Scientific Committee on Consumer Safety

SCCS

OPINION ON

Zinc oxide (nano form)

COLIPA S 76

The SCCS adopted this opinion at its 16th plenary meeting

of 18 September 2012

About the Scientific Committees

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

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

ACKNOWLEDGMENTS

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

Zinc oxide (CAS No. 1314-13-2; EC No. 215-222-5) has widespread use in cosmetic products with the following functions: bulking, skin protection and as a UV absorber, besides its authorized use in all cosmetics as a cosmetic colorant with the Colour Index No. CI 77947 in Annex IV.

Two former submissions on zinc oxide in pigmentary form as well as in the form of a nanomaterial were submitted in order to have zinc oxide approved as a UV-filter in cosmetic sunscreen products at a maximum level of 25%.

The first scientific opinion (SCCNFP/0649/03, final) on zinc oxide was adopted by the SCCNFP during its 24th plenary meeting of 24-25th June 2003 with the following conclusion: "*A considerable part of the investigations and their results submitted have been performed 15 or more years ago and consequently cannot fulfil modern requirements. However, there is a broad basic knowledge on Zn^{2+} and its compounds, e.g. ZnO.*

The physicochemical specifications of ZnO used in many of the studies are incomplete, the purity/impurities not specified. On the other hand, ZnO is practically insoluble in water. Thus, in general, ZnO may be considered as a non-toxic substance, including when used in cosmetic products.

The main concern of the present evaluation is related to the risk assessment of micronized (approximately 0.2 µm) ZnO, which may be coated by other compounds, and which is used as an ingredient in sunscreen formulations.

Micronized ZnO has been demonstrated to be photoclastogenic, possibly photo-aneugenic, and a photo-DNA damaging agent in mammalian cells cultured in vitro. The relevance of these findings needs to be clarified by appropriate investigations in vivo.

There is a lack of reliable data on the percutaneous absorption of micronized ZnO and the potential for absorption by inhalation has not been considered.

As to a safety assessment of a use of UV-filters by children over the age of 1 year, the SCCNFP issued a position statement (SCCNFP/0557/02)."

In the statement (SCCP/0932/05) on zinc oxide adopted by the SCCP on 20th September 2005, the request for a safety dossier was repeated for microfine zinc oxide.

In the general opinion (SCCP/1147/07) on the use of nanomaterials in cosmetics adopted by the SCCP on 18th December 2007 it was concluded that, "*A complete safety dossier on micron-sized and nano-sized ZnO was requested by the SCCNFP in its opinion on ZnO in 2003 (SCCNFP/0649/03). An opinion on the safety of such materials will be dependent on the availability of an adequate dossier.*"

Finally, a clarification (SCCP/1215/09) on the statement (SCCP/09328/05) on zinc oxide was adopted by the SCCP on 21st January 2009 with the clarification that:

"*The SCCP considers that on the basis of the dossier reviewed in 2003 the use of ZnO in its non-nano form (pigment grade, with particle sizes above 100 nm) is considered safe. The concern expressed in the SCCNFP opinion 0693/03 with regard to phototoxicity is not relevant for this form of ZnO due to the absence of dermal penetration.*"

According to COLIPA, the current submission III contains the information contained in the previous submissions I and II. Submission III also presents the new information that became available since the last submission and provides an overall safety assessment for this ingredient, which takes into account the entire available information.

2. TERMS OF REFERENCE

1. *Does the SCCS consider zinc oxide in its nano-form safe for use as a UV-filter with a concentration up to 25% in cosmetic products taking into account the scientific data provided?*

2. *Does the SCCS confirm that zinc oxide in its non-nano form is safe for use as a UV-filter with a concentration up to 25% as stated in the SCCP clarification (SCCP/1215/09)?*

3. *And/or does the SCCS have any further scientific concern with regard to the use of zinc oxide in cosmetic products?*

3. OPINION

3.1 Chemical and physical specifications

3.1.1 Chemical identity

3.1.1.1 Primary name and/or INCI name

Zinc oxide (INCI name)

3.1.1.2 Chemical names

Zinc oxide CI 77947; CI pigment white 4

Colipa No. S76

3.1.1.3 Trade names and abbreviations

Information has been provided on nano-sized ZnO raw materials (source materials as powder for sunscreen formulations) and some final cosmetic products containing some of the nano-sized ZnO raw materials. Information has also been provided on selected raw materials from various manufacturers, in the form of both coated and uncoated nanomaterials and products.

The following materials/products were included in the submitted dossier:

Examples of nano-sized ZnO raw materials (powder):

Z-COTE® (ZnO)	BASF SE
Nanox (ZnO)	Elementis
Nano TEC® 50 (ZnO)	Grillo Zinkoxid GmbH
Nano TEC® 60 (ZnO)	Grillo Zinkoxid GmbH
FINEX-50 (ZnO)	Sakai Chemical
MZ 30 (ZnO)	Tayca
Zinc Oxide Neutral (ZnO)	Symrise GmbH
Zano® 10 (former Zano®, ZnO)	Umicore

Z-COTE® HP1 (ZnO coated with triethoxycaprylylsilane)	BASF SE
Z-COTE® MAX (ZnO coated with dimethoxydiphenylsilanetriethoxycaprylylsilane cross-polymer)	BASF SE
Zinc Oxide NDM (ZnO coated with dimethicone)	Symrise GmbH
Zano® 10 Plus (former Zano® Plus, ZnO coated with octyl triethoxy silane)	Umicore

Examples of final cosmetic products with nano-sized ZnO raw materials:

W/O emulsion, uncoated ZnO (Sun Defense SPF 30/PA++, Ponds, 8.4% ZnO)	Unilever

W/O emulsion, coated ZnO (20% ZnO)	Umicore
O/W emulsion, coated ZnO (9% ZnO)	Umicore
O/W emulsion, coated ZnO (Perfect results SPF 15/PA++, Ponds, 2.2% (Z-COTE® HP)	Unilever
O/W emulsion, coated ZnO (all day moisture Lotion SPF 15, Olay, ZnO (Z-COTE® HP1)	Procter & Gamble

Comment

The data provided on the safety evaluation are mainly on uncoated ZnO. According to the information in submission II, the currently used coating materials are not UV absorbers and are common cosmetic ingredients that are widely used for different purposes in cosmetic products. The coatings consist of organic and inorganic materials such as silica, dimethicone, dimethoxydiphenylsilane and triethoxycaprylylsilane.

(References: 3, 4, 15, 16, 17, 18, 21, 24, 41, 51, 52, 84, 102, 103, 111, 112, 117)
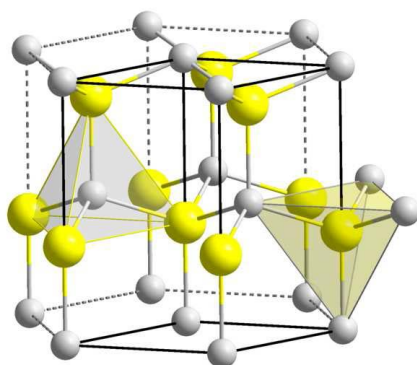
| **3.1.1.4** | **CAS/EC number** |

CAS No.: 1314-13-2

EC No.: 215-222-5

| **3.1.1.5** | **Structural formula** |

Formula: ZnO

Crystallinity: Wurtzite

| **3.1.1.6** | **Empirical formula** |

Formula: ZnO

| **3.1.2 Physical form** |

Solid, white odorless powder.

| **3.1.3 Molecular weight** |

Molecular weight: 81.38 g/mol.

3.1.4   Purity, composition and substance codes

The purity has been reported to be ≥99.0% of zinc oxide for the various nanomaterials included in the dossier.

(References: 10, 11, 51, 54, 55, 108, 112)

Comment

Two other references (52, 109) indicated for evidence of purity do not provide information on the purity of the nanomaterials.
A number of tests have been performed with a nano zinc oxide (Finex-50) with an indicated purity of ≥96%. However, in the description of the physico-chemical characteristics of the ZnO materials information on the purity has not been provided.

3.1.5   Impurities/accompanying contaminants

No information has been provided on the presence of impurities/contaminants.

Comment

As a purity of ≥99% was demonstrated in the references below, the impurities/contaminants are ≤1%.

(References: 10, 11, 51, 54, 55, 108, 112).

Two other references (52, 109) indicated do not provide information on the purity of the nanomaterials.

3.1.6   Solubility

Solubility was determined by mixing 10 grams of zinc oxide in 100 ml of demineralized water for 30 minutes at room temperature. After mixing, the suspension was centrifuged for 6 hours to separate the (undissolved) zinc oxide particles from the solution. After centrifugation the solutions were not completely clear optically, and a blue haze was noticeable. A sample of the solution was used to analyze the Zn content using Inductively Coupled Plasma (ICP) spectroscopy. Only uncoated preparations of zinc oxide were analyzed. The possible effect of coating on solubility was not determined.

The zinc concentration in the aqueous solution was:

Zinc oxide A: Zano 10 (uncoated, Umicore), batch: Z08009   42 mg/L

Zinc oxide B: NanoTec 50 (uncoated, Grillo), batch: MZ6362   20 mg/L

Zinc oxide C: Z-COTE (uncoated, BASF), batch: EHHG1407   47 mg/L

Zinc oxide D: FINEX-50 (uncoated, Sakai Chemicals, Japan), batch: 9Z22   27 mg/L

(Reference: 115)

The solubility of pigmentary, coated and uncoated ZnO was also determined in water and tissue culture medium. In these assays, atom absorption spectroscopy was used for the determination of the Zn content.

The following results were obtained:

Sample	Medium	pH	Solubility based on Zn concentration
ZnO (pigmentary, Fluka)	Aqua dest.	6.5–7	<0.1 mg/L

ZnO (pigmentary, Fluka)	DMEM/FCS**	About 7.4	35 mg/L
ZnO (uncoated, Z-COTE, BASF)	Aqua dest.	6.5–7	1.5 mg/L
ZnO (uncoated, Z-COTE, BASF)	M4 medium*	About 7.4	1.2 mg/L
ZnO (uncoated, Z-COTE, BASF	DMEM/FCS	About 7.4	37 mg/L
ZnO (coated, Z-COTE HP1, BASF)	M4 medium	About 7.4	2.4 mg/L

* M4 medium: reconstituted water containing salts and trace elements (water hardness: 2.5 mmol/L; alkalinity: 0.9 mmol/L).

** DMEM/FCS: Dulbecco/Vogt modified Eagle's minimal essential medium/fetal calf serum.

(References: 8, 9)

At low pH simulating stomach environment at pH 2.7, the solubility of particles ranged from 89.6% (particles >3 mm) to 98.5% (particles <1 mm).

(Reference: 57)

Comment

For the tests with uncoated ZnO (Reference: 115) some haze remained visible after centrifugation indicating that some non-dissolved ZnO particles remained in dispersion. The visible haze is very likely to be due to agglomerates/aggregates since dispersed nanoparticles would not be visible. These were also measured by ICP spectrometry. Thus, there is an overestimation of the solubility as determined by the method used. The data provided strongly suggest that equilibrium solubility had been reached, although it remains unclear if the solution was saturated, or if the particles remained undissolved because of the presence of some surface layer.

The data provided suggest that:

- the solubility of the nano-sized formulation in water is about 10× that of the pigmentary grade,
- in tissue culture medium the solubility of nano-sized and pigmentary grade is similar.
- the solubility of the coated and uncoated Z-COTE ZnO shows only a twofold difference, the coated ZnO being slightly more soluble. It should be noted that for the solubility of Z-COTE, two different solubility values (1.5 mg/L and 47 mg/L) have been provided and no explanation of this difference is given.

Additional information was provided on the effect of particle size on solubility in a simulated stomach environment (pH 2.7). Particles with a size <1 mm showed 98.5% dissolution. As the information on size was limited to <1 mm, no relevant information was provided specifically on nano-sized ZnO. This high solubility at low pH of both nano- and micron-sized ZnO particles was also recently demonstrated by Li et al. (Reference: AR16). In view of this, the SCCS considers that the solubility of ZnO in a stomach environment can be assumed to be high.

In conclusion, the data provided on solubility are inadequate to draw conclusions on the dissolution of ZnO nanoparticles. It is the view of the SCCS that the solubility data as presented are insufficient. Furthermore, to enable proper estimation of consumer exposure to any partially dissolved and/or insoluble fractions, the information for nanomaterials to be used as cosmetic ingredients should be provided not only on solubility, but also on the dissolution rate since equilibrium solubility is normally not achieved in the body (see SCCS Guidance on the Safety Assessment of Nanomaterials in Cosmetics SCCS/1484/12).

However, some additional information on solubility of ZnO nanoparticles in general is available in the open literature. The literature indicates that solubility of ZnO nanoparticles is in the same range as reported in the dossier (References: AR15, AR16, AR17, AR19, AR21). Some of the data indicate that the solubility of uncoated and coated ZnO (Reference: AR15), or ZnO either as nanoparticles or micron-sized particles, is similar and pH dependent (References: AR16, AR17). Also the solvent itself (water, tissue culture medium, presence of citric acid) influences the dissolution of ZnO nanoparticles (Reference: AR15, AR19, AR21). Reed et al. (Reference: AR21) compared dissolution rates between nano- and bulk-sized ZnO, and showed that the nano-form has a higher dissolution rate.

In view of the available information on the **solubility** of ZnO in aqueous media, it can be expected that very small concentrations of ZnO (e.g. <50 mg/L) will be in the form of a completely dissolved solution **under static equilibrium conditions**. This means that ZnO in a formulation will be simultaneously present as a mixture of solubilized Zn ions and a major part as undissolved ZnO particles - both with different diffusion and uptake characteristics.

The **dissolution rate**, also applicable to nanoparticles, describes the solubilization of ZnO nanoparticles in a certain fluid matrix over time, and will differ greatly depending on the fluid composition. In particular, the dissolution rate will depend critically on the fractional saturation of the matrix with Zn ions and the potential mechanisms for diffusion or active transport of Zn and O (and other) ions typical for biological processes. The dynamic nature of dissolution rate of any solubilization process implies that any mass fraction of Zn ions removed or washed away will lead to further dissolution and eventually complete solubilization of the particulate fraction of ZnO. Thus, **under non-static conditions**, e.g. in biological environments, ZnO nanoparticles will keep on releasing a certain mass fraction of Zn ions until they are completely solubilized.

(References: 8, 9, 57, 115)

3.1.7 Partition coefficient (Log P_{ow})

Log P_{ow}:   According to the applicant, measurement of the partition coefficient is not applicable as ZnO is practically insoluble in water.

Comment

This statement cannot be accepted in view of the partial solubility as presented in section 3.1.6.

Note: depending on the coating on the particles, a partitioning of particles between aqueous and octanol phases may also be expected.

3.1.8 Additional physical and chemical specifications

Melting point: 1,975°C

Boiling point: -

Flash point: -

Vapour pressure: -

Density: 5.47 g/cm^2

Viscosity: -

pKa: -

Refractive index: -

UV/vis spectrum (200–800 nm): -

(Reference: 87)

3.1.9   Particle size

The particle size distribution was provided for four uncoated ZnO nanoparticles designated A, B, C, D:

Zinc oxide A: Zano 10 (uncoated, Umicore), batch: Z08009

Zinc oxide B: NanoTec 50 (uncoated, Grillo), batch: MZ6362

Zinc oxide C: Z-COTE (uncoated, BASF), batch: EHHG1407

Zinc oxide D: FINEX-50 (uncoated, Sakai Chemicals, Japan), batch: 9Z22

To determine particle size distribution, 30 w% zinc oxide dispersions were prepared by adding zinc oxide to a mixture of water and dispersing agent. The dispersion was composed of ZnO (150 g), Tego Dispers 752W1 (30 g) and water (320 g). The dispersion was premixed in a dissolver and transferred to Dispermat SL-12 bead mill thereafter. The bead mill was operated using 0.3 mm YTZ beads at a filling ratio of 85% and a rotor speed of 4,500 rpm. The zinc oxide suspensions were circulated through the mill over a period of 60 minutes. After bead milling, a sample of the suspension was collected for particle size analysis.

The particle size distribution was measured using a CPS Disc Centrifuge (model DC20000)2, operated at a constant rotation of 10.000 rpm. 0.1 g of the 30 w% suspension was diluted in 30 ml water, after which 0.15 ml of the dilution was injected into a rotating disc and particle size measurements were recorded. The results are expressed as weight average and number average particle size.

The applicant claims that the particle size distribution (PSD) determination in aqueous environments can only be done for uncoated ZnO and considers it not relevant to determine the PSD for coated ZnO. It is argued in the submission that the coating would not change the particle size of the ZnO particle. This is supported by electron microscopy images where no differences in particle size and morphology can be identified between coated and uncoated materials. The applicant recognized that in cosmetic formulations the particle size distribution of coated and uncoated ZnO may be of interest, but the methodology will only provide information very close to the primary particle size. It is nevertheless unlikely that normal coatings will bring any significant change to particle size distribution of a nanomaterial.

Average particle size distribution based on weight measured using a CPS Disc Centrifuge is shown below.
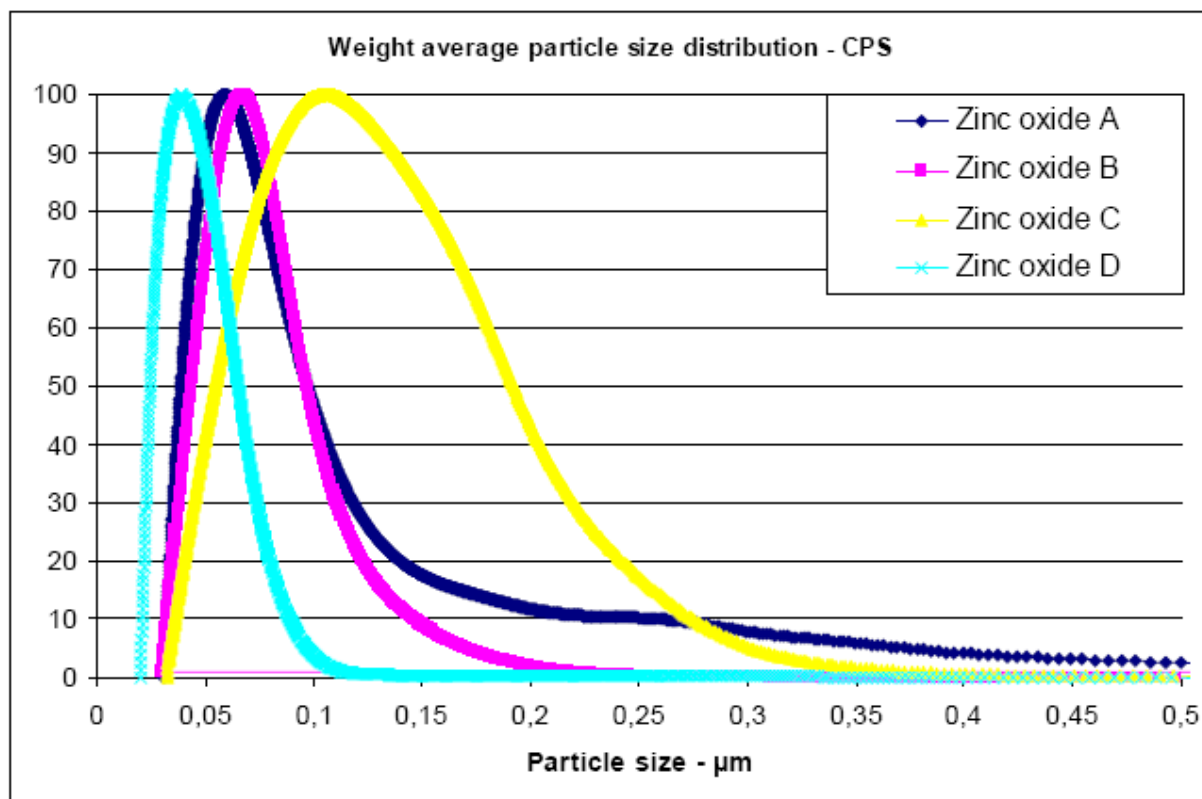
The corresponding percentile values are as follows for particle size distribution based on weight.

	ZnO A	ZnO B	ZnO C	ZnO D
d1	0.034 μm	0.034 μm	0.038 μm	0.022 μm
d10	0.042 μm	0.043 μm	0.055 μm	0.027 μm
d25	0.051 μm	0.053 μm	0.074 μm	0.032 μm
d50	0.068 μm	0.067 μm	0.105 μm	0.041 μm
d75	0.098 μm	0.085 μm	0.148 μm	0.054 μm
d90	0.187 μm	0.109 μm	0.198 μm	0.067 μm

Average particle size distribution based on number of particles measured using a CPS Disc Centrifuge is shown below.
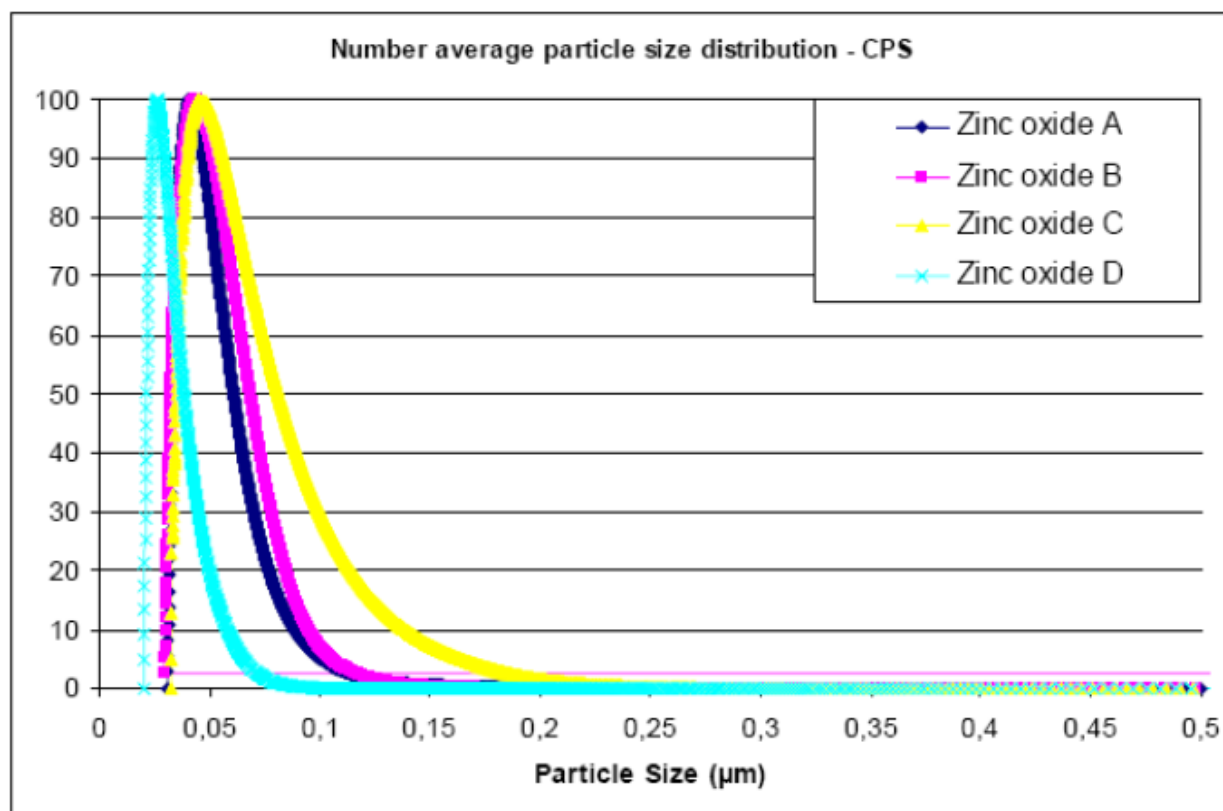
The corresponding percentile values are as follows for particle size distribution based on number of particles.

	ZnO A	ZnO B	ZnO C	ZnO D
d1	0.032 μm	0.032 μm	0.034 μm	0.021 μm
d10	0.035 μm	0.035 μm	0.038 μm	0.023 μm
d25	0.039 μm	0.040 μm	0.044 μm	0.025 μm
d50	0.046 μm	0.048 μm	0.055 μm	0.030 μm
d75	0.057 μm	0.06 μm	0.074 μm	0.036 μm
d90	0.070 μm	0.073 μm	0.098 μm	0.045 μm

Comment

For all ZnO preparations, the number distribution results show that 90% of the particles have a size below 100 nm, whereas for the weight distribution the percentage of particles with a size below 100 nm varies (ZnO A d75, ZnO B d75, ZnO C d25, ZnO D d90).

The median particle size distribution (the D50 indicates that 50% of the particles have a size below that measurement) of the four evaluated ZnO nanomaterials ranged from 30 nm (ZnO D) to 55 nm (ZnO C).

Additional parameters of the ZnO nanomaterials such as number of particles per mass unit, and surface area per mass unit were not provided.

(Reference: 114)

3.1.10 Microscopy

The ZnO nanoparticle distribution of the formulations was investigated with two different electron microscopy techniques: Cryo-SEM and ambient TEM. Several examples of both EM and TEM evaluations are presented.

3.1.10.1 TEM evaluations of uncoated ZnO

A) Z-COTE®, uncoated ZnO (BASF, left image, TEM, Reference: 13)

ZnO particles have different shapes (rod-like and isometric) and a broad size distribution range of 30–200 nm. The ZnO particles were generally present as clusters.

B) Z-COTE® HP1, coated ZnO (BASF, right image, TEM, Reference: 13)

ZnO nanoparticles have different shapes (rod-like and isometric) and a broad size distribution range of 30–200 nm. The ZnO particles were generally present as clusters.
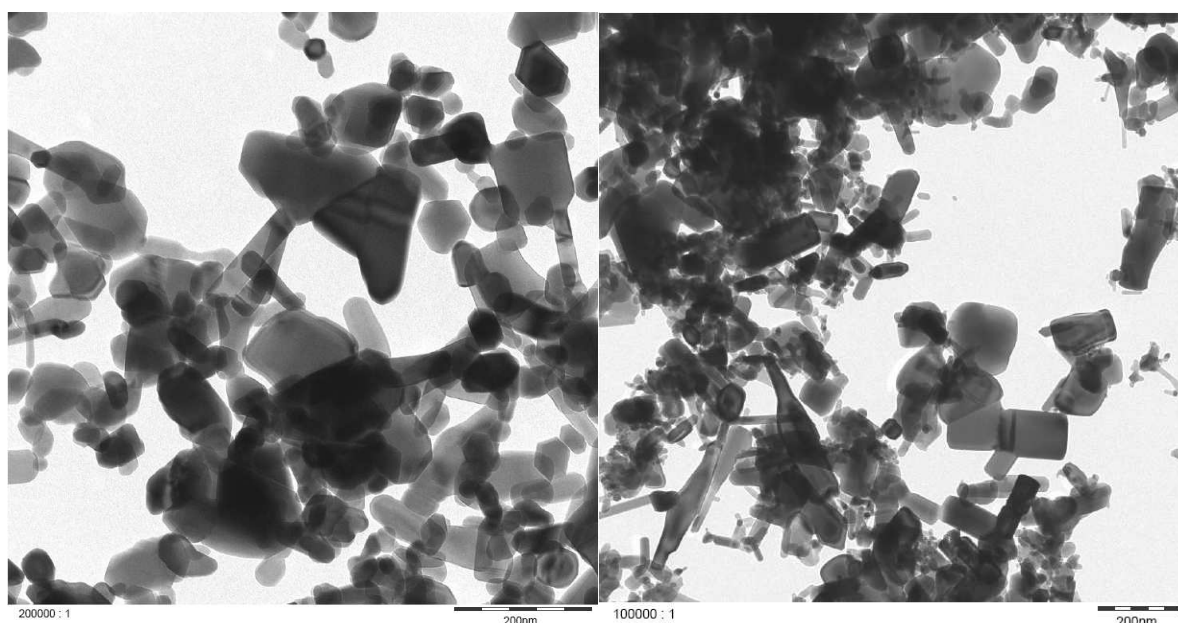
Comment

Both uncoated and coated Z-COTE® ZnO particles show similar morphology in the TEM images.

3.1.10.2 TEM evaluation of ZnO in formulations

A) W/O emulsion, coated ZnO (20% ZnO, Umicore, left image, TEM, Reference: 18)

ZnO nanoparticles have different shapes (rod-like, star-like and isometric) and a broad size distribution range of 30–150 nm. The ZnO particles were present mainly as clusters.

B) O/W emulsion, coated ZnO (9% ZnO, Umicore, right image, TEM, Reference: 25)

ZnO nanoparticles have different shapes (rod-like, star-like and isometric) and a broad size distribution range of 30–150 nm. The ZnO particles were present mainly as clusters. In addition, spherical particles with a size between 200 nm and 1 µm were present.
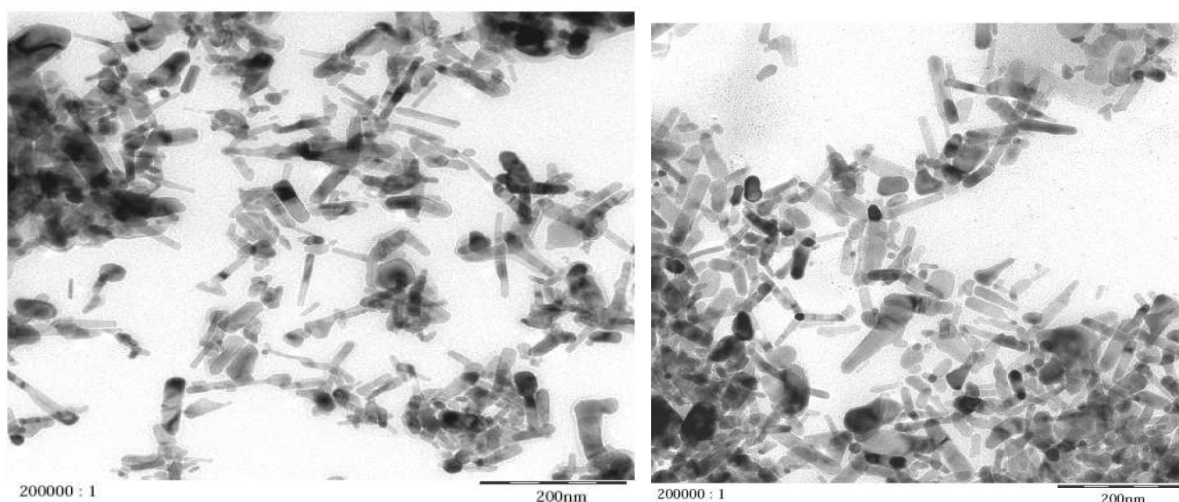
Comment

The TEM image of O/W emulsion (right image, above) as presented in the dossier could not be found in the cited reference (Reference: 24) but it is contained in Reference: 25. There is a similar discrepancy in Reference: 24 which refers to SEM images, whereas the image presented above shows TEM results.

The various formulations presented all showed the presence of ZnO particulates mainly as clusters, with size ranges as indicated for the raw materials. These clusters may be present in the original formulation, but it is also possible that it is due to the excessive load of the EM sample. Z-COTE® and Z-COTE®HP1 with a size of 30–200 nm show clusters with rod-like, star-like and isometric forms. Umicore ZnO with a size of 30–150 nm also shows clusters with rod-like, star-like and isometric forms. Similar results are shown for both O/W and W/O emulsions.

The star-like forms present in the formulations are not observed in the raw material for Z-COTE®. Both ZnO nanomaterials contain rod-shaped particles for which the Z-COTE® ZnO nanoparticles show more irregular shapes compared to the Umicore ZnO nanomaterial.

(References: 13, 18, 25)

3.1.10.3	SEM evaluation of uncoated ZnO nanomaterials

A) Zano® 10 (uncoated, Umicore, left image, Reference: 110)

B) Nano Tec® 50 (uncoated, Grillo, right image, Reference: 58)
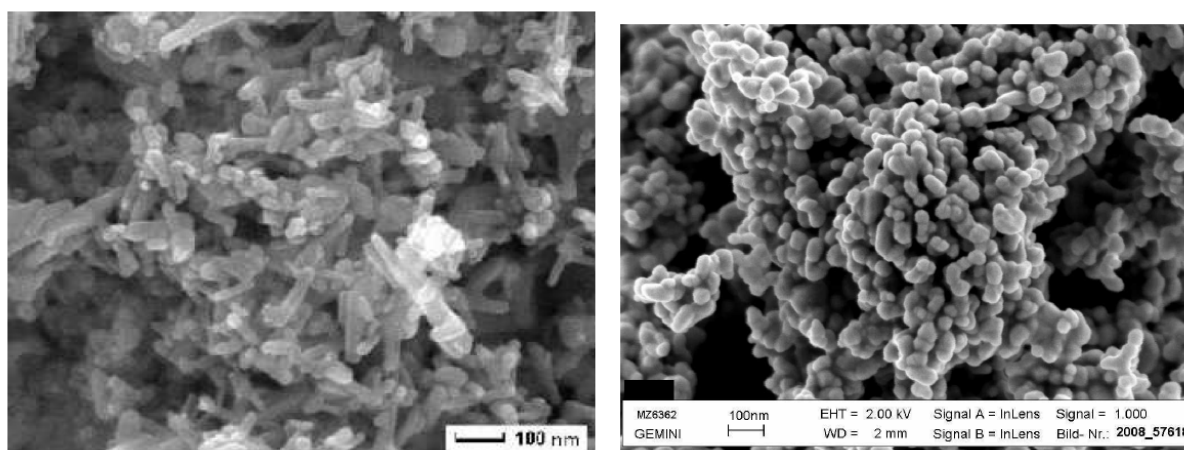
C) Nano Tec® 50 (uncoated, Grillo, Reference: 109)
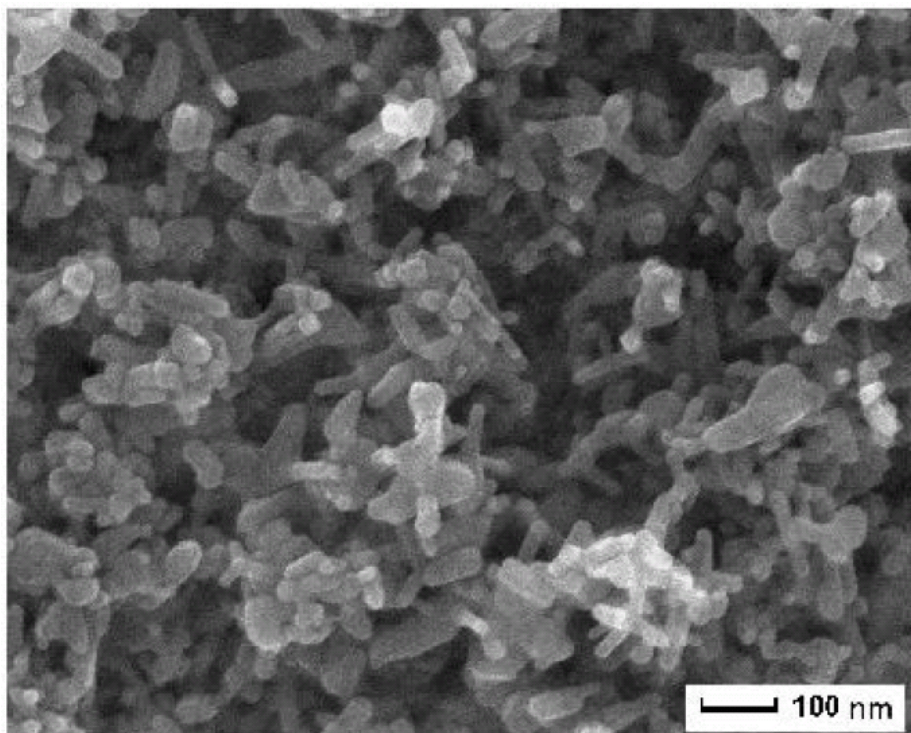
Comment

The SEM images of the three examples presented show similar morphology of the uncoated Nano Tec® 50 and Zano® 10 nanomaterials.

SEM images of ZnO nanomaterial in various formulations were included in the dossier submitted by the applicant. As these are relevant for final cosmetic products they have been considered in this opinion as additional background information but have not been further evaluated.

(References: 58, 109, 110)

3.1.11 Crystal structures

X-ray diffraction (XRD) analysis was used for determination of the crystal structure. Rietveld refinement characterization was used for analysis of crystallite size.

An example of the XRD spectrum is presented below.
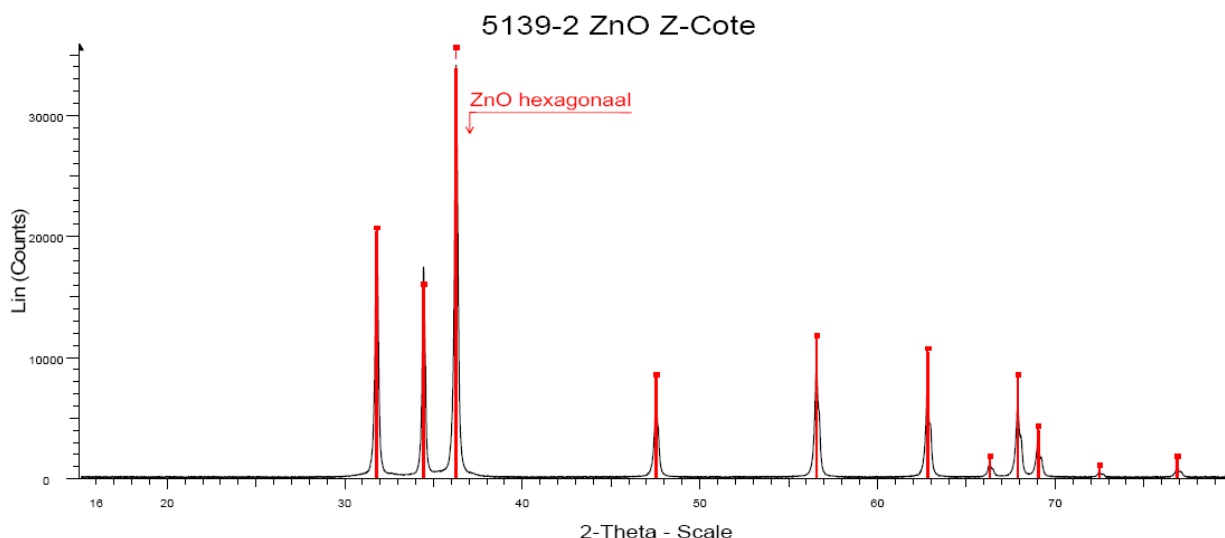
The crystal structure for all samples was identified as purely hexagonal zinc oxide. For zinc oxide this is often referred to as a "Wurtzite" structure (see also section 3.1.1.5). The crystallite sizes determined via Rietveld refinement characterization were as follows:

Zinc oxide A: Zano 10 (Umicore), batch: Z08009   48 nm

Zinc oxide B: NanoTec 50 (Grillo), batch: MZ6362   36 nm

Zinc oxide C: Z-COTE (BASF), batch: EHHG1407   67 nm

Zinc oxide D: FINEX-50 (Sakai Chemicals, Japan), batch: 9Z22 17 nm

(References: 53, 116)

3.1.12 UV absorption

UV absorption was determined for four uncoated ZnO nanomaterials. A suspension of zinc oxide samples was prepared using a mild bead mill operation to break up agglomerates and stabilize the individual particles as much as possible. These dispersions were then used to measure the UV/vis absorbance and reflection spectra. The 30 w% dispersions were diluted to a concentration of 0.1 g/L ZnO and the UV/vis absorption spectrum was measured using a Genesys 6 UV/vis spectrometer.

The following ZnO preparations were analysed:

Zinc oxide A: Zano 10 (uncoated, Umicore), batch: Z08009

Zinc oxide B: NanoTec 50 (uncoated, Grillo), batch: MZ6362

Zinc oxide C: Z-COTE (uncoated, BASF), batch: EHHG1407

Zinc oxide D: FINEX-50 (uncoated, Sakai Chemicals, Japan), batch: 9Z22
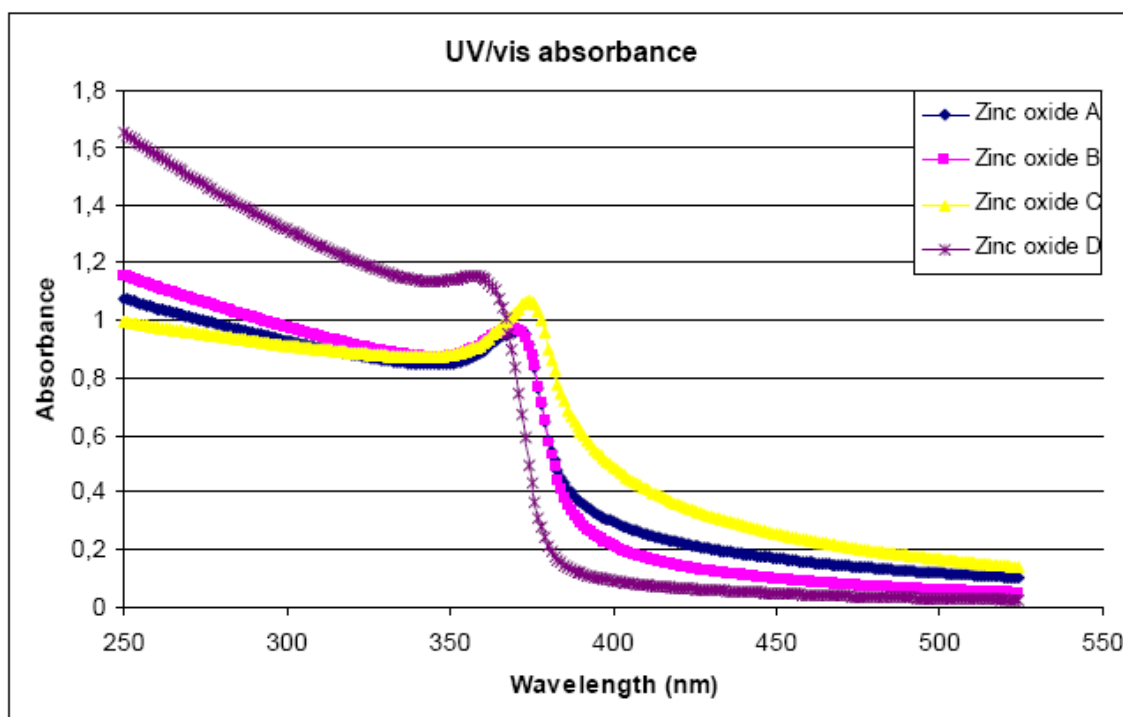
The optical properties in the UV/vis region from approximately 200 to 900 nm were also determined by generating absorption and reflection spectra using the so-called integrating sphere.
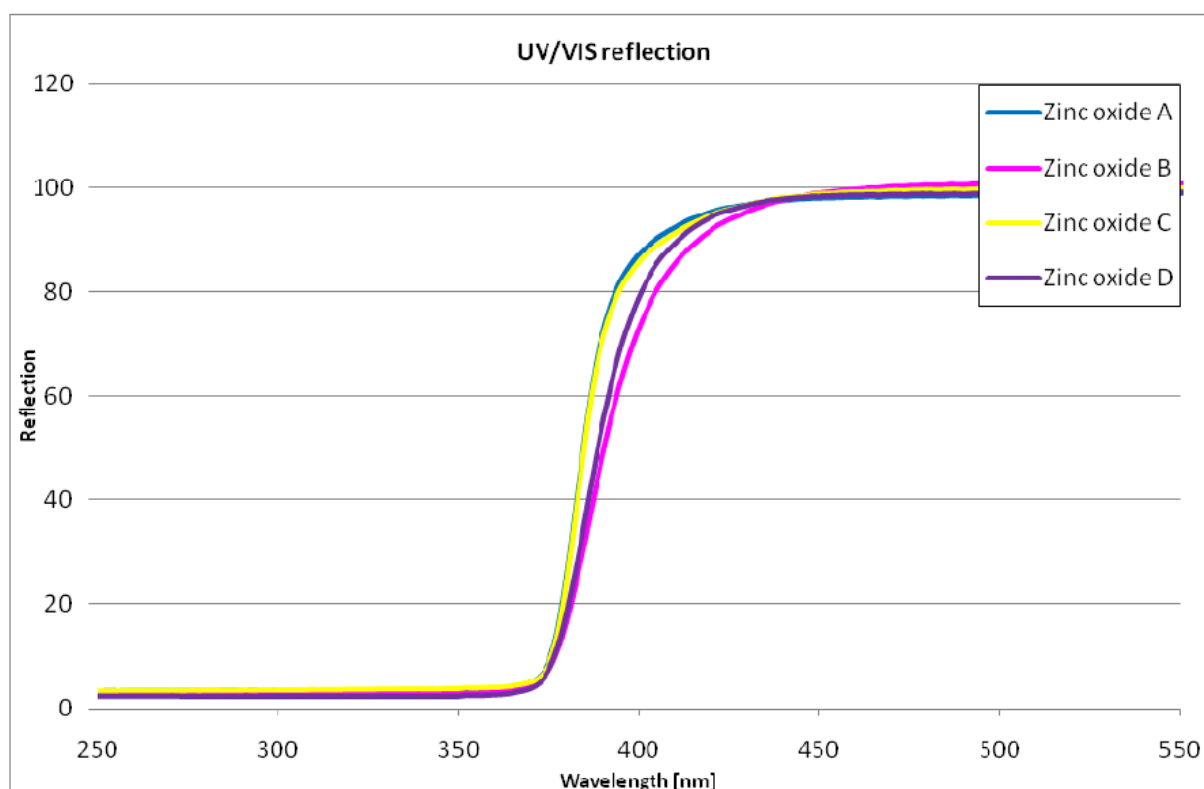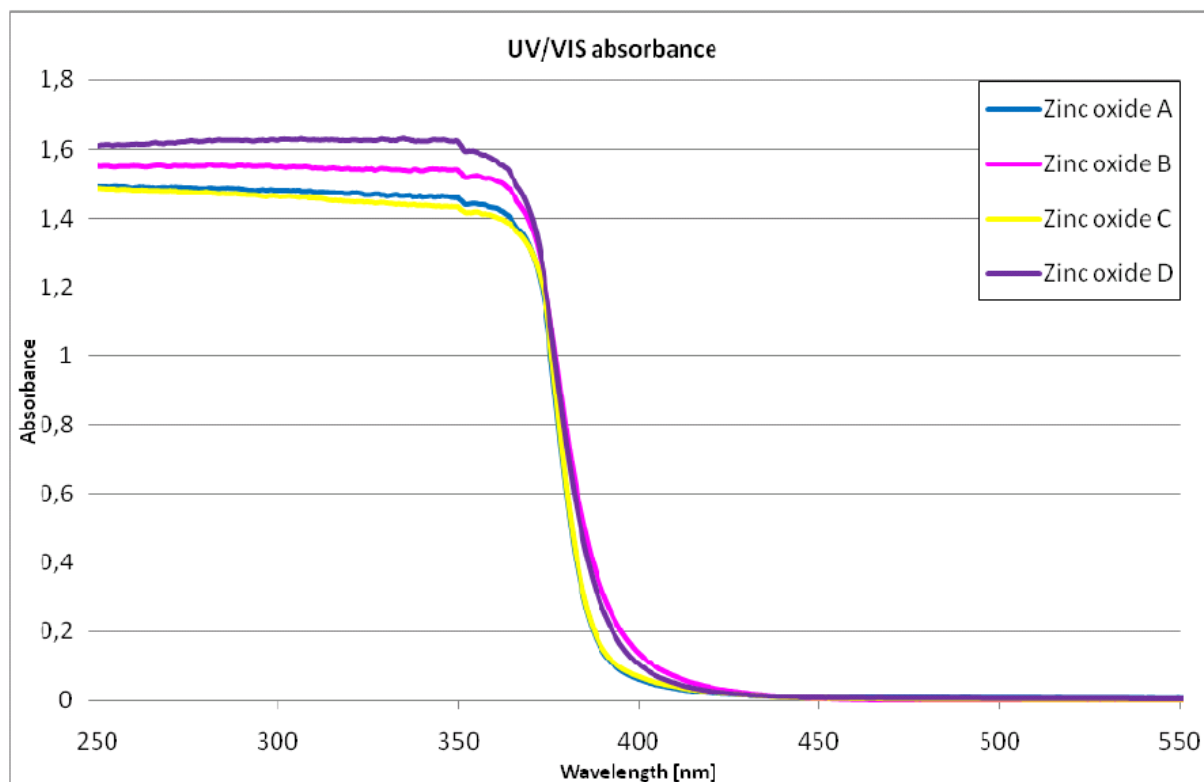
(References: 56, 114)

Comment

A similar pattern was observed regarding the UV absorption for the four evaluated ZnO nanomaterials. However, only uncoated ZnO materials were analysed.

3.1.13 Zeta potential

Zeta potential was determined using electrophoresis (Zetasizer Nano from Malvern). The pH was adjusted with NaOH.

The following samples were investigated:

FINEX-50 (Sakai Chemicals, Japan, supplied by Umicore)

Zano 10 (Umicore)

NanoTec 50 (Grillo)

Z-COTE (BASF, batch: EHH/0201)

There was no significant difference between the samples in terms of the charge.

The curve shapes are characteristic for ZnO powders. The iso electric point (IEP) was in the range of pH 9.5–10.

(Reference: 31)
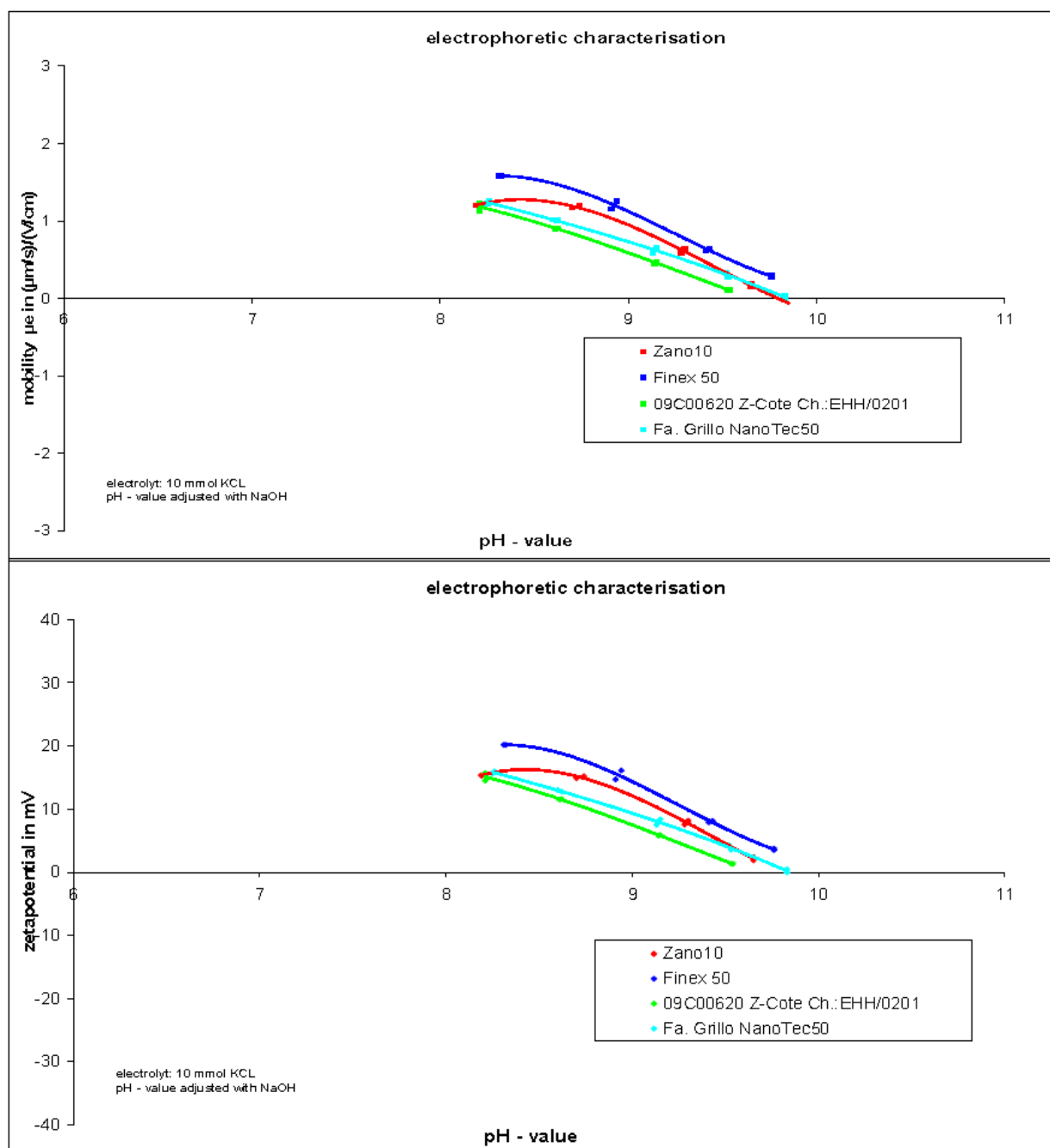
3.1.14 Droplet size in formulations

The applicant provided additional information on the effects of various formulation and technical parameters (sunscreen type, nanomaterial concentration, viscosity, propellant gas, actuator and valve type) on size and size distribution of droplets generated when a sunscreen is used as an emulsion for spray application. The information was provided as the results of an investigative study (Reference: 38).

Different types of emulsion; water in silicone (W/Si) and water in oil (W/O), were prepared using high concentrations of organic and inorganic UV-filters (ZnO as Z-COTE MAX, 1% in W/Si emulsion, 1% in W/O emulsion, among others). These formulations were incorporated in aerosol cans with gas (a blend of butane, propane and isobutane).

The size and size distribution of the droplets were analyzed by laser diffraction using a Malvern® Spraytec.

The measuring distance of the droplets was set at 11 cm for all the experiments. In general, assessments for these types of evaluations and measurements are carried out at a distance of 30 cm from the laser beam. However, the authors of the paper considered that 11 cm was a more common distance for normal use of sunscreen sprays.

A size distribution curve obtained with 22% of gas propellant for the W/Si emulsion at a–1 cm and b–11 cm is shown below.
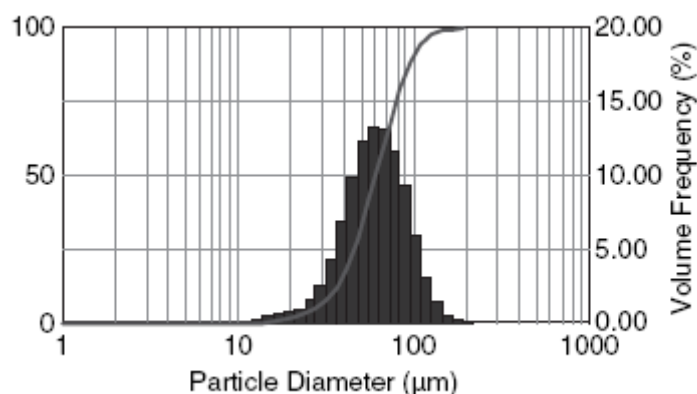
The figure below shows the influence of concentration of inorganic filters on the droplet size in the W/O emulsion.
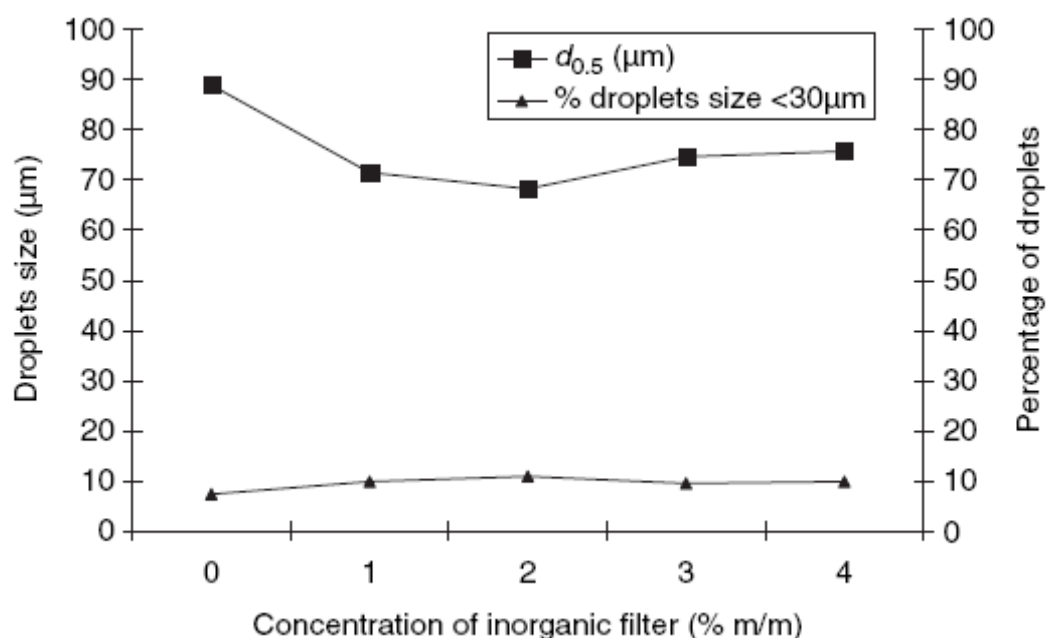
The results showed that the sprayability of the formulation and the particle size characteristics of the emitted sprays are dependent on the physicochemical properties of the formulations. Sprayable waterproof sunscreen emulsions with a high sun protection factor and a low (<5%) percentage of emitted droplets below 30 µm could be developed by optimizing formulation parameters and using appropriate actuators and valves. For all experiments, the percentage of droplets below 10 µm was less than 0.1%. Droplet size and

size distribution of the emitted droplets were highly influenced by all the parameters studied and especially by propellant concentration, viscosity, presence or absence of inorganic filters (powder) and the design of valves and actuators. Considering the aspect of the final product and droplet size, the best products (in terms of optimal droplet size) were obtained with the lower gas concentration (22%), higher concentrations of inorganic sunscreen filter and higher viscosities. The final choice of the valve and actuator was made based on the aspect of the final product after spraying. Therefore, when sunscreen-containing aerosols are formulated it is important to consider each technical and formulation parameter in order to reach the optimum size and size distribution of droplets.

(Reference: 38)

According to the applicant, no sunscreen spray products containing nano-sized ZnO are available on the EU market. It was stated that if such sprayable sunscreen products were to be produced and marketed they would comply with current standards and requirements according to the relevant guidance documents as referred to below.

- The Technical Guidance Document on Risk Assessment of the European Chemical Council emphasized that aerosols with a Mass Medium Aerodynamic Diameter (MMAD) greater than 10–15 µm are not respirable for humans, i.e. the aerosol particle sizes above this cut-off will deposit in the upper regions of the lungs due to their large particle sizes. Only particles with a size less than 10–15 µm reach the gas-exchange region of the lung.

- The U.S. Silicones Environmental, Health and Safety Council (SEHSC) recommends that, *"When considering a consumer aerosol application for any silicone-based material, regardless of the method of aerosol generation, the particle size MMAD should be at least 30 µm with no more than 1% of the particles having an aerodynamic diameter of 10 µm or less. Following this guidance ensures that virtually all aerosol particles will be trapped in the nasopharyngeal region and very few if any particles will be deposited in the tracheobronchial region."*

(References: 43, 45, 86)

Comment

The results indicate that mainly larger droplets were present when spraying a water- or silicone-based nanoparticle containing test aerosol at a distance of 11 cm. The small fraction of droplets with a diameter less than 30 µm was based on droplet size distribution measured by weight. It should be noted that even a low fraction based on aerosol weight is relevant as it contains a high number of droplets. At larger distances such as 30 cm which is normally used for such investigations, a short time lapse may occur allowing for evaporation of aerosol liquid components resulting in smaller droplet sizes than measured at 11 cm. These dried residual particles spreading in the air also need to be taken into account for possible inhalation exposure. Notably, this mechanism is even more relevant when high vapour pressure fluids such as alcohols are used in the generation of the aerosol, compared to when water or oil are used as dispersion fluids.

The report indicates that in certain tests, an inorganic filler such as TiO_2 or ZnO (Z-COTE max) was used. However, it was not indicated in the results section which inorganic filler was used in the experiments for which the data were presented. Therefore, the data cannot be exclusively ascribed to ZnO nanomaterial.

In view of the presented data, the possibility that the dried residual aerosol particles may lead to lung exposure after inhalation needs to be taken into account. Therefore the measurement of the droplet size distribution needs to be complemented by measurements of the size distribution of the dried residual aerosol particles as well.

(References: 30, 71)

3.1.15 Homogeneity and stability

Photostability

Method

Photostability was measured by UV absorption according to a BASF internal method: PMMA plates (Helioscreen HD6, 6 µm), 1.3 mg/cm^2 application rate, four plates and 11 scans/plate, measurements with Labsphere 1000S.

Samples

O/W emulsions with uncoated and coated ZnO

Formulation A: Z-COTE (10%, uncoated, BASF):   UV10037-1-1 (SPF 6.6)

Formulation B: Z-COTE HP1 (10%, coated, BASF):   UV 10037-2-2 (SPF 6.6)

Formulation C: Zano 10 Plus (20%, coated, Umicore): UM08-ZP20-OW (SPF 31.3)

Light source

Atlas CPS+ with Atlas filter solar standard according to DIN 67501.

Results/Remarks

The photostability of the uncoated ZnO is presented below.

Photostability of Z-COTE (10%, uncoated, BASF): UV10037-1-1 (SPF 6.6):
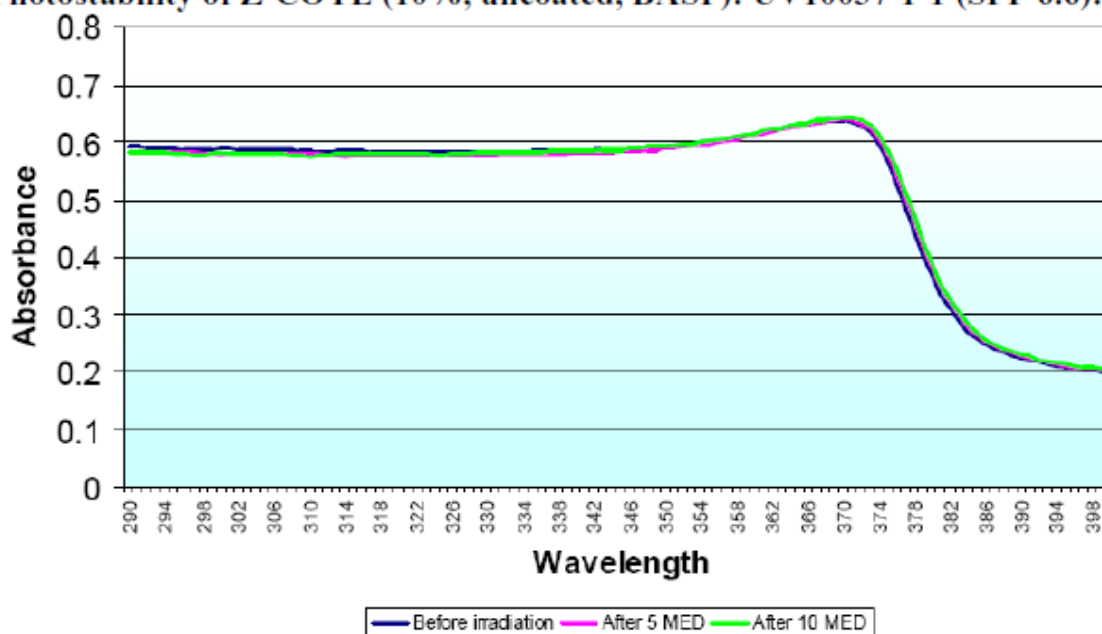
The O/W formulations were photostable over the whole UV range (from 290 to 400 nm).

However, according to the applicant, few absorbance curves were higher following irradiation as it was technically not possible to measure exactly the same point on a PMMA plate before and after irradiation, and also in case the formulation spreading of the plate

was not homogenous. This is known to result in slightly different absorbance behaviour. The observed minimal differences could be considered as an indication of variation in the measurement technique and not as variation in the photostability of the nanomaterials.

It was concluded by the applicant that all three model O/W formulations with uncoated (Z-COTE) and coated (Z-COTE HP1, Zano 10 Plus) ZnO were photostable over the whole UV range between 290 and 400 nm.

(Reference: 29)

3.1.16 Summary on supplementary physicochemical characterization

The comparative analysis and presentation of the physicochemical characteristics of the representative selected nano-sized ZnO materials shows that all nano-sized ZnO materials as described in the dossier can be considered to be broadly similar, irrespective of the manufacturing processes used (wet chemical or pyrolytic). The crystal structure of all of the ZnO nanomaterials included in the dossier is the same (wurtzite/hexagonal) and the particle shapes are roughly similar. The particle size distributions for all of the products fall within the same range and so do all other parameters tested such as solubility, zeta potential and photostability. According to the applicant, the set of physicochemical data on representative nano-sized ZnO raw materials, as well as on examples of additional nano-sized ZnO raw materials found on the EU market, indicates that the nano-sized ZnO products can be considered to be similar overall.

Comment

It should be noted that it is technically possible to produce nano-dispersed ZnO with different physicochemical properties to those described in this dossier. Therefore, this opinion specifically relates to those coated and uncoated ZnO nanomaterials for which **data have been submitted in this dossier**. It is required that manufacturers carefully analyse the raw materials which they obtain, particularly if they come in a finally stabilized formulation in fluids which may contain different forms/surface modifications of the nanomaterials. Thus, this assessment applies to ZnO nanoparticles similar to those included in this dossier, with the following characteristics:

- ZnO nanoparticles of purity ≥99%, with wurtzite crystalline structure and physical appearance as described in the dossier, i.e. clusters that are rod-like, star-like and/or isometric shapes.

- Median diameter (D50: 50% of the number below this diameter) of the particle number size distribution is between 30 nm and 55 nm, whereas the D1 (1% below this size) is above 20 nm.

- ZnO nanoparticles that are either uncoated or coated (with triethoxycaprylylsilane, dimethicone, dimethoxydiphenylsilanetriethoxycaprylylsilane cross-polymer, or octyl triethoxy silane).

- ZnO nanoparticles that have a comparable solubility to that reported in the dossier, i.e. below 50 mg/L (approximately the maximum solubility of the ZnO nanomaterials for which data are provided in the dossier).

Regarding the characterization of the ZnO nanomaterials, product parameters such as the number of particles per mass unit and surface area per mass unit were not provided. It is at the moment under consideration that mass (weight) alone may not be the best parameter for the description of a dose response relationship in toxicity studies. Therefore, it is the view of the SCCS that additional parameters, such as number of particles per mass and surface area per mass, are usefull additions to the characterization of the nanomaterials. Where such parameters are available, it is possible to evaluate dose response relationships

for toxicity by these alternative dose parameters (number of particles, total surface area administered) as well.

3.2   Function and uses

According to the European Union Risk Assessment Report (Reference: 44), zinc oxide is used in industrial settings as a rubber compounding material, and in glass and ceramic products. Zinc oxide acts as a catalyst in alkylation, oxidation, hydrogenation and desulphurisation reactions.

In paints, zinc oxide is mainly used as a corrosion inhibitor and to a lesser extent as a mildew control agent.

As zinc is an essential trace element, zinc oxide is added to fertilizers, animal feeds and human vitamin supplements. It is used in medical plasters, baby creams, calamine lotion and in dental cement (in combination with eugenol). Zinc oxide absorbs ultraviolet light, and is used as a UV-filter in pharmaceutical and cosmetic products. No specific information on the uses of nano-sized ZnO is available.

ZnO is also used as colorant according to the Cosmetics Directive 76/768/EEC, Annex IV, but no information of ZnO particle sizes used in such applications is available, and it is not clear whether it contains a nanofraction.

The present submission applies for the use of ZnO nanomaterial as a UV-filter in cosmetic sunscreen products up to 25%.

3.3   Toxicological evaluation

3.3.1   Acute toxicity

3.3.1.1   Acute oral toxicity

The acute oral toxicity of one ZnO nanoparticle product (FINEX-50) was investigated with a dose of 2,000 mg/kg.

Study Design

Guideline/method:	OECD Guideline 423 (2001).
Species/strain:	Rat/Sprague-Dawley (Crl:CD(SD)
Group size:	Three males
Test substance:	FINEX-50 ZnO (supplied by Sakai Chemical Industry Co., primary particle size: 20 nm, non-coated).
Batch:	OZ52
Purity:	≥96% as indicated in the submission
Dose levels:	2,000 mg/kg bw as 20% (W/V) aqueous suspension.
Dose volume:	10 mL/kg bw
Vehicle:	Water for injection.
Route:	Oral (gavage)
Exposure:	Single application
Observation period:	14 days
GLP:	No
Published:	No
Reference:	99

Study period   Eight days
Date of report: March 2006

The maximum dose (2,000 mg/kg body weight) as specified in OECD Guideline 423 for acute oral testing was used. The dose was administered as a 25% aqueous suspension by oral gavage. The animals were observed prior to dosing and for treatment-related effects after 2 hours and on day 1, and then at least once a day for a total of one week. Body weights were determined on days 1, 2, 3, 4, 6 and 8 after application. Gross pathology was performed in all rats at two weeks after oral administration.

Results

No animals died during the course of the study. On day 3 of observation, decreases in body weight and fecal excretion were observed in one animal, but these findings disappeared four days after dosing. There were no abnormal findings at necropsy at two weeks after oral administration.

Conclusion

The approximate acute oral toxicity (LD_{50}) was >2,000 mg/kg bw for male Sprague-Dawley rats.

(Reference: 99)

Comment

For the ZnO used, information on surface area and number of particles per mass was not provided. Also, information on the dose expressed as surface area and number of particles was not provided.

The purity of the FINEX-50 used was indicated in the submission to be ≥96%. However, data on the purity is not provided in the physico-chemical characterization of the FINEX-50 (section 3.1.4), and was also not provided in the study reference 99.

The SCCS agrees that the acute oral toxicity of the ZnO materials tested in rat is >2,000 mg/kg body weight.

Additional study

An exploratory acute oral toxicological investigation comparing the effects of nano-scale (20 nm) or pigmentary (submicron-scale 120 nm) ZnO (Produced by Jiangsu Haitai Nanomaterials Co. Ltd. Jiangsu Province, China) was performed in mice as reported by Wang et al. (Reference: 118). The primary mean size as determined by TEM was 20 ± 5 nm and 120 ± 20 nm, respectively. Surface area was 4.4×10^5 cm^2/g and 9.1×10^4 cm^2/g, respectively. The particle number concentration was 2.2×10^{16}/g and 2.0×10^{14}/g, respectively. When administered in a 1% sodium carboxymethylcellulose solution the size range of these materials was between 30 nm and 70 nm, and between 186 nm and 190 nm, respectively. Male (n=5) and female (n=5) CD-ICR mice received a single oral application of 20 nm ZnO or 120 nm ZnO powder at dose levels of 1,000, 2,000, 3,000, 4,000 and 5,000 mg/kg bw suspended in 1% sodium carboxymethylcellulose. Doses expressed as surface area were: $4.4\text{-}8.8\text{-}13.2\text{-}17.6\text{-}22 \times 10^5$ cm^2/kg bw for the 20 nm ZnO; and $9.1\text{-}18.2\text{-}27.3\text{-}36.4\text{-}45.5 \times 10^4$ cm^2/kg bw for the 120 nm ZnO. Doses expressed as number of particles were: $2.2\text{-}4.4\text{-}6.6\text{-}8.8\text{-}11 \times 10^{16}$ particles/kg bw for the 20 nm ZnO; and $2\text{-}4\text{-}6\text{-}8\text{-}10 \times 10^{14}$ particles/kg bw for the 120 nm ZnO. The control animals received the vehicle only. The animals were observed for 14 days and at termination blood was sampled for selected hematological and clinical pathology examinations. Several organs were collected for histopathological investigations and ZnO content analysis. For the 120 nm sized ZnO the

highest dose (5,000 mg/kg) induced the highest mortality; three out of five females and two out of five males died, whereas one out five females died in the 4,000 mg/kg group, and one out of five females died in the 2,000 mg/kg group. For the 20 nm sized ZnO, one out of five males died in the 5,000 mg/kg group, and one out of five females died in the 2,000 mg/kg group. In addition, effects were reported showing alterations in individual hematology, clinical pathology parameters, and pathological findings (stomach, liver, heart, spleen). Indications of Zn accumulation in selected tissues (bone, pancreas and kidney) were observed for both 20 nm and 120 nm ZnO.

(Reference: 118)

Comment

In view of the size ranges reported in Reference 118, the larger ZnO cannot be considered as pigment grade. The published paper describes the particles as submicron-sized with a mean size of 120 nm. Although not all effects showed a clear dose response relationship, many effects were noted at the highest dose administered that should be considered dose related even when they only occur at the highest dose. The pathological findings indicate a dose response relationship with the highest dose inducing the most severe alterations. The lowest dose investigated (1,000 mg/kg) also induced pathological effects in the animals. Most effects showed some minor differences between the 20 and 120 nm sized ZnO. Target organs for toxicity were liver, heart, spleen, pancreas and bone. A no observed adverse effect level (NOAEL) was not dentified because alterations were also observed at the lowest dose investigated (1,000 mg/kg).

No deaths were observed in a similar study using a single oral dose of 5 mg/kg in mice (Reference: AR16). ZnO nanoparticles were approximately 50 nm in size (TEM evaluation), which were compared to ZnO microparticles showing at least one diameter >100 nm (TEM evaluation). DLS evaluation showed an average hydrodynamic diameter of 1,226 ± 120 nm for the ZnO microparticles, and an average hydrodynamic diameter of 93 ± 14 nm for the ZnO nanoparticles. After oral administration for both ZnO nanoparticles and microparticles, Zn could be observed in serum indicating uptake from the GI–tract, either as particulate material, dissolved Zn ions, or in both forms. In liver, spleen and lung, ZnO nanoparticles showed higher Zn distribution compared to ZnO microparticles. Serum liver enzyme analysis (aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH)) indicated liver toxicity due to both ZnO nanoparticles and ZnO microparticles which was confirmed by histopathology (Reference AR16). Histopathological lesions were only observed for ZnO nanoparticles in the liver (Reference 118).

Additional studies in the open literature

An exploratory study of acute oral toxicity with a commercial ZnO nanomaterial is summarized below.

Study Design

Guideline/method:	OECD Guideline 423 (2001)
Species/strain:	Rats, Sprague-Dawley, in house animal facility.
Group size:	Five males and five females per group.
Test substance:	ZnO nanoparticles (20 nm, 63 nm in SEM) from Nanostructured and Amorphous Materials Inc., USA), surface area 50 m^2/g.
	Micro- sized ZnO from Sigma- – Aldrich (product no. ZO385).
Batch:	Stock no. 5180HT
Purity:	Not stated

Dose levels:	5, 50, 300, 1,000, 2,000 mg/kg bw as 20% (W/V) aqueous suspension, dose as surface area 0.25, 2.5, 15, 50, and 100 m^2/kg bw.
Dose volume:	10 mL/kg bw
Vehicle:	Distilled water
Route:	Oral (gavage)
Exposure:	Single application
Observation period:	14 days
GLP:	No
Published:	Yes
Study period:	14 days
Date of report:	2011

In this study, the acute oral toxicity of ZnO nanomaterial was compared to a ZnO preparation from Sigma-Aldrich. The study was performed according to the OECD guideline for acute oral toxicity OECD Guideline 423 (2001). Toxicity and animals were evaluated up to 14 days after a single administration by oral gavage.

Results

There was an inverse relationship with serum levels of liver enzymes (aspartate aminotransferase (AST) and alanine aminotransferase (ALT), the lower doses inducing the highest increase. On a weight basis the nano-sized material induced serum alterations in AST and ALT that were not observed with the commercial non-nano ZnO (Sigma-Aldrich). The incidences of microscopic lesions in the liver, pancreas, heart and stomach were higher at lower doses of nano-size zinc oxide compared to higher doses. It was concluded that nano-sized zinc oxide exhibited toxicity at lower doses probably due to lower aggregation of the material at low concentrations. No measurements of Zn ions (or particles) in blood were reported. Thus on a worst-case basis, it can be assumed that the translocating ZnO was in nanoparticulate form (SCCS Guidance on Nanomaterials in Cosmetics SCCS/1484/12). The nano-ZnO induced histopathological lesions in the liver, pancreas, stomach and heart at all the doses investigated (lowest dose 5 mg/kg), whereas the commercial ZnO did so only at the highest dose investigated (2,000 mg/kg).

Conclusion

The nano-sized ZnO induced toxicity at the lowest dose investigated (5 mg/kg body weight).

(Reference AR20)

Comment

In this study, the size of the commercial micro-ZnO is not reported. Also for the ZnO information on number of particles per mass was not provided. Information on dose expressed as number of particles was not provided. The acute toxicity evaluation was performed based on blood parameters and histopathology. Body weights were not reported.

Exploratory study

Study Design

Guideline/method:	No
Species/strain:	ICR mice
Group size:	Five males and five females.
Test substance:	ZnO nanoparticles (50 nm, TEM evaluation) and ZnO microparticles (1,226i nm, DLS measurement) from Top Nanotechnology Co. Ltd., Taiwan.
Batch:	Not stated
Purity:	Not stated
Dose levels:	1.25, 2.5, 5 g/kg bw.
Dose volume:	10 mL/kg bw
Vehicle:	Sterile water plus 1% hydroxypropyl methyl cellulose.
Route:	Oral (gavage) and intraperitoneal administration.
Exposure:	Single application
Observation period:	14 days
GLP:	No
Published:	Yes
Study period:	14 days
Date of report:	2011

In this study, ZnO nanoparticles used were approximately 50 nm in size (TEM evaluation), which were compared to ZnO microparticles showing at least one diameter >100 nm (TEM evaluation). DLS evaluation showed an average hydrodynamic diameter of 1,226 ± 120 nm for the ZnO microparticles and an average hydrodynamic diameter of 93 ± 14 nm for the ZnO nanoparticles. Toxicity was evaluated by analysing serum biochemistry parameters and histopathology. Genotoxicity was evaluated using the Ames test and the micronucleus test. Zinc absorption and tissue distribution was evaluated by ICP-MS determination of elemental Zn. In addition an *in vitro* cytotoxicity assay was performed.

Results

All animals survived a single oral or intraperitoneal dose of 5 g/kg body weight of both nano-sized ZnO (50 nm) and micro-sized ZnO (1,226 nm), although the large ZnO particles induced a body weight reduction in both male and female animals. After oral and intraperitoneal administration of a single dose of 2.5 g/kg bw for both ZnO nanoparticles and microparticles, Zn could be observed in serum indicating uptake from the GI-tract. For ZnO nanoparticles, the systemic availability was significantly higher (approximately 24% to 50%) at 2, 4 and 6 hours after administration compared to that of ZnO microparticles as indicated by Zn measurements by ICP-MS. In the liver, spleen and lung, treatment with ZnO nanoparticles showed a higher distribution of Zn compared to treatment with ZnO microparticles. Serum liver enzyme analysis (aspartate aminotransferase (AST), alanine aminotransferase (ALT) and lactate dehydrogenase (LDH)) indicated liver toxicity at 24, 48 and 72 hours after treatment, both for ZnO nanoparticles and ZnO microparticles. This was confirmed by histopathology. Histopathological lesions were only observed for ZnO nanoparticles in the liver.

The *in vitro* studies revealed a dose related induction of cytotoxicity for both nano-ZnO and micro-ZnO up to a concentration of 100 µg/mL. The Ames test (*in vitro*) and the *in vivo* micronucleus test were negative for both nano-ZnO and micro-ZnO.

(Reference: AR16)

Comment

For the ZnO nanoparticles and microparticles, information on surface area and number of particles per mass was not provided. Information on dose expressed as surface area and number of particles was not provided.

The results indicate a similar uptake, tissue distribution, and toxicity of ZnO nanoparticles and ZnO microparticles. As Zn was measured by ICP-MS, it cannot be concluded whether it was present in particulate form or as dissolved ionic Zn. Although serum levels of aspartate aminotransferase, alanine aminotransferase and lactyate dehydrogenase indicated similar liver toxicity for both ZnO nanoparticles and ZnO microparticles, histopathological lesions were only observed for ZnO nanoparticles in the liver.

3.3.1.2	Acute dermal toxicity

No information provided.

3.3.1.3	Acute inhalation toxicity

No information provided.

3.3.2	Irritation and corrosivity

Skin Irritation

The skin irritation potential was determined for one ZnO nanomaterial (FINEX-50) according to Japanese guidelines.

Study Design

Guideline/method: Guide to marketing and manufacturing of cosmetics and quasi drugs in Japan.

Species/strain: Guinea pig/Hartley

Group size: Three males per concentration.

Test substance: FINEX-50 ZnO (supplied by Sakai Chemical Industry Co., primary particle size: 20 nm, non-coated).

Batch: OZ52

Purity: ≥96% as indicated in the submission

Vehicle: Ethanol

Dose level: 25% or 40% dispersion in ethanol.

Dose volume: 0.05 mL, once a day for three consecutive days.

Exposure: Intact skin (open)

GLP: No

Study period: Up to three days

Date of report: February 2009

The cumulative acute dermal irritation potential was investigated in individually housed Hartley Guinea pigs. The hair was clipped on the dorsal area of the trunk one day prior to application and prior to examination on day 3. An amount of 0.05 mL of the test substance as a 25% or 40% ethanolic dispersion was applied to the test site (2 × 2 cm) on the right back of each of the six animals and left exposed without dressing. Percutaneous administration was used for the substance. The administration was conducted once daily for three consecutive days. The animals were examined for erythema, incrustation and edema on each day of administration.

Results

There were no signs of systemic toxicity and no mortality. Slight erythema was observed in one of three animals in the 40% test substance group on day 3 of the administration. No other abnormal dermal changes were observed at any time during the administration and observation periods. No abnormal dermal changes were observed at any time during the administration and observation periods in the 25% test substance group.

Conclusion

The three day consecutive skin irritation study in male Guinea pigs led to skin irritation scores of 0.1 and 0.0 for the 40% and 25% test substance dispersions indicating a weak irritation according to the Japanese guideline used.

(Reference: 101)

Comment

For the ZnO information on surface area and number of particles per mass was not provided. Information on dose expressed as surface area and number of particles was not provided.

The purity of the FINEX-50 used was indicated in the submission to be ≥96%. However, data on the purity is not provided in the physico-chemical characterization of the FINEX-50 (section 3.1.4), and was also not provided in the study reference 101.

This test does not correspond to OECD Guideline 404 which indicates that rabbits should be used for the *in vivo* irritation test, and gives a dermal dose of 0.5 mL per application site using a gauze patch.

Mucous membrane irritation

The mucosal irritation potential was determined for one ZnO nanomaterial (FINEX-50) according to Japanese guidelines.

Study Design

Guideline/method: Guide to marketing and manufacturing of cosmetics and quasi drugs in Japan and according to Draize (1959) and Gullot et al. (1982) in total comparable to OECD Guideline 405.

Species/strain: Rabbit/Japanese White

Group size: Three males per concentration.

Test substance:	FINEX-50 ZnO (supplied by Sakai Chemical Industry Co., primary particle size: 20 nm, non-coated).
Batch:	OZ52
Purity:	≥96% as indicated in the submission
Vehicle:	Olive oil
Dose level:	0.1 g of neat (unchanged) substance or 0.1 mL of a 25% solution in olive oil.
Dose volume:	0.1 mL
GLP:	No
Study period:	Up to seven days
Date of report:	April 2006

The potential irritant effect on the mucous membrane was investigated by instillation of either 0.1 g of the neat test substance or 0.1 mL of a 25% oily solution into the right conjunctival sac of the eye of each of the three rabbits. The 25% oily solution was chosen as the proposed consumer use concentration of the product. The eyes were not washed. The left eyes remained untreated and served as controls. Both eyes of the animals were examined within 1 and 4 hours after application and twice daily on days 1, 2, 3, 6 and 7 according to the method and scoring system of Draize.

Results

Neat test substance group

At 1 hour after administration, redness of the conjunctiva was observed in all animals, and slight or apparent edema in two of three animals and edema accompanying one-half occlusion of the eyelid in one of three animals. Slight discharge was observed in two of three animals. At 4 hours after administration, apparent or diffuse beef-like redness of the conjunctiva was observed in all three animals, and slight or apparent edema in two of three animals and edema accompanying more than one-half occlusion of the eyelid in one of three animals. Slight or apparent discharge was observed in two of three animals. On day 1, apparent redness and slight edema were observed in two of three animals, and slight discharge in one of three animals. On day 2, apparent redness was observed in two of three animals, and slight edema and slight discharge in one of three animals. On day 3, slight or apparent redness was observed in two of three animals. There were no adverse effects recorded in observations made on day 6 post-administration and thereafter.

Appendix 1-1   Eye irritation after administration of FINEX-50 zinc oxide (100%) to males

Animal No.	Organ	Lesions	Day of administration a)	Day of administration b)	Days after administration 1	2	3	6	7
7	Cornea	Opacity	0	0	0	0	0	0	0
		Area of cornea involved	0	0	0	0	0	0	0
	Iris	Abnormality	0	0	0	0	0	0	0
	Conjunctivae	Redness	2	2	0	0	0	0	0
		Chemosis	1	1	0	0	0	0	0
		Discharge	0	0	0	0	0	0	0
		Total irritation score c)	6	6	0	0	0	0	0
8	Cornea	Opacity	0	0	0	0	0	0	0
		Area of cornea involved	0	0	0	0	0	0	0
	Iris	Abnormality	0	0	0	0	0	0	0
	Conjunctivae	Redness	2	2	2	2	2	0	0
		Chemosis	2	2	1	0	0	0	0
		Discharge	1	1	0	0	0	0	0
		Total irritation score c)	10	10	6	4	4	0	0
9	Cornea	Opacity	0	0	0	0	0	0	0
		Area of cornea involved	0	0	0	0	0	0	0
	Iris	Abnormality	0	0	0	0	0	0	0
	Conjunctivae	Redness	2	3	2	2	1	0	0
		Chemosis	3	4	1	1	0	0	0
		Discharge	1	2	1	1	0	0	0
		Total irritation score c)	12	18	8	8	2	0	0
Average irritation score d)			9.3	11.3	4.7	4.0	2.0	0.0	0.0

a): 1 hour after administration.

b): 4 hours after administration.

c): Opacity x Area of cornea involved x 5 + Abnormality x 5 + (Redness + Chemosis + Discharge) x 2

d): Summation of total irritation score / number of animals

25% test substance group

Apparent redness of the conjunctiva, slight edema, and slight discharge were observed in all three animals at 1 hour after administration. At 4 hours after administration, there was slight or apparent redness of the conjunctiva in two of three animals and diffuse beef-like redness in one of three animals. In addition, slight or apparent edema was observed in two of three animals. Slight discharge was observed in one of three animals. On day 1 post-administration, slight redness was observed in one of three animals. There were no adverse effects recorded in observations made on day 2 post-administration and thereafter.

Appendix 1-2 Eye irritation after administration of FINEX-50 zinc oxide (25%) to males

Animal No.	Organ	Lesions	Day of administration a)	b)	Days after administration 1	2	3	6	7
10	Cornea	Opacity	0	0	0	0	0	0	0
		Area of cornea involved	0	0	0	0	0	0	0
	Iris	Abnormality	0	0	0	0	0	0	0
	Conjunctivae	Redness	2	1	0	0	0	0	0
		Chemosis	1	0	0	0	0	0	0
		Discharge	1	0	0	0	0	0	0
		Total irritation score c)	8	2	0	0	0	0	0
11	Cornea	Opacity	0	0	0	0	0	0	0
		Area of cornea involved	0	0	0	0	0	0	0
	Iris	Abnormality	0	0	0	0	0	0	0
	Conjunctivae	Redness	2	3	1	0	0	0	0
		Chemosis	1	2	0	0	0	0	0
		Discharge	1	1	0	0	0	0	0
		Total irritation score c)	8	12	2	0	0	0	0
12	Cornea	Opacity	0	0	0	0	0	0	0
		Area of cornea involved	0	0	0	0	0	0	0
	Iris	Abnormality	0	0	0	0	0	0	0
	Conjunctivae	Redness	2	2	0	0	0	0	0
		Chemosis	1	1	0	0	0	0	0
		Discharge	1	0	0	0	0	0	0
		Total irritation score c)	8	6	0	0	0	0	0
Average irritation score d)			8.0	6.7	0.7	0.0	0.0	0.0	0.0

a): 1 hour after administration.
b): 4 hours after administration.
c): Opacity x Area of cornea involved x 5 + Abnormality x 5 + (Redness + Chemosis + Discharge) x 2
d): Summation of total irritation score / number of animals

The calculated eye irritation indeces based on the findings at 1 hour after administration of the test substance and 25% test substance solution were 11.3 and 8.0, respectively.

Conclusion

The neat and the 25% solution of the test substance were concentration dependent, slightly and transiently irritating to the eyes of three Japanese White rabbits. Therefore, both were assessed as "mild irritant".

(Reference: 96)

Comment

For the ZnO used in this study, information on surface area and number of particles per mass was not provided. Information on dose expressed as surface area and number of particles was not provided.

The purity of the FINEX-50 used was indicated in the submission to be ≥96%. However, data on the purity is not provided in the physico-chemical characterization of the FINEX-50 (section 3.1.4), and was also not provided in the study reference 96.

In Reference 96, the number of animals investigated was six (three per group) and the numbering is from 1 to 6. However, the results are presented for animal numbers 7 to 12.

3.3.3 Skin sensitization

The skin sensitization potential of one ZnO nanomaterial (FINEX-50) was evaluated using a modification of the guinea pig maximization test as a short-term adjuvant and patch test.

Study Design

Date of report:	February 2006
Guideline/method:	Short-term adjuvant and patch test method (s-APT according to Reference: AR25).
Species/strain:	Guinea pig/Hartley
Group size:	Ten females in total (five per group).
Test substance:	FINEX-50 ZnO (supplied by Sakai Chemical Industry Co., primary particle size: 20 nm, non-coated).
Batch:	OZ52
Purity:	≥96% as indicated in the submission
Vehicle:	Distilled water
Concentration:	Induction:
	Group A) distilled water
	Group B) 40% aqueous test substance suspension
	Challenge:
	Group A) 5%, 12.5%, 25%, 40% aqueous test substance suspension.
	Group B) 1.25%, 2.5%, 5%, 12.5%, 25%, 40% aqueous test substance suspension.
Route/Exposure:	Induction:
	1st induction: intradermal injection with Freund's complete adjuvant followed by occlusive topical application of the vehicle or 40% aqueous test concentration for 72 hours.
	2nd induction: six days after 1st induction occlusive topical application of the vehicle or 40% aqueous test concentration for 48 hours.
	Challenge: 13 days after 1st induction, open application of test concentrations.
Skin evaluations:	24, 48, 72 hours after challenge.
Study period:	16 days
GLP:	No
Published:	No
Date of report:	February 2006

The sensitizing potential of the test substance was tested in female Hartley Guinea pigs according to the short-term adjuvant and patch test method (s-APT according to Yanagi et al. (Reference: AR25). In total ten animals were tested, divided in two groups of five

animals each. Prior to the 1st induction the hair was removed and the animals were shaved, followed by an intradermal injection of Freund's complete adjuvant at four sites. (The Guinea pig maximization test according to OECD Guideline 406 uses three injections as induction; one being a mixture of Freund's complete adjuvant and water, one a mixture of FCA and the test substance, and one of the test substance only). Thereafter, one group received the vehicle (group A) or a 40% aqueous test substance suspension (group B) for 72 hours under an occlusive dressing. Six days after the 1st induction, the animals were induced a 2nd time by occlusive topical application of either the vehicle or the 40% aqueous test substance concentration.

The challenge was performed by open topical application of 5%, 12.5%, 25%, 40% aqueous test substance suspension (group A) or 1.25%, 2.5%, 5%, 12.5%, 25%, 40% aqueous test substance suspension (group B), and the skin was evaluated at 24, 48 and 72 hours after the challenge.

Results

Neither the group induced with the 40% aqueous test substance suspension or with the vehicle showed any skin reaction to the challenge concentrations at the evaluation time points of 24, 48 or 72 hours.

Conclusion

It was concluded that the test substance exhibited no potential to induce dermal sensitization in Guinea pigs under the conditions of the study.

(Reference: 95)

Comment

For the ZnO used in this study, information on surface area and number of particles per mass was not provided. Information on the doses expressed as surface area and number of particles was not provided.

The purity of the FINEX-50 used was indicated in the submission to be ≥96%. However, data on the purity is not provided in the physico-chemical characterization of the FINEX-50 (section 3.1.4), and was also not provided in the study reference 95.

The sensitizing potential of the test substance was tested in female Hartley Guinea pigs according to the short-term adjuvant and patch test method (s-APT), which is not a recognized guideline assay. In the classical Guinea pig maximization test (OECD Guideline 406) the induction is performed with a mixture of the test material and FCA, and ten animals are used in the test group. Mixing of the test substance with FCA provides optimal conditions for inducing an immune response, i.e. induction of sensitization if allergic substances are present in the test material.

A concurrent positive control with a well known weak sensitizer as indicated in OECD Guideline 406 (hexylcinnamic aldehyde (CAS No. 101-86-0), mercaptobenzothiazole (CAS No. 149-30-4) and benzocaine (CAS No. 94-09-7)) was not included in this assay, so there is no certainty as to whether the test system used was able to identify weak sensitizers. However, in the paper of Yanagi et al. (Reference: AR25) eight contact sensitizers were found to show similar reactions in the GPMT performed according to the OECD Guideline 406, and in this shortened test.

The SCCS considers that the validity of this (or any) test for demonstrating sensitization potency of nanomaterials has not yet been demonstrated. The inclusion of a positive particle control might overcome this problem. However, no positive particle control has been identified thus far.

Human repeat insult patch test

Study Design

Date of report:	October 2007
Guideline/method:	According to internal laboratory methodology.
Species:	Human
Group size:	50 volunteers (six males and 44 females)
Test substance:	A) ZnO, coated (Zano 10 Plus, Umicore) (former Zano® Plus, ZnO coated with octyl triethoxysilane).
	B) ZnO uncoated (Zano 10, Umicore).
Batch:	A) 22-5D
	B) No data
Route:	Dermal occlusive application by patch.
Procedure:	Induction: Nine consecutive 24 hour exposures on Monday, Wednesday and Friday for three consecutive weeks.
Rest period:	10–14 days
Challenge:	After rest period one 24 hour exposure as during the induction period.
Reaction scoring:	24 and 48 hours after challenge application.
Concentration:	25% in corn oil (0.2 mL dispensed on occlusive, hypoallergenic patch).,
Vehicle:	Corn oil
GLP:	Yes
Published:	No

The test solutions were freshly prepared prior to applications. Of 52 volunteers enrolled for the study, 50 volunteers (six males and 44 females aged 21–66 years; Caucasian and Hispanic) completed the study.

The oily test material dispersions were dispensed onto an occlusive, hypoallergic patch. After 24 hours the patch was removed and the procedure was repeated until a series of nine consecutive 24 hour exposures were made on Monday, Wednesday and Friday for three consecutive weeks. The volunteers were given a rest period of 10–14 days after which a challenge was applied once to a previously unexposed test site. The challenge was equivalent to any of the original procedures. The skin reactions were scored 24 and 48 hours after application on a scale graded 0–4.

Results

Under the selected conditions of this study none of the 50 investigated volunteers showed any skin reaction at any time. All volunteers were consequently scored with grade 0.

Conclusion

The applicant concluded that coated and uncoated ZnO tested as 25% oily dispersion did not produce any skin irritation or skin sensitization under the conditions of this repeat human insult patch test.

(References: 105, 106)

Comment

For the ZnO used in this study, information on surface area and number of particles per mass was not provided. Information on dose expressed as surface area and number of particles was not provided.

Two volunteers discontinued the treatment at the third application after two applications without any clinical signs. Any follow-up and reasons for discontinuation were not presented in the report.

3.3.4   Dermal/percutaneous absorption

3.3.4.1   *In vitro* studies for dermal penetration of human skin

Exploratory in vitro study for percutaneous skin penetration

Study Design

Date of publication:	1997
Guideline/method:	Exploratory percutaneous skin penetration study *in vitro.*
Test system:	Abdominal human skin.
Test substance:	Titanium dioxide T805 (Degussa, Germany), and Spectra veil MOTG (Tioxide specialties, UK), a 60% dispersion of zinc oxide in mineral oil/triglyceride.
Particle size:	Mean crystalline length 116.8 nm with a standard error of 8.5 nm.
Batch:	Not stated
Dose level:	Water in oil (W/O) emulsion similar to commercial sunscreen formulation with ultrafine titanium dioxide (11% wt), and zinc oxide (2.5% wt), dose of formulation 1mg/cm^2.
Skin preparation:	Abdominal human skin from plastic surgery.
Cells:	Not stated
Skin temperature:	Room temperature
Test chamber:	Not stated
Route:	Topical administration
Exposure time:	Not stated
Sampling time points:	Not stated
GLP:	No
Published:	Yes

Characterization of the mineral content of sunscreen formulations containing both TiO_2 and ZnO was performed. The distribution of TiO_2 and ZnO at the surface of human stratum corneum was investigated *in vitro*. Human abdominal skin obtained from plastic surgery was exposed to a W/O emulsion containing 11% ultrafine titanium dioxide and 2.5% ultrafine ZnO at 1 mg/cm^2 at room temperature. Transmission electron microscopic cross-sections of the horny layer of human epidermis showed an almost regular mineral coating of the stratum corneum but neither intercellular nor intracellular ZnO penetration was noted.

Results

This study analysed the distribution of the ZnO nanoparticles in the original preparation and in the cosmetic formulation. It showed the presence of the nanomaterial on the surface of the skin and a lack of nanoparticle uptake by the cells of the stratum corneum of the skin.

(Reference: 40)

Comment

For the ZnO used in this study, information on surface area and number of particles per mass was not provided. Information on dose expressed as surface area and number of particles was not provided.

Although this study provides some evidence that there is no penetration of the nanoparticles from the formulation into the skin, the information on the study itself is rather limited, e.g. time of incubation and surface area of treated skin were not indicated. From the discussion it appears that a mixture of TiO_2 and ZnO nanoparticles was used in the formulation. This study is of no value for the evaluation of skin penetration of ZnO nanoparticles.

Exploratory in vitro study for percutaneous skin penetration

Study Design

Date of publication:	2009
Guideline/method:	Exploratory percutaneous skin penetration study *in vitro.*
Test system:	Human skin
Test substance:	Z-COTE® Max, coated with dimethoxydiphenylsilanetriethoxy-caprylylsilane, cross-polymer.
Particle size:	Not stated
Batch:	Not stated
Dose level:	Water in silicone (W/Si) emulsion and water in oil (W/O) emulsion with 1% ZnO, 2 mg/cm^2 of each emulsion.
Skin preparation:	Excised human skin from female Caucasian patients from abdominal plastic surgery, after verification of integrity and removal of subcutaneous fat, storage at –25°C for a maximum of six months prior to use.
Cells:	Six intact membranes
Skin temperature:	32±1°C
Test chamber:	Static diffusion cells (Franz-type)
Route:	Topical application
Exposure time:	24 hours

Sampling time points: 1, 2, 4, 6, 8, 12 and 24 hours

GLP: No

Published: Yes

Two different types of emulsion; water in silicone (W/Si) and water in oil (W/O), each containing 1% ZnO, were tested. Previously frozen dermatomed human Caucasian skin from surgery was put on the static diffusion cells and, after stabilization of the receptor fluid at a temperature of 37 ± 1°C to achieve a skin temperature of 32 ± 1°C, 2 mg/cm^2 of each formulation was applied to the skin membrane (six/formulation).

Receptor fluid was sampled at 1, 2, 4, 6, 8, 12 and 24 hours after application. Thereafter, the skin samples were removed, rinsed to remove residual formulation, homogenized and processed for analysis. The content of Zn++ ions in the skin was analyzed by means of Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES).

Results and conclusion

The recovery rates for ZnO after application of the W/O and W/Si emulsions after application to the skin were 76% and 86%, respectively. For the W/O emulsion, slightly more ZnO was recovered from in the skin compared to the recovery from on the skin (about 40 and 35%, respectively). For the W/Si emulsion the amount of ZnO recovered in the skin was about the same as the amount recovered from on the skin. No penetration through the skin could be observed with a limit of detection of 0.01 ppm and a limit of quantification of 0.1 ppm for Zn++, respectively in the W/O and the W/Si emulsions.

(Reference: 39)

Comment

The ZnO particles tested were not characterized, and data on particle size, surface area and number of particles per mass was not provided. Information on dose expressed as surface area and number of particles was not provided.

Exploratory in vitro study for percutaneous skin penetration

Study Design

Date of report: 2007

Guideline/method: Exploratory study on human skin penetration of sunscreen nanoparticles *in vitro.*

Test system: Human skin

Test substances: A) ZnO formulation A: dispersion with 60% of siliconate coated ZnO in caprylic capric triglyceride.

                      B) ZnO typical sunscreen formulation B: O/W emulsion sunscreen with 20% of siliconate coated ZnO in caprylic capric triglyceride.

                      C) O/W emulsion without ZnO.

Particle size: 15–40 nm

Dose level: 10 µL/cm^2.

Skin preparation: Full thickness skin from female donors following abdominoplasty cleaned by a heat-separation technique.

Replicates: A and B) eight samples, C) three samples.

Test chamber: Static glass diffusion cell (Franz type, exposed area: 1.3 cm^2, receptor phase volume: 3.5 mL).

Route: Topical application

Exposure time: 24 hours

Sampling time points: 12 and 24 hours

GLP: No

Published: Yes

Experimental design

The possible *in vitro* penetration of human skin was investigated using sunscreens (described as a novel micronized formulation with ZnO) containing nano-sized ZnO. The formulations tested included: (A) ZnO dispersion made with 60% siliconate coated ZnO in caprylic capric triglyceride; (B) a typical O/W emulsion sunscreen with 20% ZnO siliconate coated ZnO in caprylic capric triglyceride; and (C) a control (blank) O/W emulsion sunscreen without ZnO. Particle size determination was carried out before application of the coating using four different techniques identified as transmission electron microscopy (TEM), Brunauer-Emmett-Teller nitrogen-gas absorption method (BET), X-ray diffraction (XRD) and photon correlation spectroscopy (PCS). Human skin samples were from females following abdominoplasty. Epidermal samples were prepared from full thickness tissue. These membranes were mounted in static, horizontal Franz-type diffusion cells with an exposed surface area of approximately 1.3 cm^2. Treatment with the formulations A (n=8), B (n=8) and C (n=3) involved application of 10 µL/cm^2 and collection of receptor fluid samples at 12 and 24 hour intervals. The receptor fluid was analyzed for the presence of zinc by ICP-MS and electron microscopy (EM) was used to examine the tissue samples.

Results and conclusion

The analysis of particle size generation following MCP technology (high-energy dry milling) revealed similar results to the detection methods; TEM (15-40 nm), BET (30 nm), XRD (26 nm) and PCS (30 nm).

Results indicated penetration (over 24 hours) of zinc into the receptor fluid from untreated and placebo treated epidermal membranes as; 0.09 ± 0.04 and 0.22 ± 0.12 µg/cm^2, respectively. The amount of zinc found in the receptor fluid following application of the two test formulations was higher (although not statistically significant) after 24 hours exposure and the total amount found in the receptor phase was less than 0.03% of the applied dose. Analysis with electron microscopy revealed penetration of ZnO nanoparticles limited to the outer surface of the stratum corneum (SC) and loose, desquamating cells of the upper SC only. There was no evidence of penetration of nanoparticles in the lower SC layers or viable epidermis.

Fig. 2. Receptor phase penetration of zinc through human epidermal membrane as amount in microgramme per centimetre square for all treatments (a) and as percent of applied amount from the two ZnO-containing treatments (b). Columns and bars represent means and standard deviation. Grey column = 12 h, black column = 24 h; n = 8 treatments and n = 3 controls.
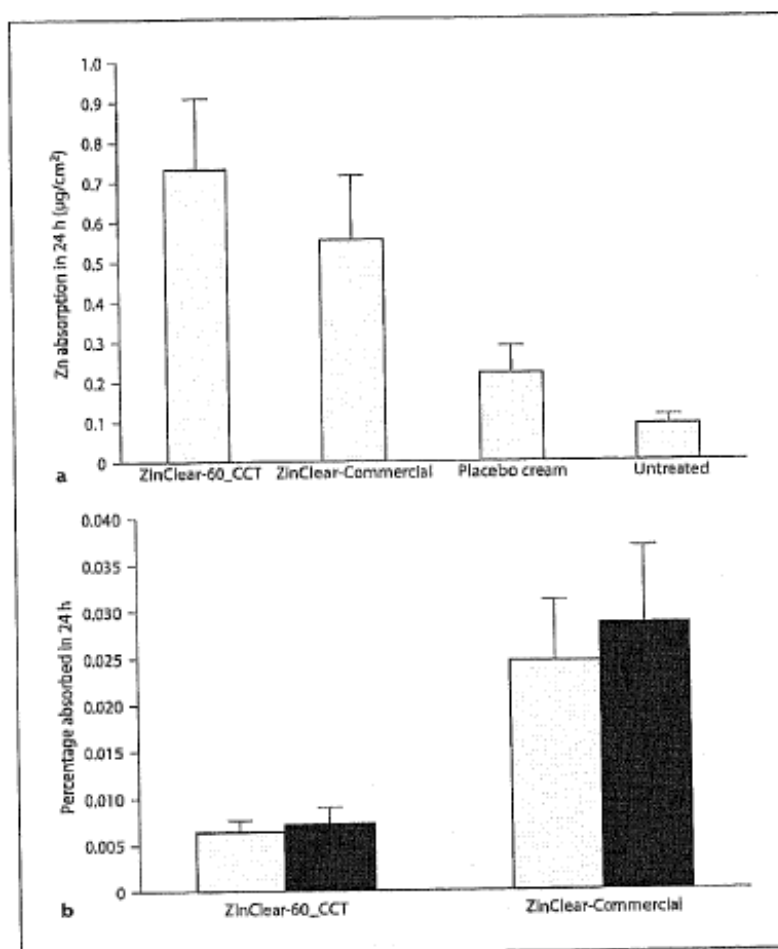
(Reference: 36)

Comment

For the ZnO used in this study, information on surface area and number of particles in dose was not provided. Information on dose expressed as surface area and number of particles was not provided.

In this publication, siliconate-coated ZnO was investigated as a 60% dispersion and as a typical sunscreen preparation containing 20% ZnO. The Zn in the receptor fluid was determined by elemental analysis of Zn using ICP-MS. Thus, it was not determined whether the low amount of Zn detected in the receptor fluid was present as solubilized Zn ions or as ZnO particles.

In the publication, the penetration of Zn++ using sunscreen formulations containing nano-particulate ZnO is reported to be very low (40–10 times lower) compared to other cosmetic formulations in the literature containing ionic zinc. It is stated in the publication that this supports the idea that nanoparticle formulation decreases the absorption of ZnO across the skin which is suggested to be due to a reduction in the amount of available solubilized Zn on the skin surface.

Based on the detection of Zn in the receptor fluid, the SCCS considers that 0.03% of the applied dose may be absorbed after topical application of ZnO on the skin.

Exploratory in vitro study for percutaneous skin

Study Design

Date of report: 2008

Guideline/method: Exploratory study on human skin penetration of sunscreen nanoparticles *in vitro.*

Test system: Human skin (excised abdominal or breast human skin from plastic surgery).

Test substance: Commercial sunscreen (Dr. Lewinn's private formula (19% W/W) with 26–30 nm mean size ZnO particles suspended in caprylic capric triglycerides (preservatives: 0.3% phenoxyethanol, 0.3% hydroxybenzoate).

Dose applied: 0.3 g of commercial product (see comment below).

Route: Topical application

Exposure time: 2–24 hours

GLP: No

Published: Yes

The distribution of topically applied nano-sized ZnO (mean primary particle size: 26–30 nm) in excised human skin after application of a commercial sunscreen was examined using multiphoton microscopy (MPM) imaging with a combination of scanning electron microscopy (SEM) and an energy-dispersive X-ray (EDX) technique. Abdominal or breast skin obtained following plastic surgery was used.

Results and conclusion

The cross-sectional imaging showed no evidence of nano-sized ZnO penetration into the cells or extracellular space.

(Reference: 120)

Comment

For the ZnO used in this study, information on surface area and number of particles per mass was not provided. Information on dose expressed as surface area and number of particles was not provided.

It is not stated in the publication whether the ZnO particles were coated or uncoated, but they appear to have been uncoated.

This paper also includes an *in vivo* experiment in humans (see 3.3.4.3). In the *in vivo* study 0.3g of the sunscreen formulation was applied to a skin surface of 50 cm^2 and rubbed in for 5 minutes. From the study description, it is not clear if an equivalent dosing was used in the *in vitro* experiment.

3.3.4.2	*In vitro* studies for dermal penetration of non-human skin

Exploratory in vitro study for percutaneous skin penetration

Study Design

Date of publication: 2006

Guideline/method:	OECD 428 and Guidance Document No. 28 and EU SCCNP opinion SCCNP/0750/03.
Test system:	Porcine skin
Test substance:	ZnO (Z-COTE uncoated), mean size of 80 nm (90% of the particles had a size below 160 nm).
Batch:	Not stated
Dose level:	Test formulation 4 mg/cm^2, ZnO 400 μg/cm^2 (Zn 360 μg/cm^2).
	Skin preparation: Skin from the lateral abdominal region of five month old domestic pigs of the Pietrain-Deutsche Landrasse-Hybrid strain, dermatomed to a thickness of around 500 μm.
Cells:	Eight intact skin preparations, each from three pigs + two vehicle controls + two untreated controls from each animal.
Skin temperature:	32 ± 1°C
Test chamber:	Static diffusion cells (Franz-type).
Route:	Topical application
Exposure time:	24 hours
Sampling time points:	3, 6, 12 and 24 hours
GLP:	Yes
Published:	Yes

The study was performed to evaluate possible skin penetration *in vitro* using porcine skin. Full skin thickness (epidermis and dermis) samples were incubated with ZnO preparations in modified Franz static dermal penetration cells.

The zinc oxide formulation was a white viscous oil/water emulsion, containing 10.3 wt% zinc oxide (corresponding to 8.3 wt% of zinc) as in an actual sunscreen formulation. The Z-COTE, uncoated microfine zinc oxide, had a mean primary particle size of 80 nm with 90% of the particles being <160 nm. Primary particles and loose agglomerates were present exclusively in the water phase and primary particles were often arranged around oil droplets.

ZnO test formulations were applied on the skin and samples of the receptor fluid were obtained at 3, 6, 12 and 24 hours. After 24 hours incubation the skin samples were removed and the presence of ZnO on the surface was evaluated using tape stripping. Zn levels in the samples (pooled tape strips, skin remaining after tape stripping, and all retained receptor fluid samples) were evaluated using Atomic Absorption Spectroscopy (AAS) or Inductively Coupled Plasma-Mass Spectroscopy (ICP-MS) depending on the concentration of the samples.

Results

The total Zn recoveries ranged from 102% to 107% with almost the total amount recovered in the first five tape strips.

The amounts of zinc found in the skin membrane and the receptor fluid were comparable in untreated, vehicle treated or zinc oxide treated skin preparations. In the receptor fluid, the levels observed for Zn for the skin samples of the three pigs were 0.8%, 0.8% and 1.4% of the applied dose, respectively. The absorbed dose was 1.5%, 1.6%, and 2.3% of the applied dose. Background levels in the receptor fluid of vehicle treated skin were similar for samples from three different pigs. In the skin, the Zn levels were 1.4%, 1.5% and 1.5%,

respectively. Untreated or vehicle treated skin preparations contained 3–5 µg of zinc corresponding to about 1% of the applied dose.

(Reference: 48)

Conclusion

From the data, the authors concluded that there was no penetration of ZnO particles or solubilized Zn through the stratum corneum of pig skin.

Comment

For the ZnO used in this study, information on surface area and number of particles per mass was not provided. Information on dose expressed as surface area and number of particles was not provided.

The mean size of the ZnO (Z-COTE uncoated) under investigation was 80 nm. It is unclear whether this mean size given is mass-based or number-based. It is also not known how the material used in this test relates to the Z-COTE formulation presented in the dossier as the size information given differs.

Exploratory in vitro study for percutaneous skin penetration

Study Design

Date of publication:	2011
Guideline/method:	Exploratory percutaneous skin penetration study *in vitro* after UVB radiation *in vivo* (sunburn simulation).
Test system:	Skin of weanling Yorkshire pigs.
Test substances:	O/W sunscreen formulations.
	A: Z-COTE HP1 (5% coated, CM 643; mean size 140 nm; SSA 12–24 m^2/g).
	B: Z-COTE (5% uncoated, CM 644; mean size 140 nm; SSA 12–24 m^2/g).
Batch:	Not stated (source: BASF SE, Germany).
UVB exposure:	Fiber optic UVB lamp (Lightningcure 200 UV-Spot light), on the day the pig was sedated and the hair clipped. The minimal erythemic dose (MED) was determined by sequential exposure to UVB light (30–120 mJ/cm^2, 6 – 24 seconds). On day 2 the pig was sedated and the exposed sites were analyzed to determine the UVB dose required to produce a minimal erythema response. The MED was determined to range between 40 and 50 mJ/cm^2.
UVB dose:	100, 110 and 120 mJ/cm^2 (to obtain a +2 erythema)
Dose level:	50 µL of each formulation on 0.64 cm^2 dermatomed pig skin A 2.5 mg dose of ZnO corresponds to a dose of 0.03–0.06 m^2, surface area on 0.64 cm².
Skin preparation:	Dermatomed pig skin (400–500 µm) biopsied 24 hours after the second UVB exposure inducing sunburn (= consistent +2 erythema).
Cells:	Formulation A and B: four with UVB exposed skin, two with unexposed skin.
Control:	Two with UVB exposed skin, two with unexposed skin.

Skin temperature: 37°C

Test chamber: Flow-through diffusion cells.

Route: Topical application

Exposure time: 24 hours

Sampling time points: Every 2 hours for the first 12 hours, every 4 hours thereafter up to 24 hours.

Examinations: Light microscopy (LM)

                        Transmission electron microscopy (TEM) plus X-ray microanalysis (EDS)

                        Scanning electron microscopy (SEM)

                        Time-of-flight secondary ion mass spectrometry (TOF-SIMS)

GLP: No

Published: Yes (abstract)

The purpose of this study was to determine whether skin damaged by UVB radiation inducing moderate sunburn with a +2 erythema reaction enhanced the penetration of TiO_2 or ZnO nanoparticles present in these sunscreen formulations. The pig was sedated and multiple sites (about 52) on the back were exposed to the UVB dose that caused a consistent +2 erythema (a pale red in a defined area of the skin). Twenty-four hours after UVB exposure, the pig was sedated, sites visually analyzed for consistency, and the skin prepared for *in vivo* or *in vitro* studies. For the *in vitro* studies, the UVB exposed and non exposed sites were dermatomed to a thickness of approximately 400–500 µm. The dermatomed skin was mounted in the flow-through diffusion cells with a dosing area of 0.64 cm^2 and maintained at 37°C. The skin was dosed with 50 µL of each formulation (CM 643: n=4 UVB exposed skin, n=2 unexposed skin; CM 644: n=4 UVB exposed skin, n=2 unexposed skin; and control: n=2 UVB exposed skin, n=2 unexposed skin). Upon completion of dosing, the perfusion was resumed and the perfusate collected every 2 hours for the first 12 hours and every 4 hours thereafter up to 24 hours. After 24 hours, the perfusion was terminated and the skin was removed from the diffusion cells. The dose site was removed with an 8 mm biopsy punch and cut into thirds. One third was placed in Trump's fixative and stored at 4°C for later processing by light microscopy (LM; flow-through 1 and 2 only) and transmission electron microscopy (TEM). The remaining third of the skin was cut in half and immediately frozen and stored at -20°C for later elemental analysis. The vials containing perfusate from each timed collection were capped and the samples immediately stored at 4°C.

Results

Light microscopy showed that UVB exposed skin showed focal intracellular epidermal edema, sunburn cells, dermal inflammation and focal microblister. In unexposed (UV light) skin residual sunscreen containing ZnO was limited to the stratum corneum. The morphology of the normal and the UVB exposed skin was not affected by topical treatment with the sunscreen formulations. The ZnO in each formulation was confirmed by TEM and elemental analysis. Energy dispersive X-ray spectroscopy (EDS) confirmed the presence of Zn indicating ZnO (from sunscreen) and Cu (from sample grids) in CM 643 and CM 644. In the pig skin from the flow-through studies, TEM/EDS found ZnO nanoparticles only on the surface of the stratum corneum on both the unexposed and UVB exposed skin, and first layers of the stratum corneum. Elemental analysis on the SEM confirmed the presence of ZnO within the stratum corneum and epidermis of the skin in the sunscreen treated samples. TOF-SIMS data indicated that ZnO did focally penetrate into the epidermis in both the normal and photo-damaged skin treated with the sunscreen formulations. However,

background Zn interference was present in some of the map overlays as determined by a region of interest analysis and the authors were not sure what caused the background in some samples.

Conclusion

Under the conditions of this study, UVB damaged skin did not enhance the penetration of Zn nanoparticles. TOF-SIMS indicated that Zn focally penetrated into the epidermis in both the UVB exposed and unexposed skin treated with the sunscreen formulations, but the observation remained inconclusive as background interference was also present. It was demonstrated by SEM and TEM that ZnO nanoparticles remained on the surface and upper stratum corneum layers in UVB exposed and unexposed skin, while ZnO particles were found to penetrate into the stratum corneum by TEM and Zn into the epidermis as demonstrated by TOF-SIMS. In any case, there was no evidence from these optical methods that the nanoparticles penetrated into the perfusate.

(References: 75, 76, AR18)

Comment

For the ZnO used in this study information on number of particles per mass was not provided. Information on dose expressed as number of particles was not provided.

It is not clear whether the mean size given is mass-based or number-based. It is also not known how the material used in this test relates to the Z-COTE formulation presented in the dossier as the size information given differs.

Exploratory in vitro study for percutaneous skin penetration

Study Design

Date of publication:	2009
Guideline/method:	Exploratory percutaneous skin penetration study *in vitro* using chemical enhancers for skin penetration.
Test system:	Skin of ten month old nude mice.
Test substances:	Purpose made ZnO nanoparticles, size ~10 nm as determined by TEM.
Batch:	Purpose made
Chemical enhancers:	Ethanol (EtOH) and oleic acid (OA) as enhancers for altering stratum corneum lipid structure; control sample, PBS.
Dose level:	50 µL of each formulation on 0.64 cm^2 dermatomed pig skin.
Skin preparation:	Removal of subcutaneous fat and mounting skin in Franz diffusion cells.
Test chamber:	Franz diffusion cells
Route:	Topical application with 400 µL of donor solution.
Exposure time:	12 hours
Examinations:	Dual channel multi photon microscopy
GLP:	No
Published:	Yes

This study investigated mechanistical aspects of chemically enhanced penetration into the stratum corneum using skin samples of four ten month old nude mice (BALB/c). ZnO nanoparticles were prepared from commercial zinc acetate dehydrate with a primary particle diameter of around 2 nm. However, an average particle diameter of 10 nm was measured. The absorption and emission peaks were reported at 370 and 525 nm. The non-linear polarization effect of second harmonic generation (SHG) was used for ZnO nanoparticles to be distinguished from the autofluorescence (AF) of the stratum corneum (SC) using dual-channel multi-photon microscopy. The study combined the SHG of ZnO nanoparticles and the AF of the SC to image the transdermal delivery of ZnO nanoparticles under the chemical enhancer conditions of oleic acid (OA), ethanol (EtOH) and oleic acid–ethanol (OA–EtOH). In addition to qualitative imaging, the microtransport properties of ZnO nanoparticles were quantified to give the enhancements of the vehicle to skin partition coefficient (K), the SHG intensity gradient (G) and the effective diffusion path length (L).

Results

The multilamellar lipid regions between the corneocytes were the pathways for ZnO nanoparticle delivery. With oleic acid, the transport of ZnO nanoparticles into the stratum corneum was facilitated by the phase-separated oleic acid domains, while the ethanol enhancer leached a significant amount of non-covalently bound amphiphilic stratum corneum lipids to modulate the skin barrier. As regards the oleic acid–ethanol donor solution, the increase of stratum corneum lipid fluidity associated with the oleic acid enhancer was offset by the effect of ethanol in loosening the stratum corneum lipid structure. Among the different chemical enhancer conditions, the oleic acid–ethanol combination was regarded as the most effective donor solution in transdermal delivery of ZnO nanoparticles into the stratum corneum.

Conclusion

The results showed that OA, EtOH and OA–EtOH were all capable of enhancing the transdermal delivery of ZnO nanoparticles compared to the PBS control by increasing the intercellular lipid fluidity or extracting lipids from the stratum corneum.

(Reference: 66)

Comment

For the ZnO used in this study information on surface area and number of particles per mass was not provided. Information on dose expressed as surface area and number of particles was not provided.

Apparently only penetration into the stratum corneum, but not into deeper skin layers was investigated.

3.3.4.3	*In vivo* studies for dermal absorption

Exploratory in vivo study for percutaneous skin penetration

Study Design

Guideline/method:	Comparative study according to internal laboratory methodology considering real use conditions, recommendation of US FDA and COLIPA SPF requirements.
Species:	Human
Group sizes:	TiA: eight volunteers (normal skin).
	TiB: nine volunteers (normal skin).

	TiHB: eight volunteers (normal skin)
	TiB: ten volunteers (stripped skin and occlusive patches).
	Six volunteers for basal elemental concentration in the skin.
Test substances:	Commercial products containing coated nano-sized ZnO:
	TiA: contained only TiO_2
	TiB: contained TiO_2 plus ZnO.
	TiHB: contained coated rutile TiO_2, size 20 nm.
Particle size:	Spherical ZnO, size 20–60 nm.
Dose applied:	Realistic use condition: 0.5–1.0 mg/cm^2 on an area of 25 cm^2.
SPF testing method:	2.0 mg/cm^2 on an area of 25 cm^2.
Skin:	Intact and tape-stripped human skin.
Skin temperature:	37°C
Exposure period:	2 hours (intact skin), 48 hours (tape-stripped skin).
GLP:	No
Date of report:	2009
Published:	Yes

The localization and possible skin penetration of nanoparticles dispersed in three sunscreen formulations under realistic use conditions was comparatively investigated in normal and altered skin. Commercial products containing nano-sized particles of coated TiO_2 and ZnO dispersed in hydrophobic emulsions were used. One product contained only TiO_2 (TiA), another TiO_2 plus ZnO (TiB) and a third material (TiHB) contained nanoparticles of the coated rutile form of TiO_2. The size and shape of nanoparticles in the three formulations were inspected with transmission electron microscopy and X-ray microanalysis. The ZnO nanoparticles were spherical and their size ranged from 20–60 nm. The application took into account relevant application conditions, such as the quantity of sunscreen applied and the duration between the applications recommended by the US FDA. The protocol consisted of an open test. The formulations were applied on the sacral region and buttocks for 2 hours using a sunscreen application rate of approximately 0.5–1.0 mg/cm^2 within an area of 25 cm^2 to reflect realistic use conditions. This condition was compared to standard FDA and COLIPA methods for the sun protection factor test (SPF), which recommend a thickness of sunscreen application of 2.0 mg/cm^2. TiB was applied to nine individuals, and TiA and TiHB to five individuals each. Penetration was also investigated in ten individuals under exaggerated exposure conditions, such as application to the skin after tape stripping and under occlusive patches (IQR chamber, 48 hour application). Tape stripping consisted of a series of strips until the tapes were free of corneocytes. A matched control group consisting of six individuals was used for the determination of basal elemental concentrations in the skin including Zn. Skin punch biopsies of 3 mm diameter were taken after application, quench-frozen and kept in containers until processing. One biopsy was taken from each volunteer. Sections of 14 μm thickness were cut from the frozen biopsy in a cryostat at -25°C. Biopsies were mounted in mounting medium for microscopy (OCT™ compound). Sections were obtained from the non-immersed portion of the tissue, and sectioning performed from inside to outside to avoid tissue contamination. Tissue integrity and the efficacy of corneocyte removal after tape stripping were checked by preparing intercalary stained sections for optical microscopy purposes. Scanning Transmission Ion Microscopy technique and Particle Induced X-ray Emission technique were used for detection of the localization of the ZnO particles by analyzing the presence of elemental Zn. The minimum detectable concentration of Zn in the skin was 0.10 μmol/g.

Results

The coverage of the outer skin layer with the TiB sunscreen formulation was homogeneously distributed. No differences were observed between the recommended SPF procedure (2.0 mg/cm^2) and the realistic use condition (0.5–1.0 mg/cm^2). Sunscreen formulations accumulated in skin wrinkles and depressions, as well as infundibulum cavities, but exogenous Zn remained on the outer layers of the keratinized tissue that enfold the follicle, i.e. outside the living skin. The penetration profiles of the nanoparticles obtained with the treated skin groups (TiA, TiB and TiHB) were all similar. The high levels of Zn observed at the outer layers of stratum corneum sharply decreased within the deeper layers to very low levels (Zn) in both protocols (SPF and realistic use condition).

By quantitatively integrating the imaged skin areas, the Zn concentration was extremely elevated in the stratum corneum (exogenous Zn). However, the Zn values decreased to physiological levels in the subcorneal epidermis and finally remained within a narrow interval of variation in this compartment with no differences between both application procedures.

The fit estimate of the Zn concentration decrease confirmed that most of the nanoparticles were confined to the stratum corneum outer layers and showed no significant penetration into the living cell layers. At approximately 80% of full stratum corneum thickness, the Zn concentration decreased to the physiological Zn level of <0.3 µmol/g. For the depth positions where Zn nanoparticles penetration ended, an estimated error of 10% was obtained which approximately corresponds to 0.5 µm for both application procedures.

Under non-physiological conditions using occlusive patches, there was no significant difference in the distribution and penetration depth profiling of the ZnO nanoparticles.

Conclusion

The results of the study demonstrated that profiles and localization of Zn nanoparticles could be accurately established *in vivo*. Following the 2 hour exposure period to a nano-sized ZnO-containing sunscreen on intact human skin using realistic use and SPF methods, detectable amounts of this physical UV-filter were only present at the skin surface and in the upper most stratum corneum regions. Layers deeper than the stratum corneum were devoid of exogenous Zn, even after 48 hours exposure to the sunscreen under occlusion. Deposition of ZnO nanoparticles in the openings of the pilosebaceous follicles was also observed but penetration of nanoparticles into viable skin tissue could not be detected.

(Reference: 46)

Comment

For the ZnO used in this study information on surface area and number of particles per mass was not provided. Information on dose expressed as surface area and number of particles was not provided.

Exploratory *in vivo* study for percutaneous skin penetration

Study Design

Guideline/method:	Exploratory study on human skin penetration of sunscreen nanoparticles.
Species:	Human
Group size:	Four volunteers (two Caucasian males, one Indian male, one Chinese female).

Test substances:	Commercial sunscreen (Dr. Lewinn's private formula (19% W/W) with 26–30 nm mean size ZnO particles suspended in caprylic capric triglycerides (preservatives: 0.3% phenoxyethanol, 0.3% hydroxybenzoate).
Dose applied:	0.3 g of commercial product.
Skin area:	50 cm^2 (forearm, cheek, shoulder or feet).
Skin:	Cleaned but otherwise untreated skin.
Route:	Topical application
Exposure time:	2–24 hours
GLP:	No
Date of report:	2008
Published:	Yes

The distribution of topically applied ZnO to human skin was examined by multiphoton microscopy (MPM) imaging with a combination of scanning electron microscopy (SEM) and an energy-dispersive X-ray (EDX) technique. A commercial sunscreen product containing nano-ZnO (mean particle size 26–30 nm) was used in this study. An area of 50 cm^2 of skin was selected on the forearm, cheek, shoulder or feet of four volunteers from different ethnic backgrounds. The selected area of skin was cleaned with ethanol prior to the investigation. An amount of approximately 0.3 g of the test sunscreen was applied to the selected skin areas and rubbed in for 5 minutes. Images were generated immediately, 4 hours after application and 24 hours after topical application. Application of the commercial sunscreen product was followed by an incubation period of 2 to 24 hours. Sections of treated skin were either tape-stripped (10–20 times) or left untouched prior to analysis.

Results

The MPM analysis showed that nano-ZnO particles stayed on the stratum corneum (SC) and accumulated into skin folds and/or hair follicle roots of human skin. The nano-sized ZnO predominantly remained on the outermost surface within a several-micrometer layer at all analysis points (at application, and after 4 and 24 hours). No penetration of nano-sized ZnO into the cells or extracellular space was observed and the 24 hour analysis showed complete removal of sunscreen from the skin. The test material localized in the hair follicle shaft did not spread to the neighbouring cells and tissue. High intensity, high resolution analysis (SEM/EDX) confirmed no noticeable presence of nano-sized ZnO in the epidermis. It was noted that nano-sized ZnO existed predominantly in an agglomerated phase.

Conclusion

In this study, it was demonstrated that after application of a commercial sunscreen formulation to human skin, the nano-sized ZnO remained either on the skin surface or stayed within the stratum corneum.

(Reference: 120)

Comment

For the ZnO used in this study information on surface area and number of particles per mass was not provided. Information on dose expressed as surface area and number of particles was not provided.

It is not stated in the publication whether the ZnO particles were coated or uncoated.

Exploratory in vivo study for percutaneous skin penetration

Study Design

Guideline/method:	Exploratory study on human skin penetration of sunscreen nanoparticles.
Species:	Human
Group size:	20 volunteers (11 in the nano-group, nine in the non-nano "bulk" group).
Test substance:	ZnO powder enriched to >99% 68Zn. Half of the stock was used to make nanoparticles with a final crystallite size of about 19 nm (± 8 nm; minimum 3 nm, maximum 60 nm) using a proprietary method based on high-energy attrition milling. Larger particles were also prepared with an average crystallite size of 110 nm (± 46 nm; minimum 25 nm, maximum 284 nm) produced by a modified version of the same method. The uncoated particles were incorporated into an oil-water formulation using a commercial process for preparing sunscreens. Both sunscreens contained ~20% wt/wt 68ZnO particles.
Dose applied:	Mean dose 4.3 mg/cm^2 (range 2.8–5.8 mg/cm^2) twice daily for five consecutive days (4.6 mg/cm^2 for the males and 3.7 mg/cm^2 for the females).
Skin area:	6 cm^2 on the back
Skin:	Back skin
Route:	Topical application
Exposure time:	Sampling at 24 hours each day and at 24 hours after day 5.
Evaluation:	Blood and urine samples, before, after and during the trial.
GLP:	No
Date of report:	2010
Published:	Yes (References: 59, 60, AR10, AR27)

An exploratory dermal penetration study was performed in human volunteers using the isotope approach for tracing potential absorption of Zn from ZnO nanoparticles applied to human skin under conditions of normal use. In total, three trials were performed. The first trial involved two male subjects with two applications to their backs of a formulation containing ZnO nanoparticles (with diameters of ~30 nm) enriched to 51% with 68Zn. The second trial involved a female in addition, and the same formulation was applied twice daily for five days. Blood was sampled in both trials at regular intervals for up to 126 days. These trials formed the basis and protocol refinement for the main trial in which particles of ZnO enriched to >99% with 68Zn were incorporated into a different formulation (Reference: AR10). Two groups, each consisting of ten people of various ages, skin classifications, and race, participated in the study at a beach. One group of ten volunteers was tested with a sunscreen containing nanoparticles of 68ZnO (about 20 nm) – the "nanoparticle" group. The other group was tested with particles of 68ZnO (>100 nm) – the "bulk" group. Sunscreen was applied to the backs of the volunteers twice daily for a period of five days and the subjects experienced a minimum of 1 hour UV exposure in two episodes following sunscreen application. Blood was sampled twice daily and urine three times daily. Blood and urine samples were also supplied before the five day beach exposure and in a follow-up period. Zinc was purified from blood and urine samples by ion exchange procedures. Changes in the isotopic abundance of 68Zn of the purified samples measured by the multi-collector were used to evaluate the dermal absorption of Zn from the sunscreens.

Results

The results from the first two trials showed changes of <0.1% in the isotope ratios in blood. The authors estimated that this limits the dermal absorption to <0.1%. In the beach trial, changes in the 68Zn/64Zn ratio in blood samples for the nanoparticle group ranged from 0.1 to 0.8% at the end of the beach trial and all subjects showed significant increases in the abundance of 68Zn six days after the completion of the trial. The changes in blood samples for the bulk group were similar to those for the nanoparticle group. Excluding the data for two outliers, there was no statistically significant difference in dermal absorption for the volunteers in the nanoparticle and the bulk groups; the mean increase was about 0.4%. However, in females a significant difference was observed between the nanoparticle and the bulk groups; the nanoparticle group absorbing more Zn. Urine samples showed larger increases in abundance of 68Zn over the same time intervals, but there was no simple relationship with changes in blood for the same volunteers. For the pretreatment (zero measurement) samples there was a very low variation between the 68Zn content in the blood between all volunteers. After ZnO exposure, an increase in variation was observed. An increase in Zn levels was also found in urine. There was a delay in the detection of 68Zn in the blood as it was detected for the first time at day 2 after the fourth application of the sunscreen in four individuals with a more rigorous follow up evaluation. The increase continued after stopping the application until at least day 11, which was the last day the samples were evaluated.

Conclusion

The authors concluded that these results provided evidence that Zn from ZnO particles in sunscreen penetrated healthy human skin. An additional factor for the Zn uptake might be the composition of the sunscreen formulation as it contained isopropyl-myristate, a known chemical enhancer of skin penetration. The total amounts of Zn absorbed are rather small when compared to the amounts of natural Zn normally present in the human body. For the male group with the highest absorption, the amount of Zn isotope tracer originating from the applied sunscreen ranged from 8.6–30.8 μg. These small amounts are in contrast to the average amount of Zn present in whole blood under physiological conditions (about 12 mg). It should also be noted that the blood values are very low in relation to the recommended daily values for the dietary intake of Zn of 8 mg for females and 11 mg for males as indicated in the paper. Overall, the amount of 68Zn tracer detected in the blood post-trial represents less than 0.001% of the applied dose. The study did not investigate whether the translocating Zn was present as nanoparticles or soluble Zn ions.

Comment

For the ZnO used in this study information on surface area and number of particles per mass was not provided. Information on dose expressed as surface area and number of particles was not provided.

The important findings of this study are: Zn uptake via healthy human skin was demonstrated; the total uptake was very low compared to the normal Zn levels in the body; there was a gender difference with females absorbing slightly more than males; and there was a delay in detection between application and detection time. The main limitation of the study is that it did not investigate whether the 68Zn was absorbed as ZnO nanoparticles or as 68Zn ions. The SCCS considers that the Zn that originated from the topically applied ZnO containin sunscreen was only a fraction of the amount of Zn present in the overall blood zinc pool. The pilot study preceding this exploratory study was published in 2012 (AR27). In the pilot study on three subjets a delayed absorption of Zn was observed which information was used for the design of the final study (AR10).

(References: 59, 60, AR10, AR27)

3.3.5   Repeated dose toxicity

3.3.5.1   Repeated dose (5 days) inhalation toxicity

Exploratory 5-day lung toxicity study

Study Design

Date of publication:	March 2010
Guideline/method:	Exploratory study with inhalation exposures according to OECD Guideline 412.
Species/strain:	Rat/Wistar
Group size:	17 males per group.
Test substance:	a) Z-COTE® HP1 coated with triethoxycaprylylsilane.
	b) Pigmentary zinc oxide powder.
Batch:	a) CNFC0701 (97.3 g Zn/100 g ZnO)
	b) S35583-206 (>99.9%, <1 µ)
Route:	Inhalation
Concentrations:	a) 0, 0.5, 2.5, 12.5 mg/m^3, corresponding to 22,126, 87,044, 233,360 particles/cm^3 (SMPS measurement during exposure).)
	Information on dose expressed as surface area was not provided.
	b) 0, 12.5 mg/m^3, corresponding to 219,031 particles/cm^3 (SMPS measurement during exposure).
Exposure period:	Five days
Frequency of exposure:	6 hours/day
Type of exposure:	Head-nose
Exposure conditions:	Head-nose exposure systems: aerodynamic exposure systems (INA 60, volume V ≈ 90 L, BASF SE).
Generator systems:	Solid particle generators and glass cyclonic separators.
Generation procedure:	The test substance was used unchanged. For each concentration, a solid particle feeder was used for generating the dust. The control group was exposed to conditioned air.
Observations:	The examinations were restricted to clinical observation, body weight data, broncho-alveolar lavage with clinico-chemical and cytological evaluation of lavage fluid, gross necropsy and histopathological examination of the respiratory tract.
Recovery period:	About 14 days
GLP:	Yes
Published:	No

The purpose of this study was to determine the pulmonary toxicity of Z-COTE® HP1 coated with triethoxycaprylylsilane in rats using a short-term bioassay including broncho-alveolar lavage with clinico-chemical and cytological evaluation of lavage fluid and serum, as well as pathological examination of the lung and pulmonary cell proliferation measurements. The

concentration response relationship should be established as well as a potential No Observed Adverse Effect Concentration (NOAEC). To elucidate the influence of particle size, pigmentary ZnO power was tested at one concentration, which is comparable to the high concentration of Z-COTE HP1. Seventeen male Wistar rats per test group and time point were head-nose exposed to respirable dusts for 6 hours per day, on five consecutive days. The target concentrations for Z-COTE® HP1 were: 0.5, 2.5 and 12.5 mg/m^3 and for pigmentary zinc oxide, powder < 1µ was: 12.5 mg/m^3. A concurrent control group was exposed to conditioned air. Animals were sacrificed on study days 4 and 25. On each sacrificing day, nine animals per group were designated for histopathological examination. Moreover, organ burdens were determined in three animals per group. On study days 7 and 28, the lungs of the five animals per group were lavaged, and the broncho-alveolar lavage fluid (BALF) was analyzed for markers indicative of injury of the broncho-alveolar region. On exposure days clinical examination was performed before, during and after exposure. During the post exposure period clinical findings were recorded once on each working day. The body weight of the animals was determined. The concentrations were produced with brush particle generators. The dust concentration was determined by gravimetrical measurements. Particle size was determined by gravimetrical cascade impactor measurements, optical particle counter and a scanning mobility particle sizer.

Results

The inhalation of Z-COTE® HP1 coated with triethoxycaprylylsilane for five days resulted in local inflammation in the lungs of the rats, indicated by changes in several parameters in the BALF and histological examinations.

Secondary to the effects in the lung, activation of the draining lymph nodes was noted. Moreover, minimal to moderate necrosis of the olfactory epithelium was observed. The effects occurred in a concentration-related manner and were reversible within the recovery period. Only a multifocal increase in alveolar macrophages was still present at the end of the recovery period. Similar effects were also observed in the animals exposed to pigmentary ZnO powder. At the lowest concentration of 0.55 mg/m^3, increased levels of a few mediators in the BALF and in serum were determined. Moreover, minimal (grade 1) multifocal necrosis of the olfactory epithelium was noted in the nasal cavity in one of the six animals treated with the lowest dose. Therefore, a No Observed Adverse Effect Concentration (NOAEC) could not be established in this study. The lowest concentration of 0.55 mg/m^3 is considered to be the Low Observed Adverse Effect Concentration (LOAEC).

Conclusion

ZnO induced a concentration-related inflammation reaction in the lung which was associated with dose-dependent increases in BALF markers. In addition to the inflammation reaction, necrosis was detected in the lung and the nose. There was no biologically relevant difference between the nano-sized and pigmentary ZnO. As ZnO is soluble in lung fluid and zinc ions are cytotoxic at higher concentrations necrosis can be attributed to the zinc ions dissolved from the ZnO particles. Likewise, elevated zinc levels were detected in various organs, most likely due to zinc ions dissolved from the ZnO particles. There was, however, no indication of systemic effects.

(References: 30, 71)

Comment

For the ZnO used in this study information on surface area and number of particles per mass was not provided. Information on dose expressed as surface area was not provided.

At the lowest dose investigated there were minimal alterations as indicated by the presence of an increased number of alveolar macrophages after the recovery period at day 25.

3.3.5.2	Repeated dose (28 days) dermal toxicity

Additional study in the open literature

Exploratory in vivo study on repeated dose dermal toxicity

Study Design

Guideline/method: Exploratory study on repeated dose dermal toxicity of zinc oxide nanoparticles based on OECD Guideline 410 with modifications for dose levels, biochemical parameters and measurement of collagen content.

Species: Sprague-Dawley rats

Group size: Ten animals (five males and five females).

Test substance ZnO nanoparticles were obtained from Nanostructured and Amorphous Materials Inc., USA, stock no. 5810HT, surface area 50 m^2 $50m^2$/g.

ZnO was obtained from Sigma-Aldrich, product no. ZO385.

Particle sizes: ZnO nanoparticles; 20 nm according to manufacturer; 63 nm by SEM evaluation; 224.7 nm by DLS in aqueous solution.

Dose applied: ZnO nanoparticle doses, 75, 180, and 360 mg/kg bw, surface area 3.75, 9, and 18 m^2/kg bw.,

ZnO 2,000 mg/kg bw.

Skin area: 10% of the total body surface

Route: Topical application

Exposure time: 6 hours per day for five days per week for a 28 day period.

Evaluation: Blood samples on day 29 and for satellite groups at day 42. Skin and tail for collagen content. Gross pathology and organ wet weight. Histopathology of organs.

GLP: No

Date of report: 2012

Published: Yes

In this exploratory study, rats were treated for five days per week at 10% of the total body skin area. Animals were treated for a total of 28 days and evaluated for toxicity at day 29. After a recovery period satellite groups were evaluated at day 42. The dosages administered were calculated to be similar to the daily uptake of nanomaterial per kg body weight per day. The average uptake (administration) for a woman was determined to be 12 mg/kg bw using a 2% concentration of ZnO in a sunscreen product, and a recommended use of 36,000 mg per day. Using conversion factors for the rat of 1, 2.5, and 5 and a body weight of approximately 180 g, the calculated doses were: 72 mg/kg bw (rounded to 75 mg/kg bw) for the low dose; 180 mg/kg bw for the intermediate dose; and 360 mg/kg bw for the high dose. Toxicity was evaluated at days 28 and 42. Hydroxyproline content of the skin and tail was estimated, and the collagen content was calculated.

Results

No significant changes were observed in the clinical chemistry parameters in both micro and nano zinc oxide treated rats. There were no statistically significant changes in the hematologic parameters compared with the control. A statistically significant increase in clotting time was observed in all treatment groups of nano zinc oxide compared to the micro-sized zinc oxide.

No gross pathology or histopathological lesions were observed in any of the organs investigated.

There was a significant decrease in the collagen content of the skin and the tail in all the nano ZnO treated groups of rats compared to the control, as well as with the micro-sized zinc oxide treated groups. The loss was higher in the skin than in the tail. There was an inverse dose relationship with the higher doses inducing a lower decrease. The decrease induced by the micro-sized ZnO (2,000 mg/kg bw) was similar to the decrease of the highest dose of nano-sized ZnO (360 mg/kg bw). A maximum decrease of more than 50% was observed in three treatment groups (skin 75 mg/kg bw males, 180 mg/kg bw males, and tail 75 mg/kg bw males).

Conclusion

The decrease in collagen content of the low dose group of nano ZnO was significant compared to the high dose group and control group in both the tail and the skin. It was suggested that this effect was due to potential skin penetration of ZnO nanoparticles due to partial dissolution, followed by induction of reactive oxygen species.

(Reference: AR23)

Comment

For the ZnO used in this study information on number of particles per mass was not provided. Information on dose expressed as number of particles was not provided.

This is the only study available on repeated dose toxicity in the skin. In general, no systemic effects were observed. The data indicate a decrease in skin collagen content. Although statistics were not presented in the paper, the data suggest statistically significant differences. The inverse dose response relationship for the decrease in the collagen content cannot be explained and needs further confirmation.

3.3.5.3	Repeated dose (30 days) oral toxicity

No data available.

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

No data available.

3.3.5.5	Chronic (>12 months) toxicity

No data available.

3.3.6	Mutagenicity/genotoxicity

3.3.6.1	Mutagenicity/genotoxicity *in vitro*

Bacterial gene mutation assays

Guideline/method: OECD Guideline 471 (1997)

Species/strain: *Salmonella typhimurium* strains TA 98, TA 100, TA 102, TA 1535, TA 1537.

Replicates: Triplicate plates, two independent experiments (SPT, PIT).

Test substance:	ZnO (Z-COTE® Max) (ZnO coated with dimethoxydiphenylsilanetriethoxy-caprylylsilane cross-polymer).
Batch:	FCGB0701
Purity:	100.4 g/100 g).
Solvent:	DMSO (standard plate test) or fetal calf serum (pre-incorporation test)
Concentrations:	Standard plate test (SPT): 0, 20, 100, 500, 2,500, 5,000 µg/plate both without and with metabolic activation (S9-mix)).
	Preincubation test (PIT): 0, 20, 100, 500, 2,500, 5,000 µg/plate (with/without metabolic activation (S9-mix)).
Treatment:	direct plate incorporation with 48 - 72 h incubation without and with S9-mix pre-incubation method was used with 20 minutes pre-incubation and at least 48 – 72 h incubation time both without and with S9-mix
GLP:	in compliance
Date of report:	March 2009

The test substance was tested for mutagenicity in the reverse mutation assay on bacteria with and without metabolic activation (S9-mix prepared from phenobarbital/β-naphthoflavone induced male Wistar rat liver) according to the standard plate test (SPT) and the plate incorporation test (PIT). The *Salmonella typhimurium* strains were exposed to the test substance dissolved in DMSO (SPT) or in fetal calf serum (PIT, added to avoid aggregate formation, and to mimic the protein containing body fluids (e.g. blood)) at concentrations ranging from 20–5,000 µg/plate. For control purposes, a sterility and solvent (DMSO) and positive controls (NOPD, MNNG, AAC, MIT.C, 2-AA) were also investigated. In the standard plate test the revertant colonies were counted after incubation at 37°C for 48 – 72 hours in the dark; in the preincubation test, the duration of preincubation was about 20 minutes whereas the revertant colonies were again counted after incubation at 37°C for 48 – 72 hours in the dark. Negative and positive controls were in accordance with the OECD guidelines.

Results

No sign of bacteriotoxicity seen as reduced his- background growth, a decrease in the number of his+ revertants, or reduction in the titer, was noted when tested up to the highest required concentration in the absence and presence of metabolic activation.
Precipitation of the test substance was recorded at and above 2,500 µg/plate with or without metabolic activation.
The test substance did not induce a biologically relevant increase in revertant colony numbers in the bacterial strains at any concentration tested in the presence or absence of metabolic activation compared to the background control. The sensitivity and validity of the test system used was demonstrated by the expected induction of a significantly increased number of revertants with the positive controls.

Conclusion

The test substance did not induce gene mutations in the bacterial strains used either in the presence or absence of S9-mix up to precipitation concentrations. Thus, it was shown to be non-mutagenic in this bacterial gene mutation test.

(Reference: 27)

Comment

For the ZnO used in this study information on surface area and number of particles per mass was not provided. For the exposures information on dose expressed as surface area and number of particles was not provided.

The positive control used represents a generally accepted chemical positive control for this test and demonstrated its performance. However it does not provide proof that a negative response of a nanomaterial/nanoparticle is really negative. There is presently no accepted nanoparticle positive control that demonstrates whether the assay is generally suitable for the mutagenicity testing of insoluble/poorly soluble nanoparticles.

The behaviour of the ZnO in the test was evaluated as indicated by the observation that there was precipitation at the two highest concentrations of ZnO investigated. Contact of bacterial DNA (*i.e.* nanoparticle uptake by bacteria) with the ZnO nanomaterials was not demonstrated. Negative findings in the Ames tests have also been shown for coated ZnO (i.e. tetramethylammoniumhydroxide-capped ZnO) (Reference: AR26). Bacterial mutagenicity assays are considered to be less appropriate for the testing of nanoparticles compared to mammalian cell systems (Reference: 72) due to the lack of endocytosis (Reference: AR4).

It is uncertain whether Zn ions that might be available from ZnO nanoparticles may cause mutagenicity in bacterial assays. Reachable intra-bacterial zinc concentrations will depend on the solubility and dissolution kinetics of ZnO nanoparticles (see section 3.1.6) and the available zinc transporter systems. Ionic zinc (e.g. zinc acetate) has tested negative in bacterial mutagenicity tests, while it tested positive in other mutagenicity tests (Reference: AR24).

The SCCS considers that the results of the used bacterial gene mutation assay can be considered negative. However, as there is no certainty on the exposure of the bacterial DNA to the added ZnO nanoparticles this negative result is of a limited value.

Comet assay in human epidermal cells

Guideline/method: According to published protocol

Species/strain: Human epidermal cell line (A431).

Replicates: Duplicate slides per tissue culture well

Test substance: ZnO nanopowder

Batch: No data

Purity: >99%), Sigma–Aldrich, St. Louis, MO, USA.

Solvent: DMEM

Concentrations: 0.001, 0.008, 0.08, 0.8, 5 µg/mL.

Exposure: cells were exposed for 6 hours

Sampling: immediately after the end of treatment

GLP: Not in compliance

Date of publication: January 2009

The DNA damaging potential of ZnO nanoparticles was investigated in a Comet assay in a human epidermal cell line (A431) according to a previously published protocol. At 24 hours after seeding, cells were exposed to different concentrations of ZnO nanoparticles (0.001,

0.008, 0.08, 0.8, 5 µg/mL) for 6 hours. Cell viability was determined with the MTT assay, LDH relase, NR uptake and trypan blue exclusion. After exposure two slides were prepared from each well (one well/concentration) for analysis. These 2 slides were exposed to alkali (pH>10), followed by electrophoresis for 25 minutes at 0.7 V/cm and 300 mA, stained with ethidium bromide and scored for comets. The Comet parameters used to measure DNA damage in the cells were % tail DNA (fraction of DNA in the tail) and Olive tail moment (OTM; arbitrary units, the product of the distance of DNA migration from the body of nuclear core and the total fraction of DNA in the tail). Images from 50 random cells (25 from each replicate slide) were analyzed for each experiment.

Results

The mean hydrodynamic diameter and zeta potential of the nanoparticle suspension in deionized water, as determined by dynamic light scattering (DLS) measurement, was 165 nm and −26 mV, respectively. The average size analyzed by transmission electron microscopy (TEM) was 30 nm. A decrease in cell viability was noted from a concentration of 0.8 µg/mL and higher at an exposure time of 24 h and 48 h. At an exposure time of 6 h, a decrease in viability occurred at 8 µg/mL and higher. The cell viability in the Comet assay exceeded 90% for all experimental groups before and after the treatment as assessed by Trypan blue dye exclusion assay.

A statistically significant and concentration dependent increase in DNA damage was observed compared to the control; in cells exposed to ZnO for 6 hours at 5 and 0.8 µg/mL. ZnO nanoparticles were also found to induce oxidative stress in cells indicated by depletion of glutathione (59% and 51%); catalase (64% and 55%) and superoxide dismutase (72% and 75%) at 0.8 and 0.08_g/ml respectively.

Effect of ZnO on Comet parameters in human epidermal cells (A431, means±SD):

Concentrations	Olive tail moment (arbitrary units)	Tail DNA (%)
Control	1.37 ± 0.12	8 ± 0.71
1 mM EMS	20.83 ± 0.53**	53.3 ± 1.2**
0.001 µg ZnO/ml	1.43 ± 0.17	9 ± 0.69
0.008 µg ZnO/ml	1.5 ± 0.1	$9,2 \pm 0.23$
0.08 µg ZnO/ml	1.83 ± 0.18	11 ± 0.91
0.8 µg ZnO/ml	2.13 ± 0.12*	10.6 ± 0.76*
5 µg ZnO/ml	2.33 ± 0.32*	13 ± 1.50*

* $p < 0.05$ when compared to control.
** $p < 0.001$ when compared to control.

Conclusion

Under the experimental conditions used ZnO nanopowder induced DNA damage in A431 cells and, consequently, ZnO nanopowder was genotoxic (clastogenic and/or mutagenic) in these cells. However, the authors emphasized that the observed genotoxic potential in human epidermal cells may be mediated through lipid peroxidation and oxidative stress.

(Reference: 94)

Comment

For the ZnO used in this study information on surface area and number of particles per mass was not provided. Information on dose expressed as surface area and number of particles was not provided.

S9-mix fraction was not mentioned and presumed not used. However, the usefulness of S9-mix fraction in assays investigating ZnO nanoparticles may be doubtful as ZnO nanoparticles will not be metabolised.

The use of two slides from a tissue culture well cannot be considered a replicate of two. It consists of one single incubation for which two read out slides were used.

The applicant stated that depletion of the anti-oxidative defence systems and cellular protection mechanisms of mitochondria *in situ* particularly under stress, is a major intracellular source of oxidative stress which is obviously no longer controlled by cellular mechanisms and therefore leads directly to DNA damage. This is therefore not related to nanoparticles *per se*, but is probably due to the imbalance of Zn ions under artificial *in vitro* conditions. The applicant thus concluded that the results are a set of cellular effects that are not linked to ZnO nanoparticles, but to Zn solubility under *in vitro* test conditions.

The authors suggest that the DNA damaging effects may be attributed to lipid peroxidation and oxidative stress as one of the probable causes. This is a plausible explanation for the induction of the observed DNA damage. However, the Zn ions were delivered by the ZnO nanoparticles in this test situation so the damage induced is of relevance for ZnO nanoformulations, irrespective of the mechanism.

In view of this, the SCCS concludes that the used ZnO nanoparticles were genotoxic in the comet assay with A431 human epidermal cells.

Additional studies in the open literature

Comet assay in human nasal mucosal cells

Guideline/method:	According to published protocol
Species/strain:	Human nasal mucosal cells obtained from ten surgery patients (three female and seven male).
Group size:	
Test substance:	ZnO nanoparticles (<100 nm, surface area 15–25 m^2/g) and ZnO powder (<5 µm) were obtained from Sigma–Aldrich (Steinheim, Germany). Sizes: mean longitudinal diameter of 86 ± 41 nm (mean ± SE) and a mean lateral diameter of 42 ± 21 nm (mean ± SE).
Batch:	Not stated
Purity:	Not stated
Dose levels:	Diluted nanoparticle or powder suspension at end concentrations of 0.01, 0.1, 5, 10 and 50 µg/mL in the well, corresponding to 0.15–-0.25, 1.5–-2.5, 75–-125, 150–-250, 750–1,250-1250 mm^2/mL in the well.
Route:	*In vitro*
Vehicle:	Distilled water
Exposure:	Single exposure of 24 hour.

Sampling: immediately after the end of treatment

GLP: Not in compliance

Date of report: 2011

The DNA damaging potential of nano-sized and micro-sized ZnO was investigated, in human mucosal cells. The cells were exposed to ZnO nanoparticles or ZnO powder dilutions for 24 h at 37°C in a humidified incubator with 5% CO_2. The MTT assay as well as the trypan blue exclusion test were performed in order to measure cell viability of human nasal mucosa cells after exposure to ZnO nanoparticles and ZnO powder. The cells were detached from the wells by trypsinization with trypsin–EDTA (0.05%) and centrifuged at 500g for 5 min. After removal of the supernatant, the cell pellet was resuspended at 10^5 cells/ml.

Slides were prepared and placed in the ice-cooled Plexiglas gel electrophoresis chamber (distance between electrodes: 30 cm) for 20 min. Electrophoresis was conducted for another 20 min at 25 V and 300 mA.. The following parameters were analyzed to quantify the induced DNA fragmentation: tail DNA (TD), tail length (TL), and Olive tail moment (OTM) as a product of the median migration distance and the percentage of DNA in the tail. The OTM was applied for statistical analysis.

Cells were prepared for TEM for ZnO particle uptake by the cells. Airway Epithelial Cell Growth-Medium (BEGM) served as negative control, and directly alkylating methyl methane sulphonate (MMS) at 100 µM was used as a reliable positive control of genotoxicity without cytotoxic effects.

Results

The frequency of cells with intracytoplasmatic ZnO nanoparticles was 10%, while particle transfer into the cell nucleus could be observed in 1.5% of the cells. While no cytotoxicity or genotoxicity was observed for the ZnO powder (<5 µm) in the tetrazolium (MTT) assay, in the trypan blue exclusion test, and in the Comet assay, cytotoxic effects were shown at a ZnO nanoparticles concentration of 50 µg/mL (P-value <0.01).
In comparison to the control, a ZnO nanoparticles concentration dependent increase in the Olive tail moment (OTM) as an indicator for genotoxic effects could be seen. The enhanced DNA migration was statistically significant at 10 µg/mL (P-value <0.05) and 50 µg/mL (P-value <0.01).
Also, the proinflammatory cytokine interleukin 8 secretion into the basolateral culture medium was increased at ZnO nanoparticles concentrations of 5 µg/mL. In contrast, the pigmentary grade ZnO did not induce cytotoxicity (MTT assay) or DNA damage (comet assay).

Conclusion

Under the experimental conditions the used ZnO nanoparticles induced DNA damage in human nasal mucosal cells and, consequently, ZnO nanoparticles was genotoxic (clastogenic and/or mutagenic) in these cells.

Under the same experimental conditions, the used ZnO powder did not induce DNA damage and, thus ZnO powder was not genotoxic (clastogenic and/or mutagenic) in these cells.

(Reference: AR11)

Comment

For the ZnO used in this study, information on number of particles per mass was not provided. Information on dose expressed as number of particles was not provided.

S9-mix fraction was not mentioned and presumably not used. However, the usefulness of S9-mix fraction in assays investigating ZnO nanoparticles may be doubtful as most ZnO nanoparticles will not be metabolised.

The SCCS agrees with the conclusion that the used ZnO nanoparticles were genotoxic in the comet assay with primary human nasal mucosal cells.

Comet assay in human nasal mucosal cells

Guideline/method:	According to published protocol
Species/strain:	Human nasal mucosal cells.
Group size:	Ten patients (seven female and three male).
Test substance:	ZnO nanoparticles (<100 nm, surface area 15–25 m^2/g) were obtained from Sigma–Aldrich (Steinheim, Germany).
Batch:	Not reported
Purity:	Not reported
Dose levels:	500 µL of diluted nanoparticle or powder suspension at end concentrations of 0.1, and 5 µg/mL in the well, corrersponding to 1.5–-2.5, and 75–-125 mm^2/mL in the well.
Route:	*In vitro*
Vehicle:	Distilled water
Exposure:	Repeated 3 consecutive exposures of 1 h with a washing step in between
Observation period:	
GLP:	Not in compliance
Published:	Yes
Study period:	27 hours
Date of report:	2011

To perform repetitive exposures, seven day old mini organ cultures (MOCs) (three-dimensional cultures of human nasal mucosa) were incubated with 0.1 and 5 µg/mL of ZnO NP suspension, without S9-mix, bronchial epithelium growth medium (BEGM), or methyl methanesulfonate (MMS) for 1 hour followed by a washing step with fresh BEGM. After the initial exposure step, the first cyto- and genotoxicity assays were performed in four MOCs for each concentration (control, 0.1 µg/mL, 5 µg/mL, MMS). The other 48 MOCs were again exposed to ZnO nanoparticles, BEGM, or MMS for another hour. This procedure was repeated one more time. Following the third exposure, the remaining 16 MOCs were further incubated in fresh BEGM for a 24 hour regeneration period. The experiment was performed on cells after 1 h exposure, after two consecutive 1 h exposures, after three consecutive 1 h exposures, and after three consecutive 1 h exposures followed by a regeneration period of 24 h.

Cells were prepared for TEM for ZnO particle uptake by the cells. Cell viability was determined by the trypan blue exclusion test.
After a 20-min DNA "unwinding"period, the electrophoresis was performed under standard conditions (25 V, 300 mA, distance between electrodes 30 cm) for 20 minutes. The following parameters were analyzed to quantify the induced DNA fragmentation: tail DNA (TD), tail length (TL), and Olive tail moment (OTM) as a product of the median

migration distance and the percentage of DNA in the tail. The OTM was applied for statistical analysis.

Results

ZnO nanoparticles had an oval shape with a mean longitudinal diameter of 76 ± 41 nm (mean ± SE) and a mean lateral diameter of 53 ± 22 nm (mean ± SE). After the exposure period it was observed that the ZnO nanoparticles were distributed to the cytoplasm and the nucleus of the cells in some of the cultures, but this was more pronounced for the 5 µg/mL exposure culture. There was no significant enhancement of DNA migration as determined by the Comet assay in nasal mucosa cells from MOCs exposed to 0.1 µg/mL of ZnO nanoparticles. At a ZnO nanoparticle concentration of 5 µg/mL, a significant increase in OTM was observed after each of one, two, and three consecutive 1 hour exposure periods compared to the control when measured directly after exposure. At both concentrations of ZnO nanoparticles, DNA fragmentation increased when measured at a 24 hours regeneration period after 3 repeated 1 hour exposures. A dose dependent increase in DNA fragmentation was present.

Conclusion

At both highest concentrations (5 µg/mL and 10 µg/mL) of ZnO-nanoparticles, DNA fragmentation increased after 24 hr of regeneration. In contrast, DNA damage which was induced by the positive control, methyl methanesulfonate, was significantly reduced after 24-hr regeneration.The authors concluded that the results suggest that repetitive exposure to low concentrations of ZnO nanoparticles results in persistent or ongoing DNA damage.

(Reference: AR12)

Comment

For the ZnO used, information on number of particles per mass was not provided. Information on dose expressed as number of particles was not provided.

Although it was suggested that a repeated exposure was used, the exposure was actually either 1, 2 or 3 hours with only a minimal non-exposed period with a washing step between the exposures.

S9-mix fraction was not mentioned and presumably not used. However, the usefulness of S9-mix fraction in assays investigating ZnO nanoparticles may be doubtful as most ZnO nanoparticles will not be metabolised.

The SCCS agrees with the conclusion that in this *in vitro* study, the ZnO exposure resulted in DNA damage. There fore, the ZnO was genotoxic in the used comet assay with human mucosal cells.

3.3.6.2	Mutagenicity/genotoxicity *in vivo*

Mouse Micronucleus Assay

Guideline/method: OECD Guideline 474 (1997), EC Regulation 440/2008

Species/strain: Mouse/NMRI

Group size: Five male mice per dose group/sacrifice time.

Test substance: ZnO (Z-COTE® HP1) (ZnO coated with triethoxycaprylylsilane).

Batch: CNFC0701

Dose levels: 0, 15, 30, 60 mg/kg bw

Application volume:	10 mL/kg body weight.
Exposure:	Single application
Route:	Intraperitoneal (ip) injection
Vehicle:	Fetal calf serum (FCS).
Sacrifice Times:	Micronucleus test: 24 hours post-dose (vehicle, positive controls, low, mid and high) and 48 hours post-dose (vehicle and high dose only).
Positive controls:	Cyclophosphamide (CPP): 20 mg/kg bw, vincristine sulfate (VCR): 0.15 mg/kg bw.
GLP:	Yes
Date of report:	August 2009

The potential of the test substance to cause chromosomal damage or spindle poison effects *in vivo* was investigated in the mouse bone marrow micronucleus test. Based on the results of a dose range-finding study, where mortality was observed at and above 80 mg/kg bw and where 60 mg/kg bw led to distinct clinical signs with no differences between sexes, each of the five male NMRI mice per dose group received a single intraperitoneal injection of the test substance suspension at 15, 30 or 60 mg/kg bw. Five male mice were used as controls receiving the vehicle (fetal calf serum), while each of the five positive control mice received 20 mg/kg bw cyclophospamide/kg bw or 0.15 mg/kg bw vincristine sulfate. In each case, the application volume was 10 mL/kg bw. All animals were observed for clinical signs of intoxication at regular intervals throughout the study period.

Bone marrow cells were harvested for evaluation of micronuclei at 24 hours post-dose (vehicle, positive controls, low, mid and high) and 48 hours post-dose (vehicle and high dose only).

Bone marrow for micronuclei examination was prepared, spread on a glass slide, fixed and stained with eosin and methylene blue, rinsed followed by staining with Giemsa solution and fixed in Corbit Balsam. Using a light microscope, the slides were evaluated. Two-thousand polychromatic erythrocytes (PCE) per animal were scored for the presence of micronuclei (10,000 per treatment group). The number of normochromatic erythrocytes (NCE) was also scored. The results obtained for the test groups, the negative and positive controls were compared to historical control data for validation purpose.

Results

The formulation analysis showed the correctness of the injected dose level and stability in the vehicle. The single intraperitoneal injection of the test substance preparations led to a dose-dependent increase in distinct clinical signs, while the animals of the negative and positive control groups revealed no findings. There were no statistical significances or biologically relevant differences in the number of erythrocytes containing micronuclei either between the vehicle control groups and the three dose groups, or between the two sacrifice intervals (24 and 48 hours). The number of normochromatic or polychromatic erythrocytes containing small micronuclei or large micronuclei did not deviate from the vehicle control values at any of the sacrifice intervals and was within the historical vehicle control data range. Both of the positive control substances, cyclophosphamide and vincristine sulfate, induced a statistically significant increase in the number of PCEs containing small and/or large micronuclei within the range of (or above) the historical positive control data indicating the suitability and sensitivity of the test system.

Conclusion

The test substance did not induce an increase in the number of polychromatic erythrocytes with micronuclei in the bone marrow of intraperitoneally treated mice and did not lead to any impairment of chromosome distribution in the course of mitosis. Thus, there was no indication for a clastogenic potential or any aneugenic activity *in vivo* under the conditions of the study.

(Reference: 26)

Comment

For the ZnO used in this study, information on surface area and number of particles per mass was not provided. Information on dose expressed as surface area and number of particles was not provided.

Intraperitoneal administration is proposed in OECD Guideline 474 but the text also states, *"If there is evidence that the test substance, or a reactive metabolite, will not reach the target tissue, it is not appropriate to use this test"*. For intraperitoneal ZnO, exposure of bone marrow to the nanoparticles has not been demonstrated, although it can be assumed that Zn ions released from the ZnO nanoparticles may have resulted in exposure of the bone marrow. The value of this test is thus limited.
The SCCS disagrees with the conclusion of the authors. This assay does not prove that ZnO nanoparticles lack clastogenic or aneugenic activity *in vivo* as exposure of the target organs was not demonstrated.

3.3.7 Carcinogenicity

No data available.

3.3.8 Reproductive toxicity

No data specific for ZnO nanoparticles have been submitted or identified from the open literature.

3.3.9 Toxicokinetics

Additional studies in the open literature

No specific data for ZnO nanoparticles have been submitted. However, an exploratory study was recently published indicating systemic availability of Zn after ZnO nanoparticles were administered orally and intraperitoneally.

ZnO nanoparticles were approximately 50 nm in size (TEM evaluation), which were compared to ZnO microparticles showing at least one diameter >100 nm (TEM evaluation). DLS evaluation for the ZnO microparticles showedi an average hydrodynamic diameter of 1,226 ± 120 nm, and for the ZnO nanoparticles an average hydrodynamic diameter of 93 ± 14 nm was reported. Information on surface area and number of particles per mass was not provided.

After oral and intraperitoneal administration of a single dose of 2.5 g/kg bw for both ZnO nanoparticles and microparticles, Zn could be observed in serum indicating uptake from the GI–tract. For ZnO nanoparticles the systemic availability was somewhat higher compared to that of ZnO microparticles as indicated by Zn measurements by ICP-MS. A higher distribution of Zn in the liver, spleen and lung was shown after treatment with ZnO NP compared to treatment with ZnO microparticles. Serum liver enzyme analysis (aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH)) indicated liver toxicity due to both ZnO NP and ZnO MP treatments which was confirmed by

histopathology. Histopathological lesions were only observed for ZnO NP treatments in the liver.

(Reference: AR16)

Comment

The results indicate a similar uptake, tissue distribution, and toxicity for ZnO nanoparticles and ZnO microparticles. As Zn was measured by ICP-MS, it cannot be concluded whether it was present as particulate material or as dissolved Zn ions. Although serum levels of aspartate aminotransferase, alanine aminotransferase, and lactyate dehydrogenase indicated similar liver toxicity for both ZnO nanoparticles and ZnO microparticles, histopathological lesions were only observed for ZnO nanoparticles in the liver.

3.3.10 Photo-induced toxicity

3.3.10.1   Phototoxicity/photoirritation and photosensitisation

Since submission I (2001) for ZnO, which served as a basis for the former opinion (SCCNFP opinion dated 24-25 June 2003, SCCNFP/0649/03, Reference: 87) and submission II (2005), no further studies have been performed or provided by the sponsors.

The studies presented in opinion SCCNFP/0649/03 (Reference: 87) did not indicate a photoirritant or photosensitizing potential of micronized ZnO (not further specified).

3.3.10.2   Phototoxicity/photomutagenicity/photoclastogenicity

Photomutagenicity in a Salmonella typhimurium Reverse Mutation Assay

Study Design

Date of report:	August 2006
Guideline/method:	Considerations on photochemical genotoxicity: Report of the International Workshop on Genotoxicity Test Procedures Working Group, 2000; Japanese Ministry of Health and Welfare: Guidelines for genotoxicity studies of drugs, 1999.
Species/strain:	*Salmonella typhimurium* TA1537, TA98, TA100, TA102.
Replicates:	Triplicate plates
Test substance:	FINEX-50 ZnO (supplied by Sakai Chemical Industry Co., primary particle size: 20 nm, non-coated).
Batch:	OZ52 (purity: ≥96% as indicated in the submission)
Concentrations:	Dose-ranging test: 5, 15, 50, 150, 500, 1,500, 5,000 µg/plate (with/without light irradiation).
Main test:	313, 625, 1,250, 2,500, 5,000 µg/plate (with/without light irradiation).
Vehicle:	DMSO
Irradiation:	
Source of light:	Sunlight simulator (SOL 500) equipped with a metal halide lamp with emission of a continuous spectrum of simulated sunlight (50% at 335 nm wavelength).
Intensity of irradiation:	1.6–1.7 mW/cm^2

Bacterial	Bacterial Strains	UVA dose (mJ/cm^2)
	TA 1537 (8 minutes 20 seconds)	0.80–0.85
	TA 98 (8 minutes 20 seconds)	0.80–0.85
	TA 100 (8 minutes 20 seconds)	0.80–0.85
	TA 102 (50 minutes)	4.8–5.1

Positive controls:	Without irradiation	TA 100: AF-2, 0.01 µg/plate
		TA 1537: 9AA, 80 µg/plate
		TA 98: AF-2, 0.1 µg/plate
		TA 102 MMC, 0.05 µg/plate
	With irradiation:	TA 98, TA 100, TA 1537: CPZ, 1.0 µg/plate
		TA 102: 8-MOP, 0.05 µg/plate

GLP:	No
Published:	No

The test substance was investigated for its potential to induce gene mutations under irradiation with simulated sunlight according to considerations on photochemical genotoxicity using the *Salmonella typhimurium strains* TA 1537, TA 98, TA 100, and TA 102. The tests were performed in the absence of S9 mix. Each concentration, including the controls, was tested in triplicate and at concentrations of 5, 15, 50, 150, 500, 1,500, 5,000 µg/plate in the dose range-finding part of the study and concentrations of 313, 625, 1,250, 2,500, 5,000 µg/plate in the main study in the absence of metabolic activation (S9 mix), under the light irradiation or without light irradiation. The test substance was dissolved in DMSO. Petri dishes containing the test substance suspension, test bacterial suspension and phosphate buffered saline were either irradiated with a sunlight simulator (SOL 500) or remained not irradiated. The irradiation time was the treatment time. After the treatments, aliquots of the mixtures were dispensed into test tubes, mixed with top agar, and spread on the minimum glucose agar plates. The plates were incubated at 37°C for 48 hours and revertant colonies on the plates were counted.

Results

The dose-range finding study was conducted at 7 dose levels, i.e., 5, 15, 50, 150, 500, 1500 and 5000µg ZnO/plate. Growth inhibition was not observed in each tester strain at any doses in both the light irradiated group and the light-unirradiated group. The plates incubated with the test item showed normal background growth up to 5,000 µg ZnO/plate in all strains used. In the main test no substantial increase in revertant colony numbers of any of the four tester strains was observed following treatment with the test substance under irradiation with simulated sunlight at any dose level. Also in the absence of irradiation no effects were found. In both the dose–range finding study and the main test, the precipitation of FINEX-50 zinc oxide was seen on the plate at 5000 and 2500 µg/plate in both the light-irradiated group and the light-unirradiated group

The sensitivity and validity of the test system used was demonstrated by the expected induction of a significantly increased number of revertants with the appropriate positive controls.

Conclusion

The test substance did not induce gene mutations both in the presence and absence of irradiation. It was therefore considered to be non-photomutagenic in this *Salmonella typhimurium* photomutagenicity test.

(Reference: 98)

Comment

For the ZnO used in this study, information on surface area and number of particles per mass was not provided. Information on dose expressed as surface area and number of particles was not provided.

The purity of the FINEX-50 used was indicated in the submission to be ≥96%. However, data on the purity is not provided in the physico-chemical characterization of the FINEX-50 (section 3.1.4), and was also not provided in the study reference 98.

S9-mix fraction was not used. However, the usefulness of S9-mix fraction in assays investigating ZnO nanoparticles may be doubtful as ZnO nanoparticles will not be metabolised.

Contact of bacterial DNA (i.e. nanoparticle uptake by bacteria) with the ZnO nanomaterials was not demonstrated. This test has been considered inappropriate for nanoparticles because bacteria do not take up nanoparticles due to the lack of endocytosis (Reference: AR4). However, Released Zn ions might be available as suggested for *in vitro* exploratory Comet assay.

The SCCS considers that the results can be considered negative in the used photomutagenicity bacterial gene mutation assay. However, as there is no certainty on the exposure of the bacterial DNA to the added ZnO nanoparticles, this negative result is of a limited value.

Photo-chromosomal aberration test with cultured mammalian cells

Study Design

Date of report:	August 2006
Guideline/method:	Considerations on photochemical genotoxicity: Report of the International Workshop on Genotoxicity Test Procedures Working Group, 2000; Japanese Ministry of Health and Welfare: Guidelines for genotoxicity studies of drugs, 1999
Species/strain:	Chinese Hamster lung fibroblasts (CHL cells).
Replicates:	Duplicate experiments.
Test substance:	FINEX-50 ZnO (supplied by Sakai Chemical Industry Co., primary particle size: 20 nm, non-coated).
Batch:	OZ52 (purity: ≥96% as indicated in the submission)
Concentrations:	Growth inhibition test: 9.77, 19.53, 39.06, 78.13, 156.25, 312.5, 625, 1,250, 2,500, 5,000 µg/mL (with/without light irradiation).
Chromosomal aberration test:	2.44, 4.88, 9.77, 19.53 µg/mL (with/without light irradiation).
Vehicle:	0.5% aqueous carboxymethyl-cellulose solution.
Irradiation:	

Source of light:	Sunlight simulator (SOL 500) equipped with a metal halide lamp with emission of a continuous spectrum of simulated sunlight (50% at 335 nm wavelength).
Intensity of irradiation:	1.6–1.7 mW/cm^2
UV doses:	4.8–5.1 J/cm^2
Preincubation time:	60 minutes
Irradiation time:	50 minutes
Recovery:	After irradiation reaction solutions were removed and the cells were further cultured for another 22 hours.
Negative control:	0.5% aqueous carboxymethyl-cellulose solution.
Positive controls:	With irradiation: 8-Methoxypsoralene (8-MOP), 0.01 µg/mL.
	Without irradiation: MNNG, 2 µg/mL.
GLP:	No
Published:	No

The cytotoxicity of the ZnO test substance was evaluated by measuring cell density. The cell density was determined as a percentage to the untreated control value (100%) by measuring the degree of staining on each dish with a single-layer culture cell densimeter. On the basis of the measured values, an approximate 50% inhibitory concentration (IC50) of the test substance was estimated. This assay was conducted in duplicate.

The test substance was investigated for its potential to induce structural chromosomal aberrations in Chinese Hamster lung fibroblasts (CHL cells) in the absence and the presence of simulated sunlight in two independent experiments. A sunlight simulator (SOL 500) equipped with a metal halide lamp with emission of a continuous spectrum of simulated sunlight (50% at 335 nm wavelength) was used as a light source. The cultures were pre-incubated with the test item for 60 minutes. After pre-incubation, the cultures were exposed to the solar simulator at an irradiation intensity of 1.6–1.7 mW/cm^2 resulting in a UVA dose of 4.8–5.1 J/cm^2. Thereafter, the cultures were washed. Corresponding cultures with the test item were kept in the dark for the 50 minute exposure period. The chromosomes were prepared 22 hours after washing. Two parallel cultures were investigated and at least 100 metaphase per plate were scored for structural chromosome aberrations in each culture.

Results

Cell growth inhibition test

The cell growth ratio was affected by precipitation of the test substance at 312.5 µg/mL and above in the cultures without irradiation, while no precipitation was observed at 156.25 µg/mL and below. The cell growth ratios by test substance were reduced to 31% at 19.5 µg/mL and 77% at 9.8 µg/mL. In the irradiated cultures, the cell growth ratio was affected by precipitation at 312.5 µg/mL and above, while no precipitation was observed at 156.25 µg/mL and below. Thus, concentrations of 19.5 µg/mL (as the maximum concentration), followed by serial 2-fold dilution to give 2.4, 4.9, 9.8 and 19.5 µg/mL were selected for the aberration test in the presence or absence of irradiation.

Chromosomal aberration test

The non-irradiated cultures revealed structural aberrations at a concentration of 19.5 µg/mL and a ratio of 13% between the non-irradiated and the irradiated groups was calculated, which was statistically significant positive. The ratios of numerical aberrations were not

statistically significant and increased at any dose level. With irradiation, the ratios of structural aberrations at 19.5 and 9.8 µg/mL were 17 and 14%, respectively, and attained statistical significance. The ratios of numerical aberrations were not affected at any dose.
In order to clarify whether the test substance was photo-clastogenic or not, the difference in the ratios of aberrations between the irradiated cultures and those which were not irradiated was analyzed, showing a significant increase at 9.8 µg/mL. The sensitivity of the system was demonstrated since the positive controls induced statistically significant increases in cells showing structural chromosome aberrations.

Conclusion

Under the conditions of the study, the test substance induced structural chromosome aberrations in the absence or presence of simulated sunlight as determined by the photo-chromosomal aberration test in Chinese Hamster lung fibroblasts. According to the authors, the test substance was also shown to be photo-clastogenic when tested up to cytotoxic concentrations. However, the study with non-, pre- or simultaneously irradiated mammalian cells (Reference: 37 below) showed that treatment with UV light resulted in an increased susceptibility to ZnO clastogenicity and thus, is not photoclastogenic *per se*, according to the applicant.

(Reference: 97)

Comment

For the ZnO used in this study, information on surface area and number of particles per mass was not provided. Information on dose expressed as surface area and number of particles was not provided.

The purity of the FINEX-50 used was indicated in the submission to be ≥96%. However, data on the purity is not provided in the physico-chemical characterization of the FINEX-50 (section 3.1.4), and was also not provided in the study reference 97.

S9-mix fraction was not mentioned and presumed not used. However, the usefulness of S9-mix fraction in assays investigating ZnO nanoparticles may be doubtful as ZnO nanoparticles will not be metabolised.

The study showed that test substance induced structural chromosome aberrations in the absence or presence of simulated sunlight. Without irradiation, the highest dose investigated (19.5 µg/mL) was positive in the test, while after irradiation a dose dependent positive response was seen for the two highest doses investigated (9.8 µg/mL and 19.5 µg/mL). Even if treatment with UV light increased the sensitivity of the cells, it is the presence of the ZnO nanoparticles that induced the chromosomal aberrations, and this cannot be ignored.

The SCCS considers that ZnO induced chromosomal aberrations in Chinese hamster lung fibroblasts both in the absence and presence of light irradiation.

Photo-chromosomal aberration test with non-, pre- or simultaneous irradiated mammalian cells

Study Design

Date of report: January 2007

Guideline/method: Requirements of the EMEA Committee for Proprietary Medicinal
                                    Products, Note for Guidance on Photosafety Testing, 27 June 2002,
                                    Considerations on Photochemical Genotoxicity: Report of the

	International Workshop on Genotoxicity Test Procedures Working Group. (Gocke et al. 2000).
Species/strain:	Chinese Hamster ovary (CHO) cells.
Replicates:	Duplicate cultures, two flasks (test substance, positive controls), four flasks (negative control).
Test substance:	ZnO, uncoated (Z-COTE®), size less than 200 nm according to materials and methods section (100 nm is mentioned in the abstract).
Batch:	EHDE0402 (purity: >99% (i.e. 100.1 g/100 g)).
Concentrations:	Phototoxicity range finder: 8.201, 13.67, 22.78, 37.97, 63.28, 105.5, 175.8, 293.0, 488.3, 813.8 µg/mL (with simultaneous and pre-imposed UV irradiation).
Chromosomal aberration test:	27.49, 34.36, 42.95, 53.69, 67.141, 83.89, 104.9, 131.1, 163.8,
	204.8, 256.0, 320.0, 400, 500 µg/mL (non-irradiated, with simultaneous and pre-imposed UV irradiation).
Vehicle:	McCoys 5A medium
Irradiation:	
Source of light:	Atlas Suntest CPS+ solar simulator (Heraeus Equipment Limited, Brentwood, UK). The intensity of UVA and UVB was measured using a Dr Gröbel RM 21 UV meter. The ratio of UVB:UVA was in the range of approximately 1:30.
UV doses:	Phototoxicity range finder: UVA: 400 and 800 mJ/cm^2.
Chromosomal aberration test:	UVA: 350 and 700 mJ/cm^2.
Groups:	
Non-irradiated:	Without irradiation
Pre-irradiated (PI):	Irradiation 2–3 hours (350 mJ/cm^2) or 1–2 hours (700 mJ/cm^2) prior to treatment with ZnO concentration.
Simultaneous irradiated (SI):	350 mJ/cm^2 and 700 mJ/cm^2 cultures received simultaneous treatment with ZnO concentration.
Incubation time:	Cells were exposed to ZnO for 3 hours, after washing, cells were harvested after a further incubation for 17 hours in tissue culture medium.
Negative control:	McCoys 5A medium.
Positive controls:	8-Methoxypsoralene (8-MOP): 0.5 and 1.0 µg/mL (with/without irradiation).
	4-Nitroquinoline-1-oxide (NQO): 0.25 and 0.3 µg/mL (without irradiation).
GLP:	Yes
Published:	Yes

ZnO was tested in an *in vitro* cytogenetic assay using duplicate cultures of Chinese Hamster Ovary (CHO) cells in the presence and absence of UV light to clarify whether the slightly pronounced clastogenicity *in vitro* in the dark is a genuine photo-genotoxic effect.. The

effect of ZnO exposure was investigated in the cells in the dark (D), under pre-imposed irradiation (PI, i.e. UV irradiation of cells followed by treatment with ZnO 1-3 hours later) and under simultaneous irradiation conditions (SI, i.e. ZnO treatment concurrent with UV irradiation).

Concentrations used in the main experiment were selected on the basis of cytotoxicity (expressed as a decrease in population doublings relative to controls). The number of cells/mL were measured in trypsinised samples of cell suspension using a Coulter counter. The highest concentration of ZnO used in the range-finder was 813.8 µg/mL (equivalent to 10 mM) for zinc. After three hours of exposure to ZnO or control chemicals, cell monolayers from all cultures were washed with sterile saline, and re-fed with fresh McCoy's 5A medium containing foetal calf serum and gentamycin, and incubated for 17 hours before cell harvesting. Approximately 1.5 h prior to harvest, colchicine was added to give a final concentration of approximately 1_g/mL to arrest dividing cells in metaphase.

At the defined sampling times, monolayers of these cultures were removed using trypsin/EDTA and a measurement of cell counts/mL was performed on an aliquot of cell suspension using a Coulter counter. The remaining cell suspensions from each flask were harvested and slides prepared for chromosome aberrations (CA) analysis using standard operating procedures.

Negative (solvent) control cultures were included in the test system under each treatment condition. 4-Nitroquinoline-1-oxide (NQO) and 8-methoxypsoralen (8-MOP) were employed as positive control chemicals in the absence and presence of UV light. The slides for CA analysis were prepared 17 hours after washing. Two parallel cultures were investigated and at least 100 metaphase plates were scored for structural chromosome aberrations in each culture.

Results

In the absence of UV light, the cytotoxicity of ZnO was concentration dependent, with 40-60% cytotoxicity observed in the concentration range 256–320 µg/mL. In SI cultures, the cytotoxicity was more pronounced than in the dark and related to the UV dose: 40-60% cytotoxicity was observed in the concentration range 131.1–256 µg/mL (350 mJ/cm^2) or 83.89–13 1.1 µg/mL (700 mJ/cm^2). Overall, cytotoxicity in SI cultures increased with ZnO concentrations, although a large variability in ZnO cytotoxicity was observed at the high UV dose (700 mJ/cm^2) and at high ZnO concentrations (≥ 204.8 µg/mL).

In PI cultures, cytotoxicity of ZnO was concentration dependent and comparable at the low UV dose (350 mJ/cm^2) to that observed in SI cultures (40-60% cytotoxicity was observed in the concentration range 104.9–256.0 µg/mL). At the high UV dose (700 mJ/cm^2) and at low ZnO concentrations (<204.8 µg/mL), cytotoxic effects of ZnO in PI cultures were intermediate between those observed in the dark and SI cultures, with 40-60% cytotoxicity observed in the concentration range 131–256 µg/mL. At higher concentrations (≥ 204.8 µg/mL), ZnO cytotoxicity was similar in the PI and SI cultures. For each individual irradiation dose and condition, the concentrations analyzed for CA (chromosome aberrations) covered a range of cytotoxicity from little or none, to maximum effects.

Treatment of cultures with ZnO in the absence of UV light resulted in statistically significant increases in the frequencies of cells with structural aberrations at 104.9 µg/mL (giving 13% cytotoxicity, as measured by population doubling) and above, in a concentration dependent manner. The frequencies of cells with structural aberrations (excluding gaps) exceeded the historical negative control (normal) range in both cultures analyzed at 163.8 µg/mL and above, and also in single cultures analyzed at several lower concentrations. These observations were considered biologically relevant and ZnO was considered clastogenic in the dark. Under SI conditions, treatment with ZnO induced biologically relevant increases in structural aberrations at 104.9 µg/mL (23% cytotoxicity) and 53.69 µg/mL (19% cytotoxicity) following 350 and 700 mJ/cm^2 UV radiation.

Under PI conditions, treatment with ZnO resulted in biologically relevant increases in the frequencies of cells with structural aberrations at 104.9 µg/mL and above (36% cytotoxicity) and at 53.69 µg/mL and above (0% cytotoxicity) following 350 and 700 mJ/cm^2 UV radiation, respectively. When compared at similar cytotoxic concentrations, the incidence of chromosome aberrations following PI or SI was generally similar at 700 mJ/cm^2.

The proportion of cells with structural aberrations treated with the vehicle (negative control) fell within historical solvent control ranges. Both treatments with the positive controls induced increases in the proportion of cells with structural aberrations. When added to cultures treated in the absence of UV light, 8-MOP induced frequencies of cells with structural aberrations that were similar to those seen in concurrent solvent control cultures (non irradiated). Thus, the validity and sensitivity of the test system was demonstrated.

Table. 5.9.2.1   Summary of results

Doses (µg/ml))	Non UV		Pre Irradiation 350 mJ/cm²		Simultaneous Irradiation 350 mJ/cm²		Pre Irradiation 700 mJ/cm²		Simultaneous Irradiation mJ/cm²	
	Tox.#	CA##	Tox.#	CA##	Tox.#	CA##	Tox.#	CA##	Tox.#	CA##
0	0	0.0100	0	0.0200	0	0.0100	0	0.0250	0	0.0600
27.49	0		12		3		0		0	
34.36	0		0		0		12		0	
42.95	0		13		19		0		0	
53.69	6	0.0350	14		13		0	0.0600*	19	0.1200*
67.11	0		21		0		29		31	
83.89	2	0.0350	18		0		14	0.1200***	38	0.2550***
104.9	13	0.0500*	36	0.0550**	23	0.1100***	31	0.1250***	58	0.2550***
131.1	25	0.0550**	28	0.1000***	37	0.1750***	41	0.270***	62	0.3500***
163.8	10	0.1200***	51	0.1150***	52	0.2500***	38	0.245***	90	0.4050***
204.8	21		56		48		43		34	
256	44	0.1400***	53	0.2300***	63	0.3450***	58	0.2700***	78	0.4500***
320	63	0.1550***	74	0.2500***	66	0.2250***	61	0.3650***	52	0.3300***
400	44		88		60		66		88	
500	100		72		100		100		77	

* p ≤ 0.05;   ** p ≤ 0.01; *** p ≤ 0.001 compared to concurrent controls; shaded concentrations analyzed, Tox# = cytotoxicity, CA## = proportion of chromosomal aberration

ZnO induced clastogenic activity in CHO cells, both in the absence and in the presence of UV light. The cytotoxicity of ZnO to CHO cells under the different irradiation conditions was as follows: SI > PI > D. The effects were more pronounced at 700 mJ/cm^2. The incidence of chromosome aberrations in SI or PI cells was generally higher than in the dark. At similar ZnO concentrations, SI conditions generally produced the greatest incidence of chromosome aberrations. However, at similar cytotoxic concentrations, the incidence of chromosome aberrations following PI or SI was generally similar for 700 mJ/cm^2.

Conclusion

The authors concluded that ZnO was clastogenic to CHO cells in the dark and after irradiation of the cellular test system, either prior to (PI conditions) or concurrently with (SI conditions). The treatment with UV light resulted in an increased susceptibility of CHO cells to ZnO clastogenicity, as indicated by clastogenic responses of higher magnitude and/or observed at lower concentrations in both SI and PI conditions when compared to those obtained in the dark.

Finally, the results provided evidence that, under conditions of *in vitro* photo-clastogenicity tests, UV irradiation of the cellular test system *per se* may produce a slight increase in the genotoxic potency of compounds that are clastogenic in the dark. Therefore, minor increases in clastogenic potency under conditions of photo-genotoxicity testing do not necessarily represent a photogenotoxic effect, but may occur due to an increased sensitivity of the test system subsequent to UV irradiation.

(References: 37, 67)

Comment

For the ZnO used in this study, information on surface area and number of particles per mass was not provided. Information on dose expressed as surface area and number of particles was not provided. The uptake of the ZnO by the exposed cells was not demonstrated.

S9-mix fraction was not mentioned and presumed not used. However, the usefulness of S9-mix fraction in assays investigating ZnO nanoparticles may be doubtful as ZnO nanoparticles will not be metabolised.

An increase in the frequency of cells with structural chromosomal aberrations was noted after UV irradiation both in simultaneous irradiation and in pre-irradiation culture conditions. In addition, clastogenic activity was also demonstrated without UV treatment. The observed clastogenic effect during irradiation may not be a photo-genotoxic effect of the ZnO as also in the pre-irradiated cultures (in which ZnO was not irradiated) a clastogenic effect was seen.

The SCCS consideres that based on the results presented ZnO has clastogenic activity in Chinese hamster ovary cells both in the presence and absence of UV.

Photo-micronucleus test in mice

Study Design

Date of report:	August 2008
Guideline/method:	Photo-micronucleus test in mice according to published methodology (Reference: AR13).
Species/strain:	Hairless mouse/HR-1
Group size:	Three male mice/group.
Test substance:	FINEX-50 ZnO (supplied by Sakai Chemical Industry Co., primary particle size: 20 nm, non-coated)
Batch:	OZ52 (purity: ≥96% as indicated in the submission)
Dose levels:	Preliminary toxicity study: 2.5%, 5%, 10%, 20% (w/v) concentration of the test sample in Aceton Olive Oil (AOO) (with irradiation).
Micronucleus test:	5%, 10%, 20% (w/v) concentration (with/without irradiation).
Vehicle:	Acetone/olive oil (4:1)
Exposure:	Once daily for two consecutive days.
Route:	Topical on the skin (open without occlusion of the test samples)
Application volume:	0.2 ml
Application area:	3 × 4 cm
Irradiation:	

Source of light:	Sunlight simulator (light emission equipment for animals (LDS-20, ABLE) with six xenon lamps) with emission of a continuous spectrum of simulated sunlight between 300–800 nm, visible light (48,800–67,600 lux).	
Intensity of irradiation:	UVA: 2.27–2.59 mW/cm^2	
	UVB: 88.6–17.2 µW/cm^2	
Irradiation time:	2 hours, once a day for two days at an interval of 24 hours.	
Negative control:	Acetone/olive oil (4:1, with/without irradiation).	
Positive controls:	With irradiation:	8-Methoxypsoralene (8-MOP), 0.0015%.
	Without irradiation:	Mitomycin C (MMC), 0.05%.
GLP:	No	

The ability of the test substance to cause photo-chromosomal damage *in vivo* was investigated in the photo-micronucleus test with mouse epidermal cells after topical application on the skin of male hairless HR-1 mice.

After a preliminary toxicity test with light irradiation to observe skin effects and to determine the maximum tolerated dose of the test article, the micronucleus test (with/without light irradiation) was carried out. In the preliminary toxicity test, concentrations of 2.5, 5, 10 and 20% (w/v) of the test article suspended in acetone/olive oil (4:1) were used.

The test article suspensions were applied on the dorsal skin of mice (3–4 cm) once a day for two days at an interval of 24 hours followed by light irradiation using a sunlight simulator (Light Emission Equipment for Animals, LDS-20: with six xenon lamps, ABLE), within 30 minutes after each application. As neither abnormal signs nor dead animals were observed in the dosing groups, concentrations of 5%, 10% and 20% were selected for the micronucleus test.

The micronucleus test was carried out with ten experimental groups of mice, five with and five without light irradiation: the negative control groups [vehicle (acetone/olive oil, 4:1)], the test article groups [5, 10 and 20 % (w/v)] and the positive control groups (with light irradiation: 8-methoxypsoralen; without light irradiation: Mitomycin C).

The dosing formulation (0.2 mL) was applied on the dorsal skin of mice once a day for two days at an interval of 24 hours. For the light-irradiated group, the mice were exposed to light from the sunlight simulator within 30 minutes after each application for 2 hours (UVA intensity: 2.27–2.59 mW/cm^2, UVB intensity: 88.6–117.2 µW/cm^2). For the non-light irradiated group, the mice were returned to their home cages immediately after application and kept in the animal room under white fluorescent light.

All animals were euthanized about 48 hours after the last treatment. The skin was processed and epidermal cell suspensions were prepared. The cell suspensions were placed on glass slides, dried and stained with acridine orange solution. Each of the 2,000 epidermal cells were examined with a fluorescence microscope and the number of micronucleated epidermal cells was recorded.

Results

In the preliminary toxicity test, after the topical application on the skin, abnormal behavioral signs or dead animals as indication for toxicity were not observed, so the test sample did not induce overt toxicity in the used application.

The fluorescence microscopic observation revealed no test substance (ZnO) related increase in the number of micronucleated epidermal cells outside the historical control data, neither with nor without UVA and UVB light irradiation.

In the vehicle group the incidence of micronucleated cells did not exceed the historical control group ranges, while the respective positive controls induced a significant increase in the number of micronucleated epidermal cells with and without simulated light irradiation indicating the suitability and sensitivity of the test system.

Conclusion

The test substance did not increase the incidence of micronucleated epidermal cells in exposed male mice with or without simulated light irradiation. From these results, it was concluded that FINEX-50 ZnO Batch OZ52 (purity: ≥96%) is not clastogenic or photo-clastogenic in epidermal cells of mice under these experimental conditions.

(Reference: 100)

Comment

For the ZnO used in this study, information on surface area and number of particles per mass was not provided. Information on dose expressed as surface area and number of particles was not provided.

The purity of the FINEX-50 used was indicated in the submission to be ≥96%. However, data on the purity is not provided in the physico-chemical characterization of the FINEX-50 (section 3.1.4). The submitted study report in reference 100 indicates a content of 100%.

A different product/batch is mentioned in the reference to that included in the dossier. Product/batch 05M099 is mentioned in the results and conclusions, but not in materials and methods. Product/batch 05M99 (lot no. 2Z51, content 100%) is mentioned in Reference: 100.

The ZnO was applied topically on the skin. Uptake by living cells in the skin was not investigated. As there is no information whether the substance reaches the target cells (or the nucleus), the value of this test is very limited.

The results show that the ZnO tested did not increase the number of micronuclei in the skin after topical application indicating that the ZnO is not a positive mutagenic substance. However, from the negative results it cannot be concluded that ZnO is non-mutagenic.

The SCCS considers the value of this test limited.

3.3.11 Human data

Comparative human inhalation study

Study Design

Date of publication:	February 2005
Guideline/method:	According to internal laboratory methodology.
Species:	Human
Group size:	12 volunteers (six males and six females, one male/female in each exposure sequence).
Test substance:	A) Fine ZnO particles (A) ZnO, coated (Zano 10 Plus, Umicore) (former Zano® Plus, ZnO coated with octyl triethoxysilane).
	B) Ultrafine ZnO particles.

ZnO particles were generated by an electric arc discharge system between two consumable zinc electrodes.

Particles sizes:	For the ultrafine particle exposures, the count median diameter was 40.4 ± 2.7 nm geometric standard deviation (GSD) 1.7, whereas for the fine particle exposures, the count median diameter was 291.2 ± 20.2 nm GSD 1.7.
Batch:	Not applicable
Route:	Inhalation (by mouth piece)
Frequency:	2 hour inhalation at rest on three exposure days.
Concentration:	500 µg/m^3 fine and ultrafine ZnO particles, or 4.6×10^{10}6x10^{10}/m^3 and $1.9 \times 10^8$9x10^8/m^3 particles, respectively, as measured during exposure.
Examinations:	Clinical symptoms, heart rate, blood pressure, mouth temperature, oxygen saturation at rest, differential blood cell counts, expression of activation markers and adhesion molecules, coagulation factors, inflammation markers, electrocardiogram (ECG) parameters, sputum induction.
GLP:	No
Published:	Yes

A comparative exploratory inhalation study was performed in 12 human volunteers (six male and six female, age range 23–52 years) with ultrafine and fine ZnO particles generated by an electric arc discharge system between two consumable zinc electrodes. The exposure response relationships for respiratory, hematologic, and cardiovascular endpoints between ultrafine and accumulation mode zinc oxide particles were compared. Twelve healthy adults inhaled 500 µg/m^3 of ultrafine zinc oxide, the same mass of fine zinc oxide, and filtered air while at rest for 2 hours. Pre-exposure and follow-up studies of symptoms, leukocyte surface markers, homeostasis, and cardiac electrophysiology were conducted up to 24 hours post-exposure. Induced sputum was sampled 24 hours after exposure.

Results

Mean ZnO inhalation exposure results (N = 12 volunteers):

Time Range (min)	TEOM Mass Concentration (µg/m³)	Filter Mass Concentration (µg/m³)	Inspired Particle No. Concentration (particles/cm³ × 10⁶)	Expired Particle No. Concentration (particles/cm³ × 10⁶)	No. Deposition Fraction
Ultrafine					
0–30	483.5 ± 89.5	536.0 ± 60.0	46.1 ± 5.6	10.0 ± 2.4	0.78
30–60	480.4 ± 81.6	516.5 ± 46.5	48.8 ± 7.6	10.1 ± 2.7	0.79
60–90	465.8 ± 60.3	515.8 ± 72.5	48.9 ± 6.9	9.8 ± 2.6	0.80
90–120	470.1 ± 70.9	517.3 ± 71.1	48.3 ± 6.8	9.6 ± 2.6	0.80
Fine					
0–30	461.1 ± 39.1	473.7 ± 49.3	0.191 ± 0.015	0.12 ± 0.11	0.33
30–60	464.1 ± 47.5	466.9 ± 73.9	0.191 ± 0.013	0.12 ± 0.10	0.35
60–90	452.5 ± 35.5	468.8 ± 58.1	0.193 ± 0.013	0.12 ± 0.07	0.35
90–120	452.9 ± 40.6	513.7 ± 60.9	0.192 ± 0.014	0.12 ± 0.07	0.36

TEOM: tapered elemental oscillating microbalance.

Frequency distribution of inhaled zinc oxide particles for the two exposures conditions (*left*: ultrafine zinc oxide; *right*: fine or accumulation mode zinc oxide) as measured by number concentration (number of particles per volume of air) measured in the inspiratory circuit of the human mouthpiece exposure system during the studies is shown below.
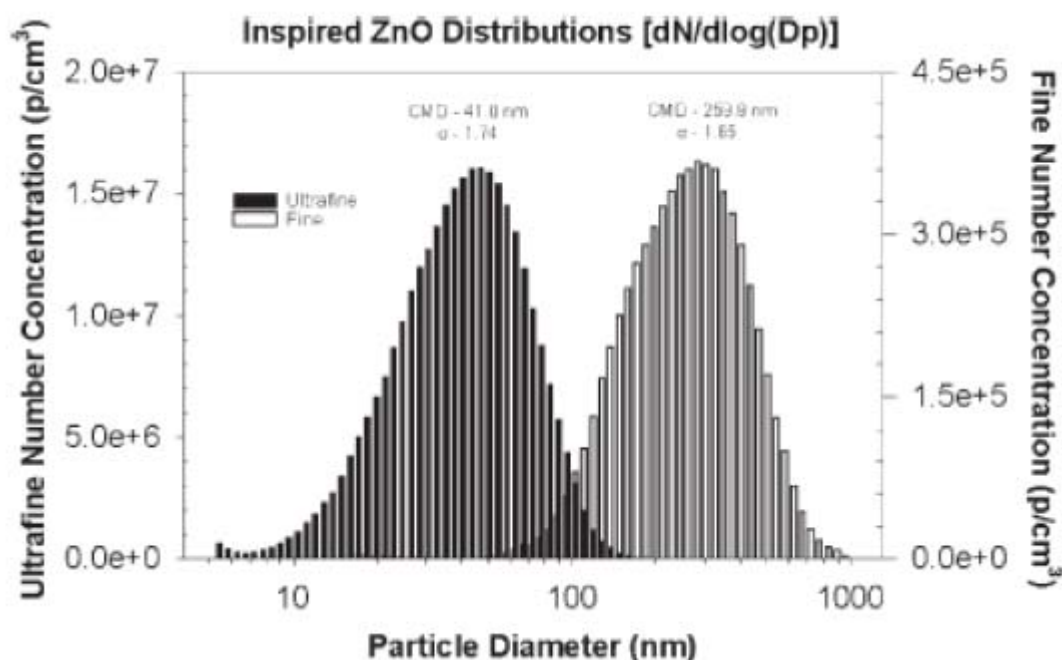
CMD: count median diameter; dN/dlog(Dp): normalized particle number concentration, P/cm^3: particles per cubic centimeter of inspired air. Overlapping distributions at a particle diameter of 100 nm indicate that in ultrafine exposures there was a small percentage by number above the ultrafine size. No differences were observed after exposure to the fine or ultrafine ZnO particles when compared to clean filtered air conditions at this level of exposure (500 µg/m^3).

Conclusion

Freshly generated zinc oxide in the fine or ultrafine fractions inhaled by healthy subjects at rest at a concentration of 500 µg/m^3 for 2 hours is below the threshold for acute systemic effects as detected by these endpoints.

(Reference: 32)

Comment

For the ZnO used in this study, information on surface area and number of particles per mass was not provided. Information on dose expressed as surface area was not provided.

The ZnO materials given as test substances in the dossier (A and B in the table) were not used in the study.

Reference 32 only describes the effect of ZnO generated at the research facility itself. Ultrafine and fine ZnO were generated by an electric arc discharge system (Palas generator; Palas, Karlsruhe, Germany) between two consumable zinc electrodes. The ZnO materials used in the reference do not relate to any of the ZnO materials submitted and included in the dossier by the applicant.

In view of the different type of ZnO used (spark generated) in the study, the SCCS considers this study not relevant for the evaluation of the use of ZnO as cosmetic ingredient.

3.3.12 Special investigations

Experimental study on toxicity after IV administration

Study Design

|	
Date of report:	December 2009
Guideline/method:	Study according to internal laboratory methodology for investigative purposes considering OECD Guideline 407, EC Commission Regulation No. 440/2008, EC Commission Directive 87/302/EEC, OECD Guideline 417
Species/strain:	Rat/Wistar (Crl:WI (Han))
Group size:	Groups of five male and five female rats.
Test substances:	a) ZnO nanoscale, coated with triethoxyoctylsilane (Z-COTE®HP1)
	b) ZnO, nanoscale uncoated (Z-COTE®)
	c) ZnO pigment
	d) Zinc sulfate
Batches:	a) CNBG1602 (ZnO >97.1%, triethoxyoctylsilane 3.4%)
	b) EHFA0907
	c) S35583-206 (>99%)
	d) 02616TC-126 (100%)
Dose levels:	a) 1 or 5 mg/kg bw (subsets 0, 1, 2), single administration day 28
	b) 1 or 5 mg/kg bw (subsets 0, 1, 2)
	c) 5 mg/kg bw (subsets 0, 1, 2)
	d) 17.6 mg/kg bw (subset 2) or 4 × 4.4 mg/kg bw on days 0, 7, 14, 21 (subsets 0, 2) as an equimolar zinc ion concentration related to the dose level of 5 mg/kg bw of ZnO.
Exposure:	I) Single application for test item groups a, b and c (subsets 0, 1, 2) and test item group d (subset 1).
	II) Four applications on study days 0, 7, 14 and 21 for test item group d (subsets 0, 2).
Route:	Intravenous
Application volume:	1 mL/kg bw
Vehicle:	a–c) Inactivated calf serum
	d) Physiological (0.9%) NaCl solution
	Application and sampling time-points: subset 0 (five males/five females per group): application on study day 0, urine sampling on study days 24/25, blood sampling and necropsy on study day 29.
	Subset 1 (five males per group): application on study day 28, blood sampling and perfusion fixation on study day 29.
	Subset 2 (five males per group): application on study day 0, perfusion fixation on study day 29.

GLP: Yes

The study was divided into separate subsets according to the dosing and sampling schedule.

- Subset 0: ZnO coated and uncoated, and pigment ZnO dosing at day 0 and sampling at days 24/25 (urine) and day 29 (blood). As control Zn sulfate was used that was administered at days 0–7–14–21 and sampled on the same days.

- Subset 1: ZnO coated and uncoated, pigment ZnO and Zn sulfate on day 28 and sampling at day 29 (day 1 after exposure).

- Subset 2: Same as subset 0 treatment at day 0, but animals were sacrificed for histopathology after perfusion fixation at day 29.

The total dose of Zn sulphate (17.6 mg/kg bw) was administered in four dosages of 4.4 mg/kg bw at days 0, 7, 14, and 28.

The treatment schedule is presented below.
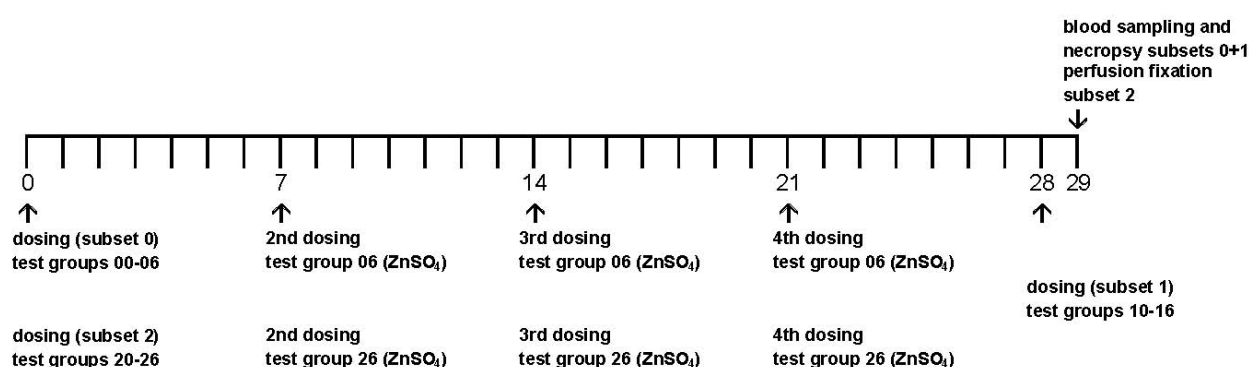
Results

The analysis confirmed the homogenous distribution of the test items in the vehicles and the correctness of the concentrations.

Significantly increased body weights were determined on study day 28 for female animals in test group 2 (zinc oxide [nanoscale, coated], 5 mg/kg bw) and test group 3 (zinc oxide [nanoscale, uncoated], 1 mg/kg bw), as well as on study days 21 and 28 for female animals in test group 5 (zinc oxide [pigment], 5 mg/kg bw) compared to the control. For male animals in test group 26 (zinc sulfate, 4 × 4.4 mg/kg bw), a significantly lower mean body weight was detected on study day 21.

Significantly lower mean body weight changes were determined on study days 7, 14 and 21 for male animals in test group 14 (zinc oxide [nanoscale, uncoated], 5 mg/kg bw).

Significantly higher mean body weight change was determined on study day 28 for female animals in test group 2 (zinc oxide [nanoscale, coated], 5 mg/kg bw).

Significant effects on body weight were noted in various groups after ZnO treatment compared to the vehicle (calf serum inactivated, 1 ml/kg bw) control animals.

Test group 2, females, ZnO nanoparticles, coated, 5 mg/kg bw, single dose day 0, increased bw day 28.

Test group 3, females, ZnO nanoparticles, uncoated, 1 mg/kg bw, single dose day 0, increased bw day 28.

Test group 5, females, ZnO-pigment, 5 mg/kg bw), single dose day 0, increased bw days 21 and 28.

Test group 26, males, Zn-sulfate, dose 4 × 4.4 mg/kg bw, decreased bw day 21.

Test group 14, males, ZnO nanoparticles, uncoated, 5 mg/kg bw, decreased bw days 7, 14, and 21.

The values reflect the normal range of biological variation inherent in the strain of rats used for this study and, therefore, were assessed as incidental.

There was no mortality, no clinical findings, no effect on food consumption or body weight gain, no impaired organ weight and no gross pathological findings in the organs of any of the investigated in either group in the respective subsets, irrespective of whether the animals were killed after one day or after four weeks following the single (ZnO) or four (zinc sulfate) intravenous test item injections. In addition, the male and female animals of subset 0 (ZnO coated and uncoated, and pigment ZnO) showed no treatment related findings in the urinalysis and the comprehensive clinical pathology examinations including additional serum parameters (haptoglobulin, alpha2-macroglobulin, troponin I) at the end of the observation period of four weeks.

However, the animals of subset 1, which were killed and investigated by clinical pathology at day 1 after injection of the test items, revealed some minor deviations in single parameters of differential blood count, enzymes, clinical chemistry and the additional serum parameter haptoglobin. See table below (5.12.1.).

Table 5.12.1   Subset 1 – Variations in clinical pathology parameters in male rats one day after intravenous injection of test items, mean±SD:

Parameter	Gr 10 Control	Gr 11 ZnO coat 1 mg/kg	Gr 12 ZnO coat 5 mg/kg	Gr 13 ZnO uncoat 1 mg/kg	Gr 14 ZnO uncoat 5 mg/kg	Gr 15 pig ZnO 5 mg/kg	Gr 16 ZnSO4 17.6 mg/kg
Monocyte giga/l	0.1±0.03	0.11±0.04	0.25**±0.08	0.17*0.06	0.14± 0.04	0.14±0.06	0.19±0.09
Monocyte %	1.5±0.4	1.9±0.6	3.8**±1.3	2.8**±0.6	2.6**±0.4	2.7±1.0	2.8±1.2
LUC giga/l	0.03±0.01	0.04±0.02	0.08**±0.02	0.05±0.04	0.06±0.04	0.05±0.02	0.04±0.01
LUC %	0.5±0.1	0.5±0.2	1.3**±0.3	0.9±0.4	1.1±0.4	1.1*±0.4	0.7±0.1
AST μkat/l	1.80±0.18	1.70±0.25	2.20**±0.15	2.03±0.24	2.32±0.41	2.56**±0.27	2.56±0.99
ALP μkat/l	1.66±0.30	1.83±0.28	2.33*±0.24	1.90±0.35	2.17±0.43	2.42*±0.44	2.83**±0.53
INP mmol/l	2.43±0.12	2.53±0.09	2.57*±0.03	2.51±0.09	2.54±0.21	2.50±0.22	2.49±0.12
Ca mmol/l	2.66±0.06	2.70±0.07	2.74±0.07	2.67±0.07	2.68±0.06	2.68±0.11	2.75*±0.04
UREA mmol/l	6.27±0.70	6.58±1.00	6.90±0.70	6.72±0.92	6.99±0.42	7.02±1.13	9.60*±2.90
CREA mmol/l	47.3±4.6	47.9±2.0	49.1±2.3	49.1±1.9	48.9±2.6	48.3±2.8	68.1*±18.6
TBIL μmol/l	2.16±0.26	2.24±0.22	2.74**±0.21	2.00±0.44	2.18±0.25	2.53±0.45	2.94±0.77
MG mmol/l	1.06±0.05	1.04±0.08	1.04±0.05	1.09±0.06	1.09±0.04	1.07±0.04	1.34**±0.22
HAPT μg/ml	0.37±0.27	1.09**±0.29	2.18**±0.69	0.74±0.57	1.58±0.83	1.81**±0.45	0.67±0.20

* p ≤ 0.05; ** p ≤ 0.01 (two-sided Wilcoxon test)

Conclusion

The comparative toxicity study of ZnO (coated or uncoated nanoparticles), ZnO (inndin pigment form) and zinc and sulfate, showed no persistent effects at the end of the observation period of four weeks when injected as a single dose intravenously at dose levels of 1 or 5 mg/kg bw.

Very mild, and in any case not persistent, deviations in single hematology parameters (monocyte, large unstained cells) and few clinical chemistry parameters, probably indicative of a minimal impairment of liver function (ZnO preparations) or of kidney function in addition (zinc sulfate) were observed one day after intravenous injection of 5 mg/kg bw pigmented or triethoxyoctylsilane coated nanoscale ZnO or an equimolar zinc sulfate dose. At 1 mg/kg bw no biologically relevant effects were observed. Among the known and very

sensitive markers of acute phase inflammatory reactions towards nanoparticles, only haptoglobulin was increased. Alpha2-macroglobulin and troponin were not affected.

Finally, the comparative screening showed only minor and in any case transient effects without toxicological relevance. There was no biologically relevant difference in the injected form of ZnO, and in particular, no enhancement of the observed effects due to the injection of nanoscale material. The equimolar injection of the zinc ion as zinc sulfate led qualitatively and quantitatively to a slight enhancement of the observed variation in homoeostasis. In any case, four weeks after treatment there was virtually no difference in comparison to the respective control group, irrespective of the injected dose level, the usage of ZnO as coated or uncoated nanoparticles, as ZnO pigment or as zinc sulfate.

(Reference: 28)

Comment

For the ZnO used in this study, information on surface area and number of particles per mass was not provided. Information on dose expressed as surface area and number of particles was not provided.

In general similar minimal effects were observed for both ZnO coated and uncoated nanoparticles. Some effects on body weight were observed, but they were not consistent. Minimal alterations were observed in blood parameters (clinical pathology) at one day after treatment. For these effects a dose response relationship could not be established as most alterations were only observed at the highest dose (5 mg/kg bw) administered.

For both coated and uncoated nano-ZnO indications for acute liver damage (alterations in blood parameters ALP, AST and bilirubin) were observed at the highest dose of 5 mg/kg bw administered.

Indications for kidney damage (increase in blood urea and creatinine) were only observed for zinc sulfate after the single high dose (17.6 mg/kg bw).

Four weeks after the administration, no alterations were observed in blood parameters evaluated for clinical pathology and in histopathology in the major organs evaluated (brain, lung, liver, spleen and kidney). Thus, there was no indication for persistent toxic effects.

The intravenous administration provided a 100% bioavailability of the nano-ZnO. A limitation of the study is that only a single administration was investigated, while for zinc sulfate the equimolar dose was administered both as a single dose or divided over four administrations in four weeks. The comparison of coated and uncoated ZnO is limited to acute reactions as indicated by several alterations in blood parameters at day 1 after treatment with 1 mg/kg bw or 5 mg/kg bw. A real comparison between coated and uncoated ZnO cannot be performed as toxic reactions were not observed.

In view of the indications for liver damage, a repeated dose toxicity study would have provided better information on potential toxicity.

In conclusion, after intravenous administration obtaining an internal dose of 5 mg/kg bw alterations in clinical pathology were observed that were indicative of liver damage. The liver damage appeared to be transient as similar alterations were not observed at four weeks after the single administration.

Based on this study, the SCCS considers a NOAEL of 1 mg/kg bw for acute toxicity of ZnO in rats after intravenous administration.

Additional studies submitted

Additional information was included in the dossier on ZnO commercially obtained from Sigma-Aldrich and not related to the ZnO presented in the dossier.

(References: 85, 94, 119)

In vitro cytotoxicity studies

Exploratory Comet assay in human epidermal cells

These studies demonstrated dose and time dependent cytotoxicity of ZnO nanoparticles. LDH release started at 5, 8 and 20 µg/mL exposure. Information on doses expressed as surface area and number of particles was not provided. NRU assay showed cytotoxicity at 8–20 µg/mL exposure. Oxidative stress markers were increased at a dose of 0.008 µg/mL exposure.

According to the applicant the cytotoxic responses in terms of disturbance of cellular homeostasis were observed at an overdosing of ZnO nanoparticles. The effect was attributed not to the nanoparticles *per se*, but to the released Zn ions as the soluble part of the ZnO nanoparticles.

(Reference: 94)

Comment

The SCCS does not consider an effect induced at doses as low as 0.08 µg/mL or even 5 µg/mL as a result of overdosing. Therefore, the SCCS considers ZnO nanoparticles at these doses cytotoxic. Even if the effects were due to the release of toxic ions, these were introduced by the added ZnO nanoparticles so the toxicity is indirectly due to the ZnO nanoparticles.

### Comparison of *in vivo* and *in vitro* studies

For the development of a predictive *in vitro* assay to assess the lung hazard potential of nanomaterials, a comparative screening study was performed. The main objectives were to compare lung toxicity impacts of nanoscale (NZnO) versus fine zinc oxide (FZnO) particles, assess predictability of *in vitro* cell culture systems, and compare the effects of instillation versus inhalation exposures in rats. The fine and nano-sized ZnO materials were obtained from Sigma-Aldrich, St. Louis, USA (purity: >99%). The surface area of the fine and nano-ZnO was 9.6 m^2/g and 12.1 m^2/g, respectively. Information on the number of particles per mass was not provided. The authors concluded that the comparisons of *in vivo* and *in vitro* toxicity measurements following nano- or fine-ZnO exposures demonstrated little convergence and few differences in potency. In addition, the implementation of the current cell culture methodologies tested in this study did not accurately predict the pulmonary hazards associated with *in vivo* exposures to ZnO.

(References: 85, 119)

Method for screening of photoactivity of nanoparticles

Undoped and doped zinc oxide was prepared by proprietary methods and characterized chemically and physically. Besides size, no other information is presented on the ZnO used. Information on surface area and number of particles per mass was not provided. In contrast to a comparative study cited in the dossier, the screening method revealed photoactivity behaviour increasing (one to twofold) with decreasing crystallite size for crystallites <100 nm. A further series of nanophase ZnO powders doped with metal ions showed that, depending on the type and/or level of dopant, the photoactivity behaviour of nanoparticles could be reduced by more than an order of magnitude. According to the dossier, this enzymatic method should be considered as an initial exploratory screening study on photoreactivity *in vitro* with questionable relevance for *in vivo* effects on the skin.

(Reference: 33)
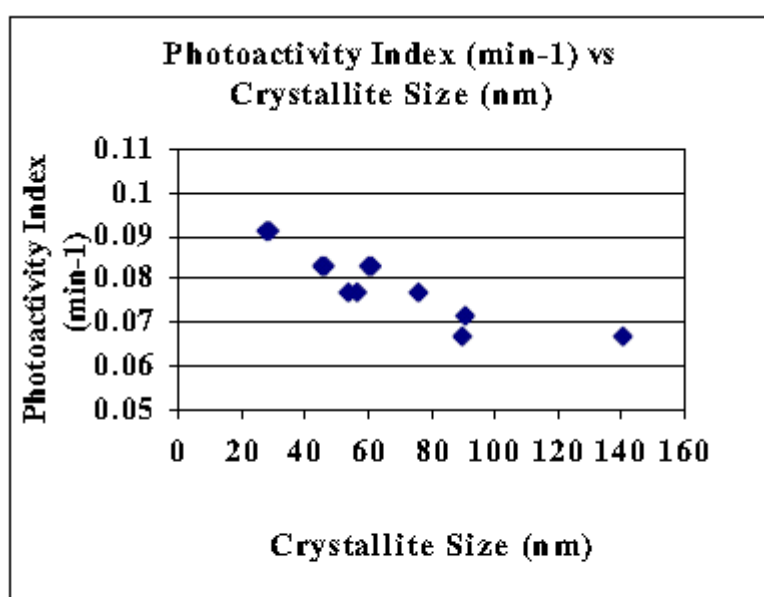
Figure 2.1a Photoactivity Index versus Crystallite Size

Comment

The paper was published in 2004 (Reference 33, submission III) and no follow up studies were cited In view of the rather limited characterization of the ZnO preparations used, the study does not contribute to the evaluation of the use of ZnO as a UV-filter in sunscreen formulations. However, scientifically the results indicate that the photoactivity of the ZnO nanoparticles can be manipulated by doping of the particles.

The SCCS considers this study not relevant for the evaluation of the use of ZnO as a cosmetic ingredient.

3.3.13 Safety evaluation (including calculation of the MoS)

The data provided, and as present in the literature, indicate that ZnO nanoparticles do not penetrate through the skin. However, some minimal absorption of zinc was demonstrated. Although the zinc was determined by methods which do not discriminate between particulate and solubilized forms, considering the dissolution rate of ZnO, it is likely that this was in the form of solubilized zinc ion. This conclusion is based on the weight of evidence from the available studies so far which suggest that only a small fraction of the nano-sized ZnO is likely to be solubilized in a formulation, and possibly when in contact with the skin, and a part of this solubilized fraction may subsequently be absorbed. The maximum solubility is estimated to be around 50 mg/L, depending on the solvent/medium under static equilibrium conditions. However, in view of the dissolution rate of ZnO, any translocating ZnO nanoparticles will also be completely solubilized due to the non-static conditions in the biological environment (see section 3.1.6). Thus, until proven otherwise, it is assumed that any transdermal penetration following application the nano-ZnO containing cosmetic product is that of Zn ions released from the ZnO nanoparticles.

As internal exposure is likely to be to ionic Zn, the safety considerations as indicated below in the EU Risk Assessment Report on Zinc Oxide (2004) (Reference 44, sub III) are relevant:

The total database available indicates that skin-bound zinc may not become systemically available in a way that it results in high peak levels of zinc in serum, but rather in a more gradual way. Given the efficient homeostatic mechanisms of mammals to maintain the total body zinc and the physiologically required levels of zinc in the various tissues constant, the anticipated slow release of zinc from the skin is not expected to disturb the homeostatic zinc balance of the body. By expert judgement, based on the aforementioned considerations, the default for dermal absorption of solutions or suspensions of zinc or zinc compounds is therefore chosen to be 2%. Based on the physical appearance, for dust exposure to zinc or zinc compounds a 10-fold lower default value of 0.2% is chosen in the risk assessment.

For sunscreen containing 10% zinc oxide the following exposure assessment was performed:

By an application of 9 g sunscreen/event, 3 events/day during 18 days/year the exposure will be 1,332 mg sunscreen/day, being 107 mg Zn2+/day. Assuming a dermal absorption of 2% the uptake is estimated to be 2.14 mg Zn2+/ day.

An NOAEL of 50 mg Zn2+/day was derived from a 10-week oral study with human volunteers and was used as a starting point for the risk characterization for repeated dose toxicity. This NOAEL of 50 mg Zn2+/day results in an internal NOAEL of 10 mg Zn2+/day by correction for oral absorption (20%; worst case, because of the homeostasis the relative absorption will be smaller by excess of Zn2+-intake). Given that this study was with women (the most sensitive population in zinc supplementation studies), and that in women clinical signs begin to appear only at a dose three times this NOAEL, a minimal MOS of 1 is considered sufficient when comparing the human NOAEL with the exposure levels for workers/consumers/general population.

The MOS between this internal NOAEL and the internal exposure as a result of exposure to sunscreen formulations is 5, which is a factor of 5 larger than the minimal MOS.

The SCCS agrees with the NOAEL indicated in the RAR statement. Therefore, this information is used together with the data from the absorption study provided in the dossier and exposure assumptions from the SCCS Notes of Guidance for the risk assessment of zinc oxide nanoparticles in sun screens as follows:

Calculation of the margin of safety for ZnO (nano)

(See section 3.1.16 for the range of nano-ZnO covered by this opinion)

Amount of sunscreen applied*	18,000 mg
Maximum concentration of ZnO	25%
Absorption through the skin	0.03% (Reference: 36)
Amount absorbed/day (18,000 × 25%/100 × 0.03%/100)	1.35 mg
Typical body weight of human	60 kg
Systemic exposure dose (1.35 mg/60 kg)	0.0225 mg/kg bw/d
No Observed Adverse Effect Level NOAEL (oral, human, sensitive subpopulation)**	0.166 mg/kg bw/d

Margin of Safety	NOAEL/SED	= 7.4

* Standard amount as indicated in the SCCS Notes of Guidance for the testing of cosmetic ingredients and their safety evaluation SCCS/1416/11

** The internal NOAEL for ZnO is 10 mg Zn^{2+}/day = 10/60= 0.166 mg /kg bw per day (Reference 44, sub III)

The calculation of the exposure via sun protection products to ZnO nanoparticles assuming Zn^{2+} uptake results in a MoS of 7.4. Given that the NOAEL is derived from a study on women (the most sensitive population in zinc supplementation studies), and that in women clinical signs begin to appear only at a dose three times this NOAEL, a minimal MOS of 1 is considered sufficient when comparing the human NOAEL with the exposure levels for workers/consumers/general population (Reference 44, sub III).

Calculation of the Margin of Safety for Lip products

According to the SCCS Notes of Guidance for the testing of cosmetic ingredients and their safety evaluation (SCCS/1416/11) the daily exposure to lipstick/lipproducts is 0.9 mg/kg/d.

Exposure dose (0.9 x 25%)	0.225 mg/kg bw/day.
No Observed Adverse Effect Level NOAEL (oral, human)*	0.83 mg/kg bw/d

Margin of Safety	NOAEL/ED**	= 3.7

* The NOAEL for ZnO is 50 mg Zn2+/day = 50/60= 0.83 mg /kg bw per day (Reference 44, sub III)

** Since route to route extrapolation is not needed, the (external) NOAEL is compared to the (external) exposure dose

It is expected that the exposure to lipstick/lip products that contain high SPF factors (eg ZnO nanomaterial) is less than the exposure to 'regular' lipstick/lip products. Typically, these products are used only in specific time frames, e.g. during outdoor holidays. Therefore 0.9 mg/kg bw/d is considered a conservative value.

The calculation of the exposure via lip products to ZnO nanoparticles assuming Zn^{2+} uptake results in a MoS of 3.7 (with a minimal MOS of 1 required, see above).

3.3.14 Discussion

Several submissions on ZnO have been received during recent years. Submission I dealt with ZnO in general, the main focus was on zinc ions as being responsible for Zn toxicity. The ZnO used was only marginally characterized. Submission II relied mainly on the report on ZnO of the regular risk assessment prepared in the context of Council Regulation (EEC) No. 793/93 on the evaluation and control of existing substances. This risk assessment was peer reviewed by the EU Commission's Scientific Committee on Toxicity, Ecotoxicity and the Environment (CSTEE). Submission III, evaluated in this opinion, deals with ZnO nanoparticles from four different sources, either coated or uncoated, with a mean size range of 30 nm to 55 nm as indicated by their number size distribution.

Regarding the dosimetry of nanoparticles it is not yet clear which metric is the best dose descriptor for nanomaterials. However, it is assumed that for evaluation of toxicity endpoints the dose metric of mass, i.e. mg/kg body weight or µg/ml in *in vitro* systems, might not be the best parameter for expression of the dose response relationship. The total surface area administered and or the number of particles have been suggested as parameters to better describe dose response relationships for nanoparticles as it is the unit of one particle that interacts with the biological system and not the number of molecules of that particle, in contrast to soluble chemicals in which the total number of molecules present can initiate such interactions. However, in the present Opinion, the dose metric used for the risk assessment has only been described in mass units.

Physicochemical properties

It should be noted that it is technically possible to produce nano-dispersed ZnO with different physicochemical properties. Therefore, this opinion relates to those coated and uncoated ZnO nanomaterials for which data have been submitted in this dossier. It is required that manufacturers carefully analyze the raw materials which they obtain, particularly if they come in a finally stabilized formulation in liquid dispersions.

It should be noted that this assessment applies to those ZnO nanoparticles that are included in this dossier, and similar materials that have the following characteristics:

- ZnO nanoparticles of purity ≥99%, with wurtzite crystalline structure, and physical appearance as described in the dossier, i.e. clusters that are rod-like, star-like and/or isometric shapes.

- ZnO nanoparticles with a median diameter (D50: 50% of the particles below this diameter) of the particle number size distribution between 30 nm and 55 nm, and D1 (1% of the particles below this diameter) above 20 nm.

- ZnO nanoparticles that are either uncoated, or coated with triethoxycaprylylsilane, dimethicone, dimethoxydiphenylsilanetriethoxycaprylylsilane cross-polymer, or octyl triethoxy silane.

- ZnO nanoparticles that have a comparable solubility to that reported in the dossier, i.e. below 50 mg/L (approximately the maximum solubility of the ZnO nanomaterials for which data are provided in the dossier).

In the submitted dossier on ZnO nanomaterials the purity of one of the ZnO nanomaterials used in some safety assays was indicated to be ≥96%. However, data on the purity is not provided in the physico-chemical characterization section of the dossier (section 3.1.4), and was also not provided in the various study reports cited although in one report a content of 100% was indicated.

As discussed in section 3.1.6, the parameter of equilibrium solubility describes the static dissolved fraction in a saturated solution. However, since ZnO nanoparticles and/or their agglomerates/aggregates in the body will not be in a static equilibrium of dissolution, Zn ions will be continuously generated and taken into the endogenous Zn pool, and the ZnO particles will continue to dissolve until completeness. Hence the parameter of dissolution

rate is more appropriate than the static solubility parameter when considering the fate of ZnO nanoparticles in the body. As the dermal penetration, if any, seems to be associated with the Zn ions released, the solubility of ZnO nanomaterials used as cosmetic ingredients should be below 50 mg/L because for this solubility level the data presented in this Opinion show an acceptable Margin of Safety.

Irritation, sensitization

A skin irritation study (not according to a guideline) was performed in which male Guinea pigs were exposed to 25% and 40% of 20 nm ZnO dispersed in ethanol. No effects were observed at any time during the administration and observation periods in the 25% test substance group. Slight erythema was observed in one of three animals in the 40% test substance group on day 3 of administration.

The ZnO nanomaterial, both as a neat dispersion and 25% solution as used in sunscreen, was slightly and transiently irritating to the eyes when tested in rabbits.

The sensitization assay was not performed according to a recognized OECD guideline. Furthermore, a concurrent positive control with a well known weak sensitizer was not included in this assay, so there is no certainty as to whether the test system used was able to identify weak sensitizers. However, for eight contact sensitizers, similar responses were found in the GPMT performed according to the OECD Guideline 406, and in a shortened test.

The validity of this (or any) test for demonstrating sensitization potency of nanomaterials has not yet been demonstrated. The inclusion of a positive particle control might overcome this problem. However, no positive particle control has been identified thus far.

Dermal absorption

The applicants have performed and provided a comprehensive review and assessment of the available *in vivo* and *in vitro* dermal penetration studies. None of the studies or projects yielded any evidence that nano-sized ZnO particles are able to cross the skin barrier in intact or compromised skin. The literature data and the data which were provided for this submission suggest that there is only minimal absorption and resulting systemic availability of zinc from application of ZnO nanoparticles containing sunscreens on the skin. Whether this zinc is available as zinc oxide nanoparticles or zinc ions has not been determined. These studies include numerous *in vitro* (using human, porcine and nude mice skin) and *in vivo* human volunteer studies. In studies that analysed particles, no penetration beyond the stratum corneum was seen. In studies that analysed Zn, small amounts were detected in deeper skin layers and receptor fluid/blood. Zn could only be detected in one out of seven of the *in vitro* studies evaluated, and was detected in the receptor liquid by elemental analysis with ICP-MS indicating some passage (maximally 0.03% of the applied dose) of the skin barrier. It was shown that some Zn may pass the skin barrier in a human volunteer study in which a small fraction of the blood Zn-pool was demonstrated to originate from a dermally applied sunscreen preparation. No major differences were seen between coated/uncoated ZnO nanomaterials, and no significant differences were observed between damaged skin versus normal healthy skin.

In view of the discussion above, it is assumed that penetration of the skin, if any, is caused by Zn ions released from ZnO nanoparticles. Therefore the solubility of ZnO is one of the critical parameters that should be considered in the characterization of ZnO used for sunscreen formulations.

General toxicity

In an acute oral toxicity study a dose of 2 g/kg body weight in rats induced some body weight decrease in one animal, although no deaths were observed. In one study all animals (mice) survived a single oral dose of 5 g/kg body weight of both nano-sized ZnO (50 nm)

and micro-sized ZnO (1,226 nm), although the large ZnO particles induced a body weight reduction in both male and female animals (Reference: AR16). After oral administration of 2.5 g/kg body weight, systemic uptake was demonstrated and indications for acute liver toxicity (at 24-72 hours after administration) were noted in serum enzyme values and histopathology (Reference: AR16). However, in another study a similar oral dose of 5 g/kg body weight of ZnO nanoparticles in mice resulted in the death of one out of five animals when ZnO of 20 nm in size was administered, whereas five out of ten animals died after administration of 120 nm diameter ZnO (Reference: 118). In addition, in several organs e.g. liver and spleen, toxicity was observed for both the 20 nm and 120 nm ZnO particles. Similar results were reported after oral administration in a recent rat study indicative of liver toxicity following oral administration of 5 mg/kg body weight (Reference: AR20). Overall, it can be concluded that after oral administration Zn can become systemically available resulting in liver toxicity.

A comparative (exploratory) intravenous toxicity study of ZnO as coated or uncoated nanoparticles, as a pigment form or as zinc sulfate was performed. No persistent effects were observed at the end of the four week observation period when the various ZnO preparations were injected intravenously as a single dose at levels of 1 or 5 mg/kg bw. Deviations in some hematology parameters (monocyte, large unstained cells) and clinical chemistry parameters (alterations in blood parameters ALP, AST and bilirubin) indicative of an effect on liver function (coated and uncoated ZnO-nano preparations) and kidney function (zinc sulfate) were observed 1 day after intravenous injection of 5 mg/kg bw pigmented or coated nanoscale ZnO or an equimolar zinc sulfate dose. As at 1 mg/kg bw, no effect was observed and a dose response relationship could not be established. There was no indication for persistent toxic effects and, as at four weeks after the administration, no alterations were observed in blood parameters evaluated for clinical pathology and for histopathology in the major organs evaluated (brain, lung, liver, spleen and kidney). In general there was no difference in the reactions between the various injected forms of ZnO. There were no indications that nanoscale ZnO induced different effects compared to pigmentary ZnO or zinc sulfate.

The intravenous administration provided 100% bioavailability of the nano-ZnO. A limitation of the study is that only a single administration was investigated, while for the Zn sulfate the equimolar dose was administered both as a single dose or divided over four administrations in four weeks. The comparison of coated and uncoated ZnO is limited to acute reactions as indicated by several alterations in blood parameters at day 1 after treatment with 1 mg/kg bw or 5 mg/kg bw. A real comparison between coated and uncoated ZnO cannot be performed as toxic reactions were not observed. In view of indications for liver damage, a repeated dose toxicity study would have provided better information on potential toxicity.

In general it can be concluded that based on the observations on serum liver enzyme levels and histopathology, the systemic availability of either ZnO nanoparticles or Zn ions has the potential to induce liver toxicity.

No oral repeated dose toxicity study with ZnO nanoparticles is available.

Inhalation

In a 5 day exposure study in rats, ZnO induced a concentration-related inflammation reaction in the lung which was associated with dose-dependent increases in BALF. In addition to the inflammation reaction, necrosis was detected in the lung and the nose. There was no difference between the nano-sized and pigmentary ZnO. Local effects in the lung were still present 25 days after the exposure. There was no indication of systemic effects after the 5 day inhalation exposure.

After inhalation exposure of human volunteers, no acute toxic effects were observed with a dose of 500 µg/m^3 air with 2 hours of exposure.

In view of the observed effects in the respiratory tract a caution is warranted against the use of ZnO nanoparticles in spray application despite the potentially low inhalation exposure.

Mutagenicity

Mutagenicity has been addressed in the dossier by both *in vitro* and *in vivo* tests.

The bacterial reverse mutation assay (according to OECD Guideline 471) was performed with dimethoxydiphenylsilanetriethoxycaprylylsilane coated ZnO nanoparticles in a panel of *Salmonella typhimurium* strains, both with and without S9-mix and was negative..The material tested negative for base pair changes and frame shift mutations, in contrast to the specific positive controls (MNNG, MIT.C, NOPD, AAC and 2-AA). Bacterial mutagenicity assays are judged less appropriate for testing of nanoparticles in comparison to mammalian cell systems in view of different internalisation pathways with eukaryotes and specific bactericidal effects of specific types of nanoparticles such as silver. According to the study provided in the dossier, no signs of bacteriology were observed. However, uptake of the nanoparticles by the bacteria and the role of Zn ions were not investigated. Negative findings in Ames tests (frame shifts, base pair substitutions) have also been reported in the literature for tetramethylammoniumhydroxide-capped ZnO nanoparticles (Reference: AR26) as well as ionic zinc (Reference: AR24).

A DNA damage potential of ZnO nanoparticles was revealed in A431 human epidermal cells by an alkakine Comet assay. Lipid peroxidation and oxidative stress induction were postulated as underlying mechanisms in the dossier. It should be emphasized however, that this does not exclude a genotoxic potential of the treatment agent, i.e. the applied ZnO nanoparticles. Several other studies in the open literature have confirmed DNA damaging effects with ZnO nanoparticles with the alkaline Comet assay (References: AR6, AR11, AR12, AR14, AR22).

The mouse micronucleus assay (according to OECD Guideline 474) using intraperitoneal administration showed an absence of clastogenic and aneugenic effects of triethoxycaprylylsilane coated ZnO nanoparticles, whereas the controls cyclophosphamide and vincristine sulfate tested positive. No evidence was provided that the ZnO nanoparticles (or dissolved ions) reached the bone marrow after the intraperitoneal applications, hence the results of this study are of limited value.

The SCCS concludes that there is evidence for *in vitro* genotoxicity of ZnO nanoparticles as demonstrated in the positive comet assay, but does not consider the negative results of the *in vivo* assay valid as exposure of the target organs was not demonstrated.

Both *in vitro* and *in vivo* data on photo-genotoxicity/mutagenicity were provided in the dossier. In *Salmonella typhimurium* mutation assays, ZnO revealed no gene mutations by base pair changes or frame shifts in the presence or absence of irradiation. In other investigations ZnO showed some clastogenic and photo-clastogenic potential. In CHO fibroblasts, increased structural chromosome aberrations were found both in the absence or presence of simulated sunlight. In CHO cells, clastogenic effects were observed after treatment with ZnO in the presence or absence of simultaneous UV irradiation. Increased chromosome aberrations were also observed in cells in which UV irradiation was performed prior to ZnO treatment, suggesting that radiation increases the susceptibility of the CHO cells to the clastogenic activity of ZnO. *In vivo,* ZnO was investigated in a photo-micronucleus test in hairless mice after topical application and did not increase the incidence of micronucleated epidermal cells in exposed animals with or without simulated light irradiation.

The SCCS is of the view that ZnO nanoparticles are (photo)clastogenic *in vitro*. The negative results in the *in vivo* micronucleus assay for epidermal cells can not be accepted as actual exposure of the living epidermal cells was not demonstrated.

Although the conclusion that UV-irridiation increases the sensitivity of the photomutagenicity/clastogenicity assay may be valid, the induction of chromosomal aberration in the absence of UV exposure should not be ignored. This should be considered a positive genotoxic activity of ZnO nanoparticles. Further studies available in the open literature have shown that ZnO nanoparticles do not cause mutations in FE1-Muta™Mouse lung epithelial cells (Reference: AR1), and induce micronuclei in A549 human lung epithelial cells only at a concentration where marked toxicity was observed (Reference: AR2).

It has been discussed in the open literature that part of the toxicity of nano ZnO is due to generation of reactive oxygen species, which has been associated with DNA damage (AR28). Scavenging of these oxygen radicals can be done by adding antioxidants or other components to the formulations. The impact of this in relation to potential harmful effects of nano ZnO in dermally applied cosmetic products is sofar not known.

Based on the available database and additional in depth evaluation of the studies, and in view of the uncertainties over whether or not nanoparticles reached the target cells/DNA in the tests, there is no conclusive evidence to conclude whether or not micro-or nano-sized ZnO particles pose a mutagenic/genotoxic, photo-toxic or photo-mutagenic/genotoxic risk to humans. However, where ZnO nanoparticles are applied on the skin in a sunscreen formulation, there is sufficient evidence to conclude that due to the very low if any systemic exposure, the risk to the consumer is negligible. The evidence from *in vitro* and *in vivo* studies presented in this dossier (section 3.3.4), and other studies on different metal/metal oxide nanoparticles (e.g. titanium dioxide – Nanoderm Project[1]) shows that penetration of nano or larger particles is generally limited to the upper few layers of the stratum corneum and there is no significant dermal penetration of the particles to systemic circulation. Whilst this leaves the possibility for nanoparticle mediated local effects, it diminishes the possibility for any harmful effects at the systemic level.

Toxicokinetics

After oral exposure there is some uptake of Zn in the systemic circulation. ZnO nanoparticles of approximately 50 nm in size (TEM evaluation) were compared to ZnO microparticles showing at least one diameter >100 nm (TEM evaluation). After oral and intraperitoneal administration for both ZnO nanoparticles and microparticles, Zn could be observed in serum indicating uptake from the GI–tract, either as particulate materials or as dissolved Zn ions. For ZnO nanoparticles the systemic availability was somewhat higher compared to that of ZnO microparticles as indicated by Zn measurements by ICP-MS. Zn showed a higher distribution in the liver, spleen and lung after treatment with ZnO nanoparticles compared to treatment with ZnO microparticles.

After inhalation exposure elevated zinc levels were detected in various organs, most likely due to zinc ions dissolved from the ZnO particles.

There are no indications for significant if any penetration of nanoparticles through the skin, most likely only a minimal amount of Zn ions released from the nanoparticles may be available for systemic exposure.

Carcinogenicity

Specific data on carcinogenicity studies of ZnO nanomaterials are not available. In view of the occurring dissolution of the ZnO nanoparticles it can be assumed that the carcinogenic risk is similar to the conventionally manufactured ZnO preparations. According to the EU

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[1]   Nanoderm   Project: http://ec.europa.eu/research/environment/pdf/env_health_projects/nanotechnology/n-nanoderm.pdf

Risk assessment report (Reference 44, submission III), there is no clear experimental or epidemiological evidence for a direct carcinogenic action of zinc or its compounds. .

4. CONCLUSION

Risk assessment of nanomaterials still has some issues to adress. At present, there are still certain gaps in the knowledge, for instance on the behaviour of nanoparticles in the test medium or in the animals. This leads to uncertainties on whether the particles remain free and hence able to reach the biological target sites and interact with various moieties, or they agglomerate/ aggregate and become less biologically available. There are also limitations in the validity of some of the tests used as they were developed for conventional (soluble) chemicals and not for nanoparticles. However, clear positive toxic responses in some of these tests clearly indicate a potential for risk to humans.

1. *Does the SCCS consider zinc oxide in its nano-form safe for use as a UV-filter with a concentration up to 25% in cosmetic products taking into account the scientific data provided?*

With regard to applications resulting in dermal exposure

From the available information, there is no indication for penetration of ZnO nanoparticles through the skin. In one study it was shown that Zn from ZnO nanoparticles in a tested sunscreen formulation made a minor contribution to the blood Zn pool of human volunteers. This shows that some Zn was absorbed from the sunscreen, although it was not known whether this was absorbed in nanoparticulate form or as solubilized Zn ions. Considering the dissolution of ZnO, it is most likely that the zinc was absorbed in ionic form. The overall weight of evidence therefore suggests that a very small proportion of Zn ions released from the ZnO nanoparticles may be available for systemic exposure when applied dermally.

With regard to applications resulting in other routes of exposure

Oral exposure

In one exploratory study in mice, systemic availability of Zn was indicated after a single oral exposure. However, no differences weren observed between ZnO administered as nanoscale or microscale particles. It is likely that absorbed Zn in the GI-tract was in the dissolved ionic form. In view of the data provided, oral exposure of nano-ZnO via applications of nano-ZnO as a cosmetic ingredient in sunscreens should be considered to be of a similar risk to micron-sized ZnO as previously evaluated in the RAR. The NOAEL for oral intake of ZnO is 50 mg /bw day (Reference 44, sub III). The oral exposure to ZnO nanoparticles as cosmetic ingredient in sunscreens is limited to accidental ingestion of small fractions of lip products and sun protection products and can be considered to be low.

Inhalation exposure

Upon inhalation of ZnO nanoparticles, serious local effects in the lung were observed. Even if this may be due to the solubilized Zn ions, the effects are a direct result of the exposure to the ZnO nanoparticles. Therefore, the SCCS is of the opinion that, on the basis of available information, the use of ZnO nanoparticles in spray products cannot be considered safe.

Overall conclusions

In summary, the evidence presented in this submission and the available literature showed that:

- There is no evidence for the absorption of ZnO nanoparticles through skin and via the oral route. Even if there was any dermal and/or oral absorption of ZnO nanoparticles, continuous dissolution of zinc ions would lead to complete solubilization of the particles in the biological environment. In the MoS calculation, the calculation of the exposure to ZnO nanoparticles assuming Zn2+ uptake results in acceptable MoSs for both the oral and dermal routes.

- Nano ZnO-containing cosmetic formulations are likely to contain a small proportion of solubilized zinc, a further small proportion of which may be absorbed through skin and other routes. The rate and amount of the absorbed zinc is, however, likely to be insignificantly small compared to the large zinc pool already present in the body.

- Although the current evidence in relation to potenial genotoxicity of ZnO is not conclusive, the use of nano ZnO in cosmetic products should not pose a risk to the consumer in the absence of a significant systemic exposure.

- Based on the parameters described in the dossier, the different particle sizes, surface modifications, and crystalline structures and morphologies investigated do not significantly alter the uptake, bioavailability and overall safety profile.

- The different typical formulations as described in this submission also do not change the overall safety profile of the tested ZnO nanoparticles.

In summary, it is concluded on the basis of available evidence that the use of ZnO nanoparticles with the characteristics as indicated below, at a concentration up to 25% as a UV-filter in sunscreens, can be considered not to pose a risk of adverse effects in humans after dermal application. This does not apply to other applications that might lead to inhalation exposure to ZnO nanoparticles (such as sprayable products). Also, this assessment only applies to ZnO nanoparticles that are included in this dossier, or are similar materials that have the following characteristics:

- ZnO nanoparticles of purity ≥99%, with wurtzite crystalline structure and physical appearance as described in the dossier, i.e. clusters that are rod-like, star-like and/or isometric shapes.

- ZnO nanoparticles with a median diameter (D50: 50% of the number below this diameter) of the particle number size distribution between 30 nm and 55 nm, and the D1 (1% below this size) above 20 nm.

- ZnO nanoparticles that are either uncoated, or coated with triethoxycaprylylsilane, dimethicone, dimethoxydiphenylsilanetriethoxycaprylylsilane cross-polymer, or octyl triethoxy silane.

- ZnO nanoparticles that have a comparable solubility to that reported in the dossier, i.e. below 50 mg/L (approximately the maximum solubility of the ZnO nanomaterials for which data are provided in the dossier).

Some tests were performed with a nano-ZnO material with a purity of ≥96% as indicated in the submission. However, data on the purity was not provided in the physico-chemical characterization section (3.1.4), and was also not provided in the various study reports cited although in one report a content of 100% was indicated.

It is worth highlighting that this opinion has considered the small proportion of the absorbed Zn following dermal application of nano ZnO to most likely be a solubilised ionic form. This is in consideration of the solubility and dissolution aspects of ZnO described in section 3.1.6. However, if any new evidence emerges in the future to show that the translocating species

were in the form of insoluble and potentially persistent nanoparticles, then the SCCS may consider revising the safety assessment of nano ZnO.

It should also be noted that the risk assessment of nanomaterials is currently evolving. In particular, the toxicokinetics aspects have not yet been fully explored in the context of nanoparticles (e.g. the size dependency). Also, long term stability of the coatings remains unclear. At the moment, testing of nanomaterials and the present assessment, are both based on the methodologies developed for substances in non-nano form, and the currently available knowledge on properties, behavior and effects of nanomaterials. Therefore this assessment is not intended to provide a blue-print for future assessments of other nanomaterials, where depending on the developments in methodological risk assessment approaches and nano-specific testing requirements, additional/different data may be required and/or requested on a case-by-case basis.

2. *Does the SCCS confirm that zinc oxide in its non-nano form is safe for use as a UV-filter with a concentration up to 25% as stated in the SCCP clarification (SCCP/1215/09)?*

The SCCS considers that the use of larger (non-nano) forms of ZnO as a UV-filter with a concentration up to 25%, as stated in the SCCP clarification (SCCP/1215/09), is safe and is not of any additional safety concern compared to the nano-forms assessed in this Opinion. This is in view of the following:

- When compared in terms of solubility, micro-sized ZnO has been shown to be less soluble (in water), and equally soluble (in tissue culture medium) compared to nano-ZnO.

- Experimental evidence shows that both nano and non-nano particulate forms of ZnO are not absorbed through the skin. Also on a theoretical ground, larger sized (non-nano) particles of ZnO are less likely to be absorbed through the skin than the nano-forms.

- As far as evaluated in the toxicity testing, micro-sized ZnO has been shown to induce either similar toxic effects (in terms of general toxicity, lung toxicity after inhalation, uptake from gastroIntestinal-tract, serum liver enzyme presence) or lower toxic effects (in terms of genotoxicity, liver histopathology) when compared to nano-sized ZnO. No toxic effects were observed at similar doses in a human volunteer inhalation study for both nano-sized ZnO (40 nm) and fine ZnO (291 nm).

Thus the calculation of MoS for nano-sized ZnO for use as a cosmetic ingredient in sunscreen formulations can also be used for the non-nano form of ZnO.

The SCCS would like to point out that a re-evaluation may be needed in the case of use of other specific coatings or specific absorption enhancers in the formulation, which can promote the dermal penetration of ZnO particles (nano or non-nano), or indications of dermal absorption after long term use of nano ZnO containing formulations.

3. And/or does the SCCS have any further scientific concern with regard to the use of *zinc* oxide in cosmetic products?

ZnO is also used as a colorant in cosmetic products. In view of the similar toxicity of both nano-sized and fine ZnO, it is considered that ZnO is safe when used as colorant in cosmetics for dermal application. However, no information is available on whether and at what concentrations ZnO is used in other cosmetic products that may lead to oral or inhalation exposure. The MoS for such uses is uncertain.
In view of the lung inflammation induced by ZnO particles after inhalation exposure, the use of ZnO in cosmetic products which may result in inhalation is of concern.

Any cosmetic products containing ZnO particles (nano or non-nano) with coatings that can promote dermal penetration will also be of concern.

5. MINORITY OPINION

6. REFERENCES

Submission I, 2001

1. Landsdown ABG (1996). Zinc in the healing wound. Lancet; 347:706-7.

2. Fergusson J, Addo HA, Jones S, Johnson BE, Frain Bell W. A study of cutaneous photosensitivity induced by amiodarone, (1985). Br J Dermatol; 113:537-49.

3. Risk assessment zinc oxide (1999). Report prepared by TNO & RIVM. Draft of 21.12.1999.

4. Berg JM, Shi Y (1996). The galvanisation of biology: a growing appreciation for the roles of zinc. Science; 271:1081-5.

5. Walsh CT, Sandstead HH, Prasad AS, Newberne PM, Fraker PJ (1994). Zinc: health effects and research priorities for the 1990s. Environ Health Perspect; 102:5-46.

6. Prasad AS (1991). Discovery of human zinc deficiency and studies in an experimental human model. Am J Clin Nutri; 53:403-12.

7. Loeser (1977) Akute orale toxizitat, Bayer AG data: Orientierende toxikol. Pruefungen, 25.4.77.

8. Dufour P (1991). Etude de la toxicite aiguë par voie cutanee chez le rat du produit oxyde de zinc lot 91.8207, EVIC-TOX, ref.: L 081/S477, EVIC-CEBA. Laboratoire de recherche et d'experimentation, November 12, 1991.

9. Scott DA, Fisher AM (1938). Studies on the pancreas and liver of normal and of zinc-fed cats, Am J Physiol, 121, 253-60.

10. Hsu FS, Krook L, Pond WG, Duncan JR (1975). Interactions of dietary calcium with toxic levels of lead and zinc in pigs. J Nutr; 105:112-8.

11. Straube EF, Schuster NH, Sinclair AJ (1980). Zinc oxide in the ferret. J Comp Path; 90:355-61.

12. Ellis TM, Masters HG, Mayberry C (1984). Examination of the susceptibility of different breeds of sheep to zinc intoxication. Australian Veterinary Journal; 61:296-8.

13. Shankar S, Ramnathan Sundaresan P, Mohla S (1986). Effect of chronic administration of excess dietary vitamin A and zinc on lipid metabolism in rats. Intern J Vit Nutr Res; 56:329-37.

14. Maita K, Hirano M, Mitsumori K, Takahashi K, Shirasu Y (1981). Subacute toxicity studies with zinc sulfate in mice and rats. J Pesticide Sci; 6:327-36.

15. Samman S, Roberts DCK (1987). The effect of zinc supplements on plasma zinc and copper levels and the reported symptoms in healthy volunteers. The Medical Journal of Australia; 146:246-9.

16. Hooper PL, Visconti L, Garry PJ, Johnson GE (1980). Zinc lowers high-density lipoprotein-cholesterol levels. JAMA; 244:1960-1.

17. Chandra RK (1984). Excessive intake of zinc impairs immune responses. JAMA; 252:1443-6.

18. Hallbook T, Lanner E (1972). Serum-zinc and healing of venous leg ulcers. The Lancet; 780-2.

19. Greaves MW, Skillen AW (1970). Effects of long-continued ingestion of zinc sulphate in patients with venous leg ulceration. The Lancet; 879-91.

20. Dufour P (1991). Evaluation de l'irritation et/ou de la corrosion cutanees chez le lapin du produit oxyde de zinc lot 91.8207, EVIC-TOX, ref.: L 082/5477. EVIC-CEBA, laboratoire de recherche et d'experimentation, November 12, 1991.

21. Lansdown ABG (1991). Interspecies variations in response to topical application of selected zinc compounds. Food Chem Toxic; 29:57-64.

22. Notox BV (1999a). Acute eye irritation/corrosion study with zinc oxide in the rabbit. Project 254352, cited in reference (4).

23. Atsunobo M (1994). Eye irritation of ultra-fine oxide (6% w/w aqueous suspension) in rabbit. Life Science Laboratory, October 19, 1994.

24. Notox BV (1999b) Assessment of contact hypersensitivity to zinc oxide in the albino guinea pig maximisation test. Project 261214, 1999b1, b2, cited in reference (4).

25. Notox BV (1999i). Assessment of contact hypersensitivity to zincweil3 pharma in the albino guinea pig maximisation test. Project 263429, 1999i, cited in reference (4).

26. Cantor S (1994). 50 humans subject repeat insult patch test skin irritation/sensitisation evaluation, ref no. WPCL 94-141Z/RIPT 00160.SSI. AMA laboratories, May 2, 1994.

27. Food and Drug Research Labs Inc. (1973). Teratologic evaluation of FDA 71-49 (zinc sulfate), PB-221 805, cited in reference (4).

28. Food and Drug Research Labs. Inc. (1974). Teratologic evaluation of compound FDA 71-49 zinc sulfate in rabbits, PB-267 191, cited in reference (4).

29. Schlicker SA, Cox DH (1968). Maternal dietary zinc and development and zinc, iron, and copper content of the rat's foetus. J Nutr; 95:287-94.

30. Derry JE, McLean WM, Freeman JB (1983). A study of the percutaneous absorption from topically applied zinc oxide ointment. Journal of Parenteral and Enteral Nutrition; 7:131-5.

31. Morgan MEI, Hughes MA, McMillan EM, King I, Mackie RM (1980). Plasma zinc in psoriatic in-patients treated with local zinc applications. British Journal of Dermatology; 102:579-83.

32. Gonül B, Söylemezoglu T, Babül A, Celebi N (1998). Effects of epidermal growth factor dosage forms onmice full-thickness skin wound zinc levels and relation to wound strength. J Pharm Pharmacol; 50:641-4.

33. Pirot F, Millet J, Kalia YN, Humbert PH (1996). In vitro study of percutaneous absorption, cutaneous bioavailability and bioequivalence of zinc and copper from five topical formulations. Skin Pharmacol; 9:259-69.

34. Labor L+S AG (1999). Cutaneous permeation of zinc oxide and zinc sulphate through pig skin in vitro, study no. 02073979/02073989, final report, cited in reference (4).

35. Leonard A, Gerber GB, Leonard F (1986). Mutagenicity, carcinogenicity and teratogenicity of zinc. Mutat Res; 168:343-53.

36. United States Environmental Protection Agency (1991). Zinc and compounds, 1-24.

37. Henry JB (1984). Clinical diagnosis and management by Laboratory methods, 7`h edition, pp. 1437.

38. Fong LY, Li JX, Farber JL, Magee PN (1996). Cell proliferation and esophageal carcinogenesis in the zinc-deficient rat. Carcinogenesis; 17:1841-8.

39. Mitchnick MA, Fairhurst D, Pinnel SR (1999). Microfine zinc oxide (Z-Cote) as a photostable UVA/UVB sunblock agent. J Am Acad Dermatol; 40:85-90.

40. Spielmann H, Balls M, Dupuis J, Pape W, De Silva O, Holzhötter HG et al. (1998). A study on UV filter chemicals from annex VII of European Union Directive 76/768/EEC, in the in vitro 3T3 NRU phototoxicity test. ATLA; 26:679-708.

41. Spielmann H, Balls M, Dupuis J, Pape G, Pechovitch G, de Silva O et al. (1998). The international EU/COLIPA in vitro phototoxicity validation study: results of phase II (Blind trial). Partl: The 3T3 NRU phototoxicity test. Toxicol In Vitro; 12:305-27.

42. Diembeck W (2001). Testing of Zinc Oxide (H&R, PN 104702) with the 3T3 Neutral Red Uptake Phototoxicity Test, Beiersdorf AG test report no. 0663-2001.

43. Fink E (1997). Repeat photopatch test for photoxicity and photoallergy of HR 96/104702 and HR 96/104702 VeK in healthy adult male and female volunteers, part 1, project no. 9066/lib, GTLF, August 1, 1997.

44. Cantor S, Lukin A, Reidy MT, Winne DR (1994). Evaluation of phototoxicity potential by UV-A irradiation on 10 human subjects. AMA Laboratories, WPCL 94147Z /PHT0016OSSI.10.

45. Cantor S, Lukin A, Reidy MT, Winne DR (1994). Photoallergy maximization test on 25 human subjects. AMA Laboratories, WPCL 94-147Z/PHT00160SSI.25.

46. The SCC Notes of Guidance for testing of cosmetic ingredients for their safety evaluation (third revision, SCCNFP/01 19/99) (1999). Annex 3, Guidelines for the in vitro assessment of the phototoxic potential of UV filters, 52-8.

47. Loprieno N (1991). In vitro assay systems for testing photomutagenic chemicals. Mutagenesis; 6:331-3.

48. Loprieno N (1992). Guidelines for safety evaluation of cosmetics ingredients in the EC countries. I'd Chem Toxic; 30:809-15.

49. RCC-CCR project, Czich, A. In vitro test on induction of chromosome aberrations in V79 cells with HR 99/ 104702 (a), 00/T00017 (b), 00/ 106407 (c), after simultaneous irradiation with artificial [Incomplete reference – Judy Burns]

50. Ballantyne M (1998). HR 96/104702: reverse mutation in three histidine-requiring strains of salmonella typhimurium and a tryptophan-requiring strain of Escherichia coli, in the presence of ultraviolet light, final report, 1-58.

51. Guidance document elaborated by the COLIPA task force "Photomutagenicity" (1995).

52. Riley S (1998). HR 96/ 104702: induction of chromosome aberrations in cultured Chinese hamster ovary (CHO) cells in the presence of ultra violet light. Covance Laboratories Ltd., Harrogate, UK, final report no. 1379/4-1052.

53. Dean SW, Lane M, Dunmore RH, Ruddock SP, Martin CN, Kirkland DJ et al. (1991). Development of assays for the detection of photomutagenicity of chemicals during exposure to UV light-I. Assay development. Mutagenesis; 6:335-41.

54. Bayer report, Brendler-Schwaab S (2001). HR 00/ 106407: Photo-comet assay in vitro, unpublished report no. T 8069508.

55. Yadrick MK, Kenney MA, Winterfeldt EA (1989). Iron, copper, and zinc status: response to a supplementation with zinc or zinc and iron in adult females. Am J Clin Nutr; 49:145-50.

56. Brusick D (1986). Genotoxic effects incultured mammalian cells produced by low pH treatment conditions and increased ion concentrations. Environ Mutagen; 8:879-86.

57. Scott D, Galloway SM, Marshall RR, Ishidate M, Brusick D, Ashby J et al. (1991). Genotoxicity under extreme culture conditions, report from ICPEMC task group 9. Mutat Res; 257:147-204.

58. Kirkland DJ (1992). Chromosomal aberration tests in vitro: problems with protocol design and interpretation of results. Mutagenesis; 7:95-106.

59. Galloway SM, Aardema MJ, Ishidate M, Ivett JL, Kirkland DJ, Morita T et al. (1994). Report from working group on in vitro tests for chromosomal aberrations. Mutat Res; 312:241-61.

60. Kirkland DJ, Muller L (2000). Interpretation of the biological relevance of genetoxicity test results: the importance of thresholds. Mutat Res; 464:137-47.

61. Ishidate M, Harnois MC, Sofuni T (1988). A comparative analysis of data on the clastogenicity of 951 chemical substances tested in mammalian cell cultures. Mutat Res; 195:151-213.

62. Hilliard CA, Armstrong MJ, Bradt CI, Hill RB, Greenwood SK, Galloway SM (1998). Chromosome aberrations in vitro related to cytotoxicity of nonmutagenic chemicals and metabolic poisons. Environ Mol Mutagen; 31:316-26.

63. Blazak WF, Stewart BE, Galperin I, Allen KL, Rudd CJ, Mitchell AD et al. (1986). Chromosome analysis of trifluorothymidine-resistant LS 178Y mouse lymphoma cell colonies. Environ Mutagen; 8:229-40.

64a. Speit G, Autrup H, Crebelli R, Henderson L, Kirsch-Volders M, Madle S et al. (2000). Thresholds in genetic toxicology - conducting remarks. Mutat Res; 464:149-53.

Submission II, 2005

64b. Gamer AO, Leibold E (2005). Study of the penetration of zinc through dermatomed pig skin in vitro after treatment with a microfine zinc oxide containing formulation (Emulsion mit 2-Cote (Zinkoxid); 10%). Report/Project number 52H0546/032193. BASF Aktiengesellschaft, Experimental Toxicology and Ecology, D-67056 Ludwigshafen, Germany, report dated 31 January 2005.

65. Russell RS, Merz RD, Sherman WT, Sivertson JN (1979). The determination of respirable particles in talcum powder. Food Cosmet Toxicol; 17:117-22.

66. Menache MG, Miller FJ, Raabe OG (1995). Particle înhaiabiiity curves for humans and small laboratory animals. Ann Occup Hyg; 39:317-28.

67. Hikiba H, Watanabe E, Barrett JC, Tsutsui T (2005). Ability of fourteen chemical agents used in dental practice to induce chromosome aberrations in Syrian hamster embryo cells. J Pharmacol Sci; 97:146-52.

68. Walter JF, DeQuoy PR (1980). The hairless mouse as a model for evaluating sunscreens. Prevention of ultraviolet B inhibition of epidermal DNA synthesis. Arch Dermatol; 116:419-21.

69. Gelis C, Girard S, Mavon A, Delverdier M, Paillous N, Vicendo P (2003). Assessment of the skin photoprotective capacities of an organo-mineral broad-spectrum sunblock on two ex vivo skin models. Photodermatol Photoimmunol Photomed; 19:242-53.

70. CSB (2003). Risk assessment zinc oxide, CAS#: 1314-13-2; EINECS#: 215-222-5. Chemical Substances Bureau, P.O. Box 1, 3720 BA Biíthoven, The Netherlands, Final draft dated October 2003.

71. CSTEE (2003). Scientific Committee on Toxicity, Ecotoxicity and the Environment (CSTEE) opinion on the results of the risk assessment of: zinc metal (CAS No. 7440-66-6); zinc chloride (CAS No. 7646-85-7); zinc sulphate (CAS No. 7733-02-0); zinc distearate (CAS No. 557-05-1, 9105-01-3); zinc phosphate (CAS No. 7779-90-0); zinc oxide (CAS No. 1314-13-2). Human health part carried out in the framework of Council Regulation (EEC) 793/93 on the evaluation and control of the risks of existing substances, adopted by the CSTEE during the 39th plenary meeting of 10 September 2003.

72. SCCNFP (2003a). Scientific Committee on Cosmetic Products and Non-Food Products intended for consumers. Opinion concerning zinc oxide. COLIPA no. S 76. SCCNFP/0649/03, final. Adopted by the SCCNFP during the 24th plenary meeting of 24-25 June 2003.

73. SCCNFP (2003b). Scientific Committee on Cosmetic Products and non-Food Products. Opinion concerning basic criteria for the in vitro assessment of dermal absorption of cosmetic ingredients. SCCNFP/0750/03, October 2003.

74. OECD (2004a). Organization for Economic Cooperation and Development. Guidance document no. 28 for the conduct of skin absorption studies. March 2004.

75. OECD (2004b). Organization for Economic Cooperation and Development. Guideline for Testing of Chemicals Method No. 428: "Skin Absorption: in vitro Method". Adopted April 13, 2004.

76. Kuschner WG, D'Alessandro A, Wintermeyer SF, Wong H, Boushey HA, Blanc PD (1995). Pulmonary responses to purified zinc oxide fume. J Investig Med; 43:371-8.

77. Kuschner WG, D'Alessandro A, Hambieton J, Blanc PD (1998). Tumor necrosis factor-alpha and interleukin-8 release from U937 human mononuclear cells exposed to zinc oxide in vitro. Mechanistic implications for metal fume fever. J Occup Environ Med; 40:454-9.

78. USP specifications, Zinc Oxide 81,39, official Monographs: 2054.

Submission III, 2010

1. Auffan M, Rose J, Bottero JY, Lowry GV, Jolivet JP, Wiesner MR (2009). Towards a definition of inorganic nanoparticles from an environmental, health and safety perspective. Nature Nanotechnol; 4:634-41.

2. Australian Government (2009). A review of the scientific literature on the safety of nanoparticulate titanium dioxide or ZnO in sunscreens. Department of Health and Aging, Therapeutic Goods Administration, Australian Government, Woden, Australia, 1-31, July 2009.

3. BASF SE (2007). Safety Data Sheet Z-COTE® HP1. BASF SE, Ludwigshafen, Germany, 18 October 2007.

4. BASF SE (2007). Safety Data Sheet Z-COTE®. BASF SE, Ludwigshafen, Germany, 18 October 2007.

5. BASF SE (2008). Composition of sun protection cream CM 643 (Z-COTE HP1®). BASF SE, Ludwigshafen, Germany, 2 September 2008, confidential.

6. BASF SE (2008). Composition of sun protection cream CM 644 (Z-COTE®). BASF SE, Ludwigshafen, Germany, 2 September 2008, confidential.

7. BASF SE (2008). SEM analytics of sun protection solutions/crèmes (German report: Sonnenschutzmittelformulierungen, Cremes), Report No. 98662. GKA Competence Center Analytics, BASF SE, Ludwigshafen, Germany, 28 October 2008.

8. BASF SE (2008). Solubility in different media (German report: Loeslichkeit in verschiedenen Medien), Report No. 08Y30980. GKA Competence Center Analytics, BASF SE, Ludwigshafen, Germany, 22 August 2008.

9. BASF SE (2008). Solubility in different media (German report: Loeslichkeit in verschiedenen Medien), Report No. 08A06855. GKA Competence Center Analytics, BASF SE, Ludwigshafen, Germany, 4 September 2008.

10. BASF SE (2008). Standard Specification, Care Materials, 1-101383, Z-COTE®, PRD 30083071. BASF SE, Ludwigshafen, Germany, 20 February 2008.

11. BASF SE (2008). Standard Specification, Care Materials, 3-103050, Z-COTE® HP1, PRD 30083072. BASF SE, Ludwigshafen, Germany, 22 October 2008.

12. BASF SE (2008). Test Method PM/00806/01e, Analysis of particle size distribution of ZCOTE and other products containing zinc by laser diffraction. GKA Competence Center Analytics, BASF SE, Ludwigshafen, Germany, distributed 5 August 2008.

13. BASF SE (2008). Z-COTE®, Z-COTE® HP1, Z-COTE® Max, T-Lite® SF, T-Lite® SF-S, T-Lite® max, Particle Morphology (German Report), GKP No. 96036. TEM laboratory, BASF SE, Ludwigshafen, Germany, 4 August 2008.

14. BASF SE (2009). Comparative table on physical-chemical properties of UV filters (ZCOTE®, Z-COTE®HP1). BASF SE, Ludwigshafen, Germany, 11 February 2009.

15. BASF SE (2009). Personal Care Ingredients, Technical Information – UV Filters. BASF SE, Ludwigshafen, Germany, 14 April 2009.

16. BASF SE (2009). Sunscreen formulations: Lotions and cremes, Fa. Procter & Gamble, all day moisture Lotion SPF 15, Olay, GKP No. 108088. SEM laboratory, BASF SE, Ludwigshafen, Germany, 15 July 2009.

17. BASF SE (2009). Sunscreen formulations: Lotions and cremes, Fa. Procter & Gamble, all day moisture Lotion SPF 15, Olay, GKP No. 108088. TEM laboratory, BASF SE, Ludwigshafen, Germany, 2 July 2009.

18. BASF SE (2009). Sunscreen formulations: Lotions and cremes, Fa. Umicore, W/O-emulsion coated ZnO (20%), GKP No. 108088. TEM laboratory, BASF SE, Ludwigshafen, Germany, 7 December 2009.

19. BASF SE (2009). Sunscreen formulations: Lotions and cremes, Fa. Umicore, O/W-emulsion uncoated ZnO (9%), GKP No. 108088. SEM laboratory, BASF SE, Ludwigshafen, Germany, 15 July 2009.

20. BASF SE (2009). Sunscreen formulations: Lotions and cremes, Fa. Umicore, W/O-emulsion coated ZnO (20%), GKP No. 108088. SEM laboratory, BASF SE, Ludwigshafen, Germany, 15 July 2009.

21. BASF SE (2009). Sunscreen formulations: Lotions and cremes, Fa. Unilever, Perfect results SPF 15/PA++, Ponds and Sun defense SPF 30/PA++, Ponds, GKP No. 108088. TEM laboratory, BASF SE, Ludwigshafen, Germany, 22 June 2009.

22. BASF SE (2009). Sunscreen formulations: Lotions and cremes, Fa. Unilever, Perfect results SPF 15/PA++, Ponds, GKP No. 108088. SEM laboratory, BASF SE, Ludwigshafen, Germany, 15 July 2009.

23. BASF SE (2009). Sunscreen formulations: Lotions and cremes, Fa. Unilever, Sun defense SPF 30/PA++, Ponds, GKP No. 108088. SEM laboratory, BASF SE, Ludwigshafen, Germany, 15 July 2009.

24. BASF SE (2009). Sunscreen formulations: Lotions and cremes, O/W-Emulsion with ZnO coated ex. Umicore, GKP No. 107220. SEM laboratory, BASF SE, Ludwigshafen, Germany, 7 May 2009.

25. BASF SE (2009). Sunscreen formulations: Lotions and cremes, O/W-Emulsion with ZnO coated ex. Umicore, GKP No. 107220. TEM laboratory, BASF SE, Ludwigshafen, Germany, 22 April 2009.

26. BASF SE (2009). Z-COTE® HP1, Micronucleus test in bone marrow cells of the mouse. Laboratory Project Identification No. 26M0488/064179. Experimental Toxicology and Ecology, BASF SE, Ludwigshafen, Germany, 24 August 2009.

27. BASF SE (2009). Z-COTE® Max, Salmonella typhimurium reverse mutation assay (standard plate test and preincubation test). Laboratory Project Identification No. 40M0620/064186. Experimental Toxicology and Ecology, BASF SE, Ludwigshafen, Germany, 9 March 2009.

28. BASF SE (2009). ZnO – Study on the toxicity and distribution of nanoscale ZnO in Wistar rats after i.v. administration and observation period of up to 4 weeks. Laboratory Project Identification No. 38C0488/06106. Experimental Toxicology and Ecology, BASF SE, Ludwigshafen, Germany, 16 December 2009.

29. BASF SE (2010). Behaviour of ZnO coated & uncoated in O/W emulsion & photostability assessment. Technical Report No. PC-10-037. Technical Service UV Protection, BASF SE, Ludwigshafen, Germany, 22 July 2010.

30. BASF SE (2010). Z-COTE® HP1, Subacute 5-day lung toxicity study in male Wistar rats, Dust aerosol exposure. Laboratory Project Identification No. 99I0488/06051. Experimental Toxicology and Ecology, BASF SE, Ludwigshafen, Germany, 10 March 2010, confidential.

31. BASF SE (2010). Zeta potential analysis. Report 118958. BASF Polymer Physics, BASF SE, Ludwigshafen, Germany, 18 March 2010.

32. Beckett WS, Chalupa DF, Pauly-Brown A, Speers DM, Stewart JC, Frampton MW, et al. (2005). Comparing inhaled ultrafine versus fine zinc oxide particles in healthy adults: a human inhalation study. Am J Respir Crit Care Med; 171:1129-35.

33. Casey P, Boskovic S, Lawrence K, Turney T (2004). Controlling the photoactivity of nanoparticles. NSTI-Nanotech; 3:370-4.

34. CEFIC (2008). Characterization of ultrafine ZnO. COLIPA/SCCP/SCENIHR meeting. Meeting presentation, 1 October 2008.

35. COLIPA (2008). Human safety assessment of nano ZnO and TiO_2 used in sunscreen. Meeting presentation, 30 September 2009.

36. Cross SE, Innes B, Roberts MS, Tsuzuki T, Robertson TA, McCormick P (2007). Human skin penetration of sunscreen nanoparticles: in-vitro assessment of a novel micronized ZnO formulation. Skin Pharmacol Physiol; 20:148-54.

37. Dufour EK, Kumaravel T, Nohynek GJ, Kirkland D, Toutain H (2006). Clastogenicity, photo-clastogenicity or pseudo-photo-clastogenicity: Genotoxic effects of zinc oxide in the dark, in pre-irradiated or simultaneously irradiated Chinese hamster ovary cells. Mutat Res; 607:215-24.

38. Durand L, Habran N, Denis C, Meulders L, Henschel V, Amighi K (2007). Influence of different parameters on droplet size and size distribution of sprayable sunscreen emulsions with high concentrations of UV-filters. Int J Cosmet Sci; 29:461-71.

39. Durand L, Habran N, Henschel V, Amighi K (2009). In vitro evaluation of the cutaneous penetration of sprayable sunscreen emulsions with high concentrations of UV filters. Int J Cosmet Sci; 31:279-92.

40. Dussert AS, Gooris E, Hemmerle J (1997). Characterization of the mineral content of a physical sunscreen emulsion and its distribution onto human stratum corneum. Int J Cosmet Sci; 19:119-29.

41. Elementis (2010). Basic physical chemical characterization of Nanox. Elementis Specialities, Cologne, Germany, personal communication, 1 September 2010.

42. EMEA (2008). Concept paper on the need for revision of the note of guidance on Photosafety testing (CPMP/SWP/398/01). European Medicines Agemcy (EMEA), Committee for medicinal products for Human use (CHMP), London, 24 January 2008.

43. European Chemicals Bureau (2003). Technical Guisdance Document on Risk Assessment, TGD, Part I. European Chemicals Bureau (ECB), European Communities, 2003.

44. European Chemicals Bureau (2004). European Union Risk Assessment Report, Zinc oxide, Volume 43, with addendum 2004, 2nd Priority list. Institute for Health and Consumer Protection, European Chemicals Bureau.

45. Federal office of Public Health (Switzerland) and Food and Consumer Product Safety Authority (The Netherlands) (2008). Guidance for industry – Recommendations on waterproofing aerosols in order to minimize consumer inhalation toxicity risks, April 2008.

46. Filipe P, Silva JN, Silva R, Cirne de Castro JL, Marques Gomes M, Alves LC et al. (2009). Stratum corneum is an effective barrier to TiO_2 and ZnO nanoparticle percutaneous absorption. Skin Pharmacol Physiol; 22:266-75.

47. FitzGeral R (2005). Review of recent literature on safety of nanomaterials in cosmetics with special references to skin absorption and resorption of ultrafine titanium dioxide and ZnO. Prepared for Physical Sunscreens Manufacturers Association (PSMA) and BASF AG, 28 September 2005.

48. Gamer AO, Leibold E, van Ravenzwaay B (2006). The in vitro absorption of microfine zinc oxide and titanium dioxide through porcine skin. Toxicol In Vitro; 20:301-7.

49. Grillo Zinkoxid GmbH (2009). Analyse Report Zinkoxid Nano Tec 50, Charge: E1047. Grillo Zinkoxid GmbH, 9 September 2009.

50. Grillo Zinkoxid GmbH (2009). Analyse Report Zinkoxid Nano Tec 60, Charge: E1046. Grillo Zinkoxid GmbH, 9 September 2009.

51. Grillo Zinkoxid GmbH (2009). Datenblatt Grillo Nano Tec 50 and Grillo Nano Tec 60. Grillo Zinkoxid GmbH, April 2009.

52. Grillo Zinkoxid GmbH (2009). Nano Tec 50 and Nano Tec 60, Summary on supplementary physico-chemical characterization, personal communication. Grillo Zinkoxid GmbH, 6 July 2009.

53. Grillo Zinkoxid GmbH (2009). Rietveld refinement of Nano Tec 60. Grillo Zinkoxid GmbH, 2 December 2009.

54. Grillo Zinkoxid GmbH (2009). Zertifikat – Zinkoxid Nano Tec 50, Charge: E1047. Betriebslaboratorium, Grillo Zinkoxid GmbH, 9 September 2009.

55. Grillo Zinkoxid GmbH (2009). Zertifikat – Zinkoxid Nano Tec 60, Charge: E1046. Betriebslaboratorium, Grillo Zinkoxid GmbH, 9 September 2009.

56. Grillo Zinkoxid GmbH (2010). Comparison of the UV/VIS spectra of nano-scale zinc oxides, personal communication. Grillo Zinkoxid GmbH, 27 August 2010.

57. Grillo Zinkoxid GmbH (2010). Research Report 02/2010 – Solubility of zinc oxide in the stomach. Grillo Zinkoxid GmbH, 18 January 2010.

58. Grillo Zinkoxid GmbH (2010). SEM images of Nano Tec 50, personal communication. Grillo Zinkoxid GmbH, 6 August 2010.

59. Gulson B, McCall M, Gomez L, Korsch M, Casey P, Kinsley L (2010). Dermal absorption of ZnO particles from sunscreens applied to humans at the beach. ICONN 2010 (abstract). Available from: URL: http://realizebeauty.files.wordpress.com/2010/02/iconn2010-abstract-gulson1.pdf (accessed 13 July 2012). [Linked checked and date of access added – Judy Burns]

60. Gulson B, Wong H, McCall M, Casey P, Trotter J, McCulloch M et al. (2008). Dermal absorption of ZnO nanoparticles using the stable isotope approach. Toxicol Lett; 180:S222, N08 (abstract).

61. Hidaka H, Kobayashi H, Koike T, Sato T, Serpone N (2006). DNA damage photoinduced by cosmetic pigments and sunscreen agents under solar exposure and artificial UV illumination. J Oleo Sci; 55:249-61.

62. ICH (2010). International Conference on Harmonization (ICH) of technical requirements for registration of pharmaceuticals for Human use. Final Concept Paper S10: Photosafety evaluation of pharmaceuticals, endorsed by ICH Steering Committee, 09 June 2010.

63. IRGC (2008). International Risk Governance Council. A report for IRGC, Risk Governance of Nanotechnology Applications in Food and Cosmetics. A report prepared for IRGC by Antje Grobe, Ortwin Renn and Alexander Jaeger Dialogik, GmbH, International Risk Governance Council, Geneva, September 2008.

64. ISO (2008). International Organization for Standardization/Technical Specification 27687, Nanotechnologies – Terminology and Definitions for Nano-objects – Nanoparticle, Nanofibre and Nanoplate, ISO/TS 27687, 2008.

65. Knorr F, Lademann J, Patzelt A, Sterry W, Blume-Peytavi U, Vogt A (2009). Follicular transport route--research progress and future perspectives. Eur J Pharm Biopharm; 71:173-80.

66. Kuo TR, Wu CL, Hsu CT, Lo W, Chiang SJ, Lin SJ, et al. (2009). Chemical enhancer induced changes in the mechanisms of transdermal delivery of zinc oxide nanoparticles. Biomaterials; 30:3002-8.

67. L'Oreal (2007). ZnO: Induction of chromosome aberrations in cultured Chinese hamster ovary (CHO) cells in the presence of ultra violet light. Covance Report No. 413/124-D6172. Covance Laboratories Ltd., Harrogate, North Yorkshire, England for L'Oreal, Asnieres sur Seine, France, January 2007.

68. Lademann J (2006). German: Translation of Title – Presentation: Risk assessment of nanotechnology in cosmetic products, Humbold University, Berlin, Germany, presented on an expert meeting at the German Federal Institute for Risk Assessment (BfR), 28 March 2006. Available from: URL: http://www.bfr.bund.de/cm/232/risikobewertung_von_nanopartikeln_in_kosmetischen_produkten.pdf (accessed 13 July 2012). [Link checked – Judy Burns]

69. Lademann J, Meinke M, Sterry W, Patzelt A (2009). Wie sicher sind Nanopartikel [German] (English abstract: How safe are nanoparticles?). Hautarzt; 60:305-9.

70. Lademann J, Richter H, Jacobi U, Patzelt A, Hueber-Becker F, Ribaud C et al. (2008). Human percutaneous absorption of a direct hair dye comparing in vitro and in vivo results: implications for safety assessment and animal testing. Food Chem Toxicol; 46:2214-23.

71. Landisedel R, Ma-Hock L, Kroll A, Hahn D, Schneckenburger J, Wiench Ket al. (2010). Testing metal-oxide nanomaterials for human safety. Adv Mater; 22:2601-27.

72. Landsiedel R, Kapp MD, Schulz M, Wiench K, Oesch F (2009). Genotoxicity investigations on nanomaterials: methods, preparation and characterization of test material, potential artifacts and limitations--many questions, some answers. Mutat Res; 681:241-58.

73. Landsiedel R, Ma-Hock L, Van Ravezwaay B, Schulz M, Wiench K, Champ S et al. (2010). Gene toxicity studies on titanium dioxide and zinc oxide nanomaterials used for UV-protection in cosmetic formulations. Nanotoxicology; 4:364-81.

74. Lynch AM, Wilcox P (2010). Review of the performance of the 3T3 NRU in vitro phototoxicity assay in the pharmaceutical industry. Exp Toxicol Pathol; 63:209-14.

75. Monteiro-Riviere NA, Inman A (2009). Flow-through studies on excised UVB-exposed porcine skin with BASF formulations. North Carolina State University, Center for Chemical Toxicology Research and Phamacokinetics, Raleigh, North Carolina, USA, 16 June 2009, confidential.

76. Monteiro-Riviere NA, Wiench K, Landsiedel R, Schulte S, Champ S, Inman AO (2010). In vitro penetration studies of sunscreen formulations containing titanium and zinc nanoparticles. SOT abstract, 2010.

77. Nash (2009). Chapter 10 – Systemic effects of topically applied sunscreen ingredients. In: Clinical Guide to Sunscreens and Photoprotection. Basic and Clinical Dermatology, Vol. 43, p. 139-54.

78. NIA (2007). Nanotechnology Industries Association (NIA). NIA Comment on SCCP Preliminary Opinion on Safety of Nanomaterials in Cosmetic Products, September 2007.

79. Nohynek GJ, Dufour EK, Roberts MS (2008). Nanotechnology, cosmetics and the skin: is there a health risk? Skin Pharmacol Physiol; 21:136-49.

80. Nohynek GJ, Lademann J, Ribaud C, Roberts MS (2007). Grey goo on the skin? Nanotechnology, cosmetic and sunscreen safety. Crit Rev Toxicol; 37:251-77.

81. OECD (2010). Organization for Economic Co-operation and Development. Environment, Health and Safety Publications, Series on the Safety of Manufactured Nanomaterials No. 21, Report of the Workshop on Risk Assessment of manufactured nanomaterials in a regulatory contex, ENV/JM/MONO(2010)10. OECD, Paris, France, 16 April 2010. Available from: URL: http://www.oecd.org/LongAbstract/0,3425,en_2649_37015404_45020877_1_1_1_1,00.html.

82. Osmond MJ, McCall (2010). Zinc oxide nanoparticles in modern sunscreens: an analysis of potential exposure and hazard. Nanotoxicology; 4:15-41.

83. Pape WJ, Maurer T, Pfannenbecker U, Steiling W (2001). The red blood cell phototoxicity test (photohaemolysis and haemoglobin oxidation): EU/COLIPA validation programme on phototoxicity (Phase II). Altern Lab Anim; 29:145-62.

84. Procter & Gamble (2009). Composition of all day moisture lotion SPF 15 Olay. Personal communication, 23 November 2009, confidential.

85. Sayes CM, Reed KL, Warheit DB (2007). Assessing toxicity of fine and nanoparticles: comparing in vitro measurements to in vivo pulmonary toxicity profiles. Toxicol Sci; 97:163-80.

86. SEHSC (2001). Silicones Environmental, Health and Safety Council of North America. Guidance for aerosol applications of silicone-based materials. Reston, VA, USA, April 2001.

87. SCCNFP (2003). The Scientific Committee on Cosmetic Products and Non-Food Products Intended for Consumers. Opinion concerning zinc oxide, COLIPA No. S76, SCCNFP/0649/03. Adopted by the SCCNFP during the 24th plenary meeting of 24-25 June 2003. Available from: URL: http://ec.europa.eu/health/ph_risk/committees/sccp/documents/out222_en.pdf (accessed 16 July 2012).

88. SCCP (2005). Scientific Committee on Consumer Products. Statement on Zinc oxide used in sunscreens, SCCP/0932/05. Adopted by the SCCP during the 5th plenary of 20 September 2005. Available from: URL: http://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_o_00m.pdf (accessed 16 July 2012).

89. SCCP (2007). Scientific Committee on Consumer Products. Opinion on the safety of nanomaterials in cosmetic products, SCCP/1147/07. Adopted by the SCCP after the public consultation on the 14th plenary of 18 December 2007. Available from: URL: http://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_o_123.pdf (accessed 16 July 2012).

90. SCCP (2009). Scientific Committee on Consumer Products. Clarification on Opinion SCCNFP/0932/05 on Zinc oxide, COLIPA No. S76, SCCP/1215/09. Adopted by the SCCP at its 19th plenary on 21 January 2009. Available from: URL: http://ec.europa.eu/health/ph_risk/committees/04_sccp/docs/sccp_o_167.pdf (accessed 16 July 2012).

91. SCENIHR (2009). Scientific Committee on Emerging and Newly Identified Health Risks. Risk Assessment of Products of Nanotechnologies. Adopted by the SCENIHR at its 28th plenary on 19 January 2009 ,

92. SCENIHR (2006). Scientific Committee on Emerging and Newly Identified Health Risks. The appropriateness of existing methodologies to assess the potential risks associated with engineered and adventitious products of nanotechnologies, SCENIHR 002/05. Adopted by the SCENIHR during the 10th plenary meeting of 10 March 2006 after public consultation. Available from: URL: http://ec.europa.eu/health/ph_risk/committees/04_scenihr/docs/scenihr_o_003b.pdf (accessed 16 July 2012).

93. Schilling K, Bradford B, Castelli D, Dufour E, Nash JF, Pape W et al. (2010). Human safety review of "nano" titanium dioxide and zinc oxide. Photochem Photobiol Sci; 9:495-509.

94. Sharma V, Shukla RF, Saxena N, Parmar D, Das M, Dhawan A (2009). DNA damaging potential of zinc oxide nanoparticles in human epidermal cells. Toxicol Lett; 185:211-8.

95. Shiseido (2006). Contact sensitization study of FINEX-50 ZnO using Guinea pigs /s-APT method). Safety Assessment Group, Quality Assessment Center, Shiseido Co., Ltd, Kanagawa, Japan, 03 February 2006.

96. Shiseido (2006). Eye irritation study of of FINEX-50 ZnO in rabbits. Safety Assessment Group, Quality Assessment Center, Shiseido Co. Ltd., Kanagawa, Japan, 18 April 2006.

97. Shiseido (2006). Photo-chromosomal aberration test of FINEX-50 ZnO with cultured mammalian cells. Safety Assessment Group, Quality Assessment Center, Shiseido Co. Ltd., Japan, 31 August 2006.

98. Shiseido (2006). Photo-reverse mutation test of FINEX-50 ZnO in bacteria. Safety Assessment Group, Quality Assessment Center, Shiseido Co. Ltd., Kanagawa, Japan, 3 August 2006.

99. Shiseido (2006). Single dose oral toxicity study of FINEX-50 ZnO in rats. Safety Assessment Group, Quality Assessment Center, Shiseido Co. Ltd., Kanagawa, Japan, 1 March 2006.

100. Shiseido (2008). Photo-micronucleus test of 05M099 with mouse epidermal cells (translated from the report in Japanese), Study No. G-07-069. Hatano Research Institute, Food and Drug Safety Center, Kanagawa, Japan for Shiseido Co. Ltd., Kanagawa, Japan, 14 August 2008.

101. Shiseido (2009). 3-day consecutive skin irritation study of FINEX-50 ZnO in Guinea pigs. Safety Assessment Group, Quality Assessment Center, Shiseido Co. Ltd., Kanagawa, Japan, 26 February 2009.

102. Symrise (2010). Data Sheet Zinc oxide NDM (product No. 106 407). Symrise GmbH & Co. KG, Holzminden, Germany, 14 October 2004.

103. Symrise (2010). Data Sheet Zinc oxide Neutral (product No. 104 702). Symrise GmbH & Co. KG, Holzminden, Germany, 14 October 2004.

104. Tsuji J, Maynard AD, Howard PC, James JT, Lam CW, Warheit DB et al. (2006). Research strategies for safety evaluation on nano materials, part IV: risk assessment of nanoparticles. Toxicol Sci; 89:42-50.

105. Umicore (2007). 50 human subject repeat insult patch test, skin irritation/sensitization evaluation (occlusive patch), Zano® 10 Plus. Report No. MS07.RIPT.L09360.50.UMI. AMA Laboratories, Inc., New City, NY, USA performed for Umicore Research, Umicore Zinc Chemicals, Angleur, Belgium, 22 October 2007.

106. Umicore (2007). 50 human subject repeat insult patch test, skin irritation/sensitization evaluation (occlusive patch), Zano®10. Report No. MS07.RIPT.L09350.50.UMI. AMA Laboratories, Inc., New City, NY, USA performed for Umicore Research, Umicore Zinc Chemicals, Angleur, Belgium, 22 October 2007.

107. Umicore (2007). Safety Data Sheet, Zano® Plus. Umicore Zinc Chemicals, Angleur, Belgium, 24 November 2007.

108. Umicore (2007). Safety Data Sheet, Zano®. Umicore Zinc Chemicals, Angleur, Belgium, 24 November 2007.

109. Umicore (2007). SEM report Zano® 10 Plus. Umicore Zinc Chemicals, Angleur, Belgium, 12 March 2007.

110. Umicore (2007). SEM report Zano® 10. Umicore Zinc Chemicals, Angleur, Belgium, 12 March 2007.

111. Umicore (2007). Technical Data Sheet Zano® 10 Plus. Umicore Zinc Chemicals, Angleur, Belgium, August 2007.

112. Umicore (2007). Technical Data Sheet Zano® 10. Umicore Zinc Chemicals, Angleur, Belgium, August 2007.

113. Umicore (2009). TEM image with regards to surface coating, Zano® 10 Plus. Umicore Zinc Chemicals, Angleur, Belgium, personal communication, 7 July 2009.

114. Umicore (2010). Particle size and UV/VIS analysis of four zinc oxide samples. Umicore Research, Umicore Zinc Chemicals, Angleur, Belgium, 16 February 2010.

115. Umicore (2010). Solubility of four zinc oxide samples. Umicore Research, Umicore Zinc Chemicals, Angleur, Belgium, 10 March 2010.

116. Umicore (2010). XRD analysis of four zinc oxide samples. Umicore Research, Umicore Zinc Chemicals, Angleur, Belgium, 16 February 2010.

117. Unilever (2010). Information on composition of representative Unilever ZnO formulations. Safety and Environmental Assurance Centre, Unilever, London, United Kingdom, personal communication, 30 July 2010, confidential.

118. Wang B, Feng W, Wang M, Wang T, Gu Y, Zhu M et al. (2008). Acute toxicological impact of nano- and submicro-scaled zinc oxide powder on healthy adult mice. J Nanopart Res; 10:263-76.

119. Warheit DB, Sayes CM, Reed KL (2009). Nanoscale and fine zinc oxide particles: can in vitro assays accurately forecast lung hazards following inhalation exposures? Environ Sci Technol; 43:7939-45.

120. Zvyagin AV, Zhao X, Gierden A, Sanchez W, Ross JA, Roberts MS (2008). Imaging of zinc nanoparticle penetration in human skin in vitro and in vivo. J Biomed Opt; 13:064031.

Additional references cited

AR1.   Beyerle A, Long AS, White PA, Kissel T, Stoeger T (2011).) Poly(ethylene imine) nanocarriers do not induce mutations nor oxidative DNA damage in vitro in MutaMouse FE1 cells. Mol Pharm; 8:,976-81.

AR2.   Corradi S, Gonzalez L, Thomassen LC, Bilaničová D, Birkedal RK, Pojana G, et al. (2012).) Influence of serum on in situ proliferation and genotoxicity in A549 human lung cells exposed to nanomaterials. Mutat Res; 745:,21-72.

AR3.   Draize JH (1959). Dermal Toxicity. In: Appraisal of the Safety of Chemicals in Foods, Drugs and Cosmetics. Assoc. Foods & Drug Official of the U. S.(ed)., Austin, Texas, St Health, pp. 46-59.

AR4.   EFSA (2011). European Food Safety Authority.) Guidance on the risk assessment of the application of nanoscience and nanotechnologies in the food and feed chain. EFSA Journal; 9:, 2140.

AR5.   EMEA Committee for Proprietary Medicinal Products (CPMP), Note for Guidance on Photosafety Testing, CPMP/SWP/398/01, London 27 June 2002

AR6.   Gerloff K, Albrecht C, Boots AW, Förster I, Schins RP (2009).) Cytotoxicity and oxidative DNA damage by nanoparticles in human intestinal Caco-2 cells. Nanotoxicology; 3:, 355-64.

AR7.   Gocke E, Muller L, Guzzie PJ, Brendler-Schwaab S, Bulera S, Chignell CF, Henderson LM, Jacobs A, Murli H, Snyder RD, Tanaka N. Considerations on Photochemical Genotoxicity: Report of the International Workshop on Genotoxicity Test Procedures Working Group Environmental and Molecular Mutagenesis 35:173-184 (2000)

AR8.   Guide to Marketing and Manufacturing of Cosmetics and Quasi-Drugs in Japan (version. 4). Yakuji Nippo Ltd. (Tokyo), p. 74-80.

AR9.   Gullot JP, Gonnet JF and Clement C (1982). Evaluation of the ocular irritation potential of 56 compounds. Food Chem.Toxicol. 20: 573-582

AR10. Gulson B, McCall M, Korsch M, Gomez L, Casey P, Oytam Y et al. (2010).) Small amounts of zinc from zinc oxide particles in sunscreens applied outdoors are absorbed through human skin. Toxicol Sci; 118:140-9.

AR11. Hackenberg S, Scherzed A, Technau A, Kessler M, Froelich K, Ginzkey C et al.(2011a).) Cytotoxic, genotoxic and pro-inflammatory effects of zinc oxide nanoparticles in human nasal mucosa cells in vitro. Toxicol In Vitro; 25:,657-63.

AR12. Hackenberg S, Zimmermann FZ, Scherzed A, Friehs G, Froelich K, Ginzkey C et al. (2011b).) Repetitive exposure to zinc oxide nanoparticles induces DNA damage in human nasal mucosa mini organ cultures. Environ Mol Mutagen; 52:, 582-9..

AR13. Hara T, Nishikawa T, Sui H, Kawakami K, Matsumoto H, Tanaka N (2007). In vivo photochemical skin micronucleus test using a sunlight simulator: detection of 8-methoxypsoralen and benzo[a]pyrene in hairless mice. Mutat Res; 631:1-8.

AR14. Karlsson HL, Cronholm P, Gustafsson J, Möller L (2008). Copper oxide nanoparticles are highly toxic: a comparison between metal oxide nanoparticles and carbon nanotubes. Chem Res Toxicol; 21:1726-32.

AR15. Kermanizadeh A, Pojana G, Gaiser BK, Birkedal R, Bilanicová D, Wallin H et al. (2012).) In vitro assessment of engineered nanomaterials using a hepatocyte cell line: cytotoxicity, pro-inflammatory cytokines and functional markers. Nanotoxicology 20 Jan. [Epub ahead of print] 2012

AR16. Li CH, Shen CC, Cheng YW, Huang SH, Wu CC, Kao CC et al. (2011).)Organ biodistribution, clearance, and genotoxicity of orally administered zinc oxide nanoparticles in mice. Nanotoxicology27 Sept. [Epub ahead of print] 2011

AR17. Miao AJA-J, Zhang XYX-Y, Luo Z, Chen CSC-S, Chin WCW-C, Santschi PH et al. (2010).) Zinc oxide- engineered nanoparticles: dissolution and toxicity to marine phytoplankton. Environ Toxicol Chem; 29:, 2814-222822.

AR18. Monteiro-Riviere NA, Wiench K, Landsiedel R, Schulte S, Inman AO, Riviere JE. (2011).) Safety evaluation of sunscreen formulations containing titanium dioxide and zinc oxide nanoparticles in UVB sunburned skin: an in vitro and in vivo study. Toxicol Sci; 123:264-80.

AR19. Mudunkotuwa IA, Rupasinghe T, Wu CMC-M, Grassian VH (2012).) Dissolution of ZnO nanoparticles at circumneutral pH: a study of size effects in the presence and absence of citric acid. Langmuir; 28:, 396-403.

AR20. Pasupuleti S, Alapati S, Ganapathy S, Anumolu G, Pully NR, Prakhya BM (2011).) Toxicity of zinc oxide nanoparticles through oral route. Toxicol Ind Health 27 Oct. [Epub ahead of print] DOI:10.1177/0748233711420473.

AR21. Reed RB, Ladner DA, Higgins CP, Westerhoff P, Ranville JF (2012).) Solubility of nano-zinc oxide in environmentally and biologically importantimpotrtant matrices. Environ Toxicol Chem; 31:, 93-999.

AR22. Sharma V, Singh SK, Anderson D, Tobin DJ, Dhawan A (2011).) Zinc oxide nanoparticle induced genotoxicity in primary human epidermal keratinocytes. J Nanosci Nanotechnol; 11:,3782-8.

AR23. Surekha P, Kishore AS, Srinivas A, Selvam G, Goparaju A, Reddy PN et al. (2012).) Repeated dose dermal toxicity of nano zinc oxide with Sprague-Dawley rats. Cutan Ocul Toxicol; 31:26-32.

AR24. Thompson ED, McDermott JA, Zerkle TB, Skare JA, Evans BL, Cody DB (1989). Genotoxicity of zinc in 4 short-term mutagenicity assays. Mutat Res; 223:, 267-72.

AR25. Yanagi M, Hoya M, Mori M, Katsumura Y (2001).) Modified short-term guinea pig sensitization tests for detecting contact allergens as an alternative to the conventional test. Contact Dermatitis;44:140-5.

AR26. Yoshida R, Kitamura D, Maenosono S (2009).) Mutagenicity of water-soluble ZnO nanoparticles in Ames test. J Toxicol Sci; 34:,119-22.

AR27. Gulson B, Wong H, Korsch M, Gomez L, Casey P, McCall M, McCulloch M, Trotter J, Stauber J, Greenoak G. (2012) Comparison of dermal absorption of zinc from different sunscreen formulations and differing UV exposure based on stable isotope tracing. Sc Total Environm 420, 313-318.

AR28. Tran DT, Salmon R. (2011) Potential photocarcinogenic effects of nanoparticle sunscreens. Australasian J Dermatol 52, 1-6.