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Cytochrome b_5 Increases Cytochrome P450 3A4-Mediated Activation of Anticancer Drug Ellipticine to 13-Hydroxyellipticine Whose Covalent Binding to DNA Is Elevated by Sulfotransferases and *N,O*-Acetyltransferases

Marie Stiborová,*[,†] Radek Indra,[†] Michaela Moserová,[†] Věra Černá,[†] Martina Rupertová,[†] Václav Martínek,[†] Tomáš Eckschlager,[‡] René Kizek,[§] and Eva Frei[∥]

[†]Department of Biochemistry, Faculty of Science, Charles University, Albertov 2030, 128 40 Prague 2, Czech Republic

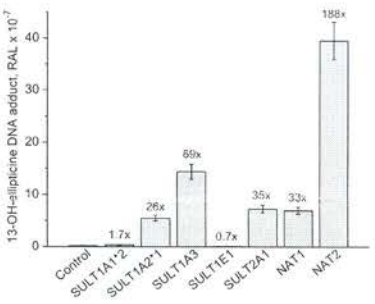
[‡]Department of Pediatric Hematology and Oncology, Charles University and University Hospital Motol, Prague, Czech Republic

[§]Department of Chemistry and Biochemistry, Faculty of Agronomy, Mendel University, Brno, Czech Republic

[∥]Division of Preventive Oncology, National Center for Tumor Diseases, German Cancer Research Center (DKFZ), 69120 Heidelberg, Germany

Supporting Information

ABSTRACT: The antineoplastic alkaloid ellipticine is a prodrug, whose pharmacological efficiency is dependent on its cytochrome P450 (P450)- and/or peroxidase-mediated activation in target tissues. The P450 3A4 enzyme oxidizes ellipticine to five metabolites, mainly to 13-hydroxy- and 12-hydroxyellipticine, the metabolites responsible for the formation of ellipticine-13-ylium and ellipticine-12-ylium ions that generate covalent DNA adducts. Cytochrome b_5 alters the ratio of ellipticine metabolites formed by P450 3A4. While the amounts of the detoxication metabolites (7-hydroxy- and 9-hydroxyellipticine) were not changed with added cytochrome b_5, 12-hydroxy- and 13-hydroxyellipticine, and ellipticine N^2-oxide increased considerably. The P450 3A4-mediated oxidation of ellipticine was significantly changed only by holo-cytochrome b_5, while apo-cytochrome b_5 without heme or Mn-cytochrome b_5 had no such effect. The change in amounts of metabolites resulted in an increased formation of covalent ellipticine-DNA adducts, one of the DNA-damaging mechanisms of ellipticine antitumor action. The amounts of 13-hydroxy- and 12-hydroxyellipticine formed by P450 3A4 were similar, but more than 7-fold higher levels of the adduct were formed by 13-hydroxyellipticine than by 12-hydroxyellipticine. The higher susceptibility of 13-hydroxyellipticine toward heterolytic dissociation to ellipticine-13-ylium in comparison to dissociation of 12-hydroxyellipticine to ellipticine-12-ylium, determined by quantum chemical calculations, explains this phenomenon. The amounts of the 13-hydroxyellipticine-derived DNA adduct significantly increased upon reaction of 13-hydroxyellipticine with either 3′-phosphoadenosine-5′-phosphosulfate or acetyl-CoA catalyzed by human sulfotransferases 1A1, 1A2, 1A3, and 2A1, or *N,O*-acetyltransferases 1 and 2. The calculated reaction free energies of heterolysis of the sulfate and acetate esters are by 10−17 kcal/mol more favorable than the energy of hydrolysis of 13-hydroxyellipticine, which could explain the experimental data.



INTRODUCTION

Ellipticine (5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole, Scheme 1), an alkaloid isolated from Apocynaceae plants, exhibits significant antitumor and anti-HIV activities [for a summary see refs 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 hematological 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 an overview, see refs 1−3].

It was suggested that the major mechanisms of antitumor, mutagenic, and cytotoxic activities of ellipticine 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 (P450) or peroxidases,[1−3,8−13] suggesting a third DNA-damaging mechanism of action. Two major DNA adducts generated from ellipticine-13-ylium and ellipticine-12-ylium (Scheme 1), by P450- and peroxidase-mediated 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]

The same DNA adducts were also detected in human cancer cells in culture, such as breast adenocarcinoma MCF-7,[14] the

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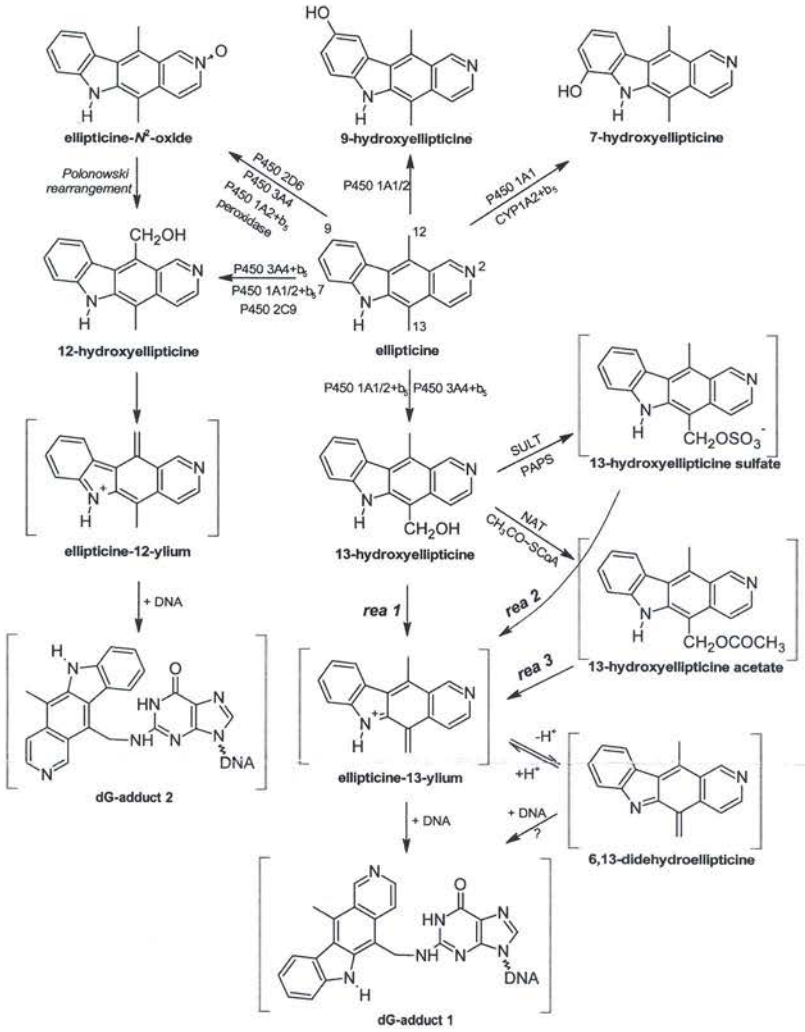
Scheme 1. Scheme of Ellipticine Metabolism by P450s and Peroxidases Showing the Identified Metabolites and Those Proposed to Form DNA Adducts[a]



[a]The compounds shown in brackets were not detected under the experimental conditions and/or not structurally characterized. The P450 enzymes predominantly oxidizing ellipticine shown in the figure were identified in this work and/or in our previous studies.[10,11,18,19].

leukemias 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 the expression of P450 1A1, 1B1, 3A4, lactoperoxidase, cyclooxygenase, or myeloperoxidase in these cells.[14−17] On the basis of these data, ellipticine might be considered a drug, whose pharmacological efficiency and/or genotoxic side effects are dependent on its activation by P450s and peroxidases in target tissues.

Because of the ellipticine-derived DNA adducts we found in healthy organs of rats, the risk of treating patients with this compound has to be considered. Our *in vivo* studies, however, demonstrated that ellipticine-DNA adducts did not persist in healthy tissues of rats, the experimental model mimicking the fate of ellipticine in humans[8,12] treated with ellipticine.[12] Therefore, these results suggest a relatively low risk of the genotoxic side effects to humans by ellipticine during the cancer treatment.

Of the P450s investigated, human P450 3A4 and rat P450 3A1 are the most active enzymes oxidizing ellipticine to 13-hydroxy- and 12-hydroxyellipticine, the reactive metabolites that dissociate to ellipticine-13-ylium and ellipticine-12-ylium which bind to DNA,[3,9,10] while the P450 1A isoforms preferentially form the other ellipticine metabolites, 9-hydroxy- and 7-hydroxyellipticine, which are the detoxication products (Scheme 1). As a consequence, ellipticine-DNA adduct levels formed by P450 1A are very low.[10,18,19] Recently, we found that cytochrome b_5 alters the ratio of ellipticine metabolites formed by isolated P450 1A1 and 1A2 reconstituted with NADPH:P450 reductase, favoring the formation of 12-hydroxy- and 13-hydroxyellipticine at the expense of 9-hydroxy- and 7-hydroxyellipticine. The change in metabolite ratio resulted in an increased formation of covalent ellipticine-DNA adducts.[19] This finding explained the previous apparent discrepancies we

found between isolated enzymes and *in vivo* studies, where P450 1A enzymatic activity correlated with ellipticine-DNA-adduct levels,[8] while isolated P450 1A1 or 1A2 in reconstituted systems was much less effective than P450 3A4.[1,10] This effect of cytochrome b_5 might be even more pronounced *in vivo* since, as we showed previously, ellipticine induces levels of both P450 1A[20] and cytochrome b_5 in rat liver[19] and in several cancer cell lines (i.e., glioblastoma and neuroblastoma cells).[16,17,21] In glioblastoma and neuroblastoma cells, ellipticine also induces P450 3A4,[17,21,22] the enzyme whose efficiency to oxidize several substrates including ellipticine is extensively influenced by cytochrome b_5.[10,23−25] Because P450 3A4 is so important for ellipticine activation, modulation of P450 3A4-catalyzed activation of ellipticine to 12-hydroxy- and 13-hydroxyellipticine by cytochrome b_5 is investigated here in detail. Mechanistic studies of the action of cytochrome b_5 on the catalysis of P450 3A4 were 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).

The *N*,*O*-acetyltransferase (NAT) and sulfotransferase (SULT) enzymes, which are expressed in several cancer cells,[26−33] can detoxify but also further activate the products of metabolism of xenobiotics. We therefore also examined whether these enzymes modify the DNA-adduct levels resulting from covalent binding of 13-hydroxyellipticine to DNA.

EXPERIMENTAL PROCEDURES

Caution: *Ellipticine is a potent mutagen and should be handled with care. Exposure to ^{32}P should be avoided, by working in a confined laboratory area, with protective clothing, plexiglass shielding, Geiger counters, and body dosimeters. Waste must be discarded according to appropriate safety procedures.*

Chemicals and Enzymes. $NADP^+$, NADPH, ellipticine, D-glucose 6-phosphate, D-glucose 6-phosphate dehydrogenase, hemin, acetyl-CoA, 3′-phosphoadenosine-5′-phosphosulfate (PAPS) and calf thymus DNA were obtained from Sigma Chemical Co. (St Louis, MO, USA); 9-hydroxyellipticine (5,11-dimethyl-9-hydroxy-6*H*-pyrido[4,3-*b*]-carbazole) was from Calbiochem (San Diego, CA, USA). All of these and other chemicals from commercial sources used in the experiments were of 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] Their authenticity was confirmed by UV spectroscopy, electrospray mass spectrometry, and high field ^1H NMR spectroscopy. The purity as analyzed by HPLC was >99.9%.[10] Supersomes isolated from insect cells transfected with baculovirus constructs containing cDNA of human P450 3A4 and expressing NADPH:P450 reductase were obtained from Gentest Corp. (Woburn, MI, USA) and tested for their efficiencies to oxidize ellipticine. Cytosolic extracts, isolated from insect cells transfected with baculovirus constructs containing human cDNA of SULT1A1*2, 1A2*1, 1A3, 1E, or 2A1 were obtained from Oxford Biomedical Research Inc. (Oxford, MA, USA), and those containing cDNA of human NAT1*4 or NAT2*4 were from Gentest (Woburn, MA, USA). Cytosolic extracts expressing SULT1A1 and SULT1A2 conjugated *p*-nitrophenol at rates of 124 and 5.5 nmol/min/mg protein, respectively; SULT1A3 conjugated dopamine at the rate of 8 nmol/min/mg protein; SULT1E conjugated estrone at the rate of 266 pmol/min/mg protein; and SULT2A1 conjugated dehydroepiandrosterone at the rate of 584 pmol/min/mg protein. Cytosolic extracts expressing NAT1 and NAT2 had a catalytic activity of 1,300 nmol/min/mg protein (substrate *p*-aminosalicylic acid) and 290 nmol/min/mg protein (substrate sulfamethazine), respectively. Enzyme activities in control cytosol were less than 10 pmol/min/mg protein. Enzymes and chemicals for the ^{32}P-postlabeling assay were obtained from the sources described.[1,10] All of these and other chemicals were of reagent grade or better.

Isolation of Cytochrome b_5 and Apo-cytochrome b_5. Cytochrome b_5 was isolated from rabbit liver microsomes by the procedure described by Roos.[34] The apo-cytochrome b_5 protein was prepared using heterologous expression in *Escherichia coli* as described in our earlier work.[35]

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 and in our previous paper.[35,36] Absorbance spectra of reconstituted cytochrome b_5 (from 350 to 500 nm) were recorded on a Hewlett-Packard 8453 UV spectrophotometer. The reconstitution of cytochrome b_5 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 an 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).[35,37]

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 $mM^{-1}\cdot cm^{-1}$,[38,39] or from the difference spectrum of reduced minus oxidized form, using molar extinction coefficient $\varepsilon_{424-409}$ = 185 $mM^{-1}\cdot cm^{-1}$.[39] The concentration of Mn-cytochrome b_5 was determined using an extinction coefficient of 57 $mM^{-1}\cdot cm^{-1}$ at 469 nm.[40]

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 NADPH of NADPH-generating system (1 mM $NADP^+$, 10 mM D-glucose 6-phosphate, and 1 U/mL D-glucose 6-phosphate dehydrogenase, to generate NADPH), human P450 3A4 in Supersomes (100 pmol) reconstituted with or without rabbit hepatic cytochrome b_5, apo-cytochrome b_5, or Mn-cytochrome b_5 (500 pmol), and 20 μM ellipticine dissolved in 5 μL methanol. In the control incubation, either P450 or ellipticine was 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[18,41]), the reaction was stopped, phenacetin added as internal standard, and metabolites extracted, separated, and quantified by HPLC as described.[10,18,41]

Incubation mixtures used to analyze DNA-adduct formation by ellipticine were as described above but contained 100 μM ellipticine dissolved in 5 μL of methanol, 1 mM NADPH instead of the NADPH generating system, and 1 mg of 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 without (i) P450, (ii) NADPH, or (iii) ellipticine. After the incubation, DNA was isolated from the residual water phase by the phenol/chloroform extraction method as described.[1] The extracted DNA was then dissolved in 200 μL of distilled water and the DNA content determined spectrophotometrically. The 260/280 nm ratios of DNA were 1.8. The isolated DNA was used to detect and quantify DNA adducts by the ^{32}P-postlabeling assay (see below).

Incubations in which 13-hydroxyellipticine was used instead of ellipticine, contained in a final volume of 500 μL: 50 mM potassium phosphate buffer (pH 5.0, 6.0, 7.0, 7.4, 8.0 and 8.4), 100 μM 13-hydroxyellipticine dissolved in 5 μL of methanol, and 1 mg of calf thymus DNA. After the 90 min incubations (37 °C) and ethyl acetate extraction, DNA was isolated from the residual water phase by the phenol/chloroform extraction method as described.[1] Incubation mixtures used to analyze the effect of SULTs and NATs on the 13-hydroxyellipticine-derived DNA adducts contained in a final volume of 500 μL, 50 mM potassium phosphate buffer (pH 7.4), 1 mg of calf

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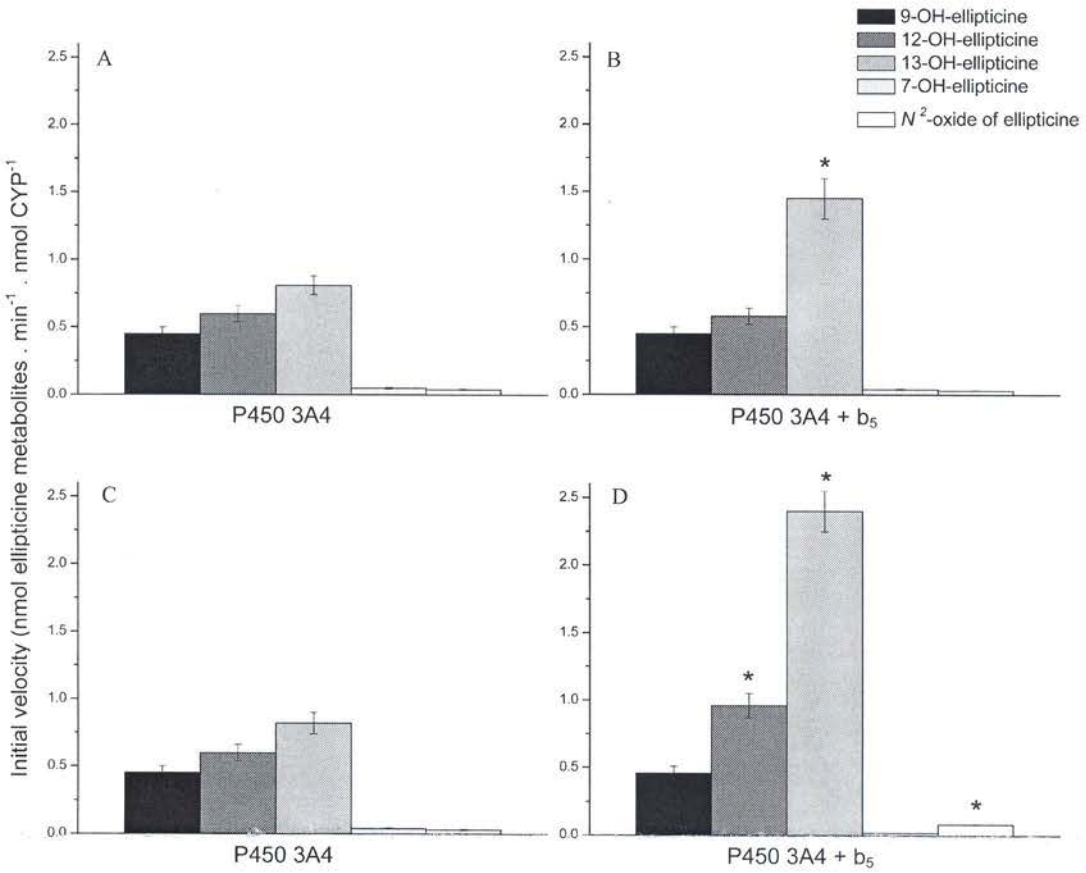

Figure 1. Effect of cytochrome b_5 (B and D) on the initial velocity of ellipticine oxidation to 9-hydroxyellipticine, 12-hydroxyellipticine, 13-hydroxyellipticine, 7-hydroxyellipticine, and ellipticine N^2-oxide by P450 3A4 in the presence of the NADPH-generating system (B) and the same enzyme in the presence of NADPH (D) compared to incubations without cytochrome b_5 (A, NADPH regenerating system; C, NADPH added). Assays were performed as described under Experimental Procedures. Average values ± SD are shown ($N = 3$). Values significantly different from the control (without cytochrome b_5); *$p < 0.001$ (Student's t-test).

thymus DNA, and 1 μM 13-hydroxyellipticine either in the presence or absence of 39 nmol human SULT1A1*2, 1A2*1, 1A3, 1E, or 2A1 and 100 μM PAPS or NAT1 or NAT2, and 2 mM acetyl-CoA. After the 90 min incubations (37 °C) and ethyl acetate extraction, DNA was isolated from the residual water phase by the phenol/chloroform extraction method as described.[1] The isolated DNA was used to detect and quantify DNA adducts by the ^{32}P-postlabeling assay (see below).

^{32}P-Postlabeling Analysis and HPLC Analysis of ^{32}P-Labeled 3′,5′-Deoxyribonucleoside Bisphosphate Adducts. The ^{32}P-postlabeling of nucleotides using the 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 analyses were done as reported recently.[1,3,8−13]

Quantum Chemical Calculations. Reaction free energies for heterolytic dissociation of 13-hydroxyellipticine and its conjugates [reactions 1−3 (rea 1, rea 2, and rea 3) in Scheme 1] and for an analogous reaction of 12-hydroxyellipticine were calculated by an *ab initio* quantum chemical approach. The accuracy of absolute reaction free energies is better, if electron correlation effects are considered during the calculation. In the present study, we evaluated the heterolytic dissociation of 12-/13-hydroxyellipticine and the 13-hydroxyellipticine conjugates, which are reactions conserving the number of paired electrons (isogyric reactions). This fact facilitates the calculation of thermochemical data even at relatively low levels of theory, lacking the electron correlation effects, Hartree−Fock (HF). The heterolytic dissociation, however, involves charged species that are best modeled with basis sets containing polarization functions since the charge distorts the electron distribution. In addition, anions require inclusion of diffuse functions. Therefore, the geometry optimizations and frequency calculations of all reactants and products studied here were performed on the HF level of theory in conjunction with the 6-31++G(d,p) basis set using the Gaussian 09 package.[42]

Accurate computation of solvation energies of charged species is still a challenge. Therefore, several approaches to estimate solvent effects were evaluated. Initially, the solvent effect was estimated by performing energy optimizations using the Conductor-like Polarizable Continuum Model (C-PCM) with default atomic radii.[43] The thermal corrected Gibbs free energies (at 298 K) were obtained from electronic calculations and harmonic vibration frequencies of these optimized structures. The resulting free energies were also corrected to represent the biochemically relevant reference state: pH 7 and 1 M concentration for all reactants except for water, for which the reference concentration was 55.3 M.[44] The reaction Gibbs free energies of individual reaction steps ($\Delta G^{0'}_{rea}$) evaluated here were calculated as the total energies of products minus the total energies of reactants.

Solvation free energies of all reactants and products were also calculated with the Langevin dipole solvation model (LD).[45] Merz−Kollman partial atomic charges obtained from *ab initio* calculations served as input for the LD model built in the ChemSol program, v2.1.[46] The standard (biochemical) free energy changes of individual

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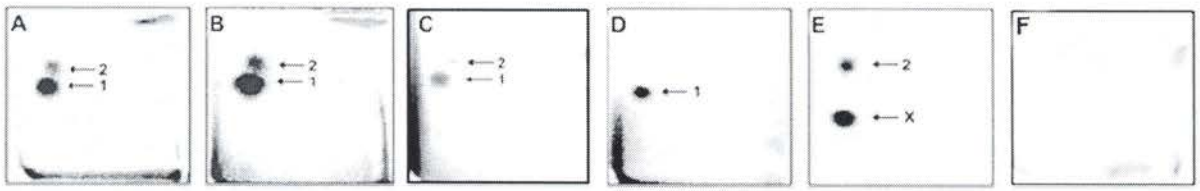

Figure 2. Autoradiographic profile of ^{32}P-labeled DNA adducts generated in calf thymus DNA by ellipticine after its activation with P450 3A4 without (A) and with cytochrome b_5 (P450 3A4/cytochrome b_5 1:5) (B); of ^{32}P-labeled digests of DNA of mammary adenocarcinoma of Wistar rats treated i.p. with 4 mg of ellipticine per kilogram body weight (C); and from calf thymus DNA reacted with 13-hydroxyellipticine (D) or 12-hydroxyelipticine (E). Autoradiographic profile of calf thymus DNA incubated with ellipticine after its activation with P450 3A4 without NADPH (control incubation) (F). Analyses were performed by the nuclease P1 version of the ^{32}P-postlabeling assay. Adduct spots 1 and 2 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*,[3,63] was generated.

reaction steps were then calculated according to a formula used previously:[47,48]

$$\Delta G_{\text{rea}}^{0'wat(LD)} = \sum \Delta G_{\text{products}}^{0'gas} - \sum \Delta G_{\text{reactants}}^{0'gas} + \sum \Delta G_{\text{products}}^{solvLD} - \sum \Delta G_{\text{reactants}}^{solvLD}$$

where $\Sigma\Delta G^{0gas}$ denotes the sum of corrected Gibbs free energies of products or reactants in gas phase, and $\Sigma\Delta G^{solvLD}$ refers to the sum of solvation free energies of the products or reactants calculated with the LD method. The similar combination of the *ab initio* (HF) gas phase free energy and LD solvation energy was successfully employed in free energy calculations of other carcinogens (aflatoxin B1, vinyl chloride, and acrylamide) and well reproduced the experimentally determined energies.[49−51]

Finally, we combined LD solvation energies with experimental solvation energies for H_2O, H_3O^+, and OH^-. A similar approach has been previously employed for the calculation of pK_a for carboxylic acids.[52]

RESULTS

Cytochrome b_5 Modulates Ellipticine Oxidation Catalyzed by Cytochromes P450 3A4. The human P450 3A4 enzyme in the presence of NADPH:P450 reductase and its cofactor NADPH oxidizes ellipticine to five metabolites, 7-hydroxy-, 9-hydroxy-, 12-hydroxy-, and 13-hydroxyellipticine and the N^2-oxide of ellipticine (Figure 1A and C and Supporting Information, Table 1), which are also formed by human, rat, rabbit, and mouse hepatic microsomes.[10,13,18,41] 13-Hydroxy- and 12-hydroxyellipticine, two reactive ellipticine metabolites forming DNA adducts,[10,11] and 9-hydroxyellipticine, a detoxication product of ellipticine,[10,18] were the predominant metabolites formed by P450 3A4. Another activation metabolite, ellipticine N^2-oxide, a precursor of 12-hydroxyellipticine,[10,11] and a further detoxication product, 7-hydroxyellipticine, were formed to a much lower extent.

The patterns and amounts of ellipticine metabolites generated by P450 3A4 changed significantly when cytochrome b_5 was present in the P450 3A4 enzymatic system (Figure 1B and D, and Supporting Information, Table 1). While the amounts of the detoxication products of ellipticine (7-hydroxy- and 9-hydroxyellipticine) were not changed with added cytochrome b_5, 12-hydroxy- and 13-hydroxyellipticine, and ellipticine N^2-oxide increased considerably. Up to a 1.6-, 2.9-, and 2.7-fold more 12-hydroxy- and 13-hydroxyellipticine and ellipticine N^2-oxide, respectively, were found with added cytochrome b_5 and NADPH (Figure 1D). A lower increase in these metabolites was, however, found when the NADPH-generating system was used instead of NADPH (Figure 1B). Glucose 6-phosphate dehydrogenase of the NADPH-generating system added to the incubation mixtures might act as a protein scavenger binding the reactive species generated from these metabolites.

Cytochrome b_5 Increases the Ellipticine-Derived DNA Adduct Formation Mediated by Cytochrome P450 3A4. 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 Scheme 1 for the structures of two deoxyguanosine adducts 1 and 2) were detected in the calf thymus DNA incubated with this drug and P450 3A4 with NADPH:P450 reductase and NADPH (Figure 2A,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 P450s,[1,9,10] or peroxidases,[11] or after treatment of cells in culture with this anticancer drug,[14−17,22] or *in vivo* in several organs of rats including mammary adenocarcinoma[3,8,12] (Figure 2C), and mice[13] exposed to this agent. These adducts are generated from ellipticine-13-ylium and ellipticine-12-ylium (Scheme 1), the reactive species formed from the corresponding hydroxyellipticines (Figure 2D,E) as confirmed by cochromatographic analysis using TLC and HPLC.[10,11,53] More than 7-fold higher levels of adduct 1 than adduct 2 are formed by the P450 3A4 enzymatic system, whereas only 1.3-fold more 13-hydroxyellipticine (species responsible for formation of adduct 1) than 12-hydroxyellipticine generating adduct 2 (compare Figure 1 and Supporting Information, Table 1) was determined in incubations with P450 3A4.

The increased levels of 12-hydroxy- and 13-hydroxyellipticine formed by the P450 3A4 enzymatic system containing cytochrome b_5 and NADPH resulted in increased levels of these two ellipticine-derived DNA adducts (Table 1). The presence of cytochrome b_5 resulted in 2.9-fold higher levels of the ellipticine-DNA adduct 1, which correlated with a 2.9-fold increased formation of 13-hydroxyellipticine (Table 1 and Figure 1). 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 P450 3A4 (Table 1, Figure 1, and Supporting Information, Table 1).

Modulation of Ellipticine Oxidation by Cytochromes P450 3A4 Is Dependent on Holo-cytochrome b_5. In order to investigate the mechanism of cytochrome b_5-mediated modulation of ellipticine oxidation by P450 3A4, we also examined the influence of apo-cytochrome b_5 and Mn-cytochrome b_5. As shown in Figure 3, the P450 3A4-mediated

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Table 1. Effect of Cytochrome b_5 on DNA Adduct Formation by Ellipticine Oxidized with Human P450 3A4 and NADPH[a]

activating system	relative adduct labeling (mean ± SD/10^7nucleotides) adduct 1	adduct 2	total
P450 3A4 + NADPH:P450 reductase	3.6 ± 0.342	0.5 ± 0.1	4.1 ± 0.4
P450 3A4 + NADPH:P450 reductase + cytochrome b_5 (1:5)	10.5 ± 0.5*	0.9 ± 0.1*	11.4 ± 0.8*

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

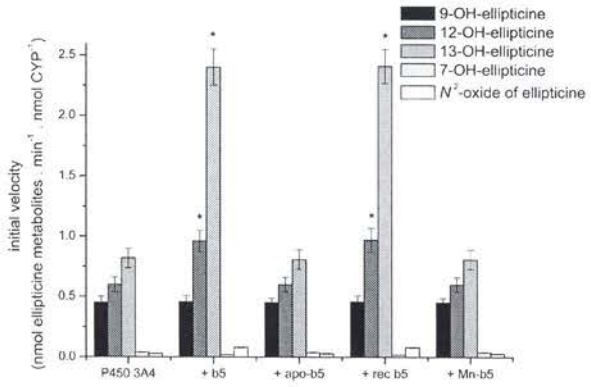

Figure 3. Effect of cytochrome b_5, apo-cytochrome b_5, apo-cytochrome b_5 reconstituted with heme, and Mn-cytochrome b_5 on the initial velocity of ellipticine oxidation to 9-hydroxyellipticine, 12-hydroxyellipticine, 13-hydroxyellipticine, 7-hydroxyellipticine, and ellipticine N^2-oxide by P450 3A4 in the presence of NADPH. Assays were performed as described under Experimental Procedures. Average values ± SD are shown ($N = 3$). Values significantly different from control (without cytochrome b_5); *$p < 0.001$ (Student's t-test).

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 the heme cofactor was without such effects (Figure 3 and Supporting Information, Table 2). We also employed a structurally similar analogue of cytochrome b_5, known to have limited electron transfer capability, Mn-cytochrome b_5.[37,54] 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 electron transfer capability.[22,54] Also in the case of Mn-cytochrome b_5, no changes in ellipticine oxidation were found (Figure 3 and Supporting Information, Table 2).

Modulation of Levels of DNA Adducts Formed by 13-Hydroxyellipticine by SULT and NAT Enzymes. Formation of the deoxyguanosine adduct generated from 13-hydroxyellipticine with DNA *in vitro* is pH-dependent; only low levels of this DNA adduct were detectable at pH 5.0, while increasing the pH resulted in a pronounced increase in the formation of this adduct (Table 2). Exposure of 13-hydroxyellipticine to either SULT or NAT enzymes in the presence of cofactors of these enzymes (PAPS, acetyl-CoA) greatly increases DNA adduction; the amount of 13-hydroxyellipticine-derived DNA adduct increased significantly. The different human SULTs showed vastly differing activities leading up to 68.5-fold more

Table 2. pH Effect on Adduct Levels Formed by Reaction of 13-Hydroxyellipticine with DNA[a], Detected by ^{32}P-Postlabeling[b]

pH	relative adduct labeling (mean ± SD/10^7 nucleotides)
5.0	0.01 ± 0.001
6.0	1.3 ± 0.3
7.0	17.0 ± 1.2
7.4	23.3 ± 2.0
8.0	41.0 ± 2.9
8.4	49.7 ± 3.2

[a]See adduct spot 1 in Figure 1. [b]Experimental conditions are as described in Experimental Procedures, except that 50 μM 13-hydroxyellipticine and 1 mg of calf thymus DNA (37 °C, 90 min) were used. Relative adduct labeling and standard deviations were obtained from triplicate determinations.

DNA adducts catalyzed by SULT1A3 than without a SULT enzyme (Figure 4). In contrast, 13-hydroxyellipticine seems not

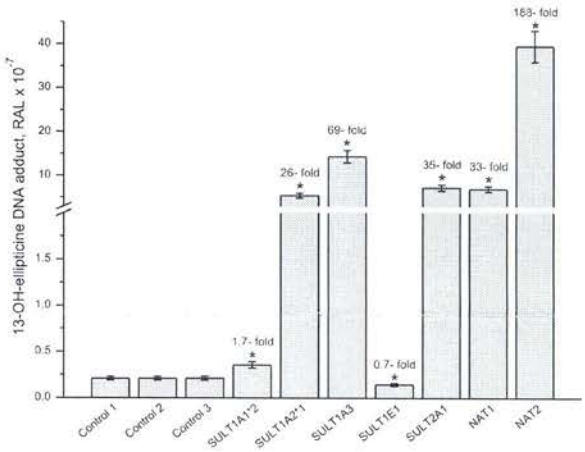

Figure 4. Effect of SULTs and NATs on DNA adduct formation by 13-hydroxyellipticine. Mean values ± standard deviations shown in the figure represent results obtained from three parallel incubations ($N = 3$). Controls 1, 2, and 3 represent the incubation without enzymes (control 1), the incubation with SULT1A3 without PAPS (control 2), and that with NAT2 without acetyl-CoA (control 3). Numbers above bars are fold increases over the control. Values significantly different from the control (without conjugation enzymes): *$p < 0.001$ (Student's t-test).

to be a substrate of SULT2E1 because this SULT did not stimulate the formation of the DNA adduct. NAT2 was by far the most active conjugation enzyme leading to 188-fold higher DNA adduct levels, while NAT1 increased these 33-fold. No increase in levels of the DNA adduct was found when enzyme cofactors PAPS or acetyl-CoA were not present in incubations (Figure 4), indicating that formation of the sulfate and acetate esters (conjugates) is responsible for the increase in levels of the 13-hydroxyellipticine-derived DNA adduct. In addition, these results suggest that sulfate and acetate esters of 13-hydroxyellipticine are more potent proximate mutagens than 13-hydroxyellipticine itself. This might be due to easier heterolytic dissociation of the conjugates in comparison to the heterolysis of parental 13-hydroxyellipticine.

Calculation of Heterolytic Cleavage of 12-Hydroxy- and 13-Hydroxyellipticine and Their Sulfate or Acetate

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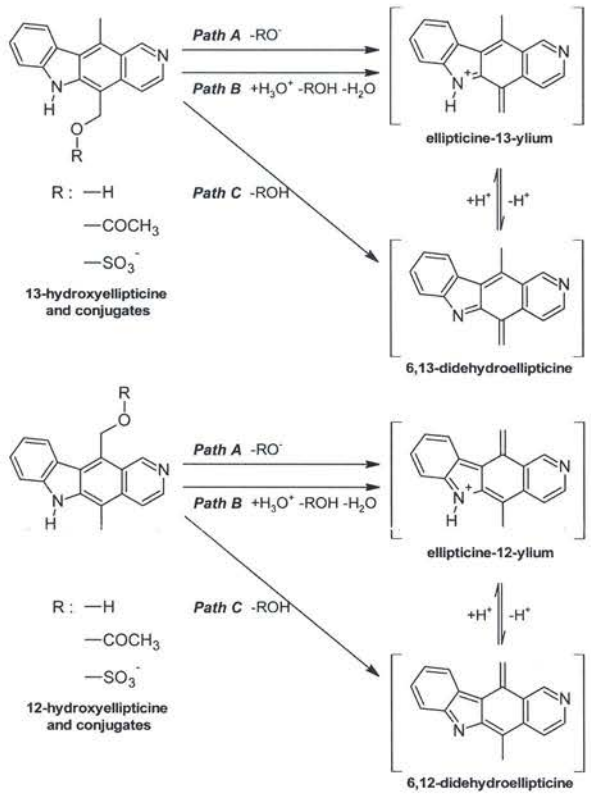
Conjugates: Thermodynamic Approaches. First, the viability of heterolytic dissociation of 12-hydroxy- and 13-hydroxyellipticine was compared using quantum chemical calculations and three different approaches to solvation modeling. For these reactions, we initially considered two paths leading directly to ellipticine-12-ylium and ellipticine-13-ylium, paths A and B in Scheme 2, and one indirect path,

Scheme 2. Proposed Paths of Heterolytic Cleavage of 12/13-Hydroxyellipticine and Their Sulfate and Acetate Conjugates That Were Considered in the Theoretical Section



denoted as path C in Scheme 2. This paths leads to the formation of 6,12-didehydroellipticine and 6,13-didehydroellipticine that represent deprotonated forms of ellipticine-12/13-ylium species. Regardless of the path, results indicated significantly higher susceptibility of 13-hydroxyellipticine toward heterolytic dissociation in comparison to 12-hydroxyellipticine (see difference in reaction free energies $\Delta\Delta G^{0,}_{rea\ 12-13OH}$ shown in Table 3). The major portion of the difference comes from the lower free energy (higher stability) of ellipticine-13-ylium over ellipticine-12-ylium, −10 and −12 kcal/mol for C-PCM and LD solvation models, respectively.

Paths A and B should in theory, after correction to pH 7, predict identical reaction free energies. They, however, vary significantly, although the discrepancy decreases when the solvation model is improved. We considered the average over paths A and B to make the best estimate of the actual reaction energy within estimated error (Table 3). The major error probably comes from the absence of electron correlation effects and from inaccuracies in the prediction of solvation free energies of charged species participating in these reactions: H_3O^+, OH^-, ellipticine-12-ylium, and ellipticine-13-ylium ions.

Reaction path C that represents the formation of 6,12- and 6,13-didehydroellipticines does not involve charged species. Thus, the absolute free energies of these reactions are almost independent of the solvation model (column C in Table 3). The small positive reaction free energy (5.9–9.3 kcal/mol) indicates that low amounts of the 6,13-didehydroellipticine metabolite in the incubation mixture are expected (0.01–0.0001% of 13-hydroxyellipticine), while the formation of 6,12-didehydroellipticine is not favored due to the high positive reaction free energy of its formation, 24–28 kcal/mol.

The results also support the generally accepted concept that proton-mediated hydrolysis of these compounds is the major mechanism leading to ellipticine-12-ylium and ellipticine-13-ylium (Scheme 2). The oxonium ion destabilizes the bond between carbon 12 or 13 and oxygen of the hydroxyl group, and two molecules of water (from the oxonium ion and the hydroxyl group) are formed. These findings indicate a preferential hydrolysis of 12-hydroxy- and 13-hydroxyellipticine under acidic conditions.

While 7-fold higher levels of adduct 1 than adduct 2 are formed by the P450 3A4 from ellipticine, levels of 13-hydroxyellipticine forming adduct 1 are only 1.3-fold higher than those of 12-hydroxyellipticine generating adduct 2 (Figure 1 and Supporting Information, Table 1). Several reasons might be responsible for the different potency of 13-hydroxyellipticine and 12-hydroxyellipticine to form DNA adducts. The dissociation (hydrolysis) of both hydroxyellipticines to the cationic yllium might be one of the reasons (Scheme 1). Indeed, as shown in Table 3, showing differences in standard reaction free energies calculated by quantum chemical approach, hydrolysis of 13-hydroxyellipticine is more favorable than that of 12-hydroxyellipticine.

In addition, the experimental data showed that reactions of 13-hydroxyellipticine with either PAPS or acetyl-CoA catalyzed by several SULTs or NATs enhanced DNA adduct 1 levels. Calculated reaction free energies of heterolysis of the sulfate

Table 3. Reaction Free Energies of Heterolytic Dissociation of 12-Hydroxy- and 13-Hydroxyellipticine Calculated by *ab Initio* Quantum Chemical Approaches in Combination with Different Solvation Models

solvation model	C-PCM		LD (ChemSol)		LD with exp. solv. for H_2O, H_3O^+ and OH^-	
reaction path (Scheme 2)	A and B average ± error	C	A and B average ± error	C	A and B average ± error	C
$\Delta G^{0,}_{rea\ 12OH}$ [a]	21 ± 40	26	9 ± 11	25	22 ± 3	28
$\Delta G^{0,}_{rea\ 13OH}$ [a]	10 ± 40	7.1	−5 ± 11	5.9	8 ± 3	9.3
$\Delta\Delta G^{0,}_{rea\ 12-13OH}$	11	19	14	19	14	19
$\Delta pK'_{eq\ 12-13OH}$ [b]	8	14	10	14	10	14

[a]Standard biochemical reaction free energies in kcal/mol. [b]Difference in equilibrium constants (12-OH- minus 13-OH-ellipticine).

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Table 4. Comparison of Reaction Free Energies of Heterolytic Dissociation of 12/13-Hydroxyellipticine, 12/13-Hydroxyellipticine Sulfate, and Acetate Conjugates Calculated by *ab Initio* Quantum Chemical Approaches in Combination with Different Solvation Models [13-Hydroxyellipticine and Its Esters (A), and 12-Hydroxyellipticine and Its Esters (B)]

(A)

solvation model	C-PCM		LD (ChemSol)		LD with exp. solv. for H_2O, H_3O^+ and OH^-	
reaction path (Scheme 2)	A and B average ± error	C	A and B average ± error	C	A and B average ± error	C
$\Delta G^{0,}_{rea\ 13OH}$ [a]	9.7 ± 40	7.1	−5.0 ± 11	5.9	8 ± 3	9.3
$\Delta G^{0,a}_{rea\ 13OSO_3^-}$	−3.8 ± 30	−1.9	−16 ± 5	−1.3	−9 ± 6	−1.3
$\Delta G^{0,a}_{rea\ 13OAc}$	−0.5 ± 30	3.7	−15 ± 3	1.1	−8 ± 8	1.1

(B)

solvation model	C-PCM		LD (ChemSol)		LD with exp. solv. for H_2O, H_3O^+ and OH^-	
reaction path (Scheme 2)	A and B average ± error	C	A and B average ± error	C	A and B average ± error	C
$\Delta G^{0,a}_{rea\ 12OH}$	21 ± 40	26	9 ± 11	25	22 ± 3	29
$\Delta G^{0,}_{rea\ 12OSO_3^-}$ [a]	4.4 ± 30	16	−1.1 ± 5	21	6.5 ± 6	21
$\Delta G^{0,}_{rea\ 12OAc}$ [a]	−8.8 ± 30	21	−4.2 ± 3	19	3.4 ± 8	19

[a]Standard biochemical reaction free energies in kcal/mol.

and acetate esters that should be formed during these reactions are by 11−17 kcal/mol more favorable than for hydrolysis of 13-hydroxyellipticine (Table 4A). Therefore, while only a small portion of 13-hydroxyellipticine could be present in the form of ellipticine-13-ylium at pH 7, the negative free energy of heterolysis indicates that under the equilibrium conditions at pH 7 the major portion of sulfate or acetate esters (more than 99%) is dissociated to ellipticine-13-ylium that forms the DNA adduct. This is consistent with the experimental finding that whereas high levels of the 13-hydroxyellipticine-derived DNA adduct were found in experimental animals exposed to ellipticine,[3,8,12,13] no 13-hydroxyellipticine sulfates and acetate conjugates were detected in these animals.[55,56]

Reaction free energies calculated for elimination (path C) of the sulfate and acetate esters are predicted to be 7−10 and 5−8 kcal/mol, respectively, lower than that of the OH group, indicating that a significant portion (10 to 90%) of these conjugates could be present as 6,13-didehydroellipticine. Although path C is less preferred at pH 7, it is virtually pH independent and could contribute to DNA adduct formation under alkaline conditions.

Heterolytic dissociation of the 12-hydroxyellipticine sulfate or acetate esters is favored by 10−30 kcal/mol in comparison to their parental compound 12-hydroxyellipticine (Table 4B). Therefore, while heterolysis of 12-hydroxyellipticine is unfavored, the dissociation of its conjugates is feasible. Free energy for the corresponding hydrolytic reaction is predicted to be around zero, indicating the presence of significant amounts of the reactive species (ellipticine-12-ylium) (Table 4B). The 12-hydroxyellipticine sulfate and acetate conjugates are, however, less prone to hydrolysis (represented by paths A and B) than 13-hydroxyellipticine conjugates, with differences ranging from 8−16 kcal/mol.

Free energy for the formation of 6,12-didehydroellipticine from the 12-hydroxyellipticine sulfate and acetate esters, reaction path C, is predicted to be unfavored (16−21 kcal/mol, Table 4B), indicating the absence of 6,12-didehydroellipticine under the conditions considered here.

DISCUSSION

The results of the present work show that P450 3A4 preferentially oxidizes ellipticine to 13-hydroxy- and 12-hydroxyellipticine, the metabolites responsible for the formation of DNA adducts.[10,11] In this study, we suggest three possible reaction paths leading to the formation of the reactive species from these metabolites; direct heterolytic dissociation (hydrolysis), heterolytic dissociation (hydrolysis) after attack by the oxonium ion; and formation of 6,12- and 6,13-didehydroellipticines (see reaction paths A−C in Scheme 2). The oxonium ion-mediated hydrolysis of these compounds is probably the favorable mechanism responsible for the formation of ellipticine-12-ylium and ellipticine-13-ylium (path B in Scheme 2).

If this path were the rate limiting step of DNA adduct formation from 13-hydroxyellipticine, then DNA adduct levels should be higher under acidic conditions. This is not the case since only low levels of this DNA adduct were detectable at pH 5.0, while under alkaline conditions considerably higher amounts of this adduct were formed. Not only the hydrolysis of 13-hydroxyellipticine but also the reactivity of ellipticine-13-ylium toward the amino group of guanine in DNA seems to be important. This finding supports the suggested structure of 13-hydroxyellipticine-mediated deoxyguanosine adduct, where ellipticine-13-ylium is bound to the amino group in position 2 of guanine (Scheme 1). A decrease in pH leads to changes in nucleobase protonation in the DNA chain, causing a decrease in its nucleophilicity, essential for binding of ellipticine-13-ylium.

In the system containing only P450 3A4 with NADPH:P450 reductase, approximately the same levels of 13-hydroxy- and 12-hydroxyellipticine metabolites were found. However, more than 7-fold higher levels of adduct 1 than adduct 2 are formed in this enzymatic system. This finding might be explained by the preference in the dissociation (hydrolysis) of 13-hydroxyellipticine to ellipticine-13-ylium in comparison to that of 12-hydroxyellipticine to ellipticine-12-ylium. The major part of the difference comes from lower (10−12 kcal/mol) free energy of carbo-cation derived from 13-hydroxyellipticine (ellipticine-13-ylium).

The results of this study also demonstrate that the presence of cytochrome b_5 leads to more DNA-adducts, predominantly the adduct with 13-hydroxyellipticine. Therefore, besides the stimulating effect of cytochrome b_5 on P450 1A1-mediated oxidation of ellipticine to 12-hydroxy- and 13-hydroxyellipticine

that was found previously,[19] this heme protein also seems to play a key role in the P450 3A4-mediated DNA-damage caused by ellipticine. In both cases, addition of cytochrome b_5 to the P450s shifts ellipticine oxidation to activation at the expense of detoxication. To further confirm these *in vitro* results, a cytochrome b_5-knockout cell model is planned to be used to analyze the oxidation products of ellipticine and ellipticine-derived DNA adducts.

The effect of cytochrome b_5 upon ellipticine activation may be even more pronounced *in vivo* because as we found previously,[19] ellipticine induces levels of cytochrome b_5 in the liver of rats treated with this drug. Moreover, ellipticine also induces expression of *P450 3A4* mRNA and P450 3A4 protein in several cancer cell lines,[17,21] and as a ligand of aryl hydrocarbon receptor,[57] it also induces P450 1A1/2,[17,20−22] the enzyme that in the presence of cytochrome b_5 also activates ellipticine.[19] Hence, all these ellipticine-mediated induction effects produce concerted regulatory effects of this drug on its own metabolism. To quantify the involvement of cytochrome b_5 in ellipticine metabolism *in vivo*, we plan to use cytochrome b_5-knockout mice.[58,59]

Generally, two mechanisms of cytochrome b_5-mediated modulation of P450 catalysis have been suggested by several authors; it can affect the P450 catalytic activities by donating the second electron to P450 in a P450 catalytic cycle and/or by acting as an allosteric modifier of the oxygenase (for reviews, see refs 23−25 and 60). The mechanism(s) underlying such allosteric effects, based on reports that apo-cytochrome b_5 can stimulate P450 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 P450 enzyme.[23−25,60−62] The results found in this study show that modulation of ellipticine oxidation by P450 3A4 is induced only by the holo-cytochrome b_5. These findings indicate a high specificity of interaction of P450 3A4 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 P450 3A4 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. We described the properties and configurations of cytochrome b_5 and its apo-form extensively in our earlier paper.[19] 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 (see Figure 7 in our previous study[19]). This structural change results in significant alterations in surface geometry and electrostatic properties of apo-cytochrome b_5, preventing adequate protein−protein interactions of cytochrome b_5 with P450 3A4. Moreover, 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 without electron transfer ability. These findings are similar to the ones with P450 1A1/2, where also the holo-cytochrome b_5 was the only active species, and neither apo-cytochrome b_5 nor the enzyme with Mn-protoporphyrin IX was active. All these results demonstrate that both the natural 3D structure of the cytochrome b_5 protein, dictating optimal conformational states of the P450-cytochrome b_5 complex, and the presence of the protoporphyrin IX bonded-Fe ions as an electron transfer agent are necessary for the observed effects.[19]

Reactions of 13-hydroxyellipticine with either PAPS or acetyl-CoA catalyzed by several SULTs and NAT1 and NAT2 leads to higher 13-hydroellipticine-DNA adduct levels. As follows from calculated reaction free energies of heterolysis of the sulfate and acetate esters of 13-hydroxyellipticine that should be formed during such reactions, this finding might be explained by substantially easier hydrolysis of these esters than 13-hydroxyellipticine itself (compare Table 4A). The human conjugation enzymes used show widely varying substrate specificities. NAT2 was the most active enzyme, while 13-hydroxyellipticine was no substrate for SULT1E1. The amount of protein added to the incubations was always the same, but the specific activities of the individual enzymes varied greatly. This variation of substrate specificity is also reflected with 13-hydroxyellipticine as substrate.

The data shown here, together with our earlier work with reconstituted P450 1A1 and 1A2,[19] emphasize the importance of investigating enzyme complexes in xenobiotic metabolism to evaluate the role of the different components in the balance between activation and detoxication. In addition, as was seen by us and others earlier,[26,28,29,47,48] the role of conjugation enzymes in activation of not only aromatic hydroxylamines but also hydroxylated methyl derivatives of aromatics has to be carefully investigated. Computational chemistry in conjunction with detailed experiments is a very valuable tool to determine thermodynamic equilibriums to explain differences in reactivity also in biological systems.

■ ASSOCIATED CONTENT

Supporting Information

Effect of cytochrome b_5 on initial velocity of ellipticine oxidation and effect of cytochrome b_5, apo-cytochrome b_5, apo-cytochrome b_5 reconstituted with heme and Mn-cytochrome b_5 on initial velocity of ellipticine oxidation. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +420 221951285. Fax: +420 221951283. E-mail: stiborov@natur.cuni.cz.

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Notes

The authors declare no competing financial interest.

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■ DEDICATION

This work is dedicated to Professor Gustav Entlicher on the occasion of his 70th birthday.

ABBREVIATIONS

HPLC, high-performance liquid chromatography; i.p., intraperitoneal; P450, cytochrome P450; PAPS, 3′-phosphoadenosine-5′-phosphosulfate; PEI-cellulose, polyethylenimine-cellulose; RAL, relative adduct labeling; r.t., retention time; TLC, thin layer chromatography

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