SHORT COMMUNICATION

Bioavailability of the glucuronide and sulfate conjugates of genistein and daidzein in Bcrp1 knockout mice

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Running title: Bioavailability of isoflavone conjugates in Bcrp1 KO mice

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Abbreviations: ABCG2, ATP-binding cassette subfamily G; BCRP, breast cancer resistance protein; daid-gluc 1, daidzein glucuronide isomer 1; daid-gluc 2, daidzein glucuronide 2; daid-sulfate, daidzein sulfate; gen-gluc 1, genistein glucuronide isomer 1; gen-gluc 2, genistein glucuronide isomer 2; gen-gluc 3, genistein glucuronide isomer 3.
ABSTRACT

The dietary polyphenols genistein and daidzein are potent effectors of biological processes. The plasma profile of both isoflavones is governed by the presence of phase II conjugates, mainly glucuronides and sulfates. Breast cancer resistance protein, (ABCG2/BCRP), interacts with genistein and daidzein, which are among the natural substrates of the transporter and competitively inhibit ABCG2-mediated drug efflux. ABCG2/BCRP can also transport glucuronide and sulfate conjugates. In this study we analyzed the plasma levels of aglycones and derived conjugated metabolites, glucuronides and sulfates, after intragastric administration of these isoflavones to wild-type and Bcrp1(-/-) knockout mice. The results show that overall plasmatic profile is mainly governed by sulfate and glucuronide derivatives whose concentration was significantly increased (7-10 fold) in Bcrp1(-/-) mice. The total AUC h nM (0-180 min), as the sum of aglycones, glucuronides and sulfates, was 901±207 in wild-type mice vs 4,988±508 in Bcrp1(-/-) mice after genistein administration (50 mg/kg body weight); 584.3±90 in wild-type mice vs 4,012±612 in Bcrp1(-/-) after daidzein administration (50 mg/kg) and 926±140 in wild-type mice vs 5,174±696 in Bcrp1(-/-) after genistein+daidzein administration (25+25 mg/kg). Therefore, our results indicate a direct and conclusive Bcrp1 efflux action on phase II metabolites of these isoflavones in vivo, and suggest a possible novel concept for ABCG2/BCRP as part of metabolism-driven efflux transport of these conjugates.
Introduction

Many phytochemicals are potent effectors of biological processes and it is well known that intake of genistein and daidzein, the main isoflavones present in soy, have important beneficial effects on health (Williams and Manach, 2005). Genistein and daidzein are isoflavones, polyphenolic compounds belonging to the flavonoid class, and represent the major active components in soy products. The bioavailability of isoflavones has been previously reported as these polyphenols might influence disease risk via several complementary and overlapping mechanisms (Lampe and Chang 2007; de Kok et al., 2008). Genistein and daidzein have low bioavailabilities due to extensive intestinal first-pass phase II metabolism and subsequent excretion of their conjugated metabolites. Bioactive conjugates, glucuronides or sulfates, are the product of activity of phase II enzymes: UDP-glucuronosyltransferases (UGTs) and sulfotransferases (SULTs), which are present mainly in intestine and liver (Williamson and Manach 2005). Despite low oral bioavailabilities, both isoflavones, and some of their conjugates, may accumulate in sufficient amounts in the body to produce their pharmacological actions (Bolca et al., 2010). Transcellular transport of several phytochemicals depends on the activity of membrane-bound, ATP-binding-cassette (ABC) transport proteins (efflux-transporters) which are able to export the compounds from cells (Brand et al., 2006; Lampe and Chang 2007). It has been hypothesized that UGT-efflux transporters coupling in both the intestine and the liver may result in enterohepatic circulation and enteric recycling, leading to longer lasting, and thus increased, phytochemical accumulation in the body, in particular with repeated intake (Wang et al., 2006).

Some plant-derived flavonoids, including the isoflavones genistein and daidzein, can competitively inhibit the drug efflux mediated by the transporter ABCG2/BCRP, a member of the ABC family of transporters (Imai et al., 2004; Katayama et al., 2007). In addition, ABCG2/BCRP can also transport glucuronide and sulfate conjugates (Xu H et al., 2009; Alfaras
et al., 2010). Recently, Zhu et al (2010) reported that ABCG2/BCRP and SULT contribute to the disposition of genistein in mouse intestine.

Taking into account the important role of ABCG2/BCRP in the transport of glucuronide and sulfate conjugates, the use of knockout mice could yield valuable information on plasma bioavailability of native forms and derived metabolites of phytochemicals.

Soy isoflavones are of particular interest. In examining the possible roles of flavonoids in human health it is important to understand the bioavailability and pharmacokinetics of these substances. The goal of this manuscript was to study the impact of ABCG2/BCRP on systemic exposure of phase II metabolites of isoflavones genistein and daidzein, after intra-gastric administration to wild-type and Bcrp1(-/-) mice. In addition ABCG2 efflux kinetics was also evaluated upon single or combined isoflavones intragastric administration.

Materials and Methods

Chemicals. Genistein and daidzein were purchased from LC Laboratories (PKC Pharmaceuticals, Inc Woburn Ma USA) and isoflurane (Isovet®) from Schering-Plough (Madrid, Spain). All the other chemicals were of analytical grade and obtained from commercial sources.

Animals. Mice (30 ± 2 g) were housed and handled according to procedures approved by the Research Committee of Animal Use of the University of Leon (Spain) and carried out according to the “Principles of Laboratory Animal Care” and the European guidelines described in EC Directive 86/609. The animals used in the experiments were Bcrp1(-/-) (n=4) and wild-type mice (n=4) (9-14 wk), all of >99% FVB genetic background. Animals were kept in a temperature-controlled environment with a 12-h light/12-h dark cycle. Mice were fed with normal rodent chow (Panlab SA, Barcelona, Spain) and water was available ad libitum.
**Isoflavone administration and sampling procedure.** Isoflavones were dissolved in a mixture containing 25% ethanol, 25% polyethyleneglycol and 50% saline. This solution was administered intragastrically (100 µl/30 g body weight, b.w.) to 4 h-fasted wild-type and Bcrp1(-/-) mice using an intragastric probe. Isoflavones were administered either separately (50 mg/kg b.w.) or together (25 mg each/kg b.w.). Blood samples were collected by orbital bleeding in isofluorane-anesthetized animals at different times. Plasma was obtained after centrifugation at 1500xg for 15 min in a Sigma 1-13 microcentrifuge (Braun Biotech. Int., Melsungen, Germany) at 4 ºC. The samples were kept at -80 ºC until analysis.

**Plasma preparation.** Plasma samples (100 µl) were mixed with 400 µl of acetonitrile, vigorously stirred in a vortex for 1 min and centrifuged at 5000xg for 10 min at 4 ºC in the microcentrifuge and the supernatant was collected. Samples were extracted twice. The supernatants were pooled and evaporated under vacuum in a SpeedVac Concentrator Savant SPD121P (Thermo Scientific, Alcobendas, Spain) equipped with a refrigerated vapor trap RVT4104 (Thermo Scientific) and a vacuum pump v-710 (BÜCHI Labortechnik AG, Postfach, Switzerland). The pellet was re-dissolved in 400 µl methanol. Methanolic samples (5 µl) were diluted with 5 µl of acidified (formic acid 1%) ultrapure Milli-Q water (Millipore Corp., Bedford, MA), filtered through a 0.45 µm membrane filter Millex-HV13 (Millipore Corp.) and analyzed (4 µl) by HPLC-DAD-MS/MS.

**HPLC-DAD-MS/MS analyses.** Plasma samples were analyzed in a 1200 series HPLC-DAD system (Agilent Technologies, Waldbronn, Germany), equipped with an HTC Ultra mass detector in series (Bruker Daltonics, Bremen, Germany). The mass detector was an ion-trap mass spectrometer equipped with an electrospray ionization (ESI, capillary voltage, 4 kV; dry temperature, 350 ºC) system. Mass scan (MS) and MS/MS daughter spectra were measured from
m/z 75 to 800 using the Ultra scan mode (26,000 m/z per second). Collision-induced fragmentation experiments were performed in the ion trap using helium as collision gas, and the collision energy was set at 50%. Mass spectrometry data were acquired in the negative ionization mode. Chromatographic separations of plasma samples were carried out on a 150 x 0.5 mm i.d., 5 μm, reverse phase SB C18 Zorbax column (Agilent) using water/formic acid (99:1, v/v) (A) and acetonitrile (B) as the mobile phases at a flow rate of 10 μL/min. The gradient started with 10% B in A to reach 40% B at 30 min, 50% B at 35 min, 90% B at 36 min for 4 min and returning to the initial conditions (10 % B). UV chromatograms of samples were recorded at 280 and 250 nm.

The isoflavones genistein, daidzein and their derived metabolites were identified by their UV spectra, ion mass, daughter ions and fragmentation pattern in the HPLC-DAD-ESI-MS/MS equipment. The compounds genistein, daidzein and their derived metabolites were quantified using UV detection at 250 nm and using commercially available genistein or daidzein standards. The recovery efficiency of daidzein and genistein from plasma was determined by spiking a plasma sample from a control with known concentrations of isoflavones. The plasma samples were processed according to the protocols described above and the recoveries calculated by comparing the peak area ratios from spiked samples to those of the corresponding concentrations of standards in methanol directly injected into the HPLC system. Linearity (r²= 0.999) was confirmed for both isoflavone-spiked plasma samples as well as isoflavones in methanol in the range from 0.01 to 5 μM. The mean recovery efficiency was 99±1% for both genistein and daidzein. The intra-day reproducibility of the HPLC–ESI method was assessed from 10 consecutive chromatographic runs using 0.5, 0.1 and 0.05 μM of each isoflavone standard in methanol:0.1% formic acid (v:v). The inter-day reproducibility of the method was assessed by analyzing the same standards and concentrations for 3 consecutive days. The relative standard deviation for peak area was in the range of 0.5–2.0% in the intra-day test and 1.0–3.1% in the
case of the inter-day test. The limit of detection (LOD) was determined as the concentration of analytes with a signal-to-noise ratio of at least 3 and the limit of quantitation (LOQ) was the lowest standard with a signal-to-noise ratio of at least 10. LODs were 6 and 5 nM for daidzein and genistein, respectively. LOQs were 18 and 16 nM for daidzein and genistein, respectively. The coefficient of variation was always lower than 10%.

**Area under the curve (AUC) and statistics.** The area under the plasma concentration-time curve from time 0 to 180 min was calculated by lineal trapezoidal rule using WinNonlin software package (WinNonlin Professional version 5.2.1, Pharsight Corp., Mountain View, CA). WinNonlin model 200 was used for the analysis. Statistical differences between AUC in wild-type and Bcrp1(-/-) mice were tested using the Student’s t test. A p value <0.05 was considered to be significant.

**Results and Discussion**

The metabolic profile of genistein and daidzein in plasma from wild-type and Bcrp1(-/-) mice is shown in Fig.1. No isoflavone-derived metabolites were detected in mice plasma prior to intragastric isoflavone administration (results not shown). In mice with intragastric genistein administration, peak 5 (at 32.2 min) was identified as genistein according to its ion mass (269 m/z), UV spectrum and coelution with authentic standard. Peaks 1-4 (at 18.4, 19.9, 21, and 23 min, respectively) were identified as genistein-derived metabolites (Fig. 1A). In mice with intragastric daidzein administration, peak 9 (at 26.5 min) was identified as daidzein according to its ion mass (255 m/z), UV spectrum and coelution with authentic standard. Peaks 6-8 (at 12.5, 16.6, and 18.9 min, respectively) were identified as daidzein-derived metabolites (Fig. 1B). Isoflavone metabolites were identified using MS analyses. The inserts in Fig 1 (A-B) show the MS$^2$ full scan for each compound detected. Metabolites 1, 2 and 3 were identified as genistein
glucuronides according to their ion mass (445 m/z) and daughter ions (269, 176 m/z). Metabolite 4 was identified as genistein sulfate with ion mass at m/z 349 and daughter ions at 269 and 80 m/z. Metabolites 6 and 7, with ion masses at 431 m/z and daughter ions at 255 and 176 m/z were identified as daidzein glucuronide conjugates, and peak 8 with ion mass at 335 m/z and daughter ions at 255 and 80 m/z was identified as a daidzein sulfate conjugate.

Fig. 2 shows the plasma concentrations (nM) of genistein, daidzein, and their metabolites after intragastric administration of 50 mg/kg of each isoflavone separately or together (25 mg genistein + 25 mg daidzein /kg b.w.) for 180 min. The AUC (h nM) for each isoflavone and derived-metabolites was calculated (Table 1).

After genistein administration, the main differences between both types of mice were observed for genistein-sulfate (Gen-sulfate) and genistein-glucuronide 1 (Gen-gluc 1), the total AUC h nM (estimated as the sum of genistein and derived-metabolites) being 5.5-fold higher in Bcrp1(-/-) than in wild-type mice (Table 1). Plasma levels of genistein slightly increased in knockout mice, which could suggest some kind of interaction of the aglicone with BCRP. However, the overall plasmatic profile was governed mainly by sulfate and glucuronide-1 derivatives whose concentrations significantly increased (7-fold and 8-fold, respectively) in Bcrp1(-/-) vs wild-type mice (Table 1). Plasma levels of genistein slightly increased in knockout mice, which could suggest some kind of interaction of the aglicone with BCRP. However, the overall plasmatic profile was governed mainly by sulfate and glucuronide-1 derivatives whose concentrations significantly increased (7-fold and 8-fold, respectively) in Bcrp1(-/-) vs wild-type mice. Zhu et al., (2010) showed a dramatically decreased excretion of genistein conjugates, mainly sulfate, in Bcrp (-/-) mouse vs wild-type in small intestine and colon. Genistein and glucuronidated genistein can be detected immediately in plasma within 5 min after oral administration in rats. Moreover, the glucuronidation occurred in the intestine, but not in the liver, upon oral administration of genistein in rats (Zhou et al., 2008; Sfakianos et al., 1997). Our results could emphasize the important role of intestinal recycling rather than enterohepatic circulation in the disposition of genistein (Chen et al., 2005).

Plasma concentration following daidzein administration showed relevant differences with respect to genistein in both wild-type and Bcrp1(-/-) mice. The plasma levels of daidzein were
higher in Bcrp1(-/-) than in wild-type mice (Fig. 2), although there were no statistical differences (Table 1). The total AUC value for daidzein (583.7±89.7 h nM; after daidzein administration) was lower than that for genistein (900.9±207.3 h nM; after genistein administration) in wild-type mice (Table 1; Fig. 2). This was related to the lack of daidzein-derived glucuronides in wild-type mice (Fig. 2). However, the presence of daidzein glucuronide 1 (977.2±196.0 h nM) in Bcrp1(-/-) mice raised the total AUC value for daidzein to close to the AUC value for genistein in Bcrp1(-/-) (Table 1; Fig. 2). Daid-sulfate levels were almost 7-fold higher in Bcrp1(-/-) than in wild-type mice. The absence of glucuronide metabolites in wild-type mice after daidzein administration could be due different activities of UGT isoforms because these isoflavones have been reported to show specific concentration-dependent patterns (Tang et al., 2009). Finally, the total AUC h nM (as the sum of daidzein and derived-metabolites) was 6.9-fold higher in Bcrp1(-/-) than in wild-type mice after daidzein administration.

When both isoflavones were administered together, the pharmacokinetic profile of the aglycones was reduced approximately by half depending on the dose administered (Fig. 2). The sulfate metabolites from both isoflavones were readily recovered from plasma in wild-type mice (Fig. 2). In Bcrp1(-/-) mice, the most relevant result was the overall increase of metabolites in plasma in a similar way to when the aglycones were administered separately. The pharmacokinetic results reflect that ABCG2 is critical for disposition of both genistein and daidzein. The co-administration of both isoflavones resulted in a tentative competition between genistein and daidzein which changed the plasma metabolic profile. In this case, only the most important metabolites (genistein, gen-sulfate, gen-gluc 1, daidzein and daid-sulfate) were detected (Fig. 2).

The role of ABCG2 in the disposition of these isoflavones should be highlighted in the context of chemopreventive approaches. Genistein and daidzein could be useful for overcoming ABCG2-mediated drug resistance in cancer cells. In this context, the intake of soy products
together with the administration of ABCG2-substrate agents (i.e. antitumoral drugs) might alter
the pharmacokinetics and the toxicity of these compounds (Sugimoto et al., 2005). For example,
the in vivo interaction of the ABCG2 substrate nitrofurantoin and soy isoflavones has been
reported in specific mice target organs such as liver and mammary glands (Merino et al., 2010).
In addition, Enokizono et al. (2007) reported that a tissue-to-plasma concentration of genistein
and daidzein of 1.5 nM in mice at steady state can interact in vivo with Bcrp1 in brain, testis,
epididymus and fetus.

From a pharmacokinetic point of view, genistein and daidzein are poorly bioavailable (less
than 15% absolute bioavailability) in rodents and humans when administered orally (Andrade et
al., 2010; Williamson and Manach, 2005). This corresponds to plasma concentration levels
significantly less than the IC\textsubscript{50} value reported for the anticancer and other beneficial in vitro
effects described for these isoflavones (de Kok et al., 2008 Imai et al., 2004). For example, Tamaki
et al. (2010) obtained genistein and daidzein IC\textsubscript{50} values of 0.24 μM and 2.4 μM, respectively.
However, the inhibitory potency of daidzein decreased 100-fold by 7-glucuronidation and was
virtually unaffected by 4’-sulfation since they obtain an IC\textsubscript{50} of 426 μM for daidzein 7-
glucuronide and IC\textsubscript{50} of 3.89 μM for daidzein-4’-sulfate.

Our results support the significant role of ABCG2 in the sulfation pathway, presumably from
the intestine, where its absence diminished completely the excretion of genistein sulfate by
enterocytes (Zhu et al., 2010). In the present study, daidzein-derived glucuronides were only
detected in plasma of Bcrp1 (-/-) mice upon intragastric administration of daidzein (Figs. 1, 2;
Table 1). These results could support previous findings from Zhu et al., (2010) who suggested a
relevant role of ABCG2 for removing sulfate-derived metabolites from the cytosolic domain. If
sulfates are not removed from the cells, they can be hydrolyzed back to aglycones by intestinal
sulfatases, which can be glucuronized to yield higher amounts of glucuronide metabolites, as
occurs in the present study. Zhang et al. (1999) found that genistein glucuronides activated...
human natural killer cells at nutritionally relevant concentrations, which suggested that genistein conjugates might also be active components. Accordingly, other glucuronides from flavonoids such as quercetin, epicatechin also show biological activities (Zhang et al 2007). Polyphenol metabolites could also exert biological activities after deconjugation at the cellular level. Along with the aforementioned intestinal sulfatase activity, a β-glucuronidase activity is present in many organs including liver and intestine (Liu and Hu, 2007). In fact, UDP-glucuronosyltransferases are present along with β-glucuronidase inside the endoplasmic reticulum (Manach 2004).

The isoflavones genistein and daidzein undergo an extensive first-pass metabolism which gives rise to the presence of a number of glucuronide and sulfate metabolites in plasma. Bcrp accepts genistein and daidzein as substrate. Impaired Bcrp-mediated efflux of genistein and daidzein could increase their exposure to UGT and SULT, and eventually produce greater amounts of metabolites. In the present study, we have shown for the first time the role of ABCG2 in the overall plasma bioavailability of these isoflavones using wild-type and knockout Bcrp1(-/-) mice. The absence of this transporter remarkably increased the presence of isoflavone conjugates in plasma which could modulate the potential biological activity of genistein and daidzein. Finally, our results suggest a possible novel concept for ABCG2/BCRP as part of metabolism-driven efflux transport.

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Authorship Contributions: Participated in research design: Álvarez, Merino and Prieto

Conducted experiments: Barrera and Vallejo.

Performed data analysis: Merino, Vallejo and Espín.

Wrote or contributed to the writing of the manuscript:

Álvarez, Tomás-Barberán and Espín.
References


Footnotes

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Legends for figures

Fig. 1. UV chromatogram and mass spectra of genistein, daidzein and derived metabolites in plasma from wild-type and Bcrp1(-/-) mice, 50 min after genistein (A) or daidzein (B) intragastric administration. (A) Genistein (peak 5) and its main metabolites, glucuronides (peaks 1-3) and sulfate (peak 4). (B) Daidzein (peak 9) and its main metabolites, glucuronides (peaks 6, 7) and sulfates (peak 8).

Fig. 2. Plasma concentration (nM) of genistein, daidzein and their derived metabolites after intragastric administration of genistein (50 mg/kg b.w.), daidzein (50 mg/kg b.w.), or both isoflavones together (25 mg genistein+25 mg daidzein/kg b.w.) in wild-type and Bcrp1(-/-) mice at 20, 50, 90 and 180 min (n=4).
### TABLE 1

Calculated AUC (area under curve) of genistein, daidzein and derived metabolites after intragastric genistein and/or daidzein administration

<table>
<thead>
<tr>
<th>Isoflavone</th>
<th>Aglycone or metabolite</th>
<th>Wild-type</th>
<th>Knockout</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genistein (50 mg/kg)</td>
<td>Genistein</td>
<td>254.7±23.9</td>
<td>371.2±31.1</td>
<td>0.097</td>
</tr>
<tr>
<td></td>
<td>Gen-sulfate</td>
<td>326.9±92.2</td>
<td>2,338.8±224.8</td>
<td>0.014</td>
</tr>
<tr>
<td></td>
<td>Gen-gluc 1</td>
<td>241.2±69.5</td>
<td>1,992.3±242.5</td>
<td>0.02</td>
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<tr>
<td></td>
<td>Gen-gluc 2</td>
<td>47.5±13.1</td>
<td>99.9±14.3</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>Gen-gluc 3</td>
<td>44.8±10.8</td>
<td>106.1±17.7</td>
<td>0.097</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td>900.9±207.3</td>
<td>4,987.7±508.4</td>
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</tr>
<tr>
<td>Daidzein (50 mg/kg)</td>
<td>Daidzein</td>
<td>222.43±32.5</td>
<td>528.9±80.2</td>
<td>0.071</td>
</tr>
<tr>
<td></td>
<td>Daid-sulfate</td>
<td>361.4±67.0</td>
<td>2,459.1±365.1</td>
<td>0.029</td>
</tr>
<tr>
<td></td>
<td>Daid-gluc 1</td>
<td>n.d.</td>
<td>977.2±196.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Daid-gluc 2</td>
<td>n.d.</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td>583.7±89.7</td>
<td>4,012.1±611.7</td>
<td>0.031</td>
</tr>
<tr>
<td>Genistein + Daidzein (25+25 mg/kg)</td>
<td>Genistein</td>
<td>149.4±12.8</td>
<td>251.5±27.8</td>
<td>0.079</td>
</tr>
<tr>
<td></td>
<td>Gen-sulfate</td>
<td>284.7±32.4</td>
<td>1,765±145.5</td>
<td>0.0099</td>
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<tr>
<td></td>
<td>Gen-gluc 1</td>
<td>162.9±33.6</td>
<td>1,155.0±192.6</td>
<td>0.0366</td>
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<tr>
<td></td>
<td>Daidzein</td>
<td>157.8±26.3</td>
<td>355.3±51.3</td>
<td>0.0756</td>
</tr>
<tr>
<td></td>
<td>Daid-sulfate</td>
<td>171.5±45.9</td>
<td>1,687.0±307.6</td>
<td>0.039</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td>926.4±138.9</td>
<td>5,174.5±696.1</td>
<td>0.026</td>
</tr>
</tbody>
</table>

n.d., not detected.
Fig. 1
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