Special Section on Prediction of Human Pharmacokinetic Parameters from In Vitro Systems

Stereoselective Inhibition of CYP2C19 and CYP3A4 by Fluoxetine and Its Metabolite: Implications for Risk Assessment of Multiple Time-Dependent Inhibitor Systems^[S]

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ABSTRACT

Recent guidance on drug-drug interaction (DDI) testing recommends evaluation of circulating metabolites. However, there is little consensus on how to quantitatively predict and/or assess the risk of in vivo DDIs by multiple time-dependent inhibitors (TDIs) including metabolites from in vitro data. Fluoxetine was chosen as the model drug to evaluate the role of TDI metabolites in DDI prediction because it is a TDI of both CYP3A4 and CYP2C19 with a circulating N-dealkylated inhibitory metabolite, norfluoxetine. In pooled human liver microsomes, both enantiomers of fluoxetine and norfluoxetine were TDIs of CYP2C19, (S)-norfluoxetine was the most potent inhibitor with time-dependent inhibition affinity constant (K_i) of 7 μM , and apparent maximum time-dependent inhibition rate ($k_{\text{inact,app}}$) of 0.059 min^{-1} . Only (S)-fluoxetine and (R)-norfluoxetine were TDIs of CYP3A4, with (R)-norfluoxetine being the most potent ($K_i = 8 \mu\text{M}$, and $k_{\text{inact,app}} = 0.011 \text{ min}^{-1}$).

Based on in-vitro-to-in-vivo predictions, (S)-norfluoxetine plays the most important role in in vivo CYP2C19 DDIs, whereas (R)-norfluoxetine is most important in CYP3A4 DDIs. Comparison of two multiple TDI prediction models demonstrated significant differences between them in in-vitro-to-in-vitro predictions but not in in-vitro-to-in-vivo predictions. Inclusion of all four inhibitors predicted an in vivo decrease in CYP2C19 (95%) and CYP3A4 (60–62%) activity. The results of this study suggest that adequate worst-case risk assessment for in vivo DDIs by multiple TDI systems can be achieved by incorporating time-dependent inhibition by both parent and metabolite via simple addition of the in vivo time-dependent inhibition rate/cytochrome P450 degradation rate constant (λ/k_{deg}) values, but quantitative DDI predictions will require a more thorough understanding of TDI mechanisms.

Introduction

Multiple inhibitor systems include administration of multiple independent inhibitors, an inhibitor with a circulating inhibitory metabolite, or a racemic inhibitor with stereoselective inhibition. Of these, inhibitors with inhibitory metabolites are well recognized. An analysis of 129 marketed in vivo cytochrome P450 (P450) inhibitors showed that 80% of these inhibitors possess circulating metabolites

(Isoherranen et al., 2009), of which some are confirmed inhibitors (Yeung et al., 2011). When chiral drugs administered as racemic mixtures (20% of the inhibitors) are accounted for, a total of 90% of P450 inhibitors are potential multiple inhibitor systems, highlighting the need for development of risk-assessment methods for multiple inhibitor systems.

Studies of in-vitro-to-in-vivo prediction of drug-drug interactions (DDIs) resulting from reversible P450 inhibition have demonstrated that incorporation of metabolites and stereoisomers (Reese et al., 2008; Templeton et al., 2010; Guest et al., 2011; Lutz and Isoherranen, 2012) increases the accuracy of the predictions. Despite increased prediction accuracy, for most of the known reversible P450 inhibitors, incorporation of metabolite inhibition did not alter DDI risk assessment (Yeung et al., 2011). The effect of metabolites in risk assessment of time-dependent inhibitors (TDIs) has not been systematically

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ABBREVIATIONS: AUC, area under the curve; CL_i , intrinsic clearance; DDI, drug-drug interaction; f_u , fraction unbound; HLM, human liver microsome; k_{deg} , P450 degradation rate constant; K_i , reversible inhibition affinity constant; K_i , time-dependent inhibition affinity constant; $k_{\text{inact,app}}$, apparent maximum time-dependent inhibition rate; K_m , Michaelis-Menten affinity constant; λ , time-dependent inhibition rate; MIC, metabolic-intermediate complex; P450, cytochrome P450; TDI, time-dependent inhibitor.

evaluated, although both static and dynamic prediction methods for TDIs are well established (Galetin et al., 2006; Ghanbari et al., 2006; Obach et al., 2007; Grimm et al., 2009; Quinney et al., 2010). Circulating metabolites are likely important in P450 time-dependent inhibition and should be characterized to improve in vivo DDI predictions and understanding (VandenBrink and Isoherranen, 2010). The U.S. Food and Drug Administration (FDA) and the European Medicines Agency recommend that in vivo circulating metabolites be characterized for in vitro P450 inhibition if the metabolite area under the curve (AUC) is $\geq 25\%$ of the parent AUC or if unbound metabolite concentrations are $>10\%$ of unbound parent concentrations, but little guidance exists on how to incorporate the contribution of TDI metabolites to prediction of DDIs. Although models have been established for the prediction of single inhibitors with multiple interaction mechanisms (Fahmi et al., 2009) or combined inhibition of transport and metabolism (Hinton et al., 2008), there are limited data on predictions for multiple inhibitors each with multiple inhibition mechanisms.

TDIs were estimated to constitute approximately 25% of in vivo P450 inhibitors in 2009 (Isoherranen et al., 2009). Since then, several new P450 TDIs have been approved, including boceprevir (Victrelis 2011; http://www.merck.com/product/usa/pi_circulars/v/victrelis/victrelis_pi.pdf), telaprevir (Incivek 2013; http://pi.vrtx.com/files/uspi_telaprevir.pdf), crizotinib (Mao et al., 2013), and erlotinib and everolimus (Kenny et al., 2012), demonstrating the continued clinical significance of TDIs. Almost half of in vitro TDIs are alkylamine drugs (VandenBrink and Isoherranen, 2010) that undergo initial N-dealkylation and subsequent metabolism to result in P450 time-dependent inhibition via a quasi-irreversible heme coordinated metabolic-intermediate complex (MIC) (Kalgutkar et al., 2007). All alkylamine TDIs possess an in vivo circulating N-dealkylated metabolite that can also inactivate P450s (VandenBrink and Isoherranen, 2010), but only a few studies have examined the role of these metabolites in in vivo DDIs. Two models have been evaluated to improve upon the underprediction of in vivo CYP3A4 inhibition using diltiazem and verapamil and their N-dealkylated metabolites as models. In these models, the TDI kinetics of the parent and metabolite were either summed up to predict total time-dependent inhibition (Wang et al., 2004; Rowland Yeo et al., 2010) or a mutual in vivo inhibitor-inhibitor interaction component was incorporated to predict in vivo interaction (Zhang et al., 2009b). Both models demonstrated improved prediction accuracy with the inclusion of metabolite time-dependent inhibition when compared with the parent alone, suggesting that incorporation of multiple inhibitors into TDI predictions and risk assessment is necessary.

This study establishes how multiple inhibitor systems, which include time-dependent inhibition, can be incorporated into DDI risk assessment. The secondary alkylamine fluoxetine was used as a model because it is a complex multiple P450 inhibitor. Fluoxetine provides both a model of a metabolite-parent pair and enantiomer mixture that incorporates combinations of time-dependent and reversible inhibition with multiple P450s. Fluoxetine and its circulating metabolite norfluoxetine are present as mixtures of stereoisomers in vivo. The (S)-enantiomers circulate at 210–280% of the (R)-enantiomers, and norfluoxetine enantiomers at 150–180% of fluoxetine enantiomers (Jannuzzi et al., 2002), hence meeting the FDA criteria for metabolite testing. In vitro, (R)- and (S)-fluoxetine are TDIs of CYP2C19 (Stresser et al., 2009) and racemic fluoxetine is a TDI of CYP3A4 (Mayhew et al., 2000). Racemic norfluoxetine causes an IC_{50} shift with CYP2C19 (Stresser et al., 2009) and appears to inhibit CYP3A4 reversibly (von Moltke et al., 1996). In this study, fluoxetine and norfluoxetine were stereoselectively characterized as CYP2C19 and CYP3A4 TDIs. The contribution of norfluoxetine to the predicted DDI was determined for

each P450, and the prediction of multiple TDIs between CYP2C19 and CYP3A4 was compared.

Materials and Methods

Chemicals and Reagents. (R)-Fluoxetine, (S)-fluoxetine, *rac*-fluoxetine, *rac*-norfluoxetine, and mass spectrometry-grade acetonitrile and water were purchased from Sigma-Aldrich (St. Louis, MO). Midazolam, 1-hydroxymidazolam, and 1-hydroxymidazolam-d₄ were purchased from Cerilliant (Round Rock, TX). 4-hydroxymephenytoin-d₃ was obtained from Toronto Research Chemicals (Toronto, ON, Canada). (S)-Mephenytoin and 4-hydroxymephenytoin were synthesized in the laboratory of Dr. William Trager (University of Washington, Seattle, WA). The (R)- and (S)-enantiomers of norfluoxetine were synthesized as previously described (Hanson et al., 2010).

Determination of Reversible and Time-Dependent Inhibition Constants. For all metabolic incubations, six different human liver microsome (HLM) donors were pooled. All donors were confirmed to be CYP2D6*1/*1, CYP2D6*1/*2, or CYP2D6*2/*2 genotype, CYP2C19*1/*1 and CYP3A5*3/*3 genotypes to limit confounding effects of genetic polymorphisms, and CYP3A substrate overlap in inhibition characterization. All pooled HLM experiments were performed in triplicate at either 0.1 mg/ml (reversible inhibition) or 1.0 mg/ml (time-dependent inhibition) microsomal protein concentration in 100 mM potassium phosphate buffer at pH = 7.4. All reversible and time-dependent inhibition was determined using 11 and 7 inhibitor concentrations (2-fold serial dilutions), respectively. Maximum inhibitor concentrations were 100 μ M, except for when determining time-dependent CYP2C19 inhibition of (R)- and (S)-fluoxetine (50 and 250 μ M, respectively) and reversible CYP3A4 inhibition of (R)- and (S)-fluoxetine (1000 μ M for both). For reversible inhibition, the substrate concentrations were approximately 5-fold below K_m : 6 μ M for (S)-mephenytoin (CYP2C19) and 1 μ M for midazolam (CYP3A4). For time-dependent inhibition, the substrate concentrations were 5-fold above K_m : 150 μ M for (S)-mephenytoin and 25 μ M for midazolam. After a 5-minute preincubation at 37°C, all HLM incubations were initiated with NADPH (1 mM final concentration) and allowed to proceed at 37°C. For the time-dependent inhibition determination, no substrate was initially present, but the aliquots were diluted 10-fold into wells containing 1 mM NADPH and substrate after 0.25, 10, 20, and 30 minutes. In the HLM incubation experiments, $<10\%$ of inhibitor depletion and a negligible accumulation of norfluoxetine ($<6\%$ of fluoxetine initial concentration) under the incubation conditions were confirmed. All HLM incubations with substrate proceeded for 3 minutes [midazolam] and 15 minutes [(S)-mephenytoin] before quenching with an equal volume of acetonitrile containing 100 nM internal standard. The inhibitor concentration at 50% maximum inhibition (IC_{50}) was determined by eq. 1:

$$v_I = v_0 \left(1 - \frac{I}{IC_{50} + I} \right) \quad (1)$$

where v_I and v_0 are the velocity of product formation at a given inhibitor concentration (I) or without the presence of inhibitor, respectively. The time-dependent inhibition affinity (K_I) and apparent maximum time-dependent inhibition rate ($k_{inact,app}$) were determined by the equation:

$$v_I = v_0 e^{-\lambda t} \quad (2)$$

where λ is:

$$\lambda = \frac{k_{inact,app} I}{K_I + I} \quad (3)$$

All velocity data were transformed to the percentage of maximum velocity (no inhibitor control). All inhibition constants are presented as mean and standard error. All in vitro constants were determined via nonlinear regression using GraphPad Prism v.5 (La Jolla, CA). Two-sided t tests were used to evaluate the statistical significance of the change in time-dependent inhibition rate between 100 μ M inhibitor and control during the initial time-dependent inhibition screening. $P < 0.05$ was considered statistically significant.

Determination of Fluoxetine Protein Binding. Using the pooled HLMs (at 1 mg/ml and 0.1 mg/ml microsomal protein in potassium phosphate buffer) or blank human plasma, (*R*)-fluoxetine, (*S*)-fluoxetine, (*R*)-norfluoxetine, or (*S*)-norfluoxetine were added to triplicate samples to make a 1 μ M final concentration. The samples were split to two ultracentrifuge tubes at equal volumes and were either centrifuged at 440,000g for 90 minutes at 37°C or incubated for 90 minutes at 37°C as previously described (Templeton et al., 2010). An aliquot was removed from both tubes, and an equal volume of acetonitrile was added; the samples were centrifuged at 3000g for 15 minutes, and the supernatant was used for analysis. For the fraction unbound (f_u), the $f_{u,HLM}$ or $f_{u,plasma}$ was determined as the quotient of determined inhibitor concentration with and without centrifugation.

Quantitation of Analytes. Concentrations of 1-hydroxymidazolam, (*S*)-4-hydroxymephenytoin, (*R*)-fluoxetine, (*S*)-fluoxetine, (*R*)-norfluoxetine, and (*S*)-norfluoxetine were analyzed using a Shimadzu Prominence ultra-high-performance liquid chromatography (Tokyo, Japan) coupled to an AB Sciex API 3200 tandem mass spectrometer (Framingham, MA). All analytes were separated using an Agilent Zorbax Eclipse XDB 2.1 $\times$ 50 mm, 5 μ m column (Santa Clara, CA) with a linear gradient elution from 95% water with 0.1% formic acid/5% acetonitrile to 50% acetonitrile over 3 minutes, 95% acetonitrile for 2 minutes, then allowed to re-equilibrate to initial conditions for 2 minutes. All analytes were detected using positive electrospray ionization with a source temperature of 500°C, ionization voltage of 5500 V, curtain gas of 10, collision activated dissociation gas of 5, source gas 1 of 80, and source gas 2 of 60. The multiple reaction monitoring transitions (m/z) used were 342 $\rightarrow$ 324 (1-hydroxymidazolam), 235 $\rightarrow$ 150 (4-hydroxymephenytoin), 310 $\rightarrow$ 44 [(*R*)- or (*S*)-fluoxetine], and 296 $\rightarrow$ 134 [(*R*)- or (*S*)-norfluoxetine]. The injection volume for all assays was 10 μ L. The lower limit of quantitation was less than 5 nM for all analytes. Interday percent coefficient of variation for all analytes at 5 nM was less than 15%. Analyst software version 1.4 (AB Sciex, Foster City, CA) was used for data analysis. All samples were protein precipitated with an equal volume of acetonitrile, centrifuged at 3000g for 15 minutes, and the supernatant was used for analysis. The organic solvent contained either 100 nM of 1-hydroxymidazolam- d_4 or 4-hydroxymephenytoin- d_3 as internal standards.

Simulations and Predictions Using In Vitro Data. The apparent time-dependent inhibition rate (λ_{app}) versus inhibitor concentration curve for *rac*-fluoxetine, *rac*-norfluoxetine, 1:1 (*R*)-fluoxetine:(*R*)-norfluoxetine, and 1:1 (*S*)-fluoxetine:(*S*)-norfluoxetine were simulated using the determined component enantiomer time-dependent inhibition rate constants and eq. 4 (additive model):

$$\lambda_{app} = \frac{k_{inact,app,1}I_1}{K_{I,1} + I_1} + \frac{k_{inact,app,2}I_2}{K_{I,2} + I_2} \quad (4)$$

Additionally, the same data were simulated using eq. 5, which incorporates competitive reversible inhibitor-inhibitor interaction (inhibitor-inhibitor interaction model):

$$\lambda_{app} = \frac{k_{inact,app,1}I_1}{K_{I,1}\left(1 + \frac{I_2}{K_{I,2}}\right) + I_1} + \frac{k_{inact,app,2}I_2}{K_{I,2}\left(1 + \frac{I_1}{K_{I,1}}\right) + I_2} \quad (5)$$

The unbound FDA *R*-value for assessing in vivo CYP2C19 and CYP3A4 DDI risk due to reversible (eq. 6) or time-dependent inhibition (eq. 7) was predicted:

$$R = \frac{I_{max}}{K_i} \quad (6)$$

$$R = \frac{\lambda}{k_{deg}} = \frac{k_{inact,app}I_{max}}{k_{deg}(K_I + I_{max})} \quad (7)$$

where I_{max} , K_i , K_I , and $k_{inact,app}$ are the unbound in vivo maximum plasma concentration of the inhibitor, in vitro unbound reversible inhibition affinity constant, in vitro unbound time-dependent inhibition affinity constant, and in vitro maximum time-dependent inhibition rate constant, respectively. For eq. 6 the unbound IC_{50} values were used instead of K_i because substrate concentrations were less than K_m for the inhibition experiments (Lutz and Isoherranen, 2012). The in vivo P450 degradation rate constants (k_{deg}) for CYP2C19 and CYP3A4 were $4.5 \times 10^{-4} \text{ min}^{-1}$ and $4.8 \times 10^{-4} \text{ min}^{-1}$,

respectively (Ghanbari et al., 2006; Obach et al., 2007). The change in the concentration of active enzyme in vivo in the presence of multiple TDIs was predicted using two models: the additive and inhibitor-inhibitor interaction models (Zhang et al., 2009b; Rowland Yeo et al., 2010).

$$\frac{CL_i}{CL_i'} = 1 + \sum_{a=1}^n \frac{k_{inact,app,a}I_{max,a}}{k_{deg}(K_{I,a} + I_{max,a})} \quad (8)$$

$$\frac{CL_i}{CL_i'} = 1 + \sum_{a=1}^n \frac{k_{inact,app,a}I_{max,a}}{k_{deg}\left(K_{I,a}\left(1 + \sum_{b \neq a}^o \frac{I_{max,b}}{K_{I,b}}\right) + I_{max,a}\right)} \quad (9)$$

The CL_i/CL_i' ratio is the ratio of the active P450 concentrations in the presence and absence of the inhibitor, respectively. Subscript *a* indicates K_I , $k_{inact,app}$, and I_{max} values for a given inhibitor of *n* number of CYP2C19 or CYP3A4 TDIs. Subscript *b* indicates K_I and I_{max} values for a given inhibitor of *o* number of CYP2C19 or CYP3A4 inhibitors. The I_{max} values used for (*R*)-fluoxetine, (*S*)-fluoxetine, (*R*)-norfluoxetine, and (*S*)-norfluoxetine after 8 days of daily oral 60 mg of fluoxetine were 130, 360, 130, and 280 nM, respectively (Bergstrom et al., 1992; Jannuzzi et al., 2002). To obtain unbound affinity constants and concentrations, all inhibitor I_{max} values were multiplied by the fraction unbound in plasma ($f_{u,p}$), and all IC_{50} and K_I values were multiplied by the fraction unbound in pooled HLMs ($f_{u,HLM}$) determined at 0.1 mg/ml and 1.0 mg/ml microsomal protein, respectively.

To determine the overprediction that would occur when predicting the in vivo CL_i/CL_i' using the additive model compared with the inhibitor-inhibitor interaction model, the fold prediction difference in CL_i/CL_i' was simulated using the quotient of eqs. 8 and 9 over a range of possible I_{max}/IC_{50} values from 0.01 to 100 for a two-inhibitor system. The two-inhibitor systems simulated were (*R*)-fluoxetine with (*R*)-norfluoxetine and (*S*)-fluoxetine with (*S*)-norfluoxetine. The in vitro reversible and time-dependent inhibition constants used for the simulation were as determined for each enantiomer. Additionally, one generic parent-metabolite inhibitor pair was simulated, with IC_{50} and K_I values for both parent and metabolite set at 10 μ M and the $k_{inact,app}$ values for parent and metabolite set at 0.1 min^{-1} and 0.01 min^{-1} , respectively.

Results

Stereoselective Time-Dependent Inhibition of CYP2C19 and CYP3A4 by Fluoxetine and Norfluoxetine. To determine which of the fluoxetine and norfluoxetine enantiomers are in vitro TDIs of CYP2C19 and CYP3A4, the time-dependent decrease in enzyme activity was first determined with 100 μ M of each inhibitor using pooled HLMs (Supplemental Fig. 1). Both fluoxetine and norfluoxetine enantiomers decreased CYP2C19 activity as a function of time ($P < 0.05$). In contrast, only (*S*)-fluoxetine and (*R*)-norfluoxetine decreased CYP3A4 activity with time ($P < 0.05$). Reversible and time-dependent in vitro inhibition parameters were then determined in pooled HLMs based on the data on which of the compounds demonstrated time-dependent inhibition of CYP3A4 or CYP2C19 in the initial screen.

In pooled HLMs, all fluoxetine and norfluoxetine enantiomers were characterized for time-dependent CYP2C19 inhibition. (*R*)-fluoxetine ($K_I = 2 \pm 1 \mu\text{M}$, and $k_{inact,app} = 0.017 \pm 0.001 \text{ min}^{-1}$) and (*S*)-norfluoxetine ($K_I = 7 \pm 1 \mu\text{M}$, and $k_{inact,app} = 0.059 \pm 0.002 \text{ min}^{-1}$) were the most efficient TDIs against CYP2C19, with $k_{inact,app}/K_I$ values of 9.4 l/min/ μmol and 8.4 l/min/ μmol , respectively (Fig. 1; Table 1). The K_I value for (*R*)-fluoxetine was 75% lower than any of the other three compounds studied. Reversible IC_{50} values against CYP2C19 were also determined in pooled HLMs (Table 1; Supplemental Fig. 2). (*R*)-Fluoxetine was also the most potent reversible inhibitor ($IC_{50} = 2 \pm 1 \mu\text{M}$) of CYP2C19. The IC_{50} values were in agreement with the K_I values (Table 1).

The in vitro time-dependent inhibition constants were determined for (*S*)-fluoxetine and (*R*)-norfluoxetine against CYP3A4 in pooled

Fig. 1. Concentration- and time-dependent inhibition of CYP2C19 by fluoxetine and norfluoxetine enantiomers in pooled HLMs. The panels depict the percentage of CYP2C19 activity remaining versus time at each inhibitor concentration and time-dependent inhibition rate versus inhibitor concentration by (R)-fluoxetine (A and B), (S)-fluoxetine (C and D), (R)-norfluoxetine (E and F), and (S)-norfluoxetine (G and H). The error bars are the standard deviation of three replicate experiments.

HLMs based on the initial time-dependent inhibition screen (Fig. 2). (R)-Norfluoxetine ($K_I = 8 \pm 3 \mu\text{M}$, and $k_{\text{inact,app}} = 0.011 \pm 0.001 \text{ min}^{-1}$) was a more efficient TDI ($k_{\text{inact,app}}/K_I$ value of $1.4 \text{ l/min}/\mu\text{mol}$) of CYP3A4 than (S)-fluoxetine ($K_I = 21 \pm 19 \mu\text{M}$, $k_{\text{inact,app}} = 0.009 \pm 0.003 \text{ min}^{-1}$, and $k_{\text{inact,app}}/K_I$ of $0.5 \text{ l/min}/\mu\text{mol}$). Overall, the studied fluoxetine and norfluoxetine enantiomers were less efficient at

inactivating CYP3A4 than CYP2C19. Interestingly, the most efficient TDIs of CYP2C19 in HLMs were (R)-fluoxetine and (S)-norfluoxetine, whereas these two compounds were the ones that did not inactivate CYP3A4. The reversible IC_{50} values for each enantiomer against CYP3A4 in pooled HLMs were also determined (Table 1; Supplemental Fig. 3). (R)- and (S)-norfluoxetine were determined to be the most potent

TABLE 1

In vitro reversible (IC_{50}) and time-dependent (K_I and $k_{inact,app}$) inhibition constants for the enantiomers of fluoxetine and norfluoxetine in HLMs

All inhibition values are expressed as mean and standard error.

	IC_{50}	K_I	$k_{inact,app}$	$k_{inact,app}/K_I$
	μM		min^{-1}	$l/min/\mu mol$
CYP2C19				
(R)-Fluoxetine	2 ± 1	2 ± 1	0.017 ± 0.001	9.4
(S)-Fluoxetine	34 ± 5	55 ± 17	0.055 ± 0.006	1.0
(R)-Norfluoxetine	15 ± 1	15 ± 3	0.050 ± 0.010	3.3
(S)-Norfluoxetine	4 ± 1	7 ± 1	0.059 ± 0.002	8.4
CYP3A4				
(R)-Fluoxetine	80 ± 9	ND	ND	ND
(S)-Fluoxetine	47 ± 5	21 ± 19	0.009 ± 0.003	0.5
(R)-Norfluoxetine	5 ± 1	8 ± 3	0.011 ± 0.001	1.4
(S)-Norfluoxetine	11 ± 1	ND	ND	ND

ND, not determined.

in vitro reversible inhibitors, with IC_{50} values of $5 \pm 1 \mu M$ and $11 \pm 1 \mu M$, respectively. The in vitro CYP3A4 IC_{50} values for (S)-fluoxetine and (R)-norfluoxetine were in agreement with their determined K_I values (Table 1).

Risk Assessment of CYP3A4 and CYP2C19 Inhibition by Fluoxetine and Norfluoxetine Enantiomers. Fraction unbound (f_u) values for both enantiomers of fluoxetine and norfluoxetine were determined in plasma and HLMs (Table 2). Protein binding between (R)-fluoxetine, (S)-fluoxetine, (R)-norfluoxetine, and (S)-norfluoxetine was similar in HLMs ($f_{u,HLM}$ 0.42–0.52 at 0.1 mg/ml, and $f_{u,HLM}$ 0.07–0.10 at 1.0 mg/ml microsomal protein) and in plasma ($f_{u,p}$ from 0.13–0.22). Using the determined in vitro parameters, the risk of in vivo inhibition (FDA unbound R-value) of CYP2C19 and CYP3A4

was predicted using I_{max}/IC_{50} (eq. 5) for reversible inhibition and λ/k_{deg} (eq. 6) for time-dependent inhibition (Table 3). The I_{max} value used was the maximum unbound plasma concentration ($f_{u,p} \cdot I_{max}$) for each of the four compounds after eight daily oral doses of 60 mg *rac*-fluoxetine (Bergstrom et al., 1992; Jannuzzi et al., 2002). For both CYP2C19 and CYP3A4, reversible inhibition was predicted to be unlikely in vivo ($I_{max}/IC_{50} < 0.1$), with the maximum predicted I_{max}/IC_{50} value of 0.03 for (R)-fluoxetine against CYP2C19 (Table 3). Both fluoxetine and norfluoxetine enantiomers are predicted to result in in vivo time-dependent inhibition of CYP2C19 ($\lambda/k_{deg} > 0.1$), with (S)-norfluoxetine predicted to result in the greatest magnitude of in vivo inhibition between the four inhibitors ($\lambda/k_{deg} = 9.4$) (Table 3). Overall, (S)-norfluoxetine and (R)-fluoxetine are predicted to play the most important roles in in vivo inhibition of CYP2C19. Based on λ/k_{deg} values, CYP3A4 is predicted to be inhibited in vivo ($\lambda/k_{deg} > 0.1$) by both (S)-fluoxetine and (R)-norfluoxetine, with (R)-norfluoxetine predicted to cause a greater in vivo DDI than (S)-fluoxetine (λ/k_{deg} values of 1.1 and 0.5, respectively). The overall role of norfluoxetine enantiomers (approximately 60% with CYP2C19 and 70% with CYP3A4 predicted contribution) indicates a significant contribution of the metabolites to in vivo P450 inhibition.

Evaluation of Inhibitor-Inhibitor Interactions in in vitro-to-in vitro Predictions of Multiple CYP2C19 TDI Systems. Mixtures of inhibitor enantiomers represent multiple independent inhibitor systems. Because both norfluoxetine and fluoxetine enantiomers are predicted to play a significant role in in vivo CYP2C19 inhibition, time-dependent inhibition by enantiomer mixtures of fluoxetine and norfluoxetine and a mixture of fluoxetine with norfluoxetine were determined to evaluate different models for the prediction of multiple independent TDI systems. The in vitro time-dependent inhibition of CYP2C19 after *rac*-fluoxetine or *rac*-norfluoxetine incubation was

Fig. 2. Time- and concentration-dependent inhibition of CYP3A4 by (S)-fluoxetine and (R)-norfluoxetine in pooled HLMs. The panels depict the percentage of CYP3A4 activity remaining versus time at each inhibitor concentration and time-dependent inhibition rate versus inhibitor concentration by (S)-fluoxetine (A and B) and (R)-norfluoxetine (C and D). The error bars are the standard deviation of three replicate experiments.

TABLE 2

The unbound fractions (f_u) for the enantiomers of fluoxetine and norfluoxetine in pooled HLMs at 0.1 mg/ml and 1.0 mg/ml microsomal protein and in plasma

	$f_{u,HLM}$		$f_{u,plasma}$
	0.1 mg/ml	1.0 mg/ml	
(<i>R</i>)-Fluoxetine	0.48	0.09	0.22
(<i>S</i>)-Fluoxetine	0.52	0.10	0.14
(<i>R</i>)-Norfluoxetine	0.42	0.07	0.20
(<i>S</i>)-Norfluoxetine	0.47	0.07	0.13

predicted using the time-dependent inhibition data by individual enantiomers, and the accuracy of these predictions was tested in pooled HLMs with *rac*-fluoxetine and *rac*-norfluoxetine (Fig. 3). Using the individual enantiomer K_i and $k_{inact,app}$ values, CYP2C19 time-dependent inhibition versus total inhibitor [(*R*)- plus (*S*)-enantiomer] concentration curves were simulated using the additive (eq. 4) and inhibitor-inhibitor interaction (eq. 5) models. Greater time-dependent inhibition was predicted with the additive model (red curve) than was observed for both *rac*-fluoxetine and *rac*-norfluoxetine (Fig. 3). The inhibitor-inhibitor interaction model underpredicted (green curve) *rac*-fluoxetine time-dependent inhibition but predicted the time-dependent inhibition by *rac*-norfluoxetine well.

To evaluate whether time-dependent inhibition by the parent plus in vivo formed metabolite can be rationalized using in vitro data, the in vitro time-dependent inhibition of CYP2C19 by a 1:1 mixture of fluoxetine:norfluoxetine individual enantiomers was predicted based on the parameters determined for each compound as independent inhibitors, and the accuracy of each prediction was evaluated. Although it is unlikely that in in vitro incubations norfluoxetine will accumulate to a 1:1 ratio with fluoxetine under normal experimental conditions, this ratio was chosen to mimic in vivo plasma ratios (Jannuzzi et al., 2002). Both the additive and inhibitor-inhibitor interaction models overpredicted time-dependent inhibition after coincubating 1:1 (*R*)-fluoxetine:(*R*)-norfluoxetine or 1:1 (*S*)-fluoxetine:(*S*)-norfluoxetine (Fig. 4). The inhibitor-inhibitor interaction model was the closest to capturing the λ versus total inhibitor concentration (fluoxetine plus norfluoxetine) profiles in both experiments. Overall, in-vitro-to-in-vitro prediction results suggest that significant inhibitor-inhibitor interaction occurs between multiple coincubated TDIs in vitro, but this model does not fully capture the complexity of the examined multiple TDI systems.

Evaluation of the Additive versus Inhibitor-Inhibitor Interaction Model in Risk Assessment of Multiple TDI Systems. Because inhibitor-inhibitor interaction can play a significant role in in vitro multiple TDI systems, it was examined whether this phenomenon is likely to play a significant role in predicting in vivo multiple TDI

DDIs. Both the additive (eq. 8) and inhibitor-inhibitor interaction (eq. 9) models were used to predict in vivo inhibition risk after *rac*-fluoxetine administration. The models were used to predict in vivo inhibition risk under different combinations of fluoxetine and norfluoxetine enantiomers and the in vivo relevant combination of all four inhibitors (Table 4). For both CYP2C19 and CYP3A4, the predicted inhibition was not different between the additive and the inhibitor-inhibitor interaction models. This similarity can be explained by the fact that in vivo unbound plasma I_{max} concentrations are at least 30-fold below the in vitro determined unbound IC_{50} values ($I_{max}/IC_{50} \leq 0.03$) (Table 3). Summation of effects of all four inhibitors yields a λ/k_{deg} of 20 and 2.5–2.6 for CYP2C19 and CYP3A4, respectively (Table 4). Because the use of the additive or inhibitor-inhibitor interaction models had little consequence in risk assessment of inhibition by fluoxetine, the overall differences in these two models were further explored via simulation of different concentrations of fluoxetine and norfluoxetine (Fig. 5). The aim was to determine what inhibitor conditions would differentiate the two models in vivo and indicate which model is more prudent to use for risk assessment of multiple TDI systems. In both multiple TDI systems, the fold prediction difference increased as either inhibitor in the system increased in I_{max}/IC_{50} . This discrepancy between the additive and inhibitor-inhibitor interaction models was more pronounced with the combination of (*R*)-fluoxetine with (*R*)-norfluoxetine than with the combination of (*S*)-fluoxetine and (*S*)-norfluoxetine (maximum fold prediction difference under the simulated conditions of 3.2-fold versus 2.6-fold).

Sensitivity analysis revealed that this difference between systems is not due to the overall magnitude of predicted interaction but is directly proportional to the differences in magnitude of $k_{inact,app}$ between parent and metabolite: the quotient of (*R*)-norfluoxetine and (*R*)-fluoxetine or (*S*)-norfluoxetine and (*S*)-fluoxetine $k_{inact,app}$ values is 2.9 and 1.1, respectively. To demonstrate this relationship, a generic metabolite and parent inhibitor pair was simulated where the parent $k_{inact,app}$ was 10-fold larger than the metabolite $k_{inact,app}$ (Fig 5., panel C). In this generic example, prediction differences up to 6.8-fold between the additive and inhibitor-inhibitor interaction models were calculated, indicating the possibility of significant differences in in vivo risk assessment between the two models when $I_{max} > IC_{50}$ for both inhibitors and the inhibitor $k_{inact,app}$ values are very different. This difference in the prediction accuracy between the models illustrates the importance in determining TDI parameters for both the metabolites and the parent drug as independent inhibitors to refine the in-vitro-to-in-vivo prediction models.

Discussion

Prediction of in vivo DDIs due to in vitro TDI is challenging (Venkatakrishnan and Obach, 2007; Grimm et al., 2009). In 2009, a survey by the Pharmaceutical Research and Manufacturers of America group found that over 75% of the researchers believe that in vivo DDIs due to in vitro TDIs cannot be accurately predicted (Grimm et al., 2009). Pertaining to TDIs that inhibit P450s via formation of an MIC, one challenge to DDI prediction is that the parent drug must undergo a series of metabolic transformations to ultimately inactivate the P450 (Mansuy et al., 1977; Jonsson and Lindeke, 1992; Ortiz de Montellano, 2005). Hence, it is unclear whether the concentration of the parent TDI can be used as a surrogate for the concentration of the metabolite that ultimately complexes with the P450 heme. In addition, MIC formation from alkylamine TDIs may arise from two competing pathways: initial *N*-dealkylation or *N*-hydroxylation (Hanson et al., 2010; Barbara et al., 2013). Thus, it is not clear whether inclusion of

TABLE 3

Stereospecific risk assessment of the inhibition of CYP2C19 and CYP3A4 after fluoxetine administration

The predicted values are shown for the change in in vivo enzyme activity from before to after fluoxetine administration due to reversible (I_{max}/IC_{50}) and time-dependent (λ/k_{deg}) inhibition.

	CYP2C19		CYP3A4	
	I_{max}/IC_{50}	λ/k_{deg}	I_{max}/IC_{50}	λ/k_{deg}
(<i>R</i>)-Fluoxetine	0.03	5.8	<0.01	NA
(<i>S</i>)-Fluoxetine	<0.01	1.2	<0.01	0.5
(<i>R</i>)-Norfluoxetine	<0.01	2.9	0.01	1.1
(<i>S</i>)-Norfluoxetine	0.02	9.4	<0.01	NA

NA, not applicable.

Fig. 3. Comparison of simulated and experimentally determined time-dependent inhibition rates of CYP2C19 by a racemic mixture of fluoxetine or norfluoxetine in pooled HLMs. Depicted is the percentage of CYP2C19 activity remaining versus time at each total inhibitor concentration (conc) and time-dependent inhibition rate versus the total concentration of a racemic mixture of (A and B) fluoxetine or (C and D) norfluoxetine. The error bars are the standard deviation of three replicate experiments. The black lines show the best fit to the experimentally determined time-dependent inhibition data, the red lines show the simulated time-dependent inhibition curve using the additive model (eq. 4), and the green lines show the simulated time-dependent inhibition curve using the inhibitor-inhibitor interaction model (eq. 5). Simulations were performed by predicting the racemic mixture time-dependent inhibition from the individual enantiomer values.

the parent drug and circulating metabolites should be considered as independent in vivo inhibitors. Our study evaluated, using fluoxetine and norfluoxetine enantiomers as a model, whether risk assessment of in vivo DDIs can be done using static models of time-dependent inhibition for multiple independent inhibitors.

Fluoxetine is a racemic secondary alkylamine, MIC-forming TDI of both CYP2C19 and CYP3A4 (Mayhew et al., 2000; Hanson et al., 2010). It also has an in vivo circulating N-dealkylated metabolite norfluoxetine (Jannuzzi et al., 2002) that is formed by CYP2D6 and other P450s (Margolis et al., 2000). In vivo, fluoxetine is a strong inhibitor of CYP2D6, causing a 7.8-fold increase in desipramine AUC (Bergstrom et al., 1992). Fluoxetine can be classified as a moderate CYP2C19 inhibitor based on the 2.9-fold increase in lansoprazole AUC (Vlase et al., 2011) after fluoxetine administration, yet in vivo CYP3A4 inhibition by fluoxetine is controversial. Multiple-dose fluoxetine increases alprazolam (40 mg/d fluoxetine for 9 days) and carbamazepine (20 mg/d fluoxetine for 7 days) AUC by 1.3-fold (Grimsley et al., 1991; Greenblatt et al., 1992) but administration of 60 mg/d for 5 days then 20 mg/d fluoxetine for 7 days resulted in no change in midazolam AUC (Lam et al., 2003). This study demonstrated that (*R*)- and (*S*)-fluoxetine enantiomers as well as (*R*)- and (*S*)-norfluoxetine metabolites are TDIs of CYP2C19, but only (*S*)-fluoxetine and (*R*)-norfluoxetine are TDIs of CYP3A4.

In vivo, norfluoxetine is formed by multiple P450 enzymes, and its formation is not dependent on CYP2C19 and CYP3A4 (Jannuzzi et al., 2002). As such, it is expected that inhibition of CYP2C19 and CYP3A4 in vivo by norfluoxetine enantiomers can be predicted based on norfluoxetine plasma concentrations, independent of fluoxetine exposure. When each of the four inhibitors is considered independently, (*S*)-norfluoxetine was predicted to contribute the most to

CYP2C19 inhibition and (*R*)-norfluoxetine to CYP3A4 inhibition. (*S*)-fluoxetine was also predicted to cause CYP3A4 inhibition in vivo but its projected contribution was less (30%) than that of (*R*)-norfluoxetine. Norfluoxetine enantiomers were predicted to be responsible for about 60% of the in vivo CYP2C19 inhibition. The importance of both norfluoxetine enantiomers in overall predicted CYP2C19 inhibition highlights the need to evaluate models for risk prediction of multiple TDIs simultaneously.

Two partially overlapping models for in-vitro-to-in-vivo prediction of DDIs due to multiple TDIs exist (Zhang et al., 2009b; Rowland Yeo et al., 2010). In-vitro-to-in-vitro predictions were done to evaluate the applicability of the additive (eq. 4) and inhibitor-inhibitor interaction (eq. 5) models in describing the effect of multiple TDIs in P450 activity. The comparison of the models showed that at low inhibitor concentrations there were no differences between the two models but significant differences were observed at inhibitor concentrations above K_i . Both models predicted well the observed decrease in CYP2C19 activity at low concentrations of the enantiomer mixtures, but did not predict the combination of the metabolite and parent. At high inhibitor concentrations, only the inhibition of CYP2C19 by norfluoxetine enantiomers could be predicted and only using the inhibitor-inhibitor interaction model. Overall, the additive model overpredicted the observed inhibition at inhibitor concentrations above the K_i while the inhibitor-inhibitor interaction model better reflected the observed CYP2C19 time-dependent inhibition. These results are in agreement with previous results of in-vitro-to-in-vitro prediction using both models with the tertiary alkylamine TDIs, erythromycin and diltiazem (with their respective *N*-dealkylated metabolites) (Zhang et al., 2009a).

Collectively, the results suggest that the multiple TDI system is more complex than either model can account for and demonstrate the

Fig. 4. Comparison of simulated and experimentally determined time-dependent inhibition rates of CYP2C19 by a 1:1 fluoxetine:norfluoxetine mixture in HLMs. The percentage of CYP2C19 activity remaining versus time at each total inhibitor concentration and time-dependent inhibition rate versus total concentration of a 1:1 mixture of (*R*)-fluoxetine:(*R*)-norfluoxetine (A and B) and (*S*)-fluoxetine:(*S*)-norfluoxetine (C and D). The error bars are the standard deviation of three replicate experiments. The black lines show the best fit to the experimentally determined time-dependent inhibition data, the red lines show the simulated time-dependent inhibition curve using the additive model (eq. 4), and the green lines show the simulated time-dependent inhibition curve using the inhibitor-inhibitor interaction model (eq. 5). Simulations were performed by predicting 1:1 fluoxetine:norfluoxetine time-dependent inhibition from the individual enantiomer values.

challenges of incorporating mechanistic complexity into mathematical models of these types of TDIs. It is possible that use of a progress curve approach could, at least for some combinations of TDIs and their metabolites, allow for better prediction of the combined effects of metabolite and parent. This approach has been suggested to provide advantages in TDI characterization for complex TDI systems (Burt et al., 2012).

Despite uncertainty in how to quantitatively predict the in vivo decrease in P450 activity due to both parent and metabolite TDIs, risk assessment of in vivo DDI liability due to multiple TDI systems is imperative at some stage during new drug development. The results of this study demonstrate that the DDI risk following fluoxetine administration could be identified for CYP2C19 using either the additive or inhibitor-inhibitor interaction model. DDI predictions of TDIs are also dependent on accurate estimation of in vivo P450 k_{deg} .

The CYP3A4 k_{deg} value ranges approximately 10-fold in the literature (Ghanbari et al., 2006; Venkatakrishnan and Obach, 2007). Using the different k_{deg} values would, however, not change the risk assessment with fluoxetine although the λ/k_{deg} values range from approximately 0.27 to 2.7 for (*S*)-fluoxetine and from 0.6 to 6.0 for (*R*)-norfluoxetine with CYP3A4. Furthermore, inclusion of CYP2C19 or CYP3A4 inhibition by norfluoxetine enantiomers did not change the predicted DDI liability as opposed to considering fluoxetine enantiomers only.

The simplest and most practical risk assessment scenario for multiple TDIs is to use the additive model (eq. 8); however, independent inhibitors are expected to interact with each other according to eq. 9. For fluoxetine, there were no differences in in-vitro-to-in-vivo predictions between the two models (Table 4). The lack of differences between the models is due to the fact that none of the inhibitors circulate at concentrations in vivo that are required to cause significant

TABLE 4
Predicted magnitude of in vivo P450 inhibition (CL_i/CL_i') by different combinations of fluoxetine and norfluoxetine enantiomers using the additive (eq. 8) and the inhibitor-inhibitor interaction (eq. 9) models

	CYP2C19		CYP3A4	
	Additive	Inhibitor-Inhibitor Interaction	Additive	Inhibitor-Inhibitor Interaction
(<i>R</i>)- and (<i>S</i>)-Fluoxetine	8	8	1.5	1.5
(<i>R</i>)- and (<i>S</i>)-Norfluoxetine	13	13	2.1	2.1
(<i>R</i>)-Enantiomers	10	10	2.1	2.1
(<i>S</i>)-Enantiomers	12	12	1.5	1.5
All enantiomers	20	20	2.6	2.5

Fig. 5. Simulation of the fold difference in prediction magnitude that would occur using the additive model instead of the inhibitor-inhibitor interaction model as a function of in vivo inhibitor potency. The simulations were for (A) (R)-fluoxetine with (R)-norfluoxetine and (B) (S)-fluoxetine with (S)-norfluoxetine inhibitor pairs and (C) an example parent-metabolite pair where the $k_{\text{inact,app}}$ for the parent is 10-fold greater than the metabolite.

reversible inhibition of the P450 of interest since eq. 9 simplifies to eq. 8 at low inhibitor concentrations. For example, with fluoxetine and norfluoxetine the I_{max}/IC_{50} values against CYP2C19 or CYP3A4 are close to zero (Table 3). Simulations of the two models with fluoxetine and norfluoxetine (Fig. 5) further supported this, indicating that minimal model discrimination (<2 -fold) occurs when $I_{\text{max}}/IC_{50} < 1$ for both fluoxetine and norfluoxetine enantiomers.

The additive model is likely to be sufficient for in-vitro-to-in-vivo risk assessment for majority of in vivo multiple TDI systems. Based on the literature data, approximately 90% of marketed TDIs circulate at unbound concentrations less than their $K_{i,u}$ (Fujioka et al., 2012). For example, both diltiazem and *N*-desalkyldiltiazem circulate at concentrations below their K_i values. The simulations of in-vitro-to-in-vivo predictions using the two models demonstrate why time-dependent in vitro inhibition of CYP3A4 by diltiazem and *N*-desalkyldiltiazem independently yield similar predicted in vivo DDIs when using the additive (Rowland Yeo et al., 2010) and inhibitor-inhibitor interaction (Zhang et al., 2009b) models. Extrapolation of this simulation to any system with two TDIs indicates that less than 2-fold difference between models will be observed when $I_{\text{max}}/IC_{50} < 1$ for both inhibitors. Some TDIs, such as ritonavir (Luo et al., 2003) and amprenavir (Ernest et al., 2005), circulate at concentrations above their in vitro inhibition affinity constants (Hsu et al., 1998; Polk et al., 2001); hence, significant differences in risk assessment between the two models would be expected, but only if the $k_{\text{inact,app}}$ values are significantly different for the independent inhibitors. In this situation, use of the inhibitor-inhibitor interaction model may be more accurate, but the additive model will always predict a larger in vivo DDI (i.e., a worst-case scenario) and hence may be more appropriate for in vivo DDI risk assessment of multiple TDI systems.

In conclusion, the results of this study show that in vitro both fluoxetine and norfluoxetine are stereoselective TDIs of CYP2C19 and CYP3A4 and that norfluoxetine is predicted to play an equal or greater role in in vivo CYP2C19 and CYP3A4 DDIs than fluoxetine. Worst-case scenario risk assessment for in vivo DDI liability of multiple TDI systems can be achieved by incorporating in vitro time-dependent inhibition information for both parent and metabolite (determined independently and stereoselectively when applicable) and can be predicted using a simple additive model of λ/k_{deg} , but this methodology will likely quantitatively overpredict the magnitude of in vivo DDIs. Our study's results indicate that a better understanding of the TDI mechanism is necessary for quantitative prediction of DDIs, so qualitative TDI risk assessment may be more appropriate at this time. Furthermore, these results provide insight into how circulating metabolites could be accounted for in in vivo DDI risk assessment for TDIs during new drug development but emphasize the need for further research to determine the most appropriate methodology and models to use for prediction of multiple TDI systems.

Authorship Contributions

Participated in research design: Lutz, Kunze, Isoherranen.

Conducted experiments: Lutz, VandenBrink, Isoherranen.

Contributed new reagents or analytic tools: Babu, Nelson.

Performed data analysis: Lutz, Kunze, Isoherranen.

Wrote or contributed to the writing of the manuscript: Lutz, Isoherranen.

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