Assessment of the Roles of P-glycoprotein and Cytochrome P450 in Triptolide-induced Liver Toxicity in Sandwich-Cultured Rat Hepatocyte Model

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Received August 6, 2013; accepted September 23, 2013

ABSTRACT

Triptolide (TP), a main bioactive component of Tripterygium wilfordii Hook F., is a promising agent for treatment of autoimmune diseases. However, a high incidence of dose-limiting hepatotoxicity was observed in the clinic. Sandwich-cultured rat hepatocyte model was used in this study to identify the involvement of P-glycoprotein (P-gp) in TP disposition and to evaluate TP-induced hepatotoxicity after modulation of P-gp by the known inhibitors, ritonavir and tariquidar, and known inducers, phenobarbital, quercetin, and H2O2. Our data showed that biliary clearance of TP reduced 73.7% and 84.2% upon treatment of ritonavir (25 μM) and tariquidar (5 μM), respectively. The TP-induced hepatotoxicity increased twofold when CYP activity was blocked by 1-aminobenzotriazole, suggesting that CYP and P-gp may both contribute to the detoxification of TP in the SCRH model. In addition, hepatotoxicity and the expression of apoptosis proteins Bax and Bcl-2 were correlated qualitatively with the TP exposure duration and its intracellular concentration, which, in turn, can be modulated by P-gp inhibitors or inducers. Our results for the first time demonstrated that in addition to CYP-mediated metabolism, P-gp also plays an important role in the disposition of TP and TP-induced hepatotoxicity. Thus, the modulation of canalicular P-gp has a potential to cause drug-drug interaction between TP and the coadministered P-gp inhibitors or inducers in the clinic.

Introduction

Triptolide (TP; PubChem CID: 107985; Fig. 1), a diterpene triepoxide, isolated from Tripterygium wilfordii Hook F, has been used for centuries in traditional Chinese medicines to treat autoimmune and inflammatory disorders such as rheumatoid arthritis, immune complex nephritis, systemic lupus erythematosus, and organ or tissue transplantation rejections (Lipsky and Tao, 1997; Panichakul et al., 2006; Lin et al., 2007; Zhuang et al., 2013). However, the clinical application of TP is greatly limited because of its narrow therapeutic window resulting from its high toxicities (Yang et al., 2012; Wang et al., 2013). Among the adverse events of TP, liver toxicity is believed to be the main cause of death based on the accumulated evidence in the clinic (Chen and Cai, 1999; Wang et al., 2007; Xue et al., 2009; Chai et al., 2011). The inhibition of mitochondrial respiratory chain (Fu et al., 2011), the excessive apoptosis of hepatocytes (Yao et al., 2008), and lipid peroxidation (Mei et al., 2005) are believed to be the main reasons involved in TP-induced hepatotoxicity. In a rat toxicokinetic study, the highest tissue concentration was found in liver (Xue et al., 2012). In addition, our study using bile duct-canalused rats indicated that about 50% of the intravenous dose was excreted into bile as TP and its metabolites (Zhuang et al., manuscript in preparation). The evidence above suggests that hepatic metabolism and excretion are important clearance routes for TP and would have impacts on its hepatotoxicity. CYP3A is responsible for the metabolism of TP, hence, inhibition or induction of CYP3A could change the exposure of TP in hepatocytes and the hepatotoxicity induced by TP (Ye et al., 2010; Xue et al., 2011). Canalicular efflux transporters such as bile salt export pump, multidrug resistance-associated protein 2 (MRP2), breast cancer resistance protein (BCRP), and P-glycoprotein (P-gp) play important roles in biliary excretion of drugs. Inhibition or induction of such transporters may impact on intracellular accumulation of drugs, and furthermore, the interplay between metabolic enzymes and transporters would complicate the disposition of a drug (Lai, 2009; De Bruyn et al., 2013). An example of the involvement of canalicular efflux transporters in the acetaminophen-induced hepatotoxicity was nicely described by Barnes et al., (2007). However, the relationship between transporter-mediated biliary clearance and hepatotoxicity of TP has not been reported.

Primary hepatocyte culture is a prominent in vitro tool to study hepatic drug metabolizing enzymes and transporters (Lu et al., 2010, Zhang et al., 2012; Ramboer et al., 2013). Several experimental models have been developed to investigate the hepatobiliary transporters, their distributions, and functions. These experimental models include liver canalicular membrane vesicles (El-Sheikh et al., 2007), isolated and cultured hepatocytes (Li et al., 1999), isolated perfused...

ABBREVIATIONS: ABT, 1-aminobenzotriazole; Bcrp, breast cancer resistance protein; BEI, biliary excretion index; CYP, cytochrome P450; HBSS, Hanks’ balanced salts buffer; LDH, lactate dehydrogenase; P-gp, P-glycoprotein; SCRH, sandwich-cultured rat hepatocytes; TP, triptolide; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry.
liver (Zamek-Gliszczynski et al., 2006), and transporter-deficient animal models (Kuroda et al., 2004). However, these models have limited utility in providing information on biliary excretion, as they are not optimal to obtain quantitative information (membrane vesicles and cultured hepatocytes) or require complicated and labor intensive experiments. Liu and colleagues (1999) first established a special format of sandwich-cultured hepatocytes (SCHR) to evaluate hepatic drug disposition into the biliary canalicular network. In that model, hepatocytes were cultured between two layers of gelled collagen in a sandwich configuration to recreate the three-dimensional cellular environment and re-establish cell polarity. It also formed canalicular membrane domains by re-establishing the tight junction and bile canalicular networks. Appropriate expression and relocalization of transport proteins in SCHR make this model an easy way to study drug disposition into bile and drug-induced liver toxicity mediated by biliary excretion transporters (Swift et al., 2010).

In this study, to investigate whether P-gp was involved in the disposition of TP, transporter-expressing Sf9 membrane vesicles were used to screen and identify the transporter involved in the biliary excretion of TP. Then, sandwich-cultured rat hepatocyte (SCHR) model was used to demonstrate the time- and dose-dependent hepatotoxicity by TP, as well as the up- and downregulation of apoptosis proteins/biomarkers (Bcl-2 and Bax) that are likely to attribute to the observed hepatotoxicity. The known P-gp inhibitors [ritonavir (PubChem CID: 53487909) and tariquidar (PubChem CID: 1482011)] and inducers [phenobarbital (PubChem CID: 4763), quercetin (PubChem CID: 5280343), and H2O2 (PubChem CID: 7841)] were applied to the SCHR to study the effects of inhibition or induction of P-gp on the intracellular concentration of TP and the consequential hepatotoxicity and changes in the apoptosis biomarkers. In addition, ABT was used in SCHR to block the CYP metabolism and to investigate the relative contributions of CYP versus P-gp toward the detoxification of TP-induced hepatotoxicity.

Materials and Methods

Chemicals. Penicillin-streptomycin solution, dexamethasone, Hanks’ balanced salts (HBSS), HBSS modified (HBSS without Ca2+ and Mg2+), collagenase (type IV), Triton X-100, phenobarbital (PB), ritonavir, tariquidar, 1-amino- benzotriazole (ABT), midazolam (PubChem CID: 4129), and quercetin were purchased from Sigma-Aldrich, Inc. (St. Louis, MO). DMEM/F12 medium was purchased from Invitrogen (Carlsbad, CA). Insulin/transferrin/selenium culture supplement and Matrigel extracellular matrix were purchased from BD Biosciences Discovery Labware (Bedford, MA). Fetal bovine serum was purchased from Gibco BRL (Gaithersburg, MD). Triptolide was purchased from Chinese National Institute of Food and Drug Control with the purity more than 99% (Beijing, China). H2O2 was purchased from SinoPharm (Beijing, China), and PB was provided by TianJin Jinyaoan Com (Tianjin, China). Rat Mdr1, Bcrp membrane, and ATPase assay kit were purchased from BD Gentest Co. (Woburn, MA). All other chemicals and reagents were of analytical grade.

Screening Assay with Rat Mdr1 and Bcrp Membranes. The ATPase assay was performed using a BD Gentest ATPase assay kit following the instruction manual. The assay mixtures, which containing rMdr1 or rBcrp membranes and TP (10 μM), were preincubated for 5 and 10 minutes, respectively. The reactions were started by addition of MgATP followed by incubation at 37°C for 10 and 30 minutes. The reactions were terminated with the stop solution (10% SDS solution). After that, color developing reagent was added to the samples and incubated for another 20 minutes before taking the absorption reading at 800 nm. To validate the membrane transporter activities from commercial sources, verapamil (60 μM) and sulfasalazine (30 μM) were included in the study as Mdr1 or Bcrp specific and positive control substrate, respectively. The stimulated ATPase activities, measured as a consequence of substrate binding to the transporters, were calculated by dividing the amount of inorganic phosphate produced by the incubation time and the amount of protein used in the incubation.

Isolation, Plating, and Maintenance of Sandwich-Cultured Rat Hepatocytes. Male SD rats weighting 200 to 250 g were maintained on a 12-hour light/dark cycle with free access to water and laboratory chow. All the animal experiments were conducted at the Beijing Center for Drug Safety Evaluation followed the protocol of the Institutional Animal Care and Use Committee of the Centre, which was in compliance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International. Rats were anesthetized with 10% chloral hydrate (3 mL/kg intraperitoneally) before surgical procedures. Rat hepatocytes were isolated using a two-step collagenase perfusion method as published (Seglen, 1976). Hepatocytes were suspended at approximately 1×106 cells/mL in DMEM-based culture medium containing 10% fetal bovine serum, 10 μM insulin, 0.1 μM dexamethasone, 2 mM l-glutamine, 100 units penicillin G, sodium, and 100 μg streptomycin sulfate. An aliquot of 0.5 mL of the hepatocyte suspension was added to each well of 24-well plates that were precoated with rat-tail tendon collagen. Hepatocytes were allowed to attach for 4–4 hours at 37°C in a humidified incubator with 95% air–5% CO2 atmosphere. Then fresh medium described above without serum was used to replace the plating medium. After 24-hour of incubation, cells were overlaid with 0.5 mL of Matrigel (0.25 mg/mL, BD Biosciences) solution prepared in culture medium; this is day 1. Culture medium was replaced every 24 hours until experiments were conducted.

Pretreatment of Ritonavir, Quercetin, phenobarbital, Quercetin, and H2O2 in SCHR. In the effect of P-gp inhibition on BEI study, culture medium containing vehicle, ritonavir (2.5, 10, and 25 μM) or tariquidar (0.5, 2.5, and 5 μM) was added to the hepatocytes on day 4 and incubated for 30 minutes prior the BEI assay. In the effect of P-gp induction on BEI study, day 2 to day 4, fresh culture medium containing vehicle, PB (0.2, 0.5, and 1 mM), quercetin (2, 10, and 20 μM), or H2O2 (0.1, 0.25, and 0.5 mM) was added to the hepatocyte culture every 24 hours to replace the old medium. TP at 0.1, 0.5, 1.0, 5.0, or 10 μM in standard or modified HBSS with Ca2+/Mg2+ was added to the culture and incubated for 30 minutes for the determination of BEI.

Co-treatment of ABT with TP to Evaluate the Relative Contributions of CYP Versus P-gp in Detoxification of TP-induced Hepatotoxicity. To elucidate the relative contributions of CYP3A and P-gp toward the detoxification of TP-induced hepatotoxicity, the SCHR pretreated 24 hours with P-gp inhibitors or 48 hours with inducers (some of them are dual CYP and P-gp modulators) were incubated with TP (10 μM) for 24 hours in presence or absence of either 50 or 250 μM of the pan-CYP inhibitor ABT. At the end of the coincubations, the TP-induced toxicities were measured using the LDH assay described in the following section. The concentrations of ABT were determined in a pilot study to achieve approximately 90% inhibition in SCHR in the presence of P-gp inhibitors (some of them are also CYP inhibitors or inducers). The remaining CYP activities were determined using CYP3A substrate midazolam (5 μM) as previous described (Lu et al., 2006).

Accumulation and Biliary Secretion Experiment. On day 4, experiments to determine TP accumulation in hepatocytes and the biliary canalicular network in the presence or absence of P-gp inducers and inhibitors were conducted. The SCHR was rinsed twice with 1 mL of warm standard HBSS solution and incubated in 0.5 mL of the same buffer for 15 minutes at 37°C. Subsequently, hepatocytes were incubated in 0.5 mL of 0.1, 0.5, 1.0, 5.0, or 10 μM TP dissolved in either standard HBSS buffer (pH 7.4) (cells + bile) or Ca2+/Mg2+-free HBSS buffer (pH 7.4) (cells) for 30 minutes and then rinsed three times with 1 mL of ice-cold standard HBSS. After the rinsing, hepatocytes were lysed with 0.2 mL of 0.5% Triton X-100 solution by placing plates on a rotator for 20 minutes at room temperature to lyse the cells. For cellular TP analysis, 100 μL cell lysate from each samples was precipitated with 200 μL of

![Fig. 1. The chemical structure of TP.](dmd.aspetjournals.org)
Acetaminophen/methanol (1:1) containing internal standard solution [propranolol (PubChem CID: 4946), 100 ng/ml]. The mixtures were centrifuged at 14,000 g at 4°C for 10 minutes, and the supernatant was then taken for LC-MS/MS analysis. The protein content of the hepatocytes in each well was measured using bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL) with 50 μl cell lysate that was set aside after lysing.

**MTT Assay.** A 0.5 mg/ml of solution of 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to rat hepatocyte culture after being treated with test compounds for 24 hours. After a 3-hour incubation, hepatocytes were checked visually for dye conversion and the medium was aspirated and rinsed twice with standard HBSS. Then the purple crystal metabolite, formazan, was dissolved in acetone, and absorption at 595 nm was measured using a microplate reader (Tecan Instrument Inc., Research Triangle Park, NC). The MTT assay results were the means of three independent experiments, each performed in replicates of six for each drug concentration. A reduced MTT reading in the test compound-treated group compared with its vehicle control group greater than 10% was considered to have cytotoxicity caused by the test compounds.

**Determination of LDH Level in Hepatocyte Culture Medium.** The LDH levels in the medium were determined in a CL8000 automated biochemical analyzer (Shimadzu, Japan) to assess hepatocyte viability. At the end of the 24-hour exposure of TP, LDH leakage into the culture medium was measured. The degree of LDH release, an indicator of cytotoxicity, was expressed as a percentage of the LDH leaked into the medium under experimental conditions to the maximum cellular release, which was measured after adding 2% Triton X-100 to release all LDH in cells + medium.

\[
\text{Cytotoxicity} = \frac{\text{LDH}_{\text{sample}} - \text{LDH}_{\text{blank}}}{\text{LDH}_{\text{Triton X-100}} - \text{LDH}_{\text{blank}}} \times 100
\]

Western Blot Analysis of P-gp, Bcl-2, and Bax Proteins. On day 5 after 24 hours of TP treatment, the medium was removed and cells were washed twice with ice-cold phosphate-buffered saline. Subsequently, cells were lysed with lysis buffer (1 mM PMSF mixed with complete protease inhibitors (Roche Diagnostics, Mannheim, Germany)). The lysates were centrifuged at 12,000 g for 20 minutes at 4°C to remove debris. Protein samples (30 μg/well for P-gp and 10 μg/well for Bcl-2 or Bax) were separated by SDS-polyacrylamide gel electrophoresis and electrotransferred to nitrocellulose membranes (0.45 μm, Millipore, Billerica, MA). Blots were blocked with 3% bovine serum albumin-Tris-buffered saline with Tween 20 for 30 minutes at room temperature. Subsequently, the membrane was incubated with appropriate primary antibodies for 10 minutes at room temperature or overnight at 4°C and then rinsed five times at 10-minute intervals with 3% bovine serum albumin-Tris-buffered saline with Tween 20. The primary antibodies used were rabbit polyclonal antibodies to P-gp (Biomol, Plymouth Meeting, PA) and rabbit antibodies to Bcl-2 or Bax (Pharmingen, San Diego, CA). The secondary antibodies used for detection were anti-rabbit IgG (H+L) HRP. Protein loading was normalized to GAPDH expression determined in the same samples. Immunoreactive protein bands were detected by chemiluminescence using a Bio-Rad VersaDoc imaging system and densitometry analysis was performed using the Quantity One software package v.4.1 (Bio-Rad Laboratories, Hercules, CA).

**Analytical Methods.** The TP concentrations in samples were quantitatively analyzed by Agilent1290 Infinity UHPLC system coupled with an Agilent 6410B triple quadrupole mass spectrometer (Agilent Technologies, Santa Clara, CA). TP and propranolol (internal standard) were eluted from an Agilent C18 column (dP = 3.5 μm, 2.1 × 50 mm, Agilent) using a mobile phase gradient (A: water with 0.1% formic acid and 2.5 mM ammonium formate; B: acetonitrile with 0.1% formic acid). The gradient was run as 0- to 2.5-minute hold at 95% B; 2.5–3 minute, a linear gradient to 10% B; 3- to 4-minute hold at 10% B. Flow rate was 0.2 ml/min. TP and propranolol were detected in positive ion mode using multiple reaction monitoring: TP, 378.1–360.1 m/z, propranolol, 260.0–116.0 m/z. The lower limit of detection was 1 nM for TP, and standard curves ranged from 1 to 1000 nM.

**Data Analysis.** The biliary excretion index (BEI) was calculated following the published method (Eq. 1, Liu et al., 1999), which represents the percentage of accumulated substrate that is excreted into bile:

\[
\text{BEI} = \frac{\text{Accumulation}_{\text{cells+ bile}} - \text{Accumulation}_{\text{cells}}}{\text{Accumulation}_{\text{cells}} \times 100}
\]

The in vitro intrinsic biliary clearance (CLbile, ml/min/mg of protein) was calculated using Eq. 2:

\[
\text{IntrinsicCL}_{\text{bile}} = \frac{\text{Accumulation}_{\text{cells+ bile}} - \text{Accumulation}_{\text{cells}}}{\text{AUC}_{\text{medium}}}
\]

where AUCmedium was the product of incubation time and the initial substrate concentration in the incubation medium. In vitro intrinsic biliary clearance values were scaled up using 200 mg protein of liver and 40 g liver/kg of rat body weight to obtain clearance values in milliliters per minute per kilogram (Liu et al., 1999; Abe et al., 2008).

**Results**

The Effects of TP on rMdr1 and rBcrp ATPase Activity. To identify the interaction of TP and P-gp at the canalicular membrane of SCRH, the ATPase screening assays with rat Mdr1- and Bcrp-expressing membranes were conducted first using verapamil as the positive control. The results demonstrated that the orthovanadate-sensitive ATPase activity of the Mdr1 membrane was significantly increased by both verapamil and TP (from 2.86 ± 0.50 to 19.8 ± 1.6 nmol/min/mg for positive control and to 42.6 ± 3.1 nmol/min/mg for TP). In the rBcrp study, although positive control, sulfasalazine, worked as expected, enhanced ATPase activity was not observed in the TP incubation (from 21.2 ± 2.2 to 44.9 ± 1.1 nmol/min/mg for positive control and to 22.1 ± 2.9 nmol/min/mg for TP). This observation suggested that TP is a substrate of rMdr1 but not a substrate of rBcrp.

**Time Course of TP Accumulation and Biliary Excretion in SCRH.** After TP was identified as a P-gp substrate, the canicular secretion of TP was investigated in the SCRH model. Figure 2 shows the time-dependent accumulation of TP in biliary canaliculic network in rat hepatocytes incubated in standard HBSS or Ca²⁺/Mg²⁺-free buffer. At different time points, the TP accumulation in bile in standard buffer (cells + bile) was significantly higher than that in Ca²⁺/Mg²⁺-free buffer (cells only), indicating TP underwent moderate excretion into bile.

![Fig. 2. TP accumulation in biliary canaliculic network over 24-hour time period in SCRH with standard (triangles symbols) or Ca²⁺/Mg²⁺-free (squares symbols) buffer. After prereincubation of SCRH with Ca²⁺ or Ca²⁺-free buffer for 15 minutes, TP (10 μM) was added. Samples were taken at different time points. The accumulation amounts of TP were measured with LC-MS/MS method (mean ± S.D., n = 3 wells/end point). *P ≤ 0.05, **P ≤ 0.01 for accumulation in standard versus Ca²⁺-free incubation buffer based on unpaired Student's t test.](https://www.dmd.aspetjournals.org/article/S0022-2828(17)31336-1/DC1/med/20160117000089047.pdf)
biliary networks. The average BEI and CL_{bile} from five different studies were 12.8 ± 1.4% and 1.59 ± 0.39 ml/min/kg, respectively, after 30-minute incubation.

Effects of P-gp Inhibitors on TP Cellular Concentration. To further confirm the involvement of P-gp in the accumulation of TP in SCRH, TP biliary excretion was evaluated in the presence of potent P-gp inhibitors ritonavir and tariquidar. Figure 3 showed that in the presence of inhibitors, TP cellular concentration was higher in the P-gp inhibitors ritonavir and tariquidar. Figure 3 showed that with the increasing concentration of P-gp inhibitors, PB, quercetin, and H2O2, the cellular concentration of TP was decreased 84.4%. As the concentration of P-gp inhibitors increased, the TP CL_{bile} in SCRH decreased dramatically as well. At the highest concentrations, ritonavir (25 µM) and tariquidar (5 µM) significantly reduced the biliary clearance of TP (CL_{bile}) by 73.7 and 84.2%, respectively. In this study, a known P-gp substrate rhodamine 123 was included as a positive control.

Effects of P-gp Inducers on TP Cellular Concentration. In this study, the effects of P-gp inducers on biliary excretion of TP in SCRH were also investigated. As expected, after 3-day treatment with P-gp inducers, PB, quercetin, or H2O2, the cellular concentration of TP was lower than the control group as the biliary excretion of TP has been enhanced (Fig. 3). The increase in accumulation rates in biliary canalicul network and the biliary clearance of TP in these studies were quantitatively presented as BEI and CL_{bile} in Table 2. The data showed that with the increasing concentration of P-gp inducers, PB, quercetin, or H2O2, both BEI and CL_{bile} of TP in SCRH enhanced. At the highest concentrations tested, BEI were up to 3.72-, 4.61-, and 3.19-fold for PB (1 mM), quercetin (20 µM), and H2O2 (0.5 mM), respectively, and CL_{bile} were up to 3.46-, 2.80-, and 2.73-fold for PB, quercetin, and H2O2, respectively. In this study, a known P-gp substrate rhodamine 123 was included as a positive control.

Alterations in the Expression of P-gp in SCRH by PB, Quercetin, and H2O2. As P-gp inducers, PB, quercetin, and H2O2 not just induced the activity as evidenced by the increase of accumulation rates of TP in biliary canalicu network, they also increased the expression of P-gp protein in the rat hepatocytes. The effects of PB, quercetin, or H2O2 on cellular P-gp expression in SCRH after 3-day of treatment were investigated by Western blot analysis. Our data showed that treatment of quercetin (20 µM), H2O2 (0.5 mM), and PB (1 mM) for 3 days resulted in expression of P-gp increases of 3.51 ± 1.41, 2.29 ± 0.32 and 4.11 ± 0.95 fold, respectively (Fig. 4).

TP-Mediated Hepatotoxicity in SCRH. TP is known to cause hepatotoxicity at certain dose levels and long-term usage. TP-mediated cytotoxicity in the SCRH was studied using the LDH release from hepatocytes. The LDH release from hepatocytes was measured using the LDH release assay. The LDH release from hepatocytes was quantitatively presented as BEI and CL_{bile} in Table 2. The data showed that with the increasing concentration of P-gp inhibitors, PB, quercetin, or H2O2, both BEI and CL_{bile} of TP in SCRH enhanced. At the highest concentrations tested, BEI were up to 3.72-, 4.61-, and 3.19-fold for PB (1 mM), quercetin (20 µM), and H2O2 (0.5 mM), respectively, and CL_{bile} were up to 3.46-, 2.80-, and 2.73-fold for PB, quercetin, and H2O2, respectively. In this study, a known P-gp substrate rhodamine 123 was included as a positive control.

**TABLE 2**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BEI % of Control</th>
<th>CL_{bile} % of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12.0 ± 1.0</td>
<td>100</td>
</tr>
<tr>
<td>Ritonavir (2.5 µM)</td>
<td>9.9 ± 1.0</td>
<td>82.5</td>
</tr>
<tr>
<td>Ritonavir (10 µM)</td>
<td>8.2 ± 0.4*</td>
<td>68.3</td>
</tr>
<tr>
<td>Ritonavir (25 µM)</td>
<td>5.7 ± 0.8*</td>
<td>47.5</td>
</tr>
<tr>
<td>Control</td>
<td>12.8 ± 1.1</td>
<td>100</td>
</tr>
<tr>
<td>Tariquidar (0.5 µM)</td>
<td>7.1 ± 1.0*</td>
<td>55.5</td>
</tr>
<tr>
<td>Tariquidar (2.5 µM)</td>
<td>4.5 ± 1.5*</td>
<td>35.2</td>
</tr>
<tr>
<td>Tariquidar (5 µM)</td>
<td>2.0 ± 0.1**</td>
<td>15.6</td>
</tr>
</tbody>
</table>

*P ≤ 0.05 and **P ≤ 0.01 indicate statistically significant differences when compared with the control group without inhibitor.
hepatocytes as an end point. Detergent Triton X-100 was used to release all intracellular LDH and that was used as a positive control for the assay. A 2-hour exposure of TP up to 10 μM was tested as a pilot study that did not result in increase of LDH release from rat hepatocytes compared with the vehicle control, suggesting TP does not cause an acute hepatotoxicity (data not shown). However, after 24-hour treatment, a dose-dependent hepatotoxicity was observed in the SCRH (Fig. 5) as the vehicle control was set at 0% (Figs. 5 and 6). These results indicated that TP induces hepatotoxicity in a concentration- and time-dependent manner. Changes in the intracellular concentration of TP can affect the hepatotoxicity. Because a 24-hour treatment of the hepatocytes with the selected concentration of TP provided a good working window for hepatotoxicity as well as other cellular effects, such as change in biliary excretion and apoptosis biomarkers, the majority of experiments in this study were conducted using 24-hour TP exposure.

**Effects of P-gp Inhibitors and Inducers on TP-Mediated Hepatotoxicity in SCRH.** To determine the effect of P-gp on TP-mediated hepatotoxicity, inducers of P-gp were incubated for 2 days prior to TP exposure (day 2 to day 4) followed by 24-hour coexposure with TP in SCRH on day 4, whereas P-gp inhibitors were pretreated for only 24 hours followed by additional 24-hour coexposure with TP. The results indicated that pretreatment of SCRH with P-gp inducer PB, quercetin, or H2O2 dramatically decreased toxicity of TP compared with the TP only control (vehicle pretreated followed by 24-hour exposure of TP; Fig. 6). The increased TP excretion via the induced transporter pump resulted in lower intracellular TP concentrations (Fig. 3) and hence led to lower hepatotoxicity. In contrast, when TP was coincubated with P-gp inhibitor ritonavir or tariquidar, the cytotoxicity was significantly increased due to the higher intracellular TP concentration resulting from a lower excretion of intracellular TP into the bile (Fig. 6). The TP control hepatotoxicity in Figs. 6 and 5 is comparable. The slight difference are attributed to the day-to-day variation and the fact that data in Fig. 5, as a 24-hour dose-dependent experiment, were measured without an additional 2 days of vehicle treatment.

**Relative Contribution of CYP3A and P-gp on TP-Mediated Hepatotoxicity in SCRH.** CYP3A and P-gp are two major detoxification mechanisms of TP-induced hepatotoxicity in an SCRH model based on the current study. In an attempt to preliminarily evaluate the relative contributions of these two mechanisms, either 50 or 250 μM ABT was coincubated with TP for 24 hours in the vehicle control, P-gp-induced, or -inhibited SCRH. With blocking of the CYP3A activity by approximately 90%, in all groups, the hepatotoxicity increased by about twofold (Table 3). This suggested that if CYP activity were added back, TP-induced hepatotoxicity would be reduced by half. Hence CYP3A and P-gp both contributed to the detoxification of TP at about equal capacity.

**Bcl-2 and Bax Protein Expressions in SCRH up on TP and P-gp Inducers Treatment.** Bcl-2 is an apoptosis inhibitor, whereas Bax is an apoptosis promoter. The ratio of Bax/Bcl-2 is often used as an indication to cell apoptosis (Raisova et al., 2001). The expression levels of Bax and Bcl-2 were detected using Western blot after SCRH were treated with vehicle, PB, quercetin, or H2O2 for 48 hours and coexposure with TP for another 24 hours. The results in Fig. 7 showed that the level of Bax expression increased in TP-only treatment group compared with its vehicle (Bax/GAPDH ~0.96 ± 0.05 versus 0.85 ± 0.05) and the P-gp inducer groups that indicate TP may play a role in promoting apoptosis. In comparison, the groups with the inducers had increased P-gp activities, and therefore reduced intracellular TP concentrations as a result showed lower Bax expression after 24-hour TP treatment. On the other hand, for the same reason, the level of Bcl-2 expression in the TP-only treated group decreased compared with its vehicle (Bcl-2/GAPDH ~0.66 ± 0.04 versus 0.92 ± 0.01) and the inducer groups. In this study, we use the ratio of Bcl-2/Bax (instead of Bax/Bcl-2) to present the protective effects of cell apoptosis under various experimental conditions. Our data showed that the mean ratio of Bcl-2/Bax reduced to 63.7% upon TP treatment. The addition of P-gp inducers PB and quercetin to the TP treatment reversed the Bcl-2/Bax ratios to 124% and 107% of the vehicle controls, respectively. Cotreatment with H2O2 showed only moderate recovery of the Bcl-2/Bax ratio to 83.2%. These results indicated that upregulation of Bax expression and downregulation of Bcl-2 expression may be one of the mechanisms that caused apoptosis/hepatotoxicity in rat hepatocytes treated with TP. The effect of P-gp inducer alone (PB, quercetin, or H2O2) on Bcl-2 or Bax was not invested in this study, and information is not available in literature.

**Discussion**

The toxicity caused by TP has drawn extensive attention for many years. Recently it was discovered that CYP3A is involved in the metabolism of TP and plays a role in the TP-induced liver injury (Ye et al., 2010; Xue et al., 2011). In this study, we present the evidence that hepatic transporter also plays a significant role in modulating the intracellular concentration of TP and, hence, the resulting hepatotoxicity. The results of our screening assay with rat Mdr1 and Bcrp...
Roles of P-gp and CYP in Triptolide-induced Liver Toxicity

membranes showed that TP is the substrate of Mrd1 but not Bcrp. The SCRH model was used to assess (1) the intracellular concentration of TP, (2) biliary excretion rates of TP under the influence of P-gp inhibitors and inducers, (3) hepatotoxicity induced by TP and the influence of P-gp inhibitors and inducers on the hepatotoxicity, (4) relative contribution of CYP3A and P-gp on the hepatotoxicity, and (5) expression of P-gp and apoptosis biomarkers Bcl-2 and Bax under the influence of P-gp inhibitors and inducers. This work represents the first report on the role of P-gp in the TP-induced hepatotoxicity and has linked the P-gp expression, P-gp inhibition and induction, intracellular TP concentration, hepatotoxicity, apoptosis biomarkers expression, and CYP-P-gp interplay in SCRH all together.

After treatment with P-gp inhibitors (ritonavir and tariquidar), the biliary excretion of TP in SCRH decreased significantly compared with the controls (Table 1). Ritonavir is not only a P-gp inhibitor but also inhibits CYP3A (Hartman et al., 2010; Ye et al., 2010), whereas tariquidar is a potent P-gp inhibitor with negligible effect on CYP (Pusztai et al., 2005). Our data showed tariquidar seemed to be a more potent inhibitor toward the biliary clearance of TP compared with ritonavir but caused less toxicity than may indicate the involvement of CYP in the disposition of TP in SCRH. In contrast to P-gp inhibition, less information is available on the mechanisms of induction of biliary canalicular P-gp (Mitsunaga et al., 2000). It has been reported that P-gp expression can be induced by compounds such as PB (Pezzotti et al., 2002), some flavonoids (Conseil et al., 1998), and reactive oxygen species such as H2O2 (Ziemann et al., 1999). In this study, PB, quercetin, and H2O2 were demonstrated to induce P-gp protein expression as well as its activity that led to the increase in biliary excretion of TP. In addition, good correlation between induced P-gp expressions and activities was observed. For example, Western blot analysis showed that the protein expression induced by PB, quercetin, and H2O2 at their highest concentration was 4.11, 3.51, and 2.29-fold higher, respectively; that is well correlated with the observed activity increase (CLbile) of 3.46-, 2.80-, and 2.73-fold for PB, quercetin, and H2O2, respectively. In this study, inhibition of P-gp was also nicely demonstrated by direct evidence of increased intracellular TP concentration and indirect evidence of increased TP-mediated hepatotoxicity, whereas the induction of P-gp also was demonstrated directly by decrease of intracellular TP concentration and indirectly by the reduced TP-mediated hepatotoxicity. In the hepatotoxicity experiments, both MTT and LDH assays were used to assess cell viability. MTT assay was first applied to screen for nontoxic concentrations for all inducers and inhibitors. The LDH assay, being a noninvasive assay showing low variability and good correlation with the cell morphologic changes (Swift et al., 2010), was used in the data presented in this report. Comparison between a treatment of TP for only 24 hours and coincubation of P-gp with inhibitors and inducers resulted in obvious alternations in the LDH levels in rat hepatocytes. For example, treatment with P-gp inducers PB, quercetin, or H2O2 showed a strong protective effect in hepatocytes from hepatotoxicity. On the other hand, P-gp inhibitors ritonavir and tariquidar aggravated the damage as a result of reduced biliary excretion and increased intracellular concentration of TP (Fig. 6). These observations were true at all levels of TP treatment from 0.5 to 10 μM.

Alteration in the levels of antiapoptotic Bax protein and proapoptotic Bcl-2 protein can affect cell survival. Several reports showed that TP

### Table 3: Hepatotoxicity of TP with P-gp inhibitors and inducers in the presence or absence of ABT

<table>
<thead>
<tr>
<th>Groups</th>
<th>ABT μM</th>
<th>% Inhibition of CYP3A by ABT</th>
<th>Cytotoxicity without ABT</th>
<th>Cytotoxicity with ABT</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP</td>
<td>50</td>
<td>85</td>
<td>19.3 ± 1.6</td>
<td>34.9 ± 3.5</td>
</tr>
<tr>
<td>TP + Ritonavir</td>
<td>50</td>
<td>91</td>
<td>38.7 ± 0.6</td>
<td>80.4 ± 1.3</td>
</tr>
<tr>
<td>TP + Tariquidar</td>
<td>50</td>
<td>90</td>
<td>28.2 ± 0.6</td>
<td>46.8 ± 3.0</td>
</tr>
<tr>
<td>TP + H2O2</td>
<td>50</td>
<td>91</td>
<td>11.5 ± 0.3</td>
<td>15.8 ± 0.8</td>
</tr>
<tr>
<td>TP + Quercetin</td>
<td>250</td>
<td>90</td>
<td>2.7 ± 0.5</td>
<td>6.0 ± 0.6</td>
</tr>
<tr>
<td>TP + PB</td>
<td>250</td>
<td>89</td>
<td>0.6 ± 1.9</td>
<td>1.9 ± 0.6</td>
</tr>
</tbody>
</table>

Fig. 6. Effect of P-gp modulators on triptolide-mediated hepatotoxicity. Cytotoxicity was determined by measuring LDH release into the culture medium after 24-hour TP (0.1–10 μM) exposure. Ritonavir (25 μM) and tariquidar (5 μM) were added to the culture medium 24 hours prior to coexposure with TP. PB (1 mM), quercetin (20 μM), and H2O2 (0.5 mM) were added to the culture medium 48 hours prior to coexposure with TP. Each bar represents the mean ± S.D. (n = 3 wells/end point) for samples from vehicle pretreatment + TP (gray bars) or P-gp modulator-pretreatment + TP (other bars); *P < 0.05; **P < 0.01.
can upregulate the expression of Bax and downregulate the expression of Bcl-2 and thus may cause apoptosis in many tissues (Shu et al., 2009; Yang et al., 2011). In the current study, it was confirmed that TP did upregulate the antiapoptosis protein Bax and downregulate the proapoptotic protein Bcl-2 in SCRH. The observed attenuation of TP-induced hepatotoxicity by PB, quercetin, and H₂O₂ also correlated with hepatocyte apoptosis as indicated by its biomarkers. These findings suggest that coadministration of P-gp inducers with TP could protect the liver from apoptosis and hepatotoxicity by enhancing TP biliary excretion via P-gp. H₂O₂ was reported to have a role of positive up regulation of mdr1 genes and the P-gp protein (Ziemann, et al., 1999). Our results also demonstrated that H₂O₂ can induce the expression of P-gp and biliary excretion of TP and thus reduce the TP-mediated hepatotoxicity like other P-gp inducers. However, H₂O₂ itself is a reactive oxygen species that could have an add-on hepatotoxicity. Comparatively, H₂O₂ was less effective than the other P-gp inducers.

It is worth noting that quantification of intracellular concentration of a drug is, in general, a difficult task. It is well known that nonspecific binding is present in many hepatocyte incubations (Witherow and Houston, 1999; Austin et al., 2005; Lu et al., 2006; Chu et al., 2013; Pfeifer et al., 2013). In addition, the presence of Matrigel in the SCH may hinder the total removal of extracellular TP, leading to an overestimation of intracellular concentrations. Nevertheless, in this study, the comparative changes in apparent intracellular TP concentrations between control group and modulator-treated groups are as informative as the true intracellular concentrations. As illustrated in Fig. 8, a plot of observed percentage of hepatotoxicity at treatment of 10 μM of TP with or without moderators (data from Fig. 6) against their apparent intracellular concentrations (data from Fig. 3) showed a good correlation with r² of 0.96. The intercept at the x-axis around 50 pmol/mg protein may partially reflect the level of nonspecific binding of TP in hepatocytes in this study.

CYP3A is the major metabolic pathway of TP. Inhibiting or inducing CYP3A affects the exposure, and toxicity of TP is well documented in preclinical species as well as in vitro studies (Ye et al., 2010; Xue et al., 2011; our ongoing studies). In the current study, the higher effectiveness of ritonavir (a dual CYP3A/P-gp inhibitor) than tariquidar (a P-gp inhibitor) and phenobarbital (a dual CYP3A/P-gp inhibitor) than quercetin (a P-gp inhibitor) on cytotoxicity may be explained by the synergistic effects between CYP3A and P-gp, given the fact that ritonavir and phenobarbital seemed to be weaker modulators of P-gp than tariquidar and quercetin, at least at comparable concentrations. The CYP and transporter interplay is a well-known phenomenon, and our results provided evidence that modulation of CYP3A and P-gp would result in an additive or synergistic effect in TP-induced hepatotoxicity. As shown in Table 3, CYP3A and P-gp likely contribute equally toward the detoxification of TP-induced hepatotoxicity in this study.

In summary, our results first demonstrate that P-gp plays an important role in the biliary excretion of TP and the consequent hepatotoxicity in addition to CYP3A. Although pathologic changes and serum markers of liver function, such as, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and lactate dehydrogenase (LDH) were found to be similar in rat and human for this class of herbal medicines (Chai et al., 2011), much human clinical toxicology data were not available. Given the narrow therapeutic index and high toxicity observed in clinical usage of TP, the dose of this widely used Chinese traditional medicine needs to be closely monitored in the clinic. The coadministration of TP with P-gp inhibitors and inducers, as well as CYP3A modulators, should also be carefully monitored.

Authorship Contributions

Participated in research design: Zhuang, Shen, Lu, Li.
Conducted experiments: Zhuang, Shen, Xiao, Tan.
Performed data analysis: Zhuang, Shen, Lu, Li.
Wrote or contributed to the writing of the manuscript: Zhuang, Li, Lu.

References


Fig. 7. Detection of Bcl-2 and Bax protein in SCRH treated with vehicle, TP, quercetin, H₂O₂, or PB. Cells were treated with vehicle, TP (10 μM), quercetin (20 μM), H₂O₂ (0.5 mM), or PB (1 mM) for 24 hours. Total proteins were analyzed by Western blotting using appropriate antibodies. GAPDH was included as an internal loading control (n = 3 wells/end point).

Fig. 8. Plot of TP-mediated hepatotoxicity against the intracellular TP concentration. Hepatocytes were treated with TP (10 μM) in the absence (control) and presence P-gp inhibitors and inducers. The x-axis of the linear regression line (r² = 0.9636) may suggest the nonspecific binding of TP to hepatocytes in this study.