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

Xiao-Mei Zhuang, Guo-Lin Shen, Wei-Bin Xiao, Yan Tan, Chuang Lu, and Hua Li

The key lab of drug metabolism and pharmacokinetics, Beijing Institute of Pharmacology and Toxicology, Beijing, 100850, China

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Millennium Pharmaceuticals, Inc. Cambridge, Massachusetts, USA (C L)
Running title

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Corresponding author:
(submission of this manuscript and future communication with readers)
Chuang Lu
Millennium Pharmaceuticals, Inc.
40 Landsdowne Street
Cambridge, MA 02139
Phone: (617) 551-8952
Fax: (617) 444-1480
E-mail: chuang.lu@mpi.com

Corresponding author:
(future communication with readers)
Hua Li
The key lab of drug metabolism and pharmacokinetics,
Beijing Institute of Pharmacology and Toxicology,
Beijing, 100850, China
Phone: +86 10 88270677
Fax: +86 10 68211656
E-mail address: amms_hli@126.com
List of Abbreviations

Abbreviation: ABT, 1-Aminobenzotriazole;  BEI, Biliary excretion index;
BSA-TBST, Bovine serum albumin-tris-buffered saline with Tween 20;  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
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 H$_2$O$_2$. 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 H$_2$O$_2$ (0.5 mM), respectively. The TP-induced hepatotoxicity increased by 2-fold 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 co-administered P-gp inhibitors or inducers in the clinic.
Introduction

Triptolide (TP, 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 et al., 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 attributed by 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 (Wang et al., 2007; Xue et al., 2009; Chen et al., 1999; Chai et al., 2011). The inhibition of mitochondrial respiratory chain (Fu et al., 2011), the excessive apoptosis of hepatocytes (Yao et al., 2008) and the 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-canulated rats indicated that about 50% of the intravenous dose was excreted into bile as TP and its metabolites (manuscript in preparation). The evidence above suggests that the 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 (BSEP), 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 further, 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 (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 (SCH) to evaluate hepatic drug disposition into the biliary canaliculular network. In that model, hepatocytes were cultured between two layers of gelled collagen in a sandwich configuration to re-create the three-dimensional cellular environment and re-establish cell polarity. It also formed canaliculular membrane domains by re-establish the tight junction and bile canaliculular networks. Appropriate expression and re-localization of transport proteins in SCH 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 (SCRH) 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) which are likely to attribute to the observed hepatotoxicity. The known P-gp inhibitors (ritonavir and tariquidar), and inducers (phenobarbital, quercetin, and H₂O₂) were applied to the SCRH 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 SCRH to block the CYP metabolism and to investigate the relative contributions of CYP vs. P-gp toward the detoxification of TP-induced hepatotoxicity.
Materials and Methods

Chemicals. Penicillin-streptomycin solution, dexamethasone (DEX), Hanks’ balanced salts (HBSS), HBSS modified (HBSS without Ca\(^{2+}\) and Mg\(^{2+}\)), collagenase (type IV), Triton X-100, Phenobarbital (PB), ritonavir, tariquidar, 1-Aminobenzotriazole (ABT), midazolam, and quercetin were purchased from Sigma-Aldrich, Inc (St. Louis, MO). DMEM/F12 medium were purchased from Invitrogen (Carlsbad, CA). Insulin/transferrin/selenium culture supplement (ITSTM) and matrigel\textsuperscript{TM} extracellular matrix were purchased from BD Biosciences Discovery Labware (Bedford, MA). Fetal bovine serum was purchased from Gibico BRL (Gaithersburg, MD). Triptolide was purchased from Chinese National Institute of Food and Drug Control with the purity more than 99% (Beijing, China). \(\text{H}_2\text{O}_2\) was purchased from SioPharm and PB was provided by TianJin Jinyaoan Com (Tianjin, China). Rat Mdr1, Bcrp membrane and ATPase assay kit were purchased from BD Genetest 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 pre-incubated for 5 and 10 minutes, respectively. The reactions were started by addition of MgATP, followed by incubation at 37°C for 10 and 30 min. 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 min 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 (SCRH).** Male SD rats weighting 200 to 250 g were maintained on a 12-hr light/dark cycle with free access to water and lab 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 (AAALAC). 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 1×10^6 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
suspendion was added to each well of 24-well plates that were pre-coated with rat-tail tendon collagen. Hepatocytes were allowed to attach for ~4 hr 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-hr 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-hr until the conduct of experiments.

**Pretreatment of Ritonavir, Tariquidar, Phenobarbital, Quercetin, and H₂O₂ in SCRH.** In the effect of P-gp inhibition on BEI study, culture medium containing vehicle, ritonavir (2.5 µM, 10 µM, and 25 µM) or tariquidar (0.5 µM, 2.5 µM, and 5 µM) was added to the hepatocytes on day 4 and incubated for 30 min 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 mM, 0.5 mM, and 1 mM), quercetin (2 µM, 10 µM, and 20 µM), or H₂O₂ (0.1 mM, 0.25 mM, and 0.5 mM) was added to the hepatocyte culture every 24 hr to replace the old medium. TP at 0.1, 0.5, 1.0, 5.0, or 10 µM in standard or modified HBSS withheld Ca²⁺/Mg²⁺ was added to the culture and incubated for 30 min for the determination of BEI.

**Co-treatment of ABT with TP to Evaluate the Relative Contributions of CYP vs. P-gp in Detoxification of TP-induced Hepatotoxicity.** In order to elucidate the relative contributions of CYP3A and P-gp toward the detoxification of TP-induced
hepatotoxicity, the SCRH pretreated 24-hr with P-gp inhibitors or 48-hr with inducers (some of them are dual CYP and P-gp modulators) were incubated with TP (10 µM) for 24-hr in presence or absence of either 50 or 250 µM of the pan-CYP inhibitor ABT. In the end of the co-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 approximate 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 CYP 3a 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 biliary canalicular 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 min 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 min, 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 min at room temperature to lyses the cells. For cellular TP analysis, 100 µl cell lysate from each samples was precipitated with 200 µl of acetonitrile/methanol (1:1) containing internal standard solution (propranolol, 100
ng/ml). The mixtures were centrifuged at 14,000 × g at 4°C for 10 min and the supernatant was then taken for LC-MS/MS analysis. The protein content of the hepatocytes in each well was measured using BCA protein assay kit (Pierce, Rockford, IL) with 50 µl cell lysate which was set aside after lyses.

**MTT Assay.** A 0.5 mg/ml of solution of MTT was added to rat hepatocyte culture after being treated with test compounds for 24 hr. After 3 hr incubation, hepatocytes were checked visually for dye conversion, the medium was aspirated, rinsed twice with standard HBSS. Then the purple crystal metabolite, formazan, was dissolved in acetonitrile, 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 to 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 with a CL8000 automated biochemical analyzer (Shimadzu, Japan) to assess hepatocyte viability. At the end of the 24 hr 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.

\[
Cytotoxicity(\%\ of\ control) = \frac{LDH_{sample} - LDH_{blank}}{LDH_{Triton\ X-100} - LDH_{blank}}
\]

**Western Blot Analysis of P-gp, Bcl-2, and Bax Proteins.** On day 5 after 24-hr TP treatment, the medium was removed and cells were washed twice with ice-cold phosphate-buffered saline (PBS). Subsequently, cells were lysed with lysis buffer [1 mM PMSF mixed with complete protease inhibitors (Roche Diagnostics, Mannheim, Germany)]. The lysates were centrifugation at 12,000× g for 20 min 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% BSA-TBST for 30 min at room temperature. Subsequently, the membrane was incubated with appropriate primary antibodies for 10 min at room temperature or over-night at 4 °C, then rinsed five times at 10-min intervals with 3% BSA-TBST. The primary antibodies used were rabbit polyclonal antibodies to P-gp (Biomol, Plymouth Meeting, PA) and rabbit antibodies to Bcl-2 and Bax (Pharmingen, San Diego, CA). The secondary antibodies used for detection was 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 Aglient 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, MA) 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: 0-2.5 min hold at 95 % B; 2.5-3 min, a linear gradient to 10 % B; 3-4 min 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 (equation 1, Liu et al., 1999), which represents the percentage of accumulated substrate that is excreted into bile:

\[
BEI(\%) = \frac{Accumulation_{cells+bile} - Accumulation_{cells}}{Accumulation_{cells+bile}} \times 100
\]
The in vitro intrinsic biliary clearance (\( \text{CL}_{\text{bile}} \), ml/min/mg of protein) was calculated using the equation 2:

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

(2)

where \( \text{AUC}_{\text{medium}} \) 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 of protein/g of liver and 40 g of liver/kg of rat body weight to obtain clearance values in ml/min/kg (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 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, while 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 canalicular secretion of TP was investigated in the SCRH model. Fig. 2 shows the time-dependent accumulation of TP in biliary canalicular network in rat hepatocytes incubated in standard HBSS or Ca^{2+}/Mg^{2+}-free buffer. At different time points, the TP accumulation in bile in standard buffer (cells + bile) was significantly higher than that in Ca^{2+}/Mg^{2+}-free buffer (cells only), indicating TP underwent moderate excretion into 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, following 30 min 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. Fig. 3 showed that in the presence of inhibitors, TP cellular concentration was higher in the Ca²⁺/Mg²⁺-free HBSS buffer. The Table 1 listed the calculated values of BEI and CL_{bile} from these studies. Both inhibitors caused a dose-dependent decrease of BEI. At 25 µM, ritonavir significantly inhibited the TP biliary excretion with BEI decreased 52.5 %, whereas tariquidar (5 µM) significantly inhibited the TP biliary excretion with BEI 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 canalicular 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 CLₘᵲ 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 CLₘᵲ 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 canaliculär 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-day resulted in expression of P-gp increases of 3.51 ± 1.41, 2.29 ± 0.32 and 4.11 ± 0.95 folds, 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 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-hr exposure of TP up to 10 µM was tested as a pilot study that didn’t result in increase of LDH release from rat hepatocytes compared to the
vehicle control suggesting TP does not cause an acute hepatotoxicity (data not shown).

However, after 24-hr treatment, a dose dependent hepatotoxicity was observed in the
SCRH (Fig. 5) as the vehicle control was set at 0 % (Fig. 5 and Fig. 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. Since a 24-hr 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-hr 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-hr
co-exposure with TP in SCRH on day 4, while P-gp inhibitors were pretreated only
for 24 hr followed by additional 24-hr co-exposure with TP. The results indicated
that pretreatment of SCRH with P-gp inducer PB, quercetin or H2O2 dramatically
decreased toxicity of TP, compared to the TP only control (vehicle-pretreated
followed by 24-hr 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 co-incubated with
P-gp inhibitor ritonavir or tariquidar, the cytotoxicity was significantly increased due
to the higher intracellular TP concentration resulted from a lower excretion of
intracellular TP into the bile (Fig. 6). When compared the TP control hepatotoxicity in Fig. 6 and Fig. 5, they are comparable. The slight difference are attributed to the day-to-day variation and the fact that data in Fig. 5, as a 24-hr dose dependent experiment, were measured without additional 2-day 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 SCRH model based on the current study. In an attempt to preliminary evaluate the relative contributions of these two mechanisms, either 50 or 250 µM ABT was co-incubated with TP for 24-hr 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 in all groups by about 2-fold (Table 3). This suggested that if CYP activity were added back, TP-induced hepatotoxicity would be reduce 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 while 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 co-exposure 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 to its vehicle (BAX/GAPDH ~ 0.96 ± 0.05 vs. 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-h TP treatment. On the other hand, for the same reason, the level of Bcl-2 expression in the TP only treated group decreased compared to its vehicle (Bcl-2/GAPDH ~ 0.66 ± 0.04 vs. 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 % up on 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. Co-treatment with H$_2$O$_2$ showed only moderate recovery of the Bcl-2/Bax ratio to 83.2 %. These results indicated that up-regulation of Bax expression and down-regulation 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 H$_2$O$_2$) on Bcl-2 or Bax was not invested in this study and information is not available in literature.
Discussions

The toxicity caused by TP has drawn extensive attention for many years. Recently it has been discovered that CYP3A 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 evidences 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, 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, and apoptosis biomarkers expression, CYP-P-gp interplay in SCRH, all together.

After treated with P-gp inhibitors (ritonavir and tariquidar), the biliary excretion of TP in SCRH decreased significantly compared to 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 negligent 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 to 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 flavonoid (Conseil et al., 1998), and reactive oxygen species (ROS) such as H$_2$O$_2$ (Ziemann et al., 1999). In this study, PB, quercetin, and H$_2$O$_2$ were demonstrated to induced 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 expresses 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 ($\text{CL}_{\text{bile}}$) 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 non-toxic concentrations for all inducers and inhibitors. The LDH assay being a non-invasive assay also 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 only for 24 hours and co-incubation 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 results 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 µM to 10 µM.

Alteration in the levels of anti-apoptotic Bax protein and pro-apoptotic Bcl-2 protein can affect cell survival. Several reports showed that TP can up-regulate the expression of Bax and down-regulate 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 up-regulate the anti-apoptosis protein BAX and down-regulate the pro-apoptotic proteins Bcl-2 in SCRH. The observed attenuation of TP-induced hepatotoxicity by PB, quercetin, and H2O2 also correlated with hepatocytes’ apoptosis as indicated by its biomarkers. These findings suggest that co-administration of P-gp inducers with TP could protect 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, H$_2$O$_2$ itself is a reactive oxygen species (ROS) that could 
have an add-on hepatotoxicity. Comparatively, H$_2$O$_2$ was less effective than the 
other P-gp inducers.

It is worth to note that quantification of intracellular concentration of a drug is in 
general a difficult task. It is well known that non-specific binding is present in many 
of 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 over estimation 
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 $r^2$ of 0.96. 
The intercept at the x-axis around 50 pmol/mg protein may partially reflect the level of 
non-specific binding of TP in hepatocytes in this study.

CYP3A is the major metabolic pathway of TP. Inhibiting or inducing of CYP3A 
affects the exposure and toxicity of TP is well documented in preclinical species as 
well as in 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 inducer) than quercetin (a P-gp inducer) 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 showed in Table 3, CYP3A and P-gp likely contribute equally toward the detoxification of TP-induced hepatotoxicity in this study.

In summary, our results firstly demonstrate that P-gp plays an important role in the biliary excretion of TP and the consequent heptotoxicity in addition to CYP3A. While pathological changes and serum marker of liver function (AST, ALT, LDH) of liver damage were found to be similar in rat and human for this class of herbal medicines (Chai et al., 2011), and 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 need to be closely monitored in the clinic. The co-administration of TP with P-gp inhibitors and inducers, as well as CYP3A modulators should also be carefully monitored.
Author Contributions

Participated in research design: Zhuang, Shen, Lu, and Li

Conducted experiments: Zhuang, Shen, Xiao, and Tan.

Performed data analysis: Zhuang, Shen, Lu, and Li.

Wrote or contributed to the writing of the manuscript: Zhuang, Li, and Lu.
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Footnotes

Xiao-Mei Zhuang and Guo-Lin Shen contributed equally to this work. This study was supported by Chinese National Science & Technology Major Special Project on Major New Drug Innovation [2008ZX09006001] and [2012ZX09301003-001].
Chemical Compounds Studied in the Article

Triptolide (PubChem CID: 107985); Ritonavir (PubChem CID: 53487909); Tariquidar (PubChem CID: 148201); Phenobarbital (PubChem CID: 4763); Quercetin (PubChem CID: 5280343); H₂O₂ (PubChem CID: 784); Propranolol (PubChem CID: 4946); Midazolam (PubChem CID: 4129).
Figure legends

FIG 1  The chemical structure of triptolide (TP)

FIG 2  TP accumulation in biliary canalicular network over 24-h time period in SCRH with standard (solid symbols) or Ca\textsuperscript{2+}-free (open symbols) buffer

After pre-incubation of SCRH with Ca\textsuperscript{2+} or Ca\textsuperscript{2+} free buffer for 15 min, TP (10 \textmu M) was added. Samples were taken at different time points. The accumulation amounts of TP were measured with LC-MS/MS method (mean ± SD, n=3 wells/end point). * P≤0.05, ** P≤0.01 for accumulation in standard versus Ca\textsuperscript{2+}-free incubation buffer based on unpaired Student’s t test.

FIG 3  Effects of P-gp inhibitors ritonavir and tariquidar and P-gp inducers PB, quercetin, and H\textsubscript{2}O\textsubscript{2} on intracellular concentration of TP in SCRH

For P-gp inhibitors, SCRH was pre-treated with tariquidar (5 \textmu M) or ritonavir (25 \textmu M) for 24 h prior co-incubated with TP (10 \textmu M). For P-gp inducers, hepatocytes were pre-treated with PB (1 mM), quercetin (20 \mu M), and H\textsubscript{2}O\textsubscript{2} (0.5 mM) for 48 hr before TP was added (10 \mu M). Then all the media was removed and Ca\textsuperscript{2+}- free buffer was added to incubate for 15 min. Finally, the cellular remain accumulation amounts of TP were measured using LC-MS/MS. Data presented as mean ± SD (n=3 wells/end point).

FIG 4  Representative Western blot of P-gp in SCRH pretreated with vehicle,
quercetin (20 µM), H$_2$O$_2$ (0.5 mM) or PB (1 mM). 30 µg protein/well of whole cell lysates (n=3 wells/end point)

**FIG 5** Triptolide (TP)-mediated cytotoxicity in SCRH model.

Cytotoxicity was determined by measuring LDH release into the culture medium after 24 hr TP exposure (0.1 - 10 µM). Each bar represents the mean ± S.D. (n=3 wells/end point). Samples from vehicle controls represented zero value on the graph are not shown. *, p < 0.05; **, p < 0.01.

**FIG 6** Effect of P-gp modulators on triptolide-mediated hepatotoxicity.

Cytotoxicity was determined by measuring LDH release into the culture medium after 24 hr TP (0.1 - 10 µM) exposure. Ritonavir (25 µM) and tariquidar (5 µM) were added to the culture medium 24 h prior to co-exposure with TP. PB (1 mM), quercetin (20 µM), and H$_2$O$_2$ (0.5 mM) were added to the culture medium 48 h prior to co-exposure 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.

**FIG 7** Detection of Bel-2 and Bax protein in SCRH treated with vehicle, TP, quercetin, H$_2$O$_2$, or PB.

Cells were treated with vehicle, TP (10 µM), quercetin (20 µM), H$_2$O$_2$ (0.5 mM), or PB (1 mM) for 24-hr. 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 (~50 pmol/mg protein) may suggest the non-specific binding of TP to hepatocytes in this study.
TABLE 1
Effects of ritonavir and tariquidar on the in vitro biliary clearance of TP in SCRH

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BEI (%)</th>
<th>% of control</th>
<th>CL_{bile} (ml/min/kg)</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12.0±1.0</td>
<td>100</td>
<td>1.9±0.2</td>
<td>100</td>
</tr>
<tr>
<td>Ritonavir (2.5 µM)</td>
<td>9.9±1.0</td>
<td>82.5</td>
<td>1.4±0.8</td>
<td>73.7</td>
</tr>
<tr>
<td>Ritonavir (10 µM)</td>
<td>8.2±0.4*</td>
<td>68.3</td>
<td>1.0±0.3</td>
<td>52.6</td>
</tr>
<tr>
<td>Ritonavir (25 µM)</td>
<td>5.7±0.8*</td>
<td>47.5</td>
<td>0.5±0.1**</td>
<td>26.3</td>
</tr>
<tr>
<td>Control</td>
<td>12.8±1.1</td>
<td>100</td>
<td>1.9±0.2</td>
<td>100</td>
</tr>
<tr>
<td>Tariquidar (0.5 µM)</td>
<td>7.1±1.0*</td>
<td>55.5</td>
<td>1.4±0.3</td>
<td>73.7</td>
</tr>
<tr>
<td>Tariquidar (2.5 µM)</td>
<td>4.5±1.5*</td>
<td>35.2</td>
<td>0.8±0.2*</td>
<td>42.1</td>
</tr>
<tr>
<td>Tariquidar (5 µM)</td>
<td>2.0±0.1**</td>
<td>15.6</td>
<td>0.3±0.1**</td>
<td>15.8</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SD (n=3 wells/end point).

Rhodamine 123 (5 µM) was used in the study as a positive control with mean values of BEI and CL_{bile} of 16.9 ± 2.6% and 15.5 ± 2.8 ml/min/kg, respectively.

* (P≤0.05) and ** (P≤0.01) indicate statistically significant differences when compared to the control group without inhibitor.

The rat hepatocytes were cultured for 4 days prior the study then ritonavir and tariquidar were added 30-min prior addition of TP.
### TABLE 2

Effects of quercetin, H$_2$O$_2$ and PB on the in vitro biliary clearance of TP in SCRH

<table>
<thead>
<tr>
<th>Treatment</th>
<th>BEI (%)</th>
<th>% of control</th>
<th>CL$_{bile}$ (ml/min/kg)</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.1±1.1</td>
<td>100</td>
<td>1.81±0.32</td>
<td>100</td>
</tr>
<tr>
<td>PB(0.2 mM)</td>
<td>18.2±0.8*</td>
<td>164</td>
<td>3.08±0.02*</td>
<td>170</td>
</tr>
<tr>
<td>PB(0.5 mM)</td>
<td>33.5±0.7**</td>
<td>301</td>
<td>5.31±0.53**</td>
<td>293</td>
</tr>
<tr>
<td>PB(1 mM)</td>
<td>41.4±1.1**</td>
<td>372</td>
<td>6.27±1.03**</td>
<td>346</td>
</tr>
<tr>
<td>Control</td>
<td>14.6±0.3</td>
<td>100</td>
<td>1.12±0.19</td>
<td>100</td>
</tr>
<tr>
<td>H$_2$O$_2$(0.1 mM)</td>
<td>24.6±1.7</td>
<td>168</td>
<td>1.42±0.25</td>
<td>127</td>
</tr>
<tr>
<td>H$_2$O$_2$(0.25 mM)</td>
<td>40.4±1.4*</td>
<td>276</td>
<td>2.17±0.95*</td>
<td>194</td>
</tr>
<tr>
<td>H$_2$O$_2$(0.5 mM)</td>
<td>46.6±1.8**</td>
<td>319</td>
<td>3.06±0.65**</td>
<td>273</td>
</tr>
<tr>
<td>Control</td>
<td>13.7±0.5</td>
<td>100</td>
<td>1.22±0.62</td>
<td>100</td>
</tr>
<tr>
<td>Quercetin(2 µM)</td>
<td>32.6±3.0*</td>
<td>237</td>
<td>1.94±2.41*</td>
<td>159</td>
</tr>
<tr>
<td>Quercetin(10 µM)</td>
<td>43.0±3.8**</td>
<td>313</td>
<td>2.95±0.00**</td>
<td>242</td>
</tr>
<tr>
<td>Quercetin(20 µM)</td>
<td>63.3±0.6**</td>
<td>461</td>
<td>3.41±0.88**</td>
<td>280</td>
</tr>
</tbody>
</table>

Data are expressed as mean±SD (n=3 wells/end point).

Rhodamine 123 (5µM) was used in the study as a positive control with mean values of BEI and CL$_{bile}$ of 16.9 ± 2.6% and 15.5 ± 2.8 ml/min/kg, respectively.

* (P≤0.05) and ** (P≤0.01) indicate statistically significant differences when compared to the control group without inhibitor.

The rat hepatocytes were cultured for 4 days prior the study and quercetin, H$_2$O$_2$ and PB were added to the culture on day 2 and 3.
<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 + 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>

After the rat hepatocytes were treated with P-gp inhibitors or inducers, TP was incubated in the presence or absence of pan-CYP inhibitor ABT. Cytotoxicity was measured at the end of the 24-hr incubation period. Remaining CYP3A activity was also measured using midazolam as the substrate.
Figure 2
Figure 3

The bar graph shows the TP accumulation (pmol/mg protein) for different treatments: Control, Quercetin, PB, H$_2$O$_2$, Ritonavir, and Tariquidar. The graph indicates that Ritonavir and Tariquidar have the highest TP accumulation, followed by H$_2$O$_2$, PB, Quercetin, and the lowest in the Control group.
Figure 4

<table>
<thead>
<tr>
<th>Control</th>
<th>Quercetin</th>
<th>H₂O₂</th>
<th>PB</th>
</tr>
</thead>
</table>

**P-gp**

**GAPDH**
Figure 5

Cytotoxicity (% of control) vs. TP Concentration (µM)

- 0.1 µM: Low cytotoxicity
- 0.5 µM: Moderate cytotoxicity
- 1.0 µM: Moderately high cytotoxicity
- 5.0 µM: High cytotoxicity
- 10.0 µM: Very high cytotoxicity

* Significant difference compared to control
** Highly significant difference compared to control
Figure 7

<table>
<thead>
<tr>
<th>Control</th>
<th>Quercetin</th>
<th>H$_2$O$_2$</th>
<th>PB</th>
<th>TP</th>
</tr>
</thead>
</table>

Bax

GAPDH

Bcl-2

GAPDH
Figure 8

The graph shows a linear relationship between TP cellular concentration (pmol/mg protein) and Hepatotoxicity (% of control). The equation of the line is:

\[ y = 0.4212x - 22.411 \]

with a coefficient of determination of:

\[ R^2 = 0.9636 \]