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Carcinogenic 3-nitrobenzanthrone but not 2-nitrobenzanthrone is metabolised to an unusual mercapturic acid in rats

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ABSTRACT

3-Nitrobenzanthrone (3-NBA) is an extremely potent mutagen and suspect human carcinogen found in diesel exhaust. Its isomer 2-nitrobenzanthrone (2-NBA) has also been found in ambient air. These isomers differ in mutagenicity in *Salmonella* by 2–3 orders of magnitude. To identify their urinary metabolites and also to assess the assumed differences in their excretion, rats were dosed orally with 2 mg/kg b.w. of either 2-NBA or 3-NBA. Their urine was collected for two consecutive days after dosage. Both LC–ESI-MS and GC–MS confirmed formation of the corresponding aminobenzanthrones (ABA). Excretion of these metabolites within the first day after dosing with 2- and 3-ABA amounted to 0.32 ± 0.06 and 0.83 ± 0.40% of the doses, respectively, while the excretion within the second day was by one order of magnitude lower. A novel mercapturic acid metabolite of 3-NBA was identified in urine by LC–ESI-MS as *N*-acetyl-*S*-(3-aminobenzanthron-2-yl)cysteine (3-ABA-MA) by comparison with the authentic standard. Its excretion amounted to 0.49 ± 0.15 and 0.02 ± 0.01% of dose within the first and second day after dosing, respectively. In contrast, no mercapturic acid was detected in the urine of rats dosed with 2-NBA. Observed difference in the mercapturic acid formation between 2- and 3-NBA is a new distinctive feature reflecting differences in the critical step of their metabolism, i.e., benzanthronylnitrenium ion formation that is intrinsically associated with biological activities of these two isomers. Moreover, 3-ABA-MA is a promising candidate biomarker of exposure to the carcinogenic 3-NBA.

1. Introduction

Both 2- and 3-nitro-7*H*-benz[*de*]anthracen-7-one (2- and 3-nitrobenzanthrone, NBA), like numerous other polycyclic nitroaromatics, are important environmental pollutants emitted from diesel engines. They are found mainly adsorbed on the surface of airborne particulate matter and are of great concern due to their mutagenic and carcinogenic potential (Enya et al., 1997; Arlt, 2005; Arlt et al., 2007). Although both are mutagenic in *Salmonella typhimurium* striking differences exist in their mutagenic potential with 3-NBA being by 2–3 orders of magnitude more mutagenic than the 2-nitro isomer (Takamura-Enya et al., 2006). Number of revertants induced in the Ames test by 3-NBA is comparable to that of 1,8-dinitropyrene, one of the most potent mutagens known (Purohit and Basu, 2000; Arlt, 2005). Carcinogenicity of 3-NBA was demonstrated in an experiment on rats following intratracheal instillation (Nagy et al., 2005). Numerous ^{32}P-postlabelling and MS studies have shown formation of DNA adducts in rats dosed with 3-NBA as well as in experiments with metabolically activated 3-NBA *in vitro* (Bieler et al., 2005, 2007; Arlt et al., 2001, 2006; da Costa et al., 2009). DNA adducts were formed also from 2-NBA in rat lungs *in vivo*, although only at very high doses of 2-NBA (Arlt et al., 2007).

3-NBA is metabolically reduced to 3-(hydroxyamino) benzanthrone (3-OH-ABA), which is either activated by *O*-acetylation and sulphate conjugation or further reduced to 3-aminobenzanthrone (3-ABA). 3-ABA was identified in the urine of mining workers heavily exposed to diesel emissions (Seidel et al., 2002). Both acetate and sulphate conjugates dissociate to electrophilic 3-benzanthronylnitrenium ion, which is the ultimate DNA reactive species (Scheme 1). Reduction of 3-NBA is catalysed mainly by NAD(P)H:quinone oxidoreductase 1 (NQO1, DT-diaphorase) (see Scheme 2).

In general, electrophilic species are detoxified by mercapturic acid pathway (MAP) initiated by conjugation with glutathione (GSH), followed by subsequent cleavage of glutamine and glycine, and terminated by acetylation to yield *S*-substituted *N*-acetylcysteines, mercapturic acids, which are excreted in urine. Mercapturic acids can serve as valuable biomarkers of exposure reflecting electrophilic reactivity of xenobiotics and/or

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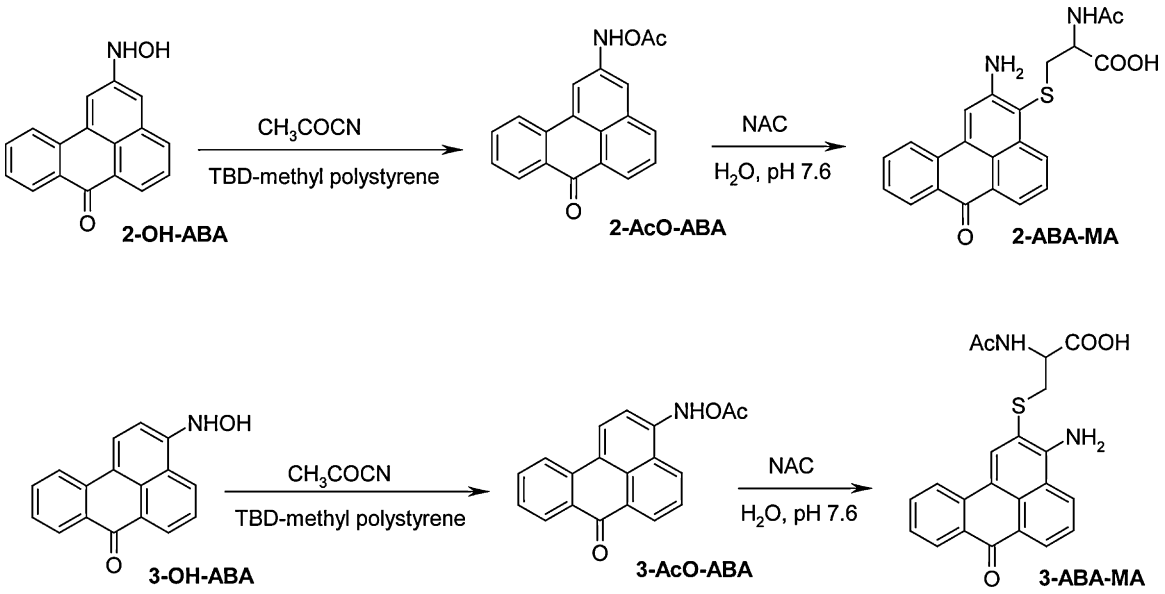

Scheme 1. Preparation of the mercapturic acids derived from 2- and 3-NBA by reaction of the corresponding activated OH-ABAs with NAC.

their metabolites (for reviews see Haufroid and Lison, 2005; Angerer et al., 2007).

Numerous *in vitro* studies on the metabolic activation of 3-NBA were published (Borlak et al., 2000; Arlt, 2005; Hansen et al., 2007; Stiborová et al., 2006), however, only one urinary metabolite, 3-aminobenzanthrone, has been identified as yet (Seidel et al., 2002).

Arylation of *N*-acetylcysteine (NAC) and GSH by activated hydroxyaminoarenes (Boyland et al., 1962; Manson, 1972; Ketterer et al., 1979) as well as by benzidine upon oxidative activation by horseradish peroxidase (Josephy and Iwaniw, 1985) has been described long ago. Therefore, it is rather surprising that, to our knowledge, very few mercapturic acids formed via arylnitrenium ions *in vivo* have been reported so far, namely, 2- and 4-aminophenylmercapturic acids derived from aniline and 2-amino-1-naphthylmercapturic acid derived from 2-naphthylamine (Boyland et al., 1963).

The aim of the present work was to identify urinary metabolites of both 2- and 3-NBA, in particular those produced through the formation of nitrenium ion intermediates. The amounts of mercapturic acids possibly found after administration of 2- and 3-NBA would reflect the extent of formation of the corresponding benzanthronylnitrenium ions and, indirectly, the extent of toxic insult caused by each NBA isomer.

2. Materials and methods

2.1. Materials

Acetonitrile for LC/MS Chromasolv was from Merck, formic acid, puriss. p.a. from Fluka. Re-distilled water was used for LC/MS and solid phase extraction. Tetrahydrofuran (THF) was dried by distillation from sodium and benzophenone. 1,5,7-Triazabicyclo[4.4.0]dec-5-ene bound to polystyrene (TBD-methyl polystyrene) was from Novabiochem, Germany. Heptafluorobutyric anhydride (HFBA) was from Fluka. Other chemicals were of synthetic or analytical grade and were used as received.

2.2. Syntheses of nitrobenzanthrones and their expected metabolites

2-Nitrobenzanthrone (2-NBA) was prepared by a published multi-step synthesis (Suzuki et al., 1997) with a modification in the key step. Instead of Ullmann reaction, which gave low yields of the key intermediate 1-(2-methoxycarbonylphenyl)-3-nitronaphthalene, Suzuki coupling (Miyaura et al., 1979) of 1-iodo-3-nitronaphthalene with 2-(methoxycarbonyl)phenylboronic acid was used.

3-Nitrobenzanthrone (3-NBA) was obtained by direct nitration of benzanthrone with fuming nitric acid as described in the literature (Enya et al., 1998).

2-Aminobenzanthrone (2-ABA). Palladium catalyst (10 mg of 10% Pd on charcoal, Degussa type containing ca. 50% of water) was activated by heating at 90 °C for 1 h in a vacuum. Argon was then introduced into the reaction flask and 2-NBA (15 mg, 54.5 μmol), diglym (30 mL) and hydrazine hydrate (75 μL, 77 μg, 1.54 μmol) were added. The reaction mixture was stirred at room temperature under argon and the course of reaction was monitored by HPLC with a PDA detector. After 2 days conversion of 2-NBA was complete. The catalyst was then filtered off and the filtrate was evaporated in a vacuum to dryness. Crystallisation of the residue from ethanol yielded 9.5 mg (71%) of orange crystals identified by comparing their ^{1}H NMR spectrum with that reported in the literature as 2-ABA (Takekawa et al., 2002).

ESI-MS: *m/z* 246 $(M+H)^+$, 268 $(M+Na)^+$. MS^2: *m/z* 246 → 228 $(MH-H_2O)^+$, 229 $(MH-NH_3)^+$; 230 $(MH-O)^+$; 217 $(MH-COH)^+$; 218 $(MH-CO)^+$, 219 $(MH-C_2H_2)^+$, 201 $(MH-CONH_3)^+$.

UV: λ_{max} = 442, 375, 303 and 230 nm.

2-Acetamidobenzanthrone (N-acetyl-2-aminobenzanthrone, 2-AcABA). 2-ABA (10 mg, 41 μmol) was refluxed in acetic anhydride (1.5 mL) for 4 h. Reaction mixture was then evaporated to dryness in a vacuum. Re-crystallisation of the residue from aqueous ethanol yielded 6.5 mg (56%) of greenish-yellow crystals.

^{1}H NMR spectrum ($CDCl_3$): δ = 2.40 (s, 3H, CH_3CO); 7.61 (td, *J* = 7.5 and 1.0 Hz, 1H, C9-H); 7.76 (td, *J* = 7.7 and 1.5 Hz, 1H, C10-H); 7.82 (d, *J* = 1.8 Hz, 1H, C3-H); 7.84 (dd, *J* = 8.2 and 7.6 Hz, 1H, C5-H); 8.19 (d, *J* = 1.9 Hz, 1H, C1-H); 8.22 (dd, *J* = 8.2 and 1.2 Hz, 1H, C11-H); 8.28 (d, *J* = 8.2 Hz, 1H, C4-H); 8.52 (dd, *J* = 7.9 and 1.1 Hz, 1H, C8-H); 8.80 (dd, *J* = 7.3 and 1.4 Hz, 1H, C6-H).

ESI-MS: *m/z* 288 $(M+H)^+$, MS^2: *m/z* 288 → 246 $(MH-CH_2CO)^+$.

UV: λ_{max} = 389, 307, 256 and 240 nm.

2-(Hydroxyamino)benzanthrone (2-OH-ABA). To a suspension of 2-NBA (15 mg, 54.5 μmol) in diglym (30 mL) palladium catalyst (15 mg of 5% Pd on charcoal) and hydrazine hydrate (75 μL, 77 μg, 1.54 μmol) was added. The reaction mixture was then stirred overnight under argon at the room temperature. Conversion of 2-NBA amounted to more than 90% as determined by HPLC with PDA detector. After filtering off the catalyst, the solvent was evaporated in a vacuum to dryness, and re-suspended in 20 mL of a 1:1 ethyl acetate–chloroform mixture. The solid was filtered off to yield 5 mg (35%) of a greenish yellow powder identified as 2-OH-ABA.

^{1}H NMR spectrum (DMSO-d_6): δ = 7.51 (bs, 1H, NH); 7.64 (t, *J* = 7.5 Hz, 1H, C9-H); 7.75 (t, *J* = 7.8 Hz, 1H, C10-H); 7.86 (t, *J* = 7 Hz, 1H, C5-H); 8.22 (d, *J* = 2 Hz, 1H, C3-H); 8.25 (d, *J* = 7.5 Hz, 1H, C4-H); 8.32 and 8.35 (d, *J* = 6.7 Hz, 2H, C6-H and C8-H); 8.41 (d, *J* = 8.2 Hz, 1H, C11-H); 8.78 (d, *J* = 1.8 Hz, 1H, C1-H); 8.88 (bs, 1H, OH).

ESI-MS: *m/z* 262 $(M+H)^+$; MS^2: 262 → 245 $(MH-NH_3)^+$, 244 $(MH-H_2O)^+$, 234 $(MH-CO)^+$, 217 $(MH-CO-NH_3)^+$.

UV: λ_{max} = 430, 375, 297 and 224 nm.

3-Aminobenzanthrone (3-ABA). 3-NBA (20 mg, 72.7 μmol) was dissolved in 20 mL of diglym, palladium catalyst (20 mg of 5% Pd on charcoal) and hydrazine hydrate (80 μL, 82 mg, 1.65 μmol) was added and the reaction mixture was stirred overnight under argon at room temperature. The catalyst was then filtered off, the filtrate was evaporated in a vacuum to dryness and the residue re-dissolved in 5 mL of ethanol. Red precipitate formed after dilution of this solution with water was filtered off and dried over P_4O_{10}. Dark red powder of 3-ABA obtained (9 mg, 51%) showed ^{1}H NMR spectrum, which was in agreement with published data (Safronov and Traven, 1993).