A Mechanistic Study of the Intestinal Absorption of Cryptotanshinone, the Major Active Constituent of Salvia miltiorrhiza

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ABSTRACT

The nature of intestinal absorption of most herbal medicine is unknown. Cryptotanshinone (CTS) is the principal active constituent of the widely used cardiovascular herb Salvia miltiorrhiza (Danshen). We investigated the oral bioavailability of CTS in rats and the mechanism for its intestinal absorption using several in vitro and in vivo models: 1) Caco-2 cell monolayers; 2) monolayers of MDCKII cells overexpressing P-glycoprotein (PgP); and 3) single-pass rat intestinal perfusion with mesenteric vein cannulation. The systemic bioavailabilities of CTS after oral and intraperitoneal administration at 100 mg/kg were 2.05 and 10.60%, respectively. In the perfused rat intestinal model, permeability coefficients based on CTS disappearance from the luminal perfusate (P_lumen) were 6.7- to 10.3-fold higher than permeability coefficients based on drug appearance in venous blood (P_blood). P_blood significantly increased in the presence of the P-gp inhibitor, verapamil. CTS transport across Caco-2 monolayers was pH-, temperature- and ATP-dependent. The transport from the apical (AP) to the basolateral (BL) side was 3- to 9-fold lower than that from the BL to the AP side. Inclusion of verapamil (50 μM) in both AP and BL sides abolished the polarized CTS transport across Caco-2 cells. Moreover, CTS was significantly more permeable in the BL to AP than in the AP to BL direction in MDCKII and MDR1-MDCKII cells. The permeability coefficients in the BL to AP direction were significantly higher in MDCKII cells overexpressing PgP. These findings indicate that CTS is a substrate for PgP that can pump CTS into the luminal side.

The efficacy of most drugs depends critically on their ability to cross cellular barriers to reach their targets. Oral administration is the most popular route for drug administration because dosing is convenient and noninvasive, and many drugs are well absorbed by the gastrointestinal tract. The small intestine represents the principal site of drug absorption. There are two principal routes by which compounds may cross the intestinal epithelium: paracellular or transcellular. A number of small hydrophilic, ionized drugs are absorbed via the paracellular pathway. Transcellular absorption from lumen to blood requires uptake across the apical membrane, followed by transport across the cytosol, then exit across the basolateral membrane and into blood. The apical efflux transporters are principally ATP-binding cassette transporter proteins, such as P-glycoprotein (PgP) and multidrug resistance-associated protein (MRP) 2, which are ideally situated to act as the first line of defense by limiting the absorption of drugs (Wacher et al., 2001; Chan et al., 2004).

In recent years, drug developers have rediscovered the potential value of herbal medicines, and their incorporation into medical care has been encouraged by the World Health Organization’s Traditional Medicines Strategy. The widely...
used traditional Chinese medicine Danshen, derived from the dried root or rhizome of *Salvia miltiorrhiza*, is an example of such a standardized medication. Approved for clinical use in China, indications for Danshen include angina pectoris, myocardial infarction, and stroke (Zhu, 1998). However, there is increased use of Danshen and its purified ingredients in the clinical management of hepatitis, menstrual disorder, miscarriage, diabetes, and chronic asthmatic bronchitis. Danshen contains diterpene quinone and phenolic acid derivatives, including tanshinones (I, IIA, and IIB), cryptotanshinone (CTS; Fig. 1), tanshinol (I and II), and salviol (Gu et al., 2004). Preclinical studies have shown that CTS has multiple pharmacological activities, including anti-inflammatory (Ryu et al., 1999), antioxidative (Wang et al., 2003), antiangiogenic (Hur et al., 2005), and cytotoxic effects (Mosaddik, 2003). It also inhibits diacylglycerol acyltransferase (Ko et al., 2002), endothelin-1 expression (Zhou et al., 2003), antiangiogenic (Hur et al., 2005), and cytotoxic effects (Mosaddik, 2003). It also inhibits diacylglycerol acyltransferase (Ko et al., 2002), endothelin-1 expression (Zhou et al., 2003), and interleukin-12 and interferon-γ production (Kang et al., 2000).

Most herbal medicines are orally administered with long-term regimens. However, the intestinal absorption properties of major ingredients of most herbal medicines are unknown, probably because of lack of sensitive analytical methods, difficulties in choice of the marker ingredients, and other confounding factors. CTS is often given orally for prolonged regimens in treatment of the aforementioned conditions. The Pharmacopoeia of the People’s Republic of China recommends dosage of 9 to 15 g daily for Danshen in decoc- tion form or up to 60 g in treatment of angina, severe arthritis, and stroke (Zhu, 1998). Because the typical content of CTS in Danshen is 0.23% (Song et al., 2005), this means approximately 21 to 138 mg of CTS is administered daily. A typical oral dose of CTS for patients is 60 to 200 mg/day (Zhu, 1998). The pharmacokinetic properties of CTS have been investigated in animals, whereas its pharmacokinetic study in humans has been hindered by the absence of a proper intravenous formulation because of its instability and low water solubility. CTS was rapidly metabolized to its active metabolite tanshinone IIA (Xue et al., 1999; Song et al., 2005). The oral bioavailability of CTS in pigs was too low to be determined as the plasma concentration was almost undetectable when CTS was given orally at 20 to 40 mg/kg (Xue et al., 1999). After i.v. injection of CTS at 10 mg/kg, CTS was rapidly distributed in the body and the elimination half-life (t_{1/2b}) was 64.8 min (Xue et al., 1999). The biliary and urinary excretion of both CTS and its metabolite tanshinone IIA was minimal (<0.3% of total dose over 48 h) after oral, i.m., or i.v. dosing, but the fecal recovery was 12% (Xue et al., 1999). The mechanism for such low bioavailability of CTS is unknown but may be related to first-pass metabolism and poor intestinal absorption. We hypothesized that the low bioavailability of CTS was associated with reduced intestinal absorption due to efflux into the lumen by active transporters. To test this hypothesis, we investigated the oral bioavailability of CTS in rats and the underlying mechanism for its intestinal absorption using several different in vitro and in vivo models: 1) Caco-2 cell monolayers; 2) monolayers of MCKII cells overexpressing PgP; and 3) single-pass rat intestinal perfusion (SSIP) with the mesenteric vein cannulated. The latter system allows the in vivo determination of intestinal absorption without concern for confounding effects from hepatic first-pass effect.

**Materials and Methods**

**Chemicals and Reagents.** CTS (purity >99%, as determined by thin-layer chromatography) and tanshinone IIA (purity >95%, as determined by thin-layer chromatography) were extracted and purified from the root of *S. miltiorrhiza* using the CH$_2$Cl$_2$ method as described previously (Hur et al., 2005) at the School of Pharmaceutical Sciences, Sun Yat-sen University, Guangzhou, People’s Republic of China. CTS and tanshinone IIA were identified by comparison of liquid chromatography-mass spectrometry and nuclear magnetic resonance spectral data with reference values (Wu et al., 2003). Phenol red was purchased from Tianjin Chemical Reagents Institute (Tianjin, People’s Republic of China). Fluoxetine (Prozac; purity >99% as determined by HPLC, used as internal standard) was purchased from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, People’s Republic of China). Dulbecco’s modified Eagle’s medium, fetal bovine serum, 0.05% trypsin-EDTA, penicillin-streptomycin, and nonessential amino acids were obtained from GibcoBRL Life Technologies (Grand Island, NY). Hank’s balanced salt solution (HBSS) at pH 6.0 contained 25 mM HEPES and 25 mM glucose. HBSS was sterilized by filtering through a 0.22-μm filter. The Krebs-Ringer buffer (pH 6.8) was used as perfusion buffer. Lucifer yellow, propranolol, dimethyl sulfoxide (DMSO), mannitol, and antipyrine were obtained from Sigma-Aldrich (Hong Kong, People’s Republic of China). The leukotriene antagonist 3-[[3-[[2-(7-chloroquinolin-2-yl)vinyl]phenyl]-(2-dimethylcarbamoylethylsulfanyl)methylsulfanyl] propionic acid (MK-571) was a gift from Dr. Ford Hutchinson (Merck Frosst Canada, Inc., Kirkland, QC, Canada). Tissue culture plastics and 0.4-μm pore-size 12 mm i.d. Transwell polycarbonate inserts were from Corning Costar Corp. (Corning, NY). The water used was of Milli-Q grade purified by a Milli-Q UV Purification System (Millipore, Bedford, MA). All other chemicals and reagents were of analytical or HPLC grade as appropriate.

**Animals.** Healthy male Sprague-Dawley rats (250–300 g) were purchased from the Animal Resources Center, Sun Yat-sen University, People’s Republic of China, and the Animal Holding Unit, National University of Singapore, Singapore. Rats were kept in a room under controlled temperature (22 ± 1°C) and automatic day-night rhythm (12 h-cycle) and housed on wire-bottom cages with paper underneath. Animals were treated humanely, and the animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health. Ethical approvals of this study were obtained from the Ethical committees of Sun Yat-sen University, Guangzhou, People’s Republic of China and the National University of Singapore, Singapore.

**Bioavailability and First-Pass Effect Study in Rats.** The animals were fasted overnight with free access to water before drug administration. CTS was freshly prepared by dissolving in distilled water. The maximum water solubility of CTS was approximately 1.0 mg/ml at 22°C room temperature, but it could be increased up to 20

![Cryptotanshinone](image_url) ![Tanshinone IIA](image_url)

Fig. 1. Chemical structures of cryptotanshinone and tanshinone IIA.
mg/ml when heated at 50°C for approximately 10 min without significantly affecting its stability. Rats were randomized to three groups (n = 6) to receive 100 mg/kg CTS by gavage (p.o., 5 ml/kg, 20 mg/ml), 100 mg/kg by i.p. injection (5 ml/kg, 20 mg/ml), or 20 mg/kg by i.v. injection (2 ml/kg, 10 mg/ml) through jugular vein cannulation. Blood samples were collected into heparinized Eppendorf tubes at the indicated times over 24 h after CTS administration. Plasma was then obtained by centrifugation at 6000 g for 5 min at 4°C and the plasma was transferred to clean 1.5-ml tubes. All samples were stored at −20°C until analysis.

SPIP Study. Animals were placed in individual cages and fasted overnight with free access to water before the experiment. Rats were anesthetized with urethane solution (20%) at a dose of 0.8 ml/100 g by i.p. injection. Animals were placed on a 37°C heating-pad, and a heating lamp was also used during surgery and throughout the in situ intestinal perfusion. Fresh heparinized blood was collected from donor rats by cardiac puncture. The surgical procedures used to prepare the SPIP with mesenteric vein cannulated were similar to those described elsewhere, with slight modifications (Fig. 2) (Singhal et al., 1998; Cummins et al., 2003). Three procedures were performed for animals undergoing in situ intestinal perfusion: jugular vein cannulation for infusion of blood collected previously from donor rats, isolation of an ileum segment for drug perfusion, and cannulation of the mesenteric vein for continuous collection of blood. After the animals were anesthetized with urethane, the right jugular vein was cannulated with a heparinized (100 IU/ml) polyethylene cannula (0.8 mm o.d.; Portex Ltd., Kent, UK). A midline incision was made and a 7- to 11-cm-long segment of ileum was chosen. Incisions were made at both ends of the segment and then gently flushed using prewarmed saline to remove intestinal contents. Two glass cannulas (4 mm o.d.) were inserted to both ends of the segment. Polyethylene tubing (5.5 mm o.d.) was connected between the inlet glass cannulas and the infusion syringe. A heparin solution (1.0 ml of 100 IU/ml) was injected into the jugular vein 10 min before the mesenteric cannulation. The mesenteric vein draining the cannulated ileum segment was cannulated using a heparinized (100 IU/ml) 22-gauge catheter (Becton Dickinson Sciences, Tokyo, Japan). The cannula was secured with a drop of cyanoacrylate adhesive (Adhesive System Co., New Hope, MN). Donor blood was infused through the right jugular vein using a peristaltic pump (BT00-300T pump; Baoding Longer Precision Pump Co., Baoding, People’s Republic of China), and the rate was adjusted based on the outflow from the mesenteric blood (∼0.3 ml/min). CTS was infused at 0.5 and 2.0 μg/ml or 2.0 μg/ml in the presence of a 50 μM concentration of the PgP inhibitor verapamil. The in situ intestinal perfusion was initiated by infusing drug solution from the syringe pump at 1.0 ml/min for 2 min followed by perfusion at 0.2 ml/min for the remainder of the experiment by a syringe pump (WZS-50F2 infusion pump; Medical Apparatus of Zhejiang University, Hanzou, People’s Republic of China). The blood from the mesenteric vein was continuously collected into a heparinized 1.5-ml tube at 5-min intervals up to 60 min. Samples were collected from the outflow of the perfusate every 5 min into 1.5-ml tubes up to 60 min. The blood samples were immediately centrifuged at 6000 g for 5 min, and the plasma was transferred to clean 1.5-ml tubes. All samples were stored at −20°C until analysis.

The perfusion solution containing 20.0 μg/ml phenol red was used as a nonabsorbable marker for measuring water flux and to correct for changes in the water flux across the intestine. Phenol red was determined using a spectrophotometric reader (model UV-1601PC; Shimadzu, Kyoto, Japan) as described previously (Cummins et al., 2003). The concentrations of CTS in the perfusate were corrected for changes in water flux at each time interval using the following equation (Singhal et al., 1998; Cummins et al., 2003):

\[
[\text{CTS}]_{\text{corrected}} = \frac{[\text{phenol red}]_{\text{in}}}{[\text{phenol red}]_{\text{out}}} \times [\text{CTS}]_{\text{measured}}
\]

Additional experiments were conducted to examine the disappearance and appearance coefficients of the passive transcellular (antipyrine, 0.2 mM) and paracellular (mannitol, 20 mM) markers using the above-mentioned SPIP model (α = 5/group). The gut does not metabolize mannitol and antipyrine and absorbs these two compounds in an unchanged form. The effects of verapamil at 50 μM on the permeability of both antipyrine and mannitol were also investigated.

Cell Culture. Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD). The control vector MDCKII cell line and its human MDR1 recombinantly transfected derivative, MDR1-MDCKII, were a kind gift from Professor Piet Borst (Netherlands Cancer Institute, Amsterdam, The Netherlands). The cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% nonessential amino acids, and 100 U/ml penicillin and gentamicin. The cells were grown in an atmosphere of 5% CO2 and 90% relative humidity at 37°C and given fresh medium every 3 to 5 days by trypsin-EDTA exposure. CTS at 0.1 to 2.0 μg/ml did not show significant cytotoxicity (<5%) to Caco-2, MDCKII, and MDR1-MDCKII cells when incubated for 48 h as determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazo-
lum bromide assay. There were no detectable metabolites when CTS at 0.5 and 2.0 µg/ml was incubated with Caco-2, MDCKII, or MDRI-MDCKII cells for up to 48 h as determined by HPLC.

For transport studies, the Caco-2 cells and MDCKII or MDRI-MDCKII cells were seeded at densities of 5 × 10⁵ and 2.5 × 10⁵ cells/insert, respectively, onto polycarbonate membrane Transwell inserts (Corning Costar Corp.) in 12-well plates. The culture medium (0.5 ml in the apical donor chamber of the insert and 1.5 ml in the basal well chamber) was replaced every 48 h for the first 6 days and every 24 h thereafter. The transepithelial electrical resistance (TEER) of the monolayers was examined routinely before and after the experiment using the EVOM (epithelial voltameter; WPI, Sarasota, FL). Caco-2 cells were used for transport experiments 21 days after seeding when the TEER values exceeded 250 Ω · cm². Caco-2 experiments were conducted on cells between passages 30 to 35. MDCKII and MDRI-MDCKII cells were used in experiments at passages 5 to 9 after receipt from the Netherlands Cancer Institute. Cells were used in transport experiments at days 4 to 6 postseeding where MDCKII TEER was typically 40 to 60 Ω · cm² and MDRI-MDCKII TEER was 120 to 150 Ω · cm². Lucifer yellow was used as a probe for paracellular transport, and a permeability of 0.1 to 0.7 × 10⁻⁶ cm/s indicated monolayer integrality.

Permeability Studies in Caco-2 and MDCKII Monolayers. The transport of CTS by Caco-2 and MDCKII and MDRI-MDCKII monolayers was investigated on an orbital shaker as described previously (Zhou et al., 2005) with some minor modifications. In brief, the cell monolayers were washed twice with warm HBSS containing 25 mM HEPES (pH 6.0) before the transport experiments. A pH of 6.0 was chosen as it stabilized CTS, and this pH resulted in maximum apical (AP) to basolateral (BL) and BL to AP transport. After each wash, the plates were incubated at 37°C for 15 min, and the TEER was measured and inserts distributed evenly between treatments on the basis of TEER values. HBSS solution on both sides of the cell monolayers was then removed by aspiration. For the measurement of AP to BL transport, 0.5 ml of HBSS containing CTS (0.1–2.0 µg/ml) was added on the AP side, and 1.5 ml of HBSS without the drug was added on the BL side. After drug loading at the AP side and incubation at 37°C, an aliquot (0.1 ml) was collected from the BL side at predetermined times (5, 10, 15, 20, 30, 45, and 60 min) after each sampling, the same volume of blank HBSS was replaced in the BL side to maintain a constant volume. For the measurement of BL to AP transport, 1.5 ml of HBSS containing CTS (0.1–2.0 µg/ml) was added on the BL side, and 0.5 ml of HBSS without the drug was added to the AP side. The inserts were then incubated at 37°C, and the incubation medium in the AP side was replaced by fresh medium at indicated times (5, 10, 15, 20, 30, 45, and 60 min) over 60 min. CTS solutions were freshly prepared by dissolving in DMSO, and the final concentration of DMSO in incubation medium was 1% (v/v). All experiments were conducted in triplicate.

The effects of pH, Na⁺, temperature, and ATP on CTS transport across Caco-2 monolayers were investigated. The effect of apical or basolateral pH (5.5–7.4) on the AP to BL and BL to AP flux of CTS at 0.5 µg/ml was examined at pH 7.4 for the receiving side. The pH was altered by substituting appropriate amounts of HEPES in the incubation medium by equimolar (25 mM) 2-(N-morpholino)ethanesulfonic acid. In experiments to investigate the effect of Na⁺ on the flux of CTS at 0.5 µg/ml across the Caco-2 cell monolayers, the sodium chloride in the HBSS was replaced by equimolar amounts (140 mM) of potassium chloride. The permeability of CTS from AP to BL and BL to AP was measured after incubation for 1 h at 37°C. To determine energy dependence of CTS flux, transport medium depleted in glucose was used in both sides of the cell monolayers. Sodium azide (10 and 2.0 mM) or 2,3-dinitrophenol (1 and 2 mM) (both ATPase inhibitors) was added to both AP and BL sides, and the monolayers were incubated for 1 h at 37°C.

In experiments to investigate the effect of MK-571 (100 µM), verapamil (50 µM), and tanshinone IIA (50 µM) in the transport of CTS (0.5 and 2.0 µg/ml) from AP to BL or BL to AP across the Caco-2 cell monolayers, the inhibitor was added to the incubation medium on both the apical and the basolateral sides of the cell monolayers throughout the experiment and also preincubated with the cells for 30 min before addition of CTS. Verapamil and tanshinone IIA were made fresh immediately before the experiment in DMSO and added to the apical and the basolateral sides with a final DMSO concentration of 1.0% (v/v). Vehicle was used for the control inserts. All samples were stored at −20°C until analysis. All incubations were performed in triplicate. To avoid interday cell-cell variations, the transport experiments for the determination of kinetics or inhibition by various compounds were always conducted on the same day using the same batch of cells.

Determination of Cryptotanshinone, Antipyrine, and Mannitol by HPLC. The concentrations of CTS in rat plasma, perfusates and cellular monolayers were determined by validated HPLC methods. A Millennium 3.2 HPLC system was used to quantify cryptotanshinone in all samples. The system consisted of an autosampler (model 717), a pump (model 510), and a double-wavelength ultraviolet detector (model 2487) at an operation wavelength of 270 nm (Waters, Milford, MA). All data were collected and processed by the Millennium 3.2 software. The analytical column was a BDS reversed-phase C₁₈ column (150 × 4.6 mm i.d., particle size 5 µm; ELITE, Dalian, People’s Republic of China). The mobile phase (flow rate of 1.2 ml/min) consisting of methanol-boric acid (4.41 g/l)-potassium chloride buffer (5.29 g/l)-triethylamine (72.28:0.05, v/v), pH 9.4, adjusted by 1.0 M HCl) was degassed immediately before use. To determine CTS in each sample, 10 µl of fluoxetine (90 µg/ml) and 300 µl of diethyl ether were added into 100 µl of each sample. The Sample was then vortexed for 1 min before centrifugation at 3000g for 12 min. The ether layer was then removed to a 1.5-ml tube, dried under negative pressure, and then reconstituted in 200 µl of MeOH-H₂O (1:1, v/v), and 20 µl was injected onto the HPLC column. Under the chromatographic conditions used for the analysis of CTS, the retention times for internal standard and CTS were 5.8 and 8.3 min, respectively. The LOQ of the assay was 5.0 ng/ml.

The antipyrine in perfusates was determined by a validated HPLC method as described previously (Hung et al., 2001), with small modifications. In brief, a 100-µl aliquot of sample was mixed with 25 µl of 20 mg/l propanolol (internal standard) in methanol and 50 µl of 10% trichloroacetic acid in an Eppendorf tube. The tube was vortexed for 1 min and centrifuged at 5000g for 10 min, and 20 µl of the resulting supernatant was injected onto the BDS reversed-phase C₁₈ column. The mobile phase was 100 mM KH₂PO₄ buffer with 25% acetonitrile at pH 3.0 at a flow rate 1.0 ml/min. The analytes were detected using a fluorescence detector with a 300 nm excitation wavelength and a 375 nm emission wavelength. The LOQ for antipyrine was 35 ng/ml. The mannitol in the perfusates was determined by a validated HPLC method with refractive index as described previously (Miki et al., 1996). An aliquot (100 µl) of perfusate was mixed with methanol and vortexed for 2 min. After centrifugation at 5000g for 10 min, 50 µl of the resulting supernatant was injected onto an amine-modified silica column (5-µm particle size, 250 × 4.6 mm; Alltech Biological Products Co. Ltd, Beijing, People’s Republic of China). The mobile phase was acetonitrile in distilled deionized water (70:30, v/v) at a flow rate of 1.0 ml/min. Detection was by a refractive index detector (LC 1240 R.I. Detector; GBC Scientific Equipment, Dandenong, Australia). The LOQ of the assay was 0.04 mM.

Pharmacokinetic Calculation. The plasma concentration-time curves of CTS were obtained by plotting the mean plasma concentrations of CTS versus time on a semilogarithmic scale. Pharmacokinetics parameters were calculated by standard model-independent pharmacokinetic formulae using WinNonlin program (Scientific Consulting Inc., Greensboro, NC). The t₁/₂ value was calculated as 0.693/β, where β is the elimination rate constant calculated from the terminal linear portion of the log plasma concentration-time curve. The total areas under the plasma concentration-time curve from time 0 to the last quantifiable time point (AUC₀₋₉) and from time 0 to
Based on the disappearance of the drug from the lumen (comparison test. Student’s parameters among the different groups was carried out by a one-way analysis of variance followed with a post hoc test (Dunnett’s multiple comparison test. Student’s t test was performed for the between-group comparisons with a significance level of P < 0.05. The permeability values of CTS across rat ileum were calculated based on the disappearance of the drug from the lumen ($P_{\text{lumen}}$) as well as the appearance of drug in the blood ($P_{\text{blood}}$) using the following equations (Singhal et al., 1998; Cummins et al., 2003; Johnson et al., 2003):

$$P_{\text{lumen}} = \frac{Q}{2mr} \ln \frac{C_{\text{lumen}}}{C_0}$$  

$$P_{\text{blood}} = \frac{\Delta X dt}{A \times C_0}$$

where $r$ is the radius of the intestinal lumen (0.18 cm), $l$ is the length of the segment (centimeters), $Q$ is the flow rate of drug through the intestine (0.2 ml/min), $C_0$ is the concentration of drug at the start of perfusion (in the syringe, micrograms per milliliter), $C_{\text{lumen}}$ is the steady-state concentration of drug exiting the lumen, $\Delta X dt$ is the rate of CTS appearance in venous blood (micrograms per second), and $A$ is the surface area of the ileum segment ($= 2mr$, square centimeters). The apparent permeability coefficient ($P_{\text{app}}$) in cellular monolayers is expressed in centimeters per second and calculated as in following equation:

$$P_{\text{app}} = \frac{\Delta Q}{\Delta t} \times \frac{1}{60} \times \frac{1}{A} \times \frac{1}{C_0}$$

where $\Delta Q/\Delta t$ is the permeability rate (micrograms per second), $A$ is the surface area of the membrane (square centimeters), and $C_0$ is the initial concentration in the donor chamber (micrograms per milliliter). Samples from the 30-min point were used for $P_{\text{app}}$ calculations. The net BL to AP efflux of CTS ($R_{\text{net}}$) was determined by calculating the ratio of $P_{\text{app}}$ in the BL to AP direction versus $P_{\text{app}}$ in the AP to BL direction ($P_{\text{appBL-AP}}/P_{\text{appAP-BL}}$) as in eq. 7 (Tang et al., 2002a,b; Zhou et al., 2005).

$$R_{\text{net}} = \frac{P_{\text{appBL-AP}}}{P_{\text{appAP-BL}}}$$

The passive diffusion flux rate (excluding the influence of efflux transporter) of CTS in Caco-2 monolayers was estimated by conducting the transport experiment in the presence of verapamil (50 µM). The active transport flux rates were then estimated by subtracting the passive diffusion rates from total flux rates. Several models to describe the kinetics of the active transport of CTS (single and two binding sites, substrate inhibition, and the sigmoid models) were fitted and compared using the Prism 3.0 program (GraphPad Software, San Diego, CA). The choice of model was confirmed by $F$ test and Akaike’s information criterion (Yamaoka et al., 1978). It was found that one-binding site model (eq. 8) was the best fit:

$$v = \frac{V_{\text{max}}[S]}{K_m + [S]}$$  

where $v$ is the apparent linear initial rate, $[S]$ is the initial concentration, $V_{\text{max}}$ is the maximum transport rate, and $K_m$ is the Michaelis-Menten constant.

The apparent inhibition constant ($K_i$) was estimated using eqs. 9 to 11 as described previously (Tang et al., 2002b; Zhou et al., 2005):

$$K_i = \frac{P_i/P_0}{1 - (P_i/P_0)}$$  

$$P_0 = P_{\text{app}} - P_{\text{appP}}$$  

$$P_1 = P_{\text{appP}} - P_{\text{app}}$$  

where $P_i$ and $P_0$ are the $P_{\text{app}}$ values of CTS in the direction of BL to AP in the presence and absence of the inhibitor, respectively, and $P_i/P_0$ is a reflection of the inhibitory effect of the test compound on the active BL to AP transport of CTS across the Caco-2 monolayers. $P_{\text{appP}}$ is the total transport in the absence of any inhibitory compound, $P_{\text{app}}$ is the total transport in the presence of a potential inhibitor, and $P_{\text{appP}}$ is the passive diffusion component.

Results

**Systemic Bioavailability of CTS in Rats.** The pharmacokinetic profiles of CTS after oral (100 mg/kg), i.p. (100 mg/kg), and i.v. (20 mg/kg) administration are shown in Fig. 3, and the pharmacokinetics parameters of CTS are listed in (Table 1). After oral administration, the $C_{\text{max}}$ of CTS was 90.31 ± 31.46 mg/ml, and this was observed at 5.19 ± 4.63 h postdosing; thereafter, it declined with a $t_{1/2p}$ of 6.64 ± 2.08 h, and the $AUC_{0-\infty}$ was 646.25 ± 238.47 mg/ml·h. Meanwhile, the $C_{\text{max}}$ of CTS after i.p. injection was 657.97 ± 229.92 mg/ml (n = 6), with a $T_{\text{max}}$ of 1.91 ± 0.20 h; it declined with a $t_{1/2p}$ of 6.88 ± 6.50 h, and the $AUC_{0-\infty}$ was 3332.40 ± 578.049 mg/ml·h. In addition, after i.v. injection of CTS at 20 mg/kg, the $t_{1/2p}$ clearance, and $AUC_{0-\infty}$ were 1.06 h, 53.0 mg/ml·h, and 578.049 mg/ml·h, respectively.

Fig. 3. The pharmacokinetics profiles (n = 6) of cryptotanshinone after oral and i.p. administration (100 mg/kg), respectively.

![Image](image-url)
ml/min/kg, and 6289.54 ± 1011.23 ng/ml · h, respectively. Thus, the $F^{p.o.}$ and $F^{p.v.}$ were 2.05 and 10.60%, respectively.

**Single-Pass Perfusion of Rat Ileum.** No significant loss of CTS (<5%) was found when the drug was perfused through the intestinal perfusion apparatus, indicating that there was no significant adsorption to the tubing. The compound was found to be stable in the perfusion buffer as well as in the intestinal perfusate at 37°C for at least 1 h. There was no significant metabolite (tanshinone IIA) formation in the perfusates or mesenteric vein blood when CTS was loaded.

For intestinal perfusions with CTS, samples were obtained from the outlet of the intestine as well as the mesenteric vein at 5-min intervals up to 60 min. The permeability values of CTS at 0.5 and 2.0 μg/ml or 2.0 μg/ml in the presence of 50 μM verapamil are shown in Table 2. The permeability of CTS based on the luminal disappearance of the drug was estimated using eq. 4 from the steady-state data (samples obtained between 45 and 60 min). The $P_{lumen}$ values of CTS were 1.50 ± 0.20 × 10⁻⁴ cm/s at 0.5 μg/ml and 1.44 ± 0.63 × 10⁻⁴ cm/s at 2.0 μg/ml. There was no statistical difference in $P_{lumen}$ of CTS at 0.5 and 2.0 μg/ml although there appeared to be a slightly decreased permeability value with increased CTS concentration. Such concentration independence of $P_{lumen}$ may reflect the relatively high intrinsic permeability of CTS and thus the permeability was insensitive to substrate concentration.

Verapamil did not significantly affect the $P_{lumen}$ value of CTS at 2.0 μg/ml ($P > 0.05$). This finding is in agreement with previous in vivo studies with verapamil (Sandstrom et al., 1999; Johnson et al., 2003). Johnson et al. (2003) reported that PSC833 (a PgP inhibitor) resulted in a significant increase in $P_{blood}$ of verapamil but did not significantly alter the disappearance of verapamil in perfused rat jejunum. Similar results were observed with verapamil in the presence of ketoconazole (a CYP3A4 and PgP inhibitor) in a human intestinal perfusion study (Sandstrom et al., 1999). These observations most likely reflect the small impact of PgP efflux on the disappearance of CTS and verapamil from the luminal perfusate relative to the high intrinsic permeability and possible substantial tissue uptake and binding of both compounds.

As for the permeability based on appearance of CTS in the mesenteric blood ($P_{blood}$), concentration-dependent changes in permeability were evident where the permeability at 2.0 μg/ml (1.40 ± 0.30 × 10⁻⁵ cm/s) was significantly lower than that at the lower concentration (0.5 μg/ml, 2.23 ± 0.57 × 10⁻⁵ cm/s, $P < 0.05$). $P_{blood}$ of CTS increased significantly in the presence of 50 μM verapamil (Fig. 4) (control versus verapamil: 1.40 ± 0.30 × 10⁻⁵ versus 3.63 ± 0.44 × 10⁻⁵ cm/s, $P < 0.01$). The ratio of $P_{lumen}$ over $P_{blood}$ of CTS at 0.5 and 2.0 μg/ml was 6.7 and 10.3, respectively. These data suggest that PgP-mediated efflux was effectively limiting the absorption of CTS across the intestine and was at least partially inhibited by verapamil. These data also illustrate the potentially different conclusions that may be drawn as to the importance of PgP efflux on drug absorption when one evaluates permeability on drug disappearance as opposed to drug appearance values.

There was no significant difference between $P_{lumen}$ and $P_{blood}$ for antipyrine (a well absorbed passive transcellular transport marker) and mannitol (a well-absorbed paracellular transport marker). The $P_{lumen}$ and $P_{blood}$ for antipyrine were 5.93 ± 1.23 × 10⁻⁵ and 6.01 ± 2.01 × 10⁻⁵ cm/s.

**Table 2**

<table>
<thead>
<tr>
<th>CTS Concentration in Perfusion</th>
<th>$P_{lumen}$</th>
<th>$P_{blood}$</th>
<th>$P_{lumen}/P_{blood}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 μg/ml</td>
<td>$1.50 ± 0.20$</td>
<td>$2.23 ± 0.57$</td>
<td>6.7</td>
</tr>
<tr>
<td>2.0 μg/ml</td>
<td>$1.44 ± 0.63$</td>
<td>$1.40 ± 0.30$</td>
<td>10.3</td>
</tr>
<tr>
<td>2.0 μg/ml + verapamil</td>
<td>$1.60 ± 0.49$</td>
<td>$3.63 ± 0.44$</td>
<td>4.4</td>
</tr>
</tbody>
</table>

* $P < 0.05$, $P_{blood}$ vs. $P_{lumen}$
* $P < 0.05$, $P_{blood}$ at 0.5 μg/ml vs. $P_{blood}$ at 2.0 μg/ml.
* $P < 0.05$, $P_{blood}$ in the presence of verapamil vs. $P_{blood}$ in the absence of verapamil.
Transport of CTS in Caco-2 Monolayers. After incubation of CTS at 0.1–2 μg/ml loaded at either the AP or BL side, the sample was collected from the receiving side for HPLC analysis. No detectable tanshinone IIA was observed when CTS concentration was over 0.1 to 2.0 μg/ml. The flux of CTS from AP to BL or BL to AP was largely proportional to CTS concentrations on the receiving side by 5 min. The flux of CTS from AP to BL or BL to AP had no significant effect on the flux of CTS for either AP to BL or BL to AP transport.

Addition of the transport buffer with sodium azide (10 mM) or 2,4-dinitrophenol (1 mM) insignificantly increased the AB to BL flux of CTS by 24.5 and 27.1%, respectively (P > 0.05). Because both sodium azide and 2,4-dinitrophenol are cellular ATPase inhibitors, their marked effect on ATP-dependent PgP-mediated efflux of CTS is expected to be seen only when intracellular ATP has been reduced.

Reducing the apical pH to 5.5 to 6.5 caused a significant (P < 0.05) increase (25–33%) in CTS flux from AP to BL or BL to AP compared with the values at pH 7.4 (Fig. 7). A maximum P_app was observed at pH 6.0 for both AP to BL and BL to AP transport. Lower pH may reduce the ionization of CTS and thus increase its intestinal transport. The substitution of sodium salts in the transport medium with potassium salts as 5,6-dimethylxanthenone-4-acetic acid (an anticancer drug and a non-PgP substrate) (Zhou et al., 2005) and several arylxoy phosphoramidate derivatives (PgP substrates) of the anti-HIV agent stavudine (Siccardi et al., 2003). This may reflect the fact that P_app is affected by many complicating factors, in particular for substrates with high intrinsic permeability and substantial tissue uptake and binding. Model fitting indicates that the BL to AP active efflux followed one binding site kinetics with a K_m of 0.24 μg/ml (0.9 μM) and V_max of 0.11 ± 0.01 nmol/min/cm² (Fig. 6). The low K_m value for CTS suggests that CTS is a high-affinity substrate for PgP.

Fig. 6. Effect of concentration on the flux of CTS from AP to BL and BL to AP sides. CTS was loaded on either the AP or BL side and incubated at 37°C. Samples from the receiving side were collected, and CTS was determined by HPLC. Data are the means ± S.D. from three to nine determinations. The curve represents the fit of model with one saturable transport system.
significantly depleted. The absence of glucose in the transport medium did not significantly affect the AP to BL flux of CTS. However, both compounds caused significant (P < 0.05) decreases (45 and 57%, respectively) in the BL to AP flux of CTS. In addition, depletion of glucose also significantly decreased the BL to AP flux of CTS by 50%, but the AP to BL flux of CTS was not significantly affected. These results indicated that the BL to AP flux of CTS was energy-dependent.

Inclusion of the PgP inhibitor verapamil (50 μM) or tanshinone IIA (50 μM) in both AP and BL sides abolished the profound polarized CTS transport, with the AP to BL P_{app} increasing approximately ~3-fold and the BL to AP P_{app} decreasing ~2.5-fold (Fig. 8). The estimated K_{app} values based on eqs. 9 to 11 for verapamil and tanshinone IIA were 9.0 and 10.8 μM, respectively. However, MK-571 just slightly increased the AP to BL P_{app} and decreased the BL to AP P_{app} values (P > 0.05, data not shown), suggesting that MRPs play a minor or negligible role in the intestinal transport of CTS.

**Transport of CTS in MDCKII Monolayers.** To further investigate the nature of the polarized transport of CTS, permeability studies were carried out in MDR1-MDCKII cells, which stably and functionally overexpress human MDR1 cells (PgP) and their wild-type parental cell line MDCKII, which expresses constitutive canine PgP at a much lower level than that in the recombinant MDR1-MDCKII cells (Polli et al., 2001). The transport data across these two MDCKII cell lines for CTS are shown in Table 3. Consistent with the data for CTS in the Caco-2 monolayer studies, CTS at 0.1, 0.5, and 2.0 μg/ml in the MDCKII monolayers showed a significantly (P < 0.05) greater permeability in the BL to AP direction compared with that in the AP to BL direction. Table 3 also shows the transport of CTS across the recombinant MDR1-MDCKII cells, which translationally and functionally overexpress PgP. Most apparent is the fact that the extent of polarized transport is now more profound, with P_{app} values in the AP to BL and BL to AP directions in both MDCKII and MDR1-MDCKII cells, indicating the substantial intrinsic permeability in MDCKII and MDR1-MDCKII cells and the presence of a barrier to the transport of CTS.

**Discussion**

Although CTS is important in human health, little is known about its intestinal absorption and the ability to reach sites of action. The extent to which a compound is absorbed by the intestinal epithelium is a critical factor in determining its overall bioavailability. In this study, we evaluated for the first time the oral and intraperitoneal bioavailability and nature of intestinal permeability of CTS using several models. These results indicate that the systemic bioavailabilities of CTS after oral and i.p. administration at 100 mg/kg were only 2.05 and 10.60%, respectively. A 4-fold higher F^{v} than P_{app,c} indicates the limiting role of the intestine as a barrier in the absorption of CTS into the blood.

Moderate to high plasma clearance (53.0 ml/min/kg) of

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**TABLE 3**

Permeability data of CTS across MDCKII and MDR1-MDCKII monolayers

<table>
<thead>
<tr>
<th>CTS Concentration and Direction</th>
<th>MDCKII P_{app}</th>
<th>MDR1-MDCKII P_{app}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0.5 μg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Verapamil (0.5 μg/ml)</td>
<td>1.92 ± 0.27</td>
<td>1.15 ± 0.19</td>
</tr>
<tr>
<td>Tanshinone IIA (0.5 μg/ml)</td>
<td>9.32 ± 1.24</td>
<td>13.34 ± 2.21</td>
</tr>
<tr>
<td>Control (2.0 μg/ml)</td>
<td>1.43 ± 0.23</td>
<td>0.83 ± 0.11</td>
</tr>
<tr>
<td>Verapamil (2.0 μg/ml)</td>
<td>8.38 ± 1.12</td>
<td>10.25 ± 2.11</td>
</tr>
<tr>
<td>Tanshinone IIA (2.0 μg/ml)</td>
<td>1.10 ± 0.18</td>
<td>0.62 ± 0.12</td>
</tr>
<tr>
<td>2.0 μg/ml (BL to AP)</td>
<td>6.54 ± 1.01</td>
<td>8.38 ± 1.12</td>
</tr>
</tbody>
</table>

* P < 0.05, AP to BL vs. BL to AP transport.

b P < 0.05, P_{app} at 0.5 μg/ml vs. P_{app} at 2.0 μg/ml.
CTS was observed in rats, which was partially attributed to partitioning into red blood cells based on an ~1.8 blood/plasma concentration ratio (Yuan et al., 2002). Therefore, blood clearance of CTS was 1.8-fold lower than plasma clearance and ~35% of hepatic blood flow in rats (~85.0 ml/min/kg). If CTS were well absorbed and cleared mainly in the liver, its oral bioavailability would be expected to be ~65%. The low and slow oral bioavailability of CTS (2.05% with a $T_{\text{max}}$ of 6.64 h) suggests that CTS was poorly absorbed and/or underwent significant extraction in the gut and/or liver. Intrapерitoneal bioavailability of CTS was 10.6%, which is most likely due to either hepatic metabolism or low solubility. The low water solubility of CTS together with first-pass metabolism is considered the major factor for its low oral bioavailability. Because intestinal drug efflux by PgP and other transporters is widely recognized as a major determinant for low or variable oral absorption and bioavailability (Wacher et al., 2001), the results of this study highlight the need to consider the role of transport proteins in attenuating the absorption of CTS. PgP (MDR1/ABCB1) located on the apical side of intestinal enterocytes can actively cause efflux of drugs from the cells back into the lumen (Borst and Elferink, 2002).

The single-pass rat intestinal perfusion model provided an isolated in vivo system in which to study the permeability and role of PgP in intestinal transport of CTS. The absorption of CTS in the single-pass perfused rat ileum was monitored by determination of disappearance permeability ($P_{\text{lumen}}$) and appearance permeability ($P_{\text{blood}}$). Single-pass intestinal perfusion experiments are often performed in the absence of mesenteric cannulation as a screening tool to estimate whether a new candidate drug will show low or high permeability in vivo (Sutton et al., 2002). From our in situ single-pass perfusion data, we found that the $P_{\text{lumen}}$ values for CTS (1.50 ± 0.20 × 10^{-4} cm/s at 0.5 µg/ml and 1.44 ± 0.63 × 10^{-4} cm/s at 2.0 µg/ml) were higher than those for verapamil (3.07 × 10^{-5} cm/s) (Johnson et al., 2003), lidocaine (7.5 × 10^{-5} cm/s) (Berggren et al., 2004), and RU60079 (a novel angiotensin II antagonist, 1.4 × 10^{-6} cm/s) (Boisset et al., 2000), but lower than that for warfarin (7.7 × 10^{-5} cm/min), a drug absorbed in unchanged form (Okudaira et al., 2000). These data may suggest that CTS is highly and well absorbed. However, the much lower $P_{\text{blood}}$ than $P_{\text{lumen}}$ of CTS indicates extensive intestinal efflux and/or gut metabolism. Such a difference between $P_{\text{blood}}$ and $P_{\text{lumen}}$ occurs because permeability calculations based only on the disappearance of drug from the lumen cannot distinguish drug losses from absorption from those from extensive metabolism (Dackson et al., 1992). However, no significant metabolite (tanshinone IIA) formation was detected in the perfusates or mesenteric vein blood when CTS was loaded, suggesting that extensive metabolism of CTS in intestine did not occur. Increased $P_{\text{blood}}$ in the presence of verapamil indicates that the compound is transported primarily via an active mechanism across the intestinal epithelium with a saturable process.

We found that the permeability of the Caco-2 monolayers to CTS is nonlinearly related to concentrations over 0.1 to 2.0 µg/ml. The deviation from linearity suggests the presence of a polarized efflux pump and/or a saturable metabolic barrier to absorption. However, the relative high intrinsic permeabilities of CTS in Caco-2 and MDCKII monolayers compared with those for most lipophilic drugs and negligible metabolite formation in Caco-2 cells indicate that the first-pass metabolism within the enterocytes plays a minor role in limiting the oral absorption of CTS.

We found that the permeability coefficients for AP to BL transport of CTS were approximately 3- to 9-fold lower than those in the BL to AP direction. The addition of the PgP inhibitor verapamil markedly reduced the transport of CTS in the BL to AP direction, resulting in a polarized permeability ratio (BL to AP/AP to BL) of ~0.70 to 0.89. The estimated $K_{i}$ for verapamil is 9.0 µM. Although verapamil is considered not to be a potent PgP inhibitor in the context of the limiting in vivo concentrations above which cardiotoxicity is observed, i.e., at concentrations >5 µM and not at the concentrations commonly used in vitro, i.e., 40 to 50 µM. Notably, verapamil can also modulate other ATP-binding cassette transporters such as MRP1 and MRP4 (Walgren et al., 2000), but it seems not to affect MRP2 or breast cancer-resistance protein activity (Borst and Elferink, 2002), the latter two being the key ATP-binding cassette efflux transporters present with PgP on the apical membrane of Caco-2 cells (Borst and Elferink, 2002). However, the MRP1–4 inhibitor, MK-571, had an insignificant effect on CTS transport in Caco-2 monolayers, excluding CTS as a substrate for MRP1–4. In addition, the AP to BL transport of CTS was significantly inhibited by tanshinone IIA, the major metabolite of CTS. It has been reported that tanshinone IIA is transported by an active mechanism across the intestine, involving a saturable process (Yuan et al., 2002). A significant increase in the AB to BL flux of CTS in the presence of sodium azide at 20 mM and 2,4-dinitrophenol at 2 mM also provided supportive evidence for the involvement of an active mechanism for CTS intestinal transport. The Caco-2 data are consistent with the presence of a PgP efflux pump on the apical membrane of Caco-2 capable of directing CTS out of the cell and back in the apical (luminal) medium.

Transport data in the MDR1-MDCKII model further supported the hypothesis that CTS is a substrate for PgP. CTS was significantly more permeable in the BL to AP than in the AP to BL direction in both cell lines. The permeability coefficients in the BL to AP direction were significantly higher in the cell line overexpressing PgP.

Because CTS is unstable in aqueous medium at physiological pH and biological matrices such as plasma, bile, and urine undergoing rapid and spontaneous degradation (Xue et al., 1999; Song et al., 2005), we chose a 30-min incubation time at pH 6.0 for the calculation of $P_{\text{app}}$ values in both Caco-2 and MDR1-MDCKII cells. Appropriate handling of biological samples containing CTS is crucial to avoid degradation and oxidation. This is particularly important for plasma samples, as CTS degradation due to unsuitable sample handling may lead to marked interindividual variation in the pharmacokinetic parameters. In this study, lowering pH and quick chilling of the samples were used to prevent degradation of CTS in all samples.

Both in vivo and in vitro studies are time-, money-, and labor-consuming. In vitro models (e.g., Caco-2 monolayers and cells overexpressing a specific transporter) have gained great attention as alternative approaches for animal studies because the resultant permeability data can be used to effectively predict the oral absorption and bioavailability of many drugs. These models can also be used to identify the transport mechanisms of new compounds. As such, in vitro studies...
should be conducted before in vivo animal studies in drug development (Artursson et al., 1996).

P-glycoproteins belong to a family of well-conserved plasma membrane proteins with two members in humans (MDR1 and MDR3), three members in mice (mdr1, mdr2, and mdr3), and three members in rats (ppg1, ppg2, and ppg3) (Borst and Elferink, 2002). Results from the present study suggest that MDR1 may play a major role in the transport of CTS, but the contribution of MDR3 cannot be excluded. It would be interesting to investigate the relative contributions of P-glycoproteins in rats and humans in the transport of CTS.

In conclusion, CTS has low systemic bioavailability after oral and i.p. administration in rats. In single-pass perfused rat ileum with mesenteric vein cannulation, the \( P_{\text{lumen}} \) values of CTS was 5.7- to 9.0-fold higher than \( P_{\text{blood}} \) values, and the \( P_{\text{blood}} \) values significantly increased in the presence of verapamil. Polarized transport of CTS was found in Caco-2 and MDR1-MDCKII cells. Addition of verapamil on both apical and basolateral sides abolished the polarized CTS transport across Caco-2 cells. These results indicate that CTS is a good substrate for PgP. The permeability data from the single-pass intestinal perfusion model and Caco-2 monolayers indicate that PgP-mediated efflux into the luminal side may play just a limited role in the low oral bioavailability of CTS. However, further work addressing the role of first-pass metabolism and other contributing factors in the low oral bioavailability of CTS is warranted.

References