Organic Anion Transporter 2 Mediates Hepatic Uptake of Tolbutamide, a CYP2C9 Probe Drug^S

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ABSTRACT

Tolbutamide is primarily metabolized by CYP2C9, and, thus, is frequently applied as a clinical probe substrate for CYP2C9 activity. However, there is a marked discrepancy in the in vitro-in vivo extrapolation of its metabolic clearance, implying a potential for additional clearance mechanisms. The goal of this study was to evaluate the role of hepatic uptake transport in the pharmacokinetics of tolbutamide and to identify the molecular mechanism thereof. Transport studies using singly transfected cells expressing six major hepatic uptake transporters showed that tolbutamide is a substrate to organic anion transporter 2 (OAT2) alone with transporter affinity [Michaelis-Menten constant (K_m)] of $19.5 \pm 4.3 \mu\text{M}$. Additionally, OAT2-specific transport was inhibited by ketoprofen (an OAT2 inhibitor) and 1 mM rifamycin SV (pan inhibitor), but not by cyclosporine and rifampicin (OAT polypeptides/ Na^+ -taurocholate cotransporting polypeptide inhibitors). Uptake studies in primary human hepatocytes confirmed the predominant role of OAT2 in the

active uptake with significant inhibition by rifamycin SV and ketoprofen, but not by the other inhibitors. Concentration-dependent uptake was noted in human hepatocytes with active transport characterized by K_m and V_{max} values of $39.3 \pm 6.6 \mu\text{M}$ and $426 \pm 30 \text{ pmol/min per milligram protein}$, respectively. Bottom-up physiologically based pharmacokinetic modeling was employed to verify the proposed role of OAT2-mediated hepatic uptake. In contrast to the rapid equilibrium (CYP2C9-only) model, the permeability-limited (OAT2-CYP2C9 interplay) model better described the plasma concentration-time profiles of tolbutamide. Additionally, the latter well described tolbutamide pharmacokinetics in carriers of CYP2C9 genetic variants and quantitatively rationalized its known drug-drug interactions. Our results provide first-line evidence for the role of OAT2-mediated hepatic uptake in the pharmacokinetics of tolbutamide, and imply the need for additional clinical studies in this direction.

Introduction

Tolbutamide is a first-generation oral sulfonylurea hypoglycemic agent used in the treatment of type 2 diabetes mellitus. It is characterized by a low clearance and good absolute oral bioavailability, and is largely metabolized by CYP2C9 to 4-hydroxytolbutamide, which is further oxidized to carboxytolbutamide (Nelson and O'Reilly, 1961; Thomas and Ikeda, 1966; Knodell et al., 1987). CYP2C9 is a genetically polymorphic enzyme involved in the clearance of drugs such as warfarin, glyburide, and phenytoin (Kirchheiner et al., 2002b; Shon et al., 2002; Kirchheiner and Brockmüller, 2005). Of the various genetic variants identified so far, the CYP2C9*2 (Arg144Cys) and CYP2C9*3 (Ile359Leu) forms have been shown to have reduced metabolic activity (Kirchheiner et al., 2002a; Schwarz, 2003; Kirchheiner and Brockmüller, 2005). Large interindividual variability in tolbutamide pharmacokinetics

observed in the clinic is suggested to be caused by CYP2C9 genetic variants (Scott and Poffenbarger, 1979; Kirchheiner et al., 2002a; Shon et al., 2002). Although in vitro studies suggested CYP2C9 involvement in the metabolism of tolbutamide (Wester et al., 2000), clinical pharmacogenomic studies implied no influence by CYP2C9 polymorphism (Kirchheiner et al., 2002a; Shon et al., 2002). Therefore, tolbutamide is considered to be the standard CYP2C9 phenotypic probe (Lee et al., 2003; <http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm292362.pdf>).

Despite predominant cytochrome P450-mediated metabolism, many studies have reported a marked disconnect in the in vitro-in vivo (IVIV) translation for tolbutamide clearance, where metabolic rates measured using human liver microsomes and human hepatocytes considerably underpredict its plasma clearance (Obach, 1999; Brown et al., 2007). It is important to understand the IVIV discrepancy and examine for potential alternative clearance mechanisms contributing to tolbutamide pharmacokinetics, given its wide application as a CYP2C9 probe substrate in drug development (Kirchheiner et al., 2002a;

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ABBREVIATIONS: CE, collision energy; CL_{int} , intrinsic clearance; DDI, drug-drug interaction; DP, declustering potential; HBSS, Hanks' balanced salt solution; HBV, hepatitis B virus; HEK293, human embryonic kidney 293; IS, internal standard; IVIV, in vitro-in vivo; K_m , Michaelis-Menten constant; LC-MS/MS, liquid chromatography-tandem mass spectroscopy; NTCP, Na^+ -taurocholate cotransporting polypeptide; OAT, organic anion transporter; OATP, organic anion-transporting polypeptide; OCT, organic cation transporter; PBPK, physiologically based pharmacokinetic; PS_{pd} , passive diffusion; REF, relative expression factor; SLC, solute carrier; Vd_{ss} , volume of distribution.

<http://www.fda.gov/downloads/drugs/guidancecompliance-regulatoryinformation/guidances/ucm292362.pdf>; Gillen et al., 2017). Reduced function variants of CYP2C9 (*3/*3) show a drop in tolbutamide clearance by approximately 85% (Kirchheiner et al., 2002a), implying that any additional liver metabolic pathways, if uncovered, will be minor and do not explain the IVIV disconnect. However, underprediction based on metabolic clearance data alone is often seen for anionic drugs, where hepatic uptake, particularly mediated by organic anion-transporting polypeptides (OATPs), is the rate-determining step in their systemic clearance (Watanabe et al., 2009; Varma et al., 2014).

The main objective of this investigation was to evaluate the role of transporter-mediated hepatic uptake in the clinical pharmacokinetics of tolbutamide. For this purpose, we studied tolbutamide transport in vitro using transporter-transfected cells and primary human hepatocytes, and employed “bottom-up” physiologically based pharmacokinetic (PBPK) modeling and simulations to evaluate the role of transporter-enzyme interplay in tolbutamide pharmacokinetics.

Materials and Methods

Chemicals and Reagents. Tolbutamide, cyclosporine A, quinidine, ketoprofen, rifampicin, and rifamycin SV were purchased from Sigma-Aldrich (St. Louis, MO). [³H]-tolbutamide was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). Hepatitis B virus (HBV) peptide was synthesized by New England Peptide (Gardner, MA). The amino acid sequence was derived from the pre-S1 region of HBV (D-type, GenBank accession number U9555.1) containing residues 2–48 and modified with N-terminal myristoylation (König et al., 2014; Yan et al., 2014). Rosuvastatin was purchased from Sequoia Research Products Ltd. (Oxford, UK). [³H]-taurocholate and [³H]-cGMP was purchased from PerkinElmer Life Sciences (Boston, MA). InVitroGRO-HT, -CP, and -HI hepatocyte media were purchased from BioreclamationIVT (Baltimore, MD). Collagen I-coated 24-well plates were obtained from BD Biosciences (Franklin Lakes, NJ). Cryopreserved human hepatocytes lots HH1027 (female, Caucasian, 59 years old, *CYP2C9**1/*1 genotype) and BOB (male, Caucasian, 61 years old, *CYP2C9**1/*1 genotype) were obtained from In Vitro ADMET Laboratories, LLC (Columbia, MD). Cryopreserved human hepatocytes lot Hu8246 (female, Caucasian, 37 years old, *CYP2C9* genotype not known) was obtained from Thermo Fisher Scientific (Carlsbad, CA). A BCA Protein Assay Kit was purchased from Pierce (Rockford, IL). NP40 protein lysis buffer was purchased from Thermo Fisher (Franklin, MA). Human embryonic kidney 293 (HEK293) cells stably transfected with human OATP1B3 or OATP2B1 were generated at Pfizer Inc. (Sandwich, UK). HEK293 cells expressing human OATP1B1 were obtained from Absorption Systems (Exton, PA). HEK293 cells stably transfected with human NTCP, OCT1, and OAT2(tv-1) were obtained from the laboratories of Per Artursson (Uppsala University, Uppsala, Sweden), Kathleen Giacomini (University of California, San Francisco, San Francisco, CA), and Ryan Pelis (Dalhousie University, Halifax, NS, Canada), respectively. Dulbecco's modified Eagle's medium, fetal bovine serum, nonessential amino acids, GlutaMAX-1, sodium pyruvate, penicillin and streptomycin solution were obtained from Invitrogen (Carlsbad, CA).

In Vitro Transport Studies Using Transporter-Transfected Cells. HEK293 cells wild type (WT) and stably transfected with NTCP, OATP1B1, OATP1B3, OATP2B1, OAT2, or OCT1 were seeded at a density of $0.5\text{--}1.2 \times 10^5$ cells/well on BioCoat 48 or 96-well poly-D-lysine-coated plates (Corning Inc., Corning, NY), grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 1% sodium pyruvate for 48 hours at 37°C, 90% relative humidity, and 5% CO₂. OATP1B1, OATP1B3, OATP2B1, and NTCP-HEK293 cells were

supplemented with NEAA and GlutaMAX. OCT1-HEK293, and OAT2-HEK293 cells were supplemented with 1% gentamycin, 1% sodium pyruvate, and 50 µg/ml hygromycin B.

For the uptake studies, HEK293 cells were washed three times with warm uptake buffer [Hanks' balanced salt solution (HBSS) with 20 mM HEPES, pH 7.4] and then incubated with uptake buffer containing tolbutamide (0.5 µM) in the absence and presence of the following inhibitors: cyclosporine (10 µM), rifamycin SV (20 and 1000 µM), ketoprofen (30, 100, and 300 µM), HBV peptide (0.1, 1 µM), and quinidine (500 µM). Based on our previous studies, ketoprofen showed concentration-dependent inhibition of OAT2 with an IC₅₀ value of ~30 µM and >80% inhibition at 300 µM (Mathialagan et al., 2017a). Therefore, three concentrations of ketoprofen in this range were studied. In the case of rifamycin SV, a 20 µM concentration inhibits only OATPs (OATP1B1/1B3/2B1), but not NTCP, OAT2, and OCT1; whereas a concentration of 1000 µM inhibits all six transporters (Bi et al., 2017). HBV peptide selectively inhibits NTCP at 0.1 µM and additionally inhibits OATP1B1 and OATP1B3 at 1 µM (König et al., 2014; Yan et al., 2014) (unpublished data). Quinidine is an OCT1 inhibitor (Letschert et al., 2006). The performance of transporter-transfected cells was validated using the following in vitro probe substrates: [³H]-cGMP (OAT2), [¹⁴C]-metformin (OCT1), [³H]-taurocholic acid (i.e., NTCP), or rosuvastatin (OATP1B1/1B3/2B1) as described by Mathialagan et al. (2017a). Cellular uptake was terminated by washing the cells four times with ice-cold transport buffer, and then the cells were lysed with 0.2 ml of 1% NP40 in water (radiolabeled compounds) or methanol containing the internal standard (IS) (non-labeled compounds). Intracellular accumulation was determined either by mixing the cell lysate with scintillation fluid followed by liquid scintillation analysis (PerkinElmer Life Sciences) for radiolabeled compounds or by liquid chromatography-tandem mass spectroscopy (LC-MS/MS) analysis for nonlabeled compounds. The total cellular protein content was determined using a Pierce BCA Protein Assay Kit according to the manufacturer's specifications. The uptake ratio was calculated as the ratio of accumulation in transfected cells to the accumulation in WT cells. Tolbutamide uptake, which was used to estimate uptake rates by linear regression, where necessary, was linear during 0.5- and 2-minute time courses.

Uptake and Inhibition Studies Using Cryopreserved Plateable Human Hepatocytes. The hepatic uptake assay was performed using short-term cultures of primary human hepatocytes as described previously with some modifications (Bi et al., 2017). Briefly, cryopreserved hepatocytes were thawed at 37°C and seeded at a density of 0.35×10^6 cells/well on 24-well collagen I-coated plates. The cells were cultured in InVitro-CP medium (BioreclamationIVT) overnight (~18 hours). Cells were preincubated with HBSS in the presence or absence of inhibitors for 10 minutes at 37°C. The preincubation buffer was aspirated, and the uptake and inhibition reaction were initiated by adding prewarmed buffer containing tolbutamide (0.5 µM) with or without inhibitors. The reactions were terminated at designated time points (0.5, 1, 2, and 5 minutes) by adding ice-cold HBSS immediately after removal of the incubation buffer. The cells were washed three times with ice-cold HBSS and lysed with 100% methanol containing IS or 0.5% Triton X-100 for radiolabeled tolbutamide. Samples were analyzed by LC-MS/MS or by liquid scintillation counting. Uptake rates were estimated from the initial time course (0.5–2 minutes) by linear regression.

Kinetic parameters of hepatic uptake in human hepatocytes were estimated using the following equation:

$$\text{Uptake rate} = PS_{pd} \cdot C + \frac{V_{max} \cdot C}{K_m + C} \quad (1)$$

Where, PS_{pd} is passive diffusion, and C is the incubation concentration. In case of transport kinetics in HEK293 cells, the uptake rate in WT cells was subtracted from the uptake rate in OAT2-transfected cells at each concentration; therefore, the PS_{pd} value in eq. 1 was assumed to be zero.

LC-MS/MS Method. LC-MS/MS analysis for tolbutamide was performed on a Triple Quad 6500 Mass Spectrometer equipped with a TurbolonSpray interface (both from Sciex, Concord, ON, Canada). The high-performance liquid chromatography systems consisted of a Model 1290 Infinity Binary Pump (Agilent Technologies, Santa Clara, CA) and the ADDA High-Speed Dual Arm Autosampling System (Apricot Designs, Covina, CA/Sound Analytics, Niantic, CT). All instruments were controlled and synchronized by Sciex Analyst software (version 1.6.2) working in tandem with the ADDA software. Mobile phases were 0.1% formic acid in water (mobile phase A) and 0.1% formic acid in acetonitrile (mobile phase B). The gradient was maintained at 5% mobile phase B for 0.2 minute, followed by a linear increase to 95% mobile phase B in 0.5 minute, and kept at 95% mobile phase B for 0.3 minute followed by a linear decrease to 5% in 0.02 minute. The column was equilibrated at 5% mobile phase B for 0.5 minute. The total run time for each injection was 1.5 minutes. The chromatographic separation was carried out on a Phenomenex (Torrance, CA) Kinetex C18 100 Å 30 × 2.1 mm column with a C18 guard column at a flow rate of 0.8 ml/min. The injection volume was 10 µl.

For mass spectrometry, the TurbolonSpray interface was operated in the positive/negative switching ion mode at 5000/–4500 V and 600°C. Quadrupoles Q1 and Q3 were set on unit resolution. Multiple reaction–monitoring mode using specific precursor/product ion transitions was used for quantification. Detection of the ions was performed by monitoring the transitions of mass/charge ratio with declustering potential (DP) and collision energy (CE) as follows: tolbutamide (negative mode, 269 → 170; DP, –65; CE, –25); and carbamazepine (positive mode, IS) (negative mode, 237 → 194; DP, 80; CE, 30).

Tolbutamide was quantitated from standard curves ranging from 0.1 to 500 nM. Linear regression was fitted to data of standard solutions using $1/X^2$ weighting. Data processing was performed using MultiQuant software (version 3.0.2; Sciex).

PBPK Modeling and Simulations of Clinical Drug-Drug Interactions. Whole-body PBPK modeling and simulations of tolbutamide were performed using a population-based ADME (absorption, distribution, metabolism, and excretion) simulator, Simcyp (version 15.1; Certara, Sheffield, UK). The virtual population (10 × 10 trials) of healthy subjects with a body weight of ~80 kg and age ranging from 18 to 65 years included both sexes. The doses, dosing intervals, and dosing durations of tolbutamide and inhibitor drugs were identical to those reported in the original clinical studies.

Bottom-up PBPK models—assuming rapid equilibrium (CYP2C9-alone) or permeability-limited (OAT2-CYP2C9 interplay) models for hepatic disposition—were developed using physicochemical properties and in vitro data (Table 1). The methodology adopted in model building for tolbutamide is similar to that applied for OATP substrates (Varma et al., 2012, 2013, 2017). The ADAM (advanced dissolution, absorption, and metabolism) model, informed with in vitro permeability data, was adopted to capture intestinal absorption and predict the oral pharmacokinetics of tolbutamide. For the formulation component, solution with no precipitation was assumed, given its oral bioavailability (*F*) is >85% (Varma et al., 2010). The full-PBPK model using the method of Rodgers et al. (default method 2), considering rapid equilibrium between blood and tissues, was adopted to obtain tolbutamide distribution into all organs. The model-predicted volume of distribution ($V_{d,ss}$) was within the range of observed values after intravenous dosing in humans (observed vs. predicted 0.12 and 0.11 l/kg) (Back et al., 1988; Tremaine et al., 1997). In parameterizing liver disposition, two scenarios were evaluated: rapid equilibrium (CYP2C9 alone); and permeability limited (OAT2-CYP2C9 interplay). For the latter, sinusoidal active uptake kinetics [J_{max} and Michaelis-Menten constant (K_m)] and PS_{pd} measured in the current in vitro plated human hepatocyte studies were employed. A recent comprehensive quantitative proteomics study (based on LC-MS/MS) showed

TABLE 1
Summary of input parameters for tolbutamide PBPK model

Parameters	Tolbutamide Inputs	Source
Physicochemical properties		
Molecular weight (g/mol)	270.3	
log P	2.34	Drugbank ^a
Compound type	Monoprotic acid	
pK _a	5.27	Calculated (MoKa 2.5.4) ^a
Plasma fraction unbound (<i>f</i> _{u,p})	0.027	Measured (Tremaine et al., 1997)
Blood/plasma ratio (R _b)	0.6	Measured ^a
Absorption		
Absorption type	ADAM	
MDCK-LE cell permeability (×10 ^{–6} cm/s)	31.3	Measured
Permeability calibrator, Propranolol (×10 ^{–6} cm/s)	23	Measured
Predicted fraction absorbed	1.0	
Unbound fraction in gut	1	Assumed
Distribution		
Distribution model	Full PBPK	Rodgers et al. (Model 2)
Predicted <i>V</i> _{d,ss} (l/kg)	0.105	Observed, 0.12 l/kg
Elimination		
CYP2C9 CL _{int,met} (µl/min per milligram microsomal protein)	1.87	Measured in human liver microsomes, (Walsky and Obach, 2004; Brown et al., 2007)
Microsomal protein binding	0.95 ^b	Obach (1999) (in-house data)
Renal CL (l/h)	0.0015	In vivo data ^a
Hepatobiliary transport		
Liver unbound fraction		
Intracellular	0.77	Model predicted
Extracellular	0.050	Model predicted
<i>PS</i> _{pd} (µl/min per 10 ^{–6} cells)	1.0	Measured (Fig. 2B) ^c
OAT2 <i>J</i> _{max} (pmol/min per 10 ^{–6} cells)	426.5	Measured (Fig. 2B) ^c
OAT2 <i>K</i> _m (µM)	39.3	Measured (Fig. 2B)
OAT2 REF	1	Measured ^d

ADAM, advanced dissolution, absorption, and metabolism model; *J*_{max}, maximum active uptake rate; K_p, tissue partition constant; P, partition coefficient; pK_a, acid dissociation constant; REF, relative expression factor.

^aInput values similar to the default tolbutamide model of Simcyp version 15.1. “Measured” implies the data were generated experimentally in-house.

^bMicrosomal protein binding was measured to be <10%, and, therefore, no corrections were applied for the intrinsic metabolic clearance.

^cAssuming that 1 mg of protein is equivalent to 1 million hepatocyte cells (Bi et al., 2017).

^dQuantitative proteomics based on LC-MS/MS showed that the OAT2 expression in three hepatocyte lots used for functional activity in this study is similar to the mean expression of 10 frozen liver samples (Vildhede et al., manuscript in preparation).

that the OAT2 expression in three hepatocyte lots used for the functional studies here is similar to the mean expression of 10 frozen liver samples (Vildhede A, Kimoto E, Rodrigues AD and Varma MV, manuscript in preparation). Therefore, the relative expression factor (REF) for IVIV scaling of OAT2-mediated transport was set at unity. In vitro intrinsic metabolic clearance measured by monitoring tolbutamide methylhydroxylase activity in pooled human liver microsomes was used to capture CYP2C9-mediated clearance (Walsky and Obach, 2004; Brown et al., 2007). Default (Simcyp version 15.1) inhibitor drug models (sulphaphenazole, fluconazole, and cimetidine) were directly implemented. Default and reported in vitro CYP2C9 K_i (inhibition constant) values were used for fluconazole and cimetidine, respectively. The average of reported competitive inhibition K_i values measured using tolbutamide as a substrate was adopted for the sulphaphenazole model. Model input parameters and the source for the values of all of the inhibitor drugs used to simulate drug-drug interactions (DDIs) are provided in Supplemental Table 1. The predictive performance of the model was assessed by the " $R_{\text{predicted/observed}}$ value" [= (mean predicted parameter)/(mean observed parameter)], with predefined acceptance criteria of 0.80–1.25 (Wagner et al., 2016).

Results

In Vitro Transport of Tolbutamide in Transfected Cells. The substrate potential of tolbutamide ($0.5 \mu\text{M}$) for hepatic uptake transporters was assessed by measuring its uptake in transporter-transfected and WT-HEK293 cells (Fig. 1). Of the six major hepatic solute carriers (SLCs) investigated, tolbutamide only showed transport by OAT2 with uptake ratios (ratio of uptake by OAT2-HEK293 cells to that by WT-HEK293 cells) of approximately 2.5 during the time course of the incubations, whereas the other five SLCs (NTCP, OATP1B1/1B3/2B1, and OCT1) did not show affinity for tolbutamide. OAT2-specific transport showed concentration dependency in HEK293 cells, with an estimated K_m value of $19.5 \pm 4.3 \mu\text{M}$ (Fig. 1B). Further, the effect of various SLC inhibitors on the transport of tolbutamide was assessed with OAT2-HEK293 cells (Fig. 1C). OATP inhibitors, cyclosporine ($10 \mu\text{M}$) and rifampicin ($20 \mu\text{M}$) (Li et al., 2014) did not show any significant impact, whereas ketoprofen, an OAT2 inhibitor (Mathialagan et al., 2017a), significantly ($P < 0.05$) reduced the uptake. Additionally, a NTCP-selective inhibitor (HBV peptide) (König et al., 2014; Yan et al., 2014) and an OCT1 inhibitor (quinidine) (Letschert et al., 2006) did not inhibit tolbutamide transport. Finally, rifampicin SV, a pan-SLC inhibitor at 1 mM (Bi et al., 2017), significantly reduced tolbutamide transport by OAT2-HEK293 cells.

In Vitro Uptake Mechanism of Tolbutamide in Human Hepatocytes. We investigated the time-dependent uptake of tolbutamide by human hepatocytes plated in short-term culture. The uptake increased over time, and the initial rates were determined by linear regression (Fig. 2A). The PS_{pd} value was estimated to be $1.0 \mu\text{L}/\text{min}$ per milligram protein after incubations in the presence of 1 mM rifampicin SV (Bi et al., 2017). Tolbutamide showed concentration-dependent uptake in human hepatocytes, with active transport characterized by K_m and V_{max} values of $39.3 \pm 6.6 \mu\text{M}$ and $426 \pm 30 \text{ pmol}/\text{min}$ per milligram protein, respectively (Fig. 2B). Therefore, the contribution of active uptake to total uptake clearance in human hepatocytes is estimated to be $\sim 90\%$ at subsaturation concentrations. Finally, tolbutamide uptake by human hepatocytes was significantly inhibited by $300 \mu\text{M}$ ketoprofen and 1 mM rifampicin SV, but

Fig. 1. Transport characteristics of tolbutamide in transporter-transfected HEK-293 cells. (A) Substrate activity of tolbutamide for six hepatic uptake transporters, measured using transporter-transfected HEK293 cells. Uptake ratio is defined as the ratio of cell accumulation in transfected cells to WT cells. The horizontal line and shaded area depict unity and arbitrary experimental error (0.5–1.5). (B) Kinetics of OAT2-mediated active uptake of tolbutamide in HEK293 cells. Shaded area depicts 95% confidence interval for the best-fit line. (C) Effect of transporter inhibitors on the uptake of tolbutamide by OAT2-HEK293 cells. Data are presented as the mean of triplicates, and, when shown, error bars capture the S.D. * $P < 0.01$, significantly different from control condition (one-way analysis of variance and Dunnett's multiple comparisons test). CSA, cyclosporine; RIF, rifampicin; RIF SV, rifampicin SV.

not by $10 \mu\text{M}$ cyclosporine, $20 \mu\text{M}$ rifampicin, and up to $1 \mu\text{M}$ HBV peptide (Fig. 2C). The effects of these inhibitors in human hepatocytes are similar to those noted with transfected cells (Fig. 1C). Collectively, OAT2 was found to play a prominent role in the uptake of tolbutamide by human hepatocytes.

Fig. 2. Uptake of tolbutamide in primary human hepatocytes. (A) Time course of tolbutamide cellular accumulation measured at 37°C in the absence and presence of 1 mM rifamycin SV. (B) Concentration-dependent uptake of tolbutamide at 37°C in the absence (filled points) and presence (open points) of 1 mM rifamycin SV. Data were fitted to eq. 1. (solid line), and the shaded area depicts the 95% confidence interval for the best-fit line. Dashed and dotted lines represent estimated active and passive uptake rates, respectively. (C) Effect of transporter inhibitors on the uptake of tolbutamide by primary human hepatocytes. (D) Uptake of tolbutamide, in the absence and presence of 1 mM rifamycin SV, in three different single-donor lots of cryopreserved primary human hepatocytes. Hepatocyte lot HH1027 was used for all uptake studies, unless stated otherwise. Data are presented as the mean of triplicates, and, when shown, error bars capture the S.D. * $P < 0.01$, significantly different from control condition (one-way analysis of variance and Dunnett's multiple comparisons test). CSA, cyclosporine A; RIF, rifamycin.

Physiologically Based Modeling of Tolbutamide Pharmacokinetics, DDIs, and CYP2C9 Pharmacogenomics.

Bottom-up PBPK models of tolbutamide, assuming either “rapid equilibrium” (CYP2C9-only) or “permeability-limited”

(OAT2-CYP2C9 interplay) hepatic disposition, were developed and evaluated using available clinical pharmacokinetic data (Fig. 3). The CYP2C9-only model considerably under-predicted intravenous and oral clearance, when employing

Fig. 3. Bottom-up PBPK model predictions of tolbutamide pharmacokinetics after intravenous (A) and oral (B) dosing. Simulated plasma concentration-time profiles based on the model assuming that the rapid equilibrium (CYP2C9 only) and the permeability-limited (OAT2-CYP2C9 interplay) hepatic disposition are depicted as dashed and solid lines, respectively. Black lines are the mean profiles of the individual trials shown in colored lines. Data points represent the mean observed data taken from separate clinical studies (Back et al., 1988; Tremaine et al., 1997; Cannady et al., 2015; Gillen et al., 2017). conc., concentration.

in vitro metabolic intrinsic clearance (CL_{int}) (CL_{int} for CYP2C9 = 1.87 $\mu\text{L}/\text{min}$ per milligram microsomal protein) measured using pooled human liver microsomes (Walsky and Obach, 2004; Brown et al., 2007). On the other hand, when hepatic uptake components (i.e., K_m , V_{max} , and PS_{pd}) estimated using human hepatocytes, and an OAT2 REF measured using LC/MS-based proteomics) were additionally incorporated into the model (OAT2-CYP2C9 interplay), tolbutamide intravenous and oral plasma concentration-time profiles of therapeutic dose (500 mg) were well described (Fig. 3). The latter model was therefore adopted for further evaluation of DDIs and the effect of CYP2C9 pharmacogenomics on tolbutamide pharmacokinetics.

Relevant clinical data were not available to directly verify the results of PBPK model simulations on the effect of OAT2 inhibition. However, the permeability-limited model well predicted the DDIs of intravenous tolbutamide with sulfaphenazole, fluconazole, and cimetidine, which are potent-to-weak CYP2C9 inhibitors (Supplemental Fig. 1; Table 2). The effect of CYP2C9 reduced-function variants on the oral tolbutamide pharmacokinetics was also reasonably predicted by the mechanistic model—assuming that the catalytic CYP2C9 activity for *1/*3, *2/*3, and *3/*3 variants are 60%, 30%, and 12% of WT (*1/*1), respectively (Fig. 4) (Scordo et al., 2002; Herman et al., 2005; Kusama et al., 2009). Predicted pharmacokinetic parameters were in good agreement with the observed values, as reflected in an $R_{\text{predicted/observed}}$ value within 0.8–1.25 for several pharmacokinetic parameters (Table 2).

Finally, a sensitivity analysis was performed to assess the influence of transport and metabolic CL_{int} values on the systemic clearance (CL_{plasma}) of tolbutamide (Fig. 5). Changes in OAT2 CL_{int} and/or CYP2C9 CL_{int} have a marked effect on tolbutamide systemic clearance, implying that the altered expression or activity of one or both can contribute to the pharmacokinetic variability of tolbutamide. Sensitivity analysis further suggests that a simultaneous change in the activity of both proteins in the same direction will have a larger impact on plasma clearance. Overall, the observed clinical pharmacokinetics, DDIs, and CYP2C9 pharmacogenomics can be quantitatively described primarily considering

all the mechanistic components (OAT2-CYP2C9 interplay) of tolbutamide hepatic disposition.

Discussion

The present study evaluated the role of transporter-mediated hepatic uptake in the systemic (plasma or blood) clearance of tolbutamide. In vitro studies using transporter-transfected cells and primary human hepatocytes provided comprehensive evidence for OAT2 being the prominent molecular mechanism involved in the hepatic uptake of tolbutamide. In addition, a bottom-up PBPK model better described the pharmacokinetics of tolbutamide when translating the in vitro data from human reagents, assuming OAT2-CYP2C9 interplay in hepatic disposition, in comparison with a CYP2C9-only mechanism. Collectively, these results suggest that OAT2-CYP2C9 interplay determines the hepatic clearance of tolbutamide, and that the inhibition of one or both pathways can lead to increased plasma exposure. This previously unrecognized role of hepatic OAT2 should be deliberated when considering tolbutamide as a clinical probe substrate for CYP2C9 activity.

OAT2 is a member of the SLC family—*SLC22*—that mediates the uptake of organic ions and is implicated to be of clinical relevance in the renal drug elimination (Sekine et al., 1998; Hosoyamada et al., 1999; Cha et al., 2001; Morrissey et al., 2013). Recent studies suggested that OAT2 contribution to the renal clearance of drugs such as penciclovir and ganciclovir (Mathialagan et al., 2017b); and to renal creatinine clearance, which is an endogenous marker for kidney function (Lepist et al., 2014; Shen et al., 2015). Although OAT2 expression is comparable in human liver and kidney, and hepatic OAT2 expression is relatively similar to other major hepatic uptake transporters such as OATPs (Nakamura et al., 2016), little is known about its functional role in the hepatic disposition/clearance of drugs (Shen et al., 2017). Using the transporter-transfected cells, we demonstrated that tolbutamide is a substrate of OAT2 but not of the other major hepatic uptake transporters (i.e., OATPs, NTCP, and OCT1). Additionally, we studied the impact of a selected set of transporter inhibitors that inhibit one or multiple uptake

TABLE 2

Summary of bottom-up PBPK (OAT2-CYP2C9 interplay) model predictions of tolbutamide pharmacokinetics, victim DDIs, and CYP2C9 pharmacogenomics

Tolbutamide Pharmacokinetic Parameters	Predicted ^a	Observed ^b	$R_{\text{predicted/observed}}$ Value	References for Observed
Pharmacokinetics				
Intravenous CL (ml/min per kilogram)	0.24	0.26	0.92	Back et al. (1988), Tremaine et al. (1997)
Intravenous V_{dss} (l/kg)	0.11	0.12	0.92	
Oral AUC 500 mg dose ($\mu\text{g}/\text{h}$ per milliliter)	681	693	0.98	Varma et al. (2010), Cannady et al. (2015),
Oral C_{max} 500 mg dose ($\mu\text{g}/\text{ml}$)	56	45	1.24	Gillen et al. (2017)
Fa.Fg	1.00	0.86	1.17	
F	0.99	0.85	1.16	
Victim DDIs				
Intravenous CL ratio with sulphaphenazole	3.6	3.1	1.16	Back et al. (1988)
Intravenous CL ratio with cimetidine	1.02	1.15	0.89	
Oral AUC ratio with fluconazole	2.4	2.1	1.14	Lazar and Wilner (1990)
Impact of CYP2C9 genotype				
CYP2C9*1/*1 Oral CL (ml/min per kilogram)	0.18	0.20	0.93	Kirchheiner et al. (2002a)
CYP2C9*1/*3 Oral CL (ml/min per kilogram)	0.13	0.12	1.10	
CYP2C9*2/*3 Oral CL (ml/min per kilogram)	0.08	0.11	0.78	
CYP2C9*3/*3 Oral CL (ml/min per kilogram)	0.05	0.03	1.42	

AUC, area under the curve.

^aMean of 10 × 10 population simulations.

^bMean value from one study. The mean of mean values was represented when data from multiple studies are available.

Fig. 4. PBPK model predictions of tolbutamide pharmacokinetics in carriers of the following *CYP2C9* genetic polymorphisms: *CYP2C9**1/*1 (A), *CYP2C9**1/*3 (B), *CYP2C9**2/*3 (C), and *CYP2C9**3/*3 (D). Simulated plasma concentration-time profiles based on the model assuming permeability-limited (OAT2-*CYP2C9* interplay) hepatic disposition are presented. Black lines are the mean profiles of individual trials shown in colored lines. Data points represent observed plasma concentrations from individual subjects (Kirchheiner et al., 2002a). conc., concentration.

transporters under the experimental conditions applied in an attempt to define the contribution of OAT2 compared with other transporters to tolbutamide hepatic uptake. Cyclosporine A (10 μ M) and rifampicin (20 μ M), known inhibitors of OATPs and NTCP at these concentrations (Li et al., 2014), did not impact tolbutamide OAT2-specific transport in transfected cells. However, an OAT2 inhibitor (ketoprofen) and a pan-SLC inhibitor (rifamycin SV 1 mM) reduced the transport significantly (Fig. 1C). A similar phenotype was observed for primary human hepatocytes (Fig. 2C), clearly implying OAT2-mediated transport as the molecular mechanism driving hepatic uptake of tolbutamide.

Tolbutamide is primarily eliminated by liver, and *CYP2C9* is the major enzyme involved in its biotransformation to the major metabolite 4-hydroxytolbutamide (Thomas and Ikeda, 1966; Relling et al., 1990). Generally, there is a higher level of confidence in the IVIV extrapolation of hepatic clearance from human liver microsomes and hepatocytes when cytochrome P450-mediated metabolism is the primary elimination pathway (Obach et al., 1997; Di et al., 2013). However, metabolic clearance values measured by monitoring 4-hydroxytolbutamide (a major metabolite) formation using liver microsomes or primary hepatocytes considerably underpredict the human clearance of tolbutamide. For instance, Obach (1999) studied tolbutamide IVIV extrapolation, along with 20 other drugs, and showed an ~5-fold disconnect for tolbutamide clearance using human liver

microsomes (Obach, 1999). Similarly, Brown et al. (2007) reported up to a 13-fold underprediction using pooled human hepatocytes. This discrepancy can be explained by considering the hepatic uptake mechanism demonstrated in the current study. A bottom-up PBPK model incorporating transporter kinetics measured using short-time culture primary human hepatocytes and intrinsic metabolic clearance measured using liver microsomes (i.e., accounting for OAT2-*CYP2C9* interplay) well recovered the plasma exposure after intravenous and oral administration of a therapeutic dose. In contrast, clearance was markedly underpredicted when only metabolic activity was considered (Fig. 3). The improved IVIV extrapolation provides a major basis for the clinical significance of OAT2-mediated hepatic uptake in the clearance of tolbutamide. DDIs involving *CYP2C9* inhibition with perpetrator drugs such as fluconazole and sulfaphenazole can also be quantitatively explained while considering OAT2-*CYP2C9* interplay (Supplemental Fig. 1; Table 2). Additionally, the plasma exposure of tolbutamide in carriers of *CYP2C9* genetic variants was well recovered (Fig. 4). Overall, consideration of the transporter-enzyme interplay in the hepatic disposition provided quantitative translation of tolbutamide clinical pharmacokinetics, and well described existing victim DDI and *CYP2C9* pharmacogenomic data.

Tolbutamide is a highly permeable drug with transcellular permeability of $\sim 31 \times 10^{-6}$ cm/s (Table 1). It is now well proven

Fig. 5. Model simulations of the effects of changes in OAT2-mediated transporter activity and CYP2C9 metabolic activity on the systemic clearance of tolbutamide (red mesh). The gray surface represents the mean observed clearance value (0.26 mL/min per kilogram). A green data point is shown to visualize the baseline values of the PBPK model in normal subjects. The blue data points depict observed oral clearance in CYP2C9 genetic variants (*1/*3, *2/*3, *3/*3) (Table 2).

that hepatic uptake (permeability-limited hepatic disposition) plays an important role in the pharmacokinetics and DDIs of several highly permeable OATP substrates, including repaglinide, cerivastatin, and montelukast (Shitara et al., 2013; Varma et al., 2015, 2017). Similarly, our study demonstrates permeability-limited hepatic disposition involving OAT2-CYP2C9 interplay as the primary clearance mechanism of tolbutamide.

A major limitation of our study is the lack of relevant clinical DDI or pharmacogenomic data to directly verify the model simulations projecting considerable increases in tolbutamide systemic exposure on OAT2 inhibition. On the other hand, as demonstrated with the mechanistic model, the existing clinical pharmacokinetic data (including reported DDIs and CYP2C9 pharmacogenomics) do not disagree with the proposed role of OAT2-mediated hepatic uptake in tolbutamide clearance. Further clinical studies designed to assess our current in vitro findings and model simulations appear warranted. However, robust hypothesis testing will be challenging in the absence of OAT2-selective inhibitors and well-defined OAT2 genotype-phenotype associations. Many of the inhibitors of OAT1 and OAT3 do not inhibit OAT2 at clinically achievable concentrations. For instance, probenecid, a recommended clinical probe inhibitor for OAT1 and OAT3, inhibits >85% of these two transporters at clinical doses, but shows weak (<5%) inhibition of OAT2-mediated transport (Mathialagan et al., 2017b). A thorough literature review suggested indomethacin as a potential probe inhibitor, although it may cause only ~30% OAT2 inhibition at its therapeutic doses (Mathialagan et al., 2017b). On the other hand, there are several nonsynonymous variants in the *SLC22A7* gene (encoding OAT2) (including Thr101Ile, Val192Ile, and Gly507Asp), but little is known about the clinical relevance of these genotypes. Indeed, studies suggested no link between renal elimination of drugs in humans and

polymorphisms in the *SLC22A7* gene (Vormfelde et al., 2006). Shin et al. (2010) studied the expression of OAT2 protein in 34 human liver samples using Western blot analysis, where the expression levels varied about 10-fold across the samples but did not show any association with the *SLC22A7* genotype. Overall, these limited reports suggest that known genetic polymorphisms may not contribute to OAT2 expression levels and its transporter activity. Due to a lack of knowledge regarding functional genetic variants and probe inhibitors, assessing OAT2 contribution to the clearance of drugs will be challenging. Investment in identifying *SLC22A7* genetic polymorphisms with altered function and screening drug libraries for clinically useful OAT2 inhibitors may help in the development of tools in this direction.

Tolbutamide is characterized by variable pharmacokinetics with a half-life of 3–10 hours in humans (Zilly et al., 1975; Jackson and Bressler, 1981; Back et al., 1988). Sensitivity analysis with the verified PBPK model implied a dependence of tolbutamide systemic clearance on the OAT2 CL_{int} and/or CYP2C9 CL_{int} (Fig. 5). Further, a simultaneous change in both uptake and metabolic clearances in the same direction could result in a marked change in pharmacokinetics. Therefore, functional variability in OAT2 and CYP2C9 activity caused by expression differences and CYP2C9 genetic polymorphism can explain the high interindividual variability in pharmacokinetics observed for tolbutamide.

In conclusion, to our knowledge, this is the first study demonstrating the potential role of OAT2-mediated hepatic uptake in drug clearance. This previously unrecognized mechanism in conjunction with CYP2C9 may determine the pharmacokinetics of tolbutamide and the optimal dose required for effective therapy. Although we demonstrated the clinical significance of OAT2-CYP2C9 interplay via quantitative IVIV extrapolation and DDIs involving CYP2C9, further clinical DDI or pharmacogenomic studies are necessary to ascertain the link between OAT2 activity and hepatic clearance of tolbutamide. Nonetheless, our mechanistic evaluation implies that OAT2-mediated hepatic uptake likely confounds the interpretation of drug interaction mechanisms of investigational drugs, when using tolbutamide as a CYP2C9 clinical probe; therefore, prior in vitro assessment of the OAT2 inhibition potential of the investigational drug is recommended.

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Authorship Contributions

Participated in research design: Bi, Mathialagan, Tylaska, Costales, Rodrigues, and Varma.

Conducted experiments: Bi, Mathialagan, Tylaska, Fu, and Keefer.

Performed data analysis: Bi, Mathialagan, Tylaska, Fu, Keefer, Vildhede, Costales, Rodrigues, and Varma.

Wrote or contributed to the writing of the manuscript: Bi, Mathialagan, Tylaska, Fu, Keefer, Vildhede, Costales, Rodrigues, and Varma.

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