Evaluation of Genotoxicity of 3-Amino-, 3-Acetylamino- and 3-Nitrobenzanthrone Using the Ames/*Salmonella* Assay and the Comet Assay

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3-Nitrobenzanthrone (3-NBA) is an extremely potent direct-acting bacterial mutagen that has been detected in diesel exhaust particles, airborne particles and so forth. Recently, 3-aminobenzanthrone (3-ABA) and 3-acetylaminobenzanthrone (3-AABA) were identified as metabolites of 3-NBA in mammalian cells. In this study, to clarify the genotoxicity of 3-ABA, 3-AABA and 3-NBA *in vitro* and *in vivo*, the mutagenicity and DNA-damaging activity of these chemicals were investigated by the Ames assay and by alkaline single gel electrophoresis (Comet assay), respectively. 3-ABA and 3-AABA were mutagenic toward four *Salmonella typhimurium* strains, *i.e.* TA98, TA100, YG1024 and YG1029, in the presence of a mammalian metabolic system (S9 mix). Both chemicals showed the highest mutagenicity toward YG1024, and induced 2180000 revertants/nmol of 3-ABA and 131000 revertants/nmol of 3-AABA. 3-NBA also showed mutagenicity toward these four strains with and without S9 mix, but the potencies of 3-NBA in these strains were decreased by the addition of S9 mix. In the presence of S9 mix, the mutagenic activities of 3-NBA in four strains were comparable to or lower than those on 3-ABA. 3-ABA, 3-AABA and 3-NBA produced statistically significant DNA damage, which was detected by an increase in the DNA tail moment, *in vivo* 3 hr after intraperitoneal injection at 160 mg/kg body weight. 3-ABA, 3-AABA and 3-NBA induced significant DNA damage in the liver, kidney, spleen and lung, spleen only, and liver, kidney, lung and bone marrow, respectively. At a lower dose (40 mg/kg), 3-NBA produced significant DNA damage in the lung. These results indicate that 3-ABA, 3-AABA and 3-NBA are not only mutagenic *in vitro* in bacteria but also genotoxic *in vivo* in mouse.

Key words ——— 3-nitrobenzanthrone, 3-aminobenzanthrone, 3-acetylaminobenzanthrone, Ames assay, Comet assay

INTRODUCTION

Environmental factors and individual susceptibility play an important role in the incidence of many human cancers.[1)] Nitrated polycyclic aromatic hydrocarbons (NO_2-PAHs) are widespread environmental pollutants that are formed during the incomplete combustion of organic substances such as fossil fuels and by the reaction of parental hydrocarbons with nitrogen oxides in ambient air.[2,3)] NO_2-PAHs are the subject of great concern because of their genotoxicity.[2,4)] 3-Nitrobenzanthrone (3-NBA, 3-nitro-7*H*-benz[*de*]anthracene-7-one) (Fig. 1), which has been detected in diesel exhaust particles,[5)] ambient air,[5,6)] rainwater[7)] and surface soil,[8,9)] is one of the most powerful known bacterial mutagens by the Ames/*Salmonella* assay.[5)] Moreover, 3-NBA induced micronuclei *in vitro* in human cell lines.[10)] By the intraperitoneal (i.p.) injection of 3-NBA, micronucleated erythrocytes were induced in mouse peripheral blood[5)] and several DNA adducts of 3-NBA were formed in multiple organs in rat.[11)] In a study of the metabolism of 3-NBA, 3-aminobenzanthrone (3-ABA) was identified as the major metabolite in cultures of primary rat alveolar type II cells, a human bronchial cell line and so forth,

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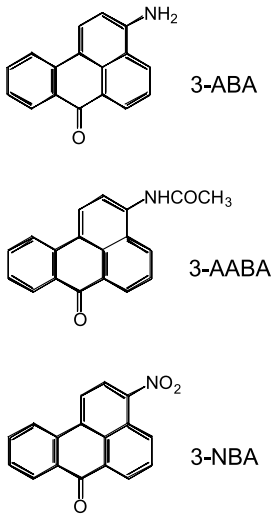

Fig. 1. Chemical Structures of 3-Aminobenzanthrone (3-ABA), 3-Acetylaminobenzanthrone (3-AABA) and 3-Nitrobenzanthrone (3-NBA)

and small amounts of 3-acetylaminobenzanthrone (3-AABA) were observed during short-term incubation of rat epithelial bronchial R3/1 and cultured primary rat alveolar type II cells.[12] Some aminoarenes and acetylamino derivatives have been shown to be genotoxic *in vitro* and *in vivo*,[13,14] and 3-ABA and 3-AABA were reported to form several DNA adducts *in vivo* in multiple organs in rat.[15] Based on these findings, 3-ABA and 3-AABA are assumed to be mutagenic and genotoxic *in vitro* and *in vivo*, but the mutagenicity of these chemicals is unclear. There have been few studies on *in vivo* DNA-damaging activity of 3-ABA, 3-AABA and 3-NBA.

Recently, the *in vivo* alkaline single cell gel electrophoresis assay, or Comet assay, has been developed as a sensitive method that is capable of detecting a wide range of DNA damage, including single- and double-strand DNA breaks, alkali-labile sites that are expressed as single-strand breaks and single-strand breaks associated with incomplete excision repair.[16] The Comet assay can be applied to any tissue in an experimental animal[17] and has been suggested to be a promising method for detecting the organ-specific genotoxicity of chemical mutagens and carcinogens, such as aromatic amines and polycyclic nitro compounds.[17]

The purpose of this study was to clarify the genotoxicity of 3-ABA, 3-AABA and 3-NBA *in vitro* and *in vivo*. We investigated the mutagenicity of 3-ABA and 3-AABA using four *Salmonella typhimurium* (*S. typhimurium*) strains by the Ames assay. Moreover, the effect of a mammalian metabolic system (S9 mix) on the bacterial mutagenicity of 3-NBA was examined, since only the direct mutagenicity of 3-NBA was determined in a previous study.[5] The DNA-damaging activities of 3-ABA, 3-AABA and 3-NBA were evaluated on multiple organs in mouse to clarify the genotoxicity *in vivo* by the Comet assay.

MATERIALS AND METHODS

Chemicals —— 3-ABA (CAS No. 13456-80-9), 3-AABA (CAS No. 53652-25-8), and 3-NBA (CAS No. 17117-34-9) were synthesized as described previously.[18] The chemical structures of 3-ABA 3-AABA, and 3-NBA are shown in Fig. 1. Ethyl methansulfonate (EMS), low melting point (LMP) agarose, and normal melting point (NMP) agarose were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Ethidium bromide was purchased from Merck (Darmstadt, Germany).

Ames/*Salmonella* Assay —— All of the samples were dissolved in dimethyl sulfoxide and assayed by the pre-incubation method[19] using *S. typhimurium* TA98,[20] TA100,[20] YG1024,[21] and YG1029.[21] S9 mix contained 0.01 ml of S9, which was prepared from livers of male Sprague-Dawley rats treated with phenobarbital and β-naphthoflavone, in a total volume of 0.5 ml. The assay was performed with four doses and duplicate plates at each dose. Mutagenic potencies of samples were calculated from linear portions of the dose-response curves. When the samples induced two-fold increases over the average yield of spontaneous revertants and showed well-behaved concentration-response patterns, the samples were judged to be positive.

Treatment of Mice —— Seven-week-old male ICR mice were supplied by Japan SLC (Hamamatsu, Japan) and used for the experiments when 8 weeks old. Five mice were assigned randomly into each group and given commercial pellets and tap water ad libitum throughout the experimental periods. 3-NBA, 3-ABA, and 3-AABA were suspended in 2% Tween 80 and intraperitoneally (i.p.) injected once to mice at doses of 40 and 160 mg/kg body weight. In the negative and positive control groups, 2% Tween 80 and EMS, which was dissolved in physiological saline, were injected i.p., respectively.

Comet Assay —— The alkaline Comet assay was performed according to a modification of the method of Tice *et al*.[22] Liver, kidney, spleen, lung, and bone

Table 1. Mutagenicities of 3-ABA, 3-AABA and 3-NBA in Four *S. typhimurium* Strains with and without S9 Mix[a)]

Compound	Revertants/nmol							
	TA98		TA100		YG1024		YG1029	
	−S9 mix	+S9 mix	−S9 mix	+S9 mix	−S9 mix	+S9 mix	−S9 mix	+S9 mix
3-ABA	ND[b)]	44200	ND	5270	316	2180000	ND	20700
3-AABA	ND	8150	ND	385	237	131000	ND	7400
3-NBA	202000 (208000)[c)]	11700	19900 (29700)[c)]	3660	5840000 (6290000)[c)]	149000	406000	22600

a) Mutagenic potencies of samples were calculated from linear portions of the dose-response curves in two independent experiments. *b*) ND: not detected. *c*) From reference [5].

marrow were removed 3 hr after injection. Each organ, except for bone marrow, was minced, suspended in chilled homogenizing buffer (pH 7.5, containing 0.075 M KCl and 0.03 M sodium EDTA), and homogenized gently with a Dounce-type homogenizer. One hundred μl of normal melting point agarose was layered as the first layer on a glass slide and then 50 μl of low melting point agarose containing about 10000 nuclei was layered on the first layer. Finally, 100 μl of normal melting point agarose was layered on the second layer. The slides were immersed in ice-cold lysing solution (pH 10, containing 2.5 M NaCl, 100 mM sodium EDTA, 10 mM Tris–HCl, 1% sodium *N*-lauryl sarcosinate, 10% DMSO and 1% Triton X-100) for 60 min. The slides were then placed on a horizontal gel electrophoresis platform and covered with chilled alkaline solution (containing 300 mM NaOH, 1 mM sodium EDTA) for 20 min to allow for the unwinding of DNA and the expression of alkali-labile sites. The nuclei were electrophoresed at 25 V for 20 min. After electrophoresis, specimens were rinsed twice with 400 mM Tris–HCl (pH 7.5) to neutralize excess alkali, stained with 50 μl of ethidium bromide solution and covered with a coverslip. One hundred nuclei were inspected per organ per animal using a fluorescence microscope (× 800) equipped with a CCD camera. The tail moment of the DNA was measured using Komet Assay software (Kinetic Imaging Ltd., Liverpool, U.K.).

Statistical Analysis ——— The *t*-test was used to test the difference in the DNA tail moment in the Comet assay between the treated and control groups; a *p*-value lower than 0.05 was considered to be statistically significant.

RESULTS

Mutagenicity of 3-ABA, 3-AABA and 3-NBA *in Vitro*

The mutagenicities of 3-ABA and 3-AABA in *S. typhimurium* strains, *i.e.* TA98, TA100, YG1024, and YG1029, are summarized in Table 1. YG1024 and YG1029 are *O*-acetyltransferase over-producing derivatives of TA98 and TA100, respectively.[21)] 3-ABA and 3-AABA were mutagenic toward these four strains in the presence of S9 mix. The mutagenic activities of 3-ABA and 3-AABA in TA98 (3-ABA, 44200 revertants/nmol; 3-AABA, 8150 revertants/nmol) were 8 and 21 times as high as those in TA100, respectively. Similarly, the mutagenic activities of these two chemicals in YG1024 were higher than those in YG1029 with S9 mix. On the other hand, the mutagenic activities of 3-ABA and 3-AABA in YG1024 (3-ABA, 2180000 revertants/nmol; 3-AABA, 131000 revertants/nmol) were 49 and 16 times as high as those in TA98. Similar tendencies were observed in the activities of these chemicals toward YG1029 and TA100 in the presence of S9 mix. The mutagenicity of 3-NBA in these four strains is also shown in Table 1. 3-NBA showed mutagenicity toward these four strains both with and without S9 mix. However, the mutagenicity of 3-NBA decreased with the addition of S9 mix, and the activities in each strain with S9 mix were 2–18% of those without S9 mix. In the presence of S9 mix, the mutagenic activities of 3-NBA in the four strains were comparable to or lower than those of 3-ABA. 3-AABA showed the lowest mutagenicities in the four strains among these three chemicals.

DNA-Damaging Activities of 3-ABA, 3-AABA and 3-NBA *in Vivo*

The DNA-damaging activities of 3-ABA, 3-AABA, and 3-NBA in multiple organs, *i.e.* liver,

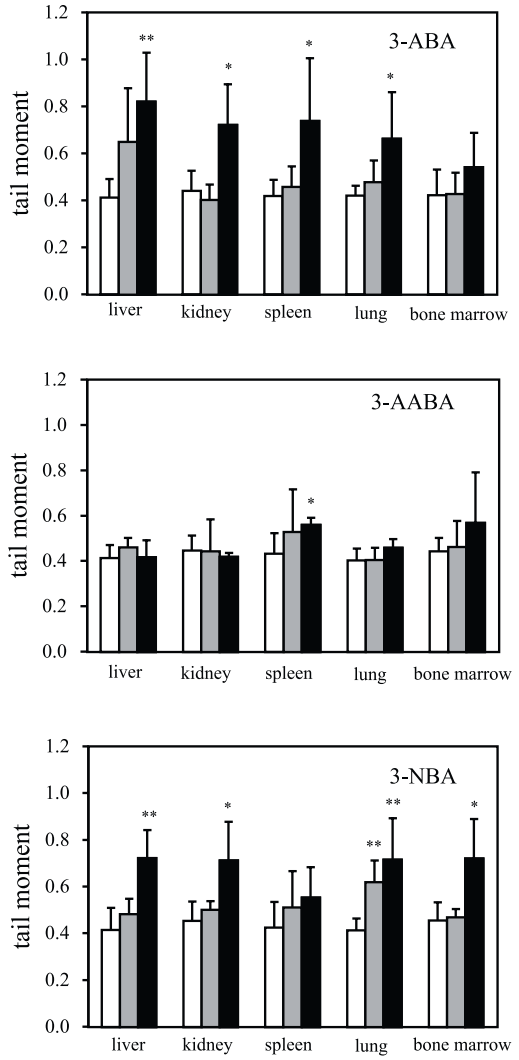

Fig. 2. DNA-Damaging Activity of 3-ABA, 3-AABA, and 3-NBA

Mice were intraperitoneally (i.p.) treated with 3-ABA at doses of 40 mg/kg (dotted column) and 160 mg/kg (closed column) body weight. Control mice (open column) were treated with 2% Tween 80 in physiological saline. Organs were removed 3 hr after injection. One hundred cells were measured per mouse. Tail moment = DNA migration × intensity. *$p < 0.05$ (*vs.* control), **$p < 0.01$ (*vs.* control).

kidney, spleen, lung and bone marrow, of mouse were determined as reflected by the DNA tail moment using the Comet assay. Mean values and standard deviations (S.D.) of tail moments of nuclear DNA in the five organs from five mice that were prepared 3 hr after the i.p. injection of 3-ABA, 3-AABA, and 3-NBA are shown in Fig. 2. As shown in Fig. 2, the DNA tail moment values increased in all of the tested organs, except for the bone marrow, with 3-ABA at 160 mg/kg body weight. A high DNA tail moment value was found in the liver by 3-ABA at 40 mg/kg body weight, but the increase of the value was not significant. With 3-AABA, a significant increase of the DNA tail moment was found in the spleen only at 160 mg/kg body weight. 3-NBA produced a significant increase in the DNA tail moment in the liver, kidney, lung, and bone marrow at 160 mg/kg body weight. The DNA tail moment in the lung was also significantly increased at a dose of 40 mg/kg body weight. The highest mean tail moment (0.82; control = 0.41) was detected in the liver of mouse treated with 3-ABA at 160 mg/kg body weight.

DISCUSSION

Previous studies have shown that 3-NBA is an extremely potent bacterial mutagen in the absence of S9 mix[5] and one major pathway for the bioactivation of 3-NBA is nitroreduction.[11,12,23] Moreover, it has been shown that phase II enzymes, *e.g. N,O*-acetyltransferase and sulfotransferase, are also involved in the formation of highly reactive arylnitrenium ions from 3-NBA, which interact with cellular DNA.[5,11,24] The present study showed that 3-ABA and 3-AABA were potent mutagens in *S. typhimurium* test strains in the presence of S9 mix, and their activities were higher in TA98 than in TA100. Similarly, 3-ABA and 3-AABA were more mutagenic in YG1024 than in YG1029. These results indicate that both 3-ABA and 3-AABA require metabolic activation by cytochrome P450 (CYP) enzymes to exert mutagenicity and both chemicals produce more frame-shifts than base substitutions. On the other hand, the mutagenic activities of 3-ABA and 3-AABA in YG1024 and YG1029 were remarkably higher than those in the original strains, *i.e.* TA98 and TA100, implying that *O*-acetyltransferase plays an important role in the metabolic activation of 3-ABA and 3-AABA. These results are consistent with those on DNA adduct formation by 3-ABA and 3-AABA in Chinese hamster V79 cells and V79 cells expressing human CYP1A2 in conjunction with human *N,O*-acetyltransferases and sulfotransferases.[25] Einistö *et al.*[26] tested 30 mutagens using YG1024, YG1029, TA98, and TA100. The *O*-acetyltransferase-overproducing strains, YG1024 and YG1029, showed a higher sensitivity to 16 chemicals than did the conventional strains, TA98 and TA100. These compounds are arylhydroxylamines, aromatic amines, and nitroarenes, and the difference in sensitivity between the *O*-acetyltransferase-overproducing strains and the conventional

strains dependent on the test compounds. 2-Aminofluorene and 2-acetylaminofluorene showed 30- and 16-fold higher mutagenicity to YG1024, respectively, compared with TA98. The mutagenic potencies of 3-NBA in *S. typhimurium* strains were decreased by the addition of S9 mix. In the presence of S9 mix, 3-ABA showed higher mutagenicity than 3-NBA in three of four strains, and the mutagenicities of 3-AABA were the lowest among these three chemicals.

Sasaki *et al.*[27] examined the *in vivo* genotoxicity of 6 heterocyclic amines, *i.e.* 3-amino-1,4-dimethyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-1), 3-amino-1-methyl-5*H*-pyrido[4,3-*b*]indole (Trp-P-2), 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]-pyridine (PhIP), in mouse liver, kidney, spleen, lung, bone marrow, stomach mucosa and brain by the Comet assay. They found that DNA damage was induced in multiple organs 3 hr after the i.p. injection of these heterocyclic amines and that the organs that were susceptible to the DNA-damaging activities of Trp-P-2, IQ, MeIQ and MeIQx were similar; *i.e.* activity was evident in the liver, kidney, lung, stomach, and brain. Our experiments indicate that 3-ABA also induced statistically significant DNA damage in multiple mouse organs, *i.e.* liver, kidney, spleen, and lung. 3-NBA also produced significant DNA damage in multiple organs, *i.e.* liver, kidney, lung and bone marrow. On the other hand, 3-AABA induced significant DNA damage only in the spleen. DNA damaging activity levels of 3-ABA and 3-NBA in these target organs were almost the same, and that of 3-AABA was the lowest. These results indicate that 3-ABA, 3-AABA, 3-NBA and/or their metabolites are distributed *via* the bloodstream. The differences in the organ specificity of the DNA-damaging activities of 3-ABA, 3-AABA, and 3-NBA might be attributed to differences in the absorption, distribution, and metabolic activation of these chemicals. Clearance of active metabolites from organs also affects DNA damage.

Arlt *et al.*[15] investigated DNA adduct formation in six organs, *i.e.* lung, liver, kidney, heart, pancreas, and colon, of rats 24 hr after the i.p. injection of 3-NBA, 3-ABA, and 3-AABA, and found that these three chemicals induced essentially the same DNA adduct pattern in all of these organs. Moreover, all of the major 3-NBA-DNA adducts were derived from reductive metabolites of 3-NBA bound to purine bases. These results suggest that 3-NBA, 3-ABA and 3-AABA are metabolically activated in different pathways and form common ultimate reactive species to show biological activities, such as DNA adduct formation. The level of total DNA adducts of 3-NBA was markedly higher than those of 3-ABA and 3-AABA, and those of latter two chemicals were almost the same. The highest DNA binding by 3-NBA was found in the pancreas followed by colon and decreased in kidney > heart = lung > liver. For 3-ABA and 3-AABA, there was less variation in the levels of DNA adducts in the tissues investigated. These organ specificities of 3-NBA, 3-ABA, and 3-AABA in DNA adduct formation did not consistent with those in DNA damaging activities detected by the Comet assay in this study. The cause of this inconsistency is uncertain, but it might be attributed to the differences of the treatment periods and end points in these two assays.

The micronucleus test is widely used as an *in vivo* genotoxicity assay because of its convenience. Previously, we reported that 3-NBA induced micronuclei in mouse peripheral blood by i.p. treatment at 25 and 50 mg/kg body weight.[5] In this study, DNA-damaging activity was detected in bone marrow by treatment with 3-NBA at 160 mg/kg body weight but not at 40 mg/kg body weight. Since it is well known that DNA-damaging activities are observed within a specific time-period after single treatment with chemicals in the Comet assay,[17] higher DNA-damaging activity might be observed in the bone marrow after a treatment period other than 3 hr.

Seidel *et al.*[28] quantified 3-ABA in urine samples of salt-mine workers who are exposed to diesel exhaust and concluded that 3-ABA appeared to be a prime candidate as a diesel-specific urinary biomarker of exposure of 3-NBA. Because 3-NBA ubiquitously present in our environment,[5–9] we may be exposed to 3-NBA and metabolize it to 3-ABA in our body system. Our present study demonstrates that both 3-NBA and 3-ABA are potent bacterial mutagens and induce DNA damage *in vivo*. To fully estimate the impact of 3-NBA and its metabolites, 3-ABA and 3-AABA, on human health, further studies on their biological activities including carcinogenicity are necessary. In addition, more detailed quantification of exposure levels to these chemicals is needed to assess their risks on human health.

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