EVIDENCE ON THE CARCINOGENICITY OF

DIBENZANTHRACENES

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PREFACE

Proposition 65[1] requires the publication of a list of chemicals "known to the state" to cause cancer or reproductive toxicity. It specifies that "a chemical is known to the state to cause cancer … if in the opinion of the state's qualified experts the chemical has been clearly shown through scientifically valid testing according to generally accepted principles to cause cancer ..." The "state's qualified experts" regarding findings of carcinogenicity are the members of the Carcinogen Identification Committee (CIC) of the OEHHA Science Advisory Board[2].

The lead agency for implementing Proposition 65 is the Office of Environmental Health Hazard Assessment (OEHHA) of the California Environmental Protection Agency. In 2011, OEHHA brought dibenzanthracenes (DBAs) to the CIC for prioritization and ranking for future listing consideration. DBAs consist of three chemicals: dibenz[*a,h*]anthracene (DB[*a,h*]A), dibenz[*a,c*]anthracene (DB[*a,c*]A), and dibenz[*a,j*]anthracene (DB[*a,j*]A). OEHHA subsequently selected DBAs for consideration for listing by the CIC. Upon selection, the public was given the opportunity to submit information relevant to the assessment of the evidence on their carcinogenicity. No information was submitted.

OEHHA developed this document with information on the evidence on the carcinogenicity of DBAs to assist the CIC in its deliberations on whether or not DBAs as a group, or chemicals within the group, should be added to the Proposition 65 list as causing cancer. The original papers discussed in the document are also provided to the CIC as part of the hazard identification materials. In addition, comments on this hazard identification document received during the public comment period also form part of the hazard identification materials, and are provided to the CIC members prior to their formal deliberations.

On November 19, 2014, the CIC is scheduled to deliberate on the carcinogenicity of DBAs as a group, and individual chemicals within the group. The CIC may choose to list under Proposition 65 the group, or individual chemicals within the group that are not already on the list. DB[*a,h*]A is already on the Proposition 65 list, so the committee deliberations and listing decisions will not affect the status of this chemical. A transcript of the meeting will be available at www.oehha.ca.gov after the meeting.

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[1] The Safe Drinking Water and Toxic Enforcement Act of 1986 (California Health and Safety Code 25249.5 *et seq*.)
[2] Title 27 Cal. Code of Regs. §25302

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1. EXECUTIVE SUMMARY

This document summarizes the evidence of carcinogenicity for the chemical group "dibenzanthracenes (DBAs)," defined in this document as consisting of three isomers: DB[*a,c*]A, DB[*a,h*]A, and DB[*a,j*]A. DB[*a,h*]A has been listed under Proposition 65 as causing cancer since 1988. Carcinogenicity evidence is being presented on DB[*a,h*]A because of its structural similarity and relevance to the determination of carcinogenicity of DBAs as a chemical group, and individual chemicals within the group that are not already on the list.

DBAs are five-ring polycyclic aromatic hydrocarbons (PAHs) containing an anthracene core (*i.e*., three linear benzene rings). DBAs are produced as products of incomplete combustion or pyrolysis of organic matter and during high temperature cooking. Human exposure to DBAs may occur in occupational settings or in general environments from contaminated air, food, water, sediment or soil.

The evidence for the carcinogenicity of DBAs as a group comes from a substantial body of evidence on DB[*a,h*]A, together with evidence on DB[*a,c*]A and DB[*a,j*]A and DBA metabolites. This evidence comes from cancer bioassays in animals, mouse skin tumor initiation-promotion studies, genotoxicity assays, DNA binding and adduct formation studies, *in vitro* cell transformation studies, *in vivo* studies of pre-neoplastic changes in subcutaneous tracheal transplants, studies on receptor binding and activation, immune function, cell proliferation and gene expression, structure-activity considerations, and Quantitative Structure Activity Relationship (QSAR) predictions of carcinogenic activity.

- DB[*a,h*]A has been listed under Proposition 65 as causing cancer since 1988. It induces malignant and benign tumors in animals, is a mouse skin tumor initiator, is genotoxic and forms DNA adducts, and is metabolized to genotoxic diols and diol epoxides that are skin tumor initiators. Regarding other findings relevant to carcinogenesis, DB[*a,h*]A induces malignant cell transformation *in vitro*, activates aryl hydrocarbon receptor (AhR), estrogen receptor (ER), and androgen receptor (AR), has immunosuppressive effects, increases cell proliferation, alters expression of genes associated with cell proliferation, apoptosis, and cell cycle regulation in mouse liver *in vivo*, and is classified as a 2A carcinogen by the International Agency for Research on Cancer (IARC).

- DB[*a,c*]A – observations following treatment include:
    - Tumor findings in a dermal application study in female mice (skin squamous cell carcinoma and combined papilloma and carcinoma), and in a 1-year neonatal *i.p*. injection study in male mice (liver adenoma)
    - Mouse skin tumor initiating activity in multiple studies and in multiple strains of mice
    - Mouse skin tumor initiating activity by its diol metabolites
    - Multiple positive genotoxicity, DNA binding and adduct formation studies of DB[*a,c*]A and its diol/diol epoxide metabolites in bacteria (mutations and DNA damage), cell-free systems (DNA binding and adduct formation), *Drosophila melanogaster* (mutations), *in vitro* mammalian systems, and mice *in vivo*
    - Multiple positive studies of *in vitro* cell transformation in Syrian hamster embryo cells
    - Findings of pre-neoplastic changes in subcutaneous tracheal transplants in mice
    - Other relevant findings including AhR agonist activity, immunosuppressive effects *in vitro,* and increased cell proliferation in a rat liver cell line
    - Predictions of carcinogenicity by several QSAR models

- DB[*a,j*]A – observations following treatment include:
    - Tumor findings in a dermal application study in female mice (skin squamous cell carcinoma and combined papilloma and carcinoma), and in a *s.c*. injection study in female mice (rare skin sarcomas in 3/15 mice)
    - Mouse skin tumor initiating activity in multiple studies in SENCAR mice by DB[*a,j*]A
    - Mouse skin tumor initiating activity by its diol/diol epoxide metabolites
    - Multiple positive genotoxicity, DNA binding and adduct formation studies of DB[*a,j*]A and its diol/diol epoxide metabolites in bacteria (mutations), cell-free systems (DNA binding and adduct formation), *in vitro* mammalian systems, and mice *in vivo*
    - Other relevant findings including AhR agonist activity and increased cell proliferation in a rat liver cell line
    - Predictions of carcinogenicity by several QSAR models

- There are strong structure-activity similarities among all three DBA isomers, and between the DBAs and six comparison PAHs listed as carcinogens under Proposition 65.

2. INTRODUCTION

This document summarizes the evidence of carcinogenicity for the chemical group "dibenzanthracenes (DBAs)," defined in this document as consisting of three isomers: namely dibenz[*a,c*]anthracene (DB[*a,c*]A), dibenz[*a,h*]anthracene (DB[*a,h*]A), and dibenz[*a,j*]anthracene (DB[*a,j*]A). DB[*a,h*]A has been listed under Proposition 65 as causing cancer since 1988. Carcinogenicity evidence is being presented on DB[*a,h*]A because of its structural similarity and relevance to the determination of carcinogenicity of the two other DBAs and for the DBAs as a chemical group.

2.1 Identity of Dibenzanthracenes (DBAs)

Dibenzanthracenes (DBAs) are five-ring polycyclic aromatic hydrocarbons (PAHs) containing an anthracene core (*i.e*., three linear benzene rings). Figure 1 shows the alphanumeric labeling of anthracene as it relates to the naming scheme of the DBAs.

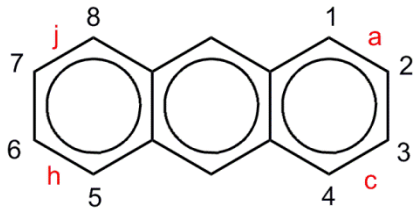
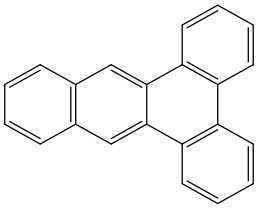
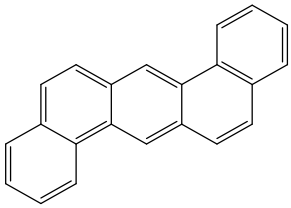
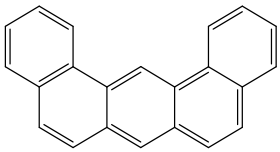


Figure 1. Chemical structure of anthracene

The structures and some chemical and physical properties of the DBAs are presented in Table 1.

Each DBA possesses two or more molecular regions referred to as bay regions. A bay region is defined as a concave area at the periphery of the PAH molecule that is bounded by three benzene rings. Bay region structures in PAHs are considered important for the formation of reactive metabolites such as diol epoxides and have been associated with mutagenicity and carcinogenicity. DB[*a,h*]A and DB[*a,j*]A also contain reactive electrophilic regions directly across from their respective bay regions, referred to as K regions (Loew *et al.*, 1979; Pullman and Pullman, 1955).

Table 1. Some chemical and physical properties of the DBAs

Chemical name[1] **(Synonyms)**	Dibenz[*a,c*]anthracene (DB[*a,c*]A; 1,2:3,4-dibenzanthracene; benzo[b]triphenylene)	Dibenz[*a,h*]anthracene[2] (DB[*a,h*]A; 1,2:5,6-dibenzanthracene)	Dibenz[*a,j*]anthracene (DB[*a,j*]A; 1,2:7,8-dibenzanthracene; 3,4,5,6-dibenzanthracene)
Molecular formula (molecular weight)	$C_{22}H_{14}$ (278.35)		
CAS No.	215-58-7	53-70-3	224-41-9
Structure			
Solubility in water at 25°C (mg/L)	0.0016	0.0005	0.00103
Melting point (°C)	205.6	269.5	269.5
Log K_{ow} (Octanol:water partition coefficient)	6.41	6.75	6.54
Henry's law constant (25°C; atm-m^3/mol)	4.89×10^{-7}	1.23×10^{-7}	4.89×10^{-7}
Vapor pressure at 25°C (mm Hg)	3.73×10^{-9}	1×10^{-10}	3.73×10^{-9}

[1] The naming of fused-ring PAHs is based on the base component of the molecule, *i.e.*, the fragment with the greatest number of rings in a linear configuration; designations of the other components are indicated in the prefix (IUPAC, 1979, Rules A-21.1-21.3). For example, a linear fragment of five benzene rings is named "pentacene" instead of "dibenzanthracene" or "benzo[*a*]naphthacene".

[2] Listed under Proposition 65 as causing cancer January 1, 1988.

2.2 Occurrence, Use and Exposure

2.2.1 Production, Use and Occurrence

Like many other PAHs, DBAs are produced as products of incomplete combustion or pyrolysis of organic matter (*e.g.*, cigarette and marijuana smoke, gasoline engine exhaust, and industrial emissions such as fuel combustion, coke oven operations, and coal-tar distillation) and during high temperature cooking (*e.g.*, grilling, broiling, roasting, baking, frying) (IARC, 1983; 2010; OEHHA, 2009). There is no commercial production of DBAs. The only known use of DBAs is for research purposes. DBAs occur in fossil fuels, such as crude oil and used motor oil, air, water, soils, sediments and food (IARC, 1983; 2010).

2.2.2 Exposure and Biomonitoring

Human exposure to DBAs may occur in occupational settings or in general environments from contaminated air, food, water, sediment or soil. With the exception of smokers and occupationally-exposed workers, most individuals are exposed to PAHs predominately from dietary sources (Alomirah *et al.*, 2010).

DBAs have poor degradability, high lipophilic solubility, and low water solubility. Therefore they tend to persist in various media, such as soils and sediment (Karcher, 1992). DBAs are present in the air (both ambient and indoor, in occupational settings and cooking fumes), in water (drinking and fresh), in dried sediments, in food (fresh and cooked), and in wildlife (*e.g.*, sea turtle eggs) (references listed in Table 2; Alomirah *et al.*, 2011; Martorell *et al.*, 2012; Sivaswamy *et al.*, 1991; Van Heddeghem *et al.*, 1980). DBAs also have been detected in human blood and breast milk (Alam and Brim, 2000; Guo *et al.*, 2012).

Environmental levels of DBAs and emissions factors for DBAs from the burning of tobacco and marijuana smoke are summarized in Table 2.

Table 2. Environmental levels and emission factors for DBAs

Medium	DBA levels[1] (mean, range or maximum)	Reference
Ambient air	Range: 0.12 - 0.22 ng/m^3	Lim *et al.* (1999)[2]
	Range: 0.01 - 174.97 ng/m^3	Hassan and Khoder (2012)[3]
Indoor air		
Occupational settings	Mean: 1.29 µg/m^3; Maximum: 12.54 µg/m^3	Verma *et al.* (1982)[4]
	DB[*a,j*]A: Mean: 0.04 µg/m^3 Range: not detected (ND) to 0.4 µg/m^3	Darby *et al.* (1986)[5]
Cooking fumes	Range: 1.1 - 8.2 µg/g	Li *et al.* (1994)
	Range: 1.9 - 2.4 µg/m^3	Wu *et al.* (1998)
	Range: 1.8 - 2.1 µg/m^3	Chiang *et al.* (1997)
	Range: 2.4 - 3.2 µg/m^3	Chiang *et al.* (1999)
Personal air	Mean: 0.055 ng/m^3 (Range: 0.015 - 0.457 ng/m^3)	Tonne *et al.* (2004)[6]
Water		
Drinking water	13.9 ± 39.94 ng/L	Wu *et al.* (2010)
Freshwater	Range: ND to 13.1 ng/L	Shi *et al.* (2011)[7]
	Range: ND (< 2 ng/L) to 40.96 ng/L	Wu *et al.* (2009)
Lake sediment	DB[*a,j*]A: Range: 1 - 309 ppb	Grimmer and Boehnke (1977)[8]
Emission factors		
Tobacco smoke	DB[*a,h*]A: 0.1 - 0.15 mg/kg cigarette condensate; 0.00115 µg/cigarette;	IARC (1983); OEHHA (2009)
	DB[*a,c*]A: present without quantification	IARC (1983)
	DB[*a,j*]A: mainstream cigarette smoke: 1.1 g/kg/cigarette sidestream cigarette smoke: 4.1 µg/kg/cigarette	IARC (1983)
Marijuana smoke	DB[*a,h*]A: 0.00141 µg/cigarette DB[*a,c*]A: present without quantification	IARC (1983); OEHHA (2009)

[1] Concentrations of DB[*a,h*]A, unless otherwise noted
[2] Source is traffic, measured in the United Kingdom
[3] Review of measurements reported in 7 studies, conducted in several different countries
[4] Sampled in Ontario foundries
[5] Sampled in workplace using bitumen or coal tar for road sealing
[6] 48-hr personal air samples of $PM_{2.5}$ for 344 non-smoking pregnant women in New York City
[7] Sampled in freshwater from various lakes and rivers in east China
[8] Sampled in Bodensee region in Germany

Hassan and Khoder (2012) indicated that the majority of DB[*a,h*]A in ambient air samples collected in Egypt was present in the particulate phase (mean / range: 171 / 51 - 337 ng/m^3), compared to the gaseous phase (mean / range: 3.4 / 2 - 5 ng/m^3). By measuring concentrations of particulate and gaseous PAHs, the authors concluded that PAHs with higher molecular weight and higher ring number, such as DBAs, are generally less volatile and tend to exist in the particulate phase (vs. gaseous phase). Studies by Ji *et al.* (2007) of urban air particulate samples collected in Kumamoto, Japan between April and May 2006 showed that DB[*a,h*]A existed in particles less than 7 µm in size, and 77% of DB[*a,h*]A existed in fine particles less than 2.1 µm in size, with an average concentration of 0.54 ng/m^3.

Several studies have reported levels of DB[*a,h*]A in food and only one study reported DB[*a,c*]A level in food. DB[*a,h*]A was detected in uncooked/fresh food (*e.g.*, sun dried Seer fish) and cooked food (*e.g.*, coconut biscuits and fried chilies) sampled in South India with concentrations ranging from 0.13 - 53.70 ppm (µg/g) (Sivaswamy *et al.*, 1991), in grilled and smoked food sampled in Kuwait at a mean concentration of 0.16 µg/kg (Alomirah *et al.*, 2011), and in various foods sampled from ten restaurants in Spain with an average concentration of 0.08 ppb (µg/kg fresh weight) (range: not detected to 0.10 ppb) (Martorell *et al.*, 2012). The levels of DB[*a,h*]A detected in various foods in Italy by Cirillo *et al.* (2010) are summarized in Table 3. Van Heddeghem *et al.* (1980) reported levels of DB[*a,c*]A (0.9 - 6.3 ppb) and DB[*a,h*]A (0.3 - 19 ppb) in smoked meat and fish in Belgium. DB[*a,h*]A levels in 280 samples of fresh seafood from Korea were all below the detection limit (0.02 ppb) (Hwang *et al.*, 2012).

Table 3. DB[*a,h*]A concentrations measured in various food types in Italy (Cirillo *et al.*, 2010)

Food type	Number of samples	Percent of samples above detection limit (= 0.5 ppb)	Maximum concentration (in ppb)
Milk	80	25	0.6
Cakes, biscuits, pastries, *etc.*	120	4	3.5
Cornflakes	81	17	2.1
Fruit juice	65	7	1.2
Ham or salami sandwiches	67	33	18.9
Chocolate	67	14	18.9
Pasta or rice with tomato sauce or legumes	203	4	7.6
Meat and meat products	126	6	24.8
Fish and fish products	58	29	12.0
Dairy products	75	5	4.7
Egg-based products	79	19	5.7
Pizza	57	20	7.0
Fresh or cooked vegetables	91	25	8.9
Bread, crackers, bread sticks, *etc.*	42	11	4.5
Fresh fruit	88	16	7.5

DBAs have been measured in two human biomonitoring studies. In a study population living near a large e-waste recycling site in Guiyu, China, DB[*a,h*]A was measured in a total of 103 neonatal umbilical cord blood samples with a median of 12.3 ppb (25th percentile: 7.4 ppb; 75th percentile: 23.2 ppb) (Guo *et al.*, 2012). No information on detectable samples or concentration range was reported in Guo's study. In a study conducted in India, DB[*a,c*]A was detected (detection limit = 0.01 ppb) in 9 of 24 samples of human placenta (mean = 0.11 ppm; range: 0.0002 - 0.6 ppm), in 7 of 24 samples of mothers' blood (mean = 0.12 ppm; range: 0.012 - 0.463 ppm), in 12 of 24 samples of umbilical cord blood (mean = 0.50 ppm; range: 0.026 - 1.361 ppm), and in 4 of 20 samples of breast milk (mean = 0.283 ppm; range: 0.013 - 0.6 ppm) (Madhavan and Naidu, 1995).

In wildlife, DB[*a,h*]A was detected in unhatched Loggerhead sea turtle eggs at two out of 20 nest sites in northwest Florida with concentrations of 0.07 - 0.077 µg/g dry weight (Alam and Brim, 2000).

3. DATA ON CARCINOGENICITY

Several PAH mixtures that contain DBAs (*i.e*., coal tar, soot, coke oven emissions, and cigarette smoke) have been shown to be carcinogenic in humans (IARC, 1984; 1985; 1986), and are listed as carcinogens under Proposition 65.

The most well studied of the DBA isomers is DB[*a,h*]A. DB[*a,h*]A was the first pure chemical compound shown to be carcinogenic in studies in experimental animals (Kennaway, 1930). See Appendix A for literature search strategy and terms. As noted above, DB[*a,h*]A has been listed as a carcinogen under Proposition 65 since 1988; thus the available data on the carcinogenicity of this isomer are presented here in summary form. Data on the carcinogenicity of DB[*a,c*]A and DB[*a,j*]A are presented in this section in detail.

3.1 Carcinogenicity Studies in Humans

No data on the long-term effects of human exposure to pure DBAs were identified in the literature search conducted by OEHHA.

3.2 Carcinogenicity Studies in Animals

The carcinogenicity of DBAs has been studied in a number of animal species, including mice, rats, hamsters, guinea pigs, chickens, pigeons and frogs. The majority of studies have been conducted with DB[*a,h*]A. The animal cancer bioassays and initiation-promotion studies of DB[*a,h*]A are briefly summarized in Section 3.2.1 (See Attachment 1 for more detailed discussion of these studies). The available animal carcinogenicity studies of DB[*a,c*]A and DB[*a,j*]A, all of which were conducted in mice, are discussed in Section 3.2.2 (cancer bioassays), Section 3.2.3 (initiation-promotion studies), and Section 3.2.4 (cocarcinogenicity studies on DB[*a,c*]A only).

3.2.1 DB[a,h]A studies

A large number (> 50) of cancer bioassays and initiation-promotion studies on DB[*a,h*]A have been conducted in mice, rats, hamsters, guinea pigs, chickens, and frogs, using a number of different routes of administration. These studies have been reviewed by IARC (1973; 2010). See Attachment 1 (IARC, 2010, pp. 254-255, 282, 305-309, 318-319, 322, 333-334, 353, 368-369, 386, 408, 428-432). Table 4, below, presents an overall summary of the positive tumor findings for DB[*a,h*]A from these studies. The table presents these findings grouped by route of administration, species, strain and sex tested.

Table 4. Overall summary of positive tumor findings for DB[*a,h*]A observed in animal studies (based on IARC, 2010)

Administration route[1]	Species	Strain	Sex[2]	Tumor type
Dermal application (Table 3.1)	Mouse	Swiss, C3H, DBA, NMRI	F	Skin papilloma/carcinoma Mammary tumor
		IF/Bcr	M	Mammary tumor
Dermal initiation-promotion, with DB[*a,h*]A as an initiator (Table 3.2)	Mouse	Swiss	M, F	Skin papilloma/carcinoma
		NMRI	F	
Subcutaneous (*s.c.*) injection (Table 3.3)	Mouse	C57BL, General Purpose/NTH	M, F	Sarcoma/fibrosarcoma Pulmonary tumor
		NMRI	F	
		CH3, B6D2F$_1$, C3H/HeJ, C57BL/6J, AKR/J, DBA/J2	M	
	Rat	NS	M, F	Skin sarcoma
		Sprague-Dawley	F	
		Albino, NS	NS	
Intrapulmonary injection (Table 3.4)	Mouse	Street	M, F	Pulmonary tumor
	Rat	Osborne/Mendel	F	Lung squamous cell adenoma/carcinoma
Oral administration (Table 3.6)	Mouse	C57BL, C3H, DBA	M, F	Small intestine adeno-carcinoma Pulmonary tumor Mammary gland tumor Hemangioendothelioma
		BALB/c	F	
Intraperitoneal (*i.p.*) injection (Table 3.7)	Mouse	B6C3F$_1$[3]	M	Liver adenoma/carcinoma
		A/J	M	Lung adenoma
		NS	NS	Peritoneal tumor
Intravenous *(i.v.)* injection (Table 3.9)	Mouse	Strain A	M, F	Pulmonary tumor
Intramuscular injection (Table 3.10)	Chicken	NS	NS	Sarcoma
	Pigeon	NS	M, F	Fibrosarcoma
Intratracheal administration (Table 3.12)	Hamster	Syrian Golden	NS	Respiratory tract tumor
Intrarenal application (Table 3.14)	Frog	*Rana pipiens*	M, F	Kidney adenocarcinoma

[1] Attachment 1 (IARC, 2010) table number given in parentheses
[2] M: male; F: female; NS: not specified
[3] Fu *et al.* (1998), not included in IARC (2010)

These bioassay data indicate that DB[*a,h*]A is a multi-species, multi-route carcinogen in both sexes inducing a variety of tumors.

3.2.2 DB[a,c]A and DB[a,j]A cancer bioassays

As shown in Table 5, nine cancer bioassays have been conducted on DB[*a,c*]A in several different strains of mice in either or both sexes, and employing various routes of administration (dermal, *s.c.* injection and *i.p.* injection). Two cancer bioassays have been conducted with DB[*a,j*]A administered by either the dermal route or *s.c.* injection to female Swiss mice. The DB[*a,c*]A studies are presented in detail in Section 3.2.2.1 and the DB[*a,j*]A studies are presented in detail in Section 3.2.2.2.

Table 5. Overview of available DB[*a,c*]A and DB[*a,j*]A mouse cancer bioassays

DBA isomer	Strain	Sex[1]	Administration route	Reference
DB[*a,c*]A	Unspecified	F	Dermal	Heidelberger *et al.* (1962)
	Swiss	M	Dermal	Finzi *et al.* (1968)
	Swiss	F	Dermal	Lijinsky *et al.* (1970)
	Unspecified	F	*S.c.* injection	Heidelberger *et al.* (1962)
	ICR/Ha Swiss	F	*S.c.* injection	Van Duuren *et al.* (1968)
	C57BL/6J	M/F combined	*S.c.* injection	Kouri *et al.* (1983)
	DBA/2J			
	$B6D2F_1$			
	$B6C3F_1$ (neonatal exposure)	M	*I.p.* injection	Von Tungeln *et al.* (1999)
DB[*a,j*]A	Swiss	F	Dermal	Lijinsky *et al.* (1970)
	Swiss	F	*S.c.* injection	

[1] M: male; F: female

3.2.2.1 DB[a,c]A mouse cancer bioassays

Dermal application studies

Heidelberger et al. (1962)
DB[*a,c*]A (purified by chromatography) was administered by dermal application to six-week-old female mice (15 animals per group, strain unspecified; from Taconic Farms) (Heidelberger *et al.*, 1962). DB[*a,c*]A (0.125 mg dissolved in 0.025 ml of benzene) was applied twice a week to the backs of mice for 56 weeks (total dose: 14 mg). The use of a control group was not reported. The proportion of mice alive at 40 weeks was 13/15. No tumors were observed in DB[*a,c*]A–treated mice at the end of 56 weeks, possibly due to the less than lifetime observation.

Finzi et al. (1968)
As part of a cocarcinogenicity study, 20 adult male Swiss mice were treated by dermal application (two drops applied every two days to the skin of the interscapular region) with 0.3% DB[*a,c*]A in benzene and observed for 25 weeks (Finzi *et al*., 1968). No tumors were found in the DB[*a,c*]A-treated group in this short 25-week study. Cocarcinogenicity study data are reported later in Section 3.2.4.

Lijinsky et al. (1970)
DB[*a,c*]A (dose: 85 µg; purity > 99%) in acetone was applied to the skin of the interscapular region of eight- to ten-week-old female Swiss mice (30 animals) twice a week for 65 weeks (total dose of DB[*a,c*]A: 11 mg), and the animals were observed until death (Lijinsky *et al*., 1970). Only the incidence of skin tumors was evaluated in the study. DB[*a,c*]A-treated mice were compared to a non-concurrent control group (20 mice) which received acetone treatment for 100 weeks. Survival was similar between the treated and control animals.

Papillomas and squamous cell carcinomas of the skin were observed in treated mice, but not in controls (Table 6). The first tumor in the DB[*a,c*]A-treated group occurred at 60 weeks, suggesting that the study duration in the previous two dermal application studies (56 weeks in Heidelberger *et al.*, 1962; 25 weeks in Finzi *et al.*, 1968) may not have been long enough to permit the observation of treatment-related tumors. Treated animals had a statistically significant increase in squamous cell carcinomas (8/16, $p < 0.01$) and combined skin carcinomas and papillomas (9/16, $p < 0.001$) compared to controls by Fisher's exact test.

Table 6. Skin tumor incidence in female Swiss mice treated dermally with DB[*a,c*]A 2x/week for 65 weeks and observed until death (Lijinsky *et al*., 1970)

Tumor Type	Control[1] (acetone)	DB[*a,c*]A (total dose: 11 mg)[2]
Papilloma	0/14	1/16 (6%)
Squamous cell carcinoma	0/14	8/16** (20%)
Combined carcinoma and papilloma	0/14	9/16*** (56%)

[1] Non-concurrent controls
[2] Treatment group tumor incidences with asterisks indicate significant results from Fisher pairwise comparison with controls (performed by OEHHA)
** $p < 0.01$; *** $p < 0.001$

S.c. injection studies

Heidelberger et al. (1962)
DB[*a,c*]A (purified by chromatography) was administered by *s.c.* injection to six-week-old female mice (15 animals per group, strain unspecified; from Taconic Farms). Mice received two injections (one month apart) of 0.2 ml DB[*a,c*]A (2 mg of DB[*a,c*]A dissolved in 0.5 ml tricaprylin; total dose: 1.6 mg) in the left hind leg through the muscle and into the subcutaneous space on the other side to prevent leakage, and were observed for 56 weeks from the time of the first injection. No tumors were observed in DB[*a,c*]A–treated mice at the end of 56 weeks.

Van Duuren et al. (1968)
As a part of DB[*a,c*]A initiation-promotion studies, a single dose of 1 mg DB[*a,c*]A dissolved in 0.1 ml benzene was administered by subcutaneous injection to female ICR/Ha Swiss mice (8-week-old; 20 animals per group). DB[*a,c*]A did not induce skin tumors during the 65-week observation period. The results of the DB[*a,c*]A initiation-promotion studies are presented in Section 3.2.3.

Kouri et al. (1983)
DB[*a,c*]A (two doses: 150 or 300 µg per mouse; purity not specified) was dissolved in 0.05 ml trioctanoin and administered once by *s.c.* injection to 5-week-old male and female mice (30 animals per dose) in three studies with B6 (C57BL/6J), D2 (DBA/2J), and B6D2F$_1$ mice. B6 inbred mice have a high-affinity aryl hydrocarbon receptor (AhR, genotype: Ah^{b}Ah^b), D2 inbred mice (Ah^{d}Ah^d) have a low-affinity AhR receptor, and B6D2F$_1$ mice are heterozygous (Ah^{b}Ah^d). Controls (10 animals per study) received the vehicle (trioctanoin) by *s.c.* injection. All animals in each study were observed for

twelve months. Tumor incidences in the control groups were not reported in the paper. No tumors were seen in animals treated with 150 µg of DB[*a,c*]A in any of the studies. Fibrosarcomas were observed in one animal treated with 300 µg DB[*a,c*]A in the study in B6 mice, and in one animal treated with 300 µg DB[*a,c*]A in the study in B6D2F_1 mice (Table 7).

Table 7. Skin fibrosarcoma incidence in mice after a single *s.c.* injection of DB[*a,c*]A and observed for 12 months (Kouri *et al*., 1983)

Strain	DB[*a,c*]A dose (µg)	Skin fibrosarcoma incidence	Tumor latency[1] (days)
B6	150	0/30	---
	300	1/30	263
D2	150	0/30	---
	300	0/30	---
B6D2F_1	150	0/30	---
	300	1/30	258

[1] Latency was defined by the authors as "the average time (in days) until the tumor is 1 cm in size".

I.p. Injection studies

Von Tungeln et al. (1999)

Twenty-four neonatal male B6C3F_1 mice were administered three *i.p.* injections of DB[*a,c*]A (purity > 99%) dissolved in 35 µl dimethyl sulfoxide (DMSO) (total DB[*a,c*]A dose: 400 nmol [111 µg]; 1/7, 2/7, and 4/7 of total dose administered on days 1, 8, and 15 of life, respectively) (Von Tungeln *et al.*, 1999). At 12 months of age, the mice were evaluated for liver and lung tumors. The vehicle control group, 24 mice, received DMSO only. Twenty-four mice administered 6-nitrochrysene (6-NC) served as positive controls.

No difference in mean body weight was observed between the treated animals and controls. Table 8 lists the liver tumor incidences of the DB[*a,c*]A-treated and control groups at 12 months. The incidence of liver adenomas in DB[*a,c*]A neonatally treated mice was significantly higher than in the vehicle controls ($p < 0.05$) using a 1-sided Fisher exact test conducted by OEHHA[3], compared with a non-significant increase using the 2-sided Fisher exact test reported by the authors. One liver carcinoma was

[3]All pairwise comparison tests conducted by OEHHA in this document are 1-sided Fisher exact tests.

observed in the vehicle controls, and none in the DB[*a,c*]A-treated mice. The positive control group showed a statistically significant increase in lung (not shown) and liver tumors compared to the vehicle control group.

Table 8. Incidence of liver tumors in male B6C3F$_1$ mice treated neonatally with DB[*a,c*]A by three *i.p.* injections and observed at age 12 months (Von Tungeln *et al.*, 1999)

Tumor Type	Vehicle controls (DMSO)	DB[*a,c*]A (total dose: 400 nmol)[1]	Positive controls (6-NC)
Adenoma	2/24 (8%)	9/24* (38%)	5/22 (23%)
Carcinoma	1/24 (4%)	0/24 (0%)	20/22 (91%)

[1] Treatment group tumor incidences with asterisks indicate significant results from Fisher pairwise comparison with vehicle controls (performed by OEHHA)
* $p < 0.05$

3.2.2.2 DB[a,j]A mouse cancer bioassays

Dermal application study

Lijinsky et al. (1970)
DB[*a,j*]A (dissolved in acetone) was applied at two doses (39 and 78 µg) to the skin of the interscapular region of eight- to ten-week-old female Swiss mice (30 animals) twice a week for 60 to 81 weeks. The animals were observed until death. Only the incidence of skin tumors was evaluated in the study. Survival of each group was reported up to 100 weeks. Total DB[*a,j*]A doses for the low- and high-dose groups were 6.3 and 9.4 mg, respectively. DB[*a,j*]A-treated mice were compared to a non-concurrent control group (20 mice) which received acetone treatment for 100 weeks. Survival in the low-dose group was significantly less than that of the control group by the log-rank test (a statistical test to compare survival curves of different groups) (Bewick *et al.*, 2004), while survival of the high-dose group was similar to that of the control group (Table 9). No explanation was given in the paper regarding the differences in survival.

Table 9. Survival in control and DB[*a,j*]A-treated animals (Lijinsky *et al*., 1970)

Week	Control (acetone)	Low-dose DB[*a,j*]A (6.3 mg)	High-dose DB[*a,j*]A (9.4 mg)
0	20 (100%)	30 (100%)	30 (100%)
20	17 (85%)	29 (97%)	30 (100%)
40	17 (85%)	18 (60%)	26 (87%)
60[1]	**14 (70%)**	**9 (30%)**	**20 (67%)**
80	9 (45%)	3 (10%)	3 (10%)
100	6 (30%)	0	1 (3%)

[1] Bold denotes the effective numbers used for statistical tests; see text for explanation.

Papillomas and squamous cell carcinomas of the skin were observed in treated mice, but not in controls (Table 10). The first skin tumors occurred at 66 weeks in the low-dose group and at 64 weeks in the high-dose group. The effective number of animals used in describing the tumor incidence of the controls and treatment groups was the number of animals surviving at 60 weeks of study. Animals in the high-dose group had a statistically significant increase in skin squamous cell carcinomas (pairwise comparison p-value < 0.05), and a significant dose-dependent trend was observed (p-value < 0.05). An increase in combined skin papillomas and carcinomas was observed in the low- and high-dose groups (low-dose pairwise p-value < 0.05; high-dose pairwise p-value < 0.01), with a significant dose-dependent trend (p-value < 0.01).

Table 10. Skin tumor incidence in female Swiss mice treated dermally with DB[*a,j*]A 2x/week for 60 - 81 weeks and observed for life (Lijinsky *et al*., 1970)

Tumor Type	Control[1] (acetone)	Low-dose DB[*a,j*]A[2] (6.3 mg)	High-dose DB[*a,j*]A[2] (9.4 mg)
Papilloma	0/14 (0%)	2/9 (22%)	2/20 (10%)
Squamous cell carcinoma	0/14* (0%)	2/9 (22%)	6/20* (30%)
Combined papilloma and carcinoma	0/14** (0%)	4/9* (44%)	8/20** (40%)

[1] Control tumor incidences with asterisks indicate significant results from exact trend test (performed by OEHHA)

[2] Treatment group tumor incidences with asterisks indicate significant results from Fisher pairwise comparison with controls (performed by OEHHA)

* $p < 0.05$; ** $p < 0.01$

***S.c.* injection study**

Lijinsky et al. (1970)
DB[*a,j*]A was injected subcutaneously into the backs of 25 female Swiss mice (eight to ten weeks old) (Lijinsky *et al.*, 1970). Each animal received one *s.c.* injection of 0.4 mg DB[*a,j*]A dissolved in 0.2 ml olive oil. Control animals (20 mice) received olive oil only. The animals were observed until death. Only the incidence of skin tumors was evaluated in the study.

Survival was similar between the control and treated animals. No tumors were observed in the control group (Table 11). The first skin tumor appeared at 73 weeks in the DB[*a,j*]A-treated group. Three sarcomas were observed in the DB[*a,j*]A-treated group compared with none in the control, but the increase was not statistically significant by the pairwise comparison conducted by OEHHA.

Table 11. Incidence of skin tumors in female Swiss mice receiving a single *s.c.* injection of DB[*a,j*]A and observed for life (Lijinsky *et al*., 1970)

Tumor Type	Control (olive oil)	DB[*a,j*]A-treated (dose: 0.4 mg)
Sarcoma	0/12 (0%)	3/15 (20%)

3.2.3 DB[a,c]A and DB[a,j]A initiation-promotion studies

All the initiation-promotion studies conducted to date with DB[*a,c*]A and DB[*a,j*]A as initiators have been based on the two-stage model of mouse skin carcinogenesis. The conceptual basis for these experiments is that normal cells are converted irreversibly into latent tumor cells generally via genetic events in the initiation stage (by applying an "initiator"), and remain dormant until further stimulation by promoting agents, usually through mechanisms other than genetic actions (Nesnow *et al.*, 1983). There are six sets of initiation-promotion studies with DB[*a,c*]A and some of its metabolites applied as initiators (discussed in Section 3.2.3.1) and four sets of studies for DB[*a,j*]A and some of its metabolites (discussed in Section 3.2.3.2), all conducted in female mice. Table 12 below presents an overview of these initiation-promotion studies, summarizing the key elements of study design, as well as the study findings.

Table 12. Skin tumor initiation-promotion studies of DB[*a,c*]A, DB[*a,j*]A, and their metabolites applied as initiators to female mice

Study design (strain, duration)	Initiator (dose)	Promoter[1] (dose)	Result[2]	Reference
DB[*a,c*]A (or its metabolites) as an initiator				
ICR/Ha Swiss, 65 weeks	DB[*a,c*]A (1 mg)	Croton resin (25 µg)	-	Van Duuren *et al.* (1968)
ICR/Ha Swiss, 58 - 60 weeks	DB[*a,c*]A (1 mg)	TPA (2.5 µg)	+	Van Duuren *et al.* (1970)
CD-1, 35 weeks	DB[*a,c*]A (2.5 µmol; 0.7 µg)	TPA (5 µmol; 3.1 µg)	+	Scribner (1973)
SENCAR, 16 weeks	DB[*a,c*]A (2 µmol; 0.6 µg)	TPA (2 µg)	+/-	Slaga *et al.* (1980)
	DB[*a,c*]A-10,11-diol (2 µmol)		-	
	DB[*a,c*]A-10,11-diol-12,13-epoxide (2 µmol)		-	
SENCAR, 28 weeks	DB[*a,c*]A (2.5 µmol; 0.7 µg)	TPA (2 µg)	+/-	Scribner and Scribner (1980)
CD-1, 68 weeks	DB[*a,c*]A (25 and 50 µg)	TPA (113 µg)	+/-	Chouroulinkov *et al.* (1983)
	DB[*a,c*]A-1,2-diol (25 µg)		+	
	DB[*a,c*]A-10,11-diol (25 µg)		+	
	DB[*a,c*]A-3,4-diol (25 µg)		+/-	

[1] TPA: 12-*o*-tetradecanoylphorbol-13-acetate; DMBA: 7,12-dimethylbenzanthracene.

[2] "+" denotes statistically significant initiating or promoting effect ($p < 0.05$); "+/-" denotes some initiating effect was observed, but the effect was either not statistically significant or statistical tests could not be conducted due to data limitations (*e.g.*, no controls or no tumor incidence data available).

Table 12 (continued)

Study design (strain, duration)	Initiator (dose)	Promoter[1] (dose)	Result[2]	Reference
DB[*a,j*]A (or its metabolites) as an initiator				
SENCAR, 19 weeks	DB[*a,j*]A (400 nmol; 111 µg)	TPA (3.4 nmol; 2.1 µg)	+	DiGiovanni *et al.* (1983)
SENCAR, 22 weeks	DB[*a,j*]A (tested in 2 separate vehicles) (400 and 800 nmol; 111 and 223 µg)	TPA (3.4 nmol; 2.1 µg)	+ (both doses)	Sawyer *et al.* (1987; 1988)
	DB[*a,j*]A-3,4-diol (tested in 2 separate vehicles) (400 and 800 nmol)		+ (both doses)	
	DB[*a,j*]A-3,4-diol-1,2-epoxide (tested in 2 separate vehicles) (400, 600 or 800 nmol)		+ (all doses)	
SENCAR, 16 weeks	DB[*a,j*]A (400 nmol; 111 µg)	TPA (3.4 nmol; 2.1 µg)	+	Harvey *et al.* (1988)
	DB[*a,j*]A-*trans*-3,4-diol (400 nmol)		+	
	DB[*a,j*]A-*trans*-3,4-diol-*anti*-1,2-epoxide-(400 nmol)		+	
SENCAR, 18 weeks	DB[*a,j*]A (400 nmol; 111 µg)	TPA (3.4 nmol; 2.1 µg)	+	Vulimiri *et al.* (1999)

[1] TPA: 12-*o*-tetradecanoylphorbol-13-acetate; DMBA: 7,12-dimethylbenzanthracene.
[2] "+" denotes statistically significant initiating or promoting effect ($p < 0.05$); "+/-" denotes some initiating effect was observed, but the effect was either not statistically significant or statistical tests could not be conducted due to data limitations (*e.g.*, no controls or no tumor incidence data available).

3.2.3.1 DB[a,c]A initiation-promotion studies

DB[*a,c*]A as an initiator with croton resin tumor promotion

Van Duuren et al. (1968)
A single dose of 1 mg DB[*a,c*]A dissolved in 0.1 ml benzene was applied by *s.c.* injection to the skin of female ICR/Ha Swiss mice (8-week-old; 20 animals per group). Two weeks later, 25 µg croton resin (a phorbol ester mixture from croton oil) in 0.1 ml acetone was applied three times a week for 63 weeks. The study included one no-treatment group and additional groups treated with the DB[*a,c*]A only, the croton resin only (one group treated for 60 weeks, another for 66 weeks), or acetone only. Tumors greater than 1 mm in diameter were recorded monthly. Incidence and latency of skin tumors in each group are listed in Table 13. DB[*a,c*]A exhibited some initiating activity for skin papillomas, as shown by the shortened papilloma latency observed in the DB[*a,c*]A/croton resin treated group (65 days) compared to the croton resin only groups (150 or 293 days), but not for squamous cell carcinomas (310 vs. 231 days). The incidence of skin tumors in the DB[*a,c*]A/croton resin treated group was not statistically different from that in the 66-week croton resin-only group (Table 13).

Table 13. Skin tumor incidence and latency in female ICR/Ha Swiss mice receiving DB[*a,c*]A as an initiator and croton resin as a promoter (Van Duuren *et al*., 1968)

Treatment		Skin tumor incidence		Days to first tumor occurrence		Weeks of treatment
Initiator (dose)	**Promoter (dose)**	**Papilloma**	**Squamous cell carcinoma**	**Papilloma**	**Squamous cell carcinoma**	
DB[*a,c*]A (1 mg)	Croton resin (25 µg)	5/20 (25%)	2/20 (10%)	65	310	65
DB[*a,c*]A (1 mg)	None	0/20	0/20	---	---	65
None[1]	**Croton resin (25 µg)**	**5/20 (25%)**	**1/20 (5%)**	**150**	**231**	**66**
None	Croton resin (25 µg)	1/20 (5%)	0/20	293	---	60
None	Acetone (vehicle)	0/20	0/20	---	---	54
None	None	0/20	0/20	---	---	59

[1] Bold denotes the group used to compare with the DB[*a,c*]A/croton resin group for pairwise comparison.

DB[*a,c*]A as an initiator with TPA tumor promotion

Van Duuren et al. (1970)
A single dose of DB[*a,c*]A (1 mg in 0.1 ml benzene) was applied to the backs of 50 female ICR/Ha Swiss mice (six- to eight-weeks-old). TPA (12-*o*-tetradecanoylphorbol-13-acetate, described by the authors as phorbol myristate acetate; 2.5 µg in 0.1 ml acetone) was then applied to the backs of mice three times a week beginning 2 weeks after DB[*a,c*]A treatment until the end of the experiments, 58 - 60 weeks (Van Duuren *et al.*, 1970). The study also included one control group (no treatment) and additional groups treated with the initiator only, the promoter only, or the vehicle (acetone) only (Table 14).

The DB[*a,c*]A/TPA-treated group had a median survival time of about one year. The median survival time for all other groups was greater than the duration of the experiments (58 – 60 weeks). Tumor incidences observed at 58 – 60 weeks are listed in Table 14. DB[*a,c*]A showed notable tumor-initiating activity, inducing 19/50 skin papillomas (vs. 1/20 in the TPA-only group; $p < 0.01$) and 4/50 skin squamous cell carcinomas (vs. none in the TPA-only group; $p = 0.25$) with earlier onset of papilloma development (74 vs. 338 days in the TPA-only group). Within 58 – 60 weeks of observation, no carcinomas were observed in the other four groups (Table 14). One mouse with two papillomas was observed in the TPA-only group.

Table 14. Skin tumor incidence observed at 58 – 60 weeks after a single dose of DB[*a,c*]A followed by TPA promotion (3x/week for 56 – 58 weeks) in female ICR/Ha Swiss mice (Van Duuren *et al*., 1970)

Treatment		Papilloma incidence	Carcinoma Incidence
Initiator (DB[*a,c*]A dose: 1 mg in 0.1 ml benzene)	**Promoter (TPA dose: 2.5 µg in 0.1 ml acetone)**		
DB[*a,c*]A[1]	TPA	19/50** (38%)	4/50 (8%)
DB[*a,c*]A	None	0/20	0/20
None[2]	**TPA**	**1/20 (5%)**	**0/20**
None	Vehicle (acetone)	0/20	0/20
None	None	0/100	0/100

[1] Treatment group tumor incidences with asterisks indicate significant results from Fisher pairwise comparison with TPA only group (performed by OEHHA)
[2] Bold denotes the group used to compare with the DB[*a,c*]A/TPA group for pairwise comparison.
** $p < 0.01$

Scribner (1973)
Eight-week-old female CD-1 mice, 30 per group, were shaved and treated with a single dose of 2.5 µmol (0.7 µg) DB[*a,c*]A in benzene. One week later, 5 µmol (3.1 µg) TPA was applied two times a week for 34 weeks. This study also characterized initiating activity of other PAHs with TPA (either at 5 or 10 µmol) promotion. Tumor data for animals treated with TPA-only was reported only at 10 µmol, not 5 µmol (Table 15). Twenty-nine DB[*a,c*]A-treated mice were alive at 35 weeks. DB[*a,c*]A exhibited statistically significant tumor-initiating activity based on increased skin papilloma incidence (Table 15; Fisher exact test, $p < 0.001$).

Table 15. Skin tumor incidence at 35 weeks after a single dose of DB[*a,c*]A followed by TPA promotion (2x/week for 34 weeks) in female CD-1 mice (Scribner, 1973)

Initiator (dose)	Dose of TPA promotion (µmol)	Average papillomas/ mouse	Papilloma incidence
DB[*a,c*]A[1] (2.5 µmol)	5	2.31	18/29*** (63%)
None	10	0	0/30

[1] Treatment group tumor incidences with asterisks indicate significant results from Fisher pairwise comparison with TPA only group (performed by OEHHA)
*** $p < 0.001$

Slaga et al. (1980)
A single dose of 2 µmol (0.6 µg) DB[*a,c*]A in acetone was applied to the skin of seven- to nine-week-old female SENCAR mice (30 animals) (Slaga *et al.*, 1980). One week later, TPA (2 µg) was applied twice a week for 15 weeks. The study included a group of 30 mice treated for 15 weeks with TPA only. The incidence of skin papillomas was recorded weekly. The SENCAR mouse was derived by crossing Charles River CD-1 mice with skin tumor-sensitive mice (originally derived from Rockland mice). The SENCAR mice are usually more sensitive to skin tumor induction than Swiss or CD-1 mice, developing more skin papillomas with a shorter latency period after initiation-promotion treatment (Nesnow *et al.*, 1983). As shown in Table 16, at the end of the 15-week TPA application, the DB[*a,c*]A/TPA-treated group had an increased skin papilloma incidence; the increase was of borderline statistical significance when compared to the TPA-only group ($p = 0.07$).

Using the protocol described above, the authors also exposed mice (29 mice/group) to 2 µmol of either DB[*a,c*]A-10,11-diol or DB[*a,c*]A-10,11-diol-12,13-epoxide (both chemicals are DB[*a,c*]A metabolites) as initiators followed by TPA promotion. No

increase in papilloma incidence was observed in groups treated with these two metabolites as initiators (Table 16).

Table 16. Skin tumor-initiating activity of DB[*a,c*]A and two metabolites followed by 15-week TPA promotion (2x/week) in female SENCAR mice (Slaga *et al*., 1980)

Initiator	Papillomas/mouse	Papilloma incidence
DB[*a,c*]A[1]	0.5	8/28 (27%)
DB[*a,c*]A-10,11-diol[1]	0.1	3/29 (10%)
DB[*a,c*]A-10,11-diol-12,13-epoxide[1]	0.1	3/29 (10%)
None	0.1	3/30 (10%)

[1] Dose: 2 µmol

Scribner and Scribner (1980)
The backs of eight-week-old female SENCAR mice (28 animals) were shaved and treated topically with a single dose of 2.5 µmol (0.7 µg) DB[*a,c*]A in acetone (Scribner and Scribner, 1980). Twenty-five days later, 2 µg TPA was applied twice a day to the back skin for 25 weeks. The study did not include a group treated with TPA only. At the end of the 25-week promotion period, 75% of the animals developed papillomas, and the average number of papillomas per mouse was approximately 1.8 - 2.

Chouroulinkov et al. (1983)
A single dose of purified DB[*a,c*]A, dissolved in acetone, was applied to the dorsal skin of female CD-1 mice (age: ~45 days) at two doses: 25 µg (39 animals) and 50 µg (40 animals). Promotion started one week later with 0.64 µg TPA (in acetone) applied twice a week for the first 29 weeks, then increased to 1 µg twice a week for the following 38 weeks, for a total dose of 113 µg (Chouroulinkov *et al*., 1983). Control animals received vehicle (acetone) only as the initiating treatment, and were subsequently treated with the same TPA dosing scheme. All skin tumors observed in animals treated with DB[*a,c*]A as an initiator were papillomas. DB[*a,c*]A showed marginal tumor-initiating activity with an increased incidence of skin tumors of borderline statistical significance for the high dose group ($p = 0.1$) level by pairwise comparison (Table 17). The *p*-value for the Fisher exact test for trend in tumor incidence with DB[*a,c*]A dose was 0.07.

The initiating activities of three purified DB[*a,c*]A metabolites (DB[*a,c*]A-1,2-diol, DB[*a,c*]A-3,4-diol and DB[*a,c*]A-10,11-diol; dose: 25 µg) were evaluated using the same TPA promotion protocol described above. DB[*a,c*]A-1,2-diol and DB[*a,c*]A-10,11-diol exposure resulted in statistically significant increases in skin tumors in initiated mice as compared with the TPA only group ($p < 0.05$ for both metabolites) (Table 17). The

tumor incidence resulting from exposure to either metabolite was approximately 2-fold greater than that observed after DB[*a,c*]A exposure.

Table 17. Skin tumor-initiating activities of DB[*a,c*]A and three DB[*a,c*]A metabolites followed by 67-week TPA promotion (2x/week) in female CD-1 mice (Chouroulinkov *et al*., 1983)

Initiator	Initiator dose (µg)	Skin tumor incidence[1,2]
DB[*a,c*]A	25	5/39 (12.8%)
DB[*a,c*]A	50	8/40 (20.0%)
DB[*a,c*]A-1,2-diol[3]	25	11/40* (27.5%)
DB[*a,c*]A-3,4-diol	25	6/37 (16.2%)
DB[*a,c*]A-10,11-diol[4]	25	10/39* (25.6%)
Vehicle (acetone)	0	3/40 (7.5%)

[1] Includes papillomas ($\geq$ 2 - 3 mm in diameter) and carcinomas
[2] Treatment group tumor incidences with asterisks indicate significant results from Fisher pairwise comparison with acetone group (performed by OEHHA)
* $p < 0.05$
[3] One carcinoma was observed in this group.
[4] Two carcinomas were observed in this group

3.2.3.2 DB[a,j]A initiation-promotion studies

DB*[a,j]*A as an initiator with TPA tumor promotion

DiGiovanni et al. (1983)
Female SENCAR mice (30 per group) were initiated with 400 nmol (111 µg) DB[*a,j*]A (in acetone) applied topically on the back. One week later, mice received a twice weekly application of 3.4 nmol (2.1 µg) TPA for 18 weeks. Control animals only received the vehicle for the initiating treatment (acetone), and were subsequently treated with the same TPA dosing scheme. As shown in Table 18, DB[*a,j*]A showed significant initiating activity for skin papillomas ($p < 0.001$).

Table 18. Skin tumor-initiating activity of DB[*a,j*]A followed by 18-week TPA promotion (2x/week) in female SENCAR mice (DiGiovanni *et al*., 1983)

Initiator	DBA dose (nmol)	Papillomas/mouse	Papilloma incidence
Acetone (vehicle)	0	0.1	3/30 (10%)
DB[*a,j*]A[1]	400	0.93	15/30*** (50%)

[1] Treatment group tumor incidences with asterisks indicate significant results from Fisher pairwise comparison with acetone group (performed by OEHHA)
*** $p < 0.001$

Sawyer et al. (1987; 1988)
DB[*a,j*]A was dissolved in 0.2 ml of two different solvents, either acetone or peroxide-free tetrahydrofuran (THF), and applied to the skin of 7 - 9 week old female SENCAR mice, 30 per group, at two doses (400 and 800 nmol; equivalent to 111 and 223 µg, respectively). Two weeks later, mice received a twice weekly application of 3.4 nmol (2.1 µg) TPA for 20 weeks. Two vehicle control groups received vehicle only as the initiating treatment (acetone controls: 30 mice per group; THF controls: 24 mice per group), and were subsequently treated with the same TPA dosing scheme. Development of skin papillomas was recorded weekly. Table 19 lists the average number of skin papillomas per animal, assessed at the time of "maximal tumor response" for each dose group, and the skin papilloma incidence. Time to reach the maximal tumor response was only reported for the groups receiving acetone or DB[*a,j*]A dissolved in acetone: 15 weeks for the vehicle control and 18 weeks for the DB[*a,j*]A-treated groups. DB[*a,j*]A was positive as a tumor-initiator, inducing a dose-dependent increase in skin papillomas when administered in either acetone or THF (trend test: $p < 0.001$) (Table 19). The increased incidences of skin papillomas were also significant by pairwise comparison ($p < 0.01$ for the low dose in THF, $p < 0.001$ for the high dose in THF and for the low and high doses in acetone) (Table 19).

The initiating activities of two DB[*a,j*]A metabolites, DB[*a,j*]A-3,4-diol and DB[*a,j*]A-3,4-diol-1,2-epoxide, were also studied using the same experimental design (Table 19). Both metabolites were positive for tumor-initiating activity, inducing statistically significant increases in skin papillomas by pairwise comparison and trend tests in both vehicles studied (Table 19). The initiating activity of DB[*a,j*]A-3,4-diol-1,2-epoxide was slightly greater when administered in the THF vehicle, as compared to the acetone vehicle, as assessed both by papilloma incidence and average number of papillomas/mouse. In contrast, the initiating activities of DB[*a,j*]A and DB[*a,j*]A-3,4-diol were greater when administered in acetone, as compared to THF.

Table 19. Skin tumor-initiating activity of DB[*a,j*]A and its metabolites (dissolved in acetone or THF) followed by 20-week TPA promotion (2x/week) in female SENCAR mice (Sawyer *et al*., 1987; 1988)

Initiator group	Dose (nmol)	Vehicle			
		Acetone (30 mice per group)		THF (24 mice per group)	
		Papillomas/ mouse	Papilloma incidence	Papillomas/ mouse	Papilloma Incidence
DB[*a,j*]A[1,2]	0	0.16	5/30*** (16%)	0.05	1/24*** (5%)
	400	1.27	21/30*** (70%)	0.86	9/24** (39%)
	800	3.00	29/30*** (97%)	1.83	16/24*** (65%)
DB[*a,j*]A-3,4-diol[1,2]	0	0.16	5/30*** (16%)	0.05	1/24*** (5%)
	400	2.38	29/30*** (97%)	0.83	12/24*** (48%)
	800	3.17	27/30*** (90%)	2.04	19/24*** (78%)
DB[*a,j*]A-3,4-diol 1,2-epoxide[1,2]	0	0.16	5/30*** (16%)	0.05	1/24*** (5%)
	400	3.55	26/30*** (86%)	5.00	23/24*** (96%)
	600	3.70	24/30*** (80%)	---	---
	800	---	---	7.63	21/24*** (87%)

[1] Vehicle tumor incidences with asterisks indicate significant results from exact trend test (performed by OEHHA)
[2] Treatment group tumor incidences with asterisks indicate significant results from Fisher pairwise comparison with vehicle group (performed by OEHHA)
** $p < 0.01$; *** $p < 0.001$

Harvey et al. (1988)
DB[*a,j*]A (400 nmol dissolved in peroxide-free THF) was applied to the skin of female SENCAR mice (24 per group). One group of 24 mice was treated with vehicle only. Two weeks later, all mice received TPA (3.4 nmol, or 2.1 µg, in 200 µl acetone) twice a week for 14 weeks. Mice treated with DB[*a,j*]A had a statistically significant increase of skin papillomas compared to the THF/TPA group ($p < 0.05$; Table 20). Initiating activities of two DB[*a,j*]A metabolites were also studied with the same TPA promotion protocol. Both of them showed stronger initiating activities than the parent compound, based on the reported papilloma incidence and average number of papillomas per mouse (Table 20).

Table 20. Skin tumor-initiating activities of DB[*a,j*]A and its metabolites followed by 14-week TPA promotion (2x/week) in female SENCAR mice (Harvey *et al*., 1988)

Initiator (dose)	Papillomas/mouse	Papilloma incidence
THF (vehicle)	0.05	1/24 (5%)
DB[*a,j*]A[1] (400 nmol)	0.58	7/24* (29%)
DB[*a,j*]A-*trans*-3,4-diol[1] (400 nmol)	0.70	9/24** (39%)
DB[*a,j*]A-*trans*-3,4-diol-*anti*-1,2-epoxide[1,2] (400 nmol)	4.00	22/24*** (92%)

[1] Treatment group tumor incidences with asterisks indicate significant results from Fisher pairwise comparison with THF group (performed by OEHHA)
* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$
[2] Referred to as *trans*-3,4-dihydroxy-*anti*-1,2-epoxy-1,2,3,4-tetrahydro-DB[*a,j*]A in the paper.

Vulimiri et al. (1999)
DB[*a,j*]A (400 nmol [111 µg]) in 0.2 ml acetone was topically applied to female SENCAR mice (30 animals per group; 6 - 7 weeks old). One group of mice was treated with vehicle only. Two weeks after initiation, 3.4 nmol (2.1 µg) TPA in 0.2 ml acetone was applied twice a week until the average number of skin papillomas per mouse reached a plateau. DB[*a,j*]A showed significant initiating activity, based on comparison of the average number of papillomas per mouse observed after 16 weeks of TPA promotion in the vehicle/TPA group vs. the DB[*a,j*]A/TPA group (0 vs. 3.08 ± 1.89) ($p < 0.05$ reported by the authors using the Mann-Whitney U-test). Information on the number of mice with papillomas was not reported, so no statistical test could be conducted based on tumor incidence.

3.2.3.3 DB[a,c]A promotion studies

Scribner and Scribner (1980)
To study DB[*a,c*]A promotion, the backs of eight-week-old female SENCAR mice (28 animals) were shaved and treated topically with a single dose of 0.1 µmol 7,12-dimethylbenzanthracene (DMBA) as an initiator. Twenty-five days later, 0.09 µmol of DB[*a,c*]A was applied to the back skin twice a day for 25 weeks. The study did not include a group treated with the initiator (DMBA) only. At the end of the 25-week DB[*a,c*]A promotion period, no skin tumors were observed in the DMBA-initiated mice. The authors stated "DB[*a,c*]A is completely lacking in promoting activity."

3.2.4 DB[a,c]A cocarcinogenicity bioassays

Finzi *et al.* (1968) studied the interaction of DB[*a,c*]A and benzpyrene (a mixture of benzo[*a*]pyrene and benzo[*e*]pyrene) on mouse skin carcinogenesis. Groups of adult male Swiss mice were treated (two drops applied every two days to the interscapular region) with one of the following dermal application dosing schemes: alternating between applications of 0.3% DB[*a,c*]A (in benzene) and benzene (20 animals); alternating between applications of 0.3% benzpyrene (in benzene) and benzene (30 animals); or alternating between applications of 0.3% benzpyrene (in benzene) and 0.3% DB[*a,c*]A (in benzene) (40 animals). Skin tumors with a diameter greater than 0.5 mm were recorded weekly. Animals were treated for 25 weeks. [No tumors were found in the group treated with DB[*a,c*]A alone in this short 25-week study, as reported in Section 3.2.2.] Co-administration of DB[*a,c*]A and benzpyrene reduced the tumor incidence seen with benzpyrene alone (co-administration: 45% vs. benzpyrene in the absence of DB[*a,c*]A: 90%) and the average number of tumors per mouse in the tumor-bearing mice (co-administration: 1 vs. benzpyrene in the absence of DB[*a,c*]A: 1.3), suggesting some competing activities for the PAHs.

Slaga and Boutwell (1977) applied DB[*a,c*]A (in 0.2 ml acetone) topically to the shaved skin of seven- to nine-week-old female Charles River CD-1 mice (30 animals per group) at four doses (5, 20, 100, or 200 nmol). Five minutes after DB[*a,c*]A treatment, an equimolar dose of DMBA was applied. An additional treatment group was exposed to 200 nmol DB[*a,c*]A followed by 5 nmol DMBA. One week later, all mice received 10 µg of TPA (in 0.2 ml acetone) applied dermally twice a week for 30 weeks. Other groups of animals received initiating doses of DMBA (5, 20, 100, or 200 nmol) followed by the TPA promotion protocol described above without DB[*a,c*]A pretreatment. Skin papilloma and carcinoma incidences were recorded weekly. Co-administration of DB[*a,c*]A reduced DMBA skin tumor initiation in this two-stage model system. A comparison of the effects of co-administration of DB[*a,c*]A and DMBA vs. DMBA only at

24 weeks after TPA promotion is shown in Table 21. Co-administration of DB[*a,c*]A and DMBA at 100 or 200 nmol resulted in a 90 or 95% reduction in the number of skin papillomas per mouse, compared to animals receiving only the corresponding dose of DMBA followed by TPA promotion (Table 21).

Table 21. Effect of DB[*a,c*]A on the initiation of skin papillomas by DMBA followed by TPA promotion (2x/week) at 24 weeks in female CD-1 mice (Slaga and Boutwell, 1977)

Pretreatment of DB[*a,c*]A (nmol)	DMBA initiation (nmol)	Papillomas/mouse (% of DMBA/TPA group)
5	5	103.0
20	20	45.0
100	100	10.5
200	5	6.5
	200	4.5

DiGiovanni *et al.* (1982) studied the impact of pre-treatment with DB[*a,c*]A on seven other PAHs in initiation-promotion studies. The other PAH initiators were DMBA, 3-methylcholanthrene (3-MC), benzo[*a*]pyrene (B[*a*]P), 7-methylbenz[*a*]anthracene (7-MBA), 12-methylbenz[*a*]anthracene (12-MBA), 5-methylchrysene (5-MeC) and DB[*a,h*]A. DB[*a,c*]A was dermally applied five minutes prior to dermal application of the "initiating" PAH to groups of 7 to 9 week-old female SENCAR mice (30 animals per group). One week after initiation, mice received 3.4 nmol TPA twice a week for 16 weeks. As shown in Table 22, a single pretreatment with DB[*a,c*]A at 200 or 400 nmol reduced the average number of skin papillomas per mouse and the skin papilloma incidence for DMBA and 7-MBA, as compared with animals not receiving DB[*a,c*]A pretreatment. A single pretreatment with DB[*a,c*]A at 200 or 400 nmol had some inhibitory effect on the average number of skin papillomas per mouse and the papilloma incidence for 3-MC and DB[*a,h*]A, and little or no effect on B[*a*]P, 12-MBA and 5-MeC initiation (Table 22).

Table 22. Effects of pretreatment of DB[*a,c*]A on skin tumor initiation by various PAHs followed by 16-week TPA promotion (2x/week) in female SENCAR mice (DiGiovanni *et al*., 1982)

DB[*a,c*]A pretreatment (dose in nmol)	Initiator (dose in nmol)	Papillomas/mouse	Papilloma incidence (%)
0	DMBA (10)	6.6	100
200		1.1	48
400		0.5	34
0	B[*a*]P (200)	3.3	88
200		3.2	86
400		2.9	82
0	3-MC (100)	4.1	90
200		3.3	82
400		2.4	80
0	7-MBA (400)	2.7	90
200		2.2	68
0	12-MBA (400)	0.7	42
200		0.8	53
0	5-MeC (400)	2.9	63
200		2.8	69
0	DB[*a,h*]A (400)	6.8	91
200		4.1	79

These authors also investigated the effect the timing of DB[*a,c*]A pretreatment had on tumor initiation with B[*a*]P (DiGiovanni *et al.*, 1982). As the time period between DB[*a,c*]A application and B[*a*]P initiation increased from 5 minutes to 36 hours, the skin papilloma incidence and number of papillomas/mouse decreased, with little effect seen at 5 minutes, and a significant inhibition of tumor formation see at 24 and 36 hours (Table 23). The authors speculated that tumor initiation inhibition could be due to the induction of aryl hydrocarbon hydroxylase (AHH) over longer time periods (> 24 hours), as DB[*a,c*]A has been shown to be a potent inducer of mouse epidermal AHH.

Table 23. Time-dependent inhibitory effect of DB[*a,c*]A on skin tumors initiated by B[*a*]P followed by 18-week TPA promotion (2x/week) in female SENCAR mice (DiGiovanni *et al*., 1982)

Pretreatment time	DB[*a,c*]A dose (nmol)	Papillomas/mouse	Papilloma incidence (%)
Control	0	2.6	88
5 min	200	2.5	86
12 h		2.1	81
24 h		1.1	42
36 h		1.0	49

3.2.5 Discussion of animal carcinogenicity studies

DB[*a,c*]A and DB[*a,j*]A have not been studied as extensively for carcinogenicity in experimental animals as has DB[*a,h*]A, which has been shown to induce tumors at a variety of sites by different routes of administration in multiple species (mouse, rat, hamster, chicken, pigeon and frog) (Table 4). The available studies of DB[*a,c*]A and DB[*a,j*]A have been conducted only in mice, and the routes of administration have been limited to the dermal, *s.c.* and/or *i.p.* routes.

DB[*a,c*]A significantly increased the incidence of skin squamous cell carcinomas in female Swiss mice in the dermal study with the longest exposure and observation period (two times per week for 65 weeks) (Lijinsky *et al.*, 1970). Statistically significant increases in tumors were not observed in the other dermal studies of DB[*a,c*]A, which were of shorter duration (56 weeks in Heidelberger *et al.*, 1962; 25 weeks in Finzi *et al.*, 1968). DB[*a,c*]A significantly increased the incidence of liver adenomas in neonatal male B6C3F$_1$ mice in the only available *i.p.* injection study (Von Tungeln *et al.*, 1999). Statistically significant increases in tumors were not observed with DB[*a,c*]A in the available *s.c.* injection studies, which generally were limited by small group sizes, administration of relatively low doses, and less than lifetime duration.

Tumors were observed in DB[*a,j*]A-treated mice in the two available studies, although the increase in tumors was statistically significant only in the dermal application study (skin squamous cell carcinomas and combined papillomas and carcinomas in female Swiss mice), and not in the *s.c.* injection study (3/15 sarcomas vs. 0/12 in the control) (Lijinsky *et al.*, 1970).

All three DBA isomers have been shown to be skin tumor initiators in mice (Tables 4, 12 - 20), as have some DB[*a,c*]A and DB[*a,j*]A metabolites (*e.g.*, DB[*a,c*]A-1,2-diol, DB[*a,c*]A-10,11-diol, DB[*a,j*]A-3,4-diol, and DB[*a,j*]A-3,4-diol-1,2-epoxide) (Table 12).

The use of benzene (a carcinogen) as the vehicle in several of the studies reviewed in this section, while potentially problematic, does not appear to confound or otherwise complicate the interpretation of these particular studies. This is because none of the DBA bioassays described above that used benzene as a vehicle was positive (Finzi *et al.*, 1968; Heidelberger *et al.*, 1962; Scribner, 1973; Van Duuren *et al.*, 1968; Van Duuren *et al.*, 1970) and only one of the initiation-promotion studies using benzene as a vehicle showed significant tumor initiating activities (for DB[*a,c*]A, Scribner, 1973), while this positive tumor initiating activity has been also observed using vehicles other than benzene (*e.g.*, Sawyer *et al.*, 1988; Slaga *et al.*, 1980).

The reduction in mouse skin papillomas after DB[*a,c*]A pretreatment followed by DMBA treatment compared to DMBA treatment alone (Slaga and Boutwell, 1977) suggests that DB[*a,c*]A administered along with other PAH carcinogens may compete for similar binding sites or target enzymes and thus decrease overall tumor formation.

3.3 Other Relevant Data

3.3.1 Genotoxicity

The genotoxicity of DBAs has been studied in multiple *in vitro* and *in vivo* studies. DB[*a,h*]A genotoxicity data are summarized briefly in Table 24. The available genotoxicity findings for DB[*a,c*]A, DB[*a,j*]A, and their metabolites are presented in Tables 25 - 34 below.

DB[*a,h*]A

Evidence of DB[*a,h*]A genotoxicity includes: positive results in bacterial DNA damage (*E. coli* and *B. subtilis*) and mutagenicity assays (TA100, TA98, TM677), positive results in mammalian cells *in vitro* in unscheduled DNA synthesis (UDS) assays in human foreskin cells and HeLa cells (a cervical epithelial cancer cell line), a forward mutation assay in Chinese hamster V79 lung cells, and a sister chromatid exchange (SCE) assay in Chinese hamster ovary (CHO) cells; positive *in vivo* results for induction of SCEs in the bone marrow of Chinese hamsters (IARC, 1983).

Table 24. Summary of DB[*a,h*]A genotoxicity findings (IARC, 1983)

Assay	Organism/cell type	Metabolic activation system added	Result
Bacteria			
DNA damage	*E. coli*	yes	+
	Bacillus subtilis	yes	+
Gene mutation	*Salmonella typhimurium* (TA100, TA98, TM677)	yes	+
Mammalian cells *in vitro*			
UDS[1]	Human foreskin cells	no	+
	HeLa cells	yes	+
	Syrian hamster embryo cells	no	–
	Primary rat hepatocytes	no	–
Gene mutation	Chinese hamster V79 cells	yes	+
SCEs[2]	Chinese hamster ovary cells	no	+
Mammalian cells *in vivo*			
SCEs	Chinese hamster bone marrow	no	+
Chromosomal breaks			–

[1] Unscheduled DNA synthesis
[2] Sister chromatid exchanges

DB[*a,c*]A, DB[*a,j*]A, and their metabolites

Bacteria

Salamone *et al.* (1979) tested DB[*a,c*]A for mutagenicity in *Salmonella* test strain TA100 in the absence of S9 metabolic activation and reported negative results. Additionally, Shimada and Nakamura (1987) found that DB[*a,c*]A did not induce mutations in *Salmonella* test strain TA1535 using polychlorinated biphenyl- or phenobarbital-induced rat liver S9 for metabolic activation, but did induce mutations in TA1535 in the presence of 3-MC-induced rat liver S9. In contrast, several studies reported that DB[*a,c*]A caused DNA damage in *Bacillus subtilis* and induced mutations in several *Salmonella* strains, including TA98, TA100, TA1535, TA1537, TA1538, MP352 and TM677 in the presence of S9 (Table 25).

Table 25. DB[*a,c*]A bacterial DNA damage and gene mutation assay results

Strain	Dose/ concentration	Result[1]		Activation System[2]	Reference
		-S9	+S9		
***Bacillus subtilis* (DNA damage)**					
H-17 *rec*$^{+}$	6-50 µg	NT	+	Aro rat liver S9	McCarroll *et al.* (1981)
M-45 *rec*$^{-}$	6-50 µg	NT	+		
***Salmonella typhimurium* (gene mutation)**					
TA98, TA100	0.1 -100 µg	NT	+	3-MC mouse liver S9	Levitt *et al.* (1979)
TA98	Up to 2000 nmol	NT	+	Aro rat liver S9	Hermann (1981)
TA98, TA100, TA1537, TA1538	10 µg	NT	+	Aro rat liver S9	McCann *et al.* (1975)
TA98, TA100, TA1537, TA1538	3.6 µM (minimum mutagenic concentration[3])	NT	+	Aro rat liver S9	Probst *et al.* (1981)
TA100	1-1000 µg	NT	+	Aro or PB rat liver S9	Andrews *et al.* (1978)
TA100	0.1-1000 µg	–	+	Aro rat liver S9	Salamone *et al.* (1979)
TA100	1-10 µM	NT	+	Aro rat liver S9	Malaveille *et al.* (1980)
TA100	~1-50 µg	NT	+	3-MC or TCDD rat/mouse liver S9	Pahlman and Pelkonen (1987)
TA100	0.3-10 µg	NT	+	3-MC rat liver microsome or S9	Lecoq *et al.* (1989)
TA100	~0.1-20 µg	NT	+	Aro rat liver S9	Kumar *et al.* (1990)
TA100	0.3 µg	NT	+	Aro rat liver S9	Lutz *et al.* (2002)
TA1535	10 µM	NT	+	3-MC rat liver S9	Shimada and Nakamura (1987)
		NT	–	PCB or PB rat liver S9	
TM677	13 µM	NT	+	Aro or PB rat liver S9	Kaden *et al.* (1979)
TM677	24 - 72 µM	NT	+	Aro rat liver S9	Skopek and Thilly (1983)
MP352	179.6 µM	NT	+	Aro rat liver S9	Pall and Hunter (1987)

[1] NT: not tested

[2] Aro: Aroclor 1254-induced; 3-MC: 3-methylcholanthrene-induced; TCDD: 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin-induced; PCB: polychlorinated biphenyls ("KC-500" containing 50% chlorine)-induced; PB: phenobarbital-induced

[3] The "minimum mutagenic concentration" is the concentration at which mutation was observed in the most sensitive tester strains

Salmonella strains vary in sensitivity for detecting different types of mutations. For example, strain(s) with sensitivity to:

- frameshift mutations are TA98, TA1537, and TA1538.
- G/C base-pair substitution mutations are TA100 and TA1535 (Ames *et al.*, 1975; Eastmond *et al.*, 2009).
- tandem duplication is MP352 (Pall and Hunter, 1987).
- forward mutations is TM677 (mating transfer of the plasmid pkM101 into a *his*$^+$ revertant of TA1535) (Shamon and Pezzuto, 1997).

Thus the results shown in Table 25 indicate that DB[*a,c*]A causes forward mutations, tandem duplication, and both base-pair and frameshift reverse mutations in *Salmonella*.

Three DB[*a,c*]A metabolites (DB[*a,c*]A-*trans*-1,2-diol, DB[*a,c*]A-*trans*-3,4-diol and DB[*a,c*]A-*trans*-10,11-diol) were tested in *Salmonella* strain TA100 with S9 (Malaveille *et al.*, 1980) and found to induce mutations. The bacterial gene mutation assay results for those DB[*a,c*]A metabolites are listed in Table 26.

Table 26. *Salmonella typhimurium* gene mutation assay results for DB[*a,c*]A metabolites

DB[*a,c*]A metabolites	Strain	Dose	Result[1]		Activation System[2]	Reference
			-S9	+S9		
DB[*a,c*]A-*trans*-1,2-diol	TA100	1-1000 µg	NT	+	Aro rat liver S9	Malaveille *et al.* (1980)
DB[*a,c*]A-*trans*-3,4-diol			NT	+		
DB[*a,c*]A-*trans*-10,11-diol			NT	+		

[1] NT: not tested
[2] Aro: Aroclor 1254-induced

DB[*a,j*]A has been tested only in one strain of *Salmonella*, TA 100, in two sets of experiments. As shown below in Table 27, DB[*a,j*]A tested positive in *Salmonella* test strain TA100 with S9 .

Table 27. DB[*a,j*]A *Salmonella typhimurium* gene mutation assay results

Strain	Concentration	Result[1]		Activation System[2]	Reference
		-S9	+S9		
TA100	1 - 1000 µg	NT	+	Aro or PB rat liver S9	Andrews *et al.* (1978)
TA100	0.3 - 10 µg	NT	+	3-MC rat liver microsomes or S9	Lecoq *et al.* (1989)

[1] NT: not tested
[2] Aro: Aroclor 1254-induced; 3-MC: 3-methylcholanthrene-induced; PB: phenobarbital-induced

Mammalian and non-mammalian genotoxicity in vitro

DB[*a,c*]A formed DNA adducts in calf thymus DNA, and tested positive for UDS in HeLa cells and primary rat hepatocytes, and in forward mutation assays in Chinese hamster V79 cells (Table 28). DB[*a,c*]A without exogenous metabolic activation did not induce SCEs in Chinese hamster ovary cells at concentrations up to 14.4 µM (Table 28), but three DB[*a,c*]A diol metabolites induced SCEs in Chinese hamster ovary cells under the same test conditions (Table 29). In addition, two DB[*a,c*]A metabolites (unspecified diol epoxide and DB[*a,c*]A-10,11-diol-12,13-epoxide), formed DNA adducts in human placenta DNA or salmon sperm DNA (Table 29).

Table 28. Cell-free and *in vitro* genotoxicity tests of DB[*a,c*]A

Endpoint	System/ cell type	Exogenous metabolic activation[1]	Dose/ Concentration	Result	Reference
DNA binding	DNA extracted from salmon testes	Rat liver microsomes	5 µg	+[2]	Grover and Sims (1968)
	Mouse embryo cells	None	0.79 or 0.94 µM	+[3]	Duncan and Brookes (1972)
		None	4.3 µM	+[3]	Duncan *et al.* (1969)
	C3H mouse embryonic cells	None	3.6 µM	+[3]	Kuroki and Heidelberger (1971)
	hamster embryonic cells			+[3]	
	C3H mouse prostate fibroblast cells (G23)			+[3]	
DNA adducts	Calf thymus DNA	DB[*a,c*]A induced CD-1 mouse epidermal homogenates[4]	100 nmol/ 0.2 ml (500 µM)	+	Buty *et al.* (1976)
			36 nmol/3 ml (12 µM)	+	Slaga *et al.* (1977)
			1 mM (200 nmol in 0.2 ml acetone)	+	Slaga *et al.* (1978)
	Salmon sperm DNA	3-MC mouse liver S9	6 µM	+	Levitt *et al.* (1979)
	Hamster embryo cells	None	3.6 µM (1 µg/ml)	+	Hewer *et al.* (1981)
	Salmon sperm DNA	3-MC rat liver S9	2 mg	+	
	Calf thymus DNA (in the dark, and under white light)	None	0.12 - 600 nmol	+	Bryla and Weyand (1992)

Table 28 (continued). Cell-free and *in vitro* genotoxicity tests of DB[*a,c*]A

Endpoint	System/ cell type	Exogenous metabolic activation[1]	Dose/ Concentration	Result	Reference
DNA bases/ nucleoside binding	Adenine, guanosine, thymine, cytosine, 5-methyl-cytosine	None	1 mM	+	Sharifian *et al.* (1985)
Unscheduled DNA Synthesis	HeLa cells	3-MC rat liver S9	0.1 - 100 µM	+	Martin *et al.* (1978)
	Primary rat hepatocytes	None	50 - 1000 µM	+	Probst *et al.* (1981)
Forward mutation (ouabain and 8-azaguanine resistance)	Chinese hamster V79 cells	Lethally irradiated Golden hamster embryo cells	3.6 µM (1 µg/ml)	+	Huberman and Sachs (1976)
Forward mutation (6-thio-guanine resistance)	Chinese hamster V79 cells	3-MC rat liver S9	up to 71.5 µM	+	Krahn and Heidelberger (1977)
SCEs	CHO cells	None	up to 14.4 µM (4 µg/ml)	–	Pal (1981)

[1] 3-MC: 3-methylcholanthrene-induced
[2] In addition to DNA binding, protein binding was also observed in an analogous experimental system under the same experimental conditions.
[3] In addition to DNA binding, RNA binding and protein binding were also observed.
[4] Epidermal homogenates were prepared by the protocol in Slaga *et al.* (1974) using mild-heat and DB[*a,c*]A pre-treatment to enhance AHH enzyme activity up to 7 times higher compared to uninduced levels.

Table 29. Cell-free and *in vitro* genotoxicity tests of DB[*a,c*]A metabolites

DB[*a,c*]A metabolites	Endpoint	System/ cell type	Dose/ concentration	Result	Reference
***anti*-DB[*a,c*]A-10,11-diol-12,13-epoxide**	DNA adducts	Salmon sperm DNA	0.5 µmol /50 µl DMSO	+	Hall *et al.* (1988)
DB[*a,c*]A diol epoxide[1]	DNA adducts	Human placenta DNA	1 mg	+	Weston *et al.* (1989)
DB[*a,c*]A-*trans*-1,2-diol	SCEs	Chinese hamster ovary cells	up to 4 µg/ml	+	Pal (1981)
DB[*a,c*]A-*trans*-3,4-diol			up to 8 µg/ml	+	
DB[*a,c*]A-*trans*-10,11-diol			up to 4 µg/ml	+	

[1] Specific form of diol epoxide not identified in the paper.

DB[*a,j*]A formed DNA adducts in mouse keratinocytes and induced forward mutation in human lymphoblastoid cells (h1A1v2) (Table 30). The h1A1v2 cell line constitutively expresses cytochrome P450 1A1, which is known to be necessary for the metabolism of DB[*a,j*]A.

Table 30. DB[*a,j*]A *in vitro* mammalian genotoxicity test results

Endpoint	System/cell types	Concentration	Result	Reference
DNA adducts	SENCAR mouse keratinocytes	1.8 µM (0.5 µg/ml)	+	Nair *et al.* (1991)
Forward mutation	Human B-lymphoblastoid cells (h1A1v2)	0.036 - 3.6 µM (10 - 1000 ng/ml)	+	Durant *et al.* (1996)

A diol epoxide metabolite of DB[*a,j*]A, DB[*a,j*]A-3,4-diol-1,2-epoxide, has been shown to form DNA adducts in calf thymus DNA (Chadha *et al.*, 1989; Nair *et al.*, 1989) (Table 31). In the studies by Chadha *et al.* (1989), the stereoselectivity of the four enantiomers of DB[*a,j*]A-3,4-diol-1,2-epoxide in forming DNA adducts was assessed. In this cell-free system, the [R,S,S,R]-isomer was shown to be the most DNA reactive.

Table 31. Cell-free genotoxicity tests of DB[*a,j*]A metabolites

Endpoint	DB[*a,j*]A metabolites	System/ cell types	Dose	Result	Reference
DNA adducts	DB[*a,j*]A-(+)-(4S,3R)-diol-(2S,1R)-epoxide	Calf thymus DNA	4 - 5 mg	+	Chadha *et al.* (1989)
	DB[*a,j*]A-(-)-(4R,3S)-diol-(2R,1S)-epoxide			+	
	DB[*a,j*]A-(-)-(4S,3R)-diol-(2R,1S)-epoxide			+	
	DB[*a,j*]A-(+)-(4R,3S)-diol-(2S,1R)-epoxide			+	
	trans-DB[*a,j*]-3,4-diol-*anti*-1,2-epoxide	^{3}H-labelled Calf thymus DNA	0.1 mg	+	Nair *et al.* (1989)

In vivo genotoxicity

In vivo genotoxicity assay data for DB[*a,c*]A, DB[*a,j*]A, and their metabolites are summarized in Tables 32 - 34. Data regarding the ability of DB[*a,c*]A to bind nonspecifically to mouse epidermis DNA and to form adducts with mouse epidermis DNA was mixed. DB[*a,c*]A was positive in a mouse micronucleus assay and increased K-*ras* mutations in mouse liver tumors at codon 13 but not H-*ras* mutations in mouse liver tumors at codon 61. DB[*a,c*]A induced somatic mutations in a *Drosophila melanogaster* insecticide-resistant (IR) phenotype, but not in the insecticide-susceptible (IS) or (IS x IR) phenotypes (Table 32).

Table 32. *In vivo* DB[*a,c*]A genotoxicity studies

Endpoint	Test system (sex/strain/ species/tissue)	Dose	Route	Result	Reference
DNA binding	Swiss or BRO mouse skin	0.1-1 µmol	Topical application	–[1]	Brookes and Lawley (1964)
	Female Swiss albino mouse epidermis and dermis	0.1-0.15 mg		+	Goshman and Heidelberger (1967)
DNA adducts	Male C57BL mouse epidermis	1 µmol	Topical application	+	Phillips *et al.* (1979)
	Mouse skin	1 µmol (in 0.15 ml acetone)		–	Hewer *et al.* (1981)
Somatic mutation (mosaic assay, eye test)	Insecticide-resistant (IR) *D. melanogaster*[2]	1 - 4 mM	Diet	+[3]	Rodriguez-Arnaiz and Tellez (2002)
	Insecticide-susceptible (IS) *D. melanogaster*[2]			–	
	IS x IR *D. melanogaster*[2]			–	
K-*ras* (codon 13) mutation	Neonatal B6C3F$_1$ mouse liver tumors	400 nmol	Intraperitoneal injection	+	Von Tungeln *et al.* (1999)
H-*ras* (codon 61) mutation				–	
Micro-nucleus	Male HOS:HR-1 hairless mouse dorsal skin	400 - 2000 µg/day for three days	Topical treatment	+	Nishikawa *et al.* (2005)

[1] Per authors, "The binding of DB[*a,c*]A to DNA was very low, and the specific radioactivity was below the level of one-half of the background count per 100 µg DNA, which was considered to be a significant level."
[2] *Drosophila melanogaster*, white/white+ (w/w+)
[3] Increased mutations were observed at three concentrations (1, 2 and 4 mM), but the increase was statistically significant only at 2 mM.

As shown in Table 33, DB[*a,j*]A induced DNA adducts in the epidermis cells of female SENCAR mice and mutations in the proto-oncogene Ha-*ras* in SENCAR mouse skin papillomas.

Table 33. *In vivo* genotoxicity studies of DB[*a,j*]A in mice exposed by topical application

Endpoint	Test system (sex/strain/ species)	Tissue	Dose	Result	Reference
DNA adducts	Female SENCAR mice	Epidermis	400 - 1600 nmol	+	Baer-Dubowska *et al.* (1995)
			400 - 1200 nmol	+	Vulimiri *et al.* (1999)
c-Ha-*ras* oncogene mutation ($A^{182} \rightarrow T$ transversion)	Proto-oncogene (Ha-*ras* codon 61) in female SENCAR mice	Skin papilloma	400 nmol	+[1]	Gill *et al.* (1992)

[1] 3.4 nmol TPA was used as a promoter

The findings from *in vivo* genotoxicity studies of several DB[*a,j*]A metabolites are presented in Table 34. Several DB[*a,j*]A metabolites formed DNA adducts in the skin of SENCAR mice and one metabolite (*anti*-DB[*a,j*]A-3,4-diol-1,2-epoxide) induced mutations in codon 61 of c-Ha-*ras* in SENCAR mice, as assessed in skin papillomas following dermal exposure.

Table 34. *In vivo* genotoxicity studies of DB[*a,j*]A metabolites in mice exposed by topical application

Endpoint / Chemicals	Test system (sex/strain/ species)	Tissue	Dose	Result	Reference
DNA adducts					
DB[*a,j*]A-*trans*-3,4-diol	Female SENCAR mice	Epidermis	400 - 1600 nmol	+	Baer-Dubowska *et al.* (1995)
***Syn/anti*-DB[*a,j*]A-3,4-diol-1,2-epoxide**				+	
DB[*a,j*]A-5,6-epoxide				+	
DB[*a,j*]A-3,4-diol	Female SENCAR mice	Epidermis	400 - 1200 nmol	+	Vulimiri *et al.* (1999)
DB[*a,j*]A-3,4-10,11-*bis*-diol			1600 nmol	+	
DB[*a,j*]A-3,4-8,9-*bis*-diol			1600 nmol	+	
10-OH-DB[*a,j*]A-3,4-diol			1600 nmol	–	
11-OH-DB[*a,j*]A-3,4-diol			1600 nmol	+	
***Anti*-DB[*a,j*]A-3,4-diol-1,2-epoxide**			400 nmol	+	
***Syn*-DB[*a,j*]A-3,4-diol-1,2-epoxide**			400 nmol	+	
c-Ha-*ras* oncogene mutation ($A^{182} \rightarrow T$ transversion)					
***Anti*-DB[*a,j*]A-3,4-diol-1,2-epoxide**	Proto-oncogene (Ha-*ras* codon 61) in female SENCAR mice	Skin papilloma	400 nmol	+[1]	Gill *et al.* (1992)

[1] 3.4 nmol TPA was used as a promoter

Summary of Genotoxicity Data for DB[*a,c*]A and DB[*a,j*]A

In summary, DB[*a,c*]A induced:

- DNA damage and gene mutations in bacteria
- Somatic mutations in *Drosophila melanogaster*
- DNA binding and DNA adducts in cell-free systems
- DNA binding, DNA adducts, mutations, and UDS in mammalian assay systems *in vitro*
- DNA binding, DNA adducts, *ras* gene mutations, and micronuclei in mice *in vivo*

DB[*a,c*]A metabolites induced:

- Bacterial gene mutation
- DNA adducts in cell-free systems
- *In vitro* chromosomal damage (SCEs)

DB[*a,j*]A induced:

- Bacterial gene mutations
- DNA adducts and gene mutation in mammalian assay systems *in vitro*
- DNA adducts in mice *in vivo*
- Mutations in *ras* oncogenes in mice *in vivo*

DB[*a,j*]A metabolites induced:

- DNA adducts in cell-free systems
- DNA adducts in mice *in vivo*
- Mutations in *ras* oncogenes in mice *in vivo*

3.3.2 In Vitro Cell Transformation Studies

Cell transformation assays are designed to detect a change in growth pattern of cells that is indicative of loss of contact inhibition, a phenotype that is characteristic of cancer cells. In general, there is a reasonably good correlation between the results of the cell transformation system and *in vivo* carcinogenesis in rodents (Creton *et al.*, 2012; Heidelberger *et al.*, 1983). DB[*a,h*]A and DB[*a,c*]A were tested in *in vitro* transformation studies.

DB[*a,h*]A tested positive at concentrations greater than 10 µg/ml in cell transformation assays in Syrian hamster embryo cells, mouse embryo fibroblast C3H10T½ cells, and mouse prostate C3H cells (US EPA, 1994). DB[*a,c*]A (up to 5 µg/ml) tested negative in a malignant transformation assay using prostate ventral cells from C3H mice in culture

(Chen and Heidelberger, 1969). DB[*a,c*]A also did not induce cell transformation in Swiss mouse embryo cells infected with AKR leukemia virus (Rhim *et al.*, 1974). However, DB[*a,c*]A was shown to induce morphological cell transformation in Syrian hamster embryo cells in two studies (DiPaolo *et al.*, 1969; Pienta, 1980; Table 35). An earlier study by Pienta *et al.* (1977) reported negative results in Syrian hamster embryo cells. This may have been due to the lower DB[*a,c*]A concentrations applied (up to 5 µg/ml) compared to the concentrations used in the later Pienta (1980) study (up to 50 µg/ml). Casto (1973) showed that adding DB[*a,c*]A (0.5 to 10 µg/ml) 18 hours prior to oncogenic simian adenovirus (SA7) inoculation increased the frequency of the viral transformation in hamster embryo cells in a dose-dependent manner.

Table 35. Cell transformation studies of DB[*a,c*]A

Test system	Concentration	Result	Reference
Syrian hamster embryo cells	10 µg/ml	+	DiPaolo *et al.* (1969)
Mouse C3H prostate cells	1 - 5 µg/ml	–	Chen and Heidelberger (1969)
Syrian hamster embryo cells with adenovirus enhancement	0.5 - 10 µg/ml	+	Casto (1973)
Swiss mouse embryo cells with AKR leukemia viral enhancement	0.1 - 5 µg/ml	–	Rhim *et al.* (1974)
Syrian hamster embryo cells	0 - 5 µg/ml	–	Pienta *et al.* (1977)
Syrian hamster embryo cells	1 - 50 µg/ml	+	Pienta (1980)

3.3.3 Pre-neoplastic in vivo morphological changes

Topping *et al.* (1978) subcutaneously transplanted tracheas from male F344 rats into isogenic recipients (two tracheas/recipient). Four weeks later, beeswax pellet implants containing 1 mg of DB[*a,c*]A were implanted intraluminally into 30 transplanted tracheas. Controls received beeswax pellets without DB[*a,c*]A. Transplanted tracheas were removed at 3 days, and 1, 2, 4 and 8 weeks after treatment initiation and evaluated histologically. DB[*a,c*]A induced epithelial hyperplasia and squamous metaplasia in the tracheal transplants. The epithelial hyperplasia was described as being “strong and widespread.” The amount of transitional epithelium (stratified hyperplastic lesions without definitive differentiation) observed increased over the eight-week treatment period. At eight weeks, about 60% of the transitional epithelium was irregular, pleomorphic and almost completely undifferentiated. Marked mononuclear

infiltration was observed in the sub-epithelial connective tissue. The authors stated, "The most striking feature of the response of tracheal tissues to carcinogens, which seems to distinguish it from the response to noncarcinogens, is the chronicity of the tissue damage", and that DB[*a,c*]A caused "severe and long-lasting epithelial and submucosal pathologic changes."

3.3.4 Pharmacokinetics and metabolism

The pharmacokinetics of DBAs have been studied *in vivo* in multiple species and multiple *in vitro* systems. The *in vivo* pharmacokinetic studies have employed various routes of administration, including dermal application, gavage, *s.c.*, *i.p.*, *i.v.*, and *i.m.* injection, and have been conducted in mice, rats and rabbits. The *in vitro* metabolism studies have been conducted using rat or human liver microsomes and mouse keratinocytes. The pharmacokinetics of DB[*a,h*]A has been studied more extensively than the other two DBA isomers.

An overview of the absorption, distribution, metabolism and excretion of PAHs in humans and animals has been summarized by IARC (2010, Section 4.1, pp. 512-538, and Section 5.4, pp. 765-771, Attachment 2). IARC (2010) also summarizes information specific to the metabolism of DB[*a,h*]A (pp. 587-594, Attachment 2) and DB[*a,j*]A (pp. 594-597, Attachment 2). Data on the metabolism of DB[*a,c*]A was not reviewed by IARC (2010).

Information specific to the absorption, distribution and excretion of the DBAs is presented below, as this information was not included in the IARC review (IARC, 2010). Also presented below:

- A brief overview of PAH metabolism, based primarily on the 2010 IARC review
- A brief summary of DB[*a,h*]A metabolism, based in part on the 2010 IARC review
- A brief summary of DB[*a,c*]A metabolism studies, none of which were reviewed by IARC (2010)
- A brief summary of DB[*a,j*]A metabolism, including studies not reviewed by IARC (2010)

Absorption and distribution

PAHs are absorbed by the skin, respiratory tract and gastrointestinal tract due to their lipophilicity. PAHs with five rings, such as the DBAs, are absorbed less rapidly than those with four or fewer rings (IARC, 2010). Here we briefly review the absorption and distribution studies of DB[*a,h*]A conducted in mice and rats. No absorption or distribution studies were identified for DB[*a,c*]A and DB[*a,j*]A. The absorption and

distribution of each of the three isomers, however, is expected to be similar, based on the similarity of the Log K_{ow} values reported for all three isomers (see Table 1).

Heidelberger and Weiss (1951) administered ^{14}C-labelled DB[*a,h*]A to female Rockland mice by either *s.c.* injection or dermal application. The authors reported that a small amount of DB[*a,h*]A was absorbed through the skin following dermal application. Twenty days after a single *s.c.* injection, over 87% of the radioactivity remained in the subcutaneous tissues. The estimated half-life of DB[*a,h*]A following *s.c.* injection or dermal application to female mice was about 12 weeks (Heidelberger and Weiss, 1951).

Heidelberger and Jones (1948) reported the distribution of ^{14}C-labelled DB[*a,h*]A in mice (unspecified strain) by *i.v.* injection, gavage and *i.p.* injection:

- By *i.v.* injection, there was extremely rapid initial uptake of DB[*a,h*]A by the liver (89%), spleen (1.9%), intestinal contents and feces (1.9%) and red blood cells (1%).
    - At 24 hours, DB[*a,h*]A was distributed mainly in the gastrointestinal (GI) tract (88.7%), feces (5.6%), liver (3.5%), lung (0.4%), urine (0.3%) and blood plasma (0.3%).
    - At 48 hours, the distribution changed to the following order: feces (40%), GI tract (14.6%), liver (11.4%), urine (7.8%), “mammary carcinoma” (4.8%, in a mouse with an existing large mammary carcinoma), heart (2.0%), salivary glands (0.9%), kidney (0.6%) and blood plasma (0.6%).
- By gavage, DB[*a,h*]A was distributed mainly to the stomach and intestines (range: 33 - 53%) after 24 hours.
- By *i.p.* injection, DB[*a,h*]A was distributed as follows after seven days: peritoneal cavity (11.8%), “carcass” (all remaining parts of the mouse not otherwise reported, 9.3%), intestines (5.6%), liver (4.5%), and excreted in feces (74%) and urine (3.9%).

Distribution studies of radiolabelled DB[*a,h*]A administered by gavage to Sprague-Dawley rats showed that after 24 hours the highest levels of radioactivity were found in the liver, followed by adrenals, ovaries, blood and fat (Daniel *et al.*, 1967). In the same study, the blood plasma concentration of radiolabelled DB[*a,h*]A peaked within 10 hours of administration and then decreased over a 72-hour period.

Metabolism

The general metabolism of PAHs is described in IARC (2010, Section 4.1, p. 512, pp. 518-538; Section 4.2, pp. 538-542, pp. 623-632, p. 637; Section 5.4, pp. 765-769,

Attachment 2), and briefly discussed here. The liver is a primary site of metabolic activation; however, many other tissues, including epithelial tissues, are capable of metabolizing PAHs. PAHs are metabolized by a number of different Phase I and Phase II enzymes and pathways. Phase I enzymes (*e.g.*, cytochrome P450s [CYP450s]) and peroxidases metabolize PAHs to form phenols and epoxides. Spontaneous aromatization of epoxides can also yield phenols (Platt and Reischmann, 1987). Phenols can be oxidized to more toxic metabolites such as phenol epoxides, phenoxy radicals, semiquinones and quinones. Epoxides are hydrolyzed to diols by epoxide hydrolase. Diols may then undergo oxidization by CYP450s to diol epoxides, which in turn can form highly reactive carbonium ions. Some diols (*i.e.*, non-K region diols) may undergo oxidation by aldo-keto reductases to form *ortho*-quinones. Quinones may in turn undergo redox cycling (*e.g.*, reduction by nicotinamide adenine dinucleotide phosphate:quinone oxidoreductase 1 [NQO1] to hydroquinone, which can be re-oxidized), generating reactive oxygen species (ROS). In addition to the above pathways, PAHs may also undergo one-electron oxidation catalyzed by CYP450s or peroxidases to form radical cations. A number of these PAH metabolites are highly reactive, and have the potential to form DNA adducts or otherwise damage DNA, including diols, diol epoxides, radical cations, quinones and ROS.

Phase II enzymes involved in the conjugation of PAH metabolites with glutathione, glucuronic acids, and sulfates include glutathione *S*-transferases, uridine 5'-diphosphate glucuronosyltransferases and sulfotransferases (IARC, 2010).

Information specific to the metabolism of the individual DBAs is discussed below.

DB[*a,h*]A

A number of cytochrome P450s metabolize DB[*a,h*]A, including CYP 1A1, 1A2, 3A3, 3A4, 3A5, 1B1, 2B6, 2C9 and 2E1 (IARC, 2010; Shimada *et al.*, 2003; Shimada and Guengerich, 2006).

In summarizing the metabolism of DB[*a,h*]A, IARC (2010) noted that more than 30 DB[*a,h*]A metabolites have been identified, and provided a partial listing of these. Additional metabolites not specifically identified by IARC (2010) include three quinone metabolites observed *in vivo* in mouse liver or skin: DB[*a,h*]A-5,6-quinone, DB[*a,h*]A-

7,14-quinone and 4,11-dihydroxy-DB[*a,h*]A-7,14-quinone (Heidelberger *et al.*, 1953[4]). Table 36 below lists several of the DB[*a,h*]A metabolites, and Figure 2 presents the structures of several confirmed and one proposed metabolite (10,11-diol-12,13-epoxide).

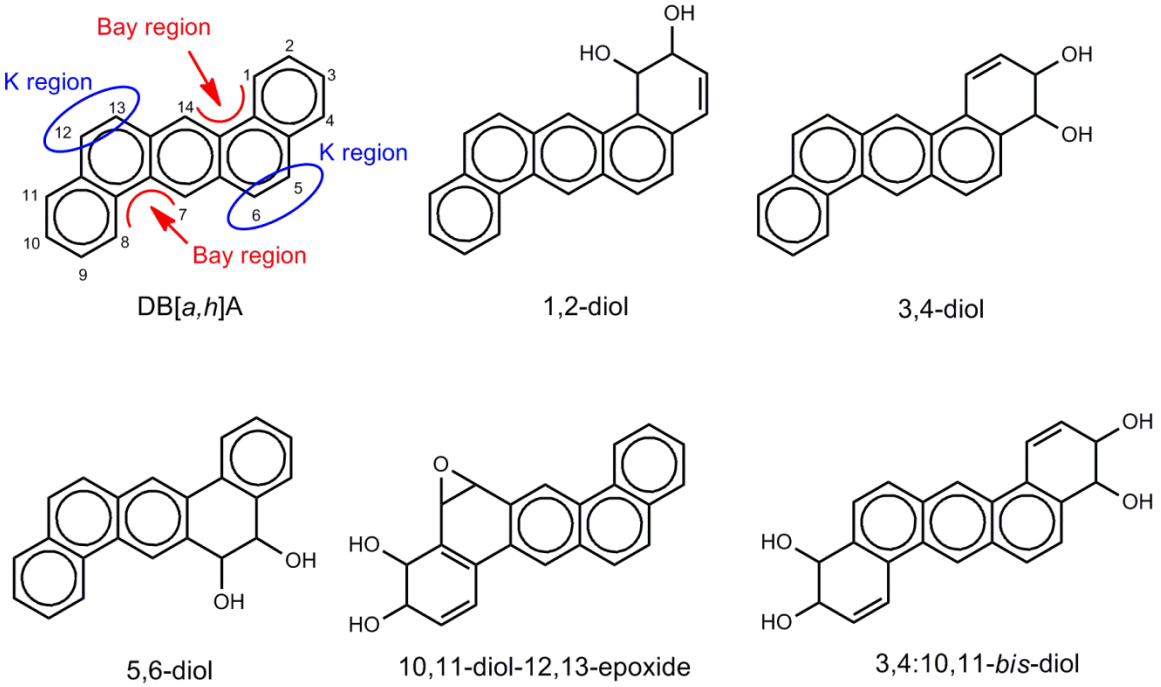


Figure 2. DB[*a,h*]A and some of its metabolites

[4]The naming scheme in this paper was different from the current rules which were established by IUPAC in 1966 (International Union of Pure and Applied Chemistry: Nomenclature of Organic Chemistry. Butterworths, London): DB[*a,h*]A-5,6-quinone was referred by Heidelberger *et al.* as "DB[*a,h*]A-3,4-anthraquinone"; DB[*a,h*]A-7,14-quinone was referred as "DB[*a,h*]A-9,10-anthraquinone"; 4,11-dihydroxy-DB[*a,h*]A-7,14-quinone was referred as "4'8'-dihydroxy-dibenz-9,10-anthraquinone".

Table 36. Summary of DB[*a,h*]A metabolites

Metabolite	Reference	Notes
DB[*a,h*]A-1,2-diol*	MacNicoll *et al*. (1979; 1980); Mushtaq *et al.* (1989); Nordqvist *et al.* (1979); Platt and Reischmann (1987)	
DB[*a,h*]A-3,4-diol*	MacNicoll *et al*. (1979; 1980); Mushtaq *et al.* (1989); Nordqvist *et al.* (1979)	Major diol metabolite (24-28%) in rat liver microsomal system
DB[*a,h*]A-5,6-diol*	MacNicoll *et al*. (1979; 1980); Mushtaq *et al.* (1989); Noor Mohammad (1986)	K-region metabolite (trace)
DB[*a,h*]A-5,6-epoxide*	Mushtaq *et al.* (1989)	K-region metabolite
DB[*a,h*]A-1,2,3,4-tetrols*	Platt and Reischmann (1987); Lecoq *et al*. (1989; 1991)	Metabolized from 3,4-diol
DB[*a,h*]A-3,4-diol-1,2-epoxide*	Noor Mohammad (1986); Nesnow *et al.* (1994); Chang *et al.* (2013)	Bay-region diol epoxide
DB[*a,h*]A-3,4:10,11-*bis*-diol* (forming 3,4:10,11-*bis*-diol-1,2-epoxide, the proposed ultimate DNA binding intermediate)	Lecoq *et al.* (1991); Carmichael *et al.* (1993); Platt and Schollmeier (1994)	The most reactive *bis* diol, metabolized from 3,4-diol
DB[*a,h*]A-3,4:8,9-*bis*-diol*	Carmichael *et al.* (1993)	Metabolized from 3,4-diol
DB[*a,h*]A-3,4:12,13-*bis*-diol*	Carmichael *et al.* (1993); Platt and Schollmeier (1994)	Metabolized from 3,4-diol
DB[*a,h*]A-1,2,3,4,8,9-hexol*	Lecoq *et al.* (1991)	Proposed to form from 3,4:8,9-*bis*-diol (an unconfirmed metabolite)
DB[*a,h*]A-1,2,3,4,10,11-hexol*	Lecoq *et al.* (1991)	Proposed to form from 3,4:8,9-*bis*-diol (an unconfirmed metabolite) or 3,4:10,11-*bis*-diol
DB[*a,h*]A-3,4,8,9,10,11-hexol*	Carmichael *et al.* (1993)	
Phenols: 1-OH-DB[*a,h*]A, 2-OH-DB[*a,h*]A, 3-OH-DB[*a,h*]A, 4-OH-DB[*a,h*]A, 5-OH-DB[*a,h*]A, 6-OH-DB[*a,h*]A*	Platt and Reischmann (1987)	
DB[*a,h*]A-5,6-quinone	Heidelberger *et al.* (1953); Nordqvist *et al.* (1979)	May lead to radical cation and ROS formation
DB[*a,h*]A-7,14-quinone	Heidelberger *et al.* (1953); Nordqvist *et al.* (1979)	May lead to radical cation and ROS formation
4,11-dihydroxy-DB[*a,h*]A-7,14-quinone	Heidelberger *et al.* (1953)	May lead to radical cation and ROS formation

*Reported in IARC (2010)

Some of the metabolic pathways of DB[*a,h*]A are presented in Figure 3, showing the enzyme-mediated formation of diol epoxides, *bis* diol epoxides and carbonium ions. A metabolic scheme depicting the formation of quinones from PAHs (using benzo[*a*]pyrene as a model PAH) is included in Attachment 2 (Figure 4.1, p. 519).

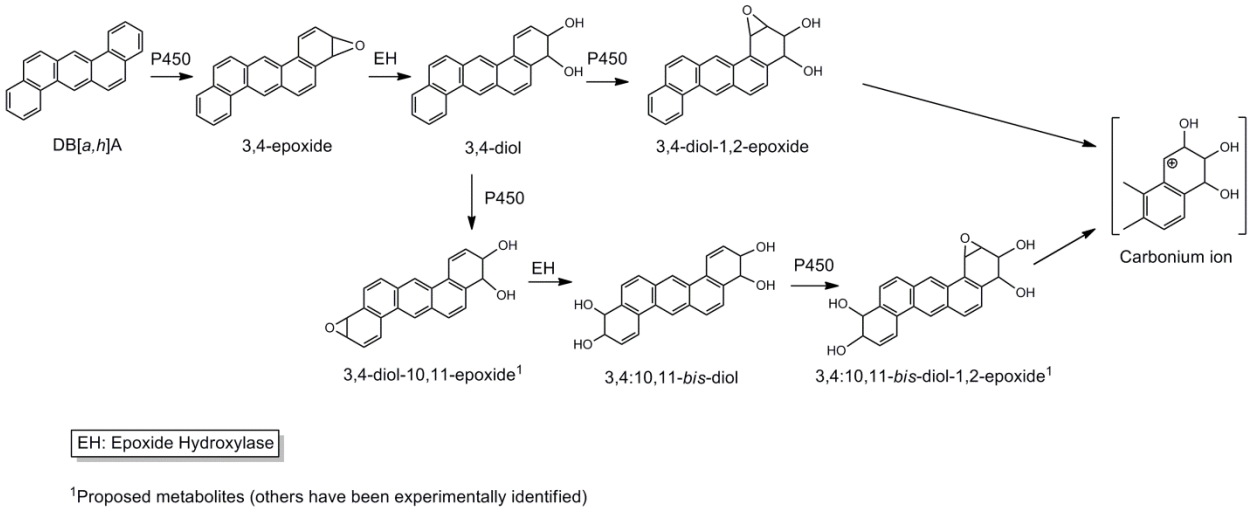


Figure 3. DB[*a,h*]A diol epoxide (Chang *et al.*, 2013), *bis* diol epoxide (Platt and Schollmeier, 1994) and carbonium ion formation (Flesher *et al.*, 2002).

DB[*a,c*]A

DB[*a,c*]A metabolism was not discussed in IARC (2010), but has been the subject of research. The results of the available studies on DB[*a,c*]A metabolism are briefly summarized below.

The following cytochrome P450s have been shown to metabolize DB[*a,c*]A: CYP 1A1, 1A2 and 1B1 (Shimada *et al.*, 2003; Shimada and Guengerich, 2006). Several metabolites of DB[*a,c*]A are shown in Figure 4. Three *trans*-diol metabolites were identified in studies using mouse skin cell cultures or rat liver microsomal systems: DB[*a,c*]A-10,11-diol (major metabolite; Sims, 1970; MacNicoll *et al.*, 1979), and DB[*a,c*]A-1,2-diol and DB[*a,c*]A-3,4-diol (both bay-region metabolites) (Chouroulinkov *et al.*, 1983). The DB[*a,c*]A-10,11-diol-12,13-epoxide was identified by Hewer *et al.* (1981), using a rat liver microsomal system. Also using a rat liver microsomal system, Lecoq *et al.* (1991) reported the formation of an additional DB[*a,c*]A metabolite, 1,2,3,4,12,13-hexol, and proposed that it may result from metabolism of the DB[*a,c*]A-12,13-diol to either the DB[*a,c*]A-1,2:12,13- or DB[*a,c*]A-3,4:12,13-*bis*-diol (the formation of these *bis* diol metabolites has not been confirmed experimentally). The formation of DB[*a,c*]A phenol metabolites has been observed in rat liver microsomal systems (Sims, 1972; MacNicoll *et al.*, 1979; Hewer *et al.*, 1984). In addition, evidence for non-

enzymatic activation of DB[*a,c*]A to DNA reactive species comes from studies by Bryla and Weyand (1992) (discussed in Section 3.3.1 Genotoxicity).

As reviewed in Section 3.3.1 Genotoxicity, several of the DB[*a,c*]A metabolites are genotoxic, inducing bacterial gene mutations, sister chromatid exchanges and DNA adducts *in vitro*.

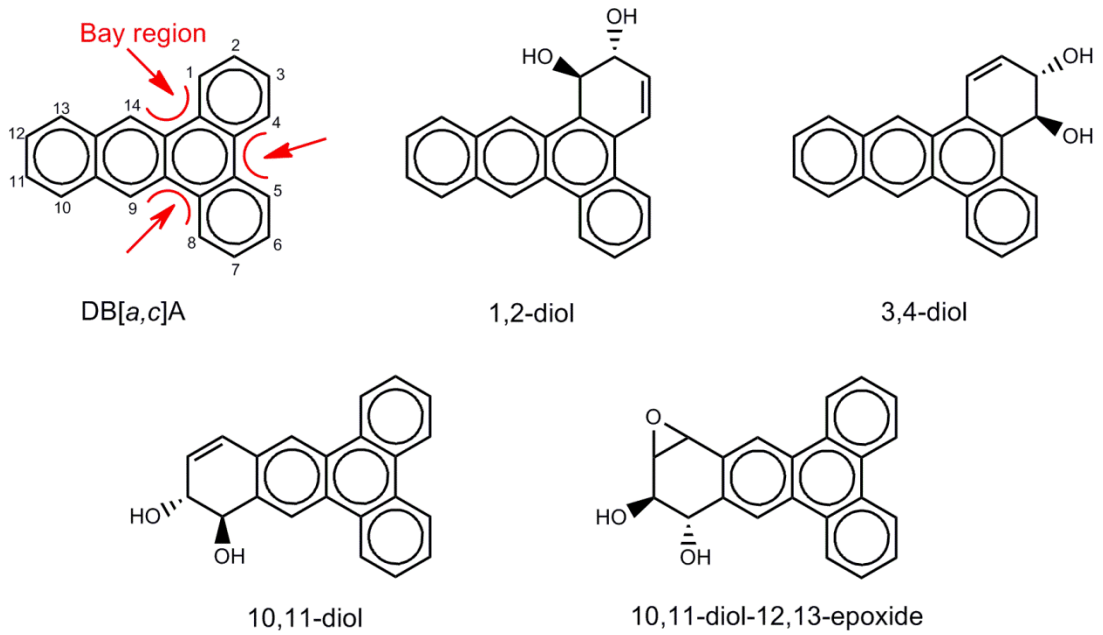


Figure 4. DB[*a,c*]A and some of its metabolites

DB[*a,j*]A

The following cytochrome P450s have been shown to metabolize DB[*a,j*]A: CYP 1A1, 1A2 and 1B1 (Shimada *et al.*, 2003; Shimada and Guengerich, 2006). In summarizing the metabolism of DB[*a,j*]A, IARC (2010) identified the following metabolites: DB[*a,j*]A-3,4-diol; DB[*a,j*]A-5,6-diol; DB[*a,j*]A-3,4-diol-1,2-epoxide (bay-region metabolite); 3,4:10,11-*bis*-diol; and 3,4:8,9-*bis*-diol (bay-region metabolite and the most reactive *bis* diol according to Lecoq *et al.*, 1991). Additional information not included in the IARC (2010) summary is discussed below.

DB[*a,j*]A-1,2,3,4-tetrahydrotetrol was identified as a metabolite in studies using rat liver microsomes (Lecoq *et al.*, 1989). DB[*a,j*]A-10,11-diol-12,13-epoxide was reported to be a metabolite by Noor Mohammad (1986), and Vulimiri *et al.* (1999) reported two more metabolites: 10-OH-DB[*a,j*]A-3,4-diol, and 11-OH-DB[*a,j*]A-3,4-diol. Several DB[*a,j*]A metabolites are shown in Figure 5.

Additional information on the metabolism of DB[*a,j*]A comes from studies conducted in primary mouse keratinocyte cultures (Nair *et al.*, 1992). The formation of several metabolites of DB[*a,j*]A was followed over the course of the 48-hour incubation period, including the 3,4- and 5,6-dihydrodiols, four unknown metabolites, and glucuronic acid conjugates of these metabolites. The 5,6-dihydrodiol was the metabolite present at the highest concentrations at 6, 12, and 24 hours, and the 3,4-dihydrodiol was the next most prevalent metabolite at those time points. By 48 hours approximately 70% of the total dose of DB[*a,j*]A added to the cell cultures had been metabolized. Excretion of metabolites into the extracellular medium, including those conjugated with glucuronic acid, increased over the 48-hour period. The concentrations of the 5,6- and 3,4-dihydrodiol metabolites decreased between 24 and 48 hours, while the concentration of one unknown metabolite (U_1) increased substantially, suggesting further metabolism of the dihydrodiols to other metabolites, including possibly U_1 (Nair *et al.*, 1992).

Similar to DB[*a,c*]A, several DB[*a,j*]A metabolites were genotoxic, inducing DNA adducts (both *in vitro* and *in vivo*) and increasing oncogene mutations in mouse skin papillomas (as reviewed in Section 3.3.1 Genotoxicity).

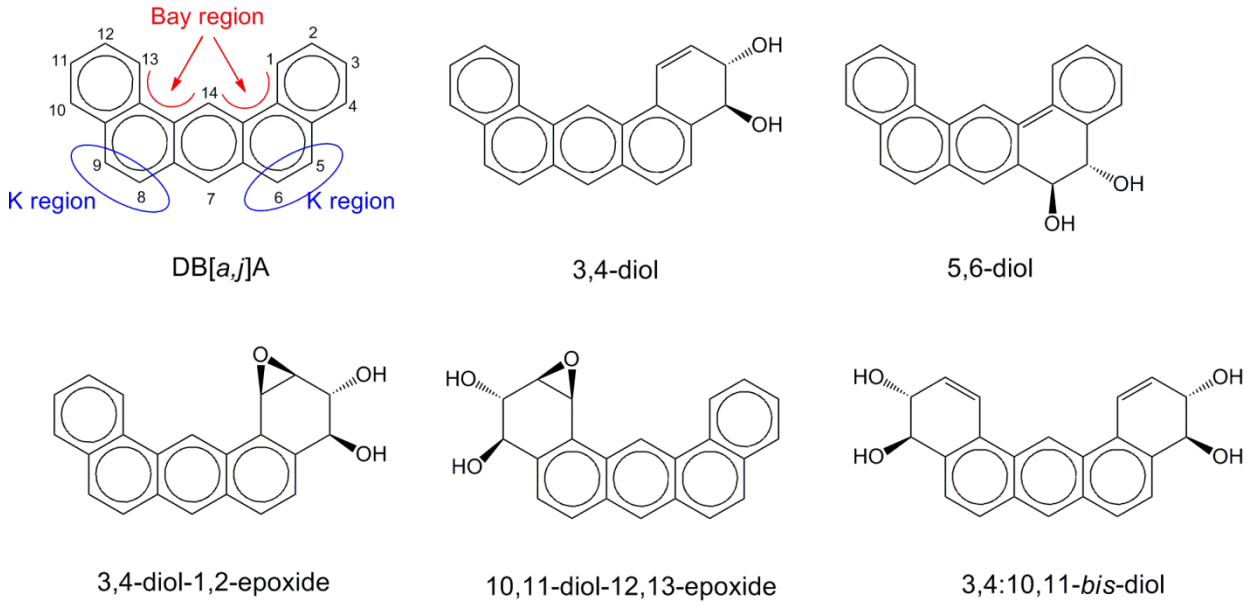


Figure 5. DB[*a,j*]A and some of its metabolites

Excretion

Direct information on excretion of the DBAs is available only for DB[*a,h*]A. Several elimination studies of DB[*a,h*]A have been reported in mice, rats and rabbits administered the compound by various routes of administration. In general, after absorption into systemic circulation, DB[*a,h*]A is excreted fairly quickly (within days), primarily in the feces, with some elimination via the urine.

DB[*a,h*]A was excreted in the form of phenolic derivatives in the urine and feces of rats, mice and rabbits administered the chemical through subcutaneous or intramuscular injections (Dobriner *et al.*, 1939). These phenolic metabolites were detected without quantification by spectroscopic analysis. Identical absorption bands were observed in the feces and urine of treated mice and rats, and these absorption bands differed from those observed in feces and urine from treated rabbits. Unchanged DB[*a,h*]A was identified in the feces of treated rats and mice, but rarely in that of treated rabbits. These results suggest that DB[*a,h*]A metabolism in the rabbit differs from that in mice and rats.

Heidelberger and Jones (1948) reported the main elimination route of DB[*a,h*]A when administered by gavage (41% at 24 hours; 79 - 83% at 48 hours), *i.p.* injection (78% at 7 days), or *i.v.* injection (48% at 48 hours) in mice was fecal excretion, with some minor excretion via the urine. Elimination in feces and urine was also reported (without quantification) following *s.c.* injection of mice; more rapid elimination in feces was observed during the first two days and then remained constant (Heidelberger and Jones, 1948). Boyland *et al.* (1941) reported that orally ingested DB[*a,h*]A in rats and rabbits was excreted in the feces and urine. Biliary excretion of DB[*a,h*]A in mice receiving *i.p.* (0.36% at 18 hours) or *i.v.* injections (53% at 24 hours) was demonstrated by Heidelberger and Jones (1948). Trace levels of radioactivity (< 0.5% of total dose) were excreted in expired air (as CO_2) in mice 24-48 hours after *i.v.* injection of ^{14}C-labelled DB[*a,h*]A (Heidelberger and Jones, 1948).

Summary of pharmacokinetics and metabolism

No information on absorption, distribution, or elimination is available for DB[*a,c*]A and DB[*a,j*]A. Evidence gathered from *in vivo* animal studies indicates that DB[*a,h*]A is absorbed into the systemic circulation very slowly following dermal application and *s.c.* injection in mice, and absorbed within hours of gavage administration in rats and mice. Once absorbed into the systemic circulation, DB[*a,h*]A is rapidly distributed within the body, with the highest levels measured in the GI tract and liver following gavage, *i.v.*, and *i.p.* administration to mice, and in the liver following gavage administration to rats.

Based on the similarity in Log K_{ow} values, similar absorption and distribution is expected for all three isomers.

The overall evidence from *in vivo* and *in vitro* studies suggests that multiple metabolic pathways involving cytochrome P450s and epoxide hydroxylase play a role in the metabolism and bioactivation of DBAs. More than 30 metabolites of DB[*a,h*]A have been identified in animal studies, cell cultures, and subcellular systems, including diols, *bis*-diols, diol epoxides, *bis*-diol epoxides, phenols, tetrols, hexols, and quinones. A number of similarly structured metabolites of DB[*a,c*]A and DB[*a,j*]A, including diols and diol epoxides, have been identified using cell cultures and subcellular systems.

DB[*a,h*]A is eliminated (within days) from the body, primarily as metabolites, following *i.v.*, *i.p.*, and gavage administration to rats and mice. DB[*a,h*]A and its metabolites are excreted into the bile, and the vast majority of the absorbed dose is eliminated in the feces, with minor amounts excreted in the urine, and one study reporting trace amounts exhaled in breath (as CO_2).

3.3.5 Animal Tumor Pathology

Information on the pathology of the tumor sites observed in animal cancer bioassays of DB[*a,h*]A (see Table 4) are not discussed in this section, since DB[*a,h*]A is already listed under Proposition 65 as causing cancer, and therefore evaluation of individual tumor findings in the DB[*a,h*]A bioassays is not the subject of this document. This section focuses on the pathology of tumor sites observed in the bioassays of DB[*a,c*]A and DB[*a,j*]A.

DB[*a,c*]A significantly increased the incidence of liver (hepatocellular) adenomas in neonatal male $B6C3F_1$ mice treated by *i.p.* injection (Von Tungeln *et al.*, 1999) and skin squamous cell carcinomas and combined papillomas and carcinomas in female Swiss mice treated by dermal application (Lijinsky *et al.*, 1970).

DB[*a,j*]A significantly increased the incidence of skin squamous cell carcinomas, and combined papillomas and carcinomas in female Swiss mice treated by dermal application. Following *s.c.* injection of DB[*a,j*]A three skin sarcomas were observed in female Swiss mice (Lijinsky *et al.*, 1970).

Skin papillomas were also observed in mice in multiple skin tumor initiation-promotion studies of DB[*a,c*]A (Van Duuren *et al.*, 1970; Scribner, 1970; Chouroulinkov *et al.*, 1983) and DB[*a,j*]A (DiGiovanni *et al.*, 1983; Sawyer *et al.*, 1987; 1988; Harvey *et al.*, 1988).

Skin tumors

Skin squamous cell papillomas frequently progress to squamous cell carcinomas (Bogovski, 1994) and are generally aggregated in evaluating carcinogenicity study results (McConnell *et al.*, 1986). Spontaneous skin tumors in mice are rare, in general not exceeding a 1% incidence (Bogovski, 1994).

Skin fibrosarcomas and sarcomas, rare in mice, are connective tissue tumors, whereas squamous cell adenomas and carcinomas are epidermal tumors (Bogovski, 1994).

Liver tumors

Hepatocellular adenomas may progress to carcinomas (Jang *et al.*, 1992; Frith *et al.*, 1994). No progression of hepatocellular adenomas to carcinomas was observed over the course of the DB[*a,c*]A study in mice by Von Tungeln *et al.* (1999); however, the short study duration (12 months) may not have been sufficiently long to observe such progression.

3.3.6 Structure-Activity Comparisons

DBAs are five-ring PAHs that contain an anthracene core and K and bay region structures. Many PAHs are carcinogenic, including several 4-, 5-, and 6-ring PAHs. For example, a total of 25 PAHs containing 4 to 6 aromatic rings are listed under Proposition 65 as causing cancer:

- The Proposition 65 listed four-ring PAHs are: benz[*a*]anthracene, chrysene, 7,12-dimethylbenz[*a*]anthracene, 3,7-dinitrofluoranthene, 3,9-dinitrofluoranthene, 1,3-dinitropyrene, 1,6-dinitropyrene, 1,8-dinitropyrene, 5-methylchrysene, 6-nitrochrysene, 1-nitropyrene, 4-nitropyrene.
- The Proposition 65 listed five-ring PAHs are: benzo[*b*]fluoranthene, benzo[*j*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, cyclopenta[*cd*]pyrene, dibenz[*a,h*]anthracene, 7H-dibenzo[*c,g*]carbazole, 3-methylcholanthrene.
- The Proposition 65 listed six-ring PAHs are: dibenzo[*a,e*]pyrene, dibenzo[*a,h*]pyrene, dibenzo[*a,i*]pyrene, dibenzo[*a,l*]pyrene, indeno[1,2,3-*cd*]pyrene.

In this section the DBAs are compared to six structurally similar carcinogenic PAHs with regard to genotoxicity and skin tumor initiating activity of the parent compounds and their diol or diol epoxide metabolites, and with respect to the target tumor sites observed

in animal studies. Predictions by several quantitative structure activity relationship (QSAR) models of the carcinogenicity of DB[*a,c*]A and DB[*a,j*]A are also presented.

Comparisons with six structurally related PAHs

Table 37 compares the three DBA isomers to six structurally related non-substituted PAHs containing four to six aromatic rings. These comparison PAHs were selected based on the following characteristics:

- Presence of at least three aromatic rings in a linear configuration
- Presence of one or more bay region structures
- Data from carcinogenicity studies in animals available

The first comparison compound is benzo[*a*]pyrene with five benzene rings, the next is benz[*a*]anthracene, with four benzene rings, and the remaining four are dibenzo[*a,e*]pyrene, dibenzo[*a,h*]pyrene, dibenzo[*a,i*]pyrene and dibenzo[*a,l*]pyrene, each with six benzene rings. Each of the comparison PAHs induces tumors in animals, is on the Proposition 65 list as causing cancer, and is classified as carcinogenic (in either Group 1, 2A or 2B) by IARC (with the exception of dibenzo[*a,e*]pyrene).

As discussed in earlier sections of this document, each of the three DBAs are genotoxic in *in vitro* and *in vivo* assays, form DNA adducts, are skin tumor initiators, and are metabolized to genotoxic diol or diol epoxide metabolites that also are skin tumor initiators.

As described in more detail below, the six structurally related carcinogenic PAHs all:

- Are genotoxic in *in vivo* (except dibenzo[*a*,*e*]pyrene and dibenzo[*a,h*]pyrene) and *in vitro* assays
- Have diol or diol epoxide metabolites (except dibenzo[*a*,*e*]pyrene) that are
    - genotoxic
    - skin tumor initiators
    - induce tumors in animals

Benzo[*a*]pyrene (B[*a*]P) is an IARC Group 1 carcinogen with five benzene rings. It induces tumors in mice, rats, and hamsters (IARC, 2010), and is positive in a range of *in vitro* and *in vivo* genotoxicity assays (IARC, 1983). Studies of the stereoisomers and enantiomers of its diol epoxide metabolites indicate that the carcinogenic potential of B[*a*]P is activated through metabolic conversion to a bay region diol epoxide (Dipple *et al.*, 1984). Many of the metabolites, such as B[*a*]P-7,8-oxide, B[*a*]P-7,8-diol, and B[*a*]P-7,8-diol-9,10-epoxide, induce skin and lung tumors in mice and are positive for genotoxicity.

Benz[*a*]anthracene is an IARC Group 2B carcinogen with four benzene rings. It induces tumors in mice, rats, and hamsters, and is positive in several *in vivo* and *in vitro* genotoxicity assays (IARC, 1983; 2010). Benz[*a*]anthracene is metabolized via the diol epoxide pathway. Benz[*a*]anthracene-3,4-diol is genotoxic in mammalian cells and is active as a tumor initiator in mouse skin and as a pulmonary carcinogen in newborn mice (IARC, 2010).

Dibenzo[*a*,*e*]pyrene is an IARC Group 3 carcinogen with six benzene rings. It induces tumors in mice and is genotoxic in bacteria and mammalian cells. It is metabolized to a bay-region diol, dibenzo[*a*,e]pyrene-3,4-diol (IARC, 2010). No genotoxicity or carcinogenicity studies are available on this metabolite.

Dibenzo[*a,h*]pyrene is an IARC Group 2B carcinogen with six benzene rings. It induces tumors in mice and rats and is genotoxic in bacteria and mammalian cells. It is metabolized to a bay-region diol, dibenzo[*a,h*]pyrene-1,2-diol. Dibenzo[*a,h*]pyrene-1,2-diol and dibenzo[*a,h*]pyrene-1,2-diol-3,4-oxide are both genotoxic in bacteria and mammalian cells in culture, are skin tumor initiators, and induce pulmonary and hepatic tumors in mice (IARC, 2010).

Dibenzo[*a,i*]pyrene is an IARC Group 2B carcinogen with six benzene rings. It induces several types of tumors in mice, rats and hamsters and is a skin tumor initiator in mice. It is genotoxic in a number of *in vivo* and *in vitro* assays. Some of its metabolites (dibenzo[*a,i*]pyrene-3,4-diol and dibenzo[*a,i*]pyrene-3,4-diol-1,2-oxide) are mutagenic and induce tumors in mice (IARC, 2010).

Dibenzo[*a*,*l*]pyrene is an IARC Group 2A carcinogen with six benzene rings. The molecule contains one bay region and one fjord region, which is defined as an area that contains five carbons and four carbon-carbon bonds (similar to a bay region, but with one more aromatic rings at its periphery) (Carrell *et al.*, 1994). Dibenzo[*a*,*l*]pyrene induces tumors in mice, rats and hamsters. It is genotoxic in several *in vivo* and *in vitro* assays. Several of its metabolites (such as the dibenzo[*a*,*l*]pyrene-11,12-diol and the dibenzo[*a*,*l*]pyrene-11,12-diol-13,14-epoxide) via the diol epoxide pathway are mutagenic and induce tumors in mice and rats (IARC, 2010).

Table 37. DBAs and structurally-related PAHs

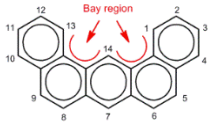
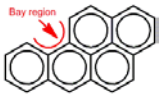
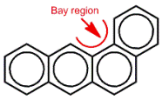
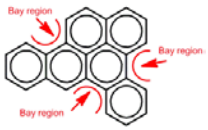
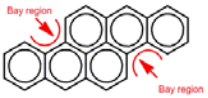
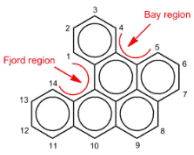
Chemical	Structure	Genotoxicity		Skin tumor initiator		Cancer Classification	
		Parent com-pound	Diol or diol epoxide meta-bolite(s)	Parent Com-pound	Diol or diol epoxide meta-bolite(s)	Proposition 65	IARC (2010)
5 rings							
DB[*a,h*]A		+	+	+	+	Listed	2A
DB[*a,c*]A		+	+	+	+	Currently under evaluation	3
DB[*a,j*]A		+	+	+	+	Currently under evaluation	3
Benzo[*a*]-pyrene		+	+	+	+	Listed	1
4 rings							
Benz[*a*]-anthracene		+	+	+	+	Listed	2B
6 rings							
Dibenzo-[*a,e*]pyrene		+	Not Tested	+	Not Tested	Listed	3
Dibenzo-[*a,h*]pyrene		+	+	+	+	Listed	2B
Dibenzo-[*a,i*]pyrene		+	+	+	+	Listed	2B
Dibenzo-[*a,l*]pyrene		+	+	+	+	Listed	2A

Table 38 compares the target tumor sites in rodents for the three DBAs and these six structurally-related PAHs. Although the available carcinogenesis studies of DB[*a,c*]A and DB[*a,j*]A are limited to studies in mice by the dermal, *s.c.*, or *i.p*. routes, Table 38 shows that skin tumors have been observed in mice treated with all three DBAs and the six structurally related PAHs. Other common tumor sites observed with DB[*a,h*]A and five of the structurally related PAHs include liver and lung.

Table 38. Comparison of target tumor sites in rodents for DBAs and structurally related PAHs (IARC, 2010)

Target tumor site / Chemical	Skin	Liver[1]	Lung[1]			Other sites[2]
Rodent species[2]	**M**	**M**	**M**	**R**	**H**	
5 rings						
DB[*a,h*]A	X	X	X	X	X	Haemangioendothelioma, mammary gland, small intestine, peritoneal, fibrosarcoma (M); sarcoma (M, R)
DB[*a,c*]A	X	X		NT	NT	
DB[*a,j*]A	X			NT	NT	
Benzo[*a*]pyrene	X	X	X	X	X	Tongue, lymphoma, lymphoreticular, anus, cervical (M); abdominal mesothelioma (R); sarcoma, mammary gland (M, R); small intestine, peritoneal (M, H); fibrosarcoma, larynx, esophagus, forestomach (M, R, H)
4 rings						
Benz[*a*]anthracene	X	X	X			Sarcoma, fibrosarcoma, bladder carcinoma (M)
6 rings						
Dibenzo[*a,e*]pyrene	X	NT	NT	NT	NT	Sarcoma (M)
Dibenzo[*a,i*]pyrene	X	X	X		X	Larynx, trachea (H); sarcoma (M); mammary gland (R); fibrosarcoma (M, R, H)
Dibenzo[*a,h*]pyrene	X	X	X		NT	Papilloma, sarcoma (M); fibrosarcoma, mammary gland (R)
Dibenzo[*a,l*]pyrene	X	X	X		NA	Oral cavity (H); ovary, uterus, lymphoma, sarcoma, fibrosarcoma (M); mammary gland, fibrosarcoma (R)

[1] NT, not tested; NA, not assessed- chemical tested for carcinogenicity but tissue not assessed
[2] Mouse (M), Rat (R), Hamster (H)

QSAR Predictions for DB[a,c]A and DB[a,j]A

Several QSAR models were used by OEHHA to predict the carcinogenicity of DB[*a,c*]A and DB[*a,j*]A. QSAR predictions were not made for the isomer DB[*a,h*]A because it is listed as a carcinogen on Proposition 65 and is included in the training sets of all QSAR

models used in this document. QSAR models predict the toxicity of chemicals by correlating physicochemical properties of related compounds to their biological activity quantitatively. Numerous QSAR models have been developed. The OECD (Organisation for Economic Co-operation and Development) and ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) guidelines were used to select QSAR models to generate predictions for DB[*a,c*]A and DB[*a,j*]A.

The models chosen for the analysis were the following:

- VEGA (a platform developed through a collaboration between the US EPA and a number of institutions from Europe under the sponsorship of ANTARES Project, CALEIDOS Project, CAESAR Project, Ministero della Salute, and ORCHESTRA Project) that contains three models:
    - CAESAR – a statistical-based system that predicts carcinogenicity toward male and female rats,
    - ToxTree – a rule-based system that predicts carcinogenicity toward humans, merging a series of rules from laboratory studies on different species, and
    - SarPy – a statistical-based model developed in Python (a general purpose programing language) to predict mutagenicity.
- Lazar, which was developed by a Swiss company (In silico toxicology GMBH, 2011) for predicting toxicological endpoints.
- QSAR Toolbox, which was developed by the Laboratory of Mathematical Chemistry with the scientific and financial assistance of OECD and the European Union. It groups chemicals into categories and fills gaps in toxicity data in order to assess chemical hazards.

In addition, predictions from two non-commercially-developed models published in the scientific literature are reported:

- Barone *et al.* (1996) developed a model using a rule-based methodology to correlate electronic properties with carcinogenicity for several PAHs.
- Vijayalakshmi and Suresh (2008) developed a model using estimates of the volume of molecular electrostatic potential at electron dense centers of the molecule to determine carcinogenic properties for several PAHs.

VEGA, Lazar, and QSAR Toolbox use carcinogenicity data from one or more of the following databases: the Distributed Structure-Searchable Toxicity (DSSTox) Public Database Network (US EPA, 2013a), the Carcinogen Potency Database (Gold *et al.*, 2011) and ISSCAN (Istituto di Sanità database on chemical carcinogens) (Benigni *et al.*, 2008a). Further details on the models used are provided in Appendix A.

QSAR modeling results

Summary results from these models are discussed below and are provided in Table 39.

Table 39. Summary of QSAR model results for DB[*a,c*]A and DB[*a,j*]A carcinogenicity

Model	DB[*a,c*]A prediction	DB[*a,j*]A prediction
VEGA ToxTree	+	+
VEGA CAESAR	+	+
Lazar	+	+
OECD QSAR Toolbox	+	+
Vijayalakshmi and Suresh (2008)	+	+
Barone *et al*. (1996)	+	±[1]

[1]±: equivocal (see discussion below on the results of Barone *et al.*, 1996)

VEGA

Results for DB[*a,c*]A and DB[*a,j*]A were positive for carcinogenicity for both the ToxTree and the CAESAR models in VEGA. The models calculate an applicability domain index, based on several different measures of reliability. The indices for all predictions were 0.948 or higher (1.0 is the best case). This indicates that the predictions are considered to be reliable because they were inside the model domain, *i.e.*, DB[*a,c*]A and DB[*a,j*]A are structurally similar to compounds in the dataset of the model (VEGA NIC v1.0.8, 2013).

Lazar

Lazar generated several positive predictions of carcinogenicity for both DB[*a,c*]A and DB[*a,j*]A. The predictions that DB[*a,c*]A and DB[*a,j*]A are carcinogenic are considered reliable based on adequate confidence for each prediction (In silico toxicology GMBH, 2011).

OECD QSAR Toolbox

The OECD QSAR Toolbox generated predictions for mouse carcinogenicity, which were positive for both DB[*a,c*]A and DB[*a,j*]A. The model gave a *p*-value that indicates the confidence of the results. *p*-Values associated with the modeling for each compound were less than 0.001, indicating that these compounds are similar to several nearest neighbors (*i.e.*, compounds that are most structurally similar to the test compound) and the predictions can be considered reliable (OECD QSAR Toolbox v. 3.2, 2013).

Published QSAR predictions based on DBA electron density

Additional QSAR models have been developed and are discussed in the literature. Two publications have reported predictive carcinogenicity results for DB[*a,c*]A and DB[*a,j*]A, using different approaches (Barone *et al.*, 1996; Vijayalakshmi and Suresh, 2008). Each model investigated a specific aspect related to the electronic properties of the molecule to determine the significance of different regions of PAHs, then used mathematical models to correlate those properties with carcinogenicity. Vijayalakshmi and Suresh (2008) incorporated information on molecular electrostatic potential at electron dense centers in the molecule to predict carcinogenicity of 28 PAHs. DB[*a,c*]A was predicted by the model to be a "mild" carcinogen (Vijayalakshmi and Suresh, 2008) since it does not have a K region. DB[*a,j*]A, like DB[*a,h*]A, has two bay regions and two K regions, and was predicted to be a strong carcinogen.

Barone *et al.* (1996) developed a rule-based approach using estimates of the local density of electronic states (LDOS) and the molecular energy difference between the highest occupied molecular orbital and the next lower level, and consideration of the peak concentration to the LDOS, to predict the carcinogenicity of 26 PAHs. DB[*a,c*]A was predicted to possess strong or moderate carcinogenic activity (Barone *et al.*, 1996). A prediction for DB[*a,j*]A was not able to be made. DB[*a,j*]A did not meet the criteria for strong or moderate carcinogenicity, nor did it meet the criteria for inactive or weak carcinogenicity, so the prediction was not clear (Barone *et al.*, 1996; Braga *et al.*, 2000).

Summary of Structure Activity Comparisons

There are numerous similarities in biologic activity between the three DBA isomers and the six structurally related non-substituted PAHs chosen for comparison (benzo[*a*]pyrene, benz[*a*]anthracene, dibenzo[*a*,*e*]pyrene, dibenzo[*a,i*]pyrene, dibenzo[*a*,*h*]pyrene, and dibenzo[*a*,*l*]pyrene). Each of these compounds has positive mutagenicity and carcinogenicity data. Each of these compounds induces skin tumors and is a skin tumor initiator in mice. All but DB[*a,j*]A and dibenzo[*a*,*e*]pyrene have been shown to induce liver tumors, and all but DB[*a,c*]A, DB[*a,j*]A, and dibenzo[*a*,*e*]pyrene have been shown to induce lung tumors[5]. In addition, all but dibenzo[*a*,*e*]pyrene has been shown to form genotoxic diol or diol epoxide metabolites that are skin tumor initiators. Additionally, DB[*a,c*]A and DB[*a,j*]A were predicted to be carcinogenic by

[5] The limited nature with respect to route of administration and study duration of the carcinogenicity studies performed to date with DB[*a,c*]A, DB[*a,j*]A, and dibenzo[*a*,*e*]pyrene raise concerns that the potential of these compounds to induce lung tumors (and liver tumors in the case of DB[*a,j*]A and dibenzo[*a*,*e*]pyrene) has not been adequately tested.

several QSAR models, including VEGA, Lazar, OECD QSAR Toolbox, and published results of Vijayalakshmi and Suresh (2008) and Barone *et al*. (1996) (DB[*a,c*]A only).

4. MECHANISMS

DBAs are likely to act through many of the same mechanisms proposed for other carcinogenic PAHs, such as benzo[*a*]pyrene (B[*a*]P), to induce tumors. These mechanisms involve metabolic activation to genotoxic species, AhR-mediated effects (including induction of xenobiotic metabolizing enzymes), other receptor-mediated effects, immunosuppression, and alterations in cell proliferation, apoptosis, and cell cycle regulation (IARC, 2010; see pp. 518-530, 538-541, 565-584, 587-597, 623-658, 765-771, Attachment 2). These mechanisms are briefly discussed below.

Metabolic activation to genotoxic species

The genotoxicity of DBAs (reviewed in Section 3.3.1) is dependent upon metabolic activation. As discussed in Section 3.3.4 (Pharmacokinetics and metabolism), DBAs are metabolized via a number of pathways to form several reactive electrophilic species, including phenols, epoxides, dihydrodiols, dihydrodiol epoxides, quinones, radical cations, and ROS.

One important metabolic pathway involves the generation of diol epoxides from the DBAs, which can yield carbonium ions to form DNA adducts and result in mutations (pp. 538-541, 590-597, 765-769, Attachment 2). There is a large body of evidence from animal and *in vitro* studies of the DBAs (reviewed in Sections 3.2.3, 3.3.1, 3.3.4, and 3.3.6, and Attachments 1 and 2) to support this genotoxic mechanism. This includes evidence that each of the DBA isomers is metabolized to diols and diol epoxides, and that diol or diol epoxide metabolites of each DBA isomer have been shown to form DNA adducts, induce mutations, and to be skin tumor initiators in the mouse.

A second metabolic pathway involves one-electron oxidation reactions catalyzed by peroxidases or CYP450s, to generate highly reactive radical cations. Radical cations form depurinating DNA adducts with guanines and adenines, which can result in mutations. These types of DNA adducts are strongly associated with the development of tumors (IARC, 2010, pp. 623-625, Attachment 2).

A third metabolic pathway involves the formation of *para*-quinones from DBAs. *para*-Quinones may bind directly to DNA, or undergo redox-cycling to generate ROS, which in turn may lead to oxidative DNA damage (Penning *et al.*, 1999; Martins *et al.*, 2013) (pp. 628-632, Attachment 2). *para*-Quinone metabolites of DB[*a,h*]A (*i.e*., DB[*a,h*]A-

7,14-quinone and 4,11-dihydroxy-DB[*a,h*]A-7,14-quinone) have been identified in rats (Nordqvist *et al.*, 1979; Heidelberger *et al.*, 1953).

Another metabolic activation pathway that may be operative is the formation of *ortho*-quinones from DBAs by the action of aldo-keto reductases that oxidize diols. Like the *para*-quinones, the *ortho*-quinones may bind to DNA directly and can also undergo redox-cycling to generate ROS. An *ortho*-quinone metabolite of DB[*a,h*]A, DB[*a,h*]A-5,6-quinone, has been identified in rats (Nordqvist *et al.*, 1979; Heidelberger *et al.*, 1953).

An additional activation pathway for the DBAs is light activation. One study reported that white light moderately increased the non-enzymatic activation of DB[*a,c*]A to DNA reactive species and formation of DNA adducts in calf thymus DNA (Bryla and Weyand, 1992) (see Section 3.3.1, Table 28).

Table 40 presents a summary of the genotoxicity, mouse skin tumor initiating activity, and animal tumorigenicity findings for each of the DBAs and several of their diol or diol epoxide metabolites.

Table 40. Summary of mutagenicity, skin tumor initiating activity, and tumorigenicity of DBAs and certain diol or diol epoxide metabolites

Chemical	Mutagenic[1]	Skin tumor initiator[1]	Carcinogenic[1]
DB[*a,h*]A[2]	+[3]	+	+
DB[*a,h*]A-1,2-diol	+	-[4]	-[4]
DB[*a,h*]A-3,4-diol	+	+	+
DB[*a,h*]A-5,6-diol	+[5]	-[4]	-[4]
DB[*a,h*]A-3,4-diol-1,2-epoxide	+[6]	+[7,8]	+[8]
DB[*a,c*]A[9]	+	+	+
DB[*a,c*]A-1,2-diol	+	+	NT
DB[*a,c*]A-3,4-diol	+	+	NT
DB[*a,c*]A-10,11-diol	+	+	NT
DB[*a,c*]A-10,11-diol-12,13-epoxide	+	-[7]	NT
DB[*a,j*]A[9]	+	+	+
DB[*a,j*]A-3,4-diol	+	+	NT
DB[*a,j*]A-5,6-diol	+	NT	NT
DB[*a,j*]A-1,2-diol-3,4-epoxide	+	NT	NT
DB[*a,j*]A-3,4-diol-1,2-epoxide	+	+	NT
DB[*a,j*]A-3,4:10,11-*bis*-diol	+	-[10]	NT

[1] +: tested positive; -: negative; NT: not tested.
[2] Unless otherwise indicated, data from IARC (2010)
[3] A dose-dependent increase in *lacZ* mutant frequency in hepatic DNA samples from DB[*a,h*]A-exposed transgenic mice (Muta™ Mouse) was observed (Malik *et al.*, 2013).
[4] Buening *et al.* (1979)
[5] Positive based on induction of UDS in HeLa cells by the metabolic precursor DB[*a,h*]A-5,6-epoxide (Martin *et al.*, 1978).
[6] The 3,4-diol-1,2-epoxide has been proposed as a probable intermediate carcinogen (Wood *et al.*, 1978; Pelkonen and Nebert, 1982; Lecoq *et al.*, 1991; Carmichael *et al.*, 1993; Nesnow *et al.*, 1994); formed DNA adducts in a dose-dependent manner in the liver of transgenic mice (Muta™ Mouse) *in vivo* (Malik *et al.*, 2013).
[7] Slaga *et al.* (1980)
[8] Chang *et al.* (2013)
[9] Unless otherwise indicated, data from Section 3 of this document and IARC (2010)
[10] Vulimiri *et al.* (1999)

Aryl hydrocarbon receptor (AhR)-mediated effects

DB[*a,h*]A and DB[*a,c*]A are high affinity aryl hydrocarbon receptor (AhR) ligands, with affinity comparable to, or greater than that of B[*a*]P (Piskorska-Pliszczynska *et al.*, 1986; Toftgard *et al.*, 1985). AhR receptor binding assays have not been reported for DB[*a,j*]A (Table 41). The AhR is widely distributed and has been detected in most cells and tissues. Activation of the AhR results in induction of the enzymes aryl hydrocarbon hydroxylase (AHH, also known as CYP 1A1), CYP 1A2 and CYP 1B1 (Shimada *et al.*, 2002). Several studies using either liver microsomal fractions, liver cells *in vitro* and rodents exposed *in vivo* have demonstrated that each of the three DBA isomers can induce the activity of CYP 1A1, CYP 1A2 and CYP 1B1 (Jones *et al.*, 2000; Roberts *et al.*, 1993; Shimada *et al.*, 2003; Skupinska *et al.*, 2007; Slaga and Boutwell, 1977). Data from AhR binding assays and AhR activation assays with the DBAs are summarized in Table 41.

Table 41. AhR: Receptor binding and activation by DBAs

Receptor binding			
Chemical	**Assay system**	**Affinity (EC_{50} or ratio to TCDD)**	**Reference**
DB[*a,h*]A	Rat liver cytosol[1]	0.025 µM	Kamps and Safe (1987)
		0.016 µM	Piskorska-Pliszczynska *et al.* (1986)
		0.006 µM	Toftgard *et al.* (1985)
		1.05 ± 0.22	Okey *et al.* (1984)
		0.97	Bigelow and Nebert (1982)
DB[*a,c*]A		0.053 µM	Kamps and Safe (1987)
		0.16 µM	Piskorska-Pliszczynska *et al.* (1986)
		0.006 µM	Toftgard *et al.* (1985)
		0.95	Bigelow and Nebert (1982)
Receptor activation			
Chemical	**Assay system**	**Activity (EC_{50} or Km or TEF[2])**	**Reference**
DB[*a,h*]A	Tox21_AhR[3]	0.073 µM	US EPA (2013b)
	ATG_AhR_CIS[3]	0.075 µM	
	AhR-CALUX[4]	0.082 ng/ml	Malik *et al.* (2013)
	AhR-CALUX[4]	5	Pieterse *et al.* (2013)
	AhR-CALUX[4]	11.46	Machala *et al.* (2001)
	AhR-RGS[4]	~1	Jones *et al.* (2000)
DB[*a,c*]A	AhR-CALUX[4]	1.75	Machala *et al.* (2001)
DB[*a,j*]A	AhR-CALUX[4]	2.16	Machala *et al.* (2001)

[1] Rat liver cytosol preparation generally contains a high concentration of AhR and is widely used to test for AhR ligands using a radioligand in the receptor binding and competitive binding assays.
[2] The EC_{50} (displayed in µM units) indicates the chemical concentration that induces a half-maximal assay response. The ToxCast database provides this same information as an "activity concentration" or AC_{50}. Km (ng/ml) refers to the predicted concentration required to elicit a response equivalent to half maximum. TEF: relative value to B[*a*]P.
[3] Reporter gene-based HepG2 cell (human hepatoma cell line) assays. The AC_{50} is more than one standard deviation below the mean of all chemicals tested, indicating that DB[*a,h*]A is a high potency AhR ligand.
[4] AhR-CALUX and AhR-RGS assay systems are rat or mouse liver tumor cell-based luciferase reporter assays that detect AhR binding activity.

The AhR-mediated induction of xenobiotic metabolizing enzymes (*e.g.*, CYP 1A1, CYP 1A2, and CYP 1B1) as a result of AhR activation by DBAs will result in an increase in DBA metabolic activation, because CYP 1A1, CYP 1A2, and CYP 1B1 are each involved in the biotransformation of DBAs (Section 3.3.4 Pharmacokinetics and Metabolism). This, in turn, can result in enhanced genotoxicity and carcinogenicity. Enhanced DNA adduct formation has been demonstrated experimentally in calf thymus DNA adduct studies of DB[*a,c*]A, where exogenous metabolic activation with DB[*a,c*]A-

induced mouse epidermal homogenates (expressing up to 7-fold higher CYP 1A1 activity than non-induced homogenates) resulted in greater adduct formation as compared to activation with non-induced homogenates (Buty *et al.*, 1976; Slaga *et al.*, 1977; 1978).

Studies in mice underscore the importance of the AhR in enzyme induction, DNA adduct formation and skin tumor induction by DB[*a,h*]A and DB[*a,c*]A. For example, Shimada *et al.* (2003) conducted studies with AhR knockout and wild type mice, and reported that AhR was required for DB[*a,h*]A induction of CYP 1A1, CYP 1A2 and CYP 1B1 mRNAs in the liver. In another example, Kleiner *et al.* (2004) found that levels of DB[*a,h*]A-derived DNA adducts in mouse skin 24 hours after dermal application were significantly lower in AhR knockout mice as compared to wild type mice. And in studies of enzyme and skin tumor induction by DB[*a,h*]A and DB[*a,c*]A in mice expressing either high- or low-affinity AhR, Kouri *et al.* (1983) found significantly lower AHH induction and tumor induction in the low-affinity AhR mice as compared to the high-affinity AhR strains.

As discussed in some detail by IARC (2010, pp. 641-658, Attachment 2), a number of additional AhR-mediated signaling pathways are thought to be involved in PAH-induced carcinogenesis. These include p53-dependent and p53-independent pathways that suppress immune function, activation of other oncogenes and tumor suppressor genes (c-Myc and HDAC6) (Hsieh *et al.*, 2012), and cross-talk with other nuclear receptors, such as the estrogen receptor (ER).

Other receptor-mediated effects

Results from ToxCast (US EPA, 2013b) indicate that DB[*a,h*]A is a high affinity ligand for the estrogen receptor (ER) and the androgen receptor (AR), as tested in reporter gene-based human cell assays (HEK293T kidney cell line and MDA-kb2 breast cancer cell line, respectively). DB[*a,h*]A has also been shown to activate human AR in reporter gene-based cell assays in CHO cells (Vinggaard *et al.*, 2000) and a human prostate cell line (Kizu *et al.*, 2004; Vinggaard *et al.*, 2000). ER and AR binding and activation studies have not been reported for DB[*a,c*]A or DB[*a,j*]A. ER-regulated genes and associated signaling pathways are widely studied, and downstream effects such as increased cell proliferation and inhibition of apoptosis can eventually lead to tumor formation in ER positive tissues (Yager and Davidson, 2006). Similarly, AR-regulated genes and signaling pathways are associated with tumor formation AR positive tissues (Balk, 2014). As reviewed in Section 3.2.1, the mammary gland and the liver, both of which express ER and AR (Li *et al.*, 2010; Nilsson and Gustafsson, 2011; Young *et al.*, 1994), are target tumor sites for DB[*a,h*]A and DB[*a,c*]A (liver only).

Immunosuppression

AhR-mediated immunosuppressive effects of DB[*a,h*]A and DB[*a,c*]A have been observed in human T-cells (Davila *et al.*, 1996, p. 651, Attachment 2). In another study, DB[*a,h*]A induced AhR-independent systemic immunosuppression in female B6C3F$_1$ mice (Smith *et al.*, 2010). A recent toxicogenomic study involving subchronic oral exposure of male transgenic mice (Muta^{TM} Mouse) to DB[*a,h*]A reported down-regulation of five genes involved in immune response and up-regulation of two pro-inflammatory genes in the liver (Malik *et al*., 2013). Immune suppression, enhanced inflammatory response and reduced immunosurveillance are associated with an increased risk of developing cancer (Penn, 2000).

Alterations in cell proliferation, apoptosis, and cell cycle regulation

Data from cell culture studies and an *in vivo* toxicogenomic study suggest that DBAs can affect cell proliferation, apoptosis, and cell cycle regulation. Specifically, each of the three DBA isomers increased cell proliferation in a dose-dependent manner in a rat liver epithelial cell line (WB-F344) (Svihalkova-Sindlerova *et al.*, 2007). *In vivo*, significantly increased cell proliferation ($p < 0.02$) was observed in the liver of male transgenic mice (Muta^{TM} Mouse) administered 6.25 mg/kg-day DB[*a,h*]A by gavage for 28 days, but not in mice receiving higher doses (12.5, or 25 mg/kg-day). Toxicogenomic analysis of hepatic gene expression in these DB[*a,h*]A-treated transgenic mice identified changes in gene expression in a number of genes, including genes involved in circadian rhythm, glucose metabolism, lipid metabolism, immune function (see above), cell cycle regulation and apoptosis (Malik *et al.*, 2013). More specifically, Malik *et al*. (2013) reported that DB[*a,h*]A treatment resulted in the induction of genes involved in cell cycle arrest, cell cycle progression from G1 to S phase (*i.e*., *Ptp4a1)* and apoptosis, and down-regulation of anti-apoptotic genes.

In summary, there is strong evidence that DBAs act through one or more genotoxic mechanisms to induce tumors, as a result of metabolic activation to form a number of different reactive metabolites capable of forming DNA adducts and causing DNA damage and mutations. Evidence from studies with DBAs and other PAHs, including B[*a*]P, suggest that other mechanisms of carcinogenesis are also likely to be involved. Table 42 summarizes the observed genotoxicity, receptor activation, and other relevant data related to mechanisms of carcinogenesis for DBAs, as well as for B[*a*]P.

Table 42. Genotoxicity, receptor activation and other data related to mechanisms of action for DBAs and B[*a*]P

Chemical	Genotoxicity	AhR	ER	AR	Immune suppression	Cell cycle, proliferation/ apoptosis
DB[*a,h*]A	X[1,2]	X[1,3]	X[1,3]	X[3,4]	X[1,5,6]	X[6,7]
DB[*a,c*]A	X[1,2]	X[1]			X[1]	X[7]
DB[*a,j*]A	X[1,2]	X[1]				X[7]
B[*a*]P	X[1,8]	X[1,8]	X[1,8]	X[4,8]	X[1,8]	X[1,8]

[1] IARC (2010)
[2] See Section 3.3.1
[3] ToxCast (US EPA, 2013b)
[4] Kizu *et al.* (2004); Vinggaard *et al.* (2000)
[5] Smith *et al.* (2010)
[6] Malik *et al*. (2013)
[7] Svihalkova-Sindlerova *et al*. (2007)
[8] Labib *et al.* (2013)

5. REVIEWS BY OTHER AGENCIES

Data on the carcinogenicity of one or more DBA isomers have been reviewed by the International Agency for Research on Cancer (IARC, 1973; 1983; 1987; 2010), the National Toxicology Program (NTP, 1981), and the US EPA (1994). None of these agencies has evaluated the carcinogenicity of DBAs as a chemical group.

DB[*a,h*]A

DB[*a,h*]A was first reviewed by IARC in 1973 and was determined to have “local and systemic carcinogenic effects” based on animal cancer bioassay data. The 1983 IARC review added additional short-term test data and concluded there is “sufficient evidence that DB[*a,h*]A is carcinogenic to experimental animals.” IARC (1987) classified DB[*a,h*]A as a Group 2A carcinogen (probably carcinogenic to humans) with supporting evidence from other relevant data for the evaluation of carcinogenicity and its mechanisms. The most recent IARC review of the carcinogenicity of DB[*a,h*]A (IARC, 2010) included additional bioassays and other relevant data available since the 1983 review, and retained the Group 2A classification.

NTP (1981) classified DB[*a,h*]A as “reasonably anticipated to be a human carcinogen” in the Second Report on Carcinogens, and that classification has remained unchanged in subsequent NTP Reports on Carcinogens.

US EPA (1994) classified DB[*a,h*]A as a B2 carcinogen (probable human carcinogen) based on sufficient evidence in experimental animals.

DB[*a,c*]A and DB[*a,j*]A

Both DB[*a,c*]A and DB[*a,j*]A were first reviewed by IARC in 1983 and both were found to have “limited evidence [on carcinogenicity] in experimental animals” based on animal bioassays and short term test data. In 1987, both DB[*a,c*]A and DB[*a,j*]A were classified as Group 3 carcinogens (not classifiable as to their carcinogenicity to humans) based on inadequate evidence of carcinogenicity in humans and limited evidence in experimental animals. The 2010 IARC review (IARC, 2010) included additional bioassays and other relevant data available since the 1983 review, and retained the Group 3 classifications of DB[*a,c*]A and DB[*a,j*]A.

Neither NTP nor US EPA have classified DB[*a,c*]A or DB[*a,j*]A as to their carcinogenicity.

6. SUMMARY AND CONCLUSIONS

6.1 Summary of Evidence

No epidemiology studies were identified that investigated the risk of cancer associated with exposure specifically to DBAs.

Tumor findings from carcinogenesis bioassays conducted in animals with the DBAs are as follows:

DB[*a,h*]A:

- Induces tumors by multiple routes of exposure, in multiple species (*i.e*., mouse, rat, hamster, chicken, pigeon, and frog) (summarized in Table 4, and Attachment 1).

DB[*a,c*]A:

- Statistically significant increases in the incidence of skin squamous cell carcinoma and combined papilloma and carcinoma in female Swiss mice in a dermal application study (Lijinsky *et al*., 1970).

- Statistically significant increase in the incidence of liver adenoma in male $B6C3F_1$ mice treated neonatally with DB[*a,c*]A by *i.p.* injection and followed for 12 months (Von Tungeln *et al.*, 1999).

DB[*a,j*]A:

- Statistically significant increases with significant dose-dependent trends in the incidence of skin squamous cell carcinoma and combined papilloma and carcinoma in female Swiss mice treated by dermal application (Lijinsky *et al*., 1970).
- Rare skin sarcomas were observed in three of 15 treated female Swiss mice following a single *s.c.* injection, and none were observed in controls (Lijinsky *et al*., 1970).

Findings of tumor-initiating activity in mouse skin tumor initiation-promotion studies with the DBAs and their diol or diol epoxide metabolites are as follows:

DB[*a,h*]A:

- Skin tumor initiating activity observed in two strains of mice (summarized in Table 4, and Attachment 1).

DB[*a,h*]A diol/diol epoxide metabolites:

- Skin tumor initiating activity observed in mice (Attachment 2)

DB[*a,c*]A:

- Initiating activity, based on statistically significant increases in skin papillomas in two studies
    - ICR/Ha Swiss mice (Van Duuren *et al*., 1970)
    - CD-1 mice (Scribner, 1973)
- Initiating activity, based on skin papillomas in 75% of treated SENCAR mice; study had no control group (Scribner and Scribner, 1980)
- Borderline statistically significant increases in skin tumors in two studies
    - Skin papillomas in SENCAR mice (Slaga *et al*., 1980)
    - Skin papillomas in CD-1 mice (Chouroulinkov *et al*., 1983)

DB[*a,c*]A metabolites (1,2-diol and 10,11-diol):

- Initiating activity, based on statistically significant increase in combined skin papillomas and carcinomas in CD-1 mice (Chouroulinkov *et al*., 1983)

DB[*a,j*]A:

- Initiating activity, based on statistically significant increases with significant dose-dependent trends in skin papillomas in two studies (using different vehicles) in SENCAR mice (Sawyer *et al.*, 1987; 1988)
- Initiating activity, based on statistically significant increases in skin papillomas in three studies in SENCAR mice (DiGiovanni *et al*., 1983; Harvey *et al.*, 1988; Vulimiri *et al.*, 1999 [findings for papilloma/mouse; incidence not reported])

DB[*a,j*]A metabolites (3,4-diol and 3,4-diol-1,2-epoxide):

- Initiating activity, based on statistically significant increases with significant dose-dependent trends in skin papillomas in two studies (using different vehicles) in SENCAR mice (Sawyer *et al*., 1987; 1988)
- Initiating activity, based on a statistically significant increase in skin papillomas in SENCAR mice (Harvey *et al.*, 1988)

Evidence of genotoxicity, DNA binding and DNA adduct formation for the DBAs and their diol or diol epoxide metabolites are as follows:

DB[*a,h*]A:

- DNA damage and mutations in bacteria; mutations, SCEs, and UDS in mammalian cells *in vitro*; DNA adducts and SCEs in mice *in vivo* (summarized in Table 24, and Attachment 2).

DB[*a,h*]A diol/diol epoxide metabolites:

- Mutations in bacteria; DNA adducts in cell-free systems, mammalian systems *in vitro,* and mice *in vivo* (Attachment 2)

DB[*a,c*]A:

- DNA damage and gene mutations in bacteria
- Somatic mutations in *Drosophila melanogaster*
- DNA binding and DNA adducts in cell-free systems
- DNA binding, DNA adducts, mutations, and UDS in mammalian assay systems *in vitro*
- DNA binding, DNA adducts, *ras* oncogene mutations, and micronuclei in mice *in vivo*

DB[*a,c*]A diol/diol epoxide metabolites:

- Bacterial gene mutation
- DNA adducts in cell-free systems
- *In vitro* chromosomal damage (SCEs)

DB[*a,j*]A:

- Bacterial gene mutations
- DNA adducts and gene mutation in mammalian assay systems *in vitro*
- DNA adducts in mice *in vivo*
- Mutations (in the *ras* oncogene) in mice *in vivo*

DB[*a,j*]A diol/diol epoxide metabolites:

- DNA adducts in cell-free systems
- DNA adducts in mice *in vivo*
- Mutations (in the *ras* oncogene) in mice *in vivo*

Findings of *in vitro* malignant cell transformation are as follows:

DB[*a,h*]A:

- Malignant cell transformation of Syrian hamster embryo cells, mouse embryo fibroblast C3H10T½ cells, and mouse prostate C3H cells *in vitro*.

DB[*a,c*]A:

- Malignant cell transformation of Syrian hamster embryo cells *in vitro* in three of four studies.

DB[*a,c*]A induced pre-neoplastic morphological changes (*i.e*., epithelial hyperplasia and squamous metaplasia) in subcutaneously transplanted rat tracheas.

DB[*a,h*]A and DB[*a,c*]A bind with high affinity to AhR, and DB[*a,h*]A, DB[*a,c*]A and DB[*a,j*]A activate AhR in reporter gene-based assays.

DB[*a,h*]A binds with high affinity and activates human ER and AR in cell lines.

DB[*a,h*]A and DB[*a,c*]A have immunosuppressive effects *in vitro* and *in vivo* (DB[*a,h*]A only).

DB[*a,h*]A, DB[*a,c*]A and DB[*a,j*]A increased cell proliferation in rat liver epithelial cells *in vitro*, and DB[*a,h*]A increased cell proliferation in mouse liver in one study at one dose level.

DB[*a,h*]A induced genes involved in cell cycle arrest, cell cycle progression from G1 to S phase, and apoptosis, and down-regulated anti-apoptotic genes in a toxicogenomic study of mouse hepatic gene expression.

DBAs share numerous similarities in biologic activity with each other and with the six structurally related non-substituted PAHs chosen for comparison (benzo[*a*]pyrene, benz[*a*]anthracene, dibenzo[*a*,*e*]pyrene, dibenzo[*a,h*]pyrene, dibenzo[*a*,*i*]pyrene, and dibenzo[*a*,*l*]pyrene). These include:

- Induction of tumors in animal bioassays
    - Increases in skin tumors in mice
    - Increases in liver tumors (with the exception of DB[*a,j*]A and dibenzo[*a,e*]pyrene)
    - Increases in lung tumors (with the exception of DB[*a,c*]A, DB[*a,j*]A and dibenzo[*a,e*]pyrene)
- Skin tumor initiating activity in mice
- Positive findings of genotoxicity and DNA binding/adduct formation

- Metabolism to diol or diol epoxides (with the exception of dibenzo[*a,e*]pyrene) with:
    - Skin tumor initiating activity in mice
    - Positive findings of genotoxicity and/or DNA binding/adduct formation
- DB[*a,h*]A and each of the six PAHs chosen for comparison are listed under Proposition 65 as causing cancer, and each (with the exception of dibenzo[*a,e*]pyrene) are classified as carcinogens by IARC (Group 1, 2A or 2B).

DB[*a,c*]A and DB[*a,j*]A are predicted to be carcinogenic by several QSAR models, including VEGA, Lazar, OECD QSAR Toolbox, and other models developed and reported by Vijayalakshmi and Suresh (2008) and Barone *et al*. (1996) (DB[*a,c*]A only).

6.2 Conclusions

The evidence for the carcinogenicity of DBAs as a group comes from a substantial body of evidence on DB[*a,h*]A, together with evidence on DB[*a,c*]A and DB[*a,j*]A and DBA metabolites. This evidence comes from cancer bioassays in animals, mouse skin tumor initiation-promotion studies, genotoxicity assays, DNA binding and adduct formation studies, *in vitro* cell transformation studies, *in vivo* studies of pre-neoplastic changes in subcutaneous tracheal transplants, studies on receptor binding, immune function, cell proliferation and gene expression, structure-activity considerations, and QSAR predictions of carcinogenic activity.

- DB[*a,h*]A has been listed under Proposition 65 as causing cancer since 1988. It induces malignant and benign tumors in animals, is a mouse skin tumor initiator, is genotoxic and forms DNA adducts, is metabolized to genotoxic diols and diol epoxides that are skin tumor initiators. Regarding other findings relevant to carcinogenesis, DB[*a,h*]A induces malignant cell transformation *in vitro*, activates AhR, ER, and AR, has immunosuppressive effects, increases cell proliferation, alters expression of genes associated with cell proliferation, apoptosis, and cell cycle regulation in mouse liver *in vivo*, and is an IARC 2A carcinogen.

- DB[*a,c*]A – observations following treatment include:
    - Tumor findings in a dermal application study in female mice (skin squamous cell carcinoma and combined papilloma and carcinoma), and in a 1-year neonatal *i.p*. injection study in male mice (liver adenoma)
    - Mouse skin tumor initiating activity in multiple studies and in multiple strains of mice
    - Mouse skin tumor initiating activity by its diol metabolites

- Multiple positive genotoxicity, DNA binding and adduct formation studies of DB[*a,c*]A and its diol/diol epoxide metabolites in bacteria (mutations and DNA damage), cell-free systems (DNA binding and adduct formation), *Drosophila melanogaster* (mutations), *in vitro* mammalian systems, and mice *in vivo*
- Multiple positive studies of *in vitro* cell transformation in Syrian hamster embryo cells
- Findings of pre-neoplastic changes in subcutaneous tracheal transplants in mice
- Other relevant findings including AhR agonist activity, immunosuppressive effects *in vitro*, and increased cell proliferation in a rat liver cell line
- Predictions of carcinogenicity by several QSAR models

- DB[*a,j*]A – observations following treatment include:
  - Tumor findings in a dermal application study in female mice (skin squamous cell carcinoma and combined papilloma and carcinoma), and in a *s.c*. injection study in female mice (rare skin sarcomas in 3/15 mice)
  - Mouse skin tumor initiating activity in multiple studies in SENCAR mice by DB[*a,j*]A
  - Mouse skin tumor initiating activity by its diol/diol epoxide metabolites
  - Multiple positive genotoxicity, DNA binding and adduct formation studies of DB[*a,j*]A and its diol/diol epoxide metabolites in bacteria (mutations), cell-free systems (DNA binding and adduct formation), *in vitro* mammalian systems, and mice *in vivo*
  - Other relevant findings including AhR agonist activity and increased cell proliferation in a rat liver cell line
  - Predictions of carcinogenicity by several QSAR models

- There are strong structure-activity similarities among all three DBA isomers, and between the DBAs and six comparison PAHs listed as carcinogens under Proposition 65.

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Appendix A. Parameters for Literature Searches on the Carcinogenicity of "Dibenzanthracenes"

General searches of the literature on the carcinogenicity of "Dibenzanthracenes" were conducted under contract by the University of California at Berkeley (Charleen Kubota, M.L.I.S.). The goal was to identify peer-reviewed open source and proprietary journal articles, print and digital books, reports and gray literature that potentially reported relevant toxicological and epidemiological information on the carcinogenicity of these chemicals. The search sought to specifically identify all literature relevant to the assessment of evidence on cancer.

Databases

The literature search utilized the following search platforms/database vendors:

- PubMed (National Library of Medicine)
- EMIC (National Library of Medicine)
- SciFinder®: CAS (Chemical Abstracts Service)
- TOXNET (National Library of Medicine): Toxicology Literature Online (TOXLINE), Genetic Toxicology Data Bank (GENE-TOX)
- Web of Knowledge: BIOSIS Previews®, Web of Science® (Thomson-Reuters, Inc.)

Search Process

Relevant subject terms were entered into the PubMed Search Builder to execute a search.

The following is a typical chemical search strategy used to search PubMed:
("*chemical name*" [MeSH] OR "CAS *registry number*"[RN]) AND ("bioassay"[MeSH] OR "carcinogenicity"[MeSH] OR "cancer"[MeSH] OR "tumor"[MeSH]) OR "neoplasm"[MeSH]) OR "genotoxicity"[MeSH]) OR "mutagenicity"[MeSH]) OR "metabolism"[MeSH]) OR "absorption"[MeSH]) OR "pharmacokinetics"[MeSH]) OR "structure activity relationship"[MeSH])

In PubMed, MeSH (Medical Subject Headings) terms at the top of hierarchical lists of subject headings are automatically "exploded" in a search to retrieve citations with more specific MeSH terms. For example, the heading "carcinogenicity" includes broad conditions that are related to cancer induction in animals and humans.

Additional databases listed above were then searched. The search strategies were tailored according to the search features unique to each database. Web of Science, for

example, was searched by entering chemical terms and refining the search by applying Web of Science categories Toxicology and/or Public, Environmental and Occupational Health. The search term used includes either the CAS registry number or the chemical name and its available synonyms. Sometimes other databases not listed here were searched as needed.

Additional focused searches for each section were performed by OEHHA and the search strategies are briefly described as follows:

Sections 3.1 - 3.3.5:

Focused searches were conducted for the DB[*a,c*]A and DB[*a,j*]A isomers, using TOXLINE and GENE-TOX. Additional relevant literature was identified from citations in individual articles.

Section 3.3.6 and Appendix B:

- PubMed and Google were used to identify QSAR models
- Information on QSAR models and modeling was found through PubMed
- PubMed search strategy: the MeSH database was used to identify relevant subject headings for QSAR information. Relevant subject terms were entered into the PubMed Search Builder to execute a PubMed search.
- The search strings applied to all databases listed above as: ("QSAR" [MeSH] OR "Quantitative Structure Activity Relationship" [MeSH] OR "Relationship, Quantitative Structure-Activity" OR “Structure-Activity Relationship, Quantitative” OR “QSAR modeling”
- 57 papers, including government reports, peer-reviewed journal articles, books, and instruction manuals were identified.

Section 4:

- Databases and other resources used: Google search engine, ChemSpider (Royal Society of Chemistry), MeSH (Medical Subject Headings) (National Library of Medicine), PubMed & PubChem BioAssay (National Library of Medicine), TOXLINE (National Library of Medicine), Web of Science® (Thomson-Reuters, Inc.), Scopus® (Elsevier B.V.), iCSS Dashboard v0.5 (US EPA ToxCast Phase II data) and CTD (Comparative Toxicogenomics Database).
- ChemSpider was searched first to gather synonyms, CAS registry number, MeSH terms and Chemical Abstracts Service headings before searching bibliographic databases.

- PubMed search strategy: the MeSH database was used to identify relevant subject headings for carcinogenic endpoints. Relevant subject terms were entered into the PubMed Search Builder to execute a PubMed search.
- The search strings applied to all databases listed above as: ("Dibenzanthracene" [MeSH] OR "polycyclic aromatic hydrocarbon" OR “other synonyms” OR “CAS registry number [RN]”) AND (“Neoplasms” [MeSH] OR “Cancer” [MeSH] OR “Mutation” [MeSH] AND “Toxicity” [MeSH] OR “Mechanism” [MeSH]).
- The DB[*a,h*]A ToxCast/Tox21 data and assay information were found through iCSS Dashboard v0.5 (US EPA ToxCast Phase II data, http://actor.epa.gov/dashboard/) and PubChem BioAssay (National Library of Medicine)
- The toxicogenomic data on DBAs were found through CTD (Comparative Toxicogenomics Database, http://ctdbase.org/) and PubMed searches.
- 48 papers related to mechanisms of action of DBAs were identified and selected using standard expert knowledge-based review practices.

In summary, 456 references, including government reports, peer-reviewed journal articles, QSAR model-instruction manuals and books, were identified through these search strategies. Among these, 193 were cited in this document.

Appendix B. Quantitative Structure Activity Relationship (QSAR) Models

QSAR models may be rule-based expert systems or statistical-based (Milan *et al.*, 2011). Rule-based expert systems codify chemical fragments into structural alerts that are responsible for the toxic effect. These models are either based on expert human knowledge or the use of data-mining methods applied to structure-specific datasets. Statistical-based models translate the properties of chemicals into molecular descriptors and use an algorithm to determine a statistical correlation with chemical toxicity (Bakhtyari *et al.*, 2013).

OEHHA obtained models in the public domain used for carcinogenicity prediction and developed predictions for DB[*a,c*]A and DB[*a,j*]A using models that were consistent with Organisation for Economic Co-operation and Development (OECD) and the International Conference on Harmonisation (ICH) guidelines for model selection, as follows:

- The ICH M7 guidelines specify that two complementary QSAR methodologies should be used to predict the outcome of a bacterial mutagenicity assay: one rule-based and the other statistical-based (ICH, 2013). OEHHA applied the same criterion to model selection for carcinogenicity.
- The OECD requirements for each QSAR model were: 1) a defined endpoint, 2) an unambiguous algorithm, 3) a defined applicability domain, and 4) appropriate measures of goodness-of-fit, robustness and predictivity (OECD, 2007).

The models chosen for the analysis were the following:

- VEGA (a platform developed through a collaboration between the US EPA and a number institutions from Europe under the sponsorship of ANTARES Project, CALEIDOS Project, CAESAR Project, Ministero della Salute, and ORCHESTRA Project) that contains three models:
    - CAESAR – a statistical-based system that predicts carcinogenicity toward male and female rats,
    - ToxTree – a rule-based system that predicts carcinogenicity toward humans, merging a series of rules from laboratory studies on different species, and
    - SarPy – a statistical-based model developed in Python to predict mutagenicity.
- Lazar, which was developed by a Swiss company (In silico toxicology GMBH, 2011) for predicting toxicological endpoints.

- QSAR Toolbox, which was developed by the Laboratory of Mathematical Chemistry with the scientific and financial assistance of OECD and the European Union. It groups chemicals into categories and filling gaps in toxicity data in order to assess the hazard of chemicals.

In addition, predictions from two published models are reported in the scientific literature:

- Barone *et al*. (1996), a non-commercially-developed model that uses a rule-based methodology to correlate electronic properties with carcinogenicity. OEHHA used the result reported in the publication.
- Vijayalakshmi and Suresh (2008), a non-commercially developed model that used the volume of molecular electrostatic potential at electron dense centers of the molecule to determine carcinogenic properties. OEHHA used the result reported in the publication.

Rule-based models reflect human mechanistic knowledge, while statistical-based models are able to assess large amounts of data to detect relationships not yet discovered by human experts. Most models clearly define the endpoint, algorithm, and various measures for validity. Table B-1 provides a comparison of the QSAR models. It is important for models to use carcinogenicity databases with large chemical inventories and that contain data from tests done on more than one species. Internal and external validation of the datasets helps to ensure that the database is adequate for the model. Additionally, an important requirement of the OECD principles is for models to have a clear definition of the applicability domain. The applicability domain is the “response and chemical structure space in which the model makes predictions with a given reliability” (Netzeva *et al.*, 2005). This parameter is particularly important because the applicability domain expresses the range of chemical structures for which the model is considered to be applicable, *i.e.*, the scope and limitations of the model (Netzeva *et al.*, 2005). Predictions are reliable only when the model’s assumptions are met; when the assumptions are violated, the prediction is unreliable (Jaworska *et al.*, 2005).

Statistical-based models, such as SarPy (VEGA NIC v1.0.8, 2013) and QSAR Toolbox (OECD QSAR Toolbox v. 3.2, 2013), commonly define the applicability domain using structural rules and/or descriptor variables in the dataset based on ranges, geometry, distances, or probability density distribution functions (Netzeva *et al.*, 2005). Another statistical-based model, CAESAR (VEGA NIC v1.0.8, 2013), evaluates the applicability domain with two methods. The general applicability domain is evaluated using a tool based on the range of chemical descriptors. Values that are predicted for chemicals outside the descriptor range are considered less reliable. To compensate for weaknesses in this general applicability domain approach, CAESAR also calculates a

similarity score that compares the compound of interest to the six most similar chemicals in the dataset (Fjodorova *et al.*, 2010).

Other models, such as Lazar (In silico toxicology GMBH, 2011), provide a statistically-calculated confidence value, dependent on the "structural density" and similarity in activity of the neighborhood (Maunz and Helma, 2008). Decision tree approaches, such as ToxTree (VEGA NIC v1.0.8, 2013), define the applicability domain in terms of structural characteristics of the chemical classes to which they apply (Benigni *et al.*, 2008b). Calculations are based on the average Euclidean distance that the descriptors of an unknown chemical are outside the range of those descriptors based on the training set (Netzeva *et al.*, 2005).

Table B-1. Comparison of QSAR models

Model	Type of model (rule-based or statistical-based)	Basis for the applicability domain	Carcinogenicity prediction measure
CAESAR	Statistical	Range of chemical descriptors and a similarity score	Carcinogen / non-carcinogen
ToxTree	Rule	Structural characteristics of the chemical classes	Carcinogen / non-carcinogen
SarPy	Statistical	Structural rules and/or descriptor variables	N/A[1]
Lazar	Statistical	Confidence value dependent on the "structural density" and similarity in activity of the neighborhood	Carcinogen / non-carcinogen
QSAR Toolbox	Statistical	Structural rules and/or descriptor variables	Positive / negative

[1] N/A = not applicable; SarPy predicts for mutagenicity, not carcinogenicity

Descriptions of Models

VEGA

VEGA, a platform based on the CAESAR model, provides a qualitative prediction of carcinogenic potency toward male and female rats. The model was built on a Counter Propagation Artificial Neural Network (CP ANN) algorithm. Output of CP ANN contains two values, Positive and Non-Positive, which sum to one, representing the degree to which the neuron that the predicted chemical falls in belongs to the class of carcinogenic or non-carcinogenic compounds. The prediction is based on whichever

value is higher. This model is based on the Distributed Structure-Searchable Toxicity (DSSTox) Public Database Network (US EPA, 2013a), which contains male and female rat data. It uses descriptors referring to topological characteristics, polarizability, and charge distribution (Fjodorova *et al.*, 2010). After the prediction has been made, specific structural alerts extracted from the ToxTree program are checked and, if a match is found, a comment is given about the presence of a fragment that may be toxic (VEGA, 2013).

The other model in the VEGA platform uses the Benigni-Bossa rulebase for mutagenicity and carcinogenicity, which was developed as a module to the ToxTree software. This expert rule-based software applies rules based on human knowledge to identify structural alerts. Each structural alert represents a chemical class that causes toxic effects through one or more shared mechanisms of action. There are 33 structural alerts included in ToxTree; 28 are based on mechanisms of genotoxic carcinogenicity and five are based on non-genotoxic mechanisms. Compounds are flagged when at least one structural alert is recognized (Benigni *et al.*, 2008b).

A third model for mutagenicity included in VEGA is the SarPy model, a statistical model developed in Python (Istituto Mario Negri *et al.*, 2013). This is based on structural alerts that were selected by the program based on their occurrence in toxic or nontoxic compounds. It extracted 112 rules from the CAESAR model's dataset, and then used these to predict mutagenicity through the VEGA platform (Bakhtyari *et al.*, 2013).

Lazar

Lazar is an open-source software program that uses statistical methods to predict toxicological endpoints by analyzing structural fragments identified in an experimentally determined training set. It first identifies similar compounds in the training dataset, *i.e.*, "neighbors", based on structural, property, biological, or activity-specific similarities. It then generates a local prediction model based on the experimental activities of neighbors and uses the model to predict properties of the compound (Maunz *et al.*, 2013).

OECD QSAR Toolbox

QSAR Toolbox combines data and tools from many sources into a logical workflow. Users apply QSAR methodologies to group chemicals into categories based on physicochemical and human/health properties that are likely to be similar. After categories are assigned, data gap filling can be completed by three different methods: 1) read-across, 2) trend analysis, and 3) QSAR models. 1) *Read-across* predicts toxicity based on nearest neighbors in the chemical category compared by prediction descriptors (OECD, 2013). The descriptor values for the target chemical and the

category members are calculated using the rule "log K_{ow}," taking the weighted average value (OECD, 2009). Log K_{ow} is the octanol:water partition coefficient, a measure of hydrophobicity, which is important for transport and distribution, thus for predicting toxicity. Members of a category are often related by a trend, such as molecular mass, carbon chain length, or other physicochemical property, for a given endpoint. 2) *Trend analysis* estimates a value for an untested chemical from an increasing, decreasing, or constant trend for an effect within a category. 3) *QSAR models* allow the user to estimate missing values from a particular statistical model for a category (OECD, 2012).

Following data gap filling, a report is generated that allows the user to evaluate the strength of the read-across prediction. The model is capable of predicting carcinogenicity and mutagenicity for multiple species. Databases include the Carcinogenic Potency Database (CPDB) (Gold *et al.*, 2011), various ISS (Istituto Superiore di Sanità) Databases (Benigni *et al.*, 2008a), the Danish EPA database (Danish QSAR Group, 2004), Micronucleus and Genotoxicity OASIS, EXCHEM, and Toxicity Japan Ministry of Health, Labor and Welfare (LMC, 2013). An applicability domain is calculated based on the descriptor range for the dataset. The application alerts users when a chemical is out of the applicability domain or when there are not enough chemicals in the category to make a prediction. Additionally, a *p*-value for the prediction confidence is calculated, allowing the user to compare different profilers to determine the best prediction.

Other models published in the scientific literature

Vijayalakshmi and Suresh (2008) used the volume of molecular electrostatic potential (MESP) at electron dense centers of the molecule to determine carcinogenic properties for a set of 28 PAHs. MESP is directly related to electron density and distinguishes the electron dense centers in a molecule into K, L, M, and N regions. Multiple linear regression analysis was conducted using the calculated MESP at these four regions for 28 PAHs. The authors' calculations suggest that the K and M regions have activating carcinogenic effects, while the L and N regions have deactivating effects. The M region is associated with the bay region and is the main activation site on the molecule, according to the bay region theory. This model combines the concepts of both the K/L region and bay region theories.

Barone *et al*. (1996) developed a rule-based methodology to correlate electronic properties with carcinogenicity. The number of electronic states per energy unit calculated over the ring that contains the highest bond order (RHBO) is the local density of states (LDOS). Barone *et al*. (1996) used estimates of the LDOS and the molecular energy difference between the highest occupied molecular orbital and the next lower level, and consideration of the peak contribution to the LDOS, to predict the

carcinogenicity of 26 PAHs. It does not use K, L, M, N, or bay regions to predict carcinogenicity activity.

Strengths and weakness of QSAR models

Rule-based models (*e.g.*, ToxTree) are advantageous in that they are transparent and the explanations reflect mechanistic knowledge (Milan *et al.*, 2011). These models, however, have several limitations. Potentially toxic chemicals are flagged, but no conclusions about nontoxic chemicals are given. The list of toxic residues is incomplete and some chemical classes exert their toxic effects through complicated mechanisms that are difficult to code through structural alerts (Benigni *et al.*, 2008b). Additionally, most known carcinogenic residues codify genotoxic activity; non-genotoxic carcinogens are underrepresented. Statistical-based models, on the other hand, are advantageous in that they are able to detect relationships not yet discovered by human experts (Milan *et al.*, 2011). Models using simple rules, such as electronic properties of compounds, present another methodology to predict carcinogenic activity. While unlikely to be capable of identifying all active PAHs, they may be useful to flag potential compounds of concern.

A number of papers assess the accuracy, sensitivity and specificity of QSAR models using external datasets. Overall, statistical models have higher accuracy than rule-based methods for both mutagenicity (Bakhtyari *et al.*, 2013) and carcinogenicity (Milan *et al.*, 2011). Some models (*e.g.*, VEGA CAESAR) have higher sensitivity, while others (*e.g.*, Lazar) have higher specificity (Milan *et al.*, 2011). Often a more conservative model with higher sensitivity is preferred to avoid false negatives (Bakhtyari *et al.*, 2013). According to Bakhtyari *et al.* (2013), the accuracy of high-performing models (CAESAR) is similar to the *in vitro* reproducibility of the Ames test.

VEGA provides transparency and allows the user to easily conduct an assessment and interpret the output. VEGA clearly defines the statistical methods used in its models, as well as the applicability domain. Lazar also clearly describes its model, but has a simpler algorithm and does not include as many datasets as VEGA. OECD QSAR Toolbox, on the other hand, requires extensive training and knowledge of the program in order to use it. It allows the user greater freedom in selecting parameters and lets the user see the chemicals used in comparisons. However, the multiple steps in the analysis lead to considerable variation in predictions made by different users. Additionally, the algorithms used in the OECD QSAR Toolbox model are not clearly described.

ATTACHMENTS

1. IARC 2010, pp. 246, 253-256, 265, 280-283, 296, 304-309, 316, 318-319, 321-322, 325, 333-334, 337, 353, 368-369, 374, 386, 402, 408, 427-433, 759, 762, 771-773
2. IARC, 2010, pp. 512-542, 565-584, 587-597, 623-658, 765-771