Drug-induced liver injury (DILI) is one of the primary reasons for the failure of pharmaceutical agents during drug development as well as the withdrawal of approved drugs from the market. Unfortunately, current in vitro screening approaches or in vivo preclinical studies do not adequately predict the DILI liability of new chemical entities. Low-incidence severe drug-related hepatotoxicity is typically not detected in the phase III clinical trials that involve a few thousand patients and may not be detected until the drug has been approved and administered to tens or hundreds of thousands of patients. These unexpected findings have led to black box warnings (e.g., bosentan, diclofenac, ketoconazole, and isoniazid), or in severe cases, withdrawal of the drug from the market (e.g., troglitazone (TGZ), lumiracoxib, ximelagatran, and bromfenac). TGZ was the first of the thiazolidinedione drugs approved in worldwide markets for the treatment of type 2 diabetes. During clinical trials, alanine transaminase (ALT) elevations >3× the upper limit of normal (ULN) in about 2% of patients, in addition to two cases of jaundice, were reported. All these patients recovered without permanent clinical complications, and TGZ was approved for marketing. However, after the broader diabetic population was exposed to TGZ, cases of liver failure associated with TGZ treatment were reported, and the drug was given a black box warning status with requirement for monthly monitoring of liver chemistries. TGZ was withdrawn from the market after rosiglitazone and pioglitazone—drugs from the same therapeutic class that demonstrated less concern about hepatotoxicity—were approved. Fourteen years have passed since the withdrawal of TGZ, but the mechanism(s) of TGZ-mediated hepatotoxicity has(ve) not been fully elucidated. Numerous mechanisms have been postulated, including inhibition of bile acid (BA) transport by TGZ and its major metabolite, TGZ sulfate (TS), which may cause hepatic accumulation of toxic BAs and subsequent liver injury (Figure 1). The bile salt export pump (BSEP) is a canalicular transporter that is predominantly responsible for biliary excretion of BAs. Impaired BSEP function due to genetic polymorphisms induces liver injury, and BSEP inhibition mediated by drugs has been associated with DILI. In vitro vesicular transport assays revealed that TGZ and TS are potent inhibitors
of BSEP and the multidrug resistance–associated protein 4 (MRP4), which are hepatic transporters that mediate biliary and basolateral efflux of BAs, respectively. However, TGZ has also been shown to inhibit sodium taurocholate cotransporting polypeptide (NTCP)-mediated BA uptake, which would reduce hepatic concentrations of BAs. In addition, hepatotoxicity signals were not detected during preclinical testing of TGZ, even though TGZ and TS are potent inhibitors of rat BSEP. Thus, the role that alteration in BA homeostasis plays in TGZ-mediated hepatotoxicity remains speculative. Although it is challenging to translate the results from isolated in vitro studies to in vivo models and from preclinical studies to humans, systems pharmacology modeling provides a useful approach to integrate data from different experimental systems, species, and biological knowledge to predict human DILI.

In the current study, a mechanistic model of DILI (DILIsym, http://www.dilisym.com, Supplementary Figure S1 online) was used to investigate (i) the role of BA transport inhibition in TGZ-mediated hepatotoxicity and (ii) the underlying mechanisms for the observed species differences. DILIsym includes submodels representing disposition of drugs and their metabolites, the physiology and pathophysiology of BAs, the hepatocyte life cycle, and liver injury biomarkers (e.g., serum ALT and bilirubin) (Figure 2). TGZ-mediated DILI responses were simulated in human and rat virtual populations (SimPops), which included variability in key model parameters. Potential risk factors for TGZ-mediated hepatotoxicity in humans in the context of BA inhibition also were assessed in human SimPops. The hepatotoxic potential of pioglitazone, a known BSEP inhibitor that is rarely associated with DILI, also was investigated as a negative control.

RESULTS
Physiologically based pharmacokinetic modeling
A physiologically based pharmacokinetic (PBPK) model was developed to describe the systemic disposition and hepatic concentrations of TGZ and TS in humans and male rats (Supplementary Figure S2a online). Simulated TGZ and TS plasma concentration–time profiles were within twofold of the mean observed concentrations in humans following a single oral dose of 400 mg TGZ (Supplementary Figure S3a online) and within threefold of the means in male rats following a single i.v. dose of 5 mg/kg TGZ (Supplementary Figure S3b online). In male rats administered a single oral dose of 5 mg/kg TGZ, the simulated TGZ plasma concentration–time profile was within 2.1-fold of the mean observed concentrations (Supplementary Figure S3b online).

Simulations of TGZ hepatotoxicity in human and rat virtual populations (SimPops)
To explore TGZ hepatotoxicity at the population level, we used previously constructed human and rat SimPops that incorporate variability in BA disposition; variability was added to six additional parameters that describe TGZ/TS disposition, body weight, and sensitivity of adenosine triphosphate (ATP) synthesis to hepatic BA accumulation. Several TGZ dose levels were simulated for the human SimPops (once-daily oral doses of 200, 400, or 600 mg for 6 months) and rat SimPops (once-daily oral doses of 5 or 25 mg/kg for 6 months). TGZ-mediated perturbations in BA disposition and DILI responses in the human and rat SimPops are presented in Figure 3. The simulated median (range) values of maximum postdose hepatic concentrations of chenodeoxycholic acid (CDCA) and lithocholic acid (LCA) species (sum of LCA, CDCA, and their conjugates) were 204 µmol/l (72–975), 273 µmol/l (99–1,539), and 314 µmol/l (118–2,610) at TGZ doses of 200, 400, and 600 mg/day, respectively, compared with a baseline value of 14 µmol/l (2–127). The baseline human hepatic ATP concentration in the current model was 4.2 mmol/l. Hepatic BA accumulation led to a decrease in both hepatic ATP levels and viable liver mass in a subset of the human SimPops; the simulated median (range) values of minimum postdose hepatic ATP concentrations were 4.13 mmol/l (3.26–4.19), 4.10 mmol/l (2.43–4.18), and 4.07 mmol/l (2.07–4.18) at TGZ doses of 200, 400, and 600 mg/day, respectively. Corresponding values for fractional viable liver mass were 1.00 (0.63–1.00), 1.00 (0.15–1.00), and 1.00 (0.15–1.00). The incidences of elevated serum ALT, serum total bilirubin, and Hy’s Law cases (serum ALT >3× the ULN and serum bilirubin >2× the ULN) in the human SimPops are summarized in Table 1; the reported incidences of ALT elevations and jaundice in the clinical trials are also listed. In the human SimPops, 200–600 mg/day TGZ induced elevations in serum ALT >3× the ULN in 0.3–5.1% of the population; Hy’s
Law cases were observed in 0.3–3.6% of the human SimPops. The incidence of ALT elevation was similar to observations from the clinical trials, in which 200–600 mg/day TGZ induced serum ALT elevations >3× the ULN in 1.9% of treated patients. The values for time to peak ALT in the human SimPops with ALT elevations >3× the ULN were 118 ± 61 and 111 ± 61 days at TGZ doses of 400 and 600 mg/day, respectively; these are comparable to the 147 ± 86 days observed during the clinical trials. Simulated time-course dynamics of serum ALT and viable liver mass in susceptible individuals (serum ALT >3× the ULN) are presented in Figure 4. In the rat SimPops, the simulated median (range) values of maximum hepatic postdose concentrations of CDCA and LCA species were 27 µmol/l (5–105) and 44 µmol/l (7–151) at TGZ doses of 5 and 25 mg/kg/day, respectively, compared with baseline values of 13.8 µmol/l (2.4–126.6). The baseline rat hepatic ATP concentration was 2.0 mmol/l. Simulated median (range) minimum hepatic ATP concentrations after TGZ doses of 5 and 25 mg/kg/day were 1.96 mmol/l (1.66–1.99) and 1.92 mmol/l (1.49–1.99), respectively. The corresponding values for fractional viable liver mass were 1.00 (0.99–1.00) and 1.00 (0.92–1.00), respectively. None of the rat SimPops exhibited serum ALT elevations >3× baseline (21 U/l).

Sensitivity analysis
To investigate the sensitivity of DILI responses to transporter inhibition constants, simulations were performed with 10-fold smaller and larger inhibition constants of TGZ/TGZ for BSEP, MRP4, and NTCP. Simulated maximum serum ALT levels in human and rat SimPops treated with TGZ (600 mg/day for humans and 5 mg/kg/day for rats) for 1 month are presented in Figure 5. In the human SimPops, serum ALT levels were sensitive to the inhibition constant ($K_i$) value of TGZ/TGZ for BSEP inhibition; when BSEP $K_i$ was decreased 10-fold (assuming 10-fold more potent inhibition), 15.7% of the population exhibited serum ALT >3× the ULN, as compared with only 3.6% of the population with the measured BSEP $K_i$. None of the individuals showed elevated serum ALT >3× the ULN when BSEP $K_i$ was increased 10-fold (assuming 10-fold less potent inhibition). The $K_i$ of TGZ/TGZ for MRP4 inhibition also influenced serum ALT elevations but to a smaller extent compared with BSEP $K_i$; the incidence of serum ALT elevations >3× the ULN ranged 2.1–3.6% when the MRP4 $K_i$ was decreased or increased by 10-fold. Modulation of the $K_i$ for NTCP inhibition by TGZ/TGZ led to opposite effects as compared with modulation of BSEP and MRP4 $K_i$; a decrease in the NTCP $K_i$ by 10-fold led to a decreased incidence of elevations in serum ALT >3× the ULN (2.1% of the population), whereas an increase in the NTCP $K_i$ by 10-fold increased the incidence of elevations in serum ALT >3× the ULN (3.9% of the population), as compared with an incidence of 3.6% with the measured NTCP $K_i$. These findings are consistent with the suggested protective role of BA uptake inhibition by some drugs in hepatic BA accumulation and subsequent DILI. In the rat SimPops, simulated serum ALT levels did not exceed 3× baseline values (21 U/l) even when $K_i$ values for BSEP or MRP4 were decreased by 10-fold or when the $K_i$ for NTCP was increased 10-fold. These results support the hypothesis that TGZ is not expected to be hepatotoxic in rats.
Hepatotoxicity was not predicted in the baseline human simulation, which did not include population variability (data not shown), whereas simulations with human SimPops revealed a subset of individuals susceptible to TGZ-mediated hepatotoxicity. To identify the most important parameters in our model in the context of BA-mediated DILI, multiple-regression analysis was performed with the lowest postdose hepatic ATP levels as the dependent variable and the 16 parameters used to develop the human SimPops as independent variables. Table 2

Figure 3 Simulated drug-induced liver injury (DILI) responses in human and rat virtual populations (SimPops) at specified troglitazone (TGZ) dose levels. Predicted maximum postdose hepatic accumulation of chenodeoxycholic acid (CDCA) and lithocholic acid (LCA) species and DILI responses (i.e., minimum hepatic ATP, minimum viable liver mass, and maximum serum ALT) in (a) human SimPops at oral doses of 200 (green triangle), 400 (blue circle), or 600 (red diamond) mg/day TGZ for 6 months, and (b) rat SimPops at oral doses of 5 (blue circle) or 25 (red diamond) mg/kg/day for 6 months. ALT, alanine transaminase; ATP, adenosine triphosphate.
lists the statistical significance (P values) and standardized coefficients of the parameters varied in human SimPops. Among the 16 parameters varied in human SimPops, 7 parameters were statistically significant predictors of hepatic ATP levels; the maximum rate of LCA sulfate biliary excretion was the most important variable influencing hepatic ATP decline, followed by the maximum rate of LCA synthesis in the intestinal lumen, the canalicular efflux regulation scaling factor, biliary clearance of TS, body weight, the toxicity $K_m$ for CDCA and LCA species, and the maximum rate of CDCA amide biliary excretion.

**DISCUSSION**

BA transport inhibition by TGZ and its major metabolite, TS, is one proposed mechanism of TGZ-mediated hepatotoxicity. Although TGZ and TS are potent inhibitors of BA transporters in isolated membrane vesicle systems, the relationship between BA transport inhibition and *in vivo* hepatotoxicity has not been evaluated. In the current study, a mechanistic model of DILI was used to investigate the hepatotoxic potential of TGZ via BA transport inhibition in humans and rats. It is important to consider population variability when predicting BA-mediated hepatotoxicity.
hepatotoxicity due to the large variability in BA exposure and the low incidence of hepatotoxicity.\textsuperscript{2,24} Nonexistence of TGZ-mediated DILI in the baseline human model in the current study also supports the necessity of population-based analysis. Therefore, human and rat SimPops that included variability in key parameters, such as BA disposition, TGZ and TS disposition, body weight, and sensitivity of ATP synthesis to hepatic BA accumulation, were used to investigate the hepatotoxic potential of TGZ at the population level.

At common clinical doses (200–600 mg/day), the simulated incidence of elevated serum ALT >3× the ULN was 0.3–5.1%, which was similar to that observed in clinical trials (1.9%) (Table 1). Hy’s Law cases were observed in 0.3–3.6% of human SimPops, whereas two cases of jaundice (0.08%, both Hy’s Law cases) relevant to TGZ treatment were reported in clinical trials (Table 1). The incidence of serum bilirubin elevations might have been overestimated in the simulations because TGZ was not discontinued even when serum ALT was increased, in contrast to the usual situation in a clinical trial. Simulations also adequately predicted the delayed time to peak ALT observed in clinical trials (Table 1). The delayed ALT elevations in the present mechanistic model were driven by a delayed buildup of toxic BAs in hepatocytes, which resulted from two factors: (i) farnesoid X receptor–mediated feedback regulation of BA synthesis/transport initially delayed BA accumulation until it could no longer compensate and (ii) competitive inhibition of biliary BA excretion by TGZ/TG’s occurred. The impact of a competitive inhibitor on BA transport decreases as hepatic BA concentrations increase and begin to outcompete the inhibitor, which slows down the rate of accumulation.\textsuperscript{20}

It should be noted that a delay in DILI presentation of weeks to months is characteristic of idiosyncratic hepatotoxicity produced by multiple drugs.\textsuperscript{25} With some drugs, the risk of DILI has been associated with specific human leukocyte antigen alleles, suggesting that latency in onset may in part reflect the time required to mount an adaptive immune response. However, it should not take several months to mount an adaptive immune response, suggesting that evolution of nonimmunological events precedes initiation of adaptive immunity in these cases. Moreover, the largest genome-wide association analysis of all-cause DILI to date did not find evidence for human leukocyte antigen associations once DILI cases attributed to flucloxacillin and amoxicillin–clavulanate were excluded.\textsuperscript{26} This observation, together with our accurate modeling of the latency associated with TGZ-mediated DILI based on altered BA homeostasis alone, supports the conclusion that adaptive immunity may not underlie most cases of idiosyncratic DILI.

Preclinical animals are less sensitive to BA-mediated DILI as compared with humans and thus do not reliably predict human hepatotoxicity that involves BA transport inhibition.\textsuperscript{23,27} Toxicity signals for TGZ were not detected during the standard preclinical toxicity testing before approval, and minimal hepatotoxicity was observed in 104 weeks of long-term toxicity studies.\textsuperscript{28} A unifying hypothesis is that differential hepatotoxicity of TGZ could be attributed to species differences in toxic BA profiles. Rats have a hydrophilic and thus less toxic BA pool; CDCA,
Table 2  List of parameters that were varied in the human and rat SimPops and results of multiple-regression analysis in human SimPops administered 600 mg/day troglitazone (TGZ) for 6 months

| Parameter name                      | Parameter description                                      | Significance | Standardized coefficienta |
|-------------------------------------|------------------------------------------------------------|--------------|----------------------------|
| Bile acid homeostasis submodel      |                                                            |              |                            |
| LCA sulfate uptake $V_{max}^a$      | Maximum velocity of hepatic uptake of LCA sulfate          | NS           | −0.08                      |
| LCA sulfate canicular efflux $V_{max}^a$ | Maximum velocity of biliary excretion of LCA sulfate   | $P < 0.001$  | 0.41                       |
| CDCA amide uptake $V_{max}^a$       | Maximum velocity of hepatic uptake of CDCA amide          | NS           | −0.01                      |
| CDCA amide canicular efflux $V_{max}^a$ | Maximum velocity of biliary excretion of CDCA amide | $P < 0.01$  | 0.14                       |
| CDCA amide basolateral efflux $V_{max}^a$ | Maximum velocity of hepatic basolateral efflux of CDCA amide | NS ($P = 0.06$) | 0.08                       |
| CDCA amidation $V_{max}^a$          | Maximum velocity of CDCA amidation in hepatocytes         | NS           | 0.06                       |
| LCA amide sulfate $V_{max}^a$       | Maximum velocity of LCA amide sulfate in hepatocytes      | NS           | −0.06                      |
| LCA synthesis $V_{max}^a$           | Maximum velocity of LCA synthesis by the gut microbiome   | $P < 0.001$  | −0.21                      |
| Uptake regulation scaling factor    | Scaling factor governing the magnitude of feedback regulation of hepatic uptake transporter function by hepatic bile acid accumulation | NS           | 0.02                       |
| Canicular efflux regulation scaling factor | Scaling factor governing the magnitude of farnesoid X receptor-mediated feedback regulation of hepatic canicular transporter function by hepatic bile acid accumulation | $P < 0.001$  | 0.2                        |
| LCA hydroxylation $V_{max}^b$       | Maximum velocity of LCA hydroxylation in hepatocytes      | NA           | NA                         |
| Drug PBPK submodel                  |                                                            |              |                            |
| TGZ intestinal absorption $K_{ab}$  | First-order rate constant for TGZ absorption from intestine | NS           | −0.05                      |
| TGZ hepatic uptake $V_{max}^a$      | Maximum velocity of TGZ hepatic uptake                    | NS           | −0.07                      |
| TGZ sulfation $V_{max}^a$           | Maximum velocity of TS formation                          | NS           | −0.06                      |
| TS biliary clearance                | Biliary clearance of TS                                   | $P < 0.001$  | 0.15                       |
| Other system-specific parameters    |                                                            |              |                            |
| Body weightc                        | Body weight                                               | $P < 0.001$  | 0.15                       |
| Toxicity $K_{in}$ for CDCA and LCA speciesc | Intracellular bile acid concentrations that induce half-maximal inhibition of ATP synthesis | $P < 0.001$  | 0.15                       |

Human and rat population samples incorporating variability in parameters governing bile acid homeostasis (bile acid SimPops) have been constructed previously.20 Four parameters in the drug PBPK submodel and two system-specific parameters also were varied. (See Supplementary Data online for methods and data used for construction of SimPops.) In the human SimPops administered 600 mg/day TGZ for 6 months, a multiple-regression analysis was performed to identify the most important parameters in TGZ-mediated hepatotoxicity using 16 varied parameters as independent variables and minimum hepatic ATP as the dependent variable. Statistical significance and standardized coefficients were calculated using JMP 10.

ATP, adenosine triphosphate; CDCA, chenodeoxycholic acid; LCA, lithocholic acid; $K_{ab}$, first-order rate constant for absorption; NA, not available; NS, not significant; PBPK, physiologically based pharmacokinetic; TGZ, troglitazone; TS, TGZ sulfate; $V_{max}$, maximum velocity.

aParameter estimates that would have resulted from the regression if all the variables had been standardized to a mean of 0 and a variance of 1. The greater the absolute value of the standardized coefficient, the greater the effects of the independent variable on the model output.26 Used in rat SimPops only.27 Used in human SimPops only.

the most widely implicated BA in cholestatic liver injury,29 is one of the dominant BAs in humans, whereas it contributes a smaller proportion of the BA pool in rats and mice.24,30 Less toxic trihydroxy BAs, such as cholic acid and muricholic acid, are more abundant in rodents.30 LCA, the most hydrophobic and potentially toxic BA, is predominately sulfated in humans, whereas LCA primarily undergoes 6β-hydroxylation to form muridexoxycholic acid in rats.31 In DILIsym, CDCA, LCA, and their conjugates were exclusively modeled as the toxic BAs.20 Simulated maximum hepatic concentrations of CDCA and LCA species in the human SimPops administered 200–600 mg/day TGZ were 72–2,610 µmol/l (Figure 3a). Although hepatic BA concentrations after administration of BA transport inhibitors to humans have not been reported, several investigations showed that concentrations of hepatic BAs increased up to 215 ± 39 and 1,961 µmol/l in patients with end-stage chronic cholestatic liver disease and hepatolithiasis, respectively,32,33 suggesting that simulated hepatic BA concentrations are not physiologically unrealistic. In rat SimPops administered 5–25 mg/kg/day TGZ, simulated maximum hepatic concentrations of CDCA and LCA species ranged from 5 to 151 µmol/l (Figure 3b). This is much lower as compared with the levels in humans due to the hydrophobic BA pool and the detoxification of LCA by hydroxylation20; hepatotoxicity was not predicted based on the rat SimPops. Sensitivity analysis also revealed that rat SimPops did not exhibit hepatotoxicity even with 10-fold lower (more potent) inhibition constants for BA efflux transporters (Figure 5). These results demonstrated that a mechanistic model that incorporates species differences in BA homeostasis correctly predicted differential hepatotoxicity of TGZ in humans vs. rats.

Only a small subset of patients treated with TGZ experienced elevated serum ALT, indicating that certain patients...
are more susceptible to TGZ-mediated toxicity. Multiple linear regression analysis identified potential risk factors for TGZ-mediated hepatotoxicity associated with BA transport inhibition (Table 2). Decreased expression and/or function of hepatic canalicular transporters may decrease biliary excretion of BAs (via BSEP) and TS (via breast cancer resistance protein and MRP2), resulting in increased hepatic exposure to toxic BAs and perpetrator drugs/metabolites. Decreased function of basolateral efflux transporters (e.g., MRP4, MRP3, and organic solute transporter α/β), which are important compensatory pathways for BA excretion when biliary excretion is impaired,33 could potentiate hepatic accumulation of toxic BAs. LCA is synthesized from CDCA in the intestine by the gut microbiome, but the rate and variability of LCA synthesis have not been well characterized. One approach to test these susceptibility factors that were identified by modeling would be to interrogate genetic polymorphisms with known functional changes.35–37 However, genomic DNA was not archived from the TGZ clinical trials, and no patient has received TGZ treatment in almost 2 decades. Therefore, cases of TGZ-mediated hepatotoxicity are not present in the current DILI registries or DNA banks. An alternative approach would be to retrospectively recruit subjects who experienced and those who did not experience TGZ-mediated hepatotoxicity for phenotyping studies using selected transporter substrates as in vivo probes38,39; next-generation sequencing also could be used to profile the gut microbiome. However, the DILI event and the passage of time could alter these phenotypes. Although it would be challenging to test the hypothesis regarding risk factors for TGZ-mediated hepatotoxicity, an important message is that these genetic and phenotyping approaches could be used to test modeling results with drugs in current and future clinical development. Body weight was identified as a significant predictor of hepatotoxicity because a fixed dose was used in SimPops, which led to different dosages per kilogram body weight. Because liver weight, blood flow, and hepatic enzyme/transporter expression were proportional to body weight in the current model, individuals with higher body weight were able to clear drugs faster, resulting in lower hepatic exposure to TGZ and TS. In the clinic, however, body weight may correlate with many other factors, and rarely has it been identified as a risk factor for DILI.

Pioglitazone, another thiazolidinedione drug that is still used in diabetic patients, is rarely associated with hepatotoxicity.40 Pioglitazone also was identified as a potent inhibitor of BSEP and MRP4 in vesicular transport assays; steady-state plasma concentrations of TGZ and pioglitazone were comparable.11 These data suggest that the hepatotoxic potential of these compounds cannot be differentiated using in vitro transporter inhibition and systemic exposure data alone. Simulations using DILIsym showed that no hepatotoxicity was predicted in the human SimPops at clinical doses of pioglitazone, mainly due to the low hepatic exposure of pioglitazone as a result of extensive hepatic metabolism (see Supplementary Data and Supplementary Figure S4 online for pioglitazone modeling results). These results re-emphasize that drug/metabolite concentrations at the site of toxicity (hepatoacellular concentrations in this case) need to be considered when predicting toxicity.41 TGZ also is metabolized extensively in the liver, but its major metabolite, TS, is a more potent BSEP inhibitor, contributing to BA accumulation and subsequent hepatotoxicity. Hepatic exposure and the inhibitory effects on hepatic transporters of pioglitazone metabolites have not been investigated experimentally and thus were not included in the current simulation. Systemic exposure of the major metabolite of pioglitazone, M-IV, is more than 10-fold smaller as compared with that of TS at their respective clinical doses.52,43 Pioglitazone metabolites may be less likely to cause hepatotoxicity, but further studies are warranted to confirm this. The current study demonstrated that systems pharmacology modeling that integrates physiological information and experimental data was able to predict differential hepatotoxicity between TGZ and pioglitazone.

Increasing evidence supports the hypothesis that drug-mediated functional disturbances in hepatic BA transporters leads to intracellular accumulation of potentially harmful BAs and subsequent hepatic injury. A systematic investigation of a panel of drugs for their inhibitory effects on BA efflux transporters using isolated membrane vesicles and hepatotoxic potential demonstrated that inhibition of BA efflux transporters is associated with DILI.11–13,44 More sophisticated model systems such as sandwich-cultured hepatocytes have been used to assess the effects of drugs and their metabolites on hepatic accumulation of BAs.33,45–47 However, results from in vitro systems may not always translate directly to in vivo hepatotoxicity risk due to the complexity of BA homeostasis (i.e., regarding vectorial transport and enterohepatic recirculation), dynamic changes in the systemic and hepatic exposures of drugs/metabolites, and feedback regulation of BA synthesis and transport as an adaptive response to hepatic BA accumulation.58,49 It is noteworthy that knowledge gaps exist in BA homeostasis, as discussed above and elsewhere,20 which may limit accurate, quantitative prediction of BA-mediated DILI. Nonetheless, systems pharmacology modeling incorporating (i) physiology/pathophysiology of BAs in humans and rats, (ii) systemic and hepatic disposition of drugs/metabolites, and (iii) in vitro inhibition potency data reasonably predicted altered BA disposition in rats administered glibenclamide20 and also adequately predicted delayed presentation and species differences in TGZ hepatotoxicity. Although effects of pioglitazone metabolites were not incorporated due to lack of data, differential hepatotoxicity between TGZ and pioglitazone was predicted correctly in the current study. It is also important to note that use of the preexisting human SimPops, with no modification except for adding variability to the parameters describing drug disposition, body weight, and sensitivity of ATP synthesis to hepatic BA accumulation, accurately predicted the incidence of TGZ hepatotoxicity; the current mechanistic model was truly predictive. These findings suggest that systems pharmacology modeling combined with population analysis may provide a useful tool to (i) integrate our current knowledge about physiological and experimental data obtained during the drug development process and (ii) prospectively predict the hepatotoxic potential of new chemical entities that are in the drug development pipeline.
METHODS

PBPK model development. A PBPK model was developed to describe the disposition of TGZ/TS in humans and male rats, and that of pioglitazone in humans (Figure 2 and Supplementary Figure S3 online). Details regarding the construction and final structure of the PBPK model are provided in Supplementary Figure S2a and Supplementary Table S1 online.

Construction of human and rat population samples (SimPops). Human (n = 331) and rat (n = 191) population samples with variability in 10 (human) or 11 (rat) parameters in the BA homeostasis submodel (BA SimPops) were constructed previously within DILIsym using the genetic algorithm in MATLAB®; these BA SimPops are system specific, and thus the same BA SimPops are used to simulate the hepatotoxic effects of different compounds. In the current study, parameters governing TGZ and TS disposition (humans and rats), pioglitazone disposition (humans), body weight (humans), and sensitivity of hepatic ATP decline to hepatic BA accumulation (humans) also were varied using the probability distribution of each parameter obtained from the literature. Parameters that varied in SimPops and the literature data used to construct human and rat SimPops are listed in Table 2 and Supplementary Table S3 online. Details related to construction of the SimPops can be found in the Supplementary Data online.

Simulation of DILI responses. Perturbation of BA disposition and DILI responses after TGZ administration in human (200, 400, or 600 mg/day (common clinical doses) for 6 months) and rat (5 (equivalent to the clinical dose) or 25 mg/kg/day for 6 months) SimPops, as well as after pioglitazone administration in human SimPops (15, 30, or 45 mg/day (common clinical doses) for 6 months) were simulated using PBPK model predictions of TGZ/TS or pioglitazone disposition, a previously developed BA homeostasis submodel, and BA transport inhibition constants for TGZ/TS or pioglitazone (i.e., K, and half-maximal inhibitory concentration) measured in isolated membrane vesicle transport systems or primary hepatocytes (Supplementary Table S2 online). To assess the sensitivity of DILI responses to inhibition constants, simulations were performed with 10-fold smaller or greater inhibition constants of TGZ/TS or pioglitazone for BSEP, MRP4, and NTCP in human (600 mg/day TGZ or 45 mg/day pioglitazone) and rat SimPops (5 mg/kg/day TGZ). Simulations for the sensitivity analyses were performed for 1 month due to the extensive computational time required for long-term simulations and also because even for the individuals with delayed presentation of hepatotoxicity, slight increases (<3× the ULN) in serum ALT could be detected within 1 month of simulation. To identify the most important parameters in the context of BA-mediated DILI in humans administered TGZ, a multiple-regression analysis was performed with minimum hepatic ATP as the dependent variable. Hepatic ATP was selected because perturbations in cellular ATP synthesis is a key step in the development of BA-mediated DILI in the current model (Figure 2) and thus is the most sensitive and variable model output as compared with other DILI responses (i.e., serum ALT and fractional viable liver mass). Sixteen parameters used to develop the human SimPops were utilized as independent variables. Because the units of independent variables were different by orders of magnitude, standardized coefficients were calculated to determine the independent variables that have a greater effect on the minimum hepatic ATP. Statistical analyses were performed using JMP 10 (SAS, Cary, NC).

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/cpt

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AUTHOR CONTRIBUTIONS

K.Y., J.L.W., P.B.W., B.A.H., and K.L.R.B wrote the manuscript. K.Y., J.L.W., P.B.W., B.A.H., and K.L.R.B designed the research. K.Y. performed the research. K.Y., J.L.W., P.B.W., B.A.H., and K.L.R.B analyzed the data.

CONFLICT OF INTEREST

The authors declared no conflict of interest.

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1. Temple, R.J. & Himmel, M.H. Safety of newly approved drugs: implications for prescribing. JAMA 287, 2273–2275 (2002).
2. Watkins, P.B. & Whitcomb, R.W. Hepatic dysfunction associated with troglitazone. N. Engl. J. Med. 338, 916–917 (1998).
3. Endocrinologic and Metabolic Drugs Advisory Committee Meeting, 26 April 1999. <http://www.fda.gov/ohrms/dockets/ac/99/transp/35499t1a.pdf>.
4. Accepted 25 January 2014.
5. Isley, W.L. Hepatotoxicity of thiazolidinediones. Expert Opin. Drug Saf. 2, 581–586 (2003).
6. Smith, M.T. Mechanisms of troglitazone hepatotoxicity. Chem. Res. Toxicol. 16, 679–687 (2003).
7. Choikier, M. Troglitazone and liver injury: in search of answers. Hepatology 41, 237–246 (2005).
8. Perez, M.J. & Britz, O. Bile-acid-induced cell injury and protection. World J. Gastroenterol. 15, 1677–1689 (2009).
9. Maillote de Buy Wenniger, L. & Beuers, U. Bile salts and cholestasis. Dig. Liver Dis. 42, 409–418 (2010).
10. Jansen, P.L. et al. Hepatocanicular bile salt export pump deficiency in patients with progressive familial intrahepatic cholestasis. Gastroenterology 141, 1370–1379 (1999).
11. van Mil, S.W. et al. Benign recurrent intrahepatic cholestasis type 2 is caused by mutations in ABCB11. Gastroenterology 127, 379–384 (2004).
12. Morgan, R.E. et al. A multifactorial approach to hepatobiliary transporter assessment enables improved therapeutic compound development. Toxicol. Sci. 136, 216–241 (2013).
13. Dawson, S., Stahl, S., Paul, N., Barber, J. & Kenna, J.G. In vitro inhibition of the bile salt export pump correlates with risk of cholestatic drug-induced liver injury in humans. Drug Metab. Dispos. 40, 130–138 (2012).
14. Pedersen, J.M. et al. Early identification of clinically relevant drug interactions with the human bile salt export pump (BSEP/ABC11). Toxicol. Sci. 136, 328–343 (2013).
15. Funk, C., Ponnelle, C., Scheuermann, G. & Pantze, M. Cholestatic potential of troglitazone as a possible factor contributing to troglitazone-induced hepatotoxicity: in vivo and in vitro interaction at the canalicular bile salt export pump (Bsep) in the rat. Mol. Pharmacol. 59, 627–635 (2001).
16. Yang, K., Yue, W., Koeck, K. & Brouwer, K.L.R. Interaction of troglitazone sulfate with hepatic basolateral and canaliculat transport proteins. 2011 AAPS Annual Meeting and Exhibition Washington, DC, 23–27 October 2011.
17. Marion, T.L., Leslie, E.M. & Brouwer, K.L.R. Use of sandwich-cultured hepatocytes to evaluate impaired bile acid transport as a mechanism of drug-induced hepatotoxicity. Mol. Pharm. 4, 911–918 (2007).
18. Shoda, L.K., Woodhead, J.L., Siler, S.Q., Watkins, P.B. & Howell, B.A. Linking physiology to toxicology using DILIsim®, a mechanistic mathematical model of drug-induced liver injury. Biopharm. Drug Dispos. 35, 33–49 (2014).
19. Howell, B.A. et al. In vitro to in vivo extrapolation and species response comparisons for drug-induced liver injury (DILI) using DILIsym™, a mechanistic mathematical model of DILI. J. Pharmacokinet. Pharmacodyn. 39, 527–541 (2012).
20. Woodhead, J.L. et al. An analysis of N-acetylcysteine treatment for acetaminophen overdose using a systems model of drug-induced liver injury. J. Pharmacol. Exp. Ther. 342, 529–540 (2012).
21. Woodhead, J.L. et al. Mechanistic modeling reveals the critical knowledge gaps in bile acid-mediated DILI. CPT Pharmacometrics Syst. Pharmacol. 3, e123 (2014).
22. Loi, C.M., Randinitis, E.J., Vassos, A.B., Kazierad, D.J., Koup, J.R. & Sedman, A.J. Lack of effect of type II diabetes on the pharmacokinetics of troglitazone in a multiple-dose study. J. Clin. Pharmacol. 37, 1114–1120 (1997).
22. Izumi, T. et al. Prediction of the human pharmacokinetics of troglitazone, a new and extensively metabolized antidiabetic agent, after oral administration, with an animal scale-up approach. *J. Pharmacol. Exp. Ther.* **277**, 1630–1641 (1996).

23. Izumi, T., Hosiyama, K., Enomoto, S., Sasahara, K. & Sugiyama, Y. Pharmacokinetics of troglitazone, an antidiabetic agent: prediction of *in vivo* stereoselective sulfation and glucuronidation from *in vitro* data. *J. Pharmacol. Exp. Ther.* **280**, 1392–1400 (1997).

24. Trottier, J., Caron, P., Straka, R.J. & Barbier, O. Profile of serum bile acids in noncholestatic volunteers: gender-related differences in response to fenofoibrate. *Clin. Pharmacol. Ther.* **90**, 279–286 (2011).

25. Watkins, P.B. Idiosyncratic liver injury: challenges and approaches. *Toxicol. Pathol.* **33**, 1–5 (2005).

26. Urban, T.J., Goldstein, D.B. & Watkins, P.B. Genetic basis of susceptibility to drug-induced liver injury: what have we learned and where do we go from here? *Pharmacogenomics* **13**, 735–738 (2012).

27. Leslie, E.M., Watkins, P.B., Kim, R.B. & Brouwer, K.L.R. Differential inhibition of rat and human Na+-dependent taurocholate cotransporting polypeptide (NTCP/SLC10A1) by bosentan: a mechanism for species differences in hepatotoxicity. *J. Pharmacol. Exp. Ther.* **321**, 1170–1178 (2007).

28. Herman, J.R. et al. Rodent carcinogenicity with the thiazolidinedione antidiabetic agent troglitazone. *Toxicol. Sci.* **68**, 226–236 (2002).

29. Greim, H. et al. Mechanism of cholestasis. 6. Bile acids in human livers with or without biliary obstruction. *Gastroenterology* **63**, 846–850 (1972).

30. Garcia-Cañaveras, J.C., Donato, M.T., Castell, J.V. & Lahoz, A. Targeted profiling of circulating and hepatic bile acids in human, mouse, and rat using a UPLC-MRM-MS-validated method. *J. Lipid Res.* **53**, 2231–2241 (2012).

31. Hofmann, A.F. Detoxyfication of lithocholic acid, a toxic bile acid: relevance to drug hepatotoxicity. *Drug Metab. Rev.* **36**, 703–722 (2004).

32. Shoda, J., Tanaka, N., He, B.F., Matsuzyaki, Y., Osuga, T. & Miyazaki, H. Alterations of bile acid composition in galatstones, bile, and liver of patients with hepatolithiasis, and their etiological significance. *Dig. Dis. Sci.* **38**, 2130–2141 (1993).

33. Fischer, S., Beuers, U., Spengler, U., Zwiebel, F.M. & Koebe, H.G. Hepatic levels of bile acids in end-stage chronic cholestatic liver disease. *Clin. Chim. Acta* **251**, 173–186 (1996).

34. Trauner, M., Wagner, M., Fickert, P. & Zollner, G. Molecular regulation of hepatobiliary transport systems: clinical implications for understanding and treating cholestasis. *J. Clin. Gastroenterol.* **39**, S111–S124 (2005).

35. leiri, I., Higuchi, S. & Sugiyama, Y. Genetic polymorphisms of uptake (OATP1B1, 1B3) and efflux (MRP2, BCRP) transporters: implications for interindividual differences in the pharmacokinetics and pharmacodynamics of statins and other clinically relevant drugs. *Expert Opin. Drug Metab. Toxicol.* **5**, 703–729 (2009).

36. Lang, C. et al. Mutations and polymorphisms in the bile salt export pump and the multidrug resistance protein 3 associated with drug-induced liver injury. *Pharmacogenet. Genomics* **17**, 47–60 (2007).

37. Meier, Y. et al. Increased susceptibility for intrahepatic cholestasis of pregnancy and contraceptive-induced cholestasis in carriers of the 131T>C polymorphism in the bile salt export pump. *World J. Gastroenterol.* **14**, 38–45 (2008).

38. Pfeifer, N.D. et al. Effect of ritonavir on (99m)technetium-mebrofenin disposition in humans: a semi-PBPK modeling and *in vitro* approach to predict transporter-mediated DDIs. *CPT Pharmacometrics Syst. Pharmacol.* **2**, e20 (2013).

39. Kusuha, H. Imaging in the study of membrane transporters. *Clin. Pharmacol. Ther.* **94**, 33–36 (2013).

40. Livertox Database: pioglitzzone. <http://livertox.nlm.nih.gov/Pioglitzzone.htm>. Accessed 4 April 2014.

41. Chu, X. et al.; International Transporter Consortium. Intracellular drug concentrations and transporters: measurement, modeling, and implications for the liver. *Clin. Pharmacol. Ther.* **94**, 126–141 (2013).

42. Pfeifer, N.D. et al. Mutations and polymorphisms in the bile salt export pump. *World J. Gastroenterol.* **14**, 38–45 (2008).

43. Eckland, D.A. & Danhof, M. Clinical pharmacokinetics of pioglitzzone. *Exp. Clin. Endocrinol. Diabetes* **2**, S234–S242 (2000).

44. Köck, K. et al. Risk factors for development of cholestatic drug-induced liver injury: inhibition of hepatic basolateral bile acid transporters multidrug resistance-associated proteins 3 and 4. *Drug Metab. Dispos.* **42**, 665–674 (2014).

45. Marion, T.L., Perry, C.H., St Claire, R.L. 3rd & Brouwer, K.L.R. Endogenous bile acid disposition in rat and human sandwich-cultured hepatocytes. *Toxicol. Appl. Pharmacol.* **261**, 1–9 (2012).

46. Ansede, J.H., Smith, W.R., Perry, C.H., St Claire, R.L. 3rd & Brouwer, K.R. An *in vitro* essay to assess transporter-based cholestatic hepatotoxicity using sandwich-cultured rat hepatocytes. *Drug Metab. Dispos.* **38**, 276–280 (2010).

47. Griffin, L.M., Watkins, P.B., Perry, C.H., St Claire, R.L. 3rd & Brouwer, K.L.R. Combination lopinavir and ritonavir alter exogenous and endogenous bile acid disposition in sandwich-cultured rat hepatocytes. *Drug Metab. Dispos.* **41**, 188–196 (2013).

48. Yang, K., Köck, K., Sedykh, A., Tropsha, A. & Brouwer, K.L.R. An updated review on drug-induced cholestasis: mechanisms and investigation of physicochemical properties and pharmacokinetic parameters. *J. Pharm. Sci.* **102**, 3037–3057 (2013).

49. Rodrigues, A.D., Lai, Y., Cvilic, M.E., Elkin, L.L., Zyaga, T. & Soars, M.G. Drug-induced perturbations of the bile acid pool, cholestasis, and hepatotoxicity: mechanistic considerations beyond the direct inhibition of the bile salt export pump. *Drug Metab. Dispos.* **42**, 566–574 (2014).