

Original Article

Intestinal absorption characteristics of imperialine: *in vitro* and *in situ* assessments

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Aim: Imperialine is an effective compound in the traditional Chinese medicine chuanbeimu (*Bulbus Fritillariae Cirrhosae*) that has been used as antitussive/expectorant in a clinical setting. In this study we investigated the absorption characteristics of imperialine in intestinal segments based on an evaluation of its physicochemical properties.

Methods: Caco-2 cells were used to examine uptake and transport of imperialine *in vitro*, and a rat *in situ* intestinal perfusion model was used to characterize the absorption of imperialine. The amount of imperialine in the samples was quantified using LC-MS/MS.

Results: The aqueous solubility and oil/water partition coefficient of imperialine were determined. This compound demonstrated a relatively weak alkalinity with a pK_a of 8.467 ± 0.028 . In Caco-2 cells, the uptake of imperialine was increased with increasing pH in medium, but not affected by temperature. The apparent absorptive and secretive coefficient was $(8.39 \pm 0.12) \times 10^{-6}$ cm/s and $(7.78 \pm 0.09) \times 10^{-6}$ cm/s, respectively. Furthermore, neither the P-glycoprotein inhibitor verapamil nor Niemann-Pick C1-Like 1 transporter inhibitor ezetimibe affected the absorption and secretion of imperialine *in vitro*. The *in situ* intestinal perfusion study showed that the absorption parameters of imperialine varied in 4 intestinal segments (duodenum, jejunum, ileum and colon) with the highest ones in the colon, where a greater number of non-ionized form of imperialine was present.

Conclusion: The intestinal absorptive characteristics of imperialine are closely related to its physicochemical properties. The passive membrane diffusion dominates the intestinal absorption of imperialine.

Keywords: imperialine; chuanbeimu; traditional Chinese medicine; antitussive/expectorant; physicochemical property; absorption; transport; Caco-2 cells; intestinal perfusion

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Introduction

Bulbus Fritillariae Cirrhosae (“Chuanbeimu” in Chinese), a traditional Chinese medicine (TCM), has been used as an anti-tussive and expectorant in China and other Asian countries for centuries^[1]. In fact, imperialine is one of the main effective compounds in Chuanbeimu and has the potential to be used as an antitussive/expectorant in a clinical setting^[2]. It is believed that oral administration is most likely preferred over intravenous administration, as oral dosing leads to obvious improved patient compliance^[3]. However, the data regarding imperialine are far from sufficient to advance the compound to clinical research for oral administration.

A previous study^[4] showed that the oral bioavailability of imperialine could be as high as 30%–50%, but how this molecule was affected by physicochemical and physiological factors was unknown.

Like most alkaloids containing a tertiary amino group, imperialine undergoes pH-dependent hydrolysis of this group (Figure 1). A relatively high pH typically leads to a greater number of non-ionized molecules and a lower overall solubility. Thus, the pH, which varies in different intestinal segments, is one of the crucial factors affecting the absorption of acidic or alkaline drugs^[5]. Due to the unknown properties of imperialine under different conditions, we undertook a study of the compound's physicochemical properties, including its solubility, *n*-octanol/water partition coefficient (*P*) and ionization constant (pK_a).

In addition to the physicochemical factors mentioned above, physiological factors can also influence the bioavailability of imperialine. One of the popularly accepted and widely used *in vitro* systems for transport studies is the Caco-2 cell monolayer^[6, 7]. These cells, originating from a human colonic adenocarcinoma line, differentiate spontaneously to resemble intestinal enterocytes in morphology and function^[8]. In the past century, Caco-2 has become an FDA approved model for bioequivalence and is widely used in transport stud-

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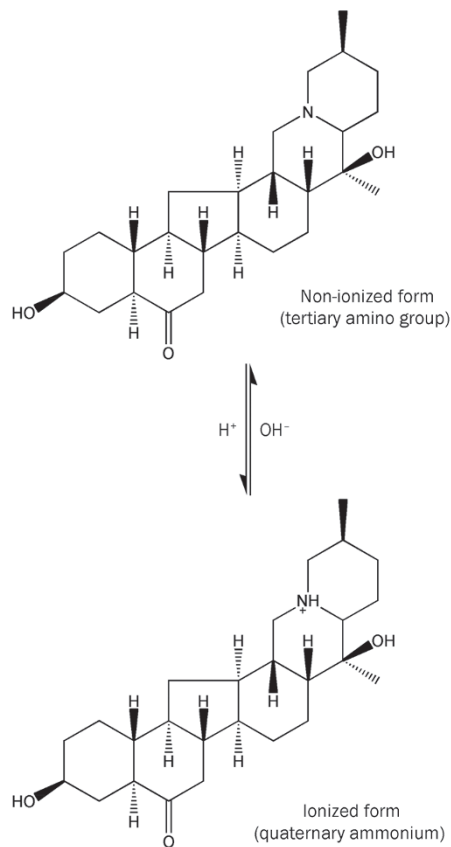


Figure 1. The chemical structure of imperialine.

ies. Dozens of transporters involved in drug uptake and efflux have been found to be expressed in Caco-2 cells, such as P-glycoprotein (P-gp)^[9] and the Niemann-Pick C1-Like 1 (NPC_{1L1}) transporter^[10, 11]. However, *in vitro* models provide limited and occasionally inaccurate information. Thus, the combination of *in vitro* models with *in situ* approaches can provide experimental conditions that are more comprehensive than those encountered following oral administration, with decreased sensitivity to pH variations. This is because the microenvironment in the intestinal segments is more closely replicated^[12, 13]. Furthermore, *in situ* techniques maintain an intact blood supply to the intestine and can be used to evaluate the influence of clearance pathways, such as enzymes and transporters present within the gut. In addition, the expression of drug metabolic enzymes and transporters differs along the intestinal tract, thereby affecting drug permeability in the various segments^[14]. NPC_{1L1} is abundantly expressed in the jejunum and proximal ileum and is specifically located adjacent to the brush border membrane of intestinal enterocytes, where cholesterol absorption takes place^[10]. By contrast, P-gp is located in nearly the whole intestinal segment. Single-pass intestinal perfusion (SPIP), with little mechanical injury to the isolated gut, is a good choice for investigating drug absorption *in situ*. In addition, it has been reported that oral drug absorption in rats closely correlates with that measured in human

beings^[15]. Thus, it is likely that intestinal perfusion studies conducted in rats, combined with studies using the Caco-2 cell model, will more precisely predict the absorption of an oral dose in humans than would an exclusively *in vitro* model.

To establish a foundation for better understanding the oral absorption and transport features of imperialine, we investigated its physicochemical properties and absorption characteristics in intestinal segments in the current study.

Materials and methods

Chemicals and reagents

Imperialine (purity >98.0%) was obtained from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). Heat-inactivated fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium (DMEM) containing 25 mmol/L *D*-glucose, 25 mmol/L HEPES, 44 mmol/L NaHCO₃, non-essential amino acid solution, *L*-glutamine and penicillin-streptomycin were purchased from Gibco Laboratory (Invitrogen Co, Grand Island, NY, USA). A 0.25% trypsin-0.02% EDTA solution was obtained from Nanjing KeyGEN Biotech Co Ltd (Nanjing, Jiangsu, China), and Hanks' balanced salt solution (HBSS, containing 137 mmol/L NaCl, 5.36 mmol/L KCl, 0.44 mmol/L KH₂PO₄, 0.34 mmol/L Na₂PO₄, 1 mmol/L CaCl₂, 1 mmol/L MgCl₂, and 5.6 mmol/L glucose) was purchased from Beijing Solarbio Science & Technology Co Ltd (Beijing, China). Verapamil was obtained from Aladdin Chemistry Co Ltd (Shanghai, China). HPLC-grade acetonitrile and *n*-octanol were purchased from Sigma-Aldrich (St Louis, MO, USA), and LC-grade formic acid was obtained from Tedia (Cincinnati, OH, USA). Ultra-pure water for the liquid chromatography (LC) mobile phase was prepared in house using a Milli-Q system (Millipore, Bedford, MA, USA). All other reagents were of analytical grade.

Quantitative determination of imperialine

In this study, we used liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) to quantify the imperialine levels in the samples. The LC-MS/MS system consisted of a Rapid Resolution Liquid Chromatography system (1200 series, Agilent Technologies, Santa Clara, CA, USA) equipped with an SL autosampler, a degasser and an SL binary pump. An Agilent 6140 triple-quadrupole mass spectrometer with electrospray ionization (ESI) interface was controlled using B01.03 software for qualitative analysis and B01.04 software for quantification.

The MS conditions were adjusted as previously reported^[4]. RP-LC separation was performed on a Diamonsil ODS column (100 mm×4.6 mm, 3 μm) with a corresponding guard column (ODS, 5 μm). The mobile phase consisted of (A) 0.1% (*v/v*) aqueous formic acid and (B) acetonitrile, and the flow rate was 0.4 mL/min. The following gradient elution was used: 0.0-1.5 min, 10.0% → 90.0% B; 1.5-2.5 min, 90.0% → 10.0% B; 2.5-4.0 min, 10.0% B. The column was maintained at 30°C, and the autosampler tray was kept at 20°C. An aliquot of 1 μL of solution was injected into the LC-MS/MS system for analysis.

Determination of physicochemical properties

Measurement of equilibrium solubility

We adopted the miniaturized shake-flask solubility method to determine the equilibrium solubility of imperialine. Pure water and a series of phosphate buffered saline (PBS) solutions with increasing pH values (pH 2.0, 3.0, 4.0, 5.0, 5.8, 6.5, 6.8, 7.0, 7.4, 7.8, and 8.5) were prepared according to Pharmacopoeia of China (Ch.P)^[16]. The pH of the buffer solutions was precisely measured using a pH meter with a combined Ag/AgCl glass electrode (FiveStar, Switzerland).

An excess of imperialine was added to 2 mL water or PBS at varying pH levels in a 5-mL Eppendorf (EP) tube, and the resulting suspension was shaken at 100 r/min, at 37°C for 24 h in a thermostated shaking incubator (17-C-1, Taicang Experimental Equipment Group Ltd, China). The values obtained were the averages of triplicate measurements. The sample solutions were filtered through a 0.45- μ m hydrophilic membrane (Tianjin Heng'ao Technological Industry Group Ltd, Tianjin, China), and the subsequent filtrates were collected. After dilution to the desired concentration range in acetonitrile, 1- μ L aliquots were injected into the LC-MS/MS system for analysis.

Measurement of the *n*-octanol/water partition coefficient

The log *P* of imperialine was determined using the shake-flask technique. A 2-mL aliquot of pure water or PBS (with different pH values) saturated with *n*-octanol was added to 2 mL imperialine-saturated *n*-octanol-saturated PBS in a 10-mL EP tube. As described previously^[17], the two phases were mutually equilibrated by shaking in a thermostated shaking incubator at a speed of 100 r/min and a temperature of 37°C for 12 h. The tubes were centrifuged at 6000 \times g for 5 min, and the aqueous and organic phases were collected. The aqueous sample solutions were then filtered through a 0.45- μ m hydrophilic membrane, and the organic solutions were filtered through a 0.45- μ m hydrophobic membrane (Tianjin Heng'ao Technological Industry Group Ltd, Tianjin, China). The resulting filtrates were diluted to the desired concentration range in acetonitrile, and 1- μ L aliquots were injected into the LC-MS/MS system for analysis. Each log *P* value reported was the average of triplicate measurements. The *n*-octanol/water partition coefficient was calculated using Eq (1),

$$P = \frac{C_o}{C_w} \quad (1)$$

Here, C_o (μ g/mL) represents the concentration of imperialine in the organic phase, and C_w (μ g/mL) represents the concentration of imperialine in the aqueous phase.

The log *P* was plotted against the pH, and the linear regression equation and correlation coefficient were calculated using a weighted ($1/x^2$) least-squares linear regression method.

Measurement of the ionization constant

The pK_a can be measured spectrophotometrically using an acid-base indicator (the spectrophotometric indicator method), which yields accurate results using equipment available in

chemistry labs. In the current study, we chose *p*-nitrophenol as the indicator. The method was validated in this study by determining the pK_a of *p*-nitrophenol and comparing the results to those previously reported (Supplementary Information S1).

Imperialine (B) solutions with different concentrations were prepared in triplicate, and 5×10^{-5} mol/L hydrochloride was then added to the solution to partially form imperialine hydrochloride (S). Then, *p*-nitrophenol was added to each solution to yield a final concentration of 5×10^{-5} mol/L. The absorbance was obtained at the selected wavelength (λ) of 401 nm using water as a blank. The pK_a of imperialine was calculated using Eq (2).

$$pK_a^T = pK_a^T(\text{Int}) - \lg \frac{[B]}{[S]} + \lg \frac{A-A_1}{A_2-A} + 2 \lg \gamma \quad (2)$$

The γ was calculated according to the Debye-Huckel Formula, Eq (3),

$$\lg \gamma = - \frac{0.5115 Z^2 \sqrt{I}}{1 + 1.5 \sqrt{I}} \quad (3)$$

where *I* represents the ionic strength of the solution, and *Z* represents the charge number of the ion.

Cell culture

The Caco-2 cell line, originating from the American Type Culture Collection, was obtained from Nanjing KeyGen Biotech Co Ltd (Nanjing, China). The cells were grown routinely in 25-cm² cell culture flasks (Catalog number: 430639; Corning Incorporated, Corning, NY, USA) in DMEM supplemented with 10% (*v/v*) FBS, 1% (*v/v*) non-essential amino acid solution, 1% (*m/v*) L-glutamine and 1% (*v/v*) penicillin-streptomycin (10 000 units/mL penicillin; 10 000 μ g/mL streptomycin) in a CO₂ incubator (MCO-15AC, SANYO, Japan) with an atmosphere of 5% CO₂, a temperature of 37°C and a relative humidity of 90%. The medium was replaced every other day after incubation. When the cells reached 80%–90% confluence, they were passaged at a ratio of 1:4 using 0.25% trypsin-0.02% EDTA.

For the uptake experiments, the cells were seeded at a density of 5×10^4 cells/cm² with 1 mL DMEM medium in 12-well tissue culture plates (Catalog No: 3415, Corning Incorporated, Corning, NY, USA)^[18]. The medium in each well was replaced every other day for the first week after seeding, after which it was replaced every day. Cells with an integrated cell-growing surface 15 d post-seeding were used for the uptake experiments.

For the transport experiments, the cells were seeded at a density of 5×10^4 cells/cm² with 0.5 mL DMEM medium onto the inserts of 12-well Costar Transwell® plates (Catalog No: 3460, Corning Incorporated, Corning, NY, USA)^[18], where the insert possessed a polyester (PET) membrane with a pore size of 0.4 μ m, a membrane area of 1.12 cm² and a nominal pore density of 4×10^6 pores/cm². The media in the insert (apical side, 0.5 mL) and in the well (basolateral side, 1.5 mL) were changed every other day for the first week after seed-

ing and then changed every day. The Caco-2 cell monolayers were used for experiments 21 d after seeding. Cells passaged between 30 and 34 times were used. The quality of the monolayers grown on the PET membranes was assessed by the trans-epithelial electrical resistance (TEER) of the monolayer and detected with a Millicell-ERS apparatus (ERS0001, Millipore, USA) at 37°C. Only those monolayers possessing TEER values higher than 1000 $\Omega \cdot \text{cm}^2$ were used.

Imperialine uptake and transport studies

Preparation of the test solutions

Imperialine was first dissolved in DMSO as a stock solution and then diluted in HBSS (pH 7.4) to yield final concentrations of 1, 2, 5, 10, 20, 50, and 100 $\mu\text{g}/\text{mL}$. For the pH course test, the stock solution was diluted in HBSS at different pH values (5.5, 6.0, 6.5, 7.0, and 7.4) to obtain a nominal concentration of 10 $\mu\text{g}/\text{mL}$. For the inhibition studies, the stock solution was diluted in HBSS containing verapamil (20 $\mu\text{g}/\text{mL}$, P-gp inhibitor) or Ezetimibe (20 $\mu\text{g}/\text{mL}$, NPC₁L₁ inhibitor) to yield final imperialine concentrations of 2, 10, and 50 $\mu\text{g}/\text{mL}$. The final DMSO concentration did not exceed 1%. The imperialine in HBSS was validated to be stable in cell tests (Supplementary Information S2).

Caco-2 cell uptake study

The uptake of imperialine by the Caco-2 cells was studied in wells containing cells that had been cultured for 15 d after seeding. The growing cell surface was gently washed twice with HBSS (pH 7.4). Afterward, the wells were preincubated with HBSS at 37°C in a thermostated shaking incubator for 20 min^[19]. For the inhibition studies, the wells were preincubated in HBSS containing 20 $\mu\text{g}/\text{mL}$ verapamil at 37°C for 20 min. Each value obtained in this section was an average of triplicate measurements.

Time course of the imperialine uptake

To measure the imperialine uptake, 1 mL HBSS containing 10 $\mu\text{g}/\text{mL}$ imperialine was added to the cells in the wells. The wells were then incubated at 37°C for 0.5, 1, 1.5, or 2 h. After the incubation period, the drug-containing HBSS was removed and 4°C HBSS was added to terminate cell uptake. The cell surface was quickly washed three times with HBSS. After the addition of normal saline (NS, 0.9% NaCl), the cells were lysed by repeated freeze-thaw cycles until the cells were completely ruptured. The ruptured cells were spun down and collected. Another aliquot of NS was added to wash the well surfaces, and the ruptured cell-containing NS was collected to obtain a ruptured cell suspension. Some of the suspension, after appropriate dilution by NS, was pretreated by adding a 4-fold volume of acetonitrile for protein precipitation. An aliquot of 1 μL supernatant was injected into the LC-MS/MS system for the determination of imperialine after centrifugation (12000 \times g for 10 min) and filtration (through a 0.22- μm hydrophobic membrane). Another part of the suspension was used for protein determination with the Bradford Method Protein Assay Kit (Catalog No: KGA803, Nanjing KeyGEN Biotech Co Ltd,

China).

The effect of pH on imperialine uptake

Samples containing 1 mL of 10 $\mu\text{g}/\text{mL}$ imperialine-HBSS at different pH values (5.5, 6.0, 6.5, 7.0, and 7.4) were added to preincubated cell wells. The wells were then incubated at 37°C for 1 h. The remaining post-treatment was performed as described in the section Time course of imperialine uptake.

The effect of concentration on imperialine uptake

Aliquots (1 mL HBSS) containing imperialine at different concentrations (1, 2, 5, 10, 20, 50, and 100 $\mu\text{g}/\text{mL}$) were added to the preincubated cell wells. The wells were then incubated at 37°C for 1 h. The post-treatment was performed as described in the section Time course of imperialine uptake.

The effect of temperature on imperialine uptake

1 mL of HBSS containing imperialine at different concentrations (2, 10, and 50 $\mu\text{g}/\text{mL}$) was added to the preincubated cell wells. The wells with different imperialine concentrations were then respectively incubated at 4, 25, and 37°C for 1 h. The post-treatment was performed as described in the section Time course of imperialine uptake.

The effect of the P-gp inhibitor on imperialine uptake

Treatments of 1 mL imperialine (2, 10, and 50 $\mu\text{g}/\text{mL}$)-HBSS (containing 20 $\mu\text{g}/\text{mL}$ verapamil) were added to the preincubated cell wells. The wells were then incubated at 37°C for 1 h. The post-treatment was performed as described in the section Time course of imperialine uptake.

The effect of the NPC1L1 inhibitor on imperialine uptake

Treatments of 1 mL imperialine (2, 10, and 50 $\mu\text{g}/\text{mL}$)-HBSS (containing 20 $\mu\text{g}/\text{mL}$ Ezetimibe) were added to the preincubated cell wells. The wells were then incubated at 37°C for 1 h. The post-treatment was performed as described in the section Time course of imperialine uptake.

Caco-2 cell transport study

The transport of imperialine across the Caco-2 cell monolayers was studied using 21-d post-seeding monolayers as described in the section Cell Culture. The monolayers were gently washed twice with HBSS (pH 7.4). After washed, the monolayers were preincubated in HBSS, both on the apical (AP) and on the basolateral (BL) sides, at 37°C in a thermostated shaking incubator for 20 min^[18]. For the P-gp inhibition study, the monolayers were preincubated in HBSS containing 20 $\mu\text{g}/\text{mL}$ Verapamil (in the donor chamber) and HBSS (in the receptor chamber); for the NPC₁L₁ transporter inhibition, the monolayers were preincubated in HBSS containing 20 $\mu\text{g}/\text{mL}$ Ezetimibe (in the donor chamber) and HBSS (in the receptor chamber). Each value obtained in this section is the average of triplicate measurements.

The effect of the apical pH on imperialine transport

Aliquots of 0.5 mL HBSS (pH 5.5, 6.0, 6.5, 7.0, and 7.4) con-

taining 10 µg/mL imperialine were added to the Transwell® AP side, and 1.5 mL HBSS (pH 7.4) was added to the BL side. The monolayers were incubated at 37°C and placed in a thermostated shaking incubator at a speed of 60 r/min during the transport process to minimize the influence of the aqueous boundary layer. Samples were taken from the receptor chamber (BL side) at 0, 30, 60, 90, and 120 min, followed by immediate replacement with the same volume of 37°C fresh HBSS. After appropriate dilution by HBSS, a 4-fold volume of acetonitrile was added. The samples were then thoroughly vortexed, and a 1 µL aliquot of the test solution (after filtration through a 0.22-µm hydrophobic membrane) was injected into the LC-MS/MS system for the measurement of imperialine.

The effect of concentration on imperialine transport

To measure the AP to BL transport, 0.5 mL HBSS containing imperialine at different concentrations (1, 2, 5, 10, 20, 50, and 100 µg/mL) was added to the AP side, and 1.5 mL HBSS was added to the BL side. The monolayers were incubated at 37°C and placed in a thermostated shaking incubator at a speed of 60 r/min during the transport process. Samples were collected from the receptor chamber (BL side) at 0, 30, 60, 90, and 120 min, followed by immediate replacement with the same volume of 37°C fresh HBSS.

For the measurement of BL to AP transport, 1.5 mL HBSS containing imperialine at different concentrations (1, 2, 5, 10, 20, 50, and 100 µg/mL) was added to the BL side, and 0.5 mL blank HBSS was added to the AP side. The monolayers were incubated at 37°C and placed in a thermostated shaking incubator at a speed of 60 r/min during the transport process. Samples were collected from the donor chamber (AP side) at 0, 30, 60, 90, and 120 min, followed by immediate replacement with the same volume of 37°C fresh HBSS. The pretreatment of samples prior to LC-MS/MS was performed as described in the section *Effect of apical pH on imperialine transport*. The transport rate was plotted against the imperialine concentration in HBSS, and the linear regression equation and correlation coefficient were calculated using a weighted ($1/x^2$) least-squares linear regression method.

The effect of the P-gp inhibitor on imperialine transport

For the measurement of AP to BL transport, 0.5 mL imperialine (2, 10, and 50 µg/mL)-HBSS (containing 20 µg/mL Verapamil) was added to the AP side, and 1.5 mL blank HBSS was added to the BL side. For the measurement of BL to AP transport, 1.5 mL imperialine (2, 10, and 50 µg/mL)-HBSS (containing 20 µg/mL verapamil) was added to the BL side, and 0.5 mL blank HBSS was added to the AP side. The post-treatment was performed as described in the section *Effect of concentration on imperialine transport*.

The effect of the NPC₁L₁ inhibitor on imperialine transport

For the measurement of AP to BL transport, 0.5 mL imperialine (2, 10, and 50 µg/mL)-HBSS (containing 20 µg/mL Ezetimibe) was added to the AP side, and 1.5 mL blank HBSS was added to the BL side. For the measurement of BL to AP trans-

port, 1.5 mL imperialine (2, 10, and 50 µg/mL)-HBSS (containing 20 µg/mL verapamil) was added to the BL side, and 0.5 mL blank HBSS was added to the AP side. The post-treatment was performed as described in the section *Effect of concentration on imperialine transport*.

Data analysis

The Caco-2 cell uptake (mg/g) was calculated using Eq (4),

$$\text{Caco-2 cell uptake (mg/kg)} = \frac{\text{Imperialine uptake (mg)}}{\text{Protein content (g)}} \quad (4)$$

The apparent permeability coefficient (P_{app} , cm/s) was calculated using Eq (5),

$$P_{\text{app}} = \frac{dQ/dt}{AC_0} \quad (5)$$

where dQ/dt (µg/s) is the drug permeation rate, A is the cross sectional area (1.12 cm²), and C_0 (µg/mL) is the initial imperialine concentration in the donor compartment.

To compensate for the dilution effect caused by fluid replacement after sampling, the cumulative transport flux TR_{cum} (µg/mL) was revised using Eq (6).

$$TR_{\text{cum}} = A_n + \frac{V_{\text{Sn}}}{V_{\text{R}}} \sum_{i=0}^{n-1} A_i \quad (6)$$

where A_n and A_i (µg) represent the detected transport flux of the n th and i th sample, respectively; V_{Sn} (mL) represents the sampling volume of the n th sample, and V_{R} (mL) is the total volume of HBSS in the receptor chamber.

If necessary, the two groups of data could be analyzed by a two-tailed t -test to investigate whether they exhibited significant differences.

Single-pass intestinal perfusion in rats

A prediction of the absorption behavior of imperialine in the human intestine is essential for the preformulation. *In vitro* assays in Caco-2 cells are routinely used for this purpose. However, *in situ* techniques combined with *in vitro* models might provide a more accurate estimation of intestinal absorption of imperialine. In the current study, we evaluated a modified gravimetric method of single-pass intestinal perfusion (SPIP) in the rat. The effective permeability coefficients (P_{eff}) for imperialine were therefore determined in rats.

Preparation of working perfusates

The intestinal perfusion solution was freshly prepared from a Krebs-Ringer (K-R) solution (containing 7.8 g/L NaCl, 0.35 g/L KCl, 0.37 g/L CaCl₂, 1.37 g/L NaHCO₃, 0.32 g/L NaH₂PO₄, 0.02 g/L MgCl₂, and 1.4 g/L glucose) with a certain amount of imperialine stock solution. Perfusates containing imperialine at different concentrations (1, 5, or 10 µg/mL) were thus obtained. For the P-gp inhibition study, 10 µg/mL verapamil was added to perfusate containing 5 µg/mL imperialine. For the NPC₁L₁ inhibition study, 10 µg/mL Ezetimibe was added to the imperialine (5 µg/mL)-containing perfusate. The imperialine in the working solutions was determined to

be sufficiently stable and thus reasonable for use after incubation with an intestinal segment for SPIP (Supplementary Information S2).

Animals and surgery

The study protocol was approved by the Animal Ethics Committee of Sichuan University (Chengdu, China). Male Sprague-Dawley rats (200±20 g) were obtained from the Experimental Animal Center of West China, Sichuan University.

The rats were raised in a controlled environment with a temperature of 23–26 °C, a relative humidity of 40%–60% and a noise level below 60 dB. Light and dark cycles were matched to the rats' natural circadian rhythms for at least 1 week to facilitate their adaptation to the housing conditions. The animals were fasted for 12 h before surgery, but water was always available.

For surgery, the rats were anesthetized and placed on a warmed pad to maintain normal body temperature under a heat lamp. A midline incision was made in the abdomen. Except for the NPC_{1L1} inhibition study, a small intestinal (duodenal, jejunal or ileal) segment of approximately 10 cm or a colonic segment of approximately 5 cm was isolated; for the NPC_{1L1} inhibition study, only jejunal and colonic segments were isolated. The final length of each segment was normalized. Semi-circular incisions were made at each end of the segment, and the lumen was rinsed with NS prewarmed to 37 °C. Both ends were cannulated using rubber tubing with an inner diameter of 2.08 mm, and then ligated.

The perfusates were infused for 5 min at a flow rate of 1 mL/min using a constant-current peristaltic pump (model number: HL-2, Shanghai Qingpu-Huxi Instrument Factory, Shanghai, China). The intestinal segments were then perfused with a test solution at 0.2 mL/min for 30 min to achieve absorption equilibrium and stable outflow rates. Then, a known-mass EP tube with a certain volume of perfusate (containing 1, 5, or 10 µg/mL imperialine) was placed at the inflow point, initiating the intestinal perfusion at a constant flow rate of 0.2 mL/min. At the other end, the outflow was gathered by an empty EP tube of known mass. Every 10 min, the EP tubes at the inflow and outflow points were simultaneously replaced by brand-new ones with or without perfusate, after which the former ones were weighed to calculate the mass of inflow and outflow. An aliquot of 0.5 mL perfusate was spiked to weigh the gain in density (ρ) of the in/out perfusate. The final perfusion was performed for a total of 60 min. After that, the tested segment was cut off and placed in NS without any stretch or crimp. The length (*l*) and width ($2\pi r$, *r* is the inner diameter) of the segments were measured. After the inflow and outflow were diluted to the desired concentration range, a 4-fold volume of acetonitrile was added and the samples were vortexed throughout. After filtration through a 0.22-µm hydrophobic membrane, an aliquot of 1 µL test solution was injected into the LC-MS/MS system for the determination of imperialine.

The value (for each segment and each perfusate with different concentrations) obtained in this section was the average of

at least five rats.

Data analysis

Because the intestine absorbs not only imperialine but also moisture, the volume of inflow perfusate (Q_{in}) and outflow solution (Q_{out}) was revised as given by Eq (7) and Eq (8), respectively,

$$Q_{in} = \frac{M_{in}}{\rho_{in}} \quad (7)$$

$$Q_{out} = \frac{M_{out}}{\rho_{out}} \quad (8)$$

where M_{in} (g) and M_{out} (g) represent the masses of the inflow and outflow perfusates, respectively, and ρ_{in} (g/mL) and ρ_{out} (g/mL) represent the densities of the inflow and outflow perfusates, respectively.

The corrected concentration of imperialine [$C_{out (corrected)}$, µg/mL] was calculated as Eq (9),

$$C_{out (corrected)} = \frac{C_{out} Q_{out}}{Q_{in}} \quad (9)$$

where Q_{in} (mL) was the measured flow entering the intestine and Q_{out} (mL) was the measured perfusate exit flow (for the specified time interval).

The effective permeability coefficient (P_{eff} , cm/min), absorption rate constant (K_a , min⁻¹) and intestinal absorption amount (M_a , µg/cm²) were calculated using Eq (10), Eq (11), and Eq (12), respectively,

$$P_{eff} = \frac{Q_{out} \left[\frac{C_{in}}{C_{out (corrected)}} \right]}{2\pi r l} \quad (10)$$

$$K_a = \frac{\left[1 - \frac{C_{out (corrected)}}{C_{in}} \right] Q}{\pi r^2 l} \quad (11)$$

$$M_a = \frac{Q [C_{in} - C_{out (corrected)}]}{2\pi r l} \quad (12)$$

where C_{in} (µg/mL) and $C_{out (corrected)}$ (µg/mL) correspond to the inlet and outlet (corrected for volume changes) imperialine concentrations, respectively, while *r* (cm) is the intestinal radius and *l* (cm) is the length of the isolated segment. *Q* (0.2 mL/min) is the flow rate through the intestinal segment.

If necessary, a two-tailed *t*-test was applied to investigate the statistical difference between two groups of data.

Results

Physicochemical properties of imperialine

The precise pH values of the water and PBS solutions were measured as 2.00, 3.00, 4.03, 5.02, 5.82, 6.48, 6.80, 7.00, 7.39, 7.80, and 8.50. The apparent solubility of imperialine decreased as the pH increased (Figure 2A). The ratio of the ionized form to the non-ionized form decreased as the pH increased.

The apparent *n*-octanol/water partition coefficient (*P*) of imperialine increased with increasing pH (Figure 2B). The obtained linear equation of log*P* against the pH of PBS was

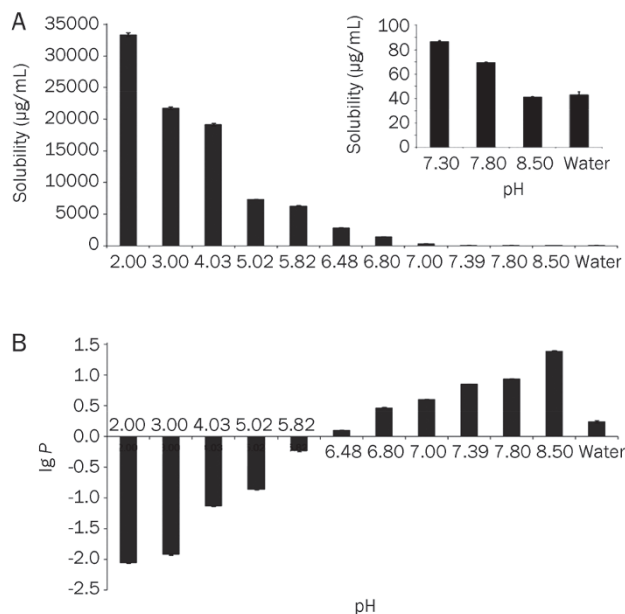


Figure 2. (A) Apparent solubility-pH curve of imperialine ($n=3$); (B) Partition coefficient-pH curve of imperialine ($n=3$).

$y=0.5653x-3.4438$, $r^2=0.9943$, where y represented $\log P$, x represented the pH and r represented the correlation coefficient. At $\log P=0$, the pH was 6.09, which suggested that imperialine behaved better in terms of solubility in the aqueous phase when the pH was <6.09 , while it exerted better solubility in the oil phase when the pH >6.09 . Its behavior was consistent with that determined in the section *Equilibrium solubility of imperialine*.

The measurements are summarized in Table 1. The calculation was carried out using Eq (2), and the pK_a of imperialine was determined to be 8.467 ± 0.028 . This indicates that imperialine was alkaline, which is consistent with the presence of a tertiary amino group in its molecular structure.

Table 1. Measurements for pK_a of imperialine^a. All results are the mean of measurements from three replicates.

$[B]\times 10^{-4}$	$[S]\times 10^{-5}$	$[B]/[S]$	A	pK_a^T
0.5218	5.0000	1.0436	0.9653 ± 0.0003	8.467 ± 0.028
0.4196	5.0000	0.8392	0.9493 ± 0.0006	
0.3174	5.0000	0.63489	0.9424 ± 0.0002	
0.2152	5.0000	0.4305	0.9128 ± 0.0002	
0.1131	5.0000	0.2261	0.8351 ± 0.0001	

^a $A_1=0.0282$, $A_2=0.9170$, $\lambda=401$ nm.

Uptake characteristics of imperialine in the Caco-2 cells

Time course of uptake

The uptake of imperialine (10 $\mu\text{g/mL}$) in the Caco-2 cell at 0.5, 1, 1.5, and 2 h is displayed in Figure 3A. Among the testing time plots, the Caco-2 cells took up imperialine to the great-

est extent at 1 h. Therefore, 1 h was selected as the incubation time for the following uptake experiment.

The effect of pH on uptake

As shown in Figure 3B, the Caco-2 cell uptake of imperialine (10 $\mu\text{g/mL}$) from HBSS at pH values of 5.5, 6.0, 6.5, 7.0, and 7.4 was tested. The values obtained were 0.17 ± 0.03 , 0.26 ± 0.03 , 0.48 ± 0.06 , 0.64 ± 0.06 , and 1.06 ± 0.03 mg/g protein, respectively. The results for the groups (pH 5.5, 6.0, 6.5, and 7.0) were compared to the results for pH 7.4, and all exhibited significant differences ($P<0.001$). Therefore, the pH obviously affected the Caco-2 cell uptake of imperialine. Because the greatest uptake occurred at pH 7.4, a pH of 7.4 was selected as the optimal pH.

The effect of concentration on uptake

We measured the Caco-2 cell uptake of imperialine in HBSS at different imperialine concentrations (1, 2, 5, 10, 20, 50, and 100 $\mu\text{g/mL}$). The results (Figure 3) show that the uptake of imperialine increased with increasing concentration in a linear manner. The linear regression equation was $y=0.0803x+0.0459$, $r^2=0.9982$, where y represents the uptake amount (mg/g), x represents the concentration of imperialine ($\mu\text{g/mL}$), and r represents the correlation coefficient. As the results showed, the imperialine uptake did not behave like a saturation phenomenon. This suggests that imperialine may have been taken up mainly by passive membrane diffusion.

The effect of temperature on uptake

We investigated the uptake of imperialine (at concentrations of 2, 10, and 50 $\mu\text{g/mL}$) at different temperatures (4, 25, or 37°C). The results are shown in Figure 3D. Significant differences were observed between groups with the same imperialine concentration but different temperatures ($P<0.05$). In addition, imperialine was taken up by the Caco-2 cells at 4°C. Although significant differences existed, we believed they were due to the enhanced drug movement and increased membrane fluidity that were physically caused by the temperature increase, instead of the energy-dependence of the process itself.

The effect of the P-gp and NPC₁L₁ inhibitor on uptake

In the current study, the P-gp inhibitor (20 $\mu\text{g/mL}$ verapamil) or NPC₁L₁ transporter inhibitor (20 $\mu\text{g/mL}$ Ezetimibe) was added in the Caco-2 cell uptake studies, and the results are shown in Figure 3E. However, similar uptake was observed in the presence of either the P-gp inhibitor or the NPC₁L₁ transporter inhibitor and in their absence. This finding implied that P-gp was not involved in the efflux of imperialine and that the NPC₁L₁ transporter did not participate in the absorption.

Transport characteristics of imperialine in Caco-2 cells

The effect of pH on transport

The varying pH in the apical-side HBSS significantly affected the imperialine transport flux from the apical (AP) to the basolateral (BL) side, as observed in Figure 4A. The cumulative transport was greatest when the apical pH was 7.4. This result suggested that when imperialine was added to apical HBSS, a

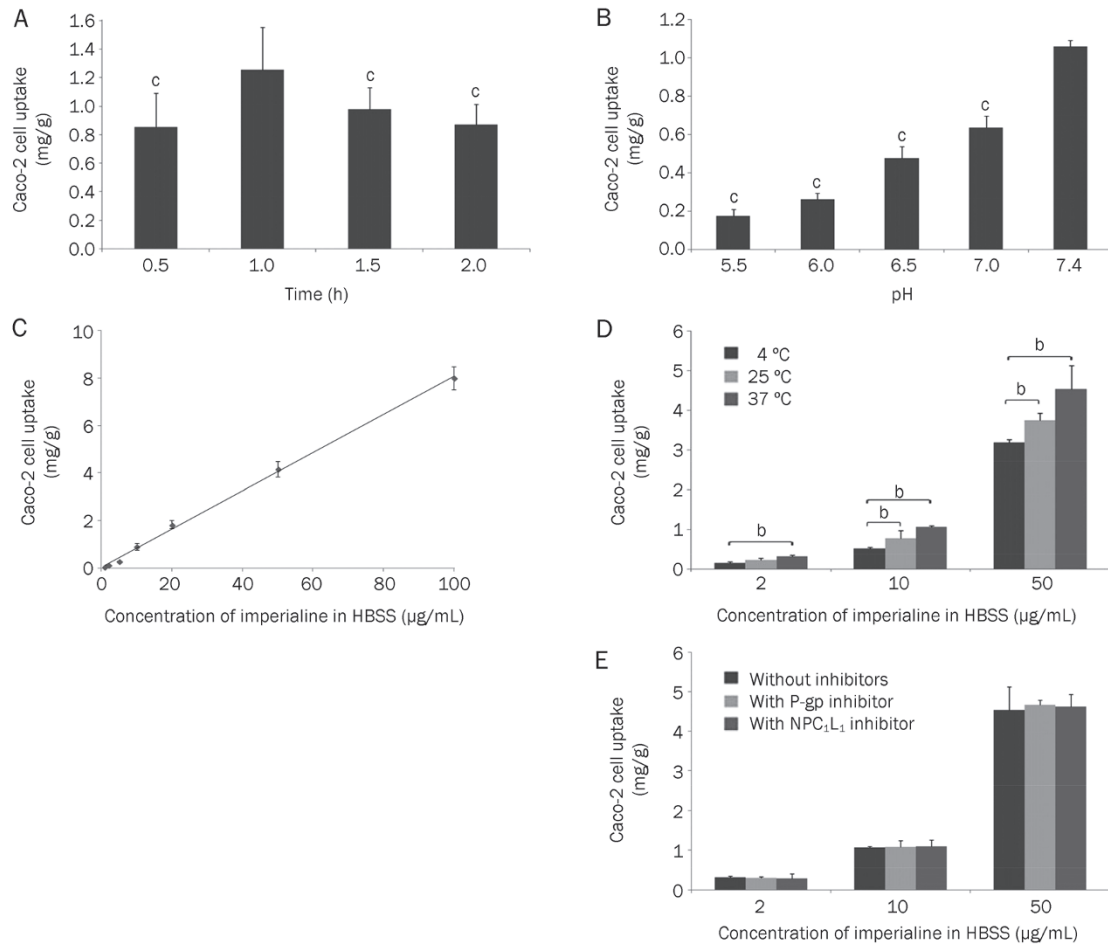


Figure 3. (A) The time course of Caco-2 cell uptake of imperialine ($n=3$): $^{\circ}P<0.01$, compared with 1 h; (B) The effect of pH on imperialine in Caco-2 cell uptake ($n=3$). $^{\circ}P<0.01$, compared with the pH 7.4 group; (C) The effect of concentration on imperialine in Caco-2 cell uptake ($n=3$); (D) The effect of temperature on imperialine in Caco-2 cell uptake ($n=3$): $^bP<0.05$; (E) The effect of the P-gp or NPC₁L₁ transporter inhibitor on Caco-2 cell uptake ($n=3$).

pH of 7.4 was optimal for the following transport experiments.

The effect of concentration on transport

We measured the absorption (AP→BL side) and secretion (BL→AP side) of imperialine in the Caco-2 cell monolayers, and the resulting data are summarized in Table 2. The transport rate was plotted against the imperialine concentration

Table 2. Transport parameters of imperialine. Mean±SD. $n=3$.

Concentration (µg/mL)	Apical to basolateral side		Basolateral to apical side	
	P_{app} ($\times 10^{-6}$ cm/s)	Transport rate (ng $\text{cm}^{-2}\cdot\text{min}^{-1}$)	P_{app} ($\times 10^{-6}$ cm/s)	Transport rate (ng $\text{cm}^{-2}\cdot\text{min}^{-1}$)
1	8.42±0.14	0.505±0.171	7.87±1.28	0.472±0.077
2	8.23±0.48	0.988±0.057	7.72±0.45	0.907±0.050
5	8.50±0.36	2.550±0.109	7.70±0.30	2.309±0.090
10	8.22±0.10	4.934±0.062	7.63±0.22	4.451±0.240
20	8.44±0.93	9.054±1.118	7.87±0.15	9.446±0.177
50	8.47±0.42	24.776±1.265	7.81±0.26	23.435±0.784
100	8.49±1.32	54.448±8.294	7.84±0.66	47.033±3.987

in HBSS (Figure 4B), and P_{app} was plotted against concentration (Figure 4C). In the concentration range of 1–100 µg/mL, both the absorption and the secretion of imperialine were well described by a straight line. The linear regression equation for absorption (AP→BL) was $y=0.5409x-0.6348$, $r^2=0.9975$, while the equation for secretion (BL→AP) was $y=0.4708x-0.0655$, $r^2=0.9999$. Here, y represents the uptake amount (mg/g), x represents the concentration of imperialine (µg/mL) and r represents the correlation coefficient. The P_{app} of absorption or secretion was basically flat in this concentration range, with an absorptive P_{app} of $(8.39\pm 0.12)\times 10^{-6}$ cm/s and a secretive P_{app} of $(7.78\pm 0.09)\times 10^{-6}$ cm/s. All results in this section corresponded to those in the Caco-2 cell uptake studies.

The effect of P-gp and the NPC₁L₁ inhibitor on transport

The effects of verapamil and Ezetimibe on imperialine absorption and secretion were studied. The addition of verapamil or Ezetimibe had no significant influence on either the absorption or the secretion (Table 3). This result further suggested that the transport of imperialine across the Caco-2 cell monolayers was not regulated by the P-gp or NPC₁L₁ transporters.

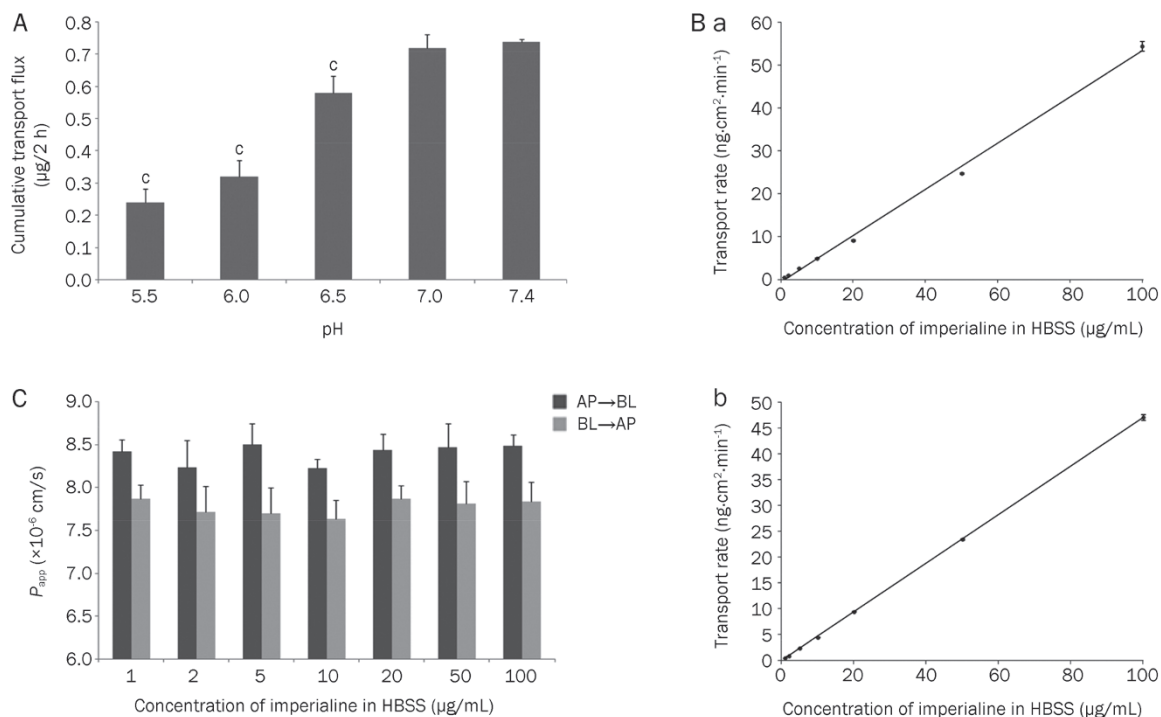


Figure 4. (A) The effect of pH on imperialine in Caco-2 cell monolayer transport ($n=3$): $^{\circ}P<0.01$ compared with the pH 7.4 group. (B) The transport rate of imperialine across the Caco-2 cell monolayer ($n=3$): (a) from the AP to the BL side and (b) from the BL to the AP side. (C) P_{app} of imperialine across the Caco-2 cell monolayers ($n=3$).

Table 3. Effect of P-gp and NPC1L1 inhibitors on the transport of imperialine across Caco-2 cell monolayers. All results are the mean of measurements from three replicates. a Mean \pm SD. bP , two-tailed t-test.

C ($\mu\text{g/mL}$)	AP \rightarrow BL P_{app} ($\times 10^{-6}$ cm/s)					BL \rightarrow AP P_{app} ($\times 10^{-6}$ cm/s)				
	Non a	Ver a	P^b	Eze a	P^b	Non a	Ver a	P^b	Eze a	P^b
2	8.81 \pm 0.81	8.23 \pm 0.48	0.35	8.98 \pm 0.45	0.36	7.72 \pm 0.45	6.70 \pm 0.83	0.18	7.72 \pm 0.92	0.80
10	8.40 \pm 0.73	8.22 \pm 0.10	0.31	8.50 \pm 0.91	0.31	7.63 \pm 0.22	7.76 \pm 0.69	0.50	7.35 \pm 0.75	0.90
50	8.19 \pm 0.32	8.47 \pm 0.42	0.60	8.22 \pm 0.11	0.66	7.81 \pm 0.26	7.38 \pm 0.42	0.20	7.60 \pm 0.56	0.58

Abbreviations: Ver, for verapamil; Eze, for Ezetimibe.

Absorptive characteristics of imperialine in rats

The modified gravimetric method was applied in a single-pass intestinal perfusion model. The cumulative absorption of imperialine (1, 5, and 10 $\mu\text{g/mL}$) in different intestinal segments is illustrated in Figure 5A, and a series of parameters obtained in various segments are summarized in Table 4. The results obtained showed that with increasing concentrations, the absorption of imperialine increased, and the saturation was not manifested at high concentrations. The imperialine absorption in the different segments varied with a constant imperialine concentration, with the colon displaying the highest level of absorption. In the same intestinal segments, the P_{eff} or K_a was basically consistent ($P>0.05$) when imperialine concentration varied. This supported the presupposition that passive diffusion was the dominant process in imperialine absorption. In addition, adding either the P-gp or the NPC₁L₁ inhibitor did not exert significant differences over absorption

without inhibitors (Figure 5B and 5C).

Discussion

The oral bioavailability of imperialine has been previously reported to be relatively high^[4]. Based on this observation, the compound's biopharmaceutical features were investigated. To clarify the relationship between the molecular properties and the absorption characteristics of imperialine, we determined the compound's physicochemical properties, including its solubility, oil/water partition coefficient and ionization constant. In addition, the *in vitro* uptake and transport mechanisms were also investigated using Caco-2 cells, and *in situ* single-pass intestinal perfusion was performed on intestinal segments.

In the current study, we quantitatively proved imperialine's aqueous solubility and oil/water partition coefficient in pure water and PBS at different pH values. The compound demon-

Table 4. Absorption parameters of imperialine in each intestinal segment. All results are the mean of measurements from five animals. Values are expressed as the mean \pm SD.

C ($\mu\text{g/mL}$)	Segment	Absorption parameters		
		P_{eff} ($\times 10^{-3}$ cm/min)	K_a ($\times 10^{-2}$ /min)	M_a ($\mu\text{g/cm}^2$)
1	Duodenum	3.68 \pm 0.81	5.19 \pm 0.92	0.28 \pm 0.06
	Jejunum	3.63 \pm 0.44	4.84 \pm 0.92	0.26 \pm 0.08
	Ileum	8.31 \pm 1.10	9.51 \pm 0.64	0.43 \pm 0.10
	Colon	15.13 \pm 1.48	17.44 \pm 2.94	0.79 \pm 0.02
5	Duodenum	3.89 \pm 0.75	4.62 \pm 1.26	1.51 \pm 0.40
	Jejunum	2.91 \pm 0.53	3.22 \pm 0.58	1.05 \pm 0.14
	Ileum	8.54 \pm 0.52	9.39 \pm 1.82	2.22 \pm 0.28
	Colon	14.00 \pm 1.64	16.58 \pm 1.65	3.22 \pm 0.34
10	Duodenum	4.41 \pm 0.69	6.51 \pm 1.95	2.22 \pm 0.74
	Jejunum	3.84 \pm 0.95	4.90 \pm 0.66	3.66 \pm 0.51
	Ileum	9.53 \pm 0.93	10.68 \pm 1.27	3.83 \pm 0.45
	Colon	15.16 \pm 2.79	10.68 \pm 1.27	7.65 \pm 0.22

strated a relatively weak alkalinity, with a pK_a of 8.467 ± 0.028 .

Furthermore, the uptake and transepithelial transport of imperialine in the Caco-2 cell model exhibited a pH-dependent effect. The main reason for this result was that most of the imperialine existing under physiological conditions, *ie*, at pH 7 or above, was in the non-ionized form, which would enhance its absorption despite the relatively low solubility of the compound. In other words, the permeability was more important than the solubility in this case. In addition, we observed that in the Caco-2 cells, both uptake and transport were concentration-dependent without saturation phenomenon in the selected concentration range, implying that passive membrane diffusion was the main mode of transport. The absorptive P_{app} was measured as $(8.39\pm 0.12)\times 10^{-6}$ cm/s and the secretory P_{app} was $(7.78\pm 0.09)\times 10^{-6}$ cm/s, which was above the recognized threshold value of 2×10^{-6} cm/s for completely absorbed drugs in Caco-2 monolayers^[19, 20], and was consistent with the relatively high bioavailability of imperialine noted in a previous pharmacokinetic study^[4]. As with the former result, the

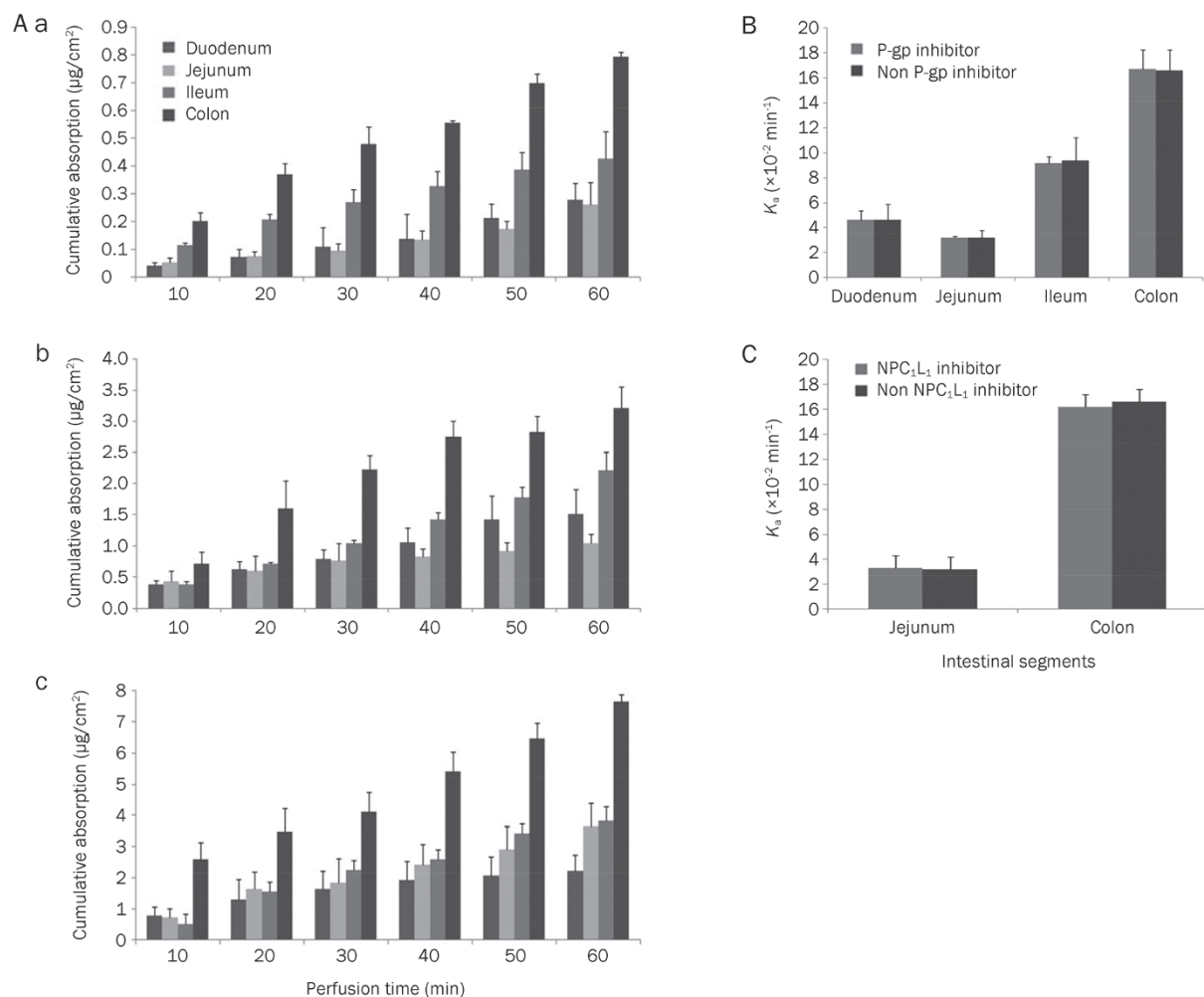


Figure 5. (A) Cumulative absorption of different concentrations of imperialine in rat intestinal segments ($n=5$). The concentration of imperialine in the perfusate was (a) 1 $\mu\text{g/mL}$, (b) 5 $\mu\text{g/mL}$, and (c) 10 $\mu\text{g/mL}$. (B) P-gp inhibition study in rat intestinal segments ($n=5$); (C) NPC₁L₁ inhibition study in rat intestinal segments ($n=5$).

inhibitory studies suggested that neither P-gp nor NPC₁L₁ was involved in the transport of imperialine. Because Verapamil is a known inhibitor of P-gp and Ezetimibe is a known inhibitor of NPC₁L₁, they did not limit the oral absorption of imperialine. The isomer of imperialine, peiminine (*ie*, verticinone), was also found to be non-P-gp-regulated^[21]. As the substrate always showed structural similarity, we firmly believed this negative result.

To further investigate the absorptive characteristics of imperialine, we performed single-pass intestinal perfusion assays combined with a modified gravimetric method in an *in situ* model. Consistent with the results obtained using Caco-2 cells *in vitro*, in a certain segment, both P_{eff} and K_a were basically consistent, supporting the conclusion that passive diffusion was the dominant process for imperialine absorption. For a given concentration, the absorption differed among the segments, with the colon absorbing the most imperialine. This may be due to the higher pH (approximately 7.4) in the colon than in the other segments, which increases the non-ionized form of imperialine and thereby enhances the imperialine permeability across the intestinal mucosa. The inhibition study again supported the prediction that imperialine was not the substrate of P-gp and that steroid compounds would not affect the absorption of imperialine through NPC₁L₁.

Conclusion

We have investigated the biopharmaceutical properties of imperialine, from its physicochemical properties to its *in vitro* and *in situ* behavior. The results presented in the current study showed that the pH of the medium affects the uptake and transport of imperialine, although the temperature does not. Furthermore, passive membrane diffusion dominated the absorptive and transport behavior of imperialine. Neither absorption nor secretion was mediated by P-gp or NPC₁L₁. These results provide a better understanding of the intestinal absorption of imperialine.

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Author contribution

Zhi-rong ZHANG and Qing LIN designed the research; Qing LIN, Li-qin LING, and Ling GUO performed the research; Zhi-rong ZHANG, Tao GONG, and Xun SUN contributed new reagents or analytic tools; Qing LIN and Ling GUO analyzed the data; and Qing LIN wrote the paper.

Supplementary information

Supplementary information is available at the *Acta Pharmacologica Sinica*'s website.

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