Committee for Risk Assessment

RAC

Annex 1

Background document

to the Opinion proposing harmonised classification and labelling at EU level of

benzo[*rst*]pentaphene

EC Number: 205-877-5
CAS Number: 189-55-9

CLH-O-0000001412-86-159/F

The background document is a compilation of information considered relevant by the dossier submitter or by RAC for the proposed classification. It includes the proposal of the dossier submitter and the conclusion of RAC. It is based on the official CLH report submitted to public consultation. RAC has not changed the text of this CLH report but inserted text which is specifically marked as 'RAC evaluation'. Only the RAC text reflects the view of RAC.

Adopted

9 June 2017

CLH report

Proposal for Harmonised Classification and Labelling

Based on Regulation (EC) No 1272/2008 (CLP Regulation),
Annex VI, Part 2

Substance Name: Benzo[rst]pentaphene

EC Number: **205-877-5**

CAS Number: **189-55-9**

Index Number: **-**

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Date: **June 2016**

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Part A.

1 PROPOSAL FOR HARMONISED CLASSIFICATION AND LABELLING

1.1 Substance

Table 1: Substance identity

Substance name:	***Benzo(r,s,t)pentaphene***
EC number:	***205-877-5***
CAS number:	***189-55-9***
Other name(s)	***Dibenzo(a,i)pyrene***
Annex VI Index number:	**-**
Degree of purity:	**-**
Impurities:	***No impurity is considered relevant for the classification of the substance Benzo(r,s,t)pentaphene***

1.2 Harmonised classification and labelling proposal

Table 2: The current Annex VI entry and the proposed harmonised classification

	CLP Regulation
Current entry in Annex VI, CLP Regulation	-
Current proposal for consideration by RAC	Carc. 1B, H350 Muta. 2, H341
Resulting harmonised classification (future entry in Annex VI, CLP Regulation)	Carc. 1B, H350 Muta. 2, H341

1.3 Proposed harmonised classification and labelling based on CLP Regulation

Table 3: Proposed classification according to the CLP Regulation

CLP Annex I ref	Hazard class	Proposed classification	Proposed SCLs and/or M-factors	Current classification [1)]	Reason for no classification [2)]
2.1.	Explosives				
2.2.	Flammable gases				
2.3.	Flammable aerosols				
2.4.	Oxidising gases				
2.5.	Gases under pressure				
2.6.	Flammable liquids				
2.7.	Flammable solids				
2.8.	Self-reactive substances and mixtures				
2.9.	Pyrophoric liquids				
2.10.	Pyrophoric solids				
2.11.	Self-heating substances and mixtures				
2.12.	Substances and mixtures which in contact with water emit flammable gases				
2.13.	Oxidising liquids				
2.14.	Oxidising solids				
2.15.	Organic peroxides				
2.16.	Substance and mixtures corrosive to metals				
3.1.	Acute toxicity - oral				
	Acute toxicity - dermal				
	Acute toxicity - inhalation				
3.2.	Skin corrosion / irritation				
3.3.	Serious eye damage / eye irritation				
3.4.	Respiratory sensitisation	**None**		None	No data available
3.4.	Skin sensitisation				
3.5.	Germ cell mutagenicity	**Muta. 2**		None	
3.6.	Carcinogenicity	**Carc. 1B**		None	
3.7.	Reproductive toxicity	**None**		None	No data available
3.8.	Specific target organ toxicity –single exposure				
3.9.	Specific target organ toxicity – repeated exposure				
3.10.	Aspiration hazard				
4.1.	Hazardous to the aquatic environment				
5.1.	Hazardous to the ozone layer				

[1)] Including specific concentration limits (SCLs) and M-factors
[2)] Data lacking, inconclusive, or conclusive but not sufficient for classification

Labelling:	Signal word:	Danger
	Hazard statements:	H341 (Suspected of causing genetic defect) H350 (May cause cancer.)
	Precautionary statements:	-

Proposed notes assigned to an entry: -

2 BACKGROUND TO THE CLH PROPOSAL

This CLH proposal aims to classify and label of benzo(r,s,t)pentaphene (dibenzo[a,i]pyrene; DB[a,i]P) for mutagenicity and carcinogenicity. So far, DB[a,i]P was not listed in any priority list of the Existing Substance Regulation (Regulation 793/93/EC).

DB[a,i]P is a polycyclic aromatic hydrocarbon (PAH). PAH constitute a large class of compounds, and hundreds of individual substances may be released during incomplete combustion or pyrolysis of organic matter, an important source of human exposure. Studies of various environmentally relevant matrices, such as coal combustion effluents, motor vehicle exhaust, used motor lubricating oil, and tobacco smoke, have shown that the PAH in these mixtures are mainly responsible for their carcinogenic potential. However, only a small number of PAH are classified according to CLP Regulation as mutagenic category 1B (benz [a]pyrene (B[a]P)), mutagenic category 2 (chrysene (CHR)) or as carcinogen category 1B (B[a]P, benzo[e]pyrene (B[e]P), benzo[a]antracene (B[a]A), CHR, benzo[b]fluoranthene (B[-b]F), benzo[j]fluoranthene (B[j]F), benzo[k]fluoranthene (B[k]F) and dibenz [a,h]antracene (DB[a,h]A)). In addition, B[a]P is a classified reprotoxicant which is classified as Repr. 1B. Lack of classification for the other congeners as CMR does not necessarily reflect absence of corresponding toxic effects. In this dossier, the experimental data of DB[a,i]P were evaluated and a classification as mutagen and carcinogen according CLP Regulation is proposed.

Human exposure to DB[a,i]P occurs primarily through the smoking of tobacco, via inhalation of polluted air, through dermal contact with vapours and also by ingestion of food and water contaminated with combustion products. Inhalation and dermal contact are the primary routes of potential human exposure to DB[a,i]P.

PAH are contained in certain elastomer/rubber materials, but potentially also in plastic materials, lacquers/varnishes, or coatings that may be encountered in or as part of consumer products, including toys. Numerous examples of such products include e.g. tool handles, bicycle handlebars, slippers, flip-flops, beach sandals, diver equipment, toy car tyres, or clay pigeons used in skeet shooting. PAH may also be contained in synthetic turf or in materials used for construction work, e.g. flooring material. During recent years, a number of laboratories have frequently identified high PAH contamination levels in a significant fraction of analysed consumer articles, such as toys, tool handles, bicycle grips, shoes, sports equipment etc. (BfR 2009; UBA 2010; Wennemer 2009).

In analysis of consumer products for their PAH contents Hutzler et al. (2011) have identified four isomeric dibenzopyrenes: DB[a,l]P; DB[a,e]P; DB[a,i]P; DB[a,h]P. The sample with the highest dibenzopyrene content (hammer grip) was presented with 7.1 mg/kg for DB[a,i]P, 5.6 mg/kg for DB[a,h]P and 2.1 mg/kg for DB[a,l]P.

2.1 History of the previous classification and labelling

DB[a,i]P is not listed in Annex VI to Regulation (EC) No 1272/2008 on Classification, Labelling and Packaging of Dangerous Substances.

Based on the results both of *in vitro* testing (bacteria; proliferating cells of cell cultures) and *in vivo* testing (soma cells) DB[a,i]P is evaluated as genotoxic by international bodies (SCF 2002; FAO/WHO 2006; EFSA 2008; IARC 2010) and by an international regulation program (IPCS 1998). Since these evaluations, no further data has become available. For the justification of classification as mutagen according to CLP Regulation a re-evaluation of the available mutagenicity/genotoxicity has been carried out.

The carcinogenic potential of DB[a,i]P was first evaluated in December 1972 by a Working Group of IARC. Bioassays in mice revealed skin papillomas and epitheliomas (the Working Group considered these tumours to be squamous-cell carcinomas) after repeated dermal application. Sarcomas were induced after single subcutaneous injection of mice and hamsters (IARC 1973). The transfer of injection-site tissues following subcutaneous injection to mice shortened the latent period of tumour appearance.

In February 1983 a Working Group of IARC re-assessed the same bioassays and concluded that there was sufficient evidence that DB[a,i]P was carcinogenic to experimental animals (IARC 1983). On the basis of these data the working group classified DB[a,i]P according to their classification system as possible carcinogenic to human beings (group 2B).

In a subsequent evaluation by IARC in 2006 additional bioassays were included. These were three initiation–promotion studies in mouse skin, one study of subcutaneous injection into mice, one study of intra-peritoneal administration to newborn mice, two studies of intra-tracheal instillation to hamsters, one study in rat mammary gland and one study with a single intrauterine administration in mice. All studies gave positive results. DB[a,i]P was confirmed as possible carcinogenic to human beings (group 2B), on the basis of sufficient evidence in animals (Straif et al. 2005; IARC 2006).

The same experimental studies on DB[a,i]P are now evaluated for classification according to CLP Regulation.

2.2 Short summary of the scientific justification for the CLH proposal

A classification of DB[a,i]P as mutagen and carcinogen is proposed.

Mutagenicity

A variety of positive genotoxicity tests both *in vitro* and *in vivo* are available. A re-evaluation of these tests taking into account the quality of the test performances leads to the conclusion that only one positive *in vitro* mutagenicity test (gene mutation test at TK locus) was carried out in accordance with the corresponding EU/OECD test guideline. Due to the lack of a positive control all other tests with a positive result have a crucial methodological shortcoming. Therefore, merely the guideline compliant positive *in vitro* gene mutation test is to be considered for justification of classification of DB[a,i]P as mutagen, although the other positive results from *in vitro*/*in vivo* testing seem to be conclusive.

A supporting criterion for justification of classification of DB[i,a]P as a mutagen is a read-across approach with B[a]P and CHR which have been already classified as mutagen. Due to chemical structures and the resulting metabolic activities of the substances a read-across approach is sufficiently justified. The read-across approach clearly supports the relevance of the positive

genotoxic effects *in vitro* and *in vivo* in soma cells induced by DB[a,i]P despite the lack of positive controls in the most positive mutagenicity tests.

Due to the currently available mutagenicity/genotoxicity data of DB[a,i]P and the read-across approach to B[a]P and CHR a classification of DB[a,i]P as a Muta. 2, H341 is proposed in accordance with CLP Regulation.

Carcinogenicity

There is sufficient evidence on carcinogenicity of DB[a,i]P by all routes tested in mice, hamsters and rats. The available experiments have demonstrated that DB[a,i]P causes tumours at several sites, by several routes of administration, in both sexes, and in several animals species and strains. Oral studies with pure DB[a,i]P are not available.

Significant tumour rates induced by DB[a,i]P were found in the skin (by repeated topical administration, and a single subcutaneous injection), in the upper respiratory tract and in the lung (by intra-tracheal instillation), in the lung and liver (by intra-peritoneal administration), and in the mammary gland (by single intra-mammary injection).

DB[a,i]P caused tumours in two rodent species (mouse and rat) and in hamster, at two different tissue sites, and by several different routes of administration. Dermal exposure to DB[a,i]P caused benign or malignant skin tumours (papilloma or epithelioma) in mice, and subcutaneous injection caused cancer at the injection site (sarcoma) in mice and hamsters. Intra-peritoneal injection of newborn mice with DB[a,i]P caused benign and malignant lung tumours in both sexes and benign and malignant liver tumours in males, and intra-tracheal instillation caused respiratory-system cancer (mostly squamous-cell carcinoma, but also adenocarcinoma and anaplastic carcinoma) in hamsters of both sexes. DB[a,i]P administered by single intra-mammary injection caused cancer of the mammary gland (fibrosarcoma and adenocarcinoma) in female rats. In initiation-promotion studies the tumour initiating activity of DB[a,i]P was demonstrated in several mouse strains. The transfer of injection-site tissues to secondary hosts shortened the latent period of tumour growth.

A dose-response relationship for tumour induction was observed in studies with single subcutaneous injection of DB[a,i]P. In mice the lowest single dose which produced fibrosarcoma in 50 % of males was 6.25 μg (equivalent to about 0.2 mg/kg bw). Almost 100 % of the animals developed tumours within 22 weeks following single injection of 50 μg DB[a,i]P (equivalent to about 1.7 mg/kg bw) in peanut oil. These tumours progressed rapidly in size, killing the host within 4 weeks from the time when the tumour first became palpable. The lowest single subcutaneous dose which produced fibrosarcomas in about 50 % of hamsters was 0.25 mg/animal (equivalent to about 3.85 mg/kg bw).

No species-specific mode of action for DB[a,i]P carcinogenesis was identified.

DB[a,i]P is possibly carcinogenic to humans. Classification as carcinogen is largely based on animal evidence. DB[a,i]P meets the criteria for classification and labelling as Category 1B carcinogen, H350 according to CLP Regulation.

2.3 Current harmonised classification and labelling

DB[a,i]P is not classified according Annex I to Regulation (EC) No 1272/2008 on Classification, Labelling and Packaging of Dangerous Substances.

2.4 Current self-classification and labelling

Notified classification and labelling according to CLP criteria

Table 4: Classification and Labelling Inventory (June 2016)

Classification		Labelling			Specific Concentration limits, M-Factors	Notes	Classification affected by Impurities / Additives	Number of Notifiers
Hazard Class and Category Code(s)	Hazard Statement Code(s)	Hazard Statement Code(s)	Supplementary Hazard Statement Code(s)	Pictograms, Signal Word Code(s)				
Carc. 2	H351	H351		GHS08 Wng				23
Carc. 1B	H350	H350		GHS09 GHS08 Dgr				4
Aquatic Acute 1	H400	H400						
Aquatic Chronic 1	H410	H410						
Not Classified								3

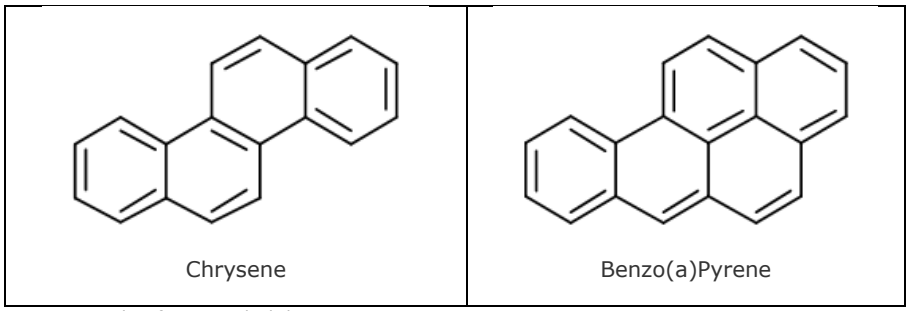
Number of Aggregated Notifications: 3

RAC general comment

Only two hazard classes were presented in the Dossier Submitter's (DS's) proposal for harmonised classification and labelling of benzo[*rst*]pentaphene: germ cell mutagenicity and carcinogenicity.

Although data from laboratory tests with this substance were presented for both hazard classes, a key supporting aspect of the proposal is its structural and biochemical similarity to other polycyclic aromatic hydrocarbons (PAHs) that are well known to possess these hazards.

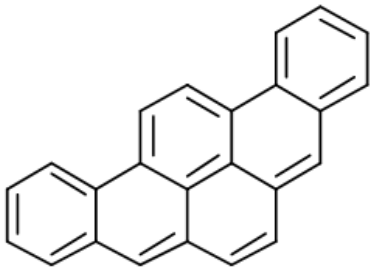
There are more than 100 substances that can be termed as PAHs. They are commonly formed by the incomplete combustion of organic substances, including the burning of wood, coal and tobacco. The main structural characteristics of PAHs are that they are planar, highly conjugated aromatic compounds. In their report, the DS presented data on chrysene (CHR) and benzo(a)pyrene (B[a]P) which are 4- and 5- membered ring structures, respectively.



Chrysene | Benzo(a)Pyrene

Note:images taken from www.lookchem.com

As can be seen from the following diagram, benzo[*rst*]pentaphene is structurally similar to both these substances (see below).



In addition, all 3 substances include at least 1 chemical sub-structure known as a bay region. These are the spaces that appear between the aromatic rings of these molecules. Here, metabolic oxidation can lead to the formation of dihydrodiols and subsequently to electrophilic diol epoxides. These structures have potential to bind with nucleophilic sites in macromolecules such as DNA, RNA and proteins. The adenine and guanine bases in single or double stranded DNA are sensitive targets, and binding at these sites has been shown to cause mutations that have been implicated in the carcinogenicity of PAHs.

Given the structural similarities of B[a]P, CHR and benzo[*rst*]pentaphene, the DS used this information in conjunction with available data to classify benzo[*rst*]pentaphene for germ cell mutagenicity and carcinogenicity.

3 JUSTIFICATION THAT ACTION IS NEEDED AT COMMUNITY LEVEL

DB[a,i]P has CMR properties that justify a harmonised classification and labelling according to article 36 of CLP.

Part B.

SCIENTIFIC EVALUATION OF THE DATA

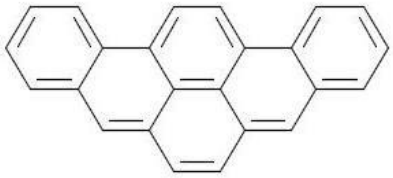
1 IDENTITY OF THE SUBSTANCE

1.1 Name and other identifiers of the substance

Table 5: Substance identity

EC number:	205-877-5
EC name:	benzo(r,s,t)pentaphene
CAS number (EC inventory):	189-55-9
CAS number:	189-55-9
CAS name:	Benzo[rst]pentaphene
IUPAC name:	benzo[rst]pentaphene
CLP Annex VI Index number:	-
Molecular formula:	$C_{24}H_{14}$
Molecular weight range:	302.37 g/mol

Structural formula:



1.2 Composition of the substance

No registration data are available for the substance DB[a,i]P (status: June 2016).

1.3 Physico-chemical properties

The term polycyclic aromatic hydrocarbon (PAH) commonly refers to a large class of organic compounds that contain only carbon and hydrogen and are comprised of two or more fused aromatic rings. DB[a,i]P is a hexacyclic polynuclear aromatic hydrocarbon.

Following five properties in particular have a decisive influence on the biological activity and on the toxicokinetics of PAH: vapour pressure, adsorption on surfaces of solid carrier particles, absorption into liquid carriers, lipid aqueous partition coefficient in tissues, and limits of solubility in the lipid and aqueous phases of tissues.

Table 6: Summary of physico - chemical properties

Property	**Value**	**Reference**	**Comment (e.g. measured or estimated)**
State of the substance at 20°C and 101,3 kPa	Greenish-yellow needles, prisms or lamellae	HSDB, 2012 (Secondary source)	-
Melting/freezing point	281.5 °C	HSDB, 2012 (Secondary source)	-
Boiling point	275 °C at 0.05 mm Hg	HSDB, 2012 (Secondary source)	-
Relative density	n.a.		
Vapour pressure	$1.8*10^{-11}$ mm Hg at 25 °C	HSDB, 2012 (Secondary source)	estimated
Surface tension	n.a.		
Water solubility	$5.5*10^{-4}$ mg/L at 25 °C	HSDB, 2012 (Secondary source)	estimated
Partition coefficient n-octanol/water	log Kow = 7.28	HSDB, 2012 (Secondary source)	estimated
Flash point		BAM, 2013	The flash point does not need to be tested because the substance is a solid.
Flammability	n.a.	BAM, 2013	Flammability upon ignition (solids): no data available Flammability in contact with water: The classification procedure needs not to be applied because the organic substance does not contain metals or metalloids. Pyrophoric properties: The classification procedure needs not to be applied because the organic substance is known to be stable into contact with air at room temperature for prolonged periods of time (days).
Explosive properties	no explosive properties	BAM, 2013	The classification procedure needs not to be applied because there are no chemical groups present in the molecule which are associated with explosive properties.
Self-ignition temperature	n.a.		
Oxidising properties	no oxidising properties	BAM, 2013	The classification procedure needs not to be applied because the organic substance does not contain oxygen or halogen atoms.
Granulometry	n.a.		
Stability in organic solvents and identity of relevant degradation products	n.a.		
Dissociation constant	n.a.		
Viscosity	n.a.		

2 MANUFACTURE AND USES

2.1 Manufacture

Not relevant for this report.

2.2 Identified uses

Not relevant for this report.

3 CLASSIFICATION FOR PHYSICO-CHEMICAL PROPERTIES

Not evaluated for this report.

4 HUMAN HEALTH HAZARD ASSESSMENT

4.1 Toxicokinetics (absorption, metabolism, distribution and elimination)

The general principles of the kinetics of PAH, in particular B[a]P, have been covered exhaustively in the published literature. Data of individual PAH are sparse. Extensive descriptions for PAH are available in the standard reviews, e.g. ATSDR (1995), IARC (1983, 2010), IPCS (1998), EFSA (2008) and WHO (1998, 2003). In this chapter, only a very brief summary is given.

4.1.1 Non-human information

4.1.2 Human information

4.1.3 Summary and discussion on toxicokinetics

Since experimental data regarding absorption, distribution, metabolism and excretion for pure DB[a,i]P are not available a general overview of the toxicokinetics of PAH is given.

Absorption

PAH are easily absorbed through the epithelia of the respiratory and gastrointestinal tract, and the skin. The absorption rate is strongly affected by various factors, such as the anatomical site, the composition of the vehicle of administration, the molecular weight of the single PAH and the dose applied. PAH adsorbed onto particulate matter are cleared from the lungs more slowly than free hydrocarbons. Absorption from the gastrointestinal tract occurs rapidly in rodents, but metabolites return to the intestine via biliary excretion. Data from both human and animal studies clearly have shown that PAH penetrate the skin and reach the systemic circulation.

Distribution

When absorbed, PAH are distributed via the bloodstream throughout all internal organs, and particularly in those with high fat contents. Intravenously injected PAH are cleared rapidly from the bloodstream of rodents but can cross the placental barrier and have been detected in fetal tissues.

Metabolism

The metabolism of PAH is complex. It starts at the moment PAH are absorbed through the epithelia of the lungs and the skin. In general, the process involves epoxidation of double bonds, a reaction catalysed by the cytochrome P-450-dependent monooxygenase system, the re-arrangement or hydration of such epoxides to yield phenols or diols, respectively, and the conjugation of the hydroxylated derivatives. Most PAH metabolised in this way are deactivated. However, PAH may also be activated to DNA-binding species, such as diol epoxides and radical cations that can initiate cancer.

Excretion

PAH metabolites and their conjugates do not persist in the body. PAH and their metabolites are rapidly excreted predominantly via the faeces and to a lesser extent in the urine. Conjugates excreted in the bile can be hydrolysed by enzymes of the gut flora and reabsorbed. This excludes those PAH moieties that become covalently bound to tissue constituents, in particular nucleic acids, and are not removed by repair.

4.2 Acute toxicity

Not evaluated for this report.

4.3 Specific target organ toxicity – single exposure (STOT SE)

Not evaluated for this report.

4.4 Irritation

Not evaluated for this report.

4.5 Corrosivity

Not evaluated for this report.

4.6 Sensitisation

Not evaluated for this report.

For the toxicological endpoint respiratory sensitisation no data are available.

4.7 Repeated dose toxicity

Not evaluated for this report.

4.8 Specific target organ toxicity (CLP Regulation) – repeated exposure (STOT RE)

Not evaluated for this report.

4.9 Germ cell mutagenicity (Mutagenicity)

Table 7: Summary table of relevant mutagenicity studies

Method	Results	Remarks	Reference
gene mutation test (TK locus) with h1A1v2 cells according to OECD guideline 476 tested concentrations: 0.3, 1.0, 10.0 and 100.0 ng/mL purity: 99.7 %	**positive** at concentrations of 10.0 and 100.0 ng/mL (MF: 4.4 and 7.5) <u>cytotoxicity:</u> doses of 10.0 and 100.0 ng/mL were clearly toxic	cells have been engineered to express cytochrome P4501A1(CYP1A1), an enzyme capable of metabolizing PAH	Durant et al. 1999
gene mutation test (HPRT locus) with V79 cells according to OECD guideline 476 tested concentrations: 0.03, 0.1 and 0.3 µg/mL purity: not known	**positive** only with use of golden hamster embryo cells from 0.03 µg/mL upwards in a dose dependent manner <u>cytotoxicity:</u> clearly cytotoxic at 0.3 µg/mL	cell mediated assay: use of golden hamster embryo cells which are capable of metabolizing PAH	Hass et al. 1982

4.9.1 Non-human information

4.9.1.1 *In vitro* data

Table 8: Gene mutations in bacteria

Test	**Bacterial strain (S. typhimurium tester strains)**	**Concentration**	**Metabol. activation**	**Result and Remarks**	**Reference**
bacterial gene mutation test (Ames test) not in accordance with OECD guideline 471 GLP: no information purity: not known	TA 98 TA 100 TA 1538	20 nmol	+	**positive** (all strains)	Mc Cann et al. 1975
bacterial gene mutation test (Ames test) not in accordance with OECD guideline 471 GLP: no information purity: > 99 %	TA 1535 TA 1537 TA 1538	50 µg/plate	+	**positive** (in TA 1535 and TA 1538)	Teranishi et al. 1975
bacterial gene mutation test (Ames test) not in accordance with OECD guideline 471 GLP: no information purity: not known	TA 100 TA 1535 TA 1537 TA 1538	1.0 - 100 µg/plate	+/-	**positive** (only in TA 1538 with metabolic activation at the highest tested doses of 10 and 100 µg/plate)	Commoner 1976

bacterial gene mutation test (Ames test) not in accordance with OECD guideline 471 GLP: no information purity: not known	TA 100	2.5 and 5.0 µg/plate	+	**positive** (at both tested concentrations)	Baker et al. 1980
bacterial gene mutation test (Ames test) not in accordance with OECD guideline 471 GLP: no information purity: not known	TA 98 TA 100	6.125 – 50 nmol	+	**positive** (both strains at all tested concentrations)	Wood et al. 1981
bacterial gene mutation test (forward mutation) no guideline available GLP: no information purity: > 98 %	TM 677	up to 30 µg/mL	+/-	**positive** (only with metabolic activation up to 30 µg/mL) weak dose-dependent increase of the mutant frequency (only graphical representation) <u>cytotoxicity:</u> no effects	Busby et al. 1995

Table 9: DNA repair in bacteria

Endpoint	Bacterial strain	Concentration	Metabol. activation	Result and remarks	Reference
DNA repair test (SOS chromo-test)	E. coli PQ37	0.156 – 10'000 µg/assay	+/-	**positive**	Mersch-Sundermann et al. 1992

no guideline available GLP: no information purity: 98 %				(only with metabolic activation from 2500 µg upwards)	
DNA repair test (Rec assay) no guideline available GLP: no information purity: not known	E. coli JC5519 rec^-	600 µg/well	+/-	**positive** (only with metabolic activation)	Ichinotsubo et al. 1977

Table 10: Gene mutations in mammalian cells

Test	Cell type	Concentration	Metabol. activation	Result and remarks	Reference
gene mutation test (HPRT locus) according to OECD guideline 476 GLP: no information purity: not known	V79 cells cell mediated assay: use of golden hamster embryo cells which are capable of metabolizing PAH	0.03, 0.1 and 0.3 µg/mL	+/- (see also column 'Cell type')	**positive** - only with use of golden hamster embryo cells - positive from 0.03 µg/mL upwards in a dose dependent manner <u>cytotoxicity:</u> - clearly cytotoxic at 0.3 µg/mL <u>controls:</u> - relevant negative and positive control	Hass et al. 1982
gene mutation test (TK locus) according to OECD guideline 476 GLP: no information purity: 99.7 %	h1A1v2 cells - cell line derived from human B-lymphoblastoid cells - cells have been engineered to express cytochrome P4501A1 (CYP1A1), an enzyme capable of metabolizing PAH	0.3, 1.0, 10.0 and 100.0 ng/mL - testing was done at low doses to measure mutagenicity at concen-trations reflecting the low levels of PAH reported in environ-mental samples	+ (see also column 'Cell type')	**positive** - positive at concentrations of 10.0 and 100.0 ng/mL (MF: 4.4 and 7.5) <u>cytotoxicity:</u> - doses of 10.0 and 100.0 ng/mL were clearly toxic <u>controls:</u> - relevant negative and positive control <u>deficiency:</u> - no differentiation of colony size for the determination small and large colonies suggestive for chromosomal aberrations or gene mutations	Durant et al. 1999

Test	Cell type	Concentration	Metabol. activation	Result and remarks	Reference
gene mutation test (TK locus) not in accordance with OECD guideline 476 GLP: no information purity: >98 %	MCL-5 cells - cell line derived from human B-lymphoblastoid cells - cells contain activity for five cytochromes P450 and microsomal epoxide hydrolase	0.1 –1.0 µg/mL	+ (see also column 'Cell type')	**negative** cytotoxicity: - higher concentrations were excessively toxic deficiency: - only graphical representation - lack of detailed experimental data - no positive control	Busby et al. 1995

Table 11: Indicator effects in mammalian cells

Test	Cell type	Concentration	Metabol. activation	Result and remarks	Reference
Unscheduled DNA synthesis (UDS test) similar to OECD guideline 482 GLP: no information purity: not known	primary rat hepatocytes	1 µmol/mL	-	**negative** cytotoxicity: - highest non toxic concentration was tested	Probst et al. 1981

4.9.1.2 *In vivo* data

Table 12: Testing in soma cells

Test	Species	Target cells	Exposure and harvest time	Result and remarks	Reference
micronucleus test GLP: no information not in accordance with OECD guideline 474 purity: not known	Sprague-Dawley rats 5 males /dose	bone marrow cells	2.5, 5.0 and 10.0 mg/kg bw - 3 intra-tracheal instillations of each dose within a 24-h period (0, 8 and 16 h) - cells were harvested 24 h after first dosing	**negative** toxicity: - cytotoxicity at 5.0 and 10.0 mg/kg bw - no information on clinical signs controls: - no positive control	Zhong et al. 1995
micronucleus test not in accordance with OECD guideline 474 GLP: no information purity: not known	Sprague-Dawley rats 5 males /dose	spleen cells	2.5, 5.0 and 10.0 mg/kg bw - 3 intra-tracheal instillations of each dose within a 24-h period (0, 8 and 16 h) - cells were harvested 24 h after first dosing	**positive** - only at the highest tested dose of 10.0 mg/kg bw; doubling of micronucleus rate compared to negative control toxicity: - no cytotoxicity - no information on clinical signs controls: - no positive control	Zhong et al. 1995
micronucleus test not in accordance with OECD guideline 474 GLP: no information purity not known	Sprague-Dawley rats 6 males /dose	lung cells	2.5, 5.0 and 10.0 mg/kg bw - 3 intra-tracheal instillations of each dose within a 24-h interval - cells were harvested 6 h after third administration - after harvesting cells were cultured in vitro	**positive** - at 5.0 and 10.0 mg/kg bw relevant increase of micronucleus rate (2.9/4.2) toxicity: - no cytotoxicity - the highest tested dose does not induce a lethal effect	Whong et al. 1994

Test	Species	Target cells	Exposure and harvest time	Result and remarks	Reference
			for the proof of micronuclei: binucleated primary lung cells (arrested dividing cells at metaphase) were used for micronuclei detection	<u>controls:</u> - no positive control	
DNA adducts (^{32}P-postlabeling analysis) no guideline available GLP: no information purity: not known	Sprague-Dawley rats 6 males /dose	lung cells	2.5, 5.0 and 10.0 mg/kg bw - 3 intra-tracheal instillations of each dose within a 24-h interval - cells were harvested 6 h after third administration	**positive** - each dose induced one adduct spot <u>toxicity</u>: - highest doses without causing a lethal effect <u>controls:</u> - no positive control	Whong et al. 1994
DNA adducts (^{32}P-postlabeling analysis) no guideline available GLP: no information purity: >99 %	Parkes mice 4 males /group	skin cells (cells of treated skin area)	application of 1.0 µmol DB[a,i]P/mouse in 200 µL tetrahydrofurane - topical treatment; shaved dorsal skin was used for application - animals were killed 7, 21, 84 days after treatment	**positive** - in dependence of different solvents 1 spot or 2 adduct spots <u>toxicity</u>: - no information <u>controls:</u> - no positive control	Hughes and Phillips 1990
DNA-adducts (^{32}P-postlabeling analysis) no guideline available GLP: no information purity: > 99 %	Parkes mice 4 males /group	lung cells	application of 1.0 µmol DB[a,i]P/mouse in 200 µL tetrahydrofurane - shaved dorsal skin was used for application - animals were killed 7, 21, 84 days after treatment	**positive** <u>toxicity:</u> - no information <u>controls:</u> - no positive control	Hughes and Philipps 1990
sister chromatid exchange (SCE test)	Sprague-Dawley rats	lung cells	2.5, 5.0 and 10.0 mg/kg bw	**positive** - dose-dependent	Whong et al. 1994

Test	Species	Target cells	Exposure and harvest time	Result and remarks	Reference
no guideline available GLP: no information (purity: not known)	6 males /dose		- 3 intra-tracheal instillations of each dose within a 24-h interval - cells were harvested 6 h after third administration	effect from 2.5 mg/kg bw upwards <u>toxicity:</u> - slight cytotoxic effect at the highest tested dose - no information on clinical signs <u>controls:</u> - no positive control	

4.9.2 Human information

No data available.

4.9.3 Other relevant information

No data available.

4.9.4 Summary and discussion of mutagenicity

<u>Experimental data *in vitro*</u>

DB[a,i]P induces mutagenic effects in bacteria and in directly exposed proliferating cells of mammalian and human cell lines. These effects were only observed in the presence of an exogenous metabolic system.

DB[a,i]P induces gene mutations in different S. typhimurium strains (Mc Cann et al. 1975; Teranishi et al. 1975; Commoner 1976; Baker et al. 1980; Wood et al. 1981; Busby et al. 1995) as well as DNA damage in E. coli tester strains (Mersch-Sundermann et al. 1992; Ichinotsubo et al. 1977).

In mammalian cell gene mutation tests DB[a,i]P induces positive effects in V79 cells and in cells of a cell line derived from human B-lymphoblastoid cells (h1A1v2 cells).
DB[a,i]P induces gene mutations in V79 cells in a cell mediated assay (Hass et al. 1982). Since V79 cells do not metabolize PAH the induction of mutagenic effects was tested in the presence and absence of golden hamster embryo cells capable of metabolizing PAH. In the presence of hamster embryo fibroblasts DB[a,i]P was mutagenic in a dose-dependent manner in the tested range from 0.03 up to 0.3 µg/mL. The highest tested concentration of 0.3 µg/mL was clearly cytotoxic. No mutagenic effect was observed in the absence of the hamster embryo cells.
In a TK-mutation test a positive effect was observed at the thymidine kinase (Tk) locus in h1A1v2 cells at the highest tested concentrations of 10 and 100 ng/mL (Durant et al. 1999). The cells had been engineered to express cytochrome P4501A1 (CYP1A1), an enzyme capable of metabolizing PAH. Clear cytotoxic effects were observed both at 10 and 100 ng/mL. The positive result cannot be

assessed in detail, because no differentiation into small colonies, suggestive of chromosomal aberrations, or large colonies, suggestive of gene mutations, was carried out. Because there is no colony sizing no conclusion can be drawn whether gene mutations or chromosomal aberrations were induced preferentially.
In a further TK-mutation test with MCL-5 cells DB[a,i]P induced no mutagenic effects up to the highest tested concentration of 1.0 µg/mL (Busby et al. 1995). Higher concentrations were characterised as excessively toxic.

DB[a,i]P did not induce DNA damage in primary rat hepatocytes (UDS test) at the only tested concentration of 1.0 µmol/mL (Probst et al. 1981). No toxic effect was observed.

Experimental data *in vivo*

DB[a,i]P induces clastogenic effects (induction of micronuclei) as well as genotoxic effects (DNA adducts, SCE's) in soma cells.

Three *in vivo* micronucleus tests using male rats are available. The induction of micronuclei was examined in different target cells (bone marrow cells, spleen cells, lung cells) under similar experimental conditions, e.g. intratracheal instillation of identical doses (2.5, 5.0 and 10.0 mg/kg bw), and three intra-tracheal instillations of each dose within a 24-h interval.
In bone-marrow cells a negative result was obtained at all tested doses (Zhong et al. 1995). Cytotoxic effects were observed at 5.0 and 10.0 mg/kg bw. No information on clinical signs was given.
In spleen cells a weak positive effect (doubling of the micronucleus rate compared to the negative control) was detected at the highest tested dose of 10 mg/kg bw (Zhong et al. 1995). No cytotoxic effects were observed. No information on clinical signs was given.

In lung cells an induction of micronuclei was observed at the highest tested doses of 5.0 and 10 mg/kg bw (Whong et al. 1994). Compared to the negative control increased micronucleus frequencies of 2.9 respectively 4.2 were found. No cytotoxic effects were observed. It was informed that the highest tested dose did not induce a lethal effect.

DB[a,i]P induces DNA adducts in lung cells of rats after intra-tracheal instillation (Whong et al. 1994). At all tested doses (2.5, 5.0 and 10.0 mg/kg bw) an identical DNA adduct was detected. Hughes and Phillips (1990) showed positive activities for DNA-adduct formation in skin cells as well as in lung cells of mice after topical treatment of shaved dorsal skin (1 µmol/mouse). Two different adducts were found in cells of the mouse skin. According to the authors these adducts were detected in much smaller quantities also in lung cells.

An SCE test in lung cells of rats was positive after intra-tracheal instillation of DB[a,i]P (Whong et al. 1994). The effect was dose-dependent in a dose-range from 2.5 up to 10 mg/kg bw. Slight cytotoxicity was observed at the highest dose tested.

Validity of the test systems

Only two positive *in vitro* mutagenicity tests (gene mutation test at Tk locus as well as at HPRT locus) were carried out in accordance with an EU/OECD test guideline.

As described previously the data for mutagenicity of DB[a,i]P were obtained from *in vitro* (bacteria; proliferating cell cultures) and *in vivo* testing (soma cells). Based on the results of these tests DB[a,i]P was evaluated as genotoxic by international bodies (SCF 2002; FAO/WHO 2006; EFSA 2008; IARC 2010) and by an international regulation program (IPCS 1998).

A re-evaluation of the available mutagenicity/genotoxicity tests taking into account the quality of the test performances leads to the conclusion that only two positive *in vitro* mutagenicity tests (gene mutation test at Tk locus (Durant et al. 1999); gene mutation test at HPRT locus (Haas et al. (1982)) were carried out in accordance with an EU/OECD test guideline. The lack of a positive control in all other positive tests is a crucial methodological shortcoming. Therefore, the results of these tests are considered as not fully reliable. Relevant controls (positive control as well as negative control) have to be considered at toxicological testing in accordance with EU/OECD guidelines. Also for internationally accepted testing procedures for which no EU/OECD guideline exist the use of controls is a standard to avoid misinterpretations of the test results.

In accordance with CLP Regulation and the ECHA guidance to CLP only fully reliable positive results of well conducted and scientifically validated tests are relevant for justification of toxicological classification of a substance. Therefore, merely the guideline compliant positive *in vitro* mutagenicity tests from Durant et al. (1999) and Haas et al. (1982) should be considered for justification of classification of DB[a,i]P as mutagen.

Read-across approach

A read-across approach between DB[a,i]P and B[a]P as well as CHR is relevant.

B[a]P and a number of further PAH, e.g. DB[a,i]P and CHR have shown mutagenic/genotoxic effects in standard assays *in vitro* and *in vivo* (IPCS 1998; SCF 2002; FAO/WHO 2006; EFSA 2008; IARC 2010; Benford et al. 2010). But so far, only B[a]P and CHR are classified as mutagenic. B[a]P (CAS 50-32-8) is classified/labelled as Cat. 1B, H340 whereas CHR (CAS 218-01-9) is classified/labelled as Cat. 2, H341 (CLP Regulation).

PAH with relatively planar, highly conjugated aromatic structures such as B[a]P and CHR have a genotoxic potential which is characterised as indirect because metabolic activation is required for the induction of genotoxic effects. Mutagenic effects are induced after biotransformation to reactive electrophilic metabolites (dihydrodiol epoxides), which bind covalently and form adducts with intracellular macromolecules such as DNA adducts. DNA adducts, which may result in gene mutations, DNA strand breaks or chromosomal aberrations, are the precursor lesions for mutations, which arise through replication of errors in the DNA during DNA synthesis.

B[a]P (five-ringed PAH) and CHR (four-ringed PAH), with respectively one reactive so-called bay-region (structural element in the PAH due to the linkage of the ring systems), are metabolised mainly through the bay-region dihydriol epoxides pathway. This pathway is catalysed by several enzymes such as CYPs and epoxide hydrolases. Metabolic activation finally leads to the formation of electrophilic diolepoxides, which belong to the most potent mutagens reported so far **(**Xue and Warshawsky 2005). The formation of diolepoxides that covalently bind to DNA appears to be the primary step in the mechanism of action of genotoxic unsubstituted PAH.

DB[a,i]P possesses two bay-regions and belongs to the group of relatively planar, highly conjugated six-ringed dibenzopyrenes. In a relevant study Hass et al. (1982) examined not only the induction of gene mutations in V79 cells but also sought to clarify the question whether the metabolic oxidation at the bay-regions is required for a mutagenic response. Since V79 cells do not metabolize PAH, mutagenesis was tested in both the presence and the absence of hamster embryo cells capable of metabolizing PAH (cell mediated assay). It was shown that DB[a,i]P was only mutagenic in the presence of hamster embryo cells. Due to this positive result the role of the bay-regions in the activation of DB[a,i]P has been investigated. First it was checked whether metabolic oxidation of DB[a,i]P at the bay-regions is necessary for the mutagenic response. Therefore the involvement of microsomal oxidation in the metabolism of DB[a,i]P was tested by using 7,8-benzoflavone (BF) a known inhibitor of mixed-function oxidases. It could be shown that the induction of mutagenic effects was prevented by additional incubation of DB[a,i]P with BF. Furthermore it could be demonstrated that the difluorinated derivate of DB[a,i]P, 2,10-difluorodibenzo[a,i]pyrene, extremely inhibited the induction of mutagenic effects in V79 cells. This effect implies the involvement of the particular fluorinated carbon atoms at positions of the bay-regions in the metabolic activation of the parent compound. Summarized it can be concluded, that a metabolic oxidation (presumably via oxidative pathway) at the bay-regions is required for a mutagenic response of DB[a,i]P in this cell-mediated assay.

Due to their chemical structures and the resulting metabolic activities at the bay-region(s) a read-across approach between DB[a,i]P, B[a]P and CHR is sufficiently justified. The read-across approach is based on the following common substance properties:

- The substances have planar, highly conjugated aromatic structures.
- The substances require metabolic activation for the induction of mutagenic/genotoxic effects.
- The bay-regions were identified as common reactive centre.
- Dihydrodiol epoxides are formed as common breakdown products via biological processes at the bay-region(s).
- The substances induce genotoxic effects *in vitro* and *in vivo* after metabolic activation at the bay-region.

Following the read-across approach it is justified to use the classification of B[a]P and CHR as mutagen as a supporting argument for justification of classification of DB[a,i]P as mutagen.

4.9.5 Comparison with criteria

Category 1 mutagen

According to the CLP Regulation (see 3.5.2.2, p. 145) substances that are known to induce heritable mutations or are to be regarded as if they induce heritable mutations in germ cells of humans are classified as Category 1 mutagen.

Category 1A: There is positive evidence from human epidemiological studies.

Category 1B: There are positive results from

- in vivo heritable germ cell mutagenicity tests in mammals or

- in vivo soma cell mutagenicity tests in mammals (in combination with evidence that the substance has potential to cause mutations to germ cells) or
- tests showing mutagenic effects in germ cells of humans without demonstration of transmission to progeny.

No data are available which justify a classification of DB[a,i]P as mutagen Category 1 in accordance with the CLP Regulation.

Category 2 mutagen

According to the CLP Regulation (see 3.5.2.2, p. 145) the classification of a substance as mutagenic Category 2 is based on: '… positive evidence obtained from experiments in mammals and/or in some cases from *in vitro* experiments, obtained from:

- somatic cell mutagenicity tests *in vivo,* in mammals or
- other *in vivo* somatic cell genotoxicity tests which are supported by positive results from *in vitro* mutagenicity assays.'

The ECHA guidance to CLP Regulation additionally explains (see 3.5.2.4, p. 385) that:

- 'Classification in Category 2 may be based on positive results of a least on *in vivo* valid mammalian somatic cell mutagenicity test, indicating mutagenic effects in soma cells.'
- 'A Category 2 mutagen classification may also be based on positive results of a least one *in vivo* valid mammalian genotoxicity test, supported by positive *in vitro* mutagenicity results.'

Taking into account these criteria neither a valid *in vivo* mammalian somatic cell mutagenicity test nor a valid *in vivo* mammalian genotoxicity test is available. The available guideline-compliant *in vitro* gene mutation tests are not sufficient as justification for classification of DB[a,i]P as Category 2 mutagen.

But the CLP Regulation (see 3.5.2.2, p.145) also notes: 'Substances which are positive in in vitro mammalian mutagenicity assays, and which also show chemical structure activity relationship to known germ cell mutagens, shall be considered for classification as Category 2 mutagens.' These requirements are fulfilled for DB[a,i]P.

In principle PAH have repeatedly shown to have genotoxic effects both *in vivo* in soma cells of rodents and *in vitro* in mammalian (inclusive human) cell lines and procaryotes. Due to the chemical structures and its resulting ability to induce mutagenic/genotoxic effects *in vitro* and *in vivo* a read-across approach between DB[a,i]P, B[a]P (Category 1B mutagen) and CHR (Category 2 mutagen) is considered of sufficient relevance. The read-across approach clearly supports the relevance of the positive mutagenic/genotoxic effects *in vitro* and *in vivo* in soma cells induced by DB[a,i]P despite the lack of positive controls in most of the positive mutagenicity tests. Therefore, it is reasonable to conclude that the classification of B[a]P and CHR as mutagen can be used as supporting criterion for justification of classification of DB[a,i]P as a mutagen.

4.9.6 Conclusions on classification and labelling

The currently available mutagenicity/genotoxicity data of DB[a,i]P, combined with the read-across approach to B[a]P and CHR, are sufficient for classification of DB[a,i]P as a Muta. 2, H341 in accordance with CLP Regulation.

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

Benzo[*rst*]pentaphene induced mutagenic effects in bacteria and in exposed proliferating cells of mammalian and human cell lines. These effects were only observed in the presence of an exogenous metabolic system.

Clastogenic effects (induction of micronuclei) were induced by benzo[*rst*]pentaphene as well as genotoxic effects (DNA adducts, sister chromatid exchange (SCE)) in somatic tissues in both regulatory and research studies.

Amongst all the studies, only two *in vitro* mammalian cell gene mutation tests were carried out in accordance with an EU/OECD test guideline (TG); one involving mutations assayed at the TK locus, the other at the HPRT locus. Both of these tests yielded positive results.

The DS commented that the lack of appropriate controls in all the other tests was a crucial methodological shortcoming. Therefore, the results of these tests were considered as not fully reliable.

In the opinion of the DS, taking into account the CLP Regulation and associated guidance, the quality and reliability of most of the available studies was below the required standard. Consequently, even though a significant number of positive *in vitro* and *in vivo* studies had been found with benzo[*rst*]pentaphene the results of these studies alone were insufficient to support classification.

To supplement these studies, the DS argued that the structural similarity of benzo[*rst*]pentaphene to B[a]P and CHR justified its classification as a mutagen. B[a]P has a harmonised classification for germ cell mutagenicity in Category 1B, CHR in Category 2. The DS cited several reviews from international bodies that concluded B[a]P and CHR to have produced mutagenic/genotoxic effects in standard assays *in vitro* and *in vivo.*

These PAHs have a relatively planar, highly conjugated aromatic structure. They require metabolic activation to dihydrodiol epoxides to induce genotoxic effects. DNA adducts, which may result in gene mutations, DNA strand breaks or chromosomal aberrations, are the precursor lesions for mutations, which arise through replication of errors in the DNA during DNA synthesis.

B[a]P (five-ringed PAH) and CHR (four-ringed PAH), each with at least one reactive bay region (structural element in the PAH due to the linkage of the ring systems), are metabolised mainly through the bay-region dihydrodiol epoxides pathway. This pathway is catalysed by several enzymes such as cytochromes P450s and epoxide hydrolases. Metabolic activation finally leads to the formation of electrophilic diol epoxides that covalently bind to DNA. Benzo[*rst*]pentaphene possesses two bay regions and is one of a number of PAHs characterised structurally as relatively planar, highly conjugated six-ringed dibenzopyrenes.

The DS described a mutation test in Chinese hamster V79 cells that investigated if metabolic oxidation at the bay regions is required for the benzo[*rst*]pentaphene mutagenicity. Since V79 cells do not metabolise PAH, mutagenesis was tested in both the presence and the absence of hamster embryo cells capable of metabolising PAH (cell mediated assay). Having shown that a positive result could only occur in the presence of hamster embryo cells, the involvement of microsomal oxidation in the metabolic activation process was confirmed using 7,8-benzoflavone (BF), a known inhibitor of mixed-function oxidases. The mutagenicity of benzo[*rst*]pentaphene was prevented by additional incubation of the treated cultures with BF. To investigate whether metabolic oxidation at the bay regions was a pre-requisite for the mutagenic response, it was shown that a difluorinated derivate of benzo[*rst*]pentaphene (2,10-difluorodibenzo[a,i]pyrene) also inhibited the induction of mutagenic effects in this test system. This inhibitory effect implies the involvement of the particular fluorinated carbon atoms at positions of the bay regions in the metabolic activation of the parent compound. The DS thus concluded that metabolic oxidation at the bay regions was required for a mutagenic response of benzo[*rst*]pentaphene in this cell-mediated assay.

Due to their chemical structures and the resulting metabolic activities at the bay region(s), the DS stated that a read-across approach between benzo[*rst*]pentaphene, B[a]P and CHR was sufficiently justified. The read-across was based on the following common properties of the 3 substances:

- Planar, highly conjugated aromatic structures.
- Metabolic activation required for the induction of mutagenic/genotoxic effects.
- The bay regions are common reactive centres. Dihydrodiol epoxides are formed as common breakdown products via biological processes at the bay region(s).
- Genotoxic effects induced *in vitro* and *in vivo* after metabolic activation at the bay region.

Given the limitations found in most of the mutagenicity/genotoxicity studies with benzo[*rst*]pentaphene, the DS proposed that it was justified to use the existing mutagenicity classifications of B[a]P (Cat. 1B) and CHR (Cat. 2) to support the classification of this substance. The studies showing mutagenicity/genotoxicity in somatic cells were generally of poor quality but, combined with read-across to the other mutagenic PAHs, the DS concluded they were sufficient to justify a Category 2 classification. However, a Category 1B classification was not considered appropriate as there were no studies of the mutagenic/genotoxic effects of benzo[*rst*]pentaphene in germ cells available.

Comments received during public consultation

Three MSCA communicated support for the proposal. They highlighted the positive results seen in the *in vitro* mutagenicity tests with benzo[*rst*]pentaphene and the structural similarity of this substance to the known mutagens B[a]P and CHR. One of these MSCA commented that the dossier lacked a critical assessment of the adequacy and quality of individual studies.

Assessment and comparison with the classification criteria

The potential mutagenicity of benzo[*rst*]pentaphene has been studied *in vitro* and *in vivo.*

Eleven *in vitro* studies were evaluated and included 5 Ames tests, 2 DNA repair tests in bacteria, 3 *in vitro* mammalian gene mutation assays and a UDS test. RAC agrees, however, that only two of the eleven *in vitro* studies can be considered as reliable when judged against current regulatory standards. Both of these tests are mammalian gene mutation assays.

The *in vivo* mutagenic potential of benzo[*rst*]pentaphene was assessed in 2 micronucleus tests, in 3 DNA adduct formation assays and 1 SCE assay in rats. None of these studies conformed to a current regulatory standard.

Additionally, there are 4 initiator-promoter assays in mice to have been conducted with benzo[*rst*]pentaphene as the initiator. Positive initiation of tumours in these tests is indicative of mutagenic activity.

Given the structural similarity to other PAHs, particularly B[a]P and CHR, RAC agrees that information on these two PAHs can support the classification of benzo[*rst*]pentaphene.

In vitro studies

Five mutagenicity assays with standard tester strains of *S. typhimurium* were summarised in the CLH report. Although the DS commented that none of these tests conformed to current regulatory standards, they all gave positive results with benzo[*rst*]pentaphene. Like other mutagenic PAHs, positive results occurred in the presence of exogenous metabolic activation. The consistent nature of the results provides compelling evidence of benzo[*rst*]pentaphene's mutagenic potential. In support of this, positive results were also reported in 2 bacterial DNA repair tests (an SOS Chromotest and the Rec A assay).

According to the DS, only two *in vitro* mammalian gene mutation studies performed with benzo[*rst*]pentaphene conform to OECD TG 476 and were considered reliable for assessment.

Human B-lymphoblastoid cells engineered to express cytochrome P450 1A1 (CYP1A1) for PAH metabolism were exposed to benzo[*rst*]pentaphene at 0.3, 1.0, 10 and 100 ng/ml in the TK-locus gene mutation test. Mutation frequency was increased at the top two doses (MF 4.4 and 7.5, respectively) and was accompanied by cytotoxicity.

A gene mutation test (HPRT locus) in V79 cells tested benzo[*rst*]pentaphene at concentrations of 0.03, 0.1 and 0.3 µg/ml with and without metabolic activation. Mutant colonies were formed at all concentrations in a dose dependent manner following metabolic activation. Within this study, the necessity for the bay regions in benzo[*rst*]pentaphene to cause mutagenicity was tested. The two bay regions were individually and in combination fluorinated and mutagenic activity was measured. Fluorination of both bay regions was required to decrease the mutagenic activity of benzo[*rst*]pentaphene to background levels in V79 cells.

In conclusion, benzo[*rst*]pentaphene showed mutagenic potential when tested in cultured mammalian in the presence of a metabolic activation system sufficient for oxidation at the bay regions and formation of reactive epoxides. It has similar activity to that of other mutagenic PAHs, including B[a]P and CHR.

An *in vitro* rat hepatocyte UDS test was also available. This reported a negative result with benzo[*rst*]pentaphene but was limited in several respects, most notably in the use of only a single test concentration at a non-toxic level. It is unclear whether higher doses could have been tested and the result is not considered reliable.

In vivo studies

Micronucleus tests were conducted to investigate the genotoxicity of benzo[*rst*]pentaphene following intra-tracheal administration to male Sprague-Dawley rats. Bone marrow cells and spleen erythrocytes were harvested from groups of 5 male rats that received 3 injections of 0, 2.5, 5 or 10 mg/kg bw test substance within a 24-h harvesting period (0, 8 and 16 h). Similarly, lung cells were sampled from group of 6 rats. No information was provided on any clinical signs of toxicity induced by benzo[*rst*]pentaphene administration and no positive controls were included. There was no increase in micronucleus frequency seen in bone marrow cells (data not presented in the CLH report), although a "cytotoxic" response was evident at the top 2 dose levels. In contrast, a two-fold increase in micronucleus formation was seen in spleen cells taken from rats at the top dose. Also, compared to the negative control value, micronucleus frequency in lung cells from the 2 highest dose groups was increased 2.9 and 4.2 fold, respectively. No significant cytotoxicity was seen in spleen or lung cells.

No explanation was provided for the apparent tissue-specific effect in these studies. Although not performed to a regulatory standard, the results provide further supporting evidence of the mutagenic potential of benzo[*rst*]pentaphene.

The study of micronuclei in lung cells was extended to include the analysis of DNA adducts in the same tissue. A clear, single, adduct spot was found in samples from each dose group using the 32P post-labelling assay. No such adduct spot was seen in DNA isolated from control rat lung cells.

Post-labelling assays were also conducted by a different laboratory in skin and lung cells taken from mice administered a single topical dose of benzo[*rst*]pentaphene. Skin cells were sampled directly from the treated area. Unique DNA adduct spots were seen on autoradiographs from treated animals providing strong evidence that this PAH can produce DNA adducts in mice.

The study that found increased micronuclei and unique DNA adduct formation in the lung cells of rats also described a dose-dependent increase in SCEs. Although a rather non-specific marker of mutagenic potential, these findings were consistent with those for the other markers.

The weight of evidence from all these *in vivo* studies is strongly suggestive of benzo[*rst*]pentaphene mutagenic potential.

There were also 4 dermal initiation-promotion studies in mice with benzo[*rst*]pentaphene employed as the initiator. All gave positive results for tumour-initiating activity regardless of single or multiple doses i.e. increased incidence of skin papilloma compared to controls. The initiation stage of these assays is strongly indicative of mutagenic activity and the

positive responses with benzo[*rst*]pentaphene support the outcomes of the *in vivo* and *in vitro* genotoxicity studies.

Similarity to B[a]P and CHR (see also RAC general comment, above)

Benzo[*rst*]pentaphene shares structural properties with the PAHs CHR and B[a]P, both of which already carry a harmonised classification for germ cell mutagenicity. Notably, all 3 substances possess planar, highly conjugated aromatic structures and common reactive centres called bay regions. They all require metabolic activation at bay regions for the induction of mutagenic/genotoxic effects. Electrophilic dihydrodiol epoxides are formed as common breakdown products via biological processes at the bay regions.

CHR and B[a]P are classified as Muta. Cat. 2 and Muta. Cat. 1B, respectively. The specific data underlying these classifications was not discussed in the CLH report, therefore, it is not possible for RAC to make a detailed comparison with the data on benzo[*rst*]pentaphene to assess whether a case for a Category 1B classification could be made. However, the proposed Category 2 classification of benzo[*rst*]pentaphene is supported by the existing classifications of CHR and B[a]P.

Classification of benzo[rst]pentaphene

There are no data on human germ cell mutagenicity with benzo[*rst*]pentaphene, therefore Category 1A is not appropriate.

The *in vitro* and *in vivo* genotoxicity data are consistently positive and reproducible across the different study types. The positive studies are further supported by 4 positive initiation-promotion assays in mice, which gave results indicative of the mutagenic activity of benzo[*rst*]pentaphene. In the absence of data from *in vivo* studies investigating the potential effects of benzo[*rst*]pentaphene on the DNA of germ cells, or demonstrating its ability to interact with the genetic material of germ cells, the criteria for Category 1B are also not met.

RAC is of the opinion that the available data from studies with benzo[*rst*]pentaphene itself are sufficient to justify at least a Category 2 classification. As indicated in Annex I to the CLP Regulation, Category 2 for this hazard class can be based on positive evidence from *in vivo* somatic cell mutagenicity/genotoxicity tests (e.g. for benzo[*rst*]pentaphene: micronucleus assay, DNA adduct formation, tumour initiating activity) supported by positive results from *in vitro* mutagenicity assay (e.g. bacterial and mammalian cell gene mutation assays).

Further justification is provided by the comparison made with the known mutagens B[a]P and CHR. In accordance with the criteria in Annex I (Table 3.5.1) to the CLP Regulation, "substances which are positive in *in vitro* mammalian mutagenicity assays, and which also show chemical structure activity relationship to known germ cell mutagens shall be considered for classification as Category 2 mutagens". This could apply even when those known mutagens have harmonised classifications of Category 1A or 1B. For benzo[*rst*]pentaphene, there are also positive indications of mutagenic potential from *in vivo* studies conducted in somatic cells, but there are no germ cell studies. The use of read-across to justify a higher classification category may therefore be possible. However, this was not considered by the DS, who simply used the existing harmonised classifications of B[a]P or CHR to justify a Category 2 classification of this substance.

Overall, the RAC recommendation is for a **Category 2 classification (H341) for germ cell mutagenicity**. A Category 1B classification may alternatively be appropriate, but the relevant supporting data from the structurally similar substance B[a]P have not been provided to RAC.

4.10 Carcinogenicity

Table 13: Summary table of relevant carcinogenicity studies

Method	Results	Remarks	Reference
carcinogenicity study by **topical** application to the skin of female Swiss albino Ha/ICR/Mil mice 20 animals per group 0.05 and 0.1 % in p-dioxan 3x/wk 12 months + 3 months treatment-free recovery period purity: not given	**0.05 % (≈0.86 mg/kg bw/d)** latency period: 336 days 16/20 (80 %; skin papillomas), 13/20 (65 %; skin epitheliomas), **0.1% (≈1.71 mg/kg bw/d)** latency period: 287 days 16/20 (80 %; skin papillomas) 15/20 (75 %; skin epitheliomas) **vs 0/20** solvent controls	**positive** Repeated dermal application by skin painting induced skin papillomas and epitheliomas at high incidences.	Hoffmann and Wynder 1966 LaVoie et al. 1979
carcinogenicity study by **subcutaneous** administration to male C57/Br/cd mice 12 animals per group dosages of 0.01–600 µg in peanut oil as a **single injection** termination: 66 wk purity: not given	fibrosarcoma (detectable by microscopy: 5 wk fully developed after 12 wk) **< 1 µg**, 0 %; **1 µg**, 9 % **2 µg**, 33 % **6.25 µg** (0.2 mg/kg bw), 50 % **12.5 µg**, 64 %; **25 µg**, 92 % **≥ 50 µg** (1.7 mg/kg bw), 100 %, dose-effect relationship	**positive** clear induction of fibrosarcomas in C57br/cd mice following a single subcutaneous injection dose-response relationship for tumour formation up to a dose of 50 µg	Homburger and Tregier 1960
carcinogenicity study by **intra-tracheal** administration to Syrian golden hamsters lifetime study 24 male / 24 female 1 mg in distilled water 1×/wk, 12 wk (total dose, 12 mg) or 500 µg in distilled water 1×/wk, 17 wk (total dose, 8.5 mg) > 99 % pure particle size: 24 % < 1 µm, 96 % < 5 µm 100 % < 25 µm no controls purity: > 99 %	**12 mg (total dose)** **≈1.14 mg/kg bw/d (m)** **≈1.3 mg/kg bw/d (f)** 6/438 respiratory tract tumours, predominantly squamous cell carcinoma bronchi, 62 %; trachea, 19 % **8.5 mg (total dose)** **≈0.57 mg/kg bw/d (m)** **≈0.65 mg/kg bw/d (f)** 31/48 respiratory tract tumours, predominantly squamous cell carcinoma bronchi, 77 %; trachea, 13 % also tumours in larynx, lung and pleura latency period: 8 wk	**positive** potent carcinogen for the hamster respiratory system; short latent period of 8 weeks high tumour incidences in bronchi, trachea and larynx	Stenbäck and Sellakumar 1974

4.10.1 Non-human information

4.10.1.1 Carcinogenicity: oral

No studies available.

4.10.1.2 Carcinogenicity: inhalation

- **Carcinogenicity studies by intra-tracheal administration**

Hamster

Carcinogenicity of DB[a,i]P in the respiratory tract was examined in Syrian golden hamsters (Sellakumar and Shubik 1974). A group of 36 male Syrian golden hamsters, 9–10 weeks of age and weighing approximately 98 g, received at weekly intervals intra-tracheal doses of 2.0 mg DB[a,i]P (purity > 99 % by thin-layer chromatography (TLC)) over four weeks (total dose, 8 mg; approximately 2.92 mg/kg bw/d), ground to a finely aggregated dust (1:1, w:w) with haematite (Fe_2O_3; particle size 94 %, < 1 μm) and then suspended in 200 μL saline (0.9 % aqueous). A second group of 48 male hamsters of the same strain and age was treated similarly with 24 weekly doses of 500 μg DB[a,i]P (total dose, 12 mg; approximately 0.73 mg/kg bw/d), and an additional group of 90 hamsters represented the untreated controls. No control group received intra-tracheal instillation of the vehicle. The animals were monitored daily, weighed once a week; they died either spontaneously or were killed when moribund. At autopsy, the trachea was ligated and the lungs were removed en bloc, still fully expanded by air, and then fixed by immersion in 10 % neutral buffered formalin. Tissue sections were prepared from each lobe of the lung, larynx, trachea, stem bronchi, and all other organs if gross pathology was present.

After 100 weeks, all treated animals and 71/90 controls had died. Respiratory insufficiency, due to extensive tumour involvement in the respiratory tract, accounted for the increased mortality rates in the treated groups. In the group treated with four doses of 2.0 mg DB[a,i]P, the incidence of tumours of the respiratory tract was 16/34 (47 %); specific incidences were one tumour of the larynx, two of the trachea, 13 of the bronchi and one of the lung; no tumours were found at other sites. In the group treated with 24 doses of 500 μg DB[a,i]P, the incidence of tumours of the respiratory tract was 39/44 (89 %); specific incidences were six tumours of the trachea, 37 of the bronchi and one of the lung. In addition, two malignant lymphomas occurred in this group. A total of 19 treatment-induced respiratory tract tumours developed in the group treated with four doses of 2.0 mg, and a total of 95 tumours in the group treated 24 times with 500 μg DB[a,i]P. Squamous-cell carcinomas were the predominant (more than 50 %) histological type, with the first tumour seen at the 27th week. Most tumours in both groups arose from the epithelium of the bronchi and trachea. No tumours of the respiratory tract were observed in the untreated group, which had an incidence of 11/82 tumours at other sites.

The test results demonstrate that DB[a,i]P is a potent carcinogen in the respiratory tract of hamsters. Both groups that received DB[a,i]P had a high incidence of carcinomas.

Lung tumour induction by DB[a,i]P in the Syrian golden hamster was studied by Stenbäck and Sellakumar (1974). Two groups of 24 male and 24 female Syrian golden hamsters, 6–7 weeks of age (weight unspecified), were treated intra-tracheally with DB[a,i]P (purity > 99 % by TLC) finely suspended in distilled water (particle size, 24 % < 1 μm, 96 % < 5 μm, 100 % < 25 μm). One group received 1.0 mg once a week for 12 weeks (total dose, 12 mg; approximately 1.14 mg/kg bw/d in

males and 1.3 mg/kg bw/d in females) and the other group received 500 µg once a week for 17 weeks (total dose, 8.5 mg; approximately 0.57 mg/kg bw/d in males and 0.65 mg/kg bw/d in females). No control group was used. Animals were monitored and weighed weekly and those in poor condition were isolated and allowed to die spontaneously or were killed when moribund (follow-up time unspecified). Autopsies were performed on all animals. At autopsy, the trachea was ligated and the lungs were removed en bloc, still fully expanded by air, and then fixed in 10 % neutral buffered formalin. Histological sections were prepared from each lobe of the lung, larynx, trachea, stem bronchi, liver and other organs showing gross pathology. Sections were stained with haematoxylin and eosin and special stains when required.

The results showed a high carcinogenic activity of DB[a,i]P in the hamsters respiratory system. The survival rates for the DB[a,i]P treated animals indicated a shortening of the lifespan. Death was most frequently attributed to respiratory insufficiency due to extensive neoplastic involvement of the respiratory system. Tumours occurred in all parts of the respiratory system, lungs, trachea, and larynx. The incidence of respiratory tumours (males and females combined) was 36/48 (75 %) in the group that received 12 doses of 1.0 mg and 31/48 (65 %) in the group that received 17 doses of 500 µg. The earliest tumours appeared in the larynx and trachea at 8 weeks. Main bronchi tumours (62 % at the 12 × 1.0 mg dose level and 77 % at the 17 × 500 µg dose level) predominated, followed by tracheal tumours (19 % at the 12 × 1.0 mg dose level and 13 % at the 17 × 500 µg dose level). Tumours of the larynx, lung and pleura were observed at lower incidences. The laryngeal tumours were mostly squamous cell carcinomas showing horn pearls, and solid areas of proliferating polymorphic squamous cells. A couple of laryngeal tumours were completely undifferentiated, devoid of keratin formation or intercellular bridges. The tracheal tumours were papillomatous, lined by cuboidal, faintly mucus producing cells or squamous cells, but also squamous cell carcinomas or anaplastic carcinomas were noted.

Overall, the results of the study have shown that DB[a,i]P is a potent carcinogen for the hamster respiratory system. The high percentage of tumours of the respiratory system (75 and 65 %) as well as the short latent period, 8 weeks for both laryngeal and tracheal tumours, emphasize the high carcinogenic activity of DB[a,i]P. Morphologically, the most common tumours were squamous-cell carcinomas. The most common location was the bronchi, followed by the trachea.

In the following table an overview of results from the two carcinogenicity studies in hamsters, in which DB[a,i]P was instilled intra-tracheally, is given.

Table 14: Results from carcinogenicity studies in the Syrian golden hamster, intra-tracheal application

Strain Sex No./sex/ group	**Dosage Purity Vehicle**	**Study Duration at death/ sacrifice**	**Results Incidence and type of tumours**	**Reference**
Syrian golden male 36 48 90 controls	Dosage: ground with haematite [1:1, < 1 µm particles] in 200 µL, 2 mg 1×/wk, 4 wk (total dose, 8 mg) 500 µg, 1×/wk, 24 wk (total dose, 12 mg) Purity: > 99 % Vehicle: 0.9 % saline	100 wk, 120 wk controls	**positive** **8 mg (toatal dose; ≈2.92 mg/kg bw/d)** 16/34 (47 %); respiratory tract tumours, predominantly squamous cell carcinoma 1/34 (3 %) larynx, 2/34 (6 %) trachea 13/34 (38 %) bronchi, 1/34 (3 %) lung **12 mg (toatal dose; ≈0.73 mg/kg bw/d)** 39/44 (89 %); respiratory tract tumours, predominantly squamous cell carcinoma 6/44 (14 %) trachea, 37/44 (84 %) bronchi 1/44 (2 %) lung 2/44 (4 %) malignant lymphoma) vs 0/82 (respiratory tract tumours)) 11/82 (13 %; tumours at other sites) in untreated controls	Sellakumar and Shubik 1974
Syrian golden male / female 24/24 no controls	Dosage: 1 mg, 1×/wk 12 wk (total dose, 12 mg) 500 µg, 1×/wk, 17 wk (total dose, 8.5 mg) Purity: > 99 % Vehicle: distilled water	lifetime study	**positive** **12 mg** (total dose), **≈1.14 mg/kg bw/d (m)** **≈1.3 mg/kg bw/d (f)** 36/48 respiratory tract tumours, predominantly squamous cell carcinoma: bronchi, 62 %; trachea, 19 % **8.5 mg** (total dose) **≈0.57 mg/kg bw/d (m)** **≈0.65 mg/kg bw/d (f)** 31/48 respiratory tract tumours, predominantly squamous cell carcinoma: bronchi, 77 %; trachea, 13 % also larynx, lung and pleura tumours	Stenbäck and Sellakumar 1974

4.10.1.3 Carcinogenicity: dermal

- **Carcinogenicity studies**

Mouse

DB[a,i]P was tested for carcinogenicity in a group of 23 male mice of the XVII strain treated twice weekly by dermal applications of a saturated solution (one drop, not specified) of DB[a,i]P in *ortho*-dichlorobenzene to the shaved backs (skin painting) for up to 14 months (Lacassagne et al. 1958). The tumour incidence was 21/23 skin papillomas (latency 83 days) and 8/23 epitheliomas (latency 219 days). No tumours were observed in the solvent control group.

These study results show that DB[a,i]P induced skin papillomas and epitheliomas in male mice after repeated dermal application for up to 14 months.

In a comparative study the carcinogenicity of higher PAH as presented in the fraction of the tobacco smoke condensate was examined (Wynder and Hoffmann 1959). DB[a,i]P is a substance found in the neutral fractions of tobacco smoke condensate (Bonnet and Neukomm 1956). The carcinogenic activity of DB[a,i]P was tested in a skin painting study in female Swiss ICR/Ha (Millerton) mice. DB[a,i]P (purified by chromatography) was topically applied to the backs of mice three times weekly in two concentrations. Groups of 10 and 20 female mice received 0.1 % and 0.01 % DB[a,i]P solution in acetone solution. A control group was not used. Mice treated with 0.1 % and 0.01 % DB[a,i]P survived for 14 and 17 months, respectively. The tumour incidence in the 0.1 % (approximately 1.71 mg/kg bw/d) DB[a,i]P dose group was 5/10 (50 %) skin papillomas and 1/10 (10 %) skin carcinomas. Skin papillomas were observed after treatment for 7 months and skin carcinomas after treatment for 14 months. In mice receiving 0.01 % (approximately 0.17 mg/kg bw/d) DB[a,i]P skin papilloma were observed after treatment over 17 months in 2/20 (10 %) mice; but no skin carcinomas were noted.

DB[a,i]P identified in tobacco smoke condensate has shown to be carcinogenic to female mice. It has induced skin papilloma and carcinoma after repeated dermal application for up to 17 months.

Nine synthesized and/or highly purified hexacyclic aromatic hydrocarbons including DB[a,l]P, DB[a,h]P and DB[a,i]P were tested for carcinogenicity and tumour-initiating potency on mouse skin (Hoffmann and Wynder 1966; LaVoie et al. 1979).

For the carcinogenicity study groups of 20 female Swiss albino Ha/ICR/Mil mice, 7–8 weeks of age, were treated thrice weekly with dermal applications (skin painting) to the shaved backs at dosages of 0.05 % or 0.1 % DB[a,i]P (purified by chromatography and recrystallized) solutions (vehicle: p-dioxan) for a period of 12 months and then allowed a 3 months treatment-free recovery period before sacrifice. A control group of 20 mice was treated with p-dioxane alone. Lesions (≥ 1 mm^3) that persisted for at least 3 weeks were diagnosed as papillomas. The animals monitored weekly by palpation for tumour development and were killed when tumours were persisted for 4-5 weeks. Autopsies were performed and all tissues suggestive of neoplasia were examined microscopically. An overview of the tumour incidences in the 0.1 % dose group as determined by weekly palpation is summarized in the following table.

Table 15: Skin tumour incidences in female Swiss albino Ha/ICR/Mil mice receiving dermal application of 0.1 % (approximately 1.71 mg/kg bw/d) DB[a,i]P for 12 months

Month	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**Total**
Survivor	20	20	20	20	19	19	19	17	14	13	8	5	4	3	2	**2**
Papilloma					1	1	3	6	7	10	15	16	16	16	**16**	**29**
Epithelioma								2	4	5	8	11	12	13	**15**	**15**

No tumours were observed in the p-dioxane-treated control group (0/20). In the test group (mice treated with 0.1 % DB[a,i]P) skin papillomas were observed in 16/20 (80 %) mice and skin epitheliomas in 15/20 (75 %) mice. A mean tumour latency period of 287 days was recorded.

An overview of the tumour incidences in the 0.05 % DB[a,i]P dose group as determined by weekly palpation is summarized in the following table.

Table 16: Skin tumour incidences in female Swiss albino Ha/ICR/Mil mice receiving dermal application of 0.05 % (approximately 0.86 mg/kg bw/d) DB[a,i]P for 12 months

Month	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**Total**
Survivor	20	20	20	20	20	19	19	19	16	14	11	9	6	3	2	**2**
Papilloma								3	6	7	10	15	16	16	**16**	**28**
Epithelioma									3	4	6	6	9	12	**13**	**13**

In the test group mice treated with 0.05 % DB[a,i]P 16/20 (80 %) developed skin papillomas and 13/20 (65 %) skin epitheliomas. A mean tumour latency period of 336 days was recorded.

Repeated dermal applications of 0.1 % (approximately 1.71 mg/kg bw/d) and 0.05 % (approximately 0.86 mg/kg bw/d) DB[a,i]P to female Swiss albino Ha/ICR/Mil mice for 12 months induced skin papillomas and epitheliomas in high incidences.

In the following table an overview of results from dermal carcinogenicity studies with DB[a,i]P in mice is given.

Table 17: Results from carcinogenicity studies in mice, dermal application

Strain Sex No./sex/ group	**Dosage Purity Vehicle**	**Study Duration at death/ sacrifice**	**Incidence and type of tumours**	**Results Specific feature**	**Reference**
XVII male 23	Dosage: 1 drop of a saturated solution (concentration, volume not specified), 2x/wk Purity: no data Vehicle: *ortho*-dichlorobenzene	14 months	21/23 (91 %); skin papillomas, latency 83 days and 8/23 epitheliomas latency 219 days vs 0 solvent control	**positive** control group not treated simultaneously no statistics	Lacassagne et al. 1958
Swiss albino Ha/ICR/Mil females 20/10	Dosage: 0.01 and 0.1 % solution (volume, not specified), 3x/wk, 17 and 14 months Purity: no data Vehicle: acetone	lifetime study (up to 17 months	**0.01 % (0.17 mg/kg bw/d)** 1/20 (skin papillomas) 0/10 (skin carcinomas) **0.1 % (1.71 mg/kg bw/d)** 5/10 (skin papillomas) 1/10 (carcinomas)	**positive** no control no statistics	Wynder and Hoffmann 1959
Swiss albino Ha/ICR/Mil female 20	Dosage: 0.05 and 0.1 % solution (volume not specified), 3x/wk 12 months + 3 months treatment-free recovery period Purity: no data (recrystallized)	15 months	**0.05 % (0.86 mg/kg bw/d)** 16/20 (80 %; skin papillomas), 13/20 (65 %; skin epitheliomas), **0.1% (1.71 mg/kg bw/d)** 16/20 (80 %; skin papillomas), 15/20 (75 %; skin epitheliomas) vs 0/20 solvent controls	**positive** no statistics	Hoffmann and Wynder 1966 LaVoie et al. 1979

- **Carcinogenicity studies by a single subcutaneous administration**

Mouse

DB[a,i]P was tested for carcinogenicity in groups of 17 male and 18 female mice of the XVII strain. Animals received a single subcutaneous injection of 0.6 mg DB[a,i]P (purity not specified) solution in *ortho*-dichlorobenzene per month for a period of 3 months (Lacassagne et al. 1958). All treated males developed local sarcomas during an average latency period of 75 days, and 16/18 females during a mean latency period of 82 days, respectively, leading to the death of all animals within 135 days.

The study results demonstrate that DB[a,i]P induces sarcomas at the injection site in mice after single subcutaneous injection.

Waravdekar and Ranadive (1958) have tested the carcinogenic activity of DB[a,i]P in hybrid mice. A group of 16 hybrid mice (second generation of hybrids raised by crossing the two inbred strains: XVII and C57BL; 8/sex; 4-5 months of age) received a single subcutaneous injection of 2.0 mg DB[a,i]P (purity not specified) suspension in 0.2 mL of propylene glycol. The site of injection was inspected daily and the appearance of the palpable tumour was recorded. When the tumours attained appreciable size, the animals were killed and the tumour dissected out and fixed in Bouin's fluid. Six sections were stained by the haematoxylin and eosin method, and Gomori's method for silver impregnation for the reticulum. All the 16 animals receiving DB[a,i]P developed tumours at the site of injection 2-3 months after treatment, within a short latent period of 74 days. No difference in the tumour incidences between male and female mice was seen. The palpable tumours grew fast and attained a fairly large size within 2 to 3 weeks of their appearance. Microscopy of the tumours revealed typical fibrosarcomas of the subcutis, varying in their histological pattern and the grade of cytologic malignancy and invasiveness. Ten of the 16 tumours were classified as typical spindle-cell sarcomas, with closely packed spindle-shaped cells. The tumour cells ranged in size from large to medium and the shape from blunt to long, narrow spindles. Mitosis was common and occasionally giant cells were present. The remaining 6 tumours exhibited significant pleomorphy of cellular as well as nuclear outlines. The tumours were extremely cellular, the cells varying in shape from fusiform spindle to oval polygonal, with abundant eosinophilic cytoplasm and oval or spherical hyperchromatic nuclei. There were many large mono- or multinucleated giant cells, and mitosis was frequent. These tumours were very active, with progressive growth indicating a high grade of malignancy. Most of these tumours infiltrated into the muscle and into the hair follicles.

The test results have shown a high carcinogenic activity of DB[a,i]P in mice. After a single subcutaneous injection of DB[a,i]P all male and female hybrid mice (100 %) developed tumours within a short latent period of 74 days. Morphologically these tumours were fibrosarcomas showing all characteristics of cytologic malignancy and invasiveness.

The minimal effective subcutaneous dose of DB[a,i]P for tumour induction determined in male C57Br/cd mice was examined by Homburger and Tregier (1960). In this study dosages of 0.01-600 µg DB[a,i]P were tested. Groups of 10, 11 or 12 animals received a single subcutaneous injection of DB[a,i]P in peanut oil. No tumours were found at dosages below 1.0 µg DB[a,i]P. The tumours developed after single subcutaneous injection of > 1.0 µg DB[a,i]P were found to be fibrosarcomas and leiomyosarcomas. Microscopically earliest detectable change in the histiogenesis of a fibrosarcoma was noted as early as 5 weeks after injection of DB[a,i]P, whereas palpable tumours appear rarely before 9 weeks after injection. Fully developed fibrosarcoma in a C57Br/cd mouse was observed 12 weeks after subcutaneous injection of DB[a,i]P. The tumour formation following single injection of DB[a,i]P with several doses to male C57Br/cd mice is given in the following table.

Table 18: Tumour formation following a single injection of DB[a,i]P to male C57Br/cd mice and the time at which these tumours occur

Doses [µg]	No. of animals	No. weeks elapsed when 50 % animals had tumours	No. weeks elapsed when all animals had tumours	No. animals dead of tumours	No. animals dead without tumours at 56 weeks	No. animals alive without tumours at 66 weeks
600	11	7	17	11	-	-
100	11	5-6	14	11	-	-
50	12	12-13	17	12	-	-
25	12	13-14	-	11	1	-
12.5	11	18-19	-	7	4	-
6.25	12	66	-	6	1	5
2.0	12	-	-	4	7	1
1.0	11	-	-	1	4	6
0.5	12	-	-	-	12	-
0.1	12	-	-	-	12	-
0.05	12	-	-	-	8	4
0.01	10	-	-	-	4	6

A dose-response relationship for tumour formation up to 50 µg DB[a,i]P was observed beyond which dosage all animals developed tumours within 17 weeks. This has been confirmed in a further study involving 8,850 males of the C57BL/6 strain. Nearly 100 % of the animals developed tumours within 22 weeks following a single injection of 500 µg DB[a,i]P in peanut oil. These tumours progressed rapidly in size, killing the host within 4 weeks from the time when the tumour become first palpable. No metastases were observed. Injections into the axilla produced tumours slightly more rapidly than injections into the groin. This difference may be due to the anatomical characteristics of these areas which permit perhaps more ready palpation of tumours in the axilla. Since tumours tend to invade and adhere to the chest wall in this site, whereas they remain more discreet in the groin.

The study results show a clear tumour induction in C57br/cd mice given a single subcutaneous injection of DB[a,i]P. Earliest occurrence of morphologically malignant cells at the injection sites were identifiable 4-5 weeks after administration. The induced tumours were fibrosarcomas of consistent morphologic and functional uniformity. A dose-response relationship for tumour formation was determined up to a dose of 50 µg DB[a,i]P.

The tumour initiating activity of DB[a,i]P was examined in a group of 50 male C57BL/6 mice receiving a single subcutaneous injection of 100 µg DB[a,i]P (purity unspecified) in peanut oil (volume unspecified). A control group of 25 mice was treated with 0.1 mg trioctanoin (CAS 538-23-8, in general used as a synthetic fiber lubricant) (Sardella et al. 1981). Survival rates after 75 weeks were 41/50 DB[a,i]P-treated animals and 24/25 controls. Tumour incidences (local sarcomas) at 25, 50 and 75 weeks were 34/50, 40/50 and 40/50 for treated mice and 0/25 in controls, respectively.

Overall, a single subcutaneous injection of 100 µg DB[a,i]P has resulted in the rapid appearance of local sarcomas in the mouse.

The influence of surgical trauma on the development and progression of experimental tumours was investigated in C57BL/6 mice given single subcutaneous injections of DB[a,i]P (Gottfried et al. 1961). In total 259 mice, 10-13 weeks old, were divided into three groups on the basis of equivalent age, weight, and sex distribution. In the first group (carcinogen control), 86 C57BL/6 mice received 0.5 mg DB[a,i]P dissolved in a 0.1 mL peanut oil suspension subcutaneously in the mid-dorsal area. To control any effects of the handling procedures employed in the treated groups, these mice were divided into two subgroups, 41 and 45 mice, respectively. One subgroup was handled in the same manner as the skin-wounded mice, i.e., shaven, skin-sterilized, immobilized as for surgery, and released. The second subgroup was, in addition to surgical preparation and handling, given Nembutal anaesthesia as a control for the mice on which laparoscopy was performed. Between the 6^{th} and 10^{th} week after injection of the compound, eight mice were sacrificed for histology of progress of induction of neoplasia at the injection site. A remainder of 78 mice comprised the experimental control group without further surgical trauma. In the second group (carcinogen and repeated skin wounding), 86 C57BL/6 mice received DB[a,i]P as described for the first group. Two mice were sacrificed after 5 weeks and two after 6 weeks for histology of the injection site. The remaining 83 mice were surgically wounded on the dorsum 15-20 mm distant from the carcinogen site, starting the 6^{th} week after carcinogen injection. The wound, created by circular skin punch, was 7-10 mm in diameter and involved the skin and subcutaneous tissue down to but not including the dorsal muscles. Wounds were allowed to heal by secondary intent (by adhesion of granulating surfaces) and surgically reopened 3 times weekly until sacrifice after the 17^{th} week. Surgical asepsis and skin disinfection with 1:5000 hyamine in 70 % isoproponal were employed in all wounding. No special procedures were employed or required to maintain noninfected wounds. In the third group (carcinogen and laparoscopy), 86 C57BL/6 mice received DB[a,i]P as described for the first group. In total, seven mice were sacrificed between the 5^{th} and 6^{th} week for histology of the carcinogen injection site. The remaining 79 mice were subjected to laparoscopy every 2 weeks beginning with the 6^{th} week after carcinogen injection. Seven mice died due to anaesthesia and are not included in the final data. A total of 72 mice comprised this group. Laparoscopies were conducted under Nembutal anaesthesia. The abdominal skin was shaven and prepared with 70 % alcohol containing 1:5000 hyamine. A 15-20 mm incision was made through skin, abdominal musculature, and peritoneum. The incision was retracted, and gentle manipulation of the intestines with blunt forceps was performed. The peritoneum was closed with silk suture, and the skin was closed with a Michel skin clamp. This surgical procedure was repeated every 2 weeks until termination at the end of the 17^{th} week. All mice were housed, five or six per pen on wood shavings, received feed and water ad libitum, weighed every 2 weeks, and the carcinogen injection site was palpated weekly. When tumours appeared, they were measured with callipers in two diameters and recorded. The time of appearance of tumours was determined by collating the palpable characteristics with the histological evidence from the sacrificed animals. In addition, the weekly size increment and growth pattern of the tumour was a further index of time of tumour initiation. The presence of all tumours was confirmed by microscopic examination of sections at death or sacrifice.

At week 12, tumours were present in 64 % of the mice subjected to laparoscopy, in 41 % of the mice subjected to skin wounding, and in 22 % of the control mice. There were no deaths in the control group until the week 15, whereas five and seven deaths with large tumours occurred prior to week 15 in the skin-wounded and laparoscopy groups, respectively. It appears that the tumours in both surgically treated groups progressed at an accelerated rate compared with the tumours in the control group. Deaths by tumour occurred in the three groups of mice by week 17 as follows: in the group subjected to laparoscopy, 38/72 mice (53 %); in the group subjected to skin-wounding, 23/82 mice (28 %); in the control group, 17/78 (22 %). Deaths with large, progressively growing tumours occurred earlier and were more numerous in the surgically treated mice compared to the controls (s. the following table).

Table 19: Number of mice dead with tumours

Weeks after a single subcutaneous injection of DB[a,i]P	Carcinogen control group Total: 78 mice	Skin-wounded group Total: 83 mice	Laparoscopy group Total: 72 mice
14	0	5	7
15	7	2	10
16	5	11	11
17	5	5	10
Cumulative total	**17 (22 %)**	**23 (28 %)**	**38 (53 %)**

The tumour incidence in each group as determined by weekly palpation is summarized in the following table. The data reveal a quantitative relationship between the degree of severity of surgical trauma and time of tumour initiation and tumour growth rate.

Table 20: Cummulative tumour incidence in C57BL/6 mice, single subcutaneous injection

Weeks after a single subcutaneous injection of DB[a,i]P	Carcinogen control group Total: 78 mice No. with tumour	Skin-wounded group Total: 83 mice No. with tumour	Laparoscopy group Total: 72 mice No. with tumour
10	1 (< 1 %)	15 (22 %)	34 (47 %)
12	17 (22 %)	34 (41 %)	46 (64 %)
13	30 (39 %)	48 (58 %)	53 (74 %)
14	43 (55 %)	54 (65 %)	59 (82 %)
15	58 (75 %)	65 (78 %)	59 (82 %)
16	61 (78 %)	76 (91 %)	61 (85 %)

The study results show a clear tumour induction in C57BL/6 mice administered a single subcutaneous injection of 0.5 mg DB[a,i]P, and in addition that surgical trauma exerted a marked effect on the latent period of DB[a,i]P tumour development. There was a markedly greater incidence of initiation of tumours in both surgically treated groups of mice as compared with the carcinogen controls.

The effects of dietary antioxidants on tumour incidence in female Swiss (ICR/Ha) random-bred and caesarean derived weanling mice, weighing 11.5-12.5 g, following a single subcutaneous injection in the nape of the neck with 100 mg DB[a,i]P solution in 0.2 mL of redistilled tricaprylin were examined by Epstein et al. (1967). A total of 1,028 animals in the treatment groups and 138 positive controls (treated with DB[a,i]P solution without antioxidants) were used in these studies. The mice were inspected daily until spontaneous death occurred; they were also weighed weekly for the first 10 weeks of each experiment, prior to which time no tumours developed. Thereafter, weekly measurements of tumour size were made. Each mouse was autopsied and samples for microscopy were taken from injection site or other tumours, metastases and any other lesions; they were fixed in Tellyesniczky fluid and sections were stained with haematoxylin and eosin. However, a list of examined organs and tissues was not available.

The average time to tumour-induced death ranged from 21-28 weeks in the treatment groups, the positive control averages were approx. 23 and 25 weeks. There were no significant histological differences in tumour development and types of tumours or other effects on organs in the various groups and controls. Fibrosarcomas at the injection site were the most common malignant neoplasm induced by DB[a,i]P. These were locally invasive and showed various degrees of differentiation, which were randomly distributed between treatment and positive control groups, and showed no

relation to the average time to tumour death. The fibrosarcoma incidence was 975/1,028 in treatment groups with antioxidants and 125/138 in positive controls, respectively. Relatively few carcinomas developed at the injection site (treatment groups: 9/1,028; positive controls: 2/138); those which occurred were squamous carcinomas of varying degrees of differentiation, with the exception of a single cystic basal cell carcinoma. The incidences of solitary and multiple pulmonary adenomas and of generalized metastases were comparatively low in all groups. The frequency of metastases was high when the local tumour at the injection site was a carcinoma, 7/11 (64 %), and low when the local tumour was a fibrosarcoma, 51/1,100 (5 %). In both cases metastases were usually confined to the lungs, 6/7 (86 %) for carcinomas, 38/51 (75 %) for fibrosarcomas.

In this lifetime study in which various antioxidants were tested none of the antioxidants, at any of the concentrations tested, produced any important effect on average body weight, on average tumour incidence or finally, any significant increases in average time to tumour-induced death.

In the following table an overview of carcinogenicity studies in mice administered DB[a,i]P by a single subcutaneous injection is given.

Table 21: Results from carcinogenicity studies in mice, subcutaneous administration

Strain Sex No./sex/ group	**Dosage Purity Vehicle**	**Study Duration at death/ sacrifice**	**Incidence and type of local tumour at the injection site**	**Results Specific feature**	**Reference**
XVII male/ female 17/18	Dosage: **0.6 mg** 1×/month, 3 months Purity: not specified Vehicle: ortho-dichlorobenzene	135 days	male, 17/17 (100 % sarcoma) latency 75 days female, 16/18 (89 % sarcoma) latency 82 days	**positive** no control limited duration no statistics	Lacassagne et al. 1958
F_2 Hybrid, XVII x C57BL male/ female 8/8	Dosage: **2.0 mg** in 200 µL **≈67 mg/kg bw (m)** **≈80 mg/kg bw(f)** 1x Purity: not specified Vehicle: propylene glycol	up to 4 months	male, 8/8 (100 % fibrosarcoma) female, 8/8 (100 % fibrosarcoma) average latency 74 days	**positive** no control limited duration no statistics	Waravdekar and Ranadive 1958
C57Br/cd male 10-12	Dosage: 0.01–600 µg (volume not specified) 1×	66 wk	fibrosarcoma **< 1 µg**, 0 % **1 µg** (0.03 mg/kg bw), 9 % **2 µg** (0.07 mg/kg bw), 33 % **6.25 µg** (0.21 mg/kg bw) 50 %	**positive** no statistics	Homburger and Tregier 1960

Strain Sex No./sex/ group	**Dosage Purity Vehicle**	**Study Duration at death/ sacrifice**	**Incidence and type of local tumour at the injection site**	**Results Specific feature**	**Reference**
	Purity: not specified Vehicle: peanut oil		**12.5 µg** (0.42 mg/kg bw), 64 % **25 µg** (0.83 mg/kg bw), 92 % **≥ 50 µg** (≥ 1.67 mg/kg bw) 100 %		
C57BL/6 Male 8,850	Dosage: **500 µg** (volume not specified), 1× Purity: not specified Vehicle: peanut oil	> 22 wk	**0.5 mg** (16.67 mg/kg bw**)** nearly 100 % fibrosarcoma	**positive** no control no statistics	Homburger and Tregier 1960
C57/BL/6 male 50 in test, 25 in control	Dosage: **100 µg** (volume not specified) in peanut oil, 1× Purity: not specified Vehicle:control group: trioctanoin	75 wk	≈3.33 mg/kg bw after 25/50/75 wk 34/50; 40/50; 40/50 68-80 %; local sarcomas vs 0/25 solvent controls	**positive** no histology no statistics	Sardella et al. 1981
C57BL/6 male and female 259 (total)	Dosage: **0.5 mg** in 0.1 mL peanut oil, 1x 16.67 mg/kg bw (m)/20 mg/kg bw (f) Putity: not known	17 wk	tumour type not specified 12th wk: 17/78 (22 %) 13th wk: 30/78 (39 %) 14th wk 43/78 (55 %) 15th wk: 58/78 (75 %) 16th wk: 61/78 (78 %)	**positive** no negative control no statistics	Gottfried et al. 1961
Swiss ICR/Ha female 138	Dosage: **100** µg in 0.2 mL 4.0 mg/kg bw 1× Purity: not specified Vehicle: tricaprylin	38 wk	125/138 (91 %; fibrosarcoma) further tumours observed: 2/138 (1 %; local carcinoma) 1/138 (1 %; lymphoma) 6/138 (4 %; solitary pulmonary adenoma) 2/138 (1 %; multiple pulmonary adenoma)	**positive** no vehicle control no statistics	Epstein et al. 1967

Hamster

The susceptibility of Syrian hamsters for induction of fibrosarcomas by a single subcutaneous injection of DB[a,i]P was studied by Wodinsky et al. (1964). DB[a,i]P doses were dissolved in 0.4 mL tri-n-caprylin ('Trioctanoin'). Five groups of 6-10 male hamsters weighing 55-75 g were randomly assigned to treatment with 2.0, 1.0, 0.5 or 0.25 mg DB[a,i]P/hamster (converted into mg/kg bw: approximately 26.67, 13.33, 6.67 or 3.33 mg/kg bw). 10 male hamsters were used in the solvent control group. Animals were injected subcutaneously at the nape of the neck.

Induction of subcutaneous tumours was observed in hamsters of all dose groups tested. At the highest dose of 2.0 mg/hamster, 4/10 animals died before the appearance of the first tumour. The tumour incidences after 17 weeks were 6/6 (100 %) at 2.0 mg, 10/10 at 1.0 mg (100 %), 9/10 at 0.5 mg (90 %), 5/9 at 0.25 mg (55 %) and 0/10 controls. The average latency period for tumour induction by DB[a,i]P was approx. 10 weeks at 2.0 mg, 10 weeks at 1.0 mg, 13 weeks at 0.5 mg, and 14 weeks at 0.25 mg. Microscopy revealed fibrosarcomas of the subcutis which were fairly uniform in their histological pattern. The tumours were classified as spindle-cell sarcomas with closely packed spindle-shaped tumour cells. Solid masses of cell alternating with large and small whorls and interlacing bundles of fibrous connective tissue were observed. The tumour cells ranged in size and varied in shape from blunt to narrow spindles. Mitoses were common, and occasionally giant cells were present.

In addition 169 female and 151 male hamsters were injected subcutaneously with a single dose of 1.0 mg DB[a,i]P/hamster. Twelve female and 13 male hamsters died without tumour development during the course of the study. The tumour incidences were 157/157 (100 %) with a mean latency period of 11 weeks in female hamsters and 138/139 (99 %) with a mean latency period of approx. 12 weeks in male hamsters.

Overall, all the tested single subcutaneous injections (approx. 26.67, 13.33, 6.67 or 3.33 mg/kg bw) of DB[a,i]P at the nape of the neck resulted in the rapid appearance of fibrosarcomas at the injection site in male and female hamsters. The incidence of tumours and duration of latency period were in a dose-related manner.

In a further study the susceptibility of the subcutaneous tissue of the cheek pouch for tumour induction by DB[a,i]P was investigated by Wodinsky et al. (1965). One hundred and twenty female Syrian hamsters, each weighing 75-100 g, were divided among six treatment groups; the animals were slightly anaesthetized and the right cheek pouches were exteriorized. Each cheek pouch received a single injection of DB[a,i]P which had dissolved in trioctanoin at doses of 0.25, 0.5, 1.0, 1.5 or 2.0 mg/hamster (converted into mg/kg bw: approx. 2.5, 5.0, 10, 15, or 20 mg/kg bw) in an injection volume of 0.2 mL. The highest dose of 2.5 mg (approx. 25 mg/kg bw) was administered at 0.4 mL. Hamsters were examined weekly until death and observed tumours were removed at autopsy for microscopy. Animals were observed up to 40 weeks after treatment with DB[a,i]P. Mortality occurred in all injected groups. The number of useable hamsters was reduced to 81 due to host mortality in all injected groups. Tumour development at the site of injection was observed from the 7^{th} week onwards. In the following table an overview of tumour production and survival of female Syrian hamsters injected with single doses of DB[a,i]P in cheek pouch is given.

Table 22: Cumulative tumour incidence, average latency period and survival of tumour-bearing female Syrian hamsters injected with a single dose of DB[a,i]P in cheek pouch

Dose DB[a,i]P/ trioctanoin	**Effective number of animals**	**No. tumours**	**Average latency period of observed tumours [weeks]**	**Mean survival time of tumour-bearing hamsters [weeks]**
0.25 mg/0.2 mL	13	11 (85 %)	25	30
0.5 mg/0.2 mL	15	13 (87 %)	20	28
1.0 mg/0.2 mL	12	12 (100 %	18	25
1.5 mg/0.2 mL	13	13 (100 %)	15	24
2.0 mg/0.2 mL	8	8 (100 %)	14	27
2.5 mg/0.4 mL	20	19 (95 %)	11	19

The cumulative tumour incidence was as follows: 0.25 mg/hamster, 11/13 (85 %); 0.5 mg/hamster, 13/15 (85 %); 1.0 mg/hamster, 12/12 (100 %); 1.5 mg/hamster, 13/13 (100 %); 2.0 mg/hamster, 8/8 (100 %); 2.5 mg/hamster, 19/20 (95 %). The average latency period of tumour development and mean survival times was related to dosage. A decreasing dose of DB[a,i]P resulted in a gradual prolongation of the average latency period and mean survival time. The average latency period for tumour induction in hamsters receiving a single injection of DB[a,i]P in the cheek pouch was 11 weeks at 2.5 mg, 14 weeks at 2.0 mg, 15 weeks at 1.5 mg, 18 weeks at 1.0 mg, 20 weeks at 0.5 mg, and 25 weeks at 0.25 mg. The tumour incidences were in close proximity for the six treatment groups. Microscopy revealed spindle cell fibrosarcomas with areas of pleomorphism. Multinucleated giant cells were seen in some areas and also mitotic figures. The average latency period of fibrosarcoma arising in the suprascapular area (Wodinsky et al. 1964) was shorter than in the cheek pouch.

In the following table an overview of results from carcinogenicity studies in Syrian hamsters, which had received single subcutaneous injections of DB[a,i]P, is given.

Table 23: Results of carcinogenicity studies in Syrian hamsters, subcutaneous administration

Strain Sex No./sex/ group	**Dosage Purity Vehicle**	**Study Duration at death/ sacrifice**	**Incidence and type of tumour**	**Results Specific feature**	**Reference**
Syrian male 6-10 10 controls	Dosage: 0.25, 0.5, 1.0, 2.0 mg in 0.4 mL tri-n-caprylin (Trioctanoin) Purity: not specified Vehicle: tri-n-caprylin	up to 17 wk	fibrosarcoma **0.25 mg (3.33 mg/kg bw)**, 5/9 (55 %) **0.5 mg (6.67 mg/kg bw)**, 9/10 (90 %) **1.0 mg (13.33 mg/kg bw)**, 10/10 (100 %) **2.0 mg (26.67 mg/kg bw)**, 6/6 (100 %) vs 0/10 controls	**positive** pilot study	Wodinsky et al. 1964
Syrian male/ female 151/169	Dosage: **1.0 mg** in 0.4 mL tri-n-caprylin (Trioctanoin) Purity: not specified Vehicle: tri-n-caprylin	up to 22 wk	**≈13.33 mg/kg bw** 13 males and 12 females died without tumours fibrosarcoma observed in male, 138/139 (99 %) mean latency: 11.6 weeks female, 157/157 (100 %) mean latency 11 weeks	**positive** no statistics	Wodinsky et al. 1964
Syrian female 120, reduced to 81 due to host mortality in all test groups	Dosage: 0.25, 0.5, 1.0, 1.5, 2.0 mg in 0.2 mL trioctanoin 2.5 mg in 0.4 mL trioctanoin Purity: not specified Vehicle: Trioctanoin	up to 40 wk	fibrosarcoma observed **0.25 mg (2.5 mg/kg bw)**, 11/13 (85 %), latency 25 wk **0.5 mg (5.0 mg/kg bw)**, 13/15 (85 %), latency 20 wk **1.0 mg (10 mg/kg bw**, 12/12 (100 %), latency 18 wk **1.5 mg (15 mg/kg bw),** 13/13 (100 %) latency 15 wk **2.0 mg (20 mg/kg bw)**, 8/8 (100 %) latency 14 wk **2.5 mg (25 mg/kg bw)**, 19/20 (95 %) latency 11 wk	**positive** no control no statistics	Wodinsky et al. 1965

- **Transplantation technique, transfer of injection-site tissues following subcutaneous injection of mice**

In the context of evaluation of accelerated carcinogen testing extensive studies in C57BL/6 mice were performed in the 1960ies. A total of 3,000 animals were used in these studies using the transplantation technique. Tumour formation in C57BL/6 mice was induced by single subcutaneous injection of 25 µg or 500 µg DB[a,i]P. After tumour development at the injection-site tissues were excised and transferred into one secondary host (Homburger and Treger 1967).

Injections of single dose of 500 µg DB[a,i]P in 0.1 mL of peanut oil were made into the left groin of C57BL/6 male mice aged 2 to 3 months, in groups of 20, 40, or 80 animals. Tumour development was observed at injection sites up to week 8, animals were then sacrificed and transplants were transferred into male C57BL/6 mice. Tumour fragments 1-2 mm in diameter were used, removed, minced in Ringer's solution and pooled, and the resulting tissue mush was injected subcutaneously into 10 mice of the same strain, sex and age. Thus, each group of 10 secondary hosts received the equivalent tissue samples from a total of 20 to 80 injected sites. The take rate was 100 %, and the growth rate of these tumours was relatively uniform. There were no regressions, and the resulting tumours killed the hosts in 2-4 weeks. Histological studies were performed on the injection sites weekly during tumour development up to week 8 in 40 males and 40 females and then in the secondary hosts. Serial histologic studies of injection sites had shown that morphologically malignant cells were identifiable 4-5 weeks after administration of DB[a,i]P. The induced fibrosarcomas were of consistent morphologic uniformity. Further examinations in mice have shown that the time of latency of tumour growth following carcinogen injection was, in general, 10 weeks; 50 % tumour incidence was reached in 14 to 15 weeks; and 100 % of the injected animals in most cases had tumours after 25 weeks. By using the transplantation technique it was shown that the transplantation of combined carcinogen injection sites from 4 animals, 5 weeks after carcinogen injection, into one secondary host, significantly accelerated tumour growth in the secondary recipient. The latency in the secondary host was 3 weeks; 50 % tumour yield was reached 4 weeks after transfer of the injection sites, with 100 % tumour yield 5 weeks later. The 50 % tumour level is thus reached 9 weeks after the original carcinogen injection, an acceleration of 5 weeks.

The transplantation technique was examined for suitability and practicability with lower doses by Homburger and Baker (1969). Groups of 40 C57BL/6J mice, 6-8 weeks old, received single subcutaneous injections of 25 µg DB[a,i]P in 0.1 mL of tricaprylin. The sites of these injections were palpated weekly and the appearance of tumours 1 cm in diameter was recorded. After 7 weeks the injection sites with tumours were removed and transferred as pooled tissues from the subcutaneous injection sites into the recipients. The tumours were removed and studied by microscopy. Another group of 50 animals was left undisturbed following the initial injection of DB[a,i]P in peanut oil, and tumours appearing at the site of injection were recorded. Tumours were fixed in formaldehyde and processed for staining by haematoxylin and eosin as well as by pyronin green method before and after exposure to ribonuclease. In this study results of microscopy were extensively reported also from studies with mice treated with 500 µg DB[a,i]P. Tumour incidence curves in primary hosts (25 µg DB[a,i]P s.c.) and secondary hosts (4 injection sites 7 weeks old) have shown an incidence of 100 % tumours at 32 weeks after DB[a,i]P injection. However, a tumour incidence of 90 % in the secondary host was already reached at 18 weeks after DB[a,i]P injection. The transfer of four carcinogen injection sites into one secondary host resulted in a shortening of the latent period independently using 500 µg or 25 µg DB[a,i]P. Microscopy of the injection site 5-6 weeks after injection of 500 µg or 25 µg DB[a,i]P in peanut oil revealed 'atypical' fibroblasts with malignant cell morphology within the tissue surrounding the injection site. Stains for ribonucleic acids showed accumulation of RNA in the perinuclear area of such cells as well as in their nuclei. Thereafter an increase in numbers of

atypical RNA-loaded fibroblasts was noted and by about the 7th week the typical pattern of fibrosarcoma was observed.

In a further study using the transplantation technique (Homburger and Treger 1970**)**, groups of 50 male C57BL/6J mice, 6-8 weeks old, received single subcutaneous injections of 25 µg DB[a,i]P in 0.1 mL of tricaprylin into the left groin and were palpated weekly for tumour developement. DB[a,i]P (purity of commercial grade) was suspended in tricaprylin by means of a magnetic stirrer and kept in a 100 °C water bath for ½ hour. Groups of 40 animals were similarly treated and after 6 weeks the injection sites were removed, pooled in Ringer's solution, cut with scissors, and made into a tissue mush. One-tenth of the volume of this tissue mush (0.5 mL) was injected into the left groin of each of 10 secondary mice of the same age and sex as the primary animals. A solvent control group of 40 male C57BL/6J mice received 0.2 mL tricaprylin subcutaneously in the left groin, and transfers of these injection sites were made into 10 animals 6 weeks later. The animals were palpated each week and time at which a tumour was first palpated was recorded and some tumours were studied histologicaly as soon as first recognized. Others were allowed to grow to 1 cm in diameter and were used for trocar transplants and microscopy. All animals were autopsied and even though some were allowed to live with large tumours for many weeks, no metastases were found. In mice given 25 µg DB[a,i]P injections, which was left in situ, the 50 % tumour level was reached in approximately 19 ½ weeks. When injection sites were transplanted after 6 weeks, the 50 % tumour level was reached within 14 ½ weeks. The first tumour with 25 µg DB[a,i]P in situ appeared 12 weeks after injection (with 500 µg DB[a,i]P, 10 weeks) and after transplantation, the first tumour appeared 9 weeks after injection (with 500 µg DB[a,i]P, 8 weeks). Microscopy showed that the tumours observed were fibrosarcomas, similar to those induced by the carcinogen left in situ. Most of these tumours were transplanted into other C57BL/6 mice and grew rapidly within 2 weeks. It was further shown that the accelerated tumour growth occurs only in presence of living cells and that dead (boiled) tissue actually slows down carcinogenesis. The site-transfer studies with the solvent tricaprylin failed to yield any tumours 107 weeks after the first injection. These negative results of transplanting pooled tricaprylin injection sites indicate that the procedure itself has no carcinogenic effect.

Additionally in one of these studies responses of DB[a,i]P-induced subcutaneous sarcomas and their transplants to various chemotherapeutic agents were examined **(**Homburger et al. 1962**)**. Male C57BL/6 mice were injected with single doses of 500 µg DB[a,i]P in peanut oil. Tumour fragments 1-2 mm in diameter were used and transferred into the recipients by the usual trocar method. The take rate was 100 %, and the growth rate of these tumours was relatively uniform. There were no regressions, and the tumours killed the hosts in 2-4 weeks. Results of experimental chemotherapy in DB[a,i]P-induced mouse tumours and their transplants are not disscused here.

In another study the influence of intrinsic factor such as sex, age, nutritional status, and connective-tissue reactivity which determine the rate of formation and subsequent behaviour of DB[a,i]P-induced tumours in C57BL/6 Jax mice were investigated **(**Homburger et al. 1963**)**. A dose of 500 µg DB[a,i]P (commercial grade) was suspended in peanut oil by stirring for 8 hours at 80-100 °C or for 2-4 hours at 150-175 °C. Injections of single doses of 500 µg DB[a,i]P in 0.1 mL of peanut oil were made into the left groin of male and female C57BL/6 Jax mice aged 2 to 3 months. A total of 250-300 animals were used in these studies. Tumour incidence was studied by weekly palpation of the injection site, and, when the tumour had reached 1 cm in diameter, the time elapsed since injection was recorded. At approximately weekly intervals, from 2 to 16 weeks after injection, 5-10 males and 5-10 females

were killed and the injection site was excised and fixed in 4 % formaldehyde for histology and fluorescence microscopic studies. Transplant characteristics were ascertained by trocar transplantation of numerous induced tumours into C57BL/6 and other strains (not specified) and by comparison in hosts of both sexes of tumour growth rates in the resulting tumour lines. Tumours which had arisen 10-13weeks after DB[a,i]P injection were transplanted, and their growth rate was compared with that of transplants from tumours that had arisen 23-25 weeks after injection of DB[a,i]P. Transplantation results, with small versus large donor tumours, were compared. The growth rates of the established tumours (1 cm in diameter) were constant for about 2 weeks, but varied considerably thereafter. The rate of tumour formation was lower in females than in males, but it was independent of body weight at the time of carcinogen injection, subsequent growth rate, and (in males only) of the age at the time of injection. For the males the average time of latency was 14 ½ weeks, with a standard error of the mean of 0.8; for the females, 18 weeks, with a standard error of the mean of 1.4. At microscopy two weeks after injection, large subcutaneous cysts and Langhans' type giant cells were seen at the injection sites. These cysts divided into smaller ones at about 8 weeks, at which time the number of Langhans' cells reached its maximum. Severe inflammation was found in about half of the mice of both sexes at 2 weeks; in all males by week 8; and in all females by weeks 10-11. Hypertrophy and increasing number of atypical fibroblasts were seen. The first fibrosarcomas were observed after 8 weeks in males and a little later in females. All tumours examined in approx. 200 mice of both sexes were fibrosarcomas. Tumours in the males seemed to be surrounded by a more intense connective-tissue inflammative reaction compared to females. Tumours in females had a slightly greater tendency to invade skin and muscle. Caloric restriction severe enough to prevent significant weight gains during the induction period prolonged latency period of tumour development significantly. Severe caloric restriction maintained for 7–23 days and resulting in marked weight losses was without effect on the growth rate of induced tumours measuring 1 cm in diameter. However, the survival time of animals thus treated was significantly prolonged.

Overall the results of the site transfer studies have shown that the transfer of four carcinogen injection sites into one secondary host resulted in a shortening of the latency period in tumour developing independently of doses (500 µg or 25 µg DB[a,i]P). Thus, accelerated growth of DB[a,i]P induced tumours was noted at all tested doses.

In the following table an overview of results from carcinogenicity studies in mice using the transplantation technique is given.

Table 24: Results from carcinogenicity studies in mice using the transplantation technique

Strain Sex No./sex/ group	**Dosage Vehicle Purity**	**Study Duration at death/ sacrifice**	**Results Incidence and type of tumour**	**Reference**
C57 BL/6 male 20-80	Dosage: **500 µg** in 100 µL peanut oil (≈16.67 mg/kg bw), 1×, followed by transplantation of injection sites to secondary hosts 1–8 wk later Purity: not specified	26 wk	**positive** fibrosarcoma identifiable 4-5 weeks after injection tumour induction: earliest onset: 10 wk 50 %, 14-15 wk; 100 %, 25 wk tumour induction in secondary host: earliest onset: 3 wk 50 %, 4 wk; 100 %, 5 wk no control	Homburger and Treger 1967
C57 BL/6J male 40, 50 for histology	Dosage: **25 µg** in 0.1 mL tricaprylin (0.83 mg/kg bw), 1×, followed by transplantation of injection sites to secondary hosts 7 wk later Purity: not specified	34 wk	**positive** 100 % fibrosarcoma at 32 wk with transplant, 90 % fibrosarcoma at 18 wk	Homburger and Baker 1969
C57BL/6J male 50, 40 for trans-plantation, 40 solvent control	Dosage: **25 µg** in 100 µL tricaprylin (0.83 mg/kg bw), 1×, followed by transplantation of injection sites to secondary hosts 6 wk later Purity: not specified (commercial grade)	34 wk	**positive** no transplant: 50 % fibrosarcoma at 19.5 wk; 92 % fibrosarcoma at 34 wk with transplant: 50 % fibrosarcoma at 14.5 wk 100 % fibrosarcoma at 31 wk vs 0 % in transplanted vehicle controls no statistics	Homburger and Treger 1970
C57BL/6 male	Dosage: **500 µg** (volume not specified) peanut oil (16.67 mg/kg), 1× Vehicle: not specified	> 22 wk	**positive** 100 % fibrosarcomas of consistent morphologic and functional uniformity; malignant cells identifiable after 4-5 wk	Homburger et al. 1962
C57BL/6 male/ female total 250-300	Dosage: **500 µg** in 100 µL peanut oil (m: 16.67 mg/kg bw, f: 20 mg/kg bw), 1× Vehicle: not specified (commercial grade)	up to 46 wk	**positive** fibrosarcomas, males, 8 wk, females, later latency period, male, 14.5 ± 0.8 wk; female, 18 ± 1.4 wk	Homburger et al. 1963

- **Dermal initiation–promotion studies in mice**

Chemical promoters enhance the effects of carcinogens when administered subsequently to the initiators. Their relative effect is linked to their ability to increase the progeny of initiated cell populations. The mouse dermal initiation/promotion models have been used routinely to identify chemicals with promoting potential and to study the process by which normal tissue becomes a tumour. These studies have used highly variable methodologies that differ by the manner of duration of treatment, mouse strain, number of mice and endpoint measured. In one model, a topical subcarcinogenic dose of a chemical is first applied to the back of the skin (initiation) followed by repeated topical applications of one or more chemicals (promotion) and the skin is monitored for tumour development. The mouse skin has been shown to be more responsive (i.e., develops higher rates of tumours using this protocol) than other commonly used laboratory rodent models. Since not all mouse strains are equally sensitive, the tumour-initiating activity of DB[a,i]P was tested in several mouse strains carried out at different durations and several laboratories and under different protocols.

Nine synthesized and/or highly purified hexacyclic aromatic hydrocarbons including DB[a,l]P, DB[a,h]P and DB[a,i]P were tested for tumour-initiating potency on mouse skin (Hoffmann and Wynder 1966; LaVoie et al. 1979). 2.5 % croton oil was used as promoter. Groups of 30 female Swiss albino Ha/ICR/Mil mice, 7–8 weeks of age, received 10 applications of 0.1 % DB[a,i]P (purified by chromatography and recrystallized; total dose 25 µg/animal) dissolved in p-dioxan over 20 days on the shaved back skin. The compounds tested were applied by skin painting. Eight days after initiation, promotion followed with thrice-weekly dermal applications of 2.5 % (2.3 mg) croton oil in acetone. A control group of 30 mice was treated with 2.5 % croton oil solution. The animals were monitored weekly by palpation for tumour development and were killed when tumours persisted for 4-5 weeks. Autopsies were performed and all tissues suggestive of neoplasia were examined microscopically. The experiment was terminated after 6 months. In the following table an overview of study results examining the tumour-initiating activity of DB[a,i]P (total dose 25 µg/animal) with 2.5 % croton oil as promoter on mouse skin is given.

Table 25: Tumour- initiating activity of DB[a,i]P in female Swiss albino Ha/ICR/Mil mice, 2.5 % croton oil as promoter

Months after treatment	**1**	**2**	**3**	**4**	**5**	**6**
Survivors	30	30	28	28	28	27
Papilloma		3 (10 %)	6 (21 %)	8 (28 %)	10 (36 %)	12 (44 %)

After 6 months skin papillomas have been developed at the injection site in about half of the treated mice. The mean latency period for papilloma development was 98 days. In the control group, mice treated with 2.5 % croton oil in acetone alone developed 2/30 (7 %) skin papillomas.

The tumour-initiating activities of DB[a,i]P on mouse skin were examined by Hecht et al. (1981, 1981a). Groups of 20 female Ha/ICR Swiss albino mice, 50–55 days of age (body weight unspecified), received 10 dermal applications of DB[a,i]P (95 % pure by HPLC) dissolved in 100 µL acetone on the shaved back once on alternate days with total doses of 100 µg in the first assay and 500 µg in the second assay. Controls received acetone only. Ten days after initiation promotion followed with thrice-weekly applications of 2.5 µg 12-ortho-tetradecanoylphorbol-19-acetate (TPA) in 100 µL acetone for 20 weeks. Mice were shaved as necessary. Tumours were (macroscopically)

counted weekly and at autopsy, skin tumours were processed by standard techniques and examined microscopically, however skin tumour types were not reported. In the following table the results of tumour-initiating activity of DB[a,i]P testing is given.

Table 26: Tumour-initiating activity of DB[a,i]Pin Ha/ICR Swiss albino mice, 2.5 µg TPA as promotor

Compound	Assay	skin tumour-bearing animals [%]	No. of skin tumours/animal
DB[a,i]P	1: total dose 100 µg	40	0.5
DB[a,i]P	2: total dose 500 µg	85	5.8
Acetone control	1	0	0
Acetone control	2	0	0

Significant tumour-initiating activity was observed in mice received initiating doses of 100 µg and 500 µg DB[a,i]P. Mice treated with 100 µg DB[a,i]P had a 40 % skin tumour incidence (average of 0.5 skin tumours/mouse), whereas the group treated with 500 µg DB[a,i]P had an 85 % skin tumour incidence (average of 5.8 skin tumours/mouse). No skin tumours were observed in the vehicle-control group.

The tumour-initiating activity of DB[a,i]P on mouse skin was tested by Chang et al. (1982). Groups of 30 female CD-1 mice, 7–8 weeks of age (body weight unspecified), were treated on the shaved dorsal surface with a single dermal application of 50, 200 or 600 nmol (15, 60 or 180 µg) DB[a,i]P ('essentially pure' on the basis of chromatography, mass spectral, and nuclear magnetic resonance analysis) in 200 µL 10 % DMSO. Controls were treated with the solvent alone. Seven days later, the mice received twice-weekly applications of 16 nmol (10 µg) of the tumour promoter TPA in 200 µL acetone for 16 weeks. Papillomas greater than 2 mm in diameter were included in the cumulative total when they persisted for 2 weeks and longer. DB[a,i]P had significant tumour-initiating activity at all doses tested. After a 16-week period of promotion, skin tumour incidence was 28, 67 and 79 % in the low-, mid- and high-dose groups, respectively. The corresponding numbers of skin papillomas/mouse (no histology) were 0.52, 5.33 and 5.25. No skin tumours were observed in the solvent control group.

In a separate experiment, promotion with TPA was continued for 24 weeks. In a group of 30 female CD-1 mice previously treated with 50 nmol (15 µg) DB[a,i]P a skin tumour incidence of 69 % was determined. The number of skin papillomas/mouse (no histology) was 2.07 ± 0.44 (mean ± SE). No skin tumours were observed in the solvent control group.

In both experiments DB[a,i]P has shown significant tumour-initiating activity on mouse skin at all doses tested.

Comparative studies of tumour-initiating activity on mouse skin were conducted with several dibenzo[a]pyrenes, one of them was DB[a,i]P (Cavalieri et al. 1989). The SENCAR mouse strain[1]

[1] The SENCAR mouse strain was selectively bred for eight generations for sensitivity to skin tumour induction by the two-stage tumourigenesis protocol using 7,12-dimethylbenz(a)anthracene (DMBA) as the initiator and 12-0-tetradecanoylphorbol-13-acetate

(derived from SENsitivity to CARcinogenesis) was used in these studies. This mouse strain has been used extensively for skin carcinogenesis experiments. SENCAR mice were initiated with DB[a,i]P and promoted with tetrade-canoyl-phorbol acetate. Groups of 24 female SENCAR mice, 8 weeks of age (body weight unspecified), received a single dermal application of 800 nmol (242 µg) DB[a,i]P (purity > 99 % by HPLC) in 100 µL dioxane:DMSO (75:25) on a shaved area of dorsal skin. The control group was treated with 100 µL dioxane:DMSO only. One week later, all mice were treated with TPA (4.26 nmol (2.6 µg)/100 µL acetone) twice a week for 25 weeks, and were killed after the 25th week of promotion. The number of skin tumours was charted weekly, complete necropsies were performed, and tissues were fixed in 10 % buffered formalin.

At the end of the experiment, 15/24 (63 %) mice treated with DB[a,i]P had developed 63 skin papillomas (2.6 papillomas/mouse). In the controls skin tumours were observed in 2/23 mice (9 %; 0.1 papillomas/mouse). The first skin papillomas appeared after 12 weeks in the DB[a,i]P-treated group compared to 20 weeks in the control group.

In the following table an overview of results from dermal initiation–promotion studies in mice is presented.

(TPA) as the promoter. The SENCAR mouse was derived from crossing Charles River CD-1 mice with skin tumour-sensitive mice (STS) (Slaga 1986; Lynch et al. 2007).

Table 27: Overview of results from dermal initiation–promotion studies in mice

Strain **Sex** **No./sex/ group**	**Dosage** **Purity** **Vehicle**	**Study Duration at death/ sacrifice**	**Results** **Incidence and type of tumour**	**Reference**
Swiss albino Ha/ICR/Mil female 30	Dosage: 0, **25 µg** of 0.1 % solution, 10x/20 days, followed by 2.5 % (2.3 mg) croton oil in acetone (volume not specified), 3x/wk Purity: not specified (recrystallized) Vehicle: dioxane	6 months	**positive** 12/27 (44 %; skin papillomas) vs 2/30 (7 %; skin papillomas) in croton oil control ($p < 0.01$)	Hoffmann and Wynder 1966 LaVoie et al. 1979
Swiss albino Ha/ICR female 20	Dosage: 0, 10, 50 µg in 100 µL (total dose, 100, 500 µg), 10×/20 days, followed 10 days later by 2.5 µg TPA in 100 µL acetone 3×/wk 20 wk Purity: 95 % Vehicle: acetone	not specified	**positive** skin tumours (not specified) **100 µg:** 40 % (0.5 tumour/mouse) **500 µg:** 85 % (5.8 tumour/mouse) vs 0 % solvent controls	Hecht et al. 1981, 1981a
CD-1 female 30	Dosage: 0, 15, 60, 180 µg in 200 µL 1×, followed 1 wk later by 10 µg TPA in 200 µL, 2×/wk, 16 wk (all dose groups) 24 wk (15 µg group) Purity: not specified 'essentially pure' Vehicle: 10 % DMSO in THF	not specified	**positive** **15 µg** (16 wk): 28 % (0.52 skin papillomas/mouse) vs 0 % in TPA controls; **60 µg** (16 wk)**:** 67 % (5.33 skin papillomas/mouse) **180 µg** (16 wk)**:** 79 % (5.25 skin papillomas/mouse) vs 0 % in solvent controls; **15 µg** (24 wk): 69 % (2.07 ± 0.44 (mean ± SE) skin papillomas/mouse) vs 0 % in solvent controls no histology results were reported.	Chang et al. 1982
SENCAR female 24, 23 controls	Dosage: 0, **242 µg** (800 nmol) in 100 µL, 1×, followed 1 wk later by 2.6 ìg TPA in 100 ìL acetone, 2×/wk, 25 wk Purity: > 99 % Vehicle: dioxane:DMSO (75:25)	26 wk	**positive** 15/24 (63 %; 2.6 skin papillomas/mouse) vs 2/23 (9 %) in solvent controls	Cavalieri et al. 1989

Carcinogenicity studies with other application routes

- **Intra-peritoneal administration in mice**

The carcinogenicity of DB[a,i]P was examined in groups of newborn male and female Swiss-Webster (BLU:Ha(ICR)) mice (Chang et al. 1982). Mice received three intra-peritoneal injections of 12.5, 25 and 50 nmol (3.8, 7.6 and 15.1 µg) DB[a,i]P ('essentially pure' on the basis of chromatography, mass spectral and nuclear magnetic resonance analysis) dissolved in 5, 10 and 20 µL DMSO, respectively, on days 1, 8 and 15 of life (total dose, 87.5 nmol (26.5 µg)). Control mice received injections of DMSO alone. The mice were weaned at 25 days of age, and killed at 49–54 weeks. At necropsy, the major organs of each animal were examined grossly, tumours were macroscopically counted, and tissue samples were fixed in 10 % buffered formalin. A representative number of observed pulmonary tumours and all hepatic tumours, and all other tissues with gross lesions were examined microscopically.

In the lungs of mice treated with DB[a,i]P multiple nodules were found that were primary adenomatous alveolar lung tumours that arose from type 2 pneumocyte cells (also called granular pneumocytes) of the alveolar epithelium. Some larger tumours have been diagnosed as adenocarcinomas with structural and cellular anaplasia, increased number of mitotic figures, and invasion in surrounding tissue (Shimkin and Stoner 1975).

Two hepatic tumours types were observed: (1) a simple nodular growth of the liver parenchymal cells, and (2) areas of papiliform and adenoid growth of tumour cells, sometimes accompanied by metastases to the lung (Walker et al. 1973; Wiliams at al. 1979).

The ability of DB[a,i]P to produce tumours in newborn mice at a total dose of 87.5 nmol (26.5 µg) is shown in the following table.

Table 28: Tumour induction of DB[a,i]P in newborn Swiss-Webster mice, intraperitoneal injections

Tissues with tumours Sex of mice	**Number of mice alive at 49 to 54 weeks of age**	**Percent of mice with tumours**	**Average number of tumours/mouse**
Lung			
Male	**39**	**95 %**	**3.64**
Male control	32	22 %	0.80
Female	**21**	**100 %**	**5.80**
Female control	39	28 %	0.44
Liver			
Male	**39**	**54 %**	**0.82**
Male control	32	0 %	0

DB[a,i]P was clearly tumourigenic in newborn mice. 97 % of the mice (males and females combined) receiving three intra-peritoneal injections of a total dose of 26.5 µg DB[a,i]P developed benign and malign tumours in the lungs with an average of 4.40 tumours/mouse. In contrast, only 27 % of the mice (males and females combined) of the control group developed pulmonary tumours with an average number of 0.61 tumours/mouse. DB[a,i]P administered by intraperitoneal injection (thrice) induced benign and malign liver tumours only in male mice (54 %; 0.82 tumours/mouse). No liver tumours were found in the male controls.

- **Administration to rat mammary gland**

The carcinogenicity of DB[a,i]P (purity > 99 % by HPLC; recrystallised from xylenes) in mammary gland was examined in female Sprague-Dawley rats (Cavalieri et al. 1989). A group of 19 female rats, 8-week-old (weight unspecified), received single injections of 4 μmol (1.2 mg) DB[a,i]P dissolved in 100 μL trioctanoin per mammary gland (eight glands; total dose, 32 μmol (9.6 mg). Following injection it was necessary to pinch the treated mammary gland for 20 sec to prevent the solution from leaking. One control group of 21 female rats was treated with 100 μL trioctanoin and the other (20 female rats) was untreated. The animals were monitored weekly for tumour development by palpation. Animals were killed when tumours were 2 cm or larger in diameter, and complete necropsy was performed. All the remaining animals were killed at 40 weeks. Mammary tumours, as well as other grossly abnormal tissues were fixed in 10 % buffered formalin, sectioned and stained with haematoxylin and eosin for microscopy. The mean survival time was 30 ± 5 weeks in the DB[a,i]P-treated group. In the untreated and vehicle control groups mean survival times were 37 ± 4 and 40 ± 0 weeks, respectively. In the DB[a,i]P-treated group, a mean tumour latency of 19 ± 2 weeks was recorded. Tumour latency was 25 ± 13 weeks in the untreated control group. At the end of the study, 18/19 rats in the DB[a,i]P-treated group had developed fibrosarcomas (2.4 tumours/tumour-bearing rat), 11/19 (58 %) rats had mammary adenocarcinomas (1.4 tumours/tumour-bearing rat) and 1/19 had mammary adenofibromas (two tumours). In contrast, 2/20 (10 %) rats in the untreated group had developed mammary epithelial tumours (one adenofibroma and one adenocarcinoma) but no fibrosarcomas. No tumours were observed in the vehicle control group treated with trioctanoin.

The results of the carcinogenicity study with DB[a,i]P by direct application to the mammary gland of female rats have shown a strong carcinogenic activity. DB[a,i]P has significantly induced fibrosarcomas and adenocarcinomas in the mammary gland of rats.

- **Intrauterine administration in mice**

In contrast to the great susceptibility of subcutaneous tissue and epidermis to DB[a,i]P carcinogenicity, no tumour development from the endometrial lining appears. A group of 30 female mice (strain, age and weight unspecified) received a single intrauterine injection of 0.5 mg DB[a,i]P (vehicle, purity and volume unspecified). After 32 weeks, none of the 30 treated animals developed tumours (Homburger and Tregier 1960).

4.10.2 Human information

No epidemiological studies on exposure to the individual DB[a,i]P were identified. Individual PAH are found in the environment not in isolation but as components of highly complex mixtures of chemicals. PAH are very widespread environmental contaminants, because they are formed during incomplete combustion of materials such as coal, oil, gas, wood, or garbage or during pyrolysis of other organic material, such as tobacco or charbroiled meat. Data on the carcinogenicity of PAH in humans are available only for mixtures containing PAH. Evidence that mixtures of PAH are carcinogenic to humans is primarily derived from occupational studies of workers following inhalation and dermal exposure, especially from coke oven workers and aluminium smelters. The data clearly suggest lung and bladder cancer. Skin cancer in man is well known occure following exposure to poorly refined lubricating and cutting oils. No data were located regarding cancer in humans following inhalation and dermal exposure of individual PAH compounds. It is difficult to ascertain the carcinogenicity of the single component PAH in these mixtures because of the presence

of other carcinogenic substances in the mixtures. In 2005, IARC re-evaluated PAH. Although certain occupations with high PAH exposure (e.g., coal gasification and coke production) were classified as carcinogenic in humans, the roles of individual PAH could not be defined **(**IARC 2010**)**.

No case reports or epidemiological studies on the significance of DB[a,i]P exposure to man are available. However, coal-tar and other materials which are known to be carcinogenic to man may contain DB[a,i]P.

The general population may be exposed to DB[a,i]P primarily through the smoking of tobacco, via inhalation of polluted air, dermal contact with vapours and also by ingestion of food and water contaminated with combustion products. However, inhalation and/or dermal contact are the primary routes of potential human exposure to DB[a,i]P.

PAH are contained in certain elastomer/rubber materials, and potentially also in plastic materials, lacquers/varnishes, or coatings that may be encountered in or part of consumer products, including toys. Numerous examples of such products include e.g. tool handles, bicycle handlebars, slippers, flip-flops, beach sandals, diver equipment, toy car tyres, or clay pigeons used in skeet shooting. PAH may also be contained in synthetic turf or in materials used for construction work, e.g. flooring material. During recent years, a number of laboratories have frequently identified high PAH contamination levels including DB[a,i]P in a significant fraction of analysed consumer articles, such as toys, tool handles, bicycle grips, shoes, sports equipment etc. (BfR 2009; UBA 2010; Wennemer 2009).

In analysis of consumer products for their PAH contents Hutzler et al. (2011) have identified four isomeric dibenzopyrenes (DB[a,l]P; DB[a,e]P; DB[a,i]P; DB[a,h]P). The sample with the highest dibenzopyrene content (hammer grip) was presented with 7.1 mg/kg for DB[a,i]P, 5.6 mg/kg for DB[a,h]P and 2.1 mg/kg for DB[a,l]P.

4.10.3 Other relevant information

DB[a,i]P is a PAH. PAH constitute a large class of compounds, and hundreds of individual substances may be released during incomplete combustion or pyrolysis of organic matter, an important source of human exposure. PAH are found in many consumer products. PAH are not intentionally synthesised for use in consumer articles, but they may enter such products when certain plasticisers (‘extender oils’) or carbon black (soot) are used as additives in the production of rubber or other elastomer materials. Further human exposure pathways are e.g. inhalation of tobacco smoke or ingestion of barbecued food. Studies of various environmentally relevant matrices, such as coal combustion effluents, motor vehicle exhaust, used motor lubricating oil, and tobacco smoke, have shown that the PAH in these mixtures are mainly responsible for their carcinogenic potential. Numerous animal studies have been published on the carcinogenic effects of PAH, as single compounds or as mixtures, by various routes of exposure. A number of individual PAH are carcinogenic to experimental animals indicating that they are potentially carcinogenic to humans. However, only a small number of PAH are classified according to CLP Regulation as Category 1B carcinogen. These are B[a]P, B[e]P, B[a]A, CHR, B[f]F, B[j]F, B[k]F and DB[a,h]A. B[a]P and CHR also are legally classified mutagens (B[a]P: Muta.1B; CHR: Muta. 2). In addition, B[a]P is a classified as Category 1B reproductive toxicant.

4.10.4 Summary and discussion of carcinogenicity

The carcinogenic potential of DB[a,i]P has been studied in numerous studies in three species under different protocols.

Although the majority of the studies with DB[a,i]P were conducted decades before standard test guidelines were adopted and no standard carcinogenicity study is available, it is thought that the consistency of carcinogenic action from a broad series of studies are sufficient to proof the evidence of its carcinogenicy. The carcinogenic evidence from these studies is considered sufficiently robust to propose classification of DB[a,i]P as a carcinogen.

There is sufficient evidence of carcinogenicity of DB[a,i]P in experimental animals. DB[a,i]P was carcinogenic by all routes tested in mice, hamsters and rats. The available experiments have demonstrated that DB[a,i]P causes tumours at several sites, by several routes of administration, in both sexes, and in several animal species and strains. Oral studies with pure DB[a,i]P are not available.

Significant tumour rates induced by DB[a,i]P have been found in the skin (by repeated topical administration, and a single subcutaneous injection), in the upper respiratory tract and in the lung (by intra-tracheal instillation), in the lung and liver (by intra-peritoneal administration), and mammary gland (by single intra-mammary injection).

DB[a,i]P was tested for carcinogenicity in the respiratory tract in two studies with intra-tracheal instillation into hamsters, three studies with dermal application in mice, and four studies with single subcutaneous injection in mice and two studies in hamsters. In two further studies on mice the effects of surgical trauma or of dietary antioxidants on the development and progression of experimental tumours was tested which were pre-administered with a single subcutaneous injection. The influence of transfer of injection-site tissues following subcutaneous injection to mice (three studies) was also analysed. In a further study on mice responses of DB[a,i]P-induced subcutaneous sarcomas and their transplants to various chemotherapeutic agents were examined and in another study the influence of intrinsic factors such as sex, age, nutritional status, and connective-tissue reactivity were investigated. In addition, DB[a,i]P was tested for carcinogenicity in four initiation–promotion studies on mouse skin, one study of intra-peritoneal administration into newborn mice, one study of intra-mammary administration to rats, and one study of intrauterine administration in mice.

The longtime studies have shown induction of skin papillomas and epitheliomas (considered as squamous-cell carcinomas) after repeated dermal application of rather low concentrations in mice and hamsters and sarcomas after single subcutaneous injection; in addition it was noted that transfer of injection-site tissues to secondary hosts shortened the latent period for producing tumours.

Tumour development in the upper respiratory tract and the lungs was induced in male and female hamster after weekly instillation of low intra-tracheal doses of DB[a,i]P.

Intra-peritoneal injection of newborn mice with DB[a,i]P on three days of life caused benign and malignant lung tumours in both sexes and benign and malignant liver tumours in males. DB[a,i]P administered by single intra-mammary injection caused cancer of the mammary gland (fibrosarcoma and adenocarcinoma) in female rats. In contrast none of the female mice have developed tumours following single intrauterine injection of DB[a,i]P. In initiation-promotion studies the tumour initiating activity of DB[a,i]P was demonstrated in several mouse strains.

No data are available in humans exposed to pure DB[a,i]P. There are a number of epidemiologic and mortality studies that show increased incidences of cancer in humans exposed to mixtures of PAH (IARC 2006; US EPA 1984; WHO 1987, 1998, 2000, 2003; Armstrong et al. 1994, 2003, 2004, 2009;

Boffetta et al. 1997; Bosetti et al. 2007; Costantino et al. 1995; Mastrangelo et al. 1996; Moolgavkar et al. 1998). Most of the PAH have been shown to be initiators of skin and lung cancer (IARC 1983, 2010). This feature was also provided for DB[a,i]P in several studies in mice of different strains and in hamsters.

No species-specific mode of action for DB[a,i]P carcinogenesis was identified.

According to today's state of knowledge the potential mechanisms behind chemical carcinogenesis are highly complex involving genotoxic events (mutations), altered gene expression at the transcriptional translational, and posttranslational levels (epigenetic events), and altered cell survival (proliferation and apoptosis) (Hanahan and Weinberg 2000).

Tumour initiation encompasses several distinct requirements, which for chemical carcinogens include the compound (reactive per se or reactive following metabolism) reacting with and thus causing changes in DNA. In many cases these changes consist of adducts (Boström et al. 2002).

In the literature it is discussed that PAH may exert both mutagenic (genotoxic) and epigenetic (nongenotoxic) actions.

PAH are a class of chemical carcinogens which undergo metabolic transformations to yield active products capable of forming covalent adducts at nucleophilic sites in tissue constituents such as DNA. PAH are metabolized to reactive intermediates which bind covalently to critical cellular constituents such as DNA, RNA, and protein, causing mutations and initiating other cellular changes that result in the development of cancer (Miller et al. 1980; Miller 1970).

It has been proposed that PAH share a similar mechanism of carcinogenic action. In general, PAH are converted to oxides and dihydrodiols, which in turn are oxidized to diol epoxides. Both oxides and diol epoxides are ultimate DNA-reactive metabolites. PAH oxides can form stable DNA adducts, and diol epoxides can form stable and depurinating adducts with DNA through formation of electrophilic carbonium ions.

There is some evidence that DB[a,i]P can be activated metabolically to the diol epoxide. DB[a,i]P was metabolized to the proximate bay-region diol, dibenzo[a,i]pyrene-3,4-diol, by rat liver preparations. Dibenzo[a,i]pyrene-3,4-diol was mutagenic to bacteria and was a tumour initiator in mouse skin. It induced pulmonary and hepatic tumours in newborn mice. Although DNA adducts from antidibenzo[a,i]pyrene-3,4-diol-1,2-oxide have not been identified, synthetic anti-dibenzo-[a,i]pyrene-3,4-diol-1,2-oxide was genotoxic in bacteria and mammalian cells in culture, was a tumour initiator in mouse skin and induced pulmonary and hepatic tumours in newborn mice (IARC 2010).

It is concluded that DB[a,i]P has carcinogenic properties that justify a harmonised classification and labelling as Carc. 1B, H350 according to CLP Regulation.

4.10.5 Comparison with criteria

According to the CLP directive a substance shall be classified as carcinogenic if:

Category 1A carcinogen

'It is known to have carcinogenic potential for humans; classification is largely based on human evidence.'

DB[a,i]P is possibly carcinogenic to humans. The available human data do not allow an allocation of PAH-related carcinogenicity to a single PAH. Therefore classification as Category 1A carcinogen is not appropriate.

Category 1B carcinogen

'It is presumed to have carcinogenic potential for humans; classification is largely based on animal evidence.'

This category depends on the strength of evidence, which consists of animal experiments for which there is sufficient evidence to demonstrate animal carcinogenicity. This means a causal relationship has been established between the agent and an increased incidence of malignant neoplasms or of an appropriate combination of benign and malignant neoplasms in

(a) two or more species of animals or in two or more independent studies in one species carried out at different times or in different laboratories or under different protocols;

(b) in both sexes of a single species;

(c) occurrence of malignant neoplasm to an unusual degree with regard to the incidence, site, type of tumour or age at onset, or when there are strong findings of tumours at multiple sites.

In comparison to the given criteria for the CLP Regulation DB[a,i]P fulfils the criteria for Category 1B carcinogen with regard to:

There is sufficient evidence of carcinogenicity from studies in experimental animals. In a review of the data related to DB[a,i]P-induced cancer sufficient evidence is available that DB[a,i]P is carcinogenic when administered in mice, hamster and rats.

DB[a,i]P caused tumours in two rodent species, at two different tissue sites, and by several different routes of administration. Dermal exposure to DB[a,i]P caused benign or malignant skin tumours (papilloma or epithelioma) in mice, and subcutaneous injection caused cancer at the injection site (sarcoma) in mice and hamsters.

Intra-peritoneal injection of newborn mice with DB[a,i]P caused benign and malign lung tumours in both sexes and benign and malign liver tumours in males. Intra-tracheal instillation of DB[a,i]P has caused respiratory-system cancer (mostly squamous-cell carcinoma, but also adenocarcinoma and anaplastic carcinoma) in hamsters of both sexes. DB[a,i]P administered by intra-mammary injection has caused cancer of the mammary gland (fibrosarcoma and adenocarcinoma) in female rats.

Tumours induced by DB[a,i]P have been found in the skin (by topical administration), respiratory tract and lung (by intra-tracheal instillation), lung and liver (by intra-peritoneal administration), and mammary gland (by intra-mammary injection).

Single subcutaneous injections of DB[a,i]P resulted in the rapid appearance of local sarcomas in the hamster and the mouse. In these studies the time of latency for tumour formation following carcinogen injection was found to be in general 10 weeks; 50 % tumour incidence is reached in 14 to 15 weeks; and 100 % of the injected animals have developed tumours after 25 weeks.

A dose-response relationship for tumour induction on the skin was observed in studies with single subcutaneous injection of DB[a,i]P in mice and hamsters.

Category 2 carcinogen

'It is a suspected human carcinogen, but the evidence is not sufficient for Category 1A or 1B.'

Following consideration would lead to classification as category 2:

(a) the evidence is limited to a single experiment;

(b) there are unresolved questions regarding the adequacy of the design, conduct or interpretation of the studies;

(c) the agent increases the incidence only of benign neoplasm or lesions of uncertain neoplastic potential; or

(d) the evidence of carcinogenicity is restricted to studies that demonstrate only promoting activity in a narrow range of tissues or organs.

Category 2 is not appropriate, because the criteria are not fulfilled. The evidence is neither limited to a single experiment, nor limited with regard to benign neoplasms, and nor limited to only promoting activity.

Conclusion

DB[a,i]P meets the criteria for classification and labelling as Category 1B carcinogen, H350 (CLP Regulation). The classification in category 1B is based on animal experiments for which there are sufficient evidence to demonstrate animal carcinogenicity. A causal relationship has been established between DB[a,i]P and an increased incidence of a combination of benign and malignant tumours in three species of animals (mouse, hamster, rat), in several studies from different laboratories and under different protocols. Further an increased incidence of tumours in both sexes of mice and hamsters and in female rats was observed in well-conducted studies. Tumour development was noted by the dermal route of administration of small doses in mice and hamster. Significantly increased tumour incidences were also seen in studies using single subcutaneous, intra-peritoneal, intra-mammary injections and intra-tracheal instillations of very small doses of DB[a,i]P.

This classification is in accordance with the classification system of IARC (2010), who ranked DB[a,i]P in group 2B (possibly carcinogenic to humans).

4.10.6 Conclusions on classification and labelling

Due to clear evidence of carcinogenic activity in animals the following classification and labelling is proposed:

According to CLP Regulation, DB[a,i]P should be classified and labelled as Category 1B carcinogen, H350 (May cause cancer).

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

The carcinogenic potential of benzo[*rst*]pentaphene has been studied in numerous studies in three species under different protocols. Although the majority of these studies were conducted decades before standard test guidelines were adopted and no standard carcinogenicity study is available, it is thought that the consistency of carcinogenic action from a broad series of studies are sufficient to prove the evidence of its carcinogenicity.

In laboratory animals, significant tumour rates induced by benzo[*rst*]pentaphene have been found in the skin (by repeated topical administration, and a single subcutaneous injection), in the upper respiratory tract and in the lung (by intra-tracheal instillation), in the lung and liver (by intra-peritoneal administration), and mammary gland (by single intra-mammary injection).

Benzo[*rst*]pentaphene was tested for carcinogenicity in the respiratory tract in two studies with intra-tracheal instillation into hamsters. Tumour development in the upper respiratory tract and the lungs was induced in males and females after weekly instillation of low intra-tracheal doses were administered.

The long-term studies have shown induction of skin papilloma and epithelioma (considered as squamous-cell carcinoma) after repeated dermal application in mice and hamsters and increased sarcoma after single subcutaneous injection; in addition it was noted that transfer of injection-site tissues to secondary hosts shortened the latent period for producing tumours.

Three intra-peritoneal injections of benzo[*rst*]pentaphene to newborn mice caused benign and malignant lung tumours in both sexes and benign and malignant liver tumours in males. Administration by single intra-mammary injection caused cancer of the mammary gland (fibrosarcoma and adenocarcinoma) in female rats. In contrast, none of the female mice receiving benzo[*rst*]pentaphene by single intrauterine injection developed treatment-related tumours.

In addition, benzo[*rst*]pentaphene was tested for tumour initiating potential in four initiation–promotion studies on mouse skin. All four studies reported positive responses by the increased frequency of skin papilloma when compared to negative controls.

No data are available in humans exposed to benzo[*rst*]pentaphene itself. There are a number of epidemiological studies that show increased incidences of cancer in humans exposed to mixtures of PAH. Most of the PAHs have been shown to be initiators of skin and lung cancers. This feature was also provided for benzo[*rst*]pentaphene in several studies in mice and in hamsters. No species-specific mode of action for benzo[*rst*]pentaphene carcinogenesis has been identified.

There is some direct evidence that benzo[*rst*]pentaphene can be activated metabolically to the diol epoxide. Benzo[*rst*]pentaphene was metabolised to the proximate bay-region diol, dibenzo[a,i]pyrene-3,4-diol, by rat liver preparations. Dibenzo[a,i]pyrene-3,4-diol was mutagenic to bacteria and was a tumour initiator in mouse skin. It induced pulmonary and hepatic tumours in newborn mice. Although DNA adducts from antidibenzo[a,i]pyrene-3,4-

diol-1,2-oxide have not been identified, synthetic anti-dibenzo-[a,i]pyrene-3,4-diol-1,2-oxide was genotoxic in bacteria and mammalian cells in culture, was a tumour initiator in mouse skin and induced pulmonary and hepatic tumours in newborn mice.

The DS concluded that benzo[*rst*]pentaphene has carcinogenic properties that justify a harmonised classification and labelling as Carc. 1B, H350.

Comments received during public consultation

Three MSCA communicated their support for the proposal. They observed how benzo[*rst*]pentaphene had been found to induce cancer at various sites in different animal species and strains, and by different routes of exposure.

Assessment and comparison with the classification criteria

There are no standard, regulatory studies to inform on the carcinogenicity of benzo[*rst*]pentaphene, but there were studies in mice and/or hamsters by the intra-tracheal, dermal and subcutaneous routes. Other less conventional studies include intra-peritoneal administration, injection into rat mammary gland and mouse uterus. In addition, tumours induced by benzo[*rst*]pentaphene have been transplanted into secondary hosts by injection.

Dermal studies

Of all the available dermal studies, only one included concurrent control animals. In this study, which was conducted in the 1950s, female Swiss mice were administered solvent alone (p-dioxan), 0.05 or 0.1% benzo[*rst*]pentaphene three times a week for 12 months and then allowed a recovery period of 3 months. Skin papillomas and epitheliomas were reported at 0.05 and 0.1% benzo[*rst*]pentaphene (see below) but were not observed in solvent control animals.

Month	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	Total
	0.05% (approx. 0.86 mg/kg bw)															
Survivors	20	20	20	20	20	19	19	19	16	14	11	9	6	3	2	2
Papilloma								3	6	7	10	15	16	16	16	28
Epithelioma									3	4	6	6	9	12	13	13
	0.1% (approx. 1.71 mg/kg bw)															
Survivors	20	20	20	20	19	19	19	17	14	13	8	5	4	3	2	2
Papilloma					1	1	3	6	7	10	15	16	16	16	16	29
Epithelioma								2	4	5	8	11	12	13	15	15

At 0.1%, skin papillomas were found in 16/20 mice and skin epithelioma in 15/20 mice. In the lower dose group, skin papillomas and epitheliomas were found in 16 and 13 of the 20 test mice, respectively. The mean tumour latency period was 336 days at 0.05% and 287 days at 0.1% benzo[*rst*]pentaphene. These results provide a strong indication of the carcinogenic potential of benzo[*rst*]pentaphene.

The remaining two dermal studies, one conducted in male mice and the other in females, also reported skin papillomas, epitheliomas or carcinomas in treated animals. However, they are of limited value because they lacked concurrent controls or relevant historical control information.

Although strictly not carcinogenicity studies, 4 mouse dermal initiation-promotion studies were included in the CLH report. They appear to have been well conducted, with the inclusion of appropriate controls, and all 4 gave clear positive results for tumour formation regardless of single or multiple administrations of benzo[*rst*]pentaphene as the initiator compound.

Intra-tracheal studies

Two studies in the hamster reported respiratory tract tumours, predominantly squamous cell carcinoma, when benzo[*rst*]pentaphene was administered intra-tracheally.

In one study, 2 groups of male Syrian golden hamsters were administered approx. 2.92 mg/kg bw benzo[*rst*]pentaphene weekly over 4 weeks (total dose 8 mg) or approx. 0.73 mg/kg bw over 24 weeks (total dose 12 mg). The test substance was ground to a finely aggregated dust with haematite before administration. Control hamsters were left untreated and did not receive the vehicle (0.9% saline). After 100 weeks, all treated animals and 71/90 controls had died. Respiratory insufficiency, due to extensive tumour involvement in the respiratory tract, accounted for the increased mortality in the treated hamsters. In total, at the end of the study period, the frequency of respiratory tract tumours was 0/82, 16/34 and 39/44 in control, 8 mg and 12 mg dose groups, respectively. The majority of tumours were found in the bronchi and trachea (see below).

Dose (mg)	Number of animals with respiratory tract tumours/total (%)	Administration protocol	Number with tumour/total (%)			
			Larynx	Trachea	Bronchi	Lung
0	0/82	-	-	-	-	-
8	16/34 (47%)	2 mg fine dust with haematite resuspended in 0.9% saline and given once weekly over 4 weeks	1/34 (3%)	2/34 (6%)	13/34 (38%)	1/34 (3%)
12	39/44 (89%)	500 µg fine dust with haematite resuspended in 0.9% saline and given once weekly for 24 weeks	-	6/44 (14%)	37/44 (84%)	1/44 (2%)

Additionally, two malignant lymphomas occurred in the hamsters that received 12 mg benzo[*rst*]pentaphene. No additional tumours were seen in the 8 mg dose group. In controls, the frequency of tumours at other sites was 11/82.

Interpretation of this study is compromised by the non-physiological dose-route, which may not have been a good model for human exposure. The nature of the formulation used for dosing further complicates assessment, given that comparable solid control material was not employed. However, the results are consistent with those from the dermal studies, showing increased tumour frequency at the site of contact with the body.

A similar study was conducted in male and female hamsters (24/sex/group) at total doses of 8.5 mg (0.57 mg/kg bw/d males, 0.65 mg/kg bw/d females) and 12 mg (1.14 mg/kg bw/d males, 1.3 mg/kg bw/d females). The reported incidences of respiratory tumours was 31/48 (65%) and 36/48 (75%) at 8.5 mg and 12 mg, respectively. The majority of tumours

were observed in the bronchi and trachea (see below) but tumours were present in the larynx, lung and pleura at lower incidences (specific values not given in the report). This study was limited, however, by the lack of concurrent controls or historical control data to reinforce the apparent positive outcome with benzo[*rst*]pentaphene.

Total Dose (mg)	Administration protocol	Number of animals with tumours/total (%)	% tracheal tumours*	% bronchial tumours*
8.5	500 µg in distilled water once per week over 17 weeks	31/48 (65%)	13	77
12	1 mg in distilled water once per week over 12 weeks	36/48 (75%)	19	62

* it is unclear from the CLH report by the DS whether the % is of total animals or those with tumours

Other routes of exposure

Seven mouse subcutaneous studies with benzo[*rst*]pentaphene were reported in the dossier.

In the best of these studies, groups of 12 male C57Br/cd mice were administered a single dose of benzo[*rst*]pentaphene (0.01-600 µg in peanut oil) and observed for up to 66 weeks. The incidence of fibrosarcoma at the site of injection increased with dose up to 50 µg (see below) and all animals developed tumours by week 17 in the 50, 100 and 600 µg dose groups. Palpable masses were evident by week 9 and fully developed fibrosarcomas were observed 12 weeks after injection.

Dose of Benzo[*rst*]pentaphene (µg)	Incidence of fibrosarcoma (%)
< 1	0
1	9
2	33
6.25	50
12.5	64
25	92
50	100

This study clearly demonstrates the carcinogenicity of benzo[*rst*]pentaphene administered by subcutaneous injection.

The remaining 6 subcutaneous studies in mice were all positive for local tumour formation at the site of injection. The findings of these studies support those of the study described above but they were limited by their lack of controls or short study duration. Equally, three subcutaneous studies in hamsters similarly reported fibrosarcomas at the injection site. These results support the outcomes observed in mice.

The non-conventional studies using transplantation techniques or intrauterine, mammary gland and intra-peritoneal administration were described as positive for tumour formation. In contrast, a limited study involving a single intrauterine administration of benzo[*rst*]pentaphene to mice did not evoke such a response. Given the limited reporting of these studies, the absence of suitable controls, test substance characterisation and/or detailed information about these tests for identifying carcinogenic substances, no firm conclusions can be derived from these studies.

Additional supporting information

Benzo[*rst*]pentaphene is metabolised to reactive diols via its bay regions. The proximate bay-region diol, dibenzo[a,i]pyrene-3,4-diol, was positive in an Ames test using strains TA98 and TA100 in the presence of an exogenous metabolic activation system. In a dermal initiation-promotion test, dibenzo[a,i]pyrene-3,4-diol was positive for tumour formation and produced liver and lung tumours in the mouse following intra-peritoneal injection.

Synthetic anti-dibenzo-[a,i]pyrene-3,4-diol-1,2-oxide was mutagenic in bacteria and mammalian cells without exogenous activation. In addition, tumours were formed in a dermal initiation-promotion assay and after intra-peritioneal injection in the liver and lung of the mouse.

Overall, the data provides evidence for genotoxicity and carcinogenicity via activation of bay regions; a mechanism shared with B[a]P and CHR.

Comparison with the criteria

No standard carcinogenicity studies were available with benzo[*rst*]pentaphene but there was evidence of tumour formation in a variety of tissues in multiple species with different routes of exposure. Also, the carcinogenic potential of benzo[*rst*]pentaphene is consistent with the positive genotoxicity studies and proposed classification as a mutagen.

As there is no evidence of carcinogenicity in humans with benzo[*rst*]pentaphene, Category 1A is not appropriate. There is evidence, however, of carcinogenicity in rodent species and thus Category 1B or 2 could be applied. When considering the final classification, there are many factors which can influence the outcome and in accordance with Annex I 3.6.2.2.6, RAC has compared the factors with the available data on benzo[*rst*]pentaphene from the dermal, intra-tracheal and subcutaneous routes of exposure.

Factor	Evidence with benzo[*rst*]pentaphene	Conclusion
Tumour type and background control	Local tumours were formed at or near the site of administration and incidence exceeded concurrent controls (when included in the study)	Tumour types are relevant to humans - Cat. 1B
Multi-site responses	Local tumours were produced at the different sites of exposure	Tumours formed at the expected sites of exposure in humans - Cat. 1B
Progression of lesions to malignancy	Malignant tumours (fibrosarcoma, malignant lymphoma, squamous cell carcinoma) were reported in mice and/or hamsters	Evidence of malignancy is sufficient for Cat. 1B
Reduced tumour latency	Latency periods were short compared to total study durations.	This factor is indicative of potency but does not allow for differentiation between classification categories.
Whether responses are in single sex or both	Both sexes of hamster and mouse reported tumours	Carcinogenic to both sexes - Cat. 1B
Whether responses are in a single species or several	Tumour formation occurred in hamsters and mice	No evidence of a species specific response so it is likely relevant to humans- Cat. 1B

Structural similarity to a substance(s) for which there is good evidence of carcinogenicity	Structural and mechanistic similarity to B[a]P and CHR which are classified as Category 1B carcinogens	Cat. 1B
Routes of exposure	Physiological (dermal) and non-physiological routes of exposure (sub-cutaneous, intra-tracheal) produced tumours	Dermal study results confirm activity after exposure by a physiological route - Cat. 1B
Comparison of ADME between test animals and humans	Not available	N/A
The possibility of a confounding effect of excessive toxicity at test doses	Tumours were not cited as a consequence of toxicity or other confounding factors by the DS	Tumours were a consequence of test substance exposure - Cat. 1B
Mode of action and its relevance for humans	Proven to be metabolised to reactive species with mutagenic activity	Mechanism is relevant to humans - Cat. 1B

Based on the available data and comparison to the criteria in Regulation (EC) 1272/2008 (see above), RAC is of the opinion that classification as **Carcinogen Category 1B (H350)** is appropriate for benzo[*rst*]pentaphene.

4.11 Toxicity for reproduction

No data are available for this toxicological endpoint.

4.12 Other effects

4.12.1 Non-human information

4.12.1.1 Neurotoxicity

Not evaluated for this report.

4.12.1.2 Immunotoxicity

Not evaluated for this report.

4.12.1.3 Specific investigations: Mechanistic studies regarding carcinogenicity

- **Metabolism and metabolic activation**

DB[a,i]P was metabolized to a 'bay-region' diol, trans-3,4-dihydroxy-3,4-dihydroDB[a,i]P (dibenzo[a,i]pyrene-3,4-diol), and trans-1,2-dihydroxy-1,2-dihydroDB[a,i]P by an Aroclor 1254-induced rat liver preparation (Hecht et al. 1981).

- **Evidence for diol epoxide metabolic activation as a mechanism of carcinogenesis**

Genotoxicity of dibenzo[a,i]pyrene-3,4-diol

Dibenzo[a,i]pyrene-3,4-diol was mutagenic in S. typhimurium TA98 and TA100 in the presence of an Aroclor 1254-induced rat liver preparation (Wood et al. 1981).

Carcinogenicity studies of dibenzo[a,i]pyrene-3,4-diol

Groups of 30 female CD-1 mice, 51–58 days of age, received a single dermal application of 50, 200 or 600 nmol (17, 67 or 202 μg) racemic DB[a,i]P-3,4-diol in DMSO:tetrahydrofuran (1:10), followed 7 days later by 16 nmol (10 μg) TPA in acetone twice a week for 16 weeks, or 24 weeks for another group treated with 50 nmol only. Control mice were treated with solvent. The incidence of tumours (skin tumours/mouse) in the dosed groups 17 weeks after treatment was 37 % (0.6), 66 % (3.03) and 81 % (5.0), respectively. No skin tumours were observed in the solvent group. The incidence of tumours (skin tumours/mouse) 25 weeks after treatment in the group dosed with 50 nmol DB[a,i]-3,4-diol was 60 % (2.14), and no skin tumours developed in mice treated with the solvent (Chang et al. 1982).

Groups of 30 female and 21 male newborn Swiss-Webster BLU:Ha(ICR) mice received intra-peritoneal injections of racemic dibenzo[a,i]pyrene-3,4-diol (total dose, 87.5 nmol, 31 μg) in DMSO on days 1, 8 and 15 of life. Control mice (32 males, 39 females) were given injections of DMSO alone. Mice were weaned at 25 days of age, separated by sex and killed at 49–54 weeks of age. All male and all female mice developed pulmonary tumours (100 %; males: 35.0 tumours/mouse, females: 32.2 tumours/mouse). Liver tumours were observed in 6 % (0.1 tumours/mouse) females and in 67 % (4.48 tumours/mouse) males. In the solvent control group no liver tumours were observed in males and females. In contrast pulmonary tumours were observed in both sexes. From the 39 female mice 28 % (0.44 tumours/mouse) developed pulmonary tumours and from the 32 male mice 22 % (0.8 tumours/mouse) (Chang et al. 1982).

Genotoxicity of dibenzo[a,i]pyrene-3,4-diol-1,2-oxide

anti-Dibenzo[a,i]pyrene-3,4-diol-1,2-oxide was mutagenic in S. typhimurium TA98 and TA100 in the absence of exogenous metabolic activation and in Chinese hamster V79 cells in culture (8-azaguanine resistance) (Wood et al. 1981).

Carcinogenicity studies of dibenzo[a,i]pyrene-3,4-diol-1,2-oxide

Groups of 30 female CD-1 mice, 51–58 days of age, received a single dermal application of 50, 200 or 600 nmol (18, 71 or 212 μg) racemic anti-trans-3,4-dihydroxy-1,2,3,4-tetrahydrodibenzo[a,i]pyrene-1,2-oxide (anti-dibenzo[a,i]pyrene-3,4-diol-1,2-oxide) in DMSO:tetrahydrofuran (1:10), followed 7 days later by 16 nmol (10 μg) TPA in acetone twice a week for 16 weeks, or 24 weeks for another group treated with 50 nmol only. Control mice were treated with solvent. The incidence of tumours (skin tumours/mouse) in the dosed groups 17 weeks after treatment was 20 % (0.2), 43 % (0.9) and 67 % (2.03), respectively. The incidence of tumours (skin tumours/mouse) 25 weeks after treatment in the group dosed with 50 nmol dibenzo[a,i]pyrene-3,4-diol-1,2-oxide was 53 % (0.9) and no skin tumours were observed in mice treated with the solvent (Chang et al. 1982).

A group of 39 newborn Swiss-Webster BLU:Ha(ICR) mice received intra-peritoneal injections of racemic anti-dibenzo[a,i]pyrene-3,4-diol-1,2-oxide (total dose, 87.5 nmol, 31 μg) in DMSO on days 1, 8 and 15 of life. Control mice (32 males, 39 females) were given injections of DMSO alone. Mice were weaned at 25 days of age, separated by sex and killed at 49–54 weeks of age. The survived seven female mice developed pulmonary tumours (100 %; 1.57 tumours/mouse) but no liver tumours. From the eight survived male mice developed 62 % pulmonary tumours (3.13 tumours/mouse) and 25 % (0.25 tumours/mouse) liver tumours. In the solvent control group no liver tumours were observed in males and females. In contrast pulmonary tumours were observed in both sexes. From

the 39 female mice 28 % (0.44 tumours/mouse) developed pulmonary tumours and from the 32 male mice 22 % (0.8 tumours/mouse) (Chang et al., 1982).

Conclusion

DB[a,i]P was metabolized to dibenzo[a,i]pyrene-3,4-diol, by rat liver preparations. DB[a,i]P-3,4-diol was mutagenic to bacteria and was a tumour initiator in mouse skin. It induced pulmonary and hepatic tumours in newborn mice.

Synthetic anti-dibenzo-[a,i]pyrene-3,4-diol-1,2-oxide was genotoxic in bacteria and mammalian cells in culture, was a tumour initiator in mouse skin and induced pulmonary and hepatic tumours in newborn mice.

5 ENVIRONMENTAL HAZARD ASSESSMENT

Not evaluated for this report.

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