Involvement of organic anion-transporting polypeptides in the hepatic uptake of dioscin in rat and human

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ABBREVIATIONS: AP, apical; BL, basolateral; DMEM, Dulbecco’s modified Eagle’s medium; EGCG, (-)-Epigallocatechin gallate; ES, estrone-3-sulfate; HBSS, hank’s balanced salt solution; HEK293 cells, human embryonic kidney cells; IS, hydrochlorothiazide; MDCKII cells, Madin-Darby canine kidney strain II cells; MRM, Multiple reactions monitoring; Na⁺-taurocholate cotransporting polypeptide (NTCP); OAT, organic anion transporter; OATP, organic anion transporting polypeptide; OCT, organic cation transporter; PAH, p-aminohippurate; TEA, tetraethyl ammonium.
Abstract

The objective of this study was to clarify the mechanism underlying hepatic uptake of dioscin, a herbal ingredient with anti-hepatitis activity, in rat and human. The liver uptake index (LUI) in vivo, perfused rat liver in situ, rat liver slices, isolated rat hepatocytes and human organic anion-transporting polypeptide (OATP)-transfected cells in vitro were used to evaluate hepatic uptake of dioscin. Values of 11.9 ± 1.6% and 15.0 ± 0.9% of dose for uptake of dioscin were observed by LUI in vivo and perfused rat livers in situ, respectively. The time course of dioscin uptake by rat liver slices was temperature-dependent. Uptake of dioscin by rat liver slices and isolated rat hepatocytes was inhibited significantly by Oatp modulators, such as ibuprofen (Oatp1a1 inhibitor), digoxin (Oatp1a4 substrate) and glycyrrhizic acid (Oatp1b2 inhibitor), but not by tetraethyl ammonium (TEA) or p-aminohippurate (PAH). Uptake of dioscin in rat hepatocytes and OATP1B3-human embryonic kidney cells (HEK) 293 cells indicated a saturable process with $K_m$ of 3.75 ± 0.51 μM and 2.08 ± 0.27 μM, respectively. (−)-Epigallocatechin gallate (EGCG), cyclosporin A, rifampicin and telmisartan inhibited transport of dioscin in OATP1B3-HEK293 cells. However, transcellular transport of dioscin in OATP1B1- or OATP1B1/MRP2-Madin-Darby canine kidney strain (MDCK) II cells was not observed. These results indicate that hepatic uptake of dioscin is involved in OATP1B3 in human, and multiple Oatps might participate in this process in rat.
Introduction

Saponins, a family of glycoconjugates, have a broad range of pharmacological activity, including antivirus, immunomodulation and anticancer effects (Sun et al., 2011). Steroidal saponins are common in traditional medicine plants, such as *Dioscorea nipponica* Makino. For instance, di-ao-xin-xue-kang capsules prepared from *D. nipponica* Makino have been used for more than 10 years in China to treat coronary heart disease (Liu et al., 2004). The bioactivity of dioscin (a major component of di-ao-xin-xue-kang capsules) has been reported and includes anti-hepatitis (induced by CCl₄) (Lu et al., 2011), antitumor (Liu et al., 2004; Mi et al., 2002; Hu et al., 2012) and antifungal (Sautour et al., 2004) activity.

Diosgenyl 2,4-di-O-α-L-rhamnopyranosyl-α-D-glucopyranoside (dioscin; Fig. 1), a typical example of a spirostane saponin, is a natural saponin that has been isolated from a number of oriental vegetables and traditional medicinal plants. Other group (Li et al., 2005) demonstrated that after an oral dose or intravenous administration at 24 h in rats, dioscin is distributed primarily in the liver at a concentration 10- or 7-fold higher than that in plasma. However, the mechanism underlying hepatic uptake of dioscin is unclear and we focused on the hepatic uptake process to examine the pharmacokinetics of dioscin.

Several transporters, such as Na⁺-taurocholate cotransporting polypeptide (NTCP) and organic anion-transporting polypeptide (OATP) 1B1 (known also as OATP-C, OATP2, LST-1 and *SLC21A6*), OATP1B3 (known also as OATP8, LST-2
and *SLC21A8*, OATP2B1 (known also as OATP-B and *SLC21A9*), organic cation transporter 1 (*OCT1, SLC22A1*) and organic anion transporter 2 (*OAT2, SLC22A7*), are expressed on the sinusoidal membrane of hepatocytes. These proteins are involved in transport of a wide variety of compounds, including clinical drugs (such as statins) from blood into hepatocytes (Hsiang et al., 1999; De et al., 2011; König et al., 2000; Tamai et al., 2000; Shitara et al., 2006; Faber et al., 2003; Hirono et al., 2004). In particular, OATP1B1 and OATP1B3, expressed mainly on the sinusoidal membrane of human hepatocytes (Faber et al., 2003), are thought to exert a significant influence on the removal of various endogenous and xenobiotic substances from the bloodstream into hepatocytes (Smith et al., 2005; Hagenbuch and Gui, 2008; Kindla et al., 2009). The substrate specificity of OATP1B1 generally overlaps that of OATP1B3. However, there are some discrepancies between them; e.g., telmisartan (an angiotensin II antagonist) is a substrate of OATP1B3 but not OATP1B1 (International et al., 2010; Ishiguro et al., 2006).

In this study, we used the liver uptake index (LUI) method *in vivo*, perfused rat liver *in situ*, rat liver slices and isolated rat hepatocytes *in vitro* as well as human OATP1B3- and OATP1B1-transfected cells to investigate the involvement of OATPs in hepatic uptake of dioscin.
Material and methods

Chemicals. Dioscin (with purity of >99%) was kindly provided by Professor Jinyong Peng (College of Pharmacy, Dalian Medical University, Dalian, China). Telmisartan was provided by Zhejiang Kinglyuan Pharmaceutical Co., Ltd. Tetraethyl ammonium (TEA), digoxin and glycyrrhizic acid were purchased from Hubei Saibo Chemical Co., Ltd and Nanjing ZeLang Medical Technology Co., Ltd, respectively. Cyclosporin A, rifampicin and (−)-epigallocatechin gallate (EGCG) were purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). p-Aminohippurate (PAH), hydrochlorothiazide (internal standard (IS)), inulin, (RS)-2-(4-(2-methylpropyl)phenyl)propanoic acid (ibuprofen) and estrone-3-sulfate (ES) were purchased from Sigma (USA). All other chemicals were of analytical grade and were commercially available.

Cell culture. The stable transfected OATP1B3-human embryonic kidney cells (HEK) 293 cells, vector-HEK293 cells, vector-, OATP1B1- and OATP1B1/MRP2-Madin-Darby canine kidney strain (MDCK) II cells were a generous gift from Professor Yuichi Sugiyama, Graduate School of Pharmaceutical Sciences, University of Tokyo (Tokyo, Japan). HEK293 cells and MDCKII cells were grown in low-glucose Dulbecco’s modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (Invitrogen), 100 U/mL penicillin, 100 μg/mL streptomycin at 37°C with a 5%(v/v) CO₂ atmosphere and 95% relative humidity. Cell culture reagents were purchased from GIBCO® (Grand Island, NY).
**Animals.** Male Wistar rats of 220 – 250 g body weight obtained from the Experimental Animal Center of Dalian Medical University (Dalian, China; permit number SCXK 2008-0002) were allowed free access to water and chow diet but were fasted for 12 h (with water ad libitum) before the experiments. All of the animal experiments were done according to local institutional guidelines for the care and use of laboratory animals.

**In vivo LUI studies.** Under light ether anesthesia, the portal vein was cannulated with polyethylene tubing (PE-50) in male Wistar rat. A 2 mL/kg bolus of normal saline, cyclosporin A (9.6 mg/kg) or rifampicin (20 mg/kg) was given by jugular vein, 5 min before the administration of dioscin and inulin. Dioscin (0.2 mg/kg body weight) and inulin (1 mg/kg body weight) dissolved in rat plasma, which was used for the bolus intravenous administration, was injected rapidly into the portal vein immediately after ligation of the hepatic artery. After 18 s of bolus administration of compounds, which is enough time for the bolus to pass completely through the liver but brief enough to prevent recirculation of the compounds (Liu et al., 1992), the portal vein was cut, the liver was excised, weighed and stored at −20°C.

**In situ perfused rat liver experiments.** Livers of male Wistar rats were prepared by standard techniques (Meng et al., 2010). Following anesthesia, the bile duct was cannulated and the liver was perfused in situ in a single pass via the portal vein with oxygenated Krebs–Henseleit buffer (Meng et al., 2010). The liver was allowed to equilibrate for approximately 10 min before addition of the dose.
Perfusion was continued with oxygenated Krebs–Henseleit buffer containing 20% (v/v) washed bovine erythrocytes at a flow rate of 12 mL/min. The superior vena cava was cannulated and used to collect the effluent perfusate. Liver viability was determined on the basis of the initial bile flow (>2 μL/min). Following equilibration, the liver was perfused for 60 min after addition of dioscin 2.5 μM, dioscin 2.5 μM + cyclosporin A 10 μM or rifampicin 10 μM (final concentration). The effluent perfusate (~500 μL) was collected at timed intervals, and the samples were stored at −20°C.

**In vitro uptake in rat liver slices.** Rat liver slices were prepared as described (Elferink et al., 2004). In brief, after anesthetic, the liver of male Wistar rat was excised and immediately placed into ice-cold buffer, saturated with carbogen (95% O₂/5% CO₂, pH 7.4) and then cut into slices with a ZQP-86 tissue slicer (200 – 300 μm thickness; Zhixin Co., Ltd., Shanghai, China). After pre-incubation for 3 min at 37°C, the liver slices were transferred to 24-well culture plates containing fresh carbogen-saturated dioscin for further incubation. In addition, uptake of dioscin (5 μM) and digoxin (5 μM) were measured at 0, 1, 5, 15 and 30 min, rinsed with ice-cold Hanks’ balanced salt solution (HBSS; pH 7.5) and dried on filter paper. According to the result of time-dependent, 15 min was chosen as an uptake time that represented the overall rate of uptake and was used to examine the effects of inhibitors (Detailed specificities of inhibitors against rat Oatps can be found in Supplemental Table 1), including PAH (1 mM), TEA (1 mM), cyclosporin A (10 μM), ibuprofen (100 μM), digoxin (5 μM), rifampicin (10 μM) and glycyrrhizic acid (100
μM), on the uptake of dioscin. The accumulated dioscin and digoxin in homogenized liver slices were determined by liquid chromatography-tandem mass spectrometry (LC-MS/MS).

**Uptake by using isolated rat hepatocytes.** Isolated rat hepatocytes were prepared by the collagenase perfusion method in male Wistar rat as described (Maeda and Sugiyama, 2010). Isolated hepatocytes (viability >80%, determined by staining with trypan blue) were suspended in Krebs–Henseleit buffer, adjusted to 2.0 × 10^6 cells/mL and kept on ice. To examine the sodium dependence of dioscin (2.5 μM) uptake by rat hepatocytes, experiments were done in Krebs–Henseleit buffer but with sodium replaced by potassium. Before the uptake study, the cell suspension and Krebs–Henseleit buffer containing the substrate were incubated separately at 37°C for 3 min and then transport was initiated by adding an equal volume of buffer (120–200 μL) to the cell suspension. After incubation at 37°C for 0.5, 1, 2 or 5 min, the reaction was terminated by separating the cells from the substrate solution by centrifugal filtration. For this purpose, a 100 μL sample of incubation mixture was collected and placed into a 450 μL centrifuge tube (Hepatocyte Transporter Suspension Assay Kit, BD Gentest™) containing 5 M sodium acetate under a 100 μL layer of an oil mixture (density 1.015, a mixture of silicone oil and mineral oil; BD Gentest™), which was centrifuged at 10,000 g for 10 s (ST-16R, Thermo) and the hepatocytes passed through the oil layer into the aqueous solution. The tube contents were frozen in liquid nitrogen immediately after centrifugation and stored at -20°C.

**Uptake experiments using transporter expression systems.** The transport
study was done as described (Hirano et al., 2004). Briefly, the assays were done in 24-well culture plates with nearly confluent cells seeded 48 h before each experiment. Uptake was initiated by adding Krebs–Henseleit buffer (Hirano et al., 2004) containing dioscin after the cells had been washed twice and incubated with Krebs–Henseleit buffer at 37°C for 15 min. The uptake was terminated at designated times by adding ice-cold Krebs–Henseleit buffer after removal of the incubation buffer. The cells were washed twice with 1 mL of ice-cold Krebs–Henseleit buffer, lysed with 0.3 mL of 0.1% (v/v) Triton X-100, then transferred to a polythene tube for quantization by LC-MS/MS. Protein was measured by the bicinchoninic acid (BCA) procedure (Solarbio, China) using bovine serum albumin as the standard.

The uptake of dioscin (2.5 μM) was measured at designate times in OATP1B3- and vector-HEK cells. According to the results, a time of 1 min was selected as representative of the uptake rate and was used to examine the concentration-dependent uptake and the effects of transporter modulators on the uptake of dioscin.

**Transcellular transport.** The transcellular transport study was done essentially as described (Matsushima et al., 2005) but with modifications. In brief, 24-well Transwell inserts (12 mm diameter, 0.6 cm² growing surface area, 0.4 μm pore size; Corning Costar, Acton, MA) were used to seed the vector-, OATP1B1- and OATP1B1/MRP2-transfected MDCKII cells with a density of 1.4 × 10⁵ cells per well. After incubation for 3 – 5 days of MDCKII cells until confluence, the integrity of the cell monolayer was evaluated by measuring the transepithelial electrical resistance
(TEER >200 Ω·cm<sup>2</sup>) using Millicell-ERS equipment (Millipore, MA, USA). The monolayer cells were rinsed gently with transport buffer (Matsushima et al., 2005) at 37°C and the transwell chambers were incubated for 15 min. Subsequently, transepithelial transport experiments were initiated by adding incubation buffer containing dioscin (2.5 μM, final concentration) or estrone-3-sulfate (ES, 1μM) to the apical (400 μL) or basolateral (600 μL) compartment. Cells were incubated at 37°C under a 5% (v/v) CO<sub>2</sub> atmosphere, and 50 μL aliquots in the receiver compartment were sampled at 30, 60, 120 and 180 min. An equal volume of transport buffer was added to the sampling compartment immediately after sample retrieval. Intracellular accumulation was measured by aspirating the medium and washing the monolayer rapidly four times with transport buffer on each side. The inserts with a monolayer were detached from the chambers and cells were lysed by addition of 0.3 mL of 0.1% (v/v) Triton X-100. The concentration of dioscin and ES in the uptake and transepithelial transport samples was determined by LC-MS/MS.

**Determination of the plasma protein-unbound fraction and blood/plasma concentration ratio.** Binding of dioscin to plasma proteins was determined by an ultrafiltration method (Watanabe et al., 2009). To estimate the fraction not bound to plasma protein (f<sub>ub</sub>), 2.5 μM dioscin was added to rat plasma samples (obtained from male Wistar rat) and incubated at 37°C for 30 min. Then, the samples underwent ultrafiltration (Millipore Corporation, Bedford, MA). The concentrations of dioscin in the plasma and filtrate were determined by LC-MS/MS and the plasma protein unbound fraction was calculated. In a pilot study, no significant difference of protein
binding was confirmed up to its concentration of 20 μM. The adsorption of the drugs on the membrane was confirmed to be negligible.

To determine the blood/plasma concentration ratio ($R_B$) values of dioscin, dioscin was added to the blood samples, and they were incubated together at 37°C for 5 min. Plasma was prepared by centrifugation of the blood samples at 2,795g for 10 min at 4°C. The concentrations of dioscin in the blood and the plasma samples were determined by LC-MS/MS.

**Biological sample preparation.** Each liver obtained from the LUI study was weighed, added to a 3-fold volume of normal saline and homogenized (IKA-T10 homogenizer; IKA, Staufen, Germany) in an ice-bath. Inulin preparation: Inulin was measured as 5—(hydroxymethyl) furfural (HMF) after acid and heat treatment (Lin et al., 2010). Briefly, homogenate samples were precipitated with 7% (v/v) HClO₄ and then through boiling, cooling and solid phase extraction processes, the eluate was collected and dried with pure nitrogen gas. Finally, the residue was reconstituted with mobile phase. Dioscin preparation: the homogenate (50 μL) obtained from the LUI studies was precipitated with 200 μL of acetonitrile following the addition of 50 μL of IS solution (800 ng/mL). Subsequently, the mixture was vortex-mixed, centrifuged and then, the supernatant organic layer was dried with nitrogen at 37°C. The dried residue was dissolved in 200 μL of the mobile phase. The liver slice samples were weighed then mixed with 300 μL of normal saline and homogenized in an ice-bath. The other preparations were handled as described for the LUI-homogenized samples for dioscin. Cells in 5 M sodium acetate buffer from the
rat hepatocytes uptake study were taken from the centrifuge tube, placed into fresh tube and sonicated. Then 50 μL of the IS solution and 500 μL of water were added. After vortex-mixing for 10 s, 1 mL of diethyl ether, the extraction solvent, was added vortex-mixed for 3 min and then centrifuged for 10 min at 2795g. The supernatant organic layer was dried with nitrogen at 37°C and the dry residue was dissolved in 200 μL of the mobile phase. The uptake and transepithelial transport, blood and plasma samples were handled as described for LUI-homogenized samples for dioscin. The samples of digoxin and ES for liver slices and rat hepatocytes, which were used as the positive control, were handled as described for dioscin. The samples were subjected to LC-MS/MS analysis.

**LC-MS/MS analysis.** An Agilent LC system (Agilent HP1200, Agilent Technology Inc., Palo Alto, CA, USA) was used for LC-MS/MS analysis. Isocratic chromatographic separation was done by passage through a Hypersil BDS-C18 column (150 mm × 4.6 mm i.d., 5 μm; Dalian Elite Analytical Instruments Co. Ltd, China). The mobile phase consisted of 70% (v/v) acetonitrile, 30% (v/v) water with 0.1% (v/v) formic acid for inulin, 80% (v/v) acetonitrile, 10% (v/v) methyl alcohol and 10% (v/v) water with 10 mmol/L aqueous ammonium acetate for dioscin, 70% (v/v) methyl alcohol, 30% (v/v) water with 0.1% formic acid for digoxin, 70% (v/v) acetonitrile, 10% (v/v) methyl alcohol and 10% (v/v) water with 10 mmol/L aqueous ammonium acetate for ES at a flow rate of 0.5 mL/min. The column was maintained at ambient temperature. An API 3200 triple-quadruple mass spectrometer (Applied Biosystems, Concord, Ontario, Canada) was operated with a TurboIonSpray
interface in positive ion mode for inulin and in negative ion mode for dioscin, digoxin, ES and IS. Analyst 1.4.1 software (Applied Biosystems) was used to control the equipment, data acquisition and analysis. Multiple reactions monitoring (MRM) was used for data acquisition. The optimized truncated MRM fragmentation transitions were \( m/z \ 127.1 \rightarrow m/z \ 109.1 \) with a collision energy (CE) of 20 eV for 5-(hydroxymethyl) furfural (converted from inulin), \( m/z \ 867.50 \rightarrow 721.50 \) with CE of –50 eV for dioscin, \( m/z \ 779.0 \rightarrow 649.0 \) with CE of –50 eV for digoxin, \( m/z \ 348.9 \rightarrow 268.9 \) with CE of –45 eV for ES and \( m/z \ 295.60 \rightarrow 204.80 \) with CE of –50 eV for IS. The dwell time for each transition was 200 ms.

Data analysis. Data obtained in the LUI studies were expressed as percentage of LUI, which represents the ratio of the hepatic extraction of dioscin to that of inulin. The LUI was obtained as (Liu et al., 1992):

\[
\text{LUI} (%) = \left( \frac{X_{\text{dioscin}}}{{\text{dose}_{\text{dioscin}}} - \frac{X_{\text{inulin}}}{{\text{dose}_{\text{inulin}}}}} \right) \times 100
\]  

(1)

where \( X_{\text{dioscin}} \) and \( X_{\text{inulin}} \) are the amount of dioscin remaining in the liver after injection. Dioscin and inulin, taken up by the liver or in the injectate, were measured by LC-MS/MS analysis.

The hepatic extraction ratio \( (E_h) \) and clearance \( (\text{CL}_{h, \text{in situ}}) \) of dioscin in perfused rat liver were calculated as:

\[
E_h = 1 - F_h
\]

(2)

\[
F_h = \frac{C_{\text{out}}}{C_{\text{in}}}
\]

(3)

\[
\text{CL}_{h, \text{in situ}} = Q_h \times E_h
\]

(4)

\[
F_h = \frac{Q_h}{(Q_h + f/\text{CL}_{\text{int}, h})}
\]

(5)
where $C_{\text{out}}$ and $C_{\text{in}}$ are the concentration of dioscin in the effluent perfusate and the influent perfusate, respectively. $F_h$ represents the bioavailability of liver. CL$_{\text{int, h}}$ and $f$ ($f=1$) represent the intrinsic hepatic clearance and the protein unbound fraction in perfusate, respectively. Hepatic blood flow rate ($Q_h$) was set at 60 mL/min/kg (Watanabe et al., 2009; Davies et al., 1993).

The in vitro uptake clearance (CL$_{h, \text{ in vitro}}$) in rat isolated hepatocytes was calculated by dividing initial uptake velocity by the concentration of dioscin in the incubation buffer. The initial uptake velocity of dioscin was calculated as a slope of the uptake volume ($\mu$L/10$^6$ cells) at 0.5 and 2 min.

In the well-stirred model in vivo (Maeda et al., 2010), hepatic clearance (CL$_{h, \text{ predicted}}$) is expressed as a function of intrinsic hepatic clearance (CL$_{\text{int, h}}$), hepatic blood flow rate ($Q_h$), and the protein unbound fraction in blood ($f_B$):

$$CL_{h, \text{ predicted}} = Q_h \times f_B \times CL_{\text{int, h}} / (Q_h + f_B \times CL_{\text{int, h}})$$

(6)

$$f_B = f_u / R_B$$

(7)

where $f_u$ and $R_B$ represent protein unbound fraction in plasma and the blood/plasma concentration ratio ($R_B$) of drug.

$$CL_{\text{int, h}} = CL_{h, \text{ in vitro}} \times 1.25 \times 10^8 \text{ (cells/g liver)/10}^6 \times 38.3 \text{ (g liver/kg body weight)}$$

(8)

Specific uptake was obtained by subtracting the uptake into vector-transfected cells from the uptake into OATPs-transfected cells. Kinetic parameters were obtained using non-linear, least-squares regression analysis via the Michaelis–Menten equation:
\[ v = \frac{V_{\text{max}} \cdot S}{K_m + S} \] (9)

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

The calculation of IC\(_{50}\) values were performed using Prism 5 (GraphPad Software Inc., La Jolla, CA). And then inhibition constants \( (K_i) \) values were obtained as (Liu et al., 2011):

\[ \text{IC}_{50} = K_i \left(1 + \frac{[S]}{K_m}\right) \] (10)

where \( K_m \) is the Michaelis constant (µmol), \([S]\) is the substrate concentration (µmol).

Each experimental point represents the mean ± S.D. of 3 – 6 measurements. All statistical analysis was done with the SPSS11.5 package. Test results are expressed as mean ± S.D and differences with \( P \leq 0.05 \) are considered to be statically significant.
Results

**LUI study.** A LUI study was done with rats to evaluate the hepatic extraction of dioscin *in vivo*. The LUI value was obtained by injecting dioscin directly into the portal vein. By correcting for nonspecific distribution to the interstitial space and retention in the vascular space in the liver (Fig. 2A), the LUI value, calculated with Equation (1) was 11.9 ± 1.6%. Cyclosporin A and rifampicin showed the significant inhibition in hepatic extraction of dioscin with LUI value decreased to 6.9 ± 1.2% and 5.5 ± 0.7%, respectively (Fig. 2A).

**Perfused rat liver.** After perfusion of dioscin (2.5 μM) into rat liver at a constant rate, the effluent perfusate samples were collected at 1, 5, 10, 15, 20, 30, 45 and 60 min; bile samples were collected at 15 min intervals. The concentration reached a plateau after 30 min and remained constant until the end of the perfusion (Fig. 2B). The value for the hepatic extraction ratio calculated by Equation (2–3) was 15.0 ± 0.9%. Moreover, this value was reduced to 7.4 ± 1.1% and 8.2 ± 1.6% when dioscin was administered simultaneously with cyclosporin A and rifampicin (Fig. 2B). Based on the Equation 3 and 5, the intrinsic hepatic clearance *in situ* was obtained and hepatic clearance of dioscin (CL<sub>in situ, predicted</sub>, calculated by well-stirred model (equation 6–7), was 0.83 ± 0.10 mL/min/kg.

**Effects of transporter modulators on dioscin uptake in rat liver slices.** Liver slice uptake experiments were used to elucidate the target transporters involved in dioscin accumulation in the liver. The uptake of dioscin (5 μM) at 37°C was linear within the first 15 min. A significant difference was found between the amount of
dioscin uptake in the liver slices at 4°C and at 37°C (Fig. 3B). Under the present experimental conditions, digoxin was used as a positive control (Fig. 3A). We investigated the inhibitory effects of transporter modulators on dioscin uptake to determine the mechanism for dioscin uptake by the liver. The initial uptake of dioscin in rat liver slices was inhibited significantly in the presence of cyclosporin A (10 μM), ibuprofen (100 μM), digoxin (5 μM), rifampicin (10 μM) and glycyrrhizic acid (100 μM), respectively. By contrast, TEA (1 mM) of and PAH (1 mM) did not inhibit the uptake of dioscin (Fig. 3C).

Characterization of dioscin uptake by isolated rat hepatocytes. We used isolated rat hepatocytes to investigate the uptake of dioscin to further examine transporter-mediated dioscin uptake. The uptake of dioscin (2.5 μM) and digoxin (5 μM) by rat hepatocytes increased linearly up to 2 min (Fig. 4A and 4B) and the uptake of dioscin at 1 min was used in subsequent studies. The uptake of dioscin in rat hepatocytes was a saturable process with $K_m$ 3.75 ± 0.51 μM and $V_{max}$ 588 ± 12 pmol/min per 10$^6$ cells (Fig. 4C). In addition, we examined the inhibitory effects of transporter modulators. Uptake of dioscin was inhibited significantly by cyclosporin A, ibuprofen, digoxin, rifampicin and glycyrrhizic acid but not by TEA or PAH (Fig. 5). Moreover, when extracellular sodium ions were replaced with potassium ions at an equimolar concentration, the uptake of dioscin was not changed (Fig. 5).

Comparison of the hepatic clearance of dioscin obtained from in situ with that from in vitro. The hepatic clearance ($CL_{h,\text{in situ}}$) of dioscin in perfused rat liver in situ calculated by Equation (2–4) was 9.0 ± 0.5 mL/min/kg. Based on the in vitro data
in rat hepatocytes, the intrinsic uptake clearance (CL_{int,h}) was obtained and the hepatic clearance of dioscin (CL_{h, predicted}), calculated by well-stirred model with Equation (6–8), was 10.8 ± 0.7 mL/min/kg (Table 1). Therefore, the CL_{h, in situ} in situ could be well consistent with the CL_{h, predicted} in vitro.

**Uptake of dioscin by transporter-expressing HEK293 cells.** The uptake of dioscin by HEK293 cells expressing OATP1B3 and vector were measured to examine whether dioscin can be transported by human hepatic transporters. The uptake of dioscin (2.5 μM) in OATP1B3-HEK293 cells was significantly higher than that of vector-HEK293 cells (Fig. 6A). Eadie–Hofstee plot analysis indicated that the $K_m$ and $V_{max}$ values of dioscin were 2.08 ± 0.17 μM and 730 ± 17 pmol/min per mg protein, respectively (Fig. 6B).

**Inhibitory effects of drugs on uptake of dioscin in OATP1B3-HEK293 cells.** The effects of cyclosporin A, rifampicin, epigallocatechin gallate (EGCG) and telmisartan on the cellular uptake of dioscin were investigated in OATP1B3-HEK293 cells (Fig. 7). All potential inhibitors were used in a concentration range of 0.1 – 500 μM, whereas dioscin was used at a concentration of only 1 μM. All clinical drugs inhibited dioscin uptake significantly. $K_i$ values for dioscin calculated by Equation (10), which are summarized in Table 2, show the following order of potency for OATP1B3 inhibition:

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telmisartan > cyclosporin A > rifampicin > EGCG
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**Transcellular transport of dioscin in OATP1B1-MDCKII cells.** To characterize the activity of double-transfected MDCKII cells coexpressing OATP1B1
and MRP2, we evaluated the transcellular transport of ES (probe substrate of OATP1B1) across the MDCKII monolayer expressing uptake and efflux transporters (Figs. 8A-C). The basal-to-apical transport of ES was approximately 2.8 and 4.2-fold higher than that in the opposite direction in OATP1B1- and OATP1B1/MRP2-MDCKII cells, respectively (Fig. 8B and C). On the other hand, a symmetrical flux of ES was observed across the vector-MDCKII cells (Fig. 8A). It indicates that the activity of OATP1B1 was maintained in this study. The basolateral-to-apical and apical-to-basolateral vectorial transport of dioscin was similar in vector-MDCKII cells (Fig. 8D). However, no significant difference in vectorial transport was found in OATP1B1- and OATP1B1/MRP2-MDCKII cells between basolateral-to-apical and apical-to-basolateral transport (Fig. 8E and F). Moreover, cellular accumulations of dioscin in vector-, OATP1B1- and OATP1B1/MRP2- MDCKII cells (Fig. 9) corresponded well with the results of transcellular transport experiments.
Discussion

We investigated the hepatic transport mechanisms of dioscin by an in vivo hepatic extraction study using LUI method, an in situ uptake study using perfused rat livers and an in vitro uptake study using rat liver slices, isolated rat hepatocytes, OATP1B3-expressing HEK293, OATP1B1- and OATP1B1/MRP2-expressing MDCKII cells. The results provide direct evidence that uptake of dioscin by liver is mediated by transporters of the OATP family, including OATP1B3 in human (Fig. 6A; Fig. 8D-F) and multiple Oatps (Oatp1a1, Oatp1a4 and Oatp1b2) in rat (Fig. 3C; Fig. 5).

Dioscin, a spirostane saponin with anti-hepatitis activity (Lu et al., 2011), accumulates in liver after intravenous or oral administration at a concentration much higher than those in plasma and other tissues (Li et al., 2005). The value of hepatic clearance (CL_{in situ, predicted}=0.83 ± 0.10 mL/min/kg) is 21.5% of plasma clearance (3.86 ± 0.51 mL/min/kg) (Li et al., 2005). In addition, Li et al (2005) reported that dioscin might be metabolized by liver. These phenomena indicate that the liver may play an unnegligible role on the elimination of dioscin in rat. Therefore, detailed investigation of hepatic uptake is crucial for understanding the pharmacokinetics of dioscin in rat and human.

Initially, LUI study showed that the LUI of dioscin in vivo was 11.9 ± 1.6% (Fig. 2A), which corresponds well with the result (15.0 ± 0.9%) obtained by a liver perfusion study in situ (Fig. 2B). Further, we also found the significant inhibitory
effects of cyclosporin A and rifampicin on liver uptake of dioscin \textit{in situ} and \textit{in vivo} (Fig. 2), indicating that dioscin was extracted by rat liver through hepatic transporters belonging to the Oatp family. After incubation for 30 min, uptake of dioscin in rat liver slices was 3.2-fold greater at 37°C than that at 4°C (Fig. 3B). The uptake of dioscin in liver at 4°C might be explained by nonspecific membrane binding or intracellular binding after passive diffusion into the cells (Proost et al., 2006). The difference of drug uptake at 4°C and at 37°C indicates that uptake of dioscin in liver was mediated by hepatic transporter. Furthermore, there was marked inhibition of dioscin uptake (Fig. 3C) by cyclosporin A, ibuprofen, digoxin, rifampicin and glycyrrhizic acid as specific inhibitors of different Oatp isoforms in rat (Supplemental Table 1), at concentrations significantly exceeding their respective $K_i$ values (Shitara et al., 2002; Ismail et al., 2003). By contrast, a high concentration of TEA (1 mM) and PAH (1 mM) did not inhibit uptake of dioscin. These results indicate that the transporters of the OATPs family might be involved in the uptake of dioscin in rat liver slices.

We characterized the transport property of dioscin using isolated rat hepatocytes. Dioscin transport into rat hepatocytes is time- and concentration-dependent (Fig. 4B and 4C). The apparent $K_m$ of dioscin was 3.75 ± 0.51 μM in rat hepatocytes. These results indicate that uptake of dioscin in hepatocytes is a transporter-mediated process. To speculate what kind of transporters are responsible for hepatic uptake of dioscin, uptake was examined in the presence of various typical inhibitors of hepatic transporters (Fig. 5). As expected, a decrease of dioscin uptake was not observed
when sodium was replaced by potassium in incubation medium, suggesting that Na\textsuperscript+\textsuperscript--independent Oatps are involved mainly in hepatic uptake of dioscin in rat, and Na\textsuperscript+\textsuperscript--dependent Ntcp is not involved (Nakakariya et al., 2008). The lack of inhibition for dioscin uptake in rat hepatocytes by PAH and TEA corresponded well with what was found for liver slices, indicating that dioscin is not transported by Oat2 or Oct1 in liver (Ishiguro et al., 2006; Sugiyama et al., 2001). However, cyclosporin A (Oatp1a1 and Oatp1a4 inhibitor), ibuprofen (Oatp1a1 inhibitor), digoxin (Oatp1a4 substrate), rifampicin (Oatp1a4 inhibitor) and glycyrrhizic acid (Oatp1b2, Oatp1a1 and Oatp1a4 inhibitor) inhibited dioscin uptake significantly, which suggests that these individual Oatp-mediated transport processes are important in uptake of dioscin in isolated rat hepatocytes (Shitara et al., 2002; Sugiyama et al., 2001; Ismair et al., 2003). The relationship of hepatic clearance for dioscin between isolated rat hepatocytes \textit{in vitro} and perfused rat liver \textit{in situ} was also investigated. The predicted \(\text{CL}_{h,\text{predicted \textit{in vitro}}} \) (Table 1) was comparable with the \(\text{CL}_{h,\text{in situ}} \) observed in the present \textit{in situ} study, suggesting that these experimental systems were useful tool for investigating the liver extraction process of drugs. However, \(\text{CL}_{h,\text{predicted \textit{in situ}}} \) and \(\text{CL}_{h,\text{in situ}} \) are not comparable with \(\text{CL}_{\text{in situ,\text{predicted \textit{in situ}}} \) And this phenomenon was true in rats for an unknown reason. At the same time, Watanabe et al (2011) reported that the hepatic clearance of rosuvastatin as well as fluvastatin was an out liner extrapolated from \textit{in vitro} to \textit{in vivo}, and that result held true in rats for an unknown reason. Results from both series of experiments point to a possible mechanism of active dioscin uptake by liver through multiple Oatp isoforms (Oatp1a1, Oatp1a4 and
Oatp1b2) as the major underlying transporters for this liver extraction process.

In order to determine directly the potential of OATPs for transport of dioscin, we evaluated the transport properties of dioscin using HEK293 cells expressing OATP1B3 and MDCKII cells expressing OATP1B1 and OATP1B1/MRP2 systems. Initially, we investigated the function of transfected cells to confirm that this experimental system is reliable (Fig. 8A-C). In transport studies, time- and concentration-dependent uptakes of dioscin were observed in OATP1B3-HEK293 cells (Fig. 6). The $K_m$ value was $2.08 \pm 0.17 \mu M$, similar to that for uptake by rat isolated hepatocytes. However, no significant transcellular transport of dioscin was observed in OATP1B1- and OATP1B1/MRP2-expressing cells (Fig. 8E and F). These results suggest that dioscin is transported into hepatocytes by OATP1B3 rather than OATP1B1 in human. Generally, it is accepted that OATP1B1 is responsible for the hepatic uptake of several compounds, such as pitavastatin (Hirano et al., 2004), which are transported mainly by OATP1B1. Recent studies suggest that fexofenadine and telmisartan are transported by OATP1B3 rather than OATP1B1 (Ishiguro et al., 2006; Shimizu et al., 2005). Moreover, in valsartan, the contribution of OATP1B1 and OATP1B3 to hepatic uptake is estimated similar (Yamashiro et al., 2006). Therefore, to estimate whether a compound or drug is transported by OATP1B1/OATP1B3 in liver should depend on the properties and chemical structures of the compound or drug.

The effect of drugs on uptake of dioscin in OATP1B3-expressing HEK293 cells was investigated to estimate potential herb–drug interactions with co-administered
drugs that are OATP substrates or inhibitors (Fig. 7). As shown in Table 2, cyclosporin A is a potent OATP inhibitor; $K_i$ for OATP1B3-mediated dioscin uptake was 1.43 μM, which was similar to peak plasma concentrations of cyclosporin A obtained from literature (Treiber et al., 2007). This strong inhibitory effect is consistent with the literature for cyclosporin A on uptake of bosentan (IC$_{50}$ values for OATP1B1 and OATP1B3, 0.3 μM and 0.8 μM, respectively) (Treiber et al., 2007). Rifampicin inhibited the dioscin uptake mediated by OATP1B3 with $K_i$ 4.55 μM, whereas plasma concentrations of rifampicin at therapeutic dose of 600 mg/day are in the range 8 – 15 μM (Acocella, 1978; Loos et al., 1985). Assuming a free fraction of 20%, the free rifampicin serum concentration initially amounts to approximately 1.6 – 3.0 μM and, thus, lies in the similar range as the apparent rifampicin $K_i$ value for OATP1B3-mediated dioscin uptake (Table 2). In fact, rifampicin (OATPs inhibitor) could affect the hepatic uptake of drugs like pitavastatin ($K_i$ for OATP1B1, 0.5 μM) and bosentan (IC$_{50}$ values for OATP1B1 and OATP1B3, 3.2 and 1.6 μM, respectively) (Treiber et al., 2007; Hirano et al., 2006). Therefore, the hepatic uptake of dioscin might be influenced if dioscin is administered simultaneously with drugs known to be substrate or inhibitor of OATPs.

Herbal medicines are becoming popular worldwide, although their mechanisms of action are generally unknown. More than one-third of adults use herbal medicines in the hope of promoting health and to manage common maladies, such as inflammation, heart disease and diabetes (Zhou et al., 2007). Recently, clinically relevant herb–drug interactions based on cytochrome P450 enzymes/drug
transporters in the liver have been reported for St John’s Wort (*Hypericon perforatum*) and cyclosporine/digoxin (Borrelli and Izzo, 2009; Ruschitzka et al., 2000). Our results indicate that if dioscin is administered simultaneously with rifampicin or cyclosporin A, the hepatic uptake of dioscin might be influenced (Fig. 2; Table 2). Finally, anti-hepatitis activity of dioscin might be changed. To date, only a very small proportion of currently available drugs have been investigated for their potential interaction with herbs, such as St John’s Wort and ginkgo (*Ginkgo biloba*) in human (Zhou et al., 2007). Thus, further clinical studies are required to gain knowledge of transporter-based herb–drug interactions.

In conclusion, our results demonstrate for the first time that uptake of dioscin by liver is mediated by a hepatic transport system belonging to the OATP family, including OATP1B3 in human and multiple Oatps (Oatp1a1, Oatp1a4 and Oatp1b2) in rat. The hepatic uptake of dioscin might be influenced when herbs containing dioscin are administered simultaneously with drugs that are substrates or inhibitors of OATP1B3.
Acknowledgements

We especially thank Dr. Yuichi Sugiyama (Graduate School of Pharmaceutical Sciences, University of Tokyo, Tokyo, Japan) for providing OATP1B3-HEK293 cells, vector-HEK293 cells, OATP1B1-MDCKII cells, OATP1B1/MRP2-MDCKII cells and vector- MDCKII cells.

Authorship Contributions

Participated in research design: Kexin Liu, Aijie Zhang and Qi Liu

Conducted experiments: Kexin Liu, Aijie Zhang and Changyuan Wang

Performed data analysis: Qiang Meng and Xiaokui Huo

Wrote or contributed to the writing of the manuscript: Kexin Liu and Aijie Zhang

Contributed new reagents or analytical tools: Jinyong Peng, Huijun Sun and Xiaochi Ma
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Mi Q, Lantvit D, Reyes-Lim E, Chai H, Zhao W, Lee IS, Peraza-Sánchez S, Ngassapa


Watanabe T, Kusuhara H, Watanabe T, Debori Y, Maeda K, Kondo T, Nakayama H,


Footnotes

This work was supported partly by a grant from the National Natural Science Foundation of China [No. 81072694, 81273580] and Dalian Government [No. 2010E12SF060].
Legend to Figures

Fig. 1. Chemical structure of dioscin.

Fig. 2. The hepatic extraction ratio of dioscin in presence or absence of cyclosporin A/rifampicin in liver uptake index (LUI) experiment (A) and perfused rat liver experiment (B).

A: Dioscin or inulin was injected rapidly into the portal vein, liver was excised, and concentration of dioscin or inulin was measured by LC-MS/MS as described in the text. Each value was normalized by the total injected dose. B: Rat livers were perfused with 2.5 μM dioscin and the hepatic extraction ratio was calculated from the concentration of dioscin in the effluent and the influent perfusate.

Fig. 3. Time profile of digoxin (A) or dioscin uptake (B) and inhibitory effects of transporter modulators on uptake of dioscin in rat liver slices (C).

A: Digoxin (5 μM) was used as a positive control. B: Dioscin concentration was 5 μM. C: Results are shown as a percentage of dioscin uptake (control) measured in the absence of inhibitor.

Fig. 4. Time profile of digoxin (A) or dioscin (B) uptake and Eadie–Hofstee plot (C) for the uptake of dioscin by isolated rat hepatocytes.

A: Uptake of digoxin was measured as a positive control; substrate concentrations used were 5 μM (filled circles) and 200 μM (open circles). B: Uptake of dioscin (2.5 μM) by isolated rat hepatocytes was measured in the absence (filled circles) or in the presence (open circles) of 25 μM dioscin over 5 min at 37°C. C: The uptake of dioscin in isolated rat hepatocytes was measured at a concentration between 0.2 μM and 25
μM.

Fig. 5. Effects of sodium ions and transporter modulators on the uptake of dioscin in isolated rat hepatocytes.

The substrate concentration was 2.5 μM. The results are shown as a percentage of dioscin uptake measured in the absence of inhibitor and in the presence of sodium ions.

Fig. 6. Time profile (A) and Eadie–Hofstee plot (B) for the uptake of dioscin in OATP1B3-HEK293 cells.

A: The substrate concentration was 2.5 μM. B: The uptake of dioscin by OATP1B3-HEK293 cells was measured at a concentration between 0.2 μM and 25 μM. The specific OATP1B3-mediated dioscin transport was obtained by subtracting the uptake in vector-HEK293 cells from that in OATP1B3-HEK293 cells for 1 min. The continuous line represents the fitted curve.

Fig. 7. Effects of drugs on dioscin uptake by OATP1B3-HEK293 cells.

The substrate concentration was 1 μM. The results are shown as the percentage of the saturable uptake of dioscin in the absence of inhibitors. Squares, triangles, circles and diamonds represent the uptake of dioscin in the presence of rifampicin, cyclosporin A, EGCG and telmisartan, respectively. Continuous lines represent the fitted curves obtained by nonlinear regression analysis.

Fig. 8. Time profiles for the transcellular transport of ES and dioscin across MDCKII cells.

Transcellular transport of ES (1 μM) and dioscin (2.5 μM) across OATP1B1-
E) and OATP1B1/MRP2-expressing MDCKII cells (C and F) was compared with that across vector MDCKII cells (A and D).

**Fig. 9. Amount of dioscin in MDCKII cells.**

Dioscin content in cells on filters cut out of the plastic inserts after the flux measurements.
Table 1 Parameters of dioscin in rat blood and *in vitro* rat hepatocytes.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Blood</th>
<th>Hepatocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>$f_u$</td>
<td>$0.061 \pm 0.004$</td>
<td>–</td>
</tr>
<tr>
<td>$R_B$</td>
<td>$0.771 \pm 0.037$</td>
<td>–</td>
</tr>
<tr>
<td>$f_B$</td>
<td>$0.079 \pm 0.009$</td>
<td>–</td>
</tr>
<tr>
<td>$CL_{h, \text{in vitro}} \text{ (mL/min/}10^6 \text{ cells)}$</td>
<td>–</td>
<td>$0.035 \pm 0.004$</td>
</tr>
<tr>
<td>$CL_{\text{int, h}} \text{ (mL/min/kg)}$</td>
<td>–</td>
<td>$167.6 \pm 7.2$</td>
</tr>
<tr>
<td>$CL_{h, \text{predicted}} \text{ (mL/min/kg)}$</td>
<td>–</td>
<td>$10.8 \pm 0.7$</td>
</tr>
</tbody>
</table>
Table 2  $K_i$ values of dioscin uptake in the presence of cyclosporin A, rifampicin, EGCG and telmisartan in OATP1B3-HEK293 cells.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$K_i$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclosporine A</td>
<td>1.43 ± 0.12</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>4.55 ± 0.78</td>
</tr>
<tr>
<td>(−)-Epigallocatechin gallate</td>
<td>8.29 ± 0.76</td>
</tr>
<tr>
<td>Telmisartan</td>
<td>1.03 ± 0.07</td>
</tr>
</tbody>
</table>
Figure 2

A

Uptake (% of dose/liver)

- Dioscin
- Dioscin + cyclosporin A
- Dioscin + rifampicin

B

Hepatic extraction ratio (%)

- Dioscin
- Dioscin + cyclosporin A
- Dioscin + rifampicin

Time (min)
Figure 3

A

Uptake of digoxin (μL/mg liver)

- 37°C
- 4°C

Time (min)

B

Uptake of dioscin (μL/mg liver)

- 37°C
- 4°C

Time (min)

C

Uptake of dioscin (% of control)

- Control
- Cyclosporin A (10 μM)
- Ibuprofen (100 μM)
- Digoxin (10 μM)
- Rifampicin (10 μM)
- TEA (1 mM)
- PAH (1 mM)

* Indicates significant difference.
Figure 4

A

Uptake of digoxin (μL/10^6 cells)

Time (min)

B

Uptake of dioscin (μL/10^6 cells)

Time (min)

C

\[ \frac{V}{[S]} \] (μL/min/10^6 cells)

\[ V \] (pmol/min/10^6 cells)

\[ K_m = 3.75 \, \mu M \]

\[ V_m = 588 \, \text{pmol/min/10}^6 \, \text{cells} \]
Figure 5

The graph illustrates the uptake of dioscin (% of control) under different conditions. The x-axis represents various compounds: Control, Cyclosporin A (10 μM), Ibuprofen (100 μM), Digoxin (5 μM), Rifampicin (10 μM), Glycyrrhizic acid (100 μM), TEA (1 mM), and PAH (1 mM). The y-axis shows the uptake percentage ranging from 0 to 120.

- The control group shows a baseline uptake of 100%.
- Compounds like Cyclosporin A and Ibuprofen at 10 μM and 100 μM, respectively, show a significant reduction in uptake, indicated by asterisks.
- Other compounds like Digoxin at 5 μM, Rifampicin at 10 μM, Glycyrrhizic acid at 100 μM, and TEA and PAH at 1 mM show a moderate reduction in uptake.

Comparing the presence or absence of sodium ions (+ or -), the uptake changes accordingly, with the absence of sodium ions showing a higher uptake in most cases.
Figure 6

A

Uptake of dioscin (μL/mg protein)

- OATP1B3
- mock

Time (min):

0 1 2 3 4 5

B

V/S (μL/min/mg protein)

K_m = 2.08 μM
V_m = 730 pmol/min/mg protein

V (pmol/min/mg protein):

0 200 400 600 800
Figure 7

Uptake of dioscin (% of control) vs. Inhibitor conc. (μM)
Title

Involvement of organic anion-transporting polypeptides in the hepatic uptake of dioscin in rat and human

Aijie Zhang, Changyuan Wang, Qi Liu, Qiang Meng, Jinyong Peng, Huijun Sun, Xiaochi Ma, Xiaokui Huo and Kexin Liu

Detailed inhibition constants for inhibitors against rat Oatps in the reference:

Supplemental Table 1 IC\textsubscript{50}/\textit{K}_i values of inhibitors against rat Oatps.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Oatp1a1 (μM)</th>
<th>Oatp1a4 (μM)</th>
<th>Oatp1b2 (μM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ibuprofen</td>
<td>126</td>
<td>2430</td>
<td>N\textsuperscript{a}</td>
<td>Shitara et al., 2002</td>
</tr>
<tr>
<td>Digoxin</td>
<td>&gt;100</td>
<td>0.2</td>
<td>–</td>
<td>Shitara et al., 2002; Cattori et al., 2000</td>
</tr>
<tr>
<td>Rifampicin</td>
<td>18.2</td>
<td>1.5</td>
<td>N\textsuperscript{b}</td>
<td>Shitara et al., 2002</td>
</tr>
<tr>
<td>Glycyrrhizic acid\textsuperscript{c}</td>
<td>48%</td>
<td>53%</td>
<td>91%</td>
<td>Ismair et al., 2003</td>
</tr>
</tbody>
</table>

\textsuperscript{a} We could not find any reference to investigate the effect of ibuprofen on Oatp1b2.

\textsuperscript{b} We could not find any reference to evaluate the effect of rifampicin on Oatp1b2.

\textsuperscript{c} The inhibitory effect of glycyrrhizic acid (100μM) on Oatp1b2 mediated estrone-3-sulfate uptake.

Reference:


Identification of organic anion transporting polypeptide 4 (Oatp4) as a major full-length
