

Mechanism-Based Inhibition of Human Cytochrome P450 2B6 by Ticlopidine, Clopidogrel, and the Thiolactone Metabolite of Prasugrel

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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 with 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.

The thienopyridine antiplatelet agents ticlopidine and clopidogrel (Fig. 1) prevent thrombogenesis via blocking 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 with ticlopidine (Kam and Nethery, 2003).

Prasugrel (Fig. 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 efficacy superior to that of 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, P450-mediated oxidation step (Fig. 1) (Rehmel et al., 2006; Williams et al., 2008).

The thiolactone metabolites of ticlopidine and clopidogrel are produced by P450-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 P450-mediated oxidation (Yoneda et al., 2004; Kurihara et al., 2005; Rehmel et al., 2006). Because of the P450 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 P450s. Indeed, several in vitro studies indicated that ticlopidine, clopidogrel, and their thiolactone metabolites are competitive inhibitors of several P450s (Ko et al., 2000; Turpeinen et al., 2004; 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 P450 to a reactive intermediate, which can irreversibly modify the P450 protein. Thus, compared with reversible (e.g., competitive) inhibition, mechanism-based inhibition more frequently results in unfavorable drug-drug interactions because the interactions are sustained for a long time until the inactivated P450s have 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

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ABBREVIATIONS: prasugrel, (\pm)-2-[2-acetyloxy-6,7-dihydrothieno[3,2-c]pyridin-5(4H)-yl]-1-cyclopropyl-2-(2-fluorophenyl)ethanone; P450, cytochrome P450; R-95913, 2-[2-oxo-6,7-dihydrothieno[3,2-c]pyridin-5(4H)-yl]-1-cyclopropyl-2-(2-fluorophenyl)ethanone; R-138727, 2-[1-[2-cyclopropyl-1-(2-fluorophenyl)-2-oxoethyl]-4-mercapto-3-piperidinylidene]acetic acid.

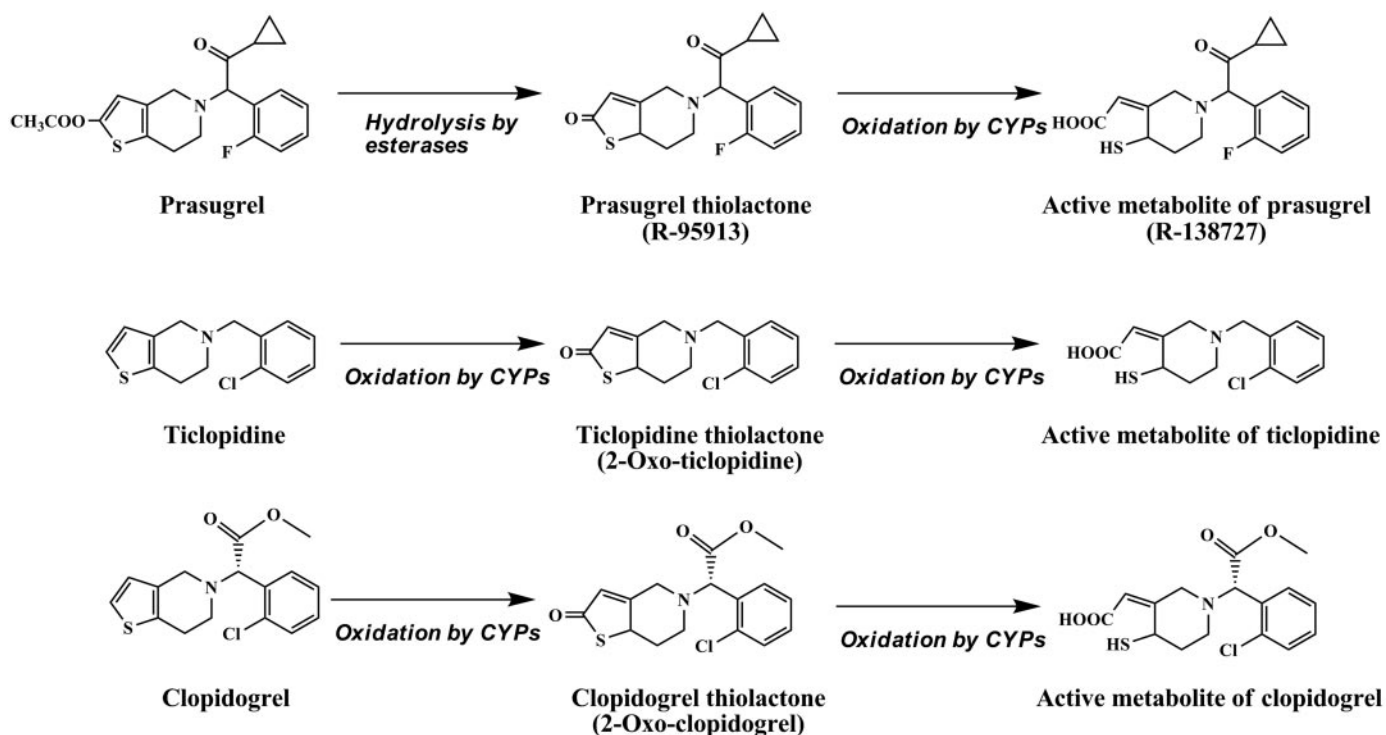


FIG. 1. Biotransformation of thienopyridine P2Y₁₂ receptor antagonists, prasugrel, ticlopidine, and clopidogrel to pharmacologically active metabolites. CYP, cytochrome P450.

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 P450 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 may 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 approximately 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 P450s 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 used 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 because 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 to determine whether any compound is a mechanism-based inhibitor of CYP2B6.

Materials and Methods

Materials. Clopidogrel (purity 99.2%), the thiolactone metabolite of clopidogrel (2-oxo-clopidogrel, purity 92.9%), and the thiolactone metabolite of prasugrel (R-95913, purity 99.4%) were synthesized at Ube Industries, Ltd. (Ube, Japan). The thiolactone metabolite of ticlopidine (2-oxo-ticlopidine, purity 99.3%) was synthesized at Daiichi Sankyo Co., Ltd. (Tokyo, Japan). Ticlopidine (purity 100%), β -NADP sodium salt, D-glucose 6-phosphate disodium salt hydrate, and glucose-6-phosphate dehydrogenase from baker's yeast were purchased from Sigma-Aldrich (St. Louis, MO). Bupropion hydrochloride was purchased from MP Biochemicals, Inc. (Solon, OH). Hydroxybupropion was purchased from BD Gentest (Woburn, MA). Phenacetin was purchased from Wako Pure Chemicals (Osaka, Japan). Three pools of human liver microsomes, prepared by combining the liver microsomal fractions from 50 donors (20 mg of protein/ml; lots 0510077, 0710403, and 0810063), were purchased from XenoTech, LLC (Lenexa, KS). 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), human liver microsomes (0.4 mg of protein/ml), and various 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 to 1 μ M for ticlopidine and the thiolactone metabolite of ticlopidine, from 0.1 to 0.4 μ M for clopidogrel, from 0.5 to 10 μ M for the thiolactone metabolite of clopidogrel, and from 1 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 glucose 6-phosphate, 0.5 unit/ml glucose-6-phosphate dehydrogenase, 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 for 0, 0.5, 1, 1.5, 2, and 2.5 min (clopidogrel). At each time point, a 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 1650g for 15 min, and the supernatant fractions were directly injected into the high-performance liquid chromatography system (Alliance 2795; Waters, Milford, MA) equipped with a Capcell Pak C18 UG120 column (5 μ m, 2.0 \times 150 mm; Shiseido Co., Ltd., Tokyo, Japan). Detection and quantitation of hydroxybupropion were performed with a mass spectrometer (Quattro micro API; Waters). 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. The proportion of solvent B in the mobile phase was increased from 5.5 to 50% linearly for 5 min, maintained at 50% from 5 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 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) 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).

$$k_{\text{obs}} = \frac{k_{\text{inact}} \times [I]}{K_I + [I]} \quad (1)$$

The k_{inact} , K_I , and k_{inact}/K_I values are expressed as mean and S.D. 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 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 that 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 with that of ticlopidine, whereas the k_{inact}/K_I ratios of the thiolactone metabolites of clopidogrel (2-oxo-clopidogrel) were small ($p < 0.001$) and comparable with that of R-95913 and were 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

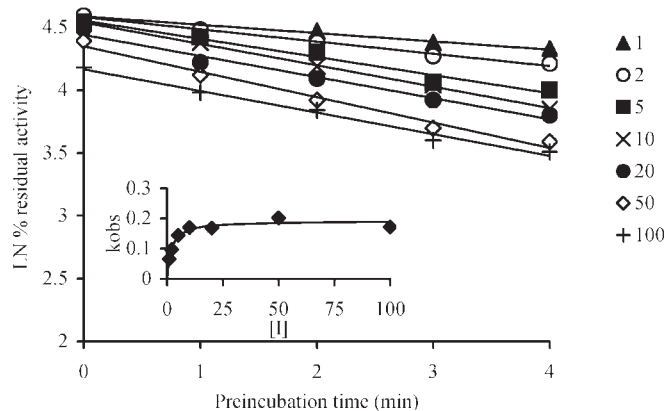
We compared the potency of ticlopidine, clopidogrel, their thiolactone metabolites, and the thiolactone metabolite of prasugrel, R-95913, in the mechanism-based inhibition of CYP2B6 in human liver microsomes. The thiolactones are the immediate metabolic precursors for the pharmacologically active metabolites of the parent thienopyridine antiplatelet prodrugs (Fig. 1). As shown in Table 1, the k_{inact} and K_I values obtained in this study were 0.762 min^{-1} and 0.928 μM for ticlopidine and 1.30 min^{-1} and 0.720 μM for clopidogrel, respectively. These values are comparable with those previously reported for ticlopidine [0.5 min^{-1} and 0.2 μM (Richter et al., 2004) and 0.32 min^{-1} and 0.43 μM (Walsky and Obach, 2007)] and clopidogrel [0.35 min^{-1} and 0.5 μM (Richter et al., 2004) and 1.9 min^{-1} and 1.4 μM (Walsky and Obach, 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 of 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 of 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 (Fig. 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, whereas 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. Because 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 Fig. 1.

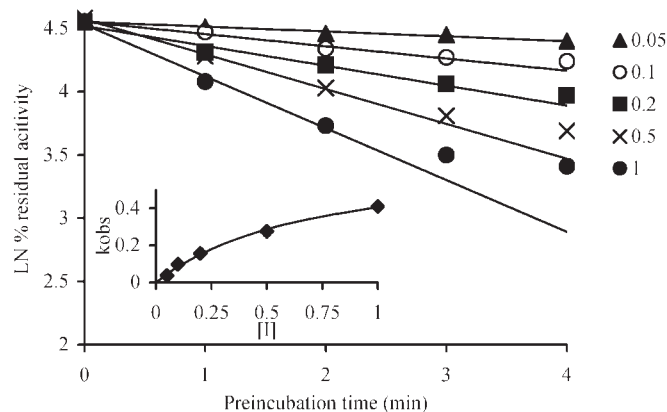
Clopidogrel is known to be substantially hydrolyzed to its inactive acid metabolite in vivo. Because clopidogrel acid metabolite showed high stability in human liver microsomes and weaker inhibitory effects on P450s compared with clopidogrel and the thiolactone metabolite of clopidogrel (Hagihara et 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 affected

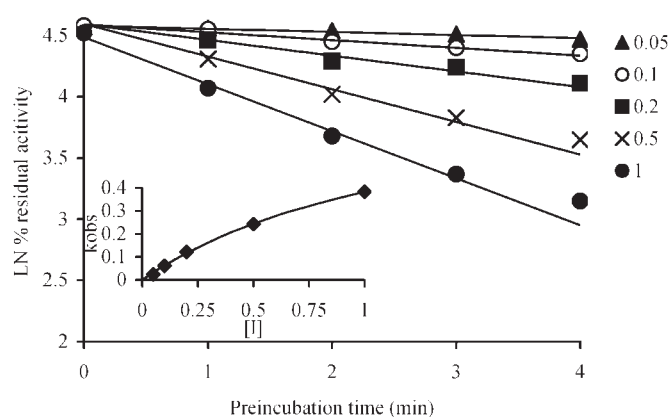
R-95913



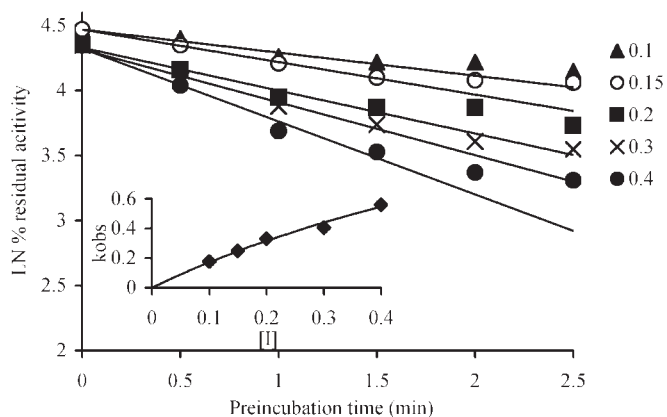
Ticlopidine



2-Oxo-ticlopidine



Clopidogrel



2-Oxo-clopidogrel

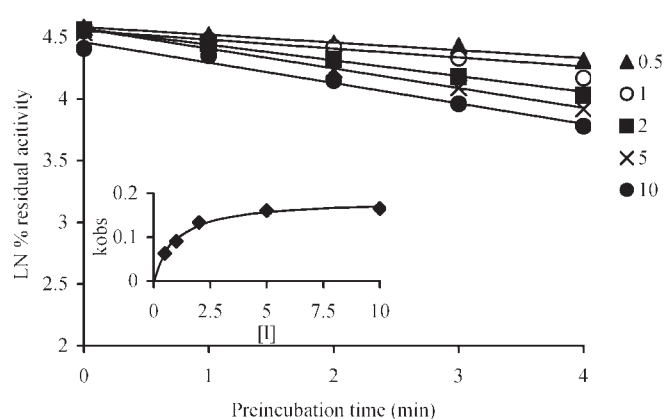


FIG. 2. Typical time- and concentration-dependent inhibition of the bupropion hydroxylation activities in human liver microsomes by R-95913, ticlopidine, clopidogrel, and their metabolites, 2-oxo-ticlopidine and 2-oxo-clopidogrel, and the corresponding plots of k_{obs} against the inhibitor concentrations to estimate the kinetic parameters. The graphs were obtained using human liver microsomes (lot 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 (micromolar) expressed next to the symbols, 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. LN % residual activity, natural logarithm of the residual activity.

TABLE 1

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

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

	k_{inact} min^{-1}	K_I μM	k_{inact}/K_I $\text{min}^{-1}\mu\text{M}^{-1}$	k_{inact}/K_I ratio to R-95913
R-95913	0.178 \pm 0.012	2.30 \pm 0.50	0.0807 \pm 0.0229	1.0
Ticlopidine	0.762 \pm 0.078	0.928 \pm 0.191	0.839 \pm 0.143*	10
2-Oxo-ticlopidine	0.766 \pm 0.116	1.19 \pm 0.23	0.655 \pm 0.122*] N.S.	8.1
Clopidogrel	1.30 \pm 0.63	0.720 \pm 0.326	1.79 \pm 0.12*] †	22
2-Oxo-clopidogrel	0.163 \pm 0.023	1.13 \pm 0.20	0.150 \pm 0.046] †	1.9

N.S., not significantly different from ticlopidine (by Tukey's test).

* $P < 0.001$, significantly different from R-95913 (by Tukey's test).

† $P < 0.001$, significantly different from clopidogrel (by Tukey's test).

the bupropion pharmacokinetics much less significantly than ticlopidine and clopidogrel.

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