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## **Genotoxicity of 3-nitrobenzanthrone and 3-aminobenzanthrone in Muta<sup>TM</sup>Mouse and lung epithelial cells derived from Muta<sup>TM</sup>Mouse**

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## Abstract

FE1 lung epithelial cells derived from Muta<sup>TM</sup>Mouse are a new model system to provide *in-vitro* mutagenicity data with the potential to predict the outcome of an *in-vivo* Muta<sup>TM</sup>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<sup>TM</sup>Mouse assay. Mice were treated with 3-NBA or 3-ABA (0, 2, or 5 mg/kg body weight/day) p.o. 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 <sup>32</sup>P-postlabelling) were found in liver (~230 adducts/10<sup>8</sup> nucleotides) with levels 20–40-fold lower in bone marrow and lung. With 3-ABA DNA adduct levels were again highest in the liver, but around 4-fold lower than for 3-NBA. FE1 cells were exposed to up to 10 µg/ml 3-NBA or 3-ABA for 6 hours 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 over 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 10–20-fold higher for 3-NBA compared to 3-ABA. Collectively our data indicate that Muta<sup>TM</sup>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<sup>TM</sup>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. Nitropolycyclic aromatic 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 suggest 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; Fig. 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 around 0.2 and 6 million revertants per 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 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; Fig. 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 *in vivo* after metabolic activation through reduction of the nitro group, which is primarily catalysed by cytosolic nitroreductase such as NAD(P)H:quinone oxidoreductase (NQO1) (Fig. 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 sulfotransferases (SULTs) [24,30,31]. The predominant DNA adducts detected by <sup>32</sup>P-postlabelling *in vivo* in rodents after treatment with either 3-NBA or 3-ABA are 2-(2'-deoxyguanosin-*N*<sup>2</sup>-yl)-3-aminobenzanthrone (dG-*N*<sup>2</sup>-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<sup>TM</sup>Mouse, BigBlue<sup>®</sup> 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 Muta<sup>TM</sup>Mouse 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 immortalised lung epithelial cell line denoted FE1 that retains certain key endogenous metabolic pathways was derived from Muta<sup>TM</sup>Mouse [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 Muta<sup>TM</sup>Mouse *in vivo* and in Muta<sup>TM</sup>Mouse-derived FE1 cells *in vitro*. In addition, DNA adduct formation was investigated using <sup>32</sup>P-postlabelling.

## 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, ON, Canada). 3-ABA (CAS No. 13456-80-9) was synthesised as described previously [31] and its authenticity was confirmed by UV spectroscopy, electrospray mass spectrometry, and high-field proton NMR spectroscopy.

### FE1 Cell Culture and Treatment

FE1 is a stable epithelial cell line derived from Muta<sup>TM</sup>Mouse lung [38]. FE1 cells were cultured in a 1:1 mixture of DMEM:F12 supplemented with 2% (v/v) FBS, 100 U/ml penicillin G, 100 mg/ml streptomycin sulphate and 1 ng/ml murine EGF (GIBCO-Invitrogen, Burlington, ON, Canada). All incubations were carried out at 37°C, 95% humidity and 5% CO<sub>2</sub>. 2–3 × 10<sup>5</sup> cells (passage 9-25) were seeded on 100 mm culture dishes and incubated overnight to ~10% confluence. The following morning, cells were exposed for 6 hours to a series of doses (0, 0.1, 1, 3, and 10 µg/ml) of 3-NBA or 3-ABA (dissolved in DMSO; 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, USA) was added to a final concentration of 0.5% v/v in the treatment medium. After chemical treatment, cells were washed with PBS (pH 7.2) and incubated for 72 hours 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, 10 mM EDTA, pH 7.6) containing 1% SDS and 1 mg/ml fresh proteinase K (GIBCO-Invitrogen, Burlington, ON, Canada). 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 2 volumes of ethanol. DNA was spooled onto a sealed Pasteur pipette, washed in 70% ethanol, and dissolved in 15–100 µl of TE buffer (10 mM Tris, 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 Muta<sup>TM</sup>Mouse, has been described in detail elsewhere [41,42]. The animals harbour a multi-copy, concatenated recombinant *λgt10* vector containing the complete *E. 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-hour light/dark cycle and provided with fresh water and Rodent Chow (Ralston Purina, Hazleton, PA, USA) *ad libitum*. These experiments involved 4 to 6 animals per group. Both 3-ABA and 3-NBA were administered in olive oil at 2 or 5 mg/kg bw 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 bw (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  $-80^{\circ}\text{C}$  until DNA isolation. To obtain bone marrow, femurs were flushed with cold PBS, the solution centrifuged at 10,000g at  $4^{\circ}\text{C}$  for 1 minute, and the pellet stored at  $-80^{\circ}\text{C}$ . Thawed lung was minced prior to cell lysis and proteinase K digestion. Liver tissue was homogenised using a conical Teflon<sup>®</sup> Duall homogeniser (Fisher Scientific, Ottawa, ON, Canada) and nuclei were isolated by differential centrifugation prior to lysis. Minced or homogenised 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 *galE* host bacterium to facilitate the isolation and enumeration of mutant copies of the *lacZ* transgene [43].  $\lambda\text{gt}10\text{lacZ}$  DNA copies were rescued from genomic Muta<sup>TM</sup>Mouse DNA (4  $\mu\text{l}$  aliquots) using the Transpack<sup>TM</sup> lambda packaging system (Stratagene, La Jolla, CA, USA). Packaged phage preparations were mixed with host bacteria (*Escherichia coli lacZ*, *galE*, *recA*<sup>-</sup>, pAA119 with *galT* and *galK*) [43,44] and allowed to adsorb for 25 minutes 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 (pfu). The remaining phage/bacteria mixture was plated on minimal agar with 0.3% w/v phenyl- $\beta$ -D-galactopyranoside (P-gal; Sigma-Aldrich). Both were incubated overnight at  $37^{\circ}\text{C}$ . MF was expressed as the ratio of mutant plaques to total pfu.

### **DNA Adduct Analysis using <sup>32</sup>P-Postlabelling**

DNA adducts were measured in each DNA sample using the butanol enrichment version of the <sup>32</sup>P-postlabelling method as described previously [30,45] with minor modifications. Enrichment by butanol extraction was used in preference to nuclease P1 digestion as it has been shown to yield a better recovery of 3-NBA-DNA adducts [22]. Briefly, DNA samples (4  $\mu\text{g}$ ) were digested with

micrococcal nuclease (120 mU, Sigma-Aldrich, Gillingham, UK) and calf spleen phosphodiesterase (40 mU, Calbiochem, Nottingham, UK), extracted with butanol, and labelled as reported. Chromatographic conditions for thin-layer chromatography (TLC) on polyethyleneimine-cellulose (PEI-cellulose) (Macherey-Nagel, Düren, Germany) were: D1, 1.0 M sodium phosphate, pH 6.0; D3, 4 M lithium formate, 7 M urea, pH 3.5; D4, 0.8 M lithium chloride, 0.5 M Tris, 8.5 M urea, pH 8.0. DNA adduct levels (RAL, relative adduct labelling) were calculated from the adduct cpm, the specific activity of [ $\gamma$ - $^{32}$ P]ATP and the amount of DNA (pmol of DNA-P) used. DNA adducts were identified using authentic standards as described previously [32]. Results were expressed as DNA adducts/ $10^8$  nucleotides (nt).

### **Statistical Analysis**

The MF was analysed by Poisson regression using SAS version 9.1 (SAS Institute, Cary, NC, USA). The natural log of total plaque count was used as an 'offset' (*i.e.*, regression variable with a constant coefficient of 1.0 for each observation). Log-linear relationships between mutant count and concentration or dose were specified by a natural log link function. Type 1, or sequential analysis, was employed to examine the statistical significance of the chemical treatment, and custom contrasts statements were employed to evaluate the statistical significance of responses at selected doses or concentrations. Pearson Correlation coefficients of MF and DNA adduct levels in FE1 cells were calculated using Microsoft Excel (Edition 2003).



## 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 (Fig. 2). For 3-NBA a clear dose-dependent increase in MF of *lacZ* was observed at doses higher than 0.1 µg/ml both in the presence or absence of an exogenous metabolic activation system (S9) (Fig. 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 higher than 1 µg/ml, but MF was much greater (up to 1.8-fold) in the presence of an exogenous S9 activation system (Fig. 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 <sup>32</sup>P-postlabelling (Fig. 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 HPLC 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-*N*<sup>6</sup>-yl)-3-aminobenzanthrone (dA-*N*<sup>6</sup>-ABA; spot 1), dG-*N*<sup>2</sup>-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 (Fig. 4). However, DNA binding by 3-NBA was much higher than by 3-ABA, with 173.1 and 18.1 adducts per 10<sup>8</sup> 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 it was detectable at a 10-fold lower dose (Fig. 4A). DNA adduct formation by both 3-NBA and 3-ABA was lower in the presence of S9 mix suggesting either that 3-NBA and 3-ABA can bind to S9 proteins or that 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;  $r > 0.900$  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<sup>TM</sup>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 Supporting Tables S1 and S2). 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 (Fig. 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 (Fig. 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<sup>TM</sup>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 Fig. 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 Supporting Table S3 and S4). No DNA adducts were observed in DNA isolated from control animals (data not shown). DNA adducts (*e.g.*, dA-*N*<sup>6</sup>-ABA, spot 1; dG-*N*<sup>2</sup>-ABA, spot 3; 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/10<sup>8</sup> nt and 55.6 adducts/10<sup>8</sup> nt for 3-NBA and 3-ABA, respectively (Fig. 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-*N*<sup>2</sup>-ABA (adduct spot 3) was the major adduct found in all tissues examined (see Supporting Table S3 and S4).

## DISCUSSION

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<sup>TM</sup>Mouse system. In the present study we used Muta<sup>TM</sup>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<sup>TM</sup>Mouse assay.

Although several other transgenic cell lines have been developed to study environmentally induced mutations [51,52], recent results have shown that Muta<sup>TM</sup>Mouse FE1 cells are a useful *in vitro* tool for assessing the mutagenic activity for a wide range of mutagens, including benzo[*a*]pyrene (BaP), 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP), 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 metabolise 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 (EROD) 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 mouse [29]. Moreover, that 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 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-*N*<sup>2</sup>-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-*N*<sup>2</sup>-ABA was the most abundant DNA adduct detected in Muta<sup>TM</sup>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<sup>TM</sup>Mouse that employed repeated 3-NBA treatments via intraperitoneal injection [33]. In the latter study it was shown that in Muta<sup>TM</sup>Mouse liver 3-NBA induces mainly GC→TA transversion mutations in the *cII* gene, and this is consistent with extensive formation of dG-*N*<sup>2</sup>-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 polymerase(s) 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 at 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 Muta<sup>TM</sup>Mouse lung epithelial cells can provide *in vitro* mutagenicity data that can reliably predict the outcome of an *in vivo* Muta<sup>TM</sup>Mouse 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-nitrobenzanthrone (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/Muta™Mouse 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 Muta™Mouse 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 Muta™Mouse, 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 27 February 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 is a promising area for further research.

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## Legend to Figures

### Figure 1:

Proposed pathways of metabolic activation and DNA adduct formation of 3-NBA and 3-ABA. See text for details. POR, cytochrome P450 oxidoreductase. R =  $-C(O)CH_3$  or  $-SO_3H$ .

### Figure 2:

Mutant frequency in the *lacZ* gene in FE1 cells treated with 3-NBA (A) or 3-ABA (B). Cells were treated without an exogenous activation system (-S9) or in the presence of an exogenous activation system (+S9). Values represent mean  $\pm$  SD of up to ten separate incubations [see Supporting Tables S5 and S6]. – = not determined. \*\*\* $p < 0.0001$ , \*\* $p < 0.01$ , \* $p < 0.05$  by comparison to controls.

### Figure 3:

Autoradiographic profiles of DNA adducts formed in FE1 cells by 3-NBA (*upper panel*) or 3-ABA (*lower panel*). Adduct profiles in FE1 cells treated with 10  $\mu\text{g/ml}$  of either 3-NBA (A) or 3-ABA (C) without an exogenous activation system (-S9), and treated with 10  $\mu\text{g/ml}$  of either 3-NBA (B) or 3-ABA (D) in the presence of an exogenous activation system (+S9).

### Figure 4:

Levels of total DNA adducts in FE1 cells after exposure to 3-NBA (A) or 3-ABA (B). Cells were treated without an exogenous activation system (-S9) or in the presence of an exogenous activation system (+S9). Values represent mean  $\pm$  SD of two separate incubations; each DNA sample was determined by two independent  $^{32}\text{P}$ -postlabelling analyses. Levels of individual DNA adducts are given in Supporting Tables S7 and S8. RAL, relative adduct labelling. ND = not detected. – = not determined.

### Figure 5:

Mutant frequency in the *lacZ* gene from various organs of Muta<sup>TM</sup>Mouse treated with 3-NBA (A) or 3-ABA (B). Results represent mean  $\pm$  SD of up to six animals. Results represent mean of two separate animal treatments [see Supporting Tables S1 and S2]. \*\*\* $p < 0.0001$ , \*\* $p < 0.01$ , \* $p < 0.05$  by comparison to controls.

### Figure 6:

Autoradiographic profiles of DNA adducts formed in liver of Muta<sup>TM</sup>Mouse treated with 3-NBA (*upper panel*) or 3-ABA (*lower panel*) [these profiles are representative of adduct profiles obtained with DNA from other mice tissue, e.g. lung and bone marrow]. Adduct profiles in Muta<sup>TM</sup>Mouse treated with a total dose of 56 mg/kg bw (A) or 140 mg/kg bw 3-NBA (B). Adduct profiles in Muta<sup>TM</sup>Mouse treated with a total dose of 56 mg/kg bw (C) or 140 mg/kg bw 3-ABA (D).

**Figure 7:**

Levels of total DNA adducts in Muta<sup>TM</sup>Mouse after exposure to 3-NBA (A) or 3-ABA (B). Values represent mean  $\pm$  SD of four animals; each DNA sample was determined by two independent <sup>32</sup>P-postlabelling analyses. Individual levels of DNA adducts are given in Supporting Tables S3 and S4). RAL, relative adduct labelling. ND = not detected.

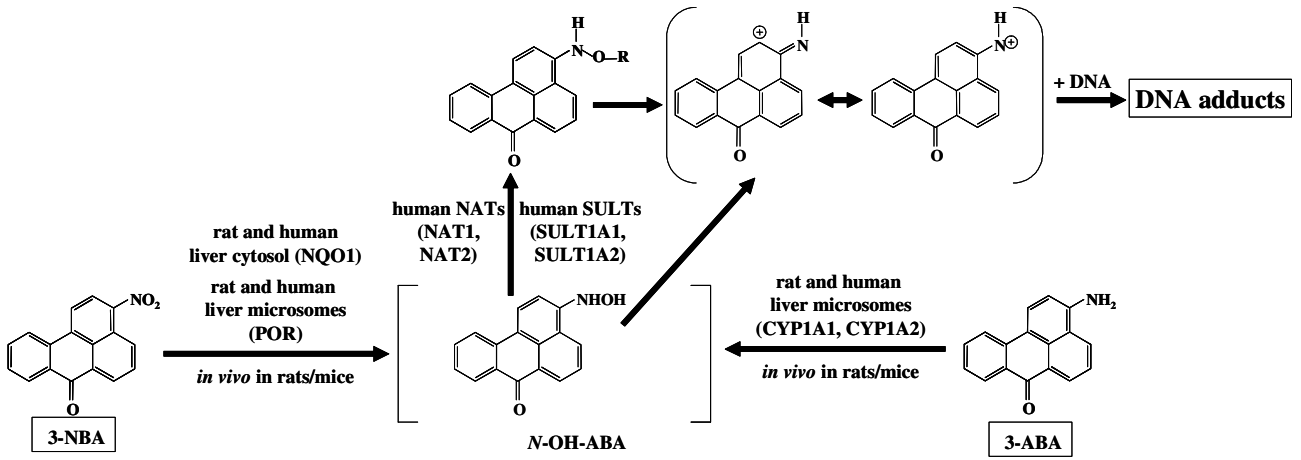


Figure 1

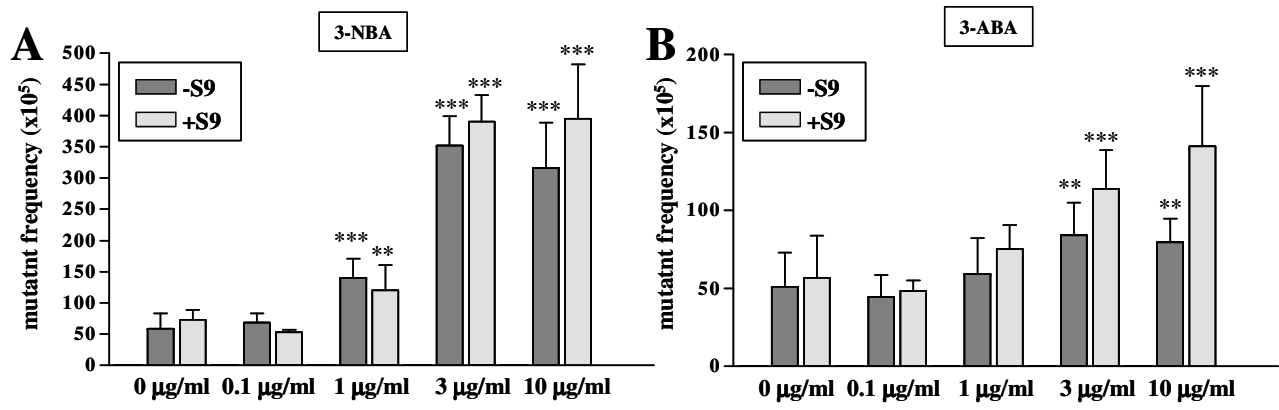


Figure 2

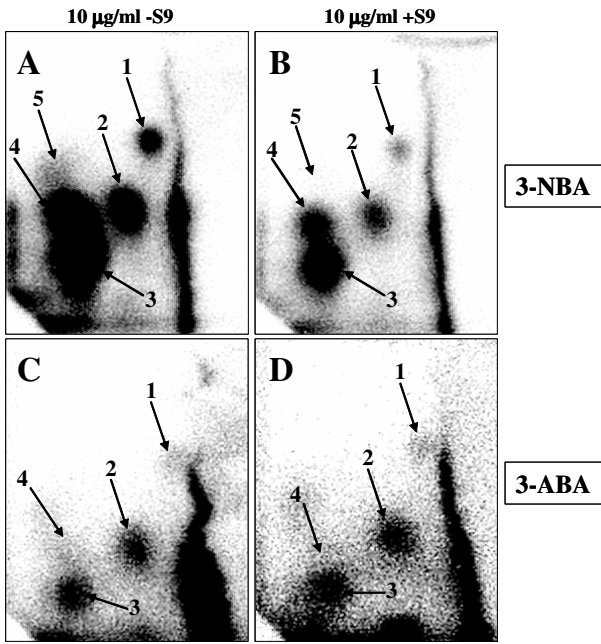


Figure 3

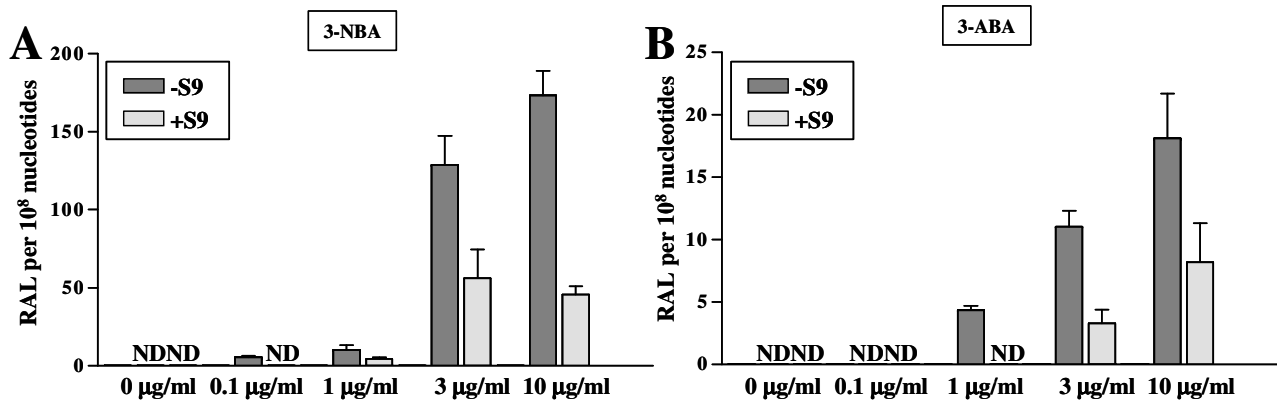


Figure 4

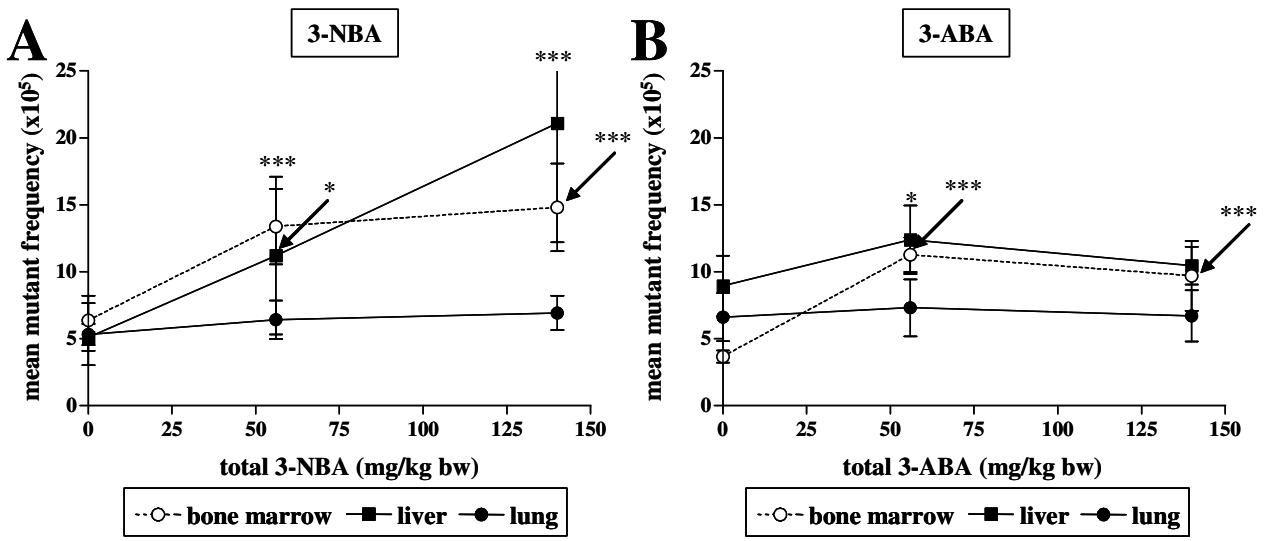


Figure 5



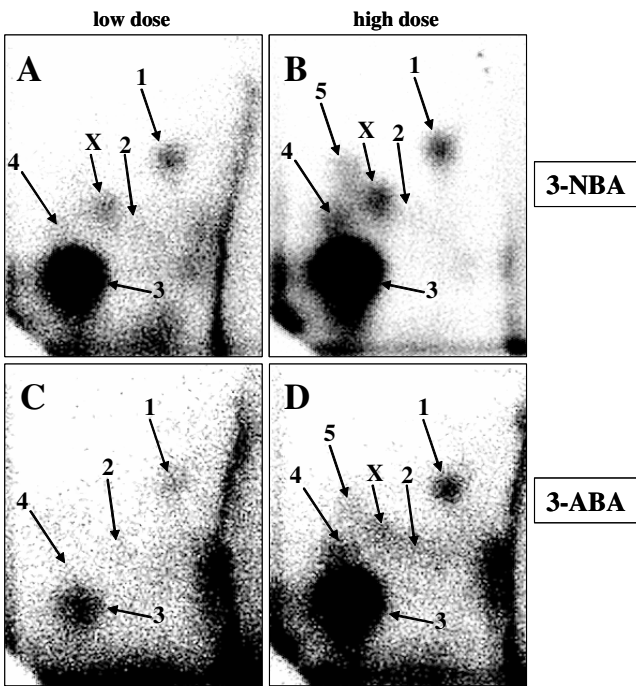


Figure 6

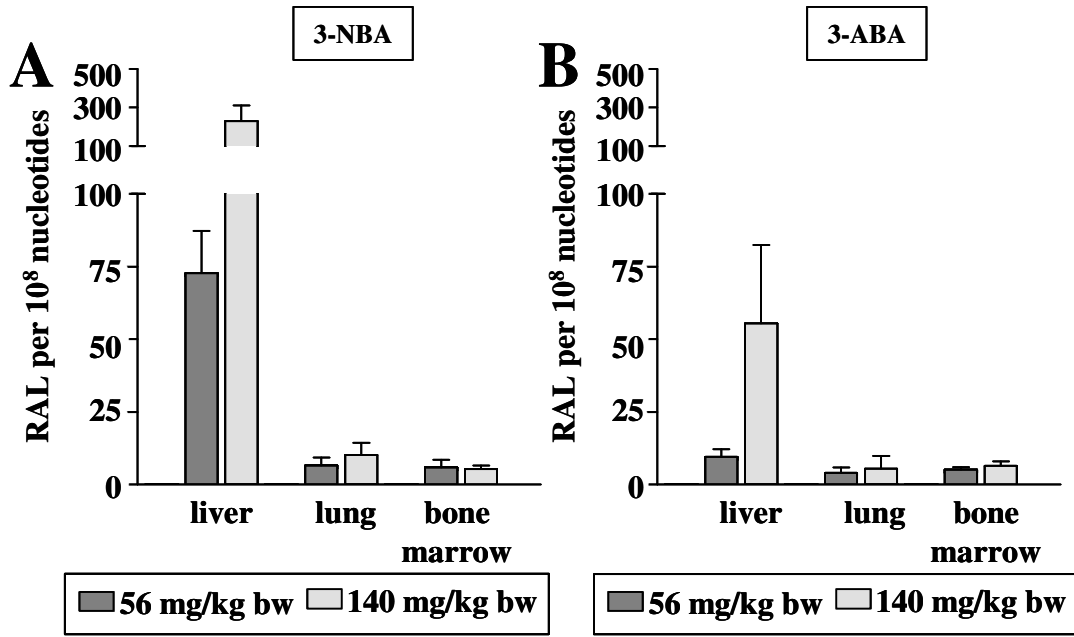


Figure 7

## **Supplementary Data**

**Table S1:** Mutant frequency in the *LacZ* gene in Muta™Mouse treated with 3-NBA

Tissue	Dose 3-NBA (mg/kg bw per day) <sup>1</sup>	Total 3-NBA (mg/kg bw)	Animals	Total mutants	Total plaques	MF (x10 <sup>5</sup> )	MF (x10 <sup>5</sup> ) (mean ± SD)
bone marrow	0	0	1	9	200,125	4.50	6.36 ± 1.83
bone marrow	0	0	2	14	151,751	9.23	
bone marrow	0	0	3	16	247,837	6.46	
bone marrow	0	0	4	12	236,572	5.07	
bone marrow	0	0	5	17	259,765	6.54	
bone marrow	2x28	56	6	53	359,165	14.76	13.37 ± 2.83
bone marrow	2x28	56	7	39	231,933	16.82	
bone marrow	2x28	56	8	23	223,981	10.27	
bone marrow	2x28	56	9	29	201,451	14.40	
bone marrow	2x28	56	10	30	282,296	10.63	
bone marrow	2x28	56	16	- <sup>2</sup>	-	-	
bone marrow	5x28	140	11	26	217,355	11.96	14.80 ± 3.27
bone marrow	5x28	140	12	12	62,788	19.11	
bone marrow	5x28	140	14	19	122,262	15.54	
bone marrow	5x28	140	15	26	206,752	12.58	
liver	0	0	1	12	233,424	5.10	5.10 ± 1.00
liver	0	0	2	14	224,147	6.20	
liver	0	0	3	3	80,348	3.70	
liver	0	0	4	16	363,473	4.40	
liver	0	0	5	6	104,039	5.80	
liver	2x28	56	6	16	161,028	9.90	11.20 ± 5.90
liver	2x28	56	7	9	138,332	6.50	
liver	2x28	56	8	8	66,764	12.00	
liver	2x28	56	9	22	280,971	7.80	
liver	2x28	56	10	17	201,616	8.40	
liver	2x28	56	16	19	83,496	22.80	
liver	5x28	140	11	23	174,778	13.20	21.10 ± 9.10
liver	5x28	140	12	13	63,947	20.30	
liver	5x28	140	14	27	79,189	34.10	
liver	5x28	140	15	57	336,303	16.90	
lung	0	0	1	14	314,767	4.45	5.33 ± 2.31
lung	0	0	2	16	208,740	7.67	
lung	0	0	3	2	89,957	2.22	
lung	0	0	4	26	343,261	7.57	
lung	0	0	5	31	653,389	4.74	
lung	2x28	56	6	31	474,469	6.53	6.42 ± 1.44
lung	2x28	56	7	26	473,144	5.50	
lung	2x28	56	8	42	490,373	8.56	
lung	2x28	56	9	38	499,982	7.60	
lung	2x28	56	10	5	104,867	4.77	
lung	2x28	56	16	37	666,643	5.55	
lung	5x28	140	11	15	178,754	8.39	6.92 ± 1.28
lung	5x28	140	12	31	522,844	5.93	
lung	5x28	140	14	20	346,409	5.77	
lung	5x28	140	15	32	421,456	7.59	

<sup>1</sup> Doses 2 and 5 mg/kg bw administered p.o. daily for 28 days, followed by a 28 day recovery period.

<sup>2</sup> Sample lost.

**Table S2:** Mutant frequency in the *LacZ* gene in Muta™Mouse treated with 3-ABA

Tissue	Dose 3-ABA (mg/kg bw per day) <sup>1</sup>	Total 3-ABA (mg/kg bw)	Animals	Total mutants	Total plaques	MF (x10 <sup>5</sup> )	MF (x10 <sup>5</sup> ) (mean ± SD)
bone marrow	0	0	2	20	580,496	3.45	3.66 ± 0.45
bone marrow	0	0	3	25	607,003	4.12	
bone marrow	0	0	4	24	579,171	4.14	
bone marrow	0	0	5	11	316,258	3.48	
bone marrow	0	0	6	16	510,253	3.14	
bone marrow	2x28	56	7	31	288,260	10.75	
bone marrow	2x28	56	8	73	657,365	11.10	
bone marrow	2x28	56	9	7	68,917	10.16	
bone marrow	2x28	56	10	31	242,536	12.78	
bone marrow	2x28	56	11	51	394,949	12.91	
bone marrow	2x28	56	12	47	474,469	9.91	
bone marrow	5x28	140	13	56	607,003	9.23	9.69 ± 2.61
bone marrow	5x28	140	14	25	431,396	5.80	
bone marrow	5x28	140	15	46	450,613	10.21	
bone marrow	5x28	140	16	34	333,984	10.18	
bone marrow	5x28	140	17	44	337,297	13.04	
liver	0	0	2	37	386,997	9.56	
liver	0	0	3	27	365,129	7.39	
liver	0	0	4	36	429,408	8.38	
liver	0	0	5	20	294,224	6.80	
liver	0	0	6	41	327,357	12.52	
liver	2x28	56	7	25	156,389	15.99	12.39 ± 2.56
liver	2x28	56	8	13	102,216	12.72	
liver	2x28	56	9	24	235,578	10.19	
liver	2x28	56	10	49	483,747	10.13	
liver	2x28	56	11	28	188,529	14.85	
liver	2x28	56	12	38	363,804	10.45	
liver	5x28	140	13	28	220,834	12.68	10.45 ± 1.42
liver	5x28	140	14	40	455,915	8.77	
liver	5x28	140	15	25	252,145	9.91	
liver	5x28	140	16	46	439,348	10.47	
liver	5x28	140	17	38	365,792	10.39	
lung	0	0	2	23	404,889	5.70	
lung	0	0	3	31	352,207	8.80	
lung	0	0	4	15	195,818	7.70	
lung	0	0	5	21	325,038	6.50	
lung	0	0	6	13	312,116	4.20	
lung	2x28	56	7	3	79,354	3.80	7.30 ± 2.13
lung	2x28	56	8	28	399,588	7.00	
lung	2x28	56	9	17	195,487	8.70	
lung	2x28	56	10	28	376,395	7.40	
lung	2x28	56	11	18	259,765	6.90	
lung	2x28	56	12	30	295,715	10.10	
lung	5x28	140	13	47	551,339	8.50	6.70 ± 1.91
lung	5x28	140	14	24	328,848	7.30	
lung	5x28	140	15	37	440,839	8.40	
lung	5x28	140	16	2	39,926	5.00	
lung	5x28	140	17	13	294,224	4.40	

<sup>1</sup> Doses 2 and 5 mg/kg bw administered p.o. daily for 28 days, followed by a 28 day recovery period.

**Table S3:** DNA adduct formation in Muta™Mouse treated with 3-NBA

Tissue	3-NBA (mg/kg bw per day) <sup>1</sup>	Total 3-NBA (mg/kg bw)	RAL <sup>2</sup> (mean ± SD/10 <sup>8</sup> nucleotides)						Total
			Spot 1	Spot 2	Spot 3	Spot 4	Spot 5	Spot X	
bone marrow	0	0	ND	ND	ND	ND	ND	ND	ND
bone marrow	2x28	56	1.2 ± 0.6	1.6 ± 1.0	2.7 ± 1.1	0.4 ± 0.2	ND	ND	5.9 ± 2.6
bone marrow	5x28	140	1.1 ± 0.3	1.0 ± 0.3	2.8 ± 0.8	0.4 ± 0.1	ND	ND	5.3 ± 1.3
liver	0	0	ND	ND	ND	ND	ND	ND	ND
liver	2x28	56	3.4 ± 0.6	2.2 ± 0.6	62.4 ± 12.0	2.4 ± 1.0	ND	2.5 ± 0.5	72.8 ± 14.5
liver	5x28	140	6.8 ± 2.1	3.8 ± 1.1	199.6 ± 69.4	11.0 ± 5.7	3.6 ± 1.7	6.8 ± 1.7	230.2 ± 81.1
lung	0	0	ND	ND	ND	ND	ND	ND	ND
lung	2x28	56	1.1 ± 0.4	0.8 ± 0.3	4.3 ± 1.7	0.5 ± 0.4	ND	ND	6.7 ± 2.7
lung	5x28	140	1.4 ± 0.6	0.9 ± 0.3	7.1 ± 3.1	0.8 ± 0.7	ND	ND	10.3 ± 4.3

<sup>1</sup> Doses 2 and 5 mg/kg bw administered p.o. daily for 28 days, 28 day recovery time.

<sup>2</sup> Mean RAL (relative adduct labelling) of four animals (each DNA sample was determined by two <sup>32</sup>P-labelling analyses); spot 1 = dA-N<sup>6</sup>-ABA, spot 3 = dG-N<sup>2</sup>-ABA, spots 4/5 = dG-C8-N-ABA. ND, not detected.

**Table S4:** DNA adduct formation in Muta™Mouse treated with 3-ABA

Tissue	3-ABA (mg/kg bw per day) <sup>1</sup>	Total 3-ABA (mg/kg bw)	RAL <sup>2</sup> (mean ± SD/10 <sup>8</sup> nucleotides)						Total
			Spot 1	Spot 2	Spot 3	Spot 4	Spot 5	Spot X	
bone marrow	0	0	ND	ND	ND	ND	ND	ND	ND
bone marrow	2x28	56	0.9 ± 0.2	1.0 ± 0.5	2.7 ± 0.6	0.6 ± 0.3	ND	ND	5.2 ± 0.9
bone marrow	5x28	140	1.2 ± 0.3	1.0 ± 0.2	3.5 ± 1.0	0.8 ± 0.3	ND	ND	6.5 ± 1.6
liver	0	0	ND	ND	ND	ND	ND	ND	ND
liver	2x28	56	1.1 ± 0.3	1.7 ± 0.5	5.6 ± 1.6	1.1 ± 0.5	ND	ND	9.5 ± 2.8
liver	5x28	140	2.9 ± 1.6	2.5 ± 0.9	44.5 ± 23.6	3.2 ± 1.3	1.0 ± 0.3	1.6 ± 0.7	55.6 ± 26.8
lung	0	0	ND	ND	ND	ND	ND	ND	ND
lung	2x28	56	0.8 ± 0.3	0.6 ± 0.2	2.2 ± 1.1	0.4 ± 0.3	ND	ND	4.1 ± 1.8
lung	5x28	140	0.9 ± 0.4	0.8 ± 0.3	3.4 ± 3.2	0.5 ± 0.4	ND	ND	5.6 ± 4.3

<sup>1</sup> Doses 2 and 5 mg/kg bw administered p.o. daily for 28 days, 28 day recovery time.

<sup>2</sup> Mean RAL (relative adduct labelling) of four animals (each DNA sample was determined by two <sup>32</sup>P-labelling analyses); spot 1 = dA-N<sup>6</sup>-ABA, spot 3 = dG-N<sup>2</sup>-ABA, spots 4/5 = dG-C8-N-ABA. ND, not detected.

**Table S5:** Mutant frequency in the *LacZ* gene in FE1 treated with 3-NBA

Trial	3-NBA ( $\mu\text{g/ml}$ )	S9 (%)	Total mutants	Total plaques	MF ( $\times 10^5$ )	MF ( $\times 10^5$ ) (mean $\pm$ SD)
A	0	0	118	296,875	39.7	58.9 $\pm$ 24.2
A	0	0	117	241,211	48.5	
B	0	0	388	434,709	89.3	
B	0	0	224	328,683	68.2	
B	0	0	348	383,021	90.9	
C	0	0	274	483,032	56.7	
C	0	0	195	331,333	58.9	
C	0	0	70	365,268	19.2	
A	0.1	0	128	162,353	78.8	
A	0.1	0	154	266,392	57.8	
A	1	0	402	394,949	101.8	140.0 $\pm$ 31.2
A	1	0	461	458,565	100.5	
B	1	0	696	380,371	183.0	
B	1	0	768	433,384	177.2	
B	1	0	848	579,171	146.4	
C	1	0	688	487,024	141.3	
C	1	0	460	305,489	150.6	
C	1	0	366	306,152	119.5	
B	3	0	700	234,584	298.4	
B	3	0	1,216	339,285	358.4	
B	3	0	1,028	288,923	355.8	
C	3	0	1,088	292,414	372.1	
C	3	0	868	204,101	425.3	
C	3	0	1,480	489,020	302.6	
A	10	0	1,240	486,397	254.9	315.6 $\pm$ 73.0
A	10	0	498	249,163	199.9	
B	10	0	552	165,667	333.2	
B	10	0	672	218,680	307.3	
C	10	0	616	168,163	366.3	
C	10	0	436	102,713	424.5	
C	10	0	1,160	359,165	323.0	
A	0	0.5	200	365,792	54.7	73.3 $\pm$ 42.1
A	0	0.5	283	625,557	45.2	
B	0	0.5	284	265,067	107.1	
B	0	0.5	332	369,768	89.8	
B	0	0.5	428	270,368	158.3	
C	0	0.5	273	694,608	39.3	
C	0	0.5	237	491,699	48.2	
C	0	0.5	128	291,416	43.9	
A	0.1	0.5	74	149,763	49.4	52.6 $\pm$ 4.5
A	0.1	0.5	290	519,531	55.8	
A	1	0.5	268	372,419	72.0	120.2 $\pm$ 40.7
A	1	0.5	469	487,723	96.2	
B	1	0.5	736	437,360	168.3	
B	1	0.5	772	422,781	182.6	
B	1	0.5	528	389,648	135.5	
C	1	0.5	664	862,272	77.0	
C	1	0.5	529	519,696	101.8	
C	1	0.5	604	470,493	128.4	
B	3	0.5	1,416	355,189	398.7	
B	3	0.5	1,232	266,392	462.5	
B	3	0.5	1,060	291,573	363.5	
C	3	0.5	1,168	315,368	370.4	
C	3	0.5	1,256	310,128	405.0	
C	3	0.5	2,120	622,752	340.4	
A	10	0.5	1,000	322,056	310.5	394.8 $\pm$ 87.7
A	10	0.5	1,088	337,960	321.9	
B	10	0.5	180	57,652	312.2	



B	10	0.5	268	72,231	371.0
C	10	0.5	872	187,624	464.8
C	10	0.5	1,008	190,848	528.2
C	10	0.5	1,792	393,624	455.3

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**Table S6:** Mutant frequency in the *LacZ* gene in FE1 treated with 3-ABA

Trial	3-ABA ( $\mu\text{g/ml}$ )	S9 (%)	Total mutants	Total plaques	MF ( $\times 10^5$ )	MF ( $\times 10^5$ ) (mean $\pm$ SD)
A	0	0	85	249,163	34.11	51.0 $\pm$ 22.0
A	0	0	74	169,643	43.62	
B	0	0	248	324,707	76.38	
B	0	0	324	400,251	80.95	
B	0	0	333	467,843	71.18	
C	0	0	96	338,126	28.39	
C	0	0	98	324,707	30.18	
C	0	0	79	357,840	22.08	
D	0	0	90	133,859	67.24	
D	0	0	52	93,436	55.65	
A	0.1	0	73	235,247	31.03	44.5 $\pm$ 14.1
A	0.1	0	75	173,619	43.20	
E	0.1	0	1	663	-	
E	0.1	0	49	82,833	59.15	
A	1	0	150	364,467	41.16	59.4 $\pm$ 22.9
A	1	0	185	352,539	52.48	
B	1	0	436	410,853	106.12	
B	1	0	384	518,205	74.10	
B	1	0	200	234,584	85.26	
C	1	0	178	417,480	42.64	
C	1	0	191	461,216	41.41	
C	1	0	255	535,435	47.62	
D	1	0	86	130,545	65.88	
D	1	0	54	144,461	37.38	
B	3	0	332	454,589	73.03	84.0 $\pm$ 21.0
B	3	0	444	373,744	118.80	
B	3	0	344	420,131	81.88	
C	3	0	411	650,739	63.16	
C	3	0	342	531,459	64.35	
C	3	0	301	446,637	67.39	
D	3	0	240	229,283	104.67	
D	3	0	106	107,352	98.74	
A	10	0	152	216,029	70.36	79.6 $\pm$ 15.2
A	10	0	189	307,477	61.47	
B	10	0	344	384,347	89.50	
B	10	0	280	333,984	83.84	
B	10	0	496	454,589	109.11	
C	10	0	407	573,869	70.92	
C	10	0	293	462,541	63.35	
C	10	0	331	485,072	68.24	
D	10	0	178	201,451	88.36	
D	10	0	141	155,064	90.93	
A	0	0.5	124	284,947	43.52	56.8 $\pm$ 27.1
A	0	0.5	88	181,571	48.47	
B	0	0.5	432	490,373	88.10	
B	0	0.5	516	528,808	97.58	
B	0	0.5	500	557,965	89.61	
C	0	0.5	189	613,629	30.80	
C	0	0.5	125	453,264	27.58	
C	0	0.5	86	360,491	23.86	
D	0	0.5	135	222,656	60.63	
D	0	0.5	138	237,566	58.09	
A	0.1	0.5	77	176,269	43.68	48.5 $\pm$ 6.6
A	0.1	0.5	31	72,396	42.82	
E	0.1	0.5	77	152,413	50.52	
E	0.1	0.5	65	113,979	57.03	
A	1	0.5	143	271,693	52.63	75.2 $\pm$ 15.5
A	1	0.5	118	233,259	50.59	

B	1	0.5	332	462,541	71.78	
B	1	0.5	322	377,720	85.25	
B	1	0.5	416	421,456	98.71	
C	1	0.5	251	344,587	72.84	
C	1	0.5	268	398,925	67.18	
C	1	0.5	313	385,672	81.16	
D	1	0.5	138	151,751	90.94	
D	1	0.5	109	133,859	81.43	
B	3	0.5	408	424,107	96.20	
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B	3	0.5	280	286,272	97.81	113.9 ± 24.7
B	3	0.5	552	426,757	129.35	
C	3	0.5	812	630,859	128.71	
C	3	0.5	776	511,579	151.69	
C	3	0.5	652	499,319	130.58	
D	3	0.5	253	254,464	99.42	
D	3	0.5	125	161,691	77.31	
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A	10	0.5	360	279,645	128.73	141.2 ± 38.6
A	10	0.5	371	292,899	126.66	
B	10	0.5	542	477,120	113.60	
B	10	0.5	528	434,709	121.46	
B	10	0.5	628	357,177	175.82	
C	10	0.5	688	361,816	190.15	
C	10	0.5	1,104	653,389	168.97	
C	10	0.5	1,096	591,099	185.42	
D	10	0.5	441	327,357	134.72	
D	10	0.5	327	490,373	66.68	

**Table S7:** DNA adduct formation in FE1 cells treated with 3-NBA

Dose [ $\mu\text{g/ml}$ ]	Metabolic activation	RAL <sup>1</sup> (mean $\pm$ SD/ $10^8$ nucleotides)					Total
		Spot 1	Spot 2	Spot 3	Spot 4	Spot 5	
0.1	-S9	1.2 $\pm$ 0.5	1.3 $\pm$ 0.7	2.3 $\pm$ 0.5	0.6 $\pm$ 0.2	ND	5.3 $\pm$ 1.0
0.1	+S9	ND	ND	ND	ND	ND	ND
1	-S9	1.0 $\pm$ 0.5	2.6 $\pm$ 0.3	5.3 $\pm$ 2.6	1.3 $\pm$ 0.8	ND	10.2 $\pm$ 3.0
1	+S9	0.7 $\pm$ 0.3	0.8 $\pm$ 0.3	2.4 $\pm$ 0.5	0.6 $\pm$ 0.2	ND	4.4 $\pm$ 1.0
3	-S9	4.1 $\pm$ 0.8	16.4 $\pm$ 3.0	61.4 $\pm$ 8.0	44.4 $\pm$ 11.1	2.3 $\pm$ 1.3	128.6 $\pm$ 18.7
3	+S9	2.9 $\pm$ 1.9	11.5 $\pm$ 5.2	27.6 $\pm$ 10.7	13.1 $\pm$ 2.0	1.0 $\pm$ 0.4	56.1 $\pm$ 18.4
10	-S9	13.5 $\pm$ 1.0	15.4 $\pm$ 5.7	93.10 $\pm$ 5.7	45.1 $\pm$ 6.3	6.0 $\pm$ 2.4	173.1 $\pm$ 15.9
10	+S9	3.5 $\pm$ 2.1	11.6 $\pm$ 1.9	21.45 $\pm$ 3.7	8.2 $\pm$ 1.7	0.8 $\pm$ 0.1	45.6 $\pm$ 5.5

<sup>1</sup> Mean RAL (relative adduct labelling) of two separate incubations (each DNA sample was determined by two <sup>32</sup>P-labelling analyses); spot 1 = dA-N<sup>6</sup>-ABA, spot 3 = dG-N<sup>2</sup>-ABA, spots 4/5 = dG-C8-N-ABA. ND, not detected.

**Table S8:** DNA adduct formation in FE1 cells treated with 3-ABA

Dose [ $\mu\text{g/ml}$ ]	Metabolic activation	RAL <sup>1</sup> (mean $\pm$ SD/ $10^8$ nucleotides)					Total
		Spot 1	Spot 2	Spot 3	Spot 4	Spot 5	
0.1	-S9	ND	ND	ND	ND	ND	ND
0.1	+S9	ND	ND	ND	ND	ND	ND
1	-S9	1.0 $\pm$ 0.4	1.0 $\pm$ 0.2	1.9 $\pm$ 0.2	0.4 $\pm$ 0.1	ND	4.3 $\pm$ 0.4
1	+S9	ND	ND	ND	ND	ND	ND
3	-S9	0.8 $\pm$ 0.2	3.5 $\pm$ 0.4	5.4 $\pm$ 0.6	1.3 $\pm$ 0.3	ND	11.0 $\pm$ 1.3
3	+S9	0.3 $\pm$ 0.1	0.9 $\pm$ 0.3	1.7 $\pm$ 0.8	0.4 $\pm$ 0.2	ND	3.3 $\pm$ 1.1
10	-S9	1.2 $\pm$ 0.4	5.2 $\pm$ 1.0	9.2 $\pm$ 2.0	2.5 $\pm$ 1.2	ND	18.1 $\pm$ 3.6
10	+S9	0.6 $\pm$ 0.2	2.5 $\pm$ 1.1	4.2 $\pm$ 1.5	0.9 $\pm$ 0.5	ND	8.2 $\pm$ 3.1

<sup>1</sup> Mean RAL (relative adduct labelling) of two separate incubations (each DNA sample was determined by two <sup>32</sup>P-labelling analyses); spot 1 = dA-N<sup>6</sup>-ABA, spot 3 = dG-N<sup>2</sup>-ABA, spots 4/5 = dG-C8-N-ABA. ND, not detected.