Molecular mechanisms governing different pharmacokinetics of ginsenosides and potential for ginsenoside-perpetrated herb-drug interactions on OATP1B3

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- Supporting Information Appendix S1 -
Supplemental methods

Cell culture and transfection

HEK293 cells were grown in a Dulbecoo's modified Eagle's medium fortified with 10% fetal calf serum in a humidified incubator at 37°C and 5% CO_2. Cells were harvested at 90% confluence and then seeded in poly-D-lysine-coated 24-well plates at a density of 2×10^5 cells/well 24 h prior to transfection. Human OATP1B1 (GeneBank accession no., NM_006446) and OATP1B3 (NM_019844) cDNA clones, purchased from Thermo Scientific (Waltham, MA, USA), were subcloned into pcDNA3.1 expression plasmid by Invitrogen Life Technologies (Shanghai, China). Plasmid expressing human OATP2B1 (NM_007256.2) was obtained from Origene (Rockville, MD). The open reading frames of rat hepatic transporters of Oatp1a1 (NM_0177111), Oatp1b2 (NM_031650), and Ntcp cDNAs (NM_017047) were synthesized and subcloned into pcDNA3.1 expression plasmid by Invitrogen Life Technologies. The expression plasmid and the empty vector were introduced separately into the HEK293 cells with Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. This produced transporter-expressing cells (TC) and mock cells (MC), respectively. Before use, the cells transfected with human OATP1B1, human OATP1B3 or rat Oatp1b2 plasmid were functionally characterized by measuring uptake of oestradiol-17β-D-glucuronide ($E_2$17βG). Those transfected with human OATP2B1, rat Oatp1a1 or rat Ntcp plasmid were functionally characterized by measuring uptake of oestrone-3-sulfate (E_1S), $E_2$17βG or taurocholate acid (TCA), respectively.

Western blotting analysis

The transfected cells and the mock cells were homogenized in homogenate buffer (250 mM sucrose, 10 mM HEPES, and 10 mM Tris-base, pH 7.4) with a protease inhibitor cocktail (1:100, *v*/*v*; Sigma, Oakville, ON, Canada) to provide crude membrane fractions of the cells. The homogenate was first centrifuged at 3,000*g* for 10 min at 4°C, and then the resultant supernatant was centrifuged at 33,000*g* for 30 min at 4°C. The formed pellet (membrane fraction) was resuspended in buffer (50 mM mannitol, 20 mM HEPES, and 20 mM Tris-base, pH 7.5) with the protease inhibitor cocktail. Immunoblotting was conducted with SDS-polyacrylamide gel electrophoresis (10% gel) and proteins were electrophoretically transferred to poly vinylidene fluoride membranes. The primary monoclonal antibody against human OATP1B1 (Abcam, Cambridge, MA, USA; dilution 1:1000) and primary polyclonal antibody against human OATP1B3 (Abcam, Cambridge, MA, USA; dilution 1:250) were used for the immunodetection of human OATP1B1 and OATP1B3. The primary monoclonal antibody against rat Oatp1b2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA; dilution 1:500) were used for the immunodetection of Oatp1b2. Blots were incubated with horseradish peroxidase-anti mouse or rabbit IgG (Bio-Rad, Richmond, CA, USA) for 1 h and signals were detected using the Immun-Star Chemiluminescent Protein Detection System according to the manufacturer's instruction (Bio-rad, Hercules, CA, USA).

Transport studies with human OATP1B1- and OATP1B3- and rat Oatp1b2-expressing HEK293 cells

Before the transport studies, the transfected HEK293 cells and mock cells were cultured with Dulbecoo's modified Eagle's medium for 48 h. The cells were then washed and preincubated at 37°C for 5 min with 250 μL of a prewarmed Krebs-Henseleit buffer (containing 142 mM NaCl, 23.8 mM $NaHCO_3$, 4.83 mM KCl, 0.96 mM KH_2PO_4, 1.20 mM $MgSO_4$,

12.5 mM HEPES, 5 mM glucose and 1.53 mM $CaCl_2$; adjusted to pH 7.4 with 1 M NaOH). After aspiration of the preincubation buffer from the cell-coated wells, 200 µL of the Krebs-Henseleit buffer fortified with the test compound or the prototypical substrate were added to initiate the transport, which were maintained by incubation for 10 min for transporter identification. The transport was terminated by aspirating the buffer from the wells. After washing three times with 400 µL of ice-cold Krebs-Henseleit buffer, the cells were lysed in water (150 µL) by freeze/thaw-enhanced ultrasonic treatment. The resulting cellular lysates (100 µL) were precipitated with 300 µL of methanol. After centrifugation at 21,100 *g* for 10 min, the supernatants were analyzed by LC-MS/MS. The total protein concentration of lysate was measured using a method first described by Bradford (1976). The transport rate of test compound in pmol·min^{-1} per mg protein was calculated using the following equation:

$$\text{Transport} = (C_L \times V_L)/T/ W_L \quad (1)$$

where C_L represents the concentration of test compound in the cellular lysate (µM), V_L is the volume of the lysate (µL), T is the incubation time (10 min) and W_L is the measured cellular protein amount of each well (mg). Differential uptake between the transfected cells (TC) and the mock cells (MC) was defined as a net transport ratio ($Transport_{TC}$/$Transport_{MC}$ ratio) and net transport ratio greater than three suggested a positive result.

Kinetics of human OATP1B3- and rat Oatp1b2-mediated cellular uptakes of ginsenoside Rg_1, ginsenoside Re and notoginsenoside R_1 were assessed with respect to Michaelis constant (K_m) and maximum velocity (V_{max}) and the incubation time was set at 5 min to ensure that the assessment was performed under linear uptake conditions (Supporting Information Appendix S3). Compound uptake during the linear phase (first 5 min) was assessed at concentrations over ranges of 3.1–200 µM. The inhibitory effect of rifampin on the activity of human OATP1B3 or rat Oatp1b2 that mediated transport of ginsenoside Rg_1, ginsenoside Re and notoginsenoside R_1 was further measured in terms of inhibition constant (K_i). The inhibitory effects of ginsenosides on the ability of human OATP1B3 and OATP1B1 to mediate transport of oestradiol-17β-D-glucuronide ($E_2$17βG) were measured in terms of half maximal inhibitory concentration (IC_{50}) and rifampin was used as positive control. The difference in the amounts of compound transported by transfected cells and the mock cells served as the transporter-dependent uptake for the kinetic and inhibition studies. All experiments were run in triplicates.

Transport studies with human MRP2-, BCRP-, BSEP- and MDR1-expressing membrane vesicles and rat Mrp2-, Bcrp- and Bsep-expressing membrane vesicles

A transport study was performed to identify the transporters that mediated the ATP-dependent cellular efflux of the test compound. A rapid filtration method was used with membrane vesicles expressing one of human MRP2, BCRP, BSEP and MDR1 and rat Mrp2, Bcrp and Bsep according to the manufacturer's protocol. In brief, a buffer [containing 50 mM 3-(*N*-morpholino) propanesulfonic acid, 70 mM KCl and 7.5 mM $MgCl_2$; adjusted to pH 7.0 with 1.7 M Tris-base] was used as transport medium, except for human MRP2 and rat Mrp2 using a buffer further containing 2 mM glutathione. The buffer was also used to dissolve the test compound and ATP (or AMP for the negative control), which was preincubated at 37°C for 5 min. The suspension of membrane vesicles was mixed with an equal volume of the buffer and preincubated at 37°C for 5 min. The transport was started by combining 20 µL of the membrane vesicle mixture with 30 µL of the test compound/ATP solution (or the test compound/AMP solution), and the resulting concentration of test compound was 20 µM in all cases. After incubation for 10 min, the transport was terminated by addition of 200 µL of an ice-cold buffer [containing 40 mM 3-(*N*-morpholino)propanesulfonic acid and 70 mM KCl; adjusted to pH 7.0 with 1.7 M Tris-base] followed by immediate transfer of the mixture into a Millipore MultiScreen-FB filtration plate (0.65 µm; Billerica, MA, USA; pre-wet with the ice-cold buffer). A Millipore MultiScreen vacuum manifold was used with a filtration plate and the membrane vesicles were washed five times with 200 µL of the ice-cold terminating buffer. Filters that retained membrane vesicles were transferred to 1.5 mL polypropylene tubes for lysis with 200 µL of 80% methanol. After centrifugation at 21,100 *g* for 10 min, the supernatants were analyzed by LC-MS/MS. The transport rate of test compound in pmol·min^{-1} per mg protein was calculated using the following equation:

$$\text{Transport} = (C_V \times V_V)/T/W_V \quad (2)$$

where C_V represents the concentration of the compound in the vesicular lysate supernatant (μM), V_V is the volume of the lysate (μL), T is the incubation time (10 min) and W_V is the amount of vesicle protein amount per well (0.05 mg). Positive results for ATP-dependent transport were defined as a net transport ratio ($Transport_{ATP}$/$Transport_{AMP}$ ratio) greater than three.

A kinetic study was performed using the transporter-expressing membrane vesicles. The transport of compounds during the linear phase (first 5 min) was assessed at concentrations over ranges of 5–300 μM. The inhibitory effects of 100 μM ginsenosides on the activities of human MRP2, BCRP, and BSEP that mediated transport of $E_2$17βG, MTX, and TCA, respectively, were also assessed. The difference between the amounts of transport in the presence of ATP and in its absence was defined as transporter-dependent uptake for the kinetic and inhibition studies. All experiments were run in triplicates.

Laboratory animals

Male Sprague-Dawley rats (230–300 g) were obtained from Sino-British SIPPR/BK Laboratory Animal Co., Ltd. (Shanghai, China). The use and treatment of rats were in compliance with the Guidance for Ethical Treatment of Laboratory Animals (The Ministry of Science and Technology of China, 2006, www.most.gov.cn/fggw/zfwj/zfwj2006). Animal studies were implemented according to the protocols approved by the Institutional Animal Care and Use Committee at Shanghai Institute of Materia Medica (Shanghai, China). China). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). All the rats were housed in cages (48 × 29 × 18 cm^3) in a room with unidirectional airflow, controlled temperature (20–25°C), relative humidity (40–70%) and a 12 h light/dark cycle. Filtered tap water was available *ad libitum*. The animals were given commercial rat chow *ad libitum* except during the night before dosing. The rats were allowed to acclimate to the facilities and environment for 5 days prior to experimental use. The femoral arteries of rats were cannulated for blood sampling, and other rats underwent bile duct cannulation for bile collection (Chen *et al.*, 2013; Cheng *et al.*, 2013). After the aseptic surgery, the rats were housed singly and allowed to regain their preoperative body weights before use. All used rats were euthanatized with CO_2 gas.

Rat studies

To determine the *in vivo* functional importance of Oatp1b2 in pharmacokinetics and disposition of ginsenosides, four rat studies were performed with the rodents treated or not treated with rifampin (i.v., 20 $mg \cdot kg^{-1}$). In the first rat study, rats were randomly assigned to nine groups. After a bolus i.v. dose of ginsenosides Rg_1, Re, Rb_1 (rifampin-free rats only), Rc (rifampin-free rats only), Rd (rifampin-free rats only) or notoginsenoside R_1 at 2.5 $\mu mol \cdot kg^{-1}$, serial blood samples (60 μL; 0, 5, 15, 30 min, 1, 2, 4, 6, 8, 10 and 24 h) were collected and heparinised. The plasma fractions were prepared by centrifugation. In the second rat study, rats were randomly assigned to nine groups to receive a bolus i.v. dose of ginsenosides Rg_1, Re, Rb_1 (rifampin-free rats only), Rc (rifampin-free rats only), Rd (rifampin-free rats only) or notoginsenoside R_1 at 2.5 $\mu mol \cdot kg^{-1}$. Bile samples were collected from rats between 0–2, 2–6, 6–12 and 12–24 h after dosing and were weighed. A sodium taurocholate solution (1.5 $mL \cdot h^{-1}$ during day time; pH 7.4) was infused into the duodenum during bile collection. In the third rat study, rats were assigned randomly into nine groups to receive a bolus i.v. dose of ginsenosides Rg_1, Re, Rb_1 (rifampin-free rats only), Rc (rifampin-free rats only), Rd (rifampin-free rats only) or notoginsenoside R_1 at 2.5 $\mu mol \cdot kg^{-1}$. The rats were housed singly in metabolic cages and the collection tubes containing their urine were frozen at −15°C during sample collection. Urine samples were collected at 0–8 and 8–24 h after dosing and were weighed. Each rat study was repeated twice. In the last study, rats were randomly assigned to nine groups to receive a bolus i.v. dose of ginsenosides Rg_1, Re, Rb_1 (rifampin-free rats only), Rc (rifampin-free rats only), Rd (rifampin-free rats only) or notoginsenoside R_1 at 2.5 $\mu mol \cdot kg^{-1}$. The rats under isoflurane anesthesia were killed by bleeding at the abdominal aorta at 5 and 30 min and at 1, 4 and 8 h (three rats per sampling time) after dosing. Selected tissues including the heart, lungs, brain, liver and kidneys were excised, rinsed in ice-cold saline, blotted, weighed and homogenized in four volumes of ice-cold saline. All rat samples were stored at −70°C until analysis.

Determination of plasma protein binding

Equilibrium dialysis was used to assess the fraction of plasma protein-unbound compound (f_u) (Guo *et al.*, 2006). The test compounds were individually added into blank human and rat plasma to generate nominal concentrations of 5 μM for ginsenoside Rg_1, ginsenoside Re and notoginsenoside R_1 and rifampin and 50 μM for ginsenosides Rb_1, Rc and Rd. The resulting plasma samples were used for 24 h equilibrium dialysis using Sepctra/Por 2 RC dialysis membranes (molecular weight cutoff, 12–14 kDa; Rancho Dominguez, CA, USA) and phosphate buffered saline (pH 7.4) as dialysate at 37°C. After completion of the dialysis, both the plasma and the dialysate were sampled (100 μL), mixed with methanol (300 and 100 μL, respectively) and centrifuged for LC-MS/MS-based analysis. The f_u value was calculated using the following equation:

$$f_u = C_d / C_p \times 100\% \quad (3)$$

where C_d represents the concentration in the dialysate (μM) after completion of dialysis and C_p is the corresponding concentration in the plasma (μM).

LC-MS/MS-based bioanalytical assays

Validated bioanalytical methods were used to measure test compounds in biomatrices using a TSQ Quantum mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) interfaced via an electrospray ionization probe with a liquid chromatography (Agilent Technologies, Waldbronn, Germany). Chromatographic separations for measurement of the ginsenosides were achieved on an Agilent Eclipse Plus 5-μm C_{18} column (50 × 2.1 mm i.d.; Santa Clara, CA, USA) and those for measurement of rifampin, E_1S, $E_217\beta G$, TCA and MTX were achieved on a Phenomenex Gemini 5-μm C_{18} column (50 × 2.0 mm i.d.; Torrance, CA, USA). Mobile phases for measurement of ginsenosides were methanol/water (1:99, v/v, containing 100 μM lithium acetate and 1 mM formic acid; solvent A) and methanol/water (99:1, v/v, containing 100 μM lithium acetate and 1 mM formic acid; solvent B). Mobile phase for measurement of rifampin, E_1S, $E_217\beta G$, TCA and MTX were methanol/water (1:99, v/v, containing 0.7 mM ammonium formate; solvent A) and methanol/water (99:1, v/v, containing 0.7 mM ammonium formate; solvent B). To simplify the assay development, a pulse gradient elution technique was used for the compound measurement (Wang *et al.*, 2007). In brief, the start proportions for measurement of ginsenosides, for measurement of rifampin, E_1S, $E_217\beta G$ and TCA and for measurement of MTX were 5% B, 50% B and 30% B, respectively, and the elution proportion segments were 3.9 min, 1.0 min and 1.0 min, respectively. The elution proportions were the same for all the analytes, i.e. 100% B, and the column equilibrium segment were normalized to 3.5 min. MS/MS was performed in the positive or negative ion mode using the precursor-to-product ion pairs *m/z* 1115→349, 1085→319, 953→773, 807→627, 953→773, 939→319, 824→151, 349→269, 447→271, 514→124 and 455→308 of ginsenosides Rb_1, Rc, Rd, Rg_1, Re, notoginsenoside R_1, rifampin, E_1S, $E_217\beta G$, TCA and MTX, respectively. The LC effluent flow was introduced into the electrospray ionization probe at a flow rate of 0.35 $mL \cdot min^{-1}$ and the data acquisition period was 2.0–4.5 min. The other effluent flows (0–2 and 5–6.5) were diverted to waste. Sample clean-up was achieved using methanol as precipitant at a methanol-to-sample volume ratio of three. After centrifugation at 21,100*g* for 10 min, the supernatant (5 μL) was applied to LC-MS/MS-based analysis. Matrix-matched calibration curves were constructed for the test compounds using weighted (1/*X*) linear regressions of the compound response (peak area; *Y*) against the corresponding nominal concentrations of compound (*X*, μM), which showed good linearity ($r^2 > 0.99$).

Data processing

GraFit software (version 5.0; Erithacus Software, Surrey, UK) was used to determine the K_m and V_{max} values by nonlinear regression analysis of initial transport rates as a function of substrate concentration. To determine the K_i and IC_{50}, the data was fitted to an appropriate equation by nonlinear regression analysis with simple weighting using the GraFit software. The calculation of K_i value was based on the following equation:

$$v = V_{max} \cdot S/[K_m(1 + I/K_i) + S] \quad (4)$$

where v represents the difference in the transport of compound transported by transfected cells and the mock cells $pmol \cdot min^{-1}$ per mg protein, I and S are the concentration of inhibitor (μM) and substrate (μM), respectively. The IC_{50} for inhibition of transport activity obtained from a plot of percentage activity remaining (relative to control) versus $\log_{10}$ inhibitor

concentration. Plasma pharmacokinetic parameters were estimated by noncompartmental analysis using Thermo Kinetica software package (version 5.0; InnaPhase, Philadelphia, PA, USA). The area under concentration-time curve (AUC_{0-t}) up to the last measured point in time was calculated using the trapezoidal rule. The value of $AUC_{0-\infty}$ was generated by extrapolating AUC_{0-t} to infinity using the elimination rate constant k and the last measured concentration C_t. The total plasma clearance ($CL_{tot,p}$) was estimated by dividing the dose by the $AUC_{0-\infty}$. The distribution volume at steady state (V_{SS}) was estimated by multiplying the $CL_{tot,p}$ by the mean residence time (MRT). The hepatobiliary excretory clearance (CL_B) or the renal excretory clearance (CL_R) was calculated by dividing the biliary or urinary cumulative amount excreted ($Cum.A_{e,0-t}$), respectively, by the plasma AUC_{0-t}. The fraction of administered ginsenoside excreted into bile (f_{e-B}) or urine (f_{e-U}) was obtained by dividing the biliary or urinary $Cum.A_{e,0-t}$ by the dose. The drug-drug interaction (DDI) index was estimated as the ratio of the maximal plasma unbound concentration after therapeutic dosing of ginsenosides divided by the *in vitro* OATP1B-specific IC_{50} (Giacomini *et al.*, 2010; Wang *et al.*, 2013). The accumulative factor (R) was calculated using the following equation:

$$R = 1/(1- e^{-k\tau}) \quad (5)$$

where k represents the elimination rate constant ($0.693/t_{1/2}$) and τ is the dosing interval (h).

All data are expressed as the mean ± standard deviation (SD). Statistical analysis was performed using IBM SPSS Statistics Software (version 19.0; IBM, Somers, NY, USA). A value of $P \leq 0.05$ was considered to be the minimum level of statistical significance.

Chemicals and Materials

Ginsenosides Rg_1, Re, Rb_1, Rc and Rd and notoginsenoside R_1 were obtained from the National Institutes for Food and Drug Control (Beijing, China). The purity of these test compounds exceeded 98%. Rifampin (rifampicin; ≥97%), oestrone-3-sulfate (E_1S; potassium salt; ≥98%), oestradiol-17β-D-glucuronide ($E_2$17βG; sodium salt; ≥98%), taurocholic acid (TCA; sodium salt hydrate; ≥95%), methotrexate (MTX; ≥99%), poly-D-lysine hydrobromide (70,000–150,000 Da) and adenosine 5′-triphosphate (ATP; disodium salt hydrate, ≥99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used for *in vitro* studies. Rifampin that was used in animal studies was freeze-dried solid available as a sterile parenteral dosage form for i.v. injection and manufactured by Huapont Pharm. (Chongqing, China) with a China Food and Drug Administration ratification number of GuoYaoZhunZi-H20041320. HPLC-grade organic solvents were purchased from Sinopharm Chemical Reagent (Shanghai, China). HPLC-grade water was prepared with a Millipore Direct-Q 3 UV water purification system (Bedford, MA, USA).

HEK-293 cells were obtained from American Type Culture Collection (Manassas, VA, USA). Inside-out membrane vesicle suspensions that expressed human MRP2, MDR1, BCRP, BSEP and rat Mrp2, Bcrp and Bsep were obtained from Genomembrane (Kanazawa, Japan). Inside-out membrane vesicle suspensions that expressed rat Mdr1a or rat Mdr1b were obtained from BD Gentest (Woburn, MA, USA).

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