

In vitro formation of metabolic-intermediate cytochrome P450 complexes in rabbit liver microsomes by tiamulin and various macrolides

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Abstract – Tiamulin and a number of macrolides were evaluated as to their ability in forming metabolic-intermediate (MI) complexes with cytochrome P450 in liver microsomes from rabbits bred for meat production. Complex formation, which occurred only in preparations where the expression of P450 3A was increased as the result of rifampicin pre-treatment and with different kinetics, was in the order tiamulin > erythromycin > TAO ≈ roxithromycin ≈ tylosin and did not take place with tilmicosin and spiramycin. Most of the tested compounds underwent an oxidative N-dealkylation and a good relationship could be found between the rate of N-dealkylase activity in induced preparations and the aptitude in generating MI complexes. Although the results from in vitro studies should be interpreted with caution, it is suggested that the potential for in vivo drug interactions also exists in the rabbit for tiamulin and for four out of the six tested macrolides.

tiamulin / macrolide / rabbit / metabolic-intermediate complex / in vitro

1. INTRODUCTION

The cytochromes P450 (P450s) represent a superfamily of enzymes mainly devoted to the oxidative biotransformation of foreign compounds. Several drugs containing an amine function can be hydroxylated by hepatic P450s and then further oxidised to a nitroso group that binds tightly to the prosthetic heme of P450 to form a metabolic-intermediate (MI) com-

plex [1]. Although this may occur with several P450 isoenzymes including P450 2B [17], 2C [18], or 2D [7], P450 3A is known to be involved in most cases [3]. This phenomenon is of practical relevance, because the complexed P450 is metabolically inactive, so that the pharmacokinetic profile of concurrently administered drugs may be altered considerably [3]. In several domestic species, tiamulin, a semisynthetic derivative of the antibiotic pleuromutilin, and

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certain macrolides are reported to give rise to a number of drug interactions with ionophores, cimetidine, sexual steroids or phenytoin, often leading to overt signs of toxicosis [1].

To a certain extent, the drug-mediated formation of MI complexes may be predicted by *in vitro* studies performed under carefully controlled conditions; however, such studies have been conducted mainly with rat or human preparations and relatively less information is available for domestic species [22, 24]. Among them, the rabbit has gained a considerable importance as a food-producing species in Italy and other Mediterranean and European countries, with the European production of rabbit meat accounting for nearly 70% of the total world production [5]. Tiamulin and tilmicosin are presently approved in the EU and extensively used in meat rabbits, especially in young weaning individuals and in breeding does and bucks, to prevent or cure a number of enteric and respiratory diseases [4]. Other macrolides, including erythromycin, tylosin and spiramycin are often prescribed for the same purposes as extra-label drugs according to the article 10 of Directive 2001/82/EC (D. Gallazzi, personal communication). Despite this, there is a paucity of knowledge about the ability displayed by such drugs in forming MI complexes in this minor species. In the present study, the *in vitro* formation of MI complexes by tiamulin and six macrolides was studied by optical difference spectroscopy using liver microsomes from commercial meat producing rabbits.

2. MATERIALS AND METHODS

2.1. Animals and treatments

Twelve healthy male crossbred rabbits (New Zealand White × California) for meat production, average weight 3.3 kg, were obtained from a local rabbitry and individ-

ually housed in a temperature-controlled room (20–22 °C and 50–60% humidity) with a 12 h dark-light cycle. The animals were allowed free access to standard pelleted food and tap water. The care, handling and sacrifice of the animals were as recommended by the Italian Law on animal experimentation (DL 116/92) and the whole study was performed according to ISO 9001:2000 requirements. In order to over express liver P450 3A, nine individuals were administered *i.p.* rifampicin (RIF) (Rifadin, Lepetit, Milan, Italy) at a dose of 50 mg/kg/day for four consecutive days [6]; three individuals remained untreated and were used as the source of uninduced (UT) hepatic microsomes.

2.2. The isolation of microsomes and Western blotting

After sacrifice, the livers were removed and homogenised with 2 volumes of ice-cold buffer (20 mM Tris-HCl, 0.5 mM EDTA, 1.15% KCl, pH 7.4) by means of a Potter-Elvehjem apparatus. Homogenates from either untreated- or induced individuals were thereafter pooled and processed in order to get one batch of UT- and three batches of RIF-preparations, respectively. Microsomes were isolated by differential ultracentrifugation and stored frozen at –80 °C as detailed elsewhere [14]. Protein concentration was measured by the Lowry method [11] using bovine serum albumin (BSA) as the standard. In order to check P450 3A induction, solubilised microsomal proteins (10 µg) from uninduced or RIF-treated rabbits were separated by 10% sodium dodecylsulphate-polyacrylamide gel electrophoresis according to Laemmli [10]. The proteins were then blotted onto nitrocellulose sheets according to the method by Towbin et al. [21] and immunostained with an anti-rat P450 3A1/2 antibody (Biotec Centre, Orleans, France). The immunochemical levels of P450 3A1/2 were quantified by densitometry using the GelDoc System (Bio-Rad, Milan, Italy).

2.3. Spectral measurements

The P450 content was determined by using the dithionite-reduced carbon monoxide difference spectrum between 450 and 490 nm [20]. The *in vitro* formation of MI complexes by tiamulin, erythromycin, triacetyloleandomycin (TAO), roxithromycin, tylosin, tilmicosin and spiramycin was followed essentially as described by Nebbia et al. [13]. Typically, microsomes were diluted in an oxygen saturated buffer (Tris-HCl 50 mM, KCl 150 mM, MgCl₂ 10 mM, pH 7.4) to a protein concentration of 2–3 mg/mL and the suspensions were equally divided into two cuvettes and placed in a thermostatted spectrophotometer (UVIKON XL, Biotek, VM) at 37 °C. After a baseline was recorded, the reaction was started by the addition of the test drug to the sample cuvette and of an equal amount of solvent (methanol or water) to the reference cuvette. After allowing 2 min for equilibration, complex formation was initiated by the addition of NADPH 1 mM to both cuvettes and monitored by repetitive scanning between 500 and 400 nm with cycles of 3 min. After baseline subtraction, the extent of formed stable complexes showing a peak at around 455 nm was quantified using the following formula:

$$\frac{\Delta \text{absorbance units (spectrum peak}_{\text{nm}} - 490_{\text{nm}}) \times 1000}{\text{nmol P450}}$$

The kinetics of complex formation was followed using the same method in the time drive mode at the spectrum peak characteristic for each drug.

2.4. Assay of N-demethylase activities

The N-demethylation rate was measured on all drugs examined except for tiamulin by incubating 1 mg of microsomal protein with various substrates dissolved in ethanol or water at a final concentration of 0.5 mM and a NADPH generating system (NADP⁺ 0.39 mM, glucose 6-phosphate 7.9 mM and 2 units of glucose 6-phosphate

dehydrogenase in Tris-HCl 0.1 M buffer, pH 7.5). The amount of the released formaldehyde was determined with Nash reagent as detailed by Belman [2] after subtracting appropriate blanks run under the same conditions but containing the solvent alone.

2.5. Chemicals

Tiamulin hydrogen fumarate was a kind gift of Dr. Renger Witkamp (Zeist, The Netherlands) and tilmicosin was kindly provided by Ely Lilly Italia (Sesto Fiorentino, Italy). Unless otherwise stated, all other drugs and chemicals were purchased from Sigma Aldrich (Milan, Italy) except for BSA, glucose 6-phosphate, glucose 6-phosphate dehydrogenase and NADP⁺ that were from Roche Diagnostics (Milan, Italy).

2.6. Statistics

Where appropriate, statistical analyses were performed using one-way ANOVA followed by the Tukey multiple comparison post-test (GraphPad InStat, San Diego, CA, USA). Differences were considered significant at *P* values lower than 0.05.

3. RESULTS

Western blotting analysis revealed that the adopted protocol was effective in increasing liver P450 3A expression in RIF-treated rabbits. Indeed, as expressed in arbitrary units, the immunochemical levels averaged 0.165 for UT-microsomes and 1.275 for RIF-microsomes.

According to the results of preliminary studies (data not shown), proper complex formation occurred only with RIF-microsomes and was maximal at a drug concentration of 125 μM. Under these conditions, all drugs but spiramycin and tilmicosin gave rise to MI complexes, with a maximum peak in the wavelength range 449–455 nm (Fig. 1). As depicted in Table I, the

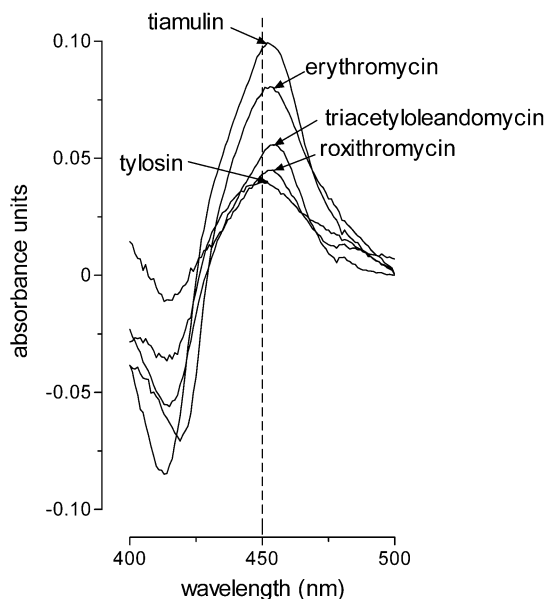


Figure 1. Representative in vitro metabolic intermediate complexes formed in hepatic microsomes from rifampicin pretreated rabbits by tiamulin and some macrolides at a concentration of 125 μ M. Absorption peaks were at 454 nm for triacetyloandomycin and roxithromycin, 453 nm for erythromycin, 452 nm for tiamulin, and 451 nm for tylosin.

ability in forming complexes was higher for tiamulin and erythromycin and comparably lower for TAO, roxithromycin, and tylosin. The kinetics of complex formation was examined for tiamulin, erythromycin, TAO and roxithromycin (Fig. 2): tiamulin showed a steeper rise than the other drugs and both TAO and erythromycin exhibited a short lag phase. In all cases, the plateau was reached after about 1 h.

The rate of N-demethylation of erythromycin in RIF-induced microsomes was about two fold higher ($P < 0.05$) than that of TAO and seven-fold higher than that of roxithromycin or tylosin; this metabolic reaction barely occurred with either tilmicosin or spiramycin (Fig. 3A) and was always of lower intensity (up to six fold) when measured in UT preparations in which only erythromycin and TAO displayed values higher than 0.5 nmol/min/mg protein (Fig. 3B).

4. DISCUSSION

In the present study the incubation of tiamulin or certain macrolides with hepatic

Table I. Extent of complex formation in hepatic microsomes from rifampicin pretreated rabbits.

Substrate	nmol complex/nmol P450
Tiamulin	20.93 \pm 3.42
Erythromycin	16.91 \pm 2.34
Triacetyloandomycin	11.02 \pm 0.58
Roxithromycin	10.29 \pm 2.68
Tylosin	9.67 \pm 1.66
Spiramycin	n.d.
Tilmicosin	n.d.

* Values are means \pm SEM ($n = 4$ to 7 assays for each substrate); n.d. not detectable.

rabbit microsomes from RIF-pretreated rabbits in the presence of NADPH gave rise to the typical MI complexes with an absorption peak at around 455 nm. Under the same conditions this did not occur with preparations from untreated individuals, thereby confirming the role played by P450 3A in the formation of such complexes with tiamulin and certain macrolides [3].

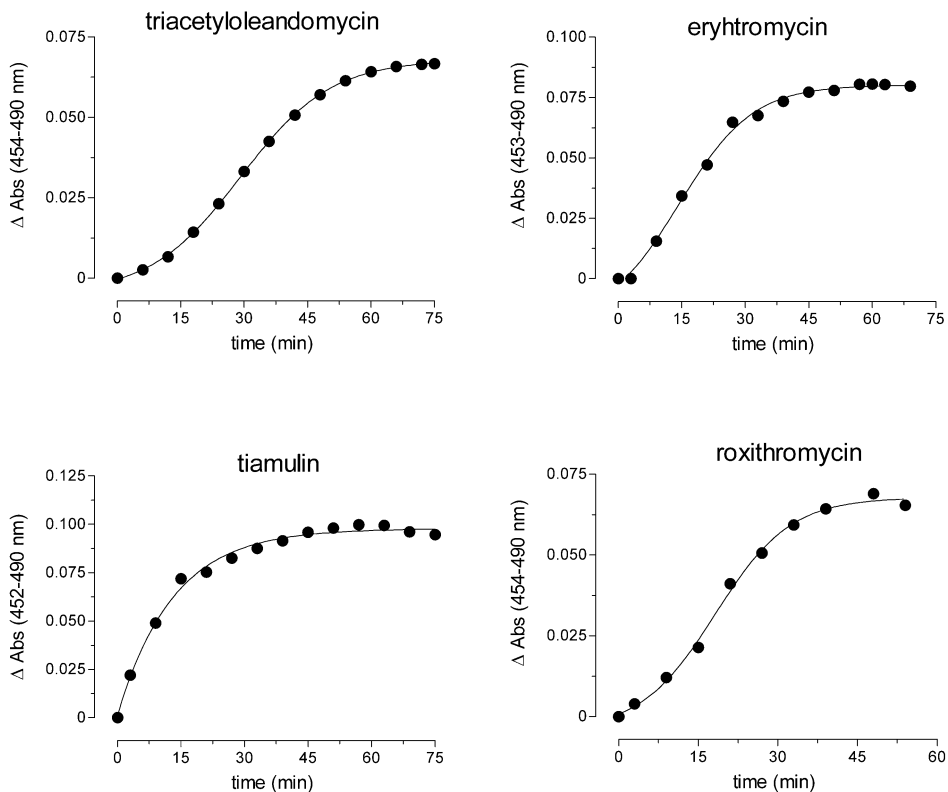


Figure 2. Kinetics of the in vitro metabolic intermediate complexes formation with tiamulin and some macrolides at a concentration of 125 μ M in liver microsomes from rifampicin pretreated rabbits.

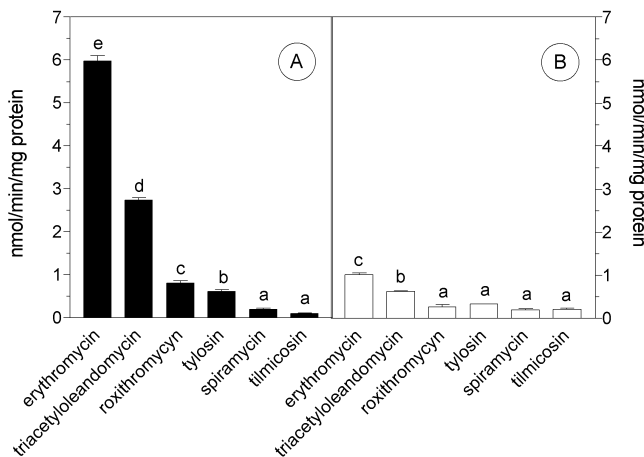


Figure 3. Rate of the in vitro N-demethylation of some macrolides (1 mM) in liver microsomes from rifampicin pretreated- (A) or untreated (B) rabbits. *N* = 4 to 6 determinations for each substrate. Bars with different superscripts differ significantly (*P* < 0.05).

Several factors are reported to influence the extent of complex formation *in vitro* including pH, cofactor and substrate concentration, and the amount of the P450 isozyme capable of being complexed [9, 19], so that it is difficult to compare the results obtained in different studies unless they are performed under similar experimental conditions. Nonetheless, in line with the results of an earlier investigation performed with liver microsomes from phenobarbital-pretreated goats [24], tiamulin and, to a lesser extent, erythromycin proved more active than TAO and tylosin in generating MI complexes, while either tilmicosin or spiramycin were apparently devoid of that property. Interestingly, tylosin has been regarded by other authors [1] as unable to produce P450 binding metabolites. Roxithromycin itself showed a weaker ability in complex formation than erythromycin, thereby matching what has been previously observed in human liver microsomes [23].

In our study most of the tested macrolides underwent an oxidative demethylation. On the whole, a good relationship was found between the ability to form MI complexes and the rate of N-demethylation, although this reaction represents only the first step in complex formation because, as mentioned above, it must be followed by a further oxidation of the N-demethylated metabolite [1]. Again, a similar trend was reported for TAO, erythromycin and roxithromycin in human liver microsomes [23].

To the best of our knowledge the formation of MI complexes has not been previously reported in liver rabbit microsomes for drugs other than erythromycin and TAO [9]. With few exceptions, the ability in forming such complexes *in vitro* exhibited by many amino drugs including tiamulin and certain macrolides may be substantially related to their inhibitory potency towards the P450-mediated biotransformation of compounds being substrates of the complexed P450s themselves [16, 22, 23, 24]. In this respect, it should be noted that liver

CYP 3A is one of the most versatile mammalian CYP isoforms being able to metabolise a large number of drugs and xenobiotics [12] and that it appears to be constitutively well expressed in rabbits [8, 15]. Taking into account that factors such as the route of exposure, the dosage and the concentration reached in liver may affect MI complex formation in living animals, our findings indicate that the potential for relevant drug-drug interactions (even at the residue level) might be expected in the rabbit mainly for tiamulin and erythromycin, and to a lesser extent for TAO, roxithromycin and tylosin, while the risk for tilmicosin and spiramycin should be reasonably very low. Owing to the extensive use of tiamulin and macrolides in rabbit-breeding industrial facilities, *in vivo* studies are in progress to assess the predictability of the results from the present work.

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