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Excipients enhance intestinal absorption of ganciclovir by P-gp inhibition: Assessed *in vitro* by everted gut sac and *in situ* by improved intestinal perfusion

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ABSTRACT

In rats we examined the effects of some common excipients on the intestinal absorption of ganciclovir (GCV), a BCS-III drug and substrate of P-gp, by assessing its *in vitro* transfer from mucosa to serosa and *in situ* transepithelial permeation. *In vitro*, all selected excipients (concentration range 0.1–1% [w/v]) could increase the transport amount of GCV in the everted gut sac model. Whereas enhancement by F-68 demonstrated regional differences like verapamil, PEG-400, Tween-80 and EL-35 exhibited no regional differences. *In situ* studies were performed by an improved perfusion model, single-pass perfusion with whole small intestine, to determine more accurately the permeability of lipophobic compounds. The permeability of GCV was significantly increased by all excipients. The effects of EL-35 and F-68 were dose-dependent but those of PEG-400 and Tween-80 were not. The results suggest that enhancements of intestinal absorption of GCV by these excipients are probably due to inhibition of P-gp-mediated drug efflux. It could be deduced from their different properties that both blocking binding sites of P-gp and altering membrane fluidity were involved in their P-gp-inhibition. The former mechanism might be involved for F-68, while the latter one might account for the effects of PEG-400, Tween-80 and EL-35.

1. Introduction

P-glycoprotein (P-gp) represents an important membrane transporter pumping a wide range of structurally unrelated drugs and xenobiotics out of cells (Ueda et al., 1999). On the intestinal level, it is located in the apical membrane of the epithelial cells and it transports drugs back into the gut lumen. Studies in animals and human have indicated that P-gp plays a major role in limiting drug absorption and consequently oral bioavailability (Terao et al., 1996; Greiner et al., 1999). These effects have restricted the clinical use of drugs as well as the development of new chemical entities (NCEs) which are substrates of P-gp. Thus, there is considerable interest in trying to enhance their absorption and oral bioavailability by inhibiting the P-gp-mediated drug efflux.

Several studies have demonstrated that using P-gp inhibitors, e.g. verapamil and cyclosporine can improve the bioavailability of a number of rewarding molecules (Chiou et al., 2002; Bansal et al., 2009). However, such inhibitors have *per se* pharmacological activities and consequently may cause toxic side effects. So far, even the third generation P-gp inhibitors like XR9576 and LY335979, which inhibit P-gp with more specificity, still have many disadvantages (Varma et al., 2003). For example, they are absorbed into the blood and will interact with P-gp in the body wherever it is expressed.

Pharmaceutical excipients, which are largely used as inert vehicles in drug formulations, are emerging as a special class of P-gp inhibitors (Varma et al., 2003). Several studies have demonstrated that some of them may disrupt the function of intestinal P-gp and thus enhance the intestinal absorption of the drugs which are substrates of P-gp. For instance, Pluronic P85 was found to increase the permeability of a broad spectrum of drugs in Caco-2 cell monolayers (Batrakova et al., 1999), and it also enhanced drug absorption in the Ussing chamber (Johnson et al., 2002).

Because excipients could inhibit P-gp-mediated drug efflux they could offer new opportunities to develop oral formulations of poorly absorbed drugs that are clinically useful. Since most P-gp-inhibiting excipients reported are non-absorbable, they will inhibit P-gp only in the gastrointestinal tract and there will be no unnecessary inhibition in other organs expressing P-gp. Furthermore, due to their outstanding safety, they have already been approved for routine use in pharmaceutical drug formulations (Varma et al., 2003). Based on these advantages and compared to other P-gp inhibitors, excipients seem to be a better choice.

Our previous study (unpublished data) has screened in Caco-2 monolayers 20 commonly used excipients, including surfactants, cyclodextrin, phospholipids, polyethylene glycol and polyvidone to select some more efficient P-gp inhibitors. Polysorbate 80 (Tween-80), Polyethylene glycol 400 (PEG-400), Cremophor EL 35 (EL-35)

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and Pluronic block copolymer F68 (F-68) were chosen for further *in vitro* and *in situ* studies.

In the present study, ganciclovir (GCV), an acyclic nucleoside analog with virostatic activity against cytomegalovirus (CMV), was selected as a model drug. GCV is not only the substrate of P-gp (Wu et al., 2004), but also has an extremely poor lipophilicify (Log $P = -1.7$). It is not metabolized in the intestine and liver (Loregian et al., 2001). Thus, it is an ideal probe for studying the effects of excipients on intestinal P-gp while avoiding the influences of intestinal CYP-enzymes. Verapamil was chosen as comparative P-gp inhibitor, as it has been used in numerous studies of P-gp-mediated drug transport (Sababi et al., 2001). Rat everted gut sac and single-pass intestinal perfusion (SPIP) were employed for *in vitro* transport and *in situ* permeation studies, respectively. The former has already been proved to be a useful *in vitro* tool to evaluate the role of P-gp in drug absorption (Barthe et al., 1998), while the latter is the best model mimicking the *in vivo* situation. Furthermore, the SPIP model has been well elucidated for its correlation with human absorption and it is well suitable for evaluating the intestinal absorption without the influence of hepatic first-pass metabolism (Lennernas, 2007; Ehrhardt and Kim, 2008). It is commonly applied for permeability study by using short length (15 cm) of small intestine (the common model). However in practice, it is difficult to detect the minute absorption of poorly permeable compounds such as BCS-III/IV drugs. Therefore, we extended the length by using the whole small intestine for a more accurate determination of permeability (the improved model). The improved model was evaluated by the determination of LDH release and electron microscopy of intestinal epithelium. Its efficiency was validated by comparison with the common model using GCV and acyclovir (ACV), another BCS-III drug.

The aims of the present work are: 1. to validate the single-pass perfusion with whole small intestine and to evaluate its efficiency; 2. to investigate the effects of excipients on the intestinal absorption of GCV *in vitro* and *in situ*; 3. to test the potential of excipients to inhibit intestinal P-gp which finally could improve oral bioavailability of certain drugs.

2. Materials and methods

2.1. Materials

Ganciclovir (GCV) and Acyclovir (ACV) were purchased from Keyi Pharmaceutical Co. Ltd. (China). Verapamil was obtained from Centralpharm Inc. (China). Pluronic F68 was from BASF China Ltd. (China). Tween-80 and Cremophor EL35 were purchased from Cognis UK Ltd. (UK). PEG-400 was obtained from Dow Chemical Ltd. (USA). Solvents were of HPLC grade. Other reagents were of AR grade.

2.2. Animals

Male Sprague–Dawley rats (200–270 g), obtained from the Experimental Animal Center of Tongji Medical College, Huazhong University of Science and Technology (China), were maintained in an animal facility with a 12–12 h light/dark cycle and free access to food and water. All animal experiments were performed in accordance with the National Guidelines for Animal Experimentation.

2.3. Assay of ganciclovir and acyclovir

GCV was measured by reverse-phase HPLC (Agilent 1100, America), using a Eurospher-100 C_{18} column (250 mm × 4.6 mm, 5 μm) at 40 °C. Analysis was carried out at a wavelength of 254 nm with ACV as internal standard. The mobile phase consisted of a mixture of methanol and water (20:400, v/v) and its flow rate was 0.8 ml/min. For the analysis of GCV in the serosal medium of everted gut sac model, direct measurement was carried out adding the internal standard followed by brief centrifugation (Fig. 1A). For analysis of the effluent perfusates in the perfusion study, a precipitation step was required: 0.1 ml of perfusates was mixed with methanol (0.3 ml) and the internal standard. The supernatant was transferred to a clean tube and evaporated to dryness under nitrogen. The residue was dissolved in the mobile phase for injection into the HPLC (Fig. 1B). The analytical procedures of ACV samples were similar to those of GCV by using GCV as the internal standard.

2.4. Everted gut sac model

2.4.1. Preparation of gut sac

The everted gut sac method was performed as previously described (Yumoto et al., 1999). Briefly, male rats fasted overnight (free access to water) were anesthetized and the intestinal segments of interest were identified (duodenum, starting 2 cm below the pylorus; jejunum, starting from 20 cm below the pylorus; ileum, starting 20 cm above ileocecal junction) and isolated. Then, the rats were sacrificed with an overdose of anesthesia. Each intestinal segment was flushed with cold saline and immediately placed in 37 °C oxygenated (O_2/CO_2, 95%:5%) buffer solution; subsequently gently everted over using a glass rod. One end of the segment was clamped

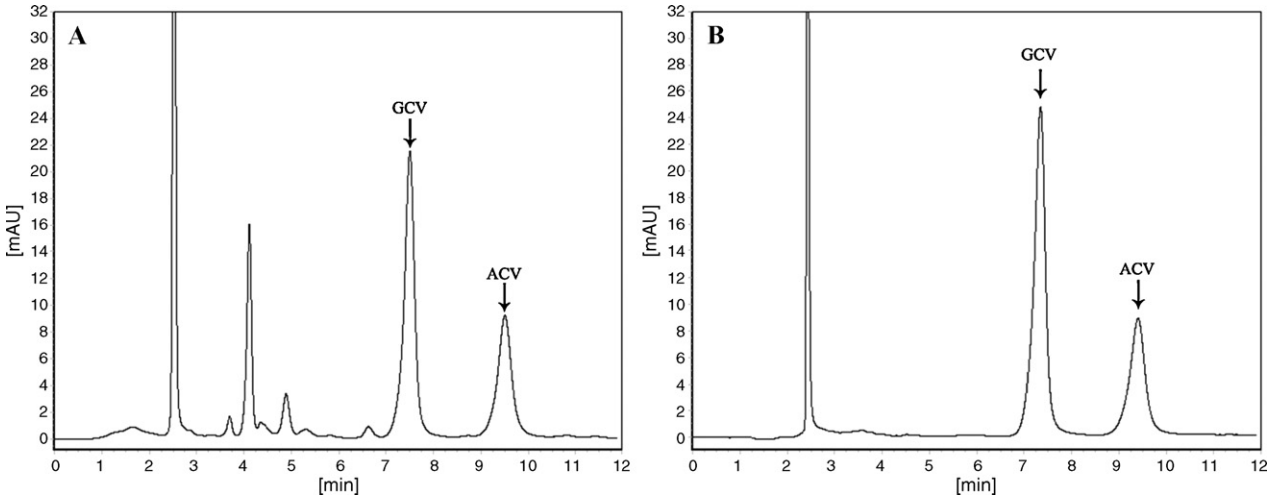

Fig. 1. Chromatograms of GCV and ACV. (A) Sample of serosal medium from everted gut sac model; (B) sample of effluent perfusates from perfusion model.

and tied with a silk thread forming a sac, while the other end was attached to a sampler. After the blank solution (1 ml) was introducing into the everted sac (serosal side), a 10-cm-long everted gut sac was prepared.

2.4.2. LDH release of the everted gut sac

To investigate the viability and the degree of damage of the gut, the release of lactate dehydrogenase (LDH), a cytosolic enzyme, was monitored (Brown et al., 2002). Its concentration in the incubation medium of mucosal side was measured with a kit supplied by Jiangcheng Biotech Institute (China). A control group was designed to test from 0 min to 240 min in intervals of 30 min the time course of LDH release without adding any enhancer. The influence of verapamil (50 μg/ml) or excipients (using the maximum concentration: 1%, w/v) on the viability of gut sac was measured at 90 min and compared to the control group.

2.4.3. Glucose transport across the everted gut sac

To verify the viability and the integrity of the gut sac incubated with verapamil or excipients, glucose concentrations in both mucosal and serosal side were measured, their ratios were calculated and compared to the control group (Ballent et al., 2006). Because glucose is actively transported in the small intestine, intact and metabolically active sacs will concentrate glucose in the serosal medium and will maintain this gradient. The sacs were incubated in the absence or presence of verapamil (50 μg/ml) or excipients (using the maximum concentration: 1%, w/v). Samples from incubation medium and contents of the sacs were collected at 0, 30, 60, 90, and 120 min. Concentrations of glucose were measured by a kit from Rongsheng Biotech Co. Ltd. (China), and a semi automatic biochemical analyzer (AVE Science & Technology Industry Co. Ltd., China) was used to detect the samples.

2.4.4. Effects of excipients on GCV transport across gut sac

The prepared sacs were pre-incubated for 5 min in blank buffer in an incubation chamber before test solution was added. Then, the sacs were incubated at 37 °C, in 80-ml oxygenated Krebs-Ringer buffer (D-glucose 7.78 mM, NaCl 133 mM, KCl 4.56 mM, NaH_2PO_4 1.50 mM, $MgCl_2$ 0.20 mM, $NaHCO_3$ 16 mM, $CaCl_2$ 3.33 mM, pH was adjusted to 7.0–7.2) containing GCV (5 μg/ml) in absence (control group) or presence of verapamil (50 μg/ml) or different excipients (0.1%/0.5%/1%, w/v). Under this condition, the transport of GCV from mucosa to serosa in each group was measured by sampling 50 μl of serosal medium at 10, 20, 30, 45, 60, and 90 min (only the final 90-min are shown).

2.5. Improved single-pass intestinal perfusion model

2.5.1. Surgical procedures

Rats were fasted for 16–18 h (free access to water) prior to the experiment. Anesthesia was induced by intraperitoneal injection of urethane (30 mg/kg body weight), and maintained throughout the experiment by intermittent intraperitoneal injection (10–15 mg/kg), as required. A midline incision was made on the abdomen. In advance, the biliary duct was ligated, the whole small intestine was isolated and gently flushed with saline (37 °C). Silicone tubes were inserted into the proximal duodenum and terminal ileum and attached to a peristaltic pump (Petro Gas Ausrüstungen Berlin GmbH, Germany). Arranged in a uniform S or multi-S pattern to avoid kinks, the small intestine was returned to the abdominal cavity to maintain its viability without disrupting blood vessels. The rat was maintained at 37 °C throughout the experiment by using an overhead lamp. The exposed area was covered with gauze, and saline (37 °C) was applied to keep it warm and moist during the experiment (shown in Fig. 2).

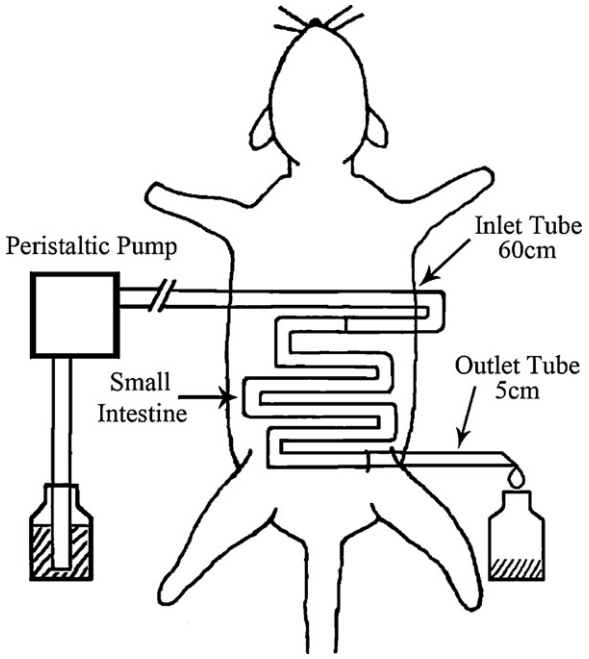

Fig. 2. Illustration of the experimental setup for *in situ* rat single-pass perfusion with whole small intestine.

The preparation was flushed with blank perfusion solution (37 °C) until the effluent perfusates were clear. The intestinal segment was perfused with test solution about 30 min to achieve absorption equilibrium and stable outflow rates. Subsequently, the effluent perfusates were collected in 10-min interval for 90 min in pre-weighed 5-ml glass vials with a lid. At the end of the experiments, the animals were euthanatized with a cardiac injection of saturated solution of KCl. The segment between two cannulas was excised without dragging and its length was measured using silk thread. The density of collected samples was determined by weighing the contents (using an electronic weighing balance) of a known volume of perfusate (using a precalibrated micropipette [Gilson, France]).

2.5.2. Preliminary studies for in vitro adsorption and stability

Before commencing the perfusion model pre-studies were necessary to ensure that loss of drug from the perfusates is due to absorption but not to other routines (e.g. nonspecific binding or degradation) (Cook and Shenoy, 2003). Adsorption studies were performed at 37 °C for 2 h by incubating drug solution in the glass vials and tubes used in the experimental setup and compared the concentration at the start. The stability of GCV and ACV in the perfusion system was tested by incubating both drugs in the influent perfusates or the freshly collected blank effluent perfusates at 37 °C for 2 h. Samples were taken at 0 and 2 h, and then analyzed by HPLC.

2.5.3. Comparison of intestinal LDH release in the two perfusion models

To evaluate the epithelium damage in the improved perfusion model, LDH release into the perfusates was monitored and compared with that in the common perfusion model. The common model using 15 cm jejunum was performed as previously described (Cook and Shenoy, 2003), with effluent perfusates sampled at 0 min, 30 min, 60 min, and 90 min. The improved model using whole small intestine was performed simultaneously and the samples were collected at 0 min, 30 min, 60 min, 90 min, and 120 min. The longer perfusion time in the improved model was selected as a longer equilibrium time was anticipated. The LDH activity

in all samples was determined by the LDH kit mentioned above (see Section 2.4.2).

2.5.4. Comparison of epithelial damage in the two perfusion models

Transmission electron micrographs of intestinal epithelium were taken to evaluate damages of tight junctions and microvilli. Specimens of perfused small intestine of the same region from the two models and from normal intestinal tissue were obtained and fixed by immersion in 2.5% glutaraldehyde for 2 h. The Department of Pathology (Tongji Medical College) performed all subsequent procedures, such as post-fixation, dehydration, infiltration, embedment, section, and stain. Specimens were characterized by Transmission Electron Microscope (FEI Tecnai G^2 12, Holland).

2.5.5. Comparison of drug absorption in the two perfusion models

To determine any changes in drug absorption when using the improved model, the permeability of GCV and ACV calculated from the two models was compared. The perfusion medium contained Krebs-Ringer Bicarbonate Buffer, with D-glucose 10 mM, NaCl 120 mM, KCl 4.56 mM, NaH_2PO_4 1.50 mM, Na_2HPO_4 0.70 mM, $MgCl_2$ 0.50 mM, and $NaHCO_3$ 15 mM. The pH was adjusted to 7.4, and the osmolality was 255–280 mOsm/kg. GCV or ACV was added to the solution to reach a final concentration of 5 μg/ml.

2.5.6. Effects of excipients on GCV transepithelial permeation

To investigate the effects of excipients on GCV transepithelial permeation, the improved perfusion model was employed. Krebs-Ringer bicarbonate buffer solution containing GCV (5 μg/ml) in absence (control group) or presence of verapamil (150 μg/ml) or different excipients (0.1%/0.5%/1%, w/v) was perfused at a constant flow rate of 0.35 ml/min. The P_{eff} values were calculated and compared.

2.5.7. Water flux correction and permeability calculation

The parameters below were calculated when the drug concentrations in the effluent perfusates were at steady state. The net water flux (NWF) was determined by a gravimetric method. C_{cor}, the drug concentration of effluent perfusates which was corrected for water flux, was calculated according to the equation:

$$C_{cor} = C_{out} \cdot \frac{Q_{out}}{Q_{in}}$$

where C_{out} is the concentration of tested drug in the effluent perfusates (μg/ml), Q_{in} and Q_{out} are the inlet and outlet flow rate, respectively, which are adjusted for liquid density (ml/min).

The effective permeability coefficients (P_{eff}) were calculated from

$$P_{eff} = \frac{Q_{in} \times \ln(C_{in}/C_{cor})}{2\pi rL}$$

where C_{in} is the concentration of tested drug in the influent perfusate; $2\pi rL$ is the area of the mass transfer surface (cm^2) within the intestinal segment which is assumed to be a cylinder area.

2.6. Statistical analysis

Data were expressed as mean ± S.D. For multiple-group comparisons, a one-way ANOVA followed by Tukey's HSD test was used. For a two-group comparison, a two-tailed nonpaired Student's *t*-test was employed. And $p < 0.05$ was considered as the level of significance.

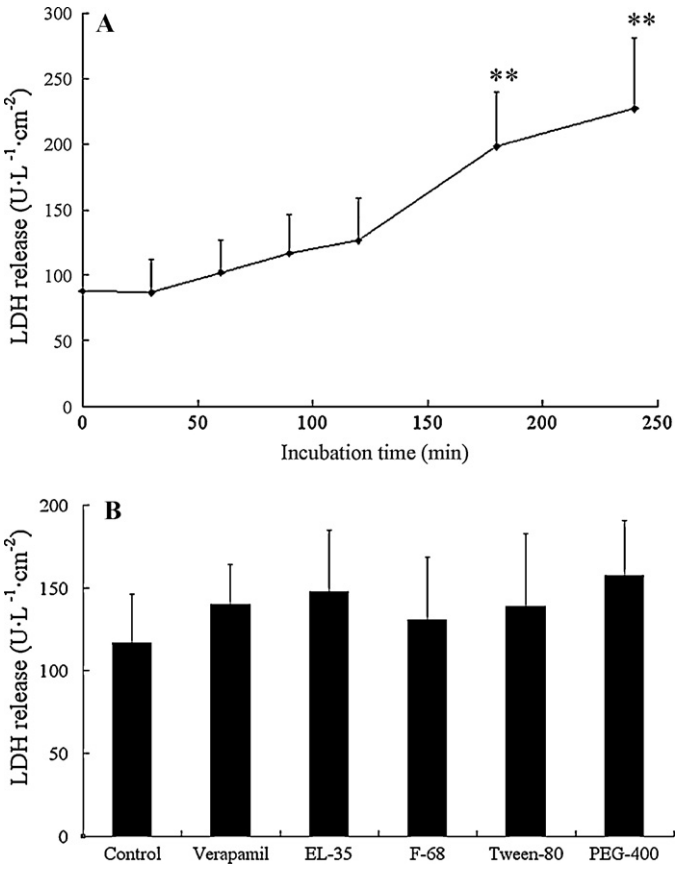

Fig. 3. LDH release in everted gut sac model. (A) The time course of LDH release in the control group; (B) LDH release in absence or presence of verapamil or excipients at 90 min. Data are shown as mean ± S.D. (n = 3–5 for each group). $^{**}p < 0.01$ compared to LDH release at 0 min.

3. Results

3.1. Everted gut sac model

3.1.1. LDH release in everted gut sac model

The LDH release indicating viability of gut sac and extent of damage of the intestinal epithelium in everted gut sac model is shown in Fig. 3A. At 0 min, LDH activity in the incubation medium was 88 U/L/cm^2. This was probably due to the surgical procedures when excising and everting the gut sac. Compared to the start, there were no significant differences of LDH activity at 30, 60, 90, or 120 min, respectively, while significant differences were found at 180 and 240 min suggesting loss of viability of the gut sac after 120 min. It was concluded that the everted gut sacs maintained their viability during the period of the experiment (90 min) and consequently this model was suitable for testing drug transport.

The effects of verapamil or excipients on the LDH release are shown in Fig. 3B. At the end (90 min) of the experiment, the amount of LDH release between test groups (verapamil and excipients) and the control group showed no significant differences. Even at the maximum concentration of each excipient (PEG-400, Tween-80, EL-35, and F-68), damage of the intestinal epithelium was very limited and did not interfere with the results.

3.1.2. Glucose transport in everted gut sac model

The concentration ratios of glucose between serosal side and mucosal side are shown in Fig. 4. It was obvious that the ratios were gradually increasing and reached a factor of 3.12–3.78 at 120 min independent of the presence of verapamil (50 μg/ml) or excipients

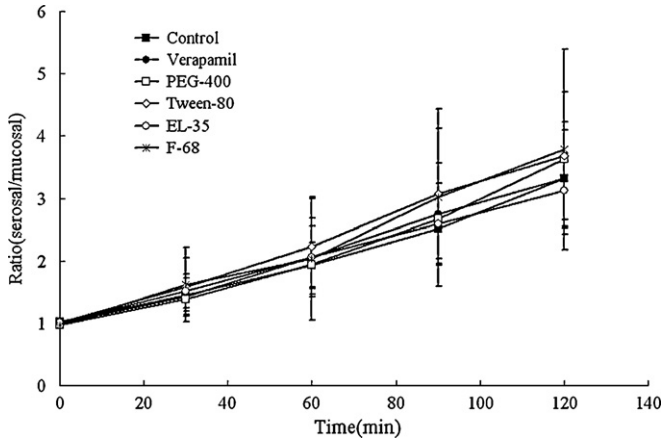

Fig. 4. Glucose transport in everted gut sac model. Data are shown as mean ± S.D. (n = 5–9 for each group). (■) control group; (●) verapamil; (□) PEG-400; (◊) Tween-80; (○) EL-35; (*) F-68.

(1%, w/v), indicating that the tissue of the gut sac was viable and well functioning. No significant differences was found in the glucose transport between excipient/verapamil groups and the control group, indicating that these compounds did not induce toxicity and had not obvious influences on the cellular, energy-related actions at any tested concentration.

3.1.3. Effects of excipients on GCV transport across gut sac

The time course of absorptive transport of GCV across small intestinal segments is illustrated in Fig. 5A. The order of GCV intestinal absorption was: ileum < jejunum < duodenum (0.63, 0.76, and 0.87 μg/10 cm-sac, respectively), but there were no significant differences among them. When the intestinal P-gp efflux was inhibited by verapamil, the order was reversed to duodenum < jejunum < ileum (1.02, 1.37, and 1.71 μg/10-cm-sac, respectively) (Fig. 5B) characterizing absorptive capacity of small intestine without the influence of P-gp efflux. Under this condition, significant regional differences of GCV absorption among the three segments were observed ($p < 0.05$). Compared to the control group, the absorbability of the verapamil group was found to be: ileum ($p < 0.05$) > jejunum ($p < 0.05$) > duodenum ($p > 0.05$). The increased absorbability which could be contributed to the inhibited function of P-gp efflux was consistent with the level of P-gp expressed in rat's small intestine.

Table 1 demonstrates that the accumulative amount of GCV in the serosal side of gut sacs after 90-min incubation with excipients represents the effects of excipients on the intestinal transport of GCV. Compared to the control group, the four excipients that passed our previous screening in Caco-2 cells (unpublished data) could all enhance significantly ($p < 0.05$) the GCV intestinal transport from mucosa to serosa, thereby no dose-dependent effect was apparent. F-68 showed a regionally different enhancement ($p < 0.05$) which was similar in the verapamil group, while other excipient groups did not show regional differences.

Table 1
Effects of excipients on the GCV transport in everted gut sac model.

Groups	Transport amount (μg/90 min/10 cm-sac)		
	Duodenum	Jejunum	Ileum
Control	0.87 ± 0.24	0.76 ± 0.12	0.62 ± 0.15
Verapamil (50 μg/ml)	1.02 ± 0.05 N.S. †	1.37 ± 0.24**,†	1.71 ± 0.12**,††
PEG400 (0.5%, w/v)	2.46 ± 0.45**	2.03 ± 0.57**	2.07 ± 0.85**
PEG400 (1%, w/v)	1.52 ± 0.44*	1.62 ± 0.41**	2.11 ± 0.71**
EL-35 (0.5%, w/v)	1.42 ± 1.42*	1.45 ± 0.26**	1.43 ± 0.23**
EL-35 (1%, w/v)	1.10 ± 0.18 N.S.	1.10 ± 0.26*	1.08 ± 0.33*
Tween-80 (0.1%, w/v)	1.57 ± 0.28**	1.15 ± 0.12**	1.68 ± 0.29**
Tween-80 (0.5%, w/v)	2.14 ± 0.89**	1.92 ± 0.67**	1.30 ± 0.42*
Tween-80 (1%, w/v)	1.12 ± 0.24 N.S.	1.26 ± 0.21**	1.09 ± 0.26**
F-68 (0.1%, w/v)	1.14 ± 0.18 N.S.	1.22 ± 0.20**	1.51 ± 0.23**,†
F-68 (0.5%, w/v)	0.99 ± 0.15 N.S.	1.22 ± 0.15**,†	1.51 ± 0.22**,††
F-68 (1%, w/v)	1.14 ± 0.18 N.S.	1.33 ± 0.27**	1.61 ± 0.34**,†

Data are shown as mean ± S.D. (n = 4–7 for each group).
N.S.: not significantly different to the same intestinal segment of control group.
* $p < 0.05$, compared to the same intestinal segment of control group.
** $p < 0.01$, compared to the same intestinal segment of control group.
† $p < 0.05$, compared to duodenum segment in the same group.
†† $p < 0.01$, compared to duodenum segment in the same group.

3.2. Improved single-pass intestinal perfusion model

3.2.1. Preliminary studies for in vitro adsorption and stability

In adsorption studies, no loss of GCV or ACV was found proofing that no drug had been attached to the glass vials and tubes in the course of incubation (data not shown). No significant differences were found between the incubation samples at 0 h and 2 h in both influent and effluent perfusates, respectively, indicating that both drugs were stable in influent and effluent perfusates during incubation (data not shown). Thus, it can be anticipated that any loss of drug in the following studies can be attributed to intestinal absorption.

3.2.2. Comparison of LDH release in the two perfusion models

Fig. 6 compares the LDH release between the improved perfusion with whole small intestine and the common perfusion with 15 cm intestine. Initially, the LDH release was at

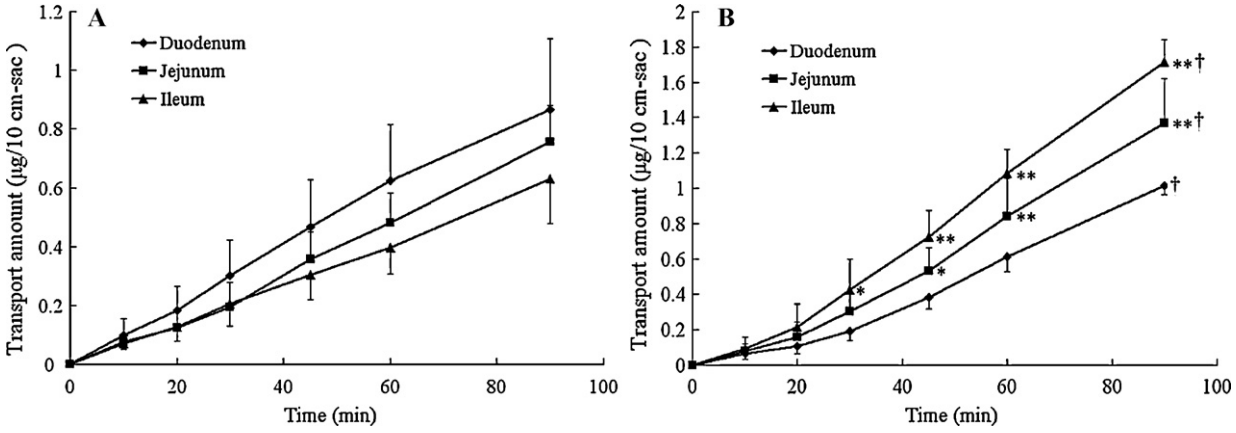

Fig. 5. The regional intestinal absorption of GCV in everted gut sac model. (A) Control group; (B) verapamil. Data are shown as mean ± S.D. (n = 5–7 for each group). *$p < 0.05$, compared to the same segment of control group; **$p < 0.01$, compared to the same segment of control group; †$p < 0.05$, compared to two other segments of verapamil group.

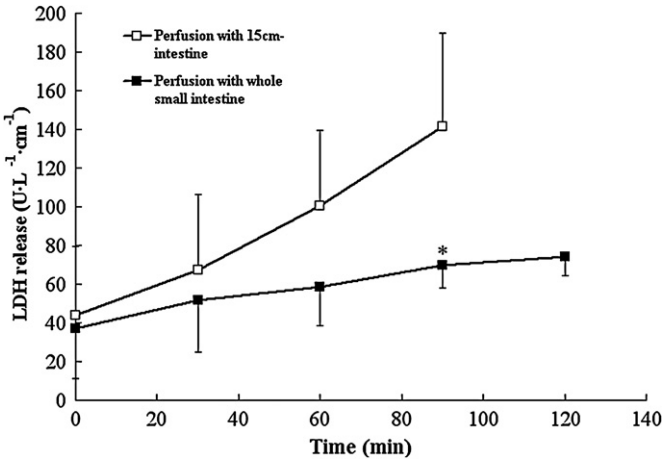

Fig. 6. Comparison of LDH release in the two perfusion models. Data are shown as mean ± S.D. (n = 4–6 for each group). *p < 0.05, compared to the same time point in the other model.

the same level in the two perfusion models. However, as the experiments continued, the release of the former reached a plateau at 70 U/L/cm, while that of the latter kept rising to 140 U/L/cm, and a significant (p < 0.05) difference was found at 90 min.

3.2.3. Comparison of epithelial damage in the two perfusion models

In Fig. 7 the morphological changes of intestinal epithelium in the two perfusion models are compared to controls. Normal structures of microvilli and tight junctions can be seen in Fig. 7A and B. In the common perfusion model, epithelial microvilli were much shorter and had some breakages (Fig. 7C), while the cellular organelles such as mitochondria (Fig. 7C) and tight junctions (Fig. 7D) were morphologically normal. In the improved perfusion model, intestinal epithelium had no obvious morphological changes compared to the normal tissue (Fig. 7E and F), suggesting that better physiological conditions were maintained in this model.

3.2.4. Comparison of drug absorption in the two perfusion models

The P_{eff} values of GCV and ACV obtained from the two perfusion models are summarized in Table 2. Compared to the common model, the mean P_{eff} value of GCV derived from the improved model decreased (85%; p < 0.001) from 1.54×10^{-5} cm/s to 0.23×10^{-5} cm/s, while that of ACV was declined (29%; p < 0.05) from 2.11×10^{-5} cm/s to 1.50×10^{-5} cm/s. It was also found that GCV which is less permeable than ACV was subject to a more intensive decrease in its P_{eff} value than ACV when it was determined by the improved model.

Table 2
Comparison of drug absorption in the single-pass perfusion with 15 cm intestine (Control) or whole small intestine (Test).

Test number	P_{eff} of ACV ($\times 10^{-5}$ cm/s)		P_{eff} of GCV ($\times 10^{-5}$ cm/s)	
	Control	Test	Control	Test
1	2.42	1.47	1.91	0.21
2	1.50	1.42	1.28	0.16
3	1.87	0.93	1.99	0.19
4	2.37	1.93	1.13	0.23
5	2.37	1.77	1.61	0.26
6			1.40	0.30
7			1.46	0.30
Mean	2.11	1.50	1.54	0.23
S.D.	0.40	0.39	0.32	0.05
p value	0.043	<0.001		

3.2.5. Effects of excipients on GCV transepithelial permeation

In Fig. 8 the effects of excipients on the permeability of GCV in the improved *in situ* perfusion model are shown. Consistent with the results from *in vitro* studies, in all groups, excipients significantly improved the permeability of GCV. EL-35 demonstrated a remarkable, dose-dependent improvement in GCV permeability in small intestine (6.7–13.4 fold larger than control group). F-68 displayed a similar improvement but its efficiency was less pronounced (1.8–5.1 fold). The effects of PEG-400 and Tween-80 were obviously dose-independent and of moderate efficiency (2.6–4.8 fold).

4. Discussion

Although some common excipients have been found to enhance drug absorption through P-gp inhibition, the underlying mechanism is still not fully understood. Most of these experiments were performed at the cellular level (e.g. Caco-2 cell lines) (Anderberg et al., 1992; Batrakova et al., 1999; Hugger et al., 2002a; Johnson et al., 2002), and only a limited number of *in vivo* or *in situ* studies have been performed (Zhang et al., 2003; Ashiru et al., 2008). In this paper, detailed *in vitro* and *in situ* studies are presented for a better understanding of the effects of P-gp-inhibiting excipients on the intestinal absorption.

The application of the everted gut sac model was validated by assessing LDH release and glucose transport. The everted gut sacs kept their viability for at least 120 min as no damages induced by excipients at their maximum test concentration were apparent (Fig. 3). The active transport of glucose in gut sac ran normally, indicating that the gut sacs were viable and well functioning during the experiments (Fig. 4). Therefore, the everted gut sac model was suitable for studying the effects of excipients on intestinal transport of drugs and the results obtained from an incubation period of 90 min can be regarded as reliable.

To estimate more accurately the extremely poor permeability of BCS-III drug, an improved rat single-pass intestinal perfusion model was established. By using the whole small intestine a major improvement was achieved. Biochemical validation was employed to ensure the feasibility of this modification, e.g. the LDH release in the improved model was significantly decreased to half of that in the common model (see Fig. 6). Furthermore, according to transmission electron micrographs, the intestinal epithelium in the common model suffered more damages than in the improved one. During perfusion, microvilli were injured to some extent, whereas no apparent damages of the tight junctions and mitochondria could be observed (see Fig. 7). Both assessments suggested that the improved model maintained better physiological condition of the intestine.

A reason for these observations might be that the real flow rates through the intestine were different between the two models although the same inlet flow rate was applied. The improved perfusion model allowed a larger amount of water absorption. The flow rate inside the intestine as well as the outflow rate was much slower; therefore, damages of the epithelium derived from a fast water flow were less. It is concluded that single-pass perfusion with whole small intestine is feasible and it has the advantage of maintaining better the physiological conditions of the intestine. Consequently, differences were observed between the two perfusion models in determination of P_{eff} (Table 2). The permeability of GCV and ACV assessed by the improved model was much smaller if compared to the standard model. This was probably due to less

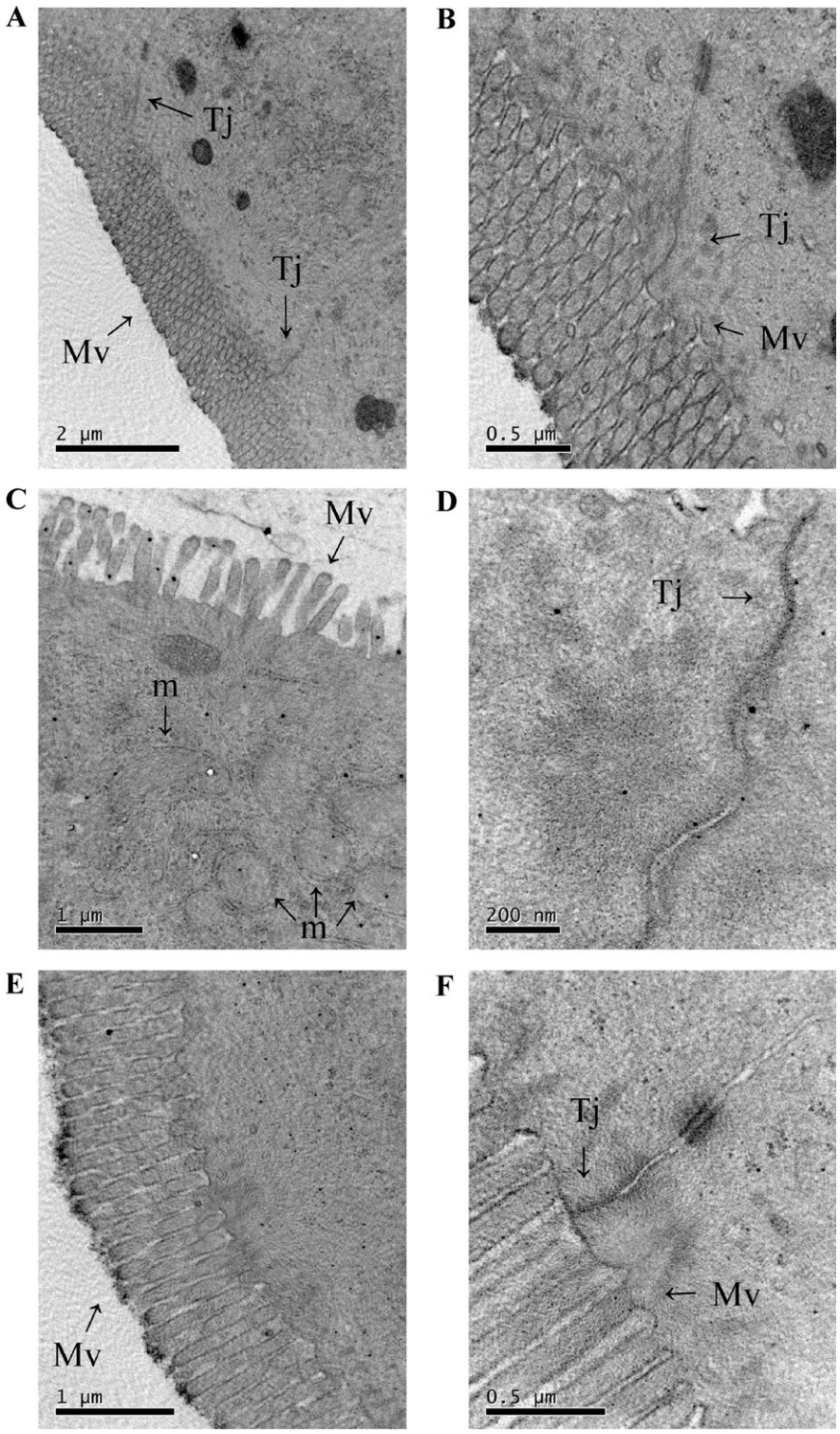

Fig. 7. Transmission Electron Micrographs of intestinal epithelium. Mv: microvillus; Tj: tight junction; m: mitochondrion. (A and B) Normal intestinal epithelium; (C and D) epithelium in perfusion with 15 cm intestine; (E and F) epithelium in perfusion with whole small intestine.

damage of the epithelium and tight junctions which both are critical in limiting the absorption of the drugs with poor permeability (BCS-III/IV). In addition, due to the longer intestine the improved model allows that from the perfused drug higher amounts can be absorbed. This might be unimportant for drugs with good permeability (BCS-I/II), but it is essential for determining accurately the poor permeability of BCS-III/IV drugs. Thus, the improved model will provide more reliable data.

In rats, no regional differences of intestinal absorption of GCV were observed (Fig. 5, control group), which is in accordance to findings in rabbit's small intestine (Yang et al., 2006). In the verapamil group, under the condition of inhibiting P-gp-mediated drug efflux we found a sequence of drug uptake: ileum > jejunum > duodenum, the significant regional differences of absorbability of small intestine to GCV were probably due to physiological differences among ileum, jejunum and duodenum. Compared to the same segment

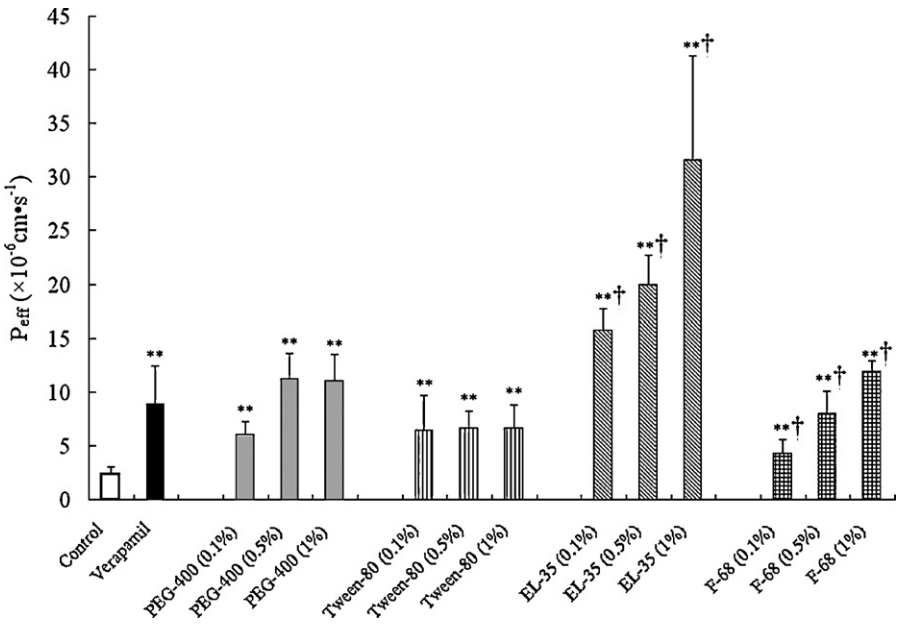

Fig. 8. Effects of excipients on the permeability of GCV. Data are shown as mean ± S.D. (n = 4–7 for each group). $^{*}p < 0.05$, compared to control group; $^{**}p < 0.05$, compared to control group; $^{\dagger}p < 0.05$, compared to two other concentration groups of the same excipient.

in control group, the increased transport amounts of GCV in verapamil group which could consider as the P-gp-mediated transport were found that ileum ($p < 0.05$) > jejunum ($p < 0.05$) > duodenum ($p > 0.05$). This order is in concordance with the expression of MDR1 mRNA in rat intestine which was moderate in the duodenum and the jejunum, and maximal in the ileum (Trezise et al., 1992). Likewise, in rats P-gp expression in the ileum was 2.31-fold higher than that in the jejunum (Tian et al., 2002). Thus, the regional differences of the increased transport amounts of GCV were consistent with the expression of P-gp in the small intestine and it confirms that GCV is a substrate of P-gp. Similar results were found for the intestinal transport of rhodamine 123, which is a typical P-gp substrate (Yumoto et al., 1999).

In the past several mechanisms have been discussed for the enhancement of absorption by excipients including (a) increasing the solubility of hydrophobic drugs (Chang et al., 1996), (b) interaction with metabolising enzymes such as CYP3A (Ren et al., 2009), (c) disruption of tight junctions (Anderberg et al., 1992; Aungst, 2000), and (d) local damage of the intestinal epithelium (Swenson et al., 1994). It should be considered that inhibition of P-gp-mediated drug efflux will also enhance absorption of P-gp substrates. To investigate this mechanism, the other possibilities need to be excluded. In the present study, GCV which is highly hydrophilic and not metabolised in the intestine and liver was chosen as a probe. Based on our results, points a and b can be easily excluded. In addition, glucose accumulated normally in gut sacs (Fig. 3), indicating that the tight junctions of intestinal epithelium remained intact. Similarly, PEG-300, EL-35, and Tween-80 did not affect the integrity of tight junctions in Caco-2 or MDR1-MDCK cells (Hugger et al., 2002a). In addition, no damages of the intestinal epithelium were apparent based on the LDH release. Therefore, points c and d cannot explain the effects of the tested excipients. Therefore, we conclude that the enhanced absorption of the P-gp substrate GCV induced by excipients is most likely due to their inhibition of P-gp.

F-68 could increase significantly the absorption of GCV and the order of regional enhancements was similar to that of verapamil (Table 1). It indicated a dose-dependent inhibition of P-gp (Fig. 8), which was consistent with the previous study of F-68 in Caco-2 cell lines (Huang et al., 2008). It was suggested that the mechanism of F-68 might be similar to that of verapamil, which inhibited P-gp by a non-competitive way (Spoelstra et al., 1994). Although PEG-400, EL-35 and Tween-80 also enhanced the absorption of GCV a regional pattern of intestinal absorption was not obvious if compared to verapamil and F-68 (Table 1). This would suggest that their action is apparently not based on a single inhibitory mechanism.

It has been reported that excipients might induce mitochondrial toxicity and subsequent ATP depletion can cause malfunction of P-gp (Regev et al., 1999; Batrakova et al., 2004). According to the normal glucose transport in gut sacs, one could indirectly assume that production and consumption of cellular energy (ATP) was not disturbed by the addition of excipients.

Changes of membrane fluidity induced by excipients have been also considered as an important inhibitory mechanism of surfactants/excipients (Dudeja et al., 1995; Rege et al., 2002). There is some controversy whether the P-gp-inhibiting excipients will increase or decrease the fluidity of epithelial membranes. Moreover, there is a close association between P-gp and cell membranes and P-gp is highly sensitive to its lipid environment (Regev et al., 1999; Ferte, 2001). The disturbance of this environment by excipients might cause changes in secondary and tertiary structure of P-gp, and this could be one reason for loss of its function. PEG-400, Tween-80, EL-35 which contained oxyethylene groups may alter the lipid phase of the membrane or they may change the fluidity of the polar head group regions of cell membranes (Hugger et al., 2002b). Therefore, such mechanisms might be involved in the malfunction of intestinal P-gp by PEG-400, Tween-80 and EL-35.

On the contrary, membrane fluidity modulation by excipients was regarded as insufficient to reduce transporter activity and for offering a generalized mechanism for their P-gp inhibition (Rege et al., 2002). We assume that the enhanced absorption induced by many P-gp inhibiting excipients such as PEG-400, Tween-80, and EL-35 might be the complex result of direct P-gp inhibition via blocking P-gp/substrate binding and indirect inhibition by altering membrane fluidity, while enhancement of F-68 is more likely due to its direct P-gp inhibition like that of verapamil. However, to determine the exact inhibitory mechanism, further biochemical experiments are mandatory.

It is of great importance to investigate the enhancing effects of excipients on intestinal absorption at the same time in *in vitro* and

in situ models. Each model has its limitations but an association of them will provide us a better understanding of the underlying mechanisms. In the *in vitro* model, the four selected excipients had the potential to enhance intestinal absorption of GCV, but apparently there were no dose-dependent effects. In the *in situ* model, the four excipients still exhibited their enhancement of intestinal absorption of GCV, but some differences could be observed. First, the enhancements were more pronounced especially that with EL-35. Second, EL-35 and F-68 showed now a dose-dependent effect whereas no dose-dependent effect of PEG-400 and Tween-80 was obvious. The differences were probably due to the design of the two models. The sample volume at the serosal side of the gut sacs was relatively small favoring rapid drug accumulation. Consequently, the concentration gradients between mucosa and serosa will become increasingly smaller which will markedly influence passive diffusion and absorption of GCV. *In situ*, the absorbed drugs will quickly distribute to other tissues and organs due to the intact blood circulation. As a result, the sink condition will be maintained and GCV can be continuously absorbed at the maximum rate. So the enhancing efficiency of excipients was higher *in situ* than *in vitro* and the absorptive characteristics such as dose-dependent effect were displayed deriving from the more sufficient absorption.

In general, in two models an enhanced absorption could be induced by four excipients. Both applied models provided comparable results. Important correlations between the validated *in vitro* and *in situ* models could be evaluated.

In conclusion, the single-pass perfusion with whole small intestine was proved to be a suitable model to determine to the poor permeability of BCS-III/IV drugs. PEG-400, Tween-80, EL-35, and F-68 could enhance the intestinal absorption of GCV via P-gp inhibition both *in vitro* and *in situ*. Due to their well tolerated and non-irritating properties, addition of these excipients to the formulation of P-gp substrates offers a promising approach to improve the oral availability of clinically important drugs.

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