Committee for Risk Assessment

RAC

Opinion

proposing harmonised classification and labelling
at EU level of

dibenzo[def,p]chrysene

EC Number: 205-886-4
CAS Number: 191-30-0

CLH-O-0000001412-86-243/F

Adopted

30 November 2018

Annankatu 18, P.O. Box 400, FI-00121 Helsinki, Finland | Tel. +358 9 686180 | Fax +358 9 68618210 | echa.europa.eu

30 November 2018

CLH-O-0000001412-86-243/F

OPINION OF THE COMMITTEE FOR RISK ASSESSMENT ON A DOSSIER PROPOSING HARMONISED CLASSIFICATION AND LABELLING AT EU LEVEL

In accordance with Article 37 (4) of Regulation (EC) No 1272/2008, the Classification, Labelling and Packaging (CLP) Regulation, the Committee for Risk Assessment (RAC) has adopted an opinion on the proposal for harmonised classification and labelling (CLH) of:

Chemical name: **dibenzo[def,p]chrysene;dibenzo[a,l]pyrene**

EC Number: **205-886-4**

CAS Number: **191-30-0**

The proposal was submitted by **Germany** and received by RAC on **23 October 2017.**

In this opinion, all classification and labelling elements are given in accordance with the CLP Regulation.

PROCESS FOR ADOPTION OF THE OPINION

Germany has submitted a CLH dossier containing a proposal together with the justification and background information documented in a CLH report. The CLH report was made publicly available in accordance with the requirements of the CLP Regulation at ***http://echa.europa.eu/harmonised-classification-and-labelling-consultation/*** on **12 February 2018**. Concerned parties and Member State Competent Authorities (MSCA) were invited to submit comments and contributions by **13 April 2018**.

ADOPTION OF THE OPINION OF RAC

Rapporteur, appointed by RAC: **Andrew Smith**

Co-Rapporteur, appointed by RAC: **Ralf Stahlmann**

The opinion takes into account the comments provided by MSCAs and concerned parties in accordance with Article 37(4) of the CLP Regulation and the comments received are compiled in Annex 2.

The RAC opinion on the proposed harmonised classification and labelling was adopted on **30 November 2018** by **consensus**.

Classification and labelling in accordance with the CLP Regulation (Regulation (EC) 1272/2008)

	Index No	International Chemical Identification	EC No	CAS No	Classification		Labelling			Specific Conc. Limits, M-factors	Notes
					Hazard Class and Category Code(s)	Hazard statement Code(s)	Pictogram, Signal Word Code(s)	Hazard state-ment Code(s)	Suppl. Hazard statement Code(s)		
Current Annex VI entry	No current Annex VI entry										
Dossier submitters proposal	-	dibenzo[def,p]chrysene	205-886-4	191-30-0	Muta. 2 Carc. 1B	H341 H350	GHS08 Dgr	H341 H350	-	Carc. SCL = 0.001 %	**-**
RAC opinion	-	dibenzo[def,p]chrysene	205-886-4	191-30-0	Muta. 2 Carc. 1B	H341 H350	GHS08 Dgr	H341 H350	-	Carc SCL 0.001%	**-**
Resulting Annex VI entry if agreed by COM	-	dibenzo[def,p]chrysene	205-886-4	191-30-0	Muta. 2 Carc. 1B	H341 H350	GHS08 Dgr	H341 H350	-	Carc SCL 0.001%	-

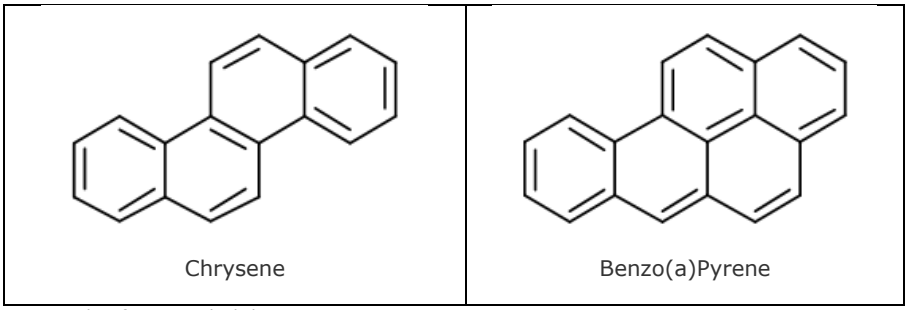
GROUNDS FOR ADOPTION OF THE OPINION

RAC general comment

Only two endpoints were presented in the Dossier Submitter's (DS) proposal for harmonised classification and labelling of dibenzo[def,p]chrysene: germ cell mutagenicity and carcinogenicity.

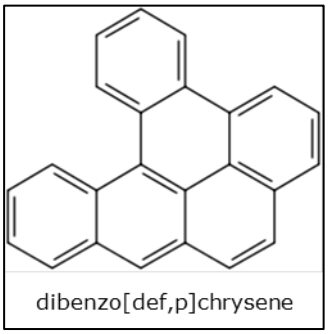
Although data from laboratory tests with this substance were presented for both endpoints, a key supporting aspect of the proposal is its structural and biochemical similarity to polycyclic aromatic hydrocarbons (PAHs) that are well known to possess these hazards.

There are more than 100 substances that can be identified 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 generally planar, highly conjugated aromatic compounds. In their report, the DS presented data on two PAHs that have been established as having carcinogenic and mutagenic potential: chrysene (4-membered benzene ring structure) and benzo(a)pyrene (B[a]P) (5-membered ring structure).

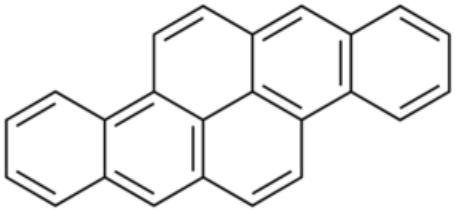


Note: images taken from www.lookchem.com

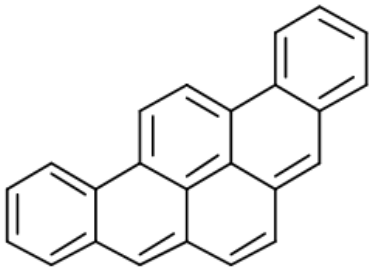
As can be seen from the following diagram, dibenzo[def,p]chrysene is a larger molecule, with 6 rings, but still has structurally similarity to both chrysene and B[a]P.



Although details are not included in the CLH report for this substance, the DS previously submitted classification proposals for 2 other PAHs: dibenzo[b,def]chrysene and benzo[rst]pentaphene. RAC agreed that these should both be classified for mutagenicity and carcinogenicity based on relevant toxicological studies and a comparison with chrysene and B[a]P. Both of dibenzo[b,def]chrysene and benzo[rst]pentaphene have a 6 ring structure.



Dibenzo[b,def]chrysene



Benzo[rst]pentaphene

The subject of this opinion, dibenzo[def,p]chrysene, differs from the others in that it has both the classical "bay region" sub-structure that is common to all these PAHs and a deeper "fjord region". These are reactive electrophilic regions that 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, chrysene and dibenzo[def,p]chrysene, the DS used this information in conjunction with available data to classify dibenzo[def,p]chrysene for germ cell mutagenicity and carcinogenicity.

HUMAN HEALTH HAZARD EVALUATION

RAC evaluation of germ cell mutagenicity

Summary of the Dossier Submitter's proposal

Dibenzo[def,p]chrysene induced mutagenic effects in bacteria and in exposed proliferating cells of mammalian and human cell lines. Positive effects were also observed using indicator tests in different cell cultures as well as on isolated calf thymus DNA. These effects were only observed in the presence of an exogenous metabolic system.

Clastogenic effects (induction of micronuclei) were induced by dibenzo[def,p]chrysene as well as genotoxic effects (DNA adducts, SCE) in somatic tissues in both conventional and research studies.

In vivo studies carried out in mice showed the induction of gene mutations in lung cells and also cells of the oral cavity (tongue cells and upper oral mucosa cells).

Amongst all the studies, only two were carried out in accordance with EU/OECD test guidelines; one was a bacterial gene mutation test in *S. typhimurium* strains TA 98, TA100 and TA104 and the other was a mammalian cell gene mutation test assayed at the TK locus. Both of these tests yielded positive results.

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. The DS considered that the lack of appropriate controls in all the other tests was a crucial methodological shortcoming. Consequently, even though a significant number of positive *in vitro* and *in vivo* studies had been conducted with dibenzo[def,p]chrysene, the results of these studies alone were insufficient to support classification.

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

B[a]P, chrysene and dibenzo[def,p]chrysene consist of four, five or six fused benzene rings (respectively). Depending of the number of benzene rings and the resulting steric effects the substances can be distinguished by the metabolic reactive centres they possess: "bay region" only (B[a]P and chrysene) or including a "fjord region" (dibenzo[def,p]chrysene). When the substances are metabolised, reactive electrophilic bay or fjord dihydro-diol epoxide enantiomers are generated that are able to bind covalently with guanine nucleotides (bay region epoxides) and adenine nucleotides (fjord region epoxides) in cellular DNA to form pre-mutagenic covalent adducts in mammalian cells and tissues that can, if not repaired, ultimately contribute to mutagenic effects.

The diol epoxide pathway is the most widely accepted pathway of PAH activation to yield DNA adducts. Such activation of PAHs occurs in the same way to produce a diol epoxide irrespective of the molecular structure and the different steric conditions at the reactive centres. The pathway is catalysed mainly by several enzymes such as cytochromes P450 and epoxide hydrolases. Metabolic activation leads to the formation of electrophilic diol epoxides, which are direct-acting mutagens.

Research studies have shown that different steric arrangements in the respective PAH ring system influence the binding affinity of a PAH to DNA. The bay region-containing PAHs (e.g., B[a]P and chrysene) are characterised by a planar aromatic ring system whereas the fjord region containing PAHs (e.g., dibenzo[def,p]chrysene) show non-planarity in the aromatic ring system. The resulting topology of DNA adducts apparently influences the distortions and stabilities of double-stranded DNA, and hence their processing by repair mechanisms. Accordingly, the adduct repair is conformation-dependent. The extent of the activity of nucleotide excision repair enzymes is influenced by the steric features at the reactive centres. DNA adducts derived from the PAHs that containing only bay regions are repaired more rapidly and effectively than adducts derived from PAHs with fjord regions. It has been shown that dibenzo[def,p]chrysene exerts a more potent genotoxic effect than B[a]P *in vivo* in an animal model system.

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

- The substances belong to the same chemical group as other PAH.
- The substances require metabolic activation for the induction of mutagenic/genotoxic effects.

- Reactive centres of the substances are the so-called fjord region (dibenzo[def,p]chrysene) or bay region (B[a]P and CHR).
- Reactive electrophilic bay or fjord dihydrodiol epoxide enantiomers are generated as metabolic products.
- The dihydrodiol epoxides bind covalently to specific targets in cellular DNA to form adducts with DNA nucleotides.
- Unrepaired DNA adducts can lead to mutagenic effects as a result of cell division.
- PAHs are known to induce mutagenic/genotoxic effects *in vitro* and *in vivo* after metabolic activation regardless of whether the reactive centre is a bay region or a fjord region.

In the view of the DS, available positive results of two guideline-compliant *in vitro* gene mutation tests of dibenzo[def,p]chrysene, combined with the read-across approach to the germ cell mutagens B[a]P (Cat. 1B) and chrysene (Cat. 2), are sufficient for classification of DB[a,l]P as Category 2 mutagen, H341 in accordance with CLP Regulation.

Comments received during public consultation

One Member State wrote in support of this proposal.

Assessment and comparison with the classification criteria

The potential mutagenicity of dibenzo[def,p]chrysene has been studied *in vitro* and *in vivo.*

Twenty-five *in vitro* studies were evaluated and included 3 Ames tests, 1 DNA repair test in bacteria, 4 *in vitro* mammalian gene mutation assays, a micronucleus test, a comet assay, an SCE test and 14 studies to determine DNA adduct formation (in both mammalian cell cultures and calf thymus DNA). Given the limited nature of the study reports in the open literature, RAC agrees that only 2 of the 25 *in vitro* studies are guideline-compliant and can be considered as reliable when judged against current regulatory standards. These tests are an Ames test and a gene mutation test (TK locus in h1A1v2 cells).

The genotoxic potential of dibenzo[def,p]chrysene was further assessed in 2 gene mutation assays in mice and 16 *in vivo* DNA adduct formation assays. None of these studies conformed to a current regulatory standard. Additionally, the CLH report included summaries of 7 tumour initiator-promoter assays in mice, in which dibenzo[def,p]chrysene was used as the initiator. Positive initiation of tumours in these tests is indicative of mutagenic activity.

Given the structural similarity to other PAHs, notably B[a]P and chrysene, RAC agrees that it is appropriate to use information on these two PAHs to further support the hazard assessment of dibenzo[def,p]chrysene.

In vitro studies

Three mutagenicity assays with standard *S.typhimurium* tester strains were summarised in the CLH report. Only one of these was performed according to OECD test guidelines but all gave positive results with dibenzo[def,p]chrysene. Like other mutagenic PAHs, positive results occurred only in the presence of exogenous metabolic activation. Following incubation with 0.1–5.0 µg/plate dibenzo[def,p]chrysene, positive results were obtained in strains TA98, TA100 and TA104. Positive and negative controls behaved accordingly. In support of this, a positive result was also reported in a bacterial DNA repair test (an SOS Chromotest).

In vitro mammalian cell tests performed with dibenzo[def,p]chrysene included four gene mutation studies. Two of the gene mutation studies were carried out at the TK locus of either MCL-5 cells or a human B-lymphoblastoid cell line (h1A1v2), and two measured mutations at HPRT locus in V79 cells. All of these gene mutation tests gave positive results.

Only the mammalian cell gene mutation test with h1A1v2 cells was performed according to the OECD test guideline. These cells are specifically engineered to express cytochrome P450 1A1 (CYP1A1), an isoenzyme especially relevant for the metabolic activation of PAHs. The cells were exposed to dibenzo[def,p]chrysene at concentrations of 0.1 - 10 ng/mL, and mutations at the TK-locus were quantified. Mutation frequency was increased from a concentration of 0.5 ng/mL in a dose-dependent manner up to the highest concentration of 10 ng/mL. Cytotoxicity was observed from a concentration of 1.0 ng/mL upwards and survival rate at the highest tested concentration was 19 %. Positive and negative controls behaved accordingly.

A non-guideline *in vitro* micronucleus test also gave a positive result. This was conducted in a Chinese hamster V79 lung cell line in which human cytochrome P450 1A1 or 1B1 had been transfected.

The consistent nature of the results provides compelling evidence of benzo[rst]pentaphene's mutagenic potential.

A comet assay and an SCE test performed with dibenzo[def,p]chrysene, both carried out in MRC-5 cells are available as well as 14 studies to determine DNA adducts; 7 of these in mammalian cell lines and 7 utilised isolated calf thymus DNA. The results of all these studies were positive, except for two DNA adduct studies in MFC-7 cells which were deemed not reliable.

In conclusion, dibenzo[def,p]chrysene has mutagenic potential in a variety of bacterial and mammalian *in vitro* test systems, in the presence of an appropriate oxidative metabolic activation system. It has similar activity to that of other mutagenic PAHs, including B[a]P and chrysene.

In vivo studies

Short summaries of 16 studies to determine the presence of DNA adducts in a variety of somatic cells in rats and mice were included in the CLH report. Animals were exposed to dibenzo[def,p]chrysene in several different ways, orally by gavage, by topical application or via intra-peritoneal (i.p.) or intra-mammary gland injection. Nine of the studies weren't deemed reliable but of the 5 that were, the results were all positive for adduct formation in the cell types tested.

Two gene mutation assays were conducted to investigate the genotoxicity of dibenzo[def,p]chrysene in B6C3F1 Big Blue® mice. Neither study was conducted in accordance with OECD test guidelines, notably because no positive control was included.

In the first study, male mice (6/group) were exposed by i.p. injection to either a single dose of 6 mg/kg bw or repeated doses of 1.2 mg/kg bw/day for 5 days dibenzo[def,p]chrysene. Lung cells were harvested 31 days after the final injection and, following the assay for mutation, a positive effect was seen following both treatment regimens. After the single dose, a 2.4-fold increase in mutation frequency was observed over that in control animals, and after repeated dosing a 2.8 fold increase in mutation frequency was seen. There was no overt toxicity or mortality produced by the test substance.

In a second study, male mice (6/group) received a topical application of 3, 6 or 12 nM dibenzo[def,p]chrysene in the oral cavity, three times/week for 38 weeks. The target cells in this study were the tongue and upper oral mucosa cells; they were harvested 38 weeks after the first administration. The results showed that gene mutations were induced in both types of cells. The effect was dose-dependent in both cell types and the increase in mutations relative to the vehicle control reached statistical significance at the highest dose tested (12 nM). At this dose the mutant fraction of cells was approximately doubled in both cell types. No changes in weight or physical behaviour of the animals were noted. There was no mortality.

Although not strictly performed to a regulatory standard protocol, these 2 studies together with the observations of DNA adducts in exposed animals, provide compelling evidence that the *in vitro* mutagenicity of dibenzo[def,p]chrysene can also occur in somatic cells *in vivo*.

The CLH report also summarised the results of 9 mouse skin tumour initiation-promotion studies in which dibenzo[def,p]chrysene was 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). Activity as an initiator in these assays is strongly indicative of mutagenic activity and the positive responses with dibenzo[def,p]chrysene support the outcomes of the *in vivo* and *in vitro* genotoxicity studies.

Overall, these *in vivo* data provide strong evidence of the mutagenicity of dibenzo[def,p]chrysene.

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

Dibenzo[def,p]chrysene shares structural and common mechanistic properties with the PAHs B[a]P and chrysene, both of which already carry a harmonised classification for germ cell mutagenicity. Notably, all 3 substances possess highly conjugated aromatic structures and common reactive centres called bay or fjord regions, depending on the steric arrangement of the PAH ring system. They all require metabolic activation at bay regions for the induction of mutagenic/ genotoxic effects. Electrophilic dihydrodiol epoxides are formed as common breakdown products of all these PAHs following oxidative metabolism at the bay and fjord regions.

<u>Classification of dibenzo[def,p]chrysene</u>

There are no data on human germ cell mutagenicity with dibenzo[def,p]chrysene, 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 9 positive initiation-promotion assays in mice, which gave results indicative of the mutagenic activity of dibenzo[def,p]chrysene. However, in the absence of data from *in vivo* studies investigating the potential effects of dibenzo[def,p]chrysene 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.

In accordance with the criteria in Annex I of the CLP Regulation, a category 2 classification is appropriate. This is based on an overwhelming weight of positive *in vitro* and *in vivo* data from mutagenicity and other relevant studies with dibenzo[def,p]chrysene itself and on its structural and mechanistic similarity to the established PAH mutagens chrysene, B[a]P, dibenzo[b,def]chrysene and benzo[rst]pentaphene.

Overall, in agreement with the DS proposal, the RAC opinion is for classification of dibenzo[def,p]chrysene in **Category 2 for germ cell mutagenicity (H341: suspected of causing genetic defects)**.

RAC evaluation of carcinogenicity

Summary of the Dossier Submitter's proposal

No data are available from humans exposed to dibenzo[def,p]chrysene alone. There are a number of epidemiological studies that show increased incidences of cancer in humans exposed to mixtures of PAH.

The carcinogenic potential of dibenzo[def,p]chrysene has been studied in numerous studies in three species under different protocols. Although no standard carcinogenicity study is available, the DS considered that the consistency of carcinogenic action from a broad series of studies are sufficient evidence of its carcinogenicity.

Repeated oral application of low doses of dibenzo[def,p]chrysene to two mouse strains induced benign and malignant tumours in various organs, e.g. ovaries, uterus, lymphoid tissues and skin; and oral squamous cell carcinoma after repeated applications of low doses on the tongue of hamsters.

Repeated dermal application of dibenzo[def,p]chrysene induced benign and malignant skin tumours (squamous cell papilloma and carcinoma) at high incidences in male and female mice. A dose-response relationship for tumour induction was observed in these studies. At low doses, tumours were observed to progress rapidly in size and killed the host within 46 weeks. In studies with higher doses, shorter latency periods were observed for tumour development in the skin of mice.

Following subcutaneous administration of dibenzo[def,p]chrysene (3 doses) to mice, sarcoma was observed at the site of administration.

Single i.p. injection dibenzo[def,p]chrysene to adult male mice induced lung adenomas in a dose-dependent manner. I.p. injections to newborn mice caused high incidences of benign and malignant lung tumours to develop in both sexes. Dibenzo[def,p]chrysene treatment also induced high incidences of benign and malignant liver tumours and tumours in other organs.

Single intra-mammary injection of dibenzo[def,p]chrysene to rats caused cancer of the mammary gland (fibrosarcoma and adenocarcinoma) in all treated animals and squamous cell carcinoma in the skin of 89 % of female rats.

In addition, dibenzo[def,p]chrysene was tested for tumour initiating potential in nine initiation–promotion studies on mouse skin. All nine studies reported positive responses by the increased frequency of skin papilloma when compared to negative controls.

The DS concluded that there was clear evidence of dibenzo[def,p]chrysene carcinogenicity in experimental animals. Together with the clear structural and mechanistic similarity of this substance to the established carcinogens chrysene and B[a]P, this supports a harmonised classification in Category 1B for carcinogenicity (H350).

As tumour development was noted following dermal administration of very small doses of dibenzo[def,p]chrysene, the T_{25} approach was used to calculate a specific concentration limit (SCL). The DS calculated a T_{25} value of 0.0005 mg/kg bw/day from the data provided in a carcinogenicity study that involved repeated topical application to the skin of female Swiss mice (Higginbotham *et al* (1993)). In accordance with the Guidance to Annex I of the CLP Regulation, this value corresponds to a high potency carcinogen (T_{25} ≤1 mg/kg/day).

Category 1B carcinogens showing high potency will normally be given a specific concentration limit an order of magnitude lower (0.01%) than the generic concentration limit (0.1%) for an ingredient to trigger classification of a mixture as a carcinogen. However, as the estimated T_{25} value for dibenzo[def,p]chrysene was considerably lower than 0.1 mg/kg, an SCL of 0.001% was proposed.

Comments received during public consultation

One Member State communicated their support for the proposal for classification as Carc. 1B and for the setting an SCL of 0.001%.

Assessment and comparison with the classification criteria

There are no standard, regulatory studies to inform on the carcinogenicity of dibenzo[def,p]chrysene, but there were two studies in mice and hamsters that involved administration by the oral route and five studies in the mouse that involved single or repeated dermal application. Other less conventional studies included i.p. or intra-mammary injection and sub-cutaneous administration to mice.

Oral studies

In a repeated dose carcinogenicity study, groups of female wild type mice (mixed genetic background of strains C57B1/6 and 129/Sv) received a dose of 0 or 1.07 mg/kg bw/day dibenzo[def,p]chrysene by gavage 5 times/week for 3 weeks. The study duration was 12 months, after which time dibenzo[def,p]chrysene was found to induce tumours in various organs (ovaries, lymphoid tissues and skin), as shown below.

The study also included an additional group of P450 1B1-null knock-out females.

Table: *Tumour findings in Wild Type and CYP1B1-knock-out mice following treatment with dibenzo[def,p]chrysene*

Finding	**Controls**	**Dibenzo[def,p]chrysene-treated groups**	
	Wild type	**Wild type**	**P450 1B1-null**
Survival rate	Not available	11/18 (61 %)	12/13 (92 %)
Total number of mice with tumours (benign + malignant)	4/27 (15 %)	17/17 (100 %)	8/13 (62%) (benign only)
Lung (adenoma)	1/27 (4 %)	0/17	5/13 (38 %)
Ovary (benign and malignant)	0/27	12/17 (72 %)	-
Skin (papilloma)	0/27	8/17 (47 %)	-
Lymphoma (malignant)	0/27	5/17 (29 %)	-
Lymphoma (follicular)	1/27 (4 %)	0/17	1/13 (8 %)
Uterus (Endometrial cystic hyperplasia)	1/27 (4 %)	0/17	5/13 (38 %)
Uterus (benign and malignant)	0/27	5/17 (29 %)	-
Liver (adenoma)	1/27 (4 %)	1/17 (6 %)	-
Liver (haemangioma)	-	-	1/13 (8 %)

In a second study, female Golden Syrian hamsters, dibenzo[def,p]chrysene was painted on to the tongue of females Golden Syrian hamsters, 5 times/week for 30 weeks (7 animals/group). Histopathology was performed on satellite groups of 3 animals after week 1, 6, 10 and 25.

After 1 week, small discrete areas of heterochromatic cells, hyperplasia, anaplasia and growth into connective tissue of the tongue mucosa were observed. After 6 weeks, moderate to severe dysplastic changes including pleomorphism, anaplasia, hyperplasia, hyperchromatism, mitotic figures and hyperkeratosis were observed. After 10 weeks these dysplastic changes were found

to be severe and extensive proliferation into the underlying connective tissue was observed. After week 25, the development of oral squamous cell carcinoma was seen in 5/7 (71 %) hamsters (a total of 15 tumours). At the end of the study, this number had increased to 6/7 (85 %) hamsters (total of 18 tumours). No tumours were observed in untreated hamsters.

The results of these two oral studies, carried out in mice and hamsters, provide strong evidence of a carcinogenic effect following treatment with dibenzo[def,p]chrysene. The finding in the knockout mice serves to illustrate the importance of metabolic activation in the carcinogenic process.

Dermal studies

Five dermal studies are available, 4 of which were carried out by repeated dosing and 1 involved just a single application of dibenzo[def,p]chrysene. The latter study did not employ a concurrent control and is therefore of limited value.

In a study from 1966, Swiss Albino mice (20 females/group) were administered a topical application of dibenzo[def,p]chrysene (0, 0.86 mg/kg bw/day or 1.71 mg/kg bw/day in p-dioxan) 3 times/week for 12 months. After 15 months the study was terminated and the mice were assessed for skin tumours. In the low dose group, squamous cell papilloma and squamous cell carcinoma were both found in 17/20 (85 %) of the mice. The mean latency period was 245 days. In the high dose group, the incidence of both tumour types was slightly higher: 18/20 (90 %). The mean latency period in this group was 210 days. No skin tumours were observed in the vehicle control group.

In a study published in 1972, Swiss Albino mice (19-21 females/group) received a topical dose of dibenzo[def,p]chrysene of 0, 0.017, 0.086, 0.17, 0.86 or 1.71 mg/kg bw/day, 3 times/week for up to 7 months:

Table*: The number of doses each animal received in each dosing group*

Dose (mg/kg bw/day):	0	0.017	0.086	0.17	0.86	1.71
No. of doses:	55	55	40	24	7	7

B[a]P (0.05 % and 0.1 %) was used as a positive control in this study.

High incidences of skin tumours were observed in the treated mice, with a latency period as short as 56 days (0.86 mg/kg bw/day). Key findings are summarised in the following table.

Table*: Number of mice with skin tumours following repeated dermal exposure of dibenzo[def,p]chrysene for up to 7 months (incidences shown by month)*

	Dibenzo[def,p]chrysene (mg/kg)					**B[a]P**	
	0.017	**0.086**	**0.17**	**0.86**	**1.71**	**0.05 %**	**0.1 %**
Total number of animals at start of study	20	19	21	20	21	20	20
	Number of animals with tumours/ number of surviving animals						
2 months	1/20	7/19	9/21	12/20	7/19	-	-
6 months	20/18	19/11	20/0	19/0	16/0	-	-
7 months	20/9	19/7	-	-	-	19/0	16/1
Mean latency	93 days	62 days	66 days	56 days	77 days	130 days	161 days

In a 1993 study (Higginbotham *et al*), dibenzo[def,p]chrysene was applied topically in acetone to the skin of female Swiss mice (22-27/group) at doses of 0, 0.3, 1.2 or 2.4 µg/day, 2 times/week for 40 weeks. High incidences of squamous cell carcinoma and/or papilloma were observed at the study close after 48 weeks. At doses of 1.2 µg/day and above, metastases were noted in the lungs and lymph nodes.

***Table**: Tumour incidence in the skin of mice treated with dibenzo[def,p]chrysene*

	Dose (µg/day)			
	0	**0.3**	**1.2**	**2.4**
No. mice with skin tumours/total no. in group	0/27	1/24 (4 %)	19/23 (83 %)	20/22 (91 %)
Squamous cell papilloma	-	1/24 (4 %)	9/23 (39 %)	16/22 (73 %)
Squamous cell carcinoma	-	-	16/23 (70%)	20/22 (91 %)
Mean latency	-	33 wks.	28±9 wks.	22±9 wks
Survival	-	47±2 wks.	46±3 wks.	43±8 wks.
Metastasis	-	-	Lung and lymph nodes (benign and malignant)	Lung and lymph nodes (benign and malignant)

Data from this study were used by the DS to derive a T_{25} value and to support their proposal for the setting of a specific concentration limit (see below).

In a fourth repeated dose dermal study (from 2004), groups of female wild-type C57BL/6J mice (17/group) and those with an aryl hydrocarbon receptor (AhR)-deficiency (AhR -/-) (15 /group) were treated first with a single dose of 1.2 mg/kg bw dibenzo[def,p]chrysene, followed by repeated doses of 34 µg/kg bw once/week for up to 20 weeks. For the wild type AhR (+/+) mice, the study duration was 24 weeks. For the AhR (-/-) mice, observations were extended for up to 2 years.

Dibenzo[def,p]chrysene induced a high incidence of skin tumours in AhR (+/+) mice following repeated dermal dosing. In AhR-deficient mice (AhR -/-), tumour development was less marked. In AhR (+/+) mice, all animals were found to have tumours by week 24. The first skin tumour appeared at week 11 and the average number of tumours per treated mouse was 2.7 ± 1.4. The tumours observed were squamous cell papilloma (76 %) and squamous cell carcinoma (24 %). In AhR (-/-) mice, 5/15 (33 %) were observed to have skin tumours by week 24. The first skin tumour was observed at week 21 and the average number of tumours per mouse was 0.46 ± 0.83. Only squamous cell papillomas were observed, no malignant tumours were seen. There were no further increases in tumour development on the skin during the follow-up period of up to 2 years. No data were available on mice treated with the vehicle control.

A study that investigated tumour formation after a single application of dibenzo[def,p]chrysene to the skin is also available. SENCAR[2] mice (24 females/group) were treated with a single dose of 30 µg of ± dibenzo[def,p]chrysene and then observed for a period of 27 weeks before sacrifice. At the end of the study, 7/24 mice were found to have skin tumours (4/7 squamous cell papilloma and 3/7 squamous cell carcinoma). It is difficult to reach any firm conclusions about the carcinogenicity of dibenzo[def,p]chrysene from this study given the absence of a negative control group, but the results are consistent with those of the other studies in mice.

Overall, these results provide a strong indication of the carcinogenic potential of dibenzo[def,p]chrysene following dermal application.

In addition, 9 studies of skin tumour initiation-promotion were included in the CLH report. They appear to have been well conducted, with the inclusion of appropriate controls, and all 9 gave clear positive results for tumour formation regardless of single or multiple administrations of dibenzo[def,p]chrysene as the initiator compound. These findings are typical of a carcinogen acting via a genotoxic mode of action.

Other routes of exposure

There were several other studies of carcinogenicity in laboratory animals included in the CLH report. Administration of dibenzo[def,p]chrysene to mice by sub-cutaneous, i.p. and intra-mammary injection has been found to increase the incidence of tumours at the application site. The results are consistent with the findings from the oral and dermal studies. However, given the non-physiological exposure routes used, these studies provide insufficient evidence for human hazard assessment and carcinogenicity classification and are not considered further in this opinion.

Comparison with the criteria

Conclusion and classification

Although no standard carcinogenicity studies are available with dibenzo[def,p]chrysene, there is consistent evidence of treatment-related tumour formation from both oral and dermal studies in mice and hamsters. Furthermore, action of this substance as an initiator in mouse skin tumour-initiation-promotion studies confirms the relevance for carcinogenicity of the positive genotoxicity studies described under Germ cell mutagenicity (above).

As there is no evidence available of carcinogenicity in humans being associated specifically with dibenzo[def,p]chrysene exposure, category 1A is not appropriate.

The observation of tumours in 2 species following treatment with dibenzo[def,p]chrysene, by 2 different routes of exposure, supported by a mechanism of action relevant to humans lead to the conclusion that a category 1B classification is justified for this substance, as shown in the following table.

Factor	Evidence with dibenzo[def,p]chrysene	Conclusion
Tumour type and background control	Mouse tumours in multiple organs, exceeding those in concurrent controls, following oral administration. In other studies, 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	Remote and local tumours were produced at different sites following multiple routes of exposure	Tumours formed at the expected sites of exposure in humans - Cat 1B
Progression of lesions to malignancy	Malignant tumours (uterus, ovary, lung, 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	Tumours increased in male and female animals.	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 chrysene which are classified as Category 1B carcinogens	Cat 1B
Routes of exposure	Physiological (oral and dermal) and non-physiological routes of exposure (sub-cutaneous, mammary gland, intra-peritoneal) produced tumours	Oral and dermal study results confirm activity after exposure by a physiological route - Cat 1B
Comparison of ADME between test animals and humans	No specific studies available, although it is well established that both animals and humans possess the metabolic capacity to activate PAHs through oxidative pathways.	Human relevance – Cat 1B
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	Postulated 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 of CLP (see above), RAC is in agreement with the DS that classification of dibenzo[def,p]chrysene for **carcinogenicity in Category 1B is appropriate**.

Calculation of SCL

The DS assessed the carcinogenic potency of dibenzo[def,p]chrysene using standard methodology, deriving an oral T_{25} value of 0.00051 mg/kg bw/day based on the skin tumour frequency seen in female mice following dermal administration (study by Higginbotham *et al*. 1993).

As mentioned by the DS, the T_{25} method for calculation of potency relies conventionally on data from studies that fulfil a number of criteria:

a) the test animals should be mammals,
b) administration of the test substance should begin early in life (preferable from time of weaning, but up to 100 days is acceptable for rats, mice and hamsters),
c) the route of administration should be via the diet, drinking water, by gavage or inhalation,
d) the test substance should be bioavailable for systemic absorption,
e) the test agent was administered alone,
f) exposure was chronic, with no more than 7 days between each dose
g) the duration of exposure was at least one-fourth of the standard study period for that species,
h) the duration of experiment was at least half of the standard lifespan for that species,

i) the study design has included a control group,
j) the study design included at least 10 animals per group,
k) the pathology data were reported for the number of animals with tumours rather than total number of tumours, and
l) the results reported were original data.

In the absence of such a complete set of information, data from experiments fulfilling as many as possible of these conditions are preferred. The assumptions made by using experiments not fulfilling regulatory guidelines should be specified and justified by toxicological considerations. Accordingly, the DS acknowledged that the study used to calculate T_{25} was a dermal study with a duration of less than ¼ of that of a standard carcinogenicity study and therefore did not strictly meet the above criteria. However, the DS made the assumption that absorption of dibenzo[def,p] chrysene would occur for all routes and considered that the sensitivity of various tissues in carcinogenicity studies involving the oral route of exposure supported the use of this approach for the estimation of potency.

This justification, together with the general observation that high incidences of tumours can be induced in animals following relatively short treatment periods involving relatively low doses (considerably less than 10 mg/kg/day over a conventional 2-year period), shows clearly that it can be regarded as a high potency carcinogen.

According to CLP, high potency carcinogens (T_{25} < 1 mg/kg bw/day) are assigned an SCL of 0.01 %. However, given that the calculated T_{25} was more than 10-fold less than the cut-off value, the DS proposed to assign an SCL of 0.001 %.

In principle, RAC agrees with this proposal. All 3 substances would appear to be high potency carcinogens as defined in the CLP Regulation – see, for example, the comparative data in the following table.

***Table**: Comparative potencies of 3 PAHs causing skin tumours in female Swiss mice following dermal treatment*

Substance	Dosing regimen	Tumour Type indicated
Dibenzo[b,def]chrysene	1.13 mg/kg bw/day 2x/week for 70 weeks	89.7 % mice with tumours - 45 % increase in squamous cell carcinoma, - 17 % increase in skin papilloma
Benzo[rst]pentaphene	~ 0.86 mg/kg bw/day 3x/week for 52 weeks	80 % increase in skin papilloma, 65 % increase in skin epitheliomas
Dibenzo[def,p]chrysene	1.2 µg/day (~0.05 mg/kg bw/day) 2x/week for 40 weeks	83 % mice with tumours - 70 % increase in squamous cell carcinoma - 39 % increase in skin papilloma

After comparing the relative potencies of the 3 PAH substances in female Swiss mice following dermal application for up to 70 weeks, it is clear that all 3 substances cause cancer at very low doses (0.05 - 1.13 mg/kw bw/day). However, the dose at which dibenzo[def,p]chrysene causes skin tumours in these studies is about 20 times lower than the other PAHs (approximately 0.05 mg/kg bw/day), and therefore on this basis it could be considered the most potent of the three.

In the recently drafted RAC opinion documents for dibenzo[b,def]chrysene and benzo[rst]pentaphene, SCLs were not proposed for this endpoint. No justification was provided by the DS as to why a SCL should not be considered for these substances. Nevertheless, given the very high potency of dibenzo[def,p]chrysene that has been seen in animals, RAC agrees with the proposal to set a SCL for this substance. RAC agrees with the DS that **an SCL of 0.001%**, which is 10-fold lower than the limit routinely set for high potency carcinogens, **is warranted**. This accounts for the exceptionally low concentrations and short exposure periods needed to produce tumours in these studies.

ANNEXES:

Annex 1 The Background Document (BD) gives the detailed scientific grounds for the opinion. The BD is based on the CLH report prepared by the Dossier Submitter; the evaluation performed by RAC is contained in 'RAC boxes'.

Annex 2 Comments received on the CLH report, response to comments provided by the Dossier Submitter and RAC (excluding confidential information).