Studies on Absorption and Tansport of Limoninoids from *Fructus Evodiae* in Caco-2 Cell Monolayer Model

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Abstract: **Objective** To study the intestinal absorption and transepithelial transport of three limoninoids: evodol (EVO), limonin (LIM), and shihulimonin A (SHIA), isolated from *Fructus Evodiae* [the unripe fruit of *Evodia rutaecarpa* and *Evodia rutaecarpa* var. *bodinieri*] in the human intestine. **Methods** The *in vitro* cultured human colon carcinoma cell line, Caco-2 cell monolayer model, was applied to studying the absorption and transepithelial transport of the three limoninoids from apical (AP) to basolateral (BL) side and from BL to AP side. The three limoninoids were measured by reversed-phase high performance liquid chromatography coupled with ultraviolet absorption detector. Transport parameters and apparent permeability coefficients (P_{app}) were then calculated and compared with those of Propranolol as a control substance of high permeability and Atenolol as a control substance of poor permeability. **Results** The P_{app} value of EVO and LIM from AP to BL side for absorption and transport were 1.78×10^{-5} cm/s and 1.16×10^{-5} cm/s, respectively, which was comparable to that of Propranolol with P_{app} 2.18×10^{-5} cm/s. **Conclusion** The absorption and transport of both EVO and LIM are main passive diffusion as the dominating process in Caco-2 cell monolayer model, and they were estimated to be high absorbed compounds. SHIA in Caco-2 cell monolayer model may be involved in metabolism in the transport processes.

Key words: absorption and transport; apparent permeability coefficients; Caco-2 cell; limoninoids
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Introduction

Fructus Evodiae [the dried unripe fruit of *Evodia rutaecarpa* (Juss.) Benth., *Evodia rutaecarpa* (Juss.) Benth. var. *officinalis* (Dode) Huang, and *Evodia rutaecarpa* (Juss.) Benth. var. *bodinieri* (Dode) Huang], well known in China as *Wuzhuyu*, has been prescribed to take orally for the treatment of gastrointestinal disorders, headache, abdominal pain, dysentery, and postpartum hemorrhage (Pharmacopoeia Committee of P. R. China, 2005). The main chemical components are indole (Zhang, Yang, and Chui, 1999; Teng and Yang, 2006a; Yang, Zhang, and Hu, 2008a; Yang and Teng, 2007; Zhao and Yang, 2008) and quinoline (Yang *et al*, 2006) alkaloids, limonoids (Yang, Zhang, and Hu, 2008b; Teng and Yang, 2006b), and essential oil (Teng *et al*, 2003), etc. A number of pharmacological effects have been attributed to such thereunto indoloquinazoline alkaloids related to body temperature maintaining effects (Kobayashi *et al*, 2001), cardioprotection (Hu *et al*, 2002), nociceptive, and anti-nociceptive action (Kobayashi, 2003). Toxicological assessment on safety of the water and 70% ethanol extracts of *Fructus Evodiae* (Yang, 2008) and assessment of acute toxicity of the quinoline alkaloids in mice (Yang *et al*, 2006) had also been reported. In recent years, the human Caco-2 cell monolayer model has gained popularity as a high-throughput *in vitro* model for some drugs intestinal absorption potential (Yang *et al*, 2007). In a previous study, we reported the permeability and the efflux of alkaloids of *Fructus Evodiae* in the human Caco-2 cell monolayer model. In this study, the

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intestinal permeability and the efflux of three limonoids, evodol (EVO), limonin (LIM), and shihulimonin A (SHIA) (Fig. 1), which are main limonoids constituents in *Fructus Evodiae*, were investigated using the human intestinal Caco-2 monolayer grown on Transwell.

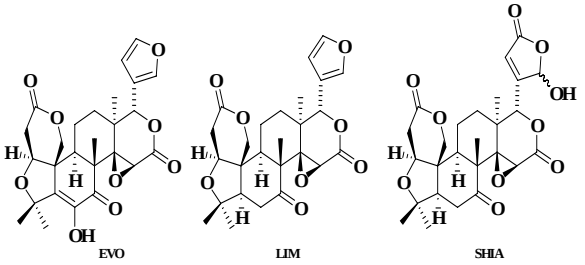

Fig. 1 Chemical structures of EVO, LIM, and SHIA

Materials and methods

Materials

Dulbecco's Modified Eagle's Medium (DMEM), Eagle's minimum essential medium (MEM) and Hank's Balanced Salts Solution (HBSS) were purchased from Gibco Laboratories (Life Technologies Inc., Grand Island, USA). Fetal bovine serum (FBS) and none-ssential amino acids (NEAA) were purchased from HyClone (Logan, USA). Propranolol, Atenolol, trypsin, dimethyl sulfoxide (DMSO), and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) were purchased from Sigma Chemical Co. (Deisenhofen, Germany). Penicillin and Streptomycin were purchased from Huabei Pharmaceutical Group Co., Ltd. Other chemicals were of analytical grade and solvents used in high-performance liquid chromatography (HPLC) were of HPLC grade. Transwell^{TM} plates of 12-well (insert diameter 12 mm, pore size 3.0 μm, insert membrane growth area 1.12 cm^2) were purchased from Corning Costar (Cambridge, USA).

EVO, LIM, and SHIA were prepared and purified from *Fructus Evodiae* described in previous report (Yang, Zhang, and Hu, 2008b; Teng and Yang, 2006b). Their purity was determined to be > 99% by HPLC. For the mobile phase, detected wavelength, and flow rate see "HPLC analysis" section.

Cell strain

The human colon adenocarcinoma cell line Caco-2 (ATCC#HTB-37) was purchased from American Type Culture Collection (ATCC, Rockville, USA).

Apparatus

A Mode HZS-H Thermostatic Water Bath Oscillator (Harbin Donglian Electronic & Technology Development Co., Ltd. of China, Harbin, China), a Mode TGL-16C High-Speed Desktop Centrifuge (Shanghai Anting Scientific Instrument Factory, Shanghai, China), a Mode GALAXY-B Carbon Dioxide Gas Incubator (RS Biotech Co., Irvine, UK), a Mode JJT–1300 Laminar Flow Cabinet (Beijing Great Wall Equipment & Engineering Co., for Air Purification, Beijing, China), a Mode LGJ0.5 Freeze Dryer (Beijing Four-Ring Scientific Instrument Co., Beijing, China), an Epithelial Voltohmmeter (EVOM, World Precision Instrument, Sarasota, USA), and a Mode XDS-1 Inverted Microscope (Chongqing Optical & Electrical Instrument Co., Ltd., Chongqing, China) were used. The analytical HPLC system consisted of a model DIONEX P680 pump, UVD 170U detector, and a Chromeleon Workstation (Version 6.50) (DIONEX Co., München, Germany), and a 7125 Rheodyne injector (Rheodyne, Cotati, USA) with a loop of 50 μL. The sample analysis was achieved on a reversed-phase Diamonsil™ C_{18} column (250 mm × 4.6 mm, 5 μm; Dikma, China) equipped with a C_{18} guard column (8 mm × 4 mm, 5 μm; Dikma, China).

Cell culture

Caco-2 cells were grown in DMEM containing *D*-glucose (4.5 g/L), supplemented with heat-inactivated 10% FBS, 1% NEAA, Penicillin (100 U/mL) and Streptomycin (100 μg/mL) in an atmosphere of 5% CO_2 and 90% relative humidity at 37 ℃. Monolayers were sub-cultured when they reached 80% confluence at a split ratio 1 : 6 using 0.25% Trypsin containing 0.01% EDTA. For the transport studies, Caco-2 cells were seeded at a density of about 6.25 × 10^4 cells/cm^2 on a 12-well Transwell insert filter and grown to late confluence (21 d). The culture medium was changed every 3–4 d after seeding. The integrity and transport

ability of the Caco-2 cell monolayer were examined by measuring the transepithelial electrical resistance (TEER) with an epithelial voltohmmeter and running standard assays using Propranolol and Atenolol as the transcellular and the paracellular flux marker, respectively. TEER was determined before the test solutions were added to the donor side and fresh HBSS was added to the receiver side of the monolayer, and also measured at the end of the transport assays. Only monolayers with a TEER value > 600 Ω/cm^2 were utilized for further experiments. All cells used in this study were as follows.

Preparation of test compounds solution

The stock solution of the test compounds was prepared in DMSO and kept at 4 ℃. The test compound solution was prepared by appropriately diluting the stock solution with HBSS (pH 7.4) to 10, 25, and 50 μmol/L. The final concentration of DMSO was less than 0.5% in all experiments, which did not have any detectable effect on the cell monolayer.

Transport experiment

HBSS supplement with 20 mmol/L *D*-glucose and 10 mmol/L HEPES (pH 7.35) was used as the transport medium. The transport experiments were undertaken to measure the flux of the test compounds. Flux describes the movement of a substance across polarized Caco-2 monolayers either in absorptive (apical→basolateral, AP→BL) or in secretory direction (basolateral→apical, BL→AP). The Caco-2 cells were rinsed thrice with pre-warmed transport medium HBSS containing 25 mmol/L HEPES (pH 7.4) and were incubated by pre-warmed transport medium 0.5 mL for AP chamber and 1.5 mL for BL chamber at 37 ℃ for 30 min. At the end of the pre-incubation, the media were removed and the appropriate test compound (EVO, LIM, SHIA, Atenolol or Propranolol) solution or transport media 0.5 mL in the AP and 1.5 mL in the BL chamber were added. After shaking at 55 r/min for different time points in a shaking water bath at 37 ℃, samples were collected from the AP chamber or BL chamber of the cell monolayer and immediately frozen, lyophilized, and preserved below −20 ℃ before analysis by HPLC. In the AP→BL directional flow assay, 400 μL from the AP chamber and 1 300 μL from BL chamber samples were collected. In the BL→AP directional flow assay, 200 μL from the AP chamber and 650 μL from BL chamber samples were collected. The TEER value was measured at the end of the experiment to check the integrity of monolayer. The dried sample in the AP→BL directional flow assay was ultrasonically treated with 400 μL MeOH for the AP chamber sample and 1300 μL for the BL chamber sample followed by centrifugation at 15 000 × *g* for 10 min. The supernatant was filtered through a 0.45 μm filter and a 50.0 μL aliquot was injected into the HPLC system. Whereas the dried sample in the BL→AP directional flow assay was ultrasonically treated with 200 μL MeOH for the AP chamber sample and 650 μL for the BL chamber sample followed by centrifugation at 15 000 × *g* for 10 min, and the supernatant was filtered through a 0.45 μm filter and a 50.0 μL aliquot was injected into the HPLC system.

HPLC analysis

The quantitative analysis of samples that permeated through the monolayer was measured by RP-HPLC. The mobile phase was composed of acetonitrile–water (52 : 48) and the wavelength of UV detector was set at 276 nm for EVO, acetonitrile–water (49 : 51) and the wavelength was at 204 nm for LIM, and acetonitrile–water (40 : 60) and the wavelength was at 206 nm for SHIA, and was delivered isocratically at a flow rate of 1.0 mL/min.

Data analysis

Based on the results of HPLC analysis, apparent permeability coefficients (P_{app}) value of across the Caco-2 cell monolayer was calculated according to the following equation:

$$P_{app} = dQ/dt \times 1/A \times 1/C_0$$

Where P_{app} is the permeability coefficient (cm/s); dQ/dt is the appearance rate of the test compound on the receiver side (μmol/s); C_0 is the initial test compound concentration on the donor side (μmol/cm^3); and A is the surface area of the insert (cm^2).

All experiments were carried out in triplicate. All data were analyzed by Microsoft Excel 2000 (Microsoft, Redmond, USA) and are expressed as $\overline{X} \pm s$.

Results

Establishment of analysis method

A rapid, simple, and reliable HPLC method has been established for the analysis of the EVO, LIM, and SHIA in the Caco-2 cell cultures. The wavelength of maximum absorbance of UV detector was set at 276 nm for EVO, 204 nm for LIM, and 206 nm for SHIA, and no interference from any Caco-2 monolayer substances was observed during the elution. The calibration curves for test compounds were constructed by plotting peak area (X) *vs* concentration (Y). The linear regression equations for calibration curves in Caco-2 cell cultures were $Y = 7 \times 10^{-6} X + 0.036\ 6$ ($r = 0.999\ 1$) for EVO, $Y = 7 \times 10^{-6} X + 0.021\ 2$ ($r = 0.999\ 1$) for LIM, and $Y = 5.7 \times 10^{-5} X + 0.015$ ($r = 0.999\ 2$) for SHIA. Calibration curves were found to be linear over the calibration range of 6.25×10^{-2}–2.50 nmol/L for both EVO and LIM, and 0.25–2.50 nmol/L for SHIA.

Validation of the Caco-2 cell monolayer system

To monitor the Caco-2 cell monolayer system, P_{app} values of Propranolol and Atenolol across Caco-2 monolayer were determined. The P_{app} value of Propranolol from the AP chamber to the BL chamber was 2.18×10^{-5} cm/s and that of Atenolol was 2.77×10^{-7} cm/s. These results were comparaed with the reported values (Yang *et al*, 2007), which indicated that Caco-2 cell monolayer model developed in this study can be used to predict the intestinal absorption potential of EVO, LIM, and SHIA.

Transport assay

The rates of membrane permeation increased linearly with the concentration within the test range of concentration (0–50 μmol/L) while the P_{app} values basically remained unchanged with concentration (Fig. 2 and Fig. 3). This bidirectional transepithelial transport suggests a passive diffusion mechanism for their transport across the Caco-2 monolayer (Yang *et al*, 2007). The bilateral P_{app} values for the EVO and LIM have been summarized in Table 1. But the P_{app} values of SHIA is not shown in Table 1 because permeation of SHIA was not detected in the AP→BL directional flow assay, suggesting the P_{app} value of SHIA is less than its limit of detection or SHIA may be involved in metabolism in the transport processes.

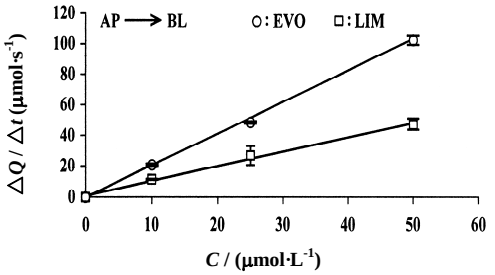

Fig. 2 Absorption transport of EVO and LIM at different concentration from AP to BL side in the Caco-2 cell monolayer model

EVO and LIM incubation with Caco-2 cells for 90 min. The values presented $\overline{X} \pm s$ ($n = 3$)

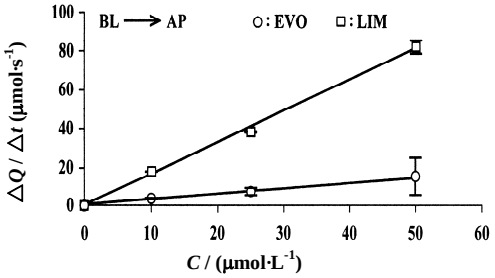

Fig. 3 Efflux-transport of EVO and LIM at different concentration from BL to AP side in the Caco-2 cell monolayer model

EVO and LIM incubation with Caco-2 cells for 90 min. The values presented $\overline{X} \pm s$ ($n = 3$)

Propranolol is a transcellular transport marker, which is a well-transported compound and has a P_{app} value of $P_{app} \geqslant 1.0 \times 10^{-5}$ cm/s (Chong, Dando, and Morrison, 1997). In contrast, Atenolol is a poorly transported compound and usually exhibits a low P_{app} value of $P_{app} \leqslant 1.0 \times 10^{-7}$ cm/s, and a moderate compound will have the P_{app} value of 1×10^{-6}–1×10^{-5} cm/s (Yang *et al*, 2007). The P_{app} values of EVO and LIM for the bidirection flux at different concentration were 10^{-5} cm/s degree. So EVO and LIM are the good absorption compounds.

Table 1 P_{app} values of EVO and LIM from bidirections at different concentration*

Compounds	Concentration / (μmol·L^{-1})	P_{app} / (× 10^{-5} cm·s^{-1})		$P_{app\ AP→BL}/P_{app\ BL→AP}$
		AP→BL	BL→AP	
EVO	10	1.835 ± 0.063	0.338 ± 0.018	5.429
	25	1.707 ± 0.032	0.246 ± 0.011	6.939
	50	1.807 ± 0.047	0.262 ± 0.016	6.897
	average	1.783 ± 0.047	0.282 ± 0.015	6.422
LIM	10	1.070 ± 0.046	1.508 ± 0.073	0.71
	25	0.933 ± 0.217	1.353 ± 0.083	0.69
	50	0.827 ± 0.045	1.581 ± 0.165	0.52
	average	1.156 ± 0.103	1.481 ± 0.107	0.64

* Each test compound incubated with Caco-2 cell monolayer was carried out for 90 min and all experiments were carried out in triplicate. Data are the $\bar{X} \pm s$ (n = 3)

$P_{app\ AP→BL}$: The test compound transport from AP to BL direction

$P_{app\ BL→AP}$: The test compound transport from BL to AP direction

$P_{app\ AP→BL}/P_{app\ BL→AP}$: The ratio of P_{app} of test compound transport from AP to BL and BL to AP direction

The ratio of $P_{app\ AP→BL}/P_{app\ BL→AP}$ for EVO was more than 5.4, suggesting greater permeability in the AP to BL direction. Whereas the bilateral permeability ratio for LIM was less than 0.7, suggesting greater permeability in the BL to AP direction.

The time course of membrane permeation

The transport amounts of AP→BL and BL→AP permeation of the EVO, LIM, and SHIA increased with incubation time (20, 40, 60, 90, and 120 min) at initial concentration of 10 μmol/L (Fig. 4 and Fig. 5). Whereas SHIA in the AP chamber was not detected in the BL→AP directional flow assay. These results further suggest that the absorption and transport of both EVO and LIM in Caco-2 cell monolayer model are main passive diffusion as the dominating process.

In conclusion, transepithelial transport across

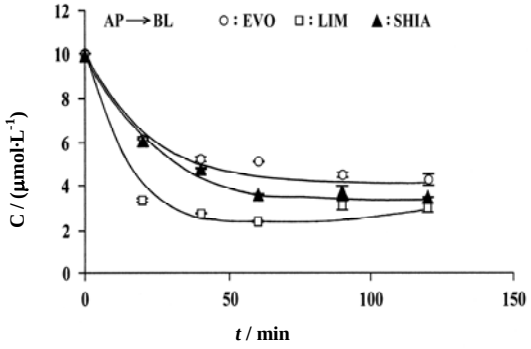

Fig. 4 Concentration-time curves of transport of EVO, LIM, and SHIA at 10 μmol/L from AP to BL side in the Caco-2 cell monolayer model

The values presented $\bar{X} \pm s$ (n = 3)

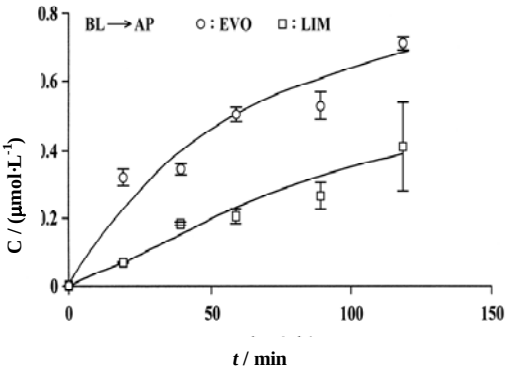

Fig. 5 Concentration-time curves of transport of EVO and LIM at 10 μmol/L from BL to AP side in the Caco-2 cell monolayer model

The values presented $\bar{X} \pm s$ (n = 3)

Caco-2 cell monolayer of EVO, LIM, and SHIA, which present in *Fructus Evodiae*, was studied for the first time. The results suggested that EVO and LIM are well absorbed compounds mainly through the transcellular pathway. The metabolism may be involved in the absorption of SHIA. EVO and LIM are likely to be the *in vivo* active components of *Fructus Evodiae*, which need further pharmacokinetic and metabolic studies *in vivo*.

Acknowledgements

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Introduction of Cover Picture

Cypripedium guttatum Sw is a plant of *Cypripedium* L. (Orchidaceae), which has biological effects such as sedation, analgesia, sweating, and antipyretic. Modern research shows that this Chinese herbal medicine can be used for the treatment of neurasthenia, epilepsy, febrile convulsion, headache, and stomachache. It is widely distributed in Northeast China, and Shandong, Shanxi, Sichuan, and Yunnan Provinces as well.

Provided by ZHOU Yao
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