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IN VITRO INTERACTION OF RAT LIVER CYTOCHROMES P-450 WITH ERYTHROMYCIN, OLEANDOMYCIN AND ERYTHRALOSAMINE DERIVATIVES

IMPORTANCE OF STRUCTURAL FACTORS

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Abstract—Several derivatives of the erythromycin, erythralosamine and oleandomycin series have been prepared. Their abilities to bind to rat liver microsomal cytochrome P-450 and to lead to the formation of stable 456 nm absorbing cytochrome P-450–metabolite complexes after their oxidative microsomal metabolism *in vitro* have been compared. The obtained data confirmed that cytochrome P-450 induced in rats either by macrolides or by 16α-pregnenolone carbonitrile were the major isozymes involved in the binding of macrolides to liver microsomes and in metabolite-complex formation. They showed that (i) hydrophobicity was in general a beneficial factor for these two properties, (ii) the presence of a bulky substituent in position 3 of erythromycin dramatically decreased their affinity for these isozymes, and (iii) the simultaneous presence of bulky substituents in position 2′ and 3 prevented iron–metabolite complex formation. These results led to the selection of two compounds, erythralosamine-2′-benzoate and erythralosamine-2′,3-diacetate, which exhibited a particularly high affinity for macrolide inducible cytochrome P-450 and were very good precursors of cytochrome P-450–iron–metabolite complex formation.

The cytochrome P-450 monooxygenase system is able to metabolize numerous different compounds, both exogenous and endogenous, having no apparent common structural or physico-chemical characteristics. Its broad specificity is due to the existence of multiple isozymes. This isozyme multiplicity is now well established on the basis of substrate or inhibitor specificities, electrophoresis, immunoreactivity and amino acid composition [1, 2]. Several compounds, such as phenobarbital (PB),† 3-methylcholanthrene (3MC), macrolide antibiotics, clofibrate or ethanol, induce the synthesis of at least one hepatic cytochrome P-450 isozyme which thus predominates and confers its own substrate specificity to the enzymatic system.

Many studies have been devoted to the understanding of the structural factors that are important for the recognition of substrates by a given cytochrome P-450 isozyme. For instance, the major cytochrome P-450 isozyme induced by polyaromatic hydrocarbons such as 3-MC was found to exhibit a high affinity for planar, rigid and polyaromatic molecules. Such molecules bind tightly to an active site close to the heme and are easily metabolized [3]. Calculations have been performed on the molecular structures of molecules, in relation to their ability to bind to the active site and to be oxidized at specific positions [4].

Much less is known about the structure of the active site of the cytochrome P-450 isozyme of rat liver which is specifically induced by some steroids such as 16α-pregnenolone carbonitrile (PCN) [1, 5]. This rat liver isozyme has been called cytochrome P-450-PB-PCN_E since it is slightly increased after PB induction and becomes the major cytochrome P-450 after induction by PCN (about 50% of the total cytochrome P-450 apoproteins) [1]. It has been shown that the administration of some macrolide antiobiotics such as troleandomycin (TAO) [6], erythromycin (E) or erythralosamine (EM) [7] to rats led to a spectacular specific increase of a cytochrome P-450 form which has characteristics identical to those of a major form induced by PCN and glucocorticoids and which should be P-450-PB-PCN_E. In a similar manner, these macrolide antibiotics act as specific inducers in rabbit of a cytochrome P-450 isozyme that is also induced by PCN [8]. In man, TAO and erythromycin have been found able to induce a cytochrome P-450 isozyme, called cytochrome P-450p that is also induced by dexamethasone [9].

Some of these macrolide antiobiotics containing an $N(CH_3)_2$ function such as TAO have a high affinity for cytochrome P-450-PB-PCN_E and act as good substrates for this isozyme [7]. The oxidative metabolism of the amino function leads to the formation of stable cytochrome P-450–Fe–metabolite complexes resulting in a strong inhibition of this isozyme [7, 10]. Such inhibitory complexes, which seem to be formed by the strong binding of a nitrosoalkane metabolite

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† Abbreviations used: PB, phenobarbital; 3MC, 3-methylcholanthrene; PCN, 16α-pregnenolone–carbonitrile; TAO, troleandomycin; E, erythromycin; EM, erythralosamine; BNF, β-naphthoflavone.

Table 1. Chemical characteristics of the erythromycin derivatives used in this study

	Abbreviations	R_1	R_2	p*Ka**	R_f†	^{1}H NMR‡ $H_{2'}$	$H_{4''}$	$N(CH_3)_2$
Erythromycin	E	H	H	8.6	0.10	3.19	2.99	2.29
Erythromycin-2′-acetate	2′MAE	CH_3CO	H	6.6	0.35	4.75	3.01	2.26
Erythromycin-2′-propionate	2′MPE	C_2H_5CO	H	6.9	0.35	4.78	3.01	2.24
Erythromycin-2′-benzoate	2′MBE	C_6H_5CO	H	6.8	0.52	5.10	3.00	2.30
Erythromycin-4″-acetate	4″MAE	H	CH_3CO	7.8	0.23	3.20	4.52	2.26
Erythromycin-2′,4″-diacetate	2′,4″DAE	CH_3CO	CH_3CO	6.4	0.42	4.75	4.55	2.28

* p*Ka* values were measured in DMF/water, 2/1 mixture.
† Retention factor on thin layer chromatography (neutral alumina, 0.25 mm) and ethyl acetate as eluent.
‡ Chemical shifts (δ in ppm relative to $SiMe_4$) of protons in position $H_{2'}$, $H_{4''}$ and $N(CH_3)_2$ measured with a Bruker 250 MHz spectrometer.

derived from the antibiotic to cytochrome P-450-iron(II), have been found *in vivo* and *in vitro* in rat [7, 10, 11] as well as in man [12]. Their formation appears to be responsible, at least in part, for many problems of drug interactions associating TAO or some erythromycin derivatives with several drugs [13]. Since these inhibitory complexes are specifically formed upon oxidation of some macrolide antibiotics by cytochrome P-450-PB-PCN_E (or its equivalent P-450p in man), one should expect a high probability of drug interaction when such a macrolide antibiotic able to form an inhibitory complex is associated with a drug mainly metabolized by this isozyme. As a first approach to determine the structural factors necessary for a compound to bind specifically to this cytochrome P-450 isozyme, we have synthesized several derivatives of oleandomycin (O), erythromycin (E) and erythralosamine (EM) and compared their ability to bind to a major liver cytochrome P-450 form induced *in vitro* either by PCN or by macrolides as well as to form cytochrome P-450–iron–metabolite complexes *in vitro*.

MATERIALS AND METHODS

Materials. Nicotinamide adenine dinucleotide reduced form (NADPH), bovine serum albumin, erythromycin base and oleandomycin were purchased from Sigma Chemical Co. (St Louis, MO). Antibiotics were kindly supplied from different sources: troleandomycin from Pfizer (Orsay, France), erythromycin propionate from Roussel-Uclaf (Romainville, France), 16α-pregnenolone-carbonitrile (PCN) from Upjohn Co. (Kalamazoo, MI).

Preparation of the macrolide derivatives. Erythralosamine was prepared according to the technique of Flynn *et al.* [14] and recrystallized three times before use. The various mono-, di- or triacetates of oleandomycin, erythromycin and erythralosamine were synthesized according to techniques described previously [14, 15]. 2′-Benzoates of O, E and EM were obtained by reaction with one equivalent of benzoyl chloride in ethyl acetate and in the presence of $NaHCO_3$. After 4 hr at room temperature, the reaction was stopped by addition of water, the organic extract washed with water and the residue obtained after solvent evaporation purified on an alumina column with ethyl acetate as solvent. The compounds were crystallized from benzene–pentane mixtures.

Erythralosamine-2′,3-dibenzoate (2′,3DBEM) was prepared by a similar procedure using an excess of benzoyl chloride in pyridine. Erythralosamine-3-benzoate (3MBEM) was obtained by treatment of the 2′,3-dibenzoate by methanol overnight in agreement with a previously reported method [14].

All the products were characterized by ^{1}H NMR and mass spectroscopy (details of their ^{1}H NMR data will be published elsewhere). For all the compounds described previously, melting points in good agreement with the literature data were obtained. Some of their physico-chemical characteristics are given in Tables 1–3. Compounds in ethanol solution (10 μl, 10^{-2} M) were spotted on a neutral alumina plate (Merck 0.25 mm). After ethyl acetate elution, compounds were located by iodine treatment and R_f measured. p*Ka* were measured with an hydrogen electrode using a 2:1 dimethylformamide:water solution [14].

Treatment of rats. Male Sprague–Dawley rats (160–200 g) were treated with phenobarbitone (80 mg/kg/day i.p. in saline), β-naphthoflavone (BNF), PCN (50 mg/kg i.p. in corn oil), or erythralosamine-2′,3-diacetate (2′,3DAEM) suspended in corn oil (200 mg/kg/day, i.p.) during 3 days. Rats were killed 24 hr after the last injection. Liver microsomes were made according to usual techniques from rats either treated as indicated previously or treated by corn oil alone for controls [16].

Assays. Proteins [17], cytochrome b_5, cytochrome P-450 [18] were determined as described previously. After treatment of rats with 2′,3DAEM, dissociation of the cytochrome P-450–iron–metabolite complex and regeneration of free cytochrome P-450 were obtained by treatment of liver microsomes with ferricyanide [11, 19]. The binding of macrolides to rat liver cytochrome P-450 was studied by difference visible spectroscopy after addition of increasing amounts of the compounds dissolved in methanol to hepatic microsomes containing 2 μM cytochrome P-450 (see for instance Refs. 7 and

Table 2. Chemical characteristics of the oleandomycin derivatives used in this study

	Abbreviations	R_1	R_2	R_3	p*Ka**	R_f†	^{1}H NMR‡ $H_{2'}$	$H_{4''}$	H_{11}	H_{13}	N≤
Oleandomycin	O	H	H	H	8.5	0.22	3.20	3.18	3.04	5.65	2.29
Oleandomycin-2'-acetate	2'MAO	CH_3CO	H	H	6.6	0.62	4.80	3.18	3.04	5.65	2.26
Oleandomycin-2'-benzoate	2'MBO	C_6H_5CO	H	H	6.7	0.81	5.10	3.20	3.05	5.55	2.30
Oleandomycin-4''-acetate	4''MAO	H	CH_3CO	H	8.1	0.35	3.15	4.55	3.05	5.60	2.26
Oleandomycin-11-acetate	11MAO	H	H	CH_3CO	8.2	0.32	3.18	3.12	5.05	4.95	2.26
Oleandomycin-4'',11-diacetate	4'',11DAO	H	CH_3CO	CH_3CO	7.6	0.58	3.19	4.59	5.05	4.86	2.26
Troleandomycin	TAO	CH_3CO	CH_3CO	CH_3CO	6.6	0.88	4.78	4.69	5.05	4.90	2.29

Notes: *†‡ as in Table 1.

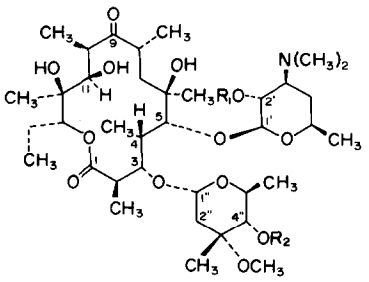

Fig. 1. Chemical characteristics of the erythromycin derivatives used in this study.

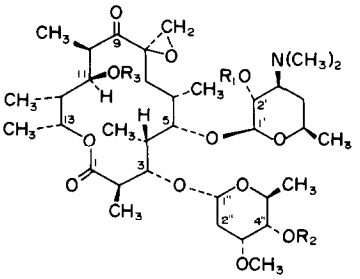

Fig. 2. Chemical characteristics of the oleandomycin derivatives used in this study.

19). *In vitro* formation of the 456 nm absorbing cytochrome P-450–metabolite complexes derived from the various macrolides was followed by difference visible spectroscopy after addition of the macrolide (30 μM) and NADPH (0.5 mM) to rat liver microsomes (2 μM cytochrome P-450) [19].

Statistical evaluations. Means, SE and regression lines were calculated on an Apple IIe microcomputer using a Microstat program modified by IUT Statistique of Paris.

RESULTS AND DISCUSSION

Different compounds studied. Six derivatives of erythromycin (Fig. 1) were prepared by specific esterification of either the 2'-OH group of the desosamine sugar or the 4''-OH group of the cladinose sugar, by using previously described procedures [14]. Some characteristics of these compounds are compared in Table 1: their p*Ka*, their R_f (retention factor) on neutral alumina thin-layer (ethyl acetate being used as eluent), and the characteristic chemical shifts of the ^{1}H NMR signals of their protons in β position of the 2'-OH and 4''-OH groups and of their $N(CH_3)_2$ function. R_f values were used in the following as parameters for macrolide polarity and hydrogen bonding ability. As shown in Fig. 1 and as indicated previously [14, 15], esterification of the OH group in β-position to the $N(CH_3)_2$ function (as in 2'MAE, 2'MPE, 2'MBE and 2',4''DAE) led to a marked decrease of the p*Ka* of erythromycin.

Derivatives of oleandomycin of increasing hydrophobicity were prepared by acetylation or benzoylation of the 2'-OH group (2'MAO, 2'MBO), by acetylation of the 4''-OH or 11-OH group (4''MAO or 11MAO) or by acetylation of both the 4''-OH and 11-OH groups (4'',11DAO) (Fig. 2). Esterification of the 2'-OH group led also to an important decrease of the p*Ka*. As shown in Table 2, the polarity and hydrogen bonding ability of these compounds varied very much when going from O to TAO or to 2'MBO (R_f from 0.22 to 0.88 and 0.81).

Moreover, some derivatives of erythralosamine, that were formed upon acidic treatment of erythromycin [14], were studied since they had a more globular and rigid structure because of internal cyclisation upon reaction of two OH groups of the macrocycle with the 9-keto function and since they were more hydrophobic because of the loss of the cladinose moiety (compare Figs 1 and 3). In fact, their R_f varied between 0.65 for EM and 0.98 for 2',3DBEM, the less polar compound that was studied in this work (Fig. 3, Table 3). These erythralosamine derivatives were also interesting since a preliminary report showed that some of them had a particularly high affinity for cytochrome P-450-PB-PCN_E [7].

Interactions of erythromycin, oleandomycin and erythralosamine derivatives with liver cytochrome P-450 from rats treated by different inducers, in the absence of NADPH. As shown in Table 4, TAO interacts with cytochromes P-450 from liver microsomes of rats pretreated with different inducers with the appearance of a difference visible spectrum

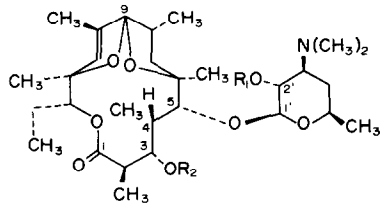

Fig. 3. Chemical characteristics of the erythralosamine derivatives used in this study.

was higher for microsomes from rats pretreated with PCN or 2',3DAEM than for microsomes from control rats or from PB-treated rats (spectral dissociation constants around 8 μM instead of 50 and 13 μM) (Table 4). Oleandomycin, which is more polar because of its three OH groups, did not give such a "type I" spectrum but, on the contrary, a "reverse type I" spectrum characterized in visible difference spectroscopy by a peak around 418 nm. This corresponds presumably to the binding of one of its OH group to the Fe(III) of cytochromes P-450 originally present in microsomes in the high-spin state. This was observed for all

Table 3. Chemical characteristics of the erythralosamine derivatives used in this study

						^{1}H NMR‡		
	Abbreviations	R_1	R_2	pKa*	R_f†	$H_{2'}$	H_3	N<
Erythralosamine	EM	H	H	8.5	0.65	3.19	4.26	2.29
Erythralosamine-2'-acetate	2'MAEM	CH_3CO	H	7.7	0.74	4.86	4.19	2.28
Erythralosamine-2',3-diacetate	2',3DAEM	CH_3CO	CH_3CO	7.3	0.89	4.85	5.30	2.28
Erythralosamine-2'-benzoate	2'MBEM	C_6H_5CO	H	6.7	0.92	5.20	4.22	2.29
Erythralosamine-3-benzoate	3MBEM	H	C_6H_5CO	7.7	0.80	3.20	5.75	2.27
Erythralosamine-2',3-dibenzoate	2',3DBEM	C_6H_5CO	C_6H_5CO	6.7	0.98	5.15	5.60	2.25

Notes: *†‡ as in Table 1.

Table 4. Characteristic of binding of TAO and oleandomycin to hepatic microsomes from rats treated with different inducing agents

	Treatment of rats	Control	PB	2',3DAEM§	PCN
TAO	Binding type*	I	I	I	I
	K_s (μM)†	50	13	8	8‖
	ΔEmax $\times 10^3$‡/nmol P-450	4	10	90	40
O	Binding type	RI	RI	RI	RI
	K_s (μM)	nm¶	nm	400	600
	ΔEmax $\times 10^3$/nmol P-450	nm	nm	17	3

* I and RI were used respectively for "type I" and "reverse type I" interactions (see text).

† K_s = apparent spectral dissociation constant.

‡ Maximum amplitudes of the difference spectra after addition of TAO or O in excess; ΔE between 390 and 420 nm for type I spectra and between 418 and 320 for reverse type I spectra.

§ After 2',3DAEM induction, microsomes were treated by 15 μM ferricyanide to dissociate the endogenous 456 nm absorbing metabolite complex [7] and restore free cytochrome P-450–Fe(III).

‖ In this case, two K_s were observed: 8 μM and 0.2 μM.

¶ nm not measured because of the very low intensities of the difference spectra.

characterized by a peak around 390 nm and a trough around 420 nm. This spectrum (called "type I") denotes a binding of TAO on a protein binding site close to the cytochrome P-450 heme which induces a low-spin to high-spin transition of a part of the microsomal cytochromes P-450. The intensity of this difference spectrum increased as a function of the P-450-PB-PCN_E isozyme content of the microsomes. It was low for control microsomes, slightly increased after PB induction and reached a maximum value with microsomes from rats treated with PCN and with 2',3DAEM which were known to contain high amounts of cytochrome P-450-PB-PCN_E [1, 7]. Moreover, the affinity of TAO the studied microsomes (Table 4). This binding occurred with a very low affinity of oleandomycin as shown by the dissociation constants measured (K_s values around 500 μM).

Because of their particular affinity to bind TAO (Table 4) and other macrolides of the E and EM series (data not shown), hepatic microsomes from rats treated with 2',3DAEM were used in the following studies.

Figure 4 gives the apparent affinity constants of various macrolides of the erythromycin, oleandomycin and erythralosamine series (indicated by their pK_s = $-\log K_s$) toward liver microsomes from 2',3DAEM-treated rats as well as their R_f

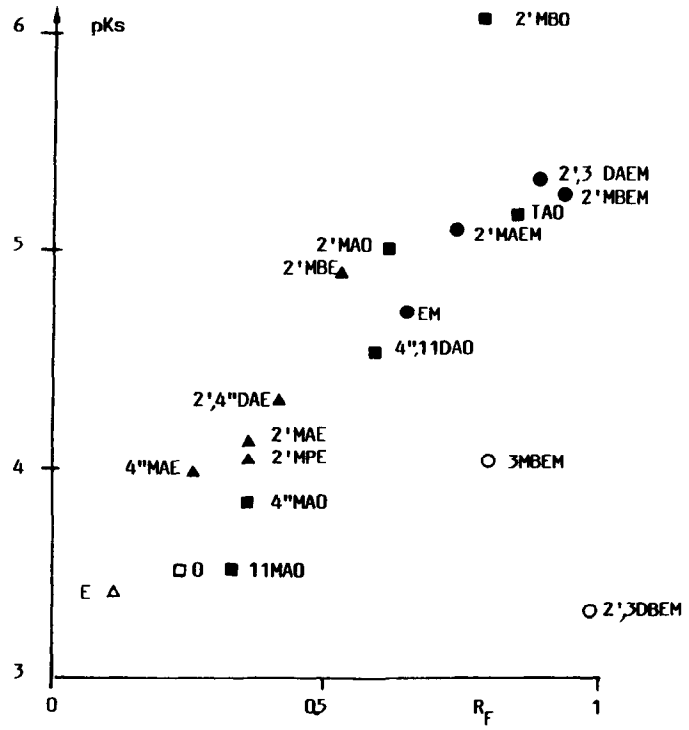

Fig. 4. Relationship between the affinity of macrolides for liver microsomal cytochromes P-450 and their hydrophobicity. Apparent spectral dissociation constants, K_s, were measured by using 10^{-7} to 10^{-3} M substrates and 2 μM liver microsomal cytochromes P-450 from rats pretreated with erythralosamine-2',3-diacetate. Hydrophobicity is referred to their retention factor R_f on neutral alumina using ethyl acetate as eluent. Black signs (▲, ■, ●) correspond to compounds giving type I spectra and white signs (△, □, ○) to those giving reverse type I spectra of the erythromycin, oleandomycin and erythralosamine series.

Equations of calculated regression lines are $pK_s = AR_f + B$

$pK_s = 3.16\ R_f + 3.05$	for E series	$r = 0.95$
$pK_s = 3.66\ R_f + 2.46$	for O series	$r = 0.92$
$pK_s = 1.75\ R_f + 3.66$	for EM series	$r = 0.90$
$pK_s = 2.72\ R_f + 3.03$	for all compounds except 3MBEM and 2',3DBEM.	$r = 0.91$

values on neutral alumina. This figure also mentions the type of interaction ("type I" or "reverse type I") that was observed by visible difference spectroscopy. None of the studied macrolides gave a "type II difference spectra" which would have resulted from the binding of their nitrogen atom to P-450-Fe(III). This was expected for such a tertiary amine function for which an approach of the nitrogen atom to the heme was prevented by steric hindrance. All the studied macrolides gave type I interactions except for four of them: E, O, 3MBEM and 2',3DBEM. This was expected for the two polar compounds, erythromycin and oleandomycin which contained several OH groups, but quite unexpected for the highly hydrophobic 3MBEM and 2',3DBEM containing a benzoyloxy group in position 3.

In the erythromycin and oleandomycin series, the affinity (pK_s) increased when the OH groups of E or O were progressively acetylated or benzoylated resulting in a decrease of their polarity (increase of R_f):

E < 4"MAE < 2'MAE < 2'MPE < 2',4"DAE < 2'MBE

O < 11MAO < 4"MAO < 4",11DAO < 2'MAO < TAO < 2'MBO

This was also true for four derivatives of EM:

EM < 2'MAEM < 2',3DAEM < 2'MBEM (Fig. 4)

Qualitatively, a decrease in the polarity and hydrogen bonding ability of all the studied macrolides except 3MBEM and 2',3DBEM was associated with an increase of their pK_s. In fact, calculations of regression lines for the 17 studied macrolides (all except 3MBEM and 2',3DBEM) ($pK_s = A + B\ R_f$) gave an r value of 0.91. Thus, there is only a very qualitative relationship between pK_s and R_f of macrolide antibiotics, but this is as expected for compounds of three different series having such large structural differences.

Similar calculations of regression lines for each

Table 5. *In vitro* formation of macrolide–metabolite–cytochrome P-450 complexes using liver microsomal preparations from rats treated with various inducers

Substrate \ Rat Treatment	% P-450 engaged in iron–metabolite complex				
	β-NF	Control	PB	PCN	2′,3DAEM*
EM†	2 ± 1	2 ± 1	5 ± 1	26 ± 4	14 ± 2
TAO†	3 ± 1	5 ± 2	7 ± 1	34 ± 4	34 ± 4
2′,3DAEM†	6 ± 1	8 ± 2	11 ± 1	41 ± 4	41 ± 4
2′MBEM†	9 ± 2	11 ± 2	14 ± 2	54 ± 5	45 ± 3
% PB/PCN$_E$‡	10	17	23	57	50

Animals have been treated as described in the experimental section. Incubations were done using 2 μM cytochrome P-450, 0.5 mM NADPH and 30 μM substrate [18].

* Microsomal preparations were pretreated with 15 μM ferricyanide for 5 min in order to dissociate the endogenous macrolide–metabolite–cytochrome P-450 complex [19].

† Percentage (mean ± SE) of cytochrome P-450 engaged in a 456 mm absorbing metabolite complex. Mean of three to six determinations.

‡ From Ref. 1 except for the case of microsomes from 2′,3DAEM-treated rats for which an immunological determination of cytochrome P-450 PB-PCN$_E$ was done by Dr P. Beaune and Guengerich (personal communication).

series of compounds, i.e. the six studied derivatives of E, for the seven studied derivatives of O and for EM, 2′MAEM, 2′,3DAEM an 2′MBEM, gave r values of 0.95, 0.92 and 0.90 respectively.

Clearly, two compounds, 3MBEM and 2′,3DBEM were out of the qualitative correlation area. These compounds which have both a benzoyloxy group in position 3 of EM were the only derivatives of erythralosamine giving rise to reverse type I spectra. They exhibited very low affinity for macrolide-inducible cytochromes P-450, well below those of other EM derivatives such as 2′MBEM or 2′,3DAEM that have comparable or higher polarity (Fig. 4).

Ability of erythromycin, oleandomycin and erythralosamine derivatives to form cytochrome P-450–Fe(II)–metabolite complexes upon in vitro *metabolism by liver microsomes.* Table 5 compares the amounts of cytochrome P-450–Fe(II)–metabolite complexes, characterized by a 456 nm peak in visible difference spectroscopy which were formed upon oxidative metabolism of four macrolides, EM, TAO, 2′,3DAEM and 2′MBEM, by liver microsomes from rats treated with several inducers. For the four macrolides, these amounts increased as a function of the content of microsomes in cytochrome P-450-PB-PCN$_E$ [1], confirming the particular ability of this cytochrome P-450 to metabolize such compounds [7]. 2′MBEM was found to be the best substrate for iron–metabolite complex formation.

In order to compare all the macrolides for their ability to form such complexes, liver microsomes from rats treated with 2′,3DAEM were used in the following part of the study. Figure 5 compares the ability of these microsomes to form 456 nm absorbing cytochrome P-450–metabolite complexes in the presence of NADPH and O_2 (indicated as the percentage of total microsomal cytochromes P-450 engaged in these complexes), as a function of the macrolide affinities previously determined (pK_s, see Fig. 4).

In a very general manner, the ability of macrolides to form iron–metabolite complexes increased with their ability to bind to cytochrome P-450 in its resting state. Only two compounds, 2′MBE and 2′MBO, were found completely unable to form an iron–metabolite complex despite their relatively good affinities for cytochrome P-450–Fe(III) (Fig. 5). Both contained a bulky benzoate function in β position of the $N(CH_3)_2$ function of the desosamine sugar, and were unable to form iron–metabolite complexes. Interestingly, 2′MBEM, which also contained a benzoate function in the same position of desosamine was the best precursor of metabolite complex formation.

Attempts to construct regression lines (% complex formation = f(pK_s)) gave satisfactory results for the oleandomycin derivatives (if one except 2′MBO) with an r value of 0.94 but poor results for the EM ($r = 0.83$) and E ($r = 0.74$) derivatives. This lack of a linear relationship between complex formation and pK_s at least for the compounds of the EM and E series is not surprising if one takes into account the various steps involved in the formation of the P-450–metabolite complexes. Moreover, it is noteworthy that the amounts of complexes formed with the E derivatives were in general low.

Structural factors important for the affinity of macrolides for cytochrome P-450 and for iron–metabolite complex formation. In a very general manner and despite the large difference of structure between the three macrolide series, a decrease of the polarity of the compounds resulted in an increase of their affinity for macrolide-inducible cytochrome P-450 and of their ability to form 456 nm absorbing iron–metabolite complexes. For the compounds of the three series obtained from acetylation of the 2′-, 3- and 4″-OH groups, there was no exception to this general rule. Among the monacetylated compounds, those having the acetoxy group in position 2′ of desosamine were the less polar ones and, at the same time, had the best affinities for macrolide-inducible cytochrome P-450 and gave the largest amounts of iron–

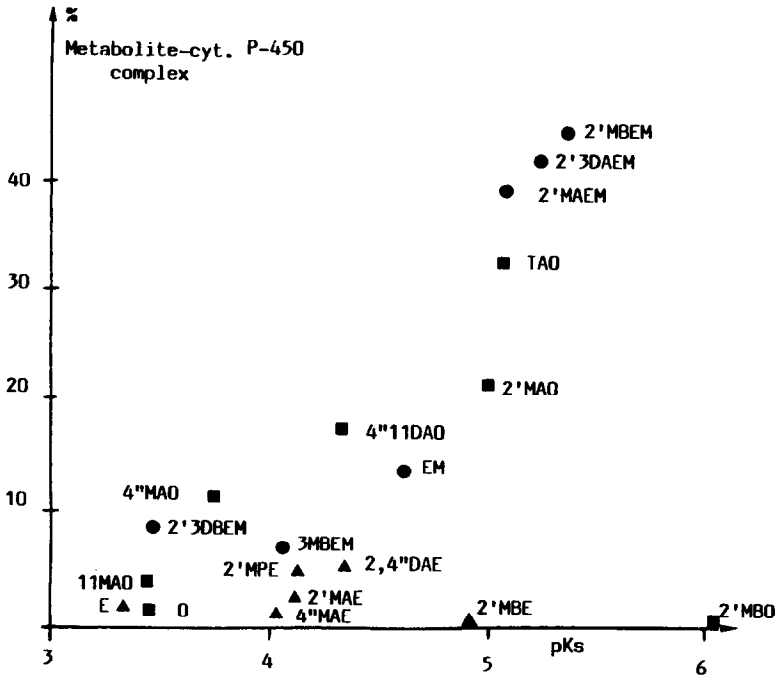

Fig. 5. Relationship between the ability of macrolides to give cytochrome P-450–metabolite complexes and their affinity for microsomal cytochrome P-450. Metabolite cytochrome P-450 complex formation was studied using 2 μM hepatic microsomal cytochrome P-450 from erythralosamine 2',3-diacetate treated rats (30 μM substrate, 0.5 mM NADPH). Values expressed as % of total cytochrome P-450 were determined as described previously [11]. ▲, Erythromycin; ■, oleandomycin; and ● erythralosamine series. Equation of the calculated regression line for the O series was % complex = 15.6 pK_s − 48.7 with $r = 0.94$.

metabolite complexes. This was at least in part due to the decrease of the p*K*a of the amine function upon acylation of 2'-OH in the β-position to the $N(CH_3)_2$ group (Tables 1–3), a well-known phenomenon for desosamine containing macrolides [15]. Upon progressive acetylation of OH functions of macrolides, a gradual decrease of polarity as well as an increase of pK_s and amounts of iron–metabolite complex was observed. This was particularly obvious in the oleandomycin series (Figs 4 and 5). For the compounds of the three series involving acetoxy groups in the same positions, the hydrophobicity, pK_s and amounts of iron–metabolite complex were higher in the erythralosamine series than in the oleandomycin and erythromycin series. This is easily understandable from a comparison of the structures of erythralosamine, oleandomycin and erythromycin. This comparison shows that, in addition to the lactone and the desosamine sugar which are present in the three compounds, erythralosamine contains only one polar OH group and a less polar ketal function whereas oleandomycin contains a very polar oleandrose sugar, an OH group on the macrocycle and a less polar keto and epoxide function. Erythromycin is even more polar with the cladinose sugar and three OH groups on the macrocycle, explaining its poor pK_s and ability to form metabolite complex.

The case of the macrolide benzoates was more complex. As far as iron–metabolite complex formation was concerned, only two compounds 2'-MBE and 2'-MBO were completely unable to form such complexes. This could be explained by an increase of steric hindrance around nitrogen upon introduction of the bulky OCOPh substituent in position 2', which prevented the approach of the nitrogen atom to the heme that was necessary for iron–metabolite complex formation. In fact, the presence of a bulky benzoate substituent in position 2' was not sufficient to prevent metabolite complex formation, since 2'MBEM was found as the best substrate for such iron–metabolite complex formation (Fig. 5). Thus, it seems that the simultaneous presence of bulky substituents in position 2' (benzoate) and 3 (sugar in 2'MBO and 2'MBE instead of H in 2'MBEM) is necessary for preventing complex formation. Accordingly, 2',3DBEM with its two benzoate groups on these positions is one of the poorest precursor of complex formation despite its very low polarity.

The two exceptions, 2',3DBEM and 3MBEM, to the global pK_s–R_f correlation (Fig. 4) are even more difficult to explain. In fact, we have no clear and complete explanation for the unability of these hydrophobic compounds to bind to the protein active site of macrolide-inducible cytochrome P-450 and to produce type I difference spectra. Rather surprisingly, they produce reverse type I difference spectra as the most hydrophilic macro-

lides such as oleandomycin and erythromycin. Preliminary studies on conformations of these compounds by ^1H NMR spectroscopy (E. Sartori, M. Delaforge, J. P. Giraud and D. Mansuy, unpublished results) indicate that, in the preferred conformation of compounds of the erythralosamine series containing a benzoate group in position 3, this benzoate is parallel to the desosamine ring and masks the $N(CH_3)_2$ function rendering its binding and recognition very difficult. This is particularly critical in the erythralosamine series because of the rigidity of the macrolide ring caused by the spiroketal function. In the oleandomycin and erythromycin series, the macrolactone ring has more possible conformations and the conformation in which the access to the $N(CH_3)_2$ function is prevented by the group in position 3 is less probable. If this masking of the $N(CH_3)_2$ function by the benzoate in position 3 of 3MBEM and 2',3DBEM was an important factor to explain the poor affinity of these macrolides for macrolide-inducible cytochrome P-450, this would suggest that the recognition of the desosamine sugar and particularly of its $N(CH_3)_2$ function could be important for the binding of macrolides to these cytochromes.

In conclusion, the aforementioned data confirm that the major cytochrome P-450 forms induced either by macrolides or by PCN (presumably P-450-PB-PCN_E or very similar isozymes [6, 7]) are the major isozymes involved in the binding of macrolides of the erythromycin, oleandomycin and erythralosamine series to liver microsomes and in metabolite-complex formation.

They gave informations on the structural factors that are important for a macrolide to bind to this P-450 isozyme(s) and to form an iron–metabolite complex, showing that (i), in general, hydrophobicity is a beneficial factor for both properties, (ii) the presence of a bulky substituent in position 3 of erythralosamine decreases dramatically their affinity, and (iii) the simultaneous presence of bulky substituents in position 2' and 3 prevents iron–metabolite complex formation.

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