Mutagenicity and DNA Adduct Formation by the Urban Air Pollutant 2-Nitrobenzantrone

Volker M. Arlt,*1 Hansruedi Glatt,† Gonçalo Gamboa da Costa,*2 Jóhannes Reynisson,‡ and David H. Phillips*†

*Section of Molecular Carcinogenesis, Institute of Cancer Research, Sutton, Surrey SM2 5NG, United Kingdom; †Department of Toxicology, German Institute of Human Nutrition, D-14555 Potsdam-Rehbruecke, Nuthetal, Germany; ‡Cancer Research UK Centre for Cancer Therapeutics, Institute of Cancer Research, Sutton, Surrey SM2 5NG, United Kingdom; and §Department of Applied Chemistry, Kanagawa Institute of Technology, 1030 Shimo-ogino, Atsugi-shi 243-0292, Kanagawa, Japan

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2-Nitrobenzantrone (2-NBA) has recently been detected in ambient air particulate matter. Its isomer 3-nitrobenzantrone (3-NBA) is a potent mutagen and suspected human carcinogen identified in diesel exhaust. The highest mutagenic activity of 2-NBA tested in Salmonella typhimurium was exhibited in strain TA1538-hSULT1A1 expressing human sulfotransferase (SULT) 1A1. 2-NBA also induced mutations in Chinese hamster lung V79 cells expressing human N-acetyltransferase 2 or SULT1A1, but no mutagenicity was observed in the parental cell line. DNA adduct formation in vitro was examined in different human cell lines by thin-layer chromatography 32P-postlabeling. Whereas 3-NBA formed characteristic DNA adducts in lung A549, liver HepG2, colon HCT116, and breast MCF-7 cells, 2-NBA–derived DNA adducts were only observed in A549 and HepG2 cells, indicating differences in the bioactivation of each isomer. The pattern of 2-NBA–derived DNA adducts in both cell lines consisted of a cluster of up to five adducts. In HepG2 cells DNA binding by 2-NBA was up to 14-fold lower than by 3-NBA. DNA adduct formation of 2-NBA was also investigated in vivo in Wistar rats treated with a single dose of 2, 10, or 100 mg/kg body weight (bw). No DNA adduct formation was detected at doses of up to 10 mg/kg bw 2-NBA, even though 3-NBA induced DNA adducts at a dose of 2 mg/kg bw. Only after administration of one high dose of 100 mg/kg bw 2-NBA was a low level of DNA adduct formation detected, and only in lung tissue. Density functional theory calculations for both NBAs revealed that the nitrenium ion of the 3-isomer is considerably more stable (~10 kcal/mol) than that of the 2-isomer, providing a possible explanation for the large differences in DNA adduct formation and mutagenicity between 2- and 3-NBA.

Key Words: 2-nitrobenzantrone; 3-nitrobenzantrone; air pollution; mutagenicity; DNA adducts; 32P-postlabeling.

Numerous epidemiological studies have shown increased mortality and morbidity from respiratory and cardiovascular diseases due to ambient air pollution (Farmer et al., 2003; Vineis et al., 2004). A complex variety of genotoxins in ambient air pollution has been detected (Kyrtopoulos et al., 2001), and high exposures are associated with an increased risk of cancer. Amongst these are nitropolycyclic aromatic hydrocarbons (nitro-PAHs) which may be present on particulate matter from direct sources, such as diesel and gasoline exhaust (Tokiwa and Ohnishi, 1986) and may be produced from gas-phase reactions of PAHs with oxides of nitrogen, with subsequent partitioning to the particulate phase (Bamford et al., 2003). Both their long persistence in the environment and the mutagenic and carcinogenic properties of certain nitro-PAHs compared has led to considerable interest in assessing their potential risk to humans (Environmental Protection Agency [EPA], 2002; International Agency for Research on Cancer [IARC], 1989; International Programme on Chemical Safety [IPCS], 2003; Tokiwa et al., 1993). Although environmental levels are lower compared to unsubstituted PAHs (IPCS, 2003), it has become clear that certain nitro-PAHs exhibit high direct-acting mutagenic potency in bacterial bioassays and in forward mutation assays based on mammalian cells (Purohit and Basu, 2000).

The aromatic nitroketone 3-nitrobenzantrone (Fig. 1B; 3-NBA, 3-nitro-7H-benz[de]anthracen-7-one) is one of the most potent mutagens and a potential human carcinogen identified in diesel exhaust and ambient air particulate matter (Arlt, 2005; Enya et al., 1997; Nagy et al., 2005a). As a likely consequence of atmospheric washout, 3-NBA has also been detected in rain water and surface soil (Arlt, 2005). 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 workers occupationally exposed to diesel emissions (Seidel et al., 2002). There is clear evidence that 3-NBA is a genotoxic mutagen forming DNA adducts after metabolic activation through reduction of the nitro group (Fig. 1B) (Arlt et al., 2001a, 2003c, 2005, 2006; Stiborova et al., 2006).
The predominant DNA adducts detected in vivo in rodents are 3-(2′-deoxyguanosin-N2-yl)-2-aminobenzanthrone and 2-(2′-deoxyguanosin-8-yl)-3-aminobenzanthrone (Arlt et al., 2006) and are most probably responsible for the GC to TA transition mutations induced by 3-NBA in vivo (Arlt et al., 2004). These DNA adducts not only represent premutagenic lesions in DNA but may also be of primary importance for tumor development in target tissues (Bieler et al., 2005, 2007).

3-NBA is a potent carcinogen after intratracheal instillation in rats, inducing mainly squamous cell carcinoma in lung (Nagy et al., 2005a). 2-Nitrobenzanthrone (2-NBA, 2-nitro-7H-benz[de]anthracen-7-one), an isomer of 3-NBA, can be formed under atmospheric conditions from benzanthrone, a widely distributed contaminant in the atmosphere, and nitrogen oxides (Fig. 1A) (Enya et al., 1997, 1998a; Suzuki et al., 1997). It has been detected in ambient air samples in the United States and Japan (Phousongphouang and Arey, 2003; Tang et al., 2004). It has been suggested that 2-NBA might be formed more specifically by atmospheric processes while 3-NBA seems to be formed preferentially by combustion processes, such as in a diesel engine (Phousongphouang and Arey, 2003). In one air sample, atmospheric levels of 2- and 3-NBA were 495 and 6.8 pg/m³, respectively, with a ratio of 2-NBA/3-NBA of around 70 (Tang et al., 2004). Although little is yet known about the toxicity of 2-NBA, it has been shown to have strand-breaking activity in the comet assay and to form DNA adducts in human A549 lung cells and in vivo in rats, as measured by high-performance liquid chromatography (HPLC) 32P-postlabeling (Nagy et al., 2005b, 2007). Although the genotoxic potential of 2-NBA in these bioassays is lower than that of 3-NBA, its higher abundance in ambient air urges further investigation to evaluate its potential human health hazard.

The aim of the present study was to determine the mutagenic activity of 2-NBA in bacteria and in mammalian cells, and to examine the metabolism and DNA adduct formation of 2-NBA and its metabolite N-hydroxy-2-aminobenzanthrone (Fig. 1A; N-OH-2-ABA) in human cells in vitro and in rats in vivo by thin-layer chromatography (TLC) 32P-postlabeling analysis. Human cells and rats were also treated with 3-NBA and N-hydroxy-3-aminobenzanthrone (Fig. 1B; N-OH-3-ABA) in order to provide a basis for assessing the genotoxic potential of 2-NBA in comparison with its isomer 3-NBA.

MATERIALS AND METHODS

Synthesis of 2-NBA, 3-NBA, N-OH-2-ABA and N-OH-3-ABA. 2-NBA was synthesized as reported (Suzuki et al., 1997) and provided by T. Takamura-Enya. 3-NBA was prepared as described (Arlt et al., 2002). N-OH-2-ABA was prepared by reduction of 2-NBA with hydrazine and Pd/C catalysis, essentially as described previously for N-OH-3-ABA (Enya et al., 1998b; Osborne et al., 2005). Their authenticities were confirmed by ultraviolet spectroscopy, electrospray mass spectrometry, and high-field proton nuclear magnetic resonance spectroscopy.
**Bacterial strains and mutagenicity experiments.** Various *Salmonella typhimurium* strains were kindly provided by colleagues: TA1538 and TA98 by B.N. Ames (Berkeley, CA), TA1538/1.8-DNP by D. Wild (Kulmbach, Germany), TA98/1.8-DNP<sub>P</sub> by S. Knasmüller (Vienna, Austria), and YG1021, YG1024, YG1041, and YG1713 by T. Nohmi (Tokyo, Japan). Strain TA1538-hSULT1A1 has been described elsewhere (Glatt and Meinl, 2004). Strain YG1713-hSULT1A1 was constructed using the same expression vector (encoding the reference type variant of the protein, *SULT1A1*). Strains TA1538-hSULT1A1 and YG1713-hSULT1A1 showed similar SULT1A1 (sulfotransferase) protein levels (approximately 0.7–1.4% of the total cytosolic protein). Bacteria were grown in Oxoid Broth No. 2 (Oxoid GmbH, Wesel, Germany) overnight at 37°C in the presence of 50 µg/ml ampicillin (TA1538-hSULT1A1 and YG1713-hSULT1A1), 25 µg/ml ampicillin plus 12.5 µg/ml tetracycline (YG1021 and YG1024), 25 µg/ml ampicillin plus 25 µg/ml kanamycin (YG1041), or in the absence of antibiotics (remaining strains). The cultures were centrifuged, suspended in medium A (1.6 g/l Bacto Nutrient Broth + 5 g/l sodium chloride), adjusted nephelometrically to a titer of 1 to 2 × 10<sup>9</sup> bacteria (colony-forming units)/ml, and kept on ice. Shortly before use they were centrifuged again and suspended at a fivefold higher density in medium A.

Mutagenicity was determined using a modified version of the liquid-preincubation assay described by Maron and Ames (1983). The bacterial suspension (100 µl) and the test compound (in 10 µl dimethyl sulfoxide [DMSO]) were added sequentially to a glass tube containing 500 µl of 100mM magnesium sulfate. After incubation for 60 min at 37°C, 2.0 ml of 45°C warm soft agar (5.5 mg/ml agar, 5.5 mg/ml sodium chloride, 30 µl of 50mM sodium phosphate buffer, pH 7.4) was added, and the mixture was poured onto a Petri dish containing 24 ml of minimal agar (15 mg/ml agar + 5 g/l sodium chloride), adjusted nephelometrically to a titer of 1 to 2 × 10<sup>9</sup> bacteria (colony-forming units)/ml, and kept on ice. Shortly before use they were centrifuged again and suspended at a fivefold higher density in medium A.

**Mammalian cell lines and mutagenicity experiments.** Isolforms of human N-acetyltransferase (NAT) 2 (*NAT2*) and SULT1A1 (*SULT1A1*) were stably expressed in Chinese hamster V79 cells (subline V79MZ) as described elsewhere (Arlt et al., 2002). The SULT1A1 protein level of V79MZ-hSULT1A1 cells was similar to that of three human liver samples studied concurrently (Meinl et al., 2006), whereas the level of NAT2 was 20 times above the highest hepatic levels found among eight subjects (H.R. Glatt, unpublished results). V79MZ cells were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub>. X-chromosomal *hprt<sup>−</sup>* cells were maintained in Eagle’s MEM supplemented with 2mM L-glutamine, 1mM penicillin (100 units/ml), and streptomycin (100 µg/ml) at 37°C in a humidified 5% CO<sub>2</sub> atmosphere at 37°C. All media were purchased from Sigma-Aldrich, UK. Subculturing was performed every 72 h when the cells were 80% confluent and incubated in a humidified 5% CO<sub>2</sub> atmosphere at 37°C. For treatment A549 (1 × 10<sup>6</sup> cells), MCF-7 (2 × 10<sup>6</sup> cells), HCT116 (2.25 × 10<sup>6</sup> cells), and HepG2 cells (4 × 10<sup>6</sup> cells) were seeded in T75 flasks in a total volume of 15 ml (Arlt et al., 2001b; Hockley et al., 2006) and after 48 h the appropriate concentrations of 2-NBA, 3-NBA, N-OH-2-ABA, or N-OH-3-ABA dissolved in DMSO were added, and incubated for a further 24 h. Controls were treated with vehicle, DMSO, only.

All incubations were carried out in duplicate and cells were harvested by trypsinisation and then washed with PBS. Cell viability was determined as described (Arlt et al., 2003a). DNA was isolated from cells by phenol extraction essentially as reported previously (Arlt et al., 2001b).

**Animal treatment for DNA adduct analysis.** Female Wistar rats (*n* = 3 per group) were treated with a single dose of 2, 10, or 100 mg/kg body weight (bw) 2-NBA dissolved in tricaprylin by intraperitoneal (ip) injection. Additional groups of rats were treated ip with a single dose of 10 mg/kg bw N-OH-2-ABA or N-OH-3-ABA dissolved in DMSO. For 3-NBA (dissolved in tricaprylin) rats (*n* = 3) were treated with 2 mg/kg bw as described previously (Arlt et al., 2003b). In a further experiment rats (*n* = 3) were treated ip with 10 mg/kg bw 2-NBA dissolved in tricaprylin daily for 5 days. Control rats (*n* = 3) were received vehicle only. Animals were killed 24 h after treatment. Five organs (liver, lung, pancreas, colon, and kidney) were removed and stored at −80°C until DNA isolation by standard phenol/chloroform extraction.

**DNA adduct analysis using the TLC ³²P-postlabeling assay.** DNA adducts were measured in each DNA sample using the butanol enrichment version of the ³²P-postlabeling method as described previously (Arlt et al., 2002) with minor modifications. Briefly, DNA samples (4 µg) were digested with micrococcal nuclease (120 µU, Sigma) and calf spleen phosphodiesterase (40 µU, Calbiochem, UK), extracted with butanol and labeled as reported. Chromatographic conditions for TLC on polyethyleneimine-cellulose were D1, 1.0M sodium phosphate, pH 6.0; D3, 4.0M lithium formate, 7M urea, pH 3.5, D4, 0.8M lithium chloride, 0.5M Tris, 8.5M urea, pH 8.0. DNA adduct levels (RAL, relative adduct labeling) were calculated from the adduct cpm, the specific activity of [³²P]-adenosine triphosphate, and the amount of DNA (pmol of DNA-P) used. 3-NBA-derived DNA adducts were identified using authentic standards as described previously (Arlt et al., 2006). Results were expressed as DNA adducts/10<sup>9</sup> nucleotides (nt).

**Quantum chemical calculations.** The energy calculations and geometry optimizations were performed with the GAUSSIAN 03 program suite (Frisch et al., 2003) utilizing restricted density function theory (DFT). The nonlocal B3LYP functional hybrid method was employed (Becke, 1988, 1993; Lee et al., 1998). The standard 6-31G(d,p) basis set (Hariharan and Pople, 1973) was used for the geometry optimization and frequency analysis. The zero-point vibrational energies were scaled according to Wong (0.9804) (Wong, 1996). Subsequent single-point energy calculations were performed with the larger 6-311G(2df,p) basis set. The electron affinities were calculated as described by Foresman and Frisch (1996) and the bond dissociation energies (BDE) as described previously (Reynisson and Steenken, 2004). The effect of the aqueous phase was simulated with Self-Consistent Isodensity Polarized Continuum model (Tomasi et al., 2005) using the same method as described above except that a slightly smaller 6-31G basis set was used for the geometry optimization and frequency analysis. In all the cases, the molecular species were reoptimized within the applied continuum except for the nitrateligandadducts of the nitrate (−NO<sub>2</sub>) derivatives where the structures, optimized in vacuum, were used. The Gibbs free energy was calculated according to the following equation: 

\[
\Delta G_m = E^{E+C+\text{H}+\text{L}+\text{P}} - E^{E+\text{C}+\text{H}+\text{L}+\text{P}}
\]

The Natural Population Analysis (NPA) (Foster and Weinhold, 1980) and Mulliken’s method (Mulliken, 1962) were used to calculate the charge densities for the molecular systems at the same level of theory as the single-point energy calculations.

**RESULTS**

**Mutagenicity in Bacteria**

2-NBA was directly mutagenic to the standard *Salmonella typhimurium* strains TA1538 and TA98 even at low substrate concentrations (Fig. 2, Table 1). These strains differ by the absence and presence, respectively, of plasmid pKM101, encoding a translesion-synthesis DNA polymerase. Since the
mutagenic activity of 2-NBA was similar in both strains, over-expression of this polymerase does not appear to be important in the fixation of 2-NBA-induced mutations in this system.

Strains lacking endogenous acetyltransferase (OAT), TA1538/1,8-DNP and TA98/1,8-DNP₆, showed reduced mutagenic responses to 2-NBA (by a factor of approximately three) compared to the parental strains TA1538 and TA98, whereas overexpression of this enzyme (in strain YG1024 compared to TA98, and YG1041 compared to YG1021) had little effect (Table 1). However, expression of another enzyme that could catalyze the formation of reactive esters from N-OH-2-ABA, human SULT1A1, enhanced the mutagenicity 2.8-fold in TA1538 background strains and 60-fold in YG7131 strains. In the latter strains the nitroreductase gene is disrupted (Table 1). This disruption led to a 200-fold decrease in the mutagenic response to 2-NBA in the absence of human SULT. The decrease resulting from nitroreductase deficiency was less, but still ninefold, when human SULT1A1 was expressed (Table 1). Plasmid-mediated overexpression of the nitroreductase enhanced the mutagenicity twofold (in strain YG1021 compared to TA98) and fourfold (in YG1041 compared to YG1024).

**Mutagenicity in Mammalian Cells**

2-NBA did not induce gene mutations (determined at the *hprt* locus) in normal Chinese hamster V79MZ cells, at any concentration used (0.03–30 μM). However, when either human NAT2 or SULT1A1 was expressed in these cells, a mutagenic effect was observed with 2-NBA even at a concentration of 0.1 μM (Fig. 3A). The concentration–response curves were similar for

<table>
<thead>
<tr>
<th>Strain Characteristics</th>
<th>Mutagenic activity of 2-NBA [revertants per nmol]</th>
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<tr>
<td>TA1538 Standard strain</td>
<td>3700</td>
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<tr>
<td>TA1538/1,8-DNP</td>
<td>TA98/1,8-DNP₆ freed from pKM101 (~TA1538 lacking OAT)</td>
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<tr>
<td>TA1538-hSULT1A1</td>
<td>TA1538 with plasmid-mediated expression of human SULT1A1</td>
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<tr>
<td>YG7131</td>
<td>TA1538 with targeted disruption NR</td>
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<tr>
<td>YG7131-hSULT1A1</td>
<td>YG7131 with plasmid-mediated expression of human SULT1A1</td>
</tr>
<tr>
<td>TA98</td>
<td>Standard strain (~TA1538 plus pKM101)</td>
</tr>
<tr>
<td>TA98/1,8-DNP₆</td>
<td>TA98-derived clone resistant to 1,8-dinitropyrene (due to lack of OAT)</td>
</tr>
<tr>
<td>YG1024</td>
<td>TA98 with plasmid-mediated strong overexpression of OAT</td>
</tr>
<tr>
<td>YG1021</td>
<td>TA98 with plasmid-mediated strong overexpression of NR</td>
</tr>
<tr>
<td>YG1041</td>
<td>TA98 with plasmid-mediated strong overexpression of NR and OAT</td>
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*a*All strains contain the same frameshift mutation in the his operon (deletion of a C in the *hisD* gene). TA98 differs from TA1538 by the presence of plasmid pKM101, which contains a gene encoding a translesion-synthesis DNA polymerase. NR, endogenous “classical” nitroreductase of *Salmonella typhimurium*; hSULT1A1, human sulfotransferase 1A1.

*b*Calculated from the initial slope of the dose–response curves (Fig. 2).
both enzymes. However, the expression level of SULT1A1 in the recombinant cell was similar to that observed in some human tissues (liver and small intestine), whereas the relative expression level of NAT2 was 20 times higher in the cell line.

In other experiments, the metabolite N-OH-2-ABA was tested for mutagenicity (Fig. 3B). Unlike 2-NBA, this metabolite was mutagenic in parental V79MZ cells. Expression of human conjugating enzymes enhanced this mutagenicity. The enhancement was stronger with NAT2 than with SULT1A1.

**DNA Adduct Formation in Mammalian Cells Expressing Recombinant Human NAT2 or SULT1A1**

For DNA adduct analysis parental and recombinant V79 cells were treated with 3μM 2-NBA. Multiple DNA adducts were detected in V79MZ-derived cells expressing human NAT2 or SULT1A1, whereas no DNA adducts were observed in the parental cell line V79MZ (Fig. 4A), suggesting that DNA adduct formation is directly linked to the observed mutagenicity in these cells (see above). The DNA adduct pattern consisted of a cluster of up to seven adducts, the major ones were also detected in human A549 and HepG2 cells treated with 2-NBA (see below; compare Fig. 6). DNA binding in V79MZ-derived cells expressing human NAT2 (140 adducts/10⁸ nt) was slightly higher than those expressing human SULT1A1 (108 adducts/10⁸ nt) (Fig. 5A).

DNA adduct formation was also investigated in V79 cells after treatment with N-OH-2-ABA. At 0.1μM multiple DNA adducts were detected in V79MZ-derived cells expressing human NAT2 or SULT1A1 similar to those observed in parental V79MZ cells treated with 2-NBA. Spot 1a (Figs. 4B and 4C) was the most abundant DNA adduct found; in the parental cell line V79MZ spot 1a was the only DNA adduct detected (Fig. 4D). DNA binding in V79MZ-derived cells expressing human NAT2 or SULT1A1 was up to sevenfold higher than DNA binding in parental V79MZ cells (Fig. 5B).

**DNA Adduct Formation in Human Cells**

Human cells (A549, HepG2, HCT116, and MCF-7) treated with 2-NBA, 3-NBA, N-OH-2-ABA, or N-OH-3-ABA were analyzed by TLC ³²P-postlabeling using butanol enrichment. In A549 and HepG2 cells, the DNA adduct pattern induced by 2-NBA consisted of a cluster of up to five adduct spots (adducts 1a–5a) (Figs. 6A, 6B, and 6C). No DNA adducts were detected in control cells (data not shown). DNA adduct formation was concentration dependent, with relative adduct levels of 165/10⁸ nt and 213/10⁸ nt in A549 and HepG2 cells at the highest 2-NBA concentration (5μM) tested, respectively (Fig. 7A). Similarly, DNA adduct formation was observed in A549 and HepG2 cells treated with 3-NBA (Figs. 6D, 6E, and 6F); however, DNA binding by 3-NBA was up to 14-fold higher than by 2-NBA in HepG2 cells (Fig. 7B). Interestingly, no 2-NBA-derived DNA adducts were observed in A549 and HepG2 cells treated with 3-ABA (Figs. 6D, 6E, and 6F).

![FIG. 3. Induction of gene mutations (at the hprt locus) by 2-NBA (A) or N-OH-2-ABA (B) in standard Chinese hamster V79MZ cells and in V79MZ-derived cell lines engineered for expression of human NAT (hNAT) 2 or SULT (hSULT) 1A1. Values are means ± SE of three cultures. Cell viability, as indicated by the number of cells harvested at the first subcultivation after treatment compared to the corresponding value of cultures treated with the solvent only was ≥70% and ≥50% after treatment with 2-NBA and N-OH-2-ABA, respectively.](https://academic.oup.com/toxsci/article-abstract/98/2/445/1709295)

![FIG. 4. Autoradiographic profiles of ³²P-postlabeled DNA adducts obtained in standard Chinese hamster V79MZ cells treated with (A) 3μM 2-NBA or (D) 0.1μM N-OH-2-ABA. Autoradiographic profiles of ³²P-postlabeled DNA adducts obtained in V79MZ-derived cell lines engineered for expression of human NAT (hNAT) 2 treated with (B) 3μM 2-NBA or (E) 0.1μM N-OH-2-ABA or SULT (hSULT) 1A1 treated with (C) 3μM 2-NBA or (F) 0.1μM N-OH-2-ABA.](https://academic.oup.com/toxsci/article-abstract/98/2/445/1709295)
adducts were found in HCT116 and MCF-7 cells (Figs. 6Ca and 6Da), whereas under the same experimental conditions DNA adduct formation by 3-NBA was observed clearly (Figs. 6Cb and 6Db).

DNA adducts were detected in all human cells treated with N-OH-2-ABA (Fig. 6Ac). The DNA adduct pattern consisted of a cluster of up to five adduct spots (adducts 1a–5a), adducts 1a, 2a, and 3a being the major ones detected, similar to the adduct pattern observed in 2-NBA–treated A549 and HepG2 cells (compare Figs. 6Aa and 6Ba). The extent of DNA binding in the latter cells treated with 2-NBA and N-OH-2-ABA was similar (Fig. 7C). The same characteristic DNA adduct pattern was found in all human cells treated with N-OH-3-ABA as that formed by 3-NBA (Fig. 6Ad). However, DNA binding by N-OH-3-ABA in human cells was lower compared with 3-NBA (Figs. 7B and 7C), whereas the extent of DNA binding by N-OH-2-ABA and N-OH-3-ABA varied markedly between cell lines (Fig. 7C).

**DNA Adduct Formation in Rats**

The formation of DNA adducts in various organs (liver, lung, pancreas, colon, and kidney) of female Wistar rats treated with 2- or 3-NBA was analyzed using the butanol enrichment version of the TLC 32P-postlabeling method (Fig. 8; Table 2). As shown in Figure 8C, the DNA adduct patterns identical to those found previously (Arlt et al., 2001a, 2003b, 2006) were detected in all organs of rats treated with 2 mg/kg bw 3-NBA. DNA binding was similar to that found in a study performed previously under the same experimental conditions, although DNA binding in pancreas was approximately threefold lower in the present study (Arlt et al., 2003b). The highest DNA binding was observed in kidney with 134 adducts/10^8 nt, followed by lung (89/10^8 nt), colon (87/10^8 nt), and pancreas (84/10^8 nt) (Table 2). In contrast, using equal amounts of 2-NBA (2 mg/kg bw; single administration) DNA adducts were not detectable in any of the organs investigated (Fig. 8B; Table 2). No DNA adducts were observed in controls (Fig. 8A). Single or repeated (once daily for 5 days) treatment of rats with 10 mg/kg bw 2-NBA also resulted in no DNA adduct formation in any of the organs (Figs. 8D and 8E; Table 2). Only a single treatment with 100 mg/kg bw 2-NBA resulted in the formation of DNA adducts at a low level (5 adducts/10^8 nt) in lung (Fig. 8Fb), but not in liver (Fig. 8Fb), pancreas, colon, or kidney (Table 2). In lung one adduct was detected (Fig. 8Fa) and comparative analyses on TLC indicated that it was chromatographically indistinguishable from adduct 2a found in DNA of human cells treated with 2-NBA (compare Fig. 6).

**FIG. 5.** Levels of total DNA adducts in standard Chinese hamster V79MZ cells and V79MZ-derived cell lines engineered for expression of human NAT (hNAT) 2 or SULT (hSULT) 1A1 after exposure to 3 μM 2-NBA (A) or 0.1 μM N-OH-2-ABA (B). Values represent mean ± SD of two separate incubations; each DNA sample was determined by two independent 32P-postlabeling analyses. ND = not detected.

**FIG. 6.** Autoradiographic profiles of 32P-postlabeled DNA adducts obtained in A549 (A), HepG2 (B), HCT116 (C), and V79MZ cells (D) treated with 5 μM 2-NBA (Aa–Da), 5 μM 3-NBA (Ab–Db), 5 μM N-OH-2-ABA (Ac–Dc), or 5 μM N-OH-3-ABA (Ad–Dd).
We also examined DNA adducts in female Wistar rats treated with 10 mg/kg bw of the reactive N-hydroxy arylamine metabolites, N-OH-2-ABA and N-OH-3-ABA (Fig. 9; Table 2). Characteristic DNA adduct patterns were observed in all organs investigated (liver, lung, pancreas, colon, and kidney) in rats treated with N-OH-3-ABA (Fig. 9C), identical to those found with the parent nitro-PAH (compare Fig. 8C). The highest DNA adduct levels were found in pancreas (430/10^8 nt), followed by lung (312/10^8 nt) and kidney (262/10^8 nt) (Table 2). In rats treated with N-OH-2-ABA DNA adducts were found in all organs investigated, although DNA binding was quite low, in the range of 1–2 adducts/10^8 nt (Table 2). The DNA adduct pattern consisted of two adducts in lung, kidney, colon, and pancreas, whereas only one adduct was detectable in liver (Fig. 9B). Comparative analyses on TLC with DNA of human cells treated with 2-NBA indicated that these two adducts were chromatographically indistinguishable from adducts 1a and 2a (compare Fig. 6).

Quantum Chemical Calculations

Previously, it has been shown that the activity of cytosolic nitroreductases toward certain nitro-aromatics is closely related to their single-electron reduction potential (Iwata et al., 1992). This implies that the rate determining step in this process is the addition of an electron to the organic molecule, in particular its nitroreduction. Therefore, as shown in Figure 1 we calculated the electron affinity for 2- and 3-NBA which reflects the energy required to reduce them. The electron addition without solvent simulation is exothermic for both isomers with /C255 42.8 and /C255 47.6 kcal/mol for 2- and 3-NBA, respectively, the 2-isomer reduction being slightly less exothermic. Taking into account the effects of the aqueous phase the exothermicity increases considerably to /C255 71.6 and /C255 77.4 kcal/mol for 2- and 3-NBA, respectively. This increase is mainly due to the enhanced solubility of the radical anions compared to their parent compounds by ~30 kcal/mol. Both isomers are easily reduced according to these calculations.
The heterolytic BDE for both N-OH-ABAs (Fig. 1) were also calculated. In vacuum N-OH-2-ABA has ~15 kcal/mol larger BDE than N-OH-3-ABA. To include the effects of the aqueous phase several aspects need to be taken into account since it involves protonation and formation of water as the leaving group. First, the \( \Delta G_{aq} \) for N-OH-2-ABA is calculated to be ~4.9 kcal/mol and ~4.8 kcal/mol for N-OH-3-ABA; the \( \Delta G_{aq} \) for their corresponding nitrenium cations are ~48.5 and ~45.6 kcal/mol, respectively. Second, for the protonation of the OH-moiety H\(^{+}\) was dehydrated from the aqueous phase. The measured hydration energy of a proton is \( \Delta G_{aq} = -263.9 \) kcal/mol (Tissandier et al., 1998). Thirdly, a heterolytic bond formation of water occurs and this energy has been experimentally determined as ~390.7 kcal/mol (Schulz et al., 1982). Combining these values, as shown in Figure 1, both isomers display moderate exothermicities in the 30 to 40 kcal/mol range, i.e., the water elimination is expected to occur and not to be the rate limiting step in this reaction cascade, although the activation energy may play a role.

The relative stability of the nitrenium intermediate appears to be a crucial factor in determining the biological activity of aromatic amines (Kadlubar and Beland, 1985). Therefore, the relative energies, or energy difference, of the nitrenium cations and their related amino compounds, 2- and 3-ABA, as a comparative model, were calculated. The energy difference between the amino compounds (2- and 3-ABA) was negligible with (0.5 kcal/mol) and without (~0.2 kcal/mol) solvent simulation (data not shown). However, a large difference was observed for the corresponding nitrenium ions, that of the 3-isomer being much more stable than its 2-isomer by ~9.4 kcal/mol with and ~12.2 kcal/mol without solvent simulation (data not shown). This observation can be explained in terms of the possible conjugation patterns of these two systems as seen in Figure 10, i.e., the 3-isomer delocalizes the nitrenium ion positive charge in a more effective way than the 2-isomer. Although the aqueous solution stabilizes the organic cations, reducing to some extent the energy difference between them, the difference is still being considerable, indicating that the nitrenium ion of the 2-isomer is conceivably more reactive than that of the 3-isomer.

The charge distribution was calculated with both the Mulliken and the NPA methods (Fig. 10). In order to elucidate the conjugation pattern of the nitrenium cations their charge
difference between them and their amino counterparts were derived. By performing this subtraction it is possible to locate the atoms that are principally affected by the introduction of a positive charge to the conjugated systems of these rigid and planar molecules. These results show that the 3-isomer delocalizes the charge more effectively than the 2-isomer, which correlates with the energy calculations. The solvent effects on the charge distribution were insignificant. The fact that both the Mulliken and NPA methods predicted similar changes in the charge distribution supports the reliability of the theoretical methods used.

**DISCUSSION**

In a recently published mutagenicity study conducted independently of the present investigations, 2-NBA was around 2000 times less potent than 3-NBA in YG1024, the strain with the highest response toward 3-NBA (Takamura-Enya et al., 2006). Some strains (TA98, TA98/1,8-DNP6, YG1021, and YG1024) have been used as well in our investigation. The largest difference between the studies is seen in strain TA98, with 160 revertants/nmol in the previous study and 3000 revertants/nmol in the present study. The higher response in our study might be due to differences in the assay conditions; we use higher cell densities, a longer liquid-preincubation time, and culture media higher in magnesium sulfate (essential for SULT-expressing strains) than other laboratories. Interestingly, deficiency of OAT reduced the response, whereas overexpression of this enzyme enhanced the activity. Strains expressing a human SULT were used only in our study. Expression of SULT1A1 enhanced the mutagenicity of 2-NBA. The increase amounted to 2.8-fold using TA1538 as the recipient strain. It was stronger (60-fold) with the nitroreductase-deficient recipient strain YG7131, starting from a much lower background level than in TA1538. Thus, it may be hypothesized that human SULT1A1 was superior to the bacterial acetyltransferase in bioactivation at the low substrate concentrations of N-OH-2-ABA intermediate expected to be present in nitroreductase-deficient strains.

In the present study it is reported for the first time that 2-NBA is capable of inducing gene mutations in mammalian cells. Mutagenicity was not observed in the parental V79MZ cell line, which is used in standard gene mutation, micronucleus and chromosomal aberration tests. However, when human NAT2 or SULT1A1 were expressed in this cell line, mutagenicity was detected even at very low concentrations of 2-NBA (0.1μM). Unlike 2-NBA, its metabolite N-OH-2-ABA was mutagenic in parental V79MZ cells. It is not known whether N-OH-2-ABA or another metabolite produced by the V79MZ cells induced the mutations. Expression of human NAT2 or SULT1A1 enhanced the mutagenicity of N-OH-2-ABA, in agreement with the effects observed on 2-NBA. Using TLC 32P-postlabeling multiple DNA adducts were detectable in V79MZ-derived cells expressing human NAT2 or SULT1A1.
treated with 2-NBA (3μM) similar to those observed in human cells (see below), whereas no DNA adducts were found in the parental cell line V79MZ. Higher DNA binding was also observed in V79MZ-derived cells expressing human conjugating enzymes compared to parental cells after treatment with N-OH-2-ABA at the low concentration tested (0.1μM). Thus, expression of human conjugating enzymes NAT2 or SUPT1A1 strongly contributes to the genotoxicity and mutagenicity of 2-NBA and N-OH-2-ABA as has been observed previously for 3-NBA (Arlt et al., 2002, 2003a, 2005). Differences in the modulating effects of the enzymes may be due to variations in the time course of the exposure to N-OH-2-ABA, i.e., initially high with a relatively rapid decrease when N-OH-2-ABA is directly used, lower but more continuous when it is produced by the cells from 2-NBA.

When human cells treated with 2-NBA were used to investigate DNA adduct formation a cluster of up to five adducts were detected using TLC 32P-postlabeling. All of them were primarily located in a diagonal zone on the TLC plates at approximately 45° to elution directions D3 and D4, similar to DNA adducts formed by 3-NBA (Arlt et al., 2001a, 2006; Osborne et al., 2005) and typical of DNA adducts derived from extracts of airborne particulate matter and diesel exhaust (Gallagher et al., 1991; Gupta, 1985). In a recent study 2-NBA-derived DNA adducts were observed in A549 and HepG2 cells by HPLC 32P-postlabeling (Arlt et al., 2007), at levels about 1/3 and 1/20, respectively, of the levels of 3-NBA–DNA adduct formation. HepG2 cells have been proven to be a sensitive cell line to examine the genotoxicity of 3-NBA as measured by the induction of single strand breaks (comet assay) and micronuclei (Lamy et al., 2004). Similarly, in the present study HepG2 cells were the most sensitive human cell line but DNA adduct levels derived from 3-NBA were up to 2.5- and 14-fold higher than by 2-NBA in A549 and HepG2 cells, respectively. In contrast to 3-NBA, surprisingly, no DNA adduct formation was observed in HCT116 and MCF-7 cells, indicating that these human cells are not metabolically competent to activate 2-NBA. Since DNA adducts were detected in all human cells exposed to N-OH-2-ABA, it seems that 2-NBA is a poor substrate for nitroreductases in HCT116 and MCF-7 cells. MCF-7 cells are capable of activating other nitro-PAHs by simple nitroreduction leading to the formation of covalent DNA adducts (Arlt et al., 2001b; Sun et al., 2004). Moreover, it has been shown that DNA binding varied among different mononitropyrene isomers tested, with 4-nitropyrene binding to DNA in MCF-7 cells to a greater extent than 1- and 2-nitropyrene (Sun et al., 2004). Thus, it remains to be investigated which enzyme(s) are responsible for the reduction and subsequent metabolic activation of 3-NBA, but not 2-NBA, in HCT116 and MCF-7 cells. Nitroreduction for 3-NBA is primarily catalyzed by cytosolic nitroreductases such as NQO1 (Arlt et al., 2005; Stiborova et al., 2006). Therefore, further studies will focus on the ability of human NQO1 to bioactivate 2-NBA. In addition, investigations on the expression of cytosolic and microsomal reductases in A549, HepG2, HCT116, and MCF-7 cells after exposure to 2- and 3-NBA may help to explain these findings.

During the preparation of this manuscript, others investigated DNA adduct formation by 2-NBA in vivo (Nagy et al., 2007). DNA adduct formation in F344 rats after a single intratracheal instillation of 5 mg/kg bw 2-NBA was examined by HPLC 32P-postlabeling. Several peaks on the HPLC related to 2-NBA–derived DNA adduct formation were found at an overall level approximately 80% of that formed by 3-NBA. In contrast, in the present study we did not detect DNA adduct formation at doses of up to 10 mg/kg bw 2-NBA after intraperitoneal treatment, after either single or repeated administration. Only after administration of one high dose of 100 mg/kg bw 2-NBA was a low level of DNA adduct formation detected, and then only in lung tissue. In the light of the detection of 2-NBA–derived DNA adducts in vivo at lower doses by Nagy and coworkers we would have expected to observe DNA adduct formation in the present study. It should be noted that in the two studies different strains of rats were used (Wistar vs. F344 rats), different routes of administration were applied (intraperitoneal vs. intratracheal administration), and DNA adducts were analyzed in different ways (TLC vs. HPLC 32P-postlabeling), but whether these or other factors account for the difference in detection of 2-NBA–derived DNA adducts between the two studies is unclear, and will require further investigation. Previously we found that 3-NBA characteristic DNA adducts were detectable by TLC 32P-postlabeling independently of the route of administration (oral, intraperitoneal, and intratracheal treatment), although DNA binding varied in different tissues (Arlt et al., 2001a, 2003b; Bieler et al., 2005). Moreover, for 3-NBA the same characteristic DNA adducts were observed by HPLC 32P-postlabeling using different routes of administration (Nagy et al., 2005a, 2006), although TLC 32P-postlabeling seems to be more sensitive (Bieler et al., 2007). Since DNA adducts were observed in vivo for 3-NBA under the same experimental conditions in the present study it seems that, similar to the observations made in HCT116 and MCF-7 cells described above, 2-NBA is also a poorer substrate for nitroreductases in vivo, at least in Wistar rats. Furthermore, although we detected DNA adduct formation in rats treated with N-OH-2-ABA, DNA binding was much lower than that of N-OH-3-ABA, suggesting that N-OH-2-ABA may also be a poorer substrate for NATs and SULTs in vivo in rats, and/or is readily metabolized to 2-ABA, a possible detoxification pathway.

Physicochemical values have been used to estimate structure-related biological activities such as mutagenicity and carcinogenicity of different isomeric compounds (Borosky, 2007; Hatch et al., 2001). Using a semiempirical quantum mechanical approach a recent study indicated that the mutagenicity of mono-nitrated benzanthrones, including 1-, 2-, 3-, 9-, and 11-NBA, depends on the reduction potential and geometry of the nitro group attached to benzanthrone, although the
differences in particular for 2- and 3-NBA were small (Takamura-Enya et al., 2006). It is also noteworthy that no differences in hydrophobicity were observed among these NBA isomers, indicating that penetration into cell membranes is expected to be similar, and therefore would not be a critical determinant for their mutagenicity or DNA adduct forming potential. Metabolic activation of nitro-PAHs involves the formation of highly reactive arylnitrenium ions capable of reacting with DNA to form covalent DNA adducts. Thus, variations in the structures of the aryl moieties of 2- and 3-NBA may have profound effects on the rates of formation and/or the reactivity of their nitrenium ions (Ford and Herman, 1992) and, consequently, on their DNA adduct forming potential and mutagenicity.

DFT is a widely applicable and robust method to calculate thermochemical parameters and charge distribution in molecular systems (Borosky, 2007; Reynisson and Steenken, 2004). Recently, it was shown that among a large group of aromatic and heterocyclic aromatic amines investigated, derivatives with the highest electron density on the exocyclic nitrogen, i.e., the most stable species, were the most mutagenic ones (Borosky, 2007). Using the DFT method we observed quite large differences in the energetics of the nitrenium ions derived from 2- and 3-NBA. Moreover, we found that the 3-isomer delocalizes the positive charge more effectively than the 2-isomer. This could mean that the greater stability of the nitrenium ion of 3-NBA provides it with more time to reach the nucleus, react with DNA and form larger amounts of DNA adducts than the nitrenium ion of 2-NBA. The latter may react more efficiently with cellular macromolecules in the immediate vicinity of its site of formation in the cytoplasm. However, other factors besides the stability of the nitrenium ions may play a role in determining the extent of DNA adduct formation.

In summary, we found that 2-NBA is mutagenic in bacteria and mammalian short-term assays. In contrast to 3-NBA, 2-NBA formed DNA adducts only in some of the human cell lines studied, indicating that differences occur in the metabolic activation pathway of each isomer. Moreover, DNA binding by 2-NBA was generally substantially lower than by 3-NBA. In contrast to a recent report we found no evidence of 2-NBA-derived DNA adduct formation in vivo in rats at comparable doses, an observation which certainly requires further investigation. Using authentic 2-NBA–DNA adduct standards in the future may be one step toward resolving these discrepancies. Structural calculations showed that physiochemical properties, or more likely a combination of both physiochemical and enzymatic properties, related to 2- and 3-NBA activation may account for their large differences in mutagenicity and DNA adduct formation.

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