AN IN VITRO STUDY ON THE METABOLISM AND POSSIBLE DRUG INTERACTIONS OF ROKITAMYCIN, A MACROLIDE ANTIBIOTIC, USING HUMAN LIVER MICROSONES

XUE-JUN ZHAO,1,2 ERIKO KOYAMA,1 AND TAKASHI ISHIZAKI1,3

Department of Clinical Pharmacology, Research Institute, International Medical Center of Japan, Tokyo, Japan

(Received October 29, 1998; accepted March 1, 1999)

This paper is available online at http://www.dmd.org

ABSTRACT:

This in vitro study was designed to identify the enzyme(s) involved in the two major metabolic pathways of rokitamycin [formations of leucymycin A7 (LMA7) from rokitamycin and of leucymycin V (LMV) from LMA7] and to assess possible drug interactions using human liver microsomes. Formation of LMA7 or LMV was NADPH-independent. Anti-rt NATDPH cytochrome P-450 (CYP) reductase serum, specific inhibitors, or substrates of CYP isoforms showed no effects on the formation of LMA7 or LMV. The mean $V_{\text{max}}$ and $K_m$ for the formation of LMA7 from rokitamycin were much greater ($P < .01$) than those for the formation of LMV from LMA7.

Two esterase inhibitors, bis-nitro-phenylphosphate and physostigmine ($100 \mu M$), inhibited the formation of LMA7 or LMV by more than 85%, whereas no appreciable inhibition occurred by several substrates of carboxylesterase (EC 3.1.1.1). Except the moderate inhibition produced by promethazine and terfenadine, theophylline, mequitazine, chlorpheniramine, and diphenhydramine showed little or no inhibition for the formation of LMA7 or LMV. Rokitamycin, LMA7, LMV, erythromycin, and clarithromycin showed somewhat little or no inhibition for the formation of LMA7 or LMV.

This in vitro study was designed to identify the enzyme(s) involved in the two major metabolic pathways of rokitamycin [formations of leucymycin A7 (LMA7) from rokitamycin and of leucymycin V (LMV) from LMA7] and to assess possible drug interactions using human liver microsomes. Formation of LMA7 or LMV was NADPH-independent. Anti-rt NATDPH cytochrome P-450 (CYP) reductase serum, specific inhibitors, or substrates of CYP isoforms showed no effects on the formation of LMA7 or LMV. The mean $V_{\text{max}}$ and $K_m$ for the formation of LMA7 from rokitamycin were much greater ($P < .01$) than those for the formation of LMV from LMA7.

Two esterase inhibitors, bis-nitro-phenylphosphate and physostigmine ($100 \mu M$), inhibited the formation of LMA7 or LMV by more than 85%, whereas no appreciable inhibition occurred by several substrates of carboxylesterase (EC 3.1.1.1). Except the moderate inhibition produced by promethazine and terfenadine, theophylline, mequitazine, chlorpheniramine, and diphenhydramine showed little or no inhibition for the formation of LMA7 or LMV. Rokitamycin, LMA7, LMV, erythromycin, and clarithromycin showed somewhat little or no inhibition for the formation of LMA7 or LMV. Rokitamycin, LMA7, LMV, erythromycin, and clarithromycin inhibited the CYP3A4-catalyzed triazolam $\alpha$-hydroxylation with IC$_{50}$ ($K_I$) values of $5.8 (2.0), 40, 33 (20), and 58 (43) \mu M$, respectively. It is concluded that the formations of LMA7 from rokitamycin and of LMV from LMA7 are catalyzed mainly by human esterase enzyme [possibly cholinesterase (EC3.1.1.8)]. However, whether rokitamycin would inhibit the CYP3A-mediated drug metabolism in vivo requires further investigations in patients.

Rokitamycin, a kitasamycin derivative, is a 16-membered ring macrolide antibiotic that has not only a more potent antibacterial activity but also a wider spectrum than other macrolides (Morohoshi et al., 1984). Rokitamycin is also relatively safe and active against Gram-positive and some Gram-negative bacteria, Mycoplasma, and Campylobacter spp (Burnie and Matthews, 1985; Hara, 1987). Unlike other macrolides that tend to be absorbed only when gastric juice is acidic, rokitamycin is absorbed even when gastric juice is hypoacidic or anacidic (Morishita et al., 1984b). Thus, it is considered to be a suitable antibiotic for the treatment of infectious respiratory diseases in the elderly whose gastric juice is often hypoacidic. In addition, the use of macrolide antibiotics has recently been extended to the treatment of Helicobacter pylori infection, which is a common cause of gastritis and peptic ulcers (Walsh and Peterson, 1995; Penston and Welfare and by a postdoctoral fellowship training program from the Bureau of McColl, 1997). Thus, these drugs, including rokitamycin, may concomitantly be administered with other clinically relevant drugs, thereby possibly causing some drug-drug interactions in humans.

The pharmacokinetic profiles of rokitamycin have been studied both in animals and humans (Morishita et al., 1984b; Sakai et al., 1988; Suzuki et al., 1987a,b). It has been reported that rokitamycin is rapidly metabolized in vivo into leucymycin A7 (LMA7),4 leucymycin V (LMV), 10'-OH-rokitamycin, and 14'-OH-rokitamycin (Fig. 1; Morishita et al., 1984a; Goto et al., 1984; Morishita et al., 1987). LMA7 and LMV are considered to be the major metabolites of rokitamycin in humans (Morishita et al., 1984b). All the metabolites have been reported to possess some antibacterial activity (Goto et al., 1984). In addition, rokitamycin was administered orally and rapidly absorbed with no accumulation in humans (Morishita et al., 1984b). The elimination half-life of rokitamycin is about 2 h in humans (Morishita et al., 1987).

To our knowledge, however, no study has been conducted with respect to the metabolism of rokitamycin and the specific enzyme(s) involved in its metabolic pathways in vitro by using human liver microsomes. In addition, the kinetic behavior of rokitamycin metabolism and rokitamycin-drug interactions have not been elucidated. Thus, the aims of this in vitro study were: 1) to investigate and compare the formation kinetics of LMA7 from rokitamycin and LMV from LMA7; 2) to identify specific enzyme(s) involved in these two major metabolic pathways of rokitamycin; and 3) to examine the

4 Abbreviations used are: LMA7, leucymycin A7; LMV, leucymycin V; CYP, cytochrome P-450; BNPP, bis-p-nitrophenyl phosphate; SKF-525A, 2-diethylaminoethyl-2,2-diphenylvalerate; FMO, flavin-containing monoxygenase.
possible rokitamycin-drug interactions in vitro using human liver microsomes

Materials and Methods

**Chemicals and Reagents.** Rokitamycin, LMA7, LMV, mequitazine, theophylline, and clarithromycin were provided by Asahi Chemical Industry Co., Ltd. (Tokyo, Japan). bis-p-Nitrophenyl phosphate (BNPP), physostigmine, terfenadine, erythromycin, ketoconazole, cimetidine, 2-diethylaminoethyl-2,2-diphenylvalerate (SKF-525A), phenacetin, acetaminophen, and desipramine were purchased from Sigma Chemical Co. (St. Louis, MO). Triazolam and its metabolite (α-hydroxytriazolam) were supplied by Nihon Pharmacia Upjohn Co. (Tokyo, Japan). 2-Hydroxydesipramine was a generous gift from Ciba-Geigy (Basel, Switzerland). Chlorpheniramine, diphenhydramine, promethazine, quinidine, clofibrate, 4-nitrophenylacetate, p-nitrophenylpropionate, α-naphthoflavone, coumarin, and p-nitrophenol were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) and sulfaphenazole was obtained from Meiji Yakuhin Co. (Tokyo, Japan). NADP$^+$, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase were obtained from Oriental Yeast (Tokyo, Japan). Racemic mephenytoin was kindly donated by Dr. Küpfner (University of Bern, Bern, Switzerland). S- and R-Mephenytoin were separated from racemic mephenytoin on a Chiralcel OJ column (10 μm, 4.6 × 250 mm; Daicel Chemical Co. Ltd., Tokyo, Japan) according to the method of Yasumori

![Diagram of metabolic pathways of rokitamycin in humans.](https://example.com/diagram.png)

**Fig. 1.** Metabolic pathways of rokitamycin in humans.

The thick arrows indicate the major metabolic pathways.
et al. (1990). Diclofenac, 4-hydroxydiclofenac, antiserum, and preimmune serum for human NADPH cytochrome P-450 (CYP) reductase were obtained from Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan). Acetonitrile, methanol, and other reagents of analytical grade were purchased from Wako Pure Chemical Industries Ltd. Other chemicals required for the study were purchased from Sigma Chemical Co.

Preparation of Microsomal Fractions. Human liver samples were obtained from six Japanese patients who underwent a partial hepatectomy for metastatic liver tumor(s) in the Division of General Surgery, International Medical Center of Japan, Tokyo, as reported previously (Chiba et al., 1993; Zhao et al., 1996; Zhao and Ishizaki, 1997b). The liver parenchyma of the nontumor-bearing part used for the study was shown later to be histopathologically normal in all the cases. Use of human samples for the study had been approved by the Institutional Ethics Committee of the International Medical Center of Japan.

Microsomal preparations from human liver tissues used herein were made according to standard procedures as described previously (Chiba et al., 1993; Echizen et al., 1993). After the determination of protein concentration (Lowry et al., 1951), the individual microsomal samples were aliquoted, frozen, and stored at −80°C until used.

Assay for Rokitamycin Metabolism with Human Liver Microsomes. Microsomal fractions were incubated in the absence or presence of an NADPH-generating system at 37°C for 5 to 10 min in test tubes. The incubation mixture consisted of 0.05 to 0.1 mg/ml microsomal protein, 100 mM potassium phosphate buffer (pH 7.4), 0.1 mM EDTA, and 0.5 to 200 μM rokitamycin or 1.0 to 600 μM LMA7, in a final volume of 250 μl. All reactions were performed in the linear range with respect to protein concentration and incubation time. After the reaction was stopped by addition of 100 μl of ice-cold acetonitrile, 25 μl of nitratrexam (25 μM in methanol) was added to the samples as an internal standard for assaying LMA7 or LMV. The mixture was centrifuged at 10,000 x g for 10 min and 50 μl of supernatant was injected onto an HPLC apparatus as described below.

HPLC Conditions. The formations of LMA7 from rokitamycin and of LMV from LMA7 were determined in the incubation mixture by an HPLC method using UV detection. The HPLC system consisted of a model L-7200 pump (Hitachi Ltd., Tokyo, Japan), a model L-7400 UV detector (Hitachi), a model L-7200 autosampler (Hitachi), a model D-7500 integrator (Hitachi), and a 4.6-× 250-mm CAPCELL PAK C18 UG120 column (Shiseido Co., Tokyo, Japan). Column temperature was maintained at 30°C with a model SM-05 water circulator (Taisei, Tokyo, Japan). The mobile phase consisted of a 33:67 (v/v) mixture of acetonitrile and 0.01 M potassium phosphate buffer, containing 5 mM 1-heptane sulfonic acid and phosphoric acid (4 ml in 2000-ml mobile phase). The mobile phase was delivered at a flow rate of 1.0 ml/min. The eluate was monitored at a wavelength of 229 nm. Interm- and intra-assay coefficients of variation for each procedure (n = 6) were <10% and the lowest limits of detection for both LMA7 and LMV, defined as the lowest concentration with a signal-to-noise ratio of 10, were 0.1 μM.

Assays for phenacetin O-deethylation (CYP1A2), diclofenac 4′-hydroxylation (CYP2C9), desipramine 2-hydroxylation (CYP2D6), and triazolam α-hydroxylation (CYP3A4) were carried out according to the respective HPLC assay methods, as reported elsewhere (Kronbach et al., 1989; Leemann et al., 1992; Tassaneeyakul et al., 1993a; Chiba et al., 1994).

Kinetics of Formations of LMA7 from Rokitamycin and LMV from LMA7. Preliminary results indicated that the formation rates of both LMA7 and LMV were linear at 37°C for incubation time up to 30 min and for microsomal protein concentration up to 0.25 mg/ml at the substrate rokitamycin or LMA7 concentration of 50 μM. Accordingly, the kinetic studies were performed at 37°C with an incubation time of 5 to 10 min at a microsomal protein concentration of 0.05 to 0.1 mg/ml.

Because the formations of LMA7 and LMV by microsomes obtained from six human livers occurred monophasically, being consistent with a simple Michaelis-Menten kinetic behavior, the one-component enzyme kinetic parameters (Km, Vmax, and Vm/Km without the numerical subindices) for the formation of LMA7 from rokitamycin (0.5–200 μM) and the formation of LMV from LMA7 (1.0–600 μM) were estimated by using the linear regression analysis of unweighted raw data. The kinetic parameters were estimated initially by the graphic analysis of Eadie-Hofstee plots and the values obtained were used as the first estimate for the nonlinear least-squares regression analysis, MULTI (Yamaoka et al., 1981), in which unweighted raw data were fitted to the model equation.

Inhibition Study. Specific inhibitors or substrates of human CYP isoforms used were α-naphthoflavone for CYP1A (Kunze and Trager, 1993), coumarin for CYP2A6 (Wrighton and Stevens, 1992), ketoconazole for CYP3A4 (Watkins et al., 1985; Newton et al., 1995), sulfaphenazole for CYP2C9 (Goldstein and de Morais, 1994), S-mephentoin for CYP2C19 (Goldstein and de Morais, 1994), quinidine for CYP2D6 (Kobayashi et al., 1989), as well as p-nitrophenol for CYP2E1 (Tassaneeyakul et al., 1993b). In addition, two nonspecific human CYP inhibitors, citimidine (Somogyi and Muirhead, 1987) and SKF-525A (Rossi et al., 1987), were also used for testing the possible inhibition for the formations of LMA7 from rokitamycin and LMV from LMA7 in four different human liver microsomes. The esterase inhibitors or substrates used were BNNP, physostigmine (latismirskiaia et al., 1997), clofibrate, 4-nitrophenoxyacetate, p-nitrophenylpropionate, procaine, caffeine, aspirin, and enalapril (Williams, 1985; Ishizaki et al., 1988; Hosokawa et al., 1995; Kamendulis et al., 1996). The concentration (5 μM) of substrate rokitamycin or LMA7 was chosen according to the usual therapeutic concentration attained in human blood (Morishita et al., 1984b) as well as the mean apparent Km values obtained from six liver microsomes tested. Rokitamycin or LMA7 was incubated with or without one of the inhibitor or substrate probes for CYP isoforms or esterases, at concentrations ranging from 1.0 to 1000 μM, under the incubation conditions as described earlier. The effects of each compound on the formation of LMA7 or LMV at the respective inhibitor or substrate probe concentration were compared with the control values determined from the incubation of rokitamycin or LMA7 alone and the inhibition values were expressed as a percentage of the respective control values. The inhibitory potency of the respective substrates/inhibitors was defined by IC50 (i.e., a 50% inhibition of LMA7 or LMV formation compared with the control values). For the identification of IC50 values, experiments were performed with microsomal preparations obtained from four different human livers.

Immunoinhibition Study. Anti-rat NADPH CYP reductase serum was used in this part of the study as an antiserum for human NADPH CYP reductase. According to the product instructions of Daiichi Pure Chemicals Co., Ltd. (Tokyo, Japan) as well as from a previous study from our laboratory (Zhao and Ishizaki, 1997a), this antiserum (50 μl) significantly (by more than 70%) inhibited human CYP reductase and potentially inhibited the metabolism of several different substrates of the respective human CYP isoforms including CYP3A4 and 2D6 in human liver microsomes.

Pooled microsomes (0.05–0.1 mg protein/ml) obtained from six different human livers were first incubated for 30 min at room temperature in the absence or presence of anti-CYP antisera (25 and 50 μl) to permit an antigen-antibody complex formation. The substrate (rokitamycin or LMA7) was then added; the assay conditions were the same as those described above.

Effects of Drugs on Rokitamycin Metabolism. Several drugs that have been used for the treatment of chronic obstructive pulmonary diseases (e.g., chronic bronchial asthma) and may be coadministered with rokitamycin in other clinical settings were assessed for their possible inhibitory effects on the formations of LMA7 from rokitamycin and LMV from LMA7 by using human liver microsomes. The tested drugs included theophylline and five H2-receptor antagonists—metizquatein, chlorpheniramine, diphenhydramine, promethazine, and terfenadine.

All of the drugs tested herein were dissolved in methanol. The inhibitory potency of the respective substrates/inhibitors was defined by IC50 and by the apparent Ki values, where appropriate. To identify the respective IC50 values, various drug concentrations ranging from 0 to 1000 μM were chosen and both rokitamycin and LMA7 concentrations were set at 5 μM, which is their near-therapeutic concentration in human plasma (Morishita et al., 1984b). For determination of the apparent Ki values, rokitamycin or LMA7 was set at different concentrations of 5, 10, 25, 50, 75, and 100 μM and the test drug concentrations were used ranging from 0 to 400 μM. Fifty microliters of each drug dissolved in methanol was evaporated to dryness before addition of the other reaction constituents. Pooled microsomes obtained from six different human livers were used to identify the apparent Ki values, which were determined graphically from the Dixon plot analysis (Dixon and Webb, 1964). Four different microsomal samples were used for determining the IC50 values. In all cases, the inhibited activities were compared with those from the respective control incubations.

778 ZHAO ET AL.
Effects of Macrolide Drugs on CYP Enzyme Activities. Macrolide drugs, including rokitamycin and its two active metabolites (LMA7 and LMV), erythromycin, and clarithromycin, were used to test the possible inhibition for the respective specific substrates of four human CYP isoforms (i.e., phenacetin O-deethylation for CYP1A2, diclofenac 4'-hydroxylation for CYP2C9, desipramine 2-hydroxylation for CYP2D6, and triazolam α-hydroxylation for CYP3A4). For determination of the IC50 values, the concentrations of these macrolide drugs ranging from 0 to 1000 μM were used and those of the probe drugs phenacetin, diclofenac, desipramine, and triazolam, were set at 10, 10, 10, and 50 μM, respectively. For determination of Ks values, triazolam was set at four different concentrations of 12.5, 25, 50, and 100 μM, whereas the tested drug concentrations (i.e., rokitamycin, LMA7, LMV, clarithromycin, and erythromycin) were used ranging from 0 to 100 μM.

Statistics. All numerical values are expressed as the mean ± S.D. throughout the text. The difference of kinetic parameters (i.e., Ks, Vmax, and Vmax/Km) between the formations of LMA7 from rokitamycin and LMV from LMA7 was compared by using a Student’s t test. P < .05 was considered statistically significant.

Results

Rokitamycin Metabolism. Two major metabolic pathways of rokitamycin (i.e., formations of LMA7 from rokitamycin and LMV from LMA7) were examined by using human liver microsomes. Preliminary studies revealed that the formation of LMA7 or LMV was NADPH-independent. Moreover, the addition of anti-rat NADPH CYP reductase serum (up to 50 μg) to the incubation mixture did not show any inhibition for the formation of LMA7 or LMV (data not shown). To further identify the possible role of CYP isoforms involved in these two metabolic pathways of rokitamycin, several relatively specific inhibitor or substrate probes of CYP isoforms (i.e., α-naphthoflavone, 1.0 μM for CYP1A2; coumarin, 100 μM for CYP2A6; sulfaphenazole, 100 μM for CYP2C9; S-mephenytoin, 100 μM for CYP2C19; quinidine, 2.0 μM for CYP2D6; p-nitrophenol, 100 μM for CYP2E1; ketoconazole, 2.0 μM for CYP3A4; and cimetidine, 100 μM for several CYP isoforms) were used to perform an inhibition study in the presence of an NADPH-generating system. However, no appreciable inhibition was observed by the addition of any of the respective inhibitor or substrate probes of CYP isoforms used (data not shown). These results strongly suggest that both the formations of LMA7 from rokitamycin and LMV from LMA7 are not catalyzed by human CYP isoform(s) and/or other NADPH-dependent enzymes(s) like flavin-containing monooxygenase (FMO).

Kinetic Study. Because rokitamycin metabolism was NADPH-independent, we performed a kinetic study in the absence of the NADPH-generating system. The typical Eadie-Hofstee and Michaelis-Menten plots for the formations of LMA7 from rokitamycin and LMV from LMA7 are shown in Fig. 2. In all of the human liver microsomes used, the Eadie-Hofstee plots for the formation of LMA7 or LMV exhibited apparently monophasic behavior, suggesting that a single enzyme may be involved in the metabolism of rokitamycin in human liver microsomes. Accordingly, a simple Michaelis-Menten kinetic analysis (i.e., one-enzyme kinetic approach) was used to estimate the kinetic parameters (i.e., Ks, Vmax, and Vmax/Km). The individual and mean kinetic parameters for the two metabolic pathways of rokitamycin obtained from six different human liver microsomes are listed in Table 1. The mean intrinsic clearance (defined as Vmax/Km) in the formation of LMA7 from rokitamycin was about 35-fold greater (P < .01) compared with that obtained from the formation of LMV from LMA7.

Inhibition Study. Because our preliminary experiments have shown not only that both the formations of LMA7 and LMV were not catalyzed by human CYP isoform(s) or by other NADPH-dependent enzyme(s), but also that heat denaturation (boiled microsomes) reduced the production of LMA7 from rokitamycin by human liver microsomes to zero, several inhibitors or substrates of esterases (see Materials and Methods) were used to perform an inhibition study with human liver microsomes. The effects of coincubation with the inhibitors of esterases on the formation of LMA7 or LMV are shown in Fig. 3. Two esterase inhibitors, BNPB and physostigmine (Iatsumi-Kaia et al., 1997), inhibited the formations of LMA7 and LMV in a concentration-related manner, with the mean IC50 values of 38 and 14 μM for LMA7 and 28 and 4.6 μM for LMV, respectively (Fig. 3).
The mean maximum inhibition produced by BNPP and physostigmine (100 μM) on the formation of LMA7 or LMV was more than 85% (Fig. 3). However, several substrates of carboxylesterase used (i.e., 4-nitrophenylacetate, p-nitrophenylpropionate, clofibrate, procaine, caffeine, aspirin, and enalapril) showed no appreciable inhibition for the formation of LMA7 or LMV (data not shown). Surprisingly, a nonspecific inhibitor of human CYP isoforms, SKF-525A (Rossi et al., 1987), dramatically inhibited the formations of both LMA7 and LMV in a concentration-related manner (Fig. 4), with the mean IC₅₀ values of 24 μM for LMA7 and 15 μM for LMV.

**Effects of Drugs on Rokitamycin Metabolism.** Possible metabolic drug interactions between rokitamycin and six other drugs that may be coadministered with rokitamycin in certain clinical settings were assessed by using human liver microsomes separately. The mean results are listed in Table 2, indicating that the two histamine H₁-receptor antagonists, promethazine and terfenadine, moderately inhibited the formation of LMA7 or LMV in human liver microsomes, with the respective mean IC₅₀ (Kᵢ) values of 83 (35) and 237 (87) μM for the formation of LMV from LMA7, respectively (Table 2). However, theophylline and three other H₁-receptor antagonists, mequitazine, chlorpheniramine, and diphenhydramine, showed little or no inhibition for the formations of both LMA7 and LMV (data not shown). Two typical Dixon plots for the inhibition of LMA7 production by promethazine and terfenadine are shown in Fig. 5.

**Effects of Macrolide Drugs on CYP Enzyme Activities.** The potential inhibitory effects of macrolide derivatives (i.e., rokitamycin, LMA7, LMV, erythromycin, and clarithromycin) on drug metabolism were evaluated by determining the IC₅₀ and Kᵢ values for metabolic reactions that are selectively catalyzed by four different CYP isoforms in human liver microsomes (Table 3 and Fig. 6). All of the five tested derivatives did not show any appreciable inhibition of phenacetin O-deethylation (CYP1A2), diclofenac 4'-hydroxylation (CYP2C9), and desipramine 2-hydroxylation (CYP2D6), with the mean IC₅₀ values greater than 500 μM (except LMV, which inhibited desipramine 2-hydroxylation with the mean IC₅₀ of 437 μM; Fig. 6, A-C). However, an inhibition of the CYP3A4-catalyzed α-hydroxytriazolam formation from triazolam was observed by addition of rokitamycin, LMA7, erythromycin, and clarithromycin (Fig. 6D), with the mean IC₅₀ (Kᵢ) values of 5.8 (2.0), 40, 33 (20), and 56 (43) μM, respectively (Table 3). The typical Dixon plots for the inhibition of triazolam α-hydroxylation

---

**TABLE 1**

<table>
<thead>
<tr>
<th>Human Liver Microsomes</th>
<th>Formation of LMA7 from Rokitamycin</th>
<th>Formation of LMV from LMA7</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Kₘ</td>
<td>Vₐₘₐₓ</td>
</tr>
<tr>
<td></td>
<td>μM</td>
<td>nmol/mg/min</td>
</tr>
<tr>
<td>HL-31</td>
<td>5.23</td>
<td>18.28</td>
</tr>
<tr>
<td>HL-32</td>
<td>7.01</td>
<td>28.60</td>
</tr>
<tr>
<td>HL-33</td>
<td>4.86</td>
<td>31.82</td>
</tr>
<tr>
<td>HL-34</td>
<td>5.33</td>
<td>32.29</td>
</tr>
<tr>
<td>HL-39</td>
<td>3.93</td>
<td>22.97</td>
</tr>
<tr>
<td>HL-40</td>
<td>2.65</td>
<td>12.21</td>
</tr>
<tr>
<td>Mean</td>
<td>4.84**</td>
<td>24.36**</td>
</tr>
<tr>
<td>± S.D.</td>
<td>1.46</td>
<td>8.04</td>
</tr>
</tbody>
</table>

**P < .01** compared with the respective mean data obtained from the formation of LMV from LMA7.

---

**FIG. 3.** Effects of BNPP and physostigmine on the formation of LMA7 from rokitamycin (A) and the formation of LMV from LMA7 (B) in human liver microsomes.

The plotted data are the mean ± S.D. for microsomes obtained from four different human livers.
by rokitamycin, erythromycin, and clarithromycin are shown in Fig. 7, A–C, respectively.

Discussion

The current in vitro study is the first to investigate the kinetic behavior of rokitamycin metabolism and to identify the enzyme involved in the metabolic pathways of rokitamycin, as well as to examine the possible rokitamycin-drug interactions with human liver microsomes. The results of this study provided in vitro evidence that both the formations of LMA7 from rokitamycin and LMV from LMA7 are mediated via human liver esterase(s) (possibly cholinesterase), but not by human CYP enzyme or other NADPH-dependent enzyme(s) (e.g., FMO). Furthermore, the present study suggested that rokitamycin and its two major metabolites (LMA7 and LMV) lacked inhibitory effects on the activities of CYP1A2, CYP2C9, and CYP2D6 (except for a high concentration of LMV, ~450 μM, Fig. 6C), but rokitamycin showed a relatively potent inhibition on CYP3A4-mediated triazolam α-hydroxylation in human liver microsomes (Fig. 6D). Thus, the likelihood of an in vivo interaction between rokitamycin and CYP3A4-metabolized drugs cannot be negated when the clinically relevant concentrations of rokitamycin in humans are taken into consideration as discussed below.

Although several macrolide antibiotics (e.g., erythromycin, troleandomycin, and clarithromycin) are catalyzed by human CYP enzyme(s), particularly CYP3A (Guengerich, 1994; Rodrigues et al., 1997), the major metabolism (formations of LMA7 and LMV) of rokitamycin was not metabolized via human CYP(s). This was shown by the NADPH-independent formations of LMA7 and LMV as well as by the lack of inhibition of the metabolic formation by anti-rat NAPDH CYP reductase serum. In addition, several specific inhibitor/substrate probes of human CYP isoforms showed no appreciable inhibition for rokitamycin metabolism. Although rokitamycin did inhibit CYP3A4-mediated triazolam α-hydroxylation, this in vitro observation does not imply that rokitamycin is metabolized by CYP3A4. It is possible for a compound to be metabolized by one particular enzyme and interact in a noncatalytic fashion with another enzyme.
as shown for the interaction between CYP2D6 and quinidine (Guengerich et al., 1986).

On the other hand, both BNPP, an inhibitor of carboxylesterases (EC 3.1.1.) and cholinesterases (EC 3.1.1.8, closely related to carboxylesterases) and physostigmine, a specific inhibitor of cholinesterases (Latsimirskaia et al., 1997), strongly suppressed the formation of LMA7 or LMV in microsomal incubations (Fig. 3). However, other substrates of carboxylesterases did not show any appreciable inhibition of rokitamycin metabolism. These observations suggest that one of the esterases (possibly cholinesterases) appears to be the main key enzyme involved in the formations of LMA7 from rokitamycin and LMV from LMA7 in human liver microsomes. However, whether other extrahepatic human tissue(s) also has (have) the ability to catalyze rokitamycin metabolism remains to be clarified, because esterases (e.g., cholinesterases) exist in several human tissues (nervous system, liver, and intestinal mucosa) as well as in the systemic circulation (Williams, 1985). In addition, a study (Tunek and Svensson, 1988) has shown that physostigmine is a systemic nonspecific inhibitor of cholinesterases and inhibits both butyrylcholine esterase and acetylcholinesterase. Nevertheless, we could not conduct the study using these specific cholinesterases. Thus, the specific cholinesterase(s) enzyme involved in the metabolism of rokitamycin could not be established in the present study.

A dramatic inhibition for the formation of LMA7 or LMV was observed by a nonspecific inhibitor of CYP isoforms, SKF-525A (Rossi et al., 1987; Fig. 4), but not by cimetidine (data not shown). However, it seems to be unnecessary to consider that rokitamycin metabolism is catalyzed by human CYP enzymes(s), because our several findings presented in this study do not support this contention as discussed above. In addition, SKF-525A is also able to inhibit aldehyde oxidase (Yoshihara and Tatsumi, 1985; Stoddart and Levine, 1993), an enzyme other than CYP isoforms. However, whether SKF-525A would be an inhibitor and/or substrate of esterases (e.g., cholinesterases) obviously remains to be scrutinized in future studies, although our data suggest this possibility. Similarly, whether the metabolism of rokitamycin would be catalyzed by aldehyde oxidase also remains to be elucidated.

Limited drug-rokitamycin interaction studies have been reported. Cazzola et al. (1991) showed that rokitamycin did not significantly alter the pharmacokinetics of theophylline, a substrate of CYP1A2 (Guengerich, 1994), in humans. However, an in vitro study with single human liver microsomes (Marre et al., 1993) revealed that rokitamycin inhibited CYP3A-mediated cyclosporin A metabolism with a \( K_i \) value of 30 \( \mu \)M, which was lower than that obtained from erythromycin (57 \( \mu \)M) or roxithromycin (113 \( \mu \)M). These data are generally in agreement with our findings that rokitamycin and erythromycin inhibited CYP3A-mediated triazolam \( \alpha \)-hydroxylation with the respective \( K_i \) values of 2.0 and 20 \( \mu \)M (Table 3). However, a marked difference in the \( K_i \) values (2.0 versus 30 \( \mu \)M for rokitamycin and 20 versus 57 \( \mu \)M for erythromycin) between our study and the Marre et al. (1993) study was observed. Although the reason for these discrepant findings remains entirely obscure, it may be explained in part by the different substrates (triazolam versus cyclosporin A) for

**TABLE 3**

<table>
<thead>
<tr>
<th>Macrolides</th>
<th>IC(_{50}) ( \mu )M</th>
<th>( K_i ) ( \mu )M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rokitamycin</td>
<td>5.8 ± 1.0(^b)</td>
<td>2(^c)</td>
</tr>
<tr>
<td>LMA7</td>
<td>40 ± 6(^d)</td>
<td>ND</td>
</tr>
<tr>
<td>LMV</td>
<td>33 ± 6(^d)</td>
<td>20(^d)</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>56 ± 5(^d)</td>
<td>43(^d)</td>
</tr>
</tbody>
</table>

\(^a\) Triazolam concentration was set at 50 \( \mu \)M for determination of IC\(_{50}\).

\(^b\) The IC\(_{50}\) values were expressed as the mean ± S.D. obtained from three different human liver microsomes.

\(^c\) The \( K_i \) values were determined by Dixon plots with a pooled microsomal sample obtained from six different human livers.

\(^d\) Interfered with the assay and therefore undetectable.

ND, not determined.

Rokitamycin concentrations were set at 5 \( \mu \)M (□), 25 \( \mu \)M (■), 50 \( \mu \)M (○), and 100 \( \mu \)M (●).

![Fig. 5. Dixon plots for the formation of LMA7 from rokitamycin by promethazine (A) and terfenadine (B) in a pooled human microsomal sample obtained from six different livers.](image_url)

FIG. 5. Dixon plots for the formation of LMA7 from rokitamycin by promethazine (A) and terfenadine (B) in a pooled human microsomal sample obtained from six different livers.

![Graph](image_url)

**TABLE 3**

<table>
<thead>
<tr>
<th>Macrolides</th>
<th>IC(_{50}) ( \mu )M</th>
<th>( K_i ) ( \mu )M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rokitamycin</td>
<td>5.8 ± 1.0(^b)</td>
<td>2(^c)</td>
</tr>
<tr>
<td>LMA7</td>
<td>40 ± 6(^d)</td>
<td>ND</td>
</tr>
<tr>
<td>LMV</td>
<td>33 ± 6(^d)</td>
<td>20(^d)</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>56 ± 5(^d)</td>
<td>43(^d)</td>
</tr>
</tbody>
</table>

\(^a\) Triazolam concentration was set at 50 \( \mu \)M for determination of IC\(_{50}\).

\(^b\) The IC\(_{50}\) values were expressed as the mean ± S.D. obtained from three different human liver microsomes.

\(^c\) The \( K_i \) values were determined by Dixon plots with a pooled microsomal sample obtained from six different human livers.

\(^d\) Interfered with the assay and therefore undetectable.

ND, not determined.
CYP3A chosen as well as by the different human liver microsomes used between these two studies.

The normal plasma or more importantly the hepatic concentration of an inhibitor in patients has been used to predict in vivo drug interactions. It is generally believed that unbound concentration of an inhibitor around the metabolic enzyme in the liver is one of key factors determining the extent of drug interactions in vivo (Ito et al., 1998). Because it is difficult to directly measure the unbound concentration in the liver, the plasma-unbound concentration at the entrance to the liver was used in the prediction (Ito et al., 1998). In addition, the use of liver versus plasma concentrations of an inhibitor to predict in vivo potential for inhibition has successfully been conducted by von Moltke et al. (1994). In humans, a single oral dose of rokitamycin (200 mg) results in a peak plasma concentration of 0.49 μg/ml (Morishita et al., 1987). Because the in vivo plasma protein binding rate of rokitamycin is about 70% (Morishita et al., 1987), the plasma-unbound concentration of rokitamycin is estimated to be about 0.15 μg/ml (0.18 μM). However, the hepatic tissue concentration of rokitamycin found in rats is 10-fold greater than that in plasma (Morishita et al., 1984a), as rokitamycin is a lipophilic drug (Morishita et al., 1987). Assuming that the concentration of rokitamycin determined in rat liver tissue would also apply to humans, the usual single oral dose (200 mg) of rokitamycin will result in a maximal rokitamycin liver concentration of about 1.8 μM, which is close to the $K_i$ value (2 μM) of rokitamycin obtained from the present study for the inhibition of CYP3A-mediated triazolam $\alpha$-hydroxylation in hu-

Fig. 6. Effects of five macrolide derivatives on four CYP activities with human liver microsomes.

Data are expressed as the mean ± S.D. from microsomes obtained from three different human livers. In D, the data from LMV could not be plotted because of the interference with the assay of $\alpha$-hydroxytriazolam (Table 3).
man liver microsomes. Thus, we assume that a rokitamycin-drug interaction might occur in humans in vivo when rokitamycin is coadministered with substrate drugs for CYP3A4. However, a clinical study is necessary to establish the extent of interactions between rokitamycin and drugs that are CYP3A4 substrates.

Although promethazine and terfenadine moderately inhibited the formations of rokitamycin and drugs that are CYP3A4 substrates.

In conclusion, our in vitro data have shown that the formations of rokitamycin and its two metabolites (LMA7 and LMV) with the $K_i$ values ranging from 22 to 87 nM (Table 2), these inhibitions should not occur in vivo when the peak plasma concentrations of promethazine ($\sim$20 nM) and terfenadine ($\sim$3.2 nM) attained after their usual oral therapeutic doses (25 and 60 mg, respectively) are taken into consideration (Periti et al., 1992; Gillum et al., 1995). On the other hand, rokitamycin and its two metabolites (LMA7 and LMV) showed no appreciable inhibition for CYP1A2-mediated phenacetin O-deethylation (Fig. 6A), which is consistent with the clinical finding that rokitamycin did not appear to alter the pharmacokinetics of theophylline, a substrate of CYP1A2 (Guengerich, 1994), in patients (Cazzola et al., 1991). Likewise, drugs that are metabolized mainly by human CYP2C9 or CYP2D6 isofrom may also not be affected by rokitamycin because our data indicated that rokitamycin had no inhibitory effects on CYP2C9-mediated diclofenac 4′-hydroxylation (Fig. 6B) and CYP2D6-mediated desipramine 2-hydroxylation (Fig. 6C). However, until more studies, particularly in vivo, are performed with rokitamycin, caution should be taken when prescribing this macrolide antibiotic in combination with any of the CYP3A4-metabolized drugs (e.g., immunosuppressants, H$_2$-receptor antagonists, carbamazepine, Ca-antagonists, triazolobenzodiazepines), commonly involved in drug interactions with macrolides in patients (Periti et al., 1992; Gillum et al., 1993; Amsden, 1995; von Rosentiel and Adam, 1995).

In conclusion, our in vitro data have shown that the formations of LMA7 from rokitamycin and LMV from LMA7 are catalyzed by human liver esterase(s) (possibly cholinesterase), but not by human CYP enzyme(s) or FMO. Because rokitamycin, like other macrolide antibiotics (e.g., erythromycin), inhibited the activity for CYP3A4 (i.e., triazolam $\alpha$-hydroxylation) in human liver microsomes, the likelihood of an in vivo interaction between rokitamycin and CYP3A4-metabolized drugs at its clinically relevant concentrations in humans cannot totally be negated and requires further investigation in patients.

Acknowledgments. We thank Dr. Meizo Kusaka for his help in the initial setup of the experiments, Dr. A. Küpper for the donation of racemic mephenytoin, and Huan Medical University, China, for supporting Dr. Xue-Jun Zhao’s research and training in Japan.


