Mechanism-Based Inactivation Of Human Cytochrome P450 2B6 By Clopidogrel: Involvement Of Both Covalent Modification Of Cysteiny1 Residue 475 And Loss of Heme

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Nonstandard abbreviations: 7-EFC, 7-ethoxy-4-trifluoromethylcoumarin; 7-HFC, 7-hydroxy-4-trifluoromethylcoumarin; CYP or P450, cytochrome P450; CPR, NADPH-dependent cytochrome P450 reductase; DTT, dithiothreitol; ESI-LC/MS, electrospray ionization liquid chromatography mass spectrometry; IBB, N-(2-(2-(2-(2-(3-(1-hydroxy-2-oxo-2-phenylethyl)phenoxy)ethoxy)ethoxy)ethyl)-5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide.
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 $k_{\text{inact}}/K_i$ 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 (CYP2B6 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 by ~350 Da corresponds to the addition of the active metabolite of clopidogrel to CYP2B6. Interestingly, 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 ESI-LC/MS/MS identified Cys 475 as the site of covalent modification by the active metabolite. This was further confirmed by the observation that mutation of Cys 475 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 Cys 475.
INTRODUCTION

Clopidogrel is a thienopyridine antiplatelet agent that is widely prescribed for the prevention of atherothrombotic events like 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 (CYPs or P450s) including CYP2B6 (Bouman et al., 2011; Kazui et al., 2010; Savi et al., 1994). The active metabolite containing a reactive thiol group prevents ADP-induced platelet aggregation by covalent modification of the Cys 17 and Cys 270 residues of human P2Y12 ADP receptor via the formation of disulfide bonds (Ding et al., 2003; Pereillo et al., 2002; Savi et al., 2000).

It is generally thought that clopidogrel is bio-activated 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 bio-activation of the thiolactone metabolite to produce the active metabolite (Dansette et al., 2009; Kazui et al., 2010). Based on in vitro studies of metabolism of clopidogrel and 2-oxo-clopidogrel, the major CYP contributors to the first oxidative step include CYP1A2, 2B6 and 2C19 (Kazui et al., 2010). However, it was recently reported that 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; Dansette et al., 2010). However, Bouman et al (2011) have questioned the role of P450s in the bio-activation 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 bio-activating clopidogrel, P450s may also be inactivated by clopidogrel. A clinical study reported that patients who received both clopidogrel and bupropion, an antidepressant drug and a specific substrate for CYP2B6, experienced an ~60% increase in the plasma concentration of bupropion and suggested that these patients may require dose adjustment when both drugs are co-administrated (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; Nishiya et al., 2009a; Nishiya et al., 2009b; Richter et al., 2004; Walsky and Obach, 2007), 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 \( \mu \text{M} \) 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 cysteiny1 residues of CYP2B6 by the active metabolite could contribute to the mechanism-based inactivation. However, no protein adducts nor modified cysteiny1 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 nmole per nmole of CYP2C19 (Ha-Duong et al., 2001). Once again, it is unclear where the sites of modification were. Despite the fact that clopidogrel and ticlopidine are potent mechanism-based inhibitors of CYPs, their mechanisms of action as inactivators remain largely unknown.

In this study, we carried out a detailed investigation in order 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 ACS reagent grade unless otherwise specified. Racemic clopidogrel and 2-oxo-clopidogrel were purchased from Toronto Research Chemicals (North York, Canada). NADPH, dithiothreitol (DTT), glutathione, and catalase were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Trifluoroacetic acid (TFA) and N-ethylmaleimide were purchased from Pierce Chemicals (Rockford, IL). 7-Ethoxy-4-trifluoromethylcoumarin (7-EFC) was purchased from Invitrogen Molecular Probes (Eugene, OR). 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-(2-(3-(1-hydroxy-2-oxo-2-phenylethyl)phenoxy)acetamido)ethoxy)ethoxy)ethyl)-5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide, was a generous gift from Dr. Daniel C. Liebler (Vanderbilt University, Nashville, TN).

**Mutagenesis, over-expression and purification of CYP2B6.** Site-directed mutagenesis was performed to prepare the C475S mutant using the Quikchange method according the manufacturer’s instructions (Agilent Technologies, Santa Clara, CA). A pair of mutagenic primers, 5’-CTGACACCCAGGATCTGGTGCGG-3’ (forward) and 5’-CCCACACCAGACTCCTGAGGTGG-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. Over-expression 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-terminal truncated form containing a (His)₄-tag at the C-terminus.
Cytochrome P450 reductase (CPR), and cytochrome b5 (cyt b5) were over-expressed and purified as previously described (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 $k_{\text{inact}}$, were determined at 37 °C in a reconstituted system as described previously (Zhang et al., 2009b). Typically, the primary reaction mixture contained CYP2B6 (1 µM), CPR (2 µM), cyt b5 (1 µM), catalase (1 unit/µL), and varying concentrations of clopidogrel (0-25 µM) or 2-oxo-clopidogrel (0-100 µM) 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 designated times, 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 (7-HFC) was measured with excitation at 410 nm and emission at 510 nm using a VictorII microtiter plate reader (Perkim Elmer, Waltham, MA). The $K_i$ and $k_{\text{inact}}$ values were obtained by fitting the rates of inactivation determined at various concentrations to the Michaelis-Menten equation using GraphPad 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 reverse-phase C3 column and eluted into a LCQ Classic ion-trap mass spectrometer (Thermo Fischer Scientific, Waltham, MA) 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 hour. The molecular mass of the DTT-treated CYP2B6 was then determined by ESI-LC/MS as described above.

**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 re-dissolved 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% of 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 and finally 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 alkylation 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 nmoles), CPR (15 nmoles) and cyt b5 (7.5 nmoles) 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 hours 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, Waltham, MA). The desalted protein mixture was then digested with at 1:25 ratio (w/w) of trypsin to protein at 37 °C for 20 hours. The tryptic digests were lyophilized to a final volume of ~1-2 µl and then re-dissolved in 0.2 ml of 0.1 M potassium phosphate solution (pH 7.0). Prior to 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, while an equal volume of water was added into the control sample. Ethylenediaminetetraacetic acid (EDTA) was added to a final concentration of 1 mM to prevent re-oxidation of the free thiols. Both samples were then incubated for 30 min at room temperature and then 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 in Ref (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 HPLC 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 HPLC 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 400-500 nm on a Shimadzu UV/Vis spectrophotometer (UV-2501PC, Shimadzu, Japan) after adding a few grains of dithionite and bubbling CO gas into the reduced sample for ~10 seconds.

**Analysis of the access channels for CYP2B6.** The solvent access channels of CYP2B6 were analyzed using Caver software (ver. 2.1.2) (Petrek et al., 2006). The calculations were performed as previously described for CYP2B1 (Zhang et al., 2009a). Briefly, the coordinates of CYP2B6 was obtained from the Protein Data Bank (PDB ID: 3IBD). The coordinates of water molecules and bound inhibitor 4-(4-chlorophenyl)imidazole were deleted prior to calculations. The initial start point of the access channel was chosen in the void region of the active site above the heme Fe. A probe molecule of 4 Å was used to probe the access channels. PYMOL (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 Figure 1A. The $K_i$ and $k_{inact}$ values were determined to be 2.4 $\mu$M and 0.17 min$^{-1}$, respectively, which gives a $k_{inact}/K_i$ ratio or inactivation efficiency of 0.071 min$^{-1}$ $\mu$M$^{-1}$. In a similar fashion, 2-oxo-clopidogrel inactivated CYP2B6 WT with $K_i$ and $k_{inact}$ values of 6.3 $\mu$M and 0.092 min$^{-1}$ respectively, as shown in Figure 1B. This gives a $k_{inact}/K_i$ ratio of 0.015 min$^{-1}$ $\mu$M$^{-1}$. Therefore, 2-oxo-clopidogrel 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 nearly 12-fold decrease 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 Figure 2. In the absence of NADPH,
CYP2B6 exhibited a molecular mass of 54416 Da, which corresponds to the mass expected for unmodified CYP2B6. The deconvoluted mass spectrum for the inactivated CYP2B6 showed two peaks at 54410 and 54758 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 of note 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 54419 Da, almost identical to that of the unmodified CYP2B6 protein, as shown in Figure 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 cysteiny1 residue of CYP2B6. In the case of 2-oxo-clopidogrel, similar results were obtained (see Figure 1S in the Supplementary Materials). Interestingly, the molecular masses of CPR and cyt b5 were not modified even though they were present in the reconstituted system (data not shown). It appears that a reactive metabolite of clopidogrel selectively modifies CYP2B6 only, even though the amino acid sequence of CPR contains seven cysteiny1 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 Figure 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 Cl 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 fragment patterns of the parent ion at m/z 661.0 can be interpreted on the basis of a disulfide bond linkage. The MS² 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 Figure 3B. The former results from the neutral loss of 129 from the parent ion, while the latter represents the metabolite fragment split from the disulfide bond. The other fragment peaks at m/z 643.0, 586.1, 211.8 can also be assigned to the GSH adduct as shown in Figure 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 Figure 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 S and –CH₂S 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 before (Talakad et al., 2011). Based on the molecular mass of the GSH adduct and the MS² and MS³ 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.** In order to select only the clopidogrel-modified peptides for peptide mapping, we employed a two-step alkylation procedure followed by an enrichment step that utilized 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, while no modified peptides were observed in the control sample. The extracted ion chromatogram and mass spectrum of this peptide are provided in the Supplementary Materials (see Figure 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 460-479 of CYP2B6, ⁴⁶⁰ASPVPEDIDLTPQECGVK⁴⁷⁹. This peptide contains one cysteinyl residue at position 475. Fragmentation of this doubly charged ion produced a series of b-ions and y-ions that are consistent with the amino acid sequence for the peptide 460-479. As shown in Figure 4, the masses of the b₄, b₅ and b₁₂ ions match with the theoretical masses for the unmodified peptide 460-479, indicating that the N-terminal end of P472 is not modified by IBB. However, the molecular mass of the b₁₈ ion exhibits an increase in mass of 57.3 Da, indicating that the covalent modification
occurs between P472 and V477. This is consistent with the observed y-ions. All the y-ions observed including the y7-ion exhibit an increase in mass of 57±1 Da, which further narrows down the modified site to \textsuperscript{473}QECGV\textsuperscript{477}. Even though the amino acid sequencing was incomplete due to the relatively large size of the peptide 460-479, it can, nonetheless, be concluded that C475 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 \textsuperscript{473}QECGV\textsuperscript{477} other than Cys 475. To further support this conclusion, we mutated Cys 475 to a serine residue and investigated the formation of protein adducts with 2-oxo-clopidogrel. As shown in the Supplementary Materials (see Figure 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 C475→S mutation on the mechanism-based inactivation of CYP2B6 by clopidogrel and 2-oxo-clopidogrel.** To investigate the role of Cys 475 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 comparison. Under the same conditions used for CYP2B6 WT, the C475S variant was no longer inactivated by 2-oxo-clopidogrel. Interestingly 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 Cys 475 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 C475→S 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, while 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-clopidogrel, CYP2B6 WT lost 56% of the activity without losing any of the native heme and the CO-detectable heme. As mentioned previously, 2-oxo-clopidogrel did not inactivate the C475S variant, and no heme loss was observed for the C475S variant.
DISCUSSIONS

In this study, we investigated the mechanisms by which clopidogrel inactivates CYP2B6 in a reconstituted system. We have, for the first time, provided evidence that the active metabolite of clopidogrel forms a covalent adduct with a cysteiny1 residue of CYP2B6 during the mechanism-based inactivation (see Figures 2 & 4). By use of a cleavable alkylation 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 460-479, \textit{ASPVPEDIDLTPQECGVGK}. Due to the relatively large size of this peptide (MW > 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 5-residue peptide \textit{QECGV}, even though the exact location of the modified residue cannot be determined by peptide sequencing alone (see Figure 4). Since the protein adduct can be cleaved by dithiothreitol, it is concluded that Cys 475 is the site of modification as Cys 475 is the only cysteiny1 residue in this 5-residue peptide \textit{QECGV}. These results indicate that the thiol group of Cys 475 forms a disulfide bond with a reactive metabolite of clopidogrel.

As to the chemical nature of the reactive metabolite, it is most likely that the pharmacologically active metabolite of clopidogrel forms the disulfide bond with Cys 475 based on several lines of evidence. First, it is well documented that metabolism of thienopyridines such as clopidogrel and ticlopidine leads to the production of the active metabolite that is capable of modifying cysteinyl residues (Ding et al., 2003; Pereillo et al., 2002; Savi et al., 2000). In fact, the pharmacological activity of clopidogrel is due to
covalent modification of the Cys 17 and Cys 270 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 the 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 Cys 475. 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 Cys 475 is the primary site for covalent modification.

It is intriguing that the active metabolite primarily modifies Cys 475, 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 Cys 475. Based on the crystal structure of CYP2B6 (Gay et al., 2010), Cys 475 is located in the β_{4-1} sheet toward the C-terminal end of CYP2B6 and is partially surface-exposed with its side chain directed toward the solvent. More importantly, Cys 475 is located in a putative access channel, referred to as Channel 1 (see Figure 5). As such, Channel 1 passes right by Cys 475. It is conceivable that the active metabolite forms a disulfide bond with Cys 475 while exiting this channel. This explains why glutathione does not protect against the
inactivation of CYP2B6 by 2-oxo-clopidogrel. Covalent modification of Cys 475 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 Figure 2C). Alignment of the amino acid sequences of the human P450s involved in clopidogrel metabolism, i.e. CYP1A2, 2B6, 2C19 and 3A4, reveals that Cys 475 is unique to CYP2B6 (data not shown). It remains to be seen whether modification of cysteinyl residues by clopidogrel occur 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 Cys 475 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 Cys 475. However, this is not the case for the mechanism-based inactivation by clopidogrel. In the case of clopidogrel, covalent modification of Cys 475, loss of the native heme and CO-detectable heme, and loss of the catalytic activity occurred simultaneously (Table 2 & Figure 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 mechanism-based inactivation of CYP2B6 by clopidogrel appears to be due to destruction of the heme. Covalent modification of Cys 475 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-clopidogrel. 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 (TSOD) (Talakad et al., 2011). We have observed the counterparts of all these five metabolites for clopidogrel in our reconstituted systems (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 likely occurs prior to the oxidation of 2-oxo-clopidogrel since 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 Ritcher et al (2004) that 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 vs supersomes) as 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 Cys 475. It is likely that loss of the heme plays a major role in inactivating the catalytic activity of CYP2B6 as loss of the heme seems to be correlated with the loss of the catalytic activity. Covalent modification of Cys 475 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 Cys 475.
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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Zhang H

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Performed data analysis: Zhang H, Amunugama H

Contributed to the writing of the manuscript: Zhang H, Hollenberg PF
REFERENCES


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FIGURE LEGENDS

Figure 1. Kinetics for the mechanism-based inactivation of CYP2B6 WT by clopidogrel (A) and 2-oxo-clopidogrel (B). The inactivation reactions were performed at 37 °C in a reconstituted system as described in Materials and Methods. Legend, (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 inserts are plots of the observed rates at various concentrations of inhibitors and they were used to calculate the kinetic parameters $K_i$ and $k_{inact}$.

Figure 2. Analysis of the protein masses of CYP2B6 by ESI-LC/MS following the inactivation by clopidogrel. CYP2B6 was inactivated at 30 °C for 5 min in the presence of 20 µM clopidogrel as described in 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.

Figure 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 in Materials and Methods. Legend: (A), the MS spectra for the GSH adduct that eluted at 26.7 min; (B), the MS² spectrum of the parent ion at m/z 661.0 and the fragment assignments; (C)
the MS³ spectrum of the m/z 354.0 ion from the parent ion at m/z 661.0 and the fragment assignments.

**Figure 4.** Peptide sequencing of the peptide 460-479 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 using the Xcalibur software as described in 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 (-NH₃) and water (-H₂O), respectively, while yo and y* ions refer to y ions that lose ammonia and water, respectively. Cys 475 is marked with an asterisk.

**Figure 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 (PDB ID: 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 Cys 475 is depicted as magenta spheres. The putative access channel passing Cys 475 is colored as cyan and referred to as Channel 1 in the text. The inset is a surface view of Channel 1 and Cys 475. CYP2B6 is presented as green surface, Cys 475 is colored in yellow and the sulfur atom of Cys 475 is colored in magenta. The figure was prepared using PYMOL (www.pymol.org).
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 in Materials and Methods. The reported data were averaged from four separate experiments.

<table>
<thead>
<tr>
<th>CYP2B6</th>
<th>Clopidogrel</th>
<th>2-oxo-Clopidogrel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_I$ (µM)</td>
<td>$k_{inact}$ (min$^{-1}$)</td>
</tr>
<tr>
<td>WT</td>
<td>2.4±0.33</td>
<td>0.17±0.031</td>
</tr>
<tr>
<td>C475S</td>
<td>4.2±0.21</td>
<td>0.12±0.015</td>
</tr>
</tbody>
</table>

*not determined because of lack of inactivation.
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 in Materials and Methods. The data were averaged from three separate experiments.

<table>
<thead>
<tr>
<th>CYP2B6</th>
<th>Clopidogrel</th>
<th>2-oxo-clopidogrel</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Activity%</td>
<td>Heme%</td>
</tr>
<tr>
<td>WT</td>
<td>28±3.3</td>
<td>38±4.2</td>
</tr>
<tr>
<td>C475S</td>
<td>20±2.5</td>
<td>35±5.1</td>
</tr>
</tbody>
</table>
Figure 1

A

Log(% Activity Remaining) vs. Concentration (μM)

Inset: Observed rate constant (k_{obs}) vs. Concentration (μM)

B

Log(% Activity Remaining) vs. Time (min)

Inset: Observed rate constant (k_{obs}) vs. Concentration (μM)