Scientific Committee on Consumer Safety

SCCS

OPINION ON

2,2'-Methylenebis-4-aminophenol HCl

COLIPA n° A155

The SCCS adopted this opinion at its 17th plenary meeting

of 11 December 2012

About the Scientific Committees

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

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

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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 2,2'-methylenebis-4-aminophenol dihydrochloride with the INCI name 2,2'-methylenebis-4-aminophenol HCl was submitted March 2003 by COLIPA[1] according to COLIPA.

The first scientific opinion (SCCP/1142/07) was adopted 16 December 2008 with the following conclusion:

"Because of the low margin of safety for the use in both oxidative and non-oxidative hair dye formulations the SCCP is of the opinion that 2,2'-methylenebis-4-aminophenol HCl as a hair dye ingredient up to a final on-head concentration of 2.0% in the presence or absence of a developer-mix, poses a risk to the health of the consumer.

In addition, a gene mutation potential of 2,2'-methylenebis-4-aminophenol HCl cannot be excluded "

In the current submission II the concentration is suggested to be reduced to 1% on-head concentration in both oxidative and non-oxidative hair dye formulations and a mammalian cell gene mutation test *in vitro* is provided.

2. TERMS OF REFERENCE

1. *Does SCCS consider 2,2'-methylenebis-4-aminophenol dihydrochloride safe for use in oxidative hair and non-oxidative hair dye formulations with a concentration on-head of maximum 1.0% taken into account the scientific data provided?*

2. *And/or does the SCCS have any scientific concern with regard to the use of 2,2'-methylenebis-4-aminophenol dihydrochloride in oxidative hair dye formulations?*

[1] COLIPA – The European Cosmetics Association

3. OPINION

3.1 Chemical and Physical Specifications

3.1.1 Chemical identity

3.1.1.1 Primary name and/or INCI name

2,2'-Methylenebis-4-aminophenol HCl (INCI)

3.1.1.2 Chemical names

Bis-(5-amino-2-hydroxyphenyl)-methane . 2 HCl

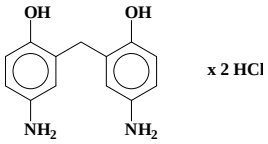
3.1.1.3 Trade names and abbreviations

Ro 1525 (SAT 980375, SAT 000344)
COLIPA n° A155

3.1.1.4 CAS / EC number

CAS: 27311-52-0 (dihydrochloride)
63969-46-0 (free base)
EC: 440-850-3 (Ro 1525)

3.1.1.5 Structural formula



3.1.1.6 Empirical formula

Formula: $C_{13}H_{14}N_2O_2$. 2 HCl

3.1.2 Physical form

Light grey powder

3.1.3 Molecular weight

Molecular weight: 230.27 (free base)

3.1.4 Purity, composition and substance codes

Batch: Ro-RN 6567-083 = SAT 980375 = SAT 000344= SAT 010232
Batch 703 = SAT 090013
Batch Ro-3962-88 = SAT 020874

Comparison of batches

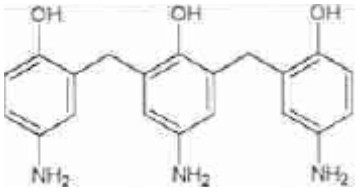
	Batch: Ro-RN 6567-083	Batch 703	Batch Ro-3962-88
Identification	NMR, LC-MS		
NMR content*	>97%	97.1%	97.2%
HPLC content, % Peak area, detection at 220 nm	99.8%	93.8	97.3
Water content, % w/w	-	1.4	0.6
Chloride content, % w/w	-	22.3	22.8
Impurities	See 3.1.5		

* The NMR content included also impurities, which were closely related to 2,2'-methylenebis-(aminophenol) (see 3.1.5), because the determination is done by the integration in the absorption range of aromatic protons.

3.1.5 Impurities / accompanying contaminants

In one experiment, 0.8% Ethanol and 0.5% acetone were determined as impurities in 2,2'-methylenebis-(aminophenol).
In one of the batches, 540 ppm *p*-aminophenol was found.

Several impurities of varying concentrations were observed in HPLC chromatograms of 2,2'-methylenebis-(aminophenol). By LC-MS, some of these (see below) were tentatively identified as shown below, but others could not be identified.



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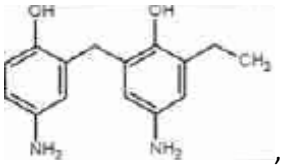

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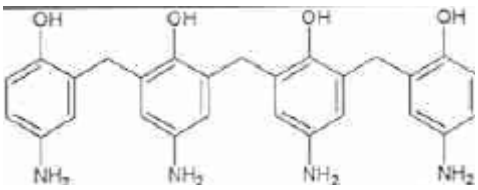

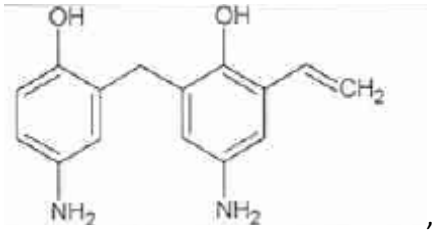

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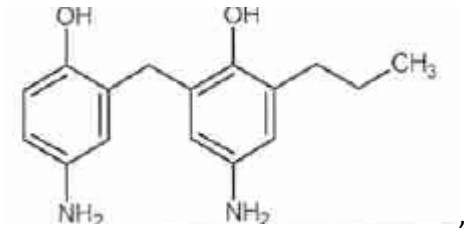

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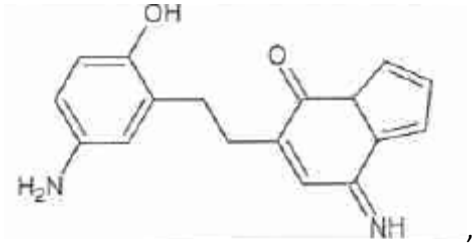

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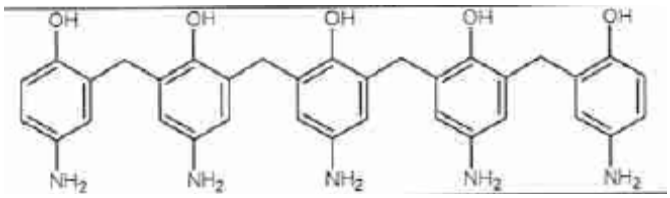

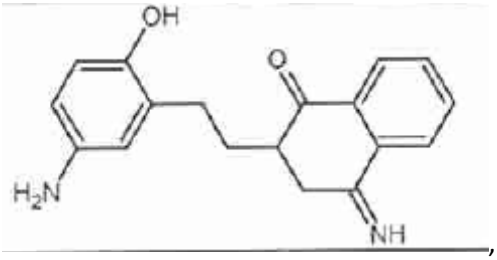

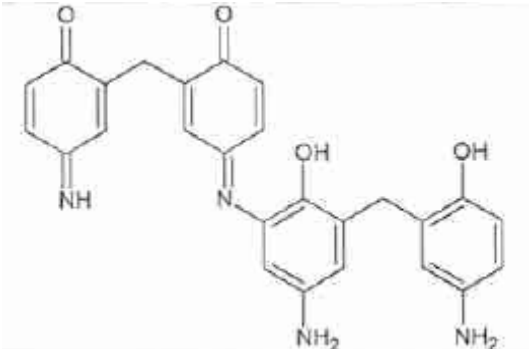

3.1.6 Solubility

Solubility			
in water	164.3 ±13 g/L	(at 20 °C after 24 h)	(Method EU A.6)
in ethanol	1 - 10 g/L	(at 22 °C after 24 h)	
in DMSO	100 - 200 g/L	(at 22 °C after 24 h)	

3.1.7 Partition coefficient (Log Pow)

Log P_{ow}: -3.01 (pH 3.6, 23°C) (Method EU A.8)

3.1.8 Additional physical and chemical specifications

Melting point:	Decomposition at >200 °C
Boiling point:	
Flash point:	
Vapour pressure:	
Density:	
Viscosity:	
pKa:	
Refractive index:	
pH:	
UV_Vis spectrum (200-800 nm):	Two different UV spectra are provided for the same batch of Ro1525: 1) Absorption peaks at 212 nm (λmax), 232nm and 300nm (in water) 2) 208nm (λmax), 277nm and 300 nm (in buffer pH 6.9)

3.1.9 Homogeneity and Stability

Stable at room temperature in the dark (no data). Solutions should be prepared fresh daily.

General Comments on physico-chemical characterisation

- No data is provided on the stability of methylenebis-4-aminophenol HCl in typical hair dye formulations.

3.2 Function and uses

2,2'-Methylenebis-4-aminophenol HCl (A 155) is used as an oxidative hair dye in cosmetic hair colouring products. It is used either with or without the addition of hydrogen peroxide.

The final concentration on head of A 155 is up to 1.0%.

3.3 Toxicological Evaluation

3.3.1 Acute toxicity

3.3.1.1 Acute oral toxicity

Guideline:	OECD 423 (1996)
Species/strain:	Sprague-Dawley rat
Group size:	3 males and 3 females
Test substance:	RO 1525
Batch:	Ro-Rn 6567-083
Purity:	not available
Dose:	0, 25, 200 and 2000 mg/kg bw; females 25 mg/kg bw only
Route:	Oral
Exposure:	single administration and 14 days observation
GLP:	in compliance
Study period:	12 May – 14 July 1998

Three female Sprague-Dawley rats were exposed to RO 1525 at the dose of 25 mg/kg. Nine male Sprague-Dawley rats were exposed to RO 1525 at the doses of 25, 200 and 2000 mg/kg, respectively. Aliquots of 20 ml/kg in water were administered orally by gavage. Animals were observed twice daily for mortality/morbidity and daily for clinical signs over a period of 14 days.

Results
At 2000 mg/kg all animals died within 1 hour after exposure. All animals exposed to 200 mg/kg died within 3 days. Sedation and unconsciousness were observed in both dose levels. Dyspnoea and tremor were observed in 200 mg/kg group. No mortality was observed at 25 mg/kg group, where clinical signs were piloerection, closed eyes, chromodacryorrhoea, pale skin, retention of faeces, discoloured urine and hunched posture. At necropsy exsiccosis, small spleen, gastric ulcers, gastric, intestinal and pulmonary haemorrhages, clear liquids in the thoracic cavity were noted in 200 mg/kg group. No differences between sexes were noted at 25 mg/kg.

Conclusions
The maximal non-lethal dose of RO 1525 was higher than 25 and lower than 200 mg/kg bw after a single oral administration in fasted rats.

Ref.: 1

3.3.1.2 Acute dermal toxicity

New study, submission II, 2010

Guideline:	OECD 402 (1987)
Species/strain:	rat, HanBrl: WIST (SPC)
Group size:	5 males and 5 females
Test substance:	Ro 1525

Batch:	Ro 3962/88
Purity:	98.4%
Vehicle:	purified water
Dose:	2000 mg/kg bw
Dosage volume:	4 mL/kg bw
Route:	dermal
GLP:	in compliance
Study period:	23 July – 13 August 2003

Five male and five female HanBRI:WIST rats were treated with Ro 1525 by dermal application during 24 hours at the dose of 2000 mg/kg bw.

Animals were examined at approximately 1, 2, 3 and 5 hours after treatment on day 1 and once daily during the next 14 days. Body weights were recorded on days 1, 8 and 15. All animals were necropsied and examined macroscopically.

No death and no clinical signs were observed during the study. Black discoloration was reported in 4 males and 2 females from test day 2 to 7 and persisted in 1 male and 2 females up to test day 8 and in one female up to test day 9. No changes in body weight and no macroscopic findings at necropsy were reported.

Conclusion
The median lethal dose of RO 1525 after single dermal administration to rats of both sexes observed over a period of 14 days is: LD50 (rat): greater than 2000 mg/kg bw.

Ref.: 10 (subm II)

3.3.1.3 Acute inhalation toxicity

No data submitted

3.3.2 Irritation and corrosivity

3.3.2.1 Skin irritation

Guideline:	OECD 404 (1992)
Species:	New Zealand White rabbits
Group:	3 females
Substance:	Ro 1525 (SAT 980375)
Batch:	Ro-Rn 6567-083
Purity:	99.8% (HPLC)
Dose:	0.5 g of Ro 1525
Vehicle:	/
GLP:	in compliance
Study period:	May 1998

Approximately 0.5 g of the test substance, spread over an area of approximately 6 cm² and moistened with 1.0 ml deionised water was applied semi-occlusive to the test site for 4 hours. The skin was examined at 1, 24, 48 and 72 hours after patch removal.

Results
No general toxic effects of the test substance were observed.
A grey staining of the skin at the application site was seen in all animals 1 and 24 h after patch removal. Except for very slight erythema, observed in 2/3 animals 1 h after patch removal, no lesions were noted at the other examination terms.

Time after patch removal	Erythema / Eschar Animal number			Oedema Animal number		
	21	22	23	21	22	23
1h	1	1	0	0	0	0
24h	0	0	0	0	0	0
48h	0	0	0	0	0	0
72h	0	0	0	0	0	0
Mean (24-72)	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**

Conclusion
The study authors concluded that the test substance "Ro 1525" did not cause any skin irritation or corrosion in this study.

Ref.: 2

Comment
The SCCP concluded that the neat test substance did cause mild and transient skin irritation.

3.3.2.2 Mucous membrane irritation

Guideline: OECD 405 (1987)
Species: New Zealand white rabbits
Group: 3 females
Substance: Ro1525
Batch: Ro-Rn 6567-083
Purity: 99.8% (HPLC)
Dose: 0.1 ml or 90 mg of undiluted test substance
Vehicle: /
GLP: in compliance
Study period: 12 May – 14 June 1998

The equivalent of 0.1 ml test substance was instilled into the conjuctival sac of one eye of each of 3 rabbits. The other eye remained untreated and served as negative control. The eyes were rinsed with warm water after 24h.

Results
The following mean scores were calculated from the individual examinations performed 24, 48 and 78h p.a.

	24	48	78
Cornea	0	1	0
Iris	0	0.33	0
Conjunctivae, redness	2	3	2.67
Conjunctivae, chemosis	2.33	4	2.67

Ocular lesions were reversible in all animals. A high inter-individual variation in the response to the test substance was seen.

Conclusion
The study authors concluded that the test substance was irritant to the rabbit eye.

Ref.: 4

HET-CAM

Guideline: /
Species: Chorionallantoic Membrane (HET-CAM)
Substance: Ro 1525

Batch: Ro-Rn 6567-083
Purity: > 98%
Concentration: neat substance
45% aqueous suspension (300 µl)
8% aqueous suspension (300 µl)
Vehicle: water
Reference: Texapon ASV (5% active substance)
GLP: in compliance
Study period: 22 – 31 March 1998

The eye irritation potential of Ro 1525 was assessed using the Hen´s-Egg-Test (HET-CAM). The undiluted as well as the diluted (45% and 8% aqueous suspension) test substance was applied on six fertilised chicken eggs per concentration. The test substance remained in contact with the CAM for 30 seconds. Then, it was rinsed off with physiological saline solution.

Results
The neat test substance caused slight to moderate haemorrhage and lysis of vessels after 30 seconds of exposure. The 45% dilution caused only slight haemorrhage and lysis of vessels.
To better estimate the irritant potential of Ro 1525, an 8% dilution was tested using the "reaction time method".

Test Substance	Relative irritation potential	Sum of individual scores		Conclusion
Ro 1525	0.07 (8% AS)	8 (98% AS)	3 (45% AS)	Moderately irritant
Reference	1.00	12		Moderately irritant

Conclusion
The study authors concluded that the test substance was moderately irritant.

Ref.: 3

Comment
The HET-CAM test is a screening test, meant to screen out severe eye irritants for the purpose of labelling or classification of chemicals, but has not been officially validated as a standalone replacement test for eye irritation. On its own it is not a suitable test for quantitative risk assessment of cosmetic ingredients. In this particular case it just confirms the previous in vivo results of a Draize eye test.

3.3.3 Skin sensitisation

Guinea Pig Maximisation Test

Guideline: OECD 406
Species: Ibm:GOHI; SPF-quality guinea pig (Himalayan spotted)
Group: 15 females (10 test and 5 control)
Test substance: Ro 1525
Batch: Ro-RN-6567-083
Purity: > 98% (HPLC)
Doses: 0.1 ml
Concentration: intradermal induction: 10% in purified water and in 1:1 (v/v) mixture of Freund's complete adjuvant
Epidermal induction: 25% in purified water
Epidermal challenge: 3% in purified water
Vehicle: purified water
GLP: in compliance
Study period: 17 July – 26 August 2002

In order to assess the cutaneous allergenic potential of Ro 1525, the Maximization-Test was performed in 15 (10 test and 5 control) female albino guinea pigs.
The intradermal induction of sensitization in the test group was performed in the nuchal region with a 10% dilution of the test item in purified water and in an emulsion of Freund's Complete Adjuvant (FCA) / physiological saline. The epidermal induction of sensitization was conducted for 48 hours under occlusion with the test item at 25% in purified water one week after the intradermal induction. The animals of the control group were intradermally induced with purified water and FCA/physiological saline and epidermally induced with purified water under occlusion.
Two weeks after epidermal induction the control and test animals were challenged by epidermal application of the test item at 3% in purified water and purified water alone under occlusive dressing. Cutaneous reactions were evaluated at 24 and 48 hours after removal of the dressing. To facilitate the reading by removing the black discoloration on the stratum corneum, the application area was stripped 4 to 5 times with Scotch Tape approximately 4 hours prior to the 24-hour reading.

Results
None of the control and test animals showed skin reactions after the challenge treatment with Ro 1525 at 3 % (w/w) in purified water.

Conclusion
The study authors concluded that the test substance was not a sensitiser.

Ref.: 5

Buehler Test

Guideline:	OECD 406 (1992)
Species:	Dunkin Hartley guinea pig, HsdPoc:DH
Group:	30 females (20 test, 10 control)
Test substance:	Ro 1525
Batch:	Ro-RN-6567-083
Purity:	/
Doses:	0.1 ml
Concentration:	1, 5, 25 and 50% Ro 1525 in white petrolatum (selection of test concentrations) Epicutaneous induction: 50% Ro 1525 in white petrolatum Epicutaneous challenge: 50% Ro 1525 in white petrolatum
Vehicle:	white petrolatum
Positive control:	α-hexylcinnamaldehyde
GLP:	in compliance
Study period:	12 May – 17 June 1998

Twenty female guinea pigs were used as a test substance group and another 10 females were used as a negative control group. There were three epicutaneous induction exposures and one epicutaneous challenge exposure. The concentration of the test substance was 50% in white petrolatum for all three induction exposures and for the challenge exposure. The areas of administration were covered occlusively for 6 hours.

Results
<u>General</u>
The test substance did not stain the skin of the guinea pigs.

<u>Skin reactions after induction exposures</u>
The application sites of all control animals were normal at each time. In the test substance group, very slight to well defined erythema and/or oedema were noted in 3/20 animals after the second and/or third induction exposure.

Skin reactions after challenge exposure
In the negative control group, the control areas and also the test substance treated areas of all animals were normal 24 and 48 hours after the end of the challenge exposure.
In the test substance group, a well-defined skin reaction was noted in 1/20 animals at the test substance treated area 24 hours and 48 hours after the end of the challenge exposure. Therefore one animal (5% of the test substance group animals) was regarded as sensitised.

Conclusion
The study authors concluded that the test substance was not a sensitiser.

Ref.: 6

Comment
As one animal was sensitised, the test substance should be considered as having skin sensitising potential.

3.3.4 Dermal / percutaneous absorption

New study, submission II, 2010

Guideline:	OECD 428 (2004)
Tissue:	dermatomed pig skin (0.79-0.80 mm), 4 pigs
Group size:	12 chambers per test run
Diffusion cells:	static diffusion cell
Skin integrity:	electrical resistance (> 4kΩ)
Test substance:	A155 (SAT 090013) [^{14}C]-A155, 0.095 mCi/mg, 29 mCi/mmol
Batch:	703 (non-labelled); CFQ11562 (labelled) CFQ 11562 (radiolabelled)
Purity:	> 99.9 area% (HPLC) 95 area% (HPLC) (radiochemical purity)
Test preparation 1:	A155 hair dye formulation containing 2% (w/w) A155 mixed 1:1 with developer *without* hydrogen peroxide
Test preparation 2:	A155 hair dye formulation containing 2% (w/w) A155 mixed 1:1 with developer *with* hydrogen peroxide
Doses:	20 mg/cm²
Receptor fluid:	phosphate buffered saline
Solubility receptor fluid:	"very high"
Method of Analysis:	liquid scintillation counting
GLP:	in compliance
Study period:	25 June – 5 August 2010

The dermal bioavailability of the test substance was investigated after open application of about 20 mg hair dye formulation per cm² pig skin. Skin discs of 3.14 cm² were exposed to the formulations for 30 minutes, and exposure was terminated by gently washing with a mild shampoo solution diluted with water (2% v/v).
The formulation was analyzed with twelve replicates for adsorbed, absorbed and penetrated amount of the test substance. The receptor fluid used was phosphate buffered saline (containing 0.9% sodium chloride).
In the static system, samples of the receptor fluid were drawn 0.5, 1, 2, 4, 6 and 24 hours after application. The removed volume was replaced by fresh receptor fluid.

Standard formulation with H_2O_2 (µg/cm²)

	Cell Number and Animal Number													
	Cell 13 *P011*	Cell 14 P011	Cell 15 P012	Cell 16 P012	Cell 17 P012	Cell 18 P010	Cell 19 P010	Cell 20 P010	Cell 21 P013	Cell 22 P013	Cell 23 P013	*Cell 24* *P010*	Mean	SD
Skin Wash 30min	*240.02*	222.31	243.53	256.13	207.85	180.10	205.94	173.74	192.09	186.11	175.81	*240.85*	204.36	28.63
Tissue Swab 30min	*20.22*	5.15	4.18	5.15	3.24	4.56	9.84	7.51	2.97	7.14	8.26	*5.15*	5.80	2.28
Pipette Tip 30min	*0.47*	0.08	0.11	0.51	0.25	0.23	0.72	0.12	0.13	0.50	0.14	*0.37*	0.28	0.22
Dislodgeable Dose 30min	*260.71*	227.54	247.82	261.79	211.34	184.90	216.50	181.38	195.18	193.75	184.21	*246.37*	210.44	23.01
Skin Wash 24h	*1.63*	2.07	1.42	1.06	1.28	0.96	0.86	1.12	1.02	0.93	0.65	*1.33*	1.14	0.39
Tissue Swab 24 h	*0.69*	2.67	0.73	0.62	0.78	0.77	0.52	0.35	0.44	0.36	0.46	*1.89*	0.77	0.69
Pipette Tip 24h	*0.02*	0.17	0.01	0.01	0.02	0.01	0.00	0.01	0.01	0.01	0.00	*0.03*	0.02	0.05
Donor Chamber Wash	*0.39*	1.01	0.08	0.35	0.20	0.10	0.17	0.18	0.10	0.14	0.15	*0.28*	0.25	0.28
Total Dislodgeable Dose	*263.44*	233.47	250.07	263.84	213.61	186.73	218.05	183.04	196.75	195.18	185.48	*249.91*	212.62	28.54
Unexposed Skin	*0.09*	0.06	0.07	0.03	0.01	0.02	0.03	0.05	*0.00	*0.00	*0.00	*0.04*	°0.03	°0.03
Total Unabsorbed	*263.53*	233.53	250.14	263.87	213.62	186.75	218.09	183.09	196.75	195.18	185.48	*249.95*	212.65	28.55
Stratum Corneum 1-5	*8.41*	2.34	1.61	4.19	2.41	3.83	6.36	3.21	0.96	3.13	0.99	*5.21*	2.90	1.64
Stratum Corneum 6-10	*1.53*	0.28	0.30	0.65	0.32	1.74	1.65	0.92	0.33	1.01	0.63	*1.70*	0.78	0.55
Stratum Corneum 11-15	*0.55*	0.14	0.11	0.41	0.19	0.69	0.71	0.58	0.47	0.45	0.20	*0.59*	0.39	0.23
Stratum Corneum 16-20	*0.27*	0.04	0.08	0.17	0.11	0.42	0.62	0.29	0.28	0.07	0.19	*0.34*	0.23	0.18
Dermal Adsorption (Total Stratum Corneum)	*10.75*	2.80	2.10	5.41	3.03	6.68	9.34	5.00	2.03	4.66	2.02	*7.84*	4.31	2.40
Dermal Absorption (Exposed Skin)	*1.54*	1.06	1.50	2.36	1.05	1.14	2.54	1.20	1.17	0.69	1.25	*1.28*	1.40	0.59
Receptor Fluid	**0.01*	**0.02	**0.00	**0.00	**0.01	**0.01	**0.01	**0.00	**0.01	**0.02	**0.01	**0.01*	°0.01	°0.01
Receptor Chamber Wash	**0.00*	**0.01	**0.00	**0.01	**0.00	**0.00	**0.00	**0.00	**0.00	**0.00	**0.00	**0.00*	°0.00	°0.00
Percutaneous Penetration	*0.01*	0.03	0.00	0.01	0.01	0.01	0.01	0.01	0.01	0.02	0.01	*0.01*	0.01	0.01
Dermal Bioavailability	*1.55*	1.08	1.50	2.37	1.06	1.15	2.56	1.21	1.19	0.71	1.26	*1.29*	1.41	0.59
Mass Balance	*275.83*	237.41	253.74	271.65	217.71	194.58	229.98	189.30	199.97	200.56	188.75	*259.08*	218.37	28.93

Cells 13 and 24 were rejected as data outwith 100±15%

Standard formulation without H_2O_2 (µg/cm²)

	Cell Number and Animal Number													
	Cell 1 P011	Cell 2 P011	Cell 3 P012	Cell 4 P012	Cell 5 P012	Cell 6 P010	Cell 7 P010	Cell 8 P010	Cell 9 P013	Cell 10 P013	Cell 11 P013	Cell 12 P010	Mean	SD
Skin Wash 30min	162.42	196.08	183.33	163.82	172.96	218.20	189.59	180.71	188.78	172.14	165.96	177.08	180.92	15.89
Tissue Swab 30min	23.10	17.24	3.98	19.81	9.36	6.87	7.97	7.50	3.15	16.94	17.33	4.49	11.48	6.94
Pipette Tip 30min	0.39	0.52	0.24	2.21	0.17	0.40	0.29	0.19	0.12	0.99	0.24	0.44	0.52	0.58
Dislodgeable Dose 30min	185.91	213.83	187.55	185.84	182.49	225.47	197.85	188.39	192.05	190.07	183.53	182.01	192.92	13.47
Skin Wash 24h	3.26	1.07	1.34	1.73	1.03	1.64	1.37	1.67	1.06	1.83	2.05	1.74	1.65	0.61
Tissue Swab 24 h	1.21	1.57	0.53	0.93	0.41	0.93	0.48	0.71	0.33	1.33	0.68	0.64	0.81	0.39
Pipette Tip 24h	0.08	0.03	0.01	0.01	0.00	0.02	0.01	0.02	0.09	0.02	0.02	0.12	0.04	0.04
Donor Chamber Wash	1.34	0.30	0.20	0.70	0.06	0.27	0.11	0.14	0.96	0.31	0.32	0.16	0.41	0.39
Total Dislodgeable Dose	191.79	216.81	189.62	189.21	183.99	228.33	199.82	190.93	194.50	193.56	186.59	184.66	195.82	13.46
Unexposed Skin	0.13	0.27	0.01	0.01	*0.01	*0.01	0.06	0.01	*0.00	*0.00	0.03	*0.01	°0.05	°0.08
Total Unabsorbed	*191.92*	*217.08*	*189.64*	*189.23*	*184.00*	*228.33*	*199.88*	*190.95*	194.50	193.56	186.62	184.67	195.86	13.49
Stratum Corneum 1-5	6.36	1.35	1.37	4.20	0.91	1.54	2.77	2.30	0.29	2.09	0.93	2.86	2.25	1.67
Stratum Corneum 6-10	2.87	1.33	0.34	0.58	0.27	0.88	1.65	1.22	0.22	0.92	0.70	1.84	1.07	0.77
Stratum Corneum 11-15	*1.22*	*0.50*	*0.11*	*0.40*	*0.11*	*0.99*	*0.63*	*0.66*	*0.10*	0.53	0.55	0.77	0.55	0.35
Stratum Corneum 16-20	0.49	0.13	0.15	0.15	0.10	0.76	0.38	0.41	0.12	0.30	0.26	0.43	0.31	0.20
Dermal Adsorption (Total Stratum Corneum)	10.94	3.31	1.97	5.33	1.39	4.18	5.44	4.60	0.73	3.84	2.44	5.90	4.17	2.70
Dermal Absorption (Exposed Skin)	2.22	0.63	1.03	1.50	1.23	2.55	1.81	2.90	2.45	3.02	2.25	1.66	1.94	0.75
Receptor Fluid	*0.01	*0.11	*0.00	*0.01	*0.01	*0.01	*0.02	*0.01	*0.01	*0.03	*0.02	*0.02	°0.02	°0.03
Receptor Chamber Wash	*0.01	*0.01	*0.01	*0.01	*0.02	*0.00	*0.02	*0.00	*0.00	*0.00	*0.00	*0.00	°0.01	°0.01
Percutaneous Penetration	0.01	0.11	0.01	0.01	0.03	0.01	0.04	0.01	0.01	0.03	0.02	0.03	0.03	0.03
Dermal Bioavailability	2.24	0.74	1.04	1.52	1.25	2.56	1.85	2.91	2.46	3.06	2.27	1.69	1.97	0.74
Mass Balance	*205.10*	*221.13*	*192.65*	*196.08*	*186.65*	235.08	207.17	198.46	197.69	200.46	191.33	192.25	202.00	13.81

Summary table

ANALYSED SAMPLE	Standard formulation **with** H_2O_2		Standard formulation **without** H_2O_2	
	[µg/cm²] ± SD	[% of dose] ± SD	[µg/cm²] ± SD	[% of dose] ± SD
Rinsings (total dislodgeable amount)	212.62 ± 28.54	97.77 ± 7.94	195.82 ± 13.46	91.86 □ 6.31
Adsorption (*stratum corneum*)	4.31 ± 2.40	1.95 ± 1.09	4.17 ± 2.70	1.96 ± 1.27
Absorption (epidermis/dermis)	1.40 ± 0.59	0.63 ± 0.27	1.94 ± 0.75	0.91 ± 0.35
Penetration (receptor fluid)	0.01 ± 0.01	0.01 ± 0.00	0.03 ± 0.03	0.01 ± 0.01
Bioavailable	1.41 ± 0.59	0.64 ± 0.27	1.97 ± 0.74	0.92 ± 0.35
Total recovery / mass balance	218.37 ± 28.93	100.37 ± 8.38	202.00 ± 13.81	94.76 ± 6.48

The amount of A 155 assumed as systemically available from standard hair dyeing formulations was 1.41 and 1.97 µg/cm² in the presence and absence of hydrogen peroxide respectively (0.64% and 0.92% of the applied amount) in this *in vitro* dermal penetration study.

Ref.: 12 (subm II)

Comment
This is a well performed study. The amount of A155 absorbed from a hair dye containing 1% A155 (on hair) *under oxidative conditions* was (mean + 1SD) 2.00 µg/cm² (0.91% of the applied dose); only 10 chambers were available. The amount of A155 absorbed from a hair dye containing 1% A155 (on hair) *under non-oxidative conditions* was (mean + 1SD) 2.71 µg/cm² (1.27% of the applied dose). These data may be used in calculating the MOS.

Guideline:	/
Tissue:	dermatomed pig skin, 2 donors (1 male and 1 female)
Group size:	6 replicates per experiment
Diffusion cells:	flow-through diffusion cell
Skin integrity:	transdermal electrical resistance (≥ 10 KΩ)
Test substance:	Ro 1525 ^{14}C-labelled Ro 1525 (95.3 µCi/mg)
Batch:	CFQ11562 (labelled)
Purity:	/ (non-labelled) 95.0% (radiochemical purity by HPLC)
Test item:	Formulation A: 1 part of the preparation (items 1 to 8) was mixed with 1 part of water for the experiments A1 and A2 Formulation B: 1 part of the preparation (items 1 to 8) was mixed with 1 part of developer for the experiments B1 and B2
Doses:	40 mg/cm² formulation (the actual dose was about 10% higher than the nominal dose of 40 mg/cm²)
Receptor fluid:	Dulbecco's phosphate buffered saline with 3% bovine serum albumin
Solubility receptor fluid:	/
Stability:	/
Method of Analysis:	liquid scintillation counter
GLP:	in compliance
Study period:	16 December 1999 – 18 February 2000

Composition of formulation

Item	Ingredient	Amount (mg)	Amount (%)
1	Ro 1525	27	3.6
2	^{14}C-labelled Ro 1525	3	0.4
3	Crème-Basis Bth 66	375	50.0
4	2-Amino-3-hydroxypyridine	10.8	1.44
5	Sodium sulphite	7.5	1
6	Ammonium sulphate	7.5	1
7	Ammonium, 25%	30	4
8	Water, dist.	289.2	38.56
Sum		750	100.00
9a	Water, for formulation A	750 µl	100
9b	Developer (containing H2O2), for formulation B	750 µl	100

The dermal absorption of Ro 1525 was studied as an ingredient of two representative formulations (A and B) on dermatomed skin preparations of two young pigs. For sensitivity

reasons and to ensure a maximum recovery (mass balance) the test substance was [14C]-labelled.

Two independent experiments were performed with each formulation using six integrity checked skin preparations in each experiment. The experiments were performed in flow through penetration cells with an application area of 0.5 cm². The non-occlusive exposure under temperature controlled conditions lasted 30 minutes before rinsing.

The test substance formulations were applied topically to the horny layer of the skin in quantities of 22.9 mg (A) and 22.2 mg (B) respectively, which corresponded to 469 μg (A) and 453 μg (B) of the test substance. 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 [14C]-amount in the fractionated receptor fluid consisting of phosphate buffered saline plus 3% bovine serum albumin. The overall amount of bioavailable test substance is defined as the sum of absorbed and penetrated quantities.

The means of test results are presented in the following table:

Parameter	Formulation A µg/cm²	Formulation A %	Formulation B µg/cm²	Formulation B %
Skin rinsings	/	89.5	/	85.2
Adsorption	31	3.3	24	2.6
Absorption	8.2	0.91	3.5	0.39
Penetration	1.0	0.11	0.085	0.009
Bioavailability	**9.2**	1.02	**3.6**	0.41
Mass balance	/	94.7	/	89.1

Individual results, experiment A1 (pig 1, female)

Skin sample	1	2	3	4	5	6	Mean (µg/cm²)
Absorption	19.40	6.17	3.39	12.89	10.69	12.62	
Penetration	0.29	0.20	2.51	0.71	1.32	3.68	
Bioavailability	**19.69**	**6.37**	**5.90**	**13.6**	**12.01**	**16.3**	**12.31 ± 5.45**

Individual results, experiment A2 (pig 2, male)

Skin sample	7	8	9	10	11	12	Mean (µg/cm²)
Absorption	12.15	0.26	13.36	0.98	0.59	5.44	
Penetration	0.42	0.20	0.07	0.69	1.69	0.62	
Bioavailability	**12.57**	**0.46**	**13.43**	**1.67**	**2.28**	**6.06**	**6.08 ± 5.68**

Individual results, experiment B1 (pig 1, female)

Skin sample	13	14	15	16	17	18	Mean (µg/cm²)
Absorption	8.92	6.39	5.07	0.13	1.06	7.82	
Penetration	0.12	0.04	0.05	0.06	0.39	0.06	
Bioavailability	**9.04**	**6.43**	**5.12**	**0.19**	**1.45**	**7.88**	**5.02 ± 3.53**

Individual results, experiment B2 (pig 2, male)

Skin sample	19	20	21	22	23	24	Mean (µg/cm²)
Absorption	1.91	2.28	1.07	1.14	3.85	2.02	

Penetration	0.02	0.01	0.11	0.05	0.10	0.01	
Bioavailability	**1.93**	**2.29**	**1.18**	**1.19**	**3.95**	**2.03**	**2.10 ± 1.02**

Conclusion
In the oxidative formulation, the amount considered absorbed was 3.56 (range 0.19 to 9.04) μg/cm² [0.41% of the applied dose]. Under non-oxidative conditions, the amount considered absorbed was 9.20 (range 0.46 to 19.69) μg/cm² [1.02% of the applied dose].
Ref.: 10

Comment
Too few replicates and an insufficient number of donors were used in this study. The amount of formulation applied (40 mg/cm²) was too high (normally 20 mg/cm²). An application area of only 0.5 cm² was used.
Under oxidative conditions, an A_{max} of 9.04 μg/cm² was obtained and under non-oxidative conditions the A_{max} was 19.69 μg/cm².

3.3.5 Repeated dose toxicity

3.3.5.1 Repeated Dose (28 days) oral toxicity

Guideline:	OECD 407
Species/strain:	Sprague-Dawley rat
Group size:	5 per sex
Test substance:	RO 1525
Batch:	Ro-Rn 6567-083
Purity:	> 98%
Dose:	0, 60, 80 and 100 mg/kg bw
Route:	gavage
Exposure:	once a day for 4 weeks
GLP:	in compliance
Study period:	21 April – 19 May 2000

The test substance, suspended in distilled water, was administered at 60, 80 and 100 mg/kg bw/day daily for 28 days by gavage. The control animals received vehicle only. All animals were observed twice daily for mortality and once daily for clinical signs. Blood samples were taken from all animals during week 4 for haematological and clinical chemistry investigations. At autopsy, organ weights were recorded and the main organs were examined macroscopically and histologically.

Results
No mortality occurred due to the test substance. There were neither treatment-related differences in body weights nor differences in haematology or clinical chemistry parameters between the exposed and control groups. The organs weight was comparable between exposed and control animals and no macroscopic changes of specific organs were observed. Brown pigmentation in the cell cytoplasm of proximal renal tubules and mild chronic interstitial inflammation, mainly localised in the cortical area, were observed in all treated animals of the high dose group. These lesions were seen, on most occasions, to be associated with tubular cell basophilia and tubular dilatation.
Moderate tubular cell basophilia associated with interstitial chronic inflammation was detected in males and females of the low and mid-dose group. In a number of males from the low-dose group, these findings were associated with vacuolation of cortical tubular cells. Brown pigmentation in the cell cytoplasm of the proximal renal tubules was again seen in animals of both sexes, particularly evident in males of the low and mid-dose groups.

Conclusions
Microscopic examination showed treatment-related changes in the kidney of animals from all treated groups (brown pigmentation in the cell cytoplasm of the proximal renal tubules)

The authors reported a significant background incidence of renal changes (tubular cell basophilia associated with interstitial chronic inflammation) also in the control animals - typical to this strain of rats - which limits the evaluation of the toxicological significance of findings.

Ref.: 11

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

Study 1

Guideline:	OECD 408
Species/strain:	Sprague-Dawley rat
Group size:	15 males and 15 females; additional 5 per sex in high dose satellite group
Test substance:	RO 1525
Batch:	Ro-Rn 6567-083
Purity:	> 98%
Dose:	0, 60, 80 and 100 mg/kg bw in water
Route:	by gavage
Exposure:	once a day for 13 weeks
GLP:	in compliance
Study period:	6 July – 6 November 2000

Three groups of 15 rats/sex received RO 1525 daily by oral gavage at doses of 0, 60, 80 and 100 mg/kg bw for 13 weeks. A control group received a vehicle only. Additionally 10 rats (5 per sex), for both the control and the high dose group, were assessed for recovery of treatment-related effects, four weeks after the last administration. Animals were observed twice daily for mortality/morbidity and once daily for clinical signs. Body weight and food consumption were determined before the exposure. Haematology, clinical chemistry and urinalysis evaluations were performed on week 13. Ophthalmologic evaluations of all animals in all groups were examined just prior to the exposure and re-examined during week 12 of treatment. All animals were subjected to a macroscopic examination and required tissues from animals in the control and exposed groups were evaluated microscopically.

Results
No deaths occurred during the course of the study. Detailed clinical signs with neurotoxicity assessment did not show any signs which could be clearly related to the treatment with the test item. Hyperaesthesia, tachypnoea and a slow arousal were occasionally noted. A slight statistically significant reduction in body weight gain was observed in the high dose females. However, this difference was no longer evident at the end of the recovery period. No findings attributed to treatment were seen at the ophthalmic examinations. A slight statistically significant decrease in haematocrit and haemoglobin were observed in the mid and high dose males on week 4. Mean corpuscular haemoglobin concentration was statistically significantly increased in high dose group. Several clinical chemistry and urinalysis parameters were affected in high dose animals. Statistically significant increase in relative kidney weight was seen in the mid and high dose males and in the high dose females. The change disappeared at the end of the recovery period. The histopathological evaluation of the kidneys in the control group and in all dose groups and in animals killed after the recovery period showed tubular degeneration. Tubular degeneration in the recovery female animals showed however a decreased incidence.

Conclusions
Renal effects in kidney are reported in the control group and at all dose levels investigated and therefore a NOAEL cannot be established.

Ref.:12

Study 2

Guideline:	OECD 408
Species/strain:	Wistar rat, Sprague-Dawley
Group size:	10 males and 10 females; additional 5 per sex in control and high dose satellite group (Wistar). Additional 5 male in control and high dose satellite groups (Sprague-Dawley)
Test substance:	RO 1525
Batch:	Ro-Rn 6567-083
Purity:	> 98%
Dose:	0, 5, 15 and 60 mg/kg bw in water
Route:	by gavage
Exposure:	once a day for 13 weeks
GLP:	in compliance
Study period:	2 April – 30 July 2001

Three groups of 10 rats/sex received RO 1525 daily by oral gavage at doses of 5, 15 and 60 mg/kg bw in distilled water for 13 weeks. An additional satellite group was set up to control and high dose groups by using five Sprague-Dawley rats/groups. Animals were observed twice daily for mortality/morbidity and once daily for clinical signs. Body weight gain and food consumption were recorded. Haematology, clinical chemistry and urinalysis evaluations were performed on week 13. Ophthalmologic evaluations of all animals in all groups were examined just prior to the exposure and re-examined during week 12 of treatment. All animals were subjected to a macroscopic examination and required tissues from animals in the control and exposed groups were evaluated microscopically.

Results
One female from the low dose and two males from the high dose groups were found dead during the study. In addition, 1 control female and 2 males from the mid dose group died due to technical problems during the bleeding procedure. Detailed clinical signs with neurotoxicity assessment did not show any signs which could be clearly related to the treatment with the test item. Body weight gain and food consumption were not affected. No findings attributed to treatment were seen at the ophthalmic examinations. Haematological parameters were not affected in any exposed groups. In clinical chemistry parameters a statistically significant increase in aspartate aminotransferase, total protein and albumin was observed in high dose males. In urinalysis parameters an increased number of epithelial cells and leukocytes were noted in all exposed groups compared to controls at the end of exposure. Statistically significant increase in relative kidney weight was seen in the high dose males (in main and satellite groups). Relative spleen weights were increased in high dose group. This change disappeared at the end of the recovery period. The histopathological evaluation of the kidneys in mid and high dose groups and in animals killed after the recovery period showed tubular degeneration and pigmentation.

Conclusion
On the basis of these results, the study authors considered the No Observed Effect Level (NOEL) to be 5 mg/kg bw per day.

Ref.: 13

Comment
In study 1 and study 2, two distinct, apparently unrelated, patterns of pathological changes were observed in the kidneys. There was a clearly spontaneous set of changes (nephropathy) seen in both control and treated Sprague Dawley rats (study 1), but not in any Wistar rat (study 2). The most important feature of this nephropathy was cast formation or thickened basement membrane and tubular basophilia. This type of spontaneous pathology has long been recognised in the Sprague Dawley rat (ref. AD1).

Based on the tubular degeneration in Wistar rats observed on the exposure level of 15 mg/kg bw /day but not at the level of 5 mg/kg/day, the NOAEL is set at 5 mg/kg/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 Test

Guideline: OECD 471
Species/strain: TA 1535, TA 1537, TA 98, TA 100 and TA 102
Replicates: Triplicates in two independent experiments
Test substance: Ro 1525
Solvent: Deionised water
Batch: Ro-Rn 6567-083
Purity: >98% by HPLC
Concentrations: 33, 100, 333, 1000, 2500, 5000 μg/plate
Treatment: Experiment 1: Standard plate incorporation assay
Experiment 2: Pre-incubation assay
Both assays with and without Phenobarbital/β-Naphthoflavone induced rat liver S9-mix
GLP: In compliance
Study period: June 1998

To evaluate the toxicity of the test article a pre-experiment was performed with strains TA 98 and TA 100. Eight concentrations between 3 and 5000 μg/plate were tested in triplicates. The plates showed normal background growth up to 5000 μg/plate in both strains.

Results
Toxic effects, evident as a reduction in the number of revertants occurred at the following concentrations:

Strain	Experiment I		Experiment II	
	without S9-mix	With S9-mix	without S9-mix	With S9-mix
TA 1535	5000	no toxic effect	1000-5000	no toxic effect
TA 1537	1000-5000	2500	1000-5000	no toxic effect
TA 98	no toxic effect	no toxic effect	2500-5000	no toxic effect
TA 100	5000	no toxic effect	5000	no toxic effect
TA 102	no toxic effect	no toxic effect	No toxic effect	no toxic effect

There were no signs of an increase in the number of revertants in any of the five tester strains at any concentration tested neither with nor without metabolic activation.

Conclusion
Under the test conditions used Ro 1525 did not induce gene mutations in bacteria.

Ref.: 7

***In vitro* chromosome aberration test**

Guideline:	OECD Guideline 473
Species/strain:	Chinese hamster cells V79
Replicates:	Two replicates in one experiment
Test substance:	Ro 1525
Solvent:	deionised water
Batch:	Ro-Rn 6567-083
Purity:	> 98% (HPLC)
Concentrations:	Without metabolic activation: 2.5, 3.0, 3.5 and 4.0 µg/ml With metabolic activation: 15, 30 and 60 µg/ml
Treatment:	Chromosomes were prepared 18 hours after start of treatment. The treatment interval was four hours with and without metabolic activation (S9-mix induced by Phenobarbital/β-Naphthoflavone)
GLP:	In compliance
Study period:	15 June – 25 August 1998

A preliminary toxicity test was conducted using eight concentrations between 39.1 and 5000 µg/ml. Reduced cell numbers below 50% of control were observed after treatment with 312.5 µg/ml and above in the presence of metabolic activation and at concentrations of ≥ 39.1 µg/ml in the absence of metabolic activation. Precipitation of the test article in culture medium was observed after treatment with ≥ 156.3 µg/ml in the absence of metabolic activation and ≥ 2500 µg/ml in the presence of metabolic activation. At each test concentration two parallel cultures were used. Appropriate negative and positive controls were included.

Results
Without metabolic activation concentrations between 1.4 and 40 µg/ml were tested and strong toxicity was observed. Therefore, another test was performed with concentrations between 1-4 µg/ml. Using these concentrations, neither mitotic indices nor cell numbers were reduced to values below 50% of the control. The test article induced concentration related and significant increases in the number of cells carrying structural chromatid and chromosome aberrations.
In the presence of metabolic activation, reduced mitotic indices below 50% of the control were observed. There were no indications of an increase in the number of cells carrying structural chromatid and chromosome aberrations at the tested concentrations.
There were no indications of increases of polyploidy metaphases neither in the absence nor in the presence of metabolic activation.

Conclusion
The conclusion is that under the test conditions used the test article Ro 1525 induced chromosome aberrations in the absence of metabolic activation and Ro 1525 is considered to be clastogenic in this *in vitro* assay.

Ref.: 8

New study, submission II, 2010

***In Vitro* Mammalian Cell Gene Mutation Test**

Guideline:	OECD 476 (1998)
Species/strain:	mouse lymphoma L5178Y cell line
Replicates:	duplicate cultures, three independent experiments
Test substance:	A155 (SAT 020874)
Batch:	Ro 3962/88
Purity:	98.4 area%
Concentrations:	Experiment I:

	without S9 mix:	3.0, 6.0, 12.0, 18.0 and 24.0 µg/ml
	with S9 mix:	3.0, 6.0, 12.0, 24.0 and 36.0 µg/ml
	Experiment IA:	
	with S9 mix:	6.0, 12.0, 24.0 and 36.0 µg/ml
	Experiment II:	
	without S9 mix:	1.6, 3.1, 6.3, 12.5 and 25.0 µg/ml
Positive control:	without S9-mix:	methyl methane sulfonate (MMS)
	With S9-mix:	cyclophosphamide (CPA)
Treatment:	Experiment I:	4 hours treatment with and without S9-mix
Experiment IA:	4 hours treatment with S9-mix	
	Experiment II:	24 hours treatment without S9-mix
GLP:	in compliance	
Study period:	29 June – 13 September 2004	

A preliminary toxicity test was performed with 8 concentrations from 24.2 to 3100 µg/ml (10mM) in the presence (4h treatment) and absence of metabolic activation (4h treatment). In all experiments, distinct toxic effects were observed at the lowest concentration of 24.2 µg/ml. The concentrations tested in the main experiment were based on the results in the main experiment. Six to seven concentrations were tested and 4-5 of these were evaluated for induction of mutations. In experiment I with metabolic activation the recommended range of 10-20% survival was not covered, therefore an additional experiment with metabolic activation was performed with an adjusted concentration range. Appropriate positive and negative controls were included in the experiments.

Results
In the first experiment the recommended toxicity was detected in both parallel cultures at 12.0 µg/ml and above in the absence and at 36.0 µg/ml in the presence of metabolic activation. In experiment IA relevant toxic effects occurred at 24.0 µg/ml and above. Although the toxic range of 10 – 20% of survival was not quite covered in both cultures of the repeat experiment IA, the data are considered acceptable. Even in the presence of very strong toxic affects below 10% of survival there was no indication of a reproducible increase of the mutation frequency.
In the second experiment severe toxic effects occurred at 12.5 µg/ml and above. At 25.0 µg/ml there was a 6.3 increase in mutant colonies in one of the two cultures. However, this result are not considered biological relevant due to excessive toxicity (below 10%).
No reproducible increase of the mutation frequency was observed in both main experiments with or without metabolic activation.
The reference mutagens used as positive controls showed a distinct increase in induced total mutant colonies and an increase of the relative quantity of small versus large colonies.

Conclusion
In conclusion, it can be stated that in the study described and under the experimental conditions reported, A 155 did not induce mutations at the mouse lymphoma thymidine kinase locus assay using the cell line L5178Y in the absence and presence of metabolic activation.

Ref.: 11 (subm II)

3.3.6.2 Mutagenicity / Genotoxicity *in vivo*

Mammalian Erythrocyte micronucleus test

Guideline:	OECD 474
Species/strain:	Mice: Crl: NMRI BR
Group size:	5 male and 5 female in each group
Test substance:	Ro 1525
Lot no:	Ro-Rn 6567-083
Purity:	Not reported

Dose level: 50, 100 and 150 mg/kg bw
Route: Oral gavage (only once)
Vehicle: Deionized water
Sacrifice times: 24 h and 48 h (highest dose only)
GLP: In compliance
Study period: 30 September – 15 November 1998

In a range-finding study, doses of 10, 50 and 200 mg test article/kg body weight were administered to two males and two females each. At the dose of 200 mg/kg bw one female was found dead after 2 hours. The rest of the animals survived 48 hours after administration. No cytotoxicity was observed in the bone marrow evaluation. Based on these findings the test doses were chosen for the main study.

For each slide the ratio of nucleated cells to erythrocytes was determined by counting at least 200 cells per slide (at least 400 cells per animal). The ratio of polychromatic to normochromatic erythrocytes was determined by counting at least 500 erythrocytes per slide (at least 1000 erythrocytes per animal). 2000 polychromatic erythrocytes per animal were counted (1000 per slide).

Results
5/15 females and 3/15 males of the highest dose groups (150 mg/kg bw) and of the high dosed spare group died within 48 hours after administration. Spare animals replaced all deceased animals of the high dose group. In the dose group (100 mg/kg bw) 1/5 females died after 24 hours. No mortality occurred in the low dose group (50 mg/kg bw). Thus, the females were slightly more sensitive to the effects of the test article regarding mortality.

There were no indications of increases in the amount of micronucleated polychromatic erythrocytes in the dosed groups compared to the control either 24 or 48 hours treatment time and neither for males nor for females. Among the males there were no differences in the PCE/NCE ratio between the tested doses and the control. Among the females, slightly lower ratios of PCE/NCE compared to the control at all three doses were observed, indicating mild cytotoxicity.

Conclusion
Under the test conditions used the test article Ro 1525 did not induce micronuclei in the bone marrow cells of mice in this *in vivo* assay.

Ref.: 9

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

Pilot study

The maternal and developmental toxicity of Ro 1525 were assessed in the rat during gestation.
Ro 1525 was administered daily by oral gavage to females from Day 6 through Day 15 of gestation at a dosage of 120 mg/kg/day. Control animals received the vehicle alone

(distilled water). The females were killed on gestation Day 20 and subjected to a post-mortem examination. The number of corpora lutea, weight of intact gravid Uterus, number and distribution of live foetuses, number and distribution of intra-uterine deaths, and individual foetal weight and sex were determined. All foetuses were examined externally. The only treatment related sign observed was a reduction in body weight, body weight gain and in corrected body weight in treated females. No foetal embryo-toxicity or teratogenicity was evident at this dose level. The data suggest that the high dosage for the main study could be 120 mg/kg/day.

Main study

Guideline:	OECD 414
Species/strain:	Sprague-Dawley rats
Group size:	mated rats in four groups (n= 25)
Test substance:	RO 1525
Batch:	Ro Rn 6567-083
Purity:	> 98%
Dose:	0, 60, 80 and 120 mg/kg bw/day
Route:	oral in distilled water
Exposure:	from day 6 through day 15 of gestation
GLP:	in compliance
Study period:	9 September – 2 October 2000

Three groups of mated rats received RO 1525 by oral gavage at doses of 60, 80 and 120 mg/kg bw day in distilled water from day 6 through day 15 of gestation. A control group of 25 mated rats received the vehicle only. The day of mating was designated as day 0 of gestation. Animals were observed twice daily for morbidity/mortality. Clinical signs were checked daily. Food consumption and body weight gain were recorded. On day 20 of gestation, the animals were killed and examined macroscopically and subjected to necropsy to determine several ovary and uterine related parameters. Foetuses were weighed, sexed and examined for possible external abnormalities.

Results
A total of four females proved not to be pregnant at necropsy (1 of the control, 1 of the high dose and 2 of the mid dose group). In addition, in the high dose group one animal showed total resorption at necropsy and four animals died between gestation Day 9 and 14. The treatment related clinical signs observed in animals of the high dose group were hunched posture, decreased activity and emaciation.
In the time period of gestation day 9 and 12, food consumption showed statistically significant reductions in the high dose group. There was a statistically significant reduction of body weight seen in animals of the high dose and mid dose groups starting from gestation day 9.
There were no treatment related macroscopic findings at necropsy of the adult females. Pre-implantation litter data did not show any dosage-related trends. No specific findings were seen in foetuses.

Conclusions
The NOAEL of materno-toxicity was 60 mg/kg bw day, while the NOAEL for embryo-toxicity/teratogenicity was 120 mg/kg bw.

Ref.: 14, 15

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)

CALCULATION OF THE MARGIN OF SAFETY

(2,2'-methylenebis-4-aminophenol HCl)

(*non-oxidative* conditions)

Absorption through the skin	**A**	**=**	**2.71 µg/cm²**
Skin Area surface	**SAS**	**=**	**580 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**1.57 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.026 mg/kg bw**
No observed adverse effect level (90-day, oral, rat)	**NOAEL**	**=**	**5 mg/kg bw**
50% bioavailability*		**=**	**2.5 mg/kg bw/d**
Margin of Safety	**NOAEL / SED**	**=**	**96**

(*oxidative* conditions)

Absorption through the skin	**A**	**=**	**2.00 µg/cm²**
Skin Area surface	**SAS**	**=**	**580 cm^2**
Dermal absorption per treatment	**SAS x A x 0.001**	**=**	**1.16 mg**
Typical body weight of human		**=**	**60 kg**
Systemic exposure dose (SED)	**SAS x A x 0.001/60**	**=**	**0.019 mg/kg bw**
No observed adverse effect level (90-day, oral, rat)	**NOAEL**	**=**	**5 mg/kg bw/d**
50% bioavailability*		**=**	**2.5 mg/kg bw/d**
Margin of Safety	**NOAEL / SED**	**=**	**132**

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

3.3.14 Discussion

Physico-chemical properties
2,2'-Methylenebis-4-aminophenol HCl is used as hair dye up to a final concentration of 1% on head in the presence or absence of a developer-mix.
No data is provided on the stability of 2,2'-methylenebis-4-aminophenol HCl in typical hair dye formulations.

General toxicity
The maximal non-lethal dose of 2,2'-methylenebis-4-aminophenol HCl was higher than 25 and lower than 200 mg/kg bw after a single oral administration in fasted rats. The LD50 was higher than 2000 mg/kg bw after single dermal administration. In the two 90-day studies in Wistar rats tubular degeneration in the kidneys at 15 mg/kg bw/day dose and above was observed. Therefore, the NOAEL was set at 5 mg/kg bw per day.
The NOAEL of materno-toxicity was 60 mg/kg bw day because of the reduced body weight gain, while the NOAEL for embryo-toxicity/teratogenicity was 120 mg/kg bw.

Irritation / sensitisation
The neat test substance did cause mild and transient skin irritation. It was irritant to the rabbit eye.
The test substance was not a sensitiser in a Guinea pig maximisation test. As one animal was sensitised in a Buehler test, the test substance should be regarded as a skin sensitiser.

Dermal absorption
In a well-performed study, the amount of 2,2'-methylenebis-4-aminophenol HCl absorbed from a hair dye containing 1% 2,2'-methylenebis-4-aminophenol HCl (on hair) *under oxidative conditions* was (mean + 1SD) 2.00 µg/cm² (0.91% of the applied dose). The amount of 2,2'-methylenebis-4-aminophenol HCl absorbed from a hair dye containing 1% 2,2'-methylenebis-4-aminophenol HCl (on hair) *under non-oxidative conditions* was (mean + 1SD) 2.71 µg/cm² (1.27% of the applied dose). These data was used in calculating the MOS.

Mutagenicity / genotoxicity
2,2'-Methylenebis-4-aminophenol HCl has been investigated for the three genetic endpoints: gene mutations, structural chromosomal aberrations and aneuploidy. 2,2'-methylenebis-4-aminophenol HCl did not induce gene mutations in bacteria or mammalian cells, but induced chromosomal aberrations in Chinese hamster V79 cells in the absence of metabolic activation. This clastogenic effect could not be confirmed in an *in vivo* micronucleus assay in the bone marrow of mice. 2,2'-methylenebis-4-aminophenol HCl did not induce mutations at the mouse lymphoma thymidine kinase locus assay using the cell line L5178Y in the absence and presence of metabolic activation.
It is therefore concluded that 2,2'-methylenebis-4-aminophenol HCl does not have genotoxic potential.

Carcinogenicity
No data submitted

4. CONCLUSION

The SCCS is of the opinion that the use of 2,2'-methylenbis 4-aminophenol HCl at a maximum on-head concentration of 1.0% in oxidative and in non-oxidative hair dye formulations does not pose a risk to the health of the consumer, apart from its sensitising potential.

5. MINORITY OPINION

Not applicable

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