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Transport of Val-Leu-Pro-Val-Pro in Human Intestinal Epithelial (Caco-2) Cell Monolayers

LIN LEI,[†,‡] HAIYAN SUN,[†] DONG LIU,[†] LIEGANG LIU,[‡] AND SHIMIN LI*[,†]

Department of Applied Biotechnology, Shenzhen Polytechnic, Shenzhen, Guangdong, People's Republic of China, and Department of Nutrition and Food Hygiene, School of Public Health, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, People's Republic of China

Angiotensin converting enzyme (ACE) inhibitory peptides are biologically active peptides that play a very important role in blood pressure regulation. In previous experiments, we obtained an ACE inhibitory peptide Val-Leu-Pro-Val-Pro (VLPVP) by DNA recombinant technology. The purpose of this study was to examine the bidirectional transport of VLPVP by using the human intestinal Caco-2 monolayers. The permeability coefficient (P_{app}) values of VLPVP over 4−8 mmol/L ranged from 7.44 $\times 10^{-8}$ to 1.35 $\times 10^{-6}$ cm/s for apical (AP) to basolateral (BL) transport, while the P_{app} values for BL to AP flux were signficantly lower than those for the AP to BL flux, with efflux ratio values of 0.74−0.13 over 4−8 mM. Preincubation of the paracellular transport enhancer (sodium deoxycholate), the inhibitor of multidrug resistant protein (MK-571), or sodium azide stimulated efflux of VLPVP significantly ($p < 0.01$); these results indicate that the transport of VLPVP across Caco-2 monolayers was involved in paracellular diffusion and MRP2 transport.

KEYWORDS: ACE; inhibitory peptide VLPVP; Caco-2; HPLC

INTRODUCTION

Angiotensin converting enzyme (ACE) inhibitory peptides are bioactive peptides with possible blood pressure lowering effects in vivo by inhibiting the angiotensin converting enzyme. Subsequently, the concentration of the vasoconstrictor angiotensin II decreases, while the concentration of the vasodilator bradykinin increases, which results in an antihypertensive effect (*1*). ACE inhibitory peptides had already been found in several food protein hydrolysates and ferments (*2, 3*) and had been shown to lower the blood pressure in spontaneously hypertensive rats and hypertensive patients (*4–6*).

Industrial production of proteins by DNA recombinant technology has been the most promising method for mass production of ACE inhibitory peptides (*7*). In our earlier studies, we developed an efficient *Escherichia coli* expression system of ACE inhibitory peptide (VLPVP) (*8, 9*). In vitro the median inhibitory concentration (IC_{50}) of this peptide was 1.7 μM, after oral administration of 400 μg/kg of body weight in a reduction of blood pressure about 5.03 ± 2.33 kPa $P < 0.01$ at 4 h in spontaneously hypertension rat.

To better understand the oral pharmacokinetic properties of VLPVP, it is important to characterize the intestinal absorption of VLPVP. We investigated in this study the mechanism of the transepithelial transport of VLPVP by using the human intestinal Caco-2 cell monolyer as a model of the intestinal epithelium (*10–12*).

MATERIALS AND METHODS

Chemicals and Reagents. The ACE peptide prepared from the *E. coli* expression system was not pure, and peptide Val-Leu-Pro-Val-Pro with a purity of 95% was therefore obtained from HD Biosciences Co., Ltd. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, 0.05% trypsin ethylenedia minetetraacetic acid, penicillin streptomycin, and nonessential amino acids (NEAA) were all obtained from Invitrogen. The MK-571 sodium azide, verapamil, phenylarsine oxide, sodium deoxycholate, Gly-Pro, dimethyl sulfoxide (DMSO), and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich. Acetonitrile (HPLC grade) was obtained from Union Hope. Hank's balanced salt solution (HBSS) was prepared by dissolving appropriate solutes in deionized water: 5.95 g of *N*-(2-hydroxyethyl)piperazine-*N*′-(4-butanesulfonic acid) (HEPES), 0.14 g of $CaCl_2$, 0.40 g of KCl, 0.06 g of KH_2PO_4, 0.047 g of $MgCl_2$, 0.10 g of $MgSO_4 \cdot 7H_2O$, 8.00 g of NaCl, 0.35 g of $NaHCO_3$, 0.048 g of Na_2HPO_4, and 4.5 g of

Table 1. Cytotoxicity of VLPVP toward Caco-2 Cells ($n = 3$)

groups	VLPVP concn(mmol/L)	absorbance	P value
1	HBSS (control)	0.756 ± 0.028	
2	2	0.740 ± 0.025	0.498
3	4	0.731 ± 0.031	0.363
4	6	0.730 ± 0.030	0.332
5	8	0.719 ± 0.039	0.251
6	10	0.714 ± 0.040	0.203
7	12	0.693 ± 0.042	0.096
8	14	0.686 ± 0.029	0.028

* Corresponding author. Telephone: 0086-0755-26019170. Fax: 0086-0755-26019262. E-mail: lsm10082003@yahoo.com.cn.
† Shenzhen Polytechnic.
‡ Huazhong University of Science and Technology.

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Table 2. Precision and Accuracy of the HPLC Method for the Analysis of VLPVP in HBSS (pH 7.4, $n = 5$ for Both Intraday and Interday Assays)

theoretical concn of VLPVP (μmol/L)	measured concn of VLPVP (μmol/L) (mean ± SD)	% recovery of theoretical concn of VLPVP	RSD (%)
intraday			
5	4.91 ± 0.05	98.20	1.02
15	14.71 ± 0.33	98.07	2.25
50	50.19 ± 2.14	100.38	4.26
interday			
5	4.89 ± 0.13	97.80	2.70
15	14.82 ± 0.48	98.80	3.26
50	48.99 ± 2.33	97.98	4.76

Table 3. Distribution of VLPVP (5 mM) and Free Amino after Incubation for 2 h in the Presence of Caco-2 Cells; Data Are the Mean ± SD of At Least Three Independent Determinations

	amount (nmol)	% of total
apical solution		
VLPVP	2404.32 ± 121.03	96.61
Val	81.14 ± 10.25	3.26
Leu	2.35 ± 0.64	0.09
cell fraction		
VLPVP	1.02 ± 0.49	0.04
Val	0.83 ± 0.37	0.03
Leu	3.30 ± 1.12	0.13
basolateral solution		
VLPVP	6.16 ± 2.17	0.25
Val	1.95 ± 0.89	0.08
Leu	4.50 ± 2.33	0.18

D-glucose, and the pH was adjusted to 7.4 using 1 M NaOH or 1 M HCl (*13*). The solution was sterilized by being filtered through a 0.22-μm filter. Transwell (Corning Costar Corp.) water was deionized and purified by a Millipore-Q UF Plus apparatus.

Cell Culture. Caco-2 cells were obtained from the American Type Culture Collection. The cells were cultured in DMEM supplemented with 10% fetal bovine serum, 1% NEAA, 100 U of penicillin, and 100 mg of streptomycin, together with an appropriate amount of sodium bicarbonate. All cultures were maintained in a humidified incubator at 37 °C with a 5% carbon dioxide in air atmosphere. The medium was changed every other day. When 80% was confluent, these cells were trypsinized with 0.25% trypsin and 0.02% EDTA solution and resuspended in medium. They were seeded in collagen-coated Transwell polycarbonate inserts (1.1 cm^2, 3.0-μm mean pore size) at a density of 2×10^5 cells/cm^3 for 21−30 days. Caco-2 cells were measured for transepithelial electrical resistances (TEER) with an epithelial voltohmeter (World Precision Instruments) at different times in culture starting from day 1 when the cells were seeded onto the inserts, up to the time (days 21−30) when they were used for experiments. The monolayers were used for the transport study when the effective TEER exceeded 300 $\Omega U/cm^2$ and the permeability of Lucifer yellow was $\leq 0.1 \times 10^6$ cm/s.

Cytotoxicity Assay. The 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was used to determine the cytotoxicity of VLPVP in Caco-2 cells as previously described (*14*). The peptide was dissolved in DMSO and then diluted in culture medium and added to the cultures 24 h after cell seeding. Cells were exposed to the drug at different concentrations (2−14 mmol/L) in culture medium for 24 h, after which 0.05 mg of MTT was added to each well, and the plates were further incubated for 4 h. Thereafter, the medium was removed, 150 μL of DMSO was added to dissolve the MTT crystals, and the optical density was read using a microplate reader (Tecan Instrument, Inc.) with 570 nm as excitation wavelength and 630 nm as the background. The viability of cells exposed to VLPVP was expressed as a percentage of the viability of cells grown in the absence of VLPVP.

Stability of VLPVP in HBSS. The stabilities of the VLPVP were examined by incubating a solution of the peptide (5 and 15 μmol/L) in HBSS buffer at different pH values (6.0, 7.0, 7.4, and 8.0) at 37 °C over 2 h. At indicated time points, an aliquot of 100 μL of the stock solution was collected and processed as standard sample. The amount of the peptide was determined at the beginning and end of the incubation period by high-performance liquid chromatography (HPLC).

Stability of VLPVP in the Presence of Caco-2 Cells. The 12-well plates were seeded with Caco-2 cells and cultured for a period of 21−30 days as described earlier. The monolayers were rinsed with the transport medium (HBSS, pH 7.4) twice and then incubated with the same media for 30 min at 37 °C, 5% CO_2. Thereafter, media was removed from the wells, replaced with VLPVP solution (5 mmol/L) in apical side, and incubated for 2 h at 37 °C, 5% CO_2. After incubation, the peptide solution was collected. The Caco-2 cell monolayers were washed with ice-cold HBSS, then trypsinized with 0.25% trypsin to suspend the cells. After being washed three times with ice-cold HBSS, the cells were homogenized by ultrasonication, and then the homogenate was centrifuged at 15 000 rpm for 30 min. The amino acid and VLPVP content of the supernatant in the apical and basolateral were measured by HPLC.

Transport Experiments. The transport of VLPVP in Caco-2 monolayers was investigated using the methods described previously with some minor modifications (*15, 16*). Before experiments, the cell monolayers were washed twice with HBSS containing 25 mmol/L of HEPES (pH 7.4). The buffer was then replaced with fresh HBSS buffer on one side of the cell layer and VLPVP in HBSS buffer on the other side. The apical side of the cell layer (insert) contained 0.5 mL, and the basolateral side (well) contained 1.5 mL.

VLPVP at specified concentrations (4, 5, 6, and 8 mmol/L) was added to either the apical (AP to BL) or basolateral side (BL to AP) of the Caco-2 cell monolayers, and an aliquot (150 μL) of sample was collected from the receiving side every 30 min for 2 h. After each sampling, an equivalent volume of HBSS buffer was added to replace the volume removed.

To identify which drug transporters were involved in the intestinal absorption of VLPVP, the inhibitory effect of sodium azide (an ATP synthesis inhibitor), verapamil (a P-glycoprotein (P-gp) inhibitor) (*17, 18*), MK-571 (a multidrug resistance associated protein (MRP) inhibitor) (*19*), sodium deoxycholate (destruction of tight junctions) (*20*), phenylarsine oxide (an endocytosis inhibitor) (*21, 22*), and Gly-Pro (a competitive peptide transporter inhibitor (PepTx)) (*23*) were added to the incubation medium on the apical side of the cell monolayers and then removed and replaced with the VLPVP (5 mmol/L) solution. All inhibitors were used at concentrations that were experimentally found not to have adverse effects on the viability of the Caco-2 cells and were freshly prepared using DMSO immediately before experiment and added to the apical side.

High-Performance Liquid Chromatography. An Agilent 1100 liquid chromatography system (Agilent Technologies) was used for all experimental determinations. This instrument was equipped with a quaternary pump, an online degasser, an autosampler, a diode-array detector, and an Agilent 1100 chemstation. The chromatographic analysis operated at 202 nm was achieved using a VYDAC 238EV54 C18 reverse-phase HPLC column. The mobile phase at a flow rate of 1.0 mL/min consisting of acetonitrile/water (22:78, vol/vol) with 0.05% TFA. VLPVP was determined by measuring the peak area.

Statistical Analysis of Data. All experiments were done in at least triplicate, and data were expressed as mean ± standard deviation. Statistical differences between the treatments were determined using a one-way analysis of variance (ANOVA) test. Probability values of $p < 0.05$ were considered to be statistically significant.

The amounts of test compounds permeated in both directions were calculated and plotted vs time. The apparent permeability coefficients (P_{app}) were calculated using eq 1:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{A} \times \frac{1}{C_0} \quad (1)$$

where dQ/dt is the permeability rate, A is the area of the membrane (cm^2), and C_0 is the initial donor concentration. Samples from all time points were calculated.

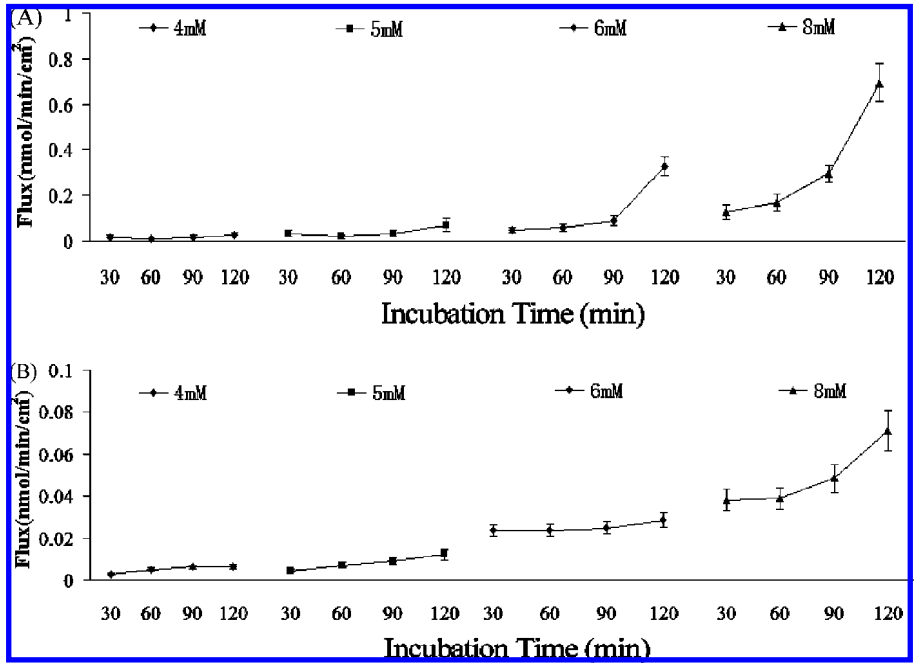

Figure 1. Cumulative transepithelial flux of VLPVP across the Caco-2 cell monolayer versus time. (**A**) Apical to basolateral flux. (**B**) Basolateral to apical flux. Data are the mean ± SD of at least three independent determinations.

The efflux ratio (*ER*) was determined by calculating the ratio of P_{app} in the secretory (ba) divided by the absorptive (ab) direction according to eq 2:

$$ER = \frac{Papp_{ba}}{Papp_{ab}} \quad (2)$$

RESULTS

Effect of VLPVP on the Growth of Caco-2 Cells. As shown in **Table 1**, VLPVP at or below a concentration of 12 mmol/L did not show significant cytotoxic effect on Caco-2 cells, but an increase in its concentration to 14 mmol/L exhibited marked cytotoxicity toward Caco-2 cells. The maximum concentration of VLPVP used for our transport studies was 8 mmo/L, which was nontoxic toward the Caco-2 cell monolayers, especially since the incubation time (up to 2 h) was shorter than the time employed for the MTT test (24 h).

Validation of HPLC Methods. Under the chromatographic conditions used for the analysis of VLPVP, the retention times for VLPVP were 5.21 min. The total chromatography run time was 8.0 min. Matrix-specific interfering peaks that required modification of the mobile phase composition were not observed. The presence of drugs such as verapamil, MK-571, sodium azide, sodium deoxycholate, Gly-Pro, and phenylarsine oxide also did not cause any interference in the assay. The linear regression of the peak area ratios versus concentrations was fitted over the concentration range of 1−100 μmol/L. A typical equation of the calibration curves was as follows: $y = 14.421x - 0.038$ ($r^2 = 0.9999$, $n = 7$), where y is the peak area and x is the concentration of VLPVP. Good linearity was seen in this concentration range.

LOD for the elaborated technique, at $S/N = 3{:}1$, was 1.0 nmol/L. On the other hand, LOQ, defined as the lowest concentration in the standard curve at which CV-defined precision ≤ 15%, was determined to be 1.5 nmol/L. This sensitivity was high enough to quantitate the low concentrations of VLPVP in the receiving pool of the monolayers when using low loading concentrations.

In this assay, the intra- and interday precisions ranged from 1.02 to 4.06% and from 2.70 to 4.76% for each QC level, respectively. The accuracy was within ±2.20%. The results, calculated using one-way ANOVA, indicated that the values were within the acceptable range and the method was accurate and precise (**Table 2**).

Stability of VLPVP. VLPVP at 5 mmol/L was added to the AP of the Caco-2 cell monolayer, and then the concentrations of intact VLPVP and free amino acids in the Caco-2 cells as well as in the apical, basolateral solutions, and cell fraction were determined. The distribution of VLPVP and free amino after incubation for 2 h is summarized in **Table 3**. As the table shows, 3.84% of VLPVP disappeared in the apical side, of which 0.04% of the VLPVP was in an intact form in the cells, 0.25% of VLPVP was transported to the basolateral intact, and about 3.54% of the VLPVP was hydrolysis. Moreover, VLPVP at 5 and 15 μmol/L was stable in HBSS at pH 6.0, 7.0, 7.4, and 8.0, whereas no significant degradation was observed over 24 h at 37 °C (data not shown). These results indicated that VLPVP has a good stability in the transport experiment.

VLPVP Transport by Caco-2 Monolayers. The influence of time and concentration on the flux of VLPVP (4−8 mM) across the Caco-2 cell monolayer is shown in **Figures 1** and **2**. **Figure 1A** shows the cumulative VLPVP transport after apical loading of VLPVP, and **Figure 1B** shows the corresponding data after basolateral loading. The apical to basolateral transport rate was significantly higher than the basolateral to apical transport rate at all time points and VLPVP concentrations. The P_{app} values of VLPVP at 4−8 mmol/L from the AP to BL side were 7.44×10^{-8} to 1.35×10^{-6} cm/s, with a marked increase in P_{app} values from AP to BL at increased VLPVP concentrations. The bidirectional transport of VLPVP was essentially linear for up to 2 h in a concentration-dependent manner with no apparent saturation, and the *ER* values were calculated to be 0.74, 0.51, 0.13, and 0.20 for VLPVP at 4, 5, 6, and 8 mM, respectively, suggesting the involvement of an active process, although the low VLPVP concentrations used (≤8 mM) did not show any saturation.

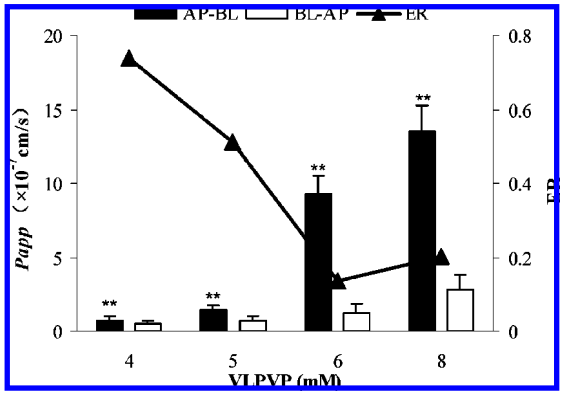

Figure 2. Permeability coefficients of VLPVP when added either from AP or from BL. The *ER* value for each concentration of VLPVP was also plotted. ** = significantly higher than the corresponding basolateral concentrations ($p < 0.01$).

Effect of Various Compounds on VLPVP Transport. In an attempt to identify the endocytosis and paracellular transport responsible for the flux of VLPVP, its permeability across Caco-2 cell monolayers in the presence of phenylarsine oxide (25 μmol/L) and sodium deoxycholate (100 μmol/L) was measured (**Figure 3A**). The results show that the phenylarsine oxide had no significant effect on the bidirectional transport. On the contrary, the bidirectional transport of VLPVP saw a notable increase in the presence of sodium deoxycholate, indicating that paracellular transport was the main mechanism for the VLPVP absorption in intestinal epithelial cells.

Verapamil (100 μmol/L), substrates for P-gp, and Gly-Pro (10 mmol/L), a competitive peptide transporter inhibitor (PepTx), did not show any effect on the transport of VLPVP across Caco-2 cell monolayers from either direction (**Figure 3B**). On the other hand, in the presence of MK-571 (50 μmol/L), substrates for MRP, the transport of VLPVP from AP to BL was changed significantly ($p < 0.01$), and a significant decrease in the *ER* values was observed. Moreover, the flux of AP to BL was significantly increased in the presence of the ATP inhibitor sodium azide (10 mmol/L). Hence, the transport of VLPVP in Caco-2 cells was dependent on MRP but not on P-gp transporters and PepTx.

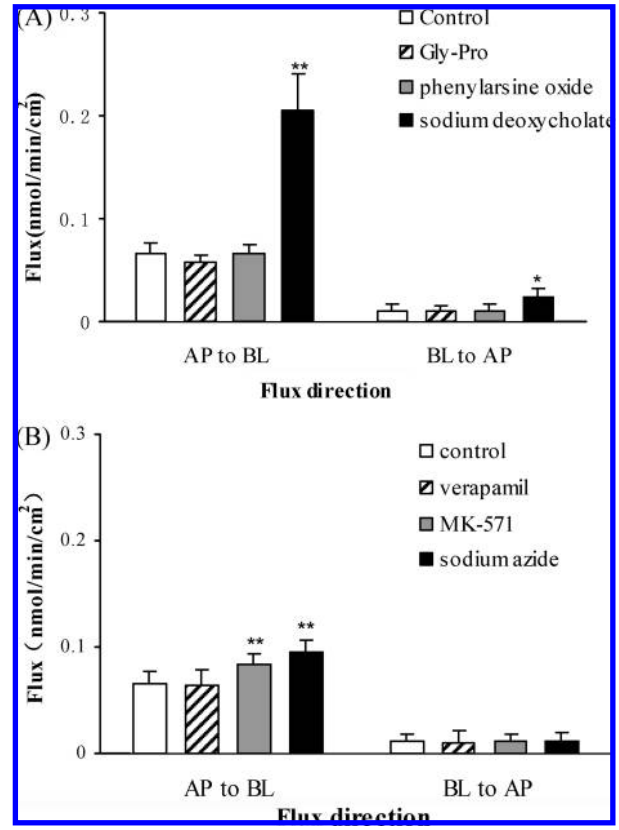

Figure 3. Effect of Gly-Pro, sodium deoxycholate, and phenylarsine oxide (**A**), verapamil, MK-571, and sodium azide (**B**) on the transport of VLPVP (5 μM) across the Caco-2 cell monolayer. Data are the mean ± SD from three determinations. *, ** = significantly different from control ($P < 0.05$ and 0.01, respectively)

DISCUSSION

Most ACE inhibitory peptides have low permeation rates across the Caco-2 monolayer, with P_{app} values in the range of 10^{-9} cm/s (*24*). Such low permeation rates are normally associated with peptides that are unstable and susceptible to enzymatic or nonenzymatic hydrolysis. In contrast, our results indicated that the VLPVP had an acceptable permeability coefficient in Caco-2 monolayers, considering its high ACE inhibitory activity. Moreover, the VLPVP was relatively stable, it was not susceptible to nonenzymatic hydrolysis, and it was reasonably stable to enzymatic degradation by Caco-2 cells. This suggested that the oral bioavailability for VLPVP in humans would be high, although it is yet to be determined clinically.

The aim of the current work was to explore the mechanism that could be operating in the transfer of VLPVP across enterocytes. The first part of this work was directed to the study of the paracellular route. In this line of investigation, tight junctions between enterocytes were opened through the use of sodium deoxycholate. The P_{app} of the VLPVP increased approximately 2−5-fold, and TEER values fell at the same time, consistent with junctional disruption. Taking into account its concentration-dependent manner and nonsaturation, we concluded that the paracellular pathway was involved in the VLPVP transport.

Further investigations indicated differences in VLPVP P_{app} values in the absorptive (AP to BL) and excretive (BL to AP) directions through Caco-2 cell monolayers. Indeed, the absorption rate was up to 5-fold higher than the excretion. Similarly, in the presence of sodium azide, the P_{app} of VLPVP from AP to BL was significantly increased, suggesting that it undergoes an active transport. Consequently, we study the role of verapamil, MK-571, and Gly-Pro in the transport of VLPVP. Indeed, only the P_{app} value of VLPVP from AP to BL increased dramatically with the presence of MK-571. Therefore, MRP2 (expressed at the AP side of the Caco-2 cells) (*25*), but not P-gp and PepTx, may be involved in the absorptive transport of VLPVP in the gut.

Concerning alternative endocytic pathways, the absence of effects induced by phenylarsine oxide, an inhibitor of receptor-mediated endocytosis, and the lack of effect on VLPVP transport are strong indications that endocytic was not mechanistically relevant.

In conclusion, the data collected in this study demonstrate that VLPVP uses both paracellular diffusion and MRP2 transport to cross a Caco-2 model of the intestinal barrier. However, to fully understand absorption properties of VLPVP in the small intestine, further research of the absorption properties of VLPVP should be undertaken.

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