STUDIES ON THE CYTOCHROME P450 (CYP)-MEDIATED METABOLIC PROPERTIES OF MILOCAMYCIN: EVALUATION OF THE POSSIBILITY OF A METABOLIC INTERMEDIATE COMPLEX FORMATION WITH CYP, AND IDENTIFICATION OF THE HUMAN CYP ISOFORMS

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ABSTRACT:

Some macrolide antibiotics cause clinical drug interactions, resulting in altered metabolism of concomitantly administered drugs, via the formation of a metabolic intermediate (MI) complex with cytochrome P450 (CYP). In this study, the possibility of MI complex formation by miocamycin (MOM) was assessed first. CYP contents and activities in rat liver microsomes were not affected and there were no detectable MI complexes after administration of MOM for either 3 or 10 days to rats. Furthermore, MOM did not form MI complexes in vitro even with microsomes from humans or dexamethasone-pretreated rats. Second, in vitro studies were conducted to identify the human CYP isoforms involved in the 14-hydroxylation reactions in the MOM metabolic pathway. The results showed that it was most likely CYP3A4 involved in the hydroxylations: 1) each hydroxylation in human liver microsomes from 10 different donors strongly correlated with testosterone 6β-hydroxylation; 2) each hydroxylation was essentially inhibited by ketoconazole and troleandomycin; 3) only cDNA-expressed CYP3A4 and CYP3A5 catalyzed the hydroxylations, and the activities of CYP3A5 were below 5% of those of CYP3A4; and 4) the apparent KM values obtained with native human liver microsomes were comparable with those obtained with cDNA-expressed CYP3A4. In conclusion, MOM is not an inhibitor of CYP via the formation of an MI complex. Moreover, CYP3A4 is mainly responsible for catalyzing the hydroxylation of MOM metabolites. Because CYP3A4 is the most abundant form of CYP in the liver and intestine, this isozyme probably accounts for the majority of drug-MOM interactions observed in clinical practice.

It is well known that macrolide antibiotics can interact adversely with commonly used drugs because in numerous occasions they are coadministered with other drugs. The metabolism of macrolide is, in most cases, mediated by cytochrome P450 (CYP)13A4, the most abundant CYP isoform in the liver. Several studies on the mechanism of these drug interactions have been carried out (Periti et al., 1992; von Rosenstiel and Adam, 1995). Fourteen-membered macrolides are known for their interaction with CYP through the formation of a metabolic intermediate (MI) complex. An important group of macrolide antibiotics including troleandomycin (TAO), erythromycin (EM), and clarithromycin (CAM) form MI complexes (Pessayre et al., 1982; Larrey et al., 1983; Periti et al., 1992). After being metabolized, these compounds form complexes with the iron(II) present in the heme group of CYP. In the absorption spectrum, the complex exhibits a peak at around 456 nm. The MI complex is enzymatically inactive and unable to bind carbon monoxide. Formation of this MI complex is thought to be initiated by CYP-dependent N-demethylation, causing these macrolides to behave as irreversible mechanism-based inhibitors. However, 16-membered macrolides [midecamycin (MDM), spiramycin, and josamycin] generally do not form the MI complex (Miura et al., 1989). It is believed that various factors, including lipophilicity, steric hindrance, and pH, play a role in MI complex formation. In fact, clinical drug interactions of 16-membered macrolides occur less frequently than those of 14-membered macrolides (Miura et al., 1989). Although it is less frequent, 16-membered macrolides also exhibit adverse interactions, which may be related to competitive inhibition of CYP3A4-mediated metabolism of coadministered drugs (Couet et al., 1990a,b, 1991).

Miocamycin (MOM) is a diacetylated derivative of MDM, a 16-membered macrolide antibiotic. MOM exerts potent antibacterial effects against Gram-positive and anaerobic bacteria, influenza bacilli, Mycoplasma, etc. (Mayama et al., 1990). Moreover, its in vivo antibacterial activity is stronger and more persistent than expected from its in vitro effects (Kawaharajo et al., 1981).

The metabolic pathway of MOM and the chemical structures of its metabolites are shown in Fig. 1. The first step in the metabolic pathway of MOM is 4-depropionylation, followed by spontaneous migration of an acyl group from the 3′ to the 4′ position, forming Mb-1. The next step is decylation of the acetyl group at the 4′ position and/or of that at the 9 position, forming Mb-2, Mb-12, and Mb-6. These three metabolites are hydroxylated at the 14 position to form the (R) or (S) epimers (Mb-3, Mb-5, Mb-9a, and Mb-13). These hydroxylations at the 14 position may be mediated by CYP.

The possible interaction of MOM with other drugs has never been

1 Abbreviations used are: CYP, cytochrome P450; MI, metabolic intermediate; MOM, miocamycin; TAO, troleandomycin; EM, erythromycin; CAM, clarithromycin; MDM, midecamycin; DMSO, dimethyl sulfoxide.

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examined. Therefore, the purpose of this study was to identify possible mechanisms of drug interaction when MOM was administered concomitantly with other drugs. For this purpose, we investigated whether MOM forms the MI complex in rats and in humans, and attempted to identify the CYP enzyme(s) that catalyzes the hydroxylation of MOM metabolites at the 14 position.

Materials and Methods

**Chemicals.** MOM and MDM were synthesized by Meiji Seika Kaisha, Ltd. (Tokyo, Japan), and used after purification. MB-2, MB-3, MB-5, MB-6, MB-9a, MB-12, and MB-13 were isolated from urine of rats and humans, rat bile, and reaction mixture of rat liver homogenate by Meiji Seika Kaisha, Ltd. (Shomura et al., 1982). Taisho Pharmaceutical Co. Ltd. (Tokyo, Japan), and used after purification. 2

**Preparation of Microsomes.** Rats were anesthetized by diethyl ether and livers were removed. Livers were perfused with ice-cold saline and weighed. Other rats received dexamethasone (50 mg/kg, i.p., daily for 3 days) and were also sacrificed 24 h after the last dosing. Other rats received dexamethasone (50 mg/kg, i.p., daily for 3 days) in 1% Tween 80 (5 ml/kg) were administered not only for 3 days but also for 10 days. Rats were sacrificed 24 h after the last dosing. Macrolides and 1% Tween 80 for control rats were administered not only for 3 days but also for 10 days. Rats were sacrificed 24 h after the last dosing.

**Measurement of Testosterone Hydroxylation.** Microsomal testosterone hydroxylation was determined as described by Funae and Imaoka (1987), with minor modifications. Incubation was carried out at 37°C in a final volume of 0.5 ml that consisted of 0.25 mg/ml of microsomal protein for rat or 0.1 mg/ml for human, testosterone, and NADPH generating system containing 6 mM magnesium chloride, 1 mM β-NAD⁺, 10 mM glucose 6-phosphate, and 0.7 U/ml glucose 6-phosphate dehydrogenase. Hydroxylation in individual liver microsomes from control rats and macrolide-pretreated rats were measured. For this experiment, testosterone was added to a final concentration of 250 μM. Moreover, the direct effects of MOM, its metabolites (MB-2, MB-6, and MB-12), or reference compounds on microsomal testosterone hydroxylation were determined. For this experiment, testosterone was added to a final concentration of 100 μM. The following compounds were used as the effector at various concentrations: MOM (1–100

**In Vivo Formation of MI Complexes.** The uncomplexed CYP, total CYP, and cytochrome b₅ contents were determined by the method of Omura and Sato (1964a,b). This technique was performed with untreated microsomes for uncomplexed CYP and with microsomes treated with 50 μM potassium ferricyanide, to dissociate the MI complex, for total CYP (complexed CYP cannot bind carbon monoxide). The complexed CYP absorbance was measured at around 456 nm in a microsomal suspension containing 2 mg protein/ml. Microsomes were placed in two cuvettes. Then 50 μM potassium ferricyanide was added to the reference cuvette to dissociate the MI complex. The wavelength scan between 370 and 500 nm was recorded on a UV-2200A spectrophotometer (Shimadzu Corporation, Kyoto, Japan). The complexed CYP was calculated with a molar extinction coefficient of 64 cm/mM (Franklin, 1991). A content of the complexed CYP was expressed as the percentage of total CYP.

**In Vitro Formation of MI Complexes.** Human liver microsomes, Pooled HepatoSomes [HBI Pool version 1.0 (12 donors)] were purchased from Human Biologics International (HBI, Scottsdale, AZ). The in vitro formation of MI complexes was measured in a microsomal suspension containing 2 mg protein/ml. Microsomes were placed in two cuvettes maintained at 37°C, which had been saturated with oxygen. Then the macrolide was added to a final concentration of 300 μM to the sample cuvette. The reaction was initiated by adding NADPH (2 mM) to both cuvettes and incubated for 15 min by gassing the cuvette contents with oxygen for 10 s at 5-min intervals. After incubation for 15 min, the complexed CYP was calculated as described for the in vivo study.

**Preparation of Microsomes.** Rats were anesthetized by diethyl ether and livers were removed. Livers were perfused with ice-cold saline and weighed. Then a 20% (w/v) homogenate was prepared using 100 mM potassium phosphate buffer containing 1.15% potassium chloride (pH 7.4). The homogenate was centrifuged at 600 g for 10 min. The supernatant was centrifuged at 12,500 g for 20 min, and the supernatant was recentrifuged at 105,000 g for 60 min. The microsomal pellet was suspended in the same buffer and centrifuged again at 105,000 g for 60 min. The washed pellet was resuspended in 100 mM potassium phosphate buffer (pH 7.4). Microsomal protein concentration was measured by the method of Lowry et al. (1951). The microsomes were stored at −80°C until use.

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µM), Mb-2, Mb-6, or Mb-12 (3–1000 µM), ketoconazole (0.003–10 µM), cimetidine (10–3000 µM), and terfenadine (1–300 µM). The maximum concentration of MOM, Mb-2, Mb-6, or Mb-12 was the highest concentration at which it could be dissolved. The substrates, except cimetidine and most other compounds, were dissolved in dimethyl sulfoxide (DMSO). The volume of DMSO added to the incubation mixtures was 1% of the incubation volume. In case of cimetidine, this was 0.5% of the incubation volume. An equivalent quantity of DMSO was added to the control incubation mixtures. IC50 values were determined by linear regression (% of control) against the logarithmic compound concentration.

2 α, β, 6 β, 7 α, ε α, ε β, and ε δ-β-hydroxytestosterone were separated and quantified by HPLC. HPLC analysis was performed with a CAPCELL PAK C18 UG120 (4.6 × 250 mm) column (SHISEIDO Co., Ltd., Tokyo, Japan), which was attached to a CAPCELL PAK C18 UG120 (4.0 × 10 mm) guard column.

The column was developed by a linear gradient from an aqueous solution of 40% methanol to 70% methanol from 0 to 30 min, at a flow rate of 1 ml/min. The column temperature was kept at 60°C, and the eluate was monitored at 254 nm. Hydrocortisone acetate was used as the internal standard.

**Measurement of Ethoxyresorufin O-Demethylation.** Microsomal ethoxyresorufin O-demethylation was determined fluorometrically by monitoring the rate of resorufin formation as described by Burke et al. (1985), with minor modifications. Reactions were carried out using 2 ml of the incubation mixture, which consisted of 0.025 mg/ml of microsomal protein, 5 µM ethoxyresorufin, and the NADPH generating system. A stock solution of the substrate was prepared by dissolving it in DMSO. The reaction was started by adding the substrate, and the reaction was monitored at 37°C in an FP-750 spectrofluorometer (JASCO Corporation, Tokyo, Japan). The excitation and emission wavelengths were set at 530 and 585 nm, respectively. The amount of product formed was calculated from the increase in fluorescence with time, using resorufin as the reference.

**Measurement of Aniline p-Hydroxylation.** Microsomal aniline p-hydroxylation was determined spectrophotometrically by monitoring the rate of aniline formation at 630 nm as described by Imai et al. (1966), with minor modifications. Reactions were carried out using 0.5 ml of the incubation mixture, which consisted of 1 mg/ml of microsomal protein, 5 mM aniline, and the NADPH generating system. The reaction was started by adding the NADPH generating system, and the mixture was incubated for 20 min.

**Statistical Analysis.** The results were expressed as the mean ± S.D. In the analysis of two groups, unpaired Student's t test was performed using a StatView (Abacus Concepts, Inc., Berkeley, CA).

**In Vitro MOM-Hydroxylated Metabolism by Human Liver Microsomes.** Human liver microsomes, Pooled HepatoSomes, and HepatoScreen Test Kit (10 donors) were purchased from Human Biologics International. Individual human liver microsomes (Lot no. HHM-0149, 0224, 0228, 0232, and 0234) were purchased from the International Institute for the Advancement of Medicine (IIAM, East, PA).

The final assay volume was 0.25 ml and consisted of the following at the indicated final concentrations: 100 mM potassium phosphate buffer (pH 7.4), 1 mM EDTA, 10 to 500 µM substrate (Mb-2, Mb-6, or Mb-12), microsomal protein, and NADPH generating system. A stock solution of substrate was prepared by dissolving it in acetone. After preincubation for 5 min at 37°C, the reaction was initiated by adding the microsomal protein, and the reaction mixture was incubated at 37°C for 20 min. The reaction was terminated by adding 0.2 ml of acetone and 50 µl of 40 µM DDM in acetone as the internal standard. The sample was centrifuged at 18000g, 4°C for 10 min. After evaporation of the supernatant under nitrogen at 40°C, the residue was dissolved in 0.1 ml of water and filtered by 4 mm Millex-HV (pore size: 0.45 µm) (Nihon Millipore Ltd., Tokyo, Japan).

**In Vitro MOM-Hydroxylation Metabolism by Microsomes Containing cDNA-Expressed Human CYP Proteins.** Microsomes prepared from human B-lymphoblastoid cells (AHH-1 TK/+) containing cDNA-expressed CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9-Cys, CYP2C9-Arg, CYP2C19, CYP2D6-Val, CYP2E1, or CYP3A4 were purchased from Gentest Corporation (Woburn, MA). Except for CYP1A1, CYP 1A2, CYP 2B6, and CYP2C19, all microsome preparations contained additional (cDNA-expressed) NADPH-CYP reductase. These reactions were carried out without agitation at 37°C for 20 min in 1.5-ml polypropylene centrifuge tubes in a final volume of 0.25 ml, as described for native human liver microsomes. For control reactions, microsomes prepared from human B-lymphoblastoid cells containing NADPH-CYP reductase or the selectable plasmid vector without the cDNA insert, were used.

Kinetic studies were carried out using microsomes prepared from B-lymphoblastoid cells and baculovirus-infected BTI-TN-SB1-4 cells, which contained overexpressed CYP2C19, CYP3A4, or CYP3A5 (Supersomes; Gentest). All insect microsome preparations contained additional (cDNA-expressed) NADPH-CYP reductase. The microsomes containing CYP3A4 contained not only coexpressed NADPH-CYP reductase but also cytochrome b5.

**HPLC Analysis of MOM Metabolites.** HPLC analysis of MOM-hydroxylated metabolites (Mb-3, Mb-5, Mb-9a, and Mb-13) was performed with an Inertis C18 (4.6 × 250 mm) column (GL Science Inc., Tokyo, Japan), which was attached to an Inertis C18 (4.0 × 10 mm) guard column cartridge. The column was developed by a linear gradient from acetonitrile/50 mM sodium acetate (pH 6.5) 35:65 to 45:55 from 0 to 20 min, and then with a linear gradient to 80:20 from 20 to 35 min, at a flow rate of 1 ml/min. The column temperature was kept at 40°C, and the eluate was monitored at 231 nm.

**Kinetic Analysis.** Kinetic parameters (apparent Kd and Vmax) values for the formation of MOM-hydroxylated metabolites were analyzed by Eadie-Hofstee plots of reaction velocity (v) against v/substrate concentration. The reaction was carried out under condition that was linear with respect to protein concentration and time of incubation.

**Univariate Regression Analysis.** The MOM-hydroxylated metabolites formation was determined in human liver microsomes prepared from 10 different organ donors (HepatoScreen Test Kit, Human Biologics International). Each hydroxylation was compared with known CYP marker activities. The following CYP marker activities were used: caffeine 3-demethylation (CYP1A2), coumarin 7-hydroxylation (CYP2A6), tolbutamide methyl-hydroxylation (CYP2C8/9), S-mephenytoin 4′-hydroxylation (CYP2C19), dextromethorphan O-demethylation (CYP2D6), chlorzoxazone 6-β-hydroxylation (CYP2E1), and testosterone 6-hydroxylation (CYP3A4/5). Pearson’s correlation coefficients (r) were determined by linear regression. The statistical significance of the correlation was denoted by *p < .01.

**CYP Isoform-Selective Inhibitors.** A series of alternative inhibitors were incubated in the presence of Mb-2, Mb-6, or Mb-12 to determine which compounds could inhibit MOM-hydroxylated metabolism. The following inhibitors, used at the designated concentrations, have been shown to selectively inhibit various CYP isoforms: α-naphthoflavone (1 µM) for CYP1A2; sultaphenazole (10 µM) for CYP2C8/9; S-mephenytoin (400 µM) for CYP2C19; quinidine (1 µM) for CYP2D6; 4-methylpyrazole (1 µM) for CYP2E1; and ketoconazole (0.5 µM) or TAO (100 µM) for CYP3A4/5. All inhibitors were dissolved in methanol. The volume of methanol added to the reaction mixtures was 0.5% of the reaction volume to avoid possible effects of the solvent on metabolism. Reaction mixtures containing TAO were preincubated with microsomes at 37°C for 15 min, and the reactions were initiated by adding the substrate. Except for reactions containing TAO, the other reactions were conducted without preincubation.

**Effects of Various Drugs on Formation of MOM-Hydroxylated Metabolites in Human Liver Microsomes.** Various drugs were incubated with Mb-2, Mb-6, or Mb-12 to determine which drugs could inhibit the formation of MOM-hydroxylated metabolites. The following drugs were used at various concentrations: bromocriptine (0.625–20 µM), carbamazepine (125–1000 µM), cyclosporine A (0.625–20 µM), diazepam (0.2–200 µM), dihydroergotamine (0.313–10 µM), disopyramide (39–10,000 µM), miconazole (0.625–20 µM), nifedipine (12.5–200 µM), terfenadine (3.125–100 µM), and theophylline (12.5–400 µM). All drugs were dissolved in methanol. The volume of methanol added to the reaction mixtures was 0.5% of the reaction volume. IC50 values were determined by linear regression (% of control) against the logarithmic drug concentration.

**Results**

**In Vivo Formation of MI Complexes after Repeated Doses of Various Macrolides to Rats.** The effects of the administration of various macrolides (0.557 mmol/kg, p.o. daily for 3 or 10 days) on rat liver CYP content and activity were compared in Tables 1 and 2.
After MOM was administered for either 3 or 10 days, the content of total CYP was not modified and MI complexes were not detectable (Table 1). After the administration of TAO, EM, or CAM, however, the content of total CYP increased, and part of that CYP was in the form of MI complexes. The levels of MI complexes increased in the following order: CAM ⩽ EM ⩽ TAO. After the administration of each macrolide, the content of cytochrome b₅ did not change (Table 1).

After MOM was administered for either 3 or 10 days, the selective monoxygenase activities for each CYP isoform were not modified, regardless of treatment of microsomes with potassium ferricyanide (Table 2). In contrast, after 3 days of treatment of rats with TAO hydroxylation of testosterone at the 2 β, 6 β, and 16 β position and aniline p-hydroxylation increased 7.3, 3.1, 3.9, and 1.3 times, respectively, in microsomes treated with potassium ferricyanide. When the microsomes were not treated with potassium ferricyanide, the levels of hydroxylations were 33, 41, 43, and 59% of that observed after treatment with potassium ferricyanide, respectively. The difference in activities between treatments with and without potassium ferricyanide indicated that activity was inhibited by formation of MI complexes. Therefore, it was obvious that CYP3A2, CYP2B1, and CYP2E1 were induced by TAO, and part of these CYP was in the form of MI complexes. The degree of induction was the largest for CYP3A2. Ethoxyresorufin O-deethylation was not modified by the administration of TAO and treatment with potassium ferricyanide (Table 4). On the other hand, 6 β-hydroxylation in human liver microsomes was clearly inhibited by 6-M or more, and the rate of caused by each of them was similar or lower than that caused by cinmetidine. In contrast, the inhibitory effect of MOM was relatively strong (IC₅₀ was 11.6 μM) and it was similar to that of terfenadine.

**Kinetics of MOM-Hydroxylated Metabolites Formation in Human Liver Microsomes.** In human liver microsomes, analysis of the data yielded a single apparent $K_M$ and $V_{max}$ (Table 5), which indicated that the formation of MOM-hydroxylated metabolites (Mb-3, Mb-5, Mb-9a, and Mb-13) conformed to a monophasic Michaelis-Menten kinetics and that there was no evidence of substrate or product inhibition. The $K_M$ values of Mb-3, Mb-5, and Mb-13 were similar (11.3–17.8 μM). However, that of Mb-9a was relatively high (46.6 μM). This result
TABLE 2
Effects of repeated doses of various macrolides on CYP activities of rat liver microsomes

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Testosterone Hydroxylation*</th>
<th>Ethynorsusarin O-deethylationb</th>
<th>Aniline p-hydroxylationc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 α (CYP2C11)</td>
<td>2 β (CYP3A2)</td>
<td>6 β (CYP3A2)</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>3 days</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.371 ± 0.243</td>
<td>1.371 ± 0.240</td>
<td>0.422 ± 0.146</td>
</tr>
<tr>
<td>TAO</td>
<td>0.192 ± 0.075*</td>
<td>0.198 ± 0.073*</td>
<td>1.033 ± 0.147*</td>
</tr>
<tr>
<td>Control</td>
<td>1.456 ± 0.300</td>
<td>1.464 ± 0.350</td>
<td>0.424 ± 0.178</td>
</tr>
<tr>
<td>EM</td>
<td>0.867 ± 0.150</td>
<td>0.872 ± 0.139</td>
<td>1.367 ± 0.590</td>
</tr>
<tr>
<td>CAM</td>
<td>0.384 ± 0.205*</td>
<td>0.385 ± 0.208*</td>
<td>2.558 ± 0.326*</td>
</tr>
<tr>
<td>10 days</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.433 ± 0.454</td>
<td>1.406 ± 0.448</td>
<td>0.287 ± 0.070</td>
</tr>
<tr>
<td>MOM</td>
<td>1.362 ± 0.235</td>
<td>1.345 ± 0.270</td>
<td>0.315 ± 0.095</td>
</tr>
<tr>
<td>TAO</td>
<td>0.192 ± 0.075*</td>
<td>0.198 ± 0.073*</td>
<td>0.006 ± 0.003</td>
</tr>
<tr>
<td>Control</td>
<td>2.036 ± 0.418</td>
<td>2.041 ± 0.478</td>
<td>0.076 ± 0.014</td>
</tr>
<tr>
<td>EM</td>
<td>1.223 ± 0.234</td>
<td>1.239 ± 0.226</td>
<td>0.108 ± 0.038</td>
</tr>
<tr>
<td>CAM</td>
<td>0.880 ± 0.3038</td>
<td>0.860 ± 0.294*</td>
<td>0.224 ± 0.031*</td>
</tr>
</tbody>
</table>

TABLE 3
In vitro formation of MI complex with various macrolides on rat and human liver microsomes

<table>
<thead>
<tr>
<th>Liver Microsomes</th>
<th>MI Complex*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MOM</td>
</tr>
<tr>
<td>Untreated rat</td>
<td>N.D.*</td>
</tr>
<tr>
<td>Dexamethasone-treated rat</td>
<td>293 ± 34.2</td>
</tr>
<tr>
<td>Human</td>
<td>112.5 ± 28.9</td>
</tr>
</tbody>
</table>

MI complex formation was measured at a final macrolide concentration of 300 μM.

*MI complex formation was not detectable. The detection limit was 25 pmol/mg protein.

N.D.: not detectable. The detection limit was 25 pmol/mg protein.

Notes:
- * Significantly different from control, P < .01 (Student’s t-test).
- † Significantly different from that without potassium ferricyanide, P < .01 (Student’s t-test).
- ‡ The limit of quantitation was 0.04 nmol/min/mg protein.
- § The limit of quantitation was 0.01 nmol/min/mg protein.
- ¶ The limit of quantitation was 0.001 nmol/min/mg protein.
- ‡‡ The limit of quantitation was 0.005 nmol/min/mg protein.

**Examined in human liver microsomes prepared from 10 different organ donors (Table 6). All four activities (formation of Mb-3, Mb-5, Mb-9a, and Mb-13) correlated well (r ≥ 0.96, P < .01) with the activity of CYP3A4/5-selective testosterone 6 β-hydroxylation. A statistically significant correlation (r ≥ 0.81, P < .01) was observed between MOM metabolism and the activity of CYP2C8/9-selective tolbutamide methyl-hydroxylation. This finding indicated that CYP3A4/5 and CYP2C8/9 were potentially involved in the formation of MOM-hydroxylated metabolites.

Studies in Human Liver Microsomes using CYP Inhibitor. Ketoconazole (0.5 μM) and TAO (100 μM) markedly inhibited (≥48% and ≥67%, respectively) the formation of all four MOM-hydroxylated metabolites (Table 2). Because both inhibitors were considered to be selective for CYP3A4/5, these data indicated that MOM metabolism was largely mediated by members of the human liver CYP3A subfamily. Some inhibition of Mb-9a and Mb-13 (14 and 22%, respectively) formation was observed with CYP2C19-selective S-mephenytoin. No significant inhibitions were detected with other inhibitors.
Inhibition of testosterone hydroxylation in rat and human liver microsomes by MOM and its metabolites

<table>
<thead>
<tr>
<th>Marker Activity</th>
<th>CYP Isoform</th>
<th>Correlation Coefficient (r)</th>
<th>Rat liver microsomes</th>
<th>Human liver microsomes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mb-3</td>
<td>Mb-5</td>
</tr>
<tr>
<td>Caffeine 3-demethylation</td>
<td>1A2</td>
<td>0.163</td>
<td>0.164</td>
<td>0.049</td>
</tr>
<tr>
<td>Coumarin 7-hydroxylation</td>
<td>2A6</td>
<td>0.197</td>
<td>0.223</td>
<td>0.143</td>
</tr>
<tr>
<td>Tolbutamide methyl-hydroxylation</td>
<td>2C8/9</td>
<td>0.834*</td>
<td>0.813*</td>
<td>0.878*</td>
</tr>
<tr>
<td>S-Mephenytoin 4'-hydroxylation</td>
<td>2C19</td>
<td>0.020</td>
<td>0.006</td>
<td>0.031</td>
</tr>
<tr>
<td>Dextromethorphan O-demethylation</td>
<td>2D6</td>
<td>0.332</td>
<td>0.319</td>
<td>0.365</td>
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<tr>
<td>Chlorzoxazone 6-hydroxylation</td>
<td>2E1</td>
<td>0.616</td>
<td>0.602</td>
<td>0.683</td>
</tr>
<tr>
<td>Testosterone 6β-hydroxylation</td>
<td>3A4/5</td>
<td>0.991*</td>
<td>0.994*</td>
<td>0.964*</td>
</tr>
</tbody>
</table>

* The statistical significance of correlation was denoted by P < .01.

MOM-Hydroxylated Metabolites Formation by cDNA-Expressed Human CYP Proteins. Human B-lymphoblastoid microsome preparations containing cDNA-expressed CYP3A4 exhibited measurable amounts of MOM-hydroxylated metabolites (Fig. 3). No hydroxylated metabolites were detected in microsomes containing cDNA-expressed CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9-Cys, CYP2C9-Arg, CYP2C19, CYP2D6-Val, or CYP2E1. Similarly, no hydroxylated metabolites were detected in control microsomes prepared from human B-lymphoblastoid cells using vector or NADPH-CYP reductase (data not shown).

To further characterize the metabolism of MOM, experiments were conducted with insect cell (BTI-TN-5B1–4) microsomes containing cDNA-expressed CYP2C19, CYP3A4, or CYP3A5. Microsomes containing CYP2C19 or CYP3A5 were expressed with NADPH-CYP reductase, and microsomes containing CYP3A4 were expressed with not only NADPH-CYP reductase but also cytochrome b₅. As a result, all four MOM-hydroxylated metabolites were detected in microsomes containing CYP3A4 or CYP3A5 (Fig. 3).

However, the amount of hydroxylated metabolites in microsomes that contained CYP3A5 was below 5% of that found in microsomes that contained CYP3A4. Similar to the case of human B-lymphoblastoid microsomes, no hydroxylated metabolites were detected in insect cell microsomes that contained CYP2C19. The results of obtained kinetic studies with insect cell microsomes that contained CYP3A4 are shown in Table 5. MOM metabolism conformed to single-enzyme Michaelis-Menten kinetics, and the apparent Kₐ values were comparable with those obtained with native human liver microsomes or human B-lymphoblastoid microsomes (data not shown). From the results of the experiment in which we used cDNA-expressed human CYP proteins, the correlation study, and the inhibi-
tion study, it was likely that CYP3A4, considered as a major CYP isofrom expressed in human livers, was mainly involved in the hydroxylation of MOM metabolites.

Drug Interactions of MOM Metabolites in Human Liver Microsomes. The effects of various drugs, whose metabolism is mediated by CYP3A, on hydroxylation of MOM metabolites, were studied in human liver microsomes (Table 7). Miconazole, dihydroergotamine, cyclosporine A, and bromocryptine markedly inhibited hydroxylation of MOM metabolites, and the IC$_{50}$ values were considerably lower than the substrate concentration of 50 mM.

On the other hand, the IC$_{50}$ values of disopyramide, carbamazepine, and theophylline were considerably higher than the substrate concentration. These four drugs also inhibited the reactions, but to a lesser extent.

Discussion

Fourteen-membered macrolides are known to interact with CYP and form stable MI complexes. However, 16-membered macrolides (MDM, spiramycin, and josamycin) generally do not form MI complexes, and they interact with other drugs less frequently than 14-membered macrolides (Miura et al., 1989). However, they also exhibit adverse interactions, which may be related to competitive inhibition of the CYP3A4-mediated metabolism of the coadministered drugs.

MOM is a 16-membered macrolide antibiotic metabolized to many metabolites, and the 14-hydroxylation of its metabolites may be mediated by CYP (Fig. 1). However, the interaction of MOM with other drugs has never been examined.

First, we investigated the formation of MI complexes in rats. CYP in rat liver was not induced and no detectable MI complexes were found after repeated administration of MOM. On the other hand, TAO, EM, and CAM induced CYP, and formed MI complexes with the CYP. Besides, similar findings that MOM did not form MI complexes were obtained from the in vitro study using human microsomes or microsomes from dexamethasone-pretreated rats and untreated rat (Table 3). In contrast, TAO, EM, or CAM formed MI complexes except for the microsomes from untreated rats. It is reported that the main constitutive CYP presenting in the male rat is CYP2C11, whereas CYP3A2 is induced by treatment with dexamethasone (Schuetz et al., 1984). MI complexes formed only in microsomes from dexamethasone-pretreated rats whose CYP3A2 in the liver were induced. Therefore, MI complexes may be formed at a part of CYP3A2. Because CYP3A4 is the most abundant CYP isoform in human liver (Shimada et al., 1994), and interactions of TAO, EM, or CAM are frequently reported with drugs whose metabolism is mediated by CYP3A4 (Periti et al., 1992), it is probable that human CYP3A4

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**Fig. 2. Effects of various CYP isoform-selective inhibitors on MOM-hydroxylated metabolites formation in human liver microsomes.**

The final assay volume was 0.25 ml containing 100 mM potassium phosphate buffer (pH 7.4), 1 mM EDTA, 50 mM substrate (Mb-2, Mb-6, or Mb-12), microsomal protein, and NADPH generating system. Reaction mixtures containing TAO were preincubated with microsomes at 37°C for 15 min. The reactions were initiated by adding the substrate, and the reaction mixtures were incubated at 37°C for 20 min. Except for those containing TAO, reactions were conducted without preincubation. Each value was expressed at the mean ± S.D. (triplicate).
forms MI complexes with these macrolides. The level of MI complexes formed with EM was lower than with CAM in vivo that was different from in vitro. This may be explained by the fact that EM is unstable at the gastric pH.

N-demethylation of most 14-membered macrolides is associated with the inhibition of CYP via the formation of MI complexes. This observation also explains why 14-membered macrolides increase the mean area under the plasma concentration of a number of coadministered drugs. The activity was measured at a final substrate concentration of 50 mM. The value could not be calculated because of obstructive peak.

Although MOM did not form MI complexes, MOM and its metabolites (Mb-2, Mb-6, and Mb-12) inhibited CYP3A4/5 in the human liver microsomes (Table 4). Especially, inhibition by MOM was strong (IC50 value: 11.6 μM) and was comparable to that by terfenadine. MOM undergoes metabolism in the gut and is not detected in plasma (Fukaya et al., 1981). Therefore, the extent of inhibition of CYP3A4/5 in the liver should be weak. However, because CYP3A4/5 are present in extrahepatic tissues, in particular the small intestine (Lown et al., 1994), metabolic inhibition by MOM is anxious in the intestine rather than in the liver.

From the above-mentioned observations, it is possible that MOM exhibits interactions that are related to competitive inhibition of CYP3A4/5-mediated metabolism of the coadministered drug. We tried to identify the CYP enzyme(s) that catalyzes the hydroxylation of MOM metabolites at the 14 position.

14-(R) or (S) hydroxylation of MOM metabolites (Mb-2, Mb-6, and Mb-12) in human liver microsomes depended on the presence of NADPH and was markedly inhibited by SKF-525A, a nonspecific CYP inhibitor, indicating that hydroxylation is CYP-dependent (data not shown).

Although a significant correlation was obtained with CYP2C8/9-selective tolbutamide methyl-hydroxylation (Table 6), CYP2C8/9-selective sulfaphenazole failed to inhibit hydroxylation in human liver microsomes (Fig. 2). Furthermore, cDNA-expressed CYP2C8 or CYP2C9 was unable to metabolize Mb-2, Mb-6, or Mb-12. This discrepancy may be related to the fact that tolbutamide methyl-hydroxylation significantly correlated with testosterone 6β-hydroxylation catalyzed by CYP3A5 (r = 0.776, P < .01, data not shown).

There is a large body of evidence that indicates that members of the CYP3A subfamily are involved in the hydroxylation of MOM metabolites: 1) four hydroxylations in a panel of human liver microsomes strongly correlated with CYP3A4/5-selective testosterone 6β-hydroxylation (Table 6); 2) each hydroxylation was essentially inhibited by ketoconazole and TAO (Fig. 2); 3) only cDNA-expressed CYP3A4 and CYP3A5 were able to catalyze the hydroxylations; and 4) similar apparent Km values were obtained with native human liver microsomes and with cDNA-expressed CYP3A4 (Table 5). As a whole, these data indicate that hydroxylations are also catalyzed by both CYP3A4 and CYP3A5. However, the activities of cDNA-expressed CYP3A5 were below 5% of those of CYP3A4, and addition of cytochrome b5 almost did not affect hydroxylation mediated by CYP3A5 (data not shown).

Furthermore, CYP3A5 is expressed in only 25% of individuals. When it is expressed, the level of CYP3A5 in CYP3A subfamily of human liver is usually lower (~30%) than that of CYP3A4 (Wrighton et al., 1989). Accordingly, it was obvious that hydroxylation of MOM metabolites was mainly mediated by CYP3A4, and CYP3A5 played a relatively minor role. Moreover, because of the presence of CYP3A4 in the gut, it is probable that the first-pass metabolism of MOM occurs in the intestine and liver.

Although MOM inhibits CYP3A, clinical drug interactions with MOM have been less frequent than those with 14-membered macrolides. MOM was shown to interfere with the kinetics of carbamazepine (Couet et al., 1990b), cyclosporine A (Couet et al., 1990a), and dihydroergotamine (Couet et al., 1991), but not with that of theophylline (Rimoldi et al., 1986; Principi et al., 1987; Dal Negro et al., 1988; Couet et al., 1989; and Pavesio et al., 1989).

Carbamazepine, commonly used as an antiepileptic drug, is usually involved in drug interactions for a number of reasons (Spina et al., 1996). Namely, carbamazepine has a narrow therapeutic range, and a relatively small change in its plasma concentration, due to induction or inhibition of metabolism, may easily result in loss of efficacy or
expression of toxicity. In this study, the IC50 values of carbamazepine were higher than the substrate concentration (Table 7), indicating a lesser extent of inhibition of MOM metabolism by carbamazepine. On the contrary, because the affinity of MOM metabolites to CYP3A4 is stronger than that of carbamazepine, and carbamazepine metabolism is mediated mainly by CYP3A4 (Kerr et al., 1994), it is possible that MOM metabolites inhibit the metabolism of carbamazepine. Although the IC50 values of theophylline were also high, there was only a small possibility for theophylline metabolism to be inhibited by MOM metabolites. Because theophylline is mainly eliminated by CYP1A2, the possibility for theophylline metabolism to be inhibited by MOM is small. However, the extent of drug interaction as a consequence of inhibition of MOM metabolism is probably lower, because: 1) MOM is metabolized to various metabolites and these metabolites have similar antibacterial activities (Yoshida et al., 1982); and 2) the therapeutic range of MOM is wide and no serious side effects have been reported so far (Mayama et al., 1990; Periti et al., 1993). Recent studies have suggested that intestinal first-pass metabolism of cyclosporine A, an immunosuppressive agent, significantly affects its bioavailability (Sketris et al., 1996).

In conclusion, the results of this study indicate that MOM does not inhibit CYP via the formation of an MI complex. CYP3A4 is responsible for catalyzing the hydroxylation of MOM metabolites. Because CYP3A4 is the most abundant isoform of CYP in the liver and intestine of adults, this enzyme probably accounts for the majority of the MOM-mediated metabolic properties of miocamycin. References


