Functional Characterization of CYP2B6 Allelic Variants in Demethylation of Antimalarial Artemether

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ABSTRACT:

Artemether (AM) is one of the most effective antimalarial drugs. The elimination half-life of AM is very short, and it shows large interindividual variability in pharmacokinetic parameters. The aim of this study was to identify cytochrome P450 (P450) isozymes responsible for the demethylation of AM and to evaluate functional differences between 26 CYP2B6 allelic variants in vitro. Of 14 recombinant P450s examined in this study, CYP2B6 and CYP3A4 were primarily responsible for production of the desmethyl metabolite dihydroartemisinin. The intrinsic clearance ($V_{max}/K_m$) of CYP2B6 was 6-fold higher than that of CYP3A4. AM demethylation activity was correlated with CYP2B6 protein levels ($P = 0.004$); however, it was not correlated with CYP3A4 protein levels ($P = 0.27$) in human liver microsomes. Wild-type CYP2B6.1 and 25 CYP2B6 allelic variants (CYP2B6.2-CYP2B6.21 and CYP2B6.23-CYP2B6.27) were heterologously expressed in COS-7 cells. In vitro analysis revealed no enzymatic activity in 5 variants (CYP2B6.8, CYP2B6.12, CYP2B6.18, CYP2B6.21, and CYP2B6.24), lower activity in 7 variants (CYP2B6.10, CYP2B6.11, CYP2B6.14, CYP2B6.15, CYP2B6.16, CYP2B6.20, and CYP2B6.27), and higher activity in 4 variants (CYP2B6.2, CYP2B6.4, CYP2B6.6, and CYP2B6.19), compared with that of wild-type CYP2B6.1. In kinetic analysis, 3 variants (CYP2B6.2, CYP2B6.4, and CYP2B6.6) exhibited significantly higher $V_{max}$, and 3 variants (CYP2B6.14, CYP2B6.20 and CYP2B6.27) exhibited significantly lower $V_{max}$ compared with that of CYP2B6.1. This functional analysis of CYP2B6 variants could provide useful information for individualization of antimalarial drug therapy.

Introduction

Malaria is a very serious problem in many countries, and there are more than 200 million cases that result in approximately 1 million deaths worldwide each year (World Health Organization, World Malaria Report 2009, http://www.who.int/malaria/world_malaria_report_2009/en/index.html). The management of malaria has traditionally relied on monotherapy with quinolines such as quinine, mefloquine, and chloroquine. However, the widespread and excessive use of these agents has resulted in drug resistance (Wernsdorfer, 1991; Price and Nosten, 2001; Le Bras and Durand, 2003). In several studies, artemisinins, unique sesquiterpene lactone endoperoxides, have been used in areas with multidrug-resistant Plasmodium falciparum malaria (Woodrow et al., 2005; Gautam et al., 2009; World Health Organization, 2010).

Artemisinin is a natural antimalarial agent derived from the Chinese medicinal plant Artemisia annua (Klayman, 1985). The artemisinin derivative artemether (AM) is the most effective antimalarial drug. AM has a fast onset of action, therapeutic efficacy against multidrug-resistant malaria, and few side effects, although neurotoxicity has been observed in experimental mammals (Hien and White, 1993; Brewer et al., 1994). AM is mainly converted to dihydroartemisinin (DHA) (Fig. 1), a desmethyl metabolite that contributes to the major-ity of the antimalarial activity. The conversion of AM to DHA is catalyzed by cytochrome P450 (P450) (van Agtmael et al., 1999b,c; Navaratnam et al., 2000). However, the elimination half-life of AM is very short, and it shows large interindividual variability in pharmacokinetic parameters (Na Bangchang et al., 1994; Mordi et al., 1997; van Agtmael et al., 1999a; Lefe`vre et al., 2002; Ali et al., 2010; Mwesigwa et al., 2010).

The P450 isozymes CYP2B6 and CYP3A4 are thought to catalyze AM demethylation (Navaratnam et al., 2000). In contrast, it has been reported that CYP2D6 and CYP2C19 make no major contribution to this reaction (van Agtmael et al., 1998), and the role of other P450s remains unclear. CYP2B6 plays a major role in the biotransformation of several therapeutically important drugs, including cyclophosph-
amide, bupropion, selegiline, efavirenz, nevirapine, and methadone (Roy et al., 1999; Hesse et al., 2000; Hiderstrand et al., 2001; Salonen et al., 2003). Many genetic polymorphisms in the CYP2B6 gene have been reported, and these are thought to be responsible for interindividual and interethnic differences in responses to CYP2B6 substrate drugs [Zanger et al., 2007; Human Cytochrome P450 (CYP) Allele Nomenclature Committee, 2008; Mo et al., 2009]. In the case of chemotherapy using cyclophosphamide, the increasing enzymatic activity of CYP2B6 variants can be associated with the increased blood concentration of the active metabolite of the drug, resulting in a heightened risk of side effects (Xie et al., 2003, 2006; Nakajima et al., 2007).

Several functional analyses of CYP2B6 variant proteins, using an in vitro expression system, have been reported. Watanabe et al. (2010) characterized the functional relevance of many CYP2B6 variants, including CYP2B6.1 to CYP2B6.28, using 7-ethoxy-4-trifluoromethylcoumarin and selegiline as substrates, and reported that CYP2B6.8, CYP2B6.11, CYP2B6.12, CYP2B6.13, CYP2B6.15, CYP2B6.18, CYP2B6.21, CYP2B6.24, and CYP2B6.28 were inactive with regard to these compounds. These results were consistent with those of a number of in vitro studies using bupropion as a substrate. In contrast, CYP2B6.16, CYP2B6.19, and CYP2B6.27 exhibited activity toward 7-ethoxy-4-trifluoromethylcoumarin and inability to detect selegiline metabolism. Several researchers have reported that these CYP2B6 variants exhibited decreased protein expression/activity when bupropion was used as the CYP2B6 substrate (Lang et al., 2004; Klein et al., 2005; Wang et al., 2006; Rotger et al., 2007). These results suggest that some allelic variants of CYP2B6 are associated with a substrate-dependent decrease in the catalytic properties of the enzyme. To date, there have been no reports of functional characterization of CYP2B6 variants in relation to AM demethylation activity.

In this study, we performed an in vitro analysis of 14 P450s (CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP2J2, CYP3A4, CYP3A5, and CYP4A11) to identify isoforms responsible for AM demethylation and evaluated functional differences among 26 CYP2B6 allelic variants (Fig. 2).

Materials and Methods

Chemicals. AM, DHA, and artemisinin (ART) were purchased from Tokyo Chemical Industry Corporation (Tokyo, Japan). Recombinant CYP1A1, CYP2A6, CYP2B6, CYP2C8, CYP2D6, and CYP4A11 Supersomes were purchased from BD Biosciences (Woburn, MA). CYP1A2, CYP2C9, CYP2E1, CYP3A4, and CYP3A5 Baculosomes were purchased from Invitrogen (Carlsbad, CA). NADPH was obtained from Oriental Yeast (Tokyo, Japan). Protease Inhibitor Cocktail Set III was purchased from Merck Chemicals (Darmstadt, Germany). Methanol (CH<sub>3</sub>OH) and acetonitrile (CH<sub>3</sub>CN) of LC-mass spectrometry grade were obtained from Wako Pure Chemical Industries (Tokyo, Japan).

DHA stock solution (5 mM) was prepared in CH<sub>3</sub>CN/H<sub>2</sub>O [50:50 (v/v)], and working solutions (1.0, 2.0, 5.0, 10, 25, 50, 100, and 200 μM) were prepared from the stock solution. These solutions were further diluted in 50 mM potassium phosphate buffer, pH 7.4, and the final calibration curves were obtained with 0.1, 0.2, 0.5, 1.0, 2.5, 5.0, 10, and 20 μM solutions. Working solutions (100 μl) were prepared in 1.5-ml plastic tubes, and 100 μl of CH<sub>3</sub>OH, 5 μl of internal standard (ART at 100 μM), and 100 μl of H<sub>2</sub>O were...
Levels of DHA were determined by the LC-MS/MS method described by Asimus and Ashton (2009), with minor modifications. AM stock solution (50 mM) was prepared in CH$_3$CN/H$_2$O [50:50 (v/v)], and a working solution (500 μM) was prepared by dilution of the stock solution in potassium phosphate buffer, pH 7.4. The incubation mixture contained AM as a substrate (1 and 50 μM), recombinant P450 enzymes (CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP2J2, CYP3A4, CYP3A5, and CYP4A11; 0.5 pmol) or human liver microsomes (50 μg), 0.5 mM NADPH, 5 mM MgCl$_2$, and 50 mM potassium phosphate buffer to a final volume of 90 μl. After preincubation (3 min at 37°C), the reaction was started by addition of NADPH. Reactions were performed for 30 min and terminated by the addition of 100 μl of methanol. Then, 5 μl of internal standard (ART at 100 μM) and 100 μl of H$_2$O were added. The resulting mixture was vortexed. After centrifugation at 12,000g for 10 min, 80 μl of the supernatant was transferred to a new plastic tube and passed through a filter (pore size: 0.2 μm; YMC). Then, 10 μl of the filtered solution was injected into the LC-MS/MS system for analysis. All peaks were integrated automatically by Xcalibur software. Levels of DHA were calculated from the calibration curves by the ratios of their peak areas to that of ART. The CYP3A4 protein was detected using the antihuman CYP3A4 antibody (Nosan, Yokohama, Japan) and horseradish peroxidase-conjugated goat anti-rabbit IgG (Dako Denmark A/S). Immuno-
isoforms with the exception of CYP2B6 and CYP3A4. At a higher concentrations used in this study, DHA was not formed by the other P450 activities of recombinant CYP2B6 and CYP3A4 were 6.61 and 2.50 pmol/min/P450.

The demethylation activities of wild-type CYP2B6 and CYP3A4 were 6.61 and 2.50 pmol/min/P450, respectively; those for CYP3A4 were 8.24 µM, 12.3 pmol·min⁻¹·pmol P450⁻¹, and 1.49 µl·min⁻¹·pmol P450⁻¹, respectively, demonstrating a higher Km and lower Vmax, which resulted in an approximately one-sixth Vmax/Km value for CYP3A4 relative to that for CYP2B6.

Comparison of AM Demethylation Activities (at 50 µM AM) to Immunoquantified CYP2B6 and CYP3A4 Protein Levels in 13 Human Liver Microsomes. As shown in Fig. 5, AM demethylation activity in 13 human liver microsomes was correlated with immunoquantified CYP2B6 content (r² = 0.548, P = 0.004) but not with immunoquantified CYP3A4 content (r² = 0.109, P = 0.272).

Enzymatic Properties for AM Demethylation by Wild-Type and 25 Variant CYP2B6s. The enzymatic activities of wild-type and 25 variant microsomal CYP2B6 proteins were determined using AM (50 µM) as a substrate (Fig. 6). For CYP2B6.8, CYP2B6.12, CYP2B6.18, CYP2B6.21, and CYP2B6.24, no AM demethylation activity was detected. The enzymatic activity of CYP2B6.3 could not be calculated because its expression level could not be determined by immunoblotting. CYP2B6.10, CYP2B6.11, CYP2B6.14, CYP2B6.15, CYP2B6.16, CYP2B6.20, and CYP2B6.27 exhibited significantly decreased activities compared with that of wild-type CYP2B6.

The Michaelis-Menten kinetics for AM demethylation were investigated for each of recombinant enzymes CYP2B6 and CYP3A4 by Michaelis-Menten plots (Fig. 4). Apparent Km, Vmax, and Vmax/Km values for CYP2B6 were estimated to be 1.95 µM, 17.9 pmol·min⁻¹·pmol P450⁻¹, and 9.19 µl·min⁻¹·pmol P450⁻¹, respectively; those for CYP3A4 were 8.24 µM, 12.3 pmol·min⁻¹·pmol P450⁻¹, and 1.49 µl·min⁻¹·pmol P450⁻¹, respectively.

As shown in Fig. 5, AM demethylation activities (at 50 µM AM) to immunoquantified CYP2B6 and CYP3A4 protein levels in 13 human liver microsomes are summarized in Table 1. The estimated kinetic parameters, apparent Km, Vmax, and Vmax/apparent Km for AM demethylation by CYP2B6.1 were 3.10 µM, 36.0 pmol·min⁻¹·pmol CYP2B6⁻¹, and 12.4 µl·min⁻¹·pmol CYP2B6⁻¹, respectively.

The Kinetic Parameters are summarized in Table 1. The estimated kinetic parameters, apparent Km, Vmax, and Vmax/apparent Km for AM demethylation by CYP2B6.1 were 3.10 µM, 36.0 pmol·min⁻¹·pmol CYP2B6⁻¹, and 12.4 µl·min⁻¹·pmol CYP2B6⁻¹.

AM Demethylation by Recombinant Human P450s. The activities of AM demethylation were measured in 14 recombinant human P450 enzymes (CYP1A1, CYP1A2, CYP1B1, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP2J2, CYP3A4, CYP3A5, and CYP4A11) at 1 and 50 µM substrate concentrations. The lower concentration used was intended to approximate one-sixth the concentration of AM substrate concentrations.

Lower substrate concentrations were used, which resulted in an approximately one-sixth Vmax/Km value for CYP3A4 relative to that for CYP2B6.

Comparison of AM Demethylation Activities (at 50 µM AM) to Immunoquantified CYP2B6 and CYP3A4 Protein Levels in 13 Human Liver Microsomes. As shown in Fig. 5, AM demethylation activity in 13 human liver microsomes was correlated with immunoquantified CYP2B6 content (r² = 0.548, P = 0.004) but not with immunoquantified CYP3A4 content (r² = 0.109, P = 0.272).

Enzymatic Properties for AM Demethylation by Wild-Type and 25 Variant CYP2B6s. The enzymatic activities of wild-type and 25 variant microsomal CYP2B6 proteins were determined using AM (50 µM) as a substrate (Fig. 6). For CYP2B6.8, CYP2B6.12, CYP2B6.18, CYP2B6.21, and CYP2B6.24, no AM demethylation activity was detected. The enzymatic activity of CYP2B6.3 could not be calculated because its expression level could not be determined by immunoblotting. CYP2B6.10, CYP2B6.11, CYP2B6.14, CYP2B6.15, CYP2B6.16, CYP2B6.20, and CYP2B6.27 exhibited significantly decreased activities compared with that of wild-type CYP2B6.

The Michaelis-Menten kinetics for AM demethylation were determined for CYP2B6.1, CYP2B6.2, CYP2B6.4, CYP2B6.5, CYP2B6.6, CYP2B6.7, CYP2B6.9, CYP2B6.10, CYP2B6.13, CYP2B6.14, CYP2B6.17, CYP2B6.19, CYP2B6.20, CYP2B6.23, CYP2B6.25, CYP2B6.26, and CYP2B6.27. The kinetic parameters are summarized in Table 1. The estimated kinetic parameters, apparent Km, Vmax, and Vmax/apparent Km for AM demethylation by CYP2B6.1 were 3.10 µM, 36.0 pmol·min⁻¹·pmol CYP2B6⁻¹, and 12.4 µl·min⁻¹·pmol CYP2B6⁻¹, respectively.

The Vmax values for CYP2B6.14, CYP2B6.20, and CYP2B6.27 were significantly decreased, whereas those for CYP2B6.2, CYP2B6.4, and CYP2B6.6 were significantly increased, relative to that for the wild-type enzyme.

Discussion

In this study, we have determined the human P450 enzymes responsible for AM demethylation. Among 14 human P450s, CYP2B6

![Fig. 4](image-url) The Michaelis-Menten curves for the demethylation of AM with recombinant CYP2B6 and CYP3A4.

![Fig. 5](image-url) Comparison of AM demethylation activities (at 50 µM AM) to immunoquantified CYP2B6 and CYP3A4 protein levels in 13 human liver microsomes. Correlation coefficients (r²) obtained in these cases are shown.
has the highest intrinsic activity for AM demethylation, followed by CYP3A4. In the kinetic parameter analysis, the affinity of CYP2B6 was 4-fold higher than that of CYP3A4, and the \( V_{\text{max/apparent}} K_{\text{m/apparent}} \) of CYP2B6 was 6-fold higher than that of CYP3A4. These results suggest that AM demethylation is likely to be mainly catalyzed by CYP2B6 in the liver. The contribution of CYP2B6 to AM demethylation by human liver microsomes was further substantiated by showing a correlation to CYP2B6 protein content \( (r^2 = 0.548) \). However, van Agtmael et al. (1999b,c) have reported that administration of AM with grapefruit juice, a CYP3A4 inhibitor, increased the blood concentration of AM and DHA but not their elimination half-life. Thus, CYP3A4 in the small intestine might also play an important role in the metabolism of AM.

CYP2B6 is a genetically polymorphic enzyme (Zanger et al., 2007; Arenaz et al., 2010). In vitro functional characterization of polymorphically expressed CYP2B6 variants revealed that CYP2B6.4 and CYP2B6.6 increased AM demethylation activity, whereas CYP2B6.8, CYP2B6.11, CYP2B6.12, CYP2B6.14, CYP2B6.15, CYP2B6.16, CYP2B6.18, CYP2B6.20, CYP2B6.21, CYP2B6.24, and CYP2B6.27 exhibited no activity or decreased activity. These alterations were consistent with those of previous in vitro studies performed using buPROPION, 7-ethoxy-4-trifluoromethylcoumarin, and selegiline as CYP2B6 substrates (Lang et al., 2004; Klein et al., 2005; Wang et al., 2006; Rotger et al., 2007; Watanabe et al., 2010). However, CYP2B6.2 exhibited increased AM demethylation activity, and the activity of CYP2B6.13 was similar to that of wild-type CYP2B6.1. There have been several reports that CYP2B6.2 exhibited no functional differences compared with CYP2B6.1 (Jinno et al., 2003; Watanabe et al., 2010) and that CYP2B6.13 had no metabolic activity toward 7-ethoxy-4-trifluoromethylcoumarin and selegiline (Watanabe et al., 2010). These results suggest that these CYP2B6 variants show substrate-dependent changes in the catalytic properties of the enzyme.

Gay et al. (2010) recently determined the crystal structure of CYP2B6, allowing the prediction of precise locations within the three-dimensional structure at which amino acid substitutions occur. They suggested that the K262R substitution on the G/H loop is responsible for reduced affinity of CYP2B6 for AM. However, a number of amino acids with altered AM demethylation activity are located far from the substrate recognition sites. Indeed, the apparent \( K_m \) values of AM demethylation were not significantly different among the CYP2B6 variants (Table 1). We hypothesize that the functional effects of these variants are transduced via long-range hydrogen-bonding networks or through subtle differences in the placement of secondary structural elements. In addition, most of the amino acid substitutions that abolished enzymatic activity are conserved among human P450s and are therefore critical for CYP2B6 activity.

This is the first study to functionally analyze CYP2B6 genetic variants with respect to AM demethylation activity. If CYP2B6 has a significant role in the metabolism of AM in vivo as well as in vitro, individuals with poor CYP2B6 metabolism might have higher plasma concentrations of AM and DHA but not their elimination half-life.

Table 1: Kinetic parameters of AM demethylation by CYP2B6 proteins expressed in COS-7 cells

<table>
<thead>
<tr>
<th>Variants</th>
<th>Apparent ( K_m ) (( \mu M ))</th>
<th>( V_{\text{max}} ) (( \text{pmol} \cdot \text{min}^{-1} \cdot \text{pmol CYP2B6}^{-1} ))</th>
<th>( V_{\text{max/apparent}} K_{\text{m/apparent}} ) (( \mu M \cdot \text{min}^{-1} \cdot \text{pmol CYP2B6}^{-1} ))</th>
<th>( V_{\text{max/apparent}} K_{\text{m/apparent}} ) Ratio</th>
<th>% CYP2B6.1</th>
</tr>
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<tbody>
<tr>
<td>CYP2B6.1</td>
<td>3.10 ± 1.1</td>
<td>36.0 ± 5.6</td>
<td>12.4 ± 4.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2B6.2</td>
<td>4.29 ± 2.7</td>
<td>64.4 ± 3.9</td>
<td>18.8 ± 9.1</td>
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<td></td>
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<tr>
<td>CYP2B6.4</td>
<td>2.73 ± 0.45</td>
<td>70.6 ± 9.29*</td>
<td>26.0 ± 2.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP2B6.5</td>
<td>6.87 ± 6.8</td>
<td>9.8 ± 3.0</td>
<td>8.91 ± 1.3</td>
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<td></td>
</tr>
<tr>
<td>CYP2B6.6</td>
<td>6.72 ± 3.0</td>
<td>150 ± 15.9*</td>
<td>24.2 ± 6.8</td>
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<tr>
<td>CYP2B6.7</td>
<td>2.80 ± 1.4</td>
<td>50.1 ± 12.3</td>
<td>19.2 ± 4.6</td>
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<tr>
<td>CYP2B6.9</td>
<td>4.44 ± 1.7</td>
<td>33.1 ± 5.2</td>
<td>8.38 ± 3.9</td>
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<tr>
<td>CYP2B6.10</td>
<td>1.93 ± 0.68</td>
<td>17.0 ± 5.0</td>
<td>9.98 ± 5.0</td>
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<tr>
<td>CYP2B6.13</td>
<td>7.33 ± 4.1</td>
<td>18.2 ± 5.5</td>
<td>2.93 ± 1.7</td>
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<tr>
<td>CYP2B6.14</td>
<td>5.06 ± 5.8</td>
<td>7.06 ± 1.63*</td>
<td>6.76 ± 5.6</td>
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<tr>
<td>CYP2B6.17</td>
<td>2.17 ± 0.4</td>
<td>21.2 ± 4.7</td>
<td>9.44 ± 2.45</td>
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<td>12.0</td>
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<tr>
<td>CYP2B6.19</td>
<td>8.06 ± 8.9</td>
<td>36.9 ± 21.9</td>
<td>7.38 ± 6.15</td>
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<td>39.4</td>
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<tr>
<td>CYP2B6.20</td>
<td>6.47 ± 9.8</td>
<td>9.85 ± 2.22*</td>
<td>8.79 ± 7.22</td>
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<td>13.1</td>
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<tr>
<td>CYP2B6.23</td>
<td>1.91 ± 0.72</td>
<td>31.4 ± 7.71</td>
<td>17.6 ± 5.93</td>
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<td>CYP2B6.25</td>
<td>2.04 ± 1.9</td>
<td>25.7 ± 7.34</td>
<td>21.6 ± 15.7</td>
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<td>CYP2B6.26</td>
<td>5.50 ± 3.4</td>
<td>37.3 ± 6.04</td>
<td>10.1 ± 8.13</td>
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<tr>
<td>CYP2B6.27</td>
<td>4.50 ± 0.98</td>
<td>10.6 ± 6.16*</td>
<td>2.59 ± 1.82</td>
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* \( P < 0.05 \) compared with CYP2B6.1.
AM concentrations than those with more active variants of this enzyme. However, because DHA also has an antimalarial effect, it would be difficult to assess the clinical outcome in subjects who polymorphically express CYP2B6 without in vivo data. To more fully understand the mechanistic basis of our findings, it would be of great value to clinically examine the relationship between CYP2B6 genotypes and the plasma concentration of AM and its metabolites.

In conclusion, demethylation of AM was mainly catalyzed by recombinant CYP2B6, although recombinant CYP3A4 also exhibited this metabolic activity. In addition, we performed a comprehensive analysis, using COS-7 cells as a heterologous expression system, to characterize nonsynonymous CYP2B6 variants. Many of the 26 variants expressed in COS-7 cells exhibited significantly altered AM demethylation activity. This study provides insights into the genotype-phenotype associations of CYP2B6 and lays a foundation for future clinical studies on interindividual variation in drug efficacy and toxicity.

Authorship Contributions

Participated in research design: Honda, Hirasawa, and Hiratsuka.
Conducted experiments: Honda, Muroi, and Tamaki.
Contributed new reagents or analytic tools: Saigusa, Suzuki, Tomioka, and Matsubara.
Performed data analysis: Honda, Oda, and Hiratsuka.
Wrote or contributed to the writing of the manuscript: Honda, Saigusa, and Hiratsuka.

References


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