Enhancement of Oral Bioavailability of 20(S)-Ginsenoside Rh2 through Improved Understanding of Its Absorption and Efflux Mechanisms

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ABSTRACT:

The development of 20(S)-ginsenoside Rh2 (Rh2s) as a chemoprevention agent is limited by its low oral bioavailability. The goals of this study were to determine the mechanisms responsible for its poor oral absorption and to improve its bioavailability by overcoming the barrier to its absorption. Comprehensive studies were conducted using the following models: 1) monolayers of Caco-2, parental, and multidrug resistance gene (MDR1)-overexpressing Madin-Darby canine kidney II (MDCKII) cells; 2) pharmacokinetics in wild-type (WT) FVB mice, MDR1a/b knockout [MDR1a/b(–/–)] FVB, and A/J mice; and 3) intestinal perfusion in WT, MDR1a/b(–/–) FVB mice, and A/J mice. Two P-glycoprotein (P-gp) inhibitors, verapamil and cyclosporine A, substantially decreased the efflux ratio of Rh2s from 28.5 to 1.0 and 1.2, respectively, in Caco-2 cells. The intracellular concentrations of Rh2s were also significantly increased (2.3- and 3.9-fold) in the presence of inhibitors. Similar results were obtained when transcellular transport of Rh2s were determined using MDR1-overexpressing MDCKII cells in the absence or presence of cyclosporine A. Compared with WT mice, the plasma Cmax and AUC0–∞ of Rh2s were substantially increased by 17- and 23-fold in MDR1a/b(–/–) FVB mice, respectively. In the A/J mice, the oral bioavailability of Rh2s (0.94% at 5 mg/kg and 0.52% at 20 mg/kg) was substantially increased by P-gp inhibitor to 33.18 and 27.14%, respectively. As expected, deletion or inhibition of P-gp significantly increased absorption and steady-state plasma concentration of Rh2s in a mouse intestinal perfusion model. In conclusion, Rh2s is a good substrate of P-gp, and inhibition of P-gp can significantly enhance its oral bioavailability.

Introduction

Ginseng is a promising candidate for cancer chemoprevention based on preclinical and epidemiological studies (Yun, 1996; Helms, 2004; Varjasi et al., 2009). In a case-controlled study including 1987 pairs of Korean subjects, the long-term consumption of ginseng was shown to be associated with a significant reduction in many different types of malignancies including lung cancer (Yun, 2003). Ginsenosides are the main active components of ginseng, and there are more than 100 ginsenosides identified so far (Christensen, 2009). The chemoprevention and anticancer mechanism of ginsenosides include mitigation of DNA damage, induction of apoptosis, inhibition of proliferation, and positive immunomodulation (Helms, 2004).

However, extensive pharmacokinetic studies indicated that many active ginsenosides have very poor oral bioavailability (much less than 5%), which has been attributed to poor oral absorption (Gu et al., 2009; Liu et al., 2009). Poor bioavailability of ginsenosides greatly impedes our ability to demonstrate the potency of ginsenosides in vivo, and our ability to overcome this impediment perhaps holds the key to advance these agents into clinical trials that will unequivocally demonstrate their clinical effectiveness (Coleman et al., 2003; Buettner et al., 2006; Jia et al., 2009).

In this study, 20(S)-ginsenoside Rh2 (Rh2s) was selected as a lead compound to demonstrate the absorption mechanisms of ginsenosides. Ginsenoside Rh2s is one of the most studied ginsenosides because it displays potent anticancer activity, especially in lung cancer cell lines (Cheng et al., 2005; Wang et al., 2006). A 9-week animal study also showed that Rh2s had a tendency to decrease lung tumor incidence in mice after its oral consumption (Yun, 2003). Like many other ginsenosides, Rh2s is a good substrate of P-gp, and inhibition of P-gp can significantly enhance its oral bioavailability.

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cesolgosides, Rh2s was reported as having low oral absorption/bioavailability (Qian et al., 2005; Gu et al., 2009). However, current understanding of the Rh2s absorption mechanism is ambiguous. Gu et al. (2010) reported that ATP-binding cassette efflux transporters May be involved in Rh2s absorption, but no actual transporter was identified. The latest publication from the same group found that Rh2 was a noncompetitive inhibitor but not a substrate of P-glycoprotein (P-gp) (Zhang et al., 2010). Therefore, identification of the predominant transporter for Rh2s and demonstration that the inhibition of relevant efflux transporters would increase oral bioavailability of Rh2s (and maybe other ginsenosides) will help us to further understand and delineate this class of compounds’ pharmacological characteristics.

P-gp, a member of the ATP-binding cassette superfamily, is one of the most prevalent efflux transporters expressed in multidrug resistance cancer cells and in several organs such as intestine, liver, kidney, and the blood-brain barrier (Sharom, 2008). P-gp plays an important role in limiting the intestinal absorption of its substrates in vivo (Kusuhara and Sugiyama, 2002), and inhibition of P-gp leads to the improvement of bioavailability of several orally administered anticancer drugs (Meerum Terwogt et al., 1998; Kemper et al., 2004; van Waterschoot et al., 2009). Most P-gp substrates are hydrophobic, and a recently published mouse P-gp crystal structure revealed that P-gp has distinct drug-binding sites (in the large internal cavity) favoring hydrophobic and aromatic interactions (Aller et al., 2009).

Therefore, the aims of this study were 1) to systematically investigate mechanisms responsible for poor absorption of Rh2s by elucidating which efflux transporter was mainly involved in the transport of Rh2s using a complementary set of in vitro, in situ, and in vivo models and 2) to demonstrate that it is possible to increase oral bioavailability of Rh2s via a mechanism-based approach (i.e., focus on inhibiting the elucidated efflux transporter derived from aim 1).

Materials and Methods

Chemicals and Reagents. Purified Rh2s (high-performance liquid chromatography purity >95%; Fig. 1) was a kind gift from Dr. M. Rousset (INSERM U178, Villejuif, France). Parental MDCKII and MDR1-MDCKII cells were provided by the Netherlands Cancer Institute (Amsterdam, Netherlands). Digoxin, cycloporsine A (Cxa), verapamil, P-gp, and MDR1-MDCKII cells were provided by the Netherlands Cancer Institute from Dr. M. Rousset (INSERM U178, Villejuit, France). Parental MDCKII cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and quality control criteria were all implemented according to a previous published report (Yang et al., 2010a). Caco-2 T7C cells were fed every other day, and cell monolayers were ready for experiments from 19 to 22 days after seeding.

Parental MDCKII and MDR1-MDCKII cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 100 U/ml penicillin, and gentamycin. Cell culture experiments from 19 to 22 days after seeding. The MDCKII or MDR1-MDCKII cell monolayers were ready for experiments from 4 to 5 days after seeding. The expression levels of MDR1 in MDR1-MDCKII were monitored by Western blotting analysis.

Saturated Aqueous Solubility Measurement. Ginsenoside Rh2s is a strong lipophilic (log P = 4.6, predicted by Molecular Operating Environment, version 8.01; Chemical Computing Group, Montreal, Canada) compound, and its solubilities in various media had not been reported. HBSS buffer was commonly used in absorption models including the Caco-2 cells and in the in situ rodent intestinal perfusion study (Yang et al., 2010b). The saturated aqueous solubility was measured in HBSS using pH values ranging from pH 5.5 to pH 8.0. The solubility of Rh2s was also determined in pure water at pH 7.4 and in simulated intestinal fluid (SIF) at pH 6.8 to mimic the in vivo environment. Stock solution of ginsenoside Rh2s was prepared in alcohol at 10 mM. Ten microliters of Rh2s was spiked into glass vials in triplicate and dried under air. One volume of 1 mL of HBSS/water/SIF at appropriate pH values was then added into a glass vial, and the mixture was shaken overnight at 37°C. After the mixture was centrifuged at 15,000 rpm for 20 min, 400 μL of supernatant was then carefully transferred to a new vial. The vial was then added with 100 μL of formononetin (10 μM) as the internal standard and dried under air. The residue was reconstituted with 200 μL of methanol and centrifuged at 15,000 rpm for 10 min. One volume of 10 μL of supernatant was injected to UPLC-MS/MS for analysis.

Transcellular Transport Study. The transcellular transport study was performed as described previously (Yang et al., 2010a). In brief, 2.5 mL of Rh2s solution was loaded onto one side of the cell monolayer, and 2.5 mL of blank HBSS was loaded onto the other side. Five sequential samples (0.5 mL) were taken at different times (0, 1, 2, 3, and 4 h) from both sides of the cell monolayer. The same volume of Rh2s solution and receiver medium (fresh HBSS) was added immediately to replace the volume lost because of sampling. The pH values of HBSS in both the apical and basolateral sides were 7.4. The apparent unidirectional permeability was obtained according to the equation $P_{app} = \frac{dC_{av}}{dV_{c}}\times V_{ch}$, where $dC_{av}/dV_{c}$ is the rate of concentration change in the receiver chamber (equals the slope of the regression line derived for the amount transported versus time profile), V is the chamber volume (2.5 mL). S is the surface area of the monolayer (4.65 cm²), and $C_{av}$ is the starting concentration in the donor side.

Both permeability from the apical to basolateral side ($P_{ab}$) and basolateral to apical side ($P_{ba}$) were calculated according to the above equation. Digoxin, a substrate of P-gp, was used as the positive control for the transport study in Caco-2 and MDR1-MDCKII cell monolayers. The efflux ratio was calculated as $P_{ba}/P_{ab}$, which represented the degrees of efflux transporter involvement. In the present study, inhibitors of efflux transporters were only added to the apical side of cell monolayers, regardless of where Rh2s was loaded.

Intracellular concentrations of Rh2s were determined at the end of a transport study. The protocol for determining Rh2s intracellular amounts in cells was the same as those described previously (Jeong et al., 2005). In brief, after 4 h of a transcellular transport study, the cell membranes were rinsed three times with HBSS, then cells were lysed with formic acid (100 μL of 5% formic acid in methanol). The concentration of Rh2s in the cell lysate was determined by high-performance liquid chromatography as described previously.

Animals. Male A/J and FVB mice (6–10 weeks old) were purchased from Harlan Laboratories (Indianapolis, IN). Male MDR1a/b knockout mice (6–10 weeks old) were purchased from Taconic Farms (Germantown, NY). They were acclimated in an environmentally controlled room (temperature, 25 ± 2°C; humidity, 50 ± 5%; 12-h dark/light cycle) for at least 1 week before experiments. The mice were fed with rodent diet (Labdiet 5001) and fasted overnight before pharmacokinetic studies.

Cell Culture. The Caco-2 cell culture has been routinely maintained in this laboratory for the last 2 decades. The culture conditions for growing Caco-2 cells were the same as those described previously (Yang et al., 2010a). Porous polycarbonate cell culture inserts (3 μm) from Corning Life Sciences (Lowell, MA) were used to seed the cells at a seeding density of 10⁴ cells/cm². Other conditions such as growth medium (Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum) and quality control criteria were all implemented according to a previous published report (Yang et al., 2010a). Caco-2 T7C cells were fed every other day, and cell monolayers were ready for experiments from 19 to 22 days after seeding.

Parental MDCKII and MDR1-MDCKII cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 100 U/ml penicillin, and gentamycin. Cell culture conditions for growing Caco-2 cells and fed every day. The MDCKII or MDR1-MDCKII cell monolayers were ready for experiments from 4 to 5 days after seeding. The expression levels of MDR1 in MDR1-MDCKII were monitored by Western blotting analysis.

![Fig. 1. Chemical structure of 20(S)-ginsenoside Rh2 (molecular weight, 622.87).](image-url)
times with ice-cold HBSS buffer, and cells attached to the polycarbonate membranes were cut off from the inserts with a sharp blade. The latter was immersed into 1 ml of HBSS and sonicated for half an hour at 4°C to break up the cells. The mixture was centrifuged at 15,000 rpm for 15 min, and 500 μl of the supernatant was recovered and air dried. The residue was reconstituted with 200 μl of methanol and analyzed by UPLC-MS/MS. The protein concentration of cell lysate was measured to normalize the accumulation of Rh2s inside the cells using the BCA protein assay kit.

In Situ Two-Site Mouse Intestinal Perfusion Study. The animal protocols used in this study were approved by the Institutional Animal Care and Use Committee at the University of Houston. The intestinal surgical procedures were described in our previous publications (Jeong et al., 2005). Two segments of intestine, upper small intestine and colon (8–12 cm each), were simultaneously cannulated. The perfusion studies were performed at a flow rate of 0.191 ml/min with 2 μM Rh2s in HBSS iso-osmotic solution, pH 7.4, using A/J, WT, and MDR1a/b(-/-) FVB mice. Cyclosporine A solution (10 μM) was mixed with 2 μM Rh2s in A/J mice to test the effects of P-gp inhibitors on Rh2s transport. To keep the temperature of the perfusate constant, the inlet cannulate was insulated and kept warm by a 37°C circulating water bath.

After a 30-min washout period with the perfusate, perfusate samples were collected every 30 min (60, 90, 120, 150 min). At the end of the perfusion experiment, the length of the each segment of intestine was measured, and each tube containing sample was weighted after perfusion. Fifty microliters of blood was collected from tail vein at the end of the perfusion experiment (2.5 h after the start). Loss of water during perfusion was measured by monitoring the net weight of the perfusate sample, and the absorption data were discarded if the water flux was more than 5% per 10 cm for the small intestine and 10% per 10 cm for the colon. The perfusate samples were immediately processed the same way as the cellular transport samples and analyzed by UPLC-MS/MS. The plasma concentrations of Rh2s were measured following the same protocol in the pharmacokinetic study. The percentages of absorption were calculated using the following equation: Absorption % = (1 - Cint/Cin) × 100, where Cin and Cin are outlet and inlet perfusion concentrations of Rh2s after correcting for water fluxes, respectively.

Stability of Rh2s in blank perfusate was measured to ensure that the concentration that disappeared from the intestinal perfusate was due to absorption. Rh2s was spiked into fresh blank perfusate (final concentration, 2 μM) collected from the upper small intestine and colon of A/J mice and immediately put into a rotating water bath (40 rpm) at 37°C to mimic in vivo conditions. After 0, 1, 2, 4, and 8 h, 0.5 ml of the incubated fluids was collected, and their concentrations were determined as described above.

Pharmacokinetic Studies of Rh2s in Wild-Type and MDR1a/b(-/-) FVB Mice. Pharmacokinetic studies of Rh2s were performed in wild-type and MDR1a/b(-/-) FVB mice to confirm the role of MDR1/P-gp in determining Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle (Supplemental Table 1) was given by gavage to each group at 20 mg/kg. Each pharmacokinetic study was performed on five mice, and 10 timed blood samples (20–25 μl) were taken by snipping its tail, after mice were anesthetized with isoflurane gas. The blood samples were collected in heparin-treated tubes and stored at −20°C until analysis.

Pharmacokinetic Studies of Rh2s Alone or with Cyclosporine A in A/J Mice. Pharmacokinetic studies of Rh2s were performed in A/J mice for both intravenous and oral administrations. Rh2s (1 mg/ml) was prepared in 20% alcohol and 20% propylene alcohol in normal saline and was injected intravenously through the lateral tail vein at the dose of 5 mg/kg. In oral dosing groups without cyclosporine A (control group), 4 mg/ml Rh2s was dispersed in oral suspending vehicle, and the content of Rh2s in oral suspending vehicle was measured before administration to make sure it was evenly suspended. The ingredients of oral suspending vehicle are shown in Supplemental Table 1. In the group with coadministration of P-gp inhibitors, 50 mg/kg solid cyclosporine A dispersed in the same oral suspending vehicle was orally administered to A/J mice 30 min before Rh2s administration, whereas the same volume of blank vehicle was given to the control group. The blood sample collection and processing procedures used for A/J mouse samples were the same as those described for the FVB mouse pharmacokinetic study.

Sample Processing and Quantitative Determination of Rh2s. An API 3200 Qtrap triple quadrupole mass spectrometer (Applied Biosystems/MDS Sciex, Foster City, CA) equipped with a Turboionspray source, operated in a negative ion mode, was used to perform the analysis of the eluent from the UPLC. The flow-dependent parameters for introduction of the samples to the mass spectrometer ionization source were set as follows: ion spray voltage, −4.5 kV; ion source temperature, 650°C; nebulizer gas (gas1), nitrogen, 50 ps.i.; turbo gas (gas2), nitrogen, 50 ps.i.; curtain gas, nitrogen, 10 ps.i.

The quantification was performed using the multiple-reactions monitoring mode with ion pair transitions to monitor Rh2s and formononetin (internal standard). The fragments for each compound detected were 621.4/160.9 (m/z) for Rh2s and 267.1/252.1 (m/z) for formononetin.

UPLC conditions for Rh2s analysis were as follows: system, Acquity Waters, Milford, MA) with a diode array detector; column, Acquity UPLC BEH C18 column (50 × 2.1 mm i.d., 1.7 μm; Waters); mobile phase A, double-distilled water; mobile phase B, 100% methanol; gradient, 0 to 0.5 min, 0% B, 0.5 to 1 min, 0 to 80% B, 1 to 2.3 min, 80 to 95% B, 2.3 to 2.9 min, 95% B, 2.9 to 3.2 min, 95 to 0% B, 3.2 to 3.7 min, 0% B; flow rate, 0.45 ml/min; column temperature, 60°C; injection volume, 10 μl. The chromatograph of Rh2s and internal standard is shown in Supplemental Fig. 1.

The standard curves of Rh2s were linear in the concentration range of 0.0195 to 10 μM, and the lower limit of quantification was 0.00975 μM in blood. The intraday and interday precision was within 15% for all quality control samples at three concentration levels (2.5, 0.3125, and 0.039 μM). The mean extraction recoveries determined using three replicates of quality control samples at three concentrations in mouse blood were from 55.8 to 67.0%.

The stability of Rh2s in mouse blood was evaluated by analyzing three replicates of quality control samples after short-term (25°C, 4 h), postprocessing (20°C, 8 h), and long-term (−20°C, 7 days) ice-cold storage and within three freeze-thaw cycles (−20 to 25°C). All the samples displayed 85 to 115% recoveries after various stability tests.

Quantification of digoxin (positive control in Rh2s transport studies) was performed in UPLC-MS/MS with the same instrumental conditions used for analysis of Rh2s. The detailed compound-dependent mass spectrometer parameters were shown in Supplemental Table 2.

Before samples could be introduced into the UPLC, they were processed to remove proteins and other related substances. One volume of 200 μl of methanol (containing 1 μM formononetin) was added into a 20-μl aliquot of mouse blood sample. The sample mixtures were vortexed for approximately 30 s, and precipitates were removed by centrifugation at 15,000 rpm for 15 min. The supernatant was transferred into a clean glass vial and evaporated to dryness at 40°C under air. The dry residue was reconstituted in 100 μl of 100% methanol (v/v), and a 10-μl aliquot of the resulting solution was injected into the UPLC-MS/MS system for analysis.

Pharmacokinetic Analysis. WinNonlin 3.3 (Pharsight, Mountain View, CA) was used for Rh2s pharmacokinetic analysis. The noncompartmental model was applied for pharmacokinetic analysis of Rh2s profiles. Pharmacokinetic parameters, including Cmax, Tmax, ke, half-life, and AUC, were directly derived from WinNonlin. The absolute oral bioavailability (F) was calculated by the following equation: F% = [(Doseo, AUCo, AUCo, AUCo, AUCo) × 100].

The clearance and apparent volume of distribution at steady state after oral administration were corrected from the original CL (CLo) and Vd (Vd), obtained from WinNonlin using the F values, as shown in the equations for CL = F × CLo/100 and Vd = F × Vd/100, where Vd is apparent volume of distribution at steady state and CL is clearance.

Statistical Analysis. The data in this study were presented as means ± S.D., if not specified otherwise. Significance differences were assessed by using Student’s t test or one-way analysis of variance. A p < 0.05 was considered as statistically significant.

Results

Saturated Aqueous Solubility. We measured the saturated aqueous solubility of Rh2s in HBSS at different pHs. The results showed that Rh2s had low aqueous solubilities in HBSS buffer (1.9 ± 0.2, 2.0 ± 0.2, 2.9 ± 0.2, and 2.4 ± 0.4 μg/ml) at four tested pH values (5.5, 6.5, 7.4, and 8.0, respectively). There was no significant difference in solubility at different pH values according to a one-way
analysis of variance. The concentrations of Rh2s in saturated solutions were 5.4 ± 0.4 μg/ml in pure water, pH 7.4, and 7.2 ± 2.5 μg/ml in simulated intestinal fluid, pH 6.8, respectively. Consistent with the solubility results, we found visible precipitates in the HBSS samples when trying to reach concentrations above 10 μM (6.22 μg/ml) after centrifugation. In contrast, the solutions were clear in the blank HBSS buffer or in samples with lower concentrations of Rh2s. The measured solubility of Rh2s in those three matrices indicated that Rh2s has a limited aqueous solubility, which also limits the maximal concentration that could be used for in vitro transcellular transport studies using the cellular models.

**Transcellular Transport of Rh2s across Caco-2 Cell Monolayers.** Before the transport study was performed, the transcellular transport of a prototypical substrate of P-glycoprotein (digoxin) was used as the positive control. The results showed that transcellular transport of digoxin displayed a significant efflux ratio (21.1) and $P_{ab}$ was 0.74 × 10^{-6} cm/s, which was similar to the published results (Xu et al., 2003). In the presence of 20 μM cyclosporine A, the efflux ratio of digoxin was decreased to 1.2.

As expected, transcellular transport of Rh2s (2 μM) across the Caco-2 monolayer from the basolateral to apical side was significantly higher than the transport from the apical to basolateral side, and the efflux ratio was decreased to close to 1. As expected, the $P_{ab}$ was significantly increased by 9- and 4-fold and the $P_{ba}$ was significantly increased by 3- and 6-fold after verapamil and cyclosporine A treatment, respectively (Table 1). Consistent with the permeability results, two P-glycoprotein inhibitors also significantly increased the intracellular accumulation of Rh2s after treatment with 50 μM verapamil (from 0.14 to 0.51 nmol/mg) or 20 μM cyclosporine A (from 0.14 to 0.54 nmol/mg).

Because there are three main efflux transporters (P-gp, MRP2, and BCRP) present at the apical membrane of the Caco-2 cells, chemical inhibitors of MRP2 (20 μM MK571) and BCRP (5 μM Ko143) were also used in the transport study to determine their possible involvement. The inhibitors and inhibitor concentrations were chosen based on the reported $K_i$ (constant of inhibition) value of 5.6 μM for MK571 (Walgren et al., 2000) and 0.6 μM for Ko143 (Brand et al., 2008) in the Caco-2 cells. Using the efflux ratio as the indicator of efflux transporter function, neither of these inhibitors was effective (Table 1). Likewise, neither Ko143 nor MK571 increased the intracellular accumulations of Rh2s, in line with the absence of the role of MRP2 or BCRP in limiting the uptake of Rh2s into Caco-2 cells (Table 1). Time-dependent transcellular transport of 2 μM Rh2s across monolayers of Caco-2 cells with different inhibitors is also presented in Supplemental Fig. 2.

**Transcellular Transport of Rh2s in MDR1-MDCKII Cells.** Human MDR1/P-gp-overexpressing MDCKII cells were used to confirm the predominant role of P-gp in the transport of Rh2s. Before the transport studies of Rh2s, 2 μM digoxin was used as a positive control in the MDR1-MDCKII cell transport study. The efflux ratio of digoxin was 64.0 in MDR1-MDCKII cells, and the $P_{ab}$ was 2.98 × 10^{-6} cm/s, which was similar to the ratio reported in a previous publication (Taub et al., 2005).

The transport of Rh2s in parental MDCKII cells was used as a negative control because the cells have very low expression of P-gp (Shirasaka et al., 2009). As expected, the efflux ratio of Rh2s was much lower in parental MDCKII cells compared with MDR1-MDCKII cells at 2 μM (28.0 versus 2.4). Likewise, the $P_{ab}$ of Rh2s was more than 7-fold higher in the parental MDCKII cells (4.97 × 10^{-6} cm/s) than MDR1-MDCKII cells (0.68 × 10^{-6} cm/s).

Four different concentrations of Rh2s were used in the bidirectional transport study in MDR1-MDCKII cells, and the efflux ratio was 65.7, 28.0, 12.4, and 6.7 at 1, 2, 3, and 5 μM, respectively (Table 1). The significantly decreased efflux ratios observed with increasing donor concentration of Rh2s (p < 0.05) was due to an increased $P_{ba}$ value from 0.49 × 10^{-6} to 3.51 × 10^{-6} cm/s (p < 0.01) and decreased $P_{ab}$ value from 32.77 × 10^{-6} to 23.5 × 10^{-6} cm/s. Similar to the results of inhibition studies in Caco-2 cells, the presence of 20 μM cyclosporine A substantially decreased $P_{ba}$ (by 16-fold) and the efflux ratio (from 28.0 to 1.2) (Table 1).

The intracellular accumulations of Rh2s were also measured, and the results showed that Rh2s accumulation was significantly higher (8.5-fold) in parental MDCKII cells (0.25 nmol/mg) than in MDR1-MDCKII cells (0.03 nmol/mg). The intracellular transporter function, neither of these inhibitors was effective (Table 1). Likewise, neither Ko143 nor MK571 increased the intracellular accumulations of Rh2s, in line with the absence of the role of MRP2 or BCRP in limiting the uptake of Rh2s into Caco-2 cells (Table 1). Time-dependent transcellular transport of 2 μM Rh2s across monolayers of Caco-2 cells with different inhibitors is also presented in Supplemental Fig. 2.

**TABLE 1**

Transcellular transport of Rh2s across monolayers of Caco-2, MDCKII, and MDR1-MDCKII cells in the absence or presence of inhibitors

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<tr>
<th>Cell Model and Substrate Concentration</th>
<th>Inhibitor</th>
<th>Inhibitor Concentration</th>
<th>$P_{ba}$</th>
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<th>Efflux Ratio ($P_{ba}/P_{ab}$)</th>
<th>Intracellular Amounts</th>
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<td>μM</td>
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<td>Caco-2 2 μM</td>
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<td>MK571 20 μM</td>
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<td>MK571 20 μM</td>
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* p < 0.05.
** p < 0.01.
*** p < 0.001.
centrations and in the absence or presence of CsA is presented in Supplemental Fig. 3.

Effects of MDR1 on the Oral Bioavailabilities of Rh2s. To investigate whether MDR1/P-gp has major effects on Rh2s oral bioavailability, plasma profiles of Rh2s were compared between MDR1a/b(−/−) and wild-type FVB mice after oral administration (20 mg/kg). In MDR1a/b(−/−) mice, the plasma $C_{max}$ was significantly increased by 17-fold ($p < 0.01$), and AUC$_{0\rightarrow\infty}$ was significantly increased by 23-fold ($p < 0.001$) compared with wild-type FVB mice (Fig. 2; Table 2).

In MDR1a/b(−/−) mice, the elimination rate constant, $K_e$, was significantly decreased by 70% (from 0.50 ± 0.25 to 0.15 ± 0.02 h$^{-1}$, $p < 0.05$) compared with wild-type FVB mice. The terminal half-life ($t_{1/2}$) of Rh2s was significantly increased by 2.5-fold (from 1.85 ± 1.27 to 4.60 ± 0.71 h, $p < 0.01$). The decreased $K_e$ and increased $t_{1/2}$ could be attributed to the decreased elimination of Rh2s as a result of the knockout of the MDR1 gene (Table 2).

Effects of Cyclosporine A on Rh2s Oral Bioavailabilities in A/J Mice. Coadministration of the P-gp inhibitor, cyclosporine A (50 mg/kg), with Rh2s significantly increased the plasma $C_{max}$ and AUC$_{0\rightarrow\infty}$ of Rh2s at both 5 and 20 mg/kg (Fig. 3). The plasma $C_{max}$ was significantly increased ($p < 0.01$) by 14-fold at 5 mg/kg and by 38-fold at 20 mg/kg. The plasma AUC$_{0\rightarrow\infty}$ was significantly increased ($p < 0.001$) by 36-fold at 5 mg/kg and 52-fold at 20 mg/kg. Pharmacokinetic parameters of Rh2s after intravenous administration were shown in Table 2, and the plasma AUC$_{0\rightarrow\infty}$ from intravenous administration was used to calculate the absolute oral bioavailability for Rh2s. The absolute oral bioavailability of Rh2s was substantially increased by 36-fold (from 0.94 to 33.18%, $p < 0.001$) and 52-fold (from 0.52 to 27.14%, $p < 0.001$) at 5 and 20 mg/kg, respectively.

Coadministration of cyclosporine A also decreased $K_e$ by 2.8-fold (from 0.48 ± 0.25 to 0.17 ± 0.04 h$^{-1}$, $p < 0.05$) and 2-fold (from 0.32 ± 0.30 to 0.16 ± 0.03 h$^{-1}$) at 5 and 20 mg/kg, respectively. The half-life of Rh2s consistently increased 78% (from 1.78 ± 0.90 to 4.41 ± 1.05 h, $p < 0.05$) and 36% (from 3.37 ± 1.78 to 4.60 ± 1.32 h) at 5 and 20 mg/kg, respectively (Table 2).

Coadministration of cyclosporine A also increased $V_m$ from 0.26 ± 0.24 to 0.49 ± 0.10 l/kg ($p < 0.05$) and 0.38 ± 0.20 to 0.51 ± 0.12 l/kg ($p = 0.2$) at 5 and 20 mg/kg, although clearance of Rh2s was not changed after cyclosporine A treatment (Table 1).

In Situ Intestinal Perfusion Study of Rh2s. An intestinal perfusion study was performed to investigate intestinal absorption of Rh2s in A/J mice and MDR1 knockout FVB mice. Stability studies showed that Rh2s is stable in the intestinal perfusate for up to 8 h (Supplemental Fig. 4). Because the UPLC-MS/MS assay has an approximate 10% S.D. based on our validation data, accurate determination of percentage absorption was difficult if the absorption was poor (i.e., less than 10%). Percentage absorption of Rh2s was higher in MDR1a/b(−/−) mice (64 and 14%) compared with WT FVB mice (<10%) in the upper small intestine and colon, respectively. Likewise, percentage absorption of Rh2s increased from <10 to 49.4% ($p < 0.05$) in the upper small intestine and from <10 to 19.8% in the colon in the presence of 10 μM cyclosporine A in A/J mice (Fig. 4A). The steady-state plasma concentration of Rh2s was consistently significantly higher (0.80 μM) in MDR1a/b(−/−) mice than in WT FVB mice (0.13 μM) at the end of a 2.5-h intestinal perfusion study. The steady-state plasma concentration of Rh2s was significantly increased by ~9-fold (from 0.12 to 1.07 μM, $p < 0.05$) in the presence of 10 μM cyclosporine A in A/J mice (Fig. 4B).

Discussion

This is the first comprehensive study that demonstrates unequivocally that Rh2s is a good substrate of P-gp and that P-gp mediates the efflux of Rh2s in vitro and in vivo. Although poor oral absorption of ginsenosides has been reported extensively, the results were mostly

<table>
<thead>
<tr>
<th>Animal Strain &amp; Dose</th>
<th>$T_{max}$ (h)</th>
<th>$C_{max}$ (μM)</th>
<th>$k_e$ (h$^{-1}$)</th>
<th>$t_{1/2}$ (h)</th>
<th>$V_m$ (l/kg)</th>
<th>CL (l/h)</th>
<th>AUC$_{0\rightarrow\infty}$ (h·μM)</th>
<th>$F$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/J</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>5 mg/kg</td>
<td>2.80 ± 0.64</td>
<td>0.31 ± 0.30</td>
<td>0.40 ± 0.09</td>
<td>1.77 ± 0.37</td>
<td>0.36 ± 0.22</td>
<td>0.13 ± 0.06</td>
<td>98.52 ± 45.58</td>
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</tr>
<tr>
<td>5 mg/kg</td>
<td>6.50 ± 1.91*</td>
<td>4.46 ± 1.20**</td>
<td>0.17 ± 0.04*</td>
<td>4.41 ± 1.05**</td>
<td>0.49 ± 0.10*</td>
<td>0.08 ± 0.00</td>
<td>32.69 ± 4.92***</td>
<td>33.18 ± 4.99***</td>
</tr>
<tr>
<td>20 mg/kg</td>
<td>4.17 ± 1.60</td>
<td>0.28 ± 0.10</td>
<td>0.32 ± 0.30</td>
<td>3.37 ± 1.78</td>
<td>0.38 ± 0.20</td>
<td>0.08 ± 0.00</td>
<td>2.04 ± 1.50</td>
<td>0.52 ± 0.38</td>
</tr>
<tr>
<td>Wild-type FVB</td>
<td>4.60 ± 1.34</td>
<td>10.66 ± 1.35***</td>
<td>0.16 ± 0.03</td>
<td>4.60 ± 1.32</td>
<td>0.51 ± 0.12</td>
<td>0.08 ± 0.00</td>
<td>106.97 ± 26.02***</td>
<td>27.14 ± 6.60***</td>
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<tr>
<td>20 mg/kg</td>
<td>3.04 ± 2.70</td>
<td>0.35 ± 0.36</td>
<td>0.50 ± 0.25</td>
<td>1.85 ± 1.27</td>
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<tr>
<td>FVB</td>
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</tr>
<tr>
<td>20 mg/kg</td>
<td>4.25 ± 2.50</td>
<td>6.01 ± 1.89**</td>
<td>0.15 ± 0.02*</td>
<td>4.60 ± 0.71*</td>
<td></td>
<td></td>
<td></td>
<td>49.90 ± 3.20***</td>
</tr>
</tbody>
</table>

* Intravenous administration.

$ * p < 0.05.$

$ ** p < 0.01.$

$ *** p < 0.001.$
The dominant role of P-gp in limiting the oral bioavailability of Rh2s was further demonstrated by the facts that oral bioavailability of Rh2s was substantially increased (from 1 to >30%) in A/J mice after cyclosporine A coadministration (Table 2) and that there were substantial increases in plasma $C_{\text{max}}$ (17.2-fold) and AUC$_{0-\infty}$ (23.0-fold) in MDR1a/b(−/−) mice compared with WT FVB mice. The improved bioavailability of Rh2s in the presence of a P-gp inhibitor was mainly attributed to enhanced intestinal absorption in mice based on the intestinal perfusion study (Fig. 4). Mechanisms responsible for increased absorption were likely to be increased permeability and elevated intracellular accumulations, as shown in both Caco-2 and MDRI-MDCKII cell models (Table 1). The improved bioavailability could also be contributed by decreased elimination, as indicated by a slower elimination rate constant ($k_e$) and a prolonged terminal half-life.

The extremely poor oral bioavailability of Rh2s (<1%) was consistent with its low solubility and low permeability observed in this study, suggesting that Rh2s belongs to the class IV compounds in the Biopharmaceutics Classification System (Dahan et al., 2009). However, the apparent low permeability of Rh2s is due to efflux by P-gp, because its intrinsic permeability (passive diffusion) is promising with the highest $P_{a,b}$ exceeding $3 \times 10^{-6}$ cm/s (obtained in the presence of P-gp inhibitors) (Table 1). The substantial increase in oral bioavailability of Rh2s in the presence of cyclosporine A to a level above 30% suggests that this compound holds great promise for oral administration (necessary for cancer chemoprevention) if it is properly formulated. Therefore, we have shown that it is possible to derive good oral bioavailability for Rh2s in animals as long as we can inhibit the function of P-gp during absorption.

We have shown that inhibition or deletion of P-gp was the main reason for improved Rh2s bioavailability in mice. In demonstrating the large changes in oral bioavailability of Rh2s, we have seen some minor inconsistencies. For example, the AUC value of Rh2s (20 mg/kg) in the cyclosporine A treatment group in A/J mice was higher than that in the MDR1a/b(−/−) FVB mice, probably because of strain differences in absorption and/or clearance of this compound. Additional factors such as difference in P450 metabolism of Rh2s were also possible. We believed that a major contribution from P450 was less likely because P450 metabolites of Rh2s were never reported, except its hydrolysis product (aglycone) in vivo (Qian et al., 2005). In addition, the large differences in $C_{\text{max}}$ and AUC values between FVB control and MDR1a/b(−/−) FVB mice were comparable with those achieved with P-gp inhibition by cyclosporine A in A/J mice. However, the contribution from P450 cannot be excluded because other ginsenosides with similar structures were reported as substrates of the P450 enzyme (Hao et al., 2010) and cyclosporine A is also a potent inhibitor of P450.

We did not observe an increase in oral bioavailability of Rh2s at the higher dose (in the absence of P-gp inhibitor) as expected because efflux ratios were smaller at higher concentrations (Table 1). We hypothesized that poor solubility was the reason that this enhancement did not occur. This is because ginsenosides with less sugar moiety (e.g., protopanaxadiol, protopanaxtriol, Rh2, and Rg3) usually have lower solubility compared to isolated ginsenosides in general. This is a critical factor that needs to be considered in the oral administration of ginsenosides in the future.
poor aqueous solubilities (Gu et al., 2010). Our solubility study showed the low aqueous solubility of Rh2s in HBSS buffer (~3 µg/ml at pH 7.4), in water (~5 µg/ml at pH 7.4), and in SIF (~7 µg/ml at pH 6.8). The poor aqueous solubility of Rh2s further substantiates the important role of P-gp efflux in vivo because Rh2s cannot fully saturate/inhibit P-gp efflux in lumina of limited and low solubility. This result did not contradict the results reported by Zhang et al. (2010), who showed that Rh2s suspended in sodium carboxymethyl cellulose could act as a P-gp inhibitor at 25 mg/kg in rats, but it did signify that unformulated Rh2s is probably not a very useful P-gp inhibitor in vivo because of solubility limit. Additional studies are planned to determine how solubilization of Rh2s could be used to further increase its bioavailability when used together with a P-gp inhibitor.

This proof-of-principle study demonstrated that oral bioavailability of Rh2s can be enhanced to a clinically acceptable level (30%) if P-gp function is completely inhibited. Because many potent ginsenosides were also shown to be substrates of P-gp in Caco-2 and MDRI-MDCKII cells (Z. Yang and M. Hu, unpublished data), P-gp efflux appeared to be an important mechanism responsible for their low bioavailabilities. These results may therefore redefine our understanding on absorption of potent ginsenosides and point to a strategy to increase their oral bioavailability so their clinical potentials can be fully evaluated. However, cyclosporine A is not an ideal P-gp inhibitor for continuous clinical usage in healthy humans because of its known therapeutic and adverse effects. Additional studies are ongoing to screen other less pharmacologically active compounds that may serve as potent P-gp inhibitors to enhance oral bioavailability of Rh2s.

In summary, we demonstrated clearly that Rh2s is a good substrate of P-gp both in vitro and in vivo and that effective and efficient inhibition of P-gp by cyclosporine A led to an increased oral bioavailability of Rh2s in A/J mice. The conclusion is supported by the facts that 1) deletion of P-gp functionality (as in MDRIa/b(−/−) FVB mice) significantly increases the oral absorption and bioavailability of Rh2s and 2) inhibition of efflux transporter P-gp substantially increases cellular uptake and transcellular transport of Rh2s in Caco-2 and MDRI-MDCKII cells. Additional studies are currently ongoing to search for P-gp inhibitors that are effective and clinically applicable for long-term use.

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Authorship Contributions
Participated in research design: Yang, Gao, and Hu. Conducted experiments: Yang, Gao, Wang, Yin, and Teng. Contributed new reagents or analytic tools: You and Jiang. Performed data analysis: Yang, Wang, Teng, Wu, and Hu. Wrote or contributed to the writing of the manuscript: Yang and Hu. Other: You, Jiang, and Hu acquired funding for the research.

References

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