DNA adduct formation and oxidative stress from the carcinogenic urban air pollutant 3-nitrobenzantrone and its isomer 2-nitrobenzantrone, *in vitro* and *in vivo*

Eszter Nagy, Shuichi Adachi¹, Takeji Takamura-Enya², Magnus Zeisig and Lennart Möller*  
Department of Biosciences and Nutrition, Karolinska Institutet, SE-141 57 Huddinge, Stockholm, Sweden, ¹Department of Public Health, Sagami Women’s University, Sagamihara, Kanagawa 228-8533, Japan and ²National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan

The carcinogenic vehicle emission product 3-nitrobenzantrone (3-NBA) is known to rearrange in the atmosphere to the isomer 2-nitrobenzantrone (2-NBA), which exists in 70-fold higher concentration in ambient air. The genotoxicity of 2-NBA and 3-NBA was studied both *in vitro* (human cell lines A549 and HepG2) and *in vivo* (F344 female rats intra-tracheally administered 5 mg/kg body weight of 3-NBA) models, using the ³²P-HPLC and the single-cell gel electrophoresis (Comet assay) methods. *In vitro*, also the parent compound benzanthrone (BA) and the metabolite 3-aminobenzanthrone (3-ABA) were evaluated. 3-NBA gave highest levels of DNA adducts in the two cell lines, but significantly higher in HepG2 (relative adduct level ~ 500 adducts/10⁸ normal nucleotides), whereas 2-NBA formed about one-third and one-twentieth of the DNA adduct amount in A549 and HepG2 cells, respectively. 3-ABA formed only minute amounts of DNA adducts and only in the A549 cells, whereas BA did not give rise to any detectable levels. The DNA adduct patterns from 3-NBA were similar between the two model systems, but differed somewhat for 2-NBA. The oxidative stress induced by BA was almost as high as what was observed for 3-NBA and 3-ABA in both cell lines, and 2-NBA induced lowest level of oxidative stress. The oxidative stress and DNA adduct level, in whole blood, was significantly increased by 3-NBA but not by 2-NBA. However, 2-NBA showed similar toxicity to 3-NBA, with respect to DNA adduct formation *in vivo*, hence it is important to further study 2-NBA as a potential contributor to health risk. While DNA adduct level in the 3-NBA-exposed animals reached a peak around 1 and 2 days after instillation, 2-NBA-treated animals showed a tendency towards a continuing increase at the end of the study.

Introduction

Vehicle-related environmental pollution is an important public health issue, due to increasing worldwide urbanization. Diesel emissions are mutagenic, which is mainly related to mono- and di-nitro polycyclic aromatic hydrocarbons (PAHs) (1). One such nitro-PAH, originating from diesel emissions and discovered in the ambient air of Tokyo, is 3-nitrobenzantrone (3-NBA) (2). 3-NBA has been ubiquitously found in the environment at levels comparable to common pollutants like 1-nitropyrene, 1,3-, 1,6- and 1,8-nitropyrenes and benzo[a]pyrene (3,4). 3-NBA has been shown to be present in urban air at levels in the range of 0.6–6.6 p.p.m., and the estimated daily human intake of 3-NBA due to inhalation is ~90 pg (2.5).

3-NBA have been shown to have similar activation pathways as 2-nitronaphthalene, 2-nitrobenzopyranone and 1,3-nitropyrene lactone, with multiple DNA adduct formation in B-lymphoblastoid cell lines (6) and mutagenicity in the Salmonella assay (7). Experiments with different cell lines have aided in the assessment of the activation pathways of 3-NBA and its metabolites, pointing out certain CYPs, such as CYP1A1, 1A2, 2B6 and 2D6, as well as different human transferases as activators (8,9).

*In vivo* short-term studies have revealed part of the mechanisms by which 3-NBA and its metabolites exert their genotoxicity, e.g. activation by peroxidases (10) and cytosolic nitroreductases rather than microsomal NADPH : cytochrome P450 oxidoreductase (POR) in hepatic POR-null mice (11). Activation of 3-NBA and its metabolites leads to formation of multiple DNA adducts in several tissues with highest levels in the primarily exposed tissues, indicating that the level of damage is route dependent and that the metabolites are readily distributed throughout the body by both extra-hepatic and as hepatic circulation (12–14). In addition, the *in vivo* mutagenicity of 3-NBA in lambda/lacZ transgenic mice after intra-peritoneal administration reveals elevated mutation frequencies as G:C → T:A transversions, as well as induction of micronuclei in peripheral blood reticulocytes (15). Apart from the DNA adduct-forming capacity and the induction of micronuclei, 3-NBA also has the potential to induce tumours in the lung of female F344 rats following intra-tracheal (i.t.) instillation, in a dose-dependent manner with squamous cell carcinoma as the predominant type of tumour (13).

3-Aminobenzanthrone (3-ABA) is a reduced metabolite of 3-NBA that has been found in the urine of mining workers, indicating human exposure of 3-NBA and/or 3-ABA (16). The metabolites of 3-NBA have been examined in different bacterial strains, revealing high sensitivity by YG1024 and YG1029 compared to the original strains TA98 and TA100, indicating the importance of O-acetyltransferase in the activation of 3-NBA, similar to what has previously been reported for 3-NBA (17). Further, cells from mice subjected to 3-NBA and 3-ABA have been analysed using the Comet assay to determine the genotoxicity of the substances, which showed that both compounds induce higher tail moments *in vivo*, revealing elevated DNA damage in different organs (18).

The level of 3-NBA is highest near the source of emission, and relatively low in general ambient air due to extensive atmospheric rearrangements to the isomer 2-nitrobenzantrone (2-NBA) (5,19). 2-NBA is about one-third as genotoxic as 3-NBA in the A549 lung cell line, and is capable of forming both DNA adducts and oxidative stress in the Comet assay (20). Since it has been shown that 2-NBA exists in 70-fold higher...
concentrations in ambient air compared to 3-NBA (5), this urges for further studies on whether 2-NBA is a significant contributor to health hazard in vivo, since no previous in vivo studies on the genotoxicity on 2-NBA have been performed.

The aim was to compare the genotoxic potential of 2-NBA and 3-NBA, both in vitro and in vivo model systems, employing the A549 and HepG2 cell lines as well as F344 female rats, and measuring DNA lesions with 32P-HPLC and the Comet assay. The A549 human lung cell line was chosen due to the assessment of the genotoxicity of a lung carcinogen and its isomer, and A549 is known to metabolize several nitro-PAHs with similar activation pathways as 3-NBA (21). The HepG2 cells were chosen since many substances that are inhaled are partly transported up from the lung and consequently swallowed and thereby enter the hepatic circulation. In addition, several studies have shown that HepG2 cells could be a suitable tool for assessing the genotoxicity of direct and indirect mutagens and for establishing the lowest genotoxic concentration (22,23).

Materials and Methods
Reagents and enzymes
The reagents and enzymes used in the study were purchased from the following sources: nucleotide P1 and RNase A (from bovine pancreas), both from Roche (Mannheim, Germany); spleen phosphodiesterase type II (SPD; from calf spleen) from Calbiochem (Darmstadt, Germany); RNase T1 (from Aspergillus oryzae), protease (from Streptomyces griseus) and micrococcocal nuclease (MN) from Sigma (St Louis, MO, USA); adenosine 5'-32P-triphosphate (32P-ATP; 3000 Ci/mmol) from Amersham International (Little Chalfont, UK); dithiothreitol (DTT) from Merck (Darmstadt, Germany) and polyonuclease kinase (PNK) from USB (Cleveland, OH, USA). All chemicals were of analytical grade.

3-NBA and 2-NBA, with purity of 99.9%, were synthesized (24,25) and kindly provided by Dr Takeji Takamura-Enya (Cancer Prevention Division, analytical grade. The substances kindly provided by Dr Takeji Takamura-Enya (Cancer Prevention Division, analytical grade.

Cell cultures
The test substances were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 2 mM. Cells (human lung cells A549, or human hepatic cells HepG2) were exposed by adding the test substances corresponding to 0.5% of the total medium volume, giving a final concentration of 10 µM of the test substances.

Both cell lines were cultured under identical conditions in Dulbecco’s modified Eagle medium (DMEM), which was supplemented to a total concentration of 10% foetal bovine serum, 100 U/ml of penicillin-streptomycin, 2 mM l-glutamine and 1 mM MEM sodium pyruvate in a 5% CO2 atmosphere at 37°C. Cells were seeded into dishes of 75 cm2 to give sufficient DNA for 32P-HPLC analysis. Duration of exposure was 18 h. Each experiment was repeated three times.

Cells were washed twice with sterile phosphate-buffered saline (PBS) and then harvested through addition of 150 µl trypsin-ethylenediaminetraetic acid (0.05%) × 10 PBS and incubated for 5 min at 37°C. The enzymatic activity of the enzyme was ceased by the addition of fresh supplemented DMEM. Cells were immediately placed on ice and cytotoxicity tests were performed on each harvested batch of cells by trypan blue staining. About 150 cells were counted and the amount of damage was on average 7% (data not shown).

The harvested cells used for the single-cell gel electrophoresis (the Comet assay) were analysed immediately, whereas cells prepared for the 32P-HPLC assay were collected, and blood from the animals was collected at days 5 and 10, and immediately frozen (–80°C), until analysis. The two time points for blood collection were chosen to allow detection of DNA damage changes over time.

Before DNA extraction, the digestive tract was divided into six sections: forestomach, glandular stomach, spleen, small intestine (20 cm), caecum and the entire colon. Each tissue was cut open and carefully rinsed with physiological saline solution. The small intestine and colon were scraped with a regular microscopic slide to obtain the soft tissue, which was used for DNA extraction. The other tissues were cut into small pieces before DNA extraction.

The tissues and blood were DNA extracted by a standard phenol–chloroform method, followed by hydrolysis of 10 µg DNA aliquots and enrichment of DNA adducts with butanol extraction. The hydrolysed and enriched aliquots were labelled using 32P-ATP and analysed with the 32P-HPLC system, as previously described (13).

Briefly, 0.2 g of liver, lung and kidney; tissues from the digestive tract and blood were homogenized and incubated with RNAse A, RNase T1 and protease (2 × 1 h at 37°C). The mixture was extracted in three consecutive steps with 1 volume of phenol, 1 volume of phenol : sevag and 1 volume of sevag. DNA was precipitated using 95% ethanol and 5 M NaCl, and the DNA pellet was washed with 70% ethanol. After the DNA pellet had dried, it was re-dissolved in water and DNA concentration and purity were determined using ultraviolet spectrometry. Aliquots of 10 µg DNA were taken out and hydrolysed through digestion with MN and SPD (2 × 2 h at 37°C).

The hydrolysed DNA and nucleotide samples were adduct enriched by butanol extraction using the phase transfer agent tetrabutyl ammonium chloride. The butanol phase was evaporated to dryness and each sample was labelled using 0.25 µl PKN buffer (400 µm biocine, 200 mM DTT, 200 mM MgCl2 and 20 mM spermidine; pH 9.6), SU T4-PNK enzyme and 1.8 µl (17.5 µCi) 32P-ATP (30 min at 37°C).

Data analysis
The 32P-HPLC system consisted of a Waters 600 E pump (Waters, Milford, MA, USA), a Hichrom RP 5-C18, K-100 guard column (Hichrom Ltd, Reading, UK), two serial reverse-phase DeltaPak C18 150 × 3.9-mm i.d., 5 µm 100 A main columns (Waters) and a Packard 500 TR flow scintillation detector (Packard Instrument Co., Meriden, CT, USA). Labelled samples were diluted with 150 µl of Milli-Q water immediately before injection into the 32P-HPLC system. Samples were analysed by flow rate, 0.5 ml/min and a linear gradient of 0–40% of 87.5% acetonitrile : water during 0–70 min in 2 M ammonium formate and 0.4 M formic acid (pH 4.5).
Most of the polar compounds in the mixture were separated by the guard column and removed by a switch valve between the guard column and the main columns, which was opened during the first 1 min after injection.

**Statistical analysis**

For the evaluation of the DNA adduct levels in the different tissues, peaks between 55 and 75 min were taken into account, excluding endogenous peaks also present in controls. Comparison between total DNA adduct levels were performed using unpaired, two-tailed Student’s t-test with unequal variance on individual values grouped according to dose and time point. DNA damage measured by the Comet assay was presented as %tail and statistically evaluated using unpaired, two-tailed Student’s t-test with unequal variances on individual values. Charts were drawn based on mean values while statistical evaluation was performed on complete data.

**Results**

**DNA adduct formation in human cell cultures**

The DNA adduct pattern of the parent compound BA and the substituted variants, 3-NBA, 3-ABA and 2-NBA, are shown in Figure 1. In the untreated and DMSO controls, in both cell lines, a single sharp peak was seen around 70 min (most clearly seen in Figure 1C), which varied inconsistently and consequently was regarded as endogenous and not counted as DNA adducts. BA did not give rise to a DNA adduct level above the background in either of the cell lines (Figure 1A and C), whereas 3-ABA, at least in the lung cell line, gave rise to a higher background level with a small peak around 65 min (Figure 1D). The DNA adduct patterns generated by all the substances were in general similar in both cell lines, although slight differences could be observed when considering smaller peaks from both 3- and 2-NBA (Figure 1B and D). The most prominent peaks were found in both cell lines.

The sum of DNA adducts between 55 and 75 min were on average about 3 DNA adducts/10⁷ normal nucleotides (NN) for the untreated and DMSO controls, as well as for BA in both cell lines and no statistically significant difference was observed (Figure 2A). In the case of the lung cell line, 3-ABA gave rise to twice as high background levels of small peaks, about 6 ± 1 DNA adducts/10⁷ NN, which was significantly higher (P < 0.05) compared to both the untreated and DMSO control. This was not observed in the HepG2 cell line.

Both 3-NBA and 2-NBA gave rise to significantly higher levels of DNA adducts (P < 0.05) compared to the controls in both cell lines (Figure 2A). The level of DNA adducts was also significantly higher for 3-NBA compared to 2-NBA in both cell lines (P < 0.05, respectively). The average levels of total DNA adducts in the lung cell line were 280 ± 40 for 3-NBA and 103 ± 30 DNA adducts/10⁸ NN for 2-NBA. In the HepG2, the total DNA adduct levels were 477 ± 67 for 3-NBA and 24 ± 8 DNA adducts/10⁸ NN for 2-NBA. There was also a significant difference (P < 0.05) between 3-ABA-treated cell lines with 6 ± 1 and 3 ± 0.4 DNA adducts/10⁸ NN for the A549 and HepG2 cell lines, respectively.

**Oxidative DNA damage in human cell cultures**

Figure 2B shows the genotoxicity (without/- FPG) of the two human cell lines exposed to different substances, whereas Figure 2C shows the oxidative DNA damage (with/+ FPG). Both cell lines displayed a significant increase in DNA damage...
(P < 0.05) after 3-NBA exposure compared to the controls (Figure 2B). There was also a statistically significant increase (P < 0.05) in DNA damage in lung cells treated with BA compared to the controls (Figure 2B).

Both cell lines showed a significant increase in oxidative DNA damage compared to the controls (P < 0.05), except HepG2 treated with 2-NBA (Figure 2C). Both cell lines displayed a significant relative increase (P < 0.05) of oxidative damage (Figure 2C) compared to total genotoxic lesions (Figure 2B), when treated with BA, 2-NBA, 3-ABA and 3-NBA in the A549 cell line. The exceptions were exposures to 2-NBA and 3-NBA of the HepG2 cell line, which were not
DNA adduct formation and oxidative stress

significantly different with or without FPG enzyme treatment, although the trend was towards an increase (Figure 2B and C).

Lung cells displayed higher oxidative damage compared to HepG2 after BA exposure. There was also a trend towards generally higher levels of oxidative damage in lung cells compared to the HepG2 after exposure to the substances.

**DNA adduct pattern in vivo**

A characteristic DNA adduct pattern with the main peaks around 60 min was observed in all the tissues after 3-NBA exposure (Figure 3A). The main peaks (shaded in the chromatograms) consisted of two fairly unresolved DNA adducts with retention times less than 30 sec apart. Similarly, rats exposed to 2-NBA displayed high levels of DNA adducts with characteristic patterns recognizable in all the tissues examined (Figure 3B). Characteristic DNA adducts after 2-NBA exposure consisted mainly of three major adducts around 58 min (shaded peaks in Figure 3B).

In the whole blood, 3-NBA gave rise to the characteristic double peaks observed in other tissues at 10 days after instillation, but at much lower levels (Figure 3C). There was no DNA adduct formation by 2-NBA above the background level, although the retention time area is shaded. The control blood displayed a few endogenous peaks, which were varying in level between different animals receiving the same treatment.

Note that the chromatograms are chosen to depict the DNA adduct pattern clearly, hence they are not from one particular time point.

**DNA adduct levels in vivo**

There were two peaks present around 65 and 70 min in controls and in the treated animals, which is why they were regarded as endogenous and not counted as DNA adducts in the retention time range of 55–75 min.

Characteristic DNA adducts after 3-NBA exposure clearly showed a rapid increase of DNA adduct levels followed by a decrease with time (Figure 4A), although some variations are present. However, the DNA adduct levels at 10 days do not exceed what was observed 1–2 days after instillation. There was an overall significant difference ($P < 0.01$) compared to the controls measured over the entire time range of 10 days. Most tissues at 1 day after instillation showed a significant increase ($P < 0.05$) compared to the controls, except for the small intestine and the glandular stomach, which displayed no and borderline significance, respectively (Figure 4A). Due to large standard deviations compared to the mean value, the kidney, spleen and forestomach display only borderline significances at 2 days after instillation in comparison to controls, whereas in the caecum and colon there was loss of significance although a trend towards higher DNA adduct levels remained. This was observed among several tissues between 3 and 10 days after exposure, where no significances were found even if the trend pointed towards an increase. In general, most tissues displayed a rapid increase followed by a decrease in DNA adduct levels. The small intestine, however, showed a continuously increasing trend, though not statistically significant (Figure 4A).

Characteristic DNA adducts following 2-NBA exposure showed a rather slow increase, but were overall significantly different from the controls ($P < 0.05$) (Figure 4B). In comparison to 3-NBA, the 2-NBA-exposed tissues do not display the same peak level at 1–2 days after instillation, but continue to increase until the end of the experiment. At individual time points, only the liver, spleen and forestomach showed significantly higher DNA adduct levels 1 day after instillation. The level of DNA adducts in lung, kidney and colon was rather high compared to the controls, but since the standard deviation was also high, only borderline significances were observed. Two days after instillation, the level of DNA adducts had increased significantly ($P < 0.05$) in the liver, lung, kidney, spleen, forestomach and small intestine. After 3 days, there was a clear drop in the level of DNA adducts in most GI tract tissues (Figure 4B). After 5 days, the levels of DNA adducts started increasing again, reaching higher levels in the liver, lung, kidney, forestomach and caecum ($P < 0.05$) and a borderline significance in the spleen. Ten days after instillation, higher levels of DNA adducts ($P < 0.05$) were observed in all tissues, except the forestomach and small intestine. However, there is a clear trend towards an increase in all tissues, quite the opposite of what was observed after 3-NBA administration.

Over all, there was a significant difference ($P < 0.001$) in DNA adduct levels between the 3-NBA- and 2-NBA-exposed tissues (except at 5 days after instillation). At 1 and 3 days after instillation, most of the tissues displayed a significant difference ($P < 0.05$) between the 3-NBA and 2-NBA groups. A drop in DNA adduct levels over time in 3-NBA-treated animals (Figure 4A) and an increase in the levels for 2-NBA-exposed animals (Figure 4B) lead to loss of significant differences ($P < 0.05$) between 5 and 10 days after instillation.

**DNA adduct and oxidative lesions in whole blood**

Blood collected from 2-NBA-treated animals displayed a significant increase in genotoxic lesions (+FPG) compared to the controls at 10 days after instillation (Figure 5A). There was no increase in genotoxic damage observed in the blood of 3-NBA-treated animals. However, following FPG enzyme treatment (+FPG) to measure oxidative damage, significant increases in DNA lesions were observed at both time points for 3-NBA only.

A significant increase in DNA adducts in whole blood was observed only for the 3-NBA-treated animals at 10 days after instillation (Figure 5B).

**Discussion**

**DNA adduct formation and oxidative damage in vitro**

There was a significant DNA adduct formation after 2-NBA and 3-NBA exposure in both cell lines (Figures 1A–D and 2A). As previously reported, the DNA adduct formation following 2-NBA exposure was about one-third of that of 3-NBA exposure in A549 cells, which is similar to the observations in this study (Figure 2A) (20).

The DNA adduct levels in the HepG2 cells were about 20-fold lower by 2-NBA compared to 3-NBA (Figure 2A). It is known that the position of the nitro-group in the BA structure plays a crucial role in the genotoxicity of different benzanthrones (19), probably influencing in part the bioavailability of the substances, and 2-NBA is seemingly less soluble and more biologically stable, when compared to 3-NBA (26). It is also possible that they have different abilities to induce enzymatic systems. This factor, however, is also dependent on the type of cell line used. Iwanari et al. (21) had examined the induction of cytochrome P540 isofoms in different human cell lines and concluded that HepG2 belonged to the type of cell lines that
Fig. 3. Representative $^{32}$P-HPLC chromatograms of the DNA adduct patterns in different tissues from 3-NBA (A) and 2-NBA (B)-treated animals and of whole blood from both 3-NBA and 2-NBA animals along with the control blood (C). Retention time areas for the major DNA adduct peaks from 3-NBA and 2-NBA, respectively, are shaded. Note that the chromatograms are not taken at specific time points of sacrifice, but rather chosen to clearly present the DNA adduct pattern. In addition, the y-axis has been altered to depict the DNA adduct patterns with best resolution, hence labels have not been added.
did induce the CYP1 family, whereas A549 did so only moderately, in the presence of both PAHs and nitro-PAHs. The significant role of the CYP1 family in the activation of 3-NBA and 3-ABA has been evaluated in a number of studies and it has been shown that induced CYP2B6/2D6 was most efficient in significantly elevating DNA adduct levels from 3-NBA and some of its metabolites, followed by CYP1A1/1A2 (27). Whether the differences in DNA adduct levels between the cell lines in this study is dependent on the cell type or the physiochemical properties of the compounds is yet to be evaluated; probably both are important. This is supported by findings showing that by using inhibitors of CYP1A1 and 1A2, such as α-naphthoflavone and furafylline, the formation of DNA adducts by human liver microsomes was reduced (8). Further, genotoxic evaluation using the Salmonella assay and hamster lung fibroblasts (V79M2) demonstrated that apart from CYPs, also N,N-acetyltransferases contribute to the high mutagenic and genotoxic potential of 3-NBA (28).

BA, the parent compound to both 3- and 2-NBA, can exert cellular toxicity and DNA damage through oxidative processes (29). This is in accordance with the findings in this study, where BA gave rise to almost equally high levels of oxidative damage as 3-NBA (Figure 2C), although no DNA adduct levels were observed above the background level (Figure 2A). The CYP1 family is also induced by PAHs via an aryl hydrocarbon receptor (AhR)-mediated mechanism (30). Binding to the AhR is a known pathway for many dioxin- and quinone-related substances, which can lead to oxidative damages (31).

The level of DNA damage induced by BA was significantly higher in lung cells (Figure 2B). Although no studies could be found regarding BA using A549 cells, a study by Singh (32) showed that i.t. instillation of BA in guinea pigs caused a hemorrhagic oedema, a feature similar to what was observed in a few cases among F344 rats following i.t-treatment with 3-NBA (13).

Elevated levels of DNA damage (Figure 2B) are considered an estimate of the background strand breaks and it seems that the HepG2, probably due to more extensive enzyme systems, is more sensitive to 3-NBA exposure compared to lung cells, which corresponds well with the DNA adduct findings (Figure 2A). This is, however, not the case when it comes to oxidative

---

**Fig. 4.** Total amount of DNA adducts at different time points (1, 2, 3, 5 and 10 days) after i.t. instillation for 3-NBA (A)- and 2-NBA (B)-treated animals. *N* = 3 at each time point in both treated groups, except for the controls (N = 5). The controls in the charts are presented as the pooled average values of the control animals (5–10 days), whereas statistical analysis was performed on the control groups separately. Treated animals were compared to the same-day controls. Statistical significance presented above the bars are *P < 0.05, **P < 0.01 and ***P < 0.001, whereas (*) represents a borderline significance (P = 0.05–0.07) with the two-tailed Student’s t-test of unequal variances. The amount of DNA damage is presented as DNA adducts/10⁸ NN, indicating the amount of damaged bases per 100 million undamaged nucleotides.
lesions only (Figure 2C), where the lung cells seem to have higher levels of oxidative damage compared to HepG2 (Figure 2C). In a similar study, HepG2 cells were exposed to different concentrations of 3-NBA and significantly increased levels of oxidative damage were observed already at 12 nM, compared to the controls (33). It is difficult to compare results between the studies, mostly because of the different concentrations and also the lack of DNA adduct data, which in this study were used together with oxidative stress to better understand the genotoxicity of the compounds tested. However, studies clearly show that HepG2 cells have a good sensitivity towards 3-NBA. Whether it is only a question of oxidative lesions such as 8-oxo-deoxyguanosine (8-oxodG) is not really known. In a previous study, we showed that although the Comet assay pointed to elevated global oxidative stress due to 2-NBA or 3-NBA exposure, the specific marker of oxidative stress 8-oxodG was not detected above the background (20). In fact, the alkaline (pH >13) version of the Comet assay (34) is recommended because it detects a broad spectrum of DNA lesions, such as DNA double-strand breaks, DNA single-strand breaks and alkali-labile sites. The FPG enzyme is used for the detection of oxidative DNA base damage, in particular, 8-oxodG (35), but it is also important to consider that it also detects a number of other DNA oxidative lesions (36).

DNA adduct formation and oxidative damage in vivo
The fingerprints of DNA adducts found in different tissues following 3-NBA treatment is very similar to those found in vitro and the number of prominent DNA adducts are the same in the tissues as well as in the cell lines (Figure 3A). The two most prominent DNA adducts by 3-NBA (shaded in Figure 3A) are those that in a previous study were characterized to be of the non-acetylated type, namely dGp-C8-N-ABA and dGp-N2-C2-ABA (13). This was also corroborated by a study in which the major DNA adducts were characterized as adducted moieties attached at the C8 and N2 positions of guanine (37).
The DNA adduct patterns in 2-NBA-exposed tissues differ somewhat from those exposed to 3-NBA, although eluted at very similar retention times (Figure 3B). There is also a slight difference in DNA adduct pattern observed in vitro and in vivo, suggesting the involvement of a different or more complex metabolic pathway in vivo. However, further analysis is needed of 2-NBA exposure before this can be concluded.

By looking at the DNA adduct levels over time, 3-NBA-exposed tissues have an increase shortly following instillation (Figure 4A), whereas the 2-NBA-treated tissues increased very slowly (Figure 2B). It is likely that the DNA adduct level induced by 2-NBA would have continued to increase if the experiment had continued longer. Overall, the amount of DNA adducts formed by 2-NBA is ~80% of adducts formed by 3-NBA, comparing the highest level of 2-NBA with highest level of 3-NBA DNA adducts in vivo (Figure 4A and B). Nota bene, this is not an absolute or certain quantification due to the varying labelling efficiency and recovery of the DNA adducts. Previously, we have shown that the recoveries for eight different 3-NBA-derived DNA adduct standards were between 4 and 55% (13). For both dGp-C8-N-ABA and dGp-N2-C2-ABA the recovery was 30%. Thus, it is difficult to quantify the DNA adducts between 2- and 3-NBA, because some DNA adducts may be present in high levels, but give low recoveries due to, for instance, low labelling efficiency. This would lead to an underestimation of the level of DNA adducts. Further, there are no pre-synthesized standards available for 2-NBA as yet, to estimate the recovery or do characterizations. Hence, the comparison in this study is only based on the relative levels observed in the tissues.

The physicochemical properties of 3- and 2-NBA showed that the latter proved to be slightly more lipophilic (26), but whether this small increase in lipophilicity has any profound impact on the bioavailability has to be further evaluated. Solubility and lipophilicity are of importance when considering bioavailability, since PAHs are differently absorbed in the intestinal tract, mainly according to their physicochemical properties, particularly their lipophilicity (38), because PAH metabolites are usually less lipophilic than the parent molecule, which can positively affect their intestinal transfer (39). In addition, lipophilic compartments in the body, such as membranes, can also affect bioavailability (40,41). In this study, the DNA adducts in the GI tract are rapidly increased similarly to the lung and kidney after 3-NBA i.t. administration in rat, whereas the less soluble and more lipophilic isomer 2-NBA gives low amounts of DNA adducts in the GI tract, supporting in part the hypothesis that solubility and lipophilicity in part delay bioavailability and DNA adduct formation from 2-NBA. This urges for further studies on 2-NBA, since a slow “leakage” from depots in the body and hence a continuous exposure to this substance may pose a health risk. Further, high lipophilicity also points to a greater ability to bioaccumulate in the adipose parts of the body.

Another important factor to consider, besides lipophilicity and bioavailability, is the repair or persistence of the DNA adducts. 3-NBA has been shown to form DNA adducts mainly at GC hotspots and induces high level of mutagenicity in frame shift sensitive bacterial strain (26,42). Early formation of DNA adducts have been correlated to tumour development later on (43). Previously, we reported that in a short-term study there were no detectable amounts of DNA adducts after 16 days following a single i.t. administration (13). However, the presence of persistent DNA adducts from 3-NBA cannot be excluded, since the method of choice for analysis has a limit of detection. More over, additional genotoxic lesions such as fixed mutations or lack of repair was not examined in that study.

In a previous study, we also noted that there was a slight dip in the level of DNA adduct after 5 days after oral administration of 3-NBA to rat (14), which was correlated to acute toxicity. The decrease of DNA adduct levels was observed in this study at 3 days, and especially clear for 2-NBA. The difference in time point can in part be explained by the different administration routes; however, evaluation of the acute toxicity was not within the scope of this study.

We have also been able to show that 3-NBA can elevate oxidative damage in vivo (Figure 5A). Previously published data have shown elevated genotoxic (FPG) effects measured by the Comet assay in liver, kidney, lung and bone marrow in treated mice, but no FPG enzyme treatment was performed to measure specific oxidative lesions (18). The results might be contradictory in that we do not find significantly elevated genotoxic damage compared to the controls in this study. However, the doses used in the previously published study correspond to 40 and 160 mg/kg body weight administered intra-peritoneally into mice, as compared to 5 mg/kg body weight in rats following i.t. instillation in this study. The level of measured oxidative lesions is rather low, but significantly different from the controls at 5 and 10 days after instillation for 3-NBA. The only significant increase in background damage, or genotoxicity, was by 2-NBA at 10 days after instillation.

DNA adduct data in whole blood confirm the genotoxicity of 3-NBA, but no DNA adduct above the background level was observed for 2-NBA at this level of exposure (Figure 5B). There is a small trend towards an increase even for 2-NBA at 10 days after instillation, but this was not validated statistically.

In summary, 2-NBA is genotoxic in vitro as well as in vivo, although not as potent as its counterpart 3-NBA, which previously has been shown to induce tumours in treated rats. 3-NBA gives rise to similar DNA adduct patterns in cell cultures as in tissues, whereas the DNA adduct pattern from 2-NBA differs somewhat between the two experimental models. 3-NBA is known to form two major DNA adducts that have previously been characterized, but the DNA adducts produced by 2-NBA remain to be identified.

Whereas DNA adduct levels from 3-NBA have a peak level between 1 and 2 days following instillation, DNA adduct levels from 2-NBA continue to increase slowly over a longer period of time and are at most ~80% of those from 3-NBA. Since 2-NBA exists in 70-fold higher concentration in ambient air, and has a similar toxicity, may be retained in the body longer compared to 3-NBA, even after a single instillation, it raises the question whether 2-NBA can bioaccumulate to a greater extent than 3-NBA and thereby pose an even greater threat. The much higher urban air concentration and seemingly similar genotoxicity indicate that 2-NBA might be the dominating isomer in its contribution to health risk, when compared to 3-NBA.

Acknowledgements

The authors wish to express their gratitude to Mary-Ann Zetterqvist for skilful technical assistance. This study was supported by the Swedish Environmental Protection Agency and Global Environment Protection from Ministry of the Environment, Japan. This work was partly supported by ECNIS (Environmental Cancer Risk, Nutrition and Individual Susceptibility), a network of excellence operating within the European Union 6th Framework Programme, Priority 5: “Food, Quality and Safety” (Contract No. 513943).
References


milk with 14C-phenanthrene, 14C-benzo[a]pyrene or 14C-TCDD in growing pigs. *Chemosphere*, 48, 843–848.


Received on September 7, 2006; revised on October 25, 2006; accepted on November 21, 2006