Fasiglifam (TAK-875) Inhibits Hepatobiliary Transporters: A Possible Factor Contributing to Fasiglifam-induced Liver Injury

Xiuli Li, Kan Zhong, Zitao Guo, Dafang Zhong, and Xiaoyan Chen

Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China (X.L., K.Z., Z.G., D.Z., X.C.)
Running Title: TAK-875 inhibits hepatobiliary transporters

Corresponding Author: Xiaoyan Chen

Shanghai Institute of Materia Medica, Chinese Academy of Sciences, 501 Haike Road, Shanghai, 201203, China
Phone/Fax: +86-21-50800738; Email: xychen@simm.ac.cn

Number of text pages: 33
Number of tables: 2
Number of figures: 8
Number of references: 42
Number of words in the Abstract: 248
Number of words in the Introduction: 747
Number of words in the Discussion: 1496

Abbreviations
ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; AUC, area under the plasma concentration–time curve; BEI, biliary excretion index; BSEP, bile salt export pump; CA, cholic acid; CDCA, chenodeoxycholic acid; CDF, 5 (and 6)-carboxy-2',7'-dichlorofluorescein; CDFDA, 5 (and 6)-carboxy-2',7'-dichlorofluorescein diacetate; CL_biliary, biliary clearance; CyA, cyclosporine A; DCA, deoxycholic acid; ER, efflux ratio; GCA, glycocholic acid; GCDCA, glycochenodeoxycholic acid; GDCA, glycodeoxycholic acid; GPR 40, G protein-coupled receptor 40; GUDCA, glycoursodeoxycholic acid; HBSS, Hanks’ balanced salt solution; HEK293, human embryonic kidney cells; HPLC, high-performance liquid chromatography; LCA, lithocholic acid; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MDCK, Madin–Darby
canine kidney cells; MRP2, multidrug resistance-associated protein 2; NTCP, Na\(^+\)-taurocholate co-transporting polypeptide; OATP, organic anion transporting polypeptide; SCRH, sandwich-cultured rat hepatocytes; TBA, total bile acid; TBIL, total bilirubin; TCA, taurocholic acid; TCDC, taurochenodeoxycholic acid; UDCA, ursodeoxycholic acid.
ABSTRACT

Fasiglifam (TAK-875), a selective G-protein-coupled receptor 40 agonist, was developed for the treatment of type 2 diabetes mellitus. However, its development was terminated in phase III clinical trials due to liver safety concerns. Our preliminary study indicated an intravenous administration of 100 mg/kg of TAK-875 increased the serum total bile acid concentration by 3 to 4 times and total bilirubin levels by 1.5 to 2.6 times in rats. In the present study, we examined the inhibitory effects of TAK-875 on hepatobiliary transporters to explore the mechanisms underlying its hepatotoxicity. TAK-875 decreased the biliary excretion index and the in vitro biliary clearance of d₈-taurocholic acid in sandwich cultured rat hepatocytes, suggesting that TAK-875 impaired biliary excretion of bile acids, possibly by inhibiting bile salt export pump (Bsep). TAK-875 inhibited the efflux transporter multidrug resistance-associated protein 2 (Mrp2) in rat hepatocytes using 5 (and 6)-carboxy-2',7'-dichlorofluorescein as a substrate. Inhibition of MRP2 was further confirmed by reduced transport of vinblastine in MDCK cells overexpressing MRP2 with IC₅₀ values of 2.41 μM. TAK-875 also inhibited the major bile acid uptake transporter Na⁺/taurocholate co-transporting polypeptide (Ntcp), which transports d₈-TCA into rat hepatocytes, with an IC₅₀ value of 10.9 μM. TAK-875 significantly inhibited atorvastatin uptake in organic anion transporter protein (OATP) 1B1 and OATP1B3 cells with IC₅₀ values of 2.28 and 3.98 μM, respectively. These results indicate that TAK-875 inhibited the efflux transporter MRP2/Mrp2 and uptake transporters Ntcp and OATP/Oatp, which may affect bile acid and bilirubin homeostasis, resulting in hyperbilirubinemia and cholestatic hepatotoxicity.
Introduction

Fasiglifam (TAK-875, Fig. 1) is an orally available, potent, and selective partial agonist of G protein-coupled receptor 40 (GPR40) that was developed for the treatment of type 2 diabetes mellitus (Negoro et al., 2010). TAK-875 markedly improves glycemic control via its glucose-dependent mechanism of action, which made it a promising candidate for the treatment of type 2 diabetes mellitus, together with its low risk of hypoglycemia compared with classic insulin secretagogues. However, the clinical development of TAK-875 was unexpectedly terminated at phase III clinical trials owing to concerns about its liver safety (Takeda, 2013). The mechanism by which TAK-875 caused liver injury still remains unclear.

After a single oral administration of 50 mg of TAK-875 to healthy subjects, TAK-875 was rapidly absorbed with a median time to the maximum concentration (i.e. $t_{\text{max}}$) ranged from 3 to 4 hours (Naik et al., 2012). The maximum plasma drug concentration (i.e. $C_{\text{max}}$) and the area under the plasma concentration–time curve from zero to infinity (i.e. $\text{AUC}_{0-\infty}$) were 2.3 $\mu$g/mL and 44.2 $\mu$g·h/mL, respectively (Naik et al., 2012). The mean percent unbound TAK-875 values in plasma were 0.17% in subjects with normal renal function (Mayer et al., 2014). The elimination of TAK-875 from the systemic circulation was relatively slow with a mean terminal elimination half-life ($t_{1/2}$) of approximately 28.1–36.6 hours (Naik et al., 2012). Furthermore, TAK-875 exhibited a longer $t_{1/2}$ (31–51 h) in diabetic patients than in healthy subjects, increasing accumulation by 2.4–3.6 times in diabetic patients (Leifke et al., 2012).

After 14 days of once-daily dosing of 50 mg TAK-875 in subjects with type 2 diabetes, the mean steady-state plasma concentration was 5.3 $\mu$g/mL (about 10.1 $\mu$M) (Leifke et al., 2012). The negligible urinary excretion values for TAK-875 ($\leq 0.3\%$) suggested that nonrenal clearance, including hepatic metabolism, was the predominant route of elimination (Naik et al., 2012). Our preliminary studies indicated that after an intravenously administration of 100 mg/kg of TAK-875 to rats, TAK-875 exhibited high concentrations even at 24 h postdose.
(Supplemental Figure 1 and Supplemental Table 1) and the parent drug was the major related compound in circulation (Supplemental Figure 2). The tissue disposition of TAK-875 in rats showed that its concentration was about 3 times greater in the liver than in plasma. If this liver/plasma ratio observed in rat was representative of humans, the concentration of TAK-875 in liver was expected to be about 30 μM after multiple doses of 50 mg TAK-875 to subjects with type 2 diabetes.

Our preliminary study also showed that the serum total bile acids (TBAs) and total bilirubin (conjugated and unconjugated, TBIL) level was increased in rats following an intravenous dose of 100 mg/kg of TAK-875. It was reported that increased serum total bile acid and conjugated bilirubin concentrations are indicators of cholestasis, which accounts for approximately 30% of all cases of drug-induced liver injury (Zamek-Gliszczynski et al., 2012). Bile acids are predominantly taken up from the sinusoidal blood into hepatocytes by the uptake transporter Na⁺/taurocholate co-transporting polypeptide (NTCP in humans/Ntcp in rodents) in a Na⁺-dependent manner (Meier et al., 1997; Trauner and Boyer, 2003; Dawson et al., 2009). Organic anion transporting proteins (OATPs in humans/Oatps in rodents) are also involved in the hepatic uptake of bile acids in a Na⁺-independent manner (Jacquemin et al., 1994; Satlin et al., 1997; Cui et al., 2001; Alrefai and Gill, 2007). After binding to intracellular binding proteins, bile acids are primarily secreted into the canaliculus via the bile salt export pump (BSEP in humans/Bsep in rodents) and multidrug resistance-associated protein (MRP in humans/Mrp in rodents) 2 on the canicular membrane (Kock and Brouwer, 2012). Bilirubin, the degradation product of heme from red blood cells, is taken up into hepatocytes by members of the OATP/Oatp family, followed by conjugation with glucuronic acid to bilirubin–glucuronide and bilirubin–diglucuronide (Cui et al., 2001). These conjugated bilirubin glucuronides are mainly excreted into bile by MRP2/Mrp2 (Chang et al., 2013).
The increase in serum TBA and TBIL concentrations may be associated with the inhibition of basolateral uptake transporters or canalicular efflux transporters. Because TAK-875 accumulates over a long time and reaches high concentrations in the liver, it may interfere with the transport of bile acids and bilirubin. Therefore, the aims of the present study were to investigate the effects of TAK-875 on the hepatobiliary disposition of bile acids and bilirubin in sandwich-cultured rat hepatocytes (SCRH) and recombinant cell lines overexpressing OATP and MRP2, and to explore the potential mechanisms for drug-induced liver injury.
Materials and Methods

Materials. The reference standard of TAK-875 was purchased from Shanghai Biochempartner Co. Ltd. (Shanghai, China). Rifampicin, atorvastatin calcium, MK-571, cyclosporine A (CyA), dexamethasone, 5 (and 6)-carboxy-2',7'-dichlorofluorescein diacetate (CDFDA), and Hanks’ balanced salt solution (HBSS, pH 7.4) were obtained from Sigma-Aldrich (St. Louis, MO). Fetal bovine serum (FBS), Dulbecco’s modified Eagle’s medium (DMEM), L-glutamine, minimum essential medium nonessential amino acids, penicillin G, and streptomycin were supplied by Invitrogen (Carlsbad, CA). d8-Taurocholic acid (d8-TCA) was purchased from Martrex, Inc. (Minnetonka, MN). Cholic acid (CA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), lithocholic acid (LCA), ursodeoxycholic acid (UDCA), taurocholic acid (TCA), taurochenodeoxycholic acid (TCDDCA), glycocholic acid (GCA), glycochenodeoxycholic acid (GCDCA), glycodeoxycholic acid (GDCA), and glycoursoxycholic acid (GUDCA) were purchased from Toronto Research Chemicals (TRC), Inc. (North York, ON, Canada). ITS+, Matrigel, and 24-well plates biocoated with collagen I or poly-D-lysine were obtained from BD Biosciences (San Jose, CA). Human embryonic kidney (HEK293) cells were individually transfected with human (h)OATP1B1, hOATP1B3, hOATP2B1, and a control vector (mock-transfected cells) and the functions of these transporters were validated using positive substrates and inhibitors. Madin–Darby canine kidney (MDCK) cells overexpressing MRP2 (MDCK-MRP2 cells) (Li et al., 2012) were a kind gift from Prof. Zhong Zuo (The Chinese University of Hong Kong, Hong Kong, China). The assay kits of total bile acid (TBA), alkaline phosphatase (ALP), alanine aminotransferase (ALT), and aspartate aminotransferase (AST) were purchased from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China). The bicinchoninic acid (BCA) protein assay kit was purchased from Beyotime (Jiangsu, China). Deionized water was generated using a Millipore Milli-Q Gradient Water Purification
System (Molsheim, France). All other solvents and reagents were of either high-performance liquid chromatography (HPLC) or analytical grade.

**In Vivo Cholestatic Effects in Rats.** All procedures involving animals were performed in accordance with *Guide for the Care and Use of Laboratory Animals* at Shanghai Institute of Materia Medica, Chinese Academy of Sciences. Male Sprague–Dawley rats weighing 200–250 g were fasted overnight before the experiments and were randomized into two groups (6 rats per group). Rats were given an intravenous administration of 20 or 100 mg/kg TAK-875 or saline as a control. Blood samples were collected before and at 15, 30, 45 min and 1, 2, 4, 8, and 24 h after the dose of TAK-875 or vehicle. Serum samples were obtained by centrifugation of blood samples at 3500 rpm for 5 min after blood samples standing for 30 min. Serum TBA, ALT, ALP, and AST concentrations were determined using the corresponding assay kits in accordance with the manufacturer’s instructions. The TBIL was measured using Hitachi 7060 automatic biochemical analyzer (Hitachi High-Technologies Co., Tokyo, Japan). The concentrations of 11 BAs in rat serum at predose and 1 h postdose were determined using the LC-MS/MS method.

**Isolation and Culture of Rat Primary Hepatocytes.** Hepatocytes from male Sprague–Dawley rats were isolated by the two-step collagenase digestion method as previously described (Annaert et al., 2001). The viability of the obtained hepatocytes was determined using trypan blue exclusion method and was routinely > 90%. Rat hepatocytes were seeded onto collagen I-coated 24-well plates at a density of $3.0 \times 10^5$ cells/well and cultured in Williams’ E medium supplemented with 0.1 μM dexamethasone, 5% FBS, 100 U penicillin, 100 μg/mL streptomycin, 2 mM glutamine, and 1% ITS*. After incubation for 4 h at 37 °C under 5% CO$_2$ and 95% humidity, the medium was replaced with serum-free medium. After incubation for 24 h, the hepatocytes were overlaid with 0.25 mg/mL of ice-cold Matrigel to form sandwich configuration and culture medium was thereafter replaced.
every 24 h. Rat hepatocytes cultured for 4 h could be used for the transporter-mediated uptake assays.

**Hepatobiliary Disposition of d₈-TCA/CDFDA in SCRH.** The uptake studies were conducted on day 4 of culture. Hepatocytes were rinsed three times with either prewarmed standard HBSS or Ca²⁺-free HBSS, and then hepatocytes were incubated with the same buffer either in the presence or absence of the test compound (20 or 100 μM TAK-875, 20 μM MK-571, or 10 μM CyA) for 10 min at 37 °C. Incubation in standard buffer maintains the integrity of the tight junctions, whereas incubation in Ca²⁺-free buffer opens the tight junctions. After the initial incubation, the incubation buffer was replaced with regular HBSS containing the test compounds. After incubation for 10 min, the hepatocytes were quickly rinsed three times with the ice-cold standard buffer. The accumulation of 5 (and 6)-carboxy-2',7'-dichlorofluorescein (CDF) in bile canaliculi was visualized under a fluorescence microscope (Olympus, Tokyo, Japan). Rat hepatocytes treated with d₈-TCA were lysed with 200 μL deionized water by three freeze–thaw cycles and the samples were stored at −20 °C until analysis. The concentration of d₈-TCA was measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis as described below. Protein concentrations were determined using a BCA protein assay kit.

**d₈-TCA Uptake Inhibition Assay.** After freshly isolated rat hepatocytes were cultured for 4 h, the cells could be used for uptake assays. To investigate the effects of TAK-875 on Na⁺-dependent uptake of d₈-TCA into rat hepatocytes, the Na⁺ in standard HBSS buffer was replaced with choline chloride. The cells were rinsed three times with standard HBSS or choline buffer and equilibrated in the same buffer with or without inhibitors (100 μM CyA, and 0–50 μM TAK-875) for 10 min. The uptake study was then initiated by adding standard HBSS containing d₈-TCA with or without the indicated inhibitors. The uptake was terminated after 5 min and the cells were quickly rinsed three times with ice-cold HBSS. The cells were
lysed by adding 200 μL of deionized water followed by three freeze–thaw cycles. The samples were stored at −20 °C until analysis. The concentration of d₈-TCA was determined using LC-MS/MS method as described below. The protein concentration was determined using a BCA assay kit.

**OATP Inhibition Assay.** OATP1B1-, OATP1B3-, OATP2B1-, and mock-transfected HEK293 cells were cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 100 U penicillin G, 100 μg/mL streptomycin, and 1% minimum essential medium nonessential amino acids at 37 °C in a humidified 5% CO₂ atmosphere. Cells were seeded on poly-D-lysine-coated 24-well plates at a density of 2 × 10⁵ cells/well. Uptake studies were performed on day 3 of culture. The cells were first washed three times with prewarmed HBSS (pH 7.4). After preincubation with HBSS in the presence or absence of various concentrations of TAK-875 for 10 min at 37 °C, the equilibration buffer was removed and uptake was initiated by adding HBSS containing the substrate atorvastatin (0.3 μM) with or without TAK-875. The uptake was terminated at the designated time by aspirating the incubation solution and washing the cells three times with ice-cold HBSS. The cells were lysed by adding 200 μL of deionized water followed by 3 freeze–thaw cycles. The protein content of the cell lysate was determined using a BCA protein assay kit. The concentrations of atorvastatin were determined by LC-MS/MS.

**MRP2 Inhibition Assay.** MDCK-MRP2 cells were cultured as described above for HEK293 cells. The cells were seeded at a density of 3 × 10⁵ cells/cm² on a polycarbonate membrane filter membrane on Transwell inserts (Millipore, Billerica, MA) and grown as monolayers for 5 days. Before starting the transport studies, the apical (A) and basolateral (B) chambers were washed twice with prewarmed HBSS, and then the cells were equilibrated for 30 min in the presence or absence of inhibitors MK-571 (20 μM) and TAK-875 (0-50 μM). HBSS containing the substrate vinblastine (Evers et al., 1998; Evers et al., 2000) was added.
to the donor side (either the apical or basolateral chamber) and the inhibitors were added to both chambers. The cells were incubated for 90 min at 37 °C, at which time aliquots (150 μL) were collected from the donor and receiver chambers for analysis. The concentrations of vinblastine in both chambers were determined by LC-MS/MS.

**Sample Preparation.** After cell lysis, 50 μL of cell lysate, 25 μL of the internal standard (10.0 ng/mL d5-atorvastatin or d5-TCA), and 150 μL of acetonitrile were added to centrifuge tubes. After vortex mixing and centrifugation at 14,000 × g for 5 min, the supernatants were injected into the LC-MS/MS system. Samples (25 μL) from the MDCK-MRP2 cells transport studies were added to a mixture of 25 μL of the internal standard vinorelbine (200 ng/mL) and 150 μL of acetonitrile, followed by vortex-mixing and centrifugation. The resultant supernatants were analyzed by LC-MS/MS.

**LC-MS/MS Analysis.** The LC system consisted of LC-30AD pumps and a SIL-30AC autosampler (Shimadzu, Kyoto, Japan). For d8-TCA inhibition assay, the analyte was separated on SB C8 (150 mm × 4.6 mm i.d., 5 μm; Agilent Technologies, Santa Clara, CA) column. The mobile phase was 5 mM ammonium acetate/acetonitrile/ammonium hydroxide (40:60:0.0004, v/v/v), and was applied at a flow rate of 0.6 mL/min. A Luna C18 column (50 mm × 2.0 mm i.d., 5 μm; Phenomenex, Torrance, CA) was used for the chromatographic analysis of atorvastatin. The mobile phase was 10 mM ammonium acetate/acetonitrile/formic acid (40:60:0.0004, v/v/v), and was applied at a flow rate of 0.6 mL/min. In the bidirectional transport inhibition assay using MDCK-MRP2 cells, the analytes were separated on a Gemini C18 column (50 mm × 2.0 mm i.d., 5 μm; Phenomenex) and eluted by a mobile phase composed of (A) 10 mM ammonium acetate and 0.1% formic acid in water and (B) methanol (constant flow rate of 0.7 mL/min). The gradient conditions were as follows: 0–0.5 min, 40% B; 0.5–1.5 min, 40%–90% B; 1.5–2.5 min, 90% B; 2.5–2.6 min, return to 40% B; and 2.6–5.0 minutes re-equilibrium.
An AB Sciex Triple Quad™ 6500 system (Applied Biosystems, Concord, Ontario, Canada) fitted with a TurboIonSpray ion source was used for MS detection. Multiple reaction monitoring (MRM) was used to quantify compounds in the positive ion mode (m/z 559 → m/z 440 for atorvastatin; m/z 564 → m/z 445 for d5-atorvastatin; m/z 811.5 → m/z 224.2 for vinblastine; and m/z 779.5 → m/z 122 for the internal standard vinorelbine) or in the negative ion mode (m/z 522 → m/z 128 for d8-TCA and m/z 519 → m/z 124 for d5-TCA).

For simultaneous quantitation of 11 bile acids in rat serum, Shimadzu LC-20AD HPLC system coupled with an AB Sciex API 4000 triple quadrupole mass spectrometer was used. The analytes were separated on XDB C18 column (5.0 mm, 4.6×150 mm, Agilent) with gradient elution of 7.5 mM ammonium acetate (pH4.0, A) and acetonitrile (B). The gradient conditions were as follows: 0–0.3 min, 30% B; 0.3–11 min, 30%–70% B; 11–14 min, 70%–100% B; 14–15 min, 100% B; 15–15.1 min, return to 30% B; and 15.1–19 minutes re-equilibrium. The flow rate was 0.8 mL/min. MRM was used to quantify compounds in the negative ion mode.

Data Analysis. Accumulation in hepatocytes plus bile (standard buffer) and hepatocytes only (Ca2+-free buffer) was calculated as the amount of d8-TCA (in picomoles per well) divided by the protein content (milligrams of protein per well). The biliary excretion index (BEI) and the in vitro biliary clearance (CL\textsubscript{biliary}) were calculated according to equations 1 and 2 (Liu et al., 1999a; Liu et al., 1999b):

\[
%\text{BEI} = \frac{(\text{Accumulation}_{\text{standard buffer}} - \text{Accumulation}_{\text{Ca}^{2+}-\text{free buffer}})}{\text{Accumulation}_{\text{standard buffer}}} \times 100
\]

(1)

\[
\text{CL}_{\text{biliary}} = \frac{(\text{Accumulation}_{\text{standard buffer}} - \text{Accumulation}_{\text{Ca}^{2+}-\text{free buffer}})}{(\text{Time} \times \text{Concentration}_{\text{media}})}
\]

(2)

The accumulation (standard and Ca2+-free buffer), BEI, and CL\textsubscript{biliary} for d8-TCA were expressed as the percentage of the control value (no inhibitor).
The permeability coefficients of vinblastine ($P_{\text{app,A to B}}$ and $P_{\text{app,B to A}}$) and the efflux ratio (ER) were calculated according to equations 3 and 4.

$$P_{\text{app}} = \frac{C_T \cdot V}{(\text{Area} \cdot C_0 \cdot t)}$$  \hspace{1cm} (3)

$$\text{ER} = \frac{P_{\text{app,B to A}}}{P_{\text{app,A to B}}}$$  \hspace{1cm} (4)

where $C_T$ = the concentration of vinblastine on the receiver side, $V$ = the loading volume on the receiver side, $\text{area} = \text{the surface area of the cell monolayer}$ ($0.33 \text{ cm}^2$ in a 24-well plate), $C_0$ = the initial concentration of vinblastine on the donor side, and $t = \text{incubation time}$. $P_{\text{app,A to B}}$ and $P_{\text{app,B to A}}$ represent the extent of permeation generated by the transport from the apical to basolateral sides and from the basolateral to apical sides, respectively.

All statistical analyses were performed using SPSS software (version 16.0 for Windows, SPSS Inc., Chicago, IL). Student’s two-tailed unpaired $t$ test was used for between-group comparisons. A $P$ value of $< 0.05$ was considered statistically significant.
Results

**In Vivo Cholestatic Effects of TAK-875 in Rats.** We first investigated the direct interactions between TAK-875 and the serum ALT, ALP, AST, TBAs, and TBIL levels in rats to evaluate the potential cholestatic effects of TAK-875. As shown in Fig. 2, compared with the control group, the serum levels of ALT, ALP, and AST at 24 h postdose were not significantly elevated at both doses. At the dose of 20 mg/kg of TAK-875, the serum TBAs levels and TBIL levels were comparable to the control within 8 h postdose. However, the TBA levels were significantly increased 3 to 4 times within 0.25 h to 4 h after an intravenous administration of 100 mg/kg of TAK-875, and were almost back to baseline after approximately 8 h postdose. The TBIL levels were increased 1.5 to 2.6 times within 0.25 h to 8 h following intravenous administration of 100 mg/kg TAK-875. The increase in the serum TBAs and TBIL was a fast response to administration of TAK-875.

The BAs, including TCA, CA, DCA, LCA, CDCA, UDCA, GCA, GCDCA, GDCA, GUDCA, and TCDCA, in rat serum were determined using LC-MS/MS (Table 1). The levels of TCA, CA, UDCA, and TCDCA in TAK-875 group were significantly increased (more than 2-fold) at 1 h postdose compared to the predose level.

**Effects of TAK-875 on the Hepatobiliary Disposition of d8-TCA in SCRH.** The effects of TAK-875 on the accumulation of d8-TCA were evaluated in SCRH (Fig. 3A). Co-incubation with 10 μM CyA, a potent inhibitor of BSEP, significantly decreased the accumulation of d8-TCA in cells incubated in the standard buffer. Incubation with 20 or 100 μM TAK-875 significantly decreased the accumulation of d8-TCA in cells incubated in the standard and Ca^{2+}-free buffers.

The effects of TAK-875 on the biliary efflux of d8-TCA were presented as BEI and CL_{biliary}. The data are shown as the percentage of the control (Fig. 3B). In the absence of inhibitors, the BEI for d8-TCA was 74.2%, indicating that 74.2% of the d8-TCA taken up by
the hepatocytes was effluxed into bile. Incubation with 10 μM CyA reduced the BEI of \( d_8 \)-TCA to 23.8% of that of control cells. TAK-875 exhibited a concentration-dependent inhibitory effect in terms of the BEI of \( d_8 \)-TCA, with BEI values of 70% and 52% of control cells at TAK-875 concentrations of 20 μM and 100 μM, respectively. Both CyA (10 μM) and TAK-875 (20 μM and 100 μM) significantly decreased the CL_biliary of \( d_8 \)-TCA to < 10% of control cells.

**Effects of TAK-875 on Ntcp and Oatp/OATP Uptake Transporters.** The inhibitory effects of TAK-875 on the uptake of \( d_8 \)-TCA by Ntcp were investigated in primary rat hepatocytes. The uptake of \( d_8 \)-TCA was approximately 17.4 times greater in the presence of Na\(^+\) than in the absence of Na\(^+\), accounting for 93.9% of the total \( d_8 \)-TCA uptake (Fig. 4A). Ntcp-mediated uptake was defined as the difference in uptake between cells incubated in Na\(^+\)-containing buffer and that in cells incubated in Na\(^+\)-free buffer. The overall Na\(^+\)-dependent uptake of \( d_8 \)-TCA by rat hepatocytes was inhibited by TAK-875 with a mean inhibitory concentration (IC\(_{50}\)) of 10.9 ± 0.7 μM (Table 2, Fig. 4B).

TAK-875 inhibited Oatp-mediated \( d_8 \)-TCA uptake (in Na\(^+\)-free buffer) in a concentration-dependent manner with an IC\(_{50}\) value of 2.94 ± 0.34 μM in primary rat hepatocytes (Table 2, Fig. 4B). The specific effects of TAK-875 on transport by OATP1B1 and OATP1B3 were then investigated using OATP1B1- and OATP1B3-expressing HEK293 cells. Atorvastatin was used as the surrogate probe substrate for OATP1B1 and OATP1B3. Significant inhibition of OATP-mediated atorvastatin uptake was observed in the presence of TAK-875 with IC\(_{50}\) values of 2.28 ± 0.17 μM and 3.98 ± 0.26 μM for OATP1B1 and OATP1B3, respectively (Table 2, Fig. 5).

**Inhibition of TAK-875 on the Mrp2/MRP2 Efflux Transporter.** CDFDA, a fluorescent bile acid analog, is hydrolyzed to CDF by esterase after its passive diffusion into hepatocytes. Then, CDF is primarily excreted into bile by MRP2/Mrp2, or effluxed back into...
blood by MRP3/Mrp3 (Zamek-Gliszczynski et al., 2003; Tian et al., 2004). Therefore, we examined the inhibitory effects of TAK-875 on Mrp2-mediated CDF accumulation in bile canaliculi using SCRHs (Fig. 6). The formation of a canalicular network was confirmed by fluorescent microscopy. The bright green spaces in Fig. 6A (small white arrows) indicate the formation of a canalicular space. MK-571, a known inhibitor of MRP2 and MRP3, and TAK-875 significantly decreased the fluorescent signal in the bile canaliculi, consistent with the inhibition of Mrp2. When administered at a high concentration, TAK-875 (100 µM) significantly increased the fluorescent signal throughout the entire microscopic field, indicating the enhanced accumulation of CDF in hepatocytes.

The inhibitory effects of TAK-875 on MRP2-mediated transport were also evaluated in MDCK-MRP2 cells using vinblastine as a substrate. The $P_{\text{app,B to A}}$ of vinblastine was much greater ($21.6 \times 10^{-6}$ cm/s) than the $P_{\text{app,A to B}}$ ($0.38 \times 10^{-6}$ cm/s), with an ER value of 56.8. The transport of vinblastine was markedly reduced by MK-571 (the ER was reduced to 5.6) and by the tested concentrations of TAK-875, indicating that these compounds inhibited MRP2-mediated transport of vinblastine. When using the reduction in the ER as an evaluation criterion, the estimated IC$_{50}$ of TAK-875 for inhibiting MDCK-MRP2 cells was $2.41 \pm 0.50$ µM (Table 2, Fig. 7).
Discussion

TAK-875 is a GPR40 agonist that was originally developed for the treatment of type 2 diabetes mellitus. However, its clinical development was unexpectedly terminated at phase III clinical trials because of liver safety concerns. Histopathological analysis revealed that compared with the normal architecture in control group, rats intravenously dosed with 100 mg/kg of TAK-875 exhibited picnotic nucleus in hepatocytes and mild bile duct dilation (Supplemental Figure 3), indicating that TAK-875 could cause cholestatic liver injury. In this study, we examined the cholestatic effects of TAK-875 by measuring the acute changes in serum enzymes (ALT, ALP, and AST), TBAs, and TBIL concentrations following an intravenous administration of TAK-875 to rats. We found that TAK-875 significantly increased the serum TBAs concentrations by 3 to 4 times compared with that in control rats, but did not change the serum levels of ALT, ALP, and AST, suggesting that TAK-875 disturbed the disposition of bile acids. Similar observations were reported with glyburide, CyA, and bosentan in rat serum (Funk et al., 2001b; Kostrubsky et al., 2003), which could cause cholestatic hepatitis in clinical settings. Considering the high accumulation of TAK-875 in liver, we speculate that TAK-875 may disturb bile acids transport, increasing the risk of hepatotoxicity. We further determined 11 BAs in rat serum at 1 h postdose after a single intravenous dose of 100 mg/kg of TAK-875 using LC-MS/MS. The levels of CA, UDCA, TCDCA, and TCA were increased > 2 times than those at predose.

Inhibition of the liver’s bile acid influx and efflux processes may cause marked changes in serum and hepatic bile acid concentrations. Because of the detergent-like properties of bile acids, an increase in the intracellular concentration of bile acids was associated with cellular necrosis and mitochondrial dysfunction during cholestasis (Maillette de Buy Wenniger and Beuers, 2010). Using d8-TCA as a probe for bile acid transport, we investigated the inhibitory effects of TAK-875 on the hepatobiliary disposition of d8-TCA in SRCHs. Of note, 20 μM
TAK-875 significantly decreased the accumulation of d₈-TCA in hepatocytes incubated in standard HBSS (representing hepatocytes plus bile) and hepatocytes incubated in Ca²⁺-free HBSS (representing hepatocytes only), leading to a decrease in the CL_biliary of d₈-TCA to < 10% of that in control cells. CL_biliary is an indicator of the overall effects of the compound on bile acids excretion. A decrease in CL_biliary can result from the inhibition of basolateral uptake transporters and/or canalicular efflux transporters.

The BEI represents the fraction of the total mass of d₈-TCA taken up that is subsequently excreted into bile. The decreased BEI of d₈-TCA in the presence of TAK-875 demonstrated that TAK-875 inhibited bile acids efflux into bile. Considering that TCA is primarily secreted into the canaliculus via Bsep, the inhibitory effects on d₈-TCA efflux by TAK-875 may be associated with the inhibition of Bsep. Many BSEP inhibitors, including troglitazone, bosentan, and glyburide, have been identified and are associated with drug-induced cholestatic hepatotoxicity (Fattinger et al., 2001; Smith, 2003). Although hepatotoxicity signals were not observed during the preclinical testing of troglitazone (Yang et al., 2014), in vitro and in vivo studies in rats demonstrated that troglitazone and its sulfate metabolite inhibited bile acids efflux by interfering with BSEP, and this interaction may contribute to troglitazone-induced hepatotoxicity in humans (Funk et al., 2001a; Funk et al., 2001b).

The decrease in the accumulation of d₈-TCA in SCRH in standard buffer to 28.7% of that in control cells suggests that TAK-875 inhibits bile acids influx into hepatocytes. Because the accumulation of d₈-TCA was inhibited to a greater extent than BEI, it seems that TAK-875 has a greater effect on hepatic bile acids uptake than on biliary bile acids efflux at the concentrations tested in rat hepatocytes. Bosentan and troglitazone exhibited similar effects on the hepatobiliary disposition of d₈-TCA in SCRH, as observed for TAK-875 (Ansede et al., 2010). However, CyA caused significant decreases in both BEI and CL_biliary, suggesting that efflux processes were inhibited to a greater extent than the uptake processes. These results are
consistent with earlier observations (Ansede et al., 2010). Bile acids uptake into hepatocytes is mediated in a Na\(^+\)-dependent manner by NTCP (predominant route) and a Na\(^+\)-independent manner by OATP (minor route). To identify the mechanisms underlying the inhibitory effects of TAK-875 on the uptake of d\(_8\)-TCA, we further investigated the effects of TAK-875 on the transporters Ntcp and Oatps/OATPs. TAK-875 exhibited more potent inhibitory effects on Oatps than on Ntcp, with IC\(_{50}\) values of 2.94 μM and 10.9 μM, respectively. The main liver OATP families in humans are OATP1B1 and OATP1B3, and in rats are Oatp1a1, Oatp1a4, and Oatp1b2 (Meier-Abt et al., 2004; Klaassen and Aleksunes, 2010). TAK-875 inhibited the transporters OATP1B1 and OATP1B3 with comparable IC\(_{50}\) values to Oatps overall.

The inhibition of NTCP could be regarded as a hepatoprotective mechanism because it reduces TBAs accumulation in hepatocytes. Bosentan exhibited hepatotoxicity in clinical settings but was not hepatotoxic in rats, a phenomenon that was attributed to its more potent inhibitory effects of rat Ntcp (IC\(_{50}\) 0.71 μM) than human NTCP (IC\(_{50}\) 24 μM) (Leslie et al., 2007). Thus, the differential inhibition of NTCP in rats/humans could explain the species differences in the susceptibility to drug-induced hepatotoxicity. Our results also suggest that TAK-875 impairs d\(_8\)-TCA uptake, primarily by inhibiting NTCP, which may be a hepatoprotective mechanism in rats. Hence, if TAK-875 inhibits human NTCP to a lesser extent than rat Ntcp or does not inhibit human NTCP, bile acids are likely to be highly accumulated in human hepatocytes through the inhibition of BSEP, increasing the likelihood of cholestatic hepatotoxicity.

The increase of serum TBIL in rat after an intravenous administration of 100 mg/kg TAK-875 also indicated that the transport of bilirubin was disturbed. Bilirubin is another potentially cytotoxic endogenous substance associated with severe liver injury. It was reported that biliary excretion is the rate-limiting step of bilirubin elimination. At high intracellular concentrations, bilirubin can uncouple oxidative phosphorylation, reduce DNA
stability, interrupt protein synthesis, and block ATPase activity in brain mitochondria (Hansen, 2001). Therefore, hyperbilirubinemia may provide an early warning of possible adverse effects such as neurotoxicity and hepatotoxicity. Bilirubin is taken up into hepatocytes via OATPs and is then excreted into bile via MRP2 (the primary route) or into plasma via MRP3 (a compensatory route) in the form of bilirubin glucuronides (Jedlitschky et al., 1997; Cui et al., 2001; Keppler, 2014). We found that TAK-875 is a strong inhibitor of OATP1B1 and OATP1B3, which suggests that TAK-875 may increase the serum concentration of unconjugated bilirubin. Indeed, in vitro, inhibition of OATP1B1 and/or OATP1B3 is associated with clinical unconjugated hyperbilirubinemia observed with several drugs, including atazanavir, indinavir, amprenavir, saquinavir, CyA, and rifampicin SV (Campbell et al., 2004; Annaert et al., 2010; Chiou et al., 2014). Furthermore, drugs that interfere with MRP2, which directly regulates the biliary excretion of bilirubin glucuronides, may contribute to clinical hyperbilirubinemia (Chang et al., 2013). When using CDF as the probe substrate of Mrp2 in the SCRH, TAK-875 significantly decreased the accumulation of CDF in bile canaliculi and increased its accumulation in hepatocytes, which indicates that TAK-875 is a potently inhibitor of Mrp2. The results of our in vitro studies using MDCK-MRP2 cells further confirmed the inhibitory effects of TAK-875 on MRP2. Therefore, by simultaneously inhibiting MRP2 and OATP1B1/3, TAK-875 may cause a marked increase in bilirubin concentrations, an event that may contribute to liver problems. In addition, impaired bile acids excretion slows bile flow, decreases biliary excretion of conjugated bilirubin, and increases bilirubin accumulation in hepatocytes (Hofmann, 1999). It was also reported that the expression of MRP2 is downregulated in cholestatic conditions (Trauner et al., 1997), which might further disrupt the elimination of bilirubin and aggravate hyperbilirubinemia.

Because TAK-875 can reach high concentrations in the liver, it might cause cholestatic
hepatotoxicity and/or hyperbilirubinemia by potently inhibiting the hepatobiliary disposition of bile acids and bilirubin (Fig. 8). Diabetic patients might therefore have a higher risk of hepatotoxicity due to the slower elimination rate and higher accumulation of TAK-875 (Leifke et al., 2012). Several transporters, including OATPs, MRPs, NTCP and BSEP are known to be polymorphic, and genetic mutations that alter the interactions between TAK-875 and either of these proteins could lead to the inter-individual differences in susceptibility to TAK-875–induced hepatotoxicity.

The rat model only evaluated the effects of TAK-875 on ALT, AST, ALP, TBA, and TBIL levels after a single administration of TAK-875, and may not be representative of drug-induced liver injury in humans which arose after long-term administration. The plasma protein binding of TAK-875 was high (>99%), thus the unbound plasma concentrations were markedly lower than the IC\textsubscript{50} values of evaluated transporters. Despite this difference, significant elevations of TBAs and TBIL levels in rat serum were observed and were speculate to be caused by the inhibition of transporters.

Overall, our observation that TAK-875 is an inhibitor of several hepatobiliary transporters, namely MRP2, NTCP, and OATP, may provide a possible mechanism of TAK-875–induced liver injury. Our results also highlight the importance of hepatobiliary transporters in drug discovery and development.
Acknowledgements

We wish to thank Dr. Xiaoyan Pang (Shanghai Institute of Materia Medica, Chinese Academy of Sciences) for helpful discussions.
Authorship contributions

Participated in research design: Li, K. Zhong, and Chen.

Conducted experiments: Li, K. Zhong, and Guo.

Contributed new reagents or analytic tools: Li, K. Zhong, and Chen.

Performed data analysis: Li, K. Zhong, and Chen.

Contributed to the writing of the manuscript: Li, K. Zhong, Chen, and D. Zhong.

Conflicts of interest

The authors declare no conflicts of interest.
References


Vinblastine and sulfynpyrazone export by the multidrug resistance protein MRP2 is associated with glutathione export. *Br J Cancer* **83**:375-383.


Scutellariae. *AAPS J* **14:**23-34.


Footnotes

This work was supported by the National Natural Science Foundation of China [81173115 and 81173117].

# Xiuli Li and Kan Zhong contributed equally to this work.
Figure legends

FIG. 1. The chemical structure of TAK-875.

FIG. 2. Effects of TAK-875 on serum ALT, ALP, AST, TBA, and TBIL levels in rats. Rats were intravenously administered with either 20 mg/kg (solid squares) or 100 mg/kg (solid triangles) of TKA-875 or saline as control (solid circles). Blood samples were collected at indicated time points and serum samples were analyzed using kits or biochemical analyzer. Data are expressed as the mean ± SD (n = 6). *p < 0.05, **p < 0.01, and ***p < 0.001 versus the control group.

FIG. 3. Inhibitory effects of TAK-875 on the accumulation, BEI, and CLbiliary of d8-TCA (1 μM) in SCRH. CyA (10 μM) was used as the positive inhibitor. (A) The accumulation of d8-TCA (1 μM) was measured in cells incubated in standard buffer (containing Ca2+, solid bar) or Ca2+-free buffer (open bar) for 10 min. (B) BEI and CLbiliary of d8-TCA in the presence of the indicated inhibitors expressed as percentages of the values in control cells. Data are shown as the mean ± SD (n = 3). **p < 0.01 and ***p < 0.001 versus control cells.

FIG. 4. (A) Inhibitory effects of TAK-875 on d8-TCA uptake into rat hepatocytes incubated in standard buffer (containing Na+, solid bar) or choline buffer (Na+–free, open bar). (B) Inhibitory effects of TAK-875 on Ntcp- and Oatp-mediated d8-TCA uptake into rat hepatocytes. Uptake was measured after incubating cells with 1 μM d8-TCA for 5 min. CyA (100 μM) was used as a positive control. Data are shown as the mean ± SD (n = 3).
FIG. 5. Inhibitory effects of TAK-875 on atorvastatin uptake into HEK293 cells overexpressing OATP1B1 (A) or OATP1B3 (B). Uptake was measured after incubating cells with 0.3 μM atorvastatin for 2 min. OATP-mediated atorvastatin uptake was corrected by subtracting the nonspecific accumulation of atorvastatin in mock-transfected HEK293 cells from that in OATP-transfected cells. Data are shown as the mean ± SD (n = 3).

FIG. 6. Inhibitory effects of TAK-875 on Mrp2-mediated accumulation of CDF in SCRHs. CDFDA (10 μM) passively diffuses into hepatocytes where it is hydrolyzed to CDF and then effluxed into bile canaliculi via MRP2/MRP2. CDF accumulation in bile canaliculi over 10 min was determined using an inverted fluorescent microscope. (A) Control cells. The white arrows point to representative bile canaliculi. (B) Cells treated with the positive inhibitor 20 μM MK-571. (C) Cells treated with 20 μM TAK-875. (D) Cells treated with 100 μM TAK-875.

FIG. 7. Inhibitory effects of TAK-875 on vinblastine transport in MDCK-MRP2 cells. Uptake was measured after incubating cells with 5 μM vinblastine for 90 min. Data are shown as the mean ± SD (n = 3).

FIG. 8. Schema showing the potential mechanisms by which TAK-875 affects the major hepatobiliary transport pathways of bile acids and bilirubin.
Table 1. The changes of serum BAs after a single intravenous dose of 100 mg/kg of TAK-875 to SD rats (each group n=6)

<table>
<thead>
<tr>
<th>No#</th>
<th>BA type</th>
<th>Control</th>
<th>TAK-875</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Predose</td>
<td>1 h Postdose</td>
<td>Fold</td>
</tr>
<tr>
<td>1</td>
<td>CA*</td>
<td>32508 ±16453</td>
<td>27242 ± 16670</td>
<td>0.870 ± 0.358</td>
</tr>
<tr>
<td>2</td>
<td>DCA</td>
<td>2796 ±1386</td>
<td>2650 ± 1140</td>
<td>0.985 ± 0.350</td>
</tr>
<tr>
<td>3</td>
<td>UDCA*</td>
<td>6654 ±2715</td>
<td>5591 ± 2917</td>
<td>0.846 ± 0.295</td>
</tr>
<tr>
<td>4</td>
<td>LCA</td>
<td>33.7 ±13.3</td>
<td>25.0 ± 5.1</td>
<td>0.798 ± 0.209</td>
</tr>
<tr>
<td>5</td>
<td>GCA</td>
<td>1700 ±780</td>
<td>1156 ± 614</td>
<td>0.713 ± 0.298</td>
</tr>
<tr>
<td>6</td>
<td>CDCA</td>
<td>4355 ± 863</td>
<td>3590 ±1188</td>
<td>0.923 ± 0.450</td>
</tr>
<tr>
<td>7</td>
<td>GDCA</td>
<td>415 ±167</td>
<td>303 ± 141</td>
<td>0.742 ± 0.198</td>
</tr>
<tr>
<td>8</td>
<td>GCDCA</td>
<td>127 ± 57</td>
<td>94 ± 47</td>
<td>0.794 ± 0.301</td>
</tr>
<tr>
<td>9</td>
<td>GUDCA</td>
<td>471 ±201</td>
<td>362 ± 286</td>
<td>0.754 ± 0.337</td>
</tr>
<tr>
<td>10</td>
<td>TCDCA*</td>
<td>79 ± 24</td>
<td>110 ± 35</td>
<td>1.40 ± 0.31</td>
</tr>
<tr>
<td>11</td>
<td>TCA*</td>
<td>238 ± 105</td>
<td>337 ± 137</td>
<td>1.45 ± 0.33</td>
</tr>
</tbody>
</table>

*indicates > 2-fold increase at 1 h postdose compared to the predose.
Table 2. The summary of IC$_{50}$ values for TAK-875 inhibition on transporters in rats and human in vitro systems

<table>
<thead>
<tr>
<th>Species</th>
<th>System</th>
<th>Transporter</th>
<th>Substrate</th>
<th>IC$_{50}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat</td>
<td>Hepatocytes</td>
<td>Ntcp</td>
<td>d$_8$-TCA</td>
<td>10.9 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>Hepatocytes</td>
<td>Oatps</td>
<td>d$_8$-TCA</td>
<td>2.94 ± 0.34</td>
</tr>
<tr>
<td>Human</td>
<td>HEK293 cells</td>
<td>OATP1B1</td>
<td>atorvastatin</td>
<td>2.28 ± 0.17</td>
</tr>
<tr>
<td></td>
<td>HEK293 cells</td>
<td>OATP1B3</td>
<td>atorvastatin</td>
<td>3.98 ± 0.26</td>
</tr>
<tr>
<td></td>
<td>MDCK cells</td>
<td>MRP2</td>
<td>vinblastine</td>
<td>2.41 ± 0.50</td>
</tr>
</tbody>
</table>
Figure 1
Figure 2
Figure 4

(A) Uptake (pmol/min/mg) of substrate with and without Na\(^+\) cotransport.

- Control
- 100 μM CyA
- 5 μM TAK-875
- 50 μM TAK-875

(B) Remaining percent (%) of substrate at different concentrations of TAK-875.

- IC\textsubscript{50} (Ntcp) = 10.9 ± 0.7 μM
- IC\textsubscript{50} (Oatps) = 2.94 ± 0.34 μM
Figure 5

(A) OATP1B1

IC$_{50}$ = 2.28 ± 0.17 μM

(B) OATP1B3

IC$_{50}$ = 3.98 ± 0.26 μM

Remaining percent (%) vs. TAK-875 Concentration (μM)
IC$_{50}$ = 2.41 ± 0.50 μM

Figure 7
Figure 8

Abbreviations:
BA: Bile acid;
Bilirubin Di-Glu: Bilirubin diglucuronide