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THE HYDROXYLATION OF THE ANTITUMOR AGENT, ELLIPTICINE, BY LIVER MICROSOMES FROM DIFFERENTLY PRETREATED RATS

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Abstract—The antitumor agent, ellipticine (5,11-dimethyl-6H-pyrido[4-3,b]carbazole) is mainly hydroxylated in position 9 by liver microsomes of differently pretreated rats, this result being in agreement with that obtained previously *in vivo*. A quick and reliable fluorometric assay, based on the differential fluorescent properties of ellipticine and 9-hydroxyellipticine, is described for the measurement of the 9-hydroxylase activity of different microsomes. This activity exhibits the usual features of the cytochrome-P450-dependent monooxygenases. Control rat liver microsomes exhibit a good affinity for ellipticine ($K_m = 3 \times 10^{-5}$ M) but a low specific activity (0.1 nmole min^{-1} mg protein $^{-1}$), perhaps related with an excess substrate inhibition. Pretreatment of rats with benzo[a]pyrene or ellipticine enhances the rate of 9-hydroxylation: pretreatment with phenobarbital does not. Metyrapone and 7,8 benzoflavone are poor inhibitors of ellipticine hydroxylation particularly in microsomes from benzo[a]pyrene- or ellipticine-pretreated rats.

Ellipticine and a number of its derivatives exert some interesting therapeutic actions. Among them, 9-hydroxyellipticine proved very active against L1210 murine leukaemia [1]. The anti-inflammatory [2], trypanocidal [3] bacteriostatic and bacteriocidal [4] properties of this compound as well as its toxicity [5] have also been studied. The physiological distribution of ellipticine in tissues, and its excretion have been studied in mice [6], dogs and rats [7]. It appears that the drug is rapidly cleared from blood, then widely distributed in tissues and finally excreted, principally in the feces through biliary secretion. A fraction of ellipticine was excreted after metabolic transformation into 9-hydroxyellipticine and eliminated as a glucuronic acid conjugate [8].

The studies on antitumor activity of ellipticine derivatives revealed that the use of 9-hydroxyellipticine instead of ellipticine enhanced the antileukaemic efficiency [9]. Moreover, it seems that some anticancerous agents such as aniline mustard [10], cyclophosphamide [11], prednisone, prednisolone and procarbazide [12] must be activated by the enzymes localized in the endoplasmic reticulum to exert their antitumor action. Consequently, it was interesting to study the metabolization of ellipticine by the microsomal cytochrome P450-dependent hydroxylation system, to compare the *in vitro* results with those obtained *in vivo* and to test the importance of the metabolism of this drug for its anticancer activity. It was important also to estimate the effect of certain drugs currently used in therapeutics, such as barbituric acid derivatives, or widespread in the environment, like polycyclic hydrocarbons, on ellipticine metabolism.

Very recently we have shown that ellipticines strongly bind to the iron of oxidized and reduced P450-cytochromes and particularly to cytochrome P448, acting consequently as good inhibitors of benzo[a]pyrene hydroxylase [13].

In this paper, we describe the hydroxylation of ellipticine by liver microsomes of control and differently pretreated rats. For the quantitative determinations, a fluorometric assay has been devised, based on the differential fluorescent properties of ellipticine and 9-hydroxyellipticine which appeared as the major metabolite. The influence, on the 9-hydroxylation of ellipticine, of known inhibitors of cytochromes P450 or P448 and of the pretreatment of rats by ellipticine itself or known inducers of cytochrome P450 or P448, is reported.

MATERIALS AND METHODS

Chemicals. Ellipticine and its 9-methoxy derivative were prepared according to the method of Dalton [14]. The synthesis of 9-hydroxyellipticine (9-OH-E) has been already described [15]. The purity of these compounds was checked by TLC and NMR spectroscopy and found to be better than 95 per cent. Metyrapone (MP) was obtained from Ciba-Geigy,

Abbreviations used are: 9-OH-E, 9 hydroxyellipticine; TLC, thin layer chromatography; NMR spectroscopy, nuclear magnetic resonance spectroscopy; MP, metyrapone; 7,8-BF, 7,8-Benzoflavone; PB, phenobarbital; BP, benzo[a]pyrene; TMS, buffer pH 7.5, 50 mM Tris–HCl, 3 mM $MgCl_2$, 200 mM sucrose; DMF, dimethylformamide.

7,8-Benzoflavone (7,8-BF) was purchased from Prolabo.

Microsomal preparations. They were obtained from male Wistar A.G. rats weighing about 150 g. When the animals were induced, they received intraperitoneally 80 mg/kg phenobarbital (PB) (one dose/day for 3 days) or 20 mg/kg benzo[a]pyrene (BP) or ellipticine dissolved in olive oil (one dose/day for 2 days). The animals were killed the day after the last injection.

Livers were removed immediately after perfusion of animals with a 0.9% sodium chloride solution, then washed and homogenized in three vol. 50 mM Tris–HCl, 3 mM $MgCl_2$ and 200 mM sucrose (TMS), pH 7.5. Preparation was carried out at 4°. The homogenate was centrifuged at 9,000 *g* for 20 min and the supernatant at 100,000 *g* for 60 min. The pellet (microsomes) was taken up in the same volume of TMS buffer, washed and recentrifuged at 100,000 *g* for 30 min. Microsomes were suspended in a small volume of TMS so that the protein concentration, determined by the method of Lowry *et al.* [16] was approximately 40 mg/ml. The microsomal cytochrome P450 content was determined according to Omura and Sato [17].

Fluorometric determination of 9-hydroxyellipticine formation. The hydroxylation of ellipticine was determined fluorometrically as follows: ellipticine (up to 188 nmoles) in 5 μl DMF was added to the reaction mixture (final volume: 1.1 ml) containing in TMS buffer pH 7.5, 1 mg NADPH and microsomal suspension (2 mg protein/ml). After a 20 min incubation at 37° the reaction was stopped by shaking the mixture for 1 min, with 2 ml ethyl acetate and 0.05 ml 1N NaOH which causes 30 per cent increase of fluorescence intensity. After centrifugation the concentration of 9-hydroxyellipticine in organic solution was determined spectrofluorometrically with excitation at 436 nm and fluorescence emission at 475 nm with the use of a Zeiss PMQ II spectrophotofluorometer: under these conditions, the extracted ellipticine exhibited a very weak fluorescence. The results were compared with those of a standard curve given by pure 9-hydroxyellipticine solution extracted under the same conditions (see below). We verified that in the used extraction conditions 9-hydroxyellipticine is completely recovered.

Isolation of ellipticine metabolites. Ellipticine metabolites were separated by TLC on silica-gel. The solution of metabolites was concentrated under vacuum then taken up in the minimum volume of ethyl acetate and chromatographed, for 30 min with a mixture benzene–chloroform–ethanol (9:6:3, v/v/v).

RESULTS

Nature of the metabolites. The main metabolite of ellipticine, obtained by incubation with hepatic microsomes of phenobarbital pretreated rat in presence of NADPH and O_2, is 9-hydroxyellipticine (Fig. 1). This result is in agreement with previous results obtained *in vivo* [8, 18]. Several characteristics of this metabolite: its R_f in thin layer chromatography (the corresponding spot being intensively red-colored

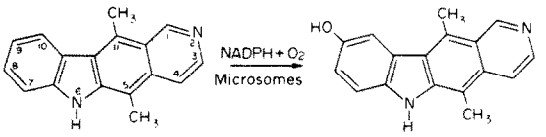

Fig. 1. Chemical structure of ellipticine and its main *in vitro* metabolite.

when exposed to iodine), its u.v. spectrum (λmax = 273 and 288 nm in ethyl acetate), its fluorescence spectra (Fig. 2a), its proton NMR and mass spectra* are identical to those of an authentic sample of 9-hydroxyellipticine. So, *in vitro*, the main microsomal oxidation of ellipticine is a hydroxylation of the aromatic nucleus in a position that is para to the indole nitrogen. A second metabolite appears in smaller quantities during microsomal NADPH dependent mixed function oxidation of ellipticine. In TLC, a second spot appears with a R_f higher than that of 9 OH-E. This other metabolite, the structure of which is under study,* was also found *in vivo* [18]. Similar results are obtained with liver microsomes from control or phenobarbital-, benzo[a]pyrene- and ellipticine-treated rats.

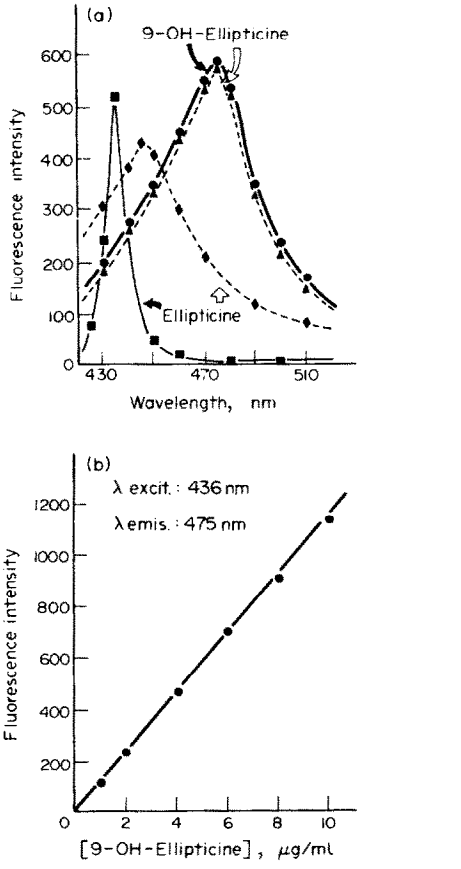

Fig. 2. Fluorometric data concerning the measurement of microsomal ellipticine hydroxylation. (a) Fluorescence emission spectra of ellipticine and 9-hydroxyellipticine solutions (5 μg/ml in 2 ml ethyl acetate + 0.05 ml NaOH 1N). – – –, excitation at 405 nm; ——, excitation at 436 nm. Slitwidth: 0.5 mm. (b) Standard curve for the determination of 9-hydroxyellipticine concentration in the fluorometric test conditions (see Materials and Methods).

* To be published.

Table 1. Effects of various incubation conditions on the 9-hydroxylation of ellipticine by control rat microsomes

Incubation system	% Activity
Complete system*	100
− NADPH	0
− O_2, + N_2	3
− NADPH + NADH (10^{-3} M)	20
+ NADH (10^{-3} M)	110
+ CO (10^{-3} M)	46
+ Deoxycholate (5×10^{-3} M)	0
Boiled microsomes	0

* The complete system contained in 1.1 ml TMS buffer pH 7.5: 2 mg of microsomal protein, 4×10^{-5} M ellipticine and 10^{-3} M NADPH. Each value represents the mean of 2–5 determinations.

Quantitative detection of 9-hydroxyellipticine. In order to compare the activities of microsomal hydroxylases under different conditions towards ellipticine, we first devised a sensitive assay for detecting ellipticine 9-hydroxylase activity, utilizing the observed differences in fluorescence between ellipticine and 9-hydroxyellipticine. The fluorescence spectra of 9-OH-E and ellipticine itself with excitation at 405 or 436 nm are compared in Fig. 2a. With a 436 nm excitation, the spectra of the two compounds are sufficiently different to permit a quantitative measurement of 9-OH-E even in the presence of ellipticine in excess. The determination of the amount of 9-OH-E formed after microsomal hydroxylation of ellipticine can then be made after extraction of the incubation mixture by ethyl acetate and NaOH (9-OH-E is extracted by ethyl acetate even from alkaline water), centrifugation of the organic phase, measurement of its fluorescence intensity at 475 nm and extrapolation from the standard curve of Fig. 2b. One then can detect 0.05–0.1 μg/ml of 9-OH-E (200–400 pmoles/ml).

Factors affecting the microsomal 9-hydroxylation of ellipticine in vitro

Cofactor requirements. Like other microsomal mixed-function oxidations the 9-hydroxylation of ellipticine requires NADPH and molecular oxygen (Table 1). NADH is far less effective as an electron donor, but used together with NADPH, it produces a slight stimulation. Heat denatured microsomes are totally inactive. Deoxycholate completely inhibits the reaction because of its denaturing effect on the enzyme organization in the microsomal membrane.

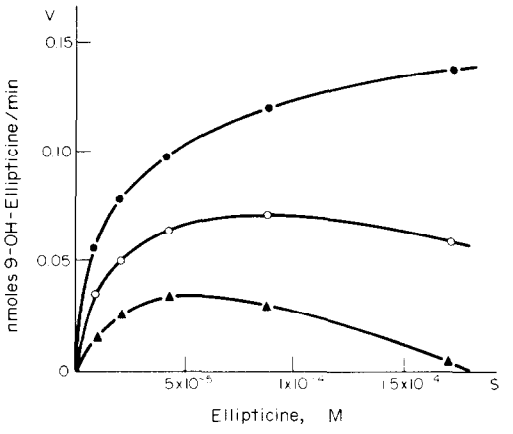

Fig. 3. Effect of microsomal protein concentrations on ellipticine hydroxylation. The amount of 9-hydroxyellipticine formed in 1 min is plotted against substrate concentration. The microsomal preparation from a control rat was used: the protein concentrations were 1.5 mg ●——●, 1 mg ○——○, 0,5 mg ▲——▲ per ml respectively. All other conditions are described in Materials and Methods.

Kinetic experiments. The reaction is linear with time during the first 20 min at 37°. A 10^{-3} M NADPH concentration is necessary to reach the maximum hydroxylation rate. Figure 3 shows the dependence of the hydroxylation reaction rate on substrate concentration, at different microsomal protein concentrations. Especially at low protein concentrations, one observed a decrease of the reaction rate for ellipticine concentrations above 10^{-4} M instead of the classical saturation effect. This may be due to an inhibitory effect of ellipticine on its own hydroxylation, as we showed that this compound has a very high affinity for cytochrome P450-Fe(III) or Fe(II) [13], or to a non specific effect of denaturation of the microsomal proteins by ellipticine which is highly hydrophobic. The dependence of ellipticine hydroxylation on microsomal protein concentrations is then linear up to 3 mg of protein/ml. The K_m value ($\simeq 2.10^{-5}$ M) (Fig. 4) indicates a good affinity of the ellipticine for the hydroxylation system. This result is in good agreement with the very high affinity found from the spectral interactions: ellipticine is a type II substrate for cytochrome P450, exhibiting a K_s of 7×10^{-7} M

Table 2. Kinetic analysis of ellipticine 9-hydroxylase activity in microsomes from variously pretreated rats

Treatment	Position of the Soret peak after CO titration (nm)	Total cytochromes-P450 content nmoles/mg protein	K_m $\times 10^{-5}$ M	V_{max} nmoles/min/mg protein	%
None	450	0.55	3.4 ± 2.2	0.09 ± 0.005	100
Phenobarbital	450	2	7.5 ± 2.5	0.08 ± 0.005	89
Benzo[a]pyrene	448	1	3.0 ± 2.1	0.14 ± 0.003	156
Ellipticine	448	1	2.8 ± 2	0.22 ± 0.004	246

The kinetic experiments were carried out with sufficiently high microsomal protein concentration ($\simeq 2$ mg per ml) to avoid the inhibition by an excess of substrate. Ellipticine concentration varies from 1×10^{-5} M to 1.7×10^{-4} M. The K_m and V_{max} were mean values from 2 to 5 experiments and were determined by the Lineweaver–Burke plot.

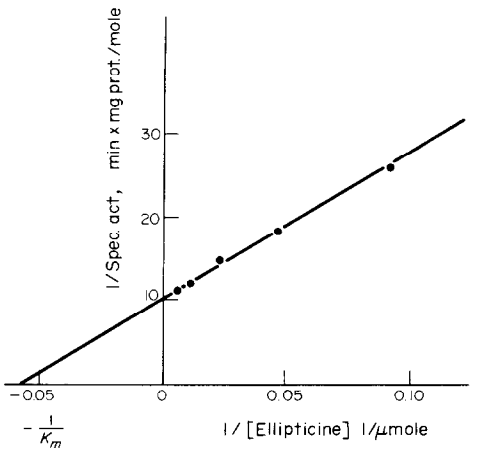

Fig. 4. Dependence of ellipticine 9-hydroxylase activity on substrate concentration (Lineweaver–Burke plot). Microsomes from a control rat were used (1.5 mg protein/ml). All other conditions are described in Materials and Methods.

[13]. The K_m for its hydroxylation is however different from the spectral K_s for its binding to cytochrome P450; this feature has been often reported for type II substrates of cytochrome P450 [19].

Effects of pretreatment. Table 2 compares the kinetic data of the 9-hydroxylation of ellipticine by microsomes of differently pretreated rats. PB induction does not increase the hydroxylating activity of microsomes towards ellipticine: the specific activity remains roughly unchanged (in fact, the rate of ellipticine hydroxylation expressed in nmoles/min/nmole cytochrome P450 even decreases from 0.16 to 0.04) and the affinity for the substrate slightly decreases. However, induction by polyaromatic compounds, BP and ellipticine itself, leads to an increase of the specific activity. It is noteworthy that ellipticine 9-hydroxylase activity is more increased after pretreatment by ellipticine itself than by BP, at least when an equal dose of both compounds which is that generally used for BP induction, is given to the rats.

Effect of inhibitors. Carbon monoxide which is able to compete for oxygen [20] for the reduced form of the heme iron inhibits the 9-hydroxylation of ellipticine (Table 1). The data shown in Table 3 indicate that metyrapone and 7,8 benzoflavone, which are known inhibitors of cytochrome P450 and cytochrome P448 dependent monooxygenases, appear as rather poor inhibitors of ellipticine hydroxylation. Cyclohexane, even up to 10^{-3} M was totally ineffective in all cases. Ellipticine [18] and BP, like the other polycyclic aromatic hydrocarbons [21] specifically induce the formation of cytochrome P448 and lead to the highest ellipticine hydroxylation activities (Table 2). On the other hand, 7,8 benzoflavone, a known inhibitor of the cytochrome P448 dependent microsomal hydroxylation of polycyclic aromatic hydrocarbons [22] does not affect ellipticine 9-hydroxylase activity in microsomes from BP- or ellipticine-pretreated rats. In Table 3, are included, for comparison, the results concerning the inhibitory effects of 7,8-BF and metyrapone on BP hydroxylase activity of the microsomes from PB- and BP-pretreated rats that were used previously for the study of ellipticine hydroxylation. One thus observes expected important inhibitions of this activity by 7,8-BF after BP induction and by metyrapone after PB induction. Metyrapone almost exclusively inhibits the hydroxylation of ellipticine by PB induced microsomes. This is easily understandable as metyrapone exhibits a particular affinity for that cytochrome P450 form which is induced by PB [23] and is a particularly good inhibitor for the hydroxylating activities dependent of that cytochrome P450 form [24, 25].

DISCUSSION

Rat liver microsomes, in the presence of NADPH, are able to hydroxylate ellipticine principally in a position that is para to the indole nitrogen. This transformation is catalyzed by cytochromes-P450 dependent monooxygenases as indicated by its cofactor requirement and its sensitivity to carbon monoxide and inducers of these monooxygenases. Phenobarbital induction generally enhances the microsomal mixed-function oxidations of exogenous compounds [26]. Ellipticine is an exception of this general rule as its microsomal hydroxylation rate decreases after PB treatment but increases significantly after benzo[a]pyrene

Table 3. Inhibitory effects of metyrapone and 7,8 benzoflavone on ellipticine hydroxylation by microsomes of control and pretreated rats

Compound added to incubation mixture	Concentration (M)	Microsomes			
		Control	PB	BP	Ellipticine
		Inhibition of ellipticine* hydroxylation (%)			
Metyrapone	10^{-3}	32	63	0	17
	10^{-4}	13	28 (80)†	0 (2)	0 (0)
	5×10^{-5}	13	16	0	0
7,8 benzoflavone	10^{-3}	0	45	0	0
	10^{-4}	0	32 (24)	0 (78)	0 (55)
	5×10^{-5}	0	27	0	0

* Substrate concentration: 8.4×10^{-5} M.

Each value represents the mean of three determinations.

† Inhibition of the BP hydroxylase activities (%) of the same microsomal preparations are indicated in parenthesis for comparison (from ref. 13).

pretreatment. In that respect, 9-hydroxylation of ellipticine resembles the 10-hydroxylation of nortriptyline and the 2-hydroxylation of desmethylimipramine [27]. Accordingly, PB induction causes a decrease of three microsomal nortriptyline, desmethylimipramine and ellipticine hydroxylase activities (respective decrease: 13, 19 and 11 per cent) though BP induction stimulates them (33, 19 and 56 per cent increase).

Compared to other substrates of the microsomal monooxygenation system [25], the affinity of ellipticine for P450-cytochromes is rather high ($K_s \simeq 5.10^{-7}$–10^{-6} M) [13]. There is nevertheless an apparent discrepancy between this affinity for cytochromes P450 and the weak specific activities exhibited by all microsomal preparations compared with those reported in the literature for numerous other drugs. The complete lack of inhibition by 7,8-BF of ellipticine 9-hydroxylase activity which is increased after BP pretreatment, is also surprising. A possible explanation of these data is based on the high affinity of ellipticine, as a pyridinic ligand of cytochromes P450-Fe(II), as shown by spectral interactions studies [13]. For this reason, ellipticine should be able to inhibit oxygen fixation and activation. Accordingly, we have shown that it is a good inhibitor of BP hydroxylase, much better than 7,8 BF (the I_{50} of ellipticine being about 10-fold smaller) [13]. For the same reason, ellipticine should inhibit its own hydroxylation, explaining the weak specific activities herein reported and the shape of the curves of Fig. 3. So it is likely that 7,8 BF, which is a weaker inhibitor of BP hydroxylase than ellipticine, is also a weaker inhibitor of ellipticine hydroxylation than ellipticine itself. This may explain the weak effect of 7,8 BF on ellipticine hydroxylation, particularly for microsomes from BP pretreated rats, the affinity of ellipticine for cytochrome P448 being especially high [13]. All these results indicate that the pyridinic compound, ellipticine, because of its polyaromatic structure, plays for cytochrome P448, the same role as metyrapone, and other pyridinic compound, for the cytochrome P450 form induced by phenobarbital. Accordingly, we showed that ellipticine inhibits strongly the cytochrome P448 dependent benzo[a]pyrene hydroxylation and its covalent binding to DNA [13].

The fluorometric test described allows a quick and reliable measurement of a microsomal activity which is stimulated by induction by polyaromatic compounds. However, its sensitivity (minimum metabolite amount detected: 200–400 pmoles of 9-OH ellipticine per ml) is not as good as those of the 7-ethoxy-coumarine dealkylation (10–20 pmoles/ml) [28] or benzo[a]pyrene hydroxylation (1 pmole/ml) [29] tests.

The observed 9-hydroxylation of ellipticine *in vitro* is in agreement with previous results obtained *in vivo* [8, 18]. The effects of inducing agents on ellipticine hydroxylation should permit to interpret the variability in therapeutic efficacy and toxicity of this drug. As 9-hydroxy-ellipticine is a better antitumor agent than ellipticine itself on L1210 mouse leukaemia [9], it seems that a part, if not the whole antitumor effect of ellipticine could be due to its 9-hydroxylation.

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