The Use of In Vitro Data and Physiologically-Based Pharmacokinetic Modeling to Predict Drug Metabolite Exposure: Desipramine Exposure in Cytochrome P4502D6 Extensive and Poor Metabolizers Following Administration of Imipramine

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ABSTRACT

Major circulating drug metabolites can be as important as the drugs themselves in efficacy and safety, so establishing methods whereby exposure to major metabolites following administration of parent drug can be predicted is important. In this study, imipramine, a tricyclic antidepressant, and its major metabolite desipramine were selected as a model system to develop metabolite prediction methods. Imipramine undergoes N-demethylation to form the active metabolite desipramine, and both imipramine and desipramine are converted to hydroxylated metabolites by the polymorphic enzyme CYP2D6. The objective of the present study is to determine whether the human pharmacokinetics of desipramine following dosing of imipramine can be predicted using static and dynamic physiologically-based pharmacokinetic (PBPK) models from in vitro input data for CYP2D6 extensive metabolizer (EM) and poor metabolizer (PM) populations. The intrinsic metabolic clearances of parent drug and metabolite were estimated using human liver microsomes (CYP2D6 PM and EM) and hepatocytes. Passive diffusion clearance of desipramine, used in the estimation of availability of the metabolite, was predicted from passive permeability and hepatocyte surface area. The predicted area under the curve (AUCm/AUCp) of desipramine/imipramine was 12- to 20-fold higher in PM compared with EM subjects following i.v. or oral doses of imipramine using the static model. Moreover, the PBPK model was able to recover simultaneously plasma profiles of imipramine and desipramine in populations with different phenotypes of CYP2D6. This example suggested that mechanistic PBPK modeling combined with information obtained from in vitro studies can provide quantitative solutions to predict in vivo pharmacokinetics of drugs and major metabolites in a target human population.

Introduction

Recent regulatory guidance from the Food and Drug Administration and International Council for Harmonisation (Guideline, 2009; http://www.fda.gov/ucm/groups/fdagov-public/@fdagov-drugs-gen/documents/document/ucm079266.pdf) proposes that any drug metabolite with exposure >10% of the parent or of the total drug-related material exposure at steady state in humans warrants further consideration with regard to safety. These guidelines recommend identifying the metabolic profile of the drug in humans, and determining systemic exposure of relevant metabolite (m) area under the concentration-time curve (AUC) relative to parent (p) AUC (AUCm/AUCp) in clinical and nonclinical studies. The metabolite/parent area under the plasma concentration versus time curve ratio (AUCm/AUCp) is also a commonly used metric in drug interaction studies involving metabolites (Yeung et al., 2011; St-Pierre et al., 1988). However, although the use of in vitro studies to predict in vivo pharmacokinetics of drugs is commonplace, the use of these approaches to predict pharmacokinetics of metabolites has not been thoroughly established due to the number of contributing variables on metabolite exposure. The equations developed by Houston (1981) for the metabolite/parent ratio (AUCm/AUCp) represent static models, which provide conceptual insight as to the determinants of metabolite exposure, including the clearance rate of the parent drug, the fraction of the dose of the parent drug that is converted to the metabolite, and the subsequent clearance of the metabolite. Another factor that can also impact metabolite disposition is its systemic availability following formation from parent drug, which depends upon sequential elimination, permeability, and transport properties of the metabolite.

A different approach to the understanding of circulating metabolite in vivo pharmacokinetic (PK) behavior is utilizing in vitro data of parent drug interactions. The metabolic profile of a drug and its metabolites is typically determined in human liver microsomes (CYP2D6 EM and PM) using in vitro studies. The metabolite/parent area under the plasma concentration versus time curve (AUCm/AUCp) can be predicted using in vitro data from human liver microsomes and hepatocytes. The metabolite/parent area under the plasma concentration versus time curve (AUCm/AUCp) represents static models, which provide conceptual insight as to the determinants of metabolite exposure, including the clearance rate of the parent drug, the fraction of the dose of the parent drug that is converted to the metabolite, and the subsequent clearance of the metabolite. Another factor that can also impact metabolite disposition is its systemic availability following formation from parent drug, which depends upon sequential elimination, permeability, and transport properties of the metabolite.
drug and metabolites in integrated dynamic physiologically-based pharmacokinetic (PBPK) models. PBPK is also potentially of value as a tool to evaluate the effect of various population factors on pharmacokinetic outcomes, including genetic polymorphism (Jamei et al., 2009; Rowland et al., 2011; Vieira et al., 2014).

In the present study, desipramine, an active metabolite of the tricyclic antidepressant imipramine, was selected as a test case to apply the approach of integrating in vitro data into static and dynamic PBPK models for metabolite exposure prediction following administration of parent drug, which was proposed in the previous study (Nguyen et al., 2016). Both imipramine and desipramine were demonstrated to undergo hydroxylation catalyzed by CYP2D6 (Brøsen and Gram, 1988; Sallee and Pollock, 1990). The differences in PK, due to variability in expression of CYP2D6, may lead to clinically significant differences in the treatment of depression with desipramine and imipramine (Dahl et al., 1992; Furman et al., 2004; Schenk et al., 2008). In this study, the impact of CYP2D6 polymorphism on metabolism and disposition of both parent drug and metabolite was investigated by simulating time-course profiles in populations of CYP2D6 extensive metabolizer (EM) and poor metabolizer (PM) genotypes. In vitro data were generated for imipramine and desipramine, including metabolic intrinsic clearance (CLint), protein binding, and membrane permeability. These data were used as input values for static and PBPK models in CYP2D6 EM and PM subjects, and compared with the PK parameters reported in the literature.

### Materials and Methods

#### Materials

Imipramine, desipramine, and amitriptyline hydrochloride were purchased from Sigma-Aldrich (St. Louis, MO). Pooled human liver microsomes (HLMs; ACD, values calculated using Advanced Chemistry Development (ACD/Laboratories) Software V12.5; CL, clearance; fu,gut, Free fraction of substrate in the enterocyte; ka, Absorption rate constant; MDCK-II, Madin-Darby canine kidney; n/a, not applicable.

**Table 1. Parameters for imipramine and active metabolite desipramine in PBPK model**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Imipramine</th>
<th>Desipramine</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight (g/mol)</td>
<td>280.4</td>
<td>266.4</td>
<td>ACD</td>
</tr>
<tr>
<td>logP</td>
<td>4.8</td>
<td>4.57</td>
<td>ACD</td>
</tr>
<tr>
<td>Compound type</td>
<td>Base</td>
<td>Base</td>
<td>ACD</td>
</tr>
<tr>
<td>pK&lt;sub&gt;a&lt;/sub&gt;</td>
<td>9.45</td>
<td>10.26</td>
<td>ACD</td>
</tr>
<tr>
<td>B/P</td>
<td>1.02</td>
<td>1.16</td>
<td>(Ciraulo et al., 1988)</td>
</tr>
<tr>
<td>fu,p</td>
<td>0.26</td>
<td>0.21</td>
<td>Measured</td>
</tr>
<tr>
<td>Absorption Model</td>
<td>First-order</td>
<td>n/a</td>
<td>(Sallee and Pollock, 1990)</td>
</tr>
<tr>
<td>Fraction absorbed</td>
<td>1</td>
<td>n/a</td>
<td>Simcyp default</td>
</tr>
<tr>
<td>k&lt;sub&gt;a&lt;/sub&gt; (h&lt;sup&gt;-1&lt;/sup&gt;)</td>
<td>1</td>
<td>n/a</td>
<td>Assumed</td>
</tr>
<tr>
<td>MDCK-II (10&lt;sup&gt;-6&lt;/sup&gt; cm/s)</td>
<td>39.3</td>
<td>n/a</td>
<td>(Mahar Doan et al., 2002)</td>
</tr>
<tr>
<td>Distribution</td>
<td>Minimal PBPK</td>
<td>Minimal PBPK</td>
<td>Predicted using method 2 (Rodgers and Rowland, 2006)</td>
</tr>
<tr>
<td>V&lt;sub&gt;ss&lt;/sub&gt; (L/kg)</td>
<td>11</td>
<td>6.5</td>
<td>(Rodgers and Rowland, 2006)</td>
</tr>
<tr>
<td>Elimination</td>
<td>Enzyme kinetics (CL&lt;sub&gt;int&lt;/sub&gt;) (µL/min/mg protein)</td>
<td>9.12</td>
<td>22</td>
</tr>
<tr>
<td>Additional CL (B&lt;sub&gt;Hep&lt;/sub&gt; CL&lt;sub&gt;int&lt;/sub&gt;) (µL/min/10&lt;sup&gt;6&lt;/sup&gt; cells)</td>
<td>8</td>
<td>0</td>
<td>Measured</td>
</tr>
<tr>
<td>f&lt;sub&gt;umic&lt;/sub&gt;</td>
<td>0.42</td>
<td>0.39</td>
<td>Measured</td>
</tr>
<tr>
<td>Renal clearance (L/hr)</td>
<td>0.15</td>
<td>1.0</td>
<td>(Ciraulo et al., 1988)</td>
</tr>
</tbody>
</table>

![Fig. 1. HPLC-UV traces of extracts of human hepatocyte incubations of imipramine. The trace on the top is the UV chromatogram of the control incubation without parent drug. The trace on the bottom is the UV chromatogram of imipramine incubation.](https://dmd.aspetjournals.org/article-pdf/1570/Nguyen-et-al/)
Metabolite Profile of Imipramine in Hepatocytes

In Vitro Incubation. Human hepatocyte (~0.75 x 10^6 cells/mL) incubations were performed in Williams E medium in a total volume of 1 mL using 10 μM imipramine concentration. Incubations were conducted at 37°C under a gas mixture of 5% CO₂/95% O₂. At time zero, 500 μL sample was quenched with 2.5 mL acetonitrile. At time 30 and 60 minutes, 250 μL sample at each time was added to the same volume of 2.5 mL acetonitrile. The precipitate was removed by centrifugation (1700g) for 5 minutes, the supernatant was decanted into a 15-mL conical glass tube, and the liquid was evaporated under a vacuum at 35°C using a Genevac evaporator. The resulting residue was reconstituted in 0.1 mL water containing 1% formic acid for HPLC-UV- tandem mass spectrometry analysis.

Metabolite Identification. The imipramine human hepatocyte extracts were analyzed by Ultra-High Performance Liquid Chromatography (UHPLC)-UV-mass spectrometry (MS) on a Thermo Orbitrap Elite coupled with Accela HPLC pump, photodiode array detector, and CTC Leap autoinjector (Thermo Fisher Scientific, Waltham, MA). Separation was effected on an Acquity BEH C18 column (2.1 x 100 mm; 1.7 μm particle size) using a mobile phase consisting of 0.1% formic acid in water (A) and acetonitrile (B) at a flow rate of 0.4 mL/min. The mobile phase composition began at 5% B, held for 0.5 minute, increased linearly to 40% B at 6 minutes, increased linearly to 80% B at 8 minutes, followed by a 1-minute wash at 95% B and 1.5-minute re-equilibration to initial conditions. The eluent passed through the photodiode array detector scanning from 200 to 400 nm and then into the source of the mass spectrometer in the positive ion mode. The source temperature was set at 400°C, and other settings and potentials were adjusted to maximize the signal for the protonated molecular ion of imipramine. The injection volume was 10 μL.

Metabolites formed in the hepatocyte incubation of imipramine were identified using UV and total ion chromatograms. The UV chromatograms were reconstructed using the wavelength maxima of the parent compound. These were then compared with UV chromatograms of the corresponding control incubation without parent drug. The UV peaks that were only present in the chromatograms of the incubation mixture but absent in the controls were identified as potential metabolites of imipramine. These were integrated, and the fractional conversion from parent drug (f_m) for desipramine formation from imipramine was estimated as the desipramine peak area divided by the sum of peak areas for all observed imipramine metabolites.

Enzyme Kinetic Study of Imipramine Metabolism in Hepatocytes

A preliminary experiment was conducted to determine linearity with respect to incubation time and hepatocyte concentration, wherein product formation was measured following substrate incubation at several different time points (2–45 minutes) and at several different hepatocyte concentrations (0.25 x 10^6 – 1 x 10^6 cells/mL). For incubation, 30 μL cell suspensions was added to a 96-well polystyrene plate with lid and incubated under a gas mixture of 5% CO₂/95% O₂ for 30 minutes. After preincubation, the reaction was commenced by adding prewarmed test compound (1 μM final concentration of desipramine). After zero, 5, 10, 20, 30, 45, and 60 minutes postcommencement of the incubation, the reactions were stopped by adding a fourfold volume of acetonitrile containing 0.05 μM amitriptyline (IS). The samples were centrifuged at 3000g for 10 minutes. The supernatants were analyzed with liquid chromatography/tandem mass spectrometry for the amount of parent compound remaining.

CL_int Determination of Desipramine in CYP2D6 EM and PM Microsomes

The CL_int of desipramine was determined in triplicate using HLMs (EM HLMs). Microsomes (0.5 mg/mL) were preincubated for 5 minutes at 37°C in 100 mM KH₂PO₄, pH 7.4, containing 3.3 mM MgCl₂, and 1.3 mM NADPH. The reactions were initiated by adding prewarmed test compound (1 μM final concentration of desipramine). After zero, 5, 10, 20, 30, 45, and 60 minutes postcommencement of the incubation, the reactions were stopped by adding a fourfold volume of acetonitrile containing 0.05 μM amitriptyline (IS). These were centrifuged at 3000g for 10 minutes. The supernatants were analyzed with liquid chromatography/tandem mass spectrometry for the amount of parent compound remaining.

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Calculation of apparent CL_int was done using the following equation:

\[
CL_{int} = \frac{0.693}{t_{1/2} \times C_{protein}} \times \frac{45 \text{ mg microsomal protein}}{g \text{ of liver weight}} \times \frac{20 \text{ g of liver}}{kg \text{ body weight}}
\]

where \(C_{protein}\) is the microsomal protein concentration in the incubation mixture and the in vitro elimination half life (\(t_{1/2}\)) was determined from the slope (~k) of the linear regression from log percentage remaining versus incubation time relationships (\(t_{1/2} = \frac{0.693}{k}\)).

TABLE 2

<table>
<thead>
<tr>
<th>Metabolite</th>
<th>UV Peak Area</th>
<th>f_m</th>
<th>f_CL,m</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desipramine</td>
<td>45,021</td>
<td>0.44</td>
<td>0.44-0.62</td>
</tr>
<tr>
<td>Imipramine N-glucuronide</td>
<td>18,764</td>
<td>0.18</td>
<td>0.18</td>
</tr>
<tr>
<td>Hydroxyimipramine</td>
<td>20,339</td>
<td>0.2</td>
<td>0.20-0.38</td>
</tr>
<tr>
<td>Hydroxydesipramine</td>
<td>18,498</td>
<td>0.18</td>
<td></td>
</tr>
<tr>
<td>All metabolites</td>
<td>102,622</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

Kinetic parameters, maximal reaction velocity (\(V_{max}\)) and Michaelis-Menten (\(K_m\)), were estimated by fitting the selected model to the in vitro data using nonlinear regression in GraphPad Prism (version 6.03).
samples of the mixtures that were not subjected to dialysis. The fraction stability through the dialysis procedure were also determined by analyzing supernatant was withdrawn for HPLC-MS analysis. Drug recovery and

\[
\text{V}_{\text{max}} = \frac{\text{Km}}{\text{Km} + [\text{S}]}
\]

where \( V_{\text{max}} \) is the maximum velocity, \( K_m \) is the Michaelis constant, and \([S]\) is the substrate concentration.

where A is the surface area of a membrane (length^2); P is the permeability (length/time); C is the concentration of a permeant (amount/length^2).

Hence, the hepatic passive transport clearance (product of \( A \times P_{\text{pass}} \)) can be predicted from passive permeability and hepatocyte surface area, as follows:

\[
\text{CL}_{\text{int,pass}} = \text{Passive permeability} \times \text{Hepatocyte Cell Surface Area} \times N \text{ cells}
\]

where passive permeability of desipramine is experimentally measured by parallel artificial membrane permeability assay from Fujikawa et al. (2007) (PPAMPA = 17.0 \times 10^{-5} \text{ cm/s}). Human hepatocyte cell surface area is 1.6 \times 10^{-5} \text{ cm}^2 (Chen et al., 2005). Number of cells is the product of 120 \times 10^6 cells/gram liver (Naritomi et al., 2003) and 20 g liver/kg body weight.

**Prediction of Metabolite Systemic Availability**

Following its formation from imipramine in liver, the systemic availability (\( F_m \)) of desipramine was estimated based on the well-stirred model (Houston, 1981), which was modified to incorporate the interplay between passive diffusion, hepatic blood flow (\( Q_h \)), and metabolic \( \text{CL}_{\text{int}} \) of desipramine (\( \text{CL}_{\text{int,met}} \)), assuming no or negligible involvement of hepatic transporters:

\[
F_m = \frac{Q_h \times \text{CL}_{\text{int,pass}} + \text{fuB} \times \text{CL}_{\text{int,met}}}{Q_h + \text{CL}_{\text{int,pass}} + \text{fuB} \times \text{CL}_{\text{int,met}}}
\]

where \( Q_h \) is \( \approx \) 21 ml/min/kg. The free fraction in blood (\( \text{fuB} \)) is the \( \text{fu}_{\text{B}} \) corrected by the blood-to-plasma ratios, which are 1.02 and 1.16 for imipramine and desipramine, correspondingly obtained as the mean values from resources, including Simcyp library and literature (Ciraulo et al., 1988; Fisar et al., 1996; Obach, 1997, 1999). \( \text{CL}_{\text{int,pass}} \) and \( \text{CL}_{\text{int,met}} \) are intrinsic passive diffusion and metabolic clearances, respectively.

**Prediction of Passive Diffusion Clearance**

The rate of total mass transport across a cellular membrane (\( dM_{\text{pass}}/dt \)) by passive transport can be described by equation below (Sugano et al., 2010):

\[
\frac{dM_{\text{pass}}}{dt} = A \times P_{\text{pass}} \times C
\]

where A is the surface area of a membrane (length^2); P is the permeability (length/time); C is the concentration of a permeant (amount/length^2).

Hence, the hepatic passive transport clearance (product of \( A \times P_{\text{pass}} \)) can be predicted from passive permeability and hepatocyte surface area, as follows:

\[
\text{CL}_{\text{int,pass}} = \text{Passive permeability} \times \text{Hepatocyte Cell Surface Area} \times N \text{ cells}
\]

where passive permeability of desipramine is experimentally measured by parallel artificial membrane permeability assay from Fujikawa et al. (2007) (PPAMPA = 17.0 \times 10^{-5} \text{ cm/s}). Human hepatocyte cell surface area is 1.6 \times 10^{-5} \text{ cm}^2 (Chen et al., 2005). Number of cells is the product of 120 \times 10^6 cells/gram liver (Naritomi et al., 2003) and 20 g liver/kg body weight.

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\[
F_m = \frac{Q_h \times \text{CL}_{\text{int,pass}} + \text{fuB} \times \text{CL}_{\text{int,met}}}{Q_h + \text{CL}_{\text{int,pass}} + \text{fuB} \times \text{CL}_{\text{int,met}}}
\]

where \( Q_h \) is \( \approx \) 21 ml/min/kg. The free fraction in blood (\( \text{fuB} \)) is the \( \text{fu}_{\text{B}} \) corrected by the blood-to-plasma ratios, which are 1.02 and 1.16 for imipramine and desipramine, correspondingly obtained as the mean values from resources, including Simcyp library and literature (Ciraulo et al., 1988; Fisar et al., 1996; Obach, 1997, 1999). \( \text{CL}_{\text{int,pass}} \) and \( \text{CL}_{\text{int,met}} \) are intrinsic passive diffusion and metabolic clearances, respectively.
Static Model of $\text{AUC}_{\text{m}}/\text{AUC}_{\text{p}}$

The in vivo ratios of area under the plasma concentration-time curve of metabolite versus parent drug after i.v. and oral administration of parent can be described by the following equations (Houston, 1981):

After an i.v. dose of parent drug:

$$\frac{\text{AUC}_{\text{m}}}{\text{AUC}_{\text{p}}} = \frac{F_m \times f_{\text{CL,m}} \times \text{CL}_{\text{p}}}{\text{CL}_{\text{m}}}$$

(6)

After an oral dose of parent drug:

$$\frac{\text{AUC}_{\text{m}}}{\text{AUC}_{\text{p}}} = \frac{F_m \times f_{\text{CL,m}} \times \text{CL}_{\text{p}}}{F_h \times \text{CL}_{\text{m}}}$$

(7)

in which, $f_{\text{CL,m}}$ is the fraction of the clearance of the parent drug that yields the metabolite. $F_m$, the metabolite systemic availability, is the portion of the total metabolite generated within an organ that is released into the systemic circulation before it is either further metabolized or secreted into bile. $\text{CL}_{\text{p}}$ is the total clearance of the parent drug, and $\text{CL}_{\text{m}}$ is the total clearance of the metabolite. In eq. 7, the fraction of imipramine that escapes first-pass elimination in the liver was estimated from in vitro total clearance of parent:

$$F_h = 1 - \frac{\text{CL}_{\text{p}}}{\text{Qh}}$$

(8)

In the static model for CYP2D6 EMs, the fraction of imipramine converted to desipramine, $f_{\text{CL,m}}$, was estimated based on imipramine metabolic profile determined from EM hepatocyte in vitro system. For PMs, desipramine $f_{\text{CL,m}}$ of 0.8 was obtained from clinical data of Brøsen and Gram (1988).

The total body clearance values of parent and metabolite were predicted from in vitro intrinsic metabolic clearances (EM and PM HLM) using the well-stirred model as follows:

$$\text{CL}_{\text{p}} = \frac{Q_h \times f_{\text{uu}} \times \text{CL}_{\text{int,p}u} \times \text{parent}}{Q_h + f_{\text{uu}} \times \text{CL}_{\text{int,p}u} \times \text{parent}}$$

(9)

$$\text{CL}_{\text{m}} = \frac{Q_h \times f_{\text{uu}} \times \text{CL}_{\text{int,m}u} \times \text{metabolite}}{Q_h + f_{\text{uu}} \times \text{CL}_{\text{int,m}u} \times \text{metabolite}}$$

(10)

**PBPK Modeling and Simulations**

Modeling and simulations of imipramine and its metabolite desipramine were performed using the population-based ADME simulator Simcyp (version 14; Simcyp, Sheffield, UK). Simulations were performed for two virtual populations of 500 (10 trials × 50 subjects each) healthy volunteers aged between 20 and 50 with a male/female ratio of 50/50, in fasted conditions, representing PM- and EM-CYP2D6 individuals receiving a normalized dose of 1 mg imipramine i.v. or orally. The PM population was generated by setting the frequency of PM CYP2D6 phenotype as 1, and other phenotypes (intermediate metabolizers, ultra-rapid metabolizers, and EM) as 0 in demographic of trial design. Meanwhile, CYP2D6 phenotype PM, IM, and UM frequencies were set to 0 for EM population.

**Results**

**Metabolic Profile of Imipramine in Human Hepatocytes to Estimate $f_m$ Values.** The metabolism of imipramine was assessed in

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$\text{CL}<em>{\text{p}}$ and $\text{CL}</em>{\text{m}}$ (mL/min/kg)</th>
<th>$F_h$</th>
<th>$\text{CL}_{\text{int,pass}}$ (mL/min/kg)</th>
<th>$F_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipramine</td>
<td>9.4</td>
<td>0.55</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Desipramine (in EM)</td>
<td>6.4</td>
<td>—</td>
<td>39.1</td>
<td>0.60</td>
</tr>
<tr>
<td>Desipramine (in PM)</td>
<td>1.0</td>
<td>—</td>
<td>39.1</td>
<td>0.93</td>
</tr>
</tbody>
</table>
human hepatocyte incubations. Chromatograms (UV traces) are shown in Fig. 1, and corresponding fractional conversion from parent drug $f_m$ is listed in Table 2 and Fig. 2. A total of four major metabolites was observed in this in vitro incubation. Primary metabolic pathways of imipramine produced desipramine ($m/z$ 267), hydroxy imipramine ($m/z$ 297), and imipramine N-glucuronide ($m/z$ 457). Hydroxy desipramine may be formed from sequential metabolism of desipramine or demethylation of hydroxy imipramine. Due to this, the fractional conversion from imipramine via N-demethylation pathway ($CL_{\text{int},m}$) was estimated as a range from 0.44 to 0.62. The fraction of imipramine that undergoes hydroxylation is in the range of 0.20–0.38. These ranges reflect extreme cases, that is, that hydroxy desipramine arose 100% via desipramine or 100% via hydroxy imipramine. Glucuronidation is responsible for 0.18 fraction of imipramine metabolism.

**Enzyme Kinetic Parameters of Desipramine Formation.** The enzyme kinetics of metabolism of imipramine to desipramine was studied in human hepatocytes. The velocity of N-demethylation was investigated using imipramine at final concentrations from 0.5 to 400 μM. The kinetic data are shown in Fig. 3 and indicate that at least two distinct enzymes are responsible for demethylation pathway. The Eadie-Hofstee plot (Fig. 3) showed that demethylation exhibited biphasic kinetics. Therefore, the total velocity for desipramine formation was described by two-enzyme model, as follows:

$$v = \frac{V_{\text{max}} \times C}{K_m + C} + CL_{\text{int}2} \times C$$  \hspace{1cm} (11)

where $V_{\text{max}}$ and $K_m$ are the apparent maximal velocity and the apparent Michaelis constant, respectively, of low $K_m$ enzyme, and $CL_{\text{int}2}$ is the $CL_{\text{int}}$ representing the high $K_m$ site. The best fit values of $V_{\text{max}}, K_m$, and $CL_{\text{int}2}$ were shown in Fig. 3.

**In Vitro $CL_{\text{int}}$ of Imipramine in Hepatocytes and Desipramine in Liver Microsomes.** The apparent $CL_{\text{int}}$ values of imipramine and its active metabolite, desipramine, were calculated from the degradation rate constants in their corresponding depletion curves (Figs. 4 and 5). For desipramine, HLMs were used because the metabolic clearance of desipramine is mediated mainly by cytochrome P450, whereas for imipramine this measurement was made in human hepatocytes because glucuronidation is also a component of clearance (Nakajima et al., 2002; Zhou et al., 2010). These apparent values were corrected for nonspecific binding of test compounds within microsomal (desipramine fraction unbound in microsomal incubation = 0.39) and hepatocyte (imipramine $fu_{\text{c}_{\text{int}}} = 0.42$) incubations. Apparent $CL_{\text{int}}$ and protein binding of imipramine and desipramine are displayed in Figs. 4 and 5, respectively. $CL_{\text{int}}$ of 2.5 ± 0.9 μL/min/mg protein of desipramine obtained in CYP2D6 PM HLMs was also determined, which is about ninefold lower than the value obtained from EM HLM incubations (22 ± 1.4 μL/min/mg protein). However, this estimate possesses uncertainty because the depletion of desipramine in PM liver microsomes is low.

**$CL$, $CL_{\text{int},\text{pass}}$, and $F_m$ Predictions.** Table 3 shows the predicted total clearance of parent drug ($CL_p$) and desipramine ($CL_m$), hepatic first-pass availability ($F_h$) of parent drug, as well as intrinsic passive diffusion ($CL_{\text{int},\text{pass}}$) and $F_m$ of desipramine. The predicted total clearance of imipramine is 9.4 mL/min/kg, and clearance values of desipramine are 6.4 and 1.0 mL/min/kg for EMs and PMs, respectively. About 55% of imipramine presented in the liver was predicted to escape the first-pass metabolism after an oral dose. Following its formation from imipramine, 60% of desipramine was estimated to leave the liver and reach the systemic circulation in EM populations. Due to its decreased activity of CYP2D6 isozyme in PMs, desipramine elimination rate is decreased, leading to increased systemic availability of this metabolite in circulation (93%) (Table 3).

**$AUC_{\text{int}}/AUC_p$.** The predicted total clearance ($CL_{\text{int}}$ and $CL_m$), $f_m$, $F_h$, and $F_m$ were used as input parameters to predict the relative exposure of desipramine compared with parent drug after i.v. and oral administration of imipramine to extensive and PM CYP2D6 healthy populations, using eqs. 6 and 7. For EM populations, the predicted $AUC_{\text{int}}/AUC_p$ ratios are
consistent with clinical data shown in Table 4. The data highlighted the clinical relevance of the CYP2D6 oxidation polymorphism in the pharmacokinetics of imipramine and desipramine. In the CYP2D6 PMs, increased desipramine plasma levels were predicted when comparing with those in EMs. Due to this, the estimated AUC ratio of desipramine versus imipramine was ~12- to 20-fold higher in PM than in EM populations, following i.v. or oral dosing of parent drug in the static and PBPK models, whereas the observed AUC ratio was 6- to 14-fold higher in PM than in EM volunteers. From PBPK simulations, the predicted mean ratios range from 0.19 to 0.28 and from 0.32 to 0.54 after i.v. and oral dose of imipramine, correspondingly. In PM populations, PBPK model predicted the range of mean ratios from 3.7 to 5.0 and from 6.5 to 9.1 after i.v. and oral dose of imipramine, respectively.

**Concentration-Time Profiles of Imipramine and Metabolite Desipramine.** The predicted means for 10 trials and observed plasma concentration-time profiles of imipramine and its active metabolite desipramine after i.v. infusion and oral administration of imipramine normalized dose to 1 mg are shown in Figs. 6-9. Predicted PK parameters for imipramine and active metabolite desipramine are listed in Tables 5 and 6. After an oral dose of 1 mg imipramine in EM, mean predicted AUC(0,∞) value of imipramine across the whole population was 13 ng/mL/h, whereas the mean value of individual trial ranged from 12 to 15 ng/mL/h. Mean predicted AUC(0,∞) value of desipramine was 5.8 ng/mL/h (mean of trials ranged from 4.3 to 6.9 ng/mL/h). In CYP2D6 PMs, predicted AUC(0,∞) value of desipramine for the whole population was 104 ng/mL/h (range from 86 to 116 ng/mL/h).

**Discussion**

Understanding the potential for contribution of a metabolite(s) to drug efficacy, toxicity, or drug-drug interactions can be challenging and requires a thorough knowledge of drug and metabolite disposition. Development of methods to accomplish this requires testing examples of drugs and metabolites that have human pharmacokinetic data and well-understood overall disposition. The example of desipramine as a metabolite which humans are exposed following administration of imipramine was selected for this study because human pharmacokinetics and metabolism are well known. Furthermore, because desipramine is cleared by CYP2D6, this example offers the opportunity to predict desipramine exposure after imipramine administration to two different population groups, that is, CYP2D6 EMs and PMs.

CYP2D6 genes are highly polymorphic, which leads to wide interindividual variation in drug clearance, induction of adverse effects, and increased potential for drug–drug interactions (Bernard et al., 2006). Because desipramine is mainly metabolized by a single enzyme, CYP2D6, it has been widely used as a probe drug for CYP2D6 activity (Ball et al., 1997; Kurtz et al., 1997; Spigset et al., 1997; Spina et al., 1997; Madani et al., 2002). High ratios of desipramine to parent drug due to impaired metabolism caused by the CYP2D6 PM phenotype have been related to increased frequency of adverse drug reactions, and even death, upon chronic administration of therapeutic doses (Swanson et al., 1997; Leuchtt et al., 2000).

In the overall metabolic profile of imipramine (Fig. 2), N-demethylation is the major pathway with the highest fraction of total clearance of the parent drug ($f_{CL,m} = 0.44–0.62$). Potter et al. (1982) demonstrated a similar finding that desipramine was the major circulating metabolite quantified in patient plasma (accounted for 67% total concentration of metabolites). By measuring the AUCs of desipramine in the PK studies whether desipramine was given as parent compound or formed from imipramine, Brosen et al. (1986) calculated the demethylation fraction of imipramine after the i.v. dose at 0.34 in CYP2D6 EMs and 0.67 in PMs. Likewise, this fraction slightly increased after an oral dose of imipramine: 0.53 in EM and 0.8 in PM populations (Brosen et al., 1986; Brosen and Gram, 1988). In CYP2D6 PMs, the decreased activity of CYP2D6 reduces the extent of formation of hydroxylimipramine from imipramine, thereby directing more of the imipramine dose to desipramine, increasing the demethylation fraction of imipramine compared with EM population. By using the HPLC/UV to
determine the fraction of metabolic clearance of parent that forms a specific metabolite, it is acknowledged that in the absence of a standard for the relevant metabolite, assumption of equivalent UV absorptivity needs to be made, even though modifications of the chemical structure can lead to changes in the UV absorbance relative to the parent drug.

To use in vitro data in a bottom-up approach to predict the exposure of a drug metabolite relative to the parent drug exposure, the following parameters must be predicted: total clearance of parent drug (CLp), total clearance of the metabolite (CLm), the fraction of the dose of the parent drug that is converted to the metabolite (Fm), and the fraction of the metabolite that once formed can enter the systemic circulation before being cleared within its organ of generation (Fm). The use of in vitro metabolism in scaling to predict human clearance has been a routine practice within pharmacological research and development organizations (Obach, 2011). In this study, the predicted CLp of imipramine of 9.4 mL/min/kg from in vitro CLint was consistent with clinical values ranging from 8 to 15 mL/min/kg (Abernethy et al., 1984; Sutfin et al., 1984; Ciraulo et al., 1988; Sallee and Pollock, 1990). The observed CLm of desipramine were 2–3 and 12 mL/min/kg in PM and EM populations, respectively (Brosen and Gram, 1988; Ciraulo et al., 1988), and, in the present study, CLm of desipramine was reasonably predicted as 1.0 mL/min/kg from in vitro CLint using HLM of PM donors and 6.4 mL/min/kg using EM HLMs. The Fm value was estimated from metabolite profiling experiment, using the UV trace to get an estimate of the percentages. Because there was a secondary metabolite also observed (2-hydroxydesipramine) that could arise via two routes, a range of Fm values was estimated. It is acknowledged that the use of a human hepatocyte metabolite profile has limitations as it does not account for other clearance mechanisms that may occur in vivo (e.g., renal, biliary, extrahepatic metabolism). However, before a new drug candidate can be administered to humans, this method is the best tool available for understanding clearance. In the case of a compound like imipramine, it is reasonable to assume that metabolism will be the clearance mechanism, due to its physicochemical properties (Varma et al., 2015).

Following its formation in liver tissues, desipramine can be subject to sequential metabolism via hydroxylation or permeate the cell membrane into the systemic circulation and the ratio of these two processes will dictate the value Fm. Because a majority of biotransformation reactions result in increased hydrophilicity, metabolites tend to decrease tissue partitioning and plasma protein binding relative to parent drugs (Smith and Obach, 2010; Obach, 2013). In this case, demethylation results in the removal of a methyl group from imipramine, which is a very minor structural change and therefore has a relatively small change in its physicochemical properties. For instance, desipramine exhibits similar lipophilicity (logP of 4.6) as imipramine (logP of 4.8) (Table 1), and this is aligned with the fact that plasma protein binding is not different between imipramine and desipramine, with measured fup of 0.26 and 0.21, respectively (Table 6). Desipramine displays high lipoidal permeability similar to its parent drug, and both imipramine and desipramine are rapidly and completely absorbed when taken orally (fraction of a dose absorbed F > 95%) (Dencker et al., 1976; Sallee and Pollock, 1990). This high human absorption fraction is correlated with their high passive permeability measured in artificial membrane parallel artificial membrane permeability assay (Avdeef et al., 2007; Chen et al., 2008). Therefore, in this study, the passive diffusion clearance of desipramine from the hepatocyte into the systemic circulation (39.1 mL/min/kg) was deemed to be reasonably predicted from passive permeability and hepatocyte surface area. The estimation of the systemic availability of the metabolite can help to elucidate the currently limited understanding regarding metabolite disposition, that is, the predominant metabolites in vitro are not the same as the predominant metabolites in vivo, or certain metabolites circulate once formed, whereas others do not (Smith and Dalvie, 2012; Zamek-Gliszczynski et al., 2014). These differences can be hypothesized to be associated with metabolic enzymes, as well as basolateral efflux in liver, intestine, and kidney. The availability of desipramine following its formation (Fm) was estimated as 60% (Table 2), which indicates that more than one half of the desipramine generated from imipramine escapes sequential metabolism.
clearance and leaves the liver to enter the systemic circulation. Using all of these values extrapolated from in vitro data, CL\text{F,pu}, CL\text{F,pv}, and F\text{pu} permitted an estimation of AUC\text{pu}/AUC\text{pv} for desipramine and imipramine that was in the range observed in clinical studies (Table 4).

The PBPK model built for imipramine was able to simulate the PK profile observed from clinical study following a 50 mg i.v. infusion of imipramine in EM healthy volunteers (Brosen and Gram, 1988). Both imipramine and desipramine are basic and lipophilic compounds and distribute widely to various tissues. The predicted V\text{ss} (11 L/kg) of imipramine using Simcyp is on the low end of reported values mostly ranging from 10 to 20 L/kg (Abemethyl et al., 1984; Ciraulo et al., 1988). The predicted clearance for imipramine is 9.4 ml/min/kg and consistent with the observed range of clinical values (8–15 ml/min/kg).

The PBPK model for metabolite desipramine was developed using desipramine in vitro data. Desipramine was predicted to also have a large volume of distribution (V\text{ss} = 5.5 L/kg) similar to its parent drug. Based on bottom-up approach with in vitro data as input parameters, the minimal PBPK model captured the shape of desipramine concentration-time curve successfully in both EM and PM populations following oral administration of imipramine (Figs. 7 and 9).

Following oral dosing of imipramine to healthy subjects genotyped as CYP2D6 EMs, the desipramine/imipramine AUC ratio was observed to range from 0.48 to 1.1 (Sutfin et al., 1984; Brosen and Gram, 1988; Kurtz et al., 1997). Both static and dynamic PBPK models successfully provided AUC\text{pu}/AUC\text{pv} ratios in estimate reasonable in agreement with reported values in EM volunteers. The AUC-desipramine/AUC-imipramine was 8 times higher in rapid EM compared with PMs with a long t\text{1/2} for desipramine in PM volunteers (more than 2 weeks) (Brosen et al., 1986). For PM populations, the static and dynamic PBPK models predicted the AUC ratios within twofold of observed values. These findings suggest the relevant impact of CYP2D6 activity on the metabolic disposition of imipramine and that in vitro methods and mechanistic modeling can reasonably predict the relative exposure of active metabolite desipramine. When considering the efficacy of imipramine in depressive patients, imipramine and desipramine concentrations should be taken as a basis for dose recommendation (Kirchheiner et al., 2001).

In conclusion, understanding sequential elimination of major metabolites is important to elucidate metabolic exposure. As shown in the present study, characterization of imipramine and its active metabolite desipramine with respect to metabolic clearance by in vitro methods, binding and membrane permeability properties, all coupled with static and dynamic PBPK models can provide mechanistic insight into overall pharmacokinetics and clinical relevance of genetic polymorphism on exposure to desipramine. The methods described in this work are currently employed to other drug and metabolite pairs wherein overall clearance pathways and dispositional properties are different from the example of imipramine and desipramine.

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Participated in research design: Nguyen, Callegari, Obach. Conducted experiments: Nguyen, Obach. Performed data analysis: Nguyen, Callegari, Obach. Wrote or contributed to the writing of: Nguyen, Callegari, Obach.

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