 10%.

The responses noted in both groups are considered positive and indicate a skin sensitising potential of 2-amino-6-chloro-4-nitrophenol.

The positive control (PPD, 1 % in DMSO) revealed a stimulation index of 7.0, demonstrating the sensitivity and validity of the test system used.

Conclusion
2-amino-6-chloro-4-nitrophenol induced a biological relevant immune response in local lymph nodes after dermal application to the mouse ear with both vehicles tested. EC3 values of 6.85 % (DMSO) and 0.68 % (acetone/water/olive oil) were calculated. The concurrent positive control demonstrated the validity and sensitivity of the assay.
Based on these findings 2-amino-6-chloro-4-nitrophenol is evaluated to be a skin sensitiser under the given test conditions.

Ref.: 18

3.3.4.	Dermal / percutaneous absorption

Guideline:	OECD 428
Species/strain:	pig
Group size:	5 skin samples, one donor
Test substance:	chlororange base (2-amino-6-chloro-4 nitrophenol)
Batch:	L-19503
Purity:	99.9 area% (HPLC)
Dose:	1 mg/cm²

The cutaneous absorption of 2-Amino-6-chloro-4-nitrophenol in a typical hair dye formulation (=test item) was investigated *in vitro*, using pig skin preparations (1 – negative control and 5 pig skin samples measuring 0.9 ± 0.1 mm in thickness, which were continuously rinsed from underneath with physiological fluid at the temperature 32 ± 2°C. After checking the skin integrity, an aliquot of the formulation was mixed with 1 volume of the 6% Welloxon (containing 3% hydrogen peroxide) and 400 mg of this final formulation (= 100mg/cm²) containing 1 mg (=1.0%) of WR 23214 was applied to the skin samples (= 1.0mg of WR23214/cm²) in the practice relevant manner for 60 minutes and subsequently washed off with water and shampoo. The determination of the amount of WR23214 in washings (= dose extractable from the skin surface) was performed by HPLC. At 16, 24, 40, 48, 64 and 72 hours, the content of WR23214 in the receptor fluid as well as after 72 hours the content extracted from the skin (epidermis and upper dermis separated) was determined in the same way.
The integrity of each skin preparation was demonstrated by examination of penetration characteristics with tritiated water resulting in 1.0 to 1.4% of the applied dose found in the receptor fluids, which was the limit of acceptance (≤ 2.0%) for all 6 skin samples.

Results of the cutaneous absorption experiment are presented in the table:

Amount of WR23214 in:	**Expressed as µg/cm² of skin surface mean ±S.D. (n=5)**	**Expressed as % of dose mean ±S.D. (n=5)**
Receptor fluid (72 h)	0.29 ± 0.11	0.03 ± 0.01
Upper dermis (72 h)	0.61 ± 0.68	0.06 ± 0.07
Epidermis (72h)	1.18 ± 0.29	0.12 ± 0.03
Rinsing solution (after 60 min.)	974.32 ± 35.00	97.43 ± 3.50
Total balance (recovery)	976.41 ± 34.46	97.64 ± 3.45

Conclusion
As the worst case assumption, considering the kinetics observed, a maximum amount of 2.08 ± 1.08 µg/cm² of Chlororange base is considered as biologically available (n = 5, one donor: receptor fluid + upper dermis + epidermis added).

Comment
According to the SCCNFP Notes of Guidance, skin samples of more than 1 donor should have been used. Therefore, the study cannot be used for a safety assessment.

Ref.: 20

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

Guideline:	OECD 408
Species/strain:	Wistar rats, strain Crl: Wi/br (SPF)
Group size:	15 animals per sex and dose, 10 per sex in a recovery group
Test Substance:	2-amino-6-chloro-4-nitrophenol
Batch:	COS 198
Purity:	100 area% (HPLC at 254 nm)
Dose:	0, 10, 30, 90 mg/kg bw/day (in 0.5% carboxymethylcellulose in water)
Route:	Oral, gavage
Exposure:	13 weeks
GLP:	in compliance

2-Amino-6-chloro-4-nitrophenol was administered, by gavage, once daily to 4 groups of SPF-Albino Wistar rats (15/sex) for 90 days. The control and high dose group included additionally 10 animals/sex as 4 weeks recovery group. The test substance was administered at dosage levels of 10, 30 or 90 mg/kg bw. The control group received the vehicle (0.5% carboxymethylcellulose in water) only. All animals were sacrificed at the end of the study.

All animals were observed daily for mortality, clinical signs and water consumption. Body weights and food consumption were recorded individually in weekly intervals. Ophthalmoscopic examination and a hearing test were performed. Blood samples were withdrawn from 10 males and 10 females of each test group for haematological and clinical chemistry investigations at week 6, 13 and 17, and urine samples were collected from 5 males and 5 females of each test group at these dates. Organ weights were measured and macroscopy and histopathology was performed on all animals.

Results
No animals died during the study. The urine of the animals of the 2 high dose groups was discoloured orange. A decrease in body weight gain was observed in the males of the high dose group whereas food consumption was not changed. Organ weight changes of the liver, lung and thymus of males and females in the high dose and recovery group were not consistent. But increases in kidney weight were found in males and females both of the high dose and recovery group.

The dose level without adverse effects was 30 mg/kg bw.

Ref.: 21

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

Guideline:	OECD 471 (1997)
Species/strain:	*Salmonella typhimurium*, TA98, TA100, TA102, TA1535, TA1537
Replicates:	Triplicates were investigated per test concentration
Assay conditions:	Plate incorporation and pre-incubation assay without and with S9 mix from rat livers (Phenobarbital/ß-Naphthoflavone induced). Three independent assays were performed
Test substance:	2-Amino-6-chloro-4-nitrophenol
Batch:	GST009-01/30-07
Purity:	99.9% (HPLC at 254 nm)
Concentrations:	3 - 5000 µg/plate, solved in DMSO, with and without metabolic activation
GLP:	in compliance

2-Amino-6-chloro-4-nitrophenol was tested for mutagenicity in the bacterial gene mutation assay (experiment 1: plate incorporation method, experiment 2 and 3: pre-incubation method) both with and without metabolic activation (S9 mix from the liver of phenobarbital/ß-naphthoflavone induced male Wistar Hanlbm rats). The *Salmonella typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537 were exposed to the test substance at concentrations ranging from 3 µg/plate to 5000 µg/plate with and without S9 mix. Test concentrations were selected based on the results obtained in a pre-experiment with all tester strains (experiment 1). Due to the observed toxicity in experiment 1, the highest test concentration in experiment 2 was 2500 µg/plate. In a confirmatory experiment a narrower concentration range (100 to 5000 µg/plate) was tested.

The following test concentrations were investigated:

Experiment 1: 10, 33, 100, 333, 1000, 2500 and 5000 µg/plate
Experiment 2: 3, 10, 33, 100, 1000 and 2500 µg/plate
Experiment 3 (TA98 only): 100, 333, 1000, 1754, 2500, 3754 and 5000 µg/plate

For control purposes, solvent (DMSO) as well as untreated negative controls and positive controls were evaluated in parallel. The following positive controls were used: Without S9 mix: 4-nitro-o-phenylenediamine for strain TA98 and TA1537; sodium azide for strain TA100 and TA1535; methyl methane sulfonate for strain TA102; with S9 mix: 2-aminoanthracene for all tester strains).

Results
Toxic effects, evident as a reduced number of revertants, were noted in all tester strains with and without S9 mix at concentrations ≥ 333 µg/plate (TA102), ≥ 2500 µg/plate (TA1535, TA1537), ≥ 3754 µg/plate (TA98) and at 5000 µg/plate (TA100). Normal background growth was noted in the experiments with the exception of experiment 2, in which a reduced background growth was noted for TA100 (2500 µg/plate with and without S9 mix) and TA102 (1000 and 2500 µg/plate with and without S9 mix).

No substantial increase in revertant colony numbers was noted in any of the five tester strains at any concentration either in the presence or in the absence of metabolic activation.

In experiment 2 a moderate but seemingly dose-related increase in revertant colonies was observed in TA98 in the presence and absence of S9 mix. At 1000 µg/plate, the number of revertants just reached the threshold of twice the solvent control. However, the increases seen were due to an unusually low solvent control and the values were in the range of the corresponding negative control and were therefore not considered relevant. A confirmatory experiment with TA98 in which neither a dose-related nor a relevant increase was noted corroborates this view. The variations noted in tester strain TA98 in experiment 2 were therefore considered irrelevant.

Reference mutagens revealed a distinct increase in revertant colonies and demonstrated the validity and sensitivity of the assay.

Conclusion
In conclusion, it can be stated that under the experimental conditions reported, 2-Amino-6-chloro-4-nitrophenol did not induce gene mutations in *Salmonella typhimurium* in any of the tester strains in the presence or absence of S9 mix.

Ref.: 22

In Vitro Mammalian Cell Gene Mutation Test

Guidelines:	OECD 476 (1997)
Species/strain:	mouse lymphoma cell line L5178Y / $TK^{+/-}$ locus
Replicates:	two independent experiments (second exp. without S9 only) two replicate cultures per concentration
Test Substance:	2-Amino-6-chloro-4-nitrophenol
Batch:	GST009-01/30-07
Purity:	99.9% (HPLC at 254 nm)
Concentrations:	30- 960 µg/ml without and 3.8 - 120 µg/ml with metabolic activation
GLP:	in compliance

2-Amino-6-chloro-4-nitrophenol was examined for its mutagenic activity in the L5178Y $TK^{+/-}$ mouse lymphoma assay in the absence and presence of metabolic activation. Mutagenicity is detected in this test system by examining the occurrence of trifluorothymidine (TFT) resistance caused by forward mutation at the thymidine kinase (TK) locus. The test was performed with and without metabolic activation (S9 mix from the liver of phenobarbital/ß-naphthoflavone induced male Wistar HanIbm rats). A range-finding test (pre-test on toxicity, measuring relative suspension growth) and two independent mutagenicity experiments were carried out. Concentrations of the test substance, which was dissolved in DMSO immediately prior to treatment, ranged from:

- 30 - 960 µg/ml without and 3.8 – 120 µg/ml with metabolic activation (4 h incubation; experiment 1), and
- 15 – 240 µg/ml without metabolic activation (24 h incubation; experiment 2).

Culture medium and DMSO were used as negative and solvent controls, while 3-Methylcholanthrene (3-MC; 3 µg/ml) and Methylmethanesulphonate (MMS; 13 µg/ml) were used as positive controls with and without metabolic activation system, respectively.

Besides the numbers of mutant colonies also the size/optical density of the colonies was determined and the ratio of small versus large colonies was calculated.

Results
In the initial range-finding study concentrations of 2-Amino-6-chloro-4-nitrophenol up to 10 mM (from 14.8 to 1900 µg/ml) were evaluated for toxicity with and without S9 mix. Toxicity was noted at 475µg/ml and above in the absence of S9 mix (relative cell growth of 37.2 % to 12.3 %) and at 59.4 µg/ml and above (relative cell growth 38.2 % to 1.4 %) in the presence of S9 mix (4 hours treatment). After 24 h of treatment in the absence of S9 mix, the cell growth was distinctly reduced (relative cell growth of 9.4 % to 4.2 %) at 237.5 µg/ml and above. Based on these findings, 6 concentrations covering a range of 30 to 960 µg/ml without and of 3.8 to 120 µg/ml with S9 mix were chosen for the main experiments, from which 4 concentrations each were evaluated.

In the first experiment, severe toxicity occurred at 960 µg/ml in both cultures in the absence of S9 mix (relative cloning efficiency 1: 12.8 to 15.3 %). In the second experiment, a steep toxicity gradient was noted with severe toxicity noted at 180 µg/ml and above for both parallel cultures (relative cloning efficiency 1: 1.5 and 1.9 %) and only a minor effect at 120 µg/ml. Figures obtained at 180 µg/ml and above were rejected as the acceptance criteria (cloning efficiency 1 ≥ 10 %) were not fulfilled at these severely toxic concentrations. In the presence of S9 mix, cloning efficiency was reduced to 22 % and 42 % of the concurrent solvent control at the highest analysable concentration of 60 µg/ml in the parallel cultures. Cultures with higher test concentrations were not continued due to severe toxicity.

In experiment 1, no relevant increase in the number of mutant colonies as compared to the concurrent controls was noted in the presence or absence of S9 mix for any test concentration investigated. In experiment 2, the mutation frequency at 120 µg/ml in the first culture just reached the threshold of twice of the concurrent solvent control value (63 per 10^6 cells). This single increase in one culture only was considered as a biological variation and not as an indication for a mutagenic effect, because this finding was not confirmed by the parallel culture (lack of reproducibility) and because the observed mutation frequency (128 per 10^6 cells) remained well within the historical range of negative and solvent controls (41-186 per 10^6 cells) of the performing laboratory.

The ratio of small to large colonies was not affected by the treatment with 2-Amino-6-chloro-4-nitrophenol.

The positive controls demonstrated that the system was able to detect known mutagens.

Conclusion
2-Amino-6-chloro-4-nitrophenol did not induce any biologically relevant increase in mutations at the mouse lymphoma thymidine kinase locus assay in L5178Y cells either in the absence or presence of metabolic activation under the described test conditions. Consequently, 2-Amino-6-chloro-4-nitrophenol is evaluated to be non-mutagenic in this *in vitro* mammalian cell mutation test.

Ref.: 23

Micronucleus assay *in vitro*

Guidelines:	OECD guideline 487 (draft)
Species/strain:	Human peripheral blood lymphocytes
Replicates:	Two cultures per concentration, 3 concentrations analysed.
Assay conditions:	Two independent experiments using pooled blood from two female donors in each trial
Test Substance:	2-Amino-6-chloro-4-nitrophenol
Batch:	GST009-01/30-07
Purity:	99.9% (HPLC at 254 nm)
Solvent:	DMSO
Concentrations:	Exp. I: with S9 mix: 1200, 1400, 1600 μg/ml; 3 h treatment 24 hours after mitogen stimulation without S9 mix: 175, 225 and 275 μg/ml, 20 h treatment 24 hours after mitogen stimulation Exp. II: with S9 mix: 1100, 1300 and 1600 μg/ml, 3 h treatment 48 hours after mitogen stimulation without S9 mix: 150, 275, 350 and 400 μg/ml, 20 h treatment 48 hours after mitogen stimulation Exp III: repeat of Exp II (without S9): 100, 150, 250, 300 μg/ml, 20 h treatment 48 hours after mitogen stimulation Exp IV: repeat of Exp II (with S9): 1200, 1300, 1400, 1500 μg/ml, 3 h treatment 48 hours after mitogen stimulation
GLP:	in compliance

2-Amino-6-chloro-4-nitrophenol was examined for its clastogenic and aneugenic potential by evaluating its ability to induce micronuclei in cultured human lymphocytes. Two independent experiments were performed with and without metabolic activation (S9 mix from the liver of Aroclor 1254 induced rats). Test concentrations were selected based on the results obtained in pre-experiments.

In experiment I, cells were treated with the test item 24 hours after mitogen stimulation with phytohaemagglutinin (PHA). In the second experiment, the treatment started 48 hours after the mitogen stimulation. The exposure times for the test item in the presence and the absence of S9 mix were 3 and 20 hours, respectively. Cytochalasin B (6 μg/ml) was added to the cultures to block cytokinesis during the recovery periods of 28 and 45 hours for the assay without and with S9 mix respectively. Cells from Experiment I were harvested at 72 h after mitogen stimulation and those from Experiment II were harvested at 96 h after mitogen stimulation. To calculate the replication index (RI), 500 cells per replicate (1000 per concentration) were examined for proportions of mononucleate, binucleate and multinucleate cells. Typically one thousand binucleate cells from each culture (2000 per concentration) are analysed for the occurrence/number of micronuclei. To further clarify some of the results, an additional 1000 binucleate cells from each culture (total 4000 cells per concentration) were analysed for Experiment I, II and III. For Experiment IV, the standard protocol of scoring 1000 binucleate cells for each culture was followed.

DMSO was used for the negative (solvent) control, and 4-nitroquinoline 1-oxide (5.00 µg/ml), vinblastine (0.08 µg/ml) and cyclophosphamide (12.5 µg/ml) were evaluated as positive controls.

Results
In Experiment I (with and without S9) and in Experiment II (with S9), the appropriate toxicity was achieved at the highest concentration tested. However, in Experiment II in the absence of S9, the highest concentration chosen for analysis (400 µg/ml) approximately induced 72% cytotoxicity. In Experiment II, in the absence of S9, the next highest dose 350 µg/ml induced 53% cytotoxicity. Since the target toxicity (60%) was not achieved in this case, the experiment was repeated (Experiment III). In Experiment III the target toxicity was achieved.

In the absence of S9 mix, in Experiment I, the frequencies of micronucleated binucleated cells (MNBN) were significantly elevated at the lowest and intermediate dose. However, there was no obvious concentration-effect-relationship. In Experiment II, the frequencies of MNBN were similar to the concurrent controls. In Experiment III (repeat of Experiment II, without S9) also, the frequencies of MNBN were within the historical vehicle control range.

In the presence of S9, in Experiment I, single cultures of the two highest concentrations (1400 and 1600 µg/ml) exceeded the historical control range. In Experiment II, all but one replicate culture showed an increase in MNBN over the historical control range. However, some of these increases were relatively small. The significance of these results was further evaluated in Experiment IV, which clearly showed a significant increase in frequencies of MNBN over the historical control range at all concentrations tested.

It is concluded that high concentrations of 2-Amino-6-chloro-4-nitrophenol have the potential to induce micronuclei in cultured human peripheral blood lymphocytes in particular in experiments with S9-mix. The potential mechanism of the mutagenic action (i. e. clastogenic vs aneugenic) has not been identified.

Ref.: 24

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

In Vivo Mammalian Erythrocyte Micronucleus Test (i.p. application)

Guideline:	OECD 474 (1997)
Species/strain:	Mice, NMRI
Group size:	6 males + 6 females per dose group and per sacrifice time (cells of 5 animals each were analysed)
Test substance:	2-Amino-6-chloro-4-nitrophenol
Batch:	GST009-01/30-07
Purity:	99.9% (HPLC at 254 nm)
Dose:	18.75, 37.5 and 75 mg/kg bw administered as single doses
Route:	i. p.
Vehicle:	1 % CMC in water
Sacrifice times:	24 and 48 hours (high dose only)
GLP:	in compliance

2-Amino-6-chloro-4-nitrophenol, (18.75, 37 and 75 mg/kg bw dissolved in 1 % CMC), was administered to groups of six male and six female NMRI mice by i. p. injection. For the high dose, two groups were treated to allow sampling after 24 and 48 hours. The doses were administered in a total volume of 10 ml/kg bw to animals. Dose selection was based on findings in the pre-experiments for toxicity in which a range of 50 to 500 mg/kg bw were administered to two female and two male mice under the same treatment procedure. Negative control groups received 1 % CMC in water and concurrent positive control groups received 10 mg/kg bw cyclophosphamide (CPA) dissolved in deionised water.

Femoral bone marrow was sampled from mice after sacrifice (24 hours after dosing for all dose groups and after 48 h for the additional high dose group). Bone marrow of the negative and concurrent positive control group animals were sampled 24 h after administration. Slides were prepared from the bone marrow preparations and stained. The slides were stained with May-Grünwald/Giemsa and evaluated (without knowledge of the dose group) for the number of polychromatic erythrocytes (PCE) with micronuclei. At least 2000 PCEs per animal were analysed. In addition, the ratio between polychromatic and total erythrocytes per animal was determined. Ten animals per test group (5 males and 5 females) were evaluated as described above.

The animals of the high dose group were examined for signs of acute toxicity several times within the first 24 hours of treatment.

Results
Stability and homogeneity data are not provided in the study. However, analytical data presented in other studies (e.g. the 90-day oral toxicity study) using a very similar vehicle (0.5 % instead of 1 % CMC) revealed a sufficient homogeneity and stability in such vehicles. Furthermore, 2-Amino-6-chloro-4-nitrophenol was shown to be stable in water for at least 7 days (Ref.: 1). Consequently, it can be assumed that the test item solutions were homogenous and stable for the required single application.

In the main study, signs of toxicity similar to those described in the pre-experiment were noted for the high dose group, up to 24 hours after substance administration, but no cases of death occurred.

The mean number of PCEs was not affected by the test item at any test concentration or sampling time as compared to the mean value of PCEs in the vehicle control. Hence, 2-Amino-6-chloro-4-nitrophenol, even tested at systemically toxic doses, showed no clear cytotoxic effect in the bone marrow. However, the occurrence of discoloured urine as well as the observed signs of toxicity demonstrate that the item was systemically distributed and bio-available. Furthermore, the toxicokinetics study performed with rats demonstrated a good bioavailability of the test item after oral administration.

There was no statistically significant or biologically relevant increase in the frequency of micronuclei in PCE of mice treated with 2-Amino-6-chloro-4-nitrophenol in comparison to the negative controls. The positive control group (CPA) produced a clear and significant increase in micronucleated PCEs and the vehicle control was well within the range of historical control data of the performing laboratory. This demonstrates the validity and sensitivity of the used test system.

Conclusion

2-Amino-6-chloro-4-nitrophenol was not mutagenic in the *in vivo* micronucleus test using NMRI mice after a single i. p. administration up to the maximum tolerated dose of 75 mg/kg bw.

Ref.: 25

In Vivo Mammalian Erythrocyte Micronucleus Test (gavage administration)

Guideline:	not indicated but performance in line with OECD 474
Species/strain:	Mice, strain NMRI
Group size:	6 males + 6 females per dose group and per sacrifice time (cells of 5 animals per sex were analysed)
Test substance:	2-Amino-6-chloro-4-nitrophenol
Batch:	COS 198
Purity:	100% (HPLC at 254 nm)
Dose:	5, 15 and 150 mg/kg bw administered as single doses
Route:	oral, gavage
Vehicle:	DMSO
Sacrifice times:	24 hours (all dose groups), 48 and 72 hours (high dose and controls only)
GLP:	in compliance

2-Amino-6-chloro-4-nitrophenol was administered by gavage to 10 to 16 week old NMRI mice at dose levels of 0, 15 or 150 mg/kg bw in DMSO at a volume of 5 ml/kg bw. Bone marrow cells were investigated 24 h after administration for all three dose and the control groups as well as 48 h and 72 h after administration for the high dose and the control groups.
Dose selection was based on findings in the pre-experiments for toxicity, in which a range of 50 to 2000 mg/kg bw was investigated. Negative control groups received DMSO alone and the concurrent positive control group received 30 mg/kg bw cyclophosphamide (CPA), also dissolved in DMSO.
Femoral bone marrow was sampled from mice after sacrifice 24 hours after dosing for all groups and after 48 and 72 h for the control and the additional high dose groups. Slides were prepared from the bone marrow preparations, stained with May-Grünwald/Giemsa and evaluated (without knowledge of the dose group) for the number of polychromatic erythrocytes (PCE) with micronuclei. At least 1000 PCEs per animal were analysed. In addition, the ratio between polychromatic (PCE) and normochromatic (NCE) erythrocytes per animal was determined. Ten animals per test group (5 males and 5 females) were evaluated as described above.

Results
In the pre-test for toxicity, one of two animals died within 1h after administering doses of 2000, 1000 and 500 mg/kg bw. At 250 mg/kg bw, 2 of 6 treated animals died within 48 h. At 200 mg/kg bw, 1 of 2 animals died within 72 h. At 150 mg/kg bw, severe clinical signs as abdominal position, tremor and convulsion were noted, but no animal died. This dose was selected as the highest test dose for the main experiment, as no lethality was observed.

The ratio of PCE/NCE did not reveal any treatment related effect. Hence 2-Amino-6-chloro-4-nitrophenol, even if tested at systemically toxic doses, showed no clear cytotoxic effect in the bone marrow. However, the occurrence of severe systemic toxic effects and the toxicokinetic study performed with rats demonstrate a good bioavailability of the test item after oral administration.

There was no statistically significant or biologically relevant increase in the number of micronuclei per 1000 PCEs in the mice of any of the 2-Amino-6-chloro-4-nitrophenol treated groups at any preparation time compared to the respective vehicle control groups.

The positive control group (CPA) produced a clear and significant increase in micronucleated PCEs, thus demonstrating the validity and sensitivity of the used test system.

Conclusion
2-Amino-6-chloro-4-nitrophenol was not mutagenic in the *in vivo* micronucleus test using NMRI mice after a single oral administration via gavage up to the maximum tolerated dose of 150 mg/kg bw, at which severe signs of toxicity were noted.

Ref.: 26

Summary mutagenicity

Several state of the art *in vitro* and *in vivo* mutagenicity tests have been performed with 2-Amino-6-chloro-4-nitrophenol. Both the Ames test and the mouse lymphoma assay were negative, indicating that 2-Amino-6-chloro-4-nitrophenol does not possess a potential to cause gene mutation *in vitro*. 2-Amino-6-chloro-4-nitrophenol did reveal a potential to induce micronuclei in cultured human lymphocytes. However, no evidence for a mutagenic/clastogenic potential was noted *in vivo*. Two micronucleus tests *in vivo* have been performed, one using the intraperitoneal route for the application of the dye, whereas in the second assay the test item was administered via gavage. None of these *in vivo* tests indicated that 2-Amino-6-chloro-4-nitrophenol might cause mutagenic/genotoxic effects *in vivo*. Therefore the genotoxic effect seen in the *in vitro* micronucleus test with human lymphocytes does not occur under appropriate *in vivo* test conditions.

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
Species/strain:	Wistar rat, strain Crl: Wi/Br (SPF)
Group size:	20 pregnant females per dose group
Test substance:	2-amino-6-chloro-4-nitrophenol
Batch:	COS 198
Purity:	100 area% (HPLC at 254 nm)
Dose:	0, 10, 30, 90 mg/kg bw/day (in 0.5% carboxymethylcellulose in water)

Treatment period: Days 6-15 of gestation
GLP: in compliance

Groups of 20 pregnant rats received 2-amino-6-chloro-4-nitrophenol by oral gavage at doses of 10, 30 or 90 mg/kg bw/day from day 5 through day 15 of gestation, the control group received the vehicle only (0.5 % carboxymethylcellulose in water). The day of positive proof for sperm in the vaginal smear was designated as day 0 of pregnancy.

Animals were checked daily for clinical signs and, in addition, modified Irwin tests were performed. Food consumption and body weight were recorded at designated intervals during pregnancy (days 0-1, 5-15, 15-20). On day 20 of pregnancy, the animals were killed and examined macroscopically. Foetuses were removed by Caesarean section. The following litter parameters were recorded: number of corpora lutea and implantation sites, number and distribution of early and late resorptions, and number and distribution of dead and live foetuses, placentae and sex. Foetuses were weighed and submitted to external, soft tissue and skeletal examinations.

Results
No treatment-related effects in dams were noted with regard to clinical observations and post-mortem findings but the urine was discoloured. Body weight gain and food consumption were reduced in the high dose group. Gross necropsy revealed no treatment related effects.
There were no treatment related effects with regard to reproduction e.g. uterus and placenta weights, the number of corpora lutea, litter size, foetal mortality, foetal body weight, birth position and sex ratio. The skeletal and visceral examination of the foetuses revealed no treatment related findings.

Conclusion
The NOAEL of maternal toxicity is 30 mg/kg bw. No teratogenic and foetotoxic effects were observed even at the highest dose 90 mg/kg bw which is the NOAEL of developmental toxicity.
Ref.: 27

3.3.9. Toxicokinetics

Biovailability across intestinal barrier in TC-7 (human intestinal epithelial) cells

Guideline: /
Cells: Human intestinal epithelial cell line TC-7
Test substance: 2-amino-6-chloro-4-nitrophenol
Batch: GST009-01/30-07
Purity: 99.9% (HPLC at 254 nm)
Test concentration: 50 µM in HBSS buffer containing 1 % DMSO
Incubation time 60 min
Number of experience: Two independent experiments
GLP: not in compliance

The bioavailability of 2-amino-6-chloro-4-nitrophenol across the intestinal barrier was investigated in human intestinal epithelial (TC-7) cells *in vitro*. The permeability from the apical (A, pH 6.5) to the basolateral (B, pH 7.4) side was investigated at 37°C in 96-well transwell plates with shaking for a 60 min contact time. Analysis of the donor (apical) and receiver

(basolateral) samples was done by means of HLPC-MS/MS and the apparent permeability coefficient (P_{app}) was calculated for two independent experiments. ^{14}C-mannitol (about 4 µM) was used to demonstrate the integrity of the cell monolayer. Only monolayers revealing a permeability of < 2.5 x 10^{-6} cm/sec were used. Propranolol, atenolol, vinblastine and ranitidine were analysed concurrently to demonstrate the validity of the test system.
According to the laboratory's classification system, a low permeability is considered for test items revealing a P_{app} < 2 x 10^{-6} cm/sec. A P_{app} of 2 - 20 x 10^{-6} cm/sec and a P_{app} ≥ 20 x 10^{-6} cm/sec classify a substance to have a moderate and a high permeability, respectively. As recommended by FDA, ranitidine (50 % absorption in humans) was used as the low permeability reference compound and propranolol (90 % absorption in humans) was used as the high permeability reference compound.

Results
The total recovery for the reference substances ranged from 82 to 96 %, and 95 % for 2-amino-6-chloro-4-nitrophenol. The figures for the reference substances propranolol (P_{app} = 47.8 x 10^{-6} cm/sec) and ranitidine (P_{app} = 0.4 x 10^{-6} cm/sec) were close to or within the acceptance range of 20 – 45 x 10^{-6} cm/sec and 0.2 – 2 x 10^{-6} cm/sec, respectively. 2-Amino-6-chloro-4-nitrophenol revealed a P_{app} of 133.2 x 10^{-6} cm/sec and was classified to be of high permeability in this test system.

Since absorption across the intestinal epithelium is considered to be a limiting factor of the uptake through the gastro-intestinal tract, the high permeability observed in this assay hints to a good absorption of 2-amino-6-chloro-4-nitrophenol after oral administration.

Ref.: 28

Toxicokinetics *in vivo*

Guideline:	/
Species/strain:	Sprague Dawley rats, strain Him:OFA, SPF
Group size:	3 males and 3 females per treatment group
Test substance:	^{14}C-2-amino-6-chloro-4-nitrophenol (ring-labelled) Radiochemical purity > 98 %, specific activity: 13.7 mCi/mmol
Batch:	O99F9213
Doses:	Exp. A, B, C, dermal application: Exp. A: Formulation without H_2O_2: 3 % formulation, 76 mg/cm^2 applied to 13.2 cm^2 corresponding to 2.27 mg dye/cm^2, Exp. B: Formulation with H_2O_2: 3 % formulation, 110 mg/cm^2 applied to 9 cm^2 corresponding to 3.26 mg dye/cm^2 Exp. C: Solution in water/DMSO (1:1): 9.99 %; 3.3 mg dye/cm^2 Exp. D, E, oral administration: 146.4 mg/kg bw as 3 % solution
Treatment period:	Exp. A, B, C: single cutaneous application in a solution of water/DMSO 1:1 (C), 34 mg/cm^2, and as part of a formulation without (A) and with (B) hydrogen peroxide for 30 min Exp. D, E: single oral application in water/DMSO 1:1
GLP:	in compliance

<u>Dermal application (experiments A, B and C)</u>

^{14}C-2-Amino-6-chloro-4-nitrophenol was applied to the clipped dorsal skin Sprague Dawley rats Him:OFA, SPF for 30 min. 3 Males and 3 females per group were used. The test substance was applied at concentrations of 9.99 % in solution (water/DMSO 1:1, experiment C) and of 3 % in formulations without (experiment A) and with hydrogen peroxide (experiment B).

After treatment, the test substance was scraped and washed off and the skin rinsed with a shampoo. During the exposure time, animals were fixed to avoid licking. After rinsing, the area was covered with gauze fixed by adhesive tape and an additional air permeable plastic cone to further prevent licking of the treated area during the 72 h in the metabolism cages.

Oral administration (experiments D and E):
146.4 mg/kg bw ^{14}C-2-amino-6-chloro-4-nitrophenol was administered in a 3% solution in water/DMSO 1:1 by gavage (approximately 1 g solution /animal) to two groups of 3 male and 3 female rats which were starved for 16 hours before treatment. In one group (experiment D), animals were placed in metabolism cages for 72 h. In experiment E, blood was taken at several time points within 24 h after administration.

During the studies, blood was taken from the retrobulbar venous plexus under light ether anaesthesia. At termination of the experiment, blood samples were taken from the aorta. Urine and faeces were collected daily (0-24, 24-48 and 48-72 h after administration) from the metabolic cages.

Animals were killed 72 hours (experiments A, B, C, and D) and 24 hours (experiment E) after the application, and the application sites (experiments A, B and C) as well as numerous organs were taken and analysed for radioactivity. The radioactivity in the remaining carcass after skin removal (experiments A, B, C and D) or after removal of the gastro-intestinal tract (experiment D) was also determined.

Results
Total recovery of the applied radioactivity for the individual animals ranged from 94.4 to 100.5% of the applied doses. After oral application (D), 2-amino-6-chloro-4-nitrophenol was mainly eliminated via urine (70 % of the applied dose within 72 h) and, to a minor extent, via the faeces (30 %). 92.7 % of radioactivity was excreted within the first 24 hours. The radioactivity found in tissues 72 h after administration was less than 0.03 % of the applied dose per g organ, with higher values noted in the kidneys, thyroids and liver. After oral application, higher residual levels were noted for several organs in females as compared to males. This is consistent with the blood level data obtained in experiment E, in which also higher blood levels were noted for females. The blood level (experiment E) reached a peak at 35 min after application and declined with an initial half-life of 1 h.

Following dermal application the majority of the dyestuff was removed by the rinsing 30 min after the application, i.e., 95.4 to 98.7 % of the applied dose was found in the washing water.
Seventy two hours after application, the amount of radioactivity remaining at the application site (skin) was less than 1 % for all three experiments. The highest value (0.82 % of the applied dose) was noted for the formulation with H_2O_2 (experiment B). For formulation without H_2O_2 (experiment A) and for the solution (DMSO/water; experiment C), the figures were 0.38 and 0.59 %, i.e. the lowest absorption rate was obtained in the formulation containing H_2O_2. The proportion of the applied dose that was absorbed was calculated by adding the amounts in urine, faeces and carcass: **A** formulation without H_2O_2 0.248 % (equal to 5.6 µg/cm²), **B** formulation

with H_2O_2 0.108 % (equal to 3.52 µg/cm²), and **C** pure dyestuff in water/DMSO 1.213 % (equal to 40.0 µg/cm²).

Comment
2-Amino-6-chloro-4-nitrophenol, given orally to rats, is quickly absorbed and excreted within 72 h, with the majority eliminated within the first 24 h after application. Excretion takes place predominantly (70 %) via urine and to a minor extent via faeces. Radioactivity was found in several organs including femur. The figures of experiment A (formulation without H_2O_2) 0.248 % (equal to 5.6 µg/cm²) may be used for safety assessment.

Ref.: 29

3.3.10. Photo-induced toxicity

3.3.10.1. Phototoxicity / photoirritation and photosensitisation

Guideline:	/
Species:	Hartley guinea pigs
Group:	two groups of 5 males (1 group each for test substance and control)
Substance:	chlororange
Batch:	R0067439
Purity:	> 97 %
Dose:	0.02 ml of 2 % chlororange
	0.02 ml of 0.1 % of 8-MOP (positive control)
GLP:	in compliance

0.02ml of 2% chlororange was applied to each side of the back of the group of 5 male guinea pigs. A similar amount of 0.1ml MOP was applied to each side of the backs of the control group. There was no occlusion.

After 30 minutes, one side of the back of each animal was covered with aluminium foil and then the backs were irradiated with UVA; dose 10J/cm^2.

Results
At 24, 48 and 72 hours there was no reaction at either the UV irradiated or unexposed sites for the chlororange treated group.
In the 8-MOP treated group there were reactions at all points at the irradiated sites but no reactions at the unirradiated sites.

Conclusion
Chlororange was not phototoxic under the conditions of the experiment.

Ref: 30

Guideline:	/
Species:	Hartley guinea pigs
Group:	two groups of 5 males (1 group each for the negative control and the positive control); 1 group of 10 (test substance)
Substance:	chlororange
Batch:	R0067439
Purity:	> 97 %
Dose:	2% chlororange for induction and challenge Water for induction and challenge with 2% chlororange (negative control) 1% tetrabromosalicylanilide (TBS) for induction and challenge (positive control)
GLP:	in compliance

The fur was clipped from the backs of the animals. 0.1 ml FCA in water was injected subcutaneously into each corner of the clipped area. The skin was then tape stripped with 5-6 applications of tape.
0.1ml of 2% chlororange (test), 0.1ml water (negative control) or 0.1ml of 1% TBS (positive control) was then applied under non-occlusive conditions. After 30 minutes the treated areas were irradiated with UVA; dose 10J/cm^2. The stripping and applications and irradiation were repeated for 5 consecutive days.
Two weeks after the final application, 0.02ml of 2% chlororange was applied to the test and negative control groups, and 0.02ml of 1% TBS to the positive control group. One half of the back of each animal was then covered by opaque material and the uncovered half irradiated with UVA; dose 10J/cm^2.

Results
The chlororange test and negative control sites showed no reaction with or without UVA exposure. The positive control group reacted as expected with UVA but there was no reaction without light.

Conclusion
Under the experimental conditions, chlororange was not shown to be a photo-allergen.

Ref.: 31

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

(2-Amino-6-chloro-4-nitrophenol)

Maximum absorption through the skin	**A (µg/cm^2)**	**=**	**5.6 µg/cm²**
Skin Area surface	**SAS (cm^2)**	**=**	**700 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**3.92 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.065 mg/kg**
No observed adverse effect level (mg/kg) (Embryo-foetal development)	**NOAEL**	**=**	**30 mg/kg**

Margin of Safety	**NOAEL / SED**	**=**	**462**

3.3.14. Discussion

Physico-chemical specifications
2-Amino-6-chloro-4-nitrophenol is intended for use at a maximum on-head concentration of 2% in semi-permanent hair dyes formulations as a "direct dye", or as a non-reactive colorant in oxidative hair-dyes formulations. In the latter case, it has been found to be stable under the conditions during oxidative hair-dyeing, with the dye and developer (hydrogen peroxide) components mixed at a ratio between 1:1 and 1:3.

General toxicity
The LD_{50} was considered to be ≥ 2000 mg/kg bw. The NOAEL was set at 30 mg/kg bw/day in a subchronic oral toxicity study in rats and for maternal toxicity. It was set at 90 mg/kg bw/day for developmental toxicity.

Irritation/sensitisation
Chororange was not photo-toxic or photo-allergenic under the conditions of the experiments. It showed to be a skin sensitiser.

Dermal absorption
According to the SCCP Notes of Guidance, skin samples of more than 1 donor should have been used. Therefore, the study cannot be used for a safety assessment.
The figures of experiment A (*in vivo* toxicokinetics study, formulation without H_2O_2) of 0.248 % (equal to 5.6 µg/cm²) is used for safety assessment.

Mutagenicity
Several state of the art *in vitro* and *in vivo* mutagenicity tests have been performed with 2-Amino-6-chloro-4-nitrophenol. Both the Ames test and the mouse lymphoma assay were negative, indicating that 2-Amino-6-chloro-4-nitrophenol does not possess a potential to cause gene mutation *in vitro*. 2-Amino-6-chloro-4-nitrophenol did reveal a potential to induce micronuclei in cultured human lymphocytes. However, no evidence for a mutagenic/clastogenic

potential was noted *in vivo*. Two micronucleus tests *in vivo* have been performed, one using the intraperitoneal route for the application of the dye, whereas in the second assay the test item was administered via gavage. None of these *in vivo* tests indicated that 2-Amino-6-chloro-4-nitrophenol might cause mutagenic/genotoxic effects *in vivo*. Therefore the genotoxic effect seen in the *in vitro* micronucleus test with human lymphocytes does not occur under appropriate *in vivo* test conditions.

4. CONCLUSION

The SCCP is of the opinion that the use of 2-Amino-6-chloro-4-nitrophenol itself as:

- as a semi-permanent hair dye, or as
- a non-reactive colorant in oxidative hair dye formulations (after mixing with hydrogen peroxide at a ratio between 1:1 and 1:3)

at a maximum concentration of 2.0 % in the finished cosmetic product does not pose a risk to the health of the consumer.

Studies on genotoxicity/mutagenicity in finished hair dye formulations should be undertaken following the relevant SCCNFP opinions and in accordance with its Notes of Guidance.

This hair dye, like many other hair dyes, is a skin sensitiser.

5. MINORITY OPINION

Not applicable

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