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The anticancer agent ellipticine on activation by cytochrome P450 forms covalent DNA adducts☆

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Abstract

Ellipticine is a potent antitumor agent whose mechanism of action is considered to be based mainly on DNA intercalation and/or inhibition of topoisomerase II. Using [^{3}H]-labeled ellipticine, we observed substantial microsome (cytochrome P450)-dependent binding of ellipticine to DNA. In rat, rabbit, minipig, and human microsomes, in reconstituted systems with isolated cytochromes P450 and in Supersomes™ containing recombinantly expressed human cytochromes P450, we could show that ellipticine forms a covalent DNA adduct detected by [^{32}P]-postlabeling. The most potent human enzyme is CYP3A4, followed by CYP1A1, CYP1A2, CYP1B1, and CYP2C9. Another minor adduct is formed independent of enzymatic activation. The [^{32}P]-postlabeling analysis of DNA modified by activated ellipticine confirms the covalent binding to DNA as an important type of DNA modification. The DNA adduct formation we describe is a novel mechanism for the ellipticine action and might in part explain its tumor specificity.

Keywords: Ellipticine; Anticancer drug; Cytochrome P450; Activation; DNA adduct; [^{32}P]-postlabeling

1. Introduction

Ellipticine (5,11-dimethyl-6H-pyrido[4,3-b]carbazole, Fig. 1) and several of its derivatives isolated from *Apocynaceae* plants (i.e. *Ochrosia borbonica*, *Excavatia coccinea*) are alkaloids exhibiting significant antitumor and anti-HIV activities [1–3]. Ellipticine and its more soluble derivatives (9-hydroxyellipticine, 2*N*-methyl-9-hydroxyellipticinium, 2*N*-methyl-9-chloroellipticinium, and 2*N*-methyl-9-methoxyellipticinium) exhibit promising results in the treatment of osteolytic breast cancer metastases, kidney sarcoma, tumors of the brain, and myeloblastic leukemia [4–7]. To increase the selectivity of ellipticine antitumor drugs, the attempts to link them to specific vectors able to direct these drugs toward target cells were performed [8–11]. One such conjugate, a heptagastrin fragment linked to ellipticine *via* a spacer has recently been synthesized and shown to be selectively taken up and to be cytotoxic to cells expressing the cholecystokinin type B receptor [11]. The main reason for the interest in ellipticine and its derivatives for clinical purposes is their high efficiencies against several types of cancer, their rather limited toxic side effects, and their complete lack of hematological toxicity [12].

The metabolism of ellipticine in organisms has been described [13]. In rats, its two major metabolites are 9-hydroxyellipticine and 7-hydroxyellipticine, as demonstrated *in vivo* and *in vitro* with microsomal preparations [12,13]. Compared to the nonhydroxylated parent compound, the 9-hydroxylated derivative exhibits a higher pharmacological efficiency, whereas the 7-hydroxylated derivative is less effective [13]. Only 9-hydroxyellipticine was formed in human liver microsomes [14]. Cytochromes P450 (CYPs) are believed to be the major enzymes catalyzing the metabolism of ellipticine and its derivatives. CYP1A1 and CYP1A2 are assumed to be the most active enzymes oxidizing these compounds, but participation of other CYPs in their metabolism cannot be excluded [12–16]. The specific

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Abbreviations: α-NF, α-naphthoflavone; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate; CYP, cytochrome P450; HPLC, high performance liquid chromatography; IC_{50}, 50% inhibitory dose; 3-IPMDIA, 3-siopropenyl-3-methyldiamantane; MDR, multidrug resistance; PEI, polyethylenimine; RAL, relative adduct labeling; and TLC, thin-layer chromatography.

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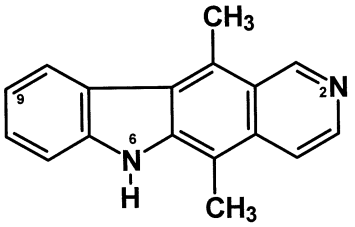

Fig. 1. Structure of ellipticine

CYP oxidizing ellipticine in humans are not known. Ellipticine is frequently used as a "selective" inhibitor of CYP1A1/2, but is also a strong inducer of CYP1A1 [17,18].

Most ellipticines are mutagenic to *Salmonella typhimurium* Ames tester strains [15,19], bacteriophage T4 [20], *Neurospora crassa* [21], and mammalian cells [19,22,23] and to induce prophage lambda in *Escherichia coli* [24,25].

Ellipticines are anticancer drugs, whose precise mechanisms of action have not yet been explained. It was suggested that the prevalent mechanisms of ellipticine antitumor activities are (a) intercalation into DNA [26,27] and (b) inhibition of DNA topoisomerase II activity [12,28–30]. Topoisomerase II inhibition by ellipticine has been extensively studied [28–30]. Ellipticine acts by stimulating topoisomerase II-mediated DNA breakage by forming a ternary complex with topoisomerase II and DNA, which leads to nucleic acid breakage and subsequent cell death. Topoisomerase II was identified as the primary cellular target of the drug [30], which did not inhibit enzyme-mediated DNA religation, suggesting that it stimulates DNA breakage by enhancing the forward rate of cleavage. Ellipticine and 9-hydroxyellipticine also cause selective inhibition of p53 protein phosphorylation in several human cancer cell lines [31], and this correlated with their cytotoxic activity. Ellipticines also uncouple mitochondrial oxidative phosphorylation [32] and thereby disrupt the energy balance of cells.

It is evident that these mechanisms of ellipticine action are not limited to cancer cells and may not sufficiently explain the specific antitumor activity of these compounds. No discrimination between healthy tissues and tumor cells in ellipticine uptake is to be expected because ellipticines are highly hydrophobic and enter cell membranes by diffusion. The specificity of the antitumor activity of ellipticines could result from other mechanisms of their action that have not been elucidated as yet.

A cancer-specific cell-kill is known to be caused by several anticancer drugs, which are almost inactive until metabolized. Several anticancer drugs (e.g. cyclophosphamide, mitomycin C) covalently bind to DNA after being enzymatically activated. To elucidate whether these features might be responsible for the antitumor specificity as well as a high efficiency of ellipticine, the potential of this anticancer agent to form DNA adducts after metabolic activation was examined. Because there is evidence that target tumors for ellipticine (i.e. breast cancer, renal cell cancer) express several drug-metabolizing CYPs (CYP1A1, CYP1B1, CYP3A4) at higher levels than peritumoral tissues [33–35], several CYPs were tested for their efficiencies to activate ellipticine.

2. Materials and methods

2.1. Chemicals and radiochemicals

Chemicals were obtained from the following sources: ellipticine, α-naphthoflavone (α-NF), nicotinamide adenine dinucleotide phosphate (NADH), NADPH, testosterone, troleandomycin, diethyldithiocarbamate, 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS), dilauroyl phosphatidylcholine, dioleyl phosphatidylcholine, dilauroyl phosphatidylserine, and quinidine from Sigma Chemical Co. (St. Louis, MO); 7-pentoxy- and 7-ethoxyresorufin from Fluka Chemie AG (Buchs, Switzerland); calf thymus DNA from Roche Diagnostics (Mannheim, Germany); and furafylline from New England Biolabs (Beverley, MA). Sulfaphenazole was kindly provided by P. Anzenbacher (Institute of Experimental Biopharmacy, Pro.Med. CS, Czech Academy of Sciences, Hradec Králové). Bicinchoninic acid was from Pierce (Rockford, IL). 3-isopropenyl-3-methyldiamantane (3-IPMDIA) was synthesized according to Olah *et al.* [36]. Enzymes and chemicals for the [^{32}P]-postlabeling assay were obtained from sources described previously [37,38]. The [^{3}H]-labeled ellipticine (24.6 GBq/mmol) was prepared by a catalytic exchange method with [^{3}H] gas [39] and stored in methanol at -18^{o}. Radiochemical purity of the compound was >98% thin-layer chromatography (TLC). All other chemicals were reagent grade or better.

2.2. Preparation of microsomes and assays

Microsomes from livers of untreated rats and rabbits were prepared as described previously [40]. Microsomes from one human liver (a 34 year-old-man, who died after a traffic accident) and from the liver of a male minipig were a gift from P. Anzenbacher were and isolated as described [41]. Supersomes™, microsomes isolated from insect cells transfected with Baculovirus constructs containing cDNA of one of the following CYPs, CYP1A1, 1A2, 1B1, 2A6, 2B6, 2C9, 2D6, 2E1, or 3A4, and expressing NADPH:CYP reductase were from Gentest Corp. Protein concentrations in the microsomal fractions were assessed by using the bicinchoninic acid protein assay with serum albumin as a standard [42]. The concentration of CYP was estimated according to Omura and Sato [43] based on the absorption of the complex of reduced CYP with carbon monoxide. Rat, rabbit, minipig, and human liver microsomes contained 0.62, 1.82, 0.89, and 0.38 nmol CYP/mg protein, respectively. Each microsomal sample was analyzed for specific CYP activities by monitoring the following reactions: ethoxyresorufin *O*-deethylation (CYP1A1/2), pentoxyresorufin

Table 1
Specific CYP activities in rat, rabbit, minipig, and human hepatic microsomes

CYP species	Substrate	Specific activity[a] nmol/min × mg protein			
		Rat	Rabbit	Minipig	Human
CYP1A1/1A2	Ethoxyresorufin	0.201	0.432	0.319	0.190
CYP2B1/2B2/2B4	Pentoxyresorufin	0.104	0.022	0.110	—[b]
CYP2B6	Benzyloxyresorufin	—[b]	—[b]	—[b]	0.061
CYP2C	Tolbutamide	2.897	2.958	0.147	0.030
CYP2D	Bufuralol	1.040	1.305	1.210	1.260
CYP2E1	Chlorzoxazone	0.540	0.920	1.067	3.116
CYP3A	Testosterone	1.430	3.604	1.093	1.501

[a] The enzyme reactions used to determine the specific activities are listed in the Materials and methods section. The values are the means of duplicate experiments.
[b] − not tested.

O-depentylation (CYP2B1/2 in rat and CYP2B4 in rabbit microsomes), [44] and benzyloxyresorufin-*O*-debenzylation (CYP2B6 in human microsomes), bufuralol 1′-hydroxylation (CYP2D), tolbutamide methyl hydroxylation (CYP2C), chlorzoxazone 6-hydroxylation (CYP2E1), and testosterone 6β-hydroxylation (CYP3A) [45; and references therein]. These activities are shown in Table 1.

2.3. Isolation of individual CYPs

CYP2B4, CYP2C3, and CYP2E1 were isolated from liver microsomes of rabbits induced with phenobarbital (2B4) and ethanol (2E1, 2C3), by procedures described by Haugen and Coon [46] and Yang *et al.* [47]. CYP3A6 was isolated from liver microsomes of a rabbit induced with rifampicin. The procedure was analogous to that used for isolation of CYP2B4. Recombinant rat CYP1A1 protein was purified to homogeneity by the procedure described previously [48] from membranes of *E. coli* transfected with a modified CYP1A1 cDNA, in the laboratory of H. W. Strobel (University of Texas, Medical School of Houston, Texas) by P. Hodek (Charles University, Prague). Recombinant human CYP1A2 was from Oxford Biomedical Research, Inc., and human recombinant CYP3A4 was a gift from P. Anzenbacher. Rabbit liver NADPH:CYP reductase was purified as described [49].

2.4. Incubations

Unless stated otherwise, incubation mixtures used for modifying DNA by ellipticine (or by [^{3}H]ellipticine) contained the following in a final volume of 750 μL:50 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, microsomal fraction containing 0.1–1 nmol CYP, 0.1–200 μM ellipticine (or [^{3}H]ellipticine) dissolved in 7.5 μL methanol and 1 mg calf thymus DNA. The reaction was initiated by adding the substrate. Control incubations were carried out either without activating system (microsomes) or without DNA or without ellipticine. Incubation mixtures, in which the efficiencies of Supersomes expressing human CYPs were tested, were the same except that 100 μM ellipticine and only 10–50 pmol of CYP were used. Incubations containing purified CYP reconstituted with NADPH:CYP reductase contained 50–500 pmol of each CYP. Briefly, CYP was reconstituted as follows (0.5 μM CYP, 0.5 μM NADPH:CYP reductase, 0.5 μg/μL CHAPS, 0.1 μg/μL liposomes [dilauroyl phosphatidylcholine, dioleyl phosphatidylcholine, dilauroyl phosphatidylserine (1:1:1)], 3 mM reduced glutathione, and 50 mM HEPES/KOH, pH 7.4) [50]. An aliquot of this mixture was then added to incubation mixtures. In the control incubation, the CYP was omitted from the reconstitution mixture. After incubation (37°, 60 min), the incubation mixtures were extracted twice with ethyl acetate (2 × 2 mL). DNA was isolated from the residual water phase by the phenol/chloroform extraction method as described earlier [38,40,51]. DNA was dissolved in 1 mL of distilled water. The [^{3}H] radioactivity of DNA modified by [^{3}H]ellipticine activated by microsomes was determined in 0.1 mL aliquots in a Packard Ultra Gold X liquid scintillation cocktail on a Packard Tri-Carb 2000 CA scintillation counter. The content of DNA was determined spectrophotometrically [52].

2.5. Inhibition studies

The following chemicals were used to inhibit the activation of ellipticine in human hepatic microsomes and in the reconstitution experiments with purified CYP: α-NF, which inhibits CYP1A1 and CYP1A2; furafylline, which inhibits CYP1A2; 3-IPMDIA, which inhibits CYP2B4 [53]; diethyldithiocarbamate, which inhibits CYP2A6 and CYP2E1; sulfaphenazole, which inhibits CYP2C; quinidine, which inhibits CYP2D6; and troleandomycin, which inhibits CYP3A4. Inhibitors were dissolved in 7.5 μL of methanol, to yield final concentrations of 1–400 μM in the incubation mixtures (except of diethyldithiocarbamate, which was dissolved in water). The mixtures containing the inhibitors were then incubated at 37° for 10 min with NADPH prior to adding the ellipticine and then for a further 60 min at 37°. An equal volume of methanol alone was added to the control incubations.

2.6. [^{32}P]-postlabeling analysis

For the standard procedure of the [^{32}P]-postlabeling method 1 μg of DNA was digested and labeled with [^{32}P] by using 100 μCi of [γ-^{32}P]-adenosine 5′-triphosphate (ATP) purchased from ICN Biochemicals GmbH with a specific activity of 7000 Ci/mmol as described previously [54]. In addition the nuclease P1 [55] and the 1-butanol extraction [56] enrichment versions of the assay were performed: DNA samples (12.5 μg) were digested with micrococcal nuclease (750 mU) and spleen phosphodiesterase (12.5 mU) in digestion buffer (20 mM sodium succinate, 8 mM $CaCl_2$, pH 6.0) for 3 h at 37^o in a total volume of 12.5 μL. Here, 2.5 μL of the digests were removed and diluted 1:1500 to determine the amount of normal nucleotides. In the nuclease P1 version, the digests (10 μL) were enriched for adducts by incubating with 5 μg (5 U) of nuclease P1 in 3 μL of a buffer containing 0.8 M sodium acetate, pH 5.0, and 2 mM $ZnCl_2$ for 30 min at 37^o. The reaction was stopped by adding 3 μL of 427 mM Tris base. The extraction with 1-butanol to enrich the adducts was carried out as described earlier [56]. In all procedures, 4 μL of labeling mix consisting of 400 mM bicine, pH 9.5, 300 mM dithiothreitol, 200 mM $MgCl_2$, 10 mM spermidine, 100 μCi [γ-^{32}P]ATP (15 pmol), 0.5 μL 90 μM ATP, and 10 U T4 polynucleotide kinase were added. After incubation for 30 min at room temperature, 20 μL were applied to a polyethylenimine (PEI)-coated cellulose TLC plate (Macherey-Nagel, Duren, Germany) and were chromatographed as described [57,58], except that D3 and D4 were adjusted to pH 4.0 and 9.1, respectively, for better resolution. To determine the amount of normal nucleotides 5 μL of the 1:1500 dilution of digests were mixed with 2.5 μL of Tris buffer (10 mM, pH 9.0) and 2.5 μL of labeling mix (see above) and incubated for 30 min at room temperature. The samples were then diluted by mixing 4 μL with 750 μL of 10 mM Tris buffer, pH 9.0. Five microliters of this solution were applied to a PEI-cellulose TLC plate and run in 0.28 M $(NH_4)_2SO_4$, 50 mM NaH_2PO_4, pH 6.5.

Adducts and normal nucleotides were detected and quantified by storage phosphor imaging on a Packard Instant Imager. Count rates of adducted fractions were determined from triplicate maps after subtraction of count rates from adjacent blank areas. Excess [γ-^{32}P]ATP after the postlabeling reaction was confirmed. Adduct levels were calculated in units of relative adduct labeling (RAL), which is the ratio of cpm of adducted nucleotides to cpm of total nucleotides in the assay.

3. Results

3.1. Evidence for covalent binding of ellipticine to DNA in vitro

Rat, rabbit, and human microsomes in the presence of NADPH are effective in activating [^{3}H]ellipticine to metabolites binding to DNA (Table 2). DNA modified by metabolites of [^{3}H]ellipticine was isolated from the reaction mixture by the procedures that included phenol/chloroform extraction [38,40,51]. This suggests that the observed DNA binding is covalent binding. Further confirmation of the covalent nature of the DNA adducts was obtained by the [^{32}P]-postlabeling assay.

Table 2
Binding of [^{3}H]ellipticine activated by microsomes to calf thymus DNA *in vitro*[a]

Activating system	DNA-binding (pmol bound/mg of DNA)
Complete microsomal systems containing 1 mM NADPH, 100 μM [^{3}H]ellipticine	
Human microsomes	
0.5 nmol CYP	17.4 ± 1.6
1.0 nmol CYP	29.6 ± 1.9
Rat microsomes	
0.5 nmol CYP	101.8 ± 8.3
1.0 nmol CYP	130.1 ± 8.9
Rabbit microsomes	
0.5 nmol CYP	258.2 ± 12.5
1.0 nmol CYP	358.2 ± 14.0
Control systems	
Without microsomes	2.1 ± 0.9
Rabbit microsomes without NADPH	4.1 ± 1.1

[a] The average binding levels and standard deviations were obtained from triplicate determinations. The DNA binding was assayed as described in Materials and methods.

With rat or human microsomes to activate ellipticine we found two adducts in the calf thymus DNA added, one major and one minor (Fig. 2, Table 3). Control incubations carried out either without ellipticine or without DNA were free of either adduct spot even after prolonged exposure times. Control incubations performed without microsomes were free of adduct Spot 1, but adduct Spot 2 was always detected (Fig. 2). Initially, all three version of the [^{32}P]-postlabeling assay (standard procedure, the nuclease P1 version and extraction with 1-butanol), were used to separate and quantify the adducts, but no differences in patterns or quantities of adducts were found (Table 3). Therefore, only one of them, the nuclease P1 version, was used in the experiments presented below.

The formation of the ellipticine-DNA adducts (assayed with rat microsomes) is dependent on incubation time and concentration of ellipticine (Figs. 3A and B). The comparison of ellipticine-DNA adduct formation catalyzed by hepatic microsomes from different species is shown in Fig. 4. Again, as in the experiments with [^{3}H]ellipticine, microsomes from rabbits were the most effective, followed by those from rats. Both human and minipig microsomes showed similar activities, but were much less active than those from lagomorphs or rodents. Increasing concentrations of microsomal CYP up to 1000 pmol increased the level of the major adduct, but had no effect on the quantities of the Adduct 2 in all species (Fig. 4). In contrast to the

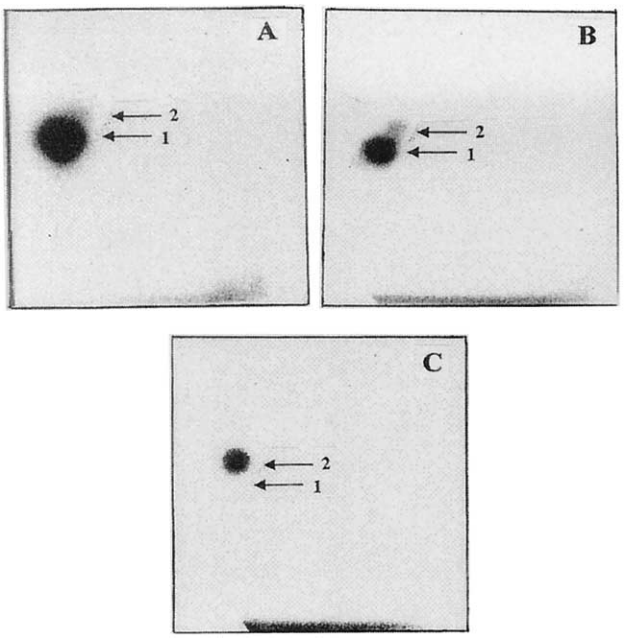

Fig. 2. Autoradiographs of PEI-cellulose TLC maps of [^{32}P]-labeled digests of calf thymus DNA reacted with ellipticine, NADPH and liver microsomes from (A) rat or (B) human. (C) is a control sample without microsomes. Analyses were performed by the nuclease P1 version of the assay. Film exposures were (A) 30 min, (B) 1.5 h, and (C) 7 h, at −80°. Origins are located at the bottom left corners (D3 from bottom to top and D4 from left to right).

major Adduct 1, Adduct 2 is, therefore, formed independently of enzymatic activation (see also Fig. 2). At CYP concentrations above 1000 pmol of rat enzymes the detectable amount of Spot 1 decreased again, probably because of binding of reactive intermediates to the microsomal proteins.

The formation of the Adduct 1 was dependent on NADPH. The level of this adduct was negligible when NADPH was omitted from the incubation mixture. The addition of NADH, which is a cofactor of the microsomal NADH: cytochrome b_5 oxidoreductase, a second electron donor for CYP-dependent systems [59], stimulates the formation of the adduct (Table 4). NADH alone is a less efficient cofactor than is NADPH (Table 4). These data indicate that CYP-catalyzed reactions are responsible for the activation of ellipticine to a reactive species forming one DNA adduct.

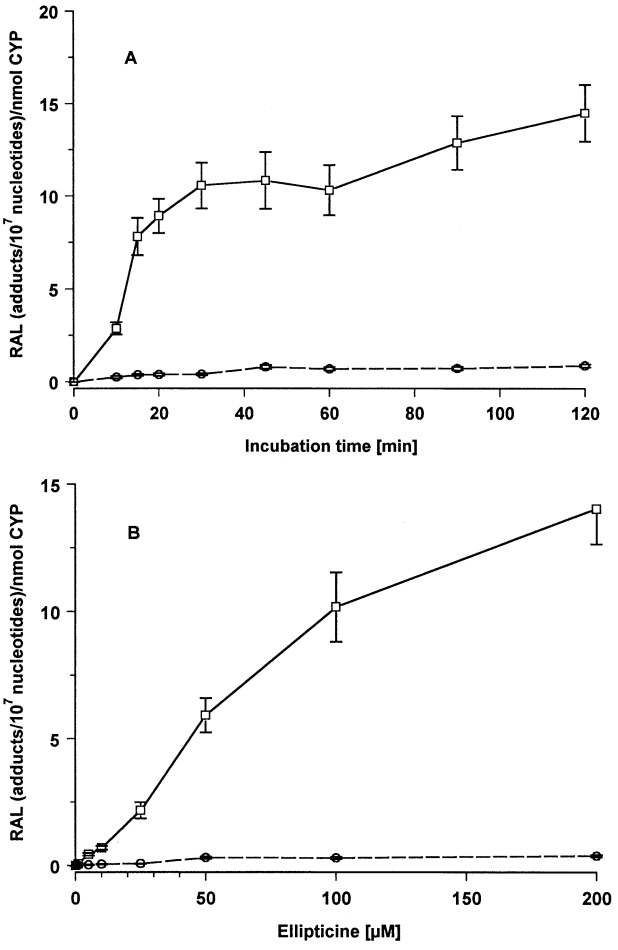

Fig. 3. Dependence of ellipticine-DNA adduct levels on incubation (A) time and on (B) ellipticine concentration in rat liver microsomes. The continuous line is the level of Spot 1; the discontinuous line the level of Spot 2 (see Fig. 2).

3.2. Elucidation of CYP species responsible for ellipticine activation

To resolve which CYPs are able to activate ellipticine, three experimental approaches were employed: (a) selective inhibition of CYP, (b) reconstituted systems with purified CYP and NADPH:CYP reductase, and (c) heterologous expression systems (Supersomes™, see Materials and Methods).

Table 3
Quantitative analysis of ellipticine-DNA adducts formed in microsomes detected by ^{32}P-postlabeling assay[a]

Microsomes from	RAL[b] (mean ± SD/10^7 nucleotides)[c] version of ^{32}P-postlabeling					
	Nuclease P1		1-Butanol extraction		Standard procedure	
	1[d]	2	1	2	1	2
Rat	10.180 ± 1.002	0.302 ± 0.030	10.190 ± 0.720	0.280 ± 0.023	10.230 ± 0.820	0.298 ± 0.029
Human	0.901 ± 0.098	0.301 ± 0.022	1.020 ± 0.051	0.295 ± 0.029	1.053 ± 0.050	0.298 ± 0.020

[a] Experimental conditions were as described in the Material and method section with 100 μM ellipticine and 1 nmol cytochromes P450 per incubation.
[b] Relative adduct labeling.
[c] Averages of three determinations in separate experiments.
[d] Adducts 1 and 2 see Fig. 2.

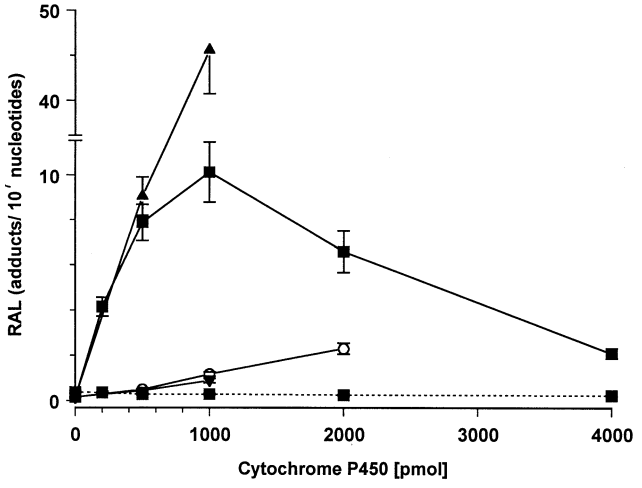

Fig. 4. Ellipticine-DNA adduct levels in relation to CYP concentrations in microsomes from different species. ▲ rabbit Spot 1, ν rat Spot 1 (continuous line) and Spot 2 (discontinuous line), ○ minipig Spot 1, ▼ human Spot 1.

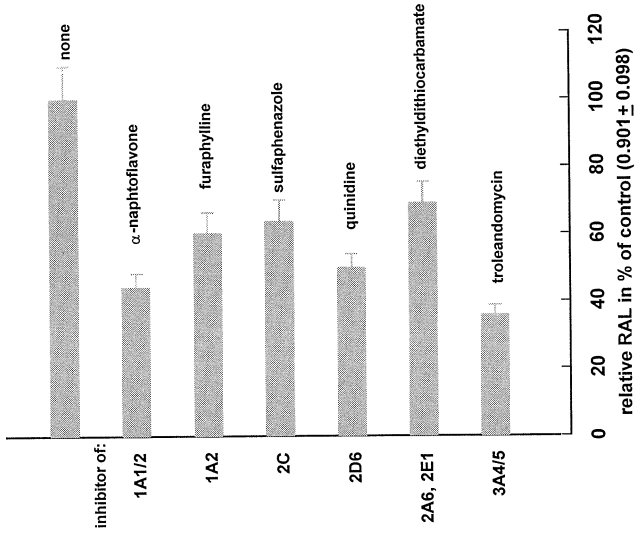

Fig. 5. Effect of CYP inhibitors on ellipticine-DNA adduct formation by human liver microsomes (100 μM ellipticine and 100 μM inhibitors were used), determined by [^{32}P]-postlabeling.

All selective inhibitors of individual CYPs tested in our experiments inhibited the formation of the major ellipticine-DNA adduct in human microsomes determined by [^{32}P]-postlabeling. If equimolar concentrations of inhibitors and ellipticine (100 μM) were used, the strongest inhibition in human microsomes was observed with an inhibitor of CYP3A4, troleandomycin [60], followed by α-NF, an inhibitor of CYP1A1/2 [60] and several reactions catalyzed by CYP3A4 [60,61], and a CYP2D6 inhibitor, quinidine [60] (Fig. 5). Likewise, the lowest 50% inhibitory dose (IC_{50}) value was obtained for troleandomycin in human microsomes (Table 5). These results indicate that numerous CYP forms participate in the activation of ellipticine to produce a DNA adduct, but that the CYP3A4 enzyme seems to be the most efficient.

To confirm the role of individual CYPs in the activation of ellipticine, several CYP enzymes were purified, reconstituted with NADPH:CYP-reductase, and used as activation system. An example of autoradiographs of adducts formed in these incubations is shown in Fig. 6 with CYP1A1 as the activation enzyme. When CYP was omitted from the incubation medium (the control sample contained liposomes, NADPH:CYP reductase, and NADPH) the adduct Spot 1 was not detected in autoradiographs, even after prolonged exposure times. Only the adduct Spot 2 was detected (Fig. 6). Among the CYP enzymes tested, human CYP3A4 (purified recombinant enzyme reconstituted with reductase) was the most efficient enzyme activating ellipticine to form DNA adducts (Fig. 7). Other CYP were less effective. In all cases, increasing concentrations of CYP reconstituted with reductase resulted in an increase of Adduct 1 formation, while the level of Adduct 2 did not change. Similar to the experiments using microsomes, selective inhibitors of individual CYP inhibited the ellipticine-DNA adduct formation in the reconstitution experiments (Fig. 7).

Table 4
The effect of cofactors on the ellipticine-DNA adduct formation determined by ^{32}P-postlabeling assay in rat liver microsomes[a]

Cofactor	RAL[b] (mean ± SD/10^7 nucleotides)[c] in adduct	
	1[d]	2
None	0.063 ± 0.008	0.293 ± 0.030
NADPH (1 mM)	10.180 ± 1.002	0.302 ± 0.030
NADPH (1 mM) + NADH (1 mM)	13.709 ± 1.105	0.308 ± 0.029
NADH (1 mM)	2.756 ± 0.253	0.323 ± 0.030

[a] Experimental conditions are described in the Material and methods section with 1 nmol of microsomal cytochome P450 and 100 μM ellipticine.
[b] Relative adduct labeling.
[c] Averages of three determinations in separate experiments.
[d] See Fig. 2.

Table 5
IC_{50} values for the inhibition of ellipticine-DNA adduct formation determined by ^{32}P-postlabeling assay in human microsomes

Inhibitor[a]	IC_{50} (μM)[b]
α-naphthoflavone (CYP1A1/2, CYP3A4)	78.0 ± 4.9[c]
Furafylline (CYP1A2)	220.0 ± 24.0
Sulfaphenazol (CYP2C)	240.0 ± 25.0
Quinidine (CYP2D6)	100.0 ± 12.0
Diethyldithicarbamate (CYP2A6,2E1)	320.0 ± 35.0
Troleandomycin (CYP3A4)	4.0 ± 0.5

[a] Isoforms of cytochrome P450 with the targets of the inhibitors in parentheses.
[b] Estimated from concentration curves from 1–400 μM inhibitors by interpolation (see Materials and methods for details). 100 μM ellipticine and 1 nmol CYP.
[c] Average of three determinations in separate experiments.

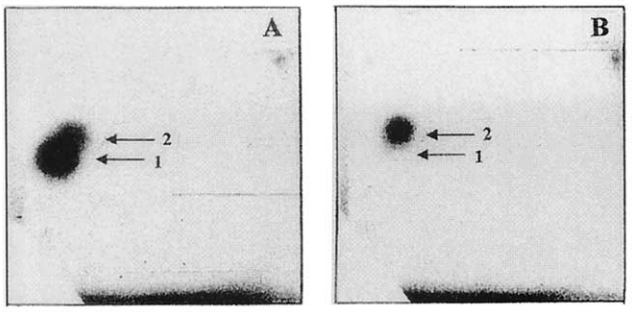

Fig. 6. Autoradiographs of PEI-cellulose TLC maps of [^{32}P]-labeled digests of calf thymus DNA treated with ellipticine, NADPH, and purified rat recombinant CYP1A1 reconstituted with NADPH: (A) CYP reductase and (B) a control sample without CYP1A1. Analyses were performed by the nuclease P1 version of the assay. Film exposure was 6.5 hrs at −80°. Origins are located at the bottom left corners (D3 from bottom to top and D4 from left to right).

Only two out of the seven purified CYP used in the experiments were human enzymes (CYP1A2 and CYP3A4). The others were rat and rabbit CYPs. Because CYPs of animal species are often not suitable models of the catalytic properties of orthologous human enzymes, we examined human CYPs to find the species responsible for activating ellipticine. For such experiments, we used microsomes of Baculovirus transfected insect cells (Supersomes™) containing recombinantly expressed human CYPs and NADPH:CYP reductase. All tested human recombinant CYPs were active in catalyzing ellipticine-DNA adduct formation (Fig. 8). The most potent human enzyme was CYP3A4, followed by CYP1A1, CYP1A2, CYP1B1, and CYP2C9. Also in this system only the formation of the Adduct 1 was dependent on enzymatic activation, and the level increased with CYP concentration.

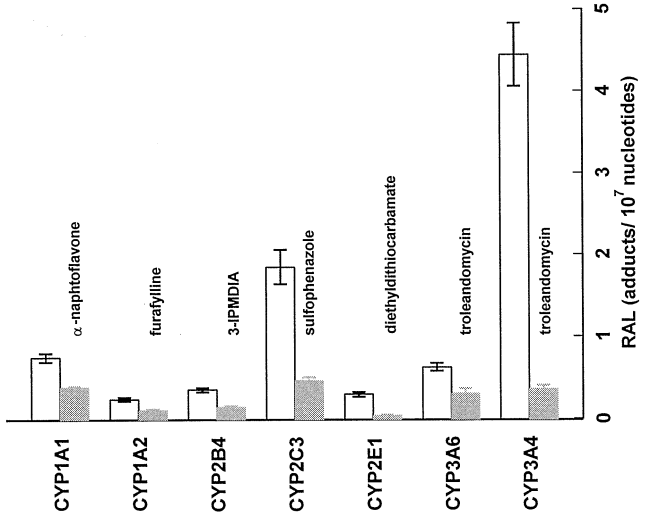

Fig. 7. Activation of ellipticine by isolated CYP reconstituted with NADPH:CYP reductase and the effect of specific inhibitors on ellipticine activation. CYP (250 pmol)/incubation and 100 μM ellipticine were used in all experiments. Concentrations of the inhibitors were 10 μM.

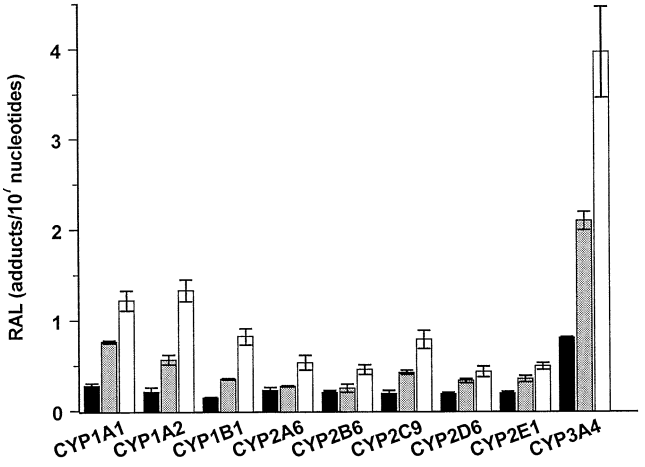

Fig. 8. Activation of ellipticine by Supersomes™ containing different amounts of human recombinant CYP. The black bars are 10 pmol, the gray bars 25 pmol, and the white bars 50 pmol CYP.

4. Discussion

We present for the first time the detection of covalent ellipticine-DNA adducts *in vitro* by using two direct methods, namely the [^{32}P]-postlabeling technique and use of tritium-labeled ellipticine. The possible formation of covalent DNA adducts by ellipticine has been postulated by DeMarini *et al.* [15], who suggested that one type of the multiple genotoxic effects of ellipticine might be a consequence of a metabolically dependent covalent binding of ellipticine to DNA [15]. Likewise, the covalent modification of DNA by an ellipticine derivative, 2*N*-methyl-9-hydroxyellipticine oxidized by peroxidase, was proposed by Auclair *et al.* [12,62,63]. But the hypothesized covalent nature of the binding was not proven in their papers [62,63].

We can only speculate on the structure of ellipticine-DNA adducts shown in the present paper. One adduct (Spot 2) is formed independent of enzyme activation (activated probably by autooxidation), whereas the major adduct on catalysis by CYP. The chromatographic conditions used to resolve the adducts were suitable to separate adducts formed from carcinogens with a pronounced hydrophobicity [40,57,58]. Although we have not yet elucidated the exact nature of the adducts, the pronounced lipophilicity of the ellipticine-DNA adducts indicates that the whole ellipticine molecule is covalently linked to DNA.

In view of possible interaction of ellipticine and its derivatives with DNA and their multiple effects on several other vital functions of cells, these drugs are representatives of antitumor compounds that have multimodal mechanisms of cytotoxicity. Two of these mechanisms that are based on DNA damage (intercalation of ellipticine into DNA and generation of DNA strand breaks by inhibiting mammalian topoisomerase II) have been considered major ones. The experimental data presented herein demonstrate a novel mechanism for ellipticine action, namely covalent binding of ellipticine to DNA. This new mechanism is another effect

based on DNA damage. At the present time, it is not possible to demonstrate that antitumor, cytostatic, or genotoxic activities of ellipticine are related to only one or several of these effects.

We suggest that the newly discovered covalent DNA binding of ellipticine metabolite(s) might in part explain its tumor specificity. Ellipticines exhibit antineoplastic activity quite selectively, e.g. against breast cancer. We showed here that CYPs are effective activators of this drug. Of the human CYPs tested, CYP3A4 had the highest activity toward ellipticine, followed by CYP1A1/2, CYP1B1, and CYP2C9. These data agree with findings by others that ellipticine is oxidized to hydroxy derivatives by numerous CYPs with differing efficiencies [12–16]. However, human microsomes, a major source of CYP3A4, show low adduct formation compared to other species (see Fig. 4). The efficiency of human microsomes and isolated CYP3A4 reconstituted with NADPH:CYP reductase to activate ellipticine cannot be compared directly. Human microsomes used in our experiments were not so rich in CYP3A4-dependent activity (Table 1) and, moreover, the specific content of CYPs in the human microsomes was low (0.38 nmol/mg protein). Human cancers, including colon, breast, lung, liver, kidney, and prostate, are known to express CYP isoforms [35]. CYP3A4 is expressed in breast cancer [35, 60] at a higher level than in the peritumoral tissues [33,35]. The CYP3A4-dependent activation of anticancer prodrug anthraquinone-di-N-oxide to its cytotoxic species in murine T50/80 mammary carcinoma is one important example showing the significance of CYP3A4 for the activation of drugs in murine mammary cancer [35]. The apparent increase in CYP3A expression under hypoxia in tumor cells underlines its importance for such tumor-specific prodrug activation [35] and could explain the efficacy of ellipticine in breast cancer.

Other enzymes efficiently activating ellipticine, namely CYP1A1 and CYP1B1, are also expressed in malignant breast cancer [33,35,60,64,65]. The levels of CYP1A1 and CYP1B1 in human breast cells are dependent not only on the constitutive gene expression, but both are induced by planar aromatic compounds binding to the aryl hydrocarbon receptor [17,18]. CYP1A1 is even strongly induced by ellipticine itself, by this mechanism [17,18]. Rekha and Sladek [66] demonstrated that human breast adenocarcinoma MCF-7/0 cells treated with 3-methylcholanthrene (CYP1A1 and/or CYP1B1 inducer) transiently expressed elevated levels of CYP1A and were transiently much more sensitive to ellipticine. Because ellipticine is a strong inducer of at least one of these CYP1 enzymes, it is probable that their expression is elevated in the target tissue breast cancer as a consequence of ellipticine treatment.

Another important feature related to expression of CYP1A1 and antineoplastic activity of ellipticine was detected in MCF-7 cells selected for resistance to adriamycin (Adr^R MCF-7), exhibiting the phenotype of multidrug resistance (MDR) [67]. Ivy et al. postulated that the MDR property of Adr^R MCF-7 cells involves several biochemical and genetic changes. One of them is a regulatory defect at the level of CYP1A1 mRNA [67] resulting in lower CYP1A1-mediated metabolism of xenobiotics such as benzo(a)pyrene. Adr^R MCF-7 cells are cross-resistant to ellipticine [67], which we would explain by a decrease in the CYP1A1-dependent activation of ellipticine. Taken together, the activities and expression levels of CYP3A4, CYP1A1, and CYP1B1, which effectively activate ellipticine to metabolites forming covalent DNA adducts, may be important factors in the specificity of ellipticine for breast cancer.

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