Biochemical Pharmacology, Vol. 29, pp. 3231–3237.

0006-2952/80/1215-3231 $02.00/0

STRUCTURE–ACTIVITY RELATIONSHIPS IN THE INHIBITORY EFFECTS OF ELLIPTICINES ON BENZO(a)PYRENE HYDROXYLASE ACTIVITY AND 3-METHYLCHOLANTHRENE MUTAGENICITY

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(*Received* 28 *April* 1980; *accepted* 22 *July* 1980)

Abstract—The structural features which determine the ability of ellipticine (5,11-dimethyl-6-H-pyrido[4-3b]carbazole) and its derivatives to interact with cytochrome P-450 and to inhibit rat liver microsomal benzo(a)pyrene hydroxylase as well as to inhibit the mutagenicity of 3-methylcholanthrene have been studied. Spectral interactions studies were carried out with either Aroclor 1254-, 3-methylcholanthrene- or phenobarbital-induced microsomes. Inhibitory activities towards benzo(a)pyrene hydroxylase and 3-methylcholanthrene mutagenicity (Ames test), were determined using Aroclor 1254-induced microsomes. It appears that every ellipticine derivative having significant inhibitory effects on hydroxylation of benzo(a)pyrene or mutagenicity of 3-methylcholanthrene also exhibits a very good affinity for microsomal cytochromes P-450. The accessibility of the pyridinic nitrogen of ellipticine derivatives appears as the most important factor for their binding to cytochromes P-450 and the presence of methyl groups in 5 and 11 positions of ellipticine derivatives is an essential condition for the expression of the inhibitory power. Various substitutions in the A ring of ellipticine appear to be of secondary importance. On the other hand the location of the pyridinic ring and consequently the arrangement of the molecule within the hydrophobic pocket of cytochrome P-450 seems also to play an important role in the inhibitory power since isoellipticines are devoid of such properties. These results should help in the design of particularly efficient inhibitors of drug and carcinogen metabolism.

The ellipticines form a class of very powerful inhibitors of the microsomal cytochrome P-450 monooxygenase catalyzed oxidative metabolism of a wide variety of drugs and carcinogens [1]. Ellipticine and its 9-hydroxy and 9-fluoro derivatives have been shown to exert a large or even complete decrease of the mutagenicity of a great number of compounds in *Salmonella* [2]. *In vivo*, 9-hydroxyellipticine is a remarkably active inhibitor of skin carcinogenesis induced by 7,12-dimethylbenz(a)antracene in mice [3]. The very interesting properties of these compounds appear to be closely linked to their high affinity for microsomal cytochromes P-450. They bind, by their pyridinic nitrogen atom, to the oxidized or reduced iron of cytochrome P-450 producing type II difference spectra, the apparent spectral dissociation constants, K_s, lying around 10^{-6} and 10^{-5} M, respectively [4]. This paper is concerned with the identification of the structural features which determine the ability of ellipticine derivatives to interact with cytochrome P-450 and to inhibit the microsomal benzo(a)pyrene hydroxylase activity as well as to inhibit the mutagenicity of 3-methylcholanthrene. We have studied the relative importance of (a) substitution in A ring, (b) the presence of methyl or other groups within C and D rings and (c) the location of the pyridinic ring in ellipticine and isomers.

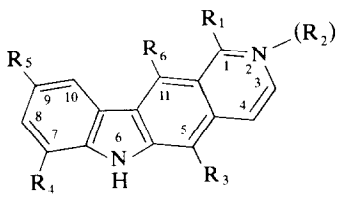
¶ *Abbreviations*—E, ellipticine; 9-FE, 9-fluoroellipticine; 9-OHE, 9-hydroxyellipticine; 7-OHE, 7-hydroxyellipticine; 7,9-(Cl_2)E, 7,9-dichloroellipticine; t.l.c., thin layer chromatography; n.m.r. spectroscopy, nuclear magnetic resonance spectroscopy; TMS, buffer pH 7.5 containing 50 mM Tris–HCl, 3 mM $MgCl_2$ and 200 mM sucrose; DMF, dimethylformamide; 3-MC, 3-methylcholanthrene; PB, phenobarbital; BP, benzo(a)pyrene.

MATERIALS AND METHODS

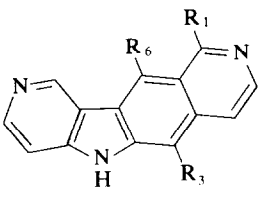
Chemicals. The ellipticines studied in this work are shown in Table 1. E¶ (I) was prepared according to the method of Dalton [5]. This method was used to synthesize 9-FE (III) from 5 fluoro-indole. 9-OHE (II) was a gift of Sanofi, Sisteron, France. 7-OHE (IV) was prepared in our laboratory from methoxy-indole, according to a method previously described [6]. The same method was used to synthesize 7,9-$(Cl)_2$E (V) from dichloroaniline. 9-OH-2-CH_3 ellipticinium acetate (VI) was a generous gift of Dr. N. Dat Xuong. Azaellipticines (X and XI) were a gift of Dr. E. Bisagni. Isoellipticines (XII–XV) were prepared in the laboratory of Prof. B. P. Roques from methoxy-indole carboxylic acid according to a method previously described [7]. 9-OH olivacine (IX) was kindly supplied by Dr. H. P. Husson. 11-desmethyl-9-OHE (VII) was a generous gift of Dr. C. Gansser and 5,11-desmethyl E (VIII) was kindly supplied by Dr. J. B. Le Pecq. The purity of these compounds was checked by t.l.c and n.m.r. spectroscopy and found to be better than 95 per cent. pK_a values were determined from the changes of light absorption spectra using acetate and cacodylate buffers of various pH values: these data have been

Table 1. Structure of ellipticine and derivatives

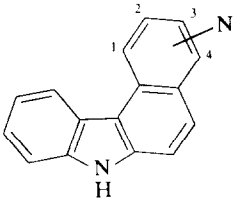
GENERAL STRUCTURES:



Ellipticines



Azaellipticines



Isoellipticines

Compounds		pK_a	R_1	R_2	R_3	R_4	R_5	R_6
Ellipticines								
I	E	6.8	H	—	CH_3	H	H	CH_3
II	9-hydroxy E	6.6	H	—	CH_3	H	OH	CH_3
III	9-fluoro E	—	H	—	CH_3	H	F	CH_3
IV	7-hydroxy E	—	H	—	CH_3	OH	H	CH_3
V	7,9-dichloro E	—	H	—	CH_3	Cl	Cl	CH_3
VI	2-methyl-9-hydroxy-ellipticinium	—	H	CH_3	CH_3	H	OH	CH_3
VII	11-desmethyl-9-hydroxy E	6.9	H	—	CH_3	H	OH	H
VIII	5,11-desmethyl E	6.4	H	—	H	H	H	H
IX	9-hydroxy olivacine	—	CH_3	—	CH_3	H	OH	H
Azaellipticines								
X	a	—	H	—	CH_3	H	—	CH_3
XI	b		$NH-(CH_2)_3-N(C_2H_5)_2$	—	CH_3	H	—	H
Isoellipticines								
XII	$a = N_1$	5.3						
XIII	$b = N_2$	6.4						
XIV	$c = N_3$	6.7						
XV	$d = N_4$	—						

kindly provided by J. B. Le Pecq. Aroclor 1254 was generously supplied by Monsanto Co. St. Louis, MO. 3-Hydroxybenzo(a)pyrene was obtained from the National Cancer Institute Chemical Repository, National Institutes of Health (Bethesda, MD) through Dr. D. G. Longfellow, Program Manager; all other chemicals were purchased from commercial sources.

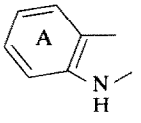
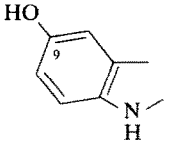
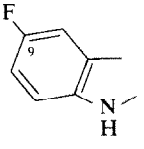
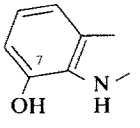
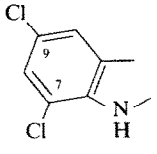
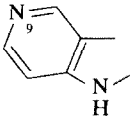
Preparation of microsomes. Male Sprague–Dawley rats weighing about 150 g were used. When the animals were treated they received i.p. either 80 mg/kg phenobarbital (one dose/day for 3 days) or 20 mg/kg 3-methylcholanthrene dissolved in olive oil (one dose/day for 2 days), or 500 mg/kg Aroclor 1254 (one dose, the animal being killed 5 days after treatment). Livers were removed and the microsomes prepared according to a previously described procedure [8]. The microsomal cytochrome P-450 content was determined according to Omura and Sato [9]. Protein was measured by the method of Lowry [10] using bovine serum albumin as a standard.

Benzo(a)pyrene hydroxylase. The hydroxylation of benzo(a)pyrene (BP) was measured according to the method of Nebert and Gelboin [11]. The incubation mixture contained in 1 ml 50 mM Tris–HCl, 3 mM $MgCl_2$, 200 mM sucrose (TMS), pH 7.5, 1 mg NADPH, 0.025 mg BP (dissolved in 5 μl acetone) and liver microsomes (0.5 mg protein/ml) from Aroclor 1254-pretreated rats. Ellipticine and derivatives were added in DMF solution (10 μl). The same amount of DMF was added to controls. The mixture was incubated for 10 min at 37° and the reaction stopped by addition of 4 ml of 25% acetone in hexane. The subsequent operations were carried out as described [11].

Binding studies. Difference spectra were recorded as previously described [4] on a Beckman Acta C-III spectrophotometer using 10 mm cuvettes containing 3 ml of a microsomal suspension in TMS buffer. Suspensions of rat liver microsomes containing 0.5–1 mg protein/ml (1.2–2.2 nmoles cytochrome P-450/ml) were employed. Ellipticine and derivatives were added (1 mM in DMF solution) in volumes of 1–40 μl. Spectral dissociation constants (K_s) which measure the strength of the binding of the ellipticines to the cytochromes of liver microsomes were determined from the abscissa intercepts of double reciprocal plots of Δ O.D. 428–490 nm (oxidized P-450) or Δ O.D. 445–490 nm (reduced P-450) vs ellipticine concentration [4].

Mutagenesis assays. Mutagenicity was measured essentially as described by Ames [12] on *Salmonella* strain TA 100. The concentration of 3-MC was 10 μg per plate. Controls were run as described previously [2] and care has been taken to ensure that toxic concentrations of ellipticine or its derivatives, as witnessed by the absence of thinning of the background lawn of auxotrophic bacteria, were not used.

Table 2. Effect of substitution within "A" ring of ellipticine on the binding properties with cytochromes P-450 and the inhibition of benzo(a)pyrene hydroxylase and 3-methylcholanthrene-induced mutagenesis

Compounds	Apparent spectral dissociation constant* $K_s(\mu M)$			Inhibition of BP hydroxylase ID_{50} (μM)†	Inhibition of 3-MC (10μg/plate) mutagenesis ID_{50} (μg/plate)‡
	Cyt P-450	Microsomes Aroclor	Microsomes 3-MC		
 Ellipticine	Oxidized	0.75	0.70	1.10	1.60
	Reduced	1.30	—		
 9-OH Ellipticine	Oxidized	0.50	0.65	0.60	1
	Reduced	1	—		
 9-F Ellipticine	Oxidized	0.80	0.45	1.50	1.30
	Reduced	2.85	—		
 7-OH Ellipticine	Oxidized	8.30	1.60	10	7
	Reduced	—	—		
 7,9-$(Cl)_2$ Ellipticine	Oxidized	10	2	8	5.50
	Reduced	—	—		
 9-Azaellipticine(*a*)	Oxidized	1	0.70	6.50	4.20
	Reduced	1	—		

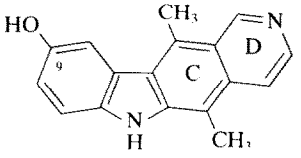
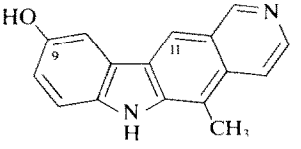
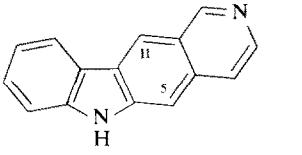
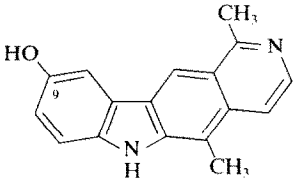
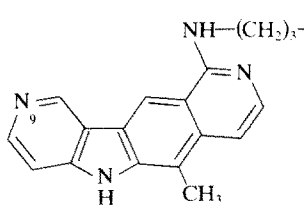
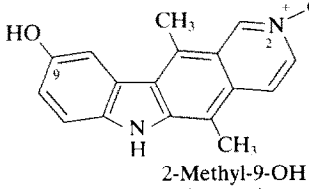
All values are the average of three independent experiments (≤ 10% variation).

* The conditions were as described in Materials and Methods. When the values are lacking with reduced microsomes the reason is due to either the absence or the hard interpretation of the spectra.

† I_{50} (ellipticine concentration giving 50 per cent inhibition of BP hydroxylation) was determined from inhibition curves obtained with six inhibitor concentrations ranging from 5×10^{-7}M to 10^{-4}M. Ellipticines were added in DMF solution (10 μl). Addition of equivalent amount of pure DMF to control does not modify the BP hydroxylation. The results are independent of order of addition of the compounds. The absolute value of benzo(a)pyrene hydroxylase activity (100%) with Aroclor 1254-induced microsomes (1.8 nmoles cytochrome P-450 per mg protein) was 1.9 ± 0.2 nmoles 3-OH benzo(a)pyrene/min/mg of protein.

‡ Concentration (μg per plate) giving 50 per cent inhibition of number of revertants per plate. I_{50} values were determined from inhibition curves obtained with 3–7 inhibitor concentrations. The absolute value of 3-MC mutagenicity (100%) with Aroclor 1254-induced microsomes was 2000 ± 200 revertants (TA 100 strain) per plate.

Table 3. Effects of suppression of methyl group in "C" ring and substitution in "D" ring on the binding properties with cytochrome P-450 and the inhibition of benzo(a)pyrene hydroxylase and 3-methylcholanthrene-induced mutagenesis

Compounds	Apparent spectral dissociation constant* K_s (μM)			Inhibition of BP hydroxylase I_{50} (μM)†	Inhibition of 3-MC (10 μg/plate) mutagenesis I_{50} (μg/plate)‡ or %
	Cyt P-450	Microsomes Aroclor	Microsomes 3-MC		
 9-Hydroxyellipticine	Oxidized	0.50	0.65	0.60	3.00
	Reduced	1.00	—		
 11-Desmethyl-9-OHE	Oxidized	4.90	2.50	50	27% at 10 μg/plate
	Reduced	25			
 5,11-Desmethyl-E	Oxidized	0.90	0.35	10	no inhibition at 10 μg/plate
	Reduced	3.85			
 9-Hydroxy olivacine	Oxidized	no spectral interaction at 13 μM		500	no inhibition at 5 μg/plate
 9-Azaellipticine (*b*)	Oxidized	no spectral interaction at 13 μM		no inhibition at 500 μM	25% at 5 μg/plate
 2-Methyl-9-OH ellipticinium (acetate)	Oxidized	no spectral interaction at 13 μM		no inhibition at 500 μM	no inhibition at 3 μg/plate

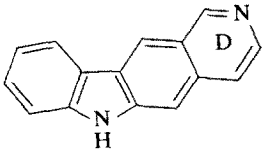
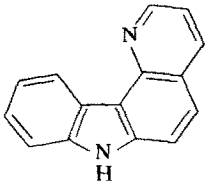
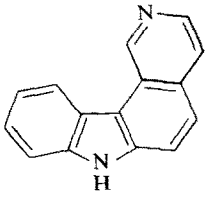
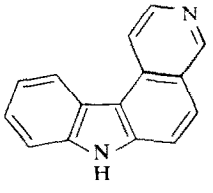
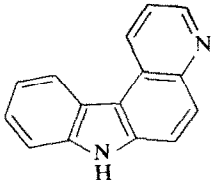
All values are the average of three independent experiments ($\leq$ 10% variation).

* The conditions were as described in Materials and Methods and in Table 2.

† I_{50} (ellipticine concentration giving 50 per cent inhibition of BP hydroxylase) was determined from inhibition curves obtained with six inhibitor concentrations ranging from 5×10^{-7}M to 5×10^{-4}M. Others conditions were as described in Table 2.

‡ Concentration (μg per plate) giving 50 per cent inhibition of number of revertants per plate. I_{50} values were determined from inhibition curves obtained with 3–7 inhibitor concentrations. Other conditions were as described in Table 2.

Table 4. Importance of the location of the pyridinic ring in the binding properties with cytochrome P-450 and the inhibition of benzo(a)pyrene hydroxylase and 3-methylcholanthrene-induced mutagenesis

Compounds	Apparent spectral dissociation constant with oxidized microsomes* K_s (μM) PB	3-MC	Inhibition of BP hydroxylase I_{50} (μM)†	Inhibition of 3-MC (10 μg/plate) mutagenesis I_{50} (μg/plate)‡ or %
 Ellipticine	0.90	0.70	1.10	1.60
 Isoellipticine *a*	no interaction		500	none (activation)
 Isoellipticine *b*	2	0.90	200	no inhibition at 5 μg/plate
 Isoellipticine *c*	1.60	0.50	200	35% inhibition at 5 μg/plate
 Isoellipticine *d*	no interaction		500	7% inhibition at 5 μg/plate

All values are the average of three independent experiments ($\leq$ 10% variation).

* The conditions were as described in Materials and Methods and in Table 2.

† I_{50} (ellipticine concentration giving 50 per cent inhibition of BP hydroxylase) was determined from inhibition curves obtained with six inhibitor concentrations ranging from 5×10^{-7}M to 5×10^{-4}M. Others conditions were as described in Table 2.

‡ Concentration (μg per plate) giving 50 per cent inhibition of number of revertants per plate. I_{50} values were determined from inhibition curves obtained with 3–7 inhibitor concentrations. Other conditions were as described in Table 2.

RESULTS

A comparison between the binding properties to cytochromes P-450 and the inhibitory effects on benzo(a)pyrene hydroxylase as well as the inhibition of mutagenesis induced by 3-MC has been established for the 15 ellipticine derivatives described in Table 1. The results are summarized in the three following tables.

Table 2 summarizes the effects of substitution within the A ring. The introduction of an hydroxy (II) or fluoro (III) group in position 9 of ellipticine

(I) does not significantly modify the apparent spectral dissociation constant, K_s, for the various microsomes. These three compounds all strongly inhibit BP hydroxylase and mutagenesis to the same extent. On the other hand when a substituent (hydroxy (IV) or chloro group (V)) is introduced in position 7 one observes an important decrease (about 10-fold) of the affinity of the ellipticine derivative for the heme iron as well as in the inhibitory effects. Finally although the presence of a nitrogen atom in place of carbon in position 9 does not change the interaction with the heme iron, this azaellipticine (X) inhibits BP-hydroxylase activity and mutagenesis less than ellipticine itself.

Table 3 illustrates the large modifications brought about either by the suppression of the methyl groups in the C ring or the introduction of a substituent in position 1 or 2 of the D ring. With regards to 9-OHE (II), the absence of the methyl group in position 11 leads to a decrease in the cytochrome binding and a loss of inhibition of BP hydroxylase activity and mutagenesis. This last effect was also observed for the 5,11-desmethylellipticine (VIII), which however maintains a good affinity for the microsomes. The pK_a of these molecules was not significantly modified (Table 1).

When a substituent is present in position 1, near the pyridinic nitrogen as for 9-hydroxyolivacine (IX) or 9-azaellipticine *b* (XI) no spectral interaction occurs and the BP hydroxylase inhibition and suppression of mutagenesis are practically absent. The quaternization of ellipticine (ellipticinium salt) (VI) also leads to a complete loss of binding affinity and inhibitory effect.

The location of pyridinic ring (ring D) and consequently the arrangement of the molecule within the hydrophobic pocket of cytochrome P-450 seems to play an important role in the inhibitory power as suggested by the results shown in Table 4. Indeed none of the four isoellipticines (XII–XV) exhibits important inhibitory properties even when it interacts strongly with the heme iron of cytochrome P-450 from PB or 3-MC induced microsomes (isoellipticine *b* and *c*) (XIII and XIV).

DISCUSSION

From the data presented in this study, it appears that every ellipticine derivative which significantly inhibits hydroxylation of benzo(a)pyrene or mutagenicity of 3-methylcholanthrene also exhibits a very good affinity for microsomal cytochromes P-450. The accessibility of the pyridinic nitrogen of ellipticine derivatives appears to be the most important factor for their binding to cytochromes P-450. When this nitrogen becomes charged after alkylation, as in the ellipticinium salts, or when a bulky substituent exists in ortho position, as in 9-hydroxyolivacine (IX) or in azaellipticine *b* (XI) cytochrome P-450 binding, as well as inhibitory power are collapsed. For the same reasons, isoellipticine *a* (XII) and *d* (XV), which have the pyridinic nitrogen buried in the α position of the C ring, are practically devoid of cytochromes P-450 binding and inhibitory power. A similar situation has been also described with some imidazole derivatives, for which the addition of an aliphatic or aryl substituent in the α position of nitrogen led to a decreased interaction with the microsomal cytochrome P-450 as well as a loss of the inhibitory effect on aldrin epoxidation [13].

The strong binding of ellipticine derivatives to cytochrome P-450 thus appears to be a necessary condition for the inhibition of BP hydroxylase activity and loss of 3-MC mutagenesis by liver microsomes. However, this condition is not sufficient to obtain good inhibitory properties. For instance, isoellipticines (*b* and *c*) (XIII and XIV), and 11- and 5,11-desmethylellipticines (VII and VIII) display good affinities for cytochromes P-450 from 3-MC induced microsomes but rather poorly inhibit benzo(a)pyrene hydroxylation or 3-MC mutagenesis.

The presence of methyl groups in 5 and 11 positions of ellipticine derivatives is therefore an essential condition for the expression of inhibitory power. Although we have presently no satisfactory explanation for the role of these methyl groups one can observe that they are also necessary for the manifestation of cytotoxic effect on mice leukemic L1210 cells, another pharmacological property of ellipticine derivatives (unpublished results).

Various substitutions on the A ring of ellipticine appear as of secondary importance for the expression of the inhibitory properties. The addition of an hydroxyl (II) or a fluoro group (III) in position nine does not significantly modify the binding properties nor the inhibitory capacities. The presence of a fluoro group in position nine brings some advantages such as a clean decrease in the mutagenicity [2] and toxicity [14] of the molecule, probably due to the reduced ability of the A ring to be oxidized. On the contrary, the introduction of an hydroxyl (IV) or a chloro group (V) in position seven leads to a decrease of the affinity for cytochromes P-450 and at the same time of the inhibitory properties. It is unlikely that these variations (about 10-fold) can be explained by modifications in the basicity or polar character of these compounds. On the other hand it is possible that this region of the molecule if engaged in a binding with the hydrophobic protein site of cytochrome P-450 as we have observed in a detailed study of inhibitory properties of ellipticines on microsomal oxidation of various substrates [1]. So slight

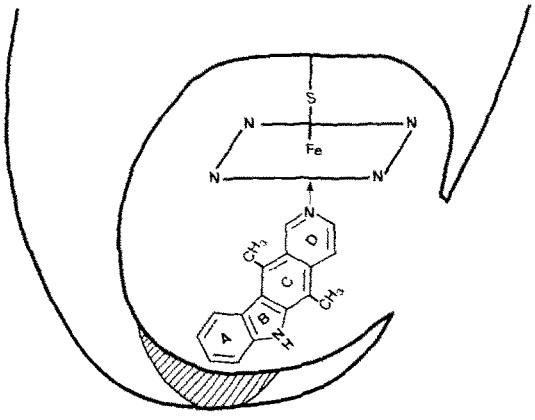


Fig. 1. Schematic representation of the binding of ellipticine to cytochrome P-450.

structural modifications in that part of the ellipticine molecule might lead to changes in the affinities for the protein and thereby indirectly alter binding to the heme iron of cytochrome P-450. From the whole set of data presented in this paper a schematic representation of the binding of ellipticine to cytochrome P-450 might be proposed (Fig. 1). This model may help to understand that slight modifications in the structure of ellipticine (substituents, steric factors) might be of importance for the inhibitory capacity of these compounds.

In conclusion, it appears that among the studied compounds, only ellipticine itself and its 9-substituted derivatives are actually good inhibitors of microsomal monooxygenases and mutagenesis. These results should help in the design of compounds particularly efficient in the inhibition of drug and carcinogen metabolism. These very potent inhibitors could be very useful in case of accidental exposure to chemical carcinogens and are presently being studied for the experimental prevention of carcinogenesis in animals [3].

Acknowledgements—This work received financial support from the Institut National de la Santé et de la Recherche Médicale (Contrat libre n° 79.4.040.2). The authors gratefully acknowledge the expert technical assistance of Mrs Josette Bonnefoux and Marcelle Vinial. We also thank Dr. Neil Johnson for his help in the preparation of the manuscript.

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