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).

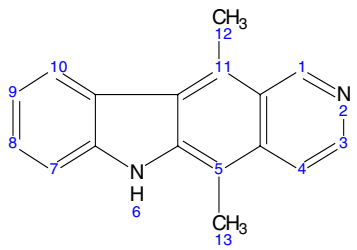

Figure 1. Structure of ellipticine.

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

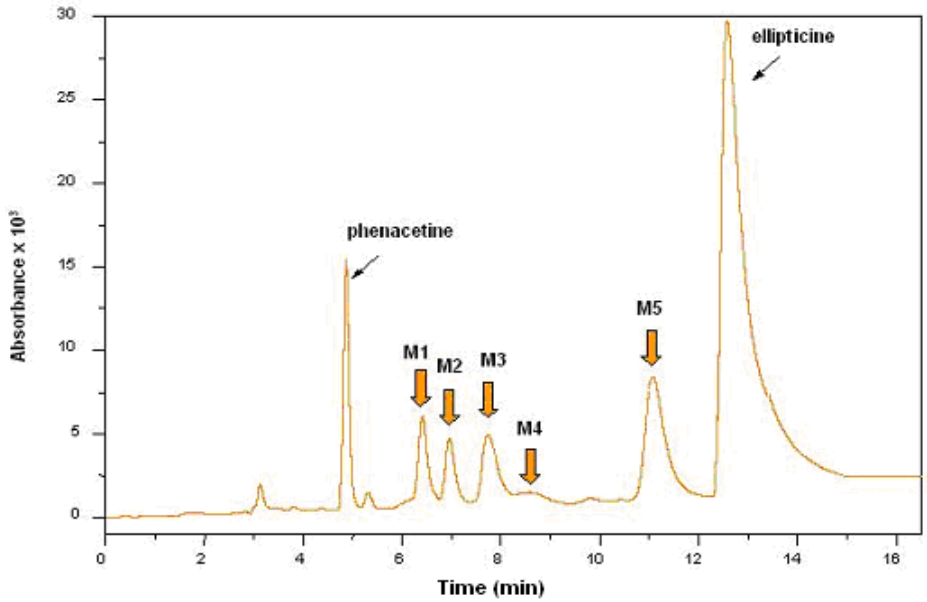

Figure 2. HPLC chromatogram of ellipticine metabolites formed by rabbit hepatic microsomes. Incubations (1 mmol·l^{-1} $NADP^+$, 10 mmol·l^{-1} D-glucose 6-phosphate, 1 U·ml^{-1} D-glucose 6-phosphate dehydrogenase, rabbit hepatic microsomes containing 0.1 nmol CYP, 10 μmol·l^{-1} ellipticine dissolved 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.

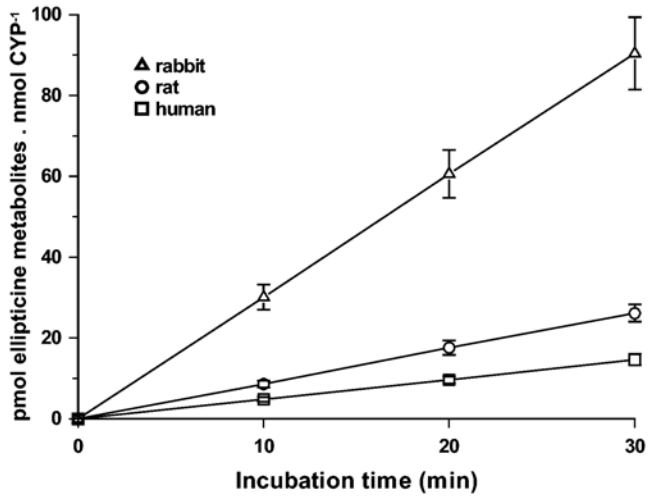

Figure 3. Time dependence of ellipticine metabolite formation by human, rat and rabbit hepatic microsomes. In all experiments, 10 μmol·l^{-1} ellipticine and microsomes containing 0.1 nmol CYP were used. Values of total ellipticine metabolite levels are averages and standard deviations of triplicate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

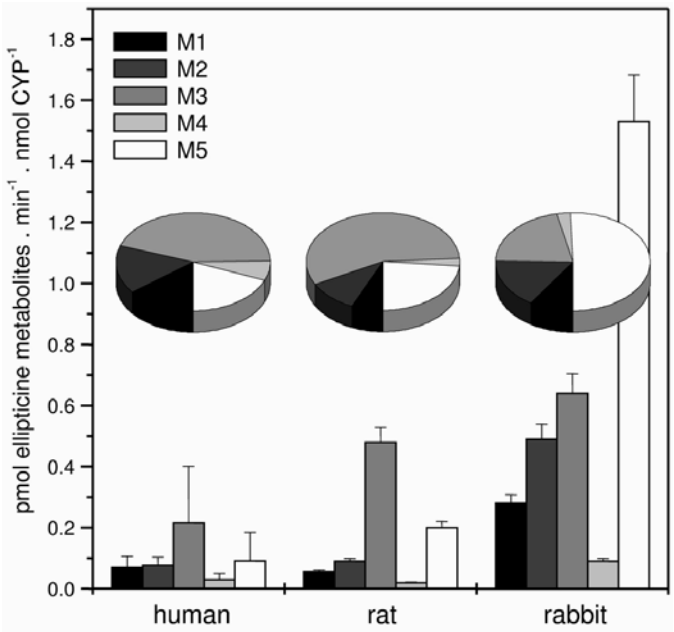

Figure 4. Oxidation of ellipticine by human, rat and rabbit hepatic microsomes. In all experiments, 10 μmol·l^{-1} ellipticine and microsomes containing 0.1 nmol CYP were used. Values of ellipticine metabolites are averages and standard deviations of triplicate incubations. Inset: percentage levels of 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).

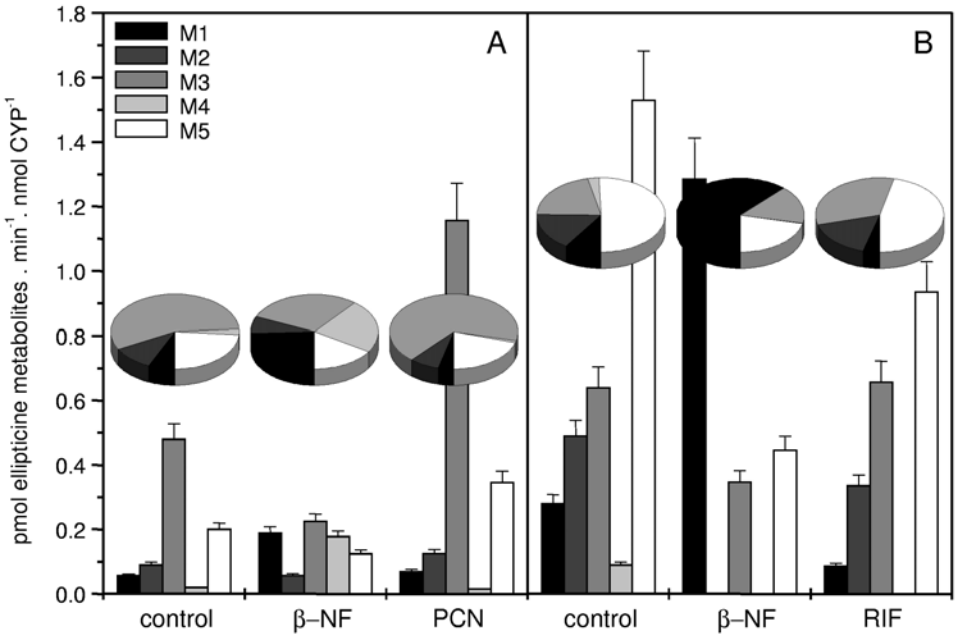

Figure 5. Ellipticine metabolism in rat (A) and rabbit (B) hepatic microsomes of control animals and those treated with inducers of CYP1A and CYP3A enzymes. Microsomes containing 0.1 nmol CYP and 10 μmmol·l^{-1} ellipticine were used in all experiments. Values of ellipticine metabolites are averages 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.

Acknowledgements. Supported by the Grant Agency of the Czech Republic (grant No. 203/06/0329) and Ministry of Education of the Czech Republic (grant MSM 0021620808).

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Final version accepted: August 1, 2006