JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Absorption Mechanism of Ginsenoside Compound K and Its Butyl and Octyl Ester Prodrugs in Caco-2 Cells

Bing Zhang,[†] Xue-Mei Zhu,[†] Jiang-Ning Hu,*[,†] Hui Ye,[†] Ting Luo,[†] Xiao-Ru Liu,[†] Hong-Yan Li,[†] Wei Li,[‡] Yi-Nan Zheng,[‡] and Ze-Yuan Deng*[,†]

[†]State Key Laboratory of Food Science and Technology, Institute for Advanced Study, Nanchang University, Nanchang, Jiangxi 330047, China

[‡]College of Chinese Material Medicine, Jilin Agricultural University, Changchun 130118, China

ABSTRACT: Ginsenoside compound K (CK) is a bioactive compound with poor oral bioavailability due to its high polarity, while its novel ester prodrugs, the butyl and octyl ester (CK-B and CK-O), are more lipophilic than the original drug and have an excellent bioavailability. The aim of this study was to examine the transport mechanisms of CK, CK-B, and CK-O using human Caco-2 cells. Results showed that CK had a low permeability coefficient (8.65×10^{-7} cm/s) for apical-to-basolated (AP-BL) transport at 10−50 μM, while the transport rate for AP to BL flux of CK-B (2.97×10^{-6} cm/s) and CK-O (2.84×10^{-6} cm/s) was significantly greater than that of CK. Furthermore, the major transport mechanism of CK was found as passive transcellular diffusion with active efflux mediated by P-glycoprotein (P-gp). In addition, it was found that CK-B and CK-O were not the substrate of efflux transporter since the selective inhibitors (verapamil and MK-571) of efflux transporter had little effects on the transport of CK-B and CK-O in the Caco-2 cells. These results suggest that improving the lipophilicity of CK by acylation can significantly improve the transport across Caco-2 cells.

KEYWORDS: *compound K, ginsenosides, butyl and octyl ester, Caco-2 cells, intestinal absorption, P-glycoprotein*

INTRODUCTION

Panax ginseng C.A. Meyer, the active components of which are mainly ginsenosides, is frequently utilized as a herbal drug in traditional oriental medicine.[1] These ginsenosides, which belong to the class of triterpene saponins, have been reported to possess various biological and pharmacological activities such as antiaging, antiinflammation and antioxidation in central nerve system, cardiovascular system and immune system.[2−4] Previous studies have shown that the pharmacological actions of ginsenosides contributed to their metabolites through biotransformation by human intestinal bacteria.[5−7] Compound K (CK; Figure 1) is one of the main pharmacologically active metabolites of protopanaxadiol ginsenosides (e.g., Rb1, Rb2 and Rc) and it was reported that, it was accumulated in the liver after absorption from the GI tract to the blood, and some CK was transformed into fatty acid esters which may be the active components of ginsenosides in the body.[8−11] Many studies revealed that most of the ginsenosides are poorly absorbed along the human intestinal tract due to a high polarity. Odani et al. have reported that the amount of ginsenoside Rg1 absorbed via oral administration was within the range of 1.9−20.0% of the dosage in animal models.[12] Other ginsenosides such as Rb1 and Rb2 were also slowly absorbed through digestive tract, and the oral bioavailabilities in rats were relatively low.[13] The biological activities of drugs depend not only on their chemical structures, but also on their degree of lipophilic and membrane permeation, which could enhance their transport across the cell membrane or influence their interaction with proteins and enzymes.[14]

Recently, considerable attention has been paid to the development of ester prodrugs, which is a widely used approach to improving overall lipophicity, membrane permeability and oral absorption of poorly absorbed drugs.[15,16] Mannich bases and N-masked prodrugs of norfloxacin (NFX)

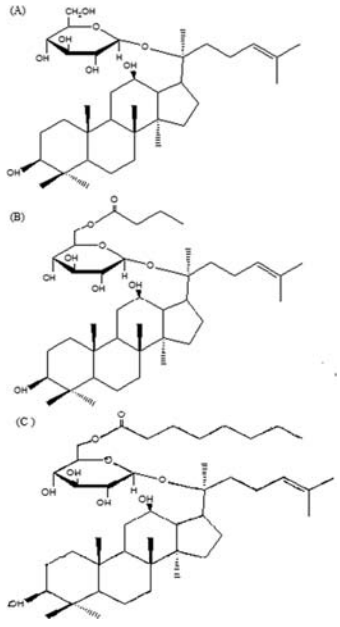

Figure 1. Chemical structures of gisenosides (A) CK, (B) CK-B, and (C) CK-O.

Received: July 20, 2012
Revised: September 25, 2012
Accepted: September 27, 2012
Published: September 27, 2012

have been reported to enhance its lipophilicity, bioavailability and *in vivo* activity.[17,18] However, to date, limited information is available concerning the mechanisms of oral absorption for CK and production of ester prodrugs to enhance the oral absorption of ginsenoside CK. To increase the oral absorption of CK, esterification provides a route to obtain more lipophilic derivatives. In addition, it has been reported that acylation of cholestane glycoside increased the antitumor potency.[19] Several acylated triterpenoid saponins isolated from the roots of *Solidago virgaurea* subsp. *virgaurea* in a low concentration also activated the metabolism of endothelial cells, which enhanced the permeability of the blood vessel walls for better adsorption of the saponins into tissues.[20] We thus speculated that the novel ester prodrugs of CK, butyl and octyl esters (CK-B and CK-O; Figure 1), which are more lipophilic than parent compound, may have an excellent oral bioavailability.

The objective of this study was to determine the transepithelial transport and absorption mechanisms of CK and its ester derivatives in the Caco-2 system. Caco-2 cell monolayers have been generally accepted as an *in vitro* model for prediction of drug absorption across human intestine and for mechanistic studies of intestinal drug transport since these cells show morphological and functional similarities to human small intestinal epithelial cells.[21−23] In this study, both ester derivatives were utilized for transepithelial transport and absorption assays in Caco-2 monolayers compared with CK to investigate whether esterification could enhance the membrane permeability of high hydrophilic compound, thus improving the intestinal absorption of drug.

MATERIALS AND METHODS

Materials and Chemicals. The human colon adenocarcinoma cell line, Caco-2, was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM), nonessential amino acids (NEAA), penicillin−streptomycin (10 000 IU/mL), Hank's balanced salt solution (HBSS), trysin and ethylenediaminetetraacetic acid (EDTA) (0.25%/0.02%) in Phosphate Buffer Saline (PBS) were from Invitrogen Corp. (Carlsbad, CA). Fetal bovine serum (FBS) was obtained from Gibco BRL (Life Technologies, Paisley, U.K.). Mannitol, verapamil and MK-571 were purchased from Sigma-Aldrich (St. Louis, MO). The CK and its ester derivatives CK-B and CK-O were kindly provided by Dr. Yinan Zheng at Jilin Agricultural University, China. Transwell cell culture chambers (pore size, 0.4 μM; diameter, 24 mm) and Millicell-ERS volt-ohmmeter with Ag/AgCl electrodes were purchased from Coster (Cambridge, MA) and from Nihon Millipore (Tokyo, Japan), respectively. Microplate reader was purchased from Thermo Fisher (Thermo Scientific Multiskan MK3).

Caco-2 Cell Culture. Caco-2 cells were cultured in DMEM containing FBS (10%, v/v), NEAA (1%, v/v), penicillin (100 U/mL), streptomycin (0.1 mg/mL), and glutamine (0.29 g/L) in a humidified atmosphere with 5% CO_2 at 37 °C. The medium was replaced every 2 days and the cells were passaged at approximately 90% confluence using a trypsin/EDTA solution (0.25%/0.02%) at a split ratio of 1−4. Passages 40−60 were used.

Cytotoxicity Assay. The cytotoxicity of CK, CK-B and CK-O to Caco-2 cells was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays.[24,25] Briefly, 0.1 mL of Caco-2 cells was seeded onto 96-well plates with a density of 10 000 cells per well in a 96-well plate. The cells were grown in an atmosphere with 5% CO_2 and 95% relative humidity. Following an incubation for 24 h, different concentrations of test compounds were added. Thereafter, 0.2 mL of 10% MTT solution was added to each well and incubated for 4 h in dark. The medium was then removed and the MTT-formazan crystals were solubilized by incubating with 0.15 mL of DMSO with gentle shaking for 10 min, and absorbance was determined at 490 nm in a microplate reader. Cells incubated without the test compounds were used as controls. In each MTT assay, every sample was tested in five replicates.

Transepithelial Transport Experiments across Caco-2 Monolayer. For transport experiments, Caco-2 cells were plated onto the 6-well transwell inserts coated with type-I collagen at a density of 4×10^5 cells/cm^2 to generate Caco-2 monolayers. Medium was changed every 2−3 days and the monolayers used for the experiments were left to differentiate for 21−27 days after postseeding. The integrity of the cell layer and the full development of the tight junctions were monitored before every experiment by measurement of transepithelial electrical resistance (TEER) of filter-grown cell monolayers with millicell-ERS equipment. Only a monolayer with a TEER value of more than 300 $\Omega \cdot cm^2$ was used for the transepithelial transport experiments.

The transport of CK, CK-B and CK-O across Caco-2 monolayers was investigated using the methods described previously with minor modifications.[26] Briefly, the monolayers were gently rinsed twice with warm HBSS (pH 7.4, 37 °C) prior to the experiments. Cell monolayers were then incubated for 30 min at 37 °C in the transport buffer. To measure the apical-to-basolated (AP-BL) permeability, 0.5 mL of mixture containing CK, CK-B or CK-O over 10−50 μM in transport buffer was added to the AP side of the transwell insert and 1.5 mL of HBSS was added to the BL chamber. The plates were then put in an incubator at 37 °C. At designated time intervals, 0.4 mL of aliquots of solution was collected from the BL side and then replaced with an equal volume of HBSS. In the direction of BL-AP, CK, CK-B or CK-O in 1.5 mL of transport buffer over 10−50 μM was added to the BL side, and 0.5 mL of the HBSS to AP side. All incubations were performed in triplicates.

For ethylenebis (oxyethylenenitrilo) tetraacetic acid (EGTA)-modulated transport experiments, the cell monolayers were pretreated with 2.5 mM of EGTA in both AP and BL sides for 15 min at 37 °C after the initial 30 min of preincubation. Cell monolayers were then washed three times with transport buffer. AP to BL transport was initiated in the same way as described above. The inhibitory effects of MK-517 and verapamil on CK, CK-B and CK-O flux by Caco-2 monolayers were examined by addition of 100 μM of each inhibitor to both AP and BL side. Thereafter, the transport study was conducted according to the method described above.

Cellular Uptake of CK, CK-B, and CK-O. Cell monolayers were prepared as described for the transport studies. Test compounds (CK, CK-B, and CK-O) were loaded onto the AP side of the cell monolayer over 2 h at 37 °C. At the selected time point, the solutions in the AP and BL side were collected and the amounts of the test compounds were then measured. The monolayers were washed five times with ice-cold HBSS and extracted with methanol containing 1% Triton X-100 for 1 h at room temperature. Cell lysates were centrifuged at 10 000*g* for 5 min and the supernatants were analyzed using HPLC. The protein concentration in cell lysates was determined using the BCA protein assay kit (Pierce, Rockford, IL) with bovine serum albumin as the standard. The uptake of the test compounds was expressed as nmoL/mg protein.

Analysis by HPLC. The analysis of ginsenoside CK and its ester derivatives (CK-B and CK-O) was performed according to a previous report by Qin et al. with a slight modification.[7] The HPLC system consisted of an Agilent HPLC series 1100 (Agilent Technologies, Little Falls, DE) with a diode array detector (DAD). The detection wavelength was set at 203 nm. A Hypersil ODS column (150 × 3.9 mm, Waters, Milford, MA) was used for sample analysis. The mobile phase was acetonitrile/water (85:15, v/v), and the flow rate was 1.0 mL/min. The standard concentrations (0.05−50 μM) of CK and ester derivatives were analyzed by HPLC and peak area measurement was used to generate calibration curves for the quantification of samples. The corresponding standards of CK, CK-B and CK-O for identification and quantification were prepared in methanol solution. The HPLC methods have been validated through accuracy, precision and recovery experiments. The method had acceptable accuracy (90−110% of true values) and precision (the intraday and interday coefficient variations less than 15%) over the concentration range

(0.10−100 μM). The recovery of samples was between 85% and 115%. The limit of quantitation (LOQ), defined as the minimum concentration which could be determined with acceptable accuracy and precision, was 0.02, 0.02, and 0.01 μM for CK, CK-B, and CK-O, respectively.

Data Analysis. The cumulative quantity of test compound permeated at each time interval was measured and plotted against time. The flux was defined as the amount of compound across the monolayers per square centimeter (nmol/cm^2). The apparent permeability coefficients (P_{app}, cm/s) were calculated using the following equation:

$$P_{app} = \left(\frac{dQ}{dt}\right)\left(\frac{1}{AC_0}\right)$$

Where dQ/dt is the rate of permeability (nmol/s); A is the surface area of the insert (cm^2); C_0 is the initial concentration. The efflux ratio (Re) was determined by calculating the ratio of P_{app} (B-A) versus P_{app} (A-B) as the following equation:

$$\mathrm{Re} = \frac{P_{app}(B-A)}{P_{app}(A-B)}$$

Student's t test was conducted with a P-value of less than 0.05 as statistically significant. All statistical analyses were carried out using SPSS 13.0 software for Windows.

RESULTS

Cytotoxicity of CK, CK-B, and CK-O. Viability of cells was directly measured using MTT test to evaluate the cytotoxicity of CK, CK-B or CK-O toward Caco-2 cells prior to transport experiments. Generally, cell viability value of less than 50% of the controls was considered as the reduction of mitochondrial activity, while a higher cell viability of more than 90% indicated that the compounds at the concentrations were nontoxic to the cells.[27] As shown in Figure 2, CK, CK-B and CK-O at 50 μM or

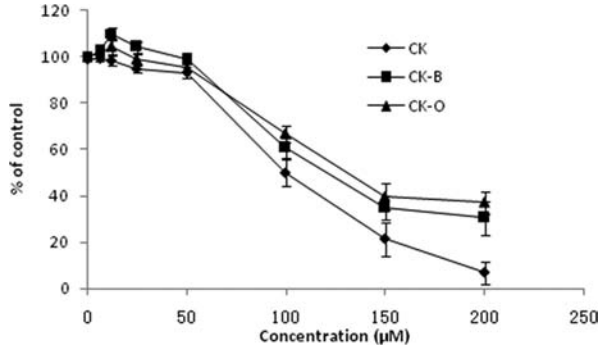

Figure 2. Cytotoxicity of CK (◆), CK-B (■) and CK-O (▲) on Caco-2 cell monolayers using the MTT assay. Data represent the mean ± SD from five replicates.

below were nontoxic to the Caco-2 cells after 2 h exposure. However, significant inhibition was observed at concentrations above 50 μM. To ensure cell viability during the permeability experiments, less than 50 μM of each compound was used for permeability studies.

Uptake of CK, CK-B, and CK-O by Caco-2 Cells. Results of the cellular uptakes of these three compounds (20 μM) into Caco-2 cell monolayers from the AP compartment over 2 h are shown in Figure 3. The uptakes of CK-B and CK-O into Caco-2 cells occurred quickly, reaching a maximum level of 0.96 and 0.74 nmoL/mg protein, respectively, after 30 min incubation. The cellular uptake of CK was much lower than that of CK-B

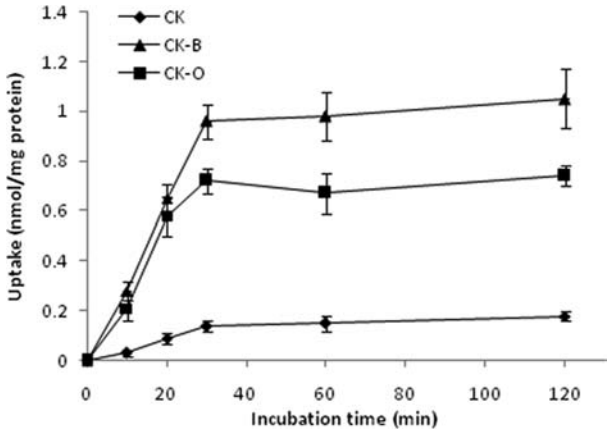

Figure 3. Time course of cellular uptake of CK (◆), CK-B (▲) and CK-O (■) at 20 μM by Caco-2 cells. The amount of compounds in cell lysates at selected time-points was measured using HPLC. The amount of protein in cell lysates was determined using the BCA protein assay kit. Data represent the mean ± SD from three replicates.

and CK-O, with a maximum uptake of 0.18 nmoL/mg protein after exposure to 20 μM CK in the AP side for 30 min. The uptake of CK, CK-B, and CK-O by Caco-2 cells accounted for 0.7%, 3.6%, and 2.8% of the initial added amounts, respectively.

Transport of CK, CK-B, and CK-O through Caco-2 Cell Monolayers. The time course of bidirectional permeability of CK, CK-B and CK-O across Caco-2 cell monolayers at 20 μM is shown in Figure 4. The bilateral flux amounts of CK increased linearly with time (Figure 4A). The apparent permeability coefficient of CK (20 μM) in AP to BL direction was determined as 8.75×10^{-7} cm/s in 2 h, which was considered to have a poor permeability and absorption rate *in vivo*.[30] The permeability in BL to AP direction was 2.6-fold greater than that in AP to BL direction. The flux amounts of CK-B and CK-O in both directions were of similar magnitude and also increased linearly over the initial 2 h (Figure 4B,C). The P_{app} values of CK-B (20 μM) in the direction of AP-BL and BL-AP were determined as 2.86×10^{-6} and 3.57×10^{-6} cm/s, respectively, and the efflux ratio (Re) was calculated as 1.2. The P_{app} (A-B) and P_{app} (B-A) of CK-O (20 μM) were 2.88×10^{-6} and 3.61×10^{-6} cm/s, respectively.

Subsequently, the effects of concentrations on the transport flux of CK, CK-B and CK-O (10−50 μM) were determined in the direction from AP to BL and BL to AP. As shown in Figure 5A, the AP to BL flux of CK was essentially linear for up to 2 h in a concentration dependent manner without any apparent saturation at the time points tested. This result indicated that the transport of CK was mainly by passive diffusion in the direction of AP to BL. The corresponding P_{app} (A-B) value, which was determined as 8.65×10^{-7} cm/s over 10−50 μM, was independent of concentration ($P > 0.05$). However, the BL to AP flux of CK was much faster than that of AP to BL direction and increased with increasing concentration, with a saturation at concentrations higher than 20 μM. The determined P_{app} (B-A) values of CK at different concentrations were significantly greater than those from AP to BL, with Re values calculated as 2.6, 2.0, 1.9, and 1.6 at 20, 30, 40, and 50 μM, respectively. The significant decrease in P_{app} (B-A) of CK at concentrations higher than 20 μM suggested the saturable flux amount of CK from the BL to AP direction. This result indicated that the permeation mechanism of CK may be passive diffusion with a BL to AP active efflux involved. The flux

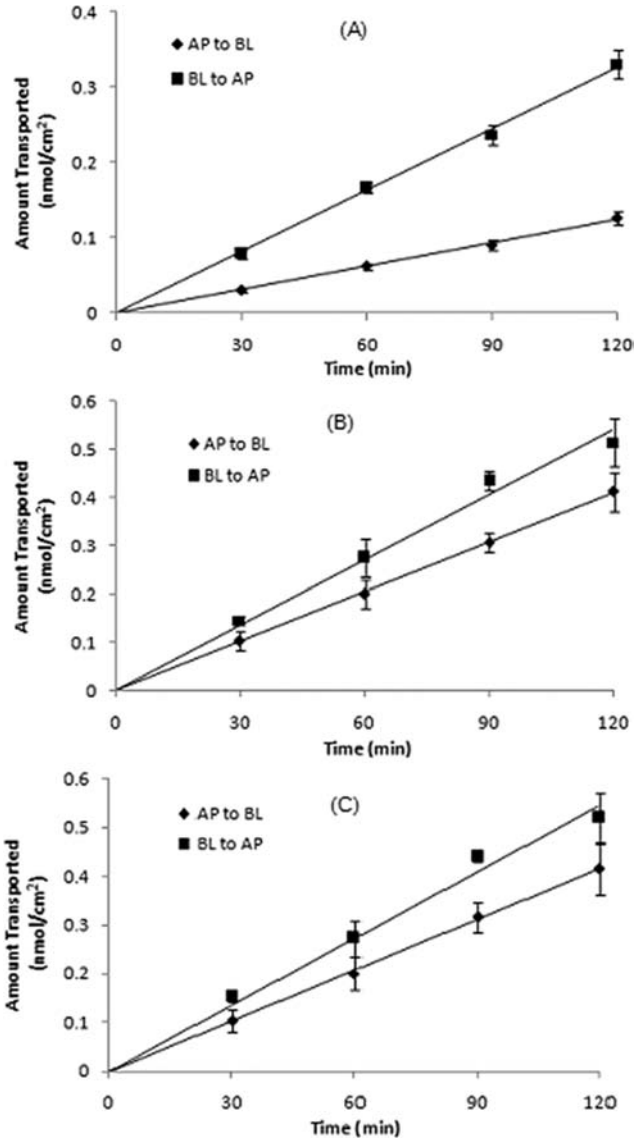

Figure 4. Time course of transport of (A) CK (20 μM), (B) CK-B (20 μM), and (C) CK-O (20 μM) across Caco-2 cell monolayers in both directions. Data represent the mean ± SD from three replicates.

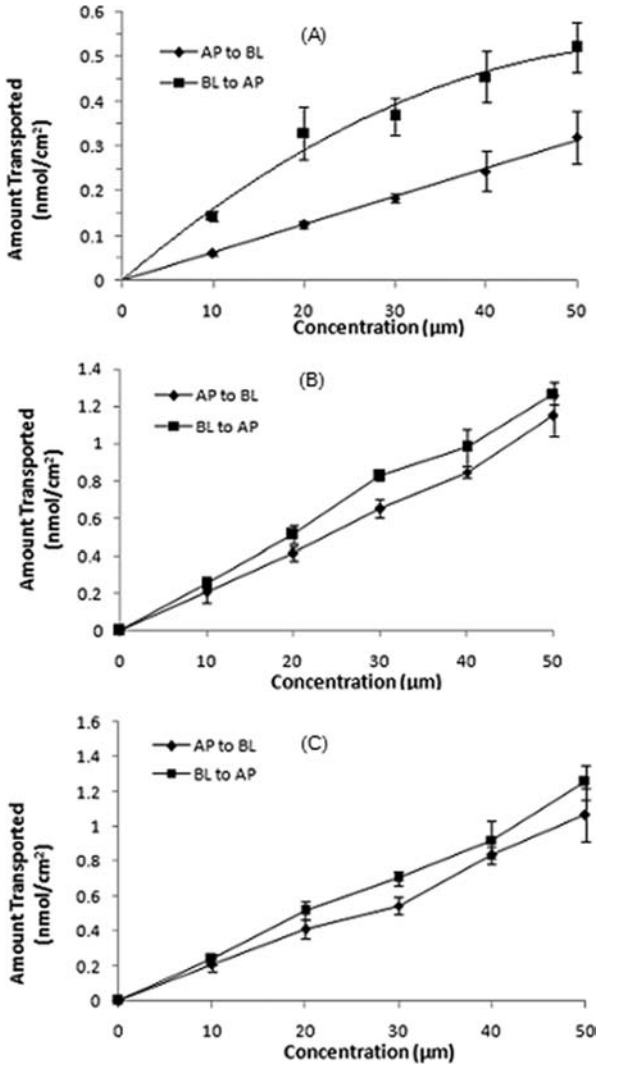

Figure 5. Effects of concentrations on the transport of (A) CK, (B) CK-B, and (C) CK-O across Caco-2 cell monolayers in both directions. Data represent the mean ± SD from three replicates.

amounts of CK-B in both directions were similar and directly proportional to CK-B concentrations over 10−50 μM (Figure 5B). The P_{app} values of CK-B determined as 2.97×10^{-6} cm/s for AP to BL and 3.58×10^{-6} cm/s for BL to AP were not dependent on concentration but of similar magnitude in both directions with Re values of 1.1−1.3. Generally, transport flux of a compound with Re values more than 1.5 was considered as an active efflux. Results obtained from the study of transport of CK-O were similar to that of CK-B (Figure 5C). P_{app} values of CK-O were 2.84×10^{-6} cm/s from AP to BL and 3.38×10^{-6} cm/s from BL to AP, which were independent of concentration and had no significant difference from the permeability of CK-B in the same direction. Therefore, the mechanism of permeation for both CK-B and CK-O in the translocation across Caco-2 cell monolayers was supposed to be passive diffusion with no active efflux involved.

Effect of Various Compounds on CK, CK-B, and CK-O Transport. EGTA, verapamil and MK-571 were investigated for their effects on the transport flux of CK, CK-B and CK-O across the Caco-2 cells. EGTA, a selective calcium chelator, is known to open the intercellular tight junctions of the Caco-2 monolayers, thus enhancing transport through the paracellular route. The Caco-2 monolayers pretreated with EGTA were employed in transport assay of CK, CK-B and CK-O to characterize the contributions of transcellular and paracellular transports of these three compounds. As shown in Figure 6A, no significant effects were observed in the P_{app} values for CK, CK-B and CK-O after pretreated with 2.5 mM of EGTA at 37 °C for 15 min, suggesting that the paracellular route had little contribution to their transport. This result indicated that the predominant transport route for these three compounds was transcellular. Mannitol is a well-known paracellular transport drug, which acted as a positive control in the EGTA-treatment experiments. The increased P_{app} value of mannitol more than 10-fold after EGTA treatment suggested the magnification of junctional pore size of monolayers in this study.

Verapamil and MK-571 are well-known as the inhibitors of P-glycoprotein (P-gp) and MRP1/2, respectively. Both P-gp and MRP1/2 overexpressing in Caco-2 cells and other cancer cell lines are considered as the most prevalent efflux transporters and possess a high capacity to efflux cytotoxic drugs.[28] To investigate the involvement of active efflux in

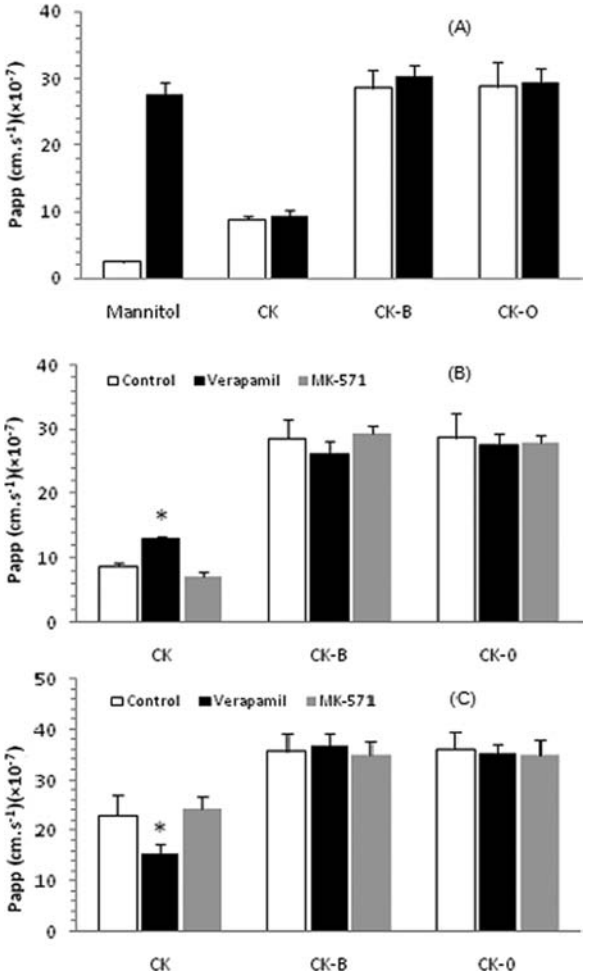

Figure 6. (A) Effects of EGTA on the apparent permeability coefficients (P_{app}) of CK, CK-B, CK-O and mannitol (paracellular transport marker). Transport experiments were conducted without EGTA pretreated (□) and with EGTA pretreated (■). (B) Effects of verapamil and MK-571 on the P_{app} values of CK, CK-B and CK-O from AP to BL direction and (C) BL to AP direction. *, $P < 0.05$ versus control.

transport for CK, CK-B and CK-O, the effects of selective inhibitors verapamil and MK-571 on the transports of these three compounds were examined. Verapamil and MK-571 showed no significant effects on the P_{app} values of CK-B and CK-O for either AP to BL or BL to AP (Figure 6B,C). Similarly, MK-571 did not significantly affect the P_{app} values of CK in both transport directions. However, verapamil significantly inhibited the BL to AP efflux of CK (by 30%), and caused a significant increase in the AP to BL flux, which indicated that CK is the substrate of P-gp.

■ DISCUSSION

The ginsenosides, including ginsenoside CK, have been reported to exhibit various biological activities. However, the poor intestinal absorption of ginsenosides after oral administration, which is considered as a limiting factor, restricts its application in medicine. Therefore, in the present study, the ester derivatives of CK were designed in order to improve its oral bioavailability. The transport mechanism of CK and its ester derivatives (CK-B and CK-O) was investigated using Caco-2 cell monolayers.

A close correlation between the permeability across Caco-2 cell monolayers and the absorption of oral administration *in vivo* has been obtained for several compounds.[29] It is well-known that compounds with P_{app} values less than 1×10^{-6} cm/s are considered to have a low absorption (<30%), while compounds with P_{app} values between 1×10^{-6} and 1×10^{-5} cm/s are considered to have a moderate absorption (30%−70%), and those with P_{app} values of more than 1×10^{-5} cm/s are considered to have a high absorption (>70%).[30] According to these criteria, compounds like CK with a P_{app} value less than 1×10^{-6} cm/s in Caco-2 cell monolayers may have a poor absorption from the intestine in human, whereas its ester derivatives (CK-B and CK-O) may have moderate absorption. Our results are consistent with the previous reports which showed that CK had a low oral bioavailability (approximately 5%) in rats.[31,32] However, as shown in our results, the low oral bioavailability of CK can be improved by esterification of CK into CK-B and CK-O.

On the other hand, in the present study, the permeation mechanism of CK, CK-B and CK-O has been determined as passive diffusion with an efflux pump (P-gp) involved for CK and without an efflux pump involved for CK-B and CK-O. Our results revealed that the efflux ratio (Re) for CK at 20 μM was 2.6, together with saturable flux in the BL to AP direction over 10−50 μM, suggesting that it might be a substrate for an efflux pump. Since the permeability of CK was significantly suppressed in BL to AP flux and increased from AP to BL flux in the presence of verapamil (a selective inhibitor of the closely related P-gp), we concluded that the efflux pump P-gp may be involved in the BL to AP transport of CK by Caco-2 cell monolayers. MRP1/2 did not appear to be involved, as indicated by the lack of effect of MK-571. Therefore, the major transport mechanism for CK was passive transcellular diffusion with the involvement of active efflux from the BL to AP flux in Caco-2 cell monolayers. Our results were similar to the report by Yang et al., which also found that the permeation mechanism of CK was passive diffusion with an efflux pump (P-gp) involved in the Caco-2 cell monolayers.[33] However, the absorption mechanism of CK was also reported as passive diffusion without an efflux pump was involved in the transport since there was no difference between bidirectional transport in Caco-2 Cells, which contradicts our results.[34] The discrepancy is probably due to the high substrate concentration (as much as 50 μM) used in their study which could have saturated the efflux process. Meanwhile, the results of our work showed that the permeabilities of CK-B and CK-O in both directions of transport were not significantly different (Re 1.2) and independent of concentrations. This along with lack of effect by transporter substrates such as verapamil and MK-571 indicated that the mechanism of permeation of CK-B and CK-O was passive diffusion with no active efflux involved.

The drug molecule can use either transcellular (passive or active) or paracellular (passive) route to cross intestinal epithelium into systemic circulation after oral administration.[35] Generally, lipophilic molecules might have a significant transport by passive diffusion through transcellular route, while hydrophilic molecules with low molecular weight cross the intestinal epithelium predominantly by paracellular passive diffusion process.[36] Because of much lower surface area available to the molecules entering the intercellular space and tight junctions in the intercellular space, the efficiency of absorption using paracellular route is lower than that of transcellular route. Therefore, one of the first transport

mechanisms to be evaluated for studying the processes involved in the absorption of compounds across monolayers was the contribution of the paracellular route. Manipulating tight junction integrity of Caco-2 monolayers, which was performed by a depletion of calcium concentration in the transport medium with a calcium chelator (EGTA), can be used as an approach to defining absorption routes.[37] Compounds fluxing across Caco-2 monolayers by the paracellular pathway are very sensitive to manipulations of tight junction integrity. In this study, the permeability of mannitol, a compound that is absorbed mainly via paracellular route, was increased significantly, indicating the opening of the tight junctions of Caco-2 monolayers. We have also characterized the transport of CK, CK-B and CK-O across Caco-2 cell monolayers. The P_{app} values of these three compounds had no significant change after opening of the tight junctions, indicating that the transports of CK, CK-B and CK-O across Caco-2 cell monolayers were predominantly via a transcellular route. The absorption route of hydrophilic compound CK was found to be transcellular. This might be due to its relative high molecular weight, which would restrict the drug molecules through the intercellular space.

In summary, we have demonstrated clearly that CK-B and CK-O exhibited better membrane permeation than CK in Caco-2 Cells. The present study may provide a useful strategy for molecular structure modification of drugs to improve oral absorption of hydrophilic compounds.

AUTHOR INFORMATION

Corresponding Author

*(Z.-Y.D.) Telephone/fax, +86 791 88304402; e-mail, zeyuandeng@hotmail.com. (J.-N.H.) Telephone, +86 791 88304449-8226; e-mail, hujiangning2005@hotmail.com .

Funding

This work was supported by the Doctoral Fund of Ministry of Education, China (No. 20103601120004), the Chinese Post-Doctorate Science Fund 47th Surface Fund (NO. 20100471424) and 4th Special Fund (NO. 201104590), and the Research Program of State Key Laboratory of Food Science and Technology, Nanchang University (Project No. SKLF-MB-201003).

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

The authors thank Dr. Yinan Zheng at Jilin Agricultural University in China for providing gift samples of CK, CK-B, and CK-O.

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■ NOTE ADDED AFTER ASAP PUBLICATION

The values in the third paragraph of the Results section were in error in the version of this paper published October 8, 2012. The correct version published October 9, 2012.