ORIGINAL ARTICLE

Mechanistic studies of the transport of peimine in the Caco-2 cell model

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Lihua Chen*, Xueping Lu, Xinli Liang, Dandan Hong, Zhiyu Guan, Yongmei Guan, Weifeng Zhu

Key Laboratory of Modern Preparation of TCM of Ministry of Education, Jiangxi University of TCM, Nanchang 330004, China

Abstract *Fritillaria thunbergii* Miq. has been widely used in traditional Chinese medicine for its expectorant, antitussive, antiinflammatory and analgesic properties. Moreover, modern pharmacological studies have demonstrated that *F. thunbergii* Miq. has efficacy in the treatment of leukemia and cancers of the liver and cervix. Although the alkaloid, peimine, is largely responsible for these pharmacological effects, it has very low oral bioavailability. The aim of this study was to investigate the intestinal absorption of peimine in Caco-2 cell monolayers. Having demonstrated that peimine is non-toxic to Caco-2 cells at concentrations < 200 µmol/L, the effect of peimine concentration, pH, temperature, efflux transport protein inhibitors and EDTA-Na$_2$ on peimine transport were studied. The results show that peimine transport is concentration-dependent; that at pH 6.0 and 7.4, the $P_{\text{app(AP-BL)}}$ of peimine is not significantly different but the $P_{\text{app(BL-AP)}}$ is; that both $P_{\text{app(AP-BL)}}$ and $P_{\text{app(BL-AP)}}$ at 4 °C are significantly higher than their corresponding values at 37 °C; that the P-glycoprotein (P-gp) inhibitors, verapamil and cyclosporin A, increase absorption of peimine; and that EDTA-Na$_2$ has no discernible effect. In summary, the results demonstrate that the intestinal absorption of peimine across Caco-2 cell monolayers involves active transport and that peimine is a substrate of P-gp.

*Corresponding author. Tel./fax: +86 791 87118658.
E-mail address: chlly98@163.com (Lihua Chen).

1. Introduction

The herb *Fritillaria thunbergii* Miq. has been widely used in traditional Chinese medicine as an antitussive and expectorant[1]. Peimine (Fig. 1) is a major bioactive alkaloid in *F. thunbergii* Miq. and is also present in other *Fritillary* species such as *F. cirrhosa* D. Don and *Bolbostemma paniculatum* (Maxim) Franquet. Pharmacological studies have confirmed peimine is an expectorant and antitussive and also revealed its antiinflammatory and analgesic properties[1]. However, it has low water solubility which limits its *in vivo* absorption and bioavailability. Our previous work[2] showed that the intestinal absorption of peimine involved both active transport and facilitated diffusion but resulted in low bioavailability in both male and female rats. To illuminate the mechanism of the intestinal absorption of peimine, we here report studies of peimine transport across Caco-2 cell monolayers.

The Caco-2 cell line is derived from a human colorectal carcinoma. It readily forms monolayers with morphological and functional similarities to the human small intestinal epithelium. In fact the FDA now recognizes the Caco-2 cell monolayer as a viable model that replicates human intestinal absorption[3–5]. It does this not only because the cells form tight intercellular junctions similar to those of the intestinal epithelium but also because they express ATP-binding cassette (ABC) membrane transporters such as P-glycoprotein (P-gp)[5–7] and multidrug resistance protein (MRP)[8–10] that act to protect the body from toxic exogenous substances. In this study the Caco-2 cell monolayer was used to study the mechanism of intestinal transport of peimine and whether P-gp plays an important role in its absorption.
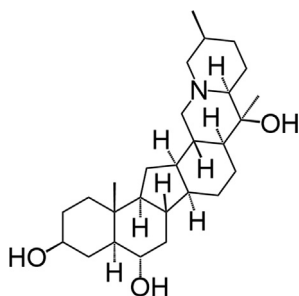

Figure 1   Chemical structure of peimine.

2. Experimental methods

2.1. Materials and reagents

Materials (suppliers) were as follows: peimine (the National Institute for Control of Pharmaceutical and Biological Products, China); the Caco-2 cell line (Shanghai Institute of Materia Medica, Chinese Academy of Sciences); fetal calf serum (FCS) and Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Thermo-Fisher Scientific); penicillin 10,000 U/mL, streptomycin 10,000 mg/mL and Hanks' balanced salt solution (HBSS) (Solarbio (Beijing Solarbio Science & Technology Co., Ltd., China); non-essential amino acids and verapamil hydrochloride (Sigma Chemical Co., USA); trypsin-EDTA solution (0.25% (*w/w*) trypsin/1 mmol/L EDTA) (Gibco Laboratories, Life Technologies Inc., USA; 24-well cell culture plate (Costar, Corning Incorporated, USA); Millicells cell culture inserts and Millipore Express PES 0.22 mm membranes (Millipore, USA); Luna 5 μ C18

(2) column (2.6 μm, 100 mm × 3 mm, I.D.) for LC–MS analysis (Phenomenex Co., Torrance, CA, USA). Water was purified using a Milli-Q water system (Millipore, Bedford, MA, USA).

2.2. Peimine cytotoxicity to Caco-2 cells

Peimine cytotoxicity was measured using the 3-(4,5-dimethythiazol-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay[11]. Caco-2 cells in DMEM culture medium were seeded in 96-well plates at a density of 5×10^4 cells/well and cultured at 37 °C for 24 h. Medium was replaced by fresh medium containing peimine at different concentrations and incubated for 4 h. After removal of medium, 20 μL 5 mg/mL MTT solution was added to each well and the plates incubated for a further 4 h. A total of 0.2 mL DMSO was then put into each well and the plate shaken for 10 min using a miniature oscillator. Finally optical density (OD) was determined at 490 nm using a microplate reader taking OD at 630 nm as the wavelength calibration control. Wells without cells were used as blank control and wells containing cells but no sample solution were used as negative control. The OD of each group was the mean of 6 replicates. Cell survival rate was calculated as (OD value of experimental group − OD value of blank group) / (OD value of negative control group − OD value of blank control group) × 100%. OD value of experimental group was calculated as (OD at 490 nm − OD at 630 nm).

2.3. Cell culture

The Caco-2 cell line was maintained at 37 °C in an atmosphere of 5% CO_2 and 95% relative humidity[12,13]. Caco-2 cells were cultured in medium containing DMEM (high D-glucose 4.5 g/L), 10% fetal bovine serum (FCS), 1% non-essential amino acids, 1% L-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin[14,15]. The cells were passaged using 0.25% EDTA-trypsin until 80%–90% confluence was reached. For transport experiments, cells were seeded onto polycarbonate filter membranes (pore size 0.4 μm, filter area 0.6 cm^2) in 24-well plates at a density of 1×10^5 cells/cm^2. The culture medium was changed every two days for the first week following seeding then every day thereafter. To monitor monolayer integrity, transepithelial electrical resistance (TEER) and alkaline phosphatase activity were measured as previously described[16,17]. Millicell membranes with monolayers TEER > 500 Ω/cm^2 were used for the transport studies. Under our culture conditions, this generally required 18–21 days[18]. Prior to experiments, monolayers were washed twice with 37 °C blank HBSS (pH 7.4) and then incubated at 37 °C for 30 min.

2.4. Transport experiments

Resistance to apical-to-basolateral (AP-to-BL) transport across cell monolayers meeting the criteria for membrane integrity was confirmed by measuring the AP-to-BL transport of fluorescein sodium. Transport experiments were conducted by adding peimine solutions to either the AP (0.4 mL) or BL (0.6 mL) side and the corresponding volume of HBSS medium to the receiving chamber. Transport plates were kept in a shaking incubator (55 rpm) at 37 °C during which samples were taken from the receiving chamber at various times up to 150 min (0.1 mL from BL or AP side) and replaced with the same volume of blank buffer. All incubations were performed in triplicate. Samples from both sides were analyzed for peimine by LC–MS/MS[19]. Data from each

experiment were used to calculate apparent permeability coefficients (P_{app}) and/or efflux ratio (ER) of peimine.

2.4.1. Effect of peimine concentration
To determine the concentration-dependence of peimine transport, solutions of peimine in DMSO/ethanol (0.1%, v/v) were diluted with HBSS solution to give concentrations of 10, 20, 50, 100 and 200 µmol/L and added to either the AP or BL side. Samples (0.1 mL) were taken from the receiver side after 150 min and analyzed by LC–MS/MS.

2.4.2. Effect of temperature
Peimine solutions containing 10, 50 and 100 µmol/L were added to the AP (0.4 mL) or BL (0.6 mL) side and HBSS solution added to the BL (0.6 mL) or AP (0.4 mL) side. Cells were then cultured at 4 or 37 °C for 150 min, after which samples (0.1 mL) were collected form AP (or BL) side and analyzed by LC–MS/MS.

2.4.3. Effect of pH
In the first experiment, peimine solutions with pH 6.0 and 7.4 were added to the AP side (0.4 mL) and HBSS solution (0.6 mL) added to BL side. In the second experiment, To the BL side transport, peimine solutions with pH 6.0 and 7.4 were added to the BL side (0.6 mL) and HBSS solution (0.4 mL) added to the AP side. After shaking at 55 rpm for 150 min at 37 °C, samples were collected from both sides and analyzed for peimine.

2.4.4. Effect of efflux transporter inhibitors
Solutions of the P-gp antagonists verapamil (100 µmol/L) and cyclosporin A (10 µmol/L)[20] were added to the AP (0.4 mL) or BL (0.6 mL) side while HBSS solution was added to the BL (0.6 mL) or AP (0.4 mL) side. Duplicate samples were collected from the chamber after 150 min incubation.

2.4.5. Effect of EDTA-Na$_2$
EDTA-Na$_2$ can increase the paracellular permeability of hydrophilic macromolecules[21]. After treating with drug solution, monolayers were cultured for 150 min before samples were collected for peimine analysis.

2.5. LC–MS/MS analysis

Samples were diluted with an equal volume of methanol, mixed with 10 µL solasodine (internal standard, IS) and centrifuged at 16,000 rpm for 10 min to precipitate proteins. The HPLC column maintained at 30 °C was attached to an Agilent 6410 triple-quadrupole mass spectrometer (Applied Biosystems/SCIEX, Carlsbad, CA, USA) equipped with an electrospray ionization (ESI) source operating in the positive ion mode. The mobile phase consisted of A (methanol)/B (0.1% formic acid water) delivered at a flow rate of 0.3 mL/min. The injection volume was 10 µL. Detection was by multiple reaction monitoring (MRM) of the transitions at m/z 432.3 → 414.3 for peimine and m/z 414.3 → 396.3 for IS. The calibration curve based on peak area ratios was linear over the concentration range 0.5–200 ng/mL with a regression equation of $y = 0.1424x - 0.0323$, $r^2 = 0.9987$ ($n = 3$). The lower limit of quantitation at a signal-noise ratio of 10 was 0.5 ng/mL. Intra- and inter-day precision were $<10.4\%$ and $<12.7\%$ respectively with accuracy in the range 92.0%–100.2%. Recoveries of peimine and IS were in the ranges 90.6%–101.4% and 89.8%–98.3% respectively. Stability studies showed that peimine

was stable in HBSS solution for 24 h at room temperature, for 30 days at -20 °C and during three freeze-thaw cycles with concentrations remaining $\pm 10.8\%$ of nominal values.

2.6. Data analysis

$P_{app(AP-BL)}$ and $P_{app(BL-AP)}$ were calculated according the following equation[22]:

$$P_{app} = \frac{V_r}{C \times S} \times \frac{dC}{dt} \tag{1}$$

where V_r is the volume of medium in the receiver chamber, C is the concentration of drug in the donor chamber, S is the surface area of the monolayer and dC/dt is the linear slope of the drug concentration–time plot in the receiver chamber after correcting for dilution. dC/dt must be a constant for accurate determination of P_{app}.

The efflux ratio (ER) was defined by the following equation[23]:

$$ER = \frac{P_{app(BL-AP)}}{P_{app(AP-BL)}} \tag{2}$$

Data are presented as means $\pm$ SD. For comparison between two groups, analysis of variance (ANOVA) was performed. Statistical significance of differences in means was determined using the Student's t-test with $P < 0.05$ taken as significant.

3. Results

3.1. Cytotoxicity of peimine to Caco-2 cells

As shown in Table 1, peimine is non-toxic to Caco-2 cells at concentrations below 200 µmol/L. Only concentrations below this limit were used in subsequent experiments.

3.2. Integrity of Caco-2 cell monolayers

Alkaline phosphatase activity, TEER values and AP-to-BL transport of fluorescein sodium are shown in Fig. 2. The results demonstrate that Caco-2 cell monolayers were suitable for peimine transport studies.

3.3. Transport experiments

3.3.1. Effect of peimine concentration
The effect of peimine concentration on its transport is shown in Fig. 3. Peimine transport is clearly concentration-dependent and

Table 1 The cytotoxicity of peimine to Caco-2 cells.

Concentration (µmol/L)	OD[a]	Survival rate (%)
0	0.29 ± 0.03	–[b]
1	0.32 ± 0.03	110.3
10	0.31 ± 0.04	106.9
20	0.32 ± 0.03	110.3
50	0.31 ± 0.04	106.9
100	0.30 ± 0.04	103.4
200	0.29 ± 0.04	100.4

[a]Date are means $\pm$ SD, $n = 6$.
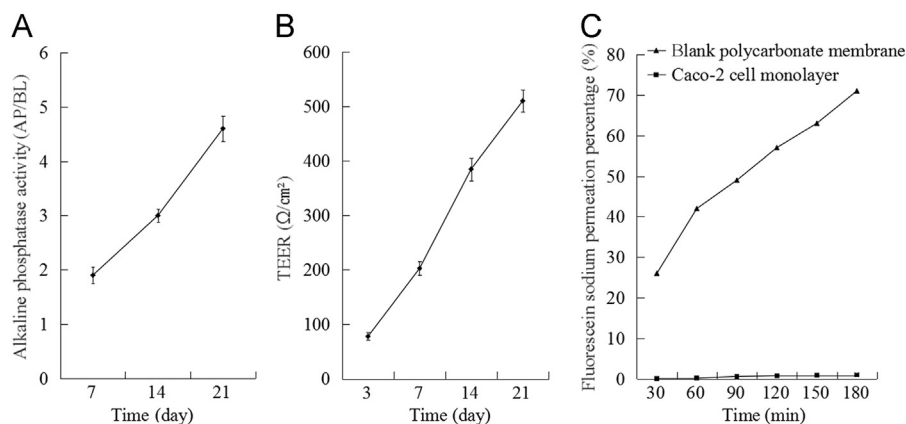[b]Not applicable.

Figure 2 (A) Alkaline phosphatase activity of AP/BL in Caco-2 cell monolayer; (B) the TEER of Caco-2 cell monolayer; (C) the AP-to-BL transport of fluorescein sodium in Caco-2 cell monolayer.
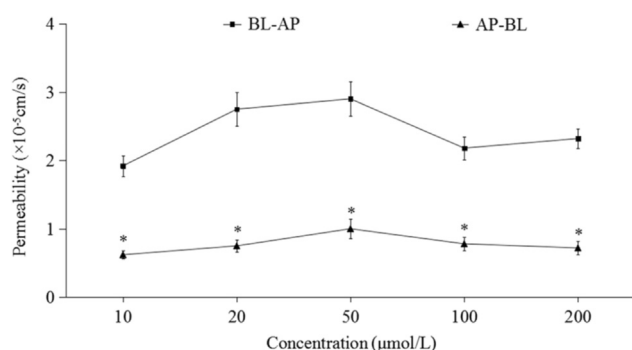

Figure 3 The effect of concentration on peimine transport. Data are means $\pm$ SD, $n = 6$. $^*P < 0.05$ compared with the $P_{app\ (BL-AP)}$.

appears to approach saturation at 100 μmol/L. ER at concentrations of 10, 20, 50, 100 and 200 μmol/L were 2.40 ± 0.31, 2.40 ± 0.77, 1.80 ± 0.46, 2.00 ± 0.67, 2.20 ± 0.10 respectively. The fact that in all but one case ER > 2 indicates that peimine undergoes active efflux transport. In addition, regardless of the direction of transport, the amount of transported peimine increased linearly with time (Fig. 4).

3.3.2. Effect of temperature
As shown in Fig. 5, ER values at 37 °C were 2.40 ± 0.31, 1.80 ± 0.46 and 2.00 ± 0.67 at the concentration of 10, 50 and 100 μmol/L, respectively. In contrast, corresponding values at 4 °C were 1.80 ± 0.50, 1.80 ± 0.50 and 0.70 ± 0.34. This is further evidence of active transport.

3.3.3. Effect of pH
The effect of pH on the transport of peimine is shown in Fig. 6. At concentrations of 10, 50 and 100 μmol/L, ER values at pH 7.4 were 2.40 ± 0.31, 1.80 ± 0.46 and 2.00 ± 0.67, respectively compared to values at pH 6.0 of 0.98 ± 0.12, 1.00 ± 0.22 and 1.10 ± 0.16. The results suggest that peimine would exhibit an absorption window in the gastrointestinal tract.

3.3.4. Effect of P-gp inhibitors
To test the possible role of intestinal P-gp in the absorption of peimine, transport was examined in the presence two P-gp inhibitors, verapamil and cyclosporin A. The results are shown in Fig. 7. In the control group, ER values at concentrations of 10,

50 and 100 μmol/L were 2.40 ± 0.31, 1.80 ± 0.46 and 2.00 ± 0.67, respectively. Corresponding values in the verapamil group were 0.67 ± 0.11, 0.88 ± 0.13 and 1.10 ± 0.23 and in the cyclosporin A group 0.60 ± 0.25, 0.61 ± 0.14 and 0.66 ± 0.17. These data clearly indicate that peimine is a substrate of P-gp.

3.3.5. Effect of EDTA-Na₂
P_{app} values are shown in Fig. 8. In the control group, ER values at concentrations of 10, 50 and 100 μmol/L were 2.40 ± 0.31, 1.80 ± 0.46 and 2.00 ± 0.67 respectively compared to corresponding values in the EDTA-Na₂ group of 1.90 ± 0.30, 1.60 ± 0.40 and 1.80 ± 0.52, respectively. The values were not significantly different.

4. Discussion

An asymmetric distribution of alkaline phosphatase activity in a Caco-2 cell monolayer is an indication of cellular differentiation. In this case, alkaline phosphatase activity was 4.6-fold higher on the BL side than on the AP side. In addition, fluorescein sodium permeation through Caco-2 cell monolayers was <1%. These results together with TEER > 500 Ω/cm² demonstrate that the Caco-2 cell model used in this study was suitable for investigating the mechanism of peimine intestinal transport.

The P_{app} values of peimine in the concentration range 10–200 μmol/L were in the range 6.2×10^{-6}–10×10^{-6} cm/s. According to the formula given by Yee et al.[24] to predict percent absorption in the gastrointestinal tract ($1.95 \times \ln(P_{app}) + 24.4$), the percent peimine absorbed is predicted to be in the range 1%–1.9%, indicating that peimine is a moderate intensity absorbed drug. In addition, since it was found that $P_{app(BL-AP)} > P_{app(AP-BL)}$ and ER > 2, it can be inferred that active efflux participates in the transport of peimine across Caco-2 cell monolayers. This is supported by the fact that at 4 °C peimine was absorbed to a greater extent than at 37 °C. Moreover, cyclosporin A and verapamil both increased $P_{app(AP-BL)}$ and decreased $P_{app(BL-AP)}$ of peimine indicating peimine is a substrate of P-gp as previously found by Li et al.[25]

In general, the degree of ionization of a drug is directly related to its absorption and bioavailability which, in turn, depend on pH. Consistent with our previous findings[2], peimine was absorbed more extensively at pH 7.4 than at pH 6.0. This is to be expected
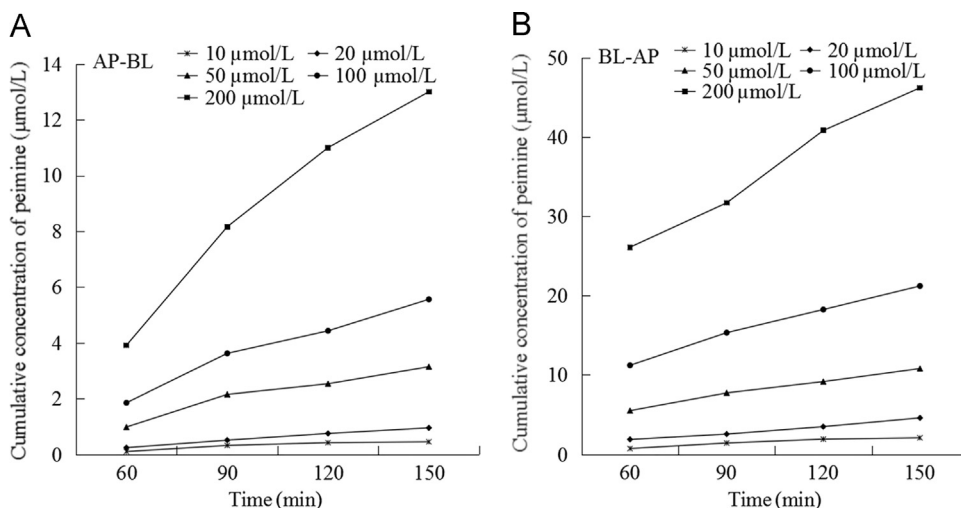
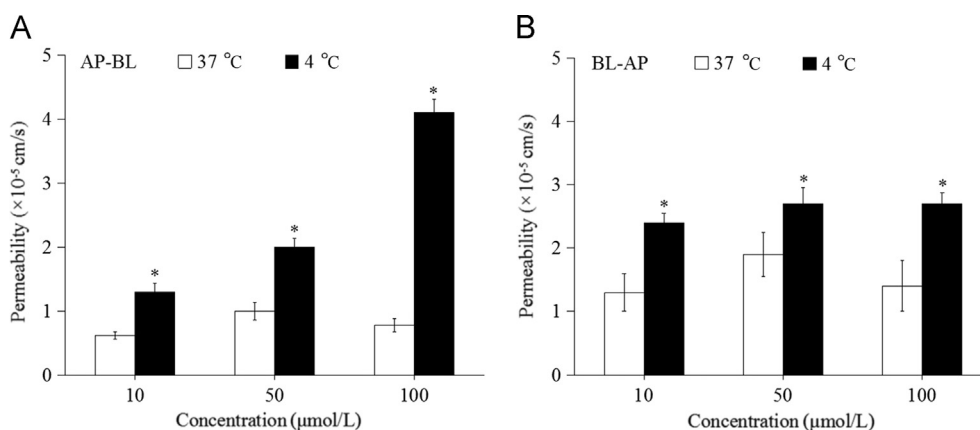**Figure 4** Accumulation of peimine in Caco-2 cells over time. Data are means $\pm$ SD, $n = 6$.

Figure 5 The effect of temperature on peimine transport. Data are means $\pm$ SD, $n = 6$. $^*P < 0.05$ compared with P_{app} determined at 37 °C.
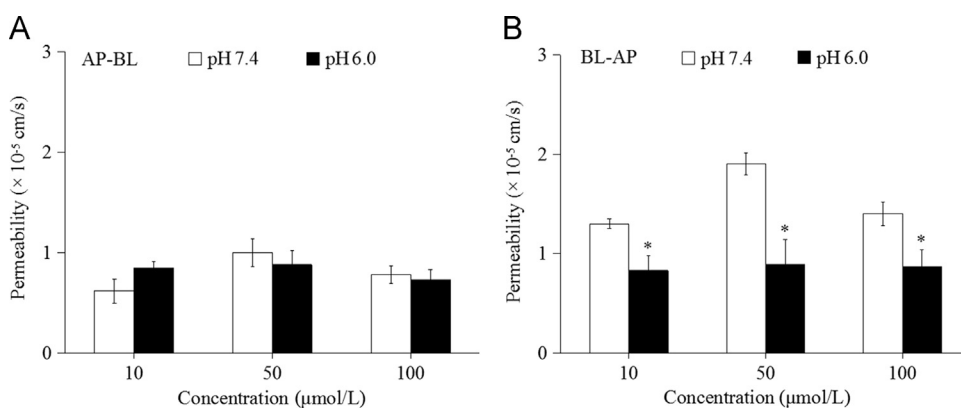

Figure 6 The effect of pH on peimine transport. Data are means $\pm$ SD, $n = 6$. $^*P < 0.05$ compared with the P_{app} at pH 7.4.

for an alkaloid where the degree of ionization decreases with increasing pH.

EDTA-Na$_2$ was found to have no effect on the transport of peimine. This demonstrates that peimine aborption through Caco-2 cell monolayers does not involve passive diffusion.

5. Conclusions

In this study, the effects of concentration, temperature, pH, efflux transporter protein inhibitors and EDTA-Na$_2$ on peimine transport across Caco-2 cell monolayers were examined to illuminate the
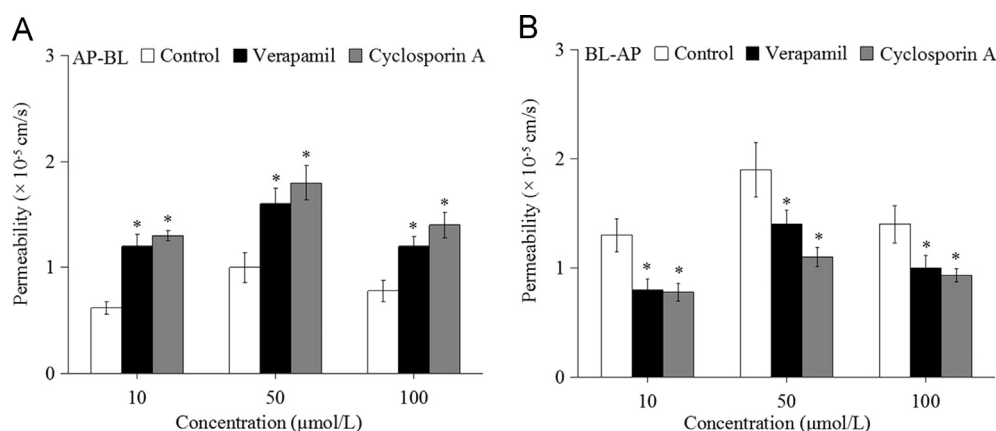
Figure 7 The effect of P-gp inhibitors on peimine transport. Data are means $\pm$ SD, $n=6$. $^{*}P<0.05$ compared with the P_{app} without verapamil or cyclosporin A.
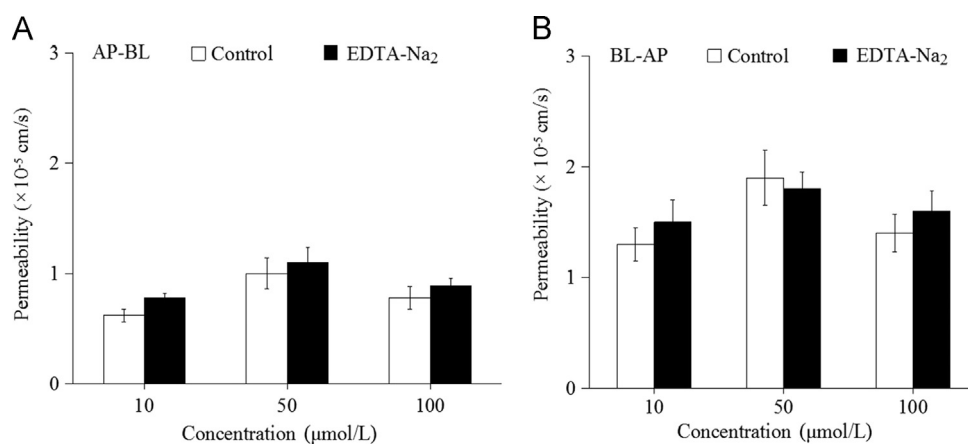

Figure 8 The effect of EDTA-Na$_2$ on peimine transport. Data are means $\pm$ SD, $n=6$.

intestinal absorption mechanism of peimine. Peimine transport was concentration-dependent and both $P_{app(AP-BL)}$ and $P_{app(BL-AP)}$ were higher at 4 °C than at 37 °C. The $P_{app(AP-BL)}$ was higher and $P_{app\,(BL-AP)}$ lower in the presence of verapamil and cyclosporin A and absorption permeability was not affected by EDTA-Na$_2$. Overall the results demonstrate that the absorption of peimine across Caco-2 cell monolayers involves active transport rather than passive diffusion and that peimine may be a substrate of P-gp.

Acknowledgments

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