

# 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

Robert L. Walsky and R. Scott Obach

*Pharmacokinetics, Pharmacodynamics, and Drug Metabolism, Pfizer, Inc., Groton, Connecticut*

Received March 22, 2007; accepted August 3, 2007

## ABSTRACT:

The use of selective chemical inhibitors of human cytochrome P450 (P450) enzymes represents a powerful method by which the relative contributions of various human P450 enzymes to the metabolism of drugs can be determined. However, the identification of CYP2B6 in the metabolism of drugs has been more challenging because of the lack of a well established inhibitor of this enzyme. In this report, we describe the selectivity of 2-phenyl-2-(1-piperidinyl)propane (PPP) as an inactivator of CYP2B6 and compare this selectivity versus other CYP2B6 inactivators: 1,1',1''-phosphinothioylidynetrisaziridine (thioTEPA), clopidogrel, and ticlopidine. Values of  $K_i$  and  $k_{inact}$  for PPP were 5.6  $\mu\text{M}$  and 0.13/min for bupropion hydroxylase catalyzed by pooled human liver microsomes, and values for thioTEPA were similar (4.8  $\mu\text{M}$  and 0.20/min, respectively). Intrinsic inactivation capability was considerably

greater for clopidogrel because of a greater  $k_{inact}$  value (1.9/min). Ticlopidine was potent with  $K_i$  and  $k_{inact}$  values of 0.32  $\mu\text{M}$  and 0.43/min, respectively. The selectivity of these four agents for CYP2B6 was determined by testing their effects on other human P450 enzyme activities using conditions that yield ~90% inactivation of CYP2B6 activity. The results showed that preincubation of human liver microsomes with PPP at 30  $\mu\text{M}$  for 30 min provided more selective inhibition for CYP2B6 than thioTEPA, clopidogrel, and ticlopidine. Furthermore, the use of clopidogrel is complicated by the observation that this agent is not stable in the presence of human liver microsomes, even without addition of NADPH. Therefore, PPP can serve as a selective chemical inactivator of CYP2B6 and be used to define the role of CYP2B6 in the metabolism of drugs.

Cytochrome P450 (P450) enzymes are the most important enzymes in drug metabolism and are responsible for clearance of a majority of drugs. Five of these enzymes, CYP1A2, CYP2C9, CYP2C19, CYP2D6, and CYP3A4, have been considered as important drug-metabolizing P450 enzymes in humans, and it has been proposed that these enzymes should be routinely examined as potential contributors to the metabolism of new drugs using in vitro approaches (Bjornsson et al., 2003). Well characterized, selective chemical inhibitors of these five enzymes have been described and applied throughout the past several years in examinations of the metabolism of drugs and other xenobiotics (Madan et al., 2002). The most frequently used chemical inhibitors are furafylline (CYP1A2 inactivator), sulfaphenazole (CYP2C9-reversible inhibitor), *S*-mephenytoin (CYP2C19-competitive substrate), quinidine (CYP2D6-reversible inhibitor), and ketoconazole (CYP3A-reversible inhibitor), although there are others that have also been successfully applied.

However, recently, some other human P450 enzymes have been shown to play a major role in the metabolism of some drugs. Of the human P450 enzymes, CYP2B6 has recently emerged as one of increasing importance. CYP2B6 has been shown to be involved in the metabolism of several drugs, including efavirenz, bupropion, cyclophosphamide, ifosfamide, and sertraline (Chang et al., 1993; Granvil et al., 1999; Kobayashi et al., 1999; Roy et al., 1999; Faucette et al., 2000; Hesse et al., 2000; Huang et al., 2000; Rae et al., 2002; Ward et al., 2003; Obach et al., 2005; Turpeinen et al., 2005). However, robust, well characterized inhibitors of CYP2B6 that are selective for this enzyme versus other human P450 enzymes have not been thoroughly evaluated. An early report on CYP2B6 described orphenadrine as a potential CYP2B6 inhibitor; however, the selectivity is not high (Guo et al., 1997). More recent work has described potent mechanism-based inactivation of CYP2B6 by clopidogrel (Richter et al., 2004), ticlopidine (Richter et al., 2004; Turpeinen et al., 2004), and 1,1',1''-phosphinothioylidynetrisaziridine (thioTEPA) (Harleton et al., 2004; Turpeinen et al., 2004; Richter et al., 2005). In studies designed to gain a better understanding of the biochemistry of CYP2B6, Chun et al. (2000) identified an analog of phencyclidine, 2-phenyl-2-(1-piperidinyl)propane (PPP) (Fig. 1), as a mechanism-based inactivator of human CYP2B6.

The potential for PPP to be used as a tool in identifying a role for CYP2B6 in the metabolism of drugs has not been explored. This requires an examination of the potential for PPP to not affect other human P450 enzymes at inhibitor concentrations that would provide 90% or more inhibition of CYP2B6. In this report, the selectivity of PPP, thioTEPA, clopidogrel, and ticlopidine as CYP2B6 inactivators has been examined in an attempt to determine which, if any, would be a superior CYP2B6 reaction phenotyping tool and to define experi-

Article, publication date, and citation information can be found at <http://dmd.aspetjournals.org>.  
doi:10.1124/dmd.107.015883.

**ABBREVIATIONS:** P450, cytochrome P450; thioTEPA, 1,1',1''-phosphinothioylidynetrisaziridine; PPP, 2-phenyl-2-(1-piperidinyl)propane; MS/MS, tandem mass spectrometry.

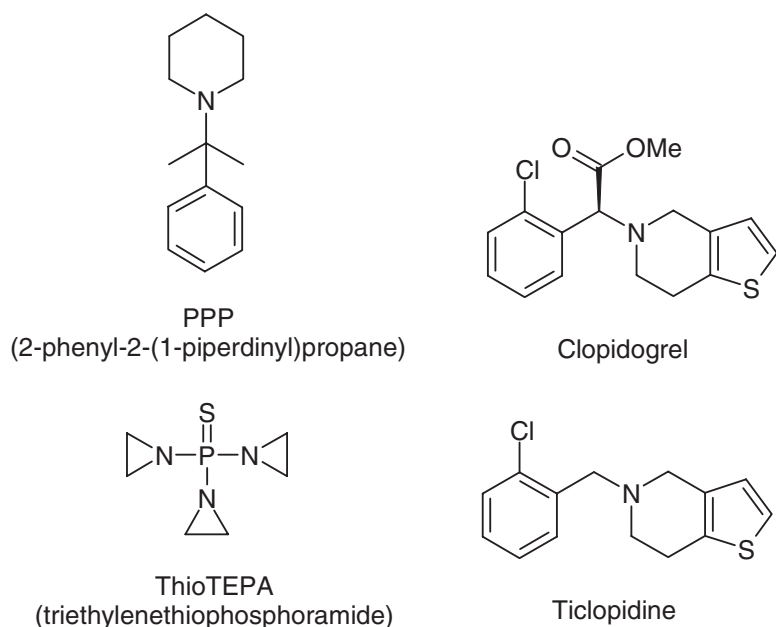


Fig. 1. Structures of the inactivators examined in this report.

mental conditions under which these agents could be used in P450 reaction phenotyping experiments.

#### Materials and Methods

**Materials.** P450 substrates, metabolite standards, and internal standards were obtained as previously described (Walsky and Obach, 2004). Ticlopidine and thioTEPA were obtained from Sigma Chemical Co. (St. Louis, MO). Clopidogrel was obtained from Sequoia Research Products (Oxford, UK). PPP was synthesized under contract by Syncom BV (Groningen, The Netherlands). Human liver microsomes pooled from 53 donors were obtained under contract from Gentest, Inc. (Woburn, MA).

**Reversible Inhibition of CYP2B6.** Specific aspects of the incubation conditions (e.g., protein concentration, incubation time, reaction termination solvent, etc.) were previously reported (Walsky and Obach, 2004). The Michaelis constant for bupropion hydroxylase was  $82 \pm 1 \mu\text{M}$  for pooled liver microsomes. In general, human liver microsomes (0.05 mg/ml) were mixed with buffer (100 mM  $\text{KH}_2\text{PO}_4$ , pH 7.4),  $\text{MgCl}_2$  (3.3 mM), and substrate (at  $K_M$ ) and then warmed to  $37^\circ\text{C}$  in a 96-well temperature-controlled heater block. Aliquots of this mixture (0.18 ml) were delivered to each well of a 96-well polypropylene polymerase chain reaction plate maintained at  $37^\circ\text{C}$ , followed by addition of the inhibitor or control solvent (mixture of water and  $\text{CH}_3\text{CN}$ ) as applicable. The inhibitors, standards, and quality controls were prepared as  $100\times$  stock solutions and  $2\text{-}\mu\text{l}$  aliquots dispensed to the appropriate incubations maintaining solvent concentrations at 1% (v/v) or less. Incubations were commenced with the addition of NADPH stock (assay concentration = 1.3 mM) to a final incubation volume of 0.2 ml and maintained at  $37^\circ\text{C}$  for 20 min. Incubations were terminated by acidification upon addition of 0.02 ml of termination solvent ( $\text{H}_2\text{O}/\text{CH}_3\text{CN}/\text{HCOOH}$ ; 92:5:3) containing [ $^2\text{H}_6$ ]hydroxybupropion as an internal standard, followed by filtration through a  $45\text{-}\mu\text{m}$  filter and analysis by high-performance liquid chromatography/tandem mass spectrometry (MS/MS) as previously described (Walsky and Obach, 2004).

**Reversible Inhibition of Other Human P450 Enzymes.** Reversible inhibition assays for CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A were conducted according to described methods (Walsky and Obach, 2004). Substrate concentrations used were equal to  $K_M$  for each reaction.

**Time-Dependent Inhibition ( $K_I/k_{\text{inact}}$ ) CYP2B6 Assay.** Specific aspects of the  $K_I/k_{\text{inact}}$  methodology used have been previously reported (Obach et al., 2007). Preincubations consisted of the following conditions: pooled human liver microsomes (0.5 mg/ml),  $\text{MgCl}_2$  (3.3 mM) in 100 mM potassium phosphate buffer, pH 7.4. Microsomes, buffer, and inhibitor were mixed, prewarmed at  $37^\circ\text{C}$  for 5 min, followed by initiation of inactivation by addition of

NADPH (1.3 mM). After preincubation periods, aliquots of  $20 \mu\text{l}$  were removed and added to a mixture of bupropion ( $800 \mu\text{M}$ ,  $10\times K_M$ ) and NADPH (1.3 mM) in 100 mM potassium phosphate buffer, pH 7.4, followed by incubation at  $37^\circ\text{C}$  for 12 min. Incubations were terminated by acidification upon addition of 0.02 ml of termination solvent ( $\text{H}_2\text{O}/\text{CH}_3\text{CN}/\text{HCOOH}$ ; 92:5:3) containing [ $^2\text{H}_6$ ]hydroxybupropion as an internal standard, followed by filtration and analysis by liquid chromatography/MS/MS.

**Time-Dependent Inhibition ( $K_I/k_{\text{inact}}$ ) CYP2B6 Assay (Clopidogrel).** To reduce an observed instability of clopidogrel in the presence of human liver microsomes, a modified experimental procedure was followed. Preincubations consisted of the following conditions:  $\text{MgCl}_2$  (3.3 mM), NADPH (1.3 mM) in 100 mM potassium phosphate buffer, pH 7.4. Buffer and inhibitor were mixed and prewarmed at  $37^\circ\text{C}$ ; preincubations were initiated by the addition of pooled human liver microsomes (0.5 mg/ml). After preincubation periods, aliquots of  $20 \mu\text{l}$  were removed and added to a mixture of bupropion ( $800 \mu\text{M}$ ,  $10\times K_M$ ) and NADPH (1.3 mM) in 100 mM potassium phosphate buffer, pH 7.4, followed by incubation at  $37^\circ\text{C}$  for 12 min. Incubations were terminated by acidification upon addition of 0.02 ml of termination solvent ( $\text{H}_2\text{O}/\text{CH}_3\text{CN}/\text{HCOOH}$ ; 92:5:3) containing [ $^2\text{H}_6$ ]hydroxybupropion as an internal standard, followed by filtration and analysis by liquid chromatography/MS/MS.

**Determination of Selectivity for CYP2B6 Inactivation and Effect of Protein Concentration.** After determination of inactivation kinetic parameters for CYP2B6, inactivators were tested for selectivity among other P450 enzymes using a concentration and incubation time that yielded 90% inactivation of CYP2B6. PPP and thioTEPA ( $30 \mu\text{M}$ ; 30 min), ticlopidine, and clopidogrel ( $3 \mu\text{M}$ ; 10 min) were incubated with NADPH and human liver microsomes at protein concentrations that were 10-fold greater than those required for the specific P450 assays. After this inactivation incubation, the mixtures were diluted 10-fold into the activity assays for CYP1A2, CYP2A6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A according to described methods (Walsky and Obach, 2004). Substrate concentrations used were equal to  $K_M$  for each reaction.

For CYP2B6 inactivation, the effect of microsomal protein concentration in the inactivation incubation was tested. PPP ( $30 \mu\text{M}$ ), thioTEPA ( $30 \mu\text{M}$ ), ticlopidine ( $3 \mu\text{M}$ ), and clopidogrel ( $3 \mu\text{M}$ ) were incubated with liver microsomes (0.5–10 mg/ml) for 10 min (ticlopidine and clopidogrel) or 30 min (PPP and thioTEPA). After this, an aliquot of the mixture was withdrawn and diluted 10-fold into the CYP2B6 activity incubation as described above. Additionally, because we had observed clopidogrel to be unstable when in the presence of liver microsomes even in the absence of NADPH, this compound was also tested as above following 20-min incubation with liver microsomes.

**Data Analysis.** Standard curve fitting was accomplished with QuanLynx (version 4.1) software (Micromass, Beverly, MA). Data were fit to quadratic

TABLE 1

Reversible inhibitory  $IC_{50}$  ( $\pm$  S.E.) values for PPP, thioTEPA, clopidogrel, and ticlopidine for several human P450 activities in pooled human liver microsomes

Enzyme	Activity	$IC_{50}$			
		PPP	ThioTEPA	Clopidogrel	Ticlopidine
		$\mu M$	$\mu M$	$\mu M$	$\mu M$
CYP1A2	Phenacetin <i>O</i> -deethylase	>300	>300	4.4 $\pm$ 0.1	12 $\pm$ 2
CYP2A6	Coumarin 7-hydroxylase	>300	240 $\pm$ 30	0.046 $\pm$ 0.010	>300
CYP2B6	Bupropion hydroxylase	5.1 $\pm$ 0.1	8.3 $\pm$ 1.3	0.046 $\pm$ 0.01	0.21 $\pm$ 0.02
CYP2C8	Amodiaquine <i>N</i> -deethylase	>300	>300	18 $\pm$ 2	100 $\pm$ 30
CYP2C9	Diclofenac 4'-hydroxylase	>300	>300	21 $\pm$ 4	51 $\pm$ 19
CYP2C19	<i>S</i> -Mephenytoin 4'-hydroxylase	>300	>300	3.7 $\pm$ 0.5	0.85 $\pm$ 0.09
CYP2D6	Dextromethorphan <i>O</i> -demethylase	74 $\pm$ 16	>300	200 $\pm$ 30	3.3 $\pm$ 0.2
CYP2E1	Chlorzoxazone 6-hydroxylase	>300	>300	>300	>300
CYP3A	Felodipine oxidase	>300	>300	66 $\pm$ 13	>300
CYP3A	Midazolam 1'-hydroxylase	200 $\pm$ 30	230 $\pm$ 60	34 $\pm$ 2	>300
CYP3A	Testosterone 6 $\beta$ -hydroxylase	>300	100 $\pm$ 40	23 $\pm$ 5	25 $\pm$ 4
Selectivity for CYP2B6		15	12	86	4

curves using  $1/x^2$  weighting. Assay run acceptance was defined by the accuracy and precision of independently prepared quality control samples at three concentrations. Substrate saturation curves and inhibition data were analyzed using the Enzyme Kinetics module of SigmaPlot version 8.0 (SPSS, Chicago, IL). Best-fit models were selected based on the Akaike Information Criterion.

Data for  $IC_{50}$  determinations were fit to the following equation:

$$\% \text{ Control Activity} = 100 \cdot \left( A - \left( \frac{B \cdot I}{I + IC_{50}} \right) \right)$$

in which  $I$  is the inhibitor concentration, the  $IC_{50}$  represents the inflection point, and the value of  $1 - (A - B)$  is the maximum percentage inhibition observed at an infinite inhibitor concentration. Determination of inactivation kinetic parameters was made using nonlinear regression of the data to the formula:

$$k_{\text{inact,obs}} = k_{\text{inact,obs},[I]=0} + \frac{k_{\text{inact}} \cdot [I]}{K_i + [I]}$$

in which  $[I]$  refers to the concentration of inactivator in the inactivation preincubation,  $k_{\text{inact,obs}}$  is the negative value of the slope of the natural logarithm of the percent activity remaining versus inactivation incubation time at various  $[I]$ ,  $k_{\text{inact,obs},[I]=0}$  is the apparent inactivation rate constant measured in the absence of inactivator,  $k_{\text{inact}}$  is the theoretical maximum inactivation rate constant at  $[I] = \infty$ , and  $K_i$  is the inactivator concentration yielding  $k_{\text{inact,obs}}$  at the sum of  $k_{\text{inact,obs},[I]=0}$  and 0.5 times  $k_{\text{inact}}$ .

## Results

**Determination of Reversible Inhibition.** PPP, thioTEPA, clopidogrel, and ticlopidine were first tested for reversible inhibition of P450 activities in pooled human liver microsomes. For PPP, the  $IC_{50}$  for CYP2B6 bupropion hydroxylase activity was 5.1  $\mu M$ , whereas the next most potently inhibited P450 activity was CYP2D6 ( $IC_{50} = 74 \mu M$ ), yielding a 15-fold split (Table 1). Other P450 activities were not significantly inhibited at PPP concentrations as high as 300  $\mu M$ . Inhibition data are shown in Fig. 2. Clopidogrel most potently inhibited CYP2B6 with an  $IC_{50}$  of 0.046  $\mu M$  and a selectivity of 86 $\times$  (to CYP2C19). ThioTEPA yielded comparable data to PPP for CYP2B6 inhibition, and the next most potently inhibited activity was CYP3A-catalyzed testosterone 6 $\beta$ -hydroxylase activity (12 $\times$ -fold split). Ticlopidine showed potent inhibition of CYP2B6 ( $IC_{50} = 0.21 \mu M$ ); however, it also potently inhibited CYP2C19 activity ( $IC_{50} = 0.85 \mu M$ ).

**Kinetics of Mechanism-Based Inactivation.** Because these compounds have been previously shown to be mechanism-based inactivators of CYP2B6 (Chun et al., 2000; Richter et al., 2004, 2005), the potential for them to inactivate human P450 enzymes was examined. Inactivation of CYP2B6 was observed when these compounds were incubated with human liver microsomes in the presence of NADPH

before the addition of bupropion as the substrate. This increase in inhibition was not observed when NADPH was not included, consistent with mechanism-based inactivation. Inactivation experiments were done to yield values for  $K_i$  and  $k_{\text{inact}}$  for CYP2B6 (Fig. 3; Table 2). Clopidogrel was the inactivator with the greatest intrinsic activity ( $k_{\text{inact}}/K_i = 1400 \text{ ml/min}/\mu\text{mol}$ ), followed by ticlopidine ( $k_{\text{inact}}/K_i = 1300 \text{ ml/min}/\mu\text{mol}$ ), thioTEPA ( $k_{\text{inact}}/K_i = 42 \text{ ml/min}/\mu\text{mol}$ ), and PPP ( $k_{\text{inact}}/K_i = 23 \text{ ml/min}/\mu\text{mol}$ ). The inactivation data for clopidogrel appeared biphasic (data not shown), and only the initial rapid inactivation phase data points occurring at less than 1.5 min were used. The reason for this is not known, but one potential explanation is offered (see below).

**Determination of P450 Selectivity.** To determine which of these four compounds would be suitable probe inhibitors for P450 reaction phenotyping protocols, they were tested for their effects on multiple human P450 activities under conditions that yield a  $\geq 90\%$  reduction in CYP2B6 activity. Results are shown in Fig. 4. Preincubation of human liver microsomes with 30  $\mu M$  PPP in the presence of NADPH for 30 min followed by 10 $\times$  dilution into marker substrate incubations yields 90% inhibition of CYP2B6, whereas other P450 activities were not inhibited (inhibition of  $\leq 10\%$ ) (Fig. 4), showing that this concentration of PPP would be adequately selective for assessing the role of CYP2B6 in human liver microsomal metabolism of substrates. With a 30-min preincubation, 30  $\mu M$  thioTEPA showed inactivation of other P450 enzymes besides CYP2B6 (consistent with previous observation in this laboratory; Obach et al., 2007). Specifically, the extent of inactivation of CYP3A and CYP2A6 by thioTEPA under conditions that provide 90% inactivation of CYP2B6 would not permit its use as a selective reaction phenotyping probe. Despite the apparent lack of selectivity of ticlopidine for CYP2B6 under reversible inhibition conditions (see above), when this agent is preincubated with human liver microsomes for 10 min at 3  $\mu M$  followed by a 10-fold dilution before assessment of P450 activities,  $>90\%$  inactivation of CYP2B6 is obtained with little effect on the other P450 enzymes. When tested as an inactivator (at 3  $\mu M$  for 10 min), clopidogrel showed selectivity for CYP2B6. However, clopidogrel possesses other challenges with its use as a reaction phenotyping probe, as discussed below.

P450 reaction phenotyping for various compounds can require different protein concentrations in the incubations to provide adequate activity for the reaction under examination. Thus, it must be known whether the potency of selective P450 probe inhibitors can be affected by different protein concentrations, which would require adjustment of the concentration of the inhibitor. Preincubation of PPP, thioTEPA,

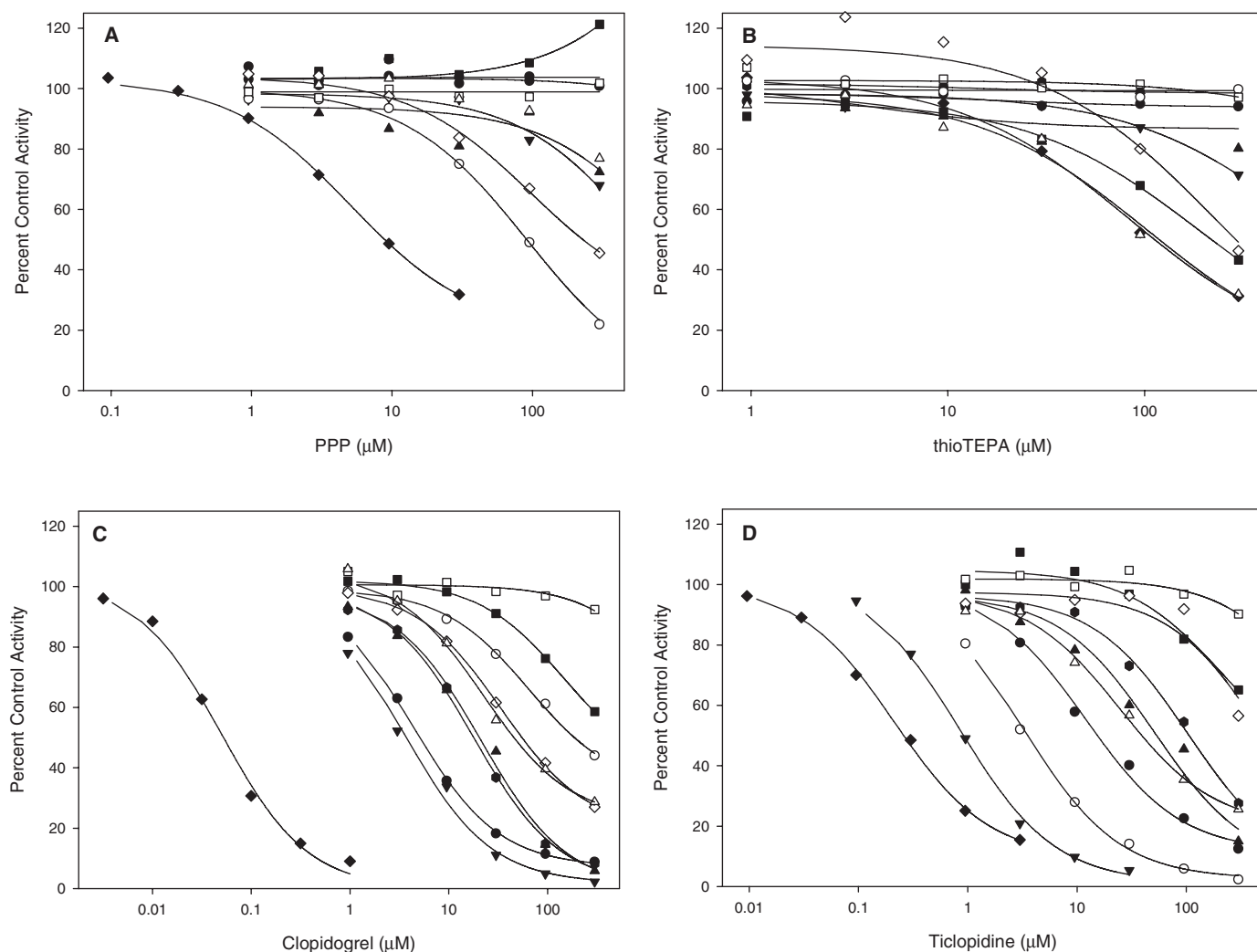


FIG. 2.  $IC_{50}$  curves for inhibition of human P450 activities by PPP (A), thioTEPA (B), clopidogrel (C), and ticlopidine (D). Symbols are CYP1A2 (●), CYP2A6 (■), CYP2B6 (◆), CYP2C8 (●), CYP2C9 (▲), CYP2C19 (▼), CYP2D6 (○), CYP2E1 (□), CYP3A4 midazolam 1'-hydroxylase (◇), and testosterone 6 $\beta$ -hydroxylase (△) activities.

clopidogrel, and ticlopidine in the presence of NADPH with human liver microsomal concentrations ranging from 0.5 to 10 mg/ml for 10 min (clopidogrel and ticlopidine) or 30 min (PPP and thioTEPA) showed that CYP2B6 inactivation is reduced as the protein concentration increases (Fig. 5). The effect was substantial for clopidogrel. It was noted that when clopidogrel was mixed with liver microsomes for 20 min before the addition of NADPH, inactivation was not observed. Incubation of clopidogrel with pooled human liver microsomes at 0.5 mg/ml at 37°C in the absence of NADPH led to a marked decrease in clopidogrel concentrations such that less than 10% remained after 10 min (Fig. 5B). This is consistent with the previous observation of hydrolysis of this compound in liver microsomes (Tang et al., 2006). Thus, the use of this agent as a CYP2B6 reaction phenotyping tool is burdened with the added complexity of instability of the reagent during incubation.

### Discussion

P450 reaction phenotyping has become a commonplace exercise in drug development and discovery, and the practice has evolved to such an extent that such data are expected in the registration dossiers for new drugs that are metabolized by P450 enzymes. The information is included in drug package insert data used to make clinical dosing

decisions to avoid/minimize drug-drug interactions. Thus, it is of utmost importance that the tools used in these studies are adequately selective so as to avoid erroneous conclusions regarding the contribution of individual P450 enzymes to drug clearance. Three approaches have been commonly applied in P450 reaction phenotyping: 1) the use of selective chemical inhibitors or inhibitory antibodies of the metabolism of the drug in human liver microsomes, 2) determination of the metabolism of the drug in heterologously expressed recombinant human P450 enzyme systems, and 3) correlation of the metabolism of the drug in a panel of liver microsome samples from individual donors to well established marker activities for individual P450 enzymes. The relative merits of each of these approaches have been described in detail in a consensus white paper (Bjornsson et al., 2003) and will not be reiterated here, but of note in this consensus is the emphasis of the use of inhibition data (using selective chemical inhibitors or inhibitory antibodies) as the approach that is most reliable and always recommended. To successfully accomplish P450 reaction phenotyping through the use of chemical inhibitors, it is imperative that these inhibitors be suitably selective for individual P450 enzymes. Selectivity has been well established for inhibitors for several of the P450 enzymes that have been the focus of investigation for years. However, for P450 enzymes that have not received as much



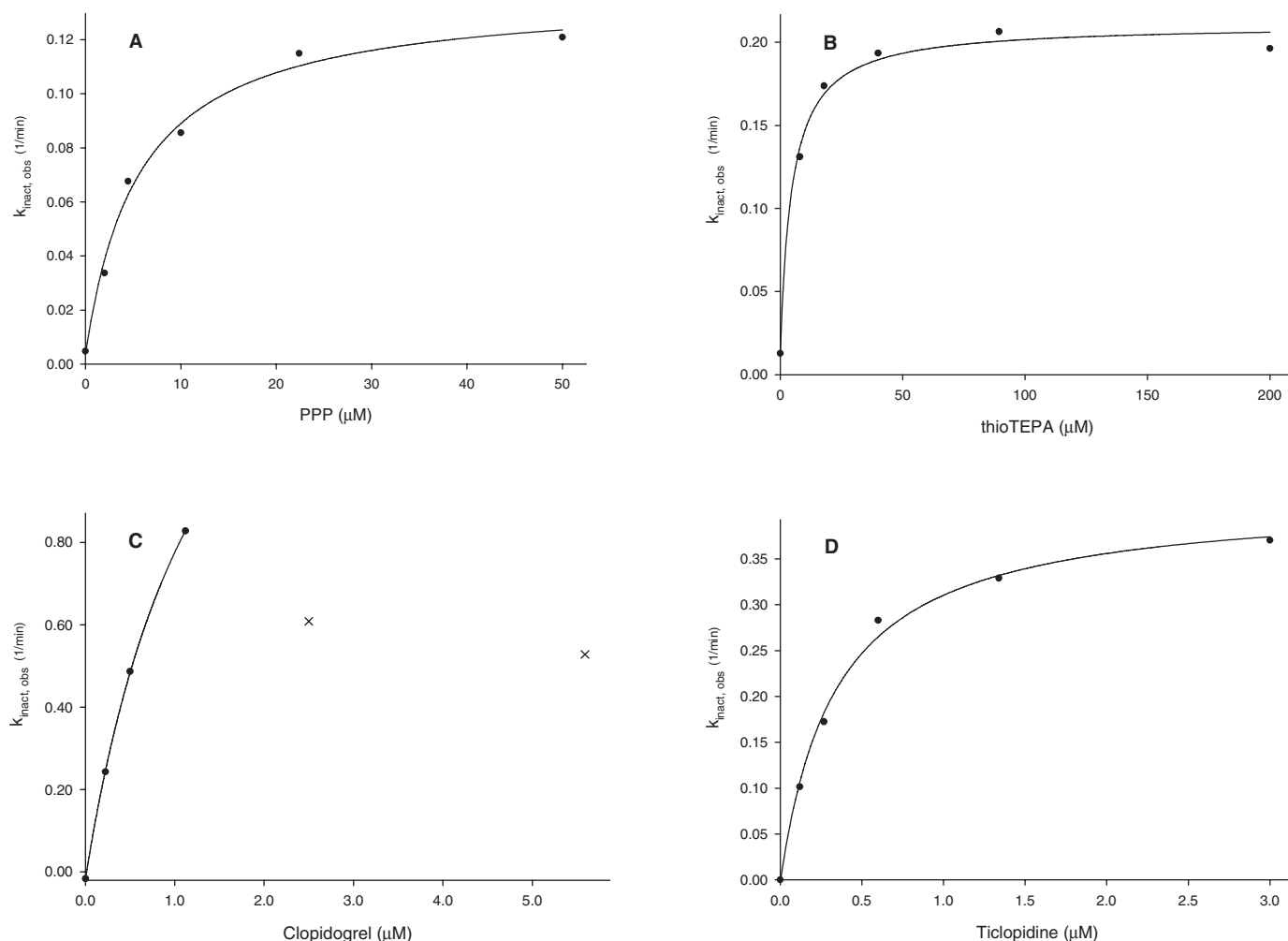


FIG. 3. Relationship between inactivator concentration and apparent inactivation rate of CYP2B6 for PPP (A), thioTEPA (B), clopidogrel (C), and ticlopidine (D). Note in C the points at [clopidogrel] > 2  $\mu$ M were not included in the determination of  $k_{\text{inact}}/K_I$ .

TABLE 2

Inactivation kinetic constants for PPP, thioTEPA, clopidogrel, and ticlopidine for CYP2B6 catalyzed bupropion hydroxylase in pooled human liver microsomes<sup>a</sup>

Parameter	PPP	ThioTEPA	Clopidogrel <sup>b</sup>	Ticlopidine
$K_I$ ( $\mu$ M)	$5.6 \pm 1.2$	$4.8 \pm 1.0$	$1.4 \pm 0.1$	$0.32 \pm 0.04$
$k_{\text{inact}}$ (1/min)	$0.13 \pm 0.01$	$0.20 \pm 0.01$	$1.9 \pm 0.1$	$0.43 \pm 0.02$
$k_{\text{inact}}/K_I$ (ml/min/ $\mu$ mol)	23	42	1400	1300

<sup>a</sup> Values represent the mean  $\pm$  S.E.

<sup>b</sup> Kinetic parameters for clopidogrel were estimated using data obtained at [clopidogrel] < 2  $\mu$ M, excluding the decrease in  $k_{\text{inact,app}}$  observed at high concentrations, and should be considered as estimates.

attention until recently (such as CYP2B6), selective inhibition tools are not as well established.

In this study, the selectivity of four compounds as potential reaction phenotyping tools for human CYP2B6 has been compared. The four compounds chosen, PPP, thioTEPA, ticlopidine, and clopidogrel, were selected for the present study because each had been recently described as a mechanism-based inactivator of CYP2B6 (Chun et al., 2000; Richter et al., 2004, 2005). The use of mechanism-based inactivators as P450 reaction phenotyping tools offers the potential advantages of being able to dilute the inactivator considerably when measuring the metabolism of the compound of interest as well as permitting the option of preparing pools of inactivated microsomes that can be well characterized and stored frozen for use at a later date.

Under a reversible inhibition experimental protocol, PPP, thioTEPA, and clopidogrel appeared to possess the greatest selectivity for CYP2B6, although the potency toward the next most potentially inhibited enzyme for PPP and thioTEPA (CYP2D6 and CYP3A, respectively) was great enough to make the selectivity marginal. Great care would be needed to use PPP and thioTEPA in this manner for CYP2B6 reaction phenotyping to avoid “spillover” into the next most potentially inhibited enzyme. Ticlopidine did not offer adequate selectivity for CYP2B6 when tested in a reversible inhibition protocol; concentrations required to effectively inhibit CYP2B6 would also partially inhibit CYP2C19 and CYP2D6. Under a reversible inhibition protocol, clopidogrel appeared to possess adequate selectivity for CYP2B6; however, this agent was observed to be considerably unstable on incubation with human liver microsomes, which would confound efforts to define its potency.

Under an experimental protocol in which these agents were used as irreversible inactivators of CYP2B6, followed by determination of selectivity for CYP2B6, PPP and ticlopidine yielded improved selectivity, whereas the selectivity of thioTEPA was diminished to such an extent to render it inadequate for P450 reaction phenotyping. PPP appeared to be slightly more selective than ticlopidine and clopidogrel, despite these latter two compounds possessing greater intrinsic inactivation capability for CYP2B6. Additionally, the potency of these inactivators can be altered if the microsomal protein concentra-

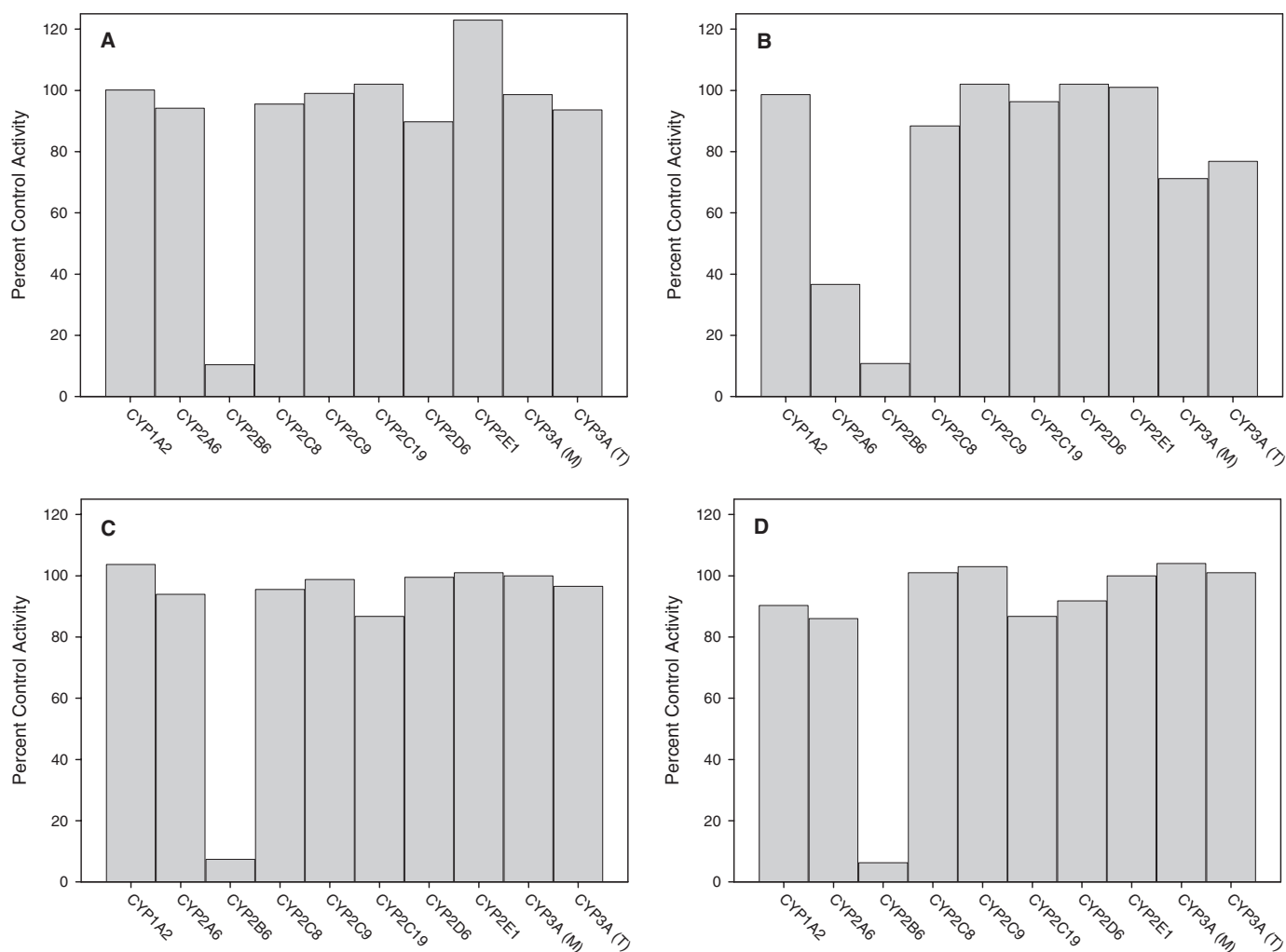


FIG. 4. Comparison of the effects of PPP (A), thioTEPA (B), clopidogrel (C), and ticlopidine (D) on CYP2B6 and other human P450 activities. Concentrations and preincubation times used were PPP, 30  $\mu$ M and 30 min; thioTEPA, 30  $\mu$ M and 30 min; clopidogrel, 3  $\mu$ M and 10 min; and ticlopidine, 3  $\mu$ M and 10 min. The designation CYP3A(M) and CYP3A(T) refers to CYP3A-catalyzed midazolam 1'-hydroxylase and testosterone 6 $\beta$ -hydroxylase activities, respectively.

tion during the inactivation incubation is elevated. Decreases in inhibitory potency with increasing microsome concentration have been observed for other agents (e.g., fluoxetine for CYP2D6 or montelukast for CYP2C8) (Margolis and Obach, 2003; Walsky et al., 2005), and this has been attributed to increased nonspecific binding of the inhibitor to phospholipid membranes, effectively decreasing the concentration available to the enzyme. Whether this is responsible for the effect of protein concentration on PPP and ticlopidine has not been determined. It was observed that incubation of clopidogrel with liver microsomes led to a marked decrease in concentration of the inactivator. This occurred even in the absence of NADPH, indicating that this instability is not caused by P450-catalyzed metabolism. Clopidogrel possesses a methyl ester moiety, which has been shown to be subject to enzymatic hydrolysis in liver microsomes (Tang et al., 2006), and it is possible that the resulting carboxylic acid does not inactivate CYP2B6. Additionally, other kinetic anomalies were observed for clopidogrel. Inactivation kinetics were particularly difficult to obtain because inactivation showed a biphasic rate over time with an extremely rapid initial phase lasting less than 1 min, followed by a considerably decreased inactivation rate. A plot of the apparent inactivation rate constant versus inactivator concentration that included clopidogrel concentrations in excess of 1.1  $\mu$ M showed a decrease in inactivation. Clearly, further mechanistic characterization

of the inactivation of CYP2B6 by clopidogrel is needed to attempt to understand these observations. Nevertheless, this kinetic behavior, whatever the reason, makes the use of clopidogrel as a CYP2B6 inactivator for reaction phenotyping less appealing because slight changes in experimental conditions would confound obtaining reproducible and reliable results.

It is important to note that this investigation describes the use of these agents as *in vitro* tools only; use as probe inhibitors *in vivo* requires other experimentation. Because of its inherent toxicity as an anticancer agent, thioTEPA would be inappropriate for use as a CYP2B6 inhibitor in healthy clinical study subjects, and additionally, the *in vitro* data suggest that it could also have problems with a lack of selectivity. PPP, although shown to be useful for *in vitro* reaction phenotyping studies, cannot be used in clinical studies at this time because it is not a substance approved for administration to humans. Clopidogrel and ticlopidine are clinical agents used to prevent the formation of blood clots and can therefore, with some care, be used in clinical pharmacokinetic drug-drug interaction studies in healthy human study subjects. In fact, both of these agents have been shown to alter the metabolism of bupropion *in vivo* (Turpeinen et al., 2005), albeit the magnitude of the interaction was not great because CYP2B6-catalyzed hydroxylation only partially contributes to the total metabolic clearance of bupropion (Welch et al., 1987). As the

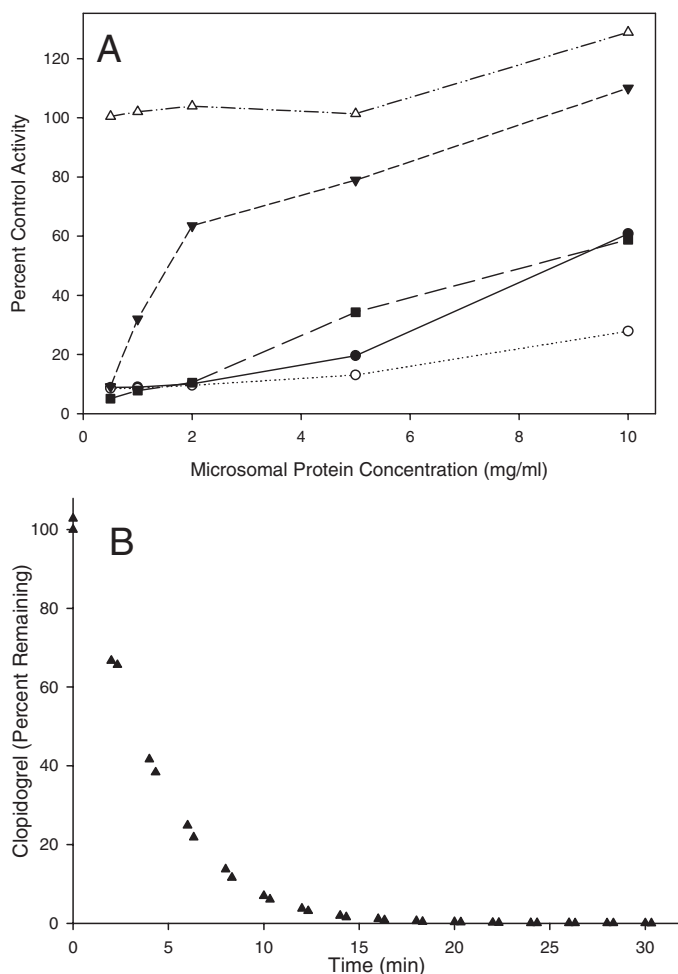


FIG. 5. Relationship between the amount of inactivation of CYP2B6 by PPP, thioTEPA, ticlopidine, and clopidogrel and the concentration of human liver microsomal protein in the inactivation incubation (A) and stability of clopidogrel (3  $\mu$ M) in human liver microsomes (0.5 mg/ml) in the absence of NADPH (B). Concentrations and preincubation times used were: PPP at 30  $\mu$ M and 30 min (●), thioTEPA at 30  $\mu$ M and 30 min (○), ticlopidine at 3  $\mu$ M and 10 min (■), clopidogrel at 3  $\mu$ M and 10 min (▲), and clopidogrel at 3  $\mu$ M and 10 min following incubation for 20 min in microsomes without NADPH (△). Binding to liver microsomes in the absence of NADPH was determined at a protein concentration of 5.0 mg/ml. Percent bound values were 22, 97, and 97 for PPP, ticlopidine, and clopidogrel, respectively. Binding for thioTEPA was negligible.

number of drugs shown to have a considerable portion of their clearance catalyzed by CYP2B6 (e.g., efavirenz) or their pharmacological activity dependent on CYP2B6-catalyzed metabolism (e.g., cyclophosphamide) increases, more clinical experimentation into the potential for drug interactions based on CYP2B6 will be needed.

In conclusion, the utility of PPP as a CYP2B6-selective inactivator that can be useful in P450 reaction phenotyping studies has been shown. Conditions under which this agent is selective for this enzyme have been described. A comparison has been made for PPP, clopidogrel, ticlopidine, and thioTEPA, and the conclusion is that PPP can serve as a selective probe inhibitor of CYP2B6 in human liver microsomes under either irreversible or reversible inhibition protocols, ticlopidine and clopidogrel could be used as a selective CYP2B6 inactivator when great care is used in study design aspects to ensure selectivity and stability, and that thioTEPA, although an inactivator of CYP2B6, lacks adequate enzyme selectivity for routine use.

**Acknowledgments.** We thank Dr. Paul Hollenberg (University of

Michigan) for initial discussions of this work and Dr. Jim Eggler (Pfizer) for coordination of the contracted preparation of PPP.

## References

- Bjornsson TD, Callaghan JT, Einolf HJ, Fischer V, Gan L, Grimm S, Kao J, King SP, Miwa G, Ni L, et al. (2003) The conduct of in vitro and in vivo drug-drug interaction studies: a Pharmaceutical Research and Manufacturers of America (PhRMA) perspective. *Drug Metab Dispos* 31:815–832.
- Chang TKH, Weber GF, Crespi CL, and Waxman DJ (1993) Differential activation of cyclophosphamide and ifosfamide by cytochromes P-450 2B and 3A in human liver microsomes. *Cancer Res* 53:5629–5637.
- Chun J, Kent UM, Moss RM, Sayre LM, and Hollenberg PF (2000) Mechanism-based inactivation of cytochromes P450 2B1 and 2B6 by 2-phenyl-2-(1-piperidinyl)propane. *Drug Metab Dispos* 28:905–911.
- 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.
- Granvil CP, Madan A, Sharkawi M, Parkinson A, and Wainer IW (1999) Role of CYP2B6 and CYP3A4 in the in vitro N-dechloroethylation of (R)- and (S)-ifosfamide in human liver microsomes. *Drug Metab Dispos* 27:533–541.
- Guo Z, Raess S, White RB, and Stevens JC (1997) Orphenadrine and methimazole inhibit multiple cytochrome P450 enzymes in human liver microsomes. *Drug Metab Dispos* 25:390–393.
- Harleton E, Webster M, Bumpus NN, Kent UM, Rae JM, and Hollenberg PF (2004) Metabolism of N,N',N"-triethylenethiophosphoramide by CYP2B1 and CYP2B6 results in the inactivation of both isoforms by two distinct mechanisms. *J Pharmacol Exp Ther* 310:1011–1019.
- Hesse LM, Venkatakrishnan K, Court MH, Von Moltke LL, Duan SX, Shader RI, and Greenblatt DJ (2000) CYP2B6 mediates the in vitro hydroxylation of bupropion: potential drug interactions with other antidepressants. *Drug Metab Dispos* 28:1176–1183.
- Huang Z, Roy P, and Waxman DJ (2000) Role of human liver microsomal CYP3A4 and CYP2B6 in catalyzing N-dechloroethylation of cyclophosphamide and ifosfamide. *Biochem Pharmacol* 59:961–972.
- Kobayashi K, Ishizuka T, Shimada N, Yoshimura Y, Kamijima K, and Chiba K (1999) Sertraline N-demethylation is catalyzed by multiple isoforms of human cytochrome P-450 in vitro. *Drug Metab Dispos* 27:763–766.
- Madan A, Usuki E, Burton LA, Oglivie BW, and Parkinson A (2002) In vitro approaches for studying the inhibition of drug metabolizing enzymes and identifying the drug metabolizing enzymes responsible for the metabolism of drugs, in *Drug-Drug Interactions* (Rodriguez AD ed) pp 217–293, Marcel Dekker, New York.
- Margolis JM and Obach RS (2003) Impact of non-specific binding to microsomes and phospholipid on the inhibition of cytochrome P4502D6: implications for relating in vitro inhibition data to in vivo drug interactions. *Drug Metab Dispos* 31:606–611.
- Obach RS, Cox LM, and Tremaine LM (2005) Sertraline is metabolized by multiple cytochrome P450 enzymes, monoamine oxidases, and glucuronyl transferases in human: an in vitro study. *Drug Metab Dispos* 33:262–270.
- Obach RS, Walsky RL, and Venkatakrishnan K (2007) Mechanism based inactivation of human cytochrome P450 enzymes and the prediction of drug-drug interactions. *Drug Metab Dispos* 35:246–255.
- Rae JM, Soukhova NV, Flockhart DA, and Desta Z (2002) Triethylenethiophosphoramide is a specific inhibitor of cytochrome P450 2B6: implications for cyclophosphamide metabolism. *Drug Metab Dispos* 30:525–530.
- Richter T, Muerdter TE, Heinkel 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.
- Richter T, Schwab M, Eichelbaum M, and Zanger UM (2005) Inhibition of human CYP2B6 by N,N',N"-triethylenethiophosphoramide is irreversible and mechanism-based. *Biochem Pharmacol* 69:517–524.
- Roy P, Yu LJ, Crespi CL, and Waxman DJ (1999) Development of a substrate-activity based approach to identify the major human liver P-450 catalysts of cyclophosphamide and ifosfamide activation based on cDNA-expressed activities and liver microsomal P-450 profiles. *Drug Metab Dispos* 27:655–666.
- Tang M, Mukundan M, Yang J, Charpentier N, LeCluyse EL, Black C, Yang D, Shi D, and Yan B (2006) Antiplatelet agents aspirin and clopidogrel are hydrolyzed by distinct carboxylesterases, and clopidogrel is transesterified in the presence of ethyl alcohol. *J Pharmacol Exp Ther* 319:1467–1476.
- 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.
- 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.
- Walsky RL and Obach RS (2004) Validated assays for human cytochrome P450 activities. *Drug Metab Dispos* 32:647–660.
- Walsky RL, Obach RS, Gaman EA, Gleeson JPR, and Proctor WR (2005) Selective inhibition of human cytochrome P4502C8 by montelukast. *Drug Metab Dispos* 33:413–418.
- Ward BA, Gorski JC, Jones DR, Hall SD, Flockhart DA, and Desta Z (2003) The cytochrome P450 2B6 (CYP2B6) is the main catalyst of efavirenz primary and secondary metabolism: implication for HIV/AIDS therapy and utility of efavirenz as a substrate marker of CYP2B6 catalytic activity. *J Pharmacol Exp Ther* 306:287–300.
- Welch RM, Lai AA, and Schroeder DH (1987) Pharmacological significance of the species differences in bupropion metabolism. *Xenobiotica* 17:287–298.

**Address correspondence to:** R. Scott Obach, Pfizer, Inc., Groton, CT 06340.  
E-mail: r.scott.obach@pfizer.com