Comparison of the inhibitory profiles of itraconazole and cimetidine in cytochrome P450 3A4 genetic variants

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Abbreviations: CYP: cytochrome P450, DDI: drug-drug interactions, TST: testosterone, ITCZ: itraconazole, CMD: cimetidine
Abstract

Cytochrome P450 (CYP) 3A4, an important drug metabolizing enzyme, is known to have genetic variants. We have previously reported that CYP3A4 variants such as CYP3A4.2, .7, .16, and .18 show different enzymatic kinetics from CYP3A4.1 (wild type). In this study, we quantitatively investigated the inhibition kinetics of two typical inhibitors, itraconazole (ITCZ) and cimetidine (CMD), on CYP3A4 variants and evaluated whether the genetic variation leads to interindividual differences in the extent of CYP3A4-mediated drug interactions. The inhibitory profiles of ITCZ and CMD on the metabolism of testosterone (TST) were analyzed by using recombinant CYP3A4 variants. The genetic variation of CYP3A4 significantly affected the inhibition profiles of the two inhibitors. In CYP3A4.7, the $K_i$ value for ITCZ was 2.4-fold higher than that for the wild type enzyme, while the $K_i$ value for CMD was 0.64 fold lower. In CYP3A4.16, the $K_i$ value for ITCZ was 0.54-fold lower than for wild type CYP3A4, while the $K_i$ value for CMD was 3.2-fold higher.

The influence of other genetic variations also differed between the two inhibitors. Docking simulations could explain the changes in the $K_i$ values based on the accessibility of TST and inhibitors to the heme moiety of the CYP3A4 molecule. In conclusion, the inhibitory effects of an inhibitor differ among CYP3A4 variants, suggesting that the genetic variation of CYP3A4 may contribute, at least in part, to interindividual differences in drug interactions mediated by CYP3A4.
inhibition, and the pattern of the influences of genetic variation differs among inhibitors as well as substrates.
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 (Shimada et al., 1994; Guengerich, 1999; Guengerich, 2008). Twenty genetic variants that lead to amino acid substitution have been identified in the CYP3A4 gene (http://www.cypalleles.ki.se/cyp3a4.htm).

In our previous study, we compared the enzymatic activities of major CYP3A4 variants from allele with relatively higher allelic frequency, such as CYP3A4*1 (wild type),*2 (2.7% in Caucasian) (Sata et al., 2000), *7 (1.4 - 3% in Caucasian) (Eiselt et al., 2001; Lamba et al., 2002), *16 (1.4-5% in Japanese) (Lamba et al., 2002; Fukushima-Uesaka et al.,2004), and *18 (1.3-2.8% in Japanese) (Fukushima-Uesaka et al.,2004) by using *Escherichia coli* expression systems (Miyazaki et al., 2008). CYP3A4.2 and CYP3A4.16 exhibited lower catalytic activity for 6-β hydroxylation of testosterone (TST), a probe substrate of CYP3A4, than CYP3A4.1 (wild type), while CYP3A4.18 had a higher activity and CYP3A4.7 had comparable activity. Other researchers have also reported similar results; the TST activity was lower in CYP3A4.2, .7, and .16 variants than CYP3A4.1 (wild type) (Sata et al., 2000; Eiselt et al., 2001; Lamba et al., 2002; Murayama et al., 2002; Fukushima-Uesaka et al.,2004) and higher with CYP3A4.18 (Kang et al., 2009). Kang et al. (2009) have recently reported the *in vivo* influence of CYP3A4*18, i.e., subjects bearing the *18 allele...
show low bone mineral density ostensibly because of the enhanced turnover of both TST and estrogen. Taken together, genetic variation of CYP3A4 is quite likely to be a key factor in interindividual differences in responses to CYP3A4 substrates (Kang et al., 2009).

On the other hand, inhibition of CYP3A4 is a major cause of drug-drug interactions (DDI) because CYP3A4 is responsible for the metabolism of many drugs that are widely used in the clinical settings (Zhou et al., 2007). Therefore, genetic variations of CYP3A4 that result in altered inhibitory kinetics might contribute to interindividual differences in the extent of CYP3A4-mediated DDI. However, the difference in the inhibition kinetics of CYP3A4 inhibitors between CYP3A4 genetic variants remains to be characterized. Moreover, no researchers have reported the clinical effect of genetic variation, i.e., CYP3A4*2, *7, *16 and *18, on the extent of drug-drug interaction.

Characterization of the nature of binding of substrate and inhibitor to CYP3A4 may also aid in understanding differences in the inhibitory kinetics among variants. After the crystal structure of CYP3A4 was determined (Williams et al., 2004; Yano et al., 2004), several docking simulations have been carried out to identify the amino acid residues responsible for ligand binding (Kriegl et al., 2005; Park et al., 2005) and to reveal and predict their binding nature (Unwalla et al., 2010; Teixeira et al., 2010).
The aims of this study were to characterize the inhibition kinetics of two typical CYP3A4 inhibitors with distinctive structures, itraconazole (ITCZ) and cimetidine (CMD), on the TST 6β-hydroxylation activity of recombinant CYP3A4.1, .2, .7, .16, .18 variants prepared from E. coli expression systems and to compare them with the results of docking simulation study for CYP3A4 variant molecules, substrates, and inhibitors.
Materials and Methods

Chemicals and Materials

TST [(8R,9S,10R,13S,14S,17S)-17-hydroxy-10,13-dimethyl-1,2,6,7,8,9,11,12,14,15,16,17-
dodecahydrocyclopenta[a]phenanthren-3-one] was purchased from Nacalai Tesque (Kyoto, Japan).

6β-hydroxytestosterone (6β-OHT) and hydrocortisone were purchased from SPIbio bertin Pharma (Bretonneux, France).

ITCZ [(2R,4S)-rel-1-(butan-2-yl)-4-{4-[4-[(2R,4S)-2-(2,4-dichlorophenyl)-2-(1H-1,2,4-triazol-1-ylmethyl)-1,3-dioxolan-4-yl]methoxy]phenyl)piperazin-1-yl]phenyl]-4,5-dihydro-1H-1,2,4-triazol-5-one] was purchased from Sigma-Aldrich (St Louis, MO).

CMD [2-cyano-1-methyl-3-(2-[(5-methyl-1H-imidazol-4-yl)methylthio]ethyl)guanidine] was purchased from Wako Pure Chemical Industries (Osaka, Japan). The membrane fractions for CYP3A4.1 (wild type), .2 (S222P), .7 (G56D), .16 (T185S), and .18 (L293P) were prepared as described previously (Miyazaki et al., 2008). All other chemicals and reagents were analytical or HPLC grade and obtained from commercial sources.

Inhibition of enzymatic activities of CYP3A4 variants by ITCZ and CMD

An aliquot of 450 µl of 200 mM potassium phosphate buffer (pH 7.4) containing substrate (TST),
inhibitor (ITCZ or CMD), 0.5 mM EDTA, and CYP3A4 membrane fraction (0.5 nmol P450/ml) was pre-incubated at 37 ºC for 10 min and reactions were initiated with 50 µl of an NADPH-generating system (50 mM glucose 6-phosphate, 5 mM NADP+, 1 unit/ml glucose 6-phosphate dehydrogenase, and 30 mM MgCl2) to start reaction. The reaction mixtures (total of 500 µl) were incubated for 20 min and the reactions were terminated by adding 450 µl of ice-cold acetonitrile, followed by spiking with 50 µl of 0.5 mM hydrocortisone in methanol (internal standard). After centrifugation at 1200 × g for 20 min at 4 ºC, 6β-OHT (in the supernatant) was determined by the HPLC-UV method described below. TST and ITCZ were dissolved in ethanol and methanol, with the final concentrations of solvent in reaction mixture of ≤1% and ≤2.5% (v/v), respectively, which have been shown not to affect CYP3A4 activity (Chauret et al., 1998; Busby et al., 1999; Easterbrook et al., 2001). The enzymatic activity of CYP3A4 was evaluated by determining the production rate of 6β-OHT from TST.

Analysis of 6β-OHT using HPLC-UV systems

Concentrations of 6 β-OHT were measured by an HPLC-UV method reported previously (Miyazaki et al., 2008). 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, Kyoto, Japan). The mobile phase consisted of methanol and water (58:42, v/v), pumped at a rate of 1.0 ml/min. The absorbance of 6β-OHT was measured at 254 nm, and the detection limit was 0.3 µM.

Analysis of metabolism and inhibition kinetics

The reaction rate (v) in the presence of a competitive inhibitor, such as ITCZ or CMD, is described by following Eq 1.

\[ v = \frac{V_{\text{max}} [S]}{[S] + K_m \cdot (1 + \frac{[I]}{K_i})} \quad \text{Eq. 1} \]

Eq 1 can be rearranged to give Eq 1'.

\[ v = \frac{V_{\text{max}}}{1 + \left\{1 + \exp(\ln[I] - \ln[K_i])\right\}\exp(\ln[K_m] - \ln[S])} \quad \text{Eq. 1'} \]

Eq 1' was simultaneously fit to the observed reaction rate at varying concentrations of substrate ([S]) and inhibitor ([I]) using nonlinear least-squares fitting with software (MLAB, Civilized Software Inc., MD) to obtain parameters of enzyme kinetics, i.e., logarithm of Michaelis constant (\(\ln K_m\)), maximum reaction rate (Vmax), and logarithm of inhibitory constant (\(\ln K_i\)), since the parameters \(K_m\) and \(K_i\) conceivably follow log-normal distribution.
Docking simulations of testosterone and inhibitors to CYP3A4 variants

Molecular docking studies were performed on two-CYP3A4 specific inhibitors with CYP3A4 variants, according to our previous reported method (Okada et al., 2009; Shimada et al., 2010). In brief, the CYP3A4 variant primary sequences were aligned with human CYP3A4 (Protein Data Bank code 1TQN) according to the reported amino acid substitution (Sata et al., 2000, Eiselt et al., 2001; Lamba et al., 2002; Murayama et al., 2002; Fukushima-Uesaka et al., 2004) using MOE software (version 2009.10; Chemical Computing Group, Montreal, Canada) for modeling of a three-dimensional structure. Docking simulations were carried out for TST (substrate) and ITCZ or CMD (inhibitor) binding to CYP3A4 using the MMFF94x force field distributed in the MOE Dock software. The docking experiments were ranked according to total interaction energy (U value) (Okada et al., 2009).

Statistical analysis

Statistical differences between the $K_{i}$ values of CYP3A4 variants were determined by ANOVA, analysis of variance followed by Dunnett’s multiple comparison tests using SPSS software for Windows (version 16 SPSS, Chicago). P values of $<0.05$ were considered statistically significant.
Results

Comparison of inhibition kinetics of CYP3A4 inhibition for CYP3A4 variants

Both ITCZ and CMD inhibited the TST 6β-hydroxylation activity of CYP3A4 variants in a concentration-dependent manner (Fig. 1). The $K_i$ value of ITCZ for CYP3A4.7 was 179 µM and significantly (2.4-fold) higher than that for CYP3A4.1 (76 µM), while that of CMD was 230 µM and significantly (1.6-fold) lower than that for CYP3A4.1 (370 µM) (Table 1). In contrast, with regard to CYP3A4.16, the $K_i$ value of ITCZ was 0.54-fold that for CYP3A4.1 and the $K_i$ value of CMD was 3.2-fold, significantly increased in comparison with that for CYP3A4.1. For CYP3A4.18, the $K_i$ value of CMD was significantly lower (0.39-fold), while for CYP3A4.2 the $K_i$ value of ITCZ was lower (0.59-fold) than that for CYP3A4.1 (Table 1).

Docking simulations

CYP3A4 variant crystal structures were generated in a homology model of CYP3A4 wild type using a MOE program. In the docking simulations with TST (substrate) and ITCZ (inhibitor) into CYP3A4 variants, the most stable energy states were in the positions shown in Figs. 2A and 2B. In consistent with the results of enzymatic study, docking simulation shows that, in CYP3A4.1, ITCZ is docked so that its azole ring is located on the vertical center line of the heme ring, whereas, in
CYP3A4.7, the position of ITCZ is far from heme ring and its orientation is also altered. Therefore, the access of ITCZ to heme region, the reaction center for TST hydroxylation, of CYP3A4.7 was impaired in the presence of TST.

For CMD, the most stable energy states in the position of TST and CMD are shown in Figs. 2C and 2D. In CYP3A4.1, CMD was shown to dock vertically with its imidazole ring in interacting with the heme ring. However, in CYP3A4.16, CMD is docked into farther position with opposite orientation so that the access of the imidazole ring to the heme region is impaired in comparison with that in CYP3A4.1. These results are also consistent with the kinetic observations in our enzymatic study (vide supra).
Discussion

Consistent with our previous report (Miyazaki et al., 2008), the $K_m$ values for TST 6β-hydroxylation were increased in CYP3A4.7 and .16, decreased in CYP3A4.18, and comparable in CYP3A4.2 (in comparison with CYP3A4.1). The observed influences of genetic variation on the enzyme catalytic efficiency ($V_{max}/K_m$) were also consistent with previous in vitro findings. (Sata et al., 2000; Eiselt et al., 2001; Lamba et al., 2002; Murayama et al., 2002; Fukushima-Uesaka et al., 2004)) The absolute $K_m$ and $V_{max}$ values in the present study were not identical to those in our previous report (Miyazaki et al., 2008). Although the cause of this discrepancy remains uncertain, minor modification of the experimental condition, such as the composition of reaction buffer to solubilize both substrate and inhibitor in our present study, may have affected the absolute value of $V_{max}/K_m$. The $K_i$ value of ITCZ for TST 6β-hydroxylation in recombinant CYP3A4.1 in this study was 76 nM (Table 1) and was comparable to $K_i$ values previously estimated in human liver microsomes, i.e., 230 nM (corrected by $f_{u,mic}$ 0.056) (Galetin et al., 2005) and 10-27 nM (Zhou et al., 2007). The observed $K_i$ value of for CMD was 370 µM (Table 1) and again consistent with the $K_i$ value of 267 µM reported by Zhou et al (Zhou et al., 2007). The $K_i$ values for ITCZ and CMD varied 4- and >8-fold among CYP3A4 variants, respectively. Moreover, the influence of genetic variations was not parallel, e.g., the $K_i$ value of ITCZ was increased in CYP3A4.7 and decreased in
CYP3A4.16, while that of CMD was decreased in CYP3A4.7 and increased in CYP3A4.16. In other words, the influence of genetic variation on the inhibition kinetics is also dependent on the inhibitors.

Nonspecific binding of a substrate or inhibitor to the in vitro microsomal preparation may lead to underestimation of $K_m$ or $K_i$ for an enzyme. Correction of a $K_i$ value for an inhibitor requires consideration of the unbound fraction ($f_{u,\text{mic}}$) in the microsomal preparation (Austin et al., 2002; Austin et al., 2005). ITCZ, used in this study as a typical inhibitor, is high lipophilic and its nonspecific binding to membrane fraction is 62-91% (Isoherranen et al., 2004) in Supersomes® and 80-88% (Isoherranen et al., 2004) and 95% (Ishigam et al., 2001) in human liver microsomes. However, the aim of present study was to investigate the relative influence of genetic variation but not to determine the absolute $K_i$ values, so accordingly we did not measure $f_{u,\text{mic}}$.

Perhaps the most plausible explanation for the differences among CYP3A4 variants is conformational changes caused by amino acid substitution that may lead altered accessibility of substrate and inhibitors to the heme iron of CYP3A4. We have previously carried out docking simulation studies for substrates and inhibitors of CYP proteins, and many of the results are consistent with the enzymatic characteristics observed in the in vitro studies (Okada et al., 2009). In this study, we constructed a model to simulate the CYP3A4 variants with amino acid
substitutions using a docking simulation program, MOE, and carried out simulation for the binding of substrates and inhibitors. As a result, CYP3A4.7 and CYP3A4.16 were shown to be structurally less suitable for access of ITCZ and CMD to the heme iron, respectively. These results are consistent with experimental observations of increased $K_i$ values. It is likely that distinctive changes in the accessibility of inhibitors to the heme iron in each variant result in the distinctive changes in the affinity of each inhibitor. Apart from the accessibility, differences in the formation of coordinate bonds between CYP3A4 and inhibitors may be also responsible for differences in the influence of genetic variation among the inhibitors. With regard to the binding of the inhibitor metyrapone to CYP3A4, it has been shown that lone electron pair of the nitrogen atom of the pyrimidine ring forms a coordinate bond with the heme iron in CYP3A4 (Park et al., 2005). Both ITCZ and CMD have a negative charge moiety (imidazole and thiazole, respectively) that can form a coordinate bond with the CYP3A4 iron, and the differences in hydrogen bonding strength may affect the affinity of inhibitors to CYP3A4.

Similar observations regarding differences in potency of inhibitors among genetic variants have been reported in CYP2C9. Kumar et al (Kumar et al., 2006) have reported that the effect of an inhibitor varies among CYP2C9 variants and that the pattern of the influence of genetic variation is also distinctive for each inhibitor. A study with a single inhibitor is not sufficient to understand the
effects of genetic variation on the potency of CYP inhibitors.

We estimated the clinical impact of CYP3A4 genetic variations on the extent of DDI by using the $K_i$ values of inhibitors (Table 1). The ratio of the AUC in the presence of an inhibitor (AUC') to that without inhibitor (AUC), a measure of DDI, can be estimated using equation 2 (Galetin et al., 2005):

$$\frac{AUC'}{AUC} = 1 + \frac{[I]}{K_i} \ldots Eq.2$$

where $[I]$ represents the concentration of inhibitor. Assuming that the concentration of each inhibitor ([I]) is fourfold of respective $K_i$ value in CYP3A4.1 (304 nM for ITCZ and 1,480 µM for CMD), the AUC of a substrate is increased 5-fold by the inhibitor in a CYP3A4.1 homozygote (AUC ratio = 5.0). With the equivalent concentration of inhibitor, the AUC ratio by ITCZ is predicted to be 8.4 for a CYP3A4.16 homozygote (increased DDI), whereas it is predicted to be only 2.7 in a CYP3A4.7 homozygote. For CMD (with the same assumption that AUC in CYP3A4.1 is increased 5-fold by CMD), the AUC ratio is predicted to be 7.3 for CYP3A4.7, 2.2 for CYP3A4.16 and 12 for CYP3A4.18, suggesting that genetic variation leads to interindividual variations in DDIs.

In conclusion, the potencies of typical inhibitors differed among wild type CYP3A4 and CYP3A4 variants. The inhibitory potencies of ITCZ and CMD varied 4- and 8-fold, respectively, among these variants. The pattern for genetic variations also differed between ITCZ and CMD.
These differences are hypothesized to result from the structural changes of CYP3A4 in each variant, which may affect the accessibility and affinity of an inhibitor to the catalytic site of CYP3A4. The genetic variation of CYP3A4 may possibly cause interindividual variations of DDI mediated by CYP3A4 inhibition.
Authorship Contributions

Participated in research design: Akiyoshi, Saito, Murayama, Yamazaki, Nakamura, Yamamoto, and Ohtani.

Conducted experiments: Akiyoshi, Saito, Murase, Miyazaki, Nakamura, Yamamoto, and Ohtani.

Contributed new reagents or analytic tools: Guengerich.

Performed data analysis: Murayama and Yamazaki.

Wrote or contributed to the writing of the manuscript: Akiyoshi, Saito, Murayama, Yamazaki, Guengerich, and Ohtani.
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Footnotes

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Legends for Figures

Figure 1. Inhibition kinetics of ITCZ or CMD for CYP3A4-mediated TST 6β-hydroxylation. (A) and (D), CYP3A4.1 (wild type). (B) and (E), CYP3A4.7. (C) and (F), CYP3A4.16. The oxidation activities represent the mean ± S.D. from independent experiments. The substrate concentration ranged from 20 to 500 µM and the ITCZ concentration ranged from 0 to 500 nM.  ○: 0 nM ITCZ; □: 50 nM ITCZ; ●: 100 nM ITCZ; ◆: 250 nM ITCZ; ▼: 500 nM ITCZ. The CMD concentration ranged from 0 to 500 µM. ○: 0 µM CMD; □: 100 µM CMD; ◆: 250 µM CMD; ●: 500 µM CMD; ▼: 1,000 µM CMD.

Figure 2. Docking simulation of TST, ITCZ, and CMD into P450 3A4 variants.

The heme group of the P450 is shown at the lower part of each panel. In the figure, oxygen, nitrogen, sulfur, and iron atoms are colored with red, blue, yellow, and light blue, respectively. (A) and (B) were based on an orientation for models of P450 3A4.1 and 7 with ITCZ interaction energies (U value) of 18.0 and 187, respectively. (C) and (D) were based on an orientation for models of P450 3A4.1 and 16 with CMD interaction energies (U value) of -19.7 and -22.7 respectively.
Table 1. Kinetic parameters of testosterone 6β-hydroxylation in CYP3A4 wild type and variants

<table>
<thead>
<tr>
<th>CYP3A4</th>
<th>n</th>
<th>$V_{max}$</th>
<th>$K_m$</th>
<th>$V_{max}/K_m$</th>
<th>$K_i$</th>
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<tr>
<td></td>
<td>nmol/min/nmol P450</td>
<td>µM</td>
<td>µl/min/nmol P450</td>
<td>ITCZ, nM</td>
<td>CMD, µM</td>
</tr>
<tr>
<td>CYP3A4.1</td>
<td>5</td>
<td>22 (18.6-25.1)</td>
<td>290 (232-372)</td>
<td>0.074 ± 0.0094</td>
<td>76 (45.1-129)</td>
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<tr>
<td>CYP3A4.2</td>
<td>5</td>
<td>6.3 (4.96-7.63)</td>
<td>220 (177-272)</td>
<td>0.029 ± 0.0073</td>
<td>45 (30.7-65.7)</td>
</tr>
<tr>
<td>CYP3A4.7</td>
<td>5</td>
<td>38 (24.9-51.5)</td>
<td>400 (243-663)</td>
<td>0.090 ± 0.018</td>
<td>180* (146-219)</td>
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<tr>
<td>CYP3A4.16</td>
<td>5</td>
<td>4.2 (3.77-4.63)</td>
<td>440 (338-555)</td>
<td>0.0099 ± 0.0028</td>
<td>41 (17.1-99.8)</td>
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<tr>
<td>CYP3A4.18</td>
<td>5</td>
<td>26 (22.0-29.3)</td>
<td>140 (83.6-223)</td>
<td>0.20 ± 0.096</td>
<td>82 (51.4-133)</td>
</tr>
</tbody>
</table>

geometric mean (-1SD - +1SD) for $V_{max}$, $K_m$, and $K_i$, arithmetic mean±SD for $V_{max}/K_m$

$n$, the number of experiment.

Values are expressed to two significant digits.

The $V_{max}/K_m$ value represents an average for each set of each data. *p<0.05 vs CYP3A4.1

DMD/2010/036780
Figure 1

**ITCZ**

a) CYP3A4.1

- Ki = 76.2 nM

b) CYP3A4.7

- Ki = 179 nM

c) CYP3A4.16

- Ki = 41.4 nM

**CMD**

d) CYP3A4.1

- Ki = 367 μM
e) CYP3A4.7

- Ki = 234 μM

f) CYP3A4.16

- Ki = 1,185 μM
Figure 2.