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METABOLIC TRANSFORMATION OF 2-METHYLELLIPTICINIUM

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SUMMARY : 2-methyellipticinium (NSC 226137) does not exhibit any spectral interaction with cytochrome p-450 ; however, it is transformed in vitro by microsomes from livers of phenobarbital induced rats. This transformation is NADPH dependant. According to presently available analytical criteria (HPLC and chromatographic behavior, UV and mass spectra), its product is very likely 2-methyl-9-hydroxyellipticinium (NSC 264137) which is an active antitumor drug in man. Two minor metabolites are also present. The same major product is found in the bile of non-induced rats after intravenous administration of 2-methylellipticinium.

INTRODUCTION : Ellipticine (Fig. 1) is a cytotoxic agent exhibiting antitumor properties. Two hydroxylated metabolites are produced by the monooxygenase : 9-hydroxyellipticine (1,2) and 7-hydroxyellipticine (3). Human and rat liver microsomes are able to perform this hydroxylation (4). The relative yield of these metabolites varies according to the type of microsomal induction. However 9-hydroxyellipticine is always the main metabolite ; this observation must be related to the fact this compound is much more cytotoxic than ellipticine itself towards L1210 cultured cells (5). 9-hydroxyellipticine is also more active than ellipticine against experimental tumors (6). 9-hydroxylated derivatives were therefore chosen for therapeutic trials in man. We were also able to demonstrate that the quaternarisation of 9-hydroxyellipticine, by addition of an alkyl or alkylamine group to the pyridinic nitrogen 2, increased the cytotoxicity and the antitumor efficiency of this compound (7). According to these data, 2-methyl-9-hydroxyellipticinium was used for phase I and phase II clinical trials. This new drug did not exhibit any noticeable hematologic toxicity. Remissions were observed for certain types of metastasis in around 20 % of otherwise chemotherapy resistant breast cancers (8,9).

Until now our research has focused on hydroxylated ellipticiniums because of their better pharmacological efficiency.

Abbreviations used are HPLC, high performance liquid chromatography ; TLC, thin layer chromatography.

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	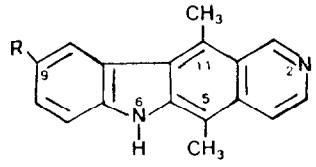	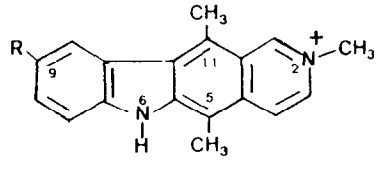
R = H	Ellipticine	2-Methylellipticinium
R = OH	9-Hydroxyellipticine	2-Methyl 9-Hydroxyellipticinium

Fig. 1 Formules of ellipticine, 2-methylellipticinium and their 9-hydroxylated derivatives.

We have reported the absence of interaction spectra of these drugs with oxidised or reduced microsomes (10). This was thought to be due to their positive charges which should preclude any binding to hydrophobic sites of iron atoms of microsomal cytochrome P-450 mixed function oxydases. Accordingly, the microsomal hydroxylation of ellipticiniums to hydroxylated compounds had been presented as very improbable (14). This prediction has not been confirmed and 2-methylellipticinium can be metabolized both in vitro in presence of rat liver microsomes and in vivo after intravenous administration. Several metabolites are produced ; the main being mostlikely 2-methyl-9-hydroxyellipticinium. Hydroxylation not accompanied by the occurence of microsomal interaction spectra has also been described for barbital and benzene (11).

MATERIAL AND METHODS

1) In vitro metabolism. We used both rat liver homogenates (9 000 g) and microsomes(2 X 100 000 g). The Wistar rats were given water to which phenobarbital (500 mg/kg) had been added. The rats were decapited and their livers, removed immediately following intracardiac perfusion of the animals with a 0,9 % sodium chloride solution. Then the livers were washed in saline buffer and homogenized in three volumes of TMS buffer pH 7,5 (Tris HCl, 50 mM ; MgCl2, 3 mM ; sucrose, 200 mM) with a potter homogeneizer (1 000 RPM). This preparation was carried out at 4°C. Microsomes were then prepared as already described (2,4). Protein concentration was determined using the method of Lowry (12). The hydroxylation capacity of microsomes was measured thanks to the hydroxylation of ellipticine (4). In vitro incubation was done at 37°C with back and forth shaking under the following conditions : 2-methylellipticinium, 2 X 10^{-5}M; NAPDH regene-

rating system (NADP, 1,3 X 10^{-3}M, glucose 6 phosphate deshydrogenase, 0,4 U/ml) ; microsomal proteins, around 2 mg/ml ; final volume with TM buffer (tris HCL 50 mM ; MgCl2, 3mM), 1 ml. The temperature was suddenly lowered to 4°C. Then 1 ml of ethyl acetate was added as well as tetraphenylborate to allow the extraction of quaternarized ammoniums. After a 30 seconds extraction, the sample was dried under nitrogen. The residue was then dissolved in a small volume of methanol.

2) In vivo metabolism. Wistar rats were anesthesized with ethyl-carbamate (1,3 g/kg). Catheters were then inserted into their bile duct. 2-methyl-ellipticinium dissolved in saline buffer (5 mg/kg) was injected intravenously. The bile was gathered for several hours. Extraction of the parent compound and of the metabolites was performed as in the in vitro experiments. Treatment with glucuronidase was no required.

3) Chemicals. 2-methylellipticinium was synthetised by Dr Dat Xuong through methylation of ellipticine with methyl iodide. The iodide anion was exchanged for an acetate anion. Metyrapone was obtained from Ciba-Geigy.NADP, Glucose 6 Phosphate deshydrogenase was purchased from Sigma.

4) Chromatographic analysis. a) HPLC : The following eluent mixture was used : methanol, 0,01 M acetate buffer (pH 5 or 7), (8,2). Detection was performed in UV (254 and 313 nm). Retention times and peak areas were obtained by a computer calculation.
b) Thin layer chromatographic on silica gel. The following systems were used : methylene chloride, methanol, ammoniac (4/1/traces) ; and butanol, acetic acid, water (5/2,5/1,5).

5) Electronic impact mass spectrometry was performed on a Ribermag R 10,10 apparatus (70 ev). Field desorption was performed on a VARIAN MAT 3 11 A, 6kV, source temperature 100° C. UV spectrum was recorded on double beam Beckman Acta III apparatus.

RESULTS AND DISCUSSION : 2-methylellipticinium is incubated one hour at 37°C in the presence of microsomes and NADPH. The HPLC chromatogram of the ethyl acetate extract discloses the presence of a new peak, which is missing in the control, incubated without NADPH. The area of the 2-methyl-ellipticinium peak is about 35 % larger in the latter experiment than in the former one. This 35 % roughly corresponds to the area of the new peak (Fig. 2). When NADPH is added to the control, after the incubation, there is no change in the chromatogram. When the incubation is performed after one minute bubbling with CO, the new peak is missing. It is 80 % inhibited in the presence of metyrapone 10^{-4}M, which is a specific inhibitor of cytochrome P-450, and in the presence of 9-hydroxyellipticine 10^{-6}M, which is a potent inhibitor of cytochrome P-448 and P-450 (13). The newly caracterized compound is therefore a metabolite of 2-methyellipticinium. The formation of this metabolite is dependant on NADPH and on the action of cytochrome P-450 enzymes. When 2-methyl-9-hydroxyellipticinium is added to the extract, the height of the new peak increases without any change in retention time (Fig. 3).

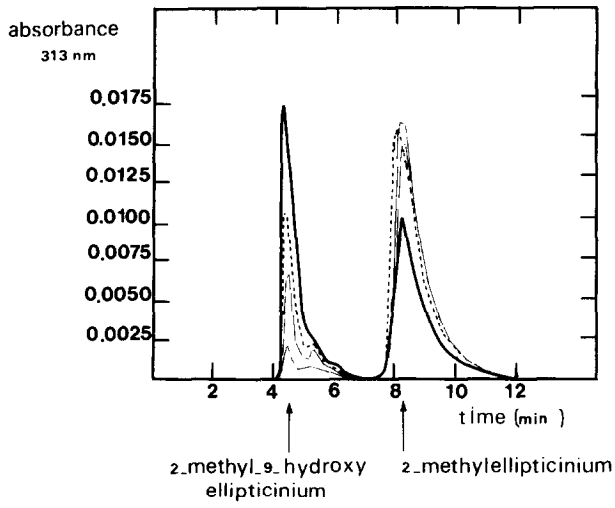

Fig. 2 Phenobarbital induced rat liver microsomal metabolism of 2-methylellipticinium. HPLC kinetic study (eluent system methanol, sodium acetate 0,01 M pH 7 (8/2)). 100 000 g protein : 1,7 mg/ml - NADPH (regenerating system) : 10^{-3}M 2-methylellipticinium 2 x 10^{-5}M. Incubation times are :

——— 0 — — — 5'----------20'——— 1 h.

Our results show that the newly caracterized compound cannot be 9-hydroxyellipticine, because this compound separates well from 2-methyl-9-hydroxyellipticine, in HPLC at pH 7 (Fig. 3).

The 2-methylellipticinium metabolite migrates like 2-methyl-9-hydroxyellipticinium in the two TLC systems used. In the first one, 9-hydroxyellipticine and 2-methyl-9-hydroxyellipticinium migrate differently (butanol, acetic acid, water). In the second one (methylene chloride, methanol, ammonia), 9-hydroxyellipticine spot turns red when exposed to iodin. No change is observed for the 2-methylellipticinium metabolite. These data establish that 2-methylellipticinium does not undergo a microsomal oxidative demethylation, followed by an hydroxylation. 2-methyl-9-hydroxyellipticinium and 2-methylellipticinium metabolite have the same optical absorption ratio, 254 nm/313 nm ; 1,62 (measured on HPLC recording). Their field desorption mass spectra, performed as already published for quaternary ammonium (15,16), are identical (molecular mass 277). Impact electronic spectra are also identical (although the molecular peak is not present) . UV spectra are identical.

Final proof will depend upon the NMR results ; however, we provisionally conclude that 2-methylellipticinium is trans-

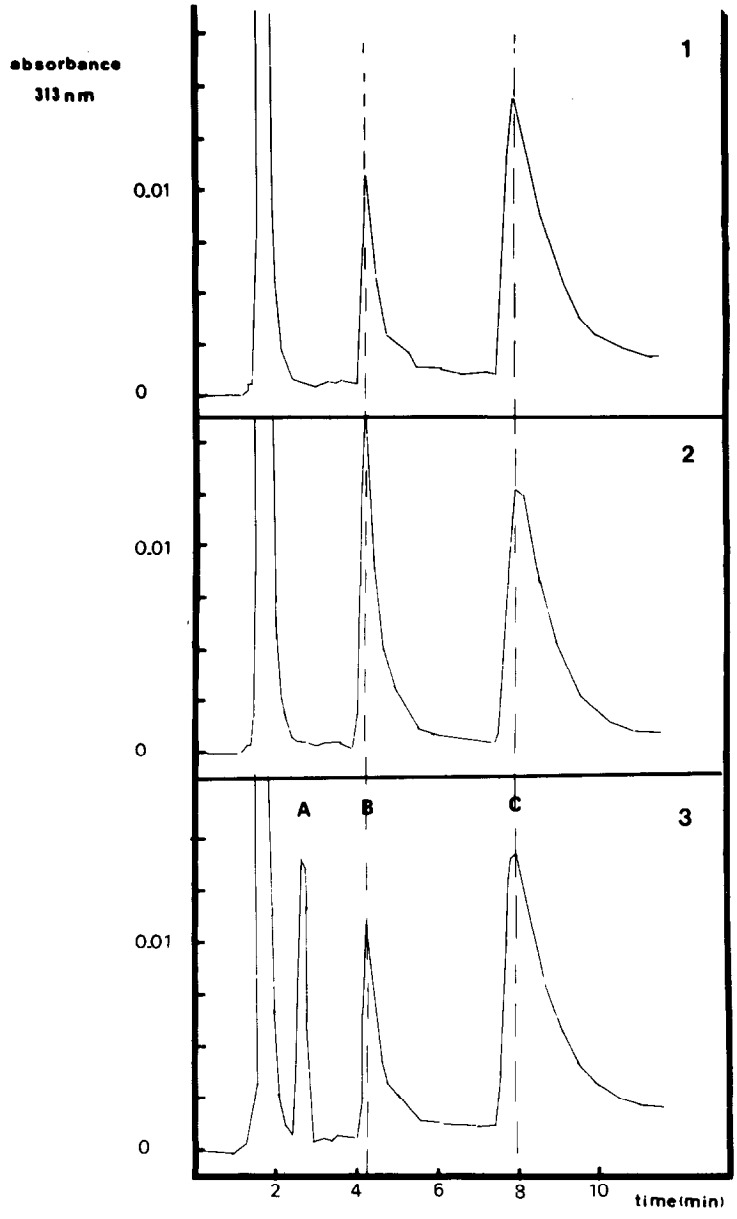

Fig. 3 HPLC chromatograms of the ethyl acetate extract of 2-methyl-ellipticinium incubated one hour with a phenobarbital induced liver 9 000 g fraction in the presence of NADPH.

1) nothing is added to the extract

2) 2-methyl-9-hydroxyellipticinium is added to the extract

3) 9- hydroxyellipticine is added to the extract

Retention times of the compounds are identified as follows :

- A 9-hydroxyellipticine.
- B 2-methyl-9-hydroxyellipticinium and metabolite of 2-methylellipticinium.
- C 2-methylellipticinium.

The first peak is methanol.

formed into 2-methyl-9-hydroxyellipticinium by phenobarbital-induced microsomes. The results of preliminary kinetic study are shown in Fig.2. The average maximum speed of 2-methylellipticinium hydroxylation is around 80 picomoles/minute/milligram of protein. This value is closed to that of ellipticine hydroxylation (4) although K_m is lower. Similar results are obtained in vivo with non-induced Wistar rats. Bile is collected 6 hours and 24 hours after the drug injection. The yield of the metabolic process is higher in vivo than in vitro : after 24 hours, 90 % of the recovered compounds are in the metabolites peaks. Similar yield have been reported in the case of ellipticine (2).

In both in vitro and in vivo experiments, two other minor metabolites were found. Since the hydroxylation of ellipticine occurs in the 7 position, one of these metabolites might be 2-methyl-7-hydroxyellipticinium. Indeed synthetic 2-methyl-7-hydroxyellipticinium and one of the minor metabolites (6' retention time, cf fig. 2) display the same retention time. All together these data establish that 2-methylellipticinium is a substrate of microsomal cytochrome P-450 mixed function oxidases. This reaction can be related to the interaction of ellipticinium derivatives with peroxydases, which are also porphyrin iron containing enzymes (17).

In the very likely hypothesis where the metabolite of 2-methylellipticinium is 2-methyl-9-hydrxyellipticinium two inferences can be drawn from our observations. On the one hand the parent compound and its metabolite could be endowed with different biodisponibilities because of the greater solubility in water of hydroxylated compounds. Hence 2-methylellipticiniuum could be toxic for tumors, which are not sensitive to 2-methyl-9-hydroxyellipticinium, provided that the tumor cells display enough cytochrome P-450 activity and are permeable to 2-methylellipticinium (without being for instance, permeable to 2-methyl-9-hydroxyellipticinium). On the other hand we can take advantage of the high yield of 2-methylellipticinium hydroxylation to prepare large amounts of 2-methyl-9-hydroxyellipticinium. Today, this drug can only be obtained through a rather difficult chemical synthesis, which is presently carried out on a large scale in order to prepare this drug for therapeutic trials.

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