Mechanism-based Inhibition Profiles of Erythromycin and Clarithromycin with Cytochrome P450 3A4 Genetic Variants

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Summary: Inhibition of cytochrome P450 (CYP) 3A4 is the major cause of drug-drug interactions (DDI). We have previously reported that the genetic variation of CYP3A4 significantly affected the inhibitory profiles of typical competitive inhibitors. In addition to competitive inhibition, some clinically significant DDI are attributable to mechanism-based inhibition (MBI). However, the differences in the MBI kinetics among CYP3A4 genetic variants remain to be characterized. In this study, we quantitatively investigated the inhibition kinetics of MBI inhibitors, erythromycin and clarithromycin, on the CYP3A4 variants CYP3A4.1, 4.2, 4.7, 4.16, and 4.18. The activity of CYP3A4 was assessed using testosterone 6β-hydroxylation with recombinant CYP3A4. Both erythromycin and clarithromycin decreased the activity of CYP3A4 in a time-dependent manner. The maximum inactivation rate constants, $k_{\text{inact,max}}$, of erythromycin for CYP3A4.2 and CYP3A4.7 were 0.5-fold that for CYP3A4.1, while that for CYP3A4.16 and CYP3A4.18 were similar to that for CYP3A4.1. The $K_i$ values of erythromycin for CYP3A4.2, 4.7, 4.16, and 4.18 were 1.2-, 0.4-, 2.2- and 0.72-fold those of CYP3A4.1, respectively. Similar results were obtained for clarithromycin. In conclusion, the inhibitory profiles of MBI inhibitors, as well as competitive inhibitors, may possibly differ among CYP3A4 variants. This difference may contribute to interindividual differences in the extent of DDI based on MBI.

Keywords: genetic variants; CYP3A4; mechanism-based inhibition; erythromycin; clarithromycin

Introduction

Cytochrome P450 (CYP) 3A enzymes are expressed in the liver, kidney, and intestine in humans and are responsible for the metabolism of >50% of clinically used drugs.1-3 Twenty genetic variants that lead to amino acid substitution have been identified in the CYP3A4 gene [http://www.cypalleles.ki.se/cyp3a4.htm].4 In our previous study, we demonstrated that enzymatic activities differed among major CYP3A4 variants, in comparison with CYP3A4.1, from alleles such as CYP3A4*2, *7, *16, and *18.5 We have also reported that the genetic variation of CYP3A4 significantly affected the inhibition profiles of two typical competitive inhibitors, cimetidine and itraconazole, and suggested that the genetic variation of CYP3A4 may contribute, at least in part, to interindividual differences in the extent of drug-drug interactions (DDI) caused by the inhibition of CYP3A4.6

Besides competitive inhibition, another mechanism of DDI involving a decrease in CYP activity is mechanism-based inactivation (MBI), where a cytochrome P450-mediated reaction produces a reactive intermediate molecule that covalently interacts with the enzyme to impair enzymatic activity. Importantly, MBI of CYP lasts even after drug is eliminated from the body, because the enzymatic activity remains inactivated until new enzyme is produced,7 so that MBI may lead to clinically significant DDI. In vitro enzymatic studies using recombinant CYP3A4 revealed that various drugs including HIV protease inhibitors, macrolide antibiotics, and verapamil cause MBI with CYP3A4.8-10 However, it remains to be determined whether the MBI kinetics differ among CYP3A4 genetic variants and whether the influences of genetic variation differ among MBI inhibitors.

With regard to CYP2D6, Lim et al.11 reported a clinical study showing that paroxetine, an MBI inhibitor for CYP2D6, showed
different MBI profiles between CYP2D6.1 and CYP2D6.10. They investigated the DDI of flecainide, a CYP2D6-substrate, and paroxetine in genotyped healthy subjects and found that treatment with paroxetine (20 mg/day for 7 days) increased the area under the plasma concentration curve (AUC) of flecainide by 1.3-fold in subjects with CYP2D6*1/*1 or CYP2D6*1/*2 but did not increase the AUC in CYP2D6*10 homozygotes or CYP2D6*10/*36 heterozygotes. This difference in the increase of AUC among genetic variants is conceivably attributable to the difference in the contribution of CYP2D6-mediated metabolism to overall clearance of flecainide among genotype. However, the difference in the mechanism-based inhibitory profile of paroxetine may have possibly contributed to the above difference among CYP2D6 genotypes.

The aim of this study was to characterize the inhibition kinetics of two typical MBI inhibitors for CYP3A4, erythromycin (EM) and clarithromycin (CAM), on the enzymatic activities [as assessed by 6β-hydroxylation of testosterone (TST)] of recombinant CYP3A4.1, 4.2, 4.7, 4.16, and 4.18 variants prepared from Escherichia coli expression systems.

Materials and Methods

Materials: TST was purchased from Nacalai Tesque (Kyoto, Japan). 6β-Hydroxytestosterone (6β-OHT) and hydrocortisone were purchased from SPI Bio Bertin Pharma (Bretonneux, France). EM was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). CAM was kindly provided by Taisho Toyama Pharmaceutical Co. (Tokyo, Japan). The E. coli membrane fractions co-expressing NADPH-CYP reductase for CYP3A4.1 (wild type), 4.2 (S222P), 4.7 (G56D), 4.16 (T185S), and 4.18 (L293P) were prepared by the method previously described. All other chemicals and reagents were analytical or HPLC grade and obtained from commercial sources.

Mechanism based inactivation of enzymatic activities of CYP3A4 variants by EM and CAM: An aliquot of 45 µl of 300 mM potassium phosphate buffer (pH 7.4) containing inhibitor (final concentration of EM 0, 0.1, 0.3, 1, 5, or 20 µM/CAM 0, 0.2, 1, 3, 10, or 50 µM), 0.5 mM EDTA, and CYP3A4 membrane fraction (0.5 pmol P450/µl) was incubated at 37°C for 10 min. Then pre-incubations were initiated by adding 5 µl of an NADPH-generating system solution (final concentration: 5 mM glucose 6-phosphate, 0.5 mM NADP+, 1 unit/ml glucose 6-phosphate dehydrogenase, and 3 mM MgCl2), for varying times. Incubations were initiated by the addition of 250 µl substrate solution (100 µM TST, containing 0.5% ethanol, v/v), 50 µl of NADPH-generating system solution, and 150 µl of 0.5 mM EDTA (final concentration: 0.01 mM). The reaction mixtures (total of 500 µl) were diluted 1:10 and incubated for 20 min, and the reactions were terminated by adding 225 µl of ice-cold acetonitrile, followed by spiking with 25 µl of 37.5 mM hydrocortisone in methanol (internal standard). After centrifugation at 1,200×g for 20 min at 4°C, 6β-OHT in the supernatant was determined by an HPLC-UV method described below.

Determination of 6β-OHT: Concentrations of 6β-OHT were measured by an HPLC-UV method reported previously. Briefly, the HPLC system consisted of a pump (LC-10AD, Shimadzu, Kyoto, Japan), a UV detector (SPD-10AV spectrophotometer, Shimadzu), and an octadecylsilane column (Cosmosil, 5C18-AR-2, 4.6 × 150 mm, Nacalai Tesque). The mobile phase consisted of methanol and water (58:42, v/v) and was pumped at a rate of 1.0 ml/min. The absorbance of 6β-OHT was measured at 254 nm. The detection limit was 0.3 µM.

Analysis of mechanism based inhibition kinetics: The observed inactivation rate constant (kobs) at each inhibitor concentration was calculated from the slope of the logarithm of remaining enzyme activity plotted against the pre-incubation time. Then, after subtracting a k inact value (apparent inactivation rate constant without inhibitors), from kobs, Δkobs values were obtained. Equation (1) was simultaneously fit to the Δkobs values at various concentrations of inhibitor ([I]) using a nonlinear least-squares program, MULTI, to obtain kinetic parameters, i.e., maximal inactivation rate constant (k inact,max) and concentration of inhibitor required for half-maximal inactivation (Ki).

\[ \Delta k_{obs} = k_{inact,max} - \frac{[I]}{[I]+K_i} \]  

Statistical analysis: Statistical differences in the logarithm of Ki values among CYP3A4 variants were determined by ANOVA (analysis of variance) followed by Bonferroni multiple comparison tests using SPSS software for Windows (version 16 SPSS, Chicago, IL). A p value of <0.05 was considered statistically significant.

Results

Both EM and CAM decreased the activity of CYP3A4 in a time-dependent manner. The inactivation rate constants of inhibitors were concentration-dependent (Figs. 1 and 2). The k inact,max values of EM for CYP3A4.2 and CYP3A4.7 were 0.54- and 0.56-fold of that for CYP3A4.1, respectively, while those for CYP3A4.16 and 4.18 were similar to those for the wild type (Table 1). The k value of EM for CYP3A4.7 and 4.18 were 0.4- and 0.72-fold of that for CYP3A4.1, respectively. In contrast, the Ki values for CYP3A4.2 and 4.16 were 1.2- and 2.2-fold higher than for CYP3A4.1. With regard to CAM, the k inact,max value for CYP3A4.2 was 0.62-fold of that for CYP3A4.1, while those for other variants were comparable (Table 1). The Ki values for CYP3A4.2, 4.7, and 4.18 were decreased to 0.4-, 0.42-, and 0.64-fold, respectively, while that for CYP3A4.16 was increased 2.4-fold (Table 1, Fig. 2). Inhibitory potencies, i.e., Ki values, of EM and CAM among CYP3A4 variants varied 5.4- and 6-fold, respectively (Table 1).

Discussion

With regard to the CYP3A4 variants, it is well known from in vitro5,6) and in vivo13-17) studies that enzymatic activities are decreased in CYP3A4.7 and 4.16, increased in CYP3A4.18, and almost unchanged in CYP3A4.2, in comparison with CYP3A4.1, at least for the substrates examined. In the present study, the influence of genetic variation on the enzymatic activities of each variant was consistent with previous findings5,13-17) (data not shown).

The Ki value of EM for CYP3A4.1 was 1.04 (0.589–1.85) µM (Table 1), consistent with the Ki values previously reported for recombinant CYP3A4.1, i.e., 0.92 µM8) and 2.3 µM.9) The Ki value of CAM for CYP3A4.1 was 0.793 (0.650–0.966) µM (Table 1), which was comparable to those previously reported, i.e., 2.25 µM8) and 4.1 µM.9) No statistically significant difference was detected in the Ki values among genetic variants. Since none of these Ki values took into account the unbound fraction (fu, unbound) in the microsomal preparation, it may not adequate to compare these values directly.18,19) However, the aim of our present study was to
investigate the relative influence of genetic variation on Ki values rather than to determine their absolute values, so that the f_{mic} value itself is not considered significant.

Inhibitory potencies of both EM and CAM differed among CYP3A4 variants. A genetic variant CYP3A4.18 with amino acid substitution (L293P) from leucine to hydrophobic proline, a well-known helix breaker, in substrate recognition site (SRS)-420,21) may conceivably affect the accessibility of substrates. In fact, the Ki values of EM and CAM for this variant in this study were about 0.7-fold of that for the wild type. Another variant CYP3A4.2 is characterized by an amino acid substitution (S222P), which is located in the membrane interaction loop22) between helices F and G from hydrophilic serine to hydrophobic and bulky proline and also seems to confer the change in accessibility of substrates. Interestingly, the Ki value for EM was increased while that for CAM was decreased in CYP3A4.2 in the present study. The cause of this opposite influence remains to be investigated. With regard to a genetic variant CYP3A4.16 with decreased enzyme activity,16,17) its amino acid substitution, T185S, is located in an allosteric site, helix E, not close to SRS.20,22–25) However, even in an allosteric site outside SRS, certain amino acid substitutions may conceivably affect the accessibility of substrates. The interaction of a ligand with an allosteric site of CYP3A4 is considered to modulate the binding of the ligand to SRS. Recently, Davydov et al.26) have directly shown using fluorescence resonance energy transfer techniques that peripheral binding of some substrates causes a substantial spin shift and serves as a prerequisite for the binding in the active site. The amino acid substitution in the allosteric site of CYP3A4.16 may possibly affect the accessibility of substrates to SRS in the same way.

Table 1. Kinetic parameters of testosterone 6β-hydroxylation in CYP3A4 wild type and variants

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>EM (min⁻¹)</th>
<th>CAM (min⁻¹)</th>
<th>K_i (µM)</th>
<th>K_i (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP3A4.1</td>
<td>5</td>
<td>0.0293 ± 0.0067</td>
<td>0.0255 ± 0.0110</td>
<td>1.04 (0.589–1.85)</td>
<td>0.793 (0.650–0.966)</td>
</tr>
<tr>
<td>CYP3A4.2</td>
<td>5</td>
<td>0.0164 ± 0.0076</td>
<td>0.0160 ± 0.0075</td>
<td>1.21 (0.344–4.29)</td>
<td>0.319 (0.0488–2.09)</td>
</tr>
<tr>
<td>CYP3A4.7</td>
<td>5</td>
<td>0.0159 ± 0.0076</td>
<td>0.0196 ± 0.0025</td>
<td>0.415 (0.124–1.38)</td>
<td>0.335 (0.222–0.507)</td>
</tr>
<tr>
<td>CYP3A4.16</td>
<td>5</td>
<td>0.0293 ± 0.0078</td>
<td>0.0216 ± 0.0098</td>
<td>2.24 (1.13–4.46)</td>
<td>1.91 (0.776–4.72)</td>
</tr>
<tr>
<td>CYP3A4.18</td>
<td>5</td>
<td>0.0248 ± 0.0055</td>
<td>0.0250 ± 0.0071</td>
<td>0.753 (0.507–1.12)</td>
<td>0.510 (0.332–0.783)</td>
</tr>
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</table>

Arithmetic mean ± S.D. for k_{max}; geometric mean (−1S.D. to +1S.D.) for K_i.
Besides the affinity of inhibitor, the extent of MBI is affected also by the turnover rate of CYP3A4, which is determined by both synthesis and degradation rate constants.\(^{27}\) To estimate the clinical influence of genetic variants on DDI, further study is required to determine the effects of CYP3A4 genetic variants on its turnover rate, preferably in the in vivo settings, since the turnover rate estimated in the in vitro study may considerably differ from that in vivo. However, low allelic frequency of CYP3A4 variants makes the in vivo study almost infeasible. While the basal level of CYP3A4 expression may also reflect the turnover rate to some extent, no study reported a quantitative difference in the protein level among subjects bearing genetic variants.

Another possible explanation for the difference in the MBI parameters is the change in the enzymatic kinetics itself. A mass-balanced model for the enzyme kinetics for MBI is represented by Eq. (2), where enzyme-inhibitor intermediate complex, [E-I], is assumed to be irreversibly transformed into an inactivated form of enzyme, [E\text{inact}], at a rate constant of \(k_o\), where [E], [I], and [P] represent active enzyme, inhibitor, and metabolite, respectively.

\[
\begin{align*}
[E] + [I] &\xrightleftharpoons[k_{-1}]{k_1} [E-I] \\
\xrightarrow{k_4} & [E] + [P] \\
\xrightarrow{k_{\text{inact}}} & [E\text{inact}]
\end{align*}
\]  

(2)

Solving the mass-balanced equations (not shown) for rate constants under the steady-state yields Eqs. (3) and (4). Equation (4) implies that the differences in the \(K_I\) values among variants are not necessarily attributable to variation of the binding or dissociation constant (\(k_{-1}\) or \(k_1\)) of an inhibitor to CYP3A4 enzyme but also attributable to formation rate constants of metabolic intermediate or metabolite (\(k_2\) or \(k_3\)) or inactivation rate constants, \(k_o\). Accordingly, a decrease in \(K_I\) value can result from a decrease in \(k_{-1}\), \(k_1\), or \(k_o\), and the decrease in \(k_{\text{inact,max}}\) value can be the result of a decrease in \(k_2\) or \(k_4\).

\[
k_{\text{inact,max}} = \frac{k_2 \cdot k_4}{k_2 + k_3 + k_4}
\]  

(3)

\[
K_I = \frac{k_3 + k_4}{k_2 + k_3 + k_4}
\]  

(4)

We have previously reported\(^{40}\) that the pattern of the influence of genetic variation on the inhibitory kinetics differs between two competitive inhibitors, cimetidine and itraconazole. Similar inconsistency in the influences of genetic variations on the inhibitory kinetics among inhibitors has been also reported for CYP2C9.\(^{28}\)

In our present study, however, the influence of genetic variations on the \(K_I\) and \(k_{\text{inact,max}}\) values was similar between EM and CAM, possibly due to the structural similarity of EM and CAM and conceivably not attributable to the inhibitory nature, i.e. MBI.

To quantitatively estimate the clinical impact of genetic variation of CYP3A4 on DDI caused by EM or CAM, the change in the plasma concentration of EM or CAM (which is metabolized by CYP3A4) by its genetic variation should be also taken into account. However, clinical studies have not been carried out to investigate the effects of CYP3A4 genetic variation on the pharmacokinetics of EM and CAM, mainly because of the low genetic frequencies of CYP3A4 variants. Another approach, e.g. in vitro study and in vitro–to–in vivo extrapolation using physiologically-based population pharmacokinetic simulation,\(^{29}\) may possibly be useful to estimate the difference in pharmacokinetics of EM and CAM among subjects bearing genetic variations for CYP3A4.

As well as MBI, competitive inhibition may simultaneously occur in some cases so that the potency of competitive inhibition should be also evaluated. In our present study, without pre-incubation (pre-incubation time = 0), the enzymatic activities remained >70% of control in all the variants even with the highest concentrations of inhibitors (2 \(\mu\)M for EM and 5 \(\mu\)M for CAM during metabolism of TST), which were far lower than competitive inhibition constants (\(K_I\)), previously reported to be 31–113 \(\mu\)M\(^{20}\) and 975 \(\mu\)M\(^{31}\), respectively, for inhibition of TST metabolism. Therefore, DDI evoked by EM and CAM is quite likely to be attributable only to MBI and not to competitive inhibition.

In conclusion, The MBI potencies of EM and CAM may possibly differ among wild type CYP3A4 and variants with differences in the inhibitory potencies, \(K_I\), of 5.4- and 6-fold, respectively, among variants. The genetic variation of CYP3A4 may possibly be one of the causes of the interindividual variations in the extent of DDI mediated by MBI of CYP3A4.

References


4) http://www.cypalleles.ki.se/cyp3a4.htm the Human Cytochrome P450 (CYP) Allele Nomenclature Committee Web Site.


