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Preneoplastic lesions, DNA adduct formation and mutagenicity of 5-, 7- and 9-hydroxy-2-nitrofluorene, metabolites of the air pollutant 2-nitrofluorene

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Abstract

The metabolites of 2-nitrofluorene (NF), 5-, 7- and 9-OH-2-nitrofluorene (OH-NF) were compared for their genotoxicity. Seventy-two hours after intraperitoneal administration of these substances individually to rats (100 mg/kg body wt.), DNA adducts in liver tissue were analyzed with ^{32}P-TLC and ^{32}P-HPLC. An in vivo liver model was used to test the initiating capacity of the said substances for the formation of preneoplastic lesions. 5-OH-NF showed low capacity to induce DNA adduct formation and low potential as initiator to induce preneoplastic lesions-foci/nodules in the liver of rats. Both 7- and 9-OH-NF induced DNA adducts and preneoplastic liver lesions but with smaller quantities compared to NF. It seems that 7- and 9-OH-NF can not be considered as detoxification products of NF. In general, the initiating capacity of these substances for the formation of preneoplastic lesions has a good correlation with their potency to form DNA adducts.

Keywords: Nitrofluorene; Hydroxy-nitrofluorene; Genotoxicity; Air pollution; Nitro-PAH; DNA adduct; Mutagenicity; Preneoplastic lesion

1. Introduction

Nitrated polycyclic aromatic hydrocarbons (nitro-PAHs) are found in urban air (Nielsen, 1983; Gorse et al., 1983; Ramdahl et al., 1982). Nitro-PAHs can be formed during incomplete combustion of organic substances such as petrol and diesel. Most of the nitro-PAHs are potent bacterial mutagens (McCoy et al., 1981; Tokiwa et al., 1981) and some of them have been shown to be carcinogenic to laboratory animals (Hirose et al., 1984; el-Bayoumy et al., 1982). 2-Nitrofluorene (NF) has been studied as a model compound for nitro-PAHs (Schuetzle and Frazier, 1986; Möller and Gustafsson, 1986; Möller et al., 1987, 1988, 1993a,1994; Beije and Möller, 1988). The bacterial mutagenicity of NF is ten thousand

Abbreviations: AAF, 2-acetylaminofluorene; AF, 2-aminofluorene; DEN, diethylnitrosamine; PAH, polycyclic aromatic hydrocarbon; MeCN, acetonitrile; NF, 2-nitrofluorene; Nitro-PAH, nitrated polycyclic aromatic hydrocarbon; 5-OH-NF, 5-hydroxy-2-nitrofluorene; 7-OH-NF, 7-hydroxy-2-nitrofluorene; 9-OH-NF, 9-hydroxy-2-nitrofluorene; PH, 2/3 partial hepatectomy

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times weaker than dinitropyrene which probably underestimate the genotoxic risk of NF (Rosenkranz et al., 1985). Experiments recently reported showed that NF induced tumors in liver, forestomach and kidney after oral administration to rats (Cui et al., 1995). By skin painting, NF has been reported to induce tumors in multiple organs (Morris et al., 1950). NF has been identified in diesel exhaust (Schulze et al., 1984; Xu et al., 1981) as well as in urban air (Iida et al., 1985) in both gas and particle phases.

After oral administration of NF to conventional rats, the major part of NF enters the metabolic pathway of 2-aminofluorene (AF) and 2-acetylaminofluorene (AAF) and is excreted mainly as OH-AAFs (Möller et al., 1988). This metabolic route is considered to be important, since AAF has been shown to be a potent carcinogen (Littlefield et al., 1980). There exists a minor metabolic route which results in the formation of hydroxylated nitrofluorenes (OH-NFs), after oral administration of NF to conventional rats. OH-NFs are, on the other hand, major metabolites of NF in germfree rats after oral administration and in perfusates from isolated rat lung and liver (Möller et al., 1987, 1988). While OH-AAFs show a low mutagenic activity (Weisburger, 1981; McCann et al., 1975), OH-NFs, although not detected individually, seem to be mutagenic metabolites of NF (Möller et al., 1988). So far, nothing is known about the carcinogenic potential of OH-NFs and they could conceivably play a role for the carcinogenicity of NF, especially since the tumor patterns are different for NF compared to AAF (Cui et al., 1995; Miller et al., 1955).

The multistage model for hepatocarcinogenesis has made it possible to study separate steps in the sequential process of carcinogenesis (Farber and Sarma, 1987). It is generally accepted that the foci and nodules of altered hepatocytes, which are observed at an early point during carcinogen treatment, are potential pre-stages for the hepatocellular carcinomas that develop at later periods (Farber, 1980; Pitot and Sirica, 1980). The foci of altered hepatocytes, which have increased levels of gamma-glutamyl transpeptidase (gamma-GT) and other tumor marker enzymes (Kalengayi et al., 1975), are presumed to be early indicators of initiation in hepatocarcinogenesis and have been used as the end point in short-term assays for the detection of chemical carcinogens (Tsuda et al., 1980).

In laboratory animal studies, carcinogens have been shown to induce DNA adducts (Smith et al., 1990; Ross et al., 1991; Wierckx et al., 1991; Möller and Zeisig, 1993). Exposures of human populations to complex mixtures with known or suspected risk of inducing cancer is also associated with formation of DNA adducts (Savela and Hemminki, 1991; Hemminki et al., 1990; Phillips et al., 1990). Correlation has been observed between the ability of PAHs and a wide range of chemical classes to form DNA adducts and their carcinogenic potency (Lutz, 1979, 1986; Pelkonen et al., 1980). It has been suggested that DNA binding and lesions induced by carcinogens occur in the early initiating phases of carcinogenesis (Berenblum, 1975; el-Bayoumy et al., 1992).

The aim of this study was to compare NF with its oxidized metabolites, 5-, 7- and 9-OH-NF, in terms of in vivo formation of DNA adducts and preneoplastic lesions in liver and in terms of their bacterial mutagenicity. The tested substances were administered intraperitoneally to simulate inhalation which excludes direct contact with bacterial nitroreductases in the gastrointestinal tract. Liver was chosen for DNA adduct analysis in this study since it is compatible with the liver foci model and, also since liver was reported to be the major tumor target tissue of NF (Miller et al., 1955; Cui et al., 1995).

2. Materials and methods

2.1. Chemicals

2-Nitrofluorene (CAS No. 607-57-8, purity > 98%) was obtained from Aldrich-Chemie (Steinhelm, Germany). 5-, 7- and 9-OH-NF were synthesized (see below) and the purities were confirmed by HPLC analyses (Fig. 1). All solvents and salts were of analytical or chromatographic grades.

2.2. Synthesis of hydroxylated nitrofluorenes (OH-NFs)

2.2.1. 9-Hydroxy-2-nitrofluorene (9-OH-NF)

It was found that 2-nitrofluorene-9-one could be efficiently transformed in a high yield to 9-hydroxy-

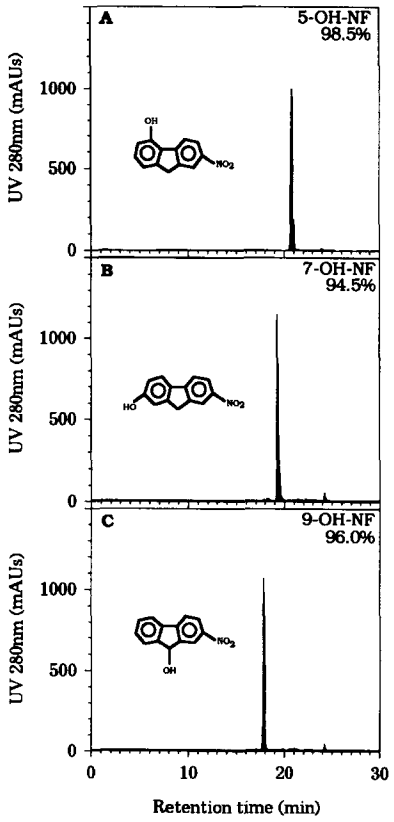


Fig. 1. The purities of 5-, 7- and 9-OH-NF used in this study were analyzed by HPLC. Solvent system used: 2 M ammonium formate buffer (pH 4.5), a linear gradient of 10–100% MeCN. The purities were 98.5%, 94.5% and 96.0% for 5-OH-NF, 7-OH-NF and 9-OHNF, respectively (the impurities were mainly traces of NF).

2-nitrofluorene under Meerwein-Ponndorf conditions with aluminium isopropylate in 2-propanol (Arcus and Coombs, 1954).

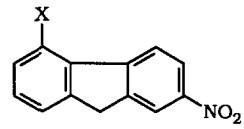
For the preparation of 9-OH-NF, 2-nitrofluorene-9-one (2.25 g, 10 mmol) (synthesized by the authors) in 2-propanol (100 ml) and aluminium isopropylate (2.5 g) was refluxed for 2 h, whereupon the mixture was concentrated and poured into ice/water containing H_2SO_4 (3 ml). The precipitate formed was collected and recrystallized from benzene.

Yield: 2.04 g (90%), m.p. 127–128°C [lit. m.p. 128–129°C (Arcus and Coombs, 1954)]

IR: 3384 (OH), 1613, 1596, 1518 (s), 1470, 1450, 1339 (s), 1184, 1075, 739 cm^{-1}.

2.2.2. 5-Hydroxy-2-nitrofluorene (5-OH-NF)

5-OH-NF was prepared in a multistep synthesis starting with 2,2′-dicarboxybiphenyl (diphenic acid), which was cyclized with polyphosphoric acid to 4-carboxy-9-fluorenone, that in turn was reduced with hydrazine under Wolff-Kishner conditions (Weisburger and Weisburger, 1955). Fluoren-4-carboxylic acid was selectively nitrated with nitric acid in acetic acid at 65–70°C to yield I, which by treatment with azide under acidic conditions gave II, whose NH_2-group was converted to the OH-group required in III by standard methods



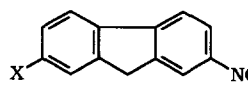
I X=COOH
II X=NH_2
III X=OH

Yield: 82%, m.p. 245–246°C [lit. m.p. 241–242°C (Weisburger and Weisburger, 1955)]

IR: 3384 (OH), 1610, 1588, 1505, 1486, 1456, 1394, 1322 (sh), 1318, 1275, 1187, 1081, 1001, 834, 815, 783, 733 cm^{-1}.

2.2.3. 7-Hydroxy-2-nitrofluorene (7-OH-NF)

7-OH-NF was prepared in a two-step synthesis from 2,7-dinitrofluorene (IV) (Janssen Chimica, Beerse, Belgium), first selectively reduced (ammonium sulfide) to V, which (6.0 g) in turn was converted to VI in the same manner as II was transformed to III (Weisburger and Weisburger, 1954).



IV X=NO_2
V X=NH_2
VI X=OH

Yield: 75%, m.p. 248–250°C [lit. m.p. 248–250°C (Weisburger and Weisburger, 1954)]

IR: 3425 (OH), 1610, 1584, 1512, 1458, 1396, 1309, 1313, 1292, 1206, 1104, 1075, 818, 759, 733 cm^{-1}.

2.3. Animals

Male Wistar rats purchased from Möllegaard Breeding Centre Ltd (Skensved, Denmark) with a

body weight of approx. 90 g per rat were used for the experiments. The rats were housed in wire-bottomed cages and maintained under standardized conditions of light (12 h light and dark cycle), humidity (55–60%) and temperature (21 ± 1°C). The rats were acclimatized for 1 week before the start of the experiments. During the entire experiment the rats had access to food (R3, Ewos, Södertälje, Sweden) and water ad libitum.

2.4. DNA adducts

The rats were divided into five groups with three rats in each group. NF, 5-, 7- and 9-OH-NF were administered i.p. (100 mg/kg bw) as emulsions in corn oil (5 ml/kg bw, i.e., 0.5 ml per animal). The control rats were administered with corn oil. The rats were killed 72 h after administration and liver tissues were collected and stored at −80°C before analyses.

Tissue from the rats was homogenized in a buffer of 1% SDS, 1 mM EDTA and 1 M Tris-HCl (pH 7.4) while kept cold on an ice bed. After RNase and proteinase K digestion, DNA was extracted by phenol and chloroform. DNA samples were pooled groupwise ($n = 3$) and then hydrolyzed by micrococcal nuclease and spleen phosphodiesterase. DNA adducts were enriched by butanol extraction in the presence of tetrabutylammonium chloride and ^{32}P-postlabelled with [γ-^{32}P]ATP and polynucleotide kinase (Beach and Gupta, 1992; Möller and Zeisig, 1993).

^{32}P-TLC analyses of postlabelled DNA adducts were performed by applying the unrefined ^{32}P-labelling mixture (6.3 μg) according to earlier published methods (Beach and Gupta, 1992) with slight modifications (Möller and Zeisig, 1993).

D1 was performed using 0.9 M sodium dihydrogen phosphate (pH 6.8). The plate was then washed by dipping twice for 7 min into water and dried with cool air.

D3. The bottom edge of the plate was dipped quickly into water and elution then performed using 8.5 M urea, 3.6 M formic acid, 3.6 M lithium hydroxide (pH 3.5). The plate was then washed by dipping twice for 7 min into water and dried with cool air.

D4. The bottom edge of the plate was dipped quickly into water and elution then performed using 8.5 M urea, 0.8 M lithium chloride, 0.5 M Tris (pH 8.0). The plate was then washed by dipping once for 7 min into water and dried with cool air.

^{32}P-HPLC-analyses of postlabelled DNA adducts were performed by injection of the total unrefined ^{32}P-labelling mixture (44 μg). A DeltaPak™ 5 μ C18-100 A column, with a flow of 0.5 ml/min of 2 M ammonium formate and 0.4 M formic acid (pH 4.5) with a linear gradient of 0–35% acetonitrile (0–70 min) was used for the separation of phosphorylated nucleotides and adducts. The ^{32}P-HPLC method for analyses of postlabelled DNA adducts is described in detail elsewhere (Möller and Zeisig, 1993; Möller et al., 1993b).

The ^{32}P-TLC and ^{32}P-HPLC methods were used in parallel. For DNA adduct quantitation, the background radioactivity was subtracted. The results were confirmed by duplicated analyses.

2.5. Preneoplastic lesions

Three sets of animal experiments were performed. NF was in each experiment compared to 5-, 7- or 9-OH-NF, respectively, for the capacity to form preneoplastic liver lesions. NF, 5-, 7- and 9-OH-NF (50 or 200 mg/kg bw in corn oil), diethylnitrosamine (DEN) (50 mg/kg bw in saline as positive control) or corn oil (5 ml/kg bw as negative control)

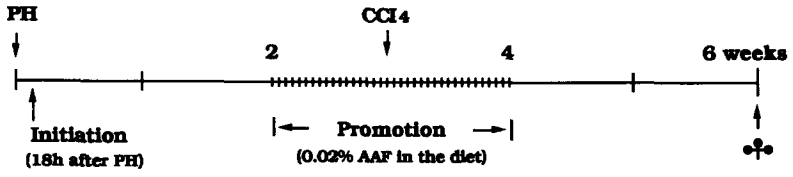


Fig. 2. Graphic presentation of the liver model used for the analyses of initiation in chemical carcinogenesis. NF, 5-, 7- and 9-OH-NF, DEN (positive control) or corn oil (negative control) were administered i.p. 18 h after partial hepatectomy (PH). For details, see Section 2: Materials and methods.

were administered i.p. to rats 18 h after partial (2/3) hepatectomy (PH). Each group contained 5–7 rats. After 2 weeks of recovery, the rats were fed a diet supplemented with 0.02% AAF (CAS No. 53-96-3, Dyets Inc., PN, USA) for 2 weeks. A dose of 2 ml/kg bw of CCl_4 (1 : 1 in corn oil) was administered intragastrically through a stomach feeding tube when the rats had been on the diet for 1 week. One week later the rats were fed basal diet (R3, Ewos, Södertälje, Sweden) until the end of the experiment, i.e., 6 weeks after initiation. The model used (Fig. 2) was concordant with that recommended by Solt and Farber (1976) and modified by Tsuda et al. (1980). The rats were starved for 24 h before they were killed. The three remaining liver lobes from each rat were transsectioned into 2–3 mm thick slices, fixed in ice-cold fresh acetone and used for morphometric analysis of liver foci and nodules after histochemical staining for gamma-GT (Rutenberg et al., 1969). Gamma-GT-positive liver lesions (diameter > 0.2 mm) were identified microscopically and quantitated using morphometric point counting on photographic magnifications of the stained sections. Number of focal lesions per cm^3 liver tissue was calculated as suggested by Wiebel et al. (1966).

2.6. Bacterial mutagenicity

To test the bacterial mutagenicity of the substances, the *Salmonella typhimurium* assay was applied. The plate incorporation test was used, with preincubation step added, and with or without metabolic activation (±S9) (Maron and Ames, 1983). The substances were solved in MeOH/DMSO and added to the top agar as 100 μl solution. The strain TA98 was used since it is most sensitive to this group of compounds (Rosenkranz and Mermelstein, 1985). Triplicate plates were poured for each dose of mutagen. The results were taken from the

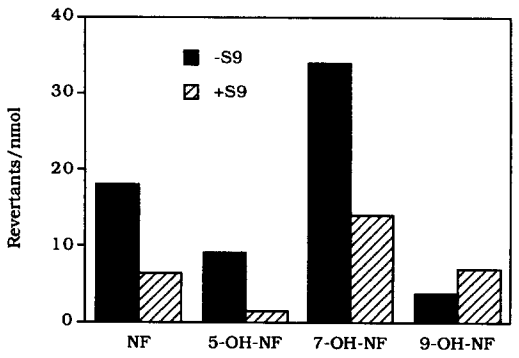


Fig. 3. Bacterial mutagenicity (*Salmonella typhimurium*), strain TA98, for NF and OH-NFs with or without metabolic activation (±S9). The results were taken from the linear portion of the dose-response curves of the mutagens.

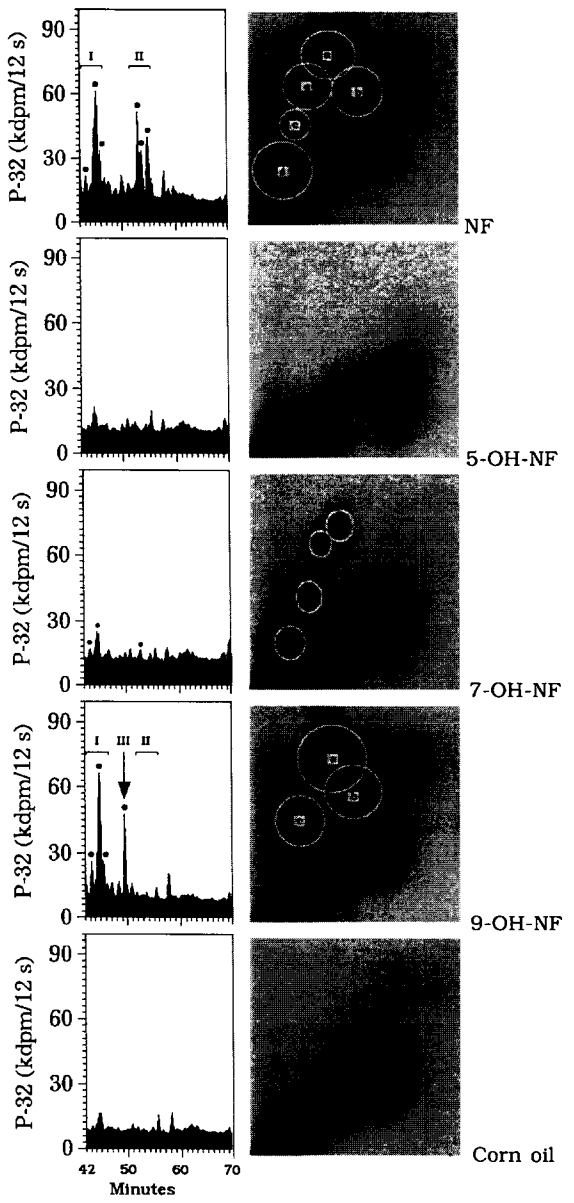


Fig. 4. ^{32}P-HPLC radioactivity chromatograms (left) and ^{32}P-TLC autoradiograms (right) of DNA adducts formed in liver tissue after NF, 5-OH-NF, 7-OH-NF, 9-OH-NF or corn oil i.p. administration.

linear portion of the dose-response curves with 8 concentrations of the mutagens ranging from 0 to 25 μg/plate. Spontaneous revertants (19–22 revertants per plate) were subtracted.

2.7. Statistics

Student's *t*-test was used for statistical calculations.

3. Results

The bacterial mutagenicity of NF and 5-, 7- and 9-OH-NF was assayed using the strain TA 98 (± S9), results shown in Fig. 3. The results indicated that 7-OH-NF was more mutagenic than NF itself (approximately two times), while 5- and 9-OH-NF were less mutagenic compared to NF (−S9). NF, 5- and 7-OH-NF had a similar pattern that showed a dominating mutagenicity without S9. 9-OH-NF had the reverse pattern displaying a dominating mutagenicity with metabolic activation (+S9). The −S9/+S9 ratios were 2.8, 7.1, 2.4 and 0.5 for NF, 5-, 7- and 9-OH-NF, respectively.

DNA adducts formed in liver tissue 72 h after the i.p. administration of the substances were analyzed with both ^{32}P-TLC and ^{32}P-HPLC (Fig. 4). In the ^{32}P-TLC autoradiograms (Fig. 4, right), 5-OH-NF was very similar to the control that only administered corn oil. 7-OH-NF showed four weak spots. NF and 9-OH-NF showed at least three ^{32}P-TLC spots (a, b, c) with similar positions on the chromatogram. NF also formed spot d and e.

In the ^{32}P-HPLC analyses (Fig. 4, left), distinct peaks were separated. The DNA adduct peaks of 9-OH-NF and NF had similar retention times in region I, although NF had peaks in region II. 9-OH-NF also had a high 'region-III' peak.

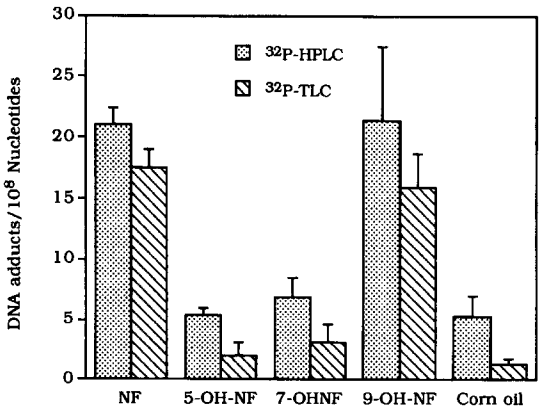


Fig. 5. Total sum of DNA adducts per 10^8 normal nucleotides in the liver of rats 72 h after i.p. administration of NF, 5-, 7- and 9-OH-NF and corn oil. The values of ^{32}P-HPLC (dotted bars) and ^{32}P-TLC (striped bars) shown were means of duplicated measurement.

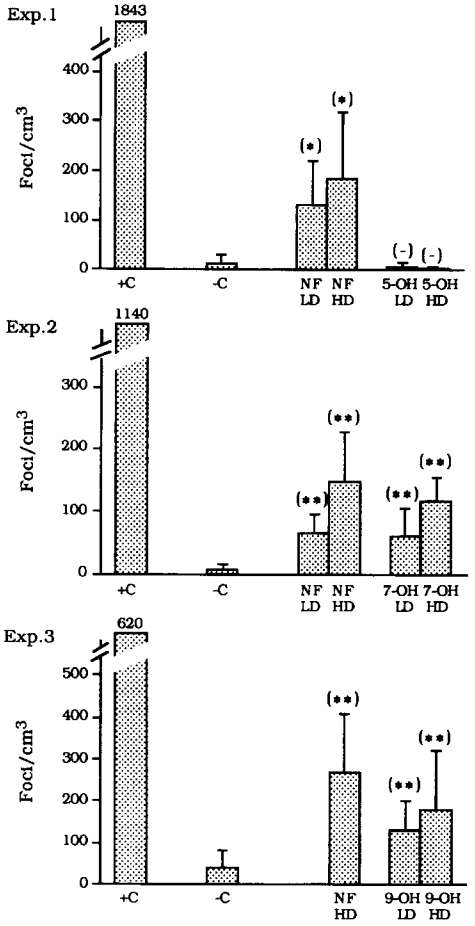


Fig. 6. Numbers of focal lesions/cm^3 liver tissue initiated by i.p. administration of 5-OH-NF (Exp. 1), 7-OH-NF (Exp. 2) and 9-OH-NF (Exp. 3) compared to NF in each experiment. Each value presented is mean of 5–7 rats. LD, low dose, 50 mg/kg bw; HD, high dose, 200 mg/kg bw. Diethylnitrosamine was administered as positive control (+C) and corn oil as negative control (−C). The statistical significance compared to the negative control is shown in parentheses, −, not significant; * $p < 0.05$, ** $p < 0.01$.

The total levels of DNA adducts are shown in Fig. 5. Both ^{32}P-TLC and ^{32}P-HPLC analysis showed similar tendencies, whereas ^{32}P-HPLC displayed generally higher levels compared to ^{32}P-TLC. NF and 9-OH-NF generated more DNA adducts per 10^8 normal nucleotides compared to 7-OH-NF and 5-OH-NF.

Three sets of animal experiments were performed to study the initiating capacities of NF and OH-NFs, in terms of the induction of preneoplastic liver lesions identified by gamma-GT positive staining (Fig. 6). Both 7- and 9-OH-NF showed significant initiating capacities compared to controls, but similar or lower initiating capacities compared to NF. On the other hand, 5-OH-NF did not show an initiating capacity compared to controls.

4. Discussion

The metabolism of NF partly enters the metabolic pathway of AAF in terms of reduction to the corresponding amine, followed by acetylation and ring-hydroxylation after oral administration to rats (Möller et al., 1988). Differences have been seen in the mutagenicity of the OH-NFs compared to OH-AAFs. OH-AAFs showed low mutagenicity (McCann et al., 1975), while the OH-NFs were potent mutagens (Möller et al., 1988). In this experiment, 5- and 9-OH-NF had a lower mutagenicity compared to NF, with 52 and 21% of the NF-mutagenicity (−S9), respectively. 7-OH-NF, on the other hand, was a more potent mutagen than the mother compound (190%). The OH-NFs differed regarding to their relative dependence on metabolic activation with 5- and 7-OH-NF being more direct acting mutagens while 9-OH-NF required metabolic activation for a higher mutagenicity (Table 1).

The OH-NFs were all lower than NF in potency as initiators to generate preneoplastic liver foci in vivo. The isomers were different when compared to each other, with no effect for 5-OH-NF (compared to the control), while 7- and 9-OH-NF had a relatively high potency to generate foci with 79 and 67% of NF, respectively (Table 1 and Fig. 6). In terms of initiating capacity, only metabolism via 5-OH-NF could be considered as a detoxification pathway.

DNA adducts are formed by carcinogens in laboratory animals (Lutz, 1979, 1986; Pelkonen et al., 1980; Möller et al., 1993c) and increased DNA adduct levels have also been found in human populations with known or suspected risk of developing cancer (Savela and Hemminki, 1991; Hemminki et al., 1990; Phillips et al., 1990). In this study, NF generates a variety of DNA adducts seen both in ^{32}P-TLC and ^{32}P-HPLC analyses. 5-OH-NF showed the lowest potency to induce DNA adducts (11 and 26% of the NF potency measured by ^{32}P-TLC and ^{32}P-HPLC, respectively), while 7-OH-NF was more potent (18 and 33%) and 9-OH-NF was similar to the potency of NF (91 and 102% of NF) (Table 1). From the results shown in Fig. 4, it is obvious that the DNA adduct patterns induced by NF and its OH-isomers were different. 9-OH-NF induced DNA adducts with similar retention time to NF at region I, but there was also a unique peak (III) formed by 9-OH-NF which was not seen in the analyses of NF.

Table 1
Comparisons of the genotoxic effects of OH-NFs versus NF (NF = 100)

Substance	Mutagenicity		Ratio	DNA adduct formation		Formation of foci
	−S9	+S9	−S9/+S9	^{32}P-TLC	^{32}P-HPLC	
NF	100	100	100	100	100	100
5-OH-NF	52	20	260	11	26	2
7-OH-NF	190	220	86	18	33	79
9-OH-NF	21	110	19	91	102	67

According to this experiment, the bacterial mutagenicity of the studied substances might be used to identify a potential risk, but not for quantitative purposes. In this respect, total amount of DNA adducts seems to be a better predictor of the potential to initiate preneoplastic lesions in vivo.

In terms of environmental exposure of NF (and nitro-PAHs in general), it is noteworthy that the primarily exposed tissues are the lung and gastrointestinal tract. Metabolism will be dominated in the lung by the oxidative pathway (OH-NFs) and by the reductive pathway in the gastrointestinal tract (reduction of NF to AF) mediated by the intestinal microflora (Möller et al., 1987, 1988). NF is not detoxified by oxidative or reductive metabolic pathway, which might therefore expose the whole body to a genotoxic risk. The carcinogenicity of NF was studied by both oral administration (Cui et al., 1995, Morris et al., 1950; Miller et al., 1955) and skin

painting (Morris et al., 1950). Although target organs were not all the same, tumors were formed in multiple organs by both routes of administration. The results indicate that NF is carcinogenic after oxidative or reductive metabolism.

NF induces a high frequency of squamous-cell carcinoma in the forestomach and renal carcinoma in rats (Cui et al., 1995), which is not the case after administration of AF or AAF (Morris et al., 1950; Miller et al., 1955). This difference could possibly relate to the genotoxic OH-derivatives of NF, such as 7- and 9-OH-NF, which are not formed after the exposure of AF or AAF.

In conclusion, metabolism of NF via OH-NFs can not be considered as a detoxification pathway. Bacterial mutagenicity was poorly correlated to preneoplastic lesions in vivo. On the other hand, DNA adducts were better correlated to in vivo generated preneoplastic lesions. 7- and 9-OH-NF were potent genotoxic agents but less potent than NF. Metabolism via 5-OH-NF could be regarded as a detoxification pathway.

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