Activation of aminophenylnorharman, aminomethylphenylnorharman and aminophenylharman to genotoxic metabolites by human N-acetyltransferases and cytochrome P450 enzymes expressed in Salmonella typhimurium umu tester strains

Yoshimitsu Oda1,*, Yukari Totsuka2, Keiji Wakabayashi2, F.Peter Guengerich3 and Tsutomu Shimada4

1Osaka Prefectural Institute of Public Health, Osaka 537-0025, Japan, 2National Cancer Center Research Institute, Tokyo 104-0045, Japan, 3Department of Biochemistry and Center in Molecular Toxicology, Vanderbilt University School of Medicine, Nashville, TN 37232, USA and 4Osaka City University Medical School, Osaka 545-8585, Japan

Norharman (9H-pyrido[3,4-b]indole) and harman (1-methyl-9H-pyrido[3,4-b]indole) contained in cigarette smoke and cooked foodstuffs, are non-mutagenic to Salmonella strains, but show co-mutagenicity with S9 mixture in the presence of aniline or o-toluidine. The resulting 9-(4′-aminophenyl)-9H-pyrido[3,4-b]indole (aminophenylnorharman, APNH), 9-(4′-amino-3′-methylphenyl)-9H-pyrido[3,4-b]indole (aminomethylphenylnorharman, AMPNH) and 9-(4′-aminophenyl)-1-methyl-9H-pyrido [3,4-b]indole (aminophenylharman, APH) are produced by coupling of norharman and aniline, norharman and o-toluidine, and harman and aniline in the presence of S9 mixture, respectively. To clarify the role of human cytochrome P450 (P450) and N-acetyltransferase (NAT) enzymes in the metabolic activation of APNH, AMPNH and APH, we determined the genotoxicity of these coupling chemicals using a variety of umu tester strains established in our laboratories. APNH, AMPNH and APH induced umuC gene expression more strongly in a bacterial O-acetyltransferase-overproducing strain than the parent strain. These chemicals were also found to induce umuC gene expression in NAT2-overexpressing strain at much higher rate than the NAT1-overexpressing strain. Among seven OY strains expressing human P450s and NADPH-P450 reductase used, the genotoxicity of APNH, AMPNH and APH was detected in OY1002/1A2 strain, OY1002/1A1 and OY1002/1A2 strains, and in OY1002/1A2 strain, respectively. From these results, it is concluded that APNH, AMPNH and APH are mainly bioactivated by P450 1A2 and NAT2, followed by NAT1 enzymes. P450 1A1 was also found to activate AMPNH at relatively slower rates.

Introduction

Epidemiological studies have shown that dietary factors and tobacco smoke greatly contribute to human cancer (1). In particular, the mutagenic and carcinogenic heterocyclic amines are of interest, since they are found in tobacco smoke condensates and cooked meat and fish. The β-carboline compounds norharman (9H-pyrido[3,4-b]indole) and harman (1-methyl-9H-pyrido[3,4-b]indole) are formed in the pyrolysis of tryptophan and are shown to be present at much higher levels than heterocyclic amines in tobacco condensates and cooked foods (2–4). Moreover, these chemicals are present in urine of patients receiving parenteral alimentation (5).

The β-carboline compound norharman and harman are not mutagenic themselves in Salmonella typhimurium TA100 and TA98 in the presence or absence of S9 mixture. Norharman becomes mutagenic to S.typhimurium TA98 and YG1024 when incubated with non-mutagenic aniline and o-toluidine in the presence of S9 mixture (6–8). Recently, the structures of the 9-(4′-aminophenyl)-9H-pyrido[3,4-b]indole (aminophenylnorharman, APNH), 9-(4′-amino-3′-methylphenyl)-9H-pyrido[3,4-b]indole (aminomethylphenylnorharman, AMPNH) and 9-(4′-aminophenyl)-1-methyl-9H-pyrido [3,4-b]indole (aminophenylharman, APH), produced by reaction with norharman and aniline, norharman and o-toluidine, and harman and aniline, respectively, were identified (9–12). APNH showed mutagenicity in S.typhimurium TA98 and YG1024 with a rat S9 mixture (10), DNA adduct formation of N4-(2′-deoxyguanosine-8-yl)-9-(4′-aminophenyl)-9H-pyrido[3,4-b]indole (dG-C8-APNH) in S.typhimurium TA98 and various organs of F344 rats (13,14), induction of sister chromatid exchange and chromosomal aberrations in cultured Chinese lung cells in vitro (15), and mutagenicity in the liver and colon of gpt delta transgenic mouse in vivo (16). In addition, we showed that human cytochrome P450 3A4 and P450 1A2 are mainly involved in the formation of APNH from norharman and aniline (17). Furthermore, APNH was demonstrated to induce liver and colon cancers in F344 rats (18).

To address the roles of human cytochrome P450 (P450) and N-acetyltransferase (NAT) enzymes in the bioactivation of genotoxic APNH, AMPNH and APH, we examined the genotoxicity of these chemicals using umu tester strains established in our laboratories (19–22). The genotoxicity was evaluated as induction of umuc gene expression, a measure of the induced cellular β-galactosidase activity when the tester strain is damaged with xenobiotic chemicals.

Materials and methods

Bacterial strains

The umu tester strains employed in this study were S.typhimurium NM2009 (O-acetyltransferase (O-AT)-overproducing strain), S.typhimurium TA1535/pSK1002 (parent strain) and S.typhimurium NM2000 (O-acetyltransferase-deficient strain) to examine the roles of O-AT with APNH, AMPNH and

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S. typhimurium NM6001 (human NAT 1-expressing strain), S. typhimurium NM6002 (human NAT 2-expressing strain) and S. typhimurium NM6000 (TA1538/1,8-DNP) (O-AT-deficient strain) were used for examining the roles of human NATs with these chemicals and the NAT enzyme activities in these strains used were described previously (21). Seven Salmonella strains—OY1002/1A1, OY1002/1A2, OY1002/1B1, OY1002/2C9, OY1002/2D6, OY1002/2E1 and OY1002/3A4—were derivatives of strain TA1535, expressing each of several human P450s and acetyltransferases.

Fig. 1. Chemical structures of APNH, AMPNH and APH and proposed mechanisms of bioactivation of these chemicals by human P450s and acetyltransferases.

Fig. 2. Induction of umuC gene expression (A, B and C) and cytotoxicity response (D, E and F) by APNH, AMPNH and APH in S. typhimurium tester strains TA1535/pSK1002 (open circles), NM2009 (filled triangles) and NM2000 (filled squares). β-Galactosidase activity (units) was determined as described in the Materials and methods. Cytotoxicity activities are expressed as % of the optical density change at 600 nm. Each point represents the means ± SD of twice or triplicate determinations.
CYP enzyme activities in each strain used were described previously (22). OY1002/pCW was the parent strain without expression of P450.

Chemicals and enzymes
APNH, AMPNH and APH were synthesized as described previously (11,12). Other chemicals used in this study were of the highest qualities commercially available. The chemical structures of three chemicals used in the present study are shown in Figure 1. Rat liver S9 fractions and cofactor for S9 mixture were obtained from Oriental Yeast Co. (Tokyo, Japan).

umu assay with addition of S9 mixture
umu assays were performed according to the procedure described by Oda et al. (20). The overnight culture was diluted 100-fold with TGA medium [1% bactotryptone (w/v), 0.5% NaCl (w/v), 0.2% glucose (w/v) and 20 µg/ml ampicillin]. The culture was incubated for 1 h at 37°C and 0.85 ml aliquots of TGA culture (OD600: 0.25–0.3), 0.15 ml of S9 mixture, and 10 µl test compound dissolved in DMSO were mixed and further incubated for 2 h.

umu assay using strains expressed human P450 and O-AT enzymes
The method described by Aryal et al. (23) was used with slight modification. The overnight culture of tester strains were diluted 100-fold in TGlyT medium [1% bactotryptone (w/v), 0.5% NaCl (w/v), 0.2% glycerol (v/v) and 1 µg tetracycline/ml] supplemented with 1.0 mM isopropyl [β-D-thiogalactoside, 0.5 mM δ-aminolevulic acid and 250 µl of trace elements/l. The culture was incubated for 2 h (37°C, 165 r.p.m.) and then 1-ml aliquots of TGlyT culture (OD600: 0.25–0.3) and 10 µl of test compound dissolved in DMSO were mixed and further incubated 3 h.

Induction of umuC gene as a response to DNA damage was determined by measuring the cellular β-galactosidase activity as described by Oda et al. (19). Cell toxicity was determined in reaction mixture by measuring the optical density change at 600 nm. The results are presented as means of two or three independent experiments.

Results
Comparison of genotoxic activities of APNH, AMPNH and APH in tester strains NM2009, TA1535/pSK1002 and NM2000
To evaluate whether or not O-AT-overexpressing NM2009 strain has a higher sensitivity than the parent TA1535/pSK1002 strain or the O-AT-deficient NM2000 strain for detecting genotoxic activities of APNH, AMPNH and APH, we compared the inducibility of umuC gene expression and

![Fig. 3. Induction of umuC gene expression (A, B and C) and cytotoxicity response (D, E and F) by APNH, AMPNH and APH in S.typhimurium tester strains NM6000 (open circles), NM6001 (filled triangles) and NM6002 (filled squares). Other details are as in the legend to Figure 2.](https://academic.oup.com/mutagenes/article-abstract/21/6/411/1396018)
cytotoxicity by three chemicals using these strains at different concentrations (Figure 2). APNH, AMPNH and APH were found to be highly genotoxic and cytotoxic in a concentration-dependent fashion in strain NM2009 after metabolic activation, followed by TA1535/pSK1002 and NM2000. In addition, the genotoxic activity with APNH was found to be more potent than those with AMPNH and APH.

Comparison of genotoxic activities of APNH, AMPNH and APH in tester strains NM6000, NM6001 and NM6002
To examine the roles of human NAT, NAT1 or NAT2 in the bioactivation of APNH, AMPNH and APH, we compared the sensitivities of two tester strains expressing NAT1-expressing strain (NM6001) and the NAT2-expressing strain (NM6002) and the parent strain (NM6000) toward the induction of the *umuC* gene expression and cytotoxicity by these chemicals (Figure 3). The NAT2-expressing strain exhibited slightly higher sensitivities for the induction of the *umuC* gene by APNH, AMPNH and APH as compared with NAT1-expressing strain. However, the NM6000 strain was found to be insensitive to these chemicals, although AMPNH induced *umuC* gene expression in this tester strain. Additionally, APNH was found to induce the *umuC* gene to a higher extent than AMPNH and APH.

Roles of P450 enzymes on the bioactivation of APNH, AMPNH and APH
We compared the sensitivity of seven tester strains (OY1002/1A1, OY1002/1A2, OY1002/1B1, OY1002/2C9, OY1002/2D6, OY1002/2E1 and OY1002/3A4) for the induction of the *umuC* gene expression by APNH, AMPNH and APH (Table I). Concentration-dependent increases in the induction of *umuC* gene expression by APNH and APH were observed in tester strain OY1002/1A2, and concentration-dependent increases in *umuC* induction by AMPNH were also observed in the OY1002/1A1 and OY1002/1A2 strains.

Effect of α-naphthoflavone on induction of *umu* operon by APNH, AMPNH and APH
Since α-naphthoflavone (α-NF) has been shown to be a selective inhibitor of P450 1A family enzymes, and we examined whether α-NF inhibits the genotoxicity induced by APNH, AMPNH and APH in strain OY1002/1A2 (Table II). The induction of *umuC* gene expression by the activated metabolites of APNH, AMPNH and APH was inhibited by 41, 58 and 61%, respectively, by the addition of α-NF to the OY1002/1A2 strain.

Discussion
APNH, AMPNH and APH, which are produced by reaction of norharman and aniline, norharman and o-toluidine, and harman and o-toluidine, respectively, in the presence of S9 mixture were reported to show mutagenic and clastogenic actions in bacterial and mammalian cells (10,11,15). In order to clarify the roles of human P450 enzymes and *N*-acetyltransferases in the metabolic activation of APNH, AMPNH and APH, we investigated the genotoxicity of these compounds by utilizing a *umu* assay using *S.typhimurium* strains, which express bacterial O-AT, different forms of human P450s, or NAT enzymes.

We demonstrated that APNH, AMPNH and APH are activated to genotoxic metabolites more strongly in O-AT-overexpressing strain NM2009 in the presence of S9 mixture than in the TA1535/pSK1002 and NM2000 strains. This activation is dependent on the presence of rat P450 enzymes. We demonstrated that APNH, AMPNH and APH were oxidized by P450 enzymes to convert an *N*-acetoxy derivative with O-acetyltransferase. Also, the genotoxic activity of APNH was found to be more potent than that of AMPNH or APH. These results show similar patterns with the results obtained in mutagenicity using *S.typhimurium* TA98 and YG1024 strains and sister chromatid exchange assay in Chinese hamster lung cells (10–12,18).

Using the O-AT-overexpressing strain NM2009, we examined the role of esterification of the activation of APNH, AMPNH and APH. In humans, two acetyltransferases (NAT1 and NAT2) are found to be present. Therefore, we investigated whether NATs activate these chemicals to genotoxic metabolites in NAT-strains. Human NAT2 showed slightly higher sensitivity than that of NAT1 to induce the cytotoxic and genotoxic effects of APNH, AMPNH and APH (Figure 3), indicating that NAT1 and NAT2 enzymes activate these compounds in tester strains.

**Table II** The effect of α-naphthoflavone on the metabolic activation of APNH, AMPNH and APH in *S.typhimurium* OY1002/1A2

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Activation of procarcinogens (BP)</th>
<th>Activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>APNH β-Galactosidase activity (units)</td>
<td>AMPNH β-Galactosidase activity (units)</td>
</tr>
<tr>
<td>None</td>
<td>329 ± 10 (100)</td>
<td>884 ± 28 (100)</td>
</tr>
<tr>
<td>α-Naphthoflavone</td>
<td>195 ± 11 (59)</td>
<td>367 ± 33 (42)</td>
</tr>
</tbody>
</table>

Control incubation were done with APNH (30 μM) and AMPNH (30 μM) or APH (100 μM). α-Naphthoflavone was added at concentration of 11 μM for APNH and AMPNH, and that of 55 μM for APH. The induced *umuC* gene expression was determined as described in Materials and Methods. Values represent means from triplicate determinations ± SD.

![Fig. 4. Formation of APNH by P450s. The two amines are proposed to be brought together in a P450 complex so that an *ipso* attack on the aniline can occur.](https://academic.oup.com/mutage/article-abstract/21/6/411/1396018)
In order to clarify the roles of human P450s in the metabolic activation of APNH, AMPNH and APH, we determined the relative genotoxic activities towards these chemicals using seven P450-expressing strains (Table I). Of the seven strains used, OY1002/1A2 (expressing P450 1A2) exhibited high activities with these chemicals, indicating that human P450 1A2 may be a major contributor to the activation of APNH, AMPNH and APH to genotoxic metabolites, although the activation of AMPNH was also catalyzed by P450 1A1. Furthermore, the induction of umuC gene expression by metabolic activation of APNH, AMPNH and APH was inhibited by 41, 58 and 61%, respectively, with α-NF in strain OY1002/1A2 (Table II). These results support the possible roles of P450 1A2 in the activation of these chemicals. However, the point should be made that P450 1A2 is predominantly a liver enzyme (24) and P450 1A1 may be more important in extrahepatic tissues (25).

Based on the present findings, we proposed a model for mechanisms of bioactivation of APNH, AMPNH and APH by human P450s and acetyltransferases in Salmonella as follows (Figure 1). First the excocyclic amino group of aminophenyl (methyl)norharman or aminophenylharman is converted to the hydroxylamine group by P450 1A enzymes followed by an O-esterification step catalyzed by N-acetyltransferase(s). Next, the ultimate reactive intermediates of these compounds are probably the nitrenium ions, which are formed after dissociation of the α-acetoxy group and these nitrenium ions react with DNA bases to form DNA adducts (e.g., dG-C8-APNH). The damaged DNA adducts induce umuC gene expression which is observed in the screen, and also presumably lead to mutation in other Salmonella typhimurium test systems.

Norharman is present at much higher concentrations than those of known mutagenic and carcinogenic heterocyclic amines in cigarette smoke condensates and cooked foods (4). Aniline and toluidine isomers are also present in cigarette smoke condensate, some vegetables and human urine (26–28), humans may be continuously exposed to these chemicals in daily life. In fact, it is reported that APNH can be detected in the urine of rats given norharman and aniline (29). In addition, APNH, a coupled compound formed from norharman and aniline (Figure 4) (30), shows carcinogenicity in the liver and colon of F344 rats (18). Therefore, it is important to determine whether APNH is produced in human body.

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