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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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Running Title: The CYP isoforms responsible for clopidogrel active metabolite formation

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Abbreviations: HPLC, high performance liquid chromatography; LC-MS/MS, liquid chromatography/tandem mass spectrometry; CYP, cytochrome P450

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Abstract

The aim of the current study is to identify the human cytochrome P450 (CYP) 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 CYP 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 CYP involved in both oxidative steps was estimated 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 CYPs, the outcomes obtained by inhibition studies were consistent with the results of CYP 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.

Introduction

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 et al., 2005). Clopidogrel is an inactive prodrug that needs to be converted to the pharmacologically active metabolite *in vivo* through the hepatic metabolism in order to exhibit the antiplatelet effect (Savi et al., 1992). Clopidogrel is first converted by the action of cytochrome P450 (CYP) to 2-oxo-clopidogrel (a thiolactone) then in a second step converted to the pharmacologically active, thiol-containing metabolite as shown in Fig. 1 (Savi et al., 2000). The CYP isoforms involved in the bioactivation of clopidogrel have been suggested to be CYP1A2 in rats (Savi et al., 1994) and CYP3A in humans (Clarke et al., 2003), although the contribution of these CYPs to produce the active metabolite was still unclear. In addition, several recent clinical studies demonstrated that CYP3A4, CYP3A5, and CYP2C19 have a significant role in the formation of the active metabolite from clopidogrel (Farid et al., 2007, Farid et al., 2008, Suh et al., 2006, Hulot et al., 2006, Brandt et al. 2007). Further, Brandt *et al*. (2007) reported that loss of function of CYP2C19 due to polymorphisms resulted in decreased exposure to clopidogrel active metabolite and hence a diminished effect of clopidogrel on platelet aggregation. Involvement of CYPs would be obvious in the first step of clopidogrel bioactivation (Fig. 1) based on the chemical structure of 2-oxo-clopidogrel showing introduction of one oxygen atom into clopidogrel. While initially 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), liver microsomes and glutathione were shown to be needed for the formation and detection of clopidogrel active metabolite (Pereillo et al., 2002). However, the CYPs needed to catalyze the formation of the active metabolite were not identified. Unlike what is currently known with respect to the

enzymes involved in the bioactivation of clopidogrel, the formation of the active metabolite of a new thienopyridine antiplatelet agent, prasugrel, from its corresponding thiolactone, 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 CYPs in the oxidation of clopidogrel to 2-oxo-clopidogrel and the formation of the active metabolite from 2-oxo-clopidogrel.

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Materials and Methods

Materials

Clopidogrel, 2-oxo-clopidogrel, the pharmacologically active metabolite of clopidogrel and R-135766 (shown in Fig. 1 and Fig. 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 LC/MS/MS. Butyl *p*-hydroxybenzoate, used as the internal standard for assay of the active metabolite by HPLC-UV, was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). Furafylline, omeprazole, (S)-N-3-benzylnirvanol, sulfaphenazole, ketoconazole, glutathione, β-nicotinamide adenine dinucleotide phosphate sodium salt (β-NADP), and D-glucose-6-phosphate were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Glucose-6-phosphate dehydrogenase was purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). The reagent used to derivatize clopidogrel active metabolite, 3'-methoxyphenacyl bromide, was purchased from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). All other chemicals, reagents and solvents used were commercially available, and of guaranteed grade or HPLC grade. Monoclonal antibodies to CYP1A2, CYP3A4, CYP2B6, and CYP2C19 were obtained from BD Biosciences Company (Woburn, MA, USA). Polyclonal antibody to CYP2C9 and the rabbit control serum were obtained from Nosan Corporation (Yokohama, Japan). The specificity and the selectivity of the monoclonal antibody to CYP2C19 and CYP3A4 were reported by Krausz 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 the selectivity of the monoclonal antibodies to CYP1A2 and CYP2B6 were published (website of BD Biosciences Company). Additionally, the specificity and the 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 CYPs

Pooled human liver microsomes from 10 donors were obtained from 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 (Woburn, MA, USA).

Production of 2-oxo-clopidogrel from clopidogrel

The assays were performed using the microsomes from baculovirus/insect cells expressing human CYPs (SUPERSOMES). The incubation mixture contained 3 mg protein/mL of SUPERSOMES, 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 of clopidogrel in a final volume of 200 μL of 50 mM potassium phosphate buffer (pH 7.4) for CYP1A1, CYP1A2, CYP2B6, CYP2C8, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 reactions, or in a final volume of 200 μL of 50 mM tris-HCl 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 threefold volumes of ethanol to terminate the reaction. The mixture was centrifuged at 20,800*g* at 4°C for 3 min, and 25 μL of the supernatant was injected into 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 using the microsomes from β-lymphoblastoid cells expressing human CYPs. The incubation mixture contained 2 mg protein/mL of the microsomes from human β-lymphoblastoid cells, NADPH-generating system, 1 mM of glutathione and 200 μM of 2-oxo-clopidogrel in a final volume of 200 μL of 50 mM potassium phosphate buffer (pH 7.4) for CYP1A1, CYP1A2, CYP2B6, CYP2C8, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 reactions, or in a final volume of 200 μL of 50 mM tris-HCl 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 threefold volumes of acetonitrile and 20 μL of a solution of butyl *p*-hydroxybenzoate as the internal standard (20 μg/mL in acetonitrile) to terminate the reaction. The mixture was centrifuged at 20,800*g* at 4°C for 3 min, and 20 μL of the supernatant was injected into 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 using the SUPERSOMES of CYP1A2, CYP2B6, CYP2C19 and the control SUPERSOMES. The incubation mixture contained 20 pmol P450/0.2 mg protein/mL of SUPERSOMES, NADPH-generating system and 0.625, 1.25, 2.5, 5, 10, 20 and 40 μM of clopidogrel in a final volume of 300 μL of 100 mM potassium phosphate 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 R-135766 as the internal standard (2 μM in acetonitrile) to terminate the reaction. The mixture was centrifuged at 20,800*g* 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 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 of glutathione and 0.625, 1.25, 2.5, 5, 10, 20 and 40 μM of 2-oxo-clopidogrel in a final volume of 300 μL of 100 mM potassium phosphate buffer (pH 7.4) for CYP2B6, CYP2C19 and CYP3A4 reactions, or in a final volume of 300 μL of 100 mM Tris-HCl buffer (pH7.5) 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'-methoxyphenacyl bromide (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* at 4°C for 3 min, and 5 μL of the supernatant was injected into LC-MS/MS system (Takahashi et al., *2008*) to determine the concentrations of an active metabolite.

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Inhibition of production of 2-oxo-clopidogrel in human liver microsomes

The K_m (Michaelis constant) value in 2-oxo-clopidogrel formation from clopidogrel was determined using 0.5 mg-protein/mL human liver microsomes as the enzyme source and clopidogrel (at 1.25, 2.5, 5, 10, 20, 40, 80 and 160 μM) as substrate in the presence of NADPH-generating system at incubation time 5 min. This assay was performed in triplicate 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_m value was calculated by WinNonlin nonlinear estimation program (version 4.0.1, Pharsight Corporation) as described below in the Data handling section. Clopidogrel concentration in the inhibition studies below was set at 4 μM which was close to K_{m1} value of high affinity component.

(1) Inhibition study using the antibodies against CYPs

The incubation mixture contained 0.5 mg-protein/mL of human liver microsomes, NADPH-generating system, 4 μM of clopidogrel and monoclonal antibody to CYP1A2, CYP2B6 and CYP2C19 in a final volume of 200 μL of 67 mM potassium phosphate buffer (pH 7.4). Five-μL 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 control were mixed and incubated for 20 min on ice. After incubation on ice, 133 μL of 100 mM potassium phosphate buffer (KPB, pH 7.4) 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 clopidogrel in methanol and carried out at 37°C for 5 min.

(2) Studies using the selective chemical inhibitors of CYPs

(S)-N-3-benzylnirvanol and omeprazole were added at a final concentration of 10 μM to the incubation medium consisting of 0.5 mg-protein/mL of human liver microsomes, 100 mM potassium phosphate buffer (pH 7.4) and 4 μM of clopidogrel, and preincubated at 37°C for 5 min. The reaction was initiated by the addition of NADPH-generating system and carried out for 5 min. In the case of furafylline, pre-incubation was performed without clopidogrel for 30 min and the reaction was initiated by the addition of NADPH-generating system and clopidogrel.

After incubation at 37°C for the appropriate times in the inhibition studies of 2-oxo-clopidogrel formation from clopidogrel, 50 μL of the incubation mixture was collected and treated as described above in the section on *Determination of the enzyme kinetics parameters for 2-oxo-clopidogrel formation.*

Inhibition of production of the active metabolite from 2-oxo-clopidogrel in human liver microsomes

The K_m value in the active metabolite formation from 2-oxo-clopidogrel was determined using 0.5 mg-protein/mL human liver microsomes as the enzyme source and 2-oxo-clopidogrel (at 1.25, 2.5, 5, 10, 20, 40, 80 and 160 μM) as substrate in the presence of NADPH-generating system and glutathione (5 mM) at incubation time 15 min. This assay was performed in triplicate 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_m value was calculated by WinNonlin nonlinear estimation program (version 4.0.1) as described below in the Data handling section. 2-Oxo-clopidogrel concentration in the inhibition studies below was set at 3 μM which was close to K_{m1} value of high affinity component.

(1) Inhibition studies using the antibodies against CYPs

The incubation mixture contained 0.5 mg-protein/mL of human liver microsomes, NADPH-generating system, 5 mM of glutathione, 3 μM of 2-oxo-clopidogrel and monoclonal antibody to CYP3A4, CYP2B6 and CYP2C19 or polyclonal antibody to CYP2C9 in a final volume of 200 μL of 67 mM potassium phosphate buffer (pH 7.4). Five-μL of microsomes (20 mg protein/mL) and 10 μL of each -monoclonal antibody (CYP3A4, CYP2B6, CYP2C19) or 25 mM Tris-HCl buffer (pH 7.5) as control were mixed and incubated for 20 min on ice. When polyclonal antibody to CYP2C9 was used, 5-μL of microsomes (20 mg protein/mL) and 10 μL of polyclonal antibody to CYP2C9 or rabbit serum as a control were mixed and incubated for 10 min at room temperature. After incubation on ice or room temperature, 113 μL of 100 mM potassium phosphate buffer (KPB, 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.

(2) Studies using the selective chemical inhibitors against CYPs

Ketoconazole, (S)-N-3-benzylnirvanol, omeprazole and sulfaphenazole were added to the incubation medium containing 0.5 mg-protein/mL of human liver microsomes, 100 mM potassium phosphate buffer (pH 7.4), 5 mM glutathione and 3 μM 2-oxo-clopidogrel at a final concentration of 2 μM for ketoconazole or 10 μM for (S)-N-3-benzylnirvanol, omeprazole and sulfaphenazole, and preincubated at 37°C for 5 min. The reaction was initiated by the addition of NADPH-generating system and carried out for 15 min.

After incubation at 37°C for 15 min in both inhibition studies of the active metabolite

formation from 2-oxo-clopidogrel, 50 μL of the incubation mixture was collected and treated as described above in the section *Determination of the enzyme kinetics parameters for the active metabolite formation from 2-oxo-clopidogrel.*

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 using a 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 using a mobile phase consisting of acetonitrile, distilled water and trifluoroacetic acid (35:65:0.02, 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 using a mobile phase consisting of acetonitrile, distilled water and trifluoroacetic acid (38:62:0.02, v/v/v) at a flow rate 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 ESI-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 using the method of Takahashi et al. (2008) with slight modification. The active metabolite of clopidogrel was derivatized with 3'-methoxyphenacyl bromide to stabilize thiol moiety in its structure. Quattro-LC MS/MS system (Micromass UK., Ltd.) was used in the positive-ion detection mode at the ESI

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interface. The peak areas of the *m/z* 338→183 for 2-oxo-clopidogrel and the *m/z* 504→354 for derivatized active metabolite were measured against the peak areas of the *m/z* 548→206 for-the internal standard (R-135766). Separation by HPLC was conducted using an Alliance 2695 Separations Module (Waters Corp.) with an ODS column (Inertsil ODS-3, 2.1 mm × 150 mm, 5 μm, GL Science Inc.) at a flow rate of 0.2 mL/min with a mobile phase consisting of methanol, distilled water and trifluoroacetic acid (TFA) (710: 290: 0.5 (v/v/v)).

Data handling

All the calculated values except for enzyme kinetics parameters below were expressed as mean ± standard deviation (SD) of three experiments throughout Results and Discussion.

(1) Estimation of the enzyme kinetics parameters for 2-oxo-clopidogrel and the active metabolite formation in the human cytochrome P450 expression systems

The reaction rate (V) was calculated according to equation 1.

$$V(pmol/pmolCYP/\min) = \frac{generated\ concentration\ (\mu M) \times 1000}{20\ (pmol\ CYP/mL) \times incubation\ time\ (\min)} \qquad (1)$$

The Michaelis-Menten constant (K_m, μM) and maximum reaction rate (V_{max}, pmol/pmol CYP/min) were calculated using WinNonlin Professional (version 4.0.1, Pharsight Corporation) 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_{max} values were expressed as mean ± standard error (SE) of parameter estimate. The intrinsic clearance (CL_{int}) was calculated as a ratio of V_{max} to K_m. The various CYPs mediated clearance ($CL_{int,\ expressed\ P450}$) in human liver microsomes was determined with equation 2. The enzyme abundance (pmol CYP/mg protein) of various CYPs in human liver microsomes was obtained from the reported data in Rowland et al., (2004).

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$$CL_{\text{int, expressed P450}}(\mu L/mg\ protein/\min) = \frac{V_{max} \times enzyme abundances(pmol CYP/mg\ protein)\ of\ \text{various CYPs}}{K_m} \quad (2)$$

The contribution ratio (f_{mcyp}, %) of each CYP which was responsible for the production of 2-oxo-clopidogrel and the active metabolite was determined with equation 3.

$$f_{mcyp}(\%) = \frac{CL_{\text{int, expressed P450 for each CYP reaction}} \times 100}{\sum CL_{\text{int, expressed P450}}} \quad (3)$$

(2) 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 using WinNonlin nonlinear estimation program (version 4.0.1) and an Eadie-Hofstee plot (x-axis: V/substrate concentration (μM), y-axis: V), where the reaction rate V (pmol/mg protein/min) was calculated according to equation 4.

$$V(pmol/mg\ protein/\min) = \frac{generated\ concentration\ (\mu M) \times 1000}{0.5\ (mg\ protein/mL) \times incubation\ time\ (\min)} \quad (4)$$

Eadie-Hofstee plots were used to visually detect deviation from linearity and to make initial estimates of kinetic constants. Data were then fitted to equation 5 using WinNonlin nonlinear estimation program. S (μM) is substrate concentration, K_{m1} and V_{max1} are the apparent K_m and V_{max} values for high affinity component and K_{m2} and V_{max2} are the apparent K_m and V_{max} values for low affinity component in equation 5.

$$V = \frac{V_{max\,1} \times S}{K_{m1} + S} + \frac{V_{max\,2} \times S}{K_{m2} + S} \quad (5)$$

The calculated K_m and V_{max} values were expressed as mean ± standard deviation (SD) of three experiments

The enzymatic activity remaining (%) in the presence of the antibody and the chemical inhibitor was calculated according to equation 6.

$$Enzymatic\ activity\ remaining\ (\%) = \frac{produced\ concentration\ (\mu M)\ with\ antibody\ or\ chemical\ inhibitor\ to\ CYPs}{produced\ concentration\ (\mu M)\ without\ antibody\ or\ chemical\ inhibitor\ to\ CYPs} \times 100 \quad (6)$$

Inhibition ratio was calculated according to equation 7.

$$Inhibition\ ratio\ (\%) = 100 - enzymatic\ activity\ remaining\ (\%) \quad (7)$$

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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 μM) 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 CYPs ranked in descending order of CYP1A2, CYP2C19 and CYP2B6. Other CYP isoforms (CYP1A1, CYP2A6, CYP2C8, CYP2C9, CYP2D6, CYP2E1, CYP3A4 and CYP4A11) showed no detectable activity towards 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 β-lymphoblastoid cells expressing the human CYPs was measured by HPLC as shown in Fig. 4, using 2-oxo-clopidogrel (200 μM) 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 and CYP3A4 converted 2-oxo-clopidogrel to the active metabolite and the activity of these CYPs ranked in descending order of CYP2C19, CYP2C9, CYP3A4, CYP 2B6. CYP1A1, CYP1A2, CYP2A6, CYP2C8, CYP2D6, CYP2E1 and CYP4A11 failed to catalyze this metabolic reaction.

The contribution of each CYP 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 SUPERSOMES containing each CYP. The K_m values for CYP1A2, CYP2B6 and CYP2C19 were 1.58±1.35, 2.08±0.73

and 1.12±0.25 μM, respectively (mean ± SE of parameter estimate) (Table 1).

Estimates using equation 3 of the contribution ratio (fm CYP) of CYP1A2, CYP2B6 and CYP2C19 to 2-oxo-clopidogrel formation were 35.8, 19.4 and 44.9%, respectively, suggesting the contribution of CYP2C19 to this oxidation process was higher compared with the other two CYPs.

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 CYP. The production of the active metabolite with CYP2C9 was not detected adequately at 15 min incubation. Therefore, we used the reaction rate of the active metabolite at 30 min for calculating the enzyme kinetic parameters for CYP2C9 reaction. The enzyme kinetics parameters for the other three CYPs reactions were calculated 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 ± SE 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/pmol CYP/min, respectively (mean ± SE of parameter estimate).

The $CL_{int,\ expressed\ P450}$ and f_{mCYP} for CYP2B6, CYP2C9, CYP2C19 and CYP3A4 reactions are shown in Table 2. Estimates of the fm CYP of CYP2B6, CYP2C9, CYP2C19 and CYP3A4 for the active metabolite formation were 32.9, 6.76, 20.6 and 39.8%, respectively, suggesting the contribution of CYP3A4 to the active metabolite formation was greater compared with the other three CYPs.

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 microsome had a biphasic

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pattern in an 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 using WinNonlin nonlinear estimation program. The apparent K_{m1} and V_{max1} values for the high affinity component were 4.70±2.62 μM and 144±61.0 pmol/mg protein/min, respectively (mean±SD, n=3). For the low-affinity component, the K_{m2} was 71.9±30.78 μM and the V_{max2} was 1230±220 pmol/mg protein/min (mean±SD, n=3). Therefore, clopidogrel concentration in the inhibition studies was set at 4 μM which was close to K_{m1} value of high affinity component.

The inhibitory effects of monoclonal antibodies to CYP1A2, CYP2B6 and CYP2C19 on the production of 2-oxo-clopidogrel were examined using human liver microsomes as the enzyme source and clopidogrel (at K_m; 4 μM) as the substrate in the presence of NADPH-generating system as shown in Table 3. The inhibition ratio of 2-oxo-clopidogrel formation with monoclonal antibodies to CYP1A2, CYP2B6 and CYP2C19 were 30.9±9.0%, 26.0±2.5% and 42.0±8.1%, respectively (mean ± SD, n=3). The inhibitory effect of monoclonal antibodies to CYP2C19 on this oxidation process was higher than that of the other two monoclonal antibodies. Moreover, the effects of chemical inhibitors on the production of 2-oxo-clopidogrel were also investigated in human liver microsomes (Fig. 6). Furafylline (10 μM), an inhibitor of CYP1A2, inhibited 2-oxo-clopidogrel formation by 34.9±0.5 %. (S)-N-3-Benzylnirvanol and omeprazole, the inhibitors of CYP2C19 at 10 μM, also inhibited the formation of 2-oxo-clopidogrel by 26.9±10.5 and 27.1±9.2%, respectively (mean ± SD, n=3).

The kinetic parameters of clopidogrel active metabolite formation from 2-oxo-clopidogrel in human liver microsomes were also estimated using WinNonlin nonlinear estimation program since 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±0.828 μM and 32.4±11.6 pmol/mg protein/min, respectively (mean±SD, n=3). For the low-affinity component, the K_{m2} was 94.9±6.05 μM and the V_{max2} was 249±41.0 pmol/mg protein/min (mean±SD, n=3). Based on these results, the 2-oxo-clopidogrel concentration in the inhibition studies was set at 3 μM which was close to 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 using human liver microsomes as the enzyme source and 2-oxo-clopidogrel (at K_m: 3 μM) as the substrate in the presence of NADPH-generating system and glutathione (5 mM) 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±3.4%, 13.5±4.4%, 32.9±4.2% and 31.3±5.3%, respectively (mean ± SD, n=3). Sulfaphenazole, the inhibitor of CYP2C9 at 10 μM also inhibited by 36.2±7.7%. (S)-N-3-benzylnirvanol and omeprazole, the inhibitors of CYP2C19 at 10 μM inhibited this reaction by 35.4±15.2 and 31.3±17.3%, respectively. Ketoconazole, an inhibitor of CYP3A4 at 2 μM, inhibited the formation of the active metabolite by 38.4±8.4 % (mean ± SD, n=3) (Fig. 6).

Discussion

This is the first report where the CYPs 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 while the remaining isoforms examined were practically inactive in this first oxidative step of clopidogrel. Savi et al. (1994) suggested that rat CYP1A was involved in the active metabolite formation from 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 as it was previously shown that formation of a thienopyridine's active metabolite required the presence of both CYPs 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, since 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 CYP2B6 and CYP2C19 inhibition observed *in vitro* by clopidogrel (Richter et al., 2004; Nishiya et al., 2007; Nishiya et al., 2009; Hagihara et al., 2008). The enzyme kinetic parameters utilized in equation 3 showed that the contribution of each CYP 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-CYP antibodies and chemical inhibitors. The inhibition

effects of the monoclonal antibodies on the first oxidation step towards 2-oxo-clopidogrel formation were consistent with the results of the contribution of each CYP involved in this oxidative step. Anti-CYP1A2, anti-CYP2B6 and anti-CYP2C19 antibodies inhibited the formation of 2-oxo-clopidogrel by 30.9±9.0%, 26.0±2.5% and 42.0±8.1%, respectively. Additionally, the inhibition levels with the chemical inhibitors on the production of 2-oxo-clopidogrel were consistent with the outcome of the antibody experiments.

Similarly, 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 since previous studies demonstrated that such an inhibition was not involved in this reaction process (Nishiya et al., 2009).

The enzyme kinetic parameters combined with equation 3 showed that the contribution of each CYP 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 metabolite 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 CYP 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 major effect on the formation of active metabolite from clopidogrel and this would explain the effect of loss of function variants of CYP2C19 on the pharmacokinetics and/or pharmacodynamic response to clopidogrel (Mega et al., 2009; Simon et al., 2009; Brandt et al., 2007; Hulot et al., 2006). 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 therapy. In addition, the major role of 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 platelets aggregation (Farid et al., 2007; Suh et al., 2006). 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. Additionally, we indicated that the contributions of CYP2C19 and CYP3A4 were more important relative to the other CYPs in the pharmacologically active metabolite formation by estimating the enzyme kinetic parameters and inhibition effects of the chemical inhibitors and the antibody to CYPs. These findings

would support the results of several clinical drug-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. (Farid et al., 2007; Farid et al., 2008; Suh et al., 2006; Hulot et al., 2006; Brandt et al., 2007; Mega et al., 2009; Simon 2009).

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FOOTNOTES

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Figure Legends

Figure 1. *Biotransformation pathway of clopidogrel leading to its pharmacologically active metabolite via 2-oxo-clopidogrel.*

Figure 2. *Structure of an internal standard in assay of 2-oxo-clopidogrel and clopidogrel active metabolite by LC-MS/MS.*

Figure 3. *Formation of 2-oxo-clopidogrel from clopidogrel in human cytochrome P450-expression system in the presence of NADPH.*

The assays were performed using the microsomes from baculovirus/insect cells expressing human cytochrome P450 (SUPERSOMES). Data were expressed as mean ± standard deviation (SD) of three experiments.

N.D., not determined.

Figure 4. *Formation of the pharmacologically active metabolite of clopidogrel from 2-oxo-clopidogrel in human cytochrome P450-expression system in the presence of NADPH and glutathione.*

The assays were performed using the microsomes from β-lymphoblastoid cells expressing human cytochrome P450. Data were expressed as mean ± standard deviation (SD) of three experiments.

N.D., not determined.

Figure 5. *Kinetic analysis of 2-oxo-clopidogrel formation from clopidogrel in human liver microsome by an Eadie-Hofstee plots and WinNonlin nonlinear estimation program.*

The human liver microsomes were incubated with 1.25 to 160 μM clopidogrel at 37°C for 5 min. Data are plotted using an Eadie-Hofstee plots (A) and Direct plots (B). Line in panel B represents the curve fit to equation 5 using WinNonlin nonlinear estimation program. Typical results from one of three experiments are shown.

Figure 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 ± standard deviation (SD) of three experiments.

Figure 7. *Kinetic analysis of the active metabolite formation from 2-oxo-clopidogrel in human liver microsome by an 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 an Eadie-Hofstee plots (A) and Direct plots (B). Line in panel B represents the curve fit to equation 5 using WinNonlin nonlinear estimation program. Typical results from one of three experiments are shown.

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Table 1 Enzyme kinetic parameters of 2-oxo-clopidogrel formation from clopidogrel

	Incubation time (min)	K_m (μM)	V_{max} (pmol/pmol CYP/min)	V_{max}/K_m (μL/pmol CYP/min)	Enzyme Abundances* (pmol CYP/mg protein)	$CL_{int,\ expressed\ P450}$ (μL/mg protein/min)	f_{mCYP} (%)
CYP1A2	1	1.58 ± 1.35	2.27 ± 0.46	1.44	52	74.9	35.8
CYP2B6	1	2.08 ± 0.73	7.66 ± 0.69	3.68	11	40.5	19.4
CYP2C19	1	1.12 ± 0.25	7.52 ± 0.36	6.71	14	93.9	44.9

The assays were performed using the microsomes baculovirus/insect cells expressing human cytochrome P450 (SUPERSOMES). K_m and V_{max} values were expressed as mean ± standard error (SE) of parameter estimate. $CL_{int,\ expressed\ P450}$ and f_{mCYP} (contribution ratio of each CYP) values were scaled to equation 2 and 3 under Materials and Method section to determine contribution.

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

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

	Incubation time (min)	K_m (μM)	V_{max} (pmol/pmol CYP/min)	V_{max}/K_m (μL/pmol CYP/min)	Enzyme Abundances* (pmol CYP/mg protein)	$CL_{int,\ expressed\ P450}$ (μL/mg protein/min)	f_{mCYP} (%)
CYP2B6	15	1.62 ± 0.08	2.48 ± 0.03	1.53	11	16.8	32.9
CYP2C9	30	18.1 ± 3.8	0.855 ± 0.084	0.0472	73	3.45	6.76
CYP2C19	15	12.1 ± 2.2	9.06 ± 0.68	0.749	14	10.5	20.6
CYP3A4	15	27.8 ± 4.2	3.63 ± 0.29	0.131	155	20.3	39.8

The assays were performed using the microsomes baculovirus/insect cells expressing human cytochrome P450 (SUPERSOMES). K_m and V_{max} values were expressed as mean ± standard error (SE) of parameter estimate. CL_{int} and f_{mCYP} (CYP contribution ratio) values were scaled to equation 2 and 3 under Materials and Method section to determine contribution.

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

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Table 3 Effect of anti-CYP antibodies on the 2-oxo-clopidogrel formation from clopidogrel and active metabolite formation from 2-oxo-clopidogrel

	Inhibition ratio (%)	
	2-oxo-clopidogrel formation	Active metabolite formation
Anti-CYP1A2	30.9 ± 9.0	
Anti-CYP2B6	26.0 ± 2.5	54.3 ± 3.4
Anti-CYP2C9		13.5 ± 4.4
Anti-CYP2C19	42.0 ± 8.1	32.9 ± 4.2
Anti-CYP3A4		31.3 ± 5.3

The assays were performed using human liver microsomes. Anti-CYP1A2, anti-CYP2B6, anti-CYP2C19 and anti-CYP3A4 were monoclonal antibodies and anti-CYP2C9 was polyclonal antibody. The inhibition ratio values were calculated with equation 4 and 5 under Materials and Method section.

Data were expressed as mean ± standard deviation (SD) of three experiments.

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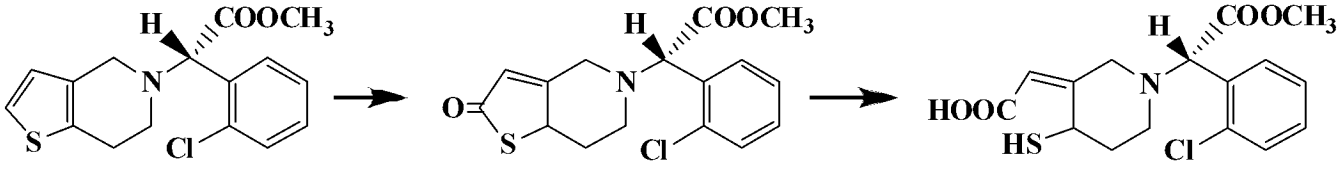
Fig. 1.



Clopidogrel **2-oxo-clopidogrel** **pharmacologically active metabolite of clopidogrel**

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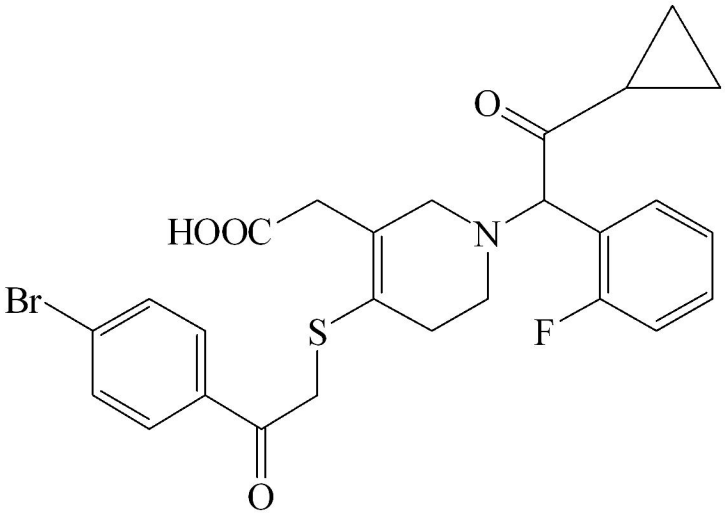
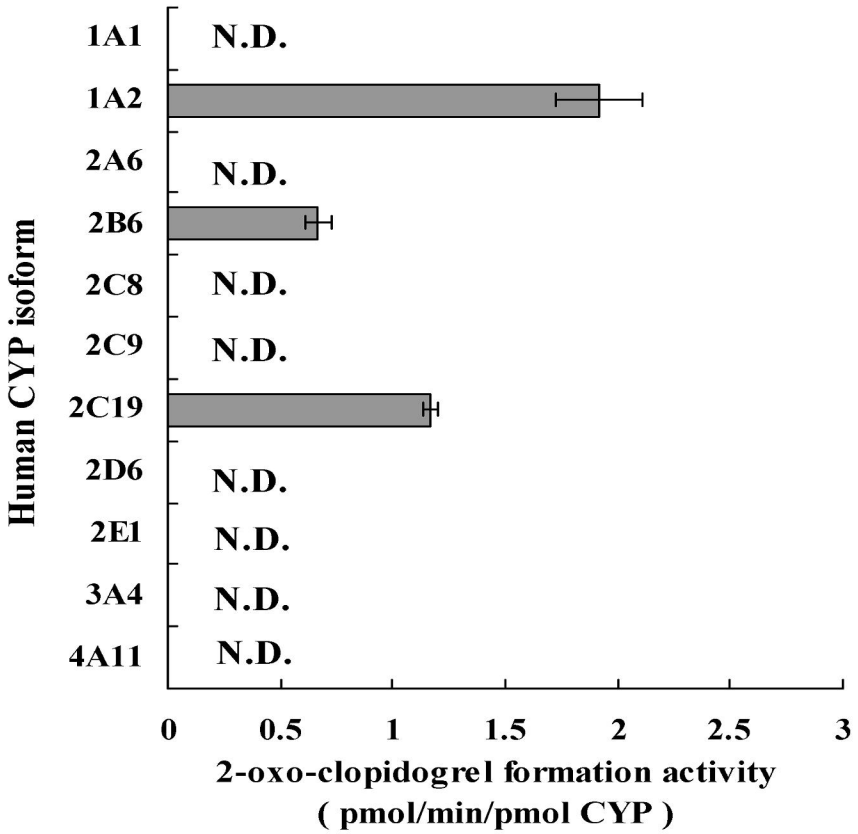
Fig. 2.



R-135766

Fig. 3.


Human CYP isoform
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1A2
2A6
2B6
2C8
2C9
2C19
2D6
2E1
3A4
4A11
N.D.
N.D.
N.D.
N.D.
N.D.
N.D.
N.D.
N.D.
0
0.5
1
1.5
2
2.5
3
2-oxo-clopidogrel formation activity
(pmol/min/pmol CYP)

Fig. 4.

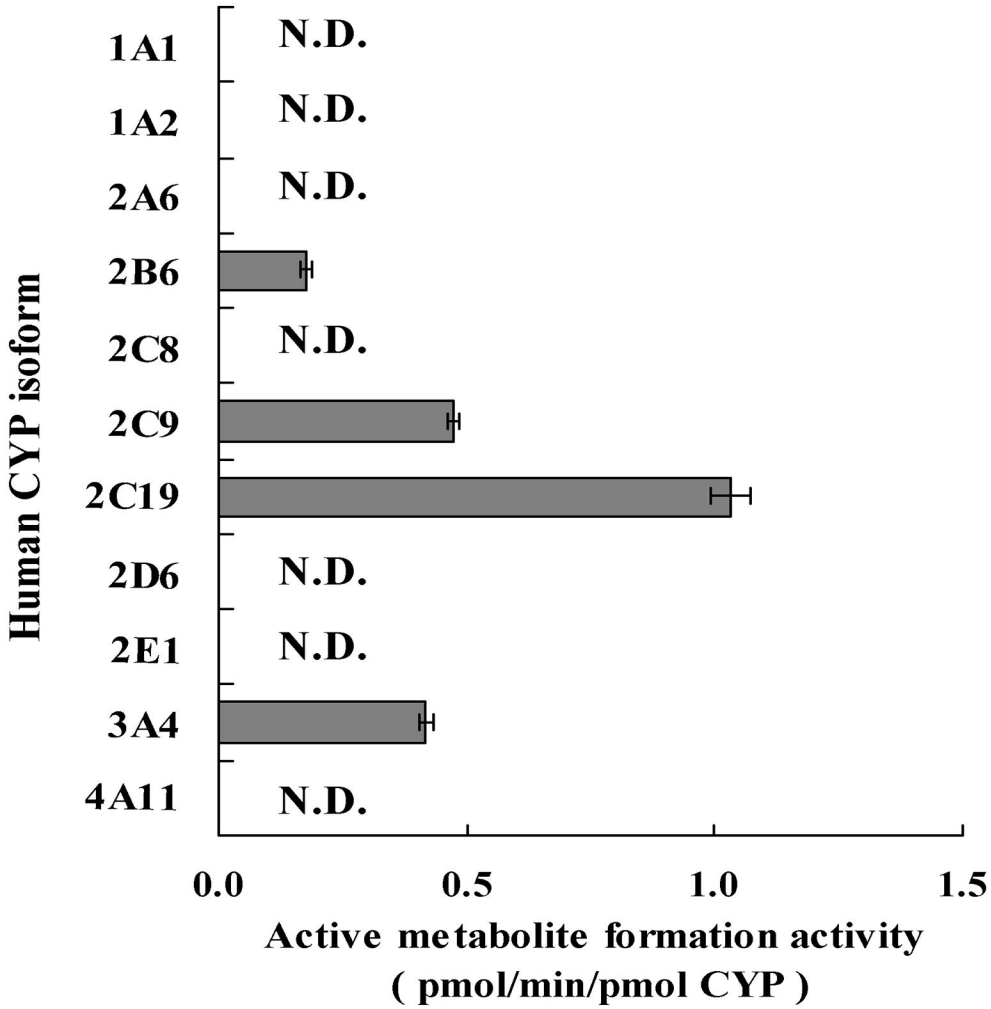
Human CYP isoform
1A1
1A2
2A6
2B6
2C8
2C9
2C19
2D6
2E1
3A4
4A11
N.D.
N.D.
N.D.
N.D.
N.D.
N.D.
0.0
0.5
1.0
1.5
Active metabolite formation activity
(pmol/min/pmol CYP)

Fig.5.

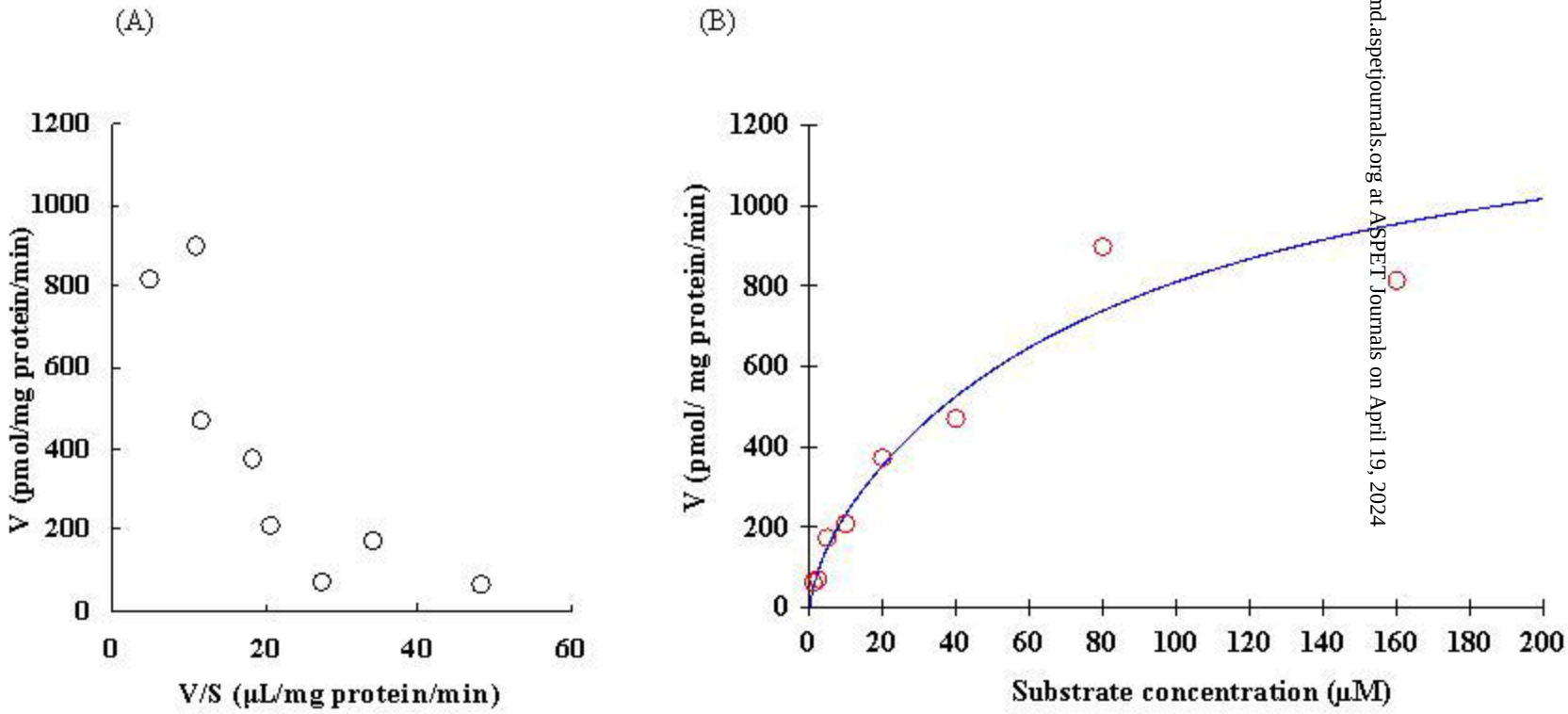

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Fig. 6.

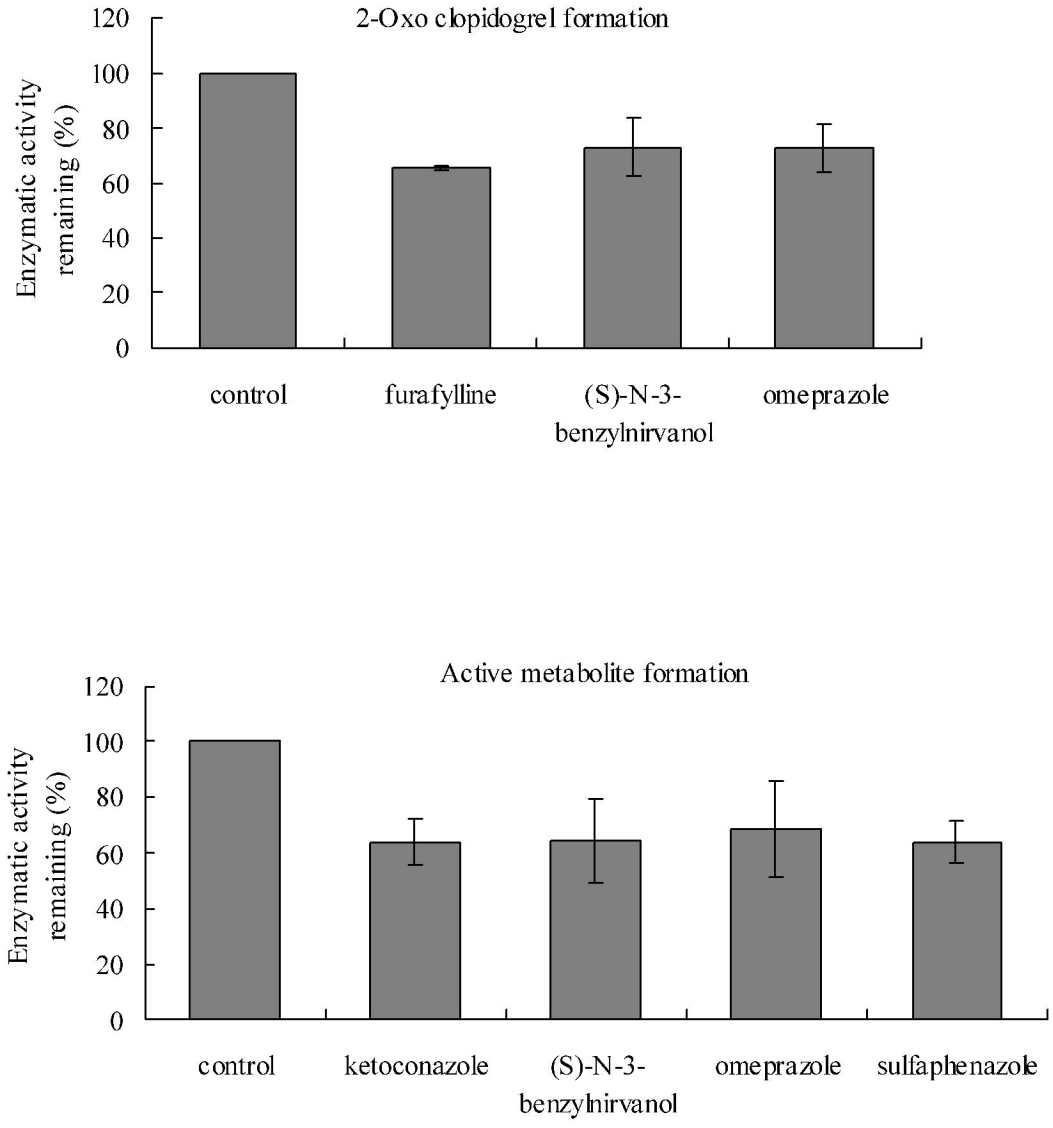

Fig.7.