Prediction of Human Serotonin and Norepinephrine Transporter Occupancy of Duloxetine by Pharmacokinetic/Pharmacodynamic Modeling in the Rat

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Non-standard Abbreviations: DASB, (3-amino-4-[2-[(di(methyl)amino)methyl]phenyl]sulfanylbenezonitrile); McN-5652, (rel-(6R,10bS)-6-[4-(Methylsulfanyl)phenyl]-1,2,3,5,6,10b-hexahydropyrrolo[2,1-a]isoquinoline); SERT, serotonin transporter; NET, norepinephrine transporter; 5-HT, serotonin; NE, norepinephrine; MDD, major depressive disorder; PET, positron emission tomography; PK/PD, pharmacokinetic/pharmacodynamic; CNS, central nervous system

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ABSTRACT
Translation of CNS occupancy and clinical effect from animal models to humans has remained elusive for many pharmacological targets. The current studies evaluate the ability of a rodent PK/PD modeling approach to translate *ex vivo* occupancy determined in rats to that observed after PET imaging in humans for the dual serotonin transporter (SERT) and norepinephrine transporter (NET) inhibitor, duloxetine. *Ex vivo* transporter occupancy in rat spinal cord was evaluated following single oral doses of 0.3 – 60 mg/kg. A novel methodology, based upon initial rates of association of transporter selective radioligands to tissue homogenates, was developed and validated for the assessment of *ex vivo* transporter occupancy. Duloxetine exhibited selectivity for occupancy of SERT over NET in rat spinal cord with ED$_{50}$ values of 1 mg/kg and 9 mg/kg, respectively. Corresponding EC$_{50}$ values for inhibition of SERT and NET based upon unbound duloxetine spinal cord concentrations were 0.5 nM and 8 nM. An effect compartment PK/PD modeling approach was utilized to analyze the relationship between the time course of duloxetine plasma concentration and SERT and NET occupancy. Duloxetine inhibited SERT and NET in rat spinal cord with a plasma EC$_{50}$ of 2.95 ng/mL and 59.0 ng/mL, respectively. Similar plasma EC$_{50}$ values for inhibition of SERT (2.29 – 3.7 ng/mL) have been reported from human PET studies. This study illustrates the value of translational PK/PD modeling approaches and suggests that the preclinical modeling approach employed in the current study is capable of predicting plasma concentrations associated with 50% occupancy of SERT in human CNS.
INTRODUCTION

Duloxetine (Cymbalta®) is a dual inhibitor of the serotonin (5-hydroxtryptamine, 5-HT) and norepinephrine (NE) transporters (SERT and NET, respectively) used for the treatment of major depressive disorder (MDD), generalized anxiety disorder and various chronic pain conditions. Evidence suggests that efficacy in the treatment of MDD is associated with a minimum SERT target occupancy of ≥ 80% at clinically efficacious doses of various selective 5-HT reuptake inhibitors (SSRIs) and tricyclic antidepressants (TCAs), based upon positron emission tomography (PET) studies (Meyer et al., 2001, 2004; Suhara et al., 2003). These target occupancy findings have recently been extended to duloxetine, a dual 5-HT and NE reuptake inhibitor (SNRI), where > 80% brain occupancy of SERT was observed at dose levels that have demonstrated efficacy in depression (Takano et al., 2006). In chronic pain conditions, duloxetine exhibits efficacy at similar dose levels and, although the site of analgesic action in humans is unclear, it is likely a result of transporter inhibition both spinally and supraspinally (Iyengar et al., 2004; Mixcoatl-Zecuatl and Jolivalt, 2011). To date, spinal cord occupancy of SERT (or NET) has not been demonstrated in either clinical or pre-clinical studies with duloxetine.

Preclinical rodent models have been utilized to assess target occupancy and may be useful in translating preclinical measurements of occupancy to observed human occupancy and clinical effect (Grimwood and Hartig, 2009). Measurements of SERT occupancy in humans and animals have historically adopted in vivo (humans and animals) or ex vivo (animals) radioligand binding methods using well-characterized radioligands such as [3H/11C]-DASB or [11C]-McN 5652 (Hughes et al., 2005; Huang et al., 2002; Liu et al., 2009). By contrast, there are fewer ligands suitable for monitoring in vivo occupancy at NET in humans, however, the identification and characterization of such radioligands is ongoing (Logan et al., 2007; Ding et al., 2010;
Hannestad et al., 2010; Gallezot et al., 2011). Studies to assess occupancy at NET have been conducted in rodents using \([^3]H\)-nisoxetine via \textit{ex vivo} binding approaches (Owens et al., 2000; Bymaster et al., 2001; Barbier et al., 2007; Lengyel et al., 2008; Aluisio et al., 2008). An assessment of transporter occupancy at both SERT and NET is required for compounds, such as duloxetine, with \textit{in vitro} profiles consistent with engagement of both transporters at therapeutically-relevant doses. Such data will enable an understanding of the relative contributions of SERT and NET occupancy to clinical efficacy and tolerability profiles.

Translation of target transporter (or receptor) occupancy from preclinical species to humans may be a viable strategy for the prediction of clinical efficacy for novel therapeutics when the relationship between target occupancy and clinical effect has been established. This approach relies upon an understanding of the relationship between drug concentrations in the plasma and CNS biophase that result in specific levels of target occupancy. The majority of studies that examine this relationship rely on single timepoint determinations of target occupancy after a single-dose administration of the test agent (Hughes et al., 2005; Liu et al., 2009). Such single timepoint determinations may misrepresent the true relationship between plasma drug concentration and CNS target occupancy at steady-state, especially in cases in which distribution of the drug from the plasma to the brain is relatively slow or the binding kinetics of the drug at the transporter (or receptor) are slow. In such cases, utilization of a direct model (e.g., simple $E_{\text{max}}$) to evaluate the relationship between occupancy and plasma concentration may lead to under or overestimation of the plasma concentrations that are required to achieve target transporter (or receptor) occupancies at steady-state. Optimal assessment requires the determination of occupancy and plasma concentration at multiple timepoints post-dose in order to fully characterize the kinetics and dynamics of changing drug concentrations in the plasma,
CNS, and occupancy at the pharmacological target. Indirect or effect compartment models may be more appropriate than direct models to describe the relationship between occupancy and plasma concentration and may lead to more accurate predictions of the steady-state relationship for drugs which exhibit delayed brain penetration and/or target pharmacodynamics (Abanades et al., 2011).

In this report, a compartmental pharmacokinetic/pharmacodynamic (PK/PD) modeling approach was utilized to describe the relationship between duloxetine plasma concentration and SERT and NET occupancy in the spinal cord after oral administration in rats. The present study describes the development and comprehensive validation of a novel *ex vivo* radioligand binding methodology for the accurate determination of SERT and NET occupancy. This is the first description of a method that utilizes initial rates of association of transporter selective radioligands to spinal cord crude homogenates for the measurement of occupancy at multiple transporters in parallel. The studies reported here provide support for the translation of preclinical to clinical SERT occupancy for duloxetine and highlight the value of utilizing PK/PD modeling approaches to predict the relationship between plasma concentration and CNS occupancy in humans from preclinical studies.
MATERIALS AND METHODS

Animals. Adult male Sprague Dawley rats (Charles River, Hollister, CA) were housed under controlled laboratory conditions (temperature at 21 ± 1°C) on a 12:12 hour light-dark cycle. Animals were given free access to food and water upon arrival to the facility and animals were acclimatized to their holding room for at least 48 hours. Animals were fasted for 15-18 hours prior to dosing. All animal experiments were approved by the Institutional Animal Care and Use Committee at Theravance, Inc.

Materials. Duloxetine [(+)-(S)-N-Methyl-3-(naphthalen-1-yloxy)-3-(thiophen-2-yl)propan-1-amine] was purchased from Waterstone Technology (Carmel, IN). [³H]-Citalopram and [³H]-nisoxetine were purchased from PerkinElmer (Waltham, MA).

Duloxetine Administration and Sampling. Rats (n = 6/timepoint/dose level) received a single oral dose of duloxetine (0.3, 1, 5, 10, 20, 30, and 60 mg/kg) and were euthanized by decapitation at specified time points (0.5, 2, 4, 6 and 8 hr for 5 and 20 mg/kg dose levels; 2 hr for 0.3, 1, 10, 30, and 60 mg/kg dose levels) post duloxetine administration. Two dose levels were selected for full time course evaluation to target spinal cord occupancies that would be changing over time for both SERT (5 mg/kg) and NET (20 mg/kg). Following decapitation, trunk blood was collected into K₂ EDTA microtainers (0.5mL blood/1mg K₂EDTA) and processed to plasma by centrifugation (at 12,000 rpm at 4°C for 4 min). Spinal cords were collected and dissected for ex vivo transporter occupancy and pharmacokinetic assessments from the same animals. Each animal thus contributed a SERT and NET occupancy determination and associated plasma concentration. The spinal cord was harvested by hydraulic extrusion using phosphate-buffered saline (PBS), and the lumbar spinal cord (approximately 1 – 1.5 cm in length) was dissected, frozen on dry ice, and stored at -80°C for ex vivo occupancy measurements (see below).
remaining spinal cord segments were collected and homogenized in 3 times volume of water (25% w/w) for PK analysis. All samples were stored at -80°C until analysis.

**Ex vivo Transporter Occupancy.** Immediately prior to initiation of the radioligand binding assays, frozen spinal cord segments were homogenized in assay buffer (50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, pH 7.4) at room temperature and at a ratio of 0.154 mL per mg of frozen tissue (approximately 250 μg protein per mL). B$_{\text{max}}$ values (± standard deviation) for [³H]-citalopram and [³H]-nisoxetine binding to SERT and NET in spinal cord homogenates were 0.55 ± 0.09 and 0.31 ± 0.04 pmol/mg protein, respectively.

For determination of *ex vivo* transporter occupancy, a kinetic radioligand binding assay was used to determine the initial rate of association of [³H]-citalopram (for SERT) or [³H]-nisoxetine (for NET) to spinal cord crude homogenates. According to the Law of Mass Action the initial rate of association (i.e. slope of the association line) is proportional to the number of free transporters (radioligand binding sites) in the incubation (see Hess et al., 2004).

Unless otherwise stated, radioligand binding assays were performed in 96-well polypropylene assay plates in a total assay volume of 200 μL containing approximately 25 μg of lumbar spinal cord membrane protein. In all cases the final assay buffer was 50 mM Tris-HCl, 120 mM NaCl, 5 mM KCl, 0.025% BSA, 100 μM ascorbic acid, pH 7.4. Total and non-specific binding (in triplicate) was determined at each time point using a single concentration of radioligand (in parentheses): [³H]-citalopram (3 nM) and [³H]-nisoxetine (5 nM) for SERT and NET, respectively. Non-specific binding for [³H]-citalopram and [³H]-nisoxetine was determined in the presence of 1 μM duloxetine or 1 μM desipramine, respectively. The *ex vivo* binding assay was initiated by the addition of spinal cord homogenate (100 μL, added manually using a multi-
channel pipet) at six time points to generate a time course (with constant shaking) of 0.4, 1.0, 1.5, 2.0, 2.5, and 3 min incubation times for SERT or 0.25, 0.5, 1.25, 1.75, and 2.25 min incubation times for NET. To minimize dissociation of unlabelled ligand during the processing steps, individual spinal cords were homogenized and assayed sequentially. Reactions were terminated by rapid filtration over 96 well GF/B glass fiber filter plates (PerkinElmer, Waltham, MA) presoaked in 0.3% polyethyleneimine. Filter plates were washed six times (approximately 0.2 mL each) with wash buffer (ice-cold 50 mM Tris-HCl, 0.9% NaCl, at 4°C) to remove unbound radioactivity. Plates were dried, 35 - 45 μL Microscint 20 liquid scintillation fluid (PerkinElmer, Waltham, MA) was added to each well, and radioactivity was measured using a Packard Topcount liquid scintillation counter (Packard BioScience Co., Meriden, CT).

Specific binding was calculated using total and non-specific binding from each timepoint on the same plate and analyzed by linear regression analysis with the GraphPad Prism Software package (GraphPad Software, Inc., San Diego, CA). The initial rates (v_i) of association of [3H]-citalopram or [3H]-nisoxetine, equal to the slope of the straight line, were determined by fitting data points, specific binding (cpm) versus time (min). The average of the initial rates of [3H]-citalopram or [3H]-nisoxetine association from vehicle-treated animals was calculated (\(v_i(\text{vehicle})\)). The % NET or SERT transporter occupancy for animals dosed with duloxetine was computed using the following equation:

\[
\% \text{Transporter Occupancy} = 100 \times \left[1 - \frac{v_i}{\text{average } v_i(\text{vehicle})}\right]
\]

Occupancy data were analyzed by non-linear regression using GraphPad Prism™ (GraphPad Software, Inc., San Diego, CA) and fit using a sigmoidal dose-response algorithm to determine SERT and NET occupancy ED_{50} values (with curve bottom fixed to 0) or EC_{50} values using rat
spinal cord concentrations (with curve bottom fixed to 0 and Hill slope set to 1). ED$_{50}$ and EC$_{50}$ values are reported with 95% confidence intervals (CI).

Prior to conduct of the in vivo studies, comprehensive in vitro studies were undertaken to validate the ex vivo occupancy assay methodology and confirm that the initial rate of radioligand binding is directly proportional to the number of available transporters in the incubation under the utilized assay conditions. In these studies, the number of available SERT or NET transporter binding sites was varied by the use of different concentrations of crude tissue homogenate or by preincubation of the homogenates with different concentrations of duloxetine to occupy the respective transporters. To vary the protein concentration, spinal cord homogenates were diluted serially in assay buffer to final assay concentrations of 25, 20, 12.5, 7.5, 2.5, and 0.3 μg protein per well. For duloxetine preincubation studies, spinal cord homogenate (~250 μg protein/mL) was incubated in the presence of duloxetine (0.2, 0.8, 2, or 79 nM for SERT; 1, 4, 9, or 396 nM for NET) at room temperature for 1 hr. Unbound compound was removed by centrifugation at 10,000 x g for 10 – 15 min followed by resuspension of the pellet in assay buffer immediately prior to initiation of the binding assay. The concentrations of duloxetine were selected to provide 20, 50, 70, or 99% inhibition of SERT and NET, as calculated using the in vitro uptake potencies determined in rat cortical synaptosomes (see Shen et al., 2010).

**Protein Binding in Rat Plasma and Brain Homogenate.** The in vitro unbound fraction of duloxetine in rat brain homogenate and in rat plasma was evaluated by equilibrium dialysis using the HT-Dialysis device (HT-Dialysis, Gayles-Ferry, CT). Rat brain was homogenized by mechanical homogenization using a Polytron Homogenizer PT10-35GT Generator in 2 times volume of PBS (33% w/w). Samples of brain homogenate or plasma were spiked with duloxetine (5 μM and 1 μM, respectively), loaded onto dialysis blocks, and dialyzed against phosphate-
buffered saline (PBS) for 5 hours at 37°C. The matrices from both sides of the dialysis membranes were equalized and extracted with acetonitrile (ACN). Unbound fractions for each compound were calculated as the ratio of peak areas from the PBS side to peak areas from the tissue or plasma side of the dialysis apparatus. Peak areas were corrected for dilution of the tissue made prior to spiking compound. Quantification was via LC-MS/MS. The unbound fraction in rat plasma and rat brain homogenate for duloxetine was 0.031 and 0.007, respectively. Unbound duloxetine spinal cord concentrations were calculated using the unbound fraction in rat brain homogenate assuming that the unbound fraction in rat brain tissue and rat spinal cord tissue are equivalent.

**Quantitative Bioanalysis.** Concentrations of duloxetine were determined in plasma and spinal cord by LC-MS/MS. Samples (10 µL) were injected on a Hypurity C18 column (50 x 2.1 mm; 3 μM) with a flow rate of 0.5 mL/min. Mobile phase A consisted of 0.2% formic acid in water and mobile phase B consisted of 0.2% formic acid in acetonitrile. The gradient elution started with a 0.5 min flow of 20% B, followed by a gradient from 20% to 75% mobile phase B for 2.5 min, followed by a gradient from 75% to 95% mobile phase B for 0.5 min, then a 0.5 min of 95% B, followed by a gradient of 90% to 20% for 0.3 min at the end, at a flow rate of 0.5 ml/min. The mass spectrometer (Sciex API5000; Applied Biosystems, Foster City, CA) was operated in positive ion multiple reaction monitoring mode. Duloxetine was monitored using mass transitions of 298.1/154.0. Proprietary compounds were used as internal standards for the bioanalysis of duloxetine and monitored using a mass transition of 328.16/180.2. The lower limit of quantification in plasma was 0.100 ng/mL. The lower limit of quantification in spinal cord was 0.4 ng/g.
Pharmacokinetic/Pharmacodynamic Modeling. Pharmacokinetic and pharmacodynamic (PK/PD) parameters were estimated by a compartmental modeling approach using WinNonlin Version 5.0.1 (Pharsight Corporation, Mountain View, CA). One and two compartment models with first-order absorption (with and without lag time) and elimination were evaluated. The pharmacodynamic model utilized in this analysis was an effect compartment $E_{\text{max}}$ model linked directly to the central pharmacokinetic compartment. Selection of models was based upon best fit in terms of visual inspection, Akaike Information Criteria (AIC), and weighted residual sum of squares using the Gauss-Newton minimization method. The following PK/PD parameters were estimated:

- $k_{01}$ (hr$^{-1}$): First-order absorption rate constant.
- V/F (L/kg): Volume of the central compartment divided by oral bioavailability.
- $k_{10}$ (hr$^{-1}$): Elimination rate constant from the central compartment.
- $E_{\text{max}}$ (% occupancy): Maximal SERT or NET transporter occupancy in spinal cord.
- EC$_{50}$ (ng/mL): Plasma duloxetine concentration associated with 50% SERT or NET transporter occupancy.
- $k_{eo}$ (hr$^{-1}$): First order equilibration rate constant between the central pharmacokinetic compartment and the pharmacodynamic effect compartment.

A direct PK/PD model that assumes instantaneous equilibration between the central pharmacokinetic compartment and the pharmacological target also was employed for analysis of the SERT and NET occupancy versus plasma concentration data using WinNonlin Version 5.0.1.
(Pharsight Corporation, Mountain View, CA). $E_{\text{max}}$ and $EC_{50}$ values as defined above were estimated in the direct PK/PD analysis.
RESULTS

Ex Vivo Transporter Occupancy Assay: Transporter Selective Radioligand Initial Association Rate Assay

To examine the relationship between available transporter binding sites and the initial rate of radioligand binding in rat spinal cord homogenates, the following studies were conducted. Firstly, the initial association rate binding assays were conducted using increasing concentrations of protein. As expected, the initial rate of [3H]-citalopram or [3H]-nisoxetine binding was proportional to the total protein concentration (Figure 1A, B). Secondly, radioligand association binding to spinal cord homogenates which had been preincubated with increasing concentrations of duloxetine to decrease the availability of unbound SERT and NET resulted in a progressive decrease in the initial rates of [3H]-citalopram or [3H]-nisoxetine binding (Figure 1C, D). A linear relationship was observed between the normalized association rates and the predicted fraction of available transporters. This was independent of the factor (i.e. protein concentration or unlabelled ligand) which modulated the number of unbound transporter binding sites (Figure 1E, F). Collectively, these data support the hypothesis that an ex vivo radioligand binding assay, in which the initial rates of association of a SERT- or a NET-selective radioligand are monitored, can be used to determine the number of available binding sites, and therefore the percent transporter occupancy.

To underscore the importance of monitoring the radioligand initial association rates, we assessed [3H]-citalopram binding to control and duloxetine-(0.8 nM; chosen to approximate 50% occupancy) treated spinal cord homogenates for up to 90 minutes (Figure 2). As observed
above, the \[^3\text{H}\]-citalopram association binding curve was approximately linear for the first 5 minutes, for both the control and duloxetine-treated homogenates. The initial association rate for the duloxetine-treated samples was 35 ± 12\% of that for the control samples, corresponding to 65\% SERT occupancy. In the control samples, as expected, \[^3\text{H}\]-citalopram continued to associate as the reaction was allowed to proceed. However, radioligand binding also increased in the duloxetine-treated homogenates, leading to a decrease in the apparent transporter occupancy. For example, at the 60 and 90 min time points, \[^3\text{H}\]-citalopram binding was inhibited 24 ± 3\% and 23 ± 3\%, respectively, in the duloxetine-treated samples. The use of extended incubation times in an \textit{ex vivo} occupancy assay therefore increases the risk that target occupancies will be underestimated.

**Ex Vivo Transporter Occupancy: Duloxetine-treated Animals**

The transporter selective radioligand initial association rate assay was used to determine the percent SERT or NET occupancy in spinal cord homogenates prepared from duloxetine-dosed animals. Occupancy was measured as function of both dose (0.3 – 60 mg/kg) and time (0.5 – 8 hr post-dose). Initial rates of radioligand binding decreased in a dose-dependent manner for both \[^3\text{H}\]-citalopram and \[^3\text{H}\]-nisoxetine, and translated to an increase in apparent occupancy at SERT and NET, respectively, when compared to vehicle-treated animals (Figure 3A and 3B). The dose response relationship demonstrated selectivity for SERT over NET with estimated \(\text{ED}_{50}\) values of 1 mg/kg (95\% CI: 0.6 - 2) and 9 mg/kg (95\% CI: 6 – 12), respectively (Figure 3C). The relationship between SERT and NET transporter occupancy and unbound duloxetine concentration in the CNS biophase (spinal cord) also was examined (Figure 3D). The occupancy-concentration relationship clearly illustrated the SERT-selectivity of duloxetine, with \(\text{EC}_{50}\) values of 0.5 nM (95\% CI: 0.3 – 0.7) and 8 nM (95\% CI: 5 – 15), respectively, consistent with its \textit{in}
vitro apparent binding affinity and potency (Bymaster et al., 2001; Tsuruda et al., 2010; Shen et al., 2010).

**Relationship between SERT and NET Transporter Occupancy and Duloxetine Plasma Concentration.**

The relationship between SERT and NET transporter occupancy and duloxetine plasma concentration is presented in Figures 4A and 4B. Data from all dose levels (0.3 – 60 mg/kg) were pooled in this analysis and depicted by the timepoint post-dose at which the occupancy and plasma concentration was measured irrespective of dose level. Full time courses of occupancy versus plasma concentration were evaluated at a low (5 mg/kg) and high (20 mg/kg) dose of duloxetine. Significant hysteresis was not identified for occupancy of either NET or SERT based on the observation that the relationship between transporter occupancy and duloxetine concentration was similar with respect to time. A slight delay was noted for occupancy of SERT (Figure 4A) and NET (Figure 4B) based upon the lower relative occupancy at the 0.5 hr timepoint. Similar plasma concentrations as those observed at 0.5 hr resulted in higher occupancy values at the 2, 4, 6, and 8 hr timepoints. However, the delay at the 0.5 hr timepoint was not apparent when the occupancy level was at a maximum (SERT; Figure 4A) or a minimum (NET; Figure 4B).

**Pharmacokinetic Modeling of Duloxetine after Oral Administration to Rats.**

A one-compartment first order absorption with first order elimination pharmacokinetic model best described the duloxetine plasma concentration versus time data after oral administration to rats (Figure 5). Other models evaluated included a one-compartment oral absorption model with a lag time and a two-compartment model; both models provided inferior
descriptions of the pharmacokinetic data (data not shown). The one-compartment model was fitted to each dose level separately for which a full plasma concentration time course was available (5 and 20 mg/kg). Parameter estimates for each of the estimated parameters are presented in Table 1. A marked decrease in the oral volume of distribution of the central compartment (V/F) with the increase in dose from 5 to 20 mg/kg was observed. A much smaller decrease in the elimination rate constant (k10) also was observed. These findings are consistent with the more than dose proportional increases in duloxetine exposure (C_{max} and AUC) observed from 5 to 20 mg/kg. Since the observed exposure at 2 hr from 0.3 mg/kg to 5 mg/kg was approximately proportional to dose, the estimated compartmental parameters at 5 mg/kg were used to simulate the pharmacokinetic profiles at 0.3 and 1 mg/kg. Similarly, the observed exposure at 2 hr from 20 – 60 mg/kg was approximately proportional to dose, and the estimated parameters at 20 mg/kg were used to simulate the PK profiles at 30 and 60 mg/kg. In each case, the model predicted the observed exposures reasonably well (Figure 5).

**Pharmacokinetic/Pharmacodynamic (PK/PD) Modeling.**

The one compartment oral absorption pharmacokinetic model was linked directly to an effect compartment sigmoidal E_{max} model and provided an adequate description of the SERT and NET occupancy versus time data. Pharmacodynamic parameter estimates derived from the effect compartment PK/PD analysis for SERT and NET are presented in Table 2. The EC_{50} for occupancy of SERT and NET in rat spinal cord was estimated to be 2.95 ng/mL and 59.0 ng/mL, respectively, based on the effect compartment modeling. The estimated PK/PD parameters were utilized to predict the SERT and NET occupancy versus time profiles over the dose range of 0.3 to 60 mg/kg (Figure 6A and 6B). SERT occupancy versus time was well-predicted over the dose range of 0.3 to 60 mg/kg (Figure 6A). Maximal SERT occupancy was predicted and observed at
doses ≥ 20 mg/kg. NET occupancy versus time was well-predicted at dose levels ≥ 10 mg/kg (Figure 6B). An under-prediction of observed NET occupancy across the dose range of 0.3 to 5 mg/kg was observed.

A direct PK/PD analysis also was conducted of the SERT and NET occupancy versus time data employing a sigmoidal E\textsubscript{max} model for comparison to the effect compartment modeling. Pharmacodynamic parameter estimates derived from the direct PK/PD analysis for SERT and NET are presented in Table 2. The EC\textsubscript{50} for occupancy of SERT and NET in rat spinal cord based on the direct model was estimated to be 2.32 ng/mL and 47.2 ng/mL, respectively.

The estimates of duloxetine plasma concentrations required for 50% SERT occupancy (EC\textsubscript{50}) derived from PK/PD modeling in the rat were directly compared to those plasma concentrations estimated for 50% SERT occupancy in humans from clinical PET studies (Table 3). The EC\textsubscript{50} values of 2.32 ng/mL and 2.95 ng/mL determined from the direct and effect compartment PK/PD modeling, respectively, in the rat compares favorably to the EC\textsubscript{50} range of 2.29 – 3.7 ng/mL determined in the human PET studies for duloxetine (Takano et al., 2006; Abanades et al., 2010).
DISCUSSION

The present study utilizes an ex vivo transporter occupancy and PK/PD modeling approach to evaluate the relationship between duloxetine SERT and NET occupancy in the spinal cord and the duloxetine plasma concentration in rats. A thorough preclinical understanding of the relationship between CNS occupancy and plasma concentration may facilitate the prediction of therapeutically effective plasma concentrations of novel agents. The current studies demonstrate an excellent concordance between the duloxetine EC50 for SERT spinal cord occupancy in rats estimated using a PK/PD modeling approach and the EC50 for SERT brain occupancy in humans as assessed in human PET studies (Takano et al., 2006; Abanades et al., 2011). These studies support the strategic use of robust methodologies for the assessment of ex vivo rodent CNS occupancy, and subsequent PK/PD analysis, as a means for the prospective prediction of CNS occupancy in humans.

Accurate assessment of transporter (or receptor) occupancy using ex vivo methodology is sensitive to many factors including the temporal effects of tissue processing (Li et al., 2003; Li et al., 2006). Time-dependent decreases in apparent transporter occupancy have been observed for SERT and NET using venlafaxine and nomifensine, respectively (Lengyel et al., 2008). As many standard SNRIs display rapid dissociation binding kinetics (e.g. dissociation half lives <10 min at room temperature) (Tsuruda et al., 2010), transporter occupancy may be underestimated under conditions which employ extended homogenate incubation times ex vivo (Lengyel et al., 2008). Here, the temporal effects of incubation time were illustrated by determination of the apparent SERT occupancy by duloxetine at different timepoints after radioligand addition. The decrease in apparent SERT occupancy with prolonged incubation time likely reflects dissociation of duloxetine from SERT such that additional transporter binding sites become available for
[\textsuperscript{3}H]-citalopram binding. Accordingly, apparent SERT occupancies by paroxetine, a compound with slower dissociation binding kinetics than duloxetine, were unaffected by incubation times up to 90 min (data not shown). It should be noted that underestimation of transporter occupancy due to compound dissociation can be affected by the tissue dilution factor, drug dose, and resulting drug concentration in the homogenate, allowing for potential re-binding to the target (in vitro) at high drug concentrations. Assay temperature may also play a role in apparent ex vivo occupancy measurements, as binding kinetic constants are generally expected to increase (shorter half lives) at elevated temperatures, leading to increased dissociation of the test compound.

Although studies of ex vivo transporter occupancy which use both 37°C and 4°C assays have been reported (Bymaster et al., 2001; Lengyl et al., 2008), we have chosen to make several ex vivo measurements over a short period of time (2 - 3 min) at room temperature, to facilitate radioligand binding while minimizing duloxetine dissociation. Compound-specific binding kinetics can be critical determinant of transporter binding in vitro, and we suggest that the binding kinetics of both the test compound and the radioligand tracer be considered in the design and interpretation of ex vivo occupancy studies. Here, we describe a transporter occupancy methodology that minimizes the confounding errors associated with ex vivo compound dissociation thus providing a reliable alternative that does not require in vivo radioligand administration and allows for occupancy measurements at multiple transporter targets in parallel.

In the current studies, duloxetine demonstrated selectivity for in vivo binding to SERT over NET in rat spinal cord. The ED\textsubscript{50} for SERT occupancy was lower (1 mg/kg versus 9 mg/kg) than that observed for NET occupancy (Figure 3C). SERT occupancy also was significantly higher at lower unbound duloxetine spinal cord concentrations and total duloxetine plasma concentrations. The EC\textsubscript{50} based on effect compartment PK/PD modeling of duloxetine plasma
concentrations was 2.95 ng/mL for SERT versus 59.0 ng/mL for NET. These findings are consistent with previous reports of the measured \textit{ex vivo} occupancy for duloxetine (Lengyel et al., 2008) and with the reported \textit{in vitro} apparent binding affinity and potency (Tsuruda et al., 2010; Shen et al., 2011).

The pharmacokinetics of duloxetine after oral dosing in rat are non-linear and exhibit more than dose-proportional increases in exposure over the dose range of 0.3 – 60 mg/kg. The duloxetine PK was modeled using a one-compartment model that incorporated first order rate constants for absorption (k01) and elimination (k10). Efforts to construct a model incorporating saturable parameters for absorption or elimination were unsuccessful and the first-order parameters were fitted individually to each dose level. This approach provided robust estimation of parameter estimates (% CV < 31) and good concordance between the observed and predicted plasma concentrations (Figure 5). Interestingly, the estimated elimination rate constant exhibited only a small decrease between the 5 and 20 mg/kg dose levels suggesting the more than dose proportional increases in exposure were not due to significantly reduced elimination (and decreased systemic clearance). Instead, the nearly 10-fold decrease in the V/F suggests that the enhanced exposure at higher doses is most likely due to a significant increase in the oral bioavailability of duloxetine assuming the volume of distribution is constant.

A minor hysteresis was observed in the relationship between SERT and NET occupancy and duloxetine plasma concentration in rats. NET and SERT occupancy measured at 0.5 hr post-dose was slightly lower than that observed at similar plasma concentrations measured at ≥ 2 hr post-dose. This observation is consistent with either delayed equilibration between plasma and CNS or slow transporter binding on/off rates (Yassen et al., 2005). An effect compartment PK/PD model was thus employed to model the relationship between NET and SERT occupancy.
and plasma concentration and account for any delay in equilibration with the CNS biophase. Although some delay was observed at the 0.5 hr timepoint, the estimates of the equilibration rate constant ($k_{eo}$) were relatively large indicating a relatively rapid equilibration with the CNS biophase (CNS equilibration $t_{1/2}$ of ~ 0.5 – 0.75 hr). Further, the estimate for EC$_{50}$ of NET and SERT occupancy derived from the direct PK/PD model (47.2 and 2.32 ng/mL, respectively) was similar to that observed using the effect compartment PK/PD model (59.0 and 2.95 ng/mL, respectively). These results appear to be consistent with the rapid kinetics of brain entry of duloxetine in humans and rapid on/off rates for duloxetine at NET and SERT (Tsuruda et al., 2010). Similar estimates of the concentration required for 50% occupancy of SERT were reported in humans after a single dose when direct or indirect models were applied to PET data suggesting that equilibration between plasma and brain is also relatively rapid for duloxetine in humans (Abanades et al., 2011).

An excellent correlation was observed in the present study between the EC$_{50}$ for duloxetine SERT occupancy in rats and the EC$_{50}$ for duloxetine SERT occupancy in humans (Takano et al., 2006; Abanades et al., 2011). This correlation suggests that development of the PK/PD relationship between plasma concentration and transporter occupancy in rats would enable a reasonable prediction of the relationship in humans for duloxetine. Several factors, however, may limit a direct translation of this relationship between rats and humans. Known species differences in transporter affinity, plasma protein binding, or CNS penetration and/or differences in regional distribution would need to be accounted for in predicting the relationship in humans. However, in the current example, duloxetine exhibits similar affinity for SERT or NET in both rats and humans [rat pK$_i$: 10.0 and 8.4 for SERT and NET, respectively; human pK$_i$: 10.1 and 8.2 for SERT and NET, respectively (Tsuruda et al., 2010 and Shen et al., 2011)].
The plasma protein binding for duloxetine in rats (96-97% bound) and humans (96-96.9% bound) also has been reported to be similar (Bymaster et al., 2005; Knadler et al., 2011). Furthermore, brain penetration is assumed to be similar in rats and humans for a highly permeable, non-P-gp substrate such as duloxetine (Theravance unpublished data). It is also possible that spinal cord occupancy may not directly reflect occupancy in the brain although prior studies have indicated equivalent CNS transporter occupancy for duloxetine in the spinal cord and brain of rats (Theravance unpublished data). Therefore, for duloxetine, a direct translation of the relationship between plasma concentration and transporter occupancy from rat to humans should be possible without correction for species differences in target affinity, plasma protein binding, brain penetration, and/or differences in spinal cord versus brain occupancy.

Direct translation of the rat PK/PD studies to humans also suggest that significantly higher duloxetine plasma concentrations (~59 ng/mL) would be required for 50% inhibition of NET in humans. The human clinical PK of duloxetine suggests that such plasma concentrations (47 – 110 ng/mL) are achieved at the higher doses (40 – 80 mg BID) of duloxetine administered in clinical trials (Knadler et al., 2011). Further, approved doses for duloxetine in MDD and chronic pain conditions range upward of 60 mg/day suggesting that NET is engaged at such dose levels although the level of occupancy is likely sub-maximal. Previous studies exploring NET target engagement by duloxetine via indirect assessments of changes in NE metabolite levels demonstrated changes consistent with moderate NET inhibition over average plasma concentrations of 40 - 70 ng/mL (Chalon et al., 2003). Moreover, the clinical efficacy and tolerability profile of duloxetine provides evidence for an effect on NE uptake (Trivedi et al., 2008). It should be noted that in the current study, the observed NET occupancy in rats was underpredicted at low dose levels (≤ 5 mg/kg) and plasma concentrations of duloxetine and this...
may limit the utility of the developed model to predict human CNS NET occupancy accurately. The inability of the developed model to robustly predict the observed NET occupancy at low doses likely is a reflection of the significant inter-animal variability observed in NET occupancy (~ 0 – 40%) over the lower concentration range, the lower density of NET binding sites in the spinal cord, and lower signal to background relative to SERT in the binding assay. Clinical PET studies will be required to clarify the NET occupancy of duloxetine in humans at approved doses. It is unknown if higher levels of NET occupancy than those engaged by duloxetine at approved doses would be advantageous in disorders, such as neuropathic pain and attention-deficit/hyperactivity disorder, that are postulated to benefit from an increase in extracellular NE.

The studies reported here provide support for the hypothesis that transporter occupancy is a translatable biomarker between animals and humans and that the relationship between plasma concentration and human transporter occupancy may be predicted from well-designed animal occupancy studies. Further, a novel methodology to accurately determine the *ex vivo* occupancy of SERT and NET in parallel was developed and validated. The utilization of similar PK/PD modeling approaches to predict drug concentrations associated with human transporter (or receptor) occupancy and clinical effect may be useful in the discovery and selection of novel therapeutics for pharmacological targets where the relationship between occupancy and clinical effect is well-understood or for novel targets where the desired level of target occupancy is assumed.
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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Bourdet, Tsuruda, Smith

Conducted experiments: Tsuruda and Obedencio

Performed data analysis: Bourdet, Tsuruda, Obedencio, and Smith

Wrote or contributed to the writing of the manuscript: Bourdet, Tsuruda, Obedencio, and Smith
References


FIGURE LEGENDS

Figure 1: Dependence of initial rate of association of [3H]citalopram (for SERT) or [3H]nisoxetine (for NET) on number of available SERT or NET transporter binding sites in rat spinal cord homogenates. Initial rates of [3H]-citalopram (A) or [3H]-nisoxetine binding (B) increase with protein concentration (μg per well). Initial rates of [3H]-citalopram (C) or [3H]-nisoxetine binding (D) decrease following preincubation of spinal cord homogenates with increasing concentrations of duloxetine (% predicted inhibition). Percent predicted inhibition by duloxetine at SERT and NET was calculated using the respective in vitro pIC50 values. Figures 1A through 1D are representative data from multiple experiments. Rates of radioligand binding at SERT (E) and NET (F) are proportional to the predicted fractional occupancy (normalized protein concentration or % inhibition by duloxetine). Figures 1E and 1F represent pooled data from multiple studies.

Figure 2: Dependence of apparent occupancy of duloxetine at SERT on duration of [3H]citalopram binding assay. Specific [3H]-citalopram binding in rat spinal cord crude homogenates was measured at various time points after pre-incubation in the absence (black circles) or presence (grey diamonds) of duloxetine (0.8 nM; chosen to achieve 50% occupancy). Increase in radioligand binding at later time points suggests a decrease in the number of duloxetine-occupied sites due to ligand dissociation.

Figure 3: Ex vivo SERT and NET occupancy by duloxetine in rat spinal cord crude homogenates. The rate of [3H]-citalopram (A) or [3H]-nisoxetine binding (B) was decreased in
spinal cord homogenates generated from duloxetine-dosed rats. The corresponding percent occupancies of SERT (black circles) and NET (grey squares) increased in a dose-dependent manner after administration of duloxetine (C). SERT and NET occupancies also increased with unbound duloxetine concentration in the spinal cord (D). Symbols represent individual animal data from all dose levels at the 2 hr timepoint. The \([\text{duloxetine}]_{\text{unbound}}\) was calculated from the measured \([\text{duloxetine}]_{\text{total}}\) in spinal cord and the fraction unbound \((f_u)\) in rat brain determined \textit{in vitro} as described in the methods.

Figure 4: Relationship between rat spinal cord occupancy at SERT (A) and NET (B) and duloxetine plasma concentration. Spinal cord transporter occupancy and duloxetine plasma concentration were determined at the indicated timepoints post-dose (0.3 to 60 mg/kg, PO). Symbols represent individual data from all dose levels at the indicated timepoints \((n = 4 - 6/\text{timepoint/dose level}).\)

Figure 5: Plasma pharmacokinetics of duloxetine after oral administration to rats. Symbols represent observed individual duloxetine plasma concentrations \((n = 4 - 6/\text{timepoint/dose level}).\) Dotted lines represent the predicted duloxetine plasma concentration versus time profile at the indicated dose levels based upon a one-compartment pharmacokinetic model. The 10 and 30 mg/kg dose levels have been omitted from the figure for clarity.

Figure 6: Predicted SERT (A) and NET (B) spinal cord transporter occupancy versus time profiles after oral administration of duloxetine to rats. Pharmacokinetic/pharmacodynamic
modeling was used to simulate the transporter occupancy versus time profile. Symbols represent the observed SERT and NET transporter occupancy. Lines indicate the predicted transporter occupancy versus time profiles. The 30 and 60 mg/kg dose levels have been omitted due to maximal SERT and NET inhibition and for clarity of the figures. Data represent mean ± S.D.; n = 4 - 6.
Table 1. Pharmacokinetic parameter estimates after oral administration of duloxetine to male Sprague-Dawley rats.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>k01 (hr⁻¹)</th>
<th>V/F (L/kg)</th>
<th>k10 (hr⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dose (mg/kg)</td>
<td>Estimate</td>
<td>% CV</td>
<td>Estimate</td>
</tr>
<tr>
<td>5</td>
<td>1.12</td>
<td>31</td>
<td>241</td>
</tr>
<tr>
<td>20</td>
<td>1.38</td>
<td>23</td>
<td>27.9</td>
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%CV: Coefficient of variation of parameter estimate
Table 2. Pharmacodynamic parameter estimates for duloxetine serotonin and norepinephrine transporter occupancy in rat spinal cord

<table>
<thead>
<tr>
<th>PK/PD Model</th>
<th>Transporter</th>
<th>Parameter</th>
<th>E&lt;sub&gt;max&lt;/sub&gt; (% Occupancy)</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (ng/mL)</th>
<th>keo (hr&lt;sup&gt;-1&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>PK/PD</td>
<td>Estimate</td>
<td>% CV</td>
<td>Estimate</td>
</tr>
<tr>
<td>Effect Compartment</td>
<td>SERT</td>
<td>88.5</td>
<td>8.2</td>
<td>2.95</td>
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</tr>
<tr>
<td></td>
<td>NET</td>
<td>96.3</td>
<td>8.1</td>
<td>59.0</td>
<td>45</td>
</tr>
<tr>
<td>Direct</td>
<td>SERT</td>
<td>85.6</td>
<td>2.8</td>
<td>2.32</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>NET</td>
<td>102</td>
<td>29</td>
<td>47.2</td>
<td>74</td>
</tr>
</tbody>
</table>

%CV: Coefficient of variation of parameter estimate

N/A: Not applicable.
Table 3. Prediction of duloxetine human serotonin and norepinephrine transporter occupancy by rat pharmacokinetic/pharmacodynamic modeling.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SERT</th>
<th>NET</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rat</td>
<td>Human</td>
</tr>
<tr>
<td>EC₅₀ (ng/mL)</td>
<td>2.32ᵃ</td>
<td>2.29ᶜ</td>
</tr>
<tr>
<td></td>
<td>2.95ᵇ</td>
<td>2.62ᵈ</td>
</tr>
<tr>
<td></td>
<td>2.29ᶜ</td>
<td>3.7ᵉ</td>
</tr>
</tbody>
</table>

ᵃ Direct PK/PD model (current study)
ᵇ Indirect (effect compartment) PK/PD model (current study)
ᶜ Direct PK/PD model (Abanades et al., 2011)
ᵈ Indirect PK/PD model (Abanades et al., 2011)
ᵉ Takano et al., 2006

N/A: Not available.