Transporter-Mediated Disposition, Clinical Pharmacokinetics and Cholestatic Potential of Glyburide and Its Primary Active Metabolites[§]

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ABSTRACT

Glyburide is widely used for the treatment of type 2 diabetes. 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 substrates to renal organic anion transporter 3. A PBPK model in combination with a 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) were higher than their free plasma concentrations. Finally, all three compounds showed bile salt export pump inhibition *in vitro*; however, significant *in vivo* inhibition was not apparent for any compound on the basis of a predicted unbound liver exposure-response effect model using measured *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 (also called glibenclamide), a second-generation sulfonylurea, is widely used for the treatment of type 2 diabetes. 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 class 1B drug (Varma et al., 2015) with evidence 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*

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ABBREVIATIONS: BCRP, breast cancer resistance protein; BSEP, bile salt export pump; CI, confidence interval; $CL_{liver,u,act}$, hepatic unbound active uptake clearance; $CL_{liver,u,bile}$, hepatic unbound biliary excretion clearance; $CL_{liver,u,efflux}$, hepatic unbound basal efflux clearance; $CL_{liver,u,pass}$, hepatic unbound passive diffusion clearance; CL_R , plasma renal clearance; $CL_{u,act}$, sandwich-cultured human hepatocyte or plated human hepatocyte unbound active uptake clearance; $CL_{u,bile}$, sandwich-cultured human hepatocyte unbound biliary excretion clearance; $CL_{u,pass}$, sandwich-cultured human hepatocyte or plated human hepatocyte unbound passive diffusion clearance; 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; $f_{u,c}$, intracellular free fraction; $f_{u,p}$, fraction unbound in plasma; HBSS, Hanks' balanced salt solution; HEK, human embryonic kidney; HLM, human liver microsome; IVIVE, *in vitro* to *in vivo* extrapolation; $k_{a,G}$, absorption rate of glyburide; Kp_{uu} , pseudo-steady-state unbound liver tissue-to-unbound plasma ratio; LC-MS/MS, liquid chromatography tandem mass spectroscopy; M1, 4-*trans*-hydroxyglyburide; M2b, 3-*cis*-hydroxyglyburide; MCMC, Markov chain Monte Carlo; MDR1, multidrug resistance protein 1; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MRP, multidrug resistance-associated protein; NTCP, sodium/taurocholate cotransporting polypeptide; OAT, organic anion transporter; OATP, organic anion transporting polypeptide; P-gp, P-glycoprotein; PHH, plated human hepatocyte; PBPK, physiologically based pharmacokinetic; SCHH, sandwich-cultured human hepatocyte.

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 modeling 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 hypoglycemic activity of the parent due to increased insulin secretion (Rydberg et al., 1994). These two major metabolites are rapidly cleared from the bloodstream when dosed intravenously (Rydberg et al., 1995) and may have higher activity 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 1) to evaluate the transport mechanisms involved in the hepatic and renal disposition of glyburide and its M1 and M2b metabolites, 2) to characterize the clinical pharmacokinetics of glyburide and the metabolites using a physiologically based pharmacokinetic (PBPK) model, and 3) 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). M1 and 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 years 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 organic anion transporter (OAT) 1 and OAT3 were obtained from Dr. Kathleen Giacomini (University of California, San Francisco, San Francisco, CA). HEK293 cells stably transfected with NTCP were obtained from Professor Per Artursson (Uppsala University, Uppsala, Sweden). Human multidrug resistance-associated protein (MRP) 2, MRP3, MRP4, and breast cancer resistance protein (BCRP) vesicles were obtained from Corning (Corning, NY). Human BSEP and multidrug resistance protein 1 (MDR1) vesicles were purchased from Solvo Biotechnology (Budapest, Hungary).

In Vitro Transport Studies

OATPs and OAT 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/well in 96-well poly-D-lysine-coated plates (OATP assays) or 300,000 cells/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 [Hanks' balanced salt solution (HBSS) with 20 mM 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 liquid chromatography tandem mass spectroscopy (LC-MS/MS). The total cellular protein content was determined by using the Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA) according to the manufacturer specifications.

MRPs, BCRP, and MDR1 Substrate Assay. M1 and M2b were evaluated for ATP-dependent transport by MRP2, MRP3, MRP4, BCRP, and MDR1 at 1 and 10 μ M concentrations. The assays were conducted in 96-well format using the rapid filtration technique. Briefly, 50 μ g 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 MOPS [3-(*N*-morpholino)propanesulfonic acid] 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 the 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 fiber filter plates, filtered, and washed four times with cold stop buffer. Accumulation of the test compound in the membrane vesicles was measured by extracting the compound with methanol containing internal standard followed by LC-MS/MS analysis.

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 μ g of BSEP vesicles were incubated with 2 μ M taurocholic acid and test compound or dimethylsulfoxide for 40 minutes 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 the 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 three times with ice-cold buffer. After the filter plate was dried, taurocholic acid was extracted from the vesicles by adding methanol and water (80:20 ratio) 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 nine 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 20 mM HEPES, pH 7.4) and then incubated for 4 minutes with uptake buffer containing 0.4 μ M ³H-taurocholic acid and test compound at 37°C and 150 rpm. Uptake was stopped by the removal

TABLE 1

Hepatic OATP-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 the mean $\pm$ SD (*n* = 3).

Compound	OATP1B1	OATP1B3	OATP2B1
Glyburide 1 μ M	1.9 $\pm$ 0.2	1.2 ^a	1.3 ^a
Glyburide 10 μ M	1.2 $\pm$ 0.1		
M1 1 μ M	26.1 $\pm$ 1.8	17.5 $\pm$ 2.6	15.2 $\pm$ 0.3
M1 10 μ M	46.7 $\pm$ 0.7	35.4 $\pm$ 3.5	21.4 $\pm$ 0.7
M2b 1 μ M	28.1 $\pm$ 0.7	22.2 $\pm$ 6.8	20.2 $\pm$ 0.3
M2b 10 μ M	39.1 $\pm$ 1.2	30.8 $\pm$ 0.8	24.8 $\pm$ 1.8
Propranolol 1 μ M ^b	1.1 $\pm$ 0.1	1.4 $\pm$ 0.1	1.3 $\pm$ 0.1
Rosuvastatin 1 μ M ^b	55.3 $\pm$ 1.1	14.2 $\pm$ 0.5	36.2 $\pm$ 2.9

^aTaken from our previous work (Varma et al., 2014).

^bPropranolol served as negative control and rosuvastatin served as positive control for all three OATPs.

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 the presence of ATP normalized to uptake in presence of AMP are presented as transport ratios. All data represent the mean $\pm$ SD ($n = 2$).

Compound	BCRP	MDR1	MRP2	MRP3	MRP4
M1 1 μ M	7.39 $\pm$ 0.66	4.89 $\pm$ 0.59	1.17 $\pm$ 0.13	1.58 $\pm$ 0.18	2.03 $\pm$ 0.08
M1 10 μ M	3.86 $\pm$ 0.27	4.30 $\pm$ 0.44	0.99 $\pm$ 0.11	1.72 $\pm$ 0.22	2.55 $\pm$ 0.15
M2b 1 μ M	2.89 $\pm$ 0.31	3.91 $\pm$ 0.24	0.85 $\pm$ 0.03	0.97 $\pm$ 0.14	1.05 $\pm$ 0.04
M2b 10 μ M	2.34 $\pm$ 0.03	3.65 $\pm$ 0.08	1.15 $\pm$ 0.16	0.94 $\pm$ 0.21	1.36 $\pm$ 0.16
Estrone sulfate 1 μ M (BCRP) ^a	14.13 $\pm$ 0.08				
N-methyl quinidine 1 μ M (hMDR1) ^a		24.70 $\pm$ 1.11			
Leukotriene C4 0.1 μ M (hMRP) ^a			30.8 $\pm$ 1.68	18.27 $\pm$ 2.54	22.54 $\pm$ 0.82

hMDR1, human MDR1; hMRP, human MRP.

^aProbe substrate was used as a positive control for the respective transporter.

of transport buffer followed by three washes with ice-cold buffer. The cells were lysed with 100 μ l of 10 mM Tris-HCL (pH 7.5), 75 mM NaCl, 125 mM NaF, 2.5 mM EDTA, and 0.5% NP40, and shaken for 45 minutes at room temperature. Accumulated radioactivity was determined by mixing 50 μ l of cell lysate with 220 μ l of scintillation fluid and analyzing the samples on a PerkinElmer (Waltham, MA) MicroBeta TriLux Liquid Scintillation Counter.

Sandwich-Cultured Human Hepatocyte and Plated Human Hepatocyte Transport Assays. The sandwich-cultured human hepatocyte (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 0.25 mg/ml Matrigel (Corning) on the second day, and the cultures were maintained in InVitroGRO-HI medium. On day 5, the cells were preincubated for 10 minutes with or without 100 μ M 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 (Framingham, MA) 5500 or 6500 Triple Quadrupole Tandem Mass Spectrometer in electrospray ionization mode. Other instrumentation consisted of Shimadzu (Kyoto, Japan) LC-20AD Solvent Delivery Units (pumps) and ADDA autosampler. Liquid chromatography was performed using a Phenomenex (Torrance, CA) Kinetex C18 or Synergi Polar-RP (30 $\times$ 2 mm) column, or a Sprite Echelon C18 column (10 $\times$ 2.1 mm) (ANALYTICAL Sales and Services, Flanders, NJ). 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.

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 the mean $\pm$ SD ($n = 3$).

Compound	OAT1	OAT3
Glyburide 1 μ M	1.24 $\pm$ 0.16	1.25 $\pm$ 0.10
Glyburide 10 μ M	1.25 $\pm$ 0.09	0.97 $\pm$ 0.07
M1 1 μ M	1.48 $\pm$ 0.20	6.22 $\pm$ 0.04
M1 10 μ M	0.37 $\pm$ 0.02	11.50 $\pm$ 0.86
M2b 1 μ M	0.64 $\pm$ 0.06	9.25 $\pm$ 0.54
M2b 10 μ M	0.96 $\pm$ 0.63	13.51 $\pm$ 0.72
³ H-para-aminohippuric acid (hOAT1) ^a	74.13 $\pm$ 5.69	
³ H-estrone sulfate (hOAT3) ^a		95.04 $\pm$ 1.98

hOAT, human OAT.

^aProbe substrate used as positive control for the respective transporter.

Mechanistic Modeling of Hepatocyte Uptake Studies. Mechanistic modeling of SCHH data to estimate in vitro unbound active uptake clearance ($CL_{u,act}$), unbound passive diffusion clearance ($CL_{u,pass}$), unbound basolateral efflux clearance ($CL_{u,efflux}$), and unbound biliary excretion clearance ($CL_{u,bile}$) 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 Material. The PHH data were analyzed using the mechanistic model developed for SCHH, $CL_{u,bile}$ set to 0. The $f_{u,c}$ 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 log₁₀ space, with 95% confidence intervals (CIs) 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 for glyburide and its two active metabolites. Details about the structural model are provided in the Supplemental Material. 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, the 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 the studies by Spraul et al. (1989) and Rydberg et al. (1995), and Neugebauer et al. (1985) can lead to different conclusions about glyburide tissue distribution. For this reason, we removed the first-hour data reported in the studies by Spraul et al., 1989) and Rydberg et al. (1995) from fitting [assuming that intravenous infusion data (Neugebauer et al., 1985) better predicts distribution volume]. Data 10 hours postdose were not simulated to avoid large errors that may incur when digitizing these extreme low concentrations from non-log-transformed plots. The physiologic parameters were the same as described previously (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 in vitro to in vivo extrapolation (IVIVE) translation (i.e., prior distribution) and clinical data for glyburide (i.e., likelihood) contributed to parameter estimates (i.e., posterior distribution). Alternatively, parameter estimation during fitting of clinical data for 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

Fig. 1. The observed and simulated intracellular accumulation of glyburide, M1, and M2b in 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. Plots represent, time course of intracellular amount of glyburide (A), M1 (D) and M2b (G) in the uptake studies; intracellular amount of glyburide (B), M1 (E) and M2b (H) in the efflux studies; and extracellular amount of glyburide (C), M1 (F) and M2b (I) in the efflux studies.

estimated using six structurally different liver transporter substrates (i.e., $10^{1.52 \pm 0.31}$, $10^{-0.875 \pm 0.52}$, and $10^{-0.857 \pm 0.27}$, as means and S.D.s in $\log_{10}$ space for active uptake, passive diffusion, and metabolism; unpublished internal data). Briefly, the Bayesian approach includes three steps. In step 1, we calculated the prior distribution of $CL_{liver,u,act}$, $CL_{liver,u,pass}$, and $CL_{liver,u,bile}$ as the products of the previously estimated IVIVE empirical scaling factors, the physiologic scaling factor of 120 million hepatocytes per gram of liver tissue, and the in vitro SCHH (or HLM) clearances (for M1 and M2b, the averaged values of the two compounds

were used). For the metabolites intrinsic biliary and basolateral efflux clearances, $CL_{liver,u,bile,M}$ and $CL_{liver,u,efflux,M}$, since we had no knowledge about their IVIVE, we assumed that their prior distributions were uniform and bounded by starting values divided and multiplied by 1000, whereas $k_{a,G}$ and F_M were upper bounded by 10 and $5/(5+1)$, respectively. In step 2, the likelihood is calculated as the sum of the squared error between pooled clinical data and simulations in $\log_{10}$ space. In step 3, the posterior distributions of the estimated parameters (i.e., parameter values specific for glyburide and its metabolites reported in this study) were

TABLE 4
Summary of estimated parameter values for glyburide and metabolites in the SCHH model (mean and 95% CI)

	$CL_{u,act}$	$CL_{u,pass}$	$CL_{u,bile}$	$CL_{u,efflux}$	$f_{u,c}$
			$\mu\text{L} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$		
Glyburide	11.8 (9.0–16)	13.8 (9.3–15)	1.9 (1.4×10^{-5} to 3.6)	5.5×10^{-5} (7.9×10^{-7} to 1.7)	0.0301 ^a (fixed)
M1	3.61 (2.4–7.2)	0.661 ^b (0.33–2.9)	0.222 (1.9×10^{-9} to 0.71)	0.634 (1.9×10^{-8} to 3.1)	0.579 ^{a,b} (fixed)
M2b	7.20 (4.6–11)		0.0866 (6.7×10^{-8} to 1.5)	1.14 (4.7×10^{-9} to 2.7)	

^a $f_{u,c}$ was estimated from PHHs after longer incubations (Supplemental Fig. 1).

^bAssumed to be the same for both metabolites to minimize overparameterization of the mechanistic model used to estimate in vitro transport kinetics in hepatocyte systems.

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 published previously (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 a higher concentration, which is consistent with our previous results (Michaelis-Menten constant, 2 μM) (Varma et al., 2014). Our previous studies (Varma et al., 2014) suggested that glyburide is not transported by OATP1B3 or OATP2B1. Metabolites M1 and 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, although 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 (Fig. 1; Supplemental Fig. 1). Rifamycin SV significantly inhibited the hepatic uptake of glyburide, M1, and M2b in both PHH and SCHH studies. SCHH data were 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 1 hour) with and without NADPH and only ~15–30% depletion at the end of 5 hours of 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 (CL_R) reasonably

described clinical pharmacokinetics of glyburide and its metabolites (Fig. 2). By combining information from in vitro SCHH data and IVIVE knowledge from other compounds, the Bayesian inference could provide reasonably confident parameter estimates (Supplemental Fig. 3; Table 5). Due to a lack of prior IVIVE knowledge, $CL_{liver,u,bile,M}$ and $CL_{liver,u,efflux,M}$ were obtained purely from fitting the clinical data. For both $CL_{liver,u,bile,M}$ and $CL_{liver,u,efflux,M}$, a value greater than 10 l/hour 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 Figs. 3 and 4. To understand the empirical IVIVE scaling factors required to bridge in vitro data and in vivo data in this study, we calculated the following ratios of posterior mean of hepatic clearance processes to physiologically scaled in vitro clearances: 44.5 for active uptake of glyburide; 0.760 for passive diffusion of glyburide; and 0.192 for metabolism of glyburide; 30.3 for active uptake of metabolites; 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 ($K_{p,uu}$) were about 32 (95% CI, 15–53) and 3.7 (95% CI, 0.092–39) 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 (Fig. 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 glyburide 10 mg/day for 3 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 were performed individually for each compound without considering the interactions among inhibitors. The simulation showed that the three compounds may cause only minimal inhibition of BSEP and NTCP in vivo (<10% inhibition) (Fig. 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 (Fig. 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 metabolized, whereas the hepatic disposition of its

Fig. 2. Observed (circles) and simulated (lines) plasma concentration-time profiles and urinary recoveries of glyburide (black), M1 (blue), and M2b (red) after 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.

active hydroxyl metabolites, M1 and M2b, are determined by hepatic uptake transporters (OATP1B1, OATP1B3 and OATP2B1) and biliary [BCRP and P-glycoprotein (P-gp)] and basolateral (MRP3 and MRP4) efflux pumps (Fig. 5). Additionally, the metabolites are substrates to the renal transporter OAT3, which is likely mediating their significant active secretion (observed human $CL_{R/f_{u,p}}$ /GFR is >25) into urine. These two metabolites of glyburide possess considerable hypoglycemic activity at

their clinically relevant plasma concentrations. After single intravenous dosing of glyburide, M1, and M2b, separately, blood glucose and serum insulin levels are significantly changed by the parent as well as the two metabolites (Rydberg et al., 1994). At an oral dose of glyburide of about 10 mg, 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

TABLE 5
Summary of PBPK model parameter estimates and their 95% CIs of glyburide and metabolites

Parameter	Unit	Value		
		Glyburide	M1	M2b
pK_a^a		5.38	5.08	5.08
$\log D_{7.4}^a$		2.1	-0.48	-0.43
$f_{u,p}$		0.0028 (0.0026–0.0030)	0.034 (0.032–0.036)	0.024 (0.023–0.025)
$R_{B/P}$		0.571	0.437 (0.42–0.45)	0.493 (0.45–0.54)
$F_a F_g^b$		1	0.5	0.5
$k_a^{c,d}$	Hour	1.85 (1.64–2.08)	0.5	0.5
$CL_R^{d,e}$	l/hour	0	7.18	7.18
$CL_{liver,u,pass}^d$	l/hour	111 (24.9–345)	0.602 (0.136–11.2)	0.602 (0.136–11.2)
$CL_{liver,u,act}^d$	l/hour	5560 (2400–11,700)	1730 (723–6620)	1730 (723–6620)
$CL_{liver,u,met}^d$	l/hour	71.9 (47.3–159)	0	0
$CL_{liver,u,bile}^{d,f}$	l/hour	0	104 (10.7–4210)	104 (10.7–4210)
$CL_{liver,u,efflux}^{d,f}$	l/hour	0	508 (28.5–12,000)	508 (28.5–12,000)
Metabolite F_{M1}^d			0.564 (0.490–0.690)	
Metabolite F_{M2b}^g				0.113
$IC_{50,BSEP}$	μM	7.45 (6.58–8.44)	34.9 (27.1–44.9)	36.7 (29.2–46.2)
$IC_{50,NTCP}$	μM	0.5 (0.37–0.66)	771 (314–1892)	8.1 (5.6–11.8)

$CL_{liver,u,met}$, hepatic unbound metabolism clearance; k_a , first-order absorption rate; $R_{B/P}$, blood-to-plasma ratio.

^a pK_a and $\log D_{7.4}$ values are generated in-house for glyburide and are predicted using ACD/Labs (Toronto, Ontario, Canada) for metabolites.

^bThe fraction absorbed and escaped from intestinal metabolism ($F_a F_g$) is fixed at 1 for glyburide based on clinical observations. Due to lack of data, the F_a for metabolites is fixed at 0.5 considering their low-to-moderate passive permeability, whereas F_g is assumed to be for their low metabolism.

^cThe absorption rate of metabolites is fixed at 0.5 arbitrarily due to limited data. This involves reabsorption of metabolites from the intestine after biliary secretion (enterohepatic recycling).

^dThe 95% CIs 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.

^eThe 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) than numerical uncertainty.

^f $CL_{liver,u,bile}$ and $CL_{liver,u,efflux}$ are correlated by fitting clinical data, whereas there is no prior information about these two parameters, as such, and the large uncertainty associated with them.

^g 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 intravenous glyburide or oral dosing, whereas equal amounts (60%) of M1 and M2b are recovered in the urine after intravenous M1 and M2b dosing (Rydberg et al., 1995).

the metabolites contribute to the hypoglycemic effect with a longer effect duration than the parent itself and that they may be leading to the long-lasting hypoglycemic 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, sex, disease, genetic variation, or DDIs could significantly alter the plasma exposure of the

metabolites and consequently modulate hypoglycemic activity, which may be of clinical importance.

Mathematical modeling 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-minute 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,

Fig. 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 the mean $\pm$ S.D. ($n = 3$). The estimated inhibition potencies [IC_{50} (95% CI)] against BSEP were 7.45 (6.58–8.44) μM , 34.9 (27.1–44.9) μM , and 36.7 (29.2–46.2) μM for glyburide, M1, and M2b, respectively. Similarly, the uptake of taurocholic acid by NTCP was inhibited with IC_{50} values of 0.5 (0.37–0.66) μM , 771 (314–1892) μM , and 8.1 (5.6–11.8) μM by glyburide, M1, and M2b, respectively.

Fig. 4. Predicted plasma (A and E) and liver tissue concentrations (B and F) of glyburide (black), M1 (blue), and M2b (red) and the fraction of inhibited BSEP (C and G) and NTCP (D and H) after 10-mg glyburide oral dosing per day for 3 days. Plots (A–D) represents 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.

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-gp (biliary) and MRP3 and/or MRP4 (basolateral) efflux pumps (Tables 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, which likely are formed in the liver after 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 the pharmacokinetics of metabolites is challenging, particularly when their disposition involves membrane transporters (Zamek-Gliszczyński 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

Fig. 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 OATP and to biliary transporters P-gp and BCRP. Additionally, M1 is a substrate to basolateral transporters MRP3 and MRP4, whereas M2b is a possible substrate to MRP4. These uptake and efflux transporters regulate the metabolite 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, whereas M1 and M2b are primarily excreted in the urine and bile.

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 (Fig. 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 with glyburide (Fig. 4A). These findings signify the potential contribution of metabolites to the pharmacodynamic activity and, additionally, their larger role in the long-lasting hypoglycemic 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 area under the curve of glyburide and consequently increases hypoglycemic effects, whereas rifampicin multiple-dose oral treatment (cytochrome P450 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 of change in glyburide pharmacokinetics caused by several cytochrome P450 inhibitors/inducers and/or OATP inhibitors (Varma et al., 2014). The current study suggests that the reduced functional activities of hepatic OATPs and/or renal OAT3 associated with DDIs, age, and genetic variation could increase, whereas those 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 in a few cases of granulomatous hepatitis (van Basten et al., 1992; Krivoy et al., 1996; Saw et al., 1996). Additionally, the 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 in vitro, although the inhibition potencies were about 5-fold lower compared with the parent (Fig. 3). We further evaluated the potential for glyburide and metabolites to inhibit BSEP in vivo considering the concentration-response effect on the basis of in vitro inhibition potencies (IC_{50}), and the PBPK model simulated unbound hepatic concentrations (Fig. 4). Although the free liver concentrations were projected to be severalfold (~4–30 times) higher than plasma free concentrations, glyburide and metabolites could not produce significant inhibition of BSEP, which implies that 1) there is a potential disconnect between in vitro and in vivo inhibition potencies and/or 2) BSEP inhibition is not the major cause of glyburide-induced cholestasis, with other mechanisms potentially 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. (2014) suggested a relationship between maximum plasma glyburide concentration and the change in bile acids exposure using DILIsym, a mechanistic model of drug-induced liver injury (Woodhead et al., 2014). We therefore evaluated in vivo BSEP inhibition assuming total hepatic concentration-response effect. Under this assumption, the model predicted up to 25% BSEP inhibition by glyburide, whereas the metabolites showed no notable inhibition (Fig. 4C). Moreover, considering the 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 regulates systemic exposure of bile acids.

The $K_{p_{uu}}$ of glyburide was predicted to be ~ 32 (95% CI = 15–53). However, the predicted metabolite $K_{p_{uu}}$ was low (~ 3.7) and with larger variability due to uncertainty in biliary and basolateral efflux parameter estimates. Although 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 (Li et al., 2016) demonstrating an accurate (but not necessarily precise) prediction of liver concentrations when fitting plasma profile data. Additionally, a 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 the 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 Fig. 4), the conclusion of minimal inhibition on NTCP and BSEP has not changed (Supplemental Fig. 5). One could argue whether a different model structure or optimization process could conclude a much more significant inhibition of BSEP. Based on simple calculations, we note that, if a 10-mg glyburide dose was injected directly into a 1.25-l liver without considering transport or metabolism, the unbound glyburide concentration would be $\sim 0.5 \mu\text{M}$, leading only to about a 6% competitive inhibition given current IC_{50} values ($\sim 7.5 \mu\text{M}$). The study did not investigate transinhibition of BSEP by the metabolites in the bile because of challenges in simulating physiologically relevant concentrations of metabolites in the bile. However, given that a significant amount of metabolites is excreted into the bile, their concentrations in bile could be higher than their concentrations in plasma and liver. Alternatively, glyburide and metabolites may have a cooperative effect leading to more severe 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 as well as its pharmacologically active metabolites. Glyburide is actively taken up by hepatocytes via OATP1B1, whereas 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 systemic and tissue exposure of parent and metabolites with implications for the pharmacodynamic drug response. Finally, this approach can be applied to other drug-metabolite pairs to predict or better characterize their pharmacokinetics/pharmacodynamics.

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Authorship Contributions

Participated in research design: Li, Bi, Vildhede, Scialis and Varma.

Conducted experiments: Bi, Vildhede, Scialis, Yang, Marroquin, and Lin.

Contributed new reagents or analytic tools: Li.

Performed data analysis: Li, Bi, Vildhede, Scialis, and Varma.

Wrote or contributed to the writing of the manuscript: Li, Bi, Vildhede, Scialis, Mathialagan, Yang, Marroquin, Lin, and Varma.

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