DOI:
10.1016/j.tox.2008.01.018

Document Version
Peer reviewed version

The environmental pollutant and carcinogen 3-nitrobenzanthrone induces cytochrome P450 1A1 and NAD(P)H:quinone oxidoreductase in rat lung and kidney, thereby enhancing its own genotoxicity*

Marie Stiborová,[a,*] Helena Dračínská[a], Jana Mizerovská[a], Eva Frei[b], Heinz H. Schmeiser[b], Jiří Hudeček[a], Petr Hodek[a], David H. Phillips[c], Volker M. Arlt[c]

[a]*Department of Biochemistry, Faculty of Science, Charles University, Albertov 2030, 128 40 Prague 2, The Czech Republic*

[b]*Division of Molecular Toxicology, German Cancer Research Center, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany*

[c]*Section of Molecular Carcinogenesis, Institute of Cancer Research, Brookes Lawley Building, Sutton, Surrey SM2 5NG, United Kingdom*

Keywords: 3-nitrobenzanthrone; 3-aminobenzanthrone; DNA adducts; NAD(P)H:quinone oxidoreductase; cytochrome P450 1A1; induction.

[*]Corresponding author at: Department of Biochemistry, Faculty of Science, Charles University, Albertov 2030, 128 40 Prague 2, Czech Republic. Tel.: +420-221951285; fax: +420-221951283. E-mail address: stiborov@natur.cuni.cz. (M. Stiborová)

Abbreviations: 3-NBA, 3-nitrobenzanthrone; 3-ABA, 3-aminobenzanthrone; COX, cyclooxygenase; c_T, cycle threshold; dA-N^6-ABA, 2-(2'-deoxyadenosin-N^6-yl)-3-aminobenzanthrone; dG-N^2-ABA, *N*-(2'-deoxyguanosin-N^2-yl)-3-aminobenzanthrone; dG-C8-*N*-ABA, *N*-(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone; EROD, 7-ethoxyresorufin *O*-deethylation; HX, hypoxanthine; LPO, lactoperoxidase; MPO, myeloperoxidase; NQO1, NAD(P)H:quinone oxidoreductase; NAT, *N*,*O*-acetyltransferase; SDS, sodium dodecyl sulphate; SULT, sulfotransferase; CYP, cytochrome P450; dA, deoxyadenosine; dG, deoxyguanosine; acetyl-CoA, acetyl coenzyme A; PAPS, 3'-phosphoadenosine-5'-phosphosulfate; TLC, thin-layer chromatography; RAL, relative adduct labelling; RT, real-time; PCR, polymerase chain reaction; XO, xanthine oxidase.

* This work was supported in part by Grant Agency of the Czech Republic, grant 303/05/2195, Ministry of Education of the Czech Republic, grant MSM0021620808 and by Cancer Research UK. V.M. Arlt and D.H. Phillips are partners of ECNIS (Environmental Cancer Risk, Nutrition and Individual Susceptibility), a network of excellence operating within the European Union 6th Framework Program, Priority 5: "Food Quality and Safety" (Contract No. 513943).

Abstract

3-Nitrobenzanthrone (3-NBA) is a carcinogen occurring in diesel exhaust and air pollution. Using the ^{32}P-postlabeling method, we found that 3-NBA and its human metabolite, 3-aminobenzanthrone (3-ABA), are activated to species forming DNA adducts by cytosols and/or microsomes isolated from rat lung, the target organ for 3-NBA carcinogenicity, and kidney. Each compound generated identical five DNA adducts. We have demonstrated the importance of pulmonary and renal NAD(P)H:quinone oxidoreductase (NQO1) to reduce 3-NBA to species that are further activated by *N*,*O*-acetyltransferases and sulfotransferases. Cytochrome P450 (CYP) 1A1 is the essential enzyme for oxidative activation of 3-ABA in microsomes of both organs, while cyclooxygenase plays a minor role. 3-NBA was also investigated for its ability to induce NQO1 and CYP1A1 in lungs and kidneys, and for the influence of such induction on DNA adduct formation by 3-NBA and 3-ABA. When cytosols from rats treated i.p. with 40 mg/kg bw of 3-NBA were incubated with 3-NBA, DNA adduct formation was up to 2.1-fold higher than in incubations with cytosols from control animals. This increase corresponded to an increase in protein level and enzymatic activity of NQO1. Incubations of 3-ABA with microsomes of 3-NBA-treated rats led to up to a 5-fold increase in DNA adduct formation relative to controls. The stimulation of DNA adduct formation correlated with the potential of 3-NBA to induce protein expression and activity of CYP1A1. These results demonstrate that 3-NBA is capable to induce NQO1 and CYP1A1 in lungs and kidney of rats thereby enhancing its own genotoxic and carcinogenic potential.

1. Introduction

Lung cancer is the most common malignant disease worldwide and is the major cause of death from cancer. Although tobacco smoking is the overwhelming cause of lung cancer, vehicular exhaust and ambient air pollution are also implicated as causative factors (IARC, 1989; Environmental Protection Agency, 2002; Vineis et al., 2004; Grashick et al., 2004). Nitro-aromatic compounds are widely distributed environmental pollutants found in exhaust from diesel and gasoline engines and on the surface of ambient air particulate matter. The increased lung cancer risk after exposure to these environmental sources and the detection of nitro-aromatics in the lungs of non-smokers with lung cancer has led to considerable interest in assessing their potential cancer risk (IARC, 1989; Environmental Protection Agency, 2002; Vineis et al., 2004; Grashick et al., 2004).

The nitroaromatic 3-nitrobenzanthrone (3-nitro-7*H*-benz[*de*]anthracen-7-one, 3-NBA, Fig. 1) occurs in diesel exhaust and in airborne particulate matter (Enya et al., 1997; Seidel et al., 2002; Nagy et al., 2005; Arlt, 2005). The main metabolite of 3-NBA, 3-aminobenzanthrone (3-ABA, Fig. 1) (Hansen et al., 2007; Svobodová et al., 2007), has been found in urine samples of salt mine workers occupationally exposed to diesel emissions (Seidel et al., 2002), demonstrating human exposure to 3-NBA. 3-NBA is carcinogenic in rats, causing lung tumours after intratracheal instillation (Nagy et al., 2005). It is also an exceptionally potent mutagen in the Ames *Salmonella typhimurium* assay, scoring more than 6 million revertants per nanomole in strain YG1024 overexpressing bacterial nitroreductase and *O*-acetyltransferase (Enya et al., 1997). 3-NBA has also been shown to be genotoxic in several other short-term tests and in the transgenic Muta Mouse assay (Arlt et al., 2004a; 2004c). Its genotoxicity has been further documented by the detection of specific DNA adducts formed *in vitro* as well as *in vivo* in rodents in various tissues (Arlt et al., 2001; 2002; 2003a; b; c; 2004a; c; 2005; 2006b; Bieler et al., 1999; 2005; 2007).

In the liver, most of the metabolic activation of 3-NBA *in vitro* is attributable to human and rat cytosolic NAD(P)H:quinone oxidoreductase (NQO1), while human *N*,*O*-acetyltransferase (NAT), NAT2, followed by NAT1, sulfotransferase (SULT), SULT1A1 and, to a lesser extent, SULT1A2

are the major activating phase II enzymes (Arlt et al., 2005; Stiborová et al., 2006). Hepatic microsomal NADPH:cytochrome P450 (CYP) reductase is also effective in the activation of 3-NBA (Arlt et al., 2003c), but in mice, 3-NBA is predominantly activated by NQO1 rather than NADPH:CYP reductase (Arlt et al., 2005) (Fig. 1). The enzymes that activate 3-NBA in the lung, the target organ for its carcinogenicity (Nagy et al., 2005), and in other extra-hepatic tissues have not yet been identified.

CYP1A1 and 1A2 (CYP1A1/2) are essential for the oxidative activation of 3-ABA in human and rat livers forming the same DNA adducts that are formed *in vivo* by 3-ABA or 3-NBA (Arlt et al., 2004b). However, other organs also have the metabolic capacity to activate 3-ABA to form DNA adducts, independent of the CYP-mediated oxidation in the liver (Arlt et al., 2006a). Previous results indicate that besides CYP enzymes expressed in several extra-hepatic tissues, peroxidases might play a role in the oxidative activation of 3-ABA (Arlt et al., 2006a). In *in-vitro* experiments, mammalian prostaglandin H synthase (cyclooxygenase, COX), lactoperoxidase (LPO) and myeloperoxidase (MPO) were found to be effective in activating 3-ABA (Arlt et al., 2006a) (Fig. 1). However, in extra-hepatic tissues the actual role of CYPs and peroxidases in the metabolic activation of 3-ABA and their relative contributions to such activation remain to be investigated. The potential of 3-NBA to influence the expression and activities of enzymes involved in its own activation and that of its metabolite 3-ABA also requires examination. Thus, although we recently found that 3-NBA induces the major enzymes activating it and its metabolite 3-ABA in rat livers (Stiborová et al., 2006), its potential to induce such enzymes in extra-hepatic organs has not yet been investigated.

The present study was undertaken to determine the capability of cytosols and microsomes isolated from two rat organs; the lung, the target organ for 3-NBA carcinogenicity, and the kidney, an important organ for excretion of xenobiotics, to activate 3-NBA and 3-ABA. A further aim was to identify enzymes involved in DNA adduct formation by 3-NBA and 3-ABA in rat lung and kidney and to evaluate whether treatment by 3-NBA induces these enzymes.

2. Material and Methods

2.1. Chemicals

NADPH, hypoxanthine (HX), dicoumarol, allopurinol, deoxyadenosine (dA) 3'-monophospate, deoxyguanosine (dG) 3'-monophosphate, acetyl coenzyme A (acetyl-CoA) 3'-phosphoadenosine-5'-phosphosulfate (PAPS), menadione (2-methyl-1,4-naphthoquinone) and calf thymus DNA were from Sigma Chemical Co (St Louis, MO, USA); Sudan I from BDH (Poole, UK); 7-ethoxyresorufin from Fluka Chemie AG (Buchs, Switzerland). Enzymes and chemicals for the ^{32}P-postlabelling assay were obtained from sources described (Phillips and Arlt, 2007). All these and other chemicals were reagent grade or better.

2.2. Synthesis of 3-NBA and 3-ABA

3-NBA and 3-ABA were synthesized as described (Arlt et al., 2003a) and their authenticity was confirmed by UV spectroscopy, electrospray mass spectra and high field proton NMR spectroscopy.

2.3. Animal experiments

The study was conducted in accordance with the Regulations for the Care and Use of Laboratory Animals (311/1997, Ministry of Agriculture, Czech Republic), which is in compliance with Declaration of Helsinki. Male Wistar rats (100-125 g, n=3 per group) were treated with a single dose of 0.4, 4 or 40 mg/kg bw of 3-NBA by intraperitoneal injection. 3-NBA was dissolved in sunflower oil at a concentration of 0.4 or 4 mg/ml. Three control animals received an equal volume of oil only. Rats were placed in cages in temperature and humidity controlled rooms. Standardised diet and water were provided *ad libitum*. The animals were killed 24 hours after treatment by cervical dislocation. Lungs and kidneys were removed immediately after death and used for isolation of mRNA and for preparation of microsomal and cytosolic fractions.

2.4. Preparation of microsomal and cytosolic fractions

Microsomal and cytosolic fractions were isolated from the lungs and kidneys of rats, either uninduced or pretreated with 3-NBA (see above) as described (Stiborová et al., 2003). Both subcellular preparations were analysed for the presence of 3-NBA using the HPLC on a CC 250/4

Nucleosil, 100-5 C18 HD column (Macherey-Nagel, 4 x 250 mm) preceded by a C-18 guard column. Eluent was 70% methanol in water, at a flow rate of 0.6 ml min^{-1}, and detection was at 254 nm. The standard of 3-NBA eluted with a retention time of 8.2 min. No 3-NBA was detectable in microsomal and cytosolic fractions from rats that had been pretreated with this compound.

2.5. Cytosolic incubations

The deaerated and argon-purged incubation mixtures, in a final volume of 750 µl, consisted of 50 mM Tris-HCl buffer (pH 7.4), containing 0.2% Tween 20, cofactors for cytosolic enzymes (1 mM NADPH, NADH or HX; 2 mM acetyl-CoA; 100 µM PAPS), pooled lung and kidney cytosolic sample from 3 rats, treated either with vehicle (control) or with 40 mg/kg bw of 3-NBA (1 mg of cytosolic protein), 30 µM 3-NBA (dissolved in 7.5 µl dimethylsulfoxide) and 0.5 mg of calf thymus DNA (2 mM dNp). The reaction was initiated by adding 3-NBA. Incubations with rat cytosols were carried out at 37°C for 3 hr; the cytosol-mediated 3-NBA-derived DNA adduct formation was found to be linear up to 4 hr (Arlt et al., 2005). Control incubations were carried out either (i) without activating system (cytosol), (ii) without cofactors (NADPH, NADH, HX), (iii) without DNA or (iv) without 3-NBA. After extraction with ethyl acetate, DNA was isolated from the residual water phase by the phenol/chloroform extraction method as described (Arlt et al., 2005).

Incubations used to evaluate the activation of 3-ABA by peroxidases in rat pulmonary and renal cytosolic samples contained, in a final volume of 750 µl, 50 mM Tris-HCl buffer (pH 7.4), containing 0.2% Tween 20, 100 µM 3-ABA (dissolved in 7.5 µl dimethylsulfoxide), 0.5 mg of calf thymus DNA and 200 µM H_2O_2.

2.6. Cytosolic inhibition studies

Dicoumarol and allopurinol were used to inhibit NQO1 and xanthine oxidase (XO), respectively, in rat pulmonary and renal cytosolic fractions (Watanabe et al., 1997; Ritter et al., 2000). Inhibitors dissolved in 7.5 µl of methanol, to yield final concentrations of 10 µM, were added to the incubation mixtures as reported previously (Arlt et al., 2005). An equal volume of methanol was added to the control incubations.

2.7. Microsomal incubations

Incubation mixtures, in a final volume of 750 µl, consisted of 50 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH or 0.1 mM arachidonic acid, pooled pulmonary and renal microsomal sample from 3 rats, either control or treated with 40 mg/kg bw of 3-NBA (0.5 mg of microsomal protein), 100 µM 3-ABA or 3-NBA (dissolved in 7.5 µl dimethylsulfoxide) and 0.5 mg of calf thymus DNA. The reaction was initiated by adding 3-ABA or 3-NBA and were carried out at 37°C for 2 hr; the microsomal-mediated 3-NBA (3-ABA)-derived DNA adduct formation was found to be linear up to 3 hr (Arlt et al., 2003c; 2004b). Control incubations were carried out either (i) without activating system (microsomes), (ii) with activating system and 3-ABA, but without DNA or (iii) with activating system and DNA but without 3-ABA. After the incubation and extraction with ethyl acetate, DNA was isolated from the residual water phase by the phenol/chloroform extraction method as described (Arlt et al., 2004b).

2.8. Microsomal inhibition studies

α-Naphthoflavone (α-NF) (Sigma), which inhibits CYP1A (Rendic and DiCarlo, 1997; Stiborová et al., 2005b), and indomethacin (Sigma), which inhibits COX (Eling et al., 1990), were used to inhibit the activation of 3-ABA by rat pulmonary and renal microsomes. Inhibitors were dissolved in 7.5 µl of methanol and were added to the incubation mixtures to yield final concentrations of 100 µM. The incubation mixtures containing the inhibitors were then incubated at 37°C for 10 min with NADPH or arachidonic acid prior to adding 3-ABA and then for a further 120 min at 37°C. An equal volume of methanol alone was added to the control incubations.

2.9. ^{32}P-Postlabelling analysis and HPLC analysis of ^{32}P-labelled 3',5'-deoxyribonucleoside bisphosphate adducts

^{32}P-Postlabelling analysis using butanol extraction, and thin layer chromatography (TLC) and HPLC were performed as described (Arlt et al., 2006b). Enrichment by butanol extraction has been shown to yield more adduct spots and a better recovery of 3-NBA (3-ABA)-derived DNA adducts than using enrichment by nuclease P1 digestion (Arlt et al., 2001; 2004b). DNA adduct spots were

numbered as reported (Arlt et al., 2006b). As reference compounds deoxyadenosine (dAp) and deoxyguanosine (dGp) 3'-monophosphates (4 μmol/ml) (Sigma) were incubated with 3-NBA (300 μM) activated by XO (1 U/ml) (Sigma) in the presence of HX and analysed as described previously (Arlt et al., 2001). DNA adduct standard samples of 3-NBA, 2-(2'-deoxyadenosin-N^6-yl)-3-aminobenzanthrone-3'-phosphate (dA3'p-N^6-ABA), *N*-(2'-deoxyguanosin-N^2-yl)-3-aminobenzanthrone-3'-phosphate (dG3'p-N^2-ABA) and *N*-(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone-3'-phosphate (dG3'p-C8-*N*-ABA), were prepared by reacting *N*-acetoxy-3-aminobenzanthrone with dAp or dGp and analysed as described recently (Arlt et al., 2006b).

2.10. Isolation of CYP1A1

Recombinant rat CYP1A1 protein was purified to homogeneity from membranes of *Escherichia coli* transfected with a modified *CYP1A1* cDNA (Stiborová et al., 2002), in the laboratory of H. W. Strobel (University of Texas, Medical School of Houston, Texas, USA) by P. Hodek (Charles University, Prague, Czech Republic).

2.11. Preparation of antibodies

Leghorn chicken were immunised subcutaneously three times a week with rat recombinant CYP1A1 and human recombinant NQO1 (Sigma) antigens (0.1 mg/animal) emulsified in complete Freund's adjuvant for the first injection and in incomplete adjuvant for boosters. Immunoglobulin fraction was purified from pooled egg yolks using fractionation by polyethylene glycol 6000 (Stiborová et al., 2002).

2.12. Estimation of CYP1A1 and NQO1 protein content in microsomes and cytosols of rat lung and kidney

Immunoquantitation of rat pulmonary and renal microsomal CYP1A1 and of cytosolic NQO1 was done by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis. Samples containing 75 μg microsomal or cytosolic proteins were subjected to electrophoresis on SDS/10% polyacrylamide gels (Stiborová et al., 2002; 2005b, 2006). After migration, proteins were transferred onto polyvinylidene difluoride membranes. Rat CYP1A1 and NQO1 proteins were

probed with the chicken polyclonal antibodies as reported elsewhere (Stiborová et al., 2002; 2005b; 2006). The antibodies against rat recombinant CYP1A1 and human NQO1 recognise these enzymes in rat pulmonary and renal microsomes as one protein band. Rat recombinant CYP1A1 (in Supersomes™, Gentest Corp., Woburn, MA, USA) and human recombinant NQO1 (Sigma Chemical Co, St Louis, MO, USA) were used as positive controls to identify the bands of CYP1A1 in microsomes and NQO1 in cytosols. The antigen-antibody complex was visualised with an alkaline phosphatase-conjugated rabbit anti-chicken IgG antibody and 5-bromo-4-chloro-3-indolylphosphate/nitrobluetetrazolium as chromogenic substrate (Stiborová et al., 2002; 2005b; 2006).

2.13. CYP1A1 and NQO1 enzyme activity assays

The microsomal samples were characterised for CYP1A1 activity using 7-ethoxyresorufin *O*-deethylation (EROD) activity and the oxidation of Sudan I (Stiborová et al., 2002; 2005b). The cytosolic samples were characterised for NQO1 activity, using menadione (2-methyl-1,4-naphthoquinone) as a substrate (Stiborová et al., 2003). NQO1 activity was determined by following the oxidation of NADPH spectrophotometrically at 340 nm. The standard assay system contained 25 mM Tris-HCl (pH 7.4), 0.2% Tween 20, 0.07% bovine serum albumin, 400 mM NADPH and 100 mM menadione dissolved in methanol.

2.14. CYP1A1 and NQO1 mRNA content in rat lungs and kidneys

Total RNA was isolated from frozen lungs and kidneys of three untreated rats and three rats pretreated with 40 mg/kg body weight of 3-NBA or 3-ABA using Trizol Reagent (Invitrogen, Carlsbad, CA, USA) according to the procedure supplied by the manufacturer. The quality of isolated RNA was verified by horizontal agarose gel electrophoresis, RNA quantity was assessed by UV-VIS spectrophotometry on a Carry 300 spectrophotometer (Varian, Palo Alto, CA, USA). RNA samples (1 μg) were reversely transcribed using 200 U of reverse transcriptase per sample with random hexamer primers utilising RevertAid™ First Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. The prepared cDNA was used for

real-time (RT) polymerase chain reaction (PCR) performed in RotorGene 2000 (Corbett Research, Sydney, Australia) under the following cycling conditions: incubation at 50ºC for 2 min and initial denaturation at 95ºC for 10 min, then 50 cycles of denaturation at 95ºC for 15 s and annealing at 60ºC for 1 min. Gain was set to 7 and fluorescence was acquired after elongation step. The PCR reaction mixtures (20 µl) contained 9 µl cDNA diluted 10-times in Milli-Q ultrapure water (Biocel A10, Millipore, Billerica, MA, USA), 10 µl TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA) and 1 µl TaqMan Gene Expression Assay Mix (commercially available unlabelled PCR primers and FAM^{TM} dye-labelled probe for rat *CYP1A1* or *NQO1* as target genes and *β-actin* as reference internal standard gene). Each sample was analysed in two parallel aliquots. Negative controls had the same compositions as samples but cDNA was omitted from the mixture. Data were analysed by the program RotorGene v6 (Corbett Research, Sydney, Australia) and evaluated by comparative cycle threshold (c_T) method for relative quantitation of gene expression. Cycle thresholds, at which a significant increase in fluorescence signal was detected, were measured for each sample. Then $\Delta\Delta c_T$ was evaluated according to following equations: $\Delta c_T = c_T$ (target) - c_T (internal standard), $\Delta\Delta c_T = \Delta c_{Ttreated} - \Delta c_{Tcontrol}$, where $\Delta c_{Ttreated}$ is Δc_T for treated rats and $\Delta c_{Tcontrol}$ is Δc_T for untreated rats. Δc_T is positive if the target is expressed at a lower level than the internal standard (*β-actin*), and negative if expressed at a higher level. The induction of mRNA expression of studied target genes in pretreated animals was evaluated as $2^{-(\Delta\Delta cT)}$.

3. Results

3.1. Activation of 3-NBA by rat pulmonary and renal cytosols and microsomes

DNA adduct formation in calf thymus DNA measured by thin-layer ^{32}P-postlabelling was used to investigate the metabolic activation of 3-NBA by cytosolic fractions. Rat pulmonary and renal cytosolic samples were capable of reductively activating 3-NBA to species forming DNA adducts (see Fig. 2A for the lung cytosol). The DNA adduct pattern generated by 3-NBA consisted of a cluster of up to five adducts (spots 1−5 in Figure 2) essentially identical to that observed *in vivo* in rats and mice treated with 3-NBA (Arlt et al., 2001; 2003b; 2005; Bieler et al., 2005; 2006), and in *in-vitro* incubations using human and rat hepatic cytosols (Arlt et al., 2005) or microsomes (Arlt et al., 2003c). Thin-layer chromatograms of ^{32}P-labelled DNA from control incubations carried out in parallel without cytosol, without DNA, or without 3-NBA were devoid of adduct spots in the region of interest (data not shown). Cochromatographic analysis of individual spots on HPLC confirmed that adduct spots 1−5 that are formed with rat pulmonary and renal cytosols are derived from 3-NBA by nitroreduction (data not shown). Three of these adducts were identified as 2-(2'-deoxyadenosin-N^6-yl)-3-aminobenzanthrone (dA-N^6-ABA; spot 1), *N*-(2'-deoxyguanosin-N^2-yl)-3-aminobenzanthrone (dG-N^2-ABA; spot 3) and *N*-(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone (dG-C8-*N*-ABA; spots 4 and 5).

We therefore analysed which cytosolic reductase is responsible for 3-NBA-DNA-adduct formation. Cytosolic fractions from both organs were capable of activating 3-NBA to form DNA adducts, even without addition of cofactors for cytosolic reductases. With kidney cytosol, up to 4-times higher adduct levels were generated than with lung cytosol. As shown in Figure 3A the formation of 3-NBA-DNA adducts by lung cytosol was stimulated by NADPH and NADH, the cofactors of NQO1, (Ross et al., 2000), and the cofactor of XO, HX (Swaminathan and Hatcher, 1986). Using renal cytosol, 3-NBA-DNA adduct formation increased 2.7-fold after the addition of NADPH or NADH, while no increase was detected after addition of HX (Fig 3B). Hence, NQO1 and XO reductively activate 3-NBA in pulmonary cytosol, although XO seems to play a minor role

in this organ and does not seem to be important in renal cytosol. This finding is also supported by the fact that 3-NBA-DNA adduct formation in cytosols of both tissues was markedly decreased by dicoumarol, an inhibitor of NQO1 (Watanabe et al., 1997; Ritter et al., 2000), whereas allopurinol, an inhibitor of XO (Watanabe et al., 1997; Ritter et al., 2000), showed no effect (Table 1).

The influence of SULTs and NATs on the activation of 3-NBA in pulmonary and renal cytosols was then investigated by adding either PAPS, the cofactor of SULTs (Glatt et al., 2001), or acetyl-CoA, the cofactor of NATs (King et al., 2000), into the incubation mixtures. The addition of PAPS and acetyl-CoA resulted in a 2.5- and 29.7-fold increase in the levels of 3-NBA adducts in uninduced cytosols, respectively, over the activity with NADPH alone (Fig. 4 and Supporting Table S1). Thus, SULTs and mainly NATs strongly contribute to the metabolic activation of 3-NBA in rat pulmonary and renal cytosols.

In further experiments we studied the formation of adducts in calf thymus DNA by 3-NBA incubated with cytosols from rats pretreated with 3-NBA (40 mg/kg bw). These pulmonary and renal cytosolic samples were more effective in formation of 3-NBA-DNA adducts in the presence of NADPH, than the pulmonary and renal cytosols of untreated (control) rats (Fig. 4 and Supporting Table S1). Formation of 3-NBA-DNA adducts was up to 2.1-fold higher in incubations with cytosols of rats treated with 3-NBA relative to those of control rats (Fig. 4). Addition of PAPS and acetyl-CoA to the incubations with induced cytosols had essentially the same stimulatory effects on 3-NBA adduct formation as in control cytosols; a 2- and 29.6-fold increase, respectively, was observed in comparison to the activity using NADPH (Fig. 4 and Supporting Table S1).

Comparison of the efficiencies of microsomes isolated from lungs and kidneys of rats either uninduced or pretreated with 3-NBA to activate this carcinogen was also performed. No 3-NBA-derived DNA adducts were detectable after incubating 3-NBA with any of these pulmonary or renal microsomes and NADPH (data not shown). Hence, it seems that microsomal NADPH:CYP reductase and/or CYP are not involved in 3-NBA activation in these organs.

3.2. Activation of 3-ABA by rat pulmonary and renal microsomes and cytosols

In contrast to 3-NBA, 3-ABA was activated by lung and kidney microsomes. Because CYPs and peroxidases were found to activate 3-ABA (Arlt et al., 2004b; 2006a), we investigated the modulation of 3-ABA-derived DNA adduct formation by cofactors and selective enzyme inhibitors. In the presence of NADPH, a cofactor of CYP-dependent enzyme systems, all pulmonary and renal microsomes were capable of oxidising 3-ABA to form DNA adducts. Microsomal activation of 3-ABA generated a pattern of DNA adducts consisting of a cluster of five adducts (see spots 1-5 in Figure 2 for the lung microsomes), identical to those formed by 3-ABA and 3-NBA *in vitro* and *in vivo* (Arlt et al., 2001; 2002; 2003a; b; c; 2004b; c; 2005; 2006a; b; Bieler et al., 2005). Arachidonic acid, a cofactor for COX-dependent oxidation (Eling et al., 1990; Stiborová et al., 2005a; 2007; Arlt et al., 2006a), also mediated formation of DNA adducts, but was less effective than NADPH (Fig. 5). In the presence of NADPH, lung and kidney microsomes from rats treated with 3-NBA (40 mg/kg bw) exhibited 5- and 3.4-fold higher efficiencies to activate 3-ABA, than microsomes from untreated rats, respectively (Fig. 5 and Supporting Table S2). Microsomes from rats treated with 3-NBA were also more effective in 3-ABA activation in the presence of arachidonic acid, but only 1.5-fold higher levels of DNA adducts were detected (Fig. 5 and Supporting Table S2). Chromatograms of DNA digests from control incubations carried out in parallel without microsomes, without NADPH, without arachidonic acid, without DNA, or without 3-ABA were all devoid of adduct spots in the region of interest (data not shown).

Addition of α-NF, an inhibitor of CYP1A1 (Rendic and DiCarlo, 1997), decreased the levels of DNA adducts generated by pulmonary and renal microsomes, while indomethacin, an inhibitor of COX (Eling et al., 1990), was less effective (Fig. 5 and Supporting Table S2). The inhibitory effect of α-NF in pulmonary and renal microsomes from pretreated rats was even higher than in microsomes of uninduced rats (Fig. 5 and Supporting Table S2). These results point to CYP1A1 having a major role in DNA adduct formation by 3-ABA in rat lungs and kidneys. Additionally, they show that treating rats with 3-NBA increases the relative contribution of this enzyme to 3-ABA activation in both organs.

In the presence of hydrogen peroxide, a cofactor for peroxidases, the pulmonary and renal cytosols from uninduced rats and animals treated with 3-NBA were capable of activating 3-ABA to form DNA adducts. In contrast to pulmonary cytosol, by which five 3-ABA-DNA adducts (spots 1-5 in Figure 2) were generated, in the renal cytosolic activation system, only four 3-ABA-DNA adducts (spots 1-4 in Figure 2) were detected. Treatment of rats with 3-NBA slightly stimulated (1.3-fold) the formation of 3-ABA-DNA adducts in cytosols of both organs (Fig 6 and Supporting Table S3). No adducts were detectable in controls without cytosols, without 3-ABA (data not shown), or without hydrogen peroxide (Fig 6 and Supporting Table S3).

3.3. The effect of 3-NBA pretreatment on expression of enzymes activating 3-NBA and 3-ABA

Because NQO1 and CYP1A1 were found to be the essential enzymes activating 3-NBA and 3-ABA with subcellular fractions from rat lungs and kidneys (present paper) and from rat and human livers (Arlt et al., 2004b; 2005), we evaluated whether treating rats with 3-NBA influences expression of these enzymes. The induction of these enzymes has already been found in the liver of rats treated with 3-NBA (Stiborová et al., 2006). In the present study, Western blots with chicken polyclonal antibodies raised against NQO1 and CYP1A1 showed that the expression of both enzymes was also induced in rat lung and kidney by 3-NBA (Fig. 7). The efficiency of 3-NBA to induce CYP1A1 expression was higher in lung than in kidney (7.4-fold *versus* 3.4-fold at 40 mg/kg) (Fig. 7). EROD activity and oxidation of Sudan I, as markers of CYP1A1 activity (Stiborová et al., 2002; 2005b), were increased in both organs of rats treated with 3-NBA (Table 2). Again, the induction in lung was higher than in kidney, although control enzyme activity in lung was only half of that in kidney.

The levels of renal and pulmonary NQO1 protein were enhanced by pretreating rats with 3-NBA (40 mg/kg bw), by 2.3- and 1.4-fold, respectively (Fig. 7). The increase in NQO1 activities in cytosols depended on the administered dose and correlated with the protein expression (Table 3 and Fig. 7). Up to a 2.5-fold increase in NQO1 activity measured with menadione as a substrate was found in tissues of rats treated with 3-NBA (Table 3).

Besides the evaluation of the effects of 3-NBA on protein levels and enzyme activities of CYP1A1 and NQO1, modulation of their mRNA expression by the compound was also investigated. The relative amounts of CYP1A1 and NQO1 mRNA were measured by RT-PCR analysis. As shown in Table 4, treatment of rats with 40 mg/kg bw of 3-NBA induced mRNA levels of CYP1A1 and NQO1 up to 3.9- and 1.4-fold, respectively, in the tissues studied.

4. Discussion

The present study has increased our knowledge on the potential of cytosolic and microsomal fractions of rat lungs and kidneys to activate 3-NBA and 3-ABA, and on the enzymes participating in their bioactivation. The rat was used as an experimental model on the basis that the same enzymes activate 3-NBA and 3-ABA in livers of both species (Arlt et al., 2003a, c, 2004b, 2005, 2006a; Stiborová et al., 2006). Therefore, the results should provide some indication of what might occur in extra-hepatic tissues of humans exposed to this pollutant.

DNA adducts formed during 3-NBA and 3-ABA activation by the cytosolic and microsomal fractions are identical to those formed *in vivo* in rats and mice treated with 3-NBA or 3-ABA (Arlt et al., 2001; 2003b; 2004; 2005; 2006a; Bieler et al., 2005; 2007), and in *in-vitro* incubations using human and rat hepatic cytosols (Arlt *et al*., 2005) or microsomes (Arlt et al., 2003c; 2004b). However, cytosols or microsomes of rat livers (Stiborová et al., 2006) were up to an order of magnitude more effective in activation of both compounds than those of lung and kidney.

In the case of 3-NBA, rat cytosolic reductase(s) of both organs studied are responsible for its reductive activation to DNA-binding products, while microsomal enzymes were ineffective under conditions used in the experiments. These results are consistent with those found *in vivo*; 3-NBA is predominantly activated by cytosolic nitroreductases rather than microsomal NADPH:CYP reductase (Arlt et al., 2005). The stimulation of 3-NBA-DNA adduct formation in rat pulmonary and renal cytosolic samples by NADPH suggested the participation of NQO1 in the reductive bioactivation of 3-NBA. Inhibition of DNA adduct formation by dicoumarol, an inhibitor of NQO1, provided additional evidence for the major role of NQO1. Hence, the same cytosolic reductase, NQO1, functions as the most effective enzyme activating 3-NBA both in the extra-hepatic organs, lung and kidney, and in the liver of rats (Arlt et al., 2005). Similarly, NQO1 is the most effective enzyme reductively activating 3-NBA in human hepatic cytosol (Arlt et al., 2005). Compared to NQO1, XO had no impact in kidney cytosol on the activation of 3-NBA to form DNA adducts. However, a low, but significant, stimulatory effect of a cofactor of this enzyme, HX, was found in

pulmonary cytosols. This is consistent with a previous observation (Borlak et al., 2000) that the reductive metabolism of 3-NBA to 3-ABA in rat alveolar type II cells was mediated, at least in part, by XO. In addition, our earlier data had shown that XO (isolated from buttermilk) was an effective activator of 3-NBA (Bieler et al., 1999), but only at unphysiological levels of enzyme.

In the present study we have demonstrated that pulmonary and renal NATs and SULTs also participate in the bioactivation of 3-NBA leading to DNA adducts. Addition of a cofactor for SULT, PAPS, increased 3-NBA-DNA-adduct levels approximately 2-fold in cytosols from both organs. A cofactor of NAT, acetyl-CoA, was even more effective in stimulating 3-NBA-DNA adduct formation; an order of magnitude higher levels of DNA adducts than without acetyl-CoA were detected. The importance of NATs and SULTs for 3-NBA activation was previously found in rat and human livers (Arlt et al., 2005; Stiborová et al., 2006) and in genetically engineered V79 cells expressing human NAT1, NAT2, SULT1A1 or SULT1A2 (Arlt et al., 2002; 2003a). Moreover, in these V79 cells, 3-NBA induced a dose-dependent increase in the mutation frequency at the *hprt* locus (H.R. Glatt, personal communication), indicating that the expression of NATs and SULTs contributes to the mutagenic potential of 3-NBA in mammalian systems.

In the case of 3-ABA, both microsomal and cytosolic enzymes of rat lung and kidney are capable of its activation to DNA binding species forming up to five DNA adducts. Stimulation of 3-ABA-DNA adduct formation by NADPH, a cofactor of CYP-dependent enzyme systems, and its inhibition by α-NF, an inhibitor of CYP1A1, indicate the predominant role of this microsomal enzyme in 3-ABA activation. The formation of 3-ABA-DNA adducts with microsomes is also mediated by arachidonic acid, a cofactor of COX, and in cytosols also by H_2O_2, a cofactor of cytosolic peroxidases. This finding supports our previous data on the role of peroxidases in the activation of 3-ABA (Arlt et al., 2006a).

Inter-individual variations in susceptibility and variations in drug-metabolizing enzyme activities in target tissues appear to be important determinants of cancer risk (Smith et al., 1995; Perera, 1997). It is known that both NQO1 and CYP1A1 are expressed not only in lungs and

kidneys of rats, but also in these tissues in humans (Rendic and DiCarlo, 1997; Ross et al., 2000). Likewise, NATs and SULTs are expressed in several human organs, including the respiratory tract (Willey et al., 1996; Mace et al., 1998; Richard et al., 2001). NAT1 mRNAs have been detected in human bronchial mucosa and peripheral lung tissue and NAT2 expression has been detected in the bronchus (Mace et al., 1998). Human bronchial epithelial cells and alveolar macrophages belong to the primary defence system against inhaled compounds and SULT1A1 is expressed in these cells (Willey et al., 1996; Richard et al., 2001). Therefore, NQO1, CYP1A1, NAT and SULT expression in the respiratory system could contribute significantly and specifically to the metabolic activation of 3-NBA and/or 3-ABA in the lung, thereby mediating 3-NBA carcinogenicity in this organ.

Expression levels and activities of NQO1, CYP1A1, NATs and SULTs differ considerably among individuals, because the enzymes are influenced by several factors, including smoking, drugs, environmental chemicals and genetic polymorphisms (Rendic and DiCarlo, 1997; Schulz et al., 1997; Chen et al., 1998). So far two polymorphisms in the human *NQO1* gene have been found in the general population, one of them being associated with an increased risk of urothelial tumours (Schulz et al., 1997) and pediatric leukaemia (Wiemels et al., 1999). The polymorphic expression of CYP1A1 has been attributed to altered expression of the aryl hydrocarbon (Ah) receptor, the transcription factor that modulates its regulation, or the Ah receptor nuclear translocator (Arnt) protein, its associated transcription factor (Rendic and DiCarlo, 1997; Hukkanen et al., 2002; Dickins, 2004). Moreover, the *CYP1A1* gene is genetically polymorphic (Rendic and DiCarlo, 1997; Hukkanen et al., 2002). So far, CYP1A1*2A, CYP1A1*2B and CYP1A1*4 polymophisms have been found that might be associated with lung, oesophageal or breast cancer and with acute myeloid leukaemia (D'Alo et al., 2004; Yang et al., 2005; Li et al., 2004; 2005). The human *NAT1* and *NAT2* genes are also genetically polymorphic, resulting in different activities of the gene product that segregate individuals into slow and rapid acetylator phenotypes (Hein et al., 2000). SULT1A1 and SULT1A2 are also polymorphic in humans (Raftogianis et al., 1999; Engelke et al., 2000) and are associated with increased cancer risk including lung cancer (Wang et al., 2002).

Thus, genetic polymorphisms in *CYP1A1, NQO1*, *NAT* and *SULT* genes could be important determinants of a possible lung cancer risk from 3-NBA.

Human and rat CYP1A1 is induced by many compounds, e.g. by planar aromatic compounds binding to the Ah receptor, e.g. 2,3,7,8-tetrachlorodibenzo[1,4]dioxine (TCDD) (Drahushuk et al., 1998), and by polycyclic hydrocarbons present in cigarette smoke (Rendic and DiCarlo, 1997; Hukkanen et al., 2002). NQO1 is inducible by a variety of agents with different mechanisms of action (see Ross et al., 2000 for a review). Among NQO1 inducers also tumour promoters and hydrogen peroxide have been reported (Prestera et al., 1993; Li and Jaiswal, 1994; Jaiswal, 1994).

Human exposure to 3-NBA is thought to occur primarily via the respiratory tract, and inhaled particles, *e.g.* derived from diesel emissions, are able to generate reactive oxygen species like hydrogen peroxide (Knaapen et al., 2004). Reactive oxygen species including hydrogen peroxide were even found to be generated by either 3-NBA itself or its metabolite 3-ABA in human A549 lung epithelial cells (Hansen et al., 2007). Hence, induction of NQO1 by hydrogen peroxide formed after exposure to particulate matter and 3-NBA might be a contributing risk factor, increasing 3-NBA activation and its binding to DNA, thereby enhancing its genotoxic potential. In addition, hydrogen peroxide produced by 3-NBA metabolite, *N*-hydroxy-3-aminobenzanthrone, was found to cause oxidative damage to DNA in human leukemia cells (Murata et al., 2006). Generation of hydrogen peroxide in the respiratory tract is also essential for peroxidases expressed in the lung (*i.e.* MPO and/or COX), which activate 3-ABA (Arlt et al., 2006a; present paper). In this context, it is noteworthy that 3-ABA is the major metabolite of 3-NBA in human fetal bronchial, human A549 lung epithelial, rat alveolar type II, rat epithelial bronchial, and rat mesenchymal lung cells (Borlak et al., 2000; Hansen et al., 2007). Furthermore, hydrogen peroxide required for peroxidase-mediated 3-ABA oxidation can also be supplied by XO, which as we show here, also contributes to the bioactivation of 3-NBA in lung cells.

In the present study, we have shown that 3-NBA acts as an inducer of the enzymes responsible for its own metabolic activation (NQO1) and its metabolite 3-ABA (CYP1A1). The expression of

NQO1 and CYP1A1 mRNAs and proteins was induced by 3-NBA in lungs and kidneys of rats treated i.p. with a single dose of 0.4, 4 or 40 mg/kg bw of 3-NBA. This induction leads to an increase in the activities of these enzymes and in their potential to activate 3-NBA in cytosols (NQO1) and 3-ABA in microsomes (CYP1A1) of both organs to species forming DNA adducts, thereby enhancing the first step of their activation. The specific mechanism(s) of induction of NQO1 and CYP1A1 by 3-NBA remain(s) to be determined.

3-NBA delivered i.p. is absorbed via the mesenteric veins and lymphatic systems, and passes through the liver. Thus, its concentration and effect in this tissue should be higher than in the distal tissues such as lung and kidney. Indeed, the induction of NQO1 and CYP1A1 in rat liver after identical i.p. treatment was up to 5- and 2-fold higher than in lungs and kidneys, respectively (Stiborová et al., 2006, present paper). Since exposure to 3-NBA occurs primarily via the respiratory tract and inhaled particles, a study evaluating the potential of 3-NBA administered via intratracheal instillation on enzymes capable of reducing 3-NBA or oxidising 3-ABA is under way in our laboratory.

In conclusion, the results of the present study show for the first time that 3-NBA is capable of inducing NQO1 and CYP1A1 in rat lung and kidney. These enzymes we found to be the predominant biotransformating enzymes involved in the metabolic activation of 3-NBA and 3-ABA not only in these organs in rats (present study), but also in the liver of both rats and humans (Arlt et al., 2004b; 2005; Stiborová et al., 2006). By 3-NBA exposure, the metabolic activation of 3-NBA itself and its reductive metabolite 3-ABA to reactive species forming DNA adducts is increased, thereby enhancing their genotoxic and carcinogenic potential.

References

Arlt, V. M., 2005. 3-Nitrobenzanthrone, a potential human cancer hazard in diesel exhaust and urban air pollution: A review of the evidence. Mutagenesis 20, 399–410.

Arlt, V. M., Bieler, C. A., Mier, W., Wiessler, M., Schmeiser, H. H., 2001. DNA adduct formation by the ubiquitous environmental contaminant 3-nitrobenzanthrone in rats determined by ^{32}P-postlabeling. Int. J. Cancer 93, 450-454.

Arlt, V. M., Cole, K. J, Phillips, D. H., 2004a. Activation of 3-nitrobenzanthrone and its metabolites to DNA-damaging species in human B-lymphoblastoid MCL-5 cells. Mutagenesis 19, 149-156.

Arlt, V. M., Glatt, H. R., Muckel, E., Pabel, U., Sorg, B. L., Schmeiser, H. H., Phillips, D. H., 2002. Metabolic activation of the environmental contaminant 3-nitrobenzanthrone by human acetyltransferases and sulfotransferase. Carcinogenesis 23, 1937–1945.

Arlt, V. M., Glatt, H., Muckel, E., Pabel, U., Sorg, B. L., Seidel, A., Frank, H., Schmeiser, H. H., Phillips, D. H., 2003a. Activation of 3-nitrobenzanthrone and its metabolites by human acetyltransferases, sulfotransferases and cytochrome P450 expressed in Chinese hamster V79 cells. Int. J. Cancer 105, 583–592.

Arlt, V. M., Henderson, C. J., Wolf, C. R., Schmeiser, H. H., Phillips, D. H., Stiborová, M, 2006a. Bioactivation of 3-aminobenzanthrone, a human metabolite of the environmental pollutant 3-nitrobenzanthrone: evidence for DNA adduct formation mediated by cytochrome P450 enzymes and peroxidases. Cancer Lett. 234, 220-231.

Arlt, V. M., Hewer, A., Sorg, B. L., Schmeiser, H. H., Phillips, D. H., Stiborová, M., 2004b. 3-Aminobenzanthrone, a human metabolite of the environmental pollutant 3-nitrobenzanthrone, forms DNA adducts after metabolic activation by human and rat liver microsomes: evidence for activation by cytochrome P450 1A1 and P450 1A2. Chem. Res. Toxicol. 17, 1092-1101.

Arlt, V. M, Schmeiser, H.H., Osborne, M. R., Kawanishi, M, Kanno, T, Yagi, T, Phillips, D. H., Takamura-Enya, T., 2006b. Identification of three major DNA adducts formed by the carcinogenic air pollutant 3-nitrobenzanthrone in rat lung at the C8 and N^2 position of guanine and at the N^6 position of adenine. Int. J. Cancer, 118, 2139-2146.

Arlt, V. M., Sorg, B. L., Osborne, M., Hewer, A., Seidel, A., Schmeiser, H. H., Phillips, D. H., 2003b. DNA adduct formation by the ubiquitous environmental pollutant 3-nitrobenzanthrone and its metabolites in rats. Biochem. Biophys. Res. Commun. 300, 107-114.

Arlt, V. M., Stiborová, M., Henderson, C. J., Osborne, M. R., Bieler, C. A., Frei, E., Martínek, V., Sopko, B., Wolf, C. R., Schmeiser, H. H., .Phillips, D. H., 2005. The environmental pollutant and potent mutagen 3-nitrobenzanthrone forms DNA adducts after reduction by NAD(P)H:quinone oxidoreductase and conjugation by acetyltransferases and sulfotransferases in human hepatic cytosols. Cancer Res. 65, 2644-2652.

Arlt, V. M., Stiborová, M., Hewer, A., Schmeiser, H. H., Phillips, D. H., 2003c. Human enzymes involved in the metabolic activation of the environmental contaminant 3-nitrobenzanthrone: evidence for reductive activation by human NADPH:cytochrome P450 reductase. Cancer Res. 63, 2752-2761.

Arlt, V. M., Zhan, L., Schmeiser, H. H., Honma, M., Hayashi, M., Phillips, D. H., Suzuki, T., 2004c. DNA adducts and mutagenic specificity of the ubiquitous environmental pollutant 3-nitrobenzanthrone in Muta Mouse. Environ. Mol. Mutagen. 43, 186-195.

Bieler, C. A., Cornelius, M., Klein, R., Arlt, V. M., Wiessler, M., Phillips, D. H., Schmeiser, H, H., 2005. DNA adduct formation by the environmental contaminant 3-nitrobenzanthrone after intratracheal instillation in rats. Int. J. Cancer 118, 833-838.

Bieler, C. A., Cornelius, M. G., Stiborova, M., Arlt, V. M., Wiessler, M., Phillips, D. H., Schmeiser, H. H., 2007. Formation and persistence of DNA adducts formed by the carcinogenic air pollutant 3-nitrobenzanthrone in target and non-target organs after intratracheal instillation in rats. Carcinogenesis 28, 1117-1121.

Bieler, C. A., Wiessler, M., Erdinger, L., Suzuki, H., Enya, T., Schmeiser, H. H., 1999. DNA adduct formation from the mutagenic air pollutant 3-nitrobenzanthrone. Mutat. Res. 439, 307-311.

Borlak, J., Hansen, T., Yuan, Z., Sikka, H. C., Kumar, S., Schmidbauer, S., Frank, H., Jacob, J., Seidel, A., 2000. Metabolism and DNA-binding of 3-nitrobenzanthrone in primary rat alveolar type II cells, in human fetal bronchial, rat epithelial and mesenchymal cell lines. Polycyclic Aromat.. Compounds 21, 73-86.

Chen, R. M., Chou, M. W., Ueng, T. H., 1998. Induction of cytochrome P450 1A in hamster liver and lung by 6-nitrochrysene. Arch. Toxicol. 72, 395-401.

D'Alo, A., Voso, M., Guidy, F., Kaseiny, G., Sica, S., Pagano, L., Hohaus, S., Leone, G., 2004. Polymorphisms of CYP1A1 and glutathione S-transferase and susceptibility to adult acute myeloid leukemia. Haematologica 89, 664-670.

Dickins , M., 2004. Induction of cytochromes P450. Curr. Top. Med. Chem. 4, 1745-1766.

Drahushuk, A. T., McGarrigle, B. P., Larsen, K. E., Stegeman, J. J., Olson, J. R., 1998. Detection of CYP1A1 protein in human liver and induction by TCDD in precision-cut liver slices incubated in dynamic organ culture. Carcinogenesis 19, 1361-1368.

Eling, T. E, Thompson, D. C., Foureman, G. L, Curtis, J. F, Hughes, M. F., 1990. Prostaglandin H synthase and xenobiotic oxidation. Annu. Rev. Pharmacol. Toxicol. 30, 1-45.

Engelke, C. E., Meinl, W., Boeing, H., Glatt, H., 2000. Association between functional genetic polymorphisms of human sulfotransferases 1A1 and 1A2. Pharmacogenetics 10, 163-169.

Environmental Protection Agency (EPA), 2002. Health Assessment Dokument for Diesel Engine Exhaust, EPA/600/8-90/057F.

Enya, T., Suzuki, H., Watanabe, T., Hirayama, T., Hisamatsu, Y., 1997. 3-Nitrobenzanthrone, a powerful bacterial mutagen and suspected human carcinogen found in diesel exhausts and airborne particulates. Environ. Sci. Technol. 31, 2772-2776.

Glatt, H., Boeing, H., Engelke, C. E. H., Ma, L., Kuhlow, A., Pabel, U., Pomplun, D., Teubner, W., Meinl, W., 2001. Human cytosolic sulphotransferases: genetics, characteristics, toxicological aspects. Mutat. Res. 482, 27-40.

Grashick, E., Laden, F., Hart, J. E., Rosner, B., Smith, T. J., Dockery, D. W., Speitzer, F. E., 2004. Lung cancer in railroad workers exposed to diesel exhaust. Environ. Health Perspect. 112, 1539-1543.

Hansen, T., Seidel, A., Borlak, J., 2007. The environmental carcinogen 3-nitrobenzanthrone and its main metabolite 3-aminobenzanthrone enhance formation of reactive oxygen intermediates in human A549 lung epithelial cells. Toxicol. Appl. Pharmacol. 221, 222-234.

Hein, D. W., Doll, M. A., Fretland, A. J., Leff, M. A., Webb, S. J., Xiao, G. H., Devanaboyina, U. S., Nangju, N. A, Feng, Y., 2000. Molecular genetics and epidemiology of the NAT1 and NAT2 acetylation polymorphisms. Cancer Epidemiol. Biomarkers Prev. 9, 29-42.

Hukkanen, J., Pelkonen, O., Hakkola, J., Raunio, H., 2002. Expression and regulation of xenobiotic-metabolizing cytochrome P450 (CYP) enzymes in human lung. Crit. Rev. Toxicol. 32, 391-411.

International Agency for Research on Cancer (IARC), 1989. Diesel and gasoline engine exhausts and some nitroarenes. IARC Monogr. Eval. Carcinog. Risks Hum. 46.

Jaiswal, A. K. (1994) Antioxidant response element. Biochem. Pharmacol. 48, 439-444.

King, R. S., Teitel, C. H., Kadlubar, F. F., 2000. *In vivo* bioactivation of *N*-hydroxy-2-amino-α-carboline. Carcinogenesis 21, 1347-1354.

Knaapen, A. M., Borm, P. J. A., Albrecht, C., Schins, R. P. F., 2004. Inhaled particles and lung cancer. Part A: mechanism. Int. J. Cancer 109,799-809.

Li, Y., Jaiswal, A. K., 1994. Human antioxidant-response-element-mediated regulation of type 1 NAD(P)H:quinone oxidoreductase gene expression. Effect of sulfhydryl modifying agents. Eur. J. Biochem. 226, 31-39.

Li, Y., Millikan, R., Bell, D., Cui, L., Tse, C., Newman, B., Conway, K., 2004. Cigarette smoking, cytochrome P450 1A1 polymorphisms, and breast cancer among African-American and white women. Breast Cancer Res. 6, R460-R473.

Li, Y., Millikan, R., Bell, D., Cui, L., Tse, C., Newman, B., Conway, K., 2005. Polychlorinated biphenyls, cytochrome P450 1A1 (CYP1A1) polymorphisms, and breast cancer risk among

African American women and white women in North Carolina: a population-based case-control study. Breast Cancer Res. 7, R12-R18.

Mace, K., Bowman, E. D., Vautravers, P., Shields, P. G., Harri, C. C., Pfeifer, A. M. A., 1998. Characterisation of xenobiotic-metabolising enzyme expression in human bronchial mucosa and peripheral lung tissue. Eur. J. Cancer 34, 914-920.

Murata, M., Tezuka T., Ohnishi, S., Takamura-Enya, T., Hisamatsu, Y., Kawanishi, S., 2006. Carcinogenic 3-nitrobenzanthrone induces oxidative damage to isolated and cellular DNA. Free Radic. Biol. Med. 40, 1242-1249.

Nagy, E., Zeisig, M., Kawamura, K., Hisumatsu, Y., Sugeta, A., Adachi, S., Moller, L., 2005. DNA-adduct and tumor formations in rats after intratracheal administration of the urban air pollutant 3-nitrobenzanthrone. Carcinogenesis 26, 1821-1828.

Perera, F. P., 1997. Environment and Cancer: Who are susceptible? Science 278, 1068-1073.

Phillips, D. H., Arlt, V. M., 2007. The ^{32}P-postlabelling assay for DNA adducts. Nat. Protoc. 2, 2772-2781.

Prestera, T., Holtzclaw, W. D., Zhang, Y., Talalay, P., 1993. Chemical and molecular regulation of enzymes that detoxify carcinogens. Proc. Natl. Acad, Sci. U.S.A. 90, 2965-2969.

Raftogianis, R. B., Wood, T. C., Weinshilboum, R. M., 1999. Human phenol sulfotransferases SULT1A2 and SULT1A1: genetic polymorphisms, allozyme properties, and human liver genotype-phenotype correlations. Biochem. Pharmacol. 58, 605-616.

Rendic, S., DiCarlo, F.J., 1997. Human cytochrome P450 enzymes: A status report summarizing their reactions, substrates, inducers, and inhibitors. Drug Metab. Rev. 29, 413-480.

Richard, K., Hume, R., Kaptein, E., Stanley, E. L., Visser, T. J, Coughtrie, M. W. H., 2001. Sulfation of thyroid hormone and dopamine during human development: ontogeny of phenol sulfotransferases and arylsulfatase in liver, lung, and brain. J. Clin. Endocrinol. Metab. 86, 2734-2742.

Ritter, C. L., Decker, R. W., Malejka-Giganti, D., 2000 Reduction of nitro and 9-oxo groups of environmental nitrofluorenes by the mammary gland in vitro. Chem. Res. Toxicol. 13, 793-800.

Ross, D., Kepa, J. K., Winski, S. L., Beall, H. D., Anwar, A., Siegel, D., 2000. NAD(P)H:quinone oxidoreductase (NQO1): chemoprotection, bioactivation, gene regulation and genetic polymorphisms. Chem.- Biol. Interact. 129, 77-97.

Schulz, W. A., Krummeck, A., Rosinger, I., Eickelman, P., Neuhaus, C., Ebert, T., Schmitz-Drager, B. J., Sies, H., 1997. Increased frequency of a null-allele for NAD(P)H–quinone oxidoreductase in patients with urological malignancies. Pharmacogenetics 7, 235-239.

Seidel, A., Dahmann, D., Krekeler, H., Jacob, J., 2002. Biomonitoring of polycyclic aromatic compounds in the urine of mining workers occupationally exposed to diesel exhaust. Int. J. Hyg. Environ. Health 204, 333-338.

Smith, G., Stanley, L. A., Sim, E., Strange, R. C., Wolf, C. R., 1995. Metabolic polymorphism and cancer susceptibility. Cancer Surv. 25, 27-65.

Stiborová, M., Dračínská, H., Hájková, J., Kadeřábková, P., Frei, E., Schmeiser, H. H., Souček, P., Phillips, D. H., Arlt, V. M., 2006. The environmental pollutant and carcinogen 3-nitrobenzanthrone and its human metabolite 3-aminobenzanthrone are potent inducers of rat hepatic cytochromes P450 1A1 and -1A2 and NAD(P)H:quinone oxidoreductase. Drug Metab. Dispos. 34, 1398-1405.

Stiborová, M., Frei, E., Hodek, P., Wiessler, M, Schmeiser, H. H., 2005a. Human hepatic and renal microsomes, cytochromes P450 1A1/2, NADPH:cytochrome P450 reductase and prostaglandin H synthase mediate the formation of aristolochic acid-DNA adducts found in patients with urothelial cancer. Int. J. Cancer 113, 189-197.

Stiborová, M, Frei, E., Sopko, B., Sopková, K., Marková, V., Laňková, M., Kumstýřová, T., Wiessler, M., Schmeiser, H. H., 2003. Human cytosolic enzymes involved in the metabolic activation of carcinogenic aristolochic acid: evidence for reductive activation by human NAD(P)H:quinone oxidoreductase. Carcinogenesis 24, 1695-1703.

Stiborová, M., Martínek, V., Rýdlová, H., Hodek, P., Frei, E., 2002. Sudan I is a potential carcinogen for humans: evidence for its metabolic activation and detoxication by human recombinant cytochrome P450 1A1 and liver microsomes. Cancer Res. 62, 5678-5684.

Stiborová, M., Martínek, V., Rýdlová, H., Koblas, T., Hodek, P., 2005b. Expression of cytochrome P450 1A1 and its contribution to oxidation of a potential human carcinogen 1-phenylazo-2-naphthol (Sudan I) in human livers. Cancer Lett. 220, 145-154.

Stiborová, M., Poljaková, J., Ryšlavá, H., Dračínský, M., Eckschlager, T., Frei, E., 2007. Mammalian peroxidases activate anticancer drug ellipticine to intermediates forming deoxyguanosine adducts in DNA identical to those found *in vivo* and generated from 12-hydroxyellipticine and 13-hydroxyellipticine. Int. J. Cancer 120, 243-251.

Svobodová, M., Šístková, J., Dračínská, H., Hudeček, J., Hodek, P., Schmeiser, H. H., Arlt, V. M., Frei, E., Stiborová, M., 2007. Reductive activation of environmental pollutants 3-nitrobenzanthrone and 2-nitrobenzanthrone. Chem. Listy, in press.

Swaminathan, S., Hatcher, J. F., 1986. Xanthine oxidase-mediated mutagenicity of bladder carcinogen 4-nitrobiphenyl. Mutat. Res. 172, 37-45.

Vineis, P., Forastiere, F., Hoek, G., Lipsett, M., 2004. Outdoor air pollution and lung cancer: recent epidemiologic evidence. Int. J. Cancer 111, 647-652.

Wang, Y., Spitz, M. R., Tsou, A. M., Zhang, K., Makan, N., Wu, X., 2002. Sulfotransferase (SULT) 1A1 polymorphism as predisposional factor for lung cancer: a case-control analysis. Lung Cancer 35, 137-142.

Watanabe, T., Kaji, H., Takashima, M., Kasai, T., Lewtes, J., Hirayama, T., 1997. Metabolic activation of 2- and 3-nitrodibenzopyranone isomers and related compounds by rat liver S9 and the effect of S9 on the mutational specificity of nitrobenzopyranones. Mutat. Res. 388, 67-78.

Wiemels, J., Pagnamenta, A., Taylor, G. M., Eden, O. B, Alexander, F. E., Greaves, M. F., 1999. A lack of functional NAD(P)H:quinone oxidoreductase allele is selectively associated with pediatric leukemias that have MLL fusions. United Kingdom Childhood Cancer Study Investigators. Cancer Res. 59, 4095-4099.

Willey, J. C. , Coy, E., Brolly, C., Utell, M. J., Frampton, M. W., Hammersley, J., Thilly, W. G., Olson, D., Cairns, K., 1996. Xenobiotic metabolism enzyme gene expression in human bronchial epithelial and alveolar macrophage cells. Am. J. Respir. Cell. Mol. Biol. 14, 262-271.

Yang, C.-X., Matsuo, K., Wang, Z., Tajima, K., 2005. Phase I/II enzyme gene polymorphisms and esophageal cancer risk: A meta-analysis of the literature. World J. Gastroenterol. 11, 2534-2538.

Table 1

Effect of the NQO1 inhibitor dicoumarol and the XO inhibitor allopurinol on DNA adduct formation by 3-NBA in rat pulmonary and renal cytosols

Cofactor/inhibitor added	Total RAL[a] (mean/10^8 nucleotides)	
	Pulmonary cytosol	Renal cytosol
NADPH only	2.69 ± 0.19	4.82 ± 0.36
NADPH + dicoumarol	0.29 ± 0.02[*]	0.33 ± 0.03[*]
HX only	0.58 ± 0.05	1.78 ± 0.12
HX + allopurinol	0.59 ± 0.05	1.79 ± 0.20

[a]RAL, relative adduct labelling. The results are presented as the mean ± SE of triplicate *in-vitro* incubations.

[*]Significantly different from control incubations without inhibitor: $p<0.001$ (Student's t-test)

Table 2

Specific CYP1A1 activities [a] in kidney and lung microsomes of control and 3-NBA-treated rats

CYP activity	Control rat		3-NBA-treated rat	
	Kidney	Lung	Kidney	Lung
EROD	9.4 ± 0.4	4.6 ± 0.5	50.2 ± 1.8 (**5.3**)[*]	29.9 ± 2.1 (**6.5**)[*]
Sudan I oxidation	9.3 ± 1.0	4.7 ± 0.5	30.3 ± 2.3 (**3.2**)[*]	29.8 ± 2.8 (**6.3**)[*]

[a]Each value (pmol of reaction product per min per mg protein) represents the mean ± SE of triplicate measurements. Numbers in parentheses represents the fold increase over the control activity caused by the pre-treatment with 3-NBA (40 mg/kg bw).

[*]Significantly different from controls: $p<0.001$ (Student's t-test).

Table 3

NQO1 specific activity in rat renal and pulmonary cytosol

	Control rat	3-NBA-treated rat		
		0.4 mg/kg	4 mg/kg	40 mg/kg
Kidney	0.09 ± 0. 01	0.15 ± 0. 01 (**1.7**) *	0.15 ± 0. 01 (**1.7**)*	0.23 ± 0. 02 (**2.5**)**
Lung	0.05 ± 0.01	0.06 ± 0.01 (**1.2**)	0.06 ± 0.01 (**1.2**)	0.09 ± 0.01 (**1.8**)*

The results (units per mg) are averages and SEs of five parallel measurements. Enzyme activities with menadione as a substrate was assayed as described in Materials and Methods. One unit of NQO1 is defined to reduce 1 μmol of NADPH per min/mg protein in the presence of menadione as substrate at 37°C.

*Significantly different from controls: $p<0.05$ (Student's t-test).

**Significantly different from controls: $p<0.01$ (Student's t-test).

Table 4

Expression of mRNA of CYP1A1 and NQO1

	CYP1A1		NQO1	
	Δc_T[a]	Fold Change	Δc_T	Fold Change
Control rats				
Kidney	4.45 ± 0.36	-	9.37 ± 0.67	-
Lung	10.48 ± 0.16	-	5.44 ± 0.34	-
3-NBA-treated rats				
Kidney	2.60 ± 0.65	3.6**	9.07 ± 0.22	1.2*
Lung	8.51 ± 0.70	3.9**	4.98 ± 0.21	1.4**

[a]Results shown are mean ± SD from data found for three rats (control and treated with 40 mg of 3-NBA/kg bw).

*Significantly different from controls: $p<0.05$ (Student's t-test).

**Significantly different from controls: $p<0.01$ (Student's t-test)

Legends to Figures

Fig. 1. Pathways of metabolic activation and DNA adduct formation of 3-nitrobenzanthrone and 3-aminobenzanthrone. See text for details. 3-NBA, 3-nitrobenzanthrone; 3-ABA, 3-aminobenzanthrone; NQO1, NAD(P)H:quinone oxidoreductase; NAT, *N*,*O*-acetyltransferases; SULT, sulfotransferase; COX-1, cyclooxygenase 1; CYP, cytochrome P450; LPO, lactoperoxidase; MPO, myeloperoxidase; POR, NADPH:cytochrome P450 oxidoreductase; R = $-COCH_3$ or $-SO_3H$; dA-N^6-ABA, 2-(2'-deoxyadenosin-N^6-yl)-3-aminobenzanthrone; dG-N^2-ABA, *N*-(2'-deoxyguanosin-N^2-yl)-3-aminobenzanthrone; dG-C8-*N*-ABA, *N*-(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone.

Fig. 2. Autoradiographic profiles of DNA adducts generated (A) by 3-NBA after its activation with cytosols isolated from lungs of rats treated with 40 mg/kg bw of 3-NBA and NADPH, and (B) by 3-ABA after its activation with microsomes isolated from lungs of rats treated with 40 mg/kg bw of 3-NBA and NADPH by using the butanol enrichment version of the ^{32}P-postlabelling assay. Spot 1 = dA-N^6-ABA, spot 3 = dG-N^2-ABA, spots 4/5 = dG-C8-*N*-ABA. (For structures of adducts see legend to Fig. 1)

Fig. 3. The effect of cofactors of NQO1 and XO on the 3-NBA-DNA adduct formation in pulmonary (A) and renal cytosols (B) of control (uninduced) rats. (F = fold increase in DNA binding in comparison to DNA binding without cofactors). Mean ± SE shown in the figure represent total levels of DNA adducts (RAL, relative adduct labelling) of three determinations. *$p<0.05$, **$p<0.01$, ***$p<0.001$ (Student's t-test). HX, hypoxanthine.

Fig. 4. DNA adduct formation by 3-NBA activated with cytosols isolated from lungs (A) and kidneys (B) of rats, control (uninduced) or pretreated with 40 mg/kg bw of 3-

NBA and the DNA-adduct levels obtained by adding either PAPS as a cofactor for SULTs or acetyl-CoA as a cofactor for NATs. Mean ± SE shown in the figure represent total levels of DNA adducts (RAL, relative adduct labelling) of three determinations. *$p<0.05$, **$p<0.01$ (Student's t-test).

Fig. 5. DNA adduct formation by 3-ABA activated with microsomes isolated from lungs (A) and kidneys (B) of rats, control (uninduced) or pretreated with 40 mg/kg bw of 3-NBA F = fold higher DNA adducts levels in microsomes from 3-NBA-treated rats compared to control (uninduced) rats. Mean ± SE shown in the figure represent total levels of DNA adducts (RAL, relative adduct labelling) of three determinations. None = without cofactor; AA = arachidonic acid; α-NF = α-naphthoflavone; IM = indomethacin. ND = not detected. *$p<0.05$, **$p<0.01$, ***$p<0.001$ (Student's t-test).

Fig. 6. DNA adduct formation by 3-ABA activated with cytosols isolated from lungs (A) and kidneys (B) of rats, control (uninduced) or pretreated with 40 mg/kg bw of 3-NBA. Mean ± SE shown in the figure represent total levels of DNA adducts (RAL, relative adduct labelling) of three determinations.

Fig. 7. Induction of CYP1A1 (A,B) and NQO1 (C,D) in lungs (A,C) and kidney (B,D) of rats treated with 0.4, 4 or 40 mg/kg bw of 3-NBA determined by Western blots as described in the Material and methods section. Values represent mean ± SD obtained from lungs and kidneys of three rats (n=3). Inset: immunoblots of microsomal CYP1A1 and cytosolic NQO1 from untreated and 3-NBA-treated rats stained with antibody against rat CYP1A1 and human NQO1. Values significantly different from control: *$p<0.05$, **$p<0.01$, ***$p<0.001$ (Student's t-test).

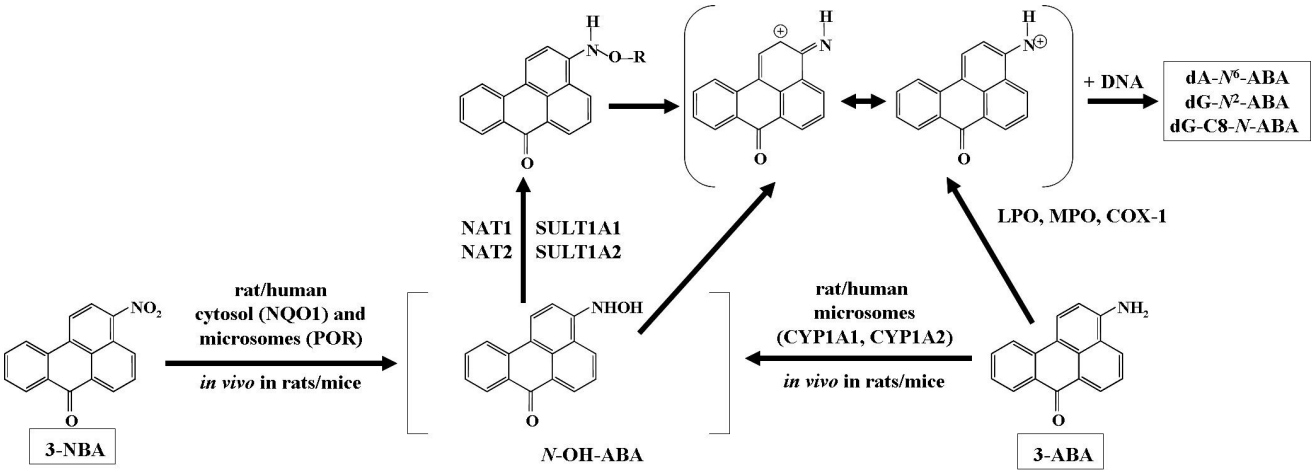
3-NBA
rat/human
cytosol (NQO1) and
microsomes (POR)
in vivo in rats/mice
N-OH-ABA
NAT1
NAT2
SULT1A1
SULT1A2
rat/human
microsomes
(CYP1A1, CYP1A2)
in vivo in rats/mice
3-ABA
LPO, MPO, COX-1
+ DNA
dA-N6-ABA
dG-N2-ABA
dG-C8-N-ABA

Figure 1

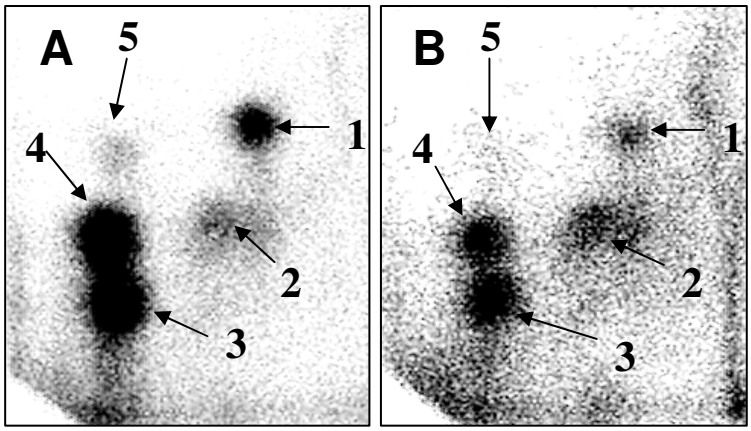

Figure 2

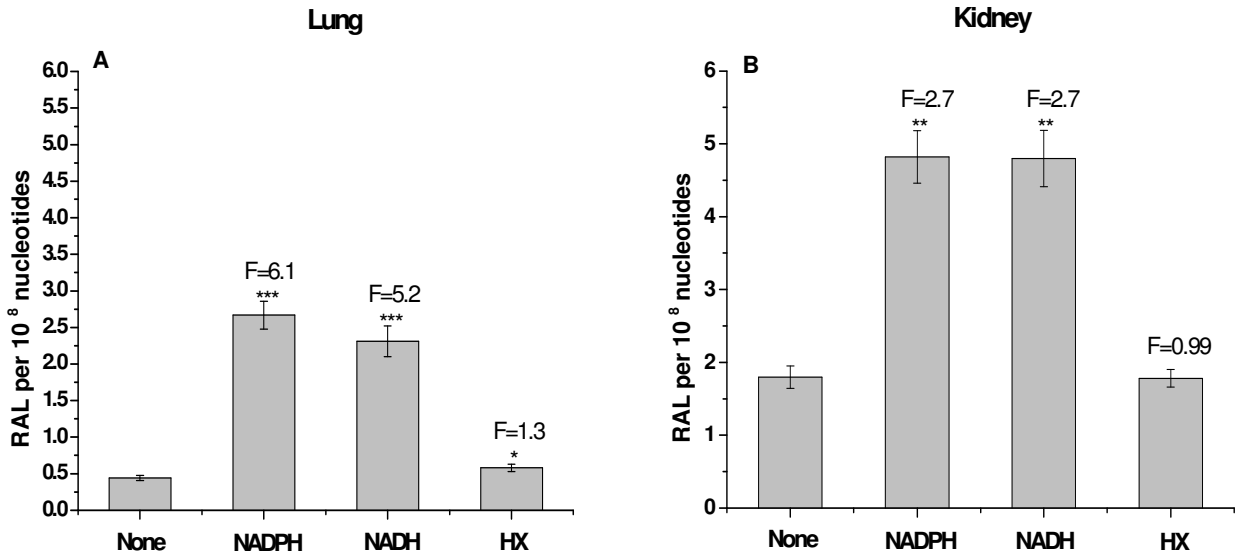
Lung
A
RAL per 10 8 nucleotides
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
F=6.1
***
F=5.2
***
F=1.3
*
None
NADPH
NADH
HX
Kidney
B
RAL per 10 8 nucleotides
6
5
4
3
2
1
0
F=2.7
**
F=2.7
**
F=0.99
None
NADPH
NADH
HX

Figure 3

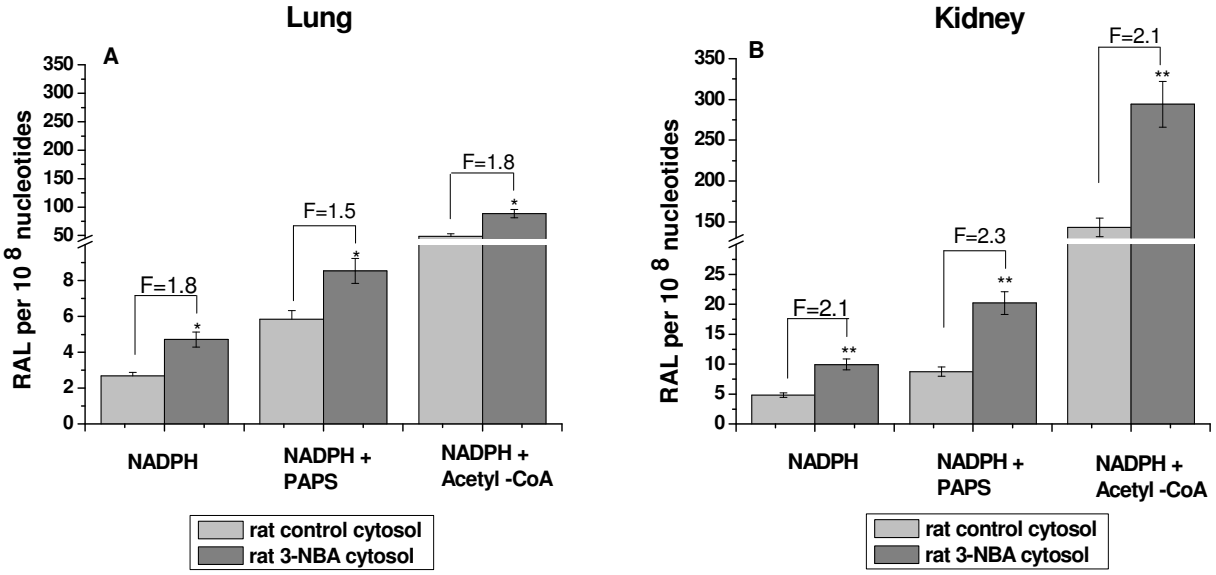
Lung
A
RAL per 10 8 nucleotides
350
300
250
200
150
100
50
8
6
4
2
0
F=1.8
F=1.5
F=1.8
*
*
*
NADPH
NADPH + PAPS
NADPH + Acetyl -CoA
rat control cytosol
rat 3-NBA cytosol
Kidney
B
RAL per 10 8 nucleotides
350
300
250
200
150
25
20
15
10
5
0
F=2.1
F=2.3
F=2.1
**
**
**
NADPH
NADPH + PAPS
NADPH + Acetyl -CoA
rat control cytosol
rat 3-NBA cytosol

Figure 4

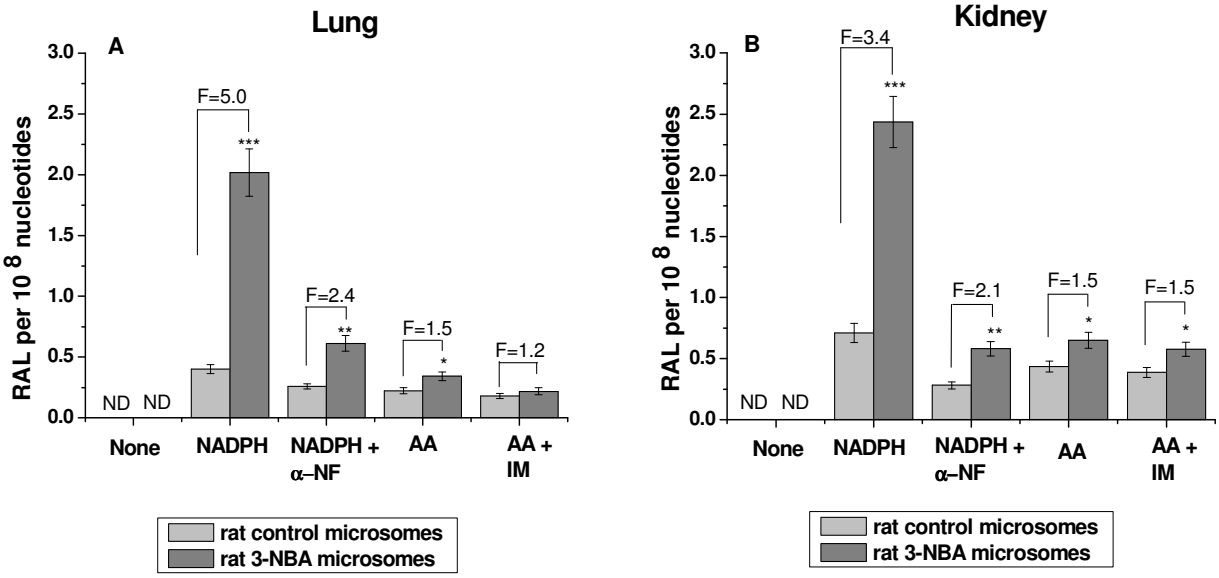
Lung
A
RAL per 10 8 nucleotides
3.0
2.5
2.0
1.5
1.0
0.5
0.0
F=5.0
***
F=2.4
**
F=1.5
*
F=1.2
ND ND
None
NADPH
NADPH + α–NF
AA
AA + IM
rat control microsomes
rat 3-NBA microsomes
Kidney
B
F=3.4
***
F=2.1
**
F=1.5
*
F=1.5
*
ND ND
None
NADPH
NADPH + α–NF
AA
AA + IM
rat control microsomes
rat 3-NBA microsomes

Figure 5

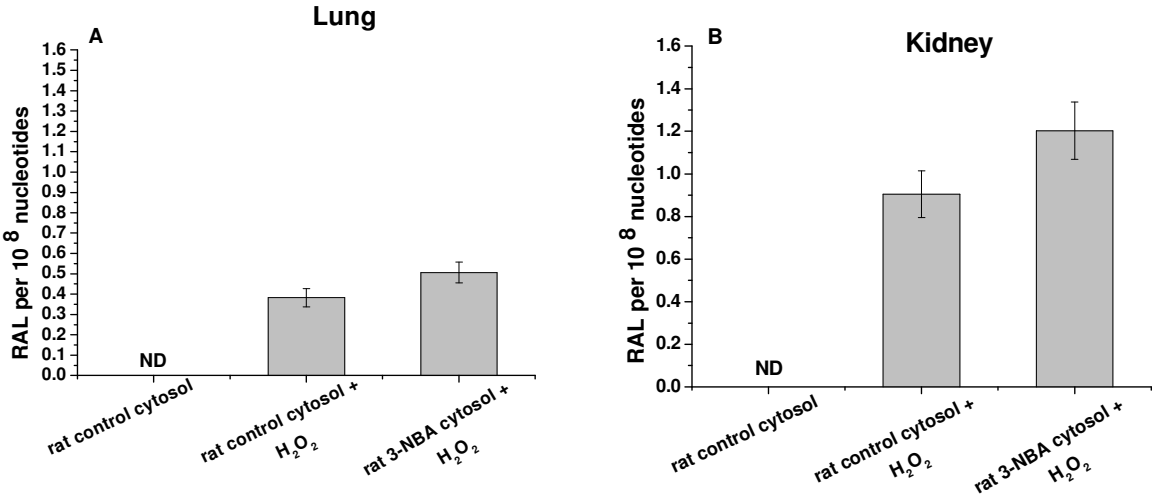
A
Lung
RAL per 10 8 nucleotides
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
ND
rat control cytosol
rat control cytosol + H2O2
rat 3-NBA cytosol + H2O2
B
Kidney
RAL per 10 8 nucleotides
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
ND
rat control cytosol
rat control cytosol + H2O2
rat 3-NBA cytosol + H2O2

Figure 6

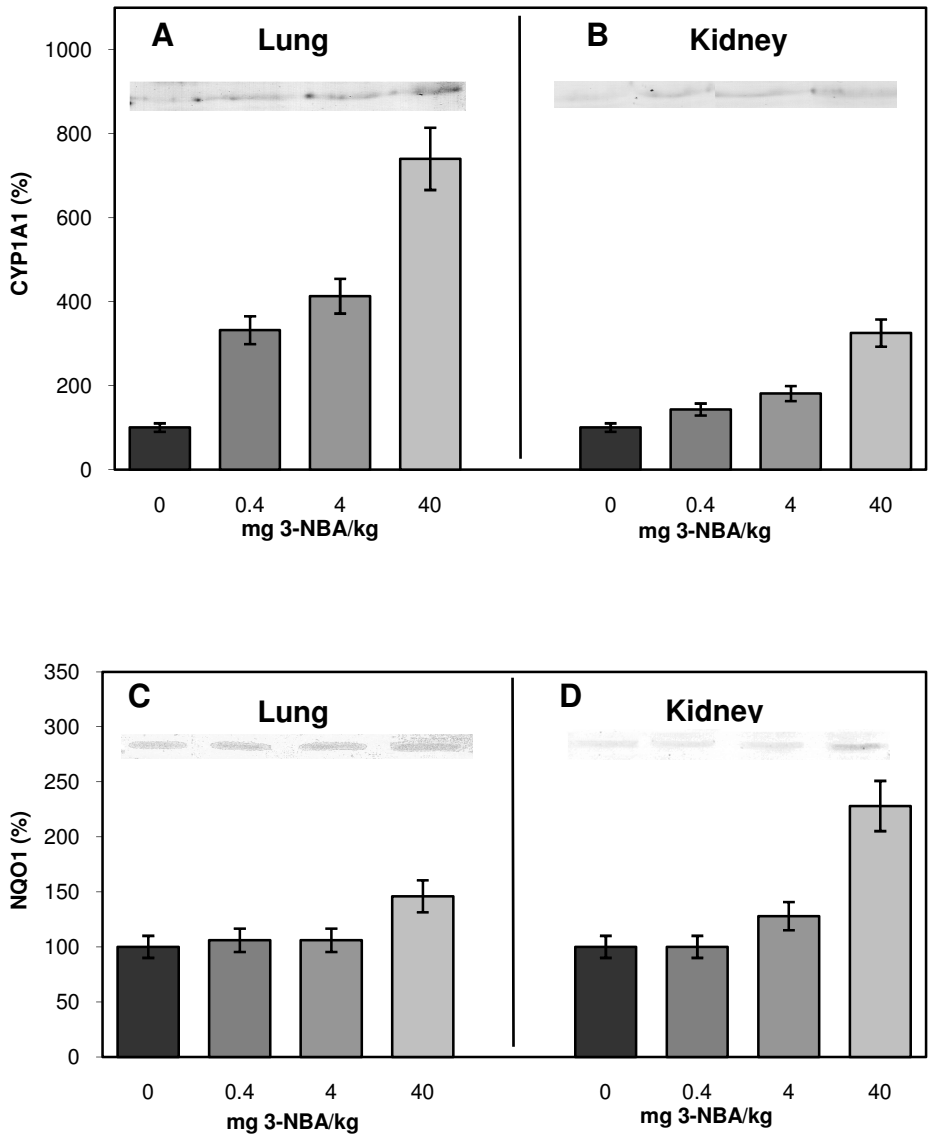
A
Lung
B
Kidney
CYP1A1 (%)
1000
800
600
400
200
0
0
0.4
4
40
mg 3-NBA/kg
C
Lung
D
Kidney
NQO1 (%)
350
300
250
200
150
100
50
0

Figure 7

Supplementary data

Supporting Table S1.

a) Effect of enzyme cofactors on DNA adduct formation by 3-NBA in rat lung cytosol

	RAL[a] (mean/10^8 nucleotides)					
	Spot 1	Spot 2	Spot 3	Spot 4	Spot 5	Total
uninduced lung cytosol without cofactor	**0.084** ± 0.011	**0.054** ± 0.009	**0.151** ± 0.012	**0.075** ± 0.010	**0.074** ± 0.010	**0.443** ± 0.034
uninduced lung cytosol + NADPH	**0.189** ± 0.015	**0.298** ± 0.021	**1.210** ± 0.102	**0.882** ± 0.068	**0.110** ± 0.011	**2.688** ± 0.190
uninduced lung cytosol + NADH	**0.199** ± 0.018	**0.317** ± 0.029	**0.799** ± 0.078	**0.844** ± 0.078	**0.170** ± 0.018	**2.309** ± 0.211
uninduced lung cytosol + HX	**0.082** ± 0.011	**0.109** ± 0.010	**0.264** ± 0.023	**0.083** ± 0.008	**0.042** ± 0.005	**0.580** ± 0.050
uninduced lung cytosol + NADPH + PAPS	**0.511** ± 0.048	**0.977** ± 0.101	**1.989** ± 0.132	**1.989** ± 0.165	**0.386** ± 0.030	**5.852** ± 0.476
uninduced lung cytosol + NADPH + acetylCoA	**5.13** ± 0.498	**7.71** ± 0.671	**13.88** ± 1.151	**17.826** ± 1.482	**3.712** ± 0.301	**48.656** ± 4.426
3-NBA lung cytosol + NADPH	**0.743** ± 0.070	**0.377** ± 0.048	**1.732** ± 0.152	**1.0596** ± 0.098	**0.7992** ± 0.081	**4.7108** ± 0.421
3-NBA lung cytosol + NADPH + PAPS	**1.10** ± 0.107	**0.99** ± 0.089	**2.616** ± 0.216	**2.921** ± 0.252	**0.912** ± 0.099	**8.545** ± 0.698
3-NBA lung cytosol + NADPH + acetylCoA	**11.836** ± 1.021	**9.885** ± 0.954	**25.819** ± 2.101	**28.92** ± 2.231	**1.223** ± 0.108	**88.75** ± 7.132

b) Effect of enzyme cofactors on DNA adduct formation by 3-NBA in rat kidney cytosol

	RAL[a] (mean/10^8 nucleotides)					
	Spot 1	Spot 2	Spot 3	Spot 4	Spot 5	Total
uninduced kidney cytosol without cofactor	**0.148** ± 0.013	**0.310** ± 0.289	**0.823** ± 0.754	**0.449** ± 0.398	**0.066** ± 0.010	**1.796** ± 0.154
uninduced kidney cytosol + NADPH	**0.467** ± 0.051	**0.698** ± 0.061	**1.797** ± 0.142	**1.483** ± 0.132	**0.392** ± 0.034	**4.82** ± 0.360
uninduced kidney cytosol + NADH	**0.464** ± 0.049	**0.690** ± 0.063	**1.788** ± 0.148	**1.459** ± 0.140	**0.400** ± 0.041	**4.80** ± 0.387
uninduced kidney cytosol + HX	**0.145** ± 0.014	**0.305** ± 0.029	**0.813** ± 0.009	**0.452** ± 0.033	**0.066** ± 0.053	**1.781** ± 0.122
uninduced kidney cytosol + NADPH + PAPS	**0.749** ± 0.064	**1.369** ± 0.327	**2.978** ± 0.234	**3.061** ± 0.300	**0.583** ± 0.052	**8.74** ± 0.782
uninduced kidney cytosol + NADPH + acetylCoA	**12.80** ± 1.001	**38.63** ± 3.123	**43.65** ± 4.176	**46.61** ± 4.101	**1.38** ± 0.151	**143.08** ± 11.201
3-NBA kidney cytosol + NADPH	**0.945** ± 0.798	**1.40** ± 0.129	**3.55** ± 0.310	**3.33** ± 0.323	**0.72** ± 0.062	**9.945** ± 0.899
3-NBA kidney cytosol + NADPH + PAPS	**2.08** ± 0.201	**2.94** ± 0.232	**8.160** ± 0.754	**7.03** ± 0.678	**1.80** ± 0.201	**20.21** ± 1.912
3-NBA kidney cytosol + NADPH + acetylCoA	**27.405** ± 2.43	**42.00** ± 4.100	**104.725** ± 9.43	**99.234** ± 9.110	**20.88** ± 2.06	**294.244** ± 28.032

[a] Mean RAL (relative adduct labelling) and standard deviation of triplicate *in-vitro* incubations; spot 1 = dA-N^6-ABA, spot 3 = dG-N^2-ABA, spots 4/5 = dG-C8-*N*-ABA.

Supporting Table S2.

a) DNA adduct formation by 3-ABA activated by lung microsomes of rats either uninduced or induced by 3-NBA

	RAL[a] (mean/10^8 nucleotides)					
	Spot 1	Spot 2	Spot 3	Spot 4	Spot 5	Total
uninduced lung microsomes without cofactor	ND[b]	ND	ND	ND	ND	ND
uninduced lung microsomes + NADPH	**0.072** ± 0.008	**0.062** ± 0.006	**0.154** ± 0.012	**0.059** ± 0.004	**0.055** ± 0.005	**0.402** ± 0.037
uninduced lung microsomes + NADPH + α-NF	**0.030** ± 0.003	**0.030** ± 0.003	**0.110** ± 0.010	**0.080** ± 0.006	**0.010** ± 0.001	**0.260** ± 0.021
uninduced lung microsomes + arachidonic acid	**0.035** ± 0.004	**0.035** ± 0.004	**0.101** ± 0.010	**0.026** ± 0.003	**0.026** ± 0.003	**0.223** ± 0.025
uninduced lung microsomes + arachidonic acid + indomethacin	**0.030** ± 0.003	**0.030** ± 0.003	**0.100** ± 0.014	**0.020** ± 0.002	ND	**0.180** ± 0.021
3-NBA lung microsomes without cofactor	ND	ND	ND	ND	ND	ND
3-NBA lung microsomes + NADPH	**0.123** ± 0.013	**0.333** ± 0.029	**0.786** ± 0.071	**0.883**± 0.092	**0.193** ± 0.015	**2.018** ± 0.194
3-NBA lung microsomes + NADPH + α-NF	**0.052** ± 0.006	**0.083** ± 0.009	**0.291** ± 0.025	**0.121** ± 0.013	**0.065** ± 0.008	**0.612** ± 0.065
3-NBA lung microsomes + arachidonic acid	**0.051** ± 0.005	**0.059** ± 0.006	**0.101** ± 0.012	**0.105** ± 0.011	**0.026** ± 0.003	**0.342** ± 0.036
3-NBA lung microsomes + arachidonic acid + indomethacin	**0.050** ± 0.005	**0.050** ± 0.005	**0.100** ± 0.012	**0.095** ± 0.010	**0.023** ± 0.003	**0.218** ± 0.029

[a] Mean RAL (relative adduct labelling) of triplicate *in-vitro* incubations; spot 1 = dA-N^6-ABA, spot 3 = dG-N^2-ABA, spots 4/5 = dG-C8-*N*-ABA.

[b] ND = not detected.

Supporting Table S2.

b) DNA adduct formation by 3-ABA activated by kidney microsomes of rats either uninduced or induced by 3-NBA

	RAL[a] (mean/10^8 nucleotides)					
	Spot 1	Spot 2	Spot 3	Spot 4	Spot 5	Total
uninduced without cofactor	ND[b]	ND	ND	ND	ND	ND
uninduced s + NADPH	**0.067** ± 0.007	**0.087** ± 0.009	**0.377** ± 0.032	**0.154** ± 0.014	**0.025** ± 0.003	**0.710** ± 0.078
uninduced + NADPH + α-NF	**0.032** ± 0.003	**0.050** ± 0.006	**0.123** ± 0.011	**0.065** ± 0.006	**0.012** ± 0.001	**0.282** ± 0.029
uninduced + arachidonic acid	**0.055** ± 0.006	**0.096** ± 0.010	**0.197** ± 0.018	**0.088** ± 0.003	ND	**0.436** ± 0.045
uninduced + arachidonic acid + indomethacin	**0.050** ± 0.006	**0.086** ± 0.010	**0.172** ± 0.018	**0.080** ± 0.002	ND	**0.388** ± 0.041
3-NBA microsomes without cofactor	ND	ND	ND	ND	ND	ND
3-NBA microsomes + NADPH	**0.179** ± 0.018	**0.303** ± 0.031	**0.991** ± 0.010	**0.808**± 0.091	**0.154** ± 0.015	**2.435** ± 0.210
3-NBA microsomes + NADPH + α-NF	**0.062** ± 0.006	**0.101** ± 0.010	**0.203** ± 0.024	**0.180** ± 0.017	**0.035** ± 0.004	**0.581** ± 0.060
3-NBA microsomes + arachidonic acid	**0.107** ± 0.011	**0.113** ± 0.012	**0.274** ± 0.025	**0.155** ± 0.018	ND	**0.649** ± 0.065
3-NBA microsomes + arachidonic acid + indomethacin	**0.100** ± 0.010	**0.080** ± 0.009	**0.265** ± 0.028	**0.130** ± 0.012	ND	**0.575** ± 0.058

[a] Mean RAL (relative adduct labelling) of triplicate *in-vitro* incubations; spot 1 = dA-N^6-ABA, spot 3 = dG-N^2-ABA, spots 4/5 = dG-C8-*N*-ABA.

[b] ND = not detected.

Supporting Table S3.

a) DNA adduct formation by 3-ABA activated by lung cytosols of rats either uninduced or induced by 3-NBA

	RAL[a] (mean/10^8 nucleotides)					
	Spot 1	Spot 2	Spot 3	Spot 4	Spot 5	Total
uninduced cytosol	ND[b]	ND	ND	ND	ND	ND
uninduced cytosol + H_2O_2	**0.057**± 0.007	**0.072** ± 0.008	**0.128** ± 0.015	**0.066** ± 0.008	**0.060** ± 0.007	**0.383** ± 0.045
3-NBA cytosol + H_2O_2	**0.099** ± 0.010	**0.086** ± 0.010	**0.114** ± 0.011	**0.114** ± 0.012	**0.093** ± 0.010	**0.506** ± 0.051

b) DNA adduct formation by 3-ABA activated by kidney cytosols of rats either uninduced or induced by 3-NBA

	RAL[a] (mean/10^8 nucleotides)					
	Spot 1	Spot 2	Spot 3	Spot 4	Spot 5	Total
uninduced cytosol	ND[b]	ND	ND	ND	ND	ND
uninduced cytosol + H_2O_2	**0.237**± 0.023	**0.233** ± 0.023	**0.272** ± 0.029	**0.163** ± 0.014	ND	**0.905** ± 0.110
3-NBA cytosol + H_2O_2	**0.393** ± 0.051	**0.210** ± 0.021	**0.400** ± 0.043	**0.200** ± 0.022	ND	**1.203** ± 0.134

[a] Mean RAL (relative adduct labelling) of triplicate *in-vitro* incubations; spot 1 = dA-N^6-ABA, spot 3 = dG-N^2-ABA, spots 4/5 = dG-C8-*N*-ABA.

[b] ND = not detected.