Mechanism-based inhibition of human cytochrome P450 2B6 by ticlopidine, clopidogrel, and the thiolactone metabolite of prasugrel


Drug Metabolism and Pharmacokinetics Research Laboratories, Daiichi Sankyo Co., Ltd., Tokyo, Japan (Y.N., K.H., T.I., M.T., S.M., and A.K.); and Department of Drug Disposition, Lilly Research Labs, Eli Lilly and Company, Indianapolis, Indiana (N.A.F.); and Association for Promoting Drug Development, Tokyo, Japan (T.I.)
**Running Title:** Mechanism-based inhibition of human CYP2B6 by antiplatelet agents

**Address correspondence to:**

Yumi Nishiya  
Drug Metabolism and Pharmacokinetics Research Laboratories, Daiichi Sankyo Co., LTD., 1-2-58 Hiromachi, Shinagawa-Ku, Tokyo, 140-8710, Japan  
Tel: +81-3-3492-3131  
Fax: +81-3-5436-8567  
E-mail: nishiya.yumi.m4@daiichisankyo.co.jp

**Text Pages:** 24  
**Table:** 1  
**Figures:** 2  
**References:** 30

**Abstract:** 147 words  
**Introduction:** 808 words  
**Discussion:** 557 words

**Abbreviations:** cytochrome P450 (CYP)
ABSTRACT

Mechanism-based inhibition of CYP2B6 in human liver microsomes by thienopyridine antiplatelet agents ticlopidine and clopidogrel and the thiolactone metabolites of those two agents plus that of prasugrel were investigated by determining the time- and concentration-dependent inhibition of the activity of bupropion hydroxylase as the typical CYP2B6 activity. By comparing the ratios of $k_{\text{inact}}$ (maximal inactivation rate constant)/ $K_I$ (the inactivator concentration producing a half-maximal rate of inactivation), it was found that the thiolactone metabolite of prasugrel is 10- and 22-fold less potent, respectively, in the mechanism-based inhibition of CYP2B6 than ticlopidine and clopidogrel. The $k_{\text{inact}}/K_I$ ratio of the thiolactone metabolite of ticlopidine was comparable to that of the parent compound, whereas this ratio for the thiolactone metabolite of clopidogrel was significantly smaller than that of clopidogrel. In conclusion, ticlopidine, its thiolactone metabolite, and clopidogrel were more potent mechanism-based inhibitors of CYP2B6 than the thiolactone metabolite of prasugrel.
INTRODUCTION

The thienopyridine antiplatelet agents ticlopidine and clopidogrel (Figure 1), prevent thrombogenesis via blocking adenosine diphosphate (ADP)-dependent activation of platelets through the P2Y\textsubscript{12} receptor, one of the ADP receptors on platelets (Sharis et al. 1998). They have been widely used for the treatment and prevention of cerebrovascular and cardiovascular diseases. Clopidogrel appears to have a relatively faster onset of action and lower incidence of adverse effects such as neutropenia and thrombotic thrombocytopenic purpura compared to ticlopidine (Kam and Nethery 2003).

Prasugrel (Figure 1), a novel thienopyridine P2Y\textsubscript{12} antagonist, demonstrated more potent antiplatelet activity and faster onset than ticlopidine and clopidogrel in preclinical and/or clinical studies (Niitsu et al. 2005; Brandt et al. 2007), and superior efficacy to clopidogrel in patients with acute coronary syndrome undergoing percutaneous coronary intervention (Wiviott et al. 2007). Prasugrel, also a prodrug, is first hydrolyzed to a thiolactone metabolite (R-95913) which is then converted to the pharmacologically active metabolite (R-138727) through a single, CYP-mediated oxidation step (Figure 1) (Rehmel et al. 2006; Wiliams et al. 2008).
The thiolactone metabolites of ticlopidine and clopidogrel are produced by CYP-mediated oxidation (Yoneda et al. 2004, Kurihara et al. 2005), whereas R-95913 is produced by esterase-mediated hydrolysis of prasugrel (Williams et al. 2008). The hydrolysis of prasugrel is very rapid both in vitro and in vivo, such that it is not detected in human plasma even at early time points after oral administration (Farid et al. 2007). The thiolactones of each of these agents are converted to the pharmacologically active metabolites, each of which possesses a thiol group, by CYP-mediated oxidation (Yoneda et al. 2004; Kurihara et al. 2005, Rehmel et al. 2006). Because of the CYP-dependency of the metabolic pathways, ticlopidine, clopidogrel and the thiolactone metabolites of ticlopidine, clopidogrel and prasugrel could have the potential to cause a drug-drug interaction through the inhibition of CYPs. Indeed, several in vitro studies indicated that ticlopidine, clopidogrel, and their thiolactone metabolites are competitive inhibitors of several CYPs (Turpeinen et al. 2004; Ko et al. 2000; Hagihara et al. 2008). R-95913 was shown not to be an inhibitor of CYP1A2, CYP3A, CYP2C9, CYP2C19, and CYP2D6 at clinical doses ($K_i$ values ranged from 7.2 to 82 µM) (Rehmel et al 2006).

Ticlopidine and clopidogrel were shown to be strong, mechanism-based inhibitors of CYP2B6 (Richter et al. 2004) and CYP2C19 for ticlopidine (Ha-Duong et al. 2001) in vitro. Mechanism-based inhibition usually involves metabolic activation of the inhibitor by CYP to
a reactive intermediate, which can irreversibly modify the CYP protein. Thus, compared to reversible (e.g. competitive) inhibition, mechanism-based inhibition more frequently results in unfavorable drug-drug interactions since the interactions are sustained for the long time until the inactivated CYPs has to be replaced by newly synthesized protein (Kalgutkar et al. 2007). Because the pharmacologically active metabolites of thienopyridine antiplatelet agents have a thiol group that has been assumed to irreversibly bind to the target P2Y12 receptor on platelets through a disulfide bond formation (Ding et al. 2003), Richter et al. (2004) suggested that a possible mechanism for the irreversible inactivation of CYP2B6 by ticlopidine and clopidogrel would be the binding of the active metabolites to the CYP protein. Hagihara et al. (2008) reported that the active, thiol-containing metabolites themselves do not inhibit CYP2B6. Thus, the possibility was raised that either clopidogrel and ticlopidine, and/or their thiolactone metabolites produced by CYP2B6 might be involved in the mechanism-based inhibition. However, there is no information about the contribution of their thiolactone metabolites to the mechanism-based inhibition of CYP2B6 by ticlopidine and clopidogrel.

CYP2B6 represents about 6% of the hepatic cytochrome P450 pool (Ekins and Wrighton 1999; Stresser and Kupfer 1999) and demonstrates more than 100-fold interindividual variability in the activity (Lamba et al. 2003). In addition, genetic polymorphism in CYP2B6 was shown to result in decreased enzyme activity (Burger et al. 2006).
Bupropion, an antismoking and antidepressant drug, is extensively metabolized by several CYPs and carbonyl reductase to threohydrobupropion and erythrohydrobupropion in addition to hydroxybupropion (Jefferson et al. 2005). The metabolic pathway to hydroxybupropion is almost exclusively catalyzed by CYP2B6 (Faucette et al. 2000), so this pathway is utilized in drug-drug interactions studies examining CYP2B6. Recent clinical studies demonstrated that ticlopidine and clopidogrel are potent inhibitors of CYP2B6 in humans as they increased the exposure to bupropion by 85% and 60%, respectively, and decreased the exposure to hydroxybupropion by 84% and 52%, respectively (Turpeinen et al. 2005). However, prasugrel was found to be a weak inhibitor of CYP2B6 in humans since the exposure to bupropion increased by 18% and that of hydroxybupropion decreased by 23% (Farid et al. 2008).

To elucidate the differences observed clinically between ticlopidine, clopidogrel, and prasugrel in the extent of inhibition of CYP2B6, this study was performed to fully evaluate the ability of ticlopidine, clopidogrel, their thiolactone metabolites, and R-95913 to inhibit CYP2B6 and if any compound is a mechanism-based inhibitor of CYP2B6.
Materials and Methods

Materials. Clopidogrel (purity 99.2%), thiolactone metabolite of clopidogrel (2-oxo-clopidogrel, purity 92.9%), and thiolactone metabolite of prasugrel (R-95913, purity 99.4%) were synthesized at Ube Industries, Ltd. (Ube, Japan). Thiolactone metabolite of ticlopidine (2-oxo-ticlopidine, purity 99.3%) was synthesized at Daiichi Sankyo Co., Ltd. (Tokyo, Japan). Ticlopidine (purity 100%), β-nicotinamide adenine dinucleotide phosphate (β-NADP) sodium salt, D-glucose 6-phosphate (G-6-P) disodium salt hydrate and glucose-6-phosphate dehydrogenase (G-6-PDH) from baker's yeast were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Bupropion hydrochloride was purchased from MP Biochemicals, Inc. (Solon, OH, USA). Hydroxybupropion was purchased from Gentest Corporation (Woburn, MA, USA). Phenacetin was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Three pools of human liver microsomes (HLM), prepared by combining the liver microsomal fractions from 50 donors (20 mg protein/mL, Lot No. 0510077, 0710403 and 0810063), were purchased from XenoTech, LLC. (Lenexa, KS, USA). All other reagents and solvents were commercially available and of the highest purity.
Assays of time- and concentration-dependent inhibition of bupropion hydroxylase in human liver microsomes. The activity of bupropion hydroxylase was determined as a typical CYP2B6 activity (Faucette et al. 2000). The experiments were designed according to Richter et al. (2004), with minor modifications. A preincubation mixture contained 0.1 M potassium phosphate buffer (pH 7.4), HLM (0.4 mg-protein/mL) and varying concentrations of the test compounds (ticlopidine, clopidogrel, their thiolactone metabolites and R-95913) in a total volume of 225 µL. The concentration of the test compounds ranged from 0.05 µM to 1 µM for ticlopidine and thiolactone metabolite of ticlopidine, from 0.1 µM to 0.5 µM for clopidogrel, from 0.5 µM to 10 µM for the thiolactone metabolite of clopidogrel, and from 1 µM to 100 µM for R-95913. Control samples containing no test compound were prepared by the addition of solvent alone. To the preincubation mixture previously maintained at 37°C were added 25 µL of an NADPH generating system containing 2.5 mM β-NADP, 25 mM G-6-P, 0.5 units/mL G-6-PDH and 10 mM MgCl₂, and the mixture was incubated further for 0, 1, 2, 3 and 4 min (ticlopidine, the thiolactone metabolites of ticlopidine and clopidogrel, and R-95913) or 0, 0.5, 1, 1.5, 2 and 2.5 min (clopidogrel). At each time point, 25 µL-aliquot of the mixture was collected and added to an assay mixture for bupropion hydroxylase activity (225 µL), consisting of 0.1 M potassium phosphate buffer (pH 7.4), 500 µM bupropion, and the NADPH generating system. After the assay mixture for bupropion
hydroxylase activity was incubated for 5 min at 37°C, a 200 µL-aliquot of the assay mixture was collected, and added to a mixture of 100 µL of methanol and 100 µL of acetonitrile, which contains 0.5 µM phenacetin as an internal standard, to terminate the bupropion hydroxylation reaction. The samples were centrifuged at 1,650 g for 15 min and the supernatant fractions were directly injected into the HPLC system (Alliance 2795; Waters Corporation, Milford, MA, USA) equipped with a Capcell pak C18 UG120 column (5 µm, 2.0 x 150 mm, Shiseido Co., Ltd., Tokyo, Japan). Detection and quantitation of hydroxybupropion was performed by a mass spectrometer (Quattro micro API, Waters Corporation). Elution was performed using a mixture of solvent A consisting of 0.1 M ammonium acetate, purified water and methanol (5:90:5, v/v) and solvent B consisting of 0.1 M ammonium acetate and methanol (5:95, v/v) as a mobile phase. Proportion of the solvent B in the mobile phase was increased from 5.5% to 50% linearly for 5 min, maintained at 50% from 5 min to 7 min, and increased to 100% thereafter. The peak areas of the m/z 256→238 product ion of hydroxybupropion were measured against the peak areas of the m/z 180→110 product ion of the internal standard.

**Data analysis.** All incubations were performed in duplicate using three human liver microsomes lots. The mean value of the bupropion hydroxylation activity expressed as the percentage against the control activity was used to estimate the kinetic parameters of
inactivation according to Silverman (1988). The natural logarithm of the residual activities (LN % residual activity) was plotted against the preincubation time to calculate the observed inactivation rate constants (k_{obs}). The hyperbolic relationship between k_{obs} and the concentrations of the test compounds was fitted by Eq. 1 using WinNonlin Professional (version 4.0.1, Pharsight Corporation, Mountain View, CA, USA) to estimate the kinetic parameters in mechanism-based inhibition, where k_{inact} is the maximum inactivation rate constant, K_I is the concentration of the test substance that produces a half-maximal rate of inactivation and [I] is the concentration of the test substance (Mayhew et al. 2000).

Eq. 1:

\[ k_{obs} = \frac{k_{inact} \times [I]}{K_I + [I]} \]

The k_{inact}, K_I and k_{inact}/K_I values are expressed as the mean and standard deviation. For the \( k_{inact}/K_I \) value, the differences between R-95913 and other compounds, ticlopidine and 2-oxo-ticlopidine, and clopidogrel and 2-oxo-clopidogrel were statistically analyzed by a Tukey's test with a significance level of 5%. The analyses were performed using the software EXSAS (Arm Co., Ltd., Osaka, Japan).
Results

Figure 2 shows the typical time- and concentration-dependent inhibition of bupropion hydroxylation activity (CYP2B6) in human liver microsomes caused by ticlopidine, clopidogrel, their thiolactone metabolites, and R-95913. The parameters of CYP2B6 inactivation, $k_{\text{inact}}$ (maximal inactivation rate constant) and $K_I$ (the inactivator concentration that produces half-maximal rate of inactivation), for each compound are summarized in Table 1. The mechanism-based inhibition of CYP2B6 by R-95913 was approximately 10- and 22-fold less potent than by ticlopidine and clopidogrel, respectively, by comparing the $k_{\text{inact}}/K_I$ ratios (Table 1). The thiolactone metabolite of ticlopidine (2-oxo-ticlopidine) also exhibited mechanism-based inhibition with a $k_{\text{inact}}/K_I$ ratio comparable to that of ticlopidine, while the $k_{\text{inact}}/K_I$ ratios of the thiolactone metabolites of clopidogrel (2-oxo-clopidogrel) was small ($p < 0.001$) and comparable to that of R-95913 and was not statistically significant (Table 1). The time-dependent inactivation of CYP2B6 by ticlopidine, clopidogrel, and R-95913 was not affected by adding 10 mM glutathione (data not shown).
Discussion

We compared the potency of ticlopidine, clopidogrel, their thiolactone metabolites, and the thiolactone metabolite of prasugrel, R-95913, in the mechanism-based inhibition of CYP2B6 in human liver microsomes. The thiolactones are the immediate metabolic precursors for the pharmacologically active metabolites of the parent thienopyridine antiplatelet prodrugs (Figure 1).

As shown in Table 1, the $k_{\text{inact}}$ and $K_I$ values obtained in this study were respectively, 0.762 min$^{-1}$ and 0.928 µM, for ticlopidine and 1.30 min$^{-1}$ and 0.720 µM for clopidogrel. These values are comparable to those previously reported for ticlopidine (0.5 min$^{-1}$ and 0.2 µM, Richter et al. 2004, 0.32 min$^{-1}$ and 0.43 µM, Walsky et al. 2007) and clopidogrel (0.35 min$^{-1}$ and 0.5 µM, Richer et al. 2004, 1.9 min$^{-1}$ and 1.4 µM, Walsky et al. 2007). The efficiency of the mechanism-based inhibition of CYP2B6 was evaluated according to the $k_{\text{inact}}/K_I$ ratio, and ticlopidine, clopidogrel, and the thiolactone metabolite of ticlopidine ($k_{\text{inact}}/K_I$ ratios: 0.839, 1.79 and 0.655 min$^{-1}$·µM$^{-1}$, respectively) were found to be more potent in CYP2B6 inactivation than R-95913 and the thiolactone metabolite of clopidogrel ($k_{\text{inact}}/K_I$ ratios: 0.0807 and 0.150 min$^{-1}$·µM$^{-1}$, respectively). These data indicated that, of the two oxidation steps in the process of producing the pharmacologically active metabolites from ticlopidine and clopidogrel (Figure 1), the first oxidation step produces chemically reactive species, most likely either an epoxide-metabolite or an S-oxide-metabolite (Ha-Duong et al. 2001), which

---

This article has not been copyedited and formatted. The final version may differ from this version.
would bind to CYP2B6 protein covalently. The results also indicate that the thiolactone metabolite of ticlopidine produces a chemically reactive metabolite, while both the thiolactone metabolite of clopidogrel and R-95913 do so to a much lesser extent.

The thiolactone metabolite of ticlopidine showed a strong mechanism-based inhibition, indicating that this metabolite undergoes the oxidation reaction that produces an unknown reactive metabolite. However, this observation does not automatically mean that the thiolactone metabolite of ticlopidine rather than ticlopidine itself is the major player in the mechanism-based inhibition of CYP2B6 caused by ticlopidine \textit{in vivo}. Since ticlopidine has been reported to be metabolized to many metabolites both \textit{in vitro} (Dalvie and O'Connell 2004) and \textit{in vivo} (Desager 1994), it is quite unlikely that the thiolactone metabolite of ticlopidine would reach levels higher than hepatic levels of the parent compound. Therefore, the data suggest that the mechanism-based inhibition of CYP2B6 by ticlopidine and clopidogrel \textit{in vivo} mainly arises from the first oxidation step to their respective thiolactones shown in Figure 1.

Clopidogrel is known to be substantially hydrolyzed to its inactive acid metabolite \textit{in vivo}. Since clopidogrel acid metabolite showed high stability in human liver microsomes and weaker inhibitory effects on CYPs compared with clopidogrel and the thiolactone metabolite of clopidogrel (Hagihara et al. 2008), it is unlikely that clopidogrel acid metabolite exhibits
mechanism-based inhibition of CYP2B6.

In summary, the *in vitro* CYP2B6 inhibition data obtained in the present study showed that ticlopidine and clopidogrel and the thiolactone metabolite of ticlopidine are more potent mechanism-based inhibitors of CYP2B6 than the thiolactones of prasugrel or clopidogrel. The data suggest that the oxidation of the thiophene moiety of ticlopidine and clopidogrel to form their respective thiolactones is the critical reaction that produces the chemically reactive metabolites causing the mechanism-based inhibition of CYP2B6. The results obtained in the present *in vitro* study help explain the clinically observed difference in drug-drug interaction in that prasugrel much less significantly affected the bupropion pharmacokinetics compared to ticlopidine and clopidogrel.
REFERENCES


receptor by thiol reagents requires interaction with both extracellular cysteine residues, Cys17 and Cys270. Blood 101: 3908-3914.


FIGURE LEGENDS

**Figure 1.** Biotransformation of thienopyridine P2Y<sub>12</sub> receptor antagonists, prasugrel, ticlopidine and clopidogrel to pharmacologically active metabolites.

CYP: cytochrome P450

**Figure 2.** Typical time- and concentration-dependent inhibition of the bupropion hydroxylation activities in human liver microsomes by R-95913, ticlopidine, clopidogrel and their metabolites, 2-oxo-ticlopidine and 2-oxo-clopidogrel, and the corresponding plots of $k_{\text{obs}}$ against the inhibitor concentrations to estimate the kinetic parameters.

The graphs were obtained using human liver micorosomes Lot No. 0510077. Pooled human liver microsomes were incubated for the indicated times in the presence of an NADPH generating system and each compound at the concentrations expressed next to the symbols (unit: µM), and then the residual CYP2B6 enzyme activity as bupropion hydroxylation was assayed. The estimates of $k_{\text{obs}}$ from the initial rates of enzyme inactivation were plotted against the inhibitor concentrations to obtain the inactivation kinetic parameters, $k_{\text{inact}}$ and $K_I$, using Eq. 1. For details on the determination of the activity and the estimation of the inactivation kinetic parameters, see *Materials and Methods*. The corresponding $k_{\text{inact}}$ and $K_I$ values are presented in Table 1.
# FIGURES and TABLE

Table 1  *Inactivation parameters of CYP2B6 by R-95913, ticlopidine and clopidogrel and their metabolites, 2-oxo-ticlopidine and 2-oxo-clopidogrel*

<table>
<thead>
<tr>
<th></th>
<th>( k_{\text{inact}} ) (min(^{-1}))</th>
<th>( K_I ) (μM)</th>
<th>( k_{\text{inact}}/K_I ) (min(^{-1})μM(^{-1}))</th>
<th>( k_{\text{inact}}/K_I ) ratio to R-95913</th>
</tr>
</thead>
<tbody>
<tr>
<td>R-95913</td>
<td>0.178 ± 0.012</td>
<td>2.30 ± 0.50</td>
<td>0.0807 ± 0.0229</td>
<td>1.0</td>
</tr>
<tr>
<td>Ticlopidine</td>
<td>0.762 ± 0.078</td>
<td>0.928 ± 0.191</td>
<td>0.839 ± 0.143***</td>
<td>10</td>
</tr>
<tr>
<td>2-Oxo-ticlopidine</td>
<td>0.766 ± 0.116</td>
<td>1.19 ± 0.23</td>
<td>0.655 ± 0.122***</td>
<td>8.1</td>
</tr>
<tr>
<td>Clopidogrel</td>
<td>1.30 ± 0.63</td>
<td>0.720 ± 0.326</td>
<td>1.79 ± 0.12***</td>
<td>22</td>
</tr>
<tr>
<td>2-Oxo-clopidogrel</td>
<td>0.163 ± 0.023</td>
<td>1.13 ± 0.20</td>
<td>0.150 ± 0.046</td>
<td>1.9</td>
</tr>
</tbody>
</table>

Data represent the mean ± S.D. of three separate determinations with human liver microsomes.

*** \( p < 0.001 \): Significantly different from R-95913 (by Tukey’s test)

††† \( p < 0.001 \): Significantly different from clopidogrel (by Tukey’s test)

N.S.: Not significantly different from ticlopidine (by Tukey’s test)
Figure 2

The graphs illustrate the effects of different concentrations of ticlopidine and 2-oxo-ticlopidine on the ln% residual activity over time. Each graph shows the preincubation time (min) on the x-axis and ln% residual activity on the y-axis. Different concentrations are represented by various symbols and line styles.

Similarly, the graphs for clopidogrel and 2-oxo-clopidogrel depict the residual activity changes with varying preincubation times and concentrations.