SCCS/1302/10
Revision of 21 June 2011

EUROPEAN COMMISSION

Directorate-General for Health & Consumers

Scientific Committee on Consumer Safety

SCCS

OPINION ON

4-Amino-2-nitrodiphenylamine-2'-carboxylic acid

COLIPA n° B87

Scientific Committees
on consumer safety
on emerging and newly identified health risks
on health and environmental risks

The SCCS adopted this opinion at its 10th plenary meeting

of 22 March 2011

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 Safety (SCCS), 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 Agency (EMA), the European Centre for Disease prevention and Control (ECDC) and the European Chemicals Agency (ECHA).

SCCS

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

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

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

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1. BACKGROUND

Submission I for 4-Amino-2-nitrodiphenylamine-2'-carboxylic acid was submitted in August 1994 by COLIPA[1].

The Scientific Committee on Cosmetic Products and Non-Food Products intended for Consumers (SCCNFP) adopted at its 23rd plenary meeting on 18 March 2003 the opinion (SCCNFP/0658/03, final) with the statement:

> *The SCCNFP is of the opinion that the information submitted is insufficient to allow an adequate risk assessment to be carried out. Accordingly, the SCCNFP considers that it is not possible to assess the safe use of the substance.*
>
> *Before any further consideration, the following information is required:*
>
> *data on the genotoxicity/mutagenicity following the relevant SCCNFP opinions and in accordance with the Notes of Guidance.*

Submission II for 4-Amino-2-nitrodiphenylamine-2'-carboxylic acid was submitted by COLIPA in July 2005. According to this submission the substance is used as a direct dye in hair dye formulations or as an ingredient in oxidative dyeing products which may or may not contain a hydrogen peroxide based developer mix up to a final concentration of 2.0% on head.

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. *Does the Scientific Committee on Consumer Safety (SCCS) consider 4-Amino-2-nitrodiphenylamine-2'-carboxylic acid safe for use as a non-oxidative hair dye with an on-head concentration of maximum 2.0 % taken into account the scientific data provided?*

2. *Does the SCCS consider 4-Amino-2-nitrodiphenylamine-2'-carboxylic acid safe for use in oxidative hair dye formulations with an on-head concentration of maximum 2.0 % taken into account the scientific data provided?*

3. *Does the SCCS recommend any restrictions with regard to the use of 4-Amino-2-nitrodiphenylamine-2'-carboxylic acid in any non-oxidative or oxidative hair dye formulations?*

[1] COLIPA - European Cosmetics Toiletry and Perfumery Association

3. OPINION

3.1. Chemical and Physical Specifications

3.1.1. Chemical identity

3.1.1.1. Primary name and/or INCI name

4-Amino-2-nitrodiphenylamine-2'-carboxylic acid

3.1.1.2. Chemical names

2-[(4-Amino-2-nitrophenyl)-amino]-benzoic acid
2-Nitro-4-amino-diphenylamine-2’-carboxylic acid

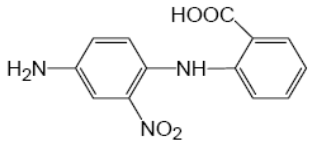
3.1.1.3. Trade names and abbreviations

RO1082
COLIPA B87

3.1.1.4. CAS / EC number

CAS: 117907-43-4
EC: 411-260-3

3.1.1.5. Structural formula



3.1.1.6. Empirical formula

Formula: $C_{13}H_{11}N_3O_4$

3.1.2. Physical form

Dark red crystals, odourless

3.1.3. Molecular weight

Molecular weight: 273.3 g/mol

3.1.4. Purity, composition and substance codes

	ANDC-012004	3279/141	raw material (ref1)	3962/46	2495/127	2495/161
HPLC-purity (area-%)	97 %	>98 %	97 %	97 %	98 %	96 %
2-aminobenzoic acid	150 ppm	0.6%	< 500 ppm	15000 ppm	1000 ppm	3000 ppm
aniline	< 50 ppm					
4-amino-2-nitrophenol	< 50 ppm					
4-fluoro-3-nitroaniline	< 40 ppm		<500 ppm	7000 ppm	1000 ppm	3000 ppm
acetic acid ethylester	0.3 % (w/w)		< 0.5 %(w/w)			
toluene	0.2 %(w/w)		< 0.3 %(w/w)			
ethanol	0.1 %(w/w)					
chloride	0.1 %(w/w)			0.005%(w/w)	0.378%(w/w)	0.033%(w/w)
water	0.4 %(w/w)		< 1.0%(w/w)	4.1 %(w/w)	2.1 %(w/w)	2.0 %(w/w)
sulphate ash	0.2 %(w/w)		< 0.5%(w/w)			
sulphate				0.016%(w/w)	< 0.1	< 0.1

Batch ANDC-012004
Identity of batch ANDC-012004 was confirmed by 1H-, 13C-, DEPT- and HSQC-NMR and IR- and UV-spectrometry. All spectra were in good accordance with the structural characteristics.
Purity was determined by HPLC-UV (276nm) and quantitative NMR-spectroscopy and was 97% (NMR) and 97.1% (HPLC-UV).
Using HPLC the batch was further analysed for impurities of 2-aminobenzoic acid (209 nm), aniline (227 nm), 4-amino-2-nitrophenol (227 nm) and 4-fluoro-3-nitroaniline (227 nm) against standard solutions of all analytes. At retention time of 21.1 minutes, an unknown impurity (1.7%) was detected. Further impurities were acetic acid ethylester, toluene, ethanol, water and chloride determined by GC-MS, Karl-Fisher-titration and ion-chromatography.

Ref.: 2

Batch 3279/141
Batch 3279/141 was analysed for purity and identity by elementary analysis, IR- and NMR-spectroscopy and HPLC. Results of the elementary analysis were 57.2% C, 4.04% H and 14.7% N as calculated.
Purity was determined as >98% by HPLC. 0.6% 2-Aminobenzoic acid was detected as an impurity; 4-Fluoro-3-nitroaniline was not detected.
TLC showed one red main spot (Rf 0.61), two weak reddish spots (RF 0.66 and 0.46) and a weak grey spot at the starting point.

Ref.: 4

Raw material
Identity and purity of an unknown batch called raw material was determined by IR, UV/VIS, NMR, IR and HPLC. Purity was > 97% (NMR, HPLC). All impurities were below the limits of detection. Heavy metal content was < 20 ppm Pb, < 10 ppm Sb, Ni, < 5 ppm As, Cd, <1 ppm Hg.

Ref.: 1

Batch 3962/46, 2495/127, 2495/161
Identity IR-spectrometry of the KBr pellet showed good accordance with the reference spectra. 1H and 13C-NMR are in accordance with the proposed structure.

Purity of batch 3962/46 was 97%, determined by HPLC-UV at 240 nm. Impurities were quantified against standard solutions of the impurities. Concentrations of chloride (0.005%) and sulphate (0.016%) were determined by ion chromatography.
Purity and impurities of batch 2495/127 and 2495/161 were determined as specified for batch 3962/46.

Ref.: 3

Comments
For the batch "raw material" no original data or spectra have been provided (ref 1) nor is it clear for what purpose this material has been used. For batch 3962/46 only the final results of the analytical methods are described. No HPLC spectra have been supplied (Ref. 3). Original data and spectra are missing for batch 3279/141 (Ref. 4).

3.1.5. Impurities / accompanying contaminants

See point 3.1.4. Purity, composition and substance codes

3.1.6. Solubility

Solubility at room temperature:

water	< 1 g/l
	< 50 mg/l (30°C) (ref 3)
ethanol	0.3-3.0 g/l
DMSO	> 100 g/l

3.1.7. Partition coefficient (Log P_{ow})

Log P_{ow}: 0.34, calculated: 0.49

3.1.8. Additional physical and chemical specifications

Melting point:	202-247 °C (thermal decomposition)
Boiling point:	/
Flash point:	> 200 °C
Vapour pressure:	/
Density:	/
Viscosity:	/
pKa:	/
Refractive index:	/
UV_Vis spectrum (200-800 nm)	peak maximum at 276 nm with a shoulder at 305 nm, a further peak at 503 nm (batch ANDC-012004).

3.1.9. Homogeneity and Stability

Stability was tested in 0.9% NaCl solution containing 0.2 mL/l DMSO at room temperature over 24 h. The authors claim that within the time no degradation could be observed.

Homogeneity of 2% B87 in PEG was determined by HPLC-Vis at 502 nm. Calibration stock solutions and standard solutions were dissolved in DMSO and diluted 1:1 with deionised water prior to analysis.
Samples of the preparation were stored at room temperature and analysed as soon as possible. Homogeneity of the formulation was determined by six aliquots each of them taken at the beginning of a penetration experiment. The aliquots were dissolved in DMSO. Mean homogeneity was 96%.

Analysis of Homogeneity of 2% and 4% B87 respectively in cream formulation was performed as described for 2% B87 in PEG. Homogeneity was 98.3% for the 2% formulation and > 81.9% for the 4% formulation respectively.

Ref.: 17

Homogeneity and stability of batch 3279/141 in 4% CMC were determined at four concentrations (0, 5, 15, 45 mg/ml) by HPLC-UV at 275 nm. Calibration was performed against standard solutions.
Three different segments of preparation (top, middle and bottom) were analyzed to determine the homogeneity of the preparation. For stability test an aliquot of the preparation was kept at room temperature for at least 3 hours.
Homogeneity varied between -1% and +2% and the preparation is stable for at least 2 hours.

Ref.: 15

Recent documents show that the following preparations are stable and homogeneous for at least 24h:

B87 in 1% carboxymethylcellulose and 0.5% Cremophor	(ref. 6)
B87 in acetone:olive oil 4:1 (v/v)	(ref. 9)
2% B87 in PEG	
B87 in 0.9% NaCl	(Ref. 20, 21)

The stability of the hair dye B 087 was tested in the presence of an aqueous dilution of hydrogen peroxide. Using UV-spectroscopy it was demonstrated that B 087 is stable in an oxidative environment, for at least 1 hour.

Ref.: 22

General Comments to physico-chemical characterisation

* According to the applicants the purity of the batches is between 96 and 98%. Taking into account the impurities, which could have been characterized, there are still about 2% of the substance which have not been characterized
* Although there are plenty of data on the purity of batches, original data like spectra are missing. This is also the case for stability and homogeneity.
* The stability in a typical hair dye formulation was not reported.

3.2. Function and uses

4-Amino-2-nitrodiphenylamine-2'-carboxylic acid is used as a direct dye in hair dye formulations or as an ingredient in oxidative dyeing products, which may or may not contain a hydrogen peroxide-based developer mix, up to a final concentration of 2% on head.

3.3. Toxicological Evaluation

3.3.1. Acute toxicity

3.3.1.1. Acute oral toxicity

Taken from SCCNFP/0658/03

Guideline:	/
Species/strain:	Wistar Albino rats
Group size:	5 males + 4 females
Test substance:	Ro 1082
Batch:	2495/127
Purity:	98%
Vehicle:	aqueous solution of 1% carboxymethylcellulose and 0.5% Cremophor
Dose:	2000 mg/kg bw
Route:	gastric gavage
Observ. Period:	14 days
GLP statement:	in compliance
Study period:	August 1987

Groups of 5 males and 4 females received a single dose of test substance at 2000 mg/kg bw by gastric gavage. The animals were observed daily and body weights were recorded on days –1, 0, 2, 7 and 14 of the study. Macroscopic examination of main organs was performed at autopsy. No histological examinations were performed.

Results
There was one death in the male group which was assumed to be treatment-related (time after dosing not specified). Autopsy observations were discoloration of the intestines, subcutis and muscles and lung oedema. Body weight gain for surviving animals was considered normal for the age and strain of rat. No abnormal findings were reported at scheduled autopsy. The LD_{50} was reported to be greater than 2000 mg/kg bw.

Ref.: 6

3.3.1.2. Acute dermal toxicity

Taken from SCCNFP/0658/03

Guideline:	OECD 402 (1987)
Species/strain:	Wistar Albino rat; Outbred, SPF
Group size:	5 male + 5 female
Test substance:	Ro 1082 suspended in 1% aqueous carboxymethylcellulose
Batch:	3962/46 (purity >95%)
Dose:	2000 mg/kg bw, occluded patch, 24 hours
Observ. period:	14 days
GLP:	in compliance

Groups of 5 male and 5 female received a single dose of test substance at 2000 mg/kg bw, applied under occlusion to an area of 25 cm² or males and 18 cm² for females. The patches were left in place for 24 hours and the residue was removed with moistened tissue. The animals were observed 1, 2 and 4 hours after dosing and thereafter daily for 14 days. Body weights were recorded on days 1, 8 and 15 of the study. Macroscopic examination of main organs was performed at autopsy. No histological examinations were performed.

Results
There were no mortalities. Lethargy was noted in the majority of animals during the first 48 hours. Body weight gain was considered to be low for the majority of animals during the first week of the study period and in one female in the second week. No skin irritation was observed on the exposed skin but discoloration due to the compound was noted throughout the study period. No abnormalities were noted at autopsy. The dermal LD_{50} was reported to be in excess of 2000 mg/kg bw in both males and females.

Ref.: 2, Subm. I

3.3.1.3. Acute inhalation toxicity

No data submitted

3.3.2 Irritation and corrosivity

3.3.2.1. Skin irritation

Taken from SCCNFP/0658/03

Single dose rabbit study

Guideline:	OECD 404 (1981)
Species/strain:	Albino rabbits, Kleinrusse strain (Chbb:HM)
Group size:	5 males
Test substance:	Ro 1082
Batch:	2495/127
Purity:	/
Dose:	0.6g
GLP:	in compliance

The substance (0.6 g moistened with water) was applied to a 6.25 cm² area of intact skin of 5 male rabbits. Semi-occlusive patches were applied and left in place for a 4-hour period. Remaining test substance was removed by swabbing with cotton wool swabs soaked in warm water. The skin was examined for erythema, eschar formation and oedema at 1, 24, 48 and 72 hours after removal of the patches and the effects were scored according to the Draize criteria.

Results
No skin reactions were observed. The neat substance was not irritating to rabbit skin.

Ref.: 7

Repeated application hairless mouse study

Guideline:	/
Species/strain:	Hairless mouse, hr/hr strain
Group size:	5 females
Test substance:	Ro 1082 in aqueous solution, adjusted to pH 8 with NaOH
Batch:	2495/127 (purity 98%)
Dose:	1-2 drops at 2% (week 1), 4% (week 2) and 8% (week 3)
GLP:	in compliance

One to two drops of the substance were applied to the same area of skin of each animal once per day, 5 days per week, with increasing concentrations in 3 subsequent weeks. Animals were examined daily for signs of erythema and oedema and the observed effects scored according to Draize.

Results
No skin reactions were noted on the skin during or after the application period.
Ref.: 5, Subm. I

Comment
The method is not according to a guideline.

3.3.2.2. Mucous membrane irritation

Taken from SCCNFP/0658/03

Guideline:	OECD 405 (1987)
Species/strain:	Albino rabbits, Kleinrusse strain (Chbb:HM)/Fa
Group size:	4 male
Test substance:	Ro 1082
Batch:	2495/127
Purity:	98%
Dose:	0.1g neat substance
GLP:	in compliance

0.1 g of the neat substance was applied once to the right eye of each animal without rinsing. The left eye served as control. Ocular reactions were recorded at 1 and 6 hours and 1, 2, 3, 7, 10, 14, 17 and 21 days after instillation. The cornea was investigated further using fluorescein at 24 hours and 7 and 21 days.

Results
Instillation affected the cornea and conjunctivae. Slightly increased opacity of the cornea was seen in 2/4 rabbits eyes, resolving in one animal by day 4, but persisting to the end of the study in the other animal. This observation was supported by the fluorescein examination which revealed slight corneal epithelial damage in these two animals. Mild to moderate irritation of the conjunctivae was seen in 4/4 animals and persisted to the end of the study period in one.
According to the defined criteria the pure test substance was classified as severely irritant to the rabbit eye.
Ref.: 8

Comment
The substance should have been tested at concentration nearer to the in-use level to establish whether persistent damage occurs.

3.3.3. Skin sensitisation

Local Lymph Node Assay (LLNA)

Guideline:	OECD 429 (2002)
Species/strain:	CBA/CaOlaHsd mice
Group size:	16 females (4 per group)
Test substance:	B87
Batch:	ANDC-012004
Purity:	98.3 (area% HPLC)
Vehicle:	acetone:olive oil, 4:1
Concentration:	2.5, 5, 10% (w/v) in acetone:olive oil, 4:1 (V/V)
Control group:	acetone:olive oil, 4:1
Positive control:	α-hexylcinnamaldehyde
GLP:	in compliance
Study period:	July 2004

Three groups each of four female mice were treated daily with B87 at concentrations of 2.5%, 5% and 10% (w/v) in acetone:olive oil, 4:1 (v/v) by topical application to the dorsum of each ear lobe (left and right) for three consecutive days. 10% was the highest technically applicable concentration in the vehicle. A control group of four mice was treated with the vehicle (acetone:olive oil, 4:1 (v/v) only. Five days after the first topical application the mice were injected intravenously into a tail vein with radio-labelled thymidine (^{3}H-methyl thymidine). Approximately five hours after intravenous injection, the mice were sacrificed, the draining auricular lymph nodes excised and pooled per group. Single cell suspensions of lymph node cells were prepared from pooled lymph nodes which were subsequently washed and incubated with trichloroacetic acid overnight. The proliferative capacity of pooled lymph node cells was determined by the incorporation of ^{3}H-methyl thymidine measured in a ß-scintillation counter.

Results
The results obtained (Stimulation Index (S.I.)) are reported in the following table.

Substance	%	SI
4-Amino-2-nitrodiphenylamine-2'-carboxylic acid	2.5	1.7
	5	1.1
	10	1.8
α-hexylcinnamaldehyde	5	1.5
	10	2.3
	15	8.4

Calculation of the EC 3 value was not done because no test concentrations produced a Stimulation Index (S.I.) of 3 or higher.
The test item B 087 was found to be a non-sensitizer when tested at up to the highest applicable concentration of 10% (w/v) in acetone:olive oil, 4:1 (v/v).

Ref.: 9

Comment
Because the test concentration was not high enough, a sensitising potential cannot be excluded.

3.3.4. Dermal / percutaneous absorption

Guideline:	OECD draft 428
Test substance:	B87
Batch:	ANDC-012004
Purity:	98.3 area% (HPLC).
Tissue:	pig skin (frozen -20ºC)
Skin integrity:	TER measurement
Method:	Static Franz diffusion cell 1 cm² / receptor compartment 8 ml
Receptor fluid:	autoclaved Dulbecco's phosphate buffered saline, pH 7.35
Formulations tested:	Cream formulation TM 0025-1a with 2% B 087 Cream formulation TM 0025-1b with 4% B 087 mixed with hydrogen peroxide containing developer (2% B 087 final conc.) B87 dissolved in polyethylene glycol 400 in a concentration of 2% w/v
Dose formulation applied:	20 mg/cm²
Replicate cells:	4 skin donors (2 males, 2 females), 4 cells/donor
Duration of the contact:	30 minutes
Duration of the diffusion:	48 hours
Analytical method:	HPLC with visible detection
Validation:	limit of detection (0.00105 mg/L) and limit of quantitation (0.00349 mg/L)
Solubility in the receptor:	/

GLP: in compliance
Study period: June 2005

The test substance was studied as an ingredient of representative formulations as well as in a solution:
Experiment A: 2% B 087 in a cream.
Experiment B: 4% B087 in a cream mixed with a hydrogen peroxide containing developer; final dye concentration of 2%.
Experiment C: 2% B 087 dissolved in polyethylene glycol 400.

Eight integrity checked dermatomed skin preparations of four young pigs were used in each experiment. Skins were inserted in static penetration cells (Franz-cells) with an application area of 1.0 cm². The non-occlusive exposure under temperature controlled conditions lasted 30 minutes before rinsing.
The test substance formulation/solution was applied topically to the horny layer of the skin in nominal quantities of 20 mg/cm^2, which corresponded to nominally 0.4 mg of the test substance per cm^2 for each experiment. 48 hours after the application, the stratum corneum was removed by repeated stripping with adhesive tapes to obtain the adsorbed test substance. The remaining skin was taken to determine the absorbed test substance. The penetration was calculated from the mass of the test substance in the receptor fluid, consisting of phosphate buffered saline. The overall amount of bioavailable test substance is defined as the sum of absorbed and penetrated quantities.

Results

	Experiment A		**Experiment B**		**Experiment C**	
	%	**µg/ cm²**	**%**	**µg/ cm²**	**%**	**µg/ cm²**
Skin rinsings	90.3 ± 1.1	394 ± 13	98.5 ± 8.4	364 ± 43	102.7 ± 4.2	402 ± 37
Adsorption	0.147 ± 0.098	0.641 ± 0.420	0.446 ± 0.249	1.652 ± 0.975	0.192 ± 0.135	0.772 ± 0.577
Absorption	0.329 ± 0.058	1.442 ± 0.290	0.430 ± 0.266	1.560 ± 0.913	0.132 ± 0.109	0.514 ± 0.410
Penetration	0.087 ± 0.042	0.382 ± 0.195	0.079 ± 0.054	0.287 ± 0.181	0.003 ± 0.004	0.011 ± 0.015
Bioavailability	0.416 ± 0.096	1.825 ± 0.465	0.509 ± 0.317	1.846 ± 1.080	0.134 ± 0.112	0.525 ± 0.424
Mass balance*	91.0 ± 1.0	/	99.5 ± 8.2	/	103.2 ± 4.0	/

* slight differences to the sum of the results may occur due to 1) rounding and 2) residual masses in the flange region of the penetration cell.

Absorption: Low absorbed test substance amounts were detected 48 h p.a. (p.a. = after the start of the topical exposure of the test substance formulation / post application) in the dermis and the residual epidermis, including remaining hair stubs and shafts. Significantly lower amounts were noticed in experiment C, compared to experiments A and B.

Penetration rate: Only in experiments with direct dye in oxidative and non-oxidative conditions, and only after 6 hours p.a., the penetration rate was above the limit of quantification.
Bioavailability: The bioavailability parallels the absorption. A significantly lower bioavailability was noticed with B87 dissolved in polyethylene glycol 400, compared to the dye in oxidative and non-oxidative conditions.

The bioavailability of the B87 in non-oxidative conditions was 1.825 ± 0.096 µg/ cm^2 (0.416% ± 0.096%) and 1.846 ± 1.080 µg/cm^2 (0.509% ± 0.317%) in oxidative conditions and only 0.525 ± 0.424 µg/ cm^2 (0.134% ± 0.112) dissolved in polyethylene glycol 400.

Ref.: 17

Comment

Due to the high CV of 59% in oxidative conditions and the low number of donors used, 4 µg/cm^2 (Mean absorption+ 2SD) will be used to calculate the margin of safety.

3.3.5. Repeated dose toxicity

3.3.5.1. Repeated Dose (14 days) oral / dermal / inhalation toxicity

No data submitted

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

Taken from SCCNFP/0658/03

Guideline:	OECD 408 (1981)
Species/strain:	Sprague Dawley rats, CD SPF stain
Group size:	10 males and 10 females + 5 males and 5 females in the recovery group
Test substance:	Ro1082
Batch:	2495/161
Purity:	96%
Vehicle:	aqueous solution of 1% Carboxymethylcellulose and 0.5% Cremophore
Dose levels:	0, 20, 60, 180 mg/kg bw/d
Dose volume:	10 ml/kg bw
Route:	gavage
Observ. Period:	13 weeks (5 days per week) + 4 weeks for the recovery group
GLP:	in compliance
Study period:	October 1988

Groups of 10 male and 10 female rats were dosed with the test substance by gavage at 20, 60 and 180 mg/kg bw/day, 5 days a week for 13 weeks. The dosing solutions were analysed during weeks 1, 12 and 13 for stability and verification of homogeneity and concentration. During the study, the animals were observed daily for clinical signs and mortality, and weekly for body weight and food and water consumption. During weeks 6 and 13, blood was sampled for haematology and blood biochemistry. At the end of the treatment period a full autopsy was conducted with recording of weights of the adrenals, thymus, spleen, heart, kidney, brain, gonads and liver, and macroscopic and microscopic examination of major organs. Ophthalmoscopy was conducted before the start of the study and at the end of the treatment period on control and high dose animals.

Results
There were no mortalities and no clinical signs of toxicity. Staining of the fur, tail and urine was reported for all treatment groups. The body weight gain and food consumption were comparable for all dose groups. The water consumption was increased in female animals in a dose-related manner at 60 and 180 mg/kg bw/day. There was a slight dose-related increase in the number of thrombocytes in male and female animals at week 13, which was significantly different from control at 180 mg/kg bw/day. Other minor significant differences in haematological parameters, as well as those seen in biochemical parameters were not dose-related and were within the normal range and therefore not considered to be of toxicological significance. No abnormal findings were reported in the ophthalmological examinations.

The absolute but not relative liver weights were increased in all female test groups without any relationship to dose. No other effects on organ weights were noted. Yellowish pigment was noted in the liver cells of some animals of all groups including the control and recovery groups. Other minor histo-pathological changes also showed a similar distribution between control and treated groups. Based on the increase in the number of thrombocytes in high dosed rats, a NOAEL of 60 mg/kg bw/day was selected by the applicant.

Ref.: 14

Comment
The adjusted calculated NOAEL based on an exposure of 7 days a week is 43 ((60x5)/7) mg/kg bw/day.

3.3.5.3. Chronic (> 12 months) toxicity

No data submitted

3.3.6. Mutagenicity / Genotoxicity

3.3.6.1 Mutagenicity / Genotoxicity *in vitro*

Bacterial Reverse Mutation Assay

Guideline:	OECD 407 (1997)
Species/Strain:	*Salmonella typhimurium* strains TA98, TA100, TA102, TA1535 and TA1537
Replicates:	3 in one experiment
Test substance:	B87
Batch:	Batch: ANDC-012004
Purity:	98.3% (area%)
Vehicle:	DMSO
Concentration:	six concentrations in the range of 33 to 5000 µg/plate
Treatment:	direct plate incorporation assay
GLP:	in compliance
Study period:	June – September 2004

B87 was tested for its ability to induce gene mutation in bacteria both with and without phenobarbital and β-naphthoflavone induced rat liver S9-mix.

Result
The plates incubated with the test item showed normal background growth up to 5000 µg/plate with and without S9-mix in all strains used.
No toxic effects, evident as a reduction in the number of revertants, were observed with and without metabolic activation.
Increases in revertant colony numbers were observed following treatment with B87 in strain TA98 in the absence and presence of metabolic activation. The required threshold of twice the number of the corresponding solvent control was exceeded in the absence and presence of metabolic activation at 2500 µg/plate and above.
Appropriate reference mutagens were used as positive controls and showed a distinct increase of induced revertant colonies.

Conclusion
In conclusion, it can be stated that under the experimental conditions reported, the test item induced gene mutations by frameshifts in the genome of the strain TA98 in the absence and presence of metabolic activation.

Ref.:10

Comment
Only one experiment was performed, which is acceptable when a clear positive result is obtained.

In vitro Mammalian Cell Gene Mutation Test

Guideline:	476 (1997)
Species/strain:	mouse lymphoma cell line L5178Y
Replicates:	Two parallel cultures in 2 independent experiments
Test substance:	B87
Batch:	ANDC-012004
Purity:	98.3 area%
Vehicle:	DMSO
Concentrations:	Experiment I 4 h, with S9-mix: 175, 350, 700, 1400, 2800 µg/ml (= 10 mM) 4 h, without S9-mix: 175, 350, 700, 1400, 2100 µg/ml Experiment II 24 h, without S9-mix: 87.5, 175, 350, 700, 1050 µg/ml
Treatment:	4 h treatment ± S9-mix and 24 h treatment without S9-mix. 72 expression period
Control:	Appropriate positive and negative controls included
GLP:	In compliance
Study period:	July – December 2004

B87 was tested in the mouse lymphoma assay for the induction of gene mutation (and chromosomal aberration). The substance was dissolved in DMSO and tested up to the maximum tested concentration (10 mM) in a pre-test. The concentrations used for valuation in the main test were based on toxicity and precipitation.

Results
In the first experiment the test item induced strong toxic effects at precipitating concentrations of 2100 µg/ml with and 2800 µg/ml without metabolic activation indicated by a relative total growth (RTG) of 20.9 and 10.2 respectively.
In the second experiment, performed solely without metabolic activation, severe toxic effects were detected in both parallel cultures at 350 µg/ml and above. The data generated at 1050 µg/ml and above are not considered acceptable since both parameters of toxicity remained far below the threshold of 10% RTG.
No reproducible increase of the mutant frequency was observed in the main experiments with and without metabolic activation. Isolated minor increases of the mutant frequency exceeding the historical background growth occurred in the first experiment with metabolic activation and in the second experiment. However, the induction of mutant frequency (IMF) exceeded 126 x 10^{-6} (Global evaluation factor) only in one culture of both experiments. In the second experiment (24 h without S9), IMF was 160 x 10^{-6} at the highest evaluated concentration, which was highly toxic (RTG =0.3%). In the first experiment with S9, the IMF was 197 x 10^{-6} in one culture at a RTG of 20.9. In both experiments the increase was not clearly concentration related. The increase was mainly in small colonies indicating a clastogenic effect.
Appropriate reference mutagens were used as positive controls and showed a distinct increase in induced total mutant colonies and an increase of the relative quantity of small versus large colonies.

Conclusion
It was concluded by the applicant that in the study described and under the experimental conditions reported, B87 was not mutagenic in this mouse lymphoma assay at the *tk* locus.
Ref.: 11

In vitro Micronucleus Test

Guideline: OECD 473 / OECD 487 (draft)

Species/strain:	V79 cells
Replicates:	Two parallel cultures in one experiment
Test item:	B87
Batch:	ANDC-012004
Purity:	98.3%
Vehicle:	DMSO
Concentrations:	525, 700, 1050 (-S9-mix) 525, 700, 1050, 1400, 2100 (+S9-mix)
Performance:	4 h treatment and 20 h recovery both with and without S9-mix
Positive controls:	Colcemid (-S9-mix) and cyclophosphmide (+S9-mix)
GLP:	In compliance
Study period:	September 2004 – February 2005

No internationally accepted guideline was available at the time of the test. Treatment conditions as time of exposure and dose selection were performed according to the OECD guideline 473 "*In vitro* Mammalian Chromosome Aberration Test". Only one experiment was performed, since the test item was considered to be mutagenic after 4 hrs treatment. The concentrations chosen for evaluation were based on results form a pre-toxicity test.

Results
In the absence of S9-mix, no toxic effects indicated by reduced XTT activity (pre-experiment) or reduced cell numbers (main experiment) were observed after treatment with the test item. In the presence of S9-mix, clear cytotoxicity with XTT activity below 40% of control was observed after treatment with the test item.
In the absence of S9-mix, no biologically relevant increase in the percentage of micronucleated cells was observed after treatment with the test item. In the presence of S9-mix, statistically significant and biologically relevant increases in the number of micronucleated cells clearly exceeding the historical control data range were observed.
The positive control substances produced a distinct increase in the number of micronucleated cells, thus demonstrating the sensitivity of the test system used for the endpoints investigated in this study.

Conclusion
In the study described and under the experimental conditions reported, B87 induced an increase in V79 cells with micronuclei in the presence of metabolic activation. It is therefore concluded that B87 is clastogenic and/or aneugenic in mammalian cells *in vitro.*

Ref.: 12

3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

In vivo Mammalian Erythrocytes Micronucleus Test

Guideline:	474 (1997)
Species/strain:	NMRI BR mice
Group size:	5 males and 5 females/dose group
Test substance:	B87
Batch:	ANDC-012004
Purity:	98.3%
Vehicle:	Corn oil
Dose level:	0, 125, 250, 250 (2 groups) mg/kg bw
Dosing volume:	10 ml/kg bw
Route:	Intraperitoneal
Sampling:	24 and 48 h (only high dose level)
Control:	Corn oil (negative), cyclophosphamide
GLP:	In compliance
Study period:	December 2004 – January 2005

B87 suspended in corn oil was administered intraperitoneally in a single dose to mice. Bone marrow of femurs was prepared 24 (all dose levels) and 48 (only for the high dose level) hours after application of the test substance. For each animal at least 2,000 polychromatic erythrocytes (PCE) obtained from femoral bone marrow were examined. The frequency of micronuclei was calculated for each animal and dose group. As estimated by a pre-experiment 500 mg per kg bw was the highest applicable dose without significant effects on the survival rates, but with clear signs of toxicity.
Blood was sampled from additional animals (for control and high dose) to be able to demonstrate the exposure of the bone marrow to B87 in case the exposure could not be demonstrated by the appearance of discoloured urine, severe toxic effects, an altered polychromatic erythrocytes / normochromatic erythrocytes ratio or positive test results.

Results
The animals in the high dose group showed the following signs of toxicity: lethargy, ataxia, red coloured urine, rough coat and hunched posture. One male animal dosed with 250 mg/kg bw was lethargic after dosing. All animals dosed with 250 and 125 mg/kg bw had red coloured urine after dosing.
No increase in the mean frequency of micronucleated polychromatic erythrocytes was observed in the polychromatic erythrocytes of the bone marrow of animals treated with B87 compared to the vehicle treated animals. One male animal of the highest dose group showed a high incidence of micronucleated polychromatic erythrocytes. Since this high incidence was only observed in one animal of the highest dose group and was still within the laboratory historical control data range, it was considered not biologically relevant.
In the high dose group, sampled after 48 hours, a decrease in the ratio of polychromatic to normochromatic erythrocytes was observed, indicating toxic effect to the bone marrow and that B87 did reach the bone marrow. No decrease in the PCE/NCE ratio was observed in the other treatment groups.
The bio-availability of the applied test substance was further demonstrated by the dose related excretion of coloured test substance or its metabolites via urine. Therefore, the blood analysis was redundant and not performed.
The positive control substance caused cytotoxicity and produced micronuclei in polychromatic erythrocytes, thus demonstrating the sensitivity of the test system used for the endpoints investigated in this study.

Conclusion
From the results obtained in this study, it was concluded that B87 did not show any evidence of mutagenic potential in this *in vivo* test for chromosomal alterations when administered intraperitoneally to mice. Under the test conditions performed B87 is not an *in vivo* clastogen or aneugen.

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

Prenatal developmental study, dose range finding study

Guideline: OECD 414 (1981)

Species/strain:	Wistar/Han rat (Kfm:WIST, outbred SPF)
Group size:	5 females (mates)
Test substance:	Ro 1082
Batch:	3279/141
Purity:	> 98%
Vehicle:	4% aqueous carboxymethylcellulose
Dose levels:	0, 50, 150 and 450 mg/kg bw/day
Dose volume:	10 ml/kg bw
Route:	gavage
Administration:	Days 6-15 of pregnancy
GLP statement:	in compliance
Study period:	March-April 1989

In this dose range-finding embryotoxicity study, 20 female rats were dosed with the test substance at 0, 50, 150 and 450 mg/kg bw/day by gavage on days 6 to 15 after mating. The dams were observed at least twice daily for clinical signs. Body weights were recorded daily from day 0 until day 21 post coitum. Food consumption was recorded on days 6, 11, 16 and 21 *post coitum*. The females were killed on day 21 post coitum and the foetuses removed by caesarean section. Post mortem examination including gross macroscopic examination of all internal organs, with emphasis upon the uterus, uterine contents, position of foetuses in the uterus and number of corpora lutea, was performed.

Results
No deaths or abortions occurred at any dose level. No clinical signs were reported except ataxia observed 3 hours after the daily test substance administration in all dams at 450 mg/kg bw/day. Red coloration of the urine of all treated dams was observed from day 6 to 16 after mating. Food consumption and body weight gain were reduced initially in the group treated at the dose of 450 mg/kg bw/day and food consumption was also reduced in the group treated at the dose of 150 mg/kg bw/day. All other maternal parameters as well as any foetal parameter were not affected by administration of the test substance.
On the basis of the results obtained from this study, dose levels of 0, 50, 150 and 450 mg/kg bw/day were chosen for the main embryotoxicity study.

Ref.: 15

Taken from SCCNFP/0658/03

Guideline:	OECD 414 (1981)
Species/strain:	Wistar/Han rat (Kfm:WIST, outbred SPF)
Group size:	25 females (mates)
Test substance:	Ro 1082
Batch:	3279/141
Purity:	> 98%
Vehicle:	4% aqueous carboxymethylcellulose
Dose levels:	0, 50, 150 and 450 mg/kg bw/day
Dose volume:	10 ml/kg bw
Route:	gavage
Administration:	Days 6-15 of pregnancy
GLP statement:	in compliance
Study period:	March-April 1989

Groups of 25 female rats were dosed with the test substance at 0, 50, 150 and 450 mg/kg bw/day by gavage on days 6 to 15 after mating. The dams were observed twice daily for clinical signs, mortality and body weight. Food consumption was recorded on days 0, 6, 11, 16 and 21. The dams were sacrificed on day 21 of pregnancy, and examined for number of corpora lutea, number and distribution of live and dead foetuses, of early or late resorptions and of implantation sites, and for macroscopic observations. The foetuses were examined

for bodyweight, sex and macroscopic external observations, and for skeletal and visceral abnormalities (half for each end point).

Results
No deaths or abortions occurred at any dose level. No clinical signs were reported except for red coloration of the urine of all treated dams from day 6 to 16. Food consumption and body weight gain were reduced initially in the group treated at 450 mg/kg bw/day (day 6-11). There was a compensatory increase from days 16-21 after cessation of dosing. At autopsy, a number of animals from all dose groups were found to have white intestinal worms. No other abnormalities were observed. The mean numbers of corpora lutea, implantation sites, post-implantation loss, live foetuses and foetal body weights were similar for control and treated groups. A small number of foetal malformations were observed which were within the normal range and treated groups did not differ significantly from control.

The test substance elicited maternal toxicity at the highest dose level tested but was not embryotoxic or teratogenic. The NOAEL for maternal toxicity was considered to be 150 mg/kg bw/day. The NOAEL for the foetal organism was considered to be 450 mg/kg bw/day.

Ref.: 16

3.3.9. Toxicokinetics

No data submitted

3.3.10. Photo-induced toxicity

3.3.10.1. Phototoxicity / photoirritation and photosensitisation

No data submitted

3.3.10.2. Phototoxicity / photomutagenicity / photoclastogenicity

No data submitted

3.3.11. Human data

No data submitted

3.3.12. Special investigations

No data submitted

3.3.13. Safety evaluation (including calculation of the MoS)

Not applicable

3.3.14. Discussion

Physico-chemical properties
4-Amino-2-nitrodiphenylamine-2'-carboxylic acid is used as a direct dye in hair dye formulations or as an ingredient in oxidative dyeing products which may or may not contain a hydrogen peroxide based developer mix up to a final concentration of 2% on head.
According to the applicants the purity of the batches is between 96 and 98%. Taking into account the impurities, which could have been characterized, there are still about 2% of the

substance which have to be characterized. Though there are plenty of data on the purity of batches original data like spectra are missing. The stability in a typical hair dye formulation was not reported.

Toxicity
The LD_{50} of 4-Amino-2-nitrodiphenylamine-2'-carboxylic acid after oral administration was reported to be greater than 2000 mg/kg bw. The dermal LD_{50} was reported to be greater than 2000 mg/kg bw in both males and females.

A 13-week oral rat study showed a few signs of systemic toxicity up to a dose of 180 mg/kg bw/day (adjusted to 128 mg/kg bw/d based on a 7 days a week exposure), the maximum dose tested.
An increase in thrombocytes in the high dose group indicated that the NOAEL should be viewed as 60 mg/kg bw/day (adjusted to 43 mg/kg bw/d based on a 7 days a week exposure).

In a prenatal developmental study on rats, the test substance elicited maternal toxicity (changes in food consumption and body weight gain) at the highest dose level tested but was not embryotoxic or teratogenic. The NOAEL for maternal toxicity was considered to be 150 mg/kg bw/day. The NOAEL for the foetal organism was considered to be 450 mg/kg bw/day.
No study on reproductive toxicity was submitted.

Skin/eye irritation and sensitisation
The neat substance was not irritating to rabbit skin. It was severely irritant to the rabbit eye.
Based on the data available, a sensitising potential cannot be excluded.

Percutaneous absorption
The bioavailability of 4-Amino-2-nitrodiphenylamine-2'-carboxylic acid under non-oxidative conditions was 1.825 ± 0.096 µg/ cm^2 (0.416% ± 0.096%) and 1.846 ± 1.080 µg/cm^2 (0.509% ± 0.317%) under oxidative conditions.
For the calculation of the margin of safety under oxidative conditions, the mean + 2SD (4 µg/cm^2) will be used.

Mutagenicity/genotoxicity
4-Amino-2-nitrodiphenylamine-2'-carboxylic acid was tested for the three genetic endpoints: gene mutations, structural and chromosomal aberrations. 4-Amino-2-nitrodiphenylamine-2'-carboxylic acid induced frameshift mutations in the *Salmonella* strain TA98 both with and without metabolic activation. An increase in mutant frequency was not observed in a mouse lymphoma assay. In an *in vitro* micronucleus assay the substance induced a clastogenic and/or aneugenic effect. The clastogenic/aneugenic effect was not confirmed in an *in vivo* micronucleus assay.
The positive results found in the *in vitro* gene mutation assay in bacteria were not confirmed nor ruled out in an appropriate *in vivo* test on the same genotoxic endpoint. Consequently, a final conclusion on the genotoxic potential of 4-Amino-2-nitrodiphenylamine-2'-carboxylic acid cannot be drawn.

Carcinogenicity
No data submitted

4. CONCLUSION

The SCCS is of the opinion that a conclusion on the gene mutation potential of 4-Amino-2-nitrodiphenylamine-2'-carboxylic acid cannot be drawn without further testing.

A sensitising potential of 4-Amino-2-nitrodiphenylamine-2'-carboxylic acid cannot be excluded.

5. MINORITY OPINION

Not applicable

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