tential of $P1H_2$ that is higher than the one-electron reduction potential of cumylperoxyl radical ($E_{red}{}^0$ = 0.65 V vs SCE) (63), the free energy changes of electron transfer from $P1H_2$ to cumylperoxyl radical are positive [$\Delta G_{et}{}^0$ (in eV) = $e(E_{ox}{}^0 - E_{red}{}^0) > 0$, where e is elementary charge]; thereby, the electron transfer step is endergonic. In such a case, the initial electron transfer rate (k_{et}) may be the rate-determining step in the overall rate of hydrogen transfer, which consists of electron and proton transfer steps. The maximum k_{et} value is evaluated from the $\Delta G_{et}{}^0$ value by eq 1, where it is assumed that the activation free energy ($\Delta G_{et}{}^{\ddagger}$) is equal to $\Delta G_{et}{}^0$ (no additional barrier is involved), Z is the frequency factor taken as 1×10^{11} M^{-1} s^{-1}, and k_B is the Boltzmann constant (64, 65).

$$k_{et} = Z \exp(-\Delta G_{et}{}^0/k_B T) \qquad (1)$$

The maximum k_{et} value is calculated as 1.2×10^2 M^{-1} s^{-1}, which is the same order of magnitude as the observed k_{HT} value (9.0×10^2 M^{-1} s^{-1}). The larger k_{HT} value than the k_{et} value indicates that the hydrogen transfer from $P1H_2$ to cumylperoxyl radical proceeds via a rate-determining electron transfer with an interaction between $P1H_2$ and cumylperoxyl radical. The formation of charge transfer complexes between cumylperoxyl radical and a variety of electron acceptors has been well-documented in the literature (66, 67). Thus, the hydrogen transfer may proceed via an inner-sphere electron transfer in the charge transfer complex formed between $P1H_2$ and cumylperoxyl radical. The acceleration of the hydrogen transfer rate in the presence of Sc^{3+} (Figure 3) is ascribed to the promoting effect of Sc^{3+} on the electron transfer step due to the strong binding of Sc^{3+} with cumylperoxyl anion produced in the electron transfer.

In conclusion, the hydrogen transfer from $P1H_2$ to cumylperoxyl radical generated in radical chain reactions proceeds via an electron transfer reaction and the rate of hydrogen transfer from $P1H_2$ to cumylperoxyl radical is faster than that from $1H_2$. The predominance of $P1H_2$ in the hydrogen transfer reaction is consistent with the electrochemical case for its one-electron oxidation potential. Since $P1H_2$ is very lipophilic as compared to (+)-catechin itself, it is proposed that $P1H_2$ interacts and penetarates the lipid bilayer giving rise to its maximized antioxidant capacity. Therefore, we believe that $P1H_2$ may be significantly more effective not only for protecting tissue from the onslaught of the radical species governing peroxidation but also for terminating the autoxidation, which plays in provoking diseased states. Studies are underway to investigate basic biochemical properties of $P1H_2$ in vivo, as well as to investigate its ability to serve as an antioxidant for the treatment of diseases associated with oxidative stress.

Acknowledgment. This work was partially supported by a Grant-in-Aid for Scientific Research Priority Area (No. 11228205) and a Grant-in-Aid for Young Scientist (B) (No. 15790032) from the Ministry of Education, Culture, Sports, Science and Technology, Japan, partly by a Grant (MF-16) from the Organization for Pharmaceutical Safety and Research, and by a Grant-in-Aid for the Scientific Research (No. 14141201) from the Ministry of Health Labor and Welfare.

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Mutation Research 559 (2004) 83–95

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In vivo mutagenicity of benzo[*f*]quinoline, benzo[*h*]quinoline, and 1,7-phenanthroline using the *lacZ* transgenic mice

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Received 25 September 2003; received in revised form 26 December 2003; accepted 26 December 2003

Abstract

Phenanthrene, a simplest angular polycyclic aromatic hydrocarbon with a bay-region in its molecule, is reported to be non-mutagenic, although most angular (non-linear) polycyclic aromatic hydrocarbons, such as benzo[*a*]pyrene and chrysene, are known to show genotoxicity after metabolic transformation into a bay-region diol epoxide. On the other hand, benzo[*f*]quinoline (BfQ), benzo[*h*]quinoline (BhQ), and 1,7-phenanthroline (1,7-Phe), which are all aza-analogs of phenanthrene, are mutagenic in the Ames test using *Salmonella typhimurium* TA100 in the presence of a rat liver S9 fraction. In this report, we undertook to investigate the in vivo mutagenicity of BfQ, BhQ and 1,7-Phe by an in vivo mutation assay system using the *lacZ* transgenic mouse (Muta^{TM}Mouse). BfQ and BhQ only slightly induced mutation in the liver and lung, respectively. BfQ- and BhQ-induced *cII* mutant spectra showed no characteristics compared with that of the control. These results suggest that the in vivo mutagenicities of BfQ and BhQ were equivocal. On the other hand, 1,7-Phe induced a potent mutation in the liver and a weak mutation in the lung. Furthermore 1,7-Phe depressed the G:C to A:T transition and increased the G:C to C:G transversion in the liver like quinoline, a hepatomutagen possessing the partial structure of 1,7-Phe, compared with the spontaneous mutation spectrum. These results suggest that the in vivo mutagenicity of 1,7-Phe might be caused by the same mechanism as that of quinoline, which induced the same mutational spectrum change (G:C to C:G transversion).
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Keywords: Tricyclic aza-arene; In vivo mutagenesis assay; Mutation spectrum

1. Introduction

Carcinogenic aza-arenes are widely distributed in the environmental pollutants such as cigarette smoke [1] and urban air [2–4]. Although numerous studies about the in vitro mutagenicity of aza-arenes have been reported, the metabolic activation mechanism of aza-arenes has not yet been elucidated, except for that of heterocyclic amines. Furthermore, there are only a few reports about the in vivo mutagenicity of aza-arenes. We have investigated the in vitro and in vivo mutagenicity of aza-arenes with special attention to their metabolic activation mechanisms. 10-Azabenzo[*a*]pyrene, a carcinogenic aza-analog [5] of benzo[*a*]pyrene, was reported to be as mutagenic as benzo[*a*]pyrene in the Ames test using *Salmonella typhimurium* TA100 in the presence of a rat liver S9 fraction [6–8]. In our previous study,

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doi:10.1016/j.mrgentox.2003.12.012

Fig. 1. Structures of BfQ, BhQ and 1,7-Phe.

10-azabenzo[a]pyrene showed signi. cant mutagenicity only in the liver and colon in an in vivo mutation assay system using the *lacZ* transgenic mouse (Muta™Mouse) [9]. We have also reported that the total dose of 200 mg/kg (50 mg/kg per day × 4 days) of quinoline, a hepatocarcinogenic [10,11] aza-analog of naphthalene, showed a potent mutagenicity and induced primarily G:C to C:G transversions in the liver of Muta™Mouse [12–14].

Phenanthrene, a simplest angular polycyclic aromatic hydrocarbon with a bay-region in its molecule, has been reported to be non-mutagenic [15], although most angular (non-linear) polycyclic aromatic hydrocarbons, such as benzo[a]pyrene and chrysene, are known to show genotoxicity after metabolic transformation into a bay-region diol epoxide. On the other hand, it was reported that benzo[f]quinoline (BfQ) and 1,7-phenanthroline (1,7-Phe) (Fig. 1), which are environmental contaminants and aza-analogs of phenanthrene, were mutagenic in the Ames test using *S. typhimurium* TA100 in the presence of a rat liver S9 fraction [16–18]. Furthermore, benzo[h]quinoline (BhQ) (Fig. 1), a positional isomer of BfQ, was reported to be weakly or equivocally mutagenic in *S. typhimurium* TA100 with a rat liver S9 fraction [19,20]. In our previous report, it was suggested that metabolic activation of these tricyclic aza-arenes might take place in the pyridine moiety, like quinoline, to form the ultimate genotoxic form, an enamine epoxide (*N*,d-hydrated a,b-epoxide) (Fig. 2) [18].

In the present study, we undertook to investigate the in vivo mutagenicity of BfQ, BhQ and 1,7-Phe by the in vivo mutation assay system using the *lacZ* transgenic mouse (Muta™Mouse).

2. Materials and methods

2.1. Materials

BfQ (CAS Registry No. 85-02-9) and BhQ (CAS Registry No. 230-27-3) were purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo), 1,7-Phe (CAS Registry No. 230-46-6) from Aldrich, phenyl-β-D-galactoside (P-gal) from Sigma Chemical Co. (St. Louis, MO, USA), proteinase K and olive oil from Wako Pure Chemicals (Osaka), and RNase from Boeringer Mannheim.

2.2. In vivo mutagenesis assay using Muta™Mouse

2.2.1. Animals and treatments

Seven-week-old male Muta™Mice, supplied by COVANCE Research Products (PA, USA), were acclimatized for 1 week before use and divided into seven groups of four mice each. BfQ, BhQ, and 1,7-Phe dissolved in olive oil (10 ml/kg body weight) were injected intraperitoneally into two, one, and two groups, respectively, at single doses of 100, 100, and 50 mg/kg, respectively, for four consecutive days. The remaining two groups were given olive oil as the control.

2.2.2. Tissues and DNA isolation

All mice were killed by cervical dislocation 14 days (BfQ-, BhQ-, 1,7-Phe- and olive oil-treated groups) or 56 days (BfQ-, 1,7-Phe- and olive oil-treated groups) after the last injection of a test chemical. The liver, spleen, lung, kidney, and bone marrow were immedi-

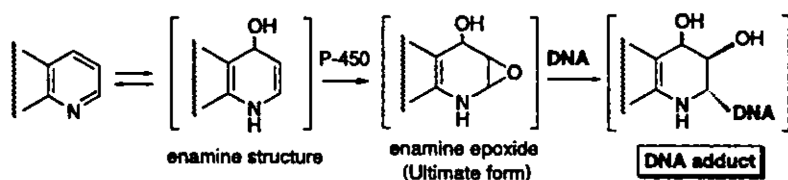

Fig. 2. Proposed metabolic activation pathway for the pyridine moiety (enamine epoxide theory).

ately extirpated, frozen in liquid nitrogen, and stored at −80 °C until DNA extraction. The genomic DNA was extracted from each tissue by the phenol/chloroform method as previously reported [12]. The isolated DNA was precipitated with ethanol, air-dried, and dissolved in an appropriate volume (20–200 μl) of TE-4 buffer (10 mM Tris–HCl at pH 8.0 containing 4 mM EDTA) at room temperature overnight. The DNA solution thus prepared was stored at 4 °C.

2.2.3. In vitro packaging

The lambda gt10/*lacZ* vector was ef. ciently recovered by the in vitro packaging reactions [21]. Our home-made (HM) packaging extract consisting of a sonic extract (SE) of *Escherichia coli* NM759 and a freeze–thaw lysate (FTL) of *E. coli* BHB2688 was prepared according to the methods of Gunther et al. [22]. As the general procedure for handling the HM extract, approximately 5 μg DNA was mixed with 15 μl of FTL and 30 μl of SE and incubated at 37 °C for 90 min. Then the SE and FTL were combined again and the mixture was incubated for another 90 min. The reaction was terminated by the addition of an appropriate volume of SM buffer (50 mM Tris–HCl at pH 7.5, 10 mM $MgSO_4$, 100 mM NaCl, 0.01% gelatin) and stored at 4 °C. By this procedure, the λgt10 vector was ef. ciently rescued from genomic DNA to form an infectious phage.

2.2.4. Mutation assays

2.2.4.1. lacZ mutant frequency determination. The positive selection for *lacZ* mutants was performed as previously reported [12,23]. Brie. y, the phage solution was absorbed to *E. coli* C (lac^- $galE^-$) at room temperature for 20–30 min. For titration, an aliquot of the phage-*E. coli* solution was mixed with LB top agar (containing 10 mM $MgSO_4$) and plated onto dishes containing bottom agar. The remaining phage-*E. coli* solution was mixed with LB top agar containing phenyl-β-D-galactoside (P-gal) (3 mg/ml) and plated as described above. The mutant frequency (MF) was calculated by the following formula:

$$\text{mutant frequency} = \left(\frac{\text{total number of plaques on selection plates}}{\text{total number of plaques on titer plates}}\right) \times \text{dilution factor}.$$

The signi. cance of differences in the mutant frequency between the treated and control groups was analyzed by using Student's *t*-test.

2.2.4.2. cII mutant frequency determination. We also examined the mutagenicity in the lambda *cII* gene integrated as a lambda vector gene, which serves as another selective marker as reported previously in the *lacI* transgenic BigBlue mouse [24]. The positive

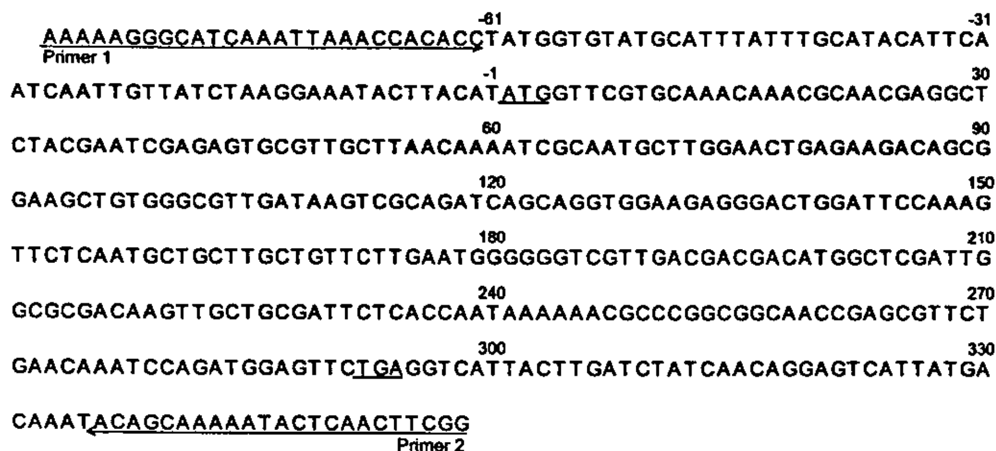

Fig. 3. Sequence map of the *cII* gene. The primers, used for PCR ampli. cation and sequencing, are shown by arrows. The PCR gives 446 bp products that involve the entire (294 bp) *cII* gene. Initiation and stop codons are underlined.

Table 1
Mutant frequencies induced by BfQ, BhQ and 1,7-Phe in . ve organs of Muta™Mouse for the expression time of 14 days

Tissue	Treatment	*lacZ* assay				*cII* assay			
		Individual animal data			Average ± S.D.	Individual animal data			Average ± S.D.
		No. of phages analyzed	No. of mutants	MF × 10^5	MF × 10^5	No. of phages analyzed	No. of mutants	MF × 10^5	MF × 10^5
Liver	Control (olive oil)	1120000	106	9.5	7.0 ± 1.6	449400	12	2.7	2.1 ± 0.6
		816000	59	7.2		938400	24	2.6	
		1198000	73	6.1		764400	11	1.4	
		791500	41	5.2		699000	11	1.6	
	BfQ	634500	32	5.0	9.6 ± 3.1	804600	12	1.5	3.8 ± 1.6
		590000	51	8.6		662400	35	5.3	
		158500	20	12.6		426300	23	5.4	
		221500	27	12.2		883200	28	3.2	
	BhQ	442000	29	6.6	5.9 ± 1.1	1188000	53	4.5	2.6 ± 1.1
		677000	39	5.8		1134600	21	1.9	
		257500	11	4.3		671700	17	2.5	
		645500	46	7.1		1011000	15	1.5	
	1,7-Phe	272000	41	15.1	15.9 ± 0.5**	813600	44	5.4	4.1 ± 0.8*
		183000	30	16.4		562800	19	3.4	
		263000	43	16.3		720000	25	3.5	
		184000	29	15.8		606000	25	4.1	
Spleen	Control (olive oil)	855500	116	13.6	7.3 ± 3.6	623100	12	1.9	2.4 ± 0.3
		533000	29	5.4		1502400	41	2.7	
		446500	25	5.6		546900	15	2.7	
		461000	22	4.8		569400	13	2.3	
	BfQ	210500	13	6.2	6.1 ± 0.6	2098200	25	1.2	3.5 ± 2.8
		244500	16	6.5		441000	13	2.9	
		403000	27	6.7		785400	65	8.3	
		256500	13	5.1		786600	13	1.7	
	BhQ	297000	12	4.0	6.5 ± 1.6	277800	10	3.6	2.9 ± 0.6
		354500	25	7.1		828300	22	2.7	
		396500	26	6.6		946200	31	3.3	
		544000	46	8.5		1608600	34	2.1	
	1,7-Phe	426500	34	8.0	7.0 ± 1.0	967200	20	2.1	2.4 ± 0.7
		502500	27	5.4		1023000	24	2.3	
		320000	24	7.5		1026900	16	1.6	
		462500	34	7.4		905400	32	3.5	
Lung	Control (olive oil)	1539500	127	8.2	6.0 ± 1.3	1027800	21	2.0	2.1 ± 0.4
		1111500	60	5.4		738000	21	2.8	
		678000	35	5.2		1142700	20	1.8	
		1473000	76	5.2		831600	15	1.8	
	BfQ	553000	39	7.1	6.0 ± 0.6	1107600	18	1.6	2.6 ± 0.6
		332000	18	5.4		903300	22	2.4	
		353000	21	5.9		1124700	36	3.2	
		266000	15	5.6		445200	14	3.1	
	BhQ	401500	51	12.7	10.8 ± 2.4*	1705500	37	2.2	3.5 ± 1.0
		481500	54	11.2		1071000	33	3.1	
		572500	72	12.6		2403000	99	4.1	
		372000	25	6.7		2083200	98	4.7	

Table 1 (*Continued*)

Tissue	Treatment	*lacZ* assay				*cII* assay			
		Individual animal data			Average ± S.D.	Individual animal data			Average ± S.D.
		No. of phages analyzed	No. of mutants	MF × 10^5	MF × 10^5	No. of phages analyzed	No. of mutants	MF × 10^5	MF × 10^5
	1,7-Phe	335500	29	8.6	10.3 ± 1.9*	1103400	26	2.4	2.9 ± 0.5
		351000	46	13.1		1012200	27	2.7	
		244500	27	11.0		909600	34	3.7	
		211000	18	8.5		892200	26	2.9	
Kidney	Control (olive oil)	219500	15	6.8	6.8 ± 1.4	551100	21	3.8	2.7 ± 1.0
		190000	17	8.9		426600	16	3.8	
		349500	17	4.9		588000	11	1.9	
		301000	20	6.6		771000	12	1.6	
	BfQ	682500	46	6.7	7.4 ± 1.1	1035000	25	2.4	3.9 ± 0.9
		550500	51	9.3		825000	36	4.4	
		474000	33	7.0		649800	30	4.6	
		484000	32	6.6		1599000	66	4.1	
	BhQ	920500	55	6.0	7.4 ± 1.2	1323600	26	2.0	2.2 ± 0.5
		622000	51	8.2		945000	27	2.9	
		113000	10	8.8		1408800	23	1.6	
		244500	16	6.5		1018200	23	2.3	
	1,7-Phe	486500	30	6.2	6.8 ± 0.5	814800	15	1.8	3.3 ± 1.6
		558000	38	6.8		660300	40	6.1	
		177000	12	6.8		520200	13	2.5	
		319500	24	7.5		1664700	48	2.9	
Bone marrow	Control (olive oil)	311000	32	10.3	7.1 ± 3.0	644100	14	2.2	1.3 ± 0.6
		465000	27	5.8		1041000	16	1.5	
		70500	2	2.8		111300	1	0.9	
		96500	9	9.3		154500	1	0.6	
	BfQ	325500	17	5.2	6.4 ± 0.9	1075200	16	1.5	2.6 ± 0.9
		256500	17	6.6		528000	12	2.3	
		326000	25	7.7		572100	22	3.8	
		708500	44	6.2		1144800	32	2.8	
	BhQ	257000	13	5.1	5.7 ± 0.5	1757100	20	1.1	1.7 ± 0.4
		617000	38	6.2		1349400	24	1.8	
		683000	41	6.0		1040400	22	2.1	
	1,7-Phe	502500	24	4.8	4.7 ± 0.5	963600	12	1.2	1.6 ± 0.6
		397500	19	4.8		962400	11	1.1	
		622000	33	5.3		1341000	21	1.6	
		332000	13	3.9		916900	24	2.6	

* Signi. cantly different from the control group, $P < 0.05$.
** Signi. cantly different from the control group, $P < 0.01$.

selection for *cII* mutants was performed according to the method of Jakubczak et al. [24] with slight modi- . cation as previously reported [14]. Brie. y, the phage solution was absorbed to *E. coli* G1225 (*h.* $^{-}$) at room temperature for 20–30 min. For titration, an appropriately diluted phage-*E. coli* solution was mixed with LB top agar (containing 10 mM $MgSO_4$), plated onto dishes containing bottom agar, and incubated for 24 h at 37 °C. The remaining phage-*E. coli* solution was mixed with LB top agar and plated onto dishes con-

Table 2
Mutant frequencies induced by BfQ and 1,7-Phe in . ve organs of Muta™Mouse for the expression time of 56 days

Tissue	Treatment	*lacZ* assay				*cII* assay			
		Individual animal data			Average ± S.D.	Individual animal data			Average ± S.D.
		No. of phages analyzed	No. of mutants	MF × 10^5	MF × 10^5	No. of phages analyzed	No. of mutants	MF × 10^5	MF × 10^5
Liver	Control (olive oil)	246500	21	8.5	7.9 ± 1.3	960700	18	1.9	1.7 ± 0.2
		168500	12	7.1		1161000	21	1.8	
		636500	39	6.1		3351900	65	1.9	
		155500	15	9.6		1978900	27	1.4	
	BfQ	259000	24	9.3	11.5 ± 2.8	543600	14	2.6	2.4 ± 0.2**
		367000	35	9.5		2746500	60	2.2	
		714000	116	16.2		3693000	97	2.6	
		180500	20	11.1		2490600	57	2.3	
	1,7-Phe	653000	63	9.6	14.8 ± 3.7*	1468200	64	4.4	4.8 ± 1.2**
		266500	35	13.1		1140000	36	3.2	
		497000	94	18.9		4469100	286	6.4	
		126000	22	17.5		306600	16	5.2	
Spleen	Control (olive oil)	608500	47	7.7	7.8 ± 0.4	1825800	53	2.9	2.9 ± 0.6
		347500	26	7.5		1304400	48	3.7	
		355500	30	8.4		1224600	36	2.9	
		389500	29	7.4		1106400	22	2.0	
	BfQ	440000	38	8.6	8.4 ± 0.3	2245800	32	1.4	3.3 ± 2.5
		242500	21	8.7		860400	14	1.6	
		354000	30	8.5		1090800	83	7.6	
		460500	36	7.8		946200	24	2.5	
	1,7-Phe	567000	81	14.3	10.0 ± 2.5	1022400	22	2.2	2.2 ± 0.04
		231500	18	7.8		976800	22	2.3	
		336000	29	8.6		1059000	23	2.2	
		253500	24	9.5		865800	19	2.2	
Lung	Control (olive oil)	390500	25	6.4	7.9 ± 1.9	657600	13	2.0	3.1 ± 0.9
		218500	21	9.6		1230600	30	2.4	
		558500	32	5.7		936000	35	3.7	
		474500	47	9.9		928800	38	4.1	
	BfQ	332500	26	7.8	7.2 ± 1.2	742200	19	2.6	3.7 ± 1.9
		554500	43	7.8		1035000	25	2.4	
		476000	39	8.2		839400	59	7.0	
		386000	20	5.2		651000	17	2.6	
	1,7-Phe	731500	56	7.7	7.7 ± 0.9	1365600	25	1.8	3.0 ± 0.9
		412000	32	7.8		728400	29	4.0	
		494500	44	8.9		966600	26	2.7	
		519000	33	6.4		946800	35	3.7	
Kidney	Control (olive oil)	442500	26	5.9	8.2 ± 2.4	1874400	64	3.4	2.5 ± 0.6
		217000	26	12.0		2313600	43	1.9	
		383000	25	6.5		1139400	25	2.2	
		596500	50	8.4		1437600	39	2.7	
	BfQ	552500	41	7.4	7.1 ± 1.0	1629600	60	3.7	2.9 ± 0.7
		479000	39	8.1		1360800	33	2.4	
		774500	56	7.2		1446000	50	3.5	
		698500	38	5.4		1277400	27	2.1	

Table 2 (*Continued*)

Tissue	Treatment	*lacZ* assay				*cII* assay			
		Individual animal data			Average ± S.D.	Individual animal data			Average ± S.D.
		No. of phages analyzed	No. of mutants	MF × 10^5	MF × 10^5	No. of phages analyzed	No. of mutants	MF × 10^5	MF × 10^5
	1,7-Phe	299500	19	6.3	7.3 ± 1.1	1201800	26	2.2	2.2 ± 0.3
		567500	50	8.8		1393200	35	2.5	
		877500	70	8.0		1409400	35	2.5	
		513500	32	6.2		1060200	19	1.8	
Bone marrow	Control (olive oil)	607500	43	7.1	7.9 ± 1.2	1334700	21	1.6	1.9 ± 0.4
		829000	57	6.9		1204800	25	2.1	
		924500	70	7.6		1441800	23	1.6	
		605500	60	9.9		1184400	30	2.5	
	BfQ	429000	26	6.1	6.9 ± 2.1	1703400	19	1.1	3.9 ± 4.1
		661500	69	10.4		1287600	15	1.2	
		893000	47	5.3		1256400	136	10.8	
		791500	45	5.7		1249200	29	2.3	
	1,7-Phe	605500	92	15.2	9.0 ± 3.8	1206600	13	1.1	1.5 ± 0.3
		447500	39	8.7		1700400	22	1.3	
		507000	30	5.9		876000	14	1.6	
		1188000	71	6.0		1444200	28	1.9	

* Signi. cantly different from the control group, $P < 0.05$.
** Signi. cantly different from the control group, $P < 0.01$.

taining bottom agar. The plates were incubated for 48 h at 25 °C for selection of *cII* mutants. The wild type phage, recovered from Muta™Mouse, has a cI^- phenotype, which permits plaque formation with the *h.* $^-$ strain at 37 °C but not at 25 °C. The mutant frequency was calculated by the following formula:

$$\text{mutant frequency} = \left(\frac{\text{total number of plaques on selection plates}}{\text{total number of plaques on titer plates}}\right) \times \text{dilution factor}.$$

The signi. cance of differences in the mutant frequency between the treated and control groups was analyzed by using Student's *t*-test.

2.2.5. Sequencing of mutants

The entire lambda *cII* region was ampli. ed directly from mutant plaques by the use of Taq DNA polymerase (Takara Shuzo, Tokyo, Japan) with primers P1; 5′-AAAAAGGGCATCAAATTAAACC-3′, and P2; 5′-CCGAAGTTGAGTATTTTTGCTGT-3′ as previously reported [14] (Fig. 3). A 446 bp PCR product was puri. ed with a microspin column (Amersham Pharmacia, Tokyo, Japan) and then used for a sequencing reaction with the Ampli Taq cycle sequencing kit (PE Biosystems, Tokyo, Japan) using the primer P1. The reaction product was puri. ed by ethanol precipitation and analyzed with the ABI PRISM™ 310 genetic analyzer (PE Biosystems).

3. Results

3.1. Mutant frequency of BfQ, BhQ, and 1,7-Phe

BfQ, BhQ, and 1,7-Phe (Fig. 1) were tested for in vivo mutagenicity using *lacZ* transgenic mice (Muta™Mice). The mutant frequencies observed in the DNA preparations extracted from the . ve organs are shown in Tables 1 and 2. Over 10 mutant plaques were analyzed in most organs. For the bone marrow in Table 1, the mutant frequency of one animal in the BhQ-treated group was missing and the number of mutants in two animals in the control group was insuf. cient because the isolated DNA was not enough

to be analyzed. The spontaneous mutant frequencies observed in the control group were similar for the . ve organs in both *lacZ* and *cII* assays regardless of the expression time (14 or 56 days), the rate ranging from 6.0 to 8.2×10^{-5} and from 1.3 to 3.1×10^{-5}, respectively. These results were similar to those of our previous studies [9,12–14].

Table 1 shows mutant frequencies with the test compounds in the . ve organs 14 days after the last injection. BfQ slightly, but not signi. cantly, increased the mutant frequency in the liver in both assays. On the other hand, BhQ signi. cantly increased the mutant frequency in the lung in the *lacZ* assay. 1,7-Phe signi. cantly increased the mutant frequency in the liver in both assays and in the lung in the *lacZ* assay.

Mutant frequencies observed in the DNA preparations extracted from the . ve organs 56 days after the last injection are shown in Table 2. BfQ signi. cantly increased the mutant frequency in the liver in the *cII* assay, whereas the mutant frequency in the *lacZ* assay was slightly, but not signi. cantly, increased. 1,7-Phe signi. cantly increased the mutant frequency in the liver in both assays like the results obtained 14 days

Table 3
Sequences of *cII* mutations in the liver of BfQ-treated Muta™Mouse for the expression time of 14 days

Mutant no.	Position	Mutation	Sequence	Amino acid change
A1	113	C to T	AAG TCG CAG	Ser to Leu
A2	99–100	GG to TT	GTG GGC GTT	Gly to Cys
A3	107	A to C	GTT GAT AAG	Asp to Ala
A4	57	C to G	CTT AAC AAA	Asn to Lys
A5	214	C to T	GCG CGA CAA	Arg to Stop
A6	181	G to T	TGG GGG GTC	Gly to Trp
A7	34	C to T	CTA CGA ATC	Arg to Stop
A8	103	G to A	GGC GTT GAT	Val to Ile
A9	196	G to T	GAC GAC ATG	Asp to Tyr
A10	129	G to C	AGG TGG AAG	Trp to Cys
A11	34	C to T	CTA CGA ATC	Arg to Stop
A12	25	G to A	AAC GAG GCT	Glu to Lys
A13	241–246	−A	AAA AAA CGC	Frameshift
A14	179–184	−G	TGG GGG GTC	Frameshift
A15	57	C to A	CTT AAC AAA	Asn to Lys
A16	35	G to T	CTA CGA ATC	Arg to Leu
A17	179–184	+G	TGG GGG GTC	Frameshift
A18	90–91	GG to TT	GCG GAA GCT	Glu to Stop
A19	94	G to T	GAA GCT GTG	Ala to Ser
A20	115	C to T	TCG CAG ATC	Gln to Stop
A21	193	G to A	GAC GAC GAC	Asp to Asn
A22	64	G to A	ATC GCA ATG	Ala to Thr
A23	103	G to A	GGC GTT GAT	Val to Ile
A24	104	T to C	GGC GTT GAT	Val to Ala
A25	89	C to T	ACA GCG GAA	Ala to Val
A26[a]	64	G to A	ATC GCA ATG	Ala to Thr
A27	175	G to T	CTT GAA TGG	Glu to Stop
A28	25	G to A	AAC GAG GCT	Glu to Lys
A29	34	C to T	CTA CGA ATC	Arg to Stop
A30	100	G to A	GTG GGC GTT	Gly to Ser
A31	62	T to C	AAA ATC GCA	Ile to Thr
A32[a]	25	G to A	AAC GAG GCT	Glu to Lys
A33	196	G to A	GAC GAC ATG	Asp to Asn
A34	179–184	−G	TGG GGG GTC	Frameshift
A35	115	C to A	TCG CAG ATC	Gln to Lys
A36	134	G to C	AAG AGG GAC	Arg to Thr

[a] Ascribable to the same mutation obtained in an identical mouse.

after the last injection. 1,7-Phe did not increase the mutant frequency in the lung for the expression time of 56 days.

3.2. Mutation spectra of BfQ, BhQ, and 1,7-Phe-induced mutations

A total of 36 BfQ-induced mutants in the liver for the expression time of 14 days, 37 BhQ-induced mutants in the lung for 14 days, and 43 1,7-Phe-induced mutants in the liver for 56 days were subjected to sequence analysis. The mutations are characterized in Tables 3–5, and summarized in Table 6. In Table 6, the same mutations in an identical mouse were treated as single events. The data of the spontaneous mutations are from our previous report [9].

1,7-Phe-induced mutations consisted mainly of base substitutions (36/39); G:C to A:T transitions (15/39) and G:C to C:G transversions (10/39) predominated. Compared with the spontaneous mutation spectrum, G:C to A:T transitions decreased and G:C to C:G transversions increased in the mutations by

Table 4
Sequences of *cII* mutations in the lung of BhQ-treated Muta^{TM}Mouse for the expression time of 14 days

Mutant no.	Position	Mutation	Sequence	Amino acid change
B1	196	G to A	GAC *GAC* ATG	Asp to Asn
B2	179–184	+G	*TGG GGG G*TC	Frameshift
B3	149	A to T	CCA A*A*G TTC	Lys to Met
B4	241–246	−A	*AAA AAA* CGC	Frameshift
B5	34	C to T	CTA *C*GA ATC	Arg to Stop
B6	113	C to T	AAG T*C*G CAG	Ser to Leu
B7	215	G to T	GCG C*G*A CAA	Arg to Leu
B8[a]	34	C to T	CTA *C*GA ATC	Arg to Stop
B9	166	G to C	CTT *G*CT GTT	Ala to Pro
B10	25	G to A	AAC *G*AG GCT	Glu to Lys
B11	34	C to T	CTA *C*GA ATC	Arg to Stop
B12	62	T to C	AAA A*T*C GCA	Ile to Thr
B13[a]	34	C to T	CTA *C*GA ATC	Arg to Stop
B14	233	T to C	ATT C*T*C ACC	Leu to Pro
B15	40	G to A	ATC *G*AG AGT	Glu to Lys
B16	212	C to T	TTG G*C*G CGA	Ala to Val
B17[a]	212	C to T	TTG G*C*G CGA	Ala to Val
B18	113	C to T	AAG T*C*G CAG	Ser to Leu
B19	46	G to C	AGT *G*CG TTG	Ala to Pro
B20	179–184	+G	*TGG GGG G*TC	Frameshift
B21	89	C to T	ACA G*C*G GAA	Ala to Val
B22	196	G to A	GAC *G*AC ATG	Asp to Asn
B23	190–198	−GAC	*GAC GAC GAC*	Frameshift
B24	34	C to T	CTA *C*GA ATC	Arg to Stop
B25	205	C to T	GCT *C*GA TTG	Arg to Stop
B26	179–184	−G	*TGG GGG G*TC	Frameshift
B27	122	G to T	ATC A*G*C AGG	Ser to Ile
B28	28	G to A	GAG *G*CT CTA	Ala to Thr
B29	52	C to G	TTG *C*TT AAC	Leu to Val
B30	197	A to G	GAC G*A*C ATG	Asp to Gly
B31	212	C to T	TTG G*C*G CGA	Ala to Val
B32	91	G to T	GCG *G*AA GCT	Glu to Stop
B33	205	C to T	GCT *C*GA TTG	Arg to Stop
B34	40	G to A	ATC *G*AG AGT	Glu to Lys
B35	34	C to T	CTA *C*GA ATC	Arg to Stop
B36[a]	40	G to A	ATC *G*AG AGT	Glu to Lys
B37	89	C to T	ACA G*C*G GAA	Ala to Val

[a] Ascribable to the same mutation obtained in an identical mouse.

Table 5
Sequences of *cII* mutations in the liver of 1,7-Phe-treated Muta™Mouse for the expression time of 56 days

Mutant no.	Position	Mutation	Sequence	Amino acid change
C1	113	C to T	AAG TCG CAG	Ser to Leu
C2	212	C to T	TTG GCG CGA	Ala to Val
C3	125	G to C	AGC AGG TGG	Arg to Thr
C4	196	G to A	GAC GAC ATG	Asp to Asn
C5	40	G to A	ATC GAG AGT	Glu to Lys
C6[a]	212	C to T	TTG GCG CGA	Ala to Val
C7	46	G to C	AGT GCG TTG	Ala to Pro
C8	94	G to C	GAA GCT GTG	Ala to Pro
C9	134	G to T	AAG AGG GAC	Arg to Met
C10	163	C to T	CCG CTT GCT	Leu to Phe
C11	34	C to T	CTA CGA ATC	Arg to Stop
C12	179–240	−62 bp		Frameshift
C13	193	G to A	GAC GAC GAC	Asp to Asn
C14	65	C to T	ATC GCA ATG	Ala to Val
C15	164–165	−T	CTT GCT GTT	Frameshift
	166	G to A		
C16	1	A to G	cat ATG GTT	Met to Val
C17	224	C to A	GTT GCT GCG	Ala to Asp
C18	196	G to A	GAC GAC ATG	Asp to Asn
C19	150	G to T	CCA AAG TTC	Lys to Asn
C20	113	C to T	AAG TCG CAG	Ser to Leu
C21[a]	150	G to T	CCA AAG TTC	Lys to Asn
C22	129	G to A	AGG TGG AAG	Trp to Stop
C23	37	A to T	CGA ATC GAG	Ile to Phe
C24	140–141	GG to CT	GAC TGG ATT	Trp to Ser
C25	89	C to A	ACA GCG GAA	Ala to Glu
C26	34	C to T	CTA CGA ATC	Arg to Stop
C27	212	C to T	TTG GCG CGA	Ala to Val
C28	233	T to C	ATT CTC ACC	Leu to Pro
C29	28	G to C	GAG GCT CTA	Ala to Pro
C30	95	C to A	GAA GCT GTG	Ala to Asp
C31	89	C to G	ACA GCG GAA	Ala to Gly
C32	100	G to C	GTG GGC GTT	Gly to Arg
C33	25	G to T	AAC GAG GCT	Glu to Stop
C34	39	C to G	CGA ATC GAG	Ile to Met
C35	103	G to C	GGC GTT GAT	Val to Leu
C36	212	C to T	TTG GCG CGA	Ala to Val
C37	64	G to A	ATC GCA ATG	Ala to Thr
C38	193	G to T	GAC GAC GAC	Asp to Tyr
C39	95	C to A	GAA GCT GTG	Ala to Asp
C40	74	G to C	CTT GGA ACT	Gly to Ala
C41	120	C to G	CAG ATC AGC	Ile to Met
C42[a]	39	C to G	CGA ATC GAG	Ile to Met
C43[a]	64	G to A	ATC GCA ATG	Ala to Thr

[a] Ascribable to the same mutation obtained in an identical mouse.

1,7-Phe. On the other hand, BfQ and BhQ-induced *cII* mutant spectra showed no characteristics compared with that of the control and consisted mainly of G:C to A:T transitions (15/34 and 18/33, respectively).

4. Discussion

In this study, we attempted to investigate the in vivo mutagenicity of three tricyclic aza-arenes, BfQ, BhQ, and 1,7-Phe. They were injected daily for 4 days

Table 6
Summary of *cII* mutation spectra in Muta™Mouse

Mutation class	Control[a] (%)	BfQ[b] (%)	BhQ[c] (%)	1,7-Phe[b] (%)
Total	32 (100)	34 (100)	33 (100)	39 (100)
Base substitution	28 (88)	28 (82)	28 (85)	36 (92)
Transitions				
GC to AT	18 (56)	15 (44)	18 (55)	15 (38)
AT to GC	1 (3)	2 (6)	3 (9)	2 (5)
Transversions				
AT to TA	3 (9)	0 (0)	1 (3)	1 (3)
AT to CG	0 (0)	1 (3)	0 (0)	1 (3)
GC to TA	5 (16)	7 (20)	3 (9)	7 (18)
GC to CG	1 (3)	3 (9)	3 (9)	10 (26)
−1 frameshifts	1 (3)	3 (9)	2 (6)	0 (0)
+1 frameshifts	2 (6)	1 (3)	2 (6)	0 (0)
Deletion	0 (0)	0 (0)	1 (3)	1 (3)
Insertion	0 (0)	0 (0)	0 (0)	0 (0)
Complex	1 (3)	2 (6)	0 (0)	2 (5)

The same mutations from an identical mouse were counted as single events.
[a] The data of the spontaneous mutations are from our previous report [9].
[b] Mutant plaques from the liver.
[c] Mutant plaques from the lung.

into Muta™Mice at the total doses of 400, 400, and 200 mg/kg intraperitoneally, respectively, based on their tolerance doses determined in preliminary tests. Although these aza-analogs of phenanthrene were weak mutagens in Muta™Mouse, different effects on the target organ speci. city and mutant spectrum were observed depending on the N-substituted position.

BfQ increased the mutant frequency in the liver for the expression times of both 14 and 56 days. On the other hand, BhQ increased mutagenicity in the lung, but not in the liver. BfQ has a nitrogen atom in the bay-region and BhQ in the non-bay-region. Therefore, the difference in the nitrogen position in the benzoquinoline molecule might alter the target organ. Quinoline has previously shown a potent in vivo mutagenicity in Muta™Mice [12–14]. These results suggest that in vivo mutagenicity is decreased by the benzene-ring fusion on the quinoline moiety. 1,7-Phe signi. cantly increased mutagenicity in the liver for the expression times of both 14 and 56 days and in the lung for the expression time of 14 days. It may be suggested that 1,7-Phe induced mutation both in the liver and lung because 1,7-Phe has a nitrogen atom in both the bay- and non-bay-regions. Our previous data indicated that metabolic activation of these phenanthrene aza-analogs might take place in the pyridine moiety [18] (Fig. 2). LaVoie and co-workers reported that BfQ might be converted to the ultimate form not only by the bay-region mechanism but also by another mechanism [17], supporting our opinion.

With regard to the suitable expression time in the evaluation of in vivo mutagenicity, different tendencies were observed between the mutagenesis of 1,7-Phe in the liver and that in the lung. 1,7-Phe showed similar mutagenicities in the liver after the expression time of both 14 and 56 days. However, in the lung, 1,7-Phe increased the mutant frequency in the lung after the expression time of 14 days, but not after 56 days. Sun and Heddle reported that mutation by ethylnitrosourea in the liver was more . rmly established after about 40 days post-treatment than after 20 days [25]. It seems that an appropriate expression time may be necessary to evaluate the in vivo mutagenicity of chemicals in each organ.

1,7-Phe also depressed the G:C to A:T transition and increased the G:C to C:G transversion like quinoline [14], a hepatomutagen possessing the partial structure of 1,7-Phe, compared with the spontaneous mutation spectrum. Therefore it may be suggested that the increase of G:C to C:G transversions might be a common feature of the quinoline-type metabolic activation in aza-arenes.

Although a major question to be answered is how the position of the nitrogen atom is responsible for the differences in mutagenicity between these tricyclic aza-arenes, the present data suggest that the position of the nitrogen atom in the polycyclic aromatic ring might in. uence in vivo mutagenicity with respect to the target organ speci. city and mutational pattern.

Acknowledgements

We thank Dr. T. Nohmi for his kind gifts of *E. coli* NM759 and BHB2688, and Dr. K. Masumura for his technical guidance in making packaging extracts. This research is supported in part by the grant from the Ministry of Environment, Japan.

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Environmental and Molecular Mutagenesis 43:186–195 (2004)

DNA Adducts and Mutagenic Specificity of the Ubiquitous Environmental Pollutant 3-Nitrobenzanthrone in Muta Mouse

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3-nitrobenzanthrone (3-NBA) is an extremely potent mutagen in the Salmonella reversion assay and a suspected human carcinogen identified in diesel exhaust and in ambient airborne particulate matter. To evaluate the in vivo mutagenicity of 3-NBA, we analyzed the mutant frequency (MF) in the *cII* gene of various organs (lung, liver, kidney, bladder, colon, spleen, and testis) in lambda/*lacZ* transgenic mice (Muta Mouse) after intraperitoneal treatment with 3-NBA (25 mg/kg body weight injected once a week for 4 weeks). Increases in MF were found in colon, liver, and bladder, with 7.0-, 4.8-, and 4.1-fold increases above the control value, respectively, whereas no increase in MF was found in lung, kidney, spleen, and testis. Simultaneously, induction of micronuclei in peripheral blood reticulocytes was observed. The sequence alterations in the *cII* gene recovered from 41 liver mutants from 3-NBA-treated mice were compared with 32 spontaneous mutants from untreated mice. Base substitution mutations predominated for both the 3-NBA-treated (80%) and the untreated (81%) groups. However, the proportion of G:C→T:A transversions in the mutants from 3-NBA-treated mice was higher (49% vs. 6%) and the proportion of G:C→A:T transitions was lower than those from untreated mice (10% vs. 66%). The increase in MF in the liver was associated with strong DNA binding by 3-NBA, whereas in lung, in which there was no increase in MF, a low level of DNA binding was observed (268.0–282.7 vs. 8.8–15.9 adducts per 10^8 nucleotides). DNA adduct patterns with multiple adduct spots, qualitatively similar to those formed in vitro after activation of 3-NBA with nitroreductases and in vivo in rats, were observed in all tissues examined. Using high-pressure liquid cochromatographic analysis, we confirmed that all major 3-NBA-DNA adducts produced in vivo in mice are derived from reductive metabolites bound to purine bases (70–80% with deoxyguanosine and 20–30% with deoxyadenosine in liver). These results suggest that G:C→T:A transversions induced by 3-NBA are caused by misreplication of adducted guanine residues through incorporation of adenine opposite the adduct (A-rule). Environ. Mol. Mutagen. 43: 186–195, 2004. © 2004 Wiley-Liss, Inc.

Key words: 3-nitrobenzanthrone; Muta Mouse; mutation spectra; *cII*; DNA adducts; ^{32}P-post-labeling; diesel exhaust; air pollution; nitropolycyclic aromatic hydrocarbon

INTRODUCTION

Air pollution from diesel exhaust is an increasing concern as an environmental risk factor for carcinogenesis [World Health Organization, 2003]. Diesel exhaust is known to induce tumors in experimental animals and epidemiological studies have shown that occupational exposure to diesel exhaust is associated with an increased risk of lung cancer in humans [International Agenecy for Research on Cancer, 1989; Boffeta et al., 2001]. Nitropolycyclic aromatic hydrocarbons (nitro-PAHs) are widely distributed environmental pollutants found in airborne particulate matter, especially that emitted from diesel and gasoline engines [Tokiwa and Ohnishi, 1986; Yaffe et al., 2001]. Many members of this class of compounds are potent mutagens and carcinogens and their detection in the lungs of nonsmokers with lung

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Grant sponsor: Cancer Research U.K.; Grant sponsor: Baden-Württemberg; Grant number: BWPLUS, BWB2003.

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Received 30 October 2003; provisionally accepted 22 November 2003; and in final form 24 January 2004

DOI 10.1002/em.20014

Published online in Wiley InterScience (www.interscience.wiley.com).

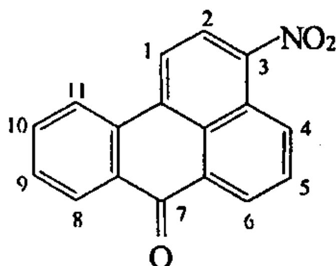

Fig. 1. Structure of 3-NBA.

cancer has led to considerable interest in assessing their potential cancer risk to humans [International Agenecy for Research on Cancer, 1989; Tokiwa et al., 1993; Purohit and Basu, 2000].

3-nitrobenzanthrone (3-NBA; 3-nitro-7*H*-benz[de]anthracen-7-one; Fig. 1) was recently detected in diesel exhaust and in airborne particulate matter and might originate both from incomplete combustion of fossil fuels and from reaction of the parent aromatic hydrocarbon with nitrogen oxides in the atmosphere [Enya et al., 1997; Feilberg et al., 2002; Seidel et al., 2002; Murahashi, 2003]. As a likely consequence of atmospheric washout, 3-NBA has also been detected more recently in surface soil and rainwater [Murahashi et al., 2003a, 2003b; Watanabe et al., 2003]. The uptake of 3-NBA in humans has been demonstrated by the detection of 3-aminobenzanthrone (3-ABA), a major metabolite of 3-NBA, in the urine of salt mine workers occupationally exposed to diesel emissions [Seidel et al., 2002]. 3-NBA is one of the most potent bacterial mutagens known to date, inducing 0.2 and 6.3 million revertants per nmol in *Salmonella typhimurium* TA98 and YG1024, respectively [Enya et al., 1997]. Moreover, 3-NBA induces micronuclei in mouse and in human cells and exhibits DNA strand-breaking activity in human cells [Enya et al., 1997; Phousongphouang et al., 2000; Arlt et al., 2004; Lamy et al., 2004]. Furthermore, 3-NBA is also an effective mutagen in human cells and preliminary data suggest that 3-NBA is carcinogenic in rats [Adachi et al., 2000; Phousongphouang et al., 2000].

3-NBA forms specific DNA adducts in different in vitro systems, in cells, and in vivo in rats [Bieler et al., 1999, 2003; Borlak et al., 2000; Kawanishi et al., 2000; Arlt et al., 2001, 2002, 2003a, 2003b, 2003c], and these adducts may play an important role in the initiation of mutagenesis and carcinogenesis. Although the structures of the DNA adducts remain to be characterized, the major DNA adducts formed in vitro and in rats are products derived from reductive metabolites bound to purine bases without carrying an *N*-acetyl group [Arlt et al., 2001, 2003a, 2003c].

Despite the strong mutagenicity of 3-NBA in bacteria, little is known about its mutagenicity in vivo. Transgenic mutation assays are a powerful tool to study chemical mutagenesis in experimental animals [Suzuki et al., 2000; Kohara et al., 2002a, 2002b; Itoh et al., 2003]. In addition, molecular analyses of induced mutations may reveal chemical-specific mutation spectra. To evaluate the mutagenicity of 3-NBA, a transgenic mouse model, Muta Mouse, was used and mutations in the *cII* gene were assessed. In addition, DNA adduct formation was investigated using ^{32}P-postlabeling.

MATERIALS AND METHODS

Synthesis of 3-NBA

3-NBA was synthesized as described recently [Arlt et al., 2002]. The authenticity of 3-NBA was confirmed by UV, electrospray mass spectra (ES-MS), and high-field proton nuclear magnetic resonance spectroscopy.

Animal Experiments

Male Muta Mouse animals were supplied by Covance Research Products (Denver, PA) and were acclimatized for 1 week before use. 3-NBA was dissolved in olive oil (2.5 mg/ml). Five mice (7- to 8-week-old, ~ 25 g body weight) were treated with 25 mg/kg body weight once a week for 4 weeks by intraperitoneal injection (10 ml/kg). Five mice received olive oil only at the same time and in the same manner. Mice were killed 3 days after the last treatment, and lung, liver, kidney, bladder, spleen, colon, and testis tissues were collected. Tissue samples were stored at −80°C until DNA isolation. DNA was extracted by a standard phenol extraction method.

Peripheral Blood Micronucleus Assay

Forty-eight hours after the first (week 1) and second injection (week 2), peripheral blood (5 μl) was collected without anticoagulant from a tail blood vessel, placed on an acridine orange-coated glass slide, covered with a coverslip, and stained [Hayashi et al., 1990]. One thousand peripheral blood reticulocytes (RETs) per animal were analyzed by fluorescence microscopy within a few days of slide preparation, and the number of cells with micronuclei was recorded.

Lambda *cII* Mutation Analysis

The MutaPlax *cII*-Select Kit (Epicentre Technologies, Madison, WI) was used for the lambda *cII* assay. The kit contained lambda packaging extracts and cultures of *hfl*$^-$ *Escherichia coli* G1225 for both recovered phage titer and selection of mutant phages. Lambda packaging and positive selection for *cII* mutants was performed according to the protocol of the manufacturer with minor modifications as described previously [Jakubczak et al., 1996]. Briefly, 500 μl of the packaged phage solution were incubated with 1 ml of G1225 cells (OD_{660} = 1.0) at room temperature for 30 min, mixed with 14 ml Luria broth top agar, and plated on five 9 cm dishes containing 10 ml bottom agar. The plates were incubated at 25°C for 48 hr. For total virus titer, a 5 μl portion of the packaged phage was mixed with 200 μl of strain G1225 cells and 6 ml LB top agar, plated on two dishes, and incubated at 37°C for 24 hr. Wild-type phage recovered from Muta Mouse has a *cI*$^-$ phenotype, which permitted plaque formation on the *hfl*$^-$ strain at 37°C but not at 25°C. The mutant frequency (MF) was determined by dividing the number of mutants plaques by the total number of recovered phage evaluated from each animal.

Sequencing of *cII* Mutants

Mutations in the lambda *cII* transgene were analyzed using the DNA cycle sequencing method described previously [Suzuki et al., 2000]. The *cII* gene region (294 base pairs) was PCR-amplified directly from mutant plaques using the primer pair 5′-AAAAAGGGCATCAAATTAAACC-3′ and 5′-CCGAAGTTGAGTATTTTTGCTGT-3′. A 446 base pair PCR product was purified and used for the sequencing reaction with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Tokyo, Japan). PCR amplification and DNA sequencing was performed using a Minicycler PTC-150-25 (MJ Research, Watertown, MA) and an ABI Prism 310 Genetic Analyzer (Applied Biosystems), respectively. Statistical analysis of the mutation spectra for differences between treated and control spectra was conducted using the Adams-Skopek algorithm [Cariello et al., 1994].

^{32}P-Postlabeling Analysis and High-Pressure Liquid Chromatography (HPLC) Analysis of ^{32}P-Labeled 3′,5′-Deoxyribonucleoside Bisphosphate Adducts

^{32}P-postlabeling analyses using nuclease P1 digestion, butanol extraction, and autoradiography were performed as described [Arlt et al., 2002]. Chromatographic conditions for thin-layer chromatography (TLC) on polyethyleneimine-cellulose (PEI-cellulose) were D1, 1.0 M sodium phosphate, pH 6.0; D3, 4 M Li-formate, 7 M urea, pH 3.5; D4, 0.8 M LiCl, 0.5 M Tris, 8.5 M urea, pH 8.0. DNA adduct levels (relative adduct labeling; RAL) were calculated from the adduct cpm, the specific activity of [γ-^{32}P]ATP and the amount of DNA (pmol of DNA-P) used. Results were expressed as DNA adducts/10^8 nucleotides. Individual adduct spots detected by the ^{32}P-postlabeling assay, or the origins after D1 only, were excised from the TLC plates, extracted, and cochromatographed on HPLC with reference bisphosphate adducts essentially as described previously [Arlt et al., 2001].

Preparation of Reference Compounds for ^{32}P-Postlabeling

Deoxyadenosine (dA) and deoxyguanosine (dG) 3′-monophosphates or calf thymus DNA (4 μmol/ml; Sigma) were incubated with 3-NBA (0.3 mM) in a reaction containing xanthine oxidase (1 U/ml; Sigma, Gillingham, U.K.) in 50 mM potassium phosphate buffer, pH 7.0, in the presence of 1 mM hypoxanthine (Sigma) as described previously [Bieler et al., 1999; Arlt et al., 2001]. The resulting adducted deoxypurine 3′-monophosphates were used as reference compounds in ^{32}P-postlabeling experiments.

RESULTS

Micronucleus Induction in Peripheral Blood of Muta Mouse

The frequencies of micronucleus formation in peripheral blood RETs after treatment of Muta Mouse with 25 mg/kg body weight 3-NBA are shown in Table I. Significant increases in the frequency of micronucleated RETs were observed 48 hr after the first (week 1) and the second (week 2) treatment. This result confirms data on micronucleus formation observed in another mouse strain using the same amount of 3-NBA [Enya et al., 1997].

TABLE I. Micronucleus Induction in Mouse Peripheral Blood Reticulocytes of Muta Mouse Treated With 3-NBA

	Micronucleated RETs per 1,000 RETs after the first and second i.p. injection of 3-NBA (mean ± SD)[a]	
Treatment	Week 1	Week 2
Control	3.0 ± 0.8	3.0 ± 1.2
3-NBA	7.5 ± 2.8[b]	8.4 ± 3.0[b]

[a]Values represent the mean ± SD of five animals.
[b]Significantly different from untreated control animals at $P < 0.05$ (t-test).

TABLE II. Mutant Frequency in the *cII* Gene From Various Organs of Muta Mouse Treated With 3-NBA

	Mean MF × 10^{-6} ± SD[a]	
Organ	Control	3-NBA
Lung	38.1 ± 24.4	38.5 ± 14.2
Liver	30.5 ± 12.1	147.4 ± 49.4[b]
Kidney	36.2 ± 13.4	37.6 ± 13.4
Colon	36.7 ± 17.6	258.7 ± 106.4[b]
Spleen	28.8 ± 9.6	34.4 ± 11.1
Testis	15.2 ± 7.4	22.8 ± 6.3
Bladder	13.1[c]	54.4[c]

[a]Results represent the mean ± SD of five animals.
[b]Significantly different from untreated control animals at $P < 0.001$ (t-test).
[c]Results represent the mean of two animals only.

Mutagenic Specificity of 3-NBA in Muta Mouse

DNA was isolated from lung, liver, kidney, bladder, colon, spleen, and testis 3 days after the last treatment. The results of the *cII* MF analyses are shown in Table II (mouse-by-mouse data are given in the Appendix). The MF was significantly increased above spontaneous levels in colon and liver, with 7.0- and 4.8-fold increases, respectively. A 4.1-fold increase was also seen for bladder, but because only two control and two treated mice were evaluated for this tissue, this difference was not tested for significance. No increase in MF above control levels was seen in the other tissues.

Since the metabolic activation of 3-NBA due to hepatic enzymes has been intensively studied, the mutagenic specificity of 3-NBA was examined in liver tissue only. Forty-one 3-NBA-induced mutants from the liver were sequenced, together with 32 spontaneous mutants from the livers of untreated mice. The mutation spectra are summarized in Table III. Spontaneous mutations consisted mainly of base substitutions (26 of 32 mutations). Among them, G:C→A:T transitions (21 of 26 transitions) predominated and almost all of them (18 of 21 mutations) occurred at CpG sites. 3-NBA-induced mutations also consisted mainly of base substitutions (33 of 41 mutations). Comparing to the control, G:C→A:T transitions were decreased (10% vs. 66%) and G:C→T:A transversions were increased (49% vs. 6%).

TABLE III. Classification of 3-NBA-Induced and Spontaneous *cII* Mutations From Liver of Muta Mouse

	Spontaneous		3-NBA-induced	
Mutation type	Number	%	Number	%
Total	32	100	41	100
Base substitutions	26	81	33	80
Transitions	21	66	6	15
G:C→A:T (at CpG)	21 (18)	66 (56)	4 (2)	10 (5)
A:T→G:C	0	0	2	5
Transversions	5	16	27	66
A:T→T:A	2	6	4	10
A:T→C:G	0	0	1	2
G:C→T:A	2	6	20	49
G:C→C:G	1	3	2	12
Frameshifts (−1)	2	6	5	12
Frameshifts (+1)	2	6	0	0
Deletions	0	0	1	2
Insertions	0	0	0	0
Complex	2	6	2	5

The distribution of mutations in the liver is shown in Figure 2. 3-NBA-induced mutations were distributed over all the *cII* gene and no apparent hot spots were observed. Statistical analysis of the mutational spectra data demonstrated that the differences between 3-NBA-treated and control spectra were significant ($P = 0.0041$).

DNA Adduct Formation of 3-NBA in Muta Mouse

DNA adduct formation in Muta Mouse was analyzed in liver, in which MF was increased, and in lung, where no increase in MF was observed. As shown in Figure 3, 3-NBA induced essentially the same DNA adduct pattern as those observed in different in vitro activation systems, including cytosolic nitroreductases and human liver microsomes, in human cells, and in rats [Arlt et al., 2001, 2003a, 2003c]. Using butanol enrichment, the observed pattern consisted of a cluster of five adducts (spots 1–5). Analyses using nuclease P1 enrichment resulted in a cluster of four adducts (spots 1–3 and 6). No DNA adducts were observed in DNA isolated from tissues of control animals treated with vehicle (olive oil) only (data not shown). Although the structures of these adducts have yet to be elucidated, all adduct spots detected in this study on TLC plates were chromatographically indistinguishable from adduct spots found in incubations with dA (adduct 1 and 2) and dG 3′-monophosphates (adduct 3, 4, and 5) generated by 3-NBA activated by xanthine oxidase [Arlt et al., 2001]. As a second, independent chromatographic procedure, we also employed reversed-phase HPLC analysis to confirm the identities of adduct spots formed by 3-NBA (Fig. 4). The results thus obtained confirmed the findings from chromatography on TLC plates. DNA binding in lung and liver ranged from 8.8 to 15.9 and from 268.0 to 282.7 adducts per 10^8 nucleotides for total DNA adducts, respectively (Fig. 5A). Levels of individual adduct spots are given in Figure 5B. In particular, adduct spot 3 was the predominant adduct formed in both tissues.

DISCUSSION

There are increasing concerns over the carcinogenic risk of diesel exhaust. 3-NBA is a potent mutagen identified in diesel exhaust and airborne particulate matter, and preliminary data indicate that 3-NBA is carcinogenic in rodents [Enya et al., 1997; Adachi et al., 2000; Seidel et al., 2002]. 3-NBA is highly mutagenic in the *Salmonella typhimurium* strains TA98 and YG1024, producing mutagenic responses comparable to those of 1,8-dinitropyrene, one of the most mutagenic nitro-PAH reported to date [Enya et al., 1997]. In addition, previous data also indicate that 3-NBA is an efficient mutagen in human lymphoblastoid-derived MCL-5 cells at the *TK* and *HPRT* loci [Phousongphouang et al., 2000]. These observations suggest that 3-NBA may also be mutagenic in vivo. Therefore, we investigated the in vivo mutagenicity of 3-NBA after intraperitoneal treatment of transgenic Muta Mouse. The mutagenicity of 3-NBA in vivo was clearly demonstrated by this assay using the *cII* gene as target sequence. Simultaneously, the clastogenicity of 3-NBA was evaluated by the peripheral blood micronucleus assay. The assay for micronuclei showed a significant increase in the frequency of micronucleated reticulocytes, confirming previous data obtained in another mouse strain and in human cells [Enya et al., 1997; Phousongphouang et al., 2000; Arlt et al., 2004; Lamy et al., 2004].

In our study, 3-NBA increased the MF in colon, liver, and bladder, whereas no increase in MF was observed in lung, kidney, spleen, and testis. Human exposure to 3-NBA is thought to occur primarily via the respiratory tract and it is possible that the tissue-specific distributions of MF would differ after inhalation or intratracheal treatment with 3-NBA. On the other hand, different tissues will respond at different rates after exposure to a mutagen, rates of metabolism aside. Mutation fixation time may be different in different tissues, depending on cell turnover rate in a tissue, with colon being a rapid responder and lung being much slower [Heddle et al., 2003; Thybaud et al., 2003]. This could explain why 3-NBA did not induce mutations in the lung in the present study. In a recent study with rats, we found that after a single dose of 3-NBA (2 mg/kg body weight, i.p.), DNA binding by 3-NBA was higher in lung compared to liver [Arlt et al., 2003a]. Moreover, preliminary results also indicate that binding by 3-NBA in rat DNA is much higher in lung compared to liver after treatment with a single dose of 3-NBA (2 mg/kg body weight) by intratracheal instillation (data not shown). Therefore, the results of the present study may suggest that tissue-specific DNA adduct formation in mice is different to those observed in the rat model, different dosing and administration aside.

Fig. 2. Mutations in the liver of *cII* gene obtained from control and 3-NBA-treated Muta Mouse. The sequence from top to bottom represents the amplified lambda *cII* region. Mutations shown above the strand were detected in control mice, whereas those below the strands (in bold) were detected in 3-NBA-treated mice. The boxes represent tandem (double) or complex mutations or deletions with undefined positions within the boxed region.

Fig. 3. Autoradiographic profiles of DNA adducts obtained from digests of liver DNA of Muta Mouse treated with 3-NBA using the nuclease P1 (A) and butanol (B) enrichment versions of the ^{32}P-postlabeling assay.

The highest induced MF was found in the colon. A strong increase in MF in colon has also been observed for other mutagens, including dinitropyrenes [Kohara et al., 2002a], suggesting that the colon is a very sensitive organ for mutagenesis in the transgenic mouse mutation assay, probably because of its high proliferation rate. When the MF induced by 3-NBA in Muta Mouse is compared with those of a mixture of dinitropyrenes administered under similar conditions [Kohara et al., 2002a], it is apparent that 3-NBA has an approximately 10-fold higher potency in the main target tissue, colon. However, it is noteworthy that enteric bacteria might also play a role in the mutagenic activation of 3-NBA in colon by nitroreduction. This is in line with earlier reports in rats of strong DNA adduct formation of 3-NBA in the gastrointestinal tract, after either oral treatment or intraperitoneal injection [Arlt et al., 2001, 2003a].

Previous studies indicated that nitroreduction catalyzed by cytosolic and microsomal nitroreductases, followed by