Genotoxic Activation of the Environmental Pollutant 3-Nitrobenzanthrone by Human Cytochrome P450 Enzymes Expressed in Salmonella typhimurium umu Tester Strains

Yoshimitsu Oda¹⁴, Tetsushi Watanabe², Hiroshi Yamazaki³ and Teruhisa Hirayama²

¹Osaka Prefectural Institute of Public Health, Osaka, Japan
²Department of Public Health, Kyoto Pharmaceutical University, Kyoto, Japan
³Laboratory of Drug Metabolism and Pharmacokinetics, Showa Pharmaceutical University, Tokyo, Japan

(Received July 4, 2007; Revised August 10, 2007; Accepted September 10, 2007)

3-Nitrobenzanthrone (3-NBA) is a mutagenic and carcinogenic compound identified in diesel exhaust, airborne particulate matter, soil, and water. To address whether 3-NBA shows genotoxic effects through any metabolic activation pathway, we determined the genotoxicity of 3-NBA using the parental strain Salmonella enterica serovar Typhimurium (S. typhimurium) TA1535/pSK1002, nitroreductase (NR)-deficient strain NM1000, bacterial O-acetyltransferase (O-AT)-deficient strain NM2000, and bacterial NR- and O-AT-overexpressing strain NM3009 established in our laboratory. In order to further clarify the role of human cytochrome P450 (P450 or CYP) enzyme in the bioactivation of 3-NBA to genotoxic metabolites, umu tester strains S. typhimurium OY1022/1A1, OY1022/1A2, OY1022/1B1, and OY1022/3A4, which express four respective human cytochrome P450 enzymes, NADPH-cytochrome P450 reductases (NPR) and O-AT were established by introducing two plasmids into S. typhimurium TA1535NR/1,8-DNP (nitroreductase-deficient and resistant to 1,8-dinitropyrene, probably due to functional loss of O-AT), one carrying both P450 and reductase cDNA in a bicistronic construct under control of an IPTG-inducible double tac promoter and the other, pOA102, carrying O-AT and umuC'-lacZ fusion genes. Induction of umuC gene expression could be monitored by measuring the cellular β-galactosidase activity produced by the umuC'-lacZ fusion gene. Although the induction of umuC by 3-NBA was weak in TA1535/pSK1002, NM1000, and NM2000 strains, the induction was considerably potent in NM2009 and NM3009 strains. 3-NBA was also found to induce umuC gene expression in OY1022/1A1 and OY1022/3A4 strains and, to a lesser extent, OY1022/1A2 and OY1022/1B1 strains, at a much higher rate than the parental OY1022/pCW strain. Collectively, these results suggest nitroreduction and O-acetylation by bacterial NR and O-AT, respectively, and nitroreduction by human CYP1A1, 3A4, 1A2, and 1B1 and NPR could contribute to the genotoxic activation of 3-NBA to produce reactive metabolites.

Key words: genotoxicity, 3-NBA, O-acetyltransferase, human cytochrome P450, SOS/umu assay

Introduction

Nitrated polycyclic aromatic hydrocarbons (nitro-PAHs) are a class of environmental contaminants that are potent mutagenic in bacterial and mammalian cells and carcinogenic in laboratory animals (1,2). Recently, 3-nitrobenzathrone (3-nitro-7H-benz[d]anthracen-7-one, 3-NBA) has been identified in diesel exhaust, airborne particulate matter, and soil and rainwater (3,5,6). 3-NBA is a powerful mutagen in the Ames test (3,4) and transgenic mouse assay (7), and genotoxic in human cells (8–10). It has also been reported to show specific DNA adduct formation in vitro, in cell culture and in vivo in rodents (11–15). Furthermore, 3-NBA is carcinogenic in F344 rats (16). 3-NBA has direct-acting mutagenicity in Salmonella enterica serovar Typhimurium (S. typhimurium) strains (3,4). This chemical is converted by bacterial nitroreductase (NR) to N-hydroxylated and then by O-acetyltransferase (O-AT) to esterified metabolites in S. typhimurium strains YG1021 overexpressing NR and YG1024 overexpressing O-AT (3,4). In contrast, although the genotoxic activation of 3-NBA in mammalian cells in vitro via nitroreduction catalyzed by cytosolic xanthine oxidase (17) and microsomal NADPH-cytochrome P450 reductase (18), and the participation of cytochrome P450 (CYP) enzymes in the oxidative metabolism of 3-NBA has been suggested (19), there is no direct evidence of the involvement of CYP enzymes in the nitroreduction of 3-NBA. The role of specific CYP enzymes in the metabolic pathways of 3-NBA has direct-acting mutagenicity in Salmonella enterica serovar Typhimurium (S. typhimurium) strains (3,4). This chemical is converted by bacterial nitroreductase (NR) to N-hydroxylated and then by O-acetyltransferase (O-AT) to esterified metabolites in S. typhimurium strains YG1021 overexpressing NR and YG1024 overexpressing O-AT (3,4). In contrast, although the genotoxic activation of 3-NBA in mammalian cells in vitro via nitroreduction catalyzed by cytosolic xanthine oxidase (17) and microsomal NADPH-cytochrome P450 reductase (18), and the participation of cytochrome P450 (CYP) enzymes in the oxidative metabolism of 3-NBA has been suggested (19), there is no direct evidence of the involvement of CYP enzymes in the nitroreduction of 3-NBA. The role of specific CYP enzymes in the metabolic pathways of 3-NBA has direct-acting mutagenicity in Salmonella enterica serovar Typhimurium (S. typhimurium) strains (3,4). This chemical is converted by bacterial nitroreductase (NR) to N-hydroxylated and then by O-acetyltransferase (O-AT) to esterified metabolites in S. typhimurium strains YG1021 overexpressing NR and YG1024 overexpressing O-AT (3,4). In contrast, although the genotoxic activation of 3-NBA in mammalian cells in vitro via nitroreduction catalyzed by cytosolic xanthine oxidase (17) and microsomal NADPH-cytochrome P450 reductase (18), and the participation of cytochrome P450 (CYP) enzymes in the oxidative metabolism of 3-NBA has been suggested (19), there is no direct evidence of the involvement of CYP enzymes in the nitroreduction of 3-NBA.
3-NBA remains unclear.

In the present study, we newly constructed the OY1022 strain by selecting resistant clones of TA1535NR capable of growth in the presence of 1,8-dinitropyrene to reduce the direct sensitivity to 3-NBA. Using this strain as a host, we established different strains expressing four recombinant human CYPs by introducing two plasmids into S. typhimurium OY1022, one carrying both CYP and NPR cDNA in a biocistron and the other, pOA102, carrying OAT and umuC- lacZ fusion genes, and investigated whether any human CYP enzymes are involved in the genotoxic activation of 3-NBA to its metabolites. Genotoxicity of 3-NBA was determined by using SOS/umu assay systems.

**Materials and Methods**

**Chemicals:** 3-Nitrobenzantrone was synthesized as described previously (20). All other chemicals and reagents were of the highest purity commercially available.

**Isolation of a dinitropyrene-resistant clone from the TA1535NR strain:** A dinitropyrene-resistant clone was isolated as described by McCoy et al. (21). Briefly, overnight cultures of Salmonella typhimurium TA1535NR (22) were plated onto Columbia base containing 5 μg of 1,8-dinitropyrene/mL. After incubation in the dark for two days, colonies were picked, recloned, and tested for a deep-rough character (sensitivity to crystal violet) (23) and sensitivity to 1,8-DNP (provided by Dr. F.P. Guengerich, Vanderbilt University School of Medicine, USA) (24, 25). These plasmids were then introduced into S. typhimurium TA1535NR/1,8-DNP (harboring pOA102) and the resulting strains were referred to as S. typhimurium OY1022/1A1, OY1022/1A2, OY1022/1B1, and OY1022/3A4, respectively (Table 1). In a similar way, the parental S. typhimurium OY1022/pCW strain was established by introducing the null vector pCW and pOA102 (Table 1).

**Determination of expression of CYP and NPR in S. typhimurium OY1022 cells:** The expression of different CYP enzymes and NADPH-cytochrome P450 reductase in S. typhimurium strains was determined according to the method previously described (26, 27). The content of CYP proteins in S. typhimurium cells on L-agar. In addition, we examined the induction of umuC gene expression by 1,8-DNP using a 1,8-DNP-resistant clone harboring pSK1002 and NM2000 (O-acetyltransferase-deficient strain). Both strains showed similar sensitivity to 1,8-DNP. Therefore, this 1,8-DNP-resistant clone appeared to have an O-acetyltransferase-deficient character. The 1,8-DNP-resistant clone (TA1535NR/1,8-DNP) was designated S. typhimurium OY1022.

**Bacterial strains and their construction:** The strains and plasmids used and the constructed strains are listed in Table 1. To establish S. typhimurium tester strains expressing human cytochrome P450 (CYP) and NADPH-cytochrome P450 reductase (NPR) genes, we employed four plasmids: pCW'/1A1/hNPR, pCW'/1A2/hNPR, pCW'/1B1/hNPR, and pCW'/3A4/hNPR (26, 27). The content of CYP proteins in S. typhimurium cells

---

**Table 1. umu tester strains and plasmids**

<table>
<thead>
<tr>
<th>S. typhimurium</th>
<th>Relevant genotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA1535</td>
<td>hisG46, gal, del (chl, uvrB, bio), tfα</td>
<td>(23)</td>
</tr>
<tr>
<td>TA1535NR</td>
<td>As TA1535 but is deficient in classical nitroreductase</td>
<td>(22)</td>
</tr>
<tr>
<td>TA1535/pSK1002</td>
<td>As TA1535 but harbors pSK1002</td>
<td>(26)</td>
</tr>
<tr>
<td>NM1000</td>
<td>As TA1535NR/pSK1002 but is deficient in classical nitroreductase</td>
<td>(34)</td>
</tr>
<tr>
<td>NM2000</td>
<td>As TA1535/1,8-DNP/pSK1002 but is deficient in O-acetyltransferase (O-AT)</td>
<td>(34)</td>
</tr>
<tr>
<td>NM3009</td>
<td>As TA1535/pSK1002 but has pNM12: O-AT-overexpressing strain</td>
<td>(34)</td>
</tr>
<tr>
<td>NM3009</td>
<td>As TA1535/pSK1002 but has pNM13:nitroreductase- and O-AT-overexpressing strain</td>
<td>(34)</td>
</tr>
<tr>
<td>OY1022</td>
<td>As TA1535NR but is resistant to 1,8-DNP probably due to O-AT deficiency</td>
<td>This work</td>
</tr>
<tr>
<td>OY1022/pCW</td>
<td>As OY1022 but harbors pCW and pOA102</td>
<td>This work</td>
</tr>
<tr>
<td>OY1022/1A1</td>
<td>As OY1022 but harbors pCW'-1A1/hNPR and pOA102</td>
<td>This work</td>
</tr>
<tr>
<td>OY1022/1A2</td>
<td>As OY1022 but harbors pCW'-1A2/hNPR and pOA102</td>
<td>This work</td>
</tr>
<tr>
<td>OY1022/1B1</td>
<td>As OY1022 but harbors pCW'-1B1/hNPR and pOA102</td>
<td>This work</td>
</tr>
<tr>
<td>OY1022/3A4</td>
<td>As OY1022 but harbors pCW'-3A4/hNPR and pOA102</td>
<td>This work</td>
</tr>
<tr>
<td>plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pACYC184</td>
<td>Tet', Cm'</td>
<td>(38)</td>
</tr>
<tr>
<td>pOA102</td>
<td>As pACYC184 but has umu lacZ gene and O-AT gene, Tet'</td>
<td>(27)</td>
</tr>
<tr>
<td>pCW</td>
<td>Amp'</td>
<td>(24)</td>
</tr>
<tr>
<td>pCW'/1A1/hNPR</td>
<td>As pCW but has human CYP 1A1 and NADPH-P450 reductase genes, Amp'</td>
<td>(25)</td>
</tr>
<tr>
<td>pCW'/1A2/hNPR</td>
<td>As pCW but has human CYP 1A2 and NADPH-P450 reductase genes, Amp'</td>
<td>(25)</td>
</tr>
<tr>
<td>pCW'/1B1/hNPR</td>
<td>As pCW but has human CYP 1B1 and NADPH-P450 reductase genes, Amp'</td>
<td>(28)</td>
</tr>
<tr>
<td>pCW'/3A4/hNPR</td>
<td>As pCW but has human CYP 3A4 and NADPH-P450 reductase genes, Amp'</td>
<td>(25)</td>
</tr>
</tbody>
</table>
Fig. 1. Cytotoxicity response and induction of *umuC* gene expression by 3-NBA in *S. typhimurium* tester strains TA1535/pSK1002 (filled circles), NM2009 (filled triangles), NM3009 (filled squares), NM1000 (open squares) and NM2000 (open triangles). Cytotoxicity activities are expressed as optical density change (z)

\[ O_D \] at 600 nm. β-Galactosidase activity (units) was determined as described in the Materials and Methods.

**umu assay**: *umu* assay was carried out according to the procedure described by Oda et al. (30). 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 1-mL aliquots of TGA culture (OD<sub>600</sub>: 0.25–0.3) and 10 μL tester compound dissolved in DMSO were mixed and further incubated for 2 h.

**umu assay using strains expressing human CYP and O-AT enzymes**: We used the method described by Aryal et al. (31) with a slight modification. The overnight culture of tester strains was 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 β-aminolevulinic acid, and 250 μL of trace elements/L (24). The culture was incubated for 2 h (37°C, 165 rpm) and then 1-mL aliquots of TGlyT culture (OD<sub>600</sub>: 0.25–0.3) and 10 μL of tester compound dissolved in DMSO were mixed and further incubated for 3 h. Induction of the *umuC* gene expression as a response to DNA damage was determined by measuring cellular β-galactosidase activity as reported by Oda et al. (30). The cytotoxic effect of the chemicals on bacterial cells was determined in the reaction mixture by measuring optical density change at 600 nm. The results are presented as the means of two or three independent experiments in Figs. 1 and 2.

**Roles of P450 enzymes in the metabolic activation of 3-NBA**: We compared the sensitivity of five tester strains (OY1022/1A1, OY1022/1A2, OY1022/1B1, OY1022/3A4, and OY1022/pCW) for the induction of *umuC* gene expression by 3-NBA (Fig. 2). Background levels of β-galactosidase activities in strains expressing hNPR-CYP were lower than in the parental strain; therefore, genotoxicity of 3-NBA in these strains was determined by Fe<sup>2+</sup> CO versus Fe<sup>2+</sup> difference spectra, according to the method of Omura and Sato (28). To determine NPR expression levels, the activity of NPR in sonicated bacterial cells was measured with cytochrome c as an electron acceptor by measuring the absorbance change at 550 nm at 20°C according to the method of Phillips and Langdon (29). The expression levels of CYP1A1, 1B1, 1A2 and 3A4 enzymes were determined to be approximately 110, 330, 110, and 110 nmol/L culture, respectively. NADPH-cytochrome c reductase activities in OY1022/1A1, OY1022/1B1, OY1022/1A2, and OY1022/3A4 strains were found to be 120, 30, 26, and 23 unit/L culture, respectively. Neither CYP protein nor NADPH-cytochrome c reductase activity was detected in *S. typhimurium* OY1022/pCW strain.

**umu assay**: The sensitivity of *S. typhimurium* TA1535/pSK1002 harboring the *umuC*-lacZ fusion gene, NM1000, NM2000, NM2009, and NM3009 strains toward cytotoxicity and genotoxicity of 3-NBA were examined (Fig. 1). 3-NBA was found to be highly cytotoxic and genotoxic in a concentration-dependent fashion in the O-AT-overexpressing strain NM2009, and NR- and O-AT-overexpressing strain NM3009. Sensitivities in cytotoxicity and genotoxicity in TA1535/pSK1002 (parental strain), NM1000 (nitroreductase-deficient strain), and NM2000 (O-AT-deficient strain) were similar (Fig. 1).
Genotoxic Activation of 3-NBA by Human CYP

Fig. 2. Induction of umuC gene expression by 3-NBA in S. typhimurium strains OY1022/pCW (open circles), OY1022/1A1 (filled circles), OY1022/1A2 (filled triangles), OY1022/1B1 (filled squares) and OY1022/3A4 (open squares). Other details are as in the legend to Fig. 1.

Table 2. Genotoxic potency of 3-NBA in S. typhimurium strains expressing human CYP enzymes

<table>
<thead>
<tr>
<th>Strains</th>
<th>β-Galactosidase activity (units)</th>
<th>Ratio Control (0 nM) to 3-NBA (1 nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OY1022/pCW</td>
<td>265 ± 25</td>
<td>451 ± 95</td>
</tr>
<tr>
<td>OY1022/1A1</td>
<td>85 ± 14</td>
<td>817 ± 71</td>
</tr>
<tr>
<td>OY1022/1A2</td>
<td>81 ± 4</td>
<td>607 ± 82</td>
</tr>
<tr>
<td>OY1022/1B1</td>
<td>101 ± 15</td>
<td>628 ± 58</td>
</tr>
<tr>
<td>OY1022/3A4</td>
<td>84 ± 9</td>
<td>820 ± 165</td>
</tr>
</tbody>
</table>

Data are the means of triplicate determinations (± S.D.). Ratio was calculated by dividing β-galactosidase activity for 3-NBA by activity in the control.

Expressed by the ratio to the control (0 nM of 3-NBA), 3-NBA at a concentration of 1 nM induced 9.8-, 9.6-, 7.5-, 6.2- and 1.7-fold increases in β-galactosidase activity in OY1022/3A4, OY1022/1A1, OY1022/1A2, OY1022/1B1 and OY1022/pCW, respectively (Table 2). These results indicated that 3-NBA was activated much more strongly in strains OY1022/3A4 and OY1022/1A1, and more strongly in OY1022/1A2 and OY1022/1B1 than the parental strain OY1022/pCW.

Discussion

We have previously reported a simple SOS/umu assay, which uses the measurement of β-galactosidase activity to detect umuC gene expression (30). This system was developed to detect a wide range of carcinogenic mutagens (30, 33) as well as the genotoxic activity of complex mixture samples such as environmental pollution (34), river and industrial wastewater (35, 36) and foodstuffs (37). Furthermore, we have shown that the umu test system, for which we constructed the nitroreductase- and O-acetyltransferase-overproducing NM3009 strain and O-acetyltransferase-overproducing NM2009 strain, is hypersensitive to the genotoxicity of nitroarenes (38) and aromatic amines (39). In this study, we determined the genotoxicity of 3-NBA by using these umu tester strains developed in our laboratory. We found that NM2009 and NM3009 strains exhibited high sensitivity to the cytotoxic and genotoxic effects of 3-NBA (Fig. 1). The results suggest that 3-NBA is bioactivated to the ultimate reactive metabolite(s) via nitroreduction followed by O-acetylation. The result is consistent with the results of an early study (4), which reported that 3-NBA is a potent mutagen in the S. typhimurium YGI024 strain overexpressing O-acetyltransferase. The order of sensitivity in the cytotoxicity of bacterial strains is almost parallel to that in genotoxicity. Molecular species that exhibit cytotoxicity might be the same as those exhibiting genotoxicity.

In the previous study, we showed that 1-nitropyrene (1-NP), a nitropolycyclic aromatic hydrocarbon, can be activated by human CYP1B1 to a genotoxic compound via nitroreduction/O-acetylation at low substrate concentrations by using a umu test system that added CYP enzymes from the outside. This influence should not be disregarded because nitroreductase is deficient in human cells, although 1-NP is activated by nitroreductase and O-AT existed in bacteria. Therefore, we developed a strain deficient in nitroreductase and O-AT. To clarify the roles of sensitivity in the cytotoxicity of bacterial strains is almost parallel to that in genotoxicity. Molecular species that exhibit cytotoxicity might be the same as those exhibiting genotoxicity.

In this study, the genotoxicity of 3-NBA was investigated in the umu assay using these strains. Among the strains used, OY1022/1A1 (expressing CYP1A1), OY1022/3A4 (expressing CYP3A4), OY1022/1A2 (expressing CYP1A2), and OY1022/1B1 (expressing CYP1B1), exhibited high genotoxic activity with 3-NBA, suggesting that human CYP1A1, 3A4, 1A2, and 1B1 are capable of efficiently bioactivating 3-NBA with co-expression of NPR (Table 2). Bieler et al. (40) have demonstrated that using the 32P-postlabeling assay, DNA binding of 3-NBA increased significantly in V79 cells expressing human CYP3A4 in conjunction with
human NPR, but not in V79 cells expressing human CYP1A1 alone. We used OY1022/1A1 strain expressing human CYP1A1 and NPR, and found that it activated 3-NBA. Thus, metabolic activation by 3-NBA could also be attributed to NPR. In fact, we observed that 3-NBA can be activated by NPR in OY1022/pOR (expressing human NPR), although the genotoxic activity of 3-NBA with the NPR-expressing strain was about 2-fold lower than with CYP3A4/hNPR- or CYP1A1/hNPR-expressing strains (data not shown). Taken together, these results supported the view that 3-NBA is mainly bioactivated by CYP1A1 and 3A4 in humans, followed by CYP1A2 and 1B1.

Human exposure to 3-NBA is thought to occur primarily through the respiratory tract. Although CYP contents in the human lung tissue are lower than in the liver, CYP enzymes in the lung may play a crucial role in extrahepatic activation of 3-NBA. Human CYP1A1 is expressed predominantly in extrahepatic organs, such as the lungs and mammary glands (41). Although human CYP3A4 is the most abundant CYP in human liver and the small intestine, it is expressed in only about 20% of individuals (42). Thus, bioactivation of 3-NBA in human lung cells may be responsible for potential human risk. Furthermore, with tester strains expressing human N,O-acetyltransferase and sulfotransferase enzymes, it is necessary to investigate whether N-OH-ABA, a 3-NBA-reduced metabolite, is further activated by acetylation or sulfation.

3-NBA is metabolized essentially via two pathways. In bacteria, 3-NBA is mainly activated by nitroreduction and O-acetylation, whereas in humans, it is probably activated by nitroreduction due to CYP1A1, 3A4, 1A2, and 1B1 and NPR, followed by O-acetylation. Based on the present findings, we proposed a model of the metabolic pathways of 3-NBA (Fig. 3).

In summary, we established new *umu* tester strains of *S. typhimurium* OY1022/1A1, OY1022/1A2, OY1022/1B1, and OY1022/3A4, which express four respective human CYP enzymes, NPR and bacterial O-AT, to

---

**Fig. 3.** Possible pathways for metabolic activation of 3-NBA.
clarify the role of human CYP enzyme in the bioactivation of 3-NBA to genotoxic metabolites. Using these tester strains, we demonstrated that genotoxic activation of 3-NBA can be catalyzed by human CYP3A4, 1A1, 1A2, and 1B1 and NPR to a genotoxin(s) in the presence of bacterial O-AT, probably via nitroreduction.

Acknowledgments: This study was supported by a Grant-in-Aid for Cancer Research from the Ministry of Health, Labour and Welfare of Japan and funds under a contract with the Ministry of the Environment of Japan.

References
1 Rosenkranz HS, Mermelstein R. Mutagenicity and genotoxicity of nitroarenes. All nitro-containing chemicals were not created equal. Mutat. Res. 1983; 114: 217–67.
24 Sandhu P, Guo Z, Baba T, Martin MV, Tukey RH, Guengerich FP. Expression of modified human cytochrome P450 1A2 in Escherichia coli: stabilization, purification, spectral characterization, and catalytic activi-


26 Oda, Y, Aryan P, Terashita T, Gillam EMJ, Guengerich FP, Shimada T. Metabolic Activation of heterocyclic amines and other procarcinogens in *Salmonella typhimurium*, *umu* tester strains expressing human cytochrome P450 A1, 1A2, 1B1, 2C9, 2D6, 2E1, and 3A4 and human cytochrome P450 reductase and bacterial O-acetylation transferase. Mutat Res. 2001; 492: 81–90.


