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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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. In contrast, increases of 346%, 280%, and 273% in biliary clearance of TP were observed with treatment of phenobarbital (1.0 mM), quercetin (20 μM), and H2O2 (0.5 mM), respectively. The TP-induced hepatotoxicity increased by 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-canaluted 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 liver, and liver canalicular membrane vesicles (Lipsky and Tao, 1997). In addition, rat primary hepatocyte model is a popular tool to study hepatobiliary transporters because of their high resemblance to human liver. However, because of limited availability, rat primary hepatocytes have limitation in use.

The development of a functional human hepatocyte model is of even greater importance, and sandwich-cultured rat hepatocytes (SCRH) is considered to be one of the most suitable in vitro models. SCRH are derived from a primary rat hepatocyte culture, which are then cultured on a collagen gel, thus allowing the recovery of cell function (Lipsky and Tao, 1997; Panichakul et al., 2006; Lin et al., 2007; Zhuang et al., 2013). SCRH is a rat hepatocyte model that mimics the human liver more closely than primary hepatocytes and could be used for toxicokinetic studies in the absence of circulating factors. In addition, SCRH can be used to study the role of transporters in drug disposition and toxicity. In a previous study, we showed that SCRH could be used to study the role of transporters in drug disposition and toxicity (Zhuang et al., 2012).

In this study, we tested whether SCRH is a useful model to study the role of P-gp in the disposition of TP and the hepatotoxicity induced by TP. We also evaluated the potential of drug-drug interaction between TP and the known P-gp inhibitors or inducers, ritonavir and tariquidar, in the SCRH model.
Roles of P-gp and CYP in Triptolide-induced Liver Toxicity

Fig. 1. The chemical structure of TP.

Materials and Methods

Chemicals. Penicillin-streptomycin solution, dexamethasone, Hanks’ balanced salts (HBSS), HBSS modified (HBSS without Ca\(^{2+}\) and Mg\(^{2+}\)), 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). H\(_2\)O\(_2\) was purchased from SioPharm (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 \(\mu\)M), 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 \(\mu\)M) and sulfasalazine (30 \(\mu\)M) 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 \(\times 10^{10}\) cells/ml in DMEM-based culture medium containing 10% fetal bovine serum, 0.1 \(\mu\)M dexamethasone, 2 mM l-glutamine, 100 units penicillin G sodium, and 100 \(\mu\)g 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 hours at 37°C in a humidified incubator with 95% air–5% CO\(_2\) 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, Tariquidar, Phenobarbital, Quercetin, and H\(_2\)O\(_2\) in SCRH. In the effect of P-gp inhibition on BEI study, culture medium containing vehicle, ritonavir (2.5, 10, and 25 \(\mu\)M) or tariquidar (0.5, 2.5, and 5 \(\mu\)M) 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 \(\mu\)M), or H\(_2\)O\(_2\) (0.1, 0.25, and 0.5 mM) was added to the hepatocytes culture every 24 hours to replace the old medium. TP at 0.1, 0.5, 1.0, 5.0, or 10 \(\mu\)M in standard or modified HBSS washed with Ca\(^{2+}\)/Mg\(^{2+}\) was added to the culture and incubated for 30 minutes for the determination of BEI.

 Cotreatment 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 SCRH 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 \(\mu\)M) for 24 hours in presence or absence of either 50 or 250 \(\mu\)M of the pan-CYP inhibitor ABT. At the end of the incubations, 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 SCRH 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 \(\mu\)M) 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 canalicularr network in the presence or absence of P-gp inducers and inhibitors were conducted. The SCRH 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 \(\mu\)M TP dissolved in either standard HBSS buffer (pH 7.4) (cells ± bile) or Ca\(^{2+}\)/Mg\(^{2+}\)-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 \(\mu\)l cell lystate from each samples was extracted with 200 \(\mu\)l of 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 study 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 Si9 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 down-regulation 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 H\(_2\)O\(_2\) (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.
The in vitro intrinsic biliary clearance (CL\textsubscript{bile}, ml/min/mg of protein) was calculated using Eq. 2:

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

(2)  

where AU_{\text{medium}} 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/kg 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 orthovandate-sensitive ATPase activity of the rMdr1 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, sulfa-lazasulfine, 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 canalicular secretion of TP was investigated in the SCRH model. Figure 2 shows the time-dependent accumulation of TP in biliary canalicular network in rat hepatocytes incubated in standard HBSS or Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-free buffer. At different time points, the TP accumulation in bile in standard buffer (cells + bile) was significantly higher than that in Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-free buffer (cells only), indicating TP underwent moderate excretion into
biliary networks. The average BEI and CL\textsubscript{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. As P-gp inhibitors increased, the TP CL\textsubscript{bile} in SCRH decreased dramatically as well. 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 H\textsubscript{2}O\textsubscript{2} (0.5 mM), respectively, and CL\textsubscript{bile} were up to 3.46-, 2.80-, and 2.73-fold for PB, quercetin, and H\textsubscript{2}O\textsubscript{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 H\textsubscript{2}O\textsubscript{2}, 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 canalicular network and the biliary clearance of TP in these studies were quantitatively presented as BEI and CL\textsubscript{bile} in Table 2. The data showed that with the increasing concentration of P-gp inducers, PB, quercetin, or H\textsubscript{2}O\textsubscript{2}, both BEI and CL\textsubscript{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 H\textsubscript{2}O\textsubscript{2} (0.5 mM), respectively, and CL\textsubscript{bile} were up to 3.46-, 2.80-, and 2.73-fold for PB, quercetin, and H\textsubscript{2}O\textsubscript{2}, 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 H\textsubscript{2}O\textsubscript{2}**. As P-gp inducers, PB, quercetin, and H\textsubscript{2}O\textsubscript{2} not just induced the activity as evidenced by the increase of accumulation rates of TP in biliary canalicular network, they also increased the expression of P-gp protein in the rat hepatocytes. The effects of PB, quercetin, or H\textsubscript{2}O\textsubscript{2} 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), H\textsubscript{2}O\textsubscript{2} (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 rat hepatocytes. Treatment BEI % of Control CL\textsubscript{bile} % of Control

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BEI % of Control</th>
<th>CL\textsubscript{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 ratio 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
membranes showed that TP is the substrate of Mdr1 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 that 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 (Penzotti et al., 2002), some flavonoids (Conseil et al., 1998), and reactive oxygen species such as H$_2$O$_2$ (Ziemann et al., 1999). In this study, PB, quercetin, and H$_2$O$_2$ were used 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 H$_2$O$_2$ at their highest concentration was 4.11, 3.51, and 2.29-fold higher, respectively; that is well correlated with the observed activity increase (CL$_{\text{bile}}$) of 3.46-, 2.80-, and 2.73-fold for PB, quercetin, and H$_2$O$_2$, 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 H$_2$O$_2$ 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 increased the levels of proapoptotic Bax protein and reduced the levels of antiapoptotic Bcl-2 protein, which resulted in the induction of apoptosis.

<table>
<thead>
<tr>
<th>Groups</th>
<th>ABT µM</th>
<th>% Inhibition of CYP3A by ABT</th>
<th>Cytotoxicity without ABT % of PC</th>
<th>Cytotoxicity with ABT % of PC</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 + H$_2$O$_2$</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 H$_2$O$_2$ (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 PBR, 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. 7, 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. 7, the comparative changes in apparent intracellular TP concentrations between control group and modulator-treated groups are as informative as the true intracellular concentrations.

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


Roles of P-gp and CYP in Triptolide-induced Liver Toxicity


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