ORIGINAL ARTICLE

Role of cytochromes P450 and peroxidases in metabolism of the anticancer drug ellipticine: additional evidence of their contribution to ellipticine activation in rat liver, lung and kidney

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Abstract

OBJECTIVE: Ellipticine is a potent antineoplastic agent exhibiting multiple mechanisms of action. This anticancer agent should be considered a pro-drug, whose pharmacological efficiency and/or genotoxic side effects are dependent on its cytochrome P450 (CYP)- and/or peroxidase-mediated activation to species forming covalent DNA adducts. The target of this study was to investigate a role of CYP and peroxidase enzymes in ellipticine oxidative activation in rats, a suitable model mimicking the fate of ellipticine in humans, in details. The contribution of pulmonary and renal CYP- and peroxidase enzymes to ellipticine metabolic activation is investigated and compared with that found in the liver. **METHODS:** Ellipticine oxidation and DNA adduct formation *in vitro* were investigated using microsomes isolated from liver, lung and kidney of rats, either control (untreated) or treated i.p. with a single dose of 40 mg of ellipticine per kg of body weight. HPLC with UV detection was employed for the separation and characterization of ellipticine metabolites. Inhibitors of CYPs and cyclooxygenase (prostaglandin H synthase, COX) were used to characterize the enzymes participating in ellipticine oxidative activation in rat liver, lung and kidney. Ellipticine-derived DNA adducts were detected by ^{32}P-postlabeling. **RESULTS:** Using α-naphthoflavone, furafylline and ketoconazole, inhibitors of CYP1A, 1A2 and 3A, respectively, we found that the CYP1A and 3A enzymes play a major role in ellipticine activation to species forming DNA adducts in liver microsomes. Because of lower expression of these enzymes in lungs and kidneys, even after their induction by ellipticine, they play a minor role in ellipticine activation in these extrahepatic tissues. Arachidonic acid, a cofactor of COX, increased ellipticine activation in the microsomes of extrahepatic tissues. In addition, indomethacin, an inhibitor of COX, efficiently inhibited formation of ellipticine-derived DNA adduct in these microsomes. Based on these results, we attribute the higher activation of ellipticine in lung and kidney microsomes to COX than to CYP enzymes. **CONCLUSION:** The results demonstrate that whereas CYP enzymes of 1A and 3A subfamilies are the major enzymes activating ellipticine in rat livers, peroxidase COX plays a significant role in this process in lungs and kidneys.

Abbreviations:

α-NF	- α-naphthoflavone
COX	- cyclooxygenase
CYP	- cytochrome P450
HPLC	- high performance liquid chromatography
LPO	- lactoperoxidase
MPO	- myeloperoxidase
S.E.M.	- standard error medium
NADPH	- nicotinamidadeninedinucleotide phosphate (reduced)
RAL	- relative adduct labeling
TLC	- thin-layer chromatography

INTRODUCTION

Ellipticine (5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole, Figure 1), an alkaloid isolated from *Apocyanaceae* plants, exhibit significant antitumor and anti-HIV activities (for a summary see Stiborova *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 Stiborova *et al.* 2001).

Ellipticine has been reported to arrest cell cycle progression by regulating the expression of cyclin B1 and Cdc2 as well as phosphorylation of Cdc2 (Kuo *et al.* 2005a,b), to induce apoptotic cell death by the generation of cytotoxic free radicals, the activation of Fas/Fas ligand system, the regulation of Bcl-2 family proteins (Kuo *et al.* 2005a,b; 2006), an increase of wild-type p53, the rescue of mutant p53 activity and the initiation of the mitochondrial apoptosis pathway (Garbett & Graves 2004; Kuo *et al.* 2005a,b; 2006). Ellipticine also activates the p53 pathway in glioblastoma cells; its impact on these cancer cells depends on the p53 status. In a U87MG glioblastoma cell line expressing p53wt, ellipticine provoked an early G0/G1 cell cycle arrest, whereas in a U373 cell line expressing p53mt it caused arrest in S and G2/M phase (Martinkova *et al.* 2010).

Ellipticine and 9-hydroxyellipticine also cause selective inhibition of p53 protein phosphorylation in several human cancer cell lines (Ohashi *et al.* 1995; Sugikawa *et al.* 1999), and this correlates with their cytotoxic activity. However, the precise molecular mechanism responsible for these effects has not been explained yet. Chemotherapy-induced cell cycle arrest was shown to result from DNA damage caused by a variety of chemotherapeutics. In the case of ellipticine, it was suggested that the prevalent DNA-mediated mechanisms of its antitumor, mutagenic and cytotoxic activities are (i) intercalation into DNA, and (ii) inhibition of DNA topoisomerase II activity (Auclair 1987; Garbett & Graves 2004; Stiborova *et al.* 2006c; 2011).

We have demonstrated that ellipticine also covalently binds to DNA after being enzymatically activated with cytochromes P450 (CYP) or peroxidases (Stiborova *et al.* 2001; 2003a,b; 2004; 2006a; 2007a,b; Poljakova *et al.* 2006), suggesting a third possible mechanism of action. Two major DNA adducts generated from 13-hydroxy- and 12-hydroxyellipticine during the ellipticine CYP- and peroxidase-mediated metabolism are formed *in vitro* and *in vivo* in rats and mice treated with this anticancer drug (Stiborova *et al.* 2001; 2003a,b; 2004; 2006a; 2007a,b; 2008, Frei *et al.* 2002; Poljakova *et al.* 2006). The same DNA adducts were also detected in cancer cells in culture, such as human breast adenocarcinoma MCF-7 cells (Borek-Dohalska *et al.* 2004), leukaemia HL-60 and CCRF-CEM cells (Poljakova *et al.* 2007), neuroblastoma cells (Poljakova *et al.* 2009) and glioblastoma cells (Martinkova *et al.* 2009) *in vitro*, and in rat breast adenocarcinoma *in vivo* (Stiborova *et al.* 2011). Toxic effects of ellipticine to these cancer cells correlate with levels of ellipticine-derived DNA adducts and are dependent on expression of CYP1A1, 1B1, 3A4 and peroxidases LPO, COX and MPO in these cells (Borek-Dohalska *et al.* 2004; Poljakova *et al.* 2007; 2009; Martinkova *et al.* 2009). 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.

All these results indicate that 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 were found to be 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) (Stiborova *et al.* 2001; 2003a,b; 2004; 2006a; 2008; Moserova *et al.* 2008). Of the mammalian peroxidases, human cyclooxygenase (COX)-2, ovine COX-1, bovine lactoperoxidase (LPO) and human myeloperoxidase (MPO) efficiently generated ellipticine-derived DNA adducts (Figure 1) (Poljakova *et al.* 2006; Stiborova *et al.* 2007a). However, the actual impacts of these enzymes *in-vivo* depend on several additional factors (Stiborova *et al.* 2008). One of them might be the presence of various patterns of individual CYP and peroxidase enzymes and/or even the presence of other proteins influencing their activities in target and non-target tissues. The CYP and peroxidase enzyme patterns depend also on a known phenomenon that ellipticine is a strong inducer of CYP1A enzymes in several tissues including cancer cells, which catalyze its own metabolism (Gasiewicz *et al.* 1996; Aimova *et al.* 2007; Martinkova *et al.* 2009). This feature might finally dictate the pharmacological efficiencies of ellipticine.

After i.p. administration of ellipticine to rats and mice, the highest levels of DNA adducts were found

in liver, followed by kidney and lung (Stiborova *et al.* 2003a; 2007b; 2008). Liver is a tissue rich in CYP enzymes, while kidney and lung contain high levels of peroxidases such as COX (Eling *et al.* 1990; 1992; Stiborova *et al.* 1991; 2005a; Culp *et al.* 1997). Knowledge about contributions of these enzymes to ellipticine activation in individual rat tissues are, however, scarce. Although previous results indicate that CYP3A1 and 1A participate in formation of DNA adducts by ellipticine in rats *in vivo* (Stiborova *et al.* 2003a), the impact of these CYPs or that of peroxidases in ellipticine activation in individual organs is not known.

In this study, we have used the Wistar rat model, known to be suitable to mimic the fate of ellipticine in humans (Stiborova *et al.* 2003; 2006a), to examine actual contributions of CYPs and peroxidases to DNA adduct formation by ellipticine in liver, lung and kidney of these rats.

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, USA); 9-hydroxyellipticine (5,11-dimethyl-9-hydroxy-6*H*-pyrido[4,3-*b*]carbazole) 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 N^2-oxide of ellipticine were synthesized as described (Wijsmuller *et al.* 1986; Boogaard *et al.* 1994) by J. Kucka (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 (Stiborova *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, Czech Republic), which is in compliance with the Declaration of Helsinki. Male Wistar rats (~100 g) were treated with a single dose of 40 mg/kg body weight (n=3) of ellipticine by intraperitoneal injection. Ellipticine was dissolved in sunflower oil/DMSO (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 hours after treatment by cervical dislocation. Livers, lungs and kidneys were removed immediately after death and used for isolation of microsomal fractions.

Preparation of microsomes

Microsomes were isolated from the livers, kidneys and lungs of rats as described (Stiborova *et al.* 2003b). Protein concentrations in the microsomal fractions were assessed using the bicinchoninic acid protein assay with bovine serum albumin as a standard (Wichelman *et al.* 1988). The concentration of CYP was estimated according to Omura and Sato (1964) based on the absorption of the complex of reduced CYP with carbon monoxide. Specific content of CYP in hepatic, pulmonary and renal microsomes of control and ellipticine-treated rats (40 mg/kg) is shown in our former work (Aimova *et al.* 2007). Hepatic, renal and pulmonary microsomal preparations from rats that had been pre-treated with ellipticine were analyzed for the presence of ellipticine or its metabolites by HPLC as described (Stiborova *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.

Microsomal incubations

Incubation mixtures used to asses DNA adducts formed by ellipticine consisted of 50 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, pooled microsomal samples from liver, kidney and lung (0.5 mg protein) from 3 male rats, either control or treated with 40 mg/kg body weight of ellipticine, 100 µM ellipticine (dissolved in 7.5 µl methanol) and 0.5 mg of calf thymus DNA in a final volume of 750 µl. The reaction was initiated by adding ellipticine. Incubations were also carried out in the presence of COX cofactors, arachidonic acid and/or hydrogen peroxide (Eling *et al.* 1992; Stiborova *et al.* 2005a). Mixtures then contained 0.1 mM arachidonic acid and/or 0.1 mM hydrogen peroxide as cofactors instead of NADPH, and additionally 5 mM magnesium chloride. Incubations were carried out at 37 °C for 30 minutes; ellipticine-DNA adduct formation was found to be linear up to 30 min of incubation (Stiborova *et al.* 2001). Control incubations were carried out (*i*) without microsomes, (*ii*) without NADPH or arachidonic acid, (*iii*) without DNA and (*iv*) without ellipticine.

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 protein of pooled hepatic microsomal fraction from 3 male rats, either untreated or treated with 40 mg/kg body weight ellipticine 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.* 2004; 2006a). After incubation, 5 µl of 1 mM phenacetine in methanol was added as an internal standard and the ellipticine metabo-

lites were extracted twice with ethyl acetate (2 × 1 ml). Analyzes of ellipticine metabolites were performed by HPLC as described (Stiborova *et al.* 2004). Recoveries of ellipticine metabolites were around 95%.

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 & DiCarlo 1997; Stiborova *et al.* 2005a,b), furafylline, which inhibits CYP1A2, ketoconazole, which inhibits CYP3A (Rendic & DiCarlo 1997; Ueng *et al.* 1997; Stiborova *et al.* 2005b) and indomethacin, which inhibits COX (Eling *et al.* 1992; Stiborova *et al.* 2005a). 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 prior to 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* (Stiborova *et al.* 2001, 2003c, 2004, 2007a, b) and *in vivo* (Stiborova *et al.* 2003a, 2007b, 2008), was employed in the experiments. The TLC and HPLC analyzes were done as reported recently (Stiborova *et al.* 2001, 2003a, c, 2004, 2007a, b).

RESULTS AND DISCUSSION

Hepatic, pulmonary and renal microsomes of rats are capable of activating ellipticine

To evaluate the role of the rat hepatic, pulmonary and renal CYPs and peroxidases in the activation of ellipticine, we performed *in vitro* experiments employing microsomes isolated from livers, lungs and kidneys of either control (untreated) rats or animals treated with 40 mg of ellipticine per kg of body weight.

The DNA adduct pattern generated by ellipticine activated with microsomes and NADPH, a cofactor of the NADPH:CYP reductase-dependent CYP enzyme system, consisted of one major and three minor adducts (see spots 1, 2, 6 and 7 in Figure 2), which were identical to those formed *in vivo* in rats treated with ellipticine (Figure 2C). Adducts spots 1 and 2 are products of 13-hydroxyellipticine (Figure 2D) and 12-hydroxyellipticine (Figure 2E) metabolites, respectively, generated by CYP and peroxidase (see scheme in Figure 1). Chromatographic analysis of spots 1 and 2 on

Tab. 1. DNA adduct formation by ellipticine activated with rat hepatic (A), pulmonary (B) and renal (C) microsomes

A	RAL[a] (mean/10^7 nucleotides)				
	Spot 1	Spot 2	Spot 6	Spot 7	Total
Untreated rats					
hepatic microsomes without cofactor	n.d.	0.08±0.01	n.d.	n.d.	0.08±0.01
hepatic microsomes + NADPH	6.84±1.31	0.26±0.03	0.29±0.02	0.28±0.03	17.6±1.39
hepatic microsomes + NADPH + a-NF	6.19±0.45	0.13±0.02	0.10±0.01	0.05±0.01	6.47±0.48
hepatic microsomes + NADPH + furafylline	7.91±0.62	0.12±0.02	0.19±0.02	0.08±0.01	8.30±0.69
hepatic microsomes + NADPH + ketoconazole	1.00±0.1	0.15±0.01	0.08±0.01	0.02±0.01	1.25±0.11
hepatic microsomes + arachidonic acid	1.01±0.1	0.015±0.01	0.017±0.01	0.017±0.01	1.06±0.12
hepatic microsomes + arachidonic acid + IM	0.22±0.02	n.d.	n.d.	n.d.	0.22±0.02
hepatic microsomes + H_2O_2	1.10 ±0.1	0.02±0.01	0.02±0.01	0.02±0.01	1.16±0.12
Rats treated with ellipticine (40 mg/kg b.w.)					
hepatic microsomes without cofactor	n.d.	0.11±0.01	n.d.	n.d.	0.11±0.01
hepatic microsomes + NADPH	33.03±2.52	0.42±0.03	0.40±0.03	0.48±0.03	34.4±2.87
hepatic microsomes + NADPH + a-NF	2.50±0.18	0.03±0.01	0.03±0.01	0.04±0.01	2.60±0.19
hepatic microsomes + NADPH + furafylline	6.28±0.51	0.08±0.01	0.07±0.01	0.07±0.01	6.50±0.59
hepatic microsomes + NADPH + ketoconazole	6.50±0.58	0.20±0.02	0.40±0.03	0.40±0.03	7.50±0.61
hepatic microsomes + arachidonic acid	0.87±0.06	0.08±0.01	0.10±0.01	0.10±0.01	1.15±0.10
hepatic microsomes + arachidonic acid + IM	0.17±0.01	0.02±0.01	0.02±0.01	0.02±0.01	0.23±0.02
hepatic microsomes + H_2O_2	0.92±0.07	0.08±0.01	0.12±0.01	0.10±0.01	1.23±0.10

B	RAL[a] (mean/10^7 nucleotides)				
	Spot 1	Spot 2	Spot 6	Spot 7	Total
Untreated rats					
lung microsomes without cofactor	0.03±0.01	0.03±0.01	0.03±0.01	0.02±0.01	0.11±0.01
lung microsomes + NADPH	0.64±0.04	0.15±0.01	0.12±0.01	0.10±0.01	1.01±0.10
lung microsomes + NADPH + a-NF	0.33±0.02	n.d.	n.d.	n.d.	0.33±0.03
lung microsomes + NADPH + ketoconazole	0.52±0.041	0.10±0.01	0.09±0.01	0.08±0.01	0.79±0.08
lung microsomes + arachidonic acid	0.03±001	0.03±0.01	0.30±0.03	0.14±0.01	0.50±0.05
lung microsomes + arachidonic acid + indomethacin	n.d.	n.d.	n.d.	n.d.	n.d.
Rats treated with ellipticine (40 mg/kg b.w.)					
lung microsomes without cofactor	0.60±0.04	0.03±0.01	0.20±0.02	0.10±0.01	0.93±0.11
lung microsomes + NADPH	1.92±0.17	0.30±0.03	0.22±0.02	0.20±0.02	2.64±0.19
lung microsomes + NADPH + a-NF	0.34±0.03	0.06±0.01	0.16±0.01	0.16±0.01	0.72±0.06
lung microsomes + NADPH + ketoconazole	1.05±0.10	0.25±0.02	0.20±0.02	0.20±0.02	1.70±0.15
lung microsomes + arachidonic acid	1.30±0.10	0.23±0.02	0.28±0.02	0.23±0.02	2.04±0.21
lung microsomes + arachidonic acid + indomethacin	0.60±0.05	0.03±0.01	0.10±0.01	0.05±0.01	1.15±0.10

C	RAL[a] (mean/10^7 nucleotides)				
	Spot 1	Spot 2	Spot 6	Spot 7	Total
Untreated rats					
kidney microsomes without cofactor	n.d.	0.004±0.001	0.01±0.01	0.01±0.01	0.02±0.01
kidney microsomes + NADPH	0.21±0.02	0.03±0.01	0.08±0.01	0.03±0.01	0.35±0.04
kidney microsomes + NADPH + a-NF	0.04±0.01	n.d.	n.d.	n.d.	0.04±0.01
kidney microsomes + NADPH + ketoconazole	0.10±0.01	0.02±0.01	0.08±0.01	0.05±0.01	0.23±0.03
kidney microsomes + arachidonic acid	n.d.	n.d.	0.02±0.01	0.02±0.01	0.04±0.01
kidney microsomes + arachidonic acid + indomethacin	n.d.	n.d.	n.d.	n.d.	n.d..
Rats treated with ellipticine (40 mg/kg b.w.)					
kidney microsomes without cofactor	0.12±0.01	0.03±0.01	0.05±0.01	0.03±0.01	0.33±0.04
kidney microsomes + NADPH	0.42±0.03	0.12±0.01	0.08±0.01	0.05±0.01	0.67±0.07
kidney microsomes + NADPH + a-NF	0.23±0.02	0.09±0.01	0.05±0.01	0.03±0.01	0.40±0.04
kidney microsomes + NADPH + ketoconazole	0.40±0.03	0.10±0.01	0.08±0.01	0.05±0.01	0.63±0.08
kidney microsomes + arachidonic acid	0.20±0.02	0.06±0.01	0.08±0.01	0.06±0.01	0.40±0.05
kidney microsomes + arachidonic acid + indomethacin	0.10±0.01	0.04±0.01	0.04±0.01	0.02±0.01	0.20±0.03

Experimental conditions are described in Materials and methods. Mean RAL ± standard deviations shown in the figure represent DNA adducts of three parallel *in vitro* incubations. ND, not detectable (the detection limit of RAL was 1 adducts/10^{10} nucleotides).

HPLC confirmed that these adducts are derived from 13-hydroxy- and 12-hydroxyellipticine, respectively, by their coelution with prepared reference compounds (data not shown). Additional minor DNA adducts formed by ellipticine are adducts 6 and 7 (Figure 2). The low levels of these adducts prevented HPLC co-chromatographic analysis or their further characterization. Control incubations without enzyme cofactors, but in the presence of hepatic microsomes, were free of adduct spots 1, 6 and 7, but adduct spot 2 was always detected (Table 1). However, in control incubations containing renal and pulmonary microsomes without enzyme cofactors, ellipticine-derived DNA adducts 1, 2, 6 and 7 were found (Table 1B,C). This finding indicates that other enzymes than only CYPs dependent on NADPH:CYP reductase might activate ellipticine in lung and kidney. This is consistent with results found in our former study with the HRN (Hepatic Cyto-

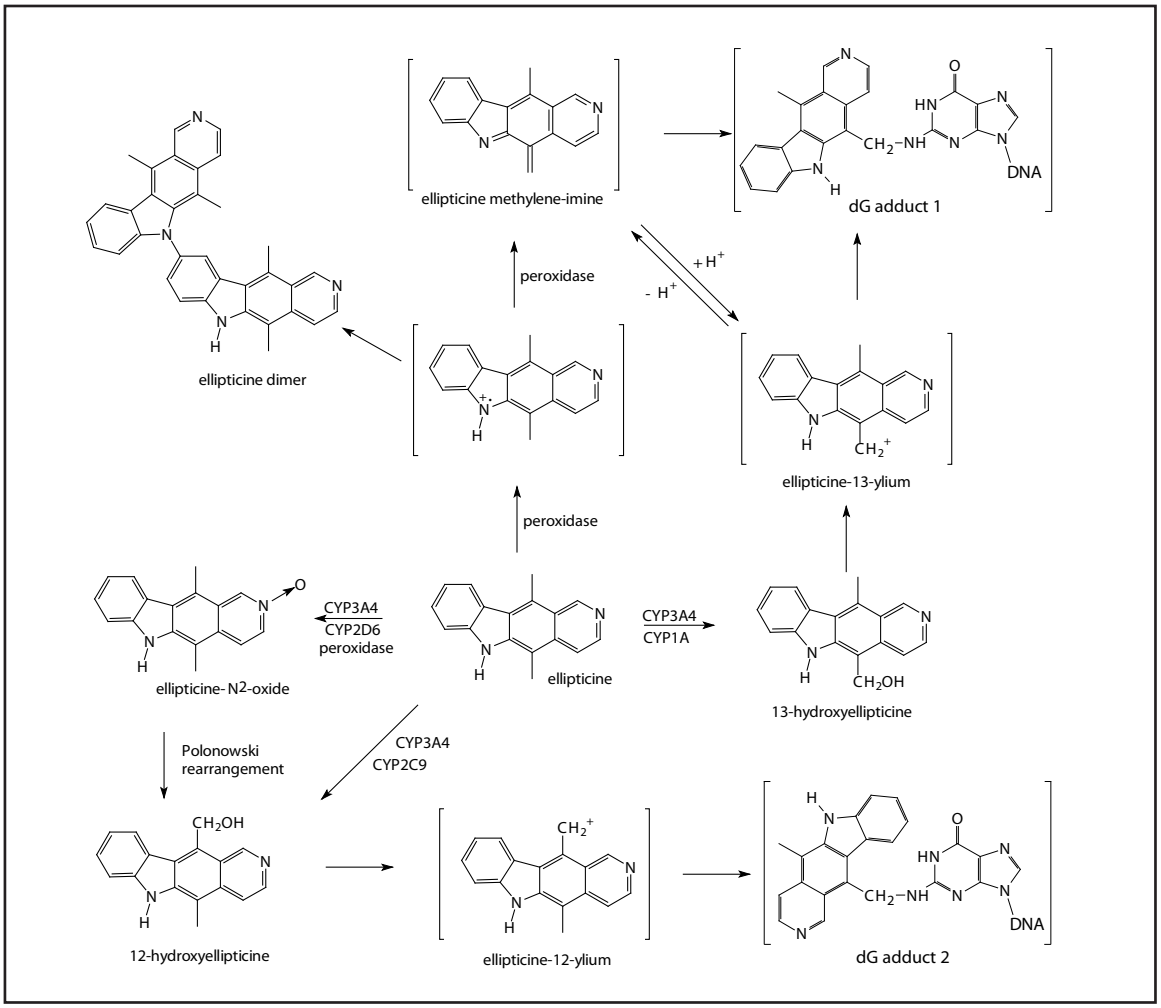

Fig. 1. Metabolism of ellipticine by peroxidases and human CYPs showing the characterized metabolites and those proposed to form DNA adducts. The compounds shown in brackets were not detected under the experimental conditions and are the electrophilic metabolites postulated as ultimate arylating species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

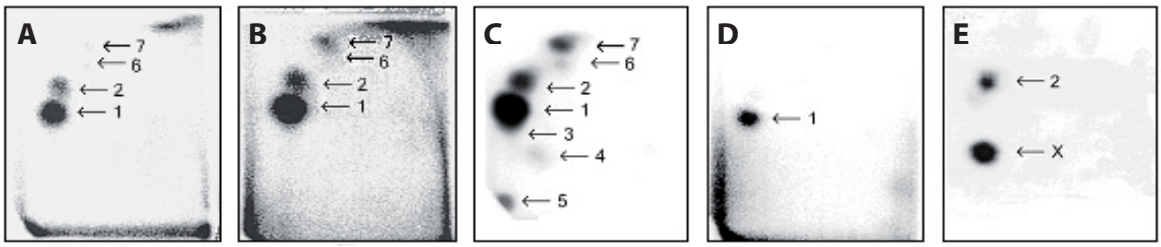

Fig. 2. Autoradiographic profile of DNA adducts generated in calf thymus DNA by ellipticine after its activation with hepatic microsomes of untreated (A) and ellipticine-treated (40 mg/kg) male rats (B), of ^{32}P-labeled digests of DNA from liver of male rats treated with the same dose of ellipticine (C), from calf thymus DNA reacted with 13-hydroxyellipticine (D) or 12-hydroxyelipticine (E). Analyses were performed by the nuclease P1 version of the ^{32}P-postlabeling assay.

chrome P450 Reductase Null) mouse model, in which NADPH:CYP reductase is deleted specifically in hepatocytes, resulting in the loss of essentially all hepatic CYP function (Stiborova *et al.* 2008), which indicate that ellipticine activation should, at least partially, be catalyzed also by enzymes, whose activities are not dependent on NADPH:CYP reductase (Stiborova *et al.* 2008). Besides peroxidases that were found to activate ellipticine (Stiborova *et al.* 2007a), the CYP2S1 enzyme, which is abundantly expressed in several tissues (Saarikoski *et al.* 2005; Downie *et al.* 2005; Bui *et al.* 2009a) might be such an enzyme. Namely, it was shown that it catalyzes the oxidation of compounds having polycyclic aromatic structures similar to ellipticine without participation of NADPH:CYP reductase (Bui *et al.* 2009a,b). While a role of peroxidases is investigated in this study, the participation of CYP2S1 in ellipticine activation still awaits further examination. Therefore, the efficiency of

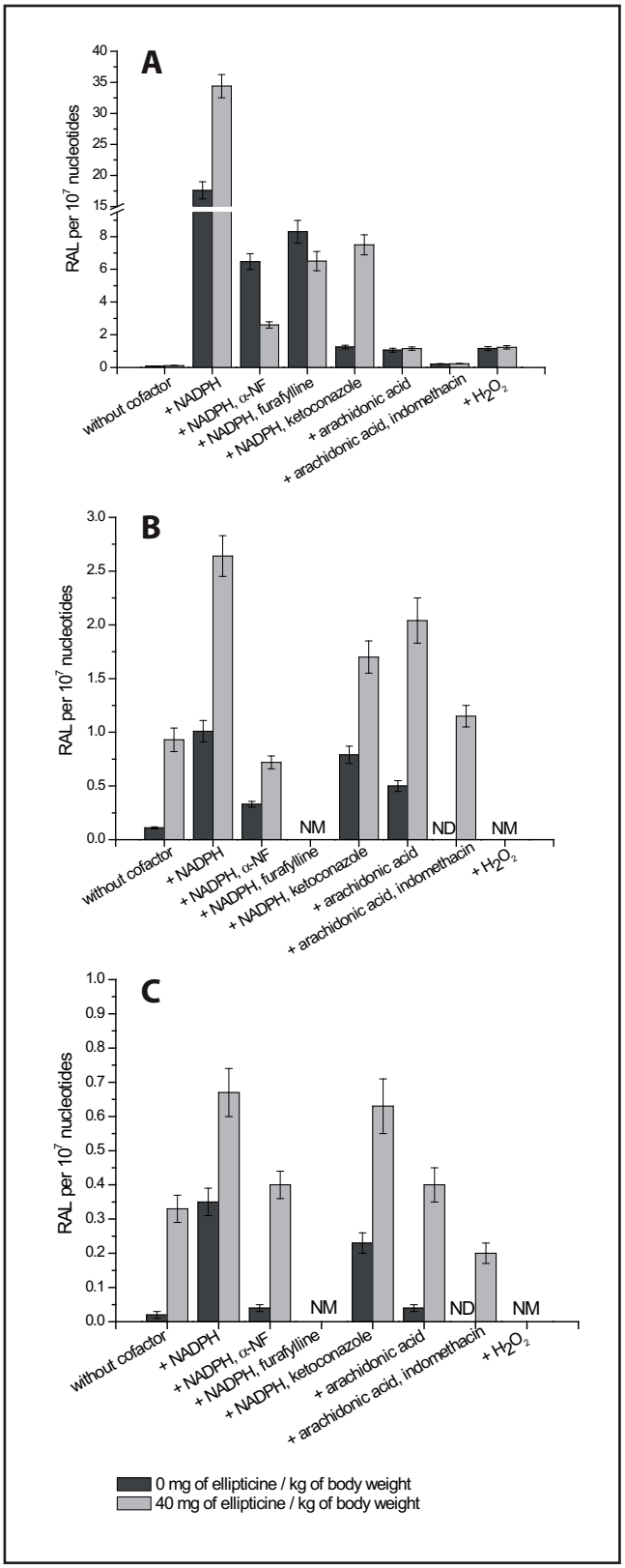

Fig. 3. DNA adduct formation by ellipticine activated with microsomes isolated from livers (**A**), lungs (**B**) and kidneys (**C**) of rats, control (uninduced) or pretreated with 40 mg/kg ellipticine and the effect of inhibitors of CYP1A, 3A and COX on ellipticine-DNA adduct formation in these microsomes. Mean RAL ± standard deviations shown in the figure represent total levels of DNA adducts of three parallel *in vitro* incubations. ND, not detectable (the detection limit of RAL was 1 adducts/10^{10} nucleotides). NM, not measured.

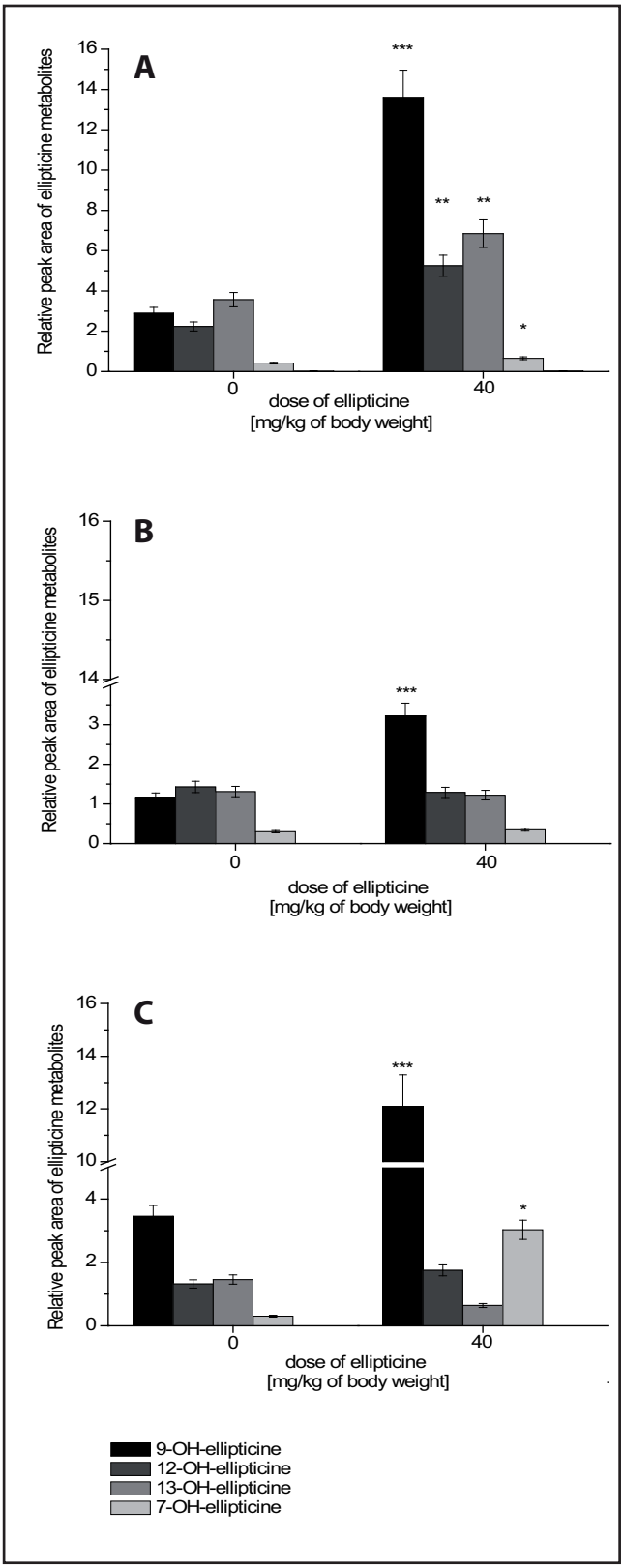

Fig 4. Ellipticine metabolism in rat liver (**A**), lung (**B**) and kidney microsomes (**C**) of control animals and those treated with 40 mg/kg ellipticine. Microsomes containing 0.2 mg microsomal protein, and 10 μM ellipticine were used in all experiments. Levels of ellipticine metabolites are averages ± standard deviations of triplicate incubations. Values significantly different from control: *p<0.05, **p<0.01, ***p<0.001 (Student's t-test).

CYP2S1 to oxidize ellipticine is planned to be investigated in our future work.

In order to evaluate the contribution of CYP1A, 3A and peroxidases, found previously to activate ellipticine *in vitro* (Stiborova *et al.* 2001, 2003b, 2004, 2008), to ellipticine activation in liver, lung and kidney, we investigated the modulation of ellipticine-DNA adduct formation by cofactors and selective inhibitors of these enzymes. Among microsomes of control (untreated) rats and those of rats pre-treated with ellipticine tested in this study, hepatic microsomes in the presence of NADPH were the most effective in activation of ellipticine. Pulmonary and renal microsomes also activated ellipticine to species forming DNA adducts, but they were more than 17- and 50-fold less efficient than microsomes isolated from livers (Figure 3, Table 1).

In the presence of NADPH, hepatic, pulmonary and renal microsomes isolated from rats treated with ellipticine were up to 2.6-fold more effective to form ellipticine-derived DNA adducts than microsomes of control (untreated) rats (Figure 3, Table 1).

In hepatic, pulmonary and renal microsomes of control (untreated) rats, ketoconazole, a selective inhibitor of CYP3A enzymes (Rendic & DiCarlo 1997; Ueng *et al.* 1997; Stiborova *et al.* 2005b), inhibited formation of ellipticine-DNA adducts mediated by the NADPH-dependent enzymatic system, by 93, 22 and 34 %, respectively (Figure 3, Table 1). However, in microsomes of rats treated with ellipticine, the impact of this inhibitor to decrease ellipticine activation to species forming DNA adducts was lower in liver and kidney. In this case, ketoconazole inhibited formation of ellipticine-derived DNA adducts by 78 and 6 % in liver and kidney, respectively (Table 1). Such findings indicate that treating rats with ellipticine shifts the CYP enzyme expression in livers and kidney to another pattern where CYP3A enzymes have lower impact on activation of this drug. Indeed, CYP1A enzymes are induced by ellipticine on mRNA and protein levels in all these tissues (Aimova *et al.* 2007), thereby increasing their own participation in ellipticine metabolism (see below).

α-Naphthoflavone (α-NF), an inhibitor of CYP1A1 and 1A2 (Rendic & DiCarlo 1997), decreased the levels of ellipticine-DNA adducts generated by hepatic, pulmonary and renal microsomes of both control and ellipticine-treated rats, but also to a different extent (Figure 3, Table 1). In hepatic and pulmonary microsomes of rats treated with ellipticine, α-NF was much more efficient to inhibit ellipticine-DNA adduct formation than in those of control rats (Table 1). Microsomes of rat kidneys were, however, the exception; α-NF was efficient inhibitor of DNA adduct formation by ellipticine also in control rats (Figure 3, Table 1). Furafylline, an inhibitor of CYP1A2 (Rendic & DiCarlo 1997), was also efficient compound decreasing activation of ellipticine by hepatic microsomes (Figure 3, Table 1). This inhibitor was, however, not tested using pulmonary and renal microsomes, because of low expression of CYP1A2 in these organs. The results found using the CYP1A inhibitors point to CYP1A enzymes as having a role in ellipticine-DNA adduct formation in rat livers, lungs and kidneys. Depending on their expression levels, they activate ellipticine to species binding to DNA. In addition, all these results indicate that in organisms treated with ellipticine, these CYP enzymes might play, because of CYP1A induction, the predominant role in the ellipticine anticancer activity based on covalent modification of DNA in target tissues.

Arachidonic acid and/or hydrogen peroxide, cofactors for peroxidase (COX)-dependent oxidation (Eling *et al.* 1990; 1992; Stiborova *et al.* 2004; 2005a; Arlt *et al.* 2006), mediated formation of DNA adducts by ellipticine in hepatic, pulmonary and renal microsomes, being increased by treating rats with ellipticine, mainly in lung (Table 1). Since COX cofactors were much less effective than NADPH in livers (Figure 3, Table 1), contribution of this enzyme to ellipticine activation in this organ is lower than that of CYPs. On the contrary, the results showing the effect of arachidonic acid and a COX inhibitor, indomethacin, on ellipticine-derived DNA adduct formation in microsomes of extrahepatic tissues indicate that COX is capable of effective ellipticine activation in these organs. It even more efficiently contributes to activation of ellipticine in these tissues of rats treated with ellipticine than in those of control (untreated) rats. Levels of adducts formed by microsomes of lung and kidney of rats treated with ellipticine in the presence of arachidonic acid were high; they represented 77 and 59 % of adduct levels formed by lung and kidnay microsomes in the presence of NADPH, respectively (Table 1). Therefore, this finding suggests a relatively high contribution COX to ellipticine activation in lung and kidney of organisms treated with this anticancer drug.

Ellipticine as a CYP inducer increases efficiencies of rat hepatic, pulmonary and renal microsomes to oxidize ellipticine to its hydroxylated metabolites

Ellipticine is oxidized by hepatic, pulmonary and renal microsomes to four metabolites, 7-hydroxy-, 9-hydroxy-, 12-hydroxy and 13-hydroxyellipticine (Figure 4). Ellipticine N^2-oxide was also produced, but this metabolite was not quantitated because of its spontaneous rearrangement to 12-hydroxyellipticine.

The increase in levels of ellipticine-DNA adducts (Figure 3, Table 1) correlates with an increase in ellipticine oxidation by hepatic microsomes isolated from rats treated with ellipticine (Figure 4). 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 (Stiborova *et al.* 2004; Kotrbova *et al.* 2006), an up to 2-fold increase in formation of 13-hydroxy- and 12-hydroxyellipticine, the metabolites generating DNA adducts 1 and 2, was found in

hepatic microsomes (Figure 4). This finding can be caused by a strong induction not only of CYP1A1/2 (Aimova *et al.* 2007), but also of CYP3A in livers of rats treated with ellipticine. Indeed, induction of CYP3A4 by ellipticine has recently been found in glioblastoma U87MG cells (Martinkova *et al.* 2009). In addition, ellipticine might also influence expression of another protein, cytochrome b_5 (Stiborova *et al.* unpublished results), which is a component of the CYP microsomal system modulating the enzymatic activities of several CYPs, including CYP3A4 (for a summary see Schenkman *et al.* 2003) and CYP1A1 (Stiborova *et al.* 2006b). Hence, its expression might also cause higher formation of 13-hydroxy- and 12-hydroxyellipticine, thereby increasing the formation of ellipticine-DNA adducts.

In contrast to liver microsomes, no increase in formation of 13-hydroxy- and 12-hydroxyellipticine was found during oxidation of ellipticine catalyzed by lung and kidney nicrosomes of rats treated with ellipticine (Figure 4). Only an increase in oxidation of ellipticine to 9-hydroxyellipticine and/or 7-hydroxyellipticine was found in lung and kidney (Figure 4). Because a more than 4- and 10-fold increase in arachidonic acid (COX)-mediated activation of ellipticine was found in lung and kidney microsomes, respectively, as compared to activation of ellipticine by liver microsomes (Table 1), the peroxidase activity of COX might be responsible for increased formation of ellipticine metabolites generating DNA adducts in these extrahepatic tissues. The question whether ellipticine might also act as an inducer of COX enzymes such as an inducible COX-2 enzyme (Soslow *et al.* 2000; Matsuo *et al.* 2001; Shono *et al.* 2001), remains to be answered.

CONCLUSIONS

The results found in this study shed more light on our previous data, showing the importance of CYP and peroxidase enzymes in ellipticine activation *in vivo* (Stiborova *et al.* 2003c; 2007a,b; 2008). The importance of CYP1A and 3A enzymes in ellipticine-derived DNA adduct formation *in vitro* and *in vivo* has already been demonstrated previously (Stiborova *et al.* 2001; 2003a; 2004; 2007a,b; 2008). Here, we show that these enzymes play a major role in ellipticine activation in the liver. However, because of their lower expression in extrahepatic organs such as lung and kidney, even after their induction by ellipticine (Aimova *et al.* 2007), they play a minor role in ellipticine activation in these extrahepatic tissues. Arachidonic acid (COX)-mediated ellipticine activation has the higher impact in this process in rat lung and kidney, predominantly after treatment of rats with ellipticine. The results also show that enzymatic activity of COX to catalyze ellipticine activation was increased in extrahepatic organs by treating rats with ellipticine. One of the COX enzymes, COX-2, was demonstrated in multiple cancer types (i.e. carcinomas and brain tumors) known to be the targets for ellipticine treatment and is even inducible by carcinogenic processes and/or by several compounds, including anticancer drugs (Soslow *et al.* 2000; Matsuo *et al.* 2001; Shono *et al.* 2001). Because of efficiency of COX-2 to mediate formation of ellipticine-derived DNA adducts *in vitro* (Stiborova *et al.* 2007a) and the results found in this work (Table 1), its participation in ellipticine activation in cells of the extrahepatic tissues, including cancer cells should be taken into account. The study of the effect of ellipticine on COX-2 expression in healthy and cancer tissues of animal models and/or cancer cells in culture is therefore under way in our laboratory.

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