Difference in Mechanism-Based Inhibition of Cytochrome P450 3A4 and 3A5 by a Series of Fluoroquinolone Antibacterial Agents

Akiko Watanabe, Hideo Takakusa, Takako Kimura, Shin-ichi Inoue, Hiroyuki Kusuhara, and Osamu Ando

ABSTRACT
A series of fluoroquinolone antibacterial compounds were found to be irreversible (compounds 1–5) and quasi-irreversible (compounds 6–9) inhibitors of CYP3A4. The purpose of this study was to evaluate their mechanism-based inhibition (MBI) potency against CYP3A5. Compounds 1–5 were also irreversible inhibitors of CYP3A5, whereas compounds 6–9 showed neither irreversible nor quasi-irreversible inhibition of CYP3A5. Compounds 6 and 8 did not form a metabolite-intermediate complex with the heme of CYP3A5 during incubation. The structural analysis of the metabolites after incubation of compounds 1 and 6 with CYP3A5 revealed that their metabolites were identical to those produced by CYP3A4, including the precursors of which are speculated to account for the MBI of CYP3A4. The homology modeling of CYP3A5 suggests that four residues around the nitroso intermediate of compound 6 in the substrate-binding pocket of CYP3A4 correspond with the bulkier residues in CYP3A5—especially Phe210 in CYP3A5—which might contribute to the steric hindrance with the nitroso intermediate of compound 6. The substrate-binding pocket structure of CYP3A5 might prevent the nitroso intermediate from coordinate binding with the heme, thereby preventing quasi-irreversible inhibition. Our study may provide new insights into the observable differences between the inhibition of CYP3A4 and CYP3A5.

Introduction
Cytochrome P450 3A is an important member of the cytochrome P450 subfamily. CYP3A4 and CYP3A5 are the major isoforms of CYP3A because they are responsible for the metabolism of more than 50% of marketed drugs (Thummel and Wilkinson, 1998; Wilkinson, 2005). CYP3A4 and CYP3A5 show an 83% homology in their amino acid sequences; therefore, the substrate specificity of these isoforms overlaps (de Wildt et al., 1999; Niwa et al., 2014). CYP3A5 is polymorphically expressed in the human liver and intestine, and thereby causes interindividual variations in the disposition of CYP3A substrates such as midazolam and nifedipine (Lamba et al., 2002). Despite the highly conserved amino acid residues of both isoforms, the effect of some of their inhibitors, especially time-dependent inhibitors, apparently differs between CYP3A4 and CYP3A5. Generally, the inhibitors have shown a weaker effect on CYP3A5 than they have on CYP3A4 (Niwa et al., 2008). Raloxifene irreversibly inhibited CYP3A4 but not CYP3A5 (Pearson et al., 2007). The reactive intermediate binds covalently to an apoprotein of CYP3A4, and the difference in the amino acid residue modified by the reactive intermediate between the two isoforms (cysteine and serine at position 239 for CYP3A4 and CYP3A5, respectively) could account for the difference in susceptibility. Erythromycin, diltiazem, nicardipine, verapamil, and lapatinib have shown quasi-irreversible inhibition of CYP3A4, in which the metabolite intermediates (MIs) form stable complexes with the heme (MI complex). However, such complexes were not observed with CYP3A5 (McConn et al., 2004; Wang et al., 2005; Takakusa et al., 2011). In the case of lapatinib, the difference in the susceptibility was attributable to their ability to generate the hydroxylamine metabolite (M3), which was followed by the generation of a nitroso intermediate forming the MI complex with the heme. However, CYP3A5 generates other metabolites (M1 and M4) at a similar or slightly lower level than does CYP3A4 (Takakusa et al., 2011). In addition to the ability to generate the metabolite intermediate, it is possible that the structure of the substrate-binding pocket of CYP3A5 limits the intermediates’ access to the heme or disturbs the formation of the appropriate configuration of the reactive intermediate required to interact with the heme.

Previously, we established an assay to distinguish between irreversible and quasi-irreversible inhibition based on the mechanism involving the dissociation of MI complexes after treatment with potassium ferricyanide, which oxidizes the heme iron to the ferric form and recovers the enzymatic activity (Buening and Franklin, 1976; Muakkassah et al., 1982; Watanabe et al., 2007). Using this mechanism-based inhibition (MBI) reversibility assay, we demonstrated that a series of fluoroquinolone antibacterial compounds (compounds 1–9) are irreversible or quasi-reversible inhibitors (compounds 1–5 and 6–9, respectively) that contain either cyclopropylamine in the pyrrolidine ring or an amine moiety in the ring form of the 7-position of fluoroquinolone, respectively. The difference in the ring structure bearing a primary amine group in quinolones may determine their MBI mechanisms. In addition, compound 10, formed by the introduction of a methyl group at the carbon atom at the root of the C7-amino moiety
of compound 8, did not inhibit CYP3A4 despite its production of a nitroso intermediate, presumably owing to the steric hindrance of the methyl moiety (Watanabe et al., 2016). In this study, we investigated the reversible or quasi-irreversible inhibition of CYP3A5 by the fluoroquinolone derivatives. We report here that while irreversible inhibition was common for CYP3A4 and CYP3A5, quasi-reversible inhibition was not, although CYP3A5 also produced nitroso intermediates.

**Materials and Methods**

**Materials.** All of the tested fluoroquinolone compounds were synthesized by Daichi Sankyo Co., Ltd. (Tokyo). The synthesis method of compound 10 as a representative fluoroquinolone compound has been described in a previous report (Odagiri et al., 2013). Midazolam maleate salt was purchased from Sigma-Aldrich (St. Louis, MO). 1’-Hydroxymidazolam was purchased from Ultrafine (Manchester, United Kingdom). Potassium phosphate buffer (0.5 M), [13C3]-hydroxymidazolam, NADPH regenerating system solutions A and B, and recombinant human CYP3A5 supersomes containing cytochrome P450 reductase and cytochrome b5 were acquired from Corning (Woburn, MA). Potassium ferricyanide was purchased from Kanto Chemical Co., Inc. (Tokyo). All other reagents and solvents were of the highest grade commercially available.

**Reversibility of MBI using Recombinant Human CYP3A5 Supersomes.** This assay was performed as described previously (Watanabe et al., 2016). Briefly, recombinant human CYP3A5 supersomes (final concentration 10 pmol/ml) were preincubated with test compound (final concentration 30 or 100 μM) for 0 or 30 minutes, the preincubation solution was mixed with or without potassium ferricyanide (final concentration 1 mM), and then incubated for 10 minutes. The metabolic activity of the incubation solution was determined by incubating it with ferricyanide (final concentration 1 mM), and then incubated for 10 minutes. The concentration of the test compounds was 100 μM except for compounds 2 and 4, the concentration of which was 30 μM. Each bar represents mean ± S.D. of triplicate experiments. *P < 0.01; NS, not significant.

**TABLE 1**

Comparison of the inhibitory potential of fluoroquinolone compounds on CYP3A4 and CYP3A5.

<table>
<thead>
<tr>
<th>Compound</th>
<th>CYP3A4 Concentration</th>
<th>% Remaining</th>
<th>CYP3A5 Concentration</th>
<th>% Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μM</td>
<td></td>
<td>μM</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>100</td>
<td>32.1 ± 4.4</td>
<td>100</td>
<td>41.3 ± 1.4</td>
</tr>
<tr>
<td>2</td>
<td>30</td>
<td>46.4 ± 1.9</td>
<td>30</td>
<td>33.9 ± 2.4</td>
</tr>
<tr>
<td>3</td>
<td>100</td>
<td>42.4 ± 1.8</td>
<td>100</td>
<td>70.5 ± 2.1</td>
</tr>
<tr>
<td>4</td>
<td>30</td>
<td>45.3 ± 2.1</td>
<td>30</td>
<td>58.0 ± 2.8</td>
</tr>
<tr>
<td>5</td>
<td>30</td>
<td>47.0 ± 1.5</td>
<td>100</td>
<td>44.3 ± 2.5</td>
</tr>
<tr>
<td>6</td>
<td>10</td>
<td>46.4 ± 1.5</td>
<td>100</td>
<td>100 ± 4</td>
</tr>
<tr>
<td>7</td>
<td>100</td>
<td>60.1 ± 3.0</td>
<td>100</td>
<td>101 ± 1</td>
</tr>
<tr>
<td>8</td>
<td>100</td>
<td>62.2 ± 2.1</td>
<td>100</td>
<td>102 ± 1</td>
</tr>
<tr>
<td>9</td>
<td>30</td>
<td>57.7 ± 1.8</td>
<td>100</td>
<td>103 ± 3</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>101 ± 13</td>
<td>100</td>
<td>106 ± 12</td>
</tr>
</tbody>
</table>

where % of control (0 minutes) = \( \frac{v(0 \text{ minutes} \times \text{inhibitor})}{v(0 \text{ minutes} \times \text{solvent})} \times 100 \)

% remaining = \( \frac{\% \text{ of control (30 minutes)}}{\% \text{ of control (0 minutes)}} \times 100 \)

Mechanism-Based Inhibition of CYP3A5 by Fluoroquinolones

Fig. 1. Reversibility of MBI of CYP3A5 by fluoroquinolone compounds in recombinant human CYP3A5 supersomes. The percentage of control data after 0-minute preincubation followed by incubation with (dark gray) or without (black) potassium ferricyanide and that after 30-minute preincubation followed by incubation with (white) or without (light gray) potassium ferricyanide were obtained. The concentration of the test compounds was 100 μM except for compounds 2 and 4, the concentration of which was 30 μM. Each bar represents mean ± S.D. of triplicate experiments. *P < 0.01; NS, not significant.
of CYP3A4, the initial CYP3A5 model was constructed by copying the coordinate of the CYP3A4 crystal structure with the mutation to the CYP3A5 amino acid in a different amino acid position. The side chains of the mutation site of the initial CYP3A5 model were refined using the Prime protein refinement module of Maestro (Schrödinger, LLC).

Results

Reversibility of MBI of CYP3A5 by Fluoroquinolones. To investigate the inhibition of CYP3A5 by a series of irreversible and quasi-irreversible inhibitors (compounds 1–5 and 6–9, respectively), and the noninhibitor or weak inhibitor (compound 10) of CYP3A4 (Watanabe et al., 2016), the MBI reversibility assay was performed in recombinant human CYP3A5 supersomes (Fig. 1). The enzymatic activity of CYP3A5 monitored as the activity of 1'-hydroxymidazolam formation from midazolam was inactivated after 30-minute preincubation with each compound (compounds 1–5), and the reduced enzymatic activity was not restored by incubation with potassium ferriyanide. This result indicates that compounds 1–5 inactivated CYP3A5 irreversibly. The inhibitory potential of CYP3A4 and CYP3A5 by these compounds was compared (Table 1). For compounds 1–5, the % remaining value after 30-minute preincubation with CYP3A5 was almost comparable with (or slightly higher than that with) CYP3A4. In contrast, the enzymatic activity of CYP3A5 was unchanged even after 30-minute preincubation with compounds 6–10, at least at the concentrations tested.

Absorption Analysis of MI Complex Formation. The formation of a MI complex with the heme of CYP3A5 was confirmed by monitoring the absorbance difference between 455 and 490 nm for 20 minutes after the addition of the NADPH-generating system in the reaction mixture containing recombinant CYP3A5 supersomes and each test compound (Fig. 2). The absorbance difference was not changed during the incubation period for compounds 6, 8, and 10. This observation suggests that compounds 6, 8, and 10 did not form a MI complex with the heme of CYP3A5.

Structural Elucidation of the Metabolites of Compounds 1 and 6 after Incubation with Recombinant CYP3A5 Supersomes. To compare the metabolic profiles of the fluoroquinolone compounds between CYP3A4 and CYP3A5, we carried out a structural analysis by liquid chromatography–tandem mass spectrometry of the metabolites after incubating CYP3A5 with compound 1, a representative irreversible inhibitor of both CYP3A4 and CYP3A5. The analysis showed four metabolites related to oxidation reactions of the cyclopropylamine moiety of compound 1 (Supplemental Fig. 1). The proposed structure of each metabolite is shown in Fig. 3. All of the metabolites of compound 1 detected after incubation with CYP3A5 were also detected following incubation with CYP3A4 (Watanabe et al., 2016). In addition, we performed a structural analysis of metabolites after incubating CYP3A5 with compound 6, a representative quasi-irreversible inhibitor of CYP3A4 but not of CYP3A5. The analysis revealed five metabolites related to oxidation reactions of the amino azaspiro[4.4]nonan moiety of compound 6 (Supplemental Fig. 2). The proposed structure of each metabolite is shown in Fig. 4. The metabolites of compound 6 detected following its metabolism by CYP3A5 were unexpectedly identical to those generated by CYP3A4 (Watanabe et al., 2016).
CYP3A5 Homology Modeling. To study the substrate-binding pocket of CYP3A5, the homology model of CYP3A5 was constructed for amino acid residues 27–495, which corresponded to the CYP3A4 crystal structure (Fig. 5A). The homology model of CYP3A5 was superposed by the docking model of compound 6 and its nitroso metabolite to CYP3A4 obtained in a previous study (Watanabe et al., 2016) (Fig. 5B and C, respectively). The model suggests that nine hydrophobic residues (Phe57, Gln79, Phe108, Ile120, Leu210, Thr224, Val240, Ile369, and Met371) around the nitroso intermediate of compound 6 in the substrate-binding pocket of CYP3A4 are different in CYP3A5, which has the following corresponding residues: Leu57, Leu79, Leu108, Leu120, Phe210, Ile224, Leu240, Val369, and Ile371. Among the different amino acid residues, four (Leu108, Leu120, Phe210, and Leu240) in CYP3A5 were bulkier than the corresponding residues in CYP3A4. Furthermore, we also speculated that Phe210 in CYP3A5 might cause steric hindrance with the nitroso intermediate, but not with compound 6. Regarding the difference in the MI complex formation of the nitroso intermediate of compound 6 between CYP3A4 and CYP3A5, we focused on the amino acid residues at position 210 of CYP3A4 and CYP3A5. Interestingly, as shown in Fig. 6, the distance between the side chain of Phe210 in CYP3A5 and the closest side chain of the nitroso intermediate was 1.35 Å, while the distance between the side chain of Leu210 in CYP3A4 and the closest side chain of the nitroso intermediate was 4.63 Å. Therefore, there would be a steric crash between Phe210 in CYP3A5 and the nitroso intermediate.

Discussion

In this study, we focused on the difference in the inhibition of CYP3A4 and CYP3A5 by a series of fluoroquinolone antibacterial compounds, which are irreversible and quasi-irreversible inhibitors (compounds 1–5 and 6–9, respectively) of CYP3A4 (Watanabe et al., 2016). We investigated the inhibition of CYP3A5 by these fluoroquinolones by evaluating their MBI reversibility using recombinant human CYP3A5 supersomes (Fig. 1) and compared the inhibitory potential between CYP3A4 and CYP3A5 at the concentration examined (Fig. 1; Table 1); however, compounds 6–9 showed no inhibition of CYP3A5 at the same or lower concentration as reported for other CYP3A4 quasi-irreversible inhibitors (McConn et al., 2004; Wang et al., 2005; Niwa et al., 2008; Takakusa et al., 2011), the effects on CYP3A5 were clearly different from the effects on CYP3A4. Consistent with the lack of quasi-irreversible inhibition, compounds 6, 8, and 10 did not form a MI complex with the heme of CYP3A5 (Fig. 2).

The metabolites generated after incubating the fluoroquinolones with recombinant human CYP3A5 supersomes were structurally analyzed to compare their profiles with those of the metabolites produced by CYP3A4. All of the metabolites of compound 1 detected following incubation with CYP3A4 (Watanabe et al., 2016) were detected after incubation with CYP3A5 supersomes (Fig. 3). We speculate that cpd1-M3 was formed via the radical intermediate that covalently binds to CYP3A4 (Watanabe et al., 2016). The generation of cpd1-M3 by CYP3A5 suggests that the same mechanism underlies the irreversible inhibition of CYP3A5 by compound 1.

In addition, the metabolites of compound 6 detected following its metabolism by CYP3A5 were also unexpectedly identical to those...
were bulkier than the corresponding residues in CYP3A4. This observation is consistent with the results indicating that the CYP3A4 pharmacophore generated based on the in vitro data of the inhibitors is larger than that of CYP3A5 and 3A7 (Ekins et al., 2003). Among these bulky amino acid residues, Phe210 in CYP3A5 might cause steric hindrance with the nitroso intermediate, but not with compound 6 (Fig. 6). The 210-amino acid residue of CYP3A4 and CYP3A5 could also be important for recognizing aflatoxin B1 (Wang et al., 1998). A mutation involving the replacement of Leu with Phe at residue 210 in CYP3A4 revealed that its regioselectivity in aflatoxin B1 metabolism was similar to that of CYP3A5.

This study highlighted the difference in the susceptibility of CYP3A4 and CYP3A5 to quasi-irreversible inhibition by compounds 6–9 despite the generation of metabolites that could potentially form the MI complex with the heme. Furthermore, it also demonstrated that in contrast, the susceptibility of the isoforms to irreversible inhibition by compounds 1–5 was similar. In addition to the ability to generate the nitroso intermediate, we speculate that the difference in the substrate-binding pocket also contributes to the differential susceptibility of the isoforms. Compound 10 did not show quasi-irreversible inhibition of CYP3A5 as was observed for CYP3A4, which was possibly owing to the steric hindrance of the methyl group with the heme (Watanabe et al., 2016). In addition to designing compounds to prevent the production of the nitroso intermediate, the introduction of a moiety to inhibit MI complex formation would be another key strategy to avoid MBI in future drug development processes.

In conclusion, we demonstrated that fluoroquinolone antibacterial compounds 1–5 irreversibly inhibited both CYP3A4 and CYP3A5, whereas compounds 6–9 showed quasi-irreversible inhibition of CYP3A4 but not CYP3A5. Our study may provide new insights into the differential effects of the tested compounds as evidenced by the quasi-irreversible inhibition of CYP3A4 but not CYP3A5.

References


Authorship Contributions
Participated in research design: Watanabe, Kusuhara.
Conducted experiments: Watanabe, Takakusa, Kimura.
Contributed new reagents or analytic tools: Watanabe, Takakusa, Kimura.
Performed data analysis: Watanabe, Takakusa, Kimura.
Wrote or contributed to the writing of the manuscript: Watanabe, Takakusa, Kimura, Inoue, Kusuhara, Ando.


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Supplemental data

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Supplemental Figure 1. Extracted ion chromatograms of the metabolites in the samples after 30-min incubation of compound 1 with recombinant CYP3A5 Supersomes in the presence of NADPH (A) and the structure of compound 1 and proposed structures of the metabolites with the fragmentation schemes (B).
Supplemental Figure 2. Extracted ion chromatograms of the metabolites in the samples after 30-min incubation of compound 6 with recombinant CYP3A5 Supersomes in the presence of NADPH (A) and the structure of compound 6 and proposed structures of the metabolites with the fragmentation schemes (B).
Supplemental Figure 2. Continued.

B