Glutaredoxin Is Involved in the Formation of the Pharmacologically Active Metabolite of Clopidogrel from Its GSH Conjugate

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ABSTRACT:

Clopidogrel is a thienopyridine antiplatelet agent that is converted to the active metabolite, R-361015, in vivo. Clopidogrel is first oxidized to a thiola lactone intermediate R-115991. R-115991 is thought to be metabolized to a GSH conjugate of R-361015 (R-361015-SG) and then is reduced to R-361015 in the presence of GSH. In this study, we investigated the enzyme-mediated formation of R-361015 from R-361015-SG in human liver microsomes and cytosols. After incubation of R-115991 in human liver microsomes, the formation of R-361015-SG, and subsequently of R-361015, was observed. The apparent formation rate of R-361015-SG was markedly decreased when human liver cytosols were added. Fitting the data to the kinetic model showed that the rate constant of R-361015-SG reduction to R-361015 in human liver microsomes was approximately 20-fold higher in the presence of human liver cytosols (5.65 min−1) than in the absence of cytosols (0.326 min−1). In addition, the formation rate of R-361015 from R-361015-SG was higher in human liver cytosols (2.84 ± 1.76 pmol · min−1 · mg−1) compared with in human liver microsomes (0.58 ± 0.96 pmol · min−1 · mg−1). The formation of R-361015 from R-361015-SG in human liver microsomes or cytosols was inhibited by anti-human glutaredoxin antibody in a concentration-dependent manner. Recombinant human glutaredoxin mediated the formation of R-361015 from R-361015-SG with Km and Vmax values of 30.0 ± 1.3 μM and 381.6 ± 209.8 pmol · min−1 · μg−1, respectively. The intrinsic clearance value (Vmax/Km) was 12.9 ± 7.5 μl · min−1 · μg−1. In conclusion, we found that human glutaredoxin is a main contributor to the formation of the pharmacologically active metabolite of clopidogrel from its GSH conjugate in human liver.

Materials and Methods

Materials. The thiola lactone metabolite of clopidogrel, which is a single enantiomer on the benzylic carbon (R-115991), the Z (cis) isomer of the active metabolite of clopidogrel (R-361015), the GSH conjugate of R-361015 (R-361015-SG), methoxyphenacetyl bromide (MPBr) derivatized R-361015 (MP-R-361015), and the internal standards (R-135766 and R-135772) were synthesized by Daiichi Sankyo Co., Ltd. (Tokyo, Japan) or Ube Industries, Ltd. (Ube, Japan) (Fig. 2). A derivatizing reagent, MPBr, was obtained from Tokyo Kasei Kogyo Co., Ltd. (Tokyo, Japan). Human liver microsomes and cytosols were purchased from XenoTech, LLC (Lenexa, KS) and the Human and Animal Bridging Research Organization (Tokyo, Japan), respectively. NADPH-gen-

ABBREVIATIONS: P450, cytochrome P450; MPBr, methoxyphenacetyl bromide; LC-MS/MS, liquid chromatography/tandem mass spectrometry; KPB, potassium phosphate buffer; PON-1, paraoxonase-1.
erating systems were purchased from BD Biosciences (San Jose, CA). Recombinant human glutaredoxin and goat anti-human glutaredoxin IgG were purchased from American Diagnostica Inc. (Greenwich, CT). All other chemicals and reagents were commercially available and of the highest purity.

Formation of R-361015-SG and R-361015 from R-115991 in Human Liver Microsomes and Cytosols. Triplicate mixtures (total volume, 990 μl each) contained potassium phosphate buffer (KPB; 36–39 mM, pH 7.4), an NADPH-generating system (1.3 mM NADP⁺, 3.3 mM glucose 6-phosphate, 0.4 units/ml glucose 6-phosphate dehydrogenase, and 3.3 mM MgCl₂), GSH (5 mM), and human liver microsomes (1 mg/ml) with/without human liver cytosols (1 mg/ml). Each mixture was preincubated at 37°C for 5 min, and 10 μl of R-115991 (final concentration, 5 μM) was added to each mixture, which was incubated at 37°C for 1, 2, 5, 10, 15, and 30 min. At each time point, 100 μl of the incubation mixture was collected, mixed with 200 μl of 5 mM MPBr acetonitrile solution and 100 μl of 100 ng/ml R-135766 acetonitrile solution to stop the reaction, and left for 10 min at room temperature to derivatize the thiol moiety of R-361015. After derivatization, the mixture was centrifuged (15,000g, 3 min, 4°C). A 10-μl aliquot of the supernatant fraction was subjected to LC-MS/MS analysis.

Inhibition of Formation of R-361015 from R-361015-SG by Anti-Human Glutaredoxin Antibody. Triplicate mixtures (total volume, 89 μl each) contained 47 to 50 mM Tris-HCl and 0.94 to 1 mM EDTA buffer (pH 7.5), human liver microsomes (0.01 mg/ml), human liver cytosols (0.01 mg/ml) or recombinant human glutaredoxin (0.05 mM human glutaredoxin, 1 μM, 75, 150, or 300 μg/ml of goat anti-human glutaredoxin antibody or goat control antibody). Each mixture was preincubated at 37°C for 30 min, and 10 μl of GSH (5 mM) and 1 μl of R-361015-SG (final concentration, 1 μM) was added to each mixture, which was incubated at 37°C for 2.5 min. A 100-μl aliquot of the incubation mixture was collected, mixed with 200 μl of 5 mM MPBr acetonitrile solution and 100 μl of 100 ng/ml R-135766 acetonitrile solution to stop the reaction, and left for 10 min at room temperature to derivatize the thiol moiety of R-361015. After derivatization, the mixture was centrifuged (15,000g, 3 min, 4°C). A 10-μl aliquot of the supernatant fraction was subjected to LC-MS/MS analysis.

Formation of R-361015 from R-361015-SG in Human Liver Microsomes or Cytosols or in the Presence of Recombinant Human Glutaredoxin. Triplicate mixtures (total volume, 990 μl each) contained KPB (40–45 mM, pH 7.4), GSH (5 mM), and human liver microsomes (0.01 mg/ml), human liver cytosols (0.01 mg/ml), or recombinant human glutaredoxin (0.0336 μg/ml). Each mixture was preincubated at 37°C for 5 min, and 10 μl of R-361015-SG (final concentration, 1 μM) was added to each mixture, which was incubated at 37°C for 2.5 min. A 100-μl aliquot of the incubation mixture was collected, mixed with 200 μl of 5 mM MPBr acetonitrile solution and 100 μl of 100 ng/ml R-135766 acetonitrile solution to stop the reaction, and left for 10 min at room temperature to derivatize the thiol moiety of R-361015. After derivatization, the mixture was centrifuged (15,000g, 3 min, 4°C). A 10-μl aliquot of the supernatant fraction was subjected to LC-MS/MS analysis.

Formation of R-361015 from R-361015-SG by Human Glutaredoxin. The incubation mixture in triplicate contained 0.5 μg/ml human glutaredoxin, 1 mM GSH, and 1.6, 3.1, 6.3, 13, 25, 50, 100, and 200 μM R-361015-SG in a final volume of 110 μl of 38 to 43 mM KPB (pH 7.4). The mixture without glutaredoxin was also prepared. A mixture without R-361015-SG was preincubated at 37°C for 5 min, and the reaction was initiated by adding 5.5 μl of R-361015-SG in KPB. After incubation at 37°C for 5 min, 10 μl of the incubation mixture was collected and mixed with 90 μl of 5 mM MPBr acetonitrile solution and 100 μl of 100 ng/ml R-135766 acetonitrile solution to stop the reaction, and left for 10 min at room temperature to derivatize the thiol moiety of R-361015. After derivatization, the mixture was centrifuged (15,000g, 3 min, 4°C). A 10-μl aliquot of the supernatant fraction was subjected to LC-MS/MS analysis.

FIG. 2. Chemical structure of the internal standards R-135766 and R-135772.
CA), and 10 μl of the supernatant was injected into an LC-MS/MS system to determine the concentration of MP-R-361015.

**Quantitation of R-361015-SG and R-361015.** The assays were performed according to methods reported previously (Takahashi et al., 2008). Quantitation was performed on an Alliance HPLC system consisting of a 2690 Separations Module (Waters, Milford, MA) coupled to a Quattro LC-MS/MS system (Waters) with the electrospray ionization source in positive ion mode. The mobile phase containing methanol, purified water, and trifluoroacetic acid (710:290:0.5, v/v/v) was applied onto the column. Each sample (10 μl) was injected onto an Inertsil ODS-3 column (150 mm × 4.6 mm, 5 μm; GL Sciences, Inc., Torrance, CA). The column temperature was maintained at 40°C. Under this condition, R-361015 [a Z (cis) isomer of clopidogrel active metabolite] was discriminated from an E (trans) isomer of R-361015 and an endo-isomer of R-361015 in which the double bond has migrated to an endocyclic position in the piperidine ring. Diastereomers of R-361015 are not separated and, thus, R-361015 was detected as a mixture of diastereomers of the Z (cis) form in this method.

The concentrations of each analyte in the samples were prepared by mixing 100 μl of a matrix (human liver microsomes and cytosols or KPB), and each equivalent volume of acetonitrile, standard solution, and R-135766 or R-135772 to the metabolites other than R-361015-SG and R-361015.

Calculation of Rate Constants for R-115991 Metabolism. The kinetic model for the formation of R-361015-SG and R-361015 from R-115991 in a closed incubation system is shown in Fig. 3. The corresponding differential equations for this model are as follows (eqs. 1–3):

\[
\frac{dZ(1)}{dt} = -(k_1 + k_4) \times Z(1) \quad (1)
\]

\[
\frac{dZ(2)}{dt} = k_1 \times Z(1) - k_2 \times Z(2) + k_3 \times Z(3) \quad (2)
\]

\[
\frac{dZ(3)}{dt} = -k_2 \times Z(2) - k_4 \times Z(3) \quad (3)
\]

where Z(1), Z(2), and Z(3) represent the concentrations of R-115991, R-361015-SG, and R-361015, respectively, and \(k_1, k_2, k_3, \) and \(k_4\) represent the rate constants of the formation of R-361015-SG from R-115991, the formation of R-361015 from R-361015-SG, and conversion of R-115991 to the metabolites other than R-361015-SG and R-361015, respectively.

The concentrations of each analyte in the samples were calculated by the calibration curve using the computer software MassLynx (version 4.0; Waters). Each calibration curve was constructed by plotting the peak area ratio of the standard compound to the internal standard versus the nominal concentrations by weighted (1/X²) linear least-squares regression.

**FIG. 3.** Schematic diagram of the model for the formation of R-361015-SG and R-361015 from R-115991 in a closed incubation system. \(k_1\), rate constant of the formation of R-361015-SG from R-115991; \(k_2\), rate constant of the formation of R-361015 from R-361015-SG; \(k_3\), rate constant of conversion of R-115991 to the metabolites other than R-361015-SG and R-361015.

**FIG. 4.** Fitted curves for the formation of R-361015-SG and R-361015 from R-115991 in human liver microsomes without (a) or with (b) human liver cytosols. R-115991 (5 μM) was incubated in 1 mg/ml human liver microsomes (a) or 1 mg/ml human liver microsomes and cytosols (b) supplemented with 5 mM GSH and the NADPH-generating system. The solid lines represent the simultaneously fitted curves of the concentration-time profiles of R-361015-SG and R-361015. The open and closed symbols represent observed individual values of R-361015-SG and R-361015, respectively.
GLUTAREDOXIN FOR CLOPIDOGREL ACTIVE METABOLITE FORMATION

TABLE 1  
Enzymatic formation rates of R-361015 from R-361015-SG in human liver microsomes and human liver cytosols and in the presence of human glutaredoxin

<table>
<thead>
<tr>
<th>Fraction or Enzyme</th>
<th>Formaion Rate of R-361015 pmol · min⁻¹ · mg⁻¹ or pmol · min⁻¹ · μg⁻¹ glutaredoxin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human liver microsomes</td>
<td>508 ± 396</td>
</tr>
<tr>
<td>Human liver cytosols</td>
<td>2843 ± 1176</td>
</tr>
<tr>
<td>Human glutaredoxin</td>
<td>130 ± 46</td>
</tr>
</tbody>
</table>

Kinetic Parameters for R-361015 Formation from R-361015-SG. The formation rates of R-361015 were fitted to eq. 4 using WinNonlin Professional (version 5.2.1; Pharsight):

\[
V = \text{Glrdx} \times V_{\text{max}} \times S(K_m + S) + k \times S \tag{4}
\]

where \( V \) (pmol/min) is the formation rate of R-361015, \( S \) (μM) is the substrate concentration, \( K_m \) (μM) and \( V_{\text{max}} \) (pmol · min⁻¹ · μg glutaredoxin⁻¹) are the kinetic parameters for the enzymatic component, which is mediated by glutaredoxin, \( k \) (μl/min) is the nonenzymatic clearance mediated by GSH, and Glrdx (μg) is the amount of glutaredoxin in the incubation system, which was 0.055 μg in this experiment (110 μl of 0.5 μg/ml glutaredoxin). The values were determined in advance by fitting the data of nonenzymatic formation by GSH to eq. 5, and the respective mean values were substituted into eq. 4.

\[
V = k \times S \tag{5}
\]

CL_{int} was calculated by dividing \( V_{\text{max}} \) by \( K_m \).

Results

Effect of Human Liver Cytosols on the Formation of R-361015-SG and R-361015 from R-115991 in Human Liver Microsomes. R-115991 (5 μM) was incubated in human liver microsomes (1 mg/ml protein) with or without human liver cytosols (1 mg/ml protein) in the presence of 5 mM GSH and an NADPH-generating system. Incubation of R-115991 in human liver microsomes showed immediate formation of R-361015-SG, followed by gradual decrease of R-361015-SG and subsequent formation of R-361015 (Fig. 4a). The apparent formation of R-361015-SG was remarkably decreased when the human liver cytosol fraction was added to human liver microsomes (Fig. 4b). The simultaneous fitting to the kinetic model (Fig. 3) exhibited a good fit to the observed data (Fig. 4). The rate constants \( k_1 \), \( k_2 \), and \( k_3 \) were 0.0275, 0.326, 0.0155, and 0.0466 min⁻¹, respectively, in human liver microsomes without a cytosol fraction, and those were 0.0307, 6.56, 0.0155, and 0.0450 min⁻¹, respectively, after the addition of cytosols to human liver microsomes (Fig. 4). The rate constant of R-361015 formation from R-361015-SG (\( k_2 \)) was increased approximately twenty times by addition of human liver cytosols. These rate constants are the values obtained in the presence of 5 mM GSH and could vary if other concentrations of GSH are used.

Formation of R-361015 from R-361015-SG in Human Liver Microsomes or Cytosols or in the Presence of Recombinant Human Glutaredoxin. R-361015-SG (1 μM) was incubated in human liver microsomes (0.01 mg/ml protein) or human liver cytosols (0.01 mg/ml protein) or in the presence of recombinant human glutaredoxin (0.0336 μg/ml) supplemented with 5 mM GSH. Both human liver microsomes and cytosols showed enzymatic formation of R-361015 from R-361015-SG. The formation rate of R-361015 from R-361015-SG was higher in human liver cytosols (2843 ± 1176 pmol · min⁻¹ · mg⁻¹) compared with that in human liver microsomes (508 ± 396 pmol · min⁻¹ · mg⁻¹), as shown in Table 1. The formation rate of R-361015 from R-361015-SG by recombinant human glutaredoxin was 130 ± 46 pmol · min⁻¹ · μg⁻¹ (Table 1). The values are expressed as mean ± S.D.

Inhibition of the formation of R-361015 from R-361015-SG by Anti-Human Glutaredoxin Antibody in Human Liver Microsomes or Cytosols or in the Presence of Recombinant Human Glutaredoxin. R-361015-SG (1 μM) was incubated in human liver microsomes (0.01 mg/ml protein) or human liver cytosols (0.01 mg/ml protein) or in the presence of recombinant human glutaredoxin (0.05 μg/ml) supplemented with 5 mM GSH and goat anti-human glutaredoxin antibody (0, 19, 0.375, 75, 150, or 300 μg/ml). Anti-human glutaredoxin antibody inhibited enzymatic-reducing activities both in human liver microsomes and in cytosols. The formation rate of R-361015 from R-361015-SG was decreased depending on the concentrations of anti-human glutaredoxin antibody, as shown in Fig. 5. The remaining activity of R-361015-SG reduction to R-361015 in human liver microsomes or human liver cytosols or in the presence of recombinant human glutaredoxin was 47.1 ± 7.3, 60.9 ± 17.7, or 74.0 ± 23.6%, respectively, at a concentration of 19 μg/ml and was 16.3 ± 23.1, 3.2 ± 2.8, or 0.0 ± 0.0%, respectively, at a concentration of 300 μg/ml of anti-human glutaredoxin antibody. The values are expressed as mean ± S.D.

Estimation of Kinetic Parameters on the Formation of R-361015 from R-361015-SG by Recombinant Human Glutaredoxin. The formation of R-361015 from R-361015-SG is mediated by two factors, an enzymatic glutaredoxin and nonenzymatic GSH. In the absence of glutaredoxin, R-361015 was proportionally formed depending on the concentrations of the substrate R-361015-SG [(Fig. 6(b)]. Nonenzymatic clearance, \( k \), was calculated to be 0.48 ±
0.06 μl/min by 1 mM GSH. The fitting profile in the presence of glutaredoxin is shown in Fig. 6(a). The enzyme kinetic parameters \( k_{\text{in}} \), \( V_{\text{max}} \), and \( CL_{\text{int}} \) by glutaredoxin were 30.0 ± 1.3 μM, 381.6 ± 209.8 pmol · min⁻¹ · μg glutaredoxin⁻¹, and 12.9 ± 7.5 μl · min⁻¹ · μg glutaredoxin⁻¹ (0.1548 ± 0.0902 μl · min⁻¹ · pmol⁻¹), respectively (Table 2). The clearance in μl · min⁻¹ · pmol⁻¹ means per picomole of glutaredoxin. Molecular weight of human glutaredoxin is 120,000. The values are expressed as mean ± S.D.

**Discussion**

The bioactivation of clopidogrel to clopidogrel thiolactone has been reported to be mediated by both P450 enzymes (Dansette et al., 2009, 2012) and paraoxonase-1 (PON-1) (Bouman et al., 2011). Dansette et al. (2009, 2012) reported that PON-1 only mediates the formation of an endo-isomer of R-361015 in which the double bond has migrated to an endocyclic position in the piperidine ring. To form the active isomer of R-361015, the thiolactone R-115991 is first oxidized to the sulfenic acid intermediate via P450 enzymes and then converted to R-361015 through the formation of a mixed disulfide with a thiol such as mercaptoethanol, N-acetyl cysteine, or GSH (Dansette et al., 2009, 2012). We studied the formation of the mixed disulfide of R-361015 and GSH (R-361015-SG) after incubation of R-115991 in human liver microsomes in the presence of GSH. The concentration-time profiles of R-361015-SG and R-361015 formation indicated that the thiolactone R-115991 is converted to R-361015-SG before R-361015 generation. These data support the concept that P450s mediate the formation of R-361015 from R-115991 and not PON-1 because hydrolysis of R-115991 by PON-1 would not result in the formation of a GSH mixed disulfide before the formation of R-361015 (Dansette et al., 2012).

In our study, we found that the addition of cytosolic fraction to liver microsomes resulted in much lower concentrations of R-361015-SG (Fig. 4) because enzymes in human liver cytosols could rapidly reduce the formed R-361015-SG to R-361015. The addition of cytosols to human liver microsomes had little effect on the formation rate of R-361015 from R-115991. This can be explained by the clearance difference in each metabolic step. Clearance of R-361015 formation from R-361015-SG in human liver cytosols was 2840 μl · min⁻¹ · mg⁻¹, which is much higher than the 3.45 to 20.3 μl · min⁻¹ · mg⁻¹ formation rate for R-361015 from R-115991 in human liver microsomes (Kazui et al., 2010). Because these data suggest that the oxidation of R-115991 by P450s seems to be rate limiting in the formation of R-361015 from R-115991, the apparent R-361015 formation rate from R-115991 was only slightly increased in the presence of human liver cytosols. Indeed, simultaneous fitting of the data to the kinetic model showed a substantial increase of the rate constant for R-361015 formation from R-361015-SG (kₐ) by human liver cytosols, whereas the rate constant of R-361015-SG formation from R-115991 (kₐ) was only slightly changed in the presence of human liver cytosols. These differences in formation rate changes in the presence of reducing enzyme(s) in human liver cytosols indicate that cytosolic enzymes can mediate the formation of R-361015 from R-361015-SG. The R-361015 formed from R-361015-SG can be partially reversed to R-361015-SG, and this step could be mediated by nonenzymatic air oxidation and/or by oxidative enzymes (P450s or other oxidases). The reverse reaction to form R-361015-SG occurs very slowly, and only small amounts of the disulfide are generated via this route.

It has been reported that a GSH conjugate of prasugrel’s active metabolite (R-138727-SG) was reduced by the cytosolic enzyme glutaredoxin to form the active metabolite (Hagihara et al., 2011). On the basis of the similarity in chemical structures between R-361015-SG and R-138727-SG, we predicted that glutaredoxin could be involved in the formation of R-361015 from R-361015-SG. Indeed, R-361015 formation was inhibited by anti-human glutaredoxin antibody in human liver microsomes or cytosols (Fig. 5), where the antibody at 300 μg/ml inhibited the reduction of R-361015-SG by 84 and 97%, respectively. Thus, human glutaredoxin appears to be a major enzyme involved in the reduction of R-361015-SG to R-361015 in human liver. The involvement of glutaredoxin was further con-

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**TABLE 2**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Formation Rate of R-361015</th>
</tr>
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<tbody>
<tr>
<td>GSH</td>
<td>0.48 ± 0.06</td>
</tr>
<tr>
<td>Glutaredoxin</td>
<td></td>
</tr>
<tr>
<td>( k_{\text{in}} ), μM</td>
<td>30.0 ± 1.3</td>
</tr>
<tr>
<td>( V_{\text{max}} ), pmol · min⁻¹ · μg glutaredoxin⁻¹</td>
<td>381.6 ± 209.8</td>
</tr>
<tr>
<td>( CL_{\text{int}} ), μl · min⁻¹ · μg glutaredoxin⁻¹</td>
<td>12.9 ± 7.5</td>
</tr>
<tr>
<td>( CL_{\text{max}} ), μl · min⁻¹ · pmol glutaredoxin⁻¹</td>
<td>0.1548 ± 0.0902</td>
</tr>
</tbody>
</table>

Fig. 6. Formation of R-361015 from R-361015-SG in the presence of glutathione with (a) or without (b) recombinant human glutaredoxin. R-361015-SG was incubated for 5 min at 37°C in KPB containing 1 mM glutathione with (a) or without (b) 0.5 μg/ml recombinant human glutaredoxin.
firmed by the kinetic study using recombinant human glutaredoxin, which reduced $R$-361015-SG to $R$-361015 in the presence of GSH, with $CL_{\text{int}}$ of 12.9 ± 7.5 μl·min⁻¹·μg⁻¹. To our knowledge only the GSH conjugate of prasugrel’s active metabolite ($R$-138727-SG) has been reported to be metabolized by human glutaredoxin in human liver (Hagihara et al., 2010). Because human glutaredoxin appears to be involved in the formation of the active metabolites of both clopidogrel and prasugrel, it is logical to suspect it is involved in the metabolism of other molecules in the thienopyridine class.

In conclusion, this is the first report to show the involvement of glutaredoxin in the formation of the pharmacologically active metabolite of clopidogrel from its GSH conjugate. This finding has led to a better understanding of the bioactivation mechanism of clopidogrel and the thienopyridine class of molecules.

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Authorship Contributions
Participated in research design: Hagihara, Kazui, and Kurihara.
Conducted experiments: Hagihara and Kazui.
Performed data analysis: Hagihara.
Wrote or contributed to the writing of the manuscript: Hagihara, Kazui, Kurihara, Ikeda, and Izumi.

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