THE EFFECT OF pH ON PEROXIDASE-MEDIATED OXIDATION OF AND DNA ADDUCT FORMATION BY ELLIPTICINE

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In order to understand the mechanism of enzymatic activation of an antineoplastic agent ellipticine, we investigated the effect of pH on the efficiency of three model peroxidases (bovine lactoperoxidase, human myeloperoxidase and horseradish peroxidase) in oxidation of ellipticine and in formation ellipticine–DNA adducts. The formation of the major ellipticine metabolite, ellipticine dimer, in which two ellipticine residues are connected through nitrogen N^6 in the pyrrole ring of one of the ellipticine moieties and carbon C9 of the other ellipticine, and formation of four ellipticine–DNA adducts were analyzed. All three peroxidases oxidize ellipticine to dimer and form ellipticine–DNA adducts, but lactoperoxidase and myeloperoxidase were less efficient in these processes than horseradish peroxidase. More than one order of magnitude higher rates of formation of dimer and amounts of the DNA adducts were found upon horseradish peroxidase than in reactions with lactoperoxidase or myeloperoxidase. An acid pH optimum was found for the formation of ellipticine dimer (pH 6.4), while the highest binding of ellipticine activated by peroxidases to DNA was detectable at pH 8.4. Likewise, the highest binding of 5-(hydroxymethyl)ellipticine, a metabolite of ellipticine generated by cytochrome P450, to DNA was found at pH 8.4. The results presented here are a contribution to the explanation of the reaction mechanism of formation of the major deoxyguanosine adduct in DNA generated from ellipticine *in vivo* and *in vitro* by its activation with cytochromes P450 and peroxidases.

Keywords: Ellipticine; Anticancer drugs; Peroxidases; Cytochrome P450; Oxidations; DNA adducts; ^{32}P-postlabeling; Mechanism of activation; Metabolism; DNA intercalation.

Ellipticine (5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole, **I**), 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 significant

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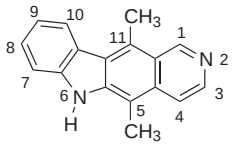
antitumor and anti-HIV activities (for a summary, see ref.[1]). The main reason for the interest in ellipticine and its derivatives for clinical purposes is their high efficiencies against several types of cancer, their rather limited toxic side effects, and their complete lack of hematological toxicity[2]. Nevertheless, ellipticine is a potent mutagen. Most ellipticine derivatives are mutagenic to *Salmonella typhimurium* strains in Ames test, bacteriophage T4, *Neurospora crassa*, and mammalian cells and induce prophage lambda in *Escherichia coli* (for a summary, see ref.[1]).



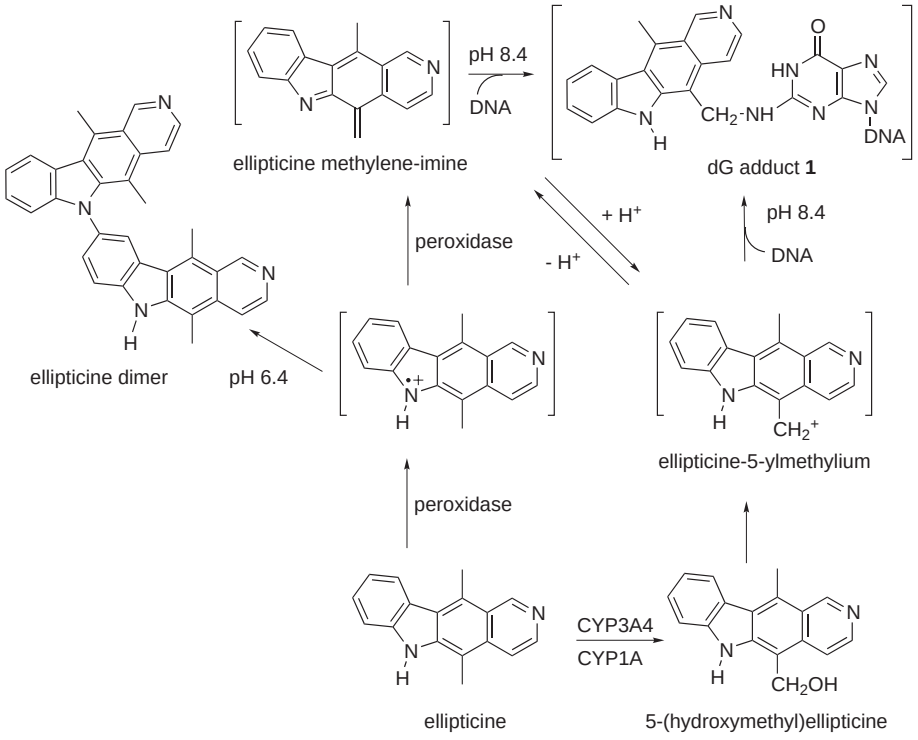
I

Ellipticines are anticancer drugs, whose precise mechanisms of action have not yet been explained. It was suggested that the prevalent mechanisms of their antitumor, mutagenic and cytotoxic activities are (i) intercalation into DNA [4,5] and (ii) inhibition of DNA topoisomerase II activity[2,6–8]. Ellipticine and 9-hydroxyellipticine also cause selective inhibition of p53 protein phosphorylation in several human cancer cell lines[9], and this correlates with their cytotoxic activity. Ellipticines also uncouple mitochondrial oxidative phosphorylation[10] and thereby disrupt the energy balance of cells.

Recently, we found another mode of the ellipticine action. We demonstrated that ellipticine covalently binds to DNA after being enzymatically activated with cytochromes P450 (CYP)$^+$ or peroxidases[1,11,12]. CYP3A4, 1A1 and 1B1 were found to be the most efficient enzymes activating ellipticine to form covalent DNA adducts *in vitro*[1,13–15]. Of the peroxidases, human cyclooxygenase (COX)-2, ovine COX-1, bovine lactoperoxidases (LPO), human myeloperoxidase (MPO) and horseradish peroxidase (HRP) efficiently generated ellipticine-derived DNA adducts[12]. These covalent DNA adducts were also detected in human breast adenocarcinoma MCF-7 cells[16], in V79 Chinese hamster lung fibroblast cells transfected with human CYP3A4, 1A1 and 1A2 (ref.[13]) and *in vivo* in rats exposed to this anticancer drug[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 enzymatic activation in target tissues.

In order to explain the mechanism of enzymatic activation of ellipticine, its oxidation by CYPs and peroxidases has been studied by us previ-

ously[11,15,18,19]. Not only the metabolites generated by both enzymatic systems, but also those responsible for the formation of two major ellipticine-derived DNA adducts, were identified[11,12,15]. CYP enzymes oxidize ellipticine to metabolites containing one atom of oxygen in their molecules; 7-hydroxy-, 9-hydroxy-, 5-(hydroxymethyl)-, 11-(hydroxymethyl)-ellipticine and ellipticine N^2-oxide are generated by this enzymatic system[15]. The major metabolite formed by peroxidases was characterized to be the ellipticine dimer, where two ellipticine residues are connected through nitrogen N^6 in the pyrrole ring of one of the ellipticine moieties and carbon C9 of the other ellipticine[11,12,19] (Scheme 1). As the minor peroxidase-mediated metabolite, the ellipticine N^2-oxide was found[11,19]. Even though, except to ellipticine N^2-oxide, the oxidative metabolites of this anticancer



SCHEME 1

Metabolism of ellipticine with peroxidases and human CYPs showing the characterized metabolites and those proposed to form the major DNA adduct **1**. The compounds shown in brackets are the electrophilic metabolites postulated as ultimate arylating species and proposed structure of the adduct **1**

drug formed by CYPs are not identical to those formed by peroxidases, both enzymes generate the same two major DNA adducts, which are also found to be formed in rats treated with ellipticine[17]. Two of the ellipticine metabolites generated by human CYP enzymes, 5-(hydroxymethyl)- and 11-(hydroxymethyl)ellipticine (the latter formed also spontaneously from ellipticine N^2-oxide by the Polonowski rearrangement[15]), are responsible for the formation of two major DNA adducts *in vitro* and *in vivo*, deoxyguanosine being identified as the target base of their binding[11,12,15,17,19]. While the ellipticine derivatives responsible for covalent modification of DNA with ellipticine were clearly established, the exact reactive species as well as the positions in guanine moieties where these species are bound remain to be elucidated. We have suggested that the reactive carbenium ions formed spontaneously from 5-(hydroxymethyl)- and 11-(hydroxymethyl)-ellipticine (ellipticin-5-ylmethylium and ellipticin-11-ylmethylium)[12,15,19] might react with the nucleophilic centers in the deoxyguanosine residue in DNA (i.e. the 2-NH_2 group of guanine) to form the adducts.

To further elucidate the reactive ellipticine intermediates formed by peroxidases, we investigated the effect of pH on peroxidase-mediated ellipticine dimer production and DNA adduct formation by ellipticine. Such a study contributes to a more detailed understanding of the mechanism of the ellipticine–DNA adduct formation.

EXPERIMENTAL

Abbreviations used

COX, cyclooxygenase; CYP, cytochrome P450; HRP, horseradish peroxidase; HPLC, high-performance liquid chromatography; LPO, lactoperoxidase; MPO, myeloperoxidase; PEI-cellulose, polyethylenimine-cellulose; RAL, relative adduct labeling; r.t., retention time; TLC, thin layer chromatography.

Chemicals and Reagents

Calf thymus DNA and ellipticine were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). The N^2-oxide of ellipticine (5,11-dimethyl-6*H*-pyrido[4,3-*b*]carbazole-*N*-oxide) was synthesized according literature[15]; its purity was >99.5% as estimated by HPLC. 5-(Hydroxymethyl)- and 11-(hydroxymethyl)ellipticine were isolated by multiple high-performance liquid chromatography (HPLC) from ethyl acetate extracts of incubations containing ellipticine and human and/or rat hepatic microsomes as described[15]. Enzymes and chemicals for the ^{32}P-postlabeling assay were obtained from sources described previously[1].

Enzyme Preparations

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

Enzyme Incubations

Incubation mixtures used to evaluate the oxidation of ellipticine with LPO, HRP and MPO contained in a final volume of 500 μl: 50 mM potassium phosphate buffer (pH 4.5–8.4), 1–100 μM ellipticine (dissolved in 5 μl methanol), 5 μg HRP and 10 μg LPO or MPO and hydrogen peroxide (40–250 μmol l^{-1}). Three control incubations (without peroxidases, without hydrogen peroxide, or without ellipticine) were done in parallel. Incubations 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 incubation, 5 μl of 1 mM salicylate or phenacetin in methanol was added as internal standards and the ellipticine metabolites were extracted with ethyl acetate (2 × 1 ml) as described[15]. The extracts were evaporated under nitrogen, dissolved in 50 μl of methanol and metabolites were separated by HPLC on a column (5 μm Ultrasphere ODS, Beckman, 4.6 × 250 mm) preceeded by a C-18 guard column. Eluents were either 45–90% methanol in 10 mM ammonium acetate (pH 2.8) (0–30 min), with a flow rate of 0.8 ml min^{-1} (method A), or 64% methanol in 5 mM heptane sulfonic acid containing 32 mM acetic acid with a flow rate of 0.8 ml min^{-1} (method B); detection was performed at 296 nm [15,18]. Recoveries of ellipticine metabolites were around 95% in the presence of enzymes without hydrogen peroxide. The major metabolite with a retention time (r.t.) 19.95 min and unconverted ellipticine with r.t. 11.85 min were separated by HPLC using method A (Fig. 1), while the minor metabolite, formed in ca. 100-fold lower levels than the major metabolite (r.t. 11.2 min) and residual ellipticine with r.t. of 12.9 min, were separated with method B (ref.[18]). The identity of both ellipticine metabolites – ellipticine dimer (major metabolite) and N^2-oxide of ellipticine (minor metabolite) was verified by mass spectroscopy[18], NMR, and/or co-chromatography on HPLC (refs[11,12,19]). The detailed data from NMR analysis are shown in our previous work[19].

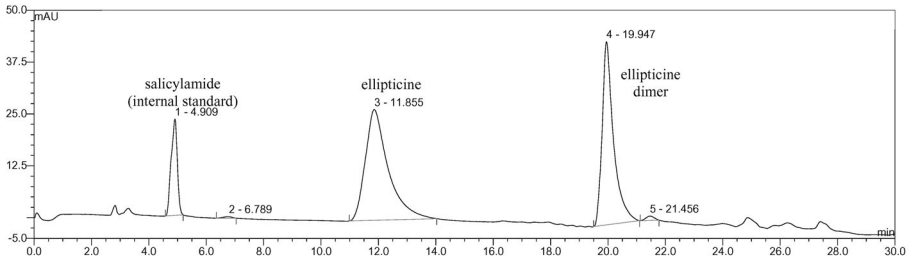


FIG. 1
HPLC chromatogram of ellipticine metabolites formed by HRP (5 μg). For details see Experimental

For DNA modification studies, the incubation mixtures contained in the final volume 750 μl: 50 mM potassium phosphate buffer (pH 5.0–9.0), 1–100 μM ellipticine dissolved in 7.5 μl of methanol, 5 μg of peroxidase (LPO, HRP or MPO), 100 μM hydrogen peroxide, and 1 mg of calf thymus DNA. Four control incubations (without peroxidases, without hydrogen peroxide, without DNA, or without ellipticine) were performed in parallel. Incubations with peroxidases were carried out at 37 °C for 5–60 min. All reactions were initiated by adding ellipticine dissolved in methanol (final methanol concentration was 1%). Incubations in which 5-(hydroxymethyl)ellipticine was used instead of ellipticine, contained in a final volume of 500 μl: 50 mM potassium phosphate buffer (pH 6.0, 7.4 and 8.4), 10 μM 5-(hydroxymethyl)ellipticine dissolved in 5 μl of methanol and 1 mg of 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]. 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).

From experiments performed earlier, calf thymus DNA (1 mg) incubated with 10 μM 5-(hydroxymethyl)- or 11-(hydroxymethyl)ellipticine in 50 mM potassium phosphate buffer (pH 7.4)[11,12,15], and liver DNA of rats treated with ellipticine (40 mg ellipticine per body weight in one dose, after two days the animals were killed and tissues analyzed for DNA adduct formation)[17] were labeled with ^{32}P to compare adduct spot patterns.

^{32}P-Postlabeling Analysis of ^{32}P-Labeled Deoxyribonucleoside 3′,5′-Bisphosphate Adducts

The ^{32}P-postlabeling of nucleotides was carried out using the nuclease P1 enrichment procedure, found previously to be appropriate for detection and quantization ellipticine-derived DNA adducts formed *in vitro*[1] and *in vivo*[17]. Separation was carried out on PEI-cellulose thin layer plates (Macherey and Nagel, Düren, Germany). Chromatographic conditions used were: D1, 1.0 M sodium phosphate, pH 6.8; D2 was omitted; D3, 3.5 M Li formiate, 8.5 M urea, pH 4.0; D4, 0.8 M LiCl, 0.5 M Tris, 8.5 M urea, pH 9.0; D5, 1.7 M sodium phosphate, pH 6.0. Normal nucleotides were separated in 280 mM $(NH_4)_2SO_4$ and 50 mM NaH_2PO_4, pH 6.5. Quantitative analysis was performed using a Canberra Packard instant imager. Adduct levels were calculated in units of relative adduct labeling (RAL) which is the ratio of cpm (counts per minute) of adducted nucleotides to cpm of total nucleotides in the assay.

HPLC Analysis of ^{32}P-Labeled Adducts

HPLC analysis was performed essentially as described previously[13–17,19]. Individual spots detected by ^{32}P-postlabeling were excised from the thin layer and extracted[13–17,19]. Cut-outs were extracted with two 800 μl portions of 6 M ammonium hydroxide/isopropanol (1:1) for 40 min. The eluent was evaporated in a Speed-Vac centrifuge. The dried extracts were dissolved in 100 μl of methanol/phosphate buffer (pH 3.5) 1:1 (v/v). Aliquots (50 μl) were analyzed on a phenyl-modified reversed-phase column (250 × 4.6 mm, 5 μm Zorbax Phenyl; Säulentechnik Knauer) with a linear gradient of methanol (from 40 to 80% in 45 min) in aqueous 0.5 M sodium phosphate and 0.5 M phosphoric acid (pH 3.5) at a flow rate of 0.9 ml/min. Radioactivity eluting from column was measured by monitoring Cerenkov radiation with a Berthold LB 506 C-I flow-through radioactivity monitor (500 μl cell, dwell time 6 s).

RESULTS

The efficiencies of three model peroxidases (HRP, bovine LPO and human MPO) in oxidation of ellipticine to its major metabolite, ellipticine dimer, was examined. HPLC (method A, see Experimental) was used to quantify the levels of this metabolite. Ellipticine dimer is eluted as a single product peak at r.t. of 19.95 min (see Fig. 1). While all three tested peroxidases oxidize ellipticine to this product, their efficiencies in oxidation of ellipticine differ considerably. LPO and MPO were less efficient in catalyzing oxidation of ellipticine to dimer than HRP (Table I). One and two orders of magnitude higher rates of formation of ellipticine dimer were found for HRP than for LPO and MPO.

TABLE I

The effect of pH on oxidation of ellipticine to the ellipticine dimer with HRP, LPO and MPO. Reaction mixtures contained in a total volume of 0.5 ml of the 50 mM sodium phosphate buffer, pH 4.6–8.4: 5 μg HRP and 10 μg LPO or MPO, 10 μM ellipticine and 0. 25 mM H_2O_2. The incubation period was 15 min.

Peroxidase	pH	Reaction rate[a] (nmol min^{-1} per μg peroxidase)
HRP	4.5	0.248 ± 0.021[b]
	5.4	0.688 ± 0.056
	6.4	1.300 ± 0.122
	7.4	0.317 ± 0.030
	8.4	0.034 ± 0.003
LPO	4.5	0.046 ± 0.005
	5.4	0.096 ± 0.009
	6.4	0.240 ± 0.021
	7.4	0.105 ± 0.013
	8.4	0.106 ± 0.014
MPO	5.4	0.011 ± 0.001
	6.4	0.020 ± 0.002
	7.4	0.005 ± 0.001

[a] Reaction rates are expressed as amounts of the ellipticine dimer production per μg peroxidase per minute. [b] The values in the table are the means ± standard errors (from three parallel experiments).

The oxidation of ellipticine with peroxidases is time- and pH-dependent. The rate of ellipticine dimer formation is linear untill 15 min of incubation, reaching a plateau after longer incubations (not shown). The rates of formation of this ellipticine metabolite were measured at pH 4.5, 5.4, 6.4, 7.4 and 8.4. For all the peroxidases used in our study, the highest reaction rate of the reaction was found at pH 6.4 (Table I).

The kinetics of ellipticine oxidation with peroxidases in the presence of hydrogen peroxide (two-substrate reaction) was examined with each of the peroxidases tested in the study, hydrogen peroxide, and increasing concentrations of ellipticine. Under the conditions used and at pH 6.4, the values of apparent Michaelis constants (K_m) for ellipticine are similar for LPO and MPO, but the value for HRP is more than 4-fold higher than for other peroxidases. The value of the maximum rate for the ellipticine oxidation (v_{max}) is highest for HRP and much lower for the other two peroxidases (Fig. 2). The lower efficiency of LPO and MPO, however, was not specific for ellipticine, we formerly observed a lower activity of these peroxidases for other substrates (i.e. *o*-anisidine, 3-aminobenzanthrone)[20,21].

During the ellipticine oxidation with peroxidases, DNA adducts are generated (Fig. 3). Except for MPO, the pattern of ellipticine–DNA adducts determined by the nuclease P1 version of the ^{32}P-postlabeling technique[1,11], consisted of two major (spots 1 and 2 in Fig. 3) and two minor (spots 6 and 7 in Fig. 3A–3C) adducts. Control incubations without peroxidases or hydrogen peroxide were free from adduct spots 1, 6 and 7, but the adduct spot 2, formed also non-enzymatically[1,11,14], was detected in all controls. All four adducts are formed also *in vivo*, in DNA of liver, lungs and kidneys of rats treated with ellipticine[17]. Two of them, adducts 1 and 2, are generated from 5-(hydroxymethyl)- and 11-(hydroxymethyl)ellipticine on deoxyguanosine in DNA, respectively[11,12,15,19]. For comparison, Figs 3D, 3E and 3F show adducts formed *in vivo*[17], and in DNA reacted with 5-(hydroxymethyl)- and 11-(hydroxymethyl)ellipticine[11,12,15,19], respectively, and Fig. 4 co-chromatography of the adducts on HPLC. Adduct spots 1 and 2 obtained from DNA reacted with ellipticine activated with peroxidases (exemplified for LPO) and those from experiments with 5-(hydroxymethyl)- and 11-(hydroxymethyl)ellipticine were excised, extracted and analyzed by co-chromatography on reversed-phase HPLC. These experiments showed that the ^{32}P-labeled ellipticine adducts were stable under the alkaline extraction conditions used and that both major adducts formed in DNA by ellipticine activated with peroxidase were indistinguishable from those generated by 5-(hydroxymethyl)- and 11-(hydroxymethyl)ellipticine. The major adduct formed in DNA by ellipticine activated with peroxidase (spot 1

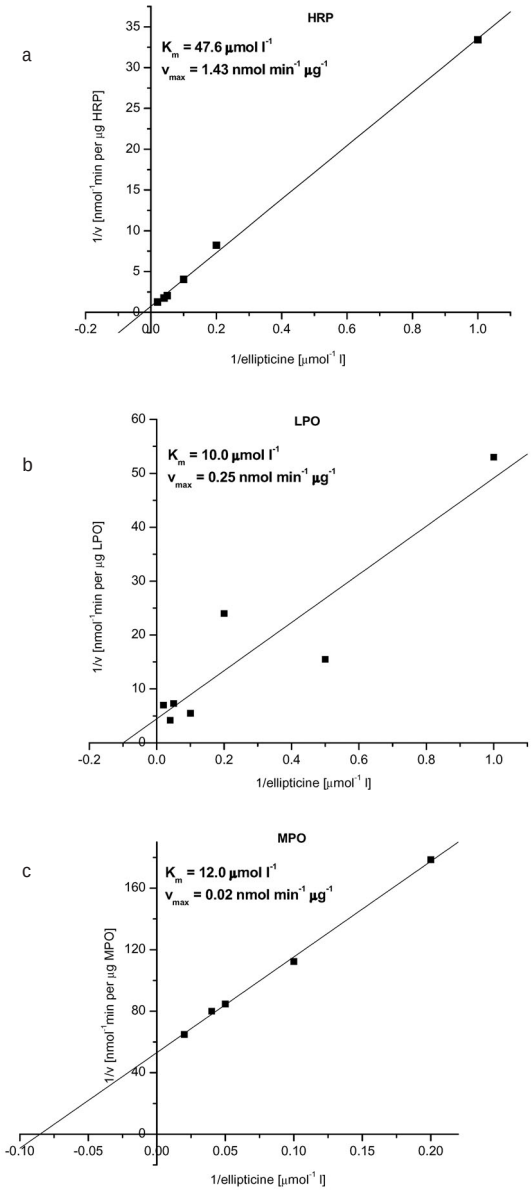


FIG. 2

Double reciprocal plots of the rate of ellipticine oxidation with HRP (a), LPO (b) and MPO (c). The experiments were performed at pH 6.4. The values in the figure are means from three parallel experiments. Standard deviations were less than 10%

in Fig. 3B) eluted with r.t. of 11.77 min (Fig. 4a), corresponding to the r.t. of 11.78 min of deoxyguanosine adduct spot 1 in DNA reacted with 5-(hydroxymethyl)ellipticine (Fig. 3E). When equal amounts of radioactivity of these adduct spots were mixed prior to analysis, a single peak was found (Fig. 4b). Adduct spot 2 of Fig. 3B, generated by ellipticine activated with peroxidase, produced a major radioactive peak (r.t. 8.88 min) (Fig. 4d) corresponding to adduct spot 2 formed by 11-(hydroxymethyl)ellipticine (Fig. 4f). A minor peak of radioactivity was eluted at 14.28 min, showing that a minor adduct is present both in DNA formed by ellipticine activated with peroxidase and in DNA reacted with 11-(hydroxymethyl)ellipticine, comigrating with adduct spot 2 on TLC (Figs 4d–4f).

The yield of ellipticine–DNA adducts is also time- and pH-dependent. Ellipticine–DNA adduct formation catalyzed by peroxidases increased with incubation time and reached also a plateau after 15 min (data not shown).

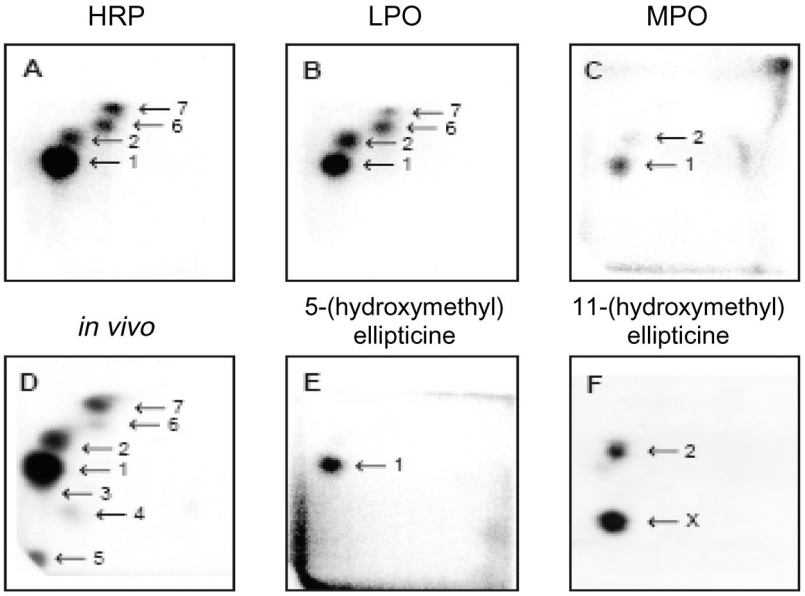


FIG. 3

Autoradiographic profiles of ellipticine–DNA adducts analyzed by ^{32}P-postlabeling assay. The adduct profiles obtained from calf thymus DNA reacted with ellipticine (100 μmol l^{-1}) and plant HRP (A), bovine LPO (B), human MPO (C) (5 μg peroxidases were used in the incubations), from liver DNA of rats treated with 40 mg ellipticine per kilogram body weight in one dose (D), from calf thymus DNA reacted with 5-(hydroxymethyl)ellipticine (E) and 11-(hydroxymethyl)ellipticine (F). For details, see Experimental

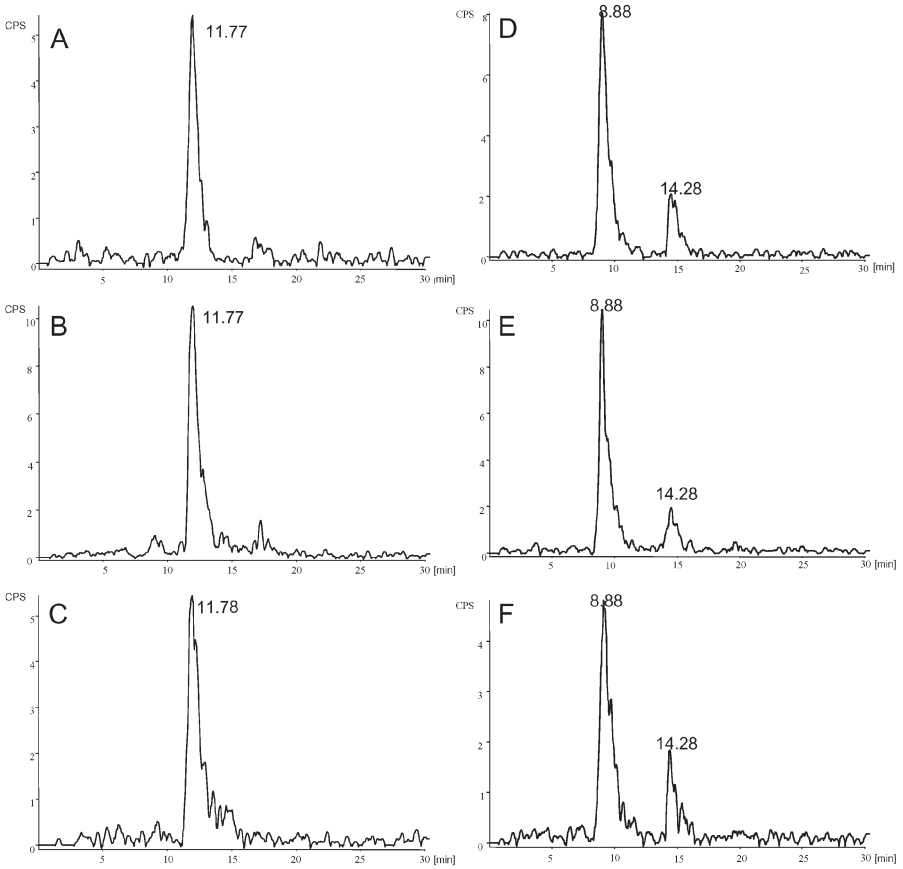


FIG. 4

Separation of ^{32}P-labeled ellipticine–nucleoside 3′,5′-bisphosphate adducts on a phenyl-modified reversed-phase column to compare adducts obtained in DNA by ellipticine activated with LPO (Fig. 3B) to those formed by 5-(hydroxymethyl)ellipticine or 11-(hydroxymethyl)ellipticine (Fig. 3E and 3F). Adduct spots were excised and extracted from PEI-cellulose TLC plates, dissolved and injected into the HPLC system. a Spot 1 from an incubation of LPO-activated ellipticine with DNA (Fig. 3B); b spot 1 from Fig. 3B mixed with spot 1 from Fig. 3E; c spot 1 from an incubation of 5-(hydroxymethyl)ellipticine with DNA (Fig. 3E); d spot 2 from an incubation of LPO-activated ellipticine with DNA (Fig. 3B); e spot 2 from Fig. 3B mixed with spot 2 from Fig. 3F; f spot 2 from an incubation of 11-(hydroxymethyl)ellipticine with DNA (Fig. 3F). The abscissa shows the retention time in min, the ordinate radioactivity in arbitrary units

However, different effects of pH on dimerization and DNA adduct formation were found. The formation of DNA adducts by ellipticine was measured at pH 5.0, 6.0, 7.4, 8.4 and 9.0. Only low amounts of DNA adducts (mainly adduct spot 2) were observed at pH 5.0 and 6.0 with HRP and LPO, while no ellipticine activation with MPO was detectable in acidic conditions. Increasing pH resulted in an increase in the yield of adducts by all test peroxidases. The highest levels of the ellipticine–DNA adducts were detected at pH 8.4 (Table II). Because the major ellipticine–DNA adduct (spot 1 in Fig. 3) is formed from reactive species generated from 5-(hydroxymethyl)-ellipticine (Figs 3 and 4), the effect of pH on formation of this adduct generated directly from DNA and 5-(hydroxymethyl)ellipticine was also

TABLE II
Quantitative analysis of adducts formed by peroxidase-activated ellipticine with DNA, detected by ^{32}P-postlabeling[a]

Peroxidase	pH	RAL[b] (mean ± SD/10^7 nucleotides)			
		Spot 1	Spot 2	Spot 6	Spot 7
HRP	5.0	n.d.	1.0 ± 0.11	n.d.	n.d.
	6.0	1.8 ± 0.2	2.1 ± 0.20	n.d.	n.d.
	7.4	132.3 ± 7.2	8.51 ± 0.42	1.28 ± 0.08	1.30 ± 0.05
	8.4	438.9 ± 34.5	11.3 ± 0.99	3.20 ± 0.30	2.50 ± 0.16
	9.0	365.7 ± 29.2	9.2 ± 0.78	1.90 ± 0.15	1.70 ± 0.12
LPO	5.0	n.d.	0.9 ± 0.10	n.d.	n.d.
	6.0	n.d.	1.9 ± 0.20	n.d.	n.d.
	7.4	15.2 ± 0.82	3.0 ± 0.11	0.83 ± 0.04	0.52 ± 0.03
	8.4	90.1 ± 8.3	3.5 ± 0.32	1.30 ± 0.11	0.90 ± 0.07
	9.0	75.0 ± 6.5	2.8 ± 0.19	0.90 ± 0.15	0.80 ± 0.08
MPO	6.0	n.d.	n.d.	n.d.	n.d.
	7.4	0.73 ± 0.04	0.28 ± 0.02	n.d.	n.d.
	8.4	3.10 ± 0.29	0.90 ± 0.11	n.d.	n.d.
	9.0	1.02 ± 0.11	0.60 ± 0.05	n.d.	n.d.

[a] Experimental conditions were as described in Experimental with 100 μM ellipticine, 5 μg peroxidases and 250 μM H_2O_2 per incubation (37 °C, 15 min). [b] RAL, relative adduct labeling, and standard deviations were obtained from triplicate determinations. n.d., not detectable (the detection limit of RAL in nucleotides was $1:10^{10}$).

investigated. Only low levels of the ellipticine–DNA adduct (spot 1) was detectable at pH 6.0, while increasing pH resulted in a pronounced increase in formation of this adduct (Table III).

The ellipticine–DNA adduct formation is dependent on ellipticine concentration. The ellipticine concentrations yielding half-maximum binding of ellipticine to DNA, characterizing the affinity of the ellipticine reactive intermediates to bind to DNA, are shown in Table IV. The highest levels of ellipticine binding to DNA were generated using HRP, followed by LPO > MPO (Table IV).

TABLE III
The effect of pH on levels of the adduct formed by reaction of 5-(hydroxymethyl)ellipticine with DNA[a], detected by ^{32}P-postlabeling[b]

pH	RAL[c] (mean ± SD/10^7 nucleotides)
6.0	1.3 ± 0.3
7.4	23.3 ± 2.0
8.4	49.7 ± 3.2

[a] See adduct spot 1 in Fig. 3. [b] Experimental conditions were as described in Experimental with 10 μM 5-(hydroxymethyl)ellipticine and 1 mg calf thymus DNA (37 °C, 90 min). [c] RAL, relative adduct labeling, and standard deviations were obtained from triplicate determinations.

TABLE IV
Concentrations of ellipticine producing half-maximum DNA adduct yields after ellipticine oxidation with peroxidases[a]

Peroxidase	Ellipticine concentration[b], μmol l^{-1}			
	pH 6.0	pH 7.4	pH 8.4	pH 9.0
HRP	9.2 ± 0.92[c]	2.1 ± 0.32	1.0 ± 0.12	3.3 ± 0.31
LPO	10.0 ± 1.2	8.0 ± 1.1	6.2 ± 0.7	8.9 ± 1.0
MPO	n.m.[d]	64.0 ± 5.1	52.3 ± 4.9	71.0 ± 6.7

[a] Experimental conditions were as described in Experimental with 1–100 μM ellipticine, 5 μg peroxidases and 400 μM H_2O_2 per incubation (37 °C, 15 min). [b] Evaluated from the formation of the ellipticine–DNA adduct spot 1 (see Fig. 3), quantitated by RAL, relative adduct labeling, see Experimental. [c] The average binding levels and standard deviations were obtained from triplicate determinations. [d] n.m., not measured.

DISCUSSION

Our recent studies indicate that ellipticine is easily and effectively oxidized with peroxidases to species that bind to DNA [11,18,19]. This process has not been fully characterized yet, but this knowledge is important for evaluation of the pharmacological efficacy and/or the genotoxic side effects of ellipticine in organisms, including humans. Therefore, we tried to increase our understanding of the mechanism of ellipticine oxidation with peroxidases. Plant HRP, bovine LPO and human MPO were used as model enzymes.

The results of the present study demonstrate that differences occur between the pH optimum of the peroxidase-mediated oxidation of ellipticine to its major metabolite, ellipticine dimer, and that of ellipticine binding to DNA. A mildly acidic pH optimum was found for the formation of ellipticine dimer (pH 6.4), while the highest binding of ellipticine activated by peroxidases to DNA reaches its maximum at pH 8.4. Both acid and alkaline pH optima were found for peroxidase-catalyzed oxidation of various other chemicals[23–26]. For example, an acid pH optimum was found for the peroxidase-catalyzed oxidation of several aromatic and heterocyclic amines[23,24,27], while peroxidase-mediated oxidation of other compounds such as Sudan I (ref.[28]) is maximal at alkaline pH. The pH optimum for peroxidases is dependent not only on the arrangement of the active centers of these enzymes and/or the structure of their substrates, but also on the redox potential of their substrates[23–29].

It is evident that the formation of DNA adducts by ellipticine activated with peroxidases is not dictated only by the optimal conditions (pH) for ellipticine oxidation with these enzymes. While acid pH facilitates ellipticine oxidation with peroxidases, an increase in pH resulted in a pronounced increase in DNA adduct formation. The most significant sensitivity to pH changes was found in the formation of the major ellipticine--derived DNA adduct, generated both in the peroxidase activation system and by 5-(hydroxymethyl)ellipticine (Tables III and IV). This adduct was proposed to be formed from the reactive species, carbenium ion (ellipticin-5-ylmethylium), formed in the ellipticine oxidation with CYPs [15] and peroxidases (through ellipticine methylene-imine)[11,12,19] (Scheme 1). Such a species was proposed to react with one of the nucleophilic centers in the deoxyguanosine residue (i.e. the 2-NH_2 group of guanine) in DNA [15,22]. The finding that the levels of the deoxyguanosine adduct formed from this ellipticine reactive species during ellipticine oxidation by peroxidases or directly in the reaction of 5-(hydroxymethyl)ellipticine with DNA signifi-

cantly decreased under acid conditions strongly supported the above suggestion. A decrease in pH leads to protonation of the NH_2 group of guanine in the DNA chain, causing a decrease in its nucleophilicity, essential for binding of ellipticin-5-ylmethylium. The basic pH might also facilitate the second electron transfer to form the ellipticine methylene-imine (Scheme 1). Nevertheless, such suggestions need to be confirmed by identification of the structure of the deoxyguanosine adduct. The preparation of the adduct from 5-(hydroxymethyl)ellipticine and deoxyguanosine (and/or deoxyguanosine 3′-monophosphate) in the amounts sufficient for its structure characterization is the aim of our further study.

In our studies HRP serves as a model peroxidase, but the findings that LPO and MPO are effective in ellipticine oxidation is of greater significance. Human MPO is expressed in acute myeloblastic leukemia[30,31], and might be involved in metabolic activation of drugs including ellipticine in leukemic myeloblasts[32,33]. Because of the high expression of this enzyme in acute myeloblastic leukemia, it is also used as a diagnostic marker in this leukemia. In addition, human MPO expressed in neutrophils and present in milk and blood[34] might, besides CYPs, participate in metabolism of ellipticine in other cancers[30,31] and/or in healthy tissues[34]. Neutrophils release MPO and undergo a respiratory burst, which is characterized by a massive increase in oxygen consumption followed by a NADPH-dependent production of hydrogen peroxide, superoxide and other free radicals. Hydrogen peroxide required for MPO-mediated ellipticine oxidation can also be supplied by xanthine oxidase[24]. LPO is secreted by human mammary ductal epithelial cells into breast ducts[24,34]. Like other lipophilic compounds, ellipticine may accumulate in fatty tissues, such as the breast and, depending on the levels of activating enzymes present (e.g. LPO and MPO), ellipticine can be oxidized to intermediates modifying key molecules such as DNA in this tissue. The elucidation of the role of peroxidases in metabolic activation of ellipticine *in vivo* is our next goal. For instance, MPO-knockout mice may help in evaluation of the involvement of this enzyme in bioactivation of ellipticine *in vivo*[35].

CONCLUSIONS

The results presented in this work confirmed that one of the multiple modes of action of ellipticine, a potent antineoplastic agent, results from its metabolic activation with peroxidases to species binding covalently to DNA. The results shed light on the mechanism of ellipticine activation. We have proposed the binding of the reactive species, the carbenium ion

ellipticin-5-ylmethylium, to the exocyclic NH_2 group of guanine in DNA as the mechanism responsible for formation of the major DNA adduct observed *in vivo* in rats treated with ellipticine. Furthermore, the study forms the basis for further prediction of human susceptibility to ellipticine.

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