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IN VIVO EFFECTS OF ERYTHROMYCIN, OLEANDOMYCIN AND ERYTHRALOSAMINE DERIVATIVES ON HEPATIC CYTOCHROME P-450

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Abstract—Rats have been treated with several derivatives of the erythromycin, erythralosamine or oleandomycin series, in order to compare their ability to induce cytochrome P-450 and to form stable 456 nm-absorbing cytochrome P-450 metabolite complexes. The data obtained confirm that the cytochromes P-450 induced in rats by various macrolides are similar to that induced by pregnenolone 16α-carbonitrile: the cytochrome P-450 IIIA$_1$ isozyme. It showed that: (i) formation of a stable inhibitory 456 nm-absorbing cytochrome P-450 complex is not a prerequisite for cytochrome P-450 induction but enhances induction by stabilization of the IIIA isozyme. Therefore, the best inducers lead also to the maximal *in vivo* amounts of cytochrome P-450 metabolite complex (except for 2'MBEM); (ii) affinity for cytochrome P-450 IIIA$_1$ is not directly involved for induction; and (iii) hydrophobicity favors induction and formation of complexes. Structural factors are also involved.

The cytochrome P-450 monooxygenase system transforms numerous different chemicals, both exogenous or endogenous, into more hydrophobic products. This broad substrate 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, cDNA and amino acid composition [1–3]. Hepatic cytochromes P-450 have been subdivided into at least five groups according to the type of inducing chemicals i.e. phenobarbital (PB*), 3-methylcholanthrene (3MC), isosafrole (Isf), pregnenolone 16α-carbonitrile (PCN), clofibrate (Clo) and ethanol. Such drugs selectively induced the synthesis of one or a few forms of the cytochromes P-450 which thus predominate and confer their own substrate specificity to the enzymatic system.

Macrolide antibiotics have been proved to display specific interactions with cytochrome P-450 similar to the IIIA$_1$ isozyme; a basal rat liver isozyme which is inducible by treatment with PB, PCN and macrolides themselves but not with other inducers (3MC, β-naphthoflavone (BNF), Clo) [2–5]. During metabolism, macrolides bearing a dimethyl amino group can form stable Fe(II)-nitrosoalkyl complexes with this form [6–8]. This macrolide- or PCN-induced cytochrome P-450 also exhibits important activity for binding and metabolizing ergotamine derivatives, cyclosporine, theophylline or some steroids [9–11]. This phenomenon also occurs in the human, since cytochrome P-450 Fe(II)–RNO complexes have been effectively observed in liver biopsy from people treated by TAO or erythromycin [7, 12]. This macrolide metabolite cytochrome P-450 complex formation may explain drug metabolism impairments and drug interactions observed after macrolide antibiotic treatments [13–16].

We have previously carried out *in vitro* studies on a large number of erythromycin and oleandomycin derivatives (Fig. 1) in order to establish relationships between chemical structure, polar character and potency for fitting into the cytochrome P-450 active sites and for being metabolized into stable cytochrome P-450–RNO inhibitory complexes [17].

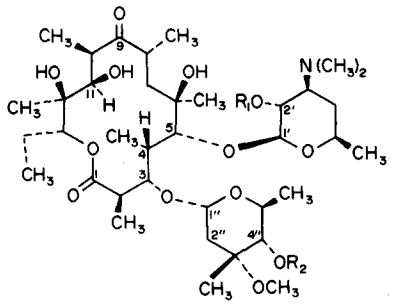
In the present work, these macrolide derivatives have been administered to rats in order to establish possible links between structural factors, their potency to induce cytochrome P-450 and the *in vivo* formation of nitroso cytochrome P-450 inhibitory complexes.

MATERIALS AND METHODS

Materials. Nicotinamide adenine dinucleotide reduced form (NADPH), bovine serum albumin, oleandomycin and PCN were purchased from the Sigma Chemical Co. (St Louis, MO). Antibiotics were kindly supplied from different sources: troleandomycin from Pfizer (Orsay, France); erythromycin base and erythromycin propionate from Roussel Uclaf (Romainville, France). Macrolide derivatives were prepared as already described [17]. All the products were characterized by their melting point, NMR and mass spectra [details of the NMR characterization will be published elsewhere (E. Sartori *et al.* manuscript in preparation)]. R_f values were determined on neutral alumina TLC plates using ethyl acetate as eluant [17]. pK_a were measured with an hydrogen electrode using a 2:1 dimethylformamide:water solution [18].

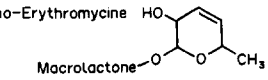
Treatment of rats. Male Sprague–Dawley rats (160–200 g) were treated for 3 days with pregnenolone 16α-carbonitrile (PCN) (50 mg/kg i.p. in

* Abbreviations: PCN, pregnenolone 16α-carbonitrile; PB, phenobarbital; BNF, β-naphthoflavone; O, oleandomycin; E, erythromycin; EM, erythralosamine; TAO, troleandomycin; 2',3DAEM, erythralosamine-2',3-diacetate; 3MC, 3 methylcholanthrene; Clo, clofibrate.

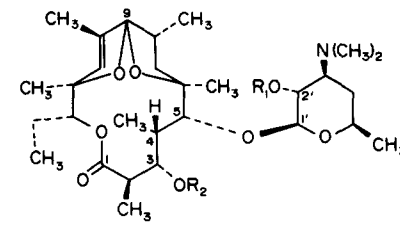


	Abbreviations	R_1	R_2
Erythromycin	E	H	H
Erythromycin-2'-acetate	2'MAE	CH_3CO	H
Erythromycin-2'-benzoate	2'MBE	C_6H_5CO	H
Erythromycin-4''-acetate	4''MAE	H	CH_3CO
Erythromycin-2',4''-diacetate	2',4'' DAE	CH_3CO	CH_3CO

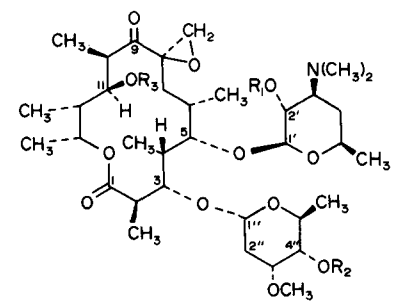
EDA: Desamino-Erythromycine



Macrolactone



	Abbreviations	R_1	R_2
Erythralosamine	EM	H	H
Erythralosamine-2'-acetate	2'MAEM	CH_3CO	H
Erythralosamine-2',3-diacetate	2',3DAEM	CH_3CO	CH_3CO
Erythralosamine-2'-benzoate	2'MBEM	C_6H_5CO	H
Erythralosamine-3-benzoate	3 MBEM	H	C_6H_5CO
Erythralosamine-2',3-dibenzoate	2',3DBEM	C_6H_5CO	C_6H_5CO



	Abbreviations	R_1	R_2	R_3
Oleandomycin	O	H	H	H
Oleandomycin-2'-acetate	2'MAO	CH_3CO	H	H
Oleandomycin-2'-benzoate	2'MBO	C_6H_5CO	H	H
Oleandomycin-4''-acetate	4''MAO	H	CH_3CO	H
Oleandomycin-11-acetate	11 MAO	H	H	CH_3CO
Oleandomycin-4'',11-diacetate	4'',11 DAC	H	CH_3CO	CH_3CO
Troleandomycin	TAO	CH_3CO	CH_3CO	CH_3CO

Fig. 1. Chemical structures of the macrolides used in this study.

corn oil), or with macrolides suspended in corn oil (200 mg/kg/day, i.p.). Rats were killed 24 hr after the last injection. Liver microsomes were made according to usual techniques from rats either treated as indicated or treated with corn oil alone for controls.

Assays. Protein [19], cytochrome P-450, cytochrome b_5 [20] and cytochrome P-450–macrolide metabolite complexes [21] were determined by published procedures. The amounts of nitroso metabolite–cytochrome P-450 inhibitory complexes were measured as the difference between the amounts of cytochrome P-450 being able to bind CO in microsomal preparations before and after 50 μM ferricyanide treatment [4, 21].

In vitro binding and the pK_s values, of macrolides to rat liver cytochromes P-450 were studied by difference visible spectroscopy after addition of increasing amounts of the compounds dissolved in methanol to 2 μM hepatic microsomes from PCN-treated rats [17]. Cytochrome P-450–2'MBEM metabolite complex formation was measured by differential spectroscopy with microsomes containing 2 μM cytochrome P-450, 0.04 mM substrate and 0.5 mM NADPH. Differential spectra were recorded between 400 and 500 nm every 2 min and maximum absorbance of the 456 nm absorbing cytochrome P-450 metabolite complex: 456–490 nm, was determined.

RESULTS AND DISCUSSION

Induction as a function of the dose of administered macrolide

As already described for TAO [21] or erythromycin, induction of rat hepatic microsomal cytochrome P-450 was dose dependent for 2',3DAEM, an acetylated macrolide deriving from erythromycin by loss of the cladinose sugar and subsequent intracyclization with dehydration (Fig. 1). In contrast to TAO or erythromycin for which high doses were required for cytochrome P-450 induction, low doses of 2',3DAEM (10 mg/kg/day, 0.015 nmole) for 3 days gave rise to a significant increase of the total

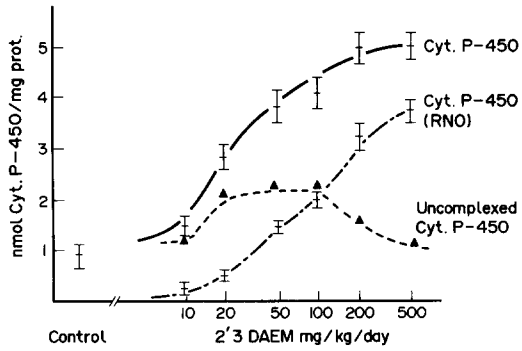


Fig. 2. Dose dependent induction of cytochrome P-450 and amount of nitroso metabolite–cytochrome P-450 complex after 2',3DAEM treatment. Rats were treated with 2',3DAEM (10 to 500 mg/kg/day, i.p. in corn oil for 3 days). Amounts of cytochrome P-450 and nitroso metabolite–cytochrome P-450 were measured in hepatic microsomes prepared on the fourth day. Control rats received corn oil. Each point is the mean ± SE of three rats.

amount of cytochrome P-450 (Fig. 2). This induction increased up to five-fold for doses of 200 or 500 mg/kg/day), at these doses 2',3DAEM being one of the best cytochrome P-450 inducers (better than arochlor).

Simultaneously the amounts of active cytochrome P-450 which were recovered uncomplexed with a 2',3DAEM nitroso metabolite, were slightly modified during the whole experiment, an important proportion of the induced cytochrome P-450 being found as cytochrome P-450-2',3DAEM metabolite complex.

Doses of 200 mg/kg/day for 3 days were thus chosen for comparison of *in vivo* effects of the different macrolides.

Induction of cytochrome P-450 and nitroso-cytochrome P-450 complex formation after rat treatment with macrolide derivatives of the O, E and EM series

After treatment of rats with macrolide derivatives (200 mg/kg/day for 3 days), the hepatic cytochrome P-450 contents were increased compared to control values (Fig. 3), except for 2',3DBEM, E and 2'MBE which are not inducers of cytochrome P-450 at the dose used. Parts of the induced cytochrome P-450 were recovered as nitroso metabolite–cytochrome P-450 complexes (Fig. 3a).

The induced cytochrome P-450 had the same characteristics as the TAO- or PCN-induced cytochrome P-450. Particularly, it exhibited a strong ability to bind and metabolize erythralosamine-2'-benzoate to nitroso metabolite cytochrome P-450 complex [5]. Upon *in vitro* incubation of microsomal preparations of macrolides- or PCN-treated rats with 2'MBEM and NADPH, nitrosometabolite cytochrome P-450 complex were formed in all the microsomal preparations (Fig. 3b). For nearly all the macrolides tested, the induced cytochrome P-450 (higher than 0.8 nmol/mg protein) was recovered, at least partly blocked as nitroso complexes.

A correlation can be found between induction and the ability of these microsomes to form nitroso metabolite–cytochrome P-450 complex (Fig. 3, inserts). The insert of Fig. 3a shows the lack of correlation between the amounts of cytochrome P-450 recovered after macrolide treatments and amounts of recovered nitroso metabolite–cytochrome P-450 complex. On the contrary, a linear relationship ($r = 0.996$) was obtained between amounts of recovered cytochrome P-450 and maximal amounts of nitroso metabolite–cytochrome P-450 complex measured after *in vitro* metabolism (Fig. 3b, insert). These amounts resulted from addition of *in vivo* remaining complex and *in vitro* metabolized 2'MBEM-cytochrome P-450 complex. This confirms that all the induced cytochromes P-450, either complexed or not after *in vivo* treatment, belong to the same form, regardless of the macrolide used for induction.

Non-macrolide compounds such as PCN or dexamethasone induced the same or very similar cytochrome P-450 isozymes [5, 22]. All these macrolide- and PCN-induced cytochromes P-450 had similar epitopes since they cross reacted with the same antibody anti-IIIA$_1$ (data not shown) [2, 5].

Involved factors for in vivo *cytochrome P-450 induction and for iron–metabolite complex formation*

Presence of the dimethyl amino function (Figs 1 and 3). The best *in vitro* precursors of nitroso metabolite–cytochrome P-450 complexes: 2',3DAEM, 2'MBEM and TAO were also the most potent inducers of isozyme IIIA$_1$ in their series. This suggests that the blockage of cytochrome P-450 by these complexes could be related to its induction. We have ruled out this possibility by treatment of rats with desaminoerythromycin (EDA, Fig. 1), which is deprived of the $N(CH_3)_2$ function and was then unable to form cytochrome P-450 complex. EDA was found to be one of the best inducers in the erythromycin series. 2'MBO, which had a dimethylamino group but for which no formation of nitrosoalkyl complex had been detected *in vitro* [17] or *in vivo*, is also a potent cytochrome P-450 inducer.

Polarity of the macrolides (Fig. 4). A correlation exists between induction of cytochrome P-450 and the polar character and hydrogen bonding ability of the administered macrolides as judged by their retention factor on neutral alumina TLC plates for the acetylated derivatives of oleandomycin: 0 <11MAO <4″MAO <4″,11DAO, <2′MAO <TAO, $r = 0.91$.

Acetylation of the 2′ position played an important role in induction ability. This can be explained by variations of pK_a (around 6.6 for 2′ esters, 8.5 to 9 for 2′hydroxy compounds). The dimethyl amino group of 2′ esters are therefore essentially not protonated at physiological pH whereas parent compounds are.

Qualitatively similar results were observed for erythromycin or erythralosamine derivatives. Acetylation of each hydroxyl group led to an increase in induction ability, the most important effect being obtained for 2′ hydroxyl acetylation.

No correlation between hydrophobic character and induction potency was observed for benzoate derivatives. 2′MBE and 2′MBEM were less potent inducers of cytochrome P-450 than the corresponding

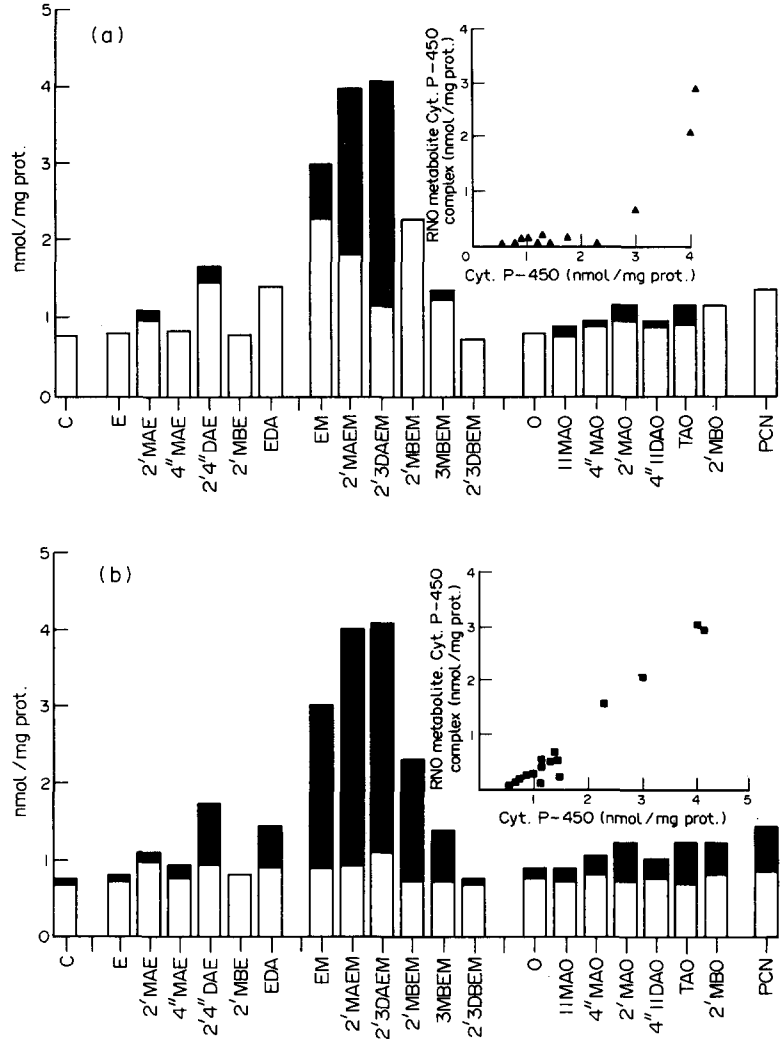


Fig. 3. *In vivo* effects of macrolides and PCN on rat liver cytochrome P-450 levels. After treatment of rats with the various compounds indicated on the abscissa, the contents of cytochrome P-450 in the liver either free (□) directly measured as CO complex, or engaged in a 456 nm absorbing Fe–nitroso metabolite complex, (■) measured after K_3FeCN_6 treatment, were measured and expressed as nmoles of cytochrome P-450 per mg microsomal protein on the ordinates. Values are means of three independent determinations on three rats. (A) Direct measurement. Insert: amount of RNO metabolite–cytochrome P-450 complex remaining in the microsomal preparation related to the amount of total recovered cytochrome P-450. (B) RNO metabolite formed *in vivo* plus *in vitro* and remaining uncomplexed cytochrome P-450 after incubation with 30 μM 2'MBEM for 30 mn and 0.5 mM NADPH. Insert: total amounts of RNO metabolite–cytochrome P-450 complex as a function of total cytochrome P-450. The equation of the calculated regression line was: RNO metabolite = 0.925 P-450 − 0.676, $r = 0.996$.

acetates. No nitroso metabolite complexes were recovered in the microsomal preparations of the rats treated with 2'MBO, 2'MBE or 2'MBEM, though 2'MBEM was the most potent *in vitro* nitroso–cytochrome P-450 complex precursor. 3MBEM was a slight inducer whereas 2',3DBEM failed to induce cytochrome P-450. This suggests involvement of steric effects, due to the hindrance of the benzoyl functions on the macrocycle or close to the $N(CH_3)_2$ group [17].

Induction as a function of in vitro *dissociation constants:* K_s. Comparison of cytochrome induction after rat treatment with macrolide derivatives to their *in vitro* dissociation constants pK_s measured on PCN-induced cytochrome P-450 showed than no correlation existed between these two effects (Fig. 5). Oleandomycin derivatives for which pK_s values were from 3.5 to 6 were found to have the same induction potencies (0.9 to 1.8 nmol/mg protein) (Fig. 3). Curiously a relationship has been obtained for erythralosamine derivatives if one excepts 2'MBEM. The induction potencies and pK_s increased in the order 2',3DAEM < 3MBEM < EM < 3MAEM < 2',3-DAEM with $r = 0.98$. This suggests that recognition of the macrolide by the cytochrome P-450 active site is not a prerequisite for induction but that similar

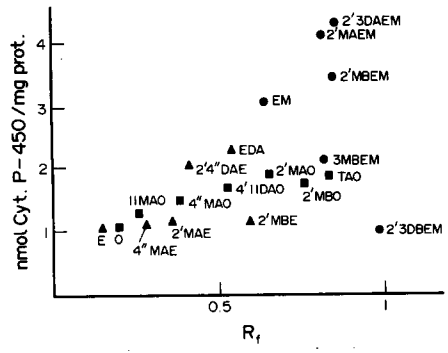


Fig. 4. Relationship between the induction of cytochrome P-450 by macrolides and their hydrophobicity. Induction was measured as total cytochrome P-450 recovered in microsomes (after K_3FeCN_6 cytochrome P-450–nitroso metabolite complex dissociation). Hydrophobicity is referred to their retention factor R_f on neutral alumina using ethyl acetate as eluent [17]. The equations of the calculated regression line were: Cyt. P-450 = 0.993 R_f + 0.757, r = 0.47 for the E series; Cyt. P-450 = −5.1 R_f + 6.8, r = 0.45 for the EM series; Cyt. P-450 = 0.974 R_f + 0.711, r = 0.913 for the O series; Cyt. P-450 = 1.72 R_f + 0.56, r = 0.44 for all compounds.

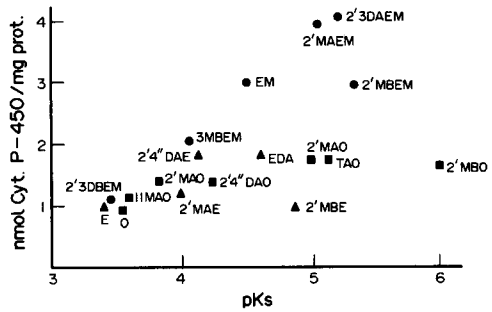


Fig. 5. Relationship between the induction of cytochrome P-450 by macrolides and their *in vitro* dissociation constants. Induction was measured as total cytochrome P-450 recovered in microsomes (after K_3FeCN_6 cytochrome P-450–nitroso metabolite complex dissociation). Dissociation constants were measured *in vitro* with PCN-induced cytochrome P-450 [17]. Induction values and pK_s are means of three independent determinations. The equations of the calculated regression lines were: Cyt. P-450 = 0.192 pK_s + 0.31, r = 0.287 for the E series; Cyt. P-450 = 1.52 pK_s − 4.38, r = 0.864 for the EM series; Cyt. P-450 = 1.878 pK_s − 5.74, r = 0.987 for the EM series −2'MBEM; Cyt. P-450 = 0.131 pK_s + 0.47, r = 0.885 for the O series; Cyt. P-450 = 0.663 pK_s − 1.37, r = 0.499 for all compounds.

factors (hydrophobicity and steric requirements around the N dimethyl amino function) are important for induction properties and accessibility to the cytochrome P-450 active site.

CONCLUSIONS

The formation of stable inhibitory cytochrome P-450 metabolite complexes upon metabolism of macrolide derivatives is a specific phenomenon which depends on the presence of active $IIIA_1$ cytochrome P-450 isozyme [7]. We tried to characterize the induction properties of macrolides and to study the links between induction and formation of cytochrome P-450 nitroso metabolite complexes.

Despite the fact that the most potent macrolide inducers of cytochrome P-450$IIIA_1$ are also the most potent *in vivo* precursors of cytochrome P-450 metabolite complexes, some macrolides (EDA, 2'MBO) were found able to induce cytochrome P-450 without formation of an inhibitory cytochrome P-450 complex. Therefore, the formation of stable nitroso-cytochrome–P-450 metabolite complex is not the cause of its induction. The increase of hepatic cytochrome P-450 after treatment with macrolide or PCN is due to two different phenomena: (i) induction of isozyme $IIIA_1$, which is observed with 2'MBO or EDA, PCN; and (ii) stabilization of this isozyme by nitroso metabolite complex formation. Such complexes have a $T_{1/2}$ of about 3 days, as determined by *in vitro* or *in vivo* studies of the TAO metabolite–cytochrome P-450 complex [21]. This is about three times the mean half-life of uncomplexed cytochrome P-450 [23, 24]. This longer half-life causes accumulation of cytochrome P-450 in the liver. Maximal inductions observed after treatment with different doses of 2'MBO or PCN are in the same range of about 1.4 nmol/mg protein, which may originate from a saturation of the induction system. About twice these amounts are obtained after treatment with potent *in vivo* precursors of nitroso metabolite complexes. This may reflect the great stability of these complexes.

Induction abilities of macrolides are also related to their hydrophobicity, pK_a, and on structural characteristics such as rigidification and conformational modifications of the macrolactone. Acetylation of hydroxyl groups yields more potent inducers and precursors of *in vivo* cytochrome P-450–metabolite complexes. Erythralosamine derivatives are more active than the corresponding erythromycin ones, the loss of the cladinose sugar and the rigidification of the macrolactone favors both complex formation and induction.

Benzoylation of position 2' abolishes the formation of cytochrome P-450–metabolite complex but not induction, either in the oleandomycin and the erythralosamine series. Benzoate in position 3 of erythralosamine decreases both induction and complex formation either *in vivo* (these data) or *in vitro* [5, 17], 2',3DBEM is fully inactive for both these activities. NMR studies show that the benzoyl group attached in position 3 of the macrolactone is parallel to the desosamine sugar and masks the dimethylamino group by steric hindrance. This is detrimental for oxidation, for metabolite complex formation and for induction when compared to EM. This phenomenon is amplified when both 2' and 3 positions are esterified (2',3DBEM).

EM is a metabolite of erythromycin [25] and has higher interaction properties either *in vitro* or *in vivo* with the cytochrome P-450 system. This compound could be at the origin of the toxic effects observed in animals after treatment with high doses of erythro-

mycin and of drug interactions reported with erythromycin [13, 14].

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