



**Scientific Committee on Consumer Safety**

**SCCS**

**OPINION**

**on Carbon Black (nano-form)**

The SCCS adopted this opinion at its 4<sup>th</sup> plenary meeting

of 12 December 2013

First revision of 27 March 2014

Second revision of 15 December 2015

### About the Scientific Committees

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

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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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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

Carbon Black, CI 77266, CAS no. 1333-86-4, EC no. 215-609-9, listed in Annex IV as reference number 126 of the Cosmetic Regulation (EC) No. 1223/2009, is used as colorant in cosmetic products.

According to the applicant the ingredient Carbon Black CI 77266 has a long history of use as a cosmetic colorant. Typical uses of Carbon Black are in different types of cosmetic products, typical use concentrations range from 0.001% to 10% with 0.001% for skin products, 5% for nail enamels and mascaras and up to 10% for other eye decorative products such as eyeliners, eye pencils and eye shadows.

As a result of the recast of the European Cosmetic Directive (76/768/EEC) into the Cosmetic Regulation (EC) No. 1223/2009 a new description will be necessary for this chemical. According to the definition established in the Cosmetic Regulation under art. 2.(k), this material is a nanomaterial. Based on this new definition, specific data on the material in its nano form was submitted by the applicant.

Reportedly, the present dossier evaluates the safety of Carbon Black/CI 77266 taking into account most recent recommendations for the safety evaluation of nanomaterials. These recommendations include the SCCS "Guidance on the safety assessment of nanomaterials in cosmetics"(SCCS/1484/12) as well as ECHA "Recommendations for nanomaterials applicable to Chapter R7a Endpoint specific guidance" (ECHA, 2012<sup>1</sup>).

Regarding the tests for nanomaterials, the review of health effects-related testing guidelines concluded that, in general, the OECD guidelines are applicable for investigating the health effects of nanomaterials, but specific attention needs to be given to the physicochemical characteristics of the test material, including such characteristics in the actual dosing solution (OECD, 2009; OECD 2010; OECD, 2012<sup>2</sup>; REACH nano-consultation, 2011<sup>3</sup>).

The current submission I- according to Cosmetics Europe - provides an overall safety assessment for this ingredient in nano-form, which takes into account the available information.

## 2. TERMS OF REFERENCE

1. *Does the SCCS consider Carbon Black, CI 77266 in its nano form safe for use as a colorant with a concentration up to 10 % in cosmetic products taking into account the scientific data provided?*
2. *Does the SCCS have any further scientific concern with regard to the use of Carbon Black, CI 77266 in its nano form as a colorant in cosmetic products?*

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<sup>1</sup> [http://echa.europa.eu/documents/10162/13632/appendix\\_r7a\\_nanomaterials\\_en.pdf](http://echa.europa.eu/documents/10162/13632/appendix_r7a_nanomaterials_en.pdf)

<sup>2</sup> <http://www.oecd.org/science/nanosafety/47104296.pdf>

<sup>3</sup> <http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=COM:2012:0572:FIN:EN:PDF>

### 3. OPINION

#### 3.1. Chemical and Physical Specifications

##### 3.1.1. Chemical identity

Carbon Black (CAS 1333-86-4) is a material consisting of elemental carbon in the form of near spherical colloidal particles and coalesced particle aggregates/agglomerates, obtained by partial combustion or thermal decomposition of hydrocarbons (Robertson and Smith, 1994).

Commercial carbon black materials generally contain more than 97% elemental carbon with variable amounts of oxygen, hydrogen and sulphur. Less than 1% of carbon black particles consist of extractable organic materials. Typical classes of organic chemicals adsorbed onto the carbon black particles surface are polycyclic aromatic hydrocarbons (PAHs), nitro-derivatives of PAHs and sulphur-containing PAHs. Examples of PAHs adsorbed onto carbon black particle surface include benzopyrenes, benzo[ghi]perylene, coronene, fluoranthene and pyrene (IARC, 2010). Several of these compounds are recognized as human carcinogens.

Carbon black materials are categorized as acetylene black, channel black, furnace black, lampblack or thermal black according to their manufacturing process. Worldwide production of carbon black in 2005 was approximately ten million tonnes. Over 90-95% of carbon black produced is of the furnace type (Robertson and Smith, 1994; IARC, 2010) and thermal black (decomposition of natural gas) follows at a distant second place (Submission 1). Carbon black is a black pigment and its principal industrial use today is based on its ability to reinforce natural and synthetic rubber including tyres, tubes, treads and other automotive products (IARC, 2010). Other uses include applications as pigments in paints, plastics, papers, inks, ceramics and cosmetics.

The different carbon black materials are characterized by their primary particles size, their aggregation and agglomeration status as well as their impurity profile. Typically, the average primary particle diameter of commercial carbon black materials range from 10 to 100 nm, while the average aggregate/agglomerate size is in the range 100-800 nm or above. Carbon black is initially formed as roughly spherical primary particles, which, in most cases, rapidly form aggregates. An aggregate is a chain of primary carbon particles that are permanently fused together in a random branching structure. The aggregate may consist of a few or hundreds of spherical particles (or, as in the case of thermal black, primarily single spheres rather than chains). Accordingly, on the basis of their primary particle size, all Carbon Black/CI 77266 materials are considered as nano-structured materials.

##### 3.1.1.1. Primary name and/or INCI name

CI 77266 [Nano] / Black 2 / D&C Black 2 (INCI)

##### 3.1.1.2. Chemical names

Carbon Black

##### 3.1.1.3. Trade names and abbreviations

*Submitted only by applicant*

- "Unipure Black LC 902" (specified as >95% pure Carbon Black) prepared from the oil furnace process is from the manufacturer LCW (Sensient)
- "Distinctive® Ink Black Lo-AQ" (a 20% aquatic dispersion of Unipure Black LC 902)
- Printex 140 (furnace blacks, Degussa AG)
- Printex G (51 nm, surface area, 30 m<sup>2</sup>/g), furnace blacks, Degussa AG)
- Spezialschwarz 4 (furnace blacks, Degussa AG)
- Carbon Black XPB 295
- Farbruss FW 200, furnace black

From open literature

- Printex 90 (14 nm, surface area 337 m<sup>2</sup>/g, specified as >99% pure Carbon Black, PAH = 0.039 ppm, furnace blacks) Degussa-Huels, Trostberg, Germany
- Monarch 880 (diameter, 16 nm; surface area, 220 m<sup>2</sup>/g, Cabot, Cambridge, MA)
- Sterling V (70 nm, surface area of 37 m<sup>2</sup>/g, PAH = 8.8 ppm Cabot, Cambridge, MA)
- Lampblack 101 (95 nm, surface area of 20 m<sup>2</sup>/g, PAH = 0.57 ppm, furnace blacks, Degussa AG)
- N330 [31 nm, PAH = 2.4 ppm, 70 – 90 m<sup>2</sup>/g]
- ASTM N375 (20 – 25 nm, surface area of 101 m<sup>2</sup>/g, BaP = 3.81 ppm, Phillips Petroleum Company, Akron, OH, USA)
- Elftex-12 furnace black (37 nm, surface area 43 m<sup>2</sup>/g, extractable organic material 0.04–0.29%) furnace blacks, Cabot, Cambridge, MA)
- FW2 (13 nm, surface area 350 m<sup>2</sup>/g, solvent extractable max 0.1%, Degussa AG)

#### 3.1.1.4. CAS / EC number

CAS: 1333-86-4  
EC: 215-609-9

#### 3.1.1.5. Structural formula

/

#### 3.1.1.6. Empirical formula

/

### 3.1.2. Physical form

Black powder

Liquid dispersions are available for most of the commercial raw materials containing carbon black

### 3.1.3. Molecular weight

12 (as carbon)

### 3.1.4. Purity, composition and substance codes

Commercial carbon black materials generally contain more than 97% elemental carbon

### 3.1.5. Impurities / accompanying contaminants

Typical classes of organic chemicals adsorbed onto the carbon black particles surface are polycyclic aromatic hydrocarbons (PAHs), nitro-derivatives of PAHs and sulphur-containing PAHs. Examples of PAHs adsorbed onto carbon black particle surface include benzopyrenes, benzo[ghi]perylene, coronene, fluoranthene and pyrene

The test item "Unipure Black LC 902" is FDA certified, and therefore meets the specifications for carbon black set by the US FDA, which are as follows:

- Total colour > 95%
- Specific surface area 200 - 260 m<sup>2</sup>/g
- Ash content < 0.15% w/w
- Total sulfur < 0.65% w/w
- Total PAH < 500 ppb
- Benzo[a]pyrene < 5 ppb, Dibenz[a,h]anthracene < 5 ppb
- As < 3 ppm, Pb < 10 ppm, Hg < 1 ppm.

Additionally, three batches of pure "Unipure Black LC 902 (4216, 4217 and 4219) underwent a full physico-chemical characterization and the following levels were observed for the main impurities:

- Individual PAHs were not detected and the detection limit of the analytical methods ranged from 0.0001 to 0.04 mg/kg (ppm)
- The maximum sulphur and ashes contents were 0.39 and 0.02% (w/w), respectively
- The heavy metals levels were approximately: As 2 ppm, Pb 2 ppm, and Hg 1 ppm.

Carbon black is regulated in EU in European Directive 2007/19/EC "Plastic materials and articles intended to come into contact with foodstuffs", with following *specifications*:

- Toluene extractables: maximum 0.1 %
- UV absorption of cyclohexane extract at 386 nm: < 0.02 AU for a 1 cm cell
- Benzo(a)pyrene content: max 0.25 mg/kg carbon black, 250 ppb.
- Maximum use level of carbon black in the polymer: 2.5 % w/w

#### *Other properties*

Three batches of the ingredient "Unipure Black LC 902" (4216, 4217 and 4219) were characterized (see Analytical file) for the following parameters:

- The pure carbon black contents range from 97.8 to 98%
- The specific surface area ranges from 209.1 to 212.2 m<sup>2</sup>/g
- The relative density ranges from 1.69 to 1.89

Additionally, the zeta potential of carbon black could not be measured or available in the published literature. Carbon black is insoluble in water and therefore the measurements of the redox potential, the partition coefficient n-Octanol/Water (Log P<sub>ow</sub>), and the pH were considered to be not relevant. Carbon black is not a doped material.

#### **SCCS comment**

Description of purity relates up to 98% of carbon black content. More information on the balancing impurities including well-known metallic contact allergens such as Co, Ni and Cr should be provided. Purity and impurity profiles of carbon black materials should comply with EU specifications of carbon black materials as used in food contact materials. Additionally, FDA specifications should also be considered. The purity / impurity profile of



the carbon black nanomaterials used in cosmetic products should be comparable with those tested for toxicity.

### 3.1.6. Solubility

Insoluble in water and other solvents

### 3.1.7. Partition coefficient (Log P<sub>ow</sub>)

Not relevant due to lack of solubility

### 3.1.8. Additional physical and chemical specifications

Melting point:	~ 3827
Boiling point:	/
Flash point:	data valid for carbon black from furnace process
Lower limit for explosion:	50g/m <sup>3</sup> , ignition temperature > 315 or > 500 C depending on test environment
Vapour pressure:	Not Applicable at typical use and storage temperatures 10 <sup>-7</sup> atm expected above 2500 K (data refers to highly Oriented Pyrolytic Graphite) Carbon Vapor Pressure and Heat of Vaporization

Ref. Marshall and Norton, J. Am. Chem. Soc., 1950, 72 (5), pp 2166–2171.

Density:	1.7 – 1.9 g/cm <sup>3</sup>
Viscosity:	Not Applicable below melting point/
pKa:	Not Applicable due to insolubility/
Refractive index:	Like for all dispersions the refractive index depends on particle size characteristics, type of matrix e.g. air, water, and further parameters of the formulation / dispersion
pH:	Not Applicable due to insolubility/
UV_Vis spectrum (200-800 nm):	/

Ref. Carbon Black User's Guide, Safety, Health, & Environmental Information, Copyright June 2004, International Carbon Black Association.

### 3.1.9. Homogeneity and Stability

/

### 3.1.10. Particle size

Table 1. presents a summary of surface area and primary particle size, aggregate diameter and agglomerate size for different types of carbon black.

**Table 1.:** Summary information on particle size (IARC, 2010)

Carbon black	Surface area (m <sup>2</sup> /g)	Approximate diameter of primary particle size (nm)	Diameter of aggregate (nm)	Size of agglomerate
Oil-furnace	12-240	10-400	50-400	Large (<2 mm)
Thermal	6-15	120-500	400-600	Large (<2 mm)
Impingement (channel)		10-30	50-200	Large (<2 mm)
Lampblack	15-25	60-200	300-600	Large (<2 mm)
Acetylene black	15-70	30-50	350-400	Pelletizes poorly

The particle size distribution of the following carbon black materials, dosing formulations and preparations was analysed:

Four batches of pure Carbon Black "Unipure Black LC 902" (C3949, 4216, 4217 and 4219) were analysed using Transmission Electron Microscopy (TEM) and Dynamic Light Scattering (DLS, see Table 2.).

**Table 2.:** Particle size distribution of batches of carbon black, powder form

Batch number	Particle size distribution by mass/volume (µm)			Particle size distribution by number (µm)		
	D10	D50	D90	D10	D50	D90
C3949	0.064	0.160	0.291	0.048	0.076	0.149
4216	0.057	0.128	0.237	0.048	0.074	0.132
4217	0.042	0.201	0.418	0.041	0.059	0.137
4219	0.052	0.137	0.269	0.044	0.067	0.134

Two raw materials (see Table 3.)

- "Distinctive<sup>®</sup> Ink Black Lo-AQ" (batch G01102213) was analysed using Transmission Electron Microscopy (TEM) and Static Light Scattering (SLS)
- "WD-CB2" (batch K-40219) was analysed using Transmission Electron Microscopy and DLS

**Table 3.:** Particle size distribution of carbon black dispersions (commercial forms)

Batch number	Particle size distribution by mass/volume (µm)			Particle size distribution by number (µm)		
	D10	D50	D90	D10	D50	D90
"Distinctive" Ink Black Lo-AQ" (batch G01102213)	0.231	4.870	27.510	0.127	0.146	0.259
"WD-CB2" (batch K-40219)	0.063	0.131	0.230	0.050	0.077	0.143

The dosing formulations used in the safety studies submitted in the present dossier, were analysed using Transmission Electron Microscopy and SLS (see Table 4.)

- Carbon black suspended in Dimethyl sulfoxide (DMSO),
- Carbon black suspended in sodium carboxymethylcellulose

**Table 4.:** Particle size distribution of dosing formulations used in safety studies

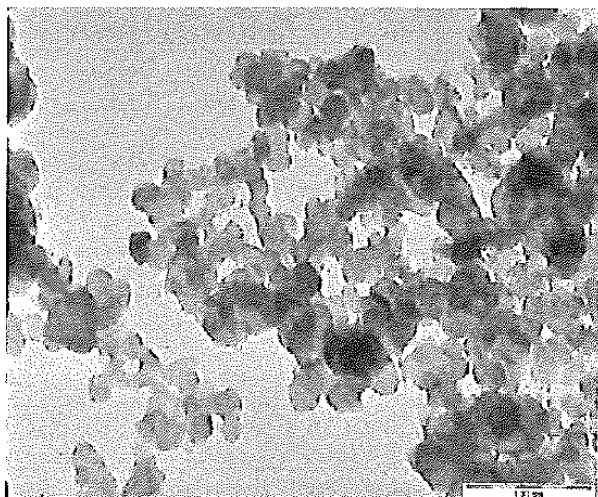
Dosing formulations	Safety studies	Particle size distribution by mass/volume ( $\mu\text{m}$ )			Particle size distribution by number ( $\mu\text{m}$ )		
		D10	D50	D90	D10	D50	D90
Unipure BlackLC 902 in sodium carboxymethyl	<ul style="list-style-type: none"> <li>• 13-week oral toxicity study in rats (Sathish, 2012)</li> <li>• Embryo-foetal development toxicity study by the oral route in rats (Ramesh, 2012)</li> </ul>	0.126	0.143	0.394	0.124	0.134	0.147
Unipure Black LC 902 in dimethyl sulfoxide	Genetic toxicity studies (Hobson, 2011; Lloyd, 2011; Lloyd 2012)	0.269	1.721	4.239	0.127	0.148	0.272

Similarly, the eyeliner formulation used in the *in vitro* percutaneous absorption studies (Johnson, 2013a; Johnson, 2013b), was analysed using Transmission Electron Microscopy and SLS. The particle size distributions results obtained in Light Scattering experiments are shown in the Table 5.

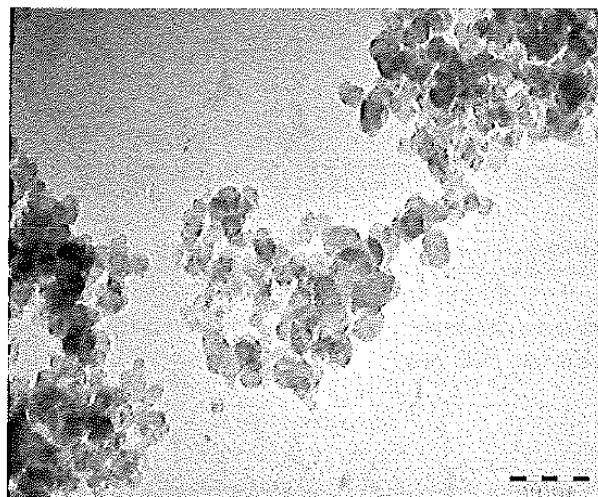
**Table 5.:** Particle size distributions of the cosmetic formulation used in percutaneous absorption studies (Johnson, 2013a; Johnson, 2013b)

Batch number	Particle size distribution by mass/volume ( $\mu\text{m}$ )			Particle size distribution by number ( $\mu\text{m}$ )		
	D10	D50	D90	D10	D50	D90
Eyeliner formulation	0.259	3.745	18.460	0.126	0.144	0.250

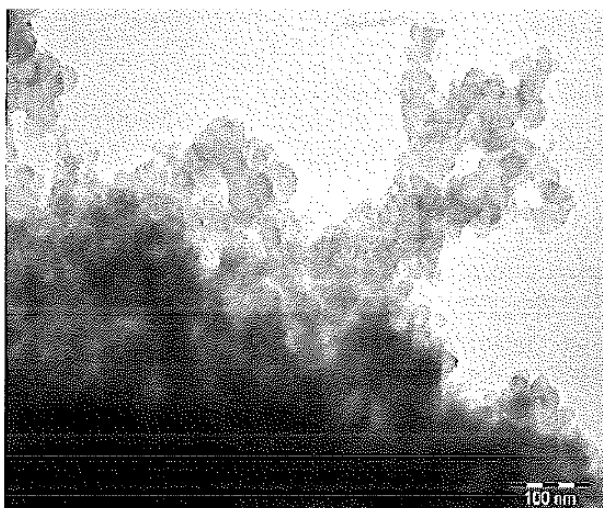
At TEM examination, the powder forms of carbon black, the commercial dispersions of carbon black, the dosing solutions used in safety studies as well as the cosmetic product used in the percutaneous absorption studies appeared overall similar (see Figs 1 to 6). Carbon black could be identified as a material composed of primary particles in the size range 20-30 nm. However, isolated carbon black particles could be sparsely/rarely/not identified, since carbon black was present as aggregates/agglomerates in the size range 0.100  $\mu\text{m}$  to 2 500  $\mu\text{m}$ .



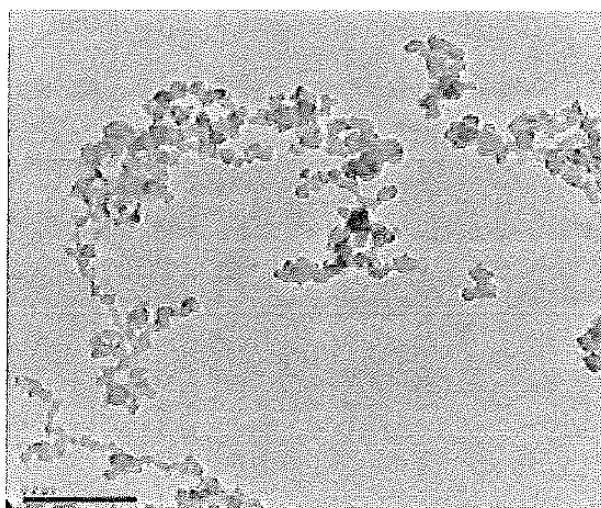
**Fig. 1.** TEM micrograph of Carbon Black powder form (batch C3949)  
Scale bar: 100 nm



**Fig. 2.** TEM micrograph of commercial dispersion WD-CB2 (batch K-40219)  
Scale bar: 100 nm

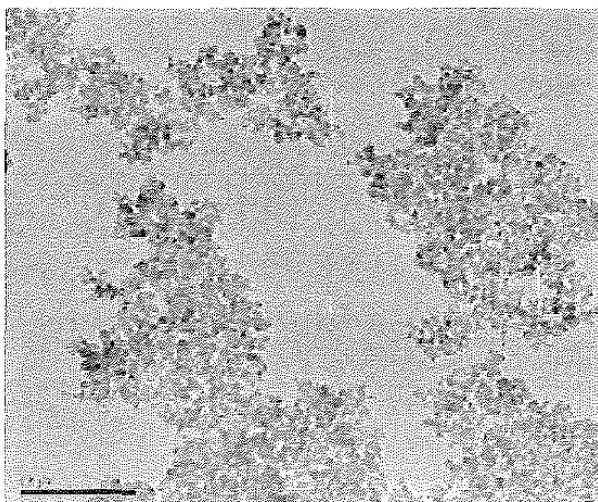


**Fig. 3.** TEM micrograph of commercial dispersion Distinctive Ink Black Lo-AQ (batch G01102213)  
Scale bar: 100 nm

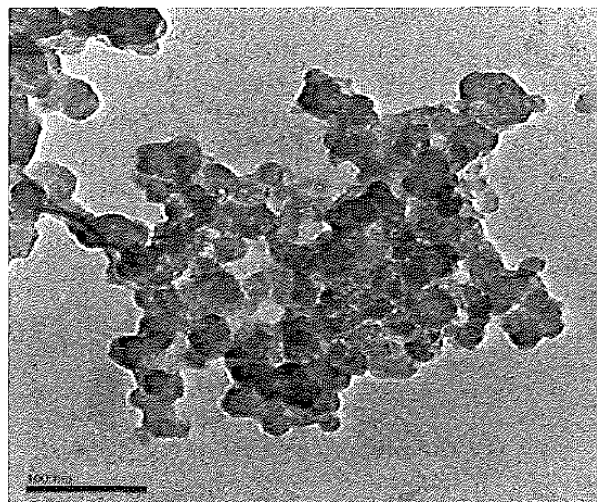


**Fig. 4.** TEM micrograph of dosing formulation used in safety studies: Unipure Black LC 902 in DMSO (Hobson, 2011; Lloyd, 2011; Lloyd 2012)  
Scale bar: 100 nm





**Fig. 5.** TEM micrograph of dosing formulation used in safety studies: Unipure Black LC 902 in sodium carboxymethylcellulose (Sathish, 2012; Ramesh, 2012)  
Scale bar: 100 nm



**Fig. 6.** TEM micrograph of the cosmetic formulation used in percutaneous absorption studies (Johnson, 2013a; Johnson, 2013b)  
Scale bar: 100 nm

At DLS/SLS examinations, a more refined evaluation of the size of the aggregates/agglomerates could be performed, and the results obtained were overall similar for the various carbon black dispersions and/or dosing formulations evaluated. Specifically, the results obtained for particle number distributions were essentially similar, with D50 and D90 values of approximately 150 nm and 200-300 nm respectively. For the powder forms of carbon black evaluated, D50 and D90 values were slightly lower i.e. about 50-100 nm and 150 nm respectively. When expressed in mass/volume distributions, the results obtained showed that larger agglomerates were observed with dispersions of carbon black in DMSO (D50 value of 1.7  $\mu\text{m}$ ) and the representative cosmetic formulation evaluated (D50 value of 3.7  $\mu\text{m}$ ), when compared to other materials evaluated.

Taken together, these particle size characterization results show that:

- consumers that use carbon black-containing cosmetic products are sparsely or not exposed to isolated nanoparticles of carbon black, but to aggregates and even large agglomerates of carbon black
- on the basis of the particle size distribution results obtained on the dosing formulations used in safety studies, the material evaluated in these studies can be considered to be overall representative of the material to which consumers are exposed externally. It may also be considered to represent a worst case scenario, since, when compared to the typical cosmetic formulation evaluated, smaller agglomerates were present in the dosing formulations used.

#### **SCCS comment**

The SCCS does not agree that the data provided presents a worst case scenario regarding the size distribution of the nanomaterial. It needs to be demonstrated that, based on the number size distribution, the nanomaterials used in the toxicity studies have the same D50 size as nanomaterials used in the cosmetic formulations.

The SCCS has accepted that nanoparticles in carbon black materials exist mainly as aggregates and agglomerates. A few tests in the presence of emulsifiers have not shown complete dispersion of the agglomerates. However, further tests would be needed to eliminate the possibility of deagglomeration to primary particles or nano-sized aggregates under other conditions, e.g. in final formulations or when in the biological environment.

### 3.2. Function and uses

The ingredient Carbon Black CI 77266 has a long history of use as a cosmetic colorant. Typical uses of carbon black are in different types of cosmetic products, specifically make-up products, and include but are not limited to eyeliners, eye pencils, eye shadows, mascaras, blushers, brush-on-brow, foundations and nail enamels, leave-on and rinse-off skin products.

Typical use concentrations range from 0.001% to 10% with 0.001% for skin products, 5% for nail enamels and mascaras and up to 10% for other eye decorative products such as eyeliners, eye pencils and eye shadows.

### 3.3. Toxicological Evaluation

Caution should be taken when testing carbon black because of its ability to adsorb colorimetric chemical markers used in biochemical assays. Carbon black may also adsorb the constituents of the growth media as well as proteins, thereby preventing the cells from receiving their proper nutrients and growth factors. Moreover, by absorbing soluble components in the growth medium the pH may be altered and thus cell viability (Monteiro-Riviere and Inman, 2006, Monteiro-Riviere *et al.*, 2009).

#### 3.3.1. Acute toxicity

##### 3.3.1.1. Acute oral toxicity

The acute toxicity of carbon black was evaluated in studies performed in rats by the oral route.

The single oral administration of carbon black (Printex-140, furnace black) at a concentration of 8 000 mg/kg bw produced no deaths, and no salient macroscopic anomalies were observed at necropsy. Accordingly, under the conditions used in that study, the maximal non-lethal dose of carbon black after single administration by the oral route was higher than 8 000 mg/kg bw (Degussa AG, 1978a).

In another acute toxicity study, carbon black (Spezialschwarz 4, furnace black) was administered once to rats by the oral route at dose levels of 6 350, 7 900 or 10 000 mg/kg bw. There were no deaths, and no salient macroscopic anomalies were observed at necropsy. Accordingly, under the conditions used in that study, the maximal non-lethal dose of carbon black was higher than 10 000 mg/kg bw (Degussa AG, 1977a).

In summary, the maximal non-lethal dose of carbon black after single administration by the oral route in rats is higher than 10 000 mg/kg bw, and carbon black is therefore considered to have no acute toxicity potential by the oral route.

**SCCS comment**

The above text is from Submission I. The acute toxicity of carbon black has only been studied in rats by the oral route. The acute oral toxicity of carbon black is unlikely to be of a concern.

**3.3.1.2. Acute dermal toxicity**

No information submitted.

**3.3.1.3. Acute inhalation toxicity**

No information submitted

**3.3.2 Irritation and corrosivity****3.3.2.1. Skin irritation**

The single application of 20% (Printex-G and Spezienschwarz 4, furnace black materials) or 27% (Printex-140, furnace black) aqueous suspensions of carbon black onto intact or abraded rabbit skin for 24 hours under occlusive conditions produced no cutaneous reactions. Accordingly, under the conditions used in those studies, carbon black was considered to be non-irritant (Degussa AG, 1977b; Degussa AG, 1977c; Degussa AG, 1978b).

Similarly, the acute skin irritation potential of undiluted carbon black moistened with water (Farbruss FW 200, furnace black) was evaluated following a single application onto intact or abraded rabbit skin for 4 hours under occlusive conditions. None of the animals exhibited any cutaneous signs of oedema or erythema, and carbon black was therefore considered to have no skin irritation potential (Degussa AG, 1984).

**SCCS comment**

The above text is from Submission I.

**Studies with Episkin<sup>SM</sup> kits**

Guideline:	ECVAM validated protocol
Test substance:	Carbon Black (CI: 77266), - Unipure LC 902
Batch:	C3949
Purity:	98%
Particle size:	Diameter; 20 to 30 nm, surface area range of 200 to 260 m <sup>2</sup> /g (see also Table 1).
Vehicle:	Sunflower oil
Dose level:	Suspended at 2.0% AM (active material) (2.04% RM), 6.0% AM (6.1%RM), and 10% (10.2% RM) in sunflower oil
Negative control:	10 µl PBS+ (Gibco, 14040-117)
Positive control:	10 µl 5% solution of Sodium Dodecyl Sulfate
GLP:	In compliance
Study period:	2011

*In vitro* evaluation of skin irritation was performed on the reconstructed human epidermis model Episkin small model (0.38 cm<sup>2</sup>). Every unit of the reconstructed epidermis consists of a stratified and differentiated epidermis obtained from human keratinocytes, seeded on a collagen matrix fixed to the bottom of the plastic insert using a toric ring.

Episkin<sup>SM</sup> kits were maintained in agar medium for transportation. They were delivered at day 13 and used at days 15 to 17.

After a 15 minutes treatment period with carbon black at room temperature, tissues were rinsed with 25 ml PBS+. The epidermis were then transferred on 2 ml/well of fresh maintenance medium and incubated for 42 hours (37°C, 5% CO<sub>2</sub>, 95% humidity).

The plates were subsequently shaken for 15 min and each epidermis unit was then transferred to another 12 well plate containing 2 ml/well of dye MTT solution and incubated for 3 h (The MTT assay is a colorimetric assay for measuring the activity of cellular enzymes that reduce the tetrazolium dye, MTT, to its insoluble derivative formazan, giving a purple color). At the end of the MTT incubation period a biopsy of the entire epidermis was taken by using a biopsy-punch and the epidermis was separated from the collagen matrix using curved forceps. Both (epidermis and collagen matrix) were transferred into a tube containing 500 µl acidified isopropanol. At the end of the extraction period, each tube was stirred and 2 x 200 µl of each extract were transferred onto a 96-wells plate. The O.D was subsequently measured at 570 nm versus acidified isopropanol.

IL-1α released in the culture medium was determined by a quantitative sandwich enzyme immunoassay technique. Monoclonal specific IL-1α antibodies were precoated onto micro-plates. 200 µl of standards or samples were added to the wells enabling IL-1α to bind to immobilized antibody. After washing, an enzyme linked polyclonal antibody specific to IL-1α was added to the wells and colour develops in proportion to the amount of IL-1α bound in the initial step. The intensity of the colour (OD values related to the IL-1α amount) was measured at 450 nm.

The study authors concluded that the *in vitro* evaluation of acute skin irritation by using the reconstructed human epidermis Episkin<sup>SM</sup> model and the ECVAM validated protocol suggests that carbon black tested at 2.0%, 6.0% and 10.0% in sunflower oil is considered as potential non-irritant.

Ref.: Brémond, 2011

### SCCS comment

The use of reconstructed human skin for irritation testing has not been validated for nanomaterials. In addition, residual carbon black may interfere with spectrometric analysis in the viability and cytokines assays. Appropriate controls should be used to evaluate possible interference of carbon black nanomaterials with the assay.

### SCCS overall conclusion on skin irritation

Under the conditions of the *in vivo* studies carbon black is not considered to be irritating to the rabbit skin. The Episkin test is inconclusive.

#### 3.3.2.2. Mucous membrane irritation

Guideline: Similar to OECD TG 405  
 Species/strain: rabbit / New Zealand white  
 Group size: 6 animals (3 males, 3 females)  
 Test substance: Printex G  
 Batch: /  
 Purity: /  
 Vehicle: /  
 Dose level: neat substance (100 mg)  
 Dose volume: /



Route: instillation in the conjunctival sac of the left eye  
 Observation: readings at 24, 48 and 72 hours  
 GLP: /  
 Study period: October 1977

The eye irritation potential of Printex G was investigated by instillation of 100 mg of the neat test substance into the conjunctival sac of the left eye of each animal. The right eye was treated with physiological saline and served as control. Six rabbits (three males, three females) were used. Both eyes were examined at 24, 48 and 72 hours after instillation and the effects on the cornea, iris and conjunctivae were scored according to the Draize scoring system.

**Results:**

No effects were observed at any of the reading times in any of the animals.

**Conclusion:**

Under the conditions of the study, Printex G was not irritating to the rabbit eye.

(Reference: Degussa AG, 1977d)

Guideline: Similar to OECD TG 405  
 Species/strain: rabbit / New Zealand white  
 Group size: 6 animals (3 males, 3 females)  
 Test substance: Spezienschwarz 4  
 Batch: /  
 Purity: /  
 Vehicle: /  
 Dose level: neat substance (100 mg)  
 Dose volume: /  
 Route: instillation in the conjunctival sac of the left eye  
 Observation: readings at 24, 48 and 72 hours  
 GLP: /  
 Study period: October 1977

The eye irritation potential of Spezienschwarz 4 was investigated by instillation of 100 mg of the neat test substance into the conjunctival sac of the left eye of each animal. The right eye was treated with physiological saline and served as control. Six rabbits (three males, three females) were used. Both eyes were examined at 24, 48 and 72 hours after instillation and the effects on the cornea, iris and conjunctivae were scored according to the Draize scoring system.

**Results:**

No effects were observed at any of the reading times in any of the animals.

**Conclusion:**

Under the conditions of the study, Printex G was not irritating to the rabbit eye.

(Reference: Degussa AG, 1977e)

Guideline: Similar to OECD TG 405  
 Species/strain: rabbit / New Zealand white  
 Group size: 8 animals  
 Test substance: Printex 140  
 Batch: /  
 Purity: /

Vehicle: /  
 Dose level: neat substance (100 mg)  
 Dose volume: /  
 Route: instillation in the conjunctival sac of the left eye  
 Observation: readings at 24, 48, 72, and 96 hours  
 GLP: /  
 Study period: June 1978

The eye irritation potential of Printex 140 was investigated by instillation of 100 mg of the neat test substance into the conjunctival sac of the left eye of each animal. The right eye was left untreated and served as control. One group (Group I) of five rabbits was treated for five minutes and then the eyes were washed. Another group (Group II) of 3 rabbits was treated for 24 hours and then the eyes were washed. Both eyes were examined at 5 minutes (Group I), 24, 48, 72 and 96 hours after instillation and the effects on the cornea, iris and conjunctivae were scored according to the Draize scoring system.

#### Results:

No effects were observed at any of the reading times in any of the animals in either group.

#### Conclusion:

Under the conditions of the study, Printex 140 was not irritating to the rabbit eye.

(Reference: Degussa AG, 1978c)

### **SCCS overall conclusion on *in vivo* eye irritation**

The acute ocular irritation potential of 3 brands of carbon black materials - furnace blacks Printex G (Degussa AG, 1977d); Spezienschwarz 4 (Degussa AG, 1977e); and Printex-140 (Degussa AG, 1978c) was evaluated following a single instillation of 100 mg of the neat material to the rabbit eye according to a test protocol similar to the OECD TG 405. No signs of ocular irritation were observed in any of the animals at any reading time. Under the conditions of these studies, the three brands of carbon black are not irritating to rabbit eyes.

### **Bovine Cornea Opacity and Permeability (BCOP) test**

Guideline: OECD 437 modified  
 Test system: Isolated bovine cornea  
 Route: Eye  
 Test substance: Carbon Black (CI: 77266), - Unipure LC 902  
 Batch: C3949  
 Purity: 98%  
 Particle size: Diameter; 20 to 30 nm, surface area range of 200 to 260 m<sup>2</sup>/g (see also Table 1).  
 Volume: 750 µl  
 Concentration: 2, 6, and 10% in sunflower oil  
 Negative control: Nutritive medium  
 Vehicle control: Sunflower oil  
 Positive control: Cetyl trimethylammonium bromure, 0.5% in distilled water  
 Temperature: 32°C  
 GLP: In compliance  
 Study period: September 2011

### Procedure

A single topical dose of 750 µl of carbon black dispersed in sunflower oil was administered to the epithelial surface of three isolated bovine corneas. The corneas were exposed to the test item for 30 minutes followed by a 10 minutes or 4-hours post incubation period. From the quantitative results obtained for each contact timepoint, a "corneal score" was calculated, enabling to classify the test item into 4 categories: slightly irritant, moderately irritant, moderately irritant to irritant, and irritant to severely irritant.

Corneal opacity was measured qualitatively as the amount of light transmission through the cornea. Permeability was measured quantitatively as the amount of Fluorescein dye that passed across the full thickness of the cornea.

### Result

The corneal scores obtained are shown in Table 6.

**Table 6.:** Corneal score

Concentrations	Score 30 min	Score 4 hours
Carbon Black		
2%	3.5±1.2	0.6±0.6
6%	4.6±0.5	1.3±3.5
10%	6.1±1.1	0.9±1.0
Negative control	1.3±0.5	0.6±0.5
Positive control	89.3±2.9	---

Slightly irritant: Corneal score; 30 min ≤ 10, 4 hours ≤ 40

Moderately irritant: Corneal score; 30 min ≤ 10, 4 hours < 40 and ≤ 100

Irritant to severe irritant: Corneal score; 30 min ≤ 10, 4 hours > 100

### Conclusion

The study authors concluded that from the results obtained under the experimental conditions adopted, the test item designated as E300281, applied to 10%, 6%, and 2% (W/W) active material in sunflower oil, is classified "slightly irritant" for the isolated bovine cornea after 30 minutes and 4 hours of contact.

Ref.: Maillet, 2011

### SCCS comment

Classification system used is not in accordance with OECD TG 437 and therefore cannot be accepted. Additionally, the used BCOP assay for eye irritation testing has not been validated for nanomaterials. In addition, residual carbon black may interfere with opacity and permeability analysis in the assay. Appropriate controls should be used to evaluate possible interference of carbon black nanomaterials with the assay.

### SCCS overall conclusion on eye irritation

Under the conditions of the three *in vivo* studies performed according to a test protocol similar to OECD TG 405 the three different brands of carbon black were not irritating to the rabbit eye. The BCOP test is inconclusive.

### 3.3.3. Skin sensitisation

#### Buehler test

The Buehler test was performed according to OECD Guideline 406 (Skin Sensitisation) and is in compliance with GLP in 2003. The test group consisted of 20 and the control group of 10 female Dunkin-Hartley guinea-pigs. Three occlusive applications over a 2-week period of carbon black (test item XPB 295) at 50% in water were performed during the induction period. After a 2-week rest period, a challenge occlusive application using the same dilution of carbon black did not elicit any skin reactions.  $\alpha$ -Hexylcinnamaldehyde was used as positive control (60% response).

The study authors concluded that carbon black was not considered to be a skin sensitizer.

Ref.: ECHA (2003a)

#### Local Lymph Node Assay (LLNA)

Guideline: OECD 429 (2010)

Species/strain: CBA/J mouse (Female, nulliparous, non-pregnant, 11 – 12 weeks old)

Group size: 4 animals per group

Test substance: Carbon Black (CI: 77266), - Unipure LC 902

Batch: C3949

Purity: 98%

Particle size: Diameter; 20 to 30 nm, surface area range of 200 to 260 m<sup>2</sup>/g (see also Table 1).

Vehicle: Propylene glycol

Concentration: 0, 0.25, 0.5, 1.0, 2.5, or 5.0% (w/v) Carbon Black in propylene glycol

Positive control: 25%  $\alpha$ -hexylcinnamaldehyde in propylene glycol

GLP: In compliance

Study period: December 2011 – January 2012

#### Procedure

The mice received topical application with 25  $\mu$ l of 0 (negative control), 0.25, 0.5, 1.0, 2.5, or 5.0% (w/v) carbon black in propylene glycol or 25%  $\alpha$ -hexylcinnamaldehyde in propylene glycol (positive control) for three consecutive days at the dorsum of each ear (25  $\mu$ l per ear). Five days after the first application, the proliferation of lymph node cells in the lymph node draining the application site was measured by incorporation of 3H-methyl thymidine. The 3H-TdR was intravenously injected into a tail vein. 5 hours later mice were sacrificed by CO<sub>2</sub> asphyxiation. The draining auricular lymph nodes were taken and pooled for each experimental group. Single cell suspensions of pooled lymph nodes were prepared. Cells were washed with PBS and precipitated with 5% trichloroacetic acid (TCA). 18 hours later the pellets were resuspended in TCA and transferred into the scintillation cocktail. The proliferation capacity of lymph node cells was determined by the incorporation of 3H-methyl thymidine.

#### Results

No treatment-related systemic clinical signs were observed in any animals from the vehicle control group as well as from treated groups at any tested concentration of carbon black. Animals from positive control group exhibited slight erythematic response at the site of application. Ear thickness measurements did not show any noteworthy increase in the animals from carbon black groups when compared to vehicle and positive control groups.

Stimulation Index (SI Value) obtained for carbon black treated groups were found to be 1.13, 1.20, 1.23, 1.26, and 1.24 for the concentrations 0.25, 0.5, 1.0, 2.5, or 5.0% (w/v)

carbon black in PG, respectively. Stimulation Index (SI Value) calculated for positive control group was found to be 3.52.

#### Conclusion

The study authors concluded that since the entire acceptance criteria were met (percentage of cell viability 70% in vehicle control group and SI for positive control higher than 3), the experiment was considered as valid. Since SI values obtained for 0.25, 0.5, 1.0, 2.5, or 5.0% (w/v) in PG of carbon black showed a less than three-fold increase over the vehicle control group, associated with a dose response relationship, it was concluded that under the above experimental conditions, carbon black is not a contact sensitizer to CBA/J strain mice in Local Lymph Node Assay.

Ref.: Vishvesh, 2012

#### Comment from the applicant. Submission I

The highest concentration used in the (aforementioned) LLNA (5%) may be considered to be rather low, even when taking into account that typical/maximal use concentrations of carbon black in cosmetic products are within the same concentration range. However, this concentration was the maximum technically achievable concentration for carbon black suspensions, therefore meeting the recommendations of the corresponding OECD Testing Guideline (OECD 429). Additionally, the appropriate dose metric for the evaluation of the skin sensitisation potential/risk of a given substance is not the concentration of the substance evaluated but rather the skin area dose (quantity of compound per skin unit surface, eg.  $\mu\text{g}/\text{cm}^2$ ). In the present case, the skin area doses of carbon black achieved in the aforementioned LLNA (up to  $1250 \mu\text{g}/\text{cm}^2$ ) were significantly higher than the skin area doses associated with the use of cosmetic products containing carbon black (range of about  $0.001 - 800 \mu\text{g}/\text{cm}^2$ ). Accordingly, the results obtained in the aforementioned LLNA are considered to be adequate to exclude any skin sensitization potential of carbon black, thus any risk of skin sensitization associated with the use of cosmetic products containing this ingredient. This conclusion takes account of the fact that the results obtained in the LLNA are consistent with those obtained in the abovementioned Buehler Test, as well as structural-activity considerations.

#### SCCS comment

The argumentation is difficult to follow, when  $25 \mu\text{l}$  is topical applied on the ear, it may be difficult to determine  $\mu\text{g}/\text{cm}^2$ . Moreover, the number  $1250 \mu\text{g}/\text{cm}^2$  is not sufficiently different from  $800 \mu\text{g}/\text{cm}^2$ . The argument that that the skin area doses of carbon black achieved in the aforementioned LLNA were significantly higher than the skin area doses associated with the use of cosmetic products containing carbon black is not accepted. The argument that higher concentrations than 5% carbon black could not be reached is not valid as products are included in the submission that contain a concentration of 20% Carbon Black. Moreover, it is not known if the carbon black particles penetrated the skin to reach the cellular targets of the immune system. In view of this, on the basis of negative outcomes of Buehler and LLNA tests, sensitisation potential of carbon black cannot be ruled out.

#### *Additional information on respiratory sensitisation*

#### **Respiratory sensitisation**

Guideline:	/
Species/strain:	Female BDF, mice aged 10 wk
Group size:	6 animals per group
Antigen:	Experiment 1: Japanese cedar pollen allergens (JCPA) Experiment 2: Native dry Japanese cedar pollen (JCP)
Test substance:	Carbon Black (no. 12)

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Batch:	/
Purity:	100%
Particle size:	30 – 200 nm; surface area 20.4 m <sup>2</sup> /g
Vehicle:	Phosphate-buffered saline solution (PBS, pH 7.2)
Concentration:	25 µl in 10 µl Carbon Black in phosphate buffer
Positive control:	Aluminum hydroxide (alum); size 260 – 2200 nm; surface area 6.2 m <sup>2</sup> /g
Route:	Dropped onto the nostrils
GLP:	/
Study period:	Before 1997

#### Procedure

*Experiment 1.* Six groups with 6 mice were immunized 8 times at one week intervals, followed by once every 3 week for the next 9 week with particles and JCPA aerosol. 25 µg of particles in 10 µl of phosphate-buffered saline solution (PBS, pH 7.2) was dropped onto the nostrils of each mouse. The following particles were tested: Carbon black, Kanto loam dust, fly ash, diesel exhaust particles [DEP], aluminum hydroxide [positive control], no particles [negative control]. Crude JCPA aerosol generated with a glass nebulizer at a total protein concentration of 1 µg/l and the mice were exposed for 30 min.

Nasal rubbing movements in mice, a parameter of allergic rhinitis, were counted for 5 min after nasal challenge for 1 week after the final immunization. The allergen used for nasal challenge was 500 µg of pollen grains suspended in PBS and administered by the same route as particles.

*Experiment 2.* Six groups of 6 mice were immunized twice a week for 16 weeks. Doses of 25 µg of the respective particles in PBS were administered to the mice by the same procedure as in experiment 1. After the administration of particles, 8 units of JCP were dropped onto the tip of the nose of each mouse by micropipet. One unit of JCP contained about 20,000 pollen grains counted by microscopy. Other experimental procedures and measurements were as described for experiment 1.

#### Results

In experiment 1, the increases in anti-JCPA IgE and IgG antibody production in mice treated with aerosolized JCPA plus respective particles were significantly greater than that found with aerosolized JCPA alone. This was associated with no marked differences in the other allergic rhinitis parameters. In experiment 2, after the administration of particles as in experiment 1, about 160,000 grains of JCP were dropped onto the tip of the nose of mice twice a week for 16 wk. Six weeks after the first immunization, the anti-JCPA IgE antibody titers of groups treated with the respective particles were greater than 1:20, whereas those of mice treated with JCP alone were 1:10. No significant differences in the anti-JCPA IgE and IgG antibody productions, nasal rubbing counts, or histopathological changes were observed after 18 wk.

#### Conclusion

The study authors noted that the results suggested that the nature of the particles, their capacity to adsorb antigens, and/or their size may not be related to enhancement of IgE antibody production nor symptoms of allergic rhinitis. However, IgE antibody production seemed to occur earlier in mice treated with particles than in mice immunized with allergens alone.

Ref.: Maejima *et al.*, 1997

#### SCCS comment

Although not specific for carbon black, as indicated in study 1, all particles showed increased IgE induction suggesting that carbon black similar to other particles used had an adjuvant activity.

### **Intranasal exposure (Non-validated test model)**

#### **Procedure**

Groups of 6 female Balb/c mice were intranasally exposed to ovalbumin (OVA) alone or in combination with ultrafine (Printex 90, 14.3 nm, surface area 253.9 m<sup>2</sup>/g) or fine (Huber 990, 260.2 nm, surface area 7.9 m<sup>2</sup>/g) carbon black particles. The induction of airway inflammation and the immune adjuvant activity were studied in the lungs and lung-draining peribronchial lymph nodes (PBLN) at day 8. OVA-specific antibodies were measured at day 21, and the development of allergic airway inflammation was studied after OVA challenges (day 28). The experiment was performed before 1997.

The carbon black particles were suspended in phosphate-buffered saline (PBS) containing 0.5 mg/ml ovalbumin at a concentration of 3.3 mg/ml, and sonicated for 2 hours.

Mice were anaesthetized by an intramuscular injection of ketamine and exposed to 20 µl of OVA (0.5 mg/ml) or particle + OVA (3.3 mg/ml and 0.5 mg/ml, respectively) in PBS by intranasal droplet application on days 0, 1 and 2 (total dose of 200 µg particles and 30 µg OVA). Mice were killed on day 8, or challenged to study asthma-like allergic airway inflammation. For the latter, mice were anaesthetized by an intramuscular ketamine injection and challenged by intranasal droplet application of 20 µl OVA (0.5 mg/ml) in PBS on days 25, 26 and 27. Mice were killed on day 28 by an overdose of pentobarbital.

#### **Results and discussion**

Ultrafine (diameter 14 nm) but not fine (diameter 260 nm) carbon black particles (200 µg) induced airway inflammation and displayed adjuvant activity. The latter was evidenced by the induction of immune sensitisation to co-administered ovalbumin and demonstrated by increased levels of cytokines associated with a Th2 immune response and by the occurrence of allergic airway inflammation after an intranasal OVA challenge. A systemic antibody response was not detected.

Allergic airway inflammation as well as a stimulation of local immune responses (changes in cellular composition of BAL and PBLN, and production of a number of type 2 cytokines in PBLN), were found after intranasal challenges with antigen.

The study authors concluded that carbon black is not sensitizing.

Ref.: ECHA (2003c)

#### **SCCS comment**

Carbon black was demonstrated to be able to act as Th2 adjuvant when used in combination with an antigen.

#### **General comment by the SCCS**

SCCS is of the opinion that it is difficult to draw a valid conclusion from the tests on skin sensitisation since it is unlikely that the carbon black particles penetrated the skin to reach the cellular targets of the immune system. In view of this, sensitisation potential of carbon black cannot be ruled out, for example in damaged skin. However, carbon black can act as adjuvant when inhalation exposure occurs in combination with an allergen.

### **3.3.4. Dermal / percutaneous absorption**

The applicant points out that with conventional cosmetic ingredients, a radiolabelled form or a chromatographic method is normally used in measurements of percutaneous absorption.



However, due to its mineral nature, there is no radioisotope for carbon black and no specific HPLC method available to perform a conventional skin penetration study. Several methods were tentatively developed for the quantitative analytical detection of carbon black including combustion combined with infrared analysis, Raman spectroscopy and spectrophotometric techniques. The results were that the desired sensitivity and specificity of carbon black detection – i.e. that would distinguish carbon (from carbon black nanoparticles) from background carbon (present in skin tissues) – cannot be obtained. A mass balance skin penetration study on carbon black using a quantitative analytical detection method was therefore not technically achievable, and imaging was the only realistic way of determining whether carbon black nanoparticles can reach the underlying viable skin compartments. In order to provide a comprehensive position on the skin penetration properties of carbon black nanoparticles a qualitative method was developed using Transmission Electron Microscopy (TEM).

### **In vitro percutaneous absorption study using intact human skin**

Guideline	/
Test substance:	Originally Carbon Black. Distinctive Ink Black LO AQ 20% (Black 2/CI 77266), prepared an eyeliner product containing 6% Carbon Black.
Test formulation:	Black 2/CI 77266 6%, water 53.83%, gums 1.8%, propylene glycol 15%, acrylates copolymer 12%, polyester 5 5%, trilaureth-4 phosphate 2%, div. preservatives 1.87%, alcohol denat 2.5%.
Purity:	Not stated
Size:	Diameter 20 – 30 nm (data on the specific batch not available, see also Table 5)
Batch no.	B01122213
Dose applied:	9.5 mg/cm <sup>2</sup> , 2 cm <sup>2</sup> exposure area, and a receptor volume of 3 ml.
Skin preparation:	Human skin sample obtained after abdominal plastic surgery. Sample thickness 1369 µm. Control sample 770 µm thickness.
Cells per application:	1 treated + 1 controls
Skin temperature:	31°C
Exposure period:	24 hours
Donor chamber:	Non-occluded
Receptor fluid:	Aqueous solution containing 9 g/l NaCl
Control:	1 controls
Particle size:	/
Skin integrity:	/
Recovery:	/
GLP:	/
Study period:	Before April 2011

#### Procedure

"Distinctive® Ink Black Lo-AQ" containing 20% pure carbon black was used in the study. Human abdominal skin samples were obtained from a single donor subjected to plastic surgery. Skin samples were mounted intact (1369 µm in thickness) in diffusion cells, using sodium chloride as the receptor fluid. Two diffusion cells (one exposed and one unexposed) were used and skin was maintained at approximately 31°C.

About 9.5 mg/cm<sup>2</sup> of a typical eyeliner formulation containing 6% carbon black was applied to the skin surface and left for 24 hours. After this time period, the skin sample was washed with 0.6 ml of a 5% sodium lauryl ether sulphate and dried with a cotton swab. The skin was rinsed a second time with the same solution and then three times with 0.6 ml water and dried with two cotton swabs.

The two skin samples (treated and control) were fixed in 2% glutaraldehyde in 0.1 M



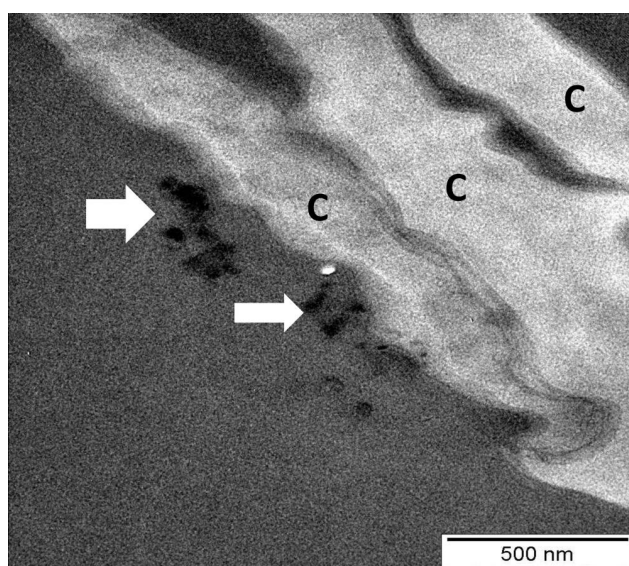
sodium cacodylate buffer (pH 7.2) for 1 hour at 4°C.

Four punch biopsies were collected from each skin samples, prepared and fixed in 1% osmium tetroxide. Subsequently, each biopsy was sliced into ultrathin microscopy grids (80 nm thickness).

The absorption of carbon black particles was estimated by Transmission Electron Microscopy (TEM) examination of multiple skin sections. In addition, toluidine blue-stained semi-thin sections of each biopsy from the control and treated skin samples were examined at light microscopy.

### Results

At TEM of the skin samples treated with carbon black containing eyeliner, particulate material identified as carbon black was only observed in the outer layers of *Stratum Corneum*. There was no evidence of deeper penetration of carbon black material into the *Stratum Corneum* or into living epidermis (Fig. 7).



**Fig. 7.** Transmission electron microscopy micrograph of eyeliner containing 6% carbon black treated skin sample demonstrating the presence of exogenous particles attributed to carbon black (arrows) on the upper layers of the *Stratum Corneum*. Three layers of corneocytes can be seen in the upper right part of the micrograph.

### Conclusion

The study authors concluded that the results obtained in the present study suggest that carbon black nanoparticles used in a typical eyeliner product have no potential to penetrate into or cross through the skin, thus no potential to produce systemic exposure of consumers.

Ref.: Hallegot and Grégoire, 2011

### SCCS comment

No accepted guideline has been used. Moreover, only one skin sample is used. Therefore the study provides supporting but not conclusive evidence on lack of skin penetration.

**In vitro percutaneous absorption study using human dermatomed skin**

Guideline	OECD 428 (2004)
Test substance:	Originally Carbon Black. Black liquid 20%, prepared as "LINER CB containing 12% w/w Carbon Black by L'Oreal with reference no. TS00204/002/001 and batch no. Fab Labo du 21/05/12 and name: 787094.
Test formulation:	Black 2/CI 77266 12%, water 64.14%, gums 3.3%, propylene glycol 15%, trilaureth-4 phosphate 2%, preservatives 1.06%, alcohol denat 2.5%.
Purity:	Not stated
Particle size:	20 – 30 nm, size distribution see Table 5.
Batch no.	B01122213
Dose applied:	10 mg/cm <sup>2</sup> , 2.54 cm <sup>2</sup> exposure area, and a receptor volume of approximately 4.5 ml. Since the test material was a thick liquid, the doses were applied to the skin and spread over the surface using pipette tips and the weight of the applied dose recorded after spreading.
Skin preparation:	Human skin samples (2 breasts and 2 abdomen, all age 76) were obtained from a tissue bank. Skin sections were cut at a thickness setting of 500 µm using an electric dermatome
Cells per application:	4 treated + 2 controls
Skin temperature:	32°C
Exposure period:	24 hours
Donor chamber:	Non-occluded
Receptor fluid:	Phosphate buffered saline (pH 7.4) 500 µL
Control:	2 controls from the same donors as was treated with Carbon Black
Skin integrity:	Skin integrity was determined by measurement of the electrical resistance across the sample. Skin with a measured resistance of <10 kΩ were regarded as having a lower integrity than normal and not used for exposure to the test material. Untreated control cells did not need to meet this requirement
Recovery:	/
GLP:	In compliance.
Study period:	September – December 2012

Ref.: Johnson, 2013a

and

Guideline	OECD 428 (2004)
Test substance:	Originally Carbon Black. Black liquid 20%, prepared as "LINER CB containing 12% w/w Carbon Black by L'Oreal with reference no. TS00204/002/001 and batch no. Fab Labo du 21/05/12 and name: 787094.
Test formulation:	Black 2/CI 77266 12%, water 64.14%, gums 3.3%, propylene glycol 15%, trilaureth-4 phosphate 2%, preservatives 1.06%, alcohol denat 2.5%.
Purity:	Not stated
Particle size:	20 – 30 nm, size distribution see Table 5.
Batch no.	B01122213 (no information on the specific batch)
Dose applied:	10 mg/cm <sup>2</sup> , 2.54 cm <sup>2</sup> exposure area, and a receptor volume of approximately 4.5 ml. Since the test material was a thick liquid, the doses were applied to the skin and spread over the surface using pipette tips and the weight of the applied dose recorded after spreading.
Skin preparation:	Human skin samples (3 breast, age 54, 70, and 70 and 1 unknown,

	age 60) were obtained from a tissue bank. Skin sections were cut at a thickness setting of 500 µm using an electric dermatome.
Cells per application:	4 treated + 2 controls
Skin temperature:	32°C
Exposure period:	24 hours
Donor chamber:	Non-occluded
Receptor fluid:	Phosphate buffered saline (pH 7.4) 500 µL
Control:	2 controls from the same donors as was treated with Carbon Black
Skin integrity:	Skin integrity was determined by measurement of the electrical resistance across the sample. Skin with a measured resistance of <10 kΩ were regarded as having a lower integrity than normal and not used for exposure to the test material. Untreated control cells did not need to meet this requirement
Recovery:	/
GLP:	In compliance.
Study period:	October – December 2012

Ref.: Johnson, 2013b

The evaluation was performed in two separate experiments that will be considered together (Johnson, 2013a and Johnson, 2013b). It is noted that tape stripping was performed in the first study. However, due to the fact that the resin, the tape strip, the tape strip adhesive and the product had overall similar electron densities it was difficult to achieve sufficient contrast for imaging. In the second study the skin surface was only washed-off and did not undergo tape stripping.

#### Methods

In the first experiments, cells were selected such that the application was represented by a total of four intact skin samples from two different donors. Two control skin samples from the same donors were also included and remained untreated for the duration of the exposure. In the second experiments, cells were selected such that the application was represented by a total of four intact skin samples from three different donors.

The receptor chambers of the cells containing small magnetic stirrer bars were filled with a recorded volume of receptor fluid (phosphate buffered saline (pH 7.4)). A pre-treatment sample (0.5 ml) was taken from each receptor chamber. Samples of receptor fluid were taken (0.5 ml), using a positive displacement pipette, 2 and 24 hours after application. An equal volume of fresh receptor fluid was added to each receptor chamber to replace the volumes removed. All receptor fluid samples were stored deep frozen. The samples were not examined.

The epidermal surface of the skin was decontaminated with a single natural sponge (approximately 1 cm<sup>3</sup>) pre-wetted with 2% solution of sodium dodecyl sulphate (SDS) in water to loosen the test material, followed by two washes of 1 ml of 2% SDS in water and aspirated with a pipette tip. The skin was rinsed with a further two washes of 1 ml of water and again aspirated with a pipette tip. The decontamination regime (sponge, SDS and water) was repeated up to twice more and complete decontamination was assessed visually. The sponges and SDS/water washes were separately combined and stored deep frozen. The sample pots were weighed before and after the samples were added to accurately assess the volume of the washes. These samples were not examined. The donor chamber was carefully removed and the underside wiped with a single sponge pre-wetted with 2% SDS in water which was added to the wash sponges. The donor chambers were not processed further.

#### First study

The skin was allowed to dry naturally. Successive layers of the skin surface were removed by the repeated application of adhesive tape (Scotch 3M Magic Tape, 1.9 cm wide), to a

maximum of 20 strips. A strip of adhesive tape was pressed onto the skin surface and then carefully peeled off to remove the *stratum corneum*. Where the epidermis tore, the process was halted at that point. The adhesive strips were stored individually, deep frozen until examination.

The first and final two tape strips, where present, were subsequently submitted for TEM examination, but the presence of residual test formulation in first tape strips prohibited sectioning of embedded tape strips. Prior to the TEM, the samples were analyzed using Scanning Electron Microscopy (SEM) in order to give good images of the area under examination. The remaining skin was carefully removed from the receptor chamber and placed in sufficient fixative (2.5% glutaraldehyde in cacodylate buffer – TS00211/001/001) to adequately cover the sample and refrigerated for at least 48 hours before further processing and TEM examination.

#### Second study

The skin was carefully removed from the receptor chamber and placed in sufficient fixative (2.5% glutaraldehyde in cacodylate buffer – TS00211/001/003) to adequately cover the sample and refrigerated for at least 48 hours before further processing and TEM examination.

### Results and Discussion

#### First study

Tape stripping was undertaken in human skin samples that were either treated with a formulation containing carbon black or were left untreated for a 24 hour exposure period. This procedure was designed to completely remove the surface layers of the skin, *i.e.* the *stratum corneum*. The Electron Microscopist observed the presence of collagen fibres on several of the skin samples but no *stratum corneum* or epidermis was observed in any of these. There was no evidence of carbon black in any of the samples, indicating that this particulate material does not penetrate into the dermis, following a 24 hour leave-on application.

#### *Tape strips*

Individual tape strips from both untreated skin and skin treated with a cosmetic formulation containing carbon black were also examined by electron microscopy. Tape strips from the outer regions of the skin (T1) were obtained (in one case T2) and from deeper in the skin sample (in most cases T8-T20) were examined. SEM was used to orientate the very thin tape strip samples in order to assist in the identification of the various biological and matrix areas of the image. It should be recognized that this lower magnification would only be able to define aggregates/agglomerates of carbon black particles rather than single particles. The similar electron densities of the resin, tape strip, adhesive and test formulation/cream resulted in poor contrast in the images which added to the challenges of defining carbon black particles in the samples by SEM.

In the majority of the tape strips samples, no *stratum corneum* was observed. It is quite possible that the fixation process for tape strips makes it difficult to maintain adherence of biological tissue to the tape, particularly in the outer strips where the residual test formulation may have still affected adhesion to the glue of the tape. In the few samples where tissue was observed, this was described as *stratum corneum*.

#### *SEM analyses*

The tape strip, the adhesive layers as well as the skin layers removed by the stripping technique could be identified on SEM pictures. No aggregates/agglomerates of carbon black particles were observed on the lower magnification SEM images captured.

### *TEM analyses*

It was not possible to obtain reliable TEM images of the processed first tape strips due to technical difficulties caused by the presence of residual test formulation/cream that had not been removed during the washing procedure prohibiting sectioning of the embedded tape strips. However, in one of the skin samples treated with cosmetic formulation containing carbon black the outer tape strip examined showed the presence of carbon black particles that appeared to be associated with test formulation cream. This, of course, is only at the surface of the *stratum corneum*. There was no evidence of carbon black particles any deeper in the skin. All the tape strips deeper into the skin from all other cells showed no evidence of carbon black particles.

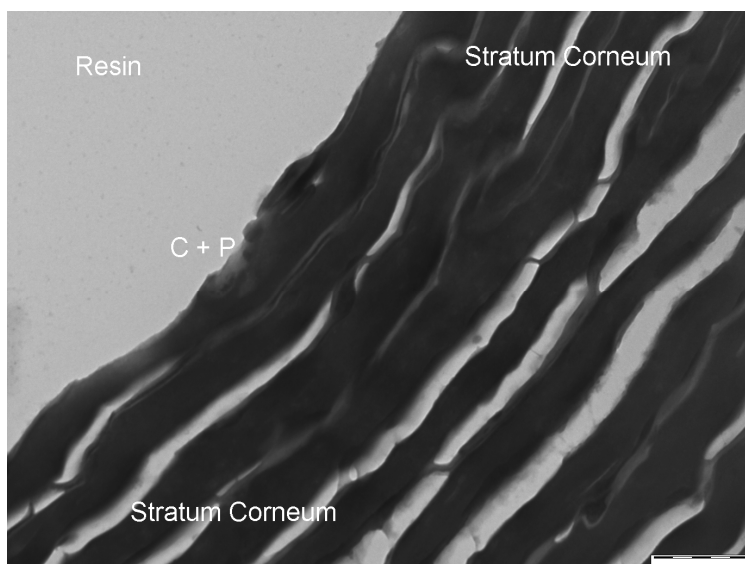
### Second study

Although the skin had not been tape stripped, delamination was seen in three of the skin samples, the two controls and one treated cell, possibly as a result of the fixation procedure. The layers of the skin easily separate and in a number of cases the *stratum corneum* had been lost during the processing of the samples.

Intact stratum corneum was seen in the remaining treated cells. There was clear evidence of carbon black particles on the skin surface of one of these samples. This was identified along the surface of the skin and was associated with the remaining formulation that had not been washed off. In the same sample, and indeed, in all the other samples examined there was no evidence of carbon black particles below the surface of the stratum corneum. In other samples where stratum corneum was present, no carbon black particles were observed on the skin surface, presumably as a consequence of the washing procedure.

### TEM micrographs

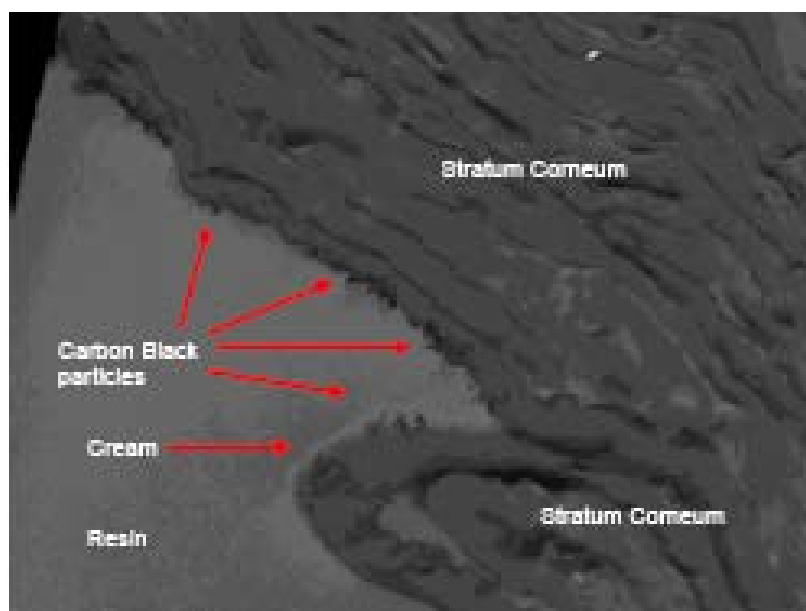
Carbon black particles associated with remaining test product at the surface of the Stratum Corneum could be observed (see Fig. 8). There was no evidence of carbon black particles in the skin layers, even in the Stratum Corneum.



**Fig. 8.** Transmission Electron Microscopy micrograph of eyeliner containing 12% carbon black treated skin sample (Johnson, 2013a). The carbon black nanoparticles appeared to be associated with the formulation on the outer layer of the Stratum Corneum. Micron bar: 500 nm. Legend: Test Formulation/cream and particulates (C+P)

Carbon Black particles (approximately sub 40 nm in size) could be observed only on the surface of the Stratum Corneum and appeared to be associated with the remaining test

product (see Fig. 9). There was no evidence of carbon black particles between the Stratum Corneum layers. In all sections evaluated, there was no evidence of carbon black particles penetrated into living skin or even into the Stratum Corneum.



**Fig. 9.** Transmission Electron Microscopy micrograph of eyeliner containing 12% carbon black treated skin sample (Johnson, 2013b). The carbon black nanoparticles were observed on the outer layer of the Stratum Corneum and not in the deeper layers. Micron bar: 500 nm

### Conclusions

The study authors concluded that carbon black particles were identified only in the outer tape strips for one of the samples and they were associated with the cream/formulation at the surface of the *Stratum Corneum*. The key observation from this part of the investigation was the absence of carbon black particles in any of the tape strip samples beyond the T2 level suggesting that carbon black particles do not penetrate into or beyond the surface layers of *Stratum Corneum*. Furthermore, carbon black was not observed either in the epidermis or dermis in the lower tape strips and remaining skin.

The absence of carbon black particles in the lower tape strips, epidermis and dermis indicates that carbon black does not penetrate into or beyond the *Stratum Corneum* following application of the cosmetic formulation.

Ref.: Johnson 2013a, 2013b

### SCCS comment

Due to the nature of the test material, imaging was considered the only practical method by the applicant. However, the use of this method is not considered as sufficiently quantitative by the SCCS. The first study is inadequate because results indicate some flaws that are typical of skin samples not stored properly. More information on the number of fields evaluated should be provided. Insofar, the test results provide some evidence to support lack of penetration of carbon black nano particles but are of limited predictive value. Other more sensitive methods may be explored. SCCS notes that the smallest particles examined are 20 nm and that no information is available in relation to smaller particles.



SCCS note that no information is available on the purity of carbon black in the submitted references. Since the test material was a thick liquid, the amount of carbon black available at the surface of the skin in the chamber is uncertain and may be lower than anticipated. Moreover, the amounts of aggregates present initially and at the end of the 24 hours are uncertain.

Based on the available data, there is no indication of carbon black particles being absorbed through the intact skin.

### 3.3.5. Repeated dose toxicity

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

#### **4-Week inhalation toxicity study in rats**

Guideline/method	/
Species:	Sprague-Dawley male rats
Age at start of study:	9 weeks
Groups:	4 groups, group size not specified
Test substance:	Carbon Black was produced from ethylene.
Batch:	/
PAH content:	/
Conc a.i.:	/
Particle size:	See Table 7
Aerodynamic diameters:	See Table 7
Dose applied:	0, 1.3, 6.2, and $42 \times 10^5$ particles/cm <sup>3</sup>
Route:	Nose-only inhalation
Exposure time:	4 h/day, 5 days/week for 4 weeks
GLP:	/
Date of report:	2011
Published:	Yes

#### Procedure

Ethylene was used as a precursor for production of carbon black. The reactant gases (C<sub>2</sub>H<sub>4</sub> and Ar) were fed into the reactor through electronic mass flow controllers. The effluent gases and carbon black were diluted in the mixing chamber with air. Concentrations used and particles size see Table 7. Approximately 61% of particles were smaller than 100 nm

**Table 7:** Distribution of Carbon Black particles (mean  $\pm$  standard deviation)

Group	Diameter* (nm)	Number (particles/cm <sup>3</sup> )	Surface (nm <sup>2</sup> /cm <sup>3</sup> )
Low	87.9 $\pm 1.47$	$1.3 \times 10^5$ $\pm 2.8 \times 10^5$	$1.1 \times 10^{10}$ $\pm 5.7 \times 10^{10}$
Middle	83.3 $\pm 1.46$	$6.2 \times 10^5$ $\pm 4.2 \times 10^5$	$2.7 \times 10^{10}$ $\pm 7.7 \times 10^{10}$
High	86.5 $\pm 1.41$	$4.2 \times 10^6$ $\pm 1.0 \times 10^6$	$1.4 \times 10^{11}$ $\pm 0.9 \times 10^{11}$

\*GM (GSD)

Pathogen-free 5-week-old Sprague-Dawley male rats were kept 4-week in quarantine for acclimatization before use. Nose-only inhalation chambers were used. The flow rate was considered sufficient for the maximum number of rodents (1.0 l/min/rat) and to monitor the particle concentration. The Sprague-Dawley male rats were exposed to carbon black for 4

hour/day, 5 days/week for 4 weeks, after which they were sacrificed on the next day. The induction of inflammation was assessed by analyzing the inflammatory cytokines in BALF. Plasma coagulability was evaluated by prothrombin time (PT) and activated partial thromboplastin time (aPTT), which were measured with a Coagulator2 coagulation analyzer.

## Results

The particle morphology was examined with transmission electron microscopy image analysis, which exhibited a mixed presence of individual particles and an aciniform of small aggregates, which is the typical shape mixture of carbon black.

Rats were exposed to carbon black nanoparticles for 4 weeks and the effects on the lungs were examined. All the rats gained weight steadily during the experiment, and no significant difference was observed among the groups. At the end of the experiment, the weight of the major organs (lung, kidney and heart) did not differ significantly among the treated groups.

To test if inhalation exposure to carbon black particles damages the lung tissue, bronchoalveolar lavage fluid (BALF) was prepared, and albumin and lactate dehydrogenase (LDH) leakage were measured. However, no detectable increase was observed in either albumin or LDH level. Consistent with these results, no pathological symptom was observed in the histological analysis of the lungs. Pulmonary inflammation was examined by analysis of inflammatory cytokines in BALF. Most of the samples, including IL-1 $\beta$ , IL-4, IL-6, TNF- $\alpha$  and INF- $\gamma$ , exhibited little change in levels. However, IL-10 level was significantly elevated in BALF from the exposed rats, and this elevation was carbon black concentration-dependent. These results suggest that exposure to nano-carbon black particles by inhalation does not damage the lung tissue and only induces minimal inflammation in the respiratory system.

Plasma coagulability was assessed with PT and aPTT measurements. Four-week exposure to carbon black nanoparticles did not affect PT. However, aPTT tended to increase in the exposed groups and this increase was dependent on the carbon concentration but was not statistically significant.

The platelet reactivity of the rats exposed to these agonists was not different from that of the control group. The blood cell numbers, including red blood cells, white blood cells and platelets, showed no significant difference among the treated groups. These results suggest that the respiratory exposure of Carbon Black nanoparticles does not affect the blood cell count and has only a minimal effect on blood functions such as plasma coagulation and platelet aggregation.

The aortas from all experimental groups exhibited normal constriction to serotonin and phenylephrine and relaxation to acetylcholine and there was no significant difference among groups, suggesting that Carbon Black nanoparticles did not affect vasomotor function.

## Conclusion

The study authors exposed male rats for nano-Carbon Black particles up to a concentration of  $4.2 \times 10^6$  particles/cm<sup>3</sup>. It was concluded that the exposure of these particles to rats did not induce any toxicity or inflammatory response in the lungs or make any functional alteration of the hemostatic and vasomotor activities.

Ref.: Kim *et al.*, 2011

## SCCS comment

Contrary to the conclusion that the exposure to carbon black did not induce any toxicity or inflammatory response or functional alteration in rat lung, the levels of IL-10 found were significantly elevated in BALF from the exposed rats. This elevation was found to be carbon black concentration-dependent.



The SCCS considers this as a poorly carried-out study because no BALF cell count was performed and no information concerning group sizes were available.

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

#### ***13-Week oral toxicity study in rats***

Guideline/method	OECD 408 (1998)
Species:	Wistar rats-HsdHan
Group size:	10 males and 10 females
Age at start of study:	8 – 9 weeks
Test substance:	Carbon Black, Unipure Black LC902, E300281
Batch:	C3949
Purity:	98%
Particle size:	Diameter; 20 to 30 nm, surface area range of 200 to 260 m <sup>2</sup> /g
Dose applied:	0 (control), 100, 300, and 1000 mg/kg bw/d
Vehicle:	0.5 % (w/v) sodium carboxymethylcellulose plus 1 % (v/v) Tween 80 in Milli-Q water
Route:	Oral gavage
Dose volume:	10 ml/kg
Exposure time:	90 consecutive days
GLP:	In compliance
Date of report:	December 2011 – March 2012
Published:	No

It was stated that the dose levels were selected on the basis of the results obtained in a preliminary 14-day oral study at the same dose levels where no adverse effects were observed (Yogesha, 2012, the study is not submitted).

#### Results

Following daily administration of carbon black, there were no deaths, no adverse clinical signs, no ocular findings or changes in body weight gain and food intake when compared to controls. Dark coloured faeces were observed in animals given carbon black at all dose levels. This finding is related to the coloured nature of the test item and hence considered to be non-adverse. There were no relevant changes in laboratory parameters. Some haematology and blood clinical chemistry parameters showed minimal changes when compared to controls. However, as they remained minor and showed no dose-relationship, these changes were considered to be unrelated to the administration of carbon black. There were no changes attributed to carbon black in any of the urinary parameters evaluated.

Dosing with carbon black did not produce any changes in organ weights. The only finding at necropsy consisted of black gastro-intestinal tract contents in all animals given carbon black, without any associated gross or microscopic changes in the gastro-intestinal mucosa apart from black discolouration. These findings were also considered to be related to the coloured nature of the test item and hence non adverse. There were no other histopathological findings attributed to carbon black.

#### Conclusion

The study authors concluded that the repeated daily oral administration to rats of carbon black for 13 weeks at 100, 300 or 1000 mg/kg/day was well tolerated, with some non-adverse findings related to the staining properties of carbon black. Accordingly, under the conditions of this study, the No Observed Adverse Effect Level (NOAEL) was 1000 mg/kg/day.

Ref.: Sathish, 2012

**13-Week inhalation (nose only) toxicity study in rats**

Guideline/method	/
Species:	Pathogen-free Sprague-Dawley male rats
Age at start of study:	7 weeks
Groups:	3 groups + control group. Group size not given.
Test substance:	Carbon Black; Printex 90 (Degussa, Frankfurt, Germany).
Batch:	/
PAH content:	/
Conc a.i.:	/
Particle size:	Aerodynamic diameters: Group 1, 1.52 µm; Group 2, 1.30 µm; Group 3, 0.97 µm
Dose applied:	The average concentrations were 8.84 mg/m <sup>3</sup> , 8.60 mg/m <sup>3</sup> and 8.97 mg/m <sup>3</sup> for group 1, group 2 and group 3.
Route:	Inhalation, nose only
Exposure time:	6 h/day, 5 days/week for 13 weeks
GLP:	/
Date of report:	2012
Published:	Yes

**Procedure**

Generation and exposure of carbon black aerosol. Printex 90 was dispersed by three methods: (1) 1.5 g of Printex 90 was added to 300 ml of distilled water and sonicated for 1 minute; (2) Printex 90 was suspended using same method as (1) and sonicated for 3 minutes in a 3 second sonication/5 second resting cycle; (3) Printex 90 was dispersed using the same method as (2) and stabilized with 2.5 mg/ml of human serum albumin (HAS).

The average diameters of Printex 90 suspensions were:

Method 1: 7140±3760 nm, mass median aerodynamic diameters 1.52 µm.

Method 2: 219±11 nm, mass median aerodynamic diameters 1.30 µm.

Method 3: 1875±409 nm, mass median aerodynamic diameters 0.97 µm.

Carbon black suspensions prepared by the three different methods were aerosolized through venturi nozzles at 8 liters of air flow per minute in a nose-only inhalation chamber. Rats were exposed to Printex 90 aerosols 6 hours a day, 5 days per week for 13 weeks.

**Results**

There were no statistical differences in body weight gain between the control group and the three agglomerated carbon black exposed groups during the 13-week exposure period. Lung weights of group 2 and group 3 were a little higher than the control group and group 1, carbon black masses were found in the lung tissues of all exposed groups. There were no statistically significant differences in pulmonary function between the control group and the three agglomerated carbon black exposed groups. No histopathological symptoms were found to be associated with carbon black exposure. The numbers of total cells, macrophages and polymorphonuclear leukocytes (PMNs) were increased by carbon black inhalation. However, statistically significant differences were found only between the control group and the Group 2 and 3 in terms of total cells and macrophages, and between the control group and Group 2 for PMNs. LDH was slightly increased in Group 3 compared with control, but no statistical significance was found. Albumin and inflammatory cytokine levels did not change following exposure to carbon black aerosols.

**Conclusion**

The study authors concluded that mild respiratory toxicity occurred in male rats exposed to nano-sized carbon black for 13 weeks at a concentration of approximately 9 mg/m<sup>3</sup> through nose-only inhalation; there were no significant differences in toxicity among the different agglomeration states.

Ref.: Lim *et al.*, 2012

**SCCS comment**

In this experiment different sized agglomerates were made in a suspension in water ranging from  $210 \pm 11$  nm to  $7140 \pm 3760$  nm. As reported above, the droplets generated (mass medium aerodynamic diameters) from these suspensions were similar ranging from 0.97 to 1.57  $\mu\text{m}$ . These data are clearly conflicting since agglomerates of 7.1  $\mu\text{m}$  diameter do not fit into droplets of 1.57  $\mu\text{m}$ . Therefore, the larger agglomerates must have de-agglomerated during aerosol generation – as also discussed by the authors. Therefore, the small (non-significant) differences seen in the different groups cannot be attributed to agglomeration differences, but are rather repetitions within the same experiment and should in fact be pooled, showing a significant influx of macrophages and neutrophils in the lung.

**13-Week inhalation toxicity study in mice, rats, and hamster**

Guideline/method	/
Species:	Female F-344 rats, female B6C3F1 mice, and female F1B Syrian golden hamsters
Age at start of study:	7 weeks
Groups:	5 – 6 females. For the histopathological and particle dosimetry analyses, groups of 6 animals were used; for all other end points, groups of 5 animals were used
Test substance:	Carbon Black; Printex 90 (Degussa-Huels, Trostberg, Germany) high surface area CB [HSCB]) for all animals, and an additional type Sterling V (Cabot, Boston, MA), low surface area CB [LSCB]; for high dose (50 mg/m <sup>3</sup> ) rats only
Batch:	/
PAH content:	0.039 mg/kg for Printex 90 and 8.8 mg/kg for Sterling V
Conc a.i.:	/
Particle size:	The HSCB particles had a primary particle size of 14 nm with a particle surface area of 300 m <sup>2</sup> /g; the LSCB particles had a primary particle size of 70 nm and a surface area of 37 m <sup>2</sup> /g
Aerodynamic diameters:	The HSCB aggregate aerosols had aerodynamic diameters ranging from 1.2 to 2.4 $\mu\text{m}$ (geometric standard deviations [GSD] = 2.0–3.1); the LSCB aggregate aerosols had aerodynamic diameters of 0.6–0.9 $\mu\text{m}$ (GSD = 3.0–3.7)
Dose applied:	Exposure levels, 0, 1, 7, and 50 mg/m <sup>3</sup> HSCB in filtered air, additional groups of rats were exposed to 50 mg/m <sup>3</sup> LSCB.
Route:	Inhalation
Exposure time:	6 h/day, 5 days/week for 13 weeks
GLP:	/
Date of report:	2005
Published:	Yes

**Procedure**

The study was designed to test the hypothesis that secondary, chronic inflammation and oxidative stress (see section 3.3.6.2.) are related events induced by subchronic (13 week) inhalation of carbon black. Previous studies had shown that the rat is the most sensitive species followed by mice and hamster. Females were chosen because they were previously shown to be more sensitive to the induction of lung tumours by poorly soluble, low toxicity particles than males. The hamster diet was supplemented with rolled oats during the post-exposure phase of the study to increase fiber content and avoid the development of diarrhea.

There were four particle exposure levels (0, 1, 7, and 50 mg/m<sup>3</sup> Printex 90, [high surface area] HSCB) for all animals and an additional particle type (Sterling V, 50 mg/m<sup>3</sup>, [low surface area] LSCB) to which only the rats were exposed. This second particle type was used to test hypotheses about size- or surface area-related variations in response. All exposures lasted for 13 weeks. After 5 weeks, groups of animals were sacrificed in order to measure the retained carbon black content in the lungs. This was done to adjust the lung burdens of the retained carbon so that the predicted burden per gram of control lung at the end of exposure was similar between the species in each dose group. For rats, the 5-week sacrifice was used to adjust the exposure concentration of Sterling V (nominal concentration: 50 mg/m<sup>3</sup>) so that the predicted lung burden at the end of exposure was similar to the Printex 90 mid-dose group (7 mg/m<sup>3</sup>) in terms of retained particle surface area. Rats were exposed first and adjustments were made to the exposure concentrations where necessary for the mice and hamsters to achieve similar retained lung doses among the three species. Groups of 15 (5 each for cellular/biochemical, carbon black lung burden, and histological analyses) animals were sacrificed immediately (1 day) post-exposure as well as 3 months and 11 months post exposure; thus, there were three post-exposure time points at which measurements were made.

#### Test concentration adaptation

5 weeks into the exposures of mice and hamsters, it was determined that the retained burden of HSCB was lower than was found for rats, which were exposed first. The concentrations for the mouse and hamster exposures were accordingly increased from 7 to 15 mg/m<sup>3</sup> for the mid-dose and from 50 to 75 mg/m<sup>3</sup> for the high-dose to produce equivalent predicted normalized lung burdens. For hamsters, the low dose also had to be increased from 1 to 1.1 mg/m<sup>3</sup>.

#### Result

**Deaths.** Rats. No rats died during exposures to carbon black. Nine died in the post-exposure phase due to a water line that was inadvertently left unconnected to the cage rack. One each in the low and high dose HSCB groups also died in the post-exposure phase. Mice. 15 Mice died during exposure, seven of which were in the control group; two were in the low dose group and three each were in the mid and high dose groups. In the post-exposure phase, four controls, three low dose, two mid dose, and five high dose mice died. Hamster. Only one hamster (high dose group) died during exposure. Two hamsters each from the control, low, and high dose groups and four from the mid dose groups died in the post-exposure phase of the study.

**Body weights.** Development of body weights showed a significant drop in body weight for rats exposed to high dose HSCB during the exposure phase of the study. Rat body weight returned to control values by the end of the exposure and remained at control levels during the recovery period. Hamsters in the high dose group also lost a significant amount of weight during the exposure period, but recovered during the post-exposure phase. There were no significant changes in body weight found for other exposure doses or in the mice.

**Lung weights.** For rats, significant elevations in lung weight (up to nearly twice control lung weight) were found at the high dose HSCB and for LSCB at the end of exposure and all post-exposure time points. The lung weights of rats exposed to LSCB were in-between those for the high and mid dose HSCB in magnitude. In mice, lung weights were found to be elevated at the end of exposure and up to 3 months of recovery in the high and mid dose groups; at the end of the recovery period (i.e., 11 months post-exposure), significant elevations were found only in the high dose group. In hamsters, significant elevations in lung weights were found only for the highest dose group and these changes persisted up to 3 months post-exposure; at 11 months post-exposure no significant changes were found.

**Lung burden.** In rats the highest lung burdens in terms of mass were achieved in the high dose HSCB and LSCB groups. These two groups were significantly different from all other groups and from each other through 3 months post-exposure; by 11 months post-exposure,

they were no longer different from each other. This was because there was lung particle clearance in the LSCB group, which was consistently higher than in the high dose HSCB group up to the 11 month time point. Both groups showed retarded clearance, as did the mid-dose HSCB rat group, whereas the low-dose group had the fastest clearance. In mice, significant elevations in lung particle burdens were found during, immediately and after exposure, and up to three months post-exposure in all dose groups. At 11 months post-exposure, significant increases were found only in the mid and high dose groups. The mice had the highest relative lung burdens of the three species when the exposures were finished, partly because the aerosol concentrations in the mid and high dose groups were over-adjusted after 5 weeks of exposure. This explains, at least in part, the higher lung burden found in mice compared to rats at the end of exposure. Despite this, the mice cleared the particles better than the rats because at 11 months post-exposure, the relative rat lung burdens in the high dose group were higher than in similarly-exposed mice. In hamsters, significant increases in lung burden were found in the mid and high dose groups during and immediately after exposure. In the post-exposure phase, significant elevations remained only for the high dose group. Thus, the hamsters appeared to have the most efficient particle clearance of the three species.

The particle retention half time for rats were 64 days for low HSCB, 115 days for mid HSCB, no significant clearance for high HSCB and 249 days for LSCB. The half times for mice were 133, 343, and 322 days for low, mid, and high HSCB exposure, respectively. For hamsters the corresponding number was 42, 53, and 309 days.

**Bronchoalveolar fluid (BAL).** The presence of polymorphonuclear leukocytes (PMN) in lavage fluid was used as a sensitive indicator of lung inflammation. All rat exposure groups except for the lowest dose level were found to have significant elevations in the percentage of PMNs after the end of the exposure, and after 3 and 11 months of recovery. Both LDH and beta-glucuronidase were significantly elevated in the high-dose HSCB and LSCB groups through 3 months of recovery. Thereafter, elevations were only found in the high-dose HSCB group. TNF-alpha and MIP-2 changes were generally in accordance with the inflammatory BAL cell responses. At the end of exposure and throughout the post-exposure phase, the percentage of lavage fluid PMNs was found to be significantly elevated in mice exposed to mid and high dose HSCB. Both LDH and beta-glucuronidase were significantly elevated in the mid- and high-dose groups through 3 months of recovery. Thereafter, elevations were only found in the high-dose group. Hamsters exposed to mid and high dose HSCB demonstrated significant elevations in total cell numbers at the end of exposure. The only animals with persistent and significant elevations during the recovery phase were those exposed to the highest concentration. Both LDH and beta-glucuronidase were significantly elevated in the mid- and high-dose groups of hamsters through 3 months of recovery. Thereafter, elevations were only found for beta-glucuronidase in the high-dose group. There were no significant elevations in cellular or biochemical parameters for any of the animals exposed to low-dose HSCB.

**Histopathology.** No exposure-related alterations were observed microscopically at any of the post-exposure time points in the lungs of control and low dose rats, mice and hamsters.

Exposure-related histopathology was present in the lung lobes of rats exposed to mid- and high-dose HSCB. At 1 day post-exposure, the principal exposure-related lung lesions were centered primarily in the centriacinar regions of the lungs with the most extensive epithelial and inflammatory responses in the alveolar ducts and the immediately surrounding alveolar parenchyma. These lesions were characterized by (1) the accumulation of large numbers of markedly hypertrophic and highly vacuolated alveolar macrophages laden with HSCB; (2) an associated mixed inflammatory cell infiltrate composed of neutrophils and lesser numbers of mononuclear cells located in the alveolar walls, alveolar air spaces, and perivascular interstitium; and (3) hyperplasia and hypertrophy of alveolar type II cells. There was only mild attenuation in the severity of the inflammatory and epithelial lesions at the later time points compared to those observed at 1 day post-exposure.

HSCB or LSCB exposed rats had significantly more macrophages than the control rats. The densities of macrophages were not significantly different between rats exposed to high-dose HSCB and LSCB at any post-exposure time point.

Exposure-related lung lesions were present in mice exposed to mid- and high-dose HSCB and sacrificed at 1 day and 3 and 11 months post-exposure. Though the lesions were similar in these two exposure groups, the magnitude of the lesions (severity) was consistently greater in mice exposed to high-dose HSCB. The most conspicuous change in the lungs of these mice was the presence of numerous carbon black particles that were widely scattered throughout the alveolar parenchyma, but more concentrated in centriacinar regions including alveolar ducts and adjacent alveoli.

Modest exposure-related increases in alveolar macrophage numbers were only evident in mice exposed to high-dose HSCB and sacrificed 3 or 11 months post-exposure and to mid-dose HSCB and sacrificed 11 months post-exposure

Hamsters exposed to the mid dose and the high dose of HSCB had widely scattered aggregates of enlarged alveolar macrophages and a few multinucleated giant cells engorged with phagocytized HSCB particles at all post-exposure time points.

#### Discussion

The results of this comparative study in rats, mice, and hamsters confirm previous findings that the rat is the most sensitive of the three species to adverse effects induced by poorly soluble particles in the respiratory tract. No adverse inflammatory or morphological changes were observed at the lowest exposure concentration (1 mg/m<sup>3</sup>) of carbon black in any of the three species. Interestingly, for the low- and mid-dose HSCB groups, the responses among the three species were similar in magnitude.

Lung inflammation and histopathology were more severe and prolonged in rats than in mice and hamsters. The magnitude of the response to LSCB in rats was between those for high- and mid-dose HSCB. The results show that hamsters have the most efficient clearance mechanisms and the least severe responses of the three species. The results from rats also show that particle surface area is an important determinant of target tissue dose and, therefore, effects. From these results, a subchronic NOAEL of 1 mg/m<sup>3</sup> respirable HSCB (Printex 90) can be assigned to female rats, mice, and hamsters.

#### Conclusion

Particle retention kinetics, inflammation, and histopathology were examined in female rats, mice, and hamsters exposed for 13 weeks to high surface area carbon black (HSCB) at doses of 0, 1, 7, and 50 mg/m<sup>3</sup>. Rats were also exposed to 50 mg/m<sup>3</sup> low surface area carbon black (LSCB). Groups of animals were sacrificed immediately after 13 weeks of exposure, and after 3 and 11 months of recovery for bronchoalveolar lavage analysis, as well as for measurements of lung burdens and lung histopathology. Additionally, groups of animals were sacrificed after 5 weeks of exposure to measure retained carbon content in the lungs and to adjust the lung burden of the retained carbon so that the predicted burden per gram of control lung at the end of the exposure was similar between the species in each dose group. For rats, the 5-week sacrifice was also used to adjust the exposure concentration of LSCB (nominal concentration 50 mg/m<sup>3</sup>) so that the predicted lung burden at the end of exposure was similar to HSCB mid-dose group (7 mg/m<sup>3</sup>) in terms of retained particle surface area. Prolonged retention of carbon black particles in the lung was found in rats and mice exposed for 13 weeks to 7 and 50 mg/m<sup>3</sup>, and in hamsters exposed for 13 weeks to 50 mg/m<sup>3</sup>. These data show that hamsters have the most efficient clearance mechanism. Low-surface carbon black (50 mg/m<sup>3</sup>) was more efficiently cleared from the lungs than was 50 mg/m<sup>3</sup> high-surface carbon black.

The study authors concluded that the NOAEL for rats, mice and hamster was 1 mg/m<sup>3</sup>.

Ref.: Elder *et al.*, 2005, Carter *et al.*, 2006



**SCCS comment**

The responses after inhalation of carbon black at 1 and 7 mg/m<sup>3</sup> among rats, mice and hamsters were similar in magnitude. The NOAEL for inhalation of carbon black nanomaterials for rats, mice and hamster was 1 mg/m<sup>3</sup>.

The mid (7 mg/m<sup>3</sup>) and high (50 mg/m<sup>3</sup>) doses of carbon black induced pathological lung alterations in mice and rats that were persistent as they were not resolved after the recovery period up to 11 months after exposure.

3.3.5.3. Chronic (> 12 months) toxicity
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Not submitted. See also Section 3.3.7. Carcinogenicity.

<b>3.3.6. Mutagenicity / Genotoxicity</b>
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Only more recent studies have been included.

3.3.6.1 Mutagenicity / Genotoxicity <i>in vitro</i>
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**Cell-free systems**

Guideline:	/
Plasmid:	Φ174 RFI
Replicates:	/
Test substance:	Fine Carbon Black Ultrafine Carbon Black (Printex 90) obtained from Degussa (Frankfurt, Germany) Batch: /
Particle size:	Fine Carbon Black: 129 nm to 592 nm, with a mean diameter of 260±13.7 nm Ultrafine Carbon Black: 7.7 nm to 28.2 nm, with a mean diameter of 14.3±0.6 nm
Purity:	Printex 90 : >99%
Culture media:	/
Concentrations:	Φ174 RFI: 1, 5 and 10 µg/290 ng of plasmid
Treatment	/
GLP:	/
Date of report:	1998

**Procedure**

The surface free radical activity of the fine carbon black and nano carbon black particles was assessed in a cell-free system using the formation of single-strand breaks in the plasmid Φ174 RFI as indicator. Particles were used at doses of 1, 5 and 10 µg carbon black per 290 ng of plasmid, in a final value of 20 ml.

**Results**

Measurement of single strand DNA breaks using supercoiled plasmid DNA as indicator, showed that nano carbon black exhibited significantly more free radical activity than fine carbon black (P <0.05). The results with fine carbon black did not differ significantly from the control.

**Conclusion**



The study authors concluded that in a cell-free system using the plasmid  $\Phi$ 174 RFI as indicator, nano carbon black caused significantly more single-strand breaks than fine carbon black particles.

Ref.: Stone *et al.*, 1998

### SCCS comment

The cell-free system used by Stone *et al.* 1998 only provides an indication of potential hazard in terms of DNA damage because, in the test, DNA was directly exposed to carbon black nanoparticles.

### **Bacterial reverse mutation assay**

Guideline:	OECD 471 (1997)
Species/Strain:	<i>Salmonella typhimurium</i> TA98, TA100, TA1535, TA1537, and TA102
Replicates:	Triplicates in two independent experiments
Test substance:	Carbon Black, E300281
Batch:	C3949 of ingredient "Unipure Black LC 902"
Purity:	98%
Particle size:	Diameter; 20 to 30 nm, surface area range of 200 to 260 m <sup>2</sup> /g.
Vehicle:	Anhydrous dimethyl sulphoxide (DMSO)
Concentration:	Experiment 1: 0.32, 1.6, 8, 40, 200, 1000, and 5000 µg/plate, without and with S9-mix plus negative (vehicle) and positive controls. Experiment 2: 156.3, 312.5, 625.0, 1250, 2500, and 5000 µg/plate, without and with S9-mix plus negative (vehicle) and positive controls
Treatment:	No pre-incubation in Experiment 1, in Experiment 2 pre-incubation with S9-mix for 1 hour at 37°C before the addition of molten agar
GLP:	In compliance
Study period:	August 2011

### Procedure

E300281 was tested for mutation (and toxicity) in five strains of *Salmonella typhimurium* (TA98, TA100, TA1535, TA1537 and TA102), in two separate experiments, at the concentrations detailed in the table above, using triplicate plates without and with S9-mix (S9-mix prepared from the liver of rats given Aroclor 1254). Negative (vehicle) controls were included in quintuplicate, and positive controls were included in triplicate in both assays without and with S9-mix. When set, the plates were inverted and incubated at 37±1°C in the dark for 2 to 3 days. Following incubation, the plates were examined for evidence of toxicity to the background lawn, and revertant colonies were counted. As the results of Experiment 1 were negative, treatments in the presence of S9-mix in Experiment 2 included a pre-incubation step.

Colonies were counted electronically using a Sorcerer Colony Counter or manually. The background lawn was inspected for signs of toxicity. Data were considered acceptable if the mean vehicle control counts fell within the historical 99% confidence intervals for group means and/or each vehicle control plate count fell within the historical 99% reference ranges, and the positive control plate counts were comparable with the historical 99% reference ranges.

### Results

Experiment 1 treatments of all the tester strains were performed in the absence and in the presence of S9-mix, using final concentrations of E300281 at 0.32, 1.6, 8, 40, 200, 1000 and 5000 µg/plate, plus negative (vehicle) and positive controls. Following these treatments, no clear evidence of toxicity was observed.

Experiment 2 treatments of all the tester strains were performed in the absence and in the presence of S9-mix. The maximum test concentration of 5000 µg/plate was retained for all strains. Narrowed concentration intervals were employed in order to examine more closely those concentrations of E300281 approaching the maximum test concentration and considered therefore most likely to provide evidence of any mutagenic activity. In addition, all treatments in the presence of S9-mix were further modified by the inclusion of a pre-incubation step. Following these treatments, evidence of toxicity in the form of a marked reduction in revertant numbers was observed at 5000 µg/plate in strain TA1537 in the presence of S9-mix. Precipitation of the test article was recorded as being visible by eye on the test plates at concentrations of 8 µg/plate and above; although as the test article was treated as a suspension and all concentrations are nominal, these observations were not used to limit concentration selection for Experiment 2.

#### Conclusion

The study authors concluded that under the conditions of the study, Carbon Black was not mutagenic in *Salmonella typhimurium* strains TA1535, TA1537, TA98, TA100 and TA102 strains either in the presence or absence of metabolic activation.

Ref.: Hobson, 2011

#### SCCS comment

SCCS note that although the Salmonella test is a reliable genotoxicity screen for the analysis of chemicals, it does not appear to be suitable for the assessment of nano materials. This might in part be related to the size of bacteria, presence of bacterial cell wall and the limited or no uptake of nanoparticles by bacteria (Doak *et al.*, 2012; Magdolenova *et al.*, 2014).

#### **Mammalian cells in vitro**

##### **DNA-adduct formation**

Guideline:	/
Cells:	A549 human type II lung alveolar epithelial cells
Replicates:	/
Test substance:	See Table 8
Batch:	/
Particle size:	See Table 8
Purity:	/
Vehicle:	Hank's balanced salt solution
Concentrations:	See Table 8
Treatment	harvest time 24 hours after the beginning of treatment
GLP:	/
Date of report:	2005

**Table 8.:** Characteristics of carbon black nanomaterials and concentrations used for in vitro studies with lung epithelial cells

Material	PAH levels <sup>a</sup> (mg/kg)	Type of treatment	Concentrations <i>in vitro</i>
Printex 90, 300 m <sup>2</sup> /g	1: 0.039 2: 0.001	Original particle Extract (DMSO)	100 µg/cm <sup>2</sup> 0 µM BaP (1 mg/cm <sup>2</sup> ) <sup>b</sup>

## Second revision of the opinion on carbon black, nano-form

	3: 0.010	Extracted particle	100 µg/cm <sup>2</sup>
	4: 0.065		
	5: ND		
	6: 0.008		
Sterling V, 30 – 40 m <sup>2</sup> /g	1: 8.8	Original particle	100 µg/cm <sup>2</sup>
	2: 0.4	Extract (DMSO)	0.84 µM BaP (3.7 mg/cm <sup>2</sup> ) <sup>b</sup>
	3: 21.5	Extracted particle	100 µg/cm <sup>2</sup>
	4: 202.5		
	5: 6.8		
	6: 89.7		
N330, 70 – 90 m <sup>2</sup> /g	1: 2.4	Original particle	100 µg/cm <sup>2</sup>
	2: 0.2	Extract (DMSO)	0.16 µM BaP (3.2 mg/cm <sup>2</sup> ) <sup>b</sup>
	3: 7.2	Extracted particle	100 µg/cm <sup>2</sup>
	4: 191		
	5: 1.4		
	6: 44.8		
Lampblack 101	1: 0.057	Original particle	100 µg/cm <sup>2</sup>
	2: 0.002	Extract DMSO	0.0006 µM BaP (1.6 mg/cm <sup>2</sup> ) <sup>b</sup>
	3: 0.05	Extracted particle	100 Ag/cm <sup>2</sup>
	4: 0.813		
	5: 0.011		
	6: 0.172		

a Individual PAHs are denominated with (1) phenanthrene, (2) anthracene, (3) fluoranthene, (4) pyrene, (5) benzo(a)pyrene, and (6) benzo (ghi)perylene, but denomination is not extensive.

b Between brackets is shown the virtual dose of particles that would have been needed to achieve this level of BaP as obtained using extracts.

### Procedure

Particle extracts were prepared by 8-h Soxhlet extraction of 7–28 g of different carbon blacks with 220 ml toluene. Extracted particles were recovered by filtration and dried after two washings in acetone for 4 h at 110 °C. The toluene extracts were transferred into DMSO (5 ml) by selective distillation in order to avoid loss of volatile PAH and used for *in vitro* experiments along with the dried extracted particles. The PAH content of the DMSO extracts was measured with high-pressure liquid chromatography (HPLC) and fluorescence detection. As positive controls, either a mix of 16 standard PAHs and a B[a]P standard.

A549 human lung epithelial cells were grown in DMEM culture medium supplemented with 10% heat-inactivated fetal calf serum. Cell studies to investigate PAH–DNA adducts with A549 cells were done at equivalent mass with all test particles and using equivalent volume (50 µl) of particle extracts reaching different PAH levels (Table 8). The particle concentration of 100 µg/cm<sup>2</sup> was used as the maximal value as determined by the absence of *in vitro* cytotoxicity of all carbon black particles. Aromatic adducts to DNA were determined by <sup>32</sup>P postlabeling method after 24 h incubation.

### Results

The four original carbon black particles as well as their extracts and the extracted particles were incubated along with positive controls. A linear relationship between B[a]P concentration in the solution (positive control) or extract (Sterling V) and the B[a]P-diol-epoxide (BPDE) adduct formation in A549 cells after 24-h incubation was found. No adduct spots were found with the other carbon black particles.

### Conclusion

The study authors concluded that PAHs were not bioavailable from three (Printex 90 [PAH = 0.039 ppm, 300 m<sup>2</sup>/g], N330 [PAH = 2.4 ppm, 70 – 90 m<sup>2</sup>/g], and Lampblack 101 [PAH =

0.057 ppm, 20 m<sup>2</sup>/g]) out of four tested commercial products that contain little PAHs. Some evidence suggests that in the carbon black sample with high PAH content (Sterling V [PAH = 8.8 ppm, 30 – 40 m<sup>2</sup>/g]) some PAHs are bioavailable for biotransformation and induction PAH–DNA adducts.

Ref.: Borm *et al.*, 2005

### **DNA strand breaks (Comet assay)**

Guideline: /  
 Cells: A549 human type II lung alveolar epithelial cells  
 Replicates: /  
 Test substance: Carbon powder from Sigma-Aldrich  
 Batch: /  
 Particle size: 20 – 40 nm  
 Purity: /  
 Vehicle: DMEM supplemented with 10% fetal bovine serum  
 Concentrations: 0, 20, and 40 µg/cm<sup>2</sup>  
 Treatment: 4 h for DNA damage (Comet assay) and intracellular ROS.  
 GLP: /  
 Date of report: 2008

#### Procedure

A selection of metal oxide nanoparticles, CuO, TiO<sub>2</sub>, ZnO, CuZnFe<sub>2</sub>O<sub>4</sub>, Fe<sub>3</sub>O<sub>4</sub>, Fe<sub>2</sub>O<sub>3</sub>, and two types of carbon based nanomaterials, carbon nanopowder (carbon) and multiwalled carbon nanotubes (nanotubes), were investigated. The carbon black particles were suspended in water and sonicated for 2 × 20 s in order to reduce agglomeration. The human lung epithelial cell line A549 was exposed to the particles, and cytotoxicity was analyzed using trypan blue staining. DNA damage and oxidative lesions were determined using the comet assay, and intracellular production of reactive oxygen species (ROS) was measured using the oxidationsensitive fluoroprobe 2',7'-dichlorofluorescein diacetate (DCFH-DA).

#### Results

There was a high variation among different nanoparticles concerning their ability to cause toxic effects. Carbon nano powder did not cause any decreased viability or any increase in DNA breaks or oxidative DNA lesions, nor in intracellular ROS.

Ref.: Karlsson *et al.*, 2008

### **SCCS comment**

The carbon nano powder is insufficiently characterized. Moreover, the sonication procedure in order to reduce agglomeration may have been too mild. Additionally, the concentrations used may have been too low and exposure time too short.

Guideline: /  
 Cells: A549 human type II lung alveolar epithelial cells  
 Replicates: /  
 Test substance: Coarse carbon black (CB: Huber 990; H. Haeffner and Co Ltd, Chepstow, UK)  
 Nanoparticulate CB (NPCB: Printex 90; Degussa, Frankfurt, Germany)  
 NPCB coated with benzo(a)pyrene (BaP-NPCB), 26 mg BaP/g Printex 90  
 Batch: /  
 Particle size: Coarse Carbon Black 260 nm  
 Nanoparticulate Carbon Black 14 nm

Purity: /  
 Vehicle: DMEM supplemented with 10% heat-inactivated foetal calf serum  
 Concentrations: 100 µg/ml (25 mg/cm<sup>2</sup>)  
 Treatment: 3 h for DNA damage (Comet assay) and intracellular ROS.  
 GLP: /  
 Date of report: 2008

#### Procedure

A549 cells were treated with tert-butyl-hyperperoxide (Tbh), urban dust (UD), coarse carbon black (CB), nanoparticulate carbon black (NPCB), benzo(a)pyrene (BaP) and NPCB coated with BaP for >24 h. The particles were suspended in foetal bovine serum-free DMEM at concentration of 100 mg/ml (25 µg/cm<sup>2</sup>) and sonicated for 20 min prior to use. For all nano particle treatments, cells were exposed to 100 µg/ml (25 µg/cm<sup>2</sup>) particles in culture medium for 0.5–24 h. Single- and double-strand breakage of DNA was determined by comet assay; cell cycle status was analysed using flow cytometry. Nuclear extracts or acid-extracted histones were used for Western blot analysis of p-ser15-p53 (p53 phosphorylated at ser15), p53 binding protein (53BP) 1, phospho-histone H2A.X (p-H2A.X) and phospho-BRCA1 (p-BRCA1). Immunoprecipitation followed by immunoblot was used to detect p53 protein phosphorylated at ser15 in cells exposed to particles and BaP for different times ranging 0.5–24 h.

#### Results

Carbon black nanoparticle exposure caused a significant increase in DNA single strand breaks and alkali-labile sites in A549 cells after 3 h exposure. Double-strand breaks, detected by neutral comet assay, occurred only in cells treated with urban dust.

The results show that p53 phosphorylation on ser15 occurred in response to carbon black nanoparticles, urban dust and BaP, but not with coarse carbon black or BaP coated nano carbon black. The highest response was achieved after 1-h exposure to Tbh (9.6-fold increase), NPCB (7.3-fold increase), UD (5.2-fold increase) and BaP (3.3-fold increase).

#### Conclusion

The study authors concluded that, carbon black nanoparticles and reactive oxidative species induce DNA damage, activating p53 and proteins related to DNA repair, mimicking irradiation-related carcinogenesis pathways.

Ref.: Mroz *et al.*, 2007

#### SCCS comment

It is noted that fine particles carbon black (260 nm) did not cause single strand DNA breaks while nano particles carbon black (14 nm) and benzo(a)pyrene coated nano particles carbon black (26 mg BaP/g) induced single strand DNA breaks after 3 h exposure. No difference was observed between uncoated and BaP coated carbon black nano particles. Uncoated nano carbon black induced ser15 phosphorylation of p53 protein, while BaP coated carbon nanoparticles did not.

Guideline: /  
 Cells: Primary mouse embryonic fibroblasts from BALB/c mice  
 Replicates: /  
 Test substance: Carbon Black nanomaterials (Nano-Innovation Co. Ltd, Shenzhen)  
 Batch: /  
 Particle size: 12.3 ± 4.1 nm  
 Purity: 99.4%  
 Vehicle: Dulbecco's modified Eagle's low glucose medium (DMEM/low). The presence of serum during the exposure was not clearly specified

Concentrations: 0, 5, 10, 20, 50 and 100 µg/ml  
 Treatment: 24 h for DNA damage (Comet assay) and intracellular ROS.  
 GLP: /  
 Date of report: 2008

#### Procedure

Manufactured nanoparticles of CB (carbon black), CNTs (single wall carbon nanotube), SiO<sub>2</sub> and ZnO were purchased from commercial suppliers and then suspended in fetal bovine serum. In order to break the agglomerate and ensure a uniform suspension, all particle samples were sonicated six times intermittently (30 s every 2 min) and characterized using TEM. Primary mouse embryo fibroblasts were freshly derived and used for each experiment. Particle suspensions were freshly prepared before the cells were exposed, and diluted to appropriate concentrations (0, 5, 10, 20, 50 and 100 µg/ml) with the culture medium, then immediately applied to the cells. Effects of nanoparticles on the viability after 24 h exposure were evaluated using two methods: the MTT assay and the WST assay. Intracellular production of reactive oxygen species (ROS) was measured using the oxidation sensitive fluoroprobe 2',7'-dichlorofluorescein diacetate (DCFH-DA).

#### Result

The cell viabilities upon incubation in the presence of carbon black, SiO<sub>2</sub> and ZnO at 20 µg/ml were inhibited by 41.5, 27.6 and 73.5%, compared with the control group. After exposure to carbon black, CNTs, SiO<sub>2</sub> and ZnO nanoparticles at 100 µg/ml, the GSH levels were reduced by 61, 50, 36 and 91%, respectively, compared with the control groups. The genotoxicity as assayed in the Comet assay can be classified from most to least toxic as follows: CNTs > ZnO > carbon black > SiO<sub>2</sub>. For example, tail DNA% was 38.2, 18.8, 12.8 and 6.8%, respectively, for the group of CNTs, ZnO, carbon black and SiO<sub>2</sub>, vs 3.26% for the untreated control.

#### Conclusion

The study authors concluded that the study clearly indicates that engineered nanoparticles of carbon black, single wall carbon nanotube, SiO<sub>2</sub> and ZnO induced statistically significant cytotoxicity through oxidative stress and DNA single strand breaks.

Ref.: Yang *et al.*, 2009

#### SCCS comment

Carbon black caused DNA damage as measured by the Comet assay in primary mouse embryo fibroblast cells after 24 h incubation.

#### Gene mutation tests

Guideline: OECD 476 (1997)  
 Species/strain: L5178Y *tk*<sup>+/-</sup> mouse lymphoma cells  
 Replicates: Duplicate cultures in two independent experiments  
 Test substance: Carbon Black, E300281  
 Batch: C3949 of ingredient "Unipure Black LC 902"  
 Particle size: Diameter; 20 to 30 nm, surface area range of 200 to 260 m<sup>2</sup>/g.  
 Purity: 98%  
 Vehicle: Anhydrous dimethyl sulphoxide (DMSO)  
 Concentrations: Experiment 1: In the absence of S9-mix: 20, 40, 60, 80, 100 and 120 µg/ml. In the presence of S9-mix 10, 20, 40, 50 and 60 µg/ml  
 Experiment 2: In the absence of S9-mix: 10, 20, 40, 60, 80, 100 and 120 µg/ml. In the presence of S9-mix: 5, 10, 20, 30, 40 and 50 µg/ml  
 Treatment: 3 hours with and without S9-mix  
 GLP: In compliance  
 Study period: August – October 2011



### Procedure

The mouse lymphoma cell line L5178Y [*hprt* locus for 6-thioguanine (6-TG) resistance] was used. The test item carbon black was evaluated in two independent experiments using duplicate cultures each (single cultures for positive controls). Both experiments used a pulse (3-hour) treatment and were conducted in the absence and presence of metabolic activation (S9-mix prepared from the liver of rats given Aroclor 1254). The concentrations selected for final tests were as follows, on the basis of precipitation criteria:

- Experiment 1
  - In the absence of S9-mix: 20, 40, 60, 80, 100 and 120 µg/ml
  - In the presence of S9-mix 10, 20, 40, 50 and 60 µg/ml
- Experiment 2
  - In the absence of S9-mix: 10, 20, 40, 60, 80, 100 and 120 µg/ml
  - In the presence of S9-mix: 5, 10, 20, 30, 40 and 50 µg/ml

Known mutagens in the presence (benzo(a)pyrene, BaP) or absence of S9-mix (4-nitroquinoline 1-oxide, NQO) were tested at two different concentrations and served as positive controls. Negative controls consisted of cultures treated with the solvent alone (DMSO). Cells were suspended in culture medium and exposed to various concentrations of the test item, solvent or positive controls. After the treatment period (3 hours), the cells were re-suspended in the culture medium. They were transferred to flasks for growth throughout the expression period (7 days) or were diluted to be plated for survival (7 days). At the end of the expression period, acceptable cultures were then plated for viability (2 plates per culture, 8 days) or 6-TG resistance (4 plates per culture, 12 to 13 days).

### Results

Mutant frequencies in solvent negative controls fell within normal ranges, and treatment with positive controls NQO and BaP yielded distinct increases in mutant frequency. Accordingly, the study was considered to be valid.

When tested up to solubility limits, carbon black did not produce reproducible increases in mutant frequency in the presence or absence of S9-mix. A slight but statistically significant linear trend was observed in the absence of S9-mix in experiment 1. However, none of the individual mutant frequencies obtained was statistically significantly different from controls, and such minor changes were not observed in Experiment 2. Accordingly, this isolated observation was considered to be of no biological significance.

### Conclusion

The study authors concluded that carbon black did not induce mutation at the *hprt* locus of L5178Y mouse lymphoma cells when tested under the conditions employed in this study. These conditions included treatments up to concentrations at which precipitating and/or undissolved test article was observed in two independent experiments in the absence and presence of a rat liver metabolic activation system.

Ref.: Lloyd, 2011

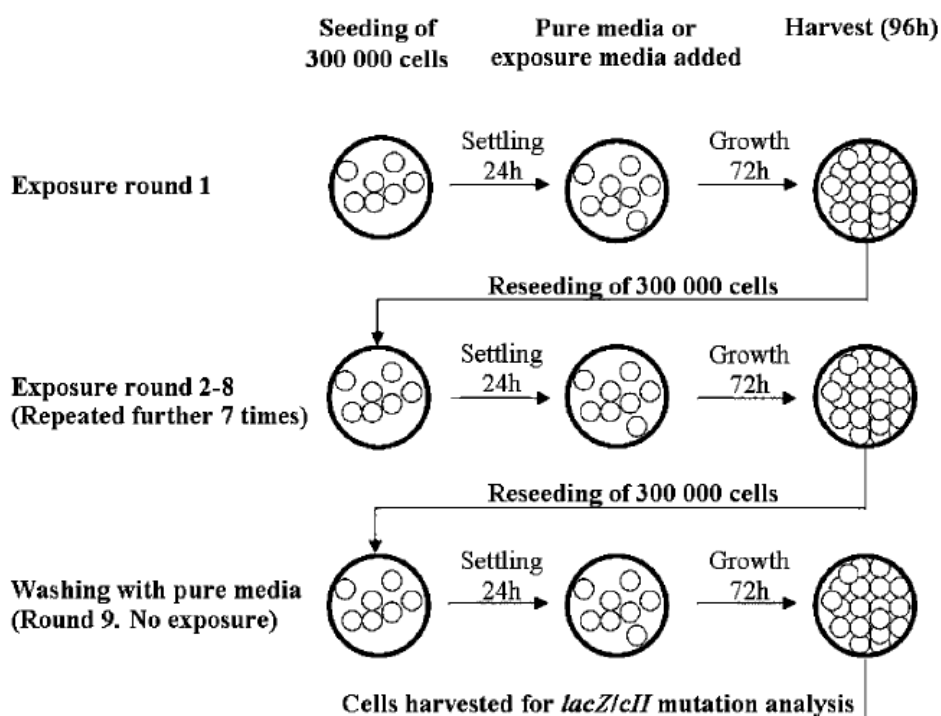
### SCCS comment

Though a 3h exposure time is commonly used for testing genotoxicity of nanomaterials with test systems such as the comet assay, it is not always sufficient for detection of mutagenicity using the *hprt* forward mutation assay. Additionally, no uptake studies were performed to confirm cellular uptake. Thus, although the study did not identify mutagenicity, an appropriate exposure time might not have been applied. Therefore, the SCCS considers this study to be of no value for this assessment.

Guideline/method	/
Cell line:	FE1 MML mouse epithelial cell line
Test substance:	Carbon Black; Printex 90 (Degussa-Huels, Trostberg, Germany)
Batch:	/
PAH content:	Organic impurity content is ~1%. The observed five PAHs sum to 74.2 ng/g. The level of detection for the 16 PAH ranged between 0.3 and 2 ng/g.
Positive control:	Benzo[a]pyrene (BaP)
Conc a.i.:	75 ug/ml
Treatment	The cells were incubated with or without carbon black in a total of eight exposure rounds making the total exposure time (8 x 72 hr) 576 hr. The cumulative dose added was 6 mg for carbon black
Particle size:	Printex 90; particle size 14 nm; surface area, 295 m <sup>2</sup> /g,
GLP:	/
Date of report:	2007
Published:	Yes

#### Procedure

The procedure is illustrated below (Fig. 10.):



**Fig. 10.:** Illustration of the repeated exposure of FE1 MML cells to pure media or media containing carbon black particles.

Exposure media containing particles was prepared freshly before every exposure round by sonicating the particles.

*Cytotoxicity.* The cells were tested in triplicates with 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100 µg/ml of carbon black. After 24 h, the media was removed and frozen at -80°C. The

cells were exposed in an identical setup as for the mutant frequency analysis as described below. They were however only exposed for three passages. Each of 26 dishes was seeded with 300,000 FE1 MML cells in 10 ml growth medium. After 24 h, medium with or without test substance was added. After 72 h, the medium was removed and frozen at  $-80^{\circ}\text{C}$ . Cytotoxicity was determined after each of the three 72-h passages of control incubations in media from untreated cells, from cells treated with  $75\ \mu\text{g/ml}$  carbon black. Cytotoxicity was determined on thawed media samples by determining lactate dehydrogenase (LDH) activity in the medium.

*Mutation analysis.* The carbon black ( $75\ \mu\text{g/ml}$ ) containing media were prepared as described above. The cells were incubated for 72 h, washed in PBS, trypsinized, and centrifuged. The cells were then resuspended in medium, counted and 300,000 cells were reseeded in a new dish. After 24 h, new medium with or without the test substance was added. The cells were incubated with or without the test article in a total of eight exposure rounds making the total exposure time ( $8 \times 72$ ) 576 h. The cumulative dose added was 6 mg for carbon black. Similar exposures were also performed with quartz particles (cumulative dose 8 mg specific surface area of  $2.9\ \text{m}^2/\text{g}$ , mean particle size of  $1.59\ \mu\text{m}$ ). After the fifth incubation with BaP, the positive control cells began to grow markedly slower than all other cells and only yielded 25% confluence compared with about 80% for all other samples. Therefore, they received pure medium for the remaining three exposure rounds. After the eight treatments the plates were washed thoroughly with PBS to remove the excess of particles. To further reduce the load of particles, and thereby easing mutation analysis, the cells were trypsinized and reseeded without test substance for 72 h, then harvested and stored at  $-80^{\circ}\text{C}$ .

Frozen cell pellets with 1.5–3 million cells were suspended in lysis buffer, and DNA was purified. The phage preparation was used to infect *Escherichia coli* G1250. Phages with mutations that inactivate the *cII* locus were identified by plaque formation under selective growth conditions. The total number of infective phages was determined by plaque formation under nonselective growth conditions.

All DNA used for the *lacZ* analysis was purified by phenol/chloroform extraction before the mutation analysis. Briefly, copies of the *lacZ* transgene were rescued from the FE1 MML cells using the Transpack™ lambda packing system, and the mutant frequency was determined using the P-gal-positive selection assay.

*Oxidatively damaged DNA.* The level of DNA damage measured by the Comet assay was assessed in five sets of experiments each conducted on a different day. The level of strand breaks and formamidopyrimidine [fapy]-DNA glycosylase (FPG) sensitive sites in FE1 MML cells were analyzed by single cell gel electrophoresis (Comet) assay. FPG sensitive sites were detected by incubation of the agarose-embedded nuclei with FPG protein. The level of DNA damage was determined by a visual scoring system.

## Results

For carbon black exposed cells, the mutant frequency was 1.40-fold (95% CI: 1.22–1.58) for *cII* and 1.23-fold (95% CI: 1.10–1.37) for *lacZ* compared with identically passaged untreated cells. Quartz did not significantly affect the mutant frequency. Carbon black also induced DNA strand breaks ( $P = 0.02$ ) and oxidized purines ( $P = 0.008$ ), as measured by the Comet assay. Quartz induced marginally more oxidized purines, but no change in strand breaks.

## Conclusions

The study authors concluded that carbon black was weakly mutagenic *in vitro* at the *cII* and *lacZ* loci. It also induced DNA strand breaks and oxidized DNA bases.

Ref.: Jacobsen *et al.*, 2007

Guideline/method	/
Cell line:	FE1 MML mouse epithelial cell line.
Test substance:	Carbon Black; Printex 90 (Degussa-Huels, Trostberg, Germany).
Batch:	/
PAH content:	Organic impurity content is ~1%. The observed five PAHs sum to 74.2 ng/g. The level of detection for the 16 PAH ranged between 0.3 and 2 ng/g.
Conc a.i.:	75 ug/ml
Treatment	The cells were incubated with or without carbon black in a total of eight exposure rounds making the total exposure time (8 x 72 hr) 576 hr. The cumulative dose added was 6 mg for carbon black.
Particle size:	Printex 90; particle size 14 nm; surface area, 295 m <sup>2</sup> /g,
GLP:	/
Date of report:	2011
Published:	Yes

This study represents a continuation of the study above (Jacobsen *et al.*, 2007). The purpose of the study was to determine the mutation spectrum in the *cII* gene of Printex 90 exposed cells.

#### Procedure

The procedure is in general described above by Jacobsen *et al.*, 2007.

*PCR and cII Sequencing.* The preparation of the material used for *cII* sequencing has been described previously (Jacobsen *et al.*, 2007). *cII* DNA was amplified in a 50 µl reaction using "PCR Core Reagents N808-009" together with forward 5'-AAA AAG GGC ATC AAA TTA ACC-3' and reverse primers 5'-CCG TTG AGT ATT TTT GCT G-3'. The purified DNA was dissolved in 20 µl TE-buffer, and the quality determined by OD260 on selected samples. The purified PCR products were sequenced by Macrogen (Rockville, Maryland).

The sequences were examined for mutations by two individuals. The first person aligned and compared the sequences with a reference sequence with the BioEdit program (Tom Hall, Ibis Therapeutics, CA). The second person examined each electropherogram manually. The results of the two analyses were compiled and any existing discrepancies reexamined. By this procedure, the authors estimate that the precision for determining the identity of a mutation is greater than 99%. Using the BioEdit procedure alone the precision was greater than 97%.

#### Results

A list of the types of mutations in the 1,026 samples analyzed is provided. The mutation spectrum in the Printex 90 exposed cells is significantly different from that observed in the unexposed control cultures ( $P < 0.0001$ ). More specifically, there were significant differences in the base substitutions alone, all mutations together [substitutions, deletions, and insertions], and the full data set.

A list of the position/mutations with the largest fold increase (Printex 90 exposed compared with controls) is presented. Eighty percent (40 out of 50 substitutions) of these were G:C→T:A transversions. G:C→A:T and G:C→C:G accounted for 16 and 4%, respectively. Position/mutations that were only observed in the Printex 90 data set is the most prominent A:T→T:A (31%), A:T→C:G (23%), G:C→T:A (19%), and G:C→C:G (15%).

#### Conclusion

The study authors conclude that the largest increases in base substitutions were observed in

G:C→T:A, G:C→C:G, and A:T→T:A transversion mutations; this is in keeping with a genetic finger print of ROS and is further substantiated by the observations that Printex 90 generates ROS and oxidatively damages DNA.

Ref.: Jacobsen *et al.*, 2011

### SCCS comment

Carbon black induced increased the mutant frequency at the *cII* locus and *lacZ* locus of FE1 MML epithelial cell line. The largest increases in base substitutions were observed in G:C→T:A, G:C→C:G, and A:T→T:A transversion mutations. Carbon black did also induce DNA strand breaks and oxidized purines.

### Micronucleus test in cultured Chinese hamster ovary (CHO) cells

Guideline:	OECD 487 (2010)
Cells:	Chinese hamster ovary (CHO) cells
Replicates:	Duplicate cultures
Test substance:	Carbon Black, E3000281
Batch:	C3949 of ingredient "Unipure Black LC 902"
Purity:	98%
Vehicle:	Anhydrous dimethyl sulphoxide (DMSO)
Particle size:	Diameter; 20 to 30 nm, surface area range of 200 to 260 m <sup>2</sup> /g.
Concentrations:	10, 20, and 30 µg/ml for pulse treatments in the absence and presence of S9-mix 10, 15 and 20 µg/ml for the continuous treatment in the absence of S9-mix
Treatment	3 h treatment; harvest time 24 hours after the beginning of treatment
GLP:	In compliance
Study period:	August – October 2011

#### Procedure

The test item carbon black was evaluated in a single experiment in the absence and presence of metabolic activation (S9-mix prepared from the livers of Aroclor 1254-treated rats). The highest concentration in each test condition was selected on the basis of solubility criteria since no cytotoxicity was observed with the test item. Top concentrations were then selected based on precipitation of the test item observed at harvest, and the following ranges of concentrations were selected for micronucleus analysis:

- 10, 20, and 30 µg/ml for pulse treatments in the absence and presence of S9-mix
- 10, 15 and 20 µg/ml for the continuous treatment in the absence of S9

Duplicate cultures were treated with each concentration of carbon black both in the presence and absence of S9-mix. Similarly, duplicate cultures were treated with known clastogens in the presence (cyclophosphamide, CPA) or absence of S9-mix (Mitomycin C, MMC) for pulse treatments and in the absence of S9-mix (vinblastine, VIN) for the continuous treatment. Solvent-treated cultures (DMSO, four replicates) were used as negative controls.

Cell preparations were stained and examined microscopically for determining the Replication Index and the proportion of micronucleated binucleate (MNBN) cells when selected. Two thousands binucleate cells per concentration were analyzed blind.

#### Results

When compared to concurrent solvent controls, treatment of cultures with positive controls CPA, MMC and VIN resulted in consistent and significant increases in MNBN frequencies, thus validating the sensitivity of the test system and procedure used.

Treatment of cultures with carbon black in the absence and presence of S9-mix resulted in MNBN cell incidences that were similar to those observed with vehicle control. The MNBN incidence (1.3%) obtained with a single culture at the highest concentration analyzed (20 µg/ml) following the continuous treatment in the absence of S9-mix was slightly high when compared to concurrent controls. However, since the observed value fell within the historical control range (0.2-1.3%), and since no such slightly increased values were observed for the replicate culture, this isolated finding was considered to be of no biological significance.

#### Conclusion

The study authors concluded that under the conditions of the study, carbon black did not produce micronuclei in cultured CHO cells either in the absence or presence of metabolic activation and was therefore considered to have no clastogenic or aneugenic potential.

Ref.: Lloyd, 2012

#### SCCS comment

The experiment was carried out with nanoparticle exposure conducted wholly in the presence of cytochalasin B<sup>4</sup>. Cytochalasin-B, formulated in DMSO, was added directly to all 24+0 hour cultures at the time of treatment. This version of the experiment often prevents the uptake of nanomaterials into cells because Cytochalasin-B inhibits endocytosis. Thus, the experimental set-up does not suit nanomaterials and it is possible that it was responsible for preventing the entry of the carbon black nanoparticles into the cells. Given this, the experimental design is inappropriate for truly reporting on carbon black genotoxicity. Also only a 3h exposure time was used (which is not always sufficient for the detection of genotoxicity using the micronucleus assay). Additionally, no uptake studies were performed to confirm cellular uptake with this short exposure time.

#### General comment by SCCS

Carbon black nano particles have been shown to induce single strand breaks both in cell-free studies as well as in mammalian cells. In addition, carbon black has been shown to induce mutations in an alveolar epithelial cell line. The genotoxic effects of nano carbon black *in vitro* are probably at least in part caused by primary genotoxicity. Conceptually, primary genotoxicity might operate via various mechanisms, such as the actions of ROS (e.g., as generated from reactive particle surfaces), or DNA-adduct formation by reactive metabolites of particle associated organic compounds (e.g., polycyclic aromatic hydrocarbons).

#### 3.3.6.2 Mutagenicity/Genotoxicity *in vivo*

#### Inhalation

##### **PAH – DNA adduct formation**

Guideline/method /  
 Species: F-344 rats, female  
 Age at start of study: /  
 Groups: 3 or more females

<sup>4</sup> During the commenting period the applicant has stated that Cytochalasin B was not present during the 3h-treatment but added immediately after



Test substance:	Carbon Black; Printex 90 (Degussa-Huels, Trostberg, Germany), Sterling V
Batch:	/
PAH content:	Printex 90, PAH = 0.039 ppm, Sterling V, PAH = 8.8 ppm
Conc a.i.:	/
Particle size:	Printex 90; particle diameter, 16 nm; surface area, 300 m <sup>2</sup> /g, Sterling V; particle diameter, 70 nm; surface area, 37 m <sup>2</sup> /g
Dose applied:	Exposure levels: Printex 90: 0, 1, 7, and 50 mg/m <sup>3</sup> Sterling V: 50 mg/m <sup>3</sup> all in filtered air
Route:	Inhalation
Exposure time:	6 h/day, 5 days/week for 13 weeks
GLP:	/
Date of report:	2005
Published:	Yes

#### Procedure

Female F344 rats were exposed by inhalation for 13 weeks. Subsequently DNA was extracted from whole lung DNA immediately after exposure. The lungs of the rats for DNA analysis were not lavaged, but the vascular system was perfused. DNA was extracted and used to determine oxidative DNA damage.

To study whether PAHs are available and subsequently transformed into DNA-binding metabolites, lungs of three animals from every exposure group were analyzed for DNA adducts immediately after exposure. For these samples, <sup>32</sup>P postlabeling was performed using either nuclease P1 enrichment or butanol extraction to enhance DNA adduct detection. Both procedures are suitable to detect PAH-related DNA adducts with its own specificities. BaP-diol-epoxide modified DNA adduct was used as positive standard.

#### Results

No adducts were found in DNA from lung homogenates isolated immediately after 13 weeks of inhalation of up to 50 mg/m<sup>3</sup> of Printex 90 and Sterling V, which resulted in lung burdens of 4.9 mg and 7.6 mg, respectively. Although the lung burden was significantly lower than the lung burden following a six or 24 month inhalation period, Sterling V contains at least 100-fold the amount of PAHs compared with Printex 90. Lung DNA from rats following inhalation of Carbon black showed no spots relating to PAH-DNA adduct formation compared to sham-exposed animals.

#### Conclusion

In the present study Printex 90 and Sterling V at the end of subchronic inhalation exposure of F344 female rats, did not induce DNA-PAH adduct formation in the lung (<sup>32</sup>P post-labeling analysis of lung samples). It was also shown that Sterling V induced PAH adducts after acute *in vitro* exposure of A549 cells (see Section 3.3.6.1). This was not found after similar *in vitro* exposures with Printex 90. These results indicate that DNA repair processes may have occurred *in vivo* in Sterling V exposed rats during and/or after subchronic inhalation exposure. Whether the greater PAH content of Sterling V may have contributed to the observed effects in the study cannot be firmly determined from the results.

Ref.: Borm et al., 2005

#### SCCS comment

The lack of detection of PAH-DNA adducts in the present experiment could be due to lack of penetration of the carbon black particles into the epithelial cells *in vivo*. Several studies have shown that alveolar macrophages take up nano-particles much faster than the epithelial cells. The amounts of particles in the nucleus of the alveolar cells may thus have been too small to induce detectable amounts of PAH-DNA adducts. *In vitro* experiments with

A549 lung alveolar epithelial cells have provided some evidence suggesting that, in a carbon black sample with high PAH content (Sterling V [PAH = 8.8 ppm]), some PAHs are bioavailable for biotransformation and induction of PAH-DNA adducts. A second explanation may be that such adducts have been repaired prior to the analyses.

#### Oxidative DNA damage

Guideline/method	/
Species:	F-344 rats, female
Age at start of study:	Weighing 200g – 250g
Groups:	5 females
Test substance:	Carbon Black; Printex 90 (Degussa-Huels, Trostberg, Germany) Sterling V
Batch:	/
PAH content:	Printex 90, PAH = 0.039 ppm, Sterling V, PAH = 8.8 ppm
Conc a.i.:	/
Particle size:	Printex 90; particle diameter, 14 nm; surface area, 300 m <sup>2</sup> /g, Sterling V; particle diameter, 70 nm; surface area, 37 m <sup>2</sup> /g
Exposure levels:	Printex 90: 1 (1.2± 0.2), 7 (7.1±1.8), and 50 (52.8±14.7) mg/m <sup>3</sup> and Sterling V: 50 (48.2) mg/m <sup>3</sup> all in filtered air
Route:	Inhalation
Exposure time:	6 h/day, 5 days/week for 13 weeks
GLP:	/
Date of report:	2003
Published:	Yes

#### Procedure

Female F344 rats were exposed by inhalation for 13 weeks and DNA was extracted from whole lung DNA immediately after the end of exposure and at 44-weeks post exposure. The lungs of the rats for DNA analysis were not lavaged but the vascular system was perfused. DNA was extracted and used to determine oxidative DNA damage. The formation of 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxo-dG), a mutagenic lesion of the C-8 position of 2'-deoxyguanosine (dG) residues, is a commonly used biomarker of free radical-induced oxidative DNA damage. Following incubation with the lysing solution, DNA was hydrolyzed and analyzed for 8-oxo-dG.

#### Results

Carbon black lung burdens were analyzed after the 13-week exposure and after 44 weeks of recovery. Exposures to Printex 90 carbon black at 1, 7, and 50 mg/m<sup>3</sup> for 13 weeks resulted in an increased lung burden of carbon black. The level after exposure to 50 mg/m<sup>3</sup> Sterling V was higher than after exposure to 50 mg/m<sup>3</sup> Printex 90. The lung burden of carbon black did not change significantly during the 44 week recovery period (see Table 9).

Carbon black induced 8-oxo-dG formation in rat lung was analyzed in tissues collected from the carbon black (Printex 90 and Sterling V) exposed animals. In the 13-week exposure group, a significant increase was observed in the 50 mg/m<sup>3</sup> carbon black compared to control animals. After the 44 week recovery period a significant increase was observed at both the 7 and 50 mg/m<sup>3</sup> Printex 90 exposure levels. Subchronic inhalation of 50 mg/m<sup>3</sup> of Sterling V carbon black did not elicit any detectable changes in the 8-oxo-dG levels relative to control animals. Additionally, levels of 8-oxo-dG did not show a time-related increase in oxidative DNA damage as evidenced by comparing 13- and 44-week recovery control animals (Table 9).

**Table 9.:** Carbon black lung burden and 8-oxo-dG formation

Exposure mg/m <sup>3</sup>	Time Weeks	Total lung burden (mg)	8-oxo-dG formation 8-oxo-dG/dG x 10 <sup>5</sup>
Control	13	-£	2.4±0.1
	44 (post exposure)	-£	2.2±0.5
Printex 90 1	13	0.09±0.04*	----†
	44 (post exposure)	0.03±0.02*	----†
7	13	0.97±0.11*	----†
	44 (post exposure)	0.70±0.13*	2.8±0.2*
50	13	4.87±0.07*	2.9±0.4*
	44 (post exposure)	5.73±1.32*	3.1±0.2*
Sterling V 50	13	7.65±0.77*	----†
	44 (post exposure)	6.34±0.79*	----†

\*Reported as significantly increased compared to control

† Data values not reported, but noted as not significant compared to control.

£ Data values not reported although visible in one figure and used in statistics.

To what extent the PAH or organic content of Printex 90 carbon black contributed to induction 8-oxo-dG cannot be ascertained, however, clearly owing to the low concentration of PAH associated with the Printex 90 carbon black (PAH = 0.039 ppm) relative to Sterling V carbon black (PAH = 8.8 ppm) for which no increase in 8-oxo-dG was measurable; lung particle overload conditions likely reflect the dominant contribution to the measured inflammatory effects as measured by 8-oxo-dG.

#### Conclusion

The study authors concluded that the findings suggest that prolonged, high-dose exposure to carbon black can promote oxidative DNA damage that is consistent with the hypothesis that inflammatory cell-derived oxidants may play a role in the pathogenesis of rat lung tumours following long-term high-dose exposure to carbon black in rats.

Ref.: Gallagher et al. 2003

#### SCCS comment

It is noted that the same animals used in the 13 week inhalation toxicity study by Elder et al., 2005, and Carter et al., 2006 were used in the two in vivo genotoxicity studies discussed above.

#### **DNA strand breaks (Comet assay)**

Guideline/method	/
Species:	female C57BL/6J mice and male Fischer F344 rats
Age at start of study:	8 weeks
Groups:	Rats, 6 animals Mice, 12 animals
Test substance:	Carbon Black, generated on-site in an argon atmosphere from graphite electrodes
Batch:	/
Conc a.i.:	/
Particle size:	57 nm
Impurity:	/
Conc a.i.:	/

Exposure levels:	Rats 159 µg/m <sup>3</sup> Mice 142 µg/m <sup>3</sup>
Negative control:	HEPA filtered air
Route:	Nose only inhalation
Exposure time:	Rats 4 h Mice 4 h or 3 times 4 h on consecutive days
GLP:	/
Date of report:	2011
Published:	Yes

#### Procedure

Rats and mice were exposed by nose-only inhalation to carbon black nanomaterials or HEPA filtered air. The carbon black nanomaterials were generated on-site in an argon atmosphere from graphite electrodes in an electric spark discharge generator. After the exposures, lungs were cannulated via the trachea and bronchoalveolar lavage was performed *in situ*. For the determination of oxidative DNA damage and mRNA expression analyses in the rats, their lung epithelial cells were isolated. Pulmonary inflammation and cytotoxicity were assessed by bronchoalveolar lavage analysis of mouse and rat lungs after exposure to CNP or air.

#### Results

In both rat and mice, no pulmonary inflammation and toxicity were detected by bronchoalveolar lavage or mRNA expression analyses. Oxidative DNA damage (measured by fpg-comet assay) was also not increased in mouse whole lung tissue or isolated lung epithelial cells from rat. In addition, the mRNA expressions of the DNA base excision repair genes OGG1, DNA Polb and XRCC1 were not altered. However, in the lung epithelial cells isolated from the nanoparticle-exposed rats a small but significant increase in APE-1 mRNA expression was measured.

#### Conclusion

The study authors concluded that short term inhalation of carbon nanoparticles under the applied exposure regimen, did not cause oxidative stress and DNA damage in the lungs of healthy mice and rats.

Refs: Wessels *et al.*, 2011

#### SCCS comment

Based on the experiments discussed above, the concentrations and time of exposure are probably not sufficient to give any results.

**DNA strand breaks** in rats were significantly elevated in lung cells of the mid dose HSCB (Printex 90) group during the inhalation exposure (i.e. rats sacrificed at 5 weeks into the exposure). Significant changes in lung tissue DNA strand breaks were not found in any other exposure group. In the lavage cells, however, there were significant elevations in DNA single strand breaks in the high dose HSCB and LSCB (Sterling V) groups in the recovery phase of the experiment. Significant elevations in the percentage of lung cells with DNA strand breaks were also found for mice exposed to high dose HSCB at the end of the recovery period. No significant changes in strand breaks in lung tissue cells were found in hamsters.

Ref.: ECHA, 2003d

In an inhalation experiment with pregnant mice, the authors reported that carbon black did not affect the level of DNA strand breaks in BAL fluid cells 5 and 24 days after exposure. However, the exposure to carbon black induced higher levels of DNA strand breaks in the liver 5 and 24 days after exposure compared to their controls (5 days: 1.3-fold increase, p

= 0.04; 24 days: 1.6-fold increase,  $p < 0.001$ ). No increase in the level of DNA strand breaks was observed on the liver of the mated mice exposed by intratracheal instillation.

In the offspring of the mice exposed to carbon black by inhalation, the level of single strand DNA strand breaks was higher in the liver at weaning and in adolescents, compared to their controls (weaning: 1.4-fold increase,  $p = 0.001$ ; adolescents: 1.5-fold increase,  $p = 0.011$ ). The level of DNA strand breaks in offspring of mice exposed to carbon black by intratracheal instillation was generally higher in liver cells from newborns, compared to tissues from older siblings at later time points ( $p < 0.001$ ).

Ref.: Jackson et al., 2012a (taken from section **3.3.8 Reproductive toxicity**)

### ***Mutation in the hprt gene***

Guideline/method	/
Species:	Fischer 344 rats, male
Age at start of study:	/
Groups:	Not specified, at least 2 animals per dose/time point
Test substance:	Carbon Black; Monarch 880 (Cabot, Cambridge,MA).
Batch:	/
Conc a.i.:	/
Particle size:	Monarch 880, particle diameter, 16 nm; surface area, 220 m <sup>2</sup> /g
Impurity:	/
Conc a.i.:	/
Exposure levels:	0 (control), 1.1±0.2, 7.1±1.8), and 52.8±14.7 mg/m <sup>3</sup> .
Route:	Inhalation.
Exposure time:	6 hr/day, 5 days/week for 13 weeks and 3 and 8 months of recovery.
GLP:	/
Date of report:	1996
Published:	Yes

### Procedure

Male Fischer 344 rats, weighing between 200 and 250 g were exposed 6 hr/day, 5 days/week for 13 weeks to air or for aerosols of carbon black (Monarch 880, particle diameter, 16 nm; surface area, 220 m<sup>2</sup>/g; Cabot, Cambridge,MA). The mean aerosol concentrations for the low, medium, and high exposure groups were 1.1±0.2, 7.1±1.8), and 52.8±14.7 mg/m<sup>3</sup>. The mass median aerodynamic diameter was 0.88 µm.

The effects on the lung were characterized after 6.5 and 13 weeks of exposure and 3 and 8 months of recovery. Endpoints characterized after carbon black exposure included mutation in the *hprt* gene of alveolar epithelial cells, changes in bronchoalveolar lavage fluid markers of lung injury and inflammation, expression of mRNA for the chemokines, MIP-2 and MCP-1, and lung histopathology. Lung burdens of carbon black were also determined.

### Results

After 13 weeks of exposure to 1.1, 7.1, and 52.8 mg/m<sup>3</sup> carbon black, lung burdens were 354, 1826, and 7861 mg carbon black, respectively. The lung clearance of carbon black appeared impaired after exposure to 7.1 and 52.8 mg/m<sup>3</sup>. Lung tissue injury and inflammation, increased chemokine expression, epithelial hyperplasia, and pulmonary fibrosis were observed after exposure to 7.1 and 52.8 mg/m<sup>3</sup> carbon black, with the effects being more pronounced at the higher exposure level. Subchronic inhalation of 1.1 mg/m<sup>3</sup> carbon black did not elicit any detectable adverse lung effects.

A significant increase in *hprt* mutant frequency in alveolar epithelial cells was detected immediately after 13 weeks of exposure to 7.1 and 52.8 mg/m<sup>3</sup> carbon black as well as after 3- and 8-month recovery periods for the group exposed to 52.8 mg/m<sup>3</sup>. No increase in *hprt* mutant frequency was observed for epithelial cells obtained from rats exposed to 1.1 mg/m<sup>3</sup> carbon black.

#### Conclusion

The study authors concluded that the observation of mutagenic effects only after carbon black exposures which produced inflammation and epithelial hyperplasia, was consistent with a mechanism of carbon black induced mutagenesis and, potentially, rat lung tumours which is dependent on inflammatory and proliferative responses.

Ref.: Driscoll *et al.*, 1996

Guideline/method	/
Species:	Female F-344 rats, female B6C3F1 mice, and female F1B Syrian golden hamsters
Age at start of study:	7 weeks
Groups:	5 females
Test substance:	Carbon Black; Printex 90 (Degussa-Huels, Trostberg, Germany)
Batch:	/
PAH content:	0.039 mg/kg for Printex 90
Conc a.i.:	/
Particle size:	The particles had a primary particle size of 14 nm with a particle surface area of 300 m <sup>2</sup> /g
Aerodynamic diameters:	Ranged during exposure from 1.2 to 1.6 µm (GSD = 2.2 - 2.8)
Dose applied:	Exposure levels, 0, 1, 7, and 50 mg/m <sup>3</sup> in filtered air
Route:	Inhalation
Exposure time:	6 h/day, 5 days/week for 13 weeks
GLP:	/
Date of report:	2006
Published:	Yes

#### Procedure

This study investigated pro- and anti-inflammatory mediators underlying species specific mechanisms in particle-induced lung inflammation using *ex vivo* mutational analysis of inflammatory cells co-incubated with lung epithelial cells.

Female F-344 rats, female B6C3F1 mice, and female F1B Syrian golden hamsters were exposed 6 hours/day 5 days/week for 13 weeks. The carbon black used during this study was Printex 90 particles, which have a primary size of 14 nm with a surface area of 300 m<sup>2</sup>/g; the aerosol aerodynamic diameters ranged during exposure from 1.2 to 1.6 µm (GSD = 2.2–2.8).

Bronchoalveolar lavage (BAL) fluid was used for cellular and biochemical analysis (superoxide, hydrogen peroxide, nitric oxide, TNF-alpha, macrophage inflammatory protein-2 (MIP-2), IL-10); superoxide dismutase, glutathione reductase, and glutathione peroxidase levels in BAL fluid and lung tissue, gamma-glutamylcysteine synthetase and manganese superoxide dismutase mRNA expression, *ex vivo* mutagenic activity of particle-elicited inflammatory cells on epithelial cells.

Cells obtained from BAL of animals after air or particle exposures were evaluated for their ability to cause mutations in rat alveolar epithelial cells *in vitro*. RLE-6TN cells, an alveolar epithelial cell line that retains characteristics of rat alveolar type II cells (ATCC-CRL 2300), were used as *in vitro* targets to assess the mutagenic activity of particle elicited BAL cells. At the end of the exposures the dishes were fed every 2 to 3 days with Ham's F-12 medium



without hypoxanthine supplemented with BPE, FBS, IGF, insulin, EGF, and 40  $\mu\text{M}$  6-thioguanine (6TG) to select for mutation in the *hprt* gene. After 14 days in culture, the cells were fixed and stained for cytokeratin and 6TG-resistant colonies were counted. Mutation frequencies were calculated as:  $(\text{no. colonies/treatment})/(\text{plating efficiency})/(10^6) = \text{mutants}/10^6 \text{ cells}$ .

## Results

*Percent of neutrophils* (PMNs) in lavage fluid was used as an indicator of lung inflammation. PMNs were elevated in the mid- and high-dose groups in rats through 3 months of recovery. PMNs remained elevated in rats in the high-dose group through the end of the study. At the end of exposure in mice and hamsters, the mid- and high-dose groups were also elevated compared with the controls and the low-dose group; the magnitudes of response were similar for the two species.

*Superoxide dismutase (SOD)* is an important indicator of antioxidant capacity in BALF and lung tissue. For SOD, all three species responded in a dose and time-related manner. The *rat* showed the least responsiveness to particle-induced inflammation, with basal levels being lowest in this species. In addition, levels of SOD activity continued to increase in the rat over the course of the study, unlike the *mouse* and *hamster*, which decreased in activity in the high-dose group over the course of the study. These data are consistent with the cytokine data, suggesting that SOD activity is responsive to the inflammatory effects related to carbon black. Overall, the levels of SOD activity were as follows: hamster > mouse > rat.

For *hydrogen peroxide* production by BAL cells, increased levels were observed in all three species at the high dose for all time points. Only the rat and mouse had significant increases at the mid-dose level, which returned to basal levels by 11 months post exposure. Hamster levels of hydrogen peroxide were significantly lower than the rat.

The *hprt* assay was used to characterize the mutagenic potential from the release of reactive oxygen and nitrogen species by particle elicited inflammatory cells (BAL cells). The hamster BAL cells did not significantly increase the mutant frequency in the *hprt* gene for any dose at any time point. Both the rat and mouse showed dose-related effects in the *hprt* mutant frequency. Although the mouse response significantly decreased by the 11-month post exposure time point, the rat response was sustained throughout the course of the study. These findings are similar to those observed for the percent PMN in BALF.

## Conclusion

The study authors concluded that the *hprt* mutant frequency of BAL cells from rats, mice and hamster exposed to 1  $\text{mg}/\text{m}^3$  was not increased; significant increases were found at 7  $\text{mg}/\text{m}^3$  (rat, mouse) and 50  $\text{mg}/\text{m}^3$  (rat, mouse). Hamster BAL cells did not significantly increase the mutant frequency in the *hprt* gene for any dose at any time point. Overall, the rat produced significantly higher levels of reactive oxygen species than either the mouse or the hamster, with the hamster producing the lowest amount.

Ref.: Carter *et al.*, 2006

## SCCS comment

It is noted that the same animals as used in the 13 week inhalation toxicity study by Elder *et al.*, 2005, and Carter *et al.*, 2006 were also used in two in vivo genotoxicity studies discussed above (Born *et al.*, 2005, Gallagher *et al.*, 2003) as well as in the study above.

## Intratracheal instillation

### **DNA strand breaks (Comet assay)**

Guideline/method	/
Species:	Female wild-type C57BL/6 (WT) and C57BL/6-Apoe <sup>tm1</sup> (ApoE <sup>-/-</sup> ).
Age at start of study:	8 weeks
Groups:	5 – 15 females
Test substance:	Carbon Black; Printex 90 (Degussa-Huels, Trostberg, Germany). SWCNT, Single-Walled Carbon Nanotubes C60, Fullerenes QD, Quantum dots Gold nano materials
Batch:	/
Conc a.i.:	/
Particle size:	Printex 90 (primary particle size 14 nm, surface area 338 m <sup>2</sup> /g, SWCNT, Single-Walled Carbon Nanotubes (primary particle size was 0.9–1.7 nm as diameter and ≤1 µm as length, surface area 731 m <sup>2</sup> /g, C60, Fullerenes (primary particle size was 0.7 nm) Gold (2 nm)
Impurity:	Printex 90, >99% pure, PAH content 75 ng/g, SWCNT, ~95% pure, PAH content 417 ng/g, C60, 99.9% pure, PAH content 0 ng/g
Conc a.i.:	/
Exposure levels:	0 (vehicle control), 0.018, or 0.054 mg 0 (clean air), 60 mg/m <sup>3</sup>
Route:	A single intratracheal instillation or inhalation
Exposure time:	Instillation: 3 and 24 h Inhalation 30 and 90 minutes
GLP:	/
Date of report:	2009
Published:	Yes

#### Procedure

The Carbon black, Printex 90 (primary particle size 14 nm, surface area 338 m<sup>2</sup>/g, >99% pure, PAH content 75 ng/g), SWCNT, Single-Walled Carbon Nanotubes (primary particle size was 0.9–1.7 nm as diameter and ≤1 µm as length, surface area 731 m<sup>2</sup>/g, 95% pure, PAH content 417 ng/g), C60, Fullerenes (primary particle size was 0.7 nm, surface area >20 m<sup>2</sup>/g, 99.9% pure, PAH content 0 ng/g), gold (2 nm) and QD, Quantum dots particles. Changes in % tail DNA in the Comet assay was used to assess DNA damage.

Female wild-type C57BL/6 (WT) and C57BL/6-Apoe<sup>tm1</sup> (ApoE<sup>-/-</sup>) mice aged 4–6 weeks. The mice were 8 weeks old at the time of the experiment. Pulmonary toxicity and DNA damage of the nano materials were studied. Moreover, the toxicity after intratracheal instillation and inhalation were compared.

#### Results

Intratracheal instillation of Printex 90 caused far more pulmonary toxicity in ApoE<sup>-/-</sup> mice than in C57 mice. In general, the exposure to Printex 90 by instillation resulted in 10 to 26-fold induction of the cytokine mRNA levels at the early time point in ApoE<sup>-/-</sup>, whereas WT mice only showed between 1.5 to 2.1-fold induction compared to their controls. Following 24 h the induction was 26- to 40-fold among ApoE<sup>-/-</sup> mice and 10 to 11-fold in C57 mice.

Instillation of Printex 90 was more toxic than inhalation of a presumed similar dose with respect to inflammation in the lungs of ApoE<sup>-/-</sup> mice.

The effects of instillation in ApoE<sup>-/-</sup> mice of three carbonaceous particles; Printex 90, fullerenes C60 (C60) and single walled carbon nanotubes (SWCNT) as well as gold particles and quantum dots (QDs) were studied. Characterization of the instillation media revealed that all particles were delivered as agglomerates and aggregates. Significant increases in *Il-6*, *Mip-2* and *Mcp-1* mRNA were detected in lung tissue, 3 h and 24 h following instillation of SWCNT, Printex 90 and QDs. DNA damage in BAL cells, the fraction of neutrophils in BAL cells and protein in BAL fluid increased statistically significantly. Gold and C60 particles caused much weaker inflammatory responses.

The Comet assay is considered to be a sensitive assay for detection of single strand DNA breaks. The DNA damage increased significantly following exposure to SWCNT, Printex 90 and quantum dots (QD), but only marginally with fullerenes C60 and gold nanoparticles. QDs were also much more genotoxic than the other particles with a more than 3-fold increase in the level of DNA damage. The high potency of QD may be a result of severe oxidative stress induced by cadmium leaked from the QDs.

#### Conclusion

The study authors concluded that the data suggest that ApoE<sup>-/-</sup> mice model is more sensitive than the C57 mice model for evaluating particle induced inflammation. Overall QDs had greatest effects followed by Printex 90 and SWCNT with C60 and gold being least inflammatory and DNA-damaging. The strong effects of QDs were likely due to Cd release. The surface area of the instilled dose correlated well with the inflammatory response for low toxicity particles.

Ref.: Jacobsen *et al.*, 2009

Guideline/method	/
Species:	C57BL/6 mice, female
Age at start of study:	8 weeks
Groups:	6 females
Test substance:	Carbon Black; Printex 90 (Degussa-Huels,Trostberg, Germany).
Batch:	/
Conc a.i.:	/
Particle size:	Printex 90; particle size 14 nm; surface area, 295 m <sup>2</sup> /g,PAH
Impurity:	Organic impurity content: ~1%. The observed five PAHs sum to 74.2 ng/g. The level of detection for the 16 PAH ranged between 0.3 and 2 ng/g.
Conc a.i.:	/
Exposure levels:	0 (vehicle control) 0.018, 0.054 or 0.162 mg of Printex 90
Route:	A single intratracheal instillation.
Exposure time:	1, 3 and 28 days
GLP:	/
Date of report:	2012
Published:	Yes

#### Procedure

Female C57BL/6 mice aged 5-6 weeks were acclimatized for 2-3 weeks before the experiment and were 8 weeks of age at the start of the study. Printex 90 was suspended in 0.9% NaCl containing 10% v/v acellular BAL from C57BL/6 mice. The particle suspensions (4.05 mg/ml) were sonicated.

The mice received a single intratracheal instillation of 0.018, 0.054 or 0.162 mg of Printex 90. Each group consisted of 6 female mice. Vehicle control solutions were prepared containing 90% 0.9% NaCl and 10% acellular BAL fluid.

One, 3 and 28 days after the instillation, the mice were anaesthetized. Immediately after withdrawing the heart blood, a bronchoalveolar lavage (BAL) was performed four times with 0.8 ml of 0.9% sterile saline through the trachea. The BAL cells were separated by centrifugation at 4°C. The BAL cells were resuspended and stored at -80°C for later analysis in the Comet assay. For differential count, cells from 50 µl were collected. The cellular composition of BAL cells was determined on 200 cells.

*DNA damage measured by the Comet assay.* Whole lung and liver were broken into specific pieces under liquid nitrogen, homogenized and filtered at 70 µm to yield individual cells. Two different Comet methods were used to process the BAL cells/liver tissue and the lung tissue samples. DNA strand breaks in liver and BAL cells were analyzed using a high throughput protocol allowing 48 samples per Gel-Bond® film. The BAL cells were quickly thawed in a 37°C water bath before being mixed with agarose. The freezing and thawing of the cells was validated not to have any effect on experimental outcome in a cell line and in primary lymphocytes. In the present experiment, the cell/agarose suspension was applied onto a GelBond(R) film with a multichannel pipette. Eight films were processed per electrophoresis, in two parallel electrophoresis tanks. Due to preparation time, the lysing procedure varied between 1-2 hours for samples in the present study (up to 3.5 hours). DNA damage was measured as Comet tail length (TL). DNA strand break in whole lung were measured as the formation of strand break and formamidopyrimidine DNA glycosylase (FPG) sensitive sites. The latter were assessed as an indicator of oxidative damaged DNA. Pulmonary and hepatic acute phase response was evaluated by *Saa3* mRNA real-time quantitative PCR.

## Result

Inflammation was strongest on 1 and 3 days post-exposure, and remained elevated for the two highest doses (i.e., 0.054 and 0.162 mg) 28 days post-exposure ( $P < 0.001$ ). DNA strand breaks were detected in lung at all doses on post-exposure day 1 ( $P < 0.001$ ) and remained elevated at the two highest doses until day 28 ( $P < 0.05$ ). BAL cell DNA strand breaks were elevated relative to controls at least at the highest dose on all post-exposure days ( $P < 0.05$ ). The level of FPG sensitive sites in lung was increased throughout with significant increases occurring on post-exposure days 1 and 3, in comparison to controls ( $P < 0.001-0.05$ ). DNA strand breaks in liver were detected on post-exposure days 1 ( $P < 0.001$ ) and 28 ( $P < 0.001$ ). Polymorphonuclear (PMN) cell counts in BAL correlated strongly with FPG sensitive sites in lung ( $r = 0.88$ ,  $P < 0.001$ ), whereas no such correlation was observed with DNA strand breaks ( $r = 0.52$ ,  $P = 0.08$ ). Printex 90 particles increased the expression of *Saa3* mRNA in lung tissue on day 1 (all doses), 3 (all doses) and 28 (0.054 and 0.162 mg), but not in liver.

## Conclusion

The study authors concluded that deposition of carbon black nanoparticles in lung induces inflammatory and genotoxic effects in mouse lung that persist considerably after the initial exposure. The results demonstrate that carbon black nanoparticles may cause genotoxicity both in the primary exposed tissue, lung and BAL cells, and in a secondary tissue, the liver.

Ref.: Bourdon *et al.*, 2012

Guideline/method	/
Species:	C57BL/6 mice, female
Age at start of study:	8 weeks
Groups:	6 females
Test substance:	Carbon Black; Printex 90 (Degussa-Huels, Trostberg, Germany). Lampblack 101
Batch:	/
Conc a.i.:	/

Particle size:	Printex 90; particle size 14 nm; surface area, 295 m <sup>2</sup> /g, PAH Lampblack 101; particle size 95 nm; surface area, 23.8 m <sup>2</sup>
Impurity:	Organic impurity content: ~1%. The observed five PAHs sum to 74.2 ng/g. The level of detection for the 16 PAH ranged between 0.3 and 2 ng/g.
Conc a.i.:	/
Exposure levels:	0 (vehicle control) 0.054 mg of Printex 90 or Lampblack 100
Route:	A single intratracheal instillation.
Exposure time:	1day
GLP:	/
Date of report:	2012
Published:	Yes

### Procedure

The study involved three titanium dioxide-based (two coated rutile; one uncoated anatase), one kaolinite clay, two silica products, and two carbon blacks (Lampblack 101, and Printex 90). Only the results with the two carbon blacks will be discussed. The procedure used is similar to that described above (Bourdon *et al.*, 2012) with the following exceptions. Only one dose was used (0.054 mg) with only one exposure time (24 h)

### Results

Exposure to Printex 90 resulted in a 2- to 3-fold increase in the total number of bronchioalveolar lavage (BAL) cells compared to vehicle exposed mice ( $p < 0.001$ ). Lampblack 101 particles did not significantly affect the total number of BAL cells.

*DNA damage.* DNA strand breaks were measured in BAL cells by the Comet assay (by tail length as compared to vehicle exposed mice). Neither Printex 90 nor Lampblack 101 induced DNA breaks in BAL cells.

### Conclusion

The study authors point out that they have previously published studies showing increased DNA damage in mice exposed to Printex 90 after 1 and 3 h (Saber *et al.* 2005; Jacobsen *et al.* 2009). Thus, the effect of Printex 90 may be transient and it is probable that the most DNA damage was repaired after 24 h.

Ref.: Saber *et al.*, 2012

### SCCS comment

The above results are in agreement with the results above (Bourdon *et al.*, 2012) where DNA strand breaks in BAL cells were only observed at a higher dose (0.162 mg, Printex 90) while DNA breaks in lung tissue were found even with 0.018 mg carbon black. In the article by Jacobsen *et al.* (2009) DNA strand breaks was found after 3 h with 0.054 mg Printex 90, while it is not clear from the study if DNA strand breaks were found after 24 h exposure with 0.054 mg carbon black.

### General discussion by SCCS

Two principle modes of genotoxic action can be considered for carbon black nanomaterials, generally referred to as primary and secondary genotoxicity. Primary genotoxicity is defined as genetic damage elicited by particles in the absence of pulmonary inflammation, whereas secondary genotoxicity implies a pathway of genetic damage resulting from the oxidative DNA attack by reactive oxygen/nitrogen species (ROS/RNS), generated during particle-elicited inflammation (Schins and Knaapen, 2007, Doak *et al.*, 2012; Magdolenova *et al.*, 2014).

Distinguishing between the production of ROS as a primary indirect DNA damage mechanism of the nanoparticles themselves and its generation as part of a secondary inflammatory reaction is difficult but can be achieved through manipulation of testing protocols. The former can only be detected in *in vitro* culture systems utilizing non-immune cell types. To detect oxidative stress induced as part of a secondary inflammatory response, *in vivo* testing would be required to establish if chronic inflammation is induced following exposure to the nanoparticles, together with simultaneous detection of elevated oxidative stress and associated DNA lesions. This would not completely rule out primary mechanisms for genotoxicity through indirect routes for the generation of oxidative stress, but would be a strong indicator for the role of a secondary inflammatory genotoxicity mechanism.

*In vivo* studies show that carbon black nanomaterials after inhalation or intratracheal instillations induce 8-oxo-dG formation and single strand DNA breaks in lungs of rats, and increase in *hprt* mutant frequency in both mice and rats. Since inflammation seems to be involved in most of these effects they may be due to a secondary effect caused by reactive oxygen species (ROS). However, the findings of DNA strand breaks in the liver of mice, exposed to carbon black by intratracheal instillations, and of mothers and their offspring upon maternal inhalation exposure (see *Reproductive toxicity and the citations of the studies in this section*) suggest that carbon black may induce genotoxic effects also by a primary genotoxic effects. This view is further supported by the *in vitro* studies of carbon black in both cell free systems and in non-immune cell types.

### 3.3.7. Carcinogenicity

#### 3.3.7.1 Long term rodent carcinogenicity studies

##### Oral

##### Mice

Guideline/method	/
Species:	CF-1 female weanling mice
Age at start of study:	Female weanling mice acclimatized for 2 weeks
Groups:	31 and 28 females
Test substance:	Carbon Black (ASTM N375)
Batch:	/
BaP content:	3.81 mg/kg
Conc a.i.:	/
Particle size:	Typical average primary particle size 20 – 25 nm. Surface area of 101 m <sup>2</sup> /g
Aerodynamic diameters:	/
Dose applied:	2.05 g per kg of ground lab chow diet. Average consumption of 100 g/kg bw/year
Route:	Oral, added to the feed
Exposure time:	2 years
GLP:	/
Date of report:	1985
Published:	Yes

Two groups of 31 and 28 female weanling CF1 mice were fed 0 (controls) or 2.05 g carbon black/kg diet (approximately 273 mg/kg bw/day) furnace Carbon Black (ASTM N375) for 2 years.



At necropsy, all tissues were examined for gross pathology. Only tissues that had macroscopically visible lesions were examined histologically. Survival at 2 years was similar in treated mice (84%) and in controls (71%). No increase in tumour incidence was observed.

Ref.: Pence and Buddingh, 1985.

### SCCS comment

The experiment is incompletely described.

#### Rats

Guideline/method	/
Species:	Sprague-Dawley female weanling rats
Age at start of study:	Female weanling rats acclimatized for 2 weeks
Groups:	29 females
Test substance:	Carbon Black (ASTM N375)
Batch:	/
BaP content:	3.81 mg/kg
Conc a.i.:	/
Particle size:	Typical average primary particle size 20 – 25 nm. Surface area of 101 m <sup>2</sup> /g
Aerodynamic diameters:	/
Dose applied:	2.05 g per kg of ground lab chow diet. Average consumption of 38 g/kg bw/year
Route:	Oral, added to the feed
Exposure time:	2 years
GLP:	/
Date of report:	1985
Published:	Yes

Two groups of 29 female weanling Sprague-Dawley rats were fed 0 (controls) or 2.05 g/kg diet (approximately 104 mg/kg bw/day) furnace Carbon Black (ASTM N375) for 2 years.

At necropsy, all tissues were examined for gross pathology. Only tissues that had macroscopically visible lesions were examined histologically. Survival at 2 years was similar in controls (45%) and treated animals (38%). No increase in tumour incidence was observed.

Ref.: Pence and Buddingh, 1985.

### SCCS comment

The experiment is incompletely described.

Guideline/method	/
Species:	Sprague-Dawley female weanling rats, 4 weeks old
Age at start of study:	6 weeks
Groups:	25 and 23 females
Test substance:	Carbon Black (ASTM N375)
Batch:	/
BaP content:	3.81 mg/kg
Conc a.i.:	/

Particle size:	Typical average primary particle size 20 – 25 nm. Surface area of 101 m <sup>2</sup> /g
Aerodynamic diameters:	/
Dose applied:	2.05 g per kg of ground lab chow diet with 20% corn oil. Average consumption of 24 g/kg bw/year
Route:	Oral, added to the feed
Exposure time:	1 year
GLP:	/
Date of report:	1987
Published:	Yes

The study examined the effect of carbon black ingestion within the context of a high fat diet, formulated to simulate the typical diet of western industrialized nations. Corn oil was added to ground commercial chow at 20% by weight and carbon black added at 2.05 g/kg diet and fed for 52 weeks to female Sprague-Dawley rats. Colon tumours were induced with 16 weekly injections of 1,2-dimethylhydrazine (DMH, 10 mg/kg body weight). The study involved 4 groups. Group 1, exposed to carbon black and DMH (25 animals, survival 64%), group 2 exposed to carbon black (25 animals, survival 100%), group 3 exposed to DMH (25 animal, survival 80%), and group 4, control no exposure (23 animals, survival 96%). The rats were maintained on the test diets for 52 weeks. At the end of this period all surviving animals were necropsied and examined for intestinal tumours and other lesions. At necropsy, all tissues were examined for gross pathology. Only tissues that had macroscopically visible lesions were examined histologically.

Tumour incidences in DMH-treated rats ingesting carbon black (group 1) were significantly ( $P < 0.05$ ) higher than in those with no carbon black added to the diet (group 3) (76% vs. 60%, tumour per rat  $1.50 \pm 0.31$  vs  $1.04 \pm 0.27$ ). No tumours were found in the group which only received carbon black (group 2) and the control group with no exposure (group 4).

The study authors concluded that the findings may implicate carbon black ingestion as a co-carcinogen for industrial workers when acting in synergism with high fat diets and other unknown colon carcinogens.

Ref.: Pence and Buddingh, 1987.

### SCCS comment

The experiment lasted only for 1 year. The result suggests that carbon black may enhance the carcinogenic effect of 1,2-dimethylhydrazine in induction of colon tumours.

### Inhalation

#### Mice

Guideline/method	Primarily as Guideline OECD Guideline 451
Species:	Female NMRI mice
Age at start of study:	7 weeks
Groups:	80 females
Test substance:	Carbon Black (Printex 90)
Batch:	/
Organic content:	0.04% organic mass could be extracted. PAH was less than 5 µg/kg and BaP was 0.6 µg/kg
Conc a.i.:	/
Particle size:	14 nm. Surface area of $227 \pm 18.8$ m <sup>2</sup> /g
Aerodynamic diameters:	Median aerodynamic diameter about in the exposure chambers, 0.64 µm.

Dose applied:	7.4 mg/m <sup>3</sup> for 4 months, followed by 12.2 mg/m <sup>3</sup> for 9.5 months, Mean concentration 10.8 mg/m <sup>3</sup> . The mice were kept in clean air for the remaining 9.5 months of the study.
Route:	Inhalation
Exposure time:	18 h/day, 5 days/wk for up to 13.5 months
GLP:	/
Date of report:	1995
Published:	Yes

#### Procedure

Groups of 80 female NMRI mice, 7 weeks of age, were exposed to carbon black (Printex 90; primary particle size, 14 nm; specific surface area, 227±18.8 m<sup>2</sup>/g; MMAD of particles in the exposure chambers, 0.64 µm). The extractable organic mass of the carbon black was 0.04%; the content of benzo[*a*]pyrene was 0.6 pg/mg and that of 1-nitropyrene was <0.5 ng/mg particle mass). The animals were exposed in whole-body exposure chambers for 18 hours per day on 5 days per week to 7.4 mg/m<sup>3</sup> carbon black for 4 months followed by 12.2 mg/m<sup>3</sup> for 9.5 months. After exposure, the mice were kept in clean air for further 9.5 months. A control group was exposed to clean air throughout the study. All animals were used in the carcinogenicity assessment. Serial histopathology was performed on the nasal and paranasal cavities, larynx, trachea and lungs on 40 mice while the other 40 mice were used for serial particle mass measurements of the lung. The experiment was terminated after 23 months.

#### Results

After 11 months and up to 17 months, body weights were significantly lower (5–7%) in the carbon black-exposed mice compared with controls. During the last months, no difference in body weight was observed between the groups. After 13.5 months, mortality was 20% in the carbon black-exposed mice and 10% in controls; 50% mortality was reached after 19 months in the carbon black-exposed group and after 20 months in the control group. In exposed mice, the lung particle burden was 0.8, 2.3 and 7.4 mg carbon black per lung after 3, 6 and 12 months, respectively; at 12 months, this corresponded to a lung particle burden of 37 mg/g clean air control lung (wet weight of control lung, 0.2 g).

Tumours were only observed in the lung, but no statistical difference was observed between experimental and control animals; 11.3% (9/80) of carbon black exposed mice had adenomas and 10% (8/80) had adenocarcinomas compared with 25% (20/80) and 15.4% (12/80) of controls, respectively.

Ref.: Heinrich *et al.*, 1995

#### Rat

Guideline/method	Primarily as Guideline OECD Guideline 451
Species:	Female Wistar rats
Age at start of study:	7 weeks
Groups:	72 females
Test substance:	Carbon Black (Printex 90)
Batch:	/
Organic content:	0.04% organic mass could be extracted. PAH was less than 5 µg/kg and BaP was 0.6 µg/kg
Conc a.i.:	/
Particle size:	15 nm. Surface area of 230 m <sup>2</sup> /g
Aerodynamic diameters:	Median aerodynamic diameter about in the exposure chambers, 1.1 µm.
Dose applied:	6.0 mg/m <sup>3</sup> .
Route:	Inhalation

Exposure time: 17 h/day, 5 days/week. One group exposed for 43 weeks and kept an additional 86 weeks in clean air, the other group exposed for 86 weeks and kept an additional 43 weeks in clean air.

GLP: /

Date of report: 1994

Published: Yes

#### Procedure

Two groups of 72 female Wistar rats, 7 weeks of age, were exposed by inhalation for 17 hours per day on 5 days per week to 6 mg/m<sup>3</sup> carbon black (Printex 90; 0.04% extractable mass of organics; benzo[a]pyrene content, 0.6 pg/mg Carbon Black; 1-nitropyrene content, <0.5 pg/mg Carbon Black; primary particle size, 15 nm; MMAD of particles in the exposure chamber, 1.1 µm; specific surface area, 230 m<sup>2</sup>/g). One of these groups was exposed for 43 weeks and kept for an additional 86 weeks in clean air and the other group was exposed for 86 weeks and housed in clean air for an additional 43 weeks. Two clean-air control groups of 72 animals were kept for 129 weeks. The respiratory tract of all animals was examined histopathologically.

#### Results

No tumour was observed in the clean-air controls. The 43-week exposure group had a lung tumour rate of 18% [13/72] (two bronchiolar/alveolar adenomas, seven benign CKSC tumours, four bronchiolar/alveolar adenocarcinomas and one squamous-cell carcinoma). The 86-week exposure group had a lung tumour rate of 8% [6/72] (one bronchiolar/alveolar adenoma, four benign CKSC tumours and one squamous-cell carcinoma). In addition to the six tumours, six other rats in the latter group developed lung lesions that were borderline between non-neoplastic and neoplastic (described as marked hyperplasia or marked squamous-cell proliferation). The difference in the tumour rates of the two exposed groups was not statistically significant.

Ref.: Dungworth *et al.*, 1994; Heinrich *et al.*, 1994.

#### SCCS note on CKSC

A lesion that is frequently seen in treated rats has been described variously as 'proliferating squamous cyst', 'proliferative keratinizing cyst', 'proliferating squamous epithelioma', 'benign cystic keratinizing squamous-cell tumour' or 'cystic keratinizing squamous-cell (CKSC) tumour'. Many authors have included this lesion in tumour counts, but the neoplastic nature of this lesion has been debated

Guideline/method: Primarily as Guideline OECD Guideline 451

Species: Female Wistar rats

Age at start of study: 7 weeks

Groups: 100 females were exposed, 220 rats exposed to clean air (controls)

Test substance: Carbon Black (Printex 90)

Batch: /

Organic content: 0.04% organic mass could be extracted. PAH was less than 5 µg/kg and BaP was 0.6 µg/kg

Conc a.i.: /

Particle size: 14 nm. Surface area of 227 ± 18.8 m<sup>2</sup>/g

Aerodynamic diameters: Median aerodynamic diameter about in the exposure chambers, 0.64 µm.

Dose applied: 7.4 mg/m<sup>3</sup> for 4 months followed by 12.2 mg/m<sup>3</sup> for 20 months

Route: Inhalation

Exposure time: 18 h/day, 5 days/week for 24 months. The rats were kept in clean air for further 6 months.

Positive control: Rats exposed to diesel exhaust.

GLP: /  
 Date of report: 1995  
 Published: Yes

#### Procedure

A group of 100 female Wistar rats, 7 weeks of age, was exposed to high-purity furnace carbon black (Printex 90; particle size 14 nm; specific surface area,  $227 \pm 18.8 \text{ m}^2/\text{g}$ ; MMAD of particles in the exposure chamber,  $0.64 \text{ }\mu\text{m}$ ). The extractable organic mass of the furnace black was 0.04%; the content of benzo[*a*]pyrene was 0.6 pg/mg and that of 1-nitropyrene was  $<0.5 \text{ ng/mg}$  particle mass. Rats were exposed in whole-body exposure chambers for 18 hours per day on 5 days per week to  $7.4 \text{ mg/m}^3$  carbon black for 4 months followed by  $12.2 \text{ mg/m}^3$  for 20 months. Dose selection rationale: the doses were chosen to obtain a similar particle lung load as in rats exposed to  $7.0 \text{ mg/m}^3$  diesel exhaust. After exposure, the rats were kept in clean air for further 6 months. Controls ( $n=220$ ) were exposed to clean air throughout the study. Eight groups of 9–21 rats (interim sacrifice groups) were also exposed to carbon black or clean air for 6, 12, 18 or 24 months. Histopathology was performed on the nasal and paranasal cavities, larynx, trachea and lung.

#### Results

Mortality in the carbon black exposed group was 56% after 24 months of exposure and 92% after 30 months. In the clean air group, mortality was 42% after 24 months and 85% after 30 months. Compared with the controls, the mean lifespan of the treated rats was significantly reduced. Mean body weights were significantly lower from day 300 to the end of exposure (carbon black-exposed, 325 g; control, 417 g). The lung burden of carbon black at 24 months was  $43.9 \pm 4.3 \text{ mg}$  per lung (equivalent to  $31.3 \text{ mg/g}$  clean-air control lung) and  $6.7 \text{ mg}$  per animal in the lung-associated lymph nodes (determined after 22 months of exposure). The differential cell count of and the concentration of lactate-dehydrogenase, beta-glucuronidase, OH-proline, and total protein in bronchoalveolar lavage showed clear exposure-related effects in the exposure group. This is important to note because the alveolar lung clearance rate was already significantly deteriorated in this group after only 3 months of exposure. One benign squamous-cell tumour (1/9; 11%) was found after 24 months compared to none lung tumour (0/10) among the controls.

The incidence of benign and malignant lung tumours was increased in the treated groups after 30 months. The numbers of rats with lung tumours are summarized in Table 10.

**Table 10.:** Lung tumour incidence in female rats exposed to carbon black by inhalation (30 month study)

	<b>Carbon black-exposed</b> (average concentration of carbon black, $11.6 \text{ mg/m}^3$ )	<b>Clean-air control</b>
Benign keratinizing cystic squamous-cell tumours	20/100	--
Adenomas	13/100	--
Squamous-cell carcinomas	4/100	--
Adenocarcinomas	13/100	1/217
No. of animals with tumours	39/100 (28/100*)	1/217

\*Count without benign keratinizing cystic squamous-cell tumours given in parentheses

Ref.: Dungworth *et al.*, 1994; Heinrich *et al.*, 1995

Guideline/method	Primarily as Guideline OECD Guideline 451
Species:	Male and female Fischer 344/N rats
Age at start of study:	7 – 9 weeks
Groups:	115-118 males and 114-116 females. Six (three female and three male) rats per exposure group were sacrificed after 3, 6, 12, 18, and 23 months of exposure. Approximately 100 rats of each gender per exposure group were maintained for life span.
Test substance:	Elftex-12 furnace black, Cabot, Boston, MA
Batch:	/
Organic content:	0.04–0.29% (mean value during the course of exposure, 0.12%).
Conc a.i.:	/
Particle size:	Surface area, 43 m <sup>2</sup> /g
Aerodynamic diameters:	2 µm MMAD (large mode); 0.1 µm MMDD (small mode).
Dose applied:	0, 2.5 or 6.5 mg/m <sup>3</sup> Carbon Black
Route:	Inhalation
Exposure time:	16 h/day, 5 days/week for up to 24 months. After exposure for 24 months, surviving rats were kept in clean air until mortality reached 90% when the experiment was terminated.
Positive control:	Rats exposed to diesel exhaust.
GLP:	/
Date of report:	1995
Published:	Yes

#### Procedure

Three groups of 114–115 female and 115–118 male Fischer 344/N specific pathogen-free rats, 7–9 weeks of age, were exposed in whole-body exposure chambers to 0, 2.5 or 6.5 mg/m<sup>3</sup> furnace carbon black (Elftex-12) for 16 hours per day on 5 days per week for up to 24 months. The carbon black aerosol was produced by an air-jet dust generator and was diluted with filtered air. The size distribution of carbon black particles in the chamber was bimodal: 67% of the particles were in the large-size mode (MMAD, 2.0 µm) and 33% in the small-size mode (mass median diffusion diameter, 0.1 µm). The level of extractable organic material was 0.04–0.29% (mean value during the course of exposure, 0.12%).

Observations were made throughout the lifespan for the majority of rats in each group (i.e. for approximately 100 males and 100 females per experimental group in total) for which bodyweight, survival and carcinogenicity were evaluated. Three males and three females were killed after 3, 6, 12, 18, or 23 months of exposure to measure lung and lung-associated lymph node burdens of particles, lung weight, bronchoalveolar lavage indicators of inflammation, DNA adducts in whole lung and alveolar type II cells, and chromosome injury in circulating lymphocytes, and to perform histopathologic assessment. After exposure for 24 months, surviving rats were kept in clean air until mortality reached 90% when the experiment was terminated.

#### Result

The high-dose exposure to carbon black significantly ( $P < 0.05$ ) reduced the median lifespan of both females and males. Survival was also significantly reduced in low-dose males. A significant reduction in the body weights of female and male rats exposed to the high dose of carbon black first occurred on days 309 and 449, respectively. This effect was seen only after day 509 of exposure for both males and females in the low-dose group. After about 22 months, the mean reduction in body weight was 16% for high-dose females and 14% for high-dose males; these figures were below 10% in low-dose animals. The exposure caused progressive, dose-related accumulation of carbon black particles in the lungs. After 23



months, the mean lung burden reached 12.4 mg/g in low-dose males, 13.9 mg/g in low-dose females, 20.2 mg/g in high-dose males and 30.0 mg/g in high-dose females.

Lung DNA adducts in type II cells were increased by carbon black exposure. The total adduct levels 20.1 per 10<sup>9</sup> were significantly higher than the adduct level in the control group (6.1 per 10<sup>9</sup> bases). None of these adducts were identified, and they did not co-migrate with an authentic benzo[a]pyrene diolepoxide adduct standard. No exposure-related chromosome damage was found in circulating lymphocytes.

Full necropsies were performed on all animals and lungs and suspected lung tumours were examined microscopically. Statistical comparisons were performed using logistic regression modeling. The incidence of adenomas and adenocarcinomas was significantly increased in females, particularly at the high-dose level. There was no significant increase in the incidence of lung tumours in males. The percentages of male and female rats with malignant lung tumours are given in Table 11.

**Table 11.:** Malignant lung tumour incidence in Fischer 344/N rats exposed to carbon black by inhalation for up to 24 months

Group	Rats with malignant neoplasms (%)	
	Females	Males
Control	0 (0/105)	1.8 (2/109) <sup>a</sup>
2.5 mg/m <sup>3</sup>	6.5 (7/107) <sup>b</sup>	0.9 (1/106) <sup>c</sup>
6.5 mg/m <sup>3</sup>	20(21/105) <sup>d</sup>	3.8 (4/106) <sup>e</sup>

<sup>a</sup>1 adenocarcinoma + 1 squamous cell carcinoma

<sup>b</sup>6 adenocarcinomas + 1 not specified

<sup>c</sup>1 adenocarcinoma

<sup>d</sup>20 adenocarcinomas + 1 squamous cell carcinoma

<sup>e</sup>1 adenocarcinoma + 2 squamous cell carcinoma + 1 adenosquamous carcinoma

Ref.: Mauderly *et al.*, 1994; Nikula *et al.*, 1995

## Intratracheal administration

### Rat

Guideline/method	/
Species:	Female Wistar rats.
Age at start of study:	15 weeks.
Groups:	Exposed group: 37 females, Control group: 39 females.
Test substance:	Printex 90
Batch:	/
Organic content:	/
Conc a.i.:	/
Particle size:	Printex 90: mean particle size, 14 nm; specific surface area, 270 m <sup>2</sup> /g
Aerodynamic diameters:	/
Dose applied:	3 mg/rat in 0.9% saline
Route:	Intratracheal instillations
Exposure time:	Once a week for 15 weeks
GLP:	/
Date of report:	1994
Published:	Yes

A group of 37 female Wistar rats, 15 weeks of age, was instilled intratracheally with 3 mg/rat furnace carbon black (Printex 90) suspended in 0.9% saline once a week for 15 weeks. A control group of 39 female rats was instilled with 0.4 ml 0.9% saline once a week for 15 weeks. The animals died spontaneously, or were killed when moribund or after 131 weeks.

More than 50% of rats in the treated and control groups survived to 100 weeks. The lungs were removed and evaluated microscopically. No primary lung tumour was found in the control group. In the treated animals, 65% [24/37] of the rats had primary lung tumours: 3 adenomas, 6 adenocarcinomas, 1 adenocarcinoma and a CKSC tumour, 4 CKSC tumours, 1 CKSC tumour and 1 adenoma, 3 squamous-cell carcinomas and 7 rats had squamous-cell carcinomas and additional lung tumours (one adenoma, one adenocarcinoma, three adenocarcinomas and CKSC tumours and one CKSC tumour).

Ref.: Pott and Roller, 1994; Pott *et al.*, 1994

Guideline/method	/
Species:	Female Wistar rats.
Age at start of study:	7 weeks.
Groups:	48 females (+ 4 spare females).
Test substance:	Carbon Black; Lampblack 101 (Degussa) Printex 90
Batch:	/
Organic content:	Toluene extract 0.1%
Conc a.i.:	/
Particle size:	Lampblack 101: mean particle size, 95 nm; density, specific surface area, 20 m <sup>2</sup> /g Printex 90: mean particle size, 14 nm; specific surface area, 300m <sup>2</sup> /g
Aerodynamic diameters:	/
Dose applied:	See Table 12
Route:	Intratracheal instillations
Exposure time:	Once a week for 16 – 17 weeks
GLP:	/
Date of report:	1996
Published:	Yes

#### Procedure

*Soot extraction.* Approximately 20 g of the carbon blacks Printex 90 and Lampblack 101, respectively, was extracted three times with boiling toluene for 30 min. After each extraction, the soot was filtered. Finally the soot was dried for 5 h under vacuum. The surface area of toluene-extracted Printex 90 and of Lamp Black 101 was almost unchanged (271m<sup>2</sup>/g and 22 m<sup>2</sup>/g, respectively) compared with the original samples.

*BaP-covered particles.* Two grams of the extracted Printex 90 were each suspended in 120 ml n-hexane containing 60 mg BaP for 10 min and were then filtered under vacuum. Thereafter the BaP-covered particles were washed once with 10 ml pentane and filtered again under vacuum until they were dry. The resulting BaP content of the treated particles was 29.5 mg BaP per g Printex 90. The corresponding relative amounts of BaP adsorbed on the particle surface were 109 µg BaP/m<sup>2</sup> extracted Printex 90.

*Treatment.* The test materials were suspended in a saline/Tween80 solution (0.9% sodium chloride and 0.25% Tween 80) and ultrasonicated for about 3 min prior to treatment. Volumes of 0.2-0.3 ml/animal were administered intratracheally (i.tr.) once per week for

16-17 weeks resulting in total doses of 15 or 30 mg particles. Fifteen milligrams each of pure BaP, and toluene-extracted carbon particles were used.

### Results

With the exception of the BaP high dose group (30 mg) where the mortality increased significantly by early lung tumour development, the survival rates of all other groups were very similar. An early development of spontaneous mammary and pituitary tumours was observed in treated as well as in control animals. A mortality rate of 50% was reached after about 18 months study duration. At the end of the instillation period, the weights of lungs in all particle groups were significantly increased. The retained mass of particles per lung was between 58% and 75% of the total administered dose.

**Table 12.:** Experimental groups and tumour formation.

Test material	Total dose	Retained mass (mg/lung)	No of rats with benign tumours	No of rats with malignant tumours	No of rats with lung tumours	% of rats with lung tumours
Vehicle	4.5 ml	-	0	0	0	0
Extracted Pr-90	15 mg	11.3±1.7 (75%)	10	4	10	21
Extracted Lampblack 101	15 mg	ND	4	0	4	8
BaP	30 mg	ND	22	38	43	90
BaP	15 mg	ND	4	10	12	25
Extracted Pr-90 + BaP	15 mg incl. 0.443 mg BaP	8.7±1.9 (58%)	12	3	13	27

The different lung tumour types and the incidences of benign and malignant (lung) tumours are given in Table 12. No lung tumour occurred in the control group. The most important tumour results were: The carcinogenic potency of extracted carbon blacks depends on the size of the primary carbon particles and on the specific surface area of the particles. Thus, the percentage of rats with lung tumours was much higher with Printex 90 than with Lampblack 101. A total dose of 15 mg pure BaP caused a lung tumour rate very similar to that of 15 mg Printex 90 carbon black extracted or covered with approximately 29.5 µg BaP per mg carbon black.

Ref.: Dasenbrock *et al.*, 1996

Guideline/method	/
Species:	Female Wistar rats
Age at start of study:	8 - 9 weeks
Groups:	21 - 48 females
Test substance:	Carbon Black; Lampblack 101 (Degussa) Printex 90
Batch:	/
Organic content:	/
Conc a.i.:	/
Particle size:	Lampblack 101: mean particle size, 95 nm; density, 1.85 g/ml; specific surface area, 18.4 m <sup>2</sup> /g

	Printex 90: mean particle size, 14 nm; specific surface area, 337 m <sup>2</sup> /g
Aerodynamic diameters:	/
Dose applied:	See Table 13
Route:	Intratracheal instillations
Exposure time:	Once a week (number of instillations given in Table 13)
GLP:	/
Date of report:	2005
Published:	Yes

#### Procedure

Groups of 21–48 female Wistar rats, 8–9 weeks of age, received intratracheal instillations at weekly intervals of one of two carbon blacks—Lampblack 101 (Degussa; mean particle size, 95 nm; density, 1.85 g/ml; specific surface area, 18.4 m<sup>2</sup>/g) and Printex 90 (mean particle size, 14 nm; specific surface area, 337 m<sup>2</sup>/g) as described in Table 13. The dusts had been suspended by ultrasonification in 0.4 ml 0.9% phosphate buffered saline solution and 0.5% Tween 80 was added to improve the homogeneity of the suspensions. A control group of 48 rats was maintained untreated. Rats were inspected for mortality and clinical signs of morbidity twice per weekday and once a day at weekends.

The experiment was terminated after 30 months unless rats were killed when moribund or diagnosed with a growing subcutaneous tumour. After death of the animals and before necropsy of the thoracic and abdominal cavity, lungs were insufflated via the trachea *in situ* with 6% neutral buffered formalin. In particular, the surface of the lung was inspected and lesions were recorded. The lungs were fixed and embedded in paraffin and sections were stained with haematoxylin–eosin. All tissues suspected of having tumours that were taken from other sites were examined for histopathological lesions, especially for primary tumours that metastasized to the lung.

The lung tumour incidence in each group is summarized in Table 13. Statistically significant increases in benign and/or malignant lung tumours were observed with both types of carbon black.

**Table 13.:** Dose schedules and incidence of lung tumours in female Wistar rats administered carbon black by intratracheal instillation.

Carbon Black	Dose instilled	Rats at risk <sup>a</sup>	50% survival (weeks) <sup>b</sup>	Malignant lung tumours (%) <sup>c</sup>	Total lung tumours (%) <sup>c</sup>
Lampblack 101	5x6 mg <sup>d</sup>	45	106	26.7	60.0
	10x6 mg <sup>e</sup>	46	104	37.0	63.0
	20x6 mg <sup>f</sup>	47	108	NH	70.2 <sup>g</sup>
Printex 90	5x1.5 mg <sup>h</sup>	46	110	37.0	67.4
	5x3 mg <sup>i,j</sup>	18	112	66.7	88.9
	5x3 mg <sup>j</sup>	27	107	55.5	77.8
	5x3 mg	45	-	60.0	82.2
	5x6 mg	48	108	68.6	83.3
	10x6 mg	47	100	NH	72.3 <sup>g</sup>
No treatment <sup>k</sup>	--	46	124	0.0	2.2

NH, no histopathology performed

<sup>a</sup> Number of rats examined that survived at least 26 weeks after first instillation

<sup>b</sup> Period after first instillation during which 50% of the animals died excluding rats that died immediately after anaesthesia

<sup>c</sup> Primary lung tumour types diagnosed as benign: adenoma and epithelioma; or malignant: adenocarcinoma and squamous-cell carcinoma; lungs with one or more malignant tumour may also have had benign tumours.

<sup>d-f,h,i</sup> One additional instillation by error. The dust volume of this instillation is included in the calculation of the total volume instilled.

<sup>d</sup> Plus 1x 2.5 mg diesel soot

<sup>e</sup> Plus 1x3 mg diesel soot

<sup>f</sup> Plus 1x 6 mg diesel soot

<sup>g</sup> Macroscopic examination

<sup>h</sup> Plus 1 ' 3 mg ultrafine hydrophilic titanium dioxide

<sup>i</sup> Plus 1 ' 6 mg ultrafine hydrophilic titanium dioxide

<sup>j</sup> These two subgroups were combined for further statistical calculations. The large difference in tumour response may be due to an inhomogeneous suspension administered to small numbers of rats per subgroup and not caused by the additional instillation of the relatively small volume of titanium dioxide (about 20% of the dose of the first subgroup).

<sup>k</sup> Altogether the experiment involved 3 control groups. The first part of the experiment started on 04.07.95, 47 rats received 20 instillations with carrier fluid, no primary lung tumours were found in the microscopic study, the second part started on 13.09.95, 46 rats received "No treatment" (described above). The third part started on 21.11.95, 46 rats received "No treatment" no primary lung tumours were found in the microscopic study.

Ref.: Pott and Roller, 2005

### **SCCS comment**

Roller (2012) made a "time-to-tumour dose threshold analysis" for intratracheal particle instillation-induced lung tumours on the basis of a large carcinogenicity study involving 16 dose groups which received intratracheal instillations of "respirable granular bio-durable particles without known significant specific toxicity" (GBP) (the above study by Pott and Roller (2005) represents 9 of the 16 dose groups). Roller concluded that retained dust volume is a highly significantly better dose measure than instilled dust mass, where particle size is taken into account and that there is no empirical support for a dose threshold from this study.

There has been considerable debate on the intratracheal instillation studies in terms of a possible overload effect of particles in the lung (e.g. Valberg *et al.*, 2009). In the three control experiments involving 139 rats only one benign lung tumour was found. No significant differences were found in the frequency of metastasis of other tumours to the lung between the exposed rats and the control rats.

The response of rats to insoluble-particle "lung overload" is stereotyped and unique to that species and when the lung-overload threshold is exceeded, rats develop lung tumours from ongoing inflammation. It is noted that the potency in terms of inducing tumours expressed as tumour/volume of dust in the lung (%/µl), i.e. relation of percentage of rats with primary lung tumours to the retained dust volume in the lung, varied with the dust type. Thus, it was 15 – 51 for quartz, 2.5 – 5.5 for Lampblack and 7.6 – 22.5 for Printex.

Intratracheal instillations cannot be used for quantitative risk characterization in relation to human exposure by inhalation. However, the studies may provide information on the inherent property of carbon black nanoparticles to induce tumours. Thus, the intratracheal instillations indicate that the smaller particles are more potent than the larger particles.

### **Dermal application**

### *Mice*

Three groups of 12, eight and eight Swiss mice [age and sex not specified] received weekly dermal applications on the clipped dorsal skin of one of three different types of furnace carbon black (Crude 'Kosmos' 40, 33 and 20) suspended in acetone [dose of carbon black not specified] containing 0.5% croton oil. A negative-control group of 20 animals was treated with acetone that contained 0.5% croton oil and a positive-control group of 15 animals was treated with a solution of 1% benzo[*a*]pyrene in acetone that contained 0.5% croton oil. The experiment lasted for 315 days. The site of application was investigated histologically. Two skin papillomas and no carcinomas were detected in the eight 'Kosmos' 33 carbon black-treated animals; no tumours were observed in the two other treated groups or the negative controls. The positive-control group had a tumour incidence of 73%: all tumours were described as squamous-cell carcinomas.

Ref.: von Haam and Mallette, 1952.

In the same study, 14 groups of Swiss mice received weekly dermal applications of 14 different concentrated extracts of carbon black [dose not specified] suspended in acetone that contained 0.5% croton oil. At the end of the experiment at 315 days, six mice with squamous-cell carcinoma with or without additional papillomas were found in four of the 14 groups treated with extracts. Seven mice with papillomas only were found in four other extract-treated groups.

Ref.: von Haam and Mallette, 1952).

In a series of experiments, a total of 240 CFW white and C3H brown mice [sex unspecified], 6–10 weeks of age, received thrice-weekly dermal applications of three types of carbon black (channel black, thermal black and furnace black) suspended in cottonseed oil, mineral oil or in carboxymethyl cellulose in water on the shaved back for 12–18 months. There was no increased incidence of skin tumours. In the same study, 32 groups of male CFW and C3H mice [number and age of the animals unspecified] received applications of furnace or thermal carbon black extracts (obtained by hot benzene extraction for 48 hours) from eight different carbon blacks for up to 12 months. All but one of the extracts were reported to show moderate to strong carcinogenicity (tumour incidence, 33–85%) [tumour type unspecified]. In an untreated control group of 943 CFW and C3H mice, 13 animals developed malignant neoplasms (six of the skin, six of the liver and one of the spleen [no further details on the histology]).

Ref.: Nau *et al.*, 1958.

### **SCCS comment**

No conclusions can be drawn from the skin painting studies. The studies are old, and important information is lacking such as the doses and type of carbon black used. Moreover, 1% benzene was used as a vehicle for some extracts.

### **General comment by SCCS**

Carbon black nanomaterials have been tested for carcinogenicity by oral administration in female rats and female mice, by inhalation exposure in rats and female mice, by intratracheal administration in female rats, and by dermal application to mice.

No carcinogenic effect was observed after oral or dermal exposure. However, the studies are old and incompletely reported. Thus, no conclusion can be drawn from the studies.

In mice, no increased tumour frequency was found in an inhalation study where female mice were exposed to 10 mg/m<sup>3</sup> carbon black (Printex 90) for 13.5 months and kept for an additional 9.5 months.



In rats, two different carbon black products (Printex 90, 14 nm and Elftex-12, 37 nm) were tested by inhalation exposure at doses of 2.5 – 6.5 mg/m<sup>3</sup> in two studies in female rats and in one study in rats of each sex. Significant increases in the incidence of malignant lung tumours or of benign and malignant lung tumours combined were observed in female rats in all three studies. No tumour increases were found among the male rats.

In two studies with intratracheal administration of female rats using two types of carbon black (Printex 90 [14 nm] and Lampblack 101 [95 nm]) (doses of 3 mg x 15 or higher), and in one study using one type (Printex 90), an increased incidence of malignant lung tumours or of benign and malignant lung tumours combined was observed. The increase in tumour frequency was dependent on the size of the particles as the smaller particles had the highest potency.

SCCS is of the opinion that carbon black can induce malignant tumours in female rats after inhalation exposure or intratracheal instillations. The potency of carbon black particles with diameter of 14 nm was higher than the potency of carbon black particle with diameter of 95 nm. Thus, the evidence presented indicates that smaller nanoparticles have a higher potency of causing tumours in lung than relatively larger nanoparticles. There is no empirical support for a dose threshold from the animal carcinogenicity studies.

### 3.3.7.2 Epidemiological carcinogenicity studies

Several epidemiological studies in relation to carbon black exposure have been performed. At this point only the three large epidemiological studies of production facilities of carbon black will be discussed. The main results are summarized in Table 14.

**Table 14.:** Overview of cohort studies with carbon black exposed workers (Taken from Roller (2009))

Authors	Area	Cohort size	Deaths		SMR lung cancer (95%CI) <sup>c</sup>	Remark (or comments of study authors)
			Overall (%) <sup>a</sup>	Lung cancer (%) <sup>b</sup>		
Dell <i>et al.</i> (2006)	United States	5011	1326 (26.5)	138 (10.4)	0.97 (0.82-1.15)	No trends with duration of employment
Wellmann <i>et al.</i> (2006)	Germany	1535	332 (21.6)	50 (15.1)	2.18 (1.61-2.87)	No apparent dose response relationship
Sorahan and Harrington	UK	1147	426 (37.1)	67 (15.7)	1.46 (1.13-1.85)	Update of Sorahan <i>et al.</i> (2001)

<sup>a</sup>In parentheses: percent of total cohort.

<sup>b</sup>In parentheses: percent of overall deaths.

<sup>c</sup>SMR = standardised mortality ratio; CI = confidence interval.

A study in the USA (Dell *et al.*, 2006) included a large cohort of workers from 18 plants with good ascertainment of cohort members and effective mortality follow-up over a long period of time. There was no indication of excess risk for cancer at any of the sites reported. There was no indication that long-service workers had higher risks than short-service workers. For most types of cancer, including lung cancer, the numbers of deaths observed did not exceed

the numbers expected on the basis of national rates. No results were provided taking into account various levels of exposure to carbon black or tobacco smoking habits.

A cohort study was conducted among blue-collar workers in a long-standing, large German carbon black production plant (Wellmann *et al.*, 2006). When mortality was compared with regional rates, there was an approximate doubling of risk for lung cancer. Exposure was assessed using full work history records from the plant and expert judgments. Furthermore, company medical records provided some information on tobacco smoking for most of the workers. Compared with the lowest exposure group, and after adjusting for smoking, there was no indication that workers with several indices of or average exposure to carbon black had higher mortality. However, the precision of these subgroup risk estimates was low. There was no excess mortality from cancer at most other sites, including oesophagus, stomach and urinary bladder, although the numbers were small.

The study of workers in five carbon black production facilities in the United Kingdom (Sorahan and Harrington, 2007) involved a large group with a long follow-up. When compared with national mortality rates, there was a clear excess of mortality from lung cancer. Although tobacco smoking histories were not known, there was no corresponding significant excess of other diseases known to be associated with smoking. The excess risk was manifest in two of five factories. Exposure was assessed using last job from worker records and a job-exposure matrix based on expert judgment and measurements from two of the five plants. When adjusted for age and divided into four subgroups based on cumulative exposure levels, relative risk did not increase monotonically with increasing exposure, although the two highest exposure categories showed higher relative risks than the two lowest categories. There was no significant excess risk for cancer at any other site.

The IARC Working Group (IARC, 2010) pointed out that the human epidemiological evidence was inconsistent. Two of the three studies of carbon black production workers observed excess risk for lung cancer and other studies provided mixed evidence for an increased risk for lung and other cancers. The few studies that assessed exposure-response for lung cancer, including the two that observed excess risks compared with the general population, provided weak or inconclusive evidence of a dose-response. Overall, these results led the Working Group to conclude that there was *inadequate evidence* from epidemiological studies to assess whether carbon black causes cancer in humans.

#### General evaluation by IARC (2010)

IARC evaluated Carbon Black in 2006.

It was concluded that "There is *inadequate evidence* in humans for the carcinogenicity of carbon black" and that "There is *sufficient evidence* in experimental animals for the carcinogenicity of carbon black.

The Overall evaluation was: Carbon black is *possibly carcinogenic to humans (Group 2B)*.

#### Rationale

In making this evaluation the Working Group considered the human and animal evidence as well as the evidence on potential mechanisms through which carbon black may cause cancer in humans.

The human epidemiological evidence was inconsistent. The IARC Working Group concluded that there was *inadequate evidence* from epidemiological studies to assess whether carbon black causes cancer in humans.

Three studies of female rats that inhaled carbon black and three additional studies of female rats exposed intratracheally found significant increases in the incidence of malignant lung tumours, providing *sufficient evidence* that carbon black can cause cancer in animals.

The Working Group considered a large body of mechanistic information. For lung cancer in rats, it was concluded that a sequence of events that starts with impaired clearance and accumulation of particles in the lung, causing inflammation, cell injury and production of reactive oxygen species that eventually lead to mutations, was well supported by experimental evidence, although some data also supported alternative pathways. High retained mass lung burdens and decreased lung clearance have been observed in coal miners, which led the Working Group to conclude that animal cancer data obtained under conditions of impaired lung clearance are relevant to humans. There was a minority opinion in the Working Group that would support the classification of carbon black in Group 2A, and invoked the analogy with quartz particles, which are carcinogenic in the lung of rats and humans. However, based on current evidence, the Working Group considered that the degree to which all elements of the above-mentioned mechanism may operate in humans is not clear and, thus, the mechanistic information did not alter the overall evaluation of Group 2B.

After the IARC evaluation the studies from USA and UK have been updated. Graber *et al.* (2014) studied the cause of death for 9033 underground coal miners from 31 US mines enrolled between 1969 and 1971. They observed an overall excess of lung cancer mortality (SMR=1.08; 95% CI 1.00 to 1.18) and a significant association with cumulative coal mine dust exposure and lung cancer. Coal mine dust exposure was positively and significantly associated with lung cancer mortality in the single exposure model (HR=1.70; 95% CI 1.02 to 2.83) and the model that included silica (HR=1.71; 95% CI 1.03 to 2.85). Log-transformed cumulative silica exposure was associated with a significant increase in lung cancer (HR= 1.76; 95% CI 1.45 to 2.14) in the single-exposure model but not when controlling for coal mine dust (HR=1.33; 95% CI 0.94 to 1.90). This association with coal mine dust was present in models with and without silica exposure. In the most recent follow-up of the British cohort (Miller and MacCalman (2010), which reports on analyses of cause-specific mortality in a cohort of almost 18000 men from 10 British collieries, an overall significant exposure-response relationship was observed for lung cancer mortality with silica but not with coal mine dust exposure.

### SCCS comment

Carbon black induced lung tumours in rats after inhalation and intratracheal instillation. The studies on the genotoxicity of carbon black suggest that the nanoparticles may induce genotoxic effects both by a primary and secondary genotoxic mechanism. Since high retained mass lung burdens and decreased lung clearance have been observed in coal miners (see section 3.3.11), SCCS is of the opinion that the animal cancer data are relevant to humans. Although the USA study has been criticized it cannot be neglected. However, based on an overall evaluation of the epidemiological data available, SCCS concur with the previous IARC conclusion that there are *inadequate evidence* from epidemiological studies.

### 3.3.8. Reproductive toxicity

Guideline/method	/
Species:	Mated ICR mice
Age at start of study:	Gestation day three (GD 3).
Groups:	Exposed group 13 mice, control group 12 mice
Test substance:	Carbon Black; Printex 90
Batch:	/
Organic content:	/
Conc a.i.:	/
Particle size:	Printex 90: mean particle size, 14 nm; specific surface area, 337 m <sup>2</sup> /g

Aerodynamic diameters: /  
 Dose applied: 50 µl carbon black suspension (1 mg/ml). Total dose 100 µg/animal  
 Route: Intranasal instillations  
 Exposure time: Gestation days 5 and 9.  
 GLP: /  
 Date of report: 2011  
 Published: Yes

#### Procedure

Pregnant ICR mice were exposed by intranasal instillations to 50 µl carbon black suspension (1 mg/ml) on gestation days 5 and 9 (total dose 100 µg/animal). Kidney and blood were collected from male offspring at week 3 and 12. Collagen expression was examined by quantitative RT-PCR and immunohistochemistry.

#### Results

There were no differences between the litter size of exposed and control mice. Exposure of pregnant ICR mice to carbon black resulted in increased expression of Collagen, type VIII, in the tubular cells in the kidney of 12 week old offspring mice, but not in the 3 week old ones. The levels of serum creatinine and blood urea nitrogen, indices of renal functions, were not different between the groups.

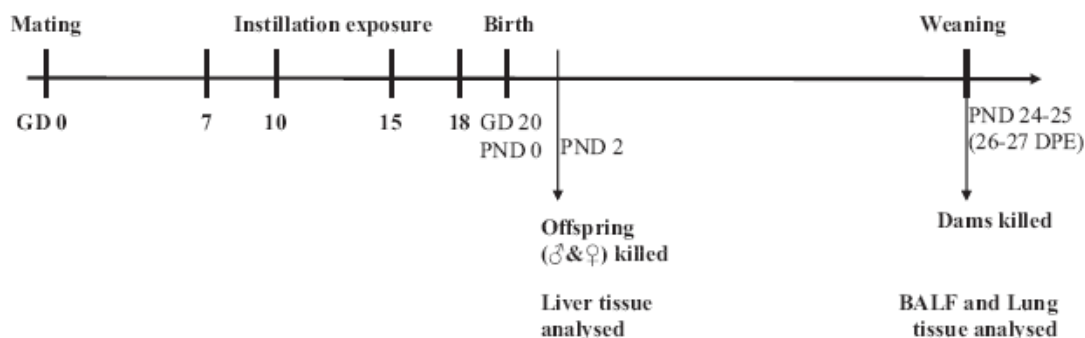
#### Conclusion

The study authors write that intranasal instillations of carbon black in pregnant mice resulted in findings in the offspring that were similar to those of tubulo-interstitial fibrosis in diabetic nephropathy.

Ref.: Umezawa *et al.*, 2011

Guideline/method /  
 Species: Mice; time-mated, nulliparous mice (C57BL/6BomTac, Taconic Europe, Ejby, DK) received  
 Age at start of study: Gestation day three (GD 3).  
 Groups: 17 – 22 mice  
 Test substance: Carbon Black; Printex 90  
 Batch: /  
 Organic content: /  
 Conc a.i.: /  
 Particle size: Printex 90: mean particle size, 14 nm; specific surface area, 337 m<sup>2</sup>/g  
 Aerodynamic diameters: /  
 Dose applied: Time-mated nulliparous C57BL/6J mice received 0, 11, 54 or 268 µg/animal subdivided in 4 instillations  
 Route: Intratracheal instillations  
 Exposure time: Gestation days 7, 10, 15 and 18.  
 GLP: /  
 Date of report: 2012  
 Published: Yes

Procedure (see fig. 11)



**Fig. 11.:** Experimental design. GD: gestational day, PND: postnatal day. Time mated mice were exposed by intratracheal instillation to total doses of 0, 11, 54 or 268  $\mu\text{g}/\text{animal}$  of Printex 90 divided over four instillations on GD 7, 10, 15 and 18. Male and female offspring were euthanized on PND 2 (4 days after the last maternal exposure). Dams were euthanized on PND 24–25 (26–27 days post-exposure) after weaning. The livers from the offspring and the lungs from the dams were analysed in the study.

## Results

Retention of carbon black particles was observed in dams as late as 26–27 days post-exposure from both the medium and the high dose groups. Persistent lung inflammation was observed only in dams exposed to the high dose of carbon black (268  $\mu\text{g}/\text{animal}$ ). Likewise, altered expression of several cytokines and chemokines, both at the transcriptional and tissue protein levels, was significant only in the high dose group.

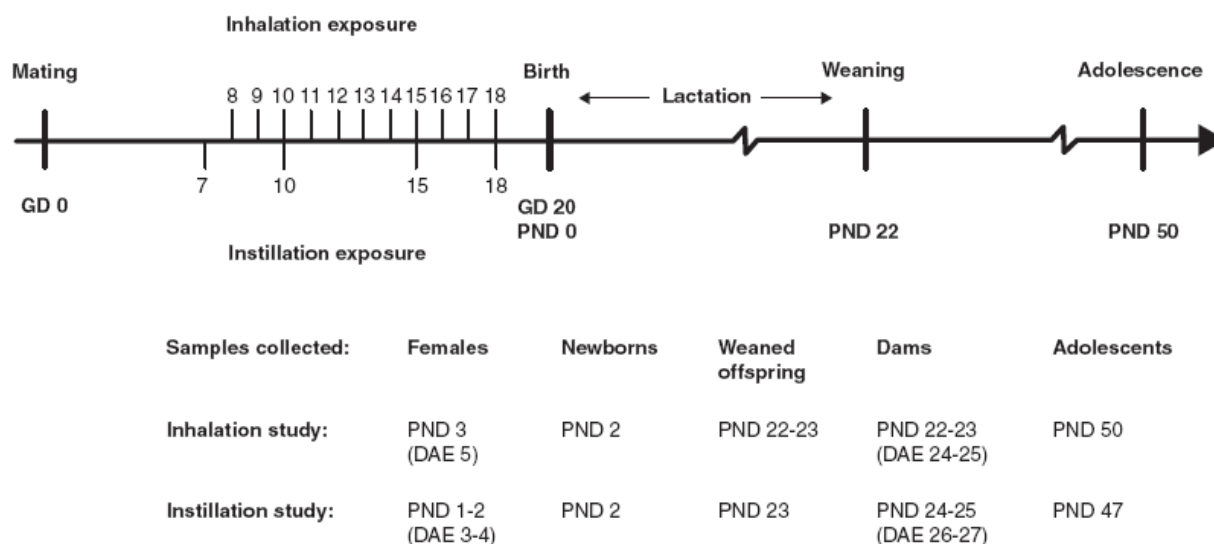
Hepatic gene expression in samples from a subset of male and female newborns collected on postnatal day 2 (4 days after the last maternal exposure) was studied. Analysis of newborn livers by DNA microarrays revealed that female offspring were more sensitive to maternal exposure than male offspring. Cellular signaling, inflammation, cell cycle and lipid metabolism were among the biological pathways affected in female offspring. Males, however, responded with subtle changes in metabolism-related genes.

Ref.: Jackson *et al.*, 2012a

Guideline/method	/
Species:	Mice; time-mated, nulliparous mice (C57BL/6BomTac, Taconic Europe, Ejby, DK)
Age at start of study:	Gestation day three (GD 3).
Groups:	80 mice exposed by intratracheal instillations and 44 mice exposed to Carbon Black by inhalation.
Test substance:	Carbon Black; Printex 90
Batch:	/
Organic content:	/
Conc a.i.:	/
Particle size:	Printex 90: mean particle size, 14 nm; specific surface area, 337 $\text{m}^2/\text{g}$
Aerodynamic diameters:	/
Dose applied:	Time-mated nulliparous C57BL/6J mice received 0, 11, 54 or 268 $\mu\text{g}/\text{animal}$ Carbon Black subdivided in 4 instillations or inhalations to 42 $\text{mg}/\text{m}^3$ Carbon Black for 1 h/day.
Route:	Intratracheal instillations Inhalation
Exposure time:	Intratracheal instillations: Gestation days 7, 10, 15 and 18.

GLP: /  
 Inhalation: Gestation days (GD) 8–18.  
 Date of report: 2012  
 Published: Yes

Procedure (see fig. 12.)



**Fig. 12.:** Experimental design. GD, gestation day (pregnancy day); PND, post natal day (days after birth); DAE, days after exposure. 'Time-mated mice' term was used for all exposed mice during gestation and when referring to results of 'females' and 'dams' together. Time-mated mice that had not given birth or had only few offspring were termed 'females'. Time-mated females that gave birth were termed 'dams'. PND 1 and PND 2 offspring were termed 'newborns'. Offspring on PND 22–23 was termed 'offspring at weaning'. Offspring on PND 50 termed 'adolescents' had not reached sexual maturity. Time-mated mice were exposed by inhalation and intratracheal instillation to Printex 90. Time-mated mice inhaled  $42 \text{ mg/m}^3$  Printex 90 or filtered air for 1 hour/day for 11 consecutive days on GD 8–18. The total instilled doses were 0, 11, 54 and  $268 \text{ } \mu\text{g/animal}$  were distributed over four instillations on GD 7, 10, 15 and 18.

**Inhalation.** The average particle size distribution was multimodal and highly dominated by sub-100 nm particles. The most abundant size number was in the order of 41 nm, which was also the average size. The average size by mass was 310 nm, and the mass size distribution was bimodal with one mode around 290 nm and a coarser mode at ca.  $1.5 \text{ } \mu\text{m}$ . Only 5% of the mass was below 100 nm, 83% of the particles were in this ultrafine size range by number.

Time-mated mice exposed to Printex 90 by inhalation indicated the presence of inflammation in the lungs. More neutrophils in BAL fluid were found in the exposed mice compared to their controls 5 and 24 days after exposure (5 days: 11.4-fold increase,  $p = 0.008$ ; 24 days: 11.6-fold increase,  $p < 0.001$ ). Also, more lymphocytes was found in BAL fluid 5 days after exposure (3.4-fold increase,  $p = 0.020$ ) and total cell counts were higher at both time-points (5 days: 1.5-fold increase,  $p = 0.032$ ; 24 days: 1.2-fold increase,  $p = 0.057$ ).

The inhalation of carbon black did not affect the level of DNA strand breaks in BAL fluid cells 5 and 24 days after exposure. The exposure to carbon black induced higher levels of DNA



strand breaks in the liver 5 and 24 days after exposure compared to their controls (5 days: 1.3-fold increase,  $p = 0.04$ ; 24 days: 1.6-fold increase,  $p < 0.001$ ).

In the offspring of the time-mated mice exposed to carbon black, the level of DNA strand breaks was higher in offspring liver at weaning and in adolescents, compared to their controls (weaning: 1.4-fold increase,  $p = 0.001$ ; adolescents: 1.5-fold increase,  $p = 0.011$ ).

The level of oxidatively generated DNA damage in the liver of offspring from the inhalation study was also determined by the level of formamidopyrimidine DNA glycosylase (FPG) enzyme sensitive sites. There was no consistent increase in oxidatively generated DNA damage in the offspring liver cells in newborns, at weaning or in adolescents (newborn exposed  $0.91 \pm 0.27$  vs. control  $0.71 \pm 0.20$ ; weaning exposed  $1.05 \pm 0.12$  vs. control  $1.28 \pm 0.13$ ; adolescents exposed  $0.87 \pm 0.10$  vs. control  $1.20 \pm 0.11$ . All data are presented as lesions per  $10^6$  base pairs).

A higher relative brain weight was found in time-mated mice 5 days after exposure to carbon black by inhalation, compared to their controls (exposed  $2.09 \pm 0.04\%$  vs. control  $1.86 \pm 0.06\%$ ,  $p = 0.005$ ). At weaning, relative lung weight was higher in exposed compared to control dams (exposed  $1.20 \pm 0.03\%$  vs. control  $1.10 \pm 0.02\%$ ,  $p = 0.005$ ). Other organs did not differ. Organ weights in offspring of dams exposed to carbon black were similar to their controls, except that the exposed female offspring had smaller relative heart weight at weaning (exposed  $0.68 \pm 0.01\%$  vs. control  $0.71 \pm 0.01\%$ ,  $p = 0.052$ ). Furthermore, exposed adolescent males had a higher relative weight of testes (exposed  $2.10 \pm 0.05\%$  vs. controls  $1.58 \pm 0.20\%$ ,  $p = 0.024$ ).

Instillation. The average size measured by zeta-sizer was approximately 140 nm and the hydrodynamic number size-distributions had a peak size between 50 and 60 nm.

Analysis of BAL fluid cell composition by differential cell count indicated the presence of inflammation in the lungs of time-mated mice exposed to carbon black.

The exposure to carbon black did not affect the level of DNA strand breaks in BAL cells in the females ( $p = 0.30$ ), while the dams exposed by instillation had significantly less DNA strand breaks in BAL cells in the high dose group 26 days after exposure compared to control dams (20% reduction,  $p = 0.007$ ). No increase in the level of DNA strand breaks was observed in the liver of time-mated mice exposed to carbon black by instillation compared to their controls.

In the offspring exposed to carbon black by maternal intratracheal instillation, the level of DNA strand breaks in liver cells was comparable to their controls ( $p = 0.8$ ). Interestingly, as we also observed in the inhalation study, the level of DNA strand breaks was generally higher in liver cells from newborns, compared to tissues from older siblings at later time points ( $p < 0.001$ ).

The thymus weight of exposed newborn and weaned offspring was similar to their controls. Other organs were not weighed.

## Conclusion

The study authors pointed out that neither inhalation nor instillation affected gestation and lactation. Maternal inhalation exposure to carbon black induced liver DNA damage in the mothers and in the *in utero* exposed offspring.

Ref.: Jackson *et al.*, 2012b

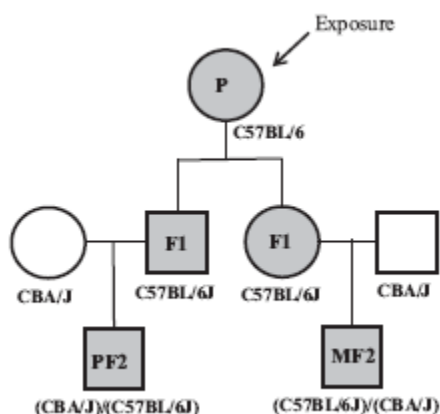
**SCCS comment**

It is noted that DNA strand breakage was found in the liver of the offspring both after inhalation exposure of the pregnant mice to carbon black. The mice receiving intratracheal instillations are probably the same animals used in the previous study by Jackson et al., 2012a.

3.3.8.1. Two generation reproduction toxicity
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Guideline/method	/
Species:	Mice; C57BL/6J C57BL/6J, BomTac, Taconic Europe, Ejby and CBA/J Charles River Wiga, Sulzfeld, Germany
Age at start of study:	/
Groups:	14 – 15 mice
Test substance:	Carbon Black; Printex 90
Batch:	/
Organic content:	/
Conc a.i.:	/
Particle size:	Printex 90: mean particle size, 14 nm; specific surface area, 337 m <sup>2</sup> /g
Aerodynamic diameters:	/
Dose applied:	Time-mated nulliparous C57BL/6J mice received 268 µg/mice subdivided in 4 instillations
Route:	Intratracheal instillations
Exposure time:	Gestation days 7, 10, 15 and 18.
GLP:	/
Date of report:	2013
Published:	Yes

Procedure  
See fig. 13.



**Fig. 13.:** Procedure used. Females: Circle, Males: Square

Animals were offspring of female mice (C57BL/6J C57BL/6J, BomTac, Taconic Europe, Ejby) exposed to nanoparticles of carbon black (Printex 90) during gestation by intratracheal instillation. To produce an F2 generation, the F1 C57BL/6J offspring were mated with CBA/J mice (Charles River Wiga, Sulzfeld, Germany). This was done to accommodate production of tissue for assessment of induction and inheritance of mutations in the germline, as this method requires that the F1 parents are of different strains.

Time-mated nulliparous C57BL/6J mice (generation P, n=15) received intratracheal instillations with nanosized carbon black on each of the gestation days 7, 10, 15 and 18 (67 µg/animal, total dose 268 µg/animal (the procedure is illustrated in fig. 13). The carbon black particles were suspended in pure water. In suspension, the peak hydrodynamic number size distribution was 50–60 nm and average zeta size was 140 nm. Control females (n=15) received vehicle without particles. Exposed C57BL/6J females from each group gave birth to a litter in the F1 generation. At 13 weeks of age, F1 male (n=24) and F1 female (n=24) C57BL/6J offspring from control and exposed litters were mated with 10 weeks old naïve CBA/J mice, producing the F2 generation. Thus, one F1 C57BL/6J male per litter cohabited with one naïve CBA/J female mouse to produce F2 litters of the mixed (CBA/J)/(C57BL/6J) strain. Likewise, one F1 C57BL/6J female from each litter were cohabiting with a naïve male CBA/J mouse, producing the F2 generation of the mixed (C57BL/6J)/(CBA/J) strain. In adulthood, testicles for assessment of sperm production were collected from one F1 C57BL/6J male per litter (age  $183 \pm 2$  days, n = 14–15), and from one F2 male per litter originating from both prenatally exposed F1 C57BL/6J males (n = 9–12) and F1 C57BL/6J females (n = 10–14) at the age of 80 days (PF2, designated (CBA/J)/(C57BL/6J) and MF2 (C57BL/6J)/(CBA/J) respectively).

Daily sperm production (DSP) The left testicle was dissected and decapsulated. The parenchyma was homogenized. Homogenization resistant (elongated) spermatids (stage 14–16) in the homogenate were stained by Trypan blue and after 30 min counted in haemocytometer under light microscope at 400× magnification. Samples were counted in two replicates. Counts from the two replicates were averaged and served as basis for calculation of the number of homogenization resistant spermatids per g testicle tissue (SC/g). The total number of spermatids in the left testicle was then calculated by multiplication with the weight of the left testicle. DSP was then calculated by dividing the total number of elongated spermatids in the testicle with the time divisor value of 4.84. This number corresponds to the time (in days) which developing spermatids spend in stage 14–16 during spermatogenesis in the mouse.

## Results

F1 males prenatally exposed to carbon black displayed no significant differences in body weight and in the assessed reproductive parameters (Testicle weight, relative testes weight, SC/g and DSP). The time it took breeding couples consisting of a prenatally carbon black exposed F1 C57BL/6J male and a naïve CBA/J female to deliver a first F2 litter was slightly extended compared to F1 control C57BL/6J males cohabiting with naïve CBA/J females ( $29.2 \pm 7.7$  and  $24.8 \pm 6.2$  days, respectively), although statistically insignificantly so. No correlation between sperm content/DSP and time-to-first F2 litter was found.

The statistical analysis revealed a highly significant influence of the genetic background for the F2 generation, depending on whether the father or the mother supplied the (C57BL/6J) allele. Absolute and relative testicle weights, SC/g and DSP were significantly higher if the paternal allele were of the C57BL/6J background (PF2, (CBA/J)/(C57BL/6J)) compared to the CBA/J genetic background (MF2, (C57BL/6J)/(CBA/J)) ( $P \leq 0.001$  for all). Furthermore, for SC/g and DSP there was statistical interaction between genetic background, i.e. whether the male or female C57BL/6J offspring were exposed during foetal life and exposure to Carbon Black (SC/g: gender × CB,  $P = 0.048$ ; CB,  $P = 0.079$ ; DSP: gender × CB,  $P = 0.050$ ). Post hoc, F2 male offspring from C57BL/6J males whose mothers were instilled with Carbon Black during pregnancy (PF2, (CBA/J)/(C57BL/6J)) had statistically significantly lower SC/g compared to controls ( $P = 0.04$ ) and tended to have lower DSP ( $P = 0.057$ ). In contrast, F2 males from C57BL/6J females exposed to Carbon Black during foetal life (MF2, (C57BL/6J)/(CBA/J)) presented similarly to corresponding controls for all studied parameters.

## Conclusion

F2 offspring, whose fathers were prenatally exposed to nanosized carbon black, showed lowered sperm production.

Ref.: Kyjovska *et al.*, 2013

### 3.3.8.2. Teratogenicity

Guideline:	OECD Guideline No. 414,
Species/strain:	Mated female Wistar rat
Group size:	24 animals
Age at start:	13 – 14 weeks
Test substance:	Carbon Black, E300281
Batch:	C3949
Purity:	98%
Particle size:	Diameter; 20 to 30 nm, surface area range of 200 to 260 m <sup>2</sup> /g
Vehicle:	0.5% sodium carboxymethylcellulose
Dose levels:	0 (control), 100, 300, or 1000 mg/kg bw/d
Dose volume:	10 ml/kg bw
Route:	Oral gavage
Administration:	Day 5 through day 19 of gestation
GLP:	In compliance
Study period:	January to June 2012

#### Procedure

The potential effects of carbon black on pregnant rats and embryo-foetal development were evaluated through daily oral gavage at 0, 100, 300 or 1 000 mg/kg/day to mated Wistar female rats (24/group) during the sensitive period of organogenesis [day 5 through day 19 of gestation, the day of detection of sperm positive vaginal smear/vaginal plug being designated as Gestation Day 0 (GD 0)]. The test item carbon black was suspended in 0.5% aqueous sodium carboxymethylcellulose and given at 10 ml/kg bw. The dose levels were selected on the basis of the results of a preliminary study performed at the same dose levels where no maternal or foetal toxic effects were observed.

Maternal evaluations and measurements included daily clinical signs and body weight/food intake measured at designated intervals. The dams were killed on GD 20 and subjected to macroscopic examination. Usual litter parameters were recorded and foetuses were sexed, weighed and submitted to external examination. About one half of the foetuses were also examined for soft tissue anomalies, and remaining foetuses were examined for skeletal anomalies.

#### Result

No deaths were observed. Dark coloured faeces were observed in all animals given carbon black and were related to the coloured nature of the test item. This finding was therefore considered to be non-adverse. When compared to controls, body weight gain was slightly but statistically significantly decreased at 100 mg/kg/day during gestation interval 5-8. As these changes were transient (only observed after the first dosing) and of minimal magnitude (+6 g for controls *versus* +3.9 g at 100 mg/kg/day), they were considered to be of no biological significance. There were no changes in litter parameters, and there were no foetuses with major malformations. Minor foetal anomalies and normal variants observed were of the type and incidences commonly observed in rats of this strain and age and hence were to be incidental.

#### Conclusion

The study authors concluded that oral administration of carbon black to pregnant rats at 100, 300 or 1 000 mg/kg bw/d during the sensitive period of organogenesis was well tolerated. There were no adverse maternal changes or any effects on embryo-foetal development. Accordingly, under the conditions of this study, The No Observed Adverse Effect Level (NOAEL) for maternal toxicity and the No Observed Effect Level (NOEL) for developmental toxicity were both set at 1 000 mg/kg bw/d.

Ref.: Ramesh (2012)

### General comments by SCCS

Five studies, four with mice and one with rats have been found in relation to reproductive toxicity of carbon black.

No effect on litter size was found in a study where mice received intranasal instillations of carbon black on gestation days 5 and 9 (total 0.1 mg). However, expression of collagen, type VIII in the tubular cells in the kidney of 12 week old offspring mice was increased. The study authors considered that the findings were similar to those of tubulo-interstitial fibrosis in diabetic nephropathy.

Time mated mice received intratracheal instillations of carbon black (altogether up to 0.268 mg, subdivided in 4 instillations). No effect was found on gestation and lactation. The results were presented in two articles. The first reported that a significant hepatic response at the mRNA level was observed in male and female offspring exposed prenatally to the highest dose of carbon black. Hepatic response was much more pronounced in the female offspring than in the male. In the second article, DNA strand breaks were studied. No effects were reported.

The second article reported also effects of time mated mice exposed to carbon black by inhalation (42 mg/m<sup>3</sup>, for altogether 10h). Maternal inhalation exposure to carbon black induced liver DNA damage in the mothers and in the *in utero* exposed offspring. Inhalation exposure did not affect gestation and lactation.

It is noted that single strand DNA breaks were found both in the livers of the offspring and the pregnant mice after exposure of the pregnant mice to carbon black by inhalation.

Time mated mice received four intratracheal instillations (altogether up to 0.268 mg) of carbon black. The F2 offspring, whose fathers were prenatally exposed to nanosized carbon black, showed lowered sperm production.

Pregnant rats received carbon black by oral administration of carbon black during the sensitive period of organogenesis. There were no adverse maternal changes or any effects on embryo-foetal development. The NOAEL for maternal toxicity and for developmental toxicity were both set at 1 000 mg/kg bw/d.

SCCS is of the opinion that oral and dermal exposure to carbon black is of little concern in relation to reproductive toxicity; however, inhalation exposure should be avoided.

### 3.3.9. Toxicokinetics

*The text is in part condensed from IARC (2010)*

#### Retention of carbon black nano particles

The lung retention of inhaled carbon black particles (Printex 90 and Sterling V) were investigated in Fischer 344 rats, B6C3F1 mice and F1B Syrian hamsters (all females) by Elder *et al.* (2005). Printex 90 was considered as a high-surface area carbon black (HSCB), while Sterling V was low-surface area carbon black (LSCB). Particle retention in the lungs was observed to be prolonged after exposure to the mid (7 mg/m<sup>3</sup>) and high (50 mg/m<sup>3</sup>) concentrations of Printex 90 in rats and mice, and also for 50 mg/m<sup>3</sup> Sterling V in rats. In hamsters, which had the most efficient clearance, pulmonary retention was prolonged only at the high dose. The particle retention half time for rats were 64 days for low HSCB (1 mg/m<sup>3</sup>), 115 days for mid HSCB, no significant clearance for high HSCB and 249 days for LSCB. The half times for mice were 133, 343, and 322 days for low, mid, and high HSCB exposure, respectively. For hamsters the corresponding number was 42, 53, and 309 days (Elder *et al.*, 2005, Carter *et al.*, 2006).

The pattern of deposition of particles depends on the particle diameter (aerodynamic or thermodynamic) and on the anatomical and physiological characteristics of the host. The deposition fraction for particles such as carbon black within the respiratory tract may vary depending on the size of the agglomerates and influences the dose to a given region of the respiratory tract. Pre-existing lung diseases or conditions can also influence deposition patterns.

Translocation of carbon black particles from the site of deposition to other tissues

Once inhaled, nanoparticles deposit on the lung surface and have first contact with the epithelial lung lining fluid (ELF) rich in proteins, which may bind to nanoparticles. In contrast to micrometer-sized particles, nanoparticles are similar in size to proteins or not much larger. These nanoparticle–protein complexes may play a crucial role in the penetration of the air–blood barrier into the circulation and the accumulation in other organs, when those proteins may serve as ferry-boats carrying the nanoparticles across body membranes through either transcellular and/or paracellular routes, and within body fluids (Fertsch-Gapp *et al.*, 2011).

Oral gavage. Female Swiss mice, aged 4 weeks and 18 months, were given 7 mg <sup>7</sup>Be-labelled furnace black particles (Elftex 8). The distribution of the isotope was determined in the animals 4 hours and 1, 2, 5 and 14 days after exposure. The authors concluded that there was uptake and distribution from the gut and that transit was more rapid in young mice. Peyer's patches (a gut-associated lymphoid tissue) of older mice took up more radiolabel than those of younger mice (LeFevre and Joel, 1986). IARC noted that it was not clear from the study whether the authors verified the stable binding of the radiolabel to the particles.

Intratracheal instillation. Oberdörster *et al.* (1992) found that the translocation of particles in rats from the alveolar lumen of the lungs was dependent on particle size. Following intratracheal instillation of 0.5 mg particles of different sizes, the smaller ultrafine particles (12 and 20 nm) penetrated the alveolar epithelial cell barrier and entered the lung interstitium to a greater extent than an equal mass of larger respirable particles (> 200 nm) within 24 hours. This proportion was shown to increase with increasing particle dose as either mass or surface area.

Inhalation. Oberdörster *et al.* (2002) showed that inhaled spark generated ultrafine <sup>13</sup>C-carbon black particles of approximately 25 nm in diameter were cleared rapidly from rat lungs and translocated to other organs (e.g. liver and spleen). Significant amounts of particles were found in the livers of rats in the high-exposure group (approximately fivefold higher amounts in the liver than in the lung at 24 hours).

Inhalation of ultrafine particles may also result in translocation of particles to the brain. Ultrafine insoluble <sup>13</sup>C-carbon black particles (CMD, 36 nm; GSD, 1.66) were found in the brains of Fischer 344 rats on days 1–7 following a 6-hour inhalation exposure to 160 µg/m<sup>3</sup> (Oberdörster *et al.*, 2004). Approximately 50% of the inhaled ultrafine particles were predicted to deposit in the olfactory mucosa (assuming equal distribution) of rats and approximately 20% of that amount was found in the olfactory bulb. On day 1 after exposure, 0.35 µg/g of added <sup>13</sup>C was detected in the olfactory bulb; the amount increased on days 3 and 5 after exposure and reached 0.43 µg/g on day 7. The cerebrum and cerebellum contained significantly increased concentrations of <sup>13</sup>C on day 1, but the levels tended to decrease subsequent to exposure. The study was not designed to distinguish between the possible paths through which <sup>13</sup>C ultrafine particles could reach the brain, including crossing the blood–brain barrier (by particles that translocated into the blood following deposition anywhere in the respiratory tract) and transport of particles that deposited in the nasal olfactory mucosa along the olfactory nerve to the olfactory bulb. However, the authors concluded that the olfactory nerve pathway was the most probable



explanation for the  $^{13}\text{C}$  found in the olfactory bulb because of the significant increase in amounts in that region.

*Intra-arterial infusion.* Khandoga *et al.* (2004) infused intra-arterially carbon black (Printex 90) in C57BL/6 mice. Phagocytic activity of Kupffer cells were analyzed by intravital video fluorescence microscopy in the liver microvasculature. Accumulation of particles in the liver exerts a strong procoagulant impact but did not trigger an inflammatory reaction and does not induce microvascular/hepatocellular tissue injury.

#### *Kinetics of carbon black-adsorbed material*

Concern have been raised that material, including carcinogenic compounds, adsorbed onto carbon black particles are retained longer in the lung upon inhalation and will subsequently lead to a greater availability of carcinogens to target cells in the lung. The size of individual ultrafine particles may allow their entry into cells and cellular organelles more readily than larger particles or agglomerates. In a study of concentrated particles from air pollution (including carbon particles) in human bronchial epithelial cells and mouse alveolar macrophages, the ultrafine fraction (< 100 nm) was found to penetrate the cells, localize in mitochondria and cause oxidative damage to mitochondrial membranes (Li *et al.*, 2003). Several studies demonstrate that carbon black administered to rats and hamsters either by inhalation or intratracheal instillation can act as a carrier of adsorbed material, which is subsequently cleared from the lung much more slowly than the material given alone.

#### *Cellular uptake of nanomaterials*

Belade *et al.* (2012) compare the intracellular uptake of three different nanomaterials, two made of carbon black (CB13 [aerodynamic diameter 13 nm, specific surface area  $373.1 \pm 10.5 \text{ m}^2/\text{g}$ , purity 99%] and CB21 [aerodynamic diameter 21 nm, specific surface area  $106.2 \pm 0.8 \text{ m}^2/\text{g}$ , purity 99%]) and one from  $\text{TiO}_2$  [aerodynamic diameter 15 nm, specific surface area  $140.9 \pm 3.3 \text{ m}^2/\text{g}$ , purity 99.7%] in the human bronchial epithelial cell line, 16HBE, and human bronchial fibroblast cell line, MRC5. Transmission electron microscopy was used to evaluate the intracellular accumulation.

The results showed that the three nanomaterials accumulated in a high percentage of cells overall (60–80%) and was close to the peak value after only 6 h. Accumulation occurred even with the lowest dose of  $0.5 \mu\text{g}/\text{cm}^2$ . The nanoparticles accumulated chiefly as aggregates in cytosolic vesicles and were absent from the mitochondria or nuclei. The differences between the results with the three nanomaterials and the two cell lines were quantitatively minor. Thus, intracellular nanoparticle accumulation seems to be a common and rapid phenomenon that occurs in both epithelial and mesenchymal cells.

In both cell types, invaginations of the plasmic membrane were observed. Together with the finding that the nanoparticles were found chiefly as aggregates located in cytosolic vesicles, these observations suggest an endocytosis-mediated mechanism of internalization occurred. In previous studies, various endocytic pathways have been suspected to be involved in nanomaterials accumulation (e.g., macropinocytosis, clathrin-mediated endocytosis, or caveolae-mediated endocytosis). No endotoxin contamination was detected in any of the three nanomaterials used by Belade *et al.* (2012).

Hussain *et al.* (2009) found in studies with 16HBE cells that carbon black nanoparticles induced oxidative stress and pro-inflammatory responses correlate not only with the surface area of the individual nano particles but also with the internalized amount of nanoparticles. Moreover, carbon black nanoparticles induced apoptotic cell death in 16HBE by a ROS dependent mitochondrial pathway (Hussain *et al.*, 2010).

It is known that at certain workplaces, exposure to carbon black nanoparticles and aromatic amines may occur simultaneously. Sanfins *et al.* (2011) studied the interactions of carbon black nanoparticles (FW2; 13 nm) with aromatic amine metabolizing enzymes in extracts from Clara cells. They found that carbon black interfered with the enzymatic acetylation of

carcinogenic aromatic amines and that that exposure to carbon black altered the acetylation of 2-aminofluorene in intact lung Clara cells by impairing the endogenous NAT-dependent pathway. These effects may represent an additional mechanism that contributes to the carcinogenicity of inhaled carbon black nanoparticles.

Saxena *et al.* (2008) studied the uptake of diesel exhaust particles (DEP) and carbon black (Printex 90) by LA4 lung epithelial cells and MHS alveolar macrophages. Both cell types ingested DEP to a similar degree; however, the MHS macrophages took up significantly more carbon black than the epithelial cells. Cytochalasin D, an agent that blocks actin polymerization in the cells, inhibited the uptake of both DEP and carbon black by both cell types, indicating that the process was actin-dependent in a manner similar to phagocytosis. In their study the uptake was assessed after 3, 7, and 24 h. The uptake of carbon black after 3 h was in both cell type about 2/3 – 3/4 of that found after 24 h.

Belade *et al.* (2012) did not find nanomaterials in the mitochondria or nuclei. However, in other studies that used primary human monocyte macrophages or mesenchymal stem cells, nanomaterials were found in these compartments (Hackenberg *et al.*, 2011; Porter *et al.*, 2006), suggesting that the site of accumulation may depend on the nanoparticles type and/or cell type. Internalised nanomaterials in target organelles such as the mitochondria or the nucleus may directly or indirectly induce DNA oxidative damages.

The ability of nanoparticles to enter the bacterial cells is likely to be less than in human cells for two reasons. Firstly, prokaryotes cannot perform endocytosis and secondly, their cell wall forms a barrier against simple diffusion of nanoparticles (particularly those in agglomerated form) into the bacterial cell – this reduced uptake could potentially lead to false negative results.

### 3.3.10. Photo-induced toxicity

No studies found

#### 3.3.10.1. Phototoxicity / photoirritation and photosensitisation

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#### 3.3.10.2. Phototoxicity / photomutagenicity / photoclastogenicity

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### 3.3.11. Human data

Condensed from IARC (2010)

#### Deposition and retention of inhaled carbon black particles in the human respiratory tract

The main exposure route for carbon black is inhalation. Although no quantitative data are available, studies of tissues from workers in carbon black factories have shown that widespread deposits of large amounts of carbon black are retained in the lungs (Beck *et al.*, 1985).

Several authors have reported that the total deposition fraction of nano aerosols in humans, increased with decreasing particle size (Jaques and Kim, 2000, Daigle *et al.*, 2003, Frampton *et al.*, 2004, Kim and Jaques, 2005). For example, the deposition fraction for particles with median sizes of 65 nm and 8.7 nm increased from 0.63 to 0.74, respectively, in healthy subjects. The deposition fraction of particles increased to a similar extent with increases in either tidal volume or respiratory period. During exercise, tidal volume

increased and residence time decreased relative to measurements taken at rest. Thus, Frampton *et al.* (2004) found that the deposition fraction increased from 0.84 to 0.94 during exercise for the same particle sizes and study group.

Gender was not found to affect the deposition fraction significantly in the Frampton *et al.* (2004) study in the two groups that had sufficient numbers to address this variable. In contrast, Jaques and Kim (2000) reported a greater total deposition fraction in women compared with men with the same breathing pattern, particularly for the smaller ultrafine particles (40 nm), although inter-subject variability was similar.

Brown *et al.* (2002) reported greater deposition of nano particles in individuals who had obstructive lung disease compared with healthy individuals. Asthmatics had an approximately 50% higher total deposition fraction of ultrafine carbon particles in the respiratory tract as either total number or mass deposited (Chalupa *et al.*, 2004; Frampton *et al.*, 2004).

Carbon black nanomaterials in cosmetic products are present as aggregates or agglomerates. The question has been raised as to whether inhaled carbon black may deagglomerate or disaggregate to either smaller aggregates or primary particles when in contact with lung fluids. This question relates to the concern that there may be additional hazards of smaller particles, such as their ability to translocate to tissues and organs beyond the lung and the ability to pass through the blood–brain barrier. Levy *et al.* (2012) have on the basis of a review of the physical characteristics of commercial carbon black and various toxicology studies concluded that commercially produced carbon black in contact with lung fluid is unlikely to deagglomerate or disaggregate into smaller aggregates or primary particles.

For particles that deposit in the alveolar region, the primary mechanism of clearance is by alveolar macrophage phagocytosis, migration to terminal bronchioles and the 'mucociliary escalator', through which particles are eventually swallowed or expectorated (Oberdörster, 1988). Particles that deposit in the alveolar region are associated with the slow clearance phase (retention half-times of months to years in humans) (Bailey *et al.*, 1985; IARC, 1996). In a study of coal miners, little or no clearance of particles was observed (by magnetopneumography) 1 year after their retirement from the mine (Freedman and Robinson, 1988; Freedman *et al.*, 1988). Carbon black nonparticles may be retained in the lungs to a greater extent than larger respirable particles because they escape alveolar macrophage phagocytosis (Renwick *et al.*, 2001). Kuempel *et al.* (2001) reported that the end-of-life lung dust burdens in the coal miners were substantially higher than expected from first-order clearance kinetics, yet lower than expected from the overloading of alveolar clearance predicted from rodent studies. Moreover, the human lung kinetic model predicts greater retained lung dust burdens from occupational exposure than predicted from current human models based on lower exposure data. They found that the mean lung dust burden at death (on the average 10 years after retirement) was 13.8 g.

In a study of healthy humans who inhaled ultrafine carbon particles (count median diameter (CMD), 25 nm; geometric standard deviation (GSD), 1.6) mild dysfunction of the small airways (increased airways resistance, was seen as reduced maximal mid-expiratory flow rate [forced expiratory flow (FEF<sub>25–75%</sub>)] (Pietropaoli *et al.*, 2004). The exposures were relatively low (single 2-hour exposures to 50 µg/m<sup>3</sup>, an ambient concentration that is found near major roads) and the individuals were healthy (no pre-existing lung disease). Bronchoconstriction was offered as the most probable mechanism, in part because pulmonary inflammation (as assessed by sputum), which would have been another possible explanation, was not observed. Reduced alveolar gas exchange (measured as reduced carbon monoxide diffusing capacity) was also observed, which was attributed to vasoconstriction. No adverse effects were observed in normal or asthmatic individuals who received single, 2-hour exposures to 10 µg/m<sup>3</sup> ultrafine carbon; the effects observed in the group exposed to 50 µg/m<sup>3</sup> were reversible.

### Extrapulmonary translocation of carbon particles in humans

The translocation of coal dust particles of respirable size [specific size not noted] from the respiratory tract to other tissue sites has been observed in coal miners. Black pigment observed in the liver and spleen was associated with years in mining and severity of coal workers' pneumoconiosis (LeFevre *et al.*, 1982). To reach the liver and spleen, the particles would have had to enter the blood circulation. It is not clear whether this was due to particles being cleared by the mucociliary clearance, being swallowed and entering the gastrointestinal tract and then being taken up in the blood, or whether the particles were able to pass through damaged epithelial and endothelial cells into the blood, as could occur under conditions of disease.

Some fraction of particles that deposit in the alveolar region may also be translocated to the lung-associated lymph nodes. This may occur by transepithelial migration of alveolar macrophages following phagocytosis of the particle or by translocation of free particles to the interstitium, where they may be phagocytosed by interstitial macrophages. Inflammation may alter mucociliary clearance, phagocytosis by alveolar macrophages and the uptake and transport of particles to and through the respiratory epithelium.

### Respiratory effects in carbon black workers

Gärtner and Brauss (1951) first described radiological changes analogous to pneumoconiosis in 31 workers in a carbon black factory. However, these individuals had no lung function abnormality. Since that time, a series of other reports have been published on pneumoconiosis in carbon black workers.

Exposure–response relationships were evident for symptoms of chronic bronchitis, small opacities on chest radiographs and several respiratory parameters (forced expiratory volume in 1 second [FEV1], FEF25–75%). Studies in Germany (Küpper *et al.*, 1996) and Poland (Szozda, 1996) provided evidence of a relationship between exposure to carbon black and lung function among smokers. The Polish studies also reported cases of hypertension and pneumoconiosis among carbon black workers. Spirometry, body plethysmography and inhalation challenge tests were conducted among employees at a German carbon black production plant to assess the impact of fine carbon black dust on pulmonary function, to determine the prevalence of obstructive airway disease among the workers and to investigate whether exposure to fine dust is related to the prevalence of bronchial hyper-responsiveness. A total of 573 exposed workers (178 nonsmokers, 107 former smokers, 288 smokers) and 99 controls (46 nonsmokers, 13 former smokers, 40 smokers) participated in the study. Measurements of dust in air showed concentrations of 0.01–9.14 mg/m<sup>3</sup> for fine dust (9–200 nm [includes fine and ultrafine sizes]) and 1.08–19.95 mg/m<sup>3</sup> for total dust (mean dust concentrations, 0.58 mg/m<sup>3</sup> for respirable dust; 1.08 mg/m<sup>3</sup> for inspirable dust). Exposure to carbon black had a small but statistically significant impact on lung function in smokers ( $P < 0.01$ ). Exposed smokers displayed signs of obstructive airway disease more frequently (7.3%) than exposed nonsmokers (3.9%). There was no effect of exposure to carbon black on lung function in former smokers or nonsmokers. Exposure to carbon black dust was not associated with an increased prevalence of bronchial hyper-reactivity (Küpper *et al.*, 1996).

The results of two studies of respiratory health of European Carbon Black workers showed exposure-related adverse effects of carbon black on the respiratory system, which were evident from an increase in the prevalence of cough and sputum production, and reductions in lung function, based on measurements of FEV1, FEF25–75% and the FEV1/forced vital capacity (FVC) ratio. An increase in exposure to inhalable dust of 1 mg/m<sup>3</sup> was associated with an increase of 80% in the prevalence of respiratory symptoms of chronic bronchitis. The prevalence of respiratory symptoms such as cough and cough and sputum production, however, was significantly affected by an increase of 1 mg/m<sup>3</sup> in exposure. Working for 40 years with a mean exposure of 1 mg/m<sup>3</sup> (480 mg.month/m<sup>3</sup>) was expected to increase the prevalence of cough by almost 70% and that of cough and sputum production by 60%.

Similarly, a 1 mg/m<sup>3</sup> increase in exposure to carbon black was associated with significant decrements in FEV1, FEF25–75% and FEV1/FVC ratio (Gardiner *et al.*, 2001).

Harber *et al.* (2003) investigated whether exposure to carbon black was associated with decrements in lung function and increased prevalence of respiratory symptoms among 1755 employees from 22 North American carbon black manufacturing plants. Multiple linear regression analyses showed that cumulative exposures to 'total' and inhalable dust were both associated with a statistically significant decrement in FEV1 and with FVC. The slopes were -2 mL and -0.7 FEV1/mg-year/m<sup>3</sup> for cumulative exposure to 'total' and inhalable dust, respectively. Cumulative exposure was also associated with an increased prevalence of chronic bronchitis in nonsmokers.

In a study from Iran, Neghab *et al.* (2011) investigated 72 male workers with a past history of and current exposure to carbon black and 69, randomly selected, healthy unexposed male office workers that served as referent group. The levels of exposure to inhalable and respirable carbon black dusts were estimated to be 6.2 ± 1.7<sup>5</sup> and 2.3 ± 0.29 mg/m<sup>3</sup> respectively (mean ± SD). Regular cough, phlegm, wheezing and shortness of breath were significantly (*p*<0.05) more prevalent among exposed workers. Furthermore, significant decreases in some pre-shift and post-shift parameters of pulmonary function of exposed workers with a spirometric pattern consistent with restrictive ventilatory disorder were found. The authors concluded that the study provide circumstantial evidence to support the notion that exposure to carbon black exceeding its current TLV is associated with a significant increase in the prevalence of respiratory symptoms along with both acute, partially reversible and chronic irreversible significant decreases in some parameters of pulmonary function.

### 3.3.12. Special investigations

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### 3.3.13. Safety evaluation (including calculation of the MoS)

The calculation of margin of safety (MoS) is not relevant for this assessment given the very low, if any, dermal penetration of nano-carbon black when applied on skin, and in consideration of the low toxicity (NOAEL for oral administration of carbon black to rats as 1000 mg/kg bw/d) observed.

The NOAEL for inhalation of carbon black nanomaterials for rats, mice and hamsters is reported to be 1 mg/m<sup>3</sup>. In view of the potential for persistent lung inflammation, similar to other nanomaterials evaluated so far, applications that might lead to inhalation exposure of the consumer to carbon black nanoparticles (such as powders or sprayable products) are not recommended.

### 3.3.14. Discussion

#### **General considerations**

Carbon black nanoparticles are non-porous materials engineered to be sorbents, but by virtue of their very small primary particle sizes, carbon black possesses significant surface areas for adsorption. Because of its ability to adsorb, caution must be taken when evaluating results from testing of carbon black nanomaterials.

<sup>5</sup> The TWA in most European countries is 3.5 mg/m<sup>3</sup>.

**Physicochemical properties**

Carbon black (CAS 1333-86-4) is a material consisting of elemental carbon in the form of near spherical colloidal particles and coalesced particle aggregates/agglomerates, obtained by partial combustion or thermal decomposition of hydrocarbons. Carbon black materials are categorized as acetylene black, channel black, furnace black, lampblack or thermal black according to their manufacturing process. Carbon black is available as a black powder or a liquid dispersion. Commercial carbon black materials generally contain more than 97% elemental carbon. Less than 1% of carbon black particles consist of extractable organic materials. Typical classes of organic chemicals adsorbed onto the carbon black particles surface are polycyclic aromatic hydrocarbons (PAHs), nitro-derivatives of PAHs and sulphur-containing PAHs.

The different carbon black materials are characterized by their primary particles size, their aggregation and agglomeration status as well as their impurity profile. Typically, the average primary particle diameter of commercial carbon black materials ranges from 10 to 100 nm, while the average aggregate/agglomerate size is in the range 100-800 nm or above. The majority of published studies reported in the present dossier were performed with carbon black materials obtained by the oil furnace process. "Non-nano" grades of carbon black have no pigmentary interest. Carbon Black nanoparticles are only used as "uncoated nanomaterials".

Typical use concentrations in cosmetic preparations range from 0.001% to 10% with 0.001% for skin products, 5% for nail enamels and mascaras and up to 10% for other eye decorative products such as eyeliners, eye pencils and eye shadows.

Description of purity relates up to 98% of carbon black content. More information on the balancing impurities including well-known metallic contact allergens such as Co, Ni and Cr should be provided. Purity and impurity profiles of carbon black materials should comply with EU specifications of carbon black materials as used in food contact materials. Additionally, the materials should comply with FDA specifications with respect to total PAH < 500 ppb and benzo(a)pyrene < 5 ppb, dibenz(a,h)anthracene < 5ppb, As < 3 ppm, Pb < 10 ppm, and Hg < 1 ppm.

**Acute toxicity**

The acute toxicity of carbon black has only been studied in rats by the oral route. The maximal non-lethal dose of carbon black after single administration by the oral route in rats is higher than 10 000 mg/kg bw.

SCCS is of the opinion that acute oral toxicity of carbon black is unlikely to be of concern.

**Irritation**

Skin irritation has been studied by applying carbon black on intact and abraded rabbit skin under occlusive conditions for up to 24 hours without any cutaneous signs of oedema or erythema.

Under the conditions of the *in vivo* studies, carbon black is not considered to be irritating to rabbit skin.

The reconstructed human epidermis Episkin<sup>SM</sup> model for skin irritation testing has not been validated for nanomaterials. In addition, residual carbon black may interfere with the spectrometric analysis in the viability and cytokine assays. Therefore, the results of Episkin assay are inconclusive.

Eye irritation potential of 3 brands of carbon black materials - furnace blacks Printex G (Degussa AG, 1977d); Spezienschwarz 4 (Degussa AG, 1977e); and Printex-140 (Degussa AG, 1978c) was evaluated following a single instillation of 100 mg of the neat material to



the rabbit eye according to a test protocol similar to the OECD TG 405. No signs of ocular irritation were observed in any of the animals at any reading time. Under the conditions of the *in vivo* studies, the three brands of carbon black are not irritating to rabbit eyes.

The BCOP assay for eye irritation testing has not been validated for nanomaterials. In addition, residual carbon black may interfere with opacity and permeability analysis in the assay. Therefore, the results of BCOP assay are inconclusive.

### **Sensitisation**

Skin sensitisation of carbon black has been tested with the *Buehler test* according to OECD guideline and did not elicit any skin reactions. Carbon black has also been tested in the *Local Lymph Node Assay* (LLNA) and did not induce any proliferative responses. Based on these studies, it was concluded by the applicant that carbon black is not a contact sensitizer.

SCCS is of the opinion that it is difficult to draw a valid conclusion from the tests on skin sensitisation since it is unlikely that the carbon black particles penetrated the skin to reach the cellular targets of the immune system. In view of this, sensitisation potential of carbon black cannot be ruled out, for example in damaged skin. However, carbon black may act as an adjuvant when inhalation exposure occurs in combination with an allergen.

### **Dermal absorption**

SCCS notes that due to the nature of the test material, no sensitive analytical method could be used. Whereas imaging is considered the only realistic method, this method is not considered as sensitive enough. Insofar, the test results have only limited predictive value. Three *in vitro* dermal penetration studies have been provided with the submission. All studies had shortcomings e.g. the amount of carbon black available at the surface of the skin in the chamber is uncertain and may be lower than anticipated, the amounts of aggregates present initially and at the end of the 24 hours are uncertain.

SCCS is of the opinion that the available data show that there is no indication of carbon black particles (> 20 nm) being absorbed through the intact skin. SCCS also notes that no information is available in relation to particles smaller than 20 nm.

### **Repeated dose toxicity**

Oral administration. In a 13-week study with rats performed according to OECD guidelines, the animals received carbon black nanomaterials (diameter 20 – 30 nm) by gavage (0, 100, 300, and 1000 mg/kg/d) for 90 consecutive days. It was concluded that carbon black was well tolerated, with only some non-adverse findings related to the staining properties of carbon black. Under the conditions of the study, the NOAEL was 1000 mg/kg/day.

Inhalation exposure. In a 4-week nose-only inhalation study (4 h/day) the levels of IL-10 were significantly elevated in BALF from the exposed rats (Kim *et al.* 2011). The SCCS considers this as a poorly carried-out study because no BALF cell count was performed and no information concerning group sizes was available. Another study (Lim *et al.* 2012) showed mild respiratory toxicity in male rats exposed to nano-sized carbon black for 13 weeks at a concentration of approximately 9 mg/m<sup>3</sup> through nose-only inhalation. Female mice, rats, and hamsters were exposed to carbon black (diameter 14 nm) by inhalation for 13 weeks) at dose levels of 0, 1, 7, and 50 mg/m<sup>3</sup>. Lung inflammation and histopathology were observed to be more severe and prolonged in rats than in mice and hamsters. Hamsters showed the most efficient clearance mechanisms and the least severe responses of the three species. In these studies, the mid (7 mg/m<sup>3</sup>) and high (50 mg/m<sup>3</sup>) doses of carbon black induced pathological lung alterations in mice and rats that were

persistent as they were not resolved after the recovery period up to 11 months after exposure.

SCCS notes that the responses after inhalation of carbon black at 1 and 7 mg/m<sup>3</sup> among rats, mice and hamsters were similar in magnitude. The NOAEL for inhalation of carbon black nanomaterials for rats, mice and hamster was 1 mg/m<sup>3</sup>, while the NOAEL for oral administration of carbon black to rats was 1000 mg/kg bw/d.

## **Mutagenicity / genotoxicity**

### In vitro studies

#### *Cell-free systems*

Measurement of single strand DNA breaks production, using supercoiled plasmid DNA as indicator, showed that nano carbon black particles (diameter 14 nm) exhibited significantly more free radical activity than fine carbon black particles (diameter 260 nm). The results with fine carbon black did not differ significantly from the control.

#### *Bacterial tests*

Bacterial tests of carbon black such as the Salmonella test are in general negative. This might in part be related to the size of bacteria, presence of bacterial cell wall in contrast to mammalian cells and the degree of nanomaterial uptake by the bacteria, which is likely to be less than in human cells. In view of this, the SCCS cannot accept negative results of Ames test where entry of nanoparticles into the bacteria has not been demonstrated.

#### *Mammalian cells*

The bioavailability of PAH to form DNA adducts was studied in A549 cells after incubation with different carbon black nano materials. PAHs were not bioavailable from three carbon black types (PAH = 0.039 ppm, PAH = 0.057 ppm, PAH = 2.4 ppm). Some evidence suggests that in the carbon black sample with high PAH content (PAH = 8.8 ppm) some PAHs are bioavailable for biotransformation and induction of PAH-DNA adducts.

The ability of carbon black to cause DNA breaks was assessed in the Comet assay with A549 cells, FE1 MML epithelial mouse cell line, and primary mouse embryo cells. It was found that carbon black nano particles (14 nm), but not fine carbon black (260 nm), induced single strand breaks. The carbon black nanoparticles did also induce ser15 phosphorylation of p53 protein in one experiment with FE1 MML cells.

Carbon black (diameter 20-30 nm) did not induce mutation at the *hprt* locus of L5178Y mouse lymphoma cells in an OECD Guideline study. However, only a 3-hr exposure time was used which may not be sufficient in the case of nano particles.

Carbon black (diameter 14 nm) exposure increased the mutant frequency of the *cII* locus and *lacZ* locus compared with identically passaged untreated cells in a study with FE1 MML cells. In a follow up study, the largest increases in base substitutions were observed in G:C→T:A, G:C→C:G, and A:T→T:A transversion mutations; this is in keeping with a genetic finger print of ROS and is further substantiated by the observations that carbon black generates ROS and oxidatively damaged DNA.

Carbon black (diameter 20-30 nm) did not produce micronuclei in cultured CHO cells either in the absence or in the presence of metabolic activation and was therefore considered to have no clastogenic or aneugenic potential. However, only a 3-hr exposure time was used and Cytochalasin B which inhibits endocytosis was present throughout the experiment.

SCCS notes that carbon black nano particles induce single strand breaks both in cell-free studies as well as in mammalian cells. In addition, carbon black has also induced mutations in an epithelial cell line. Two principle modes of genotoxic action can be considered for

carbon black nanomaterials, generally referred to as primary and secondary genotoxicity, the latter attributable to the pro-inflammatory effects of the material. The genotoxic effects of nano carbon black *in vitro* are probably at least in part caused by primary genotoxicity. Conceptually, primary genotoxicity might operate via various mechanisms, such as the actions of ROS (e.g., as generated from reactive particle surfaces), or DNA-adduct formation by reactive metabolites of particle associated organic compounds (e.g., polycyclic aromatic hydrocarbons).

### *In vivo studies*

#### *Inhalation*

Two types of nano carbon black did not induce DNA-PAH adducts in the lung ( $^{32}\text{P}$  post-labeling analysis of lung samples) of F344 female rats after a 13-week inhalation exposure. Since *in vitro* evidence suggests that one carbon black type (diameter 70 nm, PAH = 8.8 ppm), but not the other (diameter 14 nm, PAH = 0.039) induced PAH adducts after exposure of A549 cells, the present results suggest that DNA repair processes may have occurred *in vivo* during and/or after subchronic inhalation exposure.

The ability of carbon black nanomaterials to induce 8-oxo-dG formation in DNA in the lungs of female F344 rats was studied after inhalation exposure to two types of carbon black (diameter 14 nm and 70 nm) for 13 weeks. The results suggest that prolonged, high-dose exposure to the small carbon black nanomaterials, but not to the larger ones, can promote oxidative DNA damage. This is consistent with the hypothesis that inflammatory cell-derived oxidants may play a role in the pathogenesis of rat lung tumours following long-term high-dose exposure to carbon black in rats.

A significant increase in *hprt* mutation frequency was found in alveolar epithelial cells after exposure of male rats for 13 weeks to 7.1 mg/m<sup>3</sup> and 52.8 mg/m<sup>3</sup> carbon black (diameter 16 nm). In another experiment, cells obtained from bronchoalveolar lavage (BAL) of animals after carbon black (diameter 14 nm) exposure for 13 weeks were evaluated for their ability to cause *hprt* mutation in rat alveolar epithelial cells *in vitro*. The increase was larger in female rats than in female mice and was found at exposure levels of 7 and 50 mg/m<sup>3</sup> and not at 1 mg/m<sup>3</sup>. The high doses did also induce lung inflammation.

#### *Intratracheal instillation*

Three studies have been carried out on DNA strand breaks in mice after intratracheal instillation of nano carbon black particles. Characterisation of the instillation media revealed that all particles were delivered as agglomerates and aggregates. DNA strand breaks in the lungs were observed already 3 h after instillation of 54 µg carbon black (diameter 14 nm). In a second experiment, DNA strand breaks were detected in lungs even at the lowest dose of carbon black (18 µg) on post-exposure day 1 and remained elevated at the two highest doses (54 and 162 µg) until day 28. BAL cell DNA strand breaks were elevated relative to controls at least at the highest dose on all post-exposure days. DNA strand breaks were observed in the liver at all doses on day 1 and 28, but not on day 3. No dose relationship was found. In the third study, no DNA damage in BAL cells was observed at the dose tested (54 µg) after 24-h exposure. The lack of effect is in agreement with the second study where DNA strand breaks after 24 h in BAL cells were only found at a higher dose.

#### *General remarks*

*In vivo* studies show that carbon black nanomaterials after inhalation or intratracheal instillations induce 8-oxo-dG formation and single strand DNA breaks in lungs of rats, and increase in *hprt* mutation frequency in both mice and rats. Since inflammation seems to be involved in most of these effects they may be due to a secondary effect caused by reactive oxygen species (ROS). However, findings also indicate DNA strand breaks in the liver of mice exposed to carbon black by intratracheal instillations or inhalation, and in offspring of mice that were exposed by inhalation during pregnancy (see *Reproductive toxicity*). These

findings suggest that carbon black may induce genotoxic effects also by primary mechanisms. This view is further supported by the *in vitro* studies of carbon black in both cell free systems and in non-immune cell types.

### ***Carcinogenicity***

#### *Long term rodent carcinogenicity studies*

Carbon black nanomaterials have been tested for carcinogenicity by oral administration in female rats and female mice, by inhalation exposure in rats and female mice, by intratracheal administration in female rats, and by dermal administration in mice.

No carcinogenic effect was observed after oral or dermal exposure. However, the studies are old and incompletely reported. Thus, no conclusion can be drawn from the studies.

No increased tumour frequency was found in a mice inhalation study where female mice were exposed to 10 mg/m<sup>3</sup> carbon black (diameter 14 nm) for 13.5 months and kept for an additional 9.5 months.

Two different carbon black products (diameter 14 nm and 37 nm) were tested by inhalation exposure at doses of 2.5 – 6.5 mg/m<sup>3</sup> in two studies in female rats and in one study in rats of each sex. Significant increases in the incidence of malignant lung tumours or of benign and malignant lung tumours combined were observed in female rats in all three studies. No tumour increase was found among the male rats.

In two studies with intratracheal administration to female rats using two types of carbon black (diameter 14 nm and 95 nm) (doses of 3mg x 15 or higher), and in one study using one type (diameter 14 nm), an increased incidence of malignant lung tumours or of benign and malignant lung tumours combined was observed. The increase in tumour frequency was dependent on the size of the particles as the smaller particles had the highest potency.

SCCS is of the opinion that carbon black can induce malignant tumours in female rats after inhalation exposure or intratracheal instillations. The potency of carbon black particles with diameter of 14 nm was higher than the potency of carbon black particle with diameter of 95 nm. There is no empirical support for a dose threshold from the animal carcinogenicity studies.

#### *Epidemiological carcinogenicity studies*

In the evaluation of the epidemiological studies on carbon black, the IARC Working Group (IARC, 2010) pointed out that the human epidemiological evidence was inconsistent. Two of the three studies of carbon black production workers observed excess risk for lung cancer and other studies provided mixed evidence for an increased risk for lung and other cancers. The few studies that assessed exposure–response for lung cancer, including the two that observed excess risks compared with the general population, provided weak or inconclusive evidence of a dose–response. Overall, these results led the Working Group to conclude that there was *inadequate evidence* from epidemiological studies to assess whether carbon black causes cancer in humans.

#### *General evaluation*

In the IARC evaluation (IARC, 2010) it was concluded that “There is *inadequate evidence* in humans for the carcinogenicity of carbon black” and that “There is *sufficient evidence* in experimental animals for the carcinogenicity of carbon black. The overall evaluation was: Carbon black is *possibly carcinogenic to humans (Group 2B)*.”

In the evaluation, a minority group of the Working Group supported the classification of carbon black in Group 2A, and invoked the analogy with quartz particles, which are carcinogenic in the lung of rats and humans. However, based on current evidence, the Working Group considered that the degree to which all elements of the above-mentioned

mechanism may operate in humans is not clear and, thus, the mechanistic information did not alter the overall evaluation of Group 2B.

*SCCS concludes* that carbon black induced lung tumours in rats after inhalation and intratracheal instillation. The studies on the genotoxicity of carbon black suggest that the nano particles may induce genotoxic effects both by a primary and secondary genotoxic mechanism. High retained mass lung burdens and decreased lung clearance have been observed in coal miners.

*SCCS is of the opinion* that the animal cancer data are relevant to humans and that the use of nano carbon black in sprayable applications is not recommended.

### **Reproductive toxicity**

Five studies, four with mice and one with rats, have been found in relation to reproductive toxicity of carbon black.

No effect on litter size was found in a study where mice received intranasal instillations of carbon black (diameter 14 nm) on gestation days 5 and 9 (total 0.1 mg). However, collagen type VIII was increased in the kidney tubular cells of 12 week-old offspring. The study authors considered that the findings were similar to those of tubulo-interstitial fibrosis in diabetic nephropathy.

Time mated mice received intratracheal instillations of carbon black (diameter 14 nm) (altogether up to 0.268 mg, subdivided in 4 instillations). No effect was found on gestation and lactation. The results were presented in two articles. The first reported that a significant hepatic response at the mRNA level was observed in male and female offspring exposed prenatally to the highest dose of carbon black. Hepatic response was much more pronounced in the female offspring than in the male. In the second article, DNA strand breaks were studied. No effects were reported.

The second article reported also effects of time mated mice exposed to carbon black (diameter 14 nm) by inhalation (42 mg/m<sup>3</sup>, for altogether 10h). Maternal inhalation exposure to carbon black induced liver DNA damage in the mothers and in the *in utero* exposed offspring. Inhalation exposure did not affect gestation and lactation.

Time mated mice received four intratracheal instillations (altogether up to 0.268 mg) of carbon black. The F2 offspring, whose fathers were prenatally exposed to nanosized carbon black, showed lowered sperm production.

Pregnant rats received carbon black by oral administration of carbon black (diameter 20-30 nm) during the sensitive period of organogenesis. There were no adverse maternal changes or any effects on embryo-foetal development. The NOAEL for maternal toxicity and for developmental toxicity were both set at 1 000 mg/kg bw/d.

*SCCS is of the opinion that* oral exposure to carbon black is of little concern in relation to reproductive toxicity. This is also true for dermal exposure due to lack of any significant dermal penetration of carbon black nanoparticles of 20nm, or larger, in healthy intact skin. However, applications that may lead to inhalation exposure should be avoided.

### **Toxicokinetics**

The lung retention of inhaled carbon black particles has been studied in rats, mice, and hamsters. The pattern of deposition of particles depends on the particle diameter and on the anatomical and physiological characteristics of the host. The particle retention half-time increased with the exposure dose and rats had the longest retention half-time followed by

mice. Hamsters had the shortest retention half-time. Pre-existing lung diseases or conditions can also influence deposition patterns.

Once inhaled, the nanoparticles deposited in the airways have the first contact with the epithelial lung lining fluid (ELF), which is rich in proteins that may bind to nanoparticles. These nanoparticle–protein complexes may play a crucial role in the penetration from the air–blood barrier into the circulation and the accumulation in other organs; those proteins may indeed serve as ferry-boats carrying the nanoparticles across body membranes through either transcellular and/or paracellular pathways and within body fluids.

After oral administration there is uptake and distribution from the gut to gut-associated lymphoid tissue. Inhaled carbon black particles have been found to be translocated to other organs (e.g. liver and spleen) including the brain.

The intra-arterial infusion of carbon black in mice results in the accumulation of particles in the liver. The carbon black nanomaterials exerted a strong procoagulant impact but did not trigger an inflammatory reaction and did not induce microvascular/hepatocellular tissue injury.

Carbon black administered to rats and hamsters either by inhalation or intratracheal instillation can act as a carrier of adsorbed material, which is subsequently cleared from the lung much more slowly than the material given alone. In a study of intracellular uptake of carbon black nanomaterials, it was found that the nanomaterials accumulated in a high percentage of cells overall (60–80%) and reached the peak value after only 6 h. The nanoparticles accumulated chiefly as aggregates in cytosolic vesicles and were absent from mitochondria or nuclei. Studies of the mechanisms of cellular uptake suggest an endocytosis-mediated mechanism of internalization. Carbon black uptake by LA4 lung epithelial cells and MH-S alveolar macrophages showed that the macrophages took up significantly more carbon black than the epithelial cell.

*SCCS concludes that* translocation of carbon black nanomaterials after dermal exposure to other organs is unlikely. It has been reported that carbon black particles after oral administration may be distributed from the gut to lymphoid tissue. Inhaled carbon black nanomaterials may be retained in the lung for a considerable time period. However, translocation to other organs does also occur and the particles may be taken up by the cells and directly or indirectly induce DNA oxidative damages in the nuclear compartment. It has been reported that carbon black nanomaterials accumulated in the liver exert a strong procoagulant impact but do not trigger an inflammatory reaction or microvascular/hepatocellular tissue injury.

### **Human data**

Studies of tissues from workers in carbon black factories have shown that widespread deposits of large amounts of carbon black are retained in the lungs. The total deposition fraction of nano aerosols in humans increased with decreasing particle size. Greater deposition of nano particles have been found in individuals who had obstructive lung disease compared with healthy individuals. Gender does not affect the deposition fraction. According to the applicant, carbon black nanomaterials in cosmetic products will be present as aggregates or agglomerates. However, whilst the SCCS has accepted that nano particles in carbon black materials exist as aggregates and agglomerates, the applicant has not investigated the possibility that these may de-agglomerate in the presence of certain cosmetic ingredients such as emulsifiers.

For particles that deposit in the alveolar region, the primary mechanism of clearance is by alveolar macrophage phagocytosis, migration to terminal bronchioles and the 'mucociliary escalator', through which particles are eventually swallowed or expectorated. Particles that



deposit in the alveolar region are associated with the slow clearance phase (retention half-times of months to years in humans).

In a study of healthy humans, who inhaled ultrafine carbon particles, increased airways resistance, probably due to bronchoconstriction and seen as reduced maximal mid-expiratory flow rate, was observed. The exposures were relatively low (single 2-hour exposures to 50 µg/m<sup>3</sup>, an ambient concentration that is found near major roads).

The translocation of coal dust particles of respirable size from the respiratory tract to the liver and spleen has been observed in coal miners. To reach the liver and spleen, the particles would have had to enter the blood circulation. It is not clear whether this was due to particles being cleared by the mucociliary clearance, being swallowed and entering the gastrointestinal tract and then being taken up in the blood, or whether the particles were able to pass through damaged epithelial and endothelial cells into the blood, as could occur under conditions of disease. Some fraction of particles that deposit in the alveolar region may also be translocated to the lung-associated lymph nodes.

SCCS notes that studies of tissues from workers in carbon black factories have shown that widespread deposits of large amounts of carbon black are retained in the lungs. Particles that deposit in the alveolar region are associated with the slow clearance phase (retention half-times of months to years in humans). Commercially produced carbon black in contact with lung fluid is unlikely to de-agglomerate or disaggregate into smaller aggregates or primary particles. In healthy humans, who inhaled low concentrations of ultrafine carbon particles, increased airways resistance, probably due to bronchoconstriction, was observed. The translocation of coal dust particles of respirable size from the respiratory tract to the liver and spleen has been observed in coal miners.

#### 4. CONCLUSION

*1. Does the SCCS consider Carbon Black, CI 77266 in its nano form safe for use as a colorant with a concentration up to 10 % in cosmetic products taking into account the scientific data provided?*

On the basis of the available evidence, the SCCS has concluded that the use of carbon black CI 77266 in nano-structured form, with a size of 20 nm or larger at a concentration up to 10% as a colorant in cosmetic products, is considered to not pose any risk of adverse effects in humans after application on healthy, intact skin.

This opinion, however, does not apply to applications that might lead to inhalation exposure to carbon black nanoparticles, where the preparation might lead to inhalable particles.

*2. Does the SCCS have any further scientific concern with regard to the use of Carbon Black, CI 77266 in its nano form as a colorant in cosmetic products?*

The purity of carbon black nanomaterials used in cosmetic products should be >97%. The impurity profile of carbon black should be comparable with those nanomaterials tested for toxicity in this submission should comply with the EU specifications of carbon black materials as used in food contact materials, and should also comply with FDA specifications with respect to carbon black produced by furnace method<sup>6</sup>.

<sup>6</sup> Ash content ≤ 0.15%, total sulphur ≤ 0.65%, total PAH ≤ 500 ppb and benzo(a)pyrene ≤ 5ppb, dibenz(a,h)anthracene ≤ 5ppb, As ≤ 3 ppm, Pb ≤ 10 ppm, and Hg ≤ 1 ppm.

In the evidence provided in the submission, imaging was considered the only practical method by the applicant for investigating skin penetration. The use of this method is considered by the SCCS as only semi-quantitative. Other methods need to be explored.

This opinion is based on the currently available scientific evidence, which shows an overall lack of dermal absorption of carbon black nanoparticles. If any new evidence emerges in the future to show that the carbon black nanoparticles used in cosmetic products can penetrate skin (healthy, compromised, sunburnt or damaged skin) to reach viable cells, then the SCCS may consider revising this assessment.

Since the skin absorbance studies have only been performed with carbon black nanoparticles  $\geq 20$  nm, the current opinion applies to nano-structured form of carbon black with a particle size of 20 nm or larger. Additional information will be required on the use of carbon black with particles smaller than 20 nm size intended for use in cosmetic products.

## 5. MINORITY OPINION

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## 6. REFERENCES

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