Special Section on Drug Metabolism and the Microbiome

The Presystemic Interplay between Gut Microbiota and Orally Administered Calycosin-7-O-β-D-Glucoside

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ABSTRACT
Presystemic interactions with gut microbiota might play important roles in the holistic action of herbal medicines in their traditional oral applications. However, research interests usually focus on biologic activities of the in vivo available herb-derived components and their exposure in circulation. In this study, we illustrated the importance of studying the presystemic interplay with gut microbiota for understanding the holistic actions of medicinal herbs by using calycosin-7-O-β-D-glucoside (C7G), the most abundant flavonoid and chemical marker in Astragali Radix, as a model compound. When C7G was orally administrated to rats, calycosin-3′-O-glucuronide (G2) was the major circulating component in the blood together with a minor calycosin but not C7G. Rat gut microbiota hydrolyzed C7G in vitro rapidly and produced its aglycone calycosin. Calycosin exhibited higher permeability than C7G and further underwent extensive glucuronidation to yield 3′-glucuronide as the dominant metabolite. Bioactivity assays revealed that G2 exhibited similar or more potent proangiogenic effects than calycosin in human umbilical vein endothelial cells in vitro and in the vascular endothelial growth factor receptor tyrosine kinase inhibitor II–induced blood vessel loss model in zebrafish. More interestingly, the incubation of C7G with gut microbiota from both normal and colitic rats showed a probiotics-like effect through stimulating the growth of the beneficial bacteria Lactobacillus and Bifidobacterium. In conclusion, C7G interacts reciprocally with gut microbiota after oral dosing, which makes it not only an angiogenic prodrug but also a modulator of gut microbiota.

Introduction
There have been extensive research efforts to demonstrate the comprehensive involvement of gut microbiota in diverse pathophysiologic processes through interaction with host cells. Host-gut microbiota metabolic interactions start at birth and continue throughout life, maintaining host health through generating numerous endogenous cometabolites of biologic importance (Nicholson et al., 2012). Thus, manipulating the microbial balance by the use of probiotics, nutrients, and antibiotics represents a promising therapy in patients who have disorders caused by microbial imbalances (Foxx-Orenstein and Chey, 2012). On the other hand, the tremendous metabolic capability of gut microbiota is also of great importance in biotransforming structurally diverse xenobiotics into more permeable metabolites. Therefore, in some alternative medical systems where medicines are usually applied orally, the presystemic interaction with gut microbiota may define both the disposition profile and the pharmacologic activity of the medicine. However, elucidation of such presystemic interactions has generally not been included into the work portfolio of the medicine until recently.

Glycosides are major components composed of aglycone and sugar. In general, the glycosides are fairly soluble in water and possess poor membrane permeability. Among the many glycosides found in nature, flavonoid glycosides belong to an important chemical type that is ubiquitously distributed in the plant kingdom, found in fruits, vegetables, soy products, and some medicinal herbs. Some health-related benefits are associated with this group of compounds, including strengthening of the immune system, protecting against cancer, and reducing capillary fragility (Lu et al., 2013). Because many flavonoid glycosides exhibit low oral bioavailability due to the microbial hydrolysis and conjugation that occurs in the intestine and the liver, this type of compound is generally considered a natural prodrug (Arroo et al., 2008, 2009); the health benefits are attributed to the metabolites capable of reaching the circulation (Dorigochoo et al., 2012; Vissiennon et al., 2012; Romano et al., 2013). However, there have been few biologic/pharmacologic evaluations of the resultant conjugates (Terao, 1999; Koga and Meydani, 2001) because of limited
access to the pure compounds. The actions of the parent compounds before intestinal absorption are generally ignored.

Astragal Radix is a medicinal herb notable for its immunogenic effect (Zhao et al., 2008). In addition to applications for immunity enhancement, the herb is widely used in Asian countries for treatment of cardiovascular diseases such as heart failure, angina pectoris, myocardial infarction, and stroke (Zhao et al., 2008). Our recent study using a multiple reaction monitoring–based approach revealed over 400 flavonoids in the herb, among which around 130 are calycosin (7,3’-dihydroxy-4’-methoxyisoflavone) and its glycosides (Yan et al., 2014). As the most abundant and characteristic flavonoid compound in Astragal Radix (Lv et al., 2011), calycosin-7-O-β-D-glucoside (C7G) has been documented in the China Pharmacopoeia (Chinese Pharmacopoeia Commission, 2010) as one of the markers for quality control of the herb. Recently, this herb has been acclimated to European countries for phytotherapeutic purposes (Matkowski et al., 2003).

In concert with the clinical applications of Astragal Radix, C7G exhibited anti-inflammatory effects in a rabbit osteoarthritides model (Choi et al., 2007) and antioxidant and neuroprotective effects in vitro (Yu et al., 2005). The aglycone calycosin demonstrated antidiabetic (Shen et al., 2006), antioxidant and neuroprotective (Yu et al., 2005), and cardiovascular protective effects (Fan et al., 2003) in vitro. Our recent study (Tang et al., 2010; Li et al., 2011) found that calycosin acted as a selective estrogen receptor modulator to promote angiogenesis in zebrafish embryos and human umbilical vein endothelial cell (HUVEC) cultures. A pharmacutic composition consisting of calycosin and C7G has been patented for prevention and treatment of estrogen receptor-mediated diseases (Cohen 2013) and diabetic nephropathy (Zhu 2010).

However, as is widely known for flavonoids, both C7G and calycosin are likely prodrugs, and calycosin conjugates could be the pharmacologically active form. Calycosin phase II conjugates appeared to be the primary circulating forms present after an oral administration of C7G to rats (Chen et al., 2011; Zhang et al., 2012) or administration of Astragal Radix extract to a healthy volunteer (Xu et al., 2006). These findings indicate the occurrence of deglycosylation of C7G and further conjugation of calycosin. Our recent study revealed major glucuronidation and moderate sulfation of calycosin and identified 3’-O-glucuronide as the major conjugate formed in human liver microsomes (Ruan and Yan, 2014). However, the interplay between C7G and gut microbiota is largely unknown. Whether C7G, calycosin, and calycosin phase II conjugates are available in vivo in the circulating system after oral administration of C7G and, more importantly, whether the circulating metabolites of C7G in the blood possess relevant pharmacologic properties remain unclear.

Therefore, this work was designed to study the presystemic interplay between C7G and gut microbiota and the disposition of C7G after oral administration. In addition, this work evaluated the angiogenic activities of the major circulating forms of C7G using zebrafish larvae and HUVEC.

### Materials and Methods

#### Materials

Calycosin and C7G standards (purity >98%) were purchased from Forever-biotech Company (Shanghai, People’s Republic of China). Calycosin-3’-O-glucuronide (G2) (purity >98) was prepared from a large-scale reaction of calycosin with rat liver microsomes and identified using NMR analysis in our laboratory (Ruan and Yan, 2014). Endothelial cell growth supplement, heparin, gelatin, and polyethylene glycol 400 were supplied by Sigma-Aldrich (St Louis, MO). Dextran sulfate sodium salt (DSS) (molecular mass 36,000–40,000 Da) was purchased from MP Biomedicals (Santa Ana, CA). Kaighn’s modification of Ham’s F12 medium, fetal bovine serum, phosphate-buffered saline, and charcoal-stripped fetal bovine serum were all purchased from Invitrogen (Carlsbad, CA). Vascular endothelial growth factors (VEGF) were obtained from R&D Systems (Minneapolis, MN). VEGF receptor tyrosine kinase inhibitor II (VRK) was purchased from Calbiochem Company (La Jolla, CA). The GasPak EZ Anaerobe Pouch System was purchased from Becton Dickinson (Franklin Lakes, NJ). The bacterial genomic DNA extraction kit, SYBR Green Master Mix, and 16S RNA-specific primers for target bacterial genera and the universal primer for total gut bacteria were supplied by TaKaRa Biotechnology (Guanzhou, People’s Republic of China).

#### Pharmacokinetic Studies

**Animals**. Male Sprague-Dawley (SD) rats (200–250 g) were supplied by Sam Yao Hong Ltd. (Macao, People’s Republic of China). The animals were housed in an individual ventilated cage system (Tecniplast, Italy) under 12-hour light/dark cycles at a temperature of 20–23°C and were allowed to acclimate to the environment with access to water and food ad libitum for at least 1 week before the experiments. Animals were handled according to a protocol approved by the Animal Ethics Committee of Institute of Chinese Medical Sciences, University of Macau (File no. ICMS-AEC-2013-05).

**Microbial Metabolism of Calycosin-7-O-β-D-Glucoside and Calycosin**. Rat gut microbiota samples were prepared according to our previous report (Ruan et al., 2010). Briefly, a total of 10 g of fresh fecal samples were collected from five rats and were pooled at equal amounts then mixed well with 30 ml of autoclaved brain-heart infusion (BHI) medium. The resultant fecal suspension was centrifuged, and the supernatant was decanted and centrifuged at 5000 g for 30 minutes. The precipitate was resuspended with 10 ml BHI medium to provide a gut bacterial suspension (0.1 g/ml).

Biotransformation of C7G or calycosin by rat intestinal bacteria was determined in a 0.5-ml incubation system containing 25 μl of gut bacteria suspension, 10 μl of C7G or calycosin in dimethylsulfoxide (DMSO) (final concentration 25 μM) in BHI medium. Reactions were incubated anaerobically at 37°C in a GasPak EZ Anaerobe Pouch System for 0.3, 0.5, 0.75, 1, 1.5, 2, and 4 hours, respectively. Zero-minute incubations and parallel reactions without bacteria or substrate served as controls.

Reactions were stopped by the addition of 1 volume of ice-cold acetic ether followed by immediate centrifugation at 5000 g for 30 minutes to remove the bacteria. After adding 5 μl of daidzein (1 mg/ml) as an internal standard, the sample was twice extracted with 1 ml of acetic ether. The organic layers were combined and evaporated under N2 at 37°C. The residue was then reconstituted with 200 μl of methanol and filtered through a 0.45-μm membrane filter before high-pressure liquid chromatography with diode-array detection (HPLC-DAD) analysis.

**Transport of Calycosin-7-O-β-D-Glucoside and Calycosin across Caco-2 Monolayers**. Caco-2 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells at passages 30 to 40 were cultured in Dulbecco’s modified Eagle’s medium according to a protocol reported previously elsewhere (Ruan et al., 2010). Briefly, after 21 days of culture, the prepared Caco-2 monolayers were rinsed twice with Hanks’ balanced salt solution (HBSS) and preincubated in HBSS at 37°C for 30 minutes. In the absorptive transport study, 0.5 ml of HBSS containing C7G or calycosin (final concentration 12.5, 25, 50, or 100 μM) was loaded at the apical (A) side (donor chamber), and 1.5 ml of blank HBSS was placed at the basolateral (B) side (receiver chamber). In the secretory transport study, 1.5 ml of HBSS containing the test compound was added at the B side (donor chamber) and 0.5 ml of blank HBSS was placed at the A side (receiver chamber). Aliquots (100 μl) were taken from receiver chambers at different time intervals (0, 15, 30, 45, and 60 minutes) for sample analysis. After each sampling, 0.1 ml of HBSS was added to the receiver chamber to maintain a constant volume. All the experiments were performed in triplicate.

The apparent permeability coefficients (Papp) of C7G and calycosin from apical side to basolateral side (Papp A to B) or from basolateral side to apical side (Papp B to A) obtained from the bidirectional transport study using the Caco-2...
controls. All reactions were conducted in triplicate and terminated by adding acid ([UDPGA]) or with denatured proteins were performed in parallel as supernatant was filtered and subjected to high-pressure liquid chromatography.

**Hepatic Metabolic of Calycosin-7-O-β-D-Glucoside and Calycosin**

Phase II (sulfation and glucuronidation) reactions of C7G and calycosin in rat liver subcellular fractions were performed according to the method recently reported by our group (Ruan and Yan, 2014). The glucuronidation reaction was performed in rat liver microsomes at 0.1 mg protein/ml and incubated for 10 minutes. Sulfation was examined with pooled rat liver S9 at 1 mg/ml, and the reaction was allowed to proceed for 60 minutes. The concentrations of C7G and calycosin were 200 μM. Zero-minute reactions and reactions without cofactor (3’-phosphoadenosine 5’-phosphosulfate [PAPS] or UDP glucuronic acid [UDPGA]) or with denatured proteins were performed in parallel as controls. All reactions were conducted in triplicate and terminated by adding 200 μl of ice-cold methanol. After centrifugation at 20,000g for 10 minutes, the supernatant was filtered and subjected to high-pressure liquid chromatography with tandem mass spectrometry (HPLC-MS/MS) or HPLC-DAD analysis.

**Pharmacokinetic Study of Calycosin-7-O-β-D-Glucoside in the Rat**

The in vivo pharmacokinetic study was performed according to a protocol reported previously elsewhere (Yan et al., 2012) with minor modifications. In brief, a cannula was implanted into the jugular vein for blood sampling before the experiment. After cannulation, rats were allowed to recover and fasted overnight with free access to water. C7G was dissolved at 10 mg/ml in a solution consisting of polyethylene glycol 400 and water (37/3 v/v) and orally administrated to rats (n = 5) at 40 mg/kg. Blood (250 μl each) was withdrawn at appropriate time intervals (0.05, 0.10, 0.17, 0.25, 0.33, 0.5, 1, 2, 3, 4, 6, 8, 12, and 24 hours) after dosing. After each blood withdrawal, the same volume of normal saline containing heparin (20 IU/ml) was immediately injected to compensate for blood loss as well as to flush the cannula. The blood sample was transferred into heparinized tube and centrifuged for 10 minutes at 15,000 g for 5000 rpm. The resultant plasma samples were analyzed in multiple reaction monitoring mode by the ion pair 480.9/282.9, 282.9/267.9, 459.0/282.8, and 362.8/282.8 for C7G, calycosin, calycosin mono-glucuronides, and calycosin mono-sulfates, respectively.

**Bioactivity Assays**

**Effects of C7G on Growth of Gut Microbiota from Normal Rats and Rats with Experimental Colitis**

**Animal Treatment and Fecal Bacteria Preparation.** Twelve rats (200–250 g) were randomly divided into two groups (six animals each). One group received drinking water for 7 days (normal group), while the other was orally administered 5% DSS via the drinking water to induce experimental colitis (ulcerative colitis group) as described in our recent report (Huang et al., 2015). Fecal samples were freshly collected from each group on the last day of DSS treatment and were pooled within each group to prepare bacterial suspensions (0.1 g bacteria/ml medium).

**Incubation of C7G with Rat Gut Bacteria.** The effect of C7G on the growth of rat gut bacteria was examined in a mixture containing 8 μl of fecal bacterial suspension and 2 μl of C7G in DMSO (final concentration 25 μM) in BHI medium in a total volume of 0.5 ml. The mixture was anaerobically incubated at 37°C in a GasPak EZ Anaerobe Pouch System for the appropriate time intervals (0, 2, 6, and 12 hours). The reactions were terminated by an immediate centrifugation (5000g, 10 minutes, 4°C), and the bacteria were obtained for extraction of the total DNA using the MiniBEST Bacterial Genomic DNA Extraction Kit (TaKaRa Biotechnology) following the manufacturer’s instructions. DNA was stored at −80°C before the real-time polymerase chain reaction (RT-PCR) analysis.

**TABLE 1 Information for RT-PCR analysis of target gut bacteria**

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Primer Name</th>
<th>Sequence (5'-3')</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Total bacteria</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>968-F</td>
<td></td>
<td>AAAAGCAAAATCCACAC</td>
<td>Scanlan et al., 2006</td>
</tr>
<tr>
<td>1401-R</td>
<td></td>
<td>GGTGTGTTGACAGAC</td>
<td>Xu et al., 2011</td>
</tr>
<tr>
<td><strong>Bifidobacterium</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g-Bifid-F</td>
<td></td>
<td>CTTCTGGAACACAGGGTCCG</td>
<td></td>
</tr>
<tr>
<td><strong>Enterobacteriaceae</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Eco1457F</td>
<td></td>
<td>CATTGAGGTACCCCGAGAAAGAC</td>
<td></td>
</tr>
<tr>
<td>Eco1652R</td>
<td></td>
<td>CTTCAGGAGACTCAAGCTGCG</td>
<td></td>
</tr>
<tr>
<td><strong>Lactobacillus</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>g-Laboratory-F</td>
<td></td>
<td>TGGAAAACAGGTGCTAATACCG</td>
<td>Byun et al., 2004</td>
</tr>
<tr>
<td>g-Laboratory-R</td>
<td></td>
<td>GTCCATTGAGGAAGATTCCC</td>
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RT-PCR Analysis. RT-PCR analysis was performed in duplicate in 96-well plates on the ABI 7500 PCR instrument (Applied Biosystems). The target bacteria, 16S rRNA-specific primers for target bacterial genera and the universal primer targeting the conserved region of 16S rRNA gene for total gut bacteria, and the applicable literature are summarized in Table 1. The real-time amplification reactions consisted of 26 µl of 1×SYBR Green Master Mix, 1 µl of each primer (final concentration 0.2 µmol/l), 4 µl of template DNA, and 18 µl of milli-Q water. The cycling conditions consisted of preliminary denaturation (95°C for 30 seconds), followed by 40 cycles of 95°C for 5 seconds and 60°C for 34 seconds. A melting curve analysis was performed at the end of run by heating to 95°C for 15 seconds followed by cooling to 60°C for 60 seconds to verify the specificity and the identity of the PCR products. The fluorescence signal was recorded at the end of the elongation step of each cycle.

Relative Quantification of Target Genes. The comparative cycle threshold (Ct) method was used to compare the compositional alteration of each targeted bacterial genus after exposing the gut microbiota from normal or colitic rats to C7G. The DNA levels of each targeted bacterium were normalized to that of the conserved region of the 16S rRNA gene, according to the following formula: 
\[ \Delta Ct = Ct (\text{Targeted bacterial gene}) - Ct (\text{Conserved region of 16S rRNA gene}) \]
Thereafter, the relative DNA levels of each target gene at the times of 2, 6, or 12 hours were calculated using the \( \Delta \Delta Ct \) method (Livak and Schmittgen, 2001):
\[ \Delta \Delta Ct (\text{Targeted gene}) = \Delta Ct (\text{Targeted gene at 2, 6, or 12 hours}) - \Delta Ct (\text{Targeted gene at time 0}) \]
The fold changes of DNA levels were expressed as a relative expression \( 2^{-\Delta \Delta Ct} \).

Angiogenic Effects of Calycosin and Calycosin 3’-O-Glucuronide (G2). Angiogenesis Assay on HUVEC Cells. The angiogenesis assay using HUVECs was conducted as described in our previous work (Tang et al., 2010). The effect of different concentrations of calycosin or G2 on HUVECs proliferation was examined at 12.5–200 µM. Cells receiving DMSO (0.1%) served as vehicle controls, and those treated with VEGF (20 ng/ml) served as positive controls.

Angiogenesis Assay on In Vivo Zebrafish Model. Zebrafish embryos were prepared as described in our previous work (Tang et al., 2010). Briefly,

![Fig. 1.](image-url) Multiple reaction monitoring chromatograms of (A) blank rat plasma spiked with C7G, G2, and calycosin and (B) plasma sample obtained from rats after oral administration of C7G for 2 hours. (C) Plasma concentration–time profile of G2 in rats after oral administration of C7G.
zebrafish embryos were collected at 24 hours after fertilization, distributed into a 12-well microplate with 15 fish each well, and pretreated with 1 μM VEGF receptor tyrosine kinase inhibitor II (VRI) for 2 hours. The VRI was then washed out and replaced with different concentrations (12.5–50 μM) of calycosin or G2 (with 0.1% DMSO). Embryos receiving embryo water containing 0.1% DMSO served as a vehicle control and were the equivalent of no treatment. The viability and gross morphologic changes of the zebrafish embryos were evaluated.

Fig. 2. (A) Typical HPLC-UV chromatogram of incubates of C7G with rat gut microbiota for 30 minutes. (B) Mass spectra of C7G standard and the metabolite B1 (B). (C) Time courses of C7G elimination and metabolite B1 formation in rat gut microbiota.

TABLE 2

<table>
<thead>
<tr>
<th>Concentration (μM)</th>
<th>C7G P_{app} A to B (10^{-7} cm/s)</th>
<th>C7G Efflux ratio</th>
<th>Calycosin P_{app} A to B (10^{-7} cm/s)</th>
<th>Calycosin P_{app} B to A (10^{-7} cm/s)</th>
<th>Calycosin Efflux ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>12.5</td>
<td>15.2 ± 2.57</td>
<td>13.2 ± 1.43</td>
<td>307.0 ± 3.97</td>
<td>407.8 ± 10.5</td>
<td>1.33</td>
</tr>
<tr>
<td>25</td>
<td>11.0 ± 3.81</td>
<td>8.54 ± 0.61</td>
<td>263.7 ± 6.23</td>
<td>377.9 ± 16.4</td>
<td>1.43</td>
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<tr>
<td>50</td>
<td>8.28 ± 2.91</td>
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<td>291.3 ± 9.32</td>
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<tr>
<td>100</td>
<td>13.1 ± 2.09</td>
<td>8.14 ± 1.56</td>
<td>313.2 ± 3.15</td>
<td>407.8 ± 9.52</td>
<td>1.30</td>
</tr>
</tbody>
</table>
Data Analysis and Statistics

The plasma concentration-time profiles and pharmacokinetic parameters of G2 were obtained using noncompartmental analysis with WinNonlin 5.2.1 (Pharsight Corporation, Mountain View, CA). The peak plasma concentration ($C_{\text{max}}$) and the time to $C_{\text{max}}$ ($T_{\text{max}}$) were obtained directly from the concentration-time plots. The area under the plasma concentration-time curve from time 0 to infinity (AUC$_{0\rightarrow\infty}$) was calculated using the log-trapezoidal rule. The elimination half-life ($t_{1/2}$) was estimated from the terminal slope ($-ke/2.303$) of the plasma concentration–time curve.

All data were presented as mean ± S.D. unless otherwise mentioned. The differences between groups were compared using Student’s $t$ test or one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test. $P < 0.05$ was considered statistically significant.

Fig. 3. Typical HPLC-UV chromatograms of incubates of calycosin with rat liver microsomes in the presence of (A) PAPS for 60 minutes or (B) UDPGA for 10 minutes. (C) Mass spectra of calycosin standard, the metabolites S1 and G2.
Results

In Vivo Oral Pharmacokinetics of C7G in Rats. When C7G was orally administrated to the rats, the parent C7G was not detected in the plasma throughout the experimental period of 24 hours. Its aglycone calycosin could only be detected in minor amounts in plasma samples collected at 6 minutes and 10 minutes from only two rats (data not shown). In contrast, two peaks showing a mass loss of 80 and 176, respectively, together with a characteristic fragment ion at m/z 283 (corresponding to the molecular ion of calycosin) were detected, indicating the existence of monoglucuronide and monosulfate of calycosin in rat plasma. By comparing the retention time and the mass spectra of standard calycosin 3'-glucuronide (G2), the monoglucuronide in plasma was identified as calycosin-3'-O-glucuronide. The glucuronide appeared as the major circulating form in the plasma together with a less abundant monosulfate (Fig. 1B), indicating the occurrence of deglucosylation of C7G after further conjugation of calycosin in vivo.

The plasma concentration–time plot of G2 showed multiple peaks (Fig. 1C), which could be attributed to enterohepatic circulation and/or two phases of G2 formation at intestine and liver. The overall systemic exposure [area under the plasma concentration-time curve from time 0 to infinity (AUC0–∞)] of G2 was 104.8 ± 12.4 μM × h with the maximum concentration (Cmax 18.3 ± 7.30 μM) reached at 0.63 ± 0.91 hours, indicating a rapid absorption of G2. The t1/2 of G2 in rats was 8.51 ± 4.42 hours, showing a slow elimination.

In Vitro Metabolism of C7G and Calycosin by Rat Gut Microbiota. Incubation of C7G with rat gut microbiota generated an additional peak (B1), which was observed at 30.1 minutes and absent in controls (Fig. 2A). The metabolite B1 exhibited a pseudomolecular ion ([M-H]−) at m/z 283 (Fig. 2B), which was 162 mass units less than that of C7G. MS/MS analysis of m/z 283 yielded a predominant fragment ion at m/z 268 ([M-H-CH3]−), the same as that observed with calycosin. In addition, the retention time and UV spectrum of B1 were identical to those of calycosin. Thus, B1 was unambiguously identified as calycosin. In the meantime, the transformation velocity of C7G into calycosin was proportional with the substrate increase, indicating that there were transporter-mediated mechanisms involved in calycosin and C7G transport. The Phase II Metabolism of C7G and Calycosin by Liver Subcellular Fractions. C7G was not metabolized within 1 hour when incubated with rat liver subcellular fractions fortified with PAPS or UDPGA. In contrast, calycosin yielded two metabolites (Fig. 3, A and B), namely, S1 and S2, and G1 and G2, in the presence of PAPS and UDPGA, respectively.

Both S1 and S2 exhibited the molecular ions at m/z 363 ([M-H]−), which produced major fragment ions at m/z 283 ([M-H-Sul]−) and m/z 268 ([M-H-Sul-CH3]−) in their MS/MS spectra (Fig. 3C). As a result, the two metabolites were tentatively identified as the monosulfates of calycosin. The mass spectrum of G1 and G2 showed a molecular ion ([M-H]−) at m/z 459 (Fig. 3C), which gave a characteristic ion at m/z 283 ([M-H-Glc]−) in the MS/MS spectrum, indicating that they are the mono-glucuronides of calycosin. The major metabolite G2 was assigned as calycosin 3'-O-glucuronide when compared with the standard. G1 was then tentatively assigned as 7-O-glucuronide. 3'-Glucuronidation is the dominant pathway of calycosin glucuronidation as judged from the relative abundance of G2 to G1 (~200-fold) calculated from peak areas.

When estimated based on the amount of calycosin eliminated over respective incubation periods, the transformation velocity of calycosin into sulfates and glucuronides was 0.703 ± 0.071 and 6.88 ± 0.097 nmol/min/mg protein, respectively, indicating that glucuronidation is the major metabolic pathway of calycosin in the rat liver. Calycosin and G2 Promoted HUVEC Proliferation. As shown in Fig. 4, both G2 and calycosin promoted cell proliferation significantly in dose-dependent manners within 10–100 μM. Compared with vehicle control, calycosin induced a maximum of 26% increase of cell viability at 100 μM. G2 produced a comparable effect at the same concentration level. At a higher concentration (200 μM), G2 exhibited insignificant effect on HUVEC cell proliferation, whereas calycosin showed a significant inhibition, which should be resulted from the cytotoxic effect of calycosin observed at this concentration (Supplemental Fig. 1). A statistically significant (P < 0.05) increase in cell proliferation was also observed in VEGF-treated cells (57%), which served as the positive control.

Calycosin and G2 Rescued VRI-Induced Blood Vessel Loss in Zebrafish. VRI, a pyridyl-1-anthranilamide compound that displays antiangiogenic properties, has been shown to potently inhibit the kinase activities of both VEGF receptors 1 and 2 (Furet et al., 2003). The pretreatment of zebrafish embryos 24 hours after fertilization with 1 μM VRI for 2 hours followed by 24 hours of incubation in embryo medium (0.1% DMSO) at 28.5°C allowed development of blood vessel loss in regions of intersegmental vessels (ISVs) and dorsal longitudinal anastomotic vessels (DLAVs) as well as the formation of impaired subintestinal vessels (SIVs) (Fig. 5, IB). After incubation of the VRI-treated embryos with 12.5–50 μM calycosin or G2 for 24 hours, the VRI-induced blood vessel loss at ISV and DLAV regions was dose-dependently rescued whereas the impaired SIV branching.
Fig. 5. The proangiogenesis effects of calycosin and G2 on damaged blood vessel in Tg(fli-1a:EGFP)y1 zebrafish embryos. Predamaged blood vessel embryos were incubated with embryo water or 12.5, 25, or 50 μM calycosin or G2 for (I) 24 hours and (II) 48 hours. (A) Vehicle control: in embryo 52 hours after fertilization, the intersegmental blood vessels (ISVs), subintestinal vessels (SIV), and dorsal longitudinal anastomotic vessels (DLAV) appear as well-developed net structures. (B) VRI pretreatment only: embryo at 28 hours after fertilization received 2 hours of VRI treatment leading to reduced blood vessel formation in ISV, SIV, and DLAV. (C–H) Incubating VRI-pretreated embryo with 12.5, 25, or 50 μM calycosin or G2. (I, J) Healthy embryos (without VRI pretreatment) were treated with 50 μM calycosin or G2. Normal ISV, SIV, and DLAV development was similar to that observed in vehicle control. (a–j) enlarged ISV region (magnification, ×4.5) of A–J, respectively. Gray arrows indicate SIV, yellow arrows indicate DLAV, red arrows indicate ISV, and white arrows indicate the loss of blood vessels. (III) Percentage recovery of ISV of Tg(fli-1a:EGFP)y1 zebrafish. Data are plotted as mean ± S.D. (n = 3).
was partially restored (Fig. 5, IC–H, III). Moreover, G2 showed more significant rescuing effects than calycosin in the recovery of defective blood vessels in zebrafish (Fig. 5, IC–H, III) at the same dosage. Incubation of the VRI-treated embryos with calycosin or G2 for 48 hours resulted in more potent blood vessel rescuing effects (Fig. 5, IIC–H, III).

Incubation with C7G Stimulated the Growth of *Lactobacillus* and *Bifidobacterium* in Gut Bacteria from Normal and Colitic Rats. As shown in Fig. 6, when gut bacteria from normal rats were incubated in vitro, the relative contents of *Lactobacillus* and *Enterobacteriaceae* were increased, with maximum levels observed at 6 hours and 2 hours, respectively, while that of *Bifidobacterium* declined slowly with time. The presence of C7G resulted in a marked increase in the relative content of *Lactobacillus* and slightly enhanced the relative contents of *Bifidobacterium* and *Enterobacteriaceae* in gut microbiota from normal rats at early stage of incubation (2 hours). In gut microbiota from colitic rats, C7G treatment enhanced the relative content of *Lactobacillus* in a similar trend. More interestingly, the relative content of *Bifidobacterium* showed an increase at 2 hours of incubation and the presence of C7G further enhanced the relative content by 2.7-fold. The growth of *Enterobacteriaceae* from rats with experimental colitis followed a biphasic increase, and the relative content was not altered by C7G.

Discussion

Flavonoid glycosides are commonly considered as prodrugs due to low bioavailability resulting from poor oral absorption and microbial hydrolysis. However, most bioactivity assays of the parent compounds were performed on in vitro models without considering the interactions with gut microbiota in oral application and the resulting low systemic exposure. As the most abundant flavonoid and chemical marker for Astragali Radix and related supplementary products, C7G was selected as a model compound in the present study to exemplify the importance of gut microbiota in the beneficial effects of herbal medicines.
Considering the traditional oral intake of herbal medicines, we investigated the oral disposition of C7G and studied the presystemic interaction of the parent C7G with gut microbiota and the clinical relevant bioactivity of the major circulating component. It was found that calycosin-3'-O-glucuronide is the major in vivo available form and demonstrated blood vessel–rescuing effects; the parent compound, not appearing in the circulation, showed probiotics-like effects, which may contribute to the immune-enhancing effect of Astragali Radix. Our findings indicate that the pharmacologic outcome of the flavonoid glycoside after oral administration should be a summation of the activity elicited by its circulating forms and that resulting from a presystemic interplay of the parent compound with gut microbiota.

Microbial conversion as well as hepatic glucuronidation was demonstrated to be crucial for systemic exposure of the proangiogenic calycosin-3'-O-glucuronide, whereas the parent C7G was less permeable and not detectable in rat plasma. As expected, in vitro anaerobic incubation of C7G revealed a rapid and complete hydrolysis of C7G by rat gut microbiota, and the aglycone calycosin generated was highly stable in gut microbiota.

Although recent studies have reported the demethylation and dehydroxylation of C7G in rat gut microbiota (Chen et al., 2011) or human gut bacterial isolates (Zhang et al., 2014), deglucosylation seems to be the dominant metabolic pathway. In view of the high permeability (~10^{-5} \text{cm.s}^{-1}), calycosin formed is likely to stay briefly in the gut lumen, not allowing it to be further transformed by gut microbiota or produce other metabolites in significant amounts in vivo.

The aglycone calycosin was only detected in minor amount shortly after oral administration of C7G. Rapid regioselective glucuronidation is the main reason for the low exposure of the aglycone. The resultant 3'-O-glucuronide (G2) appeared as the major circulating form in the plasma and still stayed in the systemic circulation by the end of the experiment (24 hours). Although previous reports revealed calycosin conjugates as the major form in rats (Chen et al., 2011; Zhang et al., 2012) or zebrafish larvae (Hu et al., 2012) in vivo, they failed to unambiguously assign the 3'-glucuronide as the major circulating form and did not even mention the proangiogenic effects of the metabolite. The 3'-O-glucuronide was also found to be the major metabolite in human liver microsomes (Ruan and Yan, 2014), justifying the interspecies correlation.

Although C7G showed a moderate intestinal permeability, it was not detected in vivo. The rapid microbial hydrolysis of C7G and rapid absorption and conjugation, in particular glucuronidation, of the resultant calycosin account for the nil exposure of the parent C7G in vivo. It is interesting to note that incubation of the parent C7G markedly stimulated the growth of the beneficial bacteria Lactobacillus, slightly increased the relative content of Bifidobacterium, but did not affect Enterobacteriaceae in the in vitro anaerobic incubation with gut microbiota. This probiotics-like effect was more potent on the growth of the beneficial gut bacteria Lactobacillus and Bifidobacterium from colitic rats, supporting the potential of this flavonoid glycoside in reinstating microbial balance. The growth curves of three gut bacteria tested seem to be genus specific, and they varied with the origins of the gut microbiota but were not affected by C7G treatment.

The probiotics-like effects of C7G on Lactobacillus and Bifidobacterium in rat gut microbiota pools agreed well with a recent report from Zhang et al. (2014) that examined the in vitro interaction of C7G with several human gut bacterial isolates and found similar probiotics-like property of C7G. Using rat gut microbiota pools, our study better mimicked the microbial balance shift in vivo in response to C7G treatment. It has been reported that dietary polyphenols including flavonoids may contribute to the maintenance of intestinal health by maintaining/reinstating the microbial balance through stimulating the growth of beneficial bacteria and/or inhibiting pathogenic bacteria (Duenas et al., 2015). The beneficial effects of flavonoids in the supplementary foods might partially come from the modulation of the gut bacteria by the flavonoid glucosides, which has yet to be well examined. The growth-stimulating effect of C7G on Lactobacillus and Bifidobacterium might be a result of the nutritional effect of the glucose cleaved by gut microbiota from the glycoside, as many gut bacterial strains use glucose as one of the main carbon and energy sources (Macfarlane and Macfarlane, 1997). If so, one can expect that other natural glucosides that can be deglycosylated by gut microbiota will provide a sugar source that may modulate gut microbiota growth. Because C7G was depleted by gut microbiota within 2 hours of incubation while the calycosin generated was relatively stable in the gut microbiota (Fig. 2C), we speculate that calycosin may also contribute to the probiotics-like effect observed, which warrants further study.

There have been a few studies reporting on the bioactivities of flavonoid conjugates in vivo. “Phase II conjugates, sulfates or glucuronides, have been reported to have antioxidative (Terao, 1999), neuroprotective (Ho et al., 2013), and anti-inflammatory (Granica et al., 2013) effects. In addition to a notable immune-enhancing effect, Astragali Radix is also commonly used for treating cardiovascular disorders and has been shown to possess angiogenic effect in previous studies (Tang et al., 2010; Li et al., 2011). Our previous study revealed a proangiogenic activity of calycosin on HUVECs in vitro and zebrafish in vivo (Tang et al., 2010), which comes from action on estrogen receptors. When compared with calycosin, the major in vivo metabolite calycosin 3'-O-glucuronide showed a rapid and comparable or more potent effect on promoting angiogenesis in HUVEC cells and zebrafish.

In conclusion, our present study has demonstrated that the presystemic interplay between C7G and gut microbiota results in not only metabolite producing proangiogenic effects but also probiotics-like actions of the parent compound, both contributing to the beneficial activities of the glycoside. Our findings emphasize the importance of studying the disposition and actions of glucosides before their absorption for understanding the overall benefits. The obtained results also provide insight into the importance of gut microbiota in the holistic actions of medicinal herbs in traditional oral applications and warrant further investigational emphasis on reciprocal interactions between medicinal herbs and gut microbiota.

Authorship Contributions

- Participated in research design: Ruan, S.Li, Y.Li, Wu, Lee, Yan.
- Conducted experiments: Ruan, S.Li, Y.Li, Wu.
- Performed data analysis: Ruan, S.Li, Y.Li, Wu, Lee, Yan.
- Wrote or contributed to the writing of the manuscript: Ruan, S.Li, Y.Li, Wu, Lee, Yan.

References

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