Table 1. AhR Activation Potencies of Q and Its Halogenated Derivatives

Compounds	EC_{BaP50} [a] (μM)	*lacZ* units/μM [b]	Relative activity
Quinoline	>500	0.13	1.0
Monofluorinated derivatives			
2-F-Q	>250	0.80	6.2
3-F-Q	>250	1.09	8.4
5-F-Q	>250	0.63	4.8
6-F-Q	>250	0.40	3.1
7-F-Q	>250	2.03	15.6
8-F-Q	>250	0.54	4.2
Difluorinated derivatives			
3,5-diF-Q	>250	0.91	7.0
3,7-diF-Q	>250	1.78	13.7
5,6-diF-Q	>250	1.83	14.1
5,7-diF-Q	>250	1.02	7.8
5,8-diF-Q	>250	1.14	8.8
6,7-diF-Q	>250	0.77	5.9
6,8-diF-Q	>250	0.80	6.2
7,8-diF-Q	>250	1.27	9.8
Polyfluorinated derivatives			
3,5,7-triF-Q	>250	1.48	11.4
5,6,8-triF-Q	>250	1.95	15.0
6,7,8-triF-Q	158.6	3.30	25.4
5,6,7,8-tetra-F-Q	164.8	3.15	24.2
Monochlorinated derivatives			
2-Cl-Q	141.1	4.50	34.6
3-Cl-Q	>250	1.57	12.1
4-Cl-Q	>250	0.94	7.2
5-Cl-Q	>250	1.85	14.2
6-Cl-Q	>250	1.21	9.3
7-Cl-Q	>250	1.86	14.3
8-Cl-Q	>250	1.74	13.4
Dichlorinated derivatives			
5,7-diCl-Q	>50	7.68	59.1
5,8-diCl-Q	32.3	15.3	118
6,8-diCl-Q	>50	7.58	58.3
7,8-diCl-Q	28.85	22.9	176
Polychlorinated derivatives			
5,6,8-triCl-Q	14.7	56.9	438
6,7,8-triCl-Q	7.2	101.8	783
Brominated derivatives			
3-Br-Q	174.3	2.98	22.9
6-Br-Q	>250	1.81	13.9
8-Br-Q	172.9	2.71	20.8
Dibrominated derivatives			
5,8-diBr-Q	17.3	27.2	209
6,8-diBr-Q	32.1	17.5	135

Each value is the mean of at least three analyses. *a*) Concentrations producing *lacZ* units equal to 50% of the maximal response to BaP. *b*) Calculated from the slope of the linear portion of each curve near the origin. *c*) Relative values of *lacZ* units/μM of each compound compared to Q.

Table 2. AhR Activation Potencies of BhQs, BfQs, and 1,7-Phes

Compounds	EC_{BaP50} [a] (μM)	*lacZ* units/μM [b]	Relative activity
BhQ derivatives			
BhQ	>50	7.47	1.0
2-F-BhQ	29.8	16.7	2.2
3-F-BhQ	>50	8.34	1.1
5-F-BhQ	>50	11.9	1.6
6-F-BhQ	>50	6.80	0.9
7-F-BhQ	>50	11.8	1.6
9-F-BhQ	>50	12.4	1.7
10-F-BhQ	>50	2.53	0.3
3,6-diF-BhQ	>50	5.90	0.8
7,10-diF-BhQ	>50	11.2	1.5
2-Cl-BhQ	13.1	43.3	5.8
4-Cl-BhQ	33.6	22.3	3.0
6-Cl-BhQ	>50	16.3	2.2
6-Br-BhQ	32.2	20.3	2.7
BfQ derivatives			
BfQ	>50	2.62	1.0
2-F-BfQ	26.1	31.6	12.1
7-F-BfQ	30.1	17.0	6.5
7,10-diF-BfQ	16.7	28.2	10.8
2-Cl-BfQ	1.33	247	94.2
2-Br-BfQ	0.65	666	254
1,7-Phenanthroline derivatives			
1,7-Phe	>50	5.91	1.0
6-F-1,7-Phe	>50	9.74	1.6
9-F-1,7-Phe	13.8	43.4	7.3
5-Cl-1,7-Phe	0.44	1117	189
9-Cl-1,7-Phe	0.40	1227	208

Each value is the mean of at least three analyses. *a*) Concentrations producing *lacZ* units equal to 50% of the maximal response to BaP. *b*) Calculated from the slope of the linear portion of each curve near the origin. *c*) Relative values of *lacZ* units/μM of each compound compared to Q.

spective parent compound analogs.

DISCUSSION

We measured the AhR ligand activities of ten *N*-containing PAHs using the yeast AhR signaling assay to investigate the PAH ring nitrogen-substitution effect on the substrate recognition by AhR. Phenanthrene was very weak as the AhR ligand compared with BaP and chrysene, though the ligand activity was altered by nitrogen-substitution in the aromatic ring(s) position-selectively. The ligand activity of phenanthrene was significantly potentiated (about 10-fold) by nitrogen-substitution in position-4 (BhQ) and position-1 and -5 (1,7-Phe), moderately enhanced by nitrogen-substitution in position-1 (BfQ), and not at all affected by nitrogen-substitution in position-1 and -8 (4,7-Phe). BhQ and 1,7-Phe have a nitrogen atom in the bay-region of the molecule, while BfQ and 4,7-Phe do not have one in the bay-region. The result suggests that the N atom in the bay-region is more effective in enhancing the ligand activity than the non-bay-region N atom. Similar enhancing effects by the bay-region N atom(s) were observed in chrysene and BaP. 4,10-DAC, in which two N atoms are located in the bay-region, showed a higher ligand activity than chrysene and 1,10-DAC, in which one N atom is located in the bay-region and the other N atom is located in the non-bay-region. Furthermore, two azaBaPs, 6-azaBaP and 10-azaBaP, showed much higher ligand activities than BaP, and the enhancing effect on ligand activity by *N*-substitution at position-10 (bay-region) was higher than that at position-6 (non-bay-region).

Quinoline, isoquinoline, and naphthalene were about three orders of magnitude less active than BaP. The ligand activity of quinoline was augmented by halogen-substitution 3- to 783-fold. When the enhancing effect of the halogen substituent of quinoline on the AhR ligand activity was compared, chlorine and bromine atoms were more effective than the fluorine atom. Additionally, the enhancing effect on the ligand activity is dependent on the number of the halogen substituents regardless of the substituent position.

A similar result has been reported in polychlorinated naphthalene derivatives, and it is known that the AhR ligand activity of naphthalene is remarkably potentiated by substitu-

tion by more than 3 chlorine atoms.[25,26] It is well known that a majority of potent AhR ligands (*e.g.*, TCDD) are very lipophilic and could fit into a rectangle of 6.8×13.7 Å.[27,28] Quinoline is less lipophilic and smaller in molecular size than TCDD. The enhancement of the AhR ligand activity by oligochlorine- or oligobromine-substitution in quinoline might be explained by the increases of lipophilicity and molecular size.

In *N*-containing tricyclic aromatic hydrocarbons, BhQ and 1,7-Phe showed similar AhR ligand activities and BfQ showed a slightly lower ligand activity than BhQ and 1,7-Phe. However, halogen-substitution effects on the AhR ligand activity in BhQ were much lower than those in BfQ and 1,7-Phe. The AhR ligand activity of BfQ was considerably increased by 2-Cl-substitution (94-fold) and 2-Br-substitution (254-fold) and 5- and 9-Cl-substituion greatly potentiated the ligand activity of 1,7-Phe (189- and 208-fold, respectively), while the effects of Cl- and Br-substitution on the ligand activity of BhQ were only from 2.2- to 5.8-fold potentiation. Furthermore, the ligand activity of BhQ decreased by 66% (to 0.34-fold) by 10-F-substitution. These results suggest that the optimal positionalrelationship for potentiation of the ligand activity exists regarding the position of the nitrogen atom in the aromatic ring and the position of the halogen substituent in *N*-containing tricyclic aromatic hydrocarbons.

With regard to the position specific effect of halogen-substitution on the AhR ligand activity in BhQ, the halogen atom at position-2 was most effective in both cases of fluorine- and chlorine-substitution. Similarly, 2-F-BfQ showed a higher ligand activity than 7-F-BfQ and the ligand activity of 9-F-1,7-Phe was higher than that of 6-F-1,7-Phe. 2-F-BhQ, 2-F-BfQ, and 9-F-1,7-Phe have the common structure of the aza-analog of 3-fluorinated phenanthrene. This finding suggests that the position-3 of phenanthrene is best suited as the site of halogen substitution for potentiation of AhR ligand activity in azaphenanthrenes. In fact, 2-Cl-BfQ, 2-Br-BfQ, and 9-Cl-1,7-Phe showed potent AhR ligand activities (EC_{BaP50}=1.3, 0.7, and 0.4, respectively) equal to or higher than that of BaP (EC_{50}=1.4). This result indicates that the AhR ligand activity of *N*-containing polycyclic aromatics might be markedly enhanced by substitution with only one halogen atom.

In conclusion, the present study on the series of *N*-containing polycyclic aromatics and their halogen-substituted derivatives provides further basic data to better understand the ligand recognition mechanism of AhR and will be useful for prediction of the toxicity of some structurally related environmental pollutants.

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Structural activity relationship between *Salmonella*-mutagenicity and nitro-orientation of nitroazaphenanthrenes

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Abstract

Nitroazaphenanthrenes (NAphs) and their *N*-oxides (NAphOs) were synthesized as derivatives with nitrogen atoms in the 1, 4, and 9 positions of phenanthrene rings, and as nitrated derivatives substituted at the 1, 2, 3, 4, 5, 6, 7, and 8 positions of phenanthrene rings. To determine the structure activity relationship of these derivatives, all 19 isomers were bioassayed with *Salmonella* tester strains. NAphs substituted at the 4, 6, 7 and 8 positions were mutagenic for TA98, and 1-, 2-, and 3-N-9-AphOs, 6-N-1-AphO and 6-N-4-AphO were mutagenic for TA98 and TA100 without the S9 mix, while 5-N-1-AphO and 5-N-9-AphO were non- or weakly mutagenic. Nitrated derivatives, 6-N-4-Aph, 6-N-9-Aph, 6-N-1-AphO, and 6-N-4-AphO, were powerful mutagens for TA98 and TA100. Mutagenicity was enhanced by mutant strains producing nitroreductase, such as YG1021 and 1026, and by those producing *O*-acetyltransferase, such as YG1024 and 1029. Nitro derivatives substituted at positions 4 and 5 in the phenanthrene rings were perpendicular, while those at positions 2, 3, 6 and 7 were coplanar to the phenanthrene rings. NAphs substituted at the 1 and 8 positions were noncoplanar due to steric hindrance of the aromatic proton at the peri position. On the other hand, 1,5- and 1,8-dinitro-4-azaphenanthrenes showed high mutagenicity for strains TA98 and TA100 in the absence of the S9 mix, and were strongly enhanced by nitroreductase and *O*-acetyltransferase, over-producing mutants. Therefore, it was found that the mutagenic potency of NAphs and NAphOs was closely associated with the chemical properties and orientation of nitro substitution of aromatic rings.

Keywords: Nitroazaphenanthrenes; *Salmonella* mutagenicity; Nitro-orientation

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1. Introduction

Nitrophenanthrene (Nph) derivatives were synthesized by Fukuhara et al. [1,2], and their mutagenicity was investigated in *Salmonella* strains [3]. It was found that most nitro derivatives showed strong mutagenicity in *Salmonella* mutants, and that the orientation of the nitro substituent had an important structural feature affecting the mutagenic potency and metabolism of Nph [3–5]. Normally, mutagenic activity of nitroarenes is associated with structural features such as the physical dimensions of the aromatic rings, isomeric position of the nitro group, conformation of the nitro group with respect to the plane of the aromatic rings and the ability to resonance-stabilize the ultimate electrophile [6].

Aristolochic acid was used as an anti-inflammatory agent in several pharmaceutical preparations up to 1982, and consisted of several components of Nphs [7]. It was found that Aristolochic acid also contained genotoxic mutagens forming DNA adducts after metabolic activation in *Salmonella* strains and mammalian cells [8–10], as well as being was carcinogenic in rats [11–13]. It has been reported that the mutagenic activity of Nph was closely associated with reduction potentials and the dihedral angles of the nitro substituent for intercalation of chemicals into DNA [2–4,14,15].

Nitroazaphenanthrene (NAph) derivatives containing nitrogen atoms at the 1, 4, and 9 positions of phenanthrene rings were synthesized by Fukuhara et al. (unpublished data), and their reduction property was determined. Using authentic samples of Nph and NAph derivatives, *Salmonella* mutagenicity was determined, and the structural activity relationship was discussed.

2. Materials and methods

2.1. Chemicals

NAphs containing nitrogen atoms in the rings used in this study were 8-nitro-1-azaphenanthrene (8-N-1-Aph), 6- and 8-N-4-APh, 4-, 5-, 6-, and 7-N-9-Aph, 5-, 6- and 8-N-1-Aph *N*-oxide (5-, 6- and 8-N-1-AphO), 5-, 6- and 8-N-4-APhO, 1-, 2-, 3- and 5-N-9-APhO, and 1,5- and 1,8-dinitro-4-APhO (1,5- and 1,8-diN-4-AphO). These NAph derivatives were synthesized by Fukuhara et al. (unpublished data) and modified by the method described by Dewar and Warford [16].

2.2. Bacterial strains

Bacterial strains used were *Salmonella typhimurium* TA98, TA100, and TA98NR, a nitroreductase-deficient mutant of TA98, TA98/1,8DNP, an *O*-acetyltransferase-deficient mutant of TA98, YG1021 and YG1026 produced by transferring a plasmid carrying the nitroreductase gene into cells of TA98 and TA100, respectively, and strains YG1024 and 1029, carrying a plasmid of the acetyltransferase gene transferred into cells of TA98 and TA100, respectively, were generously donated by Drs T. Nohmi and M. Watanabe, National Institute of Health Sciences [17].

2.3. Electrochemical reduction by cyclic voltammetry and electronic descriptors

In dimethylformamide, teraethylammonium perchlorate was used as the supporting electrolyte at a 0.1 M concetration. The reference electrode was an Ag/Ag^+ electrode in acetonitrile with 0.1 M tetrabutylammonium perchlorate. After transfer of the solution containing the test chemical to the cell, it was purged of oxygen by bubbling with N_2 for 15 min. The cyclic voltammograms were recorded at a scan rate of 100 mV/s, while maintaining the test solution under a steady steam of N_2.

The electronic descriptor, LUMO energy levels of nitrated phenanthrenes, was calculated by MOPAC2002 (AM1), which is based on the MOPAC of the Toray System Center using the AM1 method. The initial geometries were constructed from standard bond lengths and angles. The geometries were then completely optimized using algorithms in the MOPAC program. For 5-N-4-AphO and 1,5-diN-4-AphO, LUMO energy and dihedral angle were obtained by AM1 calculations based on the structure that was optimized by PM3.

2.4. Mutagenicity test

Mutagenicity was determined by the plate incorporation test using *Salmonella* tester strains [3]. Each experiment was carried out on triplicate plates two times with or without the S9 mix, and the mean value was presented as revertants/μg of chemical.

3. Results

3.1. LUMO energy levels and dihedral angles of Nphs

To measure enzymatic reduction, the LUMO energy levels, reduction potential, and orientation of the nitro substituent to the phenanthrene rings were investigated (Table 1). Nitro substituents substituted at the 4 and 5 positions were perpendicular due to the steric effect of the bay region aromatic proton, while those at the 2, 3, 6, and 7 positions were coplanar to the phenanthrene rings (Fig. 1). In contrast, nitro substituents at the 1 and 8 positions had a nitro function with nearly perpendicular orientation to the aromatic ring system because of steric hindrance of the aromatic proton on the peri position, while the calculated dihedral angles varied between 26° and 33°. This suggested that the orientation of the nitro group generally predicts the mutagenic potency of NAphs. There was no relationship between mutagenicity, reduction potentials and LUMO energy.

Table 1
LUMO energy levels, reduction potentials, and dihedral angles of NAphs

Chemical	Epc (mV)		LUMO	Dihedral angle
	Epc1	Epc2		
8-N-1-Aph	−1042	−1631	−1429	31.5
6-N-4-Aph	−1036	−1675	−1400	0.1
8-N-4-Aph	−1058	−1555	−1372	32.2
4-N-9-Aph	−1120	−1662	−1326	62.6
5-N-9-Aph	−1085	−1615	−1219	61.7
6-N-9-Aph	−1027	−1645	−1508	0.2
7-N-9-Aph	−1004	−1664	−1436	0.0
5-N-1-APhO	−1049	−1625	−1505	61.0
6-N-1-AphO	−972	−1572	−1663	0.1
8-N-1-AphO	−988	−1641	−1623	33.3
5-N-4-APhO	−1214	−1619	−1427	32.2[a]
6-N-4-APhO	−1059	−1723	−1583	0.7
8-N-4-APhO	−1047	−1678	−1545	31.7
1-N-9-APhO	−853	−1484	−1674	26.5
2-N-9-APhO	−964	−1628	−1696	0.1
3-N-9-APhO	−893	−1463	−1733	0.1
5-N-9-APhO	−1029	−1568	−1494	60.3
1,5-diN-4-APhO	−623	−1263	−2074	30.1[b]
1,8-diN-4-APhO	−916	−1032	−2214	58.2*

[a] and [b], LUMO energy and dihedral angle were obtained by AM1 calculations based on the structure that was optimized by PM3.

* 8-NO_2, 33.5; 1-NO_2, 24.7.

3.2. Chemical properties of Nph

Fig. 1 illustrates chemical structures, consisting of Aph derivatives with a nitrogen atom at the 1, 4, and 9 positions, and NAphs, nitrated derivatives, substituted at the 1, 2, 3, 4, 5, 6, 7, and 8 positions in phenanthrene rings and their *N*-oxides.

3.3. Mutagenicity of NAph in Salmonella strains

All NAphs substituted at the 4, 6, 7, and 8 positions were mutagenic for strains TA98 and TA100 without S9 mix while 5-N-9-Aph, 5-N-1-AphO, and 5-N-9-AphO were non-mutagenic or weak mutagens. In NAphO derivatives, 6-N-1-AphO, 8-N-1-AphO, 6-N-4-AphO, and 1, 2, and 3-N-9-AphOs were mutagenic for strains TA98 and TA100, and were direct-acting mutagens, showing activity in the absence of S9 mix while 5-N-1- and 5-N-9-AphOs were non-mutagenic or weakly mutagenic (Table 2). In contrast, mutagenicity for TA98 with S9 mix was assessed, but it was not shown in any NAph derivatives (Table 2). Thus, a mutagenicity test was carried out in the absence of the S9 mix. In these nitrated derivatives, nitro derivatives substituted at the 6 position of phenanthrene rings, 6-N-4-Aph, 6-N-9-Aph, 6-N-1-AphO, and 6-N-4-AphO, showed powerful mutagenicity for strains TA98, TA100, and all YG strains used. Mutagenicity was enhanced by mutant strains producing nitroreductase such as YG1021 and 1026, and those producing *O*-acetyl-

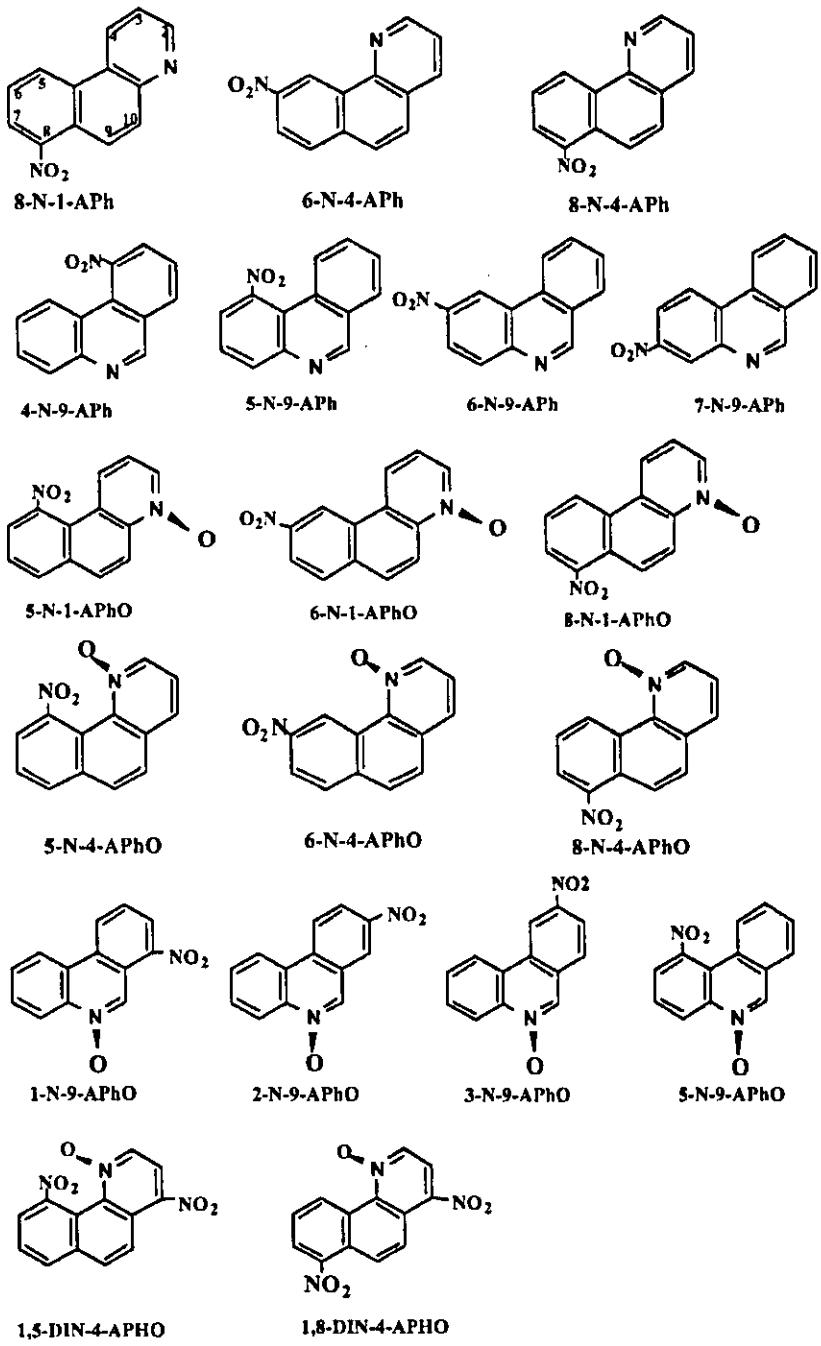

Fig. 1. Chemical structures of NAph's.

transferase such as YG1024 and YG1029. In addition, the nitroreductase activity was also confirmed by the fact that the activity was inhibited by the nitroreductase-deficient strain TA98NR, and similarly, was inhibited by the TA98/1,8-DNP$_6$, *O*-acetyltransferase-deficient strain. Nitro derivatives substituted at the 8 position of the phenanthrene rings such as 8-N-1- and 8-N-4-Aphs, were strongly reverted from autotrophy to prototrophy for strain TA100. These derivatives might arise due to base pair substitution on DNA, showing higher mutageni-

Table 2
Mutagenicity of Naphs

Chemical	Mutagenicity (revertants/μg)								
	TA98 (−S9)	TA98 (+S9)	TA98NR	TA98/1,8DNP$_6$	TA100	YG1021	YG1024	YG1026	YG1029
Spontaneous	29	28	37	11	128	67	86	178	183
8-N-1-Aph	538	8	42	0.3	1046	8479	35600	13040	1246
6-N-4-Aph	2.052	18	149	530	6060	42370	6260	46800	11020
8-N-4-Aph	828		16	141	1528	14480	7980	1282	4018
4-N-9-Aph	112	11	31	48	160	806	609	785	401
5-N-9-Aph	6	ND	2	1.6	7	47	18	53	9
6-N-9-Aph	728	13	168	98	818	1050	3478	5194	788
7-N-9-Aph	452	20	61	185	251	850	1018	918	408
5-N-1-AphO	3	ND	1	0.7	6	16	9	16	8
6-N-1-AphO	150	8	99	82	493	1247	170	5949	718
8-N-1-AphO	38	ND	3	17	113	998	79	1310	122
5-N-4-AphO	63	6	15	19	146	247	87	97	92
6-N-4-AphO	700	17	142	215	1854	6893	1818	7258	4354
8-N-4-AphO	67	ND	4	23	151	1038	786	1528	704
1-N-9-AphO	764	26	170	482	1252	1756	915	4627	897
2-N-9-AphO	213	20	20	36	148	174	639	737	106
3-N-9-AphO	634	11	141	684	173	596	3482	748	413
5-N-9-AphO	4	ND	1	0.5	5	16	12	38	7
1,5-diN-4-AphO	10460	33	6420	1440	2906	24980	11637	9541	6575
1,8-diN-4-AphO	5580	24	5240	8440	8440	21640	11002	48850	5916

ND, not detected.

city in TA100 than TA98. These nitro groups could also be due to steric hindrance of the nitro substituent on the plane of the phenanthrene rings.

Two 1,5- and 1,8-diN-4-AphOs promoted marked mutagenicity for TA98, TA100, and YG strains. Mutagenicity of 1,5- and 1,8-diN-4-AphO corresponded to 166 and 83 times, respectively, that of 5-N-4-AphO and 8-N-4-AphO for TA98, and to 19 and 55 times, respectively, that of 5-N-4- and 8-N-4-AphOs for TA100. Dinitro derivatives substituted at the 1 and 5 positions of the phenanthrene rings were strongly activated by nitroreductase, overproducing mutants more than *O*-acetyltransferase (Table 2). Both dinitro derivatives, which have relatively lower LUMO energy, showed high mutagenicity for strains TA98 and TA100, and their enzyme rich-mutants. It was found that mutagenicity of 1,5- and 1,8-diN-4-AphO was strongly activated by nitroreductase, rather than *O*-acetyltransferase, as shown in YG1021 and 1024, except for reversion for YG 1026 of 1,8-diN-4-AphO.

4. Discussion

We previously reported that the mutagenic potency of Nphs was closely associated with the chemical properties and orientation of nitro substitution of aromatic rings [3]. Nitro substituents at positions 4 and 5 in the phenanthrene rings were perpendicular, while those on positions 2, 3, 6 and 7 were nearly coplanar to the phenanthrene rings (Table 1). It has also been reported that Nphs on positions 1, 8, 9, and 10 were non-coplanar because of steric hindrance of the aromatic proton on the peri position, with dihedral angles varying from 10° to 65° [1,2].

In this study, it was found that NAphs with a nitrogen atom at the 1, 4, and 9 positions of

phenanthrene rings showed potent mutagenicity corresponding to Nphs if they had the same chemical properties as Nphs. Mutagenicity of 4-, 6-, and 7-N-9-Aphs, 1-, 2-, and 3-N-9-AphOs, was potent for *Salmonella* strains, while nitro substituents of 5-N-9-Aph, 5-N-1-AphO, and 5-N-9-AphO were non- or weakly mutagenic. The nitro substituent at the 5 position in the rings was perpendicular on the plane of phenanthrene rings, so mutagenicity was reduced markedly due to steric hindrance. This suggested that the mutagenic potency of these nitro groups closely involved the orientation of nitro substituents of the phenanthrene rings, and the inability to intercalate into the DNA helix.

Nitro substituents on positions 2, 3, 6, and 7 in the phenanthrene rings had a nitro function with coplanar orientation to the parent rings, and had relatively lower LUMO energy levels. In addition, the mutagenicity was markedly enhanced by nitroreductase-overproducing mutants such as YG1021 and YG1026, and by *O*-acetyltransferase-overproducing mutants such as YG1024 and YG1029. It was considered that these nitro groups were metabolically reduced to nitroso- and amino-derivatives by these enzymes via a hydroxylamino-intermediate.

Two 1,5- and 1,8-diNAphOs were potent mutagens for TA98 and 100, and showed strong reversion from autotrophy to prototrophy by nitroreductase and *O*-acetyltransferase. Comparing the mutagenic potency of both compounds, 1,8-diNAphO was more strongly activated by nitroreductase than 1,5-diN-4-AphO, e.g. the compound showed 48 850 revertants/μg for YG1026. From the perspective of reduction property, the reduction potentials (mV) of mono- and dinitrophenanthrenes differed; the potentials (Epc1) of mononitrophenanthrenes ranged from −853 to −1214, and those of dinitrophenanthrene (diNph) ranged from −623 to −916 (Table 1). As suggested by Fukuhara et al. [1], nitro substitution causes about a 100–300 mV positive shift in the reduction potentials. Therefore, it was found that the calculated LUMO energy levels were significantly correlated with the first reduction potentials, and the mutagenic potency of NAph was related to these chemical properties, including LUMO energy and reduction potentials.

With regard to mutagenicity and genotoxicity of nitroazabenzo[*a*]pyrene, we reported that these compounds were potent mutagens for *Salmonella* strains [18], and induced micronuclei in polychromatic erythrocytes in mice and chromosomal aberrations in Chinese hamster lung fibroblast cells [19]. Mutagenicity and genotoxicity of nitroazabenzo[*a*]pyrene *N*-oxides differed from substitution of nitro function. However, the reduction potentials of 3-nitro-6-azabenzo[*a*]pyrene *N*-oxide are not markedly different from those of 1-nitro-6-azabenzo[*a*]pyrene *N*-oxide [18]. In addition, 3,6-dinitrobenzo[*a*]pyrene was much more mutagenic than 1,6-dinitrobenzo[*a*]pyrene. This result indicates that 3,6-dinitrobenzo[*a*]pyrene was readily reduced to stronger nitroso- and amino-derivatives than 1,6-dinitrobenzo[*a*]pyrene [20].

As for a human effect of nitrated aromatic compounds, various chemicals deposited in lung tissues were determined after surgical lung resection; e.g. 1-NP, 1,3-DNP and 3-nitrofluoranthene deposited at higher levels in lung tissues decreased the 5-year survival of patients [21]. It was assumed that increasing amounts of mutagens in lung tissues might promote cell differentiation, with an increase in poor differentiation. As reported previously [3], Nph derivatives were detected in diesel emission particulates, but not from other environmental materials and human lung tissues.

Shmeiser et al. [13,22] demonstrated that aristolochic acid containing Nph carboxylic acids induced multiple tumors in the forestomach, ear duct and small intestine, and urothelial cancer in aristolochic acid nephropathy patients [8]. In addition, DNA adducts of the compound were detected in vitro by incubation of calf thymus DNA and liver homogenate, while tumor initiation by aristolochic acid was associated with carcinogen–deoxyadenosine adducts as critical lesions.

We therefore, believe that these mutagens are ubiquitous in the environment, though they have not been detected from environmental materials. It is important that the carcinogenicity of these chemicals be studied in more detail to evaluate

the structure activity relationship of NAphs and their danger to human tissues.

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Metabolic activation of 10-aza-substituted benzo[a]pyrene by cytochrome P450 1A2 in human liver microsomes

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Abstract

We previously reported that 10-azabenzo[a]pyrene (10-azaBaP), a 10-aza-analog of BaP and an environmental carcinogen, showed greater mutagenicity than BaP in the Ames test using pooled human liver S9. To investigate the cytochrome P450 (CYP) isoform involved in the activation of 10-azaBaP to the genotoxic form, the mutagenicity of 10-azaBaP using nine individual donors' and pooled human liver microsome preparations was compared with each CYP activity. Induced revertants by 2.5 nmol per plate 10-azaBaP with 0.5 mg per plate human liver microsomal protein showed a large inter-individual variation (42-fold) among the nine donors. The number of induced revertants highly correlated with the CYP1A2-selective catalytic activity from each microsome preparation, and no correlation was observed with other CYP isoform-selective catalytic activities. Moreover, recombinant human CYP1A2 contributed to the mutagenicity of 10-azaBaP more markedly than recombinant human CYP1A1. These results suggest that CYP1A2 may be the principal enzyme responsible for the metabolic activation of 10-azaBaP in human liver microsomes. With regard to the proposal that BaP may be activated by human CYP1A1, our results suggest that the nitrogen-substitution at position-10 of BaP may cause the CYP enzyme-specificity in metabolic activation to change from CYP1A1 to CYP1A2.

Keywords: Aza-substitution; Polycyclic aromatic hydrocarbon; Human liver microsome; Ames test

1. Introduction

10-azabenzo[a]pyrene (10-azaBaP) is an analog of benzo[a]pyrene (BaP) containing a nitrogen atom at position-10 (Fig. 1) and it is a carcinogenic environmental contaminant [1,2]. Kosuge et al. have isolated 7.3 mg of 10-azaBaP from 82 g of a basic fraction of coal tar [1]. This amount of 10-azaBaP indicates that its environmental content might be much lower than that of BaP. Although 10-azaBaP could not be converted to the bay-region diol epoxide (7,8-dihydrodiol-9,10-epoxide), the ultimate active form of BaP, because of the nitrogen-substitution at position-10, 10-azaBaP was also reported to be as highly mutagenic as BaP in the Ames test using *Salmonella typhimurium* TA100 in the presence of the

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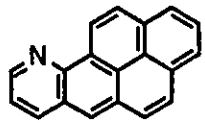

10-azabenzo[a]pyrene
(10-azaBaP)

Fig. 1. Structure of 10-azaBaP.

PCB-treated rat liver S9 [3–5]. Therefore, 10-azaBaP might be converted to the genotoxic form not by the bay-region mechanism but by other unknown mechanisms. We previously reported that 10-azaBaP was mutagenic in the liver and colon of lambda/*lacZ* transgenic mice, but the mutagenic activity of 10-azaBaP was much less than that of BaP at the same dose of 125 mg/kg/day × 5 days [6]. On the other hand, interspecies differences were observed when comparing the mutagenicity of BaP and 10-azaBaP in Ames tests using mouse, rat, and human liver homogenates, and 10-azaBaP was more mutagenic than BaP in the Ames test using pooled human liver S9 [6]. Therefore, there is the possibility that the CYP isoform responsible for the conversion of 10-azaBaP to the ultimate active form might be more abundant in human liver homogenates than that for BaP such as CYP1A1 [7–9]. However, the CYP isoform responsible for the mutagenicity of 10-azaBaP in the human liver is unknown.

In this report, we examined the in vitro mutagenicity of 10-azaBaP by the Ames test using microsome preparations from nine human livers, in order to identify the human CYP isoform responsible for the mutagenicity of 10-azaBaP.

2. Materials and methods

2.1. Materials

Individual donors' (catalog nos. HG3, HK25, HK37, HG43, HG56, HG64, HG89, HG93, and HG95) and pooled human liver microsome preparations (20 mg protein/ml) and microsome preparations from bacurovirus-infected insect cells expressing CYP1A1 and CYP1A2, coexpressed with NADPH-CYP oxidoreductase, were purchased from Gentest Co. (Woburn). Cofactor I^{TM} was purchased from Oriental Yeast Co. (Tokyo), proteinase K and olive oil from Wako Pure Chemicals (Osaka), and resorufin, 7-ethoxyresorufin, and 7-methoxyresorufin from Sigma (St. Louis). 10-AzaBaP (CAS Registry no. 189-92-4) was synthesized according to the reported method [10]. Purification of the product by recrystallization from acetone yielded 10-azaBaP as pale brown needles: mp 158–161 °C. Anal. calcd. for $C_{19}H_{11}N$: C, 90.09; H, 4.38; N, 5.53. Found: C, 89.86; H, 4.60; N, 5.40.

2.2. In vitro mutation assays (Ames tests)

10-AzaBaP was tested for mutagenicity using *S. typhimurium* TA100 (kindly provided by Dr. B.N. Ames of the University of California, Berkeley) in the presence of the microsomes and cofactors (Cofactor I^{TM}) according to the method by Ames et al. [11] with slight modification as previously reported [12–14]. Cofactor I^{TM} consisted of 4 mM NADPH, 4 mM NADH, 5 mM glucose-6-phosphate, 32.8 mM KCl, 8 mM $MgCl_2$, and 100 mM phosphate buffer (pH 7.4). Assays were carried out by preincubation of the test chemical with the microsome mix at 37 °C for 20 min. At least two independent experiments were performed. In this study, the mean number of spontaneous revertants (treated with DMSO) per plate was 222 with a S.D. of 23.

2.3. Activity of 7-ethoxyresorufin O-deethylase and 7-methoxyresorufin O-demethylase

The activities of 7-ethoxyresorufin O-deethylase (EROD) and 7-methoxyresorufin O-demethylase (MROD) were measured in hepatic microsomes according to the method of Rodrigues and Prough [15]. The assays were adapted for the use of 96-well plates and a fluorometric plate reader. The reaction components (in a total volume of 200 μl) were 0.1 M potassium phosphate buffer (pH 7.4), 1.3 mM $NADP^+$, 3.3 mM glucose-6-phasphate, 3.3 mM $MgCl_2 \cdot 6H_2O$, 0.08 units of G6PDH, 10 μM substrate (ethoxyresorufin or methoxyresorufin), and 0.04 μg of microsomal protein. After incubation at 37 °C for 30 min, 75 μl of the stop solution (80% acetonitrile/20% 0.5 M Tris base) was added. The production of fluorescent resorufin was recorded at the emission

and excitation wavelengths of 590 and 485 nm, respectively, and relative fluorescence was compared to a calibration curve. All incubations were carried out in triplicate. The activities of phenacetin O-deethylase, coumarin 7-hydroxylase, dicrofenac 4′-hydroxylase, (S)-mephenytoin 4′-hydroxylase, bufurarol 1′-hydroxylase, chlorzoxazone 6-hydroxylase, and teststerone 6β-hydroxylase were quoted from the catalog (Gentest).

3. Results

3.1. Ames tests using pooled human liver microsome

10-AzaBaP was tested for in vitro mutagenicity in the Ames test using pooled human liver microsomes (0.5 mg protein per plate) and *S. typhimurium* TA100. The dose–response curve of mutation by 10-azaBaP is shown in Fig. 2, and the maximal production of revertants per plate was obtained from the assay using 2.5 nmol of 10-azaBaP per plate. The number of revertants obtained by 10-azaBaP (2.5 nmol per plate) increased in proportion to the amount of microsomes in a range from 0 to 1 mg protein per plate (data not shown).

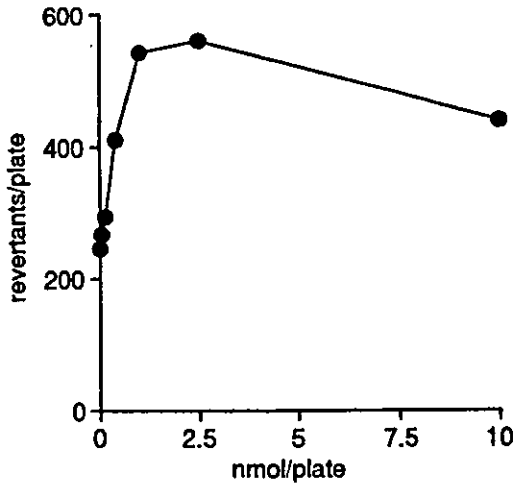

Fig. 2. The mutagenicity of 10-azaBaP in the Ames test using pooled human liver microsomes. Mutagenicity was tested by the Ames test using *S. typhimurium* TA100 in the presence of 0.5 mg of microsomal protein per plate. Each point represents the mean of duplicate analysis.

3.2. Ames tests using nine individual donors' and pooled human liver microsomes

The mutagenicity of 10-azaBaP (2.5 nmol per plate) was measured by Ames tests using nine individual donors' and pooled human liver microsomes (0.5 mg protein per plate). The mutagenicity was expressed as the number of induced revertants, which was obtained by subtracting the number of spontaneous revertants (DMSO) from the number of revertants produced by 10-azaBaP (2.5 nmol per plate) in the presence of each microsome preparation. The results shown in Fig. 3A demonstrate a large inter-individual diversity of mutagenicity, which ranged from 19 to 795 revertants, among the nine microsomes samples tested. The number of induced revertants obtained from the pooled human liver microsome preparation was approximately equal to the average level of the nine individual samples. Then the numbers of induced revertants were compared with the CYP isoform-selective catalytic activities (Table 1). As shown in Fig. 3B-D, the numbers of induced revertants significantly correlated with the CYP1A2-selective catalytic activities (phenacetin O-deethylase, 7-ethoxyresorufin O-deethylase, and 7-methoxyresorufin O-demethylase; $r^2 = 0.93$, 0.83, and 0.95, respectively) from each microsome preparation. On the other hand, no significant correlation was observed between the numbers of induced revertants and other CYP isoform-selective catalytic activities (Table 2).

3.3. Ames tests using microsomes expressing recombinant human CYP1A1 or CYP1A2

O-dealkylation of 7-methoxy- and 7-ethoxyresorufin is known to be catalyzed mainly by human CYP1A2 [16,17] and also by CYP1A1 [18]. To resolve which of these two CYPs is the predominant enzyme responsible for the activation of 10-azaBaP to the genotoxic form by human liver microsomes, we carried out the Ames test using microsomes from baculovirus-transfected insect cells expressing recombinant human CYP1A1 or 1A2. When the content of recombinant human CYP1A2 was changed from 0 to 200 pmol CYP per plate, the number of revertants by 10-azaBaP (2.5 nmol per plate) showed a dose-dependent increase (Fig. 4). On the other hand,

Table 1
Catalytic activities of nine individual donors' and pooled human liver microsome preparations with CYP isoform-selective substrates

	Assay	HG3	HK25	HK37	HG43	HG56	HG64	HG89	HG93	HG95	pooled
CYP1A2	Phenacetin O-deethylase[a]	170[b]	1100	710	590	2300	150	1500	890	280	980
	7-Ethoxyresorufin O-deethylase[c]	0.79	10.60	14.20	8.51	46.10	0.57	25.74	22.01	4.61	12.59
	7-Methoxyresorufin O-demethylase[c]	0.32	13.78	14.99	10.33	62.75	0.05	27.27	14.03	3.96	16.89
CYP2A6	Coumarin 7-hydroxylase[a]	2000	210	460	670	1400	1500	650	350	200	1000
CYP2C9	Dicrofenac 4′-hydroxylase[a]	1700	5600	1400	1700	3100	4700	2100	1700	2100	2900
CYP2C19	(*S*)-mephenytoin 4′-hydroxylase[a]	44	190	69	630	410	14	190	39	30	41
CYP2D6	Bufuralol 1′-hydoxylase[a]	110	150	130	22	100	220	13	47	160	110
CYP2E1	Chlorzoxazone 6-hydroxylase[a]	1800	2700	1700	1200	1900	3000	1700	1500	1200	2000
CYP3A4	Teststerone 6β-hydroxylase[a]	6100	3700	2500	4800	4000	2200	12000	1700	760	5600

[a] Data from the catalog (Gentest).
[b] Activities expressed as pmol product per mg protein min.
[c] 7-Alkylresorufin O-dealkylase activity was measured as described in Section 2.

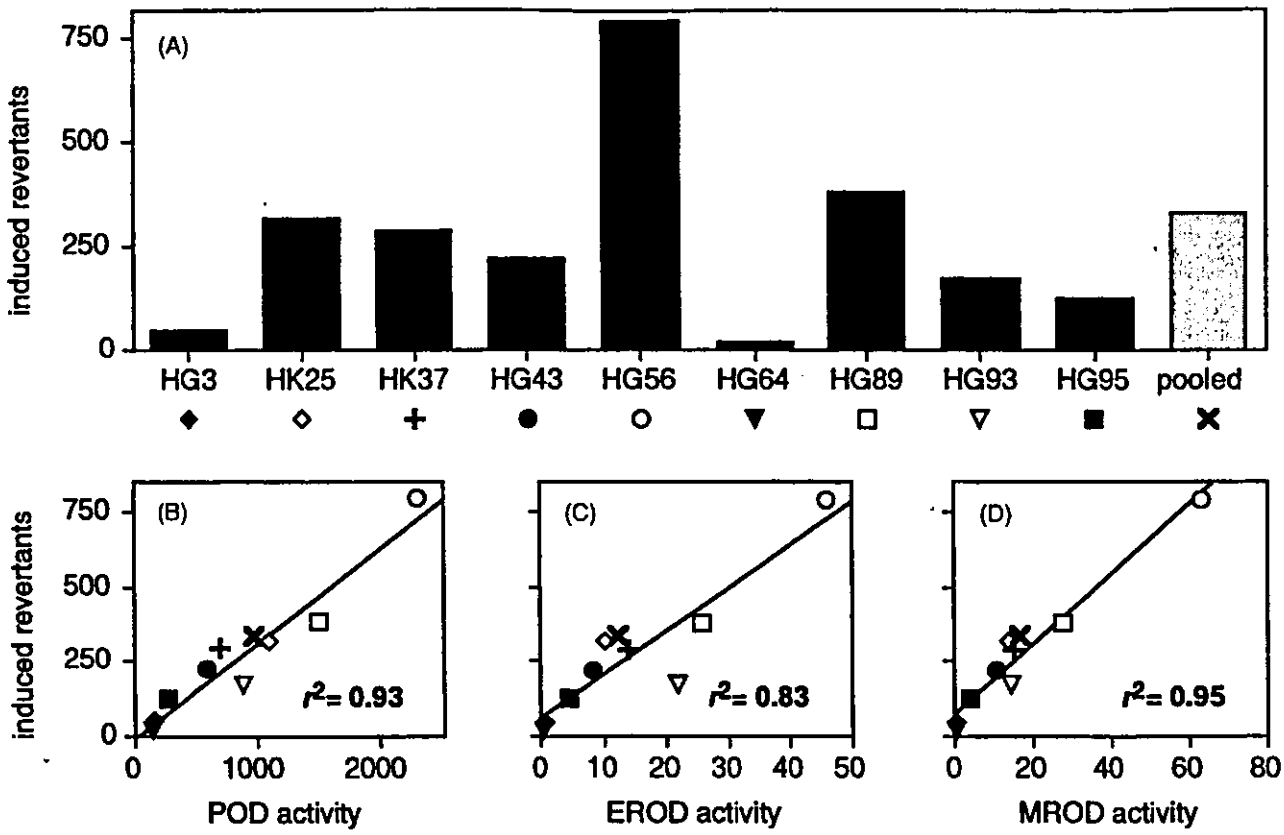

Fig. 3. The numbers of induced revertants by 10-azaBaP in 10 human livers microsome preparations (A) and relationships between the numbers of induced revertants and CYP1A2-selective catalytic activities (B–D). (A) Mutagenicity of 10-azaBaP (in terms of induced revertants) in nine individual donors' and pooled liver microsome preparations. (B) Phenacetin O-deethylase activity vs. the number of induced revertants. (C) Ethoxyresorufin O-deethylase activity vs. the number of induced revertants. (D) Methoxyresorufin O-demethylase activity vs. the number of induced revertants.

Table 2
Correlation coefficients between the number of induced revertants by 10-azaBaP and each CYP isoform-selective catalytic activity in 10 human livers microsome preparations

	Assay	r^2
CYP1A2	Phenacetin O-deethylase	0.93 ($P < 0.01$)
	7-Ethoxyresorufin O-deethylase	0.83 ($P < 0.01$)
	7-Methoxyresorufin O-demethylase	0.95 ($P < 0.01$)
CYP2A6	Coumarin 7-hydroxylase	<0.10
CYP2C9	Dicrofenac 4′-hydroxylase	<0.10
CYP2C19	(*S*)-mephenytoin 4′-hydroxylase	0.24
CYP2D6	Bufuralol 1′-hydoxylase	<0.10
CYP2E1	Chlorzoxazone 6-hydroxylase	<0.10
CYP3A4	Teststerone 6β-hydroxylase	<0.10

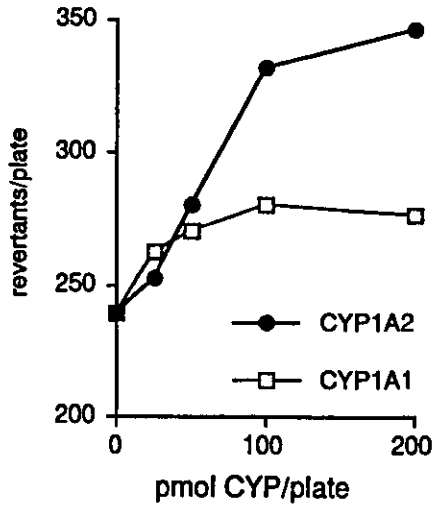

Fig. 4. The mutagenicity of 10-azaBaP (2.5 nmol per plate) in the Ames test using microsomes from baculovirus-transfected insect cells expressing recombinant human CYP1A1 or 1A2.

recombinant human CYP1A1 made a less marked contribution to the mutagenicity of 10-azaBaP than recombinant human CYP1A2.

4. Discussion

Previously one of the authors (A.H.) reported that BaP showed less mutagenicity in the Ames test with pooled human liver S9 than that with CYP1A-induced rat liver S9 [14]. Hakura et al. also reported that no direct correlation was found between the mutagenic activity of BaP in the Ames test with individual donors' human liver S9 and the CYP isozyme activities [14]. These results may be due to a low expression of CYP1A1, the principal enzyme responsible for metabolic activation of BaP, in the human liver. On the other hand, we previously demonstrated that 10-azaBaP, a 10-aza-analog of BaP, showed greater mutagenicity than BaP in the Ames test using human liver S9 [6]. Therefore there is the possibility that the CYP isoform responsible for the conversion of 10-azaBaP to the ultimate active form might be more abundant in human liver homogenates than CYP1A1, which is responsible for the conversion of BaP to the ultimate form. The purpose of the present study was to identify the CYP isoform involved in the mutagenic activation of 10-azaBaP in the Ames test using microsomes from nine human livers.

The number of induced revertants by 2.5 nmol per plate of 10-azaBaP, which produced maximal revertants per plate in the Ames test using pooled human liver microsomes, varied 42-fold (19–795) among the microsome preparations from nine human liver samples. These results suggest a large inter-individual diversity in the activity of the CYP isoform involved in the mutagenic activation of 10-azaBaP that might contribute to the variation in the number of revertants among the nine human liver microsomes preparations.

The contribution of specific CYP enzymes in human microsome samples to the mutagenicity of 10-azaBaP was investigated by correlation analysis between the CYP-catalytic activity of each microsomal sample and their mutagenic activity. The number of induced revertants was highly correlated with the activity of CYP1A2, estimated by the activities of phenacetin O-deethylase, 7-ethoxyresorufin O-deethylase, and 7-methoxyresorufin O-demethylase, and was unrelated with the activity of other CYP isoforms. Moreover, recombinant human CYP1A2 contributed to the mutagenicity of 10-azaBaP to a much higher degree than recombinant CYP1A1 in spite of as high an amino acid sequence homology as 73%. The high level of correlation between the number of induced revertants by 10-azaBaP and CYP1A2 activity and the high contribution of recombinant human CYP1A2 in 10-azaBaP mutagenicity suggest that CYP1A2 is the principal enzyme responsible for the mutagenic activation of 10-azaBaP in human liver microsomes. CYP1A1, the principal enzyme responsible for metabolic activation of BaP, is less expressed than the other isozymes in the human liver [19–21], while CYP1A2 is one of the major CYP isoforms expressed in the human liver [22]. Because of this difference in expression levels between the two CYP1A isoforms in human liver microsomes, 10-azaBaP may be more mutagenic than BaP in the Ames test using human liver homogenates (S9) [6]. With regard to a large inter-individual variation in CYP1A2 level in the human liver [22,23], the genotoxic risk of 10-azaBaP might significantly differ among individual subjects.

The metabolic activation pathway of 10-azaBaP in human liver microsomes is still unknown. We previously reported that quinoline, an aza-analog of naphthalene and one of the simplest aza-arene, may be metabolized in the pyridine moiety by the liver microsomal enzymes to the ultimate genotoxic form, enamine epoxide (*N*,4-hydrated 2,3-epoxide) [24–26], which is responsible for the mutagenic modification of DNA. There is the possibility that 10-azaBaP might be converted to mutagenic forms by metabolic activation occurring in the pyridine moiety like quinoline, and CYP1A2 might be involved in this activation pathway. Further investigation is necessary to elucidate the activation pathway of 10-azaBaP.

In conclusion, it is suggested that the nitrogen-substitution at position-10 of BaP may cause the CYP enzyme-specificity in metabolic activation to change from CYP1A1 to CYP1A2.

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In vivo transgenic mutation assays

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Abstract

Transgenic rodent gene-mutation models provide relatively quick and statistically reliable assays for gene mutations in the DNA from any tissue. This report summarizes those issues that have been agreed upon at a previous IWGT meeting [Environ. Mol. Mutagen. 35 (2000) 253], and discusses in depth those issues for which no consensus was reached before. It was previously agreed that for regulatory applications, assays should be based upon neutral genes, be generally available in several laboratories, and be readily transferable. For phage-based assays, five to ten animals per group should be analyzed, assuming a spontaneous mutant frequency (MF) of $\sim 3 \times 10^{-5}$ mutants/locus and 125,000–300,000 plaque or colony forming units (pfu or cfu) per tissue per animal. A full set of data should be generated for a vehicle control and two dose groups. Concurrent positive control animals are only necessary during validation, but positive control DNA must be included in each plating. Tissues should be processed and analyzed in a blocked design, where samples from negative control, positive control and each treatment group are processed together. The total number of pfus or cfus and the MF for each tissue and animal are reported. Statistical tests should consider the animal as the experimental unit. Nonparametric statistical tests are recommended. A positive result is a statistically significant dose–response and/or statistically significant increase in any dose group compared to concurrent negative controls using an appropriate statistical model. A negative result is a statistically non-significant change, with all mean MFs within two standard deviations of the control. During the current workshop, a general protocol was agreed in which animals are treated daily for 28 consecutive days and tissues sampled 3 days after the final treatment. This recommendation could be modified by reducing or increasing the number of treatments or the length of the treatment period, when scientifically justified. Normally male animals alone are sufficient and normally at least one rapidly proliferating and one slowly proliferating tissue should be sampled. Although, as agreed previously, sequencing data

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are not normally required, they might provide useful additional information in specific circumstances, mainly to identify and correct for clonal expansion and in some cases to determine a mechanism associated with a positive response.

1. Introduction

Subsequent to the first IWGTP workshop in Washington to discuss in vivo transgenic mutation assays [1], the working party was reconvened in order to address key issues that remained unresolved. This has resulted in the preparation of this current document, which, although based upon the original Washington report, serves to clarify important details of the treatment and sampling protocols. The detection of somatic mutation in mammals in vivo has a tremendous potential to advance our understanding of mutagenesis and of the role and source of mutations involved in cancer and, perhaps, other conditions. Unfortunately, assays involving endogenous genes are either technically challenging, as in the case of genotypic selection or PCR-based assays [2,3] or limited to particular tissues or developmental stages as in the case of phenotype-based assays [4–6]. Mutation assays using transgenic mice and rats are not subject to these limitations [7–11]. These assays permit the screening of a large number of copies of a reporter gene quickly, and thus provide statistically reliable results on the frequency of mutants/mutations in all tissues of the animal. Furthermore, these mutants can be sequenced so that the molecular nature of the mutation can be known and its origin inferred. The transgenic reporter genes have shown to be genetically neutral, which avoids the influence of in vivo selective pressures on the mutant frequency (MF), and allows the accumulation and persistence of mutations [12–14]. The possible exceptions are the plasmid-based models in which large-size-change mutants may behave differently and may not be genetically neutral. Despite the remarkable similarities in their response to mutagens and their mutation spectra compared to endogenous genes, the transgenic approach does have limitations. Transgenic reporter genes do not necessarily behave exactly the same as endogenous genes [1,15,16]. For example, they are not expressed in vivo and may be repaired differently than transcriptionally active endogenous genes. On the other hand, endogenous genes also may behave differently to each other. Mutation analysis in one endogenous gene therefore may not permit a precise extrapolation to other endogenous genes.

The availability of mouse and rat models that are able to detect gene mutations in all tissues, will not only contribute to our understanding of mutagenic and carcinogenic processes, but when used for regulatory purposes they have the potential to greatly improve cancer risk assessment. Very promising data have already been published describing the detection of mutagenicity in target tissues for which no models previously existed, such as site-of-contact tissues [17]. Good concordance with mutagenicity and carcinogenicity has also been reported [17–19]. Using a database of 155 agents, an evaluation of the ability of transgenic mouse models to predict carcinogenicity concluded that 71 out of 90 carcinogens tested gave at least one positive result in at least one transgenic mutation system (Table 1) [20]. Fourteen of the 18 non-carcinogens evaluated in these assays have produced negative results (Table 1), though the remaining four gave some positive indications [20].

Several aspects of the protocol for conducting mutagenicity assays with transgenic rodents were already agreed upon during the IWGTP meeting in Washington [1]. It was accepted that the *lacI*, *lacZ* (phage and plasmid), *cII*, and *gpt* delta assays should be considered suitable as performed under standard conditions. New versions of the assays, for example selection systems for *lacI* and *Spi*$^-$ [21–25], are now considered sufficiently developed to be regarded as standard assays. These include assays that detect not only intragenic mutations but also those which encompass flanking target gene copies or endogenous sequences (e.g. *lacZ* plasmid). In the present document, however, we will be focussing on assays for mutation within single genes. The limitations of the tests were discussed, the types of mutations detected by such systems were briefly described, and the sensitivity of the systems reviewed. It was agreed that, where available, either the mouse or the rat should be considered, as appropriate. There was agreement that a full set of data

Table 1
Summary of transgenic rodent assay results for carcinogens and non-carcinogens

Chemical	CAS number	Transgenic rodents[a]	Carcinogenicity
1,2-Dibromoethane	106-93-4	+	+
1,3-Butadiene	106-99-0	+	+
1,2:3,4-Diepoxybutane	1464-53-5	–	+
1,8-Dinitropyrene	42397-65-9	+	+
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin	1746-01-6	–	+
2,4-Diaminotoluene	95-80-7	+	+
2,6-Diaminotoluene	823-40-5	–	–
2-Acetylaminofluorene	53-96-3	+	+
2-Nitro-*p*-phenylenediamine	5307-14-2	+	+
3-Fluoroquinoline	396-31-6	–	–
3-Methylcholanthrene	56-49-5	+	+
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)	64091-91-4	+	+
4-Acetylaminofluorene	28322-02-3	+	–
4-Aminobiphenyl	92-67-1	+	+
4-Chloro-*o*-phenylenediamine	95-83-0	+	+
4-Nitroquinoline-*N*-oxide (4-NQO)	56-57-5	+	+
5-(*p*-Dimethylaminophenylazo)benzothiazole		+	–
5,9-Dimethyldibenzo[*c,g*]carbazole (DMDBC)		+	+
5-Bromo-2′-deoxyuridine	59-14-3	–	+
5-Fluoroquinoline	394-69-4	+	+
6-(*p*-Dimethylaminophenylazo)benzothiazole		+	+
7,12-Dimethylbenzanthracene	57-97-6	+	+
7*H*-Dibenzo[*c,g*]carbazole (DBC)	194-59-2	+	+
A-alpha-C	26148-68-5	+	+
Acetic acid	64-19-7	+	–
Acetone	67-64-1	–	–
Acrylamide	79-06-1	+	+
acrylonitrile	107-13-1	–	+
Aflatoxin B1	1162-65-8	+	+
Agaritine	2757-90-6	+	+
All-*trans*-retinol	68-26-8	–	–
Aristolochic acid	10190-99-5	+	+
Arsenite trioxide	1327-53-3	–	+
Azathioprine	446-86-6	+	+
Benzene	71-43-2	+	+
Benzo(*a*)pyrene	50-32-8	+	+
Beta-propiolactone	57-57-8	+	+
Carbon tetrachloride	56-23-5	–	+
CC-1065	69866-21-3	+	+
Chlorambucil	305-03-3	+	+
Chloroform	67-66-3	–	+
Coal tar	8007-45-2	+	+
Crocodolite asbestos	12001-28-4	+	+
Cyclophosphamide	50-18-0	+	+
Cyproterone acetate	427-51-0	+	+
Di(2-ethylhexyl)pthalate	117-81-7	–	+
Dichloroacetic acid	79-43-6	+	+
Diesel exhaust		+	+
Diethylnitrosamine (DEN)	55-18-5	+	+
D-Limonene	5989-27-5	–	+
Dimethylarsinic acid	75-60-5	–	+
Dimethylnitrosamine (DMN)	62-75-9	+	+
Dipropylnitrosamine (DPN)	621-64-7	+	+

Table 1 (*Continued*)

Chemical	CAS number	Transgenic rodents[a]	Carcinogenicity
Ethylene oxide	75-21-8	+	+
Ethylmethanesulfonate	62-50-0	+	+
Eugenol	97-53-0	–	–
Folic acid	59-30-3	–	–
Gamma-rays		+	+
Heptachlor	76-44-8	–	+
Hexachlorobutadiene	87-68-3	+	+
Hexavalent chromium	7440-47-3	+	+
Hydrazine sulfate	10034-93-2	–	+
2-Amino-3-methylimidazo [4,5-f] quinoline (IQ)	76180-96-6	+	+
Isopropylmethanesulfonate	926-06-7	+	+
2-Amino-3,4-dimethylimidazo [4,5-b] quinoline (MeIQ)	77094-11-2	+	+
2-Amino-3,8-dimethylimidazo [4,5-f] quinoxaline (MeIQx)	77500-04-0	+	+
Methyl bromide	74-83-9	–	+
Methyl clofenapate	21340-68-1	–	+
Methylmethanesulfonate	66-27-3	+	+
Metronidazole	443-48-1	–	+
Mitomycin-C	50-07-7	+	+
*N*7-methyldibenzo[*c*,*g*]carbazole (NMDBC)	27093-62-5	+	+
N-ethyl-*N*-nitrosourea	759-73-9	+	+
N-hydroxy-2-AAF	53-95-2	+	+
Nickel subsulfide	12035-72-2	–	+
N-methyl-*N*′-nitro-*N*-nitrosoguanidine	70-25-7	+	+
N-methyl-*N*-nitrosourea	684-93-5	+	+
N-nitrosonornicotine (NNN)	80508-23-2	+	+
N-propyl-*N*-nitrosourea	816-57-9	+	+
o-Aminoazotoluene	97-56-3	+	+
o-Anisidine	134-29-2	+	+
Oxazepam	604-75-1	+	+
p-Cresidine	120-71-8	+	+
Phenobarbital	50-06-6	+	+
PhIP	105650-23-5	+	+
Phorbol-12-myristate-13-acetate (TPA)	16561-29-8	–	+
Procarbazine.HCl	366-70-1	+	+
Quinoline	91-22-5	+	+
Sodium saccharin	128-44-9	–	+
Streptozotocin	18883-66-4	+	+
Sucrose	57-50-1	+	–
Tamoxifen	10540-29-1	+	+
Thio-TEPA	52-24-4	+	+
Toremifene citrate	89778-26-7	–	–
Trans-4-hydroxy-2-nonenal	29343-52-0	–	–
Trichloroethylene	79-01-6	–	+
Tris-(2,3-dibromopropyl)phosphate	126-72-7	+	+
Uracil	66-22-8	+	+
Urethane	51-79-6	+	+
UVB		+	+
Wyeth 14,643	50892-23-4	+	+
X-ray		+	+

Data have been extracted from reference [20].

[a] The specified chemical/radiation is positive (+) in the transgenic rodent assay when at least one positive result has been obtained, and negative (–) when all experiments carried out to date have produced negative results.