REVIEW

3-Nitrobenzanthrone, a potential human cancer hazard in diesel exhaust and urban air pollution: a review of the evidence

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Epidemiological studies have shown that exposure to diesel exhaust and urban air pollution is associated with an increased risk of lung cancer. 3-Nitrobenzanthrone [3-nitro-7H-benz[de]anthracen-7-one (3-NBA)] is an extremely potent mutagen and suspected human carcinogen identified in diesel exhaust and ambient air particulate matter. The main metabolite of 3-NBA, 3-aminobenzanthrone (3-ABA), was found in the urine of salt mine workers occupationally exposed to diesel emissions, indicating that human exposure to 3-NBA due to diesel emissions can be significant and is detectable. There is clear evidence that 3-NBA is a genotoxic mutagen forming DNA adducts after metabolic activation through simple reduction of the nitro group. Several human enzymes have been shown to activate 3-NBA and its metabolites in vitro and in cells to form electrophilic aryl nitrenium and rearranged carbenium ions, leading to the formation of purine adducts at the C8 and N2 position of guanine and at the C8 and N6 position of adenine. The predominant DNA adducts are 2-(2-deoxyguanosin-5′-yl)-3-aminobenzanthrone and N2-(2′-deoxyguanosin-8-yl)-3-aminobenzanthrone. 3-aminobenzanthrone are also the most persistent adducts in target tissue in rodents, and are most probably responsible for the induction of GC→TA transversion mutations observed in vivo. It is concluded that these adducts not only represent premutagenic lesions in DNA but are of primary importance for the initiation of the carcinogenic process and subsequent tumour formation in target tissue. Indeed, 3-NBA is carcinogenic in rats after intratracheal instillation, inducing mainly squamous cell carcinoma in lung. The intention of this article is to provide a critical review on the potential genotoxic effects of 3-NBA on human health. However, in general, there is a need for more mechanistic studies that relate 3-NBA to all processes that are considered to orchestrate tumour development and of studies on the ability of particles to promote 3-NBA genotoxicity. Because of its widespread environmental presence, 3-NBA may represent not only an occupational health hazard but also a hazard for larger sections of the general population. For an accurate risk assessment more epidemiological studies on 3-NBA-exposed individuals and a broader monitoring of environmental levels of 3-NBA are required.

Introduction

Environmental factors and an individual’s genetic susceptibility play an important role in many human cancers (1). Lung cancer is the most common malignant disease worldwide and is the major cause of death from cancer. Tobacco smoking is the overwhelming cause of lung cancer but vehicular exhaust and ambient air pollution are also implicated (2–5). Although traditional industrial emission levels are tending to decrease in Western countries, vehicular exhaust remains a continuing, even increasing, problem (6). Nitropoly cyclic aromatic hydrocarbons (nitro-PAHs) are widespread environmental contaminants found in exhausts from diesel and gasoline engine emissions, on the surface of ambient air particulate matter, in river sediments and in grilled food (7–10). The increased lung cancer risk from exposure to air pollutants and the detection of nitro-PAHs in lungs of non-smokers with lung cancer has led to considerable interest in assessing their potential risk to humans (11).

Several human biomonitoring studies using the detection of DNA adducts by the ultrasensitive 32P-postlabelling method have reported higher levels of bulky DNA adducts among subjects heavily exposed to diesel exhaust and urban air pollution (12–15). This correlates with the increased cancer risk (16). The aromatic nitroketone 3-nitrobenzanthrone [3-nitro-7H-benz[de]anthracen-7-one (3-NBA); Figure 1] is one of the most potent mutagens and a potential human carcinogen that was discovered only a few years ago in diesel exhaust and bound to the surface of airborne particulate matter (17–19). Thus, 3-NBA was described as the ‘devil in the diesel’ in the popular press (20). The main metabolite of 3-NBA, 3-aminobenzanthrone (3-ABA), has been found in workers occupationally exposed to diesel emissions (21), suggesting that exposure to 3-NBA in diesel emissions may represent a health hazard for large sections of the population. DNA adduct formation is a critical event in mutagenesis and in the initiation of carcinogenesis (22). Determining the capability of humans to activate 3-NBA to form DNA adducts and understanding which human enzymes are involved in its metabolic activation are important in the assessment of susceptibility to this environmental contaminant (23).

The intention of this article is to provide a critical review on the potential genotoxic effects of 3-NBA on human health.

Fig. 1. Structure of 3-nitrobenzanthrone (3-NBA; 3-nitro-7H-benz[de]anthracen-7-one).
As such, the article focuses on environmental levels and human sources of exposure, the potential genotoxic mechanism for 3-NBA oncogenesis in rodents, and speculates on the mechanism of 3-NBA suspected carcinogenicity in humans.

Sources of exposure to 3-nitrobenzanthrone

3-NBA was first discovered by Suzuki and co-workers (17) in organic extracts of both diesel exhaust and airborne particles. It may originate from both incomplete combustion of fossil fuels and reaction of the parent aromatic hydrocarbon [benzan-throne (BZ)] with nitrogen oxides under atmospheric conditions (17,24). 3-NBA was easily produced under an artificial atmosphere containing BZ and gaseous nitrogen dioxide along with ozone (17,25). BZ is a widely distributed contaminant in atmospheric environment. It is especially abundant in exhaust gas and particles from motor engines and in smoke and soot from burning wood (26–29).

The concentration of nitro-PAHs in urban atmospheres depends on the season, the type of heating used, the amount of traffic and the type of vehicles. Reported levels in air do not usually exceed 1 ng/m³, although concentrations up to 13 ng/m³ have been reported (10). However, levels of PAHs in urban air are in general one order of magnitude higher than those reported for nitro-PAHs (30). 3-NBA has been detected in diesel exhaust particles at concentrations of up to 6.6 µg/g particles (Table I) (17,31). Moreover, as summarized in Table I, 3-NBA has been found in ambient air particles collected in urban and semi-rural areas, and at traffic- or industrial-emissions-impacted sites at several pg/m³ levels (up to ~11.5 pg/m³ air). Higher levels (up to ~80 pg/m³ air) of 3-NBA have been observed in ambient air at workplaces subjected to high diesel emissions (Table I) (21). On the basis of the reported quantities of 3-NBA in diesel exhaust particles (17) and the daily intake via inhalation of particles of 1 µg/m³ air (LAI), a person’s intake of 3-NBA may be ~90 pg/day, based on a breath intake of about 15 m³ air/day (32). However, intake of 3-NBA by high-risk population groups may be higher (21).

2-NBA, a 3-NBA isomer, was also formed under artificial atmospheric conditions and was detected in ambient air particles collected in the USA and Japan (33,34). It was suggested that 2-NBA might be formed more specifically by atmospheric processes while 3-NBA seems to be formed preferentially by combustion processes, such as in a diesel engine (24,33,34). In one study the atmospheric concentrations of 2-NBA and 3-NBA were 495 and 6.8 pg/m³, respectively, with a ratio of 2-NBA/3-NBA of ~70 (34). A lower ratio of 2-NBA/3-NBA (~35) was found in another ambient air sample (33). Little is yet known about the toxicity of 2-NBA, but 2-NBA showed strand-breaking activity in the comet assay and induced DNA adducts in human A549 lung cells (35). Although the genotoxic potential of 2-NBA was about one-third that of 3-NBA (35), its higher abundance in ambient air does urge further investigation to evaluate its potential human health hazard.

Pollutants in the atmosphere can be removed by either dry or wet deposition. In a recent study 3-NBA was detected in rainwater (36), indicating that 3-NBA in the atmosphere can be transported into rainwater. In rainwater samples collected at a residential area in Kyoto, Japan, 3-NBA was determined in the range of 0.07–2.6 ng/l (Table I). As a likely consequence of atmospheric washout, 3-NBA was detectable in surface soil at

<table>
<thead>
<tr>
<th>Environmental source</th>
<th>Environmental 3-NBA concentration</th>
<th>Country</th>
<th>Sampling information</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diesel exhaust particle</td>
<td>&lt;0.001–6.61 µg/g particulate</td>
<td>Japan</td>
<td>Isuzu engine Model 6HEL 7127c (maximum power 250 hp/2700 rpm) working under various loading conditions</td>
<td>(17)</td>
</tr>
<tr>
<td>Diesel exhaust particle</td>
<td>0.027–0.056 µg/g particulate</td>
<td>Japan</td>
<td>Diesel engine vehicles, including heavy- and light-duty vehicles, driven at 80 km/h (n = 3)</td>
<td>(31)</td>
</tr>
<tr>
<td>Diesel exhaust particle</td>
<td>Identified by GC-MS SIM</td>
<td>USA</td>
<td>Standard reference material (SRM) 1975 obtained from the National Institute for Standards and Technology (NIST), Gaithersburg, MD</td>
<td>(33)</td>
</tr>
<tr>
<td>Airborne particle</td>
<td>5.2–11.5 pg/m³ air</td>
<td>Japan</td>
<td>Sampling point was in central Tokyo; sampling time winter 1994; same day and night time collection</td>
<td>(17)</td>
</tr>
<tr>
<td>Airborne particle</td>
<td>Tentatively identified by MS</td>
<td>USA</td>
<td>2.5 µm particles (PM2.5) were collected in Salt Lake City; sampling time was October 5, 1999 (n = 1)</td>
<td>(103)</td>
</tr>
<tr>
<td>Airborne particle</td>
<td>ND²–68.4 pg/m³ air</td>
<td>Denmark</td>
<td>Sampling site was located at Riso in a semi-rural area about 35 km west of Copenhagen; sampling time from February 1998 to February 1999 (3-NBA was present in ~25% of collected samples; n = 31)</td>
<td>(24)</td>
</tr>
<tr>
<td>Airborne particle</td>
<td>ND²–80 pg/m³ air</td>
<td>Germany</td>
<td>Samples were collected at five typical working places in an underground salt mine (n = 5)</td>
<td>(21)</td>
</tr>
<tr>
<td>Airborne particle</td>
<td>0.4 µg/m³ air</td>
<td>USA</td>
<td>Sampling point was at an industrial emission-impacted site in Concord, CA (n = 1); sampling time January 1987</td>
<td>(33)</td>
</tr>
<tr>
<td>Airborne particle</td>
<td>6.79 pg/m³ air</td>
<td>Japan</td>
<td>Sampling point was a heavy traffic road in Kanazawa (n = 1)</td>
<td>(34)</td>
</tr>
<tr>
<td>Rainwater</td>
<td>0.07–2.6 ng/l</td>
<td>Japan</td>
<td>Sampling point was a building roof in a residential area of Kyoto (n = 6); sampling time from May to July and from October to December 2001</td>
<td>(36)</td>
</tr>
<tr>
<td>Surface soil</td>
<td>1.2–1020 pg/g soil</td>
<td>Japan</td>
<td>Samples were collected in the Chiba area (n = 6)</td>
<td>(37)</td>
</tr>
<tr>
<td>Surface soil</td>
<td>144–1158 pg/g soil</td>
<td>Japan</td>
<td>Samples were collected in the Kinki region, particularly in Osaka and neighbouring cities (n = 8); sampling time between February and December 1999</td>
<td>(38)</td>
</tr>
<tr>
<td>Coal-burning-derived particle</td>
<td>0.234 µg/g particulate</td>
<td>China</td>
<td>Sampling point was a chimney of a domestic coal stove in Shenyang (n = 1)</td>
<td>(39)</td>
</tr>
</tbody>
</table>

ND, not detected.

Table I. Sources of environmental exposure to 3-nitrobenzanthrone
levels of up to ~1200 pg/g soil (Table I) (37,38). More recently, 3-NBA was detected in extracts of particles collected from a chimney of a domestic coal-burning stove at a concentration of 0.234 µg/g particles, suggesting that particles emitted from industrial and domestic coal-burning sources have to be considered as possibly minor sources of 3-NBA formation in urban air pollution (39).

**Genetic and related effects of 3-nitrobenzanthrone**

3-NBA is one of the most potent bacterial mutagens known to date, inducing 0.2 and 6.3 million revertants per nmol in *Salmonella typhimurium* strains TA98 and YG1024, respectively (Table II); numbers of revertants are comparable in each case to the number induced by the nitro-PAH 1,8-dinitropyrene (17). Of note, the contribution ratio of 3-NBA to the mutagenicity measured in the Ames test of soil extracts from the Kinki region in Japan (Table I) containing 3-NBA was estimated to be 2–38%, suggesting that 3-NBA, together with other nitro-PAHs, may be the major mutagens that contaminate surface soil in this region (38). It has also been shown that of a total of four fractions containing nitro-PAHs extracted from coal-burning-derived particulates the fraction containing 3-NBA made the largest contribution to mutagenicity as assayed by the Ames test (39).

Induction of micronuclei was observed in 3-NBA-treated human B-lymphoblastoid and hepatoma cell lines (32,40,41). In hepatoma HepG2 cells 3-NBA, at 25 nM, induced a similar micronucleus frequency as benzo(a)pyrene induced at a 2000-fold higher concentration (32). Moreover, 3-NBA exhibits DNA strand-breaking activity as measured by the comet assay in human MCL-5, HepG2 and A549 cells (32,35,41). In HepG2 cells 3-NBA at 50 nM induced approximately the same level of DNA damage as benzo(a)pyrene did at 50 µM (32). Furthermore, 3-NBA is an effective mutagen in MCL-5 and h1A1v2 human B-lymphoblastoid cells at the hprt and tk locus (40). In this context it is also noteworthy that in Chinese hamster lung V79 cells expressing human N,O-acetyltransferases (NATs) or sulphotransferases (SULTs) 3-NBA induced a dose-dependent increase in the mutation frequency at the hprt locus (H.R. Glatt, unpublished data), indicating that NATs and SULTs could contribute significantly and specifically to the metabolic activation and mutagenesis of 3-NBA (see below). Micronuclei formation of 3-NBA was also found in peripheral blood reticulocytes of mice after intraperitoneal administration (17,42). Moreover, 3-NBA was also a potent mutagen in transgenic mice (Muta ™Mouse) after intraperitoneal injection (42). Increases in mutant frequency were found in colon, liver and bladder, with 7.0-, 4.8- and 4.1-fold increases above the control value, respectively, but with no increase in mutant frequency in lung, kidney, spleen and testis.

The genotoxicity of 3-NBA was further documented by the formation of specific DNA adducts in various chemical and enzymatic in vitro assays, in cells and in vivo in rodents treated with 3-NBA (43–57). In rats, multiple DNA adducts were observed in all organs investigated (e.g. lung, liver and kidney),

<table>
<thead>
<tr>
<th>Test system</th>
<th>Results</th>
<th>Details of study</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ames test, <em>Salmonella typhimurium</em> TA98 (-S9 mix)*</td>
<td>+</td>
<td>208 000 revertants per nmol</td>
<td>(17)</td>
</tr>
<tr>
<td>Ames test, <em>Salmonella typhimurium</em> TA100 (-S9 mix)*</td>
<td>+</td>
<td>29 700 revertants per nmol</td>
<td>(17)</td>
</tr>
<tr>
<td>Ames test, <em>Salmonella typhimurium</em> YG1021 (-S9 mix)*</td>
<td>+</td>
<td>129 000 revertants per nmol</td>
<td>(17)</td>
</tr>
<tr>
<td>Ames test, <em>Salmonella typhimurium</em> YG1024 (-S9 mix)*</td>
<td>+</td>
<td>6 290 000 revertants per nmol</td>
<td>(17)</td>
</tr>
<tr>
<td>Ames test, <em>Salmonella typhimurium</em> TA1538 (-S9 mix)*</td>
<td></td>
<td>8700 revertants per nmol</td>
<td>(48)</td>
</tr>
<tr>
<td>Ames test, <em>Salmonella typhimurium</em> TA1538/1,8-DNP (-S9 mix)*</td>
<td></td>
<td>2000 revertants per nmol</td>
<td>(48)</td>
</tr>
<tr>
<td>Ames test, <em>Salmonella typhimurium</em> TA1538/1,8-DNP pNAT1 (-S9 mix)*</td>
<td>+</td>
<td>7600 revertants per nmol</td>
<td>(48)</td>
</tr>
<tr>
<td>Ames test, <em>Salmonella typhimurium</em> TA1538/1,8-DNP pNAT2 (-S9 mix)*</td>
<td>+</td>
<td>33 600 revertants per nmol</td>
<td>(48)</td>
</tr>
<tr>
<td>Mutation frequency, human B-lymphoblastoid MCL-5 cells, hprt locus in vitro</td>
<td></td>
<td>ED 3.6 µM/10 h incubation</td>
<td>(40)</td>
</tr>
<tr>
<td>Mutation frequency, human B-lymphoblastoid MCL-5 cells, tk locus in vitro</td>
<td></td>
<td>ED 3.6 µM/10 h incubation</td>
<td>(40)</td>
</tr>
<tr>
<td>Mutation frequency, human B-lymphoblastoid h1A1v2 cells, hprt locus in vitro</td>
<td></td>
<td>ED 3.6 µM/10 h incubation</td>
<td>(40)</td>
</tr>
<tr>
<td>Mutation frequency, human B-lymphoblastoid h1A1v2 cells, tk locus in vitro</td>
<td></td>
<td>ED 3.6 µM/10 h incubation</td>
<td>(40)</td>
</tr>
<tr>
<td>Mutation frequency, lambda/dacZ transgenic mice (Muta ™Mouse), i.p.*</td>
<td>+</td>
<td>ED 25 mg/kg body weight once a week for 4 weeks, sacrificed 2 days later</td>
<td>(42)</td>
</tr>
<tr>
<td>Gene mutation, lambda/dacZ transgenic mice (Muta ™Mouse), i.p. treatment, liver tissue, cII locus in vivo</td>
<td>+</td>
<td>ED 25 mg/kg body weight once a week for 4 weeks, sacrificed 2 days later</td>
<td>(42)</td>
</tr>
<tr>
<td>Micronucleus test, human B-lymphoblastoid MCL-5 cells in vitro</td>
<td></td>
<td>HID 7.3 µM/10 h incubation</td>
<td>(40)</td>
</tr>
<tr>
<td>Micronucleus test, human B-lymphoblastoid h1A1v2 cells in vitro</td>
<td>+</td>
<td>LED 5.4 µM/10 h incubation</td>
<td>(40)</td>
</tr>
<tr>
<td>Micronucleus test, human B-lymphoblastoid MCL-5 cells in vitro</td>
<td></td>
<td>LED 1.0 µM/24 h incubation</td>
<td>(41)</td>
</tr>
<tr>
<td>Micronucleus test, human hepatoma cells (HepG2) in vitro</td>
<td>+</td>
<td>LED 12 nM/24 h incubation</td>
<td>(32)</td>
</tr>
<tr>
<td>Micronucleus test, ICR mice, i.p. treatment, peripheral blood reticulocytes in vivo</td>
<td>+</td>
<td>LED 25 mg/kg body weight for 24 h</td>
<td>(17)</td>
</tr>
<tr>
<td>Micronucleus test, lambda/dacZ transgenic mice (Muta ™Mouse), i.p. treatment, peripheral blood reticulocytes in vivo</td>
<td>+</td>
<td>LED 25 mg/kg body weight for 48 h</td>
<td>(42)</td>
</tr>
<tr>
<td>Comet assay, human B-lymphoblastoid MCL-5 cells in vitro</td>
<td>+</td>
<td>LED 0.1 µM/2 h incubation</td>
<td>(41)</td>
</tr>
<tr>
<td>Comet assay, human hepatoma cells (HepG2) in vitro</td>
<td>+</td>
<td>LED 12 nM/24 h incubation</td>
<td>(32)</td>
</tr>
<tr>
<td>Comet assay, human A549 lung cells in vitro</td>
<td>+</td>
<td>LED 20 µM/24 h incubation</td>
<td>(35)</td>
</tr>
<tr>
<td>Tumour formation, F344 rat, is treatment, lung tissue in vivo</td>
<td>+</td>
<td>LED 5 mg/kg body weight once a week for 4 weeks, sacrificed after 18 months</td>
<td>(35,41,43–48,50–55,57)</td>
</tr>
<tr>
<td>DNA adduct formation, various systems in vitro</td>
<td>+</td>
<td></td>
<td>(19,42,47,49,54,56)</td>
</tr>
<tr>
<td>DNA adduct formation, various organs rat and mice in vivo</td>
<td>+</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Including preincubation step.
*Without preincubation step.
*ED, effective dose; LED, lowest effective dose; HID, highest ineffective dose.
*i.p., intraperitoneal; i.t., intratracheal.
*See Table III for details.
Adduct formation in DNA of several organs of female Sprague-Dawley rats given a single oral dose of 3-NBA

Adduct formation in DNA of several organs of female Wistar rats given a single i.p. dose of 3-NBA

Adduct formation in DNA of several organs of male lambdilac/Z transgenic mice (Muta®/Mouse) given multiple doses of 3-NBA

Adduct formation in DNA of several organs of male mice on a C57BL/6 background given i.p. a single dose of 3-NBA

Adduct formation in DNA of several organs of female Sprague-Dawley rats given a single i.t. dose of 3-NBA

Adduct formation in DNA of several organs of female F344 rats given a single i.t. dose of 3-NBA

Independent of the route of administration (oral, intraperitoneal or intratracheal) (Table III) (47,49,56). When adduct formation by 3-NBA in rat liver is compared with that of other mono nitroaromatics administered orally as a single dose, 3-NBA forms about 3-fold more DNA adducts than aristolochic acid, a plant carcinogen, and about 80-fold more than 2-nitrofluorene (47). Essentially the same DNA adducts were observed in human MCL-5 cells and in calf thymus DNA modified in vitro with 3-NBA in the presence of human hepatic microsomes or cytosols (41,51,54) indicating that 3-NBA is activated by human enzymes (see below).

In a carcinogenicity study in F344 rats, squamous cell carcinoma were found in the lungs after 7–9 months in the high dose group (total dose 2.5 mg 3-NBA; 4.5 mg/kg body weight once a week for 5 weeks) and after 10–12 months in the low dose group (total dose 1.5 mg 3-NBA; 5 mg/kg body weight once a week for 3 weeks) (19). In this study, the fraction of squamous cell carcinoma out of the total numbers of tumours observed at the end of the experiment at 18 months corresponded to 3/16 (18.8%) and 11/16 (68.7%) in the low and high dose group, respectively. A single case of adenocarcinoma was observed in each dose group. In addition, a few cases of squamous metaplasia were observed in the lungs in both groups, but not in the controls (19). No tumours were observed in control animals and in any other tissue examined. In a similar animal model using F344 rats a dose-dependent incidence of lung tumours was observed for 1,6-dinitropyrene and benzo(a)pyrene after intratracheal instillation, 1,6-dinitropyrene being the more potent carcinogen (58). However, because of the high acute toxicity of 3-NBA, a direct comparison of the carcinogenicity of 3-NBA and 1,6-dinitropyrene by the same protocol was not possible (19). Repeated administration of a suspended solution of 3-NBA induced severe fatal acute inflammation in the lungs with the consequence that lower doses had to be used. The authors concluded that the carcinogenicity of 3-NBA is similar to, or stronger than, that of 1,6-dinitropyrene (19).

**Table III. DNA adduct formation with 3-nitrobenzanthrone analysed by 32P-postlabelling in rodents in vivo**

<table>
<thead>
<tr>
<th>Details of study</th>
<th>Route*, dose and duration</th>
<th>Total DNA binding (BUT)*</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adduct formation in DNA of several organs of female Sprague-Dawley rats given a single oral dose of 3-NBA</td>
<td>Gavage; 2 mg/kg bw for 4 h</td>
<td>Small intestine (37.6/10^8 nt) &gt; forestomach (33.2/10^8 nt) &gt; glandular stomach (23.6/10^8 nt) &gt; kidney (12.6/10^8 nt) &gt; liver (10.3/10^8 nt) = lung (9.5/10^8 nt)</td>
<td>(47)</td>
</tr>
<tr>
<td>Adduct formation in DNA of several organs of female Wistar rats given a single i.p. dose of 3-NBA</td>
<td>i.p.; 2 mg/kg bw for 24 h</td>
<td>Pancreas (280.5/10^8 nt) &gt; colon (107.4/10^8 nt) &gt; lung (53.3/10^8 nt) = kidney (50.0/10^8 nt) = heart (46.0/10^8 nt) &gt; liver (14.8/10^8 nt)</td>
<td>(49)</td>
</tr>
<tr>
<td>Adduct formation in DNA of several organs of male lambdilac/Z transgenic mice (Muta®/Mouse) given multiple doses of 3-NBA</td>
<td>i.p.; 25 mg/kg bw once a week for 4 weeks</td>
<td>Liver (268.0/10^8 nt) &gt; lung (15.9/10^8 nt)</td>
<td>(42)</td>
</tr>
<tr>
<td>Adduct formation in DNA of several organs of female Sprague-Dawley rats given a single i.t. dose of 3-NBA</td>
<td>i.t.; 0.2 mg/kg bw for 48 h</td>
<td>Pancreas (55.4/10^8 nt) &gt; lung (38.7/10^8 nt) &gt; kidney (33.0/10^8 nt) = heart (26.0/10^8 nt) &gt; small intestine (19.2/10^8 nt) &gt; liver (12.1/10^8 nt) &gt; blood (9.5/10^8 nt)</td>
<td>(56)</td>
</tr>
<tr>
<td>Adduct formation in DNA of several organs of female F344 rats given a single i.t. dose of 3-NBA</td>
<td>i.t. 10 mg/kg bw for 24 h</td>
<td>Pancreas (619.9/10^8 nt) &gt; lung (349.6/10^8 nt) &gt; kidney (334.8/10^8 nt) &gt; heart (220.0/10^8 nt) = urinary bladder (215.2/10^8 nt) &gt; small intestine (98.1/10^8 nt) &gt; liver (59.1/10^8 nt) &gt; blood (41.2/10^6 nt)</td>
<td>(19)</td>
</tr>
<tr>
<td>Adduct formation in DNA of several organs of female F344 rats given a single i.t. dose of 3-NBA</td>
<td>i.t. 10 mg/kg bw for 48 h</td>
<td>Lung (268.8/10^8 nt) &gt; kidney (173.5/10^8 nt) &gt; liver (24.1/10^8 nt)</td>
<td>(57)</td>
</tr>
</tbody>
</table>

* i.p., intraperitoneal; i.t., intratracheal.

*Data using the butanol (BUT) enrichment version of the 32P-postlabelling assay are presented; nt, nucleotides. On TLC 3-NBA induced essentially the same characteristic DNA adduct pattern consisting of a cluster of up to five adducts (spots 1, 2, 3, 4 and 5) (compare Figure 4); adduct spots 1, 3 and 4/5 were identified as dA-dinitropyrene being the more potent carcinogen (58). However, being formed by 3-NBA in the presence of human hepatic microsomes or cytosols (41,51,54) indicating that 3-NBA is activated by human enzymes (see below).

Metabolic activation and DNA adducts formed by 3-nitrobenzanthrone and its metabolites

**Metabolism of 3-nitrobenzanthrone**

Although the metabolism of 3-NBA has not been extensively studied so far, 3-ABA [Note: 3-ABA was evaluated to be a suitable substrate for colouration of microporous polyethylene films, which are widely used to separate liquid mixtures, in particular, in chemical batteries (59,60). Dyes based on 3-ABA are used as disperse dyes for textiles, daylight fluorescent pigments and laser dyes (61–63). Therefore, the industrial and/or laboratory utilization of 3-ABA is another possible source of 3-ABA exposure.] (Figure 2) has been identified as the major metabolite in rat lung alveolar type II cells, rat bronchial epithelial cells (R3/1), in rat mesenchymal cells Rwd009 and human fetal bronchial cells during short-term incubations (6–24 h) with 3-NBA (46). Small amounts of 3-acetylanilinobenzanthrone (3-Ac-ABA; Figure 2) were also observed, most likely being formed by N-acetylation of 3-ABA. Moreover, 3-ABA was found in the urine of underground salt mine workers.
occupationally exposed to diesel emissions (21). No urinary analyses have been conducted in experimental animals.

DNA adduct formation by 3-nitrobenzanthonre and its metabolites

A powerful tool to elucidate the activation pathway of a carcinogen is to characterize and quantify the DNA adducts it forms and to determine what factors either enhance or inhibit adduct formation (64). The most commonly used method to detect DNA adducts is the highly sensitive 32P-postlabelling assay (65) and detection of DNA adduct formation by 3-NBA in vitro and in vivo has been by this assay exclusively.

Nitro-PAHs require metabolism to form reactive electrophilic species in order to exert their genotoxic activity. Two pathways that can activate 3-NBA leading to DNA adduct formation have been proposed (Figure 2). After nitroreduction to N-hydroxy-3-aminobenzanthrone (N-OH-ABA; Figure 2) the first pathway involves the formation of a nitrenium ion yielding non-acetylated DNA adducts. The second pathway proceeds via the formation of N-acetyl-N-hydroxy-3-aminobenzanthrone (N-Ac-N-OH-ABA) and an N-acetyl-nitrenium ion yielding acetylated DNA adducts.

Most of the adducts derived from nitroaromatics and aromatic amines so far reported are deoxyguanosine (dG) adducts with the C8 position of dG linked to the N atom of the amine or nitroaromatic or to a lesser extent with the N2 amino group of dG linked to an aromatic C atom (66,67). The formation of C8- and N'-substituted deoxyadenosine (dA) derivatives.

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![Fig. 2. Proposed pathways of metabolic activation and DNA adduct formation of 3-nitrobenzanthonre. See text for details. Ac = –C(O)CH3; R = –C(O)CH3 or –SO3H. Dotted arrow, unknown in vivo pathway.](image-url)
has also been reported. After reacting the activated ester of N-OH-ABA, N-acetoxy-3-aminobenzanthrone (N-Aco-ABA), with dG, dA or DNA in vitro a variety of DNA adducts were formed and characterized spectroscopically (53,55; T. Takamura-Enya, M. Kawanishi, Y. Nakagawa, T. Watanabe, T. Hirayama, K. Wakabayashi, Y. Hisamatsu and T. Yagi, unpublished data). As shown in Figure 3 three dG adducts have been characterized as N-(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone (dG-C8-N-ABA), 2-(2'-deoxyguanosin-N2-yl)-3-aminobenzanthrone (dG-N2-ABA) and 2-(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone (dG-C8-C2-ABA), and a dA adduct was characterized as 2-(2'-deoxyadenosin-N6-yl)-3-aminobenzanthrone (dA-N6-ABA). In addition, N-(2'-deoxyadenosin-8-yl)-3-aminobenzanthrone (dA-C8-N-ABA) was synthesized and characterized (T. Takamura-Enya, M. Kawanishi, Y. Nakagawa, T. Watanabe, T. Hirayama, K. Wakabayashi, Y. Hisamatsu and T. Yagi, unpublished data). After reacting the activated ester of N-Ac-N-0H-ABA, N-acetoxy-N-acetyl-3-aminobenzanthrone (N-Aco-N-Ac-ABA) with dG, dA or DNA, the same type of adducts in their N-acetylated forms as described above were identified (compare Figure 3; R = Ac (43,57; T. Takamura-Enya, M. Kawanishi, Y. Nakagawa, T. Watanabe, T. Hirayama, K. Wakabayashi, Y. Hisamatsu and T. Yagi, unpublished data). In addition, an unusual 3-Ac-ABA adduct of dA, which involves a double linkage between adenine and BZ (N1 to C1, N6 to C11b), creating a five-membered imidazo-type ring system [assigned dA-(N-Ac-ABA)-1], was formed (Figure 3) (57). Treatment of the latter adduct with alkali was used to generate the corresponding non-acetylated adduct (assigned dA-ABA-1) (Figure 3) (57).

Utilizing thin-layer chromatography (TLC) after 32P-postlabelling, the DNA adduct pattern in rodents in vivo consists of a characteristic cluster of up to four adducts (spots 1, 2, 3 and 6) after enrichment with nuclease P1 digestion, and of a cluster of up to five adducts (spots 1, 2, 3, 4 and 5) after enrichment by butanol extraction (Figure 4) (42,47,49,54,56). The same adducts were identified in human MCL-5 cells treated with 3-NBA (2 mg/kg body weight, i.p.) using the nuclease P1 (A) or butanol (B) enrichment version of the 32P-postlabelling assay [adapted from reference (49)]. Adduct spot 1 = dA-N6-ABA; adduct spot 3 = dG-N2-ABA; adduct 4 = dG-C8-N-ABA (compare Figure 3).
unstable at least under TLC 32P-postlabelling conditions and, therefore, it is not known which spot, adduct spot 4 or 5, represents the dG-C8-N-ABA adduct and which is a degradation product of it (55). We did not detect dG-C8-C2-ABA or dA-ABA-1 in vivo (57). These findings were in part confirmed in a separate study using HPLC 32P-postlabelling (19), 3-ABA, 3-Ac-ABA or N-Ac-N-OH-ABA induce the same adduct pattern than 3-NBA in vivo in rats (49) suggesting that 3-Ac-ABA and N-Ac-N-OH-ABA are readily deacetylated before binding to DNA and that N-OH-ABA is the critical intermediate for the formation of the electrophilic arylnitrenium and rearranged carbocation ions reacting with DNA (Figure 2) (49,54,57). More importantly, although a variety of unusual da and dG adducts were prepared and characterized after reaction with N-Ac-N-Ac-ABA in vitro, these adducts were different to those formed in vitro (47,49,57) indicating that adducts derived from N-Ac-N-Ac-ABA and activation pathways reported earlier based on in vitro synthesis (43,57; T. Takamura-Enya, M. Kawanishi, Y. Nakagawa, T. Watanabe, T. Hirayama, K. Wakabayashi, Y. Hisamatsu and T. Yagi, unpublished data) do not entirely represent the situation in vivo.

Since inhalation is the major route by which airborne materials gain access to the body, primary exposure of the lungs of rats to 3-NBA is the most suitable model system. However, because experimental inhalation studies are difficult to perform, direct intratracheal instillation of 3-NBA has been employed as an alternative exposure procedure to inhalation (19,56). In Sprague-Dawley rats treated with 0.2 or 2 mg/kg body weight for 48 h DNA adduct formation was observed in different organs (lung, liver, kidney, bladder, pancreas, heart and small intestine) and in blood (Table III). Highest total adduct levels were found in lung and pancreas, with ~350 and ~620 adducts per 10^8 nucleotides, respectively, at the higher dose (56). Similar adduct levels (~250 adducts per 10^8 nucleotides) were found in rat lung in a separate study treated with 10 mg/kg body weight 3-NBA by other authors (19). Although adduct patterns are qualitatively similar in target and non-target tissues (19,56), organ-specific DNA adduct formation by 3-NBA correlated well with the mutagenic potency of 3-NBA in a transgenic mouse mutation assay (MutaTM/Mouse; 25 mg/kg body weight once a week for 4 weeks, intraperitoneal administration) (42). In this study, the increase in mutation frequency in the liver cII gene was associated with high levels of DNA binding by 3-NBA in liver DNA, whereas in other tissues such as the lung, in which there was no increase in mutation frequency, a 20–30-fold lower level of DNA binding was observed.

Enzymatic activation of 3-nitrobenzanthrone and its human metabolite 3-aminobenzanthrone

Determining the capability of humans to metabolize the potential human carcinogen 3-NBA and understanding which human enzymes are involved in its activation are important in the assessment of an individual’s susceptibility. Using various enzymatic in vitro bioassays including rat and human hepatic microsomes and cytosols it was demonstrated that the activation of 3-NBA to N-OH-ABA is through nitroreduction catalysed primarily by cytosolic reductases, such as xanthine oxidase and NAD(P)H:quinone oxidoreductase (NQO1) (44,46,47,54). However, microsomal NADPH:cytochrome P450 oxidoreductase (POR) is also able to metabolically activate 3-NBA by simple nitroreduction (51). The role of POR in the activation of 3-NBA in vivo was evaluated by treating hepatic POR-null mice intraperitoneally with 3-NBA (54). It was shown that there was no difference in DNA binding between null and wild-type mice (54), indicating that 3-NBA is predominantly activated in vivo by cytosolic nitroreductases rather than microsomal POR. However, it was demonstrated, using human and rat hepatic microsomes, selective inhibitors and purified enzymes, that the N-oxidation of 3-Ac-ABA is catalysed mainly by cytochrome P450 (CYP) 1A1 and CYP1A2 (70,71). N-OH-ABA, formed either by nitroreduction of 3-NBA or by N-oxidation of 3-ABA, can be conjugated by phase II enzymes such as NATs and SULTs. It was shown that NAT1 and NAT2 as well as SULT1A1 and SULT1A2 can contribute substantially to the bioactivation of 3-NBA and 3-ABA, leading to reactive esters capable of forming DNA adducts (48,50,54). Moreover, recent data indicate that 3-ABA is also activated in vitro by different model peroxi-

Human exposure to 3-NBA is thought to occur primarily via the respiratory tract. Therefore, the finding that CYPs, NATs and SULTs known to be expressed in human lungs (72–76) participate in the metabolic activation of 3-NBA and its metabo-

3-Nitrobenzanthrone a potential human cancer hazard
Human biomonitoring of 3-nitrobenzantrone

Although it is clear that humans are environmentally exposed to nitro-PAHs finding sensitive and selective biomarkers is not a straightforward matter (10). A sensitive method to detect the main metabolite 3-ABA in urine was developed to biomonitor the uptake of 3-NBA (21). Biomonitoring of 18 underground salt mine workers occupationally exposed to diesel exhaust was then performed to determine their internal burden of 3-NBA exposure (21). Personal air sampling also allowed the determination of 3-NBA levels at the workplace (Table I). 3-ABA was detected in the urine at a concentration (1–143 ng/24 h urine) similar to 1-aminoypyrene (2–200 ng/24 h urine), the corresponding metabolite of 1-nitropyrene, the most abundant nitro-PAH detected in diesel exhaust particles (21). It is noteworthy that the excreted amounts of 3-ABA found as a metabolite of 3-NBA were ∼5–10-fold higher than one might expect from the airborne levels of 3-NBA at the workplace (Table I). The authors suggested that 3-NBA may have been incompletely collected by air sampling possibly as a result of decomposition (21), implying that environmental levels of 3-NBA reported thus far may be underestimated. While this is the first study to determine occupational exposure to 3-NBA further studies are needed to investigate urinary 3-ABA levels in occupational and non-occupational exposure settings.

In general, DNA adducts are useful biomarkers to detect exposures to genotoxic compounds (82,83), including exposure to complex environmental mixtures such as those in diesel emissions. Several human biomonitoring studies using the detection of DNA adducts by 32P-postlabelling in lymphocytes have reported higher levels of bulky DNA adducts among subjects heavily exposed to diesel exhaust and ambient air pollution (12–15). Distinct adduct pattern and increased adduct level were found among garage workers occupationally exposed to diesel exhaust when compared with non-exposed controls (12,13). Significantly elevated levels of DNA adducts were also reported in bus maintenance workers exposed to diesel exhaust (14). Moreover, in a meta-analysis of 13 32P-postlabelling studies on occupational cohorts exposed to air pollution a significant association between levels of DNA adducts and air pollution exposure was observed in both heavily exposed industrial workers and less severely exposed urban workers (15). After intratracheal treatment of rats with 3-NBA an overall relationship was observed between DNA adducts in blood and the lung, although adduct levels were 75–90% lower in blood (56). Thus, DNA adducts formed by 3-NBA in lymphocyte DNA may be useful biomarkers of exposure to 3-NBA in individuals exposed to it and may help to assess the effective biological dose. But it remains to be determined whether human blood will be a suitable surrogate tissue for monitoring DNA adducts formed by 3-NBA. It may also be possible to investigate exfoliated urothelial cells in urine for the presence of exposure-related DNA adducts in human fibroblast cell lines after treatment with N-AcO-N-Ac-ABA (88). A plasmid polymerase-stop assay also showed that N-AcO-N-Ac-ABA preferentially bound to guanine residues in the supF gene (88). Despite the striking similarities of this in vitro study to the in vivo data it is important to point out that none of the guanine adducts formed by 3-NBA in vivo carries an N-acetyl group. In this context, it is noteworthy that the major mutations in the lacI gene induced by diesel exhaust in lung DNA of Big Blue rats are AT→GC and GC→AT transitions (89). Therefore, as reported previously for dinitropyrenes (90), based on the present data there is no direct evidence for a contribution of 3-NBA to the mutagenicity of diesel exhaust. Although 3-NBA contributes strongly to the mutagenic activity of environmental extracts (38,39) future site-specific mutagenesis studies may provide new insights into the mutagenic activity of individual DNA adducts formed by 3-NBA.

Although it is clear that 3-NBA can dissociate from particles and become bioavailable (21), the particles themselves are thought to impact on genotoxicity as well as on cell proliferation via their ability to generate reactive oxygen species (ROS) (91). Hence, exposure to particulate matter might increase the genotoxic potential of 3-NBA. Not only can exposure to particles lead to increased levels of ROS in pulmonary tissue (91) but also 3-NBA and/or its metabolites could catalyse the generation of ROS. Indeed, increased levels of ROS were observed in vitro in human A549 lung cells after exposure to 3-NBA (35,92). Tumour development by 3-NBA in lungs may be influenced by promotional pressures like ROS in initiated cells and not only by the initial levels of DNA adducts formed by it. The most important ROS generating system in the lung is the pool of inflammatory phagocytes such as alveolar macrophages (91). Indeed, severe acute inflammation of the lung parenchyma was found in rats after intratracheal instillation of 3-NBA (19). Moreover, the formation of radicals, e.g. during metabolic activation of 3-NBA and its metabolites, may also be important in the process of tumour promotion (71). However, the exact role of ROS and particle-related effects as promotional pressures on 3-NBA-initiated genotoxicity and/or tumourigenesis remain to be explored.

Although abundant evidence suggests that 3-NBA initiates the first critical steps of chemically induced carcinogenesis, little information is yet available on the subsequent steps that lead to preneoplastic and neoplastic changes. A postulated mechanism for the carcinogenicity of 3-NBA in rodents and
humans is summarized in Figure 5. Proto-oncogenes have been identified as genetic targets that are involved in chemical carcinogenesis (93). In rodents, many chemical carcinogens activate the \( \text{ras} \) proto-oncogene by single point mutations in codons 12, 13 or 61 (94). Although, to date, we can only speculate on hotspot mutations in 3-NBA-induced tumours, future studies should focus on mutational hotspots found in those tumours and the activation of oncogenes. In some cases it has been possible to causally link sequence-specific DNA adduct formation of a carcinogen and human tumour mutations to distinct environmental exposure (95–97). As the presumed guardian of the genome, \( p53 \) is one of the most commonly mutated genes observed in human tumours and as it is mutated in over 50% of all human cancers (98). A recently developed test system uses human \( p53 \) knock-in (Hupki) mice to generate and select dysfunctional human \( p53 \) mutations experimentally (99–101). It is anticipated that such a model could explore possible links between environmental exposure to 3-NBA and mutation spectra on tumours associated with urban air pollution collected in the human tumour TP53 mutation database (102).

Conclusions

There is increasing evidence that the urban air pollutant 3-NBA is a potent mutagen and animal carcinogen, and is therefore a suspected human carcinogen. There is now a need for more mechanistic studies that relate 3-NBA to processes that orchestrate tumour development, including DNA damage, DNA repair, apoptosis, mutagenesis and proliferation as well as the ability of particles to promote 3-NBA genotoxicity. Because of its presence in diesel exhaust and ambient air pollution, exposure to 3-NBA may represent a health hazard for certain occupations and for large sections of the general population. However, for accurate risk assessment further epidemiological studies on 3-NBA-exposed individuals and a broader monitoring of environmental levels of 3-NBA are required.

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References

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