Hepatobiliary Disposition of Troglitazone and Metabolites in Rat and Human Sandwich-Cultured Hepatocytes: Use of Monte Carlo Simulations to Assess the Impact of Changes in Biliary Excretion on Troglitazone Sulfate Accumulation

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Received May 25, 2009; accepted October 1, 2009

ABSTRACT

This study examined the hepatobiliary disposition of troglitazone (TGZ) and metabolites [TGZ sulfate (TS), TGZ glucuronide (TG), and TGZ quinone (TQ)] over time in rat and human sandwich-cultured hepatocytes (SCH). Cells were incubated with TGZ; samples were analyzed for TGZ and metabolites by liquid chromatography-tandem mass spectrometry. SCH mimicked the disposition of TGZ/metabolites in vivo in rats and humans; TGZ was metabolized primarily to TS and to a lesser extent to TG and TQ. In human SCH, the biliary excretion index (BEI) was negligible for TGZ and TQ, ~16% for TS, and ~43% for TG over the incubation period; in rat SCH, the BEI for TS and TG was ~13 and ~41%, respectively. Hepatocyte accumulation of TS was extensive, with intracellular concentrations ranging from 132 to 222 μM in rat SCH; intracellular TGZ concentrations ranged from 7.22 to 47.7 μM . In human SCH, intracellular

TS and TGZ concentrations ranged from 136 to 160 μM and from 49.4 to 84.7 μM , respectively. Pharmacokinetic modeling and Monte Carlo simulations were used to evaluate the impact of modulating the biliary excretion rate constant (K_{bile}) for TS on TS accumulation in hepatocytes and medium. Simulations demonstrated that intracellular concentrations of TS may increase up to 3.1- and 5.7-fold when biliary excretion of TS was decreased 2- and 10-fold, respectively. It is important to note that altered hepatobiliary transport and the extent of hepatocyte exposure may not always be evident based on medium concentrations (analogous to systemic exposure in vivo). Pharmacokinetic modeling/simulation with data from SCH is a useful approach to examine the impact of altered hepatobiliary transport on hepatocyte accumulation of drug/metabolites.

Troglitazone (TGZ; Rezulin), a peroxisome proliferator-activated receptor γ agonist, was withdrawn from the market in 2000 due to several cases of severe idiosyncratic liver injury (Graham et al., 2003; Smith, 2003). Several mechanisms for the hepatotoxicity of TGZ have been proposed (Smith, 2003; Masubuchi, 2006), including inhibi-

tion of the bile salt export pump (Bsep, *ABCB11*), the hepatic transport protein primarily responsible for biliary excretion of bile acids, by TGZ and TGZ sulfate (TS) (Funk et al., 2001). Bsep inhibition may cause hepatic accumulation of detergent-like bile acids, thereby resulting in hepatocellular injury. It is interesting to note that TS is a more potent inhibitor of Bsep ($\text{IC}_{50} = 0.4\text{--}0.6\text{ }\mu\text{M}$) than TGZ ($\text{IC}_{50} = 3.9\text{ }\mu\text{M}$) (Funk et al., 2001), emphasizing the importance of metabolite-mediated hepatotoxicity in addition to that of the parent drug.

TGZ is metabolized extensively by the liver in humans as well as in rats. The in vivo metabolic profile and disposition

This work was supported in part by the National Institutes of Health National Institute of General Medical Sciences [Grant GM41935] (to K.L.R.B.); and the National Institutes of Health National Institute of Environmental Health Sciences [Grant T32-ES007126] (to T.L.M.).

Article, publication date, and citation information can be found at <http://jpet.aspetjournals.org>.
doi:10.1124/jpet.109.156653.

ABBREVIATIONS: TGZ, troglitazone; BSEP/Bsep, bile salt export pump; TS, troglitazone sulfate; AUC_{ss} , area under the plasma concentration versus time curve at steady state; AUC, area under the plasma concentration versus time curve; SCH, sandwich-cultured hepatocyte(s); DEX, dexamethasone; HBSS, Hanks' balanced salts solution; MEM, minimal essential medium; DMEM, Dulbecco's modified Eagle's medium; TG, troglitazone glucuronide; TQ troglitazone quinone; BEI, biliary excretion index; M, medium; C, hepatocytes; B, bile.

of TGZ in rats are similar to that in humans. In humans, TGZ is metabolized primarily to a sulfate conjugate mostly via sulfotransferase 1A3 (Honma et al., 2002) and also to a glucuronide conjugate mostly via UDP-glucuronosyltransferase 1A1 (Yoshigae et al., 2000; Watanabe et al., 2002); TGZ also is oxidized to a quinone derivative by cytochrome P450 isozymes CYP3A4 and CYP2C8 (Yamazaki et al., 1999). After oral administration of TGZ for 7 days to humans, the area under the plasma concentration versus time curve at steady state (AUC_{ss}) for TS was ~7- to 10-fold higher than that of TGZ; the plasma AUC_{ss} for TGZ quinone (TQ) was comparable with that of TGZ, but the AUC_{ss} for TG was only 20 to 40% of that for TGZ (Loi et al., 1999). Similarly, after oral administration of [^{14}C]TGZ to male rats, the AUC of TS accounted for 89% of the AUC of total plasma radioactivity, whereas the AUC of TGZ and TG was only 5 and 1% of the AUC of total plasma radioactivity, respectively (Kawai et al., 1997). After intravenous administration of [^{14}C]TGZ to male rats, the biliary excretion of TS and TG was 61 and 2% of the dose, respectively, whereas TGZ and TQ recovery in bile accounted for less than 0.1% of the dose (Kawai et al., 1997). Because TGZ is metabolized extensively and little parent compound is excreted unchanged into urine or bile (Kawai et al., 1997), the total body clearance of TGZ is governed by its metabolic clearance (Izumi et al., 1996).

Sensitive and rapid in vitro screening tools to predict hepatotoxicity are needed in the pharmaceutical industry. Primary hepatocytes remain an important tool for studying the metabolism and hepatotoxic potential of xenobiotics. Hepatocytes cultured in a single layer of collagen are suitable for cytotoxicity assays, but the use of this tool is limited due to loss of metabolic activity and short survival periods (Hewitt et al., 2007). Hepatocytes cultured in a sandwich configuration between layers of extracellular matrix have overcome many of the limitations of conventional hepatocyte culture because the cells repolarize, regain their proper morphology, and maintain liver-specific metabolic activity, including stable expression of cytochrome P450 enzymes over longer times (Liu et al., 1998, 1999a,b,c; Hamilton et al., 2001; Hewitt et al., 2007; Mingoia et al., 2007). Furthermore, sandwich-cultured hepatocytes (SCH) develop functional networks of bile canaliculi structures between cells that are sealed by tight junctions (LeCluyse et al., 1994; Liu et al., 1999b). Measurements of biliary excretion of drugs and metabolites in humans are often indirect or require invasive procedures, but cells and canaliculi networks of SCH are directly accessible, allowing the quantification of substrates and metabolites excreted into the medium, retained within the cell, or excreted into the bile (Liu et al., 1999a,c; Hoffmaster et al., 2004; Turncliff et al., 2006).

The present study used rat and human SCH to examine the metabolism of TGZ, governed primarily by phase II conjugation, and the hepatobiliary disposition of TGZ and its derived metabolites. Moreover, the ability of this in vitro system to approximate the in vivo disposition of TGZ and metabolites reported previously in rats and humans was assessed. A pharmacokinetic model was developed and used to estimate key parameters governing the hepatobiliary disposition of TGZ and metabolites in rat SCH. Because variability in results from Monte Carlo simulations may represent an approximation of variability between cells in culture, Monte Carlo simulations were conducted to explore the in-

fluence of changes in parameter estimates, analogous to hepatic transport modulation, on the simulated hepatobiliary disposition of TGZ and metabolites.

Materials and Methods

Chemicals. Penicillin-streptomycin solution, dexamethasone (DEX), Hanks' balanced salts solution (HBSS), HBSS modified (HBSS without Ca^{2+} and Mg^{2+} , with 0.38 g/l EGTA), collagenase (type IV), and Triton X-100 were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) and MEM nonessential amino acids were purchased from Invitrogen (Carlsbad, CA). Insulin/transferrin/selenium culture supplement, BioCoat culture plates, and Matrigel extracellular matrix were purchased from BD Biosciences Discovery Labware (Bedford, MA). TGZ was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). TS, TG, and TQ were kindly provided by Daiichi-Sankyo Co., Ltd. (Tokyo, Japan). All other chemicals and reagents were of analytical grade and were readily available from commercial sources.

Hepatocyte Culture. Male Wistar rats (220–300 g; Charles River Laboratories, Inc., Raleigh, NC) were maintained on a 12-h light/dark cycle with free access to water and rodent chow. The Institutional Animal Care and Use Committee of The University of North Carolina at Chapel Hill approved all procedures. Rat hepatocytes were isolated using a two-step collagenase perfusion method as described previously (Liu et al., 1999b). Hepatocytes were plated at a density of 1.75×10^6 cells/well on six-well BioCoat plates in DMEM (1.5 ml) containing 5% fetal bovine serum, 10 μ M insulin, 1 μ M DEX, 2 mM L-glutamine, 1% MEM nonessential amino acids, 100 units penicillin G sodium, and 100 μ g of streptomycin sulfate. Hepatocytes were allowed to attach ~2 h, and the medium was changed to remove unattached cells. Cells were overlaid ~24 h later with BD Matrigel at a concentration of 0.25 mg/ml in 2 ml/well ice-cold DMEM supplemented with 0.1 mM MEM nonessential amino acids, 2 mM L-glutamine, 100 units of penicillin G sodium, 100 μ g of streptomycin, 1% insulin/transferrin/selenium, and 0.1 μ M DEX. CellDirect (Durham, NC) kindly provided human hepatocytes cultured in six-well plates, seeded at a density of 1.5×10^6 cells/well and overlaid with Matrigel. Table 1 shows demographics of human liver donors. Human cells were cultured in the same medium as rat hepatocytes. Both rat and human hepatocytes were cultured at 37°C in a humidified incubator with 95% O_2 and 5% CO_2 . Medium was changed daily until the experiments were conducted.

Metabolism of TGZ and Accumulation of Parent and Metabolites in Medium, Cells, and Bile in Rat and Human SCH. Modulation of Ca^{2+} in the incubation buffer was used to manipulate the tight junctions sealing the canaliculi networks between cells. In Ca^{2+} -containing standard buffer (HBSS), tight junction integrity is preserved and substrates are excreted into the bile canaliculi; incubation of cells in Ca^{2+} -free buffer (HBSS modified) disrupts the tight junctions, opens the canaliculi spaces, and canaliculi contents are released into the incubation medium (Liu et al., 1999c). Subtraction of the amount of substrate accumulated in cells after a preincubation in Ca^{2+} -free buffer from the accumulated amount in cells + bile after preincubation with Ca^{2+} -containing buffer yields the amount of substrate excreted into the canaliculi networks.

To evaluate metabolism of TGZ, day-4 cells (rat; $n = 3$ livers) and

TABLE 1
Demographics of human liver donors

Donor	Age yr	Gender	Ethnicity	Co-Medications
1	36	Female	Caucasian	Oral contraceptive, hydrochlorothiazide
2	64	Female	Caucasian	Atorvastatin, amlodipine, benazepril, alendronate

day 8 or day 10 cells (human; $n = 2$ livers) were incubated at 37°C for 10, 20, 30, 60, 90, and 120 min for rat SCH and for 30, 60, and 120 min for human SCH, with an initial concentration of 10 μM TGZ in the culture medium. Aliquots of medium were removed at each time point, and samples were diluted with methanol [1:2 (v/v)]. At the designated time, cells were washed twice and incubated at 37°C for 5 min with HBSS with Ca^{2+} (cells + bile) or without Ca^{2+} (cells). After incubation, buffer was aspirated and cells were lysed with 1 ml of 70%:30% (v/v) methanol/water, and sonicated for 20 s. Medium, cells and cells + bile lysate samples were stored at less than -70°C until analysis.

Transport of TGZ, and Preformed TS and TG Metabolites in Rat SCH. To confirm transport of TGZ and its preformed metabolites in rat SCH, medium was aspirated from cells on day 4, and cells were rinsed twice and then preincubated for 10 min at 37°C with 2 ml of warmed standard buffer (cells + bile) or Ca^{2+} -free HBSS buffer (cells). Medium was aspirated and cells were treated with 5 μM TGZ, TS, or TG in 1.5 ml of standard buffer for 10 min. After incubation, the dosing medium was removed, and cells were washed three times with 2 ml of ice-cold standard buffer. The wash buffer was aspirated, cells were lysed by adding 1 ml of 70%:30% (v/v) methanol/water, and samples were sonicated for 20 s with a sonic dismembrator (model 100; Thermo Fisher Scientific, Waltham, MA). Cells and cells + bile lysate samples were stored at less than -70°C until analysis. Treatments were performed in triplicate in $n = 1$ experiment (TGZ) or $n = 3$ experiments (TS and TG).

Sample Analysis. Substrate accumulation was corrected for non-specific binding to the extracellular matrix by subtracting accumulation in Matrigel-coated BioCoat plates without cells. The BCA protein assay (Pierce Chemical, Rockford, IL) was used to quantify total protein concentration in cell lysate samples using bovine serum albumin as the reference standard, and accumulation was normalized to protein concentration. To account for the incompatibility of the protein assay with methanol, the average protein concentration for standard HBSS or Ca^{2+} -free HBSS incubations in a representative plate from the same liver preparation was used to normalize accumulation.

The medium and cells or cells + bile lysate samples were centrifuged at 12,000g for 2 min at 4°C, and the supernatant was diluted 1:6 (v/v) with 79%:21% (v/v) methanol/water containing an internal standard (ethyl warfarin). A solvent delivery system (Shimadzu, Columbia, MD) and a Leap HTC Pal thermostated autosampler (LEAP Technologies, Carrboro, NC) connected to an Applied Biosystems API 4000 triple quadrupole mass spectrometer with a TurboSpray ion source (Applied Biosystems, Foster City, CA) were used for analysis. Tuning, operation, integration, and data analysis were performed in negative ionization mode using multiple reaction monitoring (Analyst software version 1.4.1; Applied Biosystems). Separation was accomplished using an Aquasil C18, 50- $\times$ 2.1-mm column, with a 5- μm particle size (Thermo Fisher Scientific). Analysis required 15 μl of sample and a solvent flow of 0.75 ml/min. Initial gradient conditions (80% 10 mM ammonium acetate aqueous solution and 20% methanol) were held for 0.8 min. From 0.8 to 1.5 min, the mobile phase composition increased linearly to 60% methanol, and the eluent was directed to the mass spectrometer. From 1.5 to 4 min, the mobile phase composition increased linearly to 65% methanol. At 4.1 min, the methanol composition was increased to 90%. The flow was held at 90% methanol until 4.3 min. At 4.4 min, the column was equilibrated with 80% 10 mM ammonium acetate aqueous solution. The total run time, including equilibration, was 5 min per injection. Eight point calibration curves (5–5000 nM) were constructed as composites of TGZ (440.0 $\rightarrow$ 397.1), TS (520.2 $\rightarrow$ 440.1), TG (616.2 $\rightarrow$ 440.1), and TQ (456.1 $\rightarrow$ 413.1) by using peak area ratios of analyte and ethyl warfarin (320.8 $\rightarrow$ 160.9). All points on the curves back-calculated to within $\pm 15\%$ of the nominal value. Care was taken to minimize the light-sensitive degradation of TGZ during all experimental procedures.

B-CLEAR technology (Qualyst, Inc., Research Triangle Park, NC)

was used to calculate the biliary excretion index (BEI), which represents the percentage of accumulated substrate that is excreted into bile, using the following equation (Liu et al., 1999b):

$$\text{BEI}(\%) = \frac{\text{Accumulation}_{\text{cells+bile}} - \text{Accumulation}_{\text{cells}}}{\text{Accumulation}_{\text{cells+bile}}} \times 100 \quad (1)$$

Pharmacokinetic Modeling. The average rat SCH accumulation data for TGZ, TS, TG, and TQ were used in pharmacokinetic modeling analysis. Several structurally distinct models were evaluated, and standard model selection criteria, including Akaike's information criterion, residual distribution, and the distribution of the residual error, were used to identify the optimal structure. The model scheme shown in Fig. 1 best described the disposition of TGZ and derived metabolites in rat SCH over 120 min in the medium (M), hepatocytes (C), and bile (B). Differential equations derived from the model scheme in Fig. 1 (see legend for definition of parameters) were as follows:

$$\frac{dX_{\text{TGZ,M}}}{dt} = -(K_{\text{uptake,TGZ}} + K_{\text{f,TQ}}) \times X_{\text{TGZ,M}}; \quad X_{\text{TGZ,M}}^0 = X_0 \quad (2)$$

$$\frac{dX_{\text{TS,M}}}{dt} = K_{\text{efflux,TS}} \times X_{\text{TS,C}} - K_{\text{uptake,TS}} \times X_{\text{TS,M}}; \quad X_{\text{TS,M}}^0 = 0; \quad t \leq T_{\text{flux}} \quad (3)$$

$$\frac{dX_{\text{TS,M}}}{dt} = K_{\text{efflux,TS}} \times X_{\text{TS,C}} - K_{\text{uptake,TS}} \times X_{\text{TS,M}} + K_{\text{flux,TS}} \times X_{\text{TS,B}}; \quad t > T_{\text{flux}} \quad (4)$$

$$\frac{dX_{\text{TG,M}}}{dt} = K_{\text{efflux,TG}} \times X_{\text{TG,C}} - K_{\text{uptake,TG}} \times X_{\text{TG,M}}; \quad X_{\text{TG,M}}^0 = 0; \quad t \leq T_{\text{flux}} \quad (5)$$

$$\frac{dX_{\text{TG,M}}}{dt} = K_{\text{efflux,TG}} \times X_{\text{TG,C}} - K_{\text{uptake,TG}} \times X_{\text{TG,M}} + K_{\text{flux,TG}} \times X_{\text{TG,B}}; \quad t > T_{\text{flux}} \quad (6)$$

Fig. 1. Model scheme depicting the mass (X) of TGZ, TS, TG, and TGZ quinone (TQ) in the medium (M), hepatocytes (C), and bile (B) of rat SCH. K_{uptake} , K_{efflux} , and K_{bile} represent first-order rate constants for uptake, basolateral efflux and biliary excretion, respectively. $K_{\text{f,TS}}$, $K_{\text{f,TG}}$, and $K_{\text{f,TQ}}$ represent first-order rate constants for formation of TS, TG, and TQ, respectively. The dotted lines represent flux of TS and TG from the bile into the incubation medium, with associated first-order rate constants ($K_{\text{flux,TS}}$ and $K_{\text{flux,TG}}$, respectively), that occurs during incubation after the estimated time (T_{flux}).

$$\frac{dX_{TQ,M}}{dt} = K_{f,TQ} \times X_{TGZ,M} - K_{other} \times X_{TQ,M}; \quad X_{TQ,M}^0 = 0 \quad (7)$$

$$\frac{dX_{TGZ,C}}{dt} = K_{uptake,TGZ} \times X_{TGZ,M} - (K_{f,TS} + K_{f,TG}) \times X_{TGZ,C};$$

$$X_{TGZ,C}^0 = 0 \quad (8)$$

$$\frac{dX_{TS,C}}{dt} = K_{f,TS} \times X_{TGZ,C} + K_{uptake,TS} \times X_{TS,M} - (K_{efflux,TS} + K_{bile,TS})$$

$$\times X_{TS,C}; \quad X_{TS,C}^0 = 0 \quad (9)$$

$$\frac{dX_{TG,C}}{dt} = K_{f,TG} \times X_{TGZ,C} + K_{uptake,TG} \times X_{TG,M} - (K_{efflux,TG} + K_{bile,TG})$$

$$\times X_{TG,C}; \quad X_{TG,C}^0 = 0 \quad (10)$$

$$\frac{dX_{TS,B}}{dt} = K_{bile,TS} \times X_{TS,C}; \quad X_{TS,B}^0 = 0; \quad t \leq T_{flux} \quad (11)$$

$$\frac{dX_{TS,B}}{dt} = K_{bile,TS} \times X_{TS,C} - K_{flux,TS} \times X_{TS,B}; \quad t > T_{flux} \quad (12)$$

$$\frac{dX_{TG,B}}{dt} = K_{bile,TG} \times X_{TG,C}; \quad X_{TG,B}^0 = 0; \quad t \leq T_{flux} \quad (13)$$

$$\frac{dX_{TG,B}}{dt} = K_{bile,TG} \times X_{TG,C} - K_{flux,TG} \times X_{TG,B}; \quad t > T_{flux} \quad (14)$$

Differential equations describing the accumulation of TGZ and derived metabolites in medium, hepatocytes and bile were fit simultaneously by nonlinear least-squares regression (WinNonlin Pro version 5.0.1; Pharsight, Mountain View, CA) using a weighting scheme of $1/(Y \text{ predicted})$.

Monte Carlo Simulations. Parameter estimates with associated variance (recovered by nonlinear least-squares regression of the flux data shown above) and uncontrolled error (experimental or analytical error; 15%) served as input for Monte Carlo simulations. To explore hepatic TS disposition in response to impaired transport in a hypothetical “population” of hepatocytes, simulations were performed by modulating the rate constant for TS biliary excretion by five different scenarios [experimentally determined parameter estimates, 2-fold increase, 10-fold increase; 2-fold decrease, and 10-fold decrease].

To estimate the number of observations (sample size) necessary to detect a statistically significant difference in TS accumulation in cells or medium due to changes in the TS biliary excretion rate constant ($K_{bile,TS}$), data from Monte Carlo simulations at designated time points (10, 20, 30, 60, 90, and 120 min) were analyzed using one-way analysis of variance. Based on the effect size (the square root of the ratio of the between-group to within-group variance) calculated by Cohen’s f^2 (Cohen, 1988), a sample size for each time point was calculated to achieve a power of 0.80 ($\alpha = 0.05$) by using R packages (R Development Core Team, 2008). The relative power to detect a statistically significant difference in medium versus cell at

each time point was calculated as a ratio of the number of observations (sample size) necessary to detect a statistically significant difference in accumulation in medium to the number of observations necessary for accumulation in cells.

Results

Hepatobiliary Disposition of TGZ and Derived Metabolites in Human SCH. The disposition of TGZ and derived metabolites in the medium and hepatocytes at 30, 60, and 120 min is shown in Table 2; cumulative recovery of TGZ and derived metabolites (medium + cells + bile) was 109 to 117%. At 120 min, TGZ in the medium accounted for 64.2% of the dose. TS was the predominant metabolite of TGZ in the medium (30.2% dose; 4543 pmol) as well as in hepatocytes (5.64% dose; 846 pmol) during the 120-min incubation; TG (447 pmol) and TQ (362 pmol) were recovered predominantly in the medium (2.98 and 2.41% dose, respectively) with negligible cellular accumulation (less than 0.1% dose). Accumulation of TGZ and TQ in bile was negligible. The BEI values of TS (15–17%) and TG (41–46%) were comparable at 30, 60, and 120 min in human SCH (Table 2).

Hepatobiliary Disposition of TGZ and Metabolites in Rat SCH. Initial experiments confirmed that TGZ and two preformed metabolites of TGZ, TS and TG, were taken up into rat SCH (Fig. 2). After a 10-min incubation, average accumulation (cells + bile) of TGZ was only slightly greater than cellular accumulation (1600 versus 1560 pmol/mg protein), leading to a negligible BEI for TGZ of 2.3%. Average accumulation (cells + bile) was slightly greater than cellular accumulation for TS (2280 versus 1940 pmol/mg protein) and TG (908 versus 605 pmol/mg protein), leading to more extensive BEI values of 15 and 34% for TS and TG, respectively.

The disposition of TGZ and its derived metabolites in medium, hepatocytes, and bile are plotted in Figs. 3, 4, and 5, respectively. Cumulative recovery of TGZ and derived metabolites from medium, hepatocytes, and bile was comparable throughout the 120-min incubation period (90–104%) (Table 3). At 120 min, 35.3% of the dose remained in the medium as TGZ (Table 3). Among metabolites present in the medium, TS was predominant (37.8% dose), followed by TG (6.89% dose) and TQ (0.65% dose) at 120 min (Fig. 3; Table 2). Figure 4 depicts the hepatocyte accumulation of TGZ and derived metabolites over the 120-min incubation period. TS formation was rapid and extensive; cellular accumulation of TS exceeded TGZ even at 10 min (5.46 versus 1.50% of the dose, respectively; Table 3). The cellular accumulation of TG was <1% of the dose over the 120-min incubation. TQ accumulation in the hepatocytes was negligible. The average BEI of TS and TG was 13% and 41% over the 120-min incubation

TABLE 2

Disposition of TGZ and derived metabolites in human SCH

TGZ, TS, TG, and TQ (expressed as percentage of dose) in medium, hepatocytes, and bile at 30, 60, and 120 min after incubation in medium initially containing 10 μ M TGZ. BEI of TS and TG was calculated as described under *Materials and Methods*. Data represent mean of a single determination in $n = 2$ livers.

Time	TGZ			TS				TQ			TG				Recovery
	Medium	Cells	Bile	Medium	Cells	Bile	BEI	Medium	Cells	Bile	Medium	Cells	Bile	BEI	
	% dose			% dose				% dose			% dose				%
30 min	93.9	3.50	N.D.	3.69	6.27	1.29	17	2.28	0.07	N.D.	0.63	0.06	0.05	46	112
60 min	88.8	2.80	N.D.	13.3	6.61	1.22	17	2.23	0.07	N.D.	1.56	0.06	0.04	41	117
120 min	64.2	2.04	N.D.	30.2	5.64	0.99	15	2.41	0.07	N.D.	2.98	0.05	0.04	43	109

N.D., not detectable.

Fig. 2. Accumulation of TGZ, TS, and TG in rat SCH. Rat SCH were treated for 10 min with 5 μ M TGZ, TS, or TG and accumulation (pico-moles per milligram of protein) was measured in cells + bile (solid bars) and in cells (open bars). Values represent the mean of triplicate determinations from $n = 1$ experiment (TGZ) or mean $\pm$ S.E.M. of triplicate determinations in $n = 3$ experiments (TS and TG).

Fig. 3. Mass versus time profiles of TGZ (●), TS (○), TG (▼), and TQ (△) in the medium initially containing 10 μ M TGZ in day 4 rat SCH (mean $\pm$ S.E.M.; $n = 3$ livers in triplicate). Dashed lines represent the best fit of the pharmacokinetic model derived from the scheme depicted in Fig. 1 to the data.

time; accumulation of TGZ and TQ in bile was negligible (Fig. 5; Table 3).

Pharmacokinetic Modeling of the Hepatobiliary Disposition of TGZ and Its Derived Metabolites in Rat SCH. Because data were collected at more time points in rat SCH, a compartmental pharmacokinetic model was developed based on this data set. The model scheme depicted in Fig. 1 best described the disposition of TGZ, TS, TG, and TQ in rat SCH during the 120-min incubation. Representative descriptions of the best fit of the pharmacokinetic model to the TGZ/derived metabolite versus time data are shown as dashed lines in Figs. 3 to 5. Pharmacokinetic parameter estimates obtained by resolving the differential equations describing hepatocellular disposition of TGZ, TS, TG, and TQ versus time data are included in Table 4. The first-order rate constant for TS formation (0.383 min^{-1}) was ~ 6 -fold greater than that for TG formation (0.061 min^{-1}). The pharmacokinetic model incorporated a formation process for TQ from TGZ in the incubation medium, instead of cellular TQ formation, because accumulation of TQ in hepatocytes was negligible, whereas TQ in

TABLE 3
Disposition of TGZ and its derived metabolites in rat SCH

Time	TGZ				TS				TQ				TG				Recovery	
	Medium	Cells	Bile	% dose	Medium	Cells	Bile	BEI %	Medium	Cells	Bile	BEI %	Medium	Cells	Bile	BEI %	%	%
10 min	94.3 $\pm$ 9.3	1.50 $\pm$ 0.31	N.D.	0.48 $\pm$ 0.06	5.46 $\pm$ 0.72	7.15 $\pm$ 1.50	0.92 $\pm$ 0.47	12 $\pm$ 6	1.00 $\pm$ 0.07	N.D.	N.D.	N.D.	0.19 $\pm$ 0.02	0.39 $\pm$ 0.01	0.19 $\pm$ 0.02	33 $\pm$ 2	104	104
20 min	86.4 $\pm$ 3.4	1.97 $\pm$ 0.21	N.D.	2.97 $\pm$ 0.58	7.15 $\pm$ 1.50	7.15 $\pm$ 1.50	1.56 $\pm$ 0.15	19 $\pm$ 2	1.36 $\pm$ 0.23	N.D.	N.D.	N.D.	1.18 $\pm$ 0.10	0.51 $\pm$ 0.02	0.33 $\pm$ 0.01	40 $\pm$ 1	103	103
30 min	81.1 $\pm$ 3.7	1.57 $\pm$ 0.06	N.D.	6.14 $\pm$ 0.57	7.92 $\pm$ 2.17	7.92 $\pm$ 2.17	1.35 $\pm$ 0.26	17 $\pm$ 5	1.27 $\pm$ 0.09	N.D.	N.D.	N.D.	2.41 $\pm$ 0.16	0.43 $\pm$ 0.04	0.33 $\pm$ 0.07	42 $\pm$ 5	103	103
60 min	63.6 $\pm$ 5.0	1.38 $\pm$ 0.45	N.D.	18.6 $\pm$ 2.0	9.20 $\pm$ 1.73	9.20 $\pm$ 1.73	1.16 $\pm$ 0.45	12 $\pm$ 4	1.01 $\pm$ 0.13	N.D.	N.D.	N.D.	3.86 $\pm$ 0.78	0.31 $\pm$ 0.04	0.24 $\pm$ 0.03	44 $\pm$ 4	99	99
90 min	46.9 $\pm$ 3.1	0.73 $\pm$ 0.24	N.D.	29.9 $\pm$ 2.6	8.64 $\pm$ 1.74	8.64 $\pm$ 1.74	1.27 $\pm$ 0.34	14 $\pm$ 4	0.85 $\pm$ 0.13	N.D.	N.D.	N.D.	5.87 $\pm$ 1.15	0.24 $\pm$ 0.01	0.17 $\pm$ 0.09	37 $\pm$ 15	95	95
120 min	35.3 $\pm$ 2.8	0.30 $\pm$ 0.13	N.D.	37.8 $\pm$ 2.7	7.88 $\pm$ 1.67	7.88 $\pm$ 1.67	0.75 $\pm$ 0.13	10 $\pm$ 4	0.65 $\pm$ 0.14	N.D.	N.D.	N.D.	6.89 $\pm$ 1.47	0.16 $\pm$ 0.02	0.13 $\pm$ 0.03	45 $\pm$ 10	90	90

N.D., not detectable.

Fig. 4. Mass versus time profiles of TGZ (●), TS (○), and TG (▼) in the hepatocytes in day 4 rat SCH; incubation medium initially contained 10 μ M TGZ (mean $\pm$ S.E.M.; $n = 3$ livers in triplicate). Dashed lines represent the best fit of the pharmacokinetic model derived from the scheme depicted in Fig. 1 to the data.

Fig. 5. Mass versus time profiles of TS (○) and TG (▼) in the bile canaliculi of day 4 rat SCH; incubation medium initially contained 10 μ M TGZ (Mean $\pm$ S.E.M.; $n = 3$ livers in triplicate). Dashed lines represent the best fit of the pharmacokinetic model derived from the scheme depicted in Fig. 1 to the data.

TABLE 4

Kinetic parameters estimated from compartmental pharmacokinetic modeling analysis

TGZ (10 μ M) was incubated in day 4 rat SCH as described under *Materials and Methods*. Parameter estimates were generated by nonlinear regression of the mean TGZ, TS, TG, and TQ data in the medium, hepatocytes and bile based on the equations described under *Materials and Methods* according to the model scheme depicted in Fig. 1.

Parameter	Estimate	CV(%)
$K_{\text{uptake,TGZ}}$ (min^{-1})	0.007	2
$K_{\text{uptake,TS}}$ (min^{-1})	0.004	36
$K_{\text{uptake,TG}}$ (min^{-1})	0.001	388
$K_{\text{f,TS}}$ (min^{-1})	0.383	10
$K_{\text{f,TG}}$ (min^{-1})	0.061	11
$K_{\text{f,TQ}}$ (min^{-1})	0.001	17
K_{other} (min^{-1})	0.064	23
$K_{\text{efflux,TS}}$ (min^{-1})	0.018	39
$K_{\text{efflux,TG}}$ (min^{-1})	0.135	34
$K_{\text{bile,TS}}$ (min^{-1})	0.035	21
$K_{\text{bile,TG}}$ (min^{-1})	0.087	48
$K_{\text{flux,TS}}$ (min^{-1})	0.255	24
$K_{\text{flux,TG}}$ (min^{-1})	0.137	57
T_{flux} (min)	16.7	8

the medium represented $\sim 1\%$ of the dose throughout the 120-min incubation period. To explain the decreased accumulation of TS and TG in bile after 20 min, first-order rate constants for TS and TG flux from the bile to the incubation medium were

incorporated into the pharmacokinetic model. The first-order rate constant for TS and TG flux from the bile to the incubation medium was 0.255 and 0.137 min^{-1} , respectively. Consistent with data from the uptake study with preformed TGZ metabolites TS and TG (Fig. 2), the first-order rate constant for TS uptake ($K_{\text{uptake,TS}}$, 0.004 min^{-1}) was greater than the TG uptake rate constant ($K_{\text{uptake,TG}}$, 0.001 min^{-1}). The first-order rate constant for biliary excretion of TG (0.087 min^{-1}) was ~ 2.5 -fold higher than that for TS (0.035 min^{-1}). The first-order rate constant for basolateral efflux of TG (0.135 min^{-1}) was ~ 7.5 -fold greater than that for TS (0.018 min^{-1}).

Monte Carlo Simulations. Monte Carlo simulations were performed to assess the impact of modulation of the first-order rate constant for biliary excretion of TS ($K_{\text{bile,TS}}$) on the simulated hepatobiliary disposition of TS (Fig. 6). TS cellular exposure increased from a maximum of 1378 up to 4313 pmol when $K_{\text{bile,TS}}$ was decreased 2-fold, and up to 7832 pmol when $K_{\text{bile,TS}}$ was decreased 10-fold; TS medium exposure changed from a maximum of 5670 pmol (control) to 7579 pmol and 7219 pmol when $K_{\text{bile,TS}}$ was decreased 2- and 10-fold, respectively. Overall, measurements of TS cellular accumulation were more responsive to changes in kinetic parameters for biliary excretion than were measurements of TS in the medium (Table 5).

Discussion

This study examined the hepatobiliary disposition of TGZ and its metabolites in the SCH system. Results showing metabolism of TGZ to TS and TG confirmed that functional activity of phase II conjugation enzymes is preserved in rat and human SCH. In addition, pharmacokinetic modeling and simulation studies were used to examine the impact of impaired biliary excretion on hepatobiliary disposition of TS and TG in rat SCH.

Previously, Kostrubsky et al. (2000) measured metabolite formation in conventionally cultured human hepatocytes exposed to 10 μ M TGZ; at 1 h, concentrations of TQ were highest in medium (depending on donor), followed by TS and TG. We demonstrate that in human SCH exposed to 10 μ M TGZ, TS was most abundant in the medium followed by TQ and TG at 1 h. Apparent differences in metabolite formation observed between these studies may be attributed to differences in experimental design [e.g., culture conditions (conventional versus sandwich configuration), days in culture (day 4 versus 7–8)], and/or variability in metabolic capacity between human donors.

Transport of TGZ and its preformed metabolites was first confirmed in rat SCH in this study. TS was taken up into cells more efficiently compared with TG and TGZ, although TS is more hydrophilic than TGZ. This is consistent with the observation that TS is a higher affinity substrate than TGZ or TG for the uptake transporter organic anion-transporting polypeptide 1B1 (Nozawa et al., 2004). Further experiments in rat SCH revealed that TS was the major metabolite, and TG the next most abundant metabolite, in the medium, cells, and bile at all time points. TQ was only detected in lesser amounts in the medium, but not in cells or bile. Photooxidation of TGZ to TQ may explain this observation (Fu et al., 1996); thus, subsequent pharmacokinetic modeling incorporated conversion of TGZ to TQ in medium.

In addition, in rat and human SCH, TS and TG were

Fig. 6. Monte Carlo simulations were performed to examine the impact of modulation of TS biliary excretion on the disposition of TS in hepatocytes (A) and medium (B). The rate constant for biliary excretion of TS ($K_{\text{bile,TS}}$) was modulated by five different scenarios [experimentally determined parameter estimates (black), 2-fold increase (red), 10-fold increase (green), 2-fold decrease (yellow), and 10-fold decrease (blue)].

TABLE 5
Evaluation of the sensitivity of cell and medium accumulation to detect changes in biliary excretion of TS using data from Monte Carlo simulations

Monte Carlo simulations were performed by modulating the rate constant for biliary excretion of TS by three different scenarios (experimentally determined parameter estimates; 2-fold increase; 2-fold decrease). The relative power to detect a statistically significant difference in medium vs. cell was calculated as the ratio of the number of observations (N = sample size) necessary to detect a statistically significant difference in TS accumulation in medium to the number of observations necessary for accumulation in hepatocytes.

Time		Number of Observations to Detect a Statistically Significant Difference	
		N_{cell}	N_{medium}
TS	<i>min</i>		
	10	14	147
	20	5	10
	30	3	4
	60	3	7
	90	3	10
	120	3	17

transported into the bile, whereas transport of TGZ into the bile was negligible. These findings are consistent with published reports showing that, after intravenous administration of TGZ to rats, minimal parent TGZ is recovered in bile, whereas TS and TG (61 and 2% dose, respectively) are excreted into bile (Izumi et al., 1996; Kawai et al., 1997). The medium and biliary excretion profiles in SCH resembled the plasma concentrations (TS > TG) and biliary excretion (TS > TG), respectively, after oral dosing of TGZ to rats (Izumi et al., 1997; Kawai et al., 1997). The greater amount of TS compared with TG in rat SCH, and the greater value of $K_{\text{f,TS}}$ (0.383 min^{-1}) compared with $K_{\text{f,TG}}$ (0.061 min^{-1}) correspond to data showing that the sulfate formation clearance was 6-fold higher than the glucuronide formation clearance in rats (Izumi et al., 1997).

Accumulation of TS and TG in bile remained constant or decreased during the incubation time after 20 min in both human and rat SCH (Tables 2 and 3). To account for this, pharmacokinetic modeling was performed to estimate rate

constants for flux of TS and TG from the bile into the medium (K_{flux}), and the time of flux (T_{flux}) (Fig. 1; Table 4). Physiological mechanisms may explain this flux; actin filament-mediated contractility of bile canaliculi facilitates bile flow in vivo in rat liver (Watanabe et al., 1991), and the regular, ordered contraction of bile canaliculi has been reported previously in vitro in isolated hepatocyte couplets/hepatocyte groups (Oshio and Phillips, 1981; Phillips et al., 1982).

Accumulation of TS and TG in the medium in rat SCH (38 and 7% of the dose, respectively) was comparable with values obtained in human SCH (30 and 3% of the dose, respectively). Transport proteins responsible for the basolateral excretion of TS and TG remain to be identified. Mrp3 plays a predominant role in the basolateral excretion of glucuronide conjugates, whereas both Mrp3 and Mrp4 are involved in the basolateral excretion of sulfate conjugates along with additional as-yet-to-be-identified mechanism(s) (Zamek-Gliszczynski et al., 2006). Pharmacokinetic modeling revealed an ~7.5-fold higher first-order rate constant for basolateral efflux of TG than for TS in rat SCH. This observation may reflect more efficient basolateral efflux of TG, presumably by Mrp3. Estimates of the rate constants for hepatocyte uptake of TS and TG were very small relative to all other flux estimates (Table 4). In fact, the rate constant for hepatic uptake of TG was not recoverable with any degree of precision based on pharmacokinetic modeling of the SCH data for TG when derived from TGZ.

Monte Carlo simulations revealed that TS cellular accumulation is more responsive to changes in the rate constant for biliary excretion of TS ($K_{\text{bile,TS}}$) than medium accumulation. For example, the number of observations necessary to observe a statistically significant difference in TS medium exposure was always higher (4–147), compared with the number of observations to observe a statistically significant difference in TS hepatocyte exposure (3–14), when $K_{\text{bile,TS}}$ was modulated (Table 5). SCH data coupled with modeling and simulation are capable of estimating the impact of altered transport function on hepatic (reflected in hepatocyte accumulation) and systemic (reflected in medium accumula-

tion) exposure. Peak plasma concentrations or AUC_{plasma} have been used to examine the effect of modulation of canalicular transport proteins on drug disposition, but results of this simulation suggest that those parameters may not always reflect changes in hepatic exposure due to altered biliary excretion. For example, physiologically based pharmacokinetic modeling of pravastatin distribution also suggested that plasma concentrations are not sensitive enough to detect changes in canalicular efflux; altered canalicular efflux of pravastatin changed liver concentrations markedly but had minimal effect on plasma concentrations (Watanabe et al., 2009).

The failure of preclinical testing to predict the hepatotoxicity of TGZ emphasizes the importance of understanding the hepatobiliary disposition and toxic potential of drugs and metabolites in humans and in experimental animals. One benefit of using SCH is that accumulation of parent drug and metabolites can be measured in medium, cell, and bile compartments. Based on these data, the impact of altered transport function on medium, cell, and bile exposure to drug and metabolites can be assessed through pharmacokinetic modeling and simulation. Efflux into medium may be analogous to efflux from the hepatocytes into plasma in vivo, whereas efflux into the bile may be scaled to biliary excretion in vivo. Because data in humans regarding hepatocellular concentrations of TGZ and metabolites are lacking, as are data about efflux of TGZ or metabolites into bile, the SCH model may be a useful in vitro tool to estimate human in vivo hepatobiliary drug/metabolite disposition and the impact of impaired biliary excretion. For example, the breast cancer resistance protein, which is expressed on the canalicular membrane of hepatocytes, probably plays a role in the biliary excretion of TS in humans (Enokizono et al., 2007). Based on pharmacokinetic simulations, TS accumulation in hepatocytes may increase in subjects with functional impairment of breast cancer resistance protein. Because TGZ and TS are potent inhibitors of Bsep/BSEP (Funk et al., 2001; Yabuuchi et al., 2008), hepatocyte accumulation of TS could result in increased susceptibility of some patients to TGZ-associated liver injury. Based on the accumulated mass of TGZ and TS in SCH, and the volume of rat hepatocytes (6.2×10^{-6} $\mu\text{L/hepatocyte}$) (Uhal and Roehrig, 1982), intracellular concentrations of TGZ and TS were estimated to be 7.22 to 47.7 μM and 132 to 222 μM , respectively, whereas TGZ and TS concentrations in medium were 3.53 to 9.43 and 0.05 to 3.78 μM , respectively. These TGZ and TS intracellular concentrations were much higher than the previously reported IC_{50} value of TGZ (3.9 μM) and TS (0.4–0.6 μM) for Bsep-mediated taurocholate transport in isolated canalicular rat liver plasma membranes (Funk et al., 2001). Cellular concentrations of TS could reach >1200 μM if TS biliary excretion was impaired 10-fold based on simulations conducted in this study. In contrast, medium concentrations of TS could increase up to 4.8 μM when TS biliary excretion was decreased 10-fold. Based on data generated in human SCH, and assuming the same cellular volume for rat and human hepatocytes, intracellular concentrations of TGZ and TS were estimated to be 49.4 to 84.7 μM and 136 to 160 μM , respectively, throughout the incubation period; medium concentrations were 6.42 to 9.39 μM for TGZ and 0.37 to 3.03 μM for TS. These findings support the hypothesis that the potent inhibition of BSEP by TGZ ($IC_{50} = 20 \mu\text{M}$) (Yabuuchi et al., 2008) and/or

TS in the hepatocyte may contribute to TGZ-mediated liver injury. It is important to note that medium concentrations (analogous to systemic concentrations in vivo) considerably underestimated cellular concentrations.

In conclusion, phase II metabolites of TGZ were detected in both rat and human SCH, indicating the preservation of sulfotransferase and UDP-glucuronosyltransferase function in the SCH system. Similar TGZ metabolism and hepatobiliary disposition between rats and humans in vivo also was observed in rat and human SCH, respectively, suggesting that the SCH model is a useful in vitro tool to describe in vivo hepatobiliary disposition of drugs and derived metabolites in rats and humans. Pharmacokinetic modeling and simulation, coupled with data obtained from the SCH model, may be used as an in vitro approach to determine likely alterations in drug/metabolite disposition, including hepatic and biliary exposure in humans.

References

- Cohen J (1988) *Statistical Power Analysis for the Behavioral Sciences*, Lawrence Erlbaum, New York.
- Enokizono J, Kusuhara H, and Sugiyama Y (2007) Involvement of breast cancer resistance protein (BCRP/ABCG2) in the biliary excretion and intestinal efflux of troglitazone sulfate, the major metabolite of troglitazone with a cholestatic effect. *Drug Metab Dispos* 35:209–214.
- Fu Y, Sheu C, Fujita T, and Foote CS (1996) Photooxidation of troglitazone, a new antidiabetic drug. *Photochem Photobiol* 63:615–620.
- Funk C, Pantze M, Jehle L, Ponelle C, Scheuermann G, Lazendic M, and Gasser R (2001) Troglitazone-induced intrahepatic cholestasis by an interference with the hepatobiliary export of bile acids in male and female rats. Correlation with the gender difference in troglitazone sulfate formation and the inhibition of the canalicular bile salt export pump (Bsep) by troglitazone and troglitazone sulfate. *Toxicology* 167:83–98.
- Graham DJ, Green L, Senior JR, and Nourjah P (2003) Troglitazone-induced liver failure: a case study. *Am J Med* 114:299–306.
- Hamilton GA, Jolley SL, Gilbert D, Coon DJ, Barros S, and LeCluyse EL (2001) Regulation of cell morphology and cytochrome P450 expression in human hepatocytes by extracellular matrix and cell-cell interactions. *Cell Tissue Res* 306:85–99.
- Hewitt NJ, Lechón MJ, Houston JB, Hallifax D, Brown HS, Maurel P, Kenna JG, Gustavsson L, Lohmann C, Skonberg C, et al. (2007) Primary hepatocytes: current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. *Drug Metab Rev* 39:159–234.
- Hoffmaster KA, Zamek-Gliszczynski MJ, Pollack GM, and Brouwer KL (2004) Hepatobiliary disposition of the metabolically stable opioid peptide [D-Pen², D-Pen⁵]-enkephalin (DPDPE): pharmacokinetic consequences of the interplay between multiple transport systems. *J Pharmacol Exp Ther* 311:1203–1210.
- Honma W, Shimada M, Sasano H, Ozawa S, Miyata M, Nagata K, Ikeda T, and Yamazoe Y (2002) Phenol sulfotransferase, ST1A3, as the main enzyme catalyzing sulfation of troglitazone in human liver. *Drug Metab Dispos* 30:944–949.
- Izumi T, Enomoto S, Hosiyaama K, Sasahara K, Shibukawa A, Nakagawa T, and Sugiyama Y (1996) 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.
- Izumi T, Hosiyaama K, Enomoto S, Sasahara K, and Sugiyama Y (1997) 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.
- Kawai K, Kawasaki-Tokui Y, Odaka T, Tsuruta F, Kazui M, Iwabuchi H, Nakamura T, Kinoshita T, Ikeda T, Yoshioka T, et al. (1997) Disposition and metabolism of the new oral antidiabetic drug troglitazone in rats, mice and dogs. *Arzneimittelforschung* 47:356–368.
- Kostrubsky VE, Sinclair JF, Ramachandran V, Venkataramanan R, Wen YH, Kindt E, Galchev V, Rose K, Sinz M, and Strom SC (2000) The role of conjugation in hepatotoxicity of troglitazone in human and porcine hepatocyte cultures. *Drug Metab Dispos* 28:1192–1197.
- LeCluyse EL, Audus KL, and Hochman JH (1994) Formation of extensive canalicular networks by rat hepatocytes cultured in collagen-sandwich configuration. *Am J Physiol* 266:C1764–C1774.
- Liu X, Brouwer KL, Gan LS, Brouwer KR, Stieger B, Meier PJ, Audus KL, and LeCluyse EL (1998) Partial maintenance of taurocholate uptake by adult rat hepatocytes cultured in a collagen sandwich configuration. *Pharm Res* 15:1533–1539.
- Liu X, Chism JP, LeCluyse EL, Brouwer KR, and Brouwer KL (1999a) Correlation of biliary excretion in sandwich-cultured rat hepatocytes and in vivo in rats. *Drug Metab Dispos* 27:637–644.
- Liu X, LeCluyse EL, Brouwer KR, Gan LS, Lemasters JJ, Stieger B, Meier PJ, and Brouwer KL (1999b) Biliary excretion in primary rat hepatocytes cultured in a collagen-sandwich configuration. *Am J Physiol* 277:G12–G21.
- Liu X, LeCluyse EL, Brouwer KR, Lightfoot RM, Lee JI, and Brouwer KL (1999c)

- Use of Ca^{2+} modulation to evaluate biliary excretion in sandwich-cultured rat hepatocytes. *J Pharmacol Exp Ther* **289**:1592–1599.
- Loi CM, Alvey CW, Vassos AB, Randinitis EJ, Sedman AJ, and Koup JR (1999) Steady-state pharmacokinetics and dose proportionality of troglitazone and its metabolites. *J Clin Pharmacol* **39**:920–926.
- Masubuchi Y (2006) Metabolic and non-metabolic factors determining troglitazone hepatotoxicity: a review. *Drug Metab Pharmacokinet* **21**:347–356.
- Mingoia RT, Nabb DL, Yang CH, and Han X (2007) Primary culture of rat hepatocytes in 96-well plates: effects of extracellular matrix configuration on cytochrome P450 enzyme activity and inducibility, and its application in in vitro cytotoxicity screening. *Toxicol In Vitro* **21**:165–173.
- Nozawa T, Sugiura S, Nakajima M, Goto A, Yokoi T, Nezu J, Tsuji A, and Tamai I (2004) Involvement of organic anion transporting polypeptides in the transport of troglitazone sulfate: implications for understanding troglitazone hepatotoxicity. *Drug Metab Dispos* **32**:291–294.
- Oshio C and Phillips MJ (1981) Contractility of bile canaliculi: implications for liver function. *Science* **212**:1041–1042.
- Phillips MJ, Oshio C, Miyairi M, Katz H, and Smith CR (1982) A study of bile canicular contractions in isolated hepatocytes. *Hepatology* **2**:763–768.
- R Development Core Team (2008). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3–900051-07–0, URL <http://www.R-project.org>.
- Smith MT (2003) Mechanisms of troglitazone hepatotoxicity. *Chem Res Toxicol* **16**:679–687.
- Turncliff RZ, Hoffmaster KA, Kalvass JC, Pollack GM, and Brouwer KL (2006) Hepatobiliary disposition of a drug/metabolite pair: comprehensive pharmacokinetic modeling in sandwich-cultured rat hepatocytes. *J Pharmacol Exp Ther* **318**:881–889.
- Uhal BD and Roehrig KL (1982) Effect of dietary state on hepatocyte size. *Biosci Rep* **2**:1003–1007.
- Watanabe N, Tsukada N, Smith CR, and Phillips MJ (1991) Motility of bile canaliculi in the living animal: implications for bile flow. *J Cell Biol* **113**:1069–1080.
- Watanabe T, Kusuha H, Maeda K, Shitara Y, and Sugiyama Y (2009) Physiologically based pharmacokinetic modeling to predict transporter-mediated clearance and distribution of pravastatin in humans. *J Pharmacol Exp Ther* **328**:652–662.
- Watanabe Y, Nakajima M, and Yokoi T (2002) Troglitazone glucuronidation in human liver and intestine microsomes: high catalytic activity of UGT1A8 and UGT1A10. *Drug Metab Dispos* **30**:1462–1469.
- Yabuuchi H, Tanaka K, Maeda M, Takemura M, Oka M, Ohashi R, and Tamai I (2008) Cloning of the dog bile salt export pump (BSEP; ABCB11) and functional comparison with the human and rat proteins. *Biopharm Drug Dispos* **29**:441–448.
- Yamazaki H, Shibata A, Suzuki M, Nakajima M, Shimada N, Guengerich FP, and Yokoi T (1999) Oxidation of troglitazone to a quinone-type metabolite catalyzed by cytochrome P-450 2C8 and P-450 3A4 in human liver microsomes. *Drug Metab Dispos* **27**:1260–1266.
- Yoshigae Y, Konno K, Takasaki W, and Ikeda T (2000) Characterization of UDP-glucuronosyltransferases (UGTs) involved in the metabolism of troglitazone in rats and humans. *J Toxicol Sci* **25**:433–441.
- Zamek-Gliszczynski MJ, Nezasa K, Tian X, Bridges AS, Lee K, Belinsky MG, Kruh GD, and Brouwer KL (2006) Evaluation of the role of multidrug resistance-associated protein (Mrp) 3 and Mrp4 in hepatic basolateral excretion of sulfate and glucuronide metabolites of acetaminophen, 4-methylumbelliferone, and harmol in Abcc3^{−/−} and Abcc4^{−/−} mice. *J Pharmacol Exp Ther* **319**:1485–1491.

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