RAT LIVER MICROSOMAL METABOLISM OF *o*-AMINOPHENOL AND *N*-(2-METHOXYPHENYL)HYDROXYLAMINE, TWO METABOLITES OF THE ENVIRONMENTAL POLLUTANT AND CARCINOGEN *o*-ANISIDINE IN HUMANS

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Received Jume 24, 2010
Accepted August 17, 2010
Published online November 18, 2010

o-Aminophenol and *N*-(2-methoxyphenyl)hydroxylamine are human metabolites of the industrial and environmental pollutant and bladder carcinogen 2-methoxyaniline (*o*-anisidine). The latter one is also a human metabolite of another pollutant and bladder carcinogen, 2-methoxynitrobenzene (*o*-nitroanisole). Here, we investigated the ability of rat hepatic microsomes to metabolize these metabolites. *N*-(2-methoxyphenyl)hydroxylamine is metabolized by rat hepatic microsomes to *o*-aminophenol and predominantly *o*-anisidine, the parent carcinogen from which *N*-(2-methoxyphenyl)hydroxylamine is formed. In addition, two *N*-(2-methoxyphenyl)hydroxylamine metabolites, whose exact structures have not been identified as yet, were generated. On the contrary, no metabolites were found to be formed from *o*-aminophenol by rat hepatic microsomes. Whereas *N*-(2-methoxyphenyl)hydroxylamine is responsible for formation of three deoxyguanosine adducts in DNA, *o*-aminophenol seems to be a detoxication metabolite of *N*-(2-methoxyphenyl)hydroxylamine and/or a parental carcinogen, *o*-anisidine; no *o*-aminophenol-derived DNA adducts were found after its reaction with microsomal cytochromes P450 and peroxidases.

Keywords: *o*-Anisidine; *N*-(2-Methoxyphenyl)hydroxylamine; *o*-Aminophenol; Metabolism; Activation and detoxification.

2-Methoxyaniline (*o*-anisidine) is a potent carcinogen, causing tumors of the urinary bladder in rats and mice[1]. The International Agency for

doi:10.1135/cccc2010077

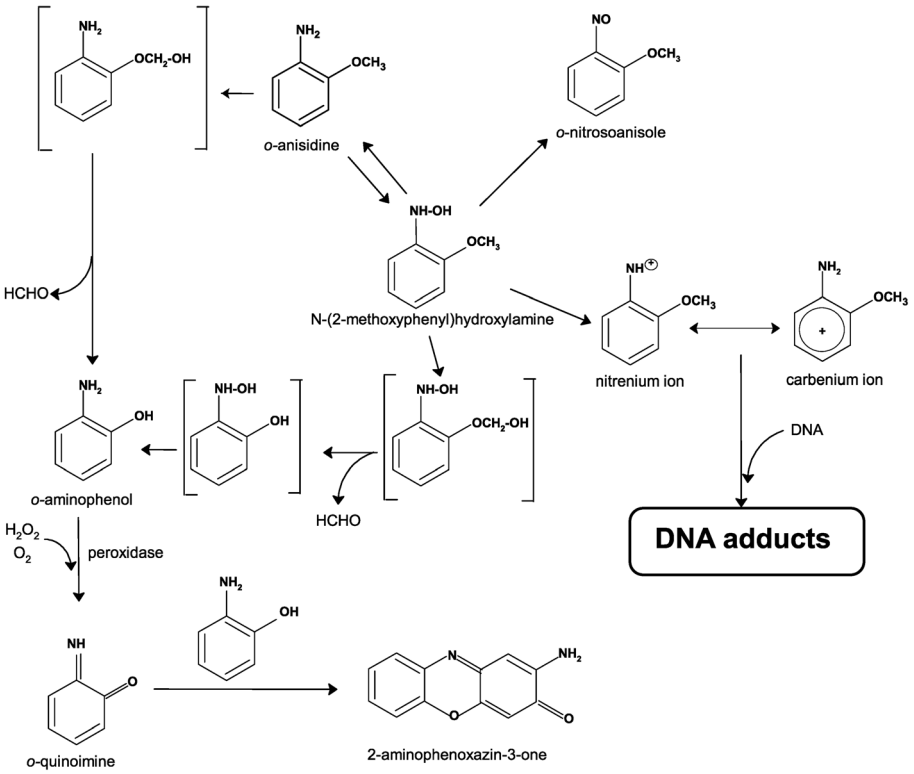
Research on Cancer (IARC) has classified *o*-anisidine as a group 2B carcinogen[1], which is possibly carcinogenic to humans. *o*-Anisidine is used as an intermediate in the manufacturing of a number of azo and naphthol pigments and dyes, which are used for printing (90%) and for paper (3%) and textile (7%) dyeing[2]. This carcinogen is also a constituent of cigarette smoke[1–3]. Such a wide use of this aromatic amine could result in occupational exposure. Hemoglobin adducts of *o*-anisidine were detected in blood samples of persons living in urban or rural areas of Germany[4]. The adducts as well as *o*-anisidine found in urine might originate not only from the sources mentioned above, but also from a possible *o*-anisidine precursor, 2-methoxynitrobenzene (*o*-nitroanisole). This chemical was released into the environment in the course of an accident in a German chemical plant, causing subsequently local and regional contamination[5].

We have found that *o*-anisidine is oxidatively activated by peroxidase and cytochrome P450 (CYP) to species binding to DNA *in vitro*[6–10]. *o*-Anisidine also forms DNA adducts *in vivo*. The same adducts as found in DNA incubated with *o*-anisidine and human microsomes *in vitro* were detected in urinary bladder, the target organ, and to a lesser extent, in liver, kidney and spleen of rats treated with *o*-anisidine[8]. The *o*-anisidine-derived DNA adducts were identified as deoxyguanosine adducts formed from a metabolite of *o*-anisidine, *N*-(2-methoxyphenyl)hydroxylamine[8–10]. The same deoxyguanosine adducts were also detected in DNA of the urinary bladder, kidney, liver and spleen of rats treated with *o*-nitroanisole[11], an oxidized counterpart of *o*-anisidine. The data found previously[8,10–12], indicate that formation of *N*-(2-methoxyphenyl)hydroxylamine, the reactive metabolite of both carcinogens, is critical for generation of DNA lesions in target organs.

Recently, we have found that *o*-anisidine is oxidized by human, rat and rabbit hepatic microsomes not only to *N*-(2-methoxyphenyl)hydroxylamine, but that this compound is a subject of complex redox cycling reactions, forming also *o*-aminophenol, *o*-nitrosoanisole and one additional metabolite, the structure of which has not been identified as yet[8,10] (Scheme 1). *N*-(2-methoxyphenyl)hydroxylamine seems to also be a subject of complex reactions, and its fate is dependent on the environment, in which it occurs. The results of preliminary experiments utilizing rabbit hepatic microsomes indicate that *N*-(2-methoxyphenyl)hydroxylamine can be further metabolized forming three metabolites. Two of them were estimated that might be *o*-aminophenol and a parent compound from which *N*-(2-methoxyphenyl)hydroxylamine is generated, *o*-anisidine[10]. When nucleophiles such as DNA or proteins are present in the incubation mix-

ture, *N*-(2-methoxyphenyl)hydroxylamine forms the adducts[8]. Nevertheless, metabolism of this compound by hepatic microsomes of rats, the animal model mimicking the metabolism of a parent carcinogen, *o*-anisidine, in human, has not been studied as yet. Therefore, metabolism of *N*-(2-methoxyphenyl)hydroxylamine by rat hepatic microsomes was investigated in the present study.

While the formation of *N*-(2-methoxyphenyl)hydroxylamine was clearly identified to be the activation pathway of *o*-anisidine and *o*-nitroanisole metabolism[8,11], biological significance of formation of another major *o*-anisidine metabolite, *o*-aminophenol, for detoxication/activation metabolism awaits further investigation. Even though *o*-aminophenol was found to be oxidized by peroxidases[13,14], a metabolic conversion of this



SCHEME 1

Pathways of *o*-anisidine, *N*-(2-methoxyphenyl)hydroxylamine and *o*-aminophenol metabolism showing the characterized metabolites and those proposed to form DNA adducts. The compounds shown in brackets were not detected under the experimental conditions

o-anisidine metabolite by microsomal CYP enzymes has not been investigated as yet. In addition, significance of its metabolism by such enzymes (CYPs and peroxidases) for activation/detoxication of this compound has not yet been examined. Therefore, investigation of potential of microsomal CYP enzymes and peroxidases to activate *o*-aminophenol is another aim of this study.

EXPERIMENTAL

Chemicals

Chemicals used were of analytical purity or better. 2-Methoxynitrosobenzene (*o*-nitrosoanisole) was synthesized as described[8,10] and identified by ^{1}H NMR [8,10]. *N*-(2-methoxyphenyl)hydroxylamine was synthesized by the procedure similar to that described earlier[15], and its identity was confirmed by electrospray mass and collision-induced dissociation spectra and high field proton NMR spectroscopy[8,10]. *o*-Aminophenol identity was confirmed by ESI mass spectra and ^{1}H and ^{13}C NMR spectroscopy[10].

Preparation of Microsomes and Assays

The study was conducted in accordance with the Regulations for the Care and Use of Laboratory Animals (311/1997, Ministry of Agriculture, Czech Republic), which complies with the Declaration of Helsinki. Adult male Wistar rats (150–200 g) were fed *ad libitum* on pellet chow and water. Microsomes from livers of ten male Wistar untreated rats or rats pre-treated with β-naphthoflavone (β-NF), phenobarbital (PB), ethanol or pregnenolone-16α-carbonitrile (3β-hydroxy-20-oxopregn-5-ene-16α-carbonitrile) (PCN) were prepared as described[8,16–18]. Hepatic microsomes of control (noninduced) rats and rats induced with β-NF, PB, ethanol and PCN contained 0.6, 1.3, 2.7, 1.8 and 1.6 nmol CYP/mg protein, respectively. The activity of NADPH:CYP reductase in rat hepatic microsomes was measured using cytochrome c as a substrate[10]. NADPH:CYP reductase activities in hepatic microsomes of control (uninduced) rats and rats induced with β-NF, PB, ethanol and PCN were 0.210, 0.199, 0.325, 0.201 and 0.210 μmol/min/mg protein, respectively.

NADPH:CYP reductase was isolated from liver microsomes of rats pre-treated with PB as described earlier[17].

Incubations

Incubation mixtures used for study of the metabolism of *N*-(2-methoxyphenyl)hydroxylamine and *o*-aminophenol contained the following concentrations in a final volume of 100 μl: 100 mM sodium phosphate buffer (pH 7.4), 1 mM $NADP^+$, 10 mM D-glucose 6-phosphate, 1 U/ml D-glucose 6-phosphate dehydrogenase (NADPH-generation system), a rat hepatic microsomal fraction containing 0.7 nmol of CYP, and 1.0 mM *N*-(2-methoxyphenyl)hydroxylamine or *o*-aminophenol dissolved in 10 μl of distilled water. The reaction was initiated by adding the substrate. After incubation in open glass tubes (37 °C, 30 min), the reactions were terminated by adding 100 μl of methanol and centrifuged at 5,000 *g* for 5 min. The supernatants were collected and 30 μl of aliquots applied onto a high-performance liquid chromatography (HPLC) column, where metabolites of *N*-(2-methoxyphenyl)hydroxylamine

were separated. Conversion of *N*-(2-methoxyphenyl)hydroxylamine with rat CYP enzymatic systems to its metabolites was linear until 40 min. Compositions of incubation mixtures used for study of the metabolism of *N*-(2-methoxyphenyl)hydroxylamine by rabbit NADPH:P450 reductase were analogous to those described above, but 2 μM NADPH:CYP reductase instead of microsomal enzymes was present. NADPH:CYP reductase was active with its substrate, cytochrome c. The HPLC was performed on a C-18 high density reversed-phase column (250 × 4.6 mm, 5 μm, Nucleosil 100-5HD, Macherey-Nagel, Duren, Germany). Metabolites were eluted with 20% methanol, 80% 7.18 μM aqueous ammonia, pH 8.0 (v/v), at a flow rate of 0.6 ml/min and monitored at 234 nm. *N*-(2-Methoxyphenyl)hydroxylamine metabolites were analyzed by mass spectrometry and by comparing their chromatographic properties on HPLC with those of synthetic standards. 2-Methoxynitrosobenzene, *o*-aminophenol, *N*-(2-methoxyphenyl)hydroxylamine and *o*-anisidine standards were eluted at retention times (r.t.) of 9.8, 10.8, 24.8 and 35.0 min, respectively.

Mass Spectrometry

Positive- and negative-ion ESI mass spectra were recorded on a Finnigan LC^Q-DECA quadrupole ion trap mass spectrometer (ThermoFinnigan, San Jose (CA), USA). Metabolites (final concentration 1 pmol/μl) dissolved in methanol–water (1:1, v/v) were continuously infused through a capillary held at 1.8 kV into the dynamic Finnigan nano-electrospray ion source via a linear syringe pump (Harvard Apparatus Model 22) at a rate of 1 μl/min. The ionizer and ion transfer optics parameters of the ion trap were as follows: spray voltage 1800 V, capillary temperature 150 °C, capillary voltage 14 V, tube lens offset –22 V, octapole 1 offset –7.4 V, lens voltage –16 V, octapole 2 offset –11.3 V, octapole r.f. amplitude 450 V peak-to-peak (pp), and entrance lens voltage –66.9 V. Helium was introduced at a pressure of 0.1 Pa to improve the trapping efficiency of the sample ions. The spectra were scanned in the m/z range of 50–800 and the gating time was set to accumulate and trap 1×10^7 ions. The mass isolation window for precursor ion selection was set to 2 amu and centered on the ^{12}C isotope of the pertinent ion. The background helium gas served as the collision gas for the collision-induced dissociation (CID) experiment. The relative activation amplitude was 35% and the activation time was 30 ms. No broadband excitations were applied. Metabolites were also characterized by the standard EI (electron impact) MS (70 eV) (FinniganMAT, San Jose (CA), USA)[10].

Covalent DNA Binding

Incubation mixtures used to study the binding of *N*-(2-methoxyphenyl)hydroxylamine or *o*-aminophenol to DNA after its metabolism with hepatic microsomes of rats pre-treated with ethanol contained in a final volume of 0.75 ml: 50 mM sodium phosphate (pH 7.4), 0.5 mM *N*-(2-methoxyphenyl)hydroxylamine or *o*-aminophenol dissolved in distilled water (10 μl/0.75 ml incubation), 1 mM NADPH, hepatic microsomes containing 1 mg of protein and 1 mg of calf thymus DNA. The reaction was initiated by adding *N*-(2-methoxyphenyl)hydroxylamine or *o*-aminophenol and reaction mixtures were incubated at 37 °C for 3 h. Control incubations were carried out either without microsomes or without DNA or without *N*-(2-methoxyphenyl)hydroxylamine or without *o*-aminophenol. In order to evaluate a spontaneous cleavage of *N*-(2-methoxyphenyl)hydroxylamine to nitrenium/carbenium ions and their binding to DNA, 0.5 mM *N*-(2-methoxyphenyl)hydroxylamine was also incubated only with DNA in the buffer mentioned above at 37 °C for 3 h. Incubation mixtures

used to study the binding of *o*-aminophenol to DNA after its oxidation with peroxidases contained in a final volume of 0.5 ml: 50 mM sodium phosphate (pH 7.4), 0.2 mM *o*-aminophenol dissolved in distilled water (5 μl/0.5 ml incubation), 5 or 10 μg of HRP or lactoperoxidase, respectively, 0.4 mM hydrogen peroxide and 1 mg of calf thymus DNA. The reaction was initiated by adding hydrogen peroxide and reaction mixture was incubated at 37 °C for 60 min. DNA was isolated from the water phase after extraction of incubations with ethyl acetate by the phenol/chloroform extraction method as described[19].

^{32}P-Postlabeling Analysis

The standard procedure[20], this procedure under the ATP-deficient conditions[21], and the nuclease P1 enrichment version[22] of the ^{32}P-postlabeling assay were performed as described[8,11,18,21]. Labeled DNA digests were separated by two chromatographic methods on polyethylenimine (PEI) cellulose plates. (i) Essentially as described[6,7,18], except that D3 solvent was 3.5 M lithium formate, 8.5 M urea (pH 3.5); D4 solvent was 0.8 M lithium chloride, 0.5 M Tris-HCl, 8.5 M urea (pH 8.0), followed by a final wash with 1.7 M sodium phosphate (pH 6.0). D2 was omitted (method *A*). (ii) ^{32}P-labeled adducts were also resolved by the modification described by Reddy et al.[22]. This procedure has been shown to be suitable for resolution of DNA adducts formed by *N*-(2-methoxyphenyl)hydroxylamine or by *o*-anisidine, reductively activated *in vitro* and *in vivo*[8,11]. The solvents used in this case were: D1, 2.3 M sodium phosphate (pH 5.77); D2 was omitted; D3, 2.7 M lithium formate, 5.1 M urea (pH 3.5); D4, 0.36 M sodium phosphate, 0.23 M Tris-HCl, 3.8 M urea (pH 8.0). After D4 development and brief water wash, the sheets were developed (along D4) in 1.7 M sodium phosphate (pH 6.0) (D5), to the top of the plate, followed by an additional 30–40 min development with the thin layer chromatography (TLC) tank partially opened, to allow the radioactive impurities to concentrate in a band close to the top edge (method *B*)[8,11]. Adduct levels were calculated in units of relative adduct labeling (RAL), which is the ratio of c.p.m. of adducted nucleotides to c.p.m. of total nucleotides in the assay.

Preparation of Reference Compounds and ^{32}P-Postlabeling Analysis of Adducts

An aliquot of 0.5 μmol of deoxyguanosine 3′-phosphate (dGp) was incubated in 50 mM Tris-HCl buffer, pH 5.0, with 20 μmol of *N*-(2-methoxyphenyl)hydroxylamine without further activation at 37 °C overnight in a total volume of 0.5 ml[8]. After incubation and extraction with ethyl acetate, 20 μl aliquots were removed from the aqueous phase and directly used for ^{32}P-postlabeling analysis; the standard procedure[21] and the nuclease P1 version of the assay was utilized[22]. Resolution of the adducts on a PEI-cellulose TLC plate was carried out by method *B*.

RESULTS

Metabolism of N-(2-Methoxyphenyl)hydroxylamine and o-Aminophenol by Rat Hepatic Microsomes

When *N*-(2-methoxyphenyl)hydroxylamine was incubated with rat hepatic microsomes in the presence of NADPH, four product peaks with r.t. of 8.2,

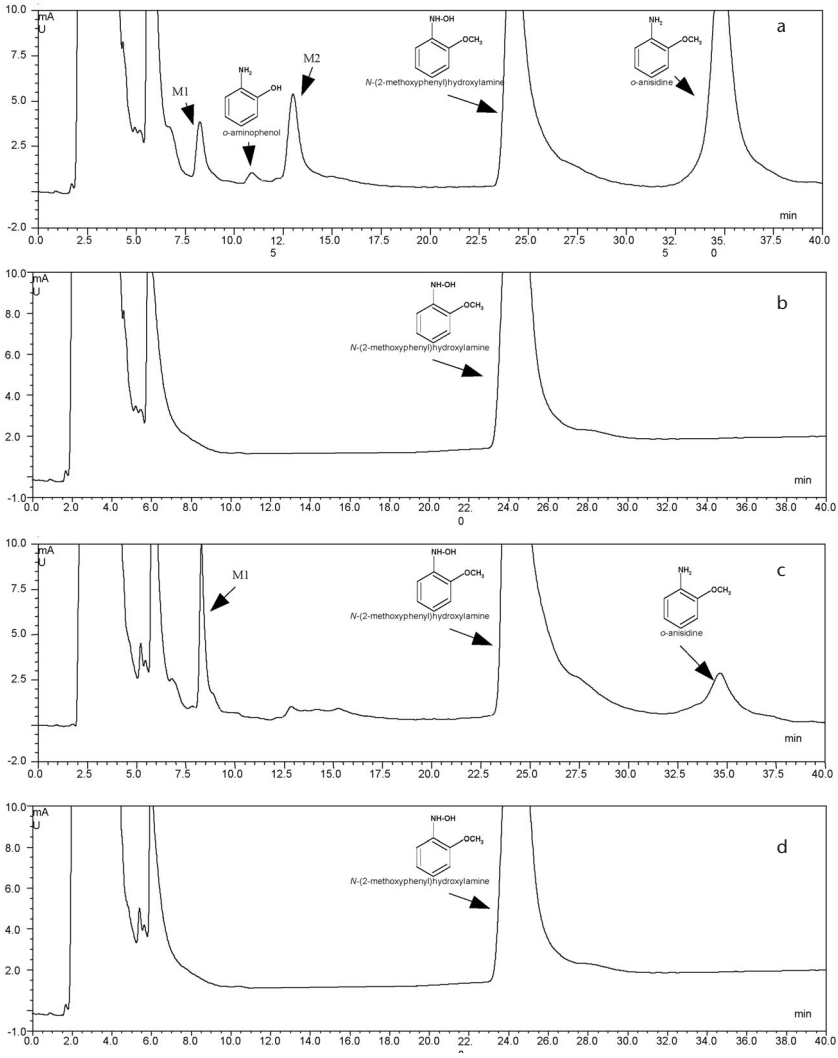


FIG. 1

HPLC elution profile of metabolites of 1 mM *N*-(2-methoxyphenyl)hydroxylamine formed during incubation with hepatic microsomes of rats pre-treated with ethanol and NADPH for 30 min (a), of the same incubation mixture in time zero (b), of metabolites of 1 mM *N*-(2-methoxyphenyl)hydroxylamine formed during incubation with NADPH:CYP reductase and NADPH for 30 min (c), and of the same incubation mixture in time zero (d). Synthetic *o*-aminophenol (e), *N*-(2-methoxyphenyl)hydroxylamine (f), and *o*-anisidine (g). For incubation conditions see Experimental. Peaks eluting between 2.0 and 5.5 min, solvent front, NADPH and protein components of microsomes and NADPH-generation system

10.8, 12.8 and 35.0 min, were separated by HPLC with UV monitoring at 234 nm (see peaks in Fig. 1a for the profile obtained with hepatic microsomes of rats pre-treated with ethanol). The chromatographic properties of an *N*-(2-methoxyphenyl)hydroxylamine metabolite with r.t. of 10.8 min corresponded to those of *o*-aminophenol (Fig. 1). The mass spectrum (Fig. 2A) is consistent with the product being *o*-aminophenol. An additional product peak detected by HPLC, eluting at r.t. of 35.0 min, was shown to be the parental compound, from which *N*-(2-methoxyphenyl)-hydroxylamine is generated, *o*-anisidine (r.t. 35.0 min) (Fig. 1). The mass spectra of this metabolite peak and *o*-anisidine were identical (not shown). Chromatographic properties of an *N*-(2-methoxyphenyl)hydroxylamine metabolite M1, eluting at r.t. of 8.2 min, did not correspond to any standard compound used for analysis. In the atmospheric pressure chemical ionization mass spectrum, this metabolite showed the mass signal at *m*/*z* 122.8 (Fig. 2b), corresponding to that of the nitrenium/carbenium ion of *N*-(2-methoxyphenyl)hydroxylamine. We have not ascertained whether

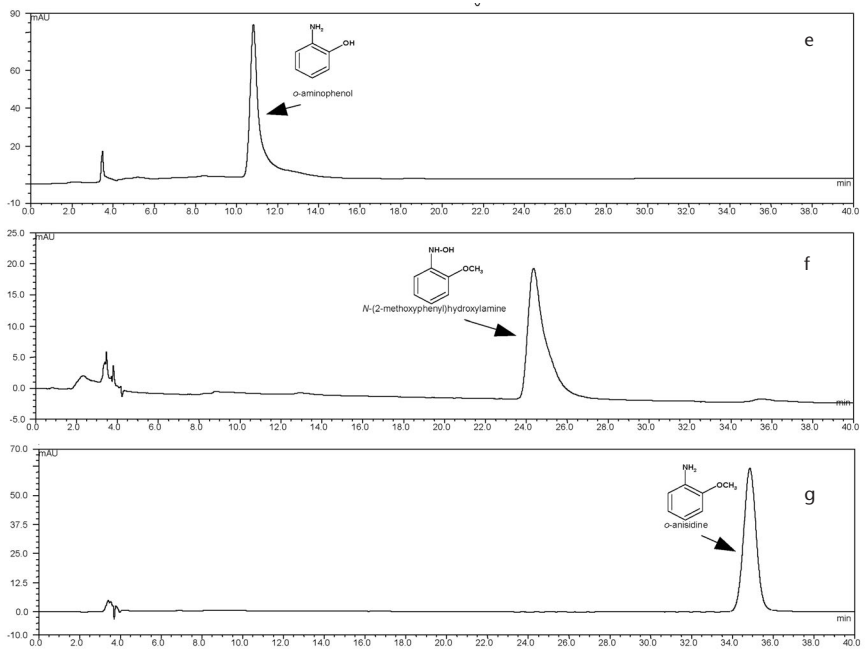


FIG. 1
(*Continued*)

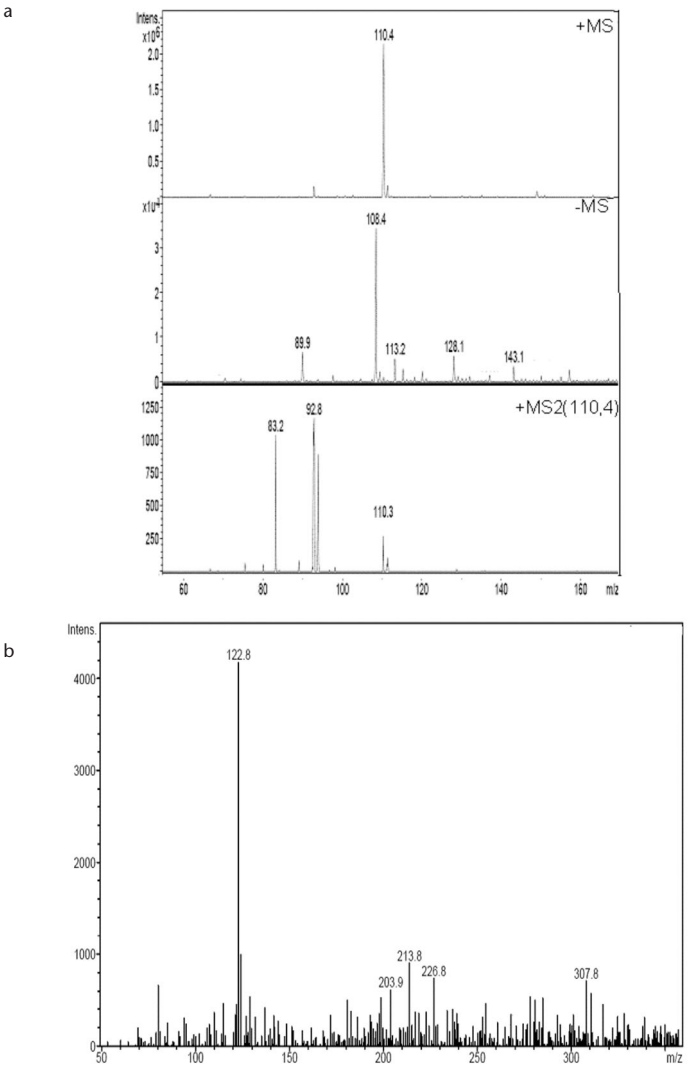


FIG. 2

a The positive- and negative-ion mass spectra of *N*-(2-methoxyphenyl)hydroxylamine metabolite with r.t. 10.8 min. The protonated ion at *m*/*z* 110.4 and the negative ion at *m*/*z* 108.4 indicate the molecular mass of *o*-aminophenol. b Atmospheric pressure chemical ionization mass spectrum of *N*-(2-methoxyphenyl)hydroxylamine metabolite M1. The ion at *m*/*z* 122.8 indicates the molecular mass of the nitrenium/carbenium ion of *N*-(2-methoxyphenyl)-hydroxylamine

this product is indeed the nitrenium/carbenium ion metabolite of *N*-(2-methoxyphenyl)hydroxylamine, formed in the incubation, or product formed in the mass spectrometer. Chromatographic properties of a product peak M2, eluting at r.t. of 12.8 min, did not correspond to any standard compound used for analysis. Its structure has not been characterized as yet.

Microsomes isolated from livers of uninduced rats and rats pre-treated with β-NF (enriched with CYP1A1/2), PB (enriched with CYP2B1/2), ethanol (enriched with CYP2E1) and PCN (enriched with CYP3A) were all capable of metabolizing *N*-(2-methoxyphenyl)hydroxylamine, participating differently in this reaction process (Table I). Among them, hepatic microsomes of rats pre-treated with ethanol were the most effective in *N*-(2-methoxyphenyl)hydroxylamine metabolism, followed by microsomes of rats pre-treated with PB, producing predominantly the reductive metabolite of *N*-(2-methoxyphenyl)hydroxylamine, *o*-anisidine (Table I).

When *N*-(2-methoxyphenyl)hydroxylamine was incubated without hepatic microsomal enzymes or without NADPH, metabolite M1, M2, *o*-aminophenol and *o*-anisidine peaks were also detectable by HPLC, but only under acidic conditions (at pH 4.5 for 60 min). At pH 7.4, used for microsomal incubations, their spontaneous formation was negligible; only a low but detectable amount of *o*-anisidine was found (Table I). In the presence of NADPH in the incubation mixture, the amount of this metabolite was increased, by 2.4-fold (Table I). These findings indicate that conversion of *N*-(2-methoxyphenyl)hydroxylamine in microsomes is mediated mainly by enzymatic reactions, but participation of NADPH in formation of *o*-anisidine cannot be excluded.

When *N*-(2-methoxyphenyl)hydroxylamine was incubated with NADPH and NADPH:CYP reductase, the enzyme that is besides CYPs the prominent component of the microsomal enzymatic system, formation of metabolite M1 and *o*-anisidine was increased (Fig. 1, Table I). Our results suggest that NADPH:CYP reductase plays a minor role in *N*-(2-methoxyphenyl)hydroxylamine metabolism in microsomes, whereas CYP enzymes are more important for this process.

In contrast to results found with *N*-(2-methoxyphenyl)hydroxylamine, no metabolites were found to be formed from *o*-aminophenol when incubated with rat hepatic microsomes and NADPH.

TABLE I
Metabolism of *N*-(2-methoxyphenyl)hydroxylamine by rat hepatic microsomes and rabbit NADPH:CYP reductase

Hepatic microsomes from rats pre-treated with[a]	*N*-(2-Methoxyphenyl)hydroxylamine metabolites[b]			
	M1	M2	*o*-Aminophenol	*o*-Anisidine
None control microsomes	2.0 ± 0.7	0.9 ± 0.8	0.6 ± 0.6	12.3 ± 6.9
β-Naphthoflavone (CYP1A1/2)	5.1 ± 2.8	4.0 ± 2.8	0.8 ± 0.2	13.3 ± 5.2
Phenobarbital (CYP2B1/2)	2.8 ± 0.9	0.7 ± 0.5	0.1 ± 0.1	40.2 ± 11.1
Ethanol (CYP2E1)	2.8 ± 0.5	n.d.	0.1 ± 0.1	55.5 ± 4.1
PCN (CYP3A)	2.9 ± 1.2	1.4 ± 0.5	1.1 ± 0.1	25.0 ± 10.6
Without microsomes, without NADPH	n.d.	n.d.	n.d.	0.7 ± 0.3
NADPH without microsomes	n.d.	n.d.	n.d.	1.7 ± 0.4
NADPH:CYP reductase	3.5 ± 0.6	n.d.	n.d.	2.2 ± 0.5

[a] Isoforms of CYP induced are shown in brackets. [b] The numbers are the peak area/min/nmol CYP (or NADPH:CYP reductase) for each metabolite; average ± SEM of three determinations in separate experiments.

DNA Adduct Formation by N-(2-Methoxyphenyl)hydroxylamine and During Its Metabolism by Hepatic Microsomes

Using the ^{32}P-postlabeling technique, we tested whether during the *N*-(2-methoxyphenyl)hydroxylamine metabolism with hepatic microsomes of rats pre-treated with ethanol, which were the most effective enzymatic system metabolizing this compound, reactive species binding to DNA are generated. DNA was isolated from incubation mixtures containing *N*-(2-methoxyphenyl)hydroxylamine, microsomes and NADPH, and analyzed for DNA adduct formation. The standard procedure and the nuclease P1 version of the ^{32}P-postlabeling assay, suitable for detection of adducts mediated in dGp by *N*-(2-methoxyphenyl)hydroxylamine (Fig. 3) or adducts in DNA of the urinary bladder of rats pre-treated with *o*-anisidine[8] were used. Using these methods, up to three DNA adducts were found when hepatic microsomes of rats treated with ethanol were incubated with DNA, *N*-(2-methoxyphenyl)hydroxylamine and NADPH, having the same chromatographic properties as adducts formed by *N*-(2-methoxyphenyl)hydroxyl-

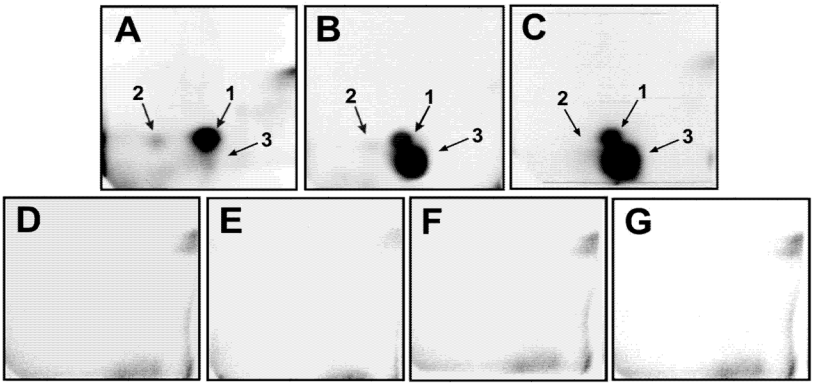


FIG. 3
Autoradiographic profiles of ^{32}P-labeled DNA adducts in calf thymus DNA formed by *N*-(2-methoxyphenyl)hydroxylamine incubated with hepatic microsomes of rats pre-treated with ethanol (A, B), in dGp incubated with *N*-(2-methoxyphenyl)hydroxylamine (C), in DNA formed by *o*-aminophenol incubated with hepatic microsomes of rats pre-treated with ethanol (D, E), and in DNA formed by *o*-aminophenol incubated with lactoperoxidase (F, G) (see Experimental. Sample C was used as a positive control. The nuclease P1 version of the ^{32}P-postlabeling assay was used for analysis shown in panels (A), (D) and (F), the standard procedure for that in panels (B) and (C), and the standard procedure under ATP-deficient conditions for that in panels (E) and (G). The method *B* (see Experimental) was utilized for resolution of adducts

amine with dGp (Fig. 3, A–C). Similar to our previous work investigating formation of DNA adducts by *o*-anisidine and *N*-(2-methoxyphenyl)-hydroxylamine[8], only two DNA adducts (adduct spots 1 and 2) were, however, clearly detectable when the nuclease P1 version of the ^{32}P-postlabeling assay was used (Fig. 3, A). This finding indicates that the major dGp adduct in DNA resulting from *N*-(2-methoxyphenyl)hydroxylamine (adduct spot 3 in Fig. 3) should to be the C8 adduct of the nitrenium ion with dGp. Namely, the C8 adducts of dGp formed by nitrenium ions from *N*-hydroxyarylamines, including those from *N*-hydroxyanilines, are frequently dephosphorylated by nuclease P1 and, therefore, their detection by the nuclease P1 version of the ^{32}P-postlabeling assay is negligible[8]. Indeed, using the standard procedure of the ^{32}P-postlabeling analysis, the adduct spot 3 (Fig. 3), accounting more than 90% of the total RAL level and exhibiting sensitivity to dephosphorylation by nuclease P1 (Table II), was de-

TABLE II
Quantitative analysis of adducts formed by *N*-(2-methoxyphenyl)hydroxylamine with DNA with or without rat hepatic microsomes

DNA adducts	DNA adduct content determined by ^{32}P-postlabeling[a] RAL (adducts/10^6 nucleotides)	
	Without microsomes	With microsomes
The standard procedure of the ^{32}P-postlabeling assay		
Spot 1[b]	0.71 ± 0.04	0.35 ± 0.03
Spot 2	0.03 ± 0.01	0.01 ± 0.01
Spot 3	8.62 ± 0.52	3.61 ± 0.22
Total	9.36 ± 0.41	3.97 ± 0.31
The nuclease P1 version of the ^{32}P-postlabeling assay		
Spot 1[b]	2.27 ± 0.19	1.18 ± 0.12
Spot 2	0.03 ± 0.01	0.01 ± 0.01
Spot 3	0.04 ± 0.01	0.01 ± 0.01
Total	2.34 ± 0.23	1.20 ± 0.12

[a] The numbers represent mean ± SEM (n = 3) of triplicate *in vitro* incubations (two postlabeling analyses of each sample). RAL, relative adduct labeling (the number of adducts per normal nucleotides in modified DNA). The total adduct content is the sum of the RAL of individual adducts. [b] see Fig. 3.

tected. Levels of DNA adducts found in this experiment were lower than those found in DNA incubated only with *N*-(2-methoxyphenyl)hydroxylamine (Table II). This finding confirms the above results, showing that *N*-(2-methoxyphenyl)hydroxylamine is metabolized in microsomes to additional metabolites, whereas when this compound is incubated with DNA it only decomposes to the nitrenium/carbenium ions that bind to DNA (Scheme 1). In addition, the nitrenium and/or carbenium ions may be scavenged by the microsomal proteins present in the incubation mixtures.

Metabolism of o-Aminophenol by Hepatic Microsomes and Peroxidases Is a Detoxication Pathway for This Compound

In additional experiments, we also analyzed whether during metabolism of *o*-aminophenol by hepatic microsomal systems (hepatic microsomes of rats pre-treated with ethanol), reactive species forming DNA adducts are generated. The standard procedure under the ATP-deficient conditions, which is more sensitive than the classical standard procedure, and the nuclease P1 version of the ^{32}P-postlabeling assay and two chromatographic methods (see methods *A* and *B* in Experimental) were utilized in these experiments. Using these ^{32}P-postlabeling assays and separation methods *A* (not shown) and *B*, no DNA adducts mediated by *o*-aminophenol metabolism in hepatic microsomes were detectable (Fig. 3).

o-Aminophenol is oxidized with other oxidative enzymes, peroxidases, forming the *o*-quinoimine metabolite[13,14] (Scheme 1), the reactive compound that might react with nucleophiles such as nucleophilic centers in DNA [7]. Therefore, we have analyzed whether during oxidation of *o*-aminophenol with peroxidases (horseradish peroxidase and lactoperoxidase were used as models), DNA adducts are formed. The same ^{32}P-postlabeling techniques we employed for the analyses of DNA adduct formation by *o*-aminophenol incubated with microsomes (the standard procedure under the ATP-deficient conditions and the nuclease P1 version of the ^{32}P-postlabeling assay and two chromatographic methods) were utilized for such a study. Using these ^{32}P-postlabeling assays and separation methods *A* (not shown) and *B*, no DNA adducts mediated by *o*-aminophenol oxidation with peroxidases were found (see Fig. 3, F and G for lactoperoxidase).

DISCUSSION

The results of this study show that rat hepatic microsomes can metabolize *N*-(2-methoxyphenyl)hydroxylamine, a reactive metabolite of carcino-

genic *o*-anisidine and *o*-nitroanisole. This compound is responsible for genotoxic effects of both carcinogens, because it is easily decomposed to the nitrenium/carbenium ion forming DNA adducts (Scheme 1)[8,10,11]. The results demonstrate that *N*-(2-methoxyphenyl)hydroxylamine is also further metabolized by this subcellular enzymatic system to *o*-aminophenol and the parent compound, *o*-anisidine. The structures of additional two metabolites formed during *N*-(2-methoxyphenyl)hydroxylamine metabolism have not been exactly characterized as yet. The formed *o*-anisidine may be *O*-demethylated again to *o*-aminophenol (Scheme 1). The question whether *o*-aminophenol is also formed from *N*-(2-methoxyphenyl)hydroxylamine by its *O*-demethylation to *N*-(2-hydroxyphenyl)hydroxylamine, which is subsequently reduced to *o*-aminophenol (Scheme 1), remains to be answered. Because one of the *N*-(2-methoxyphenyl)hydroxylamine metabolic products has not still been identified, we cannot exclude that this metabolite might be *N*-(2-hydroxyphenyl)hydroxylamine. To confirm or exclude this suggestion, this compound should be used as a standard. Therefore, its synthesis is target of our future work. In contrast to rabbit hepatic microsomes, which oxidize *N*-(2-methoxyphenyl)hydroxylamine also to a 2-methoxynitrosobenzene[10], this nitrosoderivative was not found to be generated by rat hepatic microsomes (present study).

Recently, redox cycling reactions, similar to those we found with *o*-anisidine[10] and *N*-(2-methoxyphenyl)hydroxylamine, were observed by Kim et al.[23], who studied metabolism of several aromatic and heterocyclic amines by a CYP1A2/NADPH:CYP reductase enzymatic system. They reported that the CYP system catalyzes oxidation of the *N*-hydroxylated intermediate formed from the carcinogenic heterocyclic amine 2-amino-3-methylimidazo[4,5-*f*]quinoline (IQ), to a nitrosoderivative. They demonstrated that NADPH:CYP reductase can catalyze the reduction of the IQ oxidation products, *N*-nitroso-IQ and *N*-hydroxyl-IQ, to *N*-hydroxyl-IQ and the parent amine, IQ[23]. *N*-hydroxylation products of two other aromatic amines investigated by Kim et al.[23], 2-aminofluorene and 4-aminobiphenyl, are, however, reduced non-enzymatically, by NADPH. The results of the present study show that reduction of *N*-(2-methoxyphenyl)hydroxylamine to *o*-anisidine is essentially not mediated by NADPH or NADPH:CYP reductase, but it is catalyzed by other enzymes present in hepatic microsomes used in this work. Because the efficiency of microsomes to reduce *N*-(2-methoxyphenyl)hydroxylamine to *o*-anisidine depends on levels of individual CYP enzymes present in hepatic microsomes of rats pre-treated with selective inducers of individual CYPs, the CYP enzymes should be more effective in this reduction than NADPH:CYP reductase. Nevertheless, identifi-

cation which of these CYP enzymes are responsible for this reaction awaits further investigation.

Since *N*-(2-methoxyphenyl)hydroxylamine is responsible for formation of DNA adducts, its formation is the activation pathway of *o*-anisidine and/or *o*-nitroanisole metabolism[8,11]. Because a role of *o*-aminophenol and its metabolism in detoxication and/or activation of *o*-anisidine were not known, these subjects were studied in this work. The results of this study demonstrate that metabolism of *o*-aminophenol by hepatic microsomes or peroxidases did not lead to formation of covalent DNA adducts. No metabolism of and DNA adduct formation by this compound were found in the microsomal system. No DNA adducts were also generated during its oxidation with peroxidases. This oxidation leads to formation of an *o*-quinoimine metabolite[13,14], but this intermediate seems not to be reactive enough to react with DNA. Formation of 2-aminophenoxazine-3-one that is a final metabolite of the *o*-aminophenol oxidation by peroxidases seems to be preferred (Scheme 1). This final 2-aminophenoxazine-3-one metabolite is known to have significant biological activities such as anti-inflammatory and immunomodulatory properties[24] and/or antimycobacterial[25] and anticancer activities[26]. All these findings suggest that *o*-aminophenol and its metabolism by peroxidases are not included into genotoxic processes occurring during carcinogenesis caused by *o*-anisidine. Nevertheless, *o*-aminophenol might be considered to be mutagenic, because it induces sister chromatid exchanges in a dose-dependent manner in cultured human lymphocytes *in vitro* and in Chinese hamster bone marrow cells *in vivo*[27]. In addition, Brennan and Schiestl[28] reported that *o*-aminophenol is positive in the deletion recombination assay in *Sacchromyces cerevisiae*. Furthermore, even though *o*-aminophenol has not been found to form covalent DNA adducts, it was demonstrated in *in-vitro* experiments to cause DNA damage, forming 8-oxy-7,8-dihydro-2′-deoxyguanosine in the presence of metal ions such as Cu(II)[29]. Hence, due to such processes, contribution of *o*-aminophenol to initiation of the *o*-anisidine-mediated carcinogenesis in the urinary bladder, and in the development of tumors induced by other bladder carcinogenic aromatic amines, which produce this compound as one of the metabolites, cannot be excluded[28]. Furthermore, *O*-demethylation reactions produce formaldehyde (Scheme 1), which is known to modify DNA, generating several products including hydroxymethyl adducts and cross-links[30]. Formaldehyde is mutagenic in a variety of different test systems and carcinogenic in laboratory animals[31] and has been described as "carcinogenic to human" by the IARC and "reasonably anticipated to be a human carcinogen" by the U.S. Department of Health and Human Services (2004)[32].

Therefore, on the one hand, it is plausible that formaldehyde-DNA adducts could also play a role in carcinogenesis by *o*-anisidine. On the other hand, however, formaldehyde produced in the cell is also detoxified by conjugation to glutathione and oxidized[33]; therefore, it is not likely a strong contributor to carcinogenicity caused by *o*-anisidine.

It should be noted that tumor development in a specific organ is influenced by promotional pressures on initiated cells in target organs and not only by the levels of DNA adducts formed by the compounds like *o*-anisidine. It is known that radicals formed from several carcinogens producing oxidative DNA damage, such as 8-hydroxy-2′-deoxyguanosine, are important not only in initiation, but also in promotion phases of carcinogenesis[34]. Therefore, their formation from *o*-aminophenol may be one of the factors contributing to tumor promotion in *o*-anisidine- and/or *o*-nitroanisole-mediated carcinogenesis. In addition, *o*-anisidine is oxidized by several peroxidases[6,7], which are expressed in target organs (e.g. COX), to form radicals besides DNA adducts. Hence, the production of such free radicals in or near the target cells may be another factor important in the promotional process in *o*-anisidine-mediated tumor development. However, the exact functions of such and/or other promotional pressures caused both by *o*-anisidine and *o*-aminophenol in an *o*-anisidine-mediated tumorigenesis remain to be resolved.

Abbreviations: β-NF, β-naphthoflavone; CYP, cytochrome P450; EI, electron impact; dGp, deoxyguanosine 3′-phosphate; HPLC, high performance liquid chromatography; HRP, horseradish peroxidase; $NADP^+$, nicotinamidadeninedinucleotide phosphate; NADPH, nicotinamidadeninedinucleotide phosphate reduced; PB, phenobarbital (5-ethyl-5-phenylpyrimidine-2,4,6(1H,3H,5H)-trion); PCN, pregnenolone-16α-carbonitrile (3β-hydroxy-20-oxopregn-5-ene-16α-carbonitrile); PEI-cellulose, polyethylenimine-cellulose; RAL, relative adduct labeling; r.t., retention time; RPM, rotations per minute; TLC, thin layer chromatography.

This research was supported by the Czech Science Foundation (Grant No. 303/09/0472) and the Ministry of Education, Youth and Sports of the Czech Republic (MSM0021620808 and 1M0505).

REFERENCES

1. International Agency for Research in Cancer (IARC): *IARC Monographs on the Evaluation of the Carcinogenic Risk of Chemicals to Humans*, No. 27. IARC, Lyon 1982.
2. Stabbert R., Schäfer K.-H., Biefel C., Rustemeier K.: *Rapid Commun. Mass Spectrom.* **2003**, *17*, 2125.

3. Kütting B., Göen T., Schwegler U., Fromme H., Uter W., Angerer J., Drexler H.: *Int. J. Hyg. Environ. Health* **2009**, *212*, 298.
4. Richter E., Rosler S., Scherer G., Gostomzyk J. G., Grubl A., Kamer U., Behrendt H.: *Int. Arch. Occup. Environ. Health* **2001**, *74*, 421.
5. Traupe H., Menge G., Kandt J., Karmaus W.: *Dermatology* **1997**, *195*, 112.
6. Stiborová M., Schmeiser H. H., Breuer A., Frei E.: *Gen. Physiol. Biophys.* **2001**, *20*, 267.
7. Stiborová M., Mikšanová M., Havlíček V., Schmeiser H. H., Frei E.: *Mutat. Res.* **2002**, *500*, 49.
8. Stiborová M., Mikšanová M., Šulc M., Rýdlová H., Schmeiser H. H., Frei E.: *Int. J. Cancer* **2005**, *116*, 667.
9. Rýdlová H., Mikšanová M., Ryšlavá H., Stiborová M.: *Biomed. Pap. Med. Fac. Univ. Palacky Olomouc Czech Repub.* **2005**, *49*, 441.
10. Naiman K., Dračínská H., Martínková M., Šulc M., Dračínský M., Kejíková L., Hodek P., Hudeček J., Liberda J., Schmeiser H. H., Frei E., Stiborová M.: *Chem. Res. Toxicol.* **2008**, *21*, 1610.
11. Stiborová M., Mikšanová M., Smrček S., Bieler C. A., Breuer A., Schmeiser H. H., Frei E.: *Carcinogenesis* **2004**, *25*, 833.
12. Mikšanová M., Novák P., Frei E., Stiborová M.: *Collect. Czech. Chem. Commun.* **2004**, *69*, 589.
13. Akazawa M., Takasaki M., Tomoda A.: *Tohoku J. Exp. Med.* **2000**, *192*, 301.
14. Puiu M., Răducan A., Babaligea I., Oancea D.: *Bioprocess Biosyst. Eng.* **2008**, *31*, 579.
15. Balaban A. T., Garfield R. E., Lesko M. J., Seitz W. A.: *Org. Prep. Proced. Int.* **1998**, *30*, 439.
16. Dračínská H., Mikšanová M., Svobodová M., Smrček S., Frei E., Schmeiser H. H., Stiborová M.: *Neuroendocrinol. Lett.* **2006**, *27* (Suppl. 2), 9.
17. Stiborová M., Martínek V., Rýdlová H., Hodek P., Frei E.: *Cancer Res.* **2002**, *62*, 5678.
18. Arlt V. M., Stiborová M., Henderson C. J., Thiemann M., Frei E., Aimová D., Singhs R., da Costa G. G., Schmitz O. J., Farmer P. B., Wolf C. R., Phillips D. H.: *Carcinogenesis* **2008**, *29*, 656.
19. Frei E., Bieler C. A., Arlt V. M., Wiessler M., Stiborová M.: *Biochem. Pharmacol.* **2002**, *64*, 289.
20. Gupta R. C., Reddy M. V., Randerath K: *Carcinogenesis* **1982**, *3*, 1081.
21. Randerath E., Agrawal H. P., Weaver J. A., Bordelon C. B., Randerath K.: *Carcinogenesis* **1985**, *6*, 1117.
22. Reddy M. V., Bleicher W. T., Blackburn G. R., Mackerer E. R.: *Carcinogenesis* **1990**, *11*, 1349.
23. Kim D., Kadlubar F. F., Teitel C. H., Guengerich F. P.: *Chem. Res. Toxicol.* **2004**, *17*, 529.
24. Kohno K., Miyake M., Sano O., Tanaka-Kataoka M., Yamamoto S., Koya-Miyata S., Arai N., Fujii M., Watanabe H., Ushio S., Iwaki K., Fukuda S.: *Biol. Pharm. Bull.* **2008**, *31*, 1938.
25. Uruma T., Yamaguchi H., Fukuda M., Kawakami H., Goto H., Kishimoto T., Yamamoto Y., Tomoda A., Kamiya S.: *J. Med. Microbiol.* **2005**, *54*, 1143.
26. Zheng C. L., Che X. F., Akiyama S., Miyazawa K., Tomoda A.: *Int. J. Oncol.* **2010**, *36*, 641.
27. Kirchner G., Bayer U.: *Hum. Toxicol.* **1992**, *1*, 387.
28. Brennan R. J., Schiestl R. H.: *Mutagenesis* **1997**, *12*, 215.
29. Okhuma Y., Kawanishi S.: *Arch. Biochem. Biophys.* **2001**, *389*, 49.

30. Cheng G., Shi Y., Sturla S., Jalas J., McIntee E. J., Villalta P. W., Wang M., Hecht S. S.: *Chem. Res. Toxicol.* **2003**, *16*, 145.
31. International Agency for Research in Cancer (IARC): *IARC Monographs on the Evaluation of Carcinogenic Risk to Humans*, Vol. 88, pp. 39–325. IARC, Lyon 2006.
32. U.S. Department of Health and Human Services: *11th Report on Carcinogens*. U.S. Government Printing Office, Washington, DC 2004.
33. Dhareshwar S. S., Stella V. J.: *J. Pharm. Sci.* **2008**, *97*, 4184.
34. Imaoka S., Osada M., Minamiyama Y., Yukimura T., Toyokuni S., Takemura S., Hiroi T., Funae Y.: *Cancer Lett.* **2004**, *203*, 117.