Regular Article

In vitro Intestinal Absorption and First-pass Intestinal and Hepatic Metabolism of Cycloastragenol, a Potent Small Molecule Telomerase Activator

Jing ZHU¹, Stephanie LEE¹, Maurice K. C. HO¹, Yueqing HU¹, Haihong PANG¹, Fanny C. F. Ip¹, Allison C. CHIN², Calvin B. HARLEY², Nancy Y. Ip^{1,2} and Yung H. WONG^{1,2,*}

¹Biotechnology Research Institute, Department of Biochemistry, Hong Kong University of Science and Technology, Hong Kong, China

²TA Therapeutics Limited (a Geron-BRC/HKUST Joint Venture), Hong Kong, China

Full text of this paper is available at <http://www.jstage.jst.go.jp/browse/dmpk>

Summary: Cycloastragenol (CAG) is the aglycone derivative of astragaloside IV which has recently been demonstrated to activate telomerase and represents a potential drug candidate for the treatment of degenerative diseases. In the present study, intestinal absorption and metabolism of CAG were examined using the Caco-2 model and liver microsomes, respectively. The results showed that CAG rapidly passes through the Caco-2 cell monolayer by passive diffusion. Four different glucuronide conjugates and two oxidized CAG metabolites were found in the apical and basolateral sides of Caco-2 monolayer, suggesting that first-pass intestinal metabolism of CAG might occur upon passage through the intestinal epithelium. CAG underwent extensive metabolism in rat and human liver microsomes with only 17.4% and 8.2%, respectively, of the starting amount of CAG remaining after 30 min of incubation. Monohydroxylation of the parent and oxidation of the hydroxylated CAG were found in the liver samples. The present study indicates that CAG is efficiently absorbed through intestinal epithelium. However, extensive first-pass hepatic metabolism would limit the oral bioavailability of this compound.

Keywords: Cycloastragenol; TAT2; Caco-2 cells; liver microsomes; absorption; metabolism

Introduction

Telomerase is a specific ribonucleoprotein reverse transcriptase capable of synthesizing or maintaining the length of a telomere through adding hexameric DNA repeats to telomeric ends.¹⁾ Abolition of telomerase activity results in genomic instability and cell growth arrest or apoptosis. Telomerase activation therefore emerges as a novel and attractive approach for the treatment of cancer, degenerative diseases and chronic conditions or infections which arise from accelerated cellular aging and loss of tissue homeostasis.^{2–7)}

Cycloastragenol (CAG, herein refer to as TAT2) is the aglycone derivative of astragaloside IV, a major saponin extracted from the root of *Astragalus membranaceus*.⁸⁾ CAG belongs to the group of triterpene, a naturally occurring bioactive structure. Numerous studies report the pharmacological effects of triterpenes, including anti-inflammatory,⁹⁾ anti-hepatotoxic,¹⁰⁾ antimicrobial,¹¹⁾ an-

tiviral,¹²⁾ antitumor,^{13,14)} anti-leukemia,¹⁵⁾ antinociceptive^{1–6)} and immunomodulate activities.¹⁷⁾ CAG was identified as a telomerase activator through screening a collection of Chinese medicinal extracts by the TRAP (Telomere Repeat Amplification Protocol) assay. Recently, it has been demonstrated that CAG transiently activates telomerase, slow telomere loss, increases replicative capacity, and more importantly, strengthens immune function in CD8⁺ T lymphocytes from HIV-1-infected patients.¹⁸⁾ This led to interest in developing CAG as a novel therapeutic agent for the treatment of degenerative diseases and conditions, and it is currently being advanced to clinical evaluation.

Although the *in vitro* biological activities of CAG are understood, its *in vivo* biological responses are largely dependent on pharmacokinetic process, such as intestinal absorption and first-pass metabolism, which control the access of this compound to the target tissue by dictating the amount that enters the body from the gut lumen and

Received; April 26, 2010, Accepted; July 11, 2010, J-STAGE Advance Published Date: September 22, 2010

*To whom correspondence should be addressed: Yung H. WONG, Biochemistry Department, Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong, China. Tel. +852-2358-7328, Fax. +852-2358-1552, E-mail: boyung@ust.hk

influencing the proportion that survives through first pass metabolism in both gut and liver. Therefore, it is necessary to investigate whether these processes are potential factors that would affect *in vivo* efficacy of CAG. It has been reported that the saponin astragaloside IV exhibits poor oral bioavailability which is only 2.2% in rat¹⁹⁾ and 7.4% in dog. The low bioavailability likely results from extremely limited intestinal absorption (mean P_{app} was $6.7 \pm 1.0 \times 10^{-8}$ cm/s by Caco-2 model)¹⁹⁾ and vigorous hepatic metabolism (50% of dosed amount was found metabolized in rat),²⁰⁾ indicating the adverse impact of pharmacokinetic disposition on biological benefit of this promising agent. Up to now, no data have been reported concerning the oral absorption, metabolism or bioavailability of the aglycone derivative CAG. As part of the preclinical profiling of CAG, the present study aims to characterize the intestinal permeability, cellular uptake and disposition of CAG using the human Caco-2 cell model. In addition, the hepatic biotransformation of this compound was assessed using isolated rat and human liver microsomes. Together, these two models provided important predictive information regarding the oral bioavailability of CAG and valuable reference for the *in vivo* activity study of this compound.

Materials and Methods

Materials: CAG was prepared by acid hydrolysis of astragaloside IV, purchased from Medicass Biotechnologies (Beijing, China). The structure of CAG was identified by ¹HNMR, ¹³CNMR and MS spectroscopies and the purity of CAG was determined to be over 99% by HPLC-ELSD analysis. The Caco-2 cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). Dulbecco's modified Eagle medium (DMEM), non-essential amino acids (NEAA), fetal bovine serum (FBS) were purchased from Life Technologies Corp. (Carlsbad, CA, USA). Bovine serum albumin (BSA), NADP, β -NADH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, cyclosporin A, DMSO, β -glucuronidase, and pepsin were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA). Pooled rat (male) and human (male) liver microsomes were obtained from CellzDirect Inc. (Dallas, TX, USA). All other reagents were of the highest purity.

Cell culture: Caco-2 cells were cultured in DMEM supplemented with 10% FBS, 1% NEAA, 100 units/ml of penicillin, and 0.1 mg/ml of streptomycin and were grown in a humidified atmosphere of 5% CO₂ at 37°C. Cells were harvested with trypsin-EDTA and seeded in 12 mm i.d. Transwell inserts (polycarbonate membrane, 0.4 μ m pore size, Corning Costar Corp., Corning, NY) in 12-well plates at a density of 1×10^5 cells/cm². The culture medium (0.5 ml in the insert and 1.5 ml in the well) was replaced every 48 h for the following 21 days in cul-

ture. The transepithelial electric resistance (TEER) of the monolayers was checked routinely before the experiment using a Millicell-ERS Voltammeter (Millipore Corp, Bedford, MA). The integrity of the monolayers was assessed by measuring TEER value and permeation of paracellular marker lucifer yellow. Inserts with TEER values greater than 350 Ω cm² in culture medium and lucifer yellow transport lower than 1.0×10^{-6} cm/s were selected for transport experiments.

Transport study using Caco-2 cells: Drug transport across cell monolayers was measured in apical (AP)-to-basolateral (BL) or BL-to-AP directions. Both sides of the monolayers were equilibrated with transport medium (Hank's balanced salts solution (HBSS) was supplemented with 25 mM HEPES, pH 7.4) for 30 min at 37°C before replacing the medium with transport buffer containing 50 μ M test compound to either the apical chambers (0.5 ml for AP-BL transport study) or the basolateral chambers (1 ml for BL-AP transport study). The other side was replaced by fresh transport buffer without any additive. The transport experiment was performed at 37°C with agitation at a speed of 300 rpm.

The paracellular transport marker Lucifer yellow was added to the AP side of all inserts for the assessment of monolayer integrity. Samples were collected from both sides of the cell monolayer after 1 h incubation and analyzed by LC-MS. AP-to-BL permeability or apparent permeability coefficient P_{app} (cm/s) of the test compound was calculated according to the following equation:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{A \cdot C_0}$$

where dQ/dt is the rate of appearance of test compound on the basolateral side (μ mol/s), C_0 is the initial concentration on the apical side (μ mol/ml), and A is the surface area of the monolayer (cm²).

To determine the intracellular amount of CAG, the Caco-2 cell monolayers were obtained from the filters of Transwells after each transport study and rinsed rapidly with 2 ml of ice-cold saline for 3 times. Afterward, the collected monolayers, spiked with 50 μ l testosterone (40 μ g/ml) as an internal standard, were lysed with 0.5 ml of methanol following by sonication for 5 min. The lysate was centrifuged and the supernatant was analyzed by LC-MS.

To investigate possible efflux transporters that may be involved during the transport of CAG, probenecid, cyclosporine A and quinidine were used at 100 μ M concentrations as selective inhibitors of MRP1/2 and P-glycoprotein. Each inhibitor was preloaded at both the AP and BL sides for 1 h. The transport studies were then performed in the presence of inhibitors at both AP and BL sides as described above. To test the role of the paracellular pathway in the absorption of CAG, the Caco-2 cell monolayers were incubated with transport buffer

containing 2.5 mM EGTA for 30 min. Then, bidirectional transport of CAG was carried out in the presence of 2.5 mM EGTA.

In vitro metabolism of CAG by Caco-2 cells: Caco-2 cell monolayers were exposed to 50 μ M CAG in either the AP or BL chamber. After incubation for indicated times, solutions on both sides were collected and lyophilized. The residue was dissolved with 500 μ l methanol, vortexed and centrifuged. The supernatant was collected for LC-MS analysis. For estimation of the quantities of various metabolites in Caco-2 cultures, the actual initial amounts of CAG ($[CAG]_0$) added to the chambers were experimentally determined. The amount of CAG metabolized at particular time t ($[CAG]_{metab, t}$) will be equal to the difference of the initial amount of CAG (in nmol) and sum of CAG obtained from AP and BL sides, *i.e.*

$$[CAG]_{metab, t} = [CAG]_0 - ([CAG]_{AP, t} + [CAG]_{BL, t})$$

The estimated amount of individual metabolite was calculated as the ratio of the peak area for the particular metabolite to that of the sum of peak areas of all the metabolites on the same mass spectrum (excluding the CAG peak), *i.e.*

$$[Metabolite]_t = [CAG]_{metab, t} \times \frac{\text{Individual peak area}}{\text{Sum of metabolite peak areas}}$$

The corresponding percentage of individual metabolites converted from the original amount of CAG was therefore calculated as $[Metabolite]_t/[CAG]_0 \times 100\%$.

In vitro metabolism of CAG by rat and human liver microsomes: *In vitro* metabolism of CAG was conducted in a system containing 50 μ l of NADPH regenerating solution (1 mM NADP, 1 mM NADH, 1 mM glucose-6-phosphate, 2 unit/ml glucose-6-phosphate dehydrogenase and 4 mM $MgCl_2$), 5 μ l of 1 mM CAG and 425 μ l Tris·Cl buffer (50 mM, pH 7.4). The mixture was pre-incubated at 37°C for 30 min. Reactions were initiated by adding 20 μ l rat/human liver microsomal suspensions (20 mg protein/ml) and shaken at 37°C for 30 min. The reactions were terminated by the addition of 2 ml ice-cold dichloromethane containing internal standards. After extraction by shaking the sample tube and centrifugation at 3000 rpm for 5 min, the organic phase was transferred to a new tube and evaporated under N_2 . The residue was reconstituted in 100 μ l methanol and analyzed by LC-MS system for evaluating stability and identifying metabolites.

The cytochrome P450 activities of liver microsomes were evaluated using probe substrate testosterone which bears structural similarity with CAG. The determined K_m and V_{max} for the conversion of testosterone to major metabolite 6 β -hydroxytestosterone was greater than 8.6, 19 μ M and 1.3, 4.0 nmol/min/mg for rat and human liver microsomes, respectively (reference data was supplied by

Cellzdirect).

LC-MS analysis: The samples were analyzed using a HPLC system equipped with a Finnigan P4000 quaternary pump and an AS3000 autosampler injector (Thermo Scientific, Waltham, MA, USA). An Agilent Extend C18 column (150 mm $\times$ 2.1 mm i.d., 5 μ m) was used as the analytical column. For quantitative analysis, CAG retention time was 3.2 min at a flow rate of 0.35 ml/min using a mobile phase system consisting of 65% acetonitrile and 35% water. For identification of intestinal metabolites, elution was performed using the following acetonitrile gradient conditions for a run time of 30 min: 20% at 0 min, 45% at 20 min, and 50% at 30 min. For liver microsomal metabolite analysis, an acetonitrile gradient system (0 min, 55%; 25 min, 65%) was used at a flow rate of 0.35 ml/min.

The compounds were detected with an online Thermo-Finnigan LCQ Classic ion trap mass spectrometer equipped with electrospray ionization (ESI) source. Positive and negative modes were used for the quantification and identification of liver microsomal and intestinal metabolites, respectively. Thermo-Finnigan Xcalibur software was used for system control and data acquisition. The major working parameters for mass spectrometer were as follows: sheath gas N_2 flow at 60 arbitrary units, auxiliary gas N_2 flow at 40 arbitrary units, ion-spray voltage 4.5 kV, capillary temperature at 200°C. Quantification of CAG was performed by selected ion monitoring (SIM) mode with SIM ions of m/z 513 and 1003 for CAG, and m/z 288 for internal standard testosterone. The quantification LC-MS method was validated according to FDA guidelines for bioanalytical method validation.²¹⁾ Linearity, accuracy, intra- and inter-assay precisions were all within the defined acceptance criteria. The metabolites were identified by MS full scan mode (m/z 100–1800) and MS/MS spectra were produced by collision-induced dissociation of each molecular ion of interest, using normalized collision energy of 50%.

Data analysis: All Caco-2 experiments were performed in triplicate. Data are presented as means $\pm$ SD. The student's *t*-test was adopted at a significance level of $p < 0.05$ to determine statistically significant differences among the experimental groups.

Results

Permeability of CAG across Caco-2 cells: To determine whether the transport of CAG was time-dependent, Caco-2 cells were exposed to 50 μ M CAG on the apical (AP) or basolateral (BL) side for up to 180 min at 37°C. Transport of CAG in the absorptive (AP-BL) and the secretory (BL-AP) directions are shown in **Figure 1A**. CAG permeation across Caco-2 monolayer increased linearly in the first hour. Approximately 9.1% of applied amount reached the receiver side within 10 min, and the

Fig. 1. Permeability of CAG across Caco-2 monolayer

(A) Transport of CAG (50 μM) in the absorptive (AP-BL) and secretory (BL-AP) directions were studied in time-course manner. (B) P_{app} values of CAG (50 μM) across Caco-2 cell monolayers in the absorptive (AP-BL) and secretory (BL-AP) directions in the presence of P-glycoprotein inhibitor cyclosporin A, quinidine and MRP1/2 inhibitor probenecid. Data are means $\pm$ SD of triplicates in a representative experiment.

Table 1. Apparent permeability of CAG across Caco-2 cell monolayer

[CAG] (μM)	$P_{\text{app}} \times 10^{-6}$ (cm/s)		Efflux ratio $P_{\text{app}}(\text{BL to AP})/P_{\text{app}}(\text{AP to BL})$
	AP to BL	BL to AP	
1	32.5 ± 2.3	31.0 ± 1.2	0.95
5	29.6 ± 1.9	30.1 ± 1.1	1.00
25	31.2 ± 3.5	33.4 ± 2.8	1.07
50	34.5 ± 2.3	33.1 ± 2.6	0.96
50 + EGTA	35.3 ± 3.0	36.9 ± 2.4	1.04

Different concentrations of CAG were added alone as indicated, or together with 2.5 mM EGTA. Data were averages $\pm$ SD from 3 individual experiments.

amount quickly increased to 38% after 60 min exposure. The transported amount of CAG slowed down and reached a plateau from 60 min to 180 min. This indicates a rapid and high penetration of CAG across Caco-2 cell monolayer. The BL-AP direction showed a similar transport rate for the opposite direction throughout the experiment (**Fig. 1A**), suggesting that the contribution of active transporter proteins for CAG efflux was negligible.

To elucidate the mechanism of CAG transport, the effect of initial loading concentration of CAG on the transport rate was examined after 30 min incubation. P_{app} values were found to be essentially the same at different concentrations over the range from 1 to 50 μM (**Table 1**). There were no significant differences in the efflux ratios throughout the concentration range studied, indicating a passive diffusion process for the transport of CAG across Caco-2 monolayers.

Passively absorbed compounds penetrate through Caco-2 cell monolayer by parallel transcellular and/or paracellular pathways. It has been reported that the integrity of the tight junctions in Caco-2 cells can be modu-

lated by varying the Ca^{2+} concentrations in the media.²²⁾ To determine the relative contributions of transcellular and paracellular pathways to the overall permeability of CAG, transport experiments were performed in the absence or presence of 2.5 mM EGTA to disrupt the tight junctions by Ca^{2+} chelation.²²⁾ The effect of EGTA was evaluated on the permeation of lucifer yellow (paracellular marker) in separate plates of the same source cells. A more than 20-fold increase in P_{app} value (from 1.9×10^{-7} to 5.6×10^{-6} cm/s) for lucifer yellow was observed with the presence of 2.5 mM EDTA in the transport media, indicating the positive effect of EDTA on opening cellular tight junctions. However, there was no significant change in P_{app} values of CAG with or without the Ca^{2+} -chelator EDTA (34.5×10^{-6} versus 35.3×10^{-6} cm/s; **Table 1**), indicating that the contribution of the paracellular route to the passage of CAG was minimal. CAG appeared to be transported across the Caco-2 cell monolayers predominantly via the transcellular pathway.

Additional experiments were performed to verify the lack of involvement of active transport mechanism in CAG efflux. The bidirectional transepithelial permeability of CAG was investigated in the absence or presence of cyclosporine A and quinidine (inhibitors of P-glycoprotein), as well as probenecid (an inhibitor of multidrug resistant-associated proteins MRP1/2). As shown in **Figure 1B**, CAG transport was not significantly altered in the presence of any of the inhibitors tested, suggesting that neither P-glycoprotein nor MRP1/2 was involved in CAG transport.

Intracellular accumulation of CAG: Intracellular accumulation of CAG in intact Caco-2 monolayers was measured over 6 h after AP loading of 5, 25 and 50 μM CAG. The intracellular amount of CAG increased according to the applied CAG concentrations. Uptake ki-

Fig. 2. Identification of CAG metabolites in Caco-2 culture. Representative LC-MS total ion chromatograms in negative mode of (A) CAG, (B) AP and (C) BL samples in AP-BL transport, as well as (D) AP and (E) BL samples in BL-AP transport experiment of CAG were shown. 50 μ M CAG was added to alternative side of cell monolayers and incubated for 6 h. Aliquots of buffer from apical and basolateral sides were collected and analyzed for metabolites. G1-G4 are glucuronide conjugates, and D1 and D2 are oxidized forms of CAG.

netics showed that the intracellular amount of CAG rose rapidly at the beginning of incubation and reached maxima at 10 min (0.19 ± 0.03 , 0.93 ± 0.14 and 1.79 ± 0.27 nmol/cm² culture area for 5, 25 and 50 μ M, respectively). The accumulation levels decreased afterwards and reached a steady state after 2 h with average values of 0.12, 0.61 and 0.99 nmol/cm² for 5, 25 and 50 μ M, respectively, accounting for approximately 4.8%, 4.9% and 4.0% of the initially applied CAG. Considering that transport is a dynamic process, the decline of accumulated amount of CAG may result from its transport to the basolateral chamber. Alternatively, CAG may be metabolized upon prolonged incubation with the Caco-2 cells. It should also be noted that non-specific binding was evident. At 50 μ M CAG, $2.5 \pm 0.4\%$ of applied amount was detected in a naked filter without cells. Together, these findings demonstrate that only a very small amount ($< 2.5\%$) of CAG is present in the culture medium should be used trapped in Caco-2 cells.

Metabolism of CAG in Caco-2 monolayers: The intestinal metabolism of CAG was studied using Caco-2 cell monolayers. Several phase I and phase II metabolites were observed on the AP and BL sides of the cell monolayer. LC-ESI-MS chromatograms of CAG metabolites obtained from the AP or BL sides in bidirectional

Fig. 3. ESI-mass spectra of CAG metabolites from Caco-2 cells. Metabolites illustrated in Figure 3 were subjected to negative ESI-mass spectrometry for identification. G1 and G3 with deprotonated ions m/z 663 ($487 + 176$) are glucuronide conjugates of oxidized CAG, G2 and G4 with deprotonated ions m/z 665 ($489 + 176$) are conjugates of CAG. The deprotonated molecular ions of D1 and D2 were 485 and 487 respectively, corresponding to sequential and mono-oxidized CAG.

transports are shown in **Figure 2**. Four glucuronide conjugates were detected, eluting at 6.76 (G1) and 7.86 min (G2) on the donor side, and 4.31 (G3) and 5.31 min (G4) on the receiver side, irrespective to the direction of transport. **Figure 3** shows the negative ESI-MS spectra of CAG and its metabolites. The mass of the deprotonated CAG was detected as m/z 489, and deprotonated molecular ions of the glucuronide conjugates were detected as m/z 663 (G1), m/z 665 (G2), m/z 663 (G3) and m/z 665 (G4). G2 and G4 could be identified as glucuronide conjugates of CAG, as the MS/MS fragment ions produced the CAG molecule ion at m/z 489 and a glucuronide molecule ion at m/z 176. G1 and G3 seemed to be glucuronide conjugates of the oxidized CAG, which produced daughter ions m/z 176 and 487. The daughter ion m/z 487 was 2 Da less than the CAG molecule, indicating that the oxidation of CAG ($-H_2$) occurred before glucuronide conjugation. G1-G4 could not be detected when the extracted media were pretreated with β -glucuronidase, confirming that G1-G4 were glucuronides.

Structural analysis of the conjugates was made based on MS fragment ions. However, the major identifiable fragments were those losing H_2O molecules and glucuronic acid from the original metabolites. The positions at which glucuronic acid was attached to CAG could not be determined with the mass spectra only. The amounts of G1-G4 obtained from the experiments were, however, too low for further NMR analysis.

Apart from the phase II glucuronide conjugates, two phase I oxidized CAG metabolites were detected on both sides and were eluted at 26.1 min (D1) and 26.5 min (D2), respectively (**Fig. 2B–E**). Mass spectrum data in **Figure 3** showed the deprotonated molecular ions of D1 at m/z 485 and D2 at m/z 487, suggesting that D1 was a sequential oxidation product ($-2 \times H_2$) of CAG with a 4 Da loss in molecular weight, and D2 was the

mono-oxidated metabolite ($-H_2$) of CAG. Oxidation is a common metabolic pathway for triterpene which often occurs on the hydroxyl groups on the carbon ring.^{23–25} D1 and D2 may possibly be the products of two and one hydroxyl moieties oxidation.

We also monitored the time-dependent formation and excretion of the 6 metabolites. Due to the lack of purified standards, the amounts of metabolites were estimated as fractions of the amounts of metabolized CAG in the cultures (see Methodology for the detail of the calculations). As shown in **Table 2**, a slow loss of CAG in the culture was detected, with about 87% of CAG remaining intact after 6 h incubation. Two conjugates, G3 and G4, first appeared on the basolateral side after 30 min incubation. Their amounts increased in a time-dependent manner, and corresponded up to 2.8% and 2.7% of the initially applied CAG, respectively. The occurrence of the other two conjugates, G1 and G2, was slower than G3 and G4, which started to be detectable at 2 h incubation. The amounts of G1 and G2 generated were only half (1.2% and 1.3% of the initially-applied CAG) of those of G3 and G4. Contrarily, the two oxidation products D1 and D2 showed up quickly on both AP and BL sides after only 30 min incubation. The amount of D1 increased slowly over 6 h, while D2 was generated much faster, with 0.94 nmol (3.6%) detected on both sides at 6 h, which was the most abundant metabolite of CAG detected in the reaction.

Metabolism of CAG by rat and human liver microsomes: The hepatic metabolism of CAG was investigated using rat and human liver microsomes. Total ion chromatogram and computer-reconstructed extracted ion chromatograms for CAG incubation samples are shown in **Figure 4**. Barely detectable CAG peaks were eluted at 15.8 min in the total ion chromatogram shown in **Figure 4A** (middle and lower panel), indicating that CAG was extensively metabolized in rat or human liver

Table 2. Quantities of CAG and its metabolites in Caco-2 cell monolayer culture medium after incubation at different periods

CAG and metabolites	Amount in cell culture medium (nmol)							
	30 min		2 h		4 h		6 h	
	AP	BL	AP	BL	AP	BL	AP	BL
CAG	15.4	7.5	12.1	10.4	11.3	10.7	10.5	11.2
Oxidized CAG glucuronide (G1)	n.d.	n.d.	0.12 (0.5%)	n.d.	0.22 (0.9%)	n.d.	0.31 (1.2%)	n.d.
CAG glucuronide (G2)	n.d.	n.d.	0.11 (0.4%)	n.d.	0.24 (0.9%)	n.d.	0.34 (1.3%)	n.d.
Oxidized CAG glucuronide (G3)	n.d.	0.22 (0.8%)	n.d.	0.40 (1.6%)	n.d.	0.55 (2.1%)	n.d.	0.72 (2.8%)
CAG glucuronide (G4)	n.d.	0.15 (0.6%)	n.d.	0.33 (1.3%)	n.d.	0.50 (1.9%)	n.d.	0.69 (2.7%)
Two sequential oxidized CAG (D1)	0.13 (0.5%)	0.15 (0.6%)	0.11 (0.4%)	0.12 (0.5%)	0.12 (0.5%)	0.17 (0.7%)	0.11 (0.4%)	0.18 (0.7%)
Oxidized CAG (D2)	0.30 (1.2%)	0.32 (1.2%)	0.38 (1.5%)	0.36 (1.4%)	0.42 (1.6%)	0.45 (1.7%)	0.39 (1.5%)	0.55 (2.1%)
Total metabolism of CAG	1.19 (4.6%)		2.04 (7.9%)		2.68 (10.4%)		3.3 (12.8%)	

The initial concentration of CAG added to the AP side was 50 μ M. Numbers in brackets represent the amounts of metabolites expressed as percentages of the initially applied CAG. n.d., not detectable.

Fig. 4. (A) Total ion mass chromatograms for LC-MS analysis of 10 μ M CAG after incubation with rat or human liver microsomes for 0.5 h

By comparing with the control chromatograms generated from CAG incubated with or without inactivated microsomes (uppermost), peaks of potential metabolites in the samples were identified and marked with arrows. The extent of metabolism was indicated by the diminishing peak of CAG.

(B) Extracted ion mass chromatograms of CAG metabolites formed in liver microsomes.

The potassium-cationized molecular ions of metabolites ($[M+K]^+$ m/z 543 and 545) were selected as extracted ions.

microsomes, with only 17.4% and 8.2% of CAG remaining intact in rat and human samples, respectively. The calculated *in vitro* $t_{1/2}$ values of the CAG metabolism were 12.0 ± 4.2 min and 8.4 ± 1.5 min, and *in vitro* CL_{int} were 148.1 ± 51.8 ml/min/kg and 143.7 ± 25.7 ml/min/kg for rat and human, respectively.

Compared to the blank sample without addition of microsomes and the control sample using inactivated rat microsomes, a few more peaks were observed in front of the CAG peak in the middle and lower panels of **Figure 4A**. The full scan mass experiment showed that the potassium-cationized molecular ions $[M+K]^+$ of these metabolites were m/z 543 or 545, suggesting that the molecular weight of these metabolites were 504 or 506. The extracted ion mass chromatograms with potassium-cationized molecule ions $[M+K]^+$ of m/z 543, 545 are shown in **Figure 4B**. As many as seven different metabolites, namely M1 to M7, were detected in rat and human microsomes samples.

Hydroxylation and oxidation in the carbon ring and side-chain are well-known metabolic pathways for triterpene and steroid structures.^{23–25} As shown in **Figure 5B**, M2, M3 and M6 with a molecular weight of 504 (as compared to CAG with a molecular weight of 490) were likely to be the hydroxylated and oxidized products of CAG. Other metabolites such as M1, M4, M5 and M7 all had a molecular weight of 506. The 16 Da gain in their molecular weight could be attributed to the addition of an oxygen atom, suggesting that these metabolites were potentially mono-hydroxylated products of CAG.

The metabolic profiles shown in **Figure 4B** indicate that the formation of metabolites varied between species. **Table 3** lists the relative quantity of each metabolite der-

ived from rat and human liver microsomes. In human, the metabolite M3 (m/z 504) was the most abundant (42.5%), which was probably generated by oxidation followed by hydroxylation. In contrast, M6 (m/z 504), the isomer of M3, was predominant in rat liver microsomes (26.9%). The levels of mono-hydroxylated metabolites (M1, M4 and M5) were much higher in rat than in human. The second predominant metabolite M5 (m/z 506) that accounted for 21.6% of the total metabolites in rat was almost undetectable in human (0.6%), making it a unique metabolite in rat. Moreover, the base peaks of $[M+K]^+$ were further fragmented by collision energy to provide the product ion spectrum of CAG and its metabolites (**Fig. 5**). Derivation of the ions at m/z 455, 437 and 419 was attributed to the neutral loss of $2H_2O$, $3H_2O$ and $4H_2O$ from CAG, respectively, which was consistent with the presence of four hydroxyls on CAG. The predominant ion at m/z 143 was formed by the cleavage of 2-(5-methyltetrahydrofuran-2-yl) propan-2-ol from C17 of CAG, which also yielded the substructure ion m/z 331 as shown in the same spectrum. The opening of D carbon ring yielded the characteristic ions at m/z 275. This is in agreement with data previously reported for steroids of similar structure.^{25,26} The metabolites with relatively minor structural alterations supposedly underwent similar fragmentation pathways as the parent. The MS spectral data of these metabolites are listed in **Table 3**. Some characteristic fragment ions were also observed in metabolite fragmentation, e.g. neutral loss of H_2O and m/z 143 from the cleavage on C-17. However, attempts to identify the specific positions of hydroxyl group in these metabolites using mass spectrometry were unsuccessful because no isomer-specific fragment ions were

Fig. 5. The CID mass spectrum and proposed fragmentation pathways for CAG

The molecular ions of CAG ($[M+K]^+$ m/z 529, $[M+Na]^+$ m/z 513) were fragmented by 50% collision energy to generate representative product ions. The major fragmentation pathways for CAG include the cleavage on C17 position which produced the base peak ion m/z 143 and ion m/z 331, and the loss of water which formed ions $[M-H_2O+H]^+$ m/z 473, $[M-2H_2O+H]^+$ m/z 455, $[M-3H_2O+H]^+$ m/z 437 and $[M-4H_2O+H]^+$ m/z 419. The opening of furan ring resulted in ion m/z 387, while that of the D carbon ring resulted in ions m/z 275 and 227.

Table 3. MS spectral data of CAG and phase I metabolites generated in rat and human liver microsomes

Compound	Retention Time (min)	MS spectral data in the positive ion mode	Contribution to total metabolites (%)	
			Rat	Human
CAG (MW 490)	15.8	529 $[M+K]^+$, 513 $[M+Na]^+$, 455 $[M-2H_2O+H]^+$, 437 $[M-3H_2O+H]^+$, 419 $[M-4H_2O+H]^+$, 143 [C-17 side chain]	—	—
M1	4.2	545 $[M+K]^+$	7.6	7.1
M2	6.1	543 $[M+K]^+$, 487 $[M-H_2O+H]^+$, 469 $[M-2H_2O+H]^+$, 433 $[M-4H_2O+H]^+$, 425	8.0	7.6
M3	7.7	543 $[M+K]^+$, 527 $[M+Na]^+$, 487, 469, 433, 184	13.8	42.5
M4	8.5	545 $[M+K]^+$, 417 $[M-5H_2O+H]^+$, 143	13.0	15.6
M5	8.9	545 $[M+K]^+$, 425, 391	21.6	0.6
M6	10.2	543 $[M+K]^+$, 487 $[M-H_2O+H]^+$, 468, 441, 143	26.9	18.2
M7	10.6	545 $[M+K]^+$, 453 $[M-3H_2O+H]^+$, 425, 371, 143	9.0	8.3

obtained (Table 3). Negative ion electrospray MS-MS product ion mass spectra also did not provide further insight on these metabolites.

Discussion

To investigate the oral bioavailability of CAG, *in vitro* models of intestinal absorption and metabolism (Caco-2 cells) as well as hepatic biotransformation (liver micro-

somes) were used in this study to monitor the processing of CAG. The apparent permeability of CAG was comparable to that of other known drugs such as propranolol and testosterone ($P_{app(AP-BL)}$ for these two compounds were 70.2×10^{-6} and 40.4×10^{-6} cm/s, respectively, as determined in our experiments). Approximate 87% of initially applied CAG remained unchanged after 6 h incubation with Caco-2 culture. These results suggest that CAG may have good intestinal permeation potential and the first-pass intestinal metabolism may not be extensive. However, CAG underwent extensive hepatic phase I metabolism as revealed in liver microsome-mediated degradation, which might limit the systemic bioavailability of CAG *in vivo* after oral administration.

CAG is a naturally occurring cycloartane-type triterpene. The extensive phase I metabolisms in liver microsomes resulting in low oral bioavailability has been described for other structurally similar triterpenes. Metabolic stability study of 11-keto-boswellic acid (KBA), a pentacyclic triterpene, in rat and human liver microsomes showed that >80% of KBA were metabolized after 15 min incubation,²⁷⁾ resulting in a very low human plasma bioavailability of KBA after oral administration. Oleanolic acid was also unstable in *in vitro* and *in vivo* pharmacokinetics study, with only an absolute oral bioavailability of 0.7%.²⁸⁾ Zhang and coworkers reported that the oral bioavailability of ganoderiol F, a cycloartane-type triterpene extracted from *Ganoderma lucidum*, was only 10.5% in rats.²⁹⁾ Apparently, low oral bioavailability caused by hepatic phase I metabolism was not uncommon for this class of compounds and thereby limiting their efficacy *in vivo*.

Metabolic modifications of CAG in rat and human microsomes were identified as mono-hydroxylation and hydroxylation after oxidization. The positions where the modifications occurred could not be confirmed due to the limited structural information available from MS fragmentation. A previous study reports that the biotransformation of three cycloartane-type triterpenes by the filamentous fungus *G. fusarioides* occurred mainly on their C-3 hydroxyl group for oxidation, C-17 side-chain for hydroxylation, and C-4 for demethylation.²³⁾ NMR study will be necessary to clarify whether the oxidation and hydroxylation of CAG occur at similar positions. Biotransformation of triterpenes can lead to pharmacological inactivation or cytotoxicity. Zhang *et al.*²⁴⁾ reported the *in vitro* cytotoxicity and potency of metabolites of 20-(S)-protopanaxatriol against human cancer cells, suggesting that hydroxylation at C-28 or C-29 may increase cytotoxicity, and the introduction of a hydroxyl group to C-11 β , C-15 α , or the side chain may reduce the anti-tumor activity of this substrate. Inevitably, the extensive first-pass hepatic metabolism of CAG may affect drug biological activity *in vivo*.

In conclusion, our data show that CAG is efficiently

transported across Caco-2 cells via passive transcellular diffusion. The predicted intestinal metabolism of CAG was minimal with a small percentage of conjugated and oxidized metabolites formed. Given that the expression profiles of metabolic enzymes in Caco-2 cells may be different from those in the human intestine, further *in vivo* absorption studies will be required to confirm the results from the *in vitro* models. Extensive hepatic metabolism was observed for CAG after incubation with rat and human liver microsomes, indicating that only a small fraction of orally administered CAG may be able to reach the systemic circulation. Extensive hepatic metabolism may be a primary factor precluding the pharmacological activity of CAG *in vivo*.

Acknowledgments: This project was supported in part by TA Therapeutics Limited and the Hong Kong Jockey Club.

References

- 1) Granger, M. P., Wright, W. E. and Shay, J. W.: Telomerase in cancer and aging. *Crit. Rev. Oncol. Hemat.*, **48**: 29–40 (2002).
- 2) Funk, W. D., Wang, C. K., Shelton, D. N., Harley, C. B., Pagon, G. D. and Hoeffler, W. K.: Telomerase expression restores dermal integrity to *in vitro*-aged fibroblasts in a reconstituted skin model. *Exp. Cell Res.*, **258**: 270–278 (2000).
- 3) Rudolph, K. L., Chang, S., Millard, M., Schreiber-Agus, N. and DePinho, R. A.: Inhibition of experimental liver cirrhosis in mice by telomerase gene delivery. *Science*, **287**: 1253–1258 (2000).
- 4) Dagarag, M., Ng, H. L., Lubong, R., Effros, R. B. and Yang, O. O.: Differential impairment of lytic and cytokine functions in senescent human immunodeficiency virus type 1-specific cytotoxic T lymphocytes. *J. Virol.*, **77**: 3077–3083 (2003).
- 5) Gu, X. L., Zhang, J. and Brann, D. W.: Brain and retinal vascular endothelial cells with extended life span established by ectopic expression of telomerase. *Invest. Ophthalm. Vis. Sci.*, **44**: 3219–3225 (2003).
- 6) Roy, N. S. and Nakano, T.: Telomerase immortalization of neuronally restricted progenitor cells derived from the human fetal spinal cord. *Nat. Biotech.*, **22**: 297–305 (2004).
- 7) Verra, N. C. V. and Jorritsma, A.: Human telomerase reverse transcriptase-transduced human cytotoxic T cells suppress the growth of human melanoma in immunodeficient mice. *Cancer Res.*, **64**: 2153–2161 (2004).
- 8) Chen, J., Gui, D., Chen, Y., Mou, L., Liu, Y. and Huang J.: Astragaloside IV improves high glucose-induced podocyte adhesion dysfunction via α 3 β 1 integrin upregulation and integrin-linked kinase inhibition. *Biochem Pharmacol.*, **76**: 796–804 (2008).
- 9) Safayhi, H. and Sailer, E.-R.: Anti-inflammatory actions of pentacyclic triterpenes. *Planta Med.*, **63**: 487–493 (1997).
- 10) Ahmed, B., Al-Howiriny, T. A. and Mossa, J. S.: Crotalic and emarginelic acids: two triterpenes from *Crotalaria emarginella* and anti-inflammatory and anti-hepatotoxic activity of crotalic acid. *Phytochemistry*, **67**: 956–964 (2006).
- 11) Kuete, V., Nguemaving, J. R., Beng, V. P., Azebaze, A. G. B., Bodo, B. and Nkengfack, A. E.: Antimicrobial activity of the

- methanolic extracts and compounds from *Vismia laurentii* De Wild (Guttiferae). *J. Ethnopharmacol.*, **109**: 372–379 (2007).
- 12) Zhang, Q., Jiang, Z. Y., Luo, J., Liu, J. F., Ma, Y. B., Guo, R. H. and Chen, J. J.: Anti-HBV agents. Part2: synthesis and in vitro anti-hepatitis B virus activities of alisol A derivatives. *Bioorg. & Med. Chem. Lett.*, **19**: 2148–2153 (2009).
 - 13) Kamath, S. G., Chen, N., Xiong, Y., Wenham, R. and Lancaster, J. M.: Gedunin, a novel natural substance, inhibits ovarian cancer cell proliferation. *Int. J. Gynecol. Cancer*, **19**: 1564–1569 (2009).
 - 14) Li, C. H., Chen, P. Y., Chang, U. M., Kan, L. S., Fang, W. H. and Lin, S. B.: Ganoderic acid X, a lanostanoid triterpene, inhibits topoisomerases and induces apoptosis of cancer cells. *Life Science*, **77**: 252–265 (2005).
 - 15) Hata, K., Hori, K., Ogasawara, H. and Takahashi, S.: Anti-leukemia activities of Lup-28-al-20(29)-en-3-one, a lupine triterpene. *Toxicol. Lett.*, **143**: 1–7 (2003).
 - 16) Soldi, C., Pizzolatti, M. G., Luiz, A. P., Marcon, R., Meotti, F. C. and Santos, A. R. S.: Synthetic derivatives of the α - and β -amyrin triterpenes and their antinociceptive properties. *Bioorgan & Med. Chem.*, **16**: 3377–3386 (2008).
 - 17) Rios, J.: Effects of triterpenes on the immune system. *J. of Ethnopharmacol.*, **128**: 1–14 (2010).
 - 18) Fauce, S. R., Jamieson, B. D., Chin, A. C., Mitsuyasu, R. T., Parish, S. T., Ng, H. L., Kitchen, C. M. R., Yang, O. O., Harley, C. B. and Effros, R. B.: Telomerase-Based Pharmacologic Enhancement of Antiviral Function of Human CD8⁺ T Lymphocytes. *J. Immunol.*, **181**: 7400–7406 (2008).
 - 19) Gu, Y., Wang, G., Pan, G., Fawcett, J. P., A. J. and Sun, J.: Transport and bioavailability studies of astragaloside IV, an active ingredient in *radix astragali*. *Basic Clin Pharmacol, Toxicol.*, **95**: 295–298 (2004).
 - 20) Zhang, W. D., Zhang, C., Liu, R. H., Li, H. L., Zhang, J. T., Mao, C., Moran, S. and Chen, C. L.: Preclinical pharmacokinetics and tissue distribution of a natural cardioprotective agent astragaloside IV in rats and dogs. *Life Sci.*, **79**: 808–815 (2006).
 - 21) U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM): Guidance for Industry-Bioanalytical Method Validation. (2001). <http://www.fda.gov/cder/guidance/4252fnl.pdf>.
 - 22) Artursson, P. and Magnusson, C.: Epithelial transport of drugs in cell culture. II: Effect of extracellular calcium concentration on the paracellular transport of drugs of different lipophilicities across monolayers of intestinal epithelial (Caco-2) cells. *J. Pharm. Sci.*, **79**: 595–600 (1990).
 - 23) Akihisa, T., Watanabe, K., Yoneima, R., Suzuki, T. and Kimura, Y.: Biotransformation of cycloartane-type triterpenes by the fungus *glomerella fusarioides*. *J. Nat. Prod.*, **69**: 604–607 (2006).
 - 24) Zhang, J., Guo, H. Z., Tian, Y., Liu, P., Li, N., Zhou, J. P. and Guo, D.: Biotransformation of 20 (S)-protopanaxatriol by *Mucor spinosus* and the cytotoxic structure activity relationships of the transformed products. *Phytochemistry*, **68**: 2523–2530 (2007).
 - 25) Gauthier, J., Goudreault, D., Poirier, D. and Ayotte, C.: Identification of drostanolone and 17-methyldrostanolone metabolites produced by cryopreserved human hepatocytes. *Steroids*, **74**: 306–314 (2009).
 - 26) Paulette, B., Bernard, F. M. and Prudent, P.: Sex-linked specificity of the hepatic metabolism of steroids in rats. Mass fragmentography as a method for the assay of hydrogenated metabolites of corticosterone in the liver. *Biomed. mass spectrum*, **1**: 29–39 (1974).
 - 27) Krüger, P., Daneshfar, R., Eckert, G. P., Klein, J., Volmer, D. A., Bahr, U., Müller, W. E., Karas, M. and Abdel-Tawab, M.: Metabolism of boswellic acids *in vitro* and *in vivo*. *Drug Metab. Dispos.*, **36**: 1135–1142 (2008).
 - 28) Jeong, D. W., Kim, Y. H., Kim, H. H., Choi, W. R., Lee, S. M., Han, C. K. and Lee, H. S.: Dose-linear pharmacokinetics of oleonic acid after intravenous and oral administration in rats. *Biopharm. Drug. Dispos.*, **28**: 51–57 (2007).
 - 29) Zhang, Q., Zuo, F., Nakamura, N., Ma, C. M. and Hattori, M.: Metabolism and pharmacokinetics in rats of ganoderiol F, a highly cytotoxic and antitumor triterpene from *Ganoderma lucidum*. *J. Nat. Med.*, **63**: 304–310 (2009).