Breast Cancer Resistance Protein (ABCG2) Determines Distribution of Genistein Phase II Metabolites: Reevaluation of the Roles of ABCG2 in the Disposition of Genistein

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ABSTRACT:

It was recently proposed that the improved oral bioavailability of genistein aglycone and conjugates in Bcrp1(−/−) mice is mainly due to increased intestinal absorption of aglycone and subsequent elevated exposure to conjugation enzymes. Here we tested this proposed mechanism and found that intestinal absorption of genistein aglycone did not increase in Bcrp1(−/−) mice compared with wild-type mice using an in situ mouse intestinal perfusion model and that inhibition of breast cancer resistance protein (BCRP) in Caco-2 cells also did not significantly increase permeability or intracellular concentration of aglycone. Separately, we showed that 5- to 10-fold increases in exposures of conjugates and somewhat lower fold increases (<2-fold) in exposures of aglycone were apparent after both oral and intraperitoneal administration in Bcrp1(−/−) mice. In contrast, the intestinal and biliary excretion of genistein conjugates significantly decreased in Bcrp1(−/−) mice without corresponding changes in aglycone excretion. Likewise, inhibition of BCRP functions in Caco-2 cells altered polarized excretion of genistein conjugates by increasing their basolateral excretion. We further found that genistein glucuronides could be hydrolyzed back to genistein, whereas sulfates were stable in blood. Because genistein glucuronidation rates were 110% (liver) and 50% (colon) higher and genistein sulfation rates were 40% (liver) and 42% (colon) lower in Bcrp1(−/−) mice, the changes in genistein exposures are not mainly due to changes in enzyme activities. In conclusion, improved bioavailability of genistein and increased plasma area under the curve of its conjugates in Bcrp1(−/−) mice is due to altered distribution of genistein conjugates to the systemic circulation.

Introduction

Phase II metabolizing enzymes, especially UDP-glucuronosyltransferases (UGTs) and sulfotransferases (SULTs), play an important role in the metabolism, elimination, and detoxification of phenolics (Wu et al., 2011). UGT and SULT reactions greatly increase hydrophilicity of their substrate to facilitate their ultimate elimination via bile or kidney. Because of their high polarity, phase II conjugates cannot traverse through the cellular membrane by passive diffusion in enterocytes and hepatocytes, the two main first-pass metabolic organs, and require the action of efflux transporters for their exit from the cells (Jia et al., 2004).

Genistein, a plant phenolic and one of the most commonly consumed phytoestrogens, has been extensively studied because of its pharmacological and beneficial effects (Banerjee et al., 2008; Yang et al., 2012), however, with the realization that the full biological potential of genistein (i.e., aglycone) is severely hampered by its extensive metabolism into glucuronide and sulfate conjugates in humans after oral administration (Shelnutt et al., 2000; Coldham et al., 2002; Gu et al., 2006; Hosoda et al., 2010). On the other hand, although generally less active than the aglycone, genistein glucuronides retain the ability to prevent lipid oxidation and possess modest activity to enhance human natural killer cells at nutritionally relevant concentrations (Zhang et al., 1999b; Kgomotso et al., 2008). In addition, genistein conjugates including sulfates can serve as the reservoir of parent compound in vivo by being substrates for futile (both enteric and enterohepatic) recycling and for hydrolysis in tissues (Sfakianos et al., 1997; Chen et al., 2003).

Breast cancer resistance protein (BCRP/Bcrp/ABCG2), a member of the ATP-binding cassette transporter family, is one of the most important efflux transporters for drug disposition and plays an important role in the transport of phase II conjugates (Adachi et al., 2005). BCRP is expressed not only in multidrug resistance tumor cells but also in normal human tissues, including intestine, liver, kidney, brain endothelium, and placenta. BCRP is also a highly polymorphic transporter with different expression among populations, which might result in interindividual variations in the pharmacokinetics and hyper-
sensitivity of normal cells to anticancer drugs (Imai et al., 2004; Ni et al., 2010).

Several studies showed that Bcrp1 plays an important role in the disposition of genistein, and knockout of Bcrp1 gene substantially improved oral bioavailability of genistein and its metabolites in mice (Enokizono et al., 2007; Alvarez et al., 2011). The mechanisms responsible for the improved bioavailability of genistein aglycone in Bcrp1 knockout mice were attributed to increased intestinal absorption because it is a weak substrate of BCRP (Enokizono et al., 2007). Alvarez et al. (2011) further proposed that improved bioavailability of genistein glucuronides and sulfates could be explained by an increased exposure level of genistein aglycone to UGTs and SULTs in Bcrp1 knockout mice. However, an ~10-fold increase in exposure levels of genistein conjugates did not appear to correlate with a slight increase in aglycone (~<2-fold compared with that in WT mice) in Bcrp1 knockout mice. More importantly, numerous previous studies showed that genistein is a highly permeable compound and has good oral absorption (>50%) in vivo (Coldham and Sauer, 2000; Coldham et al., 2002; Chen et al., 2005a; Zhou et al., 2008). Impaired BCRP-mediated efflux of genistein aglycone cannot explain the ~6-fold increase of oral bioavailability of total genistein in Bcrp1 knockout mice, because the efflux ratio of genistein in a pair of BCRP/Bcrp-overexpressed cell lines were less than 2.5.

This is not the only case in which conjugates showed manyfold increases in efflux transport knockout mice, whereas the aglycones were comparable with wild-type mice (Zelcer et al., 2003; Zamek-Gliszczynski et al., 2011). It is clear that increased bioavailability of conjugates cannot be attributed only to changes in aglycone absorption and alternative mechanisms should be considered instead.

To reevaluate the role of Bcrp1 on disposition of orally administered genistein and reveal the mechanism for the substantially increased exposure level of genistein and its conjugates in Bcrp1(-/-) mice, we performed pharmacokinetic and biliary excretion studies in intestinal perfusion mode in Bcrp1(-/-) and WT mice. Human Caco-2 cells were used to delineate the polarized distribution mechanism of genistein glucuronides and sulfates in intestinal epithelial cells. Hydrolysis reactions in blood were performed to test the hypothesis that increased genistein aglycone might be derived from hydrolysis of conjugates in vivo. Finally, the activities of UGTs and SULTs were measured in Bcrp1(-/-) mice. Because intestine and liver are the major metabolism organs, the study of polarized distribution of genistein and its conjugates was mainly focused on these two organs, in addition to measurements of exposures to genistein and its metabolites in the systemic circulation.

Materials and Methods

Chemicals and Reagents. Genistein and daidzein were purchased from LC Laboratories (Woburn, MA). Four genistein conjugates (Supplemental Fig. S1) were biosynthesized using mouse intestine and isolated from the mouse intestinal perfusate, as described in our previous publication (Yang et al., 2010b). Hanks’ balanced salt solution (powder form) and methotrexate were purchased from Professional Compounding Centers of America (Houston, TX). Nonsoy rat dioxo-1,2,3,4,6,7,12,12-diepoxide from Sigma-Aldrich (St. Louis, MO). Cloned Caco-2 cells (TC7) cells were a kind gift from Dr. Monique Rousset of Institut National de la Santé et de la Recherche Médicale U178 (Villejuif, France). 3-(6-Isobutyl-9-methoxy-1,4-dioxo-1,2,3,4,6,7,12,12-octahydropyrazino[1’2’:1,6]pyridin[3,4-b]indol-3-yl)propionic acid tert-butyl ester (Ko143) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The BCA protein assay kit was purchased from Thermo Fisher Scientific (Waltham, MA). Oral suspension vehicle was obtained from Professional Compounding Centers of America (Houston, TX). Nonsoy rat chow (AIN 76A) was purchased from Harlan Teklad (Madison, WI). All other materials (typically analytical grade or better) were used as received.

Animals. Male wild-type FVB mice (6–10 weeks) were from Harlan (Indianapolis, IN), and male BCRP knockout mice (6–10 weeks) were purchased from Taconic Farm, Inc. (Hudson, NY). Wistar rats were bred in-house. Mice were kept in an environmentally controlled room (temperature 25 ± 2°C, humidity 50 ± 5%, and 12-h dark/light cycle) for at least 1 week before the experiments.

Cell Culture. Caco-2 cells have been routinely cultured in our laboratory for more than 2 decades (Hu and Borchardt, 1990). The culture conditions for growing Caco-2 cells were 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 60,000 cells/cm². Caco-2 TC7 cells were fed every other day, and cell monolayers were ready for experiments from 19 to 22 days after seeding. Transepithelial electrical resistance values of the cell monolayers were measured before the experiment, and the monolayer was discarded if the net transepithelial electrical resistance values were less than 460 Ωcm² compared with those for the blank inserts.

Pharmacokinetic Studies of Genistein. The animal protocols used in this study were approved by the University of Houston’s Institutional Animal Care and Uses Committee. Mice were fed with nonsoy chow (AIN 76A) for at least 1 week before the pharmacokinetic study. Mice were fasted for 12 h with free access to water before the pharmacokinetic study and were fed again with nonsoy chow at 4 h after the commencement of a pharmacokinetic study. Bcrp1(-/-) and WT FVB mice were used in the pharmacokinetic study after oral gavages of genistein at 2 and 20 mg/kg. Intraperitoneal injection of genistein at 20 mg/kg was also performed using both Bcrp1(-/-) and WT mice. Genistein solution (2 mg/ml) was prepared in a solution of 100 mM sodium bicarbonate-polylethylene glycol 300 (7:3) at pH 10 for intraperitoneal injection. Genistein (4 mg/ml) was dispersed in oral suspending vehicle (PCCA, Houston, TX) for oral gavage. Each pharmacokinetic study was performed on five mice, and blood samples (20–25 μl) were collected by snipping the tail at 10 different time points after mice were anesthetized with isoflurane gas so each mouse had a complete pharmacokinetic profile. The blood samples collected in heparinized tubes were stored at −20°C until analysis.

Pharmacokinetic Analysis. Pharmacokinetic parameters of genistein and its four metabolites including maximum plasma concentration (Cmax), elimination rate constant (k), half-life, and area under the plasma concentration-time curve from time 0 to t (AUC0-t) were analyzed using the noncompartmental model in WinNonlin 3.3 (Pharsight, Mountain View, CA). To obtain accurate and reasonable pharmacokinetic parameters for comparison, the following criteria were adopted for noncompartmental fitting: 1) values of k and half-life were not reported when the fitting goodness (R²) in the terminal phase was less than 0.8; 2) values of k and half-life were not reported when extrapolated AUC was more than 30% of AUC0-t; 3) half-life cannot exceed 1/2 of the time of collection of the last measurable blood sample, which was 12 h in this study; and 4) elimination rate constant should be fitted on the basis of the terminal phase not on the distribution phase. Because good fitting of k and half-life were not always achievable because of the above restrictions, AUC0-t could not always be determined. Therefore, AUC0-t was presented for comparison between WT and Bcrp1(-/-) mice.

In Situ Intestinal Perfusion. Intestinal absorption of genistein was determined in WT and Bcrp1(-/-) mice (n = 4). The animal protocols used in this study were approved by the University of Houston’s Institutional Animal Care and Uses Committee. The intestinal surgical procedures were similar to those described in our previous publications (Yang et al., 2011). In brief, small intestine and colon (8–12 cm each) were simultaneously perfused with 10 μM genistein in HBSS (pH 7.4) at a flow rate of 0.191 ml/min. 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 perfusate, perfusate samples were collected every 30 min (60, 90, 120, and 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. 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 is more than 10% per 10 cm of intestine. The 500-μl perfusate samples were added with 125 μl of internal standard solution (3 μM daidzein in acetonitrile), and the mixture was vortexed and sonicated for 30 s. After centrifugation at 15,000g for 15 min, 200 μl of supernatant was injected and
analyzed by UPLC-MS/MS. The percentages of absorption were calculated using eq. 1:

\[
\text{% absorption} = \left(1 - \frac{C_{\text{out}}}{C_{\text{in}}}\right) \times 100\%
\]

In eq. 1, \(C_{\text{out}}\) and \(C_{\text{in}}\) are outlet and inlet perfusion concentrations of genistein after correction for water fluxes, respectively. To accurately compare intestinal absorption, percentage of absorption was normalized to 10 cm of intestine for all animals. After 2.5 h of intestinal perfusion, the gallbladder was dissected intact carefully. Bile samples (5 μl) were taken and diluted 20 times with HBSS to make a mixture of 100 μl. Then 200 μl of internal standard solution (3 μM daidzein in acetonitrile) was added, and the final mixture (300 μl) was vortexed and sonicated for 2 min. After centrifugation at 15,000g for 15 min, 200 μl of supernatant was collected and air-dried. It was then reconstituted in 100 μl of 15% acetonitrile, and the concentrations of genistein and its metabolites in bile were determined by UPLC-MS/MS.

**Transcellular Transport Study.** Transcellular transport of genistein was investigated in Caco-2 cells. The advantage of using the Caco-2 cell line instead of other BCRP-overexpressed cell lines is that this line contains both UGTs/SULTs and many other efflux transporters. The protocol for performing transport experiments in triplicate was the same as that described previously (Yang et al., 2010a). In brief, 2.5 ml of genistein in HBSS was loaded onto the apical side of the cell monolayer and 2.5 ml of blank HBSS was loaded onto the basolateral side. Five sequential samples (0.5 ml) were taken at different times (0, 1, 2, 3, and 4 h) from both sides. The same volume of donor genistein 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 side were 7.4. Two concentrations of genistein (2 and 10 μM) were studied, and 5 μM Ko143 was added to the apical side of the cell monolayers for BCRP inhibition studies. Methotrexate (2 μM), a well-established BCRP substrate, was used as the positive control in Caco-2 cells.

Genistein and its four metabolites were determined by UPLC-MS/MS. Rates of transport (\(B_i\)) were obtained using rates of change in concentration of transported genistein/its metabolites as a function of time (\(dC/dt\)) and volume of the chamber (\(V\)) (eq. 2). Both apical and basolateral excretion rates were calculated according to the following equation:

\[
\frac{dC}{dt}V = B_i
\]

Intracellular concentrations of genistein and its metabolites were determined at the end of the 4-h transport study. The protocol for determining intracellular amounts of genistein and its metabolite in cells was described in our previous publication (Yang et al., 2010b). The percentage of basolateral excretion, which represents basolateral distribution of genistein conjugates in Caco-2 cells, was calculated by eq. 3:

\[
\text{% basolateral excretion} = \left(\frac{\text{basolateral excretion rate}}{\text{basolateral excretion rate + apical excretion rate}}\right) \times 100\%
\]

The fraction of dose metabolized \(\left(F_{\text{met}}\right)\) was calculated as in eq. 4 to describe the extent of genistein metabolism in a Caco-2 monolayer (Pang et al., 2009), where Met\(_{\text{total}}\) is the total metabolites and Gen\(_{\text{total}}\) is the total genistein in receiver, donor, and cell compartments combined at 4 h, respectively:

\[
F_{\text{met}} = \frac{\text{Met}_{\text{total}}}{\text{dose}} = \frac{\text{Met}_{\text{total}}}{\text{Met}_{\text{total}} + \text{Gen}_{\text{total}}}
\]

Mass balance was obtained by comparing total amounts of genistein and its four metabolites at 4 h with amounts of genistein added in donor side at 0 h in the Caco-2 cell culture model.

**Deconjugation of Genistein Glucuronides and Sulfates in Blood.** Genistein glucuronides and sulfates were biosynthesized through intestinal perfusion in rat and mice, which was described in our previous publication (Yang et al., 2010b). The mixture of 3 μM genistein glucuronide (combination of G-7-G and G-4’-G) and 3.8 μM genistein was incubated in fresh blood taken from Wistar rats in a 37°C shaking water bath for up to 4 h. Blood samples (50 μl) were taken at 0, 60, 120, and 240 min, and the reaction was stopped by addition of 50 μl of acetonitrile containing 3 μM daidzein (internal standard). Likewise, the hydrolysis reactions of genistein sulfate in fresh blood [0.8 μM genistein sulfates (combination of G-7-S and G-4’-S) and 2.2 μM aglycone] were conducted using the same conditions. The concentration used for hydrolysis reaction depends on the final concentration purified from biosynthesis. We also performed the same experiments by using 50 mM potassium phosphate buffer (pH 7.4) instead of blood for incubation at the same condition as the negative control.

**Glucuronidation and Sulfation of Genistein in Hepatic and Intestinal S9 Fraction.** Hepatic and intestinal (small intestine and colon) S9 fractions from WT and Bcrp1(+/−) mice were prepared following the protocol described in our previous publication (Zhu et al., 2010). Protein concentrations of S9 fractions were determined using a protein assay kit, with bovine serum albumin as the standard.

Glucuronidation and sulfation of genistein by S9 fractions was measured using procedures described previously (Zhu et al., 2010). In brief, for the glucuronidation reaction, 10 μM genistein in 50 mM potassium phosphate buffer (pH 7.4) was added into the reaction mixture with S9 fractions (1 mg/ml), magnesium chloride (0.88 mM), saccharolactone (4.4 mM), and alamethicin (0.022 mg/ml). A solution of 3.5 mM UDPGA was added last to make a total volume of 200 μl, and the mixture was incubated in a 37°C water bath for 60 min. For the sulfation reaction, incubation conditions were the same except that 0.1 mM 3’-phosphoadenosine-5’-phosphosulfate solution was added instead of the UDPGA coenzyme system.

Both glucuronidation and sulfation reactions were stopped by addition of 50 μl of stopping solution (94% acetonitrile-6% glacial acetic acid containing 100 μM testosterone as the internal standard). After centrifugation, the supernatants of samples were analyzed by UPLC. The assay was validated in our previous publication (Zhu et al., 2010). The same mixture with water in place of 3’-phosphoadenosine-5’-phosphosulfate or UDPGA was used as the negative control.

**Quantitation of Genistein and Its Four Metabolites.** A UPLC-MS/MS assay for analysis of genistein and its four conjugated metabolites in mouse blood was developed and validated previously (Yang et al., 2010b). In brief, an API 3200 QTRAP triple quadrupole mass spectrometer Applied Biosystems/MDS Sciex (Foster City, CA) coupled with an ultra-performance liquid chromatograph (Waters, Milford, MA) was used to analyze the blood samples. Because of the limited sample volume, whole blood was used directly. The concentrations of genistein, G-7-G, G-4’-G, G-4’-S, G-7-S and daidzein (internal standard) in blood samples were determined by multiple reaction monitoring ion pair 455/0308.1 (m/z).

**Statistical Analysis.** The data in this article are presented as means ± S.D., if not specified otherwise. Significance differences were assessed by using Student’s t test. \(p < 0.05\) was considered statistically significant.

**Results**

**Impact of Bcrp1 on Pharmacokinetics of Genistein Aglycone and Conjugates after Oral Administration.** Impact of Bcrp1 on oral pharmacokinetics of aglycone. Genistein aglycone showed moderately higher blood concentrations (as expressed in AUC) in Bcrp1(−/−) mice than in WT mice at both 2 and 20 mg/kg (Fig. 1A). In Bcrp1(−/−) mice, the AUC\(_{0-\infty}\) of aglycone was increased 2.0- and 2.2-fold \((p < 0.05)\) at 2 and 20 mg/kg, respectively, and the \(C_{\text{max}}\) of aglycone was increased 2.3-fold \((p = 0.1)\) and 5.1-fold \((p < 0.01)\) at 2 and 20 mg/kg, respectively (Table 1). These results were consistent with previous reports that AUC\(_{0-\infty}\) of aglycone was ~2-fold higher in Bcrp1(−/−) mice than in WT mice after oral administration of genistein (Enokizono et al., 2007; Alvarez et al., 2011). However, it is noted that these two previous publications had very different exposure levels of genistein aglycone in plasma of Bcrp1(−/−) mice, and our results (Fig. 1) were close to what Enokizono et al. (2007) observed \((C_{\text{max}} \approx 1.1 \mu M \text{ at } 8 \text{ mg/kg oral genistein})\). The more recent study
Impact of Bcrp1 on Pharmacokinetics of Genistein Aglycone and Conjugates after Intraperitoneal Injection. To investigate the impact of hepatic Bcrp1 on pharmacokinetics of genistein aglycone and conjugates, genistein was administered intraperitoneally in WT and Bcrp1(−/−) mice, which minimizes the role of intestinal Bcrp1 in genistein disposition.

Impact of Bcrp1 on intraperitoneal pharmacokinetics of aglycone. Genistein aglycone showed higher $C_{\text{max}}$ in Bcrp1(−/−) mice compared with that in WT mice after intraperitoneal administration, but the AUC$_{0\rightarrow5}$ was not statistically increased in Bcrp1(−/−) mice compared with that in WT mice (Fig. 2A). The $k_e$ and half-life values of genistein were not significantly altered in Bcrp1(−/−)/H11002) and WT mice (Table 2).

Impact of Bcrp1 on intraperitoneal pharmacokinetics of conjugates. The blood concentrations of G-7-G and G-4'-G were below the detection limit in wild-type mice for the 2 mg/kg group and 24-h samples of BCRP(−/−) mice. The blood concentrations of G-7-G and G-4'-G were between 0.9 to 5 h · μM (or 5.5-fold) at 50 mg/kg (Alvarez et al., 2011). Although the absolute exposure levels of total genistein were significantly lower than our current finding, the effect of Bcrp1 deficiency on percent changes in AUC$_{\text{total}}$ was consistent. It was noted that genistein showed nonlinear pharmacokinetics in terms of AUC between 2 and 20 mg/kg, which is consistent with results from a previous report (Zhou et al., 2008). The exact reason for the observed nonlinear pharmacokinetic phenomenon is not known, but a possible reason is the saturation of genistein glucuronidation at the higher dose.

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TABLE 1
Pharmacokinetic parameters of genistein, G-7-G, G-4'-S, and G-7-S in WT and Bcrp1(−/−) mice after oral administration

Pharmacokinetic parameters in Bcrp1(−/−) mice were compared to WT mice at the same dose. Data are presented as means ± S.D.; n = 5.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Dose (mg/kg)</th>
<th>C_{max} (μM)</th>
<th>k_{e} (h⁻¹)</th>
<th>t_{1/2} (h)</th>
<th>AUC_{0–t} (h·μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genistein</td>
<td>FVB</td>
<td>2</td>
<td>0.10 ± 0.05</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td></td>
<td>BCRP(−/−)</td>
<td>2</td>
<td>0.23 ± 0.17</td>
<td>0.27 ± 0.20</td>
<td>3.8 ± 2.7</td>
</tr>
<tr>
<td></td>
<td>FVB</td>
<td>20</td>
<td>0.65 ± 0.22</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td></td>
<td>BCRP(−/−)</td>
<td>20</td>
<td>3.35 ± 0.65**</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td>G-7-G</td>
<td>WT</td>
<td>2</td>
<td>0.30 ± 0.16</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td></td>
<td>BCRP(−/−)</td>
<td>20</td>
<td>1.07 ± 1.03</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>20</td>
<td>9.63 ± 5.65*</td>
<td>0.16 ± 0.10</td>
<td>5.5 ± 3.3</td>
</tr>
<tr>
<td>G-4'-G</td>
<td>WT</td>
<td>2</td>
<td>0.11 ± 0.12</td>
<td>0.28 ± 0.23</td>
<td>3.7 ± 3.0</td>
</tr>
<tr>
<td></td>
<td>BCRP(−/−)</td>
<td>20</td>
<td>0.52 ± 0.37</td>
<td>0.25 ± 0.25</td>
<td>6.5 ± 6.5</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>20</td>
<td>2.14 ± 1.44*</td>
<td>0.15 ± 0.10</td>
<td>6.2 ± 3.3</td>
</tr>
<tr>
<td>G-4'-S</td>
<td>WT</td>
<td>2</td>
<td>0.04 ± 0.03</td>
<td>0.17 ± 0.01</td>
<td>4.0 ± 0.3</td>
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<tr>
<td></td>
<td>BCRP(−/−)</td>
<td>20</td>
<td>0.14 ± 0.11</td>
<td>N.A.</td>
<td>N.A.</td>
</tr>
<tr>
<td></td>
<td>WT</td>
<td>20</td>
<td>0.25 ± 0.22</td>
<td>0.32 ± 0.19</td>
<td>2.7 ± 1.3</td>
</tr>
<tr>
<td>G-7-S</td>
<td>WT</td>
<td>20</td>
<td>6.18 ± 4.41*</td>
<td>0.30 ± 0.10</td>
<td>2.4 ± 0.7</td>
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<td></td>
<td>BCRP(−/−)</td>
<td>20</td>
<td>0.08 ± 0.02</td>
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<td>2.7 ± 0.6</td>
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<tr>
<td></td>
<td>WT</td>
<td>20</td>
<td>0.17 ± 0.05</td>
<td>0.21 ± 0.03</td>
<td>3.4 ± 0.5</td>
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<tr>
<td></td>
<td>BCRP(−/−)</td>
<td>20</td>
<td>0.65 ± 0.56</td>
<td>0.21 ± 0.18</td>
<td>5.6 ± 4.5</td>
</tr>
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</table>

N.A., not available because of poor fitting value (R² < 0.8) in the terminal phase.
* p < 0.05.
** p < 0.01.
* Estimated AUC_{0–t}.

Impact of Bcrp1 on intraperitoneal pharmacokinetics of conjugates. Similar to results after oral administration, the exposure levels of genistein glucuronides and genistein sulfates were substantially higher in Bcrp1(−/−) mice than in WT mice after intraperitoneal administration at 20 mg/kg (Fig. 2, B–D). The C_{max} values of G-7-G, G-4'-S, and G-7-S were increased 4.6-, 12.8-, and 6.5-fold and their AUC_{0–t} values were increased 3.3-, 17.8-, and 7.3-fold in Bcrp1(−/−) mice, respectively (Table 2). The concentrations of G-4'-G were below the lower limit of quantification for most of the samples after intraperitoneal administration.

The k_{e} of G-7-S was significantly decreased by 85% in BCRP knockout mice at 20 mg/kg (p < 0.05) (Table 2), but the k_{e} of G-4'-S was not significantly changed in Bcrp1(−/−) mice compared with that in WT mice. Likewise, the t_{1/2} of G-7-S significantly increased 3.9-fold in Bcrp1 knockout mice, whereas that of G-4'-S did not increase significantly.

Impact of Bcrp1 on total genistein AUC in blood. We added the AUC_{0–t} values for aglycone and conjugates together (or AUC_{total}) and used this as an indicator of overall exposure after intraperitoneal administration. At 20 mg/kg, the AUC_{total} was increased from ∼19 to ∼85 h·μM (or ∼4.5-fold). This change in AUC_{total} was much greater than the change in AUC of genistein aglycone (10% higher).

Role of Bcrp1 in Intestinal Absorption of Genistein. The intestinal absorption of genistein was investigated to determine whether Bcrp1 limits intestinal absorption of genistein aglycone. Absorption of genistein was rapid with minimal effects on water flux, and percentage of absorption of 10 μM genistein was almost complete in the small intestine in WT mice (87% ± 16%) and was not increased in Bcrp1(−/−) mice (74% ± 17%) compared with that in WT mice (Fig. 3). Percentage of absorption of genistein in colon segment was consistently very similar between WT mice (46% ± 14%) and Bcrp1(−/−) knockout mice (52% ± 8%). It was comparable with our previous report that intestinal absorption of genistein in rat small intestine is 77% or greater (Chen et al., 2005b). Genistein showed higher absorption in the small intestine than in the colon in mice, but this difference were not statistically significant. These data demonstrated that intestinal absorption of genistein is expected to be complete, and Bcrp1 did not significantly affect genistein intestinal absorption, which is very different from the substantial decreases in intestinal excretion of genistein conjugates that we reported earlier (Zhu et al., 2010).

Role of Bcrp1 in Biliary Excretion of Genistein Aglycone and Conjugates. Biliary excretion is one of the major elimination pathways for genistein (Prasain et al., 2006), and therefore the role of Bcrp1 in biliary excretion of genistein aglycone and conjugates was also investigated. Our previous results showed that biliary excretion of genistein and its metabolites was in steady state after 2 h of intestinal perfusion in rats (Chen et al., 2005b). Genistein concentrations in bile were not decreased in Bcrp1(−/−) mice (9.1 ± 3.2 μM) compared with those in WT mice (9.9 ± 4.4 μM), which indicated that Bcrp1 was not involved in biliary excretion of aglycone.

Of interest, knockout of Bcrp1 showed different effects on biliary excretion of genistein glucuronides and sulfates. The concentrations of genistein glucuronides (combination of G-7-G and G-4'-G) in bile did not display significant changes in WT mice (153.6 ± 69.3 μM) and Bcrp1(−/−) mice (186.1 ± 110.7 μM). In contrast, concentrations of genistein sulfates (combination of G-4'-S and G-7-S) in bile decreased substantially (93%) from 34.0 ± 14.3 μM in WT mice to 2.3 ± 0.6 μM in Bcrp1(−/−) mice (p < 0.05). The absolute amounts of genistein conjugates recovered in bile were much higher than that of the aglycone, indicating that genistein conjugates were efficiently eliminated via biliary excretion. Significantly decreased genistein sulfates showed that Bcrp1 plays a dominant role for its biliary excretion, whereas other transporters (e.g., MRP2) may be involved in the biliary excretion of genistein glucuronides in the absence of BCRP.

Role of Bcrp1 in the Excretion of Genistein Glucuronides and Sulfates in Caco-2 Cells. To further reveal the reasons for the higher exposure levels of genistein conjugates in Bcrp1(−/−) mice after oral administration, effects of BCRP on the polarized excretion of genistein conjugates in enterocytes were investigated. Before the transport study of genistein conjugates, methotrexate, the substrate of BCRP, showed significantly higher permeability and intracellular concentrations in the presence of 5 μM Ko143 in a positive control.
study (data not shown). Two concentrations of genistein (2 and 10 µM) were used in the Caco-2 cell transport study of how BCRP inhibition by Ko143 affected polarized excretion of conjugates.

In the presence of Ko143 treatment, the apical excretion of G-7-G and G-4′-G was significantly decreased (25 and 20%, p < 0.05) at 2 µM genistein (Fig. 4, A and B) but not at 10 µM (Supplement Table S3). Compared with results for glucuronides, we found a more potent inhibitory effect on sulfate excretion by 45 and 54% at 2 and 10 µM genistein (Supplemental Fig. S1). The percentage of basolateral excretion of G-7-G was only slightly increased (10–14%, p > 0.05) at both 2 and 10 µM genistein (Fig. 5, A and B), basolateral excretion of G-4′-S and G-7-S was significantly increased (p < 0.05) at 2 µM genistein and by 28 and 46% at 10 µM genistein (Fig. 4, C and D).

In contrast to the decreased apical excretion, basolateral excretion of genistein glucuronides and sulfates increased after Ko143 treatment. Whereas basolateral excretion of G-7-G and G-4′-G was only slightly increased (10–14%, p > 0.05) at both 2 and 10 µM genistein (Fig. 5, A and B), basolateral excretion of G-4′-S and G-7-S was significantly increased (p < 0.05) 90 and 110% at 2 µM genistein and 50 and 80% at 10 µM genistein (Fig. 5, C and D).

As a consequence of the inhibitory effects of Ko143, more genistein conjugates were basolaterally excreted after Ko143 treatment in Caco-2 cells as reflected by percentage of basolateral excretion. The percentage of basolateral excretion of G-7-G was significantly increased from 53% (control) to 63% (Ko143 treatment), and G-4′-G showed a similar change (p = 0.16) at a lower dose of genistein (2 µM). The excretion pathway of genistein sulfates was significantly changed from preferring apical excretion to basolateral excretion at 2 and 10 µM, but the extent of the preference was less at the higher concentration (Supplemental Fig. S1).

Unlike that of genistein conjugates, transport of genistein aglycone from the apical to the basolateral side did not significantly increase at either 2 or 10 µM after 5 µM Ko143 treatment (Fig. 5E), which is consistent with intestinal absorption in Bcrp1(−/−) mice. Likewise, intracellular amounts of genistein aglycone in Caco-2 cells in the presence of Ko143 did not change significantly compared with the control, indicating that inhibition of BCRP did not increase steady-state accumulation of genistein aglycone (Supplemental Fig. S4). F<sub>met</sub>, a measurement of enzyme activities in intact cells, did not change at 2 or 10 µM after Ko143 treatment in terms of total and individual metabolism, indicating that inhibition of BCRP did not change the extent or the pathway of genistein metabolism in the Caco-2 cells (Supplemental Fig. S5). The recoveries of genistein and its conjugates in Caco-2 cells after 4 h were 92.3 to 108.8% in the

| TABLE 2 |

Pharmacokinetic parameters of genistein, G-7-G, G-4′-S, and G-7-S in WT and BCRP(−/−) mice after an intraperitoneal injection of 20 mg/kg

<table>
<thead>
<tr>
<th>Strain</th>
<th>C&lt;sub&gt;max&lt;/sub&gt;</th>
<th>k&lt;sub&gt;e&lt;/sub&gt;</th>
<th>t&lt;sub&gt;1/2&lt;/sub&gt;</th>
<th>AUC&lt;sub&gt;0→t&lt;/sub&gt;</th>
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<tr>
<td></td>
<td>µM</td>
<td>h&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>h</td>
<td>h·µM</td>
</tr>
<tr>
<td>Genistein</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>2.68 ± 0.86</td>
<td>0.23 ± 0.09</td>
<td>4.8 ± 4.2</td>
<td>10.0 ± 2.30</td>
</tr>
<tr>
<td>BCRP(−/−)</td>
<td>4.42 ± 1.04</td>
<td>0.52 ± 0.33</td>
<td>2.8 ± 2.8</td>
<td>11.54 ± 5.57</td>
</tr>
<tr>
<td>G-7-G</td>
<td>0.93 ± 0.38</td>
<td>N.A.</td>
<td>3.0 ± 3.0</td>
<td>10.84 ± 6.86*</td>
</tr>
<tr>
<td>BCRP(−/−)</td>
<td>4.28 ± 2.08</td>
<td>0.55 ± 0.38</td>
<td>3.1 ± 3.1</td>
<td>3.36 ± 2.38*</td>
</tr>
<tr>
<td>G-4′-S</td>
<td>0.64 ± 0.29</td>
<td>0.30 ± 0.30</td>
<td>3.1 ± 2.7</td>
<td>33.90 ± 31.78*</td>
</tr>
<tr>
<td>WT</td>
<td>8.18 ± 5.07*</td>
<td>0.35 ± 0.22</td>
<td>3.1 ± 2.7</td>
<td>3.96 ± 3.41</td>
</tr>
<tr>
<td>BCRP(−/−)</td>
<td>1.73 ± 0.85</td>
<td>0.82 ± 0.53</td>
<td>3.1 ± 2.7</td>
<td>3.96 ± 3.41</td>
</tr>
<tr>
<td>G-7-S</td>
<td>11.31 ± 6.63*</td>
<td>0.13 ± 0.03*</td>
<td>5.4 ± 1.2*</td>
<td>28.74 ± 24.13*</td>
</tr>
</tbody>
</table>

N.A., not available because of poor fitting value (R<sup>2</sup> < 0.8) in the terminal phase.

* p < 0.05.
** p < 0.01.
control group and 87.8 to 104.2% in the Ko143 treatment group, indicating that these four conjugates are the major metabolites of genistein in Caco-2 cells.

**Deconjugation of Genistein Glucuronidation and Sulfation in Blood.** The concentration of genistein glucuronides decreased and that of aglycone increased with time in fresh blood after incubation, indicating that genistein glucuronides could be hydrolyzed in vivo into the aglycone (Fig. 6A). Unlike that of genistein glucuronides, the concentrations of both sulfates and aglycone were unaltered into the aglycone (Fig. 6A). Unlike that of genistein glucuronides, indicating that genistein glucuronides could be hydrolyzed in vivo of aglycone increased with time in fresh blood after incubation, Blood.

The concentration of genistein glucuronides decreased and that genistein in Caco-2 cells.

indicating that these four conjugates are the major metabolites of Bcrp1(−/−) mice (n/3). Data are presented as means ± S.D. The perfusion of genistein was performed at a flow rate of 0.191 ml/min. SI, small intestine.

**Glucuronidation and Sulfation Activities in Bcrp1 Knockout Mice.** Besides the role of BCRP, we also tested whether enzyme activities were altered in Bcrp1(−/−) mice. We found that rates of genistein glucuronidation in the hepatic S9 fraction (combination of G-7-G and G-4'-G) from Bcrp1(−/−) mice (2737 ± 46 pmol ⋅ min⁻¹ ⋅ mg⁻¹) were significantly increased (by 110%) compared with those from WT mice (1299 ± 71 pmol ⋅ min⁻¹ ⋅ mg⁻¹). Likewise, the glucuronidation rate was increased by 50% in colon (from 78 ± 5 pmol ⋅ min⁻¹ ⋅ mg⁻¹) in WT mice to 117 ± 19 pmol ⋅ min⁻¹ ⋅ mg⁻¹ in Bcrp1(−/−) knockout mice. However, there were no significant changes in glucuronidation activities in the small intestinal S9 fraction between these two strains of mice (Fig. 7A).

Unlike genistein glucuronidation, we found that rates of genistein sulfation (combination of G-4'-S and G-7-S) from Bcrp1(−/−) mice was significantly decreased by 40% (from 13.3 ± 0.4 to 7.9 ± 0.3 pmol ⋅ min⁻¹ ⋅ mg⁻¹) in liver S9 fractions and by 42% (from 26.5 ± 0.7 to 15.2 ± 0.5 pmol ⋅ min⁻¹ ⋅ mg⁻¹) in colonic S9 fractions compared with WT FVB mice. No significant change was observed in genistein sulfation activities in small intestinal S9 fraction (Fig. 7B). Although we saw similar exposure levels of genistein glucuronides and sulfates in blood circulation (Fig. 2), the in vitro metabolism rates of glucuronidation and sulfation had large differences, and the actual reasons for this apparent discrepancy remained unknown.

**Discussion**

Our results showed that knockout of Bcrp1 led to moderately increased bioavailability of aglycone (−2-fold) and preferential distribution of genistein conjugates to plasma (as reflected by a substantial increase in plasma AUC of conjugates, usually >10-fold). Altered distribution of conjugates was the main mechanism behind the above-observed increases because the loss of Bcrp1 functionality (due to deletion of inhibition) had a substantial impact on the polarized distribution of genistein conjugates across the intestinal membrane and hepatocytes. Our results did not show increased absorption of genistein in the intestine of the knockout mouse model or across the Caco-2 cell monolayers in the presence of a potent BCRP inhibitor, Ko143.

Previous publications have proposed that increased bioavailability of genistein aglycone was due to increased intestinal absorption in
Bcrp1(−/−) mice (Enokizono et al., 2007), and elevated exposure level of genistein aglycone may be the major reason for increased AUC of genistein conjugates in Bcrp1(−/−) mice (Alvarez et al., 2011). Here, we clearly demonstrated that the intestinal absorption of genistein was not significantly altered in Bcrp1(−/−) mice nor was the absorption of genistein aglycone increased across the Caco-2 cell monolayers in the presence of a potent BCRP inhibitor, Ko143. We therefore concluded that the increased exposure of genistein glucuronides and sulfates in Bcrp1(−/−) mice was mainly caused by changes in polarized distribution of conjugates in intestine and liver. More specifically, genistein metabolites were being increasingly excreted to the systemic circulation (i.e., enhanced basolateral excretion), instead of being effluxed back to intestine and bile in Bcrp1(−/−) mice. Although increased plasma glucuronide levels were probably also aided by increased glucuronidation rates in the knockout mice, altered distribution is the major reason for the very large percentage increases in plasma AUC of genistein conjugates. We further demonstrated that increased bioavailability of genistein aglycone could be due to reconversion of glucuronides to aglycone in the blood. We propose a schematic diagram to explain the determinant impact of Bcrp1 on the distribution of genistein conjugates in enterocytes, hepatocytes, intestinal lumen, bile, and the systemic circulation (Fig. 8). Please notice the large role that Bcrp1 played in the excretion of conjugates to the apical side of the enterocytes and hepatocytes. Its deletion leads to major changes in distribution of the genistein conjugates (favoring the basolateral side), resulting in large percentage increases in plasma conjugate AUCs.

Four independent studies at in vivo, in situ, and in vitro levels were conducted to demonstrate the mechanism by which Bcrp1 deletion affected bioavailability of genistein aglycone and conjugates. In the first study, in which intestinal absorption was directly measured using an in situ intestinal perfusion model, we found that intestinal absorp-
tion of genistein aglycone did not increase in Bcrp1(/−/−) mice compared with that in wild-type mice (Fig. 3). In the second study, in which the pharmacokinetics of genistein after intraperitoneal administration were determined, we found that AUC of genistein aglycone did not significantly increase in Bcrp1(/−/−) mice but the exposure of genistein glucuronides and sulfates did (by 4–10-fold) (Fig. 2). The latter indicated that an increase in exposure of conjugates did not have to correlate with a corresponding increase in aglycone level in vivo, as postulated by an early investigation. In the third study of biliary excretion, biliary excretion of genistein aglycone did not change in Bcrp1(/−/−) mice, whereas that of genistein sulfates significantly decreased (>90%), indicating that Bcrp1 knockout could alter the polarized excretion of conjugates in the hepatocytes. In the fourth study of genistein transport in the Caco-2 cell monolayers, inhibition of BCRP did not increase permeability or intracellular concentration of genistein aglycone but altered polarized excretion of genistein conjugates (to favoring basolateral efflux) (Figs. 4 and 5). The effects of BCRP inhibition on conjugate efflux were modest in Caco-2 cells because of the presence of other efflux transporters such as MRP2/3 and relatively low expression of BCRP in Caco-2 cells (Xia et al., 2005; Maubon et al., 2007). Taken together, these results suggest that in Caco-2 cells, inhibition of BCRP did not affect rate of transport or metabolism, but affected the polarized excretion of conjugates.

It is not surprising that intestinal absorption of genistein did not increase in Bcrp1(/−/−) mice compared with that in WT mice (Fig. 3), because the compound is highly permeable and well absorbed. Genistein showed high permeability (>3 × 10⁻⁵ cm/s) in the human intestinal Caco-2 cell model (Liu and Hu, 2002; Chen et al., 2003), and the bidirectional transport study indicated that passive diffusion is the major transport mechanism of genistein in human Caco-2 cells (Liu and Hu, 2002). It was shown earlier that genistein was a weak substrate of BCRP (efflux ratio ~2) in Bcrp-overexpressed Madin-Darby canine kidney II cells (Enokizono et al., 2007). However, the efflux transporter may not have any significant in vivo effect on a highly permeable compound with a low efflux ratio (~2) because the substrate in the basolateral compartment or the mesenteric vein will be quickly removed and cannot be efflu xen back efficiently in vivo because of unfavorable mass action (Hu g et al., 2008; Zhang et al., 2009).

The reconvension of glucuronides to aglycone in blood as shown in Fig. 7 was probably responsible for the moderate increase in plasma genistein aglycone (i.e., oral bioavailability) seen in our studies. Reconversion of glucuronides to aglycone could better explain why genistein aglycone significantly increased after oral administration (for which glucuronides increased by ~14-fold) but not after intraperitoneal administration (for which glucuronides increased by only ~3-fold). Our results provide a new insight as to why increased exposure of aglycone may derive from hydrolysis of glucuronide conjugates, which is very different from the current understanding that increased levels of aglycone come directly from impaired aglycone efflux in Bcrp1(/−/−) mice (Alvarez et al., 2011).

Because Bcrp1 controls polarized excretion of conjugates in the enterocytes and hepatocytes, deletion of Bcrp1 is probably the driving force behind the large increase in the distribution of genistein conjugates to mouse plasma (a big increase in their AUC₀₋₉₀) in Bcrp1(/−/−) mice. This is consistent with our previous observation that luminal excretion of genistein glucuronides and sulfates decreased 78 and >90%, respectively, in Bcrp1(/−/−) mice compared with that in WT mice (Zhu et al., 2010). It is also consistent with results of Ko143 in Caco-2 cell monolayers, in which increased relative distribution of conjugates to the basolateral side was observed (Fig. 5). Because of unaltered intestinal absorption of genistein and slightly different enzyme activities in intestine, less luminal excretion of genistein conjugates in mouse intestine should lead to higher basolateral excretion of genistein conjugates (Fig. 5) to the systemic circulation as illustrated in Fig. 8. Likewise, Bcrp1 did not mediate the biliary excretion of genistein aglycone but played an important role in the biliary excretion of genistein sulfates. Hence, deletion of Bcrp1 would increase basolateral excretion of sulfates to the blood.

The transporter responsible for increased basolateral efflux in enterocytes and hepatocytes has not yet been defined, but the most likely candidate is MRP3, which may transport more genistein conjugates in the absence of Bcrp1 as a compensatory mechanism to prevent their accumulations inside the cell (Supplemental Fig. S4) (van de Wetering et al., 2009).

We observed a bigger impact of Bcrp1 deletion on plasma AUC of genistein and its conjugates after oral administration than after intraperitoneal administration, suggesting that Bcrp1 plays a more important role in intestinal conjugation than liver conjugation (of genistein). In liver conjugation of genistein, Bcrp1 plays a more important role in sulfonation than in glucuronidation, in that the biliary excretion of genistein glucuronides was not decreased in Bcrp1(/−/−) mice but biliary excretion of sulfate was. As a consequence, there was a bigger change in sulfate AUC than in glucuronide AUC after intraperitoneal administration. Of course, the higher glucuronidation rate and lower sulfation rate in liver of Bcrp1(/−/−) mice also probably contributed to the changes in plasma conjugate AUC.

Our result is the first report showing that knockout of Bcrp1 may also change the activities of relevant UGTs and SULTs (Fig. 7), which indicates that knockout of Bcrp1 could cause unexpected changes in SULT and UGT activities. The increased genistein glucuronidation rates and decreased sulfation rates in Bcrp1(/−/−) mice further signified the predominant role of Bcrp1 in determining the formation and distribution of sulfates in mice. A previous publication has showed that the expression levels of MRP2 and MRP3 were not changed in Bcrp1 knockout mice (Nezasa et al., 2006).

In conclusion, Bcrp1 deficiency moderately increases the oral bioavailability of genistein aglycone. More importantly, Bcrp1 defi-
ciency caused a major change in the distribution of conjugates, especially sulfates, resulting in large increases in plasma AUC of genistein glucuronides and sulfates. This impact on plasma conjugate AUC values occurred mainly in the intestine and liver and was greater after oral administration. Here, we want to advance the hypothesis that deficiency in efflux transporters could lead to altered plasma pharmacokinetics of these conjugates in Bcrp1(--/--) mice, which could increase plasma AUC of conjugates and bioavailability of genistein aglycone due to recoversion of glucuronides to aglycone in the blood.

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Authorship Contributions

Participated in research design: Yang, Zhu, and Hu.

Conducted experiments: Yang, Gao, Zhu, and Yin.

Performed data analysis: Yang, Jiang, and Hu.

Wrote or contributed to the writing of the manuscript: Yang and Hu.

References


FIG. 8. Schematic representation showing the absorption of genistein aglycone and distribution of its conjugates in intestine, bile and blood (or plasma). This is a schematic presentation and as such is an approximate representation of the major enzymes, and efflux transporters involved, and not exhaustive. Green triangle with P, genistein; magenta shape with G, genistein glucuronides; magenta diamond with s, genistein sulfates; arrows, efflux transporters for genistein conjugates transport; large green hollow arrows, proposed role of Bcrp; brown filled arrows, proposed role of other efflux transporters; small green solid arrows: aglycone transport via passive diffusion. Arrow size indicates the importance (or size) of its role in genistein conjugate transport. MRP, represented by large solid blue arrows, represents the MRP efflux transporters localized to the basolateral side. Currently, only one MRP transporter (i.e., MRP3) was shown to be involved in the basolateral efflux of conjugates (Kitamura et al., 2010), but other related MRP efflux transporters may also function. Note: Genistein has the same transport pathway in small intestine and colon. To keep the figure simple, some of the enzyme and transporter icons were omitted in the colon compartment. In the colon compartment, efflux transporters were represented by simple magenta solid arrows without identifying the actual efflux transporters, which are expected to be identical to those in the small intestine (although the expression levels may be different).


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