Comparison of *in vitro* Metabolism of Ticlopidine by Human Cytochrome P450 2B6 and Rabbit Cytochrome P450 2B4

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Abbreviations. P450, cytochrome P450; CYP2B6, an N-terminal truncated and modified and C-terminal 4-His-tagged form of cytochrome P450 2B6; CYP2B4, an N-terminal truncated and modified and C-terminal His-tagged form of cytochrome P450 2B4 with a substitution of His$^{226}$ to Tyr; 4-CPI, 4-(4-chlorophenyl)imidazole; cymal-5, 5-cyclo-hexylpentyl-β-D-maltoside; BME, 2-mercaptoethanol; PMSF, phenylmethylsulfonyl fluoride; Ni-NTA, nickel-nitrilotriacetic acid; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; TCA, trichloroacetic acid; 7-HFC, 7-hydroxy-4-(trifluoromethyl) coumarin; 7-EFC, 7-ethoxy-4-trifluoromethylcoumarin; 7-MFC, 7-methoxy-4-trifluoromethylcoumarin; NADPH, nicotinamide adenine dinucleotide phosphate; TSOD, ticlopidine S-oxide dimer; MS, mass spectrometry, HPLC, high-performance liquid chromatography; CPR, recombinant NADPH-cytochrome P450 reductase, ESI, Electrospray ionization; SRM, single reaction monitoring; amu, Atomic Mass Units.
Abstract

A recent X-ray crystal structure of a rabbit cytochrome P450 2B4 (CYP2B4) – ticlopidine complex indicated that the compound could be modeled with either the thiophene or chlorophenyl group oriented toward the heme prosthetic group. Subsequent NMR relaxation and molecular docking studies suggested that orientation with the chlorophenyl ring closer to the heme was the preferred one. To evaluate the predictive value of these findings, the oxidation of ticlopidine by reconstituted CYP2B4 was studied and compared with CYP2B6, in which the thiophene portion of the molecule likely orients toward the heme. In vitro incubation of ticlopidine with both enzymes yielded the same set of metabolites: 7-hydroxyticlopidine (M1), 2-oxoticlopidine (M2), 5-(2-chlorobenzyl)thieno[3,2-c]pyridin-5-ium metabolite (M3), 5-(2-chlorobenzyl)thieno[3,2-c]pyridin-5-ium metabolite (M4), ticlopidine N-oxide (M5) and ticlopidine S-oxide dimer (TSOD), a dimerization product of ticlopidine S-oxide (M6). The rates of metabolite formation deviated markedly from linearity with time, consistent with the known inactivation of CYP2B6 by ticlopidine. Fitting to a first order equation yielded similar rate constants ($k_{obs}$) for both enzymes. However, the amplitude ($R_{max}$) of M1 and M6 formation was 4-5 times higher for CYP2B6 than CYP2B4, indicating a greater residence time of ticlopidine with its thiophene ring closer to heme in CYP2B6. Conversely, CYP2B4 formed M4 and M5 in more abundance than CYP2B6, indicating an alternate orientation. Overall, the results suggest that the preferential orientation of ticlopidine in the active site of CYP2B4 predicted by X-ray crystallography and NMR studies is unproductive and that ticlopidine likely reorients within CYP2B4 to a more productive mode.
Introduction

Cytochromes P450 (P450s) constitute a superfamily of heme-containing proteins that play a predominant role in the oxidative metabolism of xenobiotics (Johnson and Stout, 2005). Out of the 57 identified human P450s, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1 and CYP3A4 are mainly involved in the metabolism of drugs (Lewis and Ito, 2008). CYP2B6 constitutes approximately 6% of the total human liver cytochrome P450 and metabolizes a number of important pharmaceuticals (Guengerich et al., 2005). Moreover, recent crystal structure analysis of a genetic variant of CYP2B6 in the presence of 4-(4-chlorophenyl)imidazole (4-CPI) provided the first atomic level view of this P450 (Gay et al., 2010a). In the past few years, a series of crystal structures of rabbit CYP2B4, which shares 78% sequence identity with CYP2B6, revealed the remarkable plasticity that these enzymes exhibit to accommodate various small imidazole based inhibitors such as 4-CPI or 1-(4-chlorophenyl)imidazole (1-CPI), as well as the bulkier ligands bifonazole or 1-(4-phenyl)benzylimidazole (1-PBI) (Scott et al., 2004; Zhao et al., 2006; Zhao et al., 2007; Gay et al., 2009). Very recently crystallization and structure determination of CYP2B4 in complex with the antiplatelet drugs ticlopidine and clopidogrel, which are not anchored to the heme iron by a coordinate bond, helped to further understand CYP2B plasticity and catalytic function (Gay et al., 2010b).

In addition to being a potent thienopyridine antiplatelet drug, ticlopidine is a strong mechanism-based inhibitor of CYP2B6 (Richter et al., 2004) and CYP2C19 (Ha-Duong et al., 2001). It is used to prevent atherothrombosis by irreversibly inhibiting ADP binding to the platelet receptor P2Y12 (Sharis et al., 1998; Kam and Nethery, 2003), and it also prevents
platelet aggregation and reduces the risk of cardiovascular, cerebrovascular and peripheral vascular diseases (Richter et al., 2004). However, there is substantial incidence of harmful side-effects including, but not limited to, agranulocytosis (Ono et al., 1991), aplastic anemia (Mataix et al., 1992), thrombocytopenia (Steinhubl et al., 1999) and ticlopidine induced hepatotoxicity (Grieco et al., 1998). This drug is extensively metabolized in humans and animals by cytochromes P450 (P450s) (Onyeji et al., 1999; Farid et al., 2010) via N-dealkylation, N-oxidation, S-oxidation and oxidation of the tetrahydropyridine and the thiophene ring (Tuong et al., 1981; Panak et al., 1983; Ha-Duong et al., 2001; Dalvie and O’Connell, 2004). Human metabolism of ticlopidine is primarily catalyzed by CYP3A4, CYP2C19 and CYP2B6 (Farid et al., 2010). Recent studies have indicated that the oxidation of the thiophene ring of ticlopidine is important for the formation of reactive metabolites resulting in inhibition of CYP2B6 (Nishiya et al., 2009), CYP2C19 and CYP2D6 (Ko et al., 2000; Walsky et al., 2006; Farid et al., 2010).

Despite previous studies, which inferred that the primary human P450 mediated metabolic pathway of ticlopidine is via the formation of pharmacologically active 2-oxoticlopidine (M2) and/or ticlopidine S-oxide, no formal studies have been conducted to show the specific metabolites formed by CYP2B6.

In our recent crystal structure of the CYP2B4-ticlopidine complex, the preferred orientation of the drug in the active site (Fig. 1A and 1B) was not clear from the electron density. Further studies involving NMR relaxation and molecular docking suggested that the major orientation is with the chlorophenyl ring closer to the heme (Gay et al., 2010b), which is not consistent with the major products hypothesized for CYP2B6. In the present study the specific metabolites of ticlopidine produced by CYP2B6 were identified for the first time and compared with those formed by metabolism of ticlopidine by CYP2B4. The products are correlated with
predictions from X-ray crystallography, modeling, and NMR relaxation studies, and the insights gained are used to propose chemical mechanisms for metabolite formation, including those involved in the antiplatelet action and P450 inactivation.
Materials and Methods

Materials

Ticlopidine hydrochloride, reduced β-nicotinamide adenine dinucleotide 2’-phosphate, reduced (NADPH), buspirone, bupropion and Tris-HCl were purchased from Sigma-Aldrich (St. Louis, MO). 7-Hydroxy-4-(trifluoromethyl) coumarin (7-HFC), 7-ethoxy-4-(trifluoromethyl) coumarin (7-EFC), and 7-methoxy-4-trifluoromethylcoumarin (7-MFC) were purchased from Invitrogen (Carlsbad, CA). 5-Cyclohexylpentyl-β-D-maltoside (Cymal-5) was from Anatrace (Maumee OH). Recombinant NADPH cytochrome P450 reductase (CPR) was prepared as described previously (Harlow and Halpert, 1997). Macroprep CM cation exchange resin was from Bio-Rad Laboratories (Hercules, CA). TOPP3 and JM109 cells were from Stratagene (La Jolla, CA). The molecular chaperone plasmid pGro7, which expresses GroES/EL, was obtained from TAKARA BIO (Shiba, Japan). Ni-NTA affinity resin was purchased from Qiagen (Valencia, CA). Pooled human liver microsomes were prepared under contract by BD Gentest (Woburn MA). Aliquots from the individual preparations from 60 individual human livers were pooled on the basis of equivalent protein concentrations to yield a representative microsomal pool with a protein concentration of 20.4 mg/mL (determined using the Bicinchoninic Acid Assay; Pierce, Rockford, IL). The P450 content in the pooled microsomes was 0.35 nmol/mg and was determined from the reduced CO difference spectrum (Omura and Sato, 1964). Baculovirus/insect cells expressing full length CYP2B6 enzyme (protein concentration 15 mg/mL or 100 pmol of P450/mL) were purchased from BD Biosciences Company. Ticlopidine was dissolved in methanol to prepare the stock solution. All other chemicals were of the highest grade available and were obtained from standard commercial sources.
Expression and Purification

CYP2B4 (N-terminally truncated and modified and C-terminally His-tagged form of P450 2B4 with mutation at His$^{226}$ to Tyr) and CYP2B6 (N-terminal truncated and modified and C-terminal His-tagged) were expressed in *E. coli* TOPP3 cells and *JM109* cells, respectively (Stratagene, La Jolla, CA). CYP2B6 was co-expressed with the chaperone GroES/EL (pGro7 plasmid) as described earlier (Mitsuda and Iwasaki, 2006). The expression and purification protocol used herein has been previously described (Scott et al., 2001; Kumar S, 2007). In brief, the proteins were purified on a Ni-NTA column, which was washed with buffer containing 100 mM potassium phosphate (pH 7.4 at 4 °C), 100 mM NaCl, 20% (v/v) glycerol, 10 mM BME, 0.5 mM PMSF, 4.8 mM Cymal-5, and 1 mM histidine. The protein was eluted using 40 mM histidine in the same buffer described above. Pooled P450-containing Ni fractions were diluted 10-fold in buffer containing 5 mM potassium phosphate (pH 7.4 at 4 °C), 20% (v/v) glycerol, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.2 mM dithiothreitol (DTT), 0.5 mM PMSF, and 4.8 mM Cymal-5, prior to being loaded onto a Macroprep CM cation exchange column. The column was washed using 5 mM potassium phosphate (pH 7.4 at 4 °C), 20 mM NaCl, 20% (v/v) glycerol, 1 mM EDTA, and 0.2 mM DTT, and the protein was eluted with high-salt buffer containing 50 mM potassium phosphate (pH 7.4 at 4 °C), 500 mM NaCl, 20% (v/v) glycerol, 1 mM EDTA, and 0.2 mM DTT (Gay et al., 2010a). Eluted protein was dialyzed three times at 4°C against 10 mM potassium phosphate (pH 7.4) buffer containing 10% glycerol and 1 mM EDTA. The P450 content was measured from the reduced CO-difference spectrum (Omura and Sato, 1964).

Ticlopidine Metabolism.
Incubations for metabolite identification (total volume 1.0 mL) were conducted at 37 °C for 60 min in 100 mM potassium phosphate buffer (pH 7.4) containing magnesium chloride (10 mM), NADPH (1 mM) and protein. The final concentration of the protein in the incubation mixture was as follows: human liver microsomes (1 mg/mL), baculovirus expressed full length CYP2B6, reconstituted CYP2B6 and reconstituted CYP2B4 (100 pmol/mL). The reconstituted system contained a 1:1 molar ratio of protein (CYP2B6 or CYP2B4) and cytochrome P450 reductase (CPR). Reactions were initiated by addition of ticlopidine (10 μM) after preincubation of the incubation mixture for one min. The final concentration of organic solvent in the incubation mixture was <1%. Incubations lacking NADPH served as negative controls. Reactions were terminated by addition of 1 mL of acetonitrile followed by centrifugation at 3000 g for 10 min. The supernatant was decanted to a new tube and evaporated to dryness under a stream of N₂ at 35 °C. The resulting residue was reconstituted in 30% acetonitrile containing 0.1% formic acid (0.200 mL), vortex mixed and centrifuged. Aliquots (0.035 mL) of the final reconstituted mixture were analyzed by HPLC-MS as described below.

**Kinetic Experiments**

The rates of formation of each metabolite were determined by incubating ticlopidine (1 μM) with either reconstituted CYP2B6 or reconstituted CYP2B4 (25 pmol/mL) in 100 mM phosphate buffer (pH 7.4) containing MgCl₂ (10 mM). The reconstituted system contained a 1:1 molar ratio of protein (CYP2B6 or CYP2B4) and cytochrome P450 reductase (CPR). The reactions were initiated by the addition of NADPH (1 mM) to the incubation mixture that had been preincubated for 1 min. Following addition, 0.100 mL aliquots of the mixture were drawn at 1, 2, 3, 4 and 5 min and added to tubes containing a solution of buspirone in acetonitrile (50 nM),
which was used as an internal standard. A 0.015 mL aliquot of the supernatant obtained following centrifugation of the protein was injected directly onto the HPLC-MS for determination of the peak areas. All experiments were conducted in duplicate.

**HPLC-MS Analysis**

*Metabolite Identification:* Metabolites in the CYP2B4 and CYP2B6 mediated incubation mixtures were separated on a Luna C8 (2) 100A column (3.0 μm, 150 x 2.0 mm, Phenomenex, Torrance, CA) at ambient temperature. The mobile phase consisted of 0.1% formic acid (Solvent A) and acetonitrile (Solvent B) and was delivered at 0.200 mL/min for 50 min. The initial composition of solvent B was maintained at 1% for 5 min and then increased in a linear manner as follows: 30% at 25 min; 50% at 35 min and 90% at 40 min. Solvent B was then maintained at 90% for up to 45 min and then decreased to 1% in the next 2 min. The column was allowed to equilibrate at 1% solvent B for 5 min prior to the next injection. The HPLC effluent going to the mass spectrometer was directed to waste through a divert valve for the initial 5 min after sample injection. Mass spectrometric analyses were performed on a ThermoFinnigan LTQ ion trap mass spectrometer (ThermoScientific; Waltham MA), which was interfaced to an Agilent HP-1100 HPLC system (Agilent Technologies, Palo Alto, CA) and equipped with an electrospray ionization source (ESI). The parameters for the ESI source were: capillary temperature 350 °C; spray voltage 4.5 kV; capillary voltage 39.0 V; sheath gas flow rate 30 and auxiliary gas flow rate 5.0. The mass spectrometer was operated in a positive ion mode with data-dependent scanning. The ions were monitored over a full mass range of m/z 100-1000. For a full scan, the automatic gain control was set at 5.0 x 10⁸, maximum ion time was 10 ms and the number of microscans was set at 1. For MSⁿ scanning, the automatic gain
control $1.0 \times 10^8$, maximum ion time was 100 ms and the number of microscans was set at 1. For data dependent scanning, the default charge-state was 1, default isolation width was 3.0 and the normalized collision energy was 35.0. The data obtained was analyzed using Xcalibur v2.1 software (ThermoScientific; Waltham MA).

**Kinetic Studies:** Metabolites were separated by HPLC system consisting of PAL autosampler (Leap Technologies, Carrboro, NC) and Accela pump (ThermoScientific; Waltham MA) which was connected to ThermoFinnigan LTQ Velos ion trap mass spectrometer equipped with an electrospray ionization source. Chromatography was performed by injecting 0.15 mL of the supernatant on a C18 column (3.5 μm, 150 x 2.0 mm, Phenomenex, Torrance, CA) at 8 °C. Elution was performed using a mobile phase of 0.1% formic acid (Solvent A) and acetonitrile (Solvent B) which was delivered at 0.400 mL/min for 12 min. The initial composition of solvent B was maintained at 10 % and then increased as follows: 20% at 7.25 min; 30% at 8.5 min and 90% at 8.6 min. It was then maintained at 90% for 1 min and then decreased to 10 % for the next 0.4 min. The column was allowed to equilibrate at 10 % solvent B for 2 min prior to the next injection. The HPLC effluent going to the mass spectrometer was directed to waste through a divert valve for the initial 1.25 min after sample injection. The mass spectrometer was operated in a positive ion mode. The parameters for the ESI source were: capillary temperature 300 °C; sheath and auxiliary gas flow rate were maintained at 50 and 18, respectively. The source voltage and source current was 3.0 kV and 100 μA. The normalized collision energy for MS/MS was 35.0%. The metabolites were detected by single reaction monitoring (SRM) mode. The transitions used were $m/z\ 280 \rightarrow 125$ (for 2-oxoticlopidine M2, at retention time of 4.9), $m/z\ 280 \rightarrow 262$ (for hydroxyl ticlopidine M1, at retention time of 4.56 min), $m/z\ 280 \rightarrow 171$ (for ticlopidine N-oxide M5, at retention time of 8.0 min), $m/z\ 559 \rightarrow 511$ (for TSOD M6, at
retention time of 10.3 min) and $m/z$ 386 $\rightarrow$ 122 (for buspirone at retention time of 9.7 min). Since the authentic standards of the metabolites were not available, the rate of formation each metabolite was estimated from the plot of metabolite/buspirone peak area ratio versus time. Since the amount of product formed over time was non-linear, it was fitted to a first order rate equation (Equ. 1 and 2) using Sigma Plot 11 software (Khan et al., 2002).

$$\ln \left(\frac{1-r}{R_{max}}\right) = -k_{obs}.t \hspace{50pt} (1)$$

$$r = R_{max} \left(1-e^{-k_{obs}t}\right) \hspace{50pt} (2)$$

where, $r$ and $R_{max}$ are amplitude of the product formation at a particular time $t$, and at infinity, and $k_{obs}$ is the observed rate constant.

**Isolation of Metabolites M1, M2, M5 and M6 and NMR Analysis:**

Ticlopidine metabolites were generated by the method described above except that the final volume of incubation was 60 mL. The metabolites were separated using an Agilent HP-1100 HPLC system (Agilent Technologies, Palo Alto, CA) and an Aqua C18, 125A column (5 µM, 10 x 250 mm, Phenomenex) and eluted using a linear gradient starting with acetonitrile at 6% and 0.1% formic acid (94%) and ramped to 70% acetonitrile over 50 min at a flow rate of 4 mL/min. Fractions were collected every 1 min throughout the run. All fractions were analyzed by mass spectrometry using the conditions described previously, and the fractions containing the same metabolite were pooled. The four pooled fractions were re-fractionated following HPLC separation using a Luna C8 column (4.6 x 250 mm; Phenomenex) under similar gradient conditions described above but at a flow rate of 1 mL/min. Fractions were collected every 30 s throughout the run, and those containing the metabolite were collected, combined, and
evaporated under N$_2$ using a SpeedVac concentrator (Thermo Savant, Holbrook, NY). The resulting residue was reconstituted in 0.150 mL of deuterated dimethyl sulfoxide (DMSO-d$_6$, Cambridge Isotope Laboratories, Andover, MA) and placed in 3 mm diameter tubes for NMR experiments and a sample (0.010 mL) was analyzed by LC-MS/MS to examine its purity.

NMR spectra were recorded on a Bruker Avance 600 MHz system controlled by TOPSPIN V2.1, equipped with a 5 mm TCI cryoprobe (Bruker, Rheinstetten, Germany). 1D NMR spectra were recorded using a sweep width of 12000 Hz and a total recycle time of 7.2 s. The resulting time-averaged free induction decays were transformed using an exponential line broadening of 1.0 Hz to enhance signal to noise. All spectra were referenced using residual dimethyl sulfoxide-d$_6$ ($^1$H $\delta$=2.5 ppm relative to TMS, $\delta$=0.00 and $^{13}$C $\delta$=39.5 ppm relative to TMS, $\delta$=0.00). Phasing, baseline correction and integration were all performed manually. If needed, the BIAS- and SLOPE-functions for the integral calculation were adjusted manually. COSY, multiplicity edited HSQC and HMBC data were recorded using the standard pulse sequence provided by Bruker. 2D experiments and were typically acquired using a 1K x 128 data with 16 dummy scans and a spectral width of 8000 Hz in the f2 dimension. The data was zero-filled to a size of 1K X 1K. The above process was repeated for each of the metabolites.

**Mechanism-based inactivation of CYP2B6 and CYP2B4 by Ticlopidine using Bupropion and 7-EFC as Substrates**

Assays were carried out according to Richter et al (Richter et al., 2004). The reconstituted system for the assay contained a 1:1 molar ratio of protein (CYP2B6 or CYP2B4) and cytochrome P450 reductase (CPR). The mixture was preincubated for 10 min at room temperature. Ticlopidine (100 $\mu$M) dissolved in methanol was added to the reconstituted protein
system in 100 mM potassium phosphate buffer (pH 7.4). The protein-ticlopidine mixture was further incubated for 2 min at 37°C, and the reaction was initiated by addition of NADPH (1 mM final concentration). A final concentration of 5 pmol enzyme preparation in 0.200 mL was used in all experiments. Aliquots (0.025 mL) of this mixture were withdrawn at 0, 1, 2, 3, 4 and 5 min and added to a secondary reaction mixture (0.225 mL) containing 100 mM potassium phosphate buffer (pH 7.4), 500 μM bupropion, and 1 mM NADPH prewarmed to 37 °C. After 10 min, the reactions were quenched with 0.050 mL of a 1:1 (vol/vol mixture of 2N HCl and buspirone solution (50 nM) in acetonitrile.

The samples were centrifuged at 13800g for 15 min, and an aliquot of the supernatant was analyzed by HPLC-MS for the residual hydroxybupropion peak. The conditions and the parameters for LC-MS analysis of hydroxybupropion were similar to those described in the kinetic studies section (described above) except that the metabolite was monitored in SRM mode using a transition of $m/z$ 256 → 238. For studies using 7-EFC (100 μM) as a secondary substrate, the enzyme reconstitution and preincubation conditions were essentially the same as described above. The reaction was arrested after 10 min by the addition of 20% trichloroacetic acid (TCA) followed by dilution to 1 mL with 0.1 M Tris-HCl pH 8. The concentration of the product 7-hydroxy-4-(trifluoromethyl) coumarin was measured by a fluorescence assay as previously reported (Kumar et al., 2005).
Results

Metabolism of Ticlopidine by CYP2B4 and CYP2B6

Since the metabolites formed by CYP2B6 and CYP2B4 were unknown, incubations for metabolite identification were conducted at ticlopidine concentrations of 10 μM so that all possible metabolites produced \textit{in vitro} could be detected. Reconstituted CYP2B6 and CYP2B4 showed six major peaks in the full scan chromatograms, including unchanged ticlopidine (Fig. 2). The peak at 24.5 min was a solvent related impurity and was present in the control samples (incubation without NADPH). All other peaks were drug related, since they were absent in experiments that lacked NADPH. In addition, all these peaks revealed an isotopic protonated molecular ion (MH\(^+\)) due to \(^{37}\text{Cl}\) isotope, suggesting that these were ticlopidine related products. The product profile obtained from incubations with reconstituted CYP2B6 and CYP2B4 was similar to that obtained following incubation of ticlopidine with human liver microsomes or recombinant full length CYP2B6 expressed in baculovirus infected insect cells (data not shown).

Unchanged ticlopidine eluted at 22 min and gave a protonated molecular ions (MH\(^+\)) of \(m/z\) 264 (\(^{35}\text{Cl}\)) and 266 (\(^{37}\text{Cl}\)) in the metabolic profiles that were generated following CYP2B4 and CYP2B6 incubations (Fig. 2). The mass spectrum of MH\(^+\) ion at \(m/z\) 264 gave fragment ions at \(m/z\) 154 and 125, while the corresponding mass spectrum of MH\(^+\) at \(m/z\) 266 gave fragment ions at \(m/z\) 156 and 127 (Table 1). These ions were most likely due to the chlorobenzyl group (fragment \(a\) in Table 1) and chlorobenzyl methyliminium moiety in ticlopidine (fragment \(b\) in Table 1). The peaks at 19.5, 20.8, and 23 min gave a molecular ion at \(m/z\) 280, an addition of 16 amu to the molecular ion, suggesting hydroxylation of ticlopidine. The mass spectrum...
(MS²) of hydroxyticlopidine (M1) at m/z 280 showed a major fragment ion at m/z 262 and a minor fragment ion at m/z 154 (Table 1). Further fragmentation of the ion m/z 262 in a data dependent manner (MS³) resulted in a major fragment ion at m/z 125. The ion at m/z 262 resulted from loss of a water molecule from m/z 280, suggesting that the position of hydroxylation in this metabolite was possibly on the tetrahydropyridine portion of ticlopidine. The ions at m/z 154 in the MS² spectrum and m/z 125 in the MS³ spectrum were similar to those observed in the mass spectrum of ticlopidine (Table 1). The MS² and MS³ mass spectra obtained from on MH⁺ at m/z 282 that contained 37Cl isotope showed fragment ions at m/z 264, 156 and 127, further indicating this product was hydroxylated on the tetrhydropyridine ring of ticlopidine.

For 2-oxoticlopidine (M2), the mass spectrum of MH⁺ at m/z 280 and 282 resulted in one major fragment ion at m/z 125 and 127, respectively. This addition of 16 amu indicated insertion of oxygen into the molecule, but the lack of an ion resulting from loss of a water molecule in the mass spectra suggested that either the chlorobenzyl group or the thiophene ring was modified. Modification of the chlorobenzyl group was ruled out by the presence of a fragment ion at m/z 125 (or 127) in the mass spectrum of M2. Thus, the thiophene moiety was the most likely site of modification (Table 1). Although the exact position of hydroxylation on the thiophene ring could not be ascertained from this data, the metabolite was consistent with the structure of hydroxyticlopidine that was reported previously (Dalvie and O’Connell, 2004; Shimizu et al., 2009).

The mass spectrum of ticlopidine N-oxide (M5) at m/z 280 gave fragment ions at m/z 262, 235, 170, 138, and 125. The fragment ions at m/z 262 and 125 and the presence of corresponding isotopic ions at m/z 264 and 127 in the mass spectrum of m/z 282 were similar to
those observed in the mass spectrum of hydroxyticlopidine (M1), suggesting modification of carbons or the nitrogen atom of the tetrahydropyridine ring. The fragment ions at m/z 235 and 170 in the mass spectrum of m/z 280 ion and the corresponding ions at m/z 237 and 172 in the mass spectrum of m/z 282 suggested that the nitrogen or the carbon atom at the 4 position of the tetrahydropyridine ring was modified. The structures of these metabolites were confirmed by NMR following isolation of these compounds (Table 2; supplemental Fig. S1). The peak at 20.8 min also showed two additional drug-related MH+ at m/z 262 and 260 corresponding to dihydrothienopyridinium (M3) and thienopyridinium (M4) metabolites (Fig. 2) that were 2 and 4 amu lower than the molecular ion of ticlopidine, respectively. The molecular ions and the fragments observed in the mass spectra of these two metabolites were consistent to that originally characterized (Table 1) (Dalvie and O’Connell, 2004).

The peak TSOD (M6) eluting at 26.7 min in the chromatogram gave MH+ at m/z 559. This was similar to the molecular ion of a ticlopidine S-oxide dimer (TSOD), originally proposed by Ha-Doung and co-workers (Ha-Duong et al., 2001). The peak also showed a corresponding molecular ion at m/z 561, which was ~70% of the molecular ion at m/z 559 indicating the presence of two chlorine atoms. The mass spectrum of m/z 559 showed major fragment ions at m/z 511 and 509 in the MS² spectra (Fig. 3A). The data dependent mass spectrum (MS³) of the fragment ion at m/z 511 resulted in fragment ions at m/z 493, 358, 340 and 306 (Fig. 3B). The ion 1 at m/z 511 indicated a loss 48 amu (loss of SO) from m/z 559 while the ion at m/z 509 indicated a loss of 50 amu and was formed following aromatization (2) (Fig. 3C). As shown, fragment ion 3 at m/z 493 was possibly formed following a loss of water from m/z 511 or a loss of 16 amu from m/z 509. The latter is characteristic of N-oxides (Dalvie and O’Connell, 2004) or S-oxides (Dansette et al., 2009), which loose 16 or 17 amu. The fragment ions at m/z 511 and
493 yielded the corresponding ions 4 and 5 at m/z 358 and 340 indicating loss of the 153 amu corresponding to a chlorobenzylmethylimine moiety. Finally, the fragment ion 6 at m/z 306 was the result of loss of 52 amu from m/z 358 (Fig. 3C). The mass spectrum obtained from the molecular ion that contained the 37Cl isotope (m/z 561) showed ions corresponding to fragments 1-6 that were 2 amu heavier (data not shown). This mass spectral interpretation supported the structure of TSOD. Further structure confirmation of TSOD was obtained from its NMR spectrum following isolation of the metabolite (Table 3; supplemental Fig. S1). The positional isomers of TSOD could not be differentiated from either MS or the NMR spectra. The presence of TSOD in the incubation mixtures indicated the likelihood of formation of ticlopidine S-oxide by CYP2B6 and CYP2B4. This inference was based on previous reports which indicated that the unstable ticlopidine S-oxide can readily dimerize via a cycloaddition reaction (Ha-Duong et al., 2001).

Kinetic Studies

Rates of formation of all ticlopidine metabolites observed in reactions with CYP2B4 or CYP2B6 were determined to make a quantitative comparison of the metabolism profiles of the enzymes. Quantitative incubations were performed using ticlopidine concentrations of 1 µM. Since the synthetic standards of the metabolites were not available, the ratio of the peak area of each metabolite to the peak area of the buspirone peak used as internal standard, termed peak area ratio was used to determine the kinetic constants for each product. Fig. 4 shows the peak area ratio versus time profile of each metabolite. All metabolites were formed in a non-linear fashion by both enzymes.
The parameters $R_{\text{max}}$ and $k_{\text{obs}}$ were calculated by fitting the experimental data to equation Eq.2. The rate of product formation decreased as a function of time (t), consistent with enzyme inactivation. Thus, the $k_{\text{obs}}$ obtained by this fit would be equal to the rate constant for enzyme inactivation, as shown previously with CYP3A4 and midazolam (Khan et al., 2002). For both CYP2B4 and CYP2B6, $k_{\text{obs}}$ for all metabolites (M1 through M6) ranged from 0.4 - 0.6 min$^{-1}$ (Table 2) and were comparable to inactivation constants obtained in previous studies with ticlopidine (Richter et al., 2004; Nishiya et al., 2009). The $R_{\text{max}}$ values for M1 and M6 were ~ 4 and 5 fold greater in incubations with CYP2B6 than those catalyzed by CYP2B4 (Table 4). In contrast, CYP2B4 formed ticlopidine $N$-oxide (M5) in 7-fold greater amounts than CYP2B6. Even though the thienopyridinium metabolite (M4) was detected in incubations containing CYP2B6 and CYP2B4, its $k_{\text{obs}}$ and $R_{\text{max}}$ could only be estimated in incubations containing CYP2B4 (Table 4). Since the dihydropyridinium metabolite (M3) is unstable and can be auto-oxidized to the thienopyridinium metabolite (M4) (Dalvie and O'Connell, 2004), its formation in the two incubations could not be measured accurately.

**Inactivation of CYP2B4 and CYP2B6 by Ticlopidine**

To assess inactivation of the enzymes directly, preincubations were carried out at a single concentration of ticlopidine (1 µM) and residual activity was measured using bupropion or 7-EFC (Talakad et al., 2010). Enzyme inactivation followed pseudo–first order kinetics, and the $k_{\text{inact}}$ was derived from the negative slope of the lines. The $k_{\text{inact}}$ values for CYP2B4 and CYP2B6 when bupropion was used as a substrate were 0.08 and 0.32 min$^{-1}$, respectively (Table 5). Relatively lower $k_{\text{inact}}$ values of 0.02 min$^{-1}$ for CYP2B4 and 0.1 min$^{-1}$ for CYP2B6 were observed with 7-EFC as the substrate.
Discussion

This study establishes for the first time the specific metabolites of ticlopidine produced by CYP2B6 and CYP2B4, correlates the products with predictions from X-ray crystallography, modeling, and NMR relaxation studies, and proposes chemical mechanisms for metabolite formation. The results presented here indicated that reconstituted CYP2B6 and CYP2B4 generated multiple and similar metabolites but in different ratios. Figure 5 depicts the metabolites of ticlopidine formed by these two enzymes. As shown, the major metabolic sites of ticlopidine metabolism were the tetrahydropyridine and the thiophene rings. The modification of the tetrahydropyridine ring yielded 7-hydroxyticlopidine (M1), dihydrothienopyridinium metabolite (M3), thienopyridinium metabolite (M4), and ticlopidine N-oxide (M5) (Fig. 5), while the thiophene ring was oxidized to 2-oxoticlopidine (M2) and TSOD (M6), a dimerization product of ticlopidine S-oxide (Fig. 5). The latter result also suggested that both CYP2B6 and CYP2B4 were capable of forming ticlopidine S-oxide like CYP2C19 (Ha-Duong et al., 2001).

Given the similarity in the overall metabolites produced, kinetic studies were conducted to assess the differences in the rates of each metabolite formation by the two CYPs. In all cases, time courses of product formation were non-linear (Fig. 4), which is consistent with previous studies that demonstrated inactivation of CYP2B6 by ticlopidine (Richter et al., 2004). Therefore, the data were fit to an exponential equation, from which the observed rate constants ($k_{obs}$) for formation of each metabolite and the amplitude ($R_{max}$) of each metabolite formed by these two reconstituted enzymes systems were derived. The $k_{obs}$ values were the same for all products and both enzymes, whereas examination of $R_{max}$ revealed differences in the abundances of M1, M4, M5 and M6 (Table 4). A 5-fold higher $R_{max}$ of M6 formation by CYP2B6 than
CYP2B4 (Table 4) was consistent with the increased residence time of ticlopidine in an orientation in which the thiophene ring was closer to the heme moiety in the CYP2B6 active site, as proposed by prior docking studies (Richter et al., 2004; Gay et al., 2010a). This inference was supported by the fact that M6 is a measure of ticlopidine S-oxide formation (Ha-Duong et al., 2001), which in turn is formed via oxidation of the sulfur atom in the thiophene ring (Fig. 6). The S-oxidation step is only feasible if the sulfur atom of the thiophene ring is in close proximity to the activated oxygen species in the active site.

In addition to M6, the \( R_{\text{max}} \) for hydroxyticlopidine (M1) formation also showed a four-fold difference between CYP2B6 and CYP2B4 (Table 4). A most accepted mechanism for conventional hydrocarbon hydroxylation generally involves a hydrogen atom abstraction/oxygen rebound mechanism (Fig. 6, Pathway A). Thus, formation of M1 implied that one of the binding modes in the CYP2B6 active site brings the carbon atom vicinal to the thiophene ring in close proximity of the heme iron. However, an alternative mechanism for the formation M1 could also be envisioned (Fig. 6, Pathway B). This pathway by P450 could involve initial abstraction of an electron from the thiophene sulfur atom to yield a thienyl radical cation. Subsequent rearrangement of the thienyl radical cation via deprotonation from the carbon atom vicinal to the thiophene ring could produce a carbon radical (A). This radical intermediate could then combine with the iron bound oxygen to yield the corresponding alcohol M1. Thus, this mechanism, which is analogous to reactions involving oxidation of nitrogen containing compounds, can lead to the formation of ticlopidine S-oxide or M1 (Fig. 6), depending upon the differential partitioning of the thienyl radical cation intermediate. This result therefore supports the orientation in which the thiophene ring is closer to the heme in the active site of CYP2B6.
In contrast, $R_{max}$ for the formation of M5 by CYP2B4 was 7-fold higher than CYP2B6, and M4 was only measurable in the CYP2B4 mediated incubations. These findings suggest that one of the productive binding modes of ticlopidine in the CYP2B4 active site was an orientation in which the tetrahydropyridine ring was in close proximity to the heme. As shown in Fig. 7, one possible mechanism for formation of M4 and M5 involves oxidation of the nitrogen atom to a cation radical, which could collapse to form an N-oxide (Fig. 7, Pathway A) or yield M4 (Fig. 7, Pathway B). Alternatively, carbon hydroxylation could occur by an independent hydrogen atom abstraction/oxygen rebound pathway (Fig. 7, Pathway C). In either case, this would require the nitrogen atom or its vicinal carbon to be in proximity to the activated oxygen species in the active site of CYP2B4.

As noted previously, incubations of ticlopidine with reconstituted CYP2B4 also revealed the formation of 2-oxoticlopidine (M2) and hydroxyticlopidine (M1). While $R_{max}$ of M1 formation by CYP2B4 was four-fold less than CYP2B6, the $R_{max}$ for M2 formation by the two enzymes was almost similar (0.72 and 0.94 for CYP2B4 and CYP2B6, respectively, Table 4). These results suggested that ticlopidine could also occupy the active site of CYP2B4 in a binding mode that is similar to the one described for CYP2B6, with the thiophene ring positioned down towards the heme. The results and the formation of M6 were also consistent with the previous studies, which suggested that the thiophene ring could transiently interact with the heme in the CYP2B4 active site (Gay et al., 2010b).

Interestingly, there was no indication of hydroxylation of the chlorophenyl ring by the CYP2B4. This suggested that even though ticlopidine forms a stable complex with CYP2B4 in an orientation that has its chlorophenyl ring in close proximity to the heme, this is not a
catalytically active binding mode. It is possible that in the presence of NADPH-cytochrome P450 reductase and/or following reduction of CYP2B4, ticlopidine reorients within the active site 2B4 to a more dynamic mode that brings reactive sites like the nitrogen atom of the tetrahydropyridine ring or the thiophene ring in close proximity to the activated oxygen species. Similar assumptions have been made on the binding of nicotine to P450cam (Strickler et al., 2003). Despite models that explain the distribution of monooxygenation products of nicotine, the crystal structure of the complex has indicated that the primary binding mode of nicotine is unproductive. Alternatively, the lack of oxidation products of the chlorophenyl ring of ticlopidine maybe attributed to low reactivity. Accordingly, an assessment of reactivity of ticlopidine molecule using local reactivity descriptors such as the Fukui functions (Beck, 2005) suggested that the chlorophenyl ring was a less reactive group than other atoms and functionalities (data not shown).

As mentioned in the Results section, due to observation of non-linear behavior of metabolite formation in the kinetic studies, a direct assessment of the inactivation of CYP2B4 and CYP2B6 was also conducted using bupropion and 7-EFC as substrates. Interestingly the $k_{\text{inact}}$ determined using bupropion and especially 7-EFC as the secondary substrate was found to be less than the $k_{\text{inact}}$ inferred from the time course of ticlopidine oxidation. The obtained results are consistent with an earlier report that showed differential impairment of the catalytic activity of 4-(tert-butyl)-phenylacetylene modified CYP2B4 depending on whether the secondary substrate was 7-EFC, benzphetamine, or testosterone (Zhang et al., 2009). The observed variation in the $k_{\text{inact}}$ may be due to the size or geometry of the secondary substrate in the active site. Since, the molar volumes (http://www.molinspiration.com/) of ticlopidine, 7-EFC, and bupropion are 228.8 Å$^3$, 221.7 Å$^3$ and 228.5 Å$^3$ respectively, the variation in the $k_{\text{inact}}$ is likely due
to the orientation or geometry of the secondary substrate bupropion or 7-EFC in the active site. In addition, the $k_{\text{inact}}$ values for CYP2B4 with the same substrates were ~4- and ~5-fold lower than CYP2B6, respectively. This might be related to the larger active site cavity (582 vs 253 Å) of CYP2B6 compared with CYP2B4 (Gay et al., 2010c).

In conclusion, the results from these studies are in line with the involvement of multiple binding modes of ticlopidine in CYP2B6 and CYP2B4. The data presented herein suggest that even though the experimental metabolism results correlated with the proposed binding mode of ticlopidine in the CYP2B6 active site, a major binding mode exhibited by X-ray, docking, or NMR studies for the CYP2B4-ticlopidine complex was non-productive. The study also infers that a single stable enzyme-substrate complex shown by X-ray, docking or NMR may not provide a good description of the oxidation sites and productive binding mode of the molecule. Other factors such as conformational or orientation changes following reductase binding and/or reduction of the heme iron, as well as the reactivity of the atoms in proximity to the activated oxygen species undoubtedly play a role in determining the ultimate products. This is in agreement with the results from previous studies (Oliver et al., 1997; Strickler et al., 2003). Finally, this study demonstrates the value of *in silico* tools in providing useful insight into predictive drug metabolism and in allowing elucidation of important molecular interactions between the substrate and the active site. However, these predictions can be even more valuable in enhancing the pharmacokinetic profiles and reduce toxicities of new candidates in early drug design when used in conjunction with mechanistic biotransformation studies.
References


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Zhang H, Lin H-L, Walker VJ, Hamdane D, and Hollenberg PF (2009) tert-Butylphenylacetylene is a potent mechanism-based inactivator of cytochrome P450 2B4:


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Authorship Contributions

*Participated in research design:* Halpert and Dalvie

*Conducted experiments:* Talakad, Shah, Xiang, Walker and Dalvie

*Performed data analysis:* Talakad and Dalvie

*Wrote or contributed to the writing of the manuscript:* Talakad, Shah, Halpert, and Dalvie

*Other:* Halpert acquired funding for the research
Footnotes

This work was supported by National Institutes of Health grants to James R. Halpert [ES003619].
Legends to Figure

Figure 1. Ribbon-and-stick diagram of CYP2B4-ticlopidine complex (3KW4) showing the two different orientations of ticlopidine in the active site. The heme moiety is shown in red, whereas ticlopidine is in cyan. A. thiophene ring in close proximity to the heme; B. chlorophenyl group in close proximity to the heme.

Figure 2. HPLC-MS chromatograms of mixtures following incubation of ticlopidine (10 μM) with NADPH supplemented CYP2B6 (A) and CYP2B4 (B) for 60 min. The metabolite peaks are represented as M1-M6 and the structures are shown in Fig. 1. The preparation and the experimental conditions of sample preparation are described under Materials and Methods. All the incubations contained 100 pmol of reconstituted P450.

Figure 3. Mass spectra of TSOD (M6) in a positive ion mode. A: MS/MS spectrum of m/z 559 ion (MH+ containing 35Cl isotope. B: Mass spectrum of m/z 559 ion (MS3) obtained in a data dependent mode following fragmentation of m/z 511 ion. C: Structures of fragment ions observed in the MS/MS and MS3 spectra of m/z 559.

The proposed structure of only one isomer is shown. The positional isomers of TSOD (Figure 5) could not be differentiated from the mass spectral fragment ions.

Figure 4. Kinetics of formation of ticlopidine metabolites by CYP2B6 and CYP2B4. The details of the time dependent metabolite formation by CYP2B6 and CYP2B4 are described under Materials and Methods. The solid lines through the experimental data points show the fit to the first-order exponential rate equation, \( r = R_{\text{max}} (1 - \exp(-k_{\text{obs}} t)) \). The fitting was done using Sigma Plot 11 as described in Materials and Methods.
Figure A-E represents the plot of Peak area /IS area vs Time. In particular, A: hydroxyticlopidine (M1); B: 2-oxoticlopidine (M2); C: thienopyridinium metabolite (M4); D: ticlopidine N-oxide (M5); E: TSOD (M6); (■) CYP2B6; (○) CYP2B4. All experiments were run in duplicate.

Figure 5. Metabolic scheme of ticlopidine following incubations with reconstituted CYP2B4 and CYP2B6.

The positional isomers of TSOD could not be differentiated from MS and NMR data. The metabolites detected were similar to those observed in incubations with human liver and CYP2B6 Supersomes from Gentest.

Figure 6. Mechanism for formation of hydroxyticlopidine (M1) and TSOD (M6) by CYP2B6.

Figure 7. Mechanism for formation of thienopyridinium metabolite (M4) and ticlopidine N-oxide (M5) by CYP2B4.
Table 1. Molecular ions ($^{35}$Cl or $^{37}$Cl) and mass spectral fragment ions of ticlopidine and its metabolites following incubation of ticlopidine with CYP2B4 and CYP2B6.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>Structure</th>
<th>Molecular Ions</th>
<th>Fragment Ions</th>
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<td></td>
<td>MH$^+$</td>
<td>a  b  c  d  e</td>
</tr>
<tr>
<td></td>
<td></td>
<td>264</td>
<td>125 154</td>
</tr>
<tr>
<td></td>
<td></td>
<td>266</td>
<td>127 156</td>
</tr>
<tr>
<td>M1 Hydroxyticlopidine</td>
<td></td>
<td>280</td>
<td>125 154 262</td>
</tr>
<tr>
<td></td>
<td></td>
<td>282</td>
<td>127 156 264</td>
</tr>
<tr>
<td>M2 2-Oxoticlopidine</td>
<td></td>
<td>280</td>
<td>125 154</td>
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<td></td>
<td></td>
<td>282</td>
<td>125 154</td>
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<tr>
<td>M3 Dihydropyridinium Metabolite</td>
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<tr>
<td></td>
<td></td>
<td>262</td>
<td>127</td>
</tr>
<tr>
<td>M5 Ticlopidine N-oxide</td>
<td></td>
<td>280</td>
<td>125 170 262 235 138</td>
</tr>
<tr>
<td></td>
<td></td>
<td>282</td>
<td>127 172 264 237 138</td>
</tr>
<tr>
<td>M6$^1$ TSOD</td>
<td>A OR</td>
<td>559</td>
<td></td>
</tr>
</tbody>
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$^1$ The positional isomers of TSOD could not be differentiated by the MS spectrum.
Table 2. \(^1\)H and \(^13\)C NMR (\(\delta\)) chemical shift assignments of ticlopidine, hydroxyticlopidine (M1), 2-oxoticlopidine (M2), and ticlopidine N-oxide (M5).

<table>
<thead>
<tr>
<th>Position</th>
<th>Chemical Shifts</th>
<th>1H ((\delta))</th>
<th>J (Hz)</th>
<th>13C ((\delta))</th>
<th>Chemical Shifts</th>
<th>1H ((\delta))</th>
<th>J (Hz)</th>
<th>13C ((\delta))</th>
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<th>1H ((\delta))</th>
<th>J (Hz)</th>
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<tr>
<td>2</td>
<td></td>
<td>7.45 (d, 1H)</td>
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<td>124.7</td>
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<td>131.6</td>
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s, singlet; bs, broad singlet; d, doublet; bd, broad doublet; dd, doublet of doublets; m, multiplet; t, triplet.

The \(^13\)C chemical shifts for positions 13, 14, 15 and 16 were not assigned.

\(^13\)C NMR for ticlopidine N-oxide (M5) was not assigned due to insufficient sample.
NMR spectra shown in the supplemental Fig. S1.
Table 3. $^1$H and $^{13}$C NMR ($\delta$) chemical shift assignments of TSOD (M6).

<table>
<thead>
<tr>
<th>Chemical Shifts</th>
<th>$^1$H (δ)</th>
<th>$^1$J (Hz)</th>
<th>$^{13}$C (δ)</th>
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<td>60.4</td>
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The positional isomers of TSOD could not be differentiated by NMR data.  
d, doublet; dd, doublet of doublets; m, multiplet.  
$^{13}$C chemical shifts for positions 24 to 30 were not assigned.  
NMR spectrum shown in supplemental Fig. S1.
Table 4. Observed rate constants ($k_{obs}$) and amplitude of product formation ($R_{max}$) for ticlopidine metabolites; hydroxyticlopidine (M1), 2-oxoticlopidine (M2), thienopyridinium metabolite (M4), ticlopidine $N$-oxide (M5) and TSOD (M6) following incubation of ticlopidine with CYP2B4 and CYP2B6.

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>CYP2B4</th>
<th>CYP2B6</th>
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<tbody>
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<td>$k_{obs}$</td>
<td>$R_{max}$</td>
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<tr>
<td>Hydroxyticlopidine (M1)</td>
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<td>0.74</td>
</tr>
<tr>
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</tr>
<tr>
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<td>1.00</td>
</tr>
<tr>
<td>Ticlopidine $N$-oxide (M5)</td>
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<td>1.02</td>
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<tr>
<td>TSOD (M6)</td>
<td>0.42</td>
<td>0.23</td>
</tr>
</tbody>
</table>

ND = Not determined since a trace of M4 was observed during analysis of the samples at each sampling time.
Table 5. Inactivation rate constants ($k_{inact}$) of ticlopidine, bupropion and 7-ethoxy-4-trifluoromethylcoumarin for CYP2B4 and CYP2B6.

<table>
<thead>
<tr>
<th>Compounds</th>
<th>CYP2B4</th>
<th>CYP2B6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$k_{inact}$ min$^{-1}$</td>
<td>$k_{inact}$ min$^{-1}$</td>
</tr>
<tr>
<td>Ticlopidine</td>
<td>0.50$^1$</td>
<td>0.52$^1$</td>
</tr>
<tr>
<td>Bupropion</td>
<td>0.08</td>
<td>0.32</td>
</tr>
<tr>
<td>7-Ethoxy-4-trifluoromethylcoumarin</td>
<td>0.02</td>
<td>0.10</td>
</tr>
</tbody>
</table>

$^1$ Average of $k_{obs}$ determined by fitting the non-linear time courses of metabolite formation.
Figure 1
Figure 2
Figure 3A and 3B
Figure 3C
Figure 4
Figure 5
Figure 6
Ticlopidine

Pathway A

Cation Radical

Pathway B

[Fe=OH]^+3

Dihydrothienopyridinium Metabolite (M3)

Oxidation

Thienopyridinium Metabolite (M4)

Ticlopidine N-oxide (M5)

Pathway C

Fe=O^+3

Figure 7