Identification of the Human Cytochrome P450 Enzymes Involved in the Two Oxidative Steps in the Bioactivation of Clopidogrel to Its Pharmacologically Active Metabolite

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ABSTRACT:

The aim of the current study is to identify the human cytochrome P450 (P450) isoforms involved in the two oxidative steps in the bioactivation of clopidogrel to its pharmacologically active metabolite. In the in vitro experiments using cDNA-expressed human P450 isoforms, clopidogrel was metabolized to 2-oxo-clopidogrel, the immediate precursor of its pharmacologically active metabolite, CYP1A2, CYP2B6, and CYP2C19 catalyzed this reaction. In the same system using 2-oxo-clopidogrel as the substrate, detection of the active metabolite of clopidogrel required the addition of glutathione to the system. CYP2B6, CYP2C9, CYP2C19, and CYP3A4 contributed to the production of the active metabolite. Secondly, the contribution of each P450 involved in both oxidative steps was estimated by using enzyme kinetic parameters. The contribution of CYP1A2, CYP2B6, and CYP2C19 to the formation of 2-oxo-clopidogrel was 35.8, 19.4, and 44.9%, respectively. The contribution of CYP2B6, CYP2C9, CYP2C19, and CYP3A4 to the formation of the active metabolite was 32.9, 6.76, 20.6, and 39.8%, respectively. In the inhibition studies with antibodies and selective chemical inhibitors to P450s, the outcomes obtained by inhibition studies were consistent with the results of P450 contributions in each oxidative step. These studies showed that CYP2C19 contributed substantially to both oxidative steps required in the formation of clopidogrel active metabolite and that CYP3A4 contributed substantially to the second oxidative step. These results help explain the role of genetic polymorphism of CYP2C19 and also the effect of potent CYP3A inhibitors on the pharmacokinetics and pharmacodynamics of clopidogrel in humans and on clinical outcomes.

Clopidogrel is a thienopyridine antiplatelet agent that has been widely used in the management of cardiovascular diseases, including atherothrombosis, unstable angina, and myocardial infarction (Savi and Herbert, 2005). Clopidogrel is an inactive prodrug that needs to be converted to the pharmacologically active metabolite in vivo through the hepatic metabolism to exhibit the antiplatelet effect (Savi et al., 2000). Clopidogrel is first converted by the action of cytochrome P450 (P450) to 2-oxo-clopidogrel (a thiolactone) then in a second step converted to the pharmacologically active, thiol-containing metabolite. CYP1A2, CYP2B6, and CYP2C19 catalyzed this reaction. In the second step of this pathway, where 2-oxo-clopidogrel is converted to the active metabolite, was thought to be a hydrolysis step (Savi et al., 2000). However, the role of genetic polymorphism of CYP2C19 and also the effect of potent CYP3A inhibitors on the pharmacokinetics and pharmacodynamics of clopidogrel in humans and on clinical outcomes.

ABBREVIATIONS: P450, cytochrome P450; LC/MS/MS, liquid chromatography/tandem mass spectrometry; HPLC, high-performance liquid chromatography; β-NADP, β-nicotinamide adenine dinucleotide phosphate sodium salt; Clint, intrinsic clearance.
was shown to be dependent on CYP3A, CYP2B6, CYP2C9, and CYP2C19 (Kazui et al., 2001; Rehmel et al., 2006). The aim of this study was to determine the contribution and the role of various P450 isoforms in the oxidation of clopidogrel to 2-oxo-clopidogrel and the formation of the active metabolite from 2-oxo-clopidogrel.

Materials and Methods

Materials. Clopidogrel, 2-oxo-clopidogrel, the pharmacologically active metabolite of clopidogrel and R-135766 (shown in Figs. 1 and 2) were obtained from Ube Industries, Ltd. (Ube, Japan). R-135766 was used as the internal standard for assay of the active metabolite and 2-oxo-clopidogrel by liquid chromatography/tandem mass spectrometry (LC/MS/MS). Butyl ρ-hydroxybenzoate, used as the internal standard for assay of the active metabolite by high-performance liquid chromatography (HPLC)-UV, was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Furafylline, omeprazole, (S)-N-3-benzylhydrazine, sulfaphenazole, ketoconazole, gluthathione, β-nicotinamide adenine dinucleotide phosphate sodium salt (β-NADP), and t-glucose 6-phosphate were purchased from Sigma-Aldrich (St. Louis, MO). Glucose-6-phosphate dehydrogenase was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). The reagent used to derivatize clopidogrel active metabolite, 3′-methoxymphenacyl bromide, was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). All other chemicals, reagents, and solvents used were commercially available and were of guaranteed grade or HPLC grade. Monoclonal antibodies to CYP1A2, CYP3A4, CYP2B6, and CYP2C19 were obtained from BD Biosciences Company (Woburn, MA). Polyclonal antibody to CYP2C9 and the rabbit control serum were obtained from Nason Corporation (Yokohama, Japan). The specificity and the selectivity of the monoclonal antibody to CYP2C19 and CYP3A4 were reported by Kraus et al. (2001) and Gelboin et al. (1995), respectively. The monoclonal antibody to CYP3A4 used inhibits not only the activity of CYP3A4 but that of CYP3A5 slightly. The specificity and selectivity of the monoclonal antibodies to CYP1A2 and CYP2B6 were published previously (website of BD Biosciences Company, http://wwwbdbiosciences.com/petProduct.jsp?prodId=460095 for monoclonal antibody CYP1A2 and http://wwwbdbiosciences.com/petProduct.jsp?prodId=460089 for monoclonal antibody CYP2B6). In addition, the specificity and selectivity of the polyclonal antibody to CYP2C9 were reported by Ng et al. (2003).

Microsomes from Human Liver or from Cells Expressing Isoforms of Human P450s. Pooled human liver microsomes from 10 donors were obtained from the Human and Animal Bridging Research Organization (Tokyo, Japan). Supersomes prepared from baculovirus/insect cells expressing human CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP4A11 and microsomes from human β-lymphoblastoid cell lines expressing human CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, CYP3A4, and CYP4A11 were purchased from BD Biosciences Company.

Production of 2-Oxo-clopidogrel from Clopidogrel. The assays were performed by using the microsomes from baculovirus/insect cells expressing human P450s (Supersomes). The incubation mixture contained 3 mg protein/ml of Supersomes, an NADPH-generating system (2.5 mM β-NADP, 25 mM D-glucose 6-phosphate, 10 mM magnesium chloride, and 2 units/ml of glucose-6-phosphate dehydrogenase), and 500 μM clopidogrel in a final volume of 200 μl of potassium phosphate (50 mM) buffer (pH 7.4) for CYP1A1, CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4 reactions or in a final volume of 200 μl of Tris-HCl (50 mM) buffer (pH 7.4) for CYP2A6, CYP2C9, and CYP4A11 reactions. A mixture without clopidogrel was preincubated at 37°C for 5 min, and the reaction was started by the addition of 4 μl of a solution of clopidogrel in N,N-dimethylacetamide. After incubation at 37°C for 30 min, an aliquot of the incubation mixture was collected and added to 3-fold volumes of ethanol to terminate the reaction. The mixture was centrifuged at 20,800g at 4°C for 3 min, and 25 μl of the supernant was injected into the HPLC system to determine the concentrations of 2-oxo-clopidogrel.

Production of the Active Metabolite of Clopidogrel from 2-Oxo-clopidogrel. The assays were performed by using the microsomes from β-lymphoblastoid cells expressing human P450s. The incubation mixture contained 2 mg protein/ml of the microsomes from human β-lymphoblastoid cells, an NADPH-generating system, 1 mM glutathione, and 200 μM 2-oxo-clopidogrel in a final volume of 200 μl of potassium phosphate (50 mM) buffer (pH 7.4) for CYP1A1, CYP1A2, CYP2B6, CYP2C8, CYP2C9, CYP2D6, CYP2E1, and CYP3A4 reactions or in a final volume of 200 μl of Tris-HCl (50 mM) buffer (pH 7.4) for CYP2A6, CYP2C9, and CYP4A11 reactions. A mixture without 2-oxo-clopidogrel was preincubated at 37°C for 5 min, and the reaction was started by the addition of 5 μl of a solution of 2-oxo-clopidogrel in N,N-dimethylacetamide. After the incubation at 37°C for 90 min, an aliquot of the incubation mixture was collected and mixed with 3-fold volumes of acetonitrile and 20 μl of a solution of butyl ρ-hydroxybenzoate as the internal standard (20 μg/ml in acetonitrile) to terminate the reaction. The mixture was centrifuged at 20,800g at 4°C for 3 min, and 20 μl of the supernatant was injected into the HPLC system to determine the concentrations of the active metabolite.

Determination of the Enzyme Kinetic Parameters for 2-Oxo-clopidogrel Formation. The assay was performed by using the Supersomes of CYP1A2, CYP2B6, CYP2C9, and the control Supersomes. The incubation mixture contained 20 pmol P450/0.2 mg protein/ml of Supersomes, an NADPH-generating system, and 0.625, 1.25, 2.5, 5, 10, 20, and 40 μM clopidogrel in a final volume of 300 μl of potassium phosphate (100 mM) buffer (pH 7.4). A mixture without clopidogrel was preincubated at 37°C for 5 min, and the reaction was started by the addition of 6 μl of a solution of clopidogrel in methanol. After incubation at 37°C for 0 and 1 min, 50 μl of the incubation mixture was collected and added to 200 μl of acetonitrile and 50 μl of a solution of a solution of R-135766 as the internal standard (2 μM in acetonitrile) to terminate the reaction. The mixture was centrifuged at 20,800g at 4°C for 3 min, and 5 μl of the supernatant was injected into LC/MS/MS system to determine the concentrations of 2-oxo-clopidogrel.

Determination of the Enzyme Kinetic Parameters for the Active Metabolite Formation from 2-Oxo-clopidogrel. The assay was performed by using the Supersomes of CYP3A4, CYP2B6, CYP2C9, CYP2C19, and the control. The incubation mixture contained 20 pmol P450/0.3 mg protein/ml of
Supersomes, an NADPH-generating system, 5 mM glutathione and 0.625, 1.25, 2.5, 5, 10, 20, and 40 μM 2-oxo-clopidogrel in a final volume of 300 μl of potassium phosphate (100 mM) buffer (pH 7.4) for CYP2B6, CYP2C19, and CYP3A4 reactions or in a final volume of 300 μl of Tris-HCl (100 mM) buffer (pH 7.5) for the CYP2C9 reaction. A mixture without 2-oxo-clopidogrel was preincubated at 37°C for 5 min, and the reaction was started by the addition of 6 μl of a solution of 2-oxo-clopidogrel in methanol. After incubation at 37°C for 0, 15, or 30 min, 50 μl of the incubation mixture was collected and added to 198 μl of acetonitrile, 2 μl of a solution of 3′-methoxyphenylacrylamide (500 mM in acetonitrile) as the derivatization reagent, and 50 μl of a solution of R-135766 as the internal standard (2 μM in acetonitrile) to terminate the reaction. After the mixture was allowed to stand for 10 min at room temperature, it was centrifuged at 20,800 g for 3 min, and 5 μl of the supernatant was injected into the LC/MS/MS system (Takahashi et al., 2008) to determine the concentrations of an active metabolite.

**Inhibition of Production of 2-Oxo-clopidogrel in Human Liver Microsomes.** The K<sub>m</sub> (Michaelis constant) value in 2-oxo-clopidogrel formation from clopidogrel was determined by using 0.5 mg protein/ml human liver microsomes as the enzyme source and clopidogrel (at 1.25, 2.5, 5, 10, 20, 40, and 160 μM) as substrate in the presence of an NADPH-generating system at an incubation time of 5 min. This assay was performed in triplicate by using human liver microsomes. Eadie-Hofstee plots were used to visually detect deviation from linearity and to make initial estimates of kinetic constants. The K<sub>m</sub> value was calculated by WinNonlin nonlinear estimation program (version 4.0.1; Pharsight, Mountain View, CA) as described below under Data Handling. Eadie-Hofstee concentration in the inhibition studies below was set at 4 μM, which was close to the K<sub>m</sub> value of the high-affinity component.

**Inhibition studies using the antibodies against P450s.** The incubation mixture contained 0.5 mg protein/ml of human liver microsomes, an NADPH-generating system, 4 μM clopidogrel, and monoclonal antibody to CYP1A2, CYP2B6, and CYP2C19 in a final volume of 200 μl of potassium phosphate buffer (67 mM) (pH 7.4). Five microliters of microsomes (20 mg protein/ml) and 10 μl of each monoclonal antibody (CYP1A2, CYP2B6, CYP2C19) or 25 mM Tris-HCl buffer (pH 7.5) as substrate were mixed and incubated for 20 min on ice. After incubation on ice, 133 μl of potassium phosphate (100 mM) buffer (pH 7.4), 20 μl of glutathione, and 50 μl of the NADPH-generating system were added to that mixture and preincubated at 37°C for 5 min. The reaction was started by the addition of 2 μl of a solution of 2-oxo-clopidogrel in methanol and carried out at 37°C for 15 min.

**Assay of 2-Oxo-clopidogrel and the Active Metabolite of Clopidogrel by HPLC-UV.** 2-Oxo-clopidogrel and the active metabolite produced in the human cytochrome P450 expression systems were measured by using an HPLC system (LC-VP system; Shimadzu Co., Ltd., Kyoto, Japan). Chromatographic separation of 2-oxo-clopidogrel was carried out on a YMC Pack ODS-A302 column (4.6 × 150 mm, particle size of 5 μm, YMC Co., Ltd., Tokyo, Japan) at 40°C by using a mobile phase consisting of acetonitrile, distilled water, and trifluoroacetic acid (35:65:0.2, v/v/v) at a flow rate of 1.0 ml/min. Chromatographic separation of the active metabolite of clopidogrel was carried out on the same column by using a mobile phase consisting of acetonitrile, distilled water, and trifluoroacetic acid (38:62:0.2, v/v/v) at a flow rate of 1.0 ml/min. Detection was carried out at 220 nm. The active metabolite in the analytical samples treated immediately by acetonitrile was stable at 4°C for 24 h without derivatization (data not shown).

**Assay of 2-Oxo-clopidogrel and the Active Metabolite of Clopidogrel by Electrospray Ionization-LC/MS/MS System.** In the determination of the enzymatic kinetics parameters and the inhibition studies, 2-oxo-clopidogrel and the active metabolite were measured by using the method of Takahashi et al. (2008) with slight modification. The active metabolite of clopidogrel was derivatized with 3′-methoxyphenylacrylamide to stabilize thiol moiety in its structure. Quattro-LC/MS/MS system (Micromas UK Ltd., Manchester, UK) was used in the positive-ion detection mode at the electrospray ionization interface. The peak areas of the <i>n</i>/<i>c</i> = 338→183 for 2-oxo-clopidogrel and the <i>n</i>/<i>c</i> = 504→354 for derivatized active metabolite were measured against the peak areas of the <i>n</i>/<i>c</i> = 548→206 for the internal standard (R-135766). Separation by HPLC was conducted by using an Alliance 2695 Separations Module (Waters, Milford, MA) with an ODS column (Inertisol ODS-3, 2.1 mm × 150 mm, 5 μm; GL Science Inc., Tokyo, Japan) at a flow rate of 0.2 ml/min with a mobile phase consisting of methanol, distilled water, and trifluoroacetic acid [710:290:0.5 (v/v/v)].

**Data Handling.** All of the calculated values, with the exception of enzyme kinetics parameters below, were expressed as mean ± S.D. of three experiments throughout Results and Discussion. 

**Estimation of the enzyme kinetics parameters for 2-oxo-clopidogrel and the active metabolite formation in the human P450 expression systems.** The reaction rate (V) was calculated according to eq. 1.

\[
V = \frac{\text{generated concentration (μM)} \times 1000}{20 \times \text{pmol P450 (ml)} \times \text{incubation time (min)}}
\]

The Michaelis-Menten constant (K<sub>m</sub>, μM) and maximal reaction rate (V<sub>max</sub>, pmol/pmol P450/min) were calculated by using WinNonlin Professional (version 4.0.1; Pharsight) based on a pharmacodynamic compiled model (model
no. 101) after an examination of Eadie-Hofstee plots demonstrated a linear relationship. The calculated \( K_m \) and \( V_{\text{max}} \) values were expressed as mean ± S.E. of parameter estimate. The intrinsic clearance (\( CL_{\text{int}} \)) was calculated as a ratio of \( V_{\text{max}} \) to \( K_m \). The various P450s mediated clearance (\( CL_{\text{int, expressed P450s}} \)) in human liver microsomes was determined by using eq. 2. The enzyme abundance (pmol P450/mg protein) of various P450s in human liver microsomes was obtained from the reported data in Rowland Yeo et al. (2004).

\[
CL_{\text{int, expressed P450}} (\mu l/mg protein/min) = \frac{V_{\text{max}} \times \text{enzyme abundances (pmol P450/mg protein) of various P450s}}{K_m}
\]  

(2)

The contribution ratio \( f_{\text{P450}} \) (%) of each P450 that was responsible for the production of 2-oxo-clopidogrel and the active metabolite was determined by using eq. 3.

\[
f_{\text{P450}} (\%) = \frac{CL_{\text{int, expressed P450}} \text{ for each P450 reaction} \times 100}{\Sigma CL_{\text{int, expressed P450}}}
\]  

(3)

**Inhibition of production of 2-oxo-clopidogrel or the active metabolite.** The estimation of the enzyme kinetic parameters for 2-oxo-clopidogrel or the active metabolite using human liver microsome was calculated by using WinNonlin nonlinear estimation program (version 4.0.1) and an Eadie-Hofstee plot \([x\text{-axis, } V/\text{substrate concentration } (\mu M); y\text{-axis, } V]\), where the reaction rate \( V \) (pmol/mg protein/min) was calculated according to eq. 4.

\[
V (\text{pmol/mg protein/min}) = \frac{\text{generated concentration } (\mu M) \times 1000}{0.5 (\text{mg protein/ml}) \times \text{incubation time (min)}}
\]  

(4)

Eadie-Hofstee plots were used to visually detect deviation from linearity and to make initial estimates of kinetic constants. Data were then fitted to eq. 5 by using the WinNonlin nonlinear estimation program. \( S (\mu M) \) is substrate concentration, \( K_m \) and \( V_{\text{max}} \) are the apparent \( K_m \) and \( V_{\text{max}} \) values for high-affinity component, and \( K_m \) and \( V_{\text{max}} \) values for the low-affinity component in eq. 5.

\[
V = \frac{V_{\text{max1}} \times S}{K_m + S} + \frac{V_{\text{max2}} \times S}{K_m + S}
\]  

(5)

The calculated \( K_m \) and \( V_{\text{max}} \) values were expressed as mean ± S.D. of three experiments. The enzymatic activity remaining (%) in the presence of the antibody and the chemical inhibitor was calculated according to eq. 6.

\[
\text{Enzymatic activity remaining } (\%) = \frac{\text{produced concentration (\mu M) with antibody or chemical inhibitor to P450s}}{\text{produced concentration (\mu M) without antibody or chemical inhibitor to P450s}} \times 100
\]  

(6)

The inhibition ratio was calculated according to eq. 7.

\[
\text{Inhibition ratio } (\%) = 100 - \text{enzymatic activity remaining } (\%)
\]  

(7)

**Results**

**Production of 2-Oxo-clopidogrel from Clopidogrel.** The ability to produce 2-oxo-clopidogrel in Supersomes was measured by HPLC-UV using clopidogrel (500 \( \mu M \)) as the substrate. As shown in Fig. 3, clopidogrel was metabolized to 2-oxo-clopidogrel by CYP1A2, CYP2B6, and CYP2C19, and the activity of these P450s ranked in descending order of CYP1A2, CYP2C19, and CYP2B6. Other P450 isoforms (CYP1A1, CYP2A6, CYP2C8, CYP2C9, CYP2D6, CYP2E1, CYP3A4, and CYP4A11) showed no detectable activity toward the formation of 2-oxo-clopidogrel.

**Production of the Active Metabolite of Clopidogrel from 2-Oxo-clopidogrel.** The production of the active metabolite of clopidogrel in the microsomes of \( \beta \)-lymphoblastoid cells expressing the human P450s was measured by HPLC as shown in Fig. 4, using 2-oxo-clopidogrel (200 \( \mu M \)) as the substrate. Incubation without addition of glutathione resulted in no detectable production of the active metabolite (data not shown). The isoforms CYP2B6, CYP2C9, CYP2C19, CYP3A4 converted 2-oxo-clopidogrel to the active metabolite, and the activity of these P450s ranked in descending order of CYP2C19, CYP2C9, CYP3A4, and CYP2B6. CYP1A1, CYP1A2, CYP2A6, CYP2C8, CYP2D6, CYP2E1, and CYP4A11 failed to catalyze this metabolic reaction.

**The Contribution of Each P450 Involved in the Formation of the Active Metabolite from Clopidogrel.** As the first oxidative step of clopidogrel bioactivation, the 2-oxo-clopidogrel formation rates over a range of clopidogrel concentrations were determined in Super-
TABLE 1
Enzyme kinetic parameters of 2-oxo-clopidogrel formation from clopidogrel

The assays were performed by using the microsomes baculovirus/insect cells expressing human P450 (Supersomes). $K_m$ and $V_{max}$ values were expressed as mean ± S.E. of parameter estimate. $Cl_{int, expressed}$ and $f_{adj}$ (contribution ratio of each P450) values were scaled to eqs. 2 and 3 under Materials and Methods to determine contribution.

<table>
<thead>
<tr>
<th>Incubation Time (min)</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (pmol/μmol P450/min)</th>
<th>$V_{max}/K_m$ (μmol P450/min)</th>
<th>Enzyme Abundances*</th>
<th>$Cl_{int, expressed}$ (pmol/min/μmol P450)</th>
<th>$f_{adj}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A2</td>
<td>1</td>
<td>1.58 ± 0.35</td>
<td>2.27 ± 0.46</td>
<td>1.44</td>
<td>52</td>
<td>74.9</td>
</tr>
<tr>
<td>CYP2B6</td>
<td>1</td>
<td>2.08 ± 0.73</td>
<td>7.66 ± 0.69</td>
<td>3.68</td>
<td>11</td>
<td>19.4</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>1</td>
<td>1.12 ± 0.25</td>
<td>7.52 ± 0.36</td>
<td>6.71</td>
<td>14</td>
<td>44.9</td>
</tr>
</tbody>
</table>

* The abundances of CYP1A2, CYP2B6, and CYP2C19 were obtained from the reported data in Rowland Yeo et al. (2004).

TABLE 2
Enzyme kinetic parameters of the formation of the pharmacologically active metabolite of clopidogrel from 2-oxo-clopidogrel

The assays were performed by using the microsomes baculovirus/insect cells expressing human cytochrome P450 (Supersomes). $K_m$ and $V_{max}$ values were expressed as mean ± S.E. of parameter estimate. $Cl_{int, expressed}$ and $f_{adj}$ (P450 contribution ratio) values were scaled to eqs. 2 and 3 under Materials and Methods to determine contribution.

<table>
<thead>
<tr>
<th>Incubation Time (min)</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (pmol/μmol P450/min)</th>
<th>$V_{max}/K_m$ (μmol P450/min)</th>
<th>Enzyme Abundances*</th>
<th>$Cl_{int, expressed}$ (pmol/min/μmol P450)</th>
<th>$f_{adj}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2B6</td>
<td>15</td>
<td>1.62 ± 0.08</td>
<td>2.48 ± 0.03</td>
<td>1.53</td>
<td>11</td>
<td>16.8</td>
</tr>
<tr>
<td>CYP2C9</td>
<td>30</td>
<td>18.1 ± 3.8</td>
<td>0.855 ± 0.084</td>
<td>0.072</td>
<td>73</td>
<td>3.45</td>
</tr>
<tr>
<td>CYP2C19</td>
<td>15</td>
<td>12.1 ± 2.2</td>
<td>9.06 ± 0.68</td>
<td>0.749</td>
<td>14</td>
<td>10.5</td>
</tr>
<tr>
<td>CYP3A4</td>
<td>15</td>
<td>27.8 ± 4.2</td>
<td>3.63 ± 0.29</td>
<td>0.131</td>
<td>155</td>
<td>20.3</td>
</tr>
</tbody>
</table>

* The abundances of CYP3A4, CYP2B6, CYP2C19, and CYP2C9 were obtained from the reported data in Rowland Yeo et al. (2004).

As the second oxidative step of clopidogrel, the active metabolite formation rates over a range of 2-oxo-clopidogrel concentrations were determined in Supersomes containing each P450. The kinetic parameters for the CYP2C9 reaction. The enzyme kinetics parameters for the other three P450s reactions were calculated by using the reaction rate of the active metabolite formation at 15 min. The $K_m$ values for CYP2B6, CYP2C9, CYP2C19, and CYP 3A4 were 1.62 ± 0.08, 18.1 ± 3.8, 12.1 ± 2.2, and 27.8 ± 4.2 μM, respectively (mean ± S.E. of parameter estimate) (Table 2), and the $V_{max}$ values were 2.48 ± 0.03, 0.855 ± 0.084, 9.06 ± 0.68, and 3.63 ± 0.29 pmol/μmol P450/min, respectively (mean ± S.E. of parameter estimate).

The $Cl_{int, expressed}$ and $f_{int}$ for CYP2B6, CYP2C9, CYP2C19, and CYP3A4 rections are shown in Table 2. Estimates of the $f_{int}$ P450 of CYP2B6, CYP2C9, CYP2C19, and CYP3A4 for the active metabolite formation were 32.9, 6.76, 20.6, and 39.8%, respectively, suggesting that the contribution of CYP3A4 to the active metabolite formation was greater compared with the other three P450s.

Inhibition of Production of 2-Oxo-clopidogrel or the Active Metabolite in Human Liver Microsomes. The reaction rate of 2-oxo-clopidogrel formation in human liver microsomes had a biphasic pattern in Eadie-Hofstee plots (Fig. 5), indicating the involvement of multiple enzymes. Accordingly, the kinetic parameters for 2-oxo-clopidogrel formation from clopidogrel in human liver microsomes were estimated by using the WinNonlin nonlinear estimation program.
The apparent $K_m$ and $V_{max}$ values for the high-affinity component were $4.70 \pm 2.62 \mu M$ and $144 \pm 61.0 \mathrm{pmol/mg \ protein/min}$, respectively (mean $\pm$ S.D., $n = 3$). For the low-affinity component, the $K_{m2}$ was $71.9 \pm 30.8 \mu M$ and the $V_{max2}$ was $1230 \pm 220 \mathrm{pmol/mg \ protein/min}$ (mean $\pm$ S.D., $n = 3$). Therefore, clopidogrel concentration in the inhibition studies was set at 4 $\mu M$, which was close to the $K_{m1}$ value of the high-affinity component.

The inhibitory effects of monoclonal antibodies to CYP1A2, CYP2B6, and CYP2C19 on the production of 2-oxo-clopidogrel were examined by using human liver microsomes as the enzyme source and clopidogrel were also investigated in human liver microsomes (Fig. 6). Furafylline (10 $\mu M$), an inhibitor of CYP1A2, inhibited 2-oxo-clopidogrel formation by 34.9 $\pm$ 0.5%. (S)-N-3-Benzylirvinanol and omeprazole, the inhibitors of CYP2C19 at 10 $\mu M$, also inhibited the formation of 2-oxo-clopidogrel by 26.9 $\pm$ 10.5 and 27.1 $\pm$ 9.2%, respectively (mean $\pm$ S.D., $n = 3$).

The kinetic parameters of clopidogrel active metabolite formation from 2-oxo-clopidogrel in human liver microsomes were also estimated by using WinNonlin nonlinear estimation program because the reaction rate of the active metabolite formation in human liver microsome also demonstrated a biphasic pattern in Eadie-Hofstee plots (Fig. 7), indicating the involvement of multiple enzymes. The apparent $K_{m1}$ and $V_{max1}$ values for the high-affinity component were $2.69 \pm 0.828 \mu M$ and $32.4 \pm 11.6 \mathrm{pmol/mg \ protein/min}$, respectively (mean $\pm$ S.D., $n = 3$). For the low-affinity component, the $K_{m2}$ was $94.9 \pm 6.05 \mu M$ and the $V_{max2}$ was $249 \pm 41.0 \mathrm{pmol/mg \ protein/min}$ (mean $\pm$ S.D., $n = 3$). Based on these results, the 2-oxo-clopidogrel concentration in the inhibition studies was set at 3 $\mu M$, which was close to the $K_{m1}$ value of the high-affinity component.

With respect to the second oxidation step leading to clopidogrel active metabolite formation, the inhibitory effects of monoclonal antibodies to CYP2B6, CYP2C19, CYP3A4, and polyclonal antibody to CYP2C9 on the production of the active metabolite were examined by using human liver microsomes as the enzyme source and 2-oxo-clopidogrel (at $K_{cat}$; 3 $\mu M$) as the substrate in the presence of an NADPH-generating system as shown in Table 3. The inhibition ratio of the active metabolite formation from 2-oxo-clopidogrel with antibodies to CYP2B6, CYP2C9, CYP2C19, and CYP3A4 showed 54.3 $\pm$ 3.4, 13.5 $\pm$ 4.4, 32.9 $\pm$ 4.2, and 31.3 $\pm$ 5.3%, respectively (mean $\pm$ S.D., $n = 3$).

**TABLE 3**

<table>
<thead>
<tr>
<th>Inhibition Ratio</th>
<th>2-Oxo-clopidogrel Formation</th>
<th>Active Metabolite Formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-CYP1A2</td>
<td>30.9 $\pm$ 9.0</td>
<td>9.0 $\pm$ 3.4</td>
</tr>
<tr>
<td>Anti-CYP2B6</td>
<td>26.0 $\pm$ 2.5</td>
<td>32.9 $\pm$ 4.2</td>
</tr>
<tr>
<td>Anti-CYP2C9</td>
<td>13.5 $\pm$ 4.4</td>
<td>42.0 $\pm$ 8.1</td>
</tr>
<tr>
<td>Anti-CYP2C19</td>
<td>31.3 $\pm$ 5.3</td>
<td></td>
</tr>
</tbody>
</table>

Sulfaphenazole, the inhibitor of CYP2C9 at 10 $\mu M$, inhibited the active metabolite formation by 36.2 $\pm$ 7.7%. (S)-N-3-Benzylirvinanol and omeprazole, the inhibitors of CYP2C9 at 10 $\mu M$, inhibited this reaction by 35.4 $\pm$ 15.2 and 31.3 $\pm$ 17.3%, respectively. Ketoconazole, an inhibitor of CYP3A4 at 2 $\mu M$, inhibited the formation of the active metabolite by 38.4 $\pm$ 8.4% (mean $\pm$ S.D., $n = 3$) (Fig. 6).

**Discussion**

This study is the first report in which P450s involved in the two oxidative steps required for the metabolism of clopidogrel to its active metabolite are identified and their contributions determined. CYP1A2, CYP2B6, and CYP2C19 are capable of producing 2-oxo-clopidogrel, whereas the remaining isozymes examined were practically inactive in this first oxidative step of clopidogrel. Savi et al. (1994) suggested that CYP1A2 was involved in the oxidative metabolism of clopidogrel. The present study confirmed that CYP1A2 does indeed contribute to the first oxidative step of this metabolic process. Regarding the second metabolic step, formation of clopidogrel active metabolite was catalyzed by CYP2B6, CYP2C9, CYP2C19, and CYP3A4. The reaction mixture for the active metabolite formation contained glutathione, because it was previously shown that formation of a thienopyridine’s active metabolite required the presence of both P450s and a reducing agent such as glutathione in the incubation mixture (Pereillo et al., 2002; Rehmel et al., 2006).

When the enzyme kinetic parameters of 2-oxo-clopidogrel formation by CYP1A2, CYP2B6, and CYP2C19 were calculated, we set up the incubation time at 1 min, because the reaction rate for the 2-oxo-clopidogrel formation by CYP2B6 and CYP2C19 appeared to decrease after 2-min incubation (data not shown). These decreases in the enzymatic activity are most likely attributed to mechanism-based inhibition. CYP2B6 and CYP2C19 inhibition observed in vitro by clopidogrel (Richter et al., 2004; Nishiya et al., 2007, 2009; Hagihara et al., 2008). The enzyme kinetic parameters used in eq. 3 showed that the contribution of each P450 involved in the 2-oxo-clopidogrel formation decreased in the order CYP2C19 (44.9%) $>$ CYP1A2 (35.8%) $>$ CYP2B6 (19.4%) (Table 1). To further verify the obtained contribution data from the enzyme kinetic parameters, the inhibition studies were performed with human liver microsomes, anti-P450 antibodies, and chemical inhibitors. The inhibition effects of the monoclonal antibodies on the first oxidation step toward 2-oxo-clopidogrel formation were consistent with the results of the contribution of each P450 involved in this oxidative step. Anti-CYP1A2, anti-CYP2B6, and anti-CYP2C19 antibodies inhibited the formation of 2-oxo-clopidogrel by 30.9 $\pm$ 9.0, 26.0 $\pm$ 2.5, and 42.0 $\pm$ 8.1%, respectively. In addition, the inhibition levels with the chemical inhibitors on the production of 2-oxo-clopidogrel were consistent with the outcome of the antibody experiments.

Likewise, the studies of the formation of clopidogrel active metabolite by CYP2B6, CYP2C9, CYP2C19, and CYP3A4 were performed. The possibility of mechanism-based inhibition in the second oxidation step was not allowed because previous studies demonstrated that such an inhibition was not involved in this reaction process (Nishiya et al., 2009).

The enzyme kinetic parameters combined with eq. 3 showed that the contribution of each P450 involved in the pharmacologically active metabolite formation decreased in the order CYP3A4 (39.8%) $>$ CYP2B6 (32.9%) $>$ CYP2C19 (20.6%) $>$ CYP2C9 (6.76%) (Table 2). Anti-CYP2B6, anti-CYP2C9, anti-CYP2C19, and anti-CYP3A4 antibodies inhibited the formation of the active metab-
olite by 54.3 ± 3.4, 13.5 ± 4.4, 32.9 ± 4.2, and 31.3 ± 5.3%, respectively. Moreover, the inhibition levels of the chemical inhibitors such as sulfaphenazole, (S)-N-3-benzylnirvanol, omeprazole, and ketoconazole on the production of the active metabolite from 2-oxo-clopidogrel exhibited 36.2 ± 7.7, 35.4 ± 15.2, 31.3 ± 17.3, and 38.4 ± 8.4%, respectively. The inhibition levels observed in the presence of antibodies or chemical inhibitors corresponded well to the contribution ratio of each P450 isoform in the second oxidation step. These studies clearly showed that CYP2C19 contributes substantially to both oxidative steps required to the formation of clopidogrel active metabolite and that CYP3A4 contributes only to the 2-oxo-clopidogrel to the active metabolite step. The data specifically point out that CYP2C19 would have a major effect on the formation of active metabolite from clopidogrel, and this observation would explain the effect of loss-of-function variants of CYP2C19 on the pharmacokinetics and/or pharmacodynamic response to clopidogrel (Hulot et al., 2006; Brandt et al., 2007; Mega et al., 2009; Simon et al., 2009). In these studies, common loss-of-functional polymorphisms of CYP2C19 were associated with decreased exposure in the active metabolite of clopidogrel, resulting in decreased pharmacodynamic response of clopidogrel, and patients carrying those alleles demonstrated a higher rate of cardiovascular events while on clopidogrel.

**Fig. 6.** Effects of the selective chemical inhibitors for CYP1A2 (furafylline), CYP3A4 (ketoconazole), CYP2C19 [(S)-N-3-benzylnirvanol, omeprazole], and CYP2C9 (sulfaphenazole) on the 2-oxo-clopidogrel formation and the active metabolite formation by pooled human liver microsome. Data were expressed as mean ± S.D. of three experiments.

**Fig. 7.** Kinetic analysis of the active metabolite formation from 2-oxo-clopidogrel in human liver microsome by Eadie-Hofstee plots and WinNonlin nonlinear estimation program. The human liver microsomes were incubated with 1.25 to 160 μM 2-oxo-clopidogrel at 37°C for 15 min. Data are plotted using Eadie-Hofstee plots (A) and direct plots (B). Line in B represents the curve fit to eq. 5 using WinNonlin nonlinear estimation program. Typical results from one of three experiments are shown.
therapy. In addition, the major role that CYP3A plays in the second oxidation step would explain the clinical evidence by CYP3A inhibitor on the reduction in clopidogrel active metabolite formation and the corresponding decrease in the effect of clopidogrel on platelet's aggregation (Suh et al., 2006; Farid et al., 2007). It is also likely that CYP3A5 also contributes to clopidogrel's active metabolite formation from 2-oxo-clopidogrel as was previously indicated in clinical and in vitro studies (Suh et al., 2006; Farid et al., 2007; Baker et al., 2008).

Moreover, in the present study, the inhibition ratio of the active metabolite formation from 2-oxo-clopidogrel with monoclonal antibody to CYP3A4 was lower than that with CYP3A4/5 inhibitor ketoconazole, suggesting some contribution by CYP3A5.

In conclusion, we demonstrated that the pharmacologically active metabolite of clopidogrel is produced from clopidogrel by two successive oxidation processes, with the first step from clopidogrel to 2-oxo-clopidogrel being catalyzed by CYP1A2, CYP2B6, and/or CYP2C19 and the second step from 2-oxo-clopidogrel to the active metabolite being catalyzed by CYP3A4, CYP2B6, CYP2C9, and/or CYP2C19. In addition, we indicated that the contributions of CYP2C19 and CYP3A4 were more important relative to the other P450s in the pharmacologically active metabolite formation by estimating the enzyme kinetic parameters and inhibition effects of the chemical inhibitors and the antibody to P450s. These findings would support the results of several clinical drug-interaction studies with clopidogrel, and studies on the effect of genetic polymorphism on the pharmacodynamic response of clopidogrel, and studies on the effect of genetic polymorphism of CYP2C19 on the clinical outcomes in patients treated with clopidogrel (Hulot et al., 2006; Suh et al., 2006; Brandt et al., 2007; Farid et al., 2007, 2008; Mega et al., 2009; Simon et al., 2009).

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References


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