School of Pharmacy[1], China Pharmaceutical University, Nanjing, P.R. China, Department of Pharmaceutical Chemistry[2], University of Kansas, KS, USA

Mrp2-related efflux of scutellarin in the intestinal absorption in rats

FENG CAO[1], HAIYAN ZHANG[1], JIANXIN GUO[2], QINENG PING[1]

Received May 23, 2007, accepted June 22, 2007

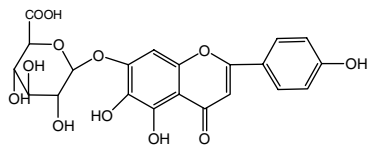
Ping Qineng, Department of Pharmaceutics, School of Pharmacy, China Pharmaceutical University, No. 24, Tongjia Xiang, Nanjing 210009, P.R. China
pingqn@cpu.edu.cn

Pharmazie 63: 75–80 (2008) *doi: 10.1691/ph.2008.7636*

This study was conducted to investigate the role of P-glycoprotein (P-gp) and Multidrug resistance-associated protein 2(Mrp2) in the rat intestinal absorption of scutellarin and explore the possible reasons for its low oral bioavailability. Verapamil had little effect on the transport amount of scutellarin shown by *in vitro* everted sac experiments and the apparent permeability of the drug demonstrated by *in situ* single-pass intestinal perfusion experiments (SPIP). Leukotriene C4 (LTC4) added to the mucosal side significantly enhanced the transport of scutellarin to the serosal side. The P_{app} value of scutellarin increased gradually on raising the L-Buthionine-[*S,R*]-sulfoximine (BSO) concentration to 0.5 mM in the perfusion solution ($P < 0.05$). When probenecid (1 mM) was coperfused, a 1.34-fold increase in the P_{app} was observed ($P < 0.05$). Coperfusion of 0.5 mM BSO and 1 mM probenecid with 4.33 μM scutellarin, the P_{app} is 2.24 times than that of the control rats ($p < 0.01$). As shown by *in silico* experiments the spatial structure of scutellarin was in good agreement with the pharmacophore of Mrp2. The efflux of Mrp2, not P-gp, in the intestinal of the rats may be one of the reasons that lead to the low oral bioavailability of scutellarin.

1. Introduction

Scutellarin, scutellarein 7-*O*-β-D-glucuronide, is the major bioactive flavonoid glucuronide isolated from a traditional Chinese medicine *Erigeron breviscapus*. It has been proved to be effective in the therapy of various ailments, such as cardiovascular diseases, sleep disorders, pain and memory impairment (Pouzet 2002; Gafner et al. 2003; Goh et al. 2005). Nowadays, it is commonly used in China in dilating blood vessels, improving hemodynamics, decreasing blood viscosity, reducing the blood platelet count and preventing platelet conglomeration (Liu et al. 2003; Chen et al. 1998; Xu and Li 1995). However, the oral bioavailability is only approximately 0.4% in beagle dogs (Ge et al. 2003). In order to improve its oral bioavailability, we (Cao et al. 2006) and others (Zhou et al. 2006) have synthesized prodrugs of scutellarin. Because the enhancement of the oral bioavailability of the prodrugs was limited, there is a growing demand for understanding the absorption mechanism of scutellarin. Until now, there are many reports on pharmacokinetics and metabolism of scutellarin in animals and humans (Gao et al. 2005; Huang et al. 2005; Chen et al. 2006). However, no study has been performed on the efflux transporters in the intestine, which might contribute to the low oral bioavailability of scutellarin. Therefore, it is necessary to study the effect of efflux transporters in the intestine of mammals to determine the possible reasons for its low oral bioavailability.



Scutellarin

P-Glycoprotein (P-gp), an ATP-binding cassette transport protein, exists constitutively in a variety of normal tissues such as the liver, kidney, small intestine, and capillary endothelium in the brain and plays an important role in the disposition of hydrophobic and cationic anticancer drugs (Raub et al. 2006; Srinivas et al. 2006). Like P-gp, multidrug resistance-associated protein 2 (Mrp2) is also expressed in almost the same tissues as P-gp. This drug-transporting protein acts as an active efflux pump for a wide range of organic anions, such as glutathione, glucuronate, and sulfate conjugates (Tian et al. 2005). Because of the anionic nature of scutellarin, it seems likely that Mrp2 contributes to the efflux of the flavonoid conjugate across the intestinal apical membrane. Mrp2 has been shown to be able to modulate the oral bioavailability of baicalein, a similar flavonoid glucuronide, by transporting it into the intestinal lumen (Akao et al. 2004).

Caco-2 cell model is a well-established model to study the absorption and related mechanisms of drugs. However, one of the major limitations of the Caco-2 model is that its expresson level of metabolizing enzymes as well as the transporters may be different from the *in vivo* situation (Sun et al. 2002). Caution should be taken when attempting to extrapolate the *in vitro* data to *in vivo* situation.

Therefore, in the present study, we explored the role of P-gp and Mrp2 in the intestinal absorption of scutellarin using everted sac systems and *in situ* intestinal perfusion experiments in male Sprague-Dawley rats. Furthermore, a computational method was used to investigate whether scutellarin is a P-gp and/or Mrp2 substrate as described by Ekins et al. (2002a) and Hirono et al. (2005).

2. Investigations, results and discussion

2.1. In vitro experiments

2.1.1. Effects of verapamil on scutellarin (0.11 mM) transport using the everted-gut sac system

We examined the effect of verapamil (P-gp inhibitor) on scutellarin (0.11 mM) transport in the jejunum and ileum (Fig. 1). Although cyclosporine A is a potent P-gp inhibitor, it may affect breast cancer resistance protein (BCRP) at the same time (Qadir et al. 2005; Gupta et al. 2006). On the other hand, verapamil did not affect the transport of BCRP substrate in human embryonic kidney (HEK) cells that overexpressed BCRP (Zhang et al. 2005). Therefore only verapamil was used as an inhibitor to determine the P-gp function of scutellarin. The amount of scutellarin transported was measured in the absence and in the presence of verapamil. No significant difference was observed with the transport of scutellarin when verapamil (200 μM) was added to the mucosal side of the jejunum, even when the concentration of verapamil was increased to 1 mM. Similarly, we did not find any difference of the transport amount when various concentrations of verapamil were added to the mucosal side of the ileum. These results indicate that P-gp has no effect on the transport of scutellarin in the intestine of rats.

2.1.2. Effects of LTC4 on scutellarin (0.11 mM) transport using the everted-gut sac system

LTC4, a high-affinity substrate of Mrp2 (Ninomiya et al. 2005), was used to study the effect of Mrp2 on the transport of scutellarin. Effects of LTC4 on scutellarin transport in the everted sacs of the rats were examined (Fig. 2). When LTC4 (0.16 μM) was added to the mucosal side of the jejunum, the increased percentages were 25%, 67%, 63%, 40% and 17% at 20, 30, 40, 50 and 60 min, respectively. A statistically significant difference was observed from 30 min to 50 min ($p < 0.05$). When the everted sacs of ileum were pre-incubated with the same concentration of LTC4, the increased percentages were 15%, 52%, 30%, 19% and 16% at 20, 30, 40, 50 and 60 min, respectively. A statistically significant difference was also observed from 30 min to 50 min ($p < 0.05$). The increased percentages of scutellarin transported through the jejunum were more than those through the ileum, which may be ascribed to the intestinal distribution of Mrp2 in the rats. Mrp2 expression level is highest in the duodenum and decreases towards ileum (Johnson et al. 2006). These results suggest that Mrp2 contributes to the scutellarin efflux from the intestinal mucosal surface of the rats.

2.2. In situ experiments

The laboratory rat intestine is one of the most absorptive organs, and encompasses *in vivo*, *in situ*, and *in vitro* experiments. *In vitro* attempts to predict *in vivo* absorption have increased by the improved Ussing Chamber (Gotoh et al. 2005). Perhaps the most widely used technique in the study of intestinal absorption of compounds is the rat SPIP model because of its proximity to *in vivo* conditions,

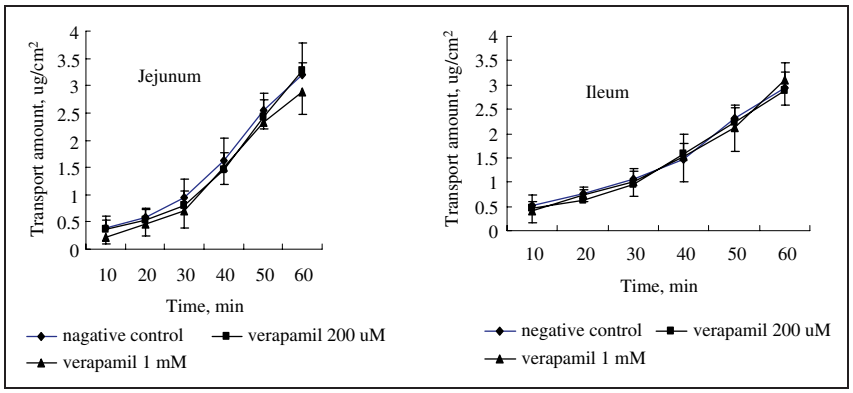


Fig. 1:
Time profile of scutellarin transport from mucosal to serosal side of the everted jejunum and ileum in the absence (◆) and presence of verapamil with a concentration of 200 μM (■) or 1 mM (▲). Each point represents the mean ± SD of three to five determinations

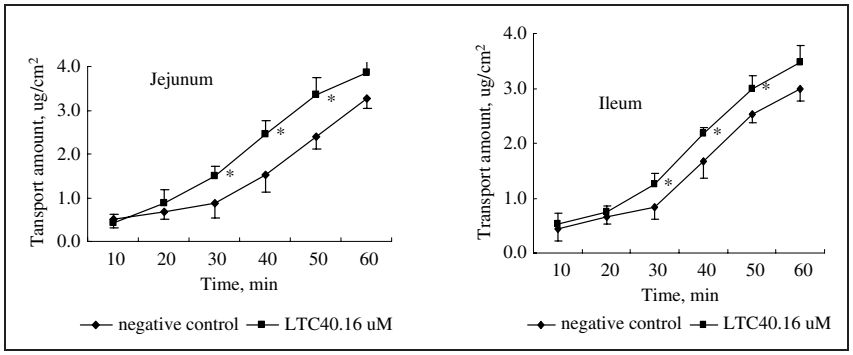


Fig. 2:
Time profile of scutellarin transport from mucosal to serosal side of the everted jejunum and ileum in the absence (◆) and presence of LTC4 at a concentration of 0.16 μM (■). Each point represents the mean ± SD of three to five determinations. * $p < 0.05$ vs the negative control

lower sensitivity to pH variations and good correlation with human absorption data. From the above *in vitro* studies, we found that Mrp2 efflux may contribute to the poor bioavailability of scutellarin. Therefore we investigated the function of P-gp and Mrp2 on scutellarin transport using the *in situ* SPIP model.

2.2.1. Physical absorption and stability of scutellarin (4.33 μM)

The physical absorption of the intestinal wall and the polyethylene tube was investigated at 37 °C for 2 h. Moreover, in order to reveal the influences of intestinal enzymes on drug, the drug's stability in the intestinal perfusate, which was prepared with fresh blank intestinal perfusate (HBSS having been perfused in the intestine of the rat for 2 h) instead of HBSS, was also studied. When the intestinal segment was added to the HBSS with scutellarin (4.33 μM), the remaining percentage of scutellarin after 2 h was $98.31 \pm 1.23\%$ ($n = 3$), indicating that the intestinal wall had almost no physical absorption of scutellarin. $99.10 \pm 1.83\%$ ($n = 3$) of scutellarin remained after 2 h incubation in the polyethylene tube. As scutellarin was incubated in the freshly prepared blank intestinal perfusate at 37 °C for 2 h, the degradation percentage of scutellarin was $2.10 \pm 0.56\%$ ($n = 3$). These results demonstrated that the loss of drug from the perfusion was due to absorption (Cook et al. 2003).

2.2.2. Effects of verapamil on scutellarin (4.33 μM) transport using the SPIP system

Because P-gp is highly expressed on the apical surface of the ileal and colonic epithelial cells, and expression gradually decreases toward the stomach (Mouly and Paine 2003), the ileum segment of the rats was selected to investigate the function of P-gp. The SPIP method is based on reaching steady-state with respect to the diffusion of compound across the intestine. Steady-state is confirmed by plotting the ratio of the outlet to inlet concentrations (corrected for water transport) versus time. In our experiments, the steady-state was usually reached within 30 min. Permeability values are calculated only from experiments where steady-state was achieved. The permeablity values for each experiment are shown in Fig. 3. There was no statistical difference in P_{app} of scutellarin in the ileum at each concentration of verapamil. From the experiments of everted intestinal sac and SPIP it can be concluded that P-gp seems to have no effect on the intestinal transport of scutellarin in the rats.

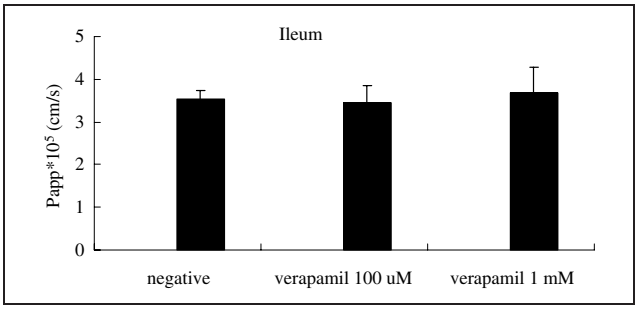


Fig. 3: The apparent permeability of scutellarin (4.33 μM) across ileum using in situ single-pass intestinal perfusion system in the absence and presence of verapamil with a concentration of 200 μM or 1 mM. Each point represents the mean ± SD of three to five determinations

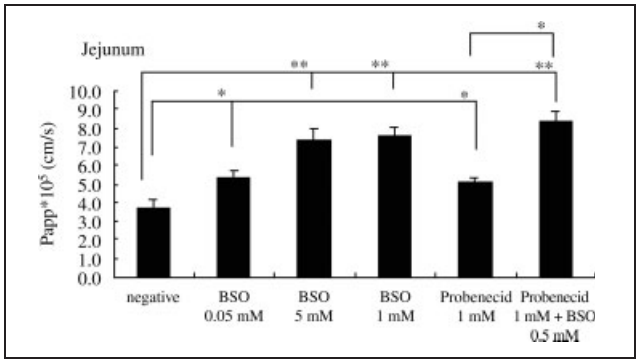


Fig. 4: The apparent permeability of scutellarin (4.33 μM) across jejunum using in situ single-pass intestinal perfusion system in the absence and presence of 0.05 mM BSO, 0.5 mM BSO, 1 mM BSO, 1 mM probenecid or 0.5 mM BSO combined with 1 mM probenecid. Each point represents the mean ± SD of three to five determinations. * $p < 0.05$, ** $p < 0.01$

2.2.3. Effects of BSO and probenecid on scutellarin (4.33 μM) transport using the SPIP system

Mrp2 is also expressed at the apical surface but its expression level is highest in the duodenum and expression decreases toward ileum (Johnson et al. 2006). The jejunum segments of the rats were selected to investigate whether scutellarin is actively secreted into the intestine by Mrp2 and the secrection is inhibited by the presence of Mrp2 inhibitors. BSO and probenecid were used as inhibitors of Mrp2. The results were summarized in Fig. 4. The P_{app} of scutellarin in the jejunum of the negative rats was 3.73×10^{-5} cm/s and the P_{app} values increased gradually when the concentration of BSO was increased to 0.5 mM ($P < 0.05$). When BSO concentration was 1 mM, the P_{app} was 7.55×10^{-5} cm/s and showed no significant difference compared with that of the rats co-perfused with BSO of 0.5 mM, suggesting a saturated efflux process. When probenecid (1 mM) was used as an Mrp2 inhibitor and co-perfused, a 1.34-fold increase in the P_{app} was observed compared to the control rats ($P < 0.05$). Coperfusion of 0.5 mM BSO and 1 mM probenecid with 4.33 μM scutellarin, the P_{app} is 2.24 times than that of the control rats ($p < 0.01$).

Taken together, these results are consistent with our observation in everted-gut sac system, where P-gp may not be involved in the transport of scutellarin in the rat small intestine. Efflux via Mrp2 is more likely the major mechanism that impedes the permeation of the drug.

2.3. In silico experiments

P-gp and Mrp2 are poorly characterized at the molecular level, in large part because of the intrinsic difficulties involved in membrane protein crystallization. As an alternative, computational modeling of transporters has aided our understanding and has significantly increased our knowledge about transporter mechanisms. The application of pharmacophore modeling to determine substrate or inhibitor specificity has greatly advanced our mechanistic understanding of drug-transporter interactions.

The recognition elements for P-gp are formed by two (type I unit) or three electron donor groups (type II unit) with distinct spatial arrangements (Seelig 1998). For the type I pattern, two electron donors are separated by 2.5 ± 0.3 Å. The type II patterns contain either two electron donor groups separated by 4.6 ± 0.6 Å or three electron donors separated by 2.5 ± 0.3 Å with a 4.6 ± 0.6 Å separa-

tion of the outer two groups. A molecule with at least one type I or type II unit is predicted to be transported by P-gp. The optimized structure of scutellarin was obtained by the Gaussian 03 W program, and the highest average frequency of the groups involved in functional units with P-gp was selected. The spatial separation of two electron donor groups, oxygen from C=O group and oxygen in pyranoid ring, is 4.12 Å, consistent with the type II pattern definition, suggested that scutellarin is a potential substrate of P-gp. With the understanding that there may be multiple binding sites within P-gp, a more detailed 3D-QSAR model contained hydrogen bond acceptors, hydrogen bond donors, hydrophobes, and ring aromatic features of the P-gp substrates was developed by Ekins et al. (2002a, 2002b). They have shown five P-gp pharmacophores, but the spatial separation of scutellarin is not consistent with any of the pharmacophores. Furthermore, this compound contains six electron donor groups (OH). According to Seelig (1998), substrate binding to P-gp increases with the number and strength of the hydrogen bonding acceptor units and these groups are not specifically favorable for an interaction with P-gp. Taken together with our *in vitro* and *in situ* results, this compound does not seem to be a substrate of P-gp.

There is only one paper describing the three-dimensional pharmacophore of ligands for rat multidrug-resistance-associated protein 2 (Hirono et al. 2005). It was shown that two hydrogen bond-acceptor groups (HA1 and HA2) and two hydrophobic groups (HP1 and HP2) are essential for the binding of ligands to rat Mrp2 and that these groups constitute the 3D pharmacophore obtained from the comparative molecular-field analysis (CoMFA) calculation. The Table shows the relative distances between the four property spheres that represent the essential functional groups for ligand binding. For scutellarin, two hydrogen bond-acceptor groups, HA1 and HA2, are the anion of the carboxyl group and the oxygen from the glycosidic bonds, respectively. Two hydrophobic groups (HP1 and HP2) are alicyclic ring of the glucuronide group and the aromatic ring neighbouring the glycosidic bonds, respectively. The spatial separations of different pharmacophore groups of scutellarin are shown in the Table and they are all consistent with spatial arrangements, which is necessary to interact with Mrp2. This computational method demonstrated that scutellarin was non-P-gp substrate, but Mrp2 substrate, and also confirmed the results of transport studies both *in vitro* and *in situ*. These results are consistent with the earlier findings with a similar flavonoid glucuronides by Akao et al. (2004).

In conclusion, the present study demonstrates that Mrp2, but not P-gp, is involved in the efflux of scutellarin in the intestine of the rats. In the everted-gut sac system, the addition of verapamil into the mucosal side did not increase the transport amount of scutellarin. LCT4, an Mrp2 inhibitor, significantly enhance the transport amount of scutellarin from mucosal side to serosal side. In the SPIP system, coperfusion verapamil with scutellarin showed no influence on the P_{app} of scutellarin. When BSO or/and probenecid was coperfused, a signifincant increase in the P_{app} of scutellarin was observed. Moreover, computational studies suggested that the spatial structure of scutellarin was consistent with the pharmacophore of Mrp2. These results demonstrated that Mrp2, not P-gp, was involved in the intestinal absorption of scutellarin in the rats, which might be one of the reasons that lead to the low oral bioavailability of the drug. On the other hand, Bcrp is expressed across the different regions of the intestine. Bcrp also has an important role in extruding glucuronide and sulfate conjugates into the intestinal lumen (Adachi et al. 2005). The substrates of Bcrp and Mrp2 often overlap and a synergistic role of these two efflux transporters exists in extruding xenobiotics. Because scutellarin is a flavonoid glucuronide, further studies should be performed to study the effect of Bcrp on the intestinal absorption of scutellarin.

Table: Relative distances of reported (Hirono et al. 2005) and calculated (for scutellarin) between the four spheres representing the functional groups

	Relative distances reported (Å)	Actual distances of scutellarin (Å)
HA1-HA2	5.0 ± 0.9	4.1–4.3
HA1-HP1	5.3 ± 0.7	5.8–6.1
HA1-HP2	5.5 ± 1.6	3.9–4.3
HP1-HA2	4.7 ± 0.8	3.7–4.0
HP1-HP2	4.8 ± 1.1	4.7–5.4
HA2-HP2	3.2 ± 1.2	2.8–3.0

3. Experimental

3.1. Materials

Scutellarin, 4′,5,6-trihydroxyflavone-7-glucuronide (purity >97%, lot number: HB 20031203) was purchased from Yunnan phytopharmaceutical CO., LTD (China). Verapamil was purchased from National institute for control of pharmaceutical and biological products. leukotriene C4 (LTC4) and L-Buthionine-[*S*,*R*]-sulfoximine (BSO) were purchased from Sigma-Aldrich (St Louis, MO). Probenecid was bought from Integrated Pharmaceutical Factory of Shanghai. All reagents were of analytical grade and were used without further purification. Distilled and deionized water was used for the preparation of all solutions. Male SD rats weighing about 250–300 g were purchased from the Laboratory Animal Center of China Pharmaceutical University. The animal experiments in this study were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals, and approved by the Institutional Animal Care and Use Committee of China Pharmaceutical University.

3.2. Transport studies using the in vitro everted-gut sac system

The studies were carried out using everted sacs prepared by a modification of a previously described procedure (Nakamaru et al. 1998). In brief, rats were fasted for 20–24 h prior to the experiments but received water *ad libitum*. They were anesthetized with diethylether and sacrificed by exsanguination via the abdominal aorta. Then, segments of the jejunum and ileum were removed from the site 5 cm away from the ligament of Treiz and above the cecum, respectively. Each segment was rinsed with cold saline, and then everted using an L-shaped stainless steel rod. One end of the everted sac was ligated with silk thread, and a polyethylene tube connected to a silicon tube was inserted into the other end and tied. The segments of approximately 5 cm in length were filled with 0.5 ml of Krebs Ringer-Henseleit bicarbonate buffer (118 mM NaCl, 4.75 mM KCl, 2.50 mM $CaCl_2$, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4$, and 25 mM $NaHCO_3$, pH 6.8 adjusted with 1 M H_3PO_4) containing 0.3% (w/v) Na_2SO_3 to improve the stability of scutellarin (The degradation percent of scutellarin in the buffer was 0.4% within 60 min at 37 °C). Subsequently, the sac was placed in 10 mL of the same buffer. Gas (95% O_2–5% CO_2) was gently bubbled into the solution on the mucosal side during the transport experiments. After a 10 min preincubation at 37 °C, a stock solution of scutellarin in the above buffer was added to the mucosal side to give a final concentration of 0.11 mM scutellarin. At 0, 10, 20, 30, 40, 60 min of incubation after the start of the experiments, 0.1 mL of solution was taken from the serosal side to determine the absorption of scutellarin and, at the same time, a similar volume of buffer solution was added to maintain the volume of the serosal side constant. All samples were stored at −20 °C until analysis. At the end of the incubation period, the sac was quickly removed, and the surface area of the sacs was measured.

In determining the function of transporters, the everted sacs were pre-incubated in the buffer containing verapamil (200 μM or 1 mM) as P-gp blocker and LTC4 (0.16 μM) as Mrp2 inhibitor (Hu et al. 2003) for 10 min at 37 °C, respectively, and then mucosal-to-serosal transport of scutellarin was examined under the presence of each inhibitor added to the mucosal side. LTC4 was dissolved in dimethylsulfoxide (the final concertration of dimethylsulfoxide in the incubation solution was 0.4%). The buffer containing 0.4% dimethylsulfoxide was also used as vehicle for the control experiments. The viability of the intestinal membrane during the test period

was monitored by measuring the liberation of the cytosolic enzyme LDH in the incubation media (Cornaire et al. 2004). There was no remarkable change of the LDH (data not shown), confirming that the viability of the intestinal membrane was maintained during the transport experiments.

3.3. Transport studies using the in situ single-pass intestinal perfusion system

3.3.1. Binding and stability studies

The following study was carried out to ensure that the loss of drug from the perfusion is due to absorption and not by other losses. Scutellarin binding studies were performed with the polyethylene tube used for perfusion directly incubated in the Hanks Balanced Salt Solution (HBSS pH 6.8 adjusted with 1 M H_3PO_4, containing 137 mM NaCl, 5.40 mM KCl, 1.30 mM $CaCl_2$, 0.80 mM $MgSO_4$, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 5.56 mM D-Glucose, 4.90 mM $NaHCO_3$ and 0.3% (w/v) Na_2SO_3 to improve the stability of scutellarin) with 4.33 μM scutellarin at 37 °C for 2 h, and the remaining scutellarin was determined by HPLC. Similarly, the physical absorption on the intestinal wall was investigated at 37 °C for 2 h. Scutellarin stability was tested in the blank perfusion HBSS. Scutellarin (4.33 μM) was incubated in the mixture at 37 °C for 2 h. Samples (0.2 ml) of the mixture were removed at 2 h, spiked with 0.2 ml ice-cold menthol with 1% acetic acid, and centrifuged (1000 × g, 10 min). The supernatant was assayed by HPLC.

3.3.2. Establishment of in situ single-pass intestinal perfusion system

In situ single-pass perfusion was conducted as described previously (Issa et al. 2003; Sutton et al. 2002). Male Sprague-Dawley rats weighing 250 to 300 g were fasted for 12 to 15 h prior to the start of the experiment. Water was allowed *ad libitum*. Anesthesia was induced and maintained for the duration of the experiment by intraperitoneal injection of urethane at a dose of 1.5 g/kg. A midline incision was made on the abdomen and an approximately 10 cm length of jejunum or ileum was selected and cannulated on both sides. The animals were maintained at 37 °C throughout the experiment by focusing a table lamp as a source of heat. The exposed segment was covered with a cotton pad soaked in normal saline and then with aluminum foil to prevent evaporation of fluids. Initially, the intestinal segment was washed with freshly prepared HBSS at 37 °C until the outlet solution was clear. Thereafter the perfusion solution (4.33 μM scutellarin in the HBSS) was perfused at a constant flow rate of 0.4 ml/min using a peristaltic pump (HL-2B, Shanghai Medical University Instrument Plant, China). The intestinal perfusate samples were collected at 15-min intervals for a duration of 105 min in preweighed 5-mL glass vials. The length and radius of intestinal segment studied was measured at the end of the experiment. Finally, the animals were sacrificed by excess of ether.

3.3.3. Inhibitory studies

In determining the function of P-gp, ileums were pre-incubated in the HBSS containing verapamil (100 μM or 1 mM) as P-gp blocker for 30 min at 37 °C. In order to study the efflux transporter of Mrp2, BSO (Dietrich et al. 2001) and probenecid (Lindahl et al. 2004) were used as inhibitors. Six jejunum segments of the rats were pre-incubated for 30 min at 37 °C in the HBSS containing: (a) BSO (0 mM) + probenecid (0 mM), (b) BSO (0.05 mM), (c) BSO (0.5 mM), (d) BSO (1 mM), (e) probenecid (1mM), (f) BSO (0.5 mM) + probenecid (1mM).

3.3.4. Sample treatment

An aliquot (0.5 ml) of each perfusate sample was mixed with 0.5 ml of methanol containing 1% acetic acid, and centrifuged (1000 × g, 10 min). The supernatant was assayed by HPLC.

3.3.5. Data analysis

The absorption/secretion of water during the experiment was studied by correcting for density changes. The density of collected samples was determined by weighing the contents (using an electronic weighing balance of a known volume of perfusate). Calculation of P_{app} (apparent permeability) was done from intestinal perfusate samples collected over 45 to 105 min with steady-state concentrations of the outlet perfusate. The P_{app} of scutellarin was calculated using Eq. (1):

$$P_{app} = [-Q_{in} \ln (C_{out(corr)}/C_{in})]/2\pi rl \quad (1)$$

where r is the radius of the intestinal lumen (cm), *l* is the length of the segment (cm), Q_{in} is the flow rate (ml/min) of inlet solution, C_{in} is the concentration (μg/ml) of drug in the entering solution, and $C_{out(corr)}$ is the concentration (μg/ml) of drug in the exiting solution corrected for water flux. The $C_{out(corr)}$ was calculated using Eq. (2):

$$C_{our(corr)} = C_{out}V_{out}/V_{in} \quad (2)$$

where C_{out} is the outlet drug concentration, V_{out} is the outlet volume, and V_{in} is the inlet volume.

Statistically significant differences between two groups was evaluated by the Student's t test. A $p < 0.05$ was considered significant for all tests.

3.4. Computational method

This study was performed according to a procedure described previously (Wiwattanawongsa et al. 2005). The three-dimensional structure of the compound was built using Chem3D Ultra version 7 software (CambridgeSoft, Cambridge, MA, USA), and molecular calculation was directly performed using the Gaussian 03 W program (Gaussian, Inc., Pittsburgh, PA, USA) on the Chem3D window. Geometry optimization was performed initially by molecular mechanics force field and subsequently using the AM1 Hamiltonian of a semiempirical method. The obtained geometries were then optimized on the basis of the *ab initio* Hartree-Fock method at the 6–21G level. The spatial separation of two electron donor groups of scutellarin was subsequently measured.

3.5. Instrumentation and HPLC conditions

The HPLC system consisted of a pump (Model LC-10A, Shimadzu, Japan), a shim-pack CLC-ODS column (150 mm × 6 mm i.d., Shimadzu) maintained at 30 °C, an UV detector (Model SPD-10A, Shimadzu) at 334 nm and a data station (Model SCL-10A, Shimadzu). This method was used to analyze the samples obtained from the studies *in vitro* and *in vivo*. The composition of the mobile phase was methanol-water-phosphoric acid-triethylamine (53 : 47 : 0.1 : 0.1, v : v : v : v). The mobile phase was delivered at a flow rate of 1 ml/min. The injection volume was 20 μl and the relative retention time was found to be about 10.0 min.

In this article, two HPLC methods with the same HPLC condition were developed. One method was used to analyze the serosal samples of the *in vitro* everted-gut sac system. Another method was used to analyze the perfusate samples of *in situ* single-pass intestinal perfusion system. Both methods were evaluated through intra-day and inter-day analysis of precision and accuracy. For both methods, all the precisions were less than 3.27%, and all the recoveries were evaluated to be 97.13–102.44%. In addition, all the linear correlation coefficients (r) were more than 0.9997, and all the samples' concentrations were in the concentration range.

Acknowledgements: The authors would like to thank Dr. Wang of Institute of Theoretical and Computational Chemistry Nanjing University for the help in the computational studies. This research was granted by NSFC with project number of 30430790.

References

Adachi Y, Suzuki H, Schinkel AH, Sugiyama Y (2005) Role of breast cancer resistance protein (Bcrp1/Abcg2) in the extrusion of glucuronide and sulfate conjugates from enterocytes to intestinal lumen. Mol Pharmacol 67: 923–928.

Akao T, Sakashita Y, Hanada M, Goto H, Shimada Y, Terasawa K (2004) Enteric excretion of baicalein, a flavone of Scutellariae Radix, via glucuronidation in rat: involvement of multidrug resistance-associated protein 2. Pharm Res 21: 2120–2126.

Cao F, Guo JX, Ping QN, Liao ZG (2006) Prodrugs of scutellarin: ethyl, benzyl and N,N-diethylglycolamide ester synthesis, physicochemical properties, intestinal metabolism and oral bioavailability in the rats. Eur J Pharm Sci 29: 385–393.

Chen X, Cui L, Duan X, Ma B, Zhong D (2006) Pharmacokinetics and metabolism of the flavonoid scutellarin in humans after a single oral administration. Drug Metab Dispos 34: 1345–1352.

Chen XX, He B (1998) Effects of breviscapine on the changes in antioxidant enzyme activity induced by cerebral ischemia reperfusion in rats. J Chi Pharm Sci 7: 91–93.

Cook TJ, Shenoy SS (2003) Intestinal permeability of chlorpyrifos using the single-pass intestinal perfusion method in the rat. Toxicology 184: 125–133.

Cornaire G, Woodley J, Hermann P, Cloarec A, Arellano C, Houin G (2004) Impact of excipients on the absorption of P-glycoprotein substrates in vitro and in vivo. Int J Pharm 278: 119–131.

Dietrich CG, de Waart DR, Ottenhoff R, Schoots IG, Elferink RP (2001) Increased bioavailability of the food-derived carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine in MRP2-deficient rats. Mol Pharmacol 59: 974–980.

Ekins S, Kim RB, Leake BF, Dantzig AH, Schuetz EG, Lan LB, Yasuda K, Shepard RL, Winter MA, Schuetz JD, Wikel JH, Wrighton SA (2002a) Application of three-dimensional quantitative structure-activity relationships of P-glycoprotein inhibitors and substrates. Mol Pharmacol 61: 974–981.

Ekins S, Kim RB, Leake BF, Dantzig AH, Schuetz EG, Lan LB, Yasuda K, Shepard RL, Winter MA, Schuetz JD, Wikel JH Wrighton SA (2002b) Three-dimensional quantitative structure-activity relationships of inhibitors of P-glycoprotein. Mol Pharmacol 61: 964–973.

Gafner S, Bergeron C, Batcha LL, Reich J, Arnason JT, Burdette JE, Pezzuto JM, Angerhofer CK (2003) Inhibition of [3H]-LSD binding to 5-HT7 receptors by flavonoids from *Scutellaria lateriflora*. J Nat Prod 66: 535–537.

Gao HM, Wang ZM, Tian J (2005) Pharmacokinetics and metabolites of scutellarin in normal and model rats. Yao Xue Xue Bao 40: 1024–1027.

Ge QH, Zhou Z, Zhi XJ, Ma LL, Chen XH (2003) Pharmacokinetics and absolute bioavailability of breviscapine in beagle dogs. Chi J Pharm 34: 618–620.

Goh D, Lee YH, Ong ES (2005) Inhibitory effects of a chemically standardized extract from Scutellaria barbata in human colon cancer cell lines, LoVo. J Agric Food Chem 53: 8197–8204.

Gotoh Y, Kamada N, Momose D (2005) The advantages of the Ussing chamber in drug absorption studies. J Biomol Screen 10: 517–523.

Gupta A, Dai Y, Vethanayagam RR, Hebert MF, Thummel KE, Unadkat JD, Ross DD, Mao Q (2006) Cyclosporin A, tacrolimus and sirolimus are potent inhibitors of the human breast cancer resistance protein (ABCG2) and reverse resistance to mitoxantrone and topotecan. Cancer Chemother Pharmacol 58: 374–383.

Hirono S, Nakagome I, Imai R, Maeda K, Kusuhara H, Sugiyama Y (2005) Estimation of the three-dimensional pharmacophore of ligands for rat multidrug-resistance-associated protein 2 using ligand-based drug design techniques. Pharm Res 22: 260–269.

Hu M, Chen J, Lin H (2003) Metabolism of flavonoids via enteric recycling: mechanistic studies of disposition of apigenin in the Caco-2 cell culture model. J Pharmacol Exp Ther 307: 314–321.

Huang JM, Weng WY, Huang XB, Ji YH, Chen E (2005) Pharmacokinetics of scutellarin and its aglycone conjugated metabolites in rats. Eur J Drug Metab Pharmacokinet 30 : 165–70.

Issa C, Gupta P, Bansal AK (2003) Implications of density correction in gravimetric method for water flux determination using rat single-pass intestinal perfusion technique: a technical note. AAPS PharmSci Tech 4: E16.

Johnson BM, Zhang P, Schuetz JD, Brouwer KL (2006) Characterization of transport protein expression in multidrug resistance-associated protein (mrp) 2-deficient rats. Drug Metab Dispos 34: 556–562.

Lindahl A, Sjoberg A, Bredberg U, Toreson H, Ungell AL, Lennernas H (2004) Regional intestinal absorption and biliary excretion of fluvastatin in the rat: possible involvement of mrp2. Mol Pharm 1: 347–356.

Liu H, Yang XL, Wang Y, Tang XQ, Jiang DY, Xu HB (2003) Protective effects of scutellarin on superoxide-induced oxidative stress in rat cortical synaptosomes. Acta Pharmacol Sin 24: 1113–1117.

Mouly S, Paine MF (2003) P-glycoprotein increases from proximal to distal regions of human small intestine. Pharm Res 20: 1595–1599.

Nakamaru M, Masubuchi Y, Narimatsu S, Awazu S, Horie T (1998) Evaluation of damaged small intestine of mouse following methotrexate administration. Cancer Chemother Pharmacol 41: 98–102.

Ninomiya M, Ito K, Horie T (2005) Functional analysis of dog multidrug resistance-associated protein 2 (Mrp2) in comparison with rat Mrp2. Drug Metab Dispos 33: 225–232.

Pouzet B (2002) SB-258741: a 5-HT7 receptor antagonist of potential clinical interest. CNS Drug Rev 8: 90–100.

Qadir M, O'Loughlin KL, Fricke SM, Williamson NA, Greco WR, Minderman H, Baer MR (2005) Cyclosporin A is a broad-spectrum multidrug resistance modulator. Clin Cancer Res 11: 2320–2326.

Raub TJ (2006) P-glycoprotein recognition of substrates and circumvention through rational drug design. Mol Pharm 3: 3–25.

Seelig A (1998) A general pattern for substrate recognition by P-glycoprotein. Eur J Biochem 251: 252–261.

Srinivas E, Murthy JN, Rao AR, Sastry GN (2006) Recent advances in molecular modeling and medicinal chemistry aspects of phospho-glycoprotein. Curr Drug Metab 7: 205–217.

Sun D, Lennernas H, Welage LS, Barnett JL, Landowski CP, Foster D, Fleisher D, Lee KD, Amidon GL (2002) Comparison of human duodenum and Caco-2 gene expression profiles for 12,000 gene sequences tags and correlation with permeability of 26 drugs. Pharm Res 19: 1400–1416.

Sutton SC, Rinaldi MT, McCarthy JM, Vukovinsky KE (2002) A statistical method for the determination of absorption rate constant estimated using the rat single pass intestinal perfusion model and multiple linear regression. J Pharm Sci 91: 1046–1053.

Tian Q, Zhang J, Chan E, Duan W, Zhou SF (2005) Multidrug Resistance Proteins (MRPs) and Implication in Drug Development. Drug Dev Res 64: 1–18.

Wiwattanawongsa K, Tantishaiyakul V, Lomlim L, Rojanasakul Y, Pinsuwan S, Keawnopparat S (2005) Experimental and computational studies of epithelial transport of mefenamic acid ester prodrugs. Pharm Res 22: 721–727.

Xu QY, Li XX (1995) Influence of DZXX injection on blood viscosity in patients with high blood viscosity. New Drugs Clin Remed 14: 233.

Zhang Y, Gupta A, Wang H, Zhou L, Vethanayagam RR, Unadkat JD, Mao Q (2005) BCRP transports dipyridamole and is inhibited by calcium channel blockers. Pharm Res 22: 2023–2034.

Zhou Q, Jiang X, Li K, Fan X (2006) Synthesis, characterization and in situ intestinal absorption of different molecular weight scutellarin-PEG conjugates. Pharmazie 61: 660–663.