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Cytochrome b_5 shifts oxidation of the anticancer drug ellipticine by cytochromes P450 1A1 and 1A2 from its detoxication to activation, thereby modulating its pharmacological efficacy

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ABSTRACT

Ellipticine is a pro-drug, whose activation is dependent on its oxidation by cytochromes P450 (CYP) and peroxidases. Cytochrome b_5 alters the ratio of ellipticine metabolites formed by isolated reconstituted CYP1A1 and 1A2, favoring formation of 12-hydroxy- and 13-hydroxyellipticine metabolites implicated in ellipticine–DNA adduct formation, at the expense of 9-hydroxy- and 7-hydroxyellipticine that are detoxication products. Cytochrome b_5 enhances the production of 12-hydroxy and 13-hydroxyellipticine. The change in metabolite ratio results in an increased formation of covalent ellipticine–DNA adducts, one of the DNA-damaging mechanisms of ellipticine antitumor action. This finding explains previous apparent discrepancies found with isolated enzymes and *in vivo*, where CYP1A enzymatic activation correlated with ellipticine–DNA-adduct levels while isolated CYP1A1 or 1A2 in reconstituted systems were much less effective than CYP3A4. The effect of cytochrome b_5 might be even more pronounced *in vivo*, since, as we show here, ellipticine increases levels of cytochrome b_5 in rat liver. Our results demonstrate that both the native 3D structure of cytochrome b_5 and the presence of the heme as an electron transfer agent in this protein enable a shift in ellipticine metabolites formed by CYP1A1/2.

1. Introduction

Ellipticine (5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole, Fig. 1), an alkaloid isolated from Apocynaceae plants, exhibits significant antitumor and anti-HIV activities (for a summary see [1–3])]. The main reasons for the interest in ellipticine and its derivatives for clinical purposes are their high efficiencies against several types of cancer, their limited toxic side effects, and their lack of haematological toxicity [4]. Nevertheless, ellipticine is a potent mutagen. Many 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 overview (for overview, see [1–3]).

The precise molecular mechanism responsible for anticancer effects of ellipticine has not yet been explained. It was suggested that the major mechanisms of its antitumor, mutagenic and cytotoxic activities are (i) intercalation into DNA [4,5], and (ii) inhibition of DNA topoisomerase II activity [2–7].

We have demonstrated that ellipticine also covalently binds to DNA after being enzymatically activated by cytochromes P450 (CYP) or peroxidases [1–3,8–13], suggesting a third DNA-damaging mechanism of action. Two major DNA adducts generated from 13-hydroxy- and 12-hydroxyellipticine (Fig. 1) during the CYP- and peroxidase-mediated ellipticine metabolism are formed *in vitro* and *in vivo* in DNA of healthy organs of rats and mice treated with this anticancer drug [1,3,8–13]. Human CYP3A4 and rat CYP3A1 are the major enzymes oxidizing ellipticine to the reactive metabolites binding to DNA (13-hydroxy- and 12-hydroxyellipticine) *in vitro* [3,9,10]. The same DNA adducts were also detected in human cancer cells in culture, such as breast adenocarcinoma MCF-7 [14], the leukaemias HL-60 and CCRF-CEM [15], neuroblastoma [16] and glioblastoma cells [17], and in rat mammary adenocarcinoma *in vivo* [3]. Toxic effects of ellipticine in these cancer cells correlated with levels of ellipticine-derived DNA adducts and were dependent on expression of CYP1A1, 1B1, 3A4 and lactoperoxidase (LPO), cyclooxygenase (COX) and myeloperoxidase (MPO) in these cells

Abbreviations: COX, cyclooxygenase; CYP, cytochrome P450; HRN, hepatic cytochrome P450 reductase null; GAPDH, glyceraldehyde phosphate dehydrogenase; HPLC, high-performance liquid chromatography; i.p., intra-peritoneal; LPO, lactoperoxidase; MPO, myeloperoxidase; PEI-cellulose, polyethylenimine-cellulose; RAL, relative adduct labeling; r.t., retention time; TLC, thin layer chromatography.

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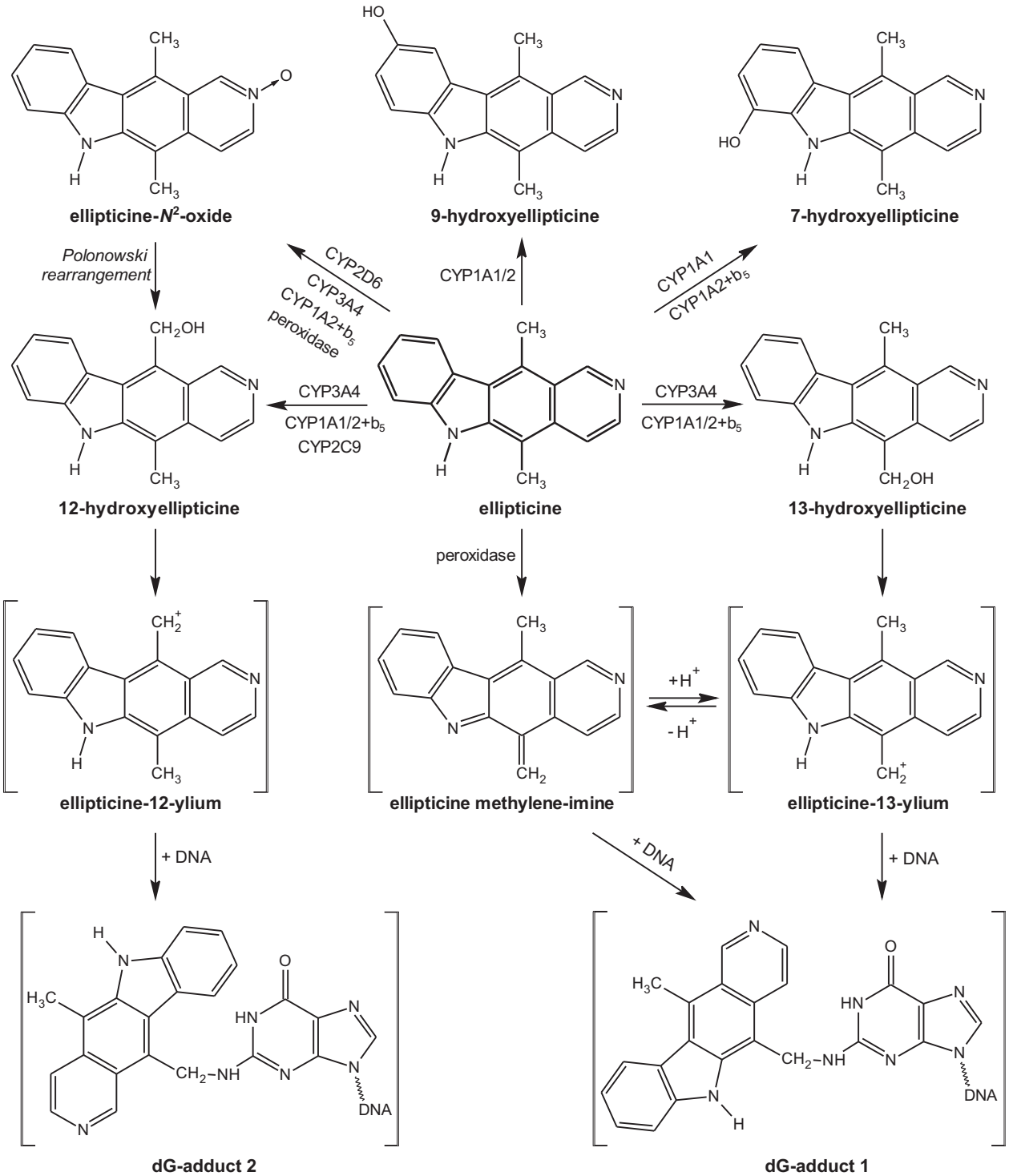

Fig. 1. Scheme of ellipticine metabolism by CYPs and peroxidases showing the identified metabolites and those proposed to form DNA adducts. The compounds shown in brackets were not detected under the experimental conditions and/or not structurally characterized. The CYP enzymes predominantly oxidizing ellipticine shown in the figure were identified in this work and/or in our previous studies [9,10,18].

[14–17]. Based on 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.

Concerning formation of ellipticine-derived DNA adducts in healthy organs, the risk of treating patients with this compound might be considered. Nevertheless, our *in vivo* studies using the rat experimental model mimicking the fate of ellipticine in human [8,12] demonstrated that ellipticine–DNA adducts did not persist in healthy tissues of rats treated with ellipticine [12]. Therefore, these results suggest a relatively low risk of the genotoxic side effects of ellipticine during the cancer treatment in human.

Studies correlating levels of individual ellipticine–DNA adducts to CYP enzyme expression determined in organs of rats treated with ellipticine showed good correlations of major adduct levels with CYP1A1/2 and CYP3A1 levels [8]. This finding is in contrast to

the metabolite patterns and DNA adduct levels seen with pure CYP enzymes of rat and human origin reconstituted with NADPH:CYP reductase [3,10,18]. Here CYP1A isoforms did not activate ellipticine, but 9-hydroxy- and 7-hydroxyellipticine, the detoxication products (Fig. 1), were the major metabolites, and as a consequence ellipticine–DNA adduct levels were very low.

In isolated microsomes, in cells and in intact organs cytochrome b_5 might influence CYP-catalyzed reactions [19]. The lack of cytochrome b_5 in our reconstituted systems could therefore be a reason for the low activating capacity of isolated CYP1A1/2. We therefore investigated the role of cytochrome b_5 on CYP catalyzed oxidation of ellipticine. A reconstituted system consisting of pure CYP1A1 or 1A2 enzymes with NADPH:CYP reductase in liposomes with or without cytochrome b_5 was used.

Many studies have investigated whether cytochrome b_5 can change the rates of CYP-mediated oxidation of substrates. Depending on the individual CYP involved, the experimental conditions and the substrate utilized, cytochrome b_5 has been shown to stimulate, inhibit or have no effect on CYP mediated reactions (for a summary, see [19–22]). This work is, to our knowledge, the first study showing that cytochrome b_5 can alter not only the amounts but also the types of metabolites produced from a single substrate by one CYP.

Mechanistic studies of the action of cytochrome b_5 on the catalysis of CYP1A1/2 were also performed using the holoprotein of cytochrome b_5, its apo-form (devoid of heme) or apo-cytochrome b_5 reconstituted with manganese protoporphyrin IX (Mn-cytochrome b_5).

2. Materials and methods

2.1. Chemicals

$NADP^+$, NADPH, ellipticine, D-glucose 6-phosphate, D-glucose 6-phosphate dehydrogenase, β-naphthoflavone (β-NF), dilauroyl phosphatidylcholine, dioleyl phosphatidylcholine, and dilauroyl-phosphatidylserine, lysozyme, hemin, cytochrome c, horse heart myoglobin, human serum albumin and calf thymus DNA were obtained from Sigma Chemical Co. (St. Louis, MO, USA); Sudan I from BDH (Poole, UK); 7-ethoxyresorufin and 7-methoxyresorufin from Fluka Chemie AG (Buchs, Switzerland); superoxide dismutase from Roche Diagnostics (Mannheim, Germany); 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 [10] by 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 [10].

2.2. 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), in compliance with the Declaration of Helsinki. Male Wistar rats (~100 g) were treated with a single dose of 4 or 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 h after treatment by cervical dislocation. Livers were removed immediately after death and immediately used for preparation of microsomal fractions.

2.3. Preparation of microsomes

Microsomes were isolated from fresh livers of rats as described [9]. Protein concentrations in the microsomal fractions were assessed using the bicinchoninic acid protein assay with bovine serum albumin as a standard [23]. Hepatic 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 [10]. Neither ellipticine nor any of its metabolites were detectable in microsomal fractions from tissues of rats that had been pretreated with ellipticine. Microsomes from the livers of three male rabbits pretreated with 80 mg/kg day of β-NF i.p. for four consecutive days were isolated as described [9].

2.4. Isolation of CYPs, NADPH:CYP reductase, cytochrome b_5 and apo-cytochrome b_5

Recombinant rat CYP1A1 protein was purified to homogeneity from membranes of *E. coli* transfected with a modified *CYP1A1* cDNA [24], in the laboratory of H.W. Strobel (University of Texas, Medical School of Houston, Texas, USA) by P. Hodek (Charles University, Prague, Czech Republic). CYP1A2 was isolated from liver microsomes of rabbits induced with β-NF by procedures described by Haugen and Coon [25]. Rabbit liver NADPH:CYP reductase was purified as described [26]. Cytochrome b_5 was isolated from rabbit liver microsomes by the procedure described by Roos [27]. The apo-cytochrome b_5 protein was prepared using heterologous expression in *E. coli* as described in our earlier work [28]. Briefly, the gene for rabbit cytochrome b_5 was constructed from synthetic oligonucleotides using polymerase chain reaction (PCR), cloned into pUC19 plasmid and amplified in DH5α cells. The gene sequence was verified by DNA sequencing. The sequence coding cytochrome b_5 was cleaved from pUC19 by NdeI and XhoI restriction endonucleases and subcloned into the expression vector pET22b. This vector was used to transform *E. coli* BL-21 (DE3) Gold cells by heat shock. Expression of cytochrome b_5 was induced with isopropyl β-D-1-thiogalactopyranoside (0.05 mM for 4 h). The cytochrome b_5 protein, produced predominantly in its apo-form, was purified to homogeneity from isolated membranes of *E. coli* cells by chromatography on a DEAE-Sepharose column [28].

2.5. Incorporation of heme into apo-cytochrome b_5

The preparation of hemin chloride solution and its incorporation into apo-cytochrome b_5 were performed by the procedure described elsewhere [29]. Solutions of heme were prepared by adding 2.6 mg of hemin chloride to a solution of 50% ethanol in water (4 ml) to give a final concentration of 1 mM. A small increment (10 μl) of 1 M NaOH was added and mixed to dissolve the hemin. The solution was then allowed to stand for several minutes so particulates could settle. A 10-μl aliquot was removed and diluted into 990 μl of 20 mM Tris, 1 mM EDTA, pH 8.0, at 25 °C and the absorbance of the Tris-ligated heme was measured at 385 nm. The process was repeated five-times, always by adding 10 μl of 1 M NaOH, until NaOH addition caused no further increase in absorbance at 385 nm. The hemin solution was further filtered through 0.2 μm Membrane MF Millipore filter (Millipore, Billerica, USA). Purified apo-cytochrome b_5 was diluted with 20 mM Tris, pH 8.0, containing 1 mM EDTA and 0.4% sodium cholate, to a protein concentration of 0.25 mg/ml. Aliquots of hemin chloride were added into the apo-cytochrome b_5 sample (1 ml) and the reconstitution of apo-cytochrome b_5 with heme was monitored

by absorbance spectroscopy. Absorbance spectra (from 350 to 500 nm) were recorded on Hewlett Packard 8453 UV spectrophotometer. The reconstitution was considered to be complete when the Soret peak of cytochrome b_5 shifted from 413 to 409 nm and an increase in absorbance at 385 nm, caused by excess of free Tris-ligated hemin, was observed in the spectrum.

Analogous procedures were utilized to incorporate manganese protoporphyrin IX (Frontier Scientific, USA) into apo-cytochrome b_5 (Mn-cytochrome b_5) [30].

2.6. Determination of reconstituted cytochrome b_5 content

The concentration of apo-cytochrome b_5 reconstituted with heme was determined spectrophotometrically (the absolute absorbance spectrum) using the molar extinction coefficient ε_{413} = 117 mM^{-1} cm^{-1} [31,32] or from the difference spectrum of reduced minus oxidized form, using molar extinction coefficient $\varepsilon_{424-409}$ = 185 mM^{-1} cm^{-1}, respectively [32]. The concentration of Mn-cytochrome b_5 was determined using an extinction coefficient of 57 mM^{-1} cm^{-1} at 469 nm [33].

2.7. Determination of cytochrome b_5 protein levels in rat liver microsomes

Immunoquantitation of rat liver microsomal cytochrome b_5 was done by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis [24,34]. Samples containing 75 μg microsomal proteins were subjected to electrophoresis on SDS/15% polyacrylamide gels. After migration, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes. Cytochrome b_5 protein was probed with a rabbit polyclonal anti-cytochrome b_5 antibody (1:750, AbCam, MA, USA) overnight at 4 °C. Antibody against glyceraldehyde phosphate dehydrogenase (GAPDH) (1:750, Millipore, MA, USA) was used as loading control. The antigen–antibody complex was visualized with an alkaline phosphatase-conjugated goat anti-rabbit IgG antibody and 5-bromo-4-chloro-3-indolylphosphate/nitrobluetetrazolium as chromogenic substrate [24,34] and scanned with a computerized image-analyzing system (ElfoMan 2.0, Ing. Semecký, Prague, Czech Republic).

2.8. Incubations

Unless stated otherwise, 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 D-glucose 6-phosphate dehydrogenase, to generate NADPH, CYP1A1 or CYP1A2 (100 pmol) reconstituted with NADPH:CYP reductase (5–150 pmol) without or with rabbit hepatic cytochrome b_5 (0–1000 pmol) and 20 μM ellipticine dissolved in 5 μl methanol. The enzyme reconstitution was performed as described [1,9,18,24,35], using different molar ratios of CYP1A1/2 to NADPH:CYP reductase and cytochrome b_5 (see Section 3). Briefly, recombinant rat CYP1A1 and rabbit CYP1A2 were reconstituted with rabbit NADPH:CYP reductase as follows (2 μM CYPs, 0.1–3 μM NADPH:CYP reductase, 0.5 μg/μl CHAPS, 0.1 μg/μl liposomes [dilauroyl phosphatidylcholine, dioleyl phosphatidylcholine, and dilauroylphosphatidylserine (1:1:1)], 3 mM reduced glutathione, and 50 mM HEPES/KOH, pH 7.4). An aliquot containing 100 pmol of reconstituted CYP enzyme was used to oxidize ellipticine. In the control incubation, either CYP or ellipticine were omitted. The reaction was initiated by adding ellipticine. After incubation at 37 °C for 20 min in open glass tubes (ellipticine oxidation was linear up to 30 min of incubation [18,36]), the reaction was stopped by adding 100 μl 2 M NaOH, then 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 a stream of nitrogen, dissolved in 50 μl of methanol and separated by HPLC (5 μm Ultrasphere ODS Beckman, 4.6 mm × 250 mm preceded by a C-18 guard column), the eluent was 64% methanol plus 36% of 5 mM heptane sulfonic acid in 32 mM acetic acid in water with a flow rate of 0.8 ml/min, detection was at 296 nm. Five ellipticine metabolites with the retention times of 6.3, 6.9, 7.8, 8.5 and 11.2 min, were separated [10,18]. Recoveries of ellipticine metabolites were approximately 95%. To characterize ellipticine metabolites, fractions containing the metabolites were collected from multiple HPLC runs, concentrated on a SpeedVac concentrator and analyzed by mass spectroscopy and/or NMR as described [10,37]. For kinetic studies 1–20 μM ellipticine was incubated with a complete reconstituted system containing CYP1A1 or CYP1A2, NADPH:CYP reductase and cytochrome b_5 (1:1:5), the metabolites analyzed as above and the kinetic parameters were determined (see Section 3).

Incubation mixtures used to analyze DNA-adduct formation by ellipticine were as described above, but contained 1 mM NADPH instead of the NADPH generating system plus 1 mg calf thymus DNA in a final volume of 750 μl. The reaction was initiated by adding ellipticine. Incubations were carried out at 37 °C for 30 min; ellipticine–DNA adduct formation was linear up to 30 min [1]. Control incubations were carried out either without (i) CYP or (ii) NADPH, or (iii) DNA, or (iv) ellipticine. After the incubation, DNA was isolated from the residual water phase by the phenol/chloroform extraction method as described [1].

2.9. CYP1A enzyme activity assays

The samples containing CYP1A1 or 1A2 reconstituted with NADPH:CYP reductase (see the procedure described above) were characterized for CYP1A1 activity using the oxidation of Sudan I for CYP1A1 [24,34] and 7-methoxyresorufin *O*-demethylation (MROD) for CYP1A2 [38,39].

2.10. ^{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* and *in vivo* [1,3,8–13], was employed in the experiments. The TLC and HPLC analyzes were done as reported recently [1,3,8–13].

2.11. Molecular modeling and visualization

Model of cytochrome b_5 holo-protein was based on coordinates of soluble domain of rat microsomal cytochrome b_5 obtained by three-dimensional NMR of ^{15}N labeled protein (PDB code 1BFX) [40]. The model of apo-cytochrome b_5 was based on coordinates of water-soluble domain of rat microsomal apo-cytochrome b_5 obtained by three-dimensional NMR of ^{13}C and ^{15}N labeled protein (PDB code 1I8C) [41]. The models were aligned and visualized in software package Discovery Studio Visualizer v2.5. Solvent accessible surface of both proteins was calculated with probe radius 1.4 Å and colored by mapping the interpolated partial atom charges to the surface.

3. Results

3.1. Cytochrome b_5 modulates ellipticine oxidation catalyzed by cytochromes P450 1A1 and 1A2

The CYP1A1 and CYP1A2 enzymes oxidize ellipticine to five metabolites, 7-hydroxy-, 9-hydroxy-, 12-hydroxy-, 13-hydroxyel-

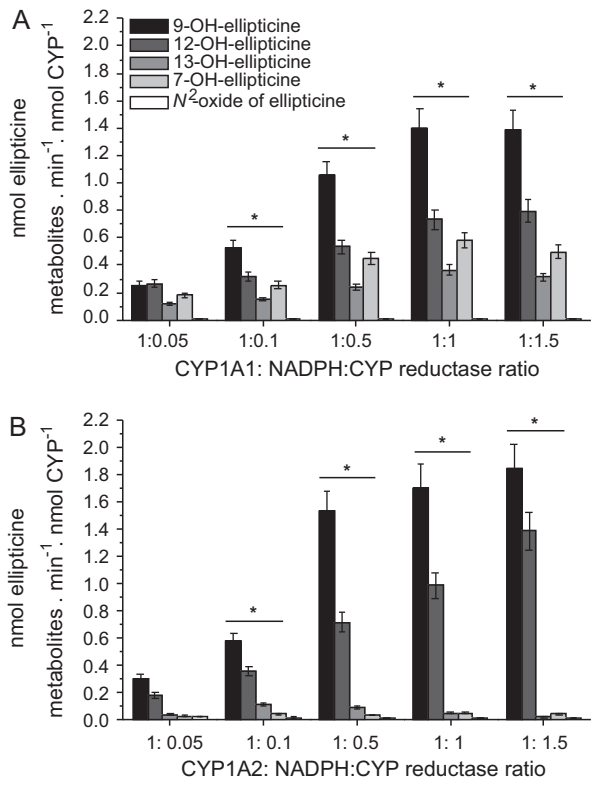

Fig. 2. The effect of increasing NADPH:CYP reductase amounts reconstituted with CYP in liposomes as described in the Materials and Methods section on rates of ellipticine oxidation (20 μM) by CYP1A1 (A) and CYP1A2 (B). Incubation time was 20 min at 37 °C, the metabolites were determined after ethyl acetate extraction of the incubations by HPLC as described. The values are averages and standard deviations of triplicate incubations. Values significantly different from incubations containing CYP1A1/2 and NADPH:CYP reductase in a ratio of 1:0.05: $^{*}p < 0.001$ (Student's *t*-test).

lipticine and N^2-oxide of ellipticine (Figs. 2 and 3), found previously to be formed by human, rat, rabbit and mouse hepatic microsomes [10,13,18,37]. Rates of ellipticine oxidation by isolated CYP1A1/2 reconstituted with NADPH:CYP reductase depend on molar ratios of CYP to NADPH:CYP reductase in the reconstituted systems. An increase in the NADPH:CYP reductase content resulted in an increased oxidation of ellipticine to all metabolites up to a molar ratio of CYP1A1/2:reductase of 1:1, with negligible changes in their yields at a ratio of 1:1.5 (Fig. 2). The yield in ellipticine metabolites was linear with increasing CYP1A1 or 1A2 concentrations up to 0.5 μM. We therefore used 0.2 μM CYP1A1/2 and reductase in further experiments.

With both CYP1A1 and 1A2, 9-hydroxyellipticine, a detoxication product of ellipticine [10,18], was the predominant metabolite (Figs. 2 and 3 and supplemental Tables 1 and 2). The other metabolites, 12-hydroxy-, 13-hydroxyellipticine and ellipticine N^2-oxide, which are responsible for formation of the major ellipticine-derived DNA adducts [10,11], and a further detoxication product, 7-hydroxyellipticine, were formed to a lower extent (Figs. 2 and 3). The levels of ellipticine N^2-oxide were slightly decreased with increasing ratios of CYP1A2:reductase, probably because of its spontaneous rearrangement to 12-hydroxyellipticine [10,11,37]. Indeed, the levels of 12-hydroxyellipticine increased accordingly (Fig. 2, supplemental Table 2).

The patterns and amounts of ellipticine metabolites generated by CYP1A1 and 1A2 changed significantly when cytochrome b_5 was present in the reconstituted system of either CYP1A1 or 1A2 with NADPH:CYP reductase under the optimal molar ratio of one. Changes in pattern of ellipticine metabolites were dependent on the molar ratios of CYP and cytochrome b_5. If CYP1A1 was used, the detoxication products of ellipticine (7-hydroxy- and 9-hydroxyellipticine) decreased with more added cytochrome b_5, while ellipticine N^2-oxide, 12-hydroxy- and 13-hydroxyellipticine increased considerably (Fig. 3A and supplemental Table 2). In the case of CYP1A2 again 12-hydroxyellipticine became the most prominent metabolite with increasing cytochrome b_5 amounts, while 9-hydroxyellipticine levels decreased. Here 7-hydroxyellipticine increased concomitant with 12-hydroxy-, 13-hydroxyellipticine and the ellipticine N^2-oxide (Fig. 3C and supplemental Table 2).

If the amount of NADPH:CYP reductase in the incubation was reduced to one tenth, the effect of cytochrome b_5 added into the reconstituted system containing CYP1A1 or 1A2 was even more dramatic, with yields of 12-hydroxyellipticine increasing 5–7-fold (Fig. 3B and D and supplemental Table 3).

Table 1
Effect of cytochrome b_5 on kinetic parameters of ellipticine oxidation by CYP1A1 and 1A2.

Ellipticine metabolites	K_m ($K_{0.5}$)[a] (μM)	V_{max} (μM/min^{-1})	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} μM^{-1})	*n*
CYP1A1 without b_5					
9-Hydroxyellipticine	0.49 ± 0.06	0.264 ± 0.004	1.32 ± 0.02	2.7 ± 0.3	1
12-Hydroxyellipticine	0.8 ± 0.1	0.102 ± 0.002	0.51 ± 0.01	0.6 ± 0.1	1
13-Hydroxyellipticine	5 ± 2	0.057 ± 0.009	0.29 ± 0.05	0.05 ± 0.03	1
7-Hydroxyellipticine	0.50 ± 0.02	0.0649 ± 0.0004	0.325 ± 0.002	0.64 ± 0.03	1
CYP1A1 with b_5					
9-Hydroxyellipticine	0.5 ± 0.1	0.100 ± 0.003	0.50 ± 0.01	1.0 ± 0.2	1
12-Hydroxyellipticine	15 ± 1[a]	3.0 ± 0.4	15 ± 2	1.0 ± 0.1	4.5 ± 0.7[a]
13-Hydroxyellipticine	13.2 ± 0.4[a]	1.8 ± 0.1	9.0 ± 0.5	0.68 ± 0.05	6.3 ± 0.9[a]
7-Hydroxyellipticine	0.4 ± 0.2	0.020 ± 0.001	0.099 ± 0.007	0.3 ± 0.2	1
CYP1A2 without b_5					
9-Hydroxyellipticine	0.24 ± 0.04	0.191 ± 0.003	0.93 ± 0.04	3.9 ± 0.7	1
12-Hydroxyellipticine	4 ± 1	0.087 ± 0.009	0.44 ± 0.02	0.12 ± 0.04	1
13-Hydroxyellipticine	8 ± 2	0.087 ± 0.009	0.43 ± 0.05	0.06 ± 0.02	1
7-Hydroxyellipticine	7 ± 1	0.0058 ± 0.0004	0.029 ± 0.002	0.0044 ± 0.0008	1
CYP1A2 with b_5					
9-Hydroxyellipticine	0.22 ± 0.09	0.115 ± 0.004	0.58 ± 0.02	3 ± 1	1
12-Hydroxyellipticine	13 ± 1	0.49 ± 0.03	2.5 ± 0.1	0.19 ± 0.02	1
13-Hydroxyellipticine	4.1 ± 0.6	0.168 ± 0.008	0.84 ± 0.04	0.21 ± 0.03	1
7-Hydroxyellipticine	4.3 ± 0.8	0.0067 ± 0.0002	0.032 ± 0.001	0.008 ± 0.002	1

Assays were performed as described under Section 2. Kinetic parameters were determined from initial velocity data as described for $N = 3$. Average values ± SE are shown. b_5, cytochrome b_5; *n*, Hill coefficient.
[a] Sigmoidal data curves were fitted using Hill equation.

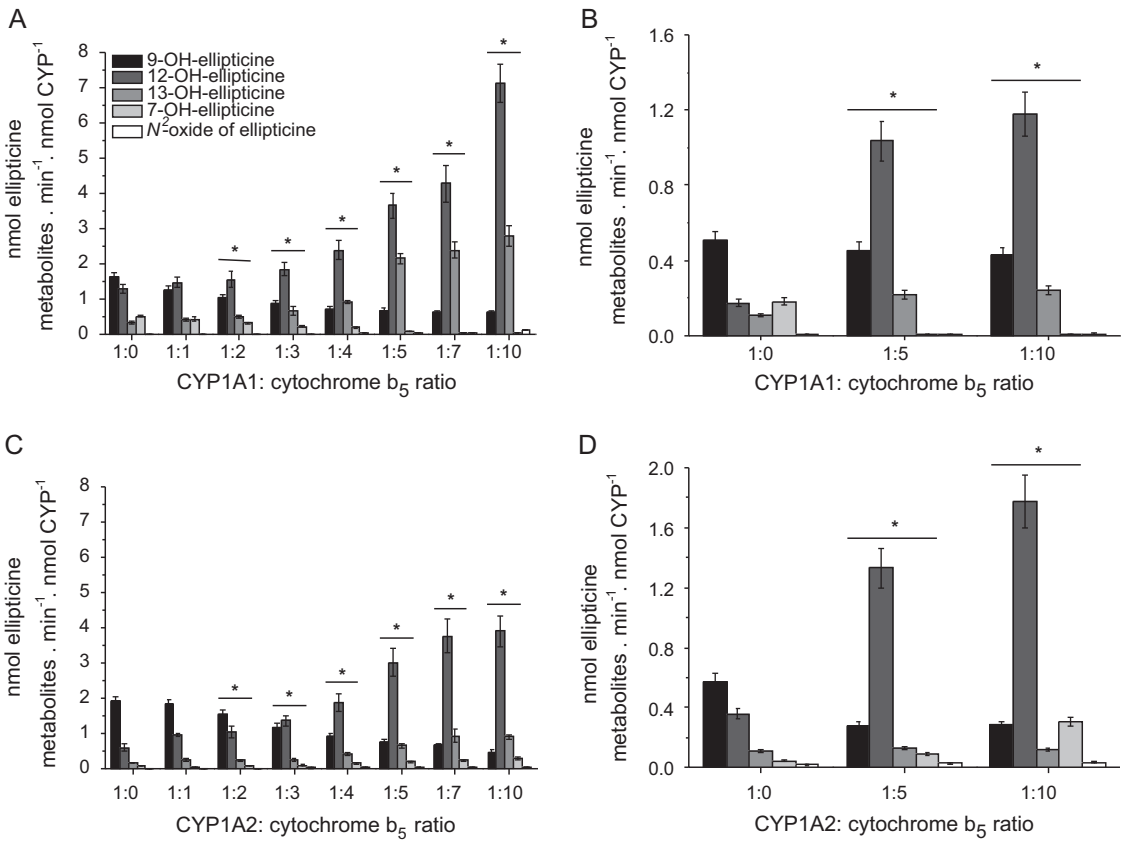

Fig. 3. Cytochrome b_5 alters patterns of ellipticine metabolites formed by its oxidation with CYP1A1 (A, B) and 1A2 (C, D) under the 1:1 (A, C) and the 1:0.1 molar ratio of CYP1A1/2:NADPH:CYP reductase (B, D). The molar ratio of CYP1A1/1A2 to cytochrome b_5 was 1:5. Conditions were as described in Fig. 2. The values are averages and standard deviations of triplicate incubations. Values significantly different from control (without cytochrome b_5); $^*p < 0.001$ (Student's *t*-test).

3.2. Cytochrome b_5 affects the kinetics of ellipticine oxidation by cytochromes P450 1A1/2

Further experiments were conducted to investigate the effects of cytochrome b_5 on kinetics of 1–20 μM ellipticine oxidation by CYP1A1 and 1A2 to its individual metabolites (Fig. 4, Table 1). The ratio of CYP to NADPH:CYP reductase used was 1:1 and the ratio of CYP to cytochrome b_5 was 1:5. Because of instability of ellipticine N^2-oxide, kinetics of its formation was not evaluated.

The presence of cytochrome b_5 increased the values of k_{cat} and the efficiencies (k_{cat}/K_m) of ellipticine oxidation to 12-hydroxy- and 13-hydroxyellipticine catalyzed by both CYP enzymes (Table 1). In addition, kinetics of ellipticine oxidation by CYP1A1, reconstituted with NADPH:CYP reductase and cytochrome b_5 changed completely from a Michaelis–Menten type saturation curve to a sigmoidal curve for 12-hydroxy- and 13-hydroxyellipticine. Indeed, Hill coefficients of 4.6 and 6.3 were determined for formation of 12-hydroxy- and 13-hydroxyellipticine, respectively (Fig. 4, Table 1). On the contrary, the kinetics of ellipticine oxidation by CYP1A2 in presence of cytochrome b_5 to these metabolites exhibited Michaelis–Menten kinetics (Fig. 4, Table 1).

3.3. Cytochrome b_5 increases the ellipticine-derived DNA adduct formation mediated by cytochromes P450 1A1 and 1A2

Using the nuclease P1 version of the ^{32}P-postlabeling assay, which was suitable to detect and quantify DNA adducts formed by ellipticine [1,8–12], two ellipticine-derived DNA adducts (see Fig. 1 for the structures of two deoxyguanosine adducts 1 and 2) were detected in the calf thymus DNA incubated with this drug and CYP1A1 or 1A2 reconstituted with NADPH:CYP reductase (Fig. 5A and B). The two adducts are identical to those found previously after *in vitro* incubation of calf thymus DNA with ellipticine, human, rat, rabbit and mouse hepatic microsomes or human and rat CYPs [1,9,10], or peroxidases [11] or after treatment of cells in culture with this anticancer drug [14–17,42] or *in vivo* (Fig. 5C), in several organs of rats including mammary adenocarcinoma [3,8,12] and mice [13] exposed to this agent. These adducts are generated from ellipticine-13-ylium and ellipticine-12-ylium (Fig. 1), the reactive species formed from the corresponding hydroxyellipticines (Fig. 5D and E) as confirmed by co-chromatographic analysis using TLC and HPLC [10,11,43]. The increased levels of 12-hydroxy- and 13-hydroxyellipticine formed by the reconstituted system containing cytochrome b_5 resulted in corresponding higher levels of these two ellipticine-derived DNA adducts (Table 2). In the case of CYP1A1, the presence of cytochrome b_5 resulted in 6.2-fold higher levels of the ellipticine–DNA adduct 1, which correlated with a 6.3-fold increased formation of 13-hydroxyellipticine, the metabolite generating this adduct (Table 2, Fig. 3). Likewise, CYP1A2 activation of ellipticine in presence of cytochrome b_5 resulted in 3.9-fold higher levels of DNA adduct 1 (Table 2) which was consistent with a similar increase (more than 3.8-fold) in 13-hydroxyellipticine metabolite formation by this system (Fig. 3). The same was true for the parallel effects of cytochrome b_5 on ellipticine–DNA-adduct 2 levels and 12-hydroxyellipticine yields in incubations with reconstituted CYP1A1 or 1A2 (Table 2, Fig. 3 and supplemental Table 2).

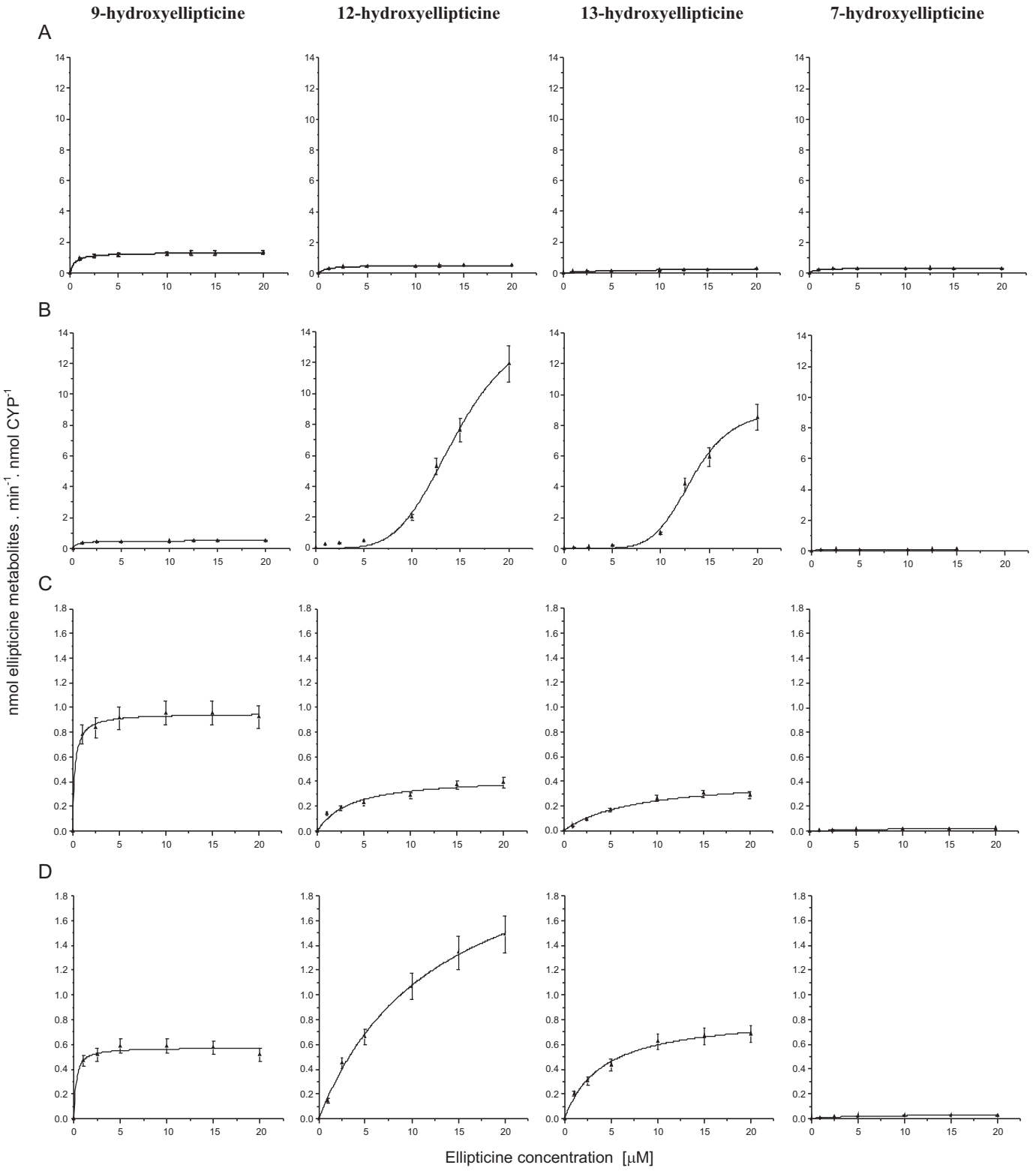

Fig. 4. Kinetics of ellipticine oxidation by CYP1A1 (A, B) and 1A2 (C, D) without (A, C) or with (B, D) cytochrome b_5. Ellipticine concentrations between 1 and 20 μM were incubated with the reconstituted liposomes containing CYP and reductase at a molar ratio of 1:1 in all incubations plus cytochrome b_5 at 5-fold higher concentrations than CYP in (B) and (D). The other conditions were as described in Fig. 2. The values are averages and standard deviations of triplicate incubations.

3.4. Modulation of ellipticine oxidation by cytochromes P450 1A1 and 1A2 is dependent on holo-cytochrome b_5

In order to investigate the mechanism of cytochrome b_5 mediated-modulation of ellipticine oxidation by CYP1A1 and 1A2, we also examined the influence of apo-cytochrome b_5 and Mn-cytochrome b_5. As shown in Fig. 6, the CYP1A1/2-mediated oxidation of ellipticine was significantly changed only by holo-cytochrome b_5 or apo-cytochrome b_5 reconstituted with heme, while apo-cytochrome b_5 without heme cofactor (see Fig. 7B and D) was essentially without such effects (Fig. 6). We also employed a structurally similar analogue of cytochrome b_5, known to have

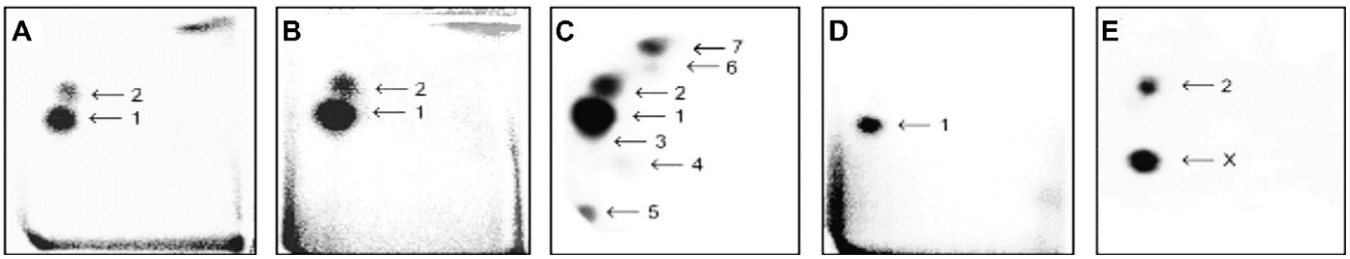

Fig. 5. Autoradiographic profile of ^{32}P-labeled DNA adducts generated in calf thymus DNA by ellipticine after its activation with reconstituted CYP1A1 without (A) and with cytochrome b_5 (CYP1A1:cytochrome b_5 1:5) (B): of ^{32}P-labeled digests of DNA from liver of a male rat treated with 40 mg ellipticine per kg body weight (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. Adduct spots 1–7 correspond to the ellipticine-derived DNA adducts. Besides adduct 2 formed by 12-hydroxyellipticine, another strong adduct (spot × in panel E), which was not found in any other activation systems or *in vivo* was generated.

Table 2
The effect of cytochrome b_5 on DNA adduct formation by ellipticine oxidized with CYP1A1 and 1A2.

Activating system	RAL (mean ± SD/10^7 nucleotides)		
	Adduct 1	Adduct 2	Total
CYP1A1 + NADPH:CYP reductase (1:1)	1.6 ± 0.2	0.8 ± 0.1	2.4 ± 0.3
CYP1A1 + NADPH:CYP reductase + cytochrome b_5 (1:1:5)	9.9 ± 0.6*	1.8 ± 0.3*	11.7 ± 0.9*
CYP1A2 + NADPH:CYP reductase (1:1)	0.9 ± 0.1	0.3 ± 0.1	1.2 ± 0.2
CYP1A2 + NADPH:CYP reductase + cytochrome b_5 (1:1:5)	3.5 ± 0.2*	0.8 ± 0.2	4.3 ± 0.3*

Values are given as means ± SD ($N = 6$). RAL, relative adduct labeling. The total RAL represents sum of RAL values of adducts 1 and 2.
* Values significantly different from control (without cytochrome b_5): $p < 0.001$ (Student's *t*-test).

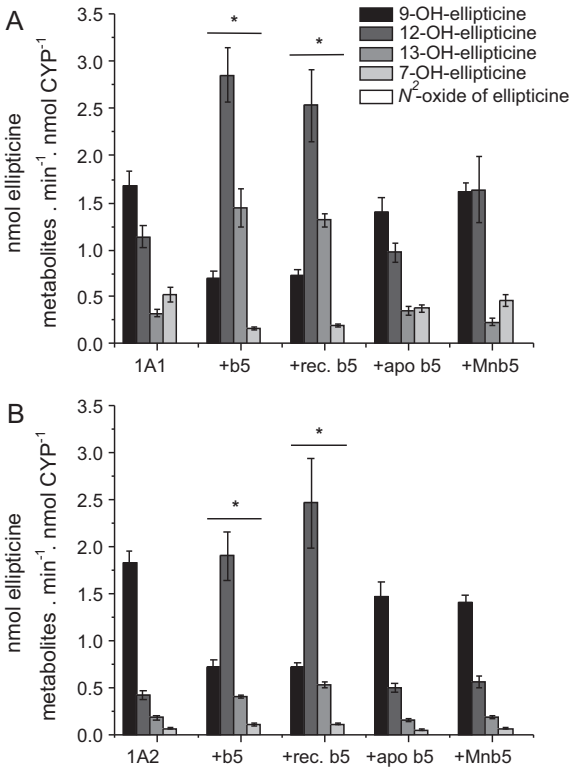

Fig. 6. The effect of cytochrome b_5, apo-cytochrome b_5 and Mn-cytochrome b_5 on rates of ellipticine oxidation by CYP1A1 (A) and 1A2 (B). Conditions were as described in Fig. 2 except that cytochrome b_5 (b_5), recombinant apo-cytochrome b_5 reconstituted with heme (rec. b_5), recombinant apo-cytochrome b_5 (apo-b_5) and recombinant apo-cytochrome b_5 reconstituted with Mn-protoporphyrin IX (Mnb_5) were added at 5-fold higher concentrations than CYP into the incubation mixtures. The values are averages and standard deviations of triplicate incubations. Values significantly different from control (without cytochrome b_5); $^*p < 0.001$ (Student's *t*-test).

limited capability of the electron transfer, Mn-cytochrome b_5 [30,42]. The apo-cytochrome b_5 reconstituted with Mn-protoporphyrin IX should adopt the same 3D conformation as the native cytochrome b_5, but it lacks the electron transfer capability [19,30,44]. Also in the case of Mn-cytochrome b_5, essentially no changes in ellipticine oxidation were found (Fig. 6).

3.5. Ellipticine increases levels of cytochrome b_5 protein in rat livers

Western blots with rabbit polyclonal antibodies raised against cytochrome b_5 were used to investigate the effect of ellipticine on cytochrome b_5 levels *in vivo*. The levels of hepatic cytochrome b_5 protein were increased 4–5-fold in male rats treated with 4 or 40 mg/kg body weight ellipticine (Fig. 8). The mechanism of this increase in cytochrome b_5 levels (*i.e.* transcriptional or translational influences, or ellipticine-induced stabilization of cytochrome b_5) was, however, not evaluated in this work and awaits further investigation.

4. Discussion

The results of this study demonstrate for the first time that cytochrome b_5 alters the ratio of ellipticine metabolites formed by CYP1A1 and 1A2, "switching" oxidation of this anticancer agent from detoxication (9-hydroxy- and/or 7-hydroxyellipticine) to DNA-forming metabolites (12-hydroxy- and 13-hydroxyellipticine). These changes resulted in higher covalent DNA adduct levels by ellipticine, one of the DNA-damaging mechanisms of ellipticine antitumor action. Therefore, besides stimulating effect of cytochrome b_5 on CYP3A4-mediated oxidation of ellipticine to 13-hydroxyellipticine that was found previously to lead to an increase in ellipticine–DNA adduct formation [3,10], this heme protein might play a key role also in the CYP1A1/2-mediated DNA-damage induced by ellipticine. To further confirm these *in vitro* results, a cytochrome b_5-knockout cell model is planned to be used to

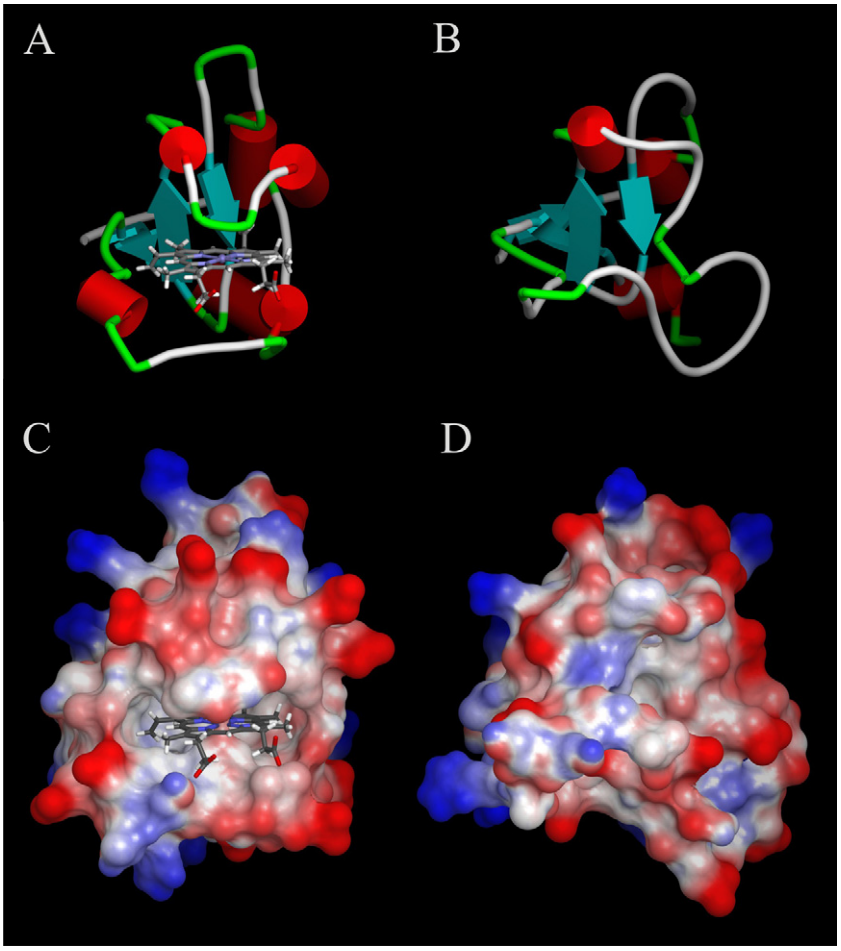

Fig. 7. Comparison of 3D structures of cytochrome b_5 (A, C) and apo-cytochrome b_5 (B, D) (based on PDB coordinates 1BFX [40] and 1I8C [41]). Heme loss results in substantial alteration of cytochrome b_5 secondary structure elements (A and B), for instance three alpha helices of apo-cytochrome b_5 are unfolded and the shape of apo-cytochrome b_5 polypeptide backbone altered (B). Colored surfaces (C and D) demonstrate the large differences in physico-chemical properties, *i.e.* surface shape and charge distribution between the holo- and apo-form of cytochrome b_5.

analyze the oxidation products of ellipticine and ellipticine-derived DNA adducts.

The findings described herein offer an explanation for previous discrepancies found in experiments with isolated CYP1A of rat and human origin reconstituted with NADPH:CYP reductase and *in vivo*. The efficiency of CYP1A in activating ellipticine to metabolites forming DNA adducts *in vivo* contrasted with the low yield of these metabolites *in vitro* [8–10]. The effect of cytochrome b_5, causing a shift in oxidation of ellipticine by CYP1A1/2 from detoxication to activation may be even more pronounced *in vivo*, because ellipticine increases levels of cytochrome b_5. Moreover, ellipticine as a ligand of aryl hydrocarbon receptor [45] also induces expression of CYP1A1/2, increasing their enzymatic activities [17,36]. Hence, both these ellipticine-mediated induction effects produce concerted regulatory effects of this drug on its own metabolism. Indeed, we have found recently that in hepatic microsomes of rats treated with ellipticine, contribution of the CYP1A enzymes to ellipticine–DNA adduct formation is higher than that of CYP3A [46]. To quantify the involvement of cytochrome b_5 in ellipticine metabolism *in vivo* we plan to use cytochrome b_5-knockout mice [47,48].

It should be noted that expression levels and activities of CYP1A1, 1A2 and 3A4 that metabolize ellipticine differ considerably among individuals, because the enzymes are influenced by several factors, including smoking, drugs and environmental chemicals [24,39,49]. Furthermore, besides induction of these enzymes by several compounds including ellipticine [17,36], different activities of CYP1A1 and 1A2 are also determined by genetic polymorphisms, which subsequently might modulate cancer development and treatment. The polymorphic expression of CYP1A1 has been attributed to altered expression of the aryl hydrocarbon (Ah) receptor, the transcription factor that modulates its regulation, or the Ah receptor nuclear translocator (Arnt) protein, its associated transcription factor [50,51]. Moreover, the *CYP1A1* and *CYP1A2* genes are polymorphic [39,49,52,53]. CYP1A1*2A, CYP1A1*2B and CYP1A1*4 polymorphisms have been found that might be associated with cancers of lung, esophagus or breast and with acute myeloid leukemia [54–57], while the CYP1A2*1F polymorphism might be associated with an increased risk of colorectal cancer [52]. In addition, the *CYP1A2*1C* allele has been associated with decreased caffeine 3-demethylation [58], and the *CYP1A2*1F* allele was correlated to increased activity [59,60]. The *CYP1A2*1F* allele has also been shown to influence the inducibility of the gene and affect the magnitude of increase of *in vivo* caffeine metabolism after both smoking [59,60] and omeprazole treatment [61,62]. Thus, genetic polymorphisms in CYP1A1 and 1A2 enzymes could be important determinants of pharmacological efficiencies of ellipticine. In contrast to CYP1A1 and 1A2, there is currently little evidence for a significant contribution of *CYP3A4* gene polymorphisms in determining CYP3A4 activity.

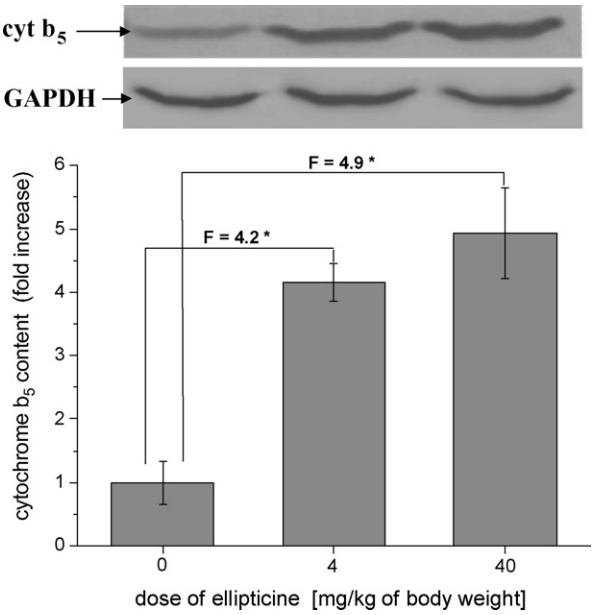

Fig. 8. Induction of cytochrome b_5 in livers of rats treated with 4 or 40 mg ellipticine per kg body weight. Liver microsomes were isolated 48 h after treatment. Mean values ± standard deviations shown in figure represent results obtained from livers of three rats ($N = 3$). Values significantly different from the control (untreated rats): $^*p < 0.001$ (Student's *t*-test). Inset: immunoblots of cytochrome b_5 from untreated and ellipticine-treated rats, respectively, stained with antibody against rat cytochrome b_5. Microsomes isolated from rat livers were subjected to SDS-PAGE, proteins transferred to PVDF membranes and probed with antibody as described in Material and Methods. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as loading control. *F*, fold increase in cytochrome b_5 expression in rats treated with ellipticine compared to control (uninduced) rats.

Besides a number of rare amino acid variants [63], no polymorphisms with a clear genotype–phenotype relationship have so far been described for the CYP3A4 gene [64,65].

It should be emphasized that the effect of cytochrome b_5 upon CYP1A1/2 catalyzed metabolism resulting in the increase of certain metabolites at the expense of others of the same substrate has to our knowledge not been reported yet. This finding indicates that cytochrome b_5 effects show specificity for different hydroxylation sites in the ellipticine molecule. Cytochrome b_5-mediated specificity for different sites of substrate oxidation seems to occur also for testosterone hydroxylation by CYP2B6; a 4- and 2.25-fold increase in 6β- and 16α-hydroxylation of testosterone was produced by cytochrome b_5, respectively [66,67]. However, in the case of this substrate, both hydroxylation reactions were stimulated by cytochrome b_5. A similar effect of cytochrome b_5 was also found for metabolism of midazolam (1- and 4-hydroxylation) and metoprolol (formation of α-hydroxymetoprolol and *O*-desmethylmetoprolol) in hepatic microsomes isolated from "hepatic cytochrome b_5 null mice"(HBN) compared to wild-type controls [47]. In such an *ex vivo* system in which several CYPs are expressed in the hepatic microsomes it is not possible to determine if cytochrome b_5 affects different CYP to different extents.

Generally, two mechanisms of cytochrome b_5-mediated modulation of CYP catalysis have been suggested by several authors; it can affect the CYP catalytic activities by donating the second electron to CYP in a CYP catalytic cycle and/or by acting as an allosteric modifier of the oxygenase (for reviews see [19–22]). The mechanism(s) underlying such allosteric effects, based on reports that apo-cytochrome b_5 can stimulate CYP catalysis, remains uncertain. It does seem clear, however, that cytochrome b_5 binding can cause conformational changes to the substrate access channel and binding pocket in the CYP enzyme [19–22,67,68].

Here, we show that addition of cytochrome b_5 to CYP1A1 alters the kinetics of ellipticine oxidation by this enzyme. For all ellipticine metabolites, with the exception of 12-hydroxy- and 13-hydroxyellipticine, the data followed Michaelis–Menten kinetics, and K_m values were essentially unchanged by the presence of cytochrome b_5 (Table 1). In contrast, the data for formation of 12-hydroxy- and 13-hydroxyellipticine did not fit Michaelis–Menten kinetics, exhibiting positively cooperative binding. These findings indicate that the presence of cytochrome b_5 can cause a conformational change in the CYP1A1 structure affecting ellipticine binding. The effect of cytochrome b_5 upon CYP1A2 catalysis was not as pronounced as the kinetics still showed Michaelis–Menten characteristics, but also here the shift to ellipticine activation from detoxication was prominent.

The results found in this study also show that modulation of ellipticine oxidation by CYP1A1/2 is induced only by the holo-cytochrome b_5. These findings indicate a high specificity of interaction of CYP1A1/2 with holo-cytochrome b_5 containing heme, which is necessary not only for electron transfer, but also for the natural conformation of the cytochrome b_5 protein. The lack of effect of apo-cytochrome b_5 on CYP1A1/2 catalysis might hence be the result of not only the loss of the electron transfer activity, but may also result from changes in 3D structure of its protein. While the holo-cytochrome b_5 contains four helixes and three loops, forming the heme binding pocket, apo-cytochrome b_5 has only one structural element, a helix of amino acids 39-42 (Fig. 7B). This structural change results in significant alterations in surface geometry and electrostatic properties of apo-cytochrome b_5 (Fig. 7) preventing adequate protein–protein interactions of cytochrome b_5 with CYP1A enzymes. Moreover, essentially no changes in ellipticine oxidation were also produced by cytochrome b_5 containing manganese protoporphyrin IX (Mn-cytochrome b_5), a structural analogue of cytochrome b_5 absenting electron transfer ability. All these results demonstrate that both the natural 3D structure of the cytochrome b_5 protein, dictating optimal conformational states of CYP1A1–cytochrome b_5 complexes, and the presence of the protoporphyrin IX bonded-Fe ion as an electron transfer agent are necessary for the observed effects.

However, it is still questionable whether Mn-cytochrome b_5, we used as a structural analogue of cytochrome b_5, is really a suitable model for mechanistic studies. It was found that affinity of Mn-protoporphyrin IX toward apo-cytochrome b_5 is substantially lower than the affinity of heme to apo-cytochrome b_5 [33]. Under the conditions typically used in a reconstituted CYP system, free Mn-protoporphyrin IX and apo-cytochrome b_5 co-exist in solution [33]. Therefore, apo-cytochrome b_5 might be present in incubations instead of Mn-cytochrome b_5. Additional studies utilizing other structural analogues of cytochrome b_5 might explain this feature. The apo-cytochrome b_5 reconstituted with porphyrins containing other metal ions with potentially higher affinity to cytochrome b_5 than Mn-protoporphyrin IX will shed further light on this question.

In conclusion, the results of this study show for the first time that cytochrome b_5 alters the CYP1A1/2-catalyzed oxidation of the antitumor agent ellipticine *in vitro*, favoring formation of the metabolites producing the reactive species generating DNA adducts and that this phenomenon leads to similar results as those seen *in vivo*. Ellipticine also induces cytochrome b_5 (present work) and CYP1A1/2 enzyme expression [17,36], thereby increasing the contribution of these enzymes to form the DNA adducts found *in vivo* [8,46]. Therefore, cytochrome b_5 in combination with CYP1A1/2 can, together with other enzymes known to activate ellipticine such as CYP3A and/or peroxidases, play a crucial role in determining the pharmacological and/or genotoxic potential of this drug.

Conflict of interest

All authors have no conflict of interest.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2011.06.003.

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