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. Nitrated polycyclic aromatic hydrocarbons (NO₂-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. NO₂-PAHs are the subject of great concern because of their genotoxicity. 3-Nitrobenzanthrone (3-NBA, 3-nitro-7H-benz[de]anthracene-7-one) (Fig. 1), which has been detected in diesel exhaust particles, ambient air, rainwater and surface soil, is one of the most powerful known bacterial mutagens by the Ames/Salmonella assay. Moreover, 3-NBA induced micronuclei in vitro in human cell lines. By the intraperitoneal (i.p.) injection of 3-NBA, micronucleated erythrocytes were induced in mouse peripheral blood and several DNA adducts of 3-NBA were formed in multiple organs in rats. 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,
and small amounts of 3-acetylanobenzanthrone (3-AABA) were observed during short-term incubation of rat epithelial bronchial R3/1 and cultured primary rat alveolar type II cells. Some aminoarenes and acetylamino derivatives have been shown to be genotoxic in vitro and in vivo, and 3-ABA and 3-AABA were reported to form several DNA adducts in vivo in multiple organs in rat. 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. The Comet assay can be applied to any tissue in an experimental animal 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.

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. 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. 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 using S. typhimurium TA98, TA100, YG1024, and YG1029. 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. Liver, kidney, spleen, lung, and bone
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 chemicals toward YG1029 and TA100 were higher than those in TA98. The mutagenicity of 3-NBA in the four strains is also shown in Table 1. 3-NBA 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,
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 and one major pathway for the bioactivation of 3-NBA is nitroreduction. Moreover, it has been shown that phase II enzymes, e.g. N-acetyltransferase and sulfotransferase, are also involved in the formation of highly reactive arylnitrenium ions from 3-NBA, which interact with cellular DNA. 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-acetyltransferases and sulfotransferases. Einistö et al. 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-Amino-
fluorene and 2-acetylaminofluorene showed 30- and
16-fold higher mutagenicity to YG1024, respect-
ively, compared with TA98. The mutagenic poten-
cies of 3-NBA in S. typhimurium strains were de-
creased 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 mutageniciti-
ies of 3-AABA were the lowest among these three
chemicals.

Sasaki et al. examined the in vivo genotoxicity
of 6 heterocyclic amines, i.e. 3-amino-1,4-dimethyl-
5H-pyrido[4,3-b]indole (Trp-P-1), 3-amino-1-methyl-
5H-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]quinoline (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. injec-
tion of these heterocyclic amines and that the or-
gans that were susceptible to the DNA-damaging
activities of Trp-P-2, IQ, MeIQ and MeIQx were simi-
lar; 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 sig-
nificant 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 blood-
stream. 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. 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
two 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 pu-
rine bases. These results suggest that 3-NBA, 3-ABA
and 3-AABA are metabolically activated in differ-
ent 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 in-
consistency 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 micro-
nuclei in mouse peripheral blood by i.p. treatment
at 25 and 50 mg/kg body weight. In this study,
DNA-damaging activity was detected in bone mar-
row 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 ob-
erved within a specific time-period after single treat-
ment with chemicals in the Comet assay, higher
DNA-damaging activity might be observed in the
bone marrow after a treatment period other than 3 hr.

Seidel et al. quantified 3-ABA in urine samples of
salt-mine workers who are exposed to diesel ex-
haust 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, we may
be exposed to 3-NBA and metabolize it to 3-AABA in
our body system. Our present study demonstrates
that both 3-NBA and 3-AABA 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 stud-
ies on their biological activities including carcino-
genicity 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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