**Short Communication**

**Differential Time- and NADPH-Dependent Inhibition of CYP2C19 by Enantiomers of Fluoxetine**

Received November 19, 2008; accepted January 12, 2009

**ABSTRACT:**
Fluoxetine ([±-N-methyl-3-phenyl-3-[(α, α- (trifluoro-p-tolyl)oxy)-propylamine]] is a widely used selective serotonin reuptake inhibitor, with more than 23 million prescriptions filled for the generic drug within the United States in 2006 (http://www.drugtopics.com/Top+200+Drugs). Racemic fluoxetine and its (R)- and (S)-enantiomers are metabolized by N-demethylation to the pharmacologically active metabolite norfluoxetine by CYP2D6 and other cytochrome P450 (P450) isoforms (Margolis et al., 2000; Mandrioli et al., 2006; refer to latter for chemical structures). Fluoxetine also undergoes CYP2C19 catalytic activity in vitro. In this study, we compared fluoxetine, its (R)- and (S)-enantiomers, ticlopidine, and S-benzyl nirvanol as potential time-dependent inhibitors of human liver microsomal CYP2C19. In a reversible inhibition protocol (30 min preincubation with liver microsomes without NADPH), we found (R)-, (S)- and racemic fluoxetine to be moderate inhibitors with IC50 values of 21, 93, and 27 μM, respectively. However, when the preincubation was supplemented with NADPH, IC50 values shifted to 4.0, 3.4, and 3.0 μM, respectively resulting in IC50 shifts of 5.2-, 28-, and 9.3-fold. Ticlopidine showed a 1.8-fold shift in IC50 value, and S-benzyl nirvanol shifted right (0.41-fold shift). Follow-up Kinact and kmax determinations with fluoxetine confirmed time-dependent inhibition [K values of 6.5, 47, and 14 μM; kmax values of 0.023, 0.085, 0.030 min⁻¹ for (R)-, (S)-, and racemate, respectively]. Although the (S)-isomer exhibits a much lower affinity for CYP2C19 inactivation relative to the (R)-enantiomer, it exhibits a more rapid rate of inactivation. Racemic norfluoxetine exhibited an 11-fold shift (18–1.5 μM) in IC50 value, suggesting that conversion of fluoxetine to this metabolite represents a metabolic pathway leading to time-dependent inhibition. These data provide an improved understanding of the drug-interaction potential of fluoxetine.

**Materials and Methods**

Materials. Pooled human liver microsomes (HLM), S-benzyl nirvanol, (S)-mephenytoin, (±)-4’-OH-mephenytoin, and stable-isotope-labeled (±)-4’-hydroxy mephenytoin-D3, were obtained from BD Biosciences (Woburn, MA). All other chemicals, including (±)-fluoxetine, (R)-fluoxetine, (S)-fluoxetine, (+)-norfluoxetine, and ticlopidine were obtained from Sigma-Aldrich (St. Louis, MO).

**S-Mephenytoin 4’-Hydroxylase IC50 Shift Assays.** Inhibition by test chemicals was determined using seven concentrations of inhibitor, separated by 0.5 log spacing, in a final volume 0.4 ml. Reactions contained 40 μM S-mephenytoin (approximately the K50), 0.3 mg/ml of pooled HLM, 1.3 mM NADP+, 3.3 mM glucose 6-phosphate, 0.4 U/ml glucose 6-phosphate dehydrogenase, 3.3 mM MgCl2, and 100 mM potassium phosphate buffer, pH 7.4. Reactions containing 5 μM HLM protein (e.g., 1.5 mg/ml) were incubated for 30 min with or without an NADPH-regenerating system before transfer of an aliquot into a secondary reaction mix containing the S-mephenytoin substrate.

**ABBREVIATIONS:** P450, cytochrome P450; HLM, human liver microsomes; MS, mass spectrometry; K50, inhibition constant; kmax, maximal inactivation rate constant.
In incubations were terminated after 10 min by transferring a 200-μl aliquot to 50 μl of formic acid (0.1%) in acetonitrile containing 0.5 μM stable-labeled isotope internal standard. After stopping the reactions, incubations were subjected to centrifugation at 4000 rpm for 20 min to compress the precipitated protein into a pellet, and the supernatants were retained for high-performance liquid chromatography/mass spectrometry (MS) analysis.

S-Mephenytoin 4'-Hydroxylase K_i and k_{inact} Assays. Incubations were performed in 0.1 M potassium phosphate (pH 7.4) with 1.5 mg/ml HLM protein with a NADPH-generating system (as described above). Solvent only and five concentrations of inhibitor (3, 10, 30, 100 and 300 μM) were tested in duplicate. After various incubation times (2, 6, 11, 17, 23, and 30 min), 80 μl was removed and added to 320 μl of a secondary S-mephenytoin assay mixture (0.1 M potassium phosphate with 1.3 mM NADP, 3.3 mM glucose 6-phosphate, 0.4 U/ml glucose-6-phosphate dehydrogenase, 3.3 mM magnesium chloride, and 200 μM S-mephenytoin). Unless otherwise indicated, after 10 min of incubation, the reaction was stopped and processed for liquid chromatography/MS analysis as described above.

Data Analysis. The IC_{50} values were calculated by linear interpolation. IC_{50} shifts were calculated by dividing the IC_{50} value in the absence of NADPH by the IC_{50} values in the presence of NADPH. For K_i/k_{inact} assays, the natural logarithm of percentage remaining activity (corrected for decrease in metabolism over time in absence of inhibitor) was plotted against preincubation time for each concentration of inhibitor tested. The slopes of the linear portion of each plot were determined, and the -slope versus inhibitor concentration dataset was fitted to a Michaelis-Menten model, k = (k_{inact} × I)/(1 + I), to obtain k_{inact} (maximum rate of inactivation) and K_i (inhibitor concentration associated with half-maximal inactivation rate) values. The K_i and k_{inact} values were determined by nonlinear regression using SigmaPlot software, version 8.0 (Systat Software, Inc., San Jose, CA), equipped with Enzyme Kinetic module, version 1.1 (SPSS Inc., Chicago, IL). Data for the 300 μM (R)- and (S)-racemic fluoroxetine were excluded for analysis because maximal rate of inactivation was consistently found at the 100 μM concentration.

Results and Discussion

Results for IC_{50} shifts, k_{inact} and K_i determinations for the inhibition of human liver microsomal CYP2C19-catalyzed S-mephenytoin 4'-hydroxylation are shown Table 1. Obach et al. (2007) demonstrated the utility of IC_{50} shift assays as an effective and simplified means for assessing the potential for drugs to inactivate P450. Indeed, testing for time- and concentration-dependent inhibition after a preincubation in the presence of NADPH has been advocated in recent U.S. Food and Drug Administration guidance (U.S. Food and Drug Administration, http://www.fda.gov/cber/gdlns/interactstud.htm). In the present study, we found that (S)-fluoxetine exhibited a mean 28-fold IC_{50} shift, whereas (R)-fluoxetine or racemic fluoroxetine exhibited lower shifts of approximately 5- and 9-fold, respectively (Fig. 1). Follow-up studies demonstrated that (S)-, (R)-, and (±)fluoxetine exhibited mean k_{inact} values of 0.085 min^{-1}, 0.023 min^{-1}, and 0.030 min^{-1}, respectively. Mean K_i values were 47, 7, and 14 μM for (S)-, (R)-, and (±), respectively. Figure 2 shows representative plots of data used to obtain K_i and k_{inact} values. Although the (S)-isomer exhibits an approximately 4-fold more rapid rate of inactivation, the lower affinity for CYP2C19 inactivation makes the (S)-isomer less efficient relative to the (R)-isomer. The K_{inact} and K_i values for racemic fluoroxetine agree well with the k_{inact} and apparent K_i values of 0.023 min^{-1} and 8 μM reported by McGinnity et al. (2006), respectively. Values were not corrected for nonspecific binding, which can be substantial for fluoroxetine (Margolis and Obach, 2003). Correcting for unbound fluoroxetine, the estimates of K_i values would be [10-fold lower (McGinnity et al., 2006)] and are within steady-state total plasma levels of fluoroxetine (ranging from 0.15 to 1.5 μM) found after therapeutic dosing of fluoroxetine (Orsulak et al., 1988). Obach et al. (2007) has suggested a relationship between the magnitude of drug interactions, I/K_i and k_{inact}/k_{deg}, where k_{deg} is the in vivo degradation rate of the P450 under investigation. Using an estimate of k_{deg} of 0.0008 min^{-1} (Mayhew et al., 2000), we would estimate k_{inact}/k_{deg} ratios of 25 to 150 and I/K_i ratios ranging from approximately 0.1 to 1 for fluoroxetine and its isomers. These data indicate the likelihood of drug interactions of fluoroxetine and drug clearance predominantly by CYP2C19 may be significant based on the contour plot model suggested by Obach et al. (2007). Indeed, there have been several reports of drugs interactions and/or inhibition by fluoroxetine of CYP2C19-mediated metabolism in vivo (Flockhart, 1995; Dingemanse et al., 1998; Harvey and Preskorn, 2001). Consistent with CYP2C19 inactivation is the previous finding that both (R)- and (S)-isomers of fluoroxetine are substrates for this enzyme (as well as CYP3A4, CYP2D6, and CYP2C9), with (S)-isomer having marginally higher intrinsic clearance (Margolis et al., 2000).

The mechanism of time-dependent inhibition was investigated by assessing the IC_{50} shift of a major fluoroxetine metabolite, norfluvoxetine racemate. We found an 11-fold shift, suggesting that metabolic conversion to this metabolite represents one pathway leading to time-dependent inhibition. Alternate metabolites may be responsible for the time-dependent inhibition found here. Indeed, a recent report suggests that the primary fluoroxetine metabolites (S)- and (R)-N-hydroxyfluvoxetine exhibit time-dependent inhibition in CYP2C19 Supersomes and are capable of forming a metabolite intermediate complex (Vanden-Brink et al., 2008).

In our hands, the CYP2C19 mechanism-based inhibitor ticlopidine exhibited a relatively weak but reproducible IC_{50} shift of 1.8 ± 0.53 and was consistent with previous reports (Obach et al., 2007). In the IC_{50} shift assay, compounds that are metabolically depleted or cause very rapid inactivation may have little or no shift or even shift in a
reverse direction. The latter occurred when we tested the competitive inhibitor (S)-benzyl nirvanol (0.41-fold shift) and was probably due to metabolic depletion.

In summary, we have shown that enantiomers of fluoxetine exhibit time-dependent inhibition of human liver microsomal CYP2C19 and differ in both affinity and rate of inactivation. These data should help in our understanding of potential drug-drug interactions elicited by fluoxetine. From a practical viewpoint, due to its large IC$_{50}$ shift value, (S)-fluoxetine seems to be a highly robust reference inhibitor for use in the routine measurement of time-dependent inhibition of liver microsomal CYP2C19.

References


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