Inhibition of P-glycoprotein leads to improved oral bioavailability of Compound K, an anti-cancer metabolite of red ginseng extract produced by gut microflora

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**Abbreviations:** C-K, Compound K; PPD, protopanaxadiol; PPT, protopanaxatriol; CsA, cyclosporine A; UPLC, ultra-performance liquid chromatography; Q-TOF-MS, quadrupole time of flight mass spectrometry; P_{a-b}, permeability from apical to basolateral side; P_{b-a}, permeability from basolateral to apical side; P-gp, p-glycoprotein; MDR1, multi-drug resistance gene 1; AUC_{0-\infty}, area under the plasma concentration-time curve from time zero to infinity; K_e, constant of elimination rate; t_{1/2}, terminal half-life; MRT, mean residence time; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
Abstract

Ginsenosides are extensively hydrolyzed by gut microflora after oral administration and their metabolites are pharmacologically active against lung cancer cells. In this study, we measured the metabolism of various ginsenosides by gut microflora, and determined mechanisms responsible for the observed pharmacokinetic behaviors of its active metabolite, Compound K (C-K). The results showed that biotransformation into C-K is the major metabolic pathway of ginsenosides after oral administration of the red ginseng extract containing both protopanaxadiol and protopanaxatriol ginsenosides. Pharmacokinetic studies in normal mice showed that C-K exhibited low oral bioavailability. To define the mechanisms responsible for this low bioavailability, two P-gp inhibitors verapamil and cyclosporine A were used, and their presence substantially decreased C-K’s efflux ratio in Caco-2 cells (from 26.6 to less than 3), and significantly increased intracellular concentrations (by as much as 40 fold). Similar results were obtained when transcellular transport of C-K was determined using MDR1-overexpressing MDCKII cells. In MDR1a/b−/− FVB mice, its plasma Cmax and AUC0-24h were substantially increased, by 4.0 and 11.7 fold, respectively. These increases appear to be due to slower elimination and faster absorption of C-K in MDR1a/b−/− mice. In conclusion, C-K is the major active metabolite of ginsenosides after microflora hydrolysis of primary ginsenosides in the red ginseng extract, and inhibition/deficiency of P-gp can lead to large enhancement of its absorption and bioavailability.
Introduction

Ginsenosides, the dammarane-type triterpene saponins, have been found to be the major components responsible for ginseng’s pharmacological activity, especially for chemoprevention and inhibition of lung cancer. A major barrier to extend ginseng or ginsenosides’ clinical use is their low oral bioavailabilities, usually less than 5% in rodents (Xu et al., 2003; Qian et al., 2005; Joo et al., 2010). Low oral bioavailability may cause large variations in the systemic exposure during clinical trials, which may lead to ambiguous results in the trials unless an extraordinarily large number of patients are enrolled. The latter is often too expensive to conduct. The low oral bioavailability of ginsenosides was previously attributed to their poor oral absorption caused by large molecular weight and bulky sugar moieties (Liu et al., 2009).

Compound K (C-K) (Fig.1) is one of the extensively investigated ginsenosides that displayed potent chemoprevention and anticancer activities in various cancer cell lines. Although C-K is of low abundance in the raw ginseng or red ginseng extract, previous studies indicated that it could be the major metabolite after oral administration of pure protopanaxadiol (PPD-type) ginsenosides (Akao et al., 1998a; Bae et al., 2000; Yoo et al., 2011). Earlier publications on ginsenoside metabolism by gut microflora usually used either pure 20(S)-protopanaxadiol (PPD) or 20(S)-protopanaxatriol (PPT) ginsenosides (Akao et al., 1998a; Akao et al., 1998b; Tawab et al., 2003; Bae et al., 2005). However, the red ginseng extract containing both series (instead of individual ginsenosides) was usually the agent used for chemoprevention, as large quantities of purified ginsenosides are too costly for long-term use. Considering the complexity of ginseng extract, its metabolism profile may be different from single ginsenosides. Therefore, a better understanding of the metabolism of both PPD and PPT ginsenosides by gut microflora is necessary for monitoring the disposition of ginsenoside by gut microflora.
Despite demonstration of the in vivo biotransformation of ginsenosides to C-K, C-K itself was reported to have low oral bioavailability (~5%) in rats (Lee et al., 2006; Paek et al., 2006). The absorption mechanism of C-K is generally considered to be passive diffusion, as one study showed that there was no difference between bidirectional transports in Caco-2 cells (Liu et al., 2009). However, there is evidence that C-K could reverse multi-drug resistance in tumor cells (Hasegawa et al., 1994). In addition, our previous study on Rh2s, a ginsenoside analog with glucose attached to the C3 position and has the same molecular weight and log P value as C-K, indicates that Rh2 underwent strong P-gp-mediated efflux both in vitro and in vivo (Yang et al., 2011).

P-gp is one of the most prevalent efflux transporters (Aller et al., 2009; Chen et al., 2011) and plays an important role in limiting the intestinal absorption of many compounds that are its substrates (Kusuhara and Sugiyama, 2002; del Amo et al., 2009). Inhibition of P-gp leads to the improvement of oral bioavailability of several anticancer drugs (Meerum Terwogt et al., 1998; Kemper et al., 2004; van Waterschoot et al., 2009) including ginsenoside Rh2 (Yang et al., 2011). A better understanding of P-gp involvement in the transport of ginsenosides, and a more quantitative measurement with regards to if and how P-gp affects their bioavailability and potential drug-drug interaction is important for the development of ginsenosides as chemopreventive agents, as these studies are recommended by FDA in a recently published Drug-Drug Interaction Guidance (February, 2012).

In this study, we first investigated the metabolism of various ginsenosides present in a red ginseng extract in gut microflora and identified the major component after biotransformation by the microflora. We continued our efforts by determining the absorption mechanism of its major metabolite C-K, to better understand the underlying mechanism responsible for its low oral bioavailability. Therefore, the aims of this study were: 1) to delineate the dominant metabolism pathway of ginsenosides in gut bacteria lysate; 2) to systemically investigate mechanisms
responsible for poor absorption of active component, C-K, by elucidating which efflux transporter was mainly responsible for the efflux of C-K using a complementary set of *in vitro* and *in vivo* models.
Materials and Methods

Chemicals and reagents. Ginsenosides Rb₁, (purity > 98%) was purchased from National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China); Rb₂, Rc, Rd, 20(S)-Rg₃, 20(R)-Rg₃, 20(S)-Rh₂, 20(R)-Rh₂ (Each purity > 98%) were purchased from Scholarbio-Tech (Chengdu, China). Compound K and F₂ (Each purity > 98%) were purchased from Must Bio-technology Co. Ltd. (Chengdu, China). Red ginseng extract was prepared by extracting red ginseng with water for three times, and then the water extracts were concentrated and finally dried to powder (red ginseng extract) under low temperature. Rk₁/Rg₅ mixture (Total purity > 90%) was purified from processed red ginseng extract by silicon column chromatography, and identified using high-resolution mass spectrometry. All other chemicals were of analytical grade and used as received.

Cells. Cloned Caco-2 cells (TC7) cells were cultured as described previously (Hu et al., 1999). Lung carcinoma LM1 cell line was provided by Dr. Ming You’s lab at Medical College of Wisconsin. Parental MDCKII and MDR1-MDCKII cells were provided by the Netherland Cancer Institute (Amsterdam, Netherlands). The inserts (Cat# 3412) were purchased from Corning Transwell® (Lowell, MA). Digoxin, cyclosporine A, verapamil and Hanks’ balanced salt solution (powder form) were purchased from Sigma-Aldrich (St. Louis, MO). Oral suspension vehicle was obtained from Professional Compounding Centers of America (Houston, TX). BCA protein assay kit was purchased from Thermo Scientific (Rockford, IL). All other materials (typically analytical grade or better) were used as received.

Animals. Male FVB and A/J mice (6-10 weeks) were purchased from Harlan Laboratory (Indianapolis, IN). Male Mdr1a/b knockout mice (6-10 weeks) were purchased from Taconic Farms (Germantown, NY). They were acclimated in an environmentally controlled room (temperature: 25 ± 2°C, humidity: 50 ± 5%, 12 h dark-light cycle) for at least 1 week prior to
experiments. The mice were fed with rodent diet (Labdiet® 5001), and fasted overnight before starting the pharmacokinetic studies.

**Cell culture.** The Caco-2 cell culture is routinely maintained in this laboratory for the last 2 decades (Hu and Borchardt, 1990; Hu and Borchardt, 1992). The culture conditions for growing Caco-2 cells were the same as those described previously (Yang et al., 2010). The TEER value >445 Ω/cm² was used as quality control to test the tightness of tight junction in Caco-2 cells. Parental MDCKII and MDR1-MDCKII cells were cultured in Dulbecco’s modified Eagle's medium supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 100U/ml penicillin and gentamicin. The expression levels of MDR1 in MDR1-MDCKII were monitored by western blotting analysis. LM1 is a metastatic cell line derived from A/J mice (Zhang et al., 2003), which is the strain used for lung cancer carcinogenesis and chemoprevention (Yan et al., 2007). LM1 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM glutamine, 50 units/ml penicillin, 50 pg/ml streptomycin and 1% nonessential amino acids. LM1 cells were seeded at 5 × 10³ per well (96 well plate). LM1 cells were fed every day and cells were ready for use after 2-3 days post-seeding.

**Hydrolysis of ginsenosides by glycosidases prepared from mouse gut microflora.** Fresh feces were collected using the A/J mice. One part of feces was mixed with ten parts (volumes) of 0.1 mM ice-cold potassium phosphate buffer. They were vortexed for 30 seconds and sonicated in an ice-cold water bath for 30 min. The suspension was subjected to centrifugation at 1,000 rpm and 4°C for 30 min. The final supernatant of fecal homogenate was stored in aliquots at -80°C until use. Protein concentrations were determined by the BCA protein assay kit, using bovine serum albumin as the protein standard.

Frozen fecal homogenate was thawed and 200 μl was taken to the disposable glass vials. The homogenate was diluted with ice-cold potassium phosphate buffer to 2 ml. Then red
ginseng extract was added to make the final concentration 1 mg/ml and the study was performed in triplicate. The mixture was incubated at 37°C and shaken at 120 rpm for 24 hrs. Samples (100 μl) were taken at 0, 4 and 24 hr, and the reaction was stopped with the addition of 500 μl 2.5 μM testosterone (internal standard) in 100% acetonitrile. After centrifugation, 10 μl of the supernatant solution was injected into the Q-TOF/MS for analysis.

**Transcellular transport study.** The transcellular transport study was performed as described previously and the permeability from apical to basolateral side (P_{a-b}) and basolateral to apical side (P_{b-a}) were calculated based on the method described in our previous publication (Yang et al., 2011). Briefly, 2.5 ml of C-K solution was loaded onto one side of the cell monolayer, and 2.5 ml of blank HBSS onto the other. Five sequential samples (0.5 ml) were taken at different times (0, 1, 2, 3 and 4 hrs) from both sides of the cell monolayer. The same volume of C-K solution and receiver medium (fresh HBSS) was added immediately to replace the volume lost as the result of sampling. The pH values of HBSS in both apical and basolateral side were 7.4.

Intracellular concentrations of C-K were determined at the end of a transport study. The protocol for determining C-K’s intracellular amounts in cells was the same as those described previously (Yang et al., 2011). The protein concentration of cell lysate was measured using the BCA protein assay kit.

**Pharmacokinetic studies of C-K in wild-type and Mdr1a/b⁻/⁻ FVB Mice.** The animal protocols used in this study were approved by the University of Houston’s Institutional Animal Care and Uses Committee. Pharmacokinetic studies of C-K were performed in wild-type and Mdr1a/b⁻/⁻ FVB mice to investigate the role of Mdr1 or P-gp in limiting the bioavailability of C-K. C-K dispersed in the oral suspending vehicle was given by gavage to each group of mice at 10 mg/kg. The ingredients of oral suspending vehicle were shown in Table S1. Each
pharmacokinetic study was performed using five mice, and 10 timed blood samples (20-25 µl) were taken by snipping its tail, after mice were anesthetized with isoflurane gas. The blood samples were collected in heparinized tubes and stored at -20°C until analysis.

**Qualitative determination of microflora-generated secondary ginsenosides.** A Waters ACQUITY UPLC™ system (Waters Corp., MA, USA) coupled to Bruker MicroTOF mass spectrometer with an ESI source was used to qualitatively identify metabolites of ginsenosides in gut microflora. Identification of ginsenosides and related metabolites was achieved by comparing retention time with authentic compounds in combination with accurate mass. The chromatograph and MS parameters were shown in Table S2.

**Quantitative determination of C-K.** An API 3200 Qtrap® triple quadrupole mass spectrometer (Applied Biosystems/MDS SCIEX, Foster City, CA, USA) equipped with a Turbolonspray™ source, operated in a positive ion mode, was used to quantitatively measure Compound K in various matrices. The quantification was performed using multiple reactions monitoring mode (MRM) with 645.0/203.0 (m/z) for C-K and 269.2/197.1 (m/z) for formononetin (internal standard). The other MS and UPLC parameters were shown in Table S3.

The standard curves of C-K were linear in the concentration range of 19.5 nM-10 µM and the LLOQ was 9.8 nM in the mouse blood. The intra-day and inter-day precision were within 15% for all QC samples at three concentration levels (2.5, 0.3125 and 0.039 µM). A volume of 200 µl methanol (containing 1 µM formononetin) was added into 20 µl aliquot of mouse blood sample. The supernatant was evaporated to dryness at 40°C under air and reconstituted in 100 µl of 100% methanol (v/v), and a 10 µl portion of each sample was injected into the UPLC-MS/MS system for analysis.

**Pharmacokinetic analysis.** WinNonlin 3.3 (Pharsight Corporation, Mountain View, California) was used for C-K and F2 pharmacokinetic analysis. The non-compartmental model
was applied for pharmacokinetic analysis of C-K profiles. Pharmacokinetic parameters, including $C_{\text{max}}$, $T_{\text{max}}$, $k_e$, half-life, MRT and AUC were directly derived from WinNonlin.

**Statistical analysis.** The data in this paper are presented as means ± S.D., if not specified otherwise. Significance differences were assessed by using Student’s t-test or one-way ANOVA. A p value of less than 0.05 was considered as statistically significant.
**Results**

**Hydrolysis of ginsenoside by glycosidases derived from gut microflora homogenate.**

After incubation with glycosidases prepared from gut microflora, the concentration of Rb1, Rb2 and Rc in red ginseng extract decreased with time (Fig. 2). Rc was completely hydrolyzed while Rb1 and Rb2 was hydrolyzed by >80% (estimated from peak area) at 24 hr (Fig. 2A & C). As a result, they were transformed into serial deglycosylated products, Rd, F2 and C-K. Both F2 and C-K kept increasing at 4 and 24 hr compared to 0 hr while Rd did not show any obvious change in term of peak area (Fig. 2A-C), probably because it is the key intermediate of hydrolysis. Blank fecal extraction did not interfere with ginsenoside analytes in chromatography (Fig. 2D). In contrast, PPT-type ginsenosides (Re, Rg1 and Rg2) were not found to be metabolized during the 24-hr incubation period (Fig. 3). In order to better visualize metabolites change with time, UPLC-TOF-MS chromatograms were overlaid from different time points (Fig. 1E) and F2 and C-K kept increasing while both Rg3 and Rh2 slightly decreased with time (the peak of Rh2 was not shown). The likely metabolic scheme of PPD-type ginsenosides Rb1, Rb2 and Rc in the presence of PPT-type ginsenosides (as in red ginseng extract) were proposed (Supplemental Figure S1), however it is noted that Rc may be converted to C-K via a different metabolic pathway (Shin et al., 2003; Yoo et al., 2011).

**Transcellular transport of C-K across Caco-2 cell monolayers.** C-K was the major metabolite formed during hydrolysis of red ginseng extract by mouse fecal homogenate. C-K has been shown to be active against multiple cancer cells (Chae et al., 2009; Kim et al., 2010; Wang et al., 2012), including against LM1 cells (Supplemental Fig. S2). Therefore, its absorption mechanisms were further investigated to better understand the reasons for its low oral bioavailability. The transcellular transport of digoxin was measured as the positive control before the transport study of C-K was performed using Caco-2 cell monolayers. The results
showed that transcellular transport of digoxin displayed significant efflux ratio (21.0) and $P_{a-b}$ was $1.48 \times 10^{-6}$ cm/s, which was similar to our previous results (Yang et al., 2011). In the presence of 20 µM cyclosporine A, the efflux ratio of digoxin was decreased from 21 to 1.2.

The results indicated that transcellular transport of C-K (2 µM) across the Caco-2 monolayers from basolateral (B) to the apical (A) side (as measured by $P_{b-a}$) was significantly higher than transport from the A to B side (Fig. 4A), and the efflux ratios ($P_{b-a} / P_{a-b}$) were 26.6 (Table 1). Two P-gp inhibitors, 50 µM verapamil or 20 µM cyclosporine A added at the apical side, were able to greatly inhibit the efflux transport of C-K (Fig. 4B&C), and the efflux ratio was decreased to 2.9 (for verapamil) and 1.1 (for cyclosporine A), respectively. In the presence of inhibitors, the $P_{a-b}$ was significantly increased by 2.9 and 2.8 fold; and the $P_{b-a}$ was significantly decreased by 3.1 and 8.5 fold after verapamil and cyclosporine A treatment, respectively (Table 1). Consistent with the permeability results, two p-glycoprotein inhibitors also significantly increased the intracellular accumulation of C-K following treatment with 50 µM verapamil (from 0.01 to 0.50 nmol/mg) or 20 µM cyclosporine A (from 0.01 to 0.15 nmol/mg).

**Transcellular transport of C-K in MDR1- MDCKII cells.** Human MDR1/P-gp over-expressing MDCKII cells were used to confirm the predominant role of P-gp in the transport of C-K. Prior to the transport studies of C-K, 2 µM digoxin was used as a positive control in MDR1-MDCKII cell transport study. The efflux ratio of digoxin was 64.0 in MDR1-MDCKII cell and the $P_{a-b}$ is $2.98 \times 10^{-7}$ cm/s showing the normal expression of P-gp in MDR1-MDCKII cells.

The transport of C-K in parental MDCKII cells was used as a negative control, because it has low expression of human P-gp. As expected, the efflux ratio of C-K was much lower in parental MDCKII cells (Fig.4D) compared to MDR1-MDCKII cells (Fig.4E) at 2 µM (1.8 vs 18.2).
However, the $P_{ab}$ of C-K was unexpectedly higher in MDR1-MDCKII cells ($1.84 \times 10^{-6}$ cm/s) than parental MDCKII cells ($0.58 \times 10^{-6}$ cm/s), which may be due to more effective tight junctions of MDCKII cell monolayers). The recoveries of C-K were within 85-115% in Caoc-2 cells and both variants of MDCKII cells. The transport data also indicated possible delay for the transcellular transport, which is not uncommon and reported in a previous publication (Hu et al., 1994). The intracellular accumulations of C-K were also measured and the results showed that C-K accumulation was significantly higher (3.1 fold) in parental MDCKII cells (0.27 nmol/mg) than in MDR1-MDCKII cells (0.08 nmol/mg, Table 1).

**Effects of Mdr1a/b deletion on the oral bioavailabilities of C-K**

In order to investigate whether MDR1/P-gp has major effects on the oral bioavailability of C-K, plasma profiles of C-K were compared between Mdr1a/b$^{-/-}$ and wild-type FVB mice after oral dosing of C-K (10 mg/kg) (Fig.5). A previous study showed that C-K exhibited linear pharmacokinetics in term of AUC, clearance and volume of distribution from 3 to 30 mg/kg (Lee et al., 2006); accordingly, the current dose (10 mg/kg) is appropriate to reveal the role of P-gp on pharmacokinetics of C-K. In Mdr1a/b$^{-/-}$ mice (on FVB background), the $T_{max}$ was significantly prolonged (from 2.8 ± 0.84 hr to 5.6 ± 2.19 hr, $p<0.05$) compared to wild-type FVB mice. More importantly, the plasma $C_{max}$ was significantly increased by 4.0 fold ($p<0.05$), and $AUC_{0,\infty}$ and $AUC_{0-t}$ was significantly increased ($p<0.001$) by 11.7 fold and 23.5 fold, respectively, when compared to wild-type FVB mice (Table 2).

In Mdr1a/b$^{-/-}$ mice, $K_e$ was significantly decreased (by 73%, from 0.13 ± 0.07 to 0.04 ± 0.01 hr$^{-1}$, $p<0.05$) compared to wild-type FVB mice. As expected, the $t_{1/2}$ of Compound K was
significantly increased (by 3.2 fold, from 5.87 ± 3.04 to 18.68 ± 6.84 hr, p<0.05). Consistently, the MRT was also significantly increased from 7.8 hr (WT) to 30.8 hr (knockout mice).
Discussion

This comprehensive study demonstrates unequivocally that C-K is a solid substrate of P-gp, and P-gp mediates the efflux of C-K in vitro and in vivo. Our results suggest that inhibition of P-gp may represent a good strategy to improve the bioavailability of C-K. Because C-K is an active ginsenoside generated by the intestinal microflora, its bioavailability may serve as a major indicator of in vivo bioavailability of PPD-type ginsenosides present in the red ginseng extract.

Our conclusion contradicts with a previous published report that suggest passive diffusion as the major absorption mechanism for C-K, which was based on comparable bidirectional permeability values in the Caco-2 cell culture model (Liu et al., 2009). The discrepancy may due to high substrate concentration (as much as 50 µM) they used which may not be soluble or could have saturated the efflux process. Another major difference between the two studies is that we used Caco-2 TC7 (cloned) cells while Liu et al used wild-type Caco-2 cells (Liu et al., 2009). Published data indicated similar expression level of p-gp in these two cell lines (Raeissi et al., 1999; Engman et al., 2001). Thus, we are not certain as to why there is this discrepancy, but our earlier paper showed that our permeability results using Rh2 (Yang et al., 2011) also did not match their results using Rh2 (Liu et al., 2009). Our in vitro results obtained using chemical inhibitors of P-gp and MDR1-overexpressed cell line support a conclusion that P-gp is the predominant efflux transporter responsible for C-K’s in vitro disposition. These in vitro results are also consistent with our studies using P-gp knockout mice.

Our study using P-gp knockout mice suggests that in vivo bioavailability of C-K can be increased by inhibiting P-gp because plasma AUC$_{0-24h}$ of C-K was 12 fold higher in P-gp knockout mice than in WT mice. Considering the oral bioavailability of C-K is reported to be
around 5% in rodents (Lee et al., 2006; Paek et al., 2006), the current study demonstrated that inhibition of P-gp should lead to significantly increased oral bioavailability of C-K. Our previous publication showed that bioavailability of Rh2s, another active ginsenoside with a similar structure and also a solid substrate of P-gp, was increased from 1% to over 30% by coadministering cyclosporine A in mice (Yang et al., 2011).

The mechanisms responsible for increased bioavailability of C-K in P-gp knockout mice appear to be decreased $k_e$ and increased $t_{1/2}$ compared to wild-type mice (Table 2) and increased absorption (Table 1). The trend of slow elimination in Mdr1a/b -/- mice was very obvious although our last sample time was not long enough ($<2$ times of $t_{1/2}$) to determine the $k_e$ and $t_{1/2}$ with high accuracy (Fig. 5). Considering the results of the transport studies using Caco-2 and MDR1-MDCK cell monolayers, increased absorption of C-K likely contributed to the increased plasma $C_{max}$ and oral bioavailability of C-K in Mdr1a/b -/- mice. Moreover, the slower elimination and longer half-life may be attributed to less biliary excretion during the terminal phase, assuming that the absorption phase would end sooner in P-gp knockout mice because of faster absorption of the same dose. Taken together, P-gp-mediated efflux appears to be responsible for C-K’s low bioavailabilities, and in absence of this efflux mechanism, C-K would have a much higher oral bioavailability. This conclusion differs significantly from previous observation that passive diffusion is the major absorption mechanism for this ginsenoside (C-K) (Liu et al., 2009).

Our results showed that PPD-type ginsenosides (Rb1, Rb2 and Rc) were extensively metabolized while PPT-type ginsenosides (Rg1 and Re) were not metabolized significantly by glycosidases derived from the intestinal microflora of A/J mice. Two pieces of evidence
demonstrate that Rg1 and Re were not metabolized. First, the peak area of Rg1 and Re stayed unchanged from 0 to 24 hrs. Second, if Rg1 and Re were metabolized, we should have observed the formation of their metabolites, Rg2, Rh1 and/or F1, but none of these metabolites was found (Fig.3). Our findings are different from previous reports on the metabolism of PPT-type ginsenosides by human intestinal microflora. Tawab et al showed that Rg1 and Re could be extensively metabolized in human intestine by microflora (Tawab et al., 2003) and Bae et al showed human fecal specimens could metabolize Re via stepwise deglycosylation to Rg1, F1 and aglycone (Bae et al., 2005). These results suggested that in A/J mouse microflora metabolism of PPD-type ginsenosides is the predominant metabolic pathway compared to PPT-type ginsenosides when both of them were present (as in the red ginseng extract). The plasma profile of C-K after oral administration of red ginseng extract at 200 mg/kg in A/J mice further confirmed that conversion of primary PPD-type ginsenosides into C-K by gut microflora in vivo (Supplemental Figure S2).

C-K showed significant anticancer activity with IC$_{50}$ = 18.45 µM while F2 and red ginseng extract did not show a significant antiproliferation effect in LM1 cells (Supplemental Figure S3). Similarly, ginsenosides with number of sugars greater than that of F2, including Rb1, Rb2, Rd, and Rc also had lower activities (against LM1 cells) than F2 (not shown). The results were consistent with several structure-activity relationship studies, which showed that anticancer activity of ginsenoside is inversely correlated to the number of sugars, which means the deglycosylation products have stronger anticancer activities than the precursor ginsenosides (Li et al., 2009; Musende et al., 2009). Because intestinal microflora is mainly responsible for this deglycosylation reaction, gut bacteria play an important role in ginsenoside effects in vivo.
In summary, we demonstrated clearly that C-K is the major metabolite of primary PPD-type ginsenosides in the red ginseng extract after they were hydrolyzed by glycosidases present in the gut microflora. C-K has low but significant oral bioavailability, and displayed high in vitro inhibitory effects against lung cancer cells. Taken together, these findings may explain why ginsenosides such as C-K are active in vivo after oral administration of the red ginseng extract that contains large quantities of primary ginsenosides that are inactive. Because C-K is a good substrate of P-gp and its absorption was greatly impeded by P-gp efflux, inhibition of P-gp via increased absorption and/or decreased elimination may offer a viable strategy to significantly increase its oral bioavailability in vivo.
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Authorship Contributions

Participated in research design: Yang, You and Hu.
Conducted experiments: Yang, Niu, Gao, Yin and Wang.
Contributed new reagents or analytic tools: You and Jiang
Performed data analysis: Yang, Wang and Hu.
Wrote or contributed to the writing of the manuscript: Yang, Wang, Niu and Hu
DMD #44008

References


prostate cancer in vivo: formulation, pharmacokinetics, biodistribution and efficacy.

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Footnotes

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Figure Legends

Figure 1. Chemical structures of ginsenosides of the protopanaxadiol (PPD) or protopanaxatriol (PPT) series. Glu=β-D-glucose; Arap=α-L-arabinose (pyranose); Araf=α-L-arabinose (furanose); Rha=α-L-rhamnose. These structures were shown here because their concentrations appear to have changed during incubation of red ginseng extract with microflora.

Figure 2. UPLC-TOF-MS chromatograms of ginsenosides present in red ginseng extract incubated in gut bacteria collected from A/J mice at 0 hr (A), 4 hr (B) and 24 hr (C), blank fecal extraction without ginsenosides (4 hr, D), and overlaid chromatograms of F2, C-K, Rg3r, and Rg3s at different time points where 0 hr was marked as blue, 4 hr was marked as green and 24 hr was marked as red (E) (n=3).

Figure 3. UPLC-TOF-MS chromatograms and peak area of PPT-type secondary ginsenosides in red ginseng extract incubated in gut bacteria collected from A/J mice at 0 hr, 4 hr and 24 hr. The top panel shows Rh1 and F1 and bottom panel shows Rg2. The table underneath the chromatogram shows the peak area of various compounds.

Figure 4. Transcellular transport of 2 µM Compound K across monolayers of Caco-2 cells, parental MDCKII and MDR1- MDCKII cells.. (A) Compound K transport alone; (B) Compound K transport with 20 µM cyclosporine A; (C) Compound K transport with 50 µM verapamil. (D) 2 µM Compound K transport in parental MDCKII cells; (E) 2 µM Compound K transport in MDR1- MDCKII cells. Transport from A to B is represented by the symbol “○” and that from B to A is represented by the symbol “●.” Data are presented as mean ± S.D; n=3.
Figure 5. The plasma concentrations of Compound K in wild-type and Mdr1a/b-/- FVB mice after oral administration of Compound K at 10 mg/kg. Data are presented as mean ± S.D.; n=5.
Table 1. Transcellular transport of C-K across monolayers of Caco-2, MDCKII, and MDR1-MDCKII cells in the absence or presence of P-gp inhibitors.

<table>
<thead>
<tr>
<th>Cell model</th>
<th>Inhibitor</th>
<th>Concentration (µM)</th>
<th>P&lt;sub&gt;a-b&lt;/sub&gt; (x10&lt;sup&gt;-6&lt;/sup&gt; cm/s)</th>
<th>P&lt;sub&gt;b-a&lt;/sub&gt; (x10&lt;sup&gt;-6&lt;/sup&gt; cm/s)</th>
<th>Efflux ratio (P&lt;sub&gt;b-a&lt;/sub&gt;/P&lt;sub&gt;a-b&lt;/sub&gt;)</th>
<th>Intracellular amounts (nmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caco-2</td>
<td>-</td>
<td></td>
<td>1.16 ± 0.33</td>
<td>30.96 ± 4.31**</td>
<td>26.6</td>
<td>0.01 ± 0</td>
</tr>
<tr>
<td></td>
<td>Verapamil</td>
<td>50</td>
<td>3.39 ± 0.41</td>
<td>9.89 ± 0.11*</td>
<td>2.9</td>
<td>0.50 ± 0.01**</td>
</tr>
<tr>
<td></td>
<td>Cyclosporin A</td>
<td>20</td>
<td>3.30 ± 0.67</td>
<td>3.62 ± 0.22</td>
<td>1.1</td>
<td>0.15 ± 0.01***</td>
</tr>
<tr>
<td>MDCK II</td>
<td>-</td>
<td></td>
<td>0.58 ± 0.12</td>
<td>1.13 ± 0.11*</td>
<td>1.8</td>
<td>0.27 ± 0.03</td>
</tr>
<tr>
<td>MDR1- MDCK II</td>
<td>-</td>
<td></td>
<td>1.84 ± 0.70</td>
<td>33.64 ± 8.08**</td>
<td>18.2</td>
<td>0.08 ± 0.01**</td>
</tr>
</tbody>
</table>

Data were presented as mean ± S.D; n=3.

“P<sub>a-b</sub>” refers to the permeability from apical to basolateral and “P<sub>b-a</sub>” refers to the permeability from basolateral to apical side. The values of permeability were compared between different direction and intracellular amounts were compared to control group. Data are the means ± S.D. of three independent experiments.

- indicated no inhibitor; * indicated p<0.05; ** indicated p<0.01; *** indicated p<0.001.
Table 2. The pharmacokinetic parameters of C-K (10 mg/kg) in FVB and Mdr1a/b −/− FVB mice after oral administration.

<table>
<thead>
<tr>
<th>Animal Strain</th>
<th>Tmax (hr)</th>
<th>Cmax (µM)</th>
<th>ke (hr⁻¹)</th>
<th>t½ (hr)</th>
<th>MRT inf (hr)</th>
<th>AUC 0-t (hr*µM)</th>
<th>AUC 0-∞ (hr*µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type FVB</td>
<td>2.8 ± 0.84</td>
<td>0.13 ± 0.03</td>
<td>0.13 ± 0.07</td>
<td>5.87 ± 3.04</td>
<td>7.82 ± 1.73</td>
<td>0.66 ± 0.19</td>
<td>0.75 ± 0.21</td>
</tr>
<tr>
<td>Mdr1a/b −/− FVB</td>
<td>5.6 ± 2.19*</td>
<td>0.53 ± 0.23*</td>
<td>0.04 ± 0.01*</td>
<td>18.68 ± 6.84*</td>
<td>30.72 ± 8.62*</td>
<td>7.70 ± 2.27***</td>
<td>17.58 ± 16***</td>
</tr>
</tbody>
</table>

Data were presented as mean ± S.D; n=5.

* indicated p<0.05; ** indicated p<0.01; *** indicated p<0.001.

“a” indicated that estimated pharmacokinetic parameters may not be very accurate due to limited sample time (24hr) in Mdr1a/b −/− mice.
Figure 1

<table>
<thead>
<tr>
<th>PPD series</th>
<th>R1</th>
<th>R2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rb1</td>
<td>Glu^2^-Glu</td>
<td>Glu^6^-Glu</td>
</tr>
<tr>
<td>Rb2</td>
<td>Glu^2^-Glu</td>
<td>Glu^-Arap</td>
</tr>
<tr>
<td>Rc</td>
<td>Glu^2^-Glu</td>
<td>Glu^6^-Araf</td>
</tr>
<tr>
<td>Rd</td>
<td>Glu^2^-Glu</td>
<td>Glu</td>
</tr>
<tr>
<td>F2</td>
<td>Glu</td>
<td>Glu</td>
</tr>
<tr>
<td>C-K</td>
<td>H</td>
<td>Glu</td>
</tr>
<tr>
<td>Rg3</td>
<td>Glu^2^-Glu</td>
<td>H</td>
</tr>
<tr>
<td>Rh2</td>
<td>Glu</td>
<td>H</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PPT series</th>
<th>R1</th>
<th>R2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rh1</td>
<td>Glu</td>
<td>H</td>
</tr>
<tr>
<td>Rg1</td>
<td>Glu</td>
<td>Glu</td>
</tr>
<tr>
<td>Re</td>
<td>Glu^-Rha</td>
<td>Glu</td>
</tr>
<tr>
<td>Rg2</td>
<td>Glu^-Rha</td>
<td>H</td>
</tr>
</tbody>
</table>
Figure 2
Figure 3

No increase of Rh1
No formation of F1 over 24 hr

No increase of Rg2 over 24 hr

<table>
<thead>
<tr>
<th>Compound</th>
<th>0hr</th>
<th>4hr</th>
<th>24hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rg1</td>
<td>4330743</td>
<td>4474314</td>
<td>4359311</td>
</tr>
<tr>
<td>Re</td>
<td>2810833</td>
<td>2787487</td>
<td>2760134</td>
</tr>
<tr>
<td>S-Rg2</td>
<td>2035730</td>
<td>2046414</td>
<td>2009334</td>
</tr>
<tr>
<td>R-Rg2</td>
<td>982891</td>
<td>983229</td>
<td>905223</td>
</tr>
<tr>
<td>S-Rh1</td>
<td>959337</td>
<td>1096862</td>
<td>1095979</td>
</tr>
<tr>
<td>R-Rh1</td>
<td>658923</td>
<td>647376</td>
<td>627316</td>
</tr>
</tbody>
</table>
Figure 4

(A) Accumulated Amounts (nmol/mg) vs. Time (min) for Cyclosporine A treatment in Caco-2 cells.

(B) Accumulated Amounts (nmol/mg) vs. Time (min) for Verapamil treatment in Caco-2 cells.

(C) Accumulated Amounts (nmol/mg) vs. Time (min) for Parental-MDCKII cell line.

(D) Accumulated Amounts (nmol/mg) vs. Time (min) for MDR1-MDCKII cell line.
Figure 5

Plasma concentrations (uM) vs Time (hr)

- FVB
- MDR1_KO
Inhibition of P-glycoprotein leads to improved oral bioavailability of Compound K, an anti-cancer metabolite of red ginseng extract produced by gut microflora

Zhen Yang, Jing-Rong Wang, Tao Niu, Song Gao, Taijun Yin, Ming You, Zhi-Hong Jiang, and Ming Hu

Table S1. The ingredients of oral suspending vehicle used in pharmacokinetic studies.

<table>
<thead>
<tr>
<th>Ingredients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Purified water</td>
</tr>
<tr>
<td>Lambda Carrageenan</td>
</tr>
<tr>
<td>Simethicone</td>
</tr>
<tr>
<td>Xanthan Gum</td>
</tr>
<tr>
<td>Microcrystalline Cellulose/Sodium CMC</td>
</tr>
<tr>
<td>Sodium Phosphate, Dibasic</td>
</tr>
<tr>
<td>Potassium Sorbate</td>
</tr>
<tr>
<td>Methylparaben</td>
</tr>
<tr>
<td>Propylparaben</td>
</tr>
</tbody>
</table>

Content information is not available
Table S2.

The chromatograph and UPLC/MS parameters of multiple ginsenosides Rg1, Re, F2 and C-K Analysis in Bruker MicroTOF mass spectrometer.

<table>
<thead>
<tr>
<th>Ginsenosides</th>
<th>[M+HCOO]’ (m/z)</th>
<th>Fragment ion (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rg1</td>
<td>845.4892</td>
<td>637, 475</td>
</tr>
<tr>
<td>Re</td>
<td>991.5504</td>
<td>783, 637, 475</td>
</tr>
<tr>
<td>F2</td>
<td>829.4913</td>
<td>621, 499</td>
</tr>
<tr>
<td>C-K</td>
<td>667.4396</td>
<td>499</td>
</tr>
</tbody>
</table>

The chromatography was performed on an Acquity BEH C18 column (2.1 × 100 mm, 1.7 μm). The mobile phase consisted of 0.1% formic acid in distilled water (A) and acetonitrile containing 0.1% formic acid (B), starting with linear gradient from 20 to 40% B within 20 min, then from 40 to 100% B at 20 - 29 min, and maintained at 100% B for 3 min at flow rate of 0.35 mL/min. Sodium formate solution was prepared by mixing 0.05 mmol NaOH solution with 0.05% formic acid in 90:10 proportion of 2-propanol/distilled water.
Supplement Table S3.

Instrument-dependent parameters for C-K and F2 in MRM mode for UPLC–MS/MS analysis in AB SCIEX 3200 Qtrap mass spectrometer.

<table>
<thead>
<tr>
<th>ESI(+)</th>
<th>Q1</th>
<th>Q3</th>
<th>Time (ms)</th>
<th>DP</th>
<th>CEP</th>
<th>CE</th>
<th>CXP</th>
</tr>
</thead>
<tbody>
<tr>
<td>CK</td>
<td>645.0</td>
<td>203.0</td>
<td>100</td>
<td>96</td>
<td>32</td>
<td>46</td>
<td>3</td>
</tr>
<tr>
<td>F2</td>
<td>807.6</td>
<td>627.5</td>
<td>100</td>
<td>125</td>
<td>50</td>
<td>53</td>
<td>5</td>
</tr>
</tbody>
</table>

UPLC gradient for C-K and F2 analysis were: gradient, 0-0.5 min, 0% B, 0.5-1 min, 0-40% B, 1-1.9 min, 40-90% B, 1.9-2.3 min, 90-95% B, 2.3-2.5 min, 95% B, 2.5-32.8 min, 95-0% B. The other UPLC conditions includes: Flow rate, 0.55 ml/min, column temperature, 45 degree; injection volume, 10 μl. System, Waters Acquity™ (Milford, MA, USA) with DAD detector; column, Acquity UPLC BEH C18 column (50×2.1mm I.D., 1.7μm, Waters); mobile phase A, 0.1% formic acid; mobile phase B, 100%, acetonitrile;
**Figure S1.** Possible Metabolic pathway of PPD-type ginsenosides Rb1, Rb2 and Rc in gut bacteria collected from A/J mice

These results suggested the tendency of stepwise de-glycosylation was terminal sugar > inner sugar (C-3) > inner sugar (C-20).
Pharmacokinetic study of red ginseng extract was performed in A/J mice to confirm the in vivo hydrolysis of ginsenosides by gut microflora. Red ginseng extract was dissolved in distilled water and was orally gavaged to A/J mice at 200 mg/kg (n=5). The blood sample collection and processing procedures used for A/J mouse samples were the same as those described for the FVB mouse pharmacokinetic study.
The cell growth inhibitory effects of ginseng powder and various metabolites generated in gut bacteria were determined using MTT assay in LM1 cell line. Cells were suspended in fresh medium at a concentration of $5 \times 10^3$ cells/ml and seeded in a 96-well flat bottomed plate in a volume of 100 $\mu$l/well. Cells were stabilized by incubation for 24 hrs at 37$^\circ$C and 100 $\mu$l aliquots of each drug with different concentrations (0.5% DMSO) ranging from 0.41 to 100 $\mu$M were added to wells. The plate was incubated at 37$^\circ$C for 48 hrs. For assay, 50 $\mu$l of MTT were added to each well and the plate was incubated for 4 hrs at 37$^\circ$C. The optical density was measured at 570 nm on a microplate reader (Bio-Rad, California). Each experiment was performed in triplicate and repeated at least twice. Antitumor activity was evaluated using IC$_{50}$ determined by non-linear regression analysis using the GraphPad PRISM 5 (San Diego, CA).