



## Enhancing effect of N-octyl-O-sulfate chitosan on etoposide absorption

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### ABSTRACT

P-glycoprotein (P-gp), expressed in the apical membranes of the epithelial cells of the intestine, can reduce the oral bioavailability of a wide range of drugs. Many surfactants/excipients have been demonstrated to potentially increase drug absorption by inhibiting P-gp. The purpose of the present study was to evaluate the effect of N-octyl-O-sulfate chitosan (NOSC) on the absorption of etoposide (VP16), a substrate of P-gp with low water solubility. The rat intestinal circulating perfusion *in situ* and Caco-2 cell uptake and monolayer membrane penetration *in vitro* were performed to investigate the enhancing ability of NOSC in comparison with some other P-gp inhibitors. The results indicated that various concentrations of NOSC all increased the intestinal absorption of VP16 in rat jejunum and ileum obviously and there was no significant difference in ileum between the enhancing effects of NOSC and other P-gp inhibitors. The VP16 uptake of Caco-2 cell was increased by NOSC solution with different concentrations. As the NOSC concentration was close to its critical micelle concentration (CMC), the cell uptake of VP16 reached to a maximum value. Both NOSC and verapamil (Ver) enhanced dramatically the transport of VP16 from apical side to basolateral side in Caco-2 cell monolayers. Moreover, they both decreased notably the transport of VP16 from basolateral side to apical side, but this effect of NOSC was weaker than that of Ver. However, transepithelial electrical resistance (TEER) of Caco-2 cell monolayers had no significant change during the study. These studies demonstrated that NOSC had the potential by inhibiting P-gp to improve the absorption of oral drugs which were P-gp substrates.

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### 1. Introduction

Oral administration is the most common route for drug administration. However, after oral administration, the absorption of some drugs may be erratic and incomplete. The different cellular efflux pumps in Caco-2 cells and their effects on drug absorption have been reported and evaluated (Tsuji et al., 1994; Döppenschmitt et al., 1998; Mizuuchi et al., 1999; Ogihara et al., 1999; Makhey et al., 1998). The P-glycoprotein (P-gp) is one of main efflux proteins in epithelial cells which extrude a wide variety of chemically different xenobiotics from cells (Mizuno et al., 2003). Besides the intestine, P-gp is widely expressed in many pharmacokinetic barriers in the body such as kidney, liver and blood-brain barrier (BBB). Therefore, it may affect significantly the pharmacokinetics of its substrate molecules.

Traditional P-gp inhibitors are almost P-gp substrates or compounds that have the similar structures to the P-gp substrates (Sridhar et al., 1992; Lum et al., 2000). They affect combination

sites of the P-gp with substrates and interfere the functions of P-gp by competitive inhibition. However, some shortages have been reported accompanying with these effects (Constantinides and Wasan, 2007), including the toxicity, adverse effect by their pharmacology activities, interaction with other drugs by co-administration, etc. In order to reduce these side effects, more attentions have been paid recently on a number of surfactants/excipients that can inhibit P-gp and thus potentially enhance drug absorption (Legen et al., 2006; Cornaire et al., 2004). These surfactants/excipients such as Cremophor EL (Shono et al., 2004) and Tween 80 (Katneni et al., 2007) inhibit P-gp by other ways directly or indirectly, enhance the intestinal permeability of the P-gp substrates, and therefore improve the bioavailability of these substrates.

Chitosan, a copolymer composed of glucosamine, N-acetylglucosamine (2-acetamido-2-deoxy- $\beta$ -D-glucose and 2-amino-2-deoxy- $\beta$ -D-glucose units linked with  $\beta$ -(1  $\rightarrow$  4) bonds), is a cationic amino polysaccharide in neutral or basic pH conditions which is easily obtained by partial deacetylation of chitin. Chitosan is generally regarded as non-toxic, biocompatible and biodegradable (Rao and Sharma, 1997; Si et al., 2010) and listed in the standard guides of American Standard Testing Materials (ASTM) for use in food preparations and in the pharmacopoeias of some countries. It has been demonstrated that chitosan can open

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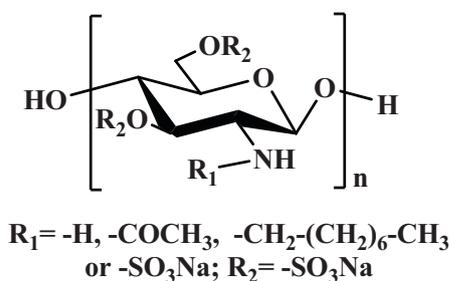


Fig. 1. The structure of N-octyl-O-sulfate chitosan (NOSC).

intercellular tight junctions to improve drug absorption (Kotzé et al., 1998), and chitosan and some of its derivatives have the effect on inhibiting P-gp (Palmerberger et al., 2008; Werle and Hoffer, 2006). However, the characteristics that chitosan dissolves only in acidic solution and poorly in water or in neutral solution greatly limit its application.

An amphiphilic chitosan derivative, N-octyl-O-sulfate chitosan (NOSC) shown in Fig. 1 was synthesized by our group (Zhang et al., 2004). NOSC can easily dissolve in aqueous solution to form micelle in self-assembly process. The NOSC micelle has great capability in solubilization of water-insoluble drugs. As a novel nanocarrier, the derivative was used to prepare the intravenous injection of paclitaxel, and its pharmacokinetics, efficacy and safety were fully investigated (Zhang et al., 2008a, 2008b). NOSC was also utilized to increase the solubility of cyclosporine, in which the equal relative bioavailability of the drug micellar system was obtained after oral administration to rats, compared with the market available capsule (Liu et al., 2007). In the present study, the rat intestinal circulating perfusion *in situ* (Michel et al., 1991) was used to verify the enhancing effect of NOSC on oral absorption of etoposide (VP16), a typical P-gp substrate with low water-solubility (Lum et al., 2000). The Caco-2 cells uptake and transport tests were carried out. The enhancing mechanism was analyzed by using various concentrations of NOSC and by comparing with some other inhibitors of P-gp, including verapamil (Ver) and Cremophor EL, in these experiments.

## 2. Materials and methods

### 2.1. Materials

Chitosan was purchased from the Shuanglin Biochemical Co. Ltd. (Nantong, China), with deacetylation degree of 92% and viscosity average molecular weight of 65 kDa. VP16 was obtained from Shanghai Natural Pharmaceutical Co. Ltd. (Shanghai, China). Verapamil was offered by Hengrui Pharmaceutical Co. Ltd. (Jiangsu, China). Cremophor EL was a gift from BASF (Germany). N-octyl-O-sulfate chitosan (NOSC) was synthesized using the chitosan as described by our group (Zhang et al., 2004), and the substitution of octyl degree and sulfonic degree were 0.38 and 2.56, respectively and viscosity average molecular weight was 65–70 kDa.

Dulbecco modified Eagle medium (DMEM), defined fetal bovine serum, nonessential amino acid solution, L-glutamine and penicillin–streptomycin were purchased from Gibco BRL (Grand Island, NY, USA). N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) was purchased from Zhuyan Biotechnology Co. Ltd. (Nanjing, China).

HPLC/spectra-grade reagents were used as the mobile phase in HPLC analysis, and all other reagents were analytical grade and used without further purification. Distilled, deionized water was produced by a Milli-Q Reagent Water System (Millipore, USA).

### 2.2. Animals and Caco-2 cell line

Sprague–Dawley (SD) rats (200–300 g) were obtained from the Shanghai Silaike Laboratory Animal Limited Liability Company. All the animals were pathogen free and allowed to access to food and water freely. Animal experiments were carried out in accordance with the Guidelines for Animal Experimentation of China Pharmaceutical University (Nanjing, China) and protocol was approved by the Animal Ethics Committee of this institution.

Caco-2 cell line (Passage: 44–54) was obtained from the cell bank of Chinese Academy of Sciences, and was frozen at  $-170^{\circ}\text{C}$  in liquid nitrogen before use.

### 2.3. VP16 solubility determination

Solubility of VP16 in NOSC solution at  $37^{\circ}\text{C}$  was evaluated. An excess of VP16 powder was dissolved and equilibrated in deionized water containing different concentrations of NOSC from 0% to 0.8% (w/v). The solutions were agitated by using a shaker under a constant rate. After 24 h, the non-dissolved solid phase was removed by filtration (0.45  $\mu\text{m}$  cellulose nitrate membrane). The filtrates were diluted with methanol and assayed by HPLC. All solubility results are the average of three replicated experiments.

### 2.4. VP16 absorption in rat intestinal circulation perfusion

#### 2.4.1. VP16 stability in intestinal circulating perfusion solution and physical absorption of rat intestinal segments

The VP16 intestinal circulating perfusion solution (50  $\mu\text{g}/\text{mL}$ ) was prepared by dissolving VP16 (5 mg) in 100 mL Hank's balanced salt solution (HBSS, pH 6.8). The solution of VP16 was incubated at  $37^{\circ}\text{C}$  with the circulating perfusion tubes for 2 h. Samples were collected at appropriate time interval (0, 30, 60, 90, 120 min) and analyzed by HPLC.

The intestinal tract was removed from rat abdomen and cut into four segments *in vitro*, which were 10 cm-long segments of duodenum (1 cm distal to pyloric sphincter), jejunum (15 cm to pyloric sphincter), ileum (20 cm proximal to cecum) and colon (2 cm distal to cecum). VP16 solutions with the segments were incubated at  $37^{\circ}\text{C}$  for 2 h, respectively. The samples solution were collected at 0, 30, 60, 90, 120 min and analyzed by HPLC. Each experiment was triplicated. The remaining ratio (%) was calculated by the following equation: remaining ratio (%) =  $C_t/C_0 \times 100\%$  where  $C_0$  and  $C_t$  were the concentrations of VP16 intestinal circulating perfusion solution at 0 min and  $t$  min, respectively, and  $t = 30, 60, 90, 120$  min.

#### 2.4.2. VP16 absorption in rat intestinal circulating perfusion test *in situ*

The experiments were carried out based on the reported method (Michel et al., 1991). Briefly, eighteen SD rats were divided randomly into 6 groups with three each. After fasted overnight with free access to water, each of rats was anesthetized, and restrained in a supine position. An incision approximately 3 cm was opened through abdominal midline. The four intestinal segments mentioned in Section 2.4.1 were exposed and incisions were made *in situ* at both sides of the segment. Then, they were gently rinsed with pre-warmed normal saline ( $37 \pm 1^{\circ}\text{C}$ ), and purged by air, followed by being connected with the catheters to the backflow peristaltic pump (HL-2, Shanghai Huxi Analysis Instrument Co., Ltd., China). 25 mL of VP16 perfusion solution (50  $\mu\text{g}/\text{mL}$ ) was used for the backflow. At the beginning, 25 mL of the solution was perfused through the segment at a flow rate of 5 mL/min. 10 min later, the solution volume in circulation was recorded as the 0 min volume and then the flow rate was adjusted to 1 mL/min for another 2 h. At the end of the experiment, all the perfusion solution was collected and about 20 mL fresh HBSS was used to wash the intestinal segment through

the catheter. The volume of sample solution was quantitatively adjusted with fresh HBSS to 50 mL. Then, the animals were sacrificed and the intestinal segments were removed. The length and radius of each intestinal segment was measured.

100  $\mu$ L sample solution was removed into a 1 mL tube with 900  $\mu$ L methanol, vortexed for 90 s, and then centrifuged at 12,000 rpm for 10 min. A 20  $\mu$ L of supernatant was injected into HPLC for VP16 determination. The mean absorption amount of VP 16 ( $I$ ) was calculated by the following equation:  $I = (25 \times C_0 - 50 \times C) / A$  where  $C_0$  and  $C$  were the concentrations ( $\mu$ g/mL) of VP16 in the perfusion solution at 0 h and 2 h, respectively and  $A$  was the surface area ( $\text{cm}^2$ ) of intestinal segment. The effect of 0.6% (w/v) NOSC in perfusion solution on VP16 absorption of the intestinal segments was examined.

#### 2.4.3. VP16 absorption of rat jejunum and ileum in presence of different concentrations of NOSC

Nine SD rats were divided randomly into 3 groups with three each and fasted overnight before experiment with free access to water. The perfusion solution of VP16 (50  $\mu$ g/mL) contains 0.1%, 0.6%, and 1.0% (w/v) NOSC, respectively. The rat treatment and perfusion method were described in Section 2.4.2, and then the mean absorption amount of VP16 with different concentrations of NOSC in rat jejunum and ileum during experiment was calculated. Each experiment was triplicated.

#### 2.4.4. VP16 absorption of rat ileum in the presence of NOSC or different P-gp inhibitors

Twelve SD rats were divided randomly into 4 groups with three each and fasted overnight before experiment with free access to water. The perfusion solution of VP16 (50  $\mu$ g/mL) contains 0.1% (w/v) Verapamil, 0.1% Cremophor EL, 0.1% Tween 80 and 0.1% NOSC, respectively. The experimental method was the same as described in Section 2.4.2, and then the mean absorption amount of VP16 in rat ileum with different P-gp inhibitors and NOSC was calculated. Each experiment was triplicated.

### 2.5. Caco-2 cells study

#### 2.5.1. Cells culture

Caco-2 cells grew routinely in plastic culture flasks (Corning). The culture medium consisted of DMEM containing 2.2 g/L  $\text{NaHCO}_3$ , 2.38 g/L HEPES and 110 mg/L sodium pyruvate, supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acids and 1% penicillin–streptomycin at 37 °C under an atmosphere of 5%  $\text{CO}_2$  and 90% relative humidity (Cell incubator, Thermo, USA). The medium was replaced every 2 days after incubation. Cells were passaged approximately every 5 days (at 80% confluence) using trypsin–EDTA at a split ratio of 1: 5.

For the transport experiments, cells were seeded at a density of  $1 \times 10^5$  cells/well on permeable polycarbonate inserts (Millicell cell culture inserts, Millipore, USA) in 24-well plates (Costar). The inserts were fed every 2 days for the first week and then daily until they were used for experiments 21–24 days after seeding. The integrity of the cell monolayers was evaluated by measuring transepithelial electrical resistance (TEER) values with MILLICELL electrical resistance system (Millipore). The cell monolayers were used in the experiments when the resistance exceeded 350  $\Omega \text{cm}^2$ , which indicated that the tight junction was well-developed and the cell monolayers were integrated (Hosoya et al., 1996).

#### 2.5.2. Cell uptake study

Cells ( $1 \times 10^5$  cells/well) were seeded into 24-well plates and incubated for 14 days. Cells were rinsed twice with 37 °C HBSS and after washing, the cells were preincubated with HBSS at 37 °C

for 20 min. Then, HBSS was removed and 400  $\mu$ L of HBSS containing VP16 (100  $\mu$ g/mL) or containing VP16 (100  $\mu$ g/mL) with NOSC was added to the cells. After 2 h incubation at 37 °C, the solutions were taken away, and then the cells were washed with 4 °C HBSS twice and 200  $\mu$ L 4 °C HBSS was added in each well of the 24-wells plates. Finally, the cells were frozen at –70 °C for 4 h and then melted at 25 °C, which was repeated four times. At the end of the experiment, the cell suspension was obtained in HBSS by blowing air repeatedly and tenderly. 100  $\mu$ L cell suspension was removed in a 500  $\mu$ L eppendorf tube with 100  $\mu$ L methanol, vortexed for 90 s, and then centrifuged at 12,000 rpm for 10 min. A 50  $\mu$ L of supernatant was injected for determination of drug by HPLC. The remained cell suspension was used for Coomassie brilliant blue method (Bradford, 1976) to analyze the amount of cell protein. The cell uptake of VP16 was calculated by the following equation: uptake ( $\mu$ g/mg) =  $C_{\text{VP16}} / C_{\text{protein}}$  where  $C_{\text{VP16}}$  and  $C_{\text{protein}}$  were the concentrations ( $\mu$ g/mL) of VP16 and the concentrations (mg/mL) of cell protein in the cell suspension, respectively.

The effect of different concentrations of VP16 (50, 75, 100, 150  $\mu$ g/mL) without NOSC and VP16 (100  $\mu$ g/mL) with various concentrations of NOSC (0.001%, 0.01%, 0.04%, 0.05%, 0.10%, (w/v)) on the uptake of VP16 was examined, respectively. Each experiment was triplicated.

#### 2.5.3. Cell monolayer transport study

After a 3-week cultivation period of Caco-2 cells on permeable polycarbonate filters, polarized monolayers with apical brush borders and well-developed tight junctions were obtained (Hunter et al., 1993). The cell monolayers were washed twice with HBSS. After washing, the monolayers were preincubated at 37 °C for 20 min, and TEER was measured. HBSS solution on both sides of the cell monolayers was then removed by aspiration. For the measurement of the apical (AP) to basolateral (BL) transport, 400  $\mu$ L of HBSS containing VP16 (100  $\mu$ g/mL) was added to the AP side, and 500  $\mu$ L blank HBSS was added to the BL side. For the measurement of the BL to AP transport, 500  $\mu$ L of HBSS containing VP16 (100  $\mu$ g/mL) was added to the BL side, and 400  $\mu$ L blank HBSS was added to the AP side. The monolayers were placed in an incubator at 37 °C. Samples were taken from the receiving chamber at 30, 60, 90 and 120 min followed by an immediate replacement of the same volume of pre-warmed fresh HBSS. The TEER was measured at the same time. At the end of the experiment, the Caco-2 cell monolayers were rinsed by 37 °C HBSS, and incubated with the culture medium at 37 °C for 24 h. The TEER was measured again to check the integrity of the monolayer. Apparent permeability coefficients ( $P_{\text{app}}$ ) and efflux ratios (ER) of VP16 were calculated according to the equations, respectively:  $P_{\text{app}} = (dQ/dt) / (A \times C_0)$ ;  $\text{ER} = P_{\text{appBL} \rightarrow \text{AP}} / P_{\text{appAP} \rightarrow \text{BL}}$ , where the  $dQ/dt$  ( $\mu$ g/s) was the drug permeation rate;  $A$  was the cross-sectional area (0.6  $\text{cm}^2$ ) and  $C_0$  ( $\mu$ g/mL) was the initial VP16 concentration in the donor compartment at  $t = 0$  min. The effect of various concentrations of NOSC (0.01%, 0.04%, 0.05%, 0.10% (w/v)) or 100  $\mu$ M verapamil on the transport of VP16 (100  $\mu$ g/mL) from the apical to the basolateral side was examined. Each experiment was triplicated.

### 2.6. HPLC analysis of the samples

VP16 samples were analyzed by a reversed phase HPLC technique. The HPLC system comprised of a SHIMADZU LC-10AT pump, SHIMADZU SPD-10A UV detector, SHIMADZU SIL-10AD auto sampler. A  $\text{C}_{18}$  column (150 mm  $\times$  4.6 mm  $\times$  5  $\mu$ m, Diamonsil) was employed for the separation of analytes.

In rat intestinal circulation perfusion, mobile phase composed of methanol: water (55:45, v/v). Flow rate was maintained at 1.0 mL/min and detection wavelength was set at 285 nm. Retention time for VP16 was about 7 min. The linear equation was

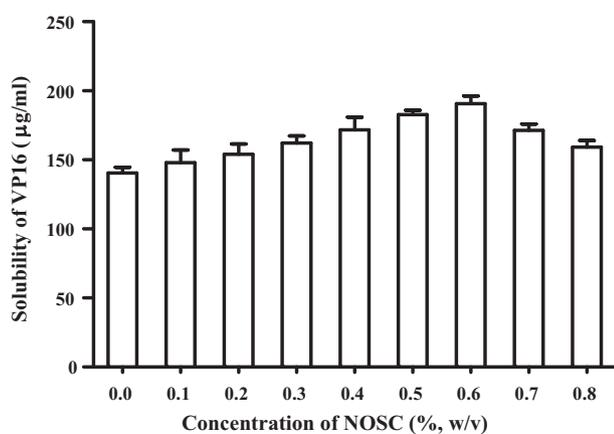


Fig. 2. VP16 solubilities in NOSC solution with different concentrations.

$A = 8436.8 \times C - 4963.8$  ( $R^2 = 0.9999$ ), where  $A$  was HPLC area and  $C$  was the concentration of VP16 ( $\mu\text{g}/\text{mL}$ ), and both intra-day and inter-day precision were lower than 3%. In Caco-2 cell studies, mobile phase composed of methanol: acetate buffer (45:55, v/v). The acetate buffer was purified water with 0.272% sodium acetate, and acetic acid was used to adjust pH of acetate buffer to 4.0. Flow rate was maintained at 1.0 mL/min and detection wavelength was set at 254 nm. Retention time for VP16 was about 13 min. The linear equation was  $A = 28952 \times C - 963.99$  ( $R^2 = 0.999$ ), and both intra-day and inter-day precision were lower than 10%.

### 2.7. Statistical analysis

Results are given as mean  $\pm$  S.D. Statistical significance was tested by two-tailed Student's  $t$ -test or one-way ANOVA. Statistical significance was set at  $P < 0.05$ , and extremely significance was set at  $P < 0.01$ .

## 3. Results

### 3.1. VP16 solubility in NOSC solutions

As the concentration of NOSC increased from 0% (w/v) to 0.8% shown in Fig. 2, firstly the solubility of VP16 raised slightly, not significantly, and then reduced, which indicated that NOSC had no obvious effect on increasing the solubility of VP16.

### 3.2. VP16 stability and physical adsorption in rat intestinal perfusion test

These studies were carried out to ensure that the loss of drug during experiment was due to absorption only and not due to other losses (e.g. nonspecific binding to the inactive intestine or chemical

Table 1

The physical adsorption of drug in four intestinal segments ( $n = 3$ ).

Drug	Mean physical adsorption (mean $\pm$ S.D. ( $\mu\text{g}/\text{cm}^2$ ))			
	Duodenum	Jejunum	Ileum	Colon
VP16	0.096 $\pm$ 0.0047	0.318 $\pm$ 0.0098	0.145 $\pm$ 0.0089	0.053 $\pm$ 0.0005

Table 2

Remaining ratio (%) of drug after incubation in intestinal circulating perfusion buffer at 37 °C for 2 h ( $n = 3$ ).

Drug	Concentration ( $\mu\text{g}/\text{mL}$ )	Remaining ratio (mean $\pm$ S.D. (%))			
		30	60	90	120 min
VP16	50	99.74 $\pm$ 0.20	99.53 $\pm$ 0.27	98.83 $\pm$ 0.09	99.11 $\pm$ 0.35

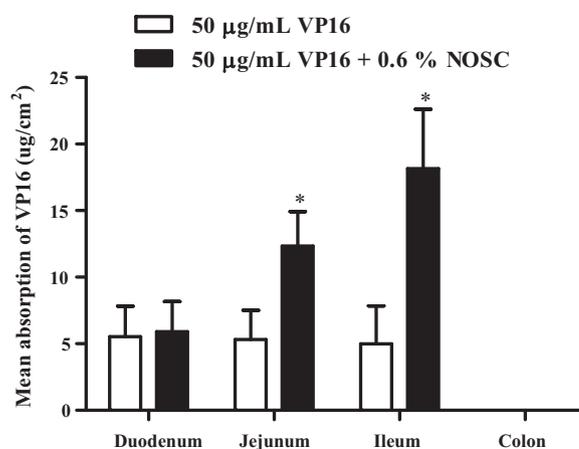


Fig. 3. The mean absorption of drug with and without NOSC ( $n = 3$ ). \* $P < 0.05$ , compared with VP16 without NOSC at the same intestinal segment.

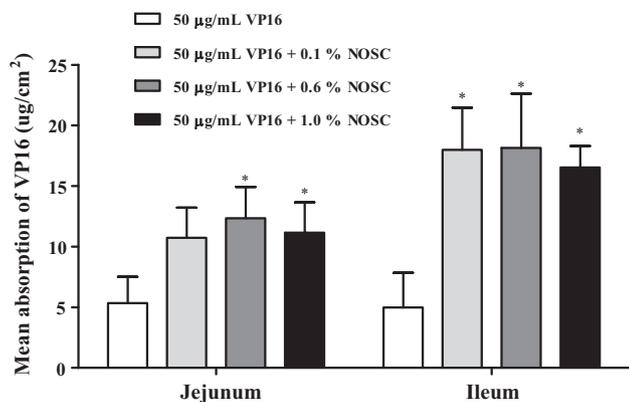
degradation). No loss of VP16 was observed during the circulating perfusion of the drug solution with the inactive intestine as shown in Table 1, and VP16 was also found to be stable in intestinal circulating perfusion buffer at 37 °C for 2 h which was shown in Table 2.

### 3.3. Absorption of VP16 with or without NOSC in rat intestinal segments

The mean absorption of VP16 with and without 0.6% (w/v) NOSC in rat four intestinal segments was shown in Fig. 3. Pre-test indicated that no loss of VP16 was observed during the circulating perfusion due to physical adsorption or chemical degradation. The mean absorption of VP16 without NOSC in rat duodenum, jejunum and ileum was  $5.52 \pm 2.29$ ,  $5.33 \pm 2.18$  and  $4.98 \pm 2.86 \mu\text{g}/\text{cm}^2$ , respectively and was not found in colon. Interestingly, NOSC improved the absorption of VP16 in both jejunum and ileum obviously ( $P < 0.05$ ). Fig. 3 showed that the mean absorption of VP16 with NOSC (0.6%) in jejunum and ileum was  $12.34 \pm 2.58$  and  $18.15 \pm 4.47 \mu\text{g}/\text{cm}^2$ , respectively, and was 2.32 and 3.64 times increase in jejunum and ileum, respectively compared with that without NOSC, while the absorption of VP16 with NOSC in duodenum was  $5.91 \pm 2.28 \mu\text{g}/\text{cm}^2$  which was almost the same with that of VP16 without NOSC ( $P > 0.05$ ).

### 3.4. The absorption of VP16 with different concentrations of NOSC in rat jejunum and ileum

Since that the mean absorption of VP16 with NOSC increased significantly in both jejunum and ileum, the effect of different concentrations of NOSC on the mean absorption of VP16 in jejunum and ileum was investigated. As shown in Fig. 4, com-



**Fig. 4.** The mean absorption of VP16 with different concentrations of NOSC in two intestinal segments ( $n=3$ ). \* $P<0.05$ , compared with VP16 without NOSC at the same intestinal segment.

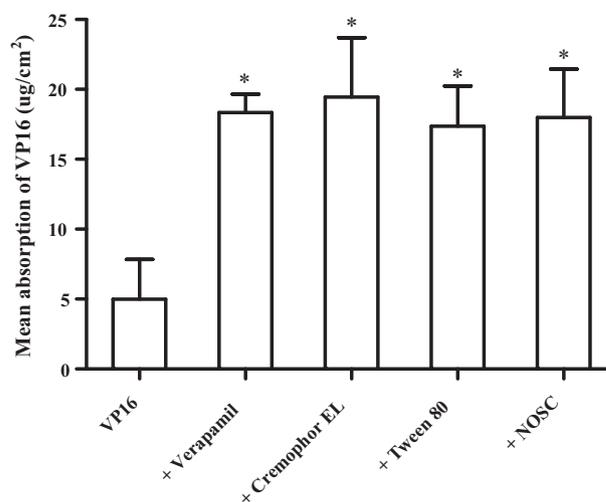
pared with the mean absorption of VP16 without NOSC in jejunum which was  $5.33 \pm 2.18 \mu\text{g}/\text{cm}^2$ , the mean absorption of VP16 with 0.1%, 0.6% and 1.0% (w/v) NOSC was  $10.23 \pm 2.49$ ,  $12.34 \pm 2.58$  and  $11.14 \pm 2.51 \mu\text{g}/\text{cm}^2$  which was 1.92, 2.32 and 2.09 times of that of VP16 without NOSC, respectively. Among that, 0.6% and 1.0% NOSC improved the mean absorption of VP16 in jejunum significantly ( $P<0.05$ ), yet 0.1% NOSC improved insignificantly ( $P>0.05$ ). Nevertheless, the concentration of NOSC from 0.1% to 1.0% increased notably ( $P<0.05$ ) the mean absorption of VP16 in ileum from  $4.98 \pm 2.86 \mu\text{g}/\text{cm}^2$  to  $17.98 \pm 3.46$ ,  $18.15 \pm 4.47$  and  $16.54 \pm 1.76 \mu\text{g}/\text{cm}^2$ , which was 3.61, 3.64 and 3.32 times of that of VP16 without NOSC, respectively.

### 3.5. The absorption of VP16 with NOSC and different P-gp inhibitors in rat ileum

On account of our tests above, the improvement of NOSC on the intestinal absorption of VP16 had been proved. VP16 was reported as a P-gp substrate (Lum et al., 2000), and so the absorption of VP16 in rat intestine was influenced by the effect of P-gp excretion. In order to find that whether the effect of NOSC on improving the intestinal absorption of VP16 was implicated to P-gp inhibition, several different P-gp inhibitors were chosen to compare the impact of them with that of NOSC on the absorption of VP16 in rat ileum in which more P-gp was reported than in jejunum (Hunter and Hirst, 1997; Varma et al., 2003). As demonstrated in Fig. 5, the mean absorption of VP16 with 0.1% (w/v) Verapamil, 0.1% Cremophor EL, 0.1% Tween 80 and 0.1% NOSC in ileum was 3.68, 3.91, 3.49 and 3.61 times of that of VP16 without them, respectively. All of them enhanced the mean absorption of VP16 in ileum ( $P<0.05$ ). However, there was no significant difference ( $P>0.05$ ) between the ability of NOSC and those P-gp inhibitors on elevating the absorption of VP16 in ileum. As a consequence of the test, it was possible for NOSC to have the same potential with other P-gp inhibitors above on inhibiting P-gp to enhance the intestinal absorption of VP16.

### 3.6. Effect of VP16 concentration and NOSC concentration on cell uptake

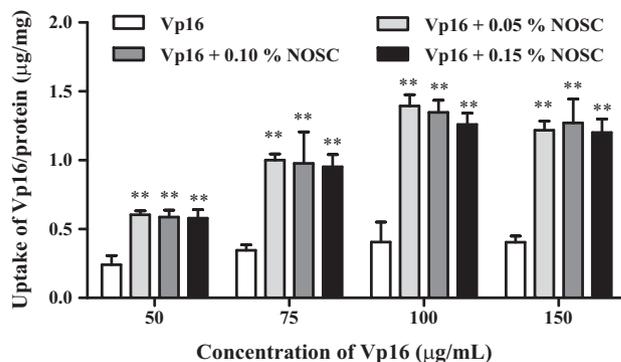
As shown in Fig. 6, the Caco-2 cell uptake was increased significantly while the concentration of VP16 was increased from  $50 \mu\text{g}/\text{mL}$  to  $150 \mu\text{g}/\text{mL}$ . In addition, the increase was not linear which showed that the Caco-2 cell uptake of VP16 had been saturated as the concentration of VP16 increased. The test



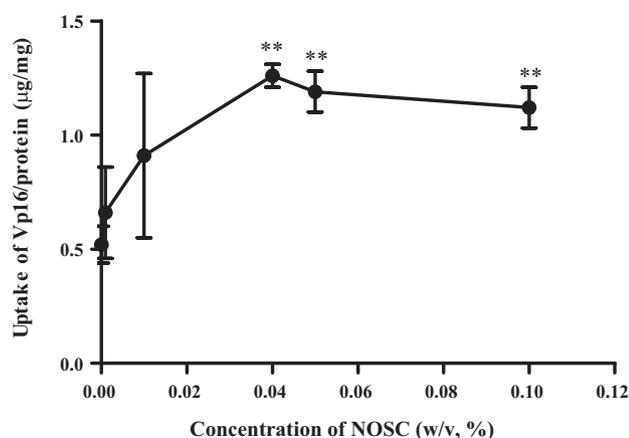
**Fig. 5.** The mean absorption of VP16 with different P-gp inhibitors in ileum ( $n=3$ ). \* $P<0.05$ , compared with VP16.

demonstrated that uptake of VP16 might be influenced by the receptor in Caco-2 cells such as P-gp. With the same concentration of VP16, the NOSC concentrations of 0.05%, 0.10% and 0.15% (w/v), all higher than its CMC value (0.045%, w/v) (Zhang et al., 2004), elevated the uptake of VP16 significantly ( $P<0.01$ ), but there was no significant difference among these increasing uptakes. The largest improvement of VP16 uptake was found at VP16 concentration of  $100 \mu\text{g}/\text{mL}$ . Interestingly, it seemed that the increasing uptake was related with VP16 concentration but was independent on NOSC concentration, which was higher than CMC value.

When the concentration of NOSC varied from 0.001% to 0.01% (w/v) spanning the CMC of NOSC, the effect on VP16 uptake was demonstrated in Fig. 7. As the NOSC concentration changed from 0.001% to 0.04% (w/v), the VP16 uptake increased and reached to the maximum level at 0.04% NOSC which was close to the CMC value. Afterwards, the uptake was slightly decreased with the increasing NOSC concentrations but there was no significant difference among these data ( $P>0.05$ ). Compared with the VP16 uptake without NOSC, the uptake with 0.04%, 0.05% and 0.10% (w/v) NOSC all increased extremely significantly ( $P<0.01$ ), and the uptake of VP16 with them were  $1.26 \pm 0.05$ ,  $1.19 \pm 0.09$  and  $1.12 \pm 0.09 \mu\text{g}/\text{mg}$ , respectively. The results were very similar to those when the NOSC concentration higher than CMC was used as described in Fig. 6.



**Fig. 6.** The effect of NOSC concentrations on VP16 uptake with different concentrations ( $n=3$ ). \*\* $P<0.01$ , compared with VP16 without NOSC at the same concentration of NOSC.



**Fig. 7.** The effect of different concentrations of NOSC on uptake of 100 µg/mL VP16 ( $n=3$ ). \*\* $P<0.01$ , compared with VP16 without NOSC.

### 3.7. Effect of NOSC on the transport of VP16 across Caco-2 cell monolayers

Based on the cell uptake studies above, the effect of NOSC and other P-gp inhibitors on the transport of VP16 was investigated. As shown in Table 3,  $Papp_{AP \rightarrow BL}$  and  $Papp_{BL \rightarrow AP}$  of VP16 were  $0.47 \pm 0.21 \times 10^{-6}$  and  $9.67 \pm 0.26 \times 10^{-6}$  cm/s, respectively and ER was 20.57, which greatly indicated the very low permeability of VP16 and its excretion by P-gp. Furthermore,  $Papp_{AP \rightarrow BL}$  of VP16 with four different concentrations of NOSC from 0.01% to 0.10% (w/v) were all increased notably and  $Papp_{BL \rightarrow AP}$  were all decreased significantly, compared with that without NOSC ( $P<0.05$ ). The results demonstrated that NOSC played an important role on inhibiting the function of P-gp and enhancing the permeability of VP16. Moreover, as the same tendency as the effect of NOSC on the cell uptake, the  $Papp_{AP \rightarrow BL}$  of VP16 was increased to the maximum level at the concentration of 0.04% NOSC, and subsequently slightly decreased. In the contrary, the  $Papp_{BL \rightarrow AP}$  of VP16 firstly decreased, and then increased. In comparison 0.04% with 0.05% NOSC or 0.04% with 0.10% NOSC, the  $Papp_{AP \rightarrow BL}$  of VP16 was no significant difference ( $P>0.05$ ), respectively. Nevertheless, the  $Papp_{BL \rightarrow AP}$  of VP16 with 0.04% NOSC is much less than that with 0.10% NOSC, which noted that as the concentration of NOSC was higher than its CMC, the ability of NOSC to inhibit the P-gp efflux attenuated.

Verapamil was reported as a typical P-gp inhibitor and widely used as the reference to identify the substrate of P-gp. In our study, 100 µM verapamil caused an obvious increase of  $Papp_{AP \rightarrow BL}$  of VP16 from  $0.47 \pm 0.21 \times 10^{-6}$  cm/s to  $1.01 \pm 0.06 \times 10^{-6}$  cm/s ( $P<0.05$ ) and a significant decrease of  $Papp_{BL \rightarrow AP}$  of VP16 from  $9.67 \pm 0.26 \times 10^{-6}$  cm/s to  $1.58 \pm 0.04 \times 10^{-6}$  cm/s ( $P<0.05$ ). ER of VP16 with verapamil was 1.56, which screened that verapamil

completely inhibited the VP16 efflux by P-gp. Our test also demonstrated that NOSC had the inhibition effect of P-gp and enhanced the AP to BL transport of VP16 across the Caco-2 cell monolayer. As presented in Table 3, when different amounts of NOSC and verapamil were added respectively to the apical compartment,  $Papp_{AP \rightarrow BL}$  of VP16 had no significant difference between them.  $Papp_{AP \rightarrow BL}$  of VP16 with 0.04% and 0.05% (w/v) NOSC were even higher than that with 100 µM verapamil. However,  $Papp_{BL \rightarrow AP}$  and ER of VP16 with any concentration of NOSC were higher than those of VP16 with verapamil. The results showed that NOSC prevented BL  $\rightarrow$  AP efflux of VP16 by inhibiting P-gp and enhanced the absorption. It was noticed that the inhibiting effect of NOSC was weaker than that of verapamil. In the presence of verapamil,  $Papp_{AP \rightarrow BL}$  ( $(1.01 \pm 0.06) \times 10^{-6}$  cm/s) was close to those with NOSC, but  $Papp_{BL \rightarrow AP}$  ( $(1.58 \pm 0.04) \times 10^{-6}$  cm/s) was much less than that with NOSC. It was suggested that the former was completely dismissed the efflux of P-gp and also decreased the diffusion effect of VP16 by unknown mechanism.

During the experiment, the TEER was also measured at 0 min, 30 min, 60 min, 120 min and after the experiment. As exhibited in Table 4, TEER of the Caco-2 cell monolayers varied slightly during the test, and at the end of experiment, after Caco-2 cells incubated with the culture medium at 37 °C for 24 h. It indicated that NOSC had the effect on inhibiting P-gp without changing TEER of the Caco-2 cell monolayers, and thus the effect on opening intercellular tight junction to enhance the transport of VP16 could be overlooked at the experimental concentration of NOSC from 0.01% to 0.1% (w/v).

## 4. Discussion

A number of studies have shown that many common pharmaceutical surfactants/excipients can modulate the activity of the efflux transporter P-gp, and possibly other transporters in recent years. Therefore, the concept that all surfactants/excipients are "inactive" has been suspected, and the idea that they were largely inactive molecules used to improve the stability and solubility of drugs has to be reevaluated (Wandel et al., 2003). In trying to assess the potential of such surfactants/excipients to improve the absorption and thus enhance the oral bioavailability of drugs, studies have been carried out with a large amount of different *in vitro* or *in vivo* systems, different drugs, and wide ranges of excipient/surfactant concentrations.

N-octyl-O-sulfate chitosan (NOSC) was a chitosan derivate newly prepared. Micelle formed by NOSC has great capability in solubilization of water-insoluble drug paclitaxel (Zhang et al., 2004, 2008a,b). Enormous attention was attracted by the potential application of NOSC as a new drug delivery system. Considering that chitosan and its some other derivatives had the effect on inhibiting P-gp or opening the cellular tight junctions to enhance the absorption of drugs (Kotzé et al., 1998; Palmberger et al., 2008; Werle and Hoffer, 2006), our tests demonstrated that NOSC improved the absorption of the water-insoluble drugs, through using the intestinal circulating perfusion technique and Caco-2 cells. In solubility studies, it was demonstrated that NOSC had little ability to increase the solubility of VP16, which removed the potentiality that NOSC improved the absorption of VP16 by elevating its solubility. In our studies, the solubility of N-octyl-O-sulfate chitosan (NOSC) is investigated in neuter pH condition, which is 10 mg/mL at least. Unlike the assay of the solubility of agents with low molecular weight, it is relatively complex to determine precisely the solubility of polymer with high molecular weight. Accordingly, we evaluated approximately the solubility of NOSC at different pH conditions. The results are listed as follows: 20 mg/mL at pH 1.2, 18 mg/mL at pH 4.5, 8 mg/mL at pH 5.5, 10 mg/mL at pH 6.8, 10 mg/mL at pH 7.4 and 20 mg/mL at deionized water. It can be seen that the good

**Table 3**  
Apparent permeability coefficients ( $Papp$  ( $\times 10^6$  cm/s)) and efflux ratios (ER) of VP16 (100 µg/mL) transport across Caco-2 cell monolayers ( $n=3$ ).

Sample	$Papp$ ( $\times 10^{-6}$ cm/s)		ER
	AP $\rightarrow$ BL	BL $\rightarrow$ AP	
Control	$0.47 \pm 0.21$	$9.67 \pm 0.26$	20.57
Verapamil (100 µM)	$1.01 \pm 0.06^*$	$1.58 \pm 0.04^*$	1.56
NOSC (w/v, %)			
0.01	$0.82 \pm 0.05^*$	$5.37 \pm 0.81^*$	6.55
0.04	$1.22 \pm 0.18^*$	$5.13 \pm 1.19^*$	4.20
0.05	$1.11 \pm 0.09^*$	$6.60 \pm 1.22^*$	5.95
0.1	$0.93 \pm 0.02^*$	$7.71 \pm 0.24^*$	8.29

\*  $P<0.05$ , compared with controlled VP16.

**Table 4**  
TEER of the Caco-2 cell monolayers in the Caco-2 transport study ( $n=3$ ).

Time (min)	0	30	60	90	120	After 24 h
AP → BL						
VP16	594 ± 6	544 ± 7*	569 ± 10	538 ± 20*	540 ± 10*	510 ± 65*
+0.01% NOSC	460 ± 10	464 ± 9	506 ± 3	502 ± 7	523 ± 3*	485 ± 10
+0.04% NOSC	556 ± 46	515 ± 35	553 ± 37	524 ± 36	515 ± 25	610 ± 38
+0.05% NOSC	429 ± 29	427 ± 21	446 ± 28	472 ± 26	476 ± 29	518 ± 5*
+0.10% NOSC	467 ± 10	480 ± 14	510 ± 4*	510 ± 4*	530 ± 21*	531 ± 10*
+100 μM Verapamil	597 ± 16	582 ± 21	634 ± 9*	631 ± 15	607 ± 14	733 ± 21*
BL → AP						
VP16	593 ± 51	555 ± 5	442 ± 67*	434 ± 77*	385 ± 73*	566 ± 111
+0.01% NOSC	472 ± 13	508 ± 23	600 ± 27	564 ± 17	592 ± 12	583 ± 13
+0.04% NOSC	604 ± 1	577 ± 76	585 ± 205	401 ± 109*	405 ± 74*	535 ± 102
+0.05% NOSC	490 ± 11	757 ± 21*	457 ± 27	406 ± 7*	373 ± 4*	506 ± 5
+0.10% NOSC	488 ± 28	383 ± 3*	177 ± 2*	228 ± 7*	246 ± 1*	423 ± 20*
+100 μM Verapamil	598 ± 23	597 ± 5	556 ± 2*	546 ± 1*	510 ± 3*	622 ± 11

\*  $P < 0.05$ , compared with 0 h.

water solubility are kept in different pH buffer solution, although the presence of octyl group results in the increase of hydrophobicity and decrease the solubility in comparison with chitosan sulfate. Furthermore, the hydrophobic group is very important for the formation of amphiphilic molecule and its micelles with solubilization capacity.

In the intestinal circulating perfusion tests, compared with the intestinal mean absorption of VP16 without NOSC in rat four intestinal segments, the mean absorption of VP16 with NOSC in rat jejunum and ileum rose dramatically ( $P < 0.05$ ), while the mean absorption of VP16 with NOSC in duodenum increased slightly ( $P > 0.05$ ). The disposition of P-gp in the intestine was reported (Hunter and Hirst, 1997; Varma et al., 2003). The rank order of the disposition of P-gp was ileum > jejunum > duodenum. Therefore, the result that NOSC increased the absorption of the P-gp substrates, VP16, much more in jejunum and ileum than in duodenum, and showed indirectly that the effect of NOSC on enhancement of the intestinal absorption of VP16 was relative to inhibition of the P-gp expressed in the intestine. On the other hand, when NOSC and a few P-gp inhibitors, such as Cremophor EL, Tween 80 and Verapamil were added in VP16 HBSS solution, the mean absorption of VP16 with them in ileum had no notable difference, which indicated that NOSC had the same mechanism with these P-gp inhibitors to improve the absorption of VP16.

According to the cell uptake studies, the uptake of various concentrations of VP16 with any concentration of NOSC from 0.05% to 0.15% (w/v) elevated extremely significantly ( $P < 0.01$ ), in comparison with that of VP16 without NOSC, which indicated that NOSC had the potent to enhance the uptake of VP16 in Caco-2 cells. In addition, when the concentration of NOSC changed extensively from 0.001% to 0.10% (w/v), the uptake of 100 μg/mL VP16 with 0.04%, 0.05% and 0.10% (w/v) NOSC all increased extremely significantly ( $P < 0.01$ ) as shown in Fig. 7.

In the transport studies, ER of VP16 was 20.57, which indicated the very low transportation of VP16 across Caco-2 cell monolayers and its efflux by P-gp. In our study, verapamil, a classic P-gp inhibitor, led to an obvious increase of  $Papp_{AP \rightarrow BL}$  of VP16 ( $P < 0.05$ ) and a significant decrease of  $Papp_{BL \rightarrow AP}$  of VP16 ( $P < 0.05$ ). ER of VP16 with verapamil was 1.56 between 1 and 2, which screened that verapamil modified the VP16 transport across Caco-2 cell monolayers by inhibiting P-gp. Our test also showed that NOSC had the same effect with verapamil on inhibiting P-gp to enhance the AP to BL transport of the P-gp substrate VP16 across the Caco-2 cell monolayer. As manifested in Table 3,  $Papp_{AP \rightarrow BL}$  of VP16 had no significant difference between them as different amounts of NOSC and verapamil were added respectively to the apical compartment. In contrary,  $Papp_{BL \rightarrow AP}$  and ER of VP16 with verapamil was lower than those of VP16 with any concentration of NOSC, which indi-

cated that the impact of verapamil on preventing BL → AP transport of VP16 and inhibiting P-gp was stronger than that of NOSC, which depended on the different ways inhibiting P-gp between NOSC and verapamil.

Otherwise,  $Papp_{AP \rightarrow BL}$  of VP16 with four different concentrations of NOSC from 0.01% to 0.10% (w/v) were all increased notably ( $P < 0.05$ ), while  $Papp_{BL \rightarrow AP}$  of VP16 with them all decreased significantly, which demonstrated that NOSC could inhibit P-gp and enhance the transport of VP16. Furthermore, as the same tendency as the effect of NOSC on the cell uptake of VP16 had that of NOSC on the transport of VP16 across Caco-2 cell monolayers, the  $Papp_{AP \rightarrow BL}$  of VP16 initially increased, and subsequently decreased. In the contrary,  $Papp_{BL \rightarrow AP}$  and ER of VP16 firstly decreased, and then increased.

NOSC is an amphipathic derivative of chitosan, which can form micelles as its concentration is higher than its CMC. The CMC of NOSC is 0.045% (w/v). When the concentration of NOSC in the solution is lower than CMC, it exists as a monomer, which makes it easier interacts with the lipid bilayers of the cell membrane. In contrast, NOSC micelle due to its larger size and the hydrophilic shell, difficultly gets into the lipid bilayers. Only when the compound is in the lipid bilayer of the cell membrane, it can interact with P-gp. Thus, the largest enhancing effect of  $Papp_{AP \rightarrow BL}$  of VP16 produced with 0.04% (w/v) NOSC. The increase of NOSC concentration above CMC cannot do the favor further for VP16 absorption.

During the transport studies, TEER was measured. Table 4 showed that when VP16 with NOSC was added, TEER of the Caco-2 cells monolayers quietly changed, and at the end of experiment, after Caco-2 cells incubated with the culture medium at 37 °C for 24 h, TEER of the Caco-2 cells monolayers recovered. The explanations for the result were that NOSC could not open the cellular tight junctions, but inhibit P-gp to enhance the permeability of water-insoluble drugs, such as VP16.

In rat intestinal circulation perfusion studies, it was indicated that when the concentration of NOSC was higher than CMC, the effect of NOSC on improving the absorption of VP16 had no change and tended to be constant. We had just investigated the effect of NOSC with concentration above CMC forming the micelles on the absorption of VP16. In Caco-2 studies, we estimated the effect of NOSC with concentration below and above CMC. The effect of NOSC with the concentration approaching CMC on the absorption of VP16 is greatest. As the concentration of NOSC was higher than CMC, there was no notable difference on the Caco-2 uptake of VP16 and the transport of VP16 across the Caco-2 monolayer, which was corresponding with *in vivo* data. In other words, the concentration of NOSC higher than CMC all has the effect on enhancing the absorption of VP16 from *in vitro* and *in vivo* data. We have not found that there

is a mathematical correlation between *ex vivo* data and *in vivo* data.

In conclusion, the results presented here show that NOSC has the effect of enhancing the absorption of several water-insoluble drugs, and the mechanism of this effect is that NOSC inhibits the P-gp located in the apical membranes of intestinal absorptive cells mainly, or opens the intestinal intercellular tight junctions. The CMC of NOSC is a very important factor for NOSC promotion for P-gp substrate drugs. When the concentration of NOSC is close to the CMC of NOSC, the strength of NOSC inhibiting P-gp is greatest. Therefore, combination anticancer drugs with NOSC may provide a useful alternative dosage form for oral administration of anticancer drugs to circumvent drug resistance in cancer chemotherapy. However, the mechanism of the surfactants/excipients' effect on inhibiting P-gp is extremely complex (e.g. changing the fluidity of the cellular membrane, inhibiting the ATP enzyme of P-gp, reducing P-gp expression), and thus the studies to investigate the effect of NOSC on inhibiting P-gp have to be carried out in future.

## 5. Conclusion

Research efforts have been devoted to demonstrating that *in situ* NOSC enhances the oral bioavailability of VP16 in rat, increases the drug permeability, and thus enhances absorption in the intestinal tract, and *in vitro* NOSC elevated the uptake and transport of VP16 through the Caco-2 cell monolayer, via interfering the activity of intestinal P-gp. The enhancement of oral absorption of VP16 formulated using NOSC further development of oral formulations for VP16 in clinical uses. NOSC can be used as a formulation excipient for the poorly orally absorbable drugs, particularly P-gp substrates.

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