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OXIDATION OF AN ANTITUMOR DRUG ELLIPTICINE BY PEROXIDASES

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Ellipticine is a potent antineoplastic agent, whose mode of action is considered to be based mainly on DNA intercalation and/or inhibition of topoisomerase II. Since we found that ellipticine also forms the cytochrome P450 (CYP)-mediated covalent DNA adducts, this anticancer drug is considered to function as a pro-drug, whose pharmacological efficiency and/or genotoxic side effects are dependent on its enzymatic activation in target tissues. Here, we demonstrate that ellipticine is also oxidized by peroxidases, which are abundantly expressed in several target tumor tissues. Lactoperoxidase, myeloperoxidase and horseradish peroxidase were used as models. Peroxidases in the presence of hydrogen peroxide oxidize ellipticine to an ellipticine dimer and N^2-oxide of ellipticine as the major and minor metabolite, respectively. Inhibition of the peroxidase-mediated ellipticine oxidation by radical scavengers ascorbate, glutathione and NADH suggests a one-electron mechanism of the oxidation. The implication of the oxidation of ellipticine by peroxidases in its mechanism of action is discussed.

INTRODUCTION

Ellipticine (5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole, Fig. 1), an alkaloid isolated from Apocyanaceae plants and several of its more soluble derivatives (9-hydroxyellipticine, 9-hydroxy-N^2-methylellipticinium, 9-chloro-N^2-methylellipticinium and 9-methoxy-N^2-methylellipticinium) exhibit promising results in the treatment of osteolytic breast cancer metastases, kidney sarcoma, tumors of brain and myeloblastic leukemia (for summary see[1]). 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[2]. Nevertheless, ellipticine is a potent mutagen. Most ellipticines 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[1]).

Ellipticine is an antineoplastic agent, whose mode of action was considered to be based mainly on DNA intercalation and/or inhibition of topoisomerase II (ref.[3–7]). Recently, we found another mode of the ellipticine action[1,8–10]. We demonstrated that ellipticine covalently binds to DNA after being enzymatically activated. Using a panel of different human recombinant cytochrome P450 (CYP) enzymes, CYP3A4, 1A1 and 1B1, those enzymes expressed at higher levels in tumors sensitive to ellipticine (i.e. breast cancer) than in peritumoral tissues[11–14], were found to be the most efficient CYP enzymes activating ellipticine to form covalent DNA adducts *in vitro*[1]. Deoxyguanosine was identified to be the target for binding of two CYP-mediated ellipticine metabolites 13-hydroxyellipticine and the N^2-oxide of ellipticine[9,10,15]. The formation of these deoxyguanosine adducts by ellipticine was also detected in V79 Chinese hamster lung fibroblast cells transfected with human CYP3A4, 1A1 and 1A2[8], in human breast adenocarcinoma MCF-7 cells[16], in human HL-60 leukemia cells[17] and *in vivo* in rats exposed to this anticancer drug[10]. On the basis of these data, ellipticine might be considered a drug, whose pharmacological efficiency and/or genotoxic side effects are dependent on its enzymatic activation in target tissues[1,8–10,15–17].

While CYP enzymes are expressed in breast cancer, their levels are several orders of magnitude less in other ellipticine sensitive tumor cells. Peroxidases, abundant in some leukemia cells [i.e. myeloperoxidase (MPO) in HL-60 cells] might be candidates for ellipticine oxidation in these cells. Likewise, lactoperoxidase (LPO) that is

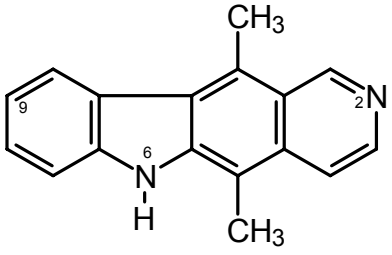

Fig. 1. Structure of ellipticine

secreted by human mammary ductal epithelial cells into the breast duct[18], might metabolized anticancer drugs. The present study was, therefore, undertaken to determine the capability of peroxidases to oxidize ellipticine.

MATERIAL AND METHODS

Enzyme preparations

Plant horseradish peroxidase (HRP; 300 purporogallin units/mg protein, 61 guaiacol units/mg protein), bovine lactoperoxidase (LPO; 117 purporogallin units/mg protein, 13 guaiacol units/mg protein), and human myeloperoxidase (MPO; 105 purporogallin units/mg protein, 11 guaiacol units/mg protein) were obtained from Sigma Chemical Co. (St. Louis, MO).

Enzyme incubations

Unless stated otherwise, incubation mixtures with peroxidases, in a final volume of 500 µl, consisted of 50 mM potassium phosphate buffer (pH 4.5 – 8.4), 1–100 µM ellipticine (dissolved in 5 µl methanol), and 1–20 µg of peroxidase (LPO, HRP, or MPO). In incubations with these peroxidases, hydrogen peroxide was added as cofactor to a final concentration of 40–250 µM. Control incubations were without peroxidases in the presence of hydrogen peroxide, or without hydrogen peroxide in the presence of peroxidases, or without ellipticine. Incubations with peroxidases were carried out at 37 °C for 10–60 min All reactions were initiated by adding ellipticine dissolved in methanol (final concentration of methanol was 1%). After incubations, 5 µl of 1 mM of salicylate or phenacetine in methanol was added as internal standards and the ellipticine metabolites were extracted twice with ethyl acetate (2 × 1 ml) from the incubations as described[15]. The extracts were evaporated under nitrogen and dissolved in 50 µl of methanol. The ellipticine metabolites were separated by HPLC. The column used was a 5 µm Ultrasphere ODS (Beckman, 4.6 × 250 mm) preceeded by a C-18 guard column. The eluents were; (i) 45% – 90% methanol in 10 mM ammonium acetate (pH 2.8), with flow rate of 0.8 ml/min, detection was at 296 nm (method A), and (ii) 64% methanol plus 36% of 5 mM heptane sulfonic acid containing 32 mM acetic acid in water with flow rate of 0.8 ml/min, detection was at 296 nm (method B)[15]. Recoveries of ellipticine metabolites were around 95% in the presence of enzymes without a peroxidase cofactor (hydrogen peroxide). One product peak with the retention time (r.t.) of 19.95 min and unconverted ellipticine with r.t. of 11.85 min were separated by HPLC using a method A, while one product peak and residual ellipticine with r.t. of 11.2 and 12.9 min, respectively, were separated with a method B.

To characterize ellipticine metabolites, fractions containing the metabolites eluting at 19.95 min (method A) and 11.2 min (method B) were collected from multiple HPLC runs, concentrated on a speed-vac evaporator and analyzed by mass spectroscopy and/or co-chromatography on HPLC as described below.

Mass Spectrometry

ESI mass spectra were recorded on a Bruker Esquire quadrupole ion trap mass spectrometers (Bruker GmbH, Bremen, Germany). Metabolites (final concentration 1 pmol/µl) dissolved in methanol/water (1:1, v/v) were continuously infused into the ion source via a linear syringe pump at a rate of 1 µl/min (Harvard Apparatus 22). The ionizer and ion transfer optics parameters of the ion trap were as follows: capillary voltage –3500 V, end plate –3000 V, capillary exit 100 V, skimmer I 35 V, skimmer II 7 V, octopole I offset 8 V, octopole II offset 8 V, octopole r.f. 100 V peak-to-peak (pp), lens I –4 V, lens II -45 V. A flow of nitrogen (drying gas at 125° C) was used to stabilize the spray. The spectra were scanned in the range *m/z* 50–2000 and the gating time was set to accumulate and trap 1×10^5 ions. Metabolites were also characterized by the MALDI-TOF (Matrix-Assisted Laser Desorption – Time of Flight) on the MALDI-TOF, Biflex (TM), Bruker-Daltonics.

RESULTS

When ellipticine was incubated with peroxidases (plant HRP, bovine LPO and human MPO were used models) in the presence of hydrogen peroxide, one major product peak, eluted at 19.95 min, was observed by HPLC analysis using the solvent system A. Using the solvent system B for resolution of peroxidase-mediated ellipticine metabolites, another minor product peak, eluted with r.t. of 11.2 min, was observed (chromatograms are not shown). On the basis of mass spectroscopy and co-chromatography with a synthetic standard, the structures of these two ellipticine metabolites were partially characterized.

In the MALDI-TOF and positive-ion electrospray mass-spectra, a major ellipticine metabolite, eluted with the system A, showed the protonated molecule at *m/z* 490 (Fig. 2), indicating the molecular mass of an ellipticine dimer. Although the molecular mass of this ellipticine dimer was clearly determined, its further structural characterization is currently being undertaken.

In the positive-ion electrospray mass-spectrum, the minor ellipticine metabolite eluting after 11.2 min with the solvent system B, showed the protonated molecule at *m/z* 263.0 (not shown), indicating the molecular mass of an oxygenated derivative of ellipticine. This minor ellipticine oxidation product was shown to be the N^2-oxide of ellipticine (5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole-*N*-oxide), because it exhibited the same chromatographic properties on HPLC as the N^2-oxide of ellipticine standard prepared synthetically, by the reaction of ellipticine with 3-chloroperbenzoic acid[15].

The rates of ellipticine oxidation by peroxidases used in the study were measured at pH 4.5, 5.4, 6.4, 7.4 and 8.4. The highest velocity of the reaction was found at pH 6.4 (see Fig. 3 for HRP). The reaction is linear till 15 min of incubation (not shown).

While all three model peroxidases used in the study oxidize ellipticine to both two oxidation products, their

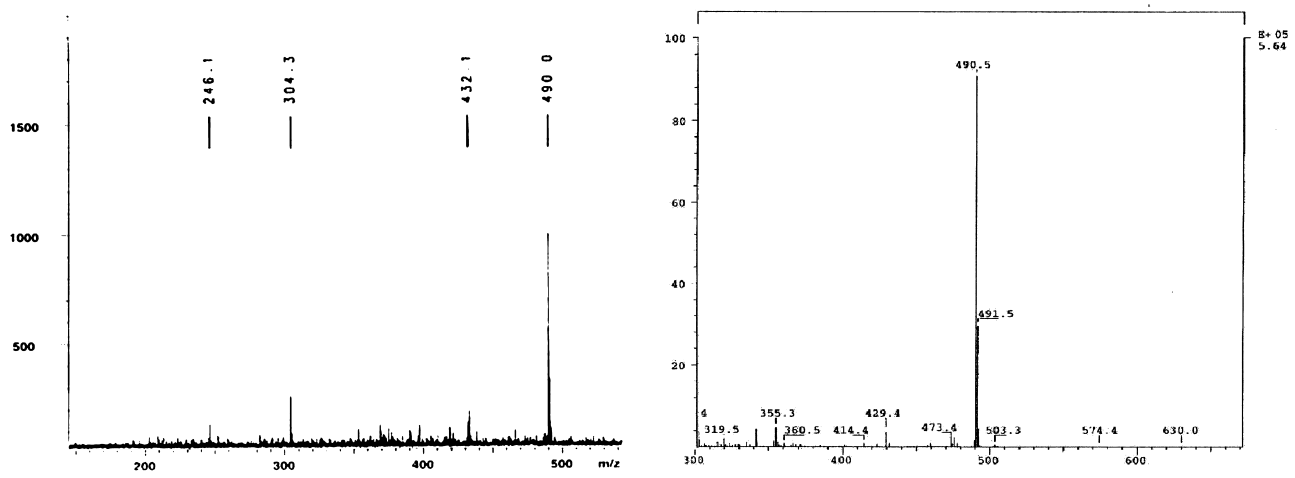

Fig. 2. Mass spectra [MALDI-TOF (A) and ESI (B)] of the major peroxidase-mediated ellipticine metabolite. The ion at *m*/*z* 490 indicates the molecular mass of an ellipticine dimer.

efficiencies to oxidize ellipticine differ considerably. LPO and MPO were less efficient in catalyzing oxidation of ellipticine than HRP. The kinetics of the two-substrate reaction of ellipticine oxidation with peroxidases in the presence of hydrogen peroxide was examined. The oxidation of ellipticine was measured in the reaction medium, which contained either of peroxidases tested in the study, hydrogen peroxide, and various concentrations of ellipticine. The reaction followed the hyperbolic kinetics for all peroxidases (measured as production of the ellipticine dimer). Under the conditions used and at pH 6.4, the values of apparent Michaelis constants (K_m) for ellipticine are of the same order for all peroxidases, while the value of the maximum rate for the ellipticine oxidation (V_{max}) is highest for HRP and decreased for other peroxidases as follows: HRP > LPO > MPO (Table 1). The lower efficiency of LPO and MPO was, however, not specific for ellipticine, we also observed lower activity of these peroxidases with other substrates (i.e. guaiacol, *o*-anisidine) (results not shown).

The oxidation of ellipticine by peroxidases (assayed with HRP and LPO) is strongly inhibited by three physiological donors, NADH, glutathione and L-ascorbic acid, which are known to be effective radical scavengers[20] (Table 2). This finding indicates that the peroxidase-mediated oxidation of ellipticine proceeds *via* a conventional peroxidase reaction, namely, by a sequential one-electron oxidation of two molecules of elliptciine with peroxidase

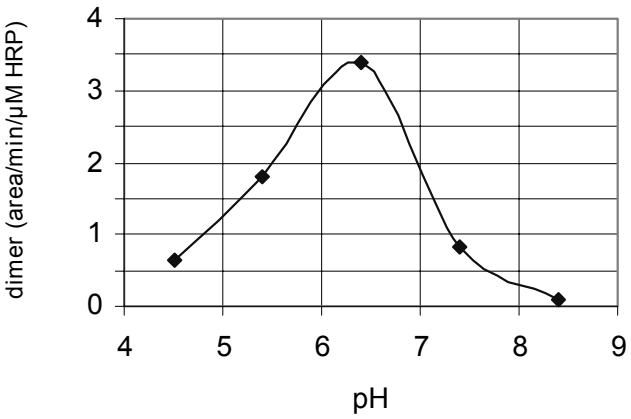

Fig. 3. pH optimum of ellipticine oxidation by HRP.

Table 1. The values of Michaelis constant (K_m) and maximum velocity (V_{max}) for elliptine oxidation with peroxidases[a]

Peroxidase	K_m (μM)[b]	V_{max} (nmol/min per μg peroxidase)[b]
HRP	47.6 ± 4.6 [c]	1.43 ± 0.14
LPO	10.0 ± 1.0	0.25 ± 0.02
MPO	12.0 ± 1.2	0.02 ± 0.002

[a]Reaction mixtures contained in a total volume of 0.5 ml of the 50 mM sodium phosphate buffer, pH 6.4: 1–10 μg peroxidases, 0.1–10 μM ellipticine and 0. 25 mM H_2O_2. The incubation period was 15 min. [b]Reactions rates are expressed as amounts of the major ellipticine metabolite, the ellipticine dimer, production per μg peroxidase per one minute. Kinetic analyses were carried out using the non-linear least-squares method as described[19]. [c]The values in the table are the means ± standard errors (from three parallel experiments).

Compounds I and II. It has already been found[19,20] that none of these scavengers is a substrate of peroxidases under the conditions used in this work. Thus, the scavengers do not act as competitors with ellipticine for binding to the active centre of the enzymes. The effect of scavengers is therefore based on reduction of the radicals formed from ellipticine back to the parent compound.

In addition, the ellipticine oxidation by peroxidases is inhibited by nucleophiles such as DNA (Table 3). Likewise, deoxyguanosine 3'-monophosphate and deoxyadenosine 3'-monophosphate inhibit the ellipticine oxidation by ~50 % in the presence of 40 μg of the deoxynucleotides. These results suggest that a primary formed ellipticine radical may bind to DNA or its deoxynucleotides.

DISCUSSION

The results of this study show that peroxidases such as bovine LPO, human MPO and plant HRP oxidize an anticancer drug ellipticine. During a radical-mechanism of the elllipticine oxidation by peroxidases, two ellipticine metabolites are generated. One of them, the minor one, is the metabolite that is also generated by ellipticine oxidation by human CYP enzymes, namely, N^2-oxide of ellipticine[15]. Such an *N*-oxidation of ellipticine was found to be responsible for formation of one of the deoxyguanosine adducts in DNA generated by CYP enzymes from ellipticine[15]. While the exact structure of the major ellipticine metabolite formed by peroxidase, the ellipticine dimer, remains to be resolved, it seems that oxidation of ellipticine to this metabolite is crucial for formation of DNA adducts, too. It follows from the strong inhibition of the formation of this metabolite by DNA and/or deoxynucleotides. Indeed, the formation of DNA adducts from ellipticine by its oxidation by peroxidases was found

Table 2. Effect of radical scavengers on oxidation of ellipticine to the ellipticine dimer with HRP and LPO[a]

Scavenger	Concentration (mM)	Ellipticine oxidation[b] (nmol/min per μg of peroxidase)	
		LPO	HRP
None		0.240 ± 0.020[c]	1.300 ± 0.130
L-Ascorbic acid	0.1	0.014 ± 0.001	ND[d]
	0.5	ND[d]	ND[d]
NADH	0.1	ND[d]	ND[d]
Glutathione	0.1	0.043 ± 0.005	ND[d]
	0.5	0.011 ± 0.001	ND[d]

[a]Reaction mixtures contained in a total volume of 0.5 ml of the 50 mM sodium phosphate buffer, pH 6.4: 5 μg HRP, 20 μg LPO, 50 μM ellipticine and 40 μM H_2O_2 and scavengers at concentrations as shown. The incubation period was 15 min. [b]Reactions rates are expressed as in Table 1. [c]The values are the means ± standard errors (from three parallel experiments). [d]Not detectable.

Table 3. Effect of calf-thymus DNA on oxidation of ellipticine to the ellipticine dimer with HRP and LPO[a]

Scavenger	Concentration (μg)	Ellipticine oxidation[b] (nmol/min per μg of peroxidase)	
		LPO	HRP
None		0.240 ± 0.020[c]	1.300 ± 0.130
DNA	20	NM[d]	0.221 ± 0.020
	40	NM[d]	0.144 ± 0.014
	60	0.160 ± 0.016	0.082 ± 0.008
	80	0.137 ± 0.014	0.069 ± 0.007

[a] Reaction mixtures contained in a total volume of 0.5 ml of the 50 mM sodium phosphate buffer, pH 6.4: 5 μg HRP, 10 μg LPO, 10 μM ellipticine and 250 μM H_2O_2 and DNA at concentrations as shown. The incubation period was 15 min. [b]Reactions rates are expressed as in Table 1. [c]The values are the means ± standard errors (from three parallel experiments). [d]Not measured.

in our preliminary studies[17,22]. Nevertheless, the confirmation of the ellipticine-derived DNA adducts formation by ellipticine by such peroxidase-catalyzed reactions remains to be confirmed.

Although HRP might only serve as a model peroxidase, the findings that MPO and LPO are effective in ellipticine oxidation may be of greater significance. MPO is expressed in several human leukemia cells and is capable of metabolizing several xenobiotics, including drugs such as ellipticine, in these cells. Furthermore, LPO, besides MPO and CYP[15], might be involved in oxidation of ellipticine in human mammary glands[18]. LPO is an enzyme present in milk, secreted by human mammary ductal epithelial cells into the breast ducts. Thus, like other lipophilic compounds ellipticine can possibly bioaccumulate in fatty tissues, such as the breast, and depending on the levels of activating enzymes present (e.g. LPO) ellipticine can be oxidized to intermediates modifying the key molecules such as DNA in this tissue. The importance of MPO and LPO in the oxidation of ellipticine is consistent with previous reports demonstrating that the enzymes metabolically activate a wide range of xenobiotics including drugs[18]. The detailed study concerning the modification of DNA molecules by ellipticine metabolites formed with peroxidases is under way in our laboratory.

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