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Mechanism-Based Inactivation of Human Cytochrome P450 2B6 by Clopidogrel: Involvement of Both Covalent Modification of Cysteinyl Residue 475 and Loss of Heme[S]

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ABSTRACT

We have investigated the mechanisms by which clopidogrel inactivates human cytochrome P450 2B6 (CYP2B6) in a reconstituted system. It was found that clopidogrel and its thiolactone metabolite, 2-oxo-clopidogrel, both inactivate CYP2B6 in a time- and concentration-dependent manner. On the basis of k_{inact}/K_I ratios, clopidogrel is approximately 5 times more efficient than 2-oxo-clopidogrel in inactivating CYP2B6. Analysis of the molecular mass of the CYP2B6 wild-type (WT) protein that had been inactivated by either clopidogrel or 2-oxo-clopidogrel showed an increase in the mass of the protein by ~350 Da. This increase in the protein mass corresponds to the addition of the active metabolite of clopidogrel to CYP2B6. It is noteworthy that this adduct can be cleaved from the protein matrix by incubation with dithiothreitol, confirming that the active metabolite is linked to a cysteinyl residue of CYP2B6 via a disulfide bond. Peptide mapping of tryptic digests of the inactivated CYP2B6 using electrospray ionization liquid chromatography-tandem mass spectrometry identified Cys475 as the site of covalent modification by the active metabolite. This was further confirmed by the observation that mutation of Cys475 to a serine residue eliminates the formation of the protein adduct and prevents the C475S variant from mechanism-based inactivation by 2-oxo-clopidogrel. However, this mutation did not prevent the C475S variant from being inactivated by clopidogrel. Furthermore, inactivation of both CYP2B6 WT and C475S by clopidogrel, but not by 2-oxo-clopidogrel, led to the loss of the heme, which accounts for most of the loss of the catalytic activity. Collectively, these results suggest that clopidogrel inactivates CYP2B6 primarily through destruction of the heme, whereas 2-oxo-clopidogrel inactivates CYP2B6 through covalent modification of Cys475.

Introduction

Clopidogrel is a thienopyridine antiplatelet agent that is widely prescribed for the prevention of atherothrombotic events such as myocardial infarction, ischemic stroke, and vascular death. Its antiplatelet activity requires metabolic biotransformation to a pharmacologically active metabolite that involves metabolism by the cytochromes P450 (P450s) including CYP2B6 (Savi et al., 1994; Kazui et al., 2010; Bouman et al., 2011). The active metabolite containing a reactive thiol group prevents ADP-induced platelet aggregation by covalent modification of the Cys17 and Cys270 residues of human P2Y12 ADP receptor via the formation of disulfide bonds (Savi et al., 2000; Pereillo et al., 2002; Ding et al., 2003).

It is generally thought that clopidogrel is bioactivated to the active metabolite in two sequential oxidative steps; the first oxidative step involves insertion of a single oxygen atom into clopidogrel only by P450s to give the thiolactone metabolite, 2-oxo-clopidogrel, and the second oxidative step involves further bioactivation of the thiolactone metabolite to produce the active metabolite (Dansette et al., 2009; Kazui et al., 2010). On the basis of in vitro studies of metabolism of clopidogrel and 2-oxo-clopidogrel, the major P450 contributors to the first oxidative step include CYP1A2, 2B6 and 2C19 (Kazui et al., 2010). However, it has been reported that

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ABBREVIATIONS: P450, cytochrome P450; DTT, dithiothreitol; 7-EFC, 7-ethoxy-4-trifluoromethylcoumarin; IBB, *N*-(2-(2-(2-(2-(3-(1-hydroxy-2-oxo-2-phenylethyl)phenoxy)acetamido)ethoxy)-ethoxy)ethyl)-5-(2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamide; WT, wild type; CPR, cytochrome P450 reductase; cyt b5, cytochrome b_5; ESI, electrospray ionization; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry.

CYP3A4 is the most efficient CYP isoform for catalyzing the first oxidative step and that CYP2C19 is not effective at all (Bouman et al., 2011). As for the second oxidative step, several lines of evidence suggest that P450s are capable of oxidizing 2-oxo-clopidogrel to give the active metabolite (Dansette et al., 2009; Kazui et al., 2010). According to Kazui et al. (2010), CYP2B6, -2C9, -2C19, and -3A4 play major roles in this step. Detailed mechanistic studies have indicated that this reaction occurs via a sulfenic acid intermediate (Dansette et al., 2009, 2010). However, Bouman et al. (2011) have questioned the role of P450s in the bioactivation of 2-oxo-clopidogrel to the active metabolite based on in vitro metabolic profiling studies. Instead, these authors have proposed that paraoxonase-1, an esterase, is the crucial enzyme for converting 2-oxo-clopidogrel to the active metabolite.

Apart from their roles in bioactivating clopidogrel, P450s may also be inactivated by clopidogrel. A clinical study reported that patients who received both clopidogrel and bupropion, an antidepressant drug and specific substrate for CYP2B6, experienced a ~60% increase in the plasma concentration of bupropion and suggested that these patients may require dose adjustment when both drugs are coadministered (Turpeinen et al., 2005). A number of in vitro studies have provided evidence supporting this suggestion. It has been reported that thienopyridine drugs such as clopidogrel and ticlopidine are potent mechanism-based inhibitors of CYP2B6 and -2C19 (Ha-Duong et al., 2001; Richter et al., 2004; Walsky and Obach, 2007; Nishiya et al., 2009a; Nishiya et al., 2009b) and may thereby cause significant adverse drug-drug interactions. In particular, clopidogrel is highly potent and selective for inhibition of CYP2B6 in human liver microsomes, with K_I and k_{inact} values of 0.5 μM and 0.35 min^{-1}, respectively (Richter et al., 2004). These findings make clopidogrel the most potent mechanism-based inhibitor known for CYP2B6. These authors hypothesized that covalent modification of cysteinyl residues of CYP2B6 by the active metabolite could contribute to the mechanism-based inactivation. However, no protein adducts or modified cysteinyl residues of CYP2B6 were reported. It was also reported that the mechanism-based inactivation of CYP2C19 by radiolabeled ticlopidine led to the adduct formation of a radioactive metabolite with the CYP2C19 protein with a stoichiometry of 1.7 nmol/nmol of CYP2C19 (Ha-Duong et al., 2001). Once again, the locations of the sites of modification were unclear. Despite the fact that clopidogrel and ticlopidine are potent mechanism-based inhibitors of P450s, their mechanisms of action as inactivators remain largely unknown.

In this study, we carried out a detailed investigation to elucidate the mechanisms by which clopidogrel inactivates CYP2B6. We investigated the formation of protein adducts as well as the loss of heme during the mechanism-based inactivation of CYP2B6 by both clopidogrel and 2-oxo-clopidogrel in the reconstituted system. Our results provide evidence showing that the mechanism-based inactivation of CYP2B6 by clopidogrel involves both covalent modification of the CYP2B6 protein and destruction of the heme.

Materials and Methods

Chemicals. All chemicals used were American Chemical Society reagent grade unless otherwise specified. Racemic clopidogrel and 2-oxo-clopidogrel were purchased from Toronto Research Chemicals (North York, ON, Canada). NADPH, dithiothreitol (DTT), glutathione, and catalase were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Trifluoroacetic acid and *N*-ethylmaleimide were purchased from Thermo Fisher Scientific (Waltham, MA). 7-Ethoxy-4-trifluoromethylcoumarin (7-EFC) was purchased from Invitrogen (Carlsbad, CA). Sequencing grade trypsin was purchased from Promega (Madison, WI). Carbon monoxide with purity >99.5% was purchased from Cryogenic Gas (Detroit, MI). Cleavable thiol-reactive biotinylating probe or IBB [*N*-(2-(2-(2-(3-(1-hydroxy-2-oxo-2-phenylethyl)phenoxy)acetamido) ethoxy)-ethoxy)ethyl)-5-(2-oxohexahydro-1*H*-thieno[3,4-*d*]imidazol-4-yl)pentanamide] was a generous gift from Dr. Daniel C. Liebler (Vanderbilt University, Nashville, TN).

Mutagenesis, Overexpression, and Purification of CYP2B6. Site-directed mutagenesis was performed to prepare the C475S mutant using the QuikChange method according to the manufacturer's instructions (Agilent Technologies, Santa Clara, CA). A pair of mutagenic primers, 5′-CTGACACCCCAGGAGTCTGGTGTGGG-3′ (forward) and 5′- CCCACACCAGACTCCTGGGGTGTCAG-3′ (reverse), and the plasmid of CYP2B6 WT (pLWCYP2B6dH) were used to amplify the plasmid of the C475S mutant. The entire mutant gene of the C475S mutant was sequenced by the Biomedical Sequence Core Facilities at the University of Michigan to confirm the mutation.

Overexpression and purification of CYP2B6 WT and the C475S variant were performed as described previously (Zhang et al., 2011). CYP2B6 used in this study was expressed as an N-terminally truncated form containing a $(His)_4$-tag at the C terminus. Cytochrome P450 reductase (CPR), and cytochrome b_5 (cyt b5) were overexpressed and purified as described previously (Zhang et al., 2009a).

Determination of the Kinetic Parameters and Partition Ratios for the Mechanism-Based Inactivation of the CYP2B6 WT and C475S Variant by Clopidogrel and 2-Oxo-clopidogrel. The kinetic parameters K_I and k_{inact} were determined at 37°C in a reconstituted system as described previously (Zhang et al., 2009b). Typically, the primary reaction mixture contained 1 μM CYP2B6, 2 μM CPR, 1 μM cyt b5, 1 unit/μl catalase, and 0 to 25 μM clopidogrel or 0 to 100 μM 2-oxo-clopidogrel in 50 mM potassium phosphate buffer, pH 7.4. The reaction was initiated by the addition of NADPH to a final concentration of 1 mM. At the designated times of inactivation, aliquots of 6 μl of the primary reaction solution were transferred to 150 μl of the secondary reaction solution that contained 0.1 mM 7-EFC and 0.3 mM NADPH in 50 mM potassium phosphate buffer, pH 7.4. The secondary reaction mixture was then incubated for 10 min at 37°C before it was terminated by the addition of 50 μl of acetonitrile. The fluorescence of 7-hydroxy-4-trifluoromethylcoumarin was measured with excitation at 410 nm and emission at 510 nm using a Victor II microtiter plate reader (PerkinElmer Life and Analytical Sciences, Waltham, MA). The K_I and k_{inact} values were obtained by fitting the rates of inactivation determined at various concentrations to the Michaelis-Mention equation using Prism 5 (GraphPad Software, La Jolla, CA).

To determine the partition ratios, primary reaction mixtures containing the molar ratios of the inactivator to the protein as indicated were incubated at 37°C for 30 min after the addition of 1 mM NADPH, and the activity remaining was measured in the secondary reaction as described above. The partition ratio was then determined as described previously (Zhang et al., 2009b).

Analysis of the Protein Adducts of the CYP2B6 WT and C475S Variant by ESI-LC/MS. To examine whether clopidogrel and 2-oxo-clopidogrel modified the apo-proteins of CYP2B6, we determined the molecular masses of the inactivated CYP2B6 proteins. CYP2B6 WT and the C475S variant were inactivated at 30°C by incubation in the primary reaction mixture in the presence of 20 μM clopidogrel or 2-oxo-clopidogrel. After 5 min of incubation, aliquots of 50 μl of the primary reaction mixture were loaded onto a reversed-phase C3 column and eluted into a LCQ Classic ion-trap mass spectrometer (Thermo Fischer Scientific) to determine the molecular masses of the inactivated CYP2B6 proteins as described previously (Zhang et al., 2009b). To remove the protein adduct from CYP2B6,

aliquots of 50 μl of the primary reaction solution that had been inactivated by clopidogrel or 2-oxo-clopidogrel were incubated with 10 mM DTT at room temperature for 1 h. The molecular mass of the DTT-treated CYP2B6 was then determined by ESI-LC/MS as described for adducted proteins.

Analysis of the GSH Adducts of Clopidogrel and 2-Oxo-clopidogrel by ESI-LC/MS/MS. To identify the reactive intermediates responsible for the modification of the apo-CYP2B6 proteins, GSH (10 mM) was added to 0.25 ml of the primary reaction solution. The reactions were incubated at 37°C for 45 min after the addition of 1 mM NADPH. Equal volumes of water were substituted for NADPH in the control samples. The reactions were terminated by the addition of 1 ml of acetonitrile. Protein precipitates were removed by centrifugation at 16,000*g* for 5 min, and the supernatants were dried under a stream of nitrogen gas. The dried samples were redissolved in 0.2 ml of 0.1% formic acid/10% acetonitrile in aqueous solution, 50 μl of which was then analyzed by ESI-LC/MS/MS on a LCQ Classic ion-trap mass spectrometer. The GSH adducts were separated on a reverse phase C18 column (Luna 100 × 4.6 mm, 3 μm; Phenomenex, Torrance, CA) and eluted with a linear gradient of solvent A (0.1% acetic acid in water) and solvent B (0.1% acetic acid in acetonitrile), starting at 20% B, increasing to 30% B at 5 min, then to 40% B at 15 min, then to 90% B at 30 min, and then held at 90% B for 20 min. The flow rate was 0.3 ml/min. The settings for the mass spectrometer were as follows: spray voltage, 4.5 kV; sheath gas flow, 90; auxiliary gas flow, 30; capillary temperature, 170°C. To record both the precursor ions and fragment ions, the mass spectrometer was set at the dependent scan mode with collision energy of 35% for fragmentation of the top two most abundant precursor ions.

Identification of the Cysteinyl Residues of CYP2B6 Modified by Clopidogrel Using ESI-LC-MS/MS. To identify the modified cysteinyl residues, we devised a two-step alkylating procedure, separated by a DTT treatment. Mechanism-based inactivation of CYP2B6 was performed in the primary reaction at 30°C as described above. Typically CYP2B6 (7.5 nmol), CPR (15 nmol), and cyt b5 (7.5 nmol) were reconstituted in 2 ml of 0.1 M potassium phosphate solution, pH 7.4, containing 20 μM clopidogrel. NADPH was added to a final concentration of 1 mM to initiate the reaction. After incubation for 25 min, the reaction was terminated by the addition of 6 M urea. In the first alkylation step, all of the unmodified cysteinyl residues in the reconstituted system were alkylated by the addition of *N*-ethylmaleimide to a final concentration of 10 mM. The reaction mixture was incubated at room temperature for 16 h to ensure complete alkylation of all free thiols. The protein mixture buffer was then exchanged to 25 mM ammonium bicarbonate, pH 7.8, using Zeba spin desalting columns (Thermo Fischer Scientific). The desalted protein mixture was then digested with at 1:25 ratio (w/w) of trypsin to protein at 37°C for 20 h. The tryptic digests were lyophilized to a final volume of ~1 to 2 μl and then redissolved in 0.2 ml of 0.1 M potassium phosphate solution, pH 7.0. Before the second alkylation reaction, the tryptic digests were split into two equal volumes (0.1 ml each). DTT was added at 1 mM concentration into one sample to cleave the clopidogrel adduct, whereas an equal volume of water was added into the control sample. EDTA was added to a final concentration of 1 mM to prevent reoxidation of the free thiols. Both samples were then incubated for 30 min at room temperature and subjected to the second alkylation with IBB followed by biotin-avidin enrichment. Alkylation of the free thiols of the modified cysteinyl residues by IBB and the enrichment of the IBB-alkylated peptide were performed as described by Lin et al. (2010). The enriched peptide was then loaded onto a reverse phase C18 column and eluted into a LCQ Deca XP ion-trap mass spectrometer for peptide sequencing as described previously (Zhang et al., 2009b).

Loss of Heme Contents As Measured by High-Performance Liquid Chromatography and Ferrous Carbonmonoxy P450. To examine whether mechanism-based inactivation of CYP2B6 by clopidogrel or 2-oxo-clopidogrel affected the heme, we measured the ferrous CO-P450 spectrum and the native heme remaining after the inactivation reaction. CYP2B6 was inactivated in 0.5 ml of 0.1 M potassium phosphate solution, pH 7.4, containing 0.1 μM CYP2B6, 0.2 μM CPR, and 20 μM clopidogrel or 2-oxo-clopidogrel. Cyt b5 and catalase were omitted from the reaction to avoid introducing additional heme other than that from CYP2B6. The inactivation reaction was initiated by the addition of NADPH to a final concentration of 1 mM, and the reaction was incubated at 37°C for 15 min. Aliquots of the reaction mixture were analyzed by high-performance liquid chromatography to determine the native heme (Lin et al., 2009), and the rest of the samples were used to determine the activity remaining in the secondary reaction, as mentioned previously, and to measure the ferrous CO-P450 spectra. The UV-visible spectra of ferrous CO-P450 were recorded from 400 to 500 nm on a Shimadzu UV/Vis spectrophotometer (UV-2501PC; Shimadzu, Kyoto, Japan) after adding a few grains of dithionite and bubbling CO gas into the reduced sample for ~10 s.

Analysis of the Access Channels for CYP2B6. The solvent access channels of CYP2B6 were analzyed using Caver software (ver. 2.1.2) (Petrek et al., 2006). The calculations were performed as described previously for CYP2B1 (Zhang et al., 2009a). In brief, the coordinates of CYP2B6 was obtained from the Protein Data Bank (3IBD). The coordinates of water molecules and bound inhibitor 4-(4-chlorophenyl)imidazole were deleted before calculations. The initial start point of the access channel was chosen in the void region of the active site above the heme iron. A probe molecule of 4 Å was used to probe the access channels. PYMOL (http://www.pymol.org) was used to visualize the access channels.

Results

Mechanism-Based Inactivation of CYP2B6 WT by Clopidogrel and 2-Oxo-clopidogrel. The kinetic parameters for the mechanism-based inactivation of CYP2B6 WT were determined in the reconstituted system. Under turnover conditions, incubation of CYP2B6 WT in the presence of clopidogrel led to the loss of the 7-EFC *O*-deethylase activity in a time- and concentration-dependent manner, as shown in Fig. 1A. The K_I and k_{inact} values were determined to be 2.4 μM and 0.17 min^{-1}, respectively, which gives a k_{inact}/K_I ratio or inactivation efficiency of 0.071 $min^{-1} \cdot \mu M^{-1}$. In a similar fashion, 2-oxo-clopidogrel inactivated CYP2B6 WT with K_I and k_{inact} values of 6.3 μM and 0.092 min^{-1}, respectively, as shown in Fig. 1B. This gives a k_{inact}/K_I ratio of 0.015 $min^{-1} \cdot \mu M^{-1}$. Therefore, 2-oxo-clopidopgrel is approximately 5-fold less efficient than clopidogrel as a mechanism-based inactivator for CYP2B6 WT, consistent with an earlier observation by Nishiya et al. (2009a), who reported a decrease of nearly 12-fold in the k_{inact}/K_I ratio in human liver microsomes. Similar results were also reported for CYP2C19 that showed clopidogrel was significantly more efficient than its thiolactone metabolite in inactivating the (*S*)-mephenytoin 4′-hydroxylase activity of human liver microsomes (Hagihara et al., 2008; Nishiya et al., 2009b). As observed earlier (Richter et al., 2004), addition of GSH to the primary reaction did not protect CYP2B6 WT from being inactivated by clopidogrel or 2-oxo-clopidogrel (data not shown).

Analysis of the Molecular Mass of the CYP2B6 WT Protein Inactivated by Clopidogrel. To examine whether the inactivation of CYP2B6 by clopidogrel led to the formation of protein adducts, we determined the molecular mass of the inactivated CYP2B6 by ESI-LC/MS. The results are shown in Fig. 2. In the absence of NADPH, CYP2B6 exhibited a molecular mass of 54,416 Da, which corresponds to the mass expected for unmodified CYP2B6. The deconvoluted

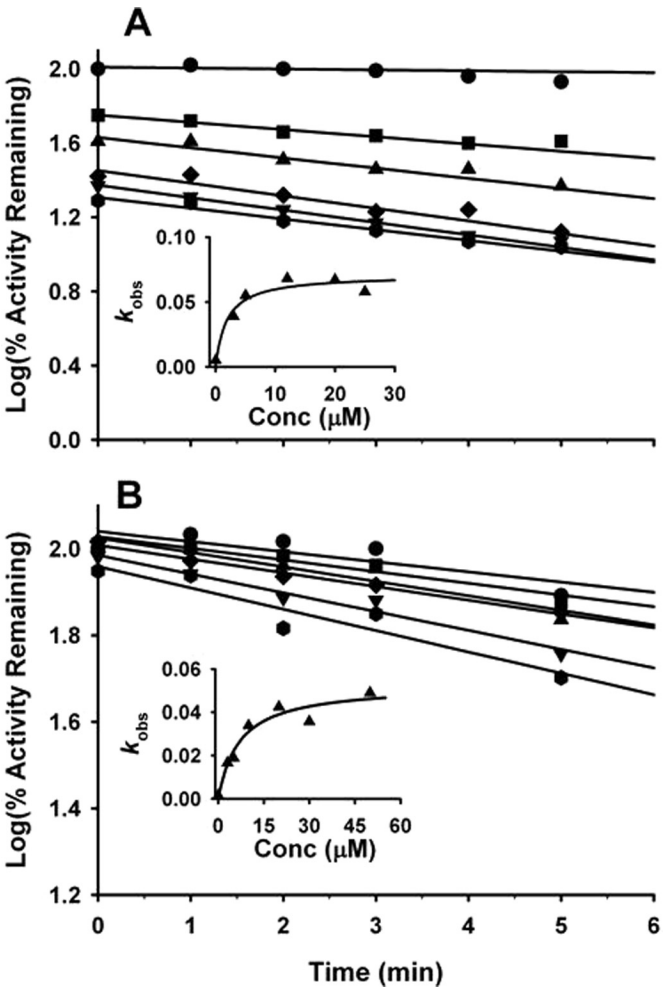

Fig. 1. Kinetics for the mechanism-based inactivation of CYP2B6 WT by clopidogrel (A) and 2-oxo-clopidogrel (B). The inactivation reactions were perfromed at 37°C in a reconstituted system as described under *Materials and Methods*. A, the concentrations of clopidogrel in the primary reaction were 0 (●), 3 (■), 5 (▲), 12 (◆), 20 (▼), and 25 (●) μM. B, the concentrations of 2-oxo-clopidogrel in the primary reaction were 0 (●), 3 (■), 5 (▲), 10 (◆), 30 (▼), and 50 (●) μM. The insets are plots of the observed rates at various concentrations of inhibitors and they were used to calculate the kinetic parameters K_I and k_{inact}.

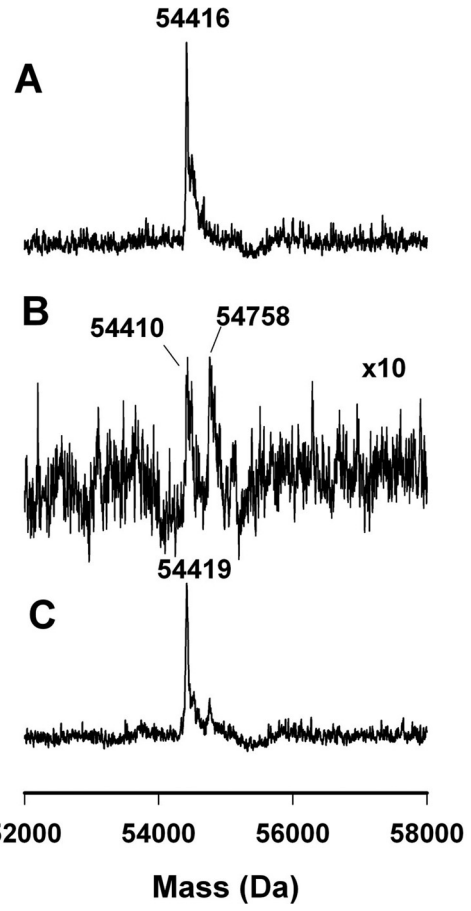

Fig. 2. Analysis of the protein masses of CYP2B6 by ESI-LC/MS after the inactivation by clopidogrel. CYP2B6 was inactivated at 30°C for 5 min in the presence of 20 μM clopidogrel as described under *Materials and Methods*. A, control CYP2B6 in the absence of NADPH. B, inactivated CYP2B6. The intensity was amplified by 10-fold for visualization. C, DTT-treated inactivated CYP2B6. The inactivated CYP2B6 from B was incubated with 10 mM DTT for 60 min at room temperature and then subjected to mass analysis by ESI-LC/MS.

mass spectrum for the inactivated CYP2B6 showed two peaks at 54,410 and 54,758 Da. The mass difference between these two peaks is 348 Da, which is approximately equal to the molecular mass of the active metabolite of clopidogrel (355 Da) within the instrumental mass accuracy of 0.1%. It is noteworthy that the intensity of the deconvoluted mass spectrum for the inactivated CYP2B6 WT was significantly decreased. This may be due to the poor ionization of the modified protein during the electrospray process.

To examine whether the adduct was linked to the inactivated CYP2B6 protein via a disulfide bond, the inactivated protein was treated with 10 mM DTT for 60 min. Analysis of the molecular mass of the DTT-treated CYP2B6 WT revealed a single mass peak at 54,419 Da, almost identical to that of the unmodified CYP2B6 protein, as shown in Fig. 2C. Apparently, this adduct was cleaved from the inactivated CYP2B6 protein by DTT. This provides strong evidence that this adduct is linked to the CYP2B6 protein via a disulfide bond. Thus, it can be concluded that inactivation of CYP2B6 by clopidogrel leads to covalent modification of a cysteinyl residue of CYP2B6. In the case of 2-oxo-clopidogrel, similar results were obtained (see Supplemental Fig. 1S). It is noteworthy that the molecular masses of CPR and cyt b5 were not modified even though they were present in the reconstituted system (data not shown). A reactive metabolite of clopidogrel selectively modifies CYP2B6 only, even though the amino acid sequence of CPR contains seven cysteinyl residues, five of which are solvent-accessible.

Analysis of the GSH Adducts of Clopidogrel by ESI-LC/MS/MS. To identify the reactive intermediate responsible for forming the protein adduct, GSH was included in the primary reaction to trap the reactive intermediate(s) of clopidogrel. After examining all the parent ions from the reaction mixture with a neutral loss of 129, we identified a GSH adduct that eluted at 26.7 min. As shown in Fig. 3A, the GSH adduct exhibits a mass peak at *m/z* 661.0 with an isotope peak at *m/z* 663.0. The presence of the isotope peak, which is 2 mass units over that of the parent mass and has a relative amplitude of ~40% of the parent ion, suggests that the GSH adduct contains a chlorine atom that presumably originated from clopidogrel. The molecular mass of the reactive intermediate deduced from the GSH adduct is 355.0 Da, which is consistent with the molecular mass of 355.1 Da for the active metabolite. Furthermore, the frag-

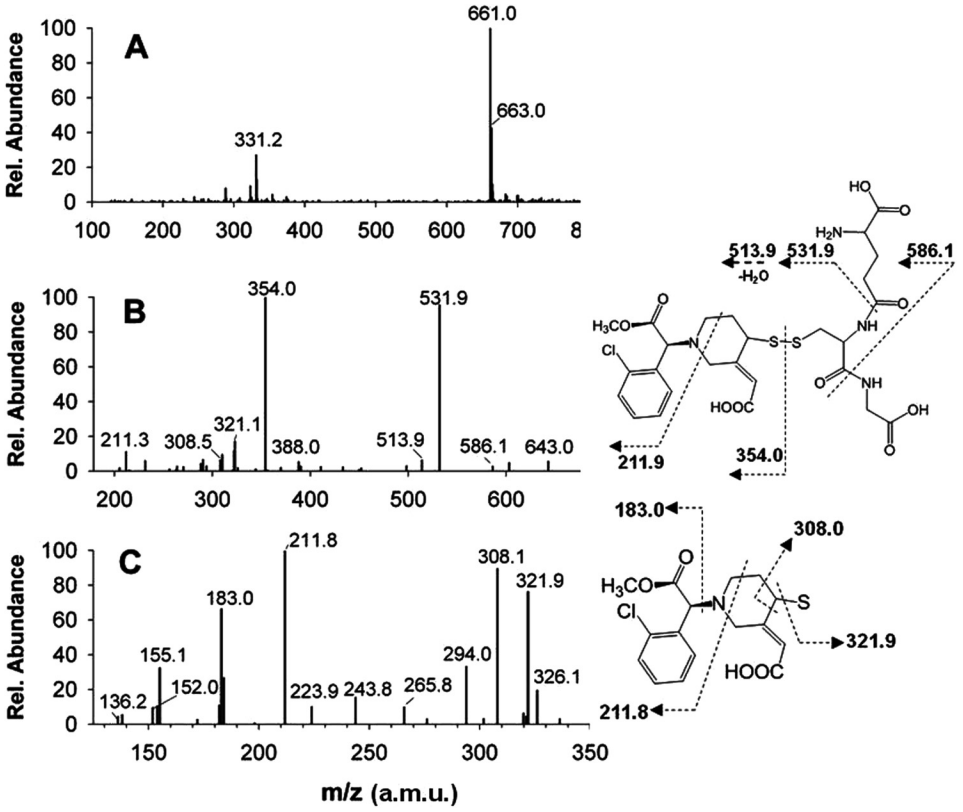

Fig. 3. Analysis of the adducts of the reactive intermediate of clopidogrel with GSH by ESI-LC/MS/MS. To generate GSH adducts, the metabolism of clopidogrel by CYP2B6 was performed in a reconstituted system in the presence of 20 μM clopidogrel and 10 mM GSH. The reaction mixture was incubated for 45 min after the addition of 1 mM NADPH. The GSH adducts were enriched and analyzed as described under *Materials and Methods*. A, the MS spectra for the GSH adduct that eluted at 26.7 min. B, the MS/MS spectrum of the parent ion at *m/z* 661.0 and the fragment assignments. C, the MS/MS/MS spectrum of the *m/z* 354.0 ion from the parent ion at *m/z* 661.0 and the fragment assignments.

ment patterns of the parent ion at *m/z* 661.0 can be interpreted on the basis of a disulfide bond linkage. The MS/MS fragment patterns of the parent ion at *m/z* 661.0 show two major fragments at *m/z* 531.9 and 354.0 as shown in Fig. 3B. The former results from the neutral loss of 129 from the parent ion, whereas the latter represents the metabolite fragment split from the disulfide bond. The other fragment peaks at *m/z* 643.0, 586.1, and 211.8 can also be assigned to the GSH adduct, as shown in Fig. 3B.

To further confirm the assignments, we analyzed the fragment patterns from the *m/z* 354.0 ion resulting from the fragmentation of the parent ion at *m/z* 661.0. As shown in Fig. 3C, the fragment pattern of the *m/z* 354.0 ion is consistent with that of the active metabolite. The fragments at *m/z* 321.9 and 308.1 most likely result from the loss of the sulfur and $-CH_2S$ groups, respectively. The fragment ions at *m/z* 211.8, 183.0, and 152.0 were also observed in the mass spectrum of the clopidogrel standard (data not shown), and they are analogous to the fragment ions of ticlopidine as reported previously (Talakad et al., 2011). On the basis of the molecular mass of the GSH adduct and the MS/MS and MS/MS/MS fragmentation patterns for the parent ion at *m/z* 661.0, it is most likely that the active metabolite of clopidogrel forms a GSH adduct via a disulfide bond. In the presence of 2-oxo-clopidogrel, we observed the identical GSH adduct (data not shown).

Identification of the Specific Cysteinyl Residues Modified by Clopidogrel through Selective Alkylation and Peptide Sequencing Using ESI-LC/MS/MS. To select only the clopidogrel-modified peptides for peptide mapping, we employed a two-step alkylation procedure followed by an enrichment step that used the tight binding of biotin-avidin. Alkylation of a cysteinyl residue by the cleavable IBB reagent would lead to an increase in mass of 57 Da according to Lin et al. (2010). Analyses of the enriched tryptic digests of the inactivated proteins yielded a doubly charged peptide ($[MH]^{2+}$ = 1042.8) that eluted at 18.6 min, whereas no modified peptides were observed in the control sample. The extracted ion chromatogram and mass spectrum of this peptide are provided in Supplemental Fig. 2S. The molecular mass of the doubly charged peptide is 2083.6 Da, which is 57 Da greater than the theoretical mass of the peptide 460 to 479 of CYP2B6, ^{460}ASPVAPEDIDLTPQECGVGK^{479}. This peptide contains one cysteinyl residue at position 475. Fragmentation of this doubly charged ion produced a series of b ions and y ions consistent with the amino acid sequence for the peptide 460 to 479. As shown in Fig. 4, the masses of the b_4, b_5, and b_{12} ions match with the theoretical masses for the unmodified peptide 460 to 479, indicating that the N-terminal end of Pro472 is not modified by IBB. However, the molecular mass of the b_{18} ion exhibits an increase in mass of 57.3 Da, indicating that the covalent modification occurs between Pro472 and Val477. This is consistent with the observed y ions. All the y ions observed, including the y_7 ion, exhibit an increase in mass of 57 $\pm$ 1 Da, which further narrows the modified site to ^{473}QECGV^{477}. Even though the amino acid sequencing was incomplete because of the relatively large size of the peptide 460 to 479, it can be concluded nonetheless that Cys475 is the site of modification by the reactive metabolite of clopidogel because it is highly unlikely that the reactive metabolite of clopidogrel would form a DTT-cleavable adduct with any of the amino acid residues in the peptide ^{473}QECGV^{477} other than Cys475. To further support this conclusion, we mutated Cys475 to a serine residue and investigated the formation of protein adducts with 2-oxo-clopidogrel. As shown in the Supplemental Fig. 3S, no protein adducts were observed for the C475S variant in the presence of 2-oxo-clopidogrel under the same conditions used for CYP2B6 WT.

Effects of the C475S Mutation on the Mechanism-Based Inactivation of CYP2B6 by Clopidogrel and 2-Oxo-clopidogrel. To investigate the role of Cys475 in the mechanism-based inactivation of CYP2B6 by clopidogrel, we determined the kinetic parameters for the mechanism-based inactivation of the C475S variant by clopidogrel and 2-oxo-clopidogrel, and the results are summarized in Table 1, together with those for the CYP2B6 WT for com-

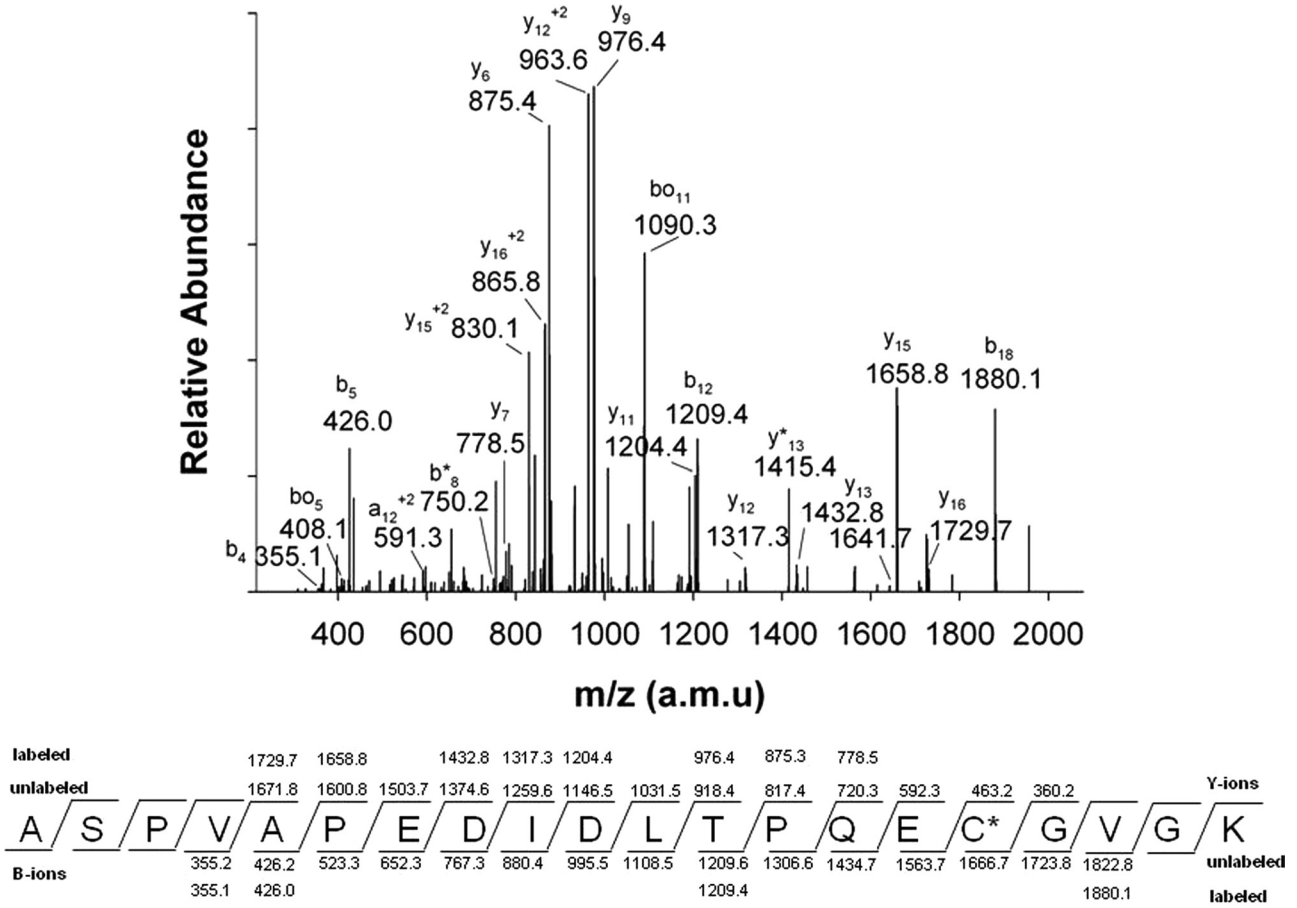

Fig. 4. Peptide sequencing of the peptide 460 to 479 by ESI-LC/MS/MS to identify the specific amino acid residue modified by the reactive metabolite of clopidogrel in CYP2B6. The observed fragment ions in the MS/MS spectrum are from the doubly charged precursor ion at m/z 1042.8 obtained in positive mode as described under *Materials and Methods*. The predicted fragment ion series (b and y ions) for the unmodified peptide 460–479 are denoted as unlabeled, whereas the fragment ions of the doubly charged precursor ion are indicated as labeled. bo and b* ions refer to b ions that lose ammonia ($-NH_3$) and water ($-H_2O$), respectively, whereas yo and y* ions refer to y ions that lose ammonia and water, respectively. Cys475 is marked with an asterisk.

TABLE 1

Summary of the K_I, k_{inact}, and partition ratios for the mechanism-based inactivation of CYP2B6 WT and C475S

Details for the measurements of the kinetic parameters and partition ratios are under *Materials and Methods*. The reported data were averaged from four separate experiments.

CYP2B6	Clopidogrel				2-Oxo-clopidogrel			
	K_I	k_{inact}	k_{inact}/K_I	Part. Ratio	K_I	k_{inact}	k_{inact}/K_I	Part. Ratio
	μM	min^{-1}			μM	min^{-1}		
WT	2.4 ± 0.33	0.17 ± 0.031	0.071	19 ± 4	6.3 ± 0.72	0.092 ± 0.015	0.015	51 ± 8
C475S	4.2 ± 0.21	0.12 ± 0.015	0.029	17 ± 2		N.D.		

N.D., not determined because of lack of inactivation.

parison. Under the same conditions used for CYP2B6 WT, the C475S variant was no longer inactivated by 2-oxo-clopidogrel. It is noteworthy that this mutation has little effect on the inactivation by clopidogrel. As shown in Table 1, the C475S variant is inactivated by clopidogrel with K_I and k_{inact} values of 4.2 μM and 0.12 min^{-1}, respectively, which are very similar to those for the CYP2B6 WT. Moreover, the partition ratio for C475S is also similar to that of the CYP2B6 WT. It is clear that mutation of Cys475 to a serine residue prevents CYP2B6 from being inactivated by 2-oxo-clopidogrel but not from being inactivated by clopidogrel.

Loss of the Heme during the Mechanism-Based Inactivation of the CYP2B6 WT and C475S Variant by Clopidogrel and 2-Oxo-clopidogrel. The inability of the C475S mutation to protect the C475S variant from being inactivated by clopidogrel prompted us to investigate the loss of the heme during the mechanism-based inactivation. The results are presented in Table 2. After 15 min of incubation in the presence of 20 μM clopidogrel, CYP2B6 WT lost 72% of the 7-EFC *O*-deethylase activity, 62% of the native heme, and 69% of the CO-detectable heme, whereas the C475S variant lost 80% of the activity, 65% of the native heme, and 88% of the CO-detectable heme. When inactivated by 2-oxo-clopidogerel, CYP2B6 WT lost 56% of the activity without losing any of the native heme and the CO-detectable heme. As mentioned previously, 2-oxo-clopdogrel did not inactivate the C475S variant, and no heme loss was observed for the C475S variant.

Discussion

In this study, we investigated the mechanisms by which clopidogrel inactivates CYP2B6 in a reconstituted system.

TABLE 2

Summary of the remaining 7-EFC *O*-deethylase activities, native heme, and CO-detectable heme following the mechanism-based inactivation of CYP 2B6 WT and the C475S variant by clopidogrel and 2-oxo-clopidogrel

CYP2B6 (1 μM) was inactivated at 37°C for 15 min in the presence of 20 μM clopidogrel or 2-oxo-clopidogrel. The activities remaining for the inactivated CYP 2B6 were determined by analyses of the 7-EFC *O*-deethylase activities in the secondary reaction mixture, the native heme was determined by HPLC, and the CO-detectable heme was measured by recording the visible spectrum of the ferrous CO-P450 after the addition of a few grains of dithionite as described under *Materials and Methods*. The data were averaged from three separate experiments.

CYP2B6	Clopidogrel			2-Oxo-clopidogrel		
	Activity	Heme	CO-P450	Activity	Heme	CO-P450
	%			%		
WT	28 ± 3.3	38 ± 4.2	31 ± 2.9	44 ± 6.2	112 ± 14	99 ± 5.8
C475S	20 ± 2.5	35 ± 5.1	12 ± 1.6	99 + 5.3	95 ± 8.9	95 ± 7.6

We have, for the first time, provided evidence that the active metabolite of clopidogrel forms a covalent adduct with a cysteinyl residue of CYP2B6 during the mechanism-based inactivation (see Figs. 2 and 4). By use of a cleavable alkylating reagent (IBB) that contains a biotin moiety, we were able to selectively enrich the modified peptide and then sequence it. It was found that the clopidogrel-modified residue resides in the peptide 460 to 479, ^{460}ASPVAPEDIDLTPQECGVGK^{479}. Because of the relatively large size of this peptide (molecular mass, >2000 Da), it could only be partially sequenced by ESI-LC/MS/MS. Nonetheless, the results from the peptide sequencing have narrowed down the identity of the modified residue to a fiv-residue peptide ^{473}QECGV^{477}, even though the exact location of the modified residue cannot be determined by peptide sequencing alone (see Fig. 4). Because the protein adduct can be cleaved by dithiothreitol, we conclude that Cys475 is the site of modification as Cys475 is the only cysteinyl residue in this 5-residue peptide ^{473}QECGV^{477}. These results indicate that the thiol group of Cys475 forms a disulfide bond with a reactive metabolite of clopidogrel.

As to the chemical nature of the reactive metabolite, on the basis of several lines of evidence, it is most likely that the pharmacologically active metabolite of clopidogrel forms the disulfide bond with Cys475. First, it is well documented that metabolism of thienopyridines such as clopidogrel and ticlopidine leads to the production of the active metabolite, which is capable of modifying cysteinyl residues (Savi et al., 2000; Pereillo et al., 2002; Ding et al., 2003). In fact, the pharmacological activity of clopidogrel is due to covalent modification of the Cys17 and Cys270 residues of the human P2Y12 ADP receptor by the active metabolite (Ding et al., 2003). Second, according to Kazui et al. (2010), CYP2B6 is capable of sequential oxidation of clopidogrel to give the active metabolite. This is supported by our observation that metabolism of 2-oxo-clopidogrel by CYP2B6 produces the active metabolite detected as a glutathione adduct. Last, substitution of 2-oxo-clopidogrel for clopidogrel in the inactivation reaction leads to the formation of a protein adduct having the same molecular mass as that formed in the presence of clopidogrel, indicating that the oxidation of 2-oxo-clopidogrel to the active metabolite is involved in protein modification. Collectively, our results show that metabolism of clopidogrel by CYP2B6 leads to formation of the pharmacologically active metabolite of clopidogrel, which in turn results in covalent modification of Cys475. Based on the observation that the C475S variant does not form a protein adduct and is not inactivated by 2-oxoclopidogrel, it is clear that Cys475 is the primary site for covalent modification.

It is intriguing that the active metabolite primarily modifies Cys475 and selectively modifies CYP2B6 but not CPR, even though CPR contains two more cysteinyl residues. The answer may be due to the unique location of Cys475. Based on the crystal structure of CYP2B6 (Gay et al., 2010), Cys475 is located in the $\beta_{4\text{-}1}$ sheet toward the C-terminal end of CYP2B6 and is partially surface-exposed, its side chain directed toward the solvent. More importantly, Cys475 is located in a putative access channel, referred to as channel 1 (see Fig. 5). As such,

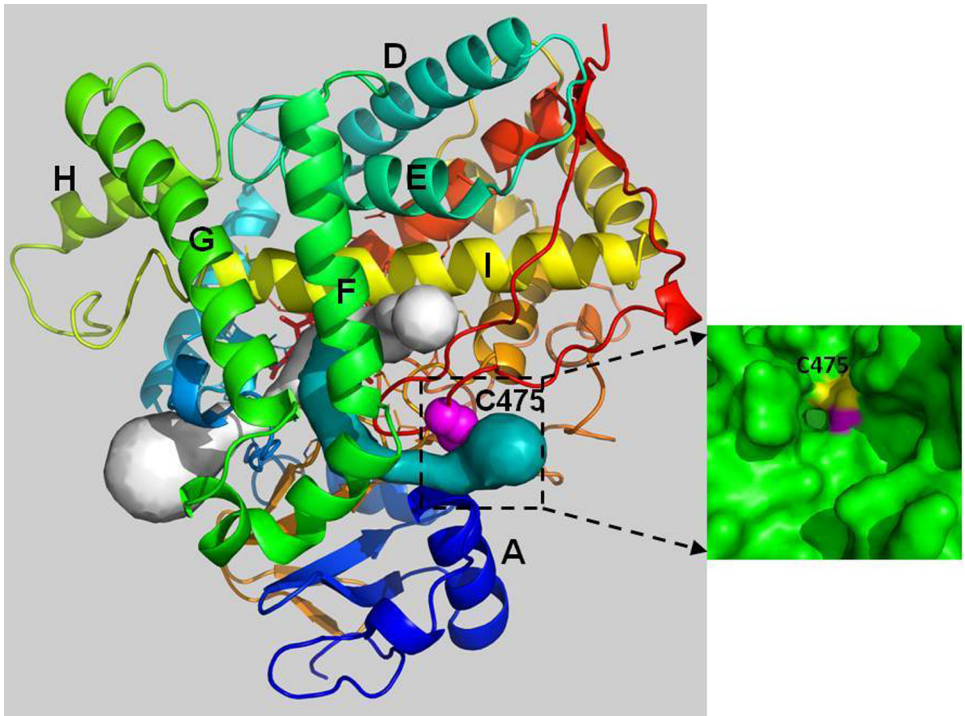

Fig. 5. Top view of the crystal structure and the solvent access channels of CYP2B6. The coordinates of CYP2B6 were obtained from the Protein Data Bank (3IBD), and the access channels were calculated using the Caver software (ver. 2.1.2). The crystal structure of CYP2B6 is presented as rainbow ribbons colored from blue (N terminus) to red (C terminus), the access channels are presented as solid surface, and Cys475 is depicted as magenta spheres. The putative access channel passing Cys475 is colored as cyan and referred to as channel 1 in the text. The inset is a surface view of channel 1 and Cys475. CYP2B6 is presented as green surface, Cys475 is yellow, and the sulfur atom of Cys475 is magenta. The figure was prepared using PYMOL (http://www.pymol.org).

channel 1 passes right by Cys475. It is conceivable that the active metabolite forms a disulfide bond with Cys475 while exiting this channel. This explains why glutathione does not protect against the inactivation of CYP2B6 by 2-oxo-clopidogrel. Covalent modification of Cys475 could block the channel or restrict passage of substrates and/or products, leading to loss of the catalytic activity. Alternatively, covalent modification of Cys475 may cause significant structural changes that adversely affect the catalytic activity. This is supported by the observation that removal of the adduct from the clopidogrel-inactivated CYP2B6 by DTT treatment fully restores the ionization to the same level as the unmodified CYP2B6 WT (see Fig. 2C). Alignment of the amino acid sequences of the human P450s involved in clopidogrel metabolism (i.e., CYP1A2, -2B6, -2C19, and -3A4) reveals that Cys475 is unique to CYP2B6 (data not shown). It remains to be seen whether modification of cysteinyl residues by clopidogrel occurs in human P450 isoforms other than CYP2B6.

Our results on the covalent modification of cysteinyl residues lend support for the hypothesis of Richter et al. (2004) that covalent modification of cysteinyl residues in CYP2B6 may play a role in the mechanism-based inactivation of CYP2B6 by clopidogrel and ticlopidine. This is clearly the case for the mechanism-based inactivation of CYP2B6 by 2-oxo-clopidogrel (see below). However, mutation of Cys475 to a serine residue has little effect on preventing the C475S variant from being inactivated by clopidogrel, even though the C475S variant is no longer inactivated by 2-oxo-clopidogrel (see Table 2). This observation indicates that other mechanism(s) may be involved. We sought to investigate whether destruction of the heme is involved by examining the heme contents of the inactivated CYP2B6. Our results shed light on the overall mechanisms by which clopidogrel inactivates CYP2B6.

Analyses of the heme contents of the CYP2B6 WT and the C475S variant that had been inactivated by 2-oxo-clopidogrel showed no losses in the native heme or CO-detectable heme (see Table 2). Lack of changes in the heme content in the inactivated CYP2B6 WT seems to suggest that 2-oxo-clopidogrel inactivates CYP2B6 WT solely by covalent modification of Cys475. However, this is not the case for the mechanism-based inactivation by clopidogrel. In the case of clopidogrel, covalent modification of Cys475, loss of the native heme and CO-detectable heme, and loss of the catalytic activity occurred simultaneously (Table 2; Fig. 2). As shown in Table 2, the loss of the native heme content accounts for most of the loss in the catalytic activity. Therefore, the primary mechanism for the inactivation of CYP2B6 by clopidogrel seems to be the destruction of the heme. Covalent modification of Cys475 may play only a minor role in the inactivation of CYP2B6 by clopidogrel. This is in clear contrast to the mechanism-based inactivation of CYP2B6 by 2-oxo-clopiodgrel. This discrepancy may arise from the fact that metabolism of thienopyridines by CYP2B6 is capable of generating multiple metabolites. A recent study of the metabolism of ticlopidine by CYP2B6 in a reconstituted system identified five metabolites, including 2-oxo-ticlopidine, hydroxyticlopidine, a tetrahydropyridinium metabolite, a thienopyridinium metabolite, and ticlopidine *S*-oxide dimer (Talakad et al., 2011). We have observed the counterparts of all five metabolites for clopidogrel in our reconstituted systems (H. Zhang and P. Hollenberg, unpublished data). In addition to the active metabolite detected as a GSH adduct, metabolism of clopidogrel by CYP2B6 yields at least six metabolites. Although the causes for the heme loss are unknown, it probably occurs before the oxidation of 2-oxo-clopidogrel, because metabolism of 2-oxo-clopidogrel does not result in heme loss. Ha-Duong et al. (2001) have suggested that the likely candidates include the *S*-oxide intermediate or a thiophene epoxide intermediate. These two intermediates are highly reactive electrophiles.

It should be noted that our observation in the reconstituted system that inactivation of CYP2B6 by clopidogrel results in loss of the CO-detectable heme does not agree with the previous study by Richter et al. (2004), which showed no loss in the CO-detectable heme in CYP2B6 supersome samples. This discrepancy is not due to different types of CYP2B6 samples used (reconstituted system versus supersomes), because we also observed loss of the heme in CYP2B6 supersome samples (data not shown). The exact cause for this discrepancy is unknown at this time. More studies are required to investigate the mechanism that leads to the loss of heme.

In summary, mechanism-based inactivation of CYP2B6 by clopidogrel leads to both loss of the heme and covalent modification of Cys475. It is likely that loss of the heme plays a major role in inactivating the catalytic activity of CYP2B6, because loss of the heme seems to be correlated with the loss of the catalytic activity. Covalent modification of Cys475 by the active metabolite of clopidogrel may also lead to loss of the catalytic activity. We hypothesize that this is due to the blockage of a substrate access or alteration of protein conformations. Collectively, these results suggest that clopidogrel inactivates CYP2B6 primarily through destruction of the heme, whereas 2-oxo-clopidogrel inactivates CYP2B6 through covalent modification of Cys475.

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Authorship Contributions

Participated in research design: Zhang.
Conducted experiments: Zhang, Amunugama, Ney, Cooper.
Performed data analysis: Zhang and Amunugama.
Wrote or contributed to the writing of the manuscript: Zhang and Hollenberg.

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