SGLT-1 transport and deglycosylation inside intestinal cells are key steps in the absorption and disposition of calycosin-7-O-β-D-glucoside in rats

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Transport of calycosin-7-O-β-D-glucoside by SGLT-1 in rats

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**List of Non-Standard Abbreviations:** LPH, lactase–phloridzin hydrolase; CG, calycosin-7-O-β-D-glucoside; GLTs, glucose transporters; SGLT-1, sodium-dependent glucose transporter 1; GLUT-2, glucose transporter 2; BSβG, broad-specific β-glucuronides; UGT, UDP-glucuronosyltranferases; RLMs, rat liver microsomes;
RIMs, rat intestinal microsomes; UDPGA, uridine diphosphate glucuronic acid; LLOQ, lower limit of quantification; BCRP, breast cancer resistance protein; MRP2, multidrug resistance-associated protein 2; TEER, transepithelial electrical resistance.
ABSTRACT

Hydrolysis by lactase–phloridzin hydrolase (LPH) is the first and critical step in the absorption of isoflavonoid glucosides. However, the absorption characteristics of calycosin-7-O-β-D-glucoside (CG) slightly differ from other isoflavonoid glucosides. In this study, we used the rat intestinal perfusion model and performed pharmacokinetic studies and *in vitro* experiments to determine the factors influencing CG absorption and disposition. After oral administration of isoflavonoid glucosides, LPH was found to play minimal or no role on the hydrolysis of CG, in contrast to that of daidzin. CG was mainly transported into the small intestinal cells by sodium-dependent glucose transporter 1 (SGLT-1) as intact. This pathway could be the main mechanism underlying the high permeability of CG in the small intestine. CG was likely to be hydrolyzed in enterocytes to its aglycone calycosin by broad-specific β-glucuronides (BSβG) and glucocerebrosidase or rapidly metabolized. Calycosin was also rapidly and extensively metabolized to 3′-glucuronide in the enterocytes and liver, and the glucuronidation rates of calycosin and CG were much higher in the former. The metabolites were also transported into lumen by breast cancer resistance protein (BCRP) and multidrug resistance-associated protein 2 (MRP2). In conclusion, the enterocytes could be an important site for CG absorption, deglycosylation, and metabolism in rats. This study could contribute to the theoretical foundation and mechanism of absorption and disposition of flavonoid compounds.
Introduction

Isoflavonoids, a class of flavonoid polyphenolic compounds, are mainly found in soy products as conjugated glucoside and confer beneficial health effects (Murphy et al., 2002; Nurmi et al., 2002). Absorption and metabolism of soy isoflavonoid glucosides have been extensively studied in recent years to elucidate their physiological functions (Walsh et al., 2007; Islam et al., 2014). Deglycosylation by lactase–phloridzin hydrolase (LPH), an enterocyte-specific enzyme localized to the brush-border membrane in small intestine, is a critical step in the absorption and metabolism of isoflavonoid glucosides. This process generates aglycones that can enter enterocytes through passive diffusion (Day et al., 2000; Walsh et al., 2007). After cellular accumulation, aglycones undergo extensive metabolism in enterocytes (Chen et al., 2005). The metabolites are either excreted into the digestive tract or passed into the portal vein by specific transporters and then metabolized by phase II enzymes in liver (Scalbert and Williamson, 2000; Del Rio et al., 2013). This pathway mainly causes low bioavailability of isoflavonoid glucosides. However, in our previous study, we found that the absorption characteristic of the main isoflavonoid glucoside in Astragali Radix differs from that of other soy isoflavonoid glucosides (Shi et al., 2015).

Astragali Radix is a medicinal herb with immunogenic properties. It is used to enhance immunity and treat cardiovascular diseases, such as myocardial infarction and heart failure (Zhao et al., 2008). More than 400 isoflavonoids have been identified in Astragali Radix (Ruan et al., 2015), including calycosin-7-O-β-D-glucoside (CG), a
marker used routinely to monitor the quality of the herb. CG exhibits several pharmacological activities, such as anti-inflammatory effect in rabbit osteoarthritis model and antioxidative and neuroprotective effects in vitro (Choi et al., 2007; Jian et al., 2015). A recent study showed that the CG metabolite selectively modulates estrogen receptors, which promote angiogenesis in zebrafish embryos (Ruan et al., 2015). The aglycone calycosin of CG also exerts antidiabetic, antioxidative, and neuroprotective effects (Fan et al., 2003; Yu et al., 2005; Shen et al., 2006). CG and calycosin are used as the main components of a pharmaceutical drug that has been patented for prevention and treatment of estrogen receptor-mediated diseases and diabetic nephropathy (Ruan et al., 2015). Nevertheless, studies on the characteristics of CG disposition remain limited.

Recently, our group studied the pharmacokinetic profiles of the active components of Astragali Radix and found that CG and its glucuronide were rapidly absorbed, with a peak concentration of about 100 ng/mL, after oral administration of the water extract of this herb (Shi et al., 2015). This suggested that CG could traverse the enterocytes intact. This finding contradicts that of a previous study, which reported that isoflavonoid glucosides cannot cross the Caco-2 monolayer (Islam et al., 2014), and hydrolysis by LPH is not essential for CG absorption. Glucosides exhibit high polarity, which allows them to diffuse across the cellular membrane. As a result, some of these compounds can be transported into enterocytes via glucose transporters (GLTs) such as sodium-dependent glucose transporter 1 (SGLT-1) and glucose transporter 2 (GLUT-2) (Wolffram et al., 2002; Day et al., 2003). Thus far, studies on
the effect of GLTs on isoflavonoid glucosides are limited and the underlying mechanism remain unclear.

Although LPH may not play a key role in CG hydrolysis, it was recently reported that calycosin glucuronide was the major component in plasma after oral administration of CG (Ruan et al., 2015). This finding suggested that most of the absorbed CG was hydrolyzed in enterocytes. Generally, after being transported into enterocytes, glucosides are deglycosylated by broad-specific β-glucuronidases (BSβG) and glucocerebrosidase, which are two major intracellular β-glucosides hydrolases (Day et al., 2003). However, studies on the characteristics of CG hydrolysis in enterocytes are limited. Although a recent study examined calycosin glucuronidation (Ruan and Yan, 2014), it only focused on liver microsomes, and its metabolic characteristics in intestinal microsomes remain unexplored. UDP-glucuronosyltransferases (UGT) enzymes are also abundantly expressed in small intestines, which are the sites of first isoflavonoids metabolism after oral administration. Therefore, it is necessary to study the corresponding metabolic characteristics of calycosin and CG in intestinal microsomes.

In this work, we investigated CG absorption, hydrolysis, and metabolism after oral administration of pure CG to determine its physiological functions. We used the rat intestinal perfusion model and performed in vivo pharmacokinetic studies with in vitro hydrolysis experiments and Caco-2 cell line to investigate CG absorption, hydrolysis, metabolism and transportation in rats. The enzyme kinetics of calycosin and CG in rat liver/intestinal microsomes (RLMs and RIMs) was also assessed to
characterize enzyme catalysis in liver and intestines.

**Materials and Methods**

**Materials**

CG, calycosin, and daidzin (≥ 98%, HPLC grade) were purchased from Chengdu Must Biotechnology Co., Ltd. (Chengdu, China) and used to prepare standard solutions of phase II metabolites in our laboratory (Shi et al., 2015). Glucurolactone, CaCl₂, MgSO₄, KCl, KH₂PO₄, NaCl, Na₂HPO₄, NaHCO₃, DMSO, uridine diphosphate glucuronic acid (UDPGA), alamethicin, MgCl₂, MK571, ko143, D-saccharic-1,4-lactone monohydrate, HEPES, and Hanks balanced salt solution were purchased from Sigma–Aldrich (St. Louis, MO, USA). Gluconolactone was obtained from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), and conduritol B epoxide was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). Erlotinib, used as internal standard (IS), was purchased from Cayman Chemical Company (Ann Arbor, MI, USA). RLMs, RIMs, and rat intestinal S9 were prepared in our laboratory by using a previously published method (Jia et al., 2004). All other chemicals (typically analytical grade) were used as received.

**Animals**

Male Sprague–Dawley rats (80–110 days old), weighing 250–300 g, were obtained from the Laboratory Animal Center of Guangzhou University of Chinese Medicine. Four rats were housed per cage in a room with unidirectional air flow under 40%–70% relative humidity, 20 °C–24 °C, and 12 h light/dark cycle. Isoflavonoids were not detected in the blank samples of plasma, bile, and perfusate, which were obtained through perfusion of a segment of the rat upper small intestine; this finding
indicates the absence of isoflavonoids in the samples. The rats were fasted overnight and given access to water prior to the experiments. The animal protocol used in the study was approved by the Southern Medical University’s Ethics Committee.

**Cell culture**

Culture conditions for Caco-2 cells have been described previously (Hu et al., 1994). Briefly, a cell monolayer was prepared by seeding $4 \times 10^5$ cells per insert (Nunc; surface area, 4.6 cm$^2$; pore size, 3 µm). Cells were maintained at 37 °C with 90% humidity and 50% CO$_2$, fed every day, and determined ready for transport experiments (19-21) d after seeding. The integrity of each monolayer was determined by measuring the transepithelial electrical resistance (TEER) (Millicell ERS®) before the experiments were conducted. Cell monolayers with TEER values less than 460 Ω/cm$^2$ were discarded (Yang et al., 2011).

**In vivo pharmacokinetic study in rats**

CG was dissolved in 20% (w/v) hydroxypropyl β-cyclodextrin aqueous solution, sterilized, and filtered through a 0.22 µm filter. Single dose of CG (0.5 mg/kg) was administered via the tail vein (i.v.). Blood samples (0.2 mL) were collected from the vein of the eye sockets and placed into dried heparinized tubes at 2, 5, 10, 20, 30, 45, 60, 90, 120, 180, 240, 360, 480, and 720 min. For the oral administration (p.o.) and intraperitoneal (i.p.) injection groups, blood samples (0.2 mL) were collected at 5, 10, 20, 30, 45, 60, 90, 120, 180, 240, 360, 480, and 720 min after administration of a single dose of CG (10 mg/kg). The blood samples were immediately centrifuged at 5510 g for 8 min, and plasma was stored at −80 °C until analysis.

Plasma samples (50 µL) were mixed with 10 µL of IS solution (5.95 ng/mL
erlotinib in methanol) and 200 μL of methanol. After vortexing to homogenize, the mixture was centrifuged at 19357 g for 30 min. The supernatant was transferred into a new tube and evaporated to dryness under a nitrogen stream. The residue was reconstituted in 100 μL of 50% methanol solution and centrifuged at 19357 g for 30 min. 1 μL of the supernatant was injected into the UPLC–MS/MS column for analysis.

Pharmacokinetic parameters were calculated using the WinNonlin® 3.3 software (Pharsight Corporation, Mountain View, CA, USA). Systemic bioavailability of CG after oral (Foral) administration was calculated as follows:

\[ F_{oral} = \frac{AUC_{oral} (0-\infty)}{Dose_{oral}} \times \frac{AUC_{i.v} (0-\infty)}{Dose_{i.v}} \times 100\% \quad (1) \]

where AUCoral (0-∞) and AUCi.v (0-∞) are areas under the plasma concentration curves and calculated after oral and intravenous (i.v.) administration, respectively.

**In situ absorption and hydrolysis experiments using the perfused rat intestinal model**

Single-pass intestinal perfusion was performed similar to previously described methods with minor modifications (Xia et al., 2012; Cai et al., 2013). The perfusate buffer, a pH 6.5 phosphate buffer, contained 1.26 mM CaCl₂, 0.81 mM MgSO₄, 5.37 mM KCl, 0.44 mM KH₂PO₄, 170.67 mM NaCl, 0.34 mM Na₂HPO₄, 4.43 mM NaHCO₃, 25.02 mM HEPES, and 2.5 or 10 μM CG. The perfusate buffer were then added with glucose or different concentrations of the inhibitors of LPH (glucurolactone), GLTs (phloridzin for SGLT-1, and phloretin for GLUT-2), or efflux transporters (ko143 for breast cancer resistance protein (BCRP), and MK571 for multidrug resistance-associated protein 2 (MRP2)) to investigate the effects of these compounds on the intestinal absorption and metabolism of CG. In addition, daidzin,
which can be hydrolyzed by LPH (Wilkinson et al., 2003), was selected as the positive control to further confirm the effect of LPH on CG. After the rats were anaesthetized by i.p. injection of 1.5 g/kg urethane (50%, w/v), four segments of the rat intestine were simultaneously perfused. The duodenum, jejunum, ileum, and colon were cannulated using 1.5–2.0 cm long cannulas, which can be easily disconnected or reconnected to the main perfusion tube. The main tube was attached to a syringe driven by an infusion pump (Model PHD2000 Harvard Apparatus, Cambridge, MA, USA), and the flow rate was maintained at 10 mL/h. The inlet cannula was insulated, stored in a circulating water bath at 37 °C, and perfused with two CG concentrations (10 and 2.5 μM). After washing for 30 min, which is sufficient to achieve steady-state absorption, the perfusate, bile, and plasma samples were collected every 30 min during the 2.5 h period. After perfusion, the length of every intestinal segment was measured, and each tube containing the sample was weighed as described previously (Xia et al., 2012).

Briefly, 50% methanol with 5.95 ng/mL IS was used to dilute the perfusate and bile samples (1:10) for detection of CG and its metabolites. Plasma samples were also prepared using the same method. The prepared samples were centrifuged at 19357 g for 30 min. 1 μL of the supernatant was injected into UPLC–MS/MS columns for analysis.

CG permeability was represented by $P^{*}_{\text{eff}}$, which can be calculated using a previously described method (Wang et al., 2006).

$$P^{*}_{\text{eff}} = \frac{1-C_{m}/C_{o}}{4G_{z}}$$ (2)

$P^{*}_{\text{eff}}$ represents the apparent permeability of the compound, which indicates the amount loss in the perfusate samples. $C_{o}$ and $C_{m}$ are the inlet and outlet concentrations, which were corrected for water flux by using the sample weight, respectively; $G_{z}$
$G_z = \pi DL/2Q$ is a scaling factor incorporating flow rate ($Q$) and intestinal length ($L$), and diffusion coefficients ($D$) to obtain a dimensionless permeability value.

The amounts of absorbed CG ($M_{ab}$) and the corresponding calycosin glucuronide excreted into the intestinal lumen ($M_{met}$) were calculated as described previously (Wang et al., 2006). Generally, $M_{ab}$ is expressed as

$$M_{ab} = Q \tau (C_o - C_m) \quad (3)$$

where $\tau$ is the sampling interval (30 min), and other parameters were identical with those defined in Eq. 1. The amounts of metabolites ($M_{met}$) were determined as

$$M_{met} = Q \tau C_{met} \quad (4)$$

where $C_{met}$ is the outlet concentration (nmol/mL) of the metabolites corrected for water flux.

**Transport experiments of analytes in cultured Caco-2 cells**

Triplicate experiments were performed in HBSS; the pH of HBSS in both apical (AP) side and the basolateral (BL) side was 7.4. Protocol for transport experiments were similar to those described previously (Ye et al., 2013). In brief, the testing solution (2 mL, containing 5 $\mu$M calycosin) was loaded onto one side of the cell monolayer and blank HBSS (2 mL) was loaded onto the other side. Five sequential samples (0.5 mL) were taken at different times (0, 0.5, 1, 1.5, and 2 h) from both sides of the cell monolayer and 250 $\mu$L of IS solution (5.95 ng/mL erlotinib in methanol) was immediately added to each sample to stabilize them. The same volume of testing solution or blank HBSS was immediately added to replace the samples obtained. Transport experiments were also performed in the presence of ko143 (10 $\mu$M) and MK571 (10 $\mu$M).

Intracellular concentrations of analytes were determined when the transport
experiments were conducted. The procedure used to determine the analytes was the same as that described previously (Jeong et al., 2005). Briefly, the cell membranes were carefully washed three times with ice-cold blank HBSS at the end of the transport study. Cells attached to the polycarbonate membranes were cut off from the inserts, immersed in 0.3 mL blank HBSS, and sonicated for 30 min in an ice bath (4 °C), and 50 μL of IS solution (5.95 ng/mL erlotinib in methanol) was immediately added to 100 μL each sample to stabilize them.

Briefly, 50% methanol was used to dilute the samples (1:5) for detection of analytes. The prepared samples were centrifuged at 19357 g for 30 min. 1 μL of the supernatant was injected into UPLC–MS/MS columns for analysis.

The rate of transport \( (B_t) \) were obtained from the rate of change in the concentration of the transported calycosin glucuronide, which can be express as a function of time and the volume of the sampling chamber \( (V) \):

\[
B_t = \frac{dC}{dt} \times V
\]  

(5)

The apparent intrinsic clearance \( (CL) \) was calculated here because the extracellular concentration of calycosin glucuronide could be very different from its intracellular concentration. CL was determined from the rate of glucuronide excretion \( (B_{ex}) \) divided by the intracellular concentration of glucuronide \( (C_i) \):

\[
CL = \frac{B_{ex}}{C_i} = \frac{B_{max}}{K_m + C_i}
\]  

(6)

where \( B_{max} \) is the excretion rate of glucuronide and \( K_m \) is the Michaelis constant. We assumed that the average volume of the engineered Caco-2 cells is 4μl/mg protein (Yamaguchi et al., 2000). The intracellular concentration of metabolite was calculated from the total amount of intracellular glucuronide divided by the total volume of intracellular protein.

The fraction of the dose metabolized \( (f_{act}) \) was calculated here as a proper
parameter to describe the extent of calycosin metabolism in the Caco-2 monolayer (Pang et al., 2009), where $M_{\text{et}}_{\text{total}}$ is the total metabolite concentration and $C_{\text{al}}_{\text{total}}$ is the total calycosin combined in the receiver side, the donor side, and within the cell at 2h:

$$f_{\text{met}} = \frac{M_{\text{et}}_{\text{total}}}{d_{\text{o}}se} = \frac{M_{\text{et}}_{\text{total}}}{M_{\text{et}}_{\text{total}} + C_{\text{al}}_{\text{total}}} \quad \text{(7)}$$

**Hydrolysis experiment using rat intestinal S9**

The hydrolysis characteristics of CG and the functions of different glucosidases were determined using rat intestinal S9. Incubation mixtures (total volume 400 μL) containing 50 mM potassium phosphate buffer (pH 7.4), CG (25, 100, and 400 μM), and 0.1 mg protein/mL rat intestinal S9 (duodenum, jejunum, and ileum) were incubated in the absence or presence of conduritol B epoxide (potent glucocerebrosidase inhibitor) or gluconolactone (specific BSβG inhibitor). Several studies reported the specificity of different enzyme inhibitors for intestinal glucosidases (Daniels et al., 1981; Day et al., 1998). The low, middle, and high concentrations of conduritol B epoxide were 1.25, 2.5, and 5 mM, whereas those of gluconolactone were 2.5, 5, and 10 mM, respectively. The reactions were performed at 37 °C for 15 min and terminated by adding 200 μL of methanol with 5.95 ng/mL IS. The reaction mixtures were centrifuged at 19357 g for 30 min, and 1 μL of the supernatant was injected into the UPLC–MS/MS for analysis. All samples were performed in triplicate.

**In vitro Enzymes Kinetic studies**

Calycosin and CG were incubated with MgCl$_2$ (0.88 mM), saccharolactone (4.4 mM), alamethicin (0.022 mg/mL), and RLMs and RIMs (final concentration =
0.01325 mg of protein per milliliter) in 50 mM potassium phosphate buffer (pH 7.4). This reaction mixture, with a total volume of 400 μL with 0.5–200 μM calycosin and 10–800 μM CG, was used to measure glucuronidation kinetics. The reaction was initiated by adding 3.5 mM UDPGA, stored for 15 min at 37 °C, and terminated by adding 200 μL of methanol with 5.95 ng/mL IS. The reaction mixtures were vortexed immediately, followed by centrifugation at 19357 g for 30 min. Subsequently, 1 μL of the supernatant was injected into the UPLC–MS/MS for analysis. All samples were performed in triplicate.

Kinetic parameters were obtained based on the profile of Eadie–Hofstee plots as described previously (Wang et al., 2006). If Eadie–Hofstee plot was linear, formation rates (V) of glucuronide of calycosin and CG at different substrate concentrations (C) were fit to the standard Michaelis–Menten equation:

\[
V = \frac{V_{\text{max}} \times C}{K_m + C}
\]  

where \( K_m \) is the Michaelis–Menten constant and \( V_{\text{max}} \) is the maximum rate of glucuronidation.

If Eadie–Hofstee plots showed characteristic profiles of atypical kinetic (Hutzler and Tracy, 2002), the data from these atypical profiles were fit to Eqs. 6, using the ADAPT II program. To confirm the best–fit model, the model candidates were discriminated using the minimum Akaike’s information criterion value, and the rule of parsimony was employed.

The following Eq. 6 describes enzyme reactions with biphasic kinetic:

\[
\text{Reaction rate} = \frac{V_{\text{max1}} \times C}{K_{m1} \times C} + \frac{V_{\text{max2}} \times C}{K_{m2} \times C}
\]  

where \( V_{\text{max1}} \) is the maximum enzyme velocity of the high-affinity phase, \( V_{\text{max2}} \) is the maximum velocity of the low-affinity phase, \( K_{m1} \) is concentration of substrate to achieve half of \( V_{\text{max1}} \) for high-affinity phase, and \( K_{m2} \) is concentration of substrate to
achieve half of \( V_{\text{max2}} \) for low-affinity phase.

**Analysis and Identification of CG and its Metabolites**

All samples were analyzed by using Agilent 6460 Triple Quadrupole mass system and 6540 accurate-mass Q-TOF MS system. The conditions were as follows: column, Zorbax SB-C18, 1.8 \( \mu \)m, 3.0 x 100 mm; mobile phase A, 100% aqueous buffer (0.1%, \( v/v \) formic acid, pH 2.5), mobile phase B, 100% acetonitrile; flow rate, 0.3 mL/min; gradient, 0–0.5 min, 15–15% B, 0.5–4 min, 15–60% B, 4–4.5 min, 60–90% B, 4.5–6 min, 90–90% B, 6–6.5 min, 90–15% B; and injection volume, 1 \( \mu \)L. Electrospray ionization was performed in positive mode.

The main mass working parameters for the mass spectrometers were set as follows: capillary voltage 3.5 kV, nozzle voltage 500 V, gas temperature 280 °C, and sheath temperature 380 °C with sheath gas flow of 11 L/min and gas flow of 5 L/min.

Data were recorded and the system was controlled using the MassHunter software (version B.06.00, Agilent Technologies).

This LC-MS/MS method has been validated. In plasma, the intra-day precision (RSD) of analysts ranged between 1.41 and 11.2%, and accuracy (RE) ranged between −12.86 and 7.92%. The inter-day precision (RSD) of analysts ranged between 4.89 and 9.21%, and accuracy (RE) ranged between −11.06 and 5.43%. The recovery ranged from 91.4 to 112.39% and the matrix effects were between 52.9 and 78.4%. In bile, the intra-day precision (RSD) of analysts ranged between 5.46 and 10.9%, and accuracy (RE) ranged between −8.44 and 10.89%. The inter-day precision (RSD) of analysts ranged between 3.32 and 8.97%, and accuracy (RE) ranged between −9.61 and 3.28%. The recovery ranged from 89.7 to 99.01% and the matrix effects were between 74.3 and 89.2%. In perfusate, the intra-day precision
(RSD) of analysts ranged between 1.96 and 12.33%, and accuracy (RE) ranged between −6.77 and 13.89%. The inter-day precision (RSD) of analysts ranged between 5.28 and 10.78%, and accuracy (RE) ranged between −10.11 and 5.87%. The recovery ranged from 91.4 to 102.41% and the matrix effects were between 87.3 and 92.4%.

The position of glucuronic acid substitution of calycosin was determined through $^1$HNMR spectroscopy. All readings were recorded on Bruker AV-500 spectrophotometer (Bruker, Faellanden, Switzerland). The purified major metabolite of calycosin (M1, 2.5 mg) was stored at −20 °C before dissolution in deuterated DMSO for $^1$HNMR analysis. Chemical shifts were given on the $\delta$ scale and referenced to tetramethylsilane at 0 ppm for $^1$HNMR (500 MHz).

Statistical Analysis

One-way ANOVA with or without Tukey–Kramer multiple comparison (post hoc) tests were used to evaluate statistical difference. Student’s $t$ test was also performed. Differences were considered significant at $p$ values < 0.05.

Results

Analysis and Identification of CG and its Metabolites

The LC–MS/MS method for CG and its metabolites exhibited a run time of 6.5 min (Fig. 1). The tested linear response range was 0.04–10 μM (for nine concentrations), with a lower limit of quantification (LLOQ) of 0.02 μM.

The retention time of CG, CG glucuronide and calycosin were 4.13, 3.12 and 5.98 min in the samples (Figs. 1A, 1B and 1C) respectively. CG concentration in the bile sample was lower than the LLOQ of the LC–MS/MS analysis method. Calycosin
was not detected in the bile and plasma samples. There were two monoglucuronides of calycosin were detected in the incubation system (Fig. 1D), which was consistent with previous reports (Ruan and Yan, 2014; Ruan et al., 2015). And the obtained monoglucuronides were eluted at 4.61 (M1) and 4.19 min (M2). However, in the perfusate, bile and plasma samples, only one monoglucuronide of calycosin was detected. High-resolution mass spectrometry was performed to determine the MS spectrum of the metabolite. The CG glucuronide showed a pseudo-molecule ion [M+H]+ of m/z 623.1600 in full-scan mass spectra and fragment ions at m/z 447.1 and 285.2; this finding indicates that this glucuronide exhibited the molecular formula of C_{28}H_{30}O_{16} (or CG-7-glucuronide). M1 and M2 showed a pseudomolecule ion [M+H]+ of m/z 461.1074 in full-scan mass spectra and fragment ions at m/z 285.0; hence, these monoglucuronides demonstrated a molecular formula of C_{22}H_{20}O_{11} (or calycosin-3’-glucuronide or calycosin-7-glucuronide). The substitution position of the proton of 3’-OH was confirmed through nuclear overhauser effect spectroscopy [correlation between Glu-H-1” (δH 5.08, 1H, d, J=7.0 Hz) and H-2’ (δH 7.28, 1H, d, J=2.0 Hz)] (Supplemental Figs. S1–S3). Therefore, the structure of M1, which was found to be the major metabolite of calycosin, was unambiguously identified as calycosin-3’-glucuronide. As such, M2 was tentatively assigned as calycosin-7-glucuronide.

**Stability of CG and its Metabolites**

The stability of CG and its metabolites in the perfusate buffer, blank bile, and blank plasma was tested to ensure that CG metabolism is not due to chemical instability. The results illustrated that the stabilities of the analytes in the blank perfusate and bile samples showed 90–110 % recoveries after incubation at 37 °C for
1, 2, 3, and 4 h (Supplemental Table S1 and S2). The stability of CG in the rat blank plasma also showed no less than 85% recoveries after the following storage conditions: 25 °C for 6 h, 37 °C for 6 h, −80 °C for 15 days, three freeze–thaw cycles (−20 °C and 25 °C), and 5 °C for 12 h (Supplemental Table S3). These results demonstrate that CG and its metabolites were stable in the perfusate buffer, blank bile and blank plasma.

In vivo Pharmacokinetic Studies of CG and its Metabolites in Rats

The mean plasma concentration–time profiles of CG and its metabolites after i.v. (0.5 mg/kg), i.p. (10 mg/kg) and p.o. (10 mg/kg) administrations of CG are shown in Fig 2. Pharmacokinetic parameters were calculated using the non-compartmental method with WinNonlin 3.3, and the results are presented in Table 1.

After p.o. administration of CG, the $C_{\text{max}}$ and AUC$_{0-\infty}$ values of calycosin-3'-glucuronide were 1189.66 ± 346.95 ng/mL and 111.16±34.18 min·μg/mL respectively, which were much higher than those of CG glucuronide and CG. After i.p. administration, CG exhibited the highest $C_{\text{max}}$ (965.24 ± 133.53 ng/mL), followed by CG glucuronide and calycosin-3'-glucuronide. However, the AUC$_{0-\infty}$ of CG (72.64±22.08 min·μg/mL) was not the highest because the elimination of CG was very fast ($t_{1/2} = 77.5 \pm 17.91$ min) and its plasma concentration was lower than 10 ng/mL after 4 h. The AUC$_{0-\infty}$ of calycosin-3'-glucuronide was 99.11±45.90 min·μg/mL. After i.v. administration of CG, the plasma concentrations of CG glucuronide and calycosin-3'-glucuronide were considerably low, and the AUC$_{0-\infty}$ of CG was 5.39±1.02 min·μg/mL. Thus, the absolute oral bioavailability of CG was 0.304%. Moreover, calycosin was not detected in the plasma after CG administrations.
Regional Transport and Metabolism of CG in a Perfused Rat Intestinal Model

After perfusion with 10 and 2.5 μM CG, it was rapidly absorbed in the duodenum, jejunum, and ileum, with rates higher than those in the colon. Moreover, $P_{\text{eff}}^*$ values of 2.5 μM in all intestinal segments were higher than those of 10 μM, but the difference was not significant (Fig. 3A). CG was converted into calycosin-3’-glucuronide and calycosin in the rat intestine, large amounts of calycosin-3’-glucuronide were detected in the perfusate sample, with the highest excretion in the jejunum and the lowest in the colon (Fig. 3C). In addition, low amounts of CG glucuronide were detected in the perfusate sample, but the concentration was lower than the LLOQ of the analytical method (0.02 μM). In the bile sample, the glucuronides of calycosin and CG were detected, and the concentration of calycosin-3’-glucuronide was higher than that of CG glucuronide. Moreover, calycosin and CG were not detected in the bile sample. Calycosin-3’-glucuronide, CG, and CG glucuronide were detected in the plasma sample, with calycosin-3’-glucuronide having the highest concentration. By contrast, calycosin was not detected in the plasma. Furthermore, CG sulfate was not detected in the perfusate, bile, and plasma samples.

Effect of LPH and Glucose in Absorption of CG and Daidzin

The LPH inhibitor glucorolactone (20 and 40 mM) was used to inhibit the hydrolysis of CG and daidzin to their corresponding aglycones and to determine the effect of this mechanism on absorption and metabolism. Surprisingly, glucorolactone had no influence on CG absorption and calycosin-3’-glucuronide excretion in the perfusate sample at two concentrations (Figs. 3D–F). In addition, the concentrations of CG and its metabolites did not change in the bile (Figs. 4C–D) and plasma (Figs.
5D–F) samples. By contrast, glucurolactone concentration-dependently reduced the absorption of daidzin, which was selected as the positive control, and the excretion of daidzein glucuronide in the perfusate sample (Figs. 3G–I). The effect of glucurolactone on the excretion of daidzein glucuronide was more remarkable in the upper small intestine than that in the colon, as evidenced by the higher LPH quantity in the small intestine. In addition, daidzein glucuronide concentration was significantly reduced in the bile (Figs. 4E) and plasma (Figs. 5G) samples. By contrast, daidzin concentration in the plasma (Figs. 5H) was significantly enhanced in the presence of glucurolactone.

Glucose, which can saturate GLTs, reduced CG absorption and calycosin-3’-glucuronide excretion in the perfusate samples of the upper small intestine (duodenum, jejunum, and ileum) (Figs. 3D–F). Glucose did not affect CG absorption in the colon but significantly reduced the concentration of CG and its metabolites in the bile and plasma samples; this finding was consistent with reduced absorption in the perfusate sample (Figs. 4C–D, and Figs. 5D–F). Glucurolactone (40 mM) was also added to the perfusate with glucose to further determine the effect of LPH on CG. Similarly, glucurolactone played minimal or no role on CG absorption and metabolism in the rat intestine (Figs. 3D–F, Figs. 4C–D, and Figs. 5D–F). As the positive control, the absorption of daidzin and the excretion of its metabolite significantly decreased in the perfusate sample added with glucose (Figs. 3G–I). The concentrations of daidzein glucuronide and daidzin in the bile and plasma samples also decreased in the presence of glucose in the perfusate sample (Fig. 4E, and Figs. 5G–H). Moreover, glucurolactone reduced daidzin absorption and daidzein glucuronide excretion in the perfusate sample containing glucose (Figs. 3G–I), which is consistent with previous reports (Liu and Hu, 2002). Daidzein glucuronide
concentration significantly decreased in the bile (Fig. 4E) and plasma samples (Fig. 5G), and daidzin concentration significantly increased in the plasma sample (Fig. 5H). These results demonstrated that LPH played minimal to no role on CG hydrolysis.

Importance of GLTs in CG Absorption

SGLT-1 and GLUT-2 are main intestinal GLTs (Zou et al., 2014). The SGLT-1 inhibitor phloridzin and the GLUT-2 inhibitor phloretin were used to determine the effects of GLTs on CG absorption and metabolism. After perfusion with phloridzin (100, 200, and 400 μM), CG absorption and calycosin-3′-glucuronide excretion were significantly and concentration-dependently reduced in the perfusate sample in duodenum, jejunum and ileum (Figs. 3J–L). Similar to glucose, phloridzin also played no role on CG absorption in the colon, as evidenced by the absence of SGLT-1 expression in the rat colon (Balen et al., 2008). Meanwhile, the concentrations of CG and its metabolites in the bile and plasma samples significantly decreased in the presence of phloridzin in the perfusate sample, which was consistent with reduced absorption in the perfusate sample (Figs. 4F–G, and Figs. 5I–J). Moreover, CG glucuronide concentration was moderately reduced in the plasma (Fig. 5K), but the effect was not statistically significant. By contrast, phloretin (50, 100, and 200 μM) had no evident effect on CG absorption and its metabolite excretion in the perfusate sample of each intestinal segment at different concentrations (Figs. 3M–O). In the bile (Figs. 4H–I) and plasma (Figs. 5L–N) samples, the concentrations of analytes remained constant in the presence of phloretin in the perfusate sample at different concentrations.

Role of efflux transporters on Absorption and Excretion of CG and its
metabolites

After perfusion with ko143 (50 μM), the inhibitor of BCRP, and MK571 (50 μM), the inhibitor of MRP2, CG absorption was enhanced in the perfusate sample of duodenum and jejunum (Figs. 3P–R), but the increase was not statistically significant. The excretion of calycosin-3’-glucuronide significantly reduced in all four segments of the intestine (Fig. 3R). Moreover, the concentrations of calycosin-3’-glucuronide and CG glucuronide in the bile significantly increased in the presence of ko143 and MK571 in the perfusate (Figs. 4J–K). By contrast, the concentrations of calycosin-3’-glucuronide, CG, and CG glucuronide only slightly (but not significantly) increased in the plasma samples (Figs. 5O–Q).

Effects of inhibitors of BCRP and MRP2 on the efflux of calycosin glucuronide in Caco-2 cells

Bidirectional calycosin 5 μM (positive control) transport across Caco-2 cells was studied in the presence of ko143 (10 μM) and MK571 (10 μM). In the presence of 10 μM ko143 or 10 μM MK571, the efflux rates of calycosin-3’-glucuronide from the cells to the AP side and BL was decreased in the bidirectional transport studies (Figs. 6A and B). Similarly, ko143 and MK571 showed inhibitory effects on the CL of calycosin glucuronide from cells to the AP side (71% for A–B, 64% for B–A by MK571; 54% for A–B, 57% for B–A by ko143) or the BL side (52% for A–B, 54% for B–A by MK571; 53% for A–B, 33% for B–A by ko143, p<0.05) in the bidirectional transport studies (Figs. 6C and D). On the other hand, the f_{net} values were significantly lower (p<0.05) and the intracellular concentrations of calycosin glucuronide were significantly higher in the presence of ko143 or MK571 in both A–B and B–A directional transports (Figs. 6E and F).
Effects of Chemical Inhibitors on the Hydrolysis of CG in S9 of Duodenum, Jejunum and Ileum

The effect of LPH on the brush border enterocytes on CG hydrolysis was negligible. The hydrolytic site of CG is likely to be inside the enterocytes. Intestinal S9 was considered as the model for intestinal deconjugation study (Islam et al., 2014). Moreover, intracellular glucosidases were found in intestinal S9. Therefore, the inhibitory effects of gluconolactone and conduritol B epoxide on CG hydrolysis in intestinal S9 of duodenum (Figs. 7A and B), jejunum (Figs. 7C and D), and ileum (Figs. 7E and F) were investigated to confirm the effect of glucocerebrosidase and BSβG on CG hydrolysis in vitro (Day et al., 1998; Islam et al., 2014). In the S9 of duodenum, jejunum, and ileum, gluconolactone significantly inhibited calycosin formation at three CG concentrations. Three concentrations of gluconolactone also inhibited more than 75% of CG hydrolysis at 25 μM. Similar results were also observed in the inhibition of conduritol B epoxide on CG hydrolysis. However, the inhibitory effect of conduritol B epoxide was weaker than that of gluconolactone.

Kinetics of Calycosin and CG Glucuronidation by RLMs and RIMs

Glucuronidation rates of calycosin and CG in RLMs and RIMs were determined using different substrate concentrations. Within the tested concentration ranges, 3’-glucuronide (M1) was found to be the major metabolite of calycosin in RLMs and RIMs (Figs. 8A and B); hence, RLMs and RIMs are preferential sites for catalysis of calycosin glucuronidation at 3’-OH rather than 7-OH (M2). Both RLMs and RIMs, which mediated M1 glucuronidation, exhibited classic Michaelis–Menten kinetic characteristics. Furthermore, M2 exhibited biphasic kinetic profile (Figs. 8A and B), as evidenced by the Eadie–Hofstee plot (Figs. 8a–d). In addition, only CG
glucuronide was formed in RLMs and RIMs. According to the Eadie–Hofstee plot (Figs. 8e and f), CG glucuronide formations by RLMs and RIMs followed classic Michaelis–Menten kinetic characteristics and substrate inhibition profile, respectively (Figs. 8C and D). All kinetic parameters of the glucuronidation of calycosin and CG are shown in Table 2.

The intrinsic clearance (CL, $V_{\text{max}}/K_m$) of M1 was much higher than that of M2 in RLMs and RIMs (M1, CL 2570.4 mL/min/mg for RLMs, CL 2955.74 mL/min/mg for RIMs; M2, CL 304.35 mL/min/mg for RLMs, CL 57.14 mL/min/mg for RIMs). RIMs exhibited lower $K_m$ and higher $V_{\text{max}}$ values for the formation of CG glucuronide than RLMs. As such, the CL of CG, through glucuronidation in RIMs, was about 30 times higher than that in RLMs (1846.39 mL/min/mg for RIMs and 67.54 mL/min/mg for RLMs).

**Discussion**

In this study, we found that CG and its metabolites rapidly appeared in the plasma sample after oral administration, which indicated that CG was not hydrolyzed by LPH and crossed the enterocytes intact (Fig. 2). This result differed from previous reports, which showed that hydrolysis by LPH was a critical first step in the disposition of isoflavonoid glucosides. We used the rat intestinal perfusion model, which has been demonstrated to be suitable for studying the role of LPH (Dai et al., 2015), and found that LPH inhibitor played minimal role in CG absorption and metabolism in the intestine (Fig. 3D–F, 4C–D, 5D–F). Daidzin, the substrate of LPH and GLTs, was chosen as positive control to ensure that the results were reliable. Consistent with a previous report (Liu and Hu, 2002), absorption and metabolism of daidzin were affected by LPH inhibitor (Fig. 3G–I, 4E, 5G–H). In addition, to determine whether
GLTs affected the role of LPH on the absorption of CG, we added glucose into perfusate buffer to saturate GLTs. We found that CG could not be hydrolyzed by LPH after the GLTs saturated (Fig. 3D–F, 4C–D, 5D–F). However, the absorption and metabolism of daidzin were affected by LPH inhibitor after the GLTs saturated (Fig. 3G–I, 4E, 5G–H). Thus, the effect of LPH was not influenced after the GLTs saturated. These results suggest that CG may not be hydrolyzed by LPH.

After oral administration of CG, calycosin-3'-glucuronide was found to be main form circulating in plasma (Fig. 2). Hence, deglycosylation of CG to calycosin was an important step before entry into systemic circulation. Since small intestine and liver can hydrolyze isoflavonoid glucosides (Day et al., 1998), we studied the pharmacokinetics of CG after i.p. administration to avoid intestinal disposition. The results showed that CG rapidly entered systemic circulation and became the main circulating component in the plasma, thereby limiting hydrolysis in the liver (Fig. 2). The gut microbiota has also been thought to hydrolyze glucosides (Bokkenheuser et al., 1987). It was reported that rutin was not absorbed until it was hydrolyzed by gut microbiota, and the absorption peak of its metabolites occurred at 7 h (He et al., 2013). However, we found that, after oral administration, CG and its metabolites were rapidly absorbed, with an absorption peak at 45 min (Fig. 2). This indicated that the role of gut microbiota in CG hydrolysis could be significantly small. Therefore, the site of CG hydrolysis is very likely to be the upper small intestine.

CG cannot easily cross cellular membranes through diffusion because of its large molecular weight. However, we found that the $P_{\text{eff}}$ values of CG in the small intestine were around 1.5 (Figs. 3A–C, 4A–B, 5A–C). Generally, $P_{\text{eff}}$ values exceeding 1.0 is considered to indicate good absorption (Johnson and Amidon, 1988). Furthermore, glucose, which exhibits high-affinity with GLTs, significantly reduced the absorption...
and metabolism rates of CG in the intestine, and the concentrations of CG and its metabolites in bile and plasma samples (Figs. 3D–F, 4C–D, 5D–F). Therefore, the most likely carrier molecule to facilitate intestinal absorption of CG could be GLTs, which could transport some flavonoid glucosides (Walgren et al., 2000). By using specific inhibitors of GLTs, our results showed that CG transport from the lumen to enterocytes was mediated by SGLT-1 (Figs. 3J–L, 4F–G, 5I–K), and the role of GLUT-2 was negligible (Figs. 3M–O, 4H–I, 5L–N). Additionally, $P_{\text{eff}}$ values of CG were the highest in the jejunum and the lowest in the colon (Figs. 3A–C). This finding could be related to the distribution of SGLT-1 in the intestine. Previous studies reported that SGLT-1 expression levels in the small intestine of rats exhibited the following order: jejunum > duodenum = ileum but was not observed in the colon (Balen et al., 2008). Therefore, the main absorption site of CG could be the upper small intestine, which should be considered in the design of CG oral preparations.

Given that CG could be hydrolyzed in enterocytes, we used intestinal (duodenum, jejunum and ileum) S9, which is an appropriate model to study intestinal deconjugation since it contains all cytosolic and membrane-bound enzymes, and inhibitors of BSβG and glucocerebrosidase (Islam et al., 2014), to identify the enzyme that hydrolyzes CG. Conduritol B epoxide, the specific inhibitor of glucocerebrosidase, showed complete inhibition at 5 mM (Day et al., 1998). Our results showed that conduritol B epoxide inhibited CG hydrolysis in the intestinal S9 in a dose-dependent manner (Fig. 7B, D, F). In contrast to conduritol B epoxide, gluconolactone was not considered a specific inhibitor; this compound completely inhibited BSβG at 10 mM and exhibited weak inhibitory effect on glucocerebrosidase (Islam et al., 2014). Gluconolactone also showed concentration-dependent effects on CG. This result indicated that CG may be hydrolyzed by BSβG and
glucocerebrosidase. However, the kinetics and inhibition mechanisms of GLT inhibitors remain unclear because we did not obtain pure enzymes of BSβG and glucocerebrosidase. Thus, studying the mechanisms by which conduritol B epoxide and gluconolactone exert inhibitory effects require further studies, and in vitro data on their inhibition should be interpreted with caution.

MRP2 and BCRP are important transporters for the efflux of glucuronides (Jiang et al., 2012). We found that the amount of calycosin-3'-glucuronide in the perfusate samples significantly decreased in the presence of MK571 and ko143 (Fig. 3R). Furthermore, experiments conducted on Caco-2 cells showed that MK571 and ko143 could decrease the efflux rates, CL and \( f_{\text{met}} \), and increase the intracellular concentrations of calycosin-3'-glucuronide (Fig. 6). Taken together, these results showed that MRP2 and BCRP were involved in the excretion of calycosin-3'-glucuronide. However, unlike MK571 and ko143 increased the concentration of metabolites of CG in the bile and plasma samples (Figs. 4J–K, 5O–Q), these inhibitors reduced the efflux rates and CL of calycosin-3'-glucuronide from Caco-2 cells to the BL side (Fig. 6). A possible reason for this could be that there was no mucus covering the Caco-2 cell; therefore the inhibitors could reach the BL side to inhibit the efflux (Liu et al., 2012). As a result, after BCRP and MRP2 were inhibited, most of calycosin-3'-glucuronide was transported to the bile and plasma in the intestinal perfusion model.

The results of the kinetic assays showed that the glucuronidation rate of calycosin at 3'-OH was higher than that at 7-OH in RLMs and RIMs. Moreover, the glucuronidation rate of calycosin at 3'-OH in RIMs was twice as high as that in RLMs (Fig. 8). Rapid glucuronidation at 3'-OH could be the reason why only calycosin-3'-glucuronide was detected in the perfusate, bile, and plasma samples, and
aglycone calycosin was not detected in the plasma sample. Similar to the case of calycosin, the glucuronidation rate of CG in RIMs was higher than that in RLMs (Fig. 8) and the CG glucuronidation by RIMs showed substrate inhibition kinetics. Such an atypical kinetics profile has been described as a two-site model in which one binding site is productive, while the other one is inhibitory at high substrate concentration, resulting in decreased velocity with increased substrate concentration (Hutzler et al., 2002). It has been reported that isoflavonoids, including calycosin, can be metabolized by multiple UGT enzymes (Ruan and Yan, 2014; Tang et al., 2009), and the rates and profiles of metabolism by individual UGT enzymes are different. Distribution of these enzymes in RLMs and RIMs are diverse and the rates and profiles of isoflavonoids glucuronidation in microsomes are determined by UGT enzymes (Zhou et al., 2010), which could explain why the profile of CG glucuronidation in RIMs was different from that of calycosin glucuronidation in RLMs and RIMs and of CG glucuronidation in RLMs. Although the liver is the main metabolic organ and several studies have focused on calycosin metabolism at this site (Ruan et al., 2015), our results indicate that, as the first site of metabolism after oral administration, more attention should be paid to the importance and characteristics of metabolism of calycosin and CG in the intestine.

In conclusion, the present study describes the characteristics of CG absorption and disposition in the rat intestine. We found that the intestine could be the main organ responsible for the hydrolysis and first-pass metabolism of CG. In contrast to the case of other structurally analogous isoflavonoid glucosides such as daidzin, hydrolysis by intestinal LPH, which was thought to be capable of hydrolyzing various flavonoids and isoflavonoid glucosides, was not necessary for CG absorption. This study is the first to determine that isoflavonoid glucosides can be transported by
SGLT-1 into enterocytes using the rat intestinal perfusion model. We also demonstrated that CG was likely to be hydrolyzed by BSβG and glucocerebrosidase in enterocytes. Thus, this study reveals a novel mechanism for the absorption and disposition of isoflavonoid glucosides in the small intestine and elucidates the underlying mechanism. Our findings contribute to improved understanding of the absorption and disposition of flavonoid compounds, which is essential for determining their potential as chemopreventive agents for LPH-deficient individuals.
Authorship contributions

Participated in research design: Jian Shi, Ming Hu, Zhongqiu Liu

Conducted experiments: Jian Shi, Haihui Zheng, Jia Yu

Contributed new reagents or analytic tools: Linlin Lu, Lijun Zhu

Performed data analysis: Jian Shi, Tongmeng Yan, Peng Wu, Ying Wang

Wrote or contributed to the writing of the manuscript: Jian Shi, Ming Hu, Zhongqiu Liu
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biomedical analysis 28:1-11.


Footnotes

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Figure legends

Figure 1. Representative MRM chromatograms and structures of CG, calycosin and their respective glucuronides. LC-MS/MS was used to separate and quantify CG and its metabolites in the experimental samples. Panels A, B, C and D represent the MRM chromatograms showing the retention time and structure of CG, calycosin and their glucuronides in perfusate, bile, plasma and incubation system samples.

Figure 2. The pharmacokinetic profile of CG and its metabolites after p.o. (10 mg/kg, A), i.p. (10 mg/kg, B) and i.v. (0.5 mg/kg, C) administration. The plasma samples were analyzed by UPLC–MS/MS. The plasma concentration–time curves of CG and its metabolites were obtained by plotting the mean plasma concentration versus time. Each data point is the average of six determinations, and the error bar represents the standard deviation of the mean (n = 6).

Figure 3. Effect of different inhibitors on the absorption and metabolism of CG and daidzin in perfusate samples. Effective permeabilities of CG or daidzin, amounts absorbed of CG or daidzin, and amounts of glucuronide of calycosin or daidzein excreted in the perfusate samples after CG was perfused alone at the concentration of 10 and 2.5 μM (A-C), CG was perfused alone or co-perfused with GCL and glucose (D-F), daidzin was perfused alone or co-perfused with GCL and glucose (G-I), CG was perfused alone or co-perfused with phloridzin (J-L), CG was perfused alone or co-perfused with phloretin (M-O) and CG was perfused alone or co-perfused with
MK571 and ko143 (P-R) were determined and normalized over a 10 cm intestinal length. Each column represents the average of four determinations, and the error bar is the S.D. (n=4). The “*” symbol means a statistically significant difference at p<0.05; and “**” means p<0.01.

Figure 4. Effect of different inhibitors on the concentration of metabolites of CG and daidzin in bile samples. The concentrations of glucuronide of calycosin or daidzein, glucuronide of CG in the bile samples after CG was perfused alone at the concentration of 10 and 2.5 μM (A-B), CG was perfused alone or co-perfused with GCL and glucose (C-D), daidzin was perfused alone or co-perfused with GCL and glucose (E), CG was perfused alone or co-perfused with phloridzin (F-G), CG was perfused alone or co-perfused with phloretin (H-I) and CG was perfused alone or co-perfused with MK571 and ko143 (J-K) were determined. Each curve represents the average of four determinations, and the error bar is the S.D. (n=4). The “*” symbol means a statistically significant difference at p<0.05; and “**” means p<0.01.

Figure 5. Effect of different inhibitors on the concentration of metabolites of CG and daidzin in plasma samples. The concentrations of glucuronide of calycosin or daidzin, CG or daidzin, and glucuronide of CG in the plasma samples after CG was perfused alone at the concentration of 10 and 2.5 μM (A-C), CG was perfused alone or co-perfused with GCL and glucose (D-F), daidzin was perfused alone or co-perfused with GCL and glucose (G-H), CG was perfused alone or co-perfused with phloridzin
(I-K), CG was perfused alone or co-perfused with phloretin (L-N) and CG was perfused alone or co-perfused with MK571 and ko143 (O-Q) were determined. Each curve represents the average of four determinations, and the error bar is the S.D. (n=4). The “*” symbol means a statistically significant difference at p<0.05; and “**” means p<0.01.

Figure 6. Effects of inhibitors on the rates of calycosin glucuronide efflux from cellular membrane to the AP side (A) or BL side (B), on the clearance of calycosin glucuronide at AP side (C) or BL side (D) of the Caco-2 cell monolayer, on fmet from the cellular membrane to the AP side or BL side (E), and on the intracellular concentration of calycosin glucuronide (F). Calycosin (5 μM) without inhibitors (open bar), with the MRP2 inhibitor MK571 at 10 μM (gray bar), and with the BCRP inhibitor ko143 (black bar) was added to the AP side (A-B) or BL side (B-A). The inhibitors were added to BL side. The “*” symbol means a statistically significant difference at p<0.05.

Figure 7. Effects of chemical inhibitors of BSβG and glucocerebrosidase on the hydrolyzation of CG in S9 of duodenum (A and B), jejunum (C and D) and ileum (E and F). Hydrolyzation of CG by S9 were measured at three concentrations (25, 100 and 400 μM) and incubated in the presence of inhibitors at 2.5, 5 and 10 mM for gluconolactone and 1.25, 2.5 and 5 mM for conduritol B epoxide. All protein concentrations were 0.1 mg/mL, incubation time for CG in S9 were range from 15 to
45 min. Control experiments were incubated without chemical inhibitors. All incubations were performed in triplicate. Each column represents mean percentage control of formation rates of these three metabolites, and error bars are standard deviations of the mean. The “*” symbol means a statistically significant difference at p<0.05.

Figure 8. Kinetics of calycosin glucuronidation by RLMs (A), RIMs (B) and CG glucuronidation by RLMs (C), RIMs (D) (n=3). The glucuronidation rates were measured at substrate concentration ranging from 1.25 to 200 μM (calycosin) and 10 to 800 μM (CG). The reaction time and protein concentration were controlled so that substrate concentration did not decrease substantially (usually less than 30%) at the end of experiments, which lasted for up to 15 min. Each data point is the average of three determinations, and the error bar represents the standard deviation of the mean. The data points in figures are observed glucuronidation rates, and the curves are plotted using the fitted kinetic parameters (Table 2).
Table 1. Pharmacokinetic parameters of CG and its metabolites in rat plasma after intravenous (i.v., 0.5 mg/kg), intraperitoneal (i.p., 10 mg/kg) and oral (p.o., 10 mg/kg) administration of CG (n=6).

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<th>C&lt;sub&gt;max&lt;/sub&gt; (ng/mL)</th>
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<td>p.o.</td>
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<td>0.32±0.11</td>
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<td>57.29±19.62</td>
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Table 2. Kinetic parameters of calycosin and CG glucuronidation obtained from RLMs and RIMs.

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<th>Compound</th>
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<th>RIMs</th>
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<td><strong>K_m (μM)</strong></td>
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Figure 1
Figure 2
Figure 3
Figure 6
Figure 7
Figure 8

A) Calycosin glucuronide by RLMs
- 7-OH (M2) ■ 3'-OH (M1)

B) Calycosin glucuronide by RIMs
- 7-OH (M2) ■ 3'-OH (M1)

C) CG glucuronide by RLMs

D) CG glucuronide by RIMs

Graphs showing the rate of glucuronide formation at different concentrations for both RLMs and RIMs.
Supplemental Data

Journal Title
Drug Metabolism and Disposition

Article Title
SGLT-1 transport and deglycosylation inside intestinal cells are key steps in the absorption and disposition of calycosin-7-O-β-D-glucoside in rats

Authors
Jian Shi, Haihui Zheng, Jia Yu, Lijun Zhu, Tongmeng Yan, Peng Wu, Linlin Lu, Ying Wang, Ming Hu, Zhongqiu Liu

Figure legends

Figure 1S 1H NMR (500M CD3OD) spectrum of Calycosin. 1H NMR was used to elucidate the structures of Calycosin, and data revealed two 1,2,4-trisubstituted benzene ring [δH 7.97 (1H, d, J = 8.5 Hz, H-5), δH 6.93 (1H, dd, J = 8.5, 2.5 Hz, H-6) and δH 6.86 (1H, d, J = 2.5 Hz, H-8)] and [δH 7.05 (1H, d, J = 1.5 Hz, H-2′), δH 6.95 (1H, overlapped, H-5′) and δH 6.94 (1H, d, overlapped, H-6′)], one olefinic proton of a trisubstituted double bond (δH 8.29, 1H, s, H-2), one methoxyls (δH 3.79, 3H, s) and two hydroxy protons [δH 9.01 (1H, s 3′-OH) and δH 10.78 (1H, br s, 7-OH)].

Figure 2S 1H NMR (500M CD3OD) spectrum of M1. 1H NMR was used to elucidate the structures of M1, and data revealed two 1,2,4-trisubstituted benzene ring [δH 7.97 (1H, d, J = 8.5 Hz, H-5), δH 6.94 (1H, dd, J = 8.5, 2.0 Hz, H-6) and δH 6.87 (1H, d, J
= 2.0 Hz, H-8)] and [δ_H 7.28 (1H, d, J = 2.0 Hz, H-2'), δ_H 7.24 (1H, dd, J = 8.5, 2.0 Hz, H-5')] and δ_H 7.04 (1H, d, J = 8.5 Hz, H-6'), one olefinic proton of a trisubstituted double bond (δ_H 8.34, 1H, s, H-2), and one methoxyls (δ_H 3.80, 3H, s). Remaining signals corresponded with those of glucuronic acid units, showing one anomeric proton at δ_H 5.08 (1H, d, J = 7.0 Hz, H-1’).

**Figure 3S** Inspection of ^1^HNMR spectroscopic data suggested that the moiety belongs to glucuronic acid, revealing that a proton of OH in Calycosin was substituted by glucuronic acid. The substitution position of the proton of 3’-OH can be confirmed by the nuclear overhauser effect spectroscopy (NOESY) correlation between Glu-H-1” (δ_H 5.08, 1H, d, J = 7.0 Hz) and H-2’ (δ_H 7.28, 1H, d, J = 2.0 Hz).
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**Table S2.** Stability of CG, CG glucuronide and calycosin-3’-glucuronide in blank bile at 37 ℃.

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Table S3. Stability of CG, CG glucuronide and calycosin-3’-glucuronide in blank plasma.

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