COMPARATIVE STUDIES OF IN VITRO INHIBITION OF CYTOCHROME P450 3A4-DEPENDENT TESTOSTERONE 6β-HYDROXYLATION BY ROXITHROMYCIN AND ITS METABOLITES, TROLEANDOMYCIN, AND ERYTHROMYCIN

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ABSTRACT:
Roxithromycin has been shown to be a relatively weak inhibitor of cytochrome P450 (P450 or CYP)-dependent drug oxidations, compared with troleandomycin. The potential for roxithromycin and its major metabolites found in human urine [namely the decladinosyl derivative (M1), O-dealkyl derivative (M2), and N-demethyl derivative (M3)] to inhibit testosterone 6β-hydroxylation after metabolic activation by CYP3A4 was examined and compared with inhibition by troleandomycin and erythromycin in vitro. Of roxithromycin and its studied metabolites, M3 was the most potent in inhibiting CYP3A4-dependent testosterone 6β-hydroxylation by human liver microsomes and was activated to the inhibitory P450Fe²⁺-metallobile complex to the greatest extent. Roxithromycin and its metabolites were N-demethylated by human liver microsomes, although the rates were slower than those measured with troleandomycin and erythromycin as substrates. Recombinant human CYP3A4 in a baculovirus system coexpressing NADPH-P450 reductase was very active in catalyzing the N-demethylation of roxithromycin, M1, and M2, as well as troleandomycin, erythromycin, and M3. The order for inhibition of CYP3A4-dependent testosterone 6β-hydroxylation activities by these macrolide antibiotics in the recombinant CYP3A4 system was estimated to be troleandomycin > erythromycin ≥ M3 ≥ M2 > M1 ≥ roxithromycin. Erythromycin, roxithromycin, and its metabolites all failed to inhibit CYP1A2-dependent (R)- warfarin 7-hydroxylation and CYP2C9-dependent (S)-warfarin 7-hydroxylation but did inhibit CYP3A4-dependent (R)- warfarin 7-hydroxylation. These results suggest that roxithromycin itself is not as potent an inhibitor of CYP3A4 activities as are troleandomycin and erythromycin, probably because of the slower metabolism of this compound to metabolites M1, M2, and M3 in humans.

Roxithromycin is a semisynthetic macrolide antibiotic that has been shown to have in vivo antibacterial activities similar to those of erythromycin but to have a longer duration of action and greater potency, compared with erythromycin (Gillum et al., 1993; Periti et al., 1992). Such pharmacokinetic differences are thought to be the result of differences in the bioavailabilities of these two chemicals in humans in vivo. Birkett et al. (1990) reported that the dose-normalized mean maximal plasma concentration for roxithromycin in 12 healthy men was approximately 5-fold greater than that for erythromycin and, as a result, the normalized AUC was 27-fold greater for roxithromycin than for erythromycin. Similar results have been reported by other groups (Koyama et al., 1988).

Roxithromycin has been shown to be metabolized in vivo to form several metabolites, including a decladinosyl derivative (M1), O-dealkyl derivative (M2), and N-monodemethyl derivative (M3), in rats, dogs, and humans (fig. 1) (Esumi et al., 1988; Koyama et al., 1988). In humans, M1, M2, and M3 have been shown to be excreted at levels of 1.2, 0.9, and 0.14% of the total dose, respectively, in urine collected between 0 and 48 hr after oral dosing with roxithromycin (Koyama et al., 1988). Small amounts (0.06% of the administered dose) of didemethylroxithromycin (M4), the oxidation product of M3, have also been detected in human urine (Koyama et al., 1988). The formation of these monomono and didemethylated metabolites of roxithromycin has been suggested to be mediated by P450 enzymes, particularly by CYP3A4/5 in humans (Delaforge et al., 1988; Yamazaki et al., 1996b; Tinel et al., 1989). In vitro experiments have suggested that CYP3A4 is a major enzyme involved in the N-demethylation of roxithromycin in human liver microsomes (Yamazaki et al., 1996a).

It was shown previously that roxithromycin inhibits the CYP3A4-dependent oxidation of testosterone and nifedipine to a lesser extent than do troleandomycin and erythromycin in human liver microsomal and recombinant human P450 systems in vitro (Yamazaki et al., 1996b). Because these latter two macrolides have been shown to cause inhibition of P450 catalytic activities by forming inhibitory P450Fe²⁺-metabolite complexes (Pessaye et al., 1982; Tinel et al., 1989; Delaforge et al., 1988), this difference may be the result of roxithromycin being metabolized relatively slowly by P450 enzymes in human liver microsomes in vitro (Yamazaki et al., 1996a,b). However, it was not determined in those studies whether the metabolites of roxithromycin are further activated by P450 enzymes to form active metabolites that inhibit P450-dependent drug oxidations in humans.

The present study was undertaken to determine whether three metabolites of roxithromycin found in vivo in humans (Koyama et al., 1988), namely decladinosylated roxithromycin (M1), O-dealkylated...
roxithromycin (M2), and N-demethylated roxithromycin (M3), inhibit CYP3A4-dependent testosterone 6β-hydroxylayion in human liver microsomes and recombinant systems. The formation of inhibitory P450-Fe²⁺-metabolite complexes from these metabolites was determined and compared with that measured using roxithromycin itself, troleandomycin, and erythromycin as substrates. N-Demethylation activities of these macrolide antibiotics were also determined in human liver microsomes. The effects of these macrolide antibiotics on (R)- and (S)-warfarin 7-hydroxylation by recombinant CYP1A2, CYP2C9, and CYP3A4 are reported.

Materials and Methods

Chemicals. Troleandomycin, erythromycin, and testosterone were purchased from Sigma Chemical Co. (St. Louis, MO). Roxithromycin and its oxidation products decladinolsylated roxithromycin (RU39001, M1), O-dealkylated roxithromycin (RU28111, M2), and N-demethylated roxithromycin (RU44981, M3) were generous gifts from Roussel Uclaf S.A. (Romainville, France). Other chemicals used were from the same sources as described previously or were obtained from local suppliers, at the highest qualities commercially available (Yamazaki et al., 1996b).

Enzyme Preparation. Human liver samples were obtained from organ donors or patients undergoing liver resection, as described previously (Shimada et al., 1994). Liver microsomes were prepared as described and suspended in 10 mM Tris-HCl buffer (pH 7.4) containing 1.0 mM EDTA and 20% (v/v) glycerol (Guengerich, 1994). Recombinant (bicistronic) human CYP2C9 and CYP3A4, in a baculovirus system that coexpresses rabbit NADPH-P450 reductase, were obtained from PanVera Co. (Madison, WI). Human (bicistronic) CYP1A2 in the baculovirus system (with human NADPH-P450 reductase) was obtained from Gentest Co. (Woburn, MA).

Enzyme Assays. Standard incubation mixtures (final volume, 0.25 ml) contained human liver microsomes (0.5 mg of protein/ml) or recombinant CYP3A4 (0.02 μM), an NADPH-generating system (consisting of 0.5 mM NADP⁺, 5 mM glucose-6-phosphate, and 0.5 unit/ml glucose-6-phosphate dehydrogenase), and 100 μM testosterone, in 100 mM potassium phosphate buffer (pH 7.4) (Yamazaki et al., 1996b). Reactions were started by the addition of NADP⁺, incubated at 37°C for 10 min, and terminated by the addition of CH₂Cl₂. Product formation was estimated by HPLC as described previously (Yamazaki et al., 1996b). (R)- and (S)-Warfarin 7-hydroxylation was determined under the same incubation conditions as described previously (Yamazaki and Shimada, 1997).

N-Demethylation of macrolide antibiotics by human liver microsomes were assessed by methods described previously (Yamazaki et al., 1996a). Briefly, macrolide antibiotics (1 mM) were incubated with liver microsomes (0.75 mg of protein/ml) in the presence of an NADPH-generating system. Incubations were conducted at 37°C for 10 min, and the formation of formylaldehyde was determined as described (Nash, 1953).

Inhibition Experiments. Two types of incubation conditions were used for the studies of the inhibition of CYP3A4-catalyzed oxidations of testosterone and (R)- and (S)-warfarin by macrolide antibiotics. In the first type, macrolide antibiotics were metabolized by human liver microsomal or recombinant P450 systems, in the presence of an NADPH-generating system, at 37°C for 20 min (preincubation). Preincubation mixtures were then mixed with the substrates and incubated at 37°C for 10 min, and the oxidation products of the substrates were determined. In the second type, macrolide antibiotics were added simultaneously with substrates to the human liver microsomal or recombinant P450 systems, and incubations were carried out at 37°C for 10 min for the determination of substrate oxidation. In both cases, control (100%) values were obtained in incubations without macrolides.

Spectral Studies. The formation of inhibitory P450-Fe²⁺-metabolite complexes by human liver microsomes was determined using troleandomycin, erythromycin, and roxithromycin and its metabolites as substrates (Larrey et al., 1983a; Franklin, 1991; Yamazaki et al., 1996b). Briefly, incubation mixtures (final volume, 2.0 ml) containing 1.0 mg of microsomal protein, 50 mM Tris-HCl (pH 7.4), 150 mM KCl, 10 mM MgCl₂, and 2 mM NADPH were divided into two cuvettes. After addition of 100 μM levels of the macrolide antibiotics to the sample cuvette (and an equal volume of solvent alone to the reference cuvette), the formation of P450-metabolite complexes was determined at 25°C by recording the increases in absorbance at 456 nm, using a Shimadzu UV-300 spectrophotometer.

Other Assays. P450 contents were estimated spectrally by the original method described (Omura and Sato, 1964). The contents of CYP3A4 in liver microsomes were estimated by coupled sodium dodecyl sulfate-polyacrylamide gel electrophoresis/immunochemical development (Western blotting) (Guengerich et al., 1982). The intensities of the immunoblots were measured with an Epson GT-8000 scanner equipped with the National Institutes of Health Image/Gel Analysis program adapted for Macintosh computers. Protein concentrations were estimated by the method of Lowry et al. (1951).

Results

Formation of Inhibitory P450-Fe²⁺-Metabolite Complexes after Incubation of Macrolide Antibiotics with Human Liver

![Chemical structures of troleandomycin (TAO), erythromycin (ERM), and roxithromycin (RXM) and its metabolites (M1, M2, M3, and M4).](image-url)
Effects of Macrolide Antibiotics on Testosterone 6β-Hydroxylation by Human Liver Microsomes. The effects of troleandomycin, erythromycin, and roxithromycin and its metabolites on the testoster-

Microsomes. Formation of inhibitory P450-Fe²⁺-metabolite complexes was determined in human liver microsomes (sample HL-4) using troleandomycin, erythromycin, and roxithromycin and its three metabolites as substrates (fig. 2). Troleandomycin was the most potent compound in producing the inhibitory P450-Fe²⁺-metabolite complex after metabolic activation by human liver microsomes, followed by erythromycin. Roxithromycin itself showed weak complex formation, but one of the metabolites (M3) caused significant complex production after metabolic activation. The other metabolites of roxithromy-

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one 6β-hydroxylation activities of human liver microsomes (sample HL-4) were examined when these antibiotics were added simultaneously with a substrate (fig. 3). As expected, troleandomycin caused a strong inhibition of testosterone 6β-hydroxylation activity, as did M3 to a lesser degree. Erythromycin, roxithromycin, M1, and M2 caused slight inhibition of testosterone 6β-hydroxylation, although to a lesser extent than did troleandomycin and M3.

The effects of preincubation of M3 with human liver microsomes, in the presence of an NADPH-generating system, on testosterone 6β-hydroxylation activities were determined (fig. 4). Testosterone 6β-hydroxylation activities were inhibited in a concentration-dependent manner by 10–100 μM M3, and these inhibitory effects were more pronounced when M3 was preincubated at 37°C for 20 min with human liver microsomes.

N-Demethylation of Macrolide Antibiotics by Human Liver Microsomes. Troleandomycin, erythromycin, and roxithromycin and its metabolites were added to liver microsomes of three human samples at 1 mM concentrations, and the formation of formaldehyde was determined (table 1). Total P450 and CYP3A4 levels for the three human samples are also included in table 1. CYP3A4 levels were the highest in sample HL-4, followed by HL-18 and HL-16. The N-demethylation activities for the six chemicals studied tended to be higher in sample HL-4, followed by HL-18 and HL-16. For the antibiotics examined, the order of N-demethylation activities of human liver microsomes was troleandomycin > erythromycin > M3 ≥ roxithromycin = M1 = M2.

Inhibition by Macrolide Antibiotics of Testosterone 6β-Hydroxylation by Recombinant CYP3A4. Recombinant CYP3A4, expressed in baculovirus
CYP3A4 caused greater inhibition of testosterone 6β-hydroxylation activities (fig. 3). In addition, the preincubation of macrolide antibiotics with recombinant CYP3A4 than in those using human liver microsomes suggested that the effects of macrolide antibiotics on testosterone 6β-hydroxylation activities were determined for these macrolide antibiotics after metabolic activation; these events have been implicated as being one of the mechanisms causing drug-drug interactions when macrolide antibiotics are administered simultaneously with other drugs to human patients (Lindstrom et al., 1993; Tinel et al., 1989; Gillum et al., 1993; Periti et al., 1992; Pessayre et al., 1982; Fisher et al., 1990).

Our previous studies of the effects of these antibiotics on CYP3A4 suggested that the order of potency with regard to the formation of 450-metabolite complexes by human liver microsomes is troleandomycin > erythromycin > roxithromycin (Yamazaki et al., 1996b).

The present studies showed that the N-demethylated product (M3) of roxithromycin was more potent in inhibiting CYP3A4-dependent testosterone 6β-hydroxylation by human liver microsomes than was the parent drug. The formation of P450-metabolite complexes with M3 was greater than with roxithromycin, M1, M2, or erythromycin.

Discussion

Several macrolide antibiotics, including troleandomycin, erythromycin, and roxithromycin, have been shown to inhibit CYP3A catalytic activities by forming inactive P450Fe2⁺-metabolite complexes after metabolic activation; these events have been implicated as being one of the mechanisms causing drug-drug interactions when macrolide antibiotics are administered simultaneously with other drugs to system, has been shown to be very active in drug monooxygenation (Shaw et al., 1997). We used this (bicistronic) CYP3A4 in studies of the effects of macrolide antibiotics on testosterone 6β-hydroxylation activities (fig. 5). The testosterone 6β-hydroxylation activity (~14 nmol/min/nmol of P450) of recombinant CYP3A4 was approximately 3-fold higher than that (~4 nmol/min/nmol of P450) of liver microsomes from HL-4, a liver sample that is high in CYP3A4 (approximately 50% of total P450 in the liver) (Shimada et al., 1994). The inhibitory effects of macrolide antibiotics on testosterone 6β-hydroxylation activities were more pronounced in experiments using recombinant CYP3A4 than in those using human liver microsomes (fig. 3). In addition, the preincubation of macrolide antibiotics with CYP3A4 caused greater inhibition of testosterone 6β-hydroxylation than was observed without preincubation in all cases. The inhibition potencies of these macrolide antibiotics were estimated to be troleandomycin > erythromycin > M3 ≈ M2 > M1 ≈ roxithromycin.

Dose-response curves for the inhibition of testosterone 6β-hydroxylation were determined for these macrolide antibiotics after metabolic activation by recombinant CYP3A4 (fig. 6). The results suggested that the effects of macrolide antibiotics on testosterone hydroxylation could be detected below 5 μM.

Effects of Macrolide Antibiotics on (R)- and (S)-Warfarin 7-Hydroxylation by Recombinant CYP1A2, CYP2C9, and CYP3A4.

Recombinant (bicistronic) CYP1A2, CYP2C9, and CYP3A4 systems were used in experiments to determine the effects of macrolide antibiotics on (R)- and (S)-warfarin 7-hydroxylation activities (fig. 7). (R)-Warfarin 7-hydroxylation by CYP1A2 and (S)-warfarin 7-hydroxylation by CYP2C9 were not inhibited by either erythromycin or roxithromycin or its metabolites M1, M2, and M3, although these macrolide antibiotics inhibited (R)-warfarin 7-hydroxylation by CYP3A4 to different extents.
but less than with troleandomycin. It has been reported that monomethylamine derivatives of macrolide antibiotics are generally more active in forming P450-metabolite complexes than are dimethylamine derivatives, in rat liver microsomes (Delaforge et al., 1983; Larrey et al., 1983b). These rat studies support our present view that M3 (roxithromycin monomethylamine) is activated by CYP3A4 to form complexes to a greater extent, compared with roxithromycin, M1, and M2, in human liver microsomes. In the present study, we also found that the order of macrolide N-demethylation activities in human liver microsomes was troleandomycin > erythromycin > M3 = roxithromycin = M1 = M2.

Recombinant systems expressing both CYP3A4 and NADPH-P450 reductase (bicistronic systems) in insect cells have been found to be very active in catalyzing the metabolism of macrolide antibiotics to active metabolites that inhibit testosterone 6β-hydroxylation. In this system, six macrolide antibiotics were found to be inhibitors of testosterone 6β-hydroxylation activities; in all cases, preincubation of the macrolide antibiotics with the CYP3A4 system caused a strong inhibition of the activities, suggesting that CYP3A4 itself catalyzes the biotransformation of these antibiotics to reactive metabolites that are harmful to the enzyme (Delaforge et al., 1983; Franklin, 1991). In this assay system, the order of potencies with respect to the inhibition of testosterone 6β-hydroxylation was relatively similar to that found in the liver microsomal system.

Erythromycin and roxithromycin and its metabolites failed to inhibit the (R)-warfarin 7-hydroxylation activities of recombinant (bicistronic) human CYP1A2 and the (S)-warfarin 7-hydroxylation activities of recombinant (bicistronic) CYP2C9 in insect cells. However, these antibiotics inhibited, to varying extents, the (R)-warfarin 7-hydroxylation and testosterone 6β-hydroxylation catalyzed by bicistronic CYP3A4. These results support the view that the major enzyme participating in the metabolism of these macrolide antibiotics in human liver microsomes is CYP3A4/5.

Koyama et al. (1988) reported that humans metabolize roxithromycin at very slow rates in vivo. When one dose of 300 mg of roxithromycin was administered orally to healthy volunteers, the excretion of M1, M2, M3, and M4 metabolites into 48-hr urine samples was only 1.2, 0.92, 0.14, and 0.06% of the total roxithromycin dose administered, respectively (Koyama et al., 1988). Those investigators also reported that the maximal plasma level of roxithromycin was 4.3-fold higher than that of erythromycin when these antibiotics were administered separately to volunteers as single oral doses of 150 mg. Birkett et al. (1990) reported that repeated oral dosing of erythromycin (250 mg, every 6 hr for 5 days) caused a 2–3-fold increase in the AUC.

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References


Henderson CR, Wrighton SA (1993) Pseudomonas aeruginosa cytochrome P-450 metabolite intermediate complexes to a greater extent, compared with roxithromycin, M1, and M2, in human liver microsomes. In the present study, we also found that the order of macrolide N-demethylation activities in human liver microsomes was troleandomycin > erythromycin > M3 = roxithromycin = M1 = M2.

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In conclusion, the present study shows that the N-demethylamine derivative (M3) of roxithromycin is a more potent inhibitor of CYP3A4, after activation by CYP3A4 itself, than is the parent drug roxithromycin. Formation of inhibitory P450–Fe3+–metabolite complexes is shown to be required for the inhibitory action of M3 and other macrolide antibiotics used in this study. The previous hypothesis, from rat studies, that formation of a nitrosoualkane derivative that attacks the P450 enzyme is critical for potent inhibition is consistent with the results of this study using human enzyme preparations. The weak abilities of roxithromycin to inhibit CYP3A4 may be the result of the slower metabolism of this compound to M3 and other metabolites in humans.