Estimation of Circulating Drug Metabolite Exposure in Human Using In Vitro Data and Physiologically Based Pharmacokinetic Modeling: Example of a High Metabolite/Parent Drug Ratio[§]

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Received August 28, 2017; accepted November 14, 2017

ABSTRACT

(*R*)-4-((4-(((4-((tetrahydrofuran-3-yl)oxy)benzo[d]isoxazol-3-yl)oxy)methyl)piperidin-1-yl)methyl)tetrahydro-2H-pyran-4-ol (TBPT), a serotonin-4 receptor partial agonist, is metabolized to two metabolites: an *N*-dealkylation product [(*R*)-3-(piperidin-4-ylmethoxy)-4-((tetrahydrofuran-3-yl)oxy)benzo[d]isoxazole (M1)] and a cyclized oxazolidine structure [7-(((4-(((*R*)-tetrahydrofuran-3-yl)oxy)benzo[d]isoxazol-3-yl)oxy)methyl)octahydro-3H (M2)]. After administration of TBPT to humans the exposure to M1 was low and the exposure to M2 was high, relative to the parent drug, despite this being the opposite in vitro. In this study, projection of the plasma metabolite/parent (M/P) ratios for M1 and M2 was attempted using in vitro metabolism, binding, and permeability data in static and dynamic physiologically based pharmacokinetic (PBPK) models. In the static model, the fraction of parent clearance yielding the metabolite (which also required taking into account secondary

metabolites of M1 and M2), the clearance of the metabolites and parent, and an estimate of the availability of the metabolites from the liver were combined to yield estimated parent/metabolite ratios of 0.32 and 23 for M1 and M2, respectively. PBPK modeling that used in vitro and physicochemical data input yielded estimates of 0.26 and 20, respectively. The actual values were 0.12 for M1/TBPT and 58 for M2/TBPT. Thus, the ratio for M1 was over-predicted, albeit at values less than unity. The ratio for M2/TBPT was underpredicted, and the high ratio of 58 may exceed a limiting ceiling of the approach. Nevertheless, when considered in the context of determining whether a potential circulating metabolite may be quantitatively important prior to administration of a drug for the first time to humans, the approaches succeeded in highlighting the importance of M2 (M/P ratio $\gg 1$) relative to M1, despite M1 being much greater than M2 in vitro.

Introduction

Circulating drug metabolites have recently become a focus in the development of new drugs regarding safety and drug interactions (<https://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM292340.pdf>; http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/07/WC500129606.pdf; Yu et al., 2015). As new chemical entities to which humans can be exposed, the metabolites of drugs have the potential to exhibit various effects that may be either beneficial (pharmacological activity) or detrimental (toxicity). Despite this possibility, the frequency with which drug metabolites cause unwanted effects unrelated to the activity of the parent drug itself is very low (Smith and Obach, 2006; Isoherranen et al., 2009; Yu and Tweedie, 2013). Nevertheless, there are a few salient instances. For example, CYP2C8 inhibition caused by the

glucuronide metabolite of gemfibrozil was shown to be responsible for a serious drug interaction with cerivastatin (Shitara et al., 2004). Valvulopathy caused by dexfenfluramine could be attributed to the interaction of the nordexfenfluramine metabolite with the serotonin 2b receptor (Rothman et al., 2000). Since human safety is at stake, the subject of drug metabolites merits attention, even if it is known that chemically stable circulating drug metabolites are rarely the culprit behind adverse drug effects.

Drug regulatory guidance on the issue of metabolites and safety has suggested that circulating metabolites comprising 10% or more of the total circulating drug-related material merit greater scrutiny with regard to safety (http://www.ich.org/fileadmin/Public_Web_Site/ICH_Products/Guidelines/Multidisciplinary/M3_R2/Step4/M3_R2_Guideline.pdf). Such metabolites need to be present in laboratory animal species used in toxicology studies at abundances that approach or exceed the exposure observed in humans. Establishing that a metabolite comprises 10% or more of total drug-related material in circulation generally requires administration of radiolabeled drug to humans and quantitative radiometric profiling of plasma samples by high-pressure liquid

<https://doi.org/10.1124/dmd.117.078279>.

[§]This article has supplemental material available at dmd.aspetjournals.org.

ABBREVIATIONS: ABT, 1-aminobenzotriazole; AUC, area under the plasma concentration vs. time curve; CL, clearance; CL_{int}, intrinsic clearance; EI, elasticity index; HBSS, Hanks' balanced salt solution; HLM, human liver microsomes; HPLC, high-performance liquid chromatography; M/P, metabolite/parent; MS, mass spectrometry; *m/z*, mass-to-charge ratio; M1, (*R*)-3-(piperidin-4-ylmethoxy)-4-((tetrahydrofuran-3-yl)oxy)benzo[d]isoxazole; M2, 7-(((4-(((*R*)-tetrahydrofuran-3-yl)oxy)benzo[d]isoxazol-3-yl)oxy)methyl)octahydro-3H,5H-spiro[oxazolo[3,2-*a*]pyridine-2,4'-pyran]; P450, cytochrome P450; PBPK, physiologically based pharmacokinetic; PK, pharmacokinetic; SCHH, sandwich-cultured human hepatocytes; SI, sensitivity index; SLE, supported liquid extraction; TBPT, (*R*)-4-((4-(((4-((tetrahydrofuran-3-yl)oxy)benzo[d]isoxazol-3-yl)oxy)methyl)piperidin-1-yl)methyl)tetrahydro-2H-pyran-4-ol; V_{ss}, volume of distribution at steady state; UHPLC, ultrahigh pressure liquid chromatography.

chromatography (HPLC). Such studies are frequently not done until well into a clinical development program, and if it is determined at this time that one or more metabolites in humans will require further analysis, this can hamper the progress of the development of the experimental drug. Therefore, a method whereby one could anticipate that a drug metabolite(s) will be major in human circulation prior to human studies of the drug would be valuable. Previous studies have shown that estimation of metabolite/parent (M/P) drug exposure ratios can be accomplished using in vitro data on the parent drug and metabolites (Lutz and Isoherranen, 2012; Nguyen et al., 2016a,b, 2017) in either a static model based on relationships first described by Houston (1981) or a dynamic physiologically based pharmacokinetic (PBPK) model. In the example drugs and metabolites used in those studies, the M/P drug ratios in plasma were under seven. In an effort to test the limits of this approach, we have attempted to apply this approach to the experimental drug (*R*)-4-((4-(((4-((tetrahydrofuran-3-yl)oxy)benzo[d]isoxazol-3-yl)-oxy)methyl)piperidin-1-yl)methyl)tetrahydro-2H-pyran-4-ol (TBPT), a partial agonist of the serotonin-4 receptor. In in vitro studies, it was shown that TBPT was metabolized by *N*-dealkylation to metabolite (*R*)-3-(piperidin-4-ylmethoxy)-4-((tetrahydrofuran-3-yl)oxy)benzo[d]isoxazole (M1) and a novel oxidative cyclization to an oxazolidine metabolite 7-(((4-(((*R*)-tetrahydrofuran-3-yl)oxy)benzo[d]isoxazol-3-yl)oxy)-methyl)octahydro-3H (M2), among others (Fig. 1) (Sawant-Basak et al., 2013). The metabolite M1 was major in vitro, whereas M2 was minor; however, a preliminary evaluation of human plasma samples from healthy volunteers dosed with TBPT showed that M2 was in vast excess relative to M1 and TBPT (at least 20-fold) (Sawant-Basak et al., 2013). The objective of the studies reported herein is to test the aforementioned static and PBPK modeling approaches to determine whether they can reasonably project that a minor in vitro metabolite (M2) can be expected to have high relative abundance in plasma.

Materials and Methods

Materials

TBPT, M1, and M2 were prepared at Pfizer Inc., Groton, CT (Brodney et al., 2012; Sawant-Basak et al., 2013). Human hepatocytes for suspension incubations were a custom pool of 10 donors (seven female; three male) prepared by Bioreclamation (Westbury, NY), and cryopreserved hepatocyte lots HH1026 and HH1027 (both lots were prepared from the same female donor) used in the sandwich-cultured system were from In Vitro ADMET (Columbia, MD). Human liver and intestinal microsomes were from 50 and 10 donors, respectively (equal number of male and female donors) and were obtained from Corning-Gentest (Woburn, MA). In VitroGro-HT (thawing), In VitroGro-CP (plating), and

In VitroGro-HI (incubation) hepatocyte media were purchased from Bioreclamation/IVT (Baltimore, MD). Hanks' balanced salt solution (HBSS) was purchased from Lonza (Walkersville, MD). BioCoat 24-well plates and Matrigel were purchased from Corning (Bedford, MA). Pierce BCA Protein Assay Kit was purchased from Thermo Fisher Scientific (Waltham, MA).

Metabolite Profile of TBPT, M1, and M2 in Human Hepatocytes

TBPT, M1, or M2 (10 μ M) were incubated with pooled cryopreserved human hepatocytes (750,000 cells/ml) in 1.0 ml Williams E medium in a humidified incubator maintained at 37°C, 75% relative humidity, and an atmosphere of 95% O₂/5% CO₂. At 0, 0.5, and 3 hours an aliquot was removed (0.3 ml) and added to CH₃CN (1.2 ml). The precipitant was removed by centrifugation (5 minutes, 1700g), and then the supernatant was transferred to a clean tube and removed by vacuum centrifugation. The resulting residue was reconstituted in 0.06 ml 1% formic acid. The reconstituted samples were injected (0.01 ml) onto a Waters Acquity HSS T3 column (2.1 $\times$ 100 mm; 1.7 μ particle size) equilibrated in 0.1% formic acid in 5% CH₃CN at a flow rate of 0.4 ml/min. This mobile phase composition was held for 0.5 minutes followed by a linear gradient to 70% CH₃CN at 4.5 minutes, 95% CH₃CN at 4.6 minutes, and held at this composition for 1 minute, followed by re-equilibration to initial conditions for 1.3 minutes. The eluent was introduced sequentially through a diode array detector (200–400 nm) and then into an ion spray source of a Thermo LTQ Orbitrap Mass Spectrometer (Thermo Fisher Scientific) operated in the positive ion mode. Various experiments were run in the mass spectrometer to gather fragmentation data in support of structural assignment of metabolites.

Enzyme Kinetics of Formation of M1 and M2 from TBPT

TBPT (0.2–200 μ M) was incubated with pooled human liver microsomes (HLM) (1.0 mg/ml) in 0.1 ml potassium phosphate buffer (100 mM, pH 7.45) containing MgCl₂ (3.3 mM) and NADPH (1.3 mM). Incubations, in triplicate, were started with the addition of NADPH and were carried out for 10 minutes at 37°C. Incubations were stopped by the addition of 0.5 ml CH₃CN containing 200 nM clozapine (as an internal standard). The mixtures were spun in a centrifuge for 5 minutes at 1700g and a portion of the supernatant (0.4 ml) was removed by vacuum centrifugation. The residues were reconstituted in 0.1 ml 1% formic acid for analysis by UHPLC mass spectrometry (MS). An identical set of incubations were done in pooled human intestinal microsomes, except that the microsomal protein concentration used was 2.0 mg/ml.

The reconstituted samples were injected (0.01 ml) onto a Waters Acquity HSS T3 column (2.1 $\times$ 100 mm; 1.7 μ particle size; Waters, Milford, MA) equilibrated in 1% formic acid in 5% CH₃CN at a flow rate of 0.4 ml/min. This mobile phase composition program was the same as previously described. The eluent was introduced into an ion spray source of the Thermo LTQ Orbitrap Mass Spectrometer (Thermo Fisher Scientific) operated in the positive ion mode. The instrument was operated in the MS¹ scanning mode [mass-to-charge ratio (*m/z*) 300–650] at a resolution of 30,000. Quantitation was done from reconstructed ion chromatograms of *m/z* 319.1660 (M1, *R*_t = 3.58 minutes), *m/z* 431.2183 (M2; *R*_t = 3.81 minutes), and 327.1377 (clozapine internal standard, *R*_t = 3.76 minutes). The standard curve range was from 5.0 to 2000 nM. Enzyme kinetic data were derived from *V* versus [*S*] data using GraphPad Prism v7.02 (GraphPad Software, Inc., La Jolla, CA).

Metabolic Lability of TBPT, M1, and M2 in Human Liver and Intestinal Microsomes

TBPT, M1, or M2 (1.0 μ M) were incubated with HLM (2.0 mg/ml), in 1.0 ml potassium phosphate buffer (100 mM, pH 7.45) containing MgCl₂ (3.3 mM) and NADPH (1.3 mM). Incubations, in triplicate, were started with the addition of NADPH and were carried out at 37°C. At 0, 2, 5, 10, 15, 20, 30, and 45 minutes an aliquot (0.1 ml) was removed and added to 0.5 ml CH₃CN containing 200 nM clozapine (as an internal standard). Samples were processed and analyzed as described previously. A similar experiment was done for TBPT in pooled human intestinal microsomes.

Permeability and Transport of TBPT, M1, and M2 in Sandwich-Cultured Human Hepatocytes (SCHH)

Plateable cryopreserved hepatocytes were thawed and plated as described previously (Kimoto et al., 2015). Briefly, hepatocytes were thawed in a water bath

Fig. 1. Structures of TBPT, M1, and M2.

at 37°C and placed on ice. The cells were then poured into In VitroGro-HT medium (Bioreclamation/IVT) at 37°C at a ratio of one vial/50 ml in a conical tube. The cells were centrifuged at 50g for 3 minutes and resuspended at 0.75×10^6 cells/ml in In VitroGro-CP medium (Bioreclamation/IVT). Cell viability was determined by Trypan Blue exclusion and exceeded 85%. On day 1, hepatocyte suspensions were plated in collagen-coated 24-well plates at a density of 0.375×10^6 cells/well in a volume of 0.5 ml/well. After 18–24 hours of incubation at 37°C, cells were overlaid with ice-cold 0.25 mg/ml Matrigel in In VitroGro-HI medium (Bioreclamation/IVT) at 0.5 ml/well and cultured for 5 days.

On day 5, the hepatocytes were first rinsed twice with $\text{Ca}^{2+}/\text{Mg}^{2+}$ containing HBSS (standard) or $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS and then preincubated for 10 minutes with standard or $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS in the absence or presence of 1 mM 1-aminobenzotriazole (ABT) or 1 mM ABT/1 mM rifamycin SV. After aspirating the preincubation buffer, 0.5 ml of incubation buffer containing substrate (1 μM) was added in the absence or presence of ABT/rifamycin SV. The uptake was terminated at designated times (0.5, 1, 2, 5, 10, and 20 minutes) by adding 0.5 ml of ice-cold standard HBSS after removal of the incubation buffer. Cells were then washed three times with 0.5 ml of ice-cold standard HBSS. The hepatocytes were lysed with methanol containing the internal standard for liquid chromatography–MS/MS quantification.

TBPT, M1, and M2 were analyzed on a Triple Quadrupole 6500 Mass Spectrometer (SCIEX, Framingham, MA) equipped with a TurboIonSpray interface connected to a SLC-30A LC system (Shimadzu, Kyoto, Japan) and HTC PAL Autosampler (LEAP Technologies, Carrboro, NC). Samples (10 μl) were injected onto a Kinetex C18 column (2.6 μm , 100 Å, 30×3.0 mm; Phenomenex, Torrance, CA) and eluted by mobile phase with initial conditions of 10% solvent B, followed by a linear gradient of 10% solvent B to 100% solvent B over 4 minutes at a flow rate of 0.4 ml/min (solvent A: 100% water with 0.1% formic acid; solvent B: 90% $\text{CH}_3\text{CN}/10\%$ water with 0.1% formic acid). The mass transitions in positive ion mode for monitoring TBPT, M1, and M2 were m/z 433 $\rightarrow$ 194, 319.1 $\rightarrow$ 98, and 431.2 $\rightarrow$ 210, respectively.

Projection of Metabolite/Parent Drug Ratios by Static Modeling

The following equation relates the M/P plasma area under the curve (AUC) ratio ($\text{AUC}_m/\text{AUC}_p$) after oral administration of the parent (Nguyen et al., 2016a,b) derived from original equations described by Houston (1981):

$$\frac{\text{AUC}_m}{\text{AUC}_p} = \frac{F_m \cdot f_{CL,m} \cdot CL_p}{F_h \cdot CL_m} + \frac{F_m \cdot F_{m,gut} \cdot f_{gut,CL,m} \cdot (1 - F_g) \cdot CL_p}{F_g \cdot F_h \cdot CL_m} \quad (1)$$

The first term represents the ratio driven by liver and the second term represents the ratio driven by the gut during first pass. The latter is needed because the metabolites being investigated are generated by CYP3A4, which has a meaningful presence in the intestine. The blood clearance (CL) values for hepatic CL_p and CL_m were generated by scaling the in vitro intrinsic CL (CL_{int}) values for parent and metabolite ($CL_{int,p}$ and $CL_{int,m}$) using factors of 45 mg microsomal protein per gram liver and 20 g of liver per kilogram of body weight, and inserting these into the well-stirred model of hepatic clearance (Pang and Rowland, 1977):

$$CL_p = \frac{Q_h \cdot f_{u,p} \cdot CL_{int,p}}{Q_h + f_{u,p} \cdot CL_{int,p}} \quad (2)$$

$$CL_m = \frac{Q_h \cdot f_{u,m} \cdot CL_{int,m}}{Q_h + f_{u,m} \cdot CL_{int,m}} \quad (3)$$

where a value of 21 ml/min/kg was used for hepatic blood flow (Q_h), and $f_{u,p}$ and $f_{u,m}$ represent the free fractions in blood for parent and metabolite, respectively. The fraction of the parent that evades first pass hepatic extraction (F_h) was estimated as

$$F_h = 1 - \frac{CL_p}{Q_h} \quad (4)$$

The values for the fraction of clearance of parent drug proceeding through a metabolite in the liver ($f_{CL,m}$) and gut ($f_{gut,CL,m}$) were estimated using two approaches. The first used the in vitro metabolite formation intrinsic clearance for each pathway directly (i.e., V_{max}/K_M) divided by the total in vitro intrinsic clearance of parent consumption determined by the in vitro half-life, $t_{1/2}$ ($CL_{int} = 0.693/\ln$ vitro

$t_{1/2}$, corrected for the concentration of microsomes). This was calculated for liver and intestinal microsomes, separately as follows:

$$f_{CL,m} = \frac{CL_{int,metabolite\ formation}}{CL_{int,parent\ consumption}} \quad (5)$$

In the second approach, secondary metabolites arising from M1 and M2 were added to their respective formation CL_{int} values. The UV peak areas of M1, M2, and their secondary metabolites from the metabolite profile (as previously described) were determined (assuming similar molar extinction coefficients for the metabolites, which is reasonable since the benzisoxazole chromophore is unaltered). The peak area for M1 and its secondary metabolites (m/z 335 and 415) (Fig. 2) were summed and the M1 CL_{int} value was multiplied by the ratio of peak areas of this sum divided by the peak area of M1 itself. The same was done for M2 by including the peak area of the secondary metabolite m/z 333 (Fig. 2). This accounts for underestimates of CL_{int} due to any further metabolism of the metabolites that were missed in the enzyme kinetic experiments. Similar data sets were used previously in the estimation of f_m values for use in projecting M/P ratios for imipramine and losartan (Nguyen et al., 2016a, 2017).

The values for F_m for metabolites, i.e. the fraction of the metabolite formed in the liver that escapes into the systemic circulation, were estimated from the following equation:

$$F_m = \frac{Q_h}{Q_h + f_{u,m} \cdot CL_{int,m}} \quad (6)$$

where the parameters are as previously described. The value for the parent availability through the gut following oral administration (F_g) was estimated as

$$F_g = \frac{Q_{gut}}{Q_{gut} + f_{u,p} \cdot CL_{int,p,gut}} \quad (7)$$

where Q_{gut} is the villous blood flow (4 ml/min/kg); $f_{u,p}$ is the unbound fraction of parent drug in blood; and $CL_{int,p,gut}$ is the intrinsic clearance for parent consumption in the intestine scaled from intestinal microsomal lability. A value of 2978 mg microsomes per intestine was used (Hatley et al., 2017). The corresponding value for $F_{m,gut}$ was assumed to be unity. The M/P ratios were adjusted to accommodate differences in molecular weight between metabolites and parent drug.

Dynamic Physiologically Based Pharmacokinetic Modeling in Simcyp

The PBPK models for TBPT and its two metabolites, M1 and M2, were developed to simulate the observed disposition profile of the metabolites after daily oral administration of TBPT (15 mg P.O.) using the population-based PBPK simulator Simcyp (version 15; Simcyp Ltd., Sheffield, United Kingdom). The simulations were performed for a virtual population of 100 healthy volunteers (10 trials $\times$ 10 subjects each) aged between 20 and 50 years with a male/female ratio of 50/50. For TBPT, a full PBPK model was developed assuming perfusion-limited distribution and using physicochemical properties (pK_a , log P), biochemical properties (human plasma f_u , blood-to-plasma ratio), and in vitro metabolic CL_{int} values. The absorption model was first order, with an absorption rate constant set at 0.6 h^{-1} based on clinical data. The F_a value was set at 0.6 based on in vitro permeability assay and preclinical PK parameters.

For M1 and M2, the volume of distribution at steady state (V_{ss}) was predicted using method 1 within the Simcyp simulator (Simcyp Ltd.), which is based on input parameters of protein binding and lipophilicity. The V_{ss} values of M1 and M2 were predicted to be 1.64 and 0.26 l/kg, respectively. The V_{ss} value for TBPT was estimated by allometric scaling of dog V_{ss} data. The M1 and M2 CL_{int} values were obtained from in vitro metabolic stability of each of the two analytes in HLM. Enzyme kinetic information using HLM was selected for TBPT metabolism.

Sensitivity analysis was performed to identify which parameters must be known with the greatest accuracy to most accurately predict the AUC_m values of TBPT metabolites. The parameters investigated included TBPT, M1, and M2 formation intrinsic clearance, elimination intrinsic clearance, plasma protein binding, blood-to-plasma ratio, and log P. The metrics examined were the AUC and C_{max} values of M1 and M2 after oral administration of TBPT at 15 mg.

Fig. 2. HPLC-UV traces of extracts of hepatocyte incubations of TBPT, M1, and M2.

The sensitivity of the M1 and M2 AUC and $C_{\max}$ values to 0.1% changes in the mean values of selected parameters using Simcyp version 15 (Simcyp Ltd.) was calculated. The elasticity index (EI) is a dimensionless expression of sensitivity [i.e., the sensitivity index (SI)] that measures the relative change in an output variable Q (e.g., AUC) for a relative change in an input parameter P . The EI was calculated as follows for each input parameter value n :

$$EI_n = \left(\frac{P_n}{Q(P_n)} \right) \cdot SI \quad (8)$$

where $Q(P_n)$ is the value of Q when $P = P_n$ and SI is a measure of the change in the output variable Q per unit change in the input parameter value P_{n+1} from its initial value P_n , calculated using the following equation:

$$SI_n = \frac{Q(P_{n+1}) - Q(P_n)}{P_{n+1} - P_n} \quad (9)$$

The EIs were used to rank the importance of parameters on the output variables.

Clinical Pharmacokinetics

This clinical study was conducted in compliance with the ethical principles originating in or derived from the Declaration of Helsinki and in compliance with all International Conference on Harmonization Good Clinical Practice Guidelines and the International Ethical Guidelines for Biomedical Research Involving Human Subjects (Council for International Organizations of Medical Sciences 2002). In addition, all local regulatory requirements were fulfilled, particularly those affording greater protection to the safety of study participants.

Dosing of Human Volunteers and Collection of Samples. The clinical pharmacokinetic (PK) data were available from a phase 1 multiple-dose escalation

study in healthy adult and elderly subjects (Nicholas et al., 2011). From this study, the present analysis was done for just the healthy subject data from the 15 mg/day group on days 1 and 14. The demographic characteristics for this group ($N = 8$; four Caucasian and four African-American subjects) were as follows: age, 31.6 years (range 21–49); weight, 83.9 kg (range 67.1–99.8); and body mass index, 27.6 (range 24.2–29.7). Predose samples were also collected on days 1, 2, 3, 4, 5, 6, 7, 8, 10, 12, and 14 to assess if steady state was reached in the study.

Assay of TBPT and Metabolites. The HPLC-MS/MS method was validated for the analysis of TBPT, M1, and M2 in human plasma treated with K_2EDTA anticoagulant. Internal standard (0.05 ml of 1.60 ng/ml in H_2O/CH_3CN) was added to the samples followed by extraction utilizing methyl-*tert* butyl ether-based supported liquid extraction (SLE) [isolute SLE + SLE plate (400 μ l); Biotage, Charlotte, NC]. The SLE extracts were evaporated under nitrogen at 35°C. The reconstituted extracts were injected (15 μ l) onto a liquid chromatographic system consisting of a Shimadzu solvent delivery system (LC-20AD), Shimadzu autosampler (Prominence SIL-20AC refrigerated to 8°C), Shimadzu column oven (CTO-20AC) operated at 30°C, and a SCIEX API 5000 tandem mass spectrometer. The chromatographic separation was achieved using a Phenomenex Luna C18(2), 50 $\times$ 2.0 mm, 5 μ m analytical column equipped with an Upchurch 0.5 μ m prefilter (IDEX Corp., Middleborough, MA). The following gradient was applied utilizing 0.1% formic acid in water and 0.1% formic acid in methanol as mobile phase A and mobile phase B (respectively: 0.01–2.00 minutes (25%–60% B); 2.00–2.20 (60%–95% B); 2.20–3.00 (hold at 95% B); 3.00–3.35 (95%–25% B); 3.35–4.00 (hold at 25% B); 4.00–5.50 (25%–65% B); 5.50–5.60 (hold at 65% B); 5.60–5.80 (65%–25% B); and 5.80–6.50 (hold at 25% B). Eluent from the liquid chromatography was analyzed by a SCIEX API 5000 tandem mass spectrometer operated in positive electrospray mode. The following transitions were monitored: TBPT (433.3 $\rightarrow$ 212.2); M1 (319.3 $\rightarrow$ 98.1); M2 (431.3 $\rightarrow$ 210.2); [2H_9]TBPT (internal standard 1, 442.3 $\rightarrow$ 221.2); and [2H_9]

Fig. 3. Enzyme kinetic plots for the formation of metabolites M1 and M2 from TBPT in human liver and intestine microsomes.

M1 (internal standard 2, 328.3 $\rightarrow$ 107.1). The approximate retention times were as follows: TBPT (1.12 minutes); M1 (1.06 minutes); M2 (1.42 minutes); [$^2\text{H}_9$]TBPT (1.11 minutes); and [$^2\text{H}_9$]M1 (1.05 minutes). The dynamic range of the assay was 5.00–7500 pg/ml for TPBT, 2.00–3000 pg/ml for M1, and 25.0–37500 pg/ml for M2. A linear ($1/x^2$) regression was applied to the analyte/internal standard peak area ratios utilizing Applied Biosystems/MDS SCIEX Analyst (version 1.5, Concord, Ontario, Canada) and Watson (version 7.3.0.01).

Pharmacokinetic Calculations. Standard PK parameters, $C_{\max}$ (maximum plasma concentration), $T_{\max}$ (time for $C_{\max}$), and AUC_τ (area under the plasma concentration vs. time curve over the dosing interval) were calculated using noncompartmental analyses. The PK calculations were done using an internal program called eNCA that uses S-PLUS scripts to calculate noncompartmental parameters. The PK data from healthy adults at 15 mg daily dose on days 1 and 14 are presented for comparison against model predictions.

Results

Metabolite Profiles of TBPT, M1, and M2 in Human Hepatocytes.

In order to know what in vitro systems would offer good quantitative representations of clearance (i.e., the types of initial metabolic reactions that TBPT, M1, and M2 undergo) must be known. Hepatocytes were selected as a suitable in vitro system since they possess a nearly complete array of drug metabolizing enzymes and cofactors. HPLC-UV profiles of extracts of hepatocyte incubations of TBPT, M1, and M2 are shown in Fig. 2. For TBPT, the vast majority of metabolism is via oxidative routes with the exception of a very small amount of direct glucuronidation. (A proposed metabolism scheme for TBPT, M1, and M2 is shown in Supplemental Fig. 1). Observation of a large peak for M1 as well as a few downstream metabolites suggests that *N*-dealkylation is the major route of TBPT metabolic clearance. For the metabolism of M1, all initial metabolic reactions are oxidative, with some subsequent conjugation. For M2, metabolism was extensive and all initial pathways also

appeared to be via oxidative biotransformation. Thus, in subsequent intrinsic clearance measurements, liver microsomes with cofactors and conditions in support of cytochrome P450 (P450) activity were used for all three compounds, since initial metabolic clearance pathways were all via P450 oxidation reactions. It is important to note that the major metabolite of M2 is denoted as oxo-M1 (m/z 333) (see Supplemental Fig. 1) and that this metabolite was also observed in the incubation of TBPT but very slightly in the incubation of M1 itself. From M2, this would arise via *N*-dealkylation of the piperidine iminium ion tautomer of M2 (i.e., metabolite designated as m/z 317 eluting at a retention time of 4.35 minutes in Fig. 2), followed by oxidation of the imine to the lactam. Thus, inclusion of oxo-M1 as an important contribution to the M2 pathway is important in estimating the formation CL_{int} for M2 (as discussed subsequently).

TABLE 1

Enzyme kinetic parameters for the metabolism of TBPT, M1, and M2 in human liver and intestinal microsomes

	$V_{\max}$	K_M	CL_{int}
	pmol/min/mg	μM	$\mu\text{L/min/mg}$
Liver microsomes			
TBPT $\rightarrow$ M1	1050 $\pm$ 38	89 $\pm$ 7	12
TBPT $\rightarrow$ M2	456 $\pm$ 33	181 $\pm$ 22	2.5
TBPT consumption	—	—	32
M1 consumption	—	—	32
M2 consumption	—	—	102
Intestinal microsomes			
TBPT $\rightarrow$ M1	385 $\pm$ 19	112 $\pm$ 11	3.4
TBPT $\rightarrow$ M2	31 $\pm$ 6	31 $\pm$ 6	1.4 ^a
TBPT consumption	—	—	13

^aIncludes additional high K_M and high $V_{\max}$ activity.

Fig. 4. Hepatic transport of TBPT, M1, and M2 in SCHH. The accumulation of TBPT, M1, and M2 was investigated at 37°C with standard HBSS (control) in the absence or presence of 1 mM rifamycin SV/1 mM ABT or $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS in hepatocyte lot HH1027 for TBPT and HH1026 for M1 and M2. Black open circle (○) represents control with 1 mM ABT, red open triangle (△) represents control with 1 mM rifamycin SV/1 mM ABT, and blue cross (×) represents $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS.

Enzyme Kinetics and Intrinsic Clearance of TBPT, M1, and M2.

Enzyme kinetics of the conversion of TBPT to M1 and M2, as well as measurement of consumption of TBPT, M1, and M2 were all needed to make estimates of the CL_{int} and $f_{\text{CL,m}}$ values. These measurements were made in liver and intestinal microsomes. Substrate saturation plots for the formation of M1 and M2 from TBPT in liver and intestinal microsomes are shown in Fig. 3. For M1, the kinetics appeared to follow simple hyperbolic Michaelis-Menten kinetics with K_M values of 89 and 112 μM in liver and intestine, respectively. The CL_{int} values were calculated to be 12 and 3.4 $\mu\text{L}/\text{min}/\text{mg}$ protein. For M2, the K_M values were 181 and 31 μM in liver and intestine, respectively, and the CL_{int} values were 2.5 and 1.4 $\mu\text{L}/\text{min}/\text{mg}$ protein in liver and intestinal microsomes, respectively. The kinetic model best describing the formation of M2 in intestinal microsomes was a two-enzyme model with a high K_M activity that could only be described as a composite slope. The kinetic parameters are listed in Table 1.

Consumption of TBPT, M1, and M2 was also measured in liver microsomes. The CL_{int} values were estimated at 32, 32, and 102 $\mu\text{L}/\text{min}/\text{mg}$ for TBPT, M1, and M2, respectively. Combining the formation kinetic data for M1 and M2 given previously with these total CL_{int} values for TBPT yielded estimates of the $f_{\text{CL,m}}$ values of 0.38 for M1 and 0.08 for M2 in liver. The total CL_{int} value for TBPT in intestinal microsomes was 13 $\mu\text{L}/\text{min}/\text{mg}$, and combining this value with the formation CL_{int} values for M1 and M2 yielded estimates of $f_{\text{gutCL,m}}$ of 0.26 and 0.11, respectively.

The estimates of formation CL_{int} values for M1 and M2 were also corrected for the potential underestimation due to sequential metabolism. In these examples, such a correction was important since the consumption CL_{int} values for M1 and M2 exceeded their CL_{int} values for formation. For M1, this offered an incremental increase by a factor of 1.1-fold (based on the relative peak areas of secondary M1 metabolites to the peak area for M1). However, for M2 such an analysis was important since the M2 consumption CL_{int} value was substantially greater than its formation CL_{int} value (102 vs. 2.5 $\mu\text{L}/\text{min}/\text{mg}$) and the peak area for the secondary metabolite m/z 333 was much larger than that for M2 itself in the HPLC trace of the metabolism of TBPT (Fig. 2). The CL_{int} value of formation for M2 was increased by a factor of 5.2. The fractions of the clearance value estimated in this manner were 0.41 and 0.41 for M1 and M2, respectively.

Hepatic Transport Study of TBPT, M1, and M2 in SCHH. The hepatic uptake of TBPT, M1, and M2 was investigated in human hepatocytes with an in vitro SCHH model. The accumulation of compounds in SCHH with standard HBSS with 1 mM ABT, which is a pan P450 inhibitor, or with 1 mM ABT/1 mM rifamycin SV, which is an OATP pan inhibitor, indicated passive diffusion or total uptake, respectively. The difference in compound accumulation in SCHH between standard HBSS and $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS represents a contribution of biliary excretion. As shown in Fig. 4, the uptake of

TBPT and M1 was not inhibited by rifamycin SV, whereas the uptake of M2 was slightly affected by rifamycin SV. The contribution of biliary excretion for all three was not determined under the test condition in the SCHH system. The in vitro CL_{int} values for total and passive transport are listed in Table 2.

Clinical Pharmacokinetic Data for TBPT, M1, and M2 Following Daily Oral Administration of TBPT. All of the subjects completed the study and were evaluated for adverse events, safety laboratory tests, and pharmacokinetics (Nicholas et al., 2011). There were no serious adverse events associated with this study. There were no vital signs, electrocardiogram, or safety laboratory test findings of potential clinical concern. Overall, TBPT was well tolerated up to a dose of 15 mg/day over 14 days of dosing; the results from the 15 mg/day dose in healthy adults are reported subsequently.

The mean plasma concentration versus time data are plotted in Fig. 5, and the mean PK parameters are listed in Table 3. TBPT demonstrated rapid absorption, with a median T_{max} occurring at 1–2 hours post dose. Following attainment of C_{max} , plasma concentrations declined in a biphasic fashion and the average CL_p/F ranged from 12 to 22 $\text{mL}/\text{min}/\text{kg}$ (correcting for a mean body weight of 83.9 kg) across a dose range of 0.1–15 mg daily in healthy adults. With repeated daily administration, plasma concentrations of TBPT were at steady state on day 14 and did not accumulate (as observed by median trough concentration vs. time profiles; data not shown).

The PK profiles of M1 and M2 following multiple daily dosing of 15 mg TBPT are summarized in Fig. 5 and Table 3. Following oral dosing of 15 mg daily TBPT, the mean C_{max} and AUC_τ values of the *N*-dealkylated metabolite M1 on day 1 were 1.7 ng/mL and 10.0 ng·h/mL, respectively, which were ~14- and ~12-fold lower than that of the parent, respectively, and there was no observable accumulation with repeated administration of TBPT. The overall ratio of M1:TBPT based

TABLE 2

In vitro intrinsic clearance values of TBPT, M1, and M2 in SCHH

The accumulation of TBPT, M1, and M2 (1 μM) was investigated at 37°C with standard HBSS in the absence or presence of 1 mM rifamycin SV/1 mM ABT or $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS in SCHH. The in vitro CL values were calculated by initial rate analysis with a linear fitting up to 2 minutes. $\text{CL}_{\text{uptake}}$, $\text{CL}_{\text{passive}}$, and CL_{bile} represent the in vitro total uptake clearance, passive clearance, and biliary clearance when incubating with standard HBSS with 1 mM ABT, standard HBSS with 1 mM rifamycin SV/1 mM ABT, and $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free HBSS, respectively. Values in parentheses are S.E.

Clearance	TBPT	M1	M2
$\mu\text{L}/\text{min}/\text{mg}$			
$\text{CL}_{\text{uptake}}$	63 (6.3)	48 (0.86)	21 (1.7)
$\text{CL}_{\text{passive}}$	83 (2.5)	49 (5.5)	16 (2.4)
CL_{bile}	ND	ND	ND

ND, not determined.

Fig. 5. Mean plasma concentration vs. time plots for TBPT, M1, and M2 in healthy human volunteers following daily oral administration of 15 mg. TBPT is in blue triangles, M1 in green squares, and M2 in red circles. Inset plots are the same data on a semilogarithmic scale.

on the $AUC_{0-\tau}$ value was 0.08–0.09 (0.11–0.12 when computed on a molar basis). In contrast, administration of TBPT led to high total plasma levels of M2 as assessed by the mean C_{max} and AUC_{τ} values on days 1 and 14. At 15 mg daily dose of TBPT the day 14 M2:TBPT exposure ratios were at least 2- to 3-fold greater than that on day 1. The half-life of M2 could not be estimated due to insufficient regression points in the elimination phase. Comparing the concentration versus time profiles for M2 and TBPT it can be observed that M2 has a prolonged elimination rate, which was greater than that of the parent. This suggests elimination rate-limited kinetics.

Projection of Metabolite/Parent Exposure Ratios from Static Modeling. The measured values from the in vitro experiments were

scaled to reflect the in vivo parameters and were used to calculate the terms needed in eq. 1 (Table 4). TBPT was projected to be a moderate clearance compound ($CL_p = 7.9$ ml/min/kg, approximately 40% of hepatic blood flow), and while metabolite M1 was also projected to have moderate clearance (11 ml/min/kg), the clearance for M2 was projected to be low (0.4 ml/min/kg). This is despite the fact that the hepatic total intrinsic clearance of M2 is greater than that for TBPT or M1 and this low scaled clearance value is driven by the substantially greater plasma protein binding of M2. The percentages of metabolic clearance of TBPT proceeding through the pathways that yield M1 and M2 were 38%–41% for M1 and 8%–41% for M2. These were estimated by dividing the formation CL_{int} values for each metabolite by the total substrate

TABLE 3

Exposure metrics for TBPT, M1, and M2 in healthy human volunteers following daily oral administration of 15 mg TBPT compared with values predicted using dynamic PBPK modeling

Metric	TBPT		M1			M2		
	Predicted	Observed	Predicted ^a	Predicted ^b	Observed	Predicted	Predicted ^b	Observed
Day 1								
C_{max} (ng/ml) ^c	32.6	23.1 (45)	5.2	5.4	1.7 (77)	36	182	148 (28)
T_{max} (h) ^d	2.9	1.5 (1–2)	3.4	3.5	1.5 (1–2)	8.9	8.9	1 (0.5–2)
$AUC_{0-\tau}$ (ng·h/ml) ^c	312	123 (51)	56	59	10.0 (99)	700	3541	2440 (35)
Day 14								
C_{max} (ng/ml) ^c	33.5	23.1 (46)	5.4	5.6	1.7 (87)	65	331	429 (33)
T_{max} (h) ^d	2.9	1 (1–2)	3.4	3.4	2 (1–2)	6.4	6.4	2 (1–4)
$AUC_{0-\tau}$ (ng·h/ml) ^c	322	134 (55)	58	62	11.6 (102)	1312	6723	7850 (39)

^a T_{max} , time to reach C_{max} .

^bPredicted using experimentally measured formation CL_{int} values.

^cPredicted using formation CL_{int} values corrected for secondary metabolism. All predicted values are point estimates of mean values.

^dValues are mean (%CV) for AUC and C_{max} .

^eValues are median (range) for T_{max} .

TABLE 4

Estimated input parameters for static model prediction of M/P drug ratios

The constants are $Q_h = 21$ ml/min/kg, $Q_{gut} = 4$ ml/min/kg; 45 mg microsomes/gm liver, 20 gm liver/kg/b.wt., and 2978 mg microsomes/whole intestine (i.e., 42 mg intestinal microsomes/kg/b.wt.).

Parameter	Input Values	Scaled Value
TBPT		
CL_p	$f_u = 0.38$; B/P = 0.86; $CL_{int} = 32$ μ l/min/mg	7.9 ml/min/kg
F_h	$CL_p = 7.9$ ml/min/kg	0.62
F_g	$f_u = 0.38$; B/P = 0.86; $CL_{int} = 13$ μ l/min/mg	0.94
Metabolite M1		
CL_m	$f_u = 0.64$; B/P = 0.88; $CL_{int} = 32$ μ l/min/mg	11 ml/min/kg
$f_{CL,m}$	$CL_{int,TBPT} = 32$ μ l/min/mg; $CL_{int,TBPT \rightarrow M1} = 12$ μ l/min/mg	0.38 or 0.41 ^a
F_m	$f_u = 0.64$; B/P = 0.88; $CL_{int} = 32$ μ l/min/mg	0.47
$f_{gutCL,m}$	$CL_{int,TBPT} = 13$ μ l/min/mg; $CL_{int,TBPT \rightarrow M1} = 3.4$ μ l/min/mg	0.26 or 0.30 ^a
$F_{m,gut}$	Assumed as unity	1
Metabolite M2		
CL_m	$f_u = 0.0025$; B/P = 0.53; $CL_{int} = 102$ μ l/min/mg	0.40 ml/min/kg
$f_{CL,m}$	$CL_{int,TBPT} = 32$ μ l/min/mg; $CL_{int,TBPT \rightarrow M2} = 2.5$ μ l/min/mg	0.08 or 0.41 ^a
F_m	$f_u = 0.0025$; B/P = 0.53; $CL_{int} = 102$ μ l/min/mg	0.98
$f_{gutCL,m}$	$CL_{int,TBPT} = 13$ μ l/min/mg; $CL_{int,TBPT \rightarrow M2} = 1.4$ μ l/min/mg	0.11 or 0.58 ^a
$F_{m,gut}$	Assumed as unity	1

B/P, blood-to-plasma ratio.

^aValues are corrected for by secondary metabolism (see *Materials and Methods*).

consumption intrinsic clearance of TBPT. The higher percentages include the correction for secondary metabolism. The fractions of each metabolite generated within the liver that evades further immediate metabolism were estimated from the projected CL_{int} values of the metabolites versus liver blood flow. For M2, it was estimated that almost all metabolites leave the liver prior to further hepatic clearance, whereas for M1 about half of the metabolites generated undergo subsequent clearance prior to release from the liver. Putting all of these factors together in the static model, eq. 1 yielded that the projected M/P exposure ratios for M1 and M2 were 0.29 and 4.2, respectively, using the measured formation CL_{int} values only, and were 0.32 and 23,

respectively, following correction of the formation CL_{int} values for unmeasured secondary metabolism (Table 7). For M2, its rapid metabolism to secondary metabolites had a large impact on the projection of the M/P ratio, whereas for M1 this factor did not have much impact. Finally, because most of TBPT is projected to evade first pass intestinal metabolism ($F_g = 0.94$) little of the exposure to metabolites M1 and M2 is due to their generation in the intestine; almost all of the exposure derives from the liver.

Projection of Metabolite/Parent Exposure Ratios from Dynamic PBPK Modeling. The input parameters used for PBPK modeling are summarized in Table 5. The predicted versus observed plasma

TABLE 5

Simcyp input parameters for TBPT, M1, and M2

Parameter	TBPT	M1	M2	Source
Molecular weight (g/mol)	432	318	430	Calculated ^c
Log P	1.68	2.19	2.08	Calculated ^c
Compound type	Monoprotic base	Monoprotic base	Monoprotic base	
pK_a	8.8	10.22	6.48	Calculated ^c
B/P	0.86	0.88	0.55 ^a	Measured
$f_{u,p}$	0.38	0.64	0.0025	Measured
Absorption				
Model	First order	NA	NA	
Fraction absorbed	1	NA	NA	Assumed
k_a (1/h)	0.6	NA	NA	Fitting
f_u (gut)	1	1	1	Assumed
Manin-Darby Canine Kidney Cell Permeability (10^{-6} cm/s)	23	NA	NA	Measured
Distribution				
Model	Minimal PBPK	Minimal PBPK	Minimal PBPK	
V_{ss} (l/kg)	2.1 ^b	1.64 ^c	0.26 ^c	Fitting
Elimination				
3A4 CL_{int} (μ l/min/mg) (forming M1)	12 or 13 ^d			Measured
3A4 CL_{int} (μ l/min/mg) (forming M2)	2.5 or 13 ^d			Measured
Additional HLM CL_{int} (μ l/min/mg)	17.5 or 6	32	102	Measured

B/P, blood-to-plasma ratio.

^aMinimum value for Simcyp is 0.55; measured value was 0.53.^bPredicted using dog PK data.^cValue from Simcyp using method 1.

^d CL_{int} values represent measured values and measured values multiplied by factors of 1.1 and 5.2 for M1 and M2, respectively, to account for secondary metabolism.

^eCalculated using ACD/Labs Software (Advanced Chemistry Software, Toronto, Ontario, Canada).

Fig. 6. Comparison of plasma concentration vs. time profiles for TBPT, M1, and M2 simulated by PBPK modeling vs. actual clinical data. Red circles represent the mean measured concentrations and green lines represent the simulated profiles.

concentration-time profiles of TBPT, M1, and M2 following oral administration of TBPT (15 mg) are displayed in Fig. 6. The predicted PK parameters are summarized and tabulated in Table 3 for comparison with the measured parameters. Overall, PBPK modeling can reasonably recover the oral profiles of TBPT and its metabolites using *in vitro* metabolic input parameters. After oral dosing of 15 mg the predicted mean $AUC_{0-\tau}$ value for TBPT was 312 ng·h/ml, which was 2.5-fold higher than the observed mean value of 123 ng·h/ml, while the mean C_{max} value was overpredicted by 1.4-fold (32.6 vs. 23.1 ng/ml). The predicted $AUC_{0-\tau}$ value of M1 was 56 ng·h/ml, which was 5.6-fold higher than the observed value of 10 ng·h/ml. Thus, for both parent drug

and M1, the exposure was somewhat overpredicted; however, the M/P ratio for M1 was better predicted since the overpredictions of exposure were partially off-setting (the predicted ratio was 0.24 while the actual ratio was 0.12). Accumulation upon repeated administration was neither predicted nor observed.

However, for M2 on day 1 the observed $AUC_{0-\tau}$ value was 2440 ng·h/ml, whereas the predicted value was only 700 ng·h/ml. Accumulation of 1.9-fold was predicted for M2 while the actual accumulation was 3.2, thus the underprediction of the M/P ratio was greater for multiple dosing, wherein the observed $AUC_{0-\tau}$ value was 7850 ng·h/ml while the projected value was 1312 ng·h/ml. However,

as was the case for the static model, taking into account the experimental underestimation of the metabolite formation CL_{int} value for M2 altered the estimation of metabolite exposures in dynamic PBPK modeling and substantially improved these. The predicted $AUC_{0-\tau}$ value increased to 3541 and 6723 ng·h/ml for days 1 and 14, respectively. The day 1 model projection was an ~ 1.5 -fold overestimate while the day 14 projection was fairly accurate (86% of the actual value). The M/P ratio at steady state was estimated at 20 compared with the actual value of 58, and this was largely driven by the overestimate of parent exposure.

The sensitivity analysis of the main PBPK model input parameters revealed that the values for plasma protein binding and intrinsic clearance for the metabolite are the two most sensitive parameters for estimates of M1 and M2 AUC values (Table 6). Clearance of formation of the metabolites was also sensitive for M2 and a bit less so for M1. The log P and blood/plasma ratios were much less sensitive parameters.

Discussion

In the development of new drug candidates, possessing an ability to predict major metabolites in human circulation prior to administration in phase 1 clinical trials would be of benefit. Such predictions could be used in identifying/predicting important circulating drug-related material and in making decisions regarding which putative metabolites to synthesize and analyze in plasma samples from preclinical toxicology studies and first-in-human studies. The pharmacokinetic principles underlying the exposure to metabolites have existed for decades (Houston, 1981; St-Pierre et al., 1988) and contributing factors include the fraction of the clearance of the parent drug that proceeds via conversion to the metabolite of interest (f_m), the relative systemic clearance rates for the metabolite and parent (CL_{parent} and $CL_{metabolite}$), and the fraction of the metabolite that enters the systemic circulation from its organ of generation (most typically the liver) prior to it being further cleared (F_m). These parameters can be used in static models and they also underlie the algorithms used in more complex PBPK models. Several in vitro experiments can be done to generate data used to estimate these input values. Despite these principles being decades old, examples of their practical application have only been recently described. Lutz and Isoherranen (2012) carried out work wherein the exposures to metabolites of omeprazole and dextromethorphan were estimated using these principles. Likewise, work from Nguyen et al. (2016a,b, 2017) applied both static and PBPK modeling to estimate plasma exposures to metabolites of midazolam, imipramine, and losartan. Such methods are challenged by the many different mechanisms of metabolite generation and clearance and how well these can be measured using in vitro approaches. Furthermore, despite the complexity of the equations and models, several assumptions regarding parent drug and metabolite disposition are necessary to simplify the approach to this challenge.

TBPT offered a unique opportunity to test these approaches because there were two metabolites measured in clinical studies, wherein one

was in great excess relative to the parent drug (M2) and the other was present at far lower concentrations than the parent drug (M1), while the opposite was observed in in vitro metabolism studies using human-derived reagents (Sawant-Basak et al., 2013). Metabolite M2 was considered unimportant from in vitro profiles and exemplifies the shortcomings of attempting to relate in vitro metabolite profile data directly to human plasma metabolite profiles without other considerations (Dalvie et al., 2009; Loi et al., 2013). The projection of the M/P exposure ratio for M1 was in reasonable agreement with the actual data (0.12 actual vs. 0.26–0.32 predicted, see Table 7). While just over 2-fold inaccurate, when taken from a perspective of drug development, correct decisions would be made regarding the lack of need to analyze and better understand this metabolite, and despite M1 being a major clearance pathway observed in vitro its exposure in vivo is low, which is likely due to subsequent systemic metabolic clearance that is faster than that of the parent drug.

The M/P AUC ratio for M2 was underestimated (Fig. 6; Table 7). Based on the in vitro data, M2 was formed via oxidation in human liver, with very little in the intestine based on the estimated F_g value of 0.94 for TBPT. M2 was marked by a rapid rise in human plasma (T_{max} of 1 hour post dose of TBPT), followed by a biphasic elimination profile where the slope of its elimination from plasma was estimated to be lower than that of TBPT, suggesting elimination rate-limited kinetics. This phenomenon could partly explain why the exposure to M2 was higher than anticipated; the day 14 plasma profile shows that it accumulated with repeat administration of TBPT, which could again be attributed to the low clearance and prolonged plasma elimination half-life. This elimination rate-limited kinetic profile is in contrast to the high total intrinsic clearance of M2 (102 μ l/min/mg) due to its rapid turnover in human hepatocytes; scaled clearance using binding parameters and intrinsic clearance in the well-stirred model predicted a CL_m value of 0.4 ml/min/kg for M2, which was more than 10-fold lower than that for TBPT (7.9 ml/min/kg). Accumulation of M2 on day 14 can be accounted for by the elimination half-life and would be independent of the half-life of the parent.

In vivo clearance values for TBPT and M2 are not available since neither has been directly administered intravenously. However, it is likely that the total clearance of M2 is lower than for TBPT, which would be due to the very high plasma protein binding of M2 relative to TBPT (150-fold higher). The in vitro intrinsic clearance of M2 is substantially more rapid than its formation intrinsic clearance. This disparity can lead to an underestimate of formation CL_{int} , even though enzyme kinetic measurements were made using conditions wherein linear rates of formation are observed experimentally. Failure to take this into account yields a large underestimate of the M2/TBPT ratio. The observation of the secondary metabolite of M2 (labeled as m/z 333 in Fig. 2) as an important metabolite in incubations starting with TBPT (present at 5-fold greater apparent abundance than M2 itself) suggests that the measured M2 formation CL_{int} value of 2.5 μ l/min/mg was underestimated. Inclusion of this factor in the prediction of the M2/TBPT ratio

TABLE 6

Normalized sensitivity coefficients (elasticity indices) for selected parameters of M1 and M2 PBPK models

	M1		M2	
	C_{max}	AUC	C_{max}	AUC
Plasma protein binding (f_u)	1.053	1.03	0.863	0.972
Blood-to-plasma ratio (B/P)	0.176	0.0003	0.801	0.007
Log P	1.097	0.002	0.412	0.214
CL_{int} (metabolite formation)	0.731	0.592	0.603	0.595
CL_{int} (metabolism of metabolite)	0.773	0.997	0.807	0.912

TABLE 7

Overall comparisons of predicted and actual M/P ratios for M1 and M2 in plasma following administration of TBPT

	M1/TBPT Ratio		M2/TBPT Ratio	
Measured steady-state value	0.12 ^a		58 ^a	
$f_{CL,m}$ value used in modeling	0.38	0.41	0.08	0.41
Static model prediction	0.29	0.32	4.2	23
Simcyp model prediction (single dose)	0.24	0.24	2.2	11
Simcyp model prediction (multiple dose)	0.24	0.26	4.1	20

^aRatios of mean AUC values.

was important and increased estimates of 4.1–4.2 to estimates of 20–23 in PBPK and static models, respectively. While the latter estimates are still smaller than the measured steady-state value of 58, these are still high enough to enable correct strategic decision making regarding the need to evaluate the pharmacokinetics and activity profile of M2. As a general concept, this example illustrates the value of comprehensive understanding of the overall metabolic pathways of the parent drug and its metabolites when making these projections.

The very high M/P ratio for M2 (58-fold) (Table 7) may represent a limitation on the use of the static and PBPK models for predicting metabolite pharmacokinetics; i.e., the M/P ratios that are in gross excess of unity may always be underpredicted. This is notable since eq. 1 is applied under the assumptions that parent and metabolite have stationary and linear pharmacokinetics, which is not the case with the current example. Nevertheless, predictions of steady-state M/P ratios for M2 were 23 and 20 for static and PBPK models, respectively, which are still well in excess of unity. The input parameter sensitivity analysis revealed that the free fraction in plasma and metabolite formation and degradation intrinsic clearances were the most important parameters in determining the projected metabolite exposure values. This makes sense since these inputs are the ones that drive clearance, which in turn drives the overall exposure.

Future efforts are underway to expand the set of drugs and metabolites used to test these methods. In the present case, as well as in the previously described examples, the metabolites were formed and further cleared by P450 enzymes, and more examples will be useful to continue to test these methods. The most challenging cases are anticipated to be those wherein the metabolite is generated by less commonly encountered clearance mechanisms (i.e., other than P450), when parent and/or metabolite clearance occurs primarily by active transport (e.g., hepatic uptake, biliary secretion, renal secretion) and when the physicochemical properties of the metabolite are vastly different from the parent drug (e.g., weakly basic parent drug metabolized to weakly acidic metabolite, marked change in molecular weight and/or total polar surface area between parent and metabolite, etc.). In the case of TBPT and M2, the greatest difference is the change in the human plasma free fraction (0.38–0.0025; 150-fold difference) and it is this difference that most likely contributes to the high M/P ratio, with f_u as a major contributor to systemic clearance. These future works could lead to refinement of the approach.

Authorship Contributions

Participated in research design: Obach, Sawant-Basak, Lin, Kimoto, Duvvuri, Nicholas.

Conducted experiments: Kimoto, Obach, Kadar.

Performed data analysis: Lin, Kimoto, Obach, Kadar, Duvvuri, Sawant-Basak.

Wrote or contributed to the writing of the manuscript: Obach, Sawant-Basak, Lin, Kimoto, Kadar, Tremaine, Duvvuri.

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