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Cytochrome b 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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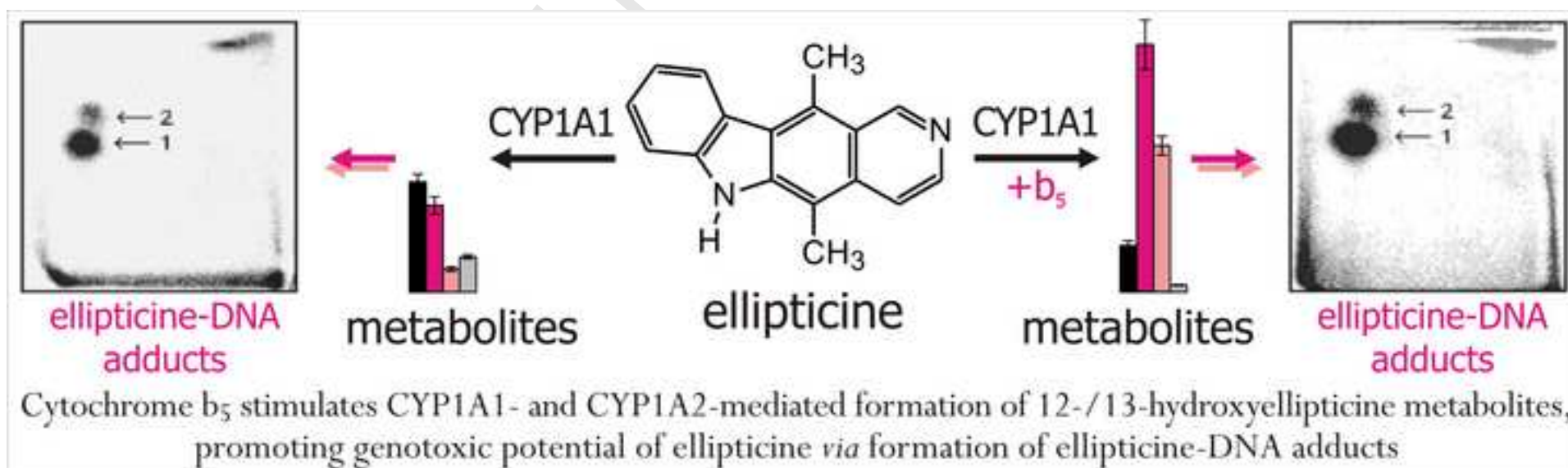
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**Cytochrome b₅ shifts oxidation of the anticancer drug ellipticine by
cytochromes P450 1A1 and 1A2 from its detoxication to activation, thereby
modulating its pharmacological efficacy**

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Key words: CYP1A1/2; cytochrome b₅; ellipticine; modulation; activation

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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

ABSTRACT

1
2 Ellipticine is a pro-drug, whose activation is dependent on its oxidation by cytochromes P450
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4 (CYP) and peroxidases. Cytochrome b₅ alters the ratio of ellipticine metabolites formed by
5
6 isolated reconstituted CYP1A1 and 1A2, favoring formation of 12-hydroxy- and 13-
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8 hydroxyellipticine metabolites implicated in ellipticine-DNA adduct formation, at the expense
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10 of 9-hydroxy- and 7-hydroxyellipticine that are detoxication products. Cytochrome b₅
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12 enhances the production of 12-hydroxy and 13-hydroxyellipticine. The change in metabolite
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14 ratio results in an increased formation of covalent ellipticine-DNA adducts, one of the DNA-
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16 damaging mechanisms of ellipticine antitumor action. This finding explains previous apparent
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18 discrepancies found with isolated enzymes and *in vivo*, where CYP1A enzymatic activation
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20 correlated with ellipticine-DNA-adduct levels while isolated CYP1A1 or 1A2 in reconstituted
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22 systems were much less effective than CYP3A4. The effect of cytochrome b₅ might be even
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24 more pronounced *in vivo*, since, as we show here, ellipticine increases levels of cytochrome b₅
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26 in rat liver. Our results demonstrate that both the native 3D structure of cytochrome b₅ and the
27
28 presence of the heme as an electron transfer agent in this protein enable a shift in ellipticine
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30 metabolites formed by CYP1A1/2.
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1. Introduction

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

10
11 The precise molecular mechanism responsible for anticancer effects of ellipticine has not
12 yet been explained. It was suggested that the major mechanisms of its antitumor, mutagenic
13 and cytotoxic activities are (i) intercalation into DNA [4,5], and (ii) inhibition of DNA
14 topoisomerase II activity [2-7].

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

1 of CYP1A1, 1B1, 3A4 and lactoperoxidase (LPO), cyclooxygenase (COX) and
2 myeloperoxidase (MPO) in these cells [14-17]. Based on these data, ellipticine might be
3 considered a drug, whose pharmacological efficiency and/or genotoxic side effects are
4 dependent on its activation by CYPs and peroxidases in target tissues.
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9 Concerning formation of ellipticine-derived DNA adducts in healthy organs, the risk of
10 treating patients with this compound might be considered. Nevertheless, our *in vivo* studies
11 using the rat experimental model mimicking the fate of ellipticine in human [8,12]
12 demonstrated that ellipticine-DNA adducts did not persist in healthy tissues of rats treated
13 with ellipticine [12]. Therefore, these results suggest a relatively low risk of the genotoxic
14 side effects of ellipticine during the cancer treatment in human.
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24 Studies correlating levels of individual ellipticine-DNA adducts to CYP enzyme
25 expression determined in organs of rats treated with ellipticine showed good correlations of
26 major adduct levels with CYP1A1/2 and CYP3A1 levels [8]. This finding is in contrast to the
27 metabolite patterns and DNA adduct levels seen with pure CYP enzymes of rat and human
28 origin reconstituted with NADPH:CYP reductase [3,10,18]. Here CYP1A isoforms did not
29 activate ellipticine, but 9-hydroxy- and 7-hydroxyellipticine, the detoxication products (Fig.
30 1), were the major metabolites, and as a consequence ellipticine-DNA adduct levels were very
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44 In isolated microsomes, in cells and in intact organs cytochrome b₅ might influence CYP-
45 catalyzed reactions [19]. The lack of cytochrome b₅ in our reconstituted systems could
46 therefore be a reason for the low activating capacity of isolated CYP1A1/2. We therefore
47 investigated the role of cytochrome b₅ on CYP catalyzed oxidation of ellipticine. A
48 reconstituted system consisting of pure CYP1A1 or 1A2 enzymes with NADPH:CYP
49 reductase in liposomes with or without cytochrome b₅ was used.
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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. Material and Methods

2.1. Chemicals

NADP⁺, NADPH, ellipticine, D-glucose 6-phosphate, D-glucose 6-phosphate dehydrogenase, β -naphthoflavone (β -NF), dilauroyl phosphatidylcholine, dioleoyl phosphatidylcholine, and dilauroylphosphatidylserine, 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-6H-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 J. Kučka (Charles University, Prague, Czech Republic); their purity was >99.5% as estimated by high-performance liquid chromatography (HPLC). Enzymatically prepared 12-hydroxy- and 13-hydroxyellipticine were obtained from multiple HPLC runs of ethyl acetate extracts of incubations of ellipticine with human and/or rat hepatic microsomes as described [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

1 animals were killed 48 hours after treatment by cervical dislocation. Livers were removed
2 immediately after death and immediately used for preparation of microsomal fractions.
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4 2.3. Preparation of microsomes

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7 Microsomes were isolated from fresh livers of rats as described [9]. Protein concentrations
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9 in the microsomal fractions were assessed using the bicinchoninic acid protein assay with
10 bovine serum albumin as a standard [23]. Hepatic microsomal preparations from rats that had
11 been pre-treated with ellipticine were analyzed for the presence of ellipticine or its
12 metabolites by HPLC as described [10]. Neither ellipticine nor any of its metabolites were
13 detectable in microsomal fractions from tissues of rats that had been pretreated with
14 ellipticine. Microsomes from the livers of three male rabbits pretreated with 80 mg/kg day of
15 β -NF i.p. for four consecutive days were isolated as described [9].
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26 2.4. Isolation of CYPs, NADPH:CYP reductase, cytochrome b_5 and apo-cytochrome b_5

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29 Recombinant rat CYP1A1 protein was purified to homogeneity from membranes of
30 *Escherichia coli* transfected with a modified *CYP1A1* cDNA [24], in the laboratory of H. W.
31 Strobel (University of Texas, Medical School of Houston, Texas, USA) by P. Hodek (Charles
32 University, Prague, Czech Republic). CYP1A2 was isolated from liver microsomes of rabbits
33 induced with β -NF by procedures described by Haugen and Coon [25]. Rabbit liver
34 NADPH:CYP reductase was purified as described [26]. Cytochrome b_5 was isolated from
35 rabbit liver microsomes by the procedure described by Roos [27]. The apo-cytochrome b_5
36 protein was prepared using heterologous expression in *Escherichia coli* as described in our
37 earlier work [28]. Briefly, the gene for rabbit cytochrome b_5 was constructed from synthetic
38 oligonucleotides using polymerase chain reaction (PCR), cloned into pUC19 plasmid and
39 amplified in DH5 α cells. The gene sequence was verified by DNA sequencing. The sequence
40 coding cytochrome b_5 was cleaved from pUC19 by NdeI and XhoI restriction endonucleases
41 and subcloned into the expression vector pET22b. This vector was used to transform *E. coli*
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1 BL-21 (DE3) Gold cells by heat shock. Expression of cytochrome b_5 was induced with
2 isopropyl β -D-1-thiogalactopyranoside (0.05 mM for 4 hours). The cytochrome b_5 protein,
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4 produced predominantly in its apo-form, was purified to homogeneity from isolated
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6 membranes of *E. coli* cells by chromatography on a DEAE-Sepharose column [28].
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9 2.5. Incorporation of heme into apo-cytochrome b_5

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11 The preparation of hemin chloride solution and its incorporation into apo-cytochrome b_5
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13 were performed by the procedure described elsewhere [29]. Solutions of heme were prepared
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15 by adding 2.6 mg of hemin chloride to a solution of 50% ethanol in water (4 ml) to give a
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17 final concentration of 1 mM. A small increment (10 μ l) of 1 M NaOH was added and mixed
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19 to dissolve the hemin. The solution was then allowed to stand for several minutes so
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21 particulates could settle. A 10- μ l aliquot was removed and diluted into 990 μ l of 20 mM Tris,
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23 1 mM EDTA, pH 8.0, at 25°C and the absorbance of the Tris-ligated heme was measured at
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25 385 nm. The process was repeated five-times, always by adding 10 μ l of 1 M NaOH, until
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27 NaOH addition caused no further increase in absorbance at 385 nm. The hemin solution was
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29 further filtered through 0.2 μ m Membrane MF Millipore filter (Millipore, Billerica,, USA).
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31 Purified apo-cytochrome b_5 was diluted with 20 mM Tris, pH 8.0, containing 1 mM EDTA
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33 and 0.4% sodium cholate, to a protein concentration of 0.25 mg/ml. Aliquots of hemin
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35 chloride were added into the apo-cytochrome b_5 sample (1 ml) and the reconstitution of apo-
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37 cytochrome b_5 with heme was monitored by absorbance spectroscopy. Absorbance spectra
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39 (from 350 to 500 nm) were recorded on Hewlett Packard 8453 UV spectrophotometer. The
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41 reconstitution was considered to be complete when the Soret peak of cytochrome b_5 shifted
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43 from 413 to 409 nm and an increase in absorbance at 385 nm, caused by excess of free Tris-
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45 ligated hemin, was observed in the spectrum.
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56 Analogous procedures were utilized to incorporate manganese protoporphyrin IX (Frontier
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58 Scientific, USA) into apo-cytochrome b_5 (Mn-cytochrome b_5) [30].
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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 $\epsilon_{413} = 117 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ [31,32] or from the difference spectrum of reduced minus oxidized form, using molar extinction coefficient $\epsilon_{424-409} = 185 \text{ mM}^{-1} \cdot \text{cm}^{-1}$, respectively [32]. The concentration of Mn-cytochrome b_5 was determined using an extinction coefficient of $57 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ 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 Results). 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 $\mu\text{g}/\mu\text{l}$ CHAPS, 0.1 $\mu\text{g}/\mu\text{l}$ liposomes [dilauroyl phosphatidylcholine, dioleoyl 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 x 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 x 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 Results).

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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 μ l. 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. ³²P-Postlabeling analysis and HPLC analysis of ³²P-labeled 3',5'-deoxyribonucleoside bisphosphate adducts

The ³²P-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 b₅ holo-protein was based on coordinates of soluble domain of rat microsomal cytochrome b₅ obtained by three-dimensional NMR of ¹⁵N labeled protein (PDB code 1BFX) [40]. The model of apo-cytochrome b₅ was based on coordinates of water-soluble domain of rat microsomal apo-cytochrome b₅ obtained by three-dimensional NMR of ¹³C and ¹⁵N 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

1 proteins was calculated with probe radius 1.4 Å and colored by mapping the interpolated
2 partial atom charges to the surface.
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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-hydroxyellipticine 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 μ M. We therefore used 0.2 μ M 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, 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

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in pattern of ellipticine metabolites were dependent on the molar ratios of CYP and cytochrome b₅. If CYP1A1 was used, the detoxication products of ellipticine (7-hydroxy- and 9-hydroxyellipticine) decreased with more added cytochrome b₅, while ellipticine N²-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 b₅ amounts, while 9-hydroxyellipticine levels decreased. Here 7-hydroxyellipticine increased concomitant with 12-hydroxy-, 13-hydroxyellipticine and the ellipticine N²-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 b₅ 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).

Cytochrome b₅ affects the kinetics of ellipticine oxidation by cytochromes P450 1A1/2

Further experiments were conducted to investigate the effects of cytochrome b₅ 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 b₅ was 1:5. Because of instability of ellipticine N²-oxide, kinetics of its formation was not evaluated.

The presence of cytochrome b₅ 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 b₅ 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

1 oxidation by CYP1A2 in presence of cytochrome b₅ to these metabolites exhibited Michaelis-
2 Menten kinetics (Fig. 4, Table 1).
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4 3.2 Cytochrome b₅ increases the ellipticine-derived DNA adduct formation mediated by 5 cytochromes P450 1A1 and 1A2 6 7

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9 Using the nuclease P1 version of the ³²P-postlabeling assay, which was suitable to detect
10 and quantify DNA adducts formed by ellipticine [1,8-12], two ellipticine-derived DNA
11 adducts (see Figure 1 for the structures of two deoxyguanosine adducts 1 and 2) were detected
12 in the calf thymus DNA incubated with this drug and CYP1A1 or 1A2 reconstituted with
13 NADPH:CYP reductase (Fig. 5A,B). The two adducts are identical to those found previously
14 after *in vitro* incubation of calf thymus DNA with ellipticine, human, rat, rabbit and mouse
15 hepatic microsomes or human and rat CYPs [1,9,10], or peroxidases [11] or after treatment of
16 cells in culture with this anticancer drug [14-17,42] or *in vivo* (Fig. 5C), in several organs of
17 rats including mammary adenocarcinoma [3,8,12] and mice [13] exposed to this agent. These
18 adducts are generated from ellipticine-13-ylidium and ellipticine-12-ylidium (Fig. 1), the reactive
19 species formed from the corresponding hydroxyellipticines (Fig. 5D,E) as confirmed by co-
20 chromatographic analysis using TLC and HPLC [10,11,43]. The increased levels of 12-
21 hydroxy- and 13-hydroxyellipticine formed by the reconstituted system containing
22 cytochrome b₅ resulted in corresponding higher levels of these two ellipticine-derived DNA
23 adducts (Table 2). In the case of CYP1A1, the presence of cytochrome b₅ resulted in 6.2-fold
24 higher levels of the ellipticine-DNA adduct 1, which correlated with a 6.3-fold increased
25 formation of 13-hydroxyellipticine, the metabolite generating this adduct (Table 2, Fig. 3).
26 Likewise, CYP1A2 activation of ellipticine in presence of cytochrome b₅ resulted in 3.9-fold
27 higher levels of DNA adduct 1 (Table 2) which was consistent with a similar increase (more
28 than 3.8-fold) in 13-hydroxyellipticine metabolite formation by this system (Fig. 3). The same
29 was true for the parallel effects of cytochrome b₅ on ellipticine-DNA-adduct 2 levels and 12-
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1 hydroxyellipticine yields in incubations with reconstituted CYP1A1 or 1A2 (Table 2, Fig. 3
2 and supplemental Table 2).
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4 3.3 Modulation of ellipticine oxidation by cytochromes P450 1A1 and 1A2 is dependent on 5 6 7 *holo-cytochrome b₅*

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9 In order to investigate the mechanism of cytochrome b₅ mediated-modulation of ellipticine
10 oxidation by CYP1A1 and 1A2, we also examined the influence of apo-cytochrome b₅ and
11 Mn-cytochrome b₅. As shown in Figure 6, the CYP1A1/2-mediated oxidation of ellipticine
12 was significantly changed only by holo-cytochrome b₅ or apo-cytochrome b₅ reconstituted
13 with heme, while apo-cytochrome b₅ without heme cofactor (see Figure 7B and 7D) was
14 essentially without such effects (Fig. 6). We also employed a structurally similar analogue of
15 cytochrome b₅, known to have limited capability of the electron transfer, Mn-cytochrome b₅
16 [30,42]. The apo-cytochrome b₅ reconstituted with Mn-protoporphyrin IX should adopt the
17 same 3D conformation as the native cytochrome b₅, but it lacks the electron transfer capability
18 [19,30,44]. Also in the case of Mn-cytochrome b₅, essentially no changes in ellipticine
19 oxidation were found (Fig. 6).
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36 3.4. Ellipticine increases levels of cytochrome b₅ protein in rat livers

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38 Western blots with rabbit polyclonal antibodies raised against cytochrome b₅ were used to
39 investigate the effect of ellipticine on cytochrome b₅ levels *in vivo*. The levels of hepatic
40 cytochrome b₅ protein were increased 4 to 5-fold in male rats treated with 4 or 40 mg/kg body
41 weight ellipticine (Fig. 8). The mechanism of this increase in cytochrome b₅ levels (*i.e.*
42 transcriptional or translational influences, or ellipticine-induced stabilization of cytochrome
43 b₅) was, however, not evaluated in this work and awaits further investigation.
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4. Discussion

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2 The results of this study demonstrate for the first time that cytochrome b₅ alters the ratio of
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4 ellipticine metabolites formed by CYP1A1 and 1A2, “switching” oxidation of this anticancer
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6 agent from detoxication (9-hydroxy- and/or 7-hydroxyellipticine) to DNA-forming
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8 metabolites (12-hydroxy- and 13-hydroxyellipticine). These changes resulted in higher
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10 covalent DNA adduct levels by ellipticine, one of the DNA-damaging mechanisms of
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12 ellipticine antitumor action. Therefore, besides stimulating effect of cytochrome b₅ on
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14 CYP3A4-mediated oxidation of ellipticine to 13-hydroxyellipticine that was found previously
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16 to lead to an increase in ellipticine-DNA adduct formation [3,10], this heme protein might
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18 play a key role also in the CYP1A1/2-mediated DNA-damage induced by ellipticine. To
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20 further confirm these *in vitro* results, a cytochrome b₅-knockout cell model is planned to be
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22 used to analyze the oxidation products of ellipticine and ellipticine-derived DNA adducts.
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29 The findings described herein offer an explanation for previous discrepancies found in
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31 experiments with isolated CYP1A of rat and human origin reconstituted with NADPH:CYP
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33 reductase and *in vivo*. The efficiency of CYP1A in activating ellipticine to metabolites
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35 forming DNA adducts *in vivo* contrasted with the low yield of these metabolites *in vitro* [8-
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37 10]. The effect of cytochrome b₅, causing a shift in oxidation of ellipticine by CYP1A1/2
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39 from detoxication to activation may be even more pronounced *in vivo*, because ellipticine
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41 increases levels of cytochrome b₅. Moreover, ellipticine as a ligand of aryl hydrocarbon
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43 receptor [45] also induces expression of CYP1A1/2, increasing their enzymatic activities
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45 [17,36]. Hence, both these ellipticine-mediated induction effects produce concerted regulatory
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47 effects of this drug on its own metabolism. Indeed, we have found recently that in hepatic
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49 microsomes of rats treated with ellipticine, contribution of the CYP1A enzymes to ellipticine
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51 DNA adduct formation is higher than that of CYP3A [46]. To quantify the involvement of
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1 cytochrome b₅ in ellipticine metabolism *in vivo* we plan to use cytochrome b₅-knockout mice
2 [47,48].
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4 It should be noted that expression levels and activities of CYP1A1, 1A2 and 3A4 that
5 metabolize ellipticine differ considerably among individuals, because the enzymes are
6 influenced by several factors, including smoking, drugs and environmental chemicals [24,39,
7 49]. Furthermore, besides induction of these enzymes by several compounds including
8 ellipticine [17,36], different activities of CYP1A1 and 1A2 are also determined by genetic
9 polymorphisms, which subsequently might modulate cancer development and treatment. The
10 polymorphic expression of CYP1A1 has been attributed to altered expression of the aryl
11 hydrocarbon (Ah) receptor, the transcription factor that modulates its regulation, or the Ah
12 receptor nuclear translocator (Arnt) protein, its associated transcription factor [50,51].
13 Moreover, the *CYP1A1* and *CYP1A2* genes are polymorphic [39,49,52,53]. CYP1A1*2A,
14 CYP1A1*2B and CYP1A1*4 polymorphisms have been found that might be associated with
15 cancers of lung, esophagus or breast and with acute myeloid leukemia [54-57], while the
16 CYP1A2*1F polymorphism might be associated with an increased risk of colorectal cancer
17 [52]. In addition, the *CYP1A2*1C* allele has been associated with decreased caffeine 3-
18 demethylation [58], and the *CYP1A2*1F* allele was correlated to increased activity [59,60].
19 The *CYP1A2*1F* allele has also been shown to influence the inducibility of the gene and
20 affect the magnitude of increase of *in vivo* caffeine metabolism after both smoking [59,60]
21 and omeprazole treatment [61,62]. Thus, genetic polymorphisms in CYP1A1 and 1A2
22 enzymes could be important determinants of pharmacological efficiencies of ellipticine. In
23 contrast to CYP1A1 and 1A2, there is currently little evidence for a significant contribution of
24 *CYP3A4* gene polymorphisms in determining CYP3A4 activity. Besides a number of rare
25 amino acid variants [63], no polymorphisms with a clear genotype–phenotype relationship
26 have so far been described for the CYP3A4 gene [64,65].
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It should be emphasized that the effect of cytochrome b₅ 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 b₅ effects show specificity for different hydroxylation sites in the ellipticine molecule. Cytochrome b₅-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 6 β - and 16 α -hydroxylation of testosterone was produced by cytochrome b₅, respectively [66,67]. However, in the case of this substrate, both hydroxylation reactions were stimulated by cytochrome b₅. A similar effect of cytochrome b₅ was also found for metabolism of midazolam (1- and 4-hydroxylation) and metoprolol (formation of α -hydroxymetoprolol and *O*-desmethylnmetoprolol) in hepatic microsomes isolated from “hepatic cytochrome b₅ 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 b₅ affects different CYP to different extents.

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Generally, two mechanisms of cytochrome b₅-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 b₅ can stimulate CYP catalysis, remains uncertain. It does seem clear, however, that cytochrome b₅ binding can cause conformational changes to the substrate access channel and binding pocket in the CYP enzyme [19-22,67,68].

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Here, we show that addition of cytochrome b₅ 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 b₅ (Table 1). In contrast, the data for

1 formation of 12-hydroxy- and 13-hydroxyellipticine did not fit Michaelis-Menten kinetics,
2 exhibiting positively cooperative binding. These findings indicate that the presence of
3 cytochrome b₅ can cause a conformational change in the CYP1A1 structure affecting
4 ellipticine binding. The effect of cytochrome b₅ upon CYP1A2 catalysis was not as
5 pronounced as the kinetics still showed Michaelis-Menten characteristics, but also here the
6 shift to ellipticine activation from detoxication was prominent.
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14 The results found in this study also show that modulation of ellipticine oxidation by
15 CYP1A1/2 is induced only by the holo-cytochrome b₅. These findings indicate a high
16 specificity of interaction of CYP1A1/2 with holo-cytochrome b₅ containing heme, which is
17 necessary not only for electron transfer, but also for the natural conformation of the
18 cytochrome b₅ protein. The lack of effect of apo-cytochrome b₅ on CYP1A1/2 catalysis might
19 hence be the result of not only the loss of the electron transfer activity, but may also result
20 from changes in 3D structure of its protein. While the holo-cytochrome b₅ contains four
21 helices and three loops, forming the heme binding pocket, apo-cytochrome b₅ has only one
22 structural element, a helix of amino acids 39-42 (Fig 7B). This structural change results in
23 significant alterations in surface geometry and electrostatic properties of apo-cytochrome b₅
24 (Fig. 7) preventing adequate protein-protein interactions of cytochrome b₅ with CYP1A
25 enzymes. Moreover, essentially no changes in ellipticine oxidation were also produced by
26 cytochrome b₅ containing manganese protoporphyrin IX (Mn-cytochrome b₅), a structural
27 analogue of cytochrome b₅ absenting electron transfer ability. All these results demonstrate
28 that both the natural 3D structure of the cytochrome b₅ protein, dictating optimal
29 conformational states of CYP1A1-cytochrome b₅ complexes, and the presence of the
30 protoporphyrin IX bonded-Fe ion as an electron transfer agent are necessary for the observed
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However, it is still questionable whether Mn-cytochrome b₅, we used as a structural analogue of cytochrome b₅, is really a suitable model for mechanistic studies. It was found that affinity of Mn-protoporphyrin IX toward apo-cytochrome b₅ is substantially lower than the affinity of heme to apo-cytochrome b₅ [33]. Under the conditions typically used in a reconstituted CYP system, free Mn-protoporphyrin IX and apo-cytochrome b₅ co-exist in solution [33]. Therefore, apo-cytochrome b₅ might be present in incubations instead of Mn-cytochrome b₅. Additional studies utilizing other structural analogues of cytochrome b₅ might explain this feature. The apo-cytochrome b₅ reconstituted with porphyrins containing other metal ions with potentially higher affinity to cytochrome b₅ 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 b₅ 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 b₅ (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 b₅ 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.

Acknowledgments

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References

- 1
2 [1] Stiborová M, Bieler CA, Wiessler M, Frei E. The anticancer agent ellipticine on activation
3
4 by cytochrome P450 forms covalent DNA adducts. *Biochem Pharmacol* 2001;62:1675-84.
5
6
- 7 [2] Stiborová M, Rupertová M, Schmeiser HH, Frei E. Molecular mechanism of
8
9 antineoplastic action of an anticancer drug ellipticine. *Biomed Pap Med Fac Univ Palacky*
10
11 Olomouc Czech Repub 2006;150:13-23.
12
13
- 14 [3] Stiborová M, Rupertová M, Frei E. Cytochrome P450- and peroxidase-mediated oxidation
15
16 of anticancer alkaloid ellipticine dictates its anti-tumor efficiency. *Biochim Biophys Acta*
17
18 2011;1814:175-85.
19
20
- 21 [4] Auclair C. Multimodal action of antitumor agents on DNA: The ellipticine series. *Arch*
22
23 *Biochem Biophys* 1987;259:1-14.
24
25
- 26 [5] Garbett NC, Graves DE. Extending nature's leads: the anticancer agent ellipticine. *Curr*
27
28 *Med Chem Anti-Cancer Agents* 2004;4:149-172.
29
30
- 31 [6] Fossé P, René B, Charra M, Paoletti C, Saucier JM. Stimulation of topoisomerase II-
32
33 mediated DNA cleavage by ellipticine derivatives: structure-activity relationships. *Mol*
34
35 *Pharmacol* 1992;42:590-95.
36
37
- 38 [7] Froelich-Ammon SJ, Patchan MW, Osheroff N, Thompson RB. Topoisomerase II binds to
39
40 ellipticine in the absence or presence of DNA. Characterization of enzyme-drug
41
42 interactions by fluorescence spectroscopy. *J Biol Chem* 1995;270:14998-5004.
43
44
- 45 [8] Stiborová M, Breuer A, Aimová D, Stiborová-Rupertová M, Wiessler M, Frei, E. DNA
46
47 adduct formation by the anticancer drug ellipticine in rats determined by ³²P-postlabeling.
48
49 *Int J Cancer* 2003;107:885-90.
50
51
- 52 [9] Stiborová M, Stiborová-Rupertová M, Bořek-Dohalská L, Wiessler M, Frei E. Rat
53
54 microsomes activating the anticancer drug ellipticine to species covalently binding to
55
56
57
58
59
60
61
62
63
64
65

deoxyguanosine in DNA are a suitable model mimicking ellipticine bioactivation in humans. *Chem Res Toxicol* 2003;16:38-47.

- [10] Stiborová M, Sejbal J, Bořek-Dohalská L, Aimová D, Poljaková J, Forsterová K, Rupertová M, Wiesner J, Hudeček J, Wiessler M, Frei E. The anticancer drug ellipticine forms covalent DNA adducts, mediated by human cytochromes P450, through metabolism to 13-hydroxyellipticine and ellipticine *N*²-oxide. *Cancer Res* 2004;64:8374-80.
- [11] Stiborová M, Poljaková J, Ryšlavá H, Dračínský M, Eckschlager T, Frei E. Mammalian peroxidases activate anticancer drug ellipticine to intermediates forming deoxyguanosine adducts in DNA identical to those found *in vivo* and generated from 12-hydroxyellipticine and 13-hydroxyellipticine. *Int J Cancer* 2007;120:243-51.
- [12] Stiborová M, Rupertová M, Aimová D, Ryšlavá H, Frei E. Formation and persistence of DNA adducts of anticancer drug ellipticine in rats. *Toxicology* 2007;236:50-60.
- [13] Stiborová M, Arlt VM, Henderson CJ, Wolf CR, Kotrbová V, Moserová M, Hudeček J, Phillips DH, Frei E. Role of hepatic cytochromes P450 in bioactivation of the anticancer drug ellipticine: studies with the hepatic NADPH:cytochrome P450 reductase null mouse. *Toxicol Appl Pharmacol* 2008;226:318-27.
- [14] Bořek-Dohalská L, Frei E, Stiborová M. DNA adduct formation by the anticancer drug ellipticine and its hydroxy derivatives in human breast adenocarcinoma MCF-7 cells. *Collect Czech Chem Commun* 2004;69:603-15.
- [15] Poljaková J, Frei E, Gomez JE, Aimová D, Eckschlager T, Hraběta J, Stiborová M. DNA adduct formation by the anticancer drug ellipticine in human leukemia HL-60 and CCRF-CEM cells. *Cancer Lett* 2007;252:270-9.
- [16] Poljaková J, Eckschlager T, Hraběta J, Hřebacková J, Smutný S, Frei E, Martínek V, Kizek R, Stiborová M. The mechanism of cytotoxicity and DNA adduct formation by the

1 anticancer drug ellipticine in human neuroblastoma cells. *Biochem Pharmacol*
2 2009;77:1466-79.
3

4 [17] Martínková E, Dontenwill M, Frei E, Stiborová M. Cytotoxicity of and DNA adduct
5 formation by ellipticine in human U87MG glioblastoma cancer cells. *Neuro Endocrinol*.
6
7 Lett 2009;30 (Suppl. 1):60-6.
8
9

10 [18] Kotrbová V, Aimová D, Březinová A, Janouchová K, Poljaková J, Hodek P, Frei E,
11 Stiborová M. Cytochromes P450 reconstituted with NADPH:P450 reductase mimic the
12 activating and detoxicating metabolism of the anticancer drug ellipticine in microsomes.
13
14 *Neuro Endocrinol Lett* 2006;27 (Suppl. 2):18-20.
15
16
17
18
19

20 [19] Schenkman JB, Jansson I. The many roles of cytochrome b₅. *Pharmacol Ther*
21 2003;9:139-52.
22
23
24

25 [20] Yamazaki, H, Gillam EM, Dong MS, Johnson WW, Guengerich FP, Shimada T.
26 Reconstitution of recombinant cytochrome P450 2C10(2C9) and comparison with
27 cytochrome P450 3A4 and other forms: effects of cytochrome P450-P450 and cytochrome
28 P450-b₅ interactions. *Arch Biochem Biophys*. 1997;342:329-37.
29
30
31
32
33

34 [21] Yamazaki H, Shimada T, Martin MV, Guengerich FP. Stimulation of cytochrome P450
35 reactions by apo-cytochrome b₅: evidence against transfer of heme from cytochrome P450
36 3A4 to apo-cytochrome b₅ or heme oxygenase, *J Biol Chem* 2001;276:30885-91.
37
38
39
40
41

42 [22] Zhang H, Myshkin E, Waskell L. Role of cytochrome b₅ in catalysis by cytochrome P450
43 2B4. *Biochem Biophys Res Commun* 2005;338:499-506.
44
45
46
47

48 [23] Wiechelmann KJ, Braun RD, Fitzpatrick JD. Investigation of the bicinchoninic acid
49 protein assay: identification of the groups responsible for color formation. *Anal Biochem*
50 1988;175:231-7.
51
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52
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56
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58
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61
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65
- [24] Stiborová M, Martínek V, Rýdlová H, Hodek P, Frei E. Sudan I is a potential carcinogen for humans: evidence for its metabolic activation and detoxication by human recombinant cytochrome P450 1A1 and liver microsomes. *Cancer Res* 2002;62:5678-84.
- [25] Haugen DA, Coon MJ. Properties of electrophoretically homogeneous phenobarbital-inducible and beta-naphthoflavone-inducible forms of liver microsomal cytochrome P-450. *J Biol Chem* 1976;251:7929-39.
- [26] Dignam JD, Strobel HW. NADPH-cytochrome P-450 reductase from rat liver: purification by affinity chromatography and characterization, *Biochemistry* 1977;6:1116-23.
- [27] Roos PH. Chromatographic separation and behavior of microsomal cytochrome P450 and cytochrome b₅. *J Chromatogr B Biomed Appl* 1996;684:107-31.
- [28] Kotrbová V, Aimová D, Ingr M, Bořek-Dohalská L, Martínek V, Stiborová M. Preparation of a biologically active apo-cytochrome b₅ via heterologous expression in *Escherichia coli*. *Protein Expr Purif* 2009;66:203-9.
- [29] Mulrooney SB, Waskell L. High-level expression in *Escherichia coli* and purification of the membrane-bound form of cytochrome b₅, *Protein Expr Purif* 2000;19:173-8.
- [30] Zhang H, Hamdane D, Im SC, Waskell L. Cytochrome b₅ inhibits electron transfer from NADPH-cytochrome P450 reductase to ferric cytochrome P450 2B4. *J Biol Chem* 2008;283:5217-25.
- [31] Strittmatter P, Velick SF. The isolation and properties of microsomal cytochrome. *J Biol Chem* 1956;221:253-64.
- [32] Estabrook RW, Werringloer J. The measurement of difference spectra: application to the cytochromes of microsomes. *Methods Enzymol* 1978;52:212-20.
- [33] Gruenke LD, Sun J, Loehr TM, Waskell L. Resonance Raman spectral properties and stability of manganese protoporphyrin IX cytochrome b₅. *Biochemistry* 1997;36:7114-25.

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- [34] Stiborová M, Martínek V, Rýdlová H, Koblas T, Hodek P. Expression of cytochrome P450 1A1 and its contribution to oxidation of a potential human carcinogen 1-phenylazo-2-naphthol (Sudan I) in human livers. *Cancer Lett* 2005;220:145-54.
- [35] Farooq Y, Gordon GCK. Kinetics of electron transfer between NADPH-cytochrome P450 reductase and cytochrome P450 3A4 *Biochem J* 2010;432:485–93.
- [36] Aimová D, Svobodová L, Kotrbová V, Mrazová B, Hodek P, Hudeček J, Vaclavikova R, Frei E, Stiborová M. The anticancer drug ellipticine is a potent inducer of rat cytochromes P450 1A1 and 1A2, thereby modulating its own metabolism. *Drug Metab Dispos* 2007;35:1926–34.
- [37] Stiborová M, Bořek-Dohalská L, Aimová D, Kotrbová V, Kukacková K, Janouchová K, Rupertová M, Ryšlavá H, Hudeček J, Frei E. Oxidation pattern of the anticancer drug ellipticine by hepatic microsomes – Similarity between human and rat systems. *Gen Physiol Biophys*. 2006;25:245-61.
- [38] Guengerich FP, Shimada T. Oxidation of toxic and carcinogenic chemicals by human cytochrome P450 enzymes. *Chem Res Toxicol* 1991;4:391-407.
- [39] Rendic S, DiCarlo FJ. Human cytochrome P450 enzymes: A status report summarizing their reactions, substrates, inducers, and inhibitors. *Drug Metab Rev* 1997;29:413-80.
- [40] Arnesano F, Banci L, Bertini I, Felli IC, Koulougliotis D. Solution structure of the B form of oxidized rat microsomal cytochrome b₅ and backbone dynamics via 15N rotating-frame NMR-relaxation measurements. Biological implications. *Eur J Biochem* 1999;260:347-54.
- [41] Falzone CJ, Wang Y, Vu BC, Scott NL, Bhattacharya S, Lecomte JT. Structural and dynamic perturbations induced by heme binding in cytochrome b₅. *Biochemistry* 2001;40:4879-91.

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- [42] Frei E, Bieler CA, Arlt VM, Wiessler M, Stiborová M. Covalent binding of the anticancer drug ellipticine to DNA in V79 cells transfected with human cytochrome P450 enzymes. *Biochem Pharmacol* 2002;64:289-95.
- [43] Moserová M, Kotrbová V, Rupertová M, Naiman K, Hudeček J, Hodek P, Frei E, Stiborová M. Isolation and partial characterization of the adduct formed by 13-hydroxyellipticine with deoxyguanosine in DNA. *Neuro Endocrinol Lett* 2008;29:728-32.
- [44] Zhang H, Im SC, Waskell L. Cytochrome b₅ increases the rate of product formation by cytochrome P450 2B4 and competes with cytochrome P450 reductase for a binding site on cytochrome P450 2B4. *J Biol Chem* 2007;282:29766-76.
- [45] Gasiewicz TA, Kende RS, Rucci G, Whitney B, Willey JJ. Analysis of structural requirements for Ah receptor antagonist activity: Ellipticines, flavones, and related compounds. *Biochem Pharmacol* 1996;52:787-830.
- [46] Stiborová M, Moserová M, Mrázová B, Kotrbová V, Frei E. 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. *Neuro Endocrinol Lett* 2010;31 (Suppl. 2):26-35.
- [47] Finn RD, McLaughlin LA, Ronseaux S, Rosewell I, Houston JB, Henderson CJ, Wolf CR. Defining the *in vivo* role for cytochrome b₅ in cytochrome P450 function through the conditional hepatic deletion of microsomal cytochrome b₅. *J Biol Chem* 2008;283:31385-93.
- [48] McLaughlin LA, Ronseaux S, Finn RD, Henderson CJ, Wolf CR. Deletion of microsomal cytochrome b₅ profoundly affects hepatic and extrahepatic drug metabolism. *Mol Pharmacol* 2010;75:269-78.
- [49] Perera FP. Environment and cancer: Who are susceptible? *Science* 1997;278:1068-73.

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- [50] Hukkanen J, Pelkonen O, Hakkola J, Raunio H. Expression and regulation of xenobiotic-metabolizing cytochrome P450 (CYP) enzymes in human lung, *Crit Rev Toxicol* 2002;32:391-411.
- [51] Dickins M. Induction of cytochromes P450. *Curr Top Med Chem* 2004;4:1745-66.
- [52] Bae SY, Choi SK, Kim KR, Park CS, Lee SK, Roh HK, Shin DW, Pie JE, Woo ZH, Kang JH. Effects of genetic polymorphisms of MDR1, FMO3 and CYP1A2 on susceptibility to colorectal cancer in Koreans. *Cancer Sci* 2006;97:774-9.
- [53] Yoshida K, Osawa K, Kasahara M, Miyaishi A, Nakanishi K, Hayamizu S, Osawa Y, Tsutou A, Tabuchi Y, Shimada E, Tanaka K, Yamamoto M, Takahashi J. Association of CYP1A1, CYP1A2, GSTM1 and NAT2 gene polymorphisms with colorectal cancer and smoking. *Asian Pac J Cancer Prev* 2007;8:438-44.
- [54] D'Alo A, Voso M, Guidy F, Kaseiny G, Sica S, Pagano L, Hohaus S, Leone G. Polymorphisms of CYP1A1 and glutathione S-transferase and susceptibility to adult acute myeloid leukaemia. *Haematologica* 2004;89:664-70.
- [55] Yang CX, Matsuo K, Wang Z, Tajima K. Phase I/II enzyme gene polymorphisms and esophageal cancer risk: A meta-analysis of the literature. *World J Gastroenterol* 2005;11:2534-8.
- [56] Li Y, Millikan R, Bell D, Cui L, Tse C, Newman B, Conway K. Cigarette smoking, cytochrome P450 1A1 polymorphisms, and breast cancer among African-American and white women. *Breast Cancer Res* 2004;6:R460-R473.
- [57] Li Y, Millikan R, Bell D, Cui L, Tse C, Newman B, Conway K. Polychlorinated biphenyls, cytochrome P450 1A1 (CYP1A1) polymorphisms, and breast cancer risk among African American women and white women in North Carolina: a population-based case-control study. *Breast Cancer Res* 2005;7:R12-R18.

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- [58] Nakajima M, Yokoi T, Mizutani M, Kinoshita M, Funayama M, Kamataki T. Genetic polymorphism in the 5'-flanking region of human CYP1A2 gene: effect on the CYP1A2 inducibility in humans. *J Biochem* 1999;125:803–8
- [59] Sachse C, Brockmoller J, Bauer S, Roots I. Functional significance of a C→A polymorphism in intron 1 of the cytochrome P450 CYP1A2 gene tested with caffeine. *Br J Clin Pharmacol* 1999;47:445–9
- [60] Ghotbi R, Christensen M, Roh HK, Ingelman-Sundberg M, Aklillu E, Bertilsson L. Comparisons of CYP1A2 genetic polymorphisms, enzyme activity and the genotype-phenotype relationship in Swedes and Koreans. *Eur J Clin Pharmacol* 2007;63:537–46
- [61] Han XM, Ouyang DS, Chen XP, Shu Y, Jiang CH, Tan ZR, Zhou HH. Inducibility of CYP1A2 by omeprazole in vivo related to the genetic polymorphism of CYP1A2. *Br J Clin Pharmacol* 2002;54:540–3.
- [62] Ingelman-Sundberg M, Sim SC, Gomez A, Rodriguez-Antona C. Influence of cytochrome P450 polymorphisms on drug therapies: pharmacogenetic, pharmacoeconomic and clinical aspects. *Pharmacol Ther* 2007;116:496-526.
- [63] Sata F, Sapone A, Elizondo G, Stocker P, Miller VP, Zheng W, Raunio H, Crespi CL, Gonzalez FJ. CYP3A4 allelic variants with amino acid substitutions in exons 7 and 12: evidence for an allelic variant with altered catalytic activity. *Clin Pharmacol Ther* 2000;67:48–56.
- [64] Schirmer M, Rosenberger A, Klein K, Kulle B, Toliat MR, Nurnberg P, Zanger UM, Wojnowski L Sex-dependent genetic markers of CYP3A4 expression and activity in human liver microsomes. *Pharmacogenomics* 2007;8:443–53
- [65] Zanger UM, Turpeinen M, Klein K, Schwab M. Functional pharmacogenetics/genomics of human cytochromes P450 involved in drug biotransformation. *Anal Bioanal Chem* 2008;392:1093-108.

1 [66] Morgan ET, Coon MJ. Effects of cytochrome b₅ on cytochrome P-450-catalyzed
2 reactions. Studies with manganese-substituted cytochrome b₅. Drug Metab Dispos
3 1984;12:358-64.
4
5
6

7 [67] Porter TD. The roles of cytochrome b₅ in cytochrome P450 reactions. J Biochem Mol
8 Toxicol 2002;16:311-6.
9

10 [68] Loughran PA, Roman LJ, Miller RT, Masters BS. The kinetic and spectral
11 characterization of the E. coli-expressed mammalian CYP4A7: cytochrome b₅ effects vary
12 with substrate. Arch Biochem Biophys 2001;385:311–21.
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Legends to figures

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4 Fig. 1. Scheme of ellipticine metabolism by CYPs and peroxidases showing the identified
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6 metabolites and those proposed to form DNA adducts. The compounds shown in
7
8 brackets were not detected under the experimental conditions and/or not structurally
9
10 characterized. The CYP enzymes predominantly oxidizing ellipticine shown in the
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12 figure were identified in this work and/or in our previous studies [9,10,18].
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17 Fig. 2. The effect of increasing NADPH:CYP reductase amounts reconstituted with CYP in
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19 liposomes as described in the Materials and Methods section on rates of ellipticine
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21 oxidation (20 μ M) by CYP1A1 (A) and CYP1A2 (B). Incubation time was 20 min at
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23 37° C, the metabolites were determined after ethyl acetate extraction of the
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25 incubations by HPLC as described. The values are averages and standard deviations of
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27 triplicate incubations. Values significantly different from incubations containing
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29 CYP1A1/2 and NADPH:CYP reductase in a ratio of 1:0.05: * $p < 0.001$ (Student's t-
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31 test).
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38 Fig. 3. Cytochrome b_5 alters patterns of ellipticine metabolites formed by its oxidation with
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40 CYP1A1 (A,B) and 1A2 (C,D) under the 1:1 (A,C) and the 1:0.1 molar ratio of
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42 CYP1A1/2:NADPH:CYP reductase (B,D). The molar ratio of CYP1A1/1A2 to
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44 cytochrome b_5 was 1:5. Conditions were as described for Figure 2. The values are
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46 averages and standard deviations of triplicate incubations. Values significantly
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48 different from control (without cytochrome b_5); * $p < 0.001$ (Student's t-test).
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54 Fig. 4. Kinetics of ellipticine oxidation by CYP1A1 (A,B) and 1A2 (C,D) without (A,C) or
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56 with (B,D) cytochrome b_5 . Ellipticine concentrations between 1 and 20 μ M were
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58 incubated with the reconstituted liposomes containing CYP and reductase at a molar
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ratio of 1:1 in all incubations plus cytochrome b₅ at five fold higher concentrations than CYP in B and D. The other conditions were as described in Figure 2. The values are averages and standard deviations of triplicate incubations.

Fig. 5. Autoradiographic profile of ³²P-labelled DNA adducts generated in calf thymus DNA by ellipticine after its activation with reconstituted CYP1A1 without (A) and with cytochrome b₅ (CYP1A1:cytochrome b₅ 1:5) (B): of ³²P-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-hydroxyellipticine (E). Analyses were performed by the nuclease P1 version of the ³²P-postlabeling assay. Adduct spots 1-7 correspond to the ellipticine-derived DNA adducts. Besides adduct 2 formed by 12-hydroxyellipticine, another strong adduct (spot X in panel E), which was not found in any other activation systems or *in vivo* was generated.

Fig. 6. The effect of cytochrome b₅, apo-cytochrome b₅ and Mn-cytochrome b₅ on rates of ellipticine oxidation by CYP1A1 (A) and 1A2 (B). Conditions were as described for Figure 2 except that cytochrome b₅ (b₅), recombinant apo-cytochrome b₅ reconstituted with heme (rec. b₅), recombinant apo-cytochrome b₅ (apo-b₅) and recombinant apo-cytochrome b₅ reconstituted with Mn-protoporphyrin IX (Mnb₅) were added at five 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 b₅); **p*<0.001 (Student's t-test).

Fig. 7. Comparison of 3D structures of cytochrome b₅ (A,C) and apo-cytochrome b₅ (B,D) (based on PDB coordinates 1BFX [40] and 1I8C [41]). Heme loss results in substantial alteration of cytochrome b₅ secondary structure elements (A and B), for instance three alpha helices of apo-cytochrome b₅ are unfolded and the shape of apo-

1 cytochrome b₅ polypeptide backbone altered (B). Colored surfaces (C and D)
2 demonstrate the large differences in physico-chemical properties, i.e. surface shape
3 and charge distribution between the holo- and apo-form of cytochrome b₅.
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8 Fig. 8. Induction of cytochrome b₅ in livers of rats treated with 4 or 40 mg ellipticine per kg
9 body weight. Liver microsomes were isolated 48 hrs after treatment. Mean values ±
10 standard deviations shown in figure represent results obtained from livers of three rats
11 (N=3). Values significantly different from the control (untreated rats): **p*<0.001
12 (Student's t-test). Inset: immunoblots of cytochrome b₅ from untreated and ellipticine-
13 treated rats, respectively, stained with antibody against rat cytochrome b₅. Microsomes
14 isolated from rat livers were subjected to SDS-PAGE, proteins transferred to PVDF
15 membranes and probed with antibody as described in Material and Methods.
16 Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as loading control. F,
17 fold increase in cytochrome b₅ expression in rats treated with ellipticine compared to
18 control (uninduced) rats.
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Table 1 - Effect of cytochrome b₅ on kinetic parameters of ellipticine oxidation by CYP1A1 and 1A2

| CYP1A1 without b₅ | | | | | |
|-------------------------------------|--------------------------------------|------------------|------------------|----------------------------------|------------|
| Ellipticine metabolites | K _m (K _{0.5}) * | V _{max} | k _{cat} | k _{cat} /K _m | n |
| | μM | $\mu M/min^{-1}$ | min^{-1} | $min^{-1} \cdot \mu M^{-1}$ | |
| 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 b₅ | | | | | |
| 9-hydroxyellipticine | 0.5 ± 0.1 | 0.100 ± 0.003 | 0.50 ± 0.01 | 1.0 ± 0.2 | 1 |
| 12-hydroxyellipticine | 15 ± 1* | 3.0 ± 0.4 | 15 ± 2 | 1.0 ± 0.1 | 4.5 ± 0.7* |
| 13-hydroxyellipticine | 13.2 ± 0.4* | 1.8 ± 0.1 | 9.0 ± 0.5 | 0.68 ± 0.05 | 6.3 ± 0.9* |
| 7-hydroxyellipticine | 0.4 ± 0.2 | 0.020 ± 0.001 | 0.099 ± 0.007 | 0.3 ± 0.2 | 1 |
| CYP1A2 without b₅ | | | | | |
| 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 b₅ | | | | | |
| 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 “Experimental Procedures.” Kinetic parameters were determined from initial velocity data as described for N = 3. Average values ± SE are shown. b₅ = cytochrome b₅; n = Hill coefficient

* sigmoidal data curves were fitted using Hill equation

Table 2 - The effect of cytochrome b₅ on DNA adduct formation by ellipticine oxidized with CYP1A1 and 1A2

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

Values are given as means \pm 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 b₅): *p<0.001 (Student's t-test).

