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Differential Time- and NADPH-dependent inhibition of CYP2C19 by enantiomers of fluoxetine.

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Running title: Time-dependent inhibition of CYP2C19 by fluoxetine enantiomers

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Number of text pages: 8

Number of tables: 1

Number of figures: 2

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Abstract: 250

Introduction: 322

Results/Discussion: 668

List of non-standard abbreviations:

CYP, cytochrome P450; HLM, human liver microsomes; K_I, Inhibition constant; k_{inact}, maximal inactivation rate constant.

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Abstract

Fluoxetine [±-N-methyl-3-phenyl-3-[(α, α, α -trifluoro-p-tolyl)oxy]propylamine)] a selective serotonin reuptake inhibitor, is widely used in treating depression and other serotonin-dependent disease conditions. Racemic, (R)- and (S)- fluoxetine are potent reversible inhibitors of CYP2D6 and the racemate has been shown to be a mechanism-based inhibitor of CYP3A4. Racemic fluoxetine also demonstrates time- and concentration-dependent inhibition of CYP2C19 catalytic activity in vitro. In this study, we compared fluoxetine, its (R)- and (S)-enantiomers, ticlopidine and S-benzylnirvanol 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 IC_{50} values of 21, 93 and 27 µM, respectively. However, when the preincubation was supplemented with NADPH, IC_{50} values shifted to 4.0, 3.4 and 3.0 µM, respectively resulting in IC_{50} shifts of 5.2, 28 and 9.3-fold. Ticlopidine showed a 1.8-fold shift in IC_{50}-value and S-benzylnirvanol shifted right (0.41-fold shift). Follow up K_I and k_{inact} determinations with fluoxetine confirmed time-dependent inhibition [K_I values of 6.5, 47 and 14 µM; k_{inact} 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 to 1.5 µM) in IC_{50} value suggesting 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.

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Introduction

Fluoxetine [±-N-methyl-3-phenyl-3-[(α, 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 (Drugtopics.com). Racemic fluoxetine and its (R)- and (S)- enantiomers are metabolized by N-demethylation to the pharmacologically active metabolite norfluoxetine by CYP2D6 and other P450 isoforms (Margolis et al, 2000; Mandrioli et al, 2006; refer to latter for chemical structure). Fluoxetine also undergoes CYP2C19-mediated O-dealkylation to the p-trifluoromethylphenol metabolite (Liu et al, 2001). In addition, racemic fluoxetine and/or its enantiomers have been shown to be reversible inhibitors of CYP2D6 (Brosen K and Skjelbo, 1991; Stevens and Wrighton, 1993), CYP2C19 (Kobayashi et al., 1995; Foti and Wahlstrom, 2008), CYP3A4 (von Moltke et al., 1994; Ring et al., 1995), and CYP2C9 (Schmider et al., 1997; Hemeryck et al., 1999). Fewer studies have been conducted examining the potential for fluoxetine to be a mechanism-based inhibitor of cytochrome P450. Mayhew et al (2000) showed fluoxetine to be a mechanism-based inhibitor of CYP3A4 and McGinnity et al (2006) recently demonstrated time- and concentration-dependent inhibition of CYP3A4 and also CYP2C19 in multiple in vitro systems, including hepatocytes. With heightened awareness of links between mechanism-based inhibitors, covalent binding and idiosyncratic toxicity (Ulrich, 2007) as well as the appearance of regulatory guidance for drug-drug interaction testing (USFDA, 2006), many laboratories are establishing or revisiting their procedures for conducting time-dependent CYP inhibition testing. In the process of augmenting our laboratory's CYP2C19 time-dependent inhibition assay, we

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tested model compounds intended to serve as reference inhibitors. In many laboratories, ticlopidine is used as a positive control inhibitor in this assay (Ha-Duong et al 2001), but we and others have found it to be only weakly inhibitory (Stresser et al, 2008), and is therefore unsatisfactory as a potent acting benchmark. In this report, we confirm the findings of McGinnity et al (2006) with racemic fluoxetine and show that the enantiomers of fluoxetine are effective, but kinetically different, time-dependent inhibitors of CYP2C19.

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Methods

Materials. Pooled human liver microsomes, S-benzylnirvanol, (S)-mephenytoin, (±)4'-OH- mephenytoin, 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 IC_{50} 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 K_M), 0.3 mg pooled HLM, 1.3 mM NADP+, 3.3 mM glucose 6-phosphate, 0.4 U/ml glucose 6-phosphate dehydrogenase, 3.3 mM $MgCl_2$ and 100 mM potassium phosphate buffer, pH 7.4. Reactions containing 5X HLM protein (e.g. 1.5 mg/ml) were incubated for 30 min with or without an NADPH regenerating system prior transfer of an aliquot into a secondary reaction mix containing the S-mephenytoin substrate. Incubations were terminated after 10 min by transferring a 200 μL aliquot to a 50 μL 0.1% formic acid 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 minutes to compress the precipitated protein into a pellet and the supernatants were retained for HPLC/MS analysis.

S-Mephenytoin 4'-hydroxylase K_I and k_{inact} assays. Incubations were performed in 0.1M potassium phosphate (pH 7.4) with 0.3 mg/ml HLM protein with an NADPH generating system (as described above). Solvent only and 5 concentrations of inhibitor (3, 10, 30, 100 and 300 μM) were tested in duplicate. After various incubation times (2, 6,

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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 minutes of incubation, the reaction was stopped and processed for LC/MS analysis as above.

Analytical methods.

The 4'-hydroxy (S)-mephenytoin metabolite was quantified using a 4000 Q-trap LC/MS/MS system (Applied Biosystems, Foster City, CA) equipped with a dual pump system (Perkin-Elmer, Wellesley, MA) and a LEAP CTC HTS PAL autosampler as previously described (Perloff et al, 2009). Mass transitions were 235.1 → 150.0 for 4'-hydroxy (S)-mephenytoin metabolite and 238.1→ 150.0 for the internal standard 4-hydroxy-S-mephenytoin-D3.

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 percent 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 vs. inhibitor concentration data set was fitted to a Michaelis-Menten model, $k = (k_{inact} \times I)/(I + K_I)$, 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

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regression using SigmaPlot software, v. 8.0, equipped with Enzyme Kinetic module v. 1.1 (SPSS, Chicago). Data for the 300 µM (R)- (S)- and racemic fluoxetine were excluded for analysis as maximal rate of inactivation was consistently found at the 100 µM concentration.

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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 cytochrome P450. Indeed, testing for time- and concentration-dependent inhibition after a preincubation in the presence of NADPH has been advocated in recent FDA guidance (USFDA, 2006). In the present study, we found (S)-fluoxetine exhibited a mean 28-fold IC_{50} shift, whereas (R)-fluoxetine or racemic fluoxetine 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 fluoxetine agree well with the k_{inact} and apparent K_I values of 0.03 $^{min-1}$ and 8 µM reported by McGinnity et al (2006), respectively. Values were not corrected for non-specific binding which can be substantial for fluoxetine (Margolis and Obach, 2003). Correcting for unbound fluoxetine, the estimates of K_I values would be ~ 10-fold lower (MgGinnity et al, 2006) and are within steady state total plasma levels of fluoxetine (ranging from 0.15 to 1.5 µM) found after therapeutic dosing of fluoxetine (Orsulak et al., 1988). Obach et al (2007) has suggested a relationship between the magnitude of drug interactions, I/K_I and k_{deg}/k_{inact}

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where k_{deg} is the in vivo degradation rate of the CYP 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 fluoxetine and its isomers. These data indicate the likelihood of drug interactions of fluoxetine and drug cleared 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 fluoxetine of CYP2C19 mediated metabolism in vivo (Harvey and Preskorn, 2001; Flockhart, 1995; Dingemanse et al, 1998). Consistent with CYP2C19 inactivation is the previous finding that both (R)- and (S)-isomers of fluoxetine 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 fluoxetine metabolite, norfluoxetine 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 fluoxetine metabolites (S)- and (R)- N-hydroxyfluoxetine exhibit time-dependent inhibition in CYP2C19 Supersomes® and are capable of forming a metabolite intermediate complex (Vandenbrink 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

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reverse direction. The latter occurred when we tested the competitive inhibitor (S)-benzylnirvanol (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 appears to be a highly robust reference inhibitor for use in the routine measurement of time-dependent inhibition of liver microsomal CYP2C19.

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Legends for Figures

Figure 1. Representative plots of IC_{50} curves for S-fluoxetine (A), R-fluoxetine (B) and racemic fluoxetine (C). Data points represent means of duplicate incubations. Calculated IC_{50} shifts for each plot were 33, 5.5 and 9.4 respectively.

Figure 2. Representative plots of inactivation rate constant (k) and inhibitor concentration for S-fluoxetine (A), R-fluoxetine (B) and racemic fluoxetine (C). The data were generated in pooled human liver microsomes by monitoring the natural log of percent S-mephenytoin 4'-hydroxylase activity remaining after preincubation times of 2, 6, 11, 17, 23 and 30 min and correction for fluoxetine-independent loss of enzyme activity. Nonlinear regression analysis using the equation $k = (k_{inact} \times I)/(I + K_I)$ was applied to determine values of k_{inact} and K_I.

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Table 1. Summary of CYP2C19 IC_{50} shift, K_i and k_{inact} values for ticlopidine, (S)-benzylnirvanol, racemic, (S)-, (R)-fluoxetine and racemic norfluoxetine in human liver microsomes. Values shown are results from experiments conducted in duplicate on independent days

Compound	IC_{50} - NADPH	IC_{50} + NADPH	IC_{50} shift	k_{inact} (min^{-1})	K_I (μM)
(±)-fluoxetine	22, 37, 22	3.3, 3.9, 1.8	6.6, 9.4, 12	0.031, 0.029, 0.031	17, 11, 13
(S)-fluoxetine	91, 79, 109	2.8, 3.3, 4.0	33, 24, 28	0.075, 0.115, 0.064	40, 54, 46
(R)-fluoxetine	20, 22, 21	3.5, 3.9, 4.7	5.6, 5.5, 4.4	0.026, 0.024, 0.018	7.5, 6.8, 5.3
(±)-norfluoxetine	18	1.5	11	N.D.	N.D.
(S)-benzylnirvanol	0.19, 0.16, 0.17	0.41, 0.43, 0.44	0.46, 0.38, 0.38	N.A.	N.A.
ticlopidine	1.14, 1.20, 1.43	0.81, 0.76, 0.59	1.4, 1.6, 2.4	N.D.	N.D.

N.A – Not applicable

N.D. – Not done.

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Figure 1

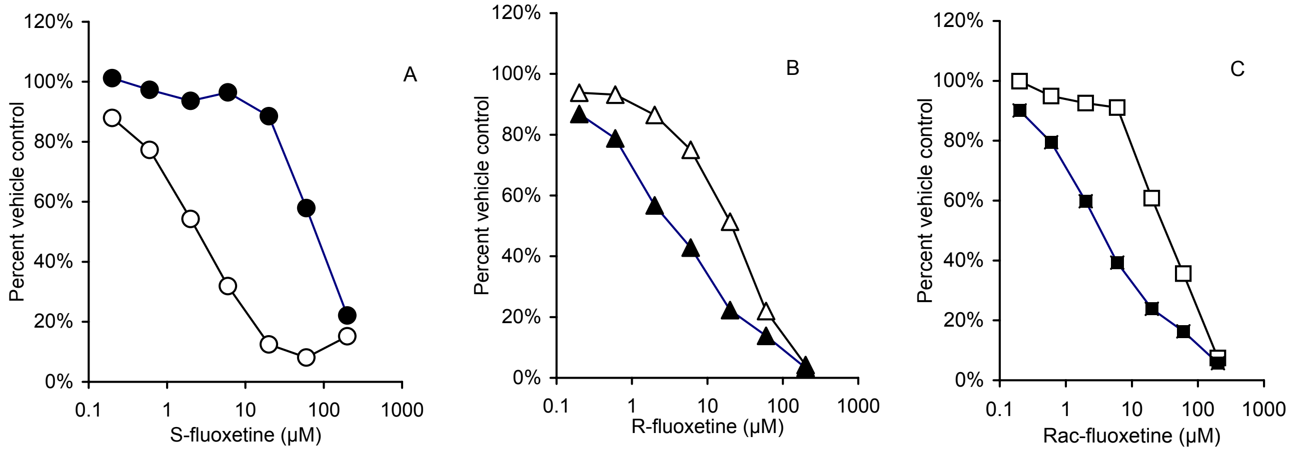

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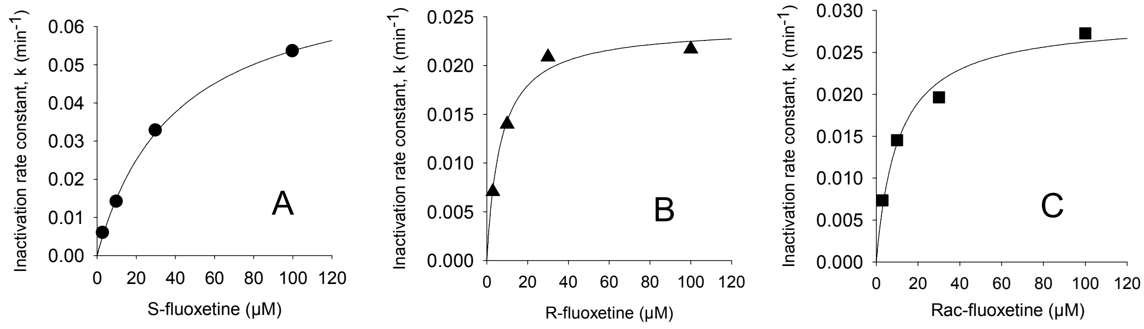
Figure 2



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