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CYP Isoforms Involved in the Metabolism of Clarithromycin in vitro: Comparison between the Identification from Disappearance Rate and that from Formation Rate of Metabolites

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Summary: To clarify whether CYP2C19 is involved in the overall metabolism of clarithromycin (CAM) or not, in vitro studies using human liver microsomes and recombinant CYPs were performed by an approach based on the disappearance rate of parent compound from the incubation mixture. In addition, the results of disappearance rate were compared with those obtained from the formation rates of the major metabolites of CAM, 14-(R)-hydroxy-CAM and N-demethyl-CAM.

The intrinsic clearance (CLint) values determined from the disappearance of CAM in nine different human liver microsomes were highly correlated with the testosterone 6β-hydroxylation activity ($r = 0.957$, $p < 0.001$). The CLint of CAM was markedly reduced by selective inhibitors of CYP3A4 (ketoconazole and troleandomycin) and by polyclonal antibodies raised against CYP3A4/W5 in human liver microsomes. Among the 11 isoforms of recombinant human CYP, only CYP3A4 revealed the metabolic activity for the disappearance of CAM. These results were fairly consistent with those obtained from the conventional approach based on the formation of major metabolites of CAM. Comparison of the kinetic parameters estimated from the disappearance rate of CAM and the formation rates of 14-(R)-hydroxy-CAM and N-demethyl-CAM indicates that N-demethylation and 14-(R)-hydroxylation account for 65% of CLint derived from the disappearance of CAM in human liver microsomes.

The findings suggest that CYP3A4 plays a predominant role in the overall metabolic clearance of CAM as well as in the formation of 14-(R)-hydroxy-CAM and N-demethyl-CAM. CYP2C19 does not appear to be involved in the overall metabolism of CAM at least in human liver microsomes. A combination of the disappearance rate of a parent compound and the formation rate of metabolites appears to be a useful approach for estimating the percentage contribution of the formation of metabolites to the overall metabolic clearance of a parent compound in vitro.

Key words: clarithromycin; CYP; CYP3A4; disappearance rate; identification

Introduction

Clarithromycin (CAM) is a 14-membered macrolide antibiotic with a broad antibacterial spectrum1,2 and widely used for the treatment of Helicobacter pylori infection such as a triple therapy with omeprazole and amoxicillin.3 Previous studies have shown that CAM is oxidized to the two major metabolites, 14-(R)-hydroxy-CAM and N-demethyl-CAM via hydroxylation at the 14-position and N-demethylation, respectively.4,5) The enzyme responsible for the formation of the two metabolites has been identified as CYP3A by the in vitro study using human liver microsomes.6) However, Furuta et al. reported that there is a gene-dose effect of CYP2C19 defective alleles on the mean area under the plasma concentration-time curves (AUC) of CAM after
the coadministration of CAM and omeprazole. They found that AUC of CAM was the highest in the poor metabolizer group, the second-highest in heterozygous extensive metabolizers group and the lowest in homozygous extensive metabolizers group of CYP2C19. They speculated that CYP2C19 is associated with the metabolism of CAM to a certain extent, otherwise omeprazole may interfere more strongly with the metabolism of CAM by CYP3A4 in the poor metabolizer group because of high concentration of omeprazole in plasma in this group.

Measuring the metabolite's formation rate with a specific metabolic pathway has generally been utilized for the identification of a CYP isoform in studies on drug metabolism. However, the usefulness of this conventional approach is limited for assessing the CYP isoform(s) responsible for the overall metabolism of drugs, since it requires information on the importance of the particular metabolic pathway in the overall metabolic clearance. Such information is not usually available. In the case of CAM, contribution of the formation of 14-(R)-hydroxy-CAM and N-demethyl-CAM from CAM to the overall metabolic clearance of CAM has not been clarified, although they are the major metabolites of CAM found in the urine of humans. Therefore, it remains possible that CYP2C19 is involved in the overall metabolism of CAM via the other metabolic pathway than 14-(R)-hydroxylation and N-demethylation.

In order to clarify this possibility, we studied the CYP isoforms involved in the overall metabolism of CAM using human liver microsomes by the disappearance rate of parent compound from the incubation mixture, and compared the results with those obtained from the conventional approach based on the formation of 14-(R)-hydroxy-CAM and N-demethyl-CAM.

Materials and Methods

Chemicals and reagents: Clarithromycin (CAM), 14-(R)-hydroxy-CAM, N-demethyl-CAM, and N-demethyl-6-O-methyl erythromycin B (internal standard for N-demethyl-CAM) were synthesized at Taisho Pharmaceutical Co. Ltd. (Saitama, Japan). Erythromycin B (internal standard for CAM) was obtained from Abbott Laboratories (North Chicago, IL, USA). A derivatization reagent, 4-fluoro-7-nitrobenzofurazan (NBD-F), was purchased from Dojindo Laboratories (Kumamoto, Japan). Testosterone and troleandomycin (TAO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 6β-Hydroxytestosterone and ketoconazole were obtained from Sumika Chemical Analysis Service (Osaka, Japan) and BIOMOL Research Lab., Inc. (Plymouth Meeting, PA, USA), respectively. Acetonitrile, methanol and other reagents of analytical grade were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Microsomal preparations from eleven different recombinant human CYP isoforms (i.e., CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9-Arg, CYP2C19, CYP2D6-Val, CYP2E1, CYP3A4, and CYP4A11) expressed in the human B lymphoblastoid cell line (AHH-1) were purchased from Gentest Corp. (Woburn, MA, USA). Microsomes derived from baculovirus infected insect cells that contained overexpressed CYP2C19 (Supersomes, Gentest Corp.) were also used for further assessing the involvement of CYP2C19. Inhibitory antibody to human CYP3A4/5 was purchased from Amersham International (Tokyo, Japan). The antibody is a polyclonal antibody raised in rabbits against rat CYP3A1 with approximately 95% inhibition of the testosterone 6β-hydroxylation catalyzed by the rat and human CYP3A subfamily, but this antibody does not inhibit the activities of CYP1A2, CYP2A6, CYP2B6, CYP2C8, 9, CYP2D6 and CYP2E1 (data provided by Amersham).

Incubation conditions with human liver microsomes and recombinant human CYP isoforms: The basic incubation medium contained 0.3 mg/mL human liver microsomes, 4 mM MgCl₂, 0.5 mM NADP, 2 mM glucose-6-phosphate, 1 IU/mL glucose-6-phosphate dehydrogenase, 100 mM potassium phosphate buffer (pH 7.4), 0.1 mM EDTA and CAM, in a final volume of 250 µL. Human liver microsomes were purchased from Gentest Corp (pooled human liver microsomes, H161) or prepared by differential centrifugation from 9 human livers obtained as an excess material during liver surgery (from five male and four female patients aged 44–67 years). Before the study, use of human liver samples for the study was approved by the institutional ethics committee/institutional review board of International Medical Center of Japan (Tokyo, Japan). The substrate concentrations were 100 µM for the formation of metabolites and 1 µM for the depression of CAM unless otherwise stated. Those concentrations were almost double or well below the reported Kₘ values of around 50 µM for N-demethylation and 14-(R)-hydroxylation of CAM.

Although data are not shown, the disappearance rate at a substrate concentration of 1 µM was not different from that of 5 µM in a preliminary study; thus, the substrate concentration used for the determination of intrinsic clearance was confirmed to be within the linearity. The mixture was incubated at 37°C in a shaking water bath for 10 or 20 min after the 1 min of preincubation. The reaction was terminated by addition of 100 µL of ice cold acetonitrile for the determination of metabolites or 100 µL of internal standard (erythromycin B, 3.125 µg/mL in acetonitrile) for the determination of CAM depression. After termination of the incubation, the mixture was centrifuged at 10,000 rpm for 10 min, and the supernatant was analyzed by HPLC.

The incubation conditions used for the recombinant...
human CYP isoforms obtained from genetically engineered B lymphoblastoid cells were essentially the same as those used for human liver microsomes, except for incubation time (120 min) and the concentration of microsomes used (1 mg/mL). The microsomes prepared from human B lymphoblastoid cells with vectors or containing cDNA-expressed NADPH-cytochrome c reductase were used for control incubations.

**HPLC assay:** The determination of CAM or its metabolites in the incubation mixture was performed by the HPLC method of Ohtake et al. with slight modification. In brief, for the determination of CAM and 14-(R)-hydroxy-CAM, an aliquot of supernatant (50 µL) prepared from the incubation mixture was directly injected into the HPLC system. The HPLC system consisted of a model LC-6A pump (Shimadzu, Kyoto, Japan), model SIL-6B autosampler (Shimadzu), model CTO-6A column oven (Shimadzu), model C-R4A integrator (Shimadzu) and NUCOSIL 100-5C18 column (150 × 4.0 mm internal diameter, GL Sciences Inc., Tokyo, Japan). The mobile phase consisted of acetonitrile: methanol: 0.05M phosphate buffer (pH 6.5) (40:15:45 by volume) delivered at 0.8 mL/min. Column temperature was maintained at 40°C. The eluate was monitored by electrochemical detection (Coulochem model 5100A, Enviromental Sciences Associates, Bedford, MA, USA) with the electric potentials of the guard cell and the first and second electrodes set at 0.95 V, 0.65 V and 0.9 V, respectively.

For the determination of N-deethylation, 100 µL of internal standard (N-demethyl-6-O-methyl erythromycin B, 2.5 µg/mL in acetonitrile), 100 µL of NBD-F (2 mg/mL in methanol), and 100 µL of 0.05M borate buffer (pH 8.5) were added to an aliquot of supernatant (50 µL) prepared from the incubation mixture and reacted for 10 min at 60°C. Then the reaction mixture was cooled on ice, mixed with 0.005M hydrochloric acid (100 µL), and an aliquot (50 µL) was injected into the HPLC system. The chromatography was conducted with a reversed-phase column, COSMOSIL 5C8 (150 × 4.0 mm in internal diameter, Nacalai Tesque Inc., Kyoto, Japan). The mobile phase consisted of acetonitrile: methanol: water: phosphoric acid (55:3:42:0.01 by v/v) delivered at 1.2 mL/min. Column temperature was maintained at 40°C. The eluate was monitored by fluorescence detection (model FP-920, Jasco, Tokyo, Japan) with excitation and emission wavelengths set at 470 and 530 nm, respectively.

The determination limit was 0.25 µM for CAM, 0.09 µM for 14-(R)-hydroxy-CAM and 0.14 µM for N-demethyl-CAM. Intra-assay coefficients of variation were <5% for all analytes.

**Intrinsic clearance of CAM:** The intrinsic clearance (CL_int) values of CAM in nine different human liver microsomes were estimated from the volume of medium (V) and the half-life of substrate disappearance (T1/2) using the equation: CL_int = V × 0.693 / T1/2. The half-life of CAM in the incubation medium was calculated by the regression analysis of semi-logarithmic plots.

Non-specific binding of CAM to microsomal protein was negligible.

**Correlation study:** The rates of N-demethylation and 14-(R)-hydroxylation or CL_int of CAM were correlated with the metabolic activities of substrates toward the respective CYP isoforms using nine different human liver microsomes.

Phenacetin O-deethylation (CYP1A2), coumarin 7-hydroxylation (CYP2A6), 7-mephobarbital N-demethylation (CYP2B6), diclofenac 4’-hydroxylation (CYP2C9), S-mephenytoin 4’-hydroxylation (CYP2C19), desipramine 2-hydroxylation (CYP2D6), chloroxazone 6-hydroxylation (CYP2E1), and testosterone 6β-hydroxylation (CYP3A4) were used for assessing the respective CYP-catalytic probe activities. Metabolites of the above in vitro probe substrates were determined using the respective HPLC assay methods, as reported elsewhere.

**Inhibition study:** Ketoconazole and TAO were used as CYP3A4 selective inhibitors. Ketoconazole (1 µM) was coincubated with CAM under the incubation conditions described above. TAO (1 µM) was preincubated with microsomes and the NADPH generating system for 15 min, before addition of CAM to initiate the reaction. The effects of inhibitors on the rates of N-demethylation and 14-(R)-hydroxylation or metabolic clearance of CAM using pooled human liver microsomes were compared with the control values and expressed as percentages of the respective control values.

**Immunoinhibition study:** The antibody used in this study was a polyclonal antibody raised in rabbits against rat CYP3A1. This antibody cross-reacts with human CYP3A4/5 and inhibits approximately 95% of the testosterone 6β-hydroxylation catalyzed by the rat and human CYP3A subfamily, but does not inhibit the activities of CYP1A2, CYP2A6, CYP2B6, CYP2C8/9, CYP2D6 and CYP2E1 (Amersham, Tokyo, Japan). The immunoinhibition studies were performed by preincubating pooled human liver microsomes (0.3 mg/mL) with various concentrations of anti-CYP3A4/5 antibodies or preimmune rabbit IgG (0–2.5 mg IgG/mg microsomal protein) in 0.1 mM potassium phosphate buffer (pH 7.4) for 30 min on ice. CAM and other components of the incubation medium were added, and the rates of N-demethylation and 14-(R)-hydroxylation or metabolic clearance of CAM were assessed as described above.

**Determination of K_m and V_max for the metabolism of CAM:** Kinetic parameters for the N-demethylation and 14-(R)-hydroxylation were obtained by incubating
Table 1. Intrinsic clearances of CAM and rates of N-demethylation and 14-(R)-hydroxylation of CAM in microsomes from nine different human livers

<table>
<thead>
<tr>
<th>Human liver microsomes</th>
<th>CL_{int} of CAM</th>
<th>Rate of N-demethylation</th>
<th>Rate of 14-(R)-hydroxylation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μL/min/mg protein</td>
<td>nmol/min/mg protein</td>
<td>nmol/min/mg protein</td>
</tr>
<tr>
<td>HL-31</td>
<td>48.0</td>
<td>0.273</td>
<td>0.201</td>
</tr>
<tr>
<td>HL-32</td>
<td>42.3</td>
<td>0.202</td>
<td>0.122</td>
</tr>
<tr>
<td>HL-33</td>
<td>68.4</td>
<td>0.485</td>
<td>0.378</td>
</tr>
<tr>
<td>HL-34</td>
<td>61.1</td>
<td>0.516</td>
<td>0.396</td>
</tr>
<tr>
<td>HL-35</td>
<td>21.7</td>
<td>0.196</td>
<td>0.117</td>
</tr>
<tr>
<td>HL-37</td>
<td>6.6</td>
<td>0.089</td>
<td>0.070</td>
</tr>
<tr>
<td>HL-38</td>
<td>75.2</td>
<td>0.611</td>
<td>0.437</td>
</tr>
<tr>
<td>HL-39</td>
<td>22.8</td>
<td>0.307</td>
<td>0.220</td>
</tr>
<tr>
<td>HL-40</td>
<td>46.5</td>
<td>0.414</td>
<td>0.320</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>43.6 ± 23.0</td>
<td>0.344 ± 0.173</td>
<td>0.251 ± 0.136</td>
</tr>
</tbody>
</table>

The intrinsic clearance (CL_{int}) of CAM was estimated from the volume of medium and the half-life of substrate disappearance as described in the text.

Table 2. Correlations between the formation rates of major metabolites or disappearance rates of CAM and different CYP marker activities in microsomes from nine different human livers

<table>
<thead>
<tr>
<th>Metabolic reaction of substrate</th>
<th>CYP isoform</th>
<th>Correlation coefficient (r) with CL_{int}</th>
<th>Correlation coefficient (r) with N-demethylation</th>
<th>Correlation coefficient (r) with 14-(R)-hydroxylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenacetin O-deethylation</td>
<td>CYP1A2</td>
<td>0.397</td>
<td>0.257</td>
<td>0.239</td>
</tr>
<tr>
<td>Coumarin 7-hydroxylation</td>
<td>CYP2A6</td>
<td>0.685*</td>
<td>0.664</td>
<td>0.633</td>
</tr>
<tr>
<td>S-Meprobital N-demethylation</td>
<td>CYP2B6</td>
<td>0.752*</td>
<td>0.582</td>
<td>0.619</td>
</tr>
<tr>
<td>Diclofenac 4'-hydroxylation</td>
<td>CYP2C9</td>
<td>0.292</td>
<td>0.000</td>
<td>-0.046</td>
</tr>
<tr>
<td>S-Mephenytoin 4'-hydroxylation</td>
<td>CYP2C19</td>
<td>0.665</td>
<td>0.825**</td>
<td>0.791*</td>
</tr>
<tr>
<td>Desipramine 2-hydroxylation</td>
<td>CYP2D6</td>
<td>0.644</td>
<td>0.576</td>
<td>0.551</td>
</tr>
<tr>
<td>Chlorzoxazone 6-hydroxylation</td>
<td>CYP2E1</td>
<td>0.172</td>
<td>0.029</td>
<td>-0.026</td>
</tr>
<tr>
<td>Testosterone 6β-hydroxylation</td>
<td>CYP3A4</td>
<td>0.957***</td>
<td>0.954***</td>
<td>0.947***</td>
</tr>
<tr>
<td>CAM N-demethylation</td>
<td>CYP3A4</td>
<td>0.895**</td>
<td>-</td>
<td>0.993***</td>
</tr>
<tr>
<td>CAM 14-(R)-hydroxylation</td>
<td>CYP3A4</td>
<td>0.881**</td>
<td>0.993***</td>
<td>-</td>
</tr>
</tbody>
</table>

* p < 0.05; ** p < 0.01; *** p < 0.001.

CAM (5–100 μM) with human liver microsomes (H161) or recombinant CYP3A4 under linear conditions with respect to protein concentration and time of incubation. Since the formation of both metabolites followed simple Michaelis-Menten kinetic behavior, K_{m} and V_{max} values were estimated by graphical analysis of Eadie-Hofstee plots.

Data analysis: Correlation coefficients (r) were determined by the least-squares linear regression analysis. The mean kinetic values were compared by Student’s t-test for unpaired data (SAS system for Windows, Ver.6.10). P values <0.05 were considered statistically significant.

Results

The formation rates of metabolites and intrinsic clearance of CAM: CL_{int} for the disappearance of CAM (1 μM) and the rates of N-demethylation and 14-(R)-hydroxylation of CAM (100 μM) are given in Table 1. The mean (±SD) value of CL_{int} was 43.6 ± 23.0 μL/min/mg, while those for N-demethylation and 14-(R)-hydroxylation were 0.344 ± 0.173 and 0.251 ± 0.136 nmol/min/mg, respectively.

Correlation study: The results of correlation between the CL_{int} of CAM and marker enzyme activities and between the formation rates of metabolites and marker enzymes are summarized in Table 2. There was an excellent correlation (r = 0.957, p < 0.001) between the CL_{int} values of CAM and testosterone 6β-hydroxylation activity (Fig. 1a). CL_{int} values of CAM were also significantly correlated with coumarin 7-hydroxylation activity (r = 0.685, p < 0.05) and S-meprobital N-demethylation activity (r = 0.752, p < 0.05), although there were no significant correlations between the CL_{int} of CAM and other metabolic activities of probe substrates used in the present study.

The formation rates of both N-demethyl-CAM and 14-(R)-hydroxyl-CAM significantly correlated with the testosterone 6β-hydroxylation activity as shown in Fig. 1b (r = 0.947–0.954, p < 0.001), and with S-
Fig. 1. Relationships between the intrinsic clearances of CAM and testosterone 6β-hydroxylation activities (a) and between formation rates of major metabolites of CAM and testosterone 6β-hydroxylation activities (b) in microsomes from nine different human livers.

Fig. 2. Relationship between N-demethylation and 14-(R)-hydroxylation activities in microsomes from nine different human livers.

mephenytoin 4′-hydroxylation activity ($r = 0.825, p < 0.01; 0.791, p < 0.05$). No statistically significant relationships were observed between N-demethylation or 14-(R)-hydroxylation of CAM and the other specific CYP enzyme activities investigated. However, the rates of N-demethylation and 14-(R)-hydroxylation of CAM were highly correlated with each other ($r = 0.993, p < 0.001$) in nine different human liver microsomes (Fig. 2), suggesting that the same CYP isoform(s) is involved in the both metabolic pathways.

**Metabolism by recombinant CYP isoform:** Among the recombinant CYP isoforms expressed in lymphoblastoid cell lines, only CYP3A4 revealed the significant metabolic clearance for CAM (Fig. 3). Similarly, with respect to the formation rates of N-demethyl-CAM and 14-(R)-hydroxyl-CAM, CYP3A4 revealed the highest catalytic activity, while CYP2D6 only showed slight activity for N-demethylation.

Although the microsomes of baculovirus-infected insect cells that contained about 10-times greater amounts of CYP2C19 than those in microsomes of lymphoblastoid cells were examined separately, they did not exhibit any appreciable activity for the N-demethylation, 14-(R)-hydroxylation or disappearance of CAM (data not shown).

**Chemical inhibition study:** The effects of ketoconazole (1 μM) and TAO (1 μM) on the metabolism of CAM in pooled human liver microsomes are shown in Fig. 4. These two widely used selective inhibitors of CYP3A4 potentially reduced the $CL_{int}$ of CAM and strongly inhibited the formation of N-demethyl-CAM and 14-(R)-hydroxyl-CAM. The reductions of $CL_{int}$ by ketoconazole and TAO were 96% and 92%, respectively. The inhibitions by ketoconazole and TAO of N-demethylation and 14-(R)-hydroxylation were $\approx 84\%$ and $\approx 80\%$, respectively.

**Immunoinhibition study:** The suppression of the metabolic clearance of CAM and the inhibition of metabolite formation by the addition of anti-CYP3A4/5 polyclonal antibody are shown in Fig. 5. The addition of antibody to the incubation medium reduced the $CL_{int}$ of CAM by 89% at a concentration of 2.5 mg IgG/mg microsomal protein. Similarly, the rates of N-demethylation and 14-(R)-hydroxylation of CAM (10 μM) were reduced markedly by the addition of antibody in a concentration-dependent manner and those activities were almost completely abolished by 1.25 and 2.5 mg IgG/mg microsomal protein, respectively.
Kinetic parameters: The kinetic parameters for \(N\)-demethylation and 14-\((R)\)-hydroxylation of CAM in human liver microsomes and in microsomes of recombinant human CYP3A4 are listed in Table 3. The apparent \(K_m\) value of 14-\((R)\)-hydroxylation in recombinant human CYP3A4 (18.6 ± 10.6 \(\mu\)M) was comparable with that obtained with human liver microsomes (25.2 ± 1.0 \(\mu\)M). However, the apparent \(K_m\) value of \(N\)-demethylation in microsomes of recombinant human CYP3A4 (14.4 ± 2.6 \(\mu\)M) was lower than that in the human liver microsomes (52.5 ± 16.6 \(\mu\)M). The sums of \(V_{\text{max}}/K_m\) values for \(N\)-demethylation and 14-\((R)\)-hydroxylation were 65% of the CL\(_{\text{int}}\) of CAM in human liver microsomes (62.9 \(\mu\)L/min/mg protein) and 83% in recombinant human CYP3A4 (19.7 \(\mu\)L/min/mg protein).

Discussion

The major human CYP isoform(s) involved in the \textit{in vitro} metabolism of CAM was identified by measuring the disappearance rate of parent compound and com-
pared with those by measuring the formation rate of metabolites. The results with respect to the disappearance of CAM indicate that CYP3A4 is a principal isoform responsible for the overall metabolic clearance of CAM in human liver microsomes. This is supported by the following evidence: 1) disappearance rate of CAM was highly correlated with the testosterone 6β-hydroxylation activity, a marker for CYP3A4, in the same panel of microsomes as those used for the metabolites formation study (Fig. 2, Table 2); 2) CYP3A4 revealed the metabolic activity for the disappearance of CAM among the 11 isoforms of recombinant human CYP, while the other CYP isoforms exhibited no significant metabolic activity (Fig. 3); 3) ketoconazole and TAO, selective inhibitors of CYP3A4, reduced the CL_int of CAM (Fig. 4); and 4) the CL_int of CAM was almost completely suppressed by the addition of anti-CYP3A4/5 antibodies to the incubation mixture (Fig. 5).

Although our study revealed the significant correlations between the CL_int of CAM and CYP2A6-selective coumarin 7-hydroxylation activity and CYP2B6-selective S-mephobarbital N-demethylation activity (Table 2), those isoforms do not appear to be involved in the clearance of CAM, since the correlation coefficient between the CAM clearance and activity of CYP2A6 or CYP2B6 was lower than that between the CAM clearance and CYP3A4 activity (Table 2). Furthermore, the correlation line between the CAM clearance and CYP3A4 activity appears to pass through the origin that has no intercept significantly larger than zero (data not shown). In addition, neither recombinant CYP2A6 nor CYP2B6 was capable of metabolizing CAM (Fig. 3). Therefore, the observed correlation between the metabolic clearance of CAM and CYP2A6 or CYP2B6 activity may be derived from the fact that CYP3A4 activity significantly correlated with both CYP2A6 (r = 0.729, p < 0.05) and CYP2B6 (r = 0.671, p < 0.05) activities in the panel of liver microsomes used in the present study.

Regarding the possibility that CYP3A5 also catalyze

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**Table 3. Kinetic parameters for N-demethylation and 14-(R)-hydroxylation of CAM in human liver microsomes and recombinant CYP3A4**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>CL_int of CAM</th>
<th>N-demethylation</th>
<th>14-(R)-hydroxylation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human liver microsomesa</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K_m (μM)</td>
<td>—</td>
<td>52.5 ± 16.6</td>
<td>25.2 ± 1.0</td>
</tr>
<tr>
<td>V_max (nmol/min/mg protein)</td>
<td>—</td>
<td>0.71 ± 0.16</td>
<td>0.68 ± 0.01</td>
</tr>
<tr>
<td>V_max/K_m (μL/min/mg protein)</td>
<td>62.9 ± 2.5</td>
<td>13.9 ± 1.6</td>
<td>26.9 ± 0.7</td>
</tr>
<tr>
<td>CYP3A4b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>K_m (μM)</td>
<td>—</td>
<td>14.4 ± 2.6</td>
<td>18.6 ± 10.6</td>
</tr>
<tr>
<td>V_max (nmol/min/mg protein)</td>
<td>—</td>
<td>0.18 ± 0.06</td>
<td>0.07 ± 0.02</td>
</tr>
<tr>
<td>V_max/K_m (μL/min/mg protein)</td>
<td>19.7 ± 1.4</td>
<td>12.4 ± 1.7</td>
<td>3.9 ± 1.6</td>
</tr>
</tbody>
</table>

a Pooled microsomes (H161). Each value is the mean ± SD of triplicate incubations.
b Recombinant human CYP3A4. Each value is the mean ± SD of triplicate incubations.
the metabolism of CAM, Williams et al. recently reported the capability of baculovirus-expressed CYP3A5 to metabolize CAM. Although CYP3A5 is generally a minor component of total CYP3A and only expressed in 25% of individuals and the expression level is usually lower as compared to CYP3A4, recent evidence indicates that CYP3A5 may represent more than 50% of the total CYP3A in some individuals possessing the CYP3A5*1 allele. However, CYP3A5 appears to play a relatively minor role based on the reported low clearance values of CYP3A5 relative to that of CYP3A4 for the metabolism of CAM. Therefore, CYP3A4 appears to be the predominant CYP isoform involved in the metabolic clearance of CAM in human liver microsomes.

The formation rates of metabolites confirmed the predominant role of CYP3A4 in two major metabolic pathways, N-demethylation and 14-(R)-hydroxylation, of CAM in human liver microsomes. In the correlation study, formation rates of both metabolites significantly correlated with the CYP3A4-selective testosterone 6β-hydroxylation activity \( r = 0.947 - 0.954, p < 0.001 \) in nine different human liver microsomes (Table 2). Although a significant correlation with CYP2C19-selective S-mephénytoin 4'-hydroxylation activity \( r = 0.791 - 0.825, p < 0.05 \) was also observed (Table 2), this isoform does not appear to be involved in the both metabolic pathways of CAM. This is because, no metabolic activity of CAM was observed with recombinant CYP2C19 from human B lymphoblastoid cells (Fig. 3) or even with overexpressed CYP2C19 from baculovirus-infected insect cells. Therefore, this correlation appears to be derived from the significant correlation between CYP2C19 and CYP3A4 activities in the panel of liver microsomes used in the present study \( r = 0.732, p < 0.05 \).

Although recombinant CYP2D6 was capable of catalyzing N-demethylation (Fig. 3), it does not appear to play a significant role in the metabolism of CAM. This is because, there was no significant correlation between N-demethylation of CAM and CYP2D6-selective desipramine 2-hydroxylation activity (Table 2), and quinidine (5 μM), a selective inhibitor of CYP2D6, did not inhibit N-demethylation of CAM (unpublished observation). Moreover, CYP2D6 in the recombinant system used in the present study is overexpressed and its content is more than 10-fold greater than that present in usual human liver microsomal preparations (data provided by Gentest). In addition, the predominant role of CYP3A4 in N-demethylation and 14-(R)-hydroxylation of CAM was further confirmed by the findings that those activities were strongly (>80%) inhibited by the CYP3A4 selective inhibitors, ketoconazole (1 μM) and TAO (1 μM), and almost completely abolished by anti-CYP3A4/5 antibody (Figs. 4 and 5).

In the present study, we found that the sum of \( V_{\text{max}} / K_a \) values for N-demethylation and 14-(R)-hydroxylation is 65% of CLint derived from the disappearance of CAM in human liver microsomes. The finding suggests that N-demethylation and 14-(R)-hydroxylation are the major metabolic pathways of CAM in human liver microsomes, and also suggests that there may be other metabolic pathways in the overall metabolism of CAM. However, the results of the present study indicate that CYP3A4 plays a predominant role in the overall metabolism of CAM as well as the N-demethylation and 14-(R)-hydroxylation of CAM. Therefore, CYP2C19 does not appear to be mainly involved, even if any, in the overall metabolism of CAM in human liver microsomes.

Based on the present results, the gene-dose effect of the defective alleles of CYP2C19 on the mean AUC of CAM reported previously could not be explained by the contribution of CYP2C19 to the metabolism of CAM. One of the possibilities to explain the gene-dose effect is the more pronounced interference with the CYP3A4 mediated CAM metabolism by high concentration of omeprazole in the CYP2C19 poor metabolizer group. Omeprazole has been found to be a weak inhibitor of CYP3A4 in vitro and does not inhibit CYP3A4 activity in vivo. The typical \( K_i \) values of omeprazole are 84.4 μM for CYP3A-mediated dextromethorphan N-demethylation and 367.5 μM for oxidation of nifedipine, though a recent report has shown relatively low \( K_i \) values of 0.4 μM and 45.6 μM for the metabolism of CYP3A substrates cisapride and simvastatin, respectively. Although the \( K_i \) value of omeprazole for the CYP3A4 mediated CAM metabolism is not known, considering the high plasma concentration of omeprazole (~5.5 μM) in the CYP2C19 poor metabolizer group, the possibility that metabolism of CAM was inhibited by omeprazole can not be ruled out.

Another possibility is the effect of omeprazole on the stability of CAM. Gustavson et al. has reported that administration of CAM with omeprazole produces higher plasma concentration of CAM with higher mean gastric pH (5.7 vs 3.1) than does the administration of CAM with placebo. CAM is more acid stable than erythromycin, however, some degradation of CAM is likely to occur in the stomach at typical gastric pH. They speculated that the increase in the plasma concentration of CAM might be due to the elevated absorption of more intact CAM by the effect of omeprazole on gastric pH. Therefore, the gene-dose effect of the defective alleles of CYP2C19 on the mean AUC of CAM reported previously might be derived from the effect of omeprazole on gastric pH which is dependent on CYP2C19 genotype status.

In conclusion, the present study using human liver
microsomes and recombinant human CYP isoforms indicates that CYP3A4 is the major isoform involved in the overall metabolic clearance of CAM as well as in the formation of 14-(R)-hydroxy-CAM and N-demethyl-CAM. CYP2C19 does not appear to be involved in the overall metabolism of CAM at least in human liver microsomes. The results also showed that the sum of $V_{\text{max}}/K_m$ values for N-demethylation and 14-(R)-hydroxylation is 65% of CL$_{\text{int}}$ derived from the disappearance of CAM in human liver microsomes. A combination of the determination of disappearance rate of a parent compound and the formation rate of metabolites may be a useful approach for estimating the percentage contribution of the formation of metabolites to the overall metabolic clearance of a parent compound in vitro.

References


CYP Phenotyping for Clarithromycin from *In Vitro* Disappearance Rate


