Regular Article

Pharmacokinetic Evidence on the Contribution of Intestinal Bacterial Conversion to Beneficial Effects of Astragaloside IV, a Marker Compound of Astragali Radix, in Traditional Oral Use of the Herb

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Summary: Astragaloside IV (AIV) is the most abundant saponin and a marker compound in Astragali Radix, a Chinese herb notable for its anti-aging and immune-enhancing effects. The present study investigated the role of intestinal bacterial conversion in the in vivo fate of AIV administered through a traditional oral route for the first time. When incubated anaerobically with rat intestinal bacteria, AIV generated five metabolites with three [monoglycosides brachyoside B and cyclogaleginoside B, the aglycone cycloastragenol (CA)] via stepwise deglycosylation and two from further epimerization (CA-iso) and dehydrogenation (CA-2H). Hydrolytic removal of C-6 glucose was a rate-limiting step for formations of CA and its derivatives. When AIV was orally administered to the rat, CA and CA-iso presented as the main components in plasma following AIV, and the AUC₀-∞ were 88.60 ± 9.66 (CA), 179.06 ± 28.53 (CA-iso) and 452.28 ± 43.33 nM·h (AIV). CA-2H was the predominant form in feces but was not detected in urine or plasma. This agreed well with in vitro data including rapid hepatic metabolism of CA-2H to form CA and CA-iso and reversible conversions between CA-2H and CA/CA-iso by intestinal bacteria. These findings support a crucial role of gut bacterial conversion of AIV in the traditional application of Astragali herb and warrant further investigational emphasis on CA and CA-iso.

Keywords: Astragali Radix; astragaloside IV; cycloastragenol; 3-epi-cycloastragenol; intestinal bacteria; oral pharmacokinetics; metabolism

Introduction

Astragali Radix originates from the dried root of Astragalus membranaceus (Fisch.) Bge. and A. membranaceus (Fisch.) Bge. var. mongholicus (Bge.) Hsiao.,¹ and is one of the common ingredients prescribed in compound formulas for medicinal purposes or available in daily diet as a functional food in China and other east Asian countries due to its remarkable immune-promoting and anti-aging effects.²,³

Astragaloside IV (AIV, 3-O-β-D-xylopyranosyl-6-O-β-D-glucopyranosyl-cycloastragenol, Fig. 1) is a typical cycloartane-type triterpene glycoside. As the most abundant saponin and a characteristic constituent of Astragali Radix,⁴ AIV was documented as the chemical marker for the quality control of the herb or Astragali-containing compound formulas in China.¹ Pharmacologic studies have revealed potent activities of AIV, including cardioprotective,⁵ angio-genesis,⁶ hepatoprotective,⁷ neuroprotective,⁸ anti-inflammatory,⁹ and immunoregulatory effects;¹⁰ therefore AIV is considered as the main active constituent that contributes to the beneficial effects of Astragali Radix. Astragali Radix and Astragali-containing formulas are traditionally taken orally for purposes of enhancing immune functions. It is interesting to note that there are also AIV or Astragali injections...
available for treatment of acute cardiovascular disorders in modern applications. So far, the rationale for these distinct applications of AIV remains unclear.

To date, the pharmacokinetics of AIV, alone or in an Astragali extract, have been reported. In contrast to a common oral use of Astragali Radix, most of the existing studies examined the intravenous pharmacokinetics of AIV. Only a few studies have determined AIV after oral dosing and revealed a poor oral bioavailability in rats (3.7% and 2.2%) and in dogs (7.4%). Transport studies of AIV using a Caco-2 cell model have revealed a poor permeability which is believed to be one of the determinant factors for its low oral bioavailability. On the other hand, it has been well established that intestinal bacteria play an important role in determining the absorption and systemic exposures of most saponin glycosides through structural modification of these components in gut lumen after oral intake. Ginsenosides serve as a good example: ginsenosides undergo stepwise deglycosylation and dehydrogenation by gut bacteria; these resultant metabolites are generally more permeable and believed to be the main active forms and now are the emphases of ginseng study. AIV has two sugar moieties, one xylose at C-3 and one glucose at C-6, in its structure. It is therefore reasonable to speculate that AIV undergoes intestinal bacterial biotransformation, and the resultant metabolites can be readily absorbed to reach the systemic circulation, and might also contribute to the health benefits of Astragali that have been observed in conventional applications of this herb. However, to date, the metabolic stability of AIV in gut bacteria and the resulting influence on its in vivo forms and systemic exposure remain to be addressed.

Therefore, in the present study, the metabolic stability of AIV was firstly characterized in vitro with rat fecal bacteria. Then, the resultant metabolites as well as the parent AIV were determined after an oral dose of AIV to the rat to estimate the contribution of gut bacteria to the health benefits of AIV in traditional use of Astragali.

Materials and Methods

**Chemicals and reagents:*** Astragaloside IV (purity >98%) was purchased from Shanghai Forever Biotech Co., Ltd. (Shanghai, China). Cycloastragenol (purity >97%) was supplied by Shanghai Tauto Biotech Co., Ltd. (Shanghai, China). BBL brain heart infusion (BHI) medium, a GasPak EZ Anaerobe Container System with Indicator and a GasPak EZ Large Incubation Container were purchased from Becton Dickinson (Franklin Lakes, NJ, USA). Glucose 6-phosphate (G-6-P), glucose-6-phosphate dehydrogenase (G-6-PD), nicotinamide adenine dinucleotide phosphate (NADP⁺), uridine 5’-diphosphoglucuronic acid (UDPGA), alamethicin, potassium phosphate, sodium carboxyl methyl cellulose, hemin bovine, heparin and vitamin K1 were obtained from Sigma-Aldrich (St. Louis, MO, USA). L-Cystine was pur-
chased from Research Organics, Inc. (Cleveland, OH, USA). HPLC-grade 1-butanol and acetonitrile were supplied by Merck (Darmstadt, Germany). Preparative thin layer chromatography plates (20 × 20 cm, silica gel) were purchased from Analtech Inc. (Newark, DE, USA). De-ionized water was obtained using a Milli-Q purification system (Millipore, Bedford, MA, USA).

Rat liver microsomes were a pool of liver microsomes from 12 untreated male Sprague Dawley rats and prepared using a standard procedure. The total content of proteins was measured using Lowry’s method.

**Preparation of rat intestinal bacteria:** One hundred milliliter of BHI medium (3.7 g/100 mL) was autoclaved and supplemented with i-cystine (50 mg), vitamin K1 (0.05 mg) and hemin bovine (0.5 mg) to obtain the culture medium. Fresh fecal samples were collected from 8 healthy SD rats and prepared as reported previously. The precipitate was suspended with BHI medium to produce the intestinal bacterial suspension at 0.2 g bacteria/mL.

**Preparation and structural identification of CA-2H:** The dehydrogenated metabolite CA-2H was prepared from a scale-up reaction of AIV with rat intestinal bacteria. Briefly, the scale-up reaction system consisted of 520-ml flasks, each of which contained 4 g intestinal bacteria and 11 mg of AIV in 150 mL of BHI media and was shaken at 180 rpm, 37°C under anaerobic conditions for 14 days. The incubates were then centrifuged and supernatants extracted thrice with 2 volumes of ethyl acetate. The organic layers were combined and concentrated by vacuum distillation. The concentrate was applied to preparative thin layer chromatography plates (20 × 20 cm, silica gel, Analtech, USA) and developed with chloroform and methanol (10:1). The band of CA-2H was scraped off the plates and the compound was recovered using chloroform. CA-2H (13 mg) was yielded after removing chloroform using vacuum evaporation and purity was determined by HPLC-MS/MS (ion pair 489.3 → 143.4). No CA or CA-iso was found in the prepared CA-2H.

**Metabolism of AIV, CA and CA-2H by rat liver microsomes:** Hepatic metabolic profiles of AIV and CA were characterized with rat liver subcellular fractions in a total of 200 µL reaction solution containing AIV or CA (final concentration: 5 µM) under the following conditions:

- Phase I: 1 mg/mL liver microsomes, NADPH-regenerating system (4 mM MgCl₂, 1 mM NADP⁺, 1 mM glucose-6-phosphate, and 1 U/mL glucose-6-phosphate dehydrogenase (G-6-PD) in 100 mM potassium phosphate buffer (pH 7.4)). Glucuronidation: 1 mg/mL liver microsomes, 8 mM MgCl₂, 2 mM uridine diphosphate glucuronic acid (UDPGA) and 25 µg alamethicin in 50 mM Tris-HCl (pH 7.4). All reactions were pre-incubated at 37°C for 5 min and initiated by adding respective cofactor G-6-PD or UDPGA and kept at 37°C for 1 h. Zero-min reactions and reactions without NADP⁺ or with denatured microsomes were performed in parallel to serve as controls. All the experiments were performed in duplicate.

- Metabolism of CA-2H in rat liver S9 or microsomes was also carried out in the presence of the NADH- (NADH 1 mM) and/or NADPH-regenerating system, to confirm formations of CA and/or CA-iso from CA-2H. Reaction systems in a total of 200 µL were pre-incubated at 37°C for 5 min and initiated by adding liver microsomes or S9 proteins (1 mg/mL). Reactions were kept at 37°C for 1 h. Zero-min reactions served as controls. All reactions were terminated by adding 400 µL of ice cold acetonitrile. After centrifugation at 15,000 × g for 10 min at 4°C, the supernatants were subjected to HPLC-MS/MS analysis.

**In vivo pharmacokinetic study:** Male SD rats (190 to 220 g, n = 5) were purchased from Beijing Laboratory Animal Research Center (Beijing, China). The animals were kept under standard conditions (12-h dark/light cycle, 22°C
and 50% humidity) for one week before study. A cannula (polyethylene, o.d. 0.8 mm, i.d. 0.4 mm) was implanted into the jugular vein of the rats. The animals were then fasted overnight with free access to water before the experiment. Astragaloside IV was dissolved in 5% sodium carboxymethyl cellulose solution at a concentration of 8 mg/mL and about 1 mL was orally administered to the rat at a dosage of 40 mg/kg. Blood samples (150 µL each) were withdrawn through the cannula at 0, 20, 40, and 60 min and 1.5, 2, 3, 4, 6, 8, 10, 12, 18, 24, and 36 h post-dosing and transferred into heparinized Eppendorf tubes. An equal volume of heparinized normal saline was injected back to the animal through the cannula immediately to compensate for blood loss. The blood sample was centrifuged at 1,500 × g for 5 min to obtain the plasma. Aliquots (50 µL) of plasma samples were mixed with 2 volumes of acetonitrile which contained 1 nM of digoxin as the internal standard. The resultant mixtures were vortex mixed for 1 min and centrifuged at 15,000 × g for 5 min. The supernatant (100 µL) was then mixed with 100 µL of water before being injected into the HPLC-MS/MS instrument.

To determine the existence of AIV and its metabolites in urine and feces, five rats were kept individually in metabolic cages and urine and feces samples were collected over 0–48 h after a single oral dose of AIV at 40 mg/kg. An aliquot (50 µL) of each sample was immediately centrifuged and processed as described under in vivo pharmacokinetic study. The supernatant (50 µL) was then reconstituted with 100 µL of culture medium. The resultant mixtures were immediately centrifuged and processed as described above for reactions. Each calibration curve contained 8 different concentrations (AIV, 7.81–10,000 nM; CA, 39.1–5,000 nM) and was performed in triplicate. The calibration curves were constructed by plotting the MS/MS peak area ratios of the analyte to the internal standard as a function of the concentration of the analyte.

**Determination of AIV and CA in in vivo pharmacokinetic study**

Serial working solutions of AIV and CA in acetonitrile were prepared from respective stock solutions (AIV 2 mM, CA 2 mM). Blank plasma was prepared from a blood sample pooled from 5 normal rats. To construct the calibration curves, 5 µL of each working solution was spiked into 45 µL of blank plasma. The resultant mixtures were processed immediately as described under in vivo pharmacokinetic study. Each calibration curve consisted of seven concentrations of the analytes, the concentration range of which were 2.5–250 nM for AIV and 1–100 nM for CA, and performed in triplicate. The calibration curves were obtained by plotting the peak area ratio of the analyte to the internal standard digoxin as a function of the plasma concentration of the analyte.

Calibration curves of AIV for both in vivo and in vitro studies fitted a linear regression mode with the weight of 1/\(x^2\), yet calibration curves of CA changed from a linear regression mode for the in vivo study to a quadratic profile over a wider concentration range tested in vitro with the weight of 1/\(x^2\). Both AIV and CA exhibited acceptable linearity (r > 0.98) and intra-day variations (<10%) within tested concentration ranges.

Extraction recovery of AIV and CA from in vitro bacterial incubates and rat plasma samples were determined to be around 70% and 90%, respectively, by comparison of peak area ratios of each analyte obtained from spiked samples with those of the analytes obtained from non-spiked samples at the same known amount. Limits of quantification (LOQ) of AIV and CA were determined with a criterion of signal-to-noise (S/N) ratios at 10. The LOQ were 3.9 nM (in vitro) and 2.5 nM (in vivo) for AIV and 1.9 nM (in vitro) and 1.0 nM (in vivo) for CA.

**HPLC-MS/MS analysis:** The Agilent series 1200 HPLC apparatus (Agilent Technologies, Santa Clara, CA, USA) was equipped with a vacuum degasser, a binary pump, an autosampler and a column oven. Samples were eluted on an Agilent ZORBAX SB-C18 column (3.5 µm, 2.1 × 100 mm, Agilent Technologies). Column temperature was kept at 35 °C. Injection volume was 5 µL for in vitro samples and 30 µL for in vivo samples. The mobile phases consisted of (A) 5 mM ammonium acetate in water and (B) acetonitrile. Different gradient elution programs were adopted for in vitro and in vivo studies respectively with details as follows:

**In vitro study:** 25% B, 0–1.5 min; 25–45% B, 1.5–5 min, 45–55% B 5–7 min, 55–80% B, 7–7.5 min, 80% B 7.5–9.5 min, 80–25% B 9.5–10 min, 25% B, 10–16 min. The flow rate started from 300 µL/min, increased to 400 µL/min in 1.5 min, then to 500 µL/min within 3.5 min, and was maintained for another 10 min before decreasing to 300 µL/min in the last minute.

**In vivo study:** 20% B, 0–2 min; 20–50% B, 2–4.5 min, 50–55% B, 4.5–7 min; 55–80% B, 7–7.5 min; 80% B, 7.5–9.5 min; 80–20% B, 9.5–10.5 min; 20% B, 10.5–
17 min. The flow rate started from 300 μL/min for first 2 min, increased to 400 μL/min in 2.5 min, then increased to 500 μL/min in 2.5 min, and was maintained for another 8.5 min before decreasing to 300 μL/min in the last minute.

The eluate was all directed into a mass spectrometer for mass analysis.

Mass spectrometry was performed on a hybrid triple quadrupole-linear ion trap tandem mass spectrometer (4000 Q TRAP, Applied BioSystems, USA) equipped with an electrospray ionization (ESI) interface. The instrument was operated in positive ion mode. Q1 scan mode was adopted in the preliminary scan of potential products and MRM operated in positive ion mode. Q1 scan mode was adopted and collision exit potential (CXP) was set at 40 psi. The mass spectrometry conditions were as follows: ion spray voltage 5,000 V, curtain gas (nitrogen) 10 psi, collision gas (nitrogen) set at medium, entrance potential 10 eV, capillary temperature 400°C, and both ion sources of gas (nitrogen) at 40 psi. The ion pair, declustering potential (DP), collision energy (CE) and collision exit potential (CXP) for each analyte are summarized in Table 1.

**NMR analysis of CA and CA-2H:** The NMR data for cycloastragenol was provided by the supplier, Shanghai Tauto Biotech Co. Ltd. The prepared CA-2H was dissolved in CDCl3 and subjected to NMR analysis (1H, 13C, ROESY and NOESY) on a Bruker AV-600 (Bruker, Newark, Germany), using TMS as the internal standard. Chemical shifts were expressed in δ and coupling constants (J) were reported in hertz (Hz).

CA-1H NMR (CDCl3): 0.37, 0.50 (2H, d, J = 4.5 Hz, H-19), 2.34 (1H, d, J = 8.0 Hz, H-17), 2.58 (2H, q, J = 12.0 Hz), 3.30 (1H, dd, J = 12.0, 4.5 Hz, H-3), 3.52 (1H, dt, J = 10.0, 3.5 Hz, H-6), 3.73 (1H, t, J = 7.5 Hz, H-24), 4.68 (1H, dt, J = 8.0, 6.0 Hz, H-16). 0.94, 0.96, 1.13, 1.19, 1.26, 1.26, 1.28 (s, 7×CH3). 13C-NMR spectrum: see Table 2.

CA-2H 1H NMR (CDCl3): 0.42, 0.65 (d, J = 4.5 Hz, H-19), 2.34 (d, J = 8.0 Hz, H-17), 2.58 (q, J = 12.0 Hz), 3.53 (dt, J = 10.0, 3.5 Hz, H-6), 3.76 (t, J = 7.5 Hz, H-24), 4.70 (dt, J = 8.0, 6.0 Hz, H-16), 0.91, 0.98, 1.16, 1.21, 1.23, 1.26, 1.28 (s, 7×CH3). 13C-NMR spectrum: see Table 2.

**Data analysis:** The in vivo plasma concentration-time data were analyzed using non-compartmental analysis with WinNonlin software (version 5.2, Pharsight Corp., CA, USA) and presented as mean±standard error of the mean (S.E.M.). In vitro data were expressed as mean±standard deviation (S.D.).

**Results**

**HPLC-MS/MS analysis of AIV and CA:** As shown in Figures 2 and 3, AIV and all its metabolites and the internal standards achieved good separation under the developed analytical conditions.
AIV was eluted at 3.9 min (analytical condition for the in vitro study) and 6.2 min (analytical condition for the in vivo study) (Figs. 2 and 3). Its MS\(^1\) spectrum exhibited characteristic ions at \(m/z\) 785 ([M+H]\(^+\)), \(m/z\) 802 ([M+Na]\(^+\)) and \(m/z\) 807 ([M+Na]\(^+\)) (Fig. 1), corresponding to a molecular weight of 784 Da. MS\(^2\) of the protonated ion at \(m/z\) 785 showed a series of typical fragment ions at \(m/z\) 767 ([M−H\(_2\)O+H]\(^+\)), \(m/z\) 749 ([M−2H\(_2\)O+H]\(^+\)), \(m/z\) 731 ([M−3H\(_2\)O+H]\(^+\)) resulting from in-source dissociation. In addition, there are another 3 clusters of fragment ions (Fig. 1): \(m/z\) 605 ([M−Glc−H\(_2\)O+H]\(^+\)), \(m/z\) 587 ([M−Glc−2H\(_2\)O+H]\(^+\)), \(m/z\) 569 ([M−Glc−3H\(_2\)O+H]\(^+\)) and \(m/z\) 551 ([M−Glc−4H\(_2\)O+H]\(^+\)), which correspond to the loss of one glucose molecule; \(m/z\) 473 ([M−Glc−Xyl−H\(_2\)O+H]\(^+\)), \(m/z\) 455 ([M−Glc−Xyl−2H\(_2\)O+H]\(^+\)), \(m/z\) 437 ([M−Glc−Xyl−3H\(_2\)O+H]\(^+\)), \(m/z\) 419 ([M−Glc−Xyl−4H\(_2\)O+H]\(^+\)) and \(m/z\) 401 ([M−Glc−Xyl−5H\(_2\)O+H]\(^+\)) due to loss of one glucose and one xylose moiety. The characteristic ion at \(m/z\) 143 corresponds to the C-17 side chain (2-(2-hydroxypropan-2-yl)-5-methyl-3,4-dihydro-2H-furanium), while \(m/z\) 125 and \(m/z\) 107 indicate loss of one and two water molecules from the C-17 side chain.

Cycloastragenol (CA), the aglycone of AIV, was eluted at 8.0 min (analytical conditions for in vitro samples) and 8.1 min (analytical conditions for in vivo samples) (Figs. 2 and 3). The fragment ions at \(m/z\) 491 ([M+H]\(^+\)), \(m/z\) 513 ([M+Na]\(^+\)), \(m/z\) 981 ([M+Na]\(^+\)) and \(m/z\) 1003 ([2M+Na]\(^+\)) in its MS\(^2\) spectrum correspond to the molecular weight of 490 Da for CA (Fig. 1). In addition, similar to that observed with AIV, the MS\(^2\) spectrum of the protonated ion of CA exhibited a cluster of characteristic ions at \(m/z\) 473

![Fig. 2. Representative MRM chromatograms of incubates of AIV with pooled rat intestinal bacteria for 0, 12, 24, and 96 h, and control reactions without drug (-) or bacteria (-) for 24 h](image)

![Fig. 3. Representative MRM chromatograms of rat plasma (A), urine (B) and fecal (C) samples collected at 8 h, 0–8 h, and 8–16 h after oral dosing of AIV at 40 mg/kg to the rat](image)
M2 exhibited a protonated ion (\([M+H]^+\)) at \(m/z\) 623, 162 mass units less than that of protonated ion of AIV, indicating a molecular weight of 622 Da due to the loss of the glucose moiety at C-6. MS2 of the protonated ion showed product ions at \(m/z\) 438 (\([M-Xyl-3H_2O+H]^+\)) and \(m/z\) 420 (\([M-Xyl-4H_2O+H]^+\)), corresponding to the loss of the xylose at C-3 and further sequential loss of water molecules. The presence of the characteristic ion at \(m/z\) 143 indicated an intact C-17 side chain. Thus M2 was tentatively identified as cyclogaleginoside B (3-O-\(\beta\)-D-xylopyranosyl-cycloastragenol).

M3 was eluted at the same time and exhibited similar characteristic ions (\(m/z\) 491 (\([M+H]^+\)) in MS1 spectrum and \(m/z\) 143 in MS2 spectrum) when compared with those of standard CA. Therefore, M3 was unambiguously identified as CA.

Similar to M3, M4 showed a characteristic ion at \(m/z\) 491 (\([M+H]^+\)) in MS1 and \(m/z\) 143 in the MS2 spectrum, indicating a molecular weight at 490 Da. Yet M4 was eluted later than M3. Thus, M4 was tentatively assigned as an isomer of CA (CA-iso) formed by intestinal bacteria.

The mass spectrum of M5 showed a characteristic ion at \(m/z\) 489 (\([M+H]^+\)), which was 2 Da less than that of CA, indicating the occurrence of dehydrogenation of CA. The presence of the characteristic ion at \(m/z\) 143 indicates an intact C-17 side chain. Thus, the potential sites for dehydrogenation of M5 were C-3, C-6 and C-16. The dehydrogenated site of M5 was further distinguished with 1H- and 13C-NMR data (Table 2). In comparison with the NMR data for CA, the 13C-NMR spectrum of M5 showed the absence of a C-3 signal at 78.3 ppm and H-3 signal at 3.30 ppm, while the presence of a signal at 217.2 ppm corresponds to a keto functional group. Furthermore, the 13C-NMR spectrum of M5 exhibited significant down-field shifts of C-2 (\(-4.4\) ppm) and C-4 (\(+8.7\) ppm) signals. Thus, the dehydrogenation should occur at the C-3 position of CA to form M5. The C-3 dehydrogenation was further confirmed by comparison of the NMR spectral data of M5 with literature-reported NMR data of 20R,24S-epoxy-3\(\beta\),16\(\alpha\),25-trihydroxy-9,19-cycloartan-3-one (CA-2H); M6, unknown.

Fig. 4. MS2 spectra of intestinal bacterial metabolites of AIV
M1, brachyoside B; M2, cyclogaleginoside B; M3, cycloastragenol (CA); M4, 3-epi-cycloastragenol (CA-iso); M5, 20R,24S-epoxy-6\(\alpha\),16\(\beta\),25-trihydroxy-9,19-cycloartan-3-one (CA-2H); M6, unknown.
corroborated an unaltered spatial configuration of C-20. Taken together, these data define M5 (CA-2H) as 20R, 24S-epoxy-6α, 16β, 25-trihydroxy-9, 19-cycloarten-3-one.

Rat intestinal bacterial biotransformation of CA and CA-2H: After 48-h incubation with rat intestinal bacteria, CA generated three metabolites by comparison with control reactions (Fig. 5). Two of them exhibited identical retention times and mass spectra to those of CA-iso and CA-2H; thus, were tentatively identified as CA-iso and CA-2H. The one (M6) that eluted at 9.0 min exhibited similar characteristic ions [m/z 489 ([M+H]+), m/z 143, m/z 125 and m/z 107] to those of CA-2H (Fig. 4), and thus might be an isomer of CA-2H. However, different from the common existence of CA-2H, M6 was not always observed in experiments with different batches of fecal samples.

Both CA and CA-iso were observed when CA-2H was incubated anaerobically with rat intestinal bacteria, yet they were absent from control reactions (Fig. 5), indicating that CA and CA-iso are epimers generated from hydrogenation of the C-3 keto group of CA-2H by gut bacteria. Thus, CA-iso was tentatively assigned as 3-epi-cycloastragenol.

Thus, the metabolic pathway of AIV in rat gut bacteria was proposed (Fig. 6).

Time course of AIV metabolism by rat intestinal bacteria: As shown in Figure 7, AIV exhibited a biphasic decline in rat intestinal bacteria: it showed an insignificant decrease in the first 8 h of the incubation, while it was depleted from the system at 24 h. Brachyoside B (M1), formed by removal of the xylose moiety at C-3, was detected immediately after the incubation, reached its maximum at 8 h, and was not detectable at 24 h. Meanwhile, cyclogaleginoside B (M2) resulting from hydrolytic removal of the glucose moiety at C-6 was observed after 8 h incubation, reached its maximum at 24 h, was eliminated slowly afterwards, and was still detectable at the end of 96 h incubation. Correspondingly, the aglycone CA (M3) was not detectable within the first 8 h, yet increased rapidly afterwards and accounted for 30% of the initial AIV at 24 h; there was still a slight increase of CA before it declined slowly after 72 h. The production of both CA-2H (M5) and CA-iso (M4) was in a similar manner to that of CA, except that the lag time for generation of these two metabolites was 12 h.
**In vitro hepatic metabolism of AIV, CA and CA-2H:** In rat liver microsomes, AIV did not undergo phase I metabolism or glucuronidation and CA was not glucuronidated according to a Q1 scan. In the presence of an NADPH-regenerating system, CA showed a slight decrease (around 5% based on the peak area data compared to that of the parallel incubation in absence of NADPH). Correspondingly, several peaks with main characteristic ions at \( m/z \) 529 or \( m/z \) 527 were observed under the selected ion monitoring mode (Fig. 8), corresponding to the sodiated adduct ion of oxidated CA \( \text{[CA+O+Na]}^+ \) or oxidated CA-2H \( \text{[CA-2H+O+Na]}^+ \). These findings indicate that CA undergoes oxidation and/or dehydrogenation after entering the rat liver.

CA-2H decreased significantly in a rat liver microsomal reaction system in the presence of NADH or NADPH and produced both CA and its isomer CA-iso (Fig. 9). CA-2H produced more CA than CA-iso as judged on the basis of the peak area of each (Fig. 9). Moreover, the amounts (peak area) of CA or CA-iso generated from the reaction system fortified with both NADH and NADPH equaled the sum of CA or CA-iso generated from reactions supplemented with NADH or NADPH alone. CA and/or CA-iso underwent further oxidation in the NADPH reaction system as evidenced by the detection of the protonated adduct ions of oxidated CA \( \text{[CA+O+H]}^+ \) or oxidated CA-iso \( \text{[CA-iso+O+H]}^+ \) at \( m/z \) 507 (data not shown). In a rat S9 reaction system fortified with NADPH, CA-2H was also able to generate CA as well as minor CA-iso, but to a lesser extent (data not shown). No phase I metabolism of CA or CA-iso was detected. In addition, there was no obvious metabolism of CA-2H in the rat liver S9 reaction system that was fortified with NADH.

**In vivo pharmacokinetics of AIV:** After an oral dosage of AIV to the rat, the parent AIV as well as the epimers CA and CA-iso produced by gut bacteria were detected in plasma samples using HPLC-MS/MS analysis (Fig. 3). The other three intestinal bacterial metabolites, namely the monoglycosides brachyoside B (M1) and cyclogaleginoside B (M2), and CA-2H (M4), were not detected in any plasma samples.

The plasma concentration-time profiles of AIV and its metabolites CA and 3-epi-cycloastragenol (CA-iso) are shown in Figure 10 and their pharmacokinetic parameters are summarized in Table 3. Since the standard compound of 3-epi-cycloastragenol was not available in the present study, the plasma levels of 3-epi-cycloastragenol were calculated from the calibration curve of CA. AIV appeared rapidly (detected at 20 min, the first time of sampling) in rat plasma after oral administration, reached its maximum level (C_max...
66.39 ± 6.00 nM) at 3.60 ± 0.4 h, and was eliminated at a half-life (t_{1/2}) of 5.94 ± 0.46 h. Yet the epimers CA and 3-epi-cycloastragenol were firstly reported in rat plasma at 3 h after dosing, reached their maximum levels (C_{max}) at 8 h, and were eliminated at similar rates as compared to that of AIV. When calculated based on a 1:1 stoichiometric conversion, the sum of AUC_{0-∞} values of CA (88.60 ± 9.66 nM·h) and its isomer (179.06 ± 28.53 nM·h) were around 59% that of AIV.

In the urine samples collected over 48 h after dosing, only AIV was detected (Fig. 3). In contrast, all five gut bacterial metabolites were detected in rat feces. These findings suggest that after entering the systemic circulation the epimers CA and 3-epi-cycloastragenol (CA-isos) either undergo further biotransformation and/or are excreted in the feces through biliary excretion or there is a sequestration of these two metabolites in some tissues/organs. Compared to the other three metabolites (CA-2H and the epimers CA and 3-epi-cycloastragenol) of AIV, the monoglycosides brachyoside B (M1) and cyclogaleginoside B (M2) were hardly detectable in fecal samples (Fig. 3); thus, they should be intermediate products of AIV formed in gut lumen.

**Discussion**

In contrast to a traditional oral use of medicinal herbs and functional foods and a well-recognized tremendous metabolic capability of gut bacteria, previous pharmacokinetic studies of herbal medicines or functional foods usually focused on measuring systemic exposures of the main components that originally exist in the herb and their hepatic metabolic stability as well. The impact of gut microbial conversion on the in vivo fate of parent components and the systemic exposure of resultant metabolites remains largely unknown; thus, its contribution to the holistic actions of the herb in conventional oral use is hard to estimate.

As one of the most famous and widely available tonic herbs in China, Astragali Radix has distinct applications which are associated with different administration routes: Astragali, alone or in compound formulas, is prepared as decoctions and known for its significant immune-enhancing effects in traditional medicinal practice, yet the modern intravenous preparations of the herb or its marker compound AIV are usually applied for cardiovascular disorders,¹¹,¹² such as angina pectoris. In the present study, the pharmacokinetic basis for the traditional oral application of the herb was provided for the first time. The contribution of gut bacteria was evidenced through characterizing structural modifications of the main and marker compound AIV by gut bacteria in vitro and measuring the systemic exposures of the resultant metabolites as well as the parent AIV following an oral dose of AIV to the rat.

Incubations of AIV with rat intestinal bacteria resulted in five metabolites, three (M1–M3) from stepwise deglycosylation of AIV. The aglycone cycloastragenol (CA, M3) was unambiguously identified with the standard compound. The monoglycosides, brachyoside B (M1) and cyclogaleginoside B (M2) were only tentatively assigned based on their mass spectral profiles and literature reports. Both these monoglycosides are naturally present in some Astragalus plants, for example, brachyoside B in *A. brachypterus*,²⁵ and Korean *A. membranaceus* (Fisch.) Bge. (*Leguminosae*)²⁸ and cyclogaleginoside B in the inflorescences of *A. galegiformis* L. (*Leguminosae*); however, none has been reported in *A. membranaceus* (Fisch.) Bge. or *A. mongholicus* (Bge.) Hsiao, the botanical origins of Astragali Radix.

Further dehydrogenation of the resultant CA at C-3 position in rat intestinal bacteria was confirmed by the generation of 20R,24S-epoxy-6α,16β,25-trihydroxy-9,19-cycloart-3-one (M5, CA-2H) on mass and NMR analyses. Similar metabolic alteration of other triterpenoid saponins, such as ginsenosides, by intestinal bacteria has been reported.²¹ The metabolic pathway of AIV by gut microbiota is thereby proposed (Fig. 6). The conversion between CA and 20R,24S-epoxy-6α,16β,25-trihydroxy-9,19-cycloart-3-one (CA-2H) was demonstrated to be reversible by the formation of CA from the incubation of the isolated CA-2H with rat intestinal bacteria.

CA also formed an isomer (M4) in rat intestinal bacteria according to the mass data. This CA isomer could also be generated directly from CA-2H in rat intestinal bacteria. Similar structural conversion has been observed with glycyrrhizins, a pentacyclic triterpenoid saponin glycoside, when it was incubated anaerobically with fresh human intestinal microflora.³⁰ Glycyrrhizin was hydrolyzed to its aglycone 18β-glycyrrhetic acid, which was then transformed reversibly to 3-epi-18β-glycyrrhetic acid via epimerization through an intermediate 3-dehydro-18β-glycyrrhetic acid. Thus, the formation of M4 from CA via a similar mechanism was proposed in the present study (Fig. 6) and CA and M4 are tentatively assigned as epimers with epimerization occurring at the C-3 position.

AIV elimination by rat intestinal bacteria exhibited a biphasic profile (Fig. 7). Formation time-courses of the two monoglycosides brachyoside B (M1) and cyclogaleginoside B (M2) and the subsequent generation of the aglycone CA and its derivatives CA-iso and CA-2H indicate that AIV deglycosylation initiate at the C-3 xylose occurs rapidly, while the hydrolytic removal of the C-6 glucose from AIV or brachyoside B formed is the rate-limiting step for the formation of the aglycone CA and its derivatives.

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**Table 3. Pharmacokinetic parameters of AIV, CA and CA-iso after oral administration of AIV at 40 mg/kg to the rat (mean ± S.E.M, n = 5)**

<table>
<thead>
<tr>
<th>Compound</th>
<th>C_{max} (nM)</th>
<th>T_{max} (h)</th>
<th>AUC_{0-∞} (nM·h)</th>
<th>t_{1/2} (h)</th>
<th>MRT (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AIV</td>
<td>66.39 ± 6.00</td>
<td>3.60 ± 0.40</td>
<td>452.28 ± 43.33</td>
<td>5.94 ± 0.46</td>
<td>9.33 ± 0.79</td>
</tr>
<tr>
<td>CA</td>
<td>11.77 ± 2.42</td>
<td>8.00 ± 0.62</td>
<td>88.60 ± 9.66</td>
<td>4.42 ± 0.81</td>
<td>11.77 ± 1.17</td>
</tr>
<tr>
<td>CA-iso</td>
<td>18.01 ± 4.52</td>
<td>8.00 ± 0.00</td>
<td>179.06 ± 28.53</td>
<td>5.70 ± 0.92</td>
<td>12.91 ± 1.56</td>
</tr>
</tbody>
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It is interesting to note that removal of the C-6 glucose from AIV or brachyoside B was only initiated after 8-h incubation. A similar phenomenon has been observed with the metabolism of the protopanaxatriol (PPT)-type ginsenosides by human intestinal bacteria. Compared to the protopanaxadiol (PPD)-type ginsenosides, which have a C-3 glucose and underwent immediate elimination in intestinal bacteria (our research group unpublished data), both AIV and the PPT-type ginsenosides have a C-6 glucose in their structures. The distinct time-course profiles of deglycosylation of these two types of compounds might be due to a C-6 steric hindrance which has been evidenced in structure-activity relationship studies of ginsenosides. The findings also indicate that different gut bacterial species might be involved in removal of sugar moieties at different positions of these triterpenoid saponins and there is a compositional/functional alteration of gut bacteria during in vitro anaerobic incubation with incubation time is prolonged.

When AIV was orally dosed to the rat, the plasma concentration-time profile of AIV (T_max of 3.60 h and t1/2 of 5.94 h) was in good agreement with the previous report. The two monoglycosides of AIV, brachyoside B and cyclogalaginoside B, which were low in intestinal bacterial incubations, were traceable in the fecal samples and not detected in plasma. Thus, these two metabolites should be transient intermediates and are unlikely to contribute significantly to the reported activities of Astragali or AIV. The dehydrogenated product 20R,24S-epoxy-6α,16β,25-trihydroxy-9,19-cycloarten-3-one (M5, CA-2H) was found in significant amount in fecal samples, yet it was not detectable in either urine or plasma samples. In contrast, both epimers, CA and 3-epi-cycloastragenol (CA-iso), presented in rat plasma in significant amounts, but were absent in urine samples. On the other hand, the in vitro metabolic studies revealed rapid formations of CA and CA-iso from CA-2H and minor oxidation and/or dehydrogenation of CA in rat liver as well as reversible conversions between CA-2H and CA/CA-iso in gut bacteria. These findings from both in vivo and in vitro studies support the following speculation on the fate of the bacterial metabolites of AIV: once CA-2H is formed and absorbed, it will be subjected to rapid hepatic metabolism to generate CA and CA-iso, then both compounds may undergo minor hepatic metabolism but extensive biliary excretion and enter the gut lumen where they form CA-2H and are excreted into feces. Formations of oxidated CA and CA-2H were found in minor amounts in vitro and not detected in vivo, suggesting that hepatic oxidation of CA/CA-2H is insignificant for the in vivo fate and actions of AIV. A further study is warranted to unravel the conversions among CA-2H, CA and 3-epi-cycloastragenol in vivo.

The present study reported the pharmacokinetic profiles of CA and its epimer 3-epi-cycloastragenol (CA-iso) following oral administration of AIV, for the first time. Neither CA nor 3-epi-cycloastragenol was detected in rat plasma until 3 h after oral dosing of AIV. This finding agreed well with their formation profiles in vitro in rat intestinal bacteria. The total AUC of both epimers was around 59% of that of AIV, indicating the clinical implications of the two gut bacterial metabolites. Interestingly, although the level of CA formed in vitro from bacterial conversion of AIV and hepatic metabolism of CA-2H was significantly higher than that of 3-epi-cycloastragenol, the latter showed a much higher C_max (18.01 ± 4.52 nM vs. 11.77 ± 2.42 nM) as well as a doubled AUC in rat plasma when compared with those of CA. These results indicate the stereoselective pharmacokinetics of the two epimers and warrant further study.

It is noteworthy that a number of A. membranaceus extracts exhibited varying degrees of capability to promote the expression of telomerase. Both AIV and CA have been marketed as telomerase activator supplements for disease treatment and longevity. Although there are many research publications related to potential benefits of AIV, none directly relates to its role as a telomerase activator. In contrast, CA exhibited moderate telomerase-increasing activity and inhibited the onset of cellular senescence of CD4+ and CD8+ T lymphocytes from HIV-1-infected persons. These activities of CA are in concert with the conventional applications of Astragalus herb in anti-aging and immune enhancement. CA is naturally absent in Astragali Radix but its systemic exposure was close to that of the parent AIV. The findings obtained from the present investigation offer the pharmacokinetic basis for beneficial actions of AIV and Astragalis Radix in a traditional oral route and warrant further investigational emphasis on gut bacterial metabolites CA and 3-epi-cycloastragenol. Furthermore, our current study with AIV also exemplifies the importance of bacterial conversion to the holistic actions of medicinal herbs or functional foods in conventional use.

References

7) Liu, H., Wei, W., Sun, W. Y. and Li, X.: Protective effects of...


