ABSTRACT

Clopidogrel, a thienopyridine antiplatelet prodrug, is metabolized by oxidation to 2-oxo-clopidogrel, followed by conversion to its pharmacologically active thiol metabolite. After oral administration of clopidogrel to humans, two thiol isomers (H3 and H4) are observed in plasma, with similar concentrations, and only H4 is active in humans. In this work, the mechanism of stereoselectivity in the formation and S-methylation of H3 and H4 was investigated in vitro. The two diastereomers of 2-oxo-clopidogrel were epimerized rapidly at physiologic pH. The intrinsic clearance (CLint) for H3 formation from 2-oxo-clopidogrel in human liver microsomes (HLMs) was 3.1-fold higher than that for H4 formation, indicating stereoselective metabolism. Kinetic studies using expressed enzymes demonstrated that the contributions of CYP2B6, CYP2C19, and CYP3A4 to the formation of H4 from 2-oxo-clopidogrel were 18.5%, 26.1%, and 53.5%, respectively. The CLint ratios of H3 formation to H4 formation from 2-oxo-clopidogrel by CYP2B6, CYP2C19, and CYP3A4 were 2.2, 1.0, and 1.7, respectively. In HLMs, H3 and H4 were further S-methylated, and the S-methylation was inhibited by 2,3-dichloromethyl benzylamine, indicating the involvement of thiol S-methyltransferase. The CLint value for the S-methylation of H3 in HLMs was 98.1-fold higher than that for H4. The stereoselective formation of H3 from 2-oxo-clopidogrel and the stereoselective S-methylation of H3 may lead to the similar exposure levels of H3 and H4 previously reported in humans. The epimerization of 2-oxo-clopidogrel and the variations of thiol S-methyltransferase may affect the exposure to H4 in humans.

INTRODUCTION

Clopidogrel (Plavix, Iscover) is a thienopyridine antiplatelet agent widely used to prevent cardiovascular events and death in patients with acute cardiovascular syndromes, particularly coronary interventions (Diener et al., 2005; Fox and Chelliah, 2007). Despite its wide use, significant interindividual variability has been shown in its efficacy with 20–40% of patients exhibiting a lower response or no response to clopidogrel therapy (Gurbel et al., 2003; Kolandaivelu and Bhatt, 2010). Only 12% of the variations in response are caused by the CYP2C19*2 mutant gene (Sulldiner et al., 2009), and the remaining factors are still unidentified.

Clopidogrel is a prodrug that requires bioactivation via a two-step cytochrome P450 (P450)–dependent oxidation to its pharmacologically active thiol 6 (Fig. 1), which relates to the variations in response to clopidogrel therapy closely (Savi et al., 2000; Kazui et al., 2010; Dansette et al., 2012). Clopidogrel is first oxidized to 2-oxo-clopidogrel (thiolactone 2). Further oxidation of thiolactone 2 leads to the formation of a sulfenic acid intermediate 4. The sulfenic acid 4 is highly unstable and can be rapidly reduced by glutathione (GSH) to form a mixed disulfide conjugate 5, which is subsequently reduced by another GSH molecule to form the active thiol 6 (Dansette et al., 2009; Zhang et al., 2012). H1 and H2 are the 3E isomers, whereas H3 and H4 are the 3Z isomers (Pereillo et al., 2002; Tuffal et al., 2011; Bluet et al., 2014). However, the absolute configurations at C4 of H1 and H2 remain unclear.

Previous studies showed the chiral instability of the diastereomers of 2 (2a and 2b) in CD3OD in the presence of K2CO3 resulted from the base-catalyzed epimerization of the chiral carbon at C4 (Fig. 1) (Dansette et al., 2012). However, whether 2a and 2b are epimerized at physiologic pH remains unclear. Due to its stereochemical structure, thiol 6 can consist of four isomers (H1–H4) in vitro (Pereillo et al., 2002; Zhang et al., 2012). H1 and H2 are the 3E isomers, whereas H3 and H4 are the 3Z isomers (Pereillo et al., 2002). The absolute configurations at C4 of H3 and H4 are S and R, respectively (Pereillo et al., 2002; Tuffal et al., 2011; Bluet et al., 2014). However, the absolute configurations at C4 of H1 and H2 remain unclear.

In vitro, H4 shows the highest biologic activity; the potency of H2 is approximately half that of H4, and H1 and H3 are inactive. After oral administration of clopidogrel to humans, only H3 and H4 could be detected, and had similar concentrations in plasma, whereas H1 and H2 were not detected (Tuffal et al., 2011; KarazNiewicz-Lada et al., 2012). Therefore, H4 is the only isoform responsible for the antiplatelet activity.
The contributions of P450 isoforms to the formation of thiol \( \text{6} \) from thiolactone \( \text{2} \) follow the order CYP3A4 > CYP2B6 > CYP2C19 > CYP2C9 (Kazui et al., 2010). Subsequently, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 were reported to be involved in the formation of active H4 from thiolactone \( \text{2} \) (Gong et al., 2012; Zhang et al., 2014). However, the contributions of these P450s to the formation of active H4 from thiolactone \( \text{2} \) remain unclear. For prasugrel, an analog of clopidogrel, each isomer of its pharmacologically active metabolite was formed by P450 isozymes and S-methylated by thiol S-methyltransferase (TMT) stereoselectively (Baker et al., 2008; Kazui et al., 2014). Therefore, clopidogrel is likely to follow a similar stereoselectivity mechanism in the formation and S-methylation of H3 and H4. A previous in vitro study showed no significant stereoselectivity in the formation of H3 and H4 from thiolactone \( \text{2} \) by CYP2C19 (Gong et al., 2012); whether other P450 isoforms are involved in the stereoselective formation of H3 and H4 remains to be investigated further, and, to the best of our knowledge, the S-methylation of thiol \( \text{6} \) has not been reported to date.

In this work, we investigated the mechanism of stereoselectivity in the formation and S-methylation of H3 and H4 of clopidogrel in humans in vitro (Fig. 2). The diastereomers of thiolactone \( \text{2} (2a \text{ and } 2b) \) were epimerized rapidly at physiologic pH. H3 and H4 were further S-methylated by TMT. The stereoselective formation of H3 from thiolactone \( \text{2} \) by CYP2B6 and CYP3A4 and the stereoselective S-methylation of H3 by TMT were observed. These stereoselective mechanisms may cause the similar exposure levels of H3 and H4 in humans. We also elucidated the absolute configurations at C4 of H1 and H2 and projected the relative contribution of each P450 isoform to the formation of active H4 from thiolactone \( \text{2} \).

Fig. 1. Bioactivation pathway of clopidogrel (Dansette et al., 2012). Owing to its stereochemical structure, compound \( \text{2} \) can exist as two epimers, whereas the active metabolite \( \text{6} \) can exist as four stereoisomers.

Fig. 2. Proposed mechanism of stereoselectivity in the formation and S-methylation of H3 and H4 in HLMs. The gray arrows represent the metabolic pathways only found in vitro. The black arrows represent the metabolic pathways both in vitro and in vivo in humans. H4 is the only isomer responsible for the antiplatelet activity in vivo in humans.
Materials and Methods

Chemicals and Materials. [75]-2-Oxo-clopidogrel (2, a 1:9 mixture of 2a and 2b); free clopidogrel trans thiol diastereomers (H1 and H2); the 3’-methoxyacetophenone derivatives of H1, H2, H3, and H4 (MP-H1, MP-H2, MP-H3, and MP-H4); the S-methylated form of H1, H2, H3, and H4 (7a, 7b, 7c, and 7d) (Fig. 2); and the 4’-bromooctophenone derivative of the analog of the clopidogrel active thiol metabolite were gifts of J. Vcare PharmaTech (Jiangsu, China). The 1H and 13C NMR chemical shifts of the 2, the derivatized thiol isomers (MP-H1 to MP-H4), and the S-methylated metabolite isomers (7a–7d) were shown in Supplemental Tables 1–3. The one-dimensional NMR spectra of 2 and the one- and two-dimensional NMR spectra of the derivatized thiol isomers (MP-H1 to MP-H4) and the S-methylated metabolite isomers (7a–7d) were shown in Supplemental Figs. 1–44. The NMR data of these standards were in accordance with their chemical structure and provided the configuration information of C5–C16 double bond of them. The 4’-bromooctophenone derivative was used as the internal standard (IS) for the assay of 7c, 7d, MP-H3, and MP-H4 by liquid chromatography–tandem mass spectrometry (LC-MS/MS). The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO): NADPH, GSH, ascorbic acid, 2-bromo-3’-methoxyacetophenone (MPB), sulfaphenazole, quinidine, ketocazolamoe, and S(5′-adenyl)-L-methionine chloride (SAM). (±)-2,3-Dichloro-4-methylbenzyldiene hydrochloride (DCMB), m-anisic acid, and omeprazole were obtained from Dalian Meilun Biotech (Liaoning, China). The monoclonal antibody to CYP2B6 was obtained from BD Gentest (Woburn, MA). All other chemicals, reagents, and solvents were of either analytical grade.

Reference standard of 7d, MP-H3, and MP-H4 by liquid chromatography–tandem mass spectrometry (LC-MS/MS). The following chemicals were purchased from Sigma-Aldrich (St. Louis, MO): NADPH, GSH, ascorbic acid, 2-bromo-3’-methoxyacetophenone (MPB), sulfaphenazole, quinidine, ketocazolamoe, and S(5′-adenyl)-L-methionine chloride (SAM). (±)-2,3-Dichloro-4-methylbenzyldiene hydrochloride (DCMB), m-anisic acid, and omeprazole were obtained from Dalian Meilun Biotech (Liaoning, China). The monoclonal antibody to CYP2B6 was obtained from BD Gentest (Woburn, MA). All other chemicals, reagents, and solvents were of either analytical grade or high-performance liquid chromatography (HPLC) grade.

Enzyme Sources. Pooled mixed-gender human liver microsomes, pooled rat liver microsomes, pooled mixed-gender human liver cytosol, and recombinant human P450 isozymes (CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5) were purchased from BD Gentest.

Separation of Thiolactone 2 Isomers and Analysis by Circular Dichroism. Reference standard 2 was separated by using a Shimadzu LC-6AD semipreparative HPLC apparatus (Shimadzu, Kyoto, Japan) equipped with a SPD-20A UV detector (Shimadzu). Separation was achieved with a YMC-Pack ODS-AQ column (250 × 20 mm I.D., 5 µm; YMC, Kyoto, Japan). Elution was performed with acetonitrile–water (52:48, v/v) at a flow rate of 8 mL/min. The detection wavelength was set at 254 nm. The eluted fractions at 40.2 and 25.4 minutes were separately collected, evaporated, and reconstituted in acetonitrile to obtain the separated standard stock solutions of H3 and H4, respectively.

Inhibition of S-Methylation of H3 and H4 in HLMs and Human Liver Cytosol. Effects of the selective chemical inhibitors for TMT (DCMB) and thiopurine S-methyltransferase (TPMT; m-anisic acid) (Lee and Kim, 1999) on the S-methylation of H3 and H4 were evaluated. DCMB was added at a final concentration of 0, 10, 100, or 1000 µM to the incubation medium consisting of HLMs (0.5 mg protein/mL); SAM (0.5 mM); and NADPH (1 mM) at 37°C for 15 minutes. Reactions were stopped by adding three volumes of ice-cold acetoni trile. After the samples were centrifuged for 15 minutes at 13,000g, the supernatant was evaporated to dryness under a nitrogen stream at 40°C. The residue was reconstituted in 100 µL mobile phase, and a 10 µL aliquot of the reconstituted solution was injected in the UPLC-Q/TOF MS system for analysis.

Preparation of Thiolactone 2 Isomers (2a and 2b) in Buffer. Either isomer of thiolactone 2 (2a or 2b) was incubated in water and 100 mM PBS (pH 4.7, 7.4, and 9) for 30 minutes at 37°C, and the peak area ratios of 2b and 2a were determined by UPLC-Q/TOF MS to analyze the chiral stability of 2a and 2b.

Inhibition of Thiolactone 2 with HLMs. Incubations were performed in 200 µL 100 mM PBS (pH 7.4) containing HLMs (0.5 mg protein/mL), 2 (3 µM, a 1:1 mixture of 2a and 2b), and GSH (5 mM) with or without NADPH (1 mM) at 37°C for 15 minutes. Reactions were stopped by adding three volumes of ice-cold acetoni trile. After the samples were centrifuged for 5 minutes at 13,000g, the supernatant was evaporated to dryness under a nitrogen stream at 40°C. The residue was reconstituted in 100 µL mobile phase, and a 10 µL aliquot of the reconstituted solution was injected into the UPLC-Q/TOF MS system for analysis.

Inhibition of Thiolactone 2 (2a and 2b) with RLMs. Incubations were typically performed in 200 µL water containing RLMs (1 mg protein/mL), 2a or 2b (10 µM), and GSH (50 µM) with or without NADPH (1 mM) at 37°C for 10 minutes. Reactions were stopped by adding three volumes of ice-cold acetoni trile. After the samples were centrifuged for 5 minutes at 13,000g, the supernatant was evaporated to dryness under a nitrogen stream at 40°C. The residue was reconstituted in 100 µL mobile phase, and a 10 µL aliquot of the reconstituted solution was injected into the UPLC-Q/TOF MS system for analysis.

S-Methylation of H1–H4 in HLMs. Incubations were typically performed in 200 µL 100 mM PBS (pH 7.4) containing HLMs (0.5 mg protein/mL); magnesium chloride (10 mM); H1, H2, H3, or H4 (2 µM); SAM (0.5 mM); and NADPH (1 mM) at 37°C for 15 minutes. Reactions were stopped by adding three volumes of ice-cold acetoni trile. After the samples were centrifuged for 5 minutes at 13,000g, the supernatant was evaporated to dryness under a nitrogen stream at 40°C. The residue was reconstituted in 100 µL mobile phase, and a 10 µL aliquot of the reconstituted solution was injected into the UPLC-Q/TOF MS system for analysis.

Inhibition of S-Methylation of H3 and H4 in HLMs and Human Liver Cytosol. Effects of the selective chemical inhibitors for TMT (DCMB) and thiopurine S-methyltransferase (TPMT; m-anisic acid) (Lee and Kim, 1999) on the S-methylation of H3 and H4 were evaluated. DCMB was added at a final concentration of 0, 10, 100, or 1000 µM to the incubation medium consisting of HLMs (0.5 mg protein/mL); SAM (0.5 mM); and 100 mM PBS (pH 7.4), and the mixtures were preincubated at 37°C for 5 minutes. Similarly, DCMB was added at a final concentration of 0, 10, 100, or 1000 µM to the incubation medium consisting of human liver cytosol (HLC; 1 mg protein/mL), SAM (0.5 mM), and 400 mM PBS (pH 6.3), and the mixtures were preincubated at 37°C for 5 minutes. m-Anisic acid was added at a final concentration of 0.2, 2, or 20 mM to the incubation medium consisting of HLC (1 mg protein/mL), SAM (0.5 mM), and 400 mM PBS (pH 6.3), and the mixtures were preincubated at 37°C for 5 minutes. Each reaction was initiated by the addition of the mixture of H3 and H4 (2 µM H3 and 2 µM H4) and terminated after incubation at 37°C for 15 minutes by adding 600 µL acetoni trile containing 10 µM IS. Proteins were removed by centrifugation at 13,000g at 4°C for 5 minutes. A 100 µL aliquot of the supernatant was diluted fourfold with the mobile phase, and then 5 µL resulting solution was injected into the LC-MS/MS system to determine the concentrations of 7c and 7d. Experiments were performed in duplicate.
Enzyme Kinetic Parameters for H3 and H4 Formation from Thiolactone 2. The conditions for incubation were optimized to be linear with respect to incubation time and protein concentration. The incubation mixture contained GSH (5 mM), NADPH (1 mM), and different enzyme sources (HLMs, 0.5 mg protein/mL, or reconstituent CYP, 20 pmol/mL) in a final volume of 200 μL 100 mM PBS (pH 7.4) for the HLMs, CYP2B6, CYP2C19, CYP2D6, CYP3A4, and CYP3A5 reactions, or in a final volume of 200 μL Tris-HCl buffer (0.1 M, pH 7.5) for the CYP2C9 reaction. The mixtures were preincubated for 5 minutes at 37°C, and each reaction was initiated by adding various concentrations of thiolactone 2 (50–100 μM, α:1 mixture of 2a and 2b). After incubation at 37°C for 0, 10, or 30 minutes, reactions were terminated by adding 600 μL acetonitrile containing 2 mM MPB as the derivatization reagent and 10 μM IS. After standing for 10 minutes at room temperature, the mixtures were centrifuged at 13,000g for 4°C for 5 minutes. A 100 μL aliquot of the supernatant was diluted fourfold with the mobile phase, and then 5 μL resulting solution was injected into the LC-MS/MS system to determine the concentrations of MP-H3 and MP-H4. Experiments were performed in duplicate.

Inhibition of H3 and H4 Formation from Thiolactone 2 in HLMs. Incubations for studies using a monoclonal antibody were performed in 200 μL 100 mM PBS (pH 7.4) containing HLMs (0.5 mg protein/mL), 2 (3 μM, α:1 mixture of 2a and 2b), GSH (5 mM), NADPH (1 mM), and the monoclonal antibody to CYP2B6. After incubation with 5 μL HLMs (20 mg protein/mL) and 10 μL monoclonal antibody to CYP2B6 or Tris-HCl buffer (25 mM, pH 7.5) used as control on ice for 20 minutes, 103 μL PBS (100 mM, pH 7.4), 40 μL GSH, and 40 μL NADPH were added to the incubations, and then the mixtures were preincubated at 37°C for 5 minutes. Each reaction was initiated by the addition of 2 μL solution of 2 in acetonitrile. Incubations for studies using chemical inhibitors were performed in 200 μL 100 mM PBS (pH 7.4) containing HLMs (0.5 mg protein/mL), 2 (3 μM, α:1 mixture of 2a and 2b), GSH (5 mM), and NADPH (1 mM) with or without a single selective chemical P450 inhibitor. The chemical inhibitors were sulfaphenazole (10 μM) for CYP2C9, omeprazole (10 μM) for CYP2C19, quinidine (8 μM) for CYP2D6, and ketoconazole (2 μM) for CYP3A4/5. The mixtures were preincubated at 37°C for 5 minutes, and the reactions were initiated by the addition of the substrate. After incubation at 37°C for 10 minutes in both studies, the reactions were terminated by adding 600 μL acetonitrile containing 2 mM MPB as the derivatization reagent and 10 μM IS. After standing for 10 minutes at room temperature, the mixtures were centrifuged at 13,000g for 4°C for 5 minutes. A 100 μL aliquot of the supernatant was diluted fourfold with the mobile phase, and then 5 μL resulting solution was injected into the LC-MS/MS system to determine the concentrations of MP-H3 and MP-H4. Experiments were performed in duplicate.

Enzyme Kinetic Parameters for S-Methylation of H3 and H4. The conditions for incubation were optimized to be linear with respect to incubation time and protein concentration. Incubations were performed in 200 μL 100 mM PBS (pH 7.4) containing HLMs (0.5 mg protein/mL), SAM (0.5 mM), GSH (5 mM), and magnesium chloride (10 mM). The mixtures were preincubated for 5 minutes at 37°C, and the reactions were initiated by adding various concentrations of H3 or H4 (0–10 μM). After incubation at 37°C for 0 or 15 minutes, the reactions were terminated by adding 600 μL acetonitrile containing 10 μM IS. Proteins were removed by centrifugation at 13,000g for 5 minutes at 4°C. A 100 μL aliquot of the supernatant was diluted fourfold with the mobile phase, and then 5 μL resulting solution was injected into the LC-MS/MS system to determine the concentrations of 7c and 7d. Experiments were performed in duplicate.

UPLC-Q/TOF MS Analysis. To determine the peak area ratio of 2b/2a or 2a/2b after incubating 2a or 2b in buffer, and to identify the thiol metabolite isomers (H1–H4) and the S-methylated metabolite isomers (7a–7d) produced in HLMs and RLMs, chromatographic analysis was performed on a Waters Acquity UPLC system (Waters, Milford, MA) equipped with a binary solvent delivery pump, column oven, and autosampler. Chromatographic separation of thiolactone 2 and the S-methylated metabolite isomers was performed on an Acquity UPLC BEH C18 column (100 × 2.1 mm ID, 1.7 μm; Waters) with an isocratic mobile phase consisting of 0.05% aqueous formic acid and acetonitrile (60:40, v/v) at a flow rate of 0.4 mL/min. Chromatographic separation of the thiol metabolite isomers was performed on an Acquity UPLC HSS T3 column (100 × 2.1 mm ID, 1.8 μm; Waters). The mobile phase was a mixture of 5 mM aqueous ammonium acetate containing 0.05% formic acid (A) and acetonitrile (B) at a flow rate of 0.45 mL/min. The gradient elution started with a 1-minute isocratic run with 5% B, followed by a linear gradient of 5% to 65% B over 14 minutes, 65% to 99% B over 1 minute, maintained for 1 minute, and then reduced to 5% B to equilibrate the column. The column and autosampler temperatures were 45°C and 4°C, respectively.

To assist peak assignment, the derivatized thiol isomers (MP-H1 to MP-H4) were analyzed using the same column (Shim-pack XR-ODS II column; 75 × 2.0 mm ID, 2.2 μm; Shimadzu) and gradient program as a previous study (Tuffail et al., 2011), except that the separation was performed on a Waters Acquity UPLC system in our study.

Mass spectrometry (MS) detection was conducted on a triple TOF 5600+ MS/MS system (AB Sciex, Concord, Ontario, Canada) in the positive electrospray ionization (ESI) mode. Mass range was set at m/z 100–1000. The following parameter settings were used: ion spray voltage, 5500 V; declustering potential, 60 V; ion source heater, 550°C; curtain gas, 40 psi; ion source gas 1, 60 psi; ion source gas 2, 60 psi. For TOF MS scans, the collision energy was 10 eV. For product ion scans, the collision energy was 35 eV, with a spread of 10 eV in the MS/MS experiment. Information-dependent acquisition (IDA) was used to trigger acquisition of MS/MS spectra for ions matching the IDA criteria. A real-time multiple mass defect filter was used in IDA criteria.

Quantitation of MP-H3, MP-H4, 7c, and 7d with LC-MS/MS. For determining the enzyme kinetics parameters and for the inhibition studies, the derivatized thiol isomers (MP-H3 and MP-H4) and the S-methylated metabolite isomers (7c and 7d) were measured by LC-MS/MS, with MP-H1 and 7c as the standards, respectively. An API 6500 triple-quadrupole mass spectrometer (AB Sciex) was used in ESI+ mode. The peak areas of the m/z 504→155 transition for the derivatized thiol isomers (MP-H1, MP-H3, and MP-H4) and the m/z 370→155 transition for the S-methylated metabolites (7c and 7d) were measured against the peak areas of the m/z 556→214 transition for IS. HPLC was performed by...
using a LC30AD liquid chromatographic system (Shimadzu). The derivatized thiol isomers were separated with an Acquity UPLC HSS T3 column (100 × 2.1 mm I.D., 1.8 μm; Waters) thermostated at 45°C at a flow rate of 0.45 mL/min with a mobile phase consisting of acetonitrile, distilled water, and formic acid (400:600:0.3, v/v/v). The S-methylated metabolite isomers were separated with an Acquity UPLC BEH C18 column (100 × 2.1 mm I.D., 1.7 μm; Waters) thermostated at 45°C at a flow rate of 0.4 mL/min with a mobile phase consisting of acetonitrile, distilled water, and formic acid (400:600:0.3, v/v/v).

### Data Analysis
The apparent kinetic parameters of the formation of H3 and H4 from thiolactone 2 and the S-methylation of H3 and H4 were determined by fitting the unweighted kinetic data from HLMs and recombinant human P450 isozymes to a one-enzyme Michaelis-Menten equation or a Hill equation \[
\frac{V}{V_{\text{max}}} = \frac{[S]}{K_{\text{m}}} + [S]
\]
and \[
\frac{V}{V_{\text{max}}} = \frac{[S]}{K_{\text{m}}} + \left[\frac{[S]}{K_{\text{m}}}\right]^n
\]
where \(V_{\text{max}}\) is the maximum rate of metabolite formation, \(K_{\text{m}}\) is the Michaelis-Menten constant (apparent \(K_{\text{m}}\), the intrinsic clearance (\(Cl_{\text{int}} = V_{\text{max}}/K_{\text{m}}\)), and Hill coefficient \(n\) (the various P450- mediated clearance (\(Cl_{\text{int, expressed P450}}\)) in HLMs was determined by the estimated \(Cl_{\text{int}}\) from each cDNA-expressed P450 isoform multiplied by the enzyme abundance of each P450 isoform. The enzyme abundance of various P450s in HLMs was obtained from previously reported data (Rodrigues, 1999). The contribution ratio \(f_{\text{P450, %}}\) of each P450 involved in the formation of H3 or H4 from thiolactone 2 was determined by the equation \(f_{\text{P450, %}} = \frac{Cl_{\text{int, expressed P450}} \times 100}{Cl_{\text{int, expressed P450}} + Cl_{\text{int, expressed P450}} - Cl_{\text{int, expressed P450}}\).

The percentage inhibition was calculated by the ratio of the rate of metabolite formation with and without the specific inhibitor. Calculations were performed with GraphPad Prism 5.0 software (La Jolla, CA).

### Results

#### Chiral Stability of Thiolactone 2 Isomers (2a and 2b) in Buffer.

The chiral stability of 2a and 2b was analyzed in water and 100 mM PBS (pH 4, 7.4, and 9). When incubated in water at 37°C for 30 minutes, 2a and 2b were stable (data not shown). When 2a or 2b was incubated in 100 mM PBS at pH 4 at 37°C, the other diastereomer was formed slowly (Fig. 3). However, when 2a or 2b was incubated in 100 mM PBS at pH values of 7.4 and 9, the other diastereomer was formed rapidly (Fig. 3). These data indicated that the base-catalyzed epimerization of the chiral carbon at C4 of 2a (Fig. 1) also occurred during the incubation. The final equilibrium mixture was approximately a 1:1 ratio of 2a and 2b, independent of whether the incubation started with 2a or 2b.

#### Elucidation of Absolute Configurations of 2a and 2b by ECD.

The absolute configurations of 2a and 2b were determined by comparing the experimental and calculated ECD spectra. Compounds 2a and 2b were a pair of epimers with the configuration differing at C4 (Fig. 1). The theoretical calculated ECD spectra of the 4R and 4S isomers were in agreement with the experimental ECD spectra of 2a and 2b, respectively (Fig. 4). Therefore, the absolute configurations of 2a and 2b at C4 were assigned as R and S, respectively.

#### Oxidation of Thiolactone 2 with HLMs.
Thiolactone 2 (a 1:1 mixture of 2a and 2b) was incubated for 15 minutes at 37°C with HLMs in the presence of NADPH and GSH (5 mM) to reduce sulfenic acid 4 to the corresponding thiols (H1–H4) (Dansette et al., 2009, 2010; Zhang et al., 2013). A UPLC-Q/TOF MS study of the incubate showed the formation of four thiol isomers exhibiting a molecular ion (ESI+) corresponding to \([M + H]^+\) that was characterized by two peaks at \(m/z = 356.073\) and 358.071 with the ratio expected for the \(^{35}\text{Cl}\) and \(^{37}\text{Cl}\) isotopes. The four thiol isomers exhibited identical MS spectra, with major fragments of the molecular ion (\(m/z = 356.073\)) at \(m/z = 322.085\) and 212.048, as previously described for thiol 6 (H1–H4) (Perello et al., 2002; Dansette et al., 2012; Zhang et al., 2012). To assist peak assignment of H1–H4, using the same chromatographic conditions as a previous study (Tuffal et al., 2011) in which the biologic activities of the individual thiol isomers (H1–H4) in vitro were tested, we obtained the extracted ion chromatograms at \(m/z\) 504.12 for the derivatized thiol isomers (MP-H1 to MP-H4) produced from derivatization with MBP of the individual thiol isomers (H1–H4). Based on the order of elution of MP-H1 to MP-H4 (Tuffal et al., 2011), we speculated that the peaks at 4.1, 5.2, 5.0, and 5.5 minutes corresponded to MP-H1, MP-H2, MP-H3, and MP-H4, respectively (data not shown). These derivatized thiol isomers (MP-H1 to MP-H4) were further identified based on the comparison of their HPLC retention times and product ion spectra with those of reference standards. Thus, the peaks of H1–H4 were assigned.

#### Oxidation of 2a and 2b with RLMs.
Because 2a and 2b were relatively stable in water, and they could be epimerized rapidly in 100 mM PBS at pH 7.4, 2a and 2b were incubated in water instead of PBS in the presence of GSH (100 μM) with RLMs. After incubation with 2a or 2b without NADPH for 10 minutes at 37°C, the peak area ratios of 2b/2a or 2a/2b were 0.08 and 0.22, respectively (data not shown). In the presence of NADPH, oxidation of 2a only produced H1 and H4, and the small amount of H2 and H3 was probably generated from 2b formed from the epimerization of 2a (Fig. 5A). Similarly, oxidation of 2b mainly produced H2 and H3 (Fig. 5B). These data indicated that H1 and H4 had a 4R configuration, and H2 and H3 had a 4S configuration (Fig. 2).

#### S-Methylation of H1–H4 with HLMs.
Incubating H1, H2, H3, and H4 with HLMs in the presence of SAM produced 7a, 7b, 7c, and 7d with retention times of 2.3, 3.0, 3.2, and 3.5 minutes, respectively.
Metabolites 7a–7d exhibited identical mass spectra with a molecular ion (ESI+) corresponding to [M + H]+ and characterized by two peaks at m/z = 370.088 and 372.085 with the ratio expected for the 35Cl and 37Cl isotopes. They also exhibited similar MS2 spectra, and the molecular ion fragment (m/z = 370.088) at m/z = 322.084 corresponded to the loss of CH3SH (Fig. 6B). The assignments for their fragmentation pattern were shown in Fig. 6C. These data indicated that 7a, 7b, 7c, and 7d were S-methylated metabolites of H1, H2, H3, and H4, respectively (Fig. 2). These metabolites were further identified based on the comparison of their HPLC retention times and product ion spectra with those of reference standards.

Inhibition of S-Methylation of H3 and H4. The S-methylation of H3 and H4 in HLMs and HLC was measured. A 1:1 mixture of H3 and H4 was used as the substrate, and the total formation of the S-methylated isomers (7c and 7d) was determined. The S-methylating activities were 14.0 pmol/min/mg protein in HLMs and 1.04 pmol/min/mg protein in HLC without chemical inhibitors (Fig. 7), indicating that the activity in HLMs was higher than that in HLC.

S-methylation is catalyzed by TMT in microsomes and TPMT in cytosol, and TMT and TPMT are inhibited by DCMB and m-anisic acid, respectively (Lee and Kim, 1999). In the cytosolic fraction, low activity of TMT was also observed, which was probably caused by...
a soluble isoform of TMT because DCMB inhibited the cytosolic S-methylating activity (Glauser et al., 1992). The inhibitory effects of DCMB on the S-methylating activity of TMT for H3 and H4 in HLMs were determined as shown in Fig. 7. DCMB inhibited the production of the S-methyl metabolites (7c and 7d) in a concentration-dependent manner in both HLMs and HLC, and the inhibition ratios of the production of the S-methyl metabolites (7c and 7d) with 1 mM DCMB were 95.5% and 78.3% in HLMs and HLC, respectively. However, m-anisic acid at a concentration of 0.2, 2, or 20 mM did not show any inhibitory effects on the production of the S-methyl metabolites (7c and 7d) in HLC. These data indicated that TMT catalyzed the S-methylation of H3 and H4 in HLMs and HLC.

Enzyme Kinetics for the Formation of H3 and H4 from Thiolactone 2. The formation rates of H3 and H4 by HLMs over a range of thiolactone 2 (a 1:1 mixture of 2a and 2b) concentrations were determined (Fig. 8A). Eadie-Hofstee plots of H3 and H4 formation by HLMs demonstrated biphasic kinetics, indicating that multiple CYP isoforms were involved in the reactions. However, the regression was not significantly improved by fitting the data to a two-

multiple CYP isoforms were involved in the reactions. Therefore, the reactions were fitted by a one-enzyme model. The $K_m$ and $V_{max}$ values for the formation of H3 were 14.6 μM and 117 pmol/min/mg protein, respectively. The $K_m$ and $V_{max}$ values for the formation of H4 were 37.8 μM and 98.7 pmol/min/mg protein, respectively. The $Cl_{int}$ values for H3 and H4 formation were 8.03 and 2.61 μL/min/mg protein, respectively, indicating stereoselective metabolism.

Kinetic analyses for the formation of H3 and H4 from thiolactone 2 were performed by using cDNA-expressed CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5 (Fig. 8; Table 1). Conversion of thiolactone 2 to H3 and H4 by CYP2B6, CYP2C9, CYP2D6, CYP3A4, and CYP3A5 followed single-enzyme Michaelis-Menten kinetics. However, the reaction mediated by CYP2C9 was best fitted by a Hill equation. Eadie-Hofstee plots of H3 and H4 formation by CYP2C9 showed a convex relationship, indicating positive cooperativity ($n > 1$). Based on the $V_{max}$ and $K_m$ values for H3 and H4 formation calculated from the expressed enzymes, CYP2B6 and CYP2C19 appear to be relative high-affinity, low-capacity enzymes, whereas CYP3A4 appears to be a relative low-affinity, high-capacity enzyme, in the formation of H3 and H4. The $Cl_{int}$ values for H3 formation by CYP2C9 and CYP3A4 were both 1.7-fold higher than those for H4 formation because of the lower $K_m$ and higher $V_{max}$ values. Although the $K_m$ value for H3 formation by CYP2B6 was greater than that for H4 formation, the $V_{max}$ value was much larger, which resulted in a 2.2-fold higher $Cl_{int}$ value for H3 formation than that for H4 formation. The $V_{max}$ and $K_m$ values for H3 formation by CYP2C19 were slightly lower, and by CYP3A5 were slightly higher, than those for H4 formation, resulting in the similar $Cl_{int}$ values for H3 and H4 formation by CYP2C19 and CYP3A5. However, the $Cl_{int}$ value for H4 formation by CYP2D6 was 3.5-fold higher than that for H3 formation due to the lower $K_m$ and higher $V_{max}$ values.

The $Cl_{int}$, expressed P450 and $f_{int}$P450 values for H3 and H4 formation from thiolactone 2 by CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5 are shown in Table 1. The $f_{int}P450$ values suggested that the contributions of CYP3A4 to both inactive H3 and active H4 formation from thiolactone 2 were greater compared with the other five P450s, and the stereoselective metabolism of thiolactone 2 to H3 and H4 was largely determined by CYP3A4. The contribution of each P450 involved in biologically active H4 formation from thiolactone 2 follows the order of CYP3A4 (53.5%) > CYP2C19 (26.1%) > CYP2B6 (18.5%).

Inhibition of H3 and H4 Formation from Thiolactone 2 in HLMs. The inhibition ratios of H3 and H4 formation from thiolactone 2 in HLMs with the selective chemical inhibitors for CYP2C9 (sulfaphenazole) and CYP2D6 (quinidine) were less than 4%, suggesting that the contributions of CYP2C9 and CYP2D6 to H3 and H4 formation were very small. The inhibitory effects of the monoclonal antibody to CYP2B6 and the selective chemical inhibitors for CYP2C19 (omeprazole) and CYP3A (ketoconazole) on the formation
of H3 and H4 in HLMs are shown in Table 2. These results confirmed the involvement of CYP2B6, CYP2C19, and CYP3A4 in the formation of H3 and H4 from thiolactone 2.

**Enzyme Kinetics in S-Methylation of H3 and H4.** The enzyme kinetic parameters for the S-methylation of H3 and H4 in HLMs were determined (Eq. 9). The S-methylation of H3 and H4 in HLMs followed single-enzyme Michaelis-Menten kinetics. The $K_m$ and $V_{max}$ values for the S-methylation of H3 were 2.07 μM and 37.1 pmol/min/mg protein, respectively. The $K_m$ and $V_{max}$ values for the S-methylation of H4 were 7.24 μM and 1.32 pmol/min/mg protein, respectively. The $C_{L_{int}}$ values for the S-methylation of H3 and H4 formation were 17.9 and 0.183 μL/min/mg protein, respectively, indicating that the S-methylation was stereoselective.

**Discussion**

Clopidogrel is a prodrug requiring oxidative bioactivation to the active thiol 6 (Savi et al., 2000; Dansette et al., 2012). In this study, we focused on the mechanism of stereoselectivity in the formation and S-methylation of H3 and H4 to further elucidate the bioactivation mechanism of clopidogrel and the pharmacokinetic variability of H3 and H4 in humans.

A previous study showed the chiral instability of 2 diastereomers in CD$_3$OD in the presence of K$_2$CO$_3$ resulted from the base-catalyzed epimerization of the chiral carbon at C4 (Dansette et al., 2012). We found that the epimerization also occurred in 100 mM PBS (Fig. 3). A previous study showed the chiral instability of 2 diastereomers in CD$_3$OD in the presence of K$_2$CO$_3$ (Dansette et al., 2012). In this study, we found that the epimerization also occurred in 100 mM PBS (Fig. 3).

### TABLE 2

<table>
<thead>
<tr>
<th>CYP Isoform</th>
<th>Thiol Isomer</th>
<th>Incubation Time (min)</th>
<th>$K_m$ (μM)</th>
<th>$V_{max}$ (pmol/min/mg P450)</th>
<th>$n^*$</th>
<th>$Cl_{int}$, in vitro (μL/min/mg P450)</th>
<th>Enzyme Abundance (pmol P450/mg protein)</th>
<th>$f_{int}$</th>
<th>$f_{act}$</th>
<th>$f_{act}$</th>
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<tr>
<td>CYP2B6</td>
<td>H3</td>
<td>10</td>
<td>2.13</td>
<td>0.737</td>
<td>—</td>
<td>0.346</td>
<td>39</td>
<td>13.5</td>
<td>25.5</td>
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<tr>
<td>H4</td>
<td>0.887</td>
<td>0.138</td>
<td>0.156</td>
<td>0.167</td>
<td>0.00243</td>
<td>96</td>
<td>0.233</td>
<td>0.141</td>
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<tr>
<td>CYP2C9</td>
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<td>28.8</td>
<td>0.0699</td>
<td>—</td>
<td>0.00147</td>
<td>19</td>
<td>8.76</td>
<td>0.141</td>
<td>0.429</td>
<td></td>
</tr>
<tr>
<td>H4</td>
<td>38.4</td>
<td>0.0564</td>
<td>—</td>
<td>2.13</td>
<td>0.461</td>
<td>19</td>
<td>8.76</td>
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<tr>
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<td>0.735</td>
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<td>0.451</td>
<td>19</td>
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<td>0.0127</td>
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<tr>
<td>H4</td>
<td>44.3</td>
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<td>0.163</td>
<td>108</td>
<td>104</td>
<td>0.144</td>
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<td>0.280</td>
<td>108</td>
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<td>57.2</td>
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</tr>
<tr>
<td>H4</td>
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<td>2.53</td>
<td>—</td>
<td>0.163</td>
<td>108</td>
<td>108</td>
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<tr>
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*Hill coefficient.

The absolute configurations at C4 of 2a and 2b were determined by experimental and theoretical ECD spectra, and were assigned as R and S, respectively (Fig. 4). The microsomal incubation of 2a or 2b in water in the presence of GSH indicated that H1 and H4 had a 4R configuration, and H2 and H3 had a 4S configuration (Figs. 2 and 5).

The absolute configurations at C4 of H3 and H4 were consistent with previous results (Bluet et al., 2014). In the presence of SAM, S-methylated metabolites 7a, 7b, 7c, and 7d were generated from H1, H2, H3, and H4 in HLMs, respectively (Fig. 6). In vitro, for the cis isomers H3 and H4, the 4R isomer (H4) was active, whereas for the trans isomers H1 and H2, the 4S isomer (H2) was active. H4 is the only active isomer in vivo in humans (Tuffal et al., 2011). Although H4 was generated from 2a, increasing the efficiency of the formation of H4 in vivo by using 2a instead of 2 (2a and 2b) is hard to achieve owing to the rapid epimerization of 2a and 2b at physiologic pH. These findings are helpful for developing new thienopyridine antiplatelet agents in the future.

Kazui et al. (2010) reported that CYP2B6, CYP2C9, CYP2C19, and CYP3A4 contributed to the formation of thiol 6 from thiolactone 2; however, thiol 6 was determined as the total amount of the mixture of H1–H4 after derivatization, meaning that the kinetics for the formation of H3 and H4 were not examined separately. The present in vitro incubation studies used HLMs and cDNA-expressed human P450s and were analyzed by LC-MS/MS under conditions allowing complete separation of the thiol isomers after derivatization. The $C_{L_{int}}$ value for H3 formation from thiolactone 2 was 3.1-fold higher than that for H4 formation (8.03 versus 2.61 μL/min/mg protein) in HLMs, indicating stereoselective metabolism. The $C_{L_{int}}$ ratios of H3 formation to H4 formation from thiolactone 2 by CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5 were 2.2, 1.7, 1.0, 0.29, 1.7, and 1.0, respectively (Table 1). These results clearly demonstrated the stereoselective formation of H3 and H4 from thiolactone 2. A previous in vitro study also showed similar $C_{L_{int}}$ values for H3 and H4 formation by CYP2C19 (Gong et al., 2012). Kinetic studies using expressed enzymes demonstrated that the contributions of CYP3A4 to both inactive H3 and active H4 formation from thiolactone 2 were greater compared with the other five P450s, and the contributions of CYP2C9 and CYP2D6 were very small (<1.5%) (Table 1). The inhibition studies with selective inhibitors and HLMs confirmed the involvement of CYP2B6, CYP2C19, and CYP3A4 in the formation of H3 and H4. Thus, the stereoselective metabolism of thiolactone 2 to H3 and H4 was largely determined by CYP3A4. However, Zhang et al. (2014) reported that the formation of H4 by CYP3A4 was negligible, probably because the catalytic efficiency of CYP3A4 was compromised, and the catalytic
isoforms involved in the formation of H4 could directly affect the

...ada et al., 2012) could be attributed to the stereoselective... configuration of the chiral carbon containing the sulfur atom were methylated by TMT (Kazui et al., 2014). The... with the observations in vivo in humans. For prasugrel, only the active

...NADPH and CYP2B6 and CYP3A4 were involved in the formation of H4 from thiolactone 2, which was consistent with our study. CYP2B6 and CYP3A4 are subjected to induction and inhibition, which may affect the metabolism and efficacy of clopidogrel.

TMT in microsomes and TPMT in cytosols catalyze S-methylation. The enzyme that catalyzed the S-methylation of H3 and H4 was identified as TMT because the S-methylation activity was predominately observed in the microsomal fraction and was sensitive to DCMB (Fig. 7). The CLint value for the S-methylation of H3 in HLMs was 98.1-fold higher than that for H4 (17.9 versus 0.183 \( \mu \)L/min/mg protein), demonstrating the stereoselectivity of the S-methylation reaction catalyzed by TMT. Interestingly, we also found that 7c was the only detectable S-methylated metabolite in plasma after single oral administration of 75 mg clopidogrel to humans (data not shown), indicating that the stereoselective S-methylation in vitro was consistent with the observations in vivo in humans. For prasugrel, only the active thiol isomers with \( S \) configuration at the chiral carbon containing the sulfur atom were methylated by TMT (Kazui et al., 2014). The configuration of the chiral carbon containing the sulfur atom of the thiol metabolites of clopidogrel and prasugrel appears to be crucial for substrate recognition in TMT.

From these data, we deduced that the similar exposure levels of H3 and H4 previously reported in humans (Tuffal et al., 2011; Karazniewicz-Lada et al., 2012) could be attributed to the stereoselective formation of H3 from thiolactone 2 by CYP2B6 and CYP3A4 and the stereoselective S-methylation of H3 by TMT. The variations of the P450 isoforms involved in the formation of H4 could directly affect the exposure to H4 in humans, which could result in the variability in response to clopidogrel therapy. The S-methylation rate of H3 was far higher than that of H4, and 7c was the only S-methylated metabolite detected in human plasma. It seems that the role of TMT in the exposure to H4 in humans is negligible. However, owing to the rapid epimerization between 2a and 2b, the S-methylation rate of H3 by TMT was likely to affect the concentration of H4 in vivo indirectly. Human hepatic microsomal TMT might be genetically polymorphic, owing to that its biochemical properties were very similar to those of genetically polymorphic human red blood cell membrane TMT (Glauser et al., 1992; Weinsilboum et al., 1999). Therefore, the exposure ratio of H3/H4 in humans was probably dependent on the exposure to thiolactone 2 and the catalytic capabilities and levels of expressions of the P450 isoforms involved in the formation of H3 and H4 from thiolactone 2 and TMT involved in the S-methylation of H3 and H4. Because of the different catalytic efficiencies of the P450 isoforms and TMT in the formation and S-methylation of H3 and H4, determining the exposure and pharmacokinetic parameters for H4 indirectly by quantifying H3 or the mixture of H3 and H4 would be unsuitable for evaluating the pharmacokinetics and pharmacodynamics relationship. This hypothesis was proven in a clinical study in which \( C_{\text{max}} \) of H4 rather than H3 was correlated with platelet aggregation in humans after oral administration of clopidogrel (Karazniewicz-Lada et al., 2014). More studies are required to investigate the role of the epimerization of thiolactone 2 and TMT in the variability in response to clopidogrel therapy.

Other thienopyridine antiplatelet prodrugs, such as prasugrel and ticlopidine, are also converted to their pharmacologically active thiol metabolites through their corresponding thiolactones (Farid et al., 2010); therefore, we expect that the thiolactone metabolites of prasugrel and ticlopidine would be also epimerized at physiologic pH. In vicagrel, a recently reported acetate analog of clopidogrel (Shan et al., 2012), the ester moiety is readily hydrolyzed to form thiolactone 2 (Qiu et al., 2014), and, consequently, it should have the same mechanism of stereoselectivity as clopidogrel for the formation of the thiol metabolite isomers from thiolactone 2 and their S-methylation. Because the formation and S-methylation of the thiol metabolite isomers of clopidogrel and prasugrel appear to be stereoselective, ticlopidine is likely to follow a similar mechanism of stereoselectivity.

In summary, we studied the mechanism of stereoselectivity in the formation and S-methylation of H3 and H4, the thiol metabolite isomers of clopidogrel, in vitro. The proposed mechanism in HLMs is as follows: 1) the diastereomers of thiolactone 2 (2a and 2b) were epimerized rapidly at physiologic pH; 2) H3 and H4 were S-methylated by TMT; and 3) the stereoselective formation of H3 from thiolactone 2 by CYP2B6 and CYP3A4 and the stereoselective S-methylation of H3 by TMT were observed and could account for the similar exposure levels of H3 and H4 reported in humans. Our findings will deepen the understanding of the bioactivation mechanism and the variability in the efficacy of clopidogrel and other thienopyridine antiplatelet prodrugs.

Acknowledgments

The authors thank the staff at Jiangsu Vcare PharmaTech (Jiangsu, China) for synthesizing the standard compounds.

Authorship Contributions


Conducted experiments: Liu, Z. Chen.


Wrote or contributed to the writing of the manuscript: Liu, Z. Chen, D. Zhong.


