ESTIMATION OF CIRCULATING DRUG METABOLITE EXPOSURE IN HUMAN USING IN VITRO DATA AND PHYSIOLOGICALLY BASED PHARMACOKINETIC MODELLING: EXAMPLE OF A HIGH METABOLITE/PARENT DRUG RATIO


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Running Title: Prediction of Metabolite Exposure

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Introduction: 549
Discussion: 1208

Number of Tables: 7
Number of Figures: 6

Abbreviations: 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]; PBPK: Physiologically-based pharmacokinetic; SCHH: Sandwich cultured human hepatocytes; TBPT: (R)-4-((4-(((tetrahydrofuran-3-yl)oxy)benzo[d]isoxazol-3-yl)oxy)methyl)piperidin-1-yl)methyl)tetrahydro-2H-pyran-4-ol;
ABSTRACT

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), a serotonin-4 receptor partial agonist, is metabolized to two metabolites: an N-dealkylation product (M1) and a cyclized oxazolidine structure (M2). After administration 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 ratios for M1 and M2 was attempted, using in vitro metabolism, binding, and permeability data in static and dynamic PBPK models. In the static model, the fraction of parent clearance yielding the metabolite (which also required accounting for 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 modelling that used in vitro and physicochemical data inputs 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 under-predicted, 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 >>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 (FDA, 2013; EMA, 2012; 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, either beneficial (pharmacological activity) or detrimental (toxicity). Despite this possibility, the frequency with which drug metabolites cause unwanted effects unrelated to activity(ies) of the parent drug itself is very low (Smith and Obach, 2006; Isoherranen, et al., 2009; Yu and Tweedie, 2012). 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 that comprise 10% or more of the total circulating drug-related material merit greater scrutiny with regard to safety (ICH, 2009). 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 radiolabelled drug to humans and quantitative radiometric profiling of plasma samples by 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 drug exposure ratios can be accomplished using in vitro data on parent drug and metabolites (Lutz and Isoherranen, 2012; Nguyen, et al., 2016a, 2016b, 2017) in either a static model based on relationships first
described by Houston (1982) or a dynamic PBPK model. In the example drugs and metabolites used in those studies, the metabolite/parent 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 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 M1 and a novel oxidative cyclization to an oxazolidine metabolite M2, among others (Figure 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 modelling 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 are a custom pool of ten donors (seven female; three male) prepared by Bioreclamation (Westbury, NY), and cryopreserved hepatocyte lot 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), InVitroGro-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 µM) were incubated with pooled cryopreserved human hepatocytes (750000 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 hr an aliquot was removed (0.3 mL) and added to CH₃CN (1.2 mL). The precipitant was removed by centrifugation (5 min, 1700 g), 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 x100 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 min followed by a linear gradient to 70% CH₃CN at 4.5 min, 95% CH₃CN at 4.6 min, held at this composition for 1 min, followed by re-equilibration to initial conditions for 1.3 min. The eluent was introduced sequentially through a diode array detector (200-400 nm) then into an ionspray source of a Thermo LTQ Orbitrap mass spectrometer 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 µM) was incubated with pooled human liver microsomes (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 min 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 min at 1700 g 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-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 x100mm; 1.7 µ particle size) 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 above. The eluent was introduced into an ionspray source of a Thermo LTQ Orbitrap mass spectrometer operated in the positive ion mode. The instrument was operated in the MS1 scanning mode (m/z 300-650) at a resolution of 30000. Quantitation was done from reconstructed ion chromatograms of m/z 319.1660 (M1, Rf = 3.58 min), m/z 431.2183 (M2; Rf = 3.81 min), and 327.1377 (clozapine internal standard, Rf = 3.76 min). The standard curve range was from 5.0 to 2000 nM. Enzyme kinetic data were derived from v vs [S] data using GraphPad Prism v7.02 (GraphPad Software, Inc).

Metabolic Lability of TBPT, M1, and M2 in Human Liver and Intestinal Microsomes. TBPT, M1 or M2 (1.0 µM) were incubated with human liver microsomes (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 min 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 above. A similar experiment was done for TBPT in pooled human intestinal microsomes.
Permeability and Transport of TBPT, M1 and M2 in Sandwich Culture 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 at 37 °C and placed on ice. The cells were then poured into In VitroGro-HT medium at 37 °C at a ratio of one vial/50 mL in a conical tube. The cells were centrifuged at 50 × g for 3 min and re-suspended at 0.75 × 10^6 cells/mL in In VitroGro-CP medium. 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 h of incubation at 37 °C, cells were overlaid with ice-cold 0.25 mg/mL Matrigel in In VitroGro-HI medium at 0.5 mL/well and cultured for 5 days.

On day 5, the hepatocytes were first rinsed twice with Ca^{2+}/Mg^{2+}-containing (standard) or Ca^{2+}/Mg^{2+}-free HBSS and then preincubated for 10 min with standard or Ca^{2+}/Mg^{2+}-free HBSS in the absence or presence of 1 mM 1-aminobenzotriazole (ABT) or 1mM 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 min) 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 LC−MS/MS quantification.

TBPT, M1 and M2 were analyzed on an SCIEX Triple Quadrupole 6500 mass spectrometer (SCIEX, Framingham, MA) equipped with TurboIonSpray interface that is 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 min (solvent A: 100% water with 0.1% formic acid; solvent B: 90% CH₃CN/10% water with 0.1% formic acid) at a flow rate of 0.4 mL/min. The mass transitions in positive ion mode for monitoring TBPT, M1 and M2 were m/z 433 → 194, 319.1 → 98 and 431.2 → 210, respectively.
Projection of Metabolite/Parent Drug Ratios by Static Modelling. The following equation relates the metabolite/parent plasma AUC ratio ($\frac{AUC_m}{AUC_p}$) after oral administration of the parent (Nguyen, et al., 2016) derived from original equations described by Houston (1981):

$$\frac{AUC_m}{AUC_p} = \frac{F_m \cdot CL_{int, p} \cdot CL_p + F_m \cdot F_{m, gut} \cdot f_{gut, CL_{int, m}} \cdot (1 - F_g) \cdot CL_p}{F_h \cdot CL_{int, m}}$$  (Eq. 1)

The first term represents the ratio driven by liver and the second 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. Values for hepatic CL_p and CL_m (blood clearance values) were generated by scaling the in vitro CL_{int} values for parent and metabolite (CL_{int, p} and CL_{int, m}) using factors 45 mg microsomal protein per gram liver and 20 grams of liver per kg body weight, and inserting these into the well-stirred model of hepatic clearance (Pang and Rowland, 1977):

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

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

wherein 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. Fraction of the parent that evades first pass hepatic extraction ($F_h$) was estimated as:

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

The values for fraction of the clearance of parent drug proceeding through a metabolite in the liver ($f_{CL_{int, m}}$) and in the gut ($f_{gut, CL_{int, m}}$) was estimated using two approaches. The first utilized 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 (CL_{int}) determined by the in vitro t$_{1/2}$ (CL_{int} = 0.693/ in vitro t$_{1/2}$, corrected for the concentration of microsomes). This was calculated for liver and intestinal microsomes, separately.
In the second approach, secondary metabolites arising from M1 and M2 were added to their respective formation intrinsic clearance values. UV peak areas of M1, M2, and their secondary metabolites from the metabolite profile (see above) 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, Figure 2) were summed and the M1 CL\text{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 (Figure 2). This accounts for underestimates of CL\text{int} due to any further metabolism of the metabolites that is missed in the enzyme kinetic experiments. Similar data sets were used previously in the estimation of f_m values for use in projecting metabolite/parent ratios for imipramine and losartan (Nguyen, et al., 2016, 2017).

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

\[ F_m = \frac{Q_h}{Q_h + f_{u,m} \cdot CL_{\text{int},m}} \]  

(Eq. 6)

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

\[ F_g = \frac{Q_{\text{gut}}}{Q_{\text{gut}} + f_{u,p} \cdot CL_{\text{int},p,gut}} \]  

(Eq. 7)

wherein Q_{\text{gut}} is villous blood flow (4 mL/min/kg), f_{u,p} is the unbound fraction of parent drug in blood, and CL_{\text{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,\text{gut}} was assumed to be unity. Metabolite to parent ratios were adjusted to accommodate differences in molecular weight between metabolites and parent drug.
Dynamic Physiologically Based Pharmacokinetic Modelling in SimCYP™. 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, PO, QD) using the population-based ADME simulator Simcyp (version 15; Simcyp Ltd., Sheffield, UK). Simulations were performed for a virtual population of 100 healthy volunteers (10 trials × 10 subjects each) aged between 20 and 50 with a male/female ratio of 50/50. For TBPT, a full PBPK model was developed assuming perfusion-limited distribution and using physicochemical properties (pKa, logP), biochemical properties (human plasma f<sub>u</sub>, blood-to-plasma ratio) and in vitro metabolic intrinsic clearance values. The absorption model was first order, with an absorption rate constant set at 0.6 hr<sup>-1</sup> based on clinical data. The F<sub>a</sub> was set at 0.6 based on in-vitro permeability assay and pre-clinical PK parameters.

For M1 and M2 the volume of distribution at steady-state V<sub>ss</sub> was predicted using Method 1 within the SimCYP simulator, which is based on input parameters of protein binding and lipophilicity. The V<sub>ss</sub> of M1 and M2 were predicted to be 1.64 and 0.26 L/kg, respectively. The V<sub>ss</sub> value for TBPT was estimated by scaling from allometric scaling of dog V<sub>ss</sub> data. M1 and M2 CL<sub>int</sub> values were obtained from in vitro metabolic stability of each of the two analytes in human liver microsomes. Enzyme kinetic information using HLM was selected for TBPT metabolism.

Sensitivity analysis was performed to identify which parameters must be known with the greatest precision to most accurately predict AUC<sub>m</sub> of TBPT metabolites. Parameters were investigated including TBPT, M1, and M2 formation intrinsic clearance, elimination intrinsic clearance, plasma protein binding, blood-to-plasma ratio, and logP. The metrics examined were AUC and C<sub>max</sub> of M1 and M2 after oral administration of TBPT at 15 mg. The sensitivity of M1 and M2 AUC and C<sub>max</sub> 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 that measures the relative change in an output variable Q (e.g., AUC) for a relative change in the input parameter P. EI was calculated as follows for each input parameter value n:
DMD Manuscript #78279

\[ EI_n = \left( \frac{P_n}{Q(P_n)} \right) \times SI \quad (\text{Eq. 8}) \]

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:

\[ SI_n = \frac{Q(P_{n+1}) - Q(P_n)}{P_{n+1} - P_n} \quad (\text{Eq. 9}) \]

The elasticity indices 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 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 (\( N=8; 4 \) Caucasian and 4 African-American) is as follows: age: 31.6 y (range 21-49); weight: 83.9 kg (range 67.1-99.8); BMI: 27.6 (range 24.2-29.7). Pre-dose 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.** An HPLC-MS/MS method was validated for the analysis of TBPT, M1, and M2 in human plasma treated with \( K_2 \)EDTA 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-\textit{tert} butyl ether (MTBE) based supported liquid extraction (SLE) (Isolute® SLE+ Supported Liquid Extraction Plate (400 \( \mu L \), Biotage). The SLE extracts were evaporated under nitrogen at 35°C. The reconstituted extracts were
injected (15 µL) onto an 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 x 2.0 mm, 5 µm analytical column equipped with an Upchurch 0.5 µm pre-filter. The following gradient was applied utilizing 0.1% formic acid in water and 0.1% formic acid in methanol as mobile phase A (MP A) and mobile phase B (MP B), respectively: 0.01 to 2.00 minutes (25 to 60% MP B), 2.00 to 2.20 (60 to 95% MP B), 2.20 to 3.00 (hold at 95% MP B), 3.00 to 3.35 (95 to 25% MP B), 3.35 to 4.00 (hold at 25% MP B), 4.00 to 5.50 (25 to 65% MP B), 5.50 to 5.60 (hold at 65% MP B), 5.60 to 5.80 (65 to 25% MP B), 5.80 to 6.50 (hold at 25% MP B). Eluent from the liquid chromatography was analyzed by a Sciex API 5000 tandem mass spectrometer operated in positive electrospray (ESI+) mode. The following transitions were monitored: TBPT (433.3→212.2), M1 (319.3→98.1), M2 (431.3→210.2), [2H9]TBPT (internal standard 1, 442.3→221.2) and [2H9]M1 (internal standard 2, 328.3→107.1). The approximate retention times were as follows: TBPT (1.12 min), M1 (1.06 min), M2 (1.42 min), [2H9]TBPT (1.11 min) and [2H9]M1 (1.05 min). The dynamic range of the assay was 5.00 to 7500 pg/mL for TPBT, 2.00 to 3000 pg/mL for M1 and 25.0 to 37500 pg/mL for M2. A linear (1/x²) regression was applied to the analyte/internal standard peak area ratios utilizing Applied Biosystems/MDS Sciex Analyst Version 1.5 and Watson Version 7.3.0.01.

**Pharmacokinetic calculations.** Standard pharmacokinetic parameters, C\text{max} (maximum plasma concentration), T\text{max} (time for C\text{max}) and AUC\text{tau} (area under the plasma concentration vs time curve over the dosing interval) were calculated using non-compartmental analyses. Pharmacokinetic calculations were done using an internal program called eNCA that uses splus scripts to calculate non-compartmental parameters. Pharmacokinetic data from healthy adults at 15 mg QD dose on Days 1 and 14 is 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, 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 Figure 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 in Supplemental Figure 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 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 Figure 1) and that this metabolite was also observed in the incubation of TBPT but very little 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 min in Figure 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 (see below).

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 CL_{int} and f_{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 Figure 3. For M1, kinetics appeared to follow simple hyperbolic Michaelis-Menten kinetics with K_{M} values of 89 and 112 µM in liver and intestine respectively. Intrinsic clearance
values were calculated to be 12 and 3.4 µL/min/mg protein. For M2, K_M values were 181 and 31 µM in liver and intestine and CL_int values were 2.5 and 1.4 µL/min/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. Kinetic parameters are listed in Table 1.

Consumption of TBPT, M1, and M2 was also measured in liver microsomes. Intrinsic clearance values were estimated at 32, 32, and 102 µL/min/mg for TBPT, M1, and M2, respectively. Combining the formation kinetic data for M1 and M2 above with these total CL_int values for TBPT yielded estimates of f_{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 µL/min/mg, and combining this value with the formation CL_int values for M1 and M2 yielded estimates of f_{gutCL,m} of 0.26 and 0.11, respectively.

The estimates of formation CL_int 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 is substantially greater than its formation CL_int (102 vs. 2.5 µL/min/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 (Figure 2). The CL_int of formation for M2 was increased by a factor of 5.2. Fraction of clearance values 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 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 of compound accumulation in SCHH between standard HBSS and Ca^{2+}/Mg^{2+}-free HBSS represents a contribution of biliary excretion. As shown in Figure 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 SCHH system. *In vitro* intrinsic clearance 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 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 below.

Mean plasma concentration versus time data are plotted in Figure 5, and mean pharmacokinetic parameters are listed in Table 3. TBPT demonstrated rapid absorption, with a median T_{\text{max}} occurring at 1-2 hrs post dose. Following attainment of C_{\text{max}}, plasma concentrations declined in a biphasic fashion and the average CL_{p}/F ranged from 12 to 22 mL/min/kg (correcting for a mean body weight of 83.9 kg) across a dose range of 0.1-15 mg QD 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 pharmacokinetic profiles of M1 and M2 following multiple daily dosing of 15 mg TBPT are summarized in Table 3 and Figure 5. Following oral dosing of 15 mg QD TBPT, mean C_{\text{max}} and AUC_{\text{tau}} of the N-dealkylated metabolite M1 on day 1 were 1.7 ng/mL and 10.0 ng•hr/mL 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 on AUC(0-τ) 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_{\text{max}} and AUC_{\text{tau}} on days 1 and 14. At 15 mg QD dose of TBPT the day 14 M2:TBPT exposure ratios were at least 2-3 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 vs 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 Modelling.** Measured values from the in
vitro experiments were scaled to reflect in vivo parameters and used to calculate the terms needed in
equation 1 (Table 4). TBPT was projected to be a moderate clearance compound (CL_{p} = 7.9 \text{ mL/min/kg},
approximately 40% of hepatic blood flow), and while metabolite M1 was also projected to have moderate
clearance (11 \text{ mL/min/kg}), the clearance for M2 was projected to be low (0.4 \text{ mL/min/kg}). This is despite
the fact that the hepatic total intrinsic clearance of M2 is greater than 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 to 41% for
M1 and 8 to 41% for M2. These were estimated by dividing the formation intrinsic clearance values for
each metabolite by the total substrate consumption intrinsic clearance of TBPT. The higher percentages
include the correction for secondary metabolism. The fractions of each metabolite generated within the
liver that evade further immediate metabolism were estimated from the projected CL_{int} values of the
metabolites vs liver blood flow. For M2, it was estimated that almost all metabolite leaves the liver prior
to further hepatic clearance whereas for M1 about half generated undergoes subsequent clearance prior to
release from the liver. Putting all of these factors together in the static model equation 1 yielded that the
projected metabolite to parent exposure ratios for M1 and M2 are 0.29 and 4.2 using measured formation
CL_{int} values only, and were 0.32 and 23 following correction of 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 metabolite to parent 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 is
derived from the liver.

**Projection of Metabolite/Parent Exposure Ratios from Dynamic PBPK Modelling.** Input parameters used
for PBPK modelling are summarized in Table 5. The predicted versus observed plasma concentration-
The time profiles of TBPT, M1, and M2 following oral administration of TBPT (15 mg) are displayed in Figure 6. The predicted pharmacokinetic parameters are summarized and tabulated in Table 3 for comparison to the measured parameters. Overall, PBPK modelling 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 $\text{AUC}_{(0-\tau)}$ value for TBPT was 312 ng$\cdot$h/\text{ml} which was 2.5 fold higher than the observed mean value of 123 ng$\cdot$h/mL, while the mean $C_{\text{max}}$ was over-predicted by 1.4 fold (32.6 vs 23.1 ng/mL). The predicted $\text{AUC}_{(0-\tau)}$ value of M1 was 56 ng$\cdot$h/mL which was 5.6 fold higher than the observed value of 10 ng$\cdot$h/mL. Thus, for both parent drug and M1, the exposure was somewhat over-predicted, however the metabolite to parent ratio for M1 was better predicted since the over-predictions of exposure were partially off-setting (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 $\text{AUC}_{0-\tau}$ was 2440 ng$\cdot$h/mL whereas the predicted value was only 700 ng$\cdot$h/mL. Accumulation of 1.9-fold was predicted for M2 while the actual accumulation was 3.2, thus the under-prediction of the metabolite-to-parent ratio was greater for multiple dosing wherein the observed $\text{AUC}_{0-\tau}$ was 7850 ng$\cdot$h/mL while the projected value was 1312 ng$\cdot$h/mL. However, as was the case for the static model, accounting for experimental underestimation of metabolite formation $CL_{\text{int}}$ value for M2 altered the estimation of metabolite exposures in dynamic PBPK modelling and substantially improved these. The predicted $\text{AUC}_{0-\tau}$ increased to 3541 and 6723 ng$\cdot$h/mL for day 1 and day 14, respectively. The day 1 model projection is a ~1.5-fold overestimate while the day 14 projection is fairly accurate (86% of actual value). Metabolite to parent ratio at steady state was estimated at 20, as compared to the actual value of 58, and this is largely driven by the overestimate of parent exposure.

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. LogP 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 making decisions regarding which putative metabolites to synthesize and analyze in plasma samples from preclinical toxicology studies and first-in-human studies. Pharmacokinetic principles underlying the exposure to metabolites have existed for decades (Houston, 1980; 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, applied both static and PBPK modelling to estimate plasma exposures to metabolites of midazolam, imipramine, and losartan (Nguyen, et al., 2016, 2017). 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. Further, 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 are two metabolites measured in clinical studies wherein one was in great excess relative to parent (M2) and the other was present at far lower concentrations than 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 (Loi, et al.,2013; Dalvie, et al., 2009). The projection of the metabolite/parent exposure ratio for M1 was in reasonable agreement with the actual data (0.12 actual vs 0.26-0.32 predicted; 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, likely due to subsequent systemic metabolic clearance that is faster than that of the parent drug.

The metabolite/parent AUC ratio for M2 was underestimated (Table 7 and Figure 6). Based on the in vitro data, M2 was formed via oxidation in human liver, with very little in the intestine based on the estimated $F_g$ of TBPT of 0.94. M2 was marked by a rapid rise in human plasma ($T_{max}$ of 1h 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 µL/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 for M2 of 0.4 mL/min/kg, which was more than 10-fold lower than that of 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 have 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 account for this yields a large underestimate of the M2/TBPT ratio. The observation of the secondary metabolite of M2 (labelled as m/z 333; Figure 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\text{int} of 2.5 µL/min/mg was underestimated. Inclusion of this factor in the prediction of M2/TBPT ratio 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 a comprehensive understanding of the overall metabolic pathways of the parent drug and its metabolites when making these projections.

The very high metabolite/parent 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. M/P ratios that are in gross excess of unity may always be under-predicted. This is notable since Equation 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, which are still well in excess of unity. The input parameter sensitivity analysis revealed that free fraction in plasma and metabolite formation and degradation intrinsic clearances were the most important 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 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 cytochrome 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 human plasma free fraction (0.38 to 0.0025; 150-fold difference) and it is this difference that most likely contributes to the high metabolite/parent ratio, with $f_o$ 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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Yu H and Tweedie D (2013) A perspective on the contribution of metabolites to drug-drug interaction potential: the need to consider both circulating levels and inhibition potency. Drug Metab. Dispos. 41: 536-540.

FIGURE LEGENDS

FIGURE 1. Structures of TBPT, M1 and M2.

FIGURE 2. HPLC-UV Traces of Extracts of Hepatocyte Incubations of TBPT, M1, and M2.

FIGURE 3. Enzyme Kinetic Plots for the Formation of Metabolites M1 and M2 from TBPT in Human Liver and Intestine Microsomes.

FIGURE 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 Ca²⁺/Mg²⁺-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 Ca²⁺/Mg²⁺-free HBSS.

FIGURE 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 semi-logarithmic scale.

FIGURE 6. Comparison of Plasma Concentrations vs Time Profiles for TBPT, M1 and M2 Simulated by PBPK Modelling vs Actual Clinical Data. Red circles represent the mean measured concentrations and green lines the simulated profiles.
TABLE 1. Enzyme Kinetic Parameters for the Metabolism of TBPT, M1, and M2 in Human Liver and Intestinal Microsomes.

<table>
<thead>
<tr>
<th></th>
<th>$V_{\text{max}}$ (pmol/min/mg)</th>
<th>$K_M$ (µM)</th>
<th>$CL_{\text{int}}$ (µL/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver Microsomes:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBPT $\rightarrow$ M1</td>
<td>1050 +/- 38</td>
<td>89 +/- 7</td>
<td>12</td>
</tr>
<tr>
<td>TBPT $\rightarrow$ M2</td>
<td>456 +/- 33</td>
<td>181 +/- 22</td>
<td>2.5</td>
</tr>
<tr>
<td>TBPT Consumption</td>
<td>--</td>
<td>--</td>
<td>32</td>
</tr>
<tr>
<td>M1 Consumption</td>
<td>--</td>
<td>--</td>
<td>32</td>
</tr>
<tr>
<td>M2 Consumption</td>
<td>--</td>
<td>--</td>
<td>102</td>
</tr>
<tr>
<td>Intestinal Microsomes:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TBPT $\rightarrow$ M1</td>
<td>385 +/- 19</td>
<td>112 +/- 11</td>
<td>3.4</td>
</tr>
<tr>
<td>TBPT $\rightarrow$ M2</td>
<td>31 +/- 6</td>
<td>31 +/- 6</td>
<td>1.4$^a$</td>
</tr>
<tr>
<td>TBPT Consumption</td>
<td>--</td>
<td>--</td>
<td>13</td>
</tr>
</tbody>
</table>

$^a$Includes additional high $K_M$ – high $V_{\text{max}}$ activity.
TABLE 2. In Vitro Intrinsic Clearance Values of TBPT, M1 and M2 in SCHH.

<table>
<thead>
<tr>
<th></th>
<th>TBPT</th>
<th>M1</th>
<th>M2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>CL_{uptake}</strong> (µL/min/mg)</td>
<td>63 (6.3)</td>
<td>48 (0.86)</td>
<td>21 (1.7)</td>
</tr>
<tr>
<td><strong>CL_{passive}</strong> (µL/min/mg)</td>
<td>83 (2.5)</td>
<td>49 (5.5)</td>
<td>16 (2.4)</td>
</tr>
<tr>
<td><strong>CL_{bile}</strong> (µL/min/mg)</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

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 Ca^{2+}/Mg^{2+} -free HBSS in SCHH. The *in vitro* CL values were calculated by initial rate analysis with a linear fitting up to 2 min. CL_{uptake}, CL_{passive}, and CL_{bile} represent *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 Ca^{2+}/Mg^{2+} -free HBSS, respectively. ND: not determined. Values in parentheses are standard errors.
TABLE 3. Exposure Metrics for TBPT, M1 and M2 in Healthy Human Volunteers Following Daily Oral Administration of 15 mg TBPT Compared to Values Predicted Using Dynamic PBPK Modelling.

<table>
<thead>
<tr>
<th>Metric</th>
<th>TBPT</th>
<th>M1</th>
<th>M2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Predicted</td>
<td>Observed</td>
<td>Predicted</td>
</tr>
<tr>
<td>Day 1:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (ng/mL)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>32.6 (45)</td>
<td>23.1</td>
<td>5.2</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt; (hr)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.9 (1-2)</td>
<td>1.5</td>
<td>3.4</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-∞&lt;/sub&gt; (ng-hr/mL)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>312 (51)</td>
<td>123</td>
<td>56</td>
</tr>
<tr>
<td>Day 14:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C&lt;sub&gt;max&lt;/sub&gt; (ng/mL)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>33.5 (46)</td>
<td>23.1</td>
<td>5.4</td>
</tr>
<tr>
<td>T&lt;sub&gt;max&lt;/sub&gt; (hr)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.9 (1-2)</td>
<td>1 (1-2)</td>
<td>3.4</td>
</tr>
<tr>
<td>AUC&lt;sub&gt;0-∞&lt;/sub&gt; (ng-hr/mL)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>322 (55)</td>
<td>134</td>
<td>58</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values are mean (%CV) for AUC and C<sub>max</sub>.<br><sup>b</sup>Values are median (range) for T<sub>max</sub>.<br><sup>c</sup>Predicted using experimentally measured formation CL<sub>int</sub> values.<br><sup>d</sup>Predicted using formation CL<sub>int</sub> values corrected for secondary metabolism. All predicted values are point estimates of mean values.
TABLE 4. Estimated Input Parameters for Static Model Prediction of Metabolite/Parent Drug Ratios

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Input Values</th>
<th>Scaled Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>TBPT:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( CL_p )</td>
<td>( f_u = 0.38; B/P = 0.86; CL_{int} = 32 \mu L/min/mg )</td>
<td>7.9 mL/min/kg</td>
</tr>
<tr>
<td>( F_h )</td>
<td>( CL_p = 7.9 \text{ mL/min/kg} )</td>
<td>0.62</td>
</tr>
<tr>
<td>( F_g )</td>
<td>( f_u = 0.38; B/P = 0.86; CL_{int} = 13 \mu L/min/mg; )</td>
<td>0.94</td>
</tr>
<tr>
<td><strong>Metabolite M1:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( CL_m )</td>
<td>( f_u = 0.64; B/P = 0.88; CL_{int} = 32 \mu L/min/mg )</td>
<td>11 mL/min/kg</td>
</tr>
<tr>
<td>( f_{CL,m} )</td>
<td>( CL_{int,TBPT} = 32 \mu L/min/mg; CL_{int,TBPT→M1} = 12 \mu L/min/mg )</td>
<td>0.38 or 0.41 (^b)</td>
</tr>
<tr>
<td>( F_m )</td>
<td>( f_u = 0.64; B/P = 0.88; CL_{int} = 32 \mu L/min/mg )</td>
<td>0.47</td>
</tr>
<tr>
<td>( f_{gut,CL,m} )</td>
<td>( CL_{int,TBPT} = 13 \mu L/min/mg; CL_{int,TBPT→M1} = 3.4 \mu L/min/mg )</td>
<td>0.26 or 0.30 (^b)</td>
</tr>
<tr>
<td>( F_{m,gut} )</td>
<td>Assumed as unity</td>
<td>1</td>
</tr>
<tr>
<td><strong>Metabolite M2:</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( CL_m )</td>
<td>( f_u = 0.0025; B/P = 0.53; CL_{int} = 102 \mu L/min/mg )</td>
<td>0.40 mL/min/kg</td>
</tr>
<tr>
<td>( f_{CL,m} )</td>
<td>( CL_{int,TBPT} = 32 \mu L/min/mg; CL_{int,TBPT→M2} = 2.5 \mu L/min/mg )</td>
<td>0.08 or 0.41 (^b)</td>
</tr>
<tr>
<td>( F_m )</td>
<td>( f_u = 0.0025; B/P = 0.53; CL_{int} = 102 \mu L/min/mg )</td>
<td>0.98</td>
</tr>
<tr>
<td>( f_{gut,CL,m} )</td>
<td>( CL_{int,TBPT} = 13 \mu L/min/mg; CL_{int,TBPT→M2} = 1.4 \mu L/min/mg )</td>
<td>0.11 or 0.58 (^b)</td>
</tr>
<tr>
<td>( F_{m,gut} )</td>
<td>Assumed as unity</td>
<td>1</td>
</tr>
</tbody>
</table>

\(^a\)Constants: \( Q_h = 21 \text{ mL/min/kg} \), \( Q_{gut} = 4 \text{ mL/min/kg} \), 45 mg microsomes/gm liver, 20 gm liver/kg body weight, 2978 mg microsomes/whole intestine (i.e. 42 mg intestinal microsomes per kg body weight).

\(^b\)Values corrected for by secondary metabolism (see Methods).
TABLE 5. Simcyp Input Parameters for TBPT, M1 and M2

<table>
<thead>
<tr>
<th>Parameter</th>
<th>TBPT</th>
<th>M1</th>
<th>M2</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular Weight (g/mol)</td>
<td>432</td>
<td>318</td>
<td>430</td>
<td>ACD</td>
</tr>
<tr>
<td>logP</td>
<td>1.68</td>
<td>2.19</td>
<td>2.08</td>
<td>ACD</td>
</tr>
<tr>
<td>Compound Type</td>
<td>Monoprotic Base</td>
<td>Monoprotic Base</td>
<td>Monoprotic Base</td>
<td></td>
</tr>
<tr>
<td>pKa</td>
<td>8.8</td>
<td>10.22</td>
<td>6.48</td>
<td>ACD</td>
</tr>
<tr>
<td>B/P</td>
<td>0.86</td>
<td>0.88</td>
<td>0.55&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Measured</td>
</tr>
<tr>
<td>fu,p</td>
<td>0.38</td>
<td>0.64</td>
<td>0.0025</td>
<td>Measured</td>
</tr>
<tr>
<td>Absorption Model</td>
<td>1st order</td>
<td>NA</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Fraction absorbed</td>
<td>1</td>
<td>NA</td>
<td>NA</td>
<td>Assumed</td>
</tr>
<tr>
<td>ka (1/h)</td>
<td>0.6</td>
<td>NA</td>
<td>NA</td>
<td>Fitting</td>
</tr>
<tr>
<td>fu(Gut)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>Assumed</td>
</tr>
<tr>
<td>MDCK Permeability (10&lt;sup&gt;-6&lt;/sup&gt; cm/s)</td>
<td>23</td>
<td>NA</td>
<td>NA</td>
<td>Measured</td>
</tr>
<tr>
<td>Distribution Model</td>
<td>Minimal PBPK</td>
<td>Minimal PBPK</td>
<td>Minimal PBPK</td>
<td></td>
</tr>
<tr>
<td>V&lt;sub&gt;ss&lt;/sub&gt; (L/kg)</td>
<td>2.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.64&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.26&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Fitting</td>
</tr>
<tr>
<td>Elimination 3A4 CL&lt;sub&gt;int&lt;/sub&gt; (µL/min/mg) (forming M1)</td>
<td>12 or 13&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Measured</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3A4 CL&lt;sub&gt;int&lt;/sub&gt; (µL/min/mg) (forming M2)</td>
<td>2.5 or 13&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Measured</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Additional HLM CL&lt;sub&gt;int&lt;/sub&gt; (µL/min/mg)</td>
<td>17.5 or 6</td>
<td>32</td>
<td>102</td>
<td>Measured</td>
</tr>
</tbody>
</table>

<sup>a</sup>Predicted using dog pharmacokinetic data.  <sup>b</sup>Value from SimCYP using Method 1.  <sup>c</sup>Minimum value for SimCYP is 0.55; measured value was 0.53.  <sup>d</sup>CL<sub>int</sub> values represent measured values and measured values multiplied by a factor of 1.1 and 5.2 for M1 and M2, respectively, to account for secondary metabolism.
TABLE 6. Normalized sensitivity coefficients (elasticity indices) for selected parameters of M1 and M2 PBPK models.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>M1</th>
<th>M2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$C_{\text{max}}$</td>
<td>AUC</td>
</tr>
<tr>
<td>Plasma Protein Binding ($f_a$)</td>
<td>1.053</td>
<td>1.03</td>
</tr>
<tr>
<td>Blood-to-Plasma Ratio (B/P)</td>
<td>0.176</td>
<td>0.0003</td>
</tr>
<tr>
<td>LogP</td>
<td>1.097</td>
<td>0.002</td>
</tr>
<tr>
<td>$CL_{\text{int}}$ (metabolite formation)</td>
<td>0.731</td>
<td>0.592</td>
</tr>
<tr>
<td>$CL_{\text{int}}$ (metabolism of metabolite)</td>
<td>0.773</td>
<td>0.997</td>
</tr>
</tbody>
</table>
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TABLE 7. Overall comparison of predicted and actual metabolite/parent ratios for M1, and M2 in plasma following administration of TBPT.

<table>
<thead>
<tr>
<th></th>
<th>M1/TBPT Ratio</th>
<th>M2/TBPT Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Measured Steady-State Value</td>
<td>0.12&lt;sup&gt;a&lt;/sup&gt;</td>
<td>58&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>f&lt;sub&gt;CL,m&lt;/sub&gt; Value Used in Modelling</td>
<td>0.38</td>
<td>0.41</td>
</tr>
<tr>
<td>Static Model Prediction</td>
<td>0.29</td>
<td>0.32</td>
</tr>
<tr>
<td>SimCYP Model Prediction (single dose)</td>
<td>0.24</td>
<td>0.24</td>
</tr>
<tr>
<td>SimCYP Model Prediction (multiple dose)</td>
<td>0.24</td>
<td>0.26</td>
</tr>
</tbody>
</table>

<sup>a</sup>Ratios of mean AUC values
Figure 2

- **TBPT-control**
- **TBPT-incubated**
- **M1-control**
- **M1-incubated**
- **M2-control**
- **M2-incubated**

- **m/z 317** (N-dealkylation)
- **m/z 445** (+O/-2H)
- **m/z 222** (O-dealkylation)
- **m/z 333** (oxo M1)
- **m/z 623** (+O/+Glc)
- **m/z 335** (oxid M1)
- **m/z 415** (+O/+SO3)
- **m/z 511** (+O/+Glc)
- **m/z 335** (oxy M1)
- **m/z 415** (M1 oxysulfate)
- **m/z 623** (+O/+Glc)
- **m/z 333** (oxo M1)

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Figure 3
Figure 4
Figure 5
Figure 6