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Permeation of astilbin and taxifolin in Caco-2 cell and their effects on the P-gp

Xiao-dan Wang[a], Min-xin Meng[a], Ling-bo Gao[a], Ting Liu[a], Qiang Xu[b], Su Zeng[a,*]

[a] College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, PR China
[b] State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing 210093, PR China

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ABSTRACT

This study was designed to understand the transport profiles of astilbin and taxifolin in Caco-2 cell model and their effects on the function and expression of P-glycoprotein. The transport studies were examined using Caco-2 cells cultured on Transwell® inserts. Their effects on the function and expression of P-glycoprotein were detected using Western Blot and RT-PCR. The transport was concentration and temperature dependent. The apparent permeability (P_{app}) of these two compounds in the secretory direction was larger than that in the absorptive direction in the concentration range of 10–1000 μM. Those compounds had no effects on the P-glycoprotein-mediated transport of Rhodamine 123. Caco-2 cells exposed to astilbin or taxifolin for 36 h exhibited higher P-glycoprotein activity through up-regulating P-glycoprotein expression at protein and mRNA levels. These results indicated that P-glycoprotein and Multidrug Resistance Protein 2 might play important roles in limiting the bioavailability of those compounds. Drugs which are the inhibitors of P-glycoprotein or Multidrug Resistance Protein 2 may increase the oral bioavailability of astilbin or taxifolin and the possibility of unwanted drug–food interactions. The increased expression of P-glycoprotein in Caco-2 cells may serve as an adaptation and defense mechanism in limiting the entry of xenobiotics into the body.

1. Introduction

Flavonoids are polyphenolic compounds present in many fruits, vegetables, and beverages (Harborne, 1986; Hertog et al., 1993). They have been strongly implicated as protectors in coronary heart disease and stroke (Puddey and Croft, 1999; Bayard et al., 2007), as well as cancer (Jiang and Lu, 2000; Spencer et al., 2003). Astilbin, 3,3′,4′,5,7-pentahydroxyflavanone-3-(6-deoxy-(l-mannopyranoside)) (Fig. 1), was isolated from the rhizome of *Smilax glabra*, a Liliaceae plant, and also has been found in many Chinese herbs, such as *Dimorphandra mollis* and *Hypericum perforatum* (Guo et al., 2004). It has been reported that astilbin can inhibit coenzyme A reductase and aldose reductase. Also, astilbin exhibits many activities in anti-bacteria, anti-oxidative, anti-inflammation, as well as hepato-protection (Moulari et al., 2006; Guo et al., 2007). Taxifolin, aglycone of astilbin (Fig. 1), is mainly found in many citrus fruits, especially grapefruit and orange. In addition to anti-oxidant activity, taxifolin has activities of protecting cerebral ischemic reperfusion injury, inhibition of triglyceride synthesis, and antimicrobial (Rice-Evans et al., 1996; Theriault et al., 2000; Wang et al., 2006; Young et al., 2007). Although astilbin and taxifolin provide many benefits to human health, the knowledge about their transport profiles in Caco-2 cell model and the interactions with drug-transporters is limited.

P-glycoprotein (P-gp) and Multidrug Resistance Protein 2 (MRP2), the important members of the ATP-binding cassette (ABC) transporter family, are the main efflux pumps for a variety of chemicals. They play important roles in the absorption and distribution of many flavonoids (Zhang and Morris, 2003; Kitagawa et al., 2005; Caia and Bennick, 2006; Brand et al., 2006; Lohner et al., 2007). It is not clear whether P-gp or MRP2 serves as the transporter of astilbin or taxifolin in Caco-2 cells. On the other hand, it has been reported that some flavonoids inhibit or up-regulate the function of P-gp through unclear mechanisms. So, it is an interesting work to examine the effects of astilbin and taxifolin on the function and expression of P-gp and explore the possible mechanism.

The monolayer of Caco-2 cells formed on microporous filters is a widely used cell model in the study of chemical transport, and it has been applied to study the transport of some flavonoids successfully, such as naringin, epicatechin, quercetin and chrysin (Vaidyanathan and Walle, 2001; Tourniaire et al., 2005; He and Zeng, 2005; Yi et al., 2006).

The aims of the present study were to understand the transport profiles of astilbin and taxifolin using Caco-2 cell model and their effects on the function and expression of P-gp in Caco-2 cells.

* Corresponding author. Tel.: +86 571 88208407; fax: +86 571 88208407.
E-mail address: zengsu@zju.edu.cn (S. Zeng).

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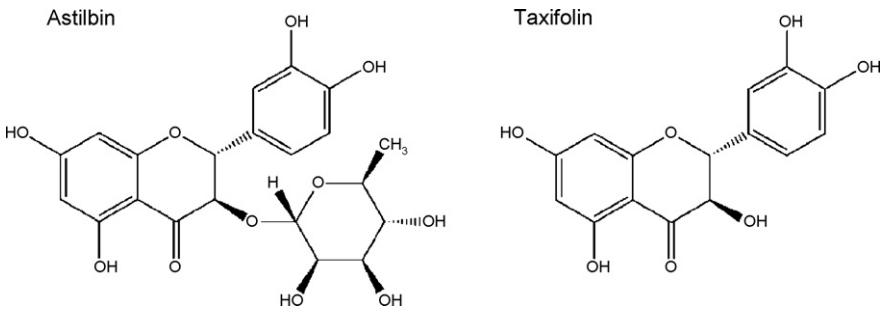

Fig. 1. Chemical structures of astilbin and taxifolin.

2. Materials and methods

2.1. Materials

Astilbin (>98% purity) and taxifolin (>98% purity) were purchased from Nanjing Sulang Medical Technology Development Co., Ltd. (Nanjing, Jiangsu, China); Verapamil, Lucifer yellow, Rhodamine 123, DMSO, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical Co. (Saint Louis, MO, USA); MK-571 was purchased from Alexis® Biochemicals Co. (Montreal, Canada); mouse monoclonal IgG_{26} and rabbit polyclonal anti-β-actin were purchased from Santa Cruz Biotechnology (CA, USA); HRP-conjugated goat anti-mouse IgG and HRP-conjugated goat anti-rabbit IgG were purchased from Boster Biological Technology (Wuhan, China); Tris, glycerin, sodium dodecyl sulphate (SDS), HEPES, PVDF membrane, dithiothreitol, bromophenol blue, Triton X100, Tween-20 were purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA); the enhanced chemiluminescence (ECL) detection system was purchased from LumiGLO, New England BioLabs (Hertfordshire, UK); TRIzol® reagent was purchased from Invitrogen Life Technologies Inc. (Carlsbad, CA, USA); M-MuLV reverse transcriptase was purchased from Fermentas Life Sciences Inc. (Vilnius, Lithuania) with Oligo $(dT)_{18}$ random primer and reaction buffer; Taq DNA polymerase with reaction buffer was purchased from Promega (Mannheim, Germany). RIPA lysis buffer and BCA protein assay kit were purchased from Beyotime Institute of Biotechnology (Haimen, Jiangsu, China). All solvents used were of HPLC grade and all chemicals were of analytical grade.

Caco-2 cells were obtained from Chinese Academy of Medical Sciences (Beijing, China). Dulbecco's modified eagle's medium (DMEM, high-glucose), fetal bovine serum, nonessential amino acids, 0.25% trypsin–EDTA solution and antibiotic–antimycotic were purchased from Gibco BRL Life Technology (Grand Island, NY, USA). Cell culture flasks and Transwell® polycarbonate inserts (12 mm diameter, 0.4 μm pore size) were obtained from Corning Costar Corp. (Bedford, MA, USA).

2.2. Cell culture

Caco-2 (passage 45–55) cells were cultured in DMEM with 10% fetal bovine serum, 1% nonessential amino acids, and 100 U/ml antibiotic–antimycotic. Cells were grown in a humidified atmosphere of 5% CO_2 at 37 °C. After reaching 80% confluence, Caco-2 cells were harvested with 0.25% trypsin–EDTA solution and seeded in 12-well plates or Transwell® inserts in 12-well plates at a seeding density of 1.0×10^5 cells/cm^2. Culture medium was replaced every other day for the first 14 days and daily thereafter for the next 7 days until the monolayer expressed properties that closely resemble morphologic and functional characteristics of normal enterocyte.

2.3. In vitro cytotoxicity studies

Cytotoxicity of astilbin and taxifolin was evaluated for Caco-2 cells using the MTT assay. Caco-2 cells were cultured in 96-well plates for 48 h at a seeding density of 1.0×10^4 cells/well before the addition of drugs. Astilbin and taxifolin dissolved in DMSO were diluted with DMEM as culture medium to different concentrations. The final concentration of DMSO was 0.3% (v/v). Control samples included 0.3% (v/v) DMSO in DMEM (vehicle), 0.1% Triton X100 in DMEM (positive control) and DMEM (negative control). Experiments were initiated by replacing the culture medium in each well with 150 μl of sample solutions at 37 °C in the CO_2 incubator. After 4 or 48 h of incubation, the medium was removed and 150 μl of MTT reagent (1 mg/ml) in the serum-free medium was added to each well. The plates were then incubated at 37 °C for another 4 h. At the end of the incubation period, the medium was removed and the intracellular formazan was solubilized with 150 μl DMSO and quantified by reading the absorbance at 590 nm on a micro-plate multi-detection instrument, SpectraMax M2 with SoftMax® Pro (Molecular Devices Corporation Sunnyvale, CA, USA). Percentage of cell viability was calculated based on the absorbance measured relative to the absorbance of cells exposed to the negative control.

2.4. Transport experiments of astilbin and taxifolin

Caco-2 cells cultured in Transwell® inserts at passage 45–55 were used for transport experiments after 21 days post seeding. The integrity of the monolayers was checked by measuring the transepithelial electrical resistance (TEER) values across the monolayers using Millicell-ERS Volt-ohmmeter (Millipore) and monitoring the permeability of the paracellular leakage marker Lucifer yellow across the monolayers. TEER was measured after subtraction of the intrinsic resistance of the cell free insert. The cell monolayers were considered tight enough for the transport experiments when the apparent permeability coefficient (P_{app}) for Lucifer yellow was less than 0.5×10^{-6} cm/s and TEER value >400 Ω cm^2. All transport studies were conducted at 37 °C. Prior to the experiment, the inserts were washed twice and equilibrated for 30 min with pre-warmed transport medium, Hank's balanced salt solution (HBSS), containing 25 mM of HEPES, pH 7.4.

Transepithelial permeability of astilbin and taxifolin was measured in the concentration range of 10–1000 μM. The stock solutions of test compounds were prepared in DMSO, diluted to the indicated final concentrations in transport buffer. The concentration of DMSO in the donor solutions was 0.3% (v/v). Transport studies were conducted in the absorptive direction and the efflux direction, separately. The transport buffer containing test compounds was added on either the apical (0.5 ml) or the basolateral (1.5 ml) side of the inserts, while the receiving compartment contained the corresponding volume of transport buffer. At the incubation of 30, 60, 90, 120 and 180 min, aliquot of 100 μl was

withdrawn from the receiver chambers and was immediately replenished with an equal volume of pre-warmed HBSS. The concentrations of the test compounds in the transport medium were immediately analyzed by a HPLC method as described later.

To evaluate the effect of temperature on the transepithelia transport of astilbin and taxifolin, the experiments were performed as described above, but the temperature was reduced to 4 °C instead of 37 °C.

The inhibition studies of astilbin and taxifolin across Caco-2 cell monolayers were investigated in the presence of inhibitors such as verapamil or MK-571. The experiments were performed by using HBSS containing 100 μM verapamil or MK-571 instead of blank HBSS.

2.5. Rhodamine 123 transport studies

The transport of Rhodamine 123 (R123) across the normal Caco-2 cell monolayers or drug-treated Caco-2 cell monolayers was investigated. For preparing the drug treated Caco-2 cell monolayers, the Caco-2 cells cultured in Transwell® inserts were sterilely supplemented with astilbin, taxifolin or DMSO on day 19 or 20. Transport experiments were conducted on day 21 post seeding.

The transport buffer containing 5 μM of R123 was added on either the apical or the basolateral side of the inserts, as described above. At 20, 40, 60, 80, 100, 120 min, aliquot of 200 μl was withdrawn from the receiver chamber, respectively, then was immediately replenished with an equal volume of pre-warmed HBSS. The concentrations of R123 were immediately analyzed by SpectraMax M2. The excitation wavelength was set at 485 nm, the emission wavelength at 535 nm. Calibration curve was constructed by performing a regression linear analysis of the Relative Fluorescence Units (RFU) versus the concentration. The calibration curve of R123 was linear over the concentration range of 0.004–0.25 μM (i.e. $y = 21354.55 \times -88.69$, $r^2 = 0.9995$).

Effects of astilbin and taxifolin on the transport of R123 across Caco-2 cell monolayers were performed on the normal Caco-2 cell monolayers cultured for 21 days. The transport experiments were performed in the presence of astilbin, taxifolin (1 μM, 10 μM, 100 μM) or verapamil (100 μM).

To evaluate the effects of astilbin and taxifolin on the function of P-gp in Caco-2 cells, cell monolayers cultured in Transwell® inserts were exposed to astilbin or taxifolin (1, 10, 100 μM), or DMSO (0.3%, v/v, vehicle) for 24 or 36 h at day 19 or 20 after seeding. Cell monolayers were washed three times and incubated for 1 h using HBSS before starting the transport experiments as described above.

2.6. P-glycoprotein expression

Caco-2 cells cultured in Transwell® inserts were supplemented with 10 μM astilbin, 100 μM taxifolin or DMSO (0.3%, v/v) as vehicle control at day 20 after seeding as described in Section 2.5. Caco-2 cells cultured as usual were used as the control. After exposure to astilbin, taxifolin or DMSO for 36 h, Caco-2 cells were washed with ice-cold HBSS (pH 7.4) and harvested by scraping with a rubber policeman into eppendorf tubes.

For Western Blot analysis, the cells were lysed by RIPA lysis buffer. The cell homogenates were centrifuged at 1.5×10^6 rcf for 30 min at 4 °C to remove nuclei and cell debris. The supernatant was carefully transferred to a new eppendorf tube and stored at −70 °C. Protein extraction was conducted on ice and protein concentrations were measured using BCA protein assay kit.

For RT-PCR analysis, total mRNA was extracted using TRIzol® reagent according to the manufacturer's protocol and dissolved in 50 μl RNase-free water.

2.7. Analysis of P-glycoprotein using Western Blot

P-gp was detected by Western Blot using 40 μg of extracted membrane protein in loading buffer (50 mM Tris, pH 6.8, 100 mM dithiothreitol, 10% glycerin, 2% SDS and 0.1% bromphenol blue). Protein samples were loaded onto 8% (v/v) SDS-PAGE and transferred onto PVDF membranes. All membranes were blocked for nonspecific binding by incubation in Tris-buffered saline (25 mM Tris, pH 6.8, TBS) containing 5% (w/v) fat-free powdered milk and 0.1% (v/v) Tween-20 overnight at 4 °C. The protein was immunodetected by incubation for 2 h with mouse monoclonal IgG_{26} (1:500) at room temperature with TBS containing 5% (w/v) fat-free powdered milk. The membrane was washed (3 × 10 min) using TBS, incubated with HRP-conjugated goat anti-mouse IgG (1:3000) for 2 h at room temperature, washed again (3 × 10 min). The proteins were then detected using an ECL system. The proteins were visualized by exposing the membrane to a Kodak film for 5 min in a dark room and scanned using a bio-imaging analyzer (Bio-Rad, Richmond, CA, USA). The density of the band was quantitated using Quantity One version 4.2.2 software (Bio-Rad, USA). β-Actin served as the internal standard, and was detected using rabbit polyclonal anti-β-actin as primary antibody and HRP-conjugated goat anti-rabbit IgG as secondary antibody. P-gp expression was presented as the ratio of P-gp band intensity to β-actin band intensity in the same blot (P-gp/β-actin).

2.8. RT-PCR analysis of MDR1 mRNA

Total cellular RNA was extracted using TRIzol® reagent and quantified at 260 nm using GeneQuant™ *pro* RNA–DNA Calculator (GE Healthcare, Stockholm, Sweden). The purity of RNA preparation was high with OD (optical density) of 260 nm/280 nm ratio in the range of 1.8–1.9. MDR1 and β-globin cDNA were synthesized using M-MuLV reverse transcriptase with Oligo $(dT)_{18}$ random primer from 5 μg total RNA. Primers (Sangon, Shanghai, China) for MDR1: MDR1-F (5′-CCC ATC ATT GCA TAT GCA GG) and MDR1-R (5′-GTT CAA ACT TCT ACT CCT GA), product size 157, annealing temperature 52 °C. Primer for β-globin: β-globin-F (5′-CAA CTT CAT CCA CGT TCA CC) and β-globin-R (5′-GAA GAG CCA AGG ACA GGT AC), product size 268, annealing temperature 53 °C. β-Globin was used as the internal control. PCR reaction was performed in a 25 μl system with Taq DNA polymerase in Eppendorf Mastercycler gradient (Eppendorf AG, Germany). After initial denaturation at 94 °C for 2 min, amplification was performed by 35 cycles using the following parameters: 94 °C for 45 s (denaturation), 52 or 53 °C (MDR1 or β-globin) for 45 s (annealing), 72 °C for 45 s (elongation) for each gene. PCR products were separated on 1.5% agarose gel containing 0.5 μg/ml ethidium bromide and photographed with a gel analysis system (Bio-Rad, Richmond, CA, USA). Band volume data were acquired using Rio-Rad Quantity One version 4.2.2 software. MDR1 expression was presented as the ratio of MDR1 band intensity to β-globin band intensity in the same blot (P-gp/β-globin).

2.9. HPLC analysis of astilbin and taxifolin

Astilbin and taxifolin in the samples were quantified by reversed-phase HPLC. The HPLC conditions were as follows: an Agilent 1200 HPLC system composed of a quaternary pump with a degasser, a thermostatted column compartment, a variable wavelength detector operated at 291 nm, an auto-injector, and Agilent ChemStation software. The HPLC separation was performed on a Waters Symmetry® C_{18} column (5 μm, 150 mm × 4.6 mm, Waters, Milford, MA, USA) with a guard C_{18} column maintained at 30 °C. The mobile phase consisted of methanol–water containing 0.3% acetic acid (35:65, v/v) at a flow rate of 1.0 ml/min. Injection volume was 20 μl.

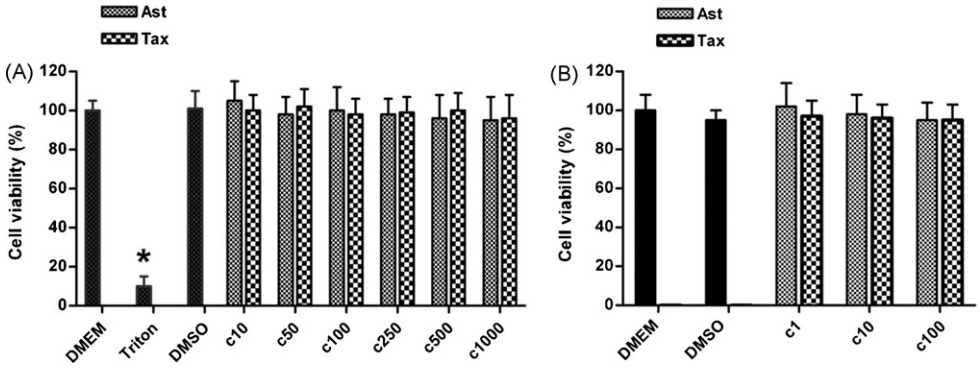

Fig. 2. Effects of astilbin and taxifolin on Caco-2 cell viability using MTT assay. (A) Exposure to drugs for 4 h. (B) Exposure to drugs for 48 h. Each column represents the mean ± SD, $n = 6$. All data were compared with that obtained in negative control. $^{*}p < 0.05$. c1: 1 μM; c10: 10 μM; c50: 50 μM; c100: 100 μM; c250: 250 μM; c500: 500 μM; c1000: 1000 μM. DMEM: negative control; DMSO: vehicle control; Triton: positive control.

2.10. Data analyses

The transport rate (V), P_{app} and efflux ratio were calculated as:

$$V = \frac{dQ}{dt \cdot A}$$

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

$$\text{Efflux ratio} = \frac{P_{app}(B–A)}{P_{app}(A–B)}$$

where dQ/dt (μmol/s) was the flux rate, A (cm^2) was the effective surface area of the cell monolayer (1.13 cm^2), and C_0 (μM) was the initial drug concentration in the donor chamber. Efflux ratio was expressed as the quotient of P_{app}(B–A) to P_{app}(A–B).

Data were expressed as the mean ± SD of six determinations. The statistical significance of difference between treatments was evaluated by using two-tailed paired Student's t tests with a significance level of $p < 0.05$. The statistical software package SAS (v8.2; SAS Institute, Inc., Cary, NC) was used for data analysis.

3. Results

3.1. In vitro cytotoxicity on Caco-2 cells

To evaluate the cytotoxicity of astilbin and taxifolin on Caco-2 cells, Caco-2 cells were incubated with those drugs at different concentrations and controls (vehicle, negative and positive). After exposure to astilbin or taxifolin at concentrations up to 1000 μM for 4 h, or 100 μM for 48 h, no toxic effect on Caco-2 cells was observed using MTT assay (Fig. 2).

Table 1
Intra- and inter-assay accuracy and precision of the method for determining astilbin and taxifolin concentrations in HBSS ($n = 5$).

Quantity spiked (μM)	Mean accuracy (μM)		Precision (RSD%)	
	Intra-assay	Inter-assay	Intra-assay	Inter-assay
Astilbin				
0.18	0.17	0.19	8.30	8.76
1.80	1.79	1.83	2.40	2.48
18.00	18.10	18.11	0.81	1.37
Taxifolin				
0.18	0.17	0.19	6.02	7.97
1.80	1.87	1.82	2.64	3.60
18.00	17.89	17.91	0.50	0.57

3.2. HPLC analysis of astilbin and taxifolin

A reversed-phase HPLC method was used in the experiments to detect astilbin and taxifolin in the samples. This method was specific for determining astilbin and taxifolin. Calibration curves were constructed by performing a regression linear analysis of the peak area versus the concentration. The calibration curves of astilbin and taxifolin were linear over the concentration range of 0.18–18 μM (i.e., $y = 54.241 \times -3.352$, $r^2 = 0.9998$ for astilbin and $y = 67.852 \times -1.945$, $r^2 = 0.9994$ for taxifolin). The intra- and inter-day precision and accuracy were analyzed at three concentrations of test compounds in five replicates within one day and on five consecutive days, respectively (data shown in Table 1). The limit of detection (LOD, signal-to-noise ration ≥ 3) was 0.05 and 0.04 μM for astilbin and taxifolin. The limit of quantitation (LOQ, signal-to-noise ratio ≥ 10) was 0.16 and 0.17 μM for astilbin and taxifolin, respectively (RSD < 9.0%, $n = 5$).

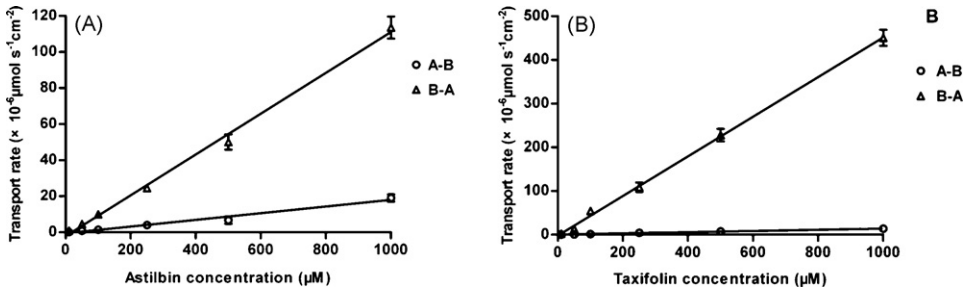

Fig. 3. Transepithelial transport rates of astilbin (A) and taxifolin (B) across Caco-2 cell monolayers in the basolateral to apical and the apical to basolateral directions. Each data point represents the mean ± SD for six independent monolayers.

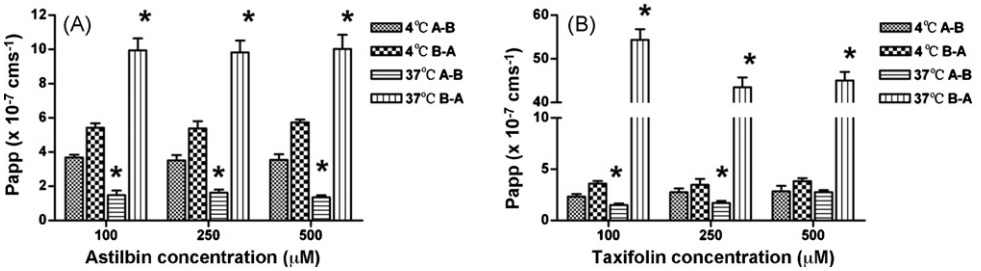

Fig. 4. Effects of temperature on the transport of astilbin (A) and taxifolin (B) across Caco-2 cell monolayers. Each column represents the mean ± SD for six independent monolayers. * $p < 0.05$, significantly different from control group.

Table 2
Effects of inhibitors (100 μM) on the transport of astilbin or taxifolin across Caco-2 cell monolayers.

Concentration (μM)	Efflux ratio		
	Control	Verapamil	MK-571
Astilbin			
10.0	6.98	0.53*	6.38
50.0	6.25	0.72*	6.29
100.0	6.74	0.60*	6.44
250.0	6.03	0.78*	6.78
500.0	7.41	0.89*	6.58
Taxifolin			
10.0	16.94	15.38	3.75*
50.0	15.41	15.94	3.84*
100.0	36.30	37.29	3.22*
250.0	25.39	24.37	3.20*
500.0	28.85	26.49	3.16*

Data were shown as mean ± SD for six independent monolayers.
* $p < 0.05$, significantly different from control group.

Table 3
Effects of drugs on the transport of R123 across Caco-2 cell monolayers.

		Papp ($\times 10^{-6}$ cm/s)		
		B-A	A-B	Efflux ratio
R123		5.97 ± 0.54	1.29 ± 0.30	4.62
R123 + astilbin	1 μM	5.46 ± 0.99	1.12 ± 0.76	4.88
	10 μM	6.16 ± 0.28	1.35 ± 0.03	4.56
	100 μM	5.83 ± 0.78	1.19 ± 0.10	4.90
R123 + taxifolin	1 μM	5.55 ± 0.89	1.26 ± 0.27	4.40
	10 μM	5.23 ± 0.51	1.19 ± 0.36	4.40
	100 μM	5.28 ± 0.15	1.48 ± 0.29	4.29
R123 + verapamil	100 μM	1.50 ± 0.44	1.23 ± 0.14	1.22*

Data were shown as mean ± SD for six independent monolayers.
* $p < 0.05$.

3.3. Transport of astilbin and taxifolin across Caco-2 cell monolayers

Transport rate of astilbin and taxifolin across Caco-2 cell monolayers in the basolateral to apical (B–A) direction was much higher than that in the apical to basolateral (A–B) direction in the concentration range of 10–1000 μM (Fig. 3). The transepithelial transport in the two directions was not saturated as shown in Fig. 3. The efflux ratios shown in Table 2 suggested that the efflux transport of astilbin and taxifolin was likely to be mediated by some transporters.

Bi-directional transport of astilbin and taxifolin across Caco-2 cell monolayers was investigated at both 37 and 4 °C to evaluate the effect of temperature on the transport of those two drugs (Fig. 4). The polarized transport that appeared obviously at 37 °C almost disappeared at 4 °C.

The inhibition effects on the efflux of astilbin and taxifolin across Caco-2 cell monolayers were investigated through incubating reaction mixtures together with P-gp inhibitor: verapamil, or MRP2 inhibitor: MK-571. Co-incubation with verapamil, the efflux ratio of astilbin was decreased significantly. Co-incubation with MK-571 did not change the transport rates and efflux ratio of astilbin. MK-571 increased the transport rate of taxifolin in A–B direction and decreased its transport in B–A direction, while verapamil seemed to have little effect on the transport of taxifolin across Caco-2 cell monolayers (Table 2). A Lineweaver–Burk plot was constructed with the reciprocal of transport rate ($1/V$) versus the reciprocal of substrate concentration ($1/s$) to determine the type of inhibition (Sha and Fang, 2004). From the Lineweaver–Burk plots (Fig. 5), it was found that verapamil acted as a competitive inhibitor in inhibiting the efflux of astilbin, and MK-571 inhibited the efflux of taxifolin in the same fashion.

3.4. Rhodamine 123 transport studies

R123 is a classical substrate of P-gp (Lee et al., 1994). In this study, R123 was used to evaluate the function of P-gp by measuring transport of R123 across Caco-2 cell monolayers. As shown in Table 3, the efflux ratios of R123 did not change after R123 was co-incubated with astilbin or taxifolin (1, 10, 100 μM), but were reduced from 4.62 to 1.22 when the efflux function of P-gp was inhibited by verapamil. It was indicated that astilbin and taxifolin did not affect the

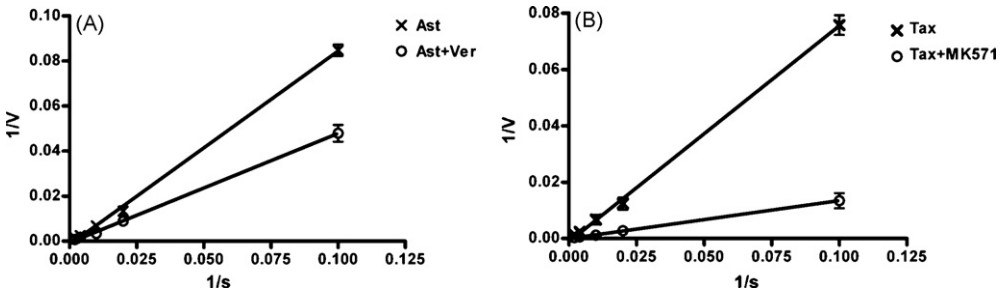

Fig. 5. Lineweaver–Burk plots of inhibition of astilbin and taxifolin efflux across Caco-2 cell monolayers by verapamil (A) or MK-571 (B). Each data point represents the mean ± SD for six independent monolayers. Ast: astilbin; Tax: taxifolin; Ver: verapamil.

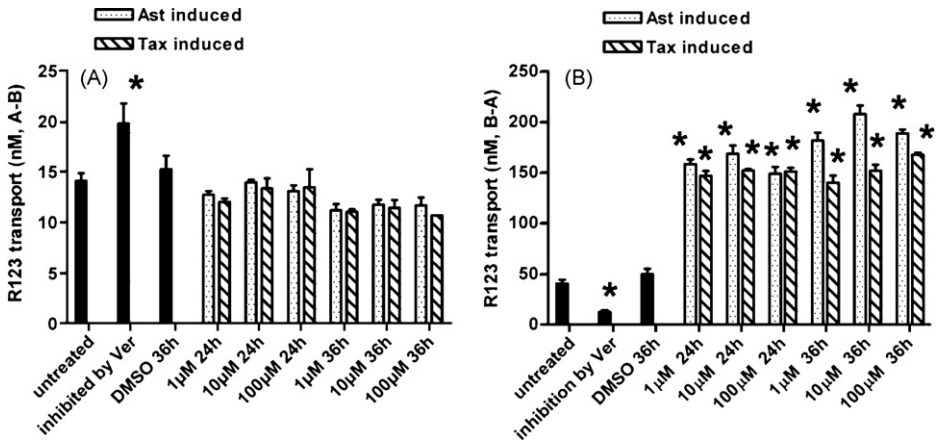

Fig. 6. Transport of R123 across drug-exposed Caco-2 cell monolayers. The cell monolayers were exposed to astilbin (Ast), taxifolin (Tax) or DMSO for 24 or 36 h, and then were used to perform the transport studies of R123 across the Caco-2 cell monolayers. (A) AP to BL direction transport; (B) BL to AP direction transport. Each column represents the mean ± SD for six independent monolayers. $^{*}p < 0.05$, significantly different from control group.

efflux of R123 mediated by P-gp across Caco-2 cell monolayers in the concentration range studied in this article.

To evaluate the effects of astilbin and taxifolin on the function of P-gp, Caco-2 cells exposed to astilbin or taxifolin at day 19 or 20 cultured on Transwell® inserts were used to perform the transport studies of R123 across the cell monolayers. TEER of Caco-2 cell monolayers exposed to drugs for 24 or 36 h had no change compared with untreated Caco-2 cell monolayers. TEER values were 545 ± 31, 552 ± 37, 590 ± 29 and $543 \pm 38\ \Omega\,cm^2$ for the cell monolayers treated by astilbin, taxifolin, DMSO or untreated, respectively.

The transport amounts of R123 in the A–B direction were marginally lower than that in untreated Caco-2 cell monolayers, but did not reach the level of significance (Fig. 6A), while the transport amounts of R123 in B–A direction increased significantly (Fig. 6B).

3.5. P-glycoprotein and MDR1 expression

Western Blot was applied to evaluate the expression level of P-gp protein in Caco-2 cells cultured in Transwell® inserts in order to assess the regulation effects of astilbin and taxifolin on the expression of P-gp. Exposure of Caco-2 cells to astilbin (10 μM) or taxifolin (100 μM) for 36 h resulted in a significant increase in P-gp protein level (Fig. 7). The P-gp protein in Caco-2 cells increased by 80% or 50% after the cells were pretreated with 10 μM astilbin or 100 μM taxifolin for 36 h compared with the untreated cells.

The expression of P-gp at mRNA level was detected using RT-PCR and suggested that astilbin or taxifolin could up-regulate the MDR1 expression in Caco-2 cells (Fig. 8). The MDR1 mRNA in Caco-2 cells exposed to astilbin or taxifolin was about 1.5-fold higher than that of the untreated Caco-2 cells.

4. Discussion

Astilbin selectively inhibits activated T lymphocytes, and this feature of astilbin provides a promising approach for the treatment of immune diseases (Guo et al., 2007). The aglycone of astilbin, taxifolin, has been reported to protect organs from ischemia-reperfusion injury through its anti-oxidative activity and may ameliorate inflammation via down-regulating integrin expression (Skaper et al., 1997; Kostyuk and Potapovich, 1998). Although there are many beneficial effects to human health and possible applications of them in disease therapy, the bioavailability of these two flavonoids is not clear. In this study, we evaluated the transport of astilbin and taxifolin across Caco-2 cell monolayers, and the effects of them on the function and expression of P-gp in the Caco-2 cells.

In the transport studies, the P_{app} values of A–B direction of both compounds was less than 1×10^{-6} cm/s, which indicated that oral bioavailability of astilbin and taxifolin may be poor (Yee, 1997). All the efflux ratios of both astilbin and taxifolin at 37 °C were larger than 2 indicating that there was one or more transporters involved in the transport process of those compounds across Caco-2 cell monolayers. Although it was difficult to discriminate facilitated diffusion from primary active transport only based on the effect of temperature, the diminished efflux ratios of astilbin and taxifolin at 4 °C indicated the efflux of astilbin and taxifolin may depend on the energy.

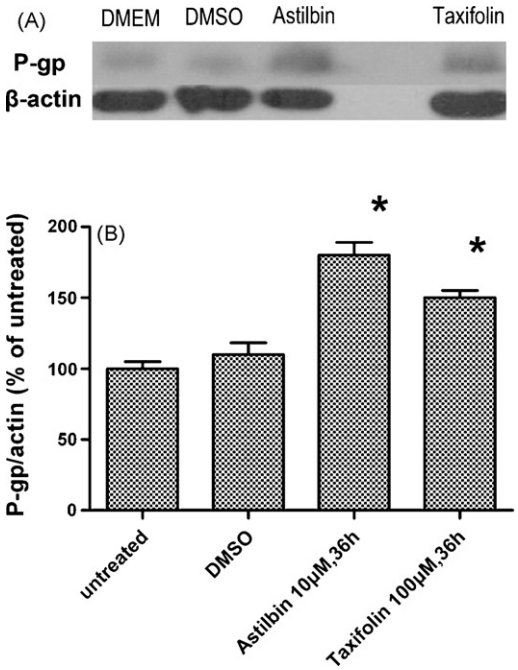

Fig. 7. P-gp and β-actin expression levels in Caco-2 cell membranes (cultured on Transwell® inserts) treated for 36 h with 10 μM astilbin or 100 μM taxifolin or exposed only to culture medium (untreated, control) or DMSO (0.3%, v/v, vehicle) as assessed by Western Blot. β-Actin was used to confirm equal protein loading. Protein were extracted as described in Section 2.7 and separated by 8% SDS-PAGE. (A) Representative experiment out of three experiments. Shown is a representative gel. Upper bands: P-gp; bottom bands: β-actin. From left to right: untreated (culture medium), DMSO (vehicle) for 36 h, 10 μM astilbin for 36 h, 100 μM taxifolin for 36 h. B: P-gp levels were quantified by densitometry in relation to β-actin. Data were expressed as mean ± SD from three independent experiments. $^{*}p < 0.05$ significantly different from control group.

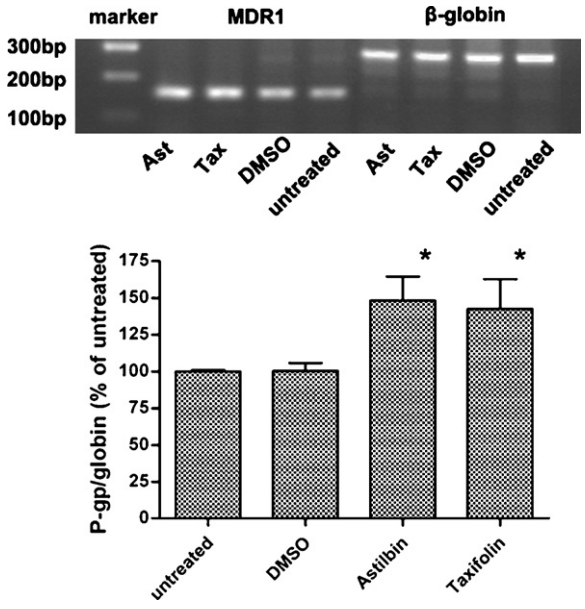

Fig. 8. MDR1 mRNA levels in Caco-2 cells (cultured on Transwell® inserts) exposed to 10 μM astilbin, 100 μM taxifolin, DMSO (0.3%, v/v, vehicle) for 36 h or culture medium (untreated, control). β-Globin was used as internal control. MDR1 mRNA levels were quantified by densitometry in relation to β-globin. Data were expressed as mean ± SD from three independent experiments. $^{*}p < 0.05$ significantly different from control group.

Verapamil, a classical inhibitor of P-gp, and MK-571, an inhibitor of MRP2, can reverse the efflux of astilbin and taxifolin, respectively. These experimental results suggest that astilbin is a substrate of P-gp, while taxifolin is a substrate of MRP2. However, in the presence of MK-571, the efflux ratio of taxifolin across the monolayer of Caco-2 cells was about 3.0 indicating that MK-571 could not inhibit the transport of taxifolin completely and there may be another transporter for taxifolin besides MRP2. In the transport studies of taxifolin across Caco-2 cell monolayers, we found that the efflux ratios increased with the increase in taxifolin concentration and reached the most at 100 μM taxifolin. This may be caused by the complex transport process of taxifolin or the complex interactions of taxifolin with MRP2 or other potential transporter(s). We also studied the effects of another inhibitor of P-gp: GF120918, and obtained the same results as verapamil. So we did not show those results in the article. Recently, some studies indicated that GF120918 was also an inhibitor of human breast cancer resistance protein (BCRP) (Allen et al., 1999; Brangi et al., 1999; Bruin et al., 1999; Maliepaard et al., 2001). So, our studies also suggested that BCRP may not play an important role in the transport of astilbin or taxifolin across the Caco-2 cell monolayers. Considering the trivial difference in chemical structure, and the significant difference in the transport mechanisms of astilbin from taxifolin, more research, such as QSAR or computer modeling, needs to be done in future for better understanding of interactions of these two flavonoids with the ABC transporters.

R123 is selectively transported in the Caco-2 cell monolayers by P-gp and has often been used to study the function of P-gp. In this study R123 was not only used to test whether Caco-2 cell monolayers expressed functional P-gp, but also used to study the effects of astilbin and taxifolin on P-gp activity. The efflux ratio of R123 was 4.62, suggesting significant P-gp efflux in the Caco-2 cell monolayers cultured in our lab. Before starting the transport of R123 across the Caco-2 cell monolayers, we detected whether astilbin, taxifolin, verapamil or HBSS had any interference in detecting fluorescence derived from R123. Under the condition described in the article, the RFU of R123 did not change with or without those compounds (100 μM). The RFU of R123 was equality in both water and HBSS at the same concentration. Those results showed that neither the biomaterial nor the compounds used in the experiments had any interference in the quantitation of R123. The addition of verapamil significantly lowered the efflux ratio of R123 from 4.62 to 1.22 indicating of P-gp suppressive activity. Astilbin and taxifolin had no effects on the transport of R123 in either direction, indicating that they may not be the inhibitors of P-gp in the concentration range studied. To date, the interactions mechanism of P-gp with its substrates or inhibitors was not understood completely because P-gp is poorly characterized at the molecular level, in large part because of the intrinsic difficulties involved in membrane protein crystallization. Some researches have proved that there are more than two drug-binding sites on P-gp (Dey et al., 1997). The substrate/inhibitor/transporter interaction is certainly more complex than that of competition for a single, or two equivalent binding sites. According to the results that 100 μM astilbin did not change the transport of R123 across the Caco-2 cell monolayers, the drug-binding sites on P-gp may be different or have little completive interaction for R123 and astilbin. However, exposing Caco-2 cells to astilbin or taxifolin for 24 or 36 h increased both the amount of R123 transported in B–A directions and the ratio of efflux significantly. When incubated with 10 μM astilbin or 100 μM taxifolin for 36 h, the transport amount of R123 in B–A direction was the most in the study conditions. This is also the reason why we chose 10 μM astilbin or 100 μM taxifolin and 36 h as incubation conditions for the Western Blot and RT-PCR studies.

With Western Blot and RT-PCR assay, we found that the mRNA and protein expression level of P-gp was higher in the Caco-2 cells exposed to the compounds compared with untreated cells. The most effective concentration of astilbin and taxfolin is 10 μM and 100 μM for the induction of P-gp expression in Caco-2 cells. Lohner et al. (2007) have previously demonstrated that taxifolin could increase P-gp protein level *in vitro* and *in vivo*. Our findings are consistent with Lohner's. Furthermore, using R123 as a probe we affirmed the up-regulation of the function of P-gp. The increased expression of P-gp caused by astilbin and taxifolin in Caco-2 cells may serve as an adaptation and defense mechanism in limiting the entry of xenobiotics into the cells. Some research has been done to investigate the signaling pathways in regulating the functions of ABC transporter systems (Han et al., 2006). For example, studies showed that pregnane X receptor had activity in regulating the expression of P-gp and some other proteins or enzymes involved in drug disposition (Geick et al., 2002). Inhibition of protein kinase C has been linked with increased drug accumulation and reversal of multidrug resistance in cells caused by P-gp (Castro et al., 1999). Other mechanisms include the activation of the NF-kB and the PI3 kinase/Akt (Thevenond et al., 2000; Nwaozuzo et al., 2003). It will be interesting to investigate the mechanism of the up-regulation P-gp expression by astilbin and taxifolin in Caco-2 cells.

In conclusion, this study provided some clues for clinical studies of astilbin and taxifolin that drugs which are either the substrates or inhibitors of P-gp or MRP2 may increase the oral bioavailability of astilbin or taxifolin. Co-administration of astilbin or taxifolin with such drugs in the clinical may cause unwanted drug–drug or drug–food interactions not only by the inhibition of P-gp or MRP2, but also by enhancing the function of P-gp through up-regulating its expression after being exposed to astilbin or taxifolin.

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