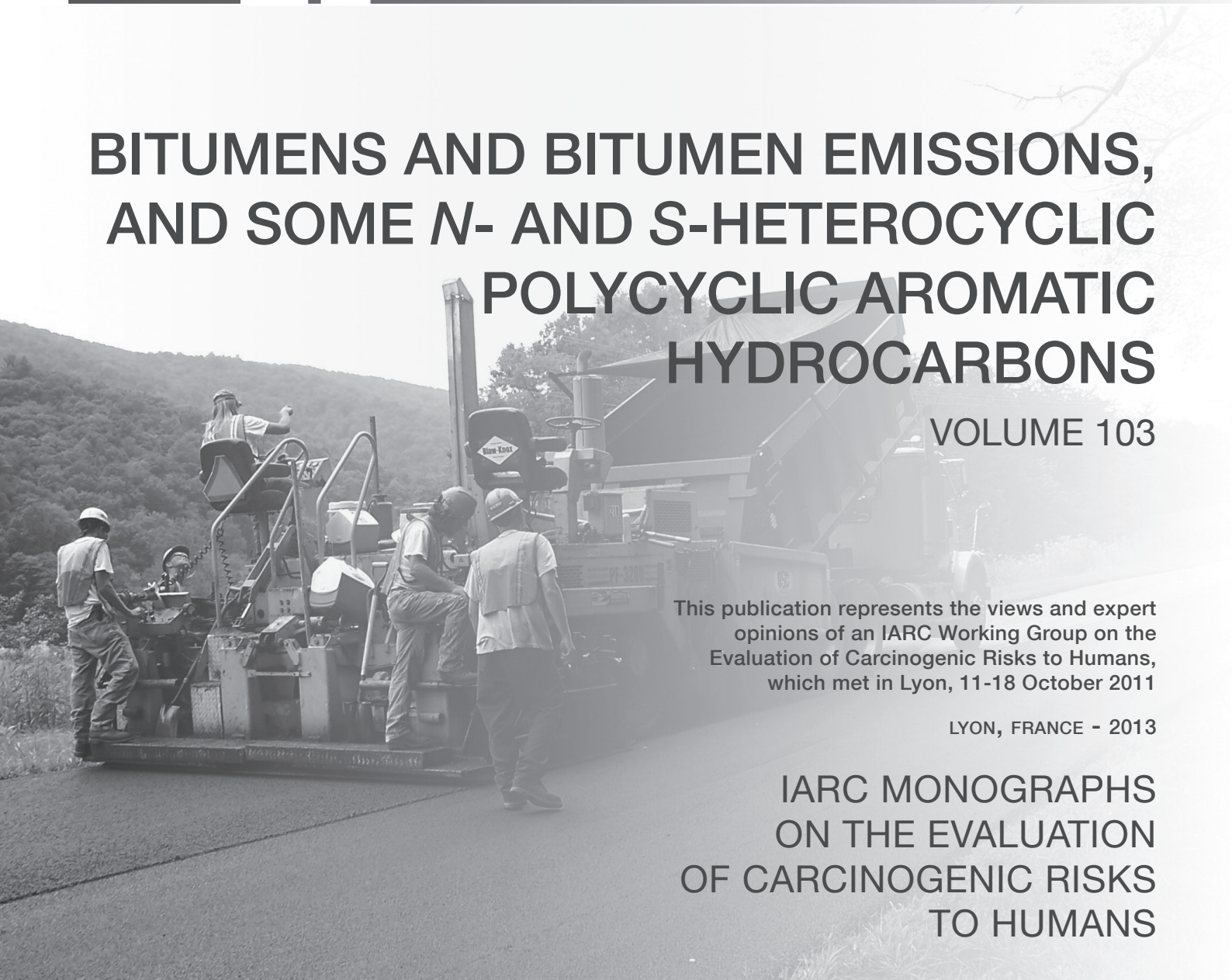IARC MONOGRAPHS

BITUMENS AND BITUMEN EMISSIONS, AND SOME *N*- AND *S*-HETEROCYCLIC POLYCYCLIC AROMATIC HYDROCARBONS

VOLUME 103

This publication represents the views and expert opinions of an IARC Working Group on the Evaluation of Carcinogenic Risks to Humans, which met in Lyon, 11-18 October 2011

LYON, FRANCE - 2013

IARC MONOGRAPHS
ON THE EVALUATION
OF CARCINOGENIC RISKS
TO HUMANS

International Agency for Research on Cancer

World Health Organization

SOME *N*- AND *S*-HETEROCYCLIC POLYCYCLIC AROMATIC HYDROCARBONS

The nine agents under review can be divided into two broad categories: *N*-heterocyclic polycyclic aromatic hydrocarbons (PAHs) – also known as azaarenes – including five acridines and two carbazoles; and *S*-heterocyclic PAHs – also known as thiaarenes – including two thiophenes [*S*-substituted cyclopentadiene moiety].

Benz[*a*]acridine was considered by previous IARC Working Groups in 1983 and 1987 (IARC, 1983, 1987). Since that time, new data have become available; these have been incorporated into the *Monograph*, and taken into consideration in the present evaluation.

Benz[*c*]acridine was considered by previous Working Groups in 1972, 1983, and 1987 (IARC, 1973a, 1983, 1987). Since that time, new data have become available; these have been incorporated into the *Monograph*, and taken into consideration in the present evaluation.

Dibenz[*a,h*]acridine was considered by previous Working Groups in 1972, 1983, and 1987 (IARC, 1973a, 1983, 1987). Since that time, new data have become available; these have been incorporated into the *Monograph*, and taken into consideration in the present evaluation.

Dibenz[*a,j*]acridine was considered by previous Working Groups in 1972, 1983, and 1987 (IARC, 1973a, 1983, 1987). Since that time, new data have become available; these have been incorporated into the *Monograph*, and taken into consideration in the present evaluation.

Dibenz[*c,h*]acridine has not previously been considered by an IARC Working Group.

Carbazole was considered by previous Working Groups in 1983, 1987, and 1998 (IARC, 1983, 1987, 1999). Since that time, new data have become available; these have been incorporated into the *Monograph*, and taken into consideration in the present evaluation.

7*H*-Dibenzo[*c,g*]carbazole (DBC) was considered by previous Working Groups in 1972, 1983, and 1987 (IARC, 1973b, 1983, 1987). Since that time, new data have become available; these have been incorporated into the *Monograph*, and taken into consideration in the present evaluation.

Dibenzothiophene has not previously been considered by an IARC Working Group.

Benzo[*b*]naphtho[2,1-*d*]thiophene has not previously been considered by an IARC Working Group.

1. Exposure Data

1.1 Identification of the agents

From Santa Cruz Biotechnology (2008), PubChem (2011a) and Sigma-Aldrich (2012a).
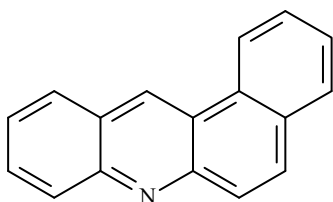
1.1.1 Benz[a]acridine

(a) Nomenclature

Chem. Abstr. Serv. Reg. No.: 225-11-6
RTECS No.: CU2700000
Synonyms: 7-Azabenz[*a*]anthracene;
1,2-benzacridine

(b) Structural and molecular formulae and relative molecular mass

$C_{17}H_{11}N$
Relative molecular mass: 229.29

(c) Chemical and physical properties of the pure substance

Description: Solid powder
Melting-point: 130 °C
Boiling-point: 446.2 °C at 760 mm Hg
Flash-point: 201.4 °C
Density: 1.239 g/cm^3
Solubility: Soluble in water
(0.000 034 g/100 mL); soluble in ethanol,
ether and acetone

1.1.2 Benz[c]acridine
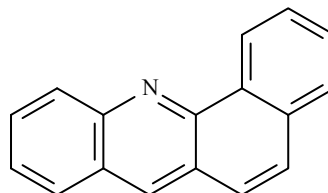
From [HSDB (2003)](#) and [PubChem (2011b)](#).

(a) Nomenclature

Chem. Abstr. Serv. Reg. No.: 225-51-4
RTECS No.: CU2975000
Synonyms: B[c]AC; 3,4-benzacridine;
α-chrysidine; α-naphthacridine

(b) Structural and molecular formulae and relative molecular mass

$C_{17}H_{11}N$
Relative molecular mass: 229.29

(c) Chemical and physical properties of the pure substance

Description: Yellow needles
Melting-point: 108 °C (needles from
aqueous ethanol)
Boiling-point: 446 °C at 760 mm Hg
Flash-point: 201 °C
Density: 1.2 g/cm^3
Solubility: Soluble in water (< 0.000035 g/
100 mL at 25 °C); soluble in ethanol, ether
and acetone

1.1.3 Dibenz[a,h]acridine

From [ChemNet (2011)](#), [Royal Society of Chemistry (2011a)](#) and [Sigma-Aldrich (2012b)](#).
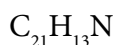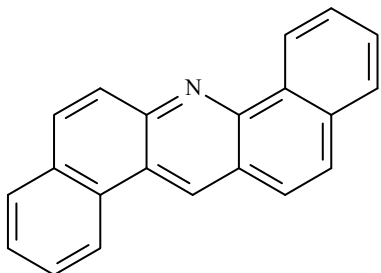
(a) Nomenclature

Chem. Abstr. Serv. Reg. No.: 226-36-8
RTECS No.: HN0875000
Synonyms: 7-Azadibenz[*a,h*]anthracene;
DB[a,h]AC; 1,2,5,6-dibenzacridine;
1,2,5,6-dinaphthacridine; former nomen-
clature: 1,2:5,6-dibenzacridine; dibenz[*a,d*]
acridine

(b) *Structural and molecular formulae and relative molecular mass*

C$_{21}$H$_{13}$N
Relative molecular mass: 279.35

(c) *Chemical and physical properties of the pure substance*

Description: Yellow crystalline solid
Melting-point: 223–224 °C
Boiling-point: 524 °C at 760 mm Hg
Flash-point: 240 °C
Density: 1.3 g/cm^3
Solubility: Soluble in water (0.00016 g/ 100 mL); soluble in acetone and cyclohexane

1.1.4 Dibenz[a,j]acridine

From CSST (2000), GSI Environmental (2010), ALS (2011a) and Sigma-Aldrich (2012c).

(a) *Nomenclature*

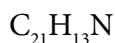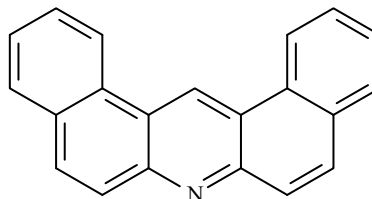*Chem. Abstr. Serv. Reg. No.:* 224-42-0
RTECS No.: HN1050000
Synonyms: 7-Azadibenz[*a,j*]anthracene; DB[*a,j*]AC; 1,2,7,8-dibenzacridine; 3,4,6,7-dinaphthacridine; former nomenclature: dibenz[*a,f*]acridine; 3,4,5,6-dibenzacridine (may correspond to dibenz[*c,h*] acridine)

(b) *Structural and molecular formulae and relative molecular mass*

C$_{21}$H$_{13}$N
Relative molecular mass: 279.35

(c) *Chemical and physical properties of the pure substance*

Description: Yellow crystalline solid
Melting-point: 219.2 °C
Boiling-point: 534 °C at 760 mm Hg
Solubility: Insoluble in water; soluble in ethanol and acetone

1.1.5 Dibenz[c,h]acridine

From Santa Cruz Biotechnology (2007a), LookChem (2008a), Royal Society of Chemistry (2011b) and Sigma-Aldrich (2012d).

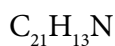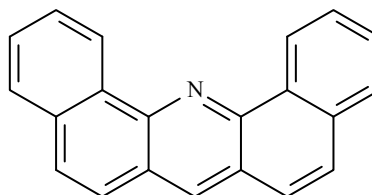(a) *Nomenclature*

Chem. Abstr. Serv. Reg. No.: 224-53-3
RTECS No.: HN1225000
Synonyms: 14-Azadibenz[*a,j*]anthracene; 3,4,5,6-dibenzacridine; former nomenclature: 3:4:5:6-dibenzacridine; 3,4,5,6 dibenzacridine

(b) *Structural and molecular formulae and relative molecular mass*

C$_{21}$H$_{13}$N
Relative molecular mass: 279.35

(c) Chemical and physical properties of the pure substance

Description: Solid
Melting-point: 190.6 °C
Boiling-point: 534 °C at 760 mm Hg
Flash-point: 240.3 °C
Density: 1.274 g/cm^3
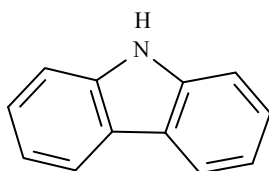*Solubility:* Soluble in water (0.00040 g/ 100 mL); soluble in ethanol and acetone

1.1.6 Carbazole

From PubChem (2011c), Sigma-Aldrich (2012e) and TCI America (2012a).

(a) Nomenclature

Chem. Abstr. Serv. Reg. No.: 86-74-8
RTECS No.: FE3150000
Synonyms: 9-Azafluorene; dibenzopyrrole; diphenylenimide; diphenylenimine

(b) Structural and molecular formulae and relative molecular mass

$C_{12}H_9N$
Relative molecular mass: 167.21

(c) Chemical and physical properties of the pure substance

Description: Yellow solid
Melting-point: 240–246 °C
Boiling-point: 355 °C at 760 mm Hg
Flash-point: 220 °C
Density: 1.1 g/cm^3
Solubility: Insoluble in water; soluble in benzene, chloroform and toluene

1.1.7 7H-*Dibenzo[c,g]carbazole*

From LookChem (2008b), ALS (2011b), Cambridge Isotope Laboratories (2012) and Sigma-Aldrich (2012f).

(a) Nomenclature

Chem. Abstr. Serv. Reg. No.: 194-59-2
RTECS No.: HO5600000
Synonyms: 7-DB[*c,g*]C; 3,4,5,6-dibenz carbazole; 3,4:5,6-dibenzocarbazole; 3,4,5,6-dibenzocarbazole; dibenzo[*c,g*] carbazole; 3,4,5,6-dinaphthacarbazole

(b) Structural and molecular formulae and relative molecular mass
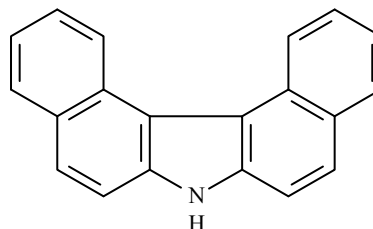
$C_{20}H_{13}N$
Relative molecular mass: 267.32

(c) Chemical and physical properties of the pure substance

Description: Yellow crystalline solid
Melting-point: 156 °C
Boiling-point: 544.1 °C at 760 mm Hg
Flash-point: 246.5 °C
Density: 1.308 g/cm^3
Solubility: Soluble in water (0.0063 g/ 100 mL); soluble in benzene, chloroform and toluene

1.1.8 Dibenzothiophene

From Royal Society of Chemistry (2011c), Sigma-Aldrich (2012 g) and TCI America (2012b).

(a) Nomenclature

Chem. Abstr. Serv. Reg. No.: 132-65-0
RTECS No.: HQ3490550

(b)   Structural and molecular formulae and relative molecular mass

C$_{12}$H$_{8}$S

Relative molecular mass: 184.26

(c)   Chemical and physical properties of the pure substance

Description: Colourless crystals
Melting-point: 97–100 °C
Boiling-point: 332–333 °C at 760 mm Hg
Density: 1.252 g/cm^3
Solubility: Insoluble in water; soluble in benzene and related solvents

*1.1.9   Benzo[*b*]naphtho[2,1-*d*]thiophene*

From Santa Cruz Biotechnology (2007b) and Chemexper (2012).

(a)   Nomenclature

Chem. Abstr. Serv. Reg. No.: 239-35-0
Synonyms: Benzo[*a*]dibenzothiophene; 3,4-benzodibenzothiophene; benzonaphtho[2,1-*d*]thiophene; 1,2-benzodiphenylene sulfide; 1,2-benzo-9-thiafluorene; naphtha[1,2:2,3]thionaphthen; naphtho[1,2-b]thianaphthene; 11-thiabenzo[*a*]fluorene

(b)   Structural and molecular formulae and relative molecular mass

C$_{16}$H$_{10}$S

Relative molecular mass: 234.32

(c)   Chemical and physical properties of the pure substance

Description: Solid
Melting-point: 188–190 °C
Boiling-point: 434.3 °C at 760 mm Hg
Flash-point: 163 °C
Density: 1.292 g/cm^3
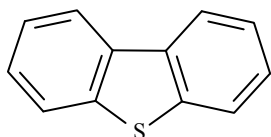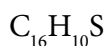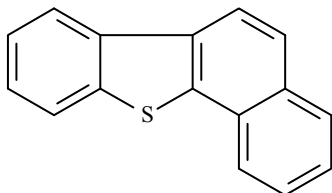*Solubility:* Insoluble in water; soluble in benzene and related solvents

1.2 Analysis

Various techniques have been described for the separation, identification and quantitative determination of *N*- and *S*-heterocyclic PAHs.

Improved isolation of benz[*a*]acridine, benz[*c*]acridine, dibenz[*a,j*]acridine, dibenzo[*c,h*]acridine and carbazole by gas chromatography from tobacco-smoke condensate has been reported (Rothwell &Whitehead, 1969).

Methods for the identification and quantitation of benz[*a*]acridine and its methyl-substituted congeners have been reviewed (Motohashi *et al.*, 1991, 1993).

High-performance liquid chromatography (HPLC) with fluorescence, and gas chromatography with mass spectrometry (GC-MS), were compared for the determination of 20 azaarenes in atmospheric particulate matter, including benz[*a*]acridine, benz[*c*]acridine, dibenz[*a,h*]acridine, dibenz[*a,j*]acridine and dibenzo[*c,h*]acridine (Delhomme & Millet, 2008). Although HPLC was proven to be the most sensitive method, GC-MS was selected in particular for the efficiency of the separation of the azaarenes.

More recently, a liquid chromatography-atmospheric pressure photoionization tandem mass-spectrometric method (LC-MS/MS) was proposed for the determination of azaarenes, including benz[*a*]acridine, benz[*c*]acridine, dibenz[*a,j*]acridine, dibenz[*a,h*]acridine and dibenzo[*c,h*]acridine in atmospheric particulate matter (Lintelmann *et al.*, 2010).

De Voogt & Laane (2009) developed a method to determine the contents of azaarenes (including benz[*a*]acridine and benz[*c*]acridine) and azaarones (oxidized azaarene derivatives) simultaneously by GC-MS in sediment.

Liquid-chromatography tandem mass spectrometry was also used to determine *N*-heterocyclic PAHs in soil (Švábenský *et al.*, 2007).

Chen & Preston (2004) described analytical procedures for the simultaneous determination of both gas- and particle-phase azaarenes of two, three and four rings. Samples of particulate material were collected on the glass-fibre filters and gas-phase material on polyurethane foam plugs. Isolated azaarene compounds were analysed by GC-MS.

1.3 Production and use

None of the heterocyclic PAHs under review are produced for commercial use (IARC, 1983; HSDB, 2009).

1.4 Occurrence and exposure

1.4.1 Occurrence

N-heterocyclic PAHs and *S*-heterocyclic PAHs generally occur as products of incomplete combustion of nitrogen- and sulfur-containing organic matter. Thermal degradation of nitrogen-containing polymers may produce *N*-heterocyclic PAHs (Wilhelm *et al.*, 2000).

(a)   Natural sources

The natural sources of *N*-heterocyclic PAHs and *S*-heterocyclic PAHs are largely analogous to those of other PAHs, namely volcanic activities, wildfires, storm events and fossil fuels (Moustafa & Andersson, 2011).

(b)   Air

N-heterocyclic PAHs and *S*-heterocyclic PAHs enter the environment as a result of natural oil seeps, oil spills, atmospheric deposition, and industrial effluents, or from incinerators (Nito & Ishizaki, 1997). Other sources are automobile exhausts (Yamauchi & Handa, 1987), coal burning, bitumen spreading and tobacco smoke (Rogge *et al.*, 1994).

The mainstream smoke of cigarettes contains dibenz[*a,h*]acridine at up to 0.1 ng per cigarette, dibenz[*a,j*]acridine at up to 10 ng per cigarette and 7*H*-dibenzo[*c,g*]carbazole (DBC) at 700 ng per cigarette (IARC, 2004). The airborne particulate Standard Reference Material (SRM 1649, NIST) contains benz[*c*]acridine at 0.26 µg/g (Durant *et al.*, 1998).

Azaarene compounds have been documented in air (Nielsen *et al.* 1986; Adams *et al.*, 1982; Cautreels *et al.*, 1977; Yamauchi & Handa, 1987; Chen & Preston, 1997, 1998, 2004), but are rarely characterized individually. One study (Delhomme & Millet, 2012) measured mean concentrations of total four-ring azaarenes, including benz[*a*]acridine and benz[*c*]acridine, of 0.007–0.72 ng/m³ in the urban atmosphere. A seasonal variation was observed, in which the maximum concentration occurred in the winter and the minimum in the summer months. [The Working Group noted that this study had sampling issues. There was an important effect of gas/particle partitioning on seasonal variability; the sampling of particulate matter (glass-fibre filter only at high sampling volume, without absorbent or foam) may have introduced some bias. A better approach is the quantitation of azaarenes (two, three and four rings) both in gas phase and particle phase (see Section 1.2; Chen & Preston, 1997, 2004).]

Table 1.1 Concentrations of selected heterocyclic polycyclic aromatic hydrocarbons in soil samples from two creosote-contaminated sites

Compound	Mean concentration ± standard error (mg/kg)	
	Site A (*n* = 3)	Site B (*n* = 3)
S-*heterocyclic PAHs*		
Dibenzothiophene	11.2 ± 0.15	12.6 ± 0.33
Benzo[*b*]naphtho[2,1-*d*]thiophene	15.8 ± 0.48	33.3 ± 0.01
Benzo[*b*]naphtho[2,3-*d*]thiophene	5.2 ± 0.13	13.4 ± 0.48
N-*heterocyclic PAHs*		
Benz[*a*]acridine	1.6 ± 0.03	4.3 ± 0.97
Benz[*c*]acridine	7.3 ± 0.42	13.3 ± 1.20
Dibenz[*a,c*]acridine	0.4 ± 0.01	0.7 ± 0.02
Carbazole	1.0 ± 0.15	0.9 ± 0.05
Dibenzo[*a,i*]carbazole	0.4 ± 0.02	< 0.2

PAH, polycyclic aromatic hydrocarbons
Adapted from Meyer *et al.* (1999)

(c) Soil and sediment

N-heterocyclic PAHs were found in soils (Kočí *et al.*, 2007; Švábenský *et al.*, 2009) and lake sediments (Wakeham, 1979); *S*-heterocyclic PAHs have been detected in fossil fuels, coal and bitumen (Vu-Duc *et al.*, 2007).

The presence of azaarenes in the Dutch surface coastal zone of the North Sea was reported by de Voogt & Laane (2009). The concentrations of acridine in the sediments varied between 9.97 and 63.5 ng/g dry weight (mean, 30.3 ± 15.2 ng/g; *n* = 48). The concentrations of the sum of benz[*a*] acridine and benz[*c*]acridine ranged between 7 and 36.1 ng/g dry weight (mean, 15.4 ng/g).

The concentrations of selected heterocyclic PAHs and metabolites at two creosote-contaminated sites are shown in Table 1.1.

(d) Water

Azaarenes and thiaarenes can be mobilized from land during storm events, transported into the aquatic environment, and contaminate drinking-water, recreation waters, fisheries, and wildlife (US EPA, 2010).

Azaarenes have been shown to dissolve more rapidly in water than homocyclic PAHs (Pearlman *et al.*, 1984). Azaarenes have been reported in rainwater (Chen & Preston, 1998).

Benzothiophene was found in one sample of stormwater runoff samples in California, at a concentration of 110 ± 13 ng/L (Zeng *et al.*, 2004).

Hamilton Harbour, located on Lake Ontario, Canada, is representative of a lake heavily polluted by industrial chemicals (Marvin *et al.*, 2000). Thiaarene profiles of reference and sediment samples showed that harbour contamination could be distinguished as arising from two primary sources of contamination: mobile emissions and emissions related to steel manufacturing.

Detailed investigation in Germany showed that the distribution of non-polar compounds (such as homocyclic PAHs) can only be detected close to the source of contamination, whereas the distribution of more polar compounds (such as azaarenes and thiaarenes) and degradation products is more widespread downstream of an aquifer (Schlanges *et al.*, 2008). Table 1.2 shows the range of concentrations of some *N*-heterocyclic PAHs and *S*-heterocyclic PAHs in groundwater samples analysed at four tar-contaminated sites in Germany (Schlanges *et al.*, 2008).

Table 1.2 Concentrations of selected heterocyclic polycyclic aromatic hydrocarbons in groundwater samples from four tar-contaminated sites in Germany

Compound	Concentration, range of means (µg/L)			
	Castrop Rauxel (n = 61)	Wülknitz (n = 8)	Stuttgart (n = 5)	Lünen (n = 14)
Carbazole	ND–101	ND–51	ND–19	ND–135
1-Benzothiophene	ND–1420	8–947	< 1–1	ND–1572
Dibenzothiophene	ND–4	ND–15	ND–2	ND–4

ND, not detected or below limit of detection (0.2–30 ng/L)

Adapted from Schlanges et al. (2008)

(e) Food

Hydroxydibenzothiophenes, including C1–C3-substituted dibenzothiophenes, were detected by GC-MS in considerable amounts in fish bile sampled in Alaska after the Exxon Valdez oil spill (Krahn et al., 1992).

The concentrations of benz[c]acridine and of dibenzacridine isomers in grilled meat were found to be in the range of 0.2 to 2.9 ng/g meat (Janoszka, 2007). Table 1.3 provides information about content of acridines in cooked meat (Blaszczyk & Janoszka, 2008).

(f) Bitumens and bitumen fume

The S-heterocyclic PAHs dibenzothiophene, benzo[b]naphtho[1,2-d]thiophene and benzo[b]naphtho[2,1-d]thiophene were detected in raw bitumen samples and in laboratory-generated bitumen fume at concentrations of 1.3–7.6 µg/g

and 15–384 µg/g, respectively, as shown in Table 1.4 (Vu-Duc et al., 2007).

(g) Crude oil

Nitrogen compounds are frequently present in fossil fuels, generally associated with the organic portion of crude material. The presence of dibenz[a,j]acridine at concentrations of 1–6.3 µg/L was reported in aviation kerosene (Rocha da Luz et al., 2009).

The S-heterocyclic PAHs dibenzothiophene, benzo[b]naphtho[1,2-d]thiophene and benzo[b]naphtho[2,1-d]thiophene were detected in petroleum crude oil (SRM 1582) at concentrations of 34, 11.4 and 3.8 ppm, respectively (Mössner & Wise, 1999).

Table 1.3 Concentrations of selected N-heterocyclic polycyclic aromatic hydrocarbons in cooked meat (pork joint)

Compound	Mean concentration ± standard error (ng/g)			
	Collar		Chop	
	Meat	Gravy	Meat	Gravy
Benz[c]acridine	0.83 ± 0.37	0.09 ± 0.01	0.99 ± 0.29	0.21 ± 0.01
Benz[a]acridine	0.54 ± 0.24	0.07 ± 0.01	0.13 ± 0.05	0.13 ± 0.03
Dibenz[a,j]acridine	0.36 ± 0.17	0.07 ± 0.01	0.09 ± 0.03	0.08 ± 0.03
Dibenz[a,h]acridine	0.52 ± 0.21	0.06 ± 0.01	0.17 ± 0.02	0.11 ± 0.04

Adapted from Blaszczyk & Janoszka (2008)

Table 1.4 Concentrations of *S*-heterocyclic polycyclic aromatic hydrocarbons in raw bitumen and in bitumen fume generated in a laboratory at 170 °C

Compound	Mean concentration ± standard error (µg/g)	
	Bitumen (*n* = 6)	Bitumen fume[a] (*n* = 6)
Dibenzothiophene	3.6 ± 0.02	384.1 ± 38
Benzo[*b*]naphtho[1,2-*d*]thiophene	1.3 ± 0.6	15.0 ± 0.9
Benzo[*b*]naphtho[2,1-*d*]thiophene	7.6 ± 0.5	54.4 ± 3.6

[a] Concentration in µg/g of collected fumes
Adapted from Vu-Duc *et al.* (2007)

(h) Coal tar

Carbazole has been reported to be a major active component of coal tar that is responsible for its antipsoriatic activity (Arbiser *et al.*, 2006).

1.4.2 Occupational exposure

No occupational exposure data concerning *N*-heterocyclic PAHs and *S*-heterocyclic PAHs specifically were available for the Working Group, except for some *S*-heterocyclic PAHs detected while generating bitumen fume in a laboratory (Binet *et al.*, 2002). However, it must be noted that *S*-heterocyclic PAHs occur in many of the same occupational settings in which exposure to other PAHs occurs. For example, *S*-heterocyclic PAHs, including dibenzothiophene, benzo[*b*]naphtha[2,1-*d*]thiophene and benzo[*b*]naphtha[1,2-*d*]thiophene, were detected in bitumen and bitumen emissions at concentrations of 1.3–7.6 and 15–384 µg/g respectively, as shown in Table 1.4.

1.5 Regulations and guidelines

No data specifically concerning *N*- or *S*-heterocyclic PAHs were available to the Working Group.

2. Cancer in Humans

No data were available to the Working Group.

3. Cancer in Experimental Animals

3.1 Benz[*a*]acridine

One study in mice treated by skin application was evaluated as inadequate by the Working Group and was not taken into consideration for the evaluation (Lacassagne *et al.*, 1956). This study is not presented in the tables.

3.1.1 Mouse

Skin application

Twelve XVII mice (age and sex not specified) were each given one drop of a 0.3% solution of benz[*a*]acridine (purity not reported) in acetone, applied to the nape of the neck, twice per week for up to 54 weeks (Lacassagne *et al.*, 1956). Six of the mice did not survive past day 90 of treatment and the remaining mice were removed from the study between days 165 and 379. None of the mice developed skin tumours. [The Working Group noted several deficiencies in this study, including the limited number of mice tested, the lack of concurrent control group, the lack of information on the age and sex of the mice, on the purity and amount of benz[*a*]acridine administered, on

Table 3.1 Study of carcinogenicity in rats given benz[*a*]acridine by intrapulmonary injection

Species, strain (sex) Duration Reference	Dosing regimen Animals/group at start	Incidence of tumours	Significance
Rat, Osborne-Mendel (F) At least 111 wk Deutsch-Wenzel *et al.* (1983)	A single pulmonary implantation of 0, 0.2, 1.0, or 5.0 mg (purity, 99.8%) in 50 µL of a 1 : 1 mixture of beeswax and tricaprylin. An additional group was untreated. Positive control groups received benzo[*a*]pyrene at 0.1, 0.3, or 1 mg. 35 rats/group	*Pleomorphic sarcoma* Not observed in untreated or benz[*a*] acridine-treated groups. Benzo[*a*]pyrene: 3/35 (9%), 0/35, 0/35 *Epidermoid carcinoma* Not observed in untreated or groups treated with benz[*a*]acridine. Benzo[*a*]pyrene: 5/35 (14%), 24/35 (69%), 27/35 (77%)	[NS]

F, female; wk, week; NS, not significant

the histological procedures employed, and on the poor survival of the dosed mice.]

3.1.2 Rat

See Table 3.1

Intrapulmonary injection

Groups of 35 female Osborne-Mendel rats (age, 3 months; mean body weight, 247 g) were given benz[*a*]acridine as a single pulmonary implantation of 0.0, 0.2, 1.0, or 5.0 mg (purity, 99.8%) in 50 µL of a 1 : 1 mixture of beeswax and tricaprylin that had been preheated to 60 °C (Deutsch-Wenzel *et al.*, 1983). One group of 35 rats was not treated. Positive control groups were also included, consisting of groups of 35 rats that were given benzo[*a*]pyrene as a pulmonary implantation of 0.1, 0.3, or 1.0 mg in beeswax and tricaprylin.

All rats survived the surgical procedure. Mean survival in rats given benz[*a*]acridine (105–111 weeks) was similar to that in rats in the control groups (103 and 110 weeks). The lungs and any other organs showing abnormalities were examined by histopathology. Lung tumours were not detected in rats given benz[*a*]acridine or in the control groups. In comparison, rats given benzo[*a*]pyrene had a dose-dependent increase in the incidence of lung epidermoid carcinoma,

with incidence being 5 out of 35 (14%) at 0.1 mg, 24 out of 35 (69%) at 0.3 mg, and 27 out of 35 (77%) at 1.0 mg.

3.2 Benz[c]acridine

Two studies using skin application in mice or lung implantation in rats were evaluated as inadequate by the Working Group and were not taken into consideration for the evaluation (Lacassagne *et al.*, 1956; Hakim, 1968). The limitations of these studies included the small number of mice tested, the lack of a concurrent vehicle-control group, lack of information on the strain, age and sex of the animals, lack of information on the purity and total amount of benz[*c*]acridine administered, and absence of any description of the histological procedures employed. These studies are not presented in the tables.

3.2.1 Mouse

See Table 3.2

(a) Skin application

Twelve XVII mice (age and sex not reported) were each given one drop of a 0.3% solution of benz[*c*]acridine (purity not reported) in acetone, applied to the nape of the neck, twice per week, for up to 54 weeks (Lacassagne *et al.*, 1956). Five

Table 3.2 Studies of carcinogenicity in mice given benz[c]acridine

Species, strain (sex) Duration Reference	Dosing regimen Animals/group at start	Incidence and multiplicity of tumours	Significance	Comments
Skin application – initiation–promotion				
Mouse, CD-1 (F) 27 wk Levin *et al.* (1983)	Single topical application of 0, 0.4, 1.0 or 2.5 µmol of benz[c]acridine in 200 µL of 5% DMSO in acetone to the shaved dorsal surface. After 12 days, topical application of 16 nmol of TPA in 200 µL of acetone, twice/wk 30 mice/group	*Skin papilloma* No. of tumour-bearing animals 15 wk: 3%, 10%, 13%, 30% 25 wk: 7%, 23%, 16%, 37% Multiplicity 15 wk: 0.03 ± 0.03, 0.10 ± 0.06, 0.19 ± 0.10, 0.77 ± 0.26 25 wk: 0.10 ± 0.06, 0.30 ± 0.12, 0.27 ± 0.17, 1.33 ± 0.38	Incidence: $P < 0.05$ for 2.5 µmol of benz[c] acridine *vs* control at 15 and 25 wk Multiplicity: $P < 0.05$ for 2.5 µmol of benz[c] acridine *vs* control at 15 and 25 wk	Purity of benz[c]acridine, NR
Mouse, CD-1 (F) 21 wk Chang *et al.* (1986)	Single topical application of 2.5 µmol of benz[c]acridine, benz[a]anthracene or 7-methylbenz[c]acridine (purity, ≥ 97%) in 200 µL of 5% DMSO in acetone to the shaved dorsal surface. Control group treated with 5% DMSO in acetone. After 9 days, all groups treated with topical applications of 16 nmol of TPA in 200 µl of acetone twice/wk for 20 wk 30 mice/group	*Skin papilloma* Incidence Benz[c]acridine: 16/30 (54%) Benz[a]anthracene: 11/30 (37%) 7-Methylbenz[c]acridine: 23/30 (77%) Control: 2/30 (7%) Multiplicity Benz[c]acridine: 0.89 ± 0.20 Benz[a]anthracene: 0.50 ± 0.14 7-Methylbenz[c]acridine: 4.47 ± 0.94 Control: 0.07 ± 0.05	Incidence: $P < 0.05$ for all three compounds; method, NR Multiplicity: $P < 0.05$ for all three compounds; method, NR	
Intraperitoneal injection				
Mouse, Swiss-Webster [Blu:Ha (ICR)] newborn (M, F) 37 wk Chang *et al.* (1984)	Injections on postnatal days 1, 8, and 15 with 150, 300 and 600 nmol (total dose, 1 050 nmol) of benz[c]acridine ("pure") in 5, 10, and 20 µl DMSO A control group was treated in similar manner with vehicle only 30 M and 30 F/group	*Lung tumours* (primarily adenoma) M: 9/13 (69%), 4/24 (17%) F: 12/20 (60%), 2/16 (12%) *Liver tumours* (mostly neoplastic nodules) M: 2/13 (15%), 0/24.	$P < 0.05$, Fisher $2 \times K$ exact test for M and F combined	

DMSO, dimethylsulfoxide; F, female; M, male; NR, not reported; NS, not significant; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; *vs, versus*; wk, week

of the mice did not survive past day 90 of treatment; the remaining mice were removed from the study between days 230 and 394. None of the mice developed skin tumours. [The Working Group noted several deficiencies in this study, including the limited number of mice tested, the lack of data concerning the concurrent control group, the age and sex of the mice, the purity and amount of benz[c]acridine administered, the histopathological procedures employed, and the poor survival of the dosed mice.]

As part of a study investigating the carcinogenicity of the alkaloid sanguinarine (Hakim, 1968), 64 Swiss mice (Haffkine, or their hybrids) (sex and age not specified) were treated by placing a drop of a 0.3% solution of benz[c]acridine (purity not reported) in benzene applied to the skin between the ears, three times per week for up to 67 weeks. Fifty mice survived 180 days and 19 survived 400 days. Five epitheliomas (squamous cell carcinoma) were found in the 19 mice surviving beyond 400 days (26%).

A second experiment was conducted in which 24 mice were treated in a manner identical to the first experiment and, in addition, were given 0.5% croton oil in acetone (volume not specified) once per week. Eighteen mice survived 180 days and only three survived until the first tumour was detected (time not specified). Two epitheliomas (squamous cell carcinoma) were found in the three surviving mice.

In a third experiment, 24 mice were treated topically twice with benz[c]acridine (the interval between treatments and amount of benz[c]acridine was not specified). After 1 month, they were treated with croton oil (amount not specified) once per week. Sixteen mice survived 180 days and four survived 400 days. No tumours were detected.

As a control, 12 mice were given croton oil once per week (Hakim, 1968). Four mice survived 180 days and two survived 400 days. No tumours were detected. [The Working Group noted several deficiencies, including the lack of a concurrent vehicle-control group for the first experiment, the lack of information on the strain, age and sex of the mice, on the purity of the benz[c]acridine, on the amount of benz[c]acridine administered, and on the histopathological procedures employed, the poor survival of the test mice, and the use of benzene, which is classified as a carcinogen (IARC Group 1), as a vehicle.]

As part of a study to determine the tumour-initiating ability of oxidized derivatives of benz[c]acridine, groups of 30 female CD-1 mice (age, 7 weeks) received a single topical application of benz[c]acridine at 0.4, 1.0, or 2.5 µmol (purity not reported), in 200 µL of 5% dimethyl sulfoxide (DMSO) in acetone, applied to the shaved dorsal surface (Levin et al., 1983). A control group of 30 mice received the solvent only. Twelve days later, all rats received topical applications of 12-O-tetradecanoylphorbol-13-acetate (TPA) at 16 nmol in 200 µL of acetone, twice per week for 25 weeks. The formation of papillomas was monitored every 2 weeks; those papillomas of 2 mm or greater in diameter and persisting more than 2 weeks were included in the final total. The tumours were not examined by histopathology. At least 28 mice in each group survived until the end of the experiment.

After 15 weeks of treatment with TPA, the percentage of tumour-bearing mice and the multiplicity of tumours were 3%, 10%, 13%, and 30%, and 0.03 ± 0.03, 0.10 ± 0.06, 0.19 ± 0.10, and 0.77 ± 0.26 (mean ± standard error) for the groups at 0.0, 0.4, 1.0, and 2.5 µmol benz[c]acridine, respectively. The comparable values after 25 weeks of treatment with TPA were 7%, 23%, 16%, and 37%, and 0.10 ± 0.06, 0.30 ± 0.12, 0.27 ± 0.17, and 1.33 ± 0.38. Compared with the control group, the incidence and multiplicity of tumours was significantly increased in the groups receiving benz[c]acridine at 2.5 µmol (fourfold contingency test and Student's t-test, respectively) at both time-points. In the same study, benz[c]acridine-3,4-dihydrodiol and benz[c]acridine-anti-3,4-dihydrodiol-1,2-epoxide were

potent initiators of skin tumours in mice and induced lung and liver tumours when administered to newborn mice.

As part of a study to compare the tumour-initiating ability of benz[*c*]acridine with that of benz[*a*]anthracene and 7-methylbenz[*c*]acridine, groups of 30 female CD-1 mice (age, 7 weeks) were given a single dose of 2.5 µmol of each compound (purity of benz[*c*]acridine, ≥ 97%) in 200 µL of 5% DMSO in acetone, applied topically to the shaved dorsal surface (Chang *et al.*, 1986). A control of 30 mice received the solvent only. Nine days later, all mice received 16 nmol of TPA in 200 µL of acetone, applied twice per week for 20 weeks. The formation of papillomas was monitored every 2 weeks; those papillomas of 2 mm or greater in diameter and persisting more than 2 weeks were included in the final total. The tumours were not examined by histopathology. The number of mice surviving until the end of the study was not indicated.

The percentage of mice with papilloma and the multiplicity of papillomas in mice treated with benz[*c*]acridine were 54% [16 out of 30] and 0.89 ± 0.20 (mean ± standard error of the mean), which were significantly greater ($P < 0.05$; statistical tests not specified) than the values observed in the control group (7% [2 out of 30] and 0.07 ± 0.05, respectively). In mice treated with benz[*a*]anthracene, the incidence and multiplicity of tumours were 37% [11 out of 30] and 0.50 ± 0.14, while in mice treated with 7-methylbenz[*c*]acridine, these values were 77% [23 out of 30] and 4.47 ± 0.94, respectively.

(b)   Intraperitoneal injection

As part of an investigation to compare the tumorigenicity of suspected benz[*c*]acridine metabolites, groups of 20–40 male and 20–40 female newborn Swiss-Webster [Blu:Ha (ICR)] mice were given intraperitoneal injections of benz[*c*]acridine, benz[*c*]acridine-1,2-dihydrodiol, benz[*c*]acridine-3,4-dihydrodiol, benz[*c*]acridine-5,6-dihydrodiol, benz[*c*]acridine-8,9-dihydrodiol, benz[*c*]acridine-10,11-dihydrodiol, benz[*c*]acridine-5,6-oxide, benz[*c*]acridine-*syn*-3,4-dihydrodiol-1,2-epoxide, benz[*c*]acridine-*syn*-8,9-dihydrodiol-10,11-epoxide, or benz[*c*]acridine-*anti*-8,9-dihydrodiol-10,11-epoxide at a dose of 150, 300, or 600 nmol (total dose, 1050 nmol), or benz[*c*]acridine-*anti*-3,4-dihydrodiol-1,2-epoxide at a dose of 70, 140, or 280 nmol (total dose, 490 nmol) in 5, 10, and 20 µL of DMSO on postnatal days 1, 8, and 15 (Chang *et al.*, 1984). All compounds were pure as determined by nuclear magnetic resonance spectroscopy. A control group consisting of 30 male and 30 female mice was treated in an identical manner with the vehicle only. Forty-six of the mice in the control group and 30–66 of the experimental mice survived until weaning at postnatal day 25. The experiment was terminated when the mice were aged 33–37 weeks. A gross necropsy was performed, and selected lung and all liver tumours were examined histologically.

The incidence of lung tumours (primarily adenoma) in female and male mice treated with benz[*c*]acridine was 60.0% [12 out of 20] and 69.2% [9 out of 13], respectively, with a multiplicity of 3.15 and 1.86 tumours per mouse. The comparable incidence values in control female and male mice were 12.5% [2 out of 16] and 16.7% [4 out of 24], with a multiplicity of 0.13 and 0.17 tumours per mouse. The incidence [63.6%; 21 out of 33] and multiplicity [2.64 tumour per mouse] of lung tumours in combined male and female mice treated with benz[*c*]acridine were statistically significantly different than the incidence ([15.0%; 6 out of 40]; $P < 0.05$, Fisher $2 \times K$ exact test) and multiplicity ([0.15 tumours per mouse]; $P < 0.05$, statistical test not specified) in combined male and female mice in the control group. The incidence of lung tumours in mice treated with benz[*c*]acridine-3,4-dihydrodiol, benz[*c*]acridine-8,9-dihydrodiol, benz[*c*]acridine-10,11-dihydrodiol, benz[*c*]acridine-*syn*-3,4-dihydrodiol-1,2-epoxide, and benz[*c*]acridine-*anti*-3,4-dihydrodiol-1,2-epoxide was

Table 3.3 Study of carcinogenicity in rats given benz[*c*]acridine by intrapulmonary implantation

Species, strain (sex) Duration Reference	Dosing regimen, Animals/group at start	Incidence of tumours
Rat, Osborne-Mendel (F) At least 116 wk Deutsch-Wenzel *et al.* (1983)	A single pulmonary implantation of 0, 0.2, 1.0, or 5.0 mg of benz[*c*]acridine (purity, 99.8%) in 50 µl of a 1 : 1 mixture of beeswax and tricaprylin. An additional group was untreated. Positive-control groups received benzo[*a*]pyrene at 0.1, 0.3, or 1.0 mg 35 rats/group	*Pleomorphic sarcoma* Untreated, 0, 0.1, 0.3, and 1 mg benz[*c*]acridine: 0/35, 0/35, 0/35, 0/35, 1/35 (3%) Benzo[*a*]pyrene: 3/35 (9%), 0/35, 0/35 *Epidermoid carcinoma* Untreated or benz[*c*]acridine-treated groups: no tumours observed Benzo[*a*]pyrene: 5/35 (14%), 24/35 (69%), 27/35 (77%)-

F, female; NR, not reported; wk, week

statistically significantly different from that in the control group, with values of 82.0%, 37.4%, 41.5%, 46.6%, and 100%, respectively.

Male mice treated with benz[*c*]acridine also developed liver tumours ("mostly type A or neoplastic nodules"), with an incidence of 15.4% [2 out of 13] and a multiplicity of 0.15 tumours per mouse. Liver tumours were not found in control male mice [0 out of 24]. The incidence of liver tumours in male mice treated with benz[*c*]acridine-3,4-dihydrodiol and benz[*c*]acridine-*anti*-3,4-dihydrodiol-1,2-epoxide was 58.6% [17 out of 29] and 81.3% [13 out of 16], values that were significantly different from those in the control group [$P < 0.0001$; one-tailed Fisher exact test]. None of the other benz[*c*]acridine dihydrodiols or dihydrodiol epoxides caused a significant increase in the incidence of liver tumours.

3.2.2 Rat

See Table 3.3

(a) Intrapulmonary implantation

Groups of 35 female Osborne-Mendel rats (age, 3 months; mean body weight, 247 g) received a single pulmonary implantation of benz[*c*]acridine (purity, 99.8%) at a dose of 0.0, 0.2, 1.0, or 5.0 mg in 50 µL of a 1 : 1 mixture of beeswax and tricaprylin that had been preheated to 60 °C (Deutsch-Wenzel *et al.*, 1983). Another group of 35 rats was not treated. Positive controls were also included, comprising groups of 35 rats receiving a pulmonary implantation of benzo[*a*]pyrene of 0.1, 0.3, or 1.0 mg in a 1 : 1 mixture of beeswax and tricaprylin.

All rats survived the surgical procedure. The mean survival in rats given benz[*c*]acridine (112–116 weeks) was similar to that in the control groups (103 and 110 weeks). The lungs and any other organs showing abnormalities were examined by histopathology. One rat given 1.0 mg of benz[*c*]acridine developed a pleomorphic sarcoma at the implantation site. None of the other rats treated with benz[*c*]acridine and none of the rats in either of the control groups developed lung tumours. In comparison, rats given benzo[*a*]pyrene had a dose-dependent increase in lung epidermoid carcinoma, with the incidence being 5 out of 35 (14%) at 0.1 mg, 24 out of 34 (69%) at 0.3 mg, and 27 out of 35 (77%) at 1.0 mg.

(b) Bladder implantation

As part of a study investigating the carcinogenicity of the alkaloid sanguinarine, 58 rats (strain, age, and sex not specified) received a paraffin pellet (~15 mg) containing an unspecified amount of benz[*c*]acridine (the pellets were prepared by dissolving benz[*c*]acridine in chloroform and mixing with paraffin in a weight ratio of 1 : 3) implanted into the bladder (Hakim, 1968).

An additional group of 64 rats was implanted with pellets not containing benz[*c*]acridine. The experiment was terminated after 16 months. In the rats implanted with benz[*c*]acridine pellets, there were 29 bladder papillomas, of which 8 were "cancers." In the control rats, there were two bladder papillomas [*P* < 0.0001; one-tailed Fisher exact test]. [The Working Group noted several deficiencies in this study, including the lack of information on the strain, age, and sex of the rats, and on the purity and amount of benz[*c*]acridine administered, the inadequate description of the histopathological procedures, and the use of chloroform, which is classified as a possible carcinogen (IARC Group 2B), to dissolve the benz[*c*]acridine.]

3.3 Dibenz[*a,h*]acridine

Several studies in mice given dibenz[*a,h*] acridine by oral administration, skin application or subcutaneous injection were evalauated as inadequate by the Working Group and were not taken into consideration for the final evaluation (Barry *et al.*, 1935; Bachmann *et al.*, 1937; Orr, 1938; Andervont & Shimkin, 1940; Badger *et al.*, 1940; Lacassagne *et al.*, 1956). The limitations of these studies included the small number of mice tested, lack of concurrent vehicle control group, lack of information on strain, age and sex of the animals, lack of information on the purity and total amount of dibenz[*a,h*]acridine administered, absence of description of the histological procedures employed, lack of description of the tumours, and the use of benzene as a vehicle. These studies are not presented in the tables.

3.3.1 Mouse

See Table 3.4

(a) Oral administration

In one experiment, a group of 10 mice (age, sex, and strain not specified) was fed dibenz[*a,h*] acridine ("pure") as a 2% or 4% solution in olive oil (volume not reported) mixed with their food (Badger *et al.*, 1940). The last mouse died 627 days after the initiation of dosing. Two of the mice developed multiple sebaceous adenomas and other tumours. In another experiment, an unspecified number of mice (age, sex, and stain not specified) was given dibenz[*a,h*]acridine ("pure") orally, either as in the previous experiment or by gavage (1 mg in 250 μL of butter or margarine, 5 days per week). Of the 12 mice that were examined from day 100 onward, 5 developed papilloma and epithelioma [squamous cell carcinoma] of the stomach. [The Working Group noted that the experimental details and results were poorly presented. The Working Group also noted several deficiencies in both experiments, including the limited number of mice tested, the lack of a concurrent control group, and the lack of information on the age, sex, and strain of the mice, on the purity and total amount of the dibenz[*a,h*]acridine administered, on the precise route of administration, and on the histopathological procedures employed.]

(b) Skin application

A group of 10 mice (age, sex, and strain not specified) was given an unspecified amount of dibenz[*a,h*]acridine (purity not reported) as a 0.3% solution in benzene applied to the interscapular region, twice per week (Barry *et al.*, 1935). Seven of the mice survived 6 months and the last mouse died after 349 days of treatment. One mouse developed an epithelioma [squamous cell carcinoma]. In a second experiment, a group of 30 mice was treated in a manner identical to the first experiment. Twenty-seven of the mice survived 6 months, 12 of the mice survived 12 months, and the last mouse died 482 days after the initiation of treatment. Four

Table 3.4 Studies of carcinogenicity in mice given dibenz[*a,h*]acridine

Species, strain (sex) Duration Reference	Dosing regimen, Animals/group at start	Incidence and multiplicity of tumours	Significance	Comments
Skin application – initiation–promotion				
Mouse, CD-1 (F) 26 wk Kumar *et al.* (2001)	Single topical application of 500 nmol of dibenz[*a,h*]acridine (purity, > 98%) in 200 μL of acetone to the shaved dorsal surface. After 9 days, treated with 16 nmol of TPA in 200 μL of acetone, twice/wk for 25 wk. Control group was treated with acetone only. 30 mice/group	*Papilloma* Dibenz[*a,h*]acridine: 24/30 (80%) Control: 0/30 Multiplicity: 3.33 ± 0.57	Incidence: $P < 0.0001$; Fisher exact test Multiplicity: $P < 0.01$; Student's *t*-test	No histopathological examination was made.
Mouse, CD-1 (F) 21 wk Kumar *et al.* (2001)	Single topical application of 50 or 175 nmol of dibenz[*a,h*]acridine (purity, > 98%) in 200 μL of acetone to the shaved dorsal surface. After 9 days, treated with 16 nmol of TPA in 200 μL of acetone, twice/wk for 20 wk. Control group was treated with acetone only. 30 mice/group	*Papilloma* 10 wk TPA 175 nmol: 6/30 (20%) Control: 0/30 Multiplicity: 0.2 ± 0.09 20 wk TPA 50 nmol: 18/30 (60%) 175 nmol: 24/30 (80%); Control: 1/30 (3%) Multiplicity: 0.04 ± 0.04; 1.6 ± 0.33, 2.3 ± 0.37	Multiplicity: $P < 0.05$; Fisher exact test (compared with controls) Multiplicity: $P < 0.01$ for both dose groups; Fisher exact test (compared with controls)	No histopathological examination was made.
Intravenous injection				
Mouse, Strain A (M, F) 20 wk Andervont & Shimkin (1940)	0, 250 μg in 250 μL of water. Controls received water only	*Pulmonary tumours* 8 wk: 1/20 (5%), 3/10 (30%) (multiplicity: 1.0, 1.3) 14 wk: 3/20 (15%), 9/13 (69%) (multiplicity, 1.0; 2.9) 20 wk: 4/19 (21%), 11/12 (92%) (multiplicity, 1.0, 2.2)	[$P = 0.0025$; one-tailed Fisher exact test] [$P = 0.0002$; one-tailed Fisher exact test]	Number at start, NR

F, female; M, male; NR, not reported; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; wk, week

(13%) mice developed epithelioma [squamous cell carcinoma] and two (7%) developed skin papilloma. [The Working Group noted that the experimental details and results were poorly presented, and there were several deficiencies in both experiments, including the limited number of mice tested in the first experiment, the lack of a concurrent control group, the lack of information on the age, sex, and strain of the mice, on the purity and amount of dibenz[*a,h*]acridine administered, and the use of benzene, which is classified as a carcinogen (IARC Group 1), as the vehicle.]

A group of 10 mice (age, sex, and strain not specified) was given "a few drops" of a saturated solution (concentration not specified) of dibenz[*a,h*]acridine (purity not reported) in acetone, applied topically to the interscapular region at weekly intervals (Orr, 1938). Two mice survived 28 weeks of treatment and one of these mice developed a skin tumour. [The Working Group noted several deficiencies including the lack of a concurrent control group, the lack of information on the age, sex and strain of the mice, and on the purity and amount of dibenz[*a,h*]acridine administered, and the poor survival of the dosed mice.]

A group of 40 mice (age, sex, and strain not specified) was given an unspecified amount of dibenz[*a,h*]acridine (purity not reported) as a 0.3% solution in benzene, applied topically twice per week (Badger *et al.*, 1940). The last mouse died after 482 days of treatment. Two mice developed papilloma and five developed epithelioma [squamous cell carcinoma]. [The Working Group noted several deficiencies in this study, including the lack of a concurrent control group, lack of information on the age, sex and strain of the mice, or on the purity and amount of dibenz[*a,h*]acridine administered, and the use of benzene, which is classified as a carcinogen (IARC Group 1), as the vehicle.]

A group of 12 XVII mice (age and sex not specified) was given one drop of dibenz[*a,h*]

acridine (purity not reported) as a 0.3% solution in acetone applied to the nape of the neck, twice per week, for up to 416 days (Lacassagne *et al.*, 1956). Six of the mice did not survive 90 days of treatment; the remaining mice were removed from the study between days 93 and 416. One mouse developed an epithelioma [squamous cell carcinoma]; at this time, three mice were still alive. [The Working Group noted several deficiencies in this study, including the limited number of mice tested, the lack of a concurrent control group, lack of information on the age and sex of the mice, or on the purity and amount of dibenz[*a,h*]acridine administered, and the poor survival of the mice tested.]

As part of a study to determine the tumour-initiating ability of a series of oxidized dibenz[*a,h*]acridine derivatives, a group of 30 female CD-1 mice (age, 7 weeks) received a single topical application of 500 nmol of dibenz[*a,h*]acridine, dibenz[*a,h*]acridine-1,2-dihydrodiol, dibenz[*a,h*]acridine-3,4-dihydrodiol, dibenz[*a,h*]acridine-8,9-dihydrodiol, dibenz[*a,h*]acridine-10,11-dihydrodiol, dibenz[*a,h*]acridine-*anti*-3,4-dihydrodiol-1,2-epoxide, dibenz[*a,h*]acridine-*syn*-3,4-dihydrodiol-1,2-epoxide, dibenz[*a,h*]acridine-*anti*-10,11-dihydrodiol-8,9-epoxide, dibenz[*a,h*]acridine-*syn*-10,11-dihydrodiol-8,9-epoxide (purity, > 98%) in 200 μL of acetone applied to the shaved dorsal surface (Kumar *et al.*, 2001). A control group of 30 mice received only the solvent. Nine days later, all mice received applications of 16 nmol of TPA in 200 μL of acetone, twice per week for 25 weeks. The formation of skin papillomas was monitored macroscopically every 2 weeks; those papillomas of 2 mm or greater in diameter and persisting more than 2 weeks were included in the final total. The tumours were not examined by histopathology. The number of mice surviving until the end of the study was not indicated.

The incidence of papilloma in mice treated with dibenz[*a,h*]acridine was 80%, with a multiplicity of 3.33 ± 0.57 tumours per mouse

(mean ± standard error). There were no tumours in the control group. Based upon the number of mice initially treated, the incidence of papillomas (24 out of 30) in the group receiving dibenz[*a,h*]acridine was statistically significantly different [*P* < 0.0001; Fisher exact test] compared with that in the control group (0 out of 30). The tumour multiplicity in the group receiving dibenz[*a,h*]acridine was also statistically significantly different compared with that in the control group (*P* < 0.01; Student's *t*-test). Mice treated with dibenz[*a,h*]acridine-3,4-dihydrodiol and dibenz[*a,h*]acridine-10,11-dihydrodiol showed significant increases in the incidence [5 out of 30, and 17 out of 30, respectively; *P* ≤ 0.03] and multiplicity (0.17 ± 0.07, and 1.23 ± 0.31, respectively; *P* < 0.01) of skin tumours compared with the control group. Mice given dibenz[*a,h*]acridine-*anti*-3,4-dihydrodiol-1,2-epoxide, dibenz[*a,h*]acridine-*anti*-10,11-dihydrodiol-8,9-epoxide, and dibenz[*a,h*]acridine-*syn*-10,11-dihydrodiol-8,9-epoxide showed significant increases in the incidence [5 out of 30, 13 out of 30, and 6 out of 30, respectively; *P* ≤ 0.03] and multiplicity (0.21 ± 0.09, 1.56 ± 0.55, and 0.20 ± 0.07, respectively; *P* < 0.05) of skin tumours compared with the control group.

In a subsequent experiment, Kumar *et al.* (2001) treated mice in an identical manner to the first experiment with 50 or 175 nmol of dibenz[*a,h*]acridine, (+)-dibenz[*a,h*]acridine-10*S*,11*S*-dihydrodiol, (–)-dibenz[*a,h*]acridine-10*R*,11*R*-dihydrodiol, (+)-dibenz[*a,h*]acridine-*syn*-10*R*,11*S*-dihydrodiol-8*R*,9*S*-epoxide, (–)-dibenz[*a,h*]acridine-*syn*-10*S*,11*R*-dihydrodiol-8*S*,9*R*-epoxide, (+)-dibenz[*a,h*]acridine-*anti*-10*S*,11*R*-dihydrodiol-8*R*,9*S*-epoxide, and (–)-dibenz[*a,h*]acridine-*anti*-10*R*,11*S*-dihydrodiol-8*S*,9*R*-epoxide. (+)-Dibenz[*a,h*]acridine-*anti*-10*S*,11*R*-dihydrodiol-8*R*,9*S*-epoxide was also given at a dose of 10 nmol. The control group (30 mice) received the solvent only. At least 26 mice in each group survived until the termination of the study after 20 weeks of treatment with TPA. When assessed after 10 weeks of promotion with TPA, there was a statistically significant increase in the incidence [6 out of 30; *P* < 0.01] and multiplicity (0.2 ± 0.09; *P* < 0.05) of skin tumours in mice receiving 175 nmol of dibenz[*a,h*]acridine compared with the control group [0 out of 30]. Likewise, after 20 weeks of promotion with TPA, 50 and 175 nmol of dibenz[*a,h*]acridine caused a significant increase in the incidence of tumours [18 out of 30 and 24 out of 30; *P* < 0.001] and multiplicity (1.6 ± 0.33 and 2.3 ± 0.37; *P* < 0.01) compared with the control group (1 out of 30 and 0.04 ± 0.04). Of all the compounds tested, the highest tumourgenicity was observed with (+)-dibenz[*a,h*]acridine-*anti*-10*S*,11*R*-dihydrodiol-8*R*,9*S*-epoxide after 10 weeks of promotion with TPA: incidence [5 out of 30, 14 out of 30, and 26 out of 30, at 10, 50 and 175 nmol] and multiplicity (0.2 ± 0.09, 0.7 ± 0.14, and 2.3 ± 0.30 at 10, 50 and 175 nmol). A similar trend (*P* < 0.01) occurred after 20 weeks of promotion with TPA.

(c) Subcutaneous administration

A group of 19 mice (age, sex, and strain not specified) received 0.9 mg of dibenz[*a,h*]acridine (purity not reported) in 300 μL of sesame oil, administered subcutaneously, every 2 weeks, for 34 weeks (Bachmann *et al.*, 1937). Thirteen mice survived more than 168 days, and of these, eight developed sarcomas at the injection site before the end of the experiment, 240 days after the initiation of treatment. [The Working Group noted several deficiencies in this study, including the lack of a concurrent control group, and the lack of information on the age, sex, and strain of the mice, on the purity and total amount of dibenz[*a,h*]acridine administered, and on the histopathological procedures employed.]

A group of 10 mice (age, sex, and strain not specified) received repeated doses of 5 mg of dibenz[*a,h*]acridine ("pure") in 200 μL of sesame oil applied subcutaneously at intervals of a few (3–5) weeks (Badger *et al.*, 1940). The last mouse

died 246 days after the initiation of treatment. Three of the mice developed sarcoma. [The Working Group noted several deficiencies in this study, including the limited number of mice tested, the lack of a concurrent control group, and the lack of information on the age, sex, and strain of the mice, on the purity and total amount of dibenz[*a,h*]acridine administered, and on the histopathological procedures employed.]

Andervont & Shimkin (1940) gave a group of male and female strain A mice (age, 2–3 months; total number, and number of each sex not specified) a single subcutaneous injection of 500 μg of dibenz[*a,h*]acridine dissolved in 100 μL of tricaprylin. Fourteen weeks after the injection, the mice were killed and their lungs were examined for pulmonary nodules; representative samples were characterized histologically as adenoma. The incidence of pulmonary tumours was 20 out of 20, with a multiplicity of 3.0 tumours per tumour-bearing mouse. There were no tumours at the injection sites.

In a subsequent experiment, strain A mice (age, 2–3 months; sex and total number not specified) were given a single subcutaneous injection of 1.0 mg of dibenz[*a,h*]acridine dissolved in 300 μL of sesame oil (Andervont & Shimkin, 1940). The mice were killed 22 weeks and 40 weeks after injection and the number of pulmonary nodules was determined. At 22 weeks, 6 out of 6 mice had pulmonary tumours. The corresponding value at 40 weeks was 14 out of 14, with a multiplicity of 70 tumours per tumour-bearing mouse. There were no tumours (0 out of 14) at the injection site. [The Working Group noted several deficiencies in this study, including the lack of a concurrent control group and the lack of information on the sex and initial number of mice treated.]

(d)   Intravenous injection

Equal numbers of male and female strain A mice (age, 2–3 months) [total number not specified] were given a single intravenous injection 0.25 mg of dibenz[*a,h*]acridine suspended in 250 μL of water (Andervont & Shimkin, 1940). A control group was injected with water only. The injections resulted in "almost no mortality," and all mice surviving the injections survived until the scheduled terminations at 8, 14, and 20 weeks. The lungs were examined for pulmonary nodules; representative samples were characterized histologically as adenoma. At 8 weeks, the incidence of lung tumours in mice receiving dibenz[*a,h*]acridine was 3 out of 10 (30%), with a multiplicity of 1.3 tumours per tumour-bearing mouse, while the incidence in the control group was 1 out of 20 (5%), with a multiplicity of 1.0 tumours per tumour-bearing mouse. At 14 weeks, the incidence of lung tumours in mice receiving dibenz[*a,h*]acridine was 9 out of 13 (69%), with a multiplicity of 2.9 tumours per tumour-bearing mouse, while the incidence in the control group was 3 out of 20 [15%; $P = 0.0025$; one-tailed Fisher exact test], with a multiplicity of 1.0 tumours per tumour-bearing mouse. At 20 weeks, the incidence of lung tumours in mice receiving dibenz[*a,h*]acridine was 11 out of 12 (92%), with a multiplicity of 2.2 tumours per tumour-bearing mouse, while the incidence in the control group was 4 out of 19 [21%; $P = 0.0002$; one-tailed Fisher exact test], with a multiplicity of 1.0 tumour per tumour-bearing mouse.

3.3.2 Rat

See Table 3.5

(a)   Subcutaneous administration

A group of 30 random-bred female Wistar albino rats (body weight, 100–110 g) were given 10 mg of dibenz[*a,h*]acridine (purity not reported) dissolved in a 3 × 10 mm disk of paraffin, as a single subcutaneous implantation to the right side of the chest (Bahna *et al.*, 1978). As a control, the rats were implanted on the left side of the chest with a paraffin disk not containing dibenz[*a,h*]acridine. The rats were monitored for 21 months, at which time 12 rats were still alive.

Table 3.5 Studies of carcinogenicity in rats given dibenz[*a,h*]acridine

Species, strain (sex) Duration Reference	Dosing regimen, Animals/group at start	Incidence of tumours	Significance	Comments
Subcutaneous administration				
Rat, Wistar (F) 84 wk Bahna *et al.* (1978)	Dibenz[*a,h*]acridine dissolved in paraffin implanted on right side of chest. As a control, the rats were implanted on left side with paraffin only 30 rats/group	*Sarcoma* Dibenz[*a,h*]acridine: 5/30 (17%) Control: 0/30	[*P* < 0.03; one-tailed Fisher exact test]	Amount of dibenz[*a,h*] acridine, not reported
Pulmonary implantation				
Rat, Osborne-Mendel (F) 113 wk Deutsch-Wenzel *et al.* (1983)	A single dose of dibenz[*a,h*]acridine at 0, 0.1, 0.3, or 1.0 mg (purity, 99.8%) in 50 µl of a 1 : 1 mixture of beeswax and tricaprylin. An additional group was untreated. Positive-control groups received benzo[*a*]pyrene at 0.1, 0.3, or 1.0 mg 35 rats/group	*Epidermoid carcinoma* Untreated, 0, 0.1, 0.03 and 1.0 mg Dibenz[*a,h*]acridine: 0/35, 0/35, 0/35, 3/35 (9%), 9/31 (29%) Benzo[*a*]pyrene: 5/35 (14%), 24/35 (69%), 27/35 (77%)	*P* = 0.0005 for 1.0 mg dibenz[*a,h*] acridine; Fisher exact test	Osteosarcoma at implantation site in one animal given 0.3 mg of dibenz[*a,h*]acridine

F, female; wk, week

Five of the 30 rats (17%) developed histologically confirmed sarcoma at the site of implantation of the dibenz[*a,h*]acridine-containing disk, with the first being diagnosed 14 months after implantation. There were no sarcomas at the site of implantation of the paraffin-only disk [*P* < 0.03 for dibenz[*a,h*]acridine implantation site *versus* paraffin-only implantation site; one-tailed Fisher exact test].

(b)   Pulmonary implantation

Groups of 35 female Osborne-Mendel rats (age, 3 months) received a single pulmonary implantation of 0.0, 0.1, 0.3, or 1.0 mg of dibenz[*a,h*]acridine (purity, 99.8%) in 50 μL of a 1 : 1 mixture of beeswax and tricaprylin that had been preheated to 60 °C (Deutsch-Wenzel *et al.*, 1983). Another group of 35 rats was not treated. Positive controls were also included, comprising groups of 35 rats receiving a pulmonary implantation of 0.1, 0.3, or 1.0 mg of benzo[*a*]pyrene in beeswax and tricaprylin.

All rats, except four that were treated with 1.0 mg of dibenz[*a,h*]acridine, survived and were evaluated. Mean survival in rats given dibenz[*a,h*] acridine (99–113 weeks) was similar to that in the negative-control groups (103 and 110 weeks). The lungs and any other organs showing abnormalities were examined by histopathology. At the end of the experiment, the incidence of lung epidermoid carcinoma was 0 out of 35 in the group receiving 0.1 mg of dibenz[*a,h*]acridine, 3 out of 35 [9%] in the group receiving 0.3 mg of dibenz[*a,h*]acridine, and 9 out of 31 [29%] in the group receiving 1.0 mg of dibenz[*a,h*]acridine. There were no tumours in either of the two control groups (0 out of 35 in each group). The incidence of epidermoid carcinoma in the group receiving 1.0 mg of dibenz[*a,h*]acridine was statistically significantly different [*P* = 0.0005; Fisher exact test] from that in either control group. One rat given 0.3 mg of dibenz[*a,h*]acridine developed an osteosarcoma at the implantation site. Rats given benzo[*a*]pyrene had a dose-dependent increase

in the incidence of lung epidermoid carcinoma, which was 5 out of 35 (14%) at 0.1 mg, 24 out of 35 (69%) at 0.3 mg, and 27 out of 35 [77%] at 1.0 mg.

3.4 Dibenz[*a,j*]acridine

Several studies using oral administration, skin application or subcutaneous injection were evaluated as inadequate by the Working Group and were not taken into consideration for the final evaluation (Barry *et al.*, 1935; Bachmann *et al.*, 1937; Andervont & Shimkin, 1940; Badger *et al.*, 1940; Lacassagne *et al.*, 1955a, b, 1956; Wynder & Hoffmann, 1964). Limitations of these studies included the small number of mice tested, the lack of concurrent vehicle control group, lack of information on strain, age and sex of the animals, lack of information on the purity and total amount of dibenz[*a,j*]acridine administered, and no description of the histological procedures employed. These studies are not presented in the tables.

3.4.1 Mouse

See Table 3.6

(a)   Oral administration

A group of 10 mice (age, sex, and strain not specified) was fed dibenz[*a,j*]acridine ("pure") as a 2% or 4% solution in olive oil (specific amount not specified) mixed with the food, or 1 mg of dibenz[*a,j*]acridine in 260 μL of butter, by stomach tube, for 5 days per week for up to 82 weeks (Badger *et al.*, 1940). The last mouse died 572 days after the initiation of dosing. No tumours were observed. [The Working Group noted several deficiencies in this study, including the limited number of mice tested, the lack of concurrent control group, and the lack of information on the age, sex, and strain of the mice, the purity and total amount of dibenz[*a,j*]acridine

Table 3.6 Studies of carcinogenicity in mice given dibenz[*a,j*]acridine

Species, strain (sex) Duration Reference	Dosing regimen, Animals/group at start	Incidence of tumours	Significance	Comments
Skin application				
Mouse, Hsd:(ICR)BR (F) 99 wk Warshawsky *et al.* (1994; 1996a)	Treated topically with 50 nmol (13.95 µg) of dibenz[*a,j*]acridine (purity, 99%) in 50 µL of acetone, or 50 µL of acetone only, or untreated; twice/wk on shaved interscapular region 50 mice/group	*Skin tumours* Untreated control, acetone control, dibenz[*a,j*]acridine 2/11 (18%), 3/11 (27%), 27/40 (68%) Squamous cell carcinoma 0/11, 1/11 (9%), 15/40 (38%) Papilloma 0/11, 2/11 (18%), 7/40 (18%) Basal cell carcinoma 0/11, 0/11, 3/40 (8%) Keratoacanthoma 0/11, 0/11, 1/40 (3%) Undifferentiated carcinoma 0/11, 0/11, 1/40 (3%)	[$P \leq 0.02$ treated *vs* either control; one-tailed Fisher exact test]	Histopathology conducted on a limited number of mice.
Mouse, C3H/Hej (M) 99 wk Warshawsky & Barkley (1987), Warshawsky *et al.* (1996a)	Treated topically with 12.5 µg dibenz[*a,j*]acridine (purity, 99%) in 50 µL of acetone, or 50 µL of acetone only, or untreated; twice/wk, in interscapular region 50 mice/group	*Skin tumours (papilloma and carcinoma combined)* Dibenz[*a,j*]acridine: 25/50 (50%) Acetone control: 0/50 Untreated control: 0/50 *Carcinoma*: 22/25 (88%)	[$P < 0.001$, for combined papilloma and carcinoma, and for malignant skin tumours only; one-tailed Fisher exact test]	
Skin application – initiation–promotion				
Mouse, Hsd(ICR)BR (F) 25 wk Warshawsky *et al.* (1992, 1996a)	Treated topically with a single dose of 200 nmol (55.8 µg) of dibenz[*a,j*] acridine (purity, 99%) in 50 µL of acetone. After 2 wk, treated with 2 µg of TPA in 50 µL of acetone. Control groups treated with acetone only, TPA only, dibenz[*a,j*]acridine only, or not treated; twice/wk, on shaved interscapular region 30 mice/group	*Skin papilloma* Dibenz[*a,j*]acridine + TPA: 17/30 (57%) Acetone: 0/30 TPA: 0/30 Dibenz[*a,j*]acridine: 0/30 Untreated: 0/30	[$P < 0.0001$; dibenz[*a,j*]acridine *vs* each control group; one-tailed Fisher exact test]	

F, female; M, male; NR, not reported; NS, not significant; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; *vs, versus*; wk, week

administered, the precise route of administration, and the histopathological procedures employed.]

(b)   Skin application

Barry *et al.* (1935) treated a group of 10 mice (age, sex, and strain not specified) with an unspecified amount of dibenz[*a,j*]acridine (purity not reported) as a 0.3% solution in benzene, applied to the interscapular region, twice per week. Six of the mice survived 6 months, three survived 12 months, and the last mouse died after 597 days of treatment. Two mice developed epithelioma [squamous cell carcinoma].

In a second experiment, Barry *et al.* (1935) treated a group of 30 mice in a manner identical to the first experiment. Twenty-eight of the mice survived 6 months, eighteen survived 1 year, and the last mouse died 551 days after the initiation of treatment. Nine mice developed epithelioma (squamous cell carcinoma) and two developed papilloma. [The Working Group noted several deficiencies in both experiments, including the limited number of mice tested in the first experiment, the lack of a concurrent control group, the lack of information on the age, sex, and strain of the mice, and on the purity and amount of dibenz[*a,j*]acridine administered, and the use of benzene, which is classified as a carcinogen (IARC Group 1), as the vehicle.]

A group of 40 mice (age, sex, and strain not specified) was given an unspecified amount of dibenz[*a,j*]acridine (purity not reported) as a 0.3% solution in benzene, applied topically twice per week (Badger *et al.*, 1940). The last mouse died after 597 days of treatment. Two mice developed papillomas and eleven developed epitheliomas (squamous cell carcinoma). [The Working Group noted several deficiencies in the study, including the lack of a concurrent control group, the lack of information on the age, sex and strain of the mice, and on the purity and amount of dibenz[*a,j*]acridine administered, and the use of benzene,

which is classified as a carcinogen (IARC Group 1), as the vehicle.]

A group of 20 XVII mice (age and sex not specified) was given one drop of a 0.3% solution of dibenz[*a,j*]acridine (purity not reported) in acetone, applied to the nape of the neck, twice per week (Lacassagne *et al.*, 1955a, 1956). Six of the mice did not survive the 90 days of treatment; the remaining mice were removed from the study between days 139 and 450. None of the mice developed epithelioma (squamous cell carcinoma). [The Working Group noted several deficiencies in this study, including the limited number of mice tested, the lack of a concurrent control group, the lack of information on the age and sex of the mice, and on the purity and amount of dibenz[*a,j*]acridine administered, and the poor survival of the dosed mice.]

Groups of 20 female Swiss mice (age not specified) were treated topically with dibenz[*a,j*]acridine (purity not reported) as a 0.5% or 1.0% solution in acetone (volume not reported) three times per week (Wynder & Hoffmann, 1964). After 12–14 months, 16 of the mice treated with 0.5% dibenz[*a,j*]acridine and 15 of the mice treated with 1.0% dibenz[*a,j*]acridine developed tumours; in both groups 60% of the tumours were carcinoma. [The Working Group noted several deficiencies in this study, including the lack of a concurrent control group, the lack of information on the age of the mice, survival, histopathology procedures, and the purity and amount of dibenz[*a,j*]acridine administered.]

Groups of 50 female carcinogen-sensitive Hsd:(ICR)BR mice (age, 7–8 weeks) were treated with 0 or 50 nmol (13.95 μg) of dibenz[*a,j*]acridine (purity, 99%) in 50 μL of acetone, applied topically on the shaved interscapular region twice per week, or were not treated (Warshawsky *et al.*, 1994, 1996a). The treatment was continued for 99 weeks. Histopathology was conducted. In mice treated with dibenz[*a,j*]acridine, the incidence of skin tumours was 27 out of 40 (68%), with the tumours being characterized as squamous cell

carcinoma (15 out of 40; 38%), squamous cell papilloma (7 out of 40; 18%), basal cell carcinoma (3 out of 40; 8%), keratoacanthoma (1 out of 40; 3%), and undifferentiated carcinoma (1 out of 40; 3%). In untreated mice, the incidence of skin tumours was 2 out of 11 (18%), while in mice treated with acetone only, the incidence was 3 out of 11 (27%). The incidence of skin tumours in mice treated with dibenz[*a,j*]acridine was statistically significantly different from both control groups [*P* ≤ 0.02; one-tailed Fisher exact test]. [The Working Group noted that histopathology was conducted only on a limited number of animals.]

Groups of 50 male C3H/Hej mice (age, 8–10 weeks) were treated with 0 or 12.5 μg of dibenz[*a,j*] acridine (purity, 99%) in 50 μL of acetone, applied topically in the interscapular region, twice per week, or were not treated (Warshawsky & Barkley, 1987; Warshawsky *et al.*, 1996a). The treatment was continued for 99 weeks. Lesions with a minimum volume of 1 mm³ and persisting for at least 1 week were classified as papilloma. Histopathology was conducted. Twenty-five of the mice treated with dibenz[*a,j*]acridine developed skin tumours, with an average latency of 80.3 weeks. Malignant skin tumours (carcinomas) occurred in 22 of the 25 (88%) mice. There were no skin tumours in either of the control groups (0 out of 50). [*P* < 0.001, for combined papilloma and carcinoma, and for malignant skin tumours only; one-tailed Fisher exact test.]

(c)   Skin application: initiation–promotion

A group of 30 female carcinogen-sensitive Hsd:(ICR)BR mice (age, 7–8 weeks) received a single treatment with 200 nmol (55.8 μg) of dibenz[*a,j*]acridine (purity, 99%) in 50 μL of acetone, applied topically to the shaved interscapular region (Warshawsky *et al.*, 1992, 1996a). Two weeks later, the group was treated topically twice per week with 2 μg of TPA in 50 μL of acetone. Control groups consisted of 30 mice that were not treated, 30 mice treated

with acetone only, 30 mice treated with TPA only, and 30 mice treated with dibenz[*a,j*]acridine only. The last mouse was removed from the study 23 weeks after the start of treatment with TPA. Histopathology was conducted. Mice given dibenz[*a,j*]acridine followed by TPA had an incidence of skin papilloma of 17 out of 30 (57%), with a multiplicity of 1.8 papillomas per tumour-bearing mouse and a mean latency of 14.5 weeks. There were no papillomas detected in any of the control groups. The incidence of skin papilloma in the groups receiving dibenz[*a,j*]acridine and TPA was statistically significantly different from the negative groups [*P* < 0.0001; one-tailed Fisher exact test].

(d)   Subcutaneous administration

Two groups of 10 mice (age, sex, and strain not specified) were given 300 μg of dibenz[*a,j*] acridine (purity not reported) in 900 μL of sesame oil by subcutaneous administration (Bachmann *et al.*, 1937). The application was repeated fortnightly [every 2 weeks]. The last mouse in the first group died 310 days after the initiation of treatment, while the last mouse in the second group died after 266 days. There were no tumours in either group. [The Working Group noted several deficiencies in this study, including the limited number of mice, the lack of a concurrent control group, the lack of information on age, sex, and strain, or on the purity and total amount of dibenz[*a,j*]acridine administered, or on the histopathological procedures employed.]

A group of 10 mice (age, sex, and strain not specified) was given 5 mg of dibenz[*a,j*]acridine (purity not reported) in 200 μL of sesame oil by subcutaneous injection (Badger *et al.*, 1940). The application was repeated at intervals of a few (3–5) weeks. The last mouse died after 583 days of treatment. Two mice developed sarcoma. [The Working Group noted several deficiencies, including the limited number of mice tested, lack of a concurrent control group, the lack of information on the age, sex and strain of the mice, the

Table 3.7 Study of carcinogenicity in rats given dibenz[*a,j*]acridine by pulmonary implantation

Species, strain (sex) Duration Reference	Dosing regimen, Animals/group at start	Incidence of tumours	Significance
Rat, Osborne-Mendel (F) 111 wk Deutsch-Wenzel *et al.* (1983)	A single pulmonary implantation of dibenz[*a,j*]acridine (purity, 99.3%) of 0, 0.1, 0.3, or 1.0 mg in 50 μL of a 1 : 1 mixture of beeswax and tricaprylin. An additional group was untreated. Positive-control groups received 0.1, 0.3, or 1.0 mg of benzo[*a*]pyrene 35 rats/group	*Pleomorphic sarcoma* Untreated, 0, 0.1, 0.3, or 1.0 mg of dibenz[*a,j*]acridine: 0/35, 0/35, 1/35 (3%), 0/35, 0/35 Benzo[*a*]pyrene: 3/35 (9%), 0/35, 0/35 *Epidermoid carcinoma* Untreated or treated with dibenz[*a,j*]acridine: no tumours reported 0.1, 0.3, and 1.0 mg of benzo[*a*] pyrene: 5/35 (14%), 24/35 (69%), 27/35 (77%)	[NS] -

F, female; NS, not significant; wk, week

purity and total amount of dibenz[*a,j*]acridine administered, and the histopathological procedures employed.]

Strain A mice (age, 2–3 months; sex and total number not specified) were given 1.0 mg of dibenz[*a,j*]acridine dissolved in 300 μL of sesame oil as a single subcutaneous injection (Andervont & Shimkin, 1940). Mice were killed 22 weeks and 40 weeks after injection to determine the number of pulmonary nodules; representative samples were characterized histologically as adenoma. At 22 weeks, six out of six mice had pulmonary tumours. The corresponding value at 40 weeks was 13 out of 13, with a multiplicity of 20 tumours per tumour-bearing mouse. There were no tumours (0 out of 13) at the injection site. [The Working Group noted several deficiencies in this study, including the lack of a concurrent control group and the lack of information on the sex and initial number of mice treated.]

Ten XVII mice (age and sex not specified) received a subcutaneous injection of 1 mg of dibenz[*a,j*]acridine (purity not reported) in 200 μL of peanut oil, three times at monthly intervals (Lacassagne *et al.*, 1955a, 1956). Five of the mice did not survive 90 days of treatment; the

remaining mice were removed from the study between day 139 and day 590. None of the mice developed sarcoma. [The Working Group noted several deficiencies in this study, including the limited number of mice tested, the poor survival of the mice, the lack of a concurrent control group, and the lack of information on the age and sex of the mice and the purity of the dibenz[*a,j*] acridine.]

3.4.2 Rat

See Table 3.7

Pulmonary implantation

Groups of 35 female Osborne-Mendel rats (age, 3 months) were given a single pulmonary implantation of 0.0, 0.1, 0.3, or 1.0 mg of dibenz[*a,j*]acridine (purity, 99.3%) in 50 μL of a 1 : 1 mixture of beeswax and tricaprylin that had been preheated to 60 °C (Deutsch-Wenzel *et al.*, 1983). Another group of 35 rats was not treated. Positive controls were also included, consisting of groups of 35 rats that were given a pulmonary implantation of 0.1, 0.3, or 1.0 mg of benzo[*a*]pyrene. The mean survival in rats given

dibenz[*a,j*]acridine (102–111 weeks) was similar to the mean survival in the negative-control groups (103 and 110 weeks). The lungs and any other organs showing abnormalities were examined by histopathology. At the end the experiment, a single pleomorphic sarcoma (1 out of 35; 3%) was observed in the group receiving 0.1 mg of dibenz[*a,j*]acridine. There were no tumours in the groups receiving 0.3 or 1.0 mg of dibenz[*a,j*] acridine, or in either of the two negative-control groups. In the groups of rats receiving benzo[*a*] pyrene, there was a dose-dependent increase in the incidence of lung epidermoid carcinoma, with the incidence being 5 out of 35 (14%) at 0.1 mg, 24 out of 35 (69%) at 0.3 mg, and 27 out of 35 (77%) at 1.0 mg.

3.5 Dibenz[*c,h*]acridine

3.5.1 Mouse

See Table 3.8

(a)   Skin application

As part of a study to determine tumour initiation by a series of oxidized derivatives of dibenz[*c,h*]acridine (Chang *et al.*, 2000), groups of 30 female CD-1 mice (age, 7 weeks) were given a single topical application of 50 or 200 nmol of dibenz[*c,h*]acridine, (+)-dibenz[*c,h*] acridine-1,2-dihydrodiol, (+)-dibenz[*c,h*] acridine-3*S*,4*S*-dihydrodiol, (–)-dibenz[*c,h*] acridine-3*R*,4*R*-dihydrodiol, (+)-dibenz[*c,h*] acridine-5,6-dihydrodiol, (+)-dibenz[*c,h*]acridine-*syn*-3*S*,4*R*-dihydrodiol-1*S*,2*R*-epoxide, (–)-dibenz[*c,h*]acridine-*syn*-3*R*,4*S*-dihydrodiol-1*R*,2*S*-epoxide, (+)-dibenz[*c,h*]acridine-*anti*-3*S*,4*R*-dihydrodiol-1*R*,2*S*-epoxide, (–)-dibenz[*c,h*]acridine-*anti*-3*R*,4*S*-dihydrodiol-1*S*,2*R*-epoxide (purity of dibenz[*c,h*]acridine not reported; purity of all other compounds, > 99%) in 200 µL of acetone, applied to the shaved dorsal surface. A control group of 30 mice received the solvent only. Nine days later, all mice received 16 nmol of TPA in 200 µL of acetone, applied twice per week for 20 weeks. The formation of papillomas was monitored every 2 weeks; those papillomas of 2 mm or greater in diameter and persisting more than 2 weeks were included in the final total. The tumours were not examined by histopathology. At least 28 mice in each group survived until the end of the study.

In the group of mice treated with 50 nmol of dibenz[*c,h*]acridine, the incidence of papilloma was 33% [10 out of 30], with a multiplicity of 0.50 ± 0.15 tumours per mouse (mean ± standard error of the mean); in the group of mice treated with 200 nmol of dibenz[*c,h*]acridine, the incidence of papilloma was 60% [18 out of 30], with a multiplicity of 1.83 ± 0.43 tumours per mouse. The incidence of papilloma in the control group was 3% [1 out of 30], with a multiplicity of 0.03 ± 0.03 tumours per mouse. The incidence and multiplicity of tumours in both groups of mice treated with dibenz[*c,h*]acridine were statistically significantly different ($P < 0.05$) from the control group (fourfold contingency test and Student's *t*-test, respectively). A significant increase in tumour incidence and multiplicity was also observed after treatment with (–)-dibenz[*c,h*]acridine-3*R*,4*R*-dihydrodiol and with each of the dibenz[*c,h*]acridine dihydrodiol epoxides.

(b)   Intraperitoneal injection

As part of an investigation to evaluate the tumorigenicity of a series of oxidized dibenz[*c,h*]acridine metabolites, groups of 80 newborn CD-1 mice (presumably 40 males and 40 females) were given intraperitoneal injections of 25, 50, and 100 nmol (total dose, 175 nmol) of dibenz[*c,h*]acridine, (+)-dibenz[*c,h*] acridine-1,2-dihydrodiol, (+)-dibenz[*c,h*] acridine-3*S*,4*S*-dihydrodiol, (–)-dibenz[*c,h*] acridine-3*R*,4*R*-dihydrodiol, (+)-dibenz[*c,h*] acridine-5,6-dihydrodiol, (+)-dibenz[*c,h*] acridine-*syn*-3*S*,4*R*-dihydrodiol-1*S*,2*R*-epoxide, (–)-dibenz[*c,h*]acridine-*syn*-3*R*,4*S*-

Table 3.8 Studies of carcinogenicity in mice given dibenz[*c,h*]acridine

Species, strain (sex) Duration Reference	Dosing regimen, Animals/group at start	Incidence and multiplicity of tumours	Significance	Comments
Skin application – initiation–promotion				
Mouse, CD-1 (F) 21 wk Chang *et al.* (2000)	A single topical application of 0, 50 or 200 nmol of dibenz[*c,h*]acridine in 200 μL of acetone, to the shaved dorsal surface. After 9 days, treated with 16 nmol of TPA in 200 μL of acetone, twice/wk. 30 mice/group	*Papilloma* Incidence 1/30 (3%), 10/30 (33%), 18/30 (60%) Multiplicity: 0.03 ± 0.03, 0.50 ± 0.15, 1.83 ± 0.43	$P < 0.05$ for both treated groups *vs* control; fourfold contingency test $P < 0.05$ for both treated groups *vs* control; Student's *t*-test	Purity of dibenz[*c,h*]acridine, NR
Intraperitoneal injection				
Mouse, CD-1 (newborn, M, F) 39 wk Chang *et al.* (2000)	Injections on postnatal days 1, 8, and 15 with 25, 50, and 100 nmol (total dose, 175 nmol) of dibenz[*c,h*]acridine (purity NR) in 5, 10, and 20 μL DMSO respectively. A control group treated in similar manner with vehicle only 80 mice/group [presumably 40 M and 40 F]	*Lung tumours (primarily adenoma)* M: 13/26 (50%), 2/33 (6%) F: 7/24 (29%), 2/36 (6%) for dibenz[*c,h*]acridine and control, respectively *Liver tumours (mostly type A or neoplastic nodules)* M: 12/26 (46%), 1/33 (3%) F: 0/24, 0/36 for dibenz[*c,h*]acridine and control, respectively	$P < 0.02$, for M, F, and M+F; one-tailed Fisher exact test $P = 0.0001$ for M, one-tailed Fisher exact test	Number of animals each sex, NR

d, day; DMSO, dimethyl sulfoxide; F, female; M, male; NR, not reported; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; *vs, versus*; wk, week

dihydrodiol-1*R*,2*S*-epoxide, (+)-dibenz[*c,h*]acridine-*anti*-3*S*,4*R*-dihydrodiol-1*R*,2*S*-epoxide, or (–)-dibenz[*c,h*]acridine-*anti*-3*R*,4*S*-dihydrodiol-1*S*,2*R*-epoxide (purity of dibenz[*c,h*]acridine not reported; purity of all other compounds, > 99%) in 5, 10, and 20 μL of DMSO, respectively, on postnatal days 1, 8, and 15 (Chang *et al.*, 2000). An additional group of 80 mice was given 10, 20, and 40 nmol (total dose, 70 nmol) of (+)-dibenz[*c,h*]acridine-*anti*-3*S*,4*R*-dihydrodiol-1*R*,2*S*-epoxide. A control group of 80 mice (presumably 40 males and 40 females) was treated in an identical manner with 5, 10, and 20 μL of the DMSO vehicle. The number of mice surviving until weaning at postnatal day 25 was 72 in the control group, and 31–71 in the treated group. The experiment was terminated when the mice were aged 36–39 weeks. A gross necropsy was performed, and selected lung and all liver tumours were examined histologically.

The incidence of lung tumours (primarily adenoma) in female, male, and combined female and male mice in the control group was 6% [2 out of 36], 6% [2 out of 33], and 6% [4 out of 69], with a multiplicity of 0.14, 0.12, and 0.13 tumours per mouse. The comparable incidence values in female, male, and combined male and female mice treated with dibenz[*c,h*]acridine were 29% [7 out of 24], 50% [13 out of 26], and 40% [20 out of 50], with a multiplicity of 3.25, 3.42, and 3.34 tumours per mouse. The incidence of lung tumours in female, male, and combined male and female mice given dibenz[*c,h*]acridine was statistically significantly different from that in the control mice [*P* < 0.02; one-tailed Fisher exact test]. Treatment with (+)-dibenz[*c,h*]acridine-3*S*,4*S*-dihydrodiol, (–)-dibenz[*c,h*]acridine-3*R*,4*R*-dihydrodiol, (+)-dibenz[*c,h*]acridine-*syn*-3*S*,4*R*-dihydrodiol-1*S*,2*R*-epoxide, and (+)-dibenz[*c,h*]acridine-*anti*-3*S*,4*R*-dihydrodiol-1*R*,2*S*-epoxide also increased the incidence of lung tumours.

In male mice treated with dibenz[*c,h*]acridine, liver tumours ("mostly type A or neoplastic nodules") developed, with an incidence of 46% [12 out of 26], and a multiplicity of 1.96 tumours per mouse. The incidence of liver tumours in male mice in the control group was 3% [1 out of 33], a difference that was statistically significant [*P* = 0.0001; one-tailed Fisher exact test], with a multiplicity of 0.63 tumours per mouse. Liver tumours were not detected in the control group [0 out of 36] or in female mice treated with dibenz[*c,h*]acridine [0 out of 24]. Treatment with (+)-dibenz[*c,h*]acridine-3*S*,4*S*-dihydrodiol, (–)-dibenz[*c,h*]acridine-3*R*,4*R*-dihydrodiol, (+)-dibenz[*c,h*]acridine-*syn*-3*S*,4*R*-dihydrodiol-1*S*,2*R*-epoxide, or (+)-dibenz[*c,h*]acridine-*anti*-3*S*,4*R*-dihydrodiol-1*R*,2*S*-epoxide also increased the incidence of liver tumours (Chang *et al.*, 2000).

3.6 Carbazole

Three studies in mice given carbazole by skin application or by subcutaneous injection were evaluated as inadequate (Kennaway, 1924; Maisin *et al.*, 1927; Schürch & Winterstein, 1935; Shear & Leiter, 1941). The limitations of these studies included the small number of mice tested, lack of concurrent vehicle control group, lack of information on strain, age and sex, lack of information on the purity and total amount of carbazole administered, and absence of description of the histological procedures employed. These studies are not presented in the tables.

3.6.1 Mouse

See Table 3.9

(a) Oral administration

Groups of 50 male and 50 female B6C3F$_1$ mice (age, 6 weeks) were fed a pellet diet containing technical-grade carbazole (purity, 96%) at a concentration of 0%, 0.15%, 0.3% or 0.6% (Tsuda *et al.*, 1982). The treatment was continued for 96 weeks, after which the mice were maintained

Table 3.9 Studies of carcinogenicity in mice given carbazole

Species, strain (sex) Duration Reference	Dosing regimen, Animals/group at start	Incidence and multiplicity of tumours	Significance	Comments
Oral administration				
Mouse, B6C3F$_1$ (M, F) 104 wk Tsuda *et al.* (1982)	Fed diet containing carbazole (purity, 96%) at 0%, 0.15%, 0.3%, or 0.6% for 96 wk, followed by 8 wk of basal diet 50 M and 50 F/group	*Hepatocellular carcinoma* M: 9/46 (20%), 12/42 (29%), 20/42 (48%), 37/48 (77%) F: 2/45 (4%), 35/49 (71%), 24/43 (56%), 30/46 (65%)	*P* < 0.001 (highest dose) *P* < 0.001 (all treated)	
		Liver neoplastic nodules [hepatocellular adenoma] M: 13/46 (28%), 30/42 (71%), 22/42 (52%), 10/48 (21%); F: 2/45 (4%), 13/49 (26%), 21/43 (49%), 16/46 (35%)	*P* < 0.001 (lowest dose) *P* < 0.001 (intermediate & highest dose)	
		Forestomach squamous cell carcinoma M: 0/46, 0/42, 0/42, 7/48 (15%) F: 0/45, 0/49, 1/43 (2%), 2/46 (4%)	*P* < 0.01 (highest dose) NS	
		Forestomach papilloma M: 0/46, 0/42, 1/42 (2%), 4/48 (8%) F: 0/45, 5/49 (10%), 7/43 (16%), 4/46 (9%)	*P* < 0.05 (highest dose) *P* < 0.01 (intermediate dose)	
Intraperitoneal administration				
Mouse, CD-1 (newborn) (M, F) 52 wk Weyand *et al.* (1993)	Injection of 5, 10 and 20 μL of either DMSO or a 50 mM solution of carbazole in DMSO on PND 1, 8 and 15, respectively. The total dose of carbazole was 1.75 μmol/mouse DMSO control, 38 M, 46 F; carbazole-treated, 34 M, 42 F.	No increase in incidence of tumours	-	Limited exposure to carbazole

d, day; DMSO, dimethyl sulfoxide, F, female; M, male; NR, not reported; PND, postnatal day; wk, week

on a basal diet for 8 weeks. Neoplastic nodules [hepatocellular adenoma] and hepatocellular carcinoma were observed in the liver; the incidence of both types of liver neoplasm in groups treated with carbazole was statistically significantly greater than that in the control group. Additionally, forestomach papilloma and forestomach squamous cell carcinoma were observed, mostly at the intermediate and highest doses, with the exception of forestomach papilloma in female mice that were also observed at the lowest dose. No tumours (squamous cell carcinoma or papilloma) were observed in the forestomach of male or female mice in the control groups.

(b) Subcutaneous administration

A group of 10 male A strain mice (age, 3–4 months), received 10 mg of crystallized carbazole moistened with glycerol, by subcutaneous injection, six times, in the left flank. All 10 mice were still alive after 1 year, and 4 were alive after 19 months. No tumours were reported at the injection site (Shear & Leiter, 1941). [The Working Group noted that the study was poorly reported; limitations included the small number of mice used and the lack of concurrent controls.]

(c) Intraperitoneal administration

Pups (CD-1 mice) were given intraperitoneal doses of 0 or 50 mM carbazole (1.75 µmol per mouse) in a volume of 5, 10 or 20 µL of DMSO on postnatal days 1, 8 and 15, respectively. The liver, lungs and any gross lesions in other tissues were examined histologically. No increase in the incidence of neoplasms was found (Weyand et al., 1993).

3.6.2 Rat

See Table 3.10

Oral administration

In a study of tumour promotion, four groups of male F344 rats were given drinking-water containing 0% or 0.05% N-butyl-N-(4-hydroxybutyl)nitrosamine [an initiator of carcinogenesis in the urinary bladder] for 2 weeks, and then fed basal diet containing carbazole at a concentration of 0% or 0.6% for 22 weeks. The incidence of urinary bladder hyperplasia was increased in carbazole-treated male F344 rats compared with controls. No neoplasia or hyperplasia was observed in the liver, kidney, or ureter (Miyata et al., 1985).

In a second study of tumour promotion, male F344 rats were given drinking-water containing N-bis(2-hydroxypropyl)nitrosamine at a concentration of 0% or 0.2% for 1 week, and 1 week later were then fed diet containing carbazole at a concentration of 0% or 0.6% for 50 weeks. Carbazole showed no promoting effect in the liver, lung, thyroid or urinary bladder. In addition, carbazole alone did not induce tumours in the lung and thyroid. An increased incidence ($P = 0.02$) of kidney (pelvic) papilloma and carcinoma combined was observed compared with initiator only (Shirai et al., 1988). [The Working Group noted that the purity of carbazole was not reported.]

3.6.3 Syrian golden hamster

See Table 3.11

Oral administration

Two groups of 12 or 18 Syrian golden hamsters (sex not reported) were fed diet containing carbazole at a concentration of 0% or 0.2% for 39 weeks (Moore et al., 1987). An increased incidence of liver foci was observed in the group receiving carbazole. [The Working Group noted the small number of hamsters tested and the short duration of exposure.]

Table 3.10 Studies of carcinogenicity in rats given drinking-water containing carbazole

Species, strain (sex) Duration Reference	Dosing regimen, Animals/group at start	Incidence of tumours	Significance	Comments
Rat, F344 (M) 24 wk Miyata *et al.* (1985)	Drinking-water containing 0% or 0.05% BBN for 2 wk followed by diet containing carbazole at 0% or 0.6% for 22 wk. On day 22 of the experiment, the left ureter of all rats was ligated. Control, 44; BBN + carbazole, 14; carbazole, 15	*Tumours of urinary bladder (papilloma)* BBN control: 0/44 Carbazole: 0/15 BBN + carbazole: 2/14 (14%) *Papillary/nodular hyperplasia* BBN control: 3/44 Carbazole: 0/15 BBN + carbazole: 5/14 (36%)*	**$P < 0.05$	
Rat, F344 (M) 52 wk Shirai *et al.* (1988)	Drinking-water containing DHPN at 0% or 0.2% for 1 wk, followed 1 wk later by diet containing carbazole at 0% or 0.6% for 50 wk 19–20 rats/group	For DHPN+carbazole, DHPN, carbazole: *Lung carcinoma*: 11/19 (58%), 16/20 (80%), 0/20 *Lung adenoma*: 17/19 (89%), 18/20 (90%), 0/20 *Thyroid carcinoma*: 15/19 (79%), 14/20 (70%), 0/20 *Thyroid adenoma*: 8/19 (42%), 7/20 (35%), 0/20 *Kidney (pelvic) papilloma and carcinoma*: 11/19 (58%), 4/20 (20%), NR *Bladder papilloma and carcinoma*: 7/19 (37%), 3/20 (15%), NR	DHPN+carbazole *vs* DHPN: NS NS NS NS $P = 0.02$ NS	No untreated controls. Purity of carbazole, NR.

BNN, *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine; d, day; DHPN, *N*-bis(2-hydroxypropyl) nitrosamine; M, male; NR, not reported; NS, not significant; wk, week.

Table 3.11 Study of carcinogenicity in hamsters given diet containing carbazole

Species, strain (sex) Duration Reference	Dosing regimen, Animals/group at start	Incidence of tumours	Significance	Comments
Hamster, Syrian golden (sex, NR) 40 wk Moore et al. (1987)	Diet containing carbazole (technical-grade, purity, 96%) at 0% or 0.2% for 39 wk 12 treated, 18 controls	*Liver foci* 0%, 0.2%: 0/18, 11/12 (92%)* *Forestomach papilloma* 0%, 0.2%: 0/18, 1/12 (8%)	*[$P < 0.0001$]	Small number of animals, short duration of exposure.

NR, not reported; wk, week

3.7 *7H*-Dibenzo[*c,g*]carbazole

Four early studies in mice given *7H*-dibenzo[*c,g*]carbazole by skin application (Boyland & Brues, 1937; Strong et al., 1938; Kirby & Peacock, 1946; Kirby, 1948), six early studies in mice given *7H*-dibenzo[*c,g*]carbazole by subcutaneous administration (Boyland & Brues, 1937; Strong et al., 1938; Andervont & Shimkin, 1940; Andervont & Edwards, 1941; Kirby, 1948; Lacassagne et al., 1955a, b) and one early study in rats given *7H*-dibenzo[*c,g*]carbazole by pulmonary implantation (Boyland & Brues, 1937) showed strong limitations and are not presented in the text or in the tables.

In addition, studies in mice given *7H*-dibenzo[*c,g*]carbazole by oral administration (Armstrong & Bonser, 1950), by skin application and by subcutaneous administration (Taras-Valéro et al., 2000), by intraperitoneal administration (Boyland & Mawson, 1938), by intravenous administration (Andervont & Shimkin, 1940), and by bladder implantation (Bonser et al., 1952), were considered by the Working Group as inadequate for evaluation, and are not presented in the tables. A study in hamsters (Sellakumar & Shubik, 1972) and in a dog (Bonser et al., 1954), although presented in the table and text, were also considered inadequate for evaluation.

Limitations of these studies included the small number of mice tested, the lack of a concurrent vehicle-control group, the lack of information on strain, age and sex, the lack of information on the purity and total amount of *7H*-dibenzo[*c,g*]carbazole administered, and absence of any description of the histological procedures employed.

3.7.1 Mouse

See Table 3.12

(a) Oral administration

In groups of male and female CBA and strong A mice (age not reported) given DBC orally at doses of 0.25–4.0 mg per week in arachis oil, for up to 59 weeks, the induction of forestomach papilloma and carcinoma, liver hepatoma and pulmonary adenoma (more efficiently in males) was reported (Armstrong & Bonser, 1950). [The Working Group noted that the study was limited by the small number of mice tested, the lack of concurrent control group, the lack of information on the purity of the DBC administered, and lack of information on the histopathological procedures employed.]

(b) Skin application

A study of carcinogenicity in skin was performed using highly purified DBC (purity, > 99%). Groups of 50 male C3H mice (age, 6–8 weeks) were treated twice per week with

12.5 μg (46.8 nmol) of DBC in 50 μL of acetone, applied to the interscapular region of the back. Topical applications were continued for 99 weeks, or until a mouse developed a tumour. Control groups included a group receiving no treatment and a group treated with solvent only. Lesions persisting for at least 1 week and with a minimum size of 1 mm^3 were diagnosed as skin papilloma. Histopathological examination was performed. The incidence of skin carcinoma was highly increased ($P < 0.0001$) in mice treated with DBC compared with either control (Warshawsky & Barkley, 1987).

In another study of complete carcinogenicity, 50 female Hsd:(ICR)BR mice (age, 5–6 weeks) were given 50 nmol of DBC in 50 μL of acetone, applied to the shaved back, twice per week, for 99 weeks or until the appearance of a tumour. Groups of untreated mice ($n = 11$), and mice treated with acetone only ($n = 11$) were used as negative controls. DBC produced skin tumours in 42 out of 50 (84%) mice, and liver neoplasms in 37 out of 50 (74%) mice (Warshawsky *et al.*, 1994). [The Working Group noted the limited number of controls evaluated by histopathology.]

In a complementary study of tumour initiation, groups of 30 female Hsd:(ICR)BR mice were given a single dose of DBC at 0 or 200 nmol, dissolved in acetone, or 200 nmol of benzo[*a*]pyrene (the positive control for initiation), applied to the shaved back. After 2 weeks, the mice were treated with 2 μg of TPA in 50 μL of acetone, applied twice per week for up 24 weeks. Skin tumours developed in 26 mice in the group receiving DBC plus TPA, and 27 mice in the group receiving benzo[*a*]pyrene plus TPA (Warshawsky *et al.*, 1992).

Female mice of the XVIInc./Z homozygous strain (age, 3 months) were given 50 μg of DBC in acetone, by skin painting, in 34 applications [interval between applications not given]. In groups of mice treated with DBC, the incidence of sarcoma was 70% (22 out of 31) at 6 months and the incidence of hepatoma was 100% (31 out

of 31) at 12–14 months (Taras-Valéro *et al.*, 2000). [The Working Group noted the poor description of experimental details.]

(c) Subcutaneous administration

Female mice of the XVIInc./Z homozygous strain (age, 3 months) were given 300 μg of DBC in 0.2 mL of olive oil by subcutaneous injection, three times, at 2-week intervals (Taras-Valéro *et al.*, 2000). In mice treated with DBC, the incidence of sarcoma was 70% (91 out of 130) at 6 months and the incidence of hepatoma was 100% (39 out of 39) at 12–14 months (162 animals in total in the experimental group). [The Working Group noted the poor description of experimental details in this study.]

(d) Intraperitoneal administration

Sixty-five mice were given DBC at a dose of 12.5 mg/kg bw in olive oil as a single intraperitoneal injection. Twenty-eight mice survived 39 days and one mouse developed a sarcoma over 200 days of observation; liver cholangiomas were also seen (Boyland & Mawson, 1938). [Study limitations included the lack of control group, lack of information on sex, age and strain used in the study, and lack of information on the histopathological methods used. The Working Group noted the poor survival of the mice.]

Groups of 20 male A/J mice (age, 6–8 weeks) were given DBC at a dose of 0, 5, 10, 20 or 40 mg/kg bw in 0.2 mL of tricaprylin, as a single intraperitoneal injection. Eight months after the injection, the mice were killed and tumours of the lung counted. Treatment with DBC resulted in a dose-related increase in the incidence (83–100%) and multiplicity (4.7–48.1 tumours per tumour-bearing mouse) of tumours of the lung compared with the controls (55% and 0.6 tumours per tumour-bearing mouse, respectively) (Warshawsky *et al.*, 1996b).

Table 3.12 Studies of carcinogenicity in mice given 7*H*-dibenzo[*c,g*]carbazole

Species, strain (sex) Duration Reference	Dosing regimen, Animals/group at start	Incidence and multiplicity of tumours	Significance	Comments
Skin application				
Mouse, C3H/Hej (M) 99 wk Warshawsky & Barkley (1987)	Treated topically with 46.8 nmol (12.5 µg) of DBC (purity, 99%) in 50 µl of acetone, or with 50 µl of acetone only, or untreated, 2×/wk, on the shaved interscapular region 50 mice/group	*Skin papillomas* 1/50 (2%), 0/50, 0/50 *Skin carcinomas* 47/50 (94%), 0/50, 0/50.	$P < 0.0001$, for skin carcinomas [one-tailed Fisher exact test]	
Mouse, Hsd:(ICR) BR (F) 99 wk Warshawsky *et al.* (1994, 1996b)	Treated topically with 50 nmol (13.4 µg) of DBC (purity, 99%) in 50 µL acetone, or 50 µL acetone only, or untreated, twice/wk on shaved interscapular region 50 mice/group	*Skin tumours* Untreated, acetone only, DBC 2/11 (18%), 3/11 (27%), 42/50 (84%) Squamous cell carcinoma: 0/11, 1/11 (9%), 27/50 (54%) Papilloma: 0/11, 2/11 (18%), 8/50 (16%) Basal cell carcinoma: 0/11, 0/11, 4/50 (16%) Keratoacanthoma: 0/11, 0/11, 2/50 (4%) Tumours in treated group: Squamous cell carcinoma: 27/50 (54%); papilloma: 8/50 (16%); basal cell carcinoma: 4/50 (8%); keratoacanthoma: 2/50 (4%) *Liver tumours* Hepatocellular carcinoma: 22/50 (44%), 0/5, 0/6 Hepatocellular adenoma: 17/50 (34%), 2/5 (40%), 1/6 (17%)	$P < 0.02$ $P < 0.001$ - - - [$P < 0.001$, squamous cell carcinoma] [$P = 0.041$, hepatocellular carcinoma] [NS]	Only 11 controls evaluated by histopathology

Table 3.12 (continued)

Species, strain (sex) Duration Reference	Dosing regimen, Animals/group at start	Incidence and multiplicity of tumours	Significance	Comments
Mouse, Hsd:(ICR) BR (F) 25 wk Warshawsky *et al.* (1992, 1996b)	Initiation–promotion Treated topically with 200 nmol of DBC (53.8 μg; purity, 99%) or BaP in 50 μL of acetone. After 2 wk, treated with 2 μg of TPA in 50 μL acetone. Control groups treated with TPA or DBC; twice/ wk on shaved interscapular region 30 mice/group	*Skin tumours* DBC+TPA, BaP+TPA, TPA, DBC: Papilloma: 26/30 (87%), 27/30 (90%), 0/30, 0/30	$P < 0.0001$; one-tailed Fisher exact test (DBC + TPA *vs* TPA)	
Intraperitoneal administration				
Mouse, A/J (M) 32 wk Warshawsky *et al.* (1996b)	Single injection at 0, 5, 10, 20 and 40 mg/kg bw of DBC in 0.2 mL of tricaprylin 55 mice/group	*Lung tumours* Incidence: 11/20 (55%), 15/18 (83%), 18/18 (100%), 12/12 (100%), 14/14 (100%) Multiplicity: 0.6, 4.7*, 13.6*, 14.2*, 48.1*	*$P < 0.05$ using Kurskal-Wallis one-way analysis (vs control group).	Study poorly reported. Limitations included lack of information on DBC purity, and lack of histopathology on organs other than lung.

BaP, benzo[*a*]pyrene; DBC, 7*H*-dibenzo[*c,g*]carbazole; F, female; M, male; NR, not reported; NS, not significant; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; vs, versus; wk, week

Table 3.13 Studies of carcinogenicity in hamsters given 7*H*-dibenzo[*c,g*]carbazole by intratracheal administration

Species, strain (sex) Duration Reference	Dosing regimen, Animals/group at start	Incidence of tumours	Significance	Comments
Hamster, Syrian (M) 30 wk Sellakumar & Shubik (1972)	Instillations of 0.5 or 3 mg of DBC suspended with an equal amount of haematite dust in 0.2 mL of saline, once/wk for 30 wk and 15 wk, respectively; control group was untreated Hamsters/group: 48 at 0.5 mg; 36 at 3 mg; 90 for controls	*Tumours of the respiratory tract* 40/45 (89%), 30/35 (86%), 0/82 (predominantly squamous cell carcinoma of the trachea, bronchi and larynx)	[*P* < 0.0001]	The study was limited by the lack of appropriate control group. Purity of DBC, NR

DBC, 7*H*-dibenzo[*c,g*]carbazole; M, male; NR, not reported; wk, week

(e) Intravenous administration

Groups of 10–12 strain A mice were given 0.25 mL of a 0.1% aqueous dispersion of DBC as a single injection; exposure duration was 8, 14, or 20 weeks. Lung tumours developed in all treated groups (Andervont & Shimkin, 1940). [A limitation of this study was the lack of concurrent control group.]

(f) Bladder implantation

Eight mice were given 1–2 mg of DBC contained in 10–20 mg paraffin-wax pellets implanted in the bladder. The treated mice showed an increase in the incidence of papilloma and metaplasia of the bladder; carcinoma was also observed (Bonser *et al.*, 1952). Twelve mice that were implanted with paraffin-wax pellets not containing DBC did not develop neoplasms of the bladder. [A limited number of animals was used.]

3.7.2 Syrian hamster

See Table 3.13

Intratracheal administration

Groups of male Syrian hamsters were given 0.5 mg (48 hamsters) or 3 mg (35 hamsters) of DBC (suspended with an equal amount of haematite dust in saline) by weekly instillation

Table 3.14 Study of carcinogenicity in a dog given 7*H*-dibenzo[*c,g*]carbazole by intravesical injection

Species, strain (sex) Duration Reference	Dosing regimen, Animals/group at start	Incidence of tumours	Significance	Comments
Dog mongrel, (F) (age NR) 168 wk Bonser *et al.* (1954)	5 mL of a 0.25% solution of DBC in arachis oil, once/wk for 12 months 1 dog	Multiple papillomas (approximately 40) and one urinary cystic transitional cell carcinoma	-	Study poorly reported, limitations included only one dog studied, no controls, purity of DBC used, NR.

DBC, 7*H*-dibenzo[*c,g*]carbazole; F, female; NR, not reported; wk, week

Table 3.15 Study of carcinogenicity in rats given benzo[*b*]naphthol[2,1-*d*]thiophene by pulmonary implantation

Species, strain (sex) Duration Reference	Dosing regimen, Animals/group at start	Incidence of tumours	Significance
Rat, Osborne-Mendel (F) 140 wk Wenzel-Hartung *et al.* (1990)	Single implantation of 0, 1, 3, or 6 mg (purity, 99.6%) in a 1 : 1 mixture of beeswax and trioctanoin. Positive-control groups given 0.03, 0.1, or 0.3 mg of benzo[*a*]pyrene 35 rats/group	*Squamous cell carcinoma of the lung* 0, 1, 3, or 6 mg of benzo[*b*]naphthol-[2,1-*d*]thiophene: 0/35, 1/35 (3%), 11/35 (31%), 11/35 (31%) 0.03, 0.1, or 0.3 mg of benzo[*a*]pyrene: 3/35 (9%), 11/35 (31%), 27/35 (77%)	[$P < 0.001$] (intermediate and highest dose)

F, female; wk, week

for 30 or 15 weeks, respectively. A group of 90 hamsters served as untreated controls. A total of 69 tumours of the respiratory tract developed in 40 out of 45 (89%) hamsters treated with 0.5 mg of DBC (multiplicity, 1.75), and 42 tumours of the respiratory tract developed in 30 out of 35 (86%) hamsters treated with 3 mg (multiplicity, 1.4). The tumours observed were predominantly squamous cell carcinomas of the trachea, bronchi and larynx. No respiratory tumours (0 out of 82) were observed in the control group (Sellakumar & Shubik, 1972). [There was no haematite control group.]

3.7.3 Dog

See Table 3.14

Intravesical injection

A dog given DBC by intravesical injection developed multiple papillomas and one urinary cystic transitional cell carcinoma (Bonser *et al.*, 1954). [The study was limited by the use of a single animal, and the absence of controls.]

3.8 Dibenzothiophene

No data on the carcinogenicity of dibenzothiophene in experimental animals were available to the Working Group.

3.9 Benzo[*b*]naphtho[2,1-*d*]thiophene

3.9.1 Rat

See Table 3.15

Pulmonary implantation

Groups of 35 inbred female Osborne-Mendel rats (age, 3 months) were given 1, 3, or 6 mg of benzo[*b*]naphtho[2,1-*d*]thiophene (purity, 99.6%) in a 1 : 1 mixture of beeswax and trioctanoin, as a single pulmonary implantation (Wenzel-Hartung *et al.*, 1990). An untreated group and a group that received the vehicle only (a mixture of beeswax and trioctanoin) served as controls. In the positive-control group, 35 rats were given 0.1, 0.3, or 1.0 mg of benzo[*a*]pyrene as a pulmonary implantation. For rats treated with benzo[*b*]naphtho[2,1-*d*]thiophene, increases in the incidence of squamous cell carcinoma were reported at doses of 1, 3 and 6 mg (2.9%, 31.4% and 31.4%, respectively). No tumours of the lung were found in the negative controls.

4. Mechanistic and Other Relevant Data

4.1 Benz[*a*]acridine

4.1.1 Metabolism and distribution

The bioconcentration and metabolism of benz[*a*]acridine in fathead minnows (*Pimephales promelas*) was investigated using ^{14}C-labelled benz[*a*]acridine. The bioconcentration factor was estimated at 106 ± 17, approximately one tenth of that predicted by octanol : water partitioning models. It was estimated that metabolism of benz[*a*]acridine reduced the extent of bioconcentration by 50–90% compared with that expected in the absence of metabolism. The rate constant for the metabolism of benz[*a*]acridine was 0.49 ± 0.07 per hour. Metabolites (not specified) accounted for the bulk of the radiolabel in fish after less than 1 day of exposure (Southworth *et al.*, 1981).

The study by Jacob *et al.* (1982) appeared to be the only comprehensive study on the metabolism of benz[*a*]acridine. Incubations were conducted with liver and lung microsomes from male Wistar rats that were previously untreated, or treated with phenobarbital or benzo[*k*]fluoranthene. The metabolite profile was analysed by GC-MS, following derivatization by silylation. A K-region 5,6-dihydrodiol and a non-K-region dihydrodiol were formed by liver and lung microsomes. Additional metabolites (number not specified) were detected, but not characterized. The K-region dihydrodiol was identified on the basis of the relative intensities of the MS fragment ions. The structure of the non-K-region dihydrodiol could not be assigned unequivocally, although the *trans*-3,4-dihydrodiol isomer was excluded by comparison with an authentic synthetic standard. Pre-treatment with phenobarbital induced K-region oxidation, while pretreatment with benzo[*k*]fluoranthene induced non-K-region oxidation. The ratios of the K-region to non-K-region metabolites were similar in liver and lung (1.8, ~6, and 0.33 for untreated, phenobarbital-treated and benzo[*k*]fluoranthene-treated rats, respectively). No evidence could be obtained for the formation of the putative ultimate carcinogen, *anti*-benz[*a*]acridine-3,4-dihydrodiol-1,2-epoxide, a bay-region diol-epoxide. The metabolic rate was low compared with that observed in concurrent incubations with benz[*c*]acridine (Jacob *et al.*, 1982).

[The bay-region diol-epoxides have yet to be unequivocally identified (either directly or indirectly) *in vivo* or in test systems *in vitro*.]

4.1.2 Genotoxicity and other relevant effects

When benz[*a*]acridine was tested for mutagenicity at concentrations of up to 0.5 mg/plate in *Salmonella typhimurium* TA98 (his⁻/his⁺) in the presence of an exogenous metabolic system, the results were inconclusive (Ho *et al.*, 1981). Contrasting with these earlier mutagenesis data, benz[*a*]acridine gave positive results a concentration of ~0.01 μM in the Mutatox test, an luminescence assay for reverse bacterial mutation in *Vibrio fischeri* (Bleeker *et al.*, 1999). The mutagenic activities of 1,2,3,4-tetrahydrobenz[*a*]acridine-1,2-epoxide and benz[*a*]acridine-3,4-dihydrodiol-1,2-epoxides were examined in bacteria and mammalian cells, to assess the potential significance of bay-region activation. The *syn-* and *anti*-benz[*a*]acridine-3,4-dihydrodiol-1,2-epoxides (racemic mixture) induced 6 and 60 his⁺ revertants/nmol, respectively, in *S. typhimurium* TA98; higher numbers of histidine autotrophs (60/nmol and 240/nmol, respectively) were induced in strain TA100. In comparison, 1,2,3,4-tetrahydrobenz[*a*]acridine-1,2-epoxide (racemic mixture) was considerably more mutagenic (800 and 3000 revertants/nmol in strains TA98 and TA100, respectively). The same trends were observed in Chinese hamster V79–6 cell lines. Benz[*a*]acridine-3,4-dihydrodiol (presumed to have a *trans* configuration) had no

intrinsic mutagenicity [in the absence of metabolic activation] and no significant increase in mutation frequency was observed in *S. typhimurium* TA100 in the presence of liver microsomes from immature male Long Evans rats treated with Aroclor 1254. However, low but statistically significant activation of the compound was observed in the same strain when the incubations were conducted in the presence of a highly purified and reconstituted mono-oxygenase system obtained from the same type of liver microsome (Wood *et al.*, 1983).

Benz[*a*]acridine and its derivatives, the *trans*-benz[*a*]acridine-3,4-dihydrodiol and the *syn*- and *anti*-benz[*a*]acridine-3,4-dihydrodiol-1,2-epoxides (as the racemic mixture), were tested for genotoxicity in two rat hepatoma cell lines, at a single concentration (250 μM) and exposure time (2 hours). The genotoxic effect was measured by alkaline elution (i.e. the appearance of alkali-labile DNA sites). The selected hepatoma cell lines were: H_5, a dedifferentiated cell line that strongly expresses PAH-inducible CYP448-dependent mono-oxygenases (CYP1A and CYP1B), but not CYP450-dependent enzymes (CYP2B); and H_{1-4}, a differentiated hybrid cell line that contains CYP448- and CYP450-dependent mono-oxygenases. The parent benz[*a*]acridine had no effect on any of the cell lines. Likewise, benz[*a*]acridine-3,4-dihydrodiol did not induce DNA-strand breaks in any of the cell lines, in contrast to the analogous benz[*c*]acridine-3,4-dihydrodiol. Each of the benz[*a*]acridine-derived diol-epoxides induced DNA damage in both cell lines. The *anti*-diol-epoxide was more potent than the *syn* isomer and was three times more potent in H_5 cells than in H_{1-4}. The genotoxicity observed with *anti*-benz[*a*]acridine-3,4-dihydrodiol-1,2-epoxide contrasted with the weak mutagenicity of the same compound in the Ames test and in Chinese hamster V79 cells (see above). [Although the authors suggested that the discrepancy between the Ames assay and this assay for genotoxicity might be due to

the antibacterial activity of benz[*a*]acridine, the Working Group noted that this would probably not explain the weak mutagenicity in V79 cells]. Overall, benz[*a*]acridine and its derivatives are not extensively metabolized to active mutagens (Loquet *et al.*, 1985).

A recombinant plasmid containing the thymidine kinase (*Tk*) gene (pAGO; 6.36 kb) was reacted *in vitro* with *syn*- and *anti*-benz[*a*]acridine-3,4-dihydrodiol-1,2-epoxide (racemic mixture). The covalent DNA binding and limited restriction by different endonucleases observed *in vitro* were correlated with biological activity by transfer of the plasmid (*Tk* gene) to TK-deficient cells. Upon transfection of mouse Ltk-cells with modified and non-modified plasmid, the benz[*a*]acridine diol-epoxides reduced the number of TK^+ clones formed to a similar, although weaker, degree than that obtained with *anti*-benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide (0.8 and 0.3 ng/10 ng DNA for the benz[*a*]acridine and benzo[*a*]pyrene derivatives, respectively). The inhibition of transformation efficiency was consistent with inactivation of the gene by chemical modification (Schaefer-Ridder *et al.*, 1984).

4.1.3 Mechanistic considerations

Several studies have addressed the induction of specific mono-oxygenases by benz[*a*]acridine. Pre-treatment of male Wistar rats with benz[*a*]acridine resulted in weak induction of liver mono-oxygenase activity, accompanied by a significant change in the microsomal metabolite profile of benz[*a*]anthracene, which favoured K-region 5,6-oxidation (Jacob *et al.*, 1983). Benz[*a*]acridine was also a weak inducer of chrysene metabolism (Jacob *et al.*, 1987). In addition, benz[*a*]acridine was found to markedly increase the rates of ethoxyresorufin and ethoxycoumarin *O*-deethylation by rat liver microsomes and to induce proteins recognized by antibodies to CYP1A1, but not CYP2B1 (Ayrton *et al.*, 1988). More recently, CYP1A1 induction by benz[*a*]

acridine was demonstrated in fish hepatoma PLHC-1 cells (Jung *et al.*, 2001).

The ability of benz[*a*]acridine to induce the aryl hydrocarbon receptor (AhR) was assessed *in vitro* in the CALUX® assay, using a rat hepatoma cell line stably transfected with a luciferase reporter gene under the control of dioxin-responsive elements. In a similar luciferase-reporter test, using the breast carcinoma MVLN cell line, benz[*a*]acridine was a weak inducer of estrogenic activity (Machala *et al.*, 2001). Quantitative structure–activity relationships for potency to activate AhR indicated ellipsoidal volume, molar refractivity, and molecular size as the best descriptors (Sovadinová *et al.*, 2006).

4.2 Benz[*c*]acridine

4.2.1 Metabolism

The study by Jacob *et al.* (1982) appears to be the only comprehensive study on the metabolism of benz[*c*]acridine. Incubations were conducted with liver microsomes from male Wistar rats that were untreated, or treated with phenobarbital, benzo[*k*]fluoranthene, or 5,6-benzoflavone. The metabolite profile was analysed by GC-MS, following derivatization by silylation. Incubation with microsomes from untreated rats yielded five different phenols (unidentified), one diphenol (unidentified) and two dihydrodiols. The major metabolite was identified as the [K-region] 5,6-dihydrodiol, on the basis of the relative intensities of the MS fragment ions. Pretreatment with phenobarbital doubled the total metabolite rate and significantly altered the metabolite profile: only one of the five phenols was detected and its amount had decreased by approximately seven times. This was accompanied by a seven-times increase in the amount of the 5,6-dihydrodiol, which was again the major metabolite. The previously detected other dihydrodiol and two additional non-K-region dihydrodiols (unidentified) were also present. Two

K-region triols (i.e. monophenolic derivatives of the K-region dihydrodiol) were also detected, but the position of the phenolic hydroxyl group was not established. Pre-treatment with benzo[*k*]fluoranthene or 5,6-benzoflavone increased the rates of total metabolism approximately 2.8 and 3.9 times, respectively. Both pre-treatments stimulated K-region oxidation and also the formation of phenols and diphenols; the 5,6-dihydrodiol was again the major metabolite. On the basis of MS fragmentation patterns, a small extent of *N*-oxidation also occurred, albeit in very small amounts, compared with a synthetic standard, *trans*-benz[*c*]acridine-3,4-dihydrodiol. Upon incubation of uninduced and benzo[*k*]fluoranthene-induced liver microsomes with the 3,4-dihydrodiol, a diphenol, assumed to be 3,4-dihydroxybenz[*c*]acridine, and a tetrol (tentatively identified as 3,4,5,6-tetrahydroxy-3,4,5,6-tetrahydrobenz[*c*]acridine) were detected. Unequivocal evidence for the formation of the putative ultimate carcinogen, *anti*-benz[*c*]acridine-3,4-dihydrodiol-1,2-epoxide (a bay-region diol-epoxide), could not be obtained (Jacob *et al.*, 1982).

4.2.2 Genotoxicity and other relevant effects

Two studies reported positive results in tests for mutagenicity with benz[*c*]acridine at a concentration of 25 µg/plate in *S. typhimurium* TA100 (his^{-}/his^{+}) in the presence of an exogenous metabolic system (Okano *et al.*, 1979; Baker *et al.*, 1980).

When tested in Chinese hamster Don (lung) cells, benz[*c*]acridine at 1–100 µM induced sister-chromatid exchange without the addition of metabolic activation from S9 (Baker *et al.*, 1983).

The mutagenic activities of 1,2,3,4-tetrahydrobenz[*c*]acridine-1,2-epoxide and of the diol-epoxide metabolites, *syn*- and *anti*-benz[*c*]acridine-3,4-dihydrodiol-1,2-epoxides, were examined in bacteria and mammalian cells, to assess the potential significance of bay-region

activation. The *syn*- and *anti*-benz[*c*]acridine-3,4-dihydrodiol-1,2-epoxides (racemic mixture) had comparable mutagenic potencies in *S. typhimurium* TA98 (250 and 300 his$^+$ revertants/nmol, respectively). In strain TA100, the *syn*-diol-epoxide induced 5100 his$^+$ revertants/nmol and was approximately twice more active than the *anti* isomer. The order of relative mutagenicities was reversed in Chinese hamster V79–6 cells, in which the anti-diol-1,2-epoxide, which induced 4.5 8-azaguanine-resistant colonies/10^5 surviving cells per nmol, was approximately twofold more active than the *syn* isomer. In both test systems (i.e. *S. typhimurium* TA98 and TA100, and V79 cells), the bay-region diol-epoxides were one to four orders of magnitude more mutagenic than their non-bay-region counterparts (i.e. racemic *anti*-1,2-dihydrodiol-3,4-epoxide, *syn*- and *anti*-8,9-dihydrodiol-10,11-epoxide, and *syn*- and *anti*-10,11-dihydrodiol-8,9-epoxide). In comparison with the analogous benz[*a*]acridine derivatives, the bay-region diol-epoxides from benz[*c*]acridine were more mutagenic by at least one order of magnitude. The bay-region 1,2,3,4-tetrahydrobenz[*c*]acridine-1,2-epoxide (racemic mixture) had high mutagenic activity, about four to eleven times greater than the corresponding benz[*a*]acridine metabolite. Neither the bay-region benz[*c*]acridine diol-epoxides nor 1,2,3,4-tetrahydrobenz[*c*]acridine-1,2-epoxide were metabolized to non-mutagenic derivatives by highly purified epoxide hydrolase. Metabolic-activation experiments were conducted in *S. typhimurium* TA100 in the presence of either liver microsomes from immature male Long Evans rats treated with Aroclor 1254, or a highly purified and reconstituted mono-oxygenase system obtained from the same type of liver microsomes. The results indicated that *trans*-benz[*c*]acridine-3,4-dihydrodiol, the putative immediate precursor of the bay-region diol-epoxides, was at least five times more active than the parent compound, and that none of the other possible *trans*-dihydrodiols (i.e. the 1,2-, 5,6-,

8,9-, and 10,11-dihydrodiols) underwent significant activation to mutagenic derivatives (Wood *et al.*, 1983).

Benz[*c*]acridine and its derivatives, the *trans*-benz[*c*]acridine-3,4-dihydrodiol and the *syn*- and *anti*-benz[*c*]acridine-3,4-dihydrodiol-1,2-epoxides (as racemic mixture), were tested for genotoxicity in two rat hepatoma cell lines, at a single concentration (250 µM) and exposure duration (2 hours). The genotoxic effect was measured by alkaline elution (i.e. the appearance of alkali-labile DNA sites). The selected cell lines were: H$_5$, a dedifferentiated cell line that strongly expresses PAH-inducible CYP448-dependent mono-oxygenases (CYP1A and CYP1B), but not CYP450-dependent enzymes (CYP2B); and H$_{1-4}$, a differentiated hybrid cell line that contains both CYP448- and CYP450-dependent mono-oxygenases. While the parent heterocycle (benz[*c*]acridine) had no effect on any of the cell lines, the 3,4-dihydrodiol induced DNA single-strand breaks at the same order of magnitude in both cell lines, with approximately 60% of the initial DNA remaining in the filter after elution. Each of the benz[*c*]acridine-derived diol-epoxides induced DNA damage in both cell lines. The *anti*-diol-epoxide was more potent than the *syn* isomer and was three times more potent in H$_{1-4}$ cells than in H$_5$. (Loquet *et al.*, 1985).

A recombinant plasmid containing the mouse thymidine kinase (*Tk*) gene (pAGO; 6.36 kb) was tested *in vitro* with *syn*- and *anti*-benz[*a*]acridine-3,4-dihydrodiol-1,2-epoxide (racemic mixture). The covalent DNA binding and limited restriction by different endonucleases observed *in vitro* were correlated with biological activity by transfer of the plasmid (*Tk* gene) to TK-deficient cells. Upon transfection of mouse LTK$^-$ cells with modified and non-modified plasmid, the benz[*a*]acridine diol-epoxides reduced the formation of TK$^+$ clones which was similar, although weaker, than that obtained with *anti*-benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide (0.8 and 0.3 ng per 10 ng DNA for the benz[*c*]acridine and benzo[*a*]

pyrene derivatives, respectively). The inhibition of transformation efficiency was consistent with inactivation of the gene by chemical modification (Schaefer-Ridder *et al.*, 1984).

4.2.3 Mechanistic considerations

Several studies have addressed the induction of specific mono-oxygenases by benz[*c*] acridine. Pretreatment of male Wistar rats with benz[*c*]acridine resulted in weak induction of liver mono-oxygenase activity, accompanied by a significant change in the microsomal metabolite profile of benz[*a*]anthracene, which favoured 5,6-oxidation. Benz[*c*]acridine was also a weak inducer of chrysene metabolism (Jacob *et al.*, 1987). The induction of CYP1A1 by benz[*c*]acridine was demonstrated in fish hepatoma PLHC-1 cells (Jung *et al.*, 2001).

The tumorigenicities of benz[*c*]acridine bay-region diol-epoxides and their putative metabolic precursors have been demonstrated (Levin *et al.*, 1983; Chang *et al.*, 1984). The substantially higher activities of the bay-region diol-epoxides from benz[*c*]acridine in bacteria and mammalian cells, compared with their benz[*a*]acridine analogues, are consistent with qualitative arguments of resonance stabilization of the carbocations stemming from epoxide ring opening (Jerina *et al.*, 1976).

The ability of benz[*c*]acridine to induce AhR was assessed in the CALUX® assay *in vitro*, using a rat hepatoma cell line stably transfected with a luciferase reporter gene under the control of dioxin-responsive elements. After exposure for 6 hours, benz[*c*]acridine was six to seven times less potent than benzo[*a*]pyrene. In a similar luciferase-reporter test, using the breast carcinoma MVLN cell line, benz[*c*]acridine did not induce estrogenic activity (Machala *et al.*, 2001). Quantitative structure–activity relationships for potency to activate AhR indicated ellipsoidal volume, molar refractivity, and molecular size as the best descriptors (Sovadinová *et al.*, 2006).

4.3 Dibenz[*a,h*]acridine

4.3.1 Metabolism

The first comprehensive study of the metabolism of dibenz[*a,h*]acridine compared the extent of conversion and metabolite patterns after incubation with liver microsomes from male Sprague-Dawley rats pre-treated with dibenz[*a,h*] acridine, 3-methylcholanthrene, phenobarbital or corn oil. After an incubation of 6 minutes, the extent of total metabolism of dibenz[*a,h*]acridine corresponded to 21, 14, 0.7, or 0.2 nmol/mg protein with microsomes from rats pre-treated with dibenz[*a,h*]acridine, 3-methylcholanthrene, phenobarbital, or corn oil, respectively.

Regardless of the type of induction, the product profiles were very similar and the major metabolites were the dihydrodiols that contained bay-region double bonds, specifically, dibenz[*a,h*]acridine-3,4-dihydrodiol and dibenz[*a,h*]acridine-10,11-dihydrodiol, each accounting for 21–23% of the total when using microsomes from rats induced with 3-methylcholanthrene. Additional metabolites included dibenz[*a,h*]acridine-1,2-dihydrodiol (about 5%), two K-region epoxides (dibenz[*a,h*]acridine-12,13-epoxide and 5,6-epoxide, at approximately 5% and 2% of the total metabolites, respectively), several unidentified polar metabolites (10–15%), and several unidentified metabolites co-eluting with 3-hydroxy-dibenz[*a,h*]acridine (20%). The 8,9-dihydrodiol was not formed (< 2%). In combination, the 3,4-dihydrodiols and 10,11-dihydrodiols accounted for 40–50% of the total metabolism, with no apparent effect of the position of the nitrogen on relative extents of formation. K-region metabolism was a minor pathway, similarly to that reported for dibenz[*a,h*] anthracene, an isosteric analogue of dibenz[*a,h*] acridine (Steward *et al.*, 1987).

A subsequent study investigated the stereoselectivity of rat-liver enzymes in the conversion of dibenz[*a,h*]acridine to its 3,4-dihydrodiol and

10,11-dihydrodiol metabolites and in the conversion of dibenz[*a,h*]acridine-10,11-dihydrodiol enantiomers to their bay-region diol-epoxides. Using liver microsomes from immature male Long-Evans rats treated with 3-methylcholanthrene, or controls, the 3,4- and the 10,11-dihydrodiols were formed predominantly as the *R,R*-enantiomers, in 38–54% enantiomeric excess. Metabolism of each of the 10,11-dihydrodiol enantiomers by liver microsomes from control rats produced predominantly bay-region diol-epoxides (characterized upon hydrolysis to the tetrols), which accounted for 46–59% of the total metabolites. In contrast, bay-region diol-epoxides accounted for only 14–17% of the total metabolites produced by liver microsomes from rats treated with 3-methylcholanthrene. In all instances, the bay-region diol-epoxides produced were predominantly of the *anti* configuration. (–)-(10*R*,11*R*)-Dibenz[*a,h*]acridine-10,11-dihydrodiol was metabolized by liver microsomes from rats treated with 3-methylcholanthrene to the highly mutagenic *anti*-(+)-(8*R*,9*S*,10*S*,11*R*) diol-epoxide in an amount that was 6.5 times more than that of the corresponding *syn*-diol-epoxide. The *anti/syn* diol-epoxide ratio was 1.7 when (–)-(10*R*,11*R*)-dibenz[*a,h*]acridine-10,11-dihydrodiol was metabolized by liver microsomes from control rats. Metabolism of (10*S*,11*S*)-dibenz[*a,h*]acridine-10,11-dihydrodiol by liver microsomes from control rats or rats treated with 3-methylcholanthrene yielded *anti/ syn* diol-epoxide ratios of 1.5 and 2.3, respectively (Kumar *et al.*, 1995).

A more recent study investigated the biotransformation of dibenz[*a,h*]acridine by recombinant human CYP1A1, 1B1, and 3A4, and rat CYP1A1, in the presence of human or rat epoxide hydrolase. Among the human isoforms, CYP1A1 was the most effective (5.38 ± 0.56 pmol/min per pmol CYP), CYP1B1 had moderate activity (0.67 ± 0.07 pmol/min per pmol CYP) and CYP3A4 was the least active (0.20 ± 0.03 pmol/ min per pmol CYP). The rate of total dibenz[*a,h*] acridine metabolism by human CYP1A1 was less than half that by rat CYP1A1. The major dibenz[*a,h*]acridine metabolites produced by human CYP1A1 and CYP1B1 were the *trans*-3,4- and *trans*-10,11-dihydrodiols. CYP1A1 gave a higher proportion of the 10,11-dihydrodiol than of the 3,4-diol (about 45% versus about 24%). In contrast, human CYP1B1 yielded a much greater proportion of 3,4-dihydrodiol than of 10,11-dihydrodiol (about 55% versus about 6%), and rat CYP1A1 did not show regioselectivity, giving nearly equal proportions of the two diols. Despite the differences in regioselectivity, human CYP1A1 and CYP1B1 and rat CYP1A1 had similar stereoselectivities for the formation of the 3,4- dihydrodiols and 10,11-dihydrodiols: in all instances, the *R,R* enantiomers were formed almost exclusively (> 91.5%) (Yuan *et al.*, 2004).

4.3.2 Genotoxicity and other relevant effects

Dibenz[*a,h*]acridine was reported to enhance viral cell transformation in immortalized rat embryo cells *in vitro* (Freeman *et al.*, 1973).

Dibenz[*a,h*]acridine was tested for clastogenicity in a Chinese hamster fibroblast cell line (CHL). Results were negative, both in the absence and in the presence of a S9 metabolic activation system, while dibenz[*a,j*]acridine and dibenz[*c,h*] acridine gave positive results in the presence of metabolic activation from S9 (see Section 4.4.2; Section 4.5.2; Matsuoka *et al.*, 1982).

Kitahara *et al.* (1978) tested the mutagenicity of dibenz[*a,h*]acridine and of the K-region dibenz[*a,h*]acridine-12,13-epoxide (racemic mixture) in *S. typhymurium* TA98 and TA100, with or without S9 from rats induced with polychlorinated biphenyls. Dibenz[*a,h*]acridine was inactive without metabolic activation, but showed mutagenicity with metabolic activation, particularly in TA100 (2.3 and 39 revertants/ µg per plate, in TA98 and TA100, respectively). The K-region 12,13-epoxide was weakly active in TA100 in the absence of metabolic activation (1.8

revertants/µg per plate). However, in the presence of metabolic activation (1.4 and 13 revertants/µg per plate in TA98 and TA100, respectively), it was less mutagenic than dibenz[a,h]acridine. These data indicated that dibenz[a,h]acridine-12,13-epoxide is a reactive metabolite, but not an intermediate in the pathway of activation of the parent compound to a mutagen (Kitahara et al., 1978).

Another mutagenicity study in S. typhimurium gave negative results at up to 1000 µg/plate in strains TA1535, TA1537, TA1538, TA98, and TA100 in the presence of microsomal S9 from rats induced with Aroclor (Salamone et al., 1979).

Dibenz[a,h]acridine was mutagenic in S. typhimurium TA100 in the presence of liver microsomes from rats co-treated with phenobarbital and 5,6-benzoflavone at 0–100 µg/plate (Karcher et al., 1985).

The mutagenicities of dibenz[a,h]acridine and dibenz[a,h]acridine-1,2-, -3,4-, -8,9-, and -10,11-dihydrodiols were assessed in S. typhimurium TA100, in the presence of a metabolic activation system from immature Long-Evans male rats pretreated with Aroclor 1254. Dibenz[a,h]acridine-10,11-dihydrodiol, the precursor of the bay-region dibenz[a,h]acridine-10,11-dihydrodiol-8,9-epoxides, was about three times more active than dibenz[a,h]acridine at 125 µM, and approximately twelve times more active than dibenz[a,h]acridine-3,4-dihydrodiol, the metabolic precursor of the dibenz[a,h]acridine-3,4-dihydrodiol-1,2-epoxides. Activation of dibenz[a,h]acridine-1,2-dihydrodiols and dibenz[a,h]acridine-8,9-dihydrodiols to mutagenic products in TA100 was almost negligible. The mutagenic activities of the four bay-region diol-epoxides from dibenz[a,h]acridine (racemic syn- and anti-3,4-dihydrodiol-1,2-epoxide; racemic syn- and anti-10,11-dihydrodiol-8,9-epoxide) were assessed in bacteria and mammalian cells. The diastereomeric 10,11-dihydrodiol-8,9-epoxides were 20–40 times more mutagenic than the corresponding 3,4-dihydrodiol-1,2-epoxides in

S. typhimurium TA98 and TA100, with the anti-10,11-dihydrodiol-8,9-epoxide being approximately 2.5 times more active in either strain than its syn diastereomer. In the Chinese hamster V79–6 cell line, which lacks the capacity for oxidative metabolism of PAHs to mutagens, the 10,11-dihydrodiol-8,9-epoxide diastereomers were 20–80 times more mutagenic than their 3,4-dihydrodiol-1,2-epoxide analogues. The anti-10,11-dihydrodiol-8,9-epoxide was twice as cytotoxic and five times more mutagenic than the syn-10,11-dihydrodiol-8,9-epoxide. Likewise, the syn-10,11-dihydrodiol-8,9-epoxide was twice as cytotoxic, and at least 20 times more mutagenic than the syn-3,4-dihydrodiol-1,2-epoxide. The anti-3,4-dihydrodiol-1,2-epoxide was the least cytotoxic of the four diol-epoxides tested (Wood et al., 1989).

In a subsequent study, the four enantiomerically pure dibenz[a,h]acridine-10,11-dihydrodiol-8,9-epoxides (Kumar et al., 1992) were also evaluated for mutagenicity in S. typhimurium TA98 and TA100 and in the Chinese hamster V79–4 cell line. The anti-(–)-(8S,9R,10R,11S) diol-epoxide was the most mutagenic of the four compounds in S. typhimurium, inducing 1200 and 6900 his⁺ revertants/nmol in strains TA98 and TA100, respectively. The mutagenic activities of the remaining three stereoisomers were 14–72% that of the (S,R,R,S) isomer, with the dose–response relationships for the induction of histidine revertants being qualitatively similar in both strains; the two anti diol-epoxides were three to seven times more mutagenic than the syn isomers. In contrast, in Chinese hamster V79 cells, the anti-(+)-(8R,9S,10S,11R) diol-epoxide, which induced 68 8-azaguanine-resistant variants/nmol per 10⁵ cells, was two to eleven times more mutagenic than the other three diol-epoxides. This is similar to what has been observed with bay-region diol-epoxides from numerous PAHs, where the (R,S,S,R) isomer may not be the most active in bacterial assays, but tends to be

the most mutagenic in mammalian cells (Chang *et al.*, 1993).

Groups of six male Sprague-Dawley rats (age, 4–6 weeks) were given dibenz[*a,h*]acridine as three equal doses of 25, 50, or 100 mg/kg bw by intratracheal instillation over 24 hours, and killed 6 hours after the third dose. ^{32}P-Postlabelling, using either butanol extraction or nuclease P$_1$ digestion enrichment procedures, detected one DNA adduct. There was a dose–response effect; at the highest dose, the number of adducts was estimated to be $1.9/10^8$ nucleotides when using butanol, and $0.7/10^8$ nucleotides when using nuclease P$_1$. Two cytogenetic end-points, sister-chromatid exchange and micronucleus formation, were also investigated. Although both assays were less sensitive than the ^{32}P-postlabelling assay, the induction of sister-chromatid exchange occurred in lung cells at the two highest doses (number of sister-chromatid exchanges, 10.6 ± 3.5 per cell and 11.2 ± 3.8 per cell with dibenz[*a,h*]acridine at a dose of 50 or 100 mg/kg bw, respectively) and micronuclei were induced at the highest dose (Whong *et al.*, 1994).

4.3.3 Mechanistic considerations and additional observations

Ionization potentials were used to predict the mechanism of metabolic activation of carcinogenic PAHs. In the case of dibenz[*a,h*]acridine, a high ionization potential (> 8.10 eV) is consistent with a mono-oxygenation pathway, rather than one-electron oxidation (Xue *et al.*, 1999).

Dibenz[*a,h*]acridine combines the structural features of benz[*a*]acridine and benz[*c*]acridine. The lack of symmetry, due to the presence of the nitrogen atom in position 7, results in two distinct bay-regions. Thus, metabolism of dibenz[*a,h*] acridine yields two pairs of bay-region diol-epoxides that are not structurally equivalent. The differences in structure result in different biological activities that differ between the diol-epoxides and their dihydrodiol precursors. The

available data in bacterial and mammalian cells indicated that the bay-region dibenz[*a,h*]acridine-10,11-dihydrodiol-8,9-epoxides and their putative metabolic 10,11-dihydrodiol precursor are considerably more mutagenic than the analogous bay-region 3,4-dihydrodiol-1,2-epoxides and their 3,4-dihydrodiol precursor. Of note, the 1,2- and 8,9-dihydrodiols, which cannot be converted to bay-region diol-epoxides, are not activated by metabolic systems to mutagenic products in *S. typhimurium* TA100 (Wood *et al.*, 1989).

A recent model computational study, using the density functional theory, yielded results generally consistent with earlier quantum mechanical calculations of the predicted ease of benzylic carbocation formation at C-1 and C-8 of dibenz[*a,h*]acridine diol-epoxides. The computational data suggested that carbocation formation at C-8 is energetically favoured over C-1, which may predict lower reactivity for the 3,4-dihydrodiol-1,2-epoxides compared with the 10,11-dihydrodiol-8,9-epoxides (Borosky & Laali, 2005). A decreased propensity for epoxide ring opening of the 3,4-dihydrodiol-1,2-epoxides may explain their lower mutagenic activity.

The data on mutagenicity in mammalian cells and on tumour initiation on mouse skin implicated *trans*-(–)-(10*R*,11*R*)-dibenz[*a,h*]acridine-10,11-dihydrodiol as the proximate carcinogen and the bay-region *anti*-(–)-(8*R*,9*S*,10*S*,11*R*) diol-epoxide as the ultimate carcinogen. The high tumorigenicity of the *R,S,S,R* diol-epoxide reveals a stereoselectivity identical to those exhibited by other homocyclic and *N*-heterocyclic PAHs, including benzo[*a*]pyrene, benz[*a*]anthracene, chrysene, benzo[*c*]phenanthrene, and dibenz[*c,h*] acridine (Chang *et al.*, 1993).

Human CYP1A1 is substantially more active in dibenz[*a,h*]acridine metabolism than human CYP1B1 and, contrary to rat CYP1A1, is regioselective for formation of the 10,11-dihydrodiol, when compared with the 3,4-dihydrodiol. In addition, the observed stereoselectivity for

production of the (–)-10*R*,11*R* isomer (the proximate carcinogen) suggests that a high expression of CYP1A1 activity may confer increased susceptibility to carcinogenesis induced by dibenz[*a*,*h*] acridine. In contrast, human CYP1B1 appears to play a minor role in the metabolic activation of dibenz[*a*,*h*]acridine (Yuan *et al.*, 2004).

Intratracheal instillation of dibenz[*a*,*h*]acridine in rats resulted in DNA adducts, sister-chromatid exchange, and the induction of micronucleus formation in lung cells. Although similar studies had not been reported with dibenz[*a*,*h*]acridine metabolites, the combined data were consistent with bioactivation of the parent compound to a genotoxicant by metabolism to the (–)-(10*R*,11*R*)-dihydrodiol and subsequent formation of the corresponding *anti* diol-epoxide (Whong *et al.*, 1994).

Dibenz[*a*,*h*]acridine was a potent inducer of mono-oxygenase activities in rat liver. The induction affected the metabolite profile of benz[*a*] anthracene in the presence of metabolic activation, suppressing 10,11-oxidation and favouring 5,6-, and 8,9- (but not bay-region) oxidation (Jacob *et al.*, 1985). Also, liver microsomes from rats pretreated with dibenz[*a*,*h*]acridine stimulate chrysene metabolism to the proximate carcinogen, *trans*-chrysene-1,2-dihydrodiol (Jacob *et al.*, 1987).

The ability of dibenz[*a*,*h*]acridine to induce AhR was quantified *in vitro* in the CALUX® assay, using a rat hepatoma cell line stably transfected with a luciferase reporter gene under the control of dioxin-responsive elements. After an exposure of 6 hours, dibenz[*a*,*h*]acridine was 2.45 times more potent than 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, and 217 times more potent than benzo[*a*] pyrene, suggesting that it may contribute significantly to overall AhR-mediated activity in the environment (e.g. river sediments). The same study did not detect statistically significant estrogenic activity with dibenzo[*a*,*h*]acridine (Machala *et al.*, 2001). Quantitative structure–activity relationships for potency in activation of

AhR indicated ellipsoidal volume, molar refractivity, and molecular size as the best descriptors (Sovadinová *et al.*, 2006).

4.4 Dibenz[*a*,*j*]acridine

4.4.1 Distribution and metabolism

(a) Distribution

When male Wistar rats were given [³H] dibenz[*a*,*j*]acridine at a dose of 0.5 mg/kg bw in DMSO by intraperitoneal administration, faecal excretion accounted for the bulk of the radiolabel and occurred essentially within 48 hours. When [³H]dibenz[*a*,*j*]acridine was given at the same dose by intravenous administration to cannulated rats, there was rapid (within 6 hours) biliary excretion. After treatment with β-glucuronidase and arylsulfatase, about 25% of the excreted radiolabel was soluble in ethyl acetate. This fraction contained 3-hydroxydibenz[*a*,*j*]acridine and 4-hydroxydibenz[*a*,*j*]acridine, polar products of secondary oxidation, and only a small amount (1–2%) of the 3,4-dihydrodiol. In the absence of enzymatic hydrolysis, the total amount of radiolabel extracted into ethyl acetate did not exceed 3% (Robinson *et al.*, 1990).

In mice, topical application of [³H]dibenz[*a*,*j*] acridine resulted in radiolabel peaks in the kidney at 6 hours and in the liver at 12 hours. The total concentration of radiolabel in the skin decreased by approximately 50% over 96 hours. After 48 hours, the parent compound accounted for 25–30% of the total radiolabel in the liver, and two metabolites, presumed to be the 1,2-diols and 3,4-diols, accounted for 2–6% of the total radiolabel. Two additional, less polar, metabolites were present at 2.5% and 5–16% in the skin and liver, respectively, but remained unidentified (Warshawsky *et al.*, 1993).

(b) Metabolism

There were numerous reports on the metabolism of dibenz[*a,j*]acridine, both *in vitro* and *in vivo*; these had been partially reviewed (Warshawsky *et al.*, 1996a).

An initial study used isolated preparations of perfused rabbit lung. The total rate of appearance of dibenz[*a,j*]acridine metabolites in the blood was lower than that for the structurally similar *N*-heterocyclic compound, 7*H*-dibenzo[*c,g*]carbazole (DBC), both in preparations from untreated rabbits (numbers not given) and from rabbits pre-treated with corn-oil (204 ± 34 ng/g lung per hour at a dose of 175 ± 12.5 μg of dibenz[*a,j*]acridine, *versus* 936 ± 144 ng/g lung per hour at a dose of 300 μg of DBC). When the rabbits were pre-treated with benzo[*a*]pyrene at a dose of 20 mg/kg bw in 3 mL of corn oil, administered intraperitoneally, 24 hours before being killed, a statistically significant increase in the metabolism of dibenz[*a,j*]acridine (to 1089 ± 235 ng/g lung per hour, $P = 0.05$) was observed. This increase was associated with a statistically significant increase ($P = 0.01$) in the production of non-extractable (i.e. conjugated) metabolites. Based upon a combination of ultraviolet (UV) and fluorescence spectroscopy and mass spectrometry, one of the major metabolites was identified as the 3,4-dihydrodiol of dibenz[*a,j*]acridine. A second major metabolite had a mass spectrum consistent with a monohydroxylated derivative of dibenz[*a,j*]acridine (Warshawsky *et al.*, 1985).

Subsequent studies provided structural proof for a variety of dibenz[*a,j*]acridine metabolites, using a combination of HPLC-UV with diode-array detection or fluorescence and mass spectrometry, and authentic standards. The metabolites identified in liver and lung microsomal preparations from Wistar rats induced with 3-methylcholanthrene were two dihydrodiols (trans-dibenz[*a,j*]acridine-3,4-dihydrodiol and trans-dibenz[*a,j*]acridine-5,6-dihydrodiol),

dibenz[*a*,]acridine-5,6-epoxide, and two phenols, 3-hydroxy- and 4-hydroxydibenz[*a,j*]acridine; the 1,2-dihydrodiol was not detected. Additional secondary metabolites, for which unequivocal characterization or strong structural evidence could be provided, included the 3,4,10,11-tetrols, 3,4,8,9-tetrols, and 1,2,3,4-tetrols, the 3,4-dihydrodiol-8,9-epoxide and the 5,6-dihydrodiol-8,9-epoxide (no stereochemical information provided), the *syn*- and *anti*-3,4-dihydrodiol-1,2-epoxide (bay-region diol-epoxide), dibenz[*a,j*]acridine-5,6,8,9-diepoxide, and incompletely characterized phenolic 3,4- and 5,6-dihydrodiols. The metabolite profiles in liver and lung microsomal preparations were very similar: *trans*-dibenz[*a,j*]acridine-3,4-dihydrodiol was the major metabolite (30–40%), dibenz[*a,j*]acridine-5,6-epoxide was the second most abundant metabolite, and the two phenols, particularly the 4-hydroxy isomer, were also present in significant proportions (Gill *et al.*, 1986, 1987). In addition to the previously mentioned metabolites, dibenz[*a,j*]acridine-*N*-oxide was detected as a minor product (about 1%) in similar incubations conducted with liver microsomes from rats that were not induced, or rats that had been induced with phenobarbital. Incubation of rat liver microsomes from uninduced or phenobarbital-induced rats in the presence of 3,3,3-trichloropropene-1,2-oxide (1.5 mM), an inhibitor of epoxide hydrolase, led to an approximately 20% decrease in the extent of total metabolism and the amount of *trans*-dibenz[*a,j*]acridine-3,4-dihydrodiol was reduced 30–40 times. Induction of epoxide hydrolase by pretreatment with *trans*-stilbene oxide failed to produce a clear decrease in the proportion of dibenz[*a,j*]acridine-5,6-epoxide compared with the control experiments (Gill *et al.*, 1987). Further metabolism of *trans*-dibenz[*a,j*]acridine-3,4-dihydrodiol in 3-methylcholanthrene-induced liver microsomes led predominantly to the 3,4-dihydrodiol-8,9-epoxide and a phenolic 3,4-dihydrodiol (44.4%, combined); the bay-region diol-epoxides

accounted for approximately 6% of the total metabolites (Gill *et al.*, 1987).

Similar studies conducted with liver microsomes from male Sprague-Dawley rats and female Hsd:(ICR)BR mice induced with 3-methylcholanthrene yielded essentially the same type of metabolic profile, although the specific metabolite distributions were not as extensively differentiated as indicated above; the only noteworthy difference was the identification of small amounts of the previously undetected 1,2-dihydrodiol (stereochemistry not specified). Treatment of rats and mice with 3-methylcholanthrene led to statistically significant increases ($P \leq 0.05$) in liver microsomal metabolism to dihydrodiols and phenols compared with animals treated with corn oil, similar to observations in parallel incubations conducted with benzo[*a*]pyrene as positive control (Wan *et al.*, 1992). The use of synchronous fluorescence spectroscopy yielded quantitative data on metabolite compositions in good agreement with those obtained from radioactivity measurements (Schneider *et al.*, 1994).

The absolute configurations of the two major dibenz[*a,j*]acridine metabolites formed with liver microsomes from uninduced, phenobarbital-induced and 3-methylcholanthrene-induced male Wistar rats or male SW mice were established on the basis of comparison with synthetic standards. About 63–70% of 3,4-dihydrodiol was in the (–)-*3R,4R* configuration regardless of species or treatment. In contrast, the 5,6-epoxide was predominantly present as the *5R,6S* isomer in uninduced and phenobarbital-induced preparations (60% and 75%, respectively, with the mouse liver microsomes; 81% and 79%, respectively, with rat liver microsomes). A reversed stereochemical preference was found in preparations from both species induced with 3-methylcholanthrene, with highly stereoselective formation of the *5S,6R* isomer dihydrodiol (91% with mouse liver microsomes and 95% with rat liver microsomes) (Duke & Holder, 1988; Duke *et al.*, 1988).

Trans-dibenz[*a,j*]acridine-3,4-dihydrodiol was also the major metabolite (57.8 ± 2.6%) produced in incubations of dibenz[*a,j*]acridine with human liver microsomes (Sugiyanto *et al.*, 1992). Human CYP1A1, CYP1A2, CYP3A4, and CYP3A5 catalysed the formation of *trans*-dibenz[*a,j*]acridine-3,4-dihydrodiol; the CYP3A4 isoform was the most selective for this metabolite, whereas CYP1A2 was selective for K-region 5,6-oxidation. Regardless of the specific CYP, the 3,4-dihydrodiol had a *3R,4R*-configuration, with an optical purity of close to 100%. Likewise, the K-region 5,6-dihydrodiol of dibenz[*a,j*]acridine was formed by CYP1A1 and CYP1A2 as the *R,R* diastereomer with an optical purity of almost 100%, while dibenz[*a,j*]acridine-5,6-epoxide was formed by CYP1A1 predominantly as the *5S,6R* isomer (80%), as observed with liver microsomes from rodents induced with 3-methylcholanthrene (Roberts-Thomson *et al.*, 1995).

Dibenz[*a,j*]acridine metabolism occurred readily *in vitro* in hepatocytes from male Wistar rats pre-treated with phenobarbital, or 3-methylcholanthrene, or untreated, with the formation of water-soluble conjugates and non-conjugated metabolites. The water-soluble metabolites accounted for > 50% of the total when 80% of the substrate had been metabolized by hepatocytes from rats induced with 3-methylcholanthrene. Hydrolysis of the cell homogenates with β-glucuronidase/aryl sulfatase before extraction with ethyl acetate resulted in a decrease of only 10% in water-soluble radiolabel, indicating that this fraction was mostly composed of thioether conjugates. This was further confirmed by preincubation of diethyl maleate with hepatocytes from rats induced with 3-methylcholanthrene, which decreased the glutathione concentrations by 56%, with a concomitant increase in the total organic solvent-soluble radioactivity (to 75% in the absence of enzymatic hydrolysis and 80% with β-glucuronidase/aryl sulfatase treatment). The major metabolites present in the organic solvent-soluble fraction, with or without

β-glucuronidase and arylsulfatase hydrolysis, were 3- and 4-hydroxy-dibenz[*a,j*]acridine and *trans*-3,4-dihydro-3,4-dihydroxydibenz[*a,j*]acridine; the 3,4-dihydrodiol accounted for 34–66% of the total organic solvent-soluble metabolites. Contrary to observations with rat liver microsomes, the K-region 5,6-epoxide and the 5,6-dihydrodiol were minor metabolites in the hepatocyte incubations. Increased hepatocyte densities (10^7 cells per mL) and prolonged incubation times led to a higher extent of metabolism, which was associated with increased DNA binding and protein binding of the radiolabel. At the end of the incubation period, the 3,4-dihydrodiol had undergone substantial metabolism, but the specific structures of the secondary metabolites were not elucidated (Robinson *et al.*, 1990).

Quantitative comparisons of total dibenz[*a,j*]acridine metabolism by preparations of liver microsomes or S9 from male Sprague-Dawley rats or female Hsd:(ICR)BR mice pretreated with different inducers (3-methylcholanthrene, Aroclor 1254, dibenz[*a,j*]acridine itself, DBC, or phenobarbital) were conducted to assess whether metabolism occurred by PAH- or aromatic amine-type biotransformation. The results indicated that with liver preparations from both species, dibenz[*a,j*]acridine was metabolized by a set of enzymes in microsomes similar to those that metabolize other PAHs (Warshawsky *et al.*, 1996a). [The Working Group noted that this reference was a review; it was not clear whether the original data were reported elsewhere].

4.4.2 Genotoxicity and other relevant effects

Dibenz[*a,j*]acridine was mutagenic in *S. typhimurium* TA98 and TA100 at concentrations as low as 5 μg/plate in the presence of an exogenous metabolic system (McCann *et al.*, 1975; Kitahara *et al.*, 1978; Baker *et al.*, 1980; Ho *et al.*, 1981); while a negative result was obtained for induction of unscheduled DNA synthesis in primary rat hepatocytes *in vitro* (Probst *et al.*, 1981).

Dibenz[*a,j*]acridine was tested for clastogenicity in a Chinese hamster fibroblast cell line. Chromosomal aberrations were produced in the presence, but not in the absence, of an S9 metabolic-activation system (Matsuoka *et al.*, 1982).

Dibenz[*a,j*]acridine and several of its metabolites were tested for mutagenicity in *S. typhimurium* TA98 and TA100, using S9 fractions from the livers of male Sprague-Dawley rats induced with Aroclor 1254 or of guinea-pigs induced with 3-methylcholanthrene. The latter was also used as the activation system for V79 Chinese hamster lung cells. Dibenz[*a,j*]acridine was mutagenic in TA100 over a dose range of 2–16 nmol/plate. Within the same dose range, 4-hydroxydibenz[*a,j*]acridine and 6-hydroxydibenz[*a,j*]acridine, the 5,6-epoxide, and the *N*-oxide were not mutagenic. Among the test compounds requiring the guinea-pig metabolic-activation system, which included 1,2-dihydrodiol, 3,4-dihydrodiol, and 5,6-dihydrodiol, the 3,4-dihydrodiol was the most mutagenic, both in TA100 and in V79 cells. No differences in mutagenicity were observed in TA100 between the 3,4-dihydrodiol enantiomers or the racemic mixture. In V79 cells, only the *3R,4R*-dihydrodiol was active, the activity being approximately three times that of the racemic mixture. The 1,2-dihydrodiol was the most mutagenic in TA98. Much weaker responses were obtained in TA100 when the guinea-pig metabolic-activation system was replaced by that from rats pre-treated with Aroclor 1254; under these conditions, no activity was detected in TA98 with any of the compounds. The most mutagenic compounds in mammalian cells and bacteria were the bay-region diol-epoxides, which did not require metabolic activation. *anti*-Dibenz[*a,j*]acridine-3,4-dihydrodiol-1,2-epoxide was more mutagenic than its *syn* isomer in all the cell systems tested. These results indicated a mutagenicity pattern comparable to those observed in PAHs (Bonin *et al.*, 1989).

Dibenz[*a,j*]acridine was analysed for cytotoxic and genotoxic effects on human lymphocytes. An

effect on the frequency of micronucleus formation above that in controls was observed only at the higher concentrations (5 and 10 μg/mL). Cytotoxicity was moderate, as indicated by a 28% decrease in the mitotic index at the highest concentrations (Warshawsky *et al.*, 1995a).

Epithelial cells from the buccal mucosa of Wistar rats were demonstrated to metabolize dibenz[*a,j*]acridine to DNA-binding species. Upon incubation with [^{14}C]dibenz[*a,j*]acridine (1.6 μM) for 18 hours, covalent binding was determined to be 4.5 ± 0.3 pmol per 10 mg DNA by liquid-scintillation counting (Autrup & Autrup, 1986).

Using ^{32}P-postlabelling, the DNA-adduct patterns and organ distributions were investigated in rodents given dibenz[*a,j*]acridine by topical application. Comparison of DNA binding in weanling female Sprague-Dawley rats, ICR mice and Syrian hamsters showed qualitatively similar profiles for the three species, with two main adducts being observed. Although liver and kidney were investigated, DNA binding occurred almost exclusively in the skin. Based upon relative adduct labelling, mice displayed the highest levels of DNA adducts (Li *et al.*, 1990).

Subsequent studies in female Hsd:(ICR)BR mice confirmed the almost exclusive formation of DNA adducts from dibenz[*a,j*]acridine in the skin, in agreement with its pattern of carcinogenicity. After topical application of the parent compound and of the *trans*-1,2-, 3,4-, and 5,6-dihydrodiols and subsequent DNA isolation, ^{32}P-postlabelling was conducted under conditions of limiting [^{32}P]ATP. The highest level of binding to skin DNA was shown by the 3,4-dihydrodiol. Dibenz[*a,j*]acridine formed two adducts in the skin, which were identical to those obtained from the 3,4-dihydrodiol. Two chromatographically different adducts, which were not produced by the parent compound, were detected upon application of the 5,6-dihydrodiol. No adducts from the 1,2-dihydrodiol were detected. When the nuclease P1 digestion enrichment procedure was used, 3,4-dihydrodiol gave rise to the formation of all four adducts. These results were consistent with formation of the 3,4-dihydrodiol as the major route of activation of dibenz[*a,j*]acridine leading to DNA binding in the skin, with subsequent metabolism to a bay-region diol-epoxide. An additional pathway to DNA-binding species may involve the 3,4,5,6-bis-dihydrodiol-1,2-oxide (Roh *et al.*, 1993; Talaska *et al.*, 1995). [The Working Group noted that routes of administration other than topical application have not been investigated for DNA adducts.]

In the presence of liver microsomes from rats treated with 3-methylcholanthrene, dibenz[*a,j*] acridine was shown to bind to calf thymus DNA, yeast RNA, and the polynucleotides polyG, polyA, polyU, and polyC. Among the polynucleotides, the greatest extent of binding was observed with polyG. The relative extents of binding of dibenz[*a,j*]acridine to the four polynucleotides were similar to those obtained with benzo[*a*]pyrene. Analysis of the effect of different modifiers (α-naphthoflavone, 3,3,3-trichloropropene-1,2-oxide, cyclohexene oxide, and styrene oxide) upon the binding levels revealed that the binding of dibenz[*a,j*]acridine to polyG was dependent upon a microsomal hydroxylating-enzyme system (Warshawsky *et al.*, 1996a).

In a more recent study, female Hsd:(ICR) BR mice were given dibenz[*a,j*]acridine (300 μg) and synthetic (+/–)-*anti*-dibenz[*a,j*]acridine-3,4-dihydrodiol-1,2-epoxide (50 μg), applied to the back. The mice were killed 48 hours later and the skin DNA was analysed by ^{32}P-postlabelling. Four adducts were formed *in vivo*. For comparison, the synthetic diol-epoxide was reacted *in vitro* with purine nucleotides (3′- and 5′-deoxyadenosine monophosphate [dAMP], 3′- and 5′-deoxyguanosine monophosphate [dGMP]) and calf thymus DNA. The synthetic 3′-dAMP adduct and 94% of the calf thymus DNA adducts formed from the diol-epoxide were chromatographically identical to the major (89%) adduct

from the same diol-epoxide *in vivo*. On the other hand, 86% of the synthetic dGMP adducts formed from the diol-epoxide were chromatographically consistent with the major (> 50%) adduct obtained *in vivo* upon application of dibenz[*a,j*] acridine (Xue *et al.*, 2001).

4.4.3 Mechanistic considerations

Data on ionization potentials were used to predict the metabolic activation of carcinogenic PAHs. In the case of dibenz[*a,j*]acridine, a high ionization potential (about 8.0 eV) is consistent with a mono-oxygenation pathway, rather than one-electron oxidation (Xue *et al.*, 1999).

Dibenz[*a,j*]acridine was found to be a moderate inducer of hepatic 7-ethoxyresorufin *O*-deethylase (EROD) activity in Ah-responsive C57BL/6J mice. EROD activity was closely related to the levels of expression of liver CYP1A1 and CYP1B1 when data from a series of 23 test PAHs were combined (Shimada *et al.*, 2003). The data on EROD activities in mice contrasted with those for recombinant human enzymes, where dibenz[*a,j*]acridine was a potent inhibitor of CYP1A1, CYP1A2, and particularly CYP1B1, with IC_{50} [concentration at which activity is inhibited by 50%] values of 56 ± 7, 41 ± 8, and 15 ± 2 nM, respectively (Shimada & Guengerich, 2006).

When dibenz[*a,j*]acridine was applied following procedures known to induce skin papilloma and carcinoma on the back of mice, A to T and G to T transversions were found in codons 12, 13, and 61 of the Ha-*Ras* gene in papillomas and carcinomas. The mutational spectra in the Ha-*Ras* gene were consistent with the observed binding of dibenz[*a,j*]acridine to dG and dA in DNA *in vivo* (Xue *et al.*, 2001).

When tested in the freshwater green alga, *Selenastrum capricornutum*, under different light sources, dibenz[*a,j*]acridine was not phototoxic. In comparison, benzo[*a*]pyrene was phototoxic,

as a result of photochemical production of quinones (Warshawsky *et al.*, 1995b).

4.5 Dibenz[*c,h*]acridine

4.5.1 Metabolism and distribution

Reports on studies of the metabolism of dibenz[*c,h*]acridine are limited. The compound has two identical bay regions expected to undergo bioactivation.

Two enantiomerically pure *trans*-3,4-dihydrodiols and the racemic mixture were assessed for metabolism by rat liver enzymes. The racemic dihydrodiol was metabolized at a rate of 2.4 nmol/nmol CYP1A1 per minute with liver microsomes from immature male Long-Evans rats treated with 3-methylcholanthrene. This rate was more than 10 times that observed with liver microsomes from uninduced rats or rats treated with phenobarbital. The major metabolites (68–83%) were a diastereomeric pair of *bis*-dihydrodiols having the new dihydrodiol group at the 8,9-position. The tetrols derived from the bay-region 3,4-dihydrodiol-1,2-epoxides accounted for 15–23% of the total metabolites. A small amount of a phenolic dihydrodiol, formed from the 3,4-dihydrodiol-8,9-epoxide, was also detected. The assignment of a phenolic structure was based on the pH-dependence of the UV spectrum and on the mass spectral information. Although the specific position of the new hydroxyl group was not assigned unequivocally, formation of the 9-hydroxy isomer was assumed, since hydroxylation at the 8-position would involve an unstable intermediate with a resonance contributor bearing a positive charge on the nitrogen. The rate of metabolite formation by a highly purified mono-oxygenase system reconstituted with CYP1A1 and epoxide hydrolase (17 nmol of metabolites/nmol of CYP1A1 per minute) was considerably higher, although the metabolite profile was very similar to that observed with liver microsomes from rats treated

wih 3-methylcholanthrene. Stereoselective formation of the 3,4-dihydrodiol-1,2-epoxide was inferred from the absolute configurations of the tetrols. The (+)-(3*S*,4*S*)-dihydrodiol yielded predominantly the *syn*-diol-epoxide, whereas the (−)-(3*R*,4*R*)-dihydrodiol gave mainly the *anti*-diol-epoxide. The major *bis*-dihydrodiol metabolites (dibenz[*c*,*h*]acridine-3,4,8,9-bis-dihydrodiol) had the same absolute configuration at the 8,9-position, assumed to be 8*R*,9*R* from analysis of the circular dichroism spectra; this implies the (8*R*,9*S*)-epoxide as their precursor (Adams *et al.*, 1999).

Microspectrofluorimetry on single living cells (mouse embryo 3T3 fibroblasts) was used to compare the metabolic profiles of dibenz[*c*,*h*]acridine, benzo[*a*]pyrene, and 6-aminochrysene. The results indicated similarities between the profiles of dibenz[*c*,*h*]acridine and benzo[*a*]pyrene, and important differences between those of dibenz[*c*,*h*]acridine and 6-aminochrysene, consistent with a PAH-type, rather than aromatic amine-type metabolism, for dibenz[*c*,*h*]acridine. Inhibition of the metabolism of dibenz[*c*,*h*]acridine occurred in the presence of benzo[*a*]pyrene, while dibenz[*c*,*h*]acridine did not inhibit the metabolism of benzo[*a*]pyrene. This indicated that benzo[*a*]pyrene is a better substrate for the metabolizing enzymes under the conditions of the assay (Lahmy *et al.*, 1987).

4.5.2 Genotoxicity and other relevant effects

The mutagenicities of dibenz[*c*,*h*]acridine and the K-region dibenz[*c*,*h*]acridine-5,6-epoxide (racemic mixture) were tested in *S. typhimurium* TA98 and TA100, both in the absence and in the presence of liver microsomal S9 from rats induced with polychlorinated biphenyls. The parent compound was inactive in the absence of metabolic activation, but showed mutagenicity in the presence of metabolic activation (11 and 95 revertants/µg per plate, in TA98 and TA100, respectively). In comparison, benzo[*a*]pyrene induced 80 revertants/µg per plate in TA100, but was more active in TA98. The 5,6-epoxide was inactive without activation, and much less mutagenic than dibenz[*c*,*h*]acridine in the presence of activation (0.7 and 8.5 revertants/µg per plate in TA98 and TA100, respectively). These data indicated that the major pathway of dibenz[*c*,*h*]acridine activation to a mutagen is not through K-region oxidation (Kitahara *et al.*, 1978).

In another study of mutagenicity, dibenz[*c*,*h*]acridine gave positive results in four strains of *S. typhimurium* (TA1535, TA1538, TA98 and TA100) in the presence of liver microsomal S9 from male Sprague-Dawley rats induced with Aroclor 1254. In TA1538, the maximum effect was a 75-times increase in the number of revertants compared with the value for the negative controls, obtained at 5000 µg/plate; in TA100, the number of revertants increased six times above background at a concentration of 4 µg/plate (Anderson & Styles, 1978).

Dibenz[*c*,*h*]acridine was mutagenic in *S. typhimurium* TA100 in the presence of liver microsomes from rats cotreated with phenobarbital and 5,6-benzoflavone at 0–100 µg/plate (Karcher *et al.*, 1985).

Dibenz[*c*,*h*]acridine was tested for clastogenicity in a Chinese hamster fibroblast cell line. Chromosomal aberrations were induced in the presence, but not in the absence, of metabolic activation from S9 (Matsuoka *et al.*, 1982).

The mutagenic activities of the enantiomers of the diastereomeric pair of bay-region dibenz[*c*,*h*]acridine-3,4-dihydrodiol-1,2-epoxides have been evaluated in *S. typhimurium* TA98 and TA100 and in the 8-azaguanine-sensitive Chinese hamster V79–6 cell line, which lacks the capacity for metabolic oxidation of PAHs to mutagens. In both strains of bacteria, the *anti*-diol-epoxide enantiomers [(+)-1*R*,2*S*,3*S*,4*R* and (−)-1*S*,2*R*,3*R*,4*S*] were two to four times more mutagenic than the *syn* [(+)-1*S*,2*R*,3*S*,4*R* and (−)-1*R*,2*S*,3*R*,4*S*] enantiomers. There was not a significant difference in mutagenicity between

the enantiomers of each pair or between each enantiomer and the corresponding racemic mixture. Contrasting with the results in bacteria, the *anti*-(+)-(1*R*,2*S*,3*S*,4*R*)-3,4-dihydrodiol-1,2-epoxide isomer was five to seven times more mutagenic in the mammalian cell line than any of the other dibenz[*c,h*]acridine-3,4-dihydro-diol-1,2-epoxides. Purified rat liver epoxide hydrolase did not catalyse the conversion of any of the 3,4-dihydrodiol-1,2-epoxide isomers to inactive products. Additional experiments on bacterial mutagenesis with dibenz[*c,h*]acridine and its derivatives requiring metabolic activation were conducted in the presence of hepatic microsomes from immature male Long-Evans rats treated with Aroclor 1254. Among the test compounds, 3,4-dihydrodibenz[*c,h*]acridine, the putative precursor of a bay-region tetrahydroepoxide, was activated to the most powerful mutagen. The second most active compound was the (−)-3*R*,4*R*-dihydrodiol. Of the three metabolically possible *trans*-dihydrodiols (at the 1,2- 3,4- and 5,6- positions; all tested as racemic mixtures), dibenz[*c,h*]acridine-3,4-dihydrodiol was the most mutagenic; it was also considerably more mutagenic than the parent compound. The 1,2,3,4-tetrahydro-3,4-diol, which lacks the bay-region 1,2 double bond, was not activated to a mutagen. These observations are consistent with metabolic activation of dibenz[*c,h*]acridine to a bay-region diol-epoxide (Wood *et al.*, 1986).

4.5.3 Mechanistic considerations

Activation of dibenz[*c,h*]acridine to a bay-region diol-epoxide is consistent with the mutagenicity data and with the relative tumorigenicities of the parent compound and several of its metabolites (see Section 3). In agreement with predictions of the bay-region theory, the 3,4-dihydrodiol is a proximate carcinogen and the bay-region 3,4-dihydrodiol-1,2-epoxides are ultimate carcinogens. The data on metabolism by rat liver microsomes suggested that a high

level of CYP1A1 activity may confer increased susceptibility to dibenz[*c,h*]acridine-induced carcinogenesis.

The rates of solvolysis of the dibenz[*c,h*]acridine-3,4-dihydrodiol-1,2-epoxides were found to be comparable to those of the corresponding diol-epoxides from dibenz[*a,j*]anthracene. This contrasted with much slower rates of solvolysis for the dibenz[*a,j*]acridine-3,4-dihydrodiol-1,2-epoxides. These observations were consistent with the fact that the benzylic cation stemming from opening of the dibenz[*a,j*]acridine-derived epoxide has a resonance contributor bearing a positive charge on the nitrogen, while this is not the case for the dibenz[*c,h*]acridine-derived epoxide (Sayer *et al.*, 1990).

The bay-region (+)-(1*R*,2*S*,3*S*,4*R*)-3,4-dihydrodiol 1,2-epoxide was the most tumorigenic of the four possible isomeric bay-region diol-epoxides from dibenz[*c,h*]acridine, both in an initiation–promotion model in mouse skin and in newborn mice. Similar observations have been reported with various carbocyclic PAHs for which the *R*,*S*,*S*,*R* bay-region diol-epoxides typically display high tumorigenic activities (Chang *et al.*, 2000).

The mutational activation of the Ha-*Ras* protooncogene in skin tumours of female CD-1 mice was investigated in an initiation–promotion model using a single application of dibenz[*c,h*] acridine (200 nmol), followed 10 days later by long-term treatment with TPA (16 nmol given twice per week for 20–25 weeks). The DNA isolated from carcinoma induced by dibenz[*c,h*]acridine efficiently transformed NIH 3T3 cells, and a high percentage of the transformed foci had an amplified Ha-*Ras* gene containing an A to T transversion in the second base of codon 61. The same mutation was detected in DNA from primary tumours in a high percentage of the carcinomas induced by dibenz[*c,h*]acridine, and also in NIH 3T3 cells transformed with DNA from benign skin papillomas induced by dibenz[*c,h*]acridine. The latter observation suggested that the mutation

is an early event in mouse skin carcinogenesis induced by dibenz[*c,h*]acridine. In a concurrent study of complete carcinogenesis in cells treated repeatedly with 7,12-dimethylbenz[*a*]anthracene, an A to T transversion in the second base of codon 61 of the Ha-*ras* gene was also identified. By analogy with 7,12-dimethylbenz[*a*]anthracene, for which metabolic activation to a bay-region diol-epoxide leads to the formation of a deoxyadenosine adduct, the bay-region diol-epoxide from dibenz[*c,h*]acridine may bind preferentially to adenine residues in DNA (Bizub *et al.*, 1986).

In a later study, Chinese hamster V79 cells were exposed to high or low concentrations of the highly carcinogenic *anti*-(+)-(*R,S,S,R*) or the less active *anti*-(–)-(*S,R,R,S*) bay-region diol-epoxides of dibenz[*c,h*]acridine. Independent 8-azaguanine-resistant clones were isolated, and base substitutions at the hypoxanthine (guanine) phosphoribosyltransferase *(Hprt)* locus were determined. While the proportion of mutations at AT base pairs increased as the concentration of the *anti*-(+)-(*R,S,S,R*) diol-epoxide decreased, concentration-dependent differences in the mutation profile were not observed for the *anti*-(–)-(*S,R,R,S*) diol-epoxide. Similar results were obtained with bay-region diol-epoxides of benzo[*a*]pyrene and benzo[*c*]phenanthrene. In a DNA repair-deficient variant of V79 cells, no concentration-dependent differences were found in the mutation profile induced by the (*R,S,S,R*) diol-epoxide of benzo[*a*]pyrene, suggesting that the occurrence of concentration-dependent differences requires an intact DNA-repair system (Conney *et al.*, 2001).

4.6 Carbazole

4.6.1 Metabolism

3-Hydroxycarbazole has been reported to be a major urinary metabolite of carbazole in rats (Johns & Wright, 1964). In a more recent study, carbazole was characterized as a noncompetitive inhibitor of CYP1A (Wassenberg *et al.*, 2005).

4.6.2 Genotoxicity and other relevant effects

In early studies, carbazole was reported to be inactive when tested for mutagenicity in *S. typhimurium* strains TA1535, TA1538, TA98 and TA100 in the presence of metabolic activation (Anderson & Styles, 1978). Similarly, other studies reported that carbazole was not mutagenic with metabolic activation from S9 in *S. typhimurium* TA98 (Ho *et al.*, 1981) and TM677 (Kaden *et al.*, 1979).

Carbazole was reported to be moderately clastogenic in the bone marrow of Swiss albino mice. At intraperitoneal doses of 25, 50, 100, 150 or 200 mg/kg bw, carbazole caused significant reductions in the mitotic index and increases in chromosomal aberrations at the two higher doses; these effects were observed after treatment for 14 hours, but not after 42 hours (Jha *et al.*, 2002).

Carbazole induced dominant lethality and sperm-head abnormalities in male Swiss albino mice (Jha & Bharti, 2002). In the former test, statistically significantly positive results were reported for mice given carbazole as five daily intraperitoneal doses at 30 or 60 mg/kg bw; in the latter test, there was a significant dose–response relationship in the range of 50–300 mg/kg bw when carbazole was given as a single dose.

Carbazole (50–500 μg/L) caused a twofold induction of EROD activity in embryos of *Fundulus heteroclitus* (killifish; saltwater minnow). The strong stimulation of EROD activity by the AhR agonist β-naphthoflavone (1 μg/L) was considerably diminished upon coincubation of the fish embryos with carbazole (Wassenberg *et al.*, 2005). Although not embryotoxic itself, carbazole enhanced the embryotoxicity of β-naphthoflavone.

4.7 *7H*-Dibenzo[*c,g*]carbazole

4.7.1 Distribution and metabolism

The toxicokinetics of DBC have been reviewed (Xue & Warshawsky, 2005).

In hamsters given a dose of 3 mg per animal by intratracheal instillation, once per week for 5 weeks, DBC passed from the lungs to the intestinal tract and was excreted mainly in the faeces (Nagel *et al.*, 1976). After inhalation as an aerosol at a concentration of 1.1–13 µg/L air for 60 minutes, [^{14}C]-labelled DBC was widely distributed in rat tissues. Within 1 hour after exposure, the highest amounts of radiolabel were observed in the respiratory tract, upper gastro-intestinal tract, liver and adrenal glands. Tissue clearance was rapid, with half-lives ranging from 1 to 16 hours. DBC was extensively metabolized, and excreted primarily in the faeces (Bond *et al.*, 1986).

When incubated with liver microsomal fractions from rats or mice pre-treated with 3-methylcholanthrene, DBC was metabolized to twelve different compounds, of which five were identified as mono-hydroxylated derivatives, namely 5-OH-DBC and 3-OH-DBC as major metabolites, and 2-OH-DBC, 4-OH-DBC and 6-OH-DBC as minor products. A dihydrodiol was tentatively identified as 3,4-dihydroxy-3,4-dihydro-DBC (Périn *et al.*, 1981). A subsequent study with rat liver microsomes identified 2-OH-DBC and 3-OH-DBC, but not 4-OH-DBC, as metabolites, while cultured rat hepatocytes also metabolized DBC predominantly to phenols (Stong *et al.*, 1989). In another study by the same research group, mouse and rat liver microsomes were reported to metabolize DBC to 5-OH-DBC, 3-OH-DBC, and 2-OH-DBC, with 1-OH-DBC being formed in trace amounts; dihydrodiols were not detected as metabolites (Wan *et al.*, 1992). On the basis of mass spectral analysis, *N*-OH-DBC was reported to be a major rat-liver microsomal metabolite; it was also formed in an isolated preparation of perfused rabbit lung (Warshawsky & Myers, 1981). However, the formation of this metabolite was not confirmed in subsequent studies (Xue *et al.*, 1993). The same group then used conventional and synchronous fluorescence spectroscopy to identify 1-OH-DBC, 3-OH-DBC, and 5-OH-DBC as metabolites of DBC formed by liver microsomes from rats induced by 3-methylcholanthrene (Schneider *et al.*, 1994).

Converting the phenolic metabolites of DBC, which are relatively unstable, to more stable acetoxy-DBC derivatives by use of acetic anhydride/pyridine facilitates their analysis (Xue *et al.*, 1993). By means of this procedure, the major DBC metabolites formed in microsomes from livers of rats induced with 3-methylcholanthrene were quantified and close agreement was found with the outcome of a radiometric analysis; the order of abundance was 5-OH-DBC > 1-OH-DBC > 3-OH-DBC.

Experiments with knockout mice in which *Cyp1a1*, *Cyp1a2* or *Cyp1b1* was deleted showed that DBC is metabolized mainly by Cyp1a1 in the liver and by Cyp1a1 and Cyp1b1 in the lung of mice induced with β-naphthoflavone, and by hepatic Cyp1a2 in non-induced mice. Comparison of metabolic profiles generated by different enzymes indicated that Cyp1a1 produces 1-OH-DBC, 2-OH-DBC, and (5+6)-OH-DBC, Cyp1a2 generates mainly (5+6)-OH-DBC, and Cyp1b1 produces 4-OH-DBC. Similar results were obtained *in vitro* with Supersomes™ [microsomes derived from baculovirus-infected insect cells] expressing human CYP1 enzymes (Shertzer *et al.*, 2007).

4.7.2 Genotoxicity and other relevant effects

(a) Mutagenicity in bacterial systems

DBC was reported to induce revertants in *S. typhimurium* TA98 in the presence of metabolic activation from S9 at 2.3 times the spontaneous reversion rate, but the results were not consistent (Salamone *et al.*, 1979). In another study in TA98,

DBC have negative results at concentrations of up to 100 μg/plate and was toxic to TA98 at 250 μg/plate, in the presence of metabolic activation (Ho *et al.*, 1981). In a subsequent study, DBC was reported to be mutagenic in TA100 with metabolic activation from mouse liver S9 (Périn *et al.*, 1988). In another test it was reported that DBC was not mutagenic in TA98 and TA100 either in the absence or the presence of metabolic activation from S9 from livers of rats, mice or hamsters pre-treated with various inducers (Schoeny & Warshawsky, 1987). However, a weakly positive response was observed with *S. typhimurium* TM677 in an assay for forward mutation. In the presence of S9 from rats pre-treated with Aroclor 1254, three phenolic metabolites were also mutagenic in TM677, in the order 3-OH-DBC > 4-OH-DBC > 2-OH-DBC.

(b) Mutagenicity in cell lines

When tested in Chinese hamster V79 cell lines expressing human CYP1A1 or CYP1A2, DBC caused an increase in mutation frequency (6-thioguanine resistance) in both cell lines (Gábelová *et al.*, 2002). In V79 cells expressing CYP1A1, DBC caused a dose-dependent increase in the frequency of micronucleus formation (Farkasová *et al.*, 2001). The effect of DBC in decreasing colony-forming ability in the same cell line, and also in HepG2 cells, correlated with its DNA-damaging activities, as measured with the alkaline DNA-unwinding assay and the modified single-cell gel electrophoresis (SCGE) assay (comet assay) (Gábelová *et al.*, 2000).

DBC and several phenolic metabolites were investigated for mutagenic activity in DPI-3 cells, an epithelial line derived from hamster embryos, co-cultured with rat liver cells. At a concentration of 40 μM, 3-OH-DBC gave 4.4 ± 0.8 mutants per 10^5 survivors, 13c-OH-DBC gave 8.0 ± 3.1 mutants, and DBC itself 8.0 ± 2.8 mutants. In the DMSO control, the number of mutants was 1.0 ± 0.2 per 10^5 survivors. Under these conditions

the metabolites 2-OH- and 4-OH-DBC were not mutagenic (Stong *et al.*, 1989).

DBC was mutagenic (inducing resistance to 6-thioguanine) in human DNA repair-deficient *Xeroderma pigmentosum* cells that were co-cultured with human Hs703T cells (an epithelial cell line derived from a human liver carcinoma) to provide a source of metabolizing activity (Parks *et al.*, 1986).

DBC induced a dose-dependent increase in the frequency of micronucleus formation in cultured lymphocytes from two donors (Warshawsky *et al.*, 1995a).

In tests for mutagenic activity *in vivo* in the Muta™Mouse, DBC induced a 30 times increase in the frequency of *LacZ* mutants in the liver, 28 days after a single subcutaneous injection, and a 3.4 times increase in the frequency of mutation in the skin, 28 days after a single topical application (Renault *et al.*, 1998).

(c) Formation of DNA adducts

Many studies have investigated the formation of DNA adducts by DBC and its metabolites, both *in vitro* and *in vivo*. Most of these studies have used sensitive ^{32}P-postlabelling analysis for detection and characterization of DNA adducts.

In one of the earliest studies of this type, female mice received DBC as a single subcutaneous injection at 44 μmol/kg bw, which resulted in very high adduct levels in the liver, relative to levels in other tissues (Schurdak & Randerath, 1985). The order of binding was liver >> kidney > lung > spleen > skin > brain, the level in liver being approximately 25 times higher than that in kidney.

In a time-course and dose–response study on the formation of DBC–DNA adducts in the lung of mice given DBC as a single intraperitoneal injection at 0, 5, 10, 20 or 40 mg/kg bw, the highest adduct levels were found at 40 mg/kg bw after 5–7 days. At lower doses, the maximum levels shifted to earlier time-points (1–3 days). Up to

seven adducts were detected by ^{32}P-postlabelling analysis (Warshawsky *et al.*, 1996b).

The patterns of DNA adducts in the skin and liver of mice treated topically or intraperitoneally with DBC, 2-OH-, 3-OH- or 4-OH-DBC were compared. In liver, the patterns from DBC and 3-OH-DBC were similar to each other, and distinct from those formed by 2-OH-DBC and 4-OH-DBC. On the other hand, in skin none of the phenolic metabolites produced an adduct pattern that resembled that of DBC; and the pattern produced by DBC in skin was different from that produced by DBC in liver. Thus DBC is activated by a pathway that involves metabolism to 3-OH-DBC in the liver, but a different pathway appears to be involved in the skin (Schurdak *et al.*, 1987a).

Levels of DNA adducts in mouse liver after topical or intraperitoneal administration of *N*-methyl-DBC were ~300 times lower than after treatment with DBC, but the difference was only about twofold in skin (Schurdak *et al.*, 1987b). The adduct patterns formed by the two compounds in liver were qualitatively similar; in skin the adduct pattern elicited by either compound was different from that seen in the liver, and the two patterns were substantially different from each other. *N*-Methyl-DBC bound preferentially to skin DNA, DBC bound preferentially to liver DNA. These results were in accordance with the target-organ specificity for carcinogenicity of the two compounds, and also indicated that a nonsubstituted nitrogen is required for genotoxicity in mouse liver, but not in mouse skin (see also Talaska *et al.*, 1994). Regardless of the route of administration (topical, oral or subcutaneous), DNA-adduct formation by DBC in mouse liver was always substantially higher (~10–140 times) than in other tissues (kidney, lung and skin) (Schurdak & Randerath, 1989). Microsomal activation of DBC *in vitro* in the presence of polynucleotides indicated that guanine moieties in DNA were the principal sites of modification and that binding of DBC can occur both via the nitrogen atom and through the 1,2,3,4-ring of the molecule (Lindquist & Warshawsky, 1989).

Among seven different phenolic derivatives of DBC, 3-OH-DBC gave rise to adducts that were similar to those formed by DBC itself; 4-OH-DBC also induced substantial adduct formation, albeit with a different pattern. In addition, 2-OH-DBC induced a low level of adducts, while 1-OH-DBC, 5-OH-DBC, 6-OH-DBC, and 13c-OH-DBC did not give rise to any detectable formation of adducts (Talaska *et al.*, 1994).

In a comparison with benzo[*a*]pyrene (a skin carcinogen), uptake of DBC from skin was found to be 70% higher than for benzo[*a*]pyrene over the first 24 hours after topical application. As a result, binding to skin protein and DNA was higher for benzo[*a*]pyrene, while binding to liver protein and DNA was higher for DBC. The amounts of protein adducts in blood were similar for the two compounds (Meier & Warshawsky, 1994).

DBC formed DNA adducts in primary mouse embryo cells (Gábelová *et al.*, 1997).

The formation of adducts by DBC was investigated in liver DNA from female mice, with separate examination of mitochondrial and nuclear DNA. At 24 hours after an intraperitoneal dose of DBC at 5 mmol/kg bw, the levels of adducts in nuclear DNA were twofold those in mitochondrial DNA; at 48 hours, the amount of adducts in nuclear DNA had decreased and that in mitochondrial DNA had increased, such that the two values were similar. At a higher dose of 15 mmol/kg bw, similar levels of adducts were observed in nuclear and mitochondrial DNA at 24 hours, although this dose and a higher dose (30 mmol/kg bw) were cytotoxic to liver cells (Périn-Roussel *et al.*, 1995). Subsequently, levels of DNA adducts were compared in parenchymal and non-parenchymal cells of the liver of mice given DBC at an intraperitoneal dose of 5 mmol/kg bw for 48 hours. Both cell types showed formation of DBC–DNA adducts, although the

amount was higher by nearly 15-fold in parenchymal cells (Périn-Roussel *et al.*, 1997).

DBC was tested for DNA-adduct formation and other DNA-damaging effects in WB-F344 progenitor cells from the rat liver (Valovicová *et al.*, 2009). Exposure to DBC at 10 μM for 24 hours led to formation of DBC–DNA adducts at a frequency of $56.3/10^8$ nucleotides, as measured by ^{32}P-postlabelling. In the dose range 0.1–20 μM, DBC induced a dose-dependent increase in DNA breakage detected in the comet assay, and caused a statistically significant increase in the frequency of micronucleus formation at concentrations of 0.5–2.5 μM. DBC did not give rise to additional DNA damage in the comet assay in the presence of formamidopyrimidine-DNA glycosylase/ AP endonuclease (Fpg endonuclease), which suggests that the lesions observed were not the result of oxidative damage.

Repeated topical administration of DBC at low doses to the dorsal skin of mice caused a steady increase in the formation of liver DNA adducts, which eventually reached a plateau. The early increase in levels of DNA adducts was not accompanied by stimulation of DNA synthesis or histological signs of cell proliferation, these effects becoming evident only after several treatments had been given and a certain level of DNA adducts had accumulated (Dorchies *et al.*, 2001).

Topical application of DBC produced significantly higher amounts of adducts in liver DNA of the Car-R mice (a mouse strain resistant to skin carcinogenesis) than in Car-S mice (a strain susceptible to skin carcinogenesis) (Périn *et al.*, 1998).

Topical application of DBC induced DNA adducts in mouse skin and liver, levels being considerably higher in the liver. DBC weakly induced CYP1A2, but had no effect on the expression of CYP1A1 in these tissues (Taras-Valéro *et al.*, 2000).

Incubation of DBC with horseradish peroxidase or rat-liver microsomes gave rise to radical cation formation and yielded, in the presence of DNA, several instable "depurinating" DNA adducts. These were identified as being guanine derivatives modified at the N7 position bound to the 5- or 6-position of DBC, or the N3 or N7 position of adenine bound to the 5-position of DBC (Chen *et al.*, 1997). The DBC–5-N7-Gua adduct was detected *in vivo* in the liver of mice treated with DBC, but it accounted for only ~0.4% of the total, the remainder being stable, covalently-modified nucleotide adducts (Dowty *et al.*, 2000).

As a step towards adduct characterization, HPLC was used to separate five of the seven DBC–DNA adducts in mouse liver that were detected by ^{32}P-postlabelling analysis and partially resolved by multidirectional thin-layer chromatography (O'Connor *et al.*, 1997). One of the DBC–DNA adducts formed in mouse liver was subsequently identified as being chromatographically identical to one of the synthetic adducts formed upon reaction between the reactive DBC-3,4-dione and nucleic acid bases and nucleotides, products that were characterized by mass spectrometry and nuclear magnetic resonance. These analyses suggested that the 4-NH2 position of cytosine was the site of adduction, and the adduct was identified as *N*4-[3,4-dione-DBC-1-yl]-Cyt (Xue *et al.*, 2002).

In Chinese hamster V79 cell lines stably expressing human metabolic enzymes, DBC induced higher levels of DNA adducts in cells that expressed CYP1A1 than in those expressing CYP1A2 (24.5 ± 7.2 *versus* 0.7 ± 0.2 adducts/10^8 nucleotides). In the parental cell lines, which are devoid of CYP activity, no DNA adducts were formed. DBC induced micronucleus formation in the CYP1A2-expressing cells in a dose-dependent manner, and gave also a positive response in the comet assay with endonucleases Fpg and EndoIII, suggesting that oxidative damage, rather than DNA-adduct formation, may be responsible for the genotoxic activity observed (Gábelová *et al.*, 2004).

In Chinese hamster V79 cells stably expressing human CYP3A4, DBC formed DNA adducts at a low level (0.25 ± 0.18 adducts/10^8 nucleotides at 10 μM). It also induced micronucleus formation and *Hprt* mutation (at the highest dose only) in these cells, which suggested that CYP3A4 plays a role in the metabolic activation of DBC (Mesárošová *et al.*, 2011).

DBC induced DNA adducts, DNA damage (as detected by the comet assay), and micronucleus formation in human hepatoma HepG2 cells. These effects were accompanied by induction of *CYP1A1/2* and *CYP1B1* mRNA (Gábelová *et al.*, 2011).

In studies *in vivo* comparing mice lacking Cyp1a2 activity (*Cyp1a2*$^{-/-}$) and mice lacking AhR activity (*Ahr*$^{-/-}$) with wildtype mice, no significant difference was found in the extent of DNA-adduct formation in lung, skin and liver after topical application of DBC. In contrast, the formation of DNA adducts was significantly reduced in both types of knockout mouse after topical application of benzo[*a*]pyrene (Talaska *et al.*, 2006). When the compound was given by intraperitoneal administration, the level of DBC–DNA adducts in liver and lung was significantly higher in *Ahr*$^{(-/-)}$ mice than in wildtype mice given the compound by intraperitoneal administration (Shertzer *et al.*, 2007).

DBC has been shown to form covalent DNA adducts in the liver of English sole (*Pleuronectes vetulus*) (Stein *et al.*, 1993) and the liver, intestinal mucosa, gills and brain of northern pike (*Esox lucius*) (Ericson *et al.*, 1999; Ericson & Balk, 2000).

(d) Cell death and cell proliferation

Dependent on concentration, DBC caused both necrosis (at ~80 μM) and apoptosis (at <1 μM) in HepG2 human hepatoma cells (O'Brien *et al.*, 2000). Subsequent studies by these authors demonstrated that human liver-cell lines differ in their ability to metabolize the compound to toxic species and that apoptosis is only observed when detectable metabolites and DNA adducts are formed (O'Brien *et al.*, 2002).

Induction of apoptosis by DBC in mouse liver *in vivo* was accompanied by an increase in expression of *Bax* mRNA and Bax protein, as well as upregulation of TGFβ1 in parenchymal cells; another change related to cell proliferation included overexpression of *Bcl2*, an anti-apoptotic gene (Martín-Burriel *et al.*, 2004).

DBC weakly induced AhR in WB-F344 rat-liver epithelial cells (a model of liver-progenitor cells *in vitro*) and it inhibited gap-junctional intercellular communication (Vondrácek *et al.*, 2006).

(e) Mutational spectrum in tumours

Tumours induced in the lungs of A/J mice given DBC at a dose of 5–40 mg/kg bw by intraperitoneal injection carried mutations in the K-*Ras* gene in 46 out of 49 cases. Of these, 35 (76%) had an AT to TA transversion in the third base of codon 61. The mutation spectrum was the same for tumours induced by DBC at a dose of 5, 20 or 40 mg/kg bw (Warshawsky *et al.*, 1996b). In a subsequent study, lung tumours in A/J mice given DBC at a dose of 10 mg/kg bw by intraperitoneal injection also had a high frequency (83%) of K-*Ras* mutations. Ten of the twelve tumours analysed had a detectable mutation; seven mutations were found at codon 61: all were AT to TA transversions (six were CAA to CAT; one was CAA to CTA) (Gray *et al.*, 2001).

When DBC was administered topically at a dose of 50 nmol or 100 nmol to the dorsal skin of Hsd:(ICR)BR mice twice per week for up to 70 weeks, the tumours induced in the skin and liver had a high frequency of Ha-*Ras* mutations (67% of skin tumours at both doses; 45% of liver tumours at the higher dose, but none [0 out of 10] at the lower dose). In all cases, the mutations were AT to TA transversions in the second base of codon 61 (CAA to CTA) (Mitchell & Warshawsky, 1999).

In Hsd:(ICR)BR mice given a single topical treatment of DBC at 200 nmol applied to the dorsal skin, followed by multiple applications of TPA at 2 mg twice per week for 28 weeks, the skin papillomas induced were frequently mutated at Ha-*Ras* ([Mitchell & Warshawsky, 2001](#)). Of the papillomas tested, 71% had mutations at codon 61, 4% had mutations at codon 12, 4% had mutations at codon 13 and 21% did not carry Ha-*Ras* mutations. CAA to CTA transversions accounted for all the mutations at codon 61. The mutation at codon 12 was a GGA to GAA transition, and the mutation at codon 13 was a GGC to GTC transversion.

Ionization potentials have been used to predict the metabolic activation of carcinogenic PAHs. Those with a high ionization potential are likely to proceed via mono-oxygenation. Carbazoles, which may be activated via one-electron transfer, mono-oxygenation or a combination of both, have lower ionization potentials than, for example, acridine derivatives, which are activated through mono-oxygenation. Thus ionization potentials predict to a certain extent the pathways of activation of carcinogenic *N*-heterocyclic PAHs ([Xue *et al.*, 1999](#)).

4.7.3 Structure–activity considerations for carbazole derivatives

[Table 4.1](#) shows the relative carcinogenic potencies of carbazole and DBC derivatives ([Warshawsky, 1992](#); see also references therein).

When administered in the diet, carbazole is a carcinogen in the liver and forestomach. None of the benzo[*a*]carbazoles are strong carcinogens: there is weak activity observed in skin for 7*H*-benzo[*a*]carbazole and slightly higher activity for the 10-methyl derivative, when injected subcutaneously. DBC is a potent skin carcinogen when administered topically or subcutaneously, and a potent liver carcinogen when given orally or subcutaneously, also inducing pulmonary and forestomach tumours.

Addition of a methyl group at the 7-position of DBC decreases the carcinogenic activity when applied topically, but not when injected subcutaneously. 5-Methyl-DBC, 6-methyl-DBC, 6,8-dimethyl-DBC and *N*-acetyl-DBC are potent carcinogens when given subcutaneously, but of these only *N*-acetyl-DBC is a liver carcinogen when injected intraperitoneally. 3-Methyl-DBC, 5,9-dimethyl-DBC and 5,9-diethyl-*N*-acetyl-DBC are active in liver, but not in subcutaneous tissue, as are 3-methoxy-DBC, 3-acetoxy-DBC and 3-hydroxy-DBC. 4-Methoxy-DBC and 4-acetoxy-DBC are active in both tissues, while 3,11-dimethyl-DBC and 5,9,*N*-trimethyl-DBC are inactive in both. These data suggested that the 5-, 6-, and 7-positions of DBC are involved in its sarcomagenic activity, while the 3- and/or the 5- and the 9-positions are involved in the hepato-carcinogenic activity of the compound.

4.8 Dibenzothiophene

4.8.1 Metabolism

The metabolism of dibenzothiophene was studied with liver microsomes from rats pre-treated with 3-methylcholanthrene, phenobarbital, dibenzothiophene or Aroclor 1254 ([Vignier *et al.*, 1985](#)). Two metabolites were identified: dibenzothiophene-5-oxide (dibenzothiophene sulfoxide), the major metabolite, and dibenzothiophene-5,5-dioxide (dibenzothiophene sulfone). No metabolites involving oxidation of carbon–carbon bonds were identified. Induction with 3-methylcholanthrene, phenobarbital, and Aroclor 1254 strongly enhanced the formation of dibenzothiophene sulfoxide, while dibenzothiophene had no effect as an inducer. In subsequent studies, the same authors showed that dibenzothiophene sulfoxide was converted to dibenzothiophene sulfone, indicating two sequential oxidation steps at the sulfur atom. The role of CYPs in dibenzothiophene oxidation reactions was also studied. Carbon monoxide, an inhibitor

Table 4.1 Relative carcinogenic activity of carbazoles in mice

Compound	Skin/subcutaneous tissue	Liver
Carbazole		++
11*H*-Benzo[*a*]carbazole	–	
8-Methyl-2-nitrobenzo[*a*]carbazole	–	
7,10-Dimethylbenzo[*a*]carbazole	–	
8-Bromobenzo[*a*]carbazole	–	
8-Chlorobenzo[*a*]carbazole	–	
2-Chlorobenzo[*a*]carbazole	–	
2-Chloro-6-methylbenzo[*a*]carbazole	–	
7*H*-Benzo[*a*]carbazole	+/–	
10-Methylbenzo[*a*]carbazole	+	
7*H*-Dibenzo[*c,g*]carbazole	+++	+++
1-Aza-7*H*-dibenzo[*c,g*]carbazole	+++	NR
4-Aza-7*H*-dibenzo[*c,g*]carbazole	++	NR
N-Methyl-7*H*-dibenzo[*c,g*]carbazole	++/+++	–
3-Methyl-7*H*-dibenzo[*c,g*]carbazole	–	+++
5-Methyl-7*H*-dibenzo[*c,g*]carbazole	+++	–
6-Methyl-7*H*-dibenzo[*c,g*]carbazole	+/++	–
3,11-Dimethyl-7*H*-dibenzo[*c,g*]carbazole	–	–
5,9-Dimethyl-7*H*-dibenzo[*c,g*]carbazole	–	+++
6,8-Dimethyl-7*H*-dibenzo[*c,g*]carbazole	+++	–
5,9,*N*-Trimethyl-7*H*-dibenzo[*c,g*]carbazole	–	–
N-Acetyl-7*H*-dibenzo[*c,g*]carbazole	+++	+++
5,9-Dimethyl-*N*-acetyl-7*H*-dibenzo[*c,g*]carbazole	–	+++
5-Acetylamino-7*H*-dibenzo[*c,g*]carbazole	+	–
5-Nitro-7*H*-dibenzo[*c,g*]carbazole	+/++	–
5,6-Dihydro-7*H*-dibenzo[*c,g*]carbazole	+/++	–
2-Methoxy-7*H*-dibenzo[*c,g*]carbazole	+/++	NR
3-Methoxy-7*H*-dibenzo[*c,g*]carbazole	–	+++
4-Methoxy-7*H*-dibenzo[*c,g*]carbazole	+++	++
6-Methoxy-7H-dibenzo[c,g]carbazole	++	–
3,11-Dimethoxy-7*H*-dibenzo[*c,g*]carbazole	–	–
3-Acetoxy-7*H*-dibenzo[*c,g*]carbazole	–	+++
4-Acetoxy-7*H*-dibenzo[*c,g*]carbazole	+	+++
6-Acetoxy-7*H*-dibenzo[*c,g*]carbazole	–	–
3-Hydroxy-7*H*-dibenzo[*c,g*]carbazole	–	++
5-Hydroxy-7*H*-dibenzo[*c,g*]carbazole	–	–
N-Ethyl-7*H*-dibenzo[*c,g*]carbazole	+	NR

–, not active; +, weakly active; ++, moderately active; +++, highly active; NR, not reported
Adapted from Warshawsky (1992)

of CYP activity, reduced sulfoxide formation by about 55% and sulfone formation by about 92% (Vignier *et al.*, 1985).

In a more recent study, the metabolism of dibenzothiophene was studied using of liver microsomes from rats pre-treated with pheno-barbital, 5,6-benzoflavone or Aroclor 1254. The same two metabolites described above were identified: dibenzothiophene sulfoxide, the major metabolite, and dibenzothiophene sulfone (Jacob *et al.*, 1991). No metabolites involving oxidation of carbon–carbon bonds were found. The yield of the sulfone metabolite was increased by each inducer, but the amount of sulfoxide formed was only marginally affected.

Dibenzothiophene was characterized as a noncompetitive inhibitor of CYP1A (Wassenberg *et al.*, 2005).

4.8.2 Genotoxicity, structure–activity relationships and other relevant data

Dibenzothiophene has been evaluated in two studies on bacterial mutagenicity in two strains of *S. typhimurium* (Table 4.2). No increase in mutagenic activity was observed for dibenzothiophene at concentrations of 0–100 µg/plate in *S. typhimurium* strain TA98 with exogenous metabolic activation (McFall *et al.*, 1984). Another study was conducted with both *S. typhimurium* strains TA98 and TA100 in the presence or absence of exogenous metabolic activation from S9 from rats induced with Aroclor 1254, with strain TA98 with metabolic activation in the pre-incubation protocol, and with strain TA100 without metabolic activation in the pre-incubation protocol. Except in the latter case, dibenzothiophene did not significantly induce mutation in these studies (Pelroy *et al.*, 1983).

Dibenzothiophene formed several unidentified DNA adducts after incubation for 24–28 hours in human HepG2 cells in culture, as determined by ^{32}P-postlabelling (Amat *et al.*, 2004).

While no formal structure–activity studies have been reported on dibenzothiophene and related three-ring thiophene-based polycyclic aromatic compounds, some information can be gleaned from the data on bacterial mutagenesis. Dibenzothiophene is a symmetrical three-ringed thiophene. Three other, asymmetric, three-ringed thiophenes were evaluated for mutagenic activity (Pelroy *et al.*, 1983). Naphtho[1,2-*b*]thiophene induced mutations in both strains, while the other isosteres, naphtho[2,3-*b*]thiophene and naphtho[2,1-*b*]thiophene, were inactive. These results suggest that a phenanthrenoid arrangement of the thio-PAH with the sulfur atom in the bay region was required for biological activity of three-ringed thiophenes.

Dibenzothiophene (10–500 µg/L) reduced EROD activity in embryos of *Fundulus heteroclitus* (killifish; saltwater minnow) by about 60%. The strong stimulation of EROD activity by the AhR agonist β-naphthoflavone (1 µg/L) was considerably diminished upon coincubation of the fish embryos with dibenzothiophene (Wassenberg *et al.*, 2005). Although not embryotoxic itself, dibenzothiophene enhanced the embryotoxicity of β-naphthoflavone.

In a study of oral toxicity, male CD-1 mice were given dibenzothiophene at a single dose of 0–1609 mg/kg bw. From the results, an LD$_{50}$ of 470 mg/kg bw was calculated. In a companion study, male CD-1 mice were pre-treated with 3-methylcholanthrene at a single dose of 80 mg/kg bw by intraperitoneal injection, and simultaneously with phenobarbital as three consecutive intraperitoneal injections at 50 mg/kg bw per day. After 24 hours, these mice were given dibenzothiophene at doses of 0–744 mg/kg bw. The LD$_{50}$ of the induced mice treated with dibenzothiophene was 335 mg/kg bw, suggesting that increased levels of CYP increased the toxicity of this compound (Leighton, 1989).

Table 4.2 Studies of mutagenicity with dibenzothiophene in bacteria

S. typhimurium strain	Concentration range (μg/plate)	Metabolic activation	Result	Reference
TA98	0–328	± S9	–	Pelroy *et al.* (1983)
TA100	0–500	± S9	–	Pelroy *et al.* (1983)
TA98	0–500	+ S9 (pre-incubation)	–	Pelroy *et al.* (1983)
TA100	0–250	– S9 (pre-incubation)	+	Pelroy *et al.* (1983)
TA98	0–100	+ S9	–	McFall *et al.* (1984)

S9, 9000 × *g* rat liver supernatant

4.9 Benzo[*b*]naphtho[2,1-*d*]thiophene

4.9.1 Metabolism

The metabolism of benzo[*b*]naphtho[2,1-*d*]thiophene upon incubation with microsomes from rat liver takes places on the sulfur atom – producing benzo[*b*]naphtho[2,1-*d*]thiophene sulfoxide and benzo[*b*]naphtho[2,1-*d*]thiophene sulfone – and on the aromatic carbons of both the benzo- and naphtha-rings – producing *trans*-benzo[*b*]naphtho[2,1-*d*]thiophene-1,2-dihydrodiol, *trans*-benzo[*b*]naphtho[2,1-*d*]thiophene-3,4-dihydrodiol, and 7-, 8-, and 9-hydroxybenzo[*b*]naphtho[2,1-*d*]thiophene (Jacob *et al.*, 1986, 1991; Misra & Amin, 1990; Murphy *et al.*, 1992). Formation of several other metabolites, including several triols, has been reported, but these were not fully characterized (Jacob *et al.*, 1986, 1991). The appearance of these metabolites by incubation with liver homogenates was dependent on the rat strain and on pre-treatment with specific inducers. In male Wistar rats, induction with Aroclor 1254 generally increased the level of the sulfone metabolite to a greater extent than that of the sulfoxide metabolite. The same effect was seen after induction with phenobarbital, while induction with 5,6-benzoflavone increased the levels of the two metabolites to a similar extent (Jacob *et al.*, 1991). Induction of Wistar rats with 1,1-bis-(*p*-chlorophenyl)-2,2,2-trichloroethane; DDT) increased the formation of a sulfone-phenol (Jacob *et al.*, 1986, 1988). When the yield of benzo[*b*]naphtho[2,1-*d*]thiophene metabolites was compared in non-induced liver homogenates from Wistar and F344 rats, microsomes from Wistar rats produced higher levels of the sulfoxide, sulfone and benzo[*b*]naphtho[2,1-*d*]thiophene-1,2-diol metabolites compared with those from F344 rats (Murphy *et al.*, 1992). Pre-treatment of F344 rats with Aroclor 1254 increased the liver microsome-mediated metabolism of benzo[*b*]naphtho[2,1-*d*]thiophene, and produced all of the known metabolites (Murphy *et al.*, 1992).

4.9.2 Genotoxicity, structure–activity relationships and other relevant data

The potential genotoxic activity of benzo[*b*]naphtho[2,1-*d*]thiophene has been evaluated in a series of studies of mutation in bacteria and one assay in mammalian cells (Table 4.3). Benzo[*b*]naphtho[2,1-*d*]thiophene did not induce mutations in *S. typhimurium* strain TA98 with or without a source of exogenous metabolic activation in a standard plate-incorporation test or in a liquid pre-incubation assay (Pelroy *et al.*, 1983; McFall *et al.*, 1984). Three studies were conducted with *S. typhimurium* strain TA100, in which benzo[*b*]naphtho[2,1-*d*]thiophene did not induce mutations in the presence or absence of exogenous metabolic activation. However, this substance was mutagenic in *S. typhimurium* TA100 in the presence of exogenous metabolic activation in the pre-incubation protocol (Pelroy *et al.*, 1983). In another study benzo[*b*]

Table 4.3 Studies of mutagenicity with benzo[*b*]naphtho[2,1-*d*]thiophene in bacteria

S. typhimurium strain or human cell line	Concentration range (µg/plate)[a]	Metabolic activation	Result	Reference
TA98	0–100	+ S9	–	McFall *et al.* (1984)
TA98	0–500	± S9	–	Pelroy *et al.* (1983)
TA100	0–500	± S9	–	Pelroy *et al.* (1983)
TA98	0–250	+ S9 (pre-incubation)	–	Pelroy *et al.* (1983)
TA100	0–250	– S9 (pre-incubation)	+	Pelroy *et al.* (1983)
TA100	0–320	+ S9	+	Misra & Amin (1990)
haA1v2 (*TK* locus)	0–10 µg/mL	NA	–	Durant *et al.* (1996)

[a] Unless otherwise specified

NA, not applicable; S9, 9000 × *g* rat liver supernatant

naphtho[2,1-*d*]thiophene was also mutagenic in *S. typhimurium* TA100 after exogenous metabolic activation (Misra & Amin, 1990). Benzo[*b*]naphtho[2,1-*d*]thiophene was not mutagenic in a human lymphoblastoid cell line (h1A1v2) known to express the metabolic enzyme CYP1A1 constitutively (Durant *et al.*, 1996).

Benzo[*b*]naphtho[2,1-*d*]thiophene formed one unidentified DNA adduct after incubation for 24–28 hours in human HepG2 cells in culture, as determined by ^{32}P-postlabelling (Amat *et al.*, 2004).

No formal structure–activity studies of four-ringed thiophenes, including benzo-naphthothiophenes, anthrathiophenes and phenanthrothiophenes, have been reported. However, results from tests of mutagenicity in bacteria provide some information on potential structure–activity relationships. Pelroy *et al.* (1983) studied the mutagenic activities of 13 four-ringed thiophenes in *S. typhimurium* TA98 and TA100 in the presence of exogenous metabolic activation. Phenanthro[3,4-*b*]thiophene was the most active compound, with a mutagenic activity in TA100 (≈195 revertants/µg) equal to that of benzo[*a*]pyrene. Anthra[2,1-*b*]thiophene induced nine TA100 revertants/µg and anthra[1,2-*b*]thiophene and anthra[2,3-*b*]thiophene each induced about four TA100 revertants/µg. In another study, phenanthro[3,4-*b*]thiophene and its isostere, phenanthro[4,3-*b*]thiophene, were compared with respect to their mutagenic activity in *S. typhimurium* TA98, TA100 and TA104 in the presence of exogenous metabolic activation from S9 from rats induced with Aroclor 1254 with the plate-incorporation protocol. Phenanthro[3,4-*b*]thiophene was mutagenic in *S. typhimurium* TA100 only (550 revertants/µg), while phenanthro[4,3-*b*]thiophene was mutagenic in TA98 (≈14 revertants/µg) and TA100 (≈13 revertants/µg) (Swartz *et al.*, 2009). All five thiophenes have phenanthrenoid structures with the thiophene ring at the distal end of the molecule in a bay or fjord configuration, a region known to enhance the mutagenic and carcinogenic activities of PAHs (Xue & Warshawsky, 2005).

4.9.3 Mechanistic considerations

The structure of benzo[*b*]naphtho[2,1-*d*]thiophene is similar to that of the carbocyclic hydrocarbon chrysene. Chrysene is metabolized to two major dihydrodiols upon incubation with liver microsomes from rats induced with 3-methylcholanthrene. Both chrysene-1,2-diol and chrysene-3,4-diol have a functionalized terminal benzo-ring. The K-region 5,6-dihydrodiol was also detected, at much lower levels (Nordqvist *et al.*, 1981). Chrysene-1,2-diol was found to be metabolized to a reactive diol epoxide, *r*-1,*t*-2-dihydroxy-*t*-3,4-oxy-1,2,3,4-tetrahydrochrysene, which forms DNA adducts in rodent and human

skin (Weston *et al.*, 1985). Benzo[*b*]naphtho[2,1-*d*]thiophene is metabolized to diols that are structurally analogous to those of chrysene: *trans*-1,2-dihydroxy-1,2-dihydrobenzo[*b*]naphtho[2,1-*d*]thiophene and *trans*-3,4-dihydroxy-3,4-dihydrobenzo[*b*]naphtho[2,1-*d*]thiophene. Both dihydrodiols were mutagenic in *S. typhimurium* TA100, with pre-incubation in the presence of a liver homogenate from rats induced with Aroclor 1254. The mutagenicity of the 3,4-dihydrodiol was comparable to that of the parent compound benzo[*b*]naphtho[2,1-*d*]thiophene, while the 1,2-dihydrodiol was a weaker mutagen (Misra & Amin, 1990). These results suggest the potential for further metabolism of benzo[*b*]naphtho[2,1-*d*]thiophene diols to diol epoxides, which could form DNA adducts and mutations, although there are no studies on benzo[*b*]naphtho[2,1-*d*]thiophene diol epoxide or DNA-adduct formation to confirm this.

5. Summary of Data Reported

5.1 Exposure data

Seven nitrogen-heterocyclic polycyclic aromatic hydrocarbons (azaarenes: benz[*a*]acridine, benz[*c*]acridine, dibenz[*a,h*]acridine, dibenz[*a,j*]acridine, dibenz[*c,h*]acridine, carbazole, 7*H*-dibenzo[*c,g*]carbazole) and two sulfur-heterocyclic polycyclic aromatic hydrocarbons (thiaarenes: dibenzothiophene and benzo[*b*]naphtho[2,1-*d*]thiophene) were reviewed. These compounds are formed during the incomplete combustion of nitrogen- and sulfur-containing organic material from natural sources (volcanic activities, wildfires, fossil fuels) and from anthropogenic sources (automobile exhausts, some industrial activities, tobacco smoke, cooking emissions). These compounds have been detected at low concentrations in the environment, in ambient air (total of four-ring azaarenes, including benz[*a*]acridine and benz[*c*]acridine, at

concentrations below the nanogram-per-cubic-metre level), water (at the microgram-per-litre level in groundwater and tar-contaminated sites) and soil (at the microgram-per-kg level). For comparison, the mainstream smoke of cigarettes contains 0.1 ng per cigarette dibenz[*a,h*]acridine, up to 10 ng per cigarette dibenz[*a,j*]acridine and 700 ng per cigarette 7*H*-dibenzo[*c,g*]carbazole.

5.2 Human carcinogenicity data

No data were available to the Working Group.

5.3 Animal carcinogenicity data

5.3.1 *Benz[*a*]acridine*

Benz[*a*]acridine has been evaluated for carcinogenicity in one study using dermal application in mice and one study of pulmonary implantation in rats. The study in mice was inadequate to evaluate the carcinogenicity of benz[*a*]acridine. Pulmonary implantation of benz[*a*]acridine did not increase the incidence of tumours of the lung in rats.

5.3.2 *Benz[*c*]acridine*

Benz[*c*]acridine has been evaluated for carcinogenicity in two studies of dermal application and four studies of dermal initiation–promotion in mice, a study of pulmonary implantation and a study of bladder implantation in rats, and a bioassay in neonatal mice.

The two studies of dermal application in mice were considered to be inadequate for evaluation of the carcinogenicity of benz[*c*]acridine. In two of the studies of initiation–promotion in mice, benz[*c*]acridine gave a positive response as an initiator; the other two initiation–promotion studies were considered to be inadequate. Benz[*c*]acridine did not increase the incidence of tumours of the lung when implanted into the lungs of rats; the study of bladder implantation in rats was

considered to be inadequate. When administered intraperitoneally to newborn mice, benz[c]acridine caused an increase in the incidence of tumours of the lung (primarily adenomas) in both sexes. The metabolites, benz[c]acridine-3,4-dihydrodiol and benz[c]acridine-*anti*-3,4-dihydrodiol-1,2-epoxide, were potent skin-tumour initiators in mice and induced tumours of the lung and liver when given to newborn mice.

5.3.3 Dibenz[a,h]acridine

Dibenz[*a,h*]acridine has been evaluated for carcinogenicity in one study of oral administration, five studies of dermal application, two studies using dermal initiation–promotion, four studies of subcutaneous injection, and a study of intravenous injection in mice. It was also tested in a study of subcutaneous injection and in a study of pulmonary implantation in rats. The studies of oral administration, dermal application, and subcutaneous injection in mice were considered to be inadequate for the evaluation of the carcinogenicity of dibenz[*a,h*]acridine. In the two initiation–promotion studies in mice, dibenz[*a,h*]acridine gave a positive response as an initiator. Dibenz[*a,h*]acridine also increased the incidence of adenoma of the lung when given to mice by intravenous injection, and of carcinoma of the lung when implanted into the lungs of rats. The metabolites dibenz[*a,h*]acridine-10,11-dihydrodiol, dibenz[*a,h*]acridine-*anti*-10,11-dihydrodiol-8,9-epoxide, and (+)-dibenz[*a,h*]acridine-*anti*-10*S*,11*R*-dihydrodiol-8*R*,9*S*-epoxide were potent skin-tumour initiators in mice.

5.3.4 Dibenz[a,j]acridine

Dibenz[*a,j*]acridine has been evaluated for carcinogenicity in one study of oral administration, seven studies of dermal application, one initiation–promotion study, and four studies of subcutaneous injection in mice, and in one study of pulmonary implantation in rats. The studies of oral administration and subcutaneous injection in mice were considered to be inadequate for the evaluation of the carcinogenicity of dibenz[*a,j*]acridine. Dibenz[*a,j*]acridine caused an increase in the incidence of skin cancer in two of the studies of dermal application and, as an initiator, in the initiation–promotion study in mice; the other studies of dermal application were considered to be inadequate. Dibenz[*a,j*]acridine did not increase the incidence of tumours of the lung when implanted into the lungs of rats.

5.3.5 Dibenz[c,h]acridine

Dibenz[*c,h*]acridine has been evaluated for carcinogenicity in one initiation–promotion study in mice and in one bioassay in neonatal mice. In the initiation–promotion study, dibenz[*c,h*]acridine, as an initiator, caused an increase in the incidence and multiplicity of skin papilloma. In the bioassay in neonatal mice, dibenz[*c,h*]acridine given by intraperitoneal injection caused an increase in the incidence of tumours of the lung (primarily adenomas) in both sexes, and in the incidence and multiplicity of liver adenoma in males. The metabolites (–)-dibenz[*c,h*]acridine-3*R*,4*R*-dihydrodiol and (+)-dibenz[*c,h*]acridine-*anti*-3*S*,4*R*-dihydrodiol-1*R*,2*S*-epoxide, and two isosteric analogues, were potent skin-tumour initiators in mice, and induced tumours of the lung and liver when given to neonatal mice.

5.3.6 Carbazole

Carbazole has been evaluated for carcinogenicity in one feeding study, three studies of dermal application and one study of subcutaneous injection in mice, in one study of intraperitoneal injection in neonatal mice, and in two studies of tumour promotion in rats. It was also tested in a feeding study in hamsters. The studies of dermal application and subcutaneous injection in mice and the feeding study in hamsters

were inadequate to evaluate the carcinogenicity of carbazole. In mice given diet containing carbazole, a dose-dependent increase in the incidence of liver neoplastic nodules (adenomas) and hepatocellular carcinoma was observed. In the forestomach of these animals, papillomas (in males and females) and carcinomas (in males only) were also detected. No increase in tumour incidence was seen in the study of intraperitoneal injection in neonatal mice. In male rats, carbazole administered in the diet did not show a promoting effect in one study, but promoted the development of kidney papilloma and carcinoma in another study.

5.3.7 7H-*Dibenzo[c,g]carbazole*

7*H*-Dibenzo[*c,g*]carbazole has been evaluated for carcinogenicity in mice in one study of oral administration, seven studies of dermal application, seven studies of subcutaneous injection, two studies of intraperitoneal injection, one study of intravenous injection, one study of bladder implantation, and one tumour-initiation study. This substance was also evaluated for carcinogenicity after intratracheal implantation in hamsters, and after intravesical injection in a dog. The oral study, five of the studies of dermal application, all studies of subcutaneous injection, one of the studies of intraperitoneal injection, the study of intravenous injection and the study of bladder implantation in mice, as well as the study of intratracheal implantation in hamsters and the study of intravesical injection in the dog were inadequate to evaluate the carcinogenicity of 7*H*-dibenzo[*c,g*]carbazole. In mice given 7*H*-dibenzo[*c,g*]carbazole by dermal application, a statistically significant increase in the incidence of skin carcinoma was observed in one study, and a statistically significantly increased incidence of skin tumours and liver neoplasms was observed in one other study. Intraperitoneal administration of 7*H*-dibenzo[*c,g*]carbazole to mice resulted in a dose-related increase in the

incidence and multiplicity of lung adenomas. A skin-painting initiation–promotion study in mice indicated that 7*H*-dibenzo[*c,g*]carbazole had tumour-initiating ability.

5.3.8 *Dibenzothiophene*

No data were available to the Working Group

5.3.9 *Benzo[b]naphto[2,1-d]thiophene*

In one study of pulmonary implantation in female rats, benzo[*b*]naphto[2,1-*d*]thiophene increased the incidence of squamous cell carcinoma of the lung.

5.4 Mechanistic and other relevant data

5.4.1 *Benz[a]acridine*

The metabolism of benz[*a*]acridine by rat liver and lung microsomes yielded benz[*a*]acridine-5,6-dihydrodiol (a K-region dihydrodiol) and an uncharacterized non-K-region dihydrodiol, which was not benz[*a*]acridine-3,4-dihydrodiol. Evidence for the formation of the bay-region diol-epoxide, *trans*-benz[*a*]acridine-3,4-dihydrodiol-1,2-oxide, has not been obtained. Benz[*a*]acridine was a weak inducer of mono-oxygenase activity in rat liver, and was shown to induce proteins recognized by antibodies to cytochrome 1A1, but not cytochrome 2B1. Mutagenicity tests conducted with benz[*a*]acridine in *Salmonella typhimurium* TA98 (his⁻/his⁺), in the presence of an exogenous metabolic system, were inconclusive. However, benz[*a*]acridine was positive in the Mutatox™ test. This assay is based on the use of a dark variant of the luminescent bacterium *Vibrio fischeri*, which can be used to detect genotoxic activity in aqueous samples; the presence of genotoxic compounds results in mutations and consequently in restoration of photoluminescence. The *cis-* and *trans*-benz[*a*]

acridine-3,4-dihydrodiol-1,2-oxides were mutagenic in *S. typhimurium* TA98 and TA100 and in Chinese hamster V79–6 cell lines. Benz[*a*]acridine-3,4-dihydrodiol was mutagenic in *S. typhimurium* TA100 in the presence of a highly purified and reconstituted mono-oxygenase system obtained from rat liver microsomes. The *cis*- and *trans*-benz[*a*]acridine-3,4-dihydrodiol-1,2-oxides induced DNA damage in two rat hepatoma cell lines. Benz[*a*]acridine itself and its metabolite *trans*-benz[*a*]acridine-3,4-dihydrodiol were inactive in the same test systems.

There is inadequate evidence for a mutagenic mechanism underlying the carcinogenicity of benz[*a*]acridine on the basis of experimental data.

5.4.2 Benz[c]acridine

The metabolism of benz[*c*]acridine by rat liver microsomes yielded several mono- and diphenols and dihydrodiols. The major metabolite was the K-region dihydrodiol, while *trans*-benz[*c*]acridine-3,4-dihydrodiol was formed in very small amounts. A small amount of *N*-oxidation products was also formed. Unequivocal evidence of the formation of *trans*-benz[*c*]acridine-3,4-dihydrodiol-1,2-epoxide was not obtained. Benz[*c*]acridine was a weak inducer of mono-oxygenase activity in rat liver.

Benz[*c*]acridine was mutagenic in *S. typhimurium* TA100 in the presence of exogenous metabolic activation. The bay-region *cis*- and *trans*-benz[*c*]acridine-3,4-dihydrodiol-1,2-epoxides were mutagenic in *S. typhimurium* TA98 and TA100 and in Chinese hamster V79–6 cells. In the same test systems, non-bay-region diol-epoxides were one to four orders of magnitude less mutagenic. *trans*-Benz[*c*]acridine-3,4-dihydrodiol, the precursor of the bay-region diol-epoxides, was at least five times more active than benz[*c*]acridine in *S. typhimurium* TA100 in the presence of exogenous metabolic activation. None of the other possible *trans*-dihydrodiols

was significantly activated under these conditions. Benz[*c*]acridine induced sister-chromatid exchange in Chinese hamster Don (lung) cells without the addition of an exogenous metabolic system. *trans*-Benz[*c*]acridine-3,4-dihydrodiol and the *cis*- and *trans*-benz[*c*]acridine-3,4-dihydrodiol-1,2-epoxides induced DNA damage in two rat hepatoma cell lines. The bay-region diol-epoxides of benz[*c*]acridine had substantially higher activities in bacterial and mammalian cells than their benz[*a*]acridine analogues. These differences are consistent with qualitative arguments regarding resonance stabilization of the carbocations resulting from opening of the epoxide ring.

There is weak evidence for a mutagenic mechanism underlying the carcinogenicity of benz[*c*]acridine on the basis of experimental data.

5.4.3 Dibenz[a,h]acridine

Dibenz[*a,h*]acridine metabolism yields two types of bay-region diol-epoxide, i.e. *cis*- and *trans*-dibenz[*a,h*]acridine-3,4-dihydrodiol-1,2-epoxide and *cis*- and *trans*-dibenz[*a,h*]acridine-10,11-dihydrodiol-8,9-epoxide. The differences in structure result in different biological activities between the diol-epoxides and between their dihydrodiol precursors. The dibenz[*a,h*]acridine-10,11-dihydrodiol-8,9-epoxides and their metabolic precursor, dibenz[*a,h*]acridine-10,11-dihydrodiol, are considerably more mutagenic in bacterial and mammalian test systems than the analogous bay-region 3,4-dihydrodiol-1,2-epoxides and their 3,4-dihydrodiol precursor. The *trans*-dibenz[*a,h*]acridine-1,2- and -8,9-dihydrodiols, which cannot be converted to bay-region diol-epoxides, are not activated to mutagenic products in *S. typhimurium* TA100. Computational data suggest that carbocation formation at C-8 is energetically favoured over that at C-1, which may determine the lower reactivity of the 3,4-dihydrodiol-1,2-epoxides in comparison

with that of the 10,11-dihydrodiol-8,9-epoxides. A decreased propensity for epoxide ring opening of the 3,4-dihydrodiol-1,2-epoxides may explain their lower mutagenic activity. Intratracheal instillation of rats with dibenz[*a,h*] acridine resulted in formation of DNA adducts, sister-chromatid exchange, and micronucleus formation in lung cells. The data on mutagenicity in mammalian cells and tumour initiation on mouse skin implicate *trans*(–)-(10*R*,11*R*)-dibenz[*a,h*]acridine-10,11-dihydrodiol as the proximate carcinogen and the bay-region *trans*(+)-(8*R*,9*S*,10*S*,11*R*) diol-epoxide as the ultimate carcinogen. The observed stereoselectivity is identical to that exhibited by other carbocyclic and aza-polycyclic aromatic hydrocarbons, including benzo[*a*]pyrene, benz[*a*]anthracene, chrysene, benzo[*c*]phenanthrene and dibenz[*c,h*] acridine. Human cytochrome 1A1 is substantially more active in metabolizing dibenz[*a,h*] acridine than human cytochrome 1B1 and, in contrast to rat cytochrome 1A1, is regioselective for the formation of dibenz[*a,h*]acridine-10,11-dihydrodiol compared with dibenz[*a,h*]acridine-3,4-dihydrodiol. In addition, stereoselectivity for the production of the proximate carcinogen, 10*R*,11*R*-dibenz[*a,h*]acridine-10,11-dihydrodiol, by cytochrome 1A1 suggests that a high expression of this enzyme activity may confer increased susceptibility to dibenz[*a,h*]acridine-induced carcinogenesis. Dibenzo[*a,h*]acridine was about 2.5 times more potent than 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and more than 200 times more potent than benzo[a]pyrene in activating the aryl hydrocarbon receptor in a rat hepatoma cell line *in vitro*.

There is moderate evidence for a mutagenic mechanism underlying the carcinogenicity of dibenz[*a,h*]acridine on the basis of experimental data.

5.4.4 Dibenz[a,j]acridine

Dibenz[*a,j*]acridine is converted by rat, mouse and human liver microsomes and by rat lung microsomes to a series of hydroxylated metabolites, including dihydrodiols, tetrahydrotetrols, phenols and diol-epoxides. *trans*-Dibenz[*a,j*]acridine-3,4-dihydrodiol is typically the major metabolite, predominantly as the *3R,4R* isomer. Human cytochrome 1A1, 1A2, 3A4 and 3A5 catalysed the formation of *trans*-dibenz[*a,j*]acridine-3,4-dihydrodiol *in vitro*; the 3A4 isoform was the most selective for this metabolite, while cytochrome 1A2 was selective for K-region 5,6-oxidation. Regardless of the specific cytochrome, the 3,4-dihydrodiol had a *3R,4R*-configuration, with almost 100% optical purity. Extensive phase-II metabolism, including glutathione conjugation, was demonstrated to occur with rat hepatocytes *in vitro*.

Dibenz[*a,j*]acridine was mutagenic in *S. typhimurium* TA98 and TA100 in the presence of an exogenous metabolic system. The compound induced chromosomal aberrations in Chinese hamster fibroblasts in the presence of exogenous metabolic activation. The most mutagenic dibenz[*a,j*]acridine metabolites in both bacterial and mammalian cells were the bay-region diol-epoxides, *cis*- and *trans*-dibenz[*a,j*] acridine-3,4-dihydrodiol-1,2-oxide, which did not require metabolic activation. The *trans* diol-epoxide was consistently more mutagenic than its *cis* isomer. Dibenz[*a,j*]acridine increased the frequency of micronucleus formation in human lymphocytes *in vitro*. In the presence of liver microsomes from rats treated with 3-methylcholanthrene, dibenz[*a,j*]acridine was shown to bind to calf thymus DNA, yeast RNA, polyG, polyA, polyU and polyC. The greatest extent of binding was observed with polyG. Epithelial cells from rat buccal mucosa metabolized dibenz[*a,j*]acridine to DNA-binding species. Upon topical application to rats, mice and hamsters, similar profiles of DNA adducts were detected by

³²P-postlabelling, almost exclusively in the skin, with higher levels of adduct being seen in mice. Topical application of the *trans*-dibenz[*a,j*]acridine-1,2-, -3,4-, and -5,6-dihydrodiols to mice, followed by ^{32}P-postlabelling analysis of skin DNA, demonstrated that the 3,4-dihydrodiol is an intermediate in the major route of dibenz[*a,j*]acridine activation leading to DNA binding in the skin. Topical application of dibenz[*a,j*]acridine and (+/–)-*trans*-benz[*a,j*]acridine-3,4-dihydrodiol-1,2-epoxide yielded four skin DNA adducts, detected by ^{32}P-postlabelling. The major adduct from dibenz[*a,j*]acridine co-eluted with a synthetic deoxyguanosine adduct and the major adduct formed by the diol-epoxide *in vivo* was a deoxyadenosine adduct. Skin papillomas and carcinomas formed after topical application of dibenz[*a,j*]acridine to mice harboured A to T and G to T transversions in codons 12, 13 and 61 of the *Hras* gene. The mutation spectra in the *Hras* gene were consistent with the observed binding of dibenz[*a,j*]acridine to deoxyguanosine or deoxyadenosine *in vivo*.

There is strong evidence for a mutagenic mechanism underlying the carcinogenicity of dibenz[*a,j*]acridine on the basis of experimental data.

5.4.5 Dibenz[c,h]acridine

Dibenz[*c,h*]acridine is mutagenic in *S. typhimurium* TA98 and TA100 in the presence of an exogenous metabolic activation system. Activation of dibenz[*c,h*]acridine to the bay-region diol-epoxide, dibenz[*c,h*]acridine-3,4-dihydrodiol-1,2-oxide, is consistent with its mutagenicity in bacterial and mammalian systems. In Chinese hamster V79–6 cells, the (+)-*anti*-(1*R*,2*S*,3*S*,4*R*)-dibenz[*c,h*]acridine-3,4-dihydrodiol-1,2-epoxide was more mutagenic than any of the other 3,4-dihydrodiol-1,2-epoxides. It was also the most tumorigenic of the four possible isomeric bay-region diol-epoxides of dibenz[*c,h*] acridine, both in an initiation–promotion model on mouse skin and in newborn mice (see above). Data on metabolism in rat liver microsomes suggest that a high expression of cytochrome 1A1 activity may confer increased susceptibility to dibenz[*c,h*]acridine-induced carcinogenesis. Exposure of Chinese hamster V79 cells to the (*R,S,S,R*) and (*S,R,R,S*) bay-region diol epoxides from dibenz[*c,h*]acridine resulted in AT base-pair mutations at the hypoxanthine (guanine) phosphoribosyltransferase locus. Dibenz[*c,h*] acridine produced chromosomal aberrations in Chinese hamster fibroblasts in the presence of exogenous metabolic activation. Following an initiation–promotion protocol, the DNA isolated from dibenz[*c,h*]acridine-induced carcinomas in female CD-1 mice efficiently transformed NIH 3T3 cells. A high percentage of the transformed foci had an amplified *Hras* gene containing an A to T transversion in the second base of codon 61. The same mutation was detected in primary tumour DNA in a high percentage of the dibenz[*c,h*]acridine-induced carcinomas and also in NIH 3T3 cells transformed with DNA from dibenz[*c,h*]acridine-induced benign skin papillomas.

There is strong evidence for a mutagenic mechanism underlying the carcinogenicity of dibenz[*c,h*]acridine, despite the absence of studies demonstrating the formation of DNA adducts induced by dibenz[*c,h*]acridine.

5.4.6 Carbazole

The major metabolite of carbazole in rats is 3-hydroxycarbazole. It is characterized as a non-competitive inhibitor of cytochrome 1A enzymes. Carbazole is not mutagenic to bacteria. It is moderately clastogenic in mice when administered intraperitoneally. It induced dominant lethality and sperm-head abnormalities in male mice. Carbazole has been reported to be a major active component of coal tar; it displays anti-angiogenic and anti-inflammatory properties *in vitro*.

There is inadequate evidence for a mutagenic mechanism underlying the carcinogenicity of carbazole on the basis of experimental data.

5.4.7 7H-*Dibenzo[c,g]carbazole*

When administered to rodents, 7*H*-dibenzo[*c,g*]carbazole is widely distributed in tissues, extensively metabolized, and excreted mainly in the faeces. In mice, 7*H*-dibenzo[*c,g*] carbazole is mainly metabolized by cytochromes 1A1 and 1A2 in the liver and by cytochromes 1A1 and 1B1 in the lung. Similar results were obtained *in vitro* in test systems expressing human cytochrome 1 enzymes. The major metabolites formed by liver cells and microsomal fractions are monohydroxylated derivatives; a dihydrodiol is also formed.

7*H*-Dibenzo[*c,g*]carbazole gave positive results in some, but not all, tests for mutagenicity in bacteria. 7*H*-Dibenzo[*c,g*]carbazole induced mutations and micronucleus formation in Chinese hamster V79 cells expressing human cytochrome 1A1 and/or 1A2. It was mutagenic in DNA repair-deficient human xeroderma pigmentosum cells, and induced micronucleus formation in cultured human lymphocytes cells *in vitro*. 7*H*-Dibenzo[*c,g*]carbazole was mutagenic in transgenic mice, inducing mutations in the liver and skin. The substance formed DNA adducts in rodent cells *in vitro* and in mice *in vivo*. The order of binding in mouse tissues after subcutaneous injection of 7*H*-dibenzo[*c,g*]carbazole was liver >> kidney > lung > spleen > skin > brain. The pattern of DNA adducts observed in the liver was different from that in skin. The pattern in the liver resembled that formed by 3-hydroxydibenzo[*a*]acridine. 7*H*-Dibenzo[*c,g*] carbazole induced DNA damage in rodent cells *in vitro,* measured as alkali-labile lesions (converted to strand breaks). There was conflicting evidence regarding its contribution to formation of oxidative damage in DNA. 7*H*-Dibenzo[c,g]carbazole caused necrosis and apoptosis in the HepG2 human hepatoma cell line, and apoptosis in mouse liver *in vivo*. It weakly induced the aryl hydrocarbon receptor and inhibited gap-junction intercellular communication in WB-F344 rat-liver epithelial cells, a property of tumour promoters. A high percentage of tumours induced in mouse lung by 7*H*-dibenzo[*c,g*]carbazole contained mutations in *Kras*, the majority of which were AT to TA transversions in the third base of codon 61. Similarly, skin tumours induced by 7*H*-dibenzo[*c,g*]carbazole frequently contained mutations in *Hras1*, mostly AT to TA transversions.

There is moderate evidence for a mutagenic mechanism underlying the carcinogenicity of 7*H*-dibenzo[*c,g*]carbazole; this compound is mutagenic by a genotoxic mechanism.

5.4.8 Dibenzothiophene

Dibenzothiophene was not mutagenic in several strains of *S. typhimurium*. Metabolism studies conducted with preparations of rat liver identified only sulfur-oxidation metabolites and a study in human liver-tumour cells indicated the formation of unidentified DNA adducts.

There is inadequate evidence for a mutagenic mechanism underlying the carcinogenicity of dibenzothiophene on the basis of experimental data.

5.4.9 Benzo[b]naphtho[2,1-d]thiophene

Benzo[*b*]naphtho[2,1-*d*]thiophene was mutagenic in two strains of S. *typhimurium*. The metabolism of this compound has been studied with preparations of rat liver microsomes, which revealed formation of two dihydrodiol metabolites, both of which were mutagenic in one strain of *S. typhimurium*. A study in HepG2 human liver-tumour cells indicated the formation of unidentified DNA adducts by benzo[*b*] naphtho[2,1-*d*]thiophene.

There is weak evidence for a mutagenic mechanism underlying the carcinogenicity of benzo[*b*]naphtho[2,1-*d*]thiophene on the basis of experimental data.

6. Evaluation

6.1 Cancer in humans

No data were available to the Working Group.

6.2 Cancer in experimental animals

There is *inadequate evidence* in experimental animals for the carcinogenicity of benz[*a*]acridine.

There is *limited evidence* in experimental animals for the carcinogenicity of benz[*c*]acridine.

There is *sufficient evidence* in experimental animals for the carcinogenicity of dibenz[*a,h*]acridine.

There is *sufficient evidence* in experimental animals for the carcinogenicity of dibenz[*a,j*]acridine.

There is *limited evidence* in experimental animals for the carcinogenicity of dibenz[*c,h*]acridine.

There is *sufficient evidence* in experimental animals for the carcinogenicity of carbazole.

There is *sufficient evidence* in experimental animals for the carcinogenicity of 7*H*-dibenzo[*c,g*]carbazole.

There is *inadequate evidence* in experimental animals for the carcinogenicity of dibenzothiophene.

There is *limited evidence* in experimental animals for the carcinogenicity of benzo[*b*]naphto[2,1-*d*]thiophene.

6.3 Overall evaluation

Benz[*a*]acridine is *not classifiable as to its carcinogenicity (Group 3)*.

Benz[*c*]acridine is *not classifiable as to its carcinogenicity (Group 3)*.

Dibenz[*a,h*]acridine is *possibly carcinogenic to humans (Group 2B)*.

Dibenz[*a,j*]acridine is *probably carcinogenic to humans (Group 2A)*. In making the overall evaluation for dibenz[*a,j*]acridine, the Working Group considered mechanistic and other relevant data.

Dibenz[*c,h*]acridine is *possibly carcinogenic to humans (Group 2B)*. In making the overall evaluation for dibenz[*c,h*]acridine, the Working Group considered mechanistic and other relevant data.

Carbazole is *possibly carcinogenic to humans (Group 2B)*.

7*H*-Dibenzo[*c,g*]carbazole is *possibly carcinogenic to humans (Group 2B)*.

Dibenzothiophene is *not classifiable as to its carcinogenicity to humans (Group 3)*.

Benzo[*b*]naphtho[2,1-*d*]thiophene is *not classifiable as to its carcinogenicity to humans (Group 3)*.

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