Oxidation Pattern of the Anticancer Drug Ellipticine by Hepatic Microsomes – Similarity Between Human and Rat Systems

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Abstract. Ellipticine is an antineoplastic agent, whose mode of action is based mainly on DNA intercalation, inhibition of topoisomerase II and formation of DNA adducts mediated by cytochrome P450 (CYP). We investigated the ability of CYP enzymes in rat, rabbit and human hepatic microsomes to oxidize ellipticine and evaluated suitable animal models mimicking its oxidation in humans. Ellipticine is oxidized by microsomes of all species to 7-hydroxy-, 9-hydroxy-, 12-hydroxy-, 13-hydroxyellipticine and ellipticine N^2-oxide. However, only rat microsomes generated the pattern of ellipticine metabolites reproducing that formed by human microsomes. While rabbit microsomes favored the production of ellipticine N^2-oxide, human and rat microsomes predominantly formed 13-hydroxyellipticine. The species difference in expression and catalytic activities of individual CYPs in livers are the cause of these metabolic differences. Formation of 7-hydroxy- and 9-hydroxyellipticine was attributable to CYP1A in microsomes of all species. However, production of 13-hydroxy-, 12-hydroxyellipticine and ellipticine N^2-oxide, the metabolites generating DNA adducts, was attributable to the orthologous CYPs only in rats and humans. CYP3A predominantly generates these metabolites in rat and human microsomes, while CYP2C3 activity prevails in microsomes of rabbits. The results underline the suitability of rat species as a model to evaluate human susceptibility to ellipticine.

Key words: Ellipticine — Anticancer drug — Metabolism — Oxidation — Cytochrome P450

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Abbreviations: α-NF, α-naphthoflavone; β-NF, β-naphthoflavone; CYP, cytochrome P450; DDTC, diethyldithiocarbamate; DMSO, dimethylsulphoxide; ESI, electron spray ionization; HPLC, high performance liquid chromatography; IC_{50}, 50 % inhibitory dose; 3-IPMDIA, 3-isopropenyl-3-methyldiamantane; NMR, nuclear magnetic resonance; PCN, pregnenolone-16α-carbonitrile; RIF, rifampicin.

Introduction

Ellipticine (5,11-dimethyl-6H-pyrido[4,3-b]carbazole; Fig. 1), an alkaloid isolated from *Apocyanaceae* plants, and several of its more soluble derivatives (9-hydroxyellipticine, 9-hydroxy-N^2-methylellipticinium, 9-chloro-N^2-methylellipticinium and 9-methoxy-N^2-methylellipticinium) exhibit significant antitumor and anti-HIV activities (for summary, see Stiborová et al. 2001). The main reason for the interest in ellipticine and its derivatives for clinical purposes is their high efficiency against several types of cancer, their rather limited toxic side effects and their complete lack of hematological toxicity (Auclair 1987). Nevertheless, ellipticine is a potent mutagen. Most ellipticines are mutagenic to *Salmonella typhimurium* Ames tester strains, bacteriophage T4, *Neurospora crassa*, and mammalian cells and induce prophage lambda in *Escherichia coli* (for an overview, see Stiborová et al. 2001).

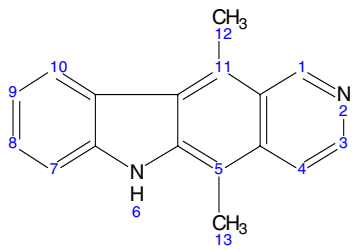

Figure 1. Structure of ellipticine.

Ellipticine is an antineoplastic agent, whose mode of action was considered to be based mainly on DNA intercalation and/or inhibition of topoisomerase II (Monnot et al. 1991; Chu and Hsu 1992; Fossé et al. 1992; Singh et al. 1994; Froelich-Ammon et al. 1995). Recently, we found another mode of the ellipticine action (Stiborová et al. 2001; 2003a,b; Frei et al. 2002), demonstrating that ellipticine covalently binds to DNA after enzymatic activation. Using a panel of different human recombinant cytochrome P450 (CYP) enzymes, CYP3A4, CYP1A1 and CYP1B1, enzymes which are expressed at higher levels in tumors sensitive to ellipticine (i.e. breast cancer) than in peritumoral tissues (Murray et al. 1993, 1995; Patterson 1999; El-Rayes 2003), were found to be the most efficient CYP enzymes activating ellipticine to form covalent DNA adducts *in vitro* (Stiborová et al. 2001). Two of the ellipticine metabolites generated by human CYP enzymes, 13-hydroxyellipticine (5-hydroxymethyl-11-methyl-6*H*-pyrido[4,3-*b*]carbazole) and

12-hydroxyellipticine (11-hydroxymethyl-5-methyl-6*H*-pyrido[4,3-*b*]carbazole) (the latter one formed also spontaneously from ellipticine N^2-oxide (5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole-*N*-oxide)) are responsible for formation of the two major DNA adducts *in vitro* and *in vivo*, deoxyguanosine was identified as the target base of their binding (Stiborová et al. 2004, 2006). Such deoxyguanosine adducts were found in numerous model systems; V79 Chinese hamster lung fibroblasts transfected with human CYP3A4, CYP1A1 and CYP1A2 (Frei et al. 2002), human breast adenocarcinoma MCF-7 cells (Bořek-Dohalská et al. 2004), human HL-60 leukemia cells (Poljaková and Stiborová 2004) and in rats exposed to this anticancer drug (Stiborová 2003a). On the basis of these data, ellipticine might be considered a drug, whose pharmacological efficiency and/or genotoxic side effects are dependent on its enzymatic activation in target tissues (Stiborová et al. 2004).

For formation of ellipticine-DNA adducts, we demonstrated recently the existence of analogy between human and rat hepatic microsomes (Stiborová et al. 2003b). As the cause of this analogy, similarities in expression and catalytic activities of CYP forms were suggested (Stiborová et al. 2003b).

While ellipticine oxidation by human CYP enzymes has been studied in detail (Stiborová et al. 2004), the knowledge of the CYP enzymatic systems of other species in this respect is incomplete. We demonstrated that ellipticine is oxidized by CYP enzymes in human hepatic microsomes to five metabolites: 7-hydroxyellipticine (5,11-dimethyl-7-hydroxy-6*H*-pyrido[4,3-*b*]carbazole), 9-hydroxyellipticine (5,11-dimethyl-9-hydroxy-6*H*-pyrido[4,3-*b*]carbazole), 12-hydroxy-, 13-hydroxyellipticine and ellipticine N^2-oxide (Stiborová et al. 2004). To our knowledge, in rats *in vivo* and in microsomal preparations, only two ellipticine metabolites, 9-hydroxy- and 7-hydroxyellipticine, were found previously (Auclair 1987; Lesca et al. 1981b), while oxidation of this anticancer agent by enzymatic systems of other animal models has not been studied as yet. Rat CYP1A1 and CYP1A2 were assumed to be the most active enzymes oxidizing ellipticine to 7-hydroxy- and 9-hydroxyellipticine, but other CYPs may be involved in the metabolism of ellipticine, too (Auclair 1987; Lesca et al. 1981a; DeMarini et al. 1992; Tassaneeyakul et al. 1993).

The present study was undertaken to investigate the metabolism of ellipticine in rat and rabbit hepatic microsomes in detail, in order to explain the molecular nature of the different activation of ellipticine by hepatic microsomal enzymes of these animal species observed in previous studies (Stiborová et al. 2001, 2003b). This knowledge underlines the suitability of rat species as a model to evaluate human susceptibility to ellipticine.

Materials and Methods

Chemicals and reagents

Chemicals were obtained from the following sources: ellipticine, diethyldithiocarbamate (DDTC), pregnenolone-16α-carbonitrile (PCN), rifampicin (RIF), furafylline,

α-naphthoflavone (α-NF), β-naphthoflavone (β-NF), sulfafenazole, $NADP^+$, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, and quinidine from Sigma Chemical Co. (St. Louis, MO, USA); bicinchoninic acid from Pierce (Rockford, IL, USA) and 9-hydroxyellipticine were from Calbiochem (San Diego, CA, USA). All these and other chemicals from commercial sources used in the experiments were reagent grade or better. 7-hydroxyellipticine and the ellipticine N^2-oxide were synthesized as described (Wijsmuller et al. 1986; Boogaard et al. 1994) by J. Kučka (Charles University, Prague, Czech Republic); their purity was >99.5 % as estimated by high performance liquid chromatography (HPLC). Rabbit polyclonal antibody raised against human CYP2C9 was a gift of P. Souček (National Institute of Public Health, Prague, Czech Republic).

Animal experiments, 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 is in compliance with Declaration of Helsinki. Microsomes from livers of ten untreated rats and three rabbits were prepared by the procedure described previously (Stiborová et al. 1988). Microsomes from the livers of ten male Wistar rats or three male rabbits pretreated with β-NF were isolated as described (Stiborová et al. 1988, 1995), those pretreated with PCN for rats and RIF for rabbits as reported by Gut et al. (1996) and Bořek-Dohalská et al. (2001), respectively. The human hepatic microsomal sample was a pooled sample of microsomes from livers of eight human donors, which were a gift of Dr. B. Szotáková (Faculty of Pharmacy, Charles University, Hradec Králové, Czech Republic). Protein concentrations in the microsomal fractions were assessed using the bicinchoninic acid protein assay with bovine serum albumin as a standard (Wiechelman et al. 1988). The concentration of CYP was estimated according to Omura and Sato (1964) by measuring the absorption of the complex of reduced CYP with carbon monoxide. Human, rat and rabbit liver microsomes contained 0.2, 0.6 and 1.8 nmol CYP·mg^{-1} protein, respectively. Hepatic microsomes of rats induced with β-NF and PCN contained 1.3 and 1.6 nmol CYP·mg^{-1} protein, respectively. Hepatic microsomes of rabbits induced with β-NF and RIF contained 3.6, and 3.7 nmol CYP·mg^{-1} protein, respectively.

Incubations

Unless stated otherwise, incubation mixtures used for study of the ellipticine metabolism contained the following concentrations in the final volume of 500 μl: 50 mmol·l^{-1} potassium phosphate buffer (pH 7.4), 1 mmol·l^{-1} $NADP^+$, 10 mmol·l^{-1} D-glucose 6-phosphate, 1 U·ml^{-1} D-glucose 6-phosphate dehydrogenase (NADPH-generation system), microsomal fraction containing 0.2 μmol·l^{-1} CYP and 5–10 μmol·l^{-1} ellipticine (ellipticine was dissolved in 5 μl methanol). The reaction was initiated by adding the substrate. In the control incubation, ellipticine was omitted from the incubation mixture. After incubation in open glass tubes (37 ℃, 20 min) the reaction was stopped by adding 100 μl of 2 mol·l^{-1} NaOH. Thereafter, 5 μl of 1 mmol·l^{-1} phenacetine in methanol was added as an internal standard and

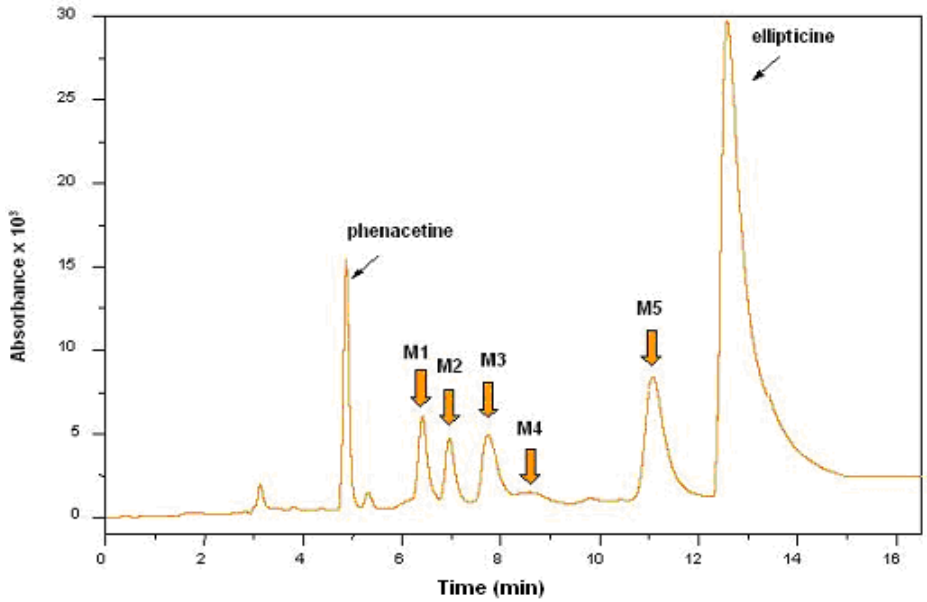

Figure 2. HPLC chromatogram of ellipticine metabolites formed by rabbit hepatic microsomes. Incubations (1 mmol·l^{-1} $NADP^+$, 10 mmol·l^{-1} D-glucose 6-phosphate, 1 U·ml^{-1} D-glucose 6-phosphate dehydrogenase, rabbit hepatic microsomes containing 0.1 nmol CYP, 10 μmol·l^{-1} ellipticine dissolved in 1 μl methanol in 50 mmol·l^{-1} potassium phosphate buffer, pH 7.4, in a final volume of 500 μl) were stopped after 20 min by extraction with ethyl acetate and extracted metabolites analyzed by HPLC (see Materials and Methods). M1, 9-hydroxyellipticine; M2, 12-hydroxyellipticine; M3, 13-hydroxyellipticine; M4, 7-hydroxyellipticine; M5, ellipticine N^2-oxide. Phenacetine (peak eluted at 4.4 min) was used as an internal standard.

the ellipticine metabolites were extracted twice with ethyl acetate (2 × 1 ml). The extracts were evaporated under nitrogen and dissolved in 50 μl of methanol. The ellipticine metabolites were separated by HPLC. The column used was a 5 μm Ultrasphere ODS (4.6 × 250 mm, Beckman) preceded by a C-18 guard column, the eluent was 64 % methanol plus 36 % of 5 mmol·l^{-1} heptane sulfonic acid containing 32 mmol·l^{-1} acetic acid in water with flow rate of 0.8 ml·min^{-1}, detection was at 296 nm. Five ellipticine metabolites with the retention times (r.t.) of 6.3, 6.9, 7.8, 8.5 and 11.2 min were separated (Fig. 2). These were collected, concentrated and analyzed. Recoveries of ellipticine metabolites were around 95 % in the presence of microsomes without a CYP cofactor (NADPH-generation system).

To characterize ellipticine metabolites, fractions containing the metabolites eluting at 6.3, 6.9, 7.8, 8.5 and 11.2 min were collected from multiple HPLC runs, concentrated on a speed-vac evaporator and analyzed by mass spectroscopy and/or nuclear magnetic resonance (NMR) as described below.

Inhibition studies

The following chemicals were used to inhibit the activation of ellipticine in rat, rabbit and human hepatic microsomes: α-NF, which inhibits CYP1A1 and CYP1A2 (Rendic and DiCarlo 1997); furafylline, which inhibits CYP1A2 (Rendic and DiCarlo 1997); 3-isopropenyl-3-methyldiamantane (3-IPMDIA), which inhibits CYP2B (Stiborová et al. 2002); DDTC, which inhibits CYP2E1 and CYP2A (Rendic and DiCarlo 1997); sulfaphenazole, which inhibits CYP2C8/9 (Rendic and DiCarlo 1997); ketoconazole, which inhibit CYP3A (Rendic and DiCarlo 1997; Ueng et al. 1997). Inhibitors were dissolved in 7.5 μl of methanol, to yield final concentrations of 0.01–1000 μmol·l^{-1} in the incubation mixtures (except of DDTC, which was dissolved in water). Three to five different concentrations of each inhibitor were used. The inhibitors-containing mixtures were then incubated at 37 ℃ for 10 min with the NADPH-generation system (see above) prior to adding the ellipticine and then for a further 20 min at 37 ℃. An equal volume of methanol alone was added to the control incubations.

Mass spectrometry

Electron spray ionization (ESI) mass spectra were recorded on a Bruker Esquire quadrupole ion trap mass spectrometer (Bruker GmbH, Bremen, Germany). Metabolites (final concentration 1 pmol/μl) dissolved in methanol/water (1 : 1, v/v) were continuously infused into the ion source *via* a linear syringe pump at a rate of 1 μl·min^{1} (Harvard Apparatus 22). The ionizer and ion transfer optics parameters of the ion trap were as follows: capillary voltage −3500 V, end plate −3000 V, capillary exit 100 V, skimmer I 35 V, skimmer II 7 V, octopole I offset 8 V, octopole II offset 8 V, octopole radio frequency magnitude 100 V peak-to-peak, lens I −4 V, lens II −45 V. A flow of nitrogen (drying gas at 125 ℃) was used to stabilize the spray. The spectra were scanned in the range m/z 50–2000 and the gating time was set to accumulate and trap 1×10^5 ions.

NMR spectrometry

NMR spectra were measured on Varian Unity Inova 400 MHz instrument (400 MHz for ^{1}H, 100, 58 MHz for ^{13}C). All samples were measured in deuterated dimethylsulphoxide (DMSO) or deuterated methanol at 25 and 35 ℃. Shigemi microtubes were used for measurements of small quantities of metabolites (150 μl of the solution). As an internal standard, the signal of the solvent was used (DMSO: $\delta = 2.50$ for ^{1}H, $\delta = 39.5$ for ^{13}C-NMR spectra; methanol: $\delta = 3.31$ for ^{1}H, $\delta = 49.1$ for ^{13}C-NMR spectra). Chemical shifts (δ, ppm) and coupling constants (Hz) were obtained by the first order analysis. COSY, NOESY, gHSQC and gHMBC spectra were recorded for all samples. COSY spectra were measured in absolute value mode using standard two-pulse sequence. NOESY spectra with mixing time of 0.3 s were taken as phase sensitive with standard three-pulse sequence. Connectivities C-H were obtained from HSQC and HMBC experiments, which were performed as gradient experiments. All two-dimensional experiments were measured in spectral windows 5000 Hz for proton and 25.000 Hz for carbon resonances.

Results

Characterization of ellipticine metabolites formed by rat, rabbit and human hepatic microsomes

When ellipticine was incubated with human, rat and rabbit hepatic microsomes in the presence of NADPH, five product peaks were observed by HPLC analysis (see Fig. 2 for rabbit hepatic microsomes). The three minor peaks eluting with r.t. of 3.1, 5.2 and 9.8 min were not derived from ellipticine, because they were also present in the chromatograms of the control incubations (without ellipticine or without NADPH). On the basis of co-chromatography with synthetic standards, mass and NMR spectroscopy, the structures of all five ellipticine metabolites were identified. In the positive-ion electrospray mass-spectrum, all ellipticine metabolites showed the protonated molecule at m/z 263.0, indicating the molecular mass of hydroxylated (oxygenated) derivatives of ellipticine. Products eluting with r.t. of 6.3 and 8.5 min (peaks M1 and M4 in Fig. 2) were identified by co-chromatography with synthetic standards as 9-hydroxy- and 7-hydroxyellipticine, respectively. The three ellipticine metabolites eluting at r.t. of 6.9, 7.8 and 11.2 min (peaks M2, M3 and M5 in Fig. 2) were characterized by NMR spectroscopy. Structure assignments of these metabolites were based on ^{1}H NMR, COSY and gHMBC spectra (Ratclife et al. 1988; Modi et al. 1991; Stiborová et al. 2004). Using this analysis, the metabolite M2 was identified as 12-hydroxyellipticine, M3 as 13-hydroxyellipticine and M5 as the ellipticine N^2-oxide (Table 1).

Table 1. ^{1}H NMR chemical shifts of ellipticine and its metabolites 12-hydroxyellipticine (M2), 13-hydroxyellipticine (M3) and ellipticine N^2-oxide (M5) in methanol-*d4* (Meth) and dimethylsulphoxide-*d6* (DMSO) at 25 °C and comparison with the data shown by Ratclife et al. (1988) and Modi et al. (1991)

H	Ellipticine		M2			M3			M5	
	Meth	DMSO	Meth	DMSO	DMSO[a]	Meth	DMSO	DMSO[b]	Meth	DMSO
H-1	9.72	9.69	9.79	9.79	9.78	9.66	9.71	9.72	9.36	9.38
H-3	8.34	8.42	8.37	8.43	8.42	8.37	8.44	8.44	8.17	8.22
H-4	8.19	7.92	8.07	7.95	7.94	8.17	8.08	8.09	8.26	8.20
H-7	7.60	7.57	7.57	7.57	7.52-7.59	7.57	7.60	7.5–7.6	7.60	7.58
H-8	7.58	7.52	7.53	7.53	7.25	7.53	7.53	7.5–7.6	7.58	7.54
H-9	7.33	7.26	7.29	7.26	7.52–7.59	7.30	7.27	7.27	7.33	7.28
H-10	8.42	8.39	8.47	8.43	8.42	8.42	8.39	8.39	8.43	8.40
H-12	3.29	3.27	5.78	5.60	5.58	3.22	– [c]	3.28	3.24	3.25
H-13	2.86	2.79	2.88	2.83	2.82	5.41	5.25	5.25	2.88	2.84
N-H	– [d]	11.40	– [d]	11.44	11.50	– [d]	11.45	11.50	– [d]	11.46

[a]data taken from Modi et al (1991), signals of H-8 and H-9 are interchanged; [b]data taken from Ratclife et al. (1988); [c]signal overlapped with solvent signal satellite; [d]N-H proton exchanged with solvent O-D.

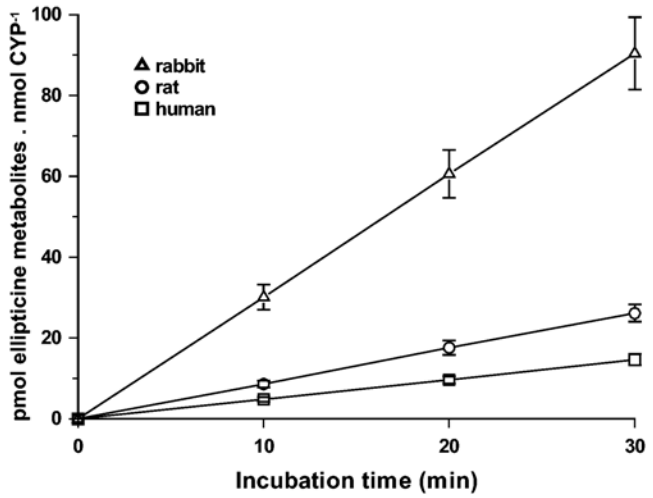

Figure 3. Time dependence of ellipticine metabolite formation by human, rat and rabbit hepatic microsomes. In all experiments, 10 μmol·l^{-1} ellipticine and microsomes containing 0.1 nmol CYP were used. Values of total ellipticine metabolite levels are averages and standard deviations of triplicate incubations.

The formation of ellipticine metabolites with microsomal systems increased with time and was linear up to 30 min (Fig. 3).

Comparison of ellipticine metabolism by human, rat and rabbit hepatic microsomes

Microsomes from rabbits were the most effective system oxidizing ellipticine, followed by those of rats and humans (Fig. 4). If we compare the ratios of ellipticine metabolites in liver microsomes of the two animal species and man, the most conspicuous finding is the high level of ellipticine N^2-oxide produced by rabbit microsomes; more than 50 % of the total levels of ellipticine metabolites are to this compound (Fig. 4 inset). In microsomes from rats and humans, ellipticine N^2-oxide is formed to a much lesser extent (∼20 %), and 13-hydroxyellipticine is the predominant metabolite; it accounts for 45 and 56 % of the total metabolite levels in human and rat microsomes, respectively (Fig. 4). The percentage of 7-hydroxy-, 9-hydroxy- and 12-hydroxyellipticine were similar in microsomes from all species. 7-hydroxyellipticine is produced at very low levels in microsomes from all species (2–6 %).

In order to explain the species differences shown above, we evaluated and compared the role of specific CYP enzymes of rat, rabbit and human hepatic microsomes in the oxidation pathways of ellipticine to individual metabolites.

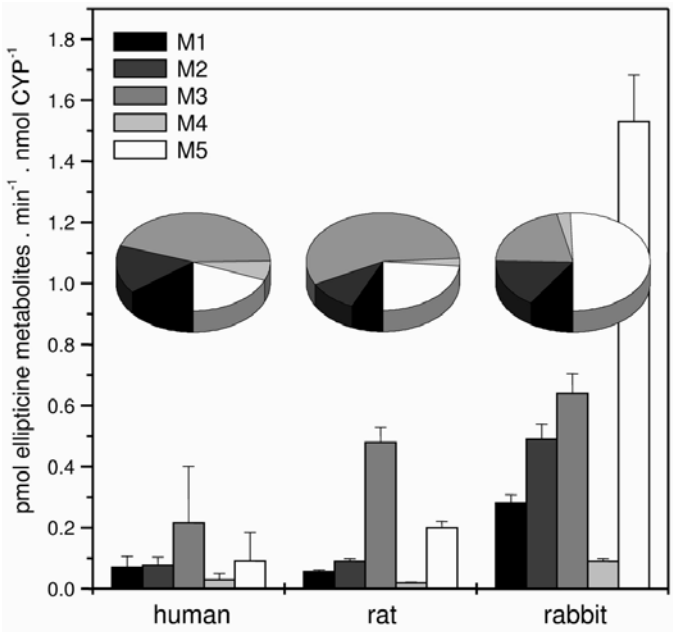

Figure 4. Oxidation of ellipticine by human, rat and rabbit hepatic microsomes. In all experiments, 10 μmol·l^{-1} ellipticine and microsomes containing 0.1 nmol CYP were used. Values of ellipticine metabolites are averages and standard deviations of triplicate incubations. Inset: percentage levels of ellipticine metabolites formed by microsomes. M1, 9-hydroxyellipticine; M2, 12-hydroxyellipticine; M3, 13-hydroxyellipticine; M4, 7-hydroxyellipticine; M5, ellipticine N^2-oxide.

Effect of CYP inhibitors on ellipticine oxidation in human, rat and rabbit microsomes

Selective inhibitors of CYP1A1/2, CYP1A2, CYP2B, CYP2C, CYP2E1 and CYP3A enzymes, α-NF, furafylline, 3-IPMDIA, sulfafenazole, DDTC and ketoconazole, respectively, were used in this study (Table 2). Quinidine, an inhibitor of CYP2D, could not be used, because most of the metabolite peaks of this compound had the same or similar r.t. on HPLC as the metabolites of ellipticine. α-NF, an inhibitor of CYP1A1/2, inhibited formation of 9-hydroxy- and 12-hydroxyellipticine in rat and human hepatic microsomes, with similar 50 % inhibitory dose (IC_{50}) values, while it stimulated the formation of 13-hydroxyellipticine 3-fold in rat and 5-fold in human microsomes. The same was found for rabbit microsomes where similar IC_{50} values were determined, but stimulatory effect of α-NF on the formation of 13-hydroxyellipticine was only 1.3-fold. No effect of α-NF was observable on ellipticine N^2-oxide formation. The inhibitor of CYP1A2, furafylline, had a much weaker inhibitory effect on oxidation of ellipticine. One hundred times higher IC_{50} values for 9-hydroxyellipticine formation (compared to α-NF) and no effect on

Table 2. Inhibition of ellipticine[a] metabolism by selective CYP inhibitors in human, rat and rabbit hepatic microsomes

H u m a n

CYP inhibitor[b]	IC_{50} (μmol·l^{-1})			
	M1	M2	M3	M5
α-NF (CYP1A1/2)	1[c]	25	stimul	n.i.[d]
furafylline (CYP1A2)	71	n.i.	95	n.i.
3-IPMDIA (CYP2B6)	110	350	270	250
sulfafenazole (CYP2C8/9)	–[e]	210	250	n.i.
DDTC (CYP2E1, CYP2A)	68	n.i.	250	70
ketoconazole (CYP3A4)	50	95	10	8

R a t

CYP inhibitor[b]	IC_{50} (μmol·l^{-1})			
	M1	M2	M3	M5
α-NF (CYP1A1/2)	3[c]	28	stimul	n.i.[d]
furafylline (CYP1A2)	139	n.i.	n.i.	n.i.
3-IPMDIA (CYP2B1/2)	93	217	234	100
sulfafenazole (CYP2C8/9)	–[e]	n.i.	n.i.	n.i.
DDTC (CYP2E1, CYP2A)	66	n.i.	202	57
ketoconazole (CYP3A1/2)	51	123	62	15

R a b b i t

CYP inhibitor[b]	IC_{50} (μmol·l^{-1})			
	M1	M2	M3	M5
α-NF (CYP1A1/2)	1[c]	20	stimul	n.i.[d]
furafylline (CYP1A2)	160	n.i.	n.i.	n.i.
3-IPMDIA (CYP2B4)	n.i.	268	294	n.i.
sulfafenazole (CYP2C8/9)	–[e]	n.i.	n.i.	n.i.
DDTC (CYP2E1, CYP2A)	79	n.i.	250	88
ketoconazole (CYP3A6)	69	n.i.	989	857

[a]ellipticine at 10 μmol.l^{-1} was used in the experiments; [b]isoforms of CYP inhibited are shown in brackets; [c]estimated from concentration-dependent inhibition of formation of ellipticine metabolites by interpolation (inhibitors were 0.01–1000 μmol·l^{-1} depending on the chemical), averages of three determinations in separate experiments, the S.E.M. values were $\leq 10\%$; [d]IC_{50} greater than 1000 μmol·l^{-1}; [e]interference of the HPLC peak of the inhibitor or of its metabolite with the ellipticine metabolite; n.i., no inhibition; stimul, a 3-fold (rat), a 5-fold (human) and a 1.3-fold (rabbit) increase in formation of 13-hydroxyellipticine; M1, 9-hydroxyellipticine; M2, 12-hydroxyellipticine; M3, 13-hydroxyellipticine; M5, ellipticine N^2-oxide.

generation of other metabolites by rat and rabbit microsomes were seen. In liver microsomes of humans, furafylline was more potent inhibitor of 9-hydroxyellipticine formation than in those of rats and rabbits and inhibited also ellipticine oxidation to 13-hydroxyellipticine (Table 2). Both α-NF and furafylline inhibited completely the oxidation of ellipticine to 7-hydroxyellipticine in microsomes of all species; because of very low initial levels of this metabolite, the inhibition was not quantified.

A selective inhibitor of CYP2B, 3-IPMDIA, was a weak inhibitor of ellipticine oxidation by microsomes of all species. It slightly inhibited formation of all ellipticine metabolites formed in human and rat hepatic microsomes, but only the production of 12-hydroxy- and 13-hydroxyellipticine in rabbit microsomes. DDTC, an inhibitor of CYP2E1 and CYP2A6, was also a weak inhibitor of ellipticine oxidation. It inhibited the formation of 9-hydroxyellipticine, ellipticine N^2-oxide and 13-hydroxyellipticine. Therefore, CYPs of CYP2B, CYP2A and CYP2E subfamilies seem to participate in ellipticine oxidation in hepatic microsomes of all species only to a lesser extent.

An efficient selective inhibitor of CYP3A, ketoconazole, inhibits effectively ellipticine oxidation mainly in human and rat microsomes, where it inhibited the formation of ellipticine N^2-oxide, 13-hydroxy-, 9-hydroxy- and 12-hydroxyellipticine. It was only a weak inhibitor in rabbit microsomes, where only 9-hydroxyellipticine formation is inhibited to a similar extent as in human and rat microsomes, 12-hydroxyellipticine formation is not inhibited by this compound at all (Table 2).

Sulfafenazole, the inhibitor of human CYP2C8 and CYP2C9, slightly inhibited 12-hydroxy- and 13-hydroxyellipticine formation in human microsomes only with IC_{50} values of 210 and 250 μmol·l^{-1} (Table 2). Because the efficiency of this inhibitor upon the oxidation of several substrates by rat and rabbit CYP2C enzymes is not clear (Rendic and DiCarlo 1997), we used an anti-CYP2C9 antibody, which was an efficient inhibitor of progesterone oxidation catalyzed CYP2C in human, rat and rabbit microsomes (results not shown). While the anti-CYP2C9 antibody inhibited the formation of 13-hydroxyellipticine, ellipticine N^2-oxide and 12-hydroxyellipticine in rabbit microsomes, by 50, 20 and 10 %, respectively, it was ineffective in rat microsomes. In human microsomes it only inhibited formation of 13-hydroxyellipticine, by 10 %, and did not inhibite formation of ellipticine N^2-oxide or 12-hydroxyellipticine.

The above results suggest that ellipticine is oxidized in hepatic microsomes mainly by CYP1A, CYP3A and/or CYP2C subfamilies. However, it should be noted that the interpretation of the results of experiments with inhibitors is sometimes difficult, because one inhibitor may be more effective with one substrate than with another. To further evaluation of the role of microsomal CYPs in ellipticine oxidation, rats and rabbits were therefore treated with specific inducers of CYPs of CYP1A and CYP3A subfamilies and hepatic microsomes isolated. These CYP subfamilies are inducible CYPs in the liver tissue, in contrast to CYP2C that is the poorly inducible and is expressed mainly constitutively in livers (Rendic and DiCarlo 1997).

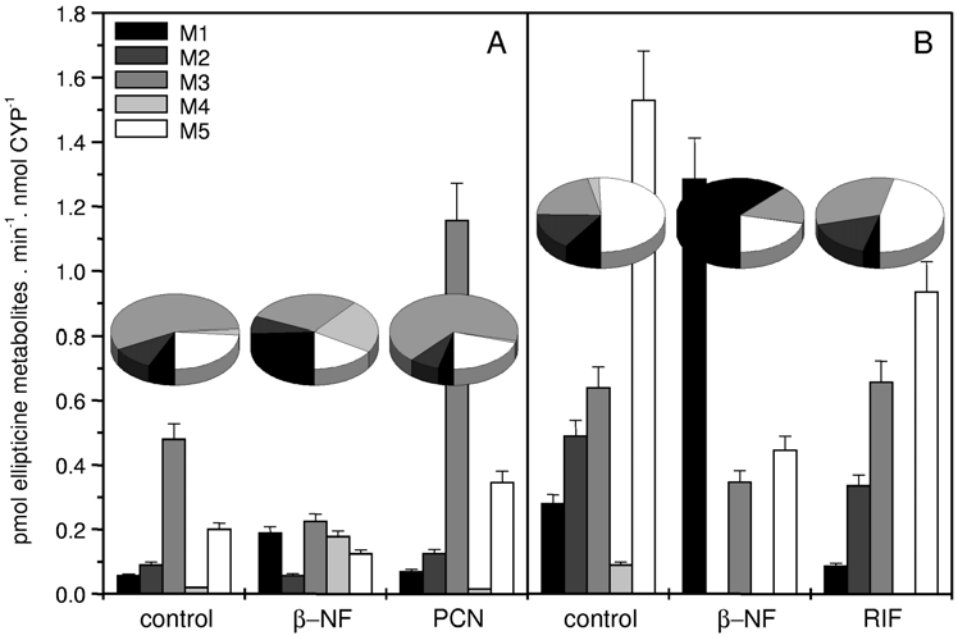

Figure 5. Ellipticine metabolism in rat (A) and rabbit (B) hepatic microsomes of control animals and those treated with inducers of CYP1A and CYP3A enzymes. Microsomes containing 0.1 nmol CYP and 10 μmmol·l^{-1} ellipticine were used in all experiments. Values of ellipticine metabolites are averages and standard deviations of triplicate incubations. β-NF, β-naphthoflavone (CYP1A1/2); PCN, pregnenolone-16α-carbonitrile (CYP3A in rats); RIF, rifampicin (CYP3A6 in rabbits). Isoforms of CYP induced are shown in brackets. Inset: percentage levels of ellipticine metabolites formed by microsomes. M1, 9-hydroxyellipticine; M2, 12-hydroxyellipticine; M3, 13-hydroxyellipticine; M4, 7-hydroxyellipticine; M5, ellipticine N^2-oxide.

Oxidation of ellipticine by hepatic microsomes of rats and rabbits treated with inducers of CYP1A and CYP3A subfamilies

Microsomes isolated from livers of uninduced animals and those induced by β-NF (enriched in CYP1A1/2), PCN in rats (enriched in CYP3A1/2) and RIF in rabbits (enriched in CYP3A6) were used in the experiments (Fig. 5). Incubations of ellipticine with rat microsomes rich in CYP1A1/2 led to a 3.3 and 8.8-fold increase in its oxidation to 9-hydroxy- and 7-hydroxyellipticine, respectively, while only formation of 9-hydroxyellipticine was increased (4.5 times) by β-NF in rabbits. 12-hydroxy- and 7-hydroxyellipticine were not detectable any more and ellipticine N^2-oxide was much lower than in uninduced rabbit microsomes. Induction of CYP3A enzymes with PCN in rats elevated levels of 13-hydroxyellipticine, 2.3 times, ellipticine N^2-oxide 1.7 times and 12-hydroxyellipticine 1.4 times, while RIF, a CYP3A6 inducer in rabbits, had no stimulatory effect on any metabolites (Fig. 5).

Discussion

The aim of this study was to explain the finding described previously (Stiborová et al. 2003b), i.e. similarities in oxidative activation of ellipticine to form DNA adducts in human and rat, but not rabbit, livers. Different levels of DNA adducts formed after activation of ellipticine by hepatic microsomes was postulated to be caused by species differences in expression levels and activities of CYP enzymes oxidizing ellipticine to metabolites responsible for binding to DNA (Stiborová et al. 2003b). Since we have shown that only some of the oxidized ellipticine metabolites bind to DNA, the pattern of metabolites generated from ellipticine by hepatic microsomes from different species is important, not only the overall oxidative metabolism. We have shown that hepatic microsomes of rats, rabbits and humans can oxidize antineoplastic agent ellipticine to the same metabolites, but the pattern as well as CYP enzymes responsible for their formation differed between rabbits on the one hand and rat and human on the other.

Ellipticine is oxidized by hepatic microsomes of rat and rabbit to 7-hydroxy-, 9-hydroxy-, 12-hydroxy-, 13-hydroxyellipticine and the ellipticine N^2-oxide, which are the same metabolites as those generated by human liver microsomes (Stiborová et al. 2004). 9-hydroxyellipticine, the metabolite excreted in urine by humans (mainly in the form of conjugates), and 7-hydroxyellipticine, were suggested to be the detoxication products (Auclair 1987). Indeed, they do not form DNA adducts found *in vivo*, in rats treated with ellipticine (Stiborová et al. 2003a; 2004; Bořek-Dohalská et al. 2004). On the contrary, 13-hydroxy-, 12-hydroxyellipticine and the ellipticine N^2-oxide, the metabolites found to generate the two major deoxyguanosine adducts in DNA of several organs of rats treated with ellipticine (Stiborová et al. 2003a), and *in vitro* in human and animal microsomal systems (Stiborová et al. 2001, 2003b, 2004, 2006), are products of the activation pathway of ellipticine. 12-hydroxyellipticine is formed in two ways, one by direct oxidation, and one by Polonowski (Hofle et al. 1999; Nicolaou et al. 2001) rearrangement of ellipticine N^2-oxide (Stiborová et al. 2004). Therefore, the formation of 12-hydroxyellipticine or ellipticine N^2-oxide, followed by its spontaneous rearrangement to 12-hydroxyellipticine, are two pathways leading to the formation of the same reactive species binding to DNA (Stiborová et al. 2006).

Comparison of the metabolism of ellipticine in human, rat and rabbit hepatic microsomes indicates that the detoxication pathway of ellipticine is analogous in all species. Ellipticine detoxication metabolites (9-hydroxy- and 7-hydroxyellipticine) are formed in similar levels in hepatic microsomes of all species and the analogous CYP enzymes (CYP1A1 and/or CYP1A2) are the major enzymes generating them. This is concluded from the effects of both CYP1A1/2 inhibitors (α-NF, furafylline) and of their inducer (β-NF). The major role of human CYP1A1/2 in the oxidation of ellipticine to these metabolites in human hepatic microsomes has already been demonstrated in our previous work, by the finding that formation of 9-hydroxy- and 7-hydroxyellipticine correlates with specific catalytic activities of CYP1A1/2 and with the levels of their expression in livers of eight human donors (Stiborová et

al. 2004). However, this is not the case for those metabolites responsible for binding to DNA, namely, 13-hydroxy-, 12-hydroxyellipticine and ellipticine N^2-oxide. Their formation and CYPs producing these metabolites in rabbit hepatic microsomes are different from those in microsomes of humans and rats.

First, 13-hydroxyellipticine is the major metabolite formed in rat and human hepatic microsomes (it accounts ∼50 % of the total metabolite levels), while ellipticine N^2-oxide is the major one in rabbit microsomes. Similar proportions of 12-hydroxyellipticine only were formed in microsomes of all species.

Second, orthologous CYP3A enzymes in human and rat, but not in rabbit hepatic microsomes are the predominant enzymes oxidizing ellipticine to 13-hydroxy-, 12-hydroxyellipticine and ellipticine N^2-oxide. This follows from the significant inhibition of their formation by ketoconazole in microsomes of humans and rats and the effect of the CYP3A inducer, PCN, in rats. In addition, formation of 13-hydroxyellipticine by CYP3A in human and rat microsomes was supported by stimulation of its production with α-NF, which is known to stimulate oxidation of several substrates catalyzed by a CYP3A subfamily (Rendic and DiCarlo 1997; Ueng et al. 1997; Bořek-Dohalská et al. 2001). We showed earlier the predominant role of human CYP3A4 in 13-hydroxyellipticine and ellipticine N^2-oxide formation in hepatic microsomes from eight donors, by the strong correlation of specific catalytic activities of CYP3A4, its expression levels and formation of these metabolites (Stiborová et al. 2004). In addition, isolated human CYP3A4 and rat CYP3A1 were found to be the principal enzymes generating ellipticine-derived DNA adducts *in vitro* (Stiborová et al. 2003a). In further experiment, the levels of the DNA adduct found from 13-hydroxyellipticine in different organs of rats treated with ellipticine correlated with CYP3A1 expression levels (Stiborová et al. 2003a; 2004). Hence, the CYP3A-mediated oxidation of ellipticine to 13-hydroxyellipticine should occur also in rats *in vivo*.

In contrast to these results, rabbit CYP2C3 seems to be the predominant enzyme generating 13-hydroxyellipticine, ellipticine N^2-oxide and 12-hydroxyellipticine (present paper) and ellipticine-DNA adducts in rabbit hepatic microsomes (Stiborová et al. 2003b). This fact follows from several findings. It is known that the CYP2C3 enzyme is one of the major CYPs expressed in rabbit livers (Haugen and Coon 1976; Rendic and DiCarlo 1997; Bořek-Dohalská et al. 2001). Such a high CYP2C3 expression corresponds to the high efficiency of hepatic microsomes of uninduced rabbits to generate these metabolites (present paper) and DNA adducts (Stiborová et al. 2003b). Furthermore, because CYP2C expression is known to may have been suppressed by compounds inducing other CYPs in livers of rabbits (Haugen and Coon 1976; Bořek-Dohalská et al. 2001), the efficiency of hepatic microsomes of induced rabbits to activate ellipticine is expected to be lower than in control microsomes. This was indeed the case in our experiment. Another finding confirming the participation of CYP2C3 in oxidation of ellipticine to 13-hydroxy-, 12-hydroxyellipticine and ellipticine N^2-oxide by rabbit hepatic microsomes is the effective inhibition of their formation by the anti-human CYP2C9 antibody, which also inhibits the catalytic activity of CYP2C3.

All these results demonstrate similarities in CYP-mediated oxidation of ellipticine to metabolites that form DNA adducts in human and rat liver, and confirm that rats, but not rabbits, may better predict human susceptibility to ellipticine.

Conclusions

The results presented in this paper show the characterization of ellipticine metabolites produced by rat, rabbit and human hepatic microsomes and demonstrate analogy between CYPs of rat and human livers oxidizing ellipticine. Therefore, studies with rats as model organisms for additional information on the oxidative activation of ellipticine *in vivo* are a prerequisite for biomonitoring studies in humans.

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ORIGINAL ARTICLE

Cytochromes P450 reconstituted with NADPH: P450 reductase mimic the activating and detoxicating metabolism of the anticancer drug ellipticine in microsomes

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Abstract

OBJECTIVES: Ellipticine is a potent antineoplastic agent exhibiting multiple action mechanisms. Recently, we found that after cytochrome P450 (CYP)-mediated oxidation ellipticine forms covalent DNA adducts. Ellipticine oxidation by isolated CYP and its binding to DNA is the target of this study.

METHODS: High performance liquid chromatography (HPLC) was employed for separation and characterization of ellipticine metabolites generated by CYPs. The ^{32}P-postlabeling technique was utilized to determine ellipticine-DNA adducts.

RESULTS: Purified CYP enzymes reconstituted with NADPH:CYP reductase oxidized ellipticine to up to five metabolites, 7-hydroxy-, 9-hydroxy-, 12-hydroxy-, 13-hydroxyellipticine and ellipticine N^2-oxide. However, only CYP1A1 was capable to form all metabolites. Using the reconstituted enzymatic system, we demonstrated that the detoxication ellipticine metabolites, 7-hydroxyellipticine and 9-hydroxyellipticine, are mainly generated by CYP1A1 and 1A2, while those responsible for DNA binding, 13-hydroxy-, 12-hydroxyellipticine and ellipticine N^2-oxide, by CYP3A1 and 2C3. Likewise, the most efficient CYPs forming DNA adducts from ellipticine were CYP3A1 and 2C3.

CONCLUSIONS: The results showed that the system of purified CYPs reconstituted with NADPH:CYP reductase proved for ellipticine oxidation provide a true reflection of the situation in the microsomal membrane.

Abbreviations

COX	- cyclooxygenase
CYP	- cytochrome P450
HPLC	- high performance liquid chromatography
$NADP^+$	- nicotine amide dinucleotide phosphate
NADPH	- nicotine amide dinucleotide phosphate reduced
U	- units

Introduction

Ellipticine (Figure 1), an alkaloid isolated from Apocyanaceae plants exhibits significant antitumor and anti-HIV activities (for a summary, see [1,5,9]). The precise mechanisms of ellipticine action have not yet been fully explained. It was suggested that the prevalent mechanisms of its antitumor, mutagenic and cytotoxic activities were (i) intercalation into DNA and (ii) inhibition of DNA topoisomerase II activity [1,5,9]. Recently we found another mode of ellipticine action. Ellipticine was shown to bind covalently to DNA after being enzymatically activated with CYP enzymes or peroxidases [4,5,8,9,11]. Human recombinant CYP3A4, 1A1 and 1B1 were found to be the most efficient enzymes activating ellipticine to form covalent DNA adducts *in vitro* [5,10,11]. Among the peroxidases, human cyclooxygenase (COX)-2, ovine COX-1, bovine lactoperoxidases, human myeloperoxidase and horseradish peroxidase efficiently generated ellipticine-derived DNA adducts [4,8]. Covalent DNA adducts were also detected in human breast adenocarcinoma MCF-7 cells [2], in V79 Chinese hamster lung fibroblasts transfected with human CYP3A4, 1A1 and 1A2 [3] and *in vivo* in rats exposed to this anticancer drug [7]. On the basis of these data, ellipticine might be considered a drug, whose pharmacological efficiency and/or genotoxic side effects are dependent on its enzymatic activation in target tissues.

In order to explain the mechanism of enzymatic activation of ellipticine, we studied previously its oxidation by human CYPs and peroxidases [4,8,10]. Not only the metabolites generated by both enzymatic systems but also those responsible for the formation of two major ellipticine-derived DNA adducts were identified [4,8,10]. Human, rat and rabbit hepatic microsomes and human recombinant CYP enzymes oxidize ellipticine to five metabolites, namely 7-hydroxy-, 9-hydroxy-, 12-hydroxy-, 13-hydroxyellipticine and ellipticine N^2-oxide [6,10]. The major metabolite formed by peroxidases was characterized to be the ellipticine dimer, where two ellipticine residues are linked through nitrogen N^6 in the pyrrole ring of one of the ellipticine moieties and carbon C9 of the other ellipticine [4,8] (Figure 1). Ellipticine N^2-oxide was found to be the minor peroxidase-mediated metabolite [4,8]. Even though, except ellipticine N^2-oxide, the oxidative metabolites of this anticancer drug formed by CYPs are not identical with those formed by peroxidases, both enzymes generate the same two major DNA adducts, which are also found in various organs of rats treated with ellipticine [7]. Two of the ellipticine metabolites generated by human CYP enzymes, 13-hydroxy- and 12-hydroxyellipticine (the latter formed also spontaneously from ellipticine N^2-oxide by the Polonowski rearrangement [10]), are responsible for the formation of two major DNA adducts *in vitro* and *in vivo*.

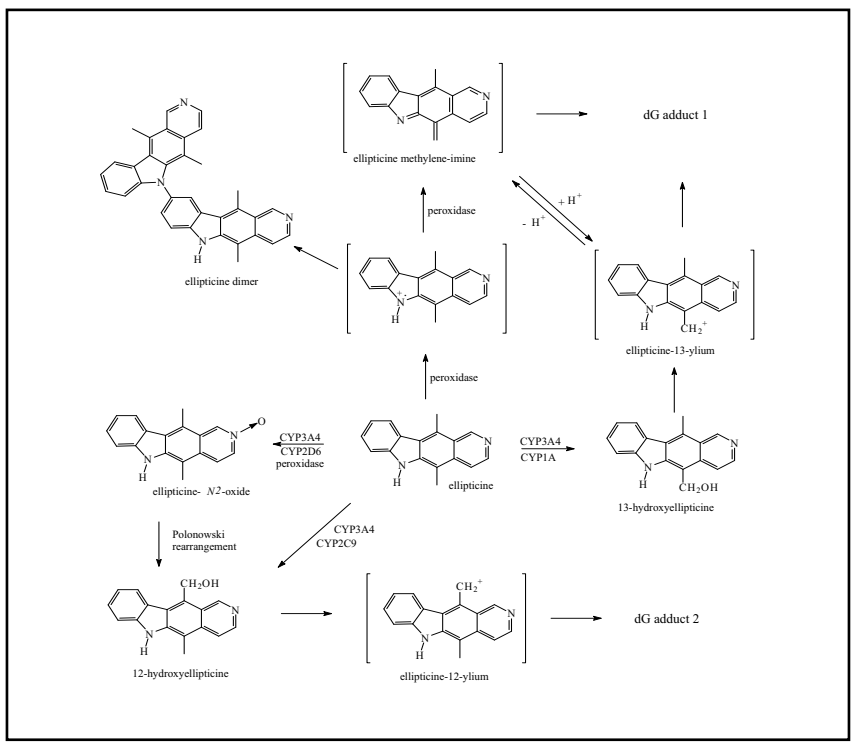

Figure 1. Metabolism of ellipticine by human CYPs and peroxidases showing the characterized metabolites forming DNA adducts.

Deoxyguanosine was identified as the target base of their binding [11] (Figure 1), but the exact structure of the reactive species and the position in guanine where these species bind remain to be elucidated. We have suggested that reactive carbenium ions formed spontaneously from 13-hydroxy- and 12-hydroxyellipticine (ellipticine-13-ylium and ellipticine-12-ylium, Figure 1) [8,10] might react with the nucleophilic centers of deoxyguanosine (e.g. the 2-NH_2 group) to form the adducts.

While ellipticine oxidation by human recombinant CYP enzymes in Supersomes™ (microsomes of Baculovirus transfected insect cells containing recombinantly expressed human CYPs and NADPH:CYP reductase) has been studied in detail [10], capacities of purified CYPs reconstituted with NADPH:CYP reductase in liposomes, which mimic the environment of the endoplasmic reticulum, are not known. Therefore we investigated the efficiency of purified CYP enzymes reconstituted with NADPH:CYP reductase to oxidize ellipticine. Moreover, we evaluated how this reconstituted enzymatic system was effective in the formation of ellipticine-DNA adducts.

Material and methods

The rat and/or rabbit CYP1A1, 1A2, 2B2, 2B4, 2C3 and 2E1, rabbit NADPH:CYP reductase and cytochrome b_5 were isolated as described [5]. Incubation mixtures used to study ellipticine metabolism contained the following in a final volume of 500 µl: 50 mM potassium phosphate buffer (pH 7.4), 1 mM $NADP^+$, 10 mM D-glucose 6-phosphate, 1 U/ml D-glucose 6-phosphate dehydrogenase, 0.1–0.2 µM CYPs reconstituted with NADPH:CYP reductase in liposomes and 5 µM ellipticine dissolved in 5 µl methanol. The enzyme reconstitution was performed as described [5,11]. After incubation (37 °C, 20 min), the reaction was stopped by adding 100 µl 2 M NaOH. Thereafter, 5 µl of 1 mM phenacetine in methanol was added as an internal standard and the ellipticine metabolites were extracted twice with ethyl acetate (2×1 ml). The extracts were evaporated under nitrogen and dissolved in 50 µl of methanol. The ellipticine metabolites were separated by HPLC as described [6,10]. Five ellipticine metabolites identified previously as 9-hydroxy-, 12-hydroxy-, 13-hydroxy-, 7-hydroxyellipticine and ellipticine N^2-oxide, were eluted at the retention times of 6.3, 6.9, 7.8, 8.5 and 11.2 min, respectively [6,10]. Incubation mixtures used for modifying DNA by ellipticine contained the following in a final volume of 750 µl:50 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, 50–250 pmol CYP reconstituted with NADPH:CYP reductase, 100 µM ellipticine dissolved in 7.5 µl methanol and 1 mg calf thymus DNA. After incubation (37 °C, 60 min), DNA was isolated by the phenol/chloroform extraction method [5,11]. ^{32}P-postlabeling analyses of ellipticine-DNA adducts were performed using nuclease P1 enrichment as described previously [5].

Results

Oxidation of ellipticine by purified CYP enzymes

To characterize the capability of the *in vitro* systems containing purified CYPs reconstituted with NADPH:CYP reductase to oxidize ellipticine, several CYP enzymes (CYP1A1, 1A2, 2B2, 2B4, 2C3, 2E1, 3A1 and 3A6) were purified and reconstituted with CYP reductase. Because cytochrome b_5 influences the oxidation of several substrates by CYP3A [12], this protein was included in the reconstitution systems containing this CYP. All reconstituted CYPs were active with their typical substrates (data not shown) and oxidized ellipticine to up to five metabolites, 7-hydroxy-, 9-hydroxy-, 12-hydroxy- , 13-hydroxyellipticine and ellipticine N^2-oxide, found previously to be formed by human, rat and rabbit hepatic microsomes [6,10]. Among the CYP enzymes tested, rat recombinant CYP1A1 followed by rabbit CYP1A2 were the most efficient enzymes oxidizing ellipticine. Both these CYP enzymes oxidized ellipticine mainly to 9-hydroxyellipticine with similar efficiencies, while 7-hydroxyellipticine was generated to a higher extent by rat CYP1A1, which was four times more efficient than rabbit CYP1A2 (Figure 2). CYP1A1 proved the only CYP enzyme studied to oxidize ellipticine to all five metabolites. Rat CYP3A1 was also a potent enzyme oxidizing ellipticine; it produced all ellipticine metabolites except 7-hydroxyellipticine, with predominance for ellipticine N^2-oxide and 13-hydroxyellipticine. In contrast, rabbit CYP3A6 was less efficient, but lead to the same metabolite pattern. Rabbit CYP2C3 was very efficient in ellipticine oxidation, producing mainly 12-hydroxy- and 13-hydroxyellipticine. Other CYPs tested in our study (rat CYP2B2, rabbit CYP2E1 and 2B4) also oxidized ellipticine, but to a much lower extent and the most prominent metabolite was ellipticine N^2-oxide.

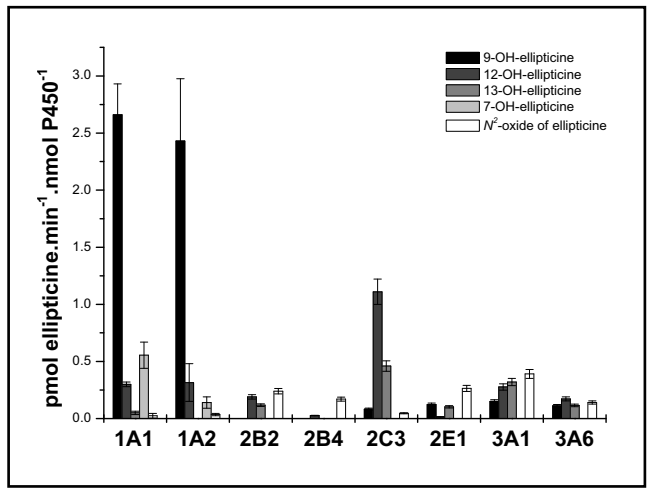

Figure 2. Oxidation of ellipticine by purified rat and rabbit CYPs reconstituted with rabbit NADPH:CYP reductase. 0.1–0.2 nmol amounts of reconstituted CYP/incubation and 5 µM ellipticine were used in all experiments. Values are averages and standard deviations of triplicate incubations.

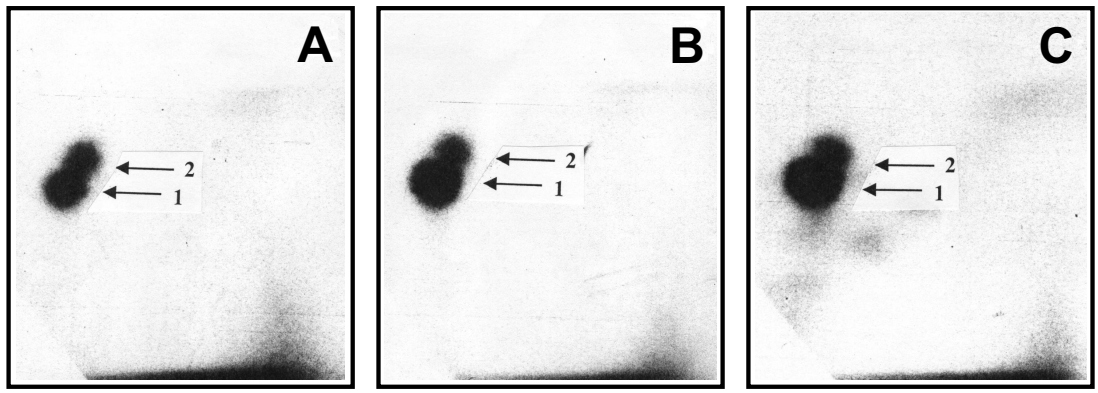

Figure 3. Ellipticine-DNA adduct levels in relation to concentration of rat CYP3A1 reconstituted with NADPH:CYP reductase. 50 pmol (A), 100 pmol (B) and 250 pmol CYP3A1 (C). Analyses were performed by the nuclease P1 version of the ^{32}P-postlabeling assay. Film exposure was 3.5 h at –80 °C. Origins are located at the bottom left corners (D3 from bottom to top and D4 from left to right).

Formation of ellipticine-DNA adducts by purified CYP enzymes

Using the same reconstituted systems we showed that all CYPs activated ellipticine to metabolites forming DNA adducts. Among the CYPs tested, the most potent enzyme generating the two major ellipticine-DNA adducts (spots 1 and 2 in Figure 3) was rat CYP3A1 (Figure 4). This was expected since the major metabolites formed by CYP3A1 were 13-hydroxy-, 12-hydroxyellipticine and N^2-oxide, which we had shown previously to form DNA adducts 1 and 2 [8]. Similar to the lower metabolite levels, isolated rabbit CYP3A6 was less efficient in activating ellipticine, forming more than four times lower levels of DNA adducts than CYP3A1, while isolated rabbit CYP2C3 was more effective than 3A6 (Figure 4). Again, the adduct levels correlated with the pattern of ellipticine metabolites formed by both CYPs. Rat CYP1A1 exhibited similar efficiency to form ellipticine-DNA adducts as CYP3A6. All the other CYPs tested in our study were less active and generated almost the same levels of both DNA adducts.

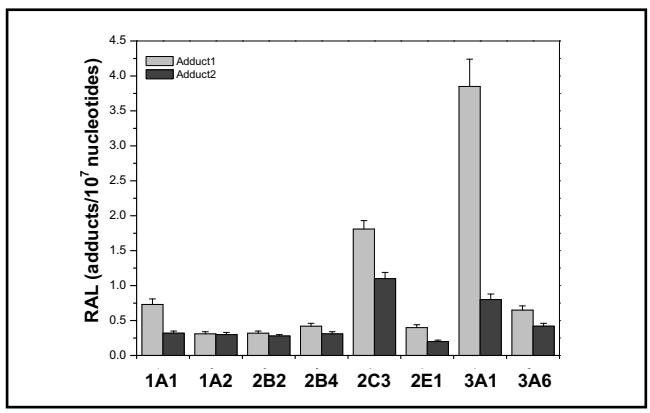

Figure 4. Activation of ellipticine by isolated CYP reconstituted with NADPH:CYP reductase determined by ^{32}P-postlabeling assay. CYP (250 pmol)/incubation and 100 μM ellipticine were used in all experiments. Values are averages and standard deviations of triplicate incubations.

Discussion

The aim of this study was to evaluate whether systems consisting of purified CYPs reconstituted with NADPH:CYP reductase in liposomes were capable of oxidizing the antineoplastic agent ellipticine. Such systems have been believed to mimic the proper structural arrangement of CYP and NADPH:CYP reductase enzymes in the membrane of the endoplasmic reticulum [12], to form the enzymatically active complex metabolizing xenobiotics. We showed that ellipticine was oxidized in reconstituted system containing individual CYP enzymes to metabolites identical to those generated in microsomes isolated from human, rat and rabbit livers [6,10]. Moreover, the same CYP enzymes in the reconstituted systems were responsible for the formation of individual ellipticine metabolites as in hepatic microsomes; CYP1A1 and/or 1A2 in the reconstituted systems as well as in rat and rabbit hepatic microsomes oxidized ellipticine to detoxication metabolites (9-hydroxy- and 7-hydroxyellipticine) [6, present paper]. The metabolites responsible for binding to DNA, 13-hydroxyellipticine, 12-hydroxyellipticine and ellipticine N^2-oxide, are mainly produced by isolated rat CYP3A1 and/or rabbit CYP2C3. CYP3A1 and CYP2C3 were also the major enzymes generating these metabolites in rat and rabbit hepatic microsomes, respectively [6]. In addition, all these results correlate with the efficiencies of CYP3A1 and 2C3 to generate ellipticine-DNA adducts in the reconstituted system. The results presented in this study demonstrate the suitability of purified CYP enzymes in the reconstituted systems for studies investigating the detoxication and activation metabolism of ellipticine *in vitro*. The data also confirm the results of our former study, in which we identified the participation of individual CYP enzymes in the formation of ellipticine metabolites in rat and rabbit microsomes [6]. Such studies are also important when microsomes are not available, e.g. from extrahepatic tissues such as nasal mucosa or tumors.

Acknowledgment

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The Anticancer Drug Ellipticine Is a Potent Inducer of Rat Cytochromes P450 1A1 and 1A2, Thereby Modulating Its Own Metabolism[S]

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ABSTRACT:

Ellipticine is an antineoplastic agent whose mode of action is based mainly on DNA intercalation, inhibition of topoisomerase II, and formation of covalent DNA adducts mediated by cytochromes P450 (P450s) and peroxidases. Here, this drug was found to induce CYP1A1 and/or 1A2 enzymes and their enzymatic activities in livers, lungs, and kidneys of rats treated (i.p.) with ellipticine. The induction is transient. In the absence of repeated administration of ellipticine, the levels and activities of the induced CYP1A decreased almost to the basal level 2 weeks after treatment. The ellipticine-mediated CYP1A induction increases the DNA adduct formation by the compound. When microsomal fractions from livers, kidneys, and lungs of rats treated with ellipticine were incubated with ellipticine, DNA adduct formation, measured by ^{32}P-postlabeling analysis, was up to 3.8-fold higher in incubations with microsomes from pretreated rats than with controls. The observed stimulation of DNA adduct formation by ellipticine was attributed to induction of CYP1A1 and/or 1A2-mediated increase in ellipticine oxidative activation to 13-hydroxy- and 12-hydroxyellipticine, the metabolites generating two major DNA adducts in human and rat livers. In addition to these metabolites, increased formation of the excretion products 9-hydroxy- and 7-hydroxyellipticine was also observed in microsomes of rats treated with ellipticine. Taken together, these results demonstrate for the first time that by inducing CYP1A1/2, ellipticine increases its own metabolism, leading both to an activation of this drug to reactive species-forming DNA adducts and to detoxication metabolites, thereby modulating to some extent its pharmacological and/or genotoxic potential.

Ellipticine (5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole; Fig. 1), an alkaloid isolated from *Apocyanaceae* plants, exhibits significant antitumor and anti-human immunodeficiency virus activities (Auclair, 1987; Stiborová et al., 2001). The main reason for the interest in ellipticine and its derivatives for clinical purposes is their high efficiencies against several types of cancer, their rather limited toxic side effects, and their complete lack of hematological toxicity (Auclair, 1987). Nevertheless, ellipticine is a potent mutagen (for a summary, see Stiborová et al., 2001).

Ellipticines are anticancer drugs whose precise mechanisms of action have not been explained yet. It was suggested that the prevalent mechanisms of their antitumor, mutagenic, and cytotoxic activities are 1) intercalation into DNA (Auclair, 1987; Singh et al., 1994), and 2) inhibition of DNA topoisomerase II activity (Auclair, 1987; Fossé et al., 1992; Froelich-Ammon et al., 1995). Ellipticine and its metabolite 9-hydroxyellipticine also cause selective inhibition of p53 protein phosphorylation in several human cancer cell lines (Sugikawa et al., 1999), and this correlates with their cytotoxic activity. Ellipticines also uncouple mitochondrial oxidative phosphorylation (Schwaller et al., 1995) and thereby disrupt the energy balance of cells.

We demonstrated that ellipticine covalently binds to DNA after being enzymatically activated with cytochromes P450 (P450s) or peroxidases (Stiborová et al., 2001, 2003c, 2004, 2007; Frei et al., 2002). Human and rat P450s of 1A and 3A subfamilies are the predominant enzymes catalyzing oxidation of ellipticine either to metabolites that are excreted from organisms (7-hydroxy- and 9-hydroxyellipticine) or form DNA adducts (12-hydroxy- and 13-hydroxyellipticine) (Stiborová et al., 2001, 2003c, 2004; Kotrbová et al., 2006). Of the peroxidases, human cyclooxygenase-2, ovine cyclooxygenase-1, bovine lactoperoxidases, human myeloperoxidase, and horseradish peroxidase efficiently generated ellipticine-

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ABBREVIATIONS: P450, cytochrome P450; AhR, aryl hydrocarbon receptor; HPLC, high-performance liquid chromatography; PVDF, polyvinylidene difluoride; EROD, 7-ethoxyresorufin *O*-deethylation; MROD, 7-methoxyresorufin *O*-deethylation; PCR, polymerase chain reaction; c_T, cycle threshold; α-NF, α-naphthoflavone; ARNT, AhR nuclear translocator.

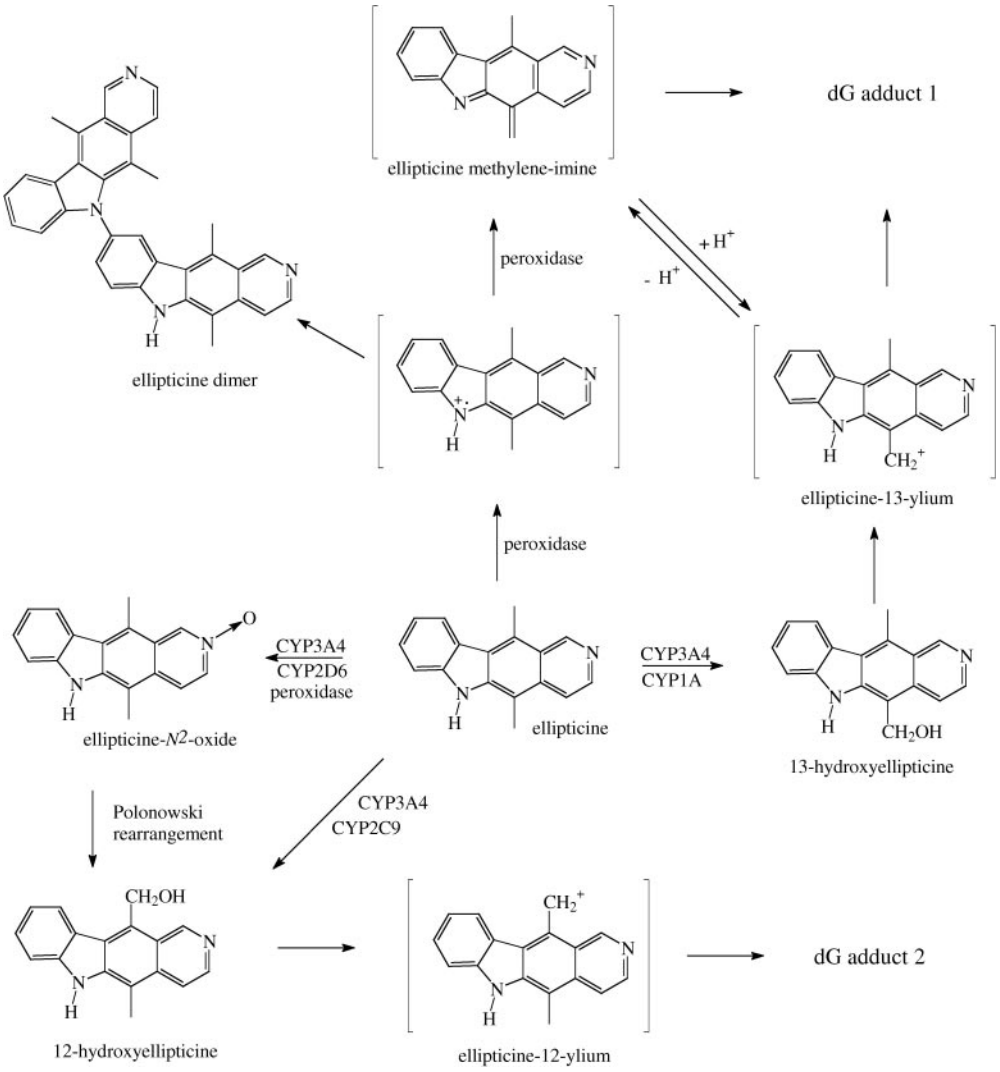

FIG. 1. Metabolism of ellipticine by peroxidases and human P450s showing the identified metabolites and those proposed to form DNA adducts. The compounds shown in brackets were not detected under the experimental conditions and are the electrophilic metabolites postulated as ultimate arylating species.

derived DNA adducts (Fig. 1) (Stiborová et al., 2007). The same DNA adducts by ellipticine were also detected in human breast adenocarcinoma MCF-7 cells (Bořek-Dohalská et al., 2004); in V79 Chinese hamster lung fibroblasts transfected with human CYP3A4, 1A1, and 1A2 (Frei et al., 2002); in leukemia HL-60 and CCRF-CEM cells (Poljaková et al., 2007); and in vivo in rats exposed to this anticancer drug (Stiborová et al., 2003a). On the basis of these data, ellipticine might be considered a drug whose pharmacological efficiency and/or genotoxic side effects are dependent on its enzymatic activation in target tissues.

Because of the important role of P450 enzymes in ellipticine metabolism, expression levels of these enzymes are crucial for antitumor, cytostatic, and/or genotoxic activities of this drug in individual tissues. Indeed, we found recently that the formation of the major ellipticine-derived DNA adduct (adduct formed in deoxyguanosine from 13-hydroxyellipticine; Fig. 1) in different organs of rats exposed to ellipticine is dependent on expression levels of CYP3A1. This is also the most efficient rat P450 enzyme-activating ellipticine to form this DNA adduct in vitro (Stiborová et al., 2001, 2003a,c; Kotrbová et al., 2006). Likewise, the orthologous human enzyme CYP3A4 in human liver microsomes is also the most efficient enzyme activating ellipticine to 13-hydroxyellipticine and DNA adducts (Stiborová et al., 2004). In rats in vivo, however, not only CYP3A1 but also P450s of the 1A subfamily seem to be important for the formation of this adduct even though in in vitro incubations CYP1A enzymes are much less active than CYP3A1 (Stiborová et al., 2001, 2003a,c; Kotrbová et al., 2006). One of the reasons for this observed discrepancy might be the possibility of ellipticine-mediated induction of CYP1A enzymes in rats, which results in their higher protein levels and activities in vivo.

CYP1A1 and 1A2 enzymes are induced by a variety of compounds, of which dioxins such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin are the most effective (Couture et al., 1990). Dioxins and related compounds induce CYP1A by binding to the ligand-activated transcription factor, aryl hydrocarbon receptor (AhR). Binding of ellipticine to this transcription factor has already been described previously (Fernandez et al., 1988), suggesting that this drug may induce CYP1A expression (Cresteil et al., 1982; Gasiewicz et al., 1996). Indeed, the previous studies of Cresteil et al. (1982) have shown that ellipticine is a CYP1A inducer, although these authors did not determine which members of this subfamily were affected. The present study was aimed at elucidating this question. Because rats were found to be suitable models mimicking the fate of ellipticine in humans (Stiborová et al., 2003a,c), they were used as model organisms in this study. Furthermore, because the constitutive and induced expression of rat CYP1A is sexually dimorphic (Iba et al., 1999), rats of both sexes were used in some experiments.

TABLE 1

Total contents of P450 in hepatic microsomes of ellipticine-treated and control rats

Results shown are mean ± S.D. from data obtained by the method of Omura and Sato (1964) for three rats.

Ellipticine Dose (mg/kg)	P450 in Hepatic Microsomes of	
	Male Rats	Female Rats
	nmol/mg protein	
0	0.32 ± 0.07	0.42 ± 0.14
4	0.33 ± 0.03	0.32 ± 0.05
40	0.35 ± 0.06	0.34 ± 0.06
80	0.61 ± 0.08*	0.21 ± 0.02*

*$P < 0.01$, significantly different from controls (Student's t test).

Materials and Methods

Chemicals. $NADP^+$, NADPH, ellipticine, D-glucose 6-phosphate, D-glucose 6-phosphate dehydrogenase, and calf thymus DNA were obtained from Sigma Chemical Co. (St. Louis, MO); Sudan I from BDH (Poole, UK); 7-ethoxyresorufin and 7-methoxyresorufin from Fluka Chemie AG (Buchs, Switzerland); and 9-hydroxyellipticine (5,11-dimethyl-9-hydroxy-6*H*-pyrido[4,3-*b*]carbazole) was from Calbiochem (San Diego, CA). All these and other chemicals from commercial sources used in the experiments were reagent grade or better. 7-Hydroxyellipticine and the N^2-oxide of ellipticine were synthesized as described previously (Wijsmuller et al., 1986; Boogaard et al., 1994) by J. Kučka (Charles University, Prague, Czech Republic); their purity was >99.5% as estimated by high-performance liquid chromatography (HPLC). Enzymatically prepared 12-hydroxy- and 13-hydroxyellipticine were obtained from multiple HPLC runs of ethyl acetate extracts of incubations of ellipticine with human and/or rat hepatic microsomes as described previously (Stiborová et al., 2004).

Animal Experiments. The study was conducted in accordance with the Regulations for the Care and Use of Laboratory Animals (311/1997, Ministry of Agriculture, Prague, Czech Republic), which is in compliance with Declaration of Helsinki. Male and female Wistar rats (~100 g) were treated with a single dose of 4, 40, or 80 mg/kg body weight ($n = 3$) of ellipticine by intraperitoneal injection. Ellipticine was dissolved in sunflower oil/dimethyl sulfoxide [1:1 (v/v)] to give a concentration of 6 mg/ml. Three control animals received an equal volume of solvent only. Rats were placed in cages in temperature and humidity-controlled rooms. Standardized diet and water were provided ad libitum. The animals were killed 48 h after treatment by cervical dislocation. Livers, lungs, and kidneys were removed immediately after death and used for isolation of mRNA and for preparation of microsomal fractions. To analyze the duration of P450 induction, 13 male rats were treated with 80 mg of ellipticine per kg body weight in one dose; rats were sacrificed 2 days (three animals), 2 weeks (two animals), 10 weeks (three animals), and 32 weeks (five animals) after the treatment by cervical dislocation and their livers used for isolation of microsomes.

Preparation of Microsomes. Microsomes were isolated from the livers, kidneys, and lungs of rats as described previously (Stiborová et al., 2003b). Protein concentrations in the microsomal fractions were assessed using the bicinchoninic acid protein assay with bovine serum albumin as a standard (Wiechelman et al., 1988). The concentration of P450 was estimated according to Omura and Sato (1964) based on the absorption of the complex of reduced P450 with carbon monoxide. The total amounts of hepatic P450 are shown in Table 1. Kidney microsomes of control and ellipticine-treated rats (40 mg/kg) contained 0.17 and 1.19 nmol of P450/mg of protein, respectively. Using the method of Omura and Sato (1964), no P450 was detectable in lung microsomes. Hepatic, renal, and pulmonary microsomal preparations from rats that had been pretreated with ellipticine were analyzed for the presence of ellipticine or its metabolites by HPLC as described previously (Stiborová et al., 2004). Neither ellipticine nor any of its metabolites were detectable in microsomal fractions from tissues of rats that had been pretreated with ellipticine.

Isolation of P450s. CYP2B4 and 2E1 enzymes were isolated from liver microsomes of rabbits induced with phenobarbital (CYP2B4) or ethanol (CYP2E1) by procedures described previously (Stiborová et al., 2002a). Recombinant rat CYP1A1 protein was purified to homogeneity from membranes of *Escherichia coli* transfected with a modified CYP1A1 cDNA (Stiborová et al., 2002b) in the laboratory of H. W. Strobel (University of Texas, Medical School of Houston, Houston, TX) by P. Hodek (Charles University, Prague, Czech Republic). Human recombinant CYP3A4 was a gift of P. Anzenbacher (Palacky University, Olomouc, Czech Republic).

Preparation of Antibodies. Leghorn chickens were immunized subcutaneously three times (at week-interval) with P450 antigens (rat recombinant CYP1A1, rabbit CYP2B4, rabbit CYP2E1, and human recombinant CYP3A4) (0.1 mg/animal) emulsified in complete Freund's adjuvant for the first injection and in incomplete adjuvant for boosters. Immunoglobulin fraction was isolated from pooled egg yolks using fractionation with polyethylene glycol 6000 (Stiborová et al., 2002b).

Determination of P450 Protein Levels in Microsomes of Rat Liver, Kidney, and Lung. Immunoquantitation of rat liver, kidney, and lung microsomal P450s (CYP1A1, 1A2, 2B, 2E1, and 3A) was done by SDS-polyacrylamide gel electrophoresis. Samples containing 75 μg of microsomal proteins were subjected to electrophoresis on SDS/10% polyacrylamide gels (Stiborová et al., 2002b, 2005). After migration, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. P450 proteins were probed with the chicken polyclonal antibodies as reported elsewhere (Stiborová et al., 2002b, 2005, 2006). The antibody against rat recombinant CYP1A1 recognizes both CYP1A1 and 1A2 in rat liver microsomes (Figs. 2 and 3) (Stiborová et al., 2002b). Rat recombinant CYP1A1 and 1A2 (in Supersomes; Gentest Corp., Woburn, MA) were used as positive controls to identify the bands of CYP1A1 and 1A2 in microsomes. The antigen-antibody complex was visualized with an alkaline phosphatase-conjugated rabbit anti-chicken IgG antibody and 5-bromo-4-chloro-3-indolylphosphate/nitrobluetetrazolium as chromogenic substrate (Stiborová et al., 2002b, 2005, 2006).

CYP1A Enzyme Activity Assays. The microsomal samples were characterized for CYP1A activity using 7-ethoxyresorufin *O*-deethylation (EROD), CYP1A2 activity using 7-methoxyresorufin *O*-demethylation (MROD) (Guengerich and Shimada, 1991) and for the oxidation of Sudan I (a marker substrate for CYP1A1) (Stiborová et al., 2002b, 2005).

CYP1A mRNA Content in Rat Livers, Kidneys, and Lungs. Total RNA was isolated from frozen livers, kidneys, and lungs of three untreated rats and three rats pretreated with 40 mg/kg body weight of ellipticine using TRIzol Reagent (Invitrogen, Carlsbad, CA) according to the procedure supplied by the manufacturer. The quality of isolated RNA was verified by horizontal agarose gel electrophoresis, and RNA quantity was assessed by UV-visible spectrophotometry on a Carry 300 spectrophotometer (Varian, Palo Alto, CA). RNA samples (1 μg) were reversely transcribed using 200 U of reverse transcriptase per sample with random hexamer primers using the RevertAid First Strand cDNA Synthesis Kit (MBI Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. The prepared cDNA was used for real-time polymerase chain reaction (PCR) performed in a RotorGene 2000 (Corbett Research, Sydney, Australia) under the following cycling conditions: incubation at 50°C for 2 min and initial denaturation at 95°C for 10 min, then 50 cycles of denaturation at 95°C for 15 s, and annealing at 60°C for 1 min. Gain was set to 7, and fluorescence was acquired after elongation step. The PCR mixtures (20 μl) contained 9 μl of cDNA diluted 10 times in Milli-Q Ultrapure water (Biocel A10; Millipore, Billerica, MA), 10 μl of TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), and 1 μl of TaqMan Gene Expression Assay Mix (commercially available unlabeled PCR primers and 5-carboxyfluorescein dye-labeled probe for rat *CYP1A1/2* as target genes and *β-actin* as reference internal standard gene). Each sample was analyzed in two parallel aliquots. Negative controls had the same composition as samples, but cDNA was omitted from the mixture. Data were analyzed by the program RotorGene v6 (Corbett Research) and evaluated by comparative cycle threshold (c_T) method for relative quantitation of gene expression. Cycle thresholds, at which a significant increase in fluorescence signal was detected, were measured for each sample. Then $\Delta\Delta c_T$ was evaluated according to the following equations: $\Delta c_T = c_T$ (target) $- c_T$ (internal standard), $\Delta\Delta c_T = \Delta c_{Ttreated} - \Delta c_{Tcontrol}$, where $\Delta c_{Ttreated}$ is Δc_T for ellipticine treated rats and $\Delta c_{Tcontrol}$ is Δc_T for untreated rats. Δc_T is positive if the target is expressed at a lower level than the internal standard (*β-actin*) and negative if expressed at a higher level. The induction of mRNA expression of the studied target genes (-fold change) in pretreated animals was evaluated as $2^{-(\Delta\Delta cT)}$.

Microsomal Incubations. Incubation mixtures used to asses DNA adducts

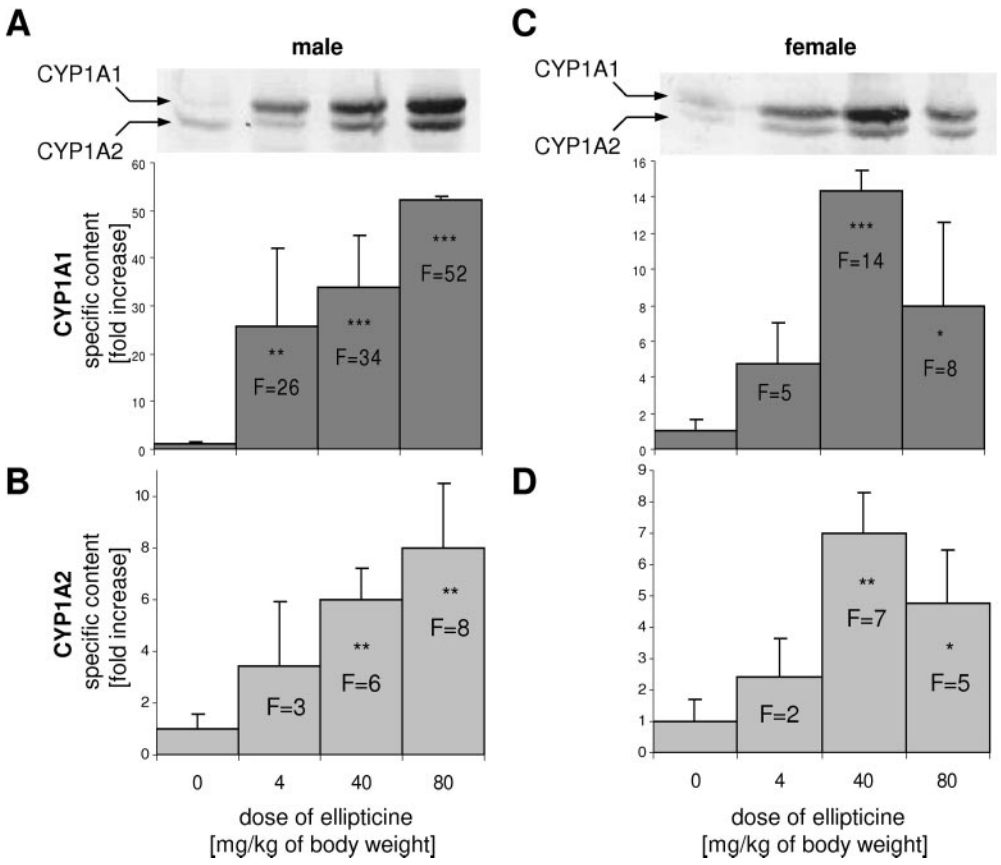

FIG. 2. Induction of CYP1A1 (A and C) and 1A2 (B and D) in livers of male (A and B) and female rats (C and D) treated with 4, 40, or 80 mg/kg body weight ellipticine. Mean values ± standard deviations shown in figure represent results obtained from livers of three rats ($n = 3$). Inset in A and C, immunoblots of microsomal CYP1A1 and 1A2 from untreated and ellipticine-treated male and female rats, respectively, stained with antibody against rat CYP1A1. Microsomes isolated from rat livers were subjected to SDS-polyacrylamide gel electrophoresis, and proteins were transferred to PVDF membranes and probed with antibody as described under *Materials and Methods*. Values significantly different from the control: *, $p < 0.05$, **, $p < 0.01$; and ***, $p < 0.001$ (Student's t test).

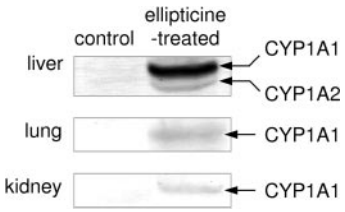

FIG. 3. Immunoblots of microsomal CYP1A1 and 1A2 from livers, lungs, and kidneys of untreated and ellipticine-treated (40 mg/kg) male rats stained with antibody against rat CYP1A1. Microsomes isolated from rat organs were subjected to SDS-polyacrylamide gel electrophoresis, and proteins were transferred to PVDF membranes and probed with antibody as described under *Materials and Methods*.

formed by ellipticine consisted of 50 mM potassium phosphate buffer, pH 7.4, 1 mM NADPH, pooled hepatic microsomal sample (0.5 mg of protein) from three male rats, either control or treated with 4, 40, and 80 mg/kg body weight of ellipticine, 100 μM ellipticine (dissolved in 7.5 μl of methanol), and 0.5 mg of calf thymus DNA in a final volume of 750 μl. The reaction was initiated by adding ellipticine. In incubations with lung and kidney microsomes, control microsomes were compared with microsomes from rats treated with 40 mg/kg body weight. Incubations were carried out at 37°C for 30 min; ellipticine-DNA adduct formation was found to be linear up to 30 min of incubation (Stiborová et al., 2001). Control incubations were carried out either 1) without microsomes or 2) without NADPH, 3) without DNA, or 4) without ellipticine. After the incubation, DNA was isolated from the residual water phase by the phenol/chloroform extraction method as described previously (Stiborová et al., 2001).

Incubation mixtures used to study the ellipticine metabolites contained 50 mM potassium 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), 0.2 mg of protein of pooled hepatic microsomal fraction from three male rats, either untreated or treated with 40 mg/kg body weight ellipticine, and 10 μM ellipticine (dissolved in 5 μl of methanol) in a final volume of 500 μl. The reaction was initiated by adding the substrate. In the control incubation, ellipticine was omitted from the incubation mixture. After incubation in open glass tubes (37°C, 20 min), the reaction was stopped by adding 100 μl of 2 M NaOH. The oxidation of ellipticine is linear up to 30 min of incubation (Stiborová et al., 2004). After incubation, 5 μl of 1 mM phenacetine in methanol was added as an internal standard and the ellipticine metabolites were extracted twice with ethyl acetate (two times, 1 ml). Analyzes of ellipticine metabolites were performed by HPLC as described previously (Stiborová et al., 2004). Recoveries of ellipticine metabolites were around 95% in the presence of microsomes without a P450 cofactor (NADPH generation system).

Inhibition Studies. The following chemicals were used to inhibit the activation of ellipticine in hepatic microsomes of male rats: α-naphthoflavone (α-NF), which inhibits CYP1A1 and 1A2, being more efficient to inhibit CYP1A1 (Rendic and DiCarlo, 1997; Stiborová et al., 2005); furafylline, which inhibits CYP1A2; and troleandomycin and ketoconazole, which inhibit CYP3A (Rendic and DiCarlo, 1997). Inhibitors were dissolved in 7.5 μl of methanol to yield final concentrations of 100 μM in the incubation mixtures used to asses DNA adducts formed by ellipticine (see above). Mixtures were then incubated at 37°C for 10 min with NADPH before adding ellipticine and then incubated for a further 30 min at 37°C. After the incubation, DNA was isolated as mentioned above.

^{32}P Postlabeling Analysis and HPLC Analysis of ^{32}P-labeled 3′,5′-Deoxyribonucleoside Bisphosphate Adducts. The ^{32}P postlabeling of nucleotides using nuclease P1 enrichment procedure, found previously to be appropriate to detect and quantify ellipticine-derived DNA adducts formed in vitro (Stiborová et al., 2001, 2003c, 2004, 2007) and in vivo (Stiborová et al., 2003a), was used in the experiments. The thin-layer chromatography and HPLC analyzes were done as reported recently (Stiborová et al., 2001, 2003a,c, 2004, 2007).

Results

Effect of Ellipticine on P450 Expression. Western blots with chicken polyclonal antibodies raised against different P450s (CYP1A1, 2B4, 2E1, and 3A4) showed that the expression of hepatic

TABLE 2

Specific CYP1A activities in hepatic microsomes of control and ellipticine-treated rats

Each value (pmol of reaction product/min/nmol P450) represents the mean ± standard deviation of data from two rats in two separate assays ($n = 4$). Numbers in parentheses represents the -fold increase over the control activity caused by the pretreatment with ellipticine (40 mg/kg).

P450 Activity	Control Rat		Ellipticine-Treated Rat	
	Male	Female	Male	Female
EROD	80.7 ± 2.0	225.8 ± 50.5	551.4 ± 92.2 (6.9)*	1737.5 ± 161.3 (7.7)*
MROD	3.28 ± 0.15	2.48 ± 0.24	19.73 ± 0.08 (6.0)*	20.07 ± 1.51 (8.0)*
Sudan I oxidation	0.125 ± 0.013	0.107 ± 0.010	1.463 ± 0.123 (12.2)*	1.491 ± 0.151 (14.0)*

* $P < 0.001$, significantly different from controls (Student's t test).

CYP1A1 and 1A2 was induced in male and female rats treated with ellipticine (Fig. 2). The expression of hepatic CYP2B, 2E1, and 3A was not altered by treating rats with ellipticine (data not shown).

Constitutive levels of CYP1A1 and 1A2 and their induction by ellipticine were sex-dependent; constitutive protein expression of CYP1A1 was higher in female rats than that of CYP1A2 in males (Fig. 2). The increase in hepatic CYP1A1 and 1A2 expression by ellipticine was dose-dependent in all cases (Fig. 2) and correlated significantly with all doses of ellipticine administered to male rats [correlation coefficients of 0.807 ($p < 0.05$, $n = 4$) for CYP1A1 and 0.896 ($p < 0.05$, $n = 4$) for CYP1A2] but only for doses of 4 and 40 mg/kg body weight administered to female rats [correlation coefficients of 0.966 ($p < 0.01$, $n = 3$) for CYP1A1 and 0.981 ($p < 0.01$, $n = 3$) for CYP1A2]. Lower induction of CYP1A in female rats treated with 80 mg/kg body weight may be caused by a higher sensitivity of female rats to the toxicity of the administered compound. Indeed, ellipticine at doses above 40 mg/kg was found to be toxic to rats (Cresteil et al., 1982). The dose of 80 mg/kg also caused a decrease in levels of total hepatic P450 in female rats but an increase in total P450 levels in male rats (Table 1). The EROD activity, a marker for CYP1A1 and 1A2, the MROD activity, a marker for CYP1A2, as well as the oxidation of Sudan I, a marker substrate of CYP1A1 (Stiborová et al., 2002b, 2005), increased in hepatic microsomes of rats treated with ellipticine (Table 2).

Ellipticine also induced CYP1A1 levels and its activities in kidneys and lungs (Fig. 3). Male rats were used in these experiments. Because of very low CYP1A1 protein levels and activities in kidneys and lungs of control animals, the ellipticine-mediated induction in these organs could not be quantified.

In addition to evaluating the effects of ellipticine on protein levels and enzyme activities of CYP1A1 and 1A2, modulation of their mRNA expression by this compound in male rats was also investigated. As shown in Table 3, treatment of male rats with 40 mg/kg body weight of ellipticine induced an increase in mRNA expression levels of CYP1A1 in livers, kidneys, and lungs, whereas no significant increase in CYP1A2 mRNA expression was observed.

Ellipticine-Mediated CYP1A1 and 1A2 Induction Is Transient. CYP1A1 and 1A2 protein expression was measured in livers of male rats 2 days and 2, 10, and 32 weeks after administration of a single i.p. dose of 80 mg of ellipticine/kg body weight. Maximum CYP1A1/2 expression levels in this organ were found 2 days after treatment, the earliest time point at which liver samples were collected. As shown in Fig. 4 a decline almost to the basal level of CYP1A1/2 protein expression and their enzymatic activities was detected 2 weeks after the dose.

Oxidation of Ellipticine by Rat Hepatic, Renal, and Pulmonary Microsomes to DNA-Binding Metabolites. We compared the formation of adducts in calf thymus DNA by ellipticine incubated with

TABLE 3

Expression of mRNA of CYP1A1 and 1A2

Results shown are mean ± standard deviation from data found for three male rats (control and treated with 40 mg/kg body weight).

	CYP1A1		CYP1A2	
	Δc_T	-Fold Change	Δc_T	-Fold Change
Control rats				
Liver	6.37 ± 0.07		−3.90 ± 0.12	
Kidney	4.45 ± 0.36		14.07 ± 0.54	
Lung	10.48 ± 0.16		13.97 ± 1.06	
Ellipticine-treated rats				
Liver	3.87 ± 1.73	5.66*	−4.04 ± 0.22	1.10
Kidney	1.12 ± 0.13	10.08**	13.80 ± 0.76	1.21
Lung	5.89 ± 0.59	24.20**	15.37 ± 0.03	0.38

* $P < 0.05$; ** $P < 0.001$ (Student's t test) significantly different from controls.

microsomes from the livers, kidneys, and lungs of treated and control male rats. The DNA adduct pattern generated by ellipticine consisted of at least two adducts (spots 1 and 2 in Fig. 5), which were identical to those formed in vivo in rats treated with ellipticine (Fig. 5C), in in vitro incubations of DNA with ellipticine and human and rat hepatic microsomes (Stiborová et al., 2001, 2003a, 2004), and in those with 13-hydroxyellipticine (Fig. 5D) or 12-hydroxyellipticine (Fig. 5E). Lung and kidney microsomes were 22.4- and 32.7-fold less efficient than microsomes isolated from livers (Fig, 6; Supplemental Tables S1 and S2).

Two additional minor ellipticine-derived DNA adducts (spots 6 and 7 in Fig. 5, A and B) were found in DNA incubated with ellipticine and rat hepatic microsomes. These adducts are also formed in vivo (Fig. 5C) and in several in vitro activation systems, such as human liver microsomes (Stiborová et al., 2004) and peroxidases (Stiborová et al., 2007), but the low adduct levels prevented HPLC co-chromatographic analyses or their further characterization. Thin-layer chromatograms of ^{32}P-labeled DNA from control incubations carried out in parallel, without ellipticine or without DNA, were devoid of all adduct spots in the region of interest (data not shown). Control incubations without microsomes or NADPH were free of adduct spots 1, 6, and 7, but the adduct spot 2 was always detected (Supplemental Table S1). This finding is consistent with our previous results showing that this adduct is formed also nonenzymatically (Stiborová et al., 2001, 2003c, 2004, 2007).

Ellipticine DNA adduct levels generated by liver, lung, and kidney microsomes isolated from male rats treated with ellipticine were higher than DNA adduct levels formed by control microsomes (Fig. 6; Supplemental Table S1). The increase in levels of DNA adducts formed in hepatic microsomes of rats treated with ellipticine might be attributed to an increase in enzymatic activity of both CYP1A1 and 1A2. Although the relative contributions of both two P450 enzymes to

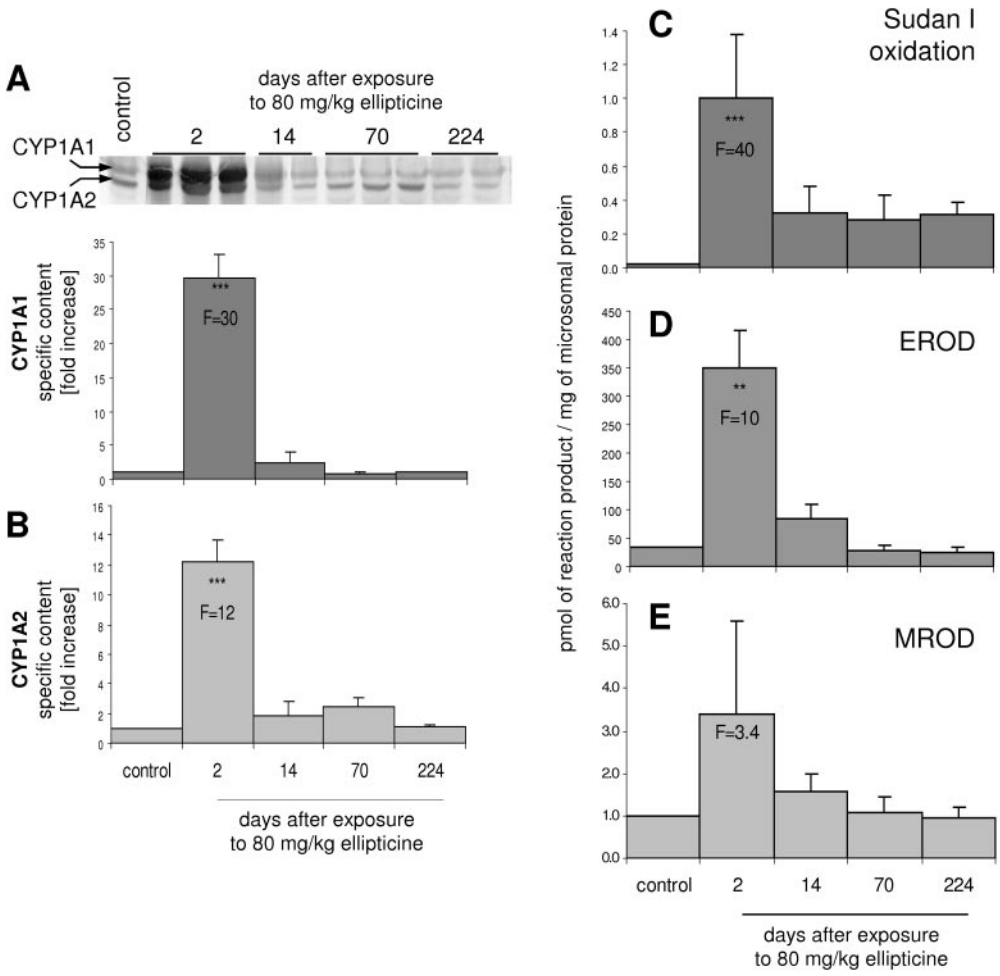

FIG. 4. Time course of ellipticine-mediated induction of CYP1A1 (A, bottom) and 1A2 protein (B) and specific CYP1A activities (C–E) in livers of male rats treated intraperitoneally with a single dose of 80 mg/kg body weight ellipticine. Results represent means ± standard deviations from three to five treated rats (see *Materials and Methods*). A, top: immunoblots of microsomal CYP1A1 and 1A2 from untreated and ellipticine-treated male rats stained with antibody against rat CYP1A1. Values significantly different from control: **, $p < 0.01$, and ***, $p < 0.001$ (Student's t test).

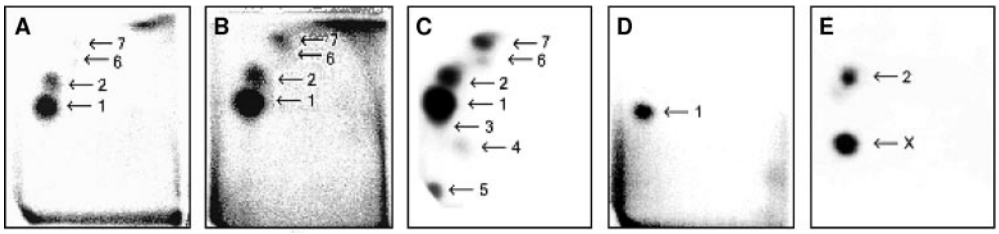

FIG. 5. Autoradiographic profile of DNA adducts generated in calf thymus DNA by ellipticine after its activation with hepatic microsomes of untreated (A) and ellipticine-treated (40 mg/kg) male rats (B), of ^{32}P-labeled digests of DNA from liver of male rats treated with the same dose of ellipticine (C), and from calf thymus DNA reacted with 13-hydroxyellipticine (D) or 12-hydroxyelipticine (E). Spots 1 to 7 and X are the ellipticine-derived DNA adducts. Analyses were performed by the nuclease P1 version of the ^{32}P postlabeling assay.

the ellipticine-DNA adduct formation in hepatic microsomes of control rats was lower than those of CYP3A enzymes, in hepatic microsomes of rats pretreated with 40 mg/kg ellipticine, their contributions increased. This follows from the effect of inhibitors of P450 enzymes of both two subfamilies (Fig. 7). α-NF, an inhibitor of CYP1A1/2 with the predominant inhibitory effect on CYP1A1, and furafylline, an inhibitor of CYP1A2, decreased the levels of ellipticine-DNA adducts generated by hepatic microsomes of control rats by 62 and 50%, respectively, whereas troleandomycin and ketoconazole, selective inhibitors of CYP3A enzymes, were more effective. These two inhibitors decreased levels of ellipticine-DNA adducts by 75 to 92% (Fig. 7). This finding indicates that CYP3A enzymes play a major role in ellipticine-DNA adduct formation in livers of control (uninduced) rats. On the contrary, the effect of CYP1A inhibitors in hepatic microsomes of rats pretreated with ellipticine was up to 4 times higher than in those of control animals, being more pronounced for α-NF than for furafylline (Fig. 7, Supplemental Table S3).

The increase in levels of ellipticine-DNA adducts correlates with an increase in ellipticine oxidation by hepatic microsomes from the 40 mg of ellipticine/kg group (Figs. 6 and 8). Besides an increase in oxidation of ellipticine to 9-hydroxyellipticine and 7-hydroxyellipticine, which was expected, because these metabolites are predominantly formed by CYP1A1/2 (Stiborová et al., 2004; Kotrbová et al., 2006), up to a 2-fold increase in formation of 13-hydroxy- and 12-hydroxyellipticine, the metabolites generating DNA adducts 1 and 2, was found (Fig. 8).

Discussion

Ellipticine is an anticancer agent whose biological effects such as pharmacological efficiencies and its potential genotoxic side effects

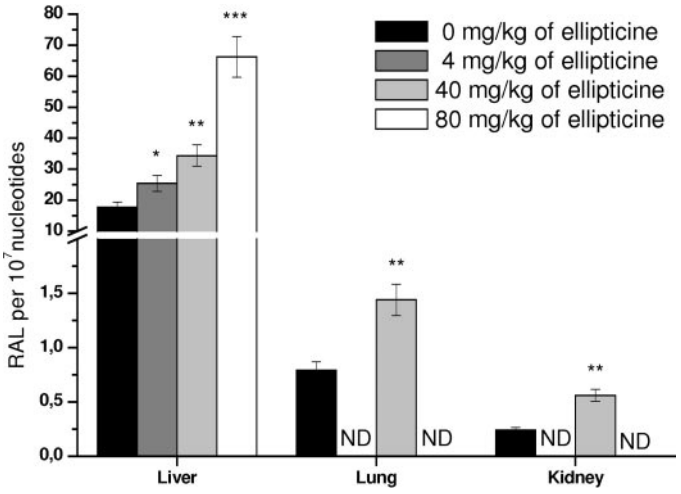

FIG. 6. DNA adduct formation by ellipticine activated with microsomes isolated from livers, lungs, and kidneys of male rats, control (uninduced) or pretreated with ellipticine as indicated. Mean relative adduct labeling ± standard deviations shown in the figure represent total levels of DNA adducts of four determinations (duplicate analyses of two independent in vitro incubations). ND, not determined. Values significantly different from control: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ (Student's t test).

depend on its P450-mediated metabolism (Stiborová et al., 2001, 2003a, 2004). Although the P450s responsible for the activation of this drug to DNA-binding species as well as those detoxifying this compound have already been identified (Stiborová et al., 2003a, 2004), knowledge about the effects of ellipticine exposure on the expression and activities of these enzymes has, until now, been limited.

In the present study, we have found that expressions of CYP1A1 and/or 1A2 proteins as well as their enzymatic activities were significantly induced by ellipticine in livers, lung, and kidneys of Wistar rats treated with ellipticine. This induction might be a consequence of the ellipticine-binding to AhR described by several authors (Fernandez et al., 1988; Gasiewicz et al., 1996). Ellipticine binding allows the cytosolic AhR to translocate into the nucleus and to dimerize with AhR nuclear translocator (ARNT). The AhR-ARNT complex functions as a transcriptional activator by binding to the Ah-responsive element in the regulatory domains of *CYP1A1* and *CYP1A2* genes (Gasiewicz et al., 1996), thus stimulating their transcription (current study).

Another mechanism of CYP1A1 induction might result from the inhibition of the oxidation of other substrates of this enzyme by ellipticine (Aimová and Stiborová, 2005). As shown by Chang and Puga (1998), a decrease in oxidation of an endogenous substrate of CYP1A1 by ellipticine results in an increase in constitutive activation of AhR-ARNT transcriptional complexes. In addition, low levels of ellipticine antagonize AhR activation by inducing ligands such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

Differences in constitutive levels and inducibility of CYP1A1 and 1A2 by ellipticine were found in male and female rats. The induction of CYP1A1/2 proteins by ellipticine, when expressed as -fold over control values, is higher in males than in females. Similar results in constitutive levels and inducibility of CYP1A1/2 by β-naphthoflavone were found in Sprague-Dawley rats (Iba et al., 1999); males were more responsive than females to induction by β-naphthoflavone. However, the absolute levels of CYP1A1/2 protein at 40 mg/kg ellipticine seem similar in male and female rat liver (see immunodetection of these P450s shown in Fig. 2). Therefore, the relative induction of CYP1A depends on the level of the controls. The CYP1A induction by 80 mg/kg body weight in females was lower than that by the 40 mg/kg ellipticine dose. Whether a higher sensitivity of female rats to the toxicity of ellipticine (Cresteil et al., 1982) may be the reason for this observation remains to be answered. Another sex-dependent difference, which awaits further investigation, was found for the CYP1A-selective activities catalyzed by hepatic microsomes. Although there is a good agreement in -fold induction between CYP1A2 protein and MROD in males and females, this relationship holds only in females for CYP1A1 protein and Sudan I oxidation. In males, -fold induction is much greater for CYP1A1 protein, perhaps reflecting the lower accuracy of the measurement in the control animals.

The CYP1A induction by ellipticine resulted in up to 3.8-fold higher ellipticine-DNA adduct levels in incubations of ellipticine with microsomes from rats treated with this compound than in incubations with control microsomes. This increase corresponded to an increase in ellipticine oxidation to 13-hydroxy- and 12-hydroxyellipticine, the metabolites generating the two major DNA adducts. However, the increased ellipticine-DNA adduct formation caused by induction of CYP1A enzymes was 9 and 1.5 times lower than the increase in CYP1A1 and 1A2 protein levels, respectively, and was also lower than the increase in CYP1A1 and/or 1A2-mediated activities. These differences might be explained by the efficiencies of induced CYP1A1/2 to oxidize ellipticine not only to 13-hydroxy- and 12-hydroxyellipticine, but also to the metabolites that do not form DNA adducts, namely, to 9-hydroxy- and 7-hydroxyellipticine (Stiborová et al., 2004).

The results found in this study, demonstrating that ellipticine-mediated induction of CYP1A leads to an increase in ellipticine activation to species-forming DNA adducts, shed light on our previous data, showing the importance of CYP1A enzymes in ellipticine activation in vivo (Stiborová et al., 2003c). The importance of CYP1A in ellipticine-derived DNA adduct formation in vivo was initially rather surprising for us because it did not correspond to the in vitro situation (Stiborová et al., 2001, 2003a). 13-Hydroxyellipticine, the metabolite responsible for generation of adduct spot 1 due to the formation of the reactive species ellipticine-13-ylium (Fig. 1), was found to be formed by CYP1A1/2 in vitro only in low amounts (Stiborová et al., 2004). Here, we show that one of the reasons for the in vivo situation might be the induction of CYP1A with ellipticine; in hepatic microsomes from rats induced with ellipticine higher levels of ellipticine-DNA adducts were generated. Indeed, the relative contributions of CYP1A (predominantly CYP1A1) to the formation of ellipticine-DNA adducts were increased in hepatic microsomes of induced rats.

The induction of CYP1A by ellipticine is transient. In the absence of repeated administration of ellipticine, the amount and activities of the induced CYP1A decreased almost to the basal level 2 weeks after treatment.

The induction of CYP1A1 protein expression and enzymatic activities by ellipticine corresponded to elevated mRNA levels of this enzyme. Levels of CYP1A2 mRNA were, however, unaffected despite the increase in both protein and activity. Similar discrepancies between induction of CYP1A mRNAs and protein levels were observed previously (Chen et al., 1998; Degawa et al., 1998; Dickins, 2004; Stiborová et al., 2006). Detailed analyses of the time dependence of the expression levels of mRNAs and proteins of the tested enzymes were not performed in this study, but they might answer the questions whether the transient induction of the mRNAs of CYP1A, or the different half-lives for their mRNAs and proteins, and/or the effects of ellipticine on the stability of mRNAs and proteins of these enzymes are the rationale for our observation.

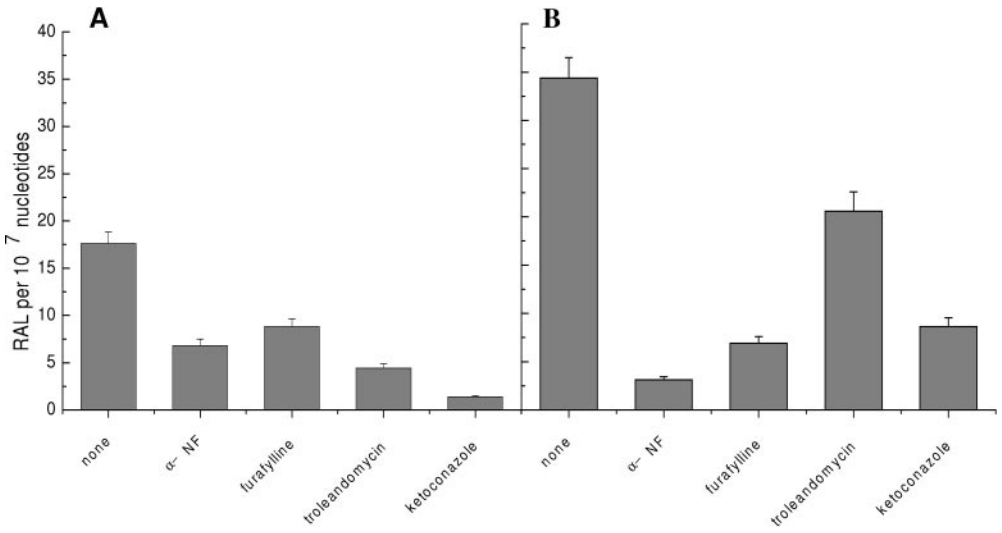

FIG. 7. Effect of CYP1A and 3A inhibitors on ellipticine-DNA adduct formation in hepatic microsomes of untreated (A) and ellipticine-treated (40 mg/kg) male rats (B). Mean relative adduct labeling ± standard deviations shown in the figure represent total levels of DNA adducts of three parallel in vitro incubations.

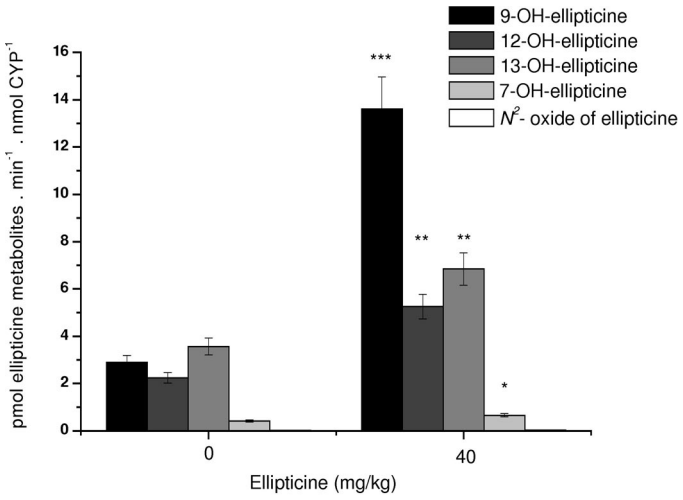

FIG. 8. Ellipticine metabolism in male rat hepatic microsomes of control animals and those treated with ellipticine. Microsomes containing 0.2 mg of microsomal protein, and 10 μM ellipticine were used in all experiments. Levels of ellipticine metabolites are averages ± standard deviations of triplicate incubations. Values significantly different from control: *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ (Student's t test).

The rat was used as an experimental model on the basis that the results should provide some indication of what might occur in patients treated with the drug. However, it should be noted that the similarities as well as differences in the CYP1A systems and their induction exist in the two species. For example, in rats CYP1A1 is often more markedly induced in liver than CYP1A2, whereas the reverse is true for humans (Dickins, 2004). Furthermore, the specificity of rat and human CYP1A1 and 1A2 is known to differ, particularly for inhibitors (Guengerich and Shimada, 1991; Stiborová et al., 2001).

Recently, we have demonstrated that ellipticine is oxidatively activated to species binding to DNA also in human cancer cells such as the breast adenocarcinoma MCF-7 (Bořek-Dohalská et al., 2004) and leukemia HL-60 and CCRF-CEM cells (Poljaková et al., 2007). CYP1A1 is expressed in all these cells and was found to be one of the enzymes responsible for ellipticine-DNA binding and its cytotoxicity against these cancer cells (Bořek-Dohalská et al., 2004; Poljaková et al., 2007). Likewise, Rekha and Sladek (1997) demonstrated that the cytotoxic activity of ellipticine to MCF-7 cells depends on the levels of CYP1A enzymes. The cited authors show that MCF-7 cells treated with another CYP1A inducer, 3-methylcholanthrene, transiently expressed elevated levels of CYP1A and were transiently much more sensitive to ellipticine. The DNA adducts we have observed in MCF cells might be responsible for the higher sensitivity observed by the above authors.

Another important feature related to the expression of CYP1A and ellipticine toxicity was detected in MCF-7 cells selected for resistance to doxorubicin hydrochloride (Adriamycin) (Adr^R MCF-7), exhibiting the phenotype of multidrug resistance (Ivy et al., 1988). Ivy et al. (1988) postulated that the resistance of Adr^R MCF-7 cells involves several biochemical and genetic changes besides multidrug resistance. One of them is a regulatory defect at the level of CYP1A1 mRNA resulting in lower CYP1A1-mediated metabolism of xenobiotics in these cells. Adr^R MCF-7 cells are cross-resistant to ellipticine (Ivy et al., 1988), which we would explain by a decrease in the CYP1A1-dependent activation of ellipticine.

Another P450 enzyme expressed in MCF-7 cells and breast cancer, CYP1B1 (Shehin et al., 2000), is known to activate ellipticine, and transcriptional activation of *Cyp1b1* is believed to involve the AhR (Kerzee and Ramos, 2001). Therefore, its induction by ellipticine should also be evaluated. Taken together, the data in these studies indicate that the expression levels, activities, and induction of CYP1A1 and 1B1, the enzymes activating ellipticine, may be an important factor in the specificity and efficiency of ellipticine for breast cancer. In this context, it will be important to evaluate whether ellipticine induces CYP1A1 and 1B1 expression in MCF-7 cells. Therefore, the study investigating the induction of CYP1A1 and 1B1 expression and their activities by ellipticine and their efficiencies to activate this chemical in MCF-7 cells is under way in our laboratory.

In conclusion, the results of the present study show for the first time

that ellipticine is capable of inducing CYP1A, an enzyme that is involved in ellipticine biotransformation in both rats and humans, in rat livers, kidneys, and lungs. By such effects, ellipticine might modulate its own disposition, pharmacological efficiency, and/or genotoxic potential. Nevertheless, this feature has to be established in vivo.

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P37

KINETICS OF ELLIPTICINE OXIDATION BY CYTOCHROMES P450 1A1 AND 1A2 RECONSTITUTED WITH NADPH:CYTOCHROME P450 REDUCTASE

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Key words: ellipticine, cytochromes P450, NADPH:cytochrome P450 reductase, kinetics

Introduction

Ellipticine (5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole, fig. 1), an alkaloid isolated from *Apocyanaceae* plants, exhibits significant antitumor and anti-HIV activities[1,2]. The main reason for the interest in ellipticine and its derivatives for clinical purposes is their high efficiencies against several types of cancer, their rather limited toxic side effects, and their complete lack of hematological toxicity[1]. Nevertheless, ellipticine is a potent mutagen (for review see[1,2]). The prevalent mechanisms of ellipticine antitumor, mutagenic and cytotoxic activities were suggested to be (*i*) intercalation into DNA (ref.[2,3]) and (*ii*) inhibition of DNA topoisomerase II activity (for review see[1,2]). We demonstrated that ellipticine covalently binds to DNA after being enzymatically activated with cytochrome P450 (CYP) enzymes or peroxidases[2–6]. Human and rat CYPs of 1A and 3A subfamilies are the predominant enzymes catalyzing oxidation of ellipticine either to metabolites that are excreted from organisms (7-hydroxy- and 9-hydroxyellipticine) or form DNA adducts (13-hydroxy- and 12-hydroxyellipticine, the latter formed also spontaneously from another ellipticine metabolite ellipticine N^2-oxide by the Polonowski rearrangement)[2–5] (fig. 1). Of the peroxidases, human cyclooxygenase (COX)-2, ovine COX-1, bovine lactoperoxidases, human myeloperoxidase and horseradish peroxidase efficiently generated ellipticine-derived DNA adducts[6]. The same DNA adducts formed by ellipticine were also detected in V79 Chinese hamster lung fibroblasts transfected with human CYP3A4, 1A1 and 1A2 (ref.[7]), in human breast adenocarcinoma MCF-7 cells[8], leukemia HL-60 and CCRF-CEM cells[9] and *in vivo* in rats exposed to this anticancer drug[4,10]. On the basis of these data, ellipticine might be considered a drug, whose pharmacological efficiency and/or genotoxic side effects are dependent on its enzymatic activation in target tissues. Here, we investigated the efficiency of purified CYP1A1 and 1A2 enzymes reconstituted with NADPH:CYP reductase to oxidize ellipticine. In addition, kinetics of ellipticine oxidation by these enzymes was evaluated.

Materials and methods

The rat CYP1A1, rabbit CYP1A2 and rabbit NADPH:CYP reductase were isolated as described[2]. Incubation mixtures used to study ellipticine metabolism contained the following in a final volume of 500 μl: 100 mM potassium phosphate buffer (pH 7.4), 1 mM $NADP^+$, 10 mM D-glucose 6-phosphate, 1 U ml^{-1} D-glucose 6-phosphate dehydrogenase, 0.01–1 μM CYPs reconstituted with NADPH:CYP reductase in liposomes and 10 μM ellipticine dissolved in 10 μl DMSO. The enzyme reconstitution was performed as described[2,3], but different ratios of

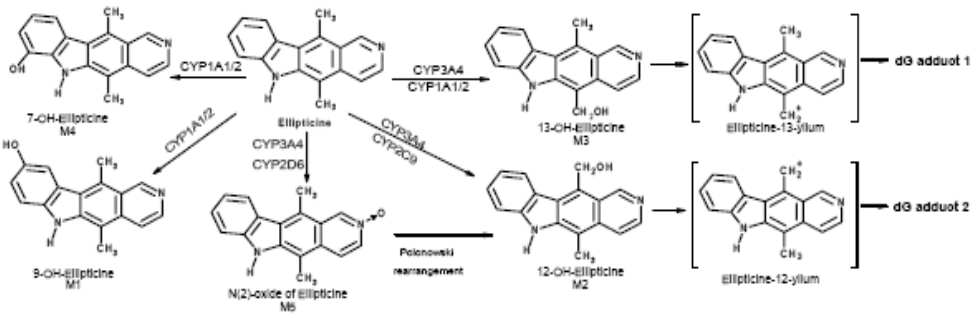

Fig. 1. **Metabolism of ellipticine by human CYPs showing the characterized metabolites found to form DNA adducts**

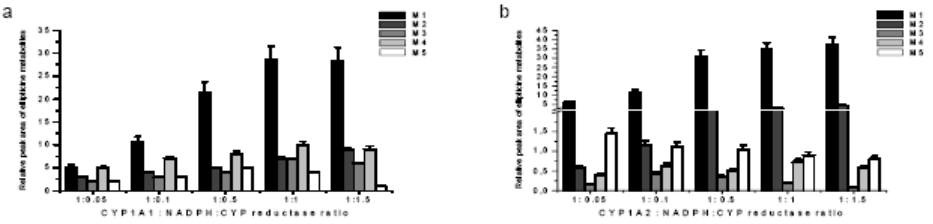

Fig. 2. **The effect of NADPH:CYP reductase on ellipticine oxidation by CYP1A1 (a) and CYP1A2 (b)**

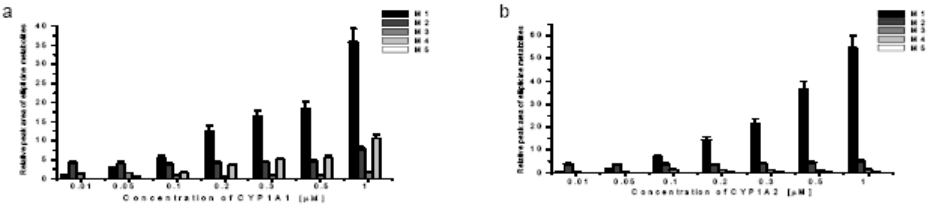

Fig. 3. **The effect of different concentrations of CYP1A1 (a) and CYP1A2 (b) on ellipticine oxidation**

CYP:reductase were utilized (fig. 2 and 3). After incubation (37 °C, 20 min), the reaction was stopped by adding ethylacetate. Thereafter, 5 μl of 1 mM phenacetine in methanol was added as an internal standard and the ellipticine metabolites were extracted twice with ethylacetate (2 × 1 ml). The extracts were evaporated under nitrogen and dissolved in 20 μl of methanol. The ellipticine metabolites were separated by HPLC as described[5]. Five ellipticine metabolites identified previously as 9-hydroxy-, 12-hydroxy-, 13-hydroxy-, 7-hydroxyellipticine and ellipticine N^2-oxide, were eluted at the retention times of 6.3, 6.9, 7.8, 8.5 and 11.2 min, respectively[5].

Results and discussion

The CYP1A1 and 1A2 enzymes reconstituted with NADPH:CYP reductase oxidized ellipticine to five metabolites: 9-hydroxy-, 12-hydroxy-, 13-hydroxy-, 7-hydroxyellipticine and ellipticine N^2-oxide (figs. 2,3), found previously to be formed by human, rat and rabbit hepatic microsomes[5]. The 9-hydroxy- and 7-hydroxyellipticine are the major ellipticine metabolites formed in the enzyme reconstituted systems containing CYP1A1 and 1A2. These results correspond to those found by us in rat, rabbit and human hepatic microsomes utilizing inducers and inhibitors of CYPs[11]. Efficiencies of CYP1A1/2 enzymes reconstituted with its reductase to oxidize ellipticine depends on the CYP:reductase ratios in the reconstitution systems. An

Table I
Kinetics parameters of ellipticine oxidation by CYP1A1 (A) and CYP1A2 (B)

CYP1A1 (A)				CYP1A2 (B)			
Ellipticine metabolites	n	V_{max} [min^{-1}]	K_m [μM]	Ellipticine metabolites	n	V_{max} [min^{-1}]	K_m [μM]
M1	1.01	1.34	0.10	M1	1.01	1.00	0.23
M2	0.91	0.08	0.82	M2	1.03	0.06	1.63
M3	0.89	0.06	3.50	M3	1.08	0.10	14.00
M4	0.98	0.22	0.51	M4	1.03	0.02	6.93

increase in the NADPH:CYP reductase content in the reconstitution systems resulted in an increase of ellipticine oxidation up to the value of the CYP:reductase ratio of 1:0.5, with negligible or low, insignificant, changes in their efficiencies up to ratios of 1:1 for CYP1A1 and 1:1.5 for CYP1A2 (fig. 2). The ratio of CYP1A1/2:reductase of 1:0.5 was used for evaluation of kinetics of ellipticine oxidation by these CYP enzymes.

An increase in the concentration of CYP1A1 and 1A2 in incubations results in an increase in formation of ellipticine metabolites, predominantly in generation of 9-hydroxyellipticine and 7-hydroxyellipticine, being linear up to CYP concentrations of 0.2 μM (fig. 3). The Michaelis-Menten kinetics was found for oxidation of ellipticine by CYP1A1 and 1A2 (data not shown). The values of Michaelis constant (K_m) and maximum velocity (V_{max}) are shown in Table I.

Conclusion

The results demonstrate that the system of purified CYP1A1 and 1A2 reconstituted with NADPH:CYP reductase oxidizes ellipticine mainly to 9-hydroxy- and 7-hydroxyellipticine, which reflects the situation of the ellipticine oxidation in human, rat and rabbit hepatic microsomes.

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Abbreviations

CYP	cytochrome P450
K_m	Michaelis constant
n	Hill coefficient
V_{max}	maximum velocity

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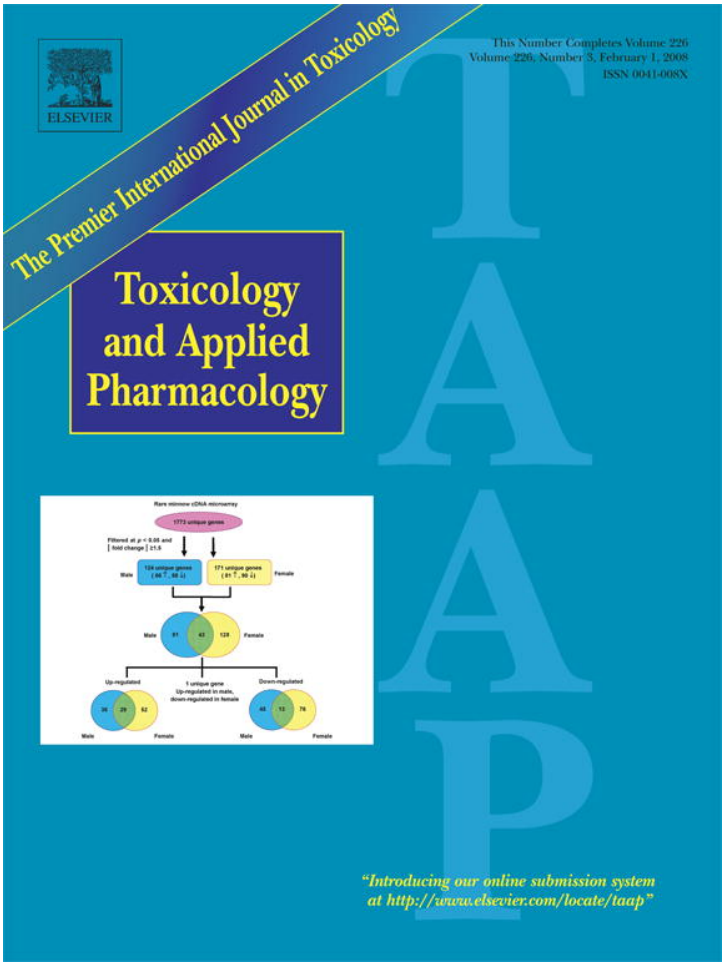

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Role of hepatic cytochromes P450 in bioactivation of the anticancer drug ellipticine: Studies with the hepatic NADPH: Cytochrome P450 reductase null mouse

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Abstract

Ellipticine is an antineoplastic agent, which forms covalent DNA adducts mediated by cytochromes P450 (CYP) and peroxidases. We evaluated the role of hepatic *versus* extra-hepatic metabolism of ellipticine, using the HRN (*H*epatic Cytochrome P450 *R*eductase *N*ull) mouse model, in which cytochrome P450 oxidoreductase (POR) is deleted in hepatocytes, resulting in the loss of essentially all hepatic CYP function. HRN and wild-type (WT) mice were treated i.p. with 1 and 10 mg/kg body weight of ellipticine. Multiple ellipticine–DNA adducts detected by ^{32}P-postlabelling were observed in organs from both mouse strains. Highest total DNA binding levels were found in liver, followed by lung, kidney, urinary bladder, colon and spleen. Ellipticine–DNA adduct levels in the liver of HRN mice were up to 65% lower relative to WT mice, confirming the importance of CYP enzymes for the activation of ellipticine in livers, recently shown *in vitro* with human and rat hepatic microsomes. When hepatic microsomes of both mouse strains were incubated with ellipticine, ellipticine–DNA adduct levels with WT microsomes were up to 2.9-fold higher than with those from HRN mice. The ratios of ellipticine–DNA adducts in extra-hepatic organs between HRN and WT mice of up to 4.7 suggest that these organs can activate ellipticine and that more ellipticine is available in the circulation. These results and the DNA adduct patterns found *in vitro* and *in vivo* demonstrate that both CYP1A or 3A and peroxidases participate in activation of ellipticine to reactive species forming DNA adducts in the mouse model used in this study.

Keywords: Anticancer drug; Ellipticine; Cytochrome P450; Peroxidase; DNA adducts; ^{32}P-postlabelling

Introduction

Ellipticine (5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole, Fig. 1), an alkaloid isolated from *Apocyanaceae* plants, exhibit significant antitumor and anti-HIV activities (for a summary see Stiborová et al., 2001). The main reason for the interest in ellipticine and its derivatives for clinical purposes is their high efficiencies against several types of cancer, their rather limited toxic side effects, and their complete lack of haematological toxicity (Auclair, 1987). Nevertheless, ellipticine is a potent mutagen. Most ellipticine derivatives are mutagenic to *Salmonella typhimurium* Ames tester strains, bacteriophage T4, *Neurospora crassa*, and mammalian cells and induce prophage lambda in *Escherichia coli* (for an overview see Stiborová et al., 2001).

The prevalent mechanisms of ellipticine antitumour, mutagenic and cytotoxic activities have been suggested to be (i) intercalation into DNA (Auclair, 1987; Singh et al., 1994) and (ii) inhibition of DNA topoisomerase II activity (Auclair, 1987; Monnot et al., 1991; Fossé et al., 1992; Froelich-Ammon et al., 1995). We have demonstrated that ellipticine also covalently

Abbreviations: α-NF, α-naphthoflavone; COX, cyclooxygenase; CYP, cytochrome P450; HRN, hepatic cytochrome P450 reductase null; HPLC, high-performance liquid chromatography; i.p., intra-peritoneal; LPO, lactoperoxidase; MPO, myeloperoxidase; PEI-cellulose, polyethylenimine-cellulose; POR, cytochrome P450 oxidoreductase; RAL, relative adduct labeling; r.t., retention time; TLC, thin layer chromatography; WT, wild-type.

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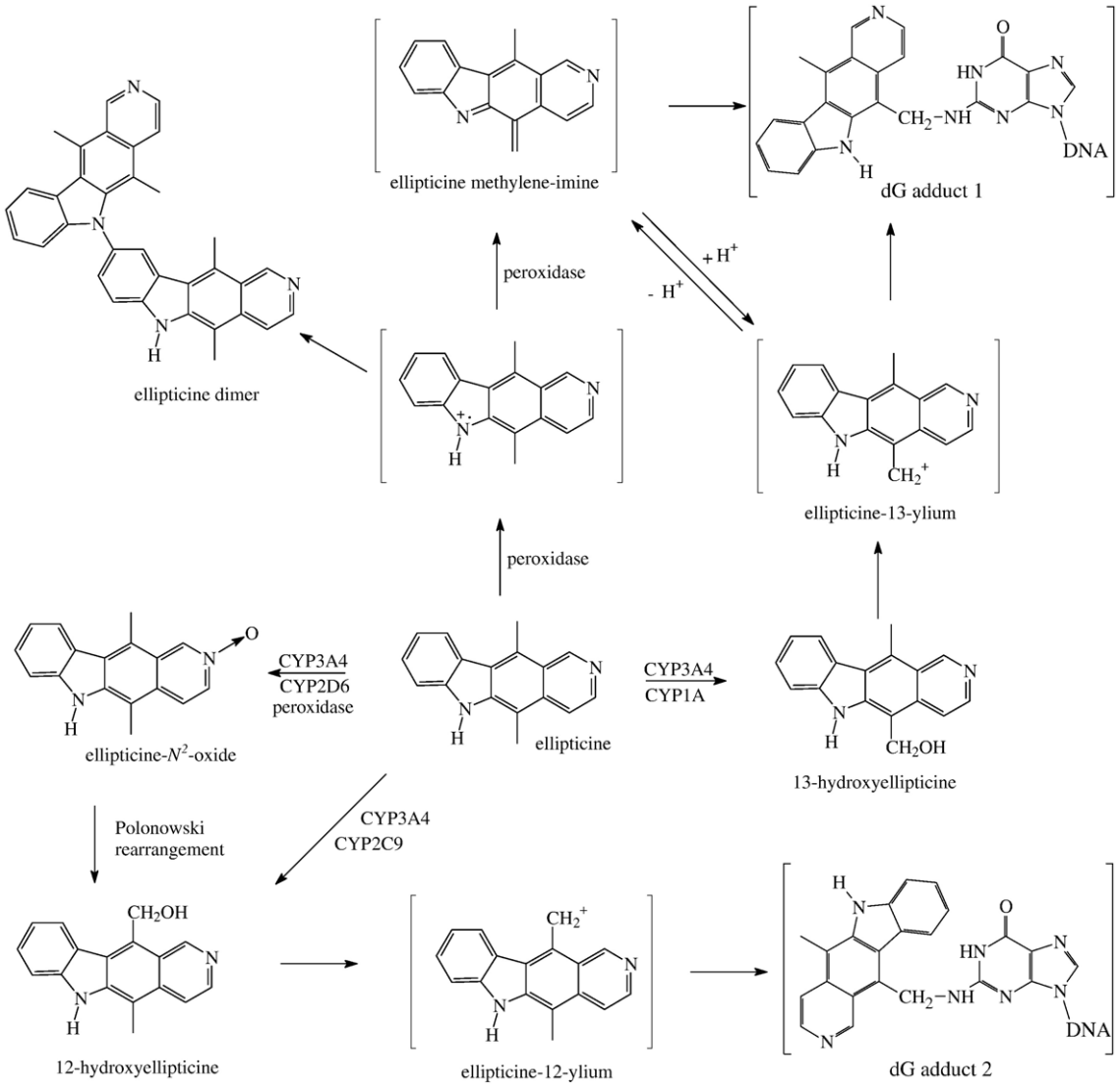

Fig. 1. Metabolism of ellipticine by peroxidases and human CYPs showing the characterized metabolites and those proposed to form DNA adducts. The compounds shown in brackets were not detected under the experimental conditions and are the electrophilic metabolites postulated as ultimate arylating species or the postulated N^2-deoxyguanosine adducts.

binds to DNA after being enzymatically activated with cytochromes P450 (CYP) or peroxidases (Stiborová et al., 2001; 2003a,b, 2004, 2006a, 2007a,b, Poljaková et al., 2006), suggesting a third possible mechanism of action.

Understanding which enzymes are involved in the metabolic activation of ellipticine is important in the assessment of susceptibility to this drug. Human and rat CYP1A and 3A are the predominant enzymes catalyzing oxidation of ellipticine *in vitro* either to metabolites that are excreted (7-hydroxy- and 9-hydroxyellipticine) or that form DNA adducts (12-hydroxy- and 13-hydroxyellipticine) (Stiborová et al., 2001, 2003a,b, 2004, 2006a). Of the peroxidases, human cyclooxygenase (COX)-2, ovine COX-1, bovine lactoperoxidase (LPO), human myeloperoxidase (MPO) and horseradish peroxidase efficiently generated ellipticine-derived DNA adducts (Fig. 1) (Poljaková et al., 2006; Stiborová et al., 2007a). The same DNA adducts were also detected in cells in culture expressing enzymes activating ellipticine (CYP1A1, COX-1 and MPO), such as human breast adenocarcinoma MCF-7 cells (Bořek-Dohalská et al., 2004), leukaemia HL-60 and CCRF-CEM cells (Poljaková et al., 2007) and V79 Chinese hamster lung fibroblasts transfected with human CYP3A4, 1A1 and 1A2 (Frei et al., 2002). On the basis of these data, ellipticine might be considered a drug, whose pharmacological efficiency and/or genotoxic side effects are dependent on its activation by CYPs and peroxidases in target tissues.

However, additional factors such as route of administration, absorption and renal clearance make it difficult to extrapolate from *in-vitro* data to the *in-vivo* situation. After i.p. administration of ellipticine to rats, the highest levels of DNA adducts were found in liver, followed by kidney and lung (Stiborová et al., 2003a, 2007b). Liver is a tissue rich in CYP enzymes, while kidney and lung contain high levels of peroxidases such as COX (Eling et al., 1990; Eling and Curtis, 1992; Stiborová et al., 1991, 2005; Culp et al., 1997). Knowledge about the participation of these enzymes in ellipticine activation in individual rat tissues is, however, scarce. Although previous

results indicate that CYP3A1 and 1A participate in formation of DNA adducts by ellipticine in rats *in vivo* (Stiborová et al., 2003a), it is unclear whether CYP or peroxidases are more important in ellipticine activation in different organs.

Gene knock-out and transgenic mice have been developed to study the role of specific enzymes in drug metabolism (Gonzalez and Kimura, 2001; Henderson and Wolf, 2003). Although CYP knock-out mouse models have yielded important data on the effect of single CYP enzymes on the metabolism of drugs and chemical carcinogens (Buters et al., 1999, 2002; Kimura et al., 1999, 2003; Tsuneoka et al., 2003; Uno et al., 2004), the functional redundancy inevitably found in the large CYP family of isoenzymes makes it difficult to determine the role of CYPs as a whole in metabolism of xenobiotics (Henderson et al., 2006). To overcome these limitations a mouse line, HRN (*H*epatic Cytochrome P450 *R*eductase *N*ull), has been developed in which cytochrome P450 oxidoreductase (POR), the unique electron donor to CYPs, is deleted specifically in hepatocytes, resulting in the loss of essentially all hepatic CYP function (Henderson et al., 2003). The HRN mouse as a model has been used to establish the role of hepatic *versus* extra-hepatic CYP-mediated metabolism and disposition of drugs such as cyclophosphamide (Pass et al., 2005; Henderson et al., 2006). With this model we found that the carcinogen 3-nitrobenzanthrone is predominantly activated *in vivo* by cytosolic nitroreductases rather than microsomal POR, whereas the oxidative activation of 3-aminobenzanthrone, the main metabolite of 3-nitrobenzanthrone, is CYP-dependent, both *in vitro* and *in vivo* (Arlt et al., 2003, 2004, 2005, 2006).

In this study, we have used the HRN mouse model to examine the hepatic CYP-dependent metabolism of ellipticine. DNA adduct formation *in vivo* in hepatic and extra-hepatic tissues was investigated using ^{32}P-postlabelling. In addition, we examined ellipticine metabolism and DNA adduct formation *in vitro* using hepatic microsomes.

Materials and methods

Animals. HRN ($Por^{lox/lox}$+Cre^{ALB}) mice on a C57BL/6 background used in this study were constructed as described previously (Henderson et al., 2003). Mice homozygous for loxP sites at the *Por* locus ($Por^{lox/lox}$) were used as wild-type (WT). All procedures were carried out under the Animal (Scientific Procedures) Act (1986) in accordance with UK law, and following local ethical review.

Treatment of HRN mice and WT mice with ellipticine. Male HRN (Henderson et al., 2003) and WT mice (25–30 g; n=3/group) were treated with a single intraperitoneal (i.p.) dose of 1 or 10 mg/kg body weight of ellipticine by intraperitoneal injection. Ellipticine was administered dissolved in 1% acetic acid at a concentration of 2.5 mg/ml. Control mice (n=3/group) received the solvent only. Animals were killed 24 h after treatment. Six organs (liver, lung, kidney, spleen, bladder and colon) were removed and stored at −80 °C until DNA isolation by standard phenol/chloroform extraction.

Measurement of ellipticine–DNA adducts. ^{32}P-postlabelling analysis with nuclease P1 enrichment, thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC) of ^{32}P-labelled 3′5′-deoxyribonucleoside bisphosphate adducts were done as reported recently (Stiborová et al., 2003a, 2004, 2007a).

Preparation of microsomes and assays. Microsomes were isolated from the pooled livers of each strain as described (Stiborová et al., 2003b). Pooled microsomal fractions were used for further analyses.

Preparation of POR antibodies. Rabbit liver NADPH:CYP reductase was purified as described (Yasukochi et al., 1979). Leghorn chickens were immunised subcutaneously three times (with one-week intervals) with rabbit hepatic POR (0.1 mg/animal) emulsified in complete Freund's adjuvant for the first injection and in incomplete adjuvant for boosters. The immunoglobulin fraction was purified from pooled egg yolks using fractionation by polyethylene glycol 6000 (Stiborová et al., 2002).

Determination of POR protein levels in hepatic microsomes. Immunoquantitation of hepatic microsomal POR was done essentially as described previously using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Stiborová et al., 2002, 2006b). The POR protein was probed with the chicken polyclonal antibodies as reported (Stiborová et al., 2002, 2006b). The antigen–antibody complex was visualised with an alkaline phosphatase-conjugated rabbit anti-chicken IgG antibody and 5-bromo-4-chloro-3-indolylphosphate/nitrobluetetrazolium as the chromogenic substrate (Stiborová et al., 2002, 2006b).

Determination of POR enzymatic activity in hepatic microsomes. Hepatic microsomal samples were characterized for POR activity using cytochrome *c* as a substrate, by the procedure reported previously (Arlt et al., 2003).

Microsomal incubations. Incubation mixtures used to generate DNA adduct formation by ellipticine *in vitro* consisted of 50 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, pooled hepatic microsomal fraction (0.5 mg protein) from HRN or WT mice, 0.1 mM ellipticine (dissolved in 7.5 µl methanol) and 0.5 mg of calf thymus DNA in a final volume of 750 µl. Incubations were also carried out in the presence of a COX cofactor, arachidonic acid (Eling and Curtis, 1992; Stiborová et al., 2005). Mixtures then contained 0.1 mM arachidonic acid as cofactor instead of NADPH, and additionally 5 mM magnesium chloride. Incubations were carried out at 37 °C for 30 min; ellipticine–DNA adduct formation was found to be linear up to 30 min of incubation (Stiborová et al., 2001). Control incubations were carried out (*i*) without microsomes, (*ii*) without NADPH or arachidonic acid, (*iii*) without DNA and (*iv*) without ellipticine. After the incubation, DNA was isolated by a standard phenol–chloroform extraction method.

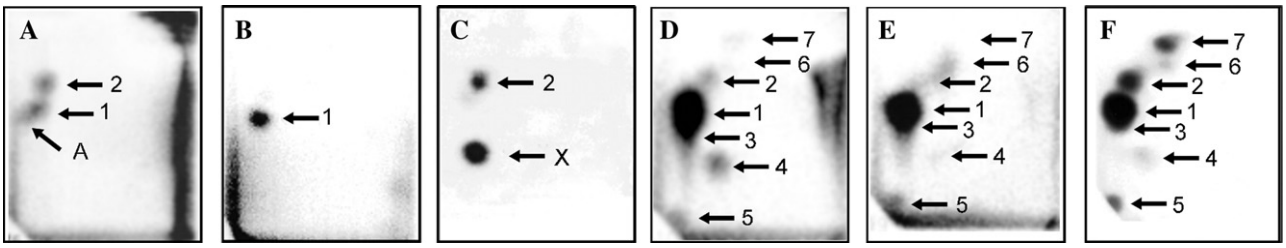

Fig. 2. Autoradiographs of TLC maps of ^{32}P-labelled digests of calf thymus DNA reacted with ellipticine and hepatic microsomes from WT mice (A), from calf thymus DNA reacted with 13-hydroxyellipticine (B) (Stiborová et al., 2004) and 12-hydroxyellipticine (C) (Stiborová et al., 2007a), of DNA from livers of WT (D) and HRN (E) mice treated with 10 mg ellipticine/kg body weight, from livers of rats treated with 40 mg ellipticine/kg body weight (F) (Stiborová et al., 2007b). Analyses were performed by the nuclease P1 version of the ^{32}P-postlabelling assay. Exposure times of the TLC plates in Instant Imager were 40 (A), 5 (B), 10 (C), 20 (D), 40 (E) and 10 min (F).

Table 1
DNA adduct formation by ellipticine activated by hepatic microsomes from HRN and WT mice

	RAL[a] (mean/10^8 nucleotides)			
	Spot 1	Spot 2	Spot A	Total
HRN mice hepatic microsomes+NADPH	5.9±0.3	2.3±0.2	6.7±0.3	14.9±0.8
HRN mice hepatic microsomes+NADPH+α-lipoic acid	1.2±0.1	1.7±0.1	0.9±0.1	3.8±0.3
HRN mice hepatic microsomes+NADPH+α-NF	2.8±0.2	2.2±0.2	1.0±0.1	6.0±0.5
HRN mice hepatic microsomes+NADPH+ketoconazole	2.5±0.2	2.0±0.2	1.0±0.1	5.5±0.4
HRN mice hepatic microsomes+arachidonic acid	2.9±0.2	2.4±0.2	ND	5.3±0.4
HRN mice hepatic microsomes without cofactor	0.2±0.04	1.9±0.2	ND	2.0±0.2
WT mice hepatic microsomes+NADPH	13.0±0.9	3.2±0.2	4.8±0.4	21.0±1.5
WT mice hepatic microsomes+NADPH+α-lipoic acid	7.6±0.6	2.7±0.2	0.7±0.1	11.0±0.9
WT mice hepatic microsomes+NADPH+α-NF	6.5±0.5	2.7±0.2	1.2±0.1	10.4±0.8
WT mice hepatic microsomes+NADPH+ketoconazole	6.0±0.5	2.1±0.2	1.0±0.1	8.1±0.7
WT mice hepatic microsomes+arachidonic acid	5.7±0.4	4.6±0.3	ND	10.3±0.8
WT mice hepatic microsomes without cofactor	0.3±0.02	2.0±0.2	ND	2.3±0.2

[a]Mean RAL (relative adduct labeling) of four determinations (duplicate analyses of two independent *in vitro* incubations). ND — not detected (the detection limit of RAL was $1/10^{10}$ nucleotides).

Incubation mixtures used to form the ellipticine metabolites contained 50 mM potassium 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), 0.2 mg protein of pooled hepatic microsomal fraction and 10 μM ellipticine (dissolved in 5 μl methanol) in a final volume of 500 μl. The reaction was initiated by adding the substrate. In the control incubation, ellipticine was omitted from the incubation mixture. After incubation in open glass tubes (37 °C, 20 min) the reaction was stopped by adding 100 μl of 2 M NaOH. The oxidation of ellipticine is linear up to 30 min of incubation (Stiborová et al., 2006a). After incubation, 5 μl of 1 mM phenacetine in methanol was added as an internal standard and the ellipticine metabolites were extracted twice with ethyl acetate (2×1 ml). Analyses of ellipticine metabolites were performed by HPLC as described (Stiborová et al., 2004, 2006a). Recoveries of ellipticine metabolites were around 95% in the presence of microsomes without a CYP cofactor (NADPH-generation system).

Inhibition studies. The following chemicals were used to inhibit the activation of ellipticine to form DNA adducts in the presence of mouse hepatic microsomes: α-naphthoflavone (α-NF), which inhibits CYP1A1 and 1A2 (Arlt et al., 2004; Rendic and DiCarlo, 1997; Stiborová et al., 2005), ketoconazole, an inhibitor of CYP3A, and α-lipoic acid, which inhibits POR (Slepneva et al., 1995). Inhibitors were dissolved in 7.5 μl of methanol, to yield final concentrations of 0.1 mM in the incubation mixtures. Mixtures were then incubated at 37 °C for 10 min with NADPH prior to adding ellipticine, and then incubated for a further 30 min at 37 °C. After the incubation, DNA was isolated as described above.

Results

Hepatic microsomes of HRN mice are capable of activating ellipticine

In order to evaluate the role of the mouse hepatic POR-dependent CYP in the activation of ellipticine, we first performed

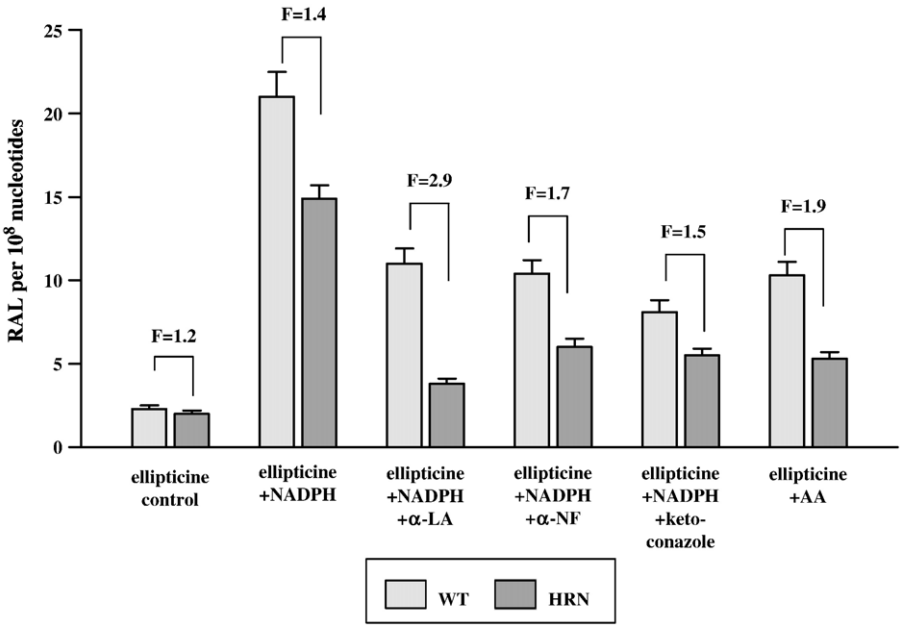

Fig. 3. DNA adduct formation by ellipticine activated with microsomes isolated from livers of HRN and WT mice as determined by TLC ^{32}P-postlabelling. *F*=fold higher DNA adducts levels in microsomes from WT mice compared to HRN mice. *Columns*: Mean RAL (relative adduct labelling)±standard deviations shown in the figure represent total levels of DNA adducts of four determinations (duplicate analyses of two independent *in vitro* incubations). Control=without cofactor; AA=arachidonic acid; α-NF=α-naphthoflavone; α-LA=α-lipoic acid. ND=not detected.

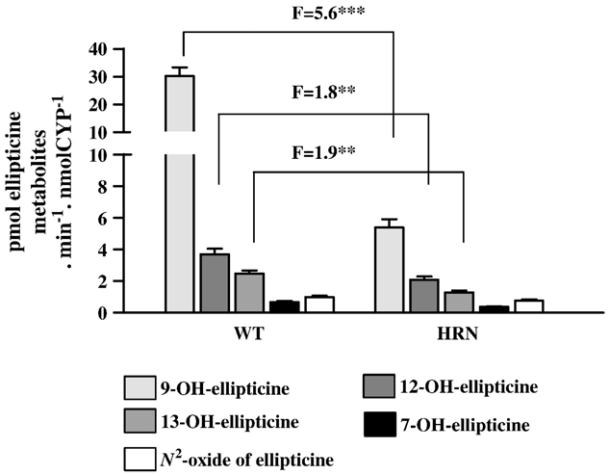

Fig. 4. Levels of ellipticine metabolites formed by hepatic microsomes (0.2 mg protein) of HRN and WT mice from 10 μM ellipticine. Levels of ellipticine metabolites were determined by HPLC (Stiborová et al., 2004, 2006a) and are averages±standard deviations of triplicate incubations. Values significantly different from HRN mice: $^{**}P<0.01$, $^{***}P<0.001$.

in-vitro experiments. Microsomes isolated from livers of HRN and WT mice used for such experiments were characterized for POR expression by Western blotting. Hepatic POR expression was detected in WT mice, while as expected, its levels in HRN mice were very low, but still detectable, by immunostaining. Hepatic POR levels in HRN mice were estimated to be less than 1.5% of the levels in WT mice. POR activity measured with cytochrome *c* as a substrate was, however, not detectable in hepatic microsomes of HRN mice.

The activation of ellipticine by mouse hepatic microsomes was determined by ^{32}P-postlabeling. The adduct pattern obtained is shown in Fig. 2A. The DNA adduct pattern generated by ellipticine consisted of at least two adducts (spots 1 and 2), which were identical to those formed *in vivo* in rats treated with ellipticine (Fig. 2F), each a product of 13-hydroxyellipticine (Fig. 2B) or 12-hydroxyellipticine (Fig. 2C) metabolites, generated by CYP and peroxidase (see scheme in Fig. 1). Chromatographic analysis of spots 1 and 2 on HPLC confirmed that these adducts are derived from 13-hydroxy- and 12-hydroxyellipticine, respectively, by their coelution with prepared reference compounds (data not shown). An additional ellipticine-derived DNA adduct, spot A, was found in DNA that had been incubated with ellipticine and mouse hepatic microsomes (Fig. 2A), predominantly in microsomes isolated from HRN mice (Table 1). This ellipticine–DNA adduct was not formed by either human or rat hepatic microsomes (Stiborová et al., 2001, 2003b, 2004) or in rats (Stiborová et al., 2003a, 2007b) (Fig. 2F) or mice (Figs. 2D, E). Control incubations without microsomes were free of adduct spots 1 and A, but adduct spot 2 was always detected (data not shown). This finding is consistent with our previous results showing that this adduct is formed also non-enzymatically (Stiborová et al., 2001, 2003b, 2004, 2007a). In the presence of microsomes without NADPH, a low but detectable amount of adduct 1 was found (Table 1).

Because CYPs and peroxidases were found to activate ellipticine (Stiborová et al., 2001; 2003b, 2004, 2007a), we investigated the modulation of ellipticine–DNA adduct formation by cofactors and selective inhibitors of these enzymes using hepatic microsomes isolated both from HRN and WT mice. In the presence of NADPH, a cofactor of POR- and CYP-dependent enzyme systems, hepatic microsomal samples of both mice strains were capable of activating ellipticine to form DNA adducts (Fig. 3). Surprisingly, levels of DNA adducts 1 and 2 formed by hepatic microsomes from HRN mice were only one half of levels formed by this hepatic subcellular fraction from WT mice (Table 1), even though POR expression in livers of HRN mice was two orders of magnitude lower.

α-Lipoic acid, a selective inhibitor of POR (Slepneva et al., 1995), decreased ellipticine–DNA adduct formation by 50–75% with hepatic microsomes from both strains of mice, with a greater extent of inhibition with HRN microsomes than with those from WT livers. Therefore, some of the activity in HRN mice is POR-dependent. Likewise, α-NF, an inhibitor of CYP1A1 and 1A2 (Rendic and DiCarlo, 1997) and ketoconazole, a selective inhibitor of CYP3A enzymes (Rendic and DiCarlo, 1997; Ueng et al., 1997), decreased the levels of ellipticine–DNA adducts generated by hepatic microsomes of both mice strains to essentially equal extent (Fig. 3, Table 1). These results point additionally to CYP enzymes, namely of the 1A and 3A subfamilies, as having a role in ellipticine–DNA adduct formation in mouse livers. Arachidonic

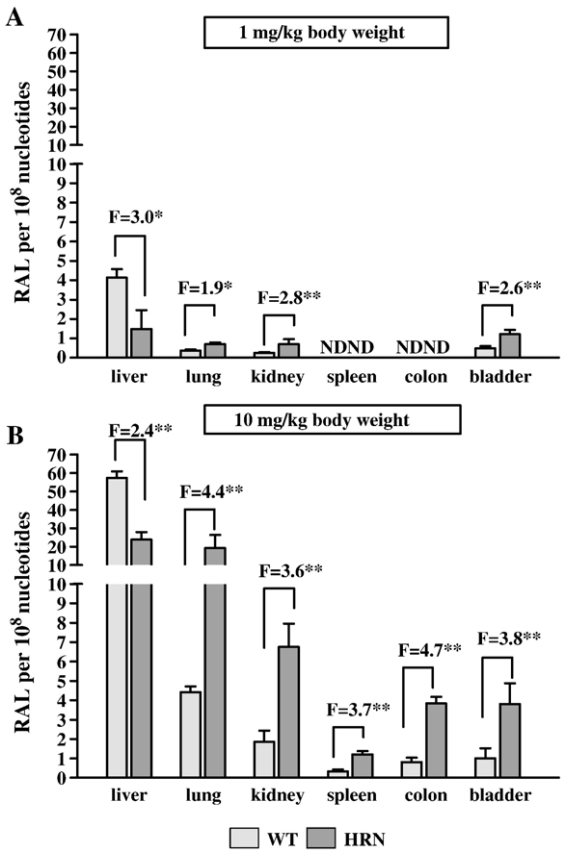

Fig. 5. Total levels of ellipticine–DNA adducts determined and quantified by ^{32}P-postlabelling analysis of DNA isolated from organs of HRN and WT mice treated i.p. with (A) 1 mg ellipticine/kg body weight or (B) 10 mg ellipticine/kg body weight. *F*=fold higher and/or lower DNA adducts in HRN than WT mice. *Columns*, mean; *bars*, S.D. (*n*=3); each DNA sample was analysed twice. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. RAL, relative adduct labelling. ND=not detected.

acid, a cofactor for COX-dependent oxidation (Eling et al., 1990; Eling and Curtis, 1992; Stiborová et al., 2004, 2005; Arlt et al., 2006), mediated formation of DNA adducts 1 and 2 by ellipticine in hepatic microsomes of both mice strains, but was less effective than NADPH (Fig. 3). The ratio of adduct levels between WT and HRN mice was similar to that observed with NADPH as cofactor.

The profile of ellipticine metabolites in hepatic microsomes from both mouse lines was similar (Fig. 4). The levels of individual metabolites were, however, different; 9-hydroxyellipticine levels were only one sixth, while the amounts of 13-hydroxy- and 12-hydroxyellipticine, were about one half in incubations with HRN microsomes compared with the levels in incubations with WT microsomes.

There might be at least two reasons for the above findings: (i) the low residual levels of POR are still sufficient to mediate CYP-catalyzed ellipticine oxidation or (ii) additional enzymes, besides CYPs, may be present in hepatic microsomes of HRN mice that activate ellipticine. Therefore, we also considered other ellipticine-activating enzymes such as COX (Stiborová et al., 2007a).

DNA adduct formation by ellipticine in HRN mice

To evaluate the importance of hepatic CYP enzymes in the oxidative activation of ellipticine to DNA adducts *in vivo* we treated HRN and WT mice i.p. with a single dose of 1 or 10 mg ellipticine/kg body weight. We chose i.p. injection as the route of administration to achieve high levels of ellipticine in the liver.

In both mouse lines treated with ellipticine essentially the same pattern of up to seven ellipticine-specific DNA adducts as that found *in vivo* in rats (Fig. 2F) (Stiborová et al., 2003a, 2007b) was observed in liver, lung, kidney and bladder (see Figs. 2D, E for liver). No ellipticine-derived DNA adducts were observed in spleen and colon at the lower dose (Fig. 5) or in any of the organs of control animals treated with vehicle only (data not shown). Adduct spots 1 and 2 (Fig. 2) were the predominant adducts in all the other tissues of mice exposed to 1 or 10 mg/kg ellipticine (Table 2). Co-chromatographic analyses of these adduct spots on HPLC confirmed that they were identical to adducts derived from 13-hydroxy- and 12-hydroxyellipticine, respectively, and deoxyguanosine.

Besides these adducts, up to five additional adducts were detected in DNA of liver, lung, kidney, colon and urinary bladder (see Fig. 2 and Table 2). The low levels of these adducts prevented HPLC co-chromatographic analysis or their further characterization.

Quantitative ^{32}P-postlabeling DNA adduct analysis is shown in Fig. 5 and Table 2. Even though the adducts 1, 2 and 3 were not well resolved on TLC plates (Fig. 2), their quantitation using different exposure times of the TLC plates on the Instant Imager allowed us to see these adducts as separated spots. DNA adduct formation was dose- and organ-specific (Fig. 5). The main target organ for DNA adduct formation was the liver in both mouse strains. The levels of three ellipticine–DNA adduct in the livers of HRN mice (adduct spots 1–3, Table 2) were substantially lower (by up to 65%) than in the livers of WT mice

Table 2
DNA adduct formation in various organs of HRN and WT mice treated with 1 or 10 mg ellipticine/kg body weight

Strain	Organ	Dose (mg/kg bw)	RAL[a] (mean/10^8 nucleotides)						
			Spot 1	Spot 2	Spot 3	Spot 4	Spot 5	Spot 6	Spot 7
WT	Liver	1	2.6±0.3	0.3±0.04	0.34±0.08	0.53±0.03	0.34±0.08	ND[b]	ND
HRN	Liver	1	1.0±0.7	0.03±0.05	0.13± 0.06	0.08±0.03	0.25±0.14	ND	ND
WT	Lung	1	0.19±0.04	0.05±0.01	ND	0.14±0.03	ND	ND	ND
HRN	Lung	1	0.47±0.09	0.06±0.01	ND	0.12±0.03	ND	ND	ND
WT	Kidney	1	0.21±0.04	0.04±0.02	ND	ND	ND	ND	ND
HRN	Kidney	1	0.65±0.24	0.05±0.03	ND	ND	ND	ND	ND
WT	Bladder	1	0.35±0.04	0.12±0.02	ND	ND	ND	ND	ND
HRN	Bladder	1	1.0±0.24	0.21±0.02	ND	ND	ND	ND	ND
WT	Spleen	1	ND	ND	ND	ND	ND	ND	ND
HRN	Spleen	1	ND	ND	ND	ND	ND	ND	ND
WT	Colon	1	ND	ND	ND	ND	ND	ND	ND
HRN	Colon	1	ND	ND	ND	ND	ND	ND	ND
WT	Liver	10	47.3±3.1	1.8±0.4	2.9±0.6	2.8±0.1	1.7±0.6	0.5±0.2	0.5±0.2
HRN	Liver	10	16.3±2.3	0.7±0.04	1.2±0.07	3.5±0.9	1.3±0.4	0.6±0.3	0.3±0.2
WT	Lung	10	3.4±0.3	0.2±0.04	0.31±0.02	0.32±0.06	0.15±0.03	ND	ND
HRN	Lung	10	15.0±5.6	0.9±0.03	0.8±0.03	1.3±0.6	0.7±0.5	0.3±0.1	0.2±0.03
WT	Kidney	10	1.51±0.45	0.23±0.09	ND	0.12±0.04	ND	ND	ND
HRN	Kidney	10	5.54±1.24	0.56±0.06	ND	0.34±0.03	ND	0.32±0.09	ND
WT	Bladder	10	0.67±0.3	0.34±0.15	ND	ND	ND	ND	ND
HRN	Bladder	10	2.4±0.6	0.88±0.4	ND	ND	ND	0.57±0.14	ND
WT	Spleen	10	0.24±0.07	0.1±0.04	ND	ND	ND	ND	ND
HR	Spleen	10	0.65±0.1	0.57±0.02	ND	ND	ND	ND	ND
WT	Colon	10	0.63±0.2	0.17±0.05	ND	ND	ND	ND	ND
HRN	Colon	10	3.0±0.4	0.5 0±0.08	ND	0.31±0.15	ND	ND	ND

[a]RAL, relative adduct labeling. All results are presented as the mean±S.D. from three mice; each DNA sample was determined by two postlabelling analyses.
[b]ND — not detected.

at both doses, indicating that CYP enzyme activity is important for the oxidative activation of ellipticine to metabolites generating these adducts. Significantly lower levels of adduct 4 were also found in livers of HRN mice treated with the lower ellipticine dose, but no significant differences in levels of adducts 4–7 were found in livers of the two mouse strains treated with the higher dose (Table 2).

Up to 4.7-fold higher levels of DNA adducts were found in extra-hepatic organs of HRN mice than of WT mice, suggesting that these tissues have the metabolic capacity to oxidize ellipticine and, more importantly, that the same reactive species forming DNA adducts are produced, probably independently of CYP-catalyzed ellipticine activation in the liver. Even though adducts 6 and 7, both known to be generated *in vitro* mainly by peroxidase-mediated oxidation (Poljaková et al., 2006; Stiborová et al., 2007a), were not found in any organ of HRN and WT mice treated with the lower dose, they were both detected in liver and lung and adduct 6 was detected in kidney and urinary bladder of HRN mice treated with 10 mg ellipticine/kg body weight. In WT mice, however, these adducts were only detected in liver (Table 2).

Discussion

Ellipticine is an anticancer agent, whose biological effects such as pharmacological efficiencies and its potential genotoxic side effects may depend on its CYP- and peroxidase mediated metabolism leading to formation of DNA adducts (Stiborová et al., 2001; 2003a,b, 2004, 2007a; Frei et al., 2002; Poljaková et al., 2006, 2007). Although the physiological disposition and DNA adduct formation by this anticancer prodrug have been studied in several animal models (Chadwick et al., 1978; Branfam et al., 1978; Stiborová et al., 2003a, 2007b), the balance between CYPs and peroxidases in the activation of ellipticine *in vivo* is not known. In order to evaluate the contribution and importance of hepatic CYP enzymes to the bioactivation of ellipticine *in vivo* we treated HRN mice with ellipticine. These mice carry a deletion of the *POR* gene in the liver (Henderson and Wolf, 2003; Henderson et al., 2003), and thus lack CYP function in hepatocytes.

We found that ellipticine is metabolized in this animal model, generating ellipticine–DNA adducts in several organs of both HRN and WT mice. Highest total DNA binding levels were always found in liver, followed by lung, kidney, urinary bladder, colon and spleen for both ellipticine doses tested. The pattern and levels of ellipticine–DNA adducts in the organs of WT mice were similar to those found in rats treated with this drug (Stiborová et al., 2003a, 2007b). These data indicate that rats and mice have a similar susceptibility to ellipticine. The adduct pattern consisted of at least two DNA adducts accounting for up to 82% of total levels in the liver of WT mice. These adducts are formed from two reactive species, ellipticine-13-ylium and ellipticine-12-ylium (Fig. 1), which we had suggested earlier to react with one of the nucleophilic centers in the deoxyguanosine residue in DNA (e.g. the exocyclic amino group of guanine, Fig. 1) (Stiborová et al., 2004, 2007a; Poljaková et al., 2006). The low amount of each DNA adduct recovered from digests of DNA treated with 13-hydroxyellipticine or 12-hydroxyellipticine (Fig. 2), however, prevented their further structural characterization. Synthetic approaches are currently being followed in our laboratory to prepare authentic ellipticine–DNA adduct standards. Besides these adducts, up to five additional adduct spots were found in some of the organs analysed.

The finding that ellipticine–DNA adducts are formed in all organs we tested in the study suggest that ellipticine or its metabolites are distributed via the blood stream to different organs and that these tissues have the metabolic capacity to oxidatively activate ellipticine. As found by Chadwick and coworkers (Chadwick et al., 1978; Branfam et al., 1978), ellipticine is very rapidly distributed from the blood, and its excretion is essentially complete by 24 h in several species including mice, rats, dogs and monkeys. The rate of ellipticine elimination from blood was found to reflect the rate of metabolism of this drug (Chadwick et al., 1978). The main organ responsible for its biotransformation was found to be the liver, forming predominantly 9-hydroxyellipticine, which is excreted mainly in bile as its glucuronide or sulfate conjugate (Chadwick et al., 1978; Branfam et al., 1978). Other *in-vivo* pathways involving hydroxylation at as yet unknown positions have also been found (Chadwick et al., 1978; Branfam et al., 1978). In *in-vitro* experiments, ellipticine was metabolized by CYP-mediated reactions by hepatic microsomes of a variety of species, such as humans, rats, rabbits (Stiborová et al., 2004; 2006a) and mice (this study) to several hydroxylated derivatives, with 9-hydroxy-, 12-hydroxy- and 13-hydroxyellipticine being the major metabolites in most species. However, because 13-hydroxy- and 12-hydroxyellipticine are reactive and have been found to form the two major ellipticine–DNA adducts (Stiborová et al., 2004, 2007a; Poljaková et al., 2006), they will not be easily detectable *in vivo*. In addition, radioactively labelled ellipticine was found to be deposited in a number of organs with the highest levels in the liver, followed by kidney, lung, intestine and spleen, and was located primarily in the nuclear fraction (Chadwick et al., 1978). One of the explanation for this may be the ellipticine–DNA binding we found in these tissues.

The levels of the two major ellipticine–DNA adducts were significantly lower in livers of HRN mice, confirming the importance of CYP enzymes in the ellipticine activation to species responsible for DNA adduct formation in this organ *in vivo*. It is remarkable from *in vitro* incubations with hepatic microsomes that the highest effect of the lack of hepatic POR was on the levels of the 9-hydroxyellipticine, the major detoxication product of ellipticine, while the metabolites which form DNA adducts were less affected. Inhibition of NADPH-dependent ellipticine activation to DNA binding species in hepatic microsomes of HRN and WT mice by α-NF and ketoconazole suggests that enzymes from the CYP1A and 3A subfamilies play a major role in this process in mouse liver, as it was found in human and rat liver (Stiborová et al., 2001, 2003b, 2004). The ratio of 13-hydroxyellipticine formed in hepatic microsomes from WT and HRN mice correlated well with the ratio of ellipticine–DNA adduct 1 formed not only *in vitro*, but also *in vivo*. A new ellipticine-derived DNA adduct (see adduct A in Fig. 2A), undetectable in incubations with hepatic microsomes from human or rat (Stiborová et al., 2001,

2003a, 2004) or *in vivo* in rats (Stiborová et al., 2003a, 2007b) or mice treated with ellipticine (present paper), was found in DNA incubated with ellipticine and mouse hepatic microsomes. The metabolite, as well as the enzymes responsible for its formation, remains to be investigated.

Although POR was deleted in hepatocytes of HRN mice the formation of DNA adducts in livers of these mice was not completely eliminated, but occurred at 35% of the level in WT mice. *In vitro* the ratio was somewhat higher with levels of the two major ellipticine–DNA adducts amounting to 50% of WT levels. In addition, inhibitors of POR and CYPs led to a decrease in levels of these two major DNA adducts in hepatic microsomes of both mouse lines of 40–65%. All these results suggest that either other enzymes also activate ellipticine in livers of the mouse model used or the substantial induction in the expression of CYP3A and CYP1A in the liver of HRN mice (Henderson et al., 2003), the enzymes that play a significant role in ellipticine activation, contributes to metabolic activation of this drug in the HRN liver. The results with added arachidonic acid confirm earlier results that *in vitro* COX might be another enzyme system capable of activating ellipticine (Poljaková et al., 2006; Stiborová et al., 2007a). It is however conspicuous that ellipticine activation with a cofactor of COX (arachidonic acid) in microsomes of HRN mice is not as high as in WT mice, on the contrary, the ratio of levels of DNA adducts was similar with arachidonic acid as with NADPH. *In vivo* a similar observation was made in that the levels of "peroxidase-catalysed" adducts 6 and 7 in livers of HRN mice were not higher than in WT mice, as might have been expected as a compensatory reaction of the organism to POR deletion. The reason for low arachidonic acid-dependent activity in hepatic POR knock-out mice is not known.

In contrast to the liver, up to 4.7-fold higher DNA adduct levels were observed in extra-hepatic tissues of HRN mice analysed in this study. Ellipticine delivered i.p. is absorbed via the mesenteric veins and lymphatic systems, and passes through the liver, where most of its metabolism occurs. In the absence of CYP-mediated oxidation in the liver, however, more ellipticine reaches the blood circulation and all distal tissues, where it is activated to DNA adducts, leading to higher levels in HRN than in WT mice. In a future study, the pharmacokinetic profiles of ellipticine in the two mouse lines shall be investigated to provide evidence of increased ellipticine plasma concentrations in HRN mice. An alternative explanation for higher DNA adduct levels in extra-hepatic tissues of HRN mice might be a possible induction of non-CYP/POR-dependent metabolic activities towards ellipticine in these tissues, such as peroxidases.

The question whether CYPs or peroxidases or both of these enzymes are responsible for ellipticine activation in extra-hepatic organs remains to be answered, but the pattern of the ellipticine–DNA adducts found in individual organs as well as knowledge on expression of CYPs and peroxidases in these tissues can help to evaluate their relative importance. In contrast to low constitutive expression of CYPs in extra-hepatic tissues, lung, kidney and urinary bladder are rich in peroxidases such as COX-1, COX-2 and LPO (Eling et al., 1990; Eling and Curtis, 1992). The role of peroxidases in ellipticine activation *in vitro* was studied by us recently, utilising both pure peroxidases (LPO, MPO, COX-1 and -2) (Poljaková et al., 2006; Stiborová et al., 2007a) and microsomes from human kidney, in which peroxidases such as COX are expressed (Stiborová et al., 2007a). All these systems activated ellipticine to ellipticine-13-ylium and ellipticine-12-ylium species generating the two major DNA adducts 1 and 2 (Fig. 1) (Poljaková et al., 2006; Stiborová et al., 2007a). Since these reactive species are the same as those formed by ellipticine oxidation by CYPs, the DNA adducts 1 and 2 cannot serve as markers for either enzyme. The formation of the two minor DNA adducts 6 and 7 (Fig. 2) *in vitro* is, however, mainly peroxidase-catalysed (Stiborová et al., 2007a). These adducts were detected in lung, kidney and urinary bladder of HRN mice treated with 10 mg ellipticine/kg body weight, but not in those of WT mice. These results suggest strongly that peroxidases such as COX and LPO might participate significantly in ellipticine activation in these organs. Therefore, the objective of our future work is to analyse the participation of peroxidases in the metabolic activation of ellipticine *in vivo*. For instance, MPO-knockout mice may help to evaluate the involvement of this enzyme in the bioactivation of ellipticine *in vivo* (Noguchi et al., 2000).

Although expression of CYP enzymes in extra-hepatic organs is much lower than in the liver (Rendic and DiCarlo, 1997), it can be induced by ellipticine not only in the liver, but also in extra-hepatic tissues such as lung and kidney, as we found in rats for CYP1A (Aimová et al., 2007). As a consequence, ellipticine oxidation both to metabolites generating DNA adducts (13-hydroxy- and 12-hydroxyellipticine) and to those leading to its excretion (9-hydroxy- and 7-hydroxyellipticine) is increased (Aimová et al., 2007). Therefore, participation of CYPs in ellipticine activation in extra-hepatic organs cannot be excluded, and awaits further investigation.

In conclusion, despite hepatic POR knock-out, ellipticine–DNA adducts are still detected *in vitro* with HRN hepatic microsomes and *in vivo* in HRN mice treated with single doses of ellipticine. The major difference between the HRN and WT lines is a considerable increase in levels of extra-hepatic DNA adducts in HRN mice. The reason for relatively high levels of liver DNA adducts could be residual POR, e.g. in non-parenchymal cells, or other enzymes like peroxidases. Furthermore, the major effect of POR is on ellipticine detoxication (formation of 9-hydroxyellipticine by microsomal CYPs), and less on its oxidative activation to 12-hydroxy and 13-hydroxyellipticine. Therefore, more unmetabolized ellipticine might be distributed among extra-hepatic tissues. Our results reveal that ellipticine metabolism and DNA adduct formation *in vitro* correspond well to levels of DNA adducts *in vivo* in the HRN mouse model used in the study.

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A COMPARATIVE STUDY

Ellipticine and benzo(a)pyrene increase their own metabolic activation via modulation of expression and enzymatic activity of cytochromes P450 1A1 and 1A2

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ABSTRACT
Two compounds known to covalently bind to DNA after their activation with cytochromes P450 (CYPs), carcinogenic benzo(a)pyrene (BaP) and an antineoplastic agent ellipticine, were investigated for their potential to induce CYP and NADPH:CYP reductase (POR) enzymes in rodent livers, the main target organ for DNA adduct formation. Two animal models were used in the study: (i) rats as animals mimicking the fate of ellipticine in humans and (ii) mice, especially wild-type (WT) and hepatic POR null (HRN™) mouse lines. Ellipticine and BaP induce expression of CYP1A enzymes in livers of experimental models, which leads to increase in their enzymatic activity. In addition, both compounds are capable of generating DNA adducts, predominantly in livers of studied organisms. As determined by ^{32}P postlabelling analysis, levels of ellipticine-derived DNA adducts formed *in vivo* in the livers of HRN™ mice were reduced (by up to 65%) relative to levels in WT mice, indicating that POR mediated CYP enzyme activity is important for the activation of ellipticine. In contrast to these results, 6.4 fold higher DNA binding of BaP was observed in the livers of HRN™ mice than in WT mice. This finding suggests a detoxication role of CYP1A in BaP metabolism *in vivo*. In *in vitro* experiments, DNA adduct formation in calf thymus DNA was up to 25 fold higher in incubations of ellipticine or BaP with microsomes from pretreated animals than with controls. This stimulation effect was attributed to induction of CYP1A1/2 enzymes, which are responsible for oxidative activation of both compounds to the metabolites generating major DNA adducts *in vitro*. Taken together, these results demonstrate that by inducing CYP1A1/2, ellipticine and BaP modulate their own enzymatic metabolic activation and detoxication, thereby modulating their either pharmacological (ellipticine) and/or genotoxic potential (both compounds).

KEY WORDS: benzo(a)pyrene; ellipticine; induction; cytochromes P450; NADPH:cytochrome P450 reductase; HRN™ mice

Introduction

Ellipticine and benzo[*a*]pyrene (BaP) are compounds exhibiting significant biological activities. Ellipticine is an efficient anticancer agent (for a summary, see Stiborová *et al.*, 2006b), while BaP is a strong carcinogen (for a summary, see Arlt *et al.*, 2008). Therefore, both two compounds were employed by us for studies concerning their phatmacological and toxicological effects.

Ellipticine, an alkaloid isolated from *Apocyanaceae* plants, and its derivatives exhibits significant antitumor and anti-HIV activities, characterized by high efficiencies against several types of cancer and rather limited toxic side effects, including complete lack of hematological toxicity. Nevertheless, ellipticines are potent mutagens. Several mechanisms of their antitumor, mutagenic and cytotoxic activities have been hitherto suggested: (i) intercalation into DNA; (ii) inhibition of DNA topoisomerase II activity; (iii) selective inhibition of p53 protein phosphorylation; (iv) disruption of the energy balance of cells by uncoupling mitochondrial oxidative phosphorylation (for a summary, see Stiborová *et al.*, 2001; 2006b). Recently, we have shown that ellipticine also binds covalently to DNA *in vitro* and *in vivo,* after being enzymatically activated with cytochromes

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P450 (CYP) (Figure 1) or peroxidases, suggesting a third possible mechanism of action (Stiborová *et al.*, 2001; 2003a,b; 2004; 2007a).

On the basis of *in vitro* studies, human and rat CYPs of 1A and 3A subfamilies seem to be the predominant enzymes oxidizing ellipticine either to metabolites that are excreted (7- or 9-hydroxyellipticine) or form DNA adducts (12- or 13-hydroxy-ellipticine) (Stiborová *et al.*, 2001; 2003b; 2004; 2006; Kotrbová *et al.*, 2006). Besides these CYPs, peroxidases such as mammalian cyclooxygenases (COX-1 and -2), lactoperoxidase and myeloperoxidase, efficiently generate the same ellipticine-derived DNA adducts *in vitro* (Stiborová *et al.*, 2007a; Poljaková *et al.*, 2006). Identical DNA adducts were also detected in cells in culture, in which both CYPs and peroxidases are expressed, such as human breast adenocarcinoma MCF-7 cells (Bořek-Dohalská *et al.*, 2004), leukemia HL-60 and CCRF-CEM cells (Poljaková *et al.*, 2007) and V79 Chinese hamster lung fibroblasts transfected with human CYP3A4, 1A1 and 1A2 (Frei *et al.*, 2002). After *i.p.* administration of ellipticine, the ellipticine-DNA adduct levels seem to be related to CYP3A1 and 1A content in different tissues of rat, but the real impact of CYPs or peroxidases in this process could not be still exactly evaluated (Stiborová *et al.*, 2003a, 2007b).

BaP, as the second model compound in this comparative study is, similarly to the other polycyclic aromatic hydrocarbons (PAHs), mutagenic and carcinogenic (IARC, 1983; Phillips 1999, 2002). PAHs are produced mainly by incomplete combustion or pyrolysis of organic matter and are ubiquitous in the environment, leading to measurable background levels of exposure in the general population (IARC, 1983). Beside the inhalation of polluted air, the main routes of exposure are through tobacco smoke, diet (Phillips 1999, 2002) and occupational exposition throughout e.g. coal, coke or coal-tar processing and use of coal-tar products (IARC, 1983). Prior to the reaction with DNA, BaP analogously to ellipticine requires metabolic activation (Figure 2), which is an essential step in the mechanism by which BaP exerts its genotoxic effects. CYP1A1 and CYP1B1 are widely accepted to be the most important enzymes in the metabolic activation of BaP (Baird *et al.*, 2005). However, current studies show that BaP-induced DNA damage was increased in mice lacking CYP1A1, indicating that *in vivo* the CYP1A1 enzyme plays a detoxification role, and protects mice against BaP toxicity (Uno *et al.*, 2004, 2006). PAHs affect the expression of numerous enzymes involved in metabolism of xenobiotics (including CYP1A1) mainly *via* the aromatic hydrocarbon receptor (AhR). AhR-dependent inducibility was correlated to the predisposion to some types of cancer (Kouri *et al.*, 1973).

The detailed knowledge on the role of CYP enzymes in activation and/or detoxication of BaP and ellipticine and that on their induction mediated by these xenobiotics, is crucial for the possibility to modify their carcinogenic and/or the therapeutic efficiency. Therefore, this field was extensively investigated in our laboratory. To investigate the real role of CYPs in metabolism of both compounds, we have used several animal models, such as rats, rabbits and/or mice in our previous studies (Stiborová *et al.*, 2001; 2003a; b; 2004; 2006; 2007b; 2008; Kotrbová *et al.*, 2006; Arlt *et al.*, 2008). In the case of mice, the HRN™ (Hepatic Cytochrome P450 Reductase Null) mice, the mouse line lacking the hepatic NADPH:CYP oxidoreductase (POR), the unique electron donor to CYPs, which results in the loss of essentially all hepatic CYP function (Henderson *et al.*, 2003, 2006), was utilized (Stiborová *et al.*, 2008; Arlt *et al.*, 2008).

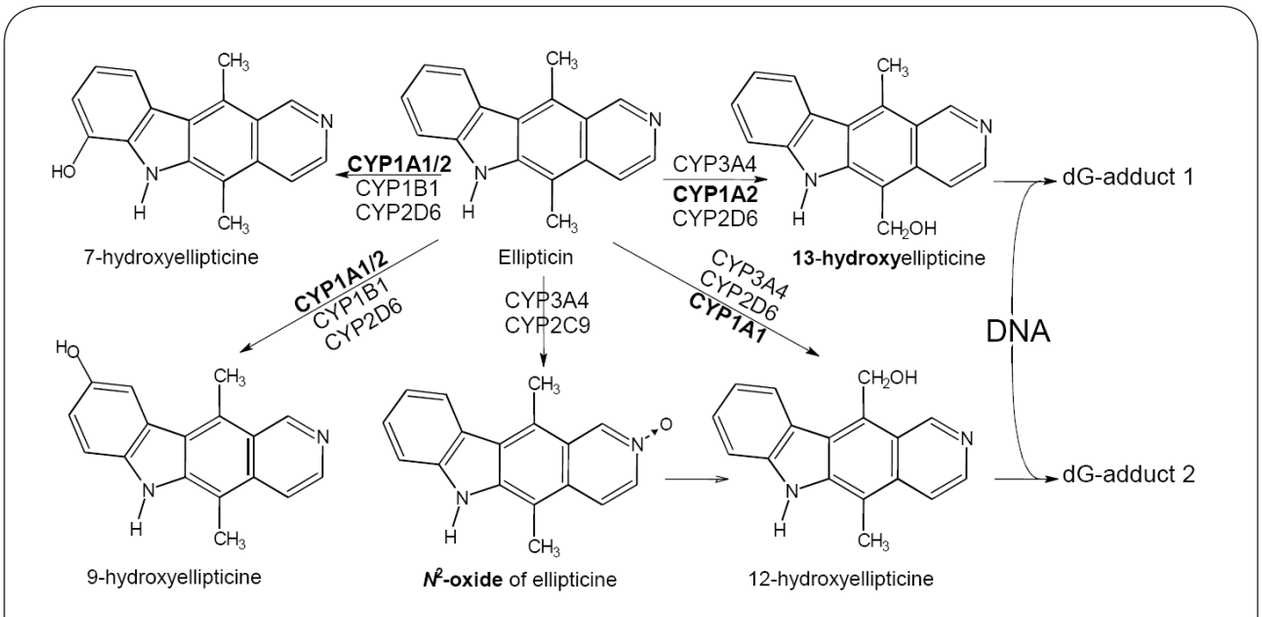

Figure 1. Metabolism and activation of ellipticine (5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole) by cytochromes P450.

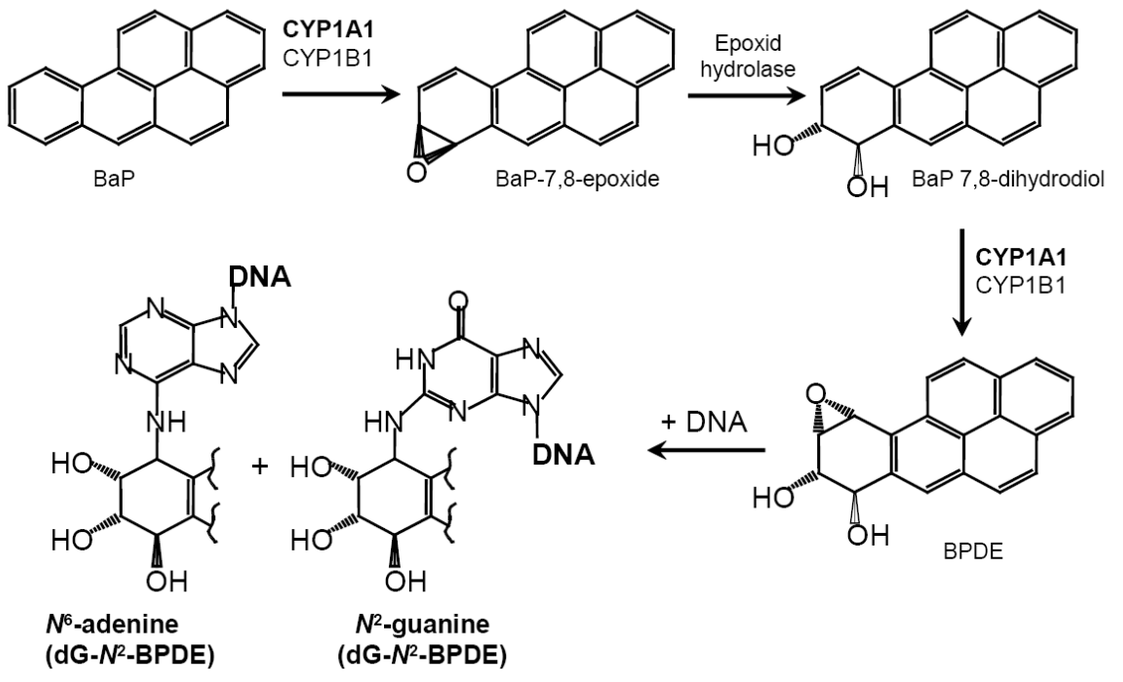

Figure 2. Metabolic activation and DNA adduct formation by benzo(a)pyrene: The typical 3-step activation process with contribution of CYP1A1 or CYP1B1 and epoxide hydrolase leads to the formation of the ultimately reactive species, benzo[a]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE) that can react with DNA, forming adducts preferentially at guanine residues.

Here, we summarize the results obtained with rats and mice previously, and present novel data obtained with these animal models. Such a study is necessary to evaluate results found till the present time and to suggest which further studies are necessary to improve our knowledge in this field.

Material and methods

Animal models

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 Declaration of Helsinki. Rats, the animal model found to be suitable to mimic the fate of ellipticine in humans (Stiborová *et al.*, 2003a; 2006), and two mouse lines, namely,.(i) „Hepatic Reductase Null" (HRN™) mice based on a C57BL/6 background (CXR Bioscience Ltd, Dundee, UK), in which NADPH:cytochrome P450 reductase (POR) is specifically deleted in the liver (*Por*$^{lox/lox}$ + *Cre*^{ALB}) (Henderson *et al.*, 2003, 2006) and (ii) mice homozygous for loxP sites at the Por locus (Por$^{lox/lox}$) as wild-type (WT) mice, were used in this study.

Treatment of animals with ellipticine and benzo(a)pyrene

Male and female Wistar rats (~100 g) were treated with a single dose of 4, 40 or 80 mg/kg body weight (n = 3) of ellipticine by intraperitoneal injection as described (Aimová *et al.*, 2007). Ellipticine was dissolved in sunflower oil/dimethyl sulphoxide (1:1, v/v) at a concentration of 6 mg/ml, control animals (n = 3) received solvent only. The doses of ellipticine used for the treatment of rats are in the range of dosage in human therapy (80–100 mg/m^2).

Groups (n = 3) of female HRN™ and WT mice (3 months old, 25–30 g) were treated intraperitoneally with a single dose of 10 mg ellipticine per kg body weight as described previously (Stiborová *et al.*, 2008). Ellipticine was administered as 10 mg/ml solution in distilled 1% acetic acid, control animals (n = 3) received solvent only.

To evaluate the BaP-mediated induction of CYP1A, groups (n = 3) of HRN™ and WT female mice were treated with 125 mg BaP per kg body weight once daily for five days by intraperitoneal injection. BaP was dissolved in corn-oil at a concentration of 12.5 mg/ml, control animals (n = 3) received solvent only (Arlt *et al.*, 2008).

Preparation of microsomes and assays

Microsomes were isolated from pooled rodent livers as described (Stiborová *et al.*, 2003b). Protein concentrations in the microsomal fractions (bicinchoninic acid protein assay with bovine serum albumin as a standard), the activities of hepatic microsomal CYP1A1/2 (7-ethoxyresorufin *O*-deethylation, EROD) and POR (using cytochrome c) as well as the protein levels of these enzymes (Western Blot) were determined as described previously (Stiborová *et al.*, 2003a,b, 2005).

Oxidation of ellipticine by hepatic microsomes. Incubation mixtures contained 50 mM potassium phosphate

buffer (*pH* 7.4), NADPH-generating system (1 mM $NADP^+$, 10 mM D-glucose 6-phosphate, 1 U/ml D-glucose 6-phosphate dehydrogenase), 0.2 mg microsomal protein, 10 μM ellipticine (dissolved in 5 μl methanol) in a final volume of 500 μl. The reaction was initiated by adding the substrate. After incubation in open glass tubes (37°C, 20 min) the reaction was stopped by adding 100 μl of 2 M NaOH, 5 μl of 1 mM phenacetine in methanol was added as an internal standard. Ellipticine metabolites were extracted twice with ethyl acetate (2 × 1 ml) and analyzed by HPLC as described (Stiborová *et al.*, 2006; 200ß).

Activation of ellipticine or BaP by hepatic microsomes

Incubation mixtures (final volume of 750 μl) used to asses DNA adduct formation consisted of 50 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, 0.5 mg of microsomal proteins and 0.5 mg of calf thymus DNA. Incubations were also carried out in the presence of a COX cofactor, 0.1 mM arachidonic acid instead of NADPH, and additionally 5 mM magnesium chloride. The reaction was initiated by adding 0.1 mM ellipticine (dissolved in 7.5 μl methanol) or 0.1 mM BaP (dissolved in 7.5 μl dimethyl sulphoxide). Incubations at 37°C were carried out for 30 or 90 min with ellipticine or BaP, respectivelly. DNA was isolated from the residual water phase by the phenol/chloroform extraction method as described (Stiborová *et al.*, 2001).

Inhibition studies

The following chemicals were used to inhibit the activation of ellipticine and BaP by mouse hepatic microsomes: α-naphthoflavone (α-NF), which inhibits CYP1A1 and 1A2; indomethacin, a selective inhibitor of COX; α-lipoic acid (α-LA), which inhibits POR; ellipticine, frequently utilized as competitive inhibitor of CYP1A1 enzyme. Inhibitors were added to incubation mixture in 7.5 μl of methanol to yield final concentrations of 0.1 mM and pre-incubated at 37 °C for 10 min with NAD*PH* prior to adding substrate (ellipticine or BaP) and then incubated for as described above. After the incubation, DNA was isolated as described above.

Measurement of DNA adducts

^{32}P-postlabeling analysis with nuclease P1 enrichment, thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC) of ^{32}P-labelled 3'5'-deoxyribonucleoside bisphosphate adducts with ellipticine were done as reported recently (Stiborová *et al.*, 2001; 2003a; 2004; 2007a). DNA adducts formed by BaP were analyzed

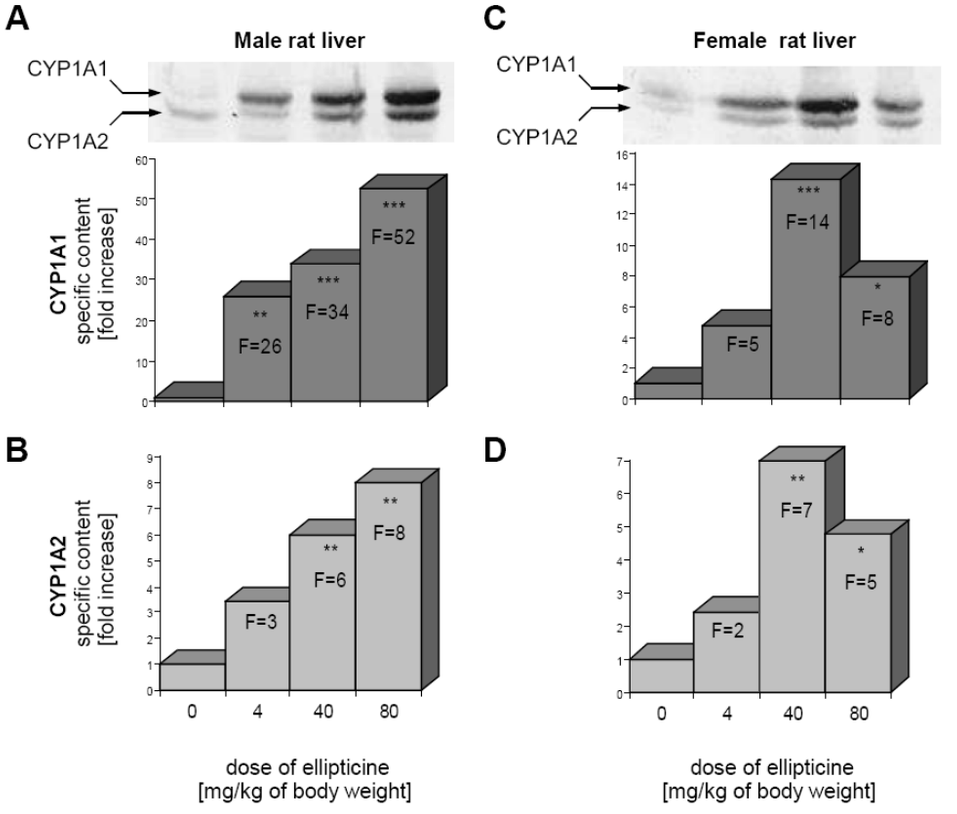

Figure 3. Induction of CYP1A1 (**A,C**) and 1A2 (**B,D**) proteins in livers of male (**A,B**) and female (**C,D**) rats, uninduced or treated with 4, 40 or 80 mg of ellipticine per kg of body weight. Inset in A and C: immunoblots of microsomal CYP1A1 and 1A2 from untreated and ellipticine-treated male and female rats, respectively, stained with antibody against rat CYP1A1. Mean values shown in figure represent results obtained from livers of three rats (n=3), SD < 15%. Values significantly different from the control: *p<0.05, **p<0.01, ***p<0.001.

analogously, using the ^{32}P-postlabeling technique as described previously (Arlt *et al*., 2008).

Results

Induction of hepatic CYP1A by ellipticine and BaP

Ellipticine and BaP induced expression of CYP1A1 and 1A2 proteins, which leads to an increase their enzymatic activities in livers of animal models used in the experiments (rats for ellipticine and mice for BaP) (Figures 3 and 4).

As shown in Figure 3, the induction of CYP1A by ellipticine was dose-dependent. The increase in expression of CYP1A proteins correlated with that in specific CYP1A-mediated activity, EROD (Table 1).

In the case of BaP, mouse models, HRN™ and its parental WT-line, were utilized for the induction experiments. HRN™ mice were found to be more susceptible to BaP-mediated CYP1A induction than the WT mouse line (Figure 4A). Using this model, lacking hepatic POR, we also evaluated whether expression of this enzyme is influenced by treating animals with BaP. Treatment of mice with BaP led to a moderate increase in expression of hepatic POR in both WT (1.2-fold increase) and HRN™ mice (1.4-fold increase). In spite of POR deficiency, CYP1A activity (EROD) was restored by BaP treatment in HRN™ mice, representing the 73-fold increase in EROD activity in microsomes of uninduced WT mice and 30 % of this activity in BaP-induced WT mice (Figure 4B).

DNA adduct formation *in vivo*

In further part of the study, we evaluated the potential of ellipticine and BaP to induce DNA adduct formation *in vivo*. Mice were used as models in these experiments. Treatment of individual mouse strains with ellipticine and BaP resulted in DNA adduct formation (Figure 5–7). The livers of all animal models were the major target organ for DNA adduct formation. Comparative analyses on TLC and HPLC have shown that DNA adduct formation *in vivo* proceeds *via* the reactive metabolite BPDE bound to the N^2 position of guanine (dG-N^2-BPDE) for BaP (Figures 2 and 6) and *via* 13-hydroxy- and 12-hydroxyellipticine in the case of the two major ellipticine-derived DNA adducts (spots 1 and 2 in Figures 1 and 5).

The experiments employing the mouse models helped us to improve our knowledge on the efficiency of CYPs to activate ellipticine and BaP. Levels of ellipticine-derived adducts in livers of HRN™ mice (Figure 5E) were reduced (by up to

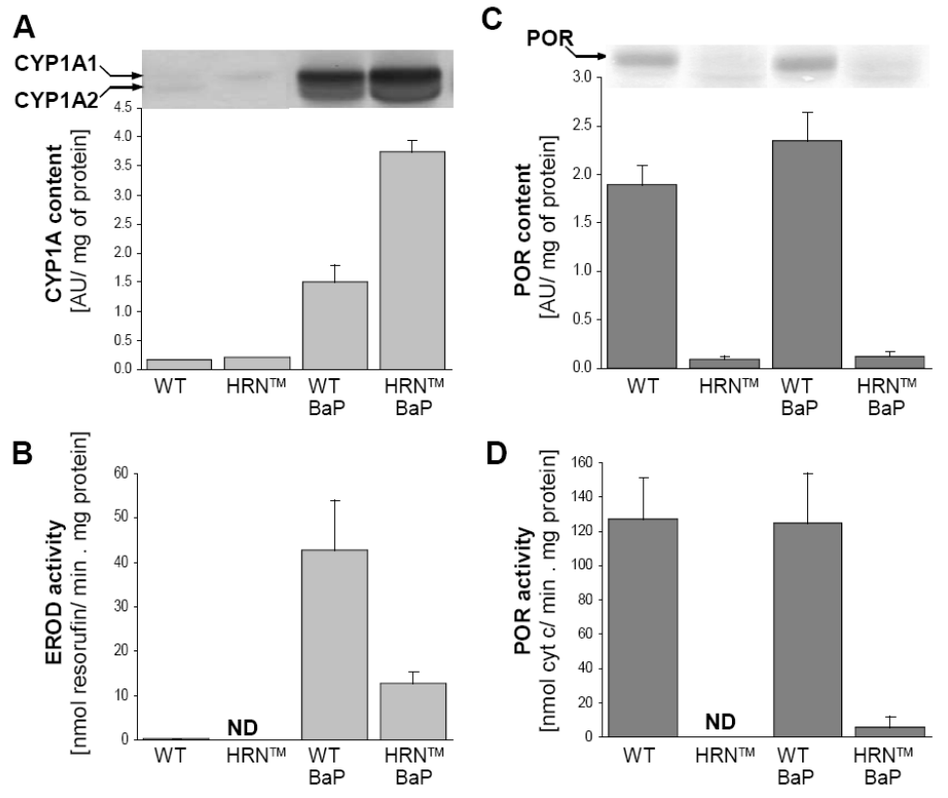

Figure 4. Induction of CYP1A (**A,B**) and POR (**C,D**) protein levels (**A,C**) and activities (**B,D**) in livers of female mice with deleted (HRN™) or intact (WT) liver POR, uninduced or treated (i.p.) with 5 × 125 mg of BaP per kg of body weight. Inset in A: immunoblots of microsomal CYP1A1/2 stained with chicken antibody against rat CYP1A1. Inset in C: immunoblots of microsomal POR stained with chicken antibody against rabbit POR. Microsomes were pooled from livers of 3 animals. Mean and SD evaluated from three separate experiments (n=3). ND = not detectable at used conditions.

65%) relative to levels in WT mice (Figure 5D), indicating that POR-mediated CYP enzyme activity is important for the oxidative activation of ellipticine to metabolites generating these adducts.

In contrast to these results, the highest DNA binding of BaP was observed in livers of HRN™ mice (Figure 6A) which was 6.4-fold ($p<0.01$) higher than DNA binding in WT mice (Figure 6B). This unexpectable finding indicates increasing the CYP-mediated activation of BaP by lack of POR in the liver.

Table 1. Specific CYP1A activity (EROD) in hepatic microsomes of control and ellipticine-treated rats.

CYP activity	Control rats		Ellipticine-treated rats	
	Male	Female	Male	Female
EROD	80.7 ± 2.0	225.8 ± 50.5	551.4 ± 92.2	1737.5 ± 161.3

[a] Each value (pmol of reaction product/min/nmol CYP) represents the mean ± standard deviation of data from two rats in two separate assays (n=4).

Activation of ellipticine and BaP by hepatic microsomes

In order to further investigate the participation of CYPs in activation of ellipticine and BaP and which of these enzymes play the major role, the *in vitro* experiments were carried out. First, incubations of DNA with BaP and/or ellipticine with microsomes isolated from livers of HRN™ and WT mice, untreated or treated with BaP, were performed. In all cases, the patterns of DNA adducts formed by ellipticine and BaP in these experiments were essentially the same as those found *in vivo* (Figures 5 and 6), generated by the pathways shown in Figures 1 and 2. The identity of adducts formed by both compounds *in vitro* with those formed *in vivo* was proved using the TLC and HPLC-cochromatography (data not shown).

Hepatic microsomes isolated from animals treated with ellipticine or BaP were always more effective to form ellipticine- and BaP-derived DNA adducts (Figures 5 and 7) than microsomes from untreated animals. A decrease in levels of ellipticine-derived adducts formed by microsomes from HRN™ mice compared with WT-mice (Figure 7C–D) correlated with almost 2-fold lower levels of 13-hydroxy- and 12-hydroxyellipticine, the metabolites generating the ellipticine-DNA adducts, formed by these microsomes (Stiborová *et al*., 2008).

NADPH-dependent activation of BaP was even 4- to 7-fold lower in HRN™ compared to WT mouse microsomes (Figure 7A–B). The study investigating the pattern of BaP metabolites formed by microsomes from livers of all mouse groups (control HRN™ and WT-mice as well as these mice treated with BaP), which might explain this feature is under way in our laboratory. Preliminary results suggest that the treatment of WT mice with BaP influenced only the relative metabolites ratio instead of the total efficiency of BaP metabolite formation.

In all model systems, the use of POR-inhibitor (α-lipoic acid), CYP1A-inhibitors (α-naphthoflavone, ellipticine) and a CYP3A-inhibitor (ketoconazole) decreased the DNA-adduct formation by both compounds (Figure 7). These results suggest that even very low levels of the POR enzyme in livers of HRN™ mice are still sufficient to mediate CYP-catalyzed activation reactions.

In order to determine which CYPs and/or other enzymes are responsible for DNA adduct formation by both compounds, modulation of microsome-mediated activation with cofactors and inhibitors of individual enzymes was utilized. Addition of NADH, a cofactor of microsomal NADH:cytochrome b_5 reductase, acting as second electron donor for CYP-dependent systems, lowered the difference between HRN™ and WT microsomal BaP-activation (Figure 7A–B). Arachidonic acid (AA), a cofactor of COX-dependent oxidation, was effective to activate ellipticine to species forming DNA adducts (Figure 7C–D), but not to mediate the BaP-DNA adduct formation (Figure 7A–B). On the contrary, an inhibitor of COX, indomethacin (IM) decreases BaP activation in incubations with hepatic microsomes from BaP-treated HRN™ mice, by 30–40% (Figure7B). These results may indicate the participation of COX in activation of both compounds, but with lower efficiency than CYPs.

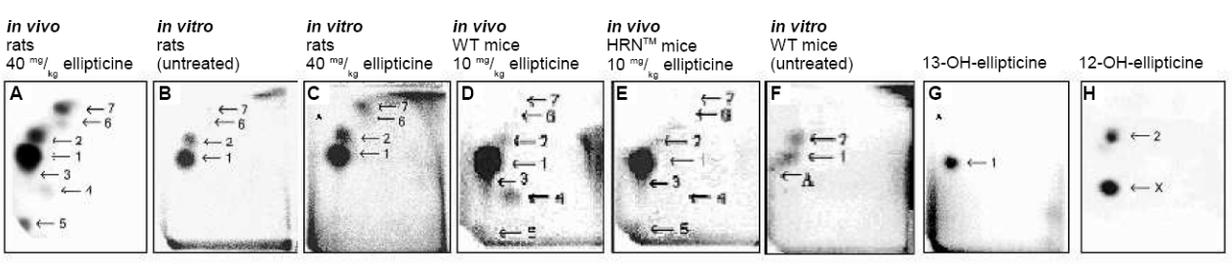

Figure 5. Autoradiographic profile of ellipticine-derived DNA adducts:
- *in vivo* in liver DNA of ellipticine-treated rats (**A**); HRN™ (**D**) and WT (**E**) mice.
- *in vitro* in calf thymus DNA after ellipticine activation with hepatic microsomes of untreated (**B**) and ellipticine-treated (**C**) male rats and wild-type mice (**F**)
- *in vitro* in calf thymus DNA reacted directly with ellipticine metabolites 13-hydroxyellipticine (**G**) or 12-hydroxyellipticine (**H**) (without enzymatic activation). Analyses were performed by the nuclease P1 version of the ^{32}P-postlabelling assay.

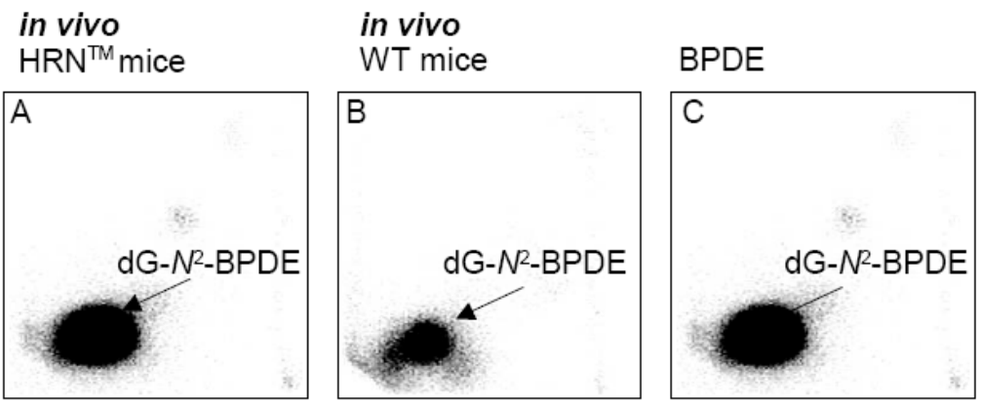

Figure 6. Autoradiographic profiles of BaP-derived DNA adducts *in vivo* in liver DNA of HRN™ (**A**) and WT (**B**) mice treated with 5 times 125 mg of BaP/kg body weight and *in vitro* in salmon testis DNA modified with BPDE (**C**) (without enzymatic activation). Analyses were performed by the nuclease P1 version of the ^{32}P-postlabelling assay.

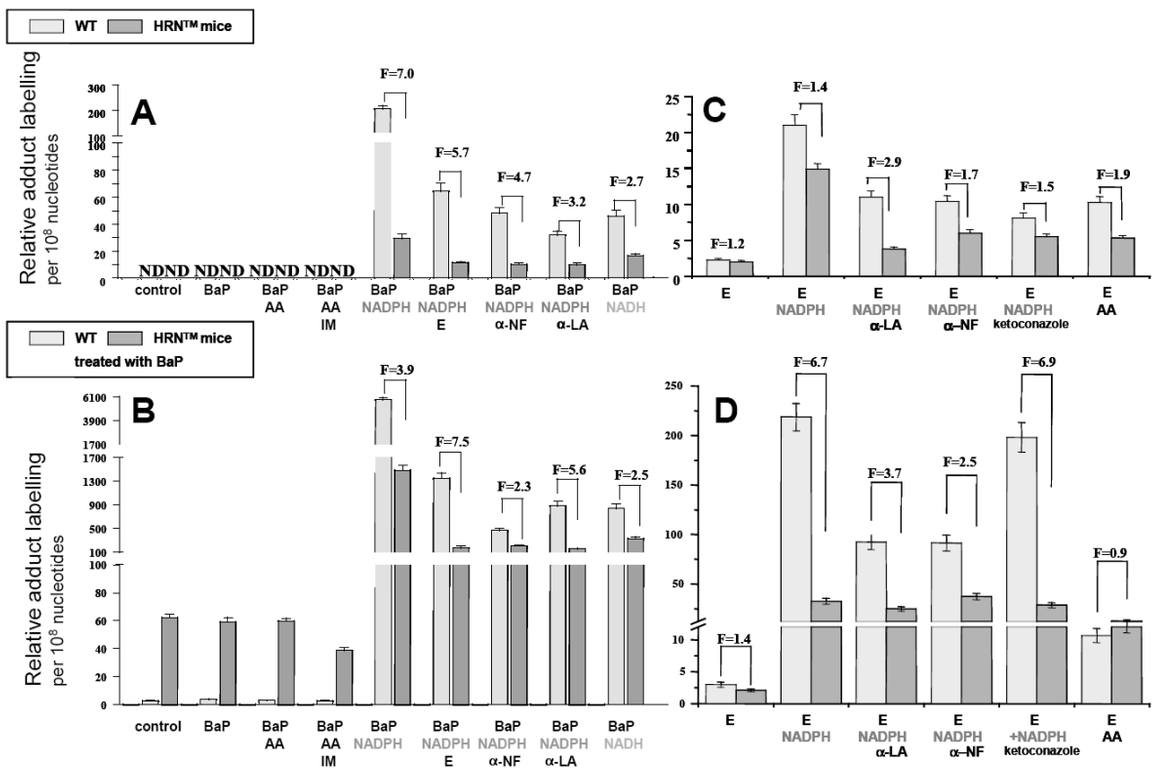

Figure 7. DNA adduct formation after activation of BaP (**A,B**) or ellipticine (**C,D**) with microsomes from livers of HRN™ and WT mice, untreated (**A,C**), or treated with 5×125 mg/kg BaP (**B,D**). Each value represents the mean of two separate analyses (n=2). F = fold increase, WT mice: HRN™ mice; ND = not detected. Control = microsomes + DNA without cofactor; AA = arachidonic acid; IM = indomethacin; E = ellipticine; α-NF = α-naphthoflavone; α-LA = α-lipoic acid.

Discussion

As shown in several studies published previously, ellipticine and BaP are two xenobiotics that react with DNA forming covalent DNA adducts (for a summary, see Stiborová *et al.*, 2006b; Arlt *et al.*, 2008). This genotoxic effect is mediated by their CYP-mediated metabolism. Although the CYP enzymes activating ellipticine and BaP to species binding to DNA *in vitro* have already been identified (Baird *et al.*, 2005; Stiborová *et al.*, 2001; 2003a; 2004; 2006a; 2008; Kotrbová *et al.*, 2006; Arlt *et al.*, 2008), the knowledge on the real impact of these CYPs on the activation of these compounds *in vivo* is limited. Likewise, the effects of repeated exposure of organisms to these compounds on enzyme-mediated activation process are scarce.

To evaluate the contribution and importance of hepatic CYP enzymes to the activation of ellipticine and BaP *in vivo,* we have used in our former and present studies the rats and especially the HRN™ mice, lacking POR and thus also POR-mediated CYP enzyme activity in the liver (Henderson *et al*., 2003; 2006), as model organisms. The use of the HRN™ mouse model has already contributed to resolve the *in vivo* enzymatic activation of several environmental toxicants, including carcinogenic 3-nitrobenzanthrone, activated by cytosolic nitroreductases rather than microsomal POR, and its reductive metabolite 3-aminobenzanthrone (Svobodová *et al*., 2007), whose activation is CYP-dependent (Arlt *et al*., 2003, 2004, 2005, 2006).

Ellipticine and BaP significantly induced expressions of CYP1A1 and 1A2 proteins as well as their enzymatic activity such as EROD in rodent livers. The CYP1A induction resulted in a significant increase in levels of ellipticine- and BaP-derived DNA adducts *in vitro*, in incubations of ellipticine or BaP with microsomes from rats treated with these compounds than in incubations with control microsomes. This is an important finding, because CYP1A enzymes are essential for ellipticine and BaP metabolism. Indeed, the importance of POR-mediated CYP1A1 activation of both compounds *in vitro* was confirmed by inhibition studies using a specific POR inhibitor, α-lipoic acid, and a CYP1A inhibitor, α-naphtoflavone.

Analogously to the results found *in vitro*, the levels of two major DNA adducts in animals treated with ellipticine were significantly decreased in liver DNA of HRN™ mice, confirming the importance of CYP enzymes in ellipticine activation in this organ *in vivo*. Inhibition of NADPH-dependent ellipticine activation in hepatic microsomes of HRN™ and WT mice by α-NF and ketoconazole suggests that CYPs of 1A and 3A subfamilies play a major role in this process in mice livers, analogously to human and rat livers (Stiborová *et al*., 2001; 2003a, b). Nevertheless, the reduction of DNA adduct formation in the liver of HRN™ mice was not absolute, being ~65%. Likewise, the decrease in levels of these two ellipticine-DNA adducts in hepatic microsomes of HRN™ mice caused by inhibitors of POR and CYPs was between 40–65%. These results suggest that other enzymes may also activate ellipticine in mice livers. A potential of arachidonic acid, a cofactor of COX enzymes, to increase the formation of these adducts in mice hepatic microsomes *in vitro* indicate that COX might be one of such enzymes.

On the contrary, BaP-induced DNA adduct formation *in vivo* was significantly increased in liver of HRN™ compared to WT mice, indicating that the real function of POR-mediated CYPs reactions is the BaP detoxification. Taken our results together with those from the CYP1-deletion studies (Uno *et al*., 2004, 2006), there is a remarkable discrepancy between the *in vivo* DNA adduct levels and *in vitro* BaP activation, which is still very difficult to explain. Although hepatic CYP enzyme activity has been essentially inactivated by the conditional deletion of hepatic POR, in non-parenchymal liver cells, the POR deletion may be incomplete. This residual POR activity, in combination with more pronounced induction of CYP enzymes (and probably also by induction of POR) in livers of HRN™ mice liver may explain these *in vitro* findings. Another explanation could be the induction of other metabolizing enzymes.

Taken together, these results show evidence of the crucial role of CYP1A enzymes in ellipticine and BaP genotoxic effect *in vivo.* By inducing CYP1A1/2, both compounds modulate their either pharmacological (ellipticine) and/or genotoxic potential (both chemicals); ellipticine increases its own metabolism leading to enhanced formation of reactive species forming DNA adducts and BaP enhances its detoxification process.

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Isolation and partial characterization of the adduct formed by 13zhydroxyellipticine with deoxyguanosine in DNA

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Abstract

OBJECTIVES: Ellipticine is a potent antineoplastic agent exhibiting multiple mechanisms of its action. Recently, we have found that 13-hydroxyellipticine, formed from ellipticine as the predominant metabolite in human livers, is bound to deoxyguanosine in DNA, generating the major DNA adduct *in vivo* and *in vitro*. The development of the methods suitable for the preparation of this adduct in the amounts sufficient for identification of its structure and those for its isolation and partial characterization is the aim of this study.

METHODS: High performance liquid chromatography (HPLC) was employed for separation of 13-hydroxyellipticine-mediated deoxyguanosine adduct. The ^{32}P-postlabeling technique was utilized to detect this adduct in DNA.

RESULTS: The formation of the 13-hydroxyellipticine-derived deoxyguanosine adduct in DNA *in vitro* was increased under the alkaline pH of the incubations and by the formation of the sulfate and acetate conjugates of 13-hydroxyellipticine generated by reactions with 3'-phosphoadenosine-5'-phosphosulfate (PAPS) or acetyl-coenzyme A (acetyl-CoA) catalyzed by human sulfotransferases (SULTs) 1A1 and 1A2 and *N,O*-acetyltransferases (NATs) 1 and 2. The HPLC method suitable for separation the 13-hydroxyellipticine-derived deoxyguanosine adduct from other reactants, deoxyguanosine and 13-hydroxyellipticine, was developed. The structure of this adduct is proposed to correspond to the product formed from ellipticine-13-ylium with the exocyclic 2-NH_2 group of guanine in DNA.

CONCLUSIONS: The data are the first report on HPLC isolation of the deoxyguanosine adduct formed by 13-hydroxyellipticine in DNA and its partial characterization.

ORIGINAL ARTICLE

Abbreviations

Acetyl-CoA -acetyl-coenzyme A
CYP - cytochrome P450
HPLC - high performance liquid chromatography
NAT - *N,O*-acetyltransferase
PAPS - 3′-phosphoadenosine-5′-phosphosulfate
RAL - relative adduct labeling
r.t. - retention time
SULT - sulfotransferase

INTRODUCTION

Ellipticine (Figure 1), an alkaloid isolated from Apocynanacea plants, exhibits significant antitumor activities (for a summary, see [10]). Ellipticine has been reported to arrest cell cycle progression, to induce apoptotic cell death by the generation of cytotoxic free radicals, an increase of wild-type p53, the rescue of mutant p53 activity and the initiation of mitochondrial pathway (for a summary, see [15]). Chemotherapy-induced cell cycle arrest was shown to result from various DNA damages caused by a variety of chemotherapeutics. In the case of ellipticine, it was suggested that the prevalent DNA-mediated mechanisms of their antitumor, mutagenic and cytotoxic activities are (i) intercalation into DNA [1,15] and (ii) inhibition of DNA topoisomerase II activity [1,15]. We have demonstrated that ellipticine also covalently binds to DNA *in vitro* and *in vivo* after being enzymatically activated with cytochromes P450 (CYP) or peroxidases [7,9–11,13,14,16,17], suggesting a third possible mechanism of action.

Human and rat CYP1A, 1B1 and 3A are the predominant enzymes catalyzing oxidation of ellipticine *in vitro* either to metabolites that are excreted (7-hydroxy- and 9-hydroxyellipticine) or that form DNA adducts (12-hydroxy- and 13-hydroxyellipticine) [6,10,11,16,17]. Of the mammalian peroxidases, cyclooxygenase-1 and -2, lactoperoxidase and myeloperoxidase efficiently generated ellipticine-derived DNA adducts (Figure 1) [7,13]. The same DNA adducts were also detected in cells in culture expressing enzymes activating ellipticine (CYP1A1, cyclooxygenase-1 and myeloperoxidase), such as human breast adenocarcinoma MCF-7 cells [2], leukemia HL-60 and CCRF-CEM cells [8] and V79 Chinese hamster lung fibroblasts transfected with human CYP3A4, 1A1 and 1A2 [5]. On the basis of these data, ellipticine might be considered a drug, whose pharmacological efficiency and/or genotoxic side effects are dependent on its activation by CYPs and peroxidases in target tissues.

13-Hydroxyellipticine formed as the predominant metabolite by CYP3A4 in human livers was identified to bind to deoxyguanosine in DNA, generating the major DNA adduct *in vitro* and *in vivo* (Figure 1) [6,9,11,13,16,17]. We have suggested earlier that the reactive carbenium ion formed spontaneously from 13-hydroxyellipticine, ellipticine-13-ylium, might react with one of the nucleophilic centers in the deoxyguanosine residue in DNA (e.g. the exocyclic amino group of guanine, Figure 1) to form the adduct [7,9,13,16]. The low amount of this DNA adduct recovered from digests of DNA treated with 13-hydroxyellipticine, however, prevented its further structural characterization. Therefore, to prepare this adduct in amounts sufficient for its further characterization, we investigated how to increase its formation *in vitro*. To increase the levels of the 13-hydroxyellipticine-derived DNA adduct, we investigated the modulation of the reaction of 13-hydroxyellipticine with DNA (or deoxyguanosine) by pH and/or by its conjugation with PAPS or acetyl-CoA catalyzed by the phase II biotransformation enzymes, human SULT1A1/2 and NAT1 and NAT2. Moreover, the HPLC isolation procedure to obtain the adduct was developed.

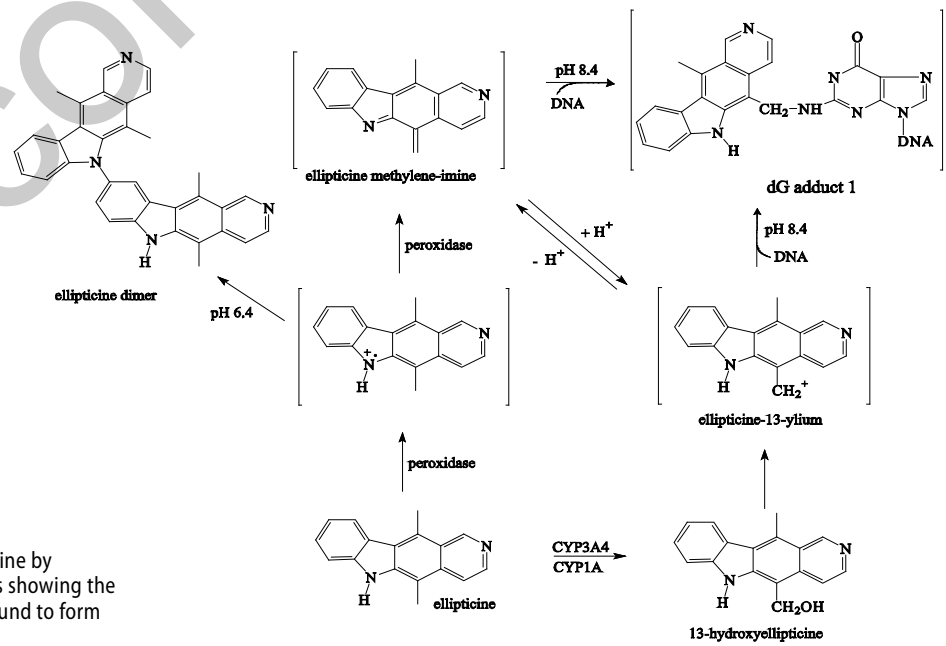

Figure 1. Metabolism of ellipticine by human CYPs and peroxidases showing the characterized metabolites found to form the major DNA adduct

MATERIAL AND METHODS

Ellipticine, deoxyguanosine, acetyl-CoA, PAPS and calf thymus DNA were from Sigma Chemical Co (St Louis, MO, USA). 13-Hydroxyellipticine was synthesized as described [4]. Cytosolic extracts, isolated from insect cells transfected with baculovirus constructs containing cDNA of human SULT1A1*2, -1A2*1 were obtained from Oxford Biomedical Research Inc. (Oxford, MA, USA), and those containing cDNA of human NAT1*4 or NAT2*4 from Gentest Corp. (Woburn, MA, USA). Enzymes and chemicals for the ^{32}P-postlabeling assay were obtained from sources described [10,12]. All these and other chemicals were reagent grade or better. The incubation mixtures in a final volume of 500 µl consisted of 0.1 mM phosphate buffer (pH 6.0, 7.4 or 8.4), 1 mg calf thymus DNA or 1 mM deoxyguanosine and 50 µM 13-hydroxyellipticine either in the presence or absence of 39 nmol human SULT1A1/2 or NAT1 or 2 and SULTs and NATs cofactors (2 mM acetyl-CoA or 100 µM PAPS). Mixtures were incubated at 37 °C for 1–24 h. Aliquots of the mixtures (50 µl) were applied onto a HPLC column, where components of the incubation mixtures were separated. The HPLC was performed with a reversed phase column (Ultrasphere, ODS, 250 × 4.6 mm, 5 µM; Beckman-Coulter, USA) using a linear methanol – acetic acid (32 mM in distilled water) gradient of 0% methanol rising to 100% in 70 min and isocratic elution of 100% methanol in 5 min (flow rate of 1 ml/min, detection at 250 and 318 nm). Deoxyguanosine, 13-hydroxyellipticine and the 13-hydroxyellipticine-derived deoxyguanosine adduct were eluted with retention times (r.t.) of 10.0, 42.2 and 23.8 min, respectively, and identified by mass spectroscopy. ^{32}P-postlabeling assays of the 13-hydroxyellipticine-mediated adduct were performed using nuclease P1 enrichment [10].

RESULTS

The effect of pH on formation of deoxyguanosine adduct from 13-hydroxyellipticine in DNA

13-Hydroxyellipticine incubated with DNA *in vitro* generates the major deoxyguanosine adduct, which was detected and quantified by the nuclease P1 version of the ^{32}P-postlabeling technique (see adduct spot 1 formed in DNA in Figure 2). The yield of formation of this ellipticine-DNA adduct is pH-dependent. Only low levels of this DNA adduct was detectable at pH 6.0, while increasing pH resulted in a pronounced increase in formation of this adduct (Figure 2, Table 1). The 13-hydroxyellipticine-derived adduct is also formed by incubation of 13-hydroxyellipticine with deoxyguanosine (Figure 3). In order to isolate the 13-hydroxyellipticine-derived deoxyguanosine adduct, a novel HPLC procedure was developed. The reversed-phase HPLC was found to be appropriate to isolate this adduct from residual deoxyguanosine and 13-hydroxyellipticine, the adduct eluted with r.t. of 23.8 min (Figure 3). An increase in pH of the incubation mixture leads again to an increase in formation of this adduct. Whereas adduct was formed at pH 8.4, no detectable levels of this adduct were found at pH 7.4 (Figure 3).

The effect of conjugation of 13-hydroxyellipticine with PAPS and acetyl-Co A catalyzed by SULT1A1/2 and NAT1/2 on formation of 13-hydroxyellipticine-derived DNAadduct

The levels of 13-hydroxyellipticine-derived DNA adduct were significantly increased by incubation of 13-hydroxyellipticine and DNA with the human SULT1A1 and 1A2 conjugation enzymes and their cofactor, PAPS, by 1.7- and 26-fold, respectively (Figure 4). Likewise, NAT1 and NAT2 in the presence of their cofactor, acetyl-CoA, stimulated the formation of the 13-hydroxyellipticine-derived DNA adduct, by 33- and 288-fold (Figure 4). An increase in its formation was detected both by the ^{32}P-postlabeling assay (Figure 4) and by HPLC (see the adduct peak eluted at 23.8 min in Figure 5).

DISCUSSION

The present paper shows the results which might increase our knowledge on the mechanism of DNA adduct formation by the anticancer drug ellipti-

Table 1. The effect of pH on levels of the adduct formed by reaction of 13-hydroxyellipticine with DNA[a] , detected by ^{32}P-postlabeling[b]

pH	RAL[c] (mean ± SD/10^7 nucleotides)
6.0	1.3 ± 0.3
7.4	23.3 ± 2.0
8.4	49.7 ± 3.2

[a]See adduct spot 1 in Figure 2. [b]Experimental conditions were as described in Material and methods excerpt that 50 µM 13-hydroxyellipticine and 1 mg calf thymus DNA (37 °C, 90 min) were used. [c]RAL, relative adduct labeling, and standard deviations were obtained from triplicate determinations.

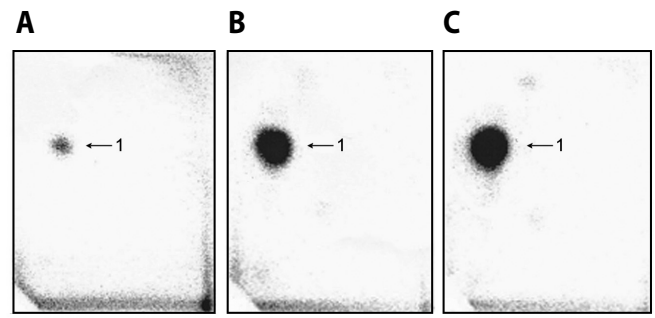

Figure 2. Autoradiographic profiles of 13-hydroxyellipticine-derived DNA adduct levels in relation to pH, analyzed with ^{32}P-postlabeling assay. (**A**) pH 6.0; (**B**) pH 7.4; (**C**) pH 8.4.

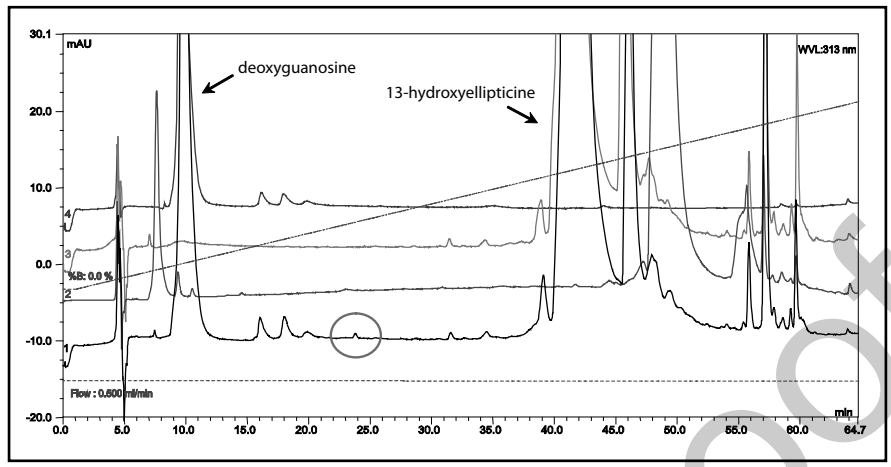

Figure 3. HPLC of 13-hydroxyellipticine-derivated DNA adduct formed by incubation of 13-hydroxyellipticine with deoxyguanosine at pH 7.4 and 8.4
1 – incubation of 13-hydroxyellipticine with deoxyguanosine at pH 8.4; **2** – incubation of 13-hydroxyellipticine with deoxyguanosine at pH 7.4; **3** – 13-hydroxyellipticine; **4** – deoxyguanosine.

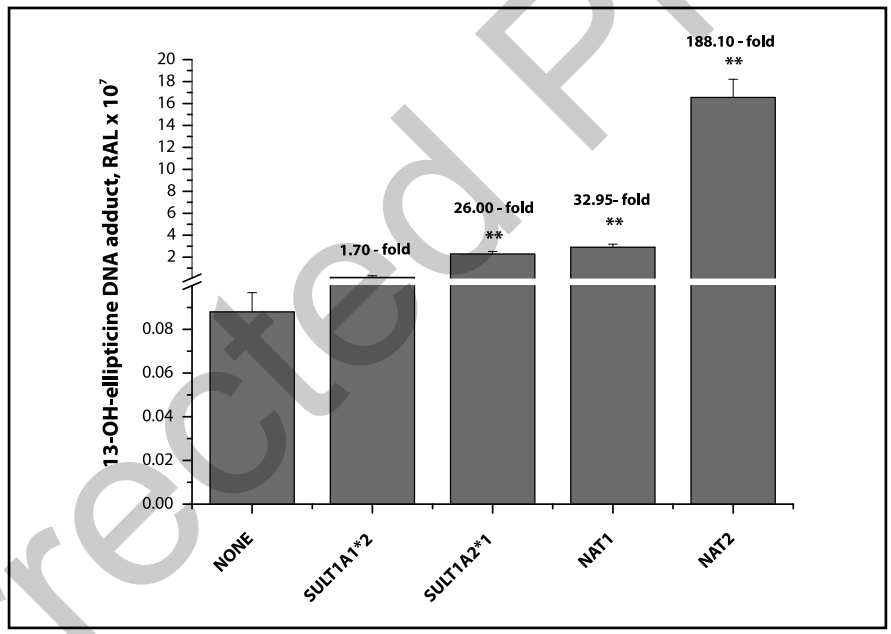

Figure 4. The effect of SULT1A1/2 and NAT1 and 2 on formation of deoxyguanosine adduct in DNA by its incubation with 13-hydroxyellipticine

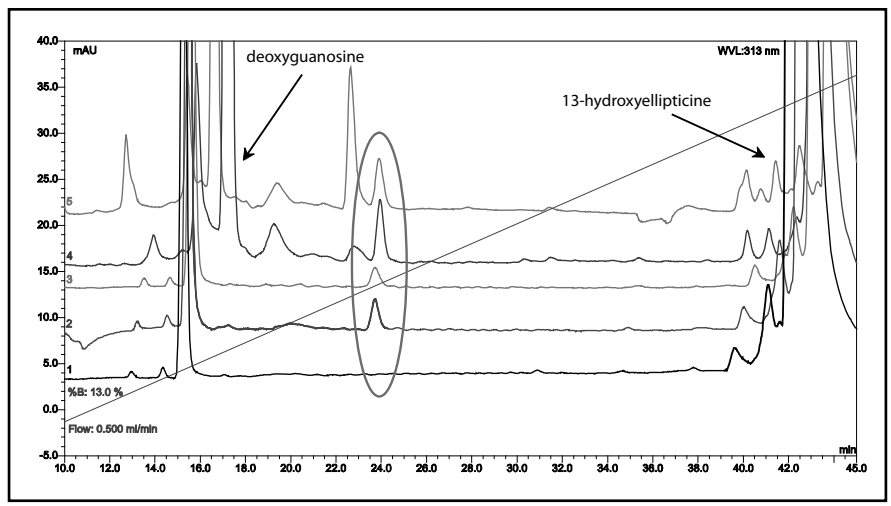

Figure 5. HPLC of 13-hydroxyellipticine-derivated DNA adduct formed by incubation of 13-hydroxyellipticine with deoxyguanosine at pH 7.4 in the presence of SULT 1A1/2 and NAT1 and NAT2 in the presence of their cofactors, PAPS and acetyl-CoA, respectively.
1 – incubation of 13-hydroxyellipticine with deoxyguanosine at pH 7.4; **2** – incubation of 13-hydroxyellipticine with deoxyguanosine at pH 7.4 with PAPS and SULT1A1; **3** – incubation of 13-hydroxyellipticine with deoxyguanosine at pH 7.4 with PAPS and SULT1A2; **4** – incubation of 13-hydroxyellipticine with deoxyguanosine at pH 7.4 with acetyl-CoA and NAT1; **5** – incubation of 13-hydroxyellipticine with deoxyguanosine at pH 7.4 with acetyl-CoA and NAT2.

cine. Here, we demonstrate that the formation of the major DNA adduct by ellipticine *in vitro* and *in vivo*, 13-hydroxyellipticine-derived DNA adduct, is increased by an increase in pH of the incubation mixture. This adduct was proposed to be formed from the reactive species, carbenium ion (ellipticine-13-ylium), formed in the ellipticine oxidation with CYPs and peroxidases (through 13-hydroxyellipticine and/or ellipticine methylene-imine) [13] (Figure 1). Such a species was proposed to react with one of the nucleophilic centers in the deoxyguanosine residue (i.e. the 2-NH_2 group of guanine) in DNA [9,12]. The finding that the levels of this deoxyguanosine adduct significantly decreased under acidic conditions strongly supported the above suggestion. A decrease in pH leads to protonation of the NH_2 group of guanine in the DNA chain, causing a decrease in its nucleophilicity, essential for binding of ellipticine-13-ylium. The basic pH might also facilitate the second electron transfer to form the ellipticine methylene-imine (Figure 1).

The formation of the 13-hydroxyellipticine-derived DNA adduct was also significantly increased by conjugation of 13-hydroxyellipticine with PAPS or acetyl-CoA to the sulfate and acetate esters catalyzed by SULTs and NATs. This finding might have physiological significance. Some of these conjugation enzymes were found to be expressed in the target tumors for ellipticine action (e.g. human breast cancer) [18]. Therefore, by stimulation of the formation of the 13-hydroxyellipticine-derived DNA, by 13-hydroxyellipticine conjugation to sulfate and acetate esters, the pharmacological efficiency of ellipticine should be increased.

We can conclude that the results found in this work support the proposed mechanism of the reaction responsible for formation of the major deoxyguanosine adduct formed in DNA by ellipticine (Figure 1). Ellipticine is bound to deoxyguanosine by its 13-methyl group, which is activated after hydroxylation due to CYP-mediated oxidation to alcohol (13-hydroxyellipticine). Namely, this hydroxylated methyl group either alone or as the sulfate or acetate ester acts as the precursor of the vinylogous imine intermediate or the carbenium ion. Michael-type addition of the intermediates to external amino group deoxyguanosine to form the adduct found in DNA [3]. The study targeted to confirm this suggestion is under way in our laboratory.

ACKNOWLEDGMENT

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Preparation of apo-cytochrome b_5 by its heterologous expression in *Escherichia coli*

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Key words: **cytochrome b_5, apo-cytochrome b_5, cytochrome P450, membrane proteins, heterologous expression, purification**

Abstract

OBJECTIVES: Cytochrome b_5 (b_5) has been shown to influence some cytochrome P450 (CYP)-mediated reactions. For elucidation of the modulation mechanism it is neccessary to evaluate not only the effect of native b_5, but also that of the apo-cytochrome b_5 (apo-b_5), on such reactions. Therefore here, this protein apo-b_5 was prepared using a heterologous expression in *Escherichia coli.*

METHODS: The gene for rabbit b_5 was prepared from synthetic oligonucleotides using PCR, cloned into pUC19 plasmid and amplified in DH5α cells. The final gene sequence was verified by DNA sequencing. The sequence coding b_5 was cleaved from pUC19 by NdeI and XhoI restriction endonucleases and re-cloned to the expression vector pET22b. This vector was used to transform *E. coli* BL-21 (DE3) Gold cells by heat shock. The cell culture was grown in Luria Broth medium at 37°C in an Orbital shaker at 200 RPM. Expression was induced with IPTG. Cytochrome b_5, produced predominantly as the apo-form, was isolated from purified membranes by ionex chromatography on column of DEAE-Sepharose.

RESULTS: Using a heterologous expression of apo-b_5 in *E. coli* and its further purification, the homogenous preparation of this protein was obtained in this work. The apo-b_5 reconstituted with heme reveals the same absorbance spectrum of its oxidized and reduced forms as native b_5. The recombinant apo-b_5 was found to be reduced with NADPH: CYP reductase.

Abbreviations

b_5 - cytochrome b_5

CYP - cytochrome P450

CPR - NADPH: CYP reductase

LB - Luria Broth medium

IPTG - isopropylthio-β-D-galaktosid

Amp - ampicillin

dNTPs - deoxynucleotidetrifosfates

PCR - polymerase chain reaction

DLPC - L-α-dilauryl-*sn*-glycero-3-phosphocholine

NADPH - nicotinamide adenine dinucleotide phosphate (reduced)

Introduction

Cytochrome b_5 (b_5) is a heme protein with molecular weight of about 17 000 [1-3], which is capable of accepting and transferring a single electron [4]. It appeared very early in evolution and is found in a wide range of phyla. Its primary structure is highly conserved. Different mammalian species show over 80% identity in the sequence of the cytosolic heme-binding domain, but reveal higher heterogeneity in the carboxy terminal domain [5, 6].

In mammals, three b_5 isoforms localized into different compartments, such as the endoplasmic reticulum, mitochondria and erythrocytes, were described [7]. The microsomal and mitochondrial b_5 are bound in a membrane and are products of two different genes [8, 9]. The erythrocyte isoform is a soluble protein thought to originate from post-translational proteolysis of the endoplasmic form [10]. Interestingly, the microsomal b_5 is translated on cytoplasmic ribosomes (independently of a signal recognition particle) and incorporated into membrane on the basis of targeting signal situated on the C-terminus of protein [11-15].

Very little is known about physiological functions of mitochondrial b_5. Probably, it works as an activator of androgenesis in rat Leydig cells [16]. The main role of erythrocyte b_5 is to reduce non-functional ferric methemoglobin to its functional oxygen binding ferrous state [17]. In microsomes, b_5 is involved in fatty acid desaturation [7, 18, 19], cholesterol [20] and plasmalogen biosyntheses [7, 21] as well as various hydroxylation reactions catalyzed by mixed function oxidase (MFO) system [22]. Cytochrome b_5 provides the reducing equivalent for all of these enzymatic activities. Cytochrome b_5 could receive electrons from either NADH cytochrome b_5 reductase [23-25] or NADPH cytochrome P450 reductase (CPR) [26, 27], as is depicted in **Fig. 1** and transfering them to cytochromes P450 (CYP) and other enzymes.

The role of microsomal b_5 in catalytic function of CYPs has not been fully understood yet. Cytochrome b_5 has been shown to stimulate, inhibit or have no effect on CYP mediated reactions. Its effect is strongly dependent on individual CYP isoforms, substrates and experimental conditions [6]. Several hypotheses to explain the influence of b_5 on CYP reactions have been suggested. The first one suggests a role of b_5 in a direct transfer of a second electron in CYP catalytic pathway. This step of the pathway seems to be a rate-limiting step in monooxygenase reaction [28]. The electron transfer from reduced b_5 to CYP is faster than the input of electron from CPR [22, 29, 30]. Due to this stimulation effect, CYPs are prevented from uncoupling of partially activated oxygen. The release of superoxide and hydrogen peroxide appears to various extend in all monooxygenase reactions, but also depend both on a CYP isoform and a substrate. Moreover, the addition of b_5 enhances coupling, therefore higher amount of activated oxygen is available to form hydroxylated product [6].

Another possible mechanism of the b_5 action is the formation of complex between b_5 and CYP, which can receive two electrons from CPR in a single step, one for reduction of

CYP and another for that of b_5 [6]. While, CYP without b_5 has to undergo two separate interactions with CPR to complete one catalytic cycle, in the casr of the b_5 presence, only single interaction of complex of CYP and b_5 with CPR is needed; b_5 provides the second electron to CYP promptly after oxygen binding.

Interaction of b_5 with CYP may also induce some conformational changes in CYP leading to breakdown of oxygenated hemoprotein to products. This hypothesis based on some controversial findings showing that not only holoprotein of b_5, but also its apo-form (*i. e. without* heme), which is not capable of electron transfer, can contribute to stimulation effect [31, 32, 33].

Cytochrome b_5 consists of two domains, the larger soluble N-terminal heme-binding core and smaller hydrophobic C-terminal tail, which anchors the protein to the membrane. 15-amino acid flexible linker connects these two domains, providing a heme domain with a sufficient mobility to bind different redox partners, whereas the protein remains in membrane. It was postulated that the linker formed from at least 7 amino acids is necessary for productive interaction [34].

The structure of full-length protein has not yet been resolved, because the hydrophobic domain constitutes an obstacle for crystallization and X-ray diffraction. Most of the structural studies were therefore made with truncated protein, where the membrane anchor is cleaved off. Nevertheless, the membrane anchor was observed to be essential for some biological activities of b_5 [35]. Namely, the soluble form of b_5 is capable to interact with soluble partners such as cytochrome c [7, 34] but does not bind to CYP and does not affect the CYP-mediated reactions at all [36, 37].

The structure of soluble domain of rabbit b_5 is illustrated in **Fig. 2.** The holoprotein structure contains two hydrophobic cores. Whereas core 1 has a clear functional role (heme binds in core 1 and is coordinated by two invariant residues His39, His 63, which protect iron

from direct interactions with molecular oxygen [6]) the highly conserved core 2 appears to have only a structural role [38-40]. The apoprotein consists of a well folded hydrophobic core 2 and 42-residue loop, which is substantially disordered in the solution. Association with the heme cofactor causes the loop to organize into a well-folded core 1[38]. The interaction with CYPs and reductases is mediated through the cluster of acidic amino acid residues, located on the protein surface close to heme *via* electrostatic forces [41-43].

Two hypothetical conformations of the anchor are shown in **Fig. 3** [5]. In the first one, the hydrophobic domain, in form of single helix, spans the lipid bilayer so the C-terminus is situated in the lumen of endoplasmic reticulum [15, 37, 44] and in second one, membrane anchor forms a hairpin loop hairpin loop structure with a carboxy terminal residue exposed to the cytoplasm [5, 15, 45]. Even though, the exact topology of membrane binding domain is still not quite clear, some contemporary evidences prefer the first possibility [15].

Our knowledge about b_5 indicates that this protein is very important molecule taking part in biotransformation of many compounds such as pharmaceuticals or procarcinogens [46-48]. Initial functional and structural studies were performed with b_5 protein purified from animal livers using its solubilisation with detergents. Intact b_5 requires detergent in order to remain in monomeric state; otherwise it forms octamers in aqueous solutions [49, 50]. Since the usage of b_5 for experiments really increases, a lot of scientists take advantage of overproduction of recombinant proteins in bacteria. For elucidation of the mechanism of the effects of b_5 on CYP mediated reactions, it is necessary to evaluate not only the effect of native b_5, but also that of the apo-cytochrome b_5 (apo-b_5). Several different approaches for b_5 preparation were utilized. One was chemical extraction of heme from purified rabbit b_5 by acid acetone treatment [32, 51]. Nevertheless, majority of protein was regrettably denaturated during this procedure. To prepare the pure apo-b_5, without altering the native protein conformation, the heme cofactor has to be gently and efficiently removed. Here, we used a

novel procedure, utilizing a heterologous expression of apo-b_5 in *E. coli*. Here, the precursor of heme biosynthesis - the δ-aminolevulinic acid was not added to bacterial culture, which leads to expression of about 90% of protein in the form of apo-b_5. Similar procedure was utilized by other authors [52, 53, 54].

Material and methods

Materials

Oligonucleotides were synthesised and dNTPs purchased from East Port (Czech Republic), Pfu polymerase was from Fermentas (Canada). *E. coli* BL-21 (DE3) Gold cells were obtained from Stratagene (USA). Restriction endonucleases (Hind III, EcoR I, Nde I, Xho I), plasmid pUC19 and T4 DNA ligase were purchased from New England Biolabs (USA). LB medium, LB agar, IPTG, DLPC, NADPH, Brij 35 and sodium cholate were obtained from Sigma-Aldrich (USA); Amp was from Duchefa Biochemie (Netherlands). Commercial kits for purification of DNA from an agarose gels and plasmid purification (Jet quick Gel Extraction Spin Kit, Jet quick Plasmid Mini and Maxiprep Spin Kit) were purchased from Genomed (Germany). Complete mini protease inhibitors tablets were obtained from Roche Diagnostic (Germany). DEAE-Sepharose CL6B was from Amersham Biosciences (Sweden). CPR was purified as described earlier [55].

Design and construction of the complete gene for rabbit cytochrome b_5

We utilized *de novo* synthesis of the gene from commercially prepared oligonucleotides, procedure similar to that described by Beck von Bodman [56]. The gene of whole amphipatic rabbit b_5 was optimized for *E. coli* codon usage and restriction endonucleases sites in DNA 2.0 program (available at www.dna20.com). Each oligonucleotide (**Fig. 4)** consists of approximately 80 base pairs with at least 18 nucleotides long overlapping area whose melting

point is higher than 50°C. Firstly, oligonucleotides 2-7 were joined together by polymerase chain reaction (PCR) using the termostable *Pfu* DNA polymerase. Amplification was performed after 10 min of denaturation at 94°C in 30 cycles at 94°C (30 s), 50°C (30 s) and 73°C (2 min) ending with a 8 min extension at 73°C. In the next PCR step, carried out at the same conditions, oligonucleotides 1 and 8 were used as primers to complete the whole sequence of b_5. The resulting PCR product was cleaved by EcoRI and Hind III endonucleases and ligated to pUC19 plasmid treated with the same enzymes. This construct was cloned into DH5α cells and amplified with 100 µg/ml ampicillin (Amp) as a selective marker. The plasmid DNA was purified by commercial kit and its correctness verified by DNA sequencing.

Protein expression and membrane isolation

The plasmid pET-22b with T7-promoter was used as an expression vector. Nde I/ Xho I digested b_5 gene followed by a stop codon from pUC19 plasmid was then ligated with Nde I/ Xho I digested pET-22b vector to give expression vector, which was used to transform *E. coli* BL-21 (DE3) Gold cells utilizing heat shock at 42°C for 30 s. Transfected cells were seeded on LB agar plates with 100 µg/ml ampicillin and grown overnight at 37°C. The next day, 500 ml of LB medium with 100 µg/ml ampicillin was inoculated. Culture was grown at 37°C and shaken in an orbital shaker at 200 RPM. When the optical density of culture at 600 nm reached a value of approximately 1.5, IPTG was added to a final concentration of 0.05 mM in order to induce the protein expression. The cells were cultivated for next 4 h and then harvested by centrifugation at 3000g for 20 min and stored at -20°C. After thawing, the pellet was re-suspended in 25 ml of cold phosphate buffer A (10 mM KH_2PO_4, pH 7.7, 1 mM EDTA), supplemented with two tablets of complete mini protease inhibitor (Roche Diagnostic, Germany). Cells were disrupted by sonication using a 1 cm diameter probe

immersed into the cell suspension and underwent eight sonication cycles with 2.5 min duration at 40% of power, using 100 W maximal setting. Cell suspension was kept on ice during sonication and after each cycle left to cool down in fridge for 15 min. Insoluble proteins and cells debris was removed by centrifugation at 3000g for 15 min at 4°C. Supernatant containing the membrane fraction was subsequently centrifuged at 105 000g on Beckman Coulter Ti 70 rotor for 70 min at 4°C. The sediment was homogenised by brief 30 s pulses in 25 ml of buffer B (10 mM KH_2PO_4, pH 7.7, 1 mM EDTA, and 20% glycerol) and left at 4°C while a protein concentration was determined. Protein content was established using bicinchoninic acid (BCA) assay with bovine serum albumin as a standard. The sample was then diluted with buffer B to yield protein concentration of 4 mg/ml. For solubilization of b_5 from membranes detergents Brij 35 and sodium cholate were added in amounts of 1 mg/mg of proteins and the mixture was stirred for at least three hours at 4°C. Solubilized membranes were centrifuged at 105 000g on Beckman Coulter Ti 70 rotor for 70 min at 4°C and then the supernatant loaded on DEAE Sepharose CL6B column equilibrated with buffer C (20 mM KH_2PO_4, pH 7.7, 1 mM EDTA, 20% glycerol, 0.6% Brij).

Protein purification

All procedures described below were performed at 4°C. DEAE Sepharose column (2.5 x 20 cm) was equilibrated with buffer C. Solution containing b_5 was applied on the column using a flow rate of 1 ml/min and washed with approximately 300 ml of buffer C to get rid of contaminant proteins not bound to the filling (until the absorbance of the eluent at 280 nm decreased to zero). The Cytochrome b_5 protein was eluted with linear gradient of KCl (0-400 mM) in buffer C. The elution profile was monitored spectrophotometrically at 280 nm. Cytochrome b_5 content and purity in collected fractions were assessed by SDS electrophoresis carried out by the method of Laemmli [57] using 15% separating gel and stained with

Coomasiie Brilliant blue. Pooled fractions were concentrated by ultrafiltration using PM 10 membrane (Millipore), dialysed overnight against 2000 ml of buffer C and loaded onto a second DEAE-Sepharose column equilibrated with buffer E (20 mM KH_2PO_4, pH 7.7, 1 mM EDTA, 20% glycerol, 0.1% sodium cholate). This chromatography step served especially for elimination of Brij detergent from b_5 preparation. After column washing with triple volume of buffer D, b_5 was obtained by isocratic elution with buffer F (200 mM KH_2PO_4, 200 mM KCl, pH 7.7, 1 mM EDTA, 20% glycerol, 0.1% sodium cholate). Sodium cholate, an ionic detergent, was removed from b_5 preparation by dialysis against 2000 ml of buffer G (50 mM KH_2PO_4, pH 7.7, 20% glycerol). After dialysis, the preparation was concentrated using an Amicon centricon apparatus, aliquoted and stored at -80°C.

Spectral characterization of recombinant cytochrome b_5

Incorporation of heme into cytochrome b_5

The preparation of hemin chloride solution and the incorporation of heme into apo-b_5 performed by the procedure described elsewhere [52]. Hemin chloride solution in 50% ethanol was prepared to yield 1 mM concentration. Small amounts of 1 M NaOH were added until the addition of NaOH caused no further hemin dissolution, *i. e.* no further increase in absorbance of Tris-liganded heme measured at 385 nm in a 10 µl aliquot 100x diluted with 20 mM Tris buffer, pH 8.0 containing 1 mM EDTA. The heme stock solution prepared with this procedure was filtered through 0.2 µm filter. Purified apo-b_5 was diluted with 20 mM Tris, pH 8.0 containing 1 mM EDTA and 0.4% sodium cholate to yield 0.25 mg/ml protein concentration. Absorbance spectra (from 350 to 500 nm) were recorded on Hewlett Packard 8453 UV spectrophotometer. Aliquots of hemin chloride were titrated into 1 ml of the apo-b_5 sample and the reconstitution of heme with apo-b_5 monitored with absorbance spectroscopy. The titration was considered to be complete when the Soret peak of b_5 shifted from 413 to 409

nm and the increase in absorbance at 385 due to excess of free Tris-liganded hemin was observed in the spectrum.

Determination of cytochrome b_5 content

The concentration of b_5 was determined from the absolute absorbance spectrum using molar extinction coefficient ε_{413} = 117 $mM^{-1}.cm^{-1}$ [58-60] or from the reduced minus oxidized difference spectrum using molar extinction coefficient $\varepsilon_{424-409}$ = 185 $mM^{-1}.cm^{-1}$, respectively [59]. The b_5 in the sample cell was reduced by the addition of approximately 1 mg of solid sodium dithionite.

Reduction of cytochrome b_5 with CPR

The reduction of b_5 with CPR was done according to the method described previously [26]. CPR and b_5 were incorporated into liposomes: 3 μM (apo-) b_5 and/or 3 μM heme, 0.1 μM CPR and liposomes prepared from DLPC in a final concentration of 0.2 mg/ml were incubated for 10 min in 495 μl of 50 mM potassium phosphate buffer, pH 7.7 at room temperature and 350 RPM. The reaction was initiated by the addition of 5 μl of 10 mM NADPH and the reduced minus oxidized difference spectrum from 400 to 500 nm was monitored on Hewlett Packard 8453 UV spectrophotomer. The reduction of b_5 causes an increase in absorbance at 424, 526 and 556 nm and a decrease in 409 nm.

Results and discussion

The gene for full length membrane rabbit microsomal b_5 was prepared from synthetic oligodeoxyribonucleotides, which are ranging in length about 80 base pairs. They were designed to comprise both sense and anti-sense strands of DNA, with 18 bases long complementary areas as shown in **figure 4** and described in Methods. This total synthetic

approach allows to design the gene suitable for expression in bacteria. We have the opportunity to choose codon sequences, which are optimal for the *E. coli* translation apparatus and enables to introduce various unique restriction sites allowing easy mutagenesis of a gene. The prepared gene was inserted into pUC19 plasmid after treating both the gene and the plasmid with EcoRI and Hind III endonucleases and *E. coli* DH5α cells were transformed by the construct. The amplified plasmid (from a single colony carving resistence to ampicilin) was completely sequenced to verify the correctness of DNA sequence. Because no mutations in the sequence were found, the expression vector using pET-22b plasmid was prepared as described in Materials and Methods and used to transform BL-21 (DE3) Gold cells utilizing heat shock at 42°C for 30 s. The growth conditions were as described (see Materils and Methods). The protein production was induced with 0.05 mM IPTG. The cells accumulated a large amount of recombinant protein, but did not show a red colour in the culture, which is an indicator of the heme presence; therefore we expected a low level of heme content in the recombinant b_5. This assumption was confirmed by absorbance spectrum of purified protein (**Fig. 7**). Cells were disrupted by sonication and b_5 was found to be present in a soluble membranous fraction, which was further gained by ultracentrifugation. Contrary to most of studies dealing with expression of b_5, we purified b_5 to homogenity as an apo-form. The homogenous apo-b_5 was purified by the procedure consisting of detergent solubilization and chromatography on a DEAE-Sepharose column (**Fig. 6**). Even though solubilization of b_5 from membranes is not complete (the part of b_5 remains in sediment after centrifugation), it leads to a significant increase in purity of b_5; only a small amount of others ballast proteins are solubilized together with b_5 (**Fig. 6**). A negligible part of sample did not bind to DEAE-Sepharose, probably due to a high content of detergent, which forms micelles under the conditions used (4°C) (**Fig. 5**). Using this purification this step homogenous apo-b_5 protein was obtained. Apo-b5 was eluted with 100 mM KCl, while balast proteins retained at a

DEAE-Sepharose column **(Fig. 5).** As shown in **Fig 7**, the prepared b_5 is predominantly in its apo-form (without heme). The amount of remaining heme protein is only about 1% compared to an apo-b_5. During the titration of apo-b_5 with hemin, the heme introduces into the apo-form protein to produce holoprotein. The formation of holoprotein (native b_5) was proven by an increase in absorbance at 413 nm (**Fig. 8**), indicating the typical Soret peak formation. The reconstitution of apo-b_5 with heme is accompanied by colour change from brown colour of hemin to red bright colour of holoprotein of b_5. The resulting heme-reconstituted b_5 has the same physical properties as native b_5 purified from rabbit livers, showing the same absorption spectra of oxidized and reduced forms of b_5 (**Fig. 9**). The Soret band at 413 nm in the oxidized form and maxima at 424, 526 and 556 nm for the dithionite-reduced form were found (**Fig. 9**). Purified recombinant b_5 was found to be reduced with CPR in the reconstituted system in liposomes after addition of NADPH. See an increase in absorbance at 424 nm and a decrease at 409 nm (**Fig. 10B**). The same spectra were found also for purified native rabbit b_5 (**Fig. 10A**), but not for hemin (**Fig. 10C**). All these findings indicate that purified apo-b_5 has its native structure without damages in the protein conformation during the purification procedure. This suggestion was confirmed by the metabolic studies with ellipticine, where apo-b_5 reconstituted with heme functions analogously to rabbit b_5 (data not shown). Further studies investigating the effect of the native b_5 and apo-b_5 on oxidation of ellipticine by CYP1A1 and 1A2 enzymes are under way in our laboratory.

Acknowledgements

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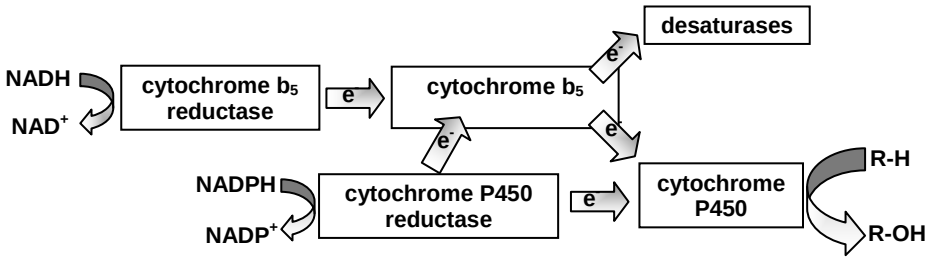

Fig. 1. Schematic demonstration of electron transfer between CYP, b_5 and their reductases (adapted from reference [1])

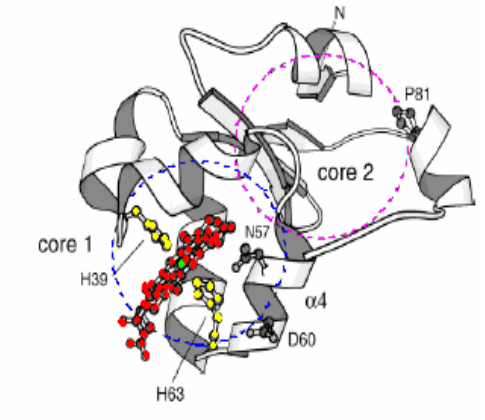

Fig. 2. Structural model of soluble domain of oxidized rat microsomal b_5 (adapted from [40]). Hydrophobic cores 1 and 2 are indicating by circles.

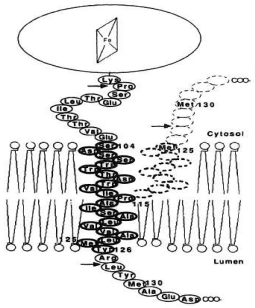

Fig. 3. Model for the possible topology of cytochrome b_5 in membrane (adapted from [15])

1. 5’-GACGAATTCATATGGCAGCGCAGAGCGATAAAGATGTGAAATATTACACCCTGGAAGAAATCAAG-3’
2. 5’-ACACCCTGGAAGAAATCAAGAAACATAACCACTCCAAAAGCACGTGGCTGATCCTGCACCACAAAGTC-3’
3. 5’-CTGTTCACGCAGAACTTCTTCACCGCCAGGGTGTTCTTCGAGGAATTTGGTGAGGTCATAGACTTTGTGGTGCAGGAT-3’
4. 5’-GAAGTTCTGCGTGAACAGGCAGGTGGCGATGCCACGGAGAACTTCGAAGACGTGGGCCACTCCACGGATGCACGCGAA-3’
5. 5’-ACGATCGTCCGGATGCAGTTCGCCGATGATGAAGGTTTTGGACAGTTCGCGTGCATCCGTGGA-3’
6. 5’-CTGCATCCGGACGATCGTAGTAAACTTAGCAAACCGATGGAAACCCTGATCACTACCGTTGAT-3’
7. 5’-CGATCAGGGCAGAAATGGCCGGGATAACCCAGTTGGTCCACCAGCTTGAGTTGCTATCAACGGTAGTGATCAGGG-3’
8. 5’-GTAAAGCTTCTCGAGTTAATCATCCGCCATATACAGACGATACATCAATGCCACGATCAGGGCAGAAATGG-3’

Fig. 4. Proposal of oligonucleotides used for b_5 gene synthesis. All of oligonucleotides are written in a 5’ to 3’ direction. At the 5’ end of the first oligonucleotide there are restriction sites for endonucleases EcoRI (GAATTC) and NdeI (CATATG), which contains an initializing codon ATG for protein translation. Similarly at the 5’ end of the last oligonucleotide there are restriction sites for endonucleases HindIII (AAGCTT) and XhoI (CTCGAG) followed by a stop codon TAA. Even oligonucleotides are complementary to the original gene sequence. Underlined areas highlight overlapping segments.

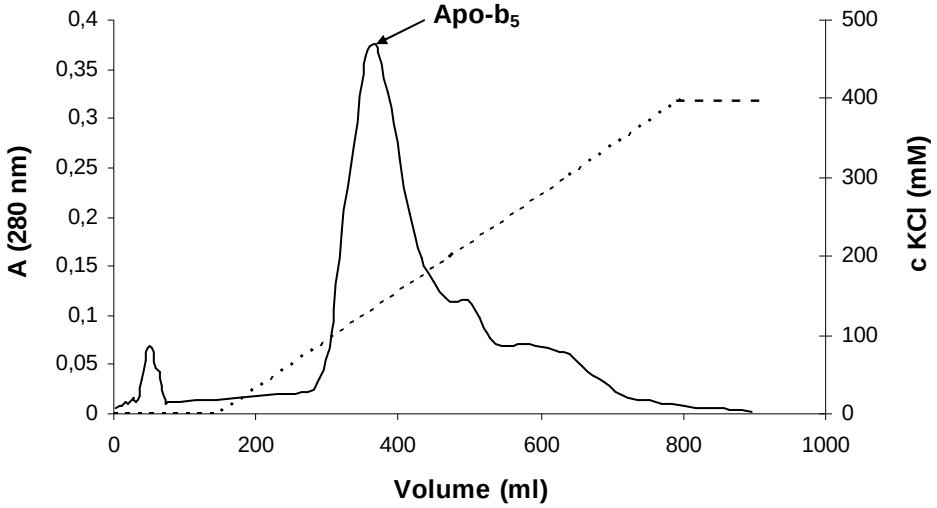

Fig. 5. Gradient elution profile of cytochrome b_5 from DEAE-Sepharose column

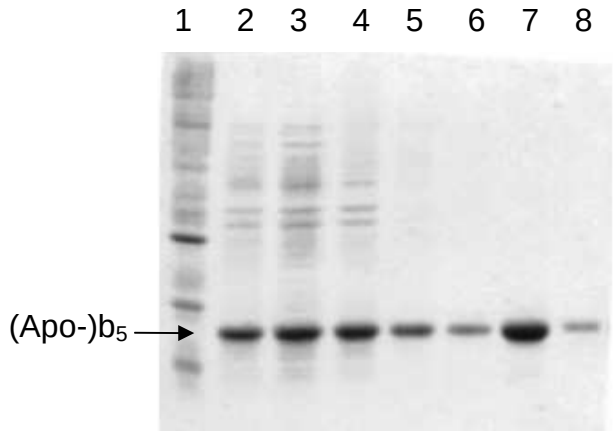

Fig. 6. SDS PAGE of b_5 samples during its purification (15% separating gel)

Lane **1,** molecular weight standard; Lane **2**, the *E. coli* cell culture; Lane **3**, supernatant of the *E. coli* cells after sonication; Lane **4**, sediment after first centrifugation at 105 000 g; Lane **5**, supernatant after solubilization; Lane **6**, sample of apo-b_5 after chromatography on DEAE-Sepharose CL6B, Lane **7**, the final apo-b_5 preparate; Lane **8**, native rabbit b_5

Table 1 Purification of membrane bound apoform of cytochrome b_5

Purification Step	Volume (ml)	Total protein [a] (mg)	Yield%
Sonicated cells [b]	25	281	100
First ultracentrifugation	27	153	54
Solubilization	67	96	34
Deae-Seppharose	25	69	25
Final preparete	3	28	10

[a] Determined by BCA assay
[b] Prepared from 5,4 g of *E. coli* cells obtained from 0.5 l of IPTG induced culture medium

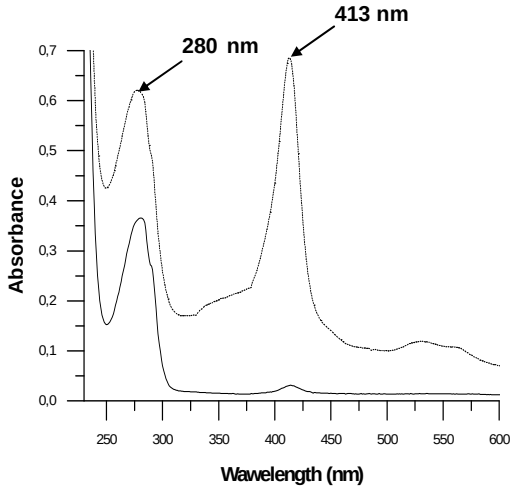

Fig. 7. Absorbance spectra of purified recombinant apo-cytochrome b_5 *(solid line).* The minimal peak at 413 nm indicates that the content of heme protein (native b_5) is less than 1% *(dashed line).*

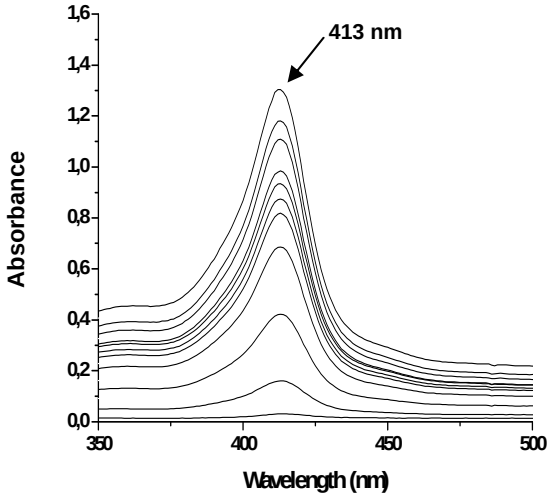

Fig. 8. Binding of heme to recombinant apo-cytochrome b_5. Each line represents the addition of 2-µl aliquot of hemin solution.

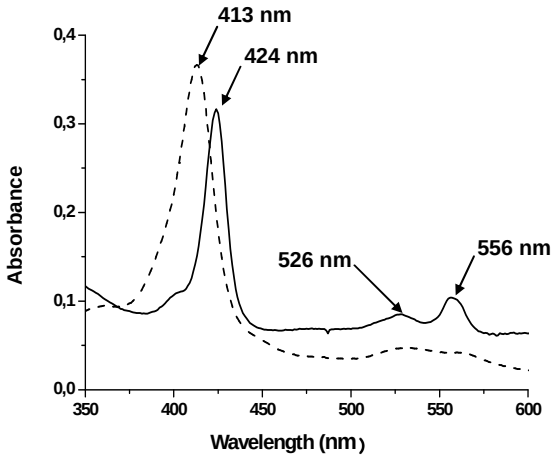

Fig. 9. Absorbance spectra of purified recombinant apo-cytochrome b_5 reconstituted with heme. *Dashed line*, oxidized form; *solid line*, sodium dithionite reduced form.

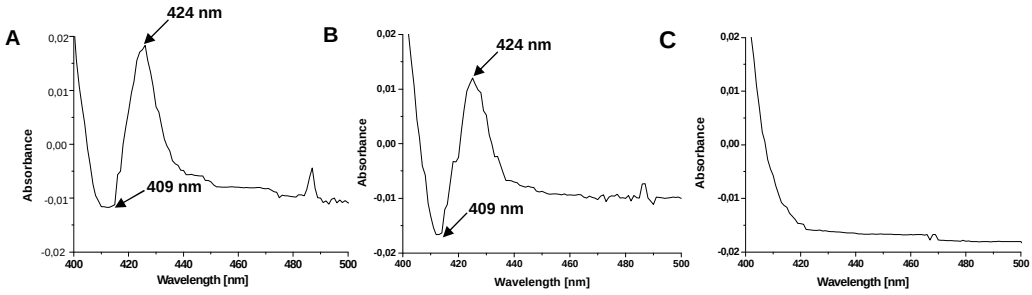

Fig. 10. Difference spectra (reduced minus oxidized) of (**A**) purified recombinant cytochrome b_5, (**B**) purified rabbit cytochrome b_5 and (**C**) hemin solution in reconstituted system with NADPH: CYP reductase.