

## Short Communication

# Pinoline May be Used as a Probe for CYP2D6 Activity

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### ABSTRACT:

Pinoline, 6-methoxy-1,2,3,4-tetrahydro- $\beta$ -carboline, is a serotonin analog that selectively inhibits the activity of monoamine oxidase-A and shows antidepressant activity. Our previous study using a panel of recombinant cytochrome P450 (P450) enzymes suggests that pinoline O-demethylation may be selectively catalyzed by polymorphic CYP2D6. The current study, therefore, aimed to delineate the impact of CYP2D6 status on pinoline metabolism. Enzyme kinetic studies using recombinant CYP2D6 allelic isozymes revealed that CYP2D6.2 exhibited 5-fold lower enzyme efficiency ( $V_{max}/K_m$ ) toward pinoline compared with CYP2D6.1, and CYP2D6.10 did not show any catalytic activity. Inhibition study showed that quinidine (1  $\mu$ M) completely blocked pinoline O-demethylase activity in human liver microsomes, whereas other P450 isoform-selective inhibitors had no or minimal effects. Pinoline O-demethylase activi-

ties in 10 human liver microsomes showed significantly strong correlation with bufuralol 1'-hydroxylase activities ( $R^2 = 0.93$ ;  $p < 0.0001$ ) and CYP2D6 contents ( $R^2 = 0.82$ ;  $p = 0.005$ ), whereas no appreciable correlations with enzymatic activities of other P450 enzymes were found. Furthermore, we compared pinoline urinary metabolic ratio (pinoline/6-hydroxy-1,2,3,4-tetrahydro- $\beta$ -carboline) between CYP2D6-humanized and wild-type control mice after intraperitoneal injection of pinoline (30 mg/kg). Results indicated that the two genotyped mice were clearly distinguished by pinoline metabolic ratio (mean  $\pm$  S.D.), which was much higher in wild-type mice ( $0.29 \pm 0.19$ ,  $n = 4$ ) than in CYP2D6-humanized transgenic mice ( $0.0070 \pm 0.0048$ ,  $n = 4$ ). Our findings suggest that pinoline O-demethylation is governed by CYP2D6 status, and pinoline, at a proper concentration or dose, may be a good probe to evaluate CYP2D6 activity.

Pinoline (6-methoxy-1,2,3,4-tetrahydro- $\beta$ -carboline), named from the words "pineal  $\beta$ -carboline," is an endogenous compound in mammalian brain and other tissues (such as retina) with a wide range of concentrations from 2 ng/g to 21  $\mu$ g/g (Pahkla et al., 1996; Herraiz and Galisteo, 2004). It may play an important role in protecting against oxidative stress in these tissues. Several studies have shown that pinoline could effectively reduce oxidative damage in the brain region and retinal homogenate, stabilize hepatic microsomal membrane, and prevent from chromium-mediated oxidative DNA damage by suppressing lipid peroxidation and scavenging hydroxyl radical (Qi et al., 2000; Pinol-Ripoll et al., 2006; Tang et al., 2007). As a serotonin (5-hydroxytryptamine) analog, pinoline selectively inhibits monoamine oxidase-A, directly binds to serotonin transporter, and competitively inhibits serotonin uptake into brain synaptosomes and platelets. As a result, pinoline exhibits antidepressant profiles in different animal models, which makes pinoline a potential antidepressant agent (Pahkla et al., 1996). In addition, pinoline has been shown to interact with imidazoline binding sites in the pancreatic  $\beta$ -cell and thus stimulate insulin secretion from isolated human islets of Langerhans, indicating that pinoline may serve as a useful prototype for the development of novel insulin secretagogues (Cooper et al., 2003).

Our recent studies have shown that pinoline mainly undergoes

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O-demethylation in human liver microsomes (HLMs), and this biotransformation is mainly catalyzed by cytochrome P450 (P450) 2D6 (CYP2D6) among 15 common human hepatic P450 enzymes (Yu et al., 2003). Note that CYP2D6 is one of the most important polymorphic phase I drug-metabolizing enzymes that is involved in the biotransformation of 20 to 30% of marketed drugs and some endogenous neuroregulators (Gonzalez and Yu, 2006; Yu, 2008). Currently, more than 90 allelic variants have been identified for the CYP2D6 gene, leading to considerable interindividual and interethnic variability in CYP2D6 drug-metabolizing activity. When individuals have impaired or deficient CYP2D6 activity, they may have increased risk of adverse drug reactions or therapeutic failure when taking a standard dose of CYP2D6-metabolized drugs. The use of appropriate phenotyping probes to define CYP2D6 activity in vitro and in vivo would be of importance to discover and develop better drugs and to achieve individualized medication (Yu et al., 2004; Zanger et al., 2004; Frank et al., 2007). Therefore, the aim of this study was to delineate the effects of CYP2D6 status on pinoline O-demethylation metabolism and to evaluate whether pinoline could be used to estimate CYP2D6 activity. We first investigated the functional difference of recombinant CYP2D6.1, CYP2D6.2, and CYP2D6.10 allelic isoforms toward pinoline O-demethylation and then conducted kinetic, inhibition, and correlation studies to define the role of CYP2D6 in pinoline O-demethylation in HLMs. Furthermore, we explored the selectivity and significance of CYP2D6 in pinoline O-demethylation in vivo by comparing pinoline urinary metabolic ratio (UMR) between the CYP2D6-humanized (Tg-CYP2D6) (Corchero et al., 2001) and wild-type control mice.

**ABBREVIATIONS:** HLMs, human liver microsomes; P450, cytochrome P450; UMR, urinary metabolic ratio; Tg-CYP2D6, CYP2D6-humanized; 6-HO-THBC, 6-hydroxy-1,2,3,4-tetrahydro-beta-carboline; HPLC, high-performance liquid chromatography; LC-MS/MS, liquid chromatography tandem mass spectrometry;  $CL_{int}$ , intrinsic clearance.

## Materials and Methods

**Chemicals and Materials.** Pinoline, harmaline,  $\beta$ -glucuronidase, and all chemical inhibitors were purchased from Sigma-Aldrich (St. Louis, MO). 6-Hydroxy-1,2,3,4-tetrahydro- $\beta$ -carboline (6-HO-THBC) was prepared as described previously (Yu et al., 2003). CYP2D6 isoforms were expressed using the baculovirus-mediated system and purified as reported previously (Yu et al., 2002, 2009). HLMs (h030, h006, h112, h066, h089, h056, h003, h088, h043, h093, h161) were purchased from BD Biosciences (Woburn, MA).

**Animals.** All mice were housed under controlled temperature ( $20 \pm 2^\circ\text{C}$ ), relative humidity (50–60%), and lighting (lights on 6:00 AM–6:00 PM) conditions, with food and water provided ad libitum. Age-matched adult (7 weeks old) wild-type FVB/N and Tg-CYP2D6 mice (Corchero et al., 2001) of 22 to 25 g were treated intraperitoneally with 30 mg/kg pinoline. Urine was collected from individual mice over 24 h after drug administration. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University at Buffalo.

**Incubation Conditions.** Incubation reactions with CYP2D6 allelic isoforms and HLMs were conducted in 100 mM potassium phosphate buffer, pH 7.4, in a final volume of 200  $\mu\text{l}$  at  $37^\circ\text{C}$ , as described previously (Yu et al., 2002; Felmlee et al., 2008; Zhang et al., 2009). In particular, each reaction consisted of 0.25 mg/ml microsomal proteins or 0.1  $\mu\text{M}$  cDNA-expressed CYP2D6, 0.2  $\mu\text{M}$  P450 reductase, and 10  $\mu\text{g}$  L- $\alpha$ -dilaurylphosphatidylcholine. All reactions were initiated by the addition of reduced nicotinamide adenine dinucleotide phosphate (1 mM final concentration). In kinetic studies, pinoline concentrations were 0.2 to 20  $\mu\text{M}$  for recombinant enzymes and 0.05 to 100  $\mu\text{M}$  for HLMs, respectively; incubations were carried out for 5 min for CYP2D6.1 and CYP2D6.2, 15 min for CYP2D6.10, and 10 min for HLMs, respectively. Pinoline concentration was fixed at 1  $\mu\text{M}$  for inhibition and correlation studies, and reactions lasted for 10 min. Incubation periods were selected so that the rates of metabolite production were within the linear ranges. Inhibitors included 2.5  $\mu\text{M}$   $\alpha$ -naphthoflavone for CYP1A2, 2.5  $\mu\text{M}$  8-methoxypsoralen for CYP2A6, 2 and 10  $\mu\text{M}$  tranylcypromine for CYP2A6/2B6/2C19, 2 and 10  $\mu\text{M}$  ticlopidine for CYP2B6/2C19, 20  $\mu\text{M}$  sulfaphenazole for CYP2C9, 1  $\mu\text{M}$  quinidine for CYP2D6, 100  $\mu\text{M}$  diethylthiocarbamate for CYP2A6/2B6/2E1, and 1  $\mu\text{M}$  ketoconazole for CYP3A4. After the reactions were terminated with 10  $\mu\text{l}$  of 60% perchloric acid, mixtures were centrifuged at 14,000g for 5 to 10 min and the supernatants were injected for high-performance liquid chromatography (HPLC) analysis. All reactions were performed in duplicate (correlation study) or triplicate (kinetic and inhibition studies).

**Quantification of Drug and Metabolite.** Analyses of in vitro incubation reactions were conducted with a Zorbax phenyl column, 5  $\mu\text{m}$ , 250 mm  $\times$  4.6 mm i.d. on an Agilent 1100 series HPLC system (Agilent Technologies, Santa Clara, CA), as described previously (Yu et al., 2003). Urine samples were diluted 10 times by blank urine. Forty microliters of diluted urine samples was incubated in the presence of 160 units of  $\beta$ -glucuronidase (80  $\mu\text{l}$ , 2000 units/ml in saline) at  $37^\circ\text{C}$  for 3 h. Reactions were terminated with 120  $\mu\text{l}$  of acetonitrile. The mixtures were centrifuged at 14,000 rpm for 5 min, and 100  $\mu\text{l}$  of supernatants were transferred to a new tube and diluted 15 times with 50% acetonitrile. The resultant mixtures were filtered with 0.2- $\mu\text{m}$  Supor Membrane filters (Pall Life Sciences, Ann Arbor, MI) and analyzed by a liquid chromatography tandem mass spectrometry (LC-MS/MS) that consisted of a Shimadzu prominence HPLC system (Shimadzu, Kyoto, Japan) and an API 3000 turbo ionspray ionization triple-quadrupole mass spectrometer (Applied Biosystems, Foster City, CA). A Luna phenyl-hexyl column (50  $\times$  4.6 mm, 3  $\mu\text{m}$ ) (Phenomenex, Torrance, CA) was used to separate pinoline, 6-HO-THBC, and harmaline (internal standard). The flow rate was 0.3 ml/min, and the mobile phase included buffer A (0.02% formic acid in water) and buffer B (0.02% formic acid in methanol). The gradient cycle consisted of a linear increase of buffer B from 5 to 80% at 0 to 9 min and an isocratic elution with 80% buffer B for 2 min followed by the initial condition (5% buffer B) from 11.5 to 15 min. The mass spectrometer was operated in positive-ion detection mode, and multiple reaction monitoring was used to analyze pinoline (with the transitions  $m/z$  203.2  $\rightarrow$  174.2), 6-HO-THBC (189.2  $\rightarrow$  160.3), and harmaline (215.2  $\rightarrow$  174.2). The instrumental parameters were tuned to maximize the multiple reaction monitoring signals. An online motorized six-port divert valve was used to introduce the HPLC eluent to the mass spectrometer over a period

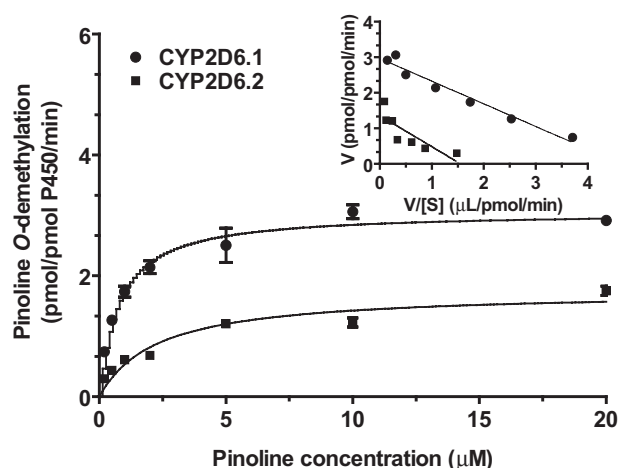


Fig. 1. Michaelis-Menten plots of pinoline *O*-demethylation catalyzed by CYP2D6.1 and CYP2D6.2 enzymes. Metabolite was undetectable in incubations with CYP2D6.10. Inserts are corresponding Eadie-Hofstee plots. Values represent the mean  $\pm$  S.E.M. of triplicate experiments. Estimated  $V_{\max}$  (pmol/pmol P450/min) and  $K_m$  ( $\mu\text{M}$ ) values were  $3.06 \pm 0.10$  and  $0.74 \pm 0.10$ , respectively, for CYP2D6.1, and  $1.75 \pm 0.13$  and  $2.28 \pm 0.55$ , respectively, for CYP2D6.2.

of 2.5 to 9 min for data acquisition, whereas the rest of flow was diverted to the waste. The calibration curve was linear from 12.5 to 500  $\mu\text{M}$  for pinoline and 250 to 5000  $\mu\text{M}$  for 6-HO-THBC, respectively.

**Data Analyses.** Values were expressed as mean  $\pm$  S.D. when experiments were conducted using different samples or mean  $\pm$  S.E.M. when using the same sample. Michaelis-Menten kinetic parameters,  $K_m$  and  $V_{\max}$ , were estimated by nonlinear regression (GraphPad Prism 5; GraphPad Software Inc., San Diego, CA). Intrinsic clearance ( $CL_{\text{int}}$ ) was calculated by dividing  $V_{\max}$  by  $K_m$ . Data were compared with unpaired two-tailed Student's *t* test (GraphPad Prism 5). Linear regression was conducted to examine the correlation of pinoline *O*-demethylase with P450 isoform-selective reactions (GraphPad Prism 5), and squared correlation coefficient ( $R^2$ ) was used to define the strength of a relationship. Statistical significance was considered if the *p* value was less than 0.05.

## Results and Discussion

To investigate the effects of CYP2D6 status on pinoline *O*-demethylation metabolism, we first compared the functional difference of recombinant CYP2D6.1, CYP2D6.2, and CYP2D6.10 allelic isoforms. CYP2D6\*2 occurs in up to 32% of whites and is usually classified as an allele with normal function as wild-type CYP2D6\*1, whereas the CYP2D6.2 protein exhibits lower or regular enzyme efficiency (Yu et al., 2002; Sakuyama et al., 2008) and shows relatively higher expression in HLMs (Zanger et al., 2001). The CYP2D6\*10 allele is present among 50% of Chinese and Japanese and is associated with reduced metabolic capacity (Yu et al., 2002; Shen et al., 2007; Sakuyama et al., 2008). Similar to what we had observed for codeine *O*-demethylation (Yu et al., 2002), CYP2D6.10 did not show any pinoline *O*-demethylase activity. In contrast, both CYP2D6.1 and CYP2D6.2 actively produced 6-HO-THBC from pinoline, showing one-enzyme Michaelis-Menten kinetics (Fig. 1). However, CYP2D6.2 had lower catalytic activity ( $CL_{\text{int}}$ ) than CYP2D6.1 (0.77 versus 4.14  $\mu\text{l/pmol}$  P450/min), which was due to its significantly higher  $K_m$  value than CYP2D6.1 ( $2.28 \pm 0.55$  versus  $0.74 \pm 0.10$   $\mu\text{M}$ ) and lower  $V_{\max}$  value than CYP2D6.1 ( $1.75 \pm 0.13$  versus  $3.06 \pm 0.10$  pmol/pmol P450/min).

To further define the role of CYP2D6 in pinoline *O*-demethylation in HLMs, we conducted inhibition and correlation studies. Among 8 chemical inhibitors, quinidine (1  $\mu\text{M}$ ) completely blocked pinoline *O*-demethylase activity in the pooled HLMs, whereas other inhibitors had no or minor effects (Fig. 2). Moreover, pinoline *O*-demethylase

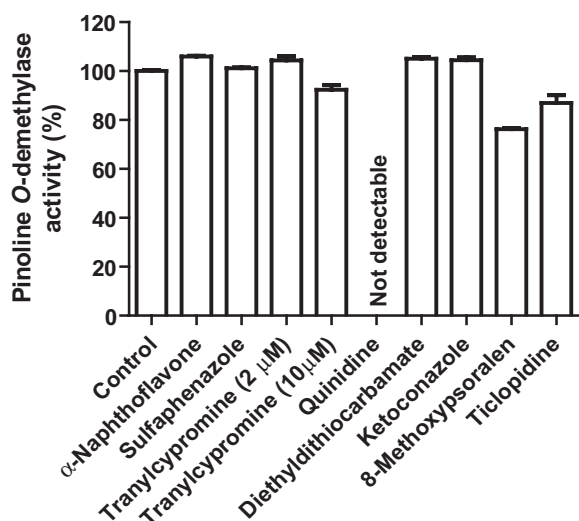


FIG. 2. Effects of chemical inhibitors on pinoline *O*-demethylase activity in HLMs. Pinoline (1 μM) was incubated with HLMs (0.25 mg/ml) for 10 min, in the absence and presence of individual inhibitors at different concentrations. Values are the mean ± S.E.M. of triplicate experiments.

activity in HLMs was strongly and significantly correlated with CYP2D6-catalyzed bufuralol 1'-hydroxylase activity ( $R^2 = 0.93$ ;  $p < 0.0001$ ) and immunoblot-estimated CYP2D6 content ( $R^2 = 0.82$ ;  $p = 0.005$ ), respectively (Table 1). Although correlation with CYP2A6 activity was statistically significant ( $p = 0.03$ ), the  $R^2$  was only 0.48 and the slope was 0.0056 to 0.065, suggesting that CYP2A6 would have limited contribution to pinoline *O*-demethylation, if there was any. Rather, no significant correlation was shown between pinoline *O*-demethylase activity and other P450 isoform-selective activity. In addition, although pinoline *O*-demethylation in the pooled HLMs showed biphasic enzyme kinetics over a wide range of substrate concentrations (0.05–100 μM), the estimated "high-affinity"  $K_m$  value was  $0.44 \pm 0.04$  μM, which is close to the  $K_m$  value for recombinant CYP2D6 (Yu et al., 2003). Of particular note, partial data (0.2–12.5 μM) were fitted well in the one-enzyme equation, as indicated by a linear Eadie-Hofstee plot and goodness of fit ( $R^2 = 0.96$ ), which gave an apparent  $K_m$   $1.30 \pm 0.14$  μM. In light of these observations and previous findings from recombinant P450s (Yu et al., 2003), pinoline *O*-demethylation may be catalyzed primarily by CYP2D6 in HLMs, and, in turn, pinoline *O*-demethylation may provide an additional means to probe CYP2D6 activity in vitro. Rather, similar to the use of other probes (e.g., dextromethorphan), an appropriate concentration (e.g., 1 μM) should be selected for pinoline because of possible involvement of "low-affinity" ( $K_m = 32 \pm 3$  μM) enzymes when higher concentrations (e.g., >10 μM) are used.

Furthermore, we used the Tg-CYP2D6 and wild-type mouse models to investigate the impact of CYP2D6 on pinoline *O*-demethylation in a whole body system. The two genotyped mice were shown to have similar debrisoquine UMR profiles as human CYP2D6 extensive metabolizers and poor metabolizers, respectively (Corchero et al., 2001). Our data showed that, 24 h after i.p. administration, approximately 55% of dosed drug (sum of pinoline and 6-HO-THBC over dose) was recovered in Tg-CYP2D6 mouse urine. This is consistent with a previous finding that approximately 70% of intraperitoneally administered [ $^{14}$ C]-labeled pinoline was recovered in the urine of male Sprague-Dawley rats (Ho et al., 1972), an animal model for CYP2D6 extensive metabolizers. Furthermore, pinoline was extensively metabolized to *O*-desmethyl metabolite in transgenic mice, which accounted for more than 99% of the excreta measured from

TABLE 1

Correlation between pinoline *O*-demethylase activity and individual P450 form-selective enzyme activities in HLMs ( $n = 10$ )

| Enzyme activity                                | P450     | $R^2$ | $p$     |
|--|----------|-------|---------|
| Phenacetin <i>O</i> -deethylase                | CYP1A2   | 0.050 | 0.535   |
| Coumarin 7-hydroxylase                         | CYP2A6   | 0.485 | 0.025   |
| ( <i>S</i> )-Mephenytoin <i>N</i> -demethylase | CYP2B6   | 0.005 | 0.841   |
| Paclitaxel 6α-hydroxylase                      | CYP2C8   | 0.089 | 0.402   |
| Diclofenac 4'-hydroxylase                      | CYP2C9   | 0.121 | 0.325   |
| ( <i>S</i> )-Mephenytoin 4'-hydroxylase        | CYP2C19  | 0.114 | 0.339   |
| Bufuralol 1'-hydroxylase                       | CYP2D6   | 0.931 | <0.0001 |
| Chlorzoxazone 6-hydroxylase                    | CYP2E1   | 0.112 | 0.345   |
| Testosterone 6β-hydroxylase                    | CYP3A4/5 | 0.041 | 0.576   |
| Lauric acid 12-hydroxylase                     | CYP4A    | 0.002 | 0.899   |
| CYP2D6 content                                 |          | 0.815 | 0.005   |

urine, resulting in an UMR value  $0.0070 \pm 0.0048$  ( $n = 4$ ). In contrast, only 14% of the administered drug was recovered in wild-type mouse urine, suggesting that compensatory metabolic pathways may occur in these mice. Indeed, approximately 28, 33, and 30% of pinoline recovered from rat urine were found to be the unchanged, 7-hydroxylated and *O*-demethylated pinoline, respectively (Ho et al., 1972). In addition, wild-type mice ( $n = 4$ ) showed a sharply higher UMR value ( $0.29 \pm 0.19$ ). These results indicate that distinct CYP2D6 status significantly alters pinoline *O*-demethylation in mouse models and pinoline UMR could serve as an indicator in assessing CYP2D6 activity in vivo. Nevertheless, at this time nothing is known about pinoline's toxicological profile in humans, although it is present in human tissues and is safe within physiological conditions, and pinoline has relatively low acute toxicity in mice ( $LD_{50}$ : 235 mg/kg i.p.; 112 mg/kg i.v.) (Airaksinen et al., 1978). Given the fact that pinoline is not currently an approved agent for use in humans, use of pinoline to probe CYP2D6 activity would be limited to preclinical settings despite its promise as a phenotyping agent in humans.

In summary, pinoline *O*-demethylation metabolism is primarily catalyzed by polymorphic CYP2D6. Compared with the wild-type CYP2D6.1, CYP2D6.2 and CYP2D6.10 showed reduced or deficient pinoline *O*-demethylase activity. The selective inhibition of pinoline *O*-demethylation by quinidine and significantly strong correlation between pinoline *O*-demethylation and CYP2D6 activity in HLMs, and the clear distinguishing of Tg-CYP2D6 from wild-type mice by urinary pinoline metabolic ratio suggest that pinoline may be an addition to the current probe drugs to evaluate CYP2D6 activity in preclinical studies.

Department of Pharmaceutical Sciences,  
School of Pharmacy and Pharmaceutical Sciences,  
University at Buffalo, the State University  
of New York, Buffalo, New York

XI-LING JIANG  
HONG-WU SHEN  
AI-MING YU

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**Address correspondence to:** Dr. Ai-Ming Yu, Department of Pharmaceutical Sciences, School of Pharmacy and Pharmaceutical Sciences, University at Buffalo, The State University of New York, NY 14260-1200. E-mail: [aimingyu@buffalo.edu](mailto:aimingyu@buffalo.edu)

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