

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

Yumi Nishiya, Katsunobu Hagihara, Takashi Ito, Masami Tajima, Shin-ichi Miura, Atsushi Kurihara, Nagy A. Farid, and Toshihiko Ikeda.

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.)

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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

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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_{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_{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 P2Y₁₂ 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 P2Y₁₂ 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; Williams 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, Rehm 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 μ M) (Rehm 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 μ L. The concentration of the test compounds ranged from 0.05 μ M to 1 μ M for ticlopidine and thiolactone metabolite of ticlopidine, from 0.1 μ M to 0.5 μ M for clopidogrel, from 0.5 μ M to 10 μ M for the thiolactone metabolite of clopidogrel, and from 1 μ M to 100 μ M 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 μ L 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 $MgCl_2$, 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 μ L-aliquot of the mixture was collected and added to an assay mixture for bupropion hydroxylase activity (225 μ L), consisting of 0.1 M potassium phosphate buffer (pH 7.4), 500 μ M bupropion, and the NADPH generating system. After the assay mixture for bupropion

hydroxylase activity was incubated for 5 min at 37°C, a 200 μ L-aliquot of the assay mixture was collected, and added to a mixture of 100 μ L of methanol and 100 μ L of acetonitrile, which contains 0.5 μ M 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 μ m, 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 \rightarrow 238 product ion of hydroxybupropion were measured against the peak areas of the m/z 180 \rightarrow 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:

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

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_{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_{inact}/K_I ratios (Table 1). The thiolactone metabolite of ticlopidine (2-oxo-ticlopidine) also exhibited mechanism-based inhibition with a k_{inact}/K_I ratio comparable to that of ticlopidine, while the k_{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_{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_{inact}/K_I ratio, and ticlopidine, clopidogrel, and the thiolactone metabolite of ticlopidine (k_{inact}/K_I ratios: 0.839, 1.79 and 0.655 $\text{min}^{-1}\cdot\mu\text{M}^{-1}$, respectively) were found to be more potent in CYP2B6 inactivation than R-95913 and the thiolactone metabolite of clopidogrel (k_{inact}/K_I ratios: 0.0807 and 0.150 $\text{min}^{-1}\cdot\mu\text{M}^{-1}$, 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

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 *in vivo*. Since ticlopidine has been reported to be metabolized to many metabolites both *in vitro* (Dalvie and O'Connell 2004) and *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 *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 *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

- Brandt, JT, Payne CD, Wiviott SD, Weerakkody G, Farid NA, Small DS, Jakubowski JA, Naganuma H and Winters KJ (2007) A comparison of prasugrel and clopidogrel loading doses on platelet function: magnitude of platelet inhibition is related to active metabolite formation. *Am Heart J* **153**: 66.e9-66.e16.
- Burger D, van der Heiden I, la Porte C, van der Ende M, Groeneveld P, Richter C, Koopmans P, Kroon F, Sprenger H, Lindemans J, Schenk P and van Schaik R (2006) Interpatient variability in the pharmacokinetics of the HIV non-nucleoside reverse transcriptase inhibitor efavirenz: the effect of gender, race, and CYP2B6 polymorphism. *Br J Clin Pharmacol* **61**: 148-154.
- Dalvie DK and O'Connell TN (2004) Characterization of novel dihydrothienopyridinium and thienopyridinium metabolites of ticlopidine in vitro: role of peroxidases, cytochromes p450, and monoamine oxidases. *Drug Metab Dispos* **32**: 49-57.
- Desager JP (1994) Clinical pharmacokinetics of ticlopidine. *Clin Pharmacokinet* **26**: 347-355.
- Ding Z, Kim S, Dorsam RT, Jin J and Kunapuli SP (2003) Inactivation of the human P2Y₁₂

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

Ekins S and Wrighton SA (1999) The role of CYP2B6 in human xenobiotic metabolism. *Drug Metab Rev* **31**: 719-754.

Farid NA Smith RL, Gillespie TA, Rash TJ, Blair PE, Kurihara A and Goldberg MJ (2007) The Disposition of Prasugrel, a Novel Thienopyridine, in Humans. *Drug Metab Dispos*: **35**:1096-1104

Farid NA, Payne CD, Ernest CS II, Li G, Winters KJ, Salazar D, and Small DS (2008) Prasugrel, a new thienopyridine antiplatelet drug, weakly inhibits cytochrome P450 2B6 in humans. *J. Clin. Pharmacol.* **48**: 53-59

Faucette SR, Hawke RL, Lecluyse EL, Shord SS, Yan B, Laethem RM and Lindley CM (2000). Validation of bupropion hydroxylation as a selective marker of human cytochrome P450 2B6 catalytic activity. *Drug Metab Dispos* **28**: 1222-1230.

Ha-Duong, NT, Dijols S, Macherey AC, Goldstein JA, Dansette PM and Mansuy D (2001)

Ticlopidine as a selective mechanism-based inhibitor of human cytochrome P450 2C19.

Biochemistry **40**: 12112-12122.

Hagihara K, Nishiya Y, Kurihara A, Kazui M, Farid NA, Ikeda T (2008) Comparison of human cytochrome P450 inhibition by the thienopyridines prasugrel, clopidogrel and ticlopidine. *Drug Metabolism and Pharmacokinetics*: In press

Jefferson JW, Pradko JF and Muir KT (2005) Bupropion for major depressive disorder: Pharmacokinetic and formulation considerations. *Clin Ther* **27**: 1685-1695.

Kalgutkar AS, Obach RS and Maurer TS (2007) Mechanism-based inactivation of cytochrome P450 enzymes: chemical mechanisms, structure-activity relationships and relationship to clinical drug-drug interaction and idiosyncratic adverse drug reactions. *Current Drug Metabolism* **8**: 407-447.

Kam PC and Nethery CM (2003) The thienopyridine derivatives (platelet adenosine diphosphate receptor antagonists), pharmacology and clinical developments. *Anaesthesia* **58**:

28-35.

Ko JW, Desta Z, Soukhova NV, Tracy T and Flockhart DA (2000) In vitro inhibition of the cytochrome P450 (CYP450) system by the antiplatelet drug ticlopidine: potent effect on CYP2C19 and CYP2D6." *Br J Clin Pharmacol* 49: 343-351.

Kurihara A, Hagihara K, Kazui M, Ozeki T, Farid NA, Ikeda T (2005) In Vitro Metabolism of Antiplatelet Agent Clopidogrel: Cytochrome P450 Isoforms Responsible for Two Oxidation Steps Involved in the Active Metabolite Formation. *Drug Metab. Rev.* 37(2): 99

Lamba V, Lamba J, Yasuda K, Strom S, Davila J, Hancock ML, Fackenthal JD, Rogan PK, Ring B, Wrighton SA and Schuetz EG Hepatic (2003) CYP2B6 expression: gender and ethnic differences and relationship to CYP2B6 genotype and CAR (constitutive androstane receptor) expression. *J Pharmacol Exp Ther* **307**: 906-922.

Mayhew BS, Jones DR and Hall SD (2000) An in vitro model for predicting in vivo inhibition of cytochrome P450 3A4 by metabolic intermediate complex formation. *Drug Metab Dispos* **28**: 1031-1037.

Niitsu Y, Jakubowski JA, Sugidachi A. and Asai F (2005) Pharmacology of CS-747 (Prasugrel, LY640315), a novel, potent antiplatelet agent with in vivo P2Y₁₂ receptor antagonist activity. *Semin Thromb Hemost* **31**: 184-194.

Rehmel J, Fayer L, Eckstein JA, Farid NA, Heim JB, Kasper SC, Kurihara A, Wrighton SA and Ring BJ (2006) Interactions of two major metabolites of prasugrel, a thienopyridine antiplatelet agent, with the cytochromes P450. *Drug Metab Dispos* **34**: 600-607.

Richter T, Mürdter TE, Heinkele G, Pleiss J, Tatzel S, Schwab M, Eichelbaum M and Zanger UM (2004) Potent mechanism-based inhibition of human CYP2B6 by clopidogrel and ticlopidine. *J Pharmacol Exp Ther* **308**: 189-197.

Sharis PJ, Cannon CP and Loscalzo J (1998) The antiplatelet effects of ticlopidine and clopidogrel. *Ann Intern Med* **129**: 394-405.

Silverman RB (1988) The potential use of mechanism-based enzyme inactivators in medicine. *J Enzyme Inhib* **2**: 73-90.

Stresser DM and Kupfer D (1999) Monospecific antipeptide antibody to cytochrome P-450

2B6. *Drug Metab Dispos* **27**: 517-525.

Turpeinen M, Tolonen A, Uusitalo J, Jalonen J, Pelkonen O and Laine K (2005). Effect of clopidogrel and ticlopidine on cytochrome P450 2B6 activity as measured by bupropion hydroxylation. *Clin Pharmacol Ther* **77**: 553-559.

Turpeinen M, Nieminen R, Juntunen T, Taavitsainen P, Raunio H and Pelkonen O (2004). Selective inhibition of CYP2B6-catalyzed bupropion hydroxylation in human liver microsomes in vitro. *Drug Metab Dispos* **32**: 626-631.

Walsky RL and Obach RS (2007) A comparison of 2-phenyl-2-(1-piperidinyl)propane (PPP), 1,1',1''-phosphinothioylidynetrisaziridine (thioTEPA), clopidogrel, and ticlopidine as selective inactivators of human cytochrome P450 2B6. *Drug Metab Dispos* **35**: 2053-2059.

Williams ET, Jones KO, Ponsler GD, Lowery SM, Perkins EJ, Wrighton SA, Ruterbories KJ, Kazui M, and Farid NA. (2008) The biotransformation of prasugrel, a new thienopyridine prodrug, by the human carboxylesterases 1 and 2. *Drug Metab. Dispos.* **36**: 1227-1232.

Wiviott SD, Braunwald E, McCabe CH, Montalescot G, Ruzyllo W, Gottlieb S, Neumann FJ, Ardissino D, Servi SD, Murphy SA, Riesmeyer J, Weerakkody G, Gibson CM, and Antman EM, for the TRITON–TIMI 38 Investigators (2007) Prasugrel versus Clopidogrel in Patients with Acute Coronary Syndromes *The New England Journal of Medicine* **357**: 2001-2015

Yoneda K, Iwamura R, Kishi H, Mizukami Y, Mogami K and Kobayashi S (2004) Identification of the active metabolite of ticlopidine from rat in vitro metabolites. *Br J Pharmacol* **142**: 551-557.

FIGURE LEGENDS

Figure 1. *Biotransformation of thienopyridine P2Y₁₂ 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_{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_{obs} from the initial rates of enzyme inactivation were plotted against the inhibitor concentrations to obtain the inactivation kinetic parameters, k_{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_{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*

	k_{inact} (min^{-1})	K_{I} (μM)	$k_{\text{inact}}/K_{\text{I}}$ ($\text{min}^{-1}\mu\text{M}^{-1}$)	$k_{\text{inact}}/K_{\text{I}}$ ratio to R-95913
R-95913	0.178 ± 0.012	2.30 ± 0.50	0.0807 ± 0.0229	1.0
Ticlopidine	0.762 ± 0.078	0.928 ± 0.191	$0.839 \pm 0.143^{***}$	N.S. 10
2-Oxo-ticlopidine	0.766 ± 0.116	1.19 ± 0.23	$0.655 \pm 0.122^{***}$	
Clopidogrel	1.30 ± 0.63	0.720 ± 0.326	$1.79 \pm 0.12^{***}$	††† 22
2-Oxo-clopidogrel	0.163 ± 0.023	1.13 ± 0.20	0.150 ± 0.046	

Data represent the mean \pm 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 1

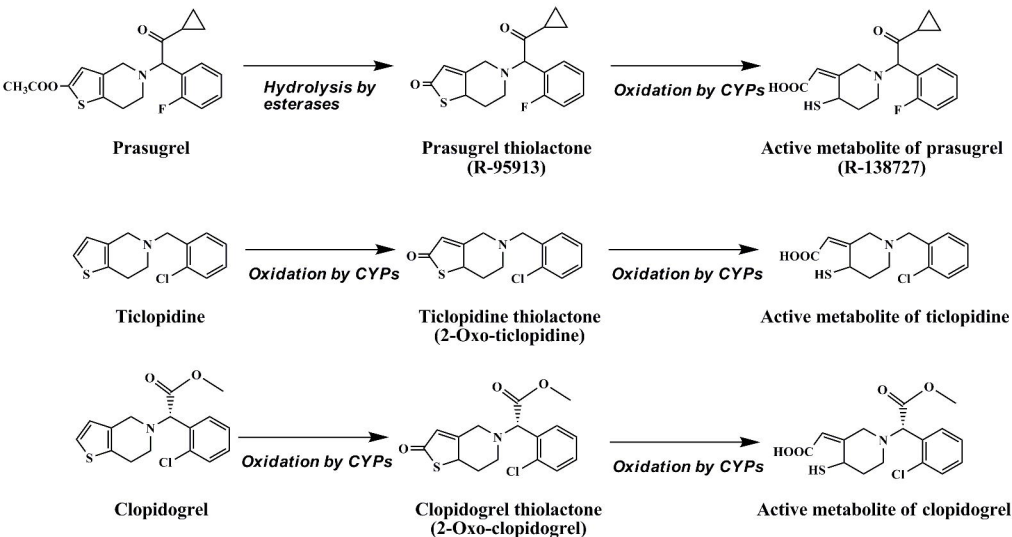
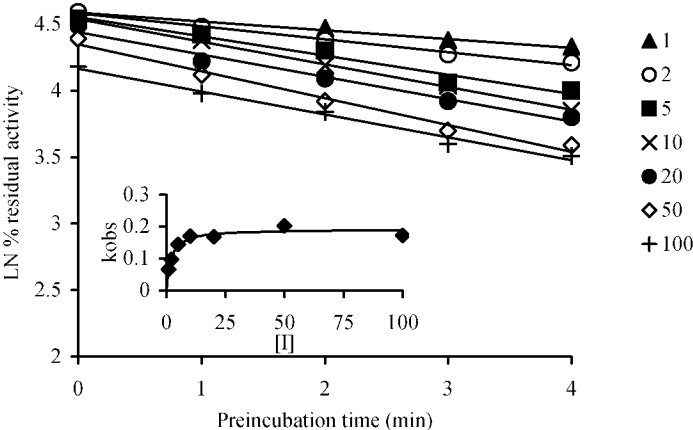
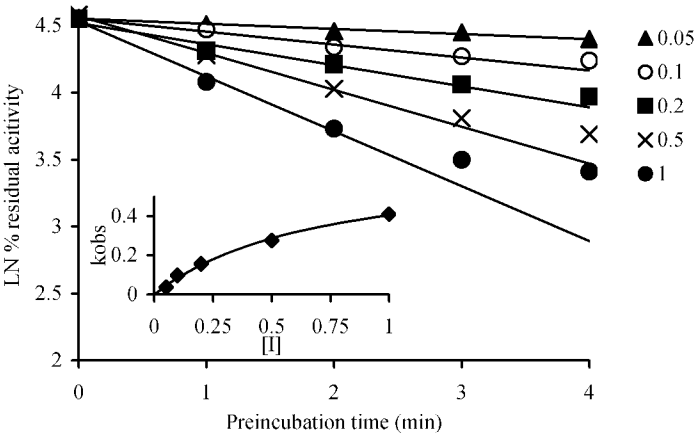


Figure 2

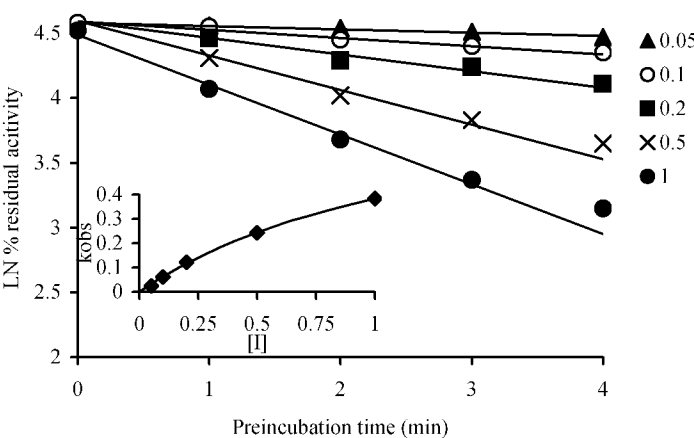
R-95913



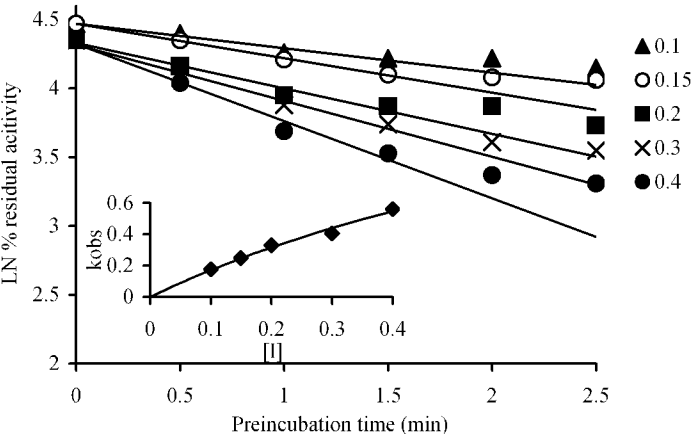
Ticlopidine



2-Oxo-ticlopidine



Clopidogrel



2-Oxo-clopidogrel

