Human Organic Anion Transporting Polypeptides 1B1, 1B3, and 2B1 Are Involved in the Hepatic Uptake of Phenolsulfonphthalein
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ABSTRACT: Phenolsulfonphthalein (PSP or phenol red), a sulfonphthalein dye, has been used as a diagnostic agent and a pH indicator in cell culture medium. After administered into the body, PSP is excreted into urine and bile. The urinary excretion of PSP is mediated by organic anion transporter 1/3 (OAT1/3) and multidrug resistance protein 2 (MRP2). In biliary excretion, PSP is effluxed from hepatocytes into the bile via MRP2. However, so far, the molecular mechanism for PSP transport from the blood into hepatocytes is unclear. In the present study, six human major hepatic uptake transporters expressed on the basolateral membrane of hepatocytes, namely, organic anion transporting polypeptide 1B1 (OATP1B1), OATP1B3, OATP2B1, Na+/taurocholate cotransporting polypeptide (NTCP), organic cation transporter 1 (OCT1), and OAT2, have been investigated to see whether they are involved in the hepatic uptake of PSP. An in vitro cell-based study demonstrated that PSP is a substrate for OATP1B1, OATP1B3, and OATP2B1, with OATP1B3 showing the highest transport efficiency. The $K_m$ values for OATP1B1-, OATP1B3-, and OATP2B1-mediated PSP uptake were 11.3 ± 1.5, 7.0 ± 1.5, and 5.1 ± 1.0 μM, respectively. PSP interacts with known OATP substrates/inhibitors. However, the presence of PSP in cell culture medium has no significant effect on OATP’s function. In vivo pharmacokinetic study in wild-type and Oatp1b2-knockout mice showed that Oatp1b2-knockout led to elevated plasma concentration and decreased liver accumulation of PSP. Taken together, the present study showed that in the liver, OATP1B1, OATP1B3, and OATP2B1 are involved in the uptake of PSP from the blood into hepatocytes, which, along with MRP2-mediated efflux of PSP from hepatocytes into the bile, constitute the vectorial transport of PSP from the blood to the bile and may play a critical role in the biliary excretion of PSP.

INTRODUCTION
Phenolsulfonphthalein (PSP or phenol red), a sulfonic acid dye, has been used as a diagnostic agent to test renal function. Nowadays, the test is still in use, albeit infrequently, to estimate the overall renal blood flow. PSP has also been used as a drug ingestion indicator, a marker in drug absorption studies. It has been reported that PSP is a substrate for organic anion transporter 1 (OAT1/SLC22A6) and OAT3 (SLC22A8) and multidrug resistance protein 2 (MRP2/ABCC2). In the kidney, OAT1 and OAT3 are expressed at the basolateral membrane of the proximal tubules, whereas MRP2 is localized to the apical membrane of proximal tubule cells. They mediate vectorial transport of PSP from the blood to the urine and thus play an important role in the tubular secretion of PSP.

Besides urinary excretion, biliary excretion is another route for the elimination of PSP in the body. Animal studies showed that when PSP was intravenously administered to rats, the biliary clearance of PSP was almost twice that of renal clearance. In humans, although the vast majority of intravenously administered PSP could be recovered in urine, there is still a significant amount of PSP being excreted into the bile. PSP is a known substrate for MRP2 which, in the liver, is localized at the canalicular membrane of hepatocytes and mediates the efflux of its substrates from hepatocytes into bile. However, so far, little is known about the transporters expressed on the sinusoidal membrane of hepatocytes that mediate uptake of PSP from the blood into hepatocytes.

In humans, six major hepatic uptake transporters belonging to solute carrier (SLC) superfamily, namely, organic anion transporting polypeptide 1B1 (OATP1B1/SLCO1B1), OATP1B3 (SLCO1B3), OATP2B1 (SLCO2B1), Na+/taurocholate cotransporting polypeptide (NTCP/SLC10A1), organic cation transporter 1 (OCT1/SLC22A1), and OAT2 (SLC22A7), play key roles in the hepatic clearance of various endogenous and xenogenous substances.
present study, we investigated these six transporters to identify and characterize the transporters that mediate hepatic uptake of PSP. Our results showed that OATP1B1, OATP1B3, and OATP2B1 are involved in the hepatic uptake of PSP with OATP1B3 showing the highest transport efficiency.

RESULTS

Characterization of Six Human Hepatic Uptake Transporters. To examine whether PSP is a substrate for hepatic uptake transporters, first, we performed expression and functional characterization of the six human major hepatic uptake transporters with their respective model substrates, namely, estradiol-17β-glucuronide (E17βG) for OATP1B1 and 1B3, 5 μM estrone-3-sulfate (E3S) for OATP2B1, 10 μM taurocholate (TCA) for NTCP, 5 μM 4-(4-(dimethylamino)styryl)-N-methylpyridinium (ASP+) for OCT1, and 5 μM penciclovir (PCV) for OAT2. Uptake of respective substrates by pcDNA5/FRT- and transporter-transfected human embryonic kidney (HEK293T) cells was carried out at 37 °C for 2 min. Data are presented as mean ± standard deviation (SD) (n = 3). *P < 0.05 vs pcDNA5/FRT control, two-tailed unpaired Student’s t test. (F) surface expression of six hepatic transporters in HEK293T cells. The numbers represent the relative expression levels of the six transporters after correction for Na+/K+ adenosine triphosphatase (ATPase) with the expression level of OATP1B1 being set to be 1. Plasma membrane proteins were isolated by surface biotinylation and analyzed by Western blot. The six transporters were detected with an anti-His antibody. Plasma membrane marker protein Na+/K+ ATPase was used as loading control.

Figure 1. Functional and expression characterization of six human hepatic uptake transporters. (A–E) transport activities of six hepatic transporters for their respective model substrates. The model substrates used were: 5 μM estradiol-17β-glucuronide (E17βG) for OATP1B1 and 1B3, 5 μM estrone-3-sulfate (E3S) for OATP2B1, 10 μM taurocholate (TCA) for NTCP, 5 μM 4-(4-(dimethylamino)styryl)-N-methylpyridinium (ASP+) for OCT1, and 5 μM penciclovir (PCV) for OAT2. Uptake of respective substrates by pcDNA5/FRT- and transporter-transfected human embryonic kidney (HEK293T) cells was carried out at 37 °C for 2 min. Data are presented as mean ± standard deviation (SD) (n = 3). *P < 0.05 vs pcDNA5/FRT control, two-tailed unpaired Student’s t test. (F) surface expression of six hepatic transporters in HEK293T cells. The numbers represent the relative expression levels of the six transporters after correction for Na+/K+ adenosine triphosphatase (ATPase) with the expression level of OATP1B1 being set to be 1. Plasma membrane proteins were isolated by surface biotinylation and analyzed by Western blot. The six transporters were detected with an anti-His antibody. Plasma membrane marker protein Na+/K+ ATPase was used as loading control.

PSP Is a Substrate for OATP1B1, OATP1B3, and OATP2B1 among the Six Human Hepatic Uptake Transporters. After the characterization of the six hepatic uptake transporters, we carried out experiments to determine whether they transport PSP. Uptake assay showed that human OATP1B1, OATP1B3, and OATP2B1 significantly transported PSP with their transport activities 2-fold greater than that of empty vector control, while human NTCP, OCT1, and OAT2 did not show significant transport for PSP (Figure 2). These results demonstrated that PSP is a substrate for human OATP1B1, OATP1B3, and OATP2B1, among which OATP1B3 showed the highest transport activity for PSP.

Time- and Concentration-Dependent Uptake of PSP by Human OATP1B1, OATP1B3, and OATP2B1. To characterize OATP1B1-, OATP1B3-, and OATP2B1-mediated PSP uptake, we performed their time-dependent and concentration-dependent uptake. Time-dependent uptake showed that OATP1B1-, 1B3-, and 2B1-mediated PSP uptake was linear up to 3 min (Figure 3). Therefore, we then performed concentration-dependent uptake at 2 min. The uptakes of PSP mediated by OATP1B1, OATP1B3, and OATP2B1 were saturable (Figure 4), and their kinetic parameters were obtained by fitting net uptake into the Michaelis–Menten equation and listed in Table 1. The $K_{m}$ and $V_{max}$ values for OATP1B1-mediated PSP uptake were 11.3 ± 1.5 μM and 55.8 ± 2.2 pmol/mg protein/min, respectively. The $K_{m}$ and $V_{max}$ values were 7.0 ± 1.5 μM and 80.0 ± 4.8 pmol/mg protein/min for OATP1B3, and 5.1 ± 1.0 μM and 23.9 ± 1.2 pmol/mg protein/min for OATP2B1. These results
indicated that OATP1B3 may play the most significant role in the hepatic uptake of PSP, although OATP1B1 and OATP2B1 are also playing important roles.

Inhibition of OATP1B1-, 1B3-, and 2B1-Mediated PSP Uptake by Their Known Substrates/Inhibitors. As PSP is transported by OATP1B1, 1B3, and 2B1, we want to check whether it interacts with their known substrates/inhibitors. Six substrates/inhibitors of OATPs, namely, bromosulfophthalein (BSP), E3S, E17β, rifampicin (RIF), rosvastatin (RSV), and TCA, had been investigated for their inhibition on OATP-mediated PSP uptake. At a concentration of 50 μM, BSP and RIF showed strong inhibition (>80%) and E17β and TCA showed moderate inhibition (50–80%) on OATP1B1-mediated PSP uptake, whereas RSV showed weak inhibition (20–50%) (Figure 5A). BSP, E17β, and RIF showed moderate inhibition on OATP1B3-mediated PSP uptake, whereas E3S, RSV, and TCA showed weak inhibition (Figure 5B). BSP and E3S showed strong and moderate inhibition on OATP2B1-mediated PSP uptake, respectively, while RIF and RSV showed weak inhibition (Figure 5C).

Inhibition of OATP1B1-, 1B3-, and 2B1-Mediated E17βG and E3S Uptake by PSP. As PSP is a substrate for OATP1B1, 1B3, and 2B1, we further examined whether PSP will inhibit OATP-mediated uptake of their model substrates. As shown in Figure 6, at a concentration of 50 μM, PSP showed moderate (50–80%) and weak (20–50%) inhibition on OATP1B1- and OATP1B3-mediated E17βG uptake, respectively, and PSP showed weak (20–50%) inhibition on OATP2B1-mediated E3S uptake.

Effect of PSP in Cell Culture Medium on the Function of OATP1B1, 1B3, and 2B1. As PSP interacts with OATP1B1, OATP1B3, and OATP2B1, and PSP is present in normal cell culture medium (~40 μM), we want to check whether the function of OATP1B1, OATP1B3, and OATP2B1 will be affected when cells were cultured and transfected in culture medium containing PSP. As shown in Figure 7A, OATP1B1 and 1B3 exhibited similar transport activities for E17βG when PSP was absent and present in cell culture medium. Similarly, OATP2B1 also showed comparable transport activities for E3S in the absence and presence of PSP in cell culture medium (Figure 7B). These results demonstrated that PSP in cell culture medium has no significant effect on the function of OATP1B1, OATP1B3, and OATP2B1.

In Vivo Pharmacokinetic Study of PSP in Wild-Type (WT) and Oatp1b2-Knockout (KO) Mice. As Oatp1b2 is the rodent ortholog of human OATP1B1 and 1B3,19,20 we used wild-type (WT) and Oatp1b2-knockout (KO) C57 mice to check the role of OATPs in the hepatic disposition of PSP in vivo. After oral administration of PSP into WT and KO mice, the blood and liver samples were collected at different times and the concentrations of PSP were measured in these samples. As shown in Figure 8 and Table 2, the plasma Cmax and AUC0–8 of PSP were 3.1- and 1.9-fold higher in KO mice than in WT mice, respectively. On the contrary, the Cmax and AUC0–8 of PSP in the liver were 1.4- and 2.1-fold lower in KO mice than in WT mice. These results demonstrated that PSP is transported by Oatp1b2 from the blood into hepatocytes and Oatp1b2 plays a significant role in the hepatic disposition of PSP in vivo.

DISCUSSION

PSP is a sulfonphthalein dye and used as a diagnostic agent and an indicator.1–3 After being intravenously administered to rats or humans, PSP is excreted into urine and bile.1,10,11 In the liver, PSP is excreted by MRP2 from hepatocytes into bile. However, so far, it is unclear whether the entry of PSP from the blood into hepatocytes is transporter-mediated and which transporter(s) is(are) involved in this process. Therefore, in the present study, we investigated six major human hepatic uptake transporters expressed on the basolateral membrane of hepatocytes, namely, OATP1B1, OATP1B3, OATP2B1,
To see whether they are involved in the hepatic uptake of PSP.

Our results showed that PSP is a substrate for human OATP1B1, 1B3, and 2B1, while it was not transported by NTCP, OCT1, and OAT2 (Figure 2). Among the three OATPs, OATP1B3 showed the highest uptake ratio (Figure 2) and transport efficiency as characterized by $V_{\text{max}}/K_{\text{m}}$ (Table 1) in vitro. As the relative expression levels of these three OATPs obtained in the present study (Figure 1F) were comparable to their abundance in human liver tissues as reported in the literature, OATP1B3 may also play the most significant role in hepatic uptake of PSP in vivo. It has been reported that Kupffer cells also express significant amounts of OATP1B1, 1B3, and 2B1 on both mRNA and protein levels. Therefore, PSP may also be taken up by Kupffer cells in the liver.

OATP-mediated PSP uptake could be inhibited by their known substrates and inhibitors (Figure 5). Reversely,
OATP1B1- and 1B3-mediated E17βG uptake and OATP2B1-mediated E3S uptake could also be inhibited by PSP (Figure 6). It was reported that OATP1B1 has two binding sites for E3S but only one binding site for E17βG.26−28 The binding site of E17βG was shared with the high-affinity binding site of E3S. However, OATP1B1-mediated PSP uptake was inhibited by E17βG but not by E3S (Figure 5A). Our explanation for this observation was that the binding site of PSP in OATP1B1 was partially overlapped with the binding site of E17βG, which in turn partially overlapped with the high-affinity binding site of E3S, but the binding sites for PSP and E3S were completely separated. OATP2B1 has only one binding site for E3S,26,29,30 which should be overlapped with the binding site for PSP but different from that for E17βG (Figure 5C).

As PSP can inhibit OATPs, we checked whether the presence of PSP in the cell culture medium would have any effect on the function of OATPs. Our results showed that the presence of PSP in the cell culture medium had no significant

Figure 6. Inhibition of (A) OATP1B1- and OATP1B3-mediated E17βG uptake and (B) OATP2B1-mediated E3S uptake by PSP. Uptake of 5 μM E17βG or E3S in the absence and presence of 50 μM PSP was measured for 2 min at 37 °C with pcDNA5/FRT- and OATP-transfected HEK293T cells. Values obtained with pcDNA5/FRT-transfected cells were subtracted from values obtained with OATP-transfected cells and were presented as percent of control. Data are given as mean ± SD (n = 3). *P < 0.05 vs control, Student’s t test.

Figure 7. Influence of PSP in cell culture medium on (A) OATP1B1- and 1B3-mediated E17βG uptake and (B) OATP2B1-mediated E3S uptake. HEK293T cells were cultured and transfected in PSP-free and PSP-containing media, respectively. Uptake of 5 μM respective substrates was carried out at 37 °C for 2 min. Data are given as mean ± SD (n = 3).

Figure 8. Plasma (A) and liver (B) concentration–time profiles of PSP after oral administration of PSP (20 mg/kg) in wild-type (WT) and Oatp1b2-knockout (KO) mice (n = 3–5).
effect on the function of OATPs as long as it was absent in the uptake buffer during functional assay (Figure 7). Therefore, for general functional assay of OATPs in which a substrate other than PSP is adopted, normal medium with PSP can be used for cell culture and transfection.

In addition to the cell-based in vitro study, in vivo study with WT and Oatp1b2-knockout mice also confirmed the significance of OATP1B transporters in the hepatic uptake of PSP. Elevated plasma concentration and decreased liver accumulation of PSP were observed in Oatp1b2-knockout mice compared to WT mice (Figure 8 and Table 2). As a rodent ortholog to human OATP1B1 and OATP1B3, 31 Oatp1b2 is also exclusively expressed in the liver.15 These results indicated the importance of Oatp1b2 in the hepatic disposition of PSP.

In summary, in the present study, our in vitro and in vivo results demonstrated that PSP is a substrate for human hepatic uptake transporters OATP1B1, OATP1B3, and OATP2B1, which are localized at the basolateral membrane of hepatocytes. Meanwhile, PSP is a substrate for MRP2, which is expressed on the apical membrane of hepatocytes. Therefore, as illustrated in Figure 9, OATP-mediated uptake and MRP2-mediated efflux constitute the vectorial transport of PSP from blood to bile and may play an essential role in the biliary excretion of PSP.

Table 2. Pharmacokinetic Parameters of PSP in Wild-Type (WT) and Oatp1b2-Knockout (KO) Mice after Oral Administration of PSP (20 mg/kg) (n = 3–5)

| PK parameters         | WT | KO |
|-----------------------|----|----|
| T<sub>max</sub> (h)   | 0.5| 0.5|
| C<sub>max</sub> (ng/mL) | 2520.0 | 7904.0 |
| AUC<sub>0−t</sub> (ng·h/mL) | 605.6 | 11 637.6 |
| AUC<sub>INF</sub> (ng·h/mL) | 6238.3 | 11 743.3 |
| t<sub>1/2</sub> (h)  | 1.5| 1.3|
| MRT (h)               | 2.0| 1.5|

Figure 9. Hepatic uptake and efflux transporters involved in the vectorial transport of PSP from the blood to the bile.

**Experimental Section**

**Chemicals and Reagents.** Phenolsulfonphthalein (PSP) was obtained from Macklin (Shanghai, China). Estradiol-17β-glucuronide (E17βG), estrone-3-sulfate (E3S), and taurocholate (TCA) were purchased from Sigma-Aldrich (St. Louis, MO). 4-(4-(Dimethylamino)styryl)-N-methylpyridinium (ASP') and penicilloyl (PCV) were from US Everbright (Suzhou, China) and Aladdin (Shanghai, China), respectively. Dulbecco’s modified Eagle’s medium (DMEM), Fetal bovine serum (FBS), trypsin, Lipofectamine 2000, sulfo-N-hydroxysuccinimide-SS-biotin, protease inhibitor tablets, and streptavidin-agrose resin were purchased from Thermo Fisher Scientific (Walther, MA). Penicillin-streptomycin was from Hyclone (Logan, UT). The bicinchoninic acid (BCA) protein assay kit was purchased from Takara (Kyoto, Japan). The first antibodies for detecting 6-His tag and Na+/K+-ATPase were from Proteintech (Rosemont, IL) and Abcam (Boston, MA), respectively. Their secondary antibodies were from Proteintech (Rosemont, IL).

**Expression Plasmids for the Six Human Hepatic Uptake Transporters.** The open reading frames of human OATP1B1, OATP1B3, OATP2B1, NTCP, OCT1, and OAT2 were incorporated with a 6-His tag in their C-terminal ends by polymerase chain reaction and cloned into the multiple cloning site of pcDNA5/FRT vector. The correctness of obtained plasmids was validated by DNA sequencing.

**Cell Culture and Protein Expression in HEK293T Cells.** HEK293T cells were cultured at 37 °C in a humidified 5% CO2 atmosphere in PSP-free DMEM unless otherwise indicated, supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Confluent cells were transiently transfected with plasmids using Lipofectamine 2000 and used after 24 h for expression and functional assay.

**Functional Assay.** One day prior to transfection, HEK293T cells were seeded in 24-well plates with a density of 2 × 10⁴ cells per well. The cells were transfected with 1 μg of DNA per well. The cells were washed three times with prewarmed uptake buffer (100 mM NaCl, 2 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, pH adjusted to 7.4 with Trizma base). Uptake was started by adding 200 μL of uptake buffer containing tested substrates. After incubation for a specific period of time, uptake was terminated by removing the uptake buffer and washing the cells four times with an ice-cold uptake buffer. The amounts of substrates E17βG, E3S, and PCV transported into cells were measured with liquid chromatography–tandem mass spectrometry (LC-MS/MS) as described previously.30,33 Total protein concentration was measured by the BCA assay and used to normalize cell number in each well.

To quantify PSP and PCV, the cells were lysed with 200 μL of ultrapure water per well by three freeze–thaw cycles and subjected to LC-MS/MS analysis. Chromatographic separation was achieved with an Agela Venusil C18 column (2.1 mm × 50 mm, 5 μm). The column temperature was maintained at 40 °C. For PSP analysis, the mobile phase consisted of 5 mM ammonium acetate aqueous solution (A) and methanol (B). The flow rate was 0.3 mL/min, and the total run time was 5.5 min with gradients: 0–0.5 min, 5% B; 0.5–1.5 min, 85% B; 1.5–3 min, 85% B; 3.0–3.2 min, 5% B; and 3.2–5.5 min, 5% B. Mass spectrometry was operated in multiple reaction monitoring (MRM) mode via negative electrospray ionization (ESI). The ion transitions for PSP and internal standard (IS) tolbutamide were m/z 353.3 → 195.0 and m/z 269.1 → 169.1, respectively. For PCV analysis, the mobile phase consisted of 0.1% formic acid aqueous solution (A) and acetonitrile (B). The flow rate was 0.5 mL/min with a 4 min gradient as follows: 0–0.5 min, 10% B; 0.5–0.8 min, 80% B; 0.8–2.8 min, 80% B; 2.8–3.1 min, 10% B; and 3.1–4 min, 10% B. Mass spectrometry was operated in MRM with positive ESI. The ion transitions for PCV and IS tolbutamide were m/z 254.1 → 152.1 and m/z 271.1 → 172.2, respectively.
For the quantification of ASP, the cells were lysed with 300 µL of 1% Triton X-100 in 20 mM Tris-HCl (pH 9) per well. Then, the fluorescence of ASP was measured with a Tecan Infinite M1000 PRO microplate reader at an excitation wavelength of 475 nm and an emission wavelength of 605 nm.

Cell Surface Biotinylation and Immunoblot Analysis. Cell surface biotinylation and immunoblot analysis were conducted as described previously. Briefly, HEK293T cells were plated in six-well plates and transfected with 5 µg of DNA plasmid per well. After 24 h of transfection, the cells were treated with sulfo-N-hydroxysuccinimide-SS-biotin in phosphate-buffered saline (PBS) and lysed with lysis buffer consisting of 10 mM Tris, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% sodium dodecyl sulfate (SDS), 1% Triton X-100, and protease inhibitors (pH 7.4) at 4 °C. The supernatant of cell lysate was incubated with streptavidin-agarose beads, and cell surface proteins were released from the beads with dithiothreitol (DTT). Protein samples were subjected to SDS-polyacrylamide gel electrophoresis and immunoblot analysis. Hepatic transporters were detected with a mouse anti-His tag antibody (1:20000 dilution, Proteintech, catalog number SA00001-1). Plasma membrane marker protein Na+/K+-ATPase was detected with a rabbit anti-Na+/K+-ATPase antibody (1:10000 dilution, Proteintech, catalog number 66005-1-Ig), followed by a horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (1:5000 dilution, Proteintech, catalog number SA00001-1). Plasma membrane marker protein Na+/K+-ATPase was detected with a rabbit anti-Na+/K+-ATPase α subunit antibody (1:10000 dilution, Abcam, catalog number ab76020), followed by an HRP-conjugated mouse anti-rabbit secondary antibody (1:10000 dilution, Proteintech, catalog number SA00001-2). Immunoblots were detected with Immobilon Chemiluminescent HRP Substrate (Millipore) and scanned with ChemiDoc MP imaging system (Bio-Rad).

In Vivo Pharmacokinetic Study of PSP in Wild-Type (WT) and Oatp1b2-knockout (KO) Mice. Male wild-type (WT) (23–28 g) and Oatp1b2-knockout (KO) (18–24 g) C57 mice were used for the study. Oatp1b2-knockout mouse model was constructed by Shanghai Model Organisms and validated by reverse transcription quantitative real-time polymerase chain reaction (PCR) (RT-qPCR) and in vivo study (unpublished data). Experimental protocols, handling, and treatment of mice were approved by the University Ethics Committee (approval number: BK20150349) and conducted according to the regulations for the Use and Care of Experimental Animals at Soochow University. WT and KO mice were administered with PSP at a dose of 20 mg/kg body weight via oral gavage, and blood samples were collected at 0.25, 0.5, 1, 2, 4, and 8 h postdose. The liver samples were harvested at 0.5, 1, 2, 4, and 8 h postdose. Plasma samples were collected by centrifugation of the blood at 16 200g. Plasma samples (25 µL) were vortexed with 100 µL of methanol-containing internal standard for 2 min to precipitate proteins and then centrifuged at 16 200g for 10 min to get the supernatants. The liver samples (100–300 mg) were homogenized in 3 vol (mass/mass) of normal saline with a GeneReady homogenizer (LifeReal, Hangzhou, China) and further prepared the same as plasma samples. PSP concentration in each sample was determined by the LC-MS/MS as described above.

Data Analysis. Uptake was performed in triplicate and repeated two to three times. Data with error bars represent mean ± standard deviation (SD). Statistical significance was determined by Student’s t test between two groups or one-way analysis of variance (ANOVA) followed by Dunnett’s test among multiple groups with Prism 7.0 (GraphPad Software). P < 0.05 was considered statistically significant. In vivo pharmacokinetic parameters were calculated using a non-compartmental model incorporated in Phoenix WinNonlin 8.2 (Certara, Princeton).

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

ASP’, 4-(4-(dimethylamino)styryl)-N-methylpyridinium; AUC, area under the plasma concentration–time curve; BCA, bicinchoninic acid; BSP, bromosulfophthalein; DMEM, Dulbecco’s modified Eagle’s medium; DTT, dithiothreitol; E17βG, estradiol-17β-glucuronide; E3S, estrone-3-sulfate; EDTA, ethylenediaminetetraacetic acid; ESI, electrospray ionization; FBS, fetal bovine serum; HEK293T, human embryonic kidney cells with large T antigen; IS, internal standard; KO, knockout; LC-MS/MS, liquid chromatography–tandem mass spectrometry; MRM, multiple reaction monitoring; MRP2, multidrug resistance protein 2; MRT, mean residence time; NTCP, Na+/taurocholate cotransporting polypeptide; OAT, organic anion transporter; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; PBS, phosphate-buffered saline; PCV, penciclovir; PSP, phenolsulphonphthalein; RIF, rifampicin; RSV, rosuvastatin;
RT-qPCR, reverse transcription quantitative real-time PCR; SD, standard deviation; SDS, sodium dodecyl sulfate; SLC, solute carrier; TCA, taurocholate; WT, wild-type

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