Genotoxicity of 3-nitrobenzanthrone and 3-aminobenzanthrone in Muta™Mouse and lung epithelial cells derived from Muta™Mouse

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FE1 lung epithelial cells derived from Muta™Mouse are a new model system to provide in vitro mutagenicity data with the potential to predict the outcome of an in vivo Muta™Mouse test. 3-Nitrobenzanthrone (3-NBA) is a potent mutagen and suspected human carcinogen identified in diesel exhaust and urban air pollution. We investigated the mutagenicity and DNA binding of 3-NBA and its main metabolite 3-aminobenzanthrone (3-ABA) in vitro and in vivo in the Muta™Mouse assay. Mice were treated with 3-NBA or 3-ABA (0, 2 or 5 mg/kg body weight/day) by gavage for 28 days and 28 days later lacZ mutant frequency (MF) was determined in liver, lung and bone marrow. For both compounds, dose-related increases in MF were seen in liver and bone marrow, but not in lung; mutagenic activity was ~2-fold lower for 3-ABA than for 3-NBA. With 3-NBA, highest DNA adduct levels (measured by 32P-post-labelling) were found in liver (~230 adducts per 10^8 nucleotides) with levels 20- to 40-fold lower in bone marrow and lung. With 3-ABA, DNA adduct levels were again highest in the liver, but ~4-fold lower than for 3-NBA. FE1 cells were exposed to up to 10 µg/ml 3-NBA or 3-ABA for 6 h with or without exogenous activation (S9) and harvested after 3 days. For 3-NBA, there was a dose-related increase in MF both with and without S9 mix, which was >10 times higher than observed in vivo. At the highest concentration of 3-ABA (10 µg/ml), we found only around a 2-fold increase in MF relative to controls. DNA adduct formation in FE1 cells was dose-dependent for both compounds, but 10- to 20-fold higher for 3-NBA compared to 3-ABA. Collectively, our data indicate that Muta™Mouse FE1 cells are well suited for cost-effective testing of suspected mutagens with different metabolic activation pathways as a guide for subsequent in vivo Muta™Mouse testing.

Introduction

Epidemiological studies have shown increased mortality and morbidity from respiratory and cardiovascular diseases associated with exposures to ambient air pollution (1,2). A complex variety of genotoxins in urban air pollution has been detected (3), and high exposures are associated with an increased risk of cancer. Nitropolyaromatic hydrocarbons (nitro-PAHs) are present on particulate matter from direct atmospheric emission, such as diesel and gasoline exhaust (4), or they can be produced from gas-phase reactions of polycyclic aromatic hydrocarbons (PAHs) with oxides of nitrogen and subsequently partition to the particulate phase (5). Nitro-PAHs often have greater mutagenic and carcinogenic properties compared to their parent PAHs, and their persistence in the environment suggests that they constitute a potential hazard to humans (6–8).

Certain nitro-PAHs exhibit high direct-acting mutagenic activity in bacterial bioassays and in forward mutation assays in mammalian cells (9). A member of this class of compounds is the aromatic nitroketone 3-nitrobenzanthrone (3-NBA; 3-nitro-7H-benz[de]anthracen-7-one; Figure 1) identified in diesel exhaust and ambient air pollution (10,11). 3-NBA is one of the most potent mutagens ever detected in the Salmonella reverse mutation assay, and it is a suspected human carcinogen (10,12). In Salmonella typhimurium, it induces ~0.2 and 6 million revertants/nmol in strains TA98 and YG1024 (without S9), respectively (10). It is a potent carcinogen after intratracheal instillation in rats, inducing mainly squamous cell carcinoma in lung (12). In mammalian cells, it induces micronuclei (13,14), DNA strand breaks (14,15), DNA adducts (15,16) as well as gene mutations (13). Recently, its isomer 2-nitrobenzanthrone (2-NBA) has been detected in urban air particulate matter leading to an even greater interest in assessing the potential health hazard of nitrobenzanthrones to humans (16–18).

The uptake of 3-NBA in humans has been demonstrated by the detection of 3-aminobenzanthrone (3-ABA; Figure 1), its main metabolite, in the urine of workers occupationally exposed to diesel emissions (19). The genotoxicity of 3-ABA has been demonstrated in several short-term assays in vitro and in vivo (15,20,21).

3-NBA forms DNA adducts in vitro and in rodents after metabolic activation through reduction of the nitro group, which is primarily catalysed by cytosolic nitroreductase such as NAD(P)H:quinone oxidoreductase (Figure 1) (22–27). 3-ABA is predominantly activated by cytochrome P450 (CYP) enzymes, namely CYP1A1 and CYP1A2 (23,28,29). Both 3-NBA and 3-ABA can be further activated by N-acetyltransferases (NATs) and sulphotransferases (SULTs) (24,30,31). The predominant DNA adducts detected by 32P-post-labelling in vivo in rodents after treatment with either 3-NBA or 3-ABA are 2-(2′-deoxyguanosin-N2-yl)-3-aminobenzanthrone (dG-N2-ABA) and N-(2′-deoxyguanosin-8-yl)-3-aminobenzanthrone (dG-C8-N-ABA) (32), and these are most probably responsible for the GC → TA transversion mutations induced by 3-NBA exposure in vivo (33). These DNA adducts not only represent premutagenic lesions in DNA but may also be of primary importance for tumour development in target tissues (34,35).

Transgenic rodent mutagenicity assays (e.g. Muta™Mouse, BigBlue® rat/mouse) are powerful tools to determine
genotoxicity in vivo (36,37). In addition, the retrievable integrated transgene target allows molecular analysis of induced mutations that may reveal chemical-specific mutation spectra. However, the high cost of in vivo assay systems such as Mutamouse can be reduced through the use of transgenic cells cultured in vitro (38). Although an in vitro model is not fully representative of the biology of the living animal, the use of a cell culture system has numerous experimental advantages. Recently, a spontaneously immortalized lung epithelial cell line denoted FE1 that retains certain key endogenous metabolic pathways was derived from Mutamouse (38). The in vitro assay provides the opportunity to rapidly generate data that can predict the outcome of an in vivo test, which, moreover, can ultimately assist in refining, reducing or replacing routine in vivo testing.

This study explores and assesses the mutagenicity of 3-NBA and its metabolite 3-ABA in Mutamouse in vivo and in Mutamouse-derived FE1 cells in vitro. In addition, DNA adduct formation was investigated using $^{32}$P-post-labeling.

### Material and Methods

#### Test compounds

**Caution.** 3-NBA is a potent mutagen, rodent carcinogen and suspected human carcinogen. 3-NBA and its derivatives should be handled with extreme care.

3-NBA (CAS No. 17117-34-9) was obtained from the Sigma Library of Rare Chemicals (Sigma-Aldrich, Oakville, Ontario, Canada). 3-ABA (CAS No. 13456-80-9) was synthesized as described previously (31) and its authenticity was confirmed by UV spectroscopy, electrospray mass spectrometry and high-field proton nuclear magnetic resonance (NMR) spectroscopy.

#### FE1 cell culture and treatment

FE1 is a stable epithelial cell line derived from Mutamouse lung (38). FE1 cells were cultured in a 1:1 mixture of Dulbecco’s modified Eagle’s medium:F12 supplemented with 2% (v/v) foetal bovine serum, 100 U/ml penicillin G, 100 mg/ml streptomycin sulphate and 1 mg/ml murine epidermal growth factor (EGF) (GIBCO–Invitrogen, Burlington, Ontario, Canada). All incubations were carried out at 37°C, 95% humidity and 5% CO$_2$. In all, 2–3 x $10^5$ cells (passages 9–25) were seeded on 100-mm culture dishes and incubated overnight to ~10% confluence. The following morning, cells were exposed for 6 h to a series of doses (0, 0.1, 1, 3 and 10 μg/ml) of 3-NBA or 3-ABA (dissolved in dimethyl sulphoxide; Sigma-Aldrich) in serum-free medium. For treatments involving the use of S9, a mixture of cofactors and Aroclor-1254-induced rat liver S9 (Moltox, Boone, NC) was added to a final concentration of 0.5% v/v in the treatment medium. After chemical treatment, cells were washed with phosphate-buffered saline (PBS) (pH 7.2) and incubated for 72 h in medium with serum for mutation expression. Following expression, genomic DNA was isolated as described previously (38–40).

Briefly, cells were incubated overnight at 37°C in lysis buffer (10 mM Tris, 100 mM sodium chloride and 10 mM ethylenediaminetetraacetic acid (EDTA), pH 7.6) containing 1% sodium dodecyl sulphate and 1 mg/ml fresh proteinase K (GIBCO–Invitrogen). Lysates were extracted with phenol–chloroform (1:1), followed by chloroform. Sodium chloride was added to a final concentration of 0.2 M and the DNA precipitated in two volumes of ethanol. DNA was dissolved onto a sealed Pasteur pipette, washed in 70% ethanol and dissolved in 15–100 μl of TE buffer (10 mM Tris and 0.1 mM EDTA, pH 7.6).

#### Animal treatment and tissue collection

The lacZ transgenic mouse strain 40.6 (BALB/c x DBA2), also known as the Mutamouse, has been described in detail elsewhere (41,42). The animals harbour a multi-copy, concatenated recombinant λgt10 vector containing the complete Escherichia coli lacZ gene (3096 bp) as a target for mutation scoring. Animals were bred, maintained and treated at Health Canada facilities under conditions approved by the Health Canada Animal Care Committee. Male mice (16–20 weeks) used in this study were maintained on a 12-h light–dark cycle and provided with fresh water and Rodent Chow (Ralston Purina, Hazleton, PA) ad libitum. These experiments involved four to six animals per group. Both 3-NBA and 3-ABA were administered in olive oil at 2 or 5 mg/kg body weight and vehicle controls received olive oil alone. The mice were treated by oral gavage (p.o.) daily for 28 days resulting in final doses of 56 (low-dose group) or 140 mg/kg body weight (high-dose group). Following the final treatment, a 28-day recovery period was allowed for mutation fixation. Mice were killed by cervical dislocation and liver, lung and bone marrow were removed, frozen in liquid nitrogen and stored at ~80°C until DNA isolation. To obtain bone marrow, femurs were flushed with cold PBS, the solution centrifuged at 10 000 x g at 4°C for 1 min and the pellet stored at ~80°C. Thawed lung was minced prior to cell lysis and proteinase K digestion. Liver tissue was homogenized using a conical Teflon® Dual homogenizer (Fisher Scientific, Ottawa, Ontario, Canada) and nuclei were isolated by differential centrifugation prior to lysis. Minced or homogenized tissues were digested overnight in lysis buffer, and DNA was extracted and handled as described above.

#### LacZ mutation analysis

Transgene mutant frequency (MF) was determined using the P-gal-positive selection assay described elsewhere (40,42). The method employs a galf$^-$ host bacterium to facilitate the isolation and enumeration of mutant copies of the lacZ transgene (43). λgt10lacZ DNA copies were rescued from genomic Mutamouse DNA (4 μl aliquots) using the Transpack® lambda packaging system (Stratagene, La Jolla, CA). Packaged phage preparations were mixed with host bacteria (E. coli lacZ, galf, recA, pAA119 with galf and galk) (43,44) and allowed to adsorb for 25 min at room temperature. An aliquot of the phage–bacteria mix was diluted with additional bacterial culture and plated on non-selective minimal agar to determine titre plaque forming unit (p.f.u.). The remaining phage–bacteria mixture was plated on minimal agar

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Fig. 1. Proposed pathways of metabolic activation and DNA adduct formation of 3-NBA and 3-ABA. See text for details. POR, CYP oxidoreductase. R = –C(O)CH$_3$ or –SO$_3$H.
Results

Mutagenesis induced by 3-NBA and 3-ABA at the lacZ transgene in FE1 cells in vitro

To examine the mutagenic effect of 3-NBA and 3-ABA in FE1 cells, cells were exposed to increasing doses (0.1–10 μg/ml) of both compounds (Figure 2). For 3-NBA, a clear dose-dependent increase in MF of lacZ was observed at doses >0.1 μg/ml both in the presence or absence of an exogenous metabolic activation system (S9) (Figure 2A). Statistical analysis looking at the concentration-specific effect of S9 showed that the mutagenic activity with S9 was greater than that without S9 at 10 μg/ml 3-NBA only (P < 0.05). For 3-ABA, a concentration-dependent increase in MF was found at doses >1 μg/ml, but MF was much greater (up to 1.8-fold) in the presence of an exogenous S9 activation system (Figure 2B). The mutagenic activity with S9 was statistically greater than without S9 at 3 and 10 μg/ml 3-ABA (P < 0.0001 in both cases). In FE1 cells, the mutagenic activity of 3-NBA was much greater than that of 3-ABA.

DNA adduct formation of 3-NBA and 3-ABA in FE1 cells in vitro

DNA from FE1 cells treated with 3-NBA or 3-ABA was analysed by TLC 32P-post-labelling (Figure 3). For both compounds, the DNA adduct pattern consisted of a cluster of up to five adducts (spots 1–5). No DNA adducts were detected in control cells (data not shown). DNA adducts were identified by cochromatographic analysis of individual spots on high-performance liquid chromatography using authentic standards (32), confirming that all DNA adducts are derived from reductive metabolites of 3-NBA bound to purines (data not shown). Three of these adducts have been identified as 2-(2'-deoxyadenosin-3'-yl)-3-aminobenzanthrone (dA-N6-ABA; spot 1), dG-8-N-ABA (spot 3) and dG-C8-N-ABA (spots 4/5).

For cells treated with either 3-NBA or 3-ABA, a dose-dependent increase in DNA adduct formation was observed.
both with and without S9 activation (Figure 4). However, DNA binding by 3-NBA was much higher than by 3-ABA, with 173.1 and 18.1 adducts per 10^8 nt being formed by 3-NBA and 3-ABA, respectively, at the highest dose (10 μg/ml; −S9). Thus, DNA adduct formation by 3-NBA was not only 10 times higher but also was detectable at a 10-fold lower dose (Figure 4A). DNA adduct formation by both 3-NBA and 3-ABA was lower in the presence of S9 mix suggesting that either 3-NBA and 3-ABA can bind to S9 proteins or metabolic enzymes present in S9 readily detoxify the compounds before they enter cells.

Pearson correlation analysis of MF with total DNA adduct levels revealed a strong correlation under all treatment conditions (r = 0.940 for 3-NBA and r = 0.900 for 3-ABA; P < 0.05).

**Mutagenesis induced by 3-NBA and 3-ABA at the lacZ transgene in vivo**

DNA was isolated from Muta™Mouse bone marrow, liver and lung 28 days after the last treatment. The results of the lacZ MF analyses are shown in Figure 5 (see also Supplementary Tables S1 and S2 are available at Mutagenesis Online). For 3-NBA, dose-related increases in MF were observed in bone marrow and liver, with up to 2.3- and 4.1-fold increases above control, respectively (Figure 5A). For 3-ABA, a significant increase in MF at both doses was found in bone marrow (up to 3-fold) and for liver at the lower dose (Figure 5B). No increases in MF above control levels were seen in lung.

**DNA adduct formation of 3-NBA and 3-ABA in vivo**

DNA adduct formation in Muta™Mouse was analysed in bone marrow, liver and lung. As shown in Figure 6, 3-NBA and 3-ABA induced essentially the same DNA adduct patterns as those observed in FE1 cells (compare Figure 3). The observed pattern in DNA of treated animals consisted of a cluster of up to five adducts (spots 1–5). In addition, a previously unobserved spot (spot X) was found in liver DNA (see also Supplementary Tables S3 and S4 are available at Mutagenesis Online). No DNA adducts were observed in DNA isolated from control animals (data not shown). DNA adducts (e.g. dA-N6-ABA, spot 1; dG-N2-ABA, spot 3 and dG-C8-N-ABA, spots 4/5) were identified as described above for FE1 cells. Highest DNA binding was observed in liver, with 230 adducts per 10^8 nt and 55.6 adducts per 10^8 nt for 3-NBA and 3-ABA, respectively (Figure 7). The liver was also the only tissue where dose-dependent increases in DNA adduct formation were found. There were no differences in DNA binding between the low- and high-dose treatment in bone marrow or lung. Levels of individual adduct spots showed that dG-N2-ABA (adduct spot 3) was the major adduct found in all tissues examined (see Supplementary Tables S3 and S4 are available at Mutagenesis Online).
DNA damage such as DNA adduct formation is an important first step in the process of mutation induction (46). DNA adducts represent premutagenic lesions, and both the initial levels of specific adducts and their persistence in the target organ contribute to their mutagenic potential and subsequent tumour development. Thus, simultaneous, tissue-specific detection of DNA adducts and mutation induction in any tissue of interest afforded by the use of transgenic animals has proved to be a powerful tool to study the genotoxic hazards of environmental contaminants (47–50). One of the convenient and effective transgenic rodent mutation assay systems is the Muta™Mouse system. In the present study, we used Muta™Mouse lung FE1 epithelial cells to investigate the mutagenicity of the urban air pollutant 3-NBA and its human metabolite 3-ABA and explored the utility of this in vitro tool as a predictive test for determining mutagenic potency in the in vivo version of the Muta™Mouse assay.

Although several other transgenic cell lines have been developed to study environmentally induced mutations (51,52), recent results have shown that Muta™Mouse FE1 cells are a useful in vitro tool for assessing the mutagenic activity for a wide range of mutagens, including benzo[a]pyrene, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine and complex mixtures such as coal tar and carbon black (38,53). In the present study, we found that treatment with 3-NBA and 3-ABA resulted in a 5- and 2-fold increase in lacZ MF over the solvent control, respectively. This response shows that FE1 cells have the metabolic capabilities to activate both compounds. Similarly, human lung epithelial A549 cells were able to metabolize 3-NBA and 3-ABA and to induce oxidative DNA damage (21). Another study showed that 3-NBA forms DNA adducts in A549 cells, although 3-ABA was not tested (16). Whereas the addition of S9 had no influence on the MF for 3-NBA, for 3-ABA its presence resulted in a 2.5-fold increase in MF. FE1 cells express CYP1A1 and exhibit ethoxyresorufin-O-deethylase activity, a measure of CYP1A1 and CYP1A2 activity (38), and it was previously demonstrated that CYPs are important in the metabolic activation of 3-ABA in mice (29). Moreover, CYP1A1 and CYP1A2 are the predominant enzymes involved in the phase I bioactivation of 3-ABA (28). Generally, these results suggest that endogenous CYP1A1 is responsible for the metabolic activation of 3-ABA in FE1 cells in the absence of exogenous S9, whereas in the presence of S9 exogenous CYPs (e.g. CYP1A1 and CYP1A2) also contributes to 3-ABA activation. It should also be noted that 3-NBA and 3-ABA are strongly activated by NATs and SULTs (24,30,31), but external activating systems (as used in the present study) usually lack the cofactors for these enzymes (54). We also noted a strong correlation between DNA adduct levels and MF in FE1 cells after treatment with 3-NBA and 3-ABA, indicating that the mutagenic effects are clearly correlated with the formation of DNA adducts. Paradoxically, although the MF in FE1 cells tended to be higher after addition of exogenous S9, DNA adduct levels were lower in the presence of S9 both for 3-NBA and 3-ABA. This could be a consequence of choosing the same time point to observe different biological/biochemical events or may be an indication of additional S9-mediated DNA damage (e.g. strand breaks), but further investigations will be required to explain this phenomenon.

Previous studies have shown that 3-NBA and 3-ABA form the same DNA adducts in tissues of rats and mice indicating that N-OH-3-ABA is the reactive intermediate (22–24,29,33). The persistence of DNA adducts has been investigated in several rat organs after a single dose of 3-NBA by intratracheal administration (35). After initial formation of DNA adducts, levels decreased rapidly during the first 2 weeks and then remained practically unchanged between 4 and 36 weeks. This pattern of adduct reduction was similar in target tissue (lung) and non-target tissues (e.g. liver and kidney). The dG-N²-ABA adduct (spot 3) was the most persistent suggesting that this DNA adduct is less amenable to repair, and more likely to be converted into mutations in critical genes for carcinogenesis (11,35). Indeed, in the present study, dG-N²-ABA was the most abundant DNA adduct detected in Muta™Mouse following a 28-day recovery period after oral treatment with 3-NBA or 3-ABA. Highest DNA binding (dose dependent) was found in liver after treatment with both 3-NBA or 3-ABA, but levels for 3-ABA were up to 8-fold lower than those of 3-NBA. We found a strong association between the formation of DNA adducts in the liver and the MF, which supports a previous Muta™Mouse that employed repeated 3-NBA treatments via intra-peritoneal injection (33). In the latter study, it was shown that in Muta™Mouse liver 3-NBA induces mainly GC→TA transversion mutations in the cII gene, and this is consistent with extensive formation of dG-N²-ABA (and dG-C8-N-ABA) in liver DNA. The induction of GC→TA transversions by 3-NBA can be explained by intrinsic properties of DNA polymerases to insert dA opposite an adduct lesion during replication, referred to as the ‘A’-rule (55). This conclusion is essentially the same as that arrived from studies with other nitro-PAH mutagens (e.g. 1,3-, 1,6- and 1,8-dinitropyrene given singly or as a mixture) that have been examined in...
transgenic mouse mutation assays (47,49). Moreover, a recent study showed that diesel exhaust particles and their extracts induce mainly GC → TA transversion mutations in the gpt delta transgenic mouse mutation assay, suggesting that nitro-PAHs present in diesel exhaust (e.g. 3-NBA) induce the same mutations and may be responsible for the observed carcinogenicity (50).

A major benefit of the present study was that we were able to demonstrate that the FE1 MutaMouse lung epithelial cells can provide in vitro mutagenicity data that can reliably predict the outcome of an in vivo MutaMouse test. The data presented in this paper clearly show that the high mutagenic potency of 3-NBA in FE1 cells is in agreement with the clear positive findings in vivo. Moreover, the weaker mutagenic activity of 3-ABA in FE1 cells in vitro, relative to 3-NBA, is in accordance with the weak positive results obtained in the in vivo assay. It should be noted that recent data demonstrated that 2-NBA, an isomer of 3-NBA, found in urban air pollution (56,57) can form DNA adducts in vitro, but is unable to bind to DNA in vivo (16). The FE1/MutaMouse assay system employed in this study can provide an ideal system to examine the mutagenic potency of 2-NBA in vitro and in vivo, and this will form the basis of a future study.

The data presented in this study collectively demonstrate that the MutaMouse FE1 cells constitute a useful cost-effective in vitro tool to screen suspected mutagens that require activation via mammalian metabolic pathways. The results obtained can subsequently be used to guide a restricted set of follow-up in vivo tests in the MutaMouse, which employ the same transgenic mutation reporting system, to confirm hazard and identify target tissues. Such a model significantly reduces assumptions in exploring from in vitro to in vivo conditions. Moreover, this type of test system can ultimately help ensure compliance with policies such as the amended Cosmetics Directive of the European Parliament (i.e. 2003/15/EC of February 27, 2003) that are aimed at restricting or eliminating the use of in vivo animal tests in human health and safety evaluations (58). The Directive banned the testing of finished cosmetic products in September 2004, and a complete test ban on cosmetic ingredients will go into effect in March 2009. Thus, the development and validation of in vitro test systems such as that examined here are essential for legislative compliance. Additional evaluations of the FE1 and similar in vitro systems using a larger set of compounds are promising areas for further research.

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Supplementary data
Supplementary Tables S1–S8 are available at Mutagenesis Online.

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