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Organic Anion Transporter 2 mediates hepatic uptake of tolbutamide, a Cytochrome P450 2C9 probe drug

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Running title: OAT2-mediated hepatic uptake of tolbutamide

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ABBREVIATIONS: AUC, area under the plasma concentration-time curve; CYP, cytochrome P-450; DDI, drug-drug interaction; IC_{50}, inhibitory potency; 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; SLC, solute carrier.

ABSTRACT

Tolbutamide is primarily metabolized by cytochrome P450 (CYP)2C9, and thus, 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 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 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 (OAT)2 alone – with transporter affinity (Km) of 19.5±4.3 μM. Additionally, OAT2-specific transport was inhibited by ketoprofen (OAT2 inhibitor) and 1mM rifamycin SV (pan inhibitor), but not by cyclosporine and rifampicin (OATPs/NTCP 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 Km and V_{max} of 39.3±6.6 μM and 426±30 pmol/min/mg-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, 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 II diabetes mellitus (T2DM). It is characterized by a low clearance and good absolute oral bioavailability; and largely metabolised by cytochrome P-450 (CYP)2C9 to 4-hydroxytolbutamide, which is further oxidised 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 Brockmöller, 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 Brockmöller, 2005). Large inter-individual variability in tolbutamide pharmacokinetics observed in the clinic is suggested to be caused by CYP2C9 genetic variants (Scott and Poffenbarger, 1978; Kirchheiner et al., 2002a; Shon et al., 2002). Although in vitro studies suggested CYP2C19 involvement in the metabolism of tolbutamide (Wester et al., 2000), clinical pharmacogenomic studies implied no influence by CYP2C19 polymorphism (Kirchheiner et al., 2002a; Shon et al., 2002). Therefore, tolbutamide is considered to be the standard CYP2C9 phenotypic probe (Lee et al., 2003; USFDA, 2012).

Despite predominant CYP-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; USFDA, 2012; 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). [^{3}H]-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 preS1 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). [^{3}H]-taurocholate and [^{3}H]-cGMP was purchased from PerkinElmer Life Sciences (Boston, MA). InVitroGro-HT, CP and HI hepatocyte media were purchased from Celsis IVT (Baltimore, MD). Collagen I coated 24-well plates were obtained from BD Biosciences (Franklin Lakes, NJ). Cryopreserved human hepatocytes lots HH1027 (female, Caucasian, 59 year old, *CYP2C9*1/*1* genotype) and BOB (male, Caucasian, 61 year old, *CYP2C9*1/*1* genotype) were obtained from *In vitro* ADMET Laboratories, LLC (Columbia, Maryland). Cryopreserved human hepatocytes lot Hu8246 (female, Caucasian, 37 year old, CYP2C9 genotype not known) was obtained from Thermo Fisher Scientific (Carlsbad, CA). BCA protein assay kit was purchased from PIERCE (Rockford, IL). NP-40 protein lysis buffer was purchased from Thermo-Fisher (Franklin, MA). Human Embryonic Kidney (HEK) 293 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, Sweden), Kathleen Giacomini (University of California, CA), and Ryan Pelis

(Dalhousie University, Canada), respectively. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, nonessential amino acids, GlutaMAX-1, sodium pyruvate, penicillin and streptomycin solution were obtained from Invitrogen.

***In vitro* Transport Studies using transporter-transfected cells**

HEK293 cells wild-type and stably transfected with NTCP, OATP1B1, OATP1B3, OATP2B1, OAT2 or OCT1 were seeded at a density of 0.5 to 1.2×10^5 cells/well on BioCoat™ 48 or 96-well poly-D-lysine coated plates (Corning Inc., Corning NY), grown in DMEM containing 10% FBS and 1% sodium pyruvate for 48 hrs at 37°C, 90% relative humidity, and 5% CO_2. 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 (HBSS with 20mM HEPES, pH 7.4) and then incubated with uptake buffer containing tolbutamide (0.5µM) in the absence and presence of inhibitors: cyclosporine (10 µM), rifamycin SV (20 and 1000 µM), ketoprofen (30, 100, 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 IC_{50} of ~30µM, and >80% inhibition at 300µM (Mathialagan et al., 2017a). Therefore, 3 concentrations of ketoprofen in this range were studied. In case of rifamycin SV, 20µM concentration only inhibit OATPs (OATP1B1/1B3/2B1), but not NTCP, OAT2 and OCT1; while, 1000µM inhibit all 6 transporters (Bi et al., 2017b). HBV peptide selectively inhibits NTCP at 0.1µM, and additionally inhibit OATP1B1 and OATP1B3 at 1µM (König et al., 2014; Yan et al., 2014)(our unpublished data). Quinidine is OCT1 inhibitor (Letschert et al., 2006).

Performance of transporter-transfected cells was validated using in vitro probe substrates: [^{3}H]-cGMP (OAT2), [^{14}C]-metformin (OCT1), [^{3}H]-taurocholic acid (NTCP), or rosuvastatin (OATP1B1/1B3/2B1) as described by Mathialagan et al. (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% NP-40 in water (radiolabelled compounds) or methanol containing internal standard (non-labelled compounds). Intracellular accumulation was determined either by mixing the cell lysate with scintillation fluid followed by liquid scintillation analysis (PerkinElmer Life Sciences, Boston, MA) for radiolabelled compounds or by LC-MS/MS analysis for non-labelled 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 a ratio of accumulation in transfected cells to the accumulation in WT cells. Tolbutamide uptake was linear during 0.5 and 2 min time-course, which was used to estimate uptake rates by linear regression, where necessary.

Uptake and Inhibition Studies using Cryopreserved Plateable Human Hepatocytes (PHH)

The hepatic uptake assay was performed using short-term culture primary human hepatocytes as described previously with some modifications (Bi et al., 2017b). 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 overnight (~18h). Cells were preincubated with HBSS in the presence or absence of inhibitors for 10min at 37°C. The preincubation buffer was aspirated, and the uptake and inhibition reaction was initiated by adding prewarmed buffer containing tolbutamide (0.5µM) with or without inhibitors. The reactions were terminated at designated time points (0.5, 1, 2, 5min) by adding ice-cold HBSS

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immediately after removal of the incubation buffer. The cells were washed three times with ice cold HBSS and lysed with 100% methanol containing internal standard or 0.5% Triton-100 for radiolabelled tolbutamide. Samples were analysed by LC-MS/MS or by liquid scintillation counting. Uptake rates were estimated from the initial time-course (0.5-2min) by linear regression.

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

$$Uptake\ rate\ = PS_{pd}.C + \frac{V_{max}\ .C}{Km + C} \qquad Eq.1$$

Where, Km and V_{max} are active transport affinity and maximum uptake rate, respectively. PS_{pd} is passive clearance, and C is the incubation concentration. In case of transport kinetics in HEK293 cells, uptake rate in wild-type cells was subtracted from uptake rate in OAT2-trasfected cells at each concentration, therefore, PS_{pd} in Eq. 1 was assumed to be zero.

LC/MS/MS Method

LC-MS/MS analysis for tolbutamide was performed on a SCIEX Triple Quad 6500 mass spectrometer (SCIEX, Ontario, Canada) equipped with TurboIonSpray interface. The HPLC systems consisted of an Agilent 1290 Infinity binary pump (Agilent Technologies, Santa Clara, CA) and ADDA autosampler (Apricot Designs, Covina, CA and 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% B for 0.2 min, followed by a linear increase to 95% B in 0.5 minutes, and kept

at 95% B for 0.3 min then a linear decrease to 5% in 0.02 minutes. The column was equilibrated at 5% B for 0.5 min. The total run time for each injection was 1.5 minutes. The chromatographic separation was carried out on a Phenomenex Kinetex C18 100Å 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 TurboIonSpray 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 (MRM) 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 (m/z) with declustering potential (DP) and collision energy (CE) as follows: tolbutamide (negative mode, 269→170, DP -65, CE -25) and carbamazepine (positive mode, internal standard, IS) (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 (DDIs)

Whole-body PBPK modeling and simulations of tolbutamide were performed using population-based ADME simulator, Simcyp (version 15.1, Certara, Sheffield, UK). The virtual population (10 x10 trials) of healthy subjects with a body weight of ~80 kg and age ranging from 18 to 65 years included both sexes. Dose, dosing interval, and dosing duration of tolbutamide and inhibitor drugs were identical to that reported in the original clinical studies.

"Bottom-up" PBPK models – assuming rapid-equilibrium (CYP2C9-alone) or permeability-limited (OAT2-CPY2C9 interplay) for hepatic disposition – were developed using physicochemical properties and in vitro data (Table 1). Methodology adopted in model building for tolbutamide is similar to that applied for OATP substrates (Varma et al., 2012; Varma et al., 2013; Varma et al., 2017). Advanced dissolution, absorption and metabolism (ADAM) model, informed with in vitro permeability data, was adopted to capture intestinal absorption and predict 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). Full-PBPK model using Rodgers et al. method (default method 2) considering rapid equilibrium between blood and tissues was adopted to obtain tolbutamide distribution into all organs. Model predicted volume of distribution (Vd_{ss}) was within the range of observed values following 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) or permeability-limited (OAT2-CYP2C9 interplay). For the latter, sinusoidal active uptake kinetics (J_{max} and K_m) and passive diffusion measured in the current in vitro plated human hepatocyte studies were employed. A recent comprehensive quantitative proteomics study (based on LC/MS/MS) showed that the OAT2 expression in 3 hepatocyte lots used for the functional studies here is similar to the mean expression of 10 frozen liver samples (Vildhede et al., Manuscript under preparation). Therefore, 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 V15.1) inhibitor drug models (sulphaphenazole, fluconazole and

cimetidine) were directly implemented. Default and reported in vitro CYP2C9 Ki values were used for fluconazole and cimetidine, respectively. Average of reported competitive inhibition Ki values measured using tolbutamide as substrate was adopted for sulphaphenazole model. Model input parameters and the source for values of all the inhibitor drugs used to simulate DDIs are provided in Supplementary Table 1. The predictive performance of model was assessed by '$R_{predicted/observed}$ 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 μM) for hepatic uptake transporters was assessed by measuring its uptake in transporter-transfected and WT-HEK293 (Figure 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 WT-HEK293 cells) of approximately 2.5 during the time-course of the incubations, while the other 5 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 Michaelis-Menten constant (K_m) of 19.5±4.3 μM (Figure 1B). Further, the effect of various SLC inhibitors on the transport of tolbutamide was assessed with OAT2-HEK293 cells (Figure 1C). OATPs inhibitors, cyclosporine (10 μM) and rifampicin (20 μM) (Li et al., 2014), did not show any significant impact, while 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 OCT1 inhibitor (quinidine (Letschert et al., 2006)) did not inhibit tolbutamide transport. Finally, rifamycin SV, a pan-SLC inhibitor at 1mM (Bi et al., 2017b), 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 (Figure 2A). The passive diffusion (PS_{pd}) was estimated to be 1.0 μL/min/mg-protein following incubations in the presence of 1mM rifamycin SV (Bi et al., 2017b).

Tolbutamide showed concentration-dependent uptake in human hepatocytes, with active transport characterized by K_m and V_{max} values of 39.3±6.6 μM and 426±30 pmol/min/mg-protein, respectively (Figure 2B). Therefore, the contribution of active uptake to total uptake clearance in human hepatocytes is estimated to be ~90% at sub-saturation concentrations. Finally, tolbutamide uptake by human hepatocytes was significantly inhibited by 300μM ketoprofen and 1mM rifamycin SV, but not by 10μM cyclosporine, 20μM rifampicin and up to 1μM HBV peptide (Figure 2C). The effects of these inhibitors in human hepatocytes are similar to that noted with transfected cells (Figure 1C). Collectively, OAT2 was found to play a prominent role in the uptake of tolbutamide by human hepatocytes.

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 (Figure 3). The CYP2C9-only model considerably underpredicted intravenous and oral clearance, when employing in vitro metabolic clearance ($CL_{int,CYP2C9}$ = 1.87 μL/min/mg-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., Km, Vmax and PS_{pd} estimated using human hepatocytes, and an OAT2 REF measured using LC/MS-based proteomics) were additionally incorporated in the model (OAT2-CYP2C9 interplay), tolbutamide intravenous and oral plasma concentration-time profiles of therapeutic dose (500mg) were well described (Figure 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 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 (Table 2, Supplementary Figure 1). 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 wild-type (**1/*1*), respectively (Figure 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 $R_{predited/observed}$ 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 intrinsic clearances on the systemic clearance (CL_{plasma}) of tolbutamide (Figure 5). Changes in OAT2 CL_{int} and/or CYP2C9 CL_{int} have a marked effect on tolbutamide systemic clearance, implying that 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 as 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 to a CYP2C9-only mechanism. Collectively, these results suggest that OAT2-CYP2C9 interplay determine the hepatic clearance of tolbutamide, and that 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 solute carrier family – *SLC22* – that mediates 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 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., 2016). 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, which inhibit one or multiple uptake transporters under the experimental conditions applied, in an attempt to define the contribution of OAT2 versus other transporters to tolbutamide hepatic uptake. Cyclosporine A (10μM) and rifampicin (20μM), known inhibitors of OATPs and NTCP at these concentrations (Li et al., 2014), did not impact tolbutamide OAT2-specific transport in transfected cells. However, OAT2 inhibitor (ketoprofen) and a pan-SLC inhibitor (rifamycin SV 1mM) reduced the transport significantly (Figure 1C). A similar phenotype was observed for primary human hepatocytes (Figure 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 CYP-mediated metabolism is the primary elimination pathway (Obach et al., 1997; Di et al., 2013). However, metabolic clearance values measured by monitoring 4-hydroxytolbutamide (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 ~5-fold disconnect for tolbutamide clearance using human liver microsomes (Obach, 1999). Similarly, Brown et al. reported up to a 13-fold underprediction using pooled human hepatocytes (Brown et al., 2007). This discrepancy can be explained by considering a 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 following intravenous and oral administration of therapeutic dose. In contrast, clearance was markedly underpredicted when only metabolic activity was considered (Figure 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 (Table 2, Supplemental Figure 1). Additionally, the plasma exposure of tolbutamide in carriers of *CYP2C9* genetic variants was well recovered (Figure 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 ~$31x10^{-6}$ cm/s (Table 1). It is now well proven that hepatic uptake (permeability-limited hepatic disposition) plays an important role in the pharmacokinetics and DDIs of several high permeable OATP substrates including repaglinide, cerivastatin and montelukast (Shitara et al., 2013; Varma et al., 2015; Varma et al., 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 increase 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 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 2 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 non-synonymous variants in *SLC22A7* gene (encoding OAT2) (including Thr101Ile, Ver192Ile and Gly507Asp), but little is known about the clinical relevance of these genotypes. Indeed, studies suggested no link between renal elimination of drugs in human and polymorphisms in *SLC22A7* gene (Vormfelde et al., 2006). Shin et al. studied the expression of OAT2 protein from 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 (Shin et al., 2010). Overall, these limited reports suggest that known genetic polymorphisms may not contribute to OAT2 expression levels and its transporter activity. Due to lack of knowledge regarding functional genetic variants and probe inhibitors, assessing OAT2 contribution to clearance of drugs will be challenging. Investment towards identifying SLC22A7 genetic polymorphisms with altered function and screening drug libraries for clinically useful OAT2 inhibitors may help develop tools in this direction.

Tolbutamide is characterized by variable pharmacokinetics with a half-life of 3-10 h in human (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} (Figure 5). Further, 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 inter-individual 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 the drug clearance. This previously unrecognized mechanism in conjunction with CYP2C9 may determine the pharmacokinetics of tolbutamide and optimal dose required for effective therapy. While 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 CYP2C9 clinical probe; and 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, Varma

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

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

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

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CONFLICT OF INTEREST

All authors are full-time employees of Pfizer Inc. The authors have no conflicts of interest that are directly relevant to this study.

Legends for Figures

Figure 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 ratio of cell accumulation in transfected cells to wild-type cells. Horizontal line and shaded area depict unity and arbitrary experimental error (0.5-1.5). B, Michaelis-Menten 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 mean of triplicates, and when shown, error bars capture s.d. *$p<0.01$, significantly different from control condition (one-way ANOVA and Dunnett's multiple comparisons test)

Figure 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 1mM rifamycin SV. B, Concentration-dependent uptake of tolbutamide at 37˚C in the absence (filled points) and presence (open points) of 1mM rifamycin SV. Data were fitted to Eq. 1. (solid line), and the shaded area depict 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 1mM rifamycin SV, in three different single-donor lots of cryopreserved primary human hepatocytes. Hepatocytes lot HH1027 was used for all uptake studies, unless stated otherwise. Data are presented as mean of triplicates, and when

shown, error bars capture s.d. *p<0.01, significantly different from control condition (one-way ANOVA and Dunnett's multiple comparisons test).

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

Figure 4. PBPK model predictions of tolbutamide pharmacokinetics in carriers of *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 mean profiles of individual trials shown in colored lines. Data points represent observed plasma concentrations from individual subjects (Kirchheiner et al., 2002a).

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

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Table 1. Summary of input parameters for tolbutamide physiologically based pharmacokinetic model.

Parameters	Tolbutamide inputs	Source
Physicochemical properties		
Molecular weight (g/mol)	270.3	
log P	2.34	Drugbank*
Compound type	Monoprotic acid	
pK_a	5.27	Calculated (MoKa 2.5.4)*
Fraction unbound ($f_{u,p}$)	0.027	Measured (Tremaine et al., 1997)
Blood/plasma ratio (Rb)	0.6	Measured*
Absorption		
Absorption type	ADAM	
MDCK-LE permeability ($\times 10^{-6}$ cm/s)	31.3	Measured
Permeability calibrator - Propranolol ($\times 10^{-6}$ cm/s)	23	Measured
Predicted Fraction absorbed	1.0	
Unbound fraction in gut (fu_{gut})	1	Assumed
Distribution		
Distribution model	Full PBPK	Rodgers et al. (Model2)
Predicted Vdss (L/kg)	0.105	Observed - 0.12 L/kg
Elimination		
CYP2C9 $CL_{int,met}$ (µL/min/mg-microsomal protein)	1.87	Measured in Human liver microsomes, (Walsky and Obach, 2004; Brown et al., 2007)
Microsomal protein binding	0.95[⊥]	(Obach, 1999) (in-house data)
Renal CL (L/h)	0.0015	In vivo data*
Hepatobiliary transport		
Liver unbound fraction		
Intracellular	0.77	Model predicted
Extracellular	0.050	Model predicted
PS_{pd} (µL/min/10^{-6}cells)	1.0	Measured (Figure 2B)[⊤]
OAT2 J_{max} (pmol/min/10^{-6}cells)	426.5	Measured (Figure 2B)[⊤]
OAT2 K_m (µM)	39.3	Measured (Figure 2B)
OAT2 relative expression factor	1	Measured[+]

*Input values similar to the default Tolbutamide model of Simcyp V15.1. Measured implies the data was generated experimentally in-house.

[⊥]Microsomal protein binding was measured to be <10%, and therefore, no correction were applied for the intrinsic metabolic clearance.

[⊤]Assuming one mg protein is equivalent to one million hepatocyte cells (Bi et al., 2017a).

[+]Quantitative proteomics based on LC/MS/MS showed that the OAT2 expression in 3 hepatocyte lots used for functional activity in this study is similar to the mean expression of 10 frozen liver samples (Vildhede et al., Manuscript under preparation).

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ADAM, Advanced dissolution, absorption and metabolism model; P, partition coefficient; pK_a, acid dissociation constant; Kp, tissue partition constant; fm, fraction metabolized; PS_{pd}, intrinsic passive uptake clearance; PS_{active}, intrinsic active uptake clearance.

Table 2. Summary of bottom-up PBPK (OAT2-CYP2C9 interplay) model predictions of tolbutamide pharmacokinetics, victim DDIs and CYP2C9 pharmacogenomics.

Tolbutamide pharmacokinetic parameters	**Predicted**[⊥]	**Observed**[⊤]	**$R_{predicted/observed}$ value**	**References for observed**
Pharmacokinetics				
Intravenous CL (mL/min/kg)	0.24	0.26	0.92	(Back et al.,
Intravenous Vdss (L/kg)	0.11	0.12	0.92	1988;
				Tremaine et al.,
				1997)
Oral AUC (μg.hr/mL) -500mg dose	681	693	0.98	(Varma et al.,
Oral C_{max} (μg/mL) -500mg dose	56	45	1.24	2010; Cannady
Fa.Fg	1.00	0.86	1.17	et al., 2015;
F	0.99	0.85	1.16	Gillen et al.,
				2017)
Victim Drug-drug interactions				
Intravenous CL ratio with Sulphaphenazole	3.6	3.1	1.16	(Back et al.,
Intravenous CL ratio with Cimetidine	1.02	1.15	0.89	1988)
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/kg)	0.18	0.20	0.93	(Kirchheiner et
*CYP2C9*1/*3* Oral CL (mL/min/kg)	0.13	0.12	1.10	al., 2002a)
*CYP2C9*2/*3* Oral CL (mL/min/kg)	0.08	0.11	0.78	
*CYP2C9*3/*3* Oral CL (mL/min/kg)	0.05	0.03	1.42	

[⊥]mean of 10x10 population simulations.
[⊤]mean value from one study. Mean of mean values was represented when data from multiple studies are available.

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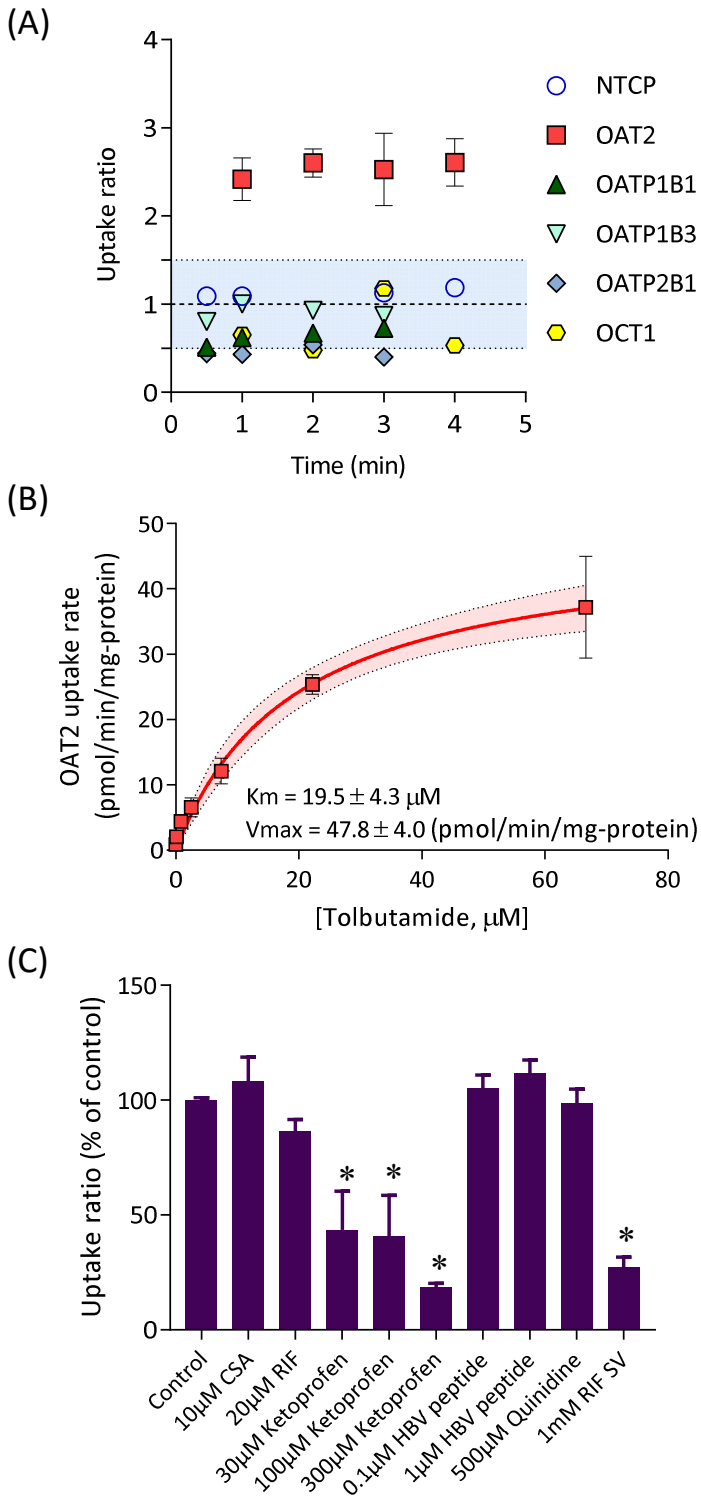

Figure 1

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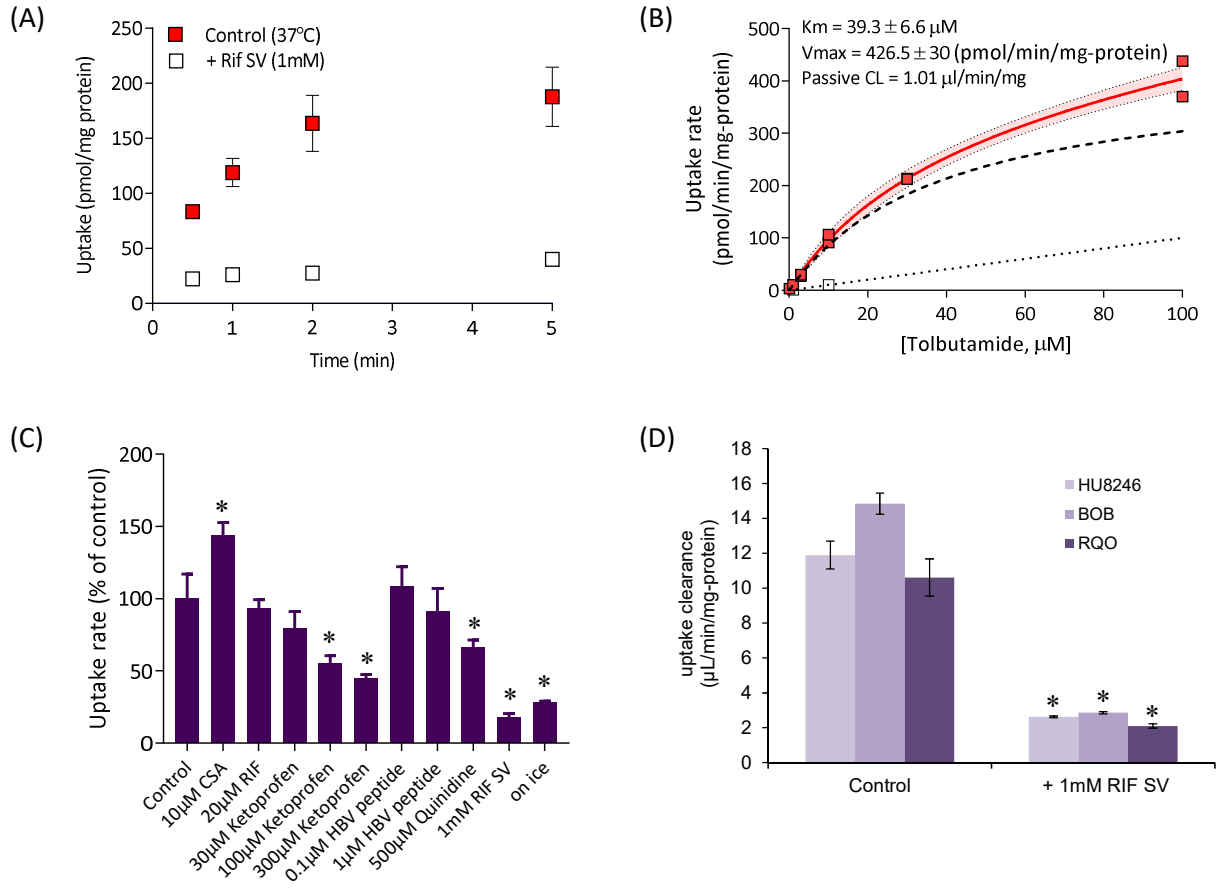

Figure 2

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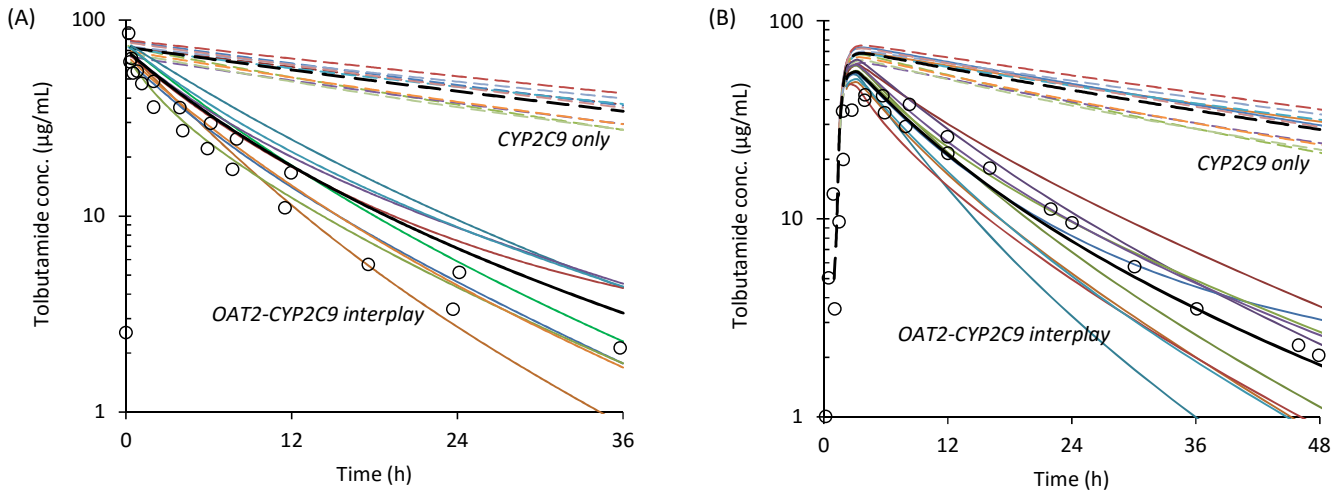

Figure 3

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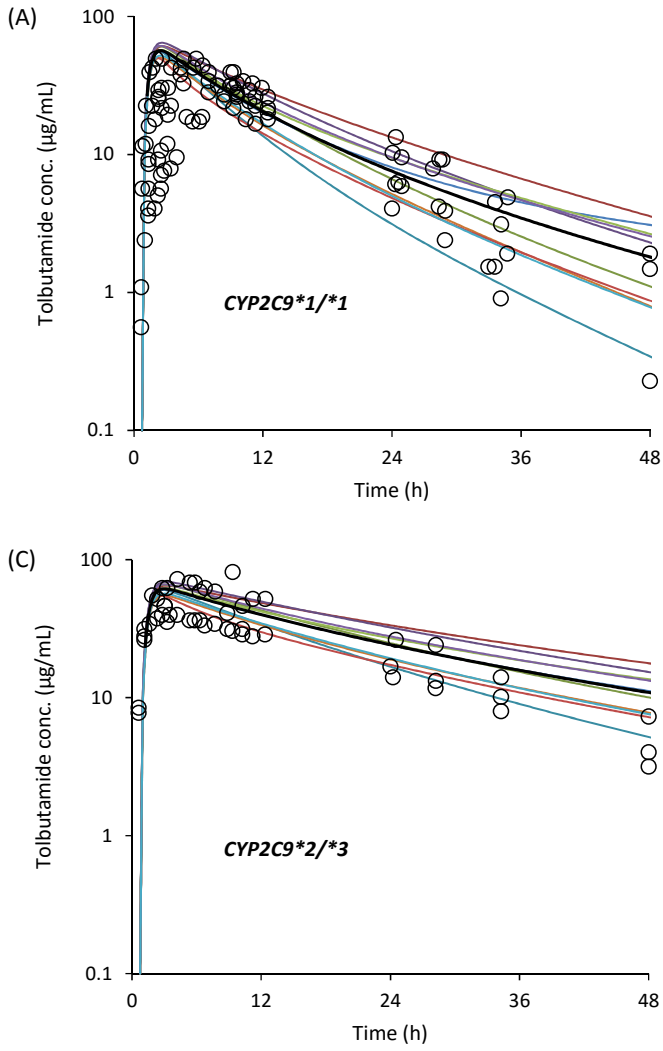

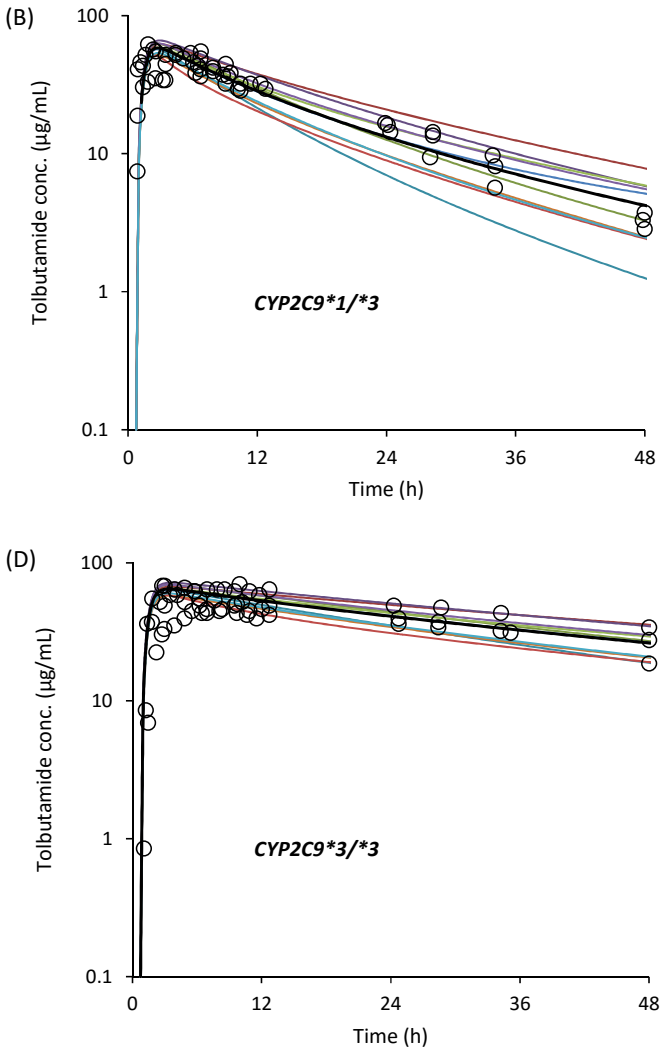

Figure 4

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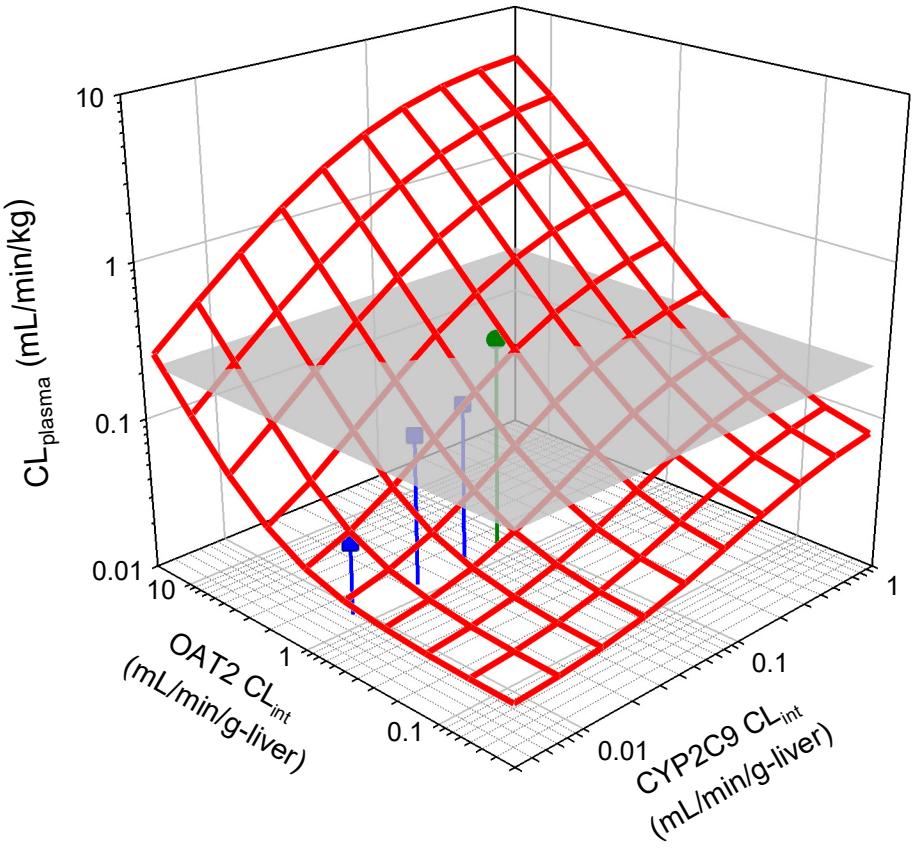

Figure 5

Organic Anion Transporter 2 mediates hepatic uptake of tolbutamide, a Cytochrome P450 2C9 probe drug

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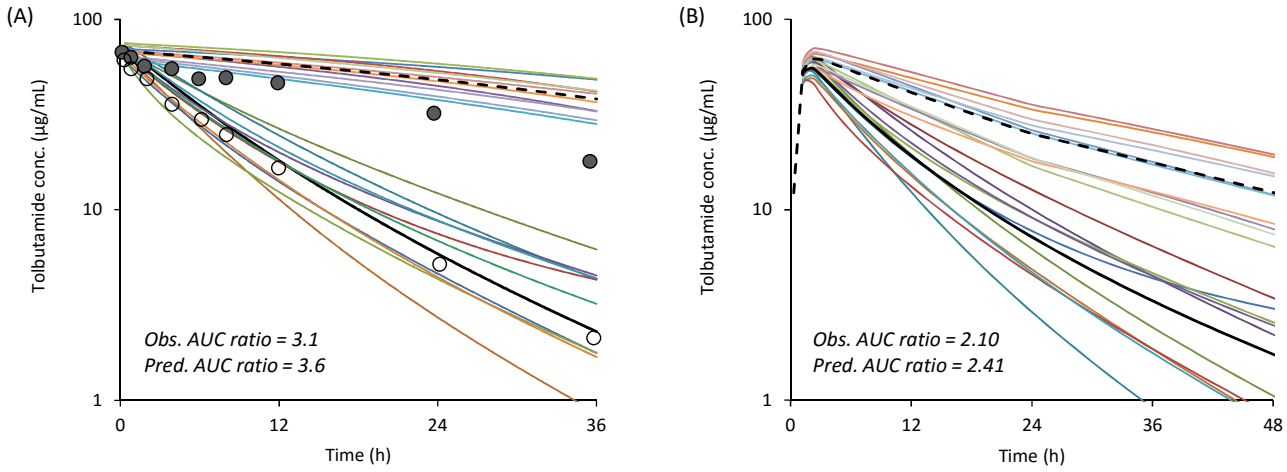
Supplementary Table 1. Summary of physiologically based pharmacokinetic model input parameters for the inhibitor drugs.

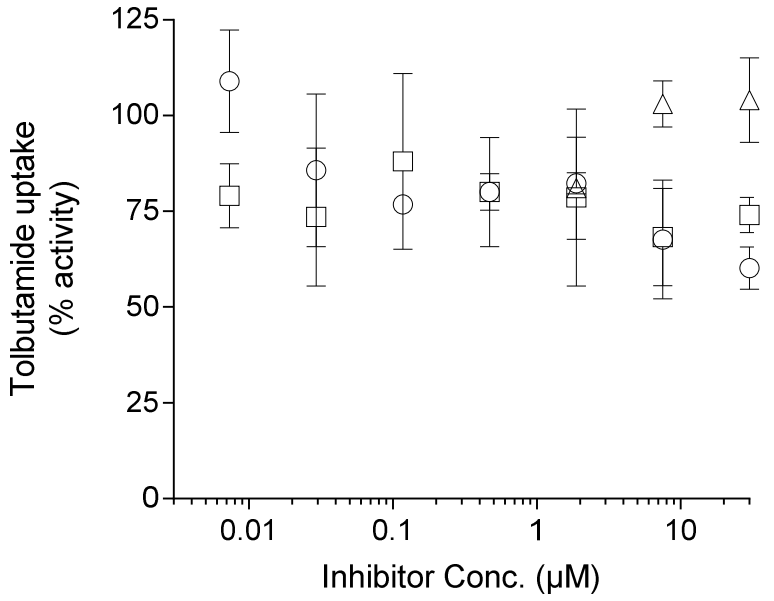
Parameters	Sulphaphenazole inputs (Simcyp V15.1 default model)	Fluconazole inputs (Simcyp V15.1 default model)	Cimetidine inputs (Simcyp V15.1 default model)
Physicochemical properties			
Molecular weight (g/mol)	314.4	306.3	252.34
log P	1.52	0.2	0.48
Compound type	Acid	Base	Base
pK_a	5.91	1.76	6.9
Fraction unbound ($f_{u,p}$)	0.04†	0.89	0.97
Blood/plasma ratio (Rb)	0.62	1	0.8
Absorption			
Absorption type	First Order	First Order	First Order
Fraction absorbed	1.00	0.98	0.92
Ka (hr-1)	1.86	1.75	0.7
Lag time (hr)			0.15
Cell permeability ($\times 10^{-6}$ cm/s)		29.8	
Unbound fraction in gut (fu_{gut})	0.0275	0.89	1
Distribution			
Distribution model	Minimal PBPK	Minimal PBPK	Full PBPK (Rodgers et al.)
Vss (L/kg)	0.162	0.748	0.58
Elimination			
CLiv or CLpo (L/hr)	0.382 (po)	1.01 (iv)	CL_{int}: 2.87(µL/min/mg-microsomal protein)
CLrenal (L/hr)	0.084	0.7	Mechanistic kidney model
Interaction			
Ki vs CYP2C9 (µM)	0.48⊤	7.92	140(Miners et al., 1988)

†Measured inhouse. Simcyp V15.1 compound file default value is 1.0.

⊤ Average of competitive inhibition Ki values measured using tolbutamide as substrate and Sulphaphenazole as inhibitor in human liver microsomes (Bourrie et al., 1996; Komatsu et al., 2000; Furuta et al., 2004; Kumar et al., 2006; Yeung and Or, 2011; Bae et al., 2014). Simcyp V15.1 compound file default value is 0.16µM.



Supplementary Figure 1. Permeability-limited (OAT2-CYP2C9 interplay) model based predictions of tolbutamide victim DDIs with (A) sulphaphenazole and (B) fluconazole. Simulated plasma concentration-time profiles in control and treatment subjects are depicted as solid and dashed lines, respectively. Black lines are mean profiles of individual trials shown in colored lines. Data points in A represent mean observed data (Back et al., 1988; Lazar and Wilner, 1990).



Supplementary Figure 2. Effect of cimetidine (triangles), fluconazole (circles) and sulphaphenazole (squares) on the tolbutamide uptake in OAT2-HEK293 cells. Mean and s.d. of triplicate measurements is shown.

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