



Application of a Mechanistic Model to Evaluate Putative Mechanisms of Tolvaptan Drug-Induced Liver Injury and Identify Patient Susceptibility Factors

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ABSTRACT

Tolvaptan is a selective vasopressin V2 receptor antagonist, approved in several countries for the treatment of hyponatremia and autosomal dominant polycystic kidney disease (ADPKD). No liver injury has been observed with tolvaptan treatment in healthy subjects and in non-ADPKD indications, but ADPKD clinical trials showed evidence of drug-induced liver injury (DILI). Although all DILI events resolved, additional monitoring in tolvaptan-treated ADPKD patients is required. *In vitro* assays identified alterations in bile acid disposition and inhibition of mitochondrial respiration as potential mechanisms underlying tolvaptan hepatotoxicity. This report details the application of DILIsym software to determine whether these mechanisms could account for the liver safety profile of tolvaptan observed in ADPKD clinical trials. DILIsym simulations included physiologically based pharmacokinetic estimates of hepatic exposure for tolvaptan and 2 metabolites, and their effects on hepatocyte bile acid transporters and mitochondrial respiration. The frequency of predicted alanine aminotransferase (ALT) elevations, following simulated 90/30 mg split daily dosing, was 7.9% compared with clinical observations of 4.4% in ADPKD trials. Toxicity was multifactorial as inhibition of bile acid transporters and mitochondrial respiration contributed to the simulated DILI. Furthermore, simulation analysis identified both pre-treatment risk factors and on-treatment biomarkers predictive of simulated DILI. The simulations demonstrated that *in vivo* hepatic exposure to tolvaptan and the DM-4103 metabolite, combined with these 2 mechanisms of toxicity, were sufficient to account for the initiation of tolvaptan-mediated DILI. Identification of putative risk-factors and potential novel biomarkers provided insight for the development of mechanism-based tolvaptan risk-mitigation strategies.

Key words: tolvaptan; ADPKD; DILI; DILIsym; quantitative systems pharmacology modeling.

Tolvaptan is a selective vasopressin V_2 receptor antagonist that blocks arginine vasopressin (AVP) binding in the distal portions of the nephron, abrogating AVP's antidiuretic activity, and inducing water diuresis, without concomitant electrolyte depletion. Tolvaptan has been approved in several countries for the treatment of hyponatremia and more recently, in Japan, Europe, Canada, and Korea for the treatment of autosomal dominant polycystic kidney disease (ADPKD).

Autosomal dominant polycystic kidney disease (ADPKD) is a genetic disease resulting from mutation in either the PKD1 or PKD2 genes. ADPKD is characterized by the development of renal cysts, which can ultimately lead to renal failure. Tolvaptan has been shown to slow ADPKD disease progression (Torres et al., 2012); however, liver safety signals were observed in the pivotal tolvaptan ADPKD clinical trial (Watkins et al., 2015). Liver signals have not been observed in other patient populations treated with tolvaptan. Understanding the mechanistic basis of tolvaptan-mediated liver injury could support the development of mechanism-based biomarkers for patient susceptibility and/or improved safety monitoring.

Drug-induced liver injury (DILI) is an important safety concern in drug development that can lead to termination of development programs, changes to the drug label, and market withdrawal (Hunter et al., 1999; Kohloser et al., 2000; Lasser et al., 2002; Guengerich, 2011). Whereas the etiology of DILI events is rarely known, several putative mechanisms have been identified. For example, it has been demonstrated that compounds that inhibit the bile salt export pump (BSEP), a hepatic bile acid efflux transporter, are disproportionately represented amongst compounds with DILI liability (Morgan et al., 2010). The association between compounds that inhibit hepatic transporters and liver safety liabilities is strengthened further by considering compound inhibition of multidrug resistance-associated proteins (MRPs) (Morgan et al., 2013). Whereas not the primary efflux pathway, MRP-mediated transport of bile acids to the basolateral space may represent a compensatory pathway to reduce bile acid accumulation when BSEP is inhibited (Köck et al., 2014; Teng and Piquette-Miller, 2007). Although the case for transporter-mediated effects is strong, bile acid accumulation is not believed to account for all DILI cases. Other potential mechanisms include mitochondrial dysfunction, reactive metabolite formation, oxidative stress, and endoplasmic reticulum (ER) stress. In fact, a recent analysis suggests that drugs that demonstrate inhibition of both mitochondrial function and BSEP are associated with a higher likelihood of severe liver injury than those that inhibit only one of these (Aleo et al., 2014). *In vitro* assays can be conducted to investigate these potential mechanisms of toxicity for tolvaptan. However, *in vitro* data alone are rarely definitive in the identification of mechanisms underlying observed human hepatotoxicity.

Quantitative and Systems Pharmacology (QSP) is one approach to evaluate *in vitro* data as a mechanism for *in vivo* hepatotoxicity. QSP has been defined by the National Institutes of Health as an approach to translational medicine that combines computational and experimental methods to elucidate, validate, and apply new pharmacological concepts to the development and use of small molecule and biologic drugs (Sorger et al., 2011).

DILIsym™, a platform QSP model of DILI, represents liver injury via an integrated system of sub-models including a physiologically based pharmacokinetic (PBPK) sub-model, mechanisms of parent compound or metabolite-mediated toxicity (bile acid transporter inhibition, mitochondrial dysfunction, and oxidative stress), hepatocyte population dynamics, and liver

inflammation (Shoda et al., 2014). New versions of DILIsym are released regularly as a product of the public-private partnership, the DILI-sim Initiative (www.dilisyms.com). DILIsym is parameterized using available experimental data, and optimized using exemplar compounds (Howell et al., 2012; Longo et al., 2016; Woodhead et al., 2014). DILIsym is designed to be customized to the compound of interest and serves as a unifying framework to investigate how *in vivo* exposure to drugs and their major metabolites induce mechanisms of toxicity leading to hepatocyte death.

In this study, putative mechanisms of tolvaptan toxicity identified through *in vitro* assays were evaluated for biological plausibility as the underlying drivers of observed liver injury using DILIsym. Further, DILIsym utilized SimPops™, populations of simulated individuals with variation in their underlying biochemistry and reflecting variable compound exposure, to explore how inter-patient variability impacted tolvaptan susceptibility to liver injury as well as the relative contribution of different mechanisms of toxicity. Finally, susceptible and resistant simulated individuals were analyzed to identify potential pre-treatment patient risk factors and to identify potential on-treatment biomarkers of impending liver injury. Together, the experimental data and mathematical modeling comprising this DILIsym QSP analysis provided a mechanistic foundation for the observed clinical hepatotoxicity, as well as support for the development of mechanism-based biomarkers.

MATERIALS AND METHODS

DILIsym overview. DILIsym has been described previously (Bhattacharya et al., 2012; Shoda et al., 2014; Woodhead et al., 2012). Briefly, DILIsym is a multi-scale and “middle-out” platform focused on the liver as the primary organ of interest, which includes detailed liver cellular dynamics and biochemistry, extends to whole body drug distribution and systemic circulation of mediators and biomarkers, and accounts for inter-individual differences through variation in anthropometrics and biochemistry. Essential processes occur in interacting sub-models: physiologically based pharmacokinetics (PBPK), bile acid transporter inhibition, mitochondrial dysfunction, oxidative stress, hepatocyte life cycle, and macrophage and endothelial cell life cycles. Parameter values are informed by experimental data, with sub-model and system-level behaviors optimized for consistency with exemplar compounds (Howell et al., 2012; Woodhead et al., 2014, 2012). Parameter solutions include those consistent with human, rat, and mouse data (Woodhead et al., 2012, 2014). Generally, simulations for the current study were conducted in the baseline simulated human, and in simulated populations (SimPops) as described previously (Longo et al., 2016; Yang et al., 2015); tolvaptan-specific adjustments are described in detail below.

Bile acid toxicity input data. The DILIsym bile acid sub-model represents bile acid enterohepatic circulation via bile acid transporters. More specifically, hepatocyte uptake of bile acids from blood occurs by the sodium taurocholate co-transporting polypeptide (NTCP) transporter. Bile acid efflux occurs via BSEP-mediated canalicular transport and MRP3- and MRP4-mediated basolateral transport. Drug-mediated inhibition of efflux transporters can result in hepatocellular bile acid accumulation, whereas drug-mediated NTCP inhibition can mitigate this effect. Elevated intracellular bile acid concentrations can mechanistically alter mitochondrial function, leading to hepatocyte death (Rolo et al., 2000; Schulz et al., 2013). Details of this

representation are provided in the [Supplementary File 1](#). Drug-mediated inhibition of transporters can be assessed in laboratory experiments using cells or membrane vesicles expressing the transporter of interest. Importantly, the hepatotoxic potential of transporter inhibition can be influenced by type of inhibition (eg, competitive vs non-competitive) as well as strength of inhibition ([Woodhead et al., 2014](#)). Inhibition type can be determined from experimental K_i data. For the parameterization of tolvaptan, K_i data were available characterizing inhibition of BSEP by tolvaptan [molecular weight (MW) = 448.94 g/mol] and 2 metabolites, DM-4103 (MW = 478.92 g/mol) and DM-4107 (MW = 480.94 g/mol). IC_{50} values for tolvaptan and the metabolites were available for NTCP ([Slizgi et al., 2016](#)).

Mitochondrial dysfunction input data. The DILIsym mitochondria sub-model represents mitochondrial bioenergetics leading to adenosine triphosphate (ATP) production in hepatocytes. Compounds may induce mitochondrial dysfunction by inhibiting the electron transport chain (ETC), by inhibiting the mitochondrial F_1F_0 ATPase, or by uncoupling mitochondrial respiration from mitochondrial ATP synthesis. Compound effects on hepatocyte mitochondrial function can be assessed in laboratory experiments by measuring hepatocyte respiration following culture with the compound in a Seahorse XF Analyzer (Seahorse Bioscience, MA) ([Nadanaciva et al., 2012](#)). For the parameterization of tolvaptan, data were obtained for the inhibition of mitochondrial respiration by tolvaptan, DM-4103, and DM-4107 ([Figure 1](#)). Experimental details are provided in [Supplementary File 2](#). A companion mechanistic mathematical model, MITOSymTM, which simulates *in vitro* hepatocellular respiration was designed to reproduce data obtained via the Seahorse assay for the purposes of deriving parameters characterizing compound induced mitochondrial dysfunction ([Yang et al., 2014](#)). Importantly, the compound concentration driving an intracellular response (ie, mitochondrial respiration) may

not be equivalent to the nominal media concentration reported in the assay protocol ([Groothuis et al., 2015](#)), where the nominal media concentration is defined as the reported (but not measured) concentration of compound in the media. To more closely describe the relationship between concentration at the site of action and effect, the intracellular concentration in the HepG2 cells used in the XF Analyzer experiments was estimated using the DILIsym PBPK simulation results; the simulated liver:plasma concentration ratio was assumed to approximate the concentration ratio between the hepatocyte and the media, thus providing a scaling factor to convert the nominal media concentration to an estimated intracellular concentration. The MITOSym optimization was conducted against both the nominal compound concentration and the estimated intracellular compound concentration. Because DILIsym seeks to represent the *in vivo* response, the parameters derived from the estimated intracellular compound concentration were selected for translation to *in vivo* DILIsym parameters.

Reactive oxygen species input data. The DILIsym oxidative stress sub-model represents the generation of reactive oxygen species (ROS) in response to compound exposure. ROS accumulation can lead to hepatocyte apoptosis or necrosis, depending on the extent of oxidative stress. Superoxide was not induced in HepG2 cells following exposure to tolvaptan and its metabolites, DM-4103, or DM-4107, as indicated by the dihydroethidium (DHE) fluorescence assay ([Supplementary Figure 3-1](#)). Experimental details are provided in [Supplementary File 3](#). Because no ROS production was observed experimentally with DHE, the DILIsym parameter values for tolvaptan did not include ROS production.

Pharmacokinetic modeling. The PBPK sub-model was parameterized in order to predict the dynamics of orally dosed tolvaptan, DM-4103, and DM-4107, in humans in both liver and blood. The goal of the model parameterization was to represent the range of observed concentrations for these 3 molecules in healthy subjects and ADPKD patients. Initially, the PBPK sub-model was parameterized based on data from healthy subjects with the understanding that it would be adjusted if necessary to reproduce the range of plasma parent concentrations for ADPKD patients.

Specific data used in optimization and validation of the PBPK sub-model are listed in [Table 1](#). The PBPK sub-model was optimized with single-dose simulations of 30, 60, and 120 mg, as well as a 7-day simulation of 60 mg daily dosing, and a 5-day simulation of both 30 and 300 mg daily dosing; these simulated dosing regimens were selected in order to match the protocols for the clinical trials listed in [Table 1](#). The parent and metabolites were all fit at once using the same optimization routine, which was a genetic algorithm using a least-squares fit to the plasma data as the fitness function. The data to which the model was optimized was weighted so that data for tolvaptan, DM-4103, and DM-4107 had equal weight. The optimization also included the total compound liver-blood ratio measured in the rat whole-body autoradiography study ([Furukawa et al., 2011](#)) in order to ensure relatively accurate liver exposure. Renal clearance of unchanged parent drug was assumed to be <5% of the total tolvaptan clearance, which is near the range suggested by the experimental data ([Shoaf et al., 2007](#)).

Parameters that were employed in optimizing the PBPK sub-model are listed in [Table 2](#). These parameters include biliary and renal clearance values for all chemical species, metabolism maximum velocity (V_{max}) values for both DM-4103 and DM-4107, and distribution parameters for all chemical species.

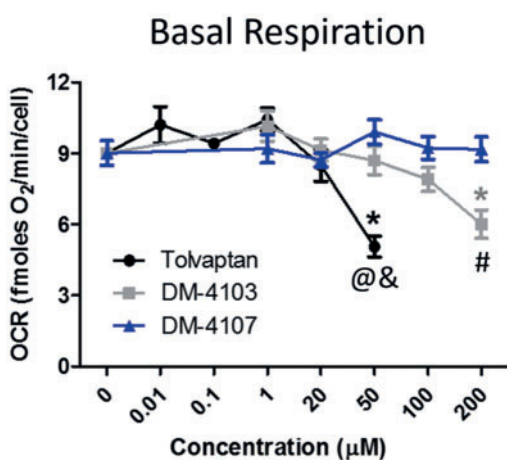


FIG. 1. The effect of 24 h treatment of tolvaptan, DM-4103 and DM-4107 on basal mitochondrial respiratory function (fmoles O_2 /min/cell) in HepG2 cells. Cells were treated with control media or compound in a 5-point dose response regimen (tolvaptan at: 0.01, 0.1, 1, 20, and 50 μ M or DM-4103 or DM-4107 at: 1, 20, 50, 100 and 200 μ M) for 24 h prior to respirometry experiments. Oxygen consumption rate (OCR) results were normalized to cell number. Data are presented as the average \pm SEM for 3 independent experiments. Statistical significance is denoted by an asterisk (* $P \leq .05$) for one-way ANOVA with Dunnett's multiple comparison test to compare each concentration to control for a given compound. Two-way ANOVAs with a Bonferroni multiple comparison test was used to compare compounds: # $P \leq .05$ (DM-4103 vs DM-4107); @ & $P \leq .05$ (tolvaptan vs DM-4103); & $P \leq .05$ (tolvaptan vs DM-4107).

TABLE 1. Concentration Data Used in the Optimization and Validation of the Baseline DILIsym PBPK Sub-Model for Tolvaptan

Analytes	Oral Dose (mg)	DILIsym Use	Protocol/Reference
Parent DM-4103	30 ^a	PBPK Optimization; Baseline Human,	(Shoaf et al., 2012a) ^b
DM-4107		Renally Sufficient SimPops	
Parent DM-4103	60 ^a	PBPK Optimization; Baseline Human,	156-05-256 (data on file) ^b
DM-4107		Renally Sufficient SimPops	
Parent DM-4107	120 ^{a,c}	PBPK Optimization; Baseline Human,	(Shoaf et al., 2007) ^{bd}
		Renally Sufficient SimPops	
Parent – –	30, 300 qd 5d	PBPK Optimization; Baseline Human,	156-03-245 (data on file) ^b
		Renally Sufficient SimPops	
Parent DM-4103 DM-4107	60 qd 7d	PBPK Validation; Renally Sufficient SimPops	(Van Wart et al., 2013) ^b
Parent – –	15	PBPK Validation; Baseline Human	156-12-202 (data on file) ^b
Parent DM-4103 DM-4107	30 ^{a,c}	PBPK Validation; Baseline Human	(Shoaf et al., 2012b) ^b
Parent – –	90/30 split daily 7d	PBPK Validation; Renally Impaired SimPops	(Boertien et al., 2013) ^e
Parent – –	90/30 split daily 7d	PBPK Validation; Renally Impaired SimPops	NCT01210560 (data on file) ^e
Parent – –	30	PBPK Optimization; Renally Impaired SimPops	156-04-001 (data on file) ^e

^aUsed in Figure 2.

^bTrial enrolled healthy subjects.

^cTwo single doses separated in time.

^dThe washout period between doses was insufficient to allow for full clearance of DM-4103, complicating the use of the DM-4103 data.

^eTrial enrolled subjects with ADPKD.

Simulated population creation. SimPops were used to understand the role of inter-individual variability in tolvaptan-mediated hepatotoxicity and to identify particular susceptibility factors. The process used to generate tolvaptan-specific SimPops is similar to the process described for troglitazone in previous DILIsym work (Yang et al., 2015). The variability in parameters specific to tolvaptan exposure in each case was superimposed upon the existing Human_mito_BA_v3A_6 SimPops included with DILIsym v3B. This SimPops covers variability in both bile acid transport and mitochondrial function. Notably, this SimPops features 17 simulated patients that were designed to exhibit mitochondrial impairment similar to that observed in NASH patients (Pérez-Carreras et al., 2003). As a result, the SimPops for this project included 229 individuals with variability in exposure parameters, bile acid transport parameters, and mitochondrial parameters. Using this method, 2 SimPops were created: a Renally Sufficient SimPops and a Renally Impaired SimPops.

The simulated human healthy volunteer population (the Renally Sufficient SimPops) was designed to recapitulate the range of tolvaptan exposures observed in healthy subjects. The PBPK sub-model for this SimPops was constructed using the parent and metabolite time course data for single 30 mg dose; single 60 mg dose; and single 120 mg dose; and for repeat doses of 60 mg for 7 days, and 30 and 300 mg for 5 days

(Table 1). As described above, the collective clinical data set was selected to provide a similar number of comparator studies for each of the 3 molecular species for the single dose case (tolvaptan, DM-4103, DM-4107), and to include both single and repeat dose studies within the optimization scheme. Several protocols not used in the optimization scheme were employed to validate the PBPK parameter values identified by the optimization scheme. The test protocols included data for 15, 30, and 60 mg single doses from clinical trials (Table 1). Finally, 90/30 mg split daily dosing was simulated in this population to compare the range of simulated tolvaptan plasma concentrations against the range of measured concentrations in ADPKD patients in 90/30 mg split daily dosing trials (Boertien et al., 2013; NCT01210560) in order to determine its validity for representing the ADPKD patient population.

To simulate certain extreme ADPKD exposure profiles that were not reproduced by the Renally Sufficient SimPops, a Renally Impaired SimPops was developed. Tolvaptan PBPK parameters that were assigned different ranges in the Renally Impaired SimPops based on their relationship to renal impairment are listed in Table 3. For example, CYP-mediated metabolism decreased in renal impairment (Michaud et al., 2008). The parameter ranges for the Renally Impaired SimPops were optimized using PK data (156-04-001, 30 mg daily in ADPKD subjects, data on file, Table 1) in which tolvaptan plasma concentrations

TABLE 2. Parameters Optimized to Data in the DILIsym PBPK Sub-Model for Tolvaptan.

Parameter Optimized in PBPK Sub-Model Fitting Process	Optimized Parameter Value	Units
Tolvaptan blood:plasma ratio	0.6	Dimensionless
Tolvaptan muscle:blood ratio	0.287	Dimensionless
Tolvaptan gut:blood ratio	0.592	Dimensionless
Tolvaptan biliary clearance	5.687	mL/h/kg ^{0.75}
DM-4103 biliary clearance	135.6	mL/h/kg ^{0.75}
DM-4107 biliary clearance	10.7	mL/h/kg ^{0.75}
DM-4103 fraction unbound in plasma	0.0245	Dimensionless
Tolvaptan liver:blood ratio	38.76	Dimensionless
DM-4103 liver:blood ratio	0.262	Dimensionless
DM-4107 liver:blood ratio	1.25	Dimensionless
DM-4107 fraction unbound in plasma	0.146	Dimensionless
Tolvaptan renal clearance	85.43	mL/h/kg ^{0.75}
DM-4103 volume of distribution	1210	mL/kg
DM-4107 volume of distribution	335	mL/kg
Tolvaptan fraction unbound in plasma	0.391	Dimensionless
Tolvaptan absorption from gut V_{max}	69.19	mg/h
Tolvaptan-DM-4103 metabolism K_m	8.60×10^{-7}	mol/mL
Tolvaptan-DM-4107 metabolism K_m	1.00×10^{-9}	mol/mL
Tolvaptan other tissue:blood ratio	0.414	Dimensionless
Tolvaptan absorption from gut K_m	0.0025	mg
Tolvaptan-DM-4103 metabolism V_{max}	5.48×10^{-4}	mol/h/kg ^{0.75}
Tolvaptan-DM-4107 metabolism V_{max}	2.56×10^{-7}	mol/h/kg ^{0.75}
Tolvaptan excretion from gut rate constant	0.4483	1/h
DM-4103 renal clearance	2.41	mL/h/kg ^{0.75}
DM-4107 renal clearance	934.3	mL/h/kg ^{0.75}

TABLE 3. Parameters Changed between the Renally Sufficient and the Renally Impaired SimPops

Parameter Name	Parameter Description	Average Fold Change from Baseline Individual PBPK Model	Rationale
Comp_W_bil_cl ^a	Tolvaptan biliary clearance	0.5	CYP activity impaired in uremic patients ^b ; biliary clearance used to describe tolvaptan metabolism to other metabolites
Comp_W_renal_cl	Tolvaptan renal clearance	0.375	Impaired renal function
CompW_Met_A_renal_cl	DM-4103 renal clearance	0.375	Impaired renal function
CompW_Met_B_renal_cl	DM-4107 renal clearance	0.375	Impaired renal function
Vmax_CompW_Met_A	DM-4103 generation V_{max}	0.5	CYP activity impaired in uremic patients ^a
Vmax_CompW_Met_B	DM-4107 generation V_{max}	0.5	CYP activity impaired in uremic patients ^a
Body_mass	Body mass	0.926	ADPKD patients more predominantly female

^aComp W = Compound W, a generic structure within DILIsym whose parameter values can be customized to represent the compound of interest.

^bMichaud et al. (2008) reports reduction of CYP expression *in vitro* as a result of incubation with uremic serum; Kirch et al. (1984) reports that non-renal clearance can be increased as much as 7-fold in individuals with moderate to severe renal impairment.

were markedly higher than those observed in the non-ADPKD PK data. Whereas this trial was conducted with ADPKD patients with preserved renal function, the data demonstrated the highest maximum concentration (C_{max}) relative to tolvaptan dose of any steady-state ADPKD clinical trial. The Renally Impaired SimPops was validated by simulating 90/30 mg split daily dosing and comparing the results against tolvaptan concentration data from ADPKD patients with moderate to severe renal dysfunction (defined by an estimated glomerular filtration rate (eGFR) of 30–60 mL/min/1.73 m² and eGFR <30 mL/min/1.73 m², respectively) given 90/30 mg tolvaptan split daily dosing (Boertien et al., 2013; NCT01210560).

Toxicity simulations. In general, tolvaptan has been regarded as safe based on clinical experience in healthy subjects as well as

in patients with hyponatremia, including patients with heart failure. Based on tolvaptan clinical trials for heart failure, a dosage regimen of 60 mg daily for 60 days was selected as a negative control that was not expected to cause hepatotoxicity.

Tolvaptan-associated liver safety signals initially were reported in ADPKD Phase III trials in which chronic tolvaptan treatment was being evaluated for its ability to slow disease progression (Torres et al., 2012). Patients were administered tolvaptan as 45/15, 60/30, or 90/30 mg split daily doses separated by 8 h. The highest dose was of the greatest interest because the 3 patients with severe liver injury (ie, that qualified as Hy's Law cases) were administered the 90/30 mg split daily doses (Watkins et al., 2015). The ADPKD Phase III trials and follow-on studies were conducted over years; one might therefore expect the simulation studies to be conducted over years. However, the

occurrence of hepatotoxicity within DILIsym is asymptotic by 6 months. Briefly, simulated hepatotoxicity emerges from the combination of hepatic exposure, mechanisms of toxicity, and inter-individual variability in both. With chronic dosing of most drugs, steady-state levels are typically reached within weeks. Mitochondrial toxicity manifests rapidly (ie, within weeks). Bile acid toxicity may manifest more slowly (ie, over months) as it is dependent on the rate of bile acid accumulation, which is itself dependent on the strength and type of transporter inhibition. Further, DILIsym is a mechanistic model, meaning that stochastic events will not alter the dynamics described above. Therefore, a 90/30 mg split daily dosage regimen was simulated for 180 days. The results were analyzed to illuminate the effect of longer treatment duration on simulated hepatotoxicity and compared against Phase III trials conducted over years.

Tolvaptan dosing was simulated at 60 mg daily for 60 days and 90/30 mg split daily dosing for 180 days to investigate hepatotoxicity using the Renally Sufficient SimPops. Simulated hepatotoxicity following 90/30 mg split daily dosing for 180 days was investigated subsequently to determine the relative importance of each toxicity mechanism and each molecular contributor to toxicity. The 90/30 mg split daily dosage regimen also was simulated for 4 different scenarios, sequentially omitting one potential mechanistic contributor to toxicity at a time: bile acid transporter inhibition effects, mitochondrial dysfunction effects, all effects due to the parent drug (tolvaptan), and all effects due to DM-4103. DM-4107 exhibited no toxic effects and so was not included in this analysis. These Mechanistic Investigation Simulations, listed in Table 4, evaluated the importance of each toxicity element to the overall DILI behavior of drug treatment.

Because the Renally Impaired SimPops was designed to represent certain exposure-related aspects of ADPKD patients (ie, a PBPK sub-model optimized for the highest levels of exposure observed in ADPKD patients), all investigations with this SimPops were conducted using the 90/30 mg split daily dosage regimen for 180 days. Mechanistic Investigative Simulations as described in the previous paragraph were conducted in the Renally Impaired SimPops.

Unlike the clinical trials, the DILIsym simulations did not include physician judgment or protocol-driven criteria to increase monitoring and/or to halt treatment when ALT elevations exceeded pre-specified thresholds. Implementing such a protocol in the SimPops simulations was unnecessary for the purpose of identifying simulated individuals at risk for hepatotoxicity and investigating the susceptibility of those individuals to toxicity. Thus, in some cases, the magnitude of the simulated

liver injury exceeded what was observed in patients whose tolvaptan treatment might have been interrupted in response to liver signals.

Susceptibility factor analysis. In order to identify potential susceptibility factors, a covariate analysis was performed with the simulation results from the Renally Sufficient and Renally Impaired SimPops (90/30 mg split daily doses for 180 days). Parameters that were varied to create the simulated individuals within the SimPops (Table 5) were used as independent variables, and simulated ALT elevations or simulated loss of liver ATP were used as dependent variables in a covariate analysis. Parameters that correlated with ALT elevations and loss of liver ATP at P values $<.05$ were identified as potential susceptibility factors. Covariate analysis was performed using JMP 9 (SAS, Cary, NC).

A further analysis of DILIsym outputs was performed in order to determine which outputs could suggest potential on-treatment biomarkers of injury due to tolvaptan. Simulated individuals with tolvaptan-mediated transaminase elevations (defined by ALT elevations greater than 3 times ULN) developing after 2 weeks of simulated tolvaptan dosing were identified from both the Renally Sufficient and Renally Impaired SimPops. The DILIsym outputs during the first 2 weeks of dosing (ie, before the onset of hepatotoxicity) were analyzed for the ability of each output to differentiate individuals who developed DILI from those who did not. The area under the receiver-operating characteristic curve (AUROC) was calculated for each output and also for each pair of outputs, and these values were used to rank order the outputs as putative biomarkers or biomarker indicators (Liu and Zhou, 2013; Vexler et al., 2014).

RESULTS

PBPK Modeling—Baseline Human

The DILIsym simulations of tolvaptan, DM-4103 and DM-4107 concentrations in renally sufficient individuals were intended to capture a range of exposures consistent with those observed in non-ADPKD individuals and ADPKD patients with well-preserved renal function. The DILIsym PBPK sub-model was optimized to single- and multiple-dose healthy subject data listed in Table 1.

PBPK Modeling—SimPops Results

The ability of the simulations to cover the measured range of tolvaptan and metabolite concentrations in healthy subjects was assessed by simulating tolvaptan dosage regimens with the

TABLE 4. List of Mechanistic Investigation Simulations and the Mechanisms that Were Turned On and Off for Each Simulation of Tolvaptan Administered 90/30 mg Daily for 180 Days

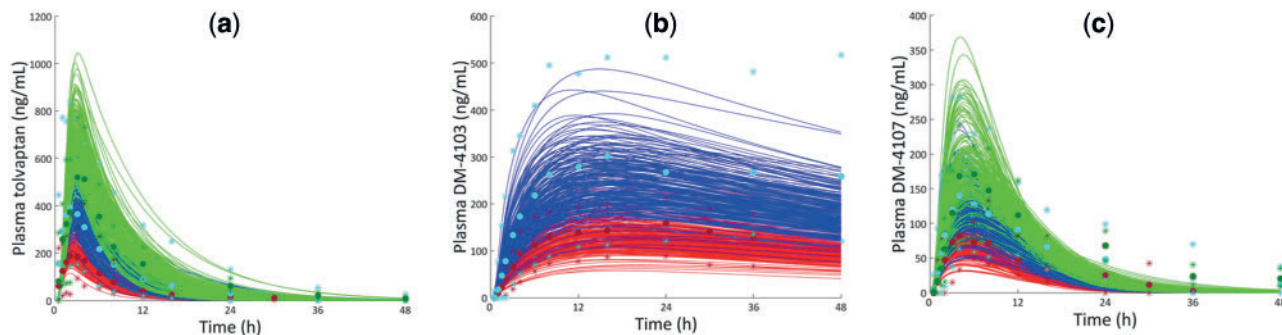
Mechanistic Investigation Simulation Name	Mechanisms On ^a	Mechanism(s) Off
–	TVP: BSEPi, NTCPi, ETCi DM-4103: BSEPi, NTCPi, ETCi	None
ETCi-Off	TVP: BSEPi, NTCPi DM-4103: BSEPi, NTCPi	ETCi
BA-Off(BSEPi, NTCPi)	TVP: ETCi DM-4103: ETCi	BA ^b (BSEPi, NTCPi)
TVP-Off	DM-4103: BSEPi, NTCPi, ETCi	TVP
DM-4103-Off	TVP: BSEPi, NTCPi, ETCi	DM-4103

^aTVP is tolvaptan; DM-4103 is a metabolite of tolvaptan; BSEPi is inhibition of BSEP; NTCPi is inhibition of NTCP; ETCi is inhibition of ETC.

^bBA is bile acids; turning this mechanism off means removing both BSEP and NTCP inhibition.

TABLE 5. List of Parameters Included in the SimPops Used for This Investigation and The Ranges For Each Parameter Relative to Their Average Values

Parameter Name	Parameter Description	Range for Renally Sufficient SimPops	Range for Renally Impaired SimPops
ATP_decr_necrosis_Vmax	V_{max} for ATP decrement to necrosis	49.1–147%	49.1–147%
Basal_Stdzd_MitoETC_Flux	Basal ETC flux	25.7–175%	25.7–175%
Body_mass	Body mass	63.0–139%	69.2–133%
CDCA_amidation_Vmax	CDCA amidation V_{max}	6.65–171%	6.65–171%
CDCAamide_baso_Vmax	CDCA-amide basolateral transport V_{max}	10.7–459%	10.7–459%
CDCAamide_canal_Vmax	CDCA-amide canalicular transport V_{max}	27.1–168%	27.1–168%
CDCAamide_uptake_Vmax	CDCA-amide uptake transport V_{max}	26.6–208%	26.6–208%
CompW_Met_A_bil_cl	DM-4103 biliary clearance	45.4–131%	56.4–153%
CompW_Met_A_renal_cl	DM-4103 renal clearance	46.6–136%	18.1–121%
CompW_Met_B_bil_cl	DM-4107 biliary clearance	50.6–139%	53.1–132%
CompW_Met_B_renal_cl	DM-4107 renal clearance	93.7–112%	15.6–126%
Comp_W_bil_cl	Tolvaptan biliary clearance	54.4–144%	17.4–126%
Comp_W_renal_cl	Tolvaptan renal clearance	57.9–135%	20.1–124%
HGF_regen_Vmax	Hepatocyte regeneration V_{max}	49.1–147%	49.1–147%
LCA_synthesis_Vmax	LCA synthesis V_{max}	6.56–1804%	6.56–1804%
LCAamide_sulfation_Vmax	LCA-amide sulfation V_{max}	13.5–349%	13.5–349%
LCA_sulfate_canal_Vmax	LCA-sulfate canalicular transport V_{max}	7.18–181%	7.18–181%
LCA_sulfate_uptake_Vmax	LCA-sulfate uptake transport V_{max}	14.6–376%	14.6–376%
Resp_Reserve_Scalar	Respiratory reserve capacity	54.7–154%	54.7–154%
Vmax_CompW_Met_A	DM-4103 formation V_{max}	37.4–231%	8.40–243%
Vmax_CompW_Met_B	DM-4107 formation V_{max}	37.4–231%	8.40–243%
Vmax_Comp_W_ab	Tolvaptan absorption from gut V_{max}	52.4–151%	45.5–132%
canal_reg_scale	FXR-mediated canalicular bile acid transporter regulation scaling factor	7.33–146%	7.33–146%
uptake_reg_scale	FXR-mediated bile acid uptake transporter regulation scaling factor	0–284%	0–284%

**FIG. 2.** Comparison between single dose clinical data for 30 mg, dark red, 60 mg, cyan, and 120 mg, dark green (Table 1), and range of Renally Sufficient SimPops simulation results for (a) tolvaptan plasma concentrations; (b) DM-4103 plasma concentrations (note: data from the 120 mg study were unavailable for this analyte; see Table 1); (c) DM-4107 plasma concentrations. Dark circles represent the average of the clinical data; stars represent the maximum and minimum value measured in the clinical trial. Simulation results for the 30 mg study are in red, simulation results for the 60 mg study are in blue, and simulation results for the 120 mg study are in green.

PBPK optimization solution in the Renally Sufficient SimPops. The Renally Sufficient SimPops simulation results extend across the range of plasma concentrations in the clinical data for the 30, 60, and 120 mg single dose studies from healthy subjects used to construct the Renally Sufficient SimPops (Figure 2).

To determine the ability of simulations to cover the measured range of tolvaptan concentrations in ADPKD patients, the simulation results in the Renally Sufficient SimPops were compared with PK data obtained from ADPKD patients treated with a 90/30 mg split daily dose of tolvaptan (Boertien *et al.*, 2013). As shown in the comparison to the validation data in Figure 3, the range of predicted variability in the Renally Sufficient SimPops reproduced most of the range of tolvaptan plasma concentrations observed in the ADPKD clinical trial, though the peak concentrations occur at a slightly later time and the highest

measured concentrations were not reproduced. Metabolites were not measured as a part of this trial's protocol.

To confirm that PBPK parameter values from the Renally Impaired SimPops met the intended objective of representing patients with relatively high exposure, seen in some patients with ADPKD, that were not represented by the Renally Sufficient SimPops (above), simulation results were compared with the clinical data from studies where ADPKD patients were given 90/30 mg split daily dosing. In the 90/30 mg split daily dosing study (Boertien *et al.*, 2013), patients were grouped according to renal function ($eGFR > 60 \text{ mL/min/1.73 m}^2$, $eGFR 30\text{--}60 \text{ mL/min/1.73 m}^2$, $eGFR < 30 \text{ mL/min/1.73 m}^2$), which allowed a comparison to patients with moderate to severe renal impairment (ie, $eGFR 30\text{--}60 \text{ mL/min/1.73 m}^2$ and $eGFR < 30 \text{ mL/min/1.73 m}^2$). Simulation results of tolvaptan from the Renally Impaired

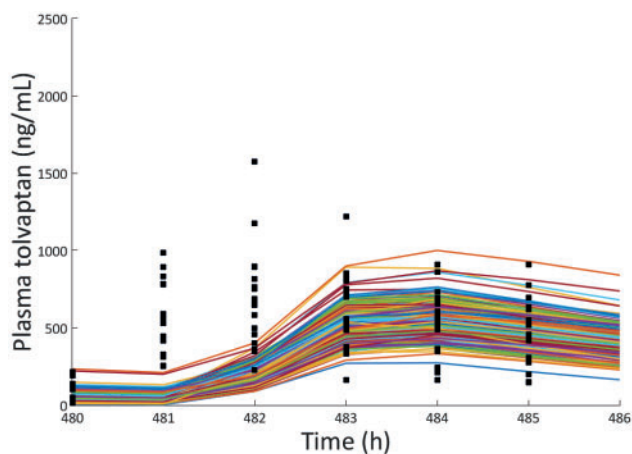


FIG. 3. Comparison between Renally Sufficient SimPops and day 21 tolvaptan plasma concentrations after 90/30 mg split daily dosing (Boertien et al., 2013). The clinical data are represented by symbols (black squares) whereas the simulation results for individual simulated patients are represented by solid lines.

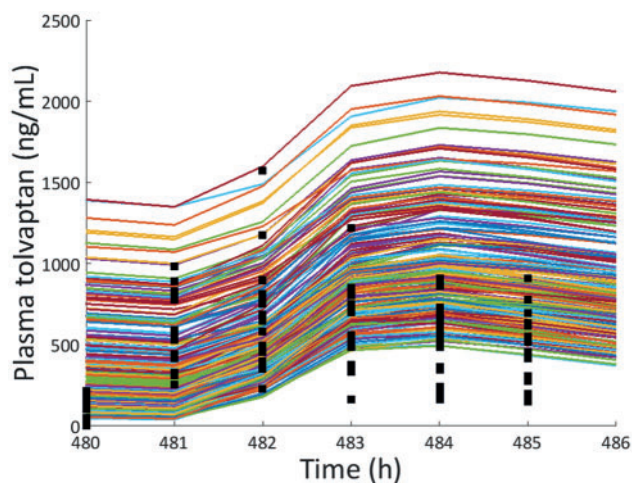


FIG. 4. Comparison between Renally Impaired SimPops and day 21 tolvaptan plasma concentrations after 90/30 mg split daily dosing (Boertien et al., 2013). The clinical data are represented by symbols (black squares) whereas the simulation results for individual simulated patients are represented by solid lines.

SimPops generally spanned the range measured for these patients with renal dysfunction in the validation data set (Figure 4). The comparison demonstrated that the Renally Impaired SimPops yielded tolvaptan plasma concentrations that met or exceeded the highest concentrations observed within the clinical trial (Boertien et al., 2013).

Toxicity Parameters

In vitro laboratory investigations were conducted to assess the potential of tolvaptan, DM-4103, or DM-4107 to induce mitochondrial dysfunction and to elicit oxidative stress. Previous research had assessed the potential of tolvaptan, DM-4103, and DM-4107 to inhibit the bile acid transporters BSEP and NTCP (Slizgi et al., 2016). These data demonstrated that both tolvaptan and DM-4103 inhibited BSEP and NTCP bile acid transporters. Mitochondrial respiration data generated in support of this project demonstrated that both tolvaptan and DM-4103 induced mitochondrial dysfunction (Figure 1). DM-4107 did not influence bile acid transporters or mitochondria, and none of the 3

chemical species induced oxidative stress as measured by cytosolic superoxide levels in HepG2 cells (Supplementary File 3).

The bile acid transporter inhibition constants were used directly in DILIsym (Table 6). BSEP inhibition was competitive based on the experimental K_i data (Slizgi et al., 2016). Because only NTCP IC_{50} data were available, either competitive or non-competitive inhibition could be assumed. For the same inhibition constant, competitive inhibition will result in more bile acid uptake than non-competitive inhibition. To allow for the greatest possible uptake (ie, the most potential toxicity), NTCP inhibition was assumed to be competitive. It should be noted that the actual level of bile acid accumulation that occurs is dependent on the net effect of tolvaptan and metabolites on the various transporters, as well as the activity of adaptive mechanisms such as farnesoid X receptor (FXR) activation.

The cellular respiration experimental data were used to optimize the MITOSym ETC inhibition parameters for tolvaptan and DM-4103. As described in the methods, both the nominal media concentration and the predicted intracellular compound concentration were considered for the optimization. As shown in Figure 5, the predicted intracellular compound concentration (based on PBPK simulation results) was markedly higher than the nominal media concentration for tolvaptan, but not DM-4103. This difference was reflected in the optimized ETC inhibition parameter value for tolvaptan, but not DM-4103. The MITOSym parameters derived when allowing the intracellular compound concentration to reflect the PBPK simulation results were transformed to DILIsym parameters (Table 6) and used for the toxicity simulations. It should be noted that the parameter values for mechanisms of hepatotoxicity for tolvaptan and metabolites are independent of the simulated patients and do not vary whether the simulations are conducted in the Renally Sufficient or the Renally Impaired SimPops.

Toxicity Investigations

To increase confidence that the DILIsym parameter values for tolvaptan reasonably captured “safe” tolvaptan scenarios (in addition to the DILI scenarios of interest), the 60 mg daily dose for 60 days tolvaptan dosage regimen was simulated in the Renally Sufficient SimPops. Nearly all simulated individuals ($n = 228/229$) were predicted to have normal ALT levels throughout; one simulated individual developed an ALT elevation (Table 7). The one simulated individual with an ALT elevation was identified as one of the aforementioned individuals with mitochondrial activity parameters characteristic of NASH patients. The fact that the other 16 NASH-like simulated individuals did not develop hepatotoxicity indicates that toxicity was not solely attributable to mitochondrial activity. Rather, this particular simulated individual also possessed bile acid transport characteristics in the lower range of normal, resulting in susceptibility to tolvaptan-mediated hepatotoxic mechanisms.

When the 90/30 mg split daily dosage regimen was simulated for 180 days in the Renally Sufficient SimPops, 7.9% of simulated individuals developed ALT elevations $>3x$ ULN. This can be compared against the clinical observation of 4.4% of patients with ALT elevations $>3x$ ULN (Watkins et al., 2015). As noted previously, the DILIsym simulations did not include physician judgment or protocol-driven criteria to increase monitoring and/or to halt treatment when ALT elevations exceeded pre-specified thresholds. Also, there were more simulated Hy’s Law cases denoting serious liver injury (ALT $>3x$ ULN and bilirubin $>2x$ ULN) (6.5%) than were observed clinically (0.2%) (Watkins et al., 2015). The average simulated time to peak ALT value was 2.5 months, which

TABLE 6. DILIsym Toxicity Parameter Values for Tolvaptan Toxicity Simulations

Compound	DILI Mechanism	DILIsym Parameter	Parameter Description	Parameter Value	Parameter Units
Tolvaptan	BSEP inhibition	Ki_noncomp_BSEP_CompW	BSEP non-competitive inhibition constant for Compound W	0.0154 ^a	mg/mL
Tolvaptan	NTCP inhibition	Ki_NTCP_CompW ^b	NTCP competitive inhibition constant for Compound W	0.0187 ^a	mg/mL
Tolvaptan	ETC inhibition	MitoS_ETC_inhib_CompW	Coefficient to quantify ETC inhibition based on compound/metabolite levels in the liver	1.09×10^{-6}	mol/mL
DM-4103	BSEP inhibition	Ki_BSEP_CompW_MetA ^b	BSEP competitive inhibition constant for Compound W metabolite A	0.002 ^a	mg/mL
DM-4103	NTCP inhibition	Ki_NTCP_CompW_MetA ^b	NTCP competitive inhibition constant for Compound W metabolite A	0.0071 ^a	mg/mL
DM-4103	ETC inhibition	MitoS_ETC_inhib	Coefficient to quantify ETC inhibition based on compound/metabolite levels in the liver	9.38×10^{-9}	mol/mL

^aValues are unit converted to mg/mL from μM IC₅₀ values. The values reflect preliminary IC₅₀ values available at the time the DILIsym modeling was conducted. The values are numerically very similar to the final values as reported recently (Slizgi et al., 2016). The numerical differences do not have any effect on the overall conclusions.

^bFor competitive inhibition of a given transporter, 6 parameters are populated. The parameters represent the different bile acid species in DILIsym (bulk bile acids, LCA, LCA amide, LCA sulfate, CDCA, CDCA amide). For example, competitive NTCP inhibition by tolvaptan is represented by using the inhibition constant for the following parameter values: (1) Ki_NTCP_CompW, (2) LCA_uptake_Ki_CompW, (3) LCAamide_uptake_Ki_CompW, (4) LCAsulfate_uptake_Ki_CompW, (5) CDCA_uptake_Ki_CompW, and (6) CDCAamide_uptake_Ki_CompW.

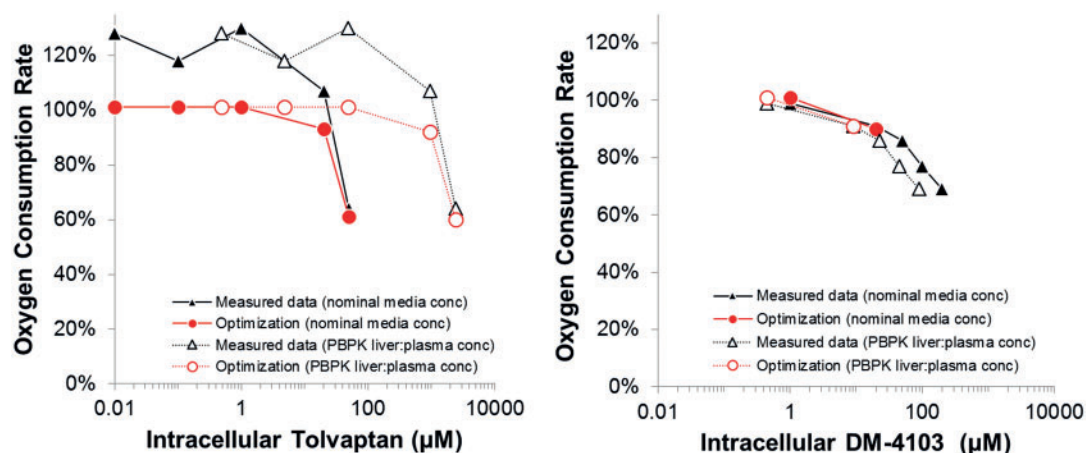


FIG. 5. Comparison of simulation results and *in vitro* data for tolvaptan- and DM-4103-induced inhibition of HepG2 oxygen consumption rate. Simulations were conducted in MITOSym and were used to identify parameter values for compound-mediated ETC inhibition that permit simulations to reproduce the *in vitro* data. The intracellular compound concentration was assumed to be equivalent to the nominal media concentration or was estimated based on the liver:plasma ratio derived from simulations using the PBPk sub-model. MITOSym parameter values identified with the estimated intracellular compound concentrations were the ones translated to DILIsym and used for toxicity simulations.

TABLE 7. Frequency of Simulated ALT Elevations in the Renally Sufficient SimPops

Toxicity Mechanisms	Dose	Simulated ALT >3x ULN	Simulated Hy's Law Cases
All ^a	60 mg daily, 60 days	1/229	1/229
All	90/30 mg daily, 180 days	18/229	15/229
TVP-Off ^b	90/30 mg daily, 180 days	0/229	0/229
DM-4103-Off	90/30 mg daily, 180 days	5/229	5/229
ETCi-Off	90/30 mg daily, 180 days	9/229	6/229
BA-Off	90/30 mg daily, 180 days	0/229	0/229

^aAll refers to simulations in which both tolvaptan and DM-4103 mediate toxicity and both bile acid and mitochondrial toxicity mechanisms are active.

^bThese simulation names refer to the Mechanistic Investigation Simulation names listed in Table 4. Mechanisms present and not present for each simulation can be found there.

is early relative to the susceptible range of 3–18 months for ALT elevations in ADPKD patients (Watkins et al., 2015).

The relative importance of parent vs. metabolite and bile acid versus mitochondrial toxicity were investigated using Mechanistic Investigation Simulations (see Materials and Methods). This exploration suggested that both mechanisms of hepatotoxicity and both molecular species were involved in the observed toxicity (Table 7). Whereas the simulations with the complete set of parameter values (all molecular species active and both mechanisms active) yielded 18/229 simulated patients with liver injury, removing bile acid-mediated toxicity (Simulation BA-Off) or parent tolvaptan-mediated toxicity (TVP-Off) eliminated the simulated hepatotoxicity altogether (0/229 simulated patients with liver injury), suggesting that these 2 elements are major contributors to the hepatotoxicity. The DM-4103-Off and ETCi-Off simulations, which remove DM-4103 toxicity or mitochondrial toxicity respectively, only attenuated the simulated toxicity, suggesting that these elements may also contribute to potential liver injury but to a lesser extent (Table 7). Together, these Mechanistic Investigation Simulations indicated that tolvaptan-mediated toxicity is likely to be multifactorial.

The results from the hepatotoxicity simulations conducted with the Renally Impaired SimPops are listed in Table 8. For the 180 days, 90/30 mg split daily dosage regimen simulation, the incidence rate of simulated ALT elevations (70/229) was much higher than the 18/229 predicted for the Renally Sufficient SimPops, indicating that changes in tolvaptan disposition similar to that observed in renal dysfunction could increase the risk of tolvaptan-mediated hepatotoxicity. Increased plasma exposure due to renal dysfunction could result from disease progression; however, this hypothesized relationship between disease progression and susceptibility to hepatotoxicity requires clinical validation, as prior clinical studies were not designed to address this question. Nevertheless, it was desirable to have a population wherein the incidence rate was inflated; more individuals with ALT elevations permitted a more robust susceptibility factor analysis and on-treatment biomarker analysis than what would have been possible with the low number of individuals with ALT elevations from the Renally Sufficient SimPops alone. The time required for the simulated individuals to reach peak ALT averaged one month. Faster timing in the Renally Impaired SimPops relative to the Renally Sufficient SimPops was attributed to increased tolvaptan exposure in these simulations.

The Mechanistic Investigation Simulation results for the Renally Impaired SimPops listed in Table 8 demonstrated a pattern similar to the Renally Sufficient SimPops. The BA-Off and TVP-Off simulations, which eliminated bile acid-mediated toxicity and parent tolvaptan-mediated toxicity, had the most precipitous decline in ALT elevation frequency relative to the case

TABLE 8. Frequency of Simulated ALT Elevations in the Renally Impaired SimPops

Toxicity Mechanisms	Dose	ALT >3x ULN	Hy's Law
All	90/30 mg daily, 180 days	70/229	66/229
TVP-Off ^a	90/30 mg daily, 180 days	3/229	0/229
DM-4103-Off	90/30 mg daily, 180 days	48/229	41/229
ETCi-Off	90/30 mg daily, 180 days	23/229	17/229
BA-Off	90/30 mg daily, 180 days	6/229	4/229

^aThese simulation names refer to the Mechanistic Investigation Simulation names listed in Table 4. Mechanisms present and not present for each simulation can be found there.

where all mechanisms were present; however, the DM-4103-Off simulation and the ETCi-Off simulation produced notably fewer ALT elevations as well. These results strongly suggest that the injury observed in the clinic is multifactorial in nature, with bile acid transport inhibition by tolvaptan the primary contributor among the mechanisms included in this model.

Susceptibility Factors

Covariate analysis was conducted to identify patient parameter values that predicted tolvaptan-mediated DILI prior to treatment initiation. Such parameters have the potential to inform the search for screening assays to prospectively identify patients at risk for tolvaptan-mediated DILI. In particular, this analysis may identify factors that predispose patients with ADPKD to liver toxicity from tolvaptan. The results of the covariate analysis for the Renally Sufficient SimPops are shown in Table 9. Of the 23 parameters varied in the SimPops, 4 showed a statistically significant correlation with both ALT elevations and liver ATP decrement. Two of these parameters are related to mitochondrial function (basal ETC flux and respiratory reserve scaling factor), one of these parameters is related to bile acid transport (CDCA-amide canalicular V_{max}), and the other is related to drug distribution (body mass). The correlation coefficient is strongest for the mitochondrial function parameters, followed by the bile acid transport parameter.

The results for the Renally Impaired SimPops are shown in Table 10. For this SimPops, there were 6 parameters that showed a statistically significant correlation with both measures of injury: 2 mitochondrial function parameters (basal ETC flux and respiratory reserve scaling factor), 3 bile acid transport parameters (CDCA-amide canalicular V_{max} , CDCA amidation V_{max} , and canalicular regulation scaling factor), and one drug distribution parameter (metabolite generation V_{max}). These parameters include the 2 mitochondrial function parameters and the bile acid transport parameter identified as correlates of injury in the Renally Sufficient SimPops; indeed, these 3 parameters—basal ETC flux, respiratory reserve capacity, and CDCA-amide canalicular transport V_{max} —are the strongest correlates of injury in the Renally Impaired SimPops as well.

The area under the receiver-operating characteristic curve (AUROC) analysis was conducted to identify simulated patient outputs that predict tolvaptan-mediated DILI prior to injury presentation. These outputs have the potential to inform the identification of patient-monitoring biomarkers to prospectively identify patients at risk for tolvaptan-mediated DILI. The list of

TABLE 9. Results of Covariate Analysis for the Renally Sufficient SimPops

Parameter ^a	Max ALT		Min ATP	
	P-value	R-value ^b	P-value	R-value ^b
Body mass	.0408	−0.1353	.0039	0.19
Basal ETC flux	.0001	−0.2541	<.0001	0.4189
CDCA-amide canalicular V_{max}	<.0001	−0.2608	<.0001	0.2984
Respiratory reserve scaling factor	.0003	−0.2354	<.0001	0.3198

^aOnly parameters meeting the statistical threshold of $P < .05$ correlation with both dependent parameters are listed. Body mass is related to ADME; basal ETC flux and respiratory reserve scaling factor are related to mitochondrial function; CDCA-amide canalicular V_{max} is related to bile acid transport.

^bCorrelations were computed in JMP 9 (SAS, Cary, NC) using the Pearson's method.

TABLE 10. Results of Covariate Analysis for the Renally Impaired SimPops

Parameter ^a	Max ALT		Min ATP	
	P-value	R-value ^b	P-value	R-value ^b
Metabolite generation V_{max}	.0033	-0.1936	.0068	0.178474
Basal ETC flux	<.0001	-0.32336	<.0001	0.372894
CDCA amidation V_{max}	.008	-0.17472	.0226	0.150631
CDCA-amide canalicular V_{max}	<.0001	-0.51093	<.0001	0.549344
Respiratory reserve scaling factor	<.0001	-0.2651	<.0001	0.295598
Canalicular regulation scaling factor	.0333	-0.14072	.0298	0.14364

^aOnly parameters meeting the statistical threshold of $P < .05$ correlation with both dependent parameters are listed. Metabolite generation V_{max} is related to ADME; basal ETC flux and respiratory reserve scaling factor are related to mitochondrial function; CDCA-amide canalicular V_{max} , CDCA amidation V_{max} , and canalicular regulation scaling factor are related to bile acid transport.

^bCorrelations were computed in JMP 9 (SAS, Cary, NC) using the Pearson's method.

TABLE 11. Results for DILIsym Outputs with an Area Under the Receiver-Operating Characteristic Curve Score > 0.75

DILIsym Outputs	AUROC ^a
Blood CDCA	0.809
Blood bulk BA	0.743
Blood CDCA-amide	0.739
Blood LCA	0.738
Bulk bile acid biliary efflux	0.859
Total bile acid biliary efflux	0.811
Midlobular ETC activity	0.901
Periportal ETC activity	0.888
Centrilobular ETC activity	0.877

Other individual blood bile acids (with scores <0.75) are listed for comparison with CDCA.

^aAUROC = area under the receiver-operating characteristic curve.

single DILIsym outputs that demonstrated an AUROC value above 0.75 for early on-treatment prediction of ALT elevations is shown in Table 11. Blood bile acids were predicted to be potential biomarkers; several blood bile acid species, as well as total blood bile acids, were predicted to be moderately good predictors of hepatic injury. Decreased bile acid efflux from the liver, as would be measured by decreased fecal bile acids (Kakiyama et al., 2014), was predicted to be an even better potential biomarker for future tolvaptan-mediated injury. DILIsym predicted that electron transport chain activity would be the best biomarker for injury susceptibility if a reliable test existed; this is based on the fact that both increasing bile acid concentrations and ETC inhibition affect ETC activity. Combining 2 potential biomarkers did not improve the predicted quality of the biomarkers in DILIsym.

DISCUSSION

About 228 of 229 simulated individuals in the Renally Sufficient SimPops experienced no ALT elevation following treatment with 60 mg tolvaptan daily for 60 days, suggesting that the DILIsym parameter values for tolvaptan are consistent with the overall safe clinical experience in healthy subjects and non-ADPKD patients. The single simulated individual with an ALT elevation had NASH-like liver biochemistry and was unlikely to be representative of patients in the clinical trials. In contrast, 7.9% of simulated patients from the Renally Sufficient SimPops treated with a 90/30 mg split daily dosage regimen experienced

ALT elevations. This is higher than the 4.4% observed in the pivotal ADPKD clinical trial (Watkins et al., 2015), and the difference may be attributed to the absence within DILIsym of some compensatory mechanisms. For example, DILIsym does not yet include mitochondrial biogenesis, a response that has been qualitatively but not quantitatively characterized (Han et al., 2012). Because tolvaptan toxicity is mediated by direct mitochondrial toxicity and bile acid accumulation, which ultimately also impacts mitochondria, the lack of compensatory mechanisms may lead to overestimated liver injury. Despite this difference, reproducing largely safe responses for 60 mg qd and a low frequency of injury for 90/30 mg split daily dosing without further re-optimization of parameter values supports the contention that DILIsym can incorporate PK and mechanistic data in a clinically relevant manner. Mechanistic Investigation Simulations indicated that both bile acid transporter inhibition and mitochondrial dysfunction induced by tolvaptan and/or its metabolites contribute to the observed liver injury in vivo. Simulation of delayed onset in ALT elevations suggested that these intrinsic mechanisms should be considered as plausible contributors to the clinical observation of delayed liver injury.

The higher frequency of simulated ALT elevations in the Renally Impaired relative to the Renally Sufficient SimPops suggested that changes in tolvaptan and metabolite disposition as observed in renal dysfunction could increase the risk of tolvaptan-mediated hepatotoxicity. Notably, although the label "Renally Impaired" was used to reflect the biological basis for parameter selection, the PBPK optimization was conducted against high exposure data measured in ADPKD patients with preserved renal function (see Materials and Methods). It is plausible to posit that higher plasma exposure is related to the effects of renal impairment. These effects, however, do not appear to be a major causal factor in liver injury. As the susceptibility factor analysis pointed out, variability in parameters related to renal impairment were not major contributors to the simulated ALT elevations. The implication is that within the clinically relevant dosing range, increased plasma tolvaptan exposure is not readily predictive of potential toxicity on an individual basis, but that increased exposure for an entire population can reveal the underlying susceptibility of certain patients within that population, leading to a potentially higher overall rate of toxicity.

The Renally Impaired SimPops could also be considered a proxy for disease progression; as ADPKD progresses, renal capacity decreases, which would be expected to alter liver exposure and/or function. Either may result in increased likelihood

of toxicity. The tolcapone pattern of hepatotoxicity includes symptoms that appear up to 18 months after the initiation of treatment in some patients; and it is tempting to speculate that disease progression could contribute to the extremely long latency to liver injury (Watkins *et al.*, 2015). However, this hypothesis remains untested as clinical studies referenced in this work were not designed to address this question.

The factorial analysis predicted that both bile acid accumulation and mitochondrial dysfunction contribute to tolcapone-mediated liver injury, and that both parent compound and DM-4103 contribute to the toxicity as well. Multifactorial tolcapone hepatotoxicity is supported by a recent study that suggested compounds inhibiting BSEP and inducing mitochondrial dysfunction were more associated with severe human DILI (Aleo *et al.*, 2014).

Notably, the predicted multifactorial nature of tolcapone hepatotoxicity stands in direct contrast to previous work using DILIsym to identify the potential mechanisms of tolcapone-induced hepatotoxicity (Longo *et al.*, 2016). Tolcapone, like tolcapone, is both a bile acid transporter inhibitor and an inducer of mitochondrial dysfunction *in vitro*. However, only the mitochondrial dysfunction contributed to tolcapone hepatotoxicity. The contrast between these 2 compounds demonstrates how QSP modeling can add value to the results of *in vitro* assays by placing their results in the proper biological context.

The susceptibility factor analysis indicated that whereas increased exposure may reveal underlying susceptibility in some patients, higher exposure alone cannot be considered a risk factor for injury, and mechanistic markers of injury need to be employed in order to prospectively identify patients at risk for hepatotoxicity. However, none of the identified parameters has a reliable, accepted test that can be used to determine the value of this parameter in patients. The results, therefore, suggest a need to develop new genetic or functional markers that can assess baseline mitochondrial function and canalicular bile acid transporter activity in the liver. The predicted multifactorial nature of the injury also suggested that the identification of at-risk individuals would likely require a combination of several markers that can assess risk in terms of both bile acid transport capability and mitochondrial function. Research to investigate potential genetic markers is ongoing.

The late onset of clinically observed tolcapone hepatotoxicity—between 3 and 18 months (Watkins *et al.*, 2015)—suggests that there is an opportunity to identify patients on-treatment that are at increased risk of developing DILI before traditional biomarkers are elevated and before patients present with symptoms. Because DILIsym mechanistically represents drug-induced changes in the liver and time to peak ALT was often delayed, it was possible to mine the simulation results for model outputs whose changes predict later hepatotoxicity. These results, summarized above as on-treatment susceptibility factors presented by simulated AUROC, suggested that novel or emerging biomarkers that are mechanistically related to mitochondrial activity could be used to monitor patients. Bile acid concentrations perform passably as biomarkers; however, the inter- and intra-individual variability in plasma bile acid concentrations confounds their usage. To incorporate both mechanisms, a more effective approach would be an on-treatment measure of mitochondrial stress, because both bile acid accumulation and electron transport chain inhibition stress the mitochondria. The results presented here suggest that this

would be a promising area for investigation into novel biomarkers of tolcapone hepatotoxicity.

Whereas both mechanisms implicated by the *in vitro* assays were shown to contribute to simulated *in vivo* toxicity, neither plasma drug nor plasma metabolite concentrations emerged from the AUROC analysis as viable biomarkers, either as individual outputs or in combination with other outputs. This is consistent with clinical observations suggesting that an individual's exposure to tolcapone or its metabolites is not a reliable predictor of whether that individual will develop toxicity (Watkins *et al.*, 2015).

DILIsym tolcapone simulations provided a biologically plausible link between *in vitro* mechanistic data and human hepatotoxicity, and allowed for identification of patient susceptibility factors; however, there are a few discrepancies between the results reported here and other previously reported findings. First, there were more simulated severe cases than clinically observed. It is likely that compensatory mechanisms like mitochondrial biogenesis described above not only affect the frequency but also the severity of liver injury. Second, our data demonstrated no induction of oxidative stress by tolcapone or its metabolites at the concentrations tested. Data from other recently published tolcapone data (Wu *et al.*, 2015) showed that concentrations of 60 μM and higher caused a significant increase in ROS in HepG2 cells. They also showed minimal ROS increases at concentrations lower than 60 μM , which were consistent with results found from our study, as we did not test tolcapone at concentrations higher than 50 μM . Interestingly, a comparison of the *in vitro* data derived 24 h area under the curve (AUC) media exposure of HepG2 cells to tolcapone and the simulated 24 h AUC tolcapone exposure in the liver indicated that liver exposure is <5% of the *in vitro* exposure needed to induce oxidative stress. These data suggest that oxidative stress, as reported by Wu *et al.* (2015), is unlikely to impact the conclusions reported here. Finally, delayed onset DILI often is attributed to an adaptive immune response (Adams *et al.*, 2010; Holt and Ju, 2006). DILIsym does not yet include a representation of the adaptive immune response. However, simulations illustrated that tolcapone-mediated bile acid transporter inhibition can result in delayed onset DILI, similar to what was reported previously for troglitazone (Yang *et al.*, 2015). The inclusion of adaptive immunity might increase the simulated frequency of hepatotoxicity but could be counter-balanced by the inclusion of compensatory mechanisms described above. Also, there are currently no clear data describing tolcapone-specific T cell responses. Taken together, it can be argued that DILIsym has integrated the available tolcapone data and our current understanding of DILI to provide *in vivo* plausibility for 2 mechanisms of hepatotoxicity and direction for the pursuit of mechanistic biomarkers.

In conclusion, the results presented herein illustrate how DILIsym can be applied to better understand clinically observed hepatotoxicity and prospectively inform patient susceptibility. Efforts currently are underway to leverage these results in the validation of biomarkers to identify individuals at risk for tolcapone hepatotoxicity.

SUPPLEMENTARY DATA

Supplementary data are available online at <http://toxsci.oxfordjournals.org/>.

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