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DNA lesions and carcinogenicity from the urban air pollutants 2- and 3-nitrobenzanthrone

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“KI TUDJA MERRE VISZ A VÉGZET,
GÖRÖNGYÖS ÚTON, SÖTÉT ÉJJELEN…”

To my family

“Who knows where fate will guide us, on a long winding path in dark night…”
(First line in the Seclar’s national anthem)

1. Abstract

In the early 1990's, Japanese doctors in urban areas discovered an increasing incidence of lung cancer among women, which was not attributed to a change in diet or smoking. This led to a growing investigation of urban air, and subsequently the discovery of 3-nitrobenzanthrone (3-NBA), a pollutant originating from diesel emissions. 3-NBA was isolated from the organic fraction of particulate matter and immediately qualified to be among the most mutagenic substances known. More over, it has the ability to rearrange into its isomer 2-NBA once it is emitted into the atmosphere. Although 2-NBA is not as genotoxic as 3-NBA, it exists in a 70-fold higher concentration, which urges for continuing investigations on its contribution to human health hazard. Extensive *in vitro* tests have been conducted to gain information about the mechanisms behind the activation of 3-NBA, and recently of 2-NBA as well. The results revealed that reactive intermediates of 3-NBA are formed through nitro-reduction, which are potent DNA damaging agents by covalently attaching to the nucleotides – the building blocks of DNA. This covalent binding between the nucleotides and a foreign substance is referred to as DNA addition products, or DNA adducts.

Different methods can be used to examine DNA adducts, and the one used in the studies in this thesis is ^{32}P-HPLC. This highly sensitive and reproducible method employs radioactive postlabelling to detect DNA adducts down to about 0.5 DNA adducts/10^8 normal nucleotides. In addition, since 3-NBA and its isomer 2-NBA belong to a group of substances called quinones, they also possess the potential to induce oxidative damage in cells. Oxidative lesions in DNA can be measured by a very sensitive method called Single cell gel electrophoresis (SCGE or Comet assay), which has been used to measure the level of damage and compared them between these substances and some of their metabolites, *in vitro* as well as *in vivo*.

The results in paper I and II, presented in this thesis, show that 3-NBA in cell cultures is more genotoxic compared to its isomer 2-NBA, metabolite 3-aminobenzanthrone (3-ABA), and parent compound benzanthrone (BA), regarding DNA adduct formation. On the other hand, 3-ABA and BA were equally potent in inducing oxidative damage, whereas 2-NBA did so the least. This was then also investigated *in vivo*. Paper II showed that DNA adduct data for 3-NBA was comparable *in vitro* and *in vivo*, but this was not true for 2-NBA. Further, the genotoxic potential of 2-NBA was not as low *in vivo* as *in vitro* compared to 3-NBA. In paper III, 3-NBA was also shown to induce massive acute toxic effects, unusual for nitro-PAHs at the levels used in these experiments. The most prominent DNA adducts formed *in vivo* were characterised using synthesised standards. Finally, the carcinogenic effect of 3-NBA was shown in paper IV, where female rats developed squamous cell carcinomas in the lungs after intratracheal administration.

The conclusions are that both 3-NBA and its isomer 2-NBA are health hazardous substances, and it is worth devoting more effort into evaluating their potential to harm humans, especially since the metabolite 3-ABA has been detected in humans occupationally exposed to diesel emission, such as mining workers.

2. Index

3. Publications

I **E. Nagy**, C. Johansson, M. Zeisig and L. Möller, Oxidative stress and DNA damage caused by the urban air pollutant 3-NBA and its isomer 2-NBA in human lung cells analyzed with three independent methods, Journal of Chromatography B 827 (2005) 94-103.

II **E. Nagy**, S. Adachi, T. Takamura-Enya, M. Zeisig and L. Möller, DNA adduct formation and oxidative stress from the carcinogenic urban air pollutant 3-nitrobenzanthrone and its isomer 2-nitrobenzanthrone, *in vitro* and *in vivo*, Mutagenesis, in press (2006).

III **E. Nagy**, S. Adachi, T. Takamura-Enya, M. Zeisig and L. Möller, DNA damage and acute toxicity caused by the urban air pollutant 3-nitrobenzanthrone in rats: characterization of DNA adducts in eight different tissues and organs with synthesized standards, Environmental Molecular Mutagenesis. 47 (2006) 541-552.

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

Additional publications – not related to the thesis

P. Jaloszynski, P. Jaruga, R. Olinski, W. Biczysko, W. Szyfter, **E. Nagy**, L. Möller and K. Szyfter, Oxidative DNA base modifications and polycyclic aromatic hydrocarbon DNA adducts in squamous cell carcinoma of larynx, Free Radical Research 37 (2003) 231-240.

E. Nagy, U.G. Norén, M. Zeisig, L.G. Ekström and L. Möller, DNA adduct formation and physiological effects from crude oil distillate and its derived base oil in isolated, perfused rat liver, Archives of Toxicology 78 (2004) 114-121.

4. Abbreviations

2-NBA: 2-nitro-7H-benz[d,e]anthracen-7-one, or 2-nitrobenzanthrone

3-NBA: 3-nitro-7H-benz[d,e]anthracen-7-one, or 3-nitrobenzanthrone

^{32}P-ATP: [gamma]32P-adenosine triphosphate

8-oxodG: 8-oxo-deoxyguanine

A549: Human lungcarcinoma cells

AAF: 2-acetylaminofluorene

AhR: Aryl hydrocarbon receptor

ALS: Alkali labile sites

AMS: Accelerated masspectrometry

BA: 7H-benz[d,e]anthracen-7-one, or Benzanthrone

BaP: Benzo[a]pyrene

BER: Base excision repair

BPDE: Benzo[a]pyrene-diol-epoxide

BuOH: Butanol

CO_X: Carbon oxides

CYP: Cytochrome P450

dA: deoxyadenosine

DE: Diesel exhaust

DEPs: Diesel exhaust particle(s)

dG: deoxyguanosine

DNA adducts: DNA addition products

DNPy: Dinitropyrene(s)

EtBr: Etidium bromide

EtOH: Ethanol

FPG: Formamidopyrimidine-DNA glycosylase

GI-tract: Gastrointestinal tract

H_2O_2: Hydrogen peroxide

HEC: Human equivalent concetration

HepG2: Human hepatocarcinoma cells

HPLC: High performance liquid chromatography

HR: Homologous recombination

ip: intraperitoneal

it: intratracheal

LOAEL: lowest observed adverse effect level

LOD: Limit of detection

MCL-5: Human lymphoblastoid cells

N-Ac-N-OH-ABA: N-acetyl-N-hydroxy-3-aminobenzanthrone

NAT: N-acetyltransferase

NER: Nucleotide excision repair

NhEJ: Non-homologous end joining

Nitro-PAH: Nitrated polycyclic aromatic hydrocarbon(s)

NOAEL: no observed adverse effect level

N-OH-ABA: N-hydroxy-3-aminobenzanthrone

NO_X: Nitrogen oxides

NPy: Nitropyrene(s)

NQO1: NAD(P)H:quinone oxidoreducatse

NuP1: Nuclease P1

O_3: Ozone

PAH: Polycyclic aromatic hydrocarbon(s)

PBPK: Physiologically based pharmacokinetic modelling

PM: Particulate matter

POR: NADPH:P450 oxidoreductase

RNS: Reactive nitrogen species

ROS: Reactive oxygen species

SCGE: Single cell gel electrophoresis, a.k.a. Comet assay

SO_X: Sulphur oxides

SSB/DSB: Single/Double strand break

TLC: Thin layer chromatography

XO: Xanthine oxidase

5. Introduction

5.1. The diversity of Black Gold: from fossil fuel to air pollution

Environmental pollution is becoming an important public health problem, due to increasing world-wide urbanisation and increasing consumption of fossil fuels [1]. Studies have shown that the fossil cumulative energy demand correlates well with many negative environmental impact categories, such as global warming, resource depletion, acidification, eutrophication*, tropospheric ozone formation, stratospheric ozone depletion, and human toxicity [2]. Several epidemiological studies have pointed out the significant impact of fossil fuel usage, which results in both economical and human health problems [3,4]. The reason to the wide spread of petroleum products in our environment is the diverse applicability of the different fractions collected from crude oil (Figure 1).

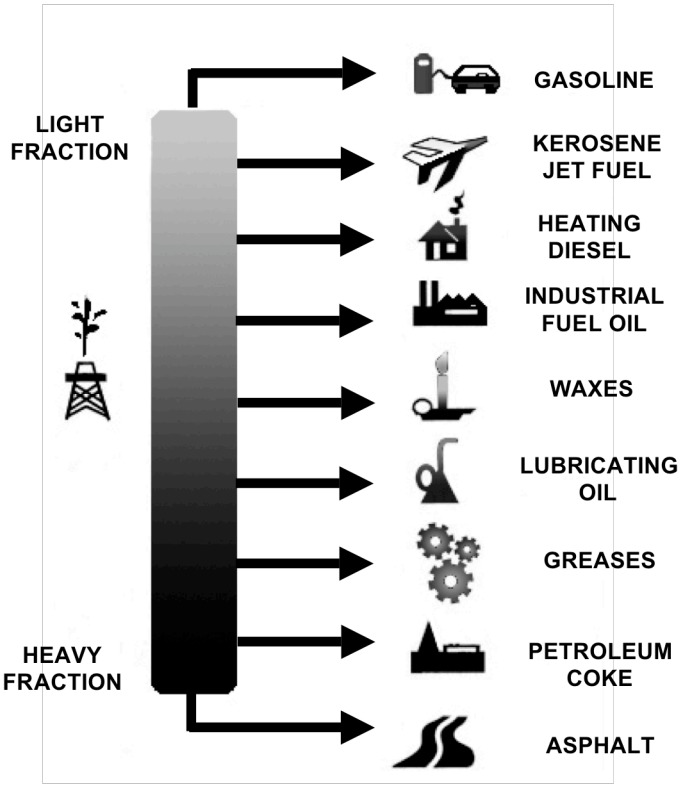


Figure 1. Crude oil refinement and the use of different fractions.
The fractions collected from crude oil are easily recognized in the gasoline we use to fuel cars and the heating oil for our homes, but less obvious are the uses of petroleum-based components of waxes, plastics, medicines, food items, and a host of other products.
Diesel is a heavier fraction then gasoline, hence it contains larger aromatic compounds and is distilled at higher temperatures. The heaviest fraction is known as bitumen and is used for road-paving processes.

* Natural eutrophication is the process by which lakes gradually age and become more productive. Cultural or anthropogenic "eutrophication" is water pollution caused by excessive plant nutrients.

Studies on crude oil and petroleum-derived products have shown that factors, apart from the source of the crude oil, such as addition of chemicals and the temperature at which the petroleum fractions are generated, greatly determine the extent of their harmfulness with respect to toxicity, mutagenicity and carcinogenicity [5,6]. As one example, heavy crude oil distillate with a 50% boiling point at 431°C, has been shown to cause bulky DNA adducts (reaction products with DNA) and DNA adduct clusters when analysed by ^{32}P-HPLC, which also correlated with mutagenicity [7]. In a liver perfusion study, the distillate was compared to a hydro-treated and saturated derivative, where the former gave rise to DNA adducts and adduct clusters, while the latter did not [8].

5.1.1. Diesel and from it derived pollutants

Diesel is fractionated from crude oil at higher temperatures than gasoline, 150-390°C compared to 20-200°C, and hence it contains more three- to four-ring aromatic compounds. Both diesel fuel and gasoline is classified by IARC as Class 2B* mixtures, whereas diesel exhaust (DE) is classified as Class 2A [9]. In contrast to gasoline exhaust, DE is also one of the major contributors to particulate matter pollution, accounting for up to 90% of the ultra-fine particulate mass (diameters below 0.1 μm) in the ambient air of many major cities of the world [10]. These diesel exhaust particles (DEPs) contain a carbonaceous core onto which combustion products are adsorbed, of which 18,000 have been characterized so far [11,12].

The soluble organic fraction on the particles, i.e. polycyclic aromatic hydrocarbons (PAHs) and their derivates, could be generated through incomplete combustion from the combustion chamber of a car, where they can also change character before emission due to processes that take place in the presence of oxygen, nitrogen and sulphur at very high temperatures. Thus the source of PAHs and particles in the emission is from the fuel, lubricating oil and fuel additives; most likely all these combine together [13,14]. Once emitted into the atmosphere, these substances are subjected to photochemical reactions, which can alter their properties and characteristics and make them potentially more hazardous [15,16].

* Class 1, known human carcinogen; Class 2A, probable and 2B, possible human carcinogen; Class 3, not classifiable for human carcinogenicity; Class 4, probably not human carcinogen.

Important factors that determine the toxicity of DEPs that are inhaled include particle deposition, clearance, retention, translocation and dissolution within the different regions of the lung. Studies conducted in an effort to evaluate the effects of DEPs on humans have suggested that exposure to DEPs may be associated with increased exacerbations of asthma, chronic bronchitis, respiratory tract infections, ischemic heart disease, stroke and different allergies [17-20]. The adverse effects of DEPs may in part be due to the variety of ways it can act upon an *in vivo* system and promote inflammation [21]. First, DEPs may act as carriers for several allergens such as Can f1 (dog), Fel d1 (cat), Der p1 (mite) and Bet v1 (birch pollen) [22-24]. Second, DEPs have been shown to have an adjuvant activity [25,26] and third, DEPs can also alter many downstream immunologic mechanisms [27,28].

Since particulate matter can act as carriers of both allergens and high molecular weight substances, investigations of ambient air has been conducted to identify some of the molecules adsorbed on the surface of DEPs. One study reports the findings of at least twelve PAHs in particulate matter fractions with the highest PAH load in the smallest fractions [29].

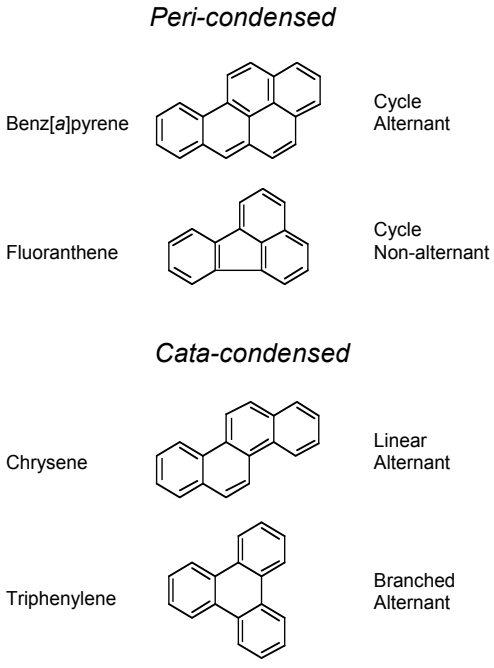


Figure 2. Structures of PAHs. Reproduced with permission from [30].

5.1.2. Polycyclic Aromatic Hydrocarbons (PAHs)

PAHs are generally divided into two categories based on their structures; these are called *peri*- or *cata*-condensed (Figure 2). Peri-condensed structures have the characteristic feature that they can form cycles and can further be subdivided into two classes; *alternants*, which are formed exclusively by six-membered rings, and *non-alternants*, that include some five-membered rings. Cata-condensed systems are always alternant and do not form cycles. They can, however, be branched or linear (non-

branched), of which the former is thermodynamically more stable and chemically less reactive then the latter [30].

The structure of single PAHs, such as carbon atom positions or structure regions, can also be divided into several categories, which in turn determine the reactivity of the substance itself [31]. This is illustrated by Benzo[*a*]pyrene (BaP) and Dibenzo[*a,l*]pyrene in Figure 3. The 'K'-region is defined as the external corner of the molecule, whereas the 'Bay'-region is an open inner corner. The 'M'-region is also referred to as distal Bay-region [30]. The 'Fjord'-region is a sterically hindered aromatic region and is of great interest because of its significantly greater tumourgenic activity compared to the planar Bay-region derivatives [32,33]. BaP can form a Bay-region diol-epoxide, and analogous diol-epoxides are now recognized as tumourgenic metabolites of numerous PAHs.

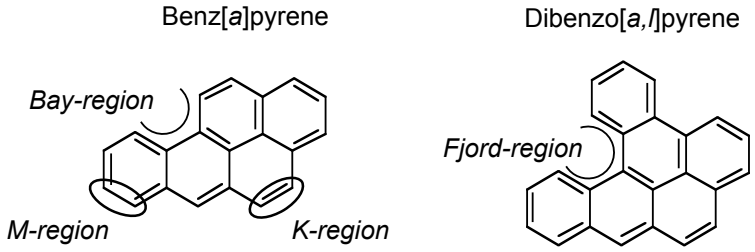


Figure 3. Structure regions of PAHs represented by Benzo[*a*]pyrene (BaP) and Dibenzo[*a,l*]pyrene. Reproduced with permission from [30].

PAHs belong to the group of contaminants that can accumulate to toxic levels in the body within a short period of time and have been implicated of being causative agents of lung [34], breast [35], esophageal [36], pancreatic [37], gastric [38], colorectal [39], bladder, skin [40], prostate [41] and cervical [42] cancers in humans.

5.1.3. Nitrated Polycyclic Aromatic Hydrocarbons (Nitro-PAHs)

It is not only the structure of the PAH molecule that greatly influences the harmfulness of the compound, but also the position and orientation of such functional groups as amino-, nitro-, halogen-, hydroxy- or alkyl-groups. For instance, studies have shown that dinitropyrenes (DNPy) are more genotoxic than nitropyrenes (NPy) [43], and that there is a structure-activity dependent hierarchy where 4-NPy is more carcinogenic than 1-NPy and additionally, 1,6- and 1,8-DNPy are more potent than 1-NPy in inducing malignant fibrous histiocytomas and leukaemia in rats [44].

These NPys and DNPys belong to a category of compounds referred to as *nitrated polycyclic aromatic hydrocarbons* (nitro-PAHs). Studies have shown that diesel engine exhaust can exert genotoxicity through oxidative processes, as well as many mono- and dinitro-PAHs [45,46]. Nitro-PAHs exist in the particulate matters emitted from diesel engines and on the surface of airborne particles, and are likely to be formed during the combustion process of fossil fuels [13] as well as via the reaction of parent hydrocarbons with nitrogen oxides in ambient air [47,48]. The nitro-PAHs are activated mainly through enzyme-catalysed reduction of the nitro group and further metabolised by different enzymes [49]. Nitro-PAHs have caused environmental concern because of their potent mutagenic activity and carcinogenicity [50]. One such substance, 3-nitrobenzanthrone, has gained much interest in recent years due to its many health hazardous properties, as discussed in the sections below [51].

5.2. PAHs and Nitro-PAHs in the environment

5.2.1. Dispersal in the environment

PAHs and nitro-PAHs can spread in the environment not only through wind and accidents, such as oil-spills at sea, but also through wet and dry depositions and diagenesis* in marine sediments, which contaminates the soil. After emission, the pollutants diffuse rapidly through the lower region of the atmosphere, called the boundary region, where they are mixed and advected by winds. In addition, cloud dynamics may lift pollutants from the boundary layer and leave them at higher levels after cloud dissipation. The pollutants can then be subjected to strong winds, which increase the potential for long-distance transportation [52]. Indeed, PAHs have been found as far away as in the Arctic regions. In the bottom sediments of different parts of the Kara Sea in northern Russia, PAHs of various origin (petrogenic, biogenic and pyrogenic) have been detected in concentrations ranging 77-537 ng/g dry weight [53]. Two- to three-ring aromatic hydrocarbons dominated in large parts of the Kara sediments, indicating petrogenic origin, whereas in the south-western part of the sea,

* The physical, chemical or biological alteration of sediments into sedimentary rock at relatively low temperatures and pressures that can result in changes to the rock's original mineralogy and texture.

four- to six-ring aromatic hydrocarbons of pyrogenic origin were the most prominent. The pollution in these areas of the Kara Sea are due to the industrial enterprises situated some 500 km up-stream from the river mouth.

5.2.2. Removal from the environment

Removal of PAHs from the atmosphere happens through several mechanisms, but the most efficient seems to be that of wet deposition or precipitation scavenging. Dry deposition has been calculated to account for a removal rate of only 3% per day. Wet deposition is the transfer of a substance from the atmosphere to the ground within or on the surface of a hydrometeor (snow, hail or rain), whereas dry deposition is the direct transfer to or absorption of gases and particles by natural surfaces (vegetation, soil, water or snow) [54].

Removal of PAHs in soil is mainly done through microbiological degradation. This factor in turn is dependent on or limited by temperature, pH, redox conditions, concentration of inorganic nutrients and metals [55].

5.3. The Benzanthrone family and their bundles of joy

5.3.1. Benzanthrone, a colourful parent

7H-benz[*d,e*]anthracen-7-one (Figure 4), or Benzanthrone (BA), is used in vat dyes (e.g. Yellow 4, Green 1), and serves also as an intermediate to combine with other dyes. Epidemiological studies indicate that skin, respiratory, gastrointestinal, genitourinary, nervous and hemopoetic systems are affected by BA exposure [56,57]. BA can also cause photosensitization [58] and many of its substituted analogues are also health hazardous [59,60].

Experimental results indicate that toxicity caused by benzanthrone-derived dyes or dye intermediates appears to be influenced by the number of carbonyl and amino-anthraquinone groups as well as by the presence of functional groups like halogen, nitro, hydroxy and methoxy attached to the parent molecule, benzanthrone [59].

Though most of these benzanthrone dyes are produced through synthesis, there are experiments suggesting that modification of parent compound could occur under certain atmospheric conditions [48]. For instance, nitro-derivatives can be formed by the hydroxyl radical-initiated reactions in the presence of ozone (O_3), nitrate radical-initiated reactions and photolysis [61]. In addition, rearrangements in the atmosphere can also give rise to new isomers [62].

5.3.2. 3-nitrobenzanthrone, a talented offspring

3-nitro-*7H*-benz[*d,e*]anthracen-7-one, or 3-nitrobenzanthrone (3-NBA), is a derivative of BA (Figure 4). 3-NBA was isolated from diesel exhaust and airborne particles, and was characterised both as a potent direct-acting mutagen in bacterial mutagenicity tests and as an inducer of micronuclei in mouse peripheral reticulocytes [51].

The mutagenicity of 3-NBA in the Ames *Salmonella typhimurium* assay was surprisingly high with 208,000±22,000 revertants/nmol in TA98 and 29,700±2,600 revertants/nmol in TA100. This is comparable to 1,8-DNPy, the most mutagenic substance in the same assay, displaying 257,000±2,873 revertants/nmol in TA98 and 55,400 (standard deviation not reported) revertants/nmol in TA100 [51].

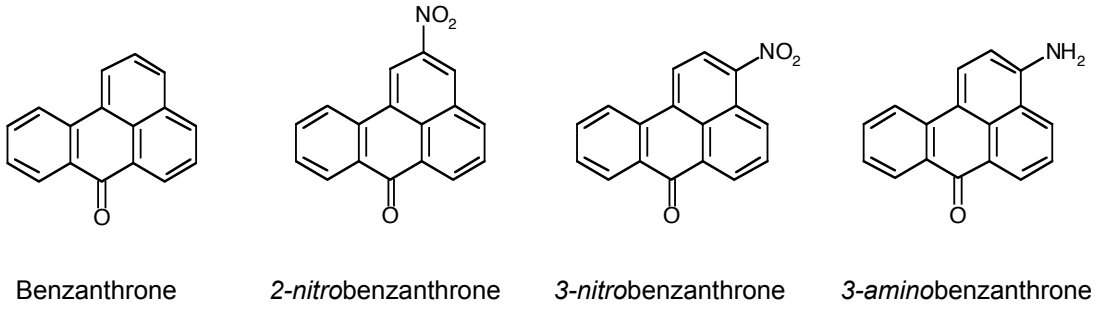
5.3.3. 3-aminobenzanthrone, a versatile progeny

3-NBA metabolises into 3-aminobenzanthrone (3-ABA, Figure 4), which has been detected in occupationally exposed humans [63]. In addition, 3-ABA is used industrially for colouration of microporous polyethylene films, which are widely used to separate liquid mixtures, in particular, in chemical batteries [64,65]. In addition, the bright colour of 3-ABA also makes it suitable as a dye for textiles, daylight fluorescent pigments and laser dyes [66-68].

5.3.4. 2-nitrobenzanthrone, a potential cousin

The level of 3-NBA is highest near the source of emission, and relatively low in the general ambient air due to extensive atmospheric rearrangements to the isomer 2-NBA (Figure 4) [62,69].

In fact, 2-NBA is about 70-fold more abundant in ambient air compared to 3-NBA [62]. *In vitro* studies have shown that in human lung and hepatocyte cell lines, 2-NBA is about 30-50% as genotoxic compared to 3-NBA and is capable of forming not only DNA lesions but also inducing oxidative stress [70,71]. The amount of DNA lesions produced by 2-NBA is not as high as those formed due to 3-NBA exposure, but 2-NBA is more lipophilic (octanol/water partition coefficient Kow 3.90 and 3.99 for 3- and 2-NBA, respectively), hence it may be metabolized more slowly and thereby subjecting the body to reactive metabolites for a longer period of time compared to 3-NBA [72].



Benzanthrone *2-nitro*benzanthrone *3-nitro*benzanthrone *3-amino*benzanthrone

Figure 4. The Benzanthrone family. Benzanthrone (BA) is the parent compound, whilst 2- and 3-nitrobenzanthrone (2- and 3-NBA) are nitrated isomers. 3-aminobenzanthrone is a metabolite of 3-NBA that has been detected in humans after occupational exposure.

5.4. The presence of 3-NBA in the environment

5.4.1. 3-NBA in the soil and sediments

Soil samples from several areas in Japan (Kanto, Chubu and Kinki) were extracted to enable mutagenicity testing with the Ames *Salmonella* assay. The concentration of 1,3-, 1,6- and 1,8-DNPy was found in the range of 12-3,270, 14-5,587 and 13-6,809 pg/g soil for the three isomers, respectively [43]. Surface soil collected from six sites in the Chubu central area was also investigated for the presence of 3-NBA. The levels obtained were in the range of 1.2-1,020 pg/g soil, which is well within the range of the dinitropyrenes (DNPys) [73].

5.4.2. 3-NBA in water and runoff

At a residential area in Kyoto, the concentration of 1-nitropyrene (1-NPy) has been determined in the range of 5.6-190 pg/L, whereas 3-NBA was present at 70-300 pg/L in rainwater [74]. In a different study, also from Japan, 1-NPy was determined in precipitation, river and seawater. The concentration was in the range of 370-3,200 pg/L in precipitation, 1-27 pg/L in river and 0.2-0.5 pg/L in seawater. For 1,3-, 1,6- and 1,8-DNPy the concentration ranged from 4.9-4,900 pg/L in precipitation [75]. Once again, 3-NBA is rather close to and comparable with the DNPys.

5.4.3. 3-NBA in air and particles

3-NBA has been found in diesel particulate in Tokyo in the concentration range of 0.6-6.6 μg/g, which was comparable to 1.8-DNPy (0.3-5.2 μg/g) in that same area [51]. In another study, several standard reference materials (SRMs) collected from different American cities, were compared to particulate matter ($PM_{2.5}$) from Baltimore [76]. The levels of 1-NPy and 1,3-, 1,6- and 1,8-DNPy for the standards ranged 72-39,640, <2-1146, <4-2,543 and <2-3,580 ng/g particulate, respectively. These values are within the range of what was detected in Japan.

Engine manufacturers have made good progress in reducing the PM emission from diesel engines. For instance, the average emission from different BMW production cars, has been reduced from 0.37 g/km to 0.05 g/km between 1985-1999. But it is not only the car engine that is important. A tax-subsidized diesel fuel used in Sweden (Urban Diesel EC1) has been compared to a European reference fuel (FTP 75). The results clearly showed a reduction of PAHs by 60 %, carbon monoxide by 44%, particulate matter by 27 % and carbon dioxide by 1.5%, when using EC1. The amount of 1-NPy, 1,3-, 1,6-, and 1,8-DNPys and 3-NBA produced by the urban diesel EC1 was approximately 0.01, 0.0010, 0.0010, 0.003 and 0.002 μg/km, respectively, which was at least 50% lower compared to the reference fuel [77]. A similar study showed that when the exhaust was directly collected from three diesel vehicles operating at 80 km/h, 3-NBA was found in the particulate matter in the concentration range of 27-56 ng/g extract [78]. In ambient particles, however, in Salt Lake City, only trace amounts of 3-NBA have been found [14].

When examining the concentration in ambient air in Concorde, California, 3-NBA was present in 0.4 pg/m^3. The concentration of the isomer 2-NBA was, however, 70-fold higher [62]. The standard diesel particle extract NIST/SRM 1975 contained more 3-NBA as compared to what was observed in ambient air. This might point to that 3-NBA shifts to 2-NBA in the presence of nitrogen oxides (NO_X) and O_3 [61,62].

Other measurements of urban air from Denmark investigated the photostability of 3-NBA and the conclusion was made that formation of 3-NBA in air is rather scarce. Moreover, the photolysis of 3-NBA was found to be somewhat smaller than for 1-NPy [15]. For 1-NPy, the major degradation pathway is believed to be photolysis and the half-life is a question of a few hours in the presence of NO_X and O_3 [79]. Photolysis of many nitro-compounds is dependent on the orientation of the nitro-group [80]. Therefore, the major source for both 3-NBA and 1-NPy is most likely the combustion processes where they form, and it is near these emission sources that the health effects should be considered. For instance, the concentration of 3-NBA at the sampling site of Risø in Denmark, was 9.8$\pm$4.2 pg/m^3 [15], whereas high levels (up to 80 pg/m^3) have been observed in underground salt mining work-places where people are subjected to large amounts of diesel exhaust almost every day [63].

In Kaohsiung City, an industrial district in Taiwan, the concentrations of 1-nitropyrene as well as 1,3-, 1,6- and 1,8-DNPy, were determined to be in the range of non-detectable (ND)-0.21, ND-0.055, ND-0.072 and ND-0.084 ng/m^3, respectively [81]. In Nagasaki city, the concentration of BaP was found in the range of 0.04-1.29 ng/m^3, while 1-NPy, 1,3-, 1,6- and 1,8-DNPys were present in the ranges of 0.21-2.87, 0.0052-0.38, 0.0065-0.41 and 0.0040-0.44 pg/m^3, respectively [82]. When examining the atmospheric concentration of 1-, 2-, 3- and 10-NBA in Kanawaza city (Osaka region) the following levels were obtained: ND, 503 pg/ m^3, 6.8 pg/ m^3 and ND, respectively. As mentioned before, the concentration of 2-NBA is higher in ambient air where 3-NBA is subjected to the atmospheric environment. Nonetheless, 3-NBA can accumulate to concentrations comparable to that of 1-NPy and the three DNPy isomers.

One can question whether it is relevant to study 3-NBA, since e.g. BaP is present at concentrations about 1000-fold higher in urban air. In a study from Copenhagen, BaP has shown to be present in concentrations of 1.4 ng/m^3 and 4.4 ng/m^3 in a city park

and busy street, respectively, and the current annual mean concentration in major European urban areas are in the range of 1-10 ng/m^3 [83]. In addition, exposure to BaP via inhalation deposits about 10-50 ng/day into the individual, whereas dietary exposure is in the magnitude of 2-500 ng/day [84]. If one assumes a daily intake of 1 µg/m^3 of particles, then the inhalation of 3-NBA would be approximately 90 pg/day, considering a breath intake of 15 m^3/day [85]. Nonetheless, when the genotoxicity of 3-NBA was compared to that of BaP, a 1,000-2,000-fold higher potency was observed for 3-NBA in a micronucleus assay. Moreover, statistically significant damages, compared to the negative DMSO control, were observed for 3-NBA-treated cells at concentrations of 12 nM [85]. Thus, even if 3-NBA is in much lower concentrations in air than BaP, its immense genotoxic potency urges for further studies and evaluations of its potential as an environmental hazard.

Text on the car: “Methanol for the sake of the environment”. Second place winner at the Swedish Touring Car Competition 2006. © Eszter Nagy

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6. Aims

6.1. General aims

The Papers I-IV in this thesis are focusing on the systemic consequences following exposure by different routes. A major contribution is the revealing of the severe acute toxicity and carcinogenicity of 3-NBA for the understanding of its toxicity on a macroscopic level.

The aim of this thesis is to present the background, the toxic potential and the present knowledge about the urban air pollutant 3-NBA, which is a suspected human carcinogen and yet to be classified by IARC. Several *in vitro* studies have opened the path to understanding the complex mechanisms behind the metabolism of 3-NBA in cell lines. However, cells are incomplete systems and may be insufficient for evaluating the complexity of substance behaviour in biological systems. For this reason, *in vivo* studies, like those presented in this thesis, are needed to provide data on substance kinetics, short-term as well long-term effects. The experiments conducted in the papers, involve mainly ingestion and intratracheal instillation in animals. All these methods and the reasons to use them are explained in later sections, along with comparisons to other methods.

6.2. Specific aims

Paper I: To evaluate and compare the toxicity of 2- and 3-NBA *in vitro*, using different analytical methods.

Paper II: To compare the DNA adduct forming capacity as well as induction of oxidative stress induced by 2- and 3-NBA *in vitro* as well as *in vivo.*

Paper III: To evaluate the DNA adduct forming capacity and acute toxicity of 3-NBA *in vivo* after ingestion, and the characterisation of DNA adducts in different tissues using pre-synthesised standards.

Paper IV: To evaluate the short-term genotoxicity effect and to characterise the main DNA adducts in question. In addition, to assess the potential of 3-NBA to form tumours in rats after intratracheal instillation.

7. Carcinogenesis

Carcinogenesis is a multi-step process as depicted in Figure 5. The scheme is simplified and not all pathways are accounted for. DNA lesions and mutations are usually considered as initiation steps, whereas as clonal expansion is the promoting step and transformation to malignant cells is the progression. A single damage is most likely not enough to cause a cell to become malignant. Several damaging steps and/or failure in repair mechanisms have to occur for the disease develops.

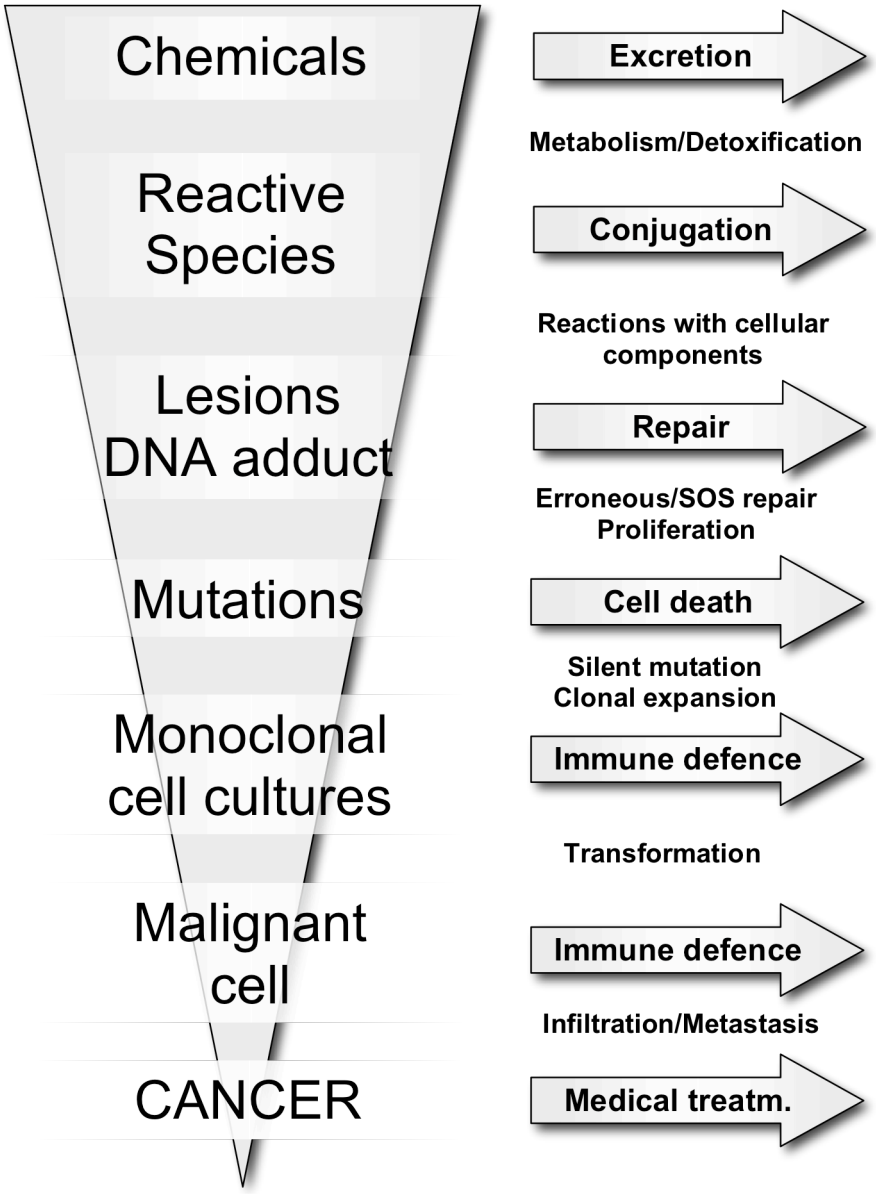


Figure 5. The multi-step process of carcinogenesis. There are many mechanisms guarding the cells from becoming malignant if they are damaged. Sometimes these mechanisms can fail and the disease may develop.

7.1. Exposure

Exposure is the first step in any hierarchy of biological reactions that may take place. PAHs can enter the body through digestion, inhalation and skin contact [86], and from mother to foetus [87]. In the following sections focus will be on exposures through ingestion and inhalation. Although the skin is the largest organ of the human body, it is rather impermeable to many substances and shields the body from an external hostile environment, whereas substances, which are inhaled or ingested, come in direct contact with the internal environment of the body.

7.1.1. Ingestion

One of the main routes to PAH-exposure is through ingestion. The dietary exposure route deposits about 10-50 times more BaP in the body compared to inhalation [84]. Oral exposure leads to the primary uptake of toxicants through the gastrointestinal tract (GI-tract). The amount of chemicals entering the systemic circulation following oral exposure depends on factors such as absorption of the chemical into the GI cells, biotransformation of the substance by the cells, extraction by the liver and excretion into bile with or without biotransformation. This phenomenon of removal of chemicals before systemic circulation is called *first-passage effect* or *presystemic elimination* [88].

Most xenobiotics are transported across the gastrointestinal mucosa via passive diffusion, through aqueous pores (hydrophilic molecules) or lipid membranes (lipophilic molecules), in which the cell expends no energy. Absorption through passive diffusion is surface area- and site-dependent, and hence is one explanation to observed species differences [88]. Absorption of chemicals can also be influenced by such factors as age [89], diet composition, like the presence and concentration of ions [90], the detoxifying effect of milk [91], starvation [92], and the metabolism by the microflora in the intestinal tract [93].

Once a chemical has been swallowed, the acidity of the stomach can change the characteristics of that chemical, perhaps an organic acid or base, and thereby making them more or less difficult to absorb. One can determine, using the Henderson-

Hasselbalch equations, how much of a chemical can exist in a non-ionised form in the different parts of the GI-tract, and thereby estimate the absorption rate [88]. Apart from the passive diffusion, which aids in absorbing and clearing chemicals and toxicants from the GI-tract, there are some active transport systems in the gut, which can remove chemicals against the electrochemical or concentration gradients. One example of an active system reducing the absorption of xenobiotica is the multi-drug-resistance transporter (p-glycoprotein), which exudes chemicals back to the intestinal lumen once they enter the enterocytes. This is to increase probability of metabolising the xenobiotica and aid secretion [94].

7.1.2. Inhalation

Absorption of xenobiotica by the lungs often leads to the chemicals directly entering *systemic circulation*, since the presystemic elimination is by-passed. One big difference in absorption of chemicals, especially gases and vapours, between the GI-tract and the lungs is that the dissociation of acids and bases, and the lipophilicity of the molecules, are of less importance, since diffusion through the cell membranes is not a rate-limiting step [88]. This is due to three major factors: First, ionised molecules have low volatility and their concentration in ambient air is insignificant. Second, the epithelial cells lining the alveoli are very thin and the capillaries are in close contact with these cells and the distance for diffusion is very short. Third, absorbed molecules are quickly removed by the rapid blood flow through the capillaries. Due to the desire to reach equilibrium with the surrounding, rapid removal results in rapid absorption [88]. High molecular weight substances are more dependent on the lymphatic flow, since the epithelial cells lining the lymphatic capillaries are permeable for large molecules [95]. Particles can also be removed by phagocytosis and via the physical transport by the mucociliary escalator to the throat [96,97].

Particles and PAHs are cleared from the respiratory tract in a variety of ways, some of which are; i) direct clearance via exhaling, the mucociliary apparatus in the nose cavity and the trachea, ii) clearance via macrophage ingestion and removal by the mucociliary apparatus, iii) macrophage ingestion and dissolution, iv) macrophage ingestion and transport to interstitial tissues of the lung, v) macrophage ingestion and

transport to lymphatic system and finally vi) direct dissolution into surrounding tissues and fluids [98].

Clearance in healthy individuals can occur within a few hours, whereas in individuals with various respiratory disorders, this mechanism can be rather slow. It has been shown that particulate matter and its organic fractions have induced inflammations, DNA adducts, mutations and cancer in *in vivo* studies, which is often due to impaired lung clearance [50]. In addition, the extent of uptake, bioavailability and metabolism of substances adhered to the surface of particles have also been studied [99]. The conclusions drawn from studies over the years are that particles in urban air can induce inflammation in the tissue where they are deposited, carry numerous substances into the lungs and alter downstream immunological mechanisms. Pollution in air is not only limited to particles, PAHs and their derivatives. Co-pollutants such as NO_X, CO_X, SO_X, O_3, metals, formaldehyde and different solvents such as toluene and phenol have also been seen to affect people's health [83,98,100,101]. Whether pollution, with regard to particulate matter and its organic fraction, can increase the prevalence of respiratory disorders in healthy persons is still under debate. A study showed that the population in East Germany, where pollution was severe, had lower prevalence of asthma and bronchial hypereactivity as compared to West Germany, where the air was cleaner [102].

7.2. Biotransformation

Some PAHs and their derivatives are deposited in the body fat [103] while others are metabolised by Phase I and Phase II xenobiotic-transforming enzymes, to produce active metabolites and conjugates [104,105]. An important consequence of substances undergoing metabolism by xenobiotic transforming enzymes is that the physiochemical properties of the xenobiotic substances are changed to aid excretion by making them more hydrophilic than the parent compound. Volatile substances are primarily exhaled [106], whereas metabolites of PAHs of two-three ring composition are preferentially excreted through the urine, and those PAHs that are of higher molecular weight are mainly excreted via the faeces [107]. However, during metabolism, a variety of unstable and reactive intermediates of PAHs are formed, and

these metabolites can attack DNA and other cellular components, causing cellular toxicity [108].

Biotransformation enzymes are present in the highest concentrations in the liver, but are also located in the skin, lung, nasal mucosa, eye and GI-tract – organs that are linked to major routes of exposure – and in tissues like the kidney, adrenal gland, pancreas, spleen, heart, brain, testis, ovary, placenta, plasma, erythrocytes, platelets, lymphocytes and aorta [109-111].

The structures and thereby the activity of these biotransformation enzymes may vary (a.k.a. *allelic variants* or *allelozymes*) between individuals or ethnic groups [112], which can have consequences for the metabolism of xenobiotics [113]. A classical example is the diminished activity of alcohol dehydrogenase in Asians, which leads to a lower tolerance to ethanol due to the accumulation of acetic aldehyde in the body [106] or polymorphisms of *N*-acetyltransferases, which subdivides the phenotypes into *slow* and *fast* acetylators that may be either beneficial or detrimental in terms of disease development due to exposure [114].

7.2.1. Phase I biotransformation

Phase I biotransformation involves hydrolysis, oxidation and reduction, and the enzymes catalysing these reaction are primarily located in the endoplasmatic reticulum (microsomes) or in the soluble fraction of the cytoplasm (cytosol), and to a lesser extent in the mitochrondria, nuclei and lysosomes. Phase I reactions include introduction of such functional groups as –OH, -NH_2, -SH or –COOH, which results in an increase in hydrophilicity [106].

Hydrolysis is mainly performed by different esterases [115] and peptidases [116], whereas oxidation is performed by a variety of amine oxidases [117], dehydrogenases, monooxygenases, peroxidases [118], and Cytochrome P450 (CYP) isozymes [119]. Reduction reactions can also be catalysed by CYPs, as well as by specific reductases that transform ketones, disulfides, aldehydes, sulfoxides, quinones, azo- and nitro compounds [106]. Nitro-reduction can also be performed by the intestinal microflora [120]. The basic reaction catalyzed by CYPs is

monooxygenation by incorporating one atom from an oxygen molecule (O_2) into a substrate and reducing the other oxygen atom to water with the reducing equivalents derived from NADPH (Figure 6). During these steps, the catalytic cycle may be interrupted and reactive oxygen species (ROS) are released, which can cause both DNA and other cellular damage that may lead to diseases [121]. ROS can also be produced by a number of biological reactions needed for cell survival. These pathways of metabolism also leads to formation of reactive nitrogen species (RNS), when, for instance $NO^{\bullet}$ reacts with H_2O_2 to form peroxynitrite ($ONOO^-$) [122]. $NO^{\bullet}$ can be produced by macrophages during inflammation, and continually by all tissues of the body, since it plays very important roles in the cardiovascular system, immune system and nervous system. NO is formed from L-arginine via the enzyme nitric oxide synthase (NOS) [106].

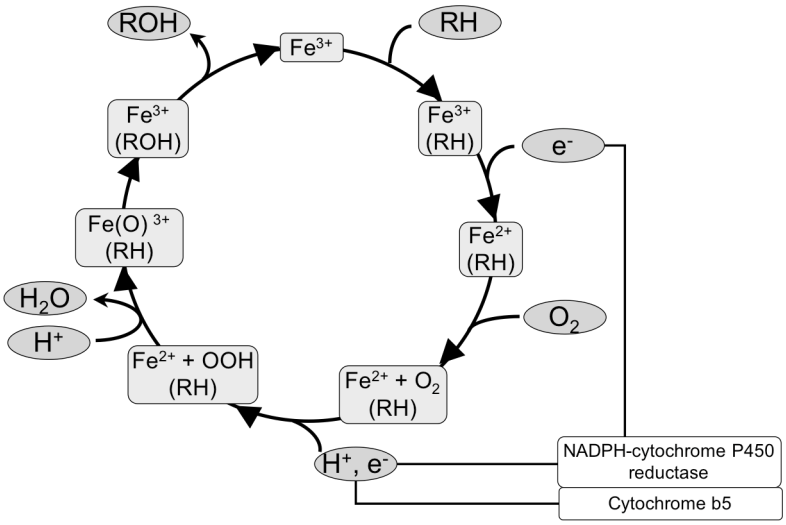


Figure 6. The Cytochrome P450 cycle. This scheme depicts the way CYP enzymes detoxify xenobiotica by incorporating one atom from an oxygen molecule (O_2) into a substrate and reducing the other oxygen atom to H_2O. Reductive elements aiding in this process are NADPH-cytochrome P450 reductase or Cytochrome b5.

Apart from ROS and RNS, isozymes of CYPs and epoxide hydrolase convert PAHs to PAH-diols, which are further metabolized to potentially carcinogenic metabolites, such as PAH diol-epoxides, or by dihydrodiol dehydrogenases to reactive PAH *o*-quinones [108,123] The human liver microsomes contain 15 or more CYP enzymes [124,125]. Those among these enzymes that are known or suspected to be active in the metabolism of PAHs and nitro-PAHs are given below.

CYP1A1: This enzyme catalyses a wide range of PAHs and nitro-PAHs and is constitutively expressed in low levels in tissues [126]. However, its induction is controlled by the aryl hydrocarbon receptor (Ah-receptor), which is a transcription factor activated by ligands such as the dioxin TCDD and other PAHs [127].

CYP1A2: The capacity of this enzyme to metabolise PAHs is less then for CYP1A1, but it has been shown to be induced by BaP, a few benzofluoranthenes and benzoanthracences [127]. However, it is a poor metaboliser of nitro-PAHs. It has been found in renal and lung carcinoma cell lines [128].

CYP1B1: This enzyme biotransforms a variety of PAHs and nitro-PAHs in most tissues [129,130].

CYP2B6: The level of this enzyme in human liver varies between individuals but can exert a significant metabolism when induced [131]. Apart from PAHs it can also metabolise certain amino-compounds such as 6-aminochrysene [132].

CYP2C8 and 2C9: These enzymes are present in the human liver with large interethnic and intraethnic variations [133]. Apart from the metabolism of several endogenous substances, such as arachidonic acid and retinoic acid, CYP2C8 can diminish the effect of the anti-cancer drug Paclitaxel. CYP2C9 is involved in metabolising several non-steroidal anti-inflammatory drugs, as well as xenobiotics like warfarin [134].

CYP2D6: This enzyme has a wide variety of known substrates, but only NKK, a tobacco specific metabolite, is suspected of inducing it [106]. Individuals lacking CYP2D6 show an unusually low incidence of certain cancers [135], however the enzyme itself appears to play little or no role in the activation of known chemical carcinogens.

CYP3A4: This enzyme is inducible by numerous substances and is known to transform steroids as well as xenobiotics such as BaP, 6-aminochrysene, 1-NP, and the mildew toxins Aflatoxin B1 and G1 [106].

Azo- and nitro-reduction (Figure 7) is performed by the intestinal microflora [49] and by certain liver enzymes; some CYPs, NADPH:P450 oxidoreductase (POR), NAD(P)H-quinone reductase (a.k.a. DT-diaphorase), xanthine oxidase (XO), and, under special conditions, aldehyde oxidase [106]. Although nitro-PAHs undergo reduction at the nitro-group, it is not necessarily the first step in metabolism. This is illustrated by the metabolism of 2,6-dinitrotoluene; oxidation and glucuronide-

conjugation occurs in the liver, followed by excretion through the bile into the intestine, where the microflora deconjugate and reduce the nitro group to an amino function. This last step aids in the re-absorption of the compound to the liver, where it is once again oxidised to form a *N*-hydroxylated compound and then conjugated with an acetate or sulfate [136].

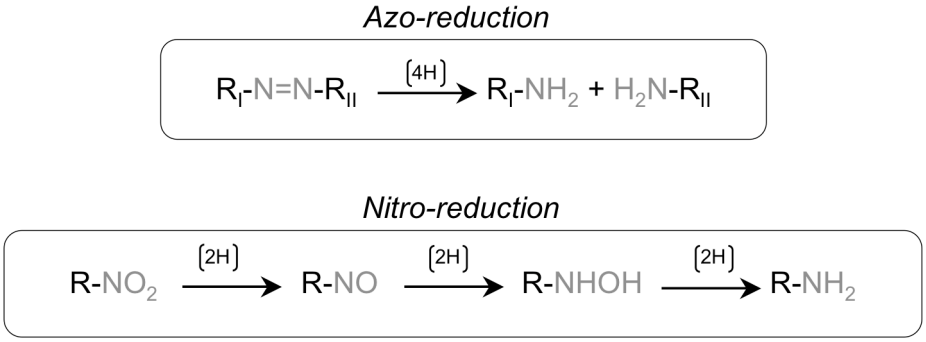


Figure 7. Azo- and Nitro-reduction in the body is done by intestineal microflora, certain CYPs, NADPH:P450 oxidoreductase (POR), DT-diaphorase, xanthine oxidase (XO), and aldehyde oxidase.

Quinones are reduced to hydroquinones primarily by DT-diaphorase and carbonyl reductase [137]. DT-diaphorase is a cytosolic flavoprotein, one of which in humans is known as NADPH-quinone oxidoreductase-1 (NQO_1), and is responsible for the majority of DT-diaphorase activity in most tissues [138]. The reaction involves a two-electron reduction of the quinone with oxidation of NAD(P)H without oxygen consumption [139]. This pathway is *not* associated with oxidative stress, unlike the one-electron reduction by NADPH-cytochrome P450 reductase, which is a microsomal flavoprotein. Oxidative stress is associated with quinones through the production of several ROS (superoxide anion, perhydroxyl radical, hydrogen peroxide and hydroxyl radical). However, quinones can also cause cellular damage through direct alkylation of proteins as well as DNA [139,140].

7.2.2. Phase II biotransformation

Phase II biotransformations include glucurinidation, sulfation, acetylation, methylation, and conjugations with glutathione and other amino acids (glycine, taurine and glutamic acid). The enzymes responsible for these reactions are located in the cytosol, with the exception of UDP-glucuronosyltransefrarses, which are

microsomal. Xenobiotics become somewhat hydrophilic when undergoing Phase I reactions, but even more so when conjugated with endogenous compounds. Phase II reactions also proceed much faster then Phase I, hence xenobiotics, which are dependent on biotransformation by CYPs to be eliminated, have a rate-limiting step in Phase I. The order of biotransformation does not necessarily follow the concept of first or second, since phase II reactions can take place before Phase I. For instance, morphine, heroin and codeine are transformed into morphine-3-glucuronide, but in the case of morphine conjugation takes place directly without any involvement of Phase I enzymes, which is required for the latter two substances [106].

N-acetylation is a major route for many compounds containing an aromatic amine (R-NH_2) or a hydrazine group (R-NH-NH_2), which are converted to aromatic amides (R-NH-$COCH_3$) and hydrazides (R-NH-NH-$COCH_3$). *N*-acetyltransferases (NATs) are cytosolic enzymes found in the liver and most other tissues in most mammals, except dog and fox, which cannot acetylate xenobiotics. In addition, in contrast to most other biotransforming enzymes, there is a limited number of NATs. Humans have only two (NAT1 and NAT2) [141].

Apart from transferring acetyl-groups, cytosolic sulphotransferases (SULTs) transfer a sulpho moiety from the cofactor 5'-phosphoadenosine-3'-phosphosulphate (PAPS) to nucleophilic groups of xenobiotics and small endogenous compounds (such as hormones and neurotransmitters). SULTs differ in their substrate specificity and tissue distribution and genetic polymorphisms have been described for three human SULTs [142].

7.3. DNA adducts

Both endogenous and exogenous substances can damage cells by binding, cross-linking, oxidising, reducing, causing breakage and distorting structures of cellular components as well as interfering with important pathways by mimicking a substrate for an enzyme or by blocking certain receptors. One type of these damages that will be explained in more detail is the formation, repair and consequences of DNA

addition products (DNA adducts), which is defined as any substance (atom or group) that is covalently bound to any part on the DNA.

Several bulky PAHs can, apart from quinone pathways, also form bay-region diol-epoxides. A feature common to all bay-region diol-epoxides is their resistance to hydrolysation by epoxide hydrolase, which results from steric hindrance from the nearby dihydrodiol group. In addition, during the formation of diol-epoxides, a carbocation is also formed, which can be stabilised through delocalisation of the positive charge over the unsaturated bonds on the molecule. Those diol-epoxides that are most sterically hindered and distort the molecule so that delocalisation is made difficult, are more prone to react with DNA than with water, compared to a fully ionised intermediate. [143].

7.3.1. DNA adduct formation

Aromatic amine generated DNA adducts can be formed through a variety of metabolic pathways; firstly conversion to *N*-hydroxyarylamines and *N*-hydroxyarylamides from amines through oxidation by CYPs and monooxygenases, further conjugation to reactive *N,O*-esters by transferases, or formation of electrophilic imines and quinones [144].

The reactive *N*-hydroxy-intermediate can also be formed through the first nitro-reduction step of nitro-PAHs, which can be performed by different CYPs and nitroreductases [145]. As with the amines, *N*-hydroxy-intermediates from reduced nitro-PAHs can undergo further conjugation, preferentially *O*-esterification. It is important to keep in mind that nitro-PAHs also have pathways that involve epoxide formation and quinone pathways. In other words, metabolism of these bulky pollutants is a complex process that can produce many reactive metabolites, hence the numerous DNA adducts that can be observed from a single substance [145]. The harmfulness of the chemical metabolised is also dependent on the position of the substituents, such as the nitro or amino group, due to structure and activity relationships [44].

There are several types of DNA adducts that can be formed by electrophilic attack on the bases at the ring nitrogens, exocyclic amino groups and carbonyl oxygens, or the phosphodiester backbone [146]. Direct alkylating agents react preferentially at the N^7 and O^6 position of the Guanine (G) base, whereas large bulky structures from PAHs and aromatic amines bind to the N^2 and C^8 position, respectively [147]. PAHs preferentially bind to the N^6 position of Adenine (Figure 8). The type of mutation induced by the DNA adducts is strongly determined by the chemical and physical properties of the adducted moiety and the position at which it is attached to the nucleotide [146].

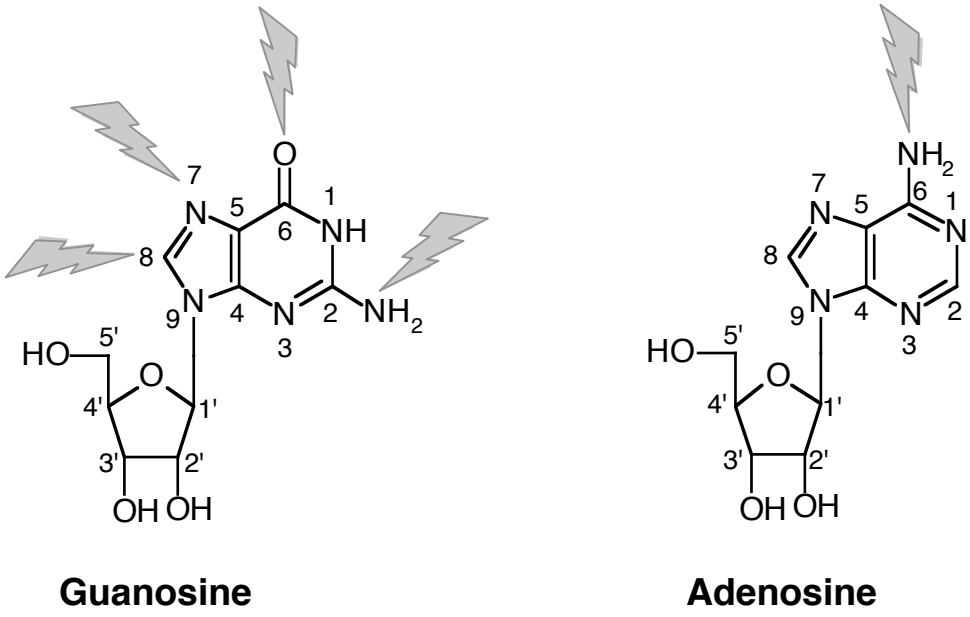


Figure 8. Reaction spots preferably attacked by PAHs on the guanosine and adenosine bases. The position where an activated substance will attach is largely determined by sterical hindrance, electrophilic groups, as well as nucleotide sequence.

7.3.2. DNA adduct repair

To maintain DNA integrity, the cells have evolved several types of repair mechanisms to correct a wide variety of damages. The repair mechanism involved is dependent on the type of damage induced. The direct type of damages to DNA is the formation of adducts by attachment of ROS or bulky PAHs and their derivatives. The indirect damages that can also be attributed to DNA adducts involve DNA strand breaks.

Base Excision Repair (BER)

BER is a major pathway by which DNA is being repaired. Enzymatic removal of the damaged base is performed by a glycosylase that causes an apurinic or apyrimidinic site, which is then removed, possibly together with neighboring nucleotides, so that the appropriate nucleotide(s) can be incorporated and sealed by DNA polymerase and ligase [148]. DNA base damage caused by oxidative processes, either endogenous or exogenous, are important substrates for BER [149].

Nucleotide Excision Repair (NER)

The NER pathway is primarily employed for the removal of bulky DNA adducts, such as those generated by PAHs and their derivatives. This pathway of repair mobilises more then twenty different subunits to be able to recognise, remove the damaged nucleotide along with some adjacent nucleotides, then to synthesise and ligate the strand [150].

O^6-Methylguanine-DNA methyltransferase repair

The main role for this O^6-Methylguanine-DNA methyltransferase is to protect cells against simple alkylations. The enzyme restores the damaged base by transferring the alkyl-group to itself, a mechanism by which the enzyme becomes inactivated [151].

Homologous recombination (HR)

There are primarily two major pathways by which DNA strand-breaks are repaired; homologous recombination and non-homologous end joining. The initial step in homologous recombination involves the production of a 3'-ended single-strand tail, which invades the undamaged homologous DNA strand on the sister chromatid and together with DNA synthesis the Holliday junction is formed. This junction is then cleaved and two DNA strands are produced with or without structural cross-over between the two chromosomes [152].

Non-homologous End Joining (NhEJ)

NhEJ is the simplest way of repairing double-strand breaks (DSB:s) and is the straightforward religation of DNA strand ends without the requirement for a template. NhEJ plays a major role in the elimination of DSBs during G_1 phases of the cell

cycle, which includes competence, entry (G_{1A}), progression (G_{1B}), and assembly (G_{1C}), based on the effects of limiting growth factors, nutrients, or inhibitors [152].

7.3.3. Consequences of DNA adducts

DNA adducts can cause a variety of insults on the DNA, by inducing mutations such as base substitutions (transition and transversions), frameshift (addition or deletion of nucleotides), chromosomal aberrations or breakage and micronuclei. Most of the damage occurs during replication at and repair of the DNA lesions, when repair is incomplete or incorrect and the damage is fixed so that it is inherited by the next generation [153]. One example of a substance that gives rise to DNA adducts, genetic alterations and finally tumours is 2-aminofluorene, an amino compound and metabolite of the air pollutant 2-nitrofluorene. It is converted during metabolism to 2-acetylaminofluorene (AAF), a metabolite that induces neoplasms of the mammary gland, ear duct and liver in rats [154], and in the bladder of mice [155]. Several studies in the 1960's demonstrated that ring-hydrolxylation as well as hydroxylation at the nitrogen occurred in the metabolic pathway of AAF [156,157], followed by esterification at the *N*-hydroxy group, which yielded a highly reactive compound capable of reacting non-enzymatically and also bind covalently to nucleophilic sites on proteins and DNA. This led to the division of chemical carcinogens into three groups; *procarcinogens* that require metabolic activation to become carcinogenic, their highly reactive metabolites were termed *ultimate* carcinogens, and the intermediates between the pro- and ultimate carcinogens were termed *proximate* carcinogens [158]. Several studies performed on AAF have shown that DNA adduct formation does occur and that some of these adducts are persistent, which correlate well with the tumours observed in the treated animals [159-161]. Thus, DNA adduct formation can be a first step in chemical carcinogenesis and can be used as biomarkers to detect at an early stage an increased risk for development of disease.

7.3.4. DNA adducts as biomarkers

When determining the extent of exposure in individuals, one has to keep in mind the different aspects of dose, such as the external, internal and effective dose, followed by early and late biological effect, and lastly the onset of disease. The external and

internal dose is not necessarily the same and depends on many different factors; for instance, the ability of the substance to bioaccumulate or polymorphisms in detoxifying enzymes, which influences individual susceptibility [162]. It is more important to measure the biological effective dose, also known as the 'tissue dose', which can be assessed by determining the reaction products of the compound at the target site. Such reaction products are protein and DNA adducts [163].

DNA adducts have been detected in several human tissues and summarised results show that lowering exposure to carcinogenic PAH results in decreasing PAH-DNA adduct levels, although dosimetry has not always been precise [164]. However, biomonitoring and molecular epidemiological studies should be directed to combine several endpoint measurements, such as adduct formation at specific sites, mutational spectra in cancer-relevant genes, and genetic markers of (cancer) susceptibility in a number of cancer-predisposing genes [164]. This is due to such limitations as very large intra- and inter-individual variations as a consequence of polymorphisms, age, sex, parallel exposures (medications), lifestyle and so on. Little is known about the relationship between DNA adducts and development of cancer several years later, since DNA adduct formation is a short-term effect, whereas cancer is a multi-stage long-term effect. Some epidemiological studies have addressed this matter and provided some information based on prospective and case-cohort evaluations [165,166]. In the study by Peluso et al, the conclusion was drawn that adducted DNA obtained from leucocytes was associated with subsequent risk of lung cancer, especially in never-smokers, and that there was a positive association between DNA adduct level and O_3 [166]. These data were supported by the Danish case-cohort study, in which bulky DNA adducts were associated with increased risk of cancer, although the association was weak [165].

7.4. Neoplastic development

7.4.1. Initiation

One of the characteristics for the initiation step is the irreversibility in the sense that the genotype/phenotype of the initiated cell is established at the time of initiation. At

least three processes are important in initiation; metabolism, DNA repair and proliferation. The molecular mechanisms involved in initiation are simple mutations in the cellular genome, such as tumour suppressor genes, oncogenes and proto-oncogenes [158]. Thus, carcinogenic agents administered at subcarcinogenic doses can cause substantial DNA lesions, such as DNA adducts, to start initiations that do not give rise to obvious or gross chromosomal changes. Also, inherited defects in tumour suppressors play an important role in the initiation of early malignancies [167].

7.4.2. Promotion

Unlike initiation, which is an irreversible step, promotion is reversible at both cellular and gene expression level. In addition, there is no evidence that promoting agents directly interact with DNA like initiating agents do [158]. For instance, TCDD is the strongest known promoting agent for liver carcinogenesis in rats, but it acts via the AhR and mediates clonal expansion of 'initiated' preneoplastic hepatocytes, identified as enzyme-altered foci by inhibiting apoptosis and bypassing AhR-mediated growth arrest [168].

Another characteristic of the promotion stage is that it is susceptible to the modulation by physiologic factors such as age, diet and hormones. Many of these endogenous factors are promoters themselves [169]. Thus, promotion results from the enhancement of signal transduction pathways induced in the initiated cell and its progeny by continuous exposure to the promoting agent.

7.4.3. Progression

The stage of progression usually develops from cells in the stage of promotion, but may develop from normal cells as well. This occurs after exposure to high or cytotoxic levels of complete carcinogens, i.e. carcinogens capable if initiating and promoting. One such complete carcinogen is 2-AAF [170]. 2-AAF induces G–T transversions, but causes also electron drainage on oxidative phosphorylation and thus interferes with the redox cycling in the mitochondria, which corresponds to the chronic toxicity observed [171].

Progression results then from continuing evolution of a basically unstable karyotype. Mechanisms that lead to instability include disruption of the mitotic apparatus, alterations in the telomere functions, DNA hypomethylation, recombination, gene amplification and gene transcription [172].

8. Metabolism of 3-NBA

Through experiments conducted on both *in vitro* and *in vivo* systems, it has been concluded thus far, that 3-NBA can be activated by two major pathways that leads to DNA adduct formation. The first step in activation is through the formation of the *N*-hydroxy-3-aminobenzanthrone (*N*-OH-ABA) common to many other nitro-PAHs discussed above. From this point the active metabolite may form a reactive nitrenium ion intermediate, which yield non-acetylated DNA adducts, or it may proceed through the formation of *N*-acetyl-*N*-hydroxy-3-aminobenzanthrone (*N*-Ac-*N*-OH-ABA) and an *N*-acetyl-nitrenium ion, yielding acetylated DNA adducts (Figure 9).

8.1. Enzymes involved in the activation of 3-NBA

After incubating calf thymus DNA with 3-NBA and xanthine oxidase, a mammalian nitro-reductase, seven different DNA adducts have been found [173]. These DNA adducts have also been observed *in vivo* in female Sprague-Dawley rats orally administered 2 mg/kg bw of 3-NBA [174]. To gain information on the type of DNA adducts formed *in vivo*, co-chromatography has been performed with DNA adduct standards. This reveals that 40% of those formed in the rats are attached to dA and 60% to dG. There is a small difference in the number of DNA adducts observed using either nuclease P1 (NuP1) or butanol for adduct enrichment. This points to that at least one of the DNA adducts generated by 3-NBA are attached at the C8-position of dG, since that kind of adducts are sensitive to NuP1 treatment [174]. In a further study, 2 mg/kg bw of 3-NBA and several of its identified metabolites, 3-ABA, 3-acetylaminobenzanthrone (3-Ac-ABA) and *N*-Ac-*N*-OH-ABA, have intraperitoneally (ip) been administered to Wistar rats [175], the results showing that the reductive metabolites of 3-NBA are bound to purine bases and lack an *N*-acetyl group. This suggests that 3-NBA metabolites undergo several biotransformations and that *N*-OH-ABA is the common reactive intermediate.

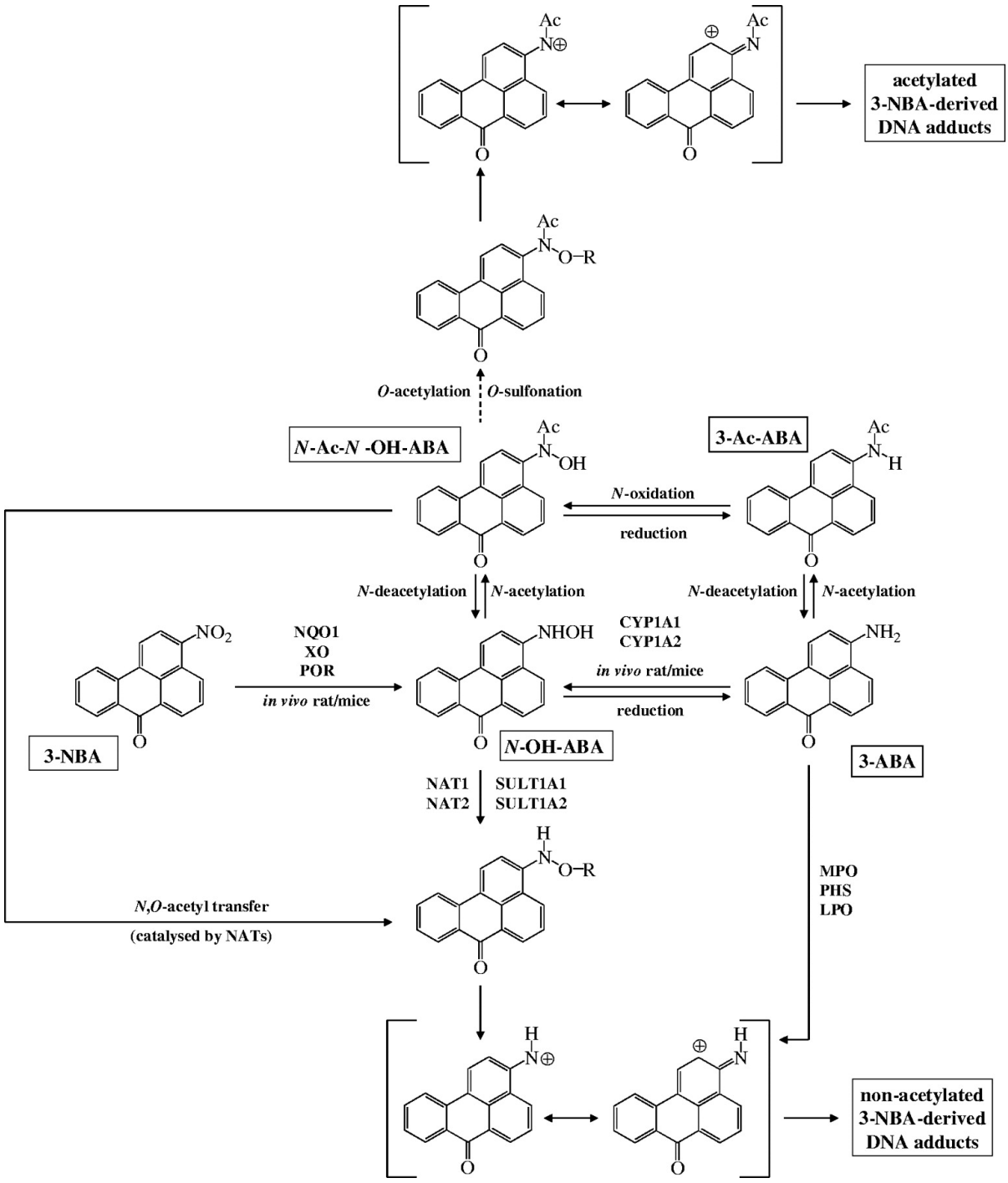


Figure 9. The suggested activation pathway of 3-NBA. The first step in the activation of 3-NBA into DNA damaging agents is nitro-reduction by NQO1, XO and/or POR. This leads to the intermediate N-OH-ABA, which can be further reduced to 3-ABA, or enter phase II biotransformation involving conjugation and de-conjugated, to form acetylated and non-acetylated DNA adducts, respectively. Permission provided by Dr V.M. Arlt.

This has been confirmed in Chinese hamster V79MZ-h1A2-derived cell lines expressing human CYP1A2 in conjunction with human NAT1, NAT2, SULT1A1 or SULT1A2, respectively [176]. Human CYP1A2 is required for the metabolic activation of 3-ABA and 3-Ac-ABA via *N*-oxidation, and enhances the activity of 3-NBA via nitroreduction, and NAT1, NAT2, SULT1A1 and SULT1A2 strongly contribute to the high genotoxicity of 3-NBA, with predominant activation by NAT2, followed by SULT1A2, NAT1, and SULT1A1 [176,177].

Several, both microsomal and cytosolic, phase I enzymes can be involved in the activation of 3-NBA and its metabolites. Chinese hamster V79 and human lymphoblastoid MCL-5 cells expressing recombinant human CYPs and POR have been investigated for 3-NBA activation [178,179]. Most of the hepatic microsomal activation of 3-NBA appears to depend on POR, and basically the same DNA adducts are formed in the two cell lines. Of the recombinant human CYPs examined, 2B6 and 2D6 are the most efficient to activate 3-NBA by nitro-reduction, followed by 1A1 and 1A2 [178]. The importance of POR has been confirmed in a different study, in which CYP3A4 has been shown to be an additional activator of 3-NBA and its metabolites [180]. The importance of CYP enzymes in the activation of 3-NBA and its metabolites has been confirmed by using enzyme inducers as well as inhibitors, which reveals that CYP1A1 and 1A2 are particularly important for the activation of 3-ABA [181]. Studies on human hepatic cytosolic samples reveal that most of the reductive activation of 3-NBA in human hepatic cytosols is attributed to NAD(P)H:quinone oxidoreductase (NQO1) rather than xanthine oxidase [177]. This observation has been confirmed by inhibiting and inducing NQO1 in hepatic cytosols [177,182]. In fact, after intaperitoneal treatment hepatic POR-null mice (HRN™ mice) and wild-type littermates with 2 mg/kg bw of 3-NBA, no difference in DNA binding has been observed, indicating that 3-NBA is predominantly activated by cytosolic nitroreductases rather than microsomal POR. Further, peroxidases such as Prostaglandin H synthase, lactoperoxidase, and myeloperoxidase have also been shown to generate DNA adducts from both 3-ABA and 3-NBA [182]. Moreover, *in-vivo* studies on rats ip-treated with either 0.4, 4 or 40 mg/kg bw of 3-NBA or 3-ABA have shown that 3-NBA and 3-ABA are efficient inducers of the enzymes NQO1, CYP1A1 and 1A2, thereby enhancing their own genotoxic potential [183]. The reductive activation of 3-NBA by hepatic cytosolic samples from rats pre-treated with

3-NBA or 3-ABA has led to 10-fold higher 3-NBA-DNA adduct levels than in hepatic cytosol from control rats, confirming the important role of NQO1 in the cytosolic activation of 3-NBA.

In a comparative study, different *Salmonella* strains were subjected to different mono- di- and tri-nitrated benzanthrones (MNBAs, DNBAs and TNBAs, respectively) [72]. Some of the strains were nitroreductase and acetyltransferase-deficient, whereas others were overproducing these enzymes. 2-NBA was the only compounds that showed decreasing or increasing mutagenicity in the nitroreductase deficient or effluent strain, respectively. 3-NBA, on the other hand, was very efficient in a strain sensitive for frame-shift mutations, confirming previous studies [51]. Generally, the mutagenicity of the MNBAs was more dependent on the reduction potential and orientation of the nitro-group, compared to the poly-nitrated variants. By comparing the physical-chemical properties of NBAs to DNPys, in addition to mutagenicity testing [51], it seems in general that NBAs are more easily reduced and may therefore be efficient direct acting mutagens [184]. This simple reduction of the nitro substituent is due to the electron-drawing effect of the keto moiety at the 7 position of the NBAs.

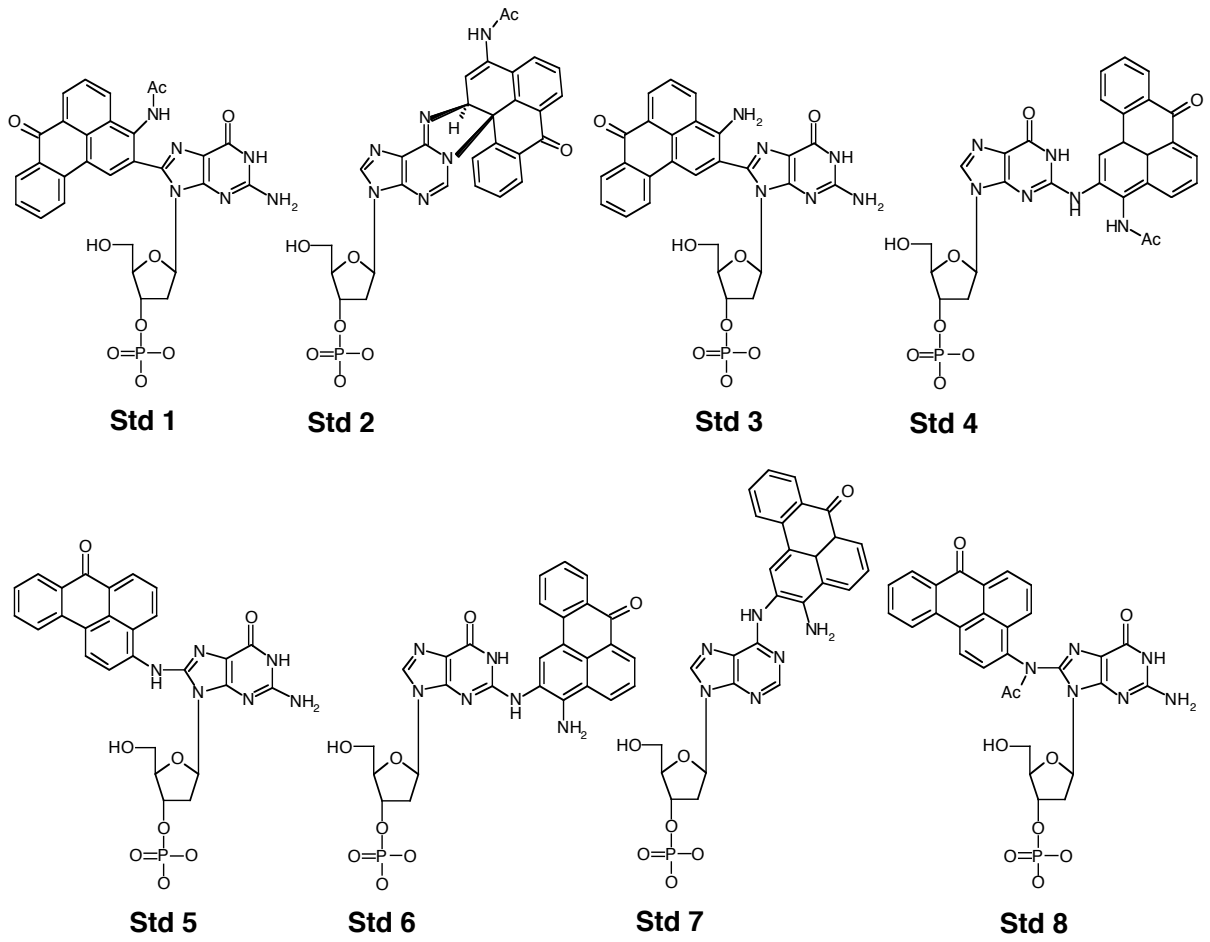
8.2. DNA adducts formed by 3-NBA

Through synthesis of a variety of DNA adduct standards (Figure 10) [185], several efforts have been made to characterize the 3-NBA-derived DNA adducts formed *in vivo*. Previous speculations had lead to the assumption that both acetylated and non-acetylated DNA adducts are formed by 3-NBA. The acetylated adducts *N*-acetyl-3-amino-2-(2'-deoxyguanosin-8-yl)benzanthrone (a C-C adduct), *N*-acetyl-*N*-(2'-deoxyguanosin-8-yl)-3-aminobenzanthrone (a C-N adduct), and an unusual 3-acetylaminobenzanthrone adduct of deoxyadenosine involving a double linkage between adenine and benzanthrone (N1 to C1, N6 to C11b), thereby creating a five-membered imidazo-type ring system, have not been detected in DNA from 3-NBA-treated rats [186]. When using eight different DNA adduct standards, including the three mentioned, only three (dGp-C8-N-ABA, dGp-N2-C2-ABA, and dAp-N6-C2-ABA) of the non-acetylated type have been unequivocally determined, suggesting that

at least part of the pathway for activation of 3-NBA proceeds through *O*-acetylation of the hydroxylamine intermediate. Two other DNA adduct standards (dGp-C8-C2-N-Ac-ABA and dGp-N2-C2-N-Ac-ABA) of the acetylated type, have not been unambiguously characterised, since they vary inconsistently between samples and also align with peaks found in controls [187].

Using the Muta™Mouse [188] model, the *in vivo* mutagenicity of 3-NBA has been analysed in various organs after intraperitoneal injection of 3-NBA (25 mg/kg bw) once a week for 4 weeks [189]. Increases in mutant frequency have been found in colon, liver, and bladder. Sequence alterations compared in the liver *cII* gene reveal that base substitution mutations predominate for both the 3-NBA-treated (80%) and the untreated (81%) groups. However, the proportion of G:C→T:A transversions in the mutants from 3-NBA-treated mice is higher (49% vs. 6%), which is probably induced by misreplication of adducted guanine residues through incorporation of adenine opposite the adduct, also known as the A-rule. This increase in mutant frequency is associated with higher DNA adduct formation in liver DNA (70-80% with dG and 20-30% with dA), with DNA adduct patterns qualitatively similar to those observed in previous studies [189]. Recently, the major guanine DNA adducts in livers of treated Muta™Mouse have been identified as dGp-C8-N-ABA and dGp-N2-C2-ABA, suggesting that these adducts are probable responsible for the induction of G:C→T:A transversion mutations observed *in vivo* [190].

In the human lung cell line A549, both 2- and 3-NBA give rise to high levels of DNA adducts, although 2-NBA is about one third as potent as 3-NBA [71], whereas the parent compounds BA and the metabolite 3-ABA do not give rise to any detectable amounts of DNA adducts [70]. When Fischer F344 female rats are it-administered 5 mg/kg bw of either 2- or 3-NBA, the results show that while 3-NBA is quickly metabolised, the level of DNA adducts generated by 2-NBA increase only slowly but the highest level of these lesions are comparable between the two substances [70]. This observation raises the question whether 2-NBA, unlike 3-NBA, has the potential to bioaccumulate.



Standard Nr.	Name	Short
Std 1	*N*-acetyl-3-amino-2-(2'-deoxyguanosin-8-yl)-benzanthrone 3'-phosphate	dGp-C8-C2-N-Ac-ABA
Std 2	9'-(2"-deoxyribofuranosyl)purino[6',1':2,3] imidazo[5,4-*p*]-1,11b-dihydro-*N*-acetyl-3-aminobenzanthrone 3'-phosphate	dAp-N-Ac-ABA
Std 3	3-amino-2-(2'-deoxyguanosin-8-yl)-benzanthrone 3'-phosphate	dGp-C8-C2-ABA
Std 4	*N*-acetyl-3-amino-2-(2'-deoxyguanosin-N^2-yl)-benzanthrone 3'-phosphate	dGp-N2-C2-N-Ac-ABA
Std 5	3-amino-N-(2'-deoxyguanosin-8-yl)-benzanthrone 3'-phosphate	dGp-C8-N-ABA
Std 6	3-amino-2-(2'-deoxyguanosin-N^2-yl)-benzanthrone 3'-phosphate	dGp-N2-C2-ABA
Std 7	3-amino-2-(2'-deoxyadenosin-N^6-yl)-benzanthrone 3'-phosphate	dAp-N6-C2-ABA
Std 8	*N*-acetyl-3-amino-*N*-(2'-deoxyguanosin-8-yl)-benzanthrone 3'-phosphate	dGp-C8-N-N-Ac-ABA

Figure 10 with corresponding table. Eight synthesised standards, kindly provided by Dr Takeji Takamura-Enya for the characterisation of 3-NBA derived DNA adducts. The standards are synthesised as 3'-monophosphates to enable labelling for ^{32}P-HPLC analysis.

8.3. Oxidative damage and other DNA lesion by 3-NBA

Apart from DNA adduct formation, 3-NBA also possesses the potential of causing DNA strand breaks and chromosomal damage in cells [85,179]. 3-NBA shows a gentotoxic potential far greater then BaP in HepG2 cells, and gives measurable DNA damage at concentration as low as 12 nM in both the single cell gel electrophoresis (SCGE, a.k.a. Comet assay) and micronucleus assay [85]. The capability of 3-NBA to cause oxidative DNA damage has also been tested in A549 cells and compared to 2-NBA [71]. With the Comet assay both 2- and 3-NBA have been shown to induce oxidative DNA damage. However, the typical marker for oxidative damage, 8-oxo-dG, has not been detected with a different analytical method (HPLC-EC; High Performance Liquid Chromatography with electrochemical detection). This could either mean that different oxidative lesions are formed in DNA, not only 8-oxo-dG, or that the HPLC-EC method, which is less sensitive than the Comet assay does not detect the possibly low level of damage produced. Recent studies have also shown that the parent compounds BA and the metabolite 3-ABA form equally high levels of oxidative DNA damage as 3-NBA in both A549 and HepG2 cells [187]. This, in part, supports the hypothesis that part of the biotransformation of these benzanthrones is via the quinone reduction pathway.

8.4. Acute toxicity by 3-NBA

Autopsy on animals orally treated with 3-NBA revealed that this pollutant does not only cause DNA adducts and oxidative lesions, but it is also acutely toxic [70]. The DNA adduct levels in the majority of organs were characterized by an increase shortly after administration, which was followed by a sharp decrease with the accumulated severity of tissue damage. The DNA adduct levels then reached a maximum at the end of experiment, when the tissues had re-generated. The tissue damage included haemorrhages, loss of villous surface structure in the small intestine, as well as intestine fragility and oedema of the adipose tissue around the gastro-intestinal-tract. These observations suggest that 3-NBA not only exerts acute toxic effects, but that the bioavailability is affected by storage in tissues and later becomes available, resulting in increased DNA adduct levels at later time points of collection [238].

In the long-term studies, it has been shown that 3-NBA gives rise to tumours in the treated rats in a dose-dependent manner. The most prominent tumours were sqamous cell carcinoma, although minor adenomas were also seen. Other observations included severe alveolitis with haemorrhages, and exudation of alveolar fluid. No other changes were seen in organs and tissues, except for the respiratory system. In the trachea and bronchus, the loss of ciliated cells and metaplastic changes were observed [191].

9. Experimental designs and methods

9.1. From *in vitro* to *in vivo*

This thesis presents studies performed on 3-NBA using *in vitro* as well as *in vivo* models. All of these experimental models have their advantages and disadvantages and, which are discussed below.

9.1.1. *In vitro* assays

Rapid *in vitro* indicators of genotoxicity continue to play a valuable role in understanding toxicity of PAHs and their derivatives as single substances and/or complex mixtures. One must, however, keep in mind that different bacterial strains may react or be differently sensitive to the substances studied. For instance, TA98 and TA1538 strains, respond differently to total extracts from mobile-source emissions. TA98 is sensitive and detects frameshift promutagens and responds regardless of substance activation by S9, while TA1538, not sensitive for total extracts, distinguishes the presence of indirect-acting mutagens and gives increased response in addition of S9 activation [192].

By using several strains, and perhaps even comparing these results to other *in vitro* assays using mammalian cells, one can obtain a more complete picture of substance behaviour. At the comparison between the metabolic activation of organic extracts from diesel, coke oven, roofing tar and cigarette smoke in microbial and mammalian assays, it has been suspected that the differences observed between the relative mutagenic activity of these emissions in the mammalian cell and microbial assays is not due to a lack of optimisation of the S9 system, but may be inherent in the different response of the indicator cells to different chemical classes [193]. Fast *in vitro* assays have four major uses: i) comparative screening, ii) analysing factors that alter xenobiotics, iii) allowing for study of single substances in several systems in parallel, and iv) analysing complex mixtures and the interaction of the substances within a fraction in several systems in parallel. *In vitro* assays are also valuable in finding links to human diseases. For instance, the mutagenicity of fifteen nitro-PAHs from ambient air particles has been tested in six Salmonella typhimurium strains (TA7001-7006)

[194] and all of the compounds tested induce CG→AT transversion, which is observed as the most frequent base-substitution mutation of p53 tumour suppressor gene in human lung cancer. Further, mouse aortic smooth muscle cells pre-treated with a CYP1B1 inhibitor prior to exposure to 3-methylcholanthrene, a known carcinogen, reveal a significant drop in DNA adduct formation indicating that CYP1B1 is the primary CYP enzyme responsible for formation of genotoxic metabolites that may play a role in the induction of atherosclerosis by this substance [195].

One is not limited to usage of bacterial, yeast or human derived tissue cell lines, also components from the immune system may be employed. Studies have shown that PAHs have the ability to alter important functions in immunological processes, such as impairing antigen presentation by mouse macrophages [196], suppression of phagocytic activity of peritoneal macrophages [197] and also diminishing Langerhans cell function after topical application of DMBA [198]. PAHs also inhibit differentiation of human monocytes into macrophages [199]. For instance, in the presence of BaP, the formation of adherent macrophagic cells, deriving from monocytes upon the action of certain cytokines, is inhibited. Also the expression of macrophagic phenotypic markers, such as CD71 and CD64, are reduced without alteration of cell viability or induction of apoptosis [199].

9.1.2. Organ perfusion

Liver perfusion, in which an isolated liver is artificially kept alive with a pump providing circulating, oxygenated blood, can be related to an *in vivo* environment and applied to the study of single substances or complex mixtures [8,200]. During liver perfusion, continuous monitoring of the condition of the liver is possible. Samples collected from the perfusate allows monitoring of lactate metabolism, and together with measurements of excreted bile, information can be gained about the metabolic capacity and viability of the liver [201,202], whereas biopsies from the organ can provide histological [203], kinetic [104], as well as DNA adduct [8] data. Similarly, lungs can also be isolated and perfusion, which is a very powerful tool in assessing a more complex metabolism of for instance air pollutants [204] or specific types of compounds not possible or ethical to subject whole animals or humans to. For this

reason, perfusion can also be performed on human placenta to obtain information on the metabolism of genotoxic substances [205]. The drawback of perfusions is that no systemic information can be obtained and metabolism is only observed for the perfused organ. Since many substances may be stored and metabolized in one part of the body but exert their toxicity on another, such data is not possible to observe.

9.1.3. Animal studies

Animal studies can be used for monitoring the contamination of the environment, as well as assessing complex metabolic pathways including pathological/morphological consequences of exposure [206,207]. *In vivo* studies can also be very expensive, laborious and time consuming. It is desirable to limit the time required in many cases, and much information can be obtained even in a short-term animal study with a small number of animals. For example, guinea pigs and mice have been short-term (2 weeks) exposed to diesel exhaust to evaluate the usefulness of exposure experiments, limited with respect to exposure time and number of animals, to assess immunotoxic and genotoxic effects [208]. Data obtained from the guinea pigs reveal strain on airways, interference with acute allergic reaction and interference with or inactivation of antibody production.

However, the choice of exposure period, type and number of animals used, the concentration of substances and over-all study design depends on many different factors, such as the type of substance(s) and the type of effect studied (genotoxicity, carcinogenicity, inflammatory processes, recovery processes etc). Also the *prevalence* of an effect is important to consider when designing a study. An effect with very high prevalence demands less animals used, as compared to an effect with very low prevalence.

The type of animals used is also to be considered, since anatomical differences may play a key role in the differences of the results obtained. Dogs and monkeys are commonly used to study chronic bronchitis due to their close resemblance to humans, but apart from some common pathological features with those observed in humans, the inflammation of airway walls is still not well characterised in neither dogs nor monkeys [209].

One major problem regarding animal models and human exposure is the extrapolation of the data obtained from "staged" conditions to real life situations that humans encounter. In the case of humans, one has to take into account the diversity in life style, behaviour, genetic susceptibility, socio-economic factors, surrounding environment etc. that cannot be experimented on in a laboratory. When humans develop a disease, there is often an underlying pathology that predispose them to infections with relatively low doses of pathogens, while normal, healthy animals lack that underlying pathology.

Complete carcinogens exert both initiating and promoting properties. The dose-response relationship for cancers, induced by PAHs at low levels, can be expressed by the multiplicative model* for cancer incidence [210]. Strong mutagens that do not act as promoters can, if animal tests are negative, be classified as non-carcinogens. These kinds of substances may instead interact with inherited or acquired promoting factors and increase the risk of tumours that can also be found in controls [211]. When combined with a strong promotor, the over-all cancer probability due to carcinogen exposure will be dependent on the initiating effect of the carcinogen rather than the strong promotor. Moreover, purely mutagenic substances can only give rise to tumours if there is a background promotion present, which is the case in real life situations. This shows that one must also consider synergistic, additive and inhibiting behaviours of substances and enzymes, especially when combined in complex mixtures.

9.1.4. Human studies

Human studies are the most relevant in the sense that they reflect real life situations, with all the factors, such as age, sex, smoking, drinking, occupational exposure, genetic susceptibility etc., included. The most easily obtained samples from humans for analysis are blood and urine. From blood and urine, one can obtain rather broad information regarding pharmacokinetics, toxicokinetics, immunological responses, genotoxicity, metabolism, the dynamic distribution of substances from organs to

* Pcan = Pinit × Pprom where Pcan stands for the probability for cancer, a non-linear curve with upward rise at high doses. Pinit is the probability of initiation, a linear non-threshold curve, and Pprom is the probability of promotion, an S-shaped function with a no-effect threshold.

blood or vice versa, etc. Other sources of samples are cells from skin, nose, mouth cavity, cells obtained through bronchoalveolar lavage, also sperm, faeces, and of course biopsies from different organs and tissues. Sperm has been used to assess the role of PAHs in infertility and the results reveal that DNA adduct levels due to PAHs correlate negatively with alcohol consumption and smoking, but positively with post-occupational exposure to PAHs, as well as abnormalities in the sperm head and cell count. The results indicate that DNA adducts in sperm could provide as a biomarker for infertility [212].

The relationship between oxidative DNA lesions, as well as aromatic DNA adducts, have been measured in cancerous and surrounding normal tissue of the larynx from 68 smokers. No associations have been found between the oxidative and aromatic DNA lesions, which points to that PAH metabolism might not be the most significant contributor to oxidative stress in this tissue. The authors suggest that perhaps the tobacco smoke is the major source for ROS instead [213]. The problem with these types of biopsy/tissue samples is that one needs sufficient amounts of them to statistically verify analysis data, and it is not always easy to obtain tissues.

9.2. Exposure techniques in animal studies

Traditional ways to examine the effects of complex mixtures of PAHs have been e.g. through *in vitro* assays, such as the modified *Salmonella* Mutagenicity Assay [214] and skin painting, usually on the dorsal part of mice and rats [215], from which the results can be correlated to mutagenicity and carcinogenicity indices. A major route of exposure to toxicants is through ingestion. Humans and rodents have analogous PAH metabolism [216], thus studies on biomarkers of exposure (concentrations of parent compounds and/or reactive metabolites, DNA and protein adducts) help to establish the link between exposure events, the resulting toxic effects and extrapolate the findings to humans [107].

Many PAHs are air pollutants and are therefore inhaled, and the lung serves as the target organ. To assess the impact a substance has on the lung, several techniques have been developed, e.g. inhalation and intratracheal administration. Hence, with

respect to the substances studied in this thesis, these two exposure techniques are described in more detail and compared.

9.2.1. Inhalation

Inhalation studies using laboratory animals are carried out under well-controlled conditions to assess the toxicity of aerosols, gases, and vapours or mixtures. Inhalation studies are non-invasive and correspond well to physiological routes of exposure in humans. The degree of complexity of inhalation studies increases when complex mixtures have to be tested. Most inhalation facilities use dynamic exposure systems where the airflow and introduction of agents into the system are continuous with a suitable online computer control system to monitor the inhalation chamber atmosphere with respect to aerosol and/or vapour concentrations, particle size, air flow rates, temperature, humidity, stability and reproducibility [217]. Still, a major obstacle is the dosimetry. For instance, the 'nominal concentration' is the mass of the test substance introduced into the system relative to the total volume of air. Loss of test substance to the wall, skin and fur on animals (whole body exposure), sedimentation and chemical reactivity, can lower the actual concentration compared to the nominal. One way to come around this is by using nose-only inhalation studies or intratracheal inhalation [218,219]. Another aspect of dosimetry that needs to be taken into account is whether the toxicity is due to the first-pass absorption at the site of entry, or by the same toxicants returning to the lung with circulation [220]. The major fraction of the toxicants are rapidly absorbed by the alveolar type I epithelium and enter systemic circulation without extensive metabolism [221], whereas the remaining smaller fraction deposited on the bronchial epithelium is subdued to intense local metabolism [99,222]. Very often, most metabolites and adducts detected following exposure have originated from the liver and this latter fraction is easily overlooked [220], although local metabolism may play an important role in the development of tumours [223].

9.2.2. Intratracheal Instillation

Instillation has certain advantages over inhalation, e.g. it is more simple, the actual dose is better controlled and can be higher in shorter time, it shields laboratory

workers from hazardous, and enables highly localized exposures to specific lobes of the lung, allowing for one lobe to serving as a control for another [218,224].

There are, however, several important disadvantages with intratracheal instillation and a number of them concerns the non-physiologic introduction of the toxicant, involving invasive delivery, usually at a dose and/or dose rate substantially greater than that which would have occurred during inhalation. In addition, the distribution and physiochemical properties of an instilled material within the respiratory tract may differ from the distribution of an inhaled material [224-226]. Furthermore, the upper respiratory tract, which can be a potentially important target site for an inhaled test material, is bypassed [224]. Moreover, anaesthesia used could influence the initial effect of the instilled material on the lung surface, as well as test material retention and clearance, or the health of animals [227].

9.2.3. Ingestion

Oral exposure can take place either via food, water, by direct instillation or by transport from the respiratory tract. One major drawback is the physicochemical modification of substances via the acidic environment, which is different between species, that can alter ionic state and thereby uptake and distribution [88]. Ingestion can also be a confounding factor when analysing effects on the lungs following inhalation.

9.3. Extrapolation from animal to human

One problem often present in animal studies is how to extrapolate animal data to human equivalent concentrations (HEC). Quantitative aspects in risk assessment include dose-response, exposure evaluation, variation in susceptibility and characterisation of uncertainty [228]. Approaches for characterising the threshold dose-response includes no observed adverse effect level (NOAEL) and lowest observed adverse effect level (LOAEL), but it is important to keep in mind that NOAEL should not be perceived as risk-free, since studies have shown that for continuous endpoints a 5% risk averages NOAEL [229].

Traditionally, NOAEL is used to calculate both reference doses and acceptable daily intake by taking into account uncertainty factors related to toxicokinetics and toxicodynamics within and between species. This approach often assumes a 10-fold variation at each step (Figure 11) [228]. Sometimes the benchmark dose (BMD) is used, which uses all the experimental data to estimate a statistical lower bound on a dose corresponding to a certain level of risk [230].

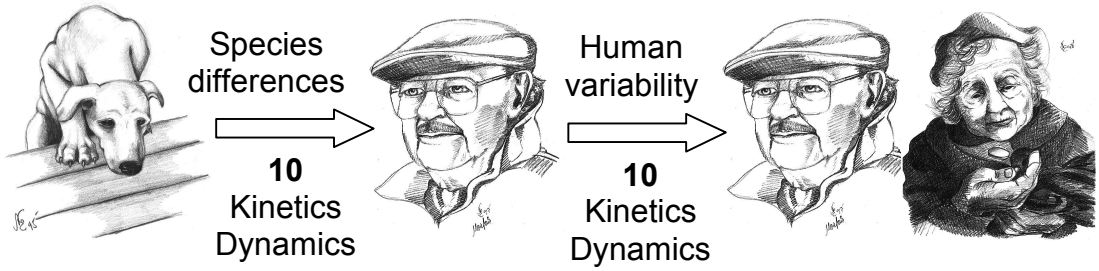


Figure 11. Toxicokinetic and dynamic inherent in interspecies and inter-individual extrapolations. Kinetics refers refers to absorption, distribution, metabolism and excretion, whereas dynamics refers to interactions of the toxicants within the organism. © Eszter Nagy.

For non-threshold responses, two general types of dose-response models exist; statistical, assuming statistically distributed individual tolerances, and mechanistic, based on the biological response mechanism [228]. Physiologically based pharmacokinetic modelling (PBPK) is a mathematical modelling where physiology and anatomy of the animal or human body, and the biochemistry of the chemical or chemicals of interest, are incorporated into a conceptual model for computer simulation [220,231].

9.4. Analytical methods

Different kinds of methods have been developed for the study of lesions like DNA adducts, including ^{32}P-postlabelling [232,233], immunoassays [234], fluorescence [235] and accelerated mass spectrometry (AMS) [236]. ^{32}P-postlabelling has a limit of detection (LOD) of DNA adducts in the range of 0.01–1 fmol but require the handling of radioactive material. Immunoassays offer LOD in the range of 1–40 fmol but the

specificity of antibodies can pose a problem [237]. Fluorescence methods offer LOD in the range of 1-20 fmol but require substances, which fluoresce, and are highly specific for specific types of DNA adducts, such as benzo[a]pyrene-diol-expoxide (BPDE) derived DNA adducts [238]. AMS alone provides structural information and an even higher sensitivity (0.001 fmol) than post-labelling, but it requires the use of isotopically labelled substances that are specifically searched for during detection [237].

The analytical methods presented below are those that have been used in the studies in this thesis: ^{32}P-HPLC, HPLC-EC/UV and Single Cell Gel Electrophoresis (SCGE, also called the Comet Assay).

9.4.1. ^{32}P-HPLC

^{32}P-postlabelling with High-Performance Liquid Chromatography (^{32}P-HPLC) separation is a good method for the detection of DNA adducts. It yields DNA adduct patterns of genotoxic substances, and characterisations can be achieved by co-chromatography with standards, where the high resolution is beneficial, especially for complex mixtures. ^{32}P-HPLC was developed in the early 1990s and proved to be somewhat less sensitive than ^{32}Postlabelling with Thin Layer Chromatography (^{32}P-TLC) with autoradiography. However, ^{32}P-HPLC is usually faster, with better separation, versatility and reproducibility compared to ^{32}P-TLC [239]. ^{32}P-HPLC, using only HPLC for separation and online detection, in contrary to variants using TLC for pre-separation and radioactivity measurement on collected eluent fractions, has since then been used to analyse many kinds of DNA adduct samples, from both *in vitro*, *in vivo* and humans [86,233,240,241]. The ^{32}P-HPLC used here is based on the extraction of DNA, enzymatic digestion to nucleotides, enrichment of adducted nucleotides, enzymatic labelling with ^{32}P-phosphate, chromatographic separation of various adducted and non-adducted nucleotides, and detection of the radioactivity from the labelled compounds.

The purpose of the DNA-extraction is to free the DNA from the nucleus and to recover it in a purified form, without protein or RNA contamination. Whether it is a question of tissues or cultured cells, DNA extraction can be performed through a

simple phenol:sevag extraction after tissue homogenisation, enzymatic RNA and protein digestion.

DNA is hydrolysed to single nucleotides so that the 3'-phosphate group is still attached to the nucleotides, a requirement for the later radioactive labelling step (Figure 12). If hydrolysis is not complete, the hydrolysate will contain di- and oligonucleotides, and adducts of these, which can confound analysis since they result in HPLC peaks that – although they may contain certain adducted moieties – will not behave like mononucleotide adducts. Di- and oligonucleotides can be eliminated using Nuclease P1 (NuP1) in the enrichment step, but NuP1-treatment also destroys DNA adducts of aromatic amines and nitro-compounds, why it was not used in these studies. Since the concentration of adducted nucleotides are much less then those that are un-damaged, the hydrolysed DNA and nucleotide samples are adduct-enriched by Butanol (BuOH) extraction (Figure 12).

The major obstacle and source of problem regarding hydrolysis is the SPD enzyme. The activity varies with supplier and sometimes even between batches from the same supplier. During a meeting in Heidelberg, 27^{th}-29^{th} of September 2006, several laboratories presented the problems with the varying results obtained, partly due to the SPD, but also due to varying protocols and work-up methodologies.

The BuOH-extracted samples are labelled with a ^{32}P-phosphate group using T4-PNK enzyme and [gamma]^{32}P-adenosine triphosphate (^{32}P-ATP). Labelling is performed by the PNK enzyme, which takes the radioactive γ-phosphate from an ATP molecule and attaches it to the 5'-end of the hydrolysed and enriched nucleotides (Figure 12). Both adducted and normal nucleotides are labelled as well as di- and oligonucleotides. By using Apyrase, an ATP diphosphohydrolase the unspecific background from ^{32}P-ATP in analysis can be reduced, but in our method we instead open a valve in the analysis system, which shunts most of the unwanted free phosphate and ATP to waste, and thereby reduces the radioactivity background.

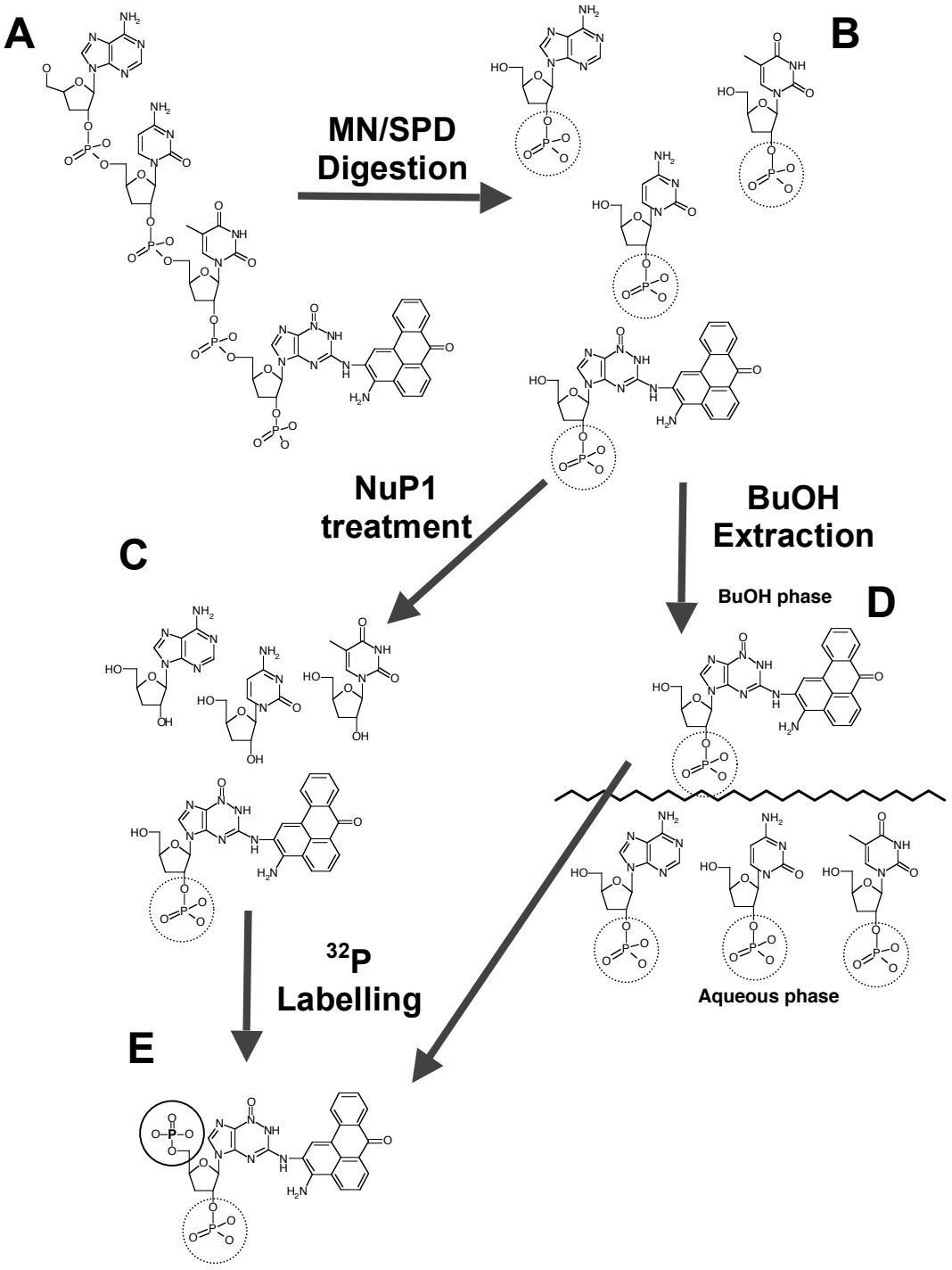


Figure 12. Schematic picture of the ^{32}P-postlabelling procedure. A) DNA is enzymatically digested, so that the 3'-photsphate on the nucleotides is still attached to the nucleotides (B). Following digestion, DNA adducts must be enriched and separated from the normal nucleotides. Nuclease P1 (NuP1) treatment (C) will result in that the 3'-phosphate is selectively removed from normal nucleotides, and some C8-coupled dG adducts. Through butanol (BuOH) extraction (D), the DNA adducts will prefer the more lipophilic BuOH phase, whereas the normal nucleotides will remain in the aqueous phase, which is discarded. For labelling to occur (E), the DNA adducts are mixed with radiolabelled ATP and a kinase, that will transfer the radioactive phosphate from the ATP molecule to the adducted nucleotide.

The HPLC consists of five major units; one (isocratic) or several (gradient) eluent reservoirs, a pump, a sample injector, one or a set of columns, and a detector (Figure 13). The ^{32}P-HPLC system used in the studies presented in this thesis use a Waters 600 E pump (Waters, Milford, MA, USA), also containing a 4-way-mixing valve, which mixes four different eluents. The sample is injected, diluted to 170 µl, into the eluent stream via the injector. The mix of eluents (in the case of gradient analysis) carries the sample to the columns, on which substance separation and resolution is obtained. The pump is adapted to work under high pressure to press the injected sample through the silica packed columns.

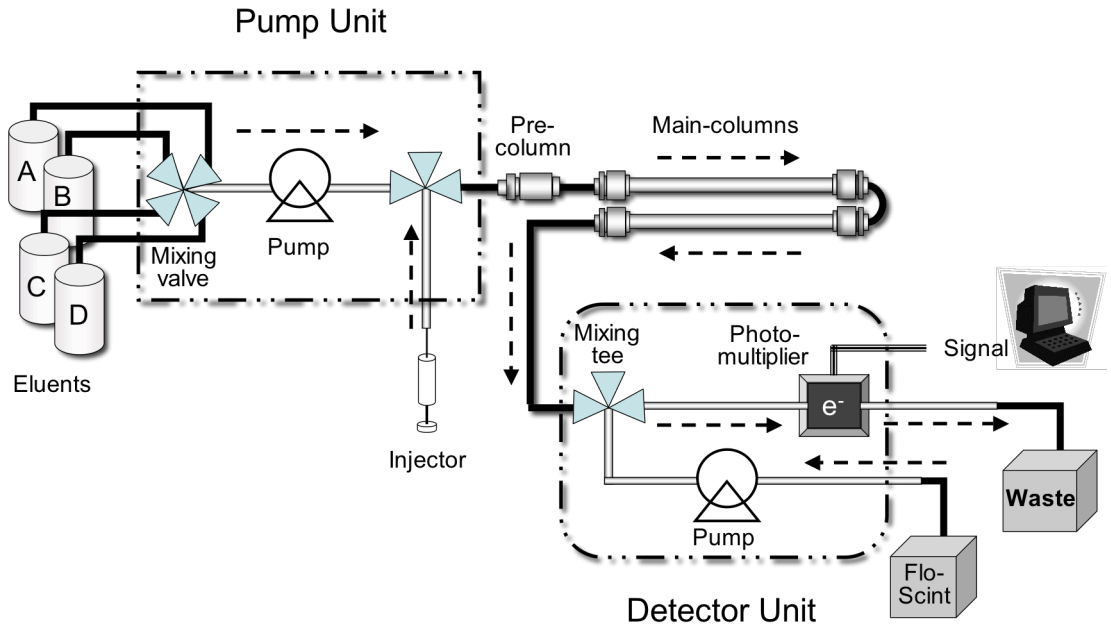


Figure 13. Scheme over the ^{32}P-HPLC system. The pump unit is where the proper mixing and pumping of eluents occur. The dashed arrows point in the direction of the eluent flow through the system. A coarse separation occurs at the pre-column and a fine separation at the serial coupled main columns. In the detector the sample is mixed with scintillation fluid before entering the photomultiplier cell, where electric signals are registered by a computer. The sample is then shunted to the waste.

The eluents used in our system are as follows; **A** consists of 2M ammonium formate (Af) and 0.4 M formic acid (Hf) (pH 4.5), **B** is made up of 50 mM Af and 20 mM Hf (pH 4.0), **C** contains 87.5% Acetonitril (MeCN):H_2O, and **D** is 100% Methanol (MeOH). In most cases, a linear gradient (Figure 14) can be used to adequately separate different analytes. The linear gradient used in our studies consists of the combination of the **A** and **C** eluent, where **C** is increased until the concentration of MeCN reaches 35% after 70 min. The **B** and **D** is used to wash out remaining substances from the columns, and **D** also for long-term storage, since MeOH is inert to column packing. During this linear gradient a constant flow of 0.5 ml/min is pumped through the system.

Sometimes resolution is very poor due to similar structures and physicochemical properties of different compounds. In those cases a so called plateau gradient can be employed (Figure 14). The plateau gradient with a flow rate of 0.5 ml/min is used as follows in this case: 0–19% of 87.5% MeCN:H_2O, during 0–33 min. A plateau follows holding this mixture for 15 min. Then the amount of 87.5% MeCN:H_2O is increased to 40% during 48–90 min.

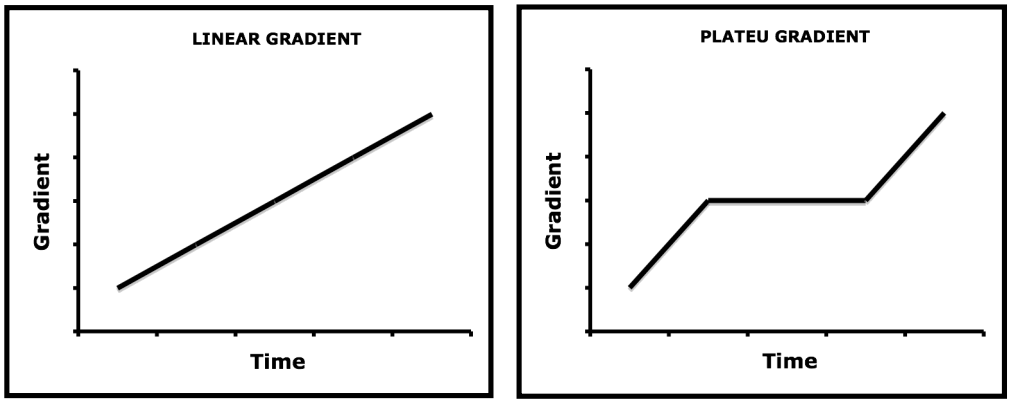


Figure 14. Schematic representation of the concept of linear and plateau gradient. During the linear conditions there is a constantly changing mix of eluents throughout the time of analysis. In the case of the plateau gradient, there is a changing mix of eluent in the beginning of analysis, then a specific mixture is kept isocratically for a certain amount of time, until the changing mix is continued for the remaining time of analysis. The plateau method is employed in order to increase separation between substances that behave very similarly in on the separation columns. Hence, in the HPLC system, separation is not solely dependent on column properties (solid phase) but also on the eluents (mobile phase).

The guard column, Hichrom, RP 5-C18, K-100 (Hichrom Ltd., Reading, UK), coarsely separates the substances, mainly free phosphates and ATP and to a certain extent normal nucleotides from the more lipophilic adducted ones. This small separation is not enough to give good resolution, but enough to be able to shunt the unwanted substances to the waste under a period of 1 min. The final separation is performed by two serial coupled main columns, reverse-phase DeltaPak 150 mm × 3.9 mm i.d., 5 μm 100 A (Waters, Milford, MA, USA).

Detection of the radioactively labelled analytes is performed by a Packard 500 TR flow scintillation detector (Packard Instrument Co., Meriden, CT, USA), a photomultiplier detector, meaning that it photoelectrically enhances the signals from radioactive decay. Sensitivity is increased by mixing the eluent with scintillation fluid (Radiomatic FloScint IV). The optimal energy window for the detection of ^{32}P-labelled samples is 8,000 – 600,000 electron-volt, which does not give rise to an increase of background radiation and detection efficiency of ^{32}P of 60%.

Data files containing raw data from the detector are processed by a script especially written for this purpose and designed by Dr Magnus Zeisig in the spreadsheet program Informix Wingz. This script corrects the data for intensity decline owing to radioactive decay over time and continuing efficiency in the detector, and detects, validates and quantifies peaks based on radioactivity statistics, automatically printing a report containing the chromatograms, run data and level of peaks.

The strength of this method is its sensitivity and low LOD. The postlabelling method employing the TLC, is about an order of magnitude more sensitive, but then again detection with autoradiography normally requires at least 24 h exposure, whereas the time for detection in the detector cell is 12 seconds. The method has also good reproducibility, but the entire work-up procedure is dependent on sometimes instable enzymatic reactions. Small and polar adducts are also a problem to separate well from normal DNA compounds.

During data processing, some problems can arise from local elevations of chromatogram background or partly unresolved peaks, requiring manual intervention.

9.4.2. HPLC-EC/UV

HPLC–EC/UV is a method for measurement of oxidative base damage to DNA, especially 8-oxo-deoxyguanine (8-oxo-dG). An extensive effort has been done within the ESCODD network (European Standards Committee on Oxidative DNA Damage) to optimize and validate different methods for measuring DNA damage [242,243]. The absolute background level of 8-oxodG is difficult to estimate because it is easily formed during the isolation and hydrolysis of DNA, leading to overestimated levels [244-247]. Also, 8-oxodG is more easily oxidized than guanine and can be further oxidized into secondary oxidation products [244,248]. In order to reduce oxidation during sample preparations, samples should be kept chilled and only high purity chemicals and enzymes should be used. Furthermore, the antioxidant desferoxamine mesylate, which is a Fe3+ chelator, also seems to reduce artifactual oxidation and Chelex 100 resin can be used to remove metal ions [245].

Procedures are performed rapidly and on ice, and all aqueous solutions are treated with Chelex 100 resin for 1 h, to remove metal ions, and then filtered through a CN 0.45 μm filter. Desferoxamine mesylate is added to isotonic buffers along with Triton X-100, after which the buffers are kept on ice. Cells and tissue, that have been kept on ice are thawed, homogenised centrifuged and washed to obtain the crude nuclei pellets. The pellets are dissolved and incubated in 3 M guanidine thiocyanate and transferred to pre-centrifuged Phase Lock Gel™ (PLG) tubes, where the nucleic acid is separated and then purified by a Sevag extraction and washing. DNA is then dissolved, enzymatically digested and filtered to remove enzymes.

The HPLC-EC/UV system consists an isocratic Scantec 650 pump (Scantec, Partille, Sweden), a 1 mm RP-C18 Opti-Guard column (Optimize, Portland, OR), two serial coupled DeltaPak 150 mm × 3.9 mm i.d., 5 μm 100 A main columns (Waters, Milford, MA, USA), an electrochemical detector Coulochem II (origin) and a UV detector 486 (Waters, Milford, MA, USA). Isocratic elution with 0.75 mL/min of 10% v/v MeOH:20 mM sodium acetate (pH 5.3) are used for separation of the analytes. The HPLC–EC/UV system is washed overnight with MeOH:H_2O (4:1) at a flow rate of 0.08 mL/min, and then re-equilibrated with the main eluent for at least 30 min the day of analysis. New calibration curves for 8-oxodG and dG are created each day of

analysis. The amount of oxidative damage is measured as the ratio between the level of 8-oxodG and dG. Hence, both have to be detected and this occurs at the same time. 8-oxodG is detected by the electrochemical detector, with the screen and analytical electrodes operating at +200 mV and +350 mV, respectively. dG is measured with the UV detector set at 290 nm. The detection limit is approximately 5 fmol for pure standard and 20 fmol for 50-100 µg of DNA)

One advantage is that with the use of standards, one can calibrate and precisely determine the amount of oxidative damage as 8-oxodG. The disadvantages are that if one is not careful during work-up, artifactual oxidation occurs rather easily and gives false positive results. This method also requires rather large amounts of DNA, since small amounts of DNA can give rise to very large deviation in 8-oxodG. Moreover, the LOD is several orders of magnitude higher then for ^{32}P-HPLC or the Comet assay.

9.4.3. Single Cell Gel Electrophoresis or Comet assay

The recommended alkaline (pH above 13) version of the Comet assay [249] is capable of detecting single-strand breaks (SSB), alkali-labile sites (ALS) DNA–DNA/DNA–protein cross-linking and SSB associated with incomplete excision repair sites. By using formamidopyrimidine-DNA glycosylase (FPG) treatment, the types of damage detected are increased: (i) open ring forms of 7-methylguanine, including 2,6-diamino-4-hydroxy-5-N-methylformamidopyrimidine and 4,6-diamino-5-amido-pyrimidine, (ii) 8-oxoguanine, (iii) 5-hydroxycytidine, (iv) 5-hydroxyuracil, (v) aflaoxin-bound imidazole-ring-opened guanine and (vi) damage induced by amino compounds such as imidazole-ring-opened-N2-aminofluorene-C8-guanine [250-252].

The triangular shaped A549 cell lines were originally obtained from the American Type Culture Collection [253]. Those cultured for the experiments for these articles have been provided by Prof. Ian Cotgreave, from the Dept. of Environmental Medicine at the Karolinska Institutet, Stockholm, Sweden. The rounded HepG2 cell lines have been provided by Prof. Joseph Rafter from the Dept. of Biosciences and Nutrition at the Karolinska Institutet, Stockholm, Sweden. When both A549 cells and HepG2 are cultured in parallel, the DMEM is supplemented with 2 mM L-Glutamate, since HepG2 has a somewhat slower generation cycle then A549.

Exposure is carried out for 24 h, after which the cells are washed and harvested by trypsination. Enzymatic activity is ceased by the addition of fresh supplemented DMEM, and cells are immediately placed on ice. Cytotoxicity tests are performed on each harvested batch by Trypan Blue staining, where up to 10% damaged cells are deemed acceptable. Performing a dose response for testing the maximum amount of 3-NBA that could be added to cells, it is clear that exceeding 50 µM give around 15-20% staining with Trypan Blue. Thus, the maximum concentration of 20 µM of the test substances is selected because pilot studies have indicated that this concentration is the minimum required by the method least sensitive in detecting effects by 2-NBA and 3-NBA, and the maximum allowed by those most sensitive.

Harvested cells are washed and centrifuged several times, so as to remove the remaining medium, then dissolved in PBS and added to 0.75% agarose, which is spread over an agarose pre-coated microscope slide. When the gels solidify, the slides are placed in an alkaline (pH 10) lysis buffer for 1 h on ice and in dark. DNA unwinding and washing is performed in alkaline buffer (pH>13) for 40 min, on ice and in dark. Electrophoresis is performed, after which the slides are washed and then dried over night.

When DNA is damaged, strand breaks occur or can be induced by such enzymes as FPG. The cells are moulded into a gel on a microscopic slide. The cell membranes and other cellular components are lysed and washed off, exposing the DNA and to the influence of the electric current imposed upon the slide. Since the gel is a polymer, which forms a mesh around the cells, there will be a certain restraint for the DNA to travel through the medium. Cells with intact DNA will not be able to move through the medium. However, fragmented DNA can move across the medium in the direction of the cathode, since DNA is negatively charged. Thus, the nucleus where the DNA is un-damaged will be well-defined and round, but will display a tail, which is both longer and more prominent as DNA damage increases.

Following this protocol, without FPG-enzyme treatment, will give information about general or background genotoxicity levels, but it doesn't say anything about oxidative damage. In order to detect in particular oxidative lesions, one has to treat the cells with FPG. This gives rise to additional DNA strand breaks from oxidative and some

other lesions, from which the background genotoxcity can be subtracted to obtain the oxidative lesions only. After the lysis treatment above, the slides are placed in enzyme buffer and FPG-treated in a humidity chamber at 37 °C for 30 min, and following this the slides are alkaline treated and electrophoresis is performed as previously described. This is followed by fixation in methanol for 5 min, drying of slides and then treatment with Ethidium bromide (EtBr) solution for 5 min. Longer time does not give any better staining results, thus excess EtBr is washed away and a cover slip is placed over the slides for cell counting.

By using the Komet™ Imaging Software, installed on a computer linked to a microscope with CCD camera, quantification of DNA damage and repair in single cell preparations can be performed. The borders and intensity of the tail and nucleus is measured automatically, but the program also allows the viewer to perform manual adjustments if needed. One way to present data is by using the % Tail DNA parameter, which is simply the amount of DNA in the tail compared to the head (or nucleus) of the comet (Figure 15).

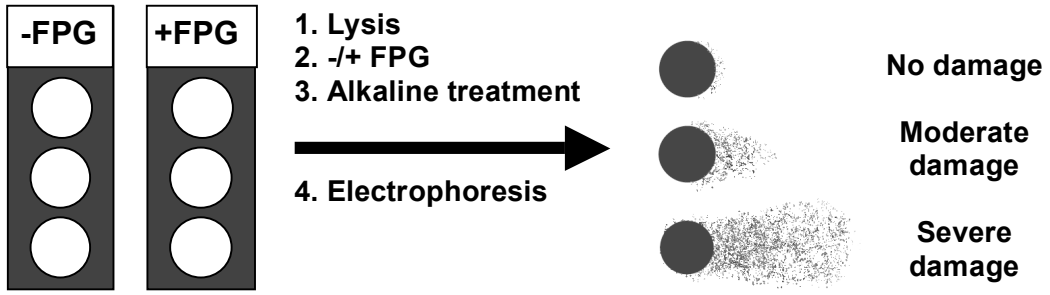


Figure 15. The principal steps in the Comet assay. Analysis on -/+ FPG are done in parallel. Lysis treatment destroys the membranes and other cell components to release DNA. FPG enzyme is added to enhance oxidative lesions and alkaline causes DNA to unwind. After electrophoresis and depending on the degree of DNA damage, the cells will display a "tail" which varies with the amount of DNA strand breaks.

An alternative parameter called Olive Tail moment can be used, which represents the product of the percentage of total DNA in the tail and the distance between the centres of mass of the head and tail regions [254]. This parameter takes into account the distance that the DNA has migrated and thus provides an indication of the pattern of

DNA damage in an individual cell. Olive Tail Moment = ($Tail_{mean}$ - $Head_{mean}$) X (% Tail DNA/100).

The Comet assay is a very sensitive method since only a few hundred cells are needed to obtain results; and usually preparing triplicates of samples does not demand more effort then preparing singlets. The limit is often only the size of the electrophoresis chamber, which can take only 18 slides at most. The system is also quite fast and results can be obtained over a day. Indeed, there are also some research adapting this method to a high throughput screening assay [255]. However, a certain amount of cells need to be counted and this if often done by humans, not machines. Cell counting takes time and there is always the risk of being biased by e.g. apoptotic cells and cells with deviating morphology.

Other things worth taking into consideration is that the Comet assay is often used to measure oxidative damage, but it should not be confused with solely 8-oxodG, although it is recognised as a marker for oxidative stress. FPG enzyme treatment can reveal other lesions as well, thus further evaluation of this is needed of the types of damages one is looking at.

10. Discussions on 3-NBA and its isomers

10.1. Paper I and II – *IN VITRO*

There are numerous studies revealing the genotoxic potential of 3-NBA *in vitro*. Some deal with mutagenicity and induction of micronuclei (MN), [85,256], whereas many of them inform on such genotoxic potential as DNA adduct formation [71,176,179,181,186,257,258]. In Papers I and II, the DNA adduct forming capacity of 3-NBA (16A), along with BA, 2-NBA and 3-ABA, was determined, also the non-oxidative (16B) and oxidative (16C) DNA lesions detected by single-cell gel electrophoresis (Comet assay) were determined.

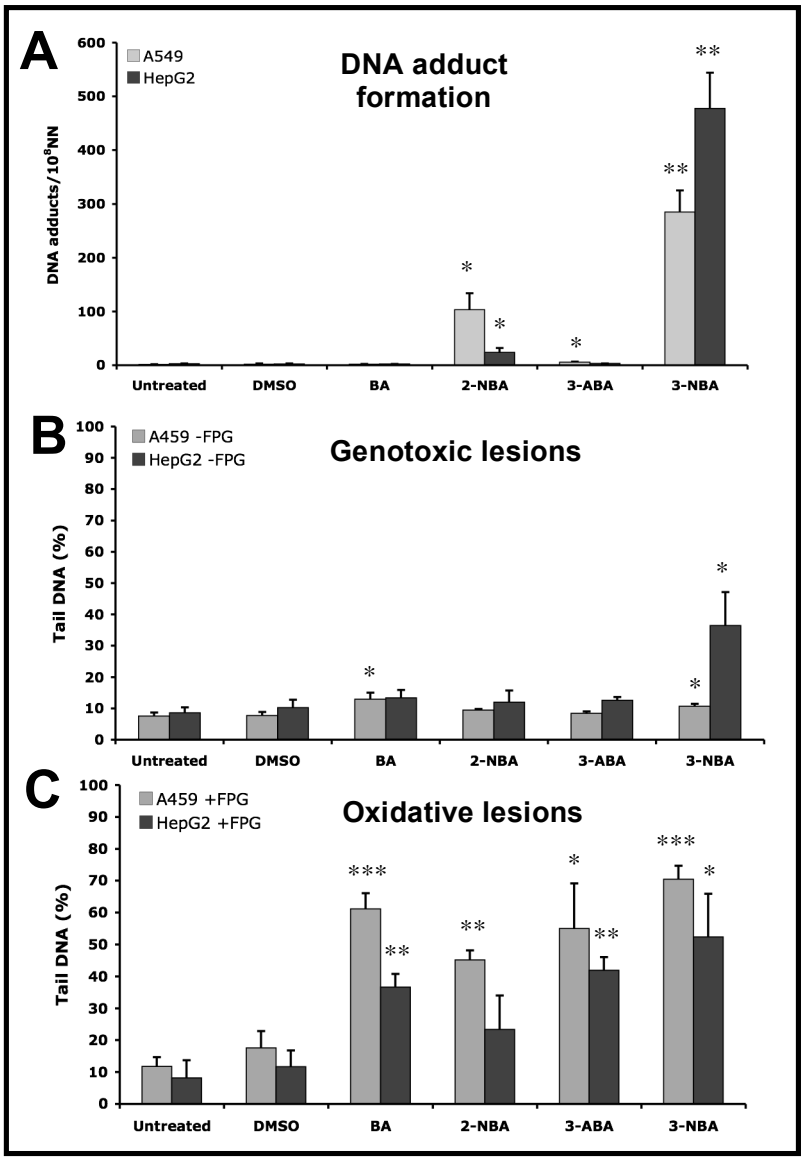


Figure 16A-C. Different genotoxic insults on DNA, caused by a variety of benzanthrone compounds.

Induction of MN has been observed for 3-NBA treated human B-lymphoblastoid (MCL-5) and HepG2 cells [85,179,256]. In the HepG2 cells, a 2,000-fold lower concentration of 3-NBA was required to induce a similar MN frequency as BaP did at 50 μM. In addition, 3-NBA also exhibited a strand breaking activity measured by the Comet assay at a 1,000-fold lower concentration as compared to BaP [85]. In Paper I and II, no positive controls were used, since experience as well as previously presented studies on human cell lines clearly revealed the undoubted genotoxic potential of 3-NBA with regard to DNA adduct formation as well as strand breaks [70,187]. In both papers, 2-NBA was about 30% as genotoxic with regard to DNA adduct formation (Figure 16A) as 3-NBA, and about 40-60% (Figure 16B and 16C) as genotoxic with regard to oxidative DNA damage. The level of DNA adducts from 2- and 3-NBA in Paper I and II, is basically the same. In Paper II, the lack of difference, although the concentration is 10 and 20 μM in the two studies, suggests that there might be a saturation of the enzymatic systems either activating the substance or detoxifying the cell and/or DNA, similar to what we have previously observed for crude oil distillates [8]. While we could detect adducts and measure oxidative damage by the Comet assay, HPLC-EC did not reveal any elevated 8-oxodG level. However, it has previously been reported that in the case of peroxynitrite, the levels of 8-oxodG were low and even dropped, whereas other base oxidations increased [248]. Moreover, 8-oxodG is also more easily oxidized than dG itself and can be further oxidized [244,248,259]. At the 50 μM level, elevated levels of 8-oxodG could be detected by HPLC-EC/UV, but this concentration was cytotoxic. The high levels of oxidative damage in both cell lines by all four samples (Figure 16C) suggests that the NBAs and 3-ABA in part would go through the quinone-reduction pathway just as the parent compound BA (Paper II). The oxidative processes could occur either by the induction of CYP1A1 or via the AhR mediated mechanism [260-262]. The level of DNA damage induced by BA was significantly higher in lung cells (Figure 16C). Although no studies could be found regarding BA using A549 cells, a study by Singh et al (1971) showed that (it) instillation of BA in guinea pigs caused a hemorrhagic oedema [263], a feature similar to what was observed in a few cases among F344 rats following it-treatment with 3-NBA (Paper IV) [191].

Interestingly, the difference in DNA adducts and oxidative lesions between A549 and HepG2 is quite large with the highest level in the liver cell line after 3-NBA exposure (Figure 16A and 16B), but the case was quite the opposite regarding oxidative lesions (Figure 16C) measured by the Comet assay (Paper II) [70]. The DNA adduct pattern, however, is very similar between both cell lines regarding DMSO, BA, 2-NBA, 3-ABA and 3-NBA (Figure 17A and 17B). With the ^{32}P-HPLC at least 10 different peaks could be seen from 3-NBA, but there were some five major ones that dominated in both cell lines (Paper I and II). Utilising the ^{32}P-TLC method with postlabelling, other laboratories have shown that autoradiographic profiles of 3-NBA derived DNA adducts express six major peaks; five after BuOH enrichment, but only four when using NuP1, which suggests that at least one of the detected spots is a C8-coupling. In addition, the autoradiographic profiles are virtually the same *in vitro* as those as generated *in vivo*.

Previously reported findings of DNA adducts from 3-ABA [181], could not be confirmed in the A549 and HepG2 cell lines with the ^{32}P-HPLC system. A possible explanation is the difference between cell lines. Using recombinant human CYP1A1 and 1A2 expressed in Chinese hamster V79 cells and microsomes of baculovirus-transfected insect cells (Supersomes), it was found that these enzymes are required for the activation of 3-ABA and the formation of DNA adducts as a consequence. Essentially the same DNA adduct pattern was detected in the metabolically competent human lymphoblastoid MCL-5 cells expressing P450 1A1 and 1A2 [181]. HepG2 cells are known to have nitro reductase activity [264], and reduction of nitro-heterocycles in A549 cells occurs mainly through the catalysis by NADPH-requiring enzymes [265]. It has been shown that NADPH:P450 reductase, which is expressed in bronchial and bronchiolar epithelium, alveolar lining cells and alveolar macrophages, does reduce nitrated xenobiotica and thereby activate it [266,267], but there can be large differences between native and immortalised cell lines in the expression of specific activating enzymes. In hamster lung fibroblasts, the strongest expression, due to exposure to 3-NBA, has been observed for CYP1A1, -1A2, -2B6 and -2D6, although others have been seen as well (CYP1B1, -2A6, -2C9, -2E1, -3A4) [178]. The induction of CYP1A1 and -1B1 has been observed in A549 cells treated with different kinds of nitrated polyaromatic hydrocarbons [128]. However, the induction of CYP1A2 was not induced to any greater extent in that study, most likely because in

humans this enzyme is said to be liver-specific [130] and its expression is exceptionally low, if not absent, in tissue-derived cell lines [268]. Therefore, it is difficult to compare results obtained from different cell lines. It should be noted, however, that the expression of CYP1A2 has been detected in some level in lung carcinoma cell lines, which are not entirely alike native cells [128].

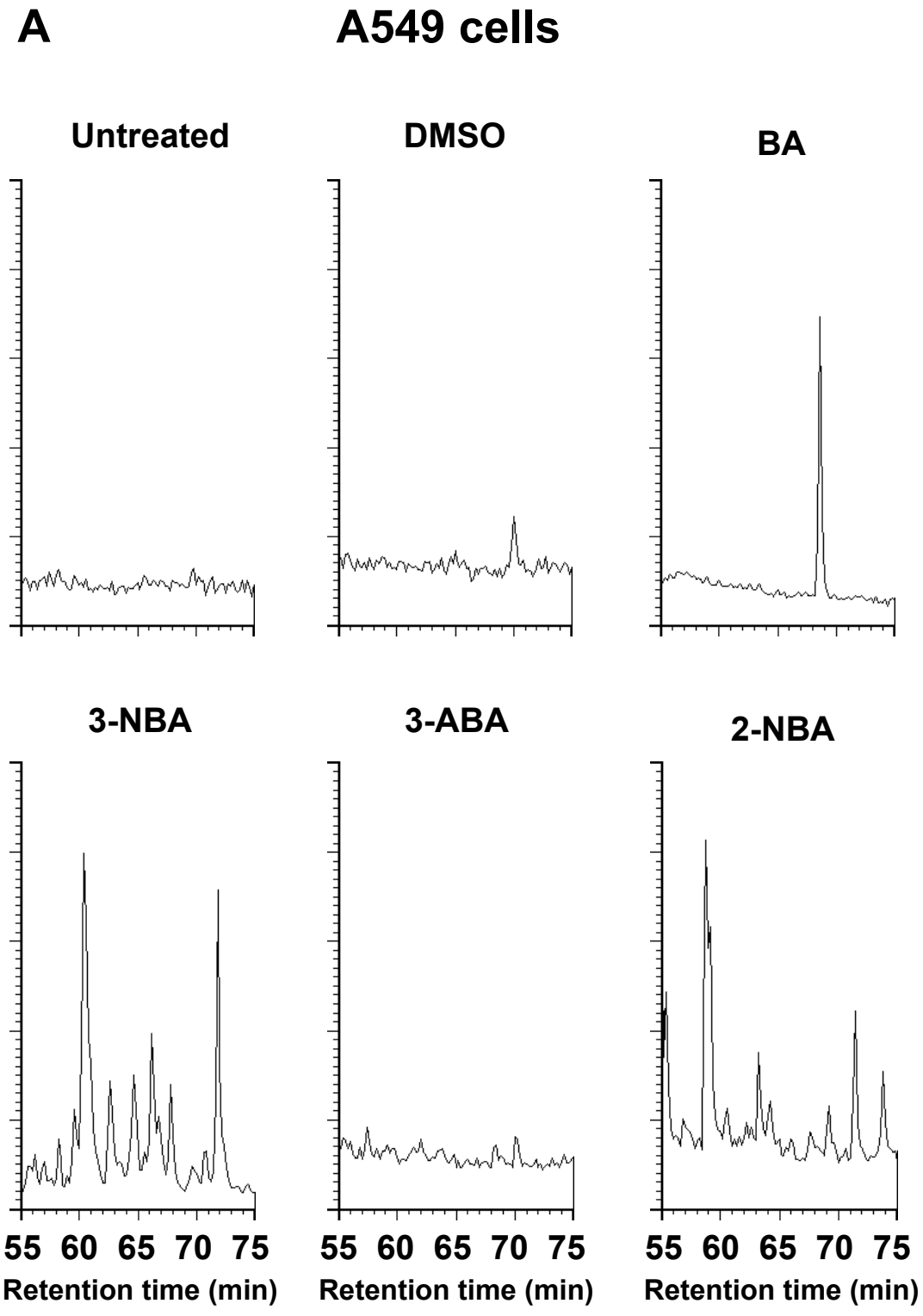


Figure 17A. The figures depict representative ^{32}P-HPLC chromatograms of DNA adduct pattern from A549 cells untreated or treated with 0.5% DMSO as well as 10µM BA, 3-NBA, 3-ABA and 2-NBA. Note: The labels on the y-axis are not presented, since the chromatograms are adjusted to clearly depict the DNA adduct pattern, rather then the intensity.

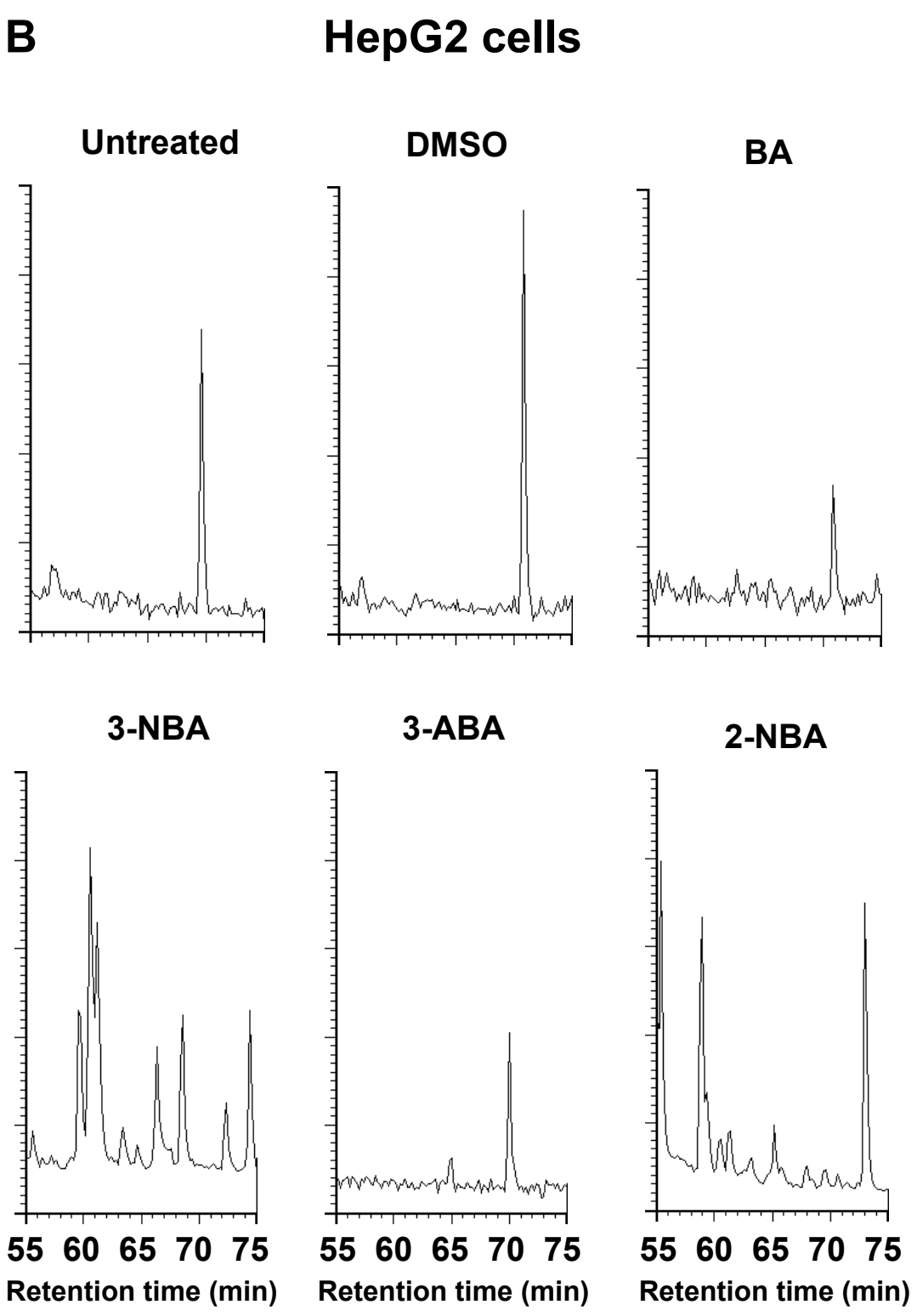


Figure 17B. The figures depict representative ^{32}P-HPLC chromatograms of DNA adduct pattern from HepG2 cells untreated or treated with 0.5% DMSO as well as 10μM BA, 3-NBA, 3-ABA and 2-NBA. Note: The labels on the y-axis are not presented, since the chromatograms are adjusted to clearly depict the DNA adduct pattern, rather then the intensity.

10.2. Paper II and III – *IN VIVO*

The wide range of DNA adducts from 3-NBA, more than 10, has been observed in both Paper I – and II) and many of these are very similar to what has been seen *in vivo* (Paper II-IV) [70,187,191]. The similarities between the DNA adduct patterns *in vitro* and *in vivo* (Figure 17A-B, and 18A-B) has also been observed by other laboratories [174,175,183,189,269]. In addition, the DNA adduct pattern for 3-NBA has generally been the same between tissues, although with varying levels, regardless of administration route (Figure 18A) [70,174,175,187,191,269]. However, there are some discrepancies between *in vitro* and *in vivo* DNA adduct patterns regarding 2-NBA (Figure 18B) [70]. This small variation could be a question of the more extensive metabolism in animals compared to selected cells. It could also depend on the visibility of peaks: At higher doses the prominent peaks tend to over-shadow the smaller ones, which are at lower doses quite distinguishable from the background and adjacent signals. In the opinion of the authors, however, it is more likely to depend on the different cell systems rather then chromatographic interpretations, since *in vivo* systems involve a more extensive metabolism then specific cells.

In vivo, DNA adduct level generated by 3-NBA shows rapid increase with time, with a subsequent decrease (Paper II-IV), while the 2-NBA generated DNA adduct levels continue to increase throughout the experiment (Paper II) (Figure 18C and D) [70,187,191].

This "lagging" effect of 2-NBA is believed to be a consequence of its higher lipophilicity and lower solubility compared to 3-NBA (Paper II). *In vivo* studies have shown that PAHs are differently absorbed in the intestinal tract, mainly according to their physicochemical properties, particularly their lipophilicity [270]. For instance, it is suggested that since PAH metabolites are usually less lipophilic compared to the parent molecule, PAH metabolism in enterocytes can positively affect their intestinal transfer [271]. In addition, it has also been shown in the case of 2-acetylaminofluorene that lipophilic compartments in the body, such as membranes, can also affect bioavailability [272,273]. This potential slow "leakage" urges for further studies on 2-NBA, since the continuous exposure to this substance may pose a health risk.

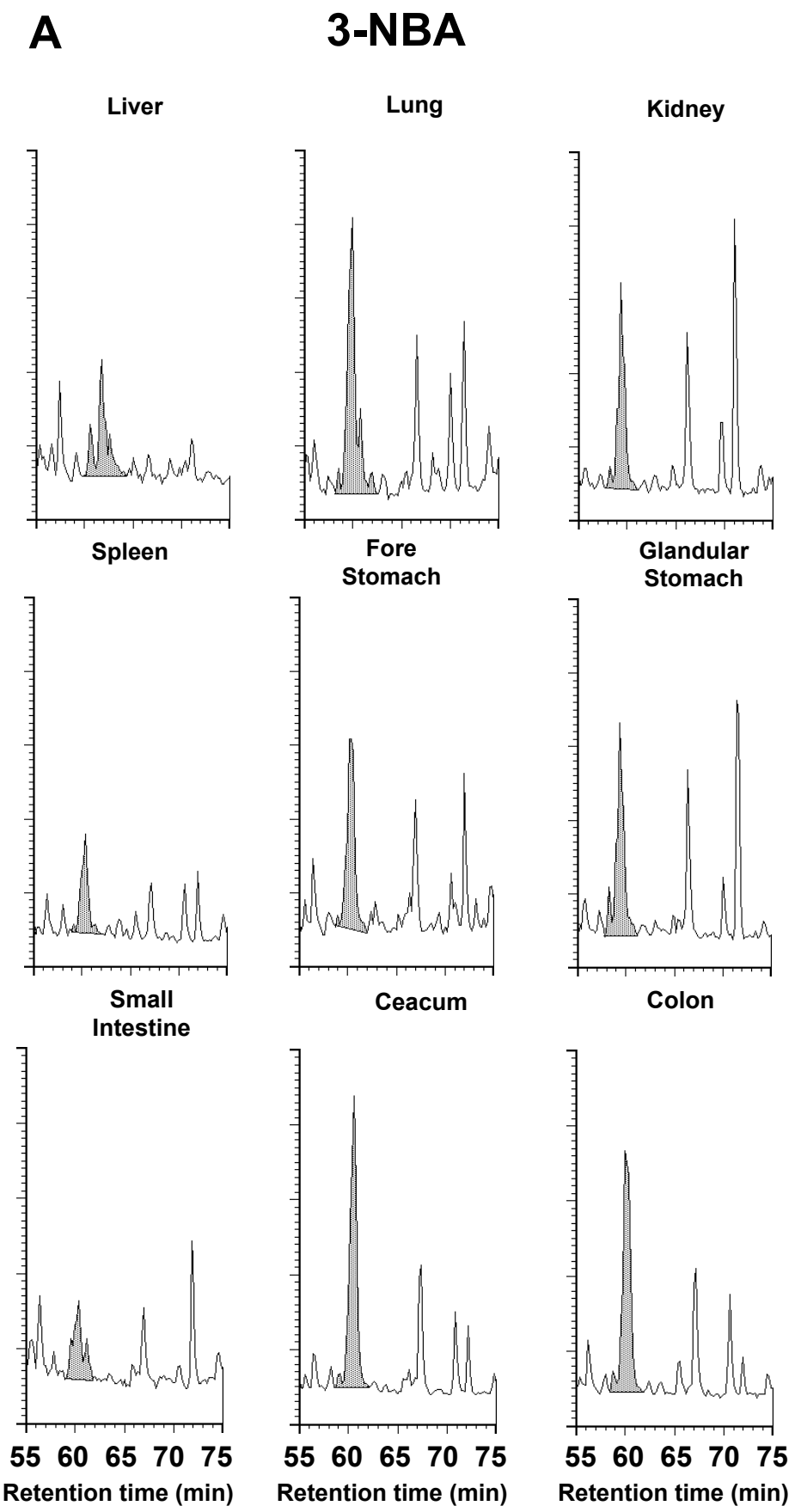


Figure 18A. Representative chromatograms of different tissues following intratracheal exposure to 5 mg/kg bw of 3-NBA. Note: The labels on the y-axis are not presented, since the chromatograms are adjusted to clearly depict the DNA adduct pattern, rather then the intensity.

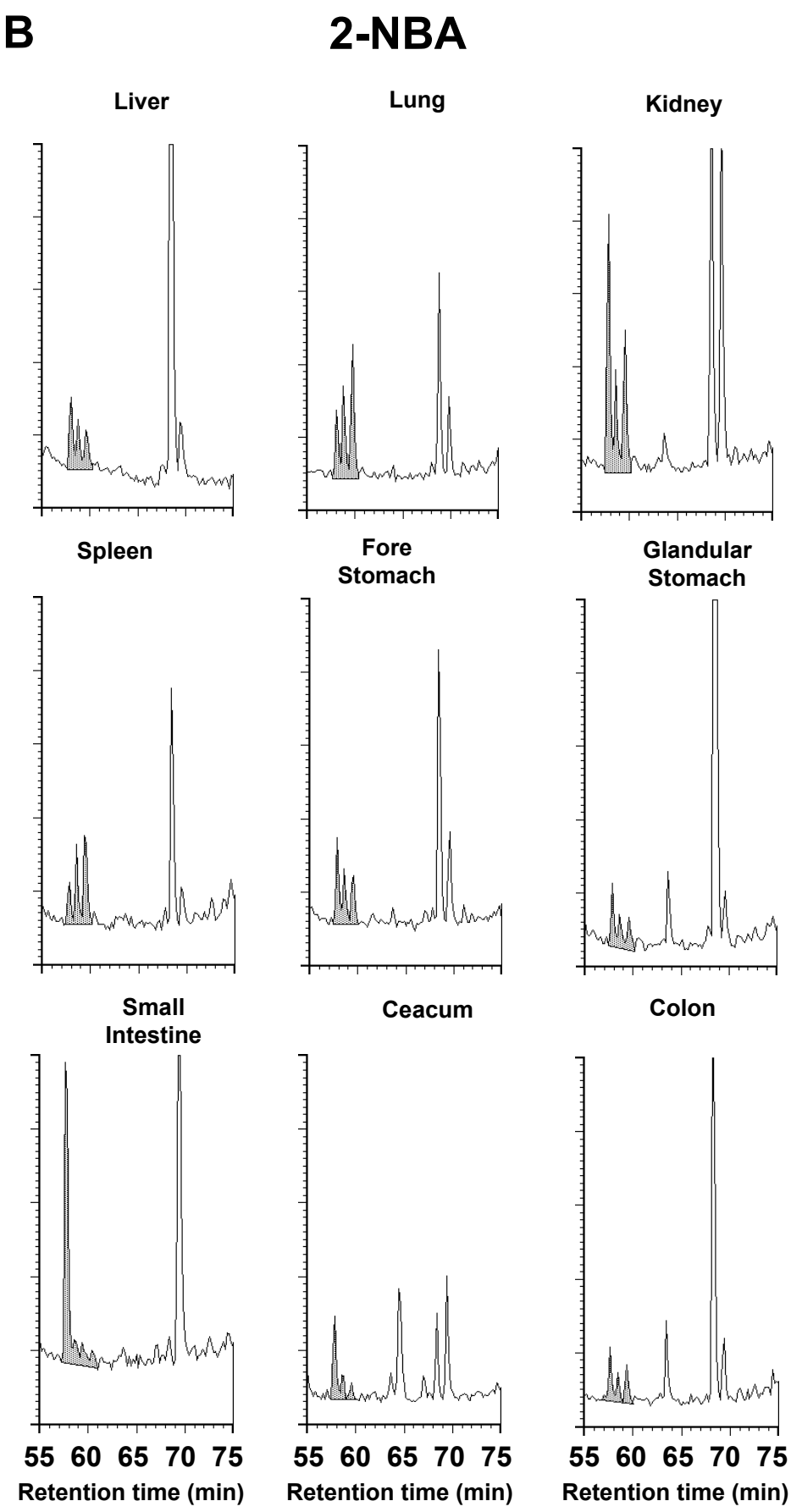


Figure 18B. Representative chromatograms of the DNA adducts patterns in different tissues following intratracheal exposure to 5 mg/kg bw of 2-NBA. Note: The labels on the y-axis are not presented, since the chromatograms are adjusted to clearly depict the DNA adduct pattern, rather then the intensity.

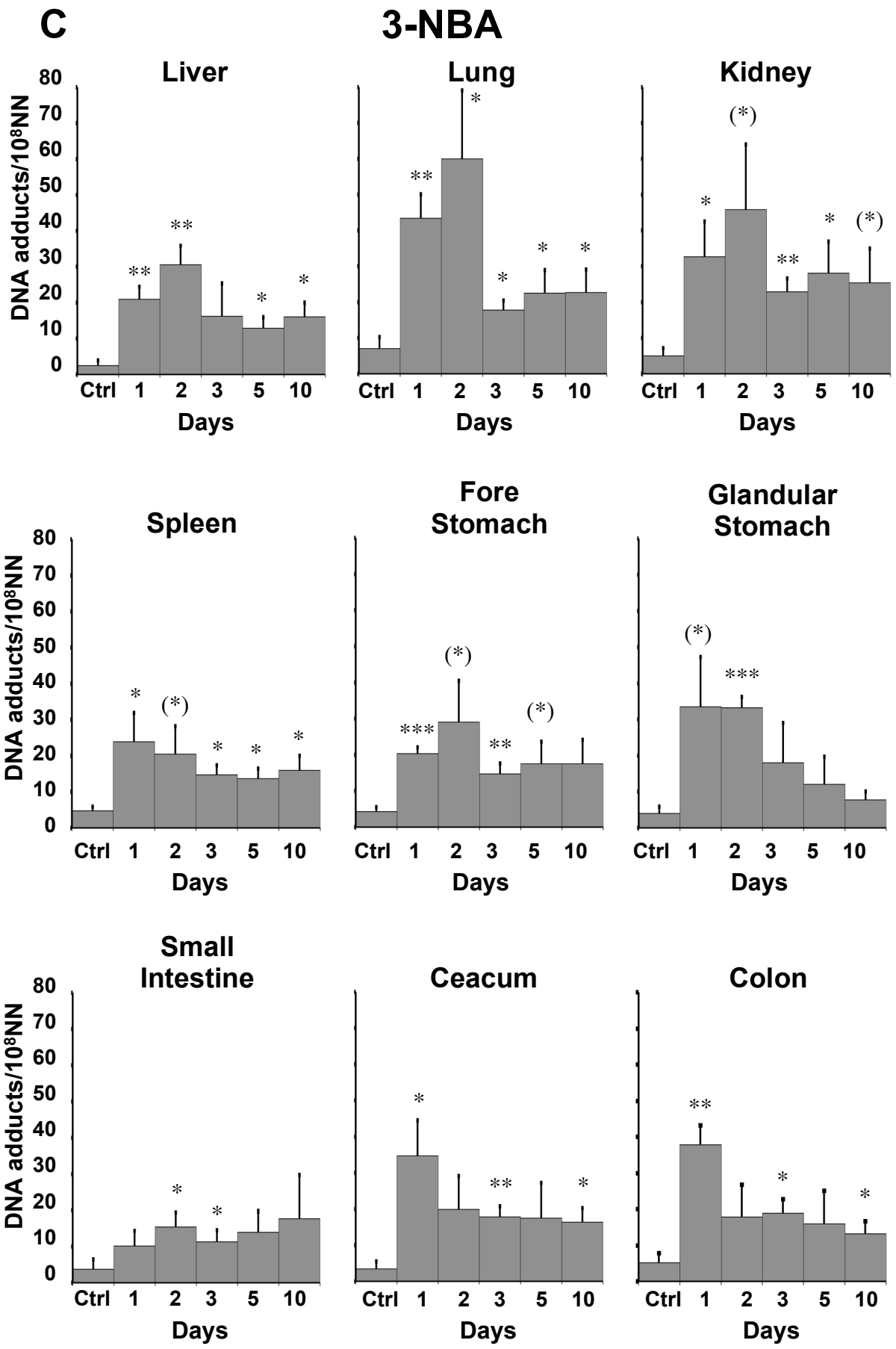


Figure 18C. The diagrams depict 3-NBA (5 mg/kg bw) derived DNA adduct levels varying with time. N=3 at each time point, except for control where N=5. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and (*) borderline significance p= 0.05-0.07, all compared with control.

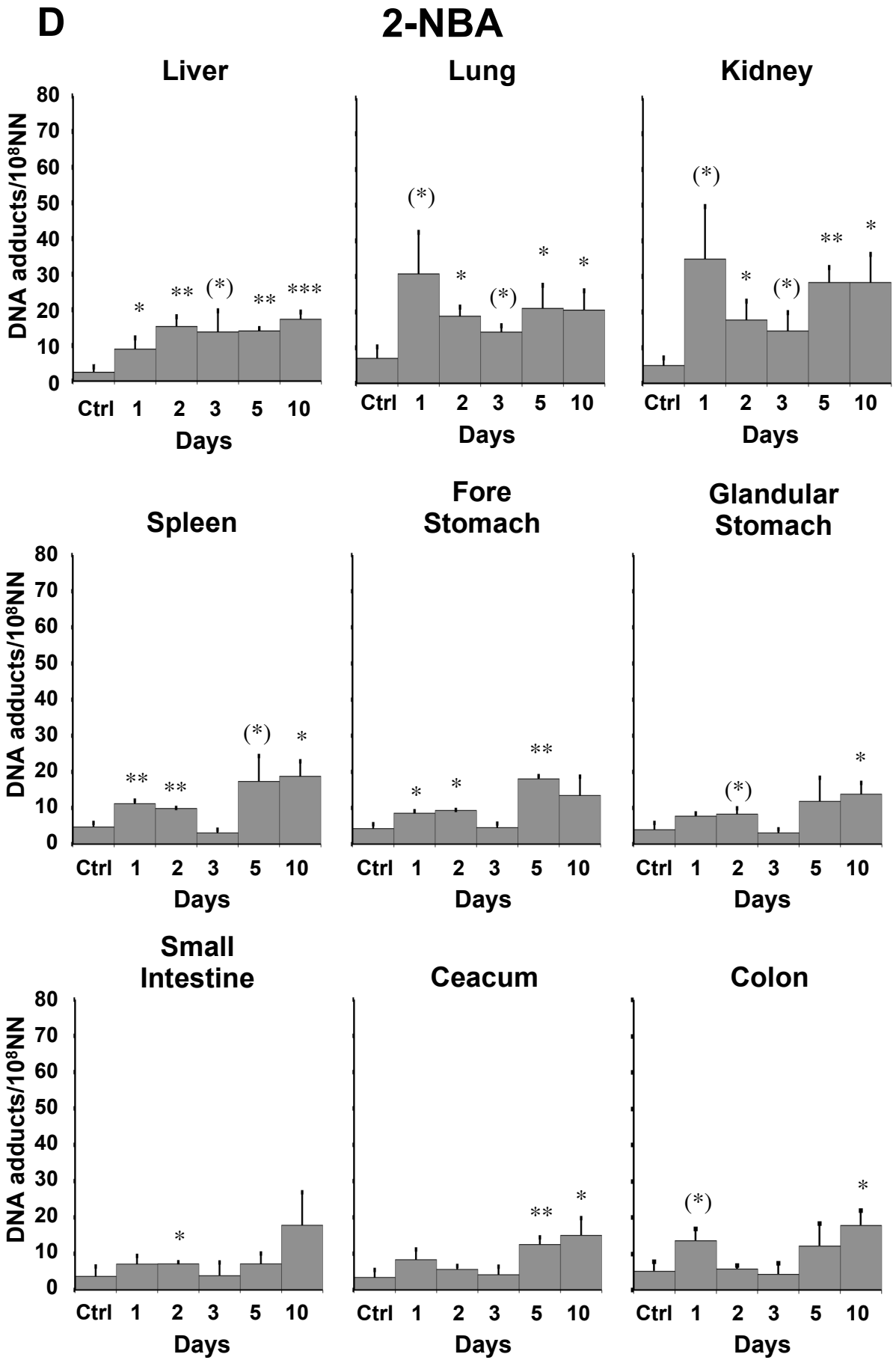


Figure 18D. The diagrams depict 2-NBA (5 mg/kg bw) derived DNA adduct levels varying with time. N=3 at each time point, except for control where N=5. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and (*) borderline significance p= 0.05-0.07, all compared with control.

Also, the amount of DNA adducts formed by 2-NBA are approximately 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 18C and D), which is far more then observed *in vitro* (Paper I and II). Granted, it 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 4-55% (Paper III). 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-synthesised standards available for 2-NBA as yet, to estimate the recovery or do characterisations. Hence, the comparison is only based on the relative levels observed in the tissues.

A sudden drop in DNA adduct level was observed for 2-NBA [70] and 3-NBA [187] 3-5 days after intratracheal administration. The difference in decrease in DNA adduct levels between the two studies can in part be explained by the different administration routes. However, the drop in the DNA adduct level was also consistent with the acute toxicity and tissue damage observed following administration. As the tissue damage accumulates, the DNA adduct level drops and once re-generation occurs, the DNA adduct levels start increasing again (Figure 19).

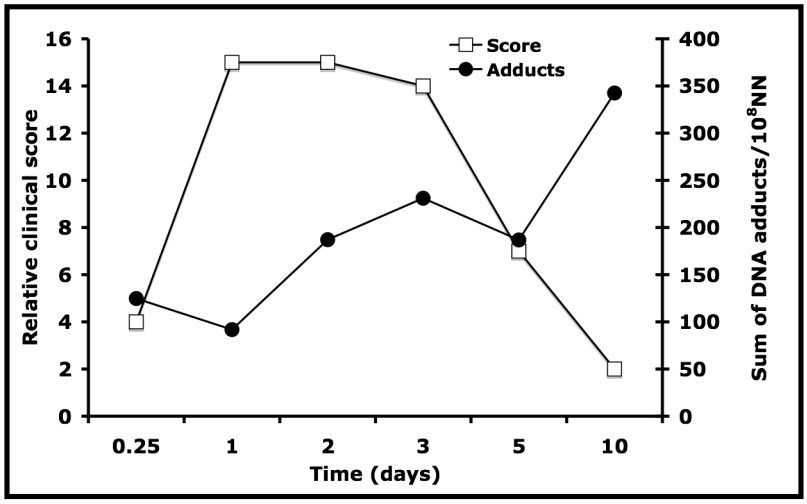


Figure 19. Clinical score depicted against DNA adduct formation over time. This diagram shows how DNA adduct formation is affected by tissue damage.

In a number of *in vivo* studies with 3-NBA administration by different routes [70,174,175,177,187,189,191,269], 3-NBA specific DNA adducts were detected at varying levels, but with similar profiles in all organs analyzed.

Another effect that has been measured is oxidative damage *in vivo* (Paper II) [70], which was elevated in blood at both 5 and 10 days post intratracheal instillation, but only at 10 days regarding DNA adduct formation for 3-NBA. The peaks detected in blood were dG-N2-C2-ABA and dG-C8-N2-ABA. 2-NBA did not give rise to any elevated level of oxidative damage or detectable amounts of DNA adducts.

10.3. Paper IV - CARCINOGENICITY

In Paper II-IV it is rather evident that the majority of the DNA adducts are cleared from the DNA within a few days. However, this does not mean that there are no persistent DNA adducts present in the tissues. In paper IV, it was mentioned that since the DNA adduct level was at a background level after 16 days, perhaps there were no persistent adducts present [191]. This should be interpreted as no persistent DNA adducts present at levels detectable with ^{32}P-HPLC. Persistent 3-NBA adducts in the lungs of Sprague-Dawley rats were detected 36 weeks after single intratracheal instillation of only 0.2 mg/kg bw (Bieler CA. unpublished data). Humans are often subjected to lower levels of substances, but repeatedly and during a longer period of time. Repeated dosing of 3-NBA (2 mg/kg bw once a week for 6 weeks) has shown that maximum levels were reached after 1 week, and even after 18 weeks the level of DNA adducts (~100 DNA adducts/10^8NN in the lung and kidney) were as high as when administration stopped and there was no trend towards decrease at the end of the experiment (Nagy E. unpublished data).

3-NBA is known to induce frame-shift mutations [51], GC→TA transversions [189], micronuclei [85] and DNA strand breaks [70,71,85,179]. If some of these modifications of DNA occur at critical genes, e.g. oncogenes, then initiated cells can develop [274]. Ergo, there is abundant evidence showing that 3-NBA can initiate cells. Which of these mechanisms that are the most important is hard to say. It is a fact that 3-NBA gives rise to lung cancer in treated animals within seven months,

which classifies 3-NBA as a lung-carcinogen, unusual for nitro-PAHs (Paper IV) [191]. 3-NBA gives rise to squamous cell carcinoma as the predominant tumour type, although a few cases of squamous metaplasia and adenocarcinoma were also observed. However, due to the acute toxicity, explained above, similar doses as those used in studies on 1,6-DNPy [275], could not be employed due to induction of fatal inflammations in the airways of the exposed animals (Paper IV).

Car of the future (hopefully mine some day), SAAB Aero X Prototype, with advanced V6-twin turbo BioPower engine that can deliver 400 bhp at 5,000 rpm. Runs on 100% Bioethanol for a clean conscience. Photo taken at the International Car Fair 2006. © Michael Johansson.

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11. Conclusions

DNA adducts are believed to be an important step in chemical carcinogenesis. Some of them are not formed by natural processes, and among these substances is 3-NBA, a combustions product from Diesel fuelled engines, which earlier have been shown to have a high genotoxic potential.

The ^{32}P-HPLC method was successfully employed in both evaluating the variation of the level of DNA adducts over time, and help revealing certain systemic features, such as the possible retaining of substances in the adipose tissue and the connection between the degree of inflammation and retarded metabolism was shown. The short-term studies on 3-NBA have shown that besides genotoxicity, such as DNA adducts and oxidative DNA lesions, it also induces acute toxicity at levels not seen by other nitro-PAHs.

3-NBA is known to rearrange in the atmosphere to its isomer 2-NBA. *In vitro* studies have shown that 2-NBA is about 1/3 as genotoxic as 3-NBA, but in animal studies it can form about 80% as high levels of DNA adducts as 3-NBA. Since 2-NBA exists in a 70-fold higher concentration in ambient air, compared to 3-NBA, it is worth asking whether this isomer can be a significant contributor to toxicity from Diesel emission polluted air.

The DNA adducts levels can very seldom be measured in tumour tissues due to the dilution by a growing cell mass. Long-term studies have shown that 3-NBA is a lung carcinogen in rats, at least as or perhaps even more potent than 1,6-DNPy. This urges for further studies and assessment of human exposures.

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