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, MDR1a/b knockout [MDR1a/b(−/−)] FVB, and A/J mice; and 3) intestinal perfusion in WT, MDR1a/b(−/−), 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 C_{max} and AUC_{0–t} 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; Varjas 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 had 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 has a poor oral absorption and to improve its bioavailability by overcoming the barrier to its absorption.

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senosides, 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 Rh2s 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) would 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 (Sharam, 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 activity cavity) favoring hydrophobic and aromatic interactions (Aller et al., 2009).

Therefore, the aims of this study were 1) to systemically 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 prepared by the laboratories of author Z.J. at Hong Kong Baptist University. Cloned Caco-2 (TC7) cells were 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, cyclosporine A (CsA), verapamil, and MDR1-MDCKII cells were provided by the Netherlands Cancer Institute from Dr. M. Rousset (INSERM U178, Villejuit, France). Parental MDCKII 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 10^5 cells/cm^2. 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 TC7 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 gentamicin. Cell culture was maintained at 5% CO2 and 90% relative humidity at 37°C. They were seeded at 2.0 × 10^6 cells/well into the same cell culture inserts as those used for 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.

Saturation Aqueous Solubility Measurement. Ginsenoside Rh2s is a strong lipophilic (log P = 4.0, predicted by Molecular Operating Environment, version 2008.10; 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 the supernatant was added with 100 μl of acetonitrile/water/acidic acid (100:50:0.1, v/v/v, under air). The aqueous phase 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 the supernatant was then 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 $P_{app} = \frac{dC_{in}}{dV} \times (VSC_0)$, where $dC_{in}$ 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 area of the monolayer (4.65 cm^2), and $C_0$ is the starting concentration in the donor side.

Both permeability from the apical to basolateral side ($P_{a-b}$) and basolateral to apical side ($P_{b-a}$) 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 $P_{a-b} / P_{b-a}$, 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 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 Uses 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 AJ, WT, and MDR1a/b(-/-) FVB mice. Cyclosporin A solution (10 µM) was mixed with 2 µM Rh2s in AJ mice to test the effects of P-gp efflux pumps on Rh2s transport. To keep the temperature of the perfusate constant, the inlet cannula 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 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 monitored by measuring 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.

**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 was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral suspending vehicle was given by gavage to each group at 20 mg/kg. Each Rh2s bioavailability in vivo. Rh2s dispersed in the oral sus...
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$_{a-b}$ 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 ratios (P$_{b-a}$/P$_{a-b}$) were 28.5 (Table 1). Use of two MDR1/P-gp inhibitors alone at the apical side, 50 µM verapamil or 20 µM cyclosporine A, was able to completely inhibit the efflux transport of Rh2s, and the efflux ratio was decreased to close to 1. As expected, the P$_{b-a}$ was significantly increased by 9- and 4-fold and the P$_{a-b}$ was significantly decreased 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 0.31 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 Ko143) 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$_{a-b}$ 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$_{a-b}$ 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, 3.3, 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$_{a-b}$ value from 0.49 × 10$^{-6}$ to 3.51 × 10$^{-6}$ cm/s (p < 0.01) and decreased P$_{b-a}$ 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$_{b-a}$ (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 accumulation of Rh2s was consistently significantly increased by 20-fold (p < 0.001), after coinubcation with CsA in MDR1-MDCKII cells (Table 1). Transcellular transport of Rh2s as a function of time across monolayers of parental MDCKII and MDR1-MDCKII cells at different conditions is shown in Table 1.

**TABLE 1**

<table>
<thead>
<tr>
<th>Cell Model and Substrate Concentration</th>
<th>Inhibitor</th>
<th>Inhibitor Concentration</th>
<th>P$_{a-b}$</th>
<th>P$_{b-a}$</th>
<th>Efflux Ratio (P$<em>{a-b}$/P$</em>{b-a}$)</th>
<th>Intracellular Amounts</th>
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<tr>
<td>Caco-2</td>
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<tr>
<td>2 µM</td>
<td></td>
<td></td>
<td>0.37 ± 0.01</td>
<td>10.66 ± 1.74</td>
<td>28.5 ± 4.7</td>
<td>0.14 ± 0.02</td>
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<td>2 µM</td>
<td>Verapamil</td>
<td>50</td>
<td>3.21 ± 0.35*</td>
<td>3.25 ± 0.46*</td>
<td>1.0 ± 0.1*</td>
<td>0.31 ± 0.04**</td>
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<td>2 µM</td>
<td>Cyclosporine A</td>
<td>20</td>
<td>1.42 ± 0.18*</td>
<td>1.71 ± 0.23*</td>
<td>1.2 ± 0.2*</td>
<td>0.54 ± 0.08**</td>
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<tr>
<td>2 µM</td>
<td>Ko143</td>
<td>5</td>
<td>0.39 ± 0.13</td>
<td>8.97 ± 2.40</td>
<td>22.7 ± 6.1</td>
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<td>MK571</td>
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<td>16.01 ± 1.46</td>
<td>41.1 ± 4.0</td>
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<td>2 µM</td>
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<td>4.97 ± 0.69</td>
<td>11.97 ± 2.40</td>
<td>2.4 ± 0.5</td>
<td>0.25 ± 0.09</td>
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<td>1 µM</td>
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<td></td>
<td>0.49 ± 0.06</td>
<td>32.77 ± 3.33</td>
<td>65.7 ± 6.7</td>
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<td>2 µM</td>
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<td></td>
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<td>19.16 ± 4.92</td>
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<td>3.3 µM</td>
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<td>12.4 ± 1.3</td>
<td>0.19 ± 0.05</td>
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<tr>
<td>5 µM</td>
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<td>3.51 ± 0.73</td>
<td>23.50 ± 1.93</td>
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<td>0.67 ± 0.14</td>
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<tr>
<td>1 µM</td>
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<td></td>
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<td>19.16 ± 2.13</td>
<td>4.0 ± 0.16</td>
<td>0.23 ± 0.05</td>
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</tbody>
</table>

*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−∞} 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−∞} 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−∞} 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−∞} 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_{ss} 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 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 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

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**TABLE 2**

<table>
<thead>
<tr>
<th>Animal Strain &amp; Dose</th>
<th>Inhibitor (CsA)</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_{ss} (l/kg)</th>
<th>CL (l/h)</th>
<th>AUC_{0−∞} (μM h)</th>
<th>F (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/J</td>
<td></td>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>5 mg/kg</td>
<td></td>
<td>2.80 ± 0.84</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>
<td></td>
</tr>
<tr>
<td>5 mg/kg</td>
<td></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></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>20 mg/kg</td>
<td></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>
</tr>
<tr>
<td>Wild-type FVB</td>
<td></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>
<td>2.17 ± 3.42</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>20 mg/kg</td>
<td></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>
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</table>

* Intravenous administration.
* p < 0.05.
** p < 0.01.
*** p < 0.001.
INCREASED ORAL BIOAVAILABILITY OF Rh2 BY INHIBITION OF P-gp

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 $P_{app}$ 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 $C_{max}$ 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

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**Fig. 3.** Plasma concentrations of Rh2s alone or with 50 mg/kg cyclosporine A treatment after oral administrations at 5 and 20 mg/kg in A/J mice. Data are presented as mean ± S.D.; $n = 5$. Note that 12-h samples for 20 mg/kg with the CsA treatment group were not collected. The point in the circle represents the average concentration, which is an approximate value because not all samples contained quantifiable Rh2s.

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**Fig. 4.** A, absorption percentages of Rh2s (2 µM) in intestinal perfusion in WT, MDR1a/b(−/−) FVB, and A/J mice. B, plasma concentrations of Rh2s after intestinal perfusion in WT, MDR1a/b(−/−) FVB, and A/J mice. A concentration of 10 µM CsA was added in perfusate to inhibit P-gp function in A/J mice. Data are presented as mean ± S.D.; $n = 4$. *, $p < 0.05$; #, absorption percentages of Rh2s were less than 10%.
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 lumen of intestine because of its low 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 MDR1-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 MDR1a/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 MDR1-MDCKII cells. Additional studies are currently ongoing to search for P-gp inhibitors that are effective and clinically applicable compounds that may serve as potent P-gp inhibitors to enhance oral bioavailability of Rh2s.

Acknowledgments

The pharmacokinetic data analysis was assisted by Dr. Diana Chow (College of Pharmacy, University of Houston).

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


Meerum Terwogt MJ, Beijnen JH, and van Bladeren PJ (2002) Pharmacokinetics of 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 MDR1a/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 MDR1-MDCKII cells. Additional studies are currently ongoing to search for P-gp inhibitors that are effective and clinically applicable compounds that may serve as potent P-gp inhibitors to enhance oral bioavailability of Rh2s.

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Supplement Materials
Drug Metabolism and Disposition

Enhancement of oral bioavailability of ginsenoside 20(s)-Rh2 through improved understanding of its absorption and efflux mechanisms

Zhen Yang, Song Gao, Jingrong Wang, Taijun Yin, Yang Teng, Baojian Wu, Ming You,
Zhihong Jiang, and Ming Hu

Supplement Table S1. The ingredients of oral suspending vehicle used in pharmacokinetic studies.

<table>
<thead>
<tr>
<th>Ingredients</th>
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<tbody>
<tr>
<td>Purified water</td>
</tr>
<tr>
<td>Lambda Carrageenan</td>
</tr>
<tr>
<td>Simethicone</td>
</tr>
<tr>
<td>Xanthan Gum</td>
</tr>
<tr>
<td>Microcrystalline Cellulose/Sodium CMC</td>
</tr>
<tr>
<td>Sodium Phosphate, Dibasic</td>
</tr>
<tr>
<td>Potassium Sorbate</td>
</tr>
<tr>
<td>Methylparaben</td>
</tr>
<tr>
<td>Propylparaben</td>
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</table>

Content information is not available
**Supplement Table S2.** Compound-dependent parameters for digoxin and formononetin (I.S.) in MRM mode for UPLC–MS/MS analysis.

<table>
<thead>
<tr>
<th></th>
<th>Q1 (m/z)</th>
<th>Q3 (m/z)</th>
<th>Time (ms)</th>
<th>DP (V)</th>
<th>CEP (V)</th>
<th>CE (V)</th>
<th>CXP (V)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digoxin</td>
<td>780.1</td>
<td>85.0</td>
<td>100</td>
<td>-90</td>
<td>-36</td>
<td>-85</td>
<td>-2</td>
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<tr>
<td>I.S.</td>
<td>267.1</td>
<td>252.1</td>
<td>100</td>
<td>-47</td>
<td>-23</td>
<td>-30</td>
<td>-1</td>
</tr>
</tbody>
</table>

DP: declustering potential; CEP: collision cell entrance potential; CE: collision energy; CXP: collision cell exit potential.
Supplement Figure S1. UPLC-MS/MS Chromatographs of Rh2s (A) and I.S. (B) in blank plasma.

A
Rh2s (1 µM)
MRM: 621.4/160.9

B
I.S. (1 µM)
MRM: 267.1/252.1
**Supplement Figure S2.** Transcellular transport of 2 µM Rh2s across monolayers of Caco-2 cells with different inhibitors.

(A) Rh2s transport alone; (B) Rh2s transport with 50 µM verapamil; (C) Rh2s transport with 20 µM cyclosporin A; (D) Rh2s transport with 20 µM MK571; (E) Rh2s transport with 5 µM Ko143. Transport from A to B is represented by ○ and that from B to A is represented by ●. Data are presented as mean ± S.D; n=3.
**Supplement Figure S3.** Transcellular transport of Rh2s across monolayers of parental MDCKII and MDR1- MDCKII cells with different concentrations and in the presence or absence of CsA.

(A) 2 µM Rh2s transport in parental MDCKII cells; (B) 1 µM Rh2s transport in MDR1- MDCKII cells; (C) 2 µM Rh2s transport in MDR1- MDCKII cells; (D) 3.3 µM Rh2s transport in MDR1- MDCKII cells; (E) 5 µM Rh2s transport in MDR1- MDCKII cells; (F) 2 µM Rh2s transport in MDR1- MDCKII cells with cyclosporin A. Transport from A to B is represented by ○ and that from B to A is represented by ●. Data are presented as mean ± S.D; n=3.
Supplement Figure S4. Stability of Rh2s (2 µM) in fresh intestinal perfusate collected from A/J mice.