The hepatobiliary disposition of timosaponin B2 is highly dependent on influx/efflux transporters but not metabolism

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ABBREVIATIONS: TB-2, timosaponin B2; OATP/Oatp, organic anion-transporting polypeptide; HEK, human embryonic kidney; SCRHs, sandwich-cultured rat hepatocytes; Bcrp/BCRP, breast cancer resistance protein; Mrp2/MRP2, multidrug resistance-associated protein 2; DDI, drug-drug interaction; HBSS, Hanks’ balanced salt solution; DMSO, dimethyl sulfoxide; SD, Sprague-Dawley; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; BSP, bromosulfophthalein; DMEM, Dulbecco’s modified essential medium; BEI, biliary excretion index; Ko143, (3S,6S,12aS)-1,2,3,4,6,7,12,12a-Octahydro-9-methoxy-6-(2-methylpropyl)-1,4–dioxopyrazino[1’,2’:1,6]pyrido[3,4-b]indole-3-propanoic acid 1,1-dimethylethyl ester; MK-571, 3-[[3-[(1E)-2-(7-chloro-2-quinolinyl)ethenyl]phenyl][3-(dimethylamino)-3-oxopropyl]thio)methyl]thio]; ADME, absorption, distribution, metabolism, and excretion; SNPs, single-nucleotide polymorphisms.
Abstract

The purpose of this study was to characterize the hepatobiliary disposition of timosaponin B2 (TB-2), a natural saponin. Although TB-2 has multiple pharmacological activities, the mechanism of its hepatobiliary disposition has not been explored. Because the metabolism of TB-2 is limited and the accumulation of TB-2 in primary hepatocytes is highly temperature dependent (93% of its accumulation is due to active uptake), the contribution of hepatic transporters was investigated. Organic anion-transporting polypeptide (OATP)1B1- and OATP1B3-transfected human embryonic kidney (HEK) 293 cells were employed. TB-2 serves as a substrate for OATP1B1 and OATP1B3, with the former playing a predominant role in the hepatic uptake of TB-2. An inhibition study in sandwich-cultured rat hepatocytes (SCRHs) suggested that TB-2 is a substrate for both breast cancer resistance protein (Bcrp) and multidrug resistance-associated protein 2 (Mrp2), consistent with its high biliary excretion index (BEI) (43.1% - 44.9%). This hypothesis was further verified in BCRP and MRP2 membrane vesicles. The cooperation of uptake and efflux transporters in TB-2 hepatic disposition could partially explain the double-peak phenomenon observed in rat plasma and liver and biliary clearance, which accounted for 70% of the total TB-2 clearance. Moreover, TB-2 significantly increased the rosuvastatin concentration in rat plasma in a concentration-dependent manner and decreased its biliary excretion, which corresponded to reductions in rosuvastatin accumulation in hepatocytes and the BEI in SCRHs, representing a perfect example of a potential saponin-statin drug-drug interaction (DDI). These studies demonstrate that transporters (Oatp, Bcrp/Mrp2), but not metabolism, contribute significantly to rat TB-2 hepatobiliary disposition.
Introduction

Saponins, a group of amphiphilic glycosides containing sugar chains, are responsible for the pharmacological activities of many herbal medicines and dietary supplements (Vincken et al., 2007). For example, Timosaponin B-II (TB-2), also known as (25S)-26-O-\(\beta\)-D-glucopyranosyl-22-hydroxy-5\(\beta\)-furost-3\(\beta\),26-diol-3-O-\(\beta\)-D-glucopyranosyl(1 → 2)-\(\beta\)-D-galactopyranoside (Figure 1), is a major bioactive steroid saponin originally isolated from *Anemarrhena asphodeloides* (Meng et al., 1999). TB-2 has multiple reported pharmacological activities, such as protecting against high glucose-induced apoptosis (Guo et al., 2014b) and inhibiting superoxide generation in human neutrophils (Zhang et al., 1999), as well as antiplatelet, antithrombotic (Lu et al., 2011), and anti-inflammatory (Lu et al., 2009) activities. Interestingly, TB-2 also promotes learning and memorization in memory-deficit rat models (Li et al., 2007), making it a potential candidate for anti-dementia treatment.

Despite wide interest in the pharmacological effects and mechanisms of TB-2, its pharmacokinetic behavior and the mechanism of its disposition have not been fully explored. The reported absolute bioavailability of TB-2 in rats is only 1.1 ± 0.3%, with a double peak in the plasma concentration (Cai et al., 2008). The biliary excretion of TB-2 after intravenous injection was found to be comparable to urinary excretion but was greater than fecal excretion (Xu et al., 2014). Thus, based on reports for several other saponins with similar pharmacokinetic behavior, we hypothesized that the disposition of TB-2 might be influenced by hepatic transporters. For example, dioscin, a steroid saponin with very low bioavailability (0.2%), is a substrate of organic anion-transporting polypeptides (Oatp), which are responsible for the extraction of substrates from blood into the liver; thus, the concentration of dioscin in the liver is high after intravenous
injection (Zhang et al., 2013). Glycyrrhizin, a triterpene saponin with low bioavailability (4%), is a substrate of multidrug resistance-associated protein 2 (Mrp2), which mediates biliary excretion (Makino et al., 2008), consistent with 98% of unmodified glycyrrhizin being excreted into the bile after intravenous administration (Yamamura et al., 1991). However, to the best of our knowledge, there is no published study to clarify the contribution of hepatic transporters to the hepatobiliary disposition of TB-2, and the metabolic capacity of TB-2 in the liver has not been characterized.

In this study, the metabolic and liver disposition of TB-2 was thoroughly investigated from multiple perspectives using in vitro tools. The concentration of TB-2 in rat plasma, liver and bile after oral administration was also assessed. Furthermore, the drug-drug interaction (DDI) between rosvastatin and TB-2 was examined to verify the role of transporters in the hepatobiliary disposition of TB-2.
Materials and Methods

Chemicals and reagents. TB-2 and TB-4 (a TB-2 derivative that was used as an internal standard) were provided by Professor Chenggang Huang (Shanghai Institute of Materia Medica, China). Rosuvastatin, bromosulfophthalein (BSP), tolbutamide, testosterone and probenecid were purchased from Sun Chemical Technology Co., Ltd. (Shanghai, China). S9 fractions and microsomal enzymes were purchased from Research Institute for Liver Diseases Co., Ltd. (Shanghai, China). Fetal bovine serum, insulin, William’s E medium, Hanks’ balanced salt solution (HBSS), penicillin-streptomycin, and L-glutamine were obtained from Invitrogen (Carlsbad, CA). Dexamethasone, dimethyl sulfoxide (DMSO), collagenase (type IV), reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) and glutathione were purchased from Sigma-Aldrich (St. Louis, MO). BD Matrigel® Basement Membrane Matrix, rat tail collagen (type I), and ITS (insulin, transferrin, and selenium) premix were obtained from BD Biosciences (Palo Alto, CA). A BSA protein assay kit was obtained from Pierce Chemical (Rockford, IL).

Animals. The animals used in the study were purchased from Shanghai SLAC Laboratory Animal Co. (Shanghai, China). The experiments were performed according to protocols provided by the Institutional Animal Care and Use Committee of Shanghai Institute of Materia Medica, China Academic Science. Male Sprague-Dawley (SD) rats (250 ± 20 g) were housed in air-conditioned animal quarters under the following conditions: a controlled temperature of 23 ± 2 °C, a relative humidity of 50 ± 10%, and a 12 h/12 h light/dark cycle. The rats were acclimated for 7 days before the experiment was conducted. The rats were fed a standard diet and provided water ad libitum.
Metabolism of TB-2 in rat S9 fractions, microsomes, and primary hepatocytes. The metabolic profile of TB-2 was investigated using rat S9 fractions, microsomes, and freshly isolated primary rat hepatocytes. Hepatocytes were isolated from male SD rats by a previously described two-step perfusion (Shen et al., 2012). The hepatocytes were incubated in six-well plates at a density of $0.5 \times 10^6$/per well, followed by the immediate addition of 1 $\mu$M TB-2. The cells were incubated in an orbital shaker at 37 °C for 3 h. Cell suspensions (300 µl) were collected at 0 and 3 h, and ice-cold methanol (300 µl) was added immediately to terminate potential reactions. The cell samples were then lysed by sonication, and 200 µl samples were extracted by liquid-liquid extraction. To observe TB-2 (1 $\mu$M) metabolism in S9 fractions and microsomes, we adjusted the protein density of the rat hepatic S9 fractions and microsomal enzymes systems to 1 mg/ml with 100 mM of phosphate buffer before sample incubation. Bovine serum albumin was used as a control treatment. The samples were supplemented with 1 mM NADPH and 4 mM glutathione, as previously reported (Wolf et al., 1986). Testosterone (5 µM) was used as a positive control. The systems were then incubated in a water bath (37 °C) for 3 h. Samples (300 µl) were collected at 0 and 3 h, and ice-cold methanol (300 µl) was added immediately to terminate potential reactions.

Accumulation of TB-2 in primary hepatocytes. The following two protocols were performed to evaluate the hepatocellular accumulation of TB-2.

Protocol 1. The accumulation study was conducted according to a previously described method, with slight modifications (Swift and Brouwer, 2010; Marion et al., 2011; Guo et al., 2014a). The cells were cultured in a Matrigel collagen-sandwich configuration, rinsed twice and incubated in 1 ml of HBSS in six-well plates at 37 °C for 15 min. After incubation, HBSS was
removed, and the hepatocytes were incubated with 2 ml of dosing solution (standard HBSS containing the test compounds) for another 15 min. The test compounds included the following: (a) TB-2 with or without Oatp inhibitors, i.e., rifampicin (20 μM), probenecid (20 μM) and BSP (20 μM); (b) TB-2 with or without rifampicin (5, 10, 20 μM). Accumulation was terminated by aspirating the dosing solution and rinsing the hepatocytes twice with 1 ml of ice-cold phosphate buffer. The plate was then stored at -80 °C for future analysis. The cell samples were lysed by sonication in 1 ml of water after three freeze-thaw cycles, and approximately 20 μl of the sample was used to determine protein concentrations.

Protocol 2. Temperature-dependent accumulation was determined by incubating the hepatocytes with warm (37 °C) and cold (4 °C) dosing solution, as previously described (Sharma et al., 2013). In brief, hepatocytes were incubated in warm (37 °C) and cold (4 °C) media with 1 ml of dosing solution (HBSS containing 10 μM TB-2). The remainder of the protocol was the same as described for protocol 1.

Accumulation of TB-2 in OATP1B1- and OATP1B3-transfected HEK-293 systems.

OATP1B1-, OATP1B3- and Mock-transfected human embryonic kidney (HEK) 293 cells were kindly supplied by Professor Da-fang Zhong (Shanghai Institute of Materia Medica). These cells were cultured at 37 °C in a humidified atmosphere of 5% CO₂. The cells were maintained under sub-confluent conditions and split twice weekly (at a ratio of 1:3 to 1:10, depending on their density). The cells were then cultured in 24-well plates (BD, Catalog# 356414) containing poly-D-lysine for the assay and plated with 5 mM sodium butyrate for 24 h before the experiments were performed. The accumulation experiments were conducted by Parma Resources Co., Ltd. (Shanghai). The following were examined: (a) the intracellular accumulation of TB-2 (1 or 10 μM)
in Mock-control, OATP1B1-, and OATP1B3-transfected cells after 15 min of incubation with rosvastatin (1 or 10 μM) as the positive control; (b) the intracellular accumulation of TB-2 (10 μM) in Mock-control and OATP1B1-transfected cells after 2 min of incubation; (c) the intracellular accumulation of TB-2 (0.5-100 μM) in Mock-control and OATP1B1-transfected cells after 2 min of incubation. The subsequent incubation and analysis were similar to that employed in the hepatocyte accumulation studies. The Michaelis-Menten equation was used with Prism 5 (Graph Pad Software Inc., La Jolla, CA) to determine the kinetic parameters of the uptake transporters, as follows:

\[ v = \frac{V_{\text{max}} \cdot S}{K_m + S} \]  

(1)

where \( V_{\text{max}} \) is the maximum uptake rate (pmol/min per mg of protein), \( K_m \) is the Michaelis constant (μM) and \( S \) is the substrate concentration (pmol/mg of protein).

The contribution of OATP1B1 and OATP1B3 to compound uptake in hepatocytes was evaluated according to previously described methods (Kitamura et al., 2008). In brief, the relative active factor (\( R \) value) was the reported ratio of the uptake of rosvastatin in human hepatocytes (\( C_{L, \text{hepatocyte}} \)) to that in transporter-expressing cells (\( C_{L, \text{transporter}} \)) (Kitamura et al., 2008). The calculation was as follows:

\[ C_{L, \text{OATP}} = \frac{A_{\text{OATP}}}{T} \]  

(2)

Contribution of OATP1B1 = \[ \frac{R_{\text{OATP1B1}} \times C_{L, \text{OATP1B1}}}{R_{\text{OATP1B1}} \times C_{L, \text{OATP1B1}} + R_{\text{OATP1B3}} \times C_{L, \text{OATP1B3}}} \]  

\[ = \frac{R_{\text{OATP1B1}}}{R_{\text{OATP1B1}} \times A_{\text{OATP1B1}} + R_{\text{OATP1B3}} \times A_{\text{OATP1B3}}} \]  

(3)

Contribution of OATP1B3 = \[ \frac{R_{\text{OATP1B3}} \times C_{L, \text{OATP1B3}}}{R_{\text{OATP1B3}} \times C_{L, \text{OATP1B3}} + R_{\text{OATP1B3}} \times C_{L, \text{OATP1B3}}} \]  

\[ = \frac{R_{\text{OATP1B3}}}{R_{\text{OATP1B1}} \times A_{\text{OATP1B1}} + R_{\text{OATP1B3}} \times A_{\text{OATP1B3}}} \]  

(4)

The ratio of rosvastatin accumulation between HEK293-OATP1B1 and HEK293-OATP1B3
was 0.32-0.36 in our study, consistent with a previously reported value (0.32) for rosuvastatin (Kitamura et al., 2008). Therefore, the reported values of $R_{\text{OATP1B1}}$ (the average of the reported values of 1.15, 1.40 and 0.601) and $R_{\text{OATP1B3}}$ (the average of the reported values of 0.188, 0.0835 and 0.0482) (Kitamura et al., 2008) were used to evaluate the contribution of OATP1B1 and OATP1B3 to TB-2 uptake. $A_{\text{OATP}}$ is the amount accumulated in transporter-expressing cells, and $T$ is the time for accumulation in transfected cells.

**Biliary excretion of TB-2 in sandwich-cultured rat hepatocytes (SCRHs).** SCRHs were established according to previously described methods with minor modifications (Chandra et al., 2005). In brief, after primary rat hepatocytes were isolated and plated in 24-well plates, the cells were washed and cultured with William’s E medium containing fetal bovine serum (5%). After two days, 0.25 mg/ml BD Matrigel dissolved in ice-cold William’s E medium without fetal bovine serum was overlaid on the plates. The culture medium was changed daily, and the experiment was performed on day 5 of culture (day 1 was the time of plating). In brief, SCRHs were pre-incubated in 300 µl of warm HBSS with or without Ca$^{2+}$ at 37 °C for 15 min; the tight junctions of the bile canaliculi open temporally following exposure to HBSS without Ca$^{2+}$. At the end of pre-incubation, the buffer was aspirated, and the hepatocytes were incubated with 300 µl of dosing solution (HBSS with Ca$^{2+}$) to initiate uptake (15 min, 37 °C). The tested compounds included TB-2 (5 µM) with or without the inhibitors Ko143 (100, 250, 500 µM) or MK-571 (10, 100 µM). The dosing solution was aspirated from the plates after incubation, and uptake was terminated by washing the cells twice with ice-cold phosphate buffer. After the remaining buffer was aspirated, the cell samples were lysed by sonication in 1 ml of water after three freeze-thaw cycles, and approximately 20 µl of the cell sample was used to determine the protein concentration. The
biliary excretion index (BEI) was calculated using B-CLEAR® technology (Qualyst Transporter Solutions, Durham, NC) according to the following equation (Liu et al., 1999; Pan et al., 2012):

\[
\text{BEI} = \frac{\text{Accumulation}_{(\text{cell}+\text{bile})} - \text{Accumulation}_{(\text{cell})}}{\text{Accumulation}_{(\text{cell}+\text{bile})}} \times \text{cell} \quad (5)
\]

**Transport study of TB-2 with human BCRP- and MRP2-expressing membrane vesicles.**

Inside-out membrane vesicles were purchased from Genomembrane (Kanazawa, Japan). The vesicular transport study was performed using a rapid filtration technique according to the manufacturer’s protocol with slight modifications. Briefly, 30 µl of vesicle suspension (50 mM MOPS-Tris, 70 mM KCl and 7.5 mM MgCl₂) containing MRP2- or Mock-expressing membrane vesicles (50 µg of protein) was pre-incubated at 37 °C for 5 min and then rapidly mixed with 30 µl of the reaction mixture (100 µM TB-2, 4 mM glutathione and 8 mM ATP after mixing). The reactions proceeded at 37 °C for 10 min and were stopped by the addition of 200 µl of chilled wash buffer (40 mM MOPS-Tris and 70 mM KCl). The reaction mixture was then transferred to a Millipore filtration plate (0.65 µm; Billerica, MA, USA), and the filters were washed 5 times with 200 µl of the wash buffer. The protocol for the BCRP study was as the same as that for the MRP2 study, except that glutathione was omitted. The concentration of TB-2 on the filter was determined by LC-MS/MS.

**Pharmacokinetics of TB-2 in rats.** Rats were randomly assigned to two groups and fasted overnight with free access to water before dosing. All rats were administered an oral dose of TB-2 (30 mg/kg, dissolved in saline). At 0.167 h, 0.5 h, 1 h, 2 h, and 6 h, 25 rats in group A (n = 5 for each time point) were anesthetized with urethane (1.4 g/kg, dissolved in saline) and sacrificed to collect hepatic portal venous plasma, abdominal aorta plasma and the liver. Five rats in group B were cannulated with PE-10 polyethylene tubing under anesthesia, and bile was collected at 0-2 h,
2-4 h, 4-6 h, 6-8 h, 8-10 h, and 10-24 h after dosing. The blood and bile samples were centrifuged at 10000 rpm for 5 min, and all samples were stored at -20 °C until analysis.

The fraction \( f_{\text{bile}} \) of biliary clearance (CL_{\text{bile}}) of the total clearance (CL_{\text{total}}) was calculated as follows:

\[
\text{CL}_{\text{total}} = \frac{D_{\text{oral}} \times F}{AUC_{\text{oral}}} \tag{6}
\]

\[
\text{CL}_{\text{bile}} = \frac{M_{\text{bile}}}{AUC_{\text{oral}}} \tag{7}
\]

\[
f_{\text{bile}} = \frac{CL_{\text{bile}}}{CL_{\text{total}}} = \frac{M_{\text{bile}}}{D_{\text{oral}} \times F} \tag{8}
\]

where \( D_{\text{oral}} \) is the oral dose of TB-2; \( AUC_{\text{oral}} \) is the area under time-concentration in plasma after oral administration; \( M_{\text{bile}} \) is the amount of TB-2 excreted into bile; and \( F \) is the bioavailability of TB-2, for which the reported value of 1.1% was used (Cai et al., 2008).

**DDI between TB-2 and rosuvastatin in hepatocytes and SCRHs.** The accumulation study of DDI between TB-2 and rosuvastatin was as same as protocol 1 of the uptake study on TB-2, except that the victim compound used was rosuvastatin and TB-2 was employed as the perpetrator. The impact of TB-2 on rosuvastatin biliary excretion was also assessed in SCRHs.

**DDI between TB-2 and rosuvastatin in rats.** Rats were assigned to six groups (\( n = 4 \) for each group). Rosuvastatin (5 mg/kg, dissolved in saline) was administered to the rats in group C by oral gavage. Rosuvastatin (5 mg/kg, dissolved in saline) and TB-2 (30, 60, and 180 mg/kg, dissolved in saline) were co-administered to the rats by oral gavage in groups D, E, and F, respectively. Approximately 300 µl of blood sample was collected from the orbital vein at 0.08, 0.25, 0.5, 1, 2, 3, 4, 6, 8, and 10 h under light ether anesthesia. Food was withheld during this process, but water was freely provided. Plasma samples were obtained after centrifugation at 10000 rpm for 5 min and stored at -20 °C until analysis.
For the biliary excretion analysis, rosuvastatin (1 mg/kg, dissolved in saline) was injected 0.5 h in advance into the rat tail vein after oral administration with TB-2 (180 mg/kg, in group G) or saline (in group H). The fasted rats were anesthetized by intraperitoneal injection of urethane. A polyethylene tube (PE-10) was inserted into the common bile duct for bile collection, and bile samples were collected at 0-0.25 h, 0.25-0.5 h, 0.5-1 h, 1-1.5 h, 1.5-2 h, and 2-3 h after oral administration. All the samples were stored at -20 °C until analysis.

**Quantification by LC-MS/MS.** All samples were analyzed using a Shimadzu LCMS-8030 triple quadrupole system (Shimadzu Corp., Japan) equipped with electrospray ionization (ESI). Data processing was performed using Shimadzu LC-MS Lab Solution System version 5.42 SP4 (Shimadzu Corp., Japan). Chromatographic separation was performed on an ACE C18 column (100 mm × 2.1 mm, 3.0 μm, ACT, Scotland, UK). The injection volume was 10 μl, and the column temperature was maintained at 40 °C. All quantification was monitored in positive mode, and the mass transitions were monitored in MRM mode. The optimized monitored ions and collision energy (CE) were as follows: m/z 903.30-417.40 and CE 26 V for TB-2; m/z 593.40-413.30 and CE 24 V for TB-4 (internal standard of TB-2); m/z 482.15-258.0 and CE 35 V for rosuvastatin; and m/z 271.50-154.70 and CE 25 V for tolbutamide (internal standard of rosuvastatin). The mobile phase consisted of acetonitrile (A) and 0.1% formic acid in water (B), and the flow rate was 0.2 ml/min. The gradient parameters for TB-2 were 88% B to 70% B at 0 min to 0.1 min, 70% B at 0.1 min to 2.0 min, 70% B to 10% B at 2.0 min to 2.5 min, 10% B at 2.5 min to 3.0 min, 10% B to 88% B at 3.0 min to 3.5 min, and 88% B at 3.5 min to 7.5 min. The gradient parameters for rosuvastatin were 62% B at 0 min to 2.0 min, 62% B to 25% B at 2.0 min to 3.5 min, and 62% B at 3.51 min to 7.5 min. All test compounds showed good linearity, with an
R² > 0.99.

**Data analysis.** A non-compartmental analysis utilizing Phoenix WinNonlin software (Pharsight 6.2, NC, USA) was performed. The data are expressed as the mean ± S.D. A two-tailed Student’s t test was used to assess the significance of the results.
Results

Metabolism of TB-2 in rat S9 fractions, microsomes, and primary hepatocytes. The metabolism of the reference compound testosterone was higher than 90% in rat S9 fractions (Figure 2A), microsomes (Figure 2B), and primary hepatocytes (Figure 2C). However, no significant difference in the concentration of TB-2 was observed in the presence or absence of S9 fractions, microsomes or hepatocytes (Figure 2D - 2E), indicating that metabolism is not the major clearance pathway for TB-2 in the liver. Moreover, no metabolites were detected in the TB-2-containing samples after a full scan by HPLC-Q-TOF (Agilent Corp, USA) (data not shown).

Accumulation of TB-2 in primary rat hepatocytes. To determine the contributions of passive diffusion and active uptake to TB-2 hepatic transport, the effect of temperature was evaluated in primary hepatocytes. As shown in Figure 3A, the concentration of TB-2 in hepatocytes was reduced to approximately 6.7% in cold (4 °C) medium compared to warm (37 °C) medium (2.96 ± 0.31 vs. 44.18 ± 1.75 ng/mg protein), indicating that the transport of TB-2 in primary hepatocytes is mainly dependent on active transport. Furthermore, the Oatp inhibitors rifampicin (P < 0.001), probenecid (P < 0.01), and BSP (P < 0.01) significantly reduced the uptake of TB-2 (20 μM) by rat primary hepatocytes (Figure 3B). The most prominent inhibition was induced by rifampicin: TB-2 uptake was reduced up to 90.7% by rifampicin in a dose-dependent manner (Figure 3C).

Accumulation of TB-2 in OATP1B1- and OATP1B3- transfected HEK293 systems. Because Oatp inhibitors inhibited TB-2 accumulation in hepatocytes, we suspected that TB-2 was a substrate for Oatp. To test this hypothesis, TB-2 uptake in OATP1B1- and OATP1B3-HEK293 systems was investigated. As depicted in Figure 4A, rosuvastatin accumulation increased
in a concentration-dependent manner in HEK293-OATP1B1 and HEK293-OATP1B3 cells compared to HEK293-Mock cells, confirming the activity of OATP1B1 and OATP1B3 in these cells. As expected, TB-2 uptake was increased 2- to 7-fold in HEK293-OATP1B1 cells and 2- to 5-fold in HEK293-OATP1B3 cells compared with HEK293-Mock cells (Figure 4B). TB-2 accumulation in HEK293-OATP1B1 cells was 300% of that in HEK293-OATP1B3 cells, whereas rosvastatin accumulation in HEK293-OATP1B1 cells was only 32-36% of that in HEK293-OATP1B3 cells. In addition, the uptake of TB-2 reached a plateau after approximately 10 min (Figure 5A). OATP1B1-mediated uptake exhibited a $K_m$ of 3.69 ± 0.46 µM and a $V_{max}$ of 12.94 ± 0.40 pmol/min/mg of protein (Figure 5B).

**Biliary excretion of TB-2 in SCRHs and BCRP- and MRP2-expressing membrane vesicles.** Figure 6 shows that after a 15-min incubation in SCRHs, the BEI of TB-2 ranged from 43.1% to 44.9%, indicating that biliary excretion plays an important role in TB-2 hepatobiliary disposition. The Bcrp inhibitor Ko143 and Mrp2 inhibitor MK-571(Yamazaki et al., 2005; Weiss et al., 2007) significantly decreased the TB-2 BEI values from 44.9% to 12.7% (Figure 6A) and 43.1% to 15.2% (Figure 6B), respectively. In addition, the concentrations of TB-2 were significantly higher in both BCRP- and MRP2-expressing membrane vesicles than in Mock-expressing membrane vesicles (the control group) ($P < 0.05$) (Figure 6C).

**Pharmacokinetic study of TB-2 in rats.** As shown in Figure 7A, the concentration of TB-2 was approximately 1.5-fold higher in portal vein plasma than in abdominal aorta plasma, in agreement with the corresponding AUC values (55.2 ± 18.2 vs. 24.6 ± 6.6 h*ng/ml). Significant double peaks were observed in both plasma and liver samples (Figure 7A and 7B).

After oral administration of a 30 mg/kg dose of TB-2, the highest concentration in bile was
11.2 ± 4.1 µg/ml at 2-6 h, and the accumulated biliary excretion of TB-2 was as high as 59.7 ± 11.8 µg (Figure 8A and 8B). Considering that the bioavailability of TB-2 is 1.1% (Cai et al., 2008), this biliary clearance value represents 70% of the absorbed oral dose (eqs. 6-8).

**DDI between TB-2 and rosuvastatin in hepatocytes.** The accumulation of rosuvastatin (5 µM) was reduced in a concentration-dependent manner by TB-2 (IC_{50} = 5.27 µM) in hepatocytes after a 15-min incubation (Figure 9A). Moreover, rosuvastatin accumulation in hepatocytes was a concentration-dependent process that was partly inhibited by TB-2 (20 µM) in a concentration range of 1-15 µM (Figure 9B). Although the \( V_{\text{max}} \) of rosuvastatin uptake in hepatocytes was not significantly influenced by TB-2 (11.0 vs. 10.8 pmol/min/mg of protein), the \( K_{\text{m}} \) did increase from 2.8 µM to 7.8 µM (Table 1).

TB-2 decreased the BEI of rosuvastatin in SCRHs as well. In particular, the BEI of rosuvastatin was reduced from 42.8% to 18.7% as the TB-2 concentration increased from 0 to 20 µM (Figure 10).

**DDI between TB-2 and rosuvastatin in rats.** The plasma concentration of rosuvastatin increased significantly when co-administered with TB-2. After co-administration with TB-2 at doses of 30-180 mg/kg, \( C_{\text{max}} \) increased from 46.1 ± 13.9 to 97.0 ± 22.8 ng/ml, and the AUC of rosuvastatin increased in a dose-dependent manner from 113.0 ± 7.8 to 282.2 ± 74.4 h* ng/ml (Figure 11, Table 2).

The rosuvastatin concentration in bile decreased (Figure 12A) and the total biliary excretion decreased by 50% (1566.8 ± 391.4 ng VS 884.8 ± 174.1 ng) (Figure 12B) in the presence of TB-2 (180 mg/kg).
Discussion

In this study, the hepatobiliary disposition and metabolism of TB-2 were explored to characterize the absorption, distribution, metabolism, and excretion (ADME) profile and identify potential DDIs of this promising drug candidate.

Because no metabolism was observed in the rat S9 fractions, the microsomes, or the primary hepatocytes (Figure 2), TB-2 metabolism may not play an important role in its elimination. Because TB-2 uptake by hepatocytes was highly temperature dependent (active uptake accounted for 93% of TB-2 accumulation) (Figure 3A), the connection between TB-2 and liver uptake transporters was further investigated. The accumulation of TB-2 in rat primary hepatocytes was significantly inhibited by multiple Oatp inhibitors, including rifampicin, probenecid and BSP (Figure 3B). For example, 20 μM rifampicin reduced TB-2 accumulation in primary hepatocytes by 90.7% (Figure 3C), indicating that its accumulation is highly dependent on Oatp transporters. In particular, OATP1B1 and OATP1B3 are expressed in the human liver and play significant roles in the uptake of substrates into hepatocytes (Kindla et al., 2009). Additionally, a comparison of TB-2 accumulation in OATP1B1- and OATP1B3-transfected HEK293 cells revealed that OATP1B1 and OATP1B3 could significantly increase TB-2 uptake into these cells (Figure 4). Calculations of the effects of these polypeptides on TB-2 (eqs. 2-4) revealed that the contribution of OATP1B1 to TB-2 uptake reached 92%-98%, suggesting that it plays a dominant role in this process in hepatocytes. The cellular kinetic parameters of OATP1B1 were also determined ($K_m = 3.69 \pm 0.46 \mu M$, $V_{\text{max}} = 12.94 \pm 0.40 \text{ pmol/min/mg of protein}$) (Figure 5). Considering that Oatp1b2 in rats has an expression profile similar to that of human OATP1B1/1B3 (Hagenbuch and Meier, 2003), Oatp1b2 is expected to be the uptake transporter for TB-2 in the rat liver.
Bcrp and Mrp2 are expressed in rat hepatocytes on the canalicular membrane side and are responsible for the biliary excretion of various substrates. The BEI of TB-2 in SCRHSs was significantly reduced by Ko143 (a Bcrp inhibitor) and MK-571 (an Mrp2 inhibitor), suggesting that TB-2 is a substrate of Bcrp and Mrp2. The concentration of TB-2 in human BCRP- or MRP2-expressing membrane vesicles was significantly higher than that in Mock-expressing membrane vesicles (Figure 6), further verifying that TB-2 is a substrate of human BCRP and MRP2. Because the substrate and inhibitor specificities of human and rat BCRP/MRP2 are similar (Grime and Paine, 2013), TB-2 may also be a substrate of Bcrp/Mrp2 in rats.

Obvious double peaks in rat plasma were previously observed after oral administration of TB-2 (Cai et al., 2008) or herbal medicine (Cai et al., 2010). In this study, distinct double peaks were observed not only in portal vein plasma and abdominal aorta plasma but also in the liver after oral administration (Figure 7). TB-2 was excreted into the bile, and the highest concentration in the bile was of 11.2 ± 4.1 µg/ml. Moreover, biliary excretion accounted for up to 70% of the absorbed oral dose (Figure 8), consistent with its high BEI values (43.1% to 44.9%). Another group has reported that the distribution of TB-2 in the small intestine achieves a maximum concentration at 60 min after intravenous dosage compared to 10 min for other organs, providing further evidence of active TB-2 biliary excretion (Xu et al., 2014) that can be attributed to the cooperation of the hepatic uptake (Oatp) and efflux transporters (Bcrp/Mrp2). In fact, in transwell studies, the permeation of Bcrp- or Mrp2-transfected MDCK cell monolayers by TB-2 was limited in the absence of an auxiliary uptake transporter (data not shown). Therefore, we speculate that TB-2 hepatobiliary disposition occurs as follows: TB-2 is absorbed into the portal vein and then extracted into hepatocytes primarily by the uptake transporter (Oatps), resulting in the first peaks
in the rat plasma (10 min) and liver (30 min). A small fraction of TB-2 accumulates in the liver in
the absence of metabolism, but it is mainly excreted into bile ($f_{\text{ bile}} = 70\%$) via efflux transporters
(Bcrp/Mrp2), thus leading to enterohepatic circulation and contributing to the second peaks in the
liver (2 h) and plasma (1 h). Indeed, biliary excretion may not be the only reason for its second
absorption peak. Different TB-2 absorption sites in the gut may also have contributed to the
second plasma peak, and the remaining TB-2 in the gut and the biliary-excreted TB-2 could have
been absorbed together. Otherwise, it is difficult to explain why the second peak was as high as
the first one (Figure 7). Further investigation is needed to verify this hypothesis. Although
evidence suggests that Oatp or Mrp2 alone may contribute to liver accumulation or biliary
excretion of saponins such as dioscin and glycyrrhizin (Makino et al., 2008; Zhang et al., 2013),
this is the first report of cooperation by uptake transporters (Oatp) and efflux transporters
(Bcrp/Mrp2) in the hepatobiliary disposition of saponins.

Although 70% of the absorbed TB-2 was excreted into the bile and a significant double-peak
phenomenon was observed, the bioavailability of TB-2 was only 1.1% after oral administration of
180 mg/kg (Cai et al., 2008). Another group has reported that the bioavailabilities of TB-2 were
0.62% and 0.55% after oral administration of 30 and 180 mg/kg, respectively, implying the
linearity of TB-2 pharmacokinetics within the tested dose range (Xu, 2013). Considering that
passive diffusion accounts for only approximately 7% of TB-2 uptake across the hepatic
membrane (Figure 3A) and that TB-2 is a substrate of Bcrp and Mrp2, its low permeability in the
gut is not surprising. This low passive permeability may prevent its absorption, and efflux
transporters (Bcrp/Mrp2) may pump it back into the intestinal tract. The low passive permeability
of TB-2 may result from its unfavorable physicochemical traits, including its large molecular mass.
(921, favorable value \(< 500\) Da), high hydrogen-bonding capacity (31, favorable value \(< 12\)) and high molecular flexibility (25, favorable value \(< 10\)), which lead to poor membrane permeability and hinder intestinal absorption (Yu et al., 2012). Moreover, metabolism in the gastrointestinal tract may also impede TB-2 absorption, as the deglycosylation of many saponins occurs in the gastrointestinal tract via the action of colonic microflora (Hasegawa et al., 1996). Indeed, because deglycosylated metabolites of TB-2 have been detected in the gastrointestinal contents and urine of rats (Liu et al., 2012; Xu, 2013), gastrointestinal metabolism should be further explored to clarify its contribution to the poor bioavailability of TB-2.

Rosuvastatin is a substrate of Oatp, Bcrp, and Mrp2, and its metabolism is limited (Nezasa et al., 2002; Kitamura et al., 2008), which highly overlaps with the ADME spectrum of TB-2. Thus, rosuvastatin was chosen as a probe to investigate the potential DDI between rosuvastatin (victim) and TB-2 (perpetrator). TB-2 reduced the accumulation of rosuvastatin in primary hepatocytes \((IC_{50} = 5.27\ \mu M)\) (Figure 9A) and the BEI of rosuvastatin in SCRHs (42.8% vs. 18.7%) (Figure 10). Moreover, the significant change in the \(K_m\) of rosuvastatin (2.8 \(\mu M\) vs. 7.8 \(\mu M\)) without a significant change in its \(V_{max}\) implies a competitive process (Table 1). These results are consistent with the in vivo observations that TB-2 increased the AUC of rosuvastatin from 113.0 ± 7.8 to 282.2 ± 74.4 h*ng/ml dose dependently (Figure 11, Table 2) and decreased its biliary excretion by approximately 50% (Figure 12B). These data confirm the previous hypothesis that TB-2 and rosuvastatin share similar influx and efflux transporters. The transporter spectrum of some saponins may overlap with those of statins. For example, ginsenoside Rh2 isolated from ginsenoside is a substrate of BCRP (Jin et al., 2006), and glycyrrhetic acid derived from licorice is a moderate inhibitor of MRP2 and BCRP (Yoshida et al., 2008). Although this overlap may lead to
potential DDI issues, no studies or clinical reports examining these issues have been published. Indeed, this is the first comprehensive study of saponin-statin DDIs, and the results indicate a potential future clinical risk.

The involvement of multiple transporters complicates TB-2-related pharmacokinetic and toxicity processes due to the potential for interindividual variability. For example, some single-nucleotide polymorphisms (SNPs) of the genes encoding OATP, BCRP and MRP2 have been associated with alterations in the PK profiles of statins (Niemi et al., 2006; Xiang et al., 2006; Hua et al., 2012; Konig et al., 2013). The increased expression of OATP and decreased expression of MRP2 may also alter pharmacokinetic parameters and disposition in patients with diabetes (Jung et al., 2001; Kast et al., 2002; Hasegawa et al., 2010). Because TB-2 is a substrate of both OATP and MRP2 and has potential value for preventing diabetic cardiovascular complications (Guo et al., 2014b), it should be used judiciously in diabetes patients, particularly when co-administered with a statin.

In summary, our study demonstrates that influx (Oatp) and efflux transporters (Bcrp/Mrp2), but not metabolic enzymes, contribute significantly to rat TB-2 hepatobiliary disposition. Moreover, TB-2 interferes with the rosuvastatin PK profile and biliary excretion, as they share similar transporters. Additional work is needed to clarify the reasons for the poor bioavailability of TB-2, and a schematic representation of this process is presented in Supplemental Figure 1.
Acknowledgements. We thank Dr. Weiwei Jia from Dr. Chuan Li’s lab (Shanghai Institute of Materia Medica, Chinese Academy of Sciences) for their help in conducting the BCRP and MRP2 vesicle studies and Dr. Peihua Sun from Pharma Resources Co., Ltd. for help with the studies in OATP1B1- and OATP1B3- transfected cell lines.
Authorship Contributions

Participated in the research design: Tian, Sheng, Xu, Wu, Huang and Pan

Conducted experiments: Sheng, Tian, Chen, Wang and Pan.

Contributed new reagents or analytic tools: Huang, Xu and Pan

Performed data analyses: Tian, Sheng and Pan,

Wrote or contributed to the writing of the manuscript: Tian, Sheng, Huang and Pan
References


Biochim Biophys Acta **1609**:1-18.


Swift B and Brouwer KL (2010) Influence of seeding density and extracellular matrix on bile Acid


and glycyrrhetic acid-3-O-glucuronide in biological fluids: application of ion-pair extraction and fluorescence labelling agent. *J Chromatogr* **567**:151-160.


Yu K, Chen F, and Li C (2012) Absorption, Disposition, and Pharmacokinetics of Saponins from Chinese Medicinal Herbs: What Do We Know and What Do We Need to Know More? *Curr Drug Metab* **13**:577-598.


Footnotes

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# Jingjing Sheng and Xiaoting Tian contributed equally to this work.

# Corresponding author: Prof Guoyu Pan and Chenggang Huang contributed equally to this work.
Legends for Figures

Fig. 1. Structure of Timosaponin B2 (TB-2)

Fig. 2. TB-2 (1 μM) was incubated with S9 fractions (D), microsomes (E) or primary hepatocytes (F). The concentration of TB-2 in the suspension was detected at 0 h (white bars) and 3 h (black bars); testosterone (5 μM) was used as a positive control (A - C). Each point presents the mean ± S.D. (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 compared with the control.

Fig. 3. Intracellular accumulation of TB-2 (10 μM) in primary rat hepatocytes at 4 °C and 37 °C (A). Effects of rifampicin (20 μM), probenecid (20 μM), and BSP (20 μM) on the accumulation of TB-2 (10 μM) in primary rat hepatocytes (B). Effects of rifampicin (0, 5, 10, 20 μM) on the accumulation of TB-2 (10 μM) in primary rat hepatocytes after 15 min of incubation (C). Each point indicates the mean ± S.D. (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 compared with the control.

Fig. 4. Intracellular accumulation of TB-2 (1 and 10 μM) in Mock-control, OATP1B1-transfected, and OATP1B3-transfected cells after 15 min of incubation (B); rosuvastatin was used as a positive control (A). Each point indicates the mean ± S.D. (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 compared with the Mock-control.

Fig. 5. Time curves of TB-2 (10 μM) in Mock-control and OATP1B1-transfected cells after 15 min of incubation (A). The intracellular accumulation of different doses of TB-2 in OATP1B1-transfected cells after 2 min of incubation was measured to calculate K_m and V_max (B). Each point indicates the mean ± S.D. (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 compared with the Mock-control.

Fig. 6. The effect of different concentrations of Ko143 (A) and MK571 (B) on TB-2 (5 μM)
accumulation in SCRHs after 15 min of incubation. Accumulated TB-2 was pre-incubated with
calcium-containing buffer (black bars) or calcium-free buffer (white bars). The numbers above the
bars correspond to the BEIs. The intracellular accumulation of TB-2 in Mock-control, human
BCRP or MRP2-expressing membrane vesicles was measured after 10 min of incubation (C).
Each point indicates the mean ± S.D. (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 compared with
the Mock control.

Fig. 7. The concentration of TB-2 in rat portal vein plasma and abdominal aortic plasma (A) and
liver (B) at 0.167, 0.5, 1, 2, and 6 h after oral administration of TB-2 (30 mg/kg). Each point
indicates the mean ± S.D. (n = 5).

Fig. 8. The concentration (A) and excreted amount (B) of TB-2 in rat bile after oral administration
of TB-2 (30 mg/kg) at 0-24 h. Each point presents the mean ± S.D. (n = 5).

Fig. 9. Profile of the concentration-dependent inhibition of the uptake of rosuvastatin (5 μM) by
various concentrations of TB-2 (0-50 μM) in primary rat hepatocytes (A). The intracellular
accumulation of rosuvastatin (1-15 μM) in primary rat hepatocytes with or without TB-2 (20 μM)
was measured to calculate the Km and Vmax of rosuvastatin (B). Each point indicates the mean ±
S.D. (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 compared with the control.

Fig. 10. The accumulation of rosuvastatin (5 μM) in the presence of TB-2 (0, 5, 10, and 20 μM)
was measured in SCRHs pre-incubated with calcium-containing buffer (black bars) or
calcium-free buffer (white bars). The numbers on the top of the bars correspond to the BEIs. Each
point indicates the mean ± S.D. (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 compared with the
control.

Fig. 11. Mean plasma concentration-time profiles of rosuvastatin (5 mg/kg) after oral
administration of TB-2 (0, 30, 60, and 180 mg/kg) in SD rats. Each point indicates the mean ± S.D. 

(n = 4).

**Fig. 12.** The mean concentration (A) and excreted amount (B) of rosuvastatin (1 mg/kg) after tail vein injection in the presence of TB-2 (0 or 180 mg/kg, oral administration, 0.5 h in advance) in SD rats. Each point indicates the mean ± S.D. (n = 4). *P < 0.05, **P < 0.01, ***P < 0.001 compared with the control.
Table 1. Apparent kinetic parameters ($K_m$ and $V_{max}$) of rosuvastatin with or without TB-2 in primary rat hepatocytes. Each value is the mean ± S.D. ($n = 3$).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Rosuvastatin</th>
<th>Rosuvastatin +TB-2</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{max}$ (pmol/min/mg protein)</td>
<td>11.0 ± 0.5</td>
<td>10.8 ± 1.4</td>
</tr>
<tr>
<td>$K_m$ (μM)</td>
<td>2.8 ± 0.3</td>
<td>7.8 ± 2.1</td>
</tr>
</tbody>
</table>
Table 2. The main pharmacokinetic parameters of rosuvastatin (5 mg/kg) after oral administration with or without TB-2 (30, 60, 180 mg/kg). Each value is the mean ± S.D. (n = 4).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Rosuvastatin</th>
<th>Rosuvastatin +TB-2(30 mg/kg)</th>
<th>Rosuvastatin +TB-2(60 mg/kg)</th>
<th>Rosuvastatin +TB-2(180 mg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC_{all} (h ng/ml)</td>
<td>113.0 ± 7.8</td>
<td>154.3 ± 12.3</td>
<td>227.2 ± 52.8</td>
<td>282.2 ± 74.4</td>
</tr>
<tr>
<td>C_{max} (ng/ml)</td>
<td>46.1 ± 13.9</td>
<td>99.2 ± 16.5</td>
<td>102.7 ± 26.4</td>
<td>97.0 ± 22.8</td>
</tr>
</tbody>
</table>
Figure 2

A. Concentration of testosterone (ng/ml) at 0h and 3h for S9 fraction (-) and (+).

B. Concentration of testosterone (ng/ml) at 0h and 3h for microsomes (-) and (+).

C. Concentration of testosterone (ng/ml) at 0h and 3h for heparocytes (-) and (+).

D. Concentration of TB-2 (ng/ml) at 0h and 3h for S9 fraction (-) and (+).

E. Concentration of TB-2 (ng/ml) at 0h and 3h for microsomes (-) and (+).

F. Concentration of TB-2 (ng/ml) at 0h and 3h for heparocytes (-) and (+).
Figure 4

(A) Concentration of rosvastatin (pmol/mg protein) vs. Mock, OATP1B1, and OATP1B3 at different concentrations of rosvastatin (µM). (B) Concentration of TB-2 (pmol/mg protein) vs. Mock, OATP1B1, and OATP1B3 at different concentrations of TB-2 (µM).
Figure 5

(A) Concentration of TB-2 (pmol/mg protein) over time (min) for 293-Mock and 293-OATP1B1.

(B) Concentration of TB-2 (pmol/mg protein) at different concentrations of TB-2 (µM) for 293-Mock, 293-OATP1B1, and OATP1B1-Mediated uptake.

- $K_m = 3.69 \pm 0.46 \mu M$
- $V_{max} = 12.94 \pm 0.40 \text{ pmol/min/mg}$
Figure 6

(A) Concentration of TB-2 (ng/mg protein) at different concentrations of KO143 (nM).

(B) Concentration of TB-2 (ng/mg protein) at different concentrations of MK571 (μM).

(C) Transport of TB-2 (pmol/mg protein/min) for MOCK, BCRP, and MRP2.
Figure 8

A

Conc of TB-2 in bile (ng/ml)

0 5000 10000 15000 20000

0.2 2-4 4-6 6-8 8-10 10-24

Time (h)

B

Biliary excretion of TB-2 (ng)

0 20000 40000 60000 80000

0 2 4 6 8 10 12 14 16 18 20 22 24

Time (h)
Figure 9

A

Conc. of rosvastatin (ng/mg protein)

IC$_{50}$ = 5.27 µM

B

Conc. of rosvastatin (ng/mg protein)

Conc. of TB-2 (µM)

Conc. of rosvastatin (µM)
Figure 10

The bar graph shows the concentration of rosvastatin (ng/mg protein) in the presence of different concentrations of TB-2 (µM) under two conditions: Ca²⁺ (+) and Ca²⁺ (-).

- The control group shows a 42.8% decrease in concentration.
- The 5 µM concentration shows a 28.6% decrease.
- The 10 µM concentration shows an 18.7% decrease.
- The 20 µM concentration shows a 21.9% decrease.

The y-axis represents the concentration of rosvastatin, while the x-axis represents the concentration of TB-2 (µM). The error bars indicate the variability of the data.
Figure 11
Figure 12

A

Conc. of rosvastatin in bile (ng/ml)

0-0.25 0.25-0.5 0.5-1 1-1.5 1.5-2 2-3h

Rosuvastatin Rosuvastatin + TB-2

*** *** *** **

B

Biliary excretion of rosvastatin (ng)

Time (h)

Rosuvastatin Rosuvastatin + TB-2

* * ** **
Supplemental data:

The hepatobiliary disposition of timosaponin B2 is highly dependent on influx/efflux transporters but not metabolism

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Drug Metabolism and Disposition.
Supplemental Figure 1: Schematic figure give an integral instruction to this study