Transporter-mediated disposition, clinical pharmacokinetics and cholestatic potential of glyburide and its primary active metabolites

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Running title: Disposition of glyburide and its active metabolites

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ABBREVIATIONS:
BCRP, breast cancer resistance protein;
BSEP, bile salt export pump;
$CL_{\text{liver},u,\text{act}}$, hepatic unbound active uptake clearance;
$CL_{\text{liver},u,\text{met}}$, hepatic unbound metabolism clearance;
$CL_{\text{liver},u,bile}$, hepatic unbound biliary excretion clearance;
$CL_{\text{liver},u,\text{efflux}}$, hepatic unbound basal efflux clearance;
$CL_{u,\text{act}}$, SCHH or PHH unbound active uptake clearance;
$CL_{u,\text{pass}}$, SCHH or PHH unbound passive diffusion clearance;
$CL_{u,bile}$, SCHH unbound biliary excretion clearance;
$CL_{R}$, plasma clearance due to renal excretion;
$CL_{\text{liver},u,\text{pass}}$, hepatic unbound passive diffusion clearance;
CYP, Cytochrome P-450;
DDI, drug-drug interaction;
$F_{a}$, fraction of drug absorbed;
$F_{g}$, fraction of drug escaping gut-wall extraction;
$F_{M1}$, fraction of glyburide converted to M1;
$F_{M2b}$, fraction of glyburide converted to M2b;
$fu,c$, intracellular free fraction;
$fu,p$, fraction unbound in plasma;
HLM, human liver microsome;
HHEP, suspended human hepatocytes;
$IC_{50}$, concentration of inhibitor associated with half-maximum inhibition;
$k_{a}$, first order absorption rate;
$Kp_{uu}$, pseudo steady state unbound liver tissue to unbound plasma ratio;
M1, 4-trans-hydroxyglyburide;
M2b, 3-cis-hydroxyglyburide;
MDR, multidrug resistance protein;
MRP, multidrug resistance-associated protein;
NTCP, sodium/taurocholate co-transporting polypeptide;
OATP, organic anion transporting polypeptide;
OAT, organic anion transporter;
PHH, plated human hepatocytes;
PBPK, physiologically-based pharmacokinetic;
$R_{BP}$, blood-to-plasma ratio;
SCHH, sandwich cultured human hepatocytes;
ABSTRACT

Glyburide is widely used for the treatment of type 2 diabetes mellitus. We studied the mechanisms involved in the disposition of glyburide and its pharmacologically active hydroxy metabolites, M1 and M2b; and evaluated their clinical pharmacokinetics and the potential role in glyburide-induced cholestasis employing physiologically based pharmacokinetic (PBPK) modeling. Transport studies of parent and metabolites in human hepatocytes and transfected cell systems imply hepatic uptake mediated by organic anion transporting polypeptides. Metabolites are also subjected to basolateral and biliary efflux by P-glycoprotein, breast cancer resistance protein and multidrug resistance-associated proteins; and are also substrates to renal organic anion transporter 3. A PBPK model in combination with Bayesian approach was developed considering the identified disposition mechanisms. The model reasonably described plasma concentration-time profiles and urinary recoveries of glyburide and the metabolites, implying the role of multiple transport processes in their pharmacokinetics. Predicted free liver concentrations of the parent (~30-fold) and metabolites (~4-fold) are higher than their free plasma concentrations. Finally, all three compounds showed bile salt export pump (BSEP) inhibition in vitro; however, significant in vivo inhibition was not apparent for any compound on the basis of predicted unbound liver exposure-response effect model. Interestingly, assuming all the glyburide and metabolites reach highest possible concentrations in liver, they are unlikely to induce significant in vivo BSEP inhibition with the obtained in vitro IC50 values. In conclusion, this study demonstrates the important role of multiple drug transporters in the disposition of glyburide and its active metabolites, suggesting that variability in the function of these processes may lead to pharmacokinetic variability in the parent and the metabolites, potentially translating to pharmacodynamic variability.
INTRODUCTION

Glyburide [INN, glibenclamide], a second generation sulfonylurea, is widely used for the treatment of type II diabetes mellitus (T2DM). It is a potent stimulator of pancreatic insulin secretion, and may additionally decrease the resistance of muscle and liver to the action of insulin (Feldman, 1985). Glyburide is an extended clearance classification system (ECCS) class 1B drug (Varma et al., 2015), with evidences for active hepatic uptake via organic anion transporting polypeptide (OATP)1B1 and subsequent metabolism (Naritomi et al., 2004; Zhou et al., 2010; Varma et al., 2014). CYP2C9 is thought to be primarily responsible for the biotransformation of glyburide in vivo, with CYP3A4 playing a minor role. Several clinical studies demonstrate an association between glyburide pharmacokinetics and genetic polymorphism of CYP2C9, where the carriers of CYP2C9*3 variants show reduced clearance (Kirchheiner et al., 2002; Niemi et al., 2002; Ravindran et al., 2006). On the contrary, in vitro enzymology studies suggest CYP3A4 to be the major metabolizing enzyme, with CYP2C9 playing a minimal role (Zharikova et al., 2009; Zhou et al., 2010). Based on the in vitro studies and mechanistic modelling of clinical pharmacokinetics and drug-drug interactions (DDIs) of glyburide as a victim drug, we recently assessed the quantitative role of the transporter-enzyme (OATP1B1-CYP2C9/3A4) interplay in its hepatic clearance (Varma et al., 2014).

Glyburide is excreted as hydroxy metabolites with 50% of the dose in the urine and about 50% of the dose in the bile (Feldman, 1985). The two major circulating metabolites, 4-trans-hydroxyglyburide (M1) and 3-cis-hydroxyglyburide (M2b), were shown to have ~50-75% of the hypoglycaemic activity of the parent due to increased insulin secretion (Rydberg et al., 1994). These two major metabolites are rapidly cleared from the blood stream when dosed intravenously (Rydberg et al., 1995); and may have higher activities at low concentrations with
longer effect duration than the parent drug (Rydberg et al., 1997; Jonsson et al., 2001). However, the transport and metabolic processes involved in the disposition of these metabolites are not characterized.

Drug induced cholestasis is often noted and associated with Bile salt export pump (BSEP) inhibition (Rodrigues et al., 2014). Examples of drugs that are known to induce cholestasis and cholestatic or mixed hepatitis potentially via inhibition of BSEP include cyclosporine A, rifampicin, bosentan and glyburide. Bosentan induces a dose-dependent liver injury and increased serum bile salts and alkaline phosphatase levels in a significant proportion of treated patients (Fattinger et al., 2001). Additionally, the cholestatic potency of bosentan is elevated when combined with glyburide in humans (Mylona and Cleland, 1999; Fattinger et al., 2001). As a result, this combination is contraindicated in clinical practice (Bosentan product label). Bosentan and its metabolites and glyburide are known to inhibit BSEP, which is believed to be a major cause of the observed cholestatic findings (Fattinger et al., 2001). The contribution of glyburide major metabolites (M1 and M2b) to the glyburide-induced liver injury has not been studied.

The goals of this study were, (i) to evaluate the transport mechanisms involved in the hepatic and renal disposition of glyburide and its 4-trans-hydroxy (M1) and 3-cis-hydroxy (M2b) metabolites, (ii) to characterize the clinical pharmacokinetics of glyburide and the metabolites using a physiologically based pharmacokinetic (PBPK) model, and (iii) to quantitate the hepatic exposure of the parent and metabolites and project their quantitative role in the glyburide-induced cholestasis.
MATERIALS AND METHODS

Chemicals and Reagents.

Glyburide and rifamycin SV were purchased from Sigma-Aldrich (St. Louis, MO). 4-trans-hydroxyglyburide (M1) and 3-cis-hydroxyglyburide (M2b) were obtained from Medical Isotopes (Pelham, NH). InVitroGRO-HT, CP and HI hepatocyte media were purchased from Celsis IVT (Baltimore, MD). Cryopreserved human hepatocytes from donor HH1025 and HH1026 (Caucasian female, 59-year-old) were purchased from In vitro ADMET Laboratories, LLC. (Columbia, MD). Human Embryonic Kidney (HEK)293 cells stably transfected with human OATP1B1, OATP1B3, or OATP2B1 were generated at Pfizer Inc (Sandwich, UK). HEK293 cells transfected with OAT1 and OAT3 were obtained from Dr. Kathleen Giacomini (UCSF, CA). HEK293 cells stably transfected with NTCP were obtained from Prof. Per Artursson (Uppsala University, Sweden). Human MRP2, MRP3, MRP4, and BCRP vesicles were obtained from Corning (Corning, NY). Human BSEP and MDRI vesicles were purchased from Solvo Biotechnology (Budapest, Hungary).

In vitro transport studies

Organic anion transporting polypeptides (OATPs) and organic anion transporter (OATs) substrate assay

HEK-OATP1B1, HEK-OATP1B3, HEK-OATP2B1, HEK-OAT1, HEK-OAT2, HEK-OAT3 and HEK-mock cells were seeded at densities of 60,000-90,000 cells per well in 96-well poly-D-lysine coated plates (OATP assays) or 300,000 cells per well in 24-well plates (OAT assays) and cultured for 48 hours. For the uptake assays, the cells were washed three times with uptake
buffer (HBSS with 20mM HEPES, pH 7.4) and then incubated with uptake buffer containing test compound, at 37°C and 150 rpm. Cellular uptake was terminated by quickly washing the cells three or four times with ice-cold uptake buffer. The cells were then lysed with methanol containing internal standard and the samples were quantified by LC-MS/MS. The total cellular protein content was determined by using the Pierce BCA Protein Assay kit according to the manufacturer’s specifications.

*Multidrug resistance-associated proteins (MRPs), breast cancer resistance protein (BCRP), and multidrug resistance protein 1 (MDR1) substrate assay*

M1 and M2b were evaluated for ATP-dependent transport by MRP2, MRP3, MRP4, BCRP, and MDR1 at 1 and 10 μM concentrations. The assays were conducted in 96-well format using the rapid filtration technique. Briefly, 50 μg of membrane vesicles were incubated with test compound for 5 minutes at 37°C in the presence of 5 mM ATP or 5 mM AMP in buffer containing 2.5 mM glutathione, 70 mM KCl, 7.5 mM MgCl₂, and 50 mM 3-(N-Morpholino)propanesulfonic acid (MOPS) adjusted to pH 7.4 with Tris (MRP2 and MRP3) or 250 mM sucrose, 10 mM MgCl₂, and 10 mM Tris adjusted to pH 7.4 with HCl (MRP4, BCRP, and MDR1). The transport reaction was stopped by addition of cold stop buffer (70 mM KCl and 40 mM MOPS adjusted to pH 7.4 with Tris for MRP2 and MRP3 and 100 mM NaCl in assay buffer for MRP4, BCRP, and MDR1). Samples were transferred to 96-well glass fibre filter plates, filtered and washed four times with cold stop buffer. Accumulation of test compound in the membrane vesicles was measured by extracting the compound with methanol containing internal standard followed by LC-MS/MS analysis.

*Bile salt export pump (BSEP) inhibition assay*
Glyburide, M1 and M2b were evaluated for inhibition of BSEP-mediated ATP-dependent transport of taurocholic acid using membrane vesicles. The assay was conducted in a 384-well format at 11 concentrations per compound. The rapid filtration method was used as described previously with some modifications (Dawson et al., 2012). Briefly, 16 µg of BSEP vesicles were incubated with 2 µM taurocholic acid and test compound or dimethyl sulfoxide (DMSO) for 40 min at 25°C in buffer containing 4 mM ATP, 100 mM KNO₃, 10 mM Mg(NO₃)₂, 50 mM sucrose and 50 mM HEPES, pH 7.4. The transport reaction was stopped by addition of cold 0.5 M EDTA and cold stop buffer (10 mM Tris pH 7.4, 100 mM KNO₃, 10 mM Mg(NO₃)₂, and 50 mM sucrose). The samples were rapidly filtered and washed 3 times with ice-cold buffer. After the filter plate was dried, taurocholic acid was extracted from the vesicles by adding methanol:water (80:20) to the filter plate and its concentration was measured by LC-MS/MS.

**NTCP inhibition assay**

Glyburide, M1 and M2b were evaluated for inhibition of NTCP-mediated transport of taurocholic acid at 9 concentrations per compound. HEK-NTCP cells were seeded at a density of 60,000 cells/well in poly-D-lysine coated 96-well plates and cultured for 48 hours. The cells were washed three times with uptake buffer (HBSS with 20mM HEPES, pH 7.4) and then incubated for 4 min with uptake buffer containing 0.4 µM ³H-taurocholic acid and test compound at 37°C and 150 rpm. Uptake was stopped by the removal of transport buffer followed by three washes with ice-cold buffer. The cells were lysed with 100 µl of 10 mM Tris-HCl pH 7.5, 75 mM NaCl, 125 mM NaF, 2.5 mM EDTA, and 0.5% NP40, shaking for 45 minutes at room temperature. Accumulated radioactivity was determined by mixing 50 µl of cell lysate with 220 µl of scintillation fluid and analyzing the samples on a Perkin Elmer MicroBeta TriLux Liquid Scintillation Counter.
**Sandwich-cultured human hepatocyte (SCHH) and plated human hepatocyte (PHH) transport assays**

The SCHH methodology was described previously (Bi et al., 2006). Briefly, cryopreserved human hepatocytes were thawed and seeded in 24-well collagen coated plates using InVitroGRO-HT and InVitroGRO-CP media. The plates were overlaid with 0.25mg/ml matrigel on the second day and the cultures were maintained in InVitroGRO-HI medium. On day 5, the cells were preincubated for 10 min with or without 100 µM rifamycin SV (to determine the rates of passive diffusion and total uptake, respectively), in buffer with or without Ca^{++} (to determine biliary clearance). The reactions were terminated at specified time points by washing the cells three times with ice-cold HBSS. The cells were lysed with methanol containing internal standard and intracellular concentrations were determined by LC-MS/MS.

The plated human hepatocyte (PHH) uptake study was conducted to determine the intracellular free fraction ($f_{u,c}$) with a longer incubation time. The assay was conducted 6 hours after seeding (without overlaying with matrigel) as described for the SCHH assay with the exception of Ca^{++}-free incubations.

**LC-MS/MS analysis**

LC-MS/MS analysis was conducted for all *in vitro* samples using a Sciex 5500 or 6500 triple quadrupole tandem mass spectrometer in electrospray ionization (ESI) mode. Other instrumentation consisted of Shimadzu LC-20AD pumps and ADDA autosampler. Liquid chromatography was performed using either a Phenomenex Kinetex C18 or a Synergi Polar-RP (30x2mm), or Sprite Echelon C18 (10x2.1mm) analytical column. Analytes were eluted with a
gradient profile starting with 0.1% formic acid in water and increasing concentration of 0.1% formic acid in acetonitrile.

Mechanistic modelling of hepatocyte uptake studies

Mechanistic modelling of SCHH data to estimate in vitro unbound active uptake ($CL_{u,act}$), passive diffusion ($CL_{u,pass}$), basolateral efflux ($CL_{u,efflux}$) and biliary excretion ($CL_{u,bile}$) clearances of glyburide and metabolites M1 and M2b were performed as described previously (Kimoto et al., 2015). The detailed model structure is provided in the Supplemental Materials. The PHH data was analysed using the mechanistic model developed for SCHH, $CL_{SCHH,bile}$ set to 0. The intracellular free fraction ($f_{u,c}$) was estimated along with other parameters during PHH data fitting. $CL_{u,pass}$ and $f_{u,c}$ were assumed to be the same for the two metabolites (configurational isomers). Parameter estimation was performed using a global optimization algorithm (differential evolution) in $\log_{10}$ space, with 95% confidence intervals quantified by the residual bootstrap. All models in this study were implemented in MATLAB (version 2016a, Mathworks, Natick, MA).

PBPK modeling of glyburide and its active metabolites

A previously published PBPK model for liver transporter substrates (Li et al., 2014a) was used to model the human plasma data of glyburide and its two active metabolites. Details about the structural model are provided in the Supplemental Materials. Given that the physiochemical properties and in vitro uptake characteristics of the two metabolites estimated in SCHH were reasonably close, and that the clinical pharmacokinetic data of the two metabolites are also similar (Rydberg et al., 1995), we assumed that hepatic active uptake ($CL_{liver,u,act}$), passive diffusion ($CL_{liver,u,pass}$), and biliary excretion ($CL_{liver,u,bile}$) were similar for the two metabolites to decrease the number of fitted parameters. Biliary excretion and basolateral efflux ($CL_{liver,u,efflux}$)
of glyburide, as well as further metabolism of the metabolites, were assumed to be zero based on our in vitro studies. The unbound hepatic clearance processes, fraction of glyburide converted to M1 ($F_{M1}$, with the ratio between $F_{M1}$ and $F_{M2b}$ fixed at 5 based on clinical observation), and the absorption rate of glyburide ($k_{a,G}$) were initially estimated using the global optimization (i.e. differential evolution) in log10 space to determine one set of values that best described the pooled clinical data from six independent studies with healthy participants (Neugebauer et al., 1985; Chalk et al., 1986; Spraul et al., 1989; Rydberg et al., 1995; Niemi et al., 2001; Lilja et al., 2007). Although most data are reasonably consistent, the first hour data reported in (Spraul et al., 1989; Rydberg et al., 1995) and in (Neugebauer et al., 1985) can lead to different conclusion about glyburide tissue distribution. For this reason, we removed the first hour data reported in (Spraul et al., 1989; Rydberg et al., 1995) from fitting (assuming intravenous infusion data (Neugebauer et al., 1985) better predicts distribution volume). Data 10 hours post dose were not simulated to avoid large errors that may incur when digitizing these extreme low concentrations from non-log-transformed plots. The physiological parameters were the same as described elsewhere (Rodgers and Rowland, 2006; Li et al., 2014b).

The distributions of nine fitted parameters were estimated using a Bayesian inference, where both previous knowledge about IVIVE translation (i.e. prior distribution) and clinical data of glyburide (i.e. likelihood) contributed to parameter estimates (i.e. posterior distribution). Alternatively speaking, parameter estimation during fitting clinical data of glyburide is constrained by our best guess about IVIVE learned from other compounds. With the “middle-out” approach described previously (Li et al., 2014b), the distributions IVIVE empirical scaling factors (for SCHH, lot HH1025) have been estimated using six structurally different liver transporter substrates (i.e. $10^{1.52±0.31}$, $10^{-0.875±0.52}$, and $10^{-0.857±0.27}$, as means and standard
deviations in $\log_{10}$ space for active uptake, passive diffusion, and metabolism, unpublished internal data). Briefly, the Bayesian approach includes three steps. Step 1, we calculated the prior distribution of $CL_{\text{liver, u, act}}$, $CL_{\text{liver, u, pass}}$, and $CL_{\text{liver, u, bile}}$ as the products of the previously estimated IVIVE empirical scaling factors, the physiological scaling factor of 120 million hepatocytes per gram liver tissue, and the in vitro SCHH (or HLM) clearances (for M1 and M2b, the averaged values of the two compounds were used). As to $CL_{\text{liver, u, bile, M}}$ and $CL_{\text{liver, u, efflux, M}}$, since we had no knowledge about their IVIVE, we assumed that their prior distributions of were uniform and bounded by starting values divided and multiplied by 1000, while $k_{a,G}$ and $F_M$ were upper bounded by 10 and $5 / (5 + 1)$, respectively. Step 2, the likelihood is calculated as the sum of the squared error between pooled clinical data and simulations in $\log_{10}$ space. Step 3, the posterior distributions of the estimated parameters (i.e. parameter values specific for glyburide and its metabolites reported in this study) were generated after combining priors from step 1 and likelihood from step 2, by using an adaptive Markov chain Monte Carlo (MCMC) approach. The adaptive MCMC has been previously published (Haario et al., 2006) and implemented in the MCMC toolbox for MATLAB (http://helios.fmi.fi/~lainema/mcmc/#sec-4). The starting position of MCMC chains and initial error variance were set with the globally optimized values.
RESULTS

Substrate affinity of glyburide and metabolites to hepatic and renal transporters

Glyburide is transported by OATP1B1, with the uptake by HEK293 cells transfected with OATP1B1 being significantly higher (P<0.05) than by HEK-mock cells (Table 1). The uptake ratio at 10 µM is lower than that at 1 µM, indicating saturation at higher concentration – consistent with our previous results (Km of 2 µM) (Varma et al., 2014). Our previous studies suggested that glyburide is not transported by OATP1B3 or OATP2B1 (Varma et al., 2014). Metabolites, 4-trans-hydroxyglyburide (M1) and 3-cis-hydroxyglyburide (M2b), showed substrate affinity to all three hepatic OATP isoforms with uptake ratios generally over 20. Both metabolites were identified as substrates of the canalicular efflux transporters BCRP and MDR1, while neither metabolite showed affinity to MRP2 (Table 2). M1 was also a substrate of the basolateral efflux transporters MRP3 and MRP4, with the transport into the membrane vesicles being significantly higher (P<0.05) in the presence of ATP than in the presence of AMP. M2b, on the other hand, was not transported by MRP3 but showed significant transport by MRP4 at 10 µM substrate concentration. Neither parent nor the metabolites showed substrate affinity to renal OAT1; however, both metabolites were transported by OAT3 (Table 3).

Hepatic disposition of glyburide and metabolites using human hepatocyte assays

Primary human hepatocytes in plated culture were used to assess the involvement of active uptake in the hepatic disposition and to determine the hepatobiliary transport kinetics (Figure 1, Supplemental Figure 1). Rifamycin SV significantly inhibited the hepatic uptake of glyburide, M1 and M2b in both PHH and SCHH studies. SCHH data was simultaneously fitted to estimate in vitro transport parameters using mathematical models (Table 4). While statistically significant
active uptake was discerned for the three compounds, due to limited data points during the efflux phase estimated basal efflux and biliary excretion were associated with large uncertainties. The metabolic stabilities of M1 and M2b were assessed using HLM and suspension human hepatocytes; wherein, neither metabolite showed any measurable turnover in HLM incubations (up to 1h) with and without NADPH, and only ~15-30% depletion at the end of 5-hour incubation in human hepatocytes. M1 and M2b did not convert to each other or to the parent in these studies. Based on these findings, we assumed further metabolism of M1 and M2b to be negligible for PBPK modeling and simulations.

**Pharmacokinetic characterization of glyburide and metabolites**

The whole-body PBPK model implementing the multiple hepatic transport processes and observed renal clearance reasonably described clinical pharmacokinetics of glyburide and its metabolites (Figure 2). By combining information from *in vitro* SCHH data and IVIVE knowledge from other compounds, the Bayesian inference could provide reasonably confident parameter estimates (Table 5, Supplemental Figure 3). Due to lack of prior IVIVE knowledge, $CL_{\text{liver,u,bile,M}}$ and $CL_{\text{liver,u,efflux,M}}$ were obtained purely from fitting the clinical data. For both $CL_{\text{liver,u,bile,M}}$ and $CL_{\text{liver,u,efflux,M}}$, a value greater than 10 L·hour$^{-1}$ seemed to be necessary to describe the clinical data. To show the importance of prior information in decreasing estimation uncertainty, the estimated (posterior) parameter distributions with and without prior information are provided in Supplemental Figures 3 and 4. To understand empirical IVIVE scaling factors required to bridge *in vitro* data and *in vivo* data in this study, we calculated ratios of posterior mean of hepatic clearance processes to physiologically scaled *in vitro* clearances: 44.5 for active uptake, 0.760 for passive diffusion, and 0.192 for metabolism of glyburide, and 30.3 for active uptake and 0.0860 for passive diffusion of metabolites. With the posterior distributions of fitted
parameters, we simulated liver concentrations of glyburide, M1 and M2b. The predicted pseudo steady-state unbound liver to unbound plasma ratios ($K_{puu}$) were about 32 (95% confidence interval as 15 and 53) and 3.7 (0.092 and 39) for glyburide and metabolites, respectively.

**Inhibition of BSEP and NTCP by glyburide and metabolites**

The uptake of taurocholic acid by human BSEP and NTCP was inhibited in the presence of glyburide and metabolites in a dose-dependent manner (Figure 3). Glyburide was a more potent inhibitor against both BSEP and NTCP than its metabolites. Interestingly, although the two metabolites showed similar inhibition potencies ($IC_{50}$) against BSEP, M1 was less potent than M2b against NTCP.

**Predicted inhibition of BSEP and NTCP in human liver**

Based on the PBPK model and the posterior distributions of fitted parameters, we prospectively simulated the liver intracellular free concentrations of glyburide and metabolites after oral dosing of 10 mg glyburide per day for three days. Assuming that the inhibition follows a free concentration-direct response model (i.e. $C_{liver,free} / (C_{liver,free} + IC_{50})$), we simulated the BSEP and NTCP inhibition *in vivo* for the parent and metabolites using mean $IC_{50}$ values determined in the *in vitro* assay. The simulations are performed individually for each compound without considering interactions among inhibitors. The simulation showed that the three compounds may cause only minimal inhibition of BSEP and NTCP *in vivo* (<10% inhibition) (Figure 4 A – D). In addition, we simulated the inhibition based on total liver concentrations, where only glyburide showed a stronger inhibition – up to 25% BSEP inhibition and 75% NTCP inhibition (Figure 4 E – H).
DISCUSSION

Collective data from this study depict that glyburide is primarily cleared from the blood compartment by hepatic uptake via OATP1B1 and subsequently metabolised; while, the hepatic disposition of its active hydroxyl metabolites, M1 and M2b, are determined by hepatic uptake transporters (OATP1B1, OATP1B3 and OATP2B1) and biliary (BCRP and P-gp) and basolateral (MRP3 and MRP4) efflux pumps (Figure 5). Additionally, the metabolites are substrates to the renal transporter OAT3, which is likely mediating their significant active secretion (observed human CLR/fu,p,GFR is >25) into urine. These two metabolites of glyburide possess considerable hypoglycaemic activity at their clinically relevant plasma concentrations. Following single intravenous dosing of glyburide, M1 and M2b, separately, blood glucose and serum insulin levels are significantly changed by parent, as well as, the two metabolites (Rydberg et al., 1994). At about 10 mg oral dose of glyburide, metabolite levels are higher than those of glyburide, with high metabolite levels found at least 10-16 hours after glyburide intake (Jonsson et al., 2001) in patients with type 2 diabetes. This implies that the metabolites contribute to hypoglycaemic effect with longer effect duration than the parent itself, and may be leading to the long-lasting hypoglycaemic events noted with glyburide (Asplund et al., 1983; Rydberg et al., 1997). Overall, this study demonstrates for the first time an important role for hepatic and renal transporters in the pharmacokinetics of glyburide active metabolites; suggesting that the functional changes in these processes due to age, gender, disease, genetic variation or DDIs could significantly alter the plasma exposure of the metabolites and consequently modulate hypoglycaemic activity, which may be of clinical importance.

Mathematical modelling was employed for SCHH data to evaluate hepatocyte vectorial transport and further estimate the intrinsic transport rates to execute mechanistic PBPK modeling. SCHH
(as well as 75-min plated hepatocyte) studies suggested significant active uptake for all three compounds, which is associated with OATP substrate activity as demonstrated using transporter-transfected cells. However, SCHH could not discern statistically significant basolateral efflux or biliary clearance for any compound, leading to uncertain estimates for both parameters, although membrane vesicle studies suggested that metabolites are transported by BCRP and P-glycoprotein (biliary) and MRP3 and/or MRP4 (basolateral) efflux pumps (Table 2 and 4). This may be attributed to limited sensitivity for these possibly slow efflux processes and the experimental variability in the SCHH system. However, the pharmacokinetics of the metabolites were best described by the PBPK model with the estimated active basolateral efflux clearance (i.e. total efflux minus passive and biliary clearances) higher than the biliary clearance implying that metabolites are preferentially pumped into blood (Table 5). These hydrophilic metabolites, likely formed in the liver following glyburide dosing, are primarily eliminated by the kidneys supporting the importance of basolateral efflux in their hepatic handling (Rydberg et al., 1995). Evidently, the interplay of uptake and biliary and basolateral efflux transporters are key determinants of the pharmacokinetics of these metabolites.

Predicting or evaluating pharmacokinetics of metabolites is challenging, particularly when their disposition involve membrane transporters (Zamek-Gliszczynski et al., 2014; Kimoto et al., 2015; Templeton et al., 2016). Here, we developed a PBPK model considering hepatobiliary transport and metabolism to characterize the pharmacokinetics of glyburide and its metabolites. Our group previously estimated system-specific empirical scaling factors for hepatic active and passive transport, and metabolism by simultaneously fitting clinical observations of seven compounds with global optimization methods (Li et al., 2014b). We employed the same mechanistic model combined with a Bayesian approach, which naturally combines in vitro data,
IVIVE scaling factors determined previously (prior knowledge) and the clinical data (likelihood), to characterize the plasma pharmacokinetics of parent and metabolites, and to further effectively decrease the uncertainty in the parameter estimations and liver concentration predictions. The model described the plasma concentration-time profiles of the parent and metabolites and recovered the renal excretion profiles reasonably well (Figure 3). On the basis of the PBPK model simulations, the following mechanistic information can be derived. First, hepatic uptake transporters play a predominant role in the systemic clearance of glyburide and metabolites. Second, due to higher plasma protein binding of the parent, our PBPK model simulations suggested comparable unbound plasma concentrations and a higher unbound trough concentration of metabolites, particularly of M1, compared to glyburide (Figure 4A). These findings signify the potential contribution of metabolites to the pharmacodynamic activity, and additionally their larger role in the long-lasting hypoglycaemic effects of glyburide (Asplund et al., 1983; Rydberg et al., 1997; Jonsson et al., 2001).

The role of transporter-enzyme interplay in the hepatic clearance of glyburide can be corroborated by its clinical DDIs. For instance, coadministration of a single intravenous dose of rifampicin (OATP inhibitor) increases the plasma AUC of glyburide and consequently increases hypoglycaemic effects, while rifampicin multiple-dose oral treatment (CYP induction and OATP inhibition) shows minimal impact on glyburide exposure and pharmacodynamics (Zheng et al., 2009). Using a similar PBPK approach, we previously rationalized the magnitude change in glyburide pharmacokinetics caused by several CYP inhibitors/inducers and/or OATP inhibitors (Varma et al., 2014). The current study suggests that reduced functional activities of hepatic OATPs and/or renal OAT3 associated with DDIs, age and genetic variation could elevate, while
that of MRP3/4 may decrease, systemic exposure of M1 and M2b leading to variability in pharmacodynamic response.

Glyburide is a relatively safe drug; however, it has been implicated in occasional cases of cholestatic jaundice and hepatocellular disease, and a few cases of granulomatous hepatitis (van Basten et al., 1992; Krivoy et al., 1996; Saw et al., 1996). Additionally, bosentan-glyburide combination is contraindicated as emphasized with a black box warning on the bosentan product label. Here, we showed that glyburide major metabolites inhibit BSEP \textit{in vitro}, although the inhibition potencies were about 5-fold lower compared to the parent (Figure 3). We further evaluated the potential for glyburide and metabolites to inhibit BSEP \textit{in vivo} considering concentration-response effect on the basis of \textit{in vitro} inhibition potencies ($IC_{50}$) and the PBPK model simulated unbound hepatic concentrations (Figure 4). Although the free liver concentrations were projected to be several fold (~4-30 times) higher than plasma free concentrations, glyburide and metabolites could not produce significant inhibition of BSEP, which implies that (i) there is a potential disconnect between \textit{in vitro} and \textit{in vivo} inhibition potencies, and/or (ii) BSEP inhibition is not the major cause of glyburide-induced cholestasis with potentially other mechanisms involved. Generally, BSEP inhibition is one of numerous potential mechanisms leading to drug induced cholestasis and evaluation of this liability in isolation may not provide an overall assessment of toxicity (Rodrigues et al., 2014; Shon and Abernethy, 2014). Alternatively, Woodhead et al. suggested a relationship between maximum plasma glyburide concentration and change in bile acids exposure using DILIsym, a mechanistic model of DILI (Woodhead et al., 2014). We therefore evaluated \textit{in vivo} BSEP inhibition assuming total hepatic concentration-response effect. Under this assumption, the model predicted up to 25% BSEP inhibition by glyburide, while the metabolites showed no notable inhibition.
Moreover, considering free or total concentration-response effect, glyburide, but not the metabolites, showed notable inhibition of NTCP, which plays a key role in the hepatic uptake and regulate systemic exposure of bile acids.

\(K_{puu}\) (i.e., unbound liver to unbound plasma concentration ratio) of glyburide was predicted to be \(~32\) (95% CI = 15-53). However, predicted metabolites \(K_{puu}\) was low (~3.7) and with larger variability due to uncertainty in biliary and basolateral efflux parameter estimates. While it is not easy to verify the human liver exposure predictions due to limitations in obtaining appropriate clinical data (i.e., in vivo liver concentration of parent and metabolites), our rationale for using the current PBPK approach comes from a previous study demonstrating accurate (but may not necessarily precise) prediction of liver concentrations, when fitting plasma profile data (Li et al., 2016). Additionally, Bayesian approach was applied to improve the precision in this study. However, in Bayesian we cannot rule out the possibility that our prior IVIVE knowledge is biased resulting in underprediction of liver exposure. As such, we simulated liver exposure, BSEP and NTCP inhibition again after removing priors from MCMC. Although the new prediction bands are wider (i.e. more likely to cover real exposure, Supplemental Figure 4), the conclusion of minimal inhibition on NTCP and BSEP has not changed (Supplemental Figure 5). One could argue – if a different model structure or optimization process could conclude a much more significant inhibition of BESP? Based on simple calculations, we note that, if a 10 mg glyburide was dosed directly into a 1.25 L liver without considering transport or metabolism, the unbound glyburide concentration would be around ~0.5µM, only leading to about 6% competitive inhibition given current IC50 values (~7.5µM). The study did not investigate \(trans\)-inhibition of BSEP by the metabolites in the bile, due to challenges in simulating physiologically relevant concentrations of metabolites in the bile. However, given a significant amount of
metabolites is excreted into the bile, their concentrations in bile could be higher than concentrations in plasma and liver. Alternatively, glyburide and metabolites may have a cooperative effect leading to severer BSEP inhibition in vivo. Further understanding in the area concerning disconnect in exposure-response effect with the free drug hypothesis is warranted to rationalize the role of BSEP inhibition in glyburide-induced cholestasis.

In conclusion, we characterized the mechanisms involved in the disposition and pharmacokinetics of glyburide and its pharmacologically active metabolites. Glyburide is actively taken up by hepatocytes via OATP1B1, while both metabolites were identified as substrates of multiple hepatic and renal transporters. A PBPK model with Bayesian analysis verified the clinical relevance of these multiple transporter processes in determining parent and metabolites systemic and tissue exposure with implications for the pharmacodynamic drug response. Finally, this approach can be applied to other drug-metabolite pairs in order to predict or better characterize their pharmacokinetics/pharmacodynamics.
ACKNOWLEDGMENTS

Authors would like to thank Tristan Maurer, Hugh Barton, David Rodrigues and Larry Tremaine for valuable inputs during this work.
AUTHORSHIP CONTRIBUTIONS

Participated in research design: RL, YB, AV, MV.

Conducted experiments: YB, AV, RS, XY, LM, JL.

Contributed new reagents or analytic tools: RL.

Performed data analysis: RL, YB, AV, RS, MV.

Wrote or contributed to the writing of the manuscript: RL, YB, AV, RS, SM, XY, LM, JL, MV.
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.
Reference


Figure Captions

Figure 1. The observed and simulated intracellular accumulation of glyburide, M1 and M2b in sandwich cultured human hepatocytes (SCHH). The red circles and lines represent data and simulations in the control condition; the blue squares and lines represent data and simulations with rifamycin SV; and the black triangles and lines represent data and simulations in the absence of Ca/Mg.

Figure 2. Observed (circles) and simulated (lines) plasma concentration-time profiles and urinary recoveries of glyburide (black), M1 (blue), and M2b (red), following intravenous dosing of glyburide (A and B), oral dosing of glyburide (C and D), intravenous dosing of M1 (E and F), and intravenous dosing of M2b (G and H). Green and black in (A) indicate infusion and bolus dosing, respectively.

Figure 3. Inhibition of BSEP-mediated transport of taurocholic acid (A) and NTCP-mediated transport of taurocholic acid (B) by glyburide (circles), M1 (diamonds) and M2b (triangles). Data points are mean±s.d. (n=3). The estimated inhibition potencies, IC50 (95% CI), against BSEP were 7.45 (6.58 to 8.44) µM, 34.9 (27.1 to 44.9) µM and 36.7 (29.2 to 46.2) µM for glyburide, M1 and M2b, respectively. Similarly, the uptake of taurocholic acid by NTCP was inhibited with IC50 values of 0.5 (0.37 to 0.66) µM, 771 (314 to 1892) µM and 8.1 (5.6 to 11.8) µM by glyburide, M1 and b respectively.
Figure 4. Predicted plasma (A and E) and liver tissue concentrations (B and F) of glyburide (black), M1 (blue), and M2b (red), and fraction of inhibited BSEP (C and G) and NTCP (D and H) after 10 mg glyburide oral dosing per day for three days. Plots A-D represent unbound concentrations and inhibition based on unbound concentrations. Plots E-H represent total concentrations and inhibition based on total concentrations. The solid and dotted lines represent median predictions and 2.5 and 97.5 percentiles.

Figure 5. Schematic diagram of hepatic and renal disposition of glyburide (G) and its hydroxy metabolites. Glyburide is taken up into the hepatocytes across the sinusoidal membrane by passive diffusion and active uptake via OATP1B1. Glyburide is primarily metabolized by CYP2C9 and CYP3A4 to form M1 and M2b. Both metabolites are substrates to all three isoforms of OATPs and to biliary transporters, P-gp and BCRP. Additionally, M1 is a substrate to basolateral transporters MRP3 and MRP4, while M2b is a possible substrate to MRP4. These uptake and efflux transporters regulate the metabolites exposure in the blood and hepatocyte compartment. Additionally, M1 and M2b are actively secreted in the urine via OAT3 on the basolateral membrane of the kidney proximal tubule cells. Parent and metabolites inhibit BSEP and NTCP mediated transport of bile acids with varying inhibition potencies. The majority of the parent is metabolized to M1, M2b and other metabolites, while M1 and M2b are primarily excreted in the urine and bile.
Table 1. Hepatic OATPs mediated transport of glyburide and metabolites. Uptake by HEK cells stably transfected with human OATP1B1, OATP1B3 and OATP2B1, normalized to uptake by wild-type HEK cells, are presented as uptake ratios. All data represent mean±SD (n=3).

<table>
<thead>
<tr>
<th>Compound</th>
<th>OATP1B1</th>
<th>OATP1B3</th>
<th>OATP2B1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± S.D.</td>
<td>Mean ± S.D.</td>
<td>Mean ± S.D.</td>
</tr>
<tr>
<td>Glyburide 1 µM</td>
<td>1.9 ± 0.2</td>
<td>1.2±</td>
<td>1.3±</td>
</tr>
<tr>
<td>Glyburide 10 µM</td>
<td>1.2 ± 0.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>M1 1 µM</td>
<td>26.1 ± 1.8</td>
<td>17.5 ± 2.6</td>
<td>15.2 ± 0.3</td>
</tr>
<tr>
<td>M1 10 µM</td>
<td>46.7 ± 0.7</td>
<td>35.4 ± 3.5</td>
<td>21.4 ± 0.7</td>
</tr>
<tr>
<td>M2b 1 µM</td>
<td>28.1 ± 0.7</td>
<td>22.2 ± 6.8</td>
<td>20.2 ± 0.3</td>
</tr>
<tr>
<td>M2b 10 µM</td>
<td>39.1 ± 1.2</td>
<td>30.8 ± 0.8</td>
<td>24.8 ± 1.8</td>
</tr>
<tr>
<td>Propranolol 1 µM*</td>
<td>1.1 ± 0.1</td>
<td>1.4 ± 0.1</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>Rosuvastatin 1 µM*</td>
<td>55.3 ± 1.1</td>
<td>14.2 ± 0.5</td>
<td>36.2 ± 2.9</td>
</tr>
</tbody>
</table>

*Propranolol served as negative control and rosuvastatin served as positive control for all three OATPs.

†Taken from our previous work (Varma et al., 2014)
Table 2. Hepatic BCRP, MDR1, and MRPs mediated transport of glyburide metabolites. Uptake by inverted membrane vesicles overexpressing human BCRP, MDR1, MRP2, MRP3, and MRP4 in presence of ATP normalized to uptake in presence of AMP are presented as transport ratios. All data represent mean±SD (n=2).

<table>
<thead>
<tr>
<th>Compound</th>
<th>BCRP Transport ratio</th>
<th>MDR1 Transport ratio</th>
<th>MRP2 Transport ratio</th>
<th>MRP3 Transport ratio</th>
<th>MRP4 Transport ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± S.D.</td>
<td>Mean ± S.D.</td>
<td>Mean ± S.D.</td>
<td>Mean ± S.D.</td>
<td>Mean ± S.D.</td>
</tr>
<tr>
<td>M1 1 µM</td>
<td>7.39 ± 0.66</td>
<td>4.89 ± 0.59</td>
<td>1.17 ± 0.13</td>
<td>1.58 ± 0.18</td>
<td>2.03 ± 0.08</td>
</tr>
<tr>
<td>M1 10 µM</td>
<td>3.86 ± 0.27</td>
<td>4.30 ± 0.44</td>
<td>0.99 ± 0.11</td>
<td>1.72 ± 0.22</td>
<td>2.55 ± 0.15</td>
</tr>
<tr>
<td>M2b 1 µM</td>
<td>2.89 ± 0.31</td>
<td>3.91 ± 0.24</td>
<td>0.85 ± 0.03</td>
<td>0.97 ± 0.14</td>
<td>1.05 ± 0.04</td>
</tr>
<tr>
<td>M2b 10 µM</td>
<td>2.34 ± 0.03</td>
<td>3.65 ± 0.08</td>
<td>1.15 ± 0.16</td>
<td>0.94 ± 0.21</td>
<td>1.36 ± 0.16</td>
</tr>
<tr>
<td>Estrone sulfate 1 µM (BCRP)*</td>
<td>14.13 ± 0.08</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>N-methyl quinidine 1 µM (hMDR1)*</td>
<td>-</td>
<td>24.70 ± 1.11</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Leukotriene C4 0.1 µM (hMRPs)*</td>
<td>-</td>
<td>-</td>
<td>30.8 ± 1.68</td>
<td>18.27 ± 2.54</td>
<td>22.54 ± 0.82</td>
</tr>
</tbody>
</table>

*Probe substrate used as positive control for the respective transporter.
Table 3. Renal OATs mediated transport of glyburide and metabolites. Uptake by HEK cells stably transfected with human OAT1 and OAT3, normalized to uptake by wild-type HEK cells, are presented as uptake ratios. All data represent mean±SD (n=3).

<table>
<thead>
<tr>
<th>Compound</th>
<th>OAT1</th>
<th>OAT3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Uptake ratio</td>
<td>Uptake ratio</td>
</tr>
<tr>
<td></td>
<td>Mean ± S.D.</td>
<td>Mean ± S.D.</td>
</tr>
<tr>
<td>Glyburide 1 µM</td>
<td>1.24 ± 0.16</td>
<td>1.25 ± 0.10</td>
</tr>
<tr>
<td>Glyburide 10 µM</td>
<td>1.25 ± 0.09</td>
<td>0.97 ± 0.07</td>
</tr>
<tr>
<td>M1 1 µM</td>
<td>1.48 ± 0.20</td>
<td>6.22 ± 0.04</td>
</tr>
<tr>
<td>M1 10 µM</td>
<td>0.37 ± 0.02</td>
<td>11.50 ± 0.86</td>
</tr>
<tr>
<td>M2b 1 µM</td>
<td>0.64 ± 0.06</td>
<td>9.25 ± 0.54</td>
</tr>
<tr>
<td>M2b 10 µM</td>
<td>0.96 ± 0.63</td>
<td>13.51 ± 0.72</td>
</tr>
<tr>
<td>^3H-para aminohippuric acid (hOAT1)*</td>
<td>74.13 ± 5.69</td>
<td>-</td>
</tr>
<tr>
<td>^3H-Estrone Sulfate (hOAT3)*</td>
<td>-</td>
<td>95.04 ± 1.98</td>
</tr>
</tbody>
</table>

*Probe substrate used as positive control for the respective transporter.
Table 4. Summary of estimated parameter values for glyburide and metabolites in sandwich culture human hepatocyte model (mean and 95% confidence interval).

<table>
<thead>
<tr>
<th></th>
<th>$\text{CL}_{u,\text{act}}$</th>
<th>$\text{CL}_{u,\text{pass}}$</th>
<th>$\text{CL}_{u,\text{bile}}$</th>
<th>$\text{CL}_{u,\text{efflux}}$</th>
<th>$f_u,c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>($\mu$L·min$^{-1}$·mg protein$^{-1}$)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glyburide</td>
<td>11.8 (9.0, 16)</td>
<td>13.8 (9.3, 15)</td>
<td>1.9 (1.4×10$^{-5}$, 3.6)</td>
<td>5.5×10$^{-5}$ (7.9×10$^{-7}$, 1.7)</td>
<td>0.0301$\dagger$ (fixed)</td>
</tr>
<tr>
<td>M1</td>
<td>3.61 (2.4, 7.2)</td>
<td>0.222</td>
<td>(1.9×10$^{-7}$, 0.71)</td>
<td>0.634 (1.9×10$^{-8}$, 3.1)</td>
<td>0.579$\dagger$ (fixed)</td>
</tr>
<tr>
<td>M2b</td>
<td>7.20 (4.6, 11)</td>
<td>0.0866</td>
<td>(6.7×10$^{-8}$, 1.5)</td>
<td>1.14 (4.7×10$^{-9}$, 2.7)</td>
<td></td>
</tr>
</tbody>
</table>

*Assumed same for both metabolites in order to minimize over parameterization of the mechanistic model used to estimate in vitro transport kinetics in hepatocyte systems.

$\dagger f_u,c$ was estimated from plated human hepatocytes following longer incubations (Supplemental Figure 1).
Table 5. Summary of PBPK model parameter estimates and their 95% confidence intervals of glyburide and metabolites.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Unit</th>
<th>Glyburide</th>
<th>Trans-4-hydroxy glyburide (M1)</th>
<th>Cis-3-hydroxy glyburide (M2b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pKa</td>
<td></td>
<td>5.38</td>
<td>5.08</td>
<td>5.08</td>
</tr>
<tr>
<td>logD&lt;sub&gt;7.4&lt;/sub&gt;</td>
<td></td>
<td>2.1</td>
<td>-0.48</td>
<td>-0.43</td>
</tr>
<tr>
<td>(f_{\text{up}})</td>
<td></td>
<td>0.0028</td>
<td>(0.0026, 0.0030)</td>
<td>(0.032, 0.036)</td>
</tr>
<tr>
<td>(R_{\text{BP}})</td>
<td></td>
<td>0.571</td>
<td>(0.42, 0.45)</td>
<td>0.493</td>
</tr>
<tr>
<td>(F_d F_g)</td>
<td></td>
<td>1</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>(k_a)</td>
<td>(\text{hour}^{-1})</td>
<td>1.85</td>
<td>(1.64, 2.08)</td>
<td>0.5</td>
</tr>
<tr>
<td>(CL_R)</td>
<td>(\text{L} \cdot \text{hour}^{-1})</td>
<td>0</td>
<td>7.18</td>
<td>7.18</td>
</tr>
<tr>
<td>(CL_{\text{liver,u,pass}})</td>
<td>(\text{L} \cdot \text{hour}^{-1})</td>
<td>111</td>
<td>(24.9, 345)</td>
<td>(0.136, 11.2)</td>
</tr>
<tr>
<td>(CL_{\text{liver,u,act}})</td>
<td>(\text{L} \cdot \text{hour}^{-1})</td>
<td>5560</td>
<td>(2400, 11700)</td>
<td>(723, 6620)</td>
</tr>
<tr>
<td>(CL_{\text{liver,u,met}})</td>
<td>(\text{L} \cdot \text{hour}^{-1})</td>
<td>71.9</td>
<td>(47.3, 159)</td>
<td>0</td>
</tr>
<tr>
<td>(CL_{\text{liver,u,bile}})</td>
<td>(\text{L} \cdot \text{hour}^{-1})</td>
<td>0</td>
<td>104</td>
<td>(10.7, 4210)</td>
</tr>
<tr>
<td>(CL_{\text{liver,u,efflux}})</td>
<td>(\text{L} \cdot \text{hour}^{-1})</td>
<td>0</td>
<td>508</td>
<td>(28.5, 12000)</td>
</tr>
<tr>
<td>Metabolite (F_{M1})</td>
<td></td>
<td>0.564</td>
<td>(0.490, 0.690)</td>
<td>0.113</td>
</tr>
<tr>
<td>Metabolite (F_{M2b})</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(IC_{50,BSEP})</td>
<td>(\mu\text{M})</td>
<td>7.45</td>
<td>34.9</td>
<td>36.7</td>
</tr>
<tr>
<td>(IC_{50,NTCP})</td>
<td>(\mu\text{M})</td>
<td>0.5</td>
<td>(0.37, 0.66)</td>
<td>(314, 1892)</td>
</tr>
</tbody>
</table>

1. \(pKa\) and \(\log D_{7.4}\) values are generated in house for glyburide, and predicted using ACD/Labs (Ontario, Canada) for metabolites.
2. The fraction absorbed and escaped from intestinal metabolism \((F_d F_g)\) is fixed at 1 for glyburide based on clinical observations. Due to lack of data, \(F_d\) for metabolites is fixed at 0.5 considering their low-to-moderate passive permeability, while \(F_g\) is assumed to be for their low metabolism.
3. The absorption rate of metabolites is fixed at 0.5 arbitrarily due to limited data. This involves reabsorption of metabolites from the intestine following biliary secretion (entero-hepatic recycling).
4. The plasma renal clearance \((CL_R)\) is directly determined from the ratio of amount of urinary excretion and plasma exposure. As such, its variability is more of the individual variability (not addressed here) rather than numerical uncertainty.
5. The 95% confidence intervals are approximated as 2.5 and 97.5 percentiles of values generated in Bayesian inference. M1 and M2b are assumed to share the same hepatic uptake characteristics as indicated in the text.
6. $F_{M2b}$ is fixed as one fifth of $F_{M1}$ based on clinical observations. About 25-30% and 5-6% of M1 and M2b, respectively, are recovered in the urine after glyburide IV or oral dosing, while equal mounts (60%) of M1 and M2b are recovered in the urine following M1 and M2b IV dose (Rydberg et al., 1995).

7. $CL_{\text{liver, bile}}$ and $CL_{\text{liver, efllux}}$ are correlated by fitting clinical data, while there is no prior information about these two parameters, as such, large uncertainty are associated with them.
Figure 1. The observed and simulated intracellular accumulation of glyburide, M1 and M2b in the sandwich cultured human hepatocyte (SCHH) uptake studies. The red circles and lines represent data and simulations in the control condition; the blue squares and lines represent data and simulations with RSV; and the black triangles and lines represent data and simulations in the absence of Ca/Mg.
Figure 2. Observed (circles) and simulated (lines) clinical pharmacokinetic time courses of glyburide (black), M1 (blue), and M2b (red), following intravenous dosing of glyburide (A) and (B), oral dosing of glyburide (C) and (D), intravenous dosing of M1 (E) and (F), and intravenous dosing of M2b (G) and (H). Green and black in (A) indicate infusion and bolus dosing.
Figure 3. Inhibition of hBSEP-mediated transport of taurocholic acid (A) and hNTCP-mediated transport of taurocholic acid (B) by glyburide (circles), M1 (diamonds) and M2b (triangles). Data points are mean ± s.d. (n=3). The estimated inhibition potency, IC50 (95% CI), against BSEP are 7.45 (6.58 to 8.44) µM, 34.9 (27.1 to 44.9) µM and 36.7 (29.2 to 46.2) µM for glyburide, M1 and M2a, respectively. Similarly, the uptake of taurocholic acid by NTCP was inhibited with IC50 values of 0.5 (0.37 to 0.66) µM, 771 (314 to 1892) µM and 8.1 (5.6 to 11.8) µM by glyburide, M1 and M2a, respectively.
Figure 4. Predicted plasma (A and E) and unbound liver tissue concentrations (B and F) of glyburide (black), M1 (blue), and M2b (red), and fraction of inhibited BSEP (C and G) and NTCP (D and H) after 10 mg glyburide oral dosing per day for three days. Plots A, B, C, and D represent unbound concentrations and inhibition based on unbound concentrations. Plots E, F, G, and H represent total concentrations and inhibition based on total concentrations. The solid and dotted lines represent median predictions and 2.5 and 97.5 percentiles.
Figure 5.

- **M1** = ~40% of IV dose
- **M2b** = ~40% of IV dose

- **M1** = ~60% of IV dose
- **M2b** = ~60% of IV dose