The presystemic interplay between gut microbiota and orally administered calycosin-7-O-β-D-glucoside

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List of Non-Standard Abbreviations:  C7G, calycosin-7-O-β-D-glucoside; G2, calycosin-3′-O-glucuronide; VRI, VEGFR tyrosine kinase inhibitor II; DSS, dextran sulfate sodium salt; LC/MS, Liquid Chromatography/Mass Spectrometry; LC/MS/MS, Liquid Chromatography/Mass Spectrometry/Mass Spectrometry.
Abstract

Pre-systemic 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 biological 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, 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, further underwent extensive glucuronidation to yield 3′-glucuronide as the dominant metabolite. Bioactivity assays revealed that G2 exhibited similar or more potent pro-angiogenic effects than calycosin in human umbilical vein endothelial cells in vitro and in the VEGFR 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 pro-drug, but also a modulator of gut microbiota.
INTRODUCTION

There have been extensive research efforts to demonstrate comprehensive involvement of gut microbiota in diverse pathophysiological processes through interact with host cells. The host-gut microbiota metabolic interactions start at our birth and continue throughout our life, and maintain host health through generating numerous endogenous co-metabolites of biological importance (Nicholson et al., 2012). Thus manipulating microbial balance using probiotics, nutrients, and antibiotics represent promising therapies in patients with 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 the medicines are usually applied orally, the pre-systemic interaction with gut microbiota may define both the disposition profile and the pharmacological activity of the medicine. However, elucidation of the presystemic interactions has generally not been included into the work portfolio of the medicine until recently.

Glycosides are major components composed by aglycone and. In general, the glycosides are fairly soluble in water and possess poor membrane permeability. Among many glycosides found in nature, flavonoid glycosides belong to an important chemical type which ubiquitously distribute in the plant kingdom, including fruits, vegetables, soy products and some medicinal herbs. Some health related benefits associated with this group of compounds include strengthening of the immune system,
protecting against cancer, and reducing capillary fragility (Lu MF et al., 2013). Since many flavonoid glycosides exhibit low oral bioavailability due to microbial hydrolysis and conjugation that occurs in the intestine and the liver, this type of compounds are generally considered as natural pro-drugs (Arroo et al, 2008, 2009), and their health benefits are attributed to the metabolites capable of reaching the circulation (Dorjgochoo et al., 2012; Vissiennon et al., 2012; Romano et al., 2013). However, biological/pharmacological evaluation of the resultant conjugates was rather few (Terao, 1999; Koga and Meydani, 2001) due to limited access to the pure compounds. The actions of the parent compounds prior to intestinal absorption are generally ignored.

Astragali 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 utilized for treatment of cardiovascular diseases such as heart failure, angina pectoris, myocardial infarction and stroke in Asian countries (Zhao et al., 2008). Our recent study using a MRM-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 Astragali Radix (Lv et al., 2011), calycosin-7-O-β-D-glucoside (C7G) has been documented in 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 Astragali Radix, C7G exhibited anti-inflammatory effect in rabbit osteoarthritis model (Choi et al., 2007) and anti-oxidative and neuroprotective effects \textit{in vitro} (Yu et al., 2005). The aglycone calycosin demonstrated anti-diabetic (Shen et al., 2006), anti-oxidative and neuroprotective (Yu et al., 2005), and cardiovascular protective effects (Fan et al., 2003) \textit{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 pharmaceutical composition consisting of calycosin and C7G has been patented for prevention and treatment of estrogen receptor-mediated diseases (US2009/0258942 A2) and diabetic nephropathy (WO2010/081264 A1).

However, as is widely known for flavonoids, both C7G and calycosin are likely pro-drugs and calycosin conjugates could be the pharmacologically active form. Calycosin phase II conjugates appeared to be the primary circulating forms present following an oral administration of C7G to rats (Chen et al., 2011; Zhang et al., 2012) or administration of Astragali 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 \textit{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 pharmacological properties, remain unclear.

Therefore, this work was designed to study the presystemic interplay between C7G and gut microbiota and 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, 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 (ECGS), heparin, gelatin, and PEG 400 were supplied by Sigma (St Louis, MO). Dextran sulfate sodium salt (DSS) (MW 36,000-40,000 Da) was purchased from MP Biomedicals (Santa Ana, CA). Kaighn’s modification of Ham’s F12 medium (F-12K), fetal bovine serum (FBS), phosphate-buffered saline (PBS), charcoal-stripped fetal bovine serum (CS-FBS), were all purchased from Invitrogen (Carlsbad, CA). Vascular endothelial growth factors (VEGF) were obtained from R&D Systems (Minneapolis, MN). VEGFR tyrosine kinase inhibitor II (VRI) 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 rRNA-specific primers for target bacterial genera and the universal primer for total gut bacteria were supplied by TaKaRa Biotechnology Co. Ltd. (Guangzhou, China).

Pharmacokinetic Studies

Animals

Male Sprague-Dawley (SD) rats (200~250 g) were supplied by Sam Yao Hong Ltd. (Macao, China). The animals were housed in an individual ventilated cage system (Tecniplast, Italy) under 12-h light/dark cycles at a temperature of
20~23°C and 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 grams of fresh fecal samples were collected from 5 rats and pooled at equal amounts, then mixed well with 30 mL of autoclaved BHI medium. The resultant fecal suspension was centrifuged and the supernatant was decanted and centrifuged at 5,000 g for 30 min. The precipitate was re-suspended with 10 mL BHI medium to provide 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 gut bacteria suspension, 10 μL C7G or calycosin in DMSO (final concentration 25 μM) in BHI medium. Reactions were incubated anaerobically at 37°C in a GasPak™ EZ Anaerobe Pouch System (Franklin Lakes, NJ) for 0.33, 0.5, 0.75, 1, 1.5, 2, 4h, 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 5,000 g for 30 min 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 acetic ether. The organic layers were combined and evaporated under N₂ at 37°C. The residue was then reconstituted with 200 μL
methanol and filtered through a 0.45 μm membrane filter prior to 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. Cells at passage 30-40 were cultured in DMEM according to a protocol reported previously (Ruan et al., 2010). Briefly, after 21 days of culture, the prepared Caco-2 monolayers were rinsed twice with HBSS and pre-incubated in HBSS at 37°C for 30 min. In the absorptive transport study, 0.5 mL of HBSS solutions containing C7G or calycosin (final concentration 12.5, 25, 50, or 100 μM) were loaded at the apical (A) side (donor chamber), and 1.5 mL of blank HBSS was placed at 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, 60 min) for sample analysis. After each sampling, 0.1 mL HBSS was added to the receiver chamber to maintain a constant volume. All the experiments were performed in triplicate.

The apparent permeability coefficients ($P_{app}$) of C7G and calycosin from apical side to basolateral side ($P_{app \text{ A to B}}$) or from basolateral side to apical side ($P_{app \text{ B to A}}$) obtained from the bidirectional transport study using the Caco-2 cell model were calculated using the following equation (Schrickx and Fink-Gremmels, 2007):

$$P_{app} = \frac{(dC/dt \times V)}{(C_0 \times A)}$$
where \( \frac{dC}{dt} \) is the rate of the tested compound appearing in the receiver chamber, \( V \) is the volume of the solution in the receiver chamber, \( C_0 \) is the initial concentration of the tested compound added in the donor chamber, and \( A \) is the cell monolayer surface area.

**Hepatic metabolism 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 carried out according to the method recently reported by our group (Ruan and Yan, 2014). Glucuronidation reaction was carried out in rat liver microsomes at 0.1 mg protein/mL and incubated for 10 min. Sulfation was examined with pooled rat liver S9 at 1 mg/mL and the reaction was allowed to proceed for 60 min. The concentrations of C7G and calycosin were 200 \( \mu \)M. Zero-min reactions, reactions without co-factor (PAPS or UDPGA) or with denatured proteins were performed in parallel as controls. All reactions were conducted in triplicate and terminated by adding 200 \( \mu \)L of ice-cold methanol. After centrifugation at 20,000 \( g \) for 10 min, the supernatant was filtered and subjected to 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 (Yan et al., 2012) with minor modifications. In brief, a cannula was implanted into the jugular vein for blood sampling prior to 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 PEG 400 and water.
(3/7, 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 h) 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 min at 15,000 g to obtain plasma. An aliquot (100 μL) of plasma sample was mixed with 2 μL of puerarin (internal standard) followed by adding three volumes of methanol to precipitate proteins. After centrifugation (20,000 g, 10 min), the supernatant was filtered and subjected to HPLC-MS/MS analysis.

**Calibration curves of C7G, calycosin and G2 in rat plasma**

Stock solutions of C7G, calycosin and G2 were prepared in methanol and diluted to 8 different concentrations. An aliquot of each solution was spiked with pooled rat plasma to achieve final concentrations of C7G, calycosin and G2 within 4.88-5000, 4-88-5000, 2.44-40000 nM, respectively. The resultant mixture was processed as described above and analytes were determined by LC-MS/MS. The calibration curves were constructed by plotting peak area ratio of the analyte to the internal standard as a function of the concentration of the analyte. Intra- (3 replicates within a day) and inter-day (3 replicates per day over 3 days) variations were measured at two concentration levels to determine the precision and accuracy. The limit of detection (LOD) and limit of quantification (LOQ) were defined as the lowest concentration of the analyte resulting in a signal-to-noise ratio of 3:1 and 10:1, respectively. The results
indicated a good linearity ($r^2 > 0.999$), high sensitivity and good reproducibility of the analytical method (Supplemental Tables 1 and 2).

**HPLC-DAD analysis** Samples from the transport study and *in vitro* metabolic studies were analyzed on an Agilent 1200 series HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with a vacuum degasser, a binary pump, an autosampler and DAD. Samples were separated on an Agilent ODS reversed-phase C$_{18}$ column (250 × 4.6 mm, 5 μm) coupled with an ODS reversed-phase C$_{18}$ guard column (12.5 × 4.6 mm, 5 μm). The column was maintained at 25°C and the flow rate set at 1.0 mL/min. The injection volume was 70 μL. C7G, calycosin and their metabolites were monitored at 254 nm and the UV absorption spectra were recorded over 210-400 nm. The mobile phases consisted of 0.1% aqueous formic acid (A) and ACN (B) and eluted as follows: 0-33min, 5%-39% B; 33-40min, 39%-100% B.

**HPLC-MS/MS analysis** HPLC-MS/MS analysis was performed on an 4000 Q-Trap HPLC-MS/MS system (Applied Biosystems, Foster City, CA) consisting of an HP 1200 series binary pump RPLC system and an mass spectrometry coupled with an electrospray (TurboIonSpray) ionization source. Sample analysis was performed on an Agilent ZORBAX SB C$_{18}$ column (100 × 2.1 mm, 3.5 μm) coupled with a SB C$_{18}$ guard column (15 × 2.1 mm, 3.5 μm). The mobile phase and the gradient elution were the same as that used for HPLC-DAD analysis. Negative ionization mode showed a higher sensitivity and thus, was chosen for sample analysis in the present study. The TurboIonSpray ion source conditions were as follows: curtain gas (CUR) 10, ionspray voltage (IS) -4000 V, temperature (TEM) 450°C, ion source gas 1 (GS1)
Plasma samples were analyzed in MRM mode by monitoring 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 (200~250g) were randomly divided into two groups (6 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 (UC 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 pooled each within 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, 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 appropriate time intervals (0, 2, 6, and 12 h). Reactions were terminated by an immediate centrifugation (5,000 g, 10 min, 4°C) and the bacteria were obtained for extraction of the total DNA using the MiniBEST Bacterial Genomic DNA Extraction Kit following the manufacturer’s instruction.
DNA was stored at -80°C prior to RT-PCR analysis.

**RT-PCR analysis** RT-PCR analysis was performed in duplicate in 96-well plates on the ABI 7500 PCR instrument (Applied Biosystems Inc., USA.). 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 referenced literature were 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 mili-Q water. The cycling conditions consisted of preliminary denaturation (95°C for 30 s), followed by 40 cycles of 95°C for 5 s and 60°C for 34 s. Melting curve analysis was performed at the end of run by heating to 95°C for 15 s followed by cooling to 60°C for 60 s 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** Comparative cycle threshold (Ct) method was used to compare the compositional alteration of each target 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 16S rRNA gene, according to the following formula: \( \Delta C_t = C_t (\text{targeted bacterial gene}) - C_t (\text{conserved region of 16S rRNA gene}) \). Thereafter, the relative DNA levels of each target gene at time 2, 6 or 12 h were calculated using the \( \Delta \Delta C_t \) method (Livak and Schmittgen, 2001): \( \Delta \Delta C_t (\text{targeted gene}) = \Delta C_t (\text{targeted gene at 2, 6 or 12 h}) - \Delta C_t \)
(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, Zebrafish embryos were collected at 24 h post fertilization (hpf), distributed into a 12-well microplate with 15 fish each well and pretreated with 1 μM VEGFR tyrosine kinase inhibitor II (VRI) for 2 h. 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 equivalent to no treatment. The viability and gross morphological changes of zebrafish embryos were evaluated.

**Data analysis and statistics** The plasma concentration-time profiles and pharmacokinetic parameters of G2 were obtained using non-compartmental 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 zero to infinity (AUC$_{0-\infty}$) was calculated using 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 means ± SD unless otherwise mentioned. The differences between groups were compared using Student’s t-test or one-way ANOVA followed by Tukey’s multiple comparison test. $P<0.05$ was deemed significant.
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 24 h. Its aglycone calycsoin can only be detected in minor amounts in plasma samples collected at 6 min and 10 min from only two rats (data not shown). In contrast, two peaks showing 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 mono-sulfate (Figure 1B), indicating the occurrence of deglycosylation of C7G following further conjugation of calycosin in vivo.

The plasma concentration-time plot of G2 showed multiple peaks (Figure 1C), which could be attributed to enterohepatic circulation and/or two phases of G2 formation at intestine and liver. The overall systemic exposure (AUC₀-∞) was 104.8 ± 12.4 μM×h with the maximum concentration (Cmax 18.3 ± 7.30 μM) reached at 0.63 ± 0.91 h, indicating a rapid absorption of G2. The t₁/₂ of G2 in rats was 8.51±4.42 h, 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 min and absent in controls (Figure 2A). The metabolite B1 exhibited a pseudo-molecular ion ([M-H]-) at $m/z$ 283 (Figure 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-CH$_3$]-), 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. C7G was time dependently decreased by the gut microbiota and was depleted within 2 h (Figure 2C). Meanwhile, B1 showed a quick increase, reached a plateau at about 2 h and remained constant afterwards. When incubated alone, calycosin remained intact in rat gut microbiota within 2 h. These results indicated that calycosin is quite stable in rat gut bacteria.

Transport of C7G and calycosin across Caco-2 cell monolayer The $P_{app}$ values from the bidirectional transport of C7G and calycosin across Caco-2 cell monolayers are summarized in Table 2. The $P_{app}$ values of C7G were ~ $1.0 \times 10^{-6}$ cm/s over the examined concentrations, predicting a moderate oral absorption (20-70%) in human body. The $P_{app}$ values (~ $3.5 \times 10^{-5}$ cm/s) of calycosin were 20~40 times those of C7G, indicating a much higher intestinal permeability of the aglycone than C7G. The efflux ratios ($P_{app}$ B to A/$P_{app}$ A to B) of both C7G and calycosin were less than 1.5, suggesting that both compounds transport across intestinal epithelial cells mainly via passive diffusion. Furthermore, the $P_{app}$ values did not change proportionally with the substrate increase, indicating that there were no transporter-mediated mechanisms involved in calycosin and C7G transport.
**Phase II Metabolism of C7G and calycosin by liver subcellular fractions**

C7G was not metabolized within 1 h when incubated with rat liver subcellular fractions fortified with PAPS or UDPGA. In contrast, calycosin yielded two metabolites (Figure 3A & 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-CH$_3$]$^-$) in their MS/MS spectra (Figure 3C). As a result, the two metabolites were tentatively identified as the mono-sulfates of calycosin. The mass spectrum of G1 and G2 showed a molecular ion ([M-H]$^-$) at $m/z$ 459 (Figure 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 rat liver.

**Calycosin and G2 promoted HUVEC proliferation** As shown in Figure 4, both
G2 and calycosin promoted cell proliferation significantly in dose-dependent manners within 10-100 μM. Compared to 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, while calycosin showed a significant inhibition, which should be resulted from the cytotoxic effect of calycosin observed at this concentration (Supplemental Figure 1). A 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 pyridinyl-anthranilamide compound that displays anti-angiogenic properties, has been shown to potently inhibit the kinase activities of both VEGFRs 1 and 2 (Furet et al., 2003). The pretreatment of 24 hpf zebrafish embryos with 1 μM VRI for 2 h followed by 24 h incubation in embryos 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) (Figure 5I-B). Following incubation of the VRI-treated embryos with 12.5 - 50 μM calycosin or G2 for 24 h, the VRI-induced blood vessel loss at ISV and DLAV regions were dose-dependently rescued while the impaired SIV branching was partially restored (Figure 5I (C-H), 5III). Moreover, G2 showed more significant rescuing effects than calycosin in the recovery of defective blood vessels in zebrafish (Figure 5I (C-H), 5III) at the same dosage. Incubation of the VRI-treated
embryos with calycosin or G2 for 48 h resulted in more potent blood vessel rescuing effects (Figure 5II (C-H), 5III).

*Incubation with C7G stimulated the growth of Lactobacillus and Bifidobacterium in gut bacteria from normal and colitic rats.* As shown in Figure 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 6h and 2h, 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 h). 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 h 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.
Discussions

Flavonoid glycosides are commonly considered as pro-drugs 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 pre-systemic 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 pharmacological 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 pro-angiogenic calycosin-3′-O-glucuronide, while
the parent C7G was poorly 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, while the aglycone calycosin generated was highly stable in gut microbiota. Although recent studies reported demethylation and dehydroxylation of C7G in rat gut microbiota (Chen et al., 2011) or human gut bacterial isolates (Zhang et al., 2014), deglycosylation seems to be the dominant metabolic pathway. In view of the high permeability (~10⁻⁵ cm.s⁻¹), calycosin formed is likely to stay shortly in 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 main circulating form in the plasma and still stayed in the systemic circulation by the end of the experiment (24h). 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, not even to mention the pro-angiogenic 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 inter-species 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 \textit{in vivo}. It’s interesting to note that incubation of the parent C7G markedly stimulated the growth of the beneficial bacteria \textit{Lactobacillus}, slightly increased the relative content of \textit{Bifidobacterium}, but not affected \textit{Enterobacteriaceae} in the \textit{in vitro} anaerobic incubation with gut microbiota. This probiotics-like effect was more potent on the growth of the beneficial gut bacteria \textit{Lactobacillus} and \textit{Bifidobacterium} from colitic rats, supporting a potential of this flavonoid glycoside in reinstating microbial balance. The growth curves of three gut bacteria tested seem to be genus specific and varied with origins of gut microbiota, but not affected by C7G treatment. The probiotics-like effects of C7G on \textit{Lactobacillus} and \textit{Bifidobacterium} in rat gut microbiota pools agreed well with a recent report from Zhang and co-workers (Zhang et al., 2014), which examined the \textit{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 mimic the microbial balance shift \textit{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, however, have been addressed scarcely. The growth stimulating effect of C7G on \textit{Lactobacillus} and \textit{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 which can be deglycosylated by gut microbiota to provide sugar source to the later may modulate gut microbiota growth. Since C7G was depleted by gut microbiota within 2h of incubation while the calycosin generated was relatively stable in gut microbiota (Figure 2C), so we speculate that calycosin may also contribute to the probiotics-like effect observed, which warrants further study.

There are a few studies reporting bioactivities of flavonoid conjugates in vivo. The phase II conjugates, sulfates or glucuronides, have been reported antioxidation (Terao, 1999), neuroprotective (Ho et al., 2013), anti-inflammation (Granica et al., 2013). 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 has revealed a pro-angiogenic activity of calycosin on HUVECs in vitro and zebrafish in vivo (Tang et al., 2010) which comes from an action on estrogen receptors. When compared with calycosin, the major in vivo metabolite calycosin 3′-O-glucuronide showed rapid and comparable or more potent effect on promoting angiogenesis in HUVEC cells and zebrafish.

In conclusion, the present study demonstrated that the presystemic interplay between C7G and gut microbiota resulted in not only the metabolite producing pro-angiogenic effects, but also the probiotics-like actions of the parent compound,
both contributing to the beneficial activities of the glycoside. Our findings exemplify the importance of studying the disposition and actions of glycosides prior to 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 herb in traditional oral applications and warrant further investigational emphasis on reciprocal interactions between medicinal herbs and gut microbiota.
Authorship contributions

Participated in research design: Ru Yan, Jianqing Ruan, Shang Li, Yaping Li, Wenjin Wu, Simon Ming-Yuen Lee

Conducted experiments: Jianqing Ruan, Shang Li, Yaping Li, Wenjin Wu

Performed data analysis: Jianqing Ruan, Shang Li, Yaping Li, Wenjin Wu, Simon Ming-Yuen Lee, Ru Yan

Wrote or contributed to the writing of the manuscript: Jianqing Ruan, Ru Yan, Shang Li, Yaping Li
References


Choi SI, Heo TR, Min BH, Cui JH, Choi BH, and Park SR (2007) Alleviation of osteoarthritis by calycosin-7-O-beta-D-glucopyranoside (CG) isolated from


*Fitoterapia* 91:180-8.


Scanlan PD, Shanahan F, O'Mahony C, and Marchesi JR (2006) Culture-independent


Footnotes

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**Figure Legends**

Figure 1 MRM chromatograms of (A) blank rat plasma spiked with C7G, G2 and calycosin, (B) plasma sample obtained from rats following oral administration of C7G for 2 h; and the plasma concentration-time profile of G2 in rats after oral administration of C7G (C).

Figure 2 Typical HPLC-UV chromatogram of incubates of C7G with rat gut microbiota for 30 min (A), mass spectra of C7G standard and the metabolite B1 (B), and time courses of C7G elimination and metabolite B1 formation in rat gut microbiota (C).

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

Figure 4 Effects of calycosin and G2 on proliferation of HUVEC by XTT assay. VEGF treatment (20 ng/mL) serves as positive control. Results are expressed as percentage of control (100%). Data are means ± SEM of at least five independent experiments.

Figure 5 The pro-angiogenesis effects of calycosin and G2 on damaged blood vessel in Tg(fli-1a:EGFP)y1 zebrafish embryos. Pre-damaged blood vessel embryos were
incubated with embryo water or 12.5, 25 and 50μM calycosin or G2 for 24h (Figure I) and 48h (Figure II). (A) Vehicle control: 52 hpf embryo, intersegmental blood vessels (ISVs), subintestinal vessels (SIV) and dorsal longitudinal anastomotic vessels (DLAV) appear as well developed net structure. (B) VRI pre-treatment only: 28 hpf embryo received 2 h VRI treatment lead to reduced blood vessel formation in ISV, SIV and DLAV; (C-H) Incubating VRI pre-treated embryo with 12.5, 25 and 50 μM calycosin or G2. (I, J) Health embryos (without VRI pretreatment) were treated with 50 μM calycosin or G2: Normal ISV, SIV and DLAV development were similar to that observed in vehicle control. (a-j) enlarged ISV region (× 4.5) of A-J respectively. Gray arrows indicate SIV, Yellow arrows indicate DLAV, Red arrows indicate ISV and White arrows indicate the lost blood vessels. (III) Percentage recovery of ISV of Tg(fli-1a:EGFP)y1 zebrafish. Data are plotted as the means ± SD (n = 3).

Figure 6 Effects of C7G on relative contents of target bacteria in gut microbiota from normal rats and rats with colitis induced by dextran sulfate sodium.
<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Primer name</th>
<th>Sequence (5’-3’)</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td>total bacteria</td>
<td>968-F</td>
<td>AACGCGAAGAACCTTAC</td>
<td>(Scanlan et al., 2006)</td>
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<tr>
<td></td>
<td>1401-R</td>
<td>CGGTGTGTACAAGACCC</td>
<td>al., 2006</td>
</tr>
<tr>
<td>Bifidobacterium</td>
<td>g-Bifid-F</td>
<td>CTCCTGGAAACGGGTGG</td>
<td>(Xu et al., 2011)</td>
</tr>
<tr>
<td></td>
<td>g-Bifid-R</td>
<td>GGTGTTCTTCCCCGATATCTACA</td>
<td>2011</td>
</tr>
<tr>
<td>Enterobacteriaceae</td>
<td>Eco1457F</td>
<td>CATTGACGTTACCCGCAAGAGAAGC</td>
<td>(Bartosch et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>Eco1652R</td>
<td>CTCTACGAGACTCAAGCTTGC</td>
<td>al., 2004</td>
</tr>
<tr>
<td>Lactobacillus</td>
<td>g-Lab-F</td>
<td>TGGAAACAGTTGCTAATACCG</td>
<td>(Byun et al., 2004)</td>
</tr>
<tr>
<td></td>
<td>g-Lab-R</td>
<td>GTCCATTGTGGAAGATTCCC</td>
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Table 2  Apparent permeability coefficients ($P_{\text{app}}$) of C7G and calycosin at different concentrations on a Caco-2 cell model.

<table>
<thead>
<tr>
<th>Conc. (μM)</th>
<th>C7G</th>
<th>calycosin</th>
<th>Efflux ratio</th>
<th>C7G</th>
<th>calycosin</th>
<th>Efflux ratio</th>
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<tr>
<td></td>
<td>$P_{\text{app}}$ A to B ($10^{-7}$ cm/s)</td>
<td>$P_{\text{app}}$ B to A ($10^{-7}$ cm/s)</td>
<td>Efflux ratio</td>
<td>$P_{\text{app}}$ A to B ($10^{-7}$ cm/s)</td>
<td>$P_{\text{app}}$ B to A ($10^{-7}$ cm/s)</td>
<td>Efflux ratio</td>
</tr>
<tr>
<td>12.5</td>
<td>15.2±2.57</td>
<td>13.2±1.43</td>
<td>0.86</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>0.78</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>
<td>8.16±1.05</td>
<td>0.99</td>
<td>291.3±9.32</td>
<td>338.1±24.3</td>
<td>1.16</td>
</tr>
<tr>
<td>100</td>
<td>13.1±2.09</td>
<td>8.14±1.56</td>
<td>0.62</td>
<td>313.2±3.15</td>
<td>407.8±9.52</td>
<td>1.30</td>
</tr>
</tbody>
</table>
Figure 1
Figure 3
Figure 4
Figure 5
Figure 6