Influence of Chitosan Nanoparticles as the Absorption Enhancers on Salvianolic acid B In vitro and In vivo Evaluation

Xin Jin, Shi-bing Zhang, Shi-meng Li, Ke Liang, Zeng-yong Jia

Department of Pharmacy, The Suqian First Hospital, Suqian, Jiangsu 223800, Department of Pharmacy, The First Affiliated Hospital of Soochow University, Suzhou, Jiangsu 215006, China

ABSTRACT

Background: Salvianolic acid B (SalB) represents the most abundant and bio-active phenolic constituent among the water-soluble compounds of Salvia miltiorrhiza. But the therapeutic potential of SalB has been significantly restricted by its poor absorption. Methods: In this study, chitosans (CS) and CS nanoparticles (NPs) with different molecular weights (MWs), which have influence on the absorption of SalB, was also investigated. Results: As a preliminary study, watersoluble CS with various MWs (3, 30, 50, and 100 kDa) was chosen. We investigated the MW-dependent Caco-2 cell layer transport phenomena in vitro of CS and NPs at concentrations (4 μg/ml, w/v). SalB, in presence CS or NPs has no significant toxic effect on Caco-2 cell. As the MW increases, the absorption enhancing effect of CS increases. However, as the MW decreases, the absorption enhancing effect of NPs increases. The AUC of the SalB-100 kDa CS was 4.25 times greater than that of free SalB. And the AUC of the SalB-3 kDa NPs was 16.03 times greater than that of free SalB. Conclusion: CS and NPs with different MWs as the absorption enhancers can promote the absorption of SalB. And the effect on NPs is better than CS. Key words: Absorption enhancer, bioavailability, Caco-2 cell model, chitosan, chitosan nanoparticles, molecular weight, salvianolic acid B

SUMMARY

Formation mechanism for NPs

INTRODUCTION

Danshen, the dried root of Salvia miltiorrhiza, is a widely used traditional herb in China to improve body functions. Salvianolic acid B [SalB, structure shown in Figure 1a] represents the most abundant and bioactive phenolic constituent among the water-soluble compounds of Danshen. Studies have shown that SalB can elicit endothelium-dependent vasodilation, lower blood pressure in hypertension, and improve regional cerebral blood flow and prohibit platelet aggregation. SalB has attractive pharmacological activities; however, the treatment of SalB has been significantly restricted by its poor bioavailability. Generally, hydrophilic compounds transport across the intestinal barrier via paracellular pathway. The presence of tight junctions between the epithelial cells will limit absorption of SalB when the paracellular pathway occupies less than 0.1% of the total surface area of the intestine epithelium. It is difficult to find the suitable delivery to increase SalB absorption due to its hydrophilic. Due to the special features of adhering to the mucosal surface and transiently opening the tight junctions between epithelial cells, Chitosan [CS, Figure 1b] have been used as an absorption assistance of hydrophilic molecules. Meanwhile CS can be ionically cross linked with multivalent anions such as triply phosphate [TPP, Figure 1c] to form CS nanoparticles (NPs). This process, known as ionic gelation, has some advantages since it is a mild process resulting in NPs with low sizes and has been proven to encapsulate different lipophilic, biological, and active compounds.

Although numerous literatures are available on CS and NPs, the absorption relationships between NPs with different molecular weights (MWs) have not been reported. Meanwhile, to the best of our knowledge, NPs was first chosen as the absorption enhancer to absorb the hydrophilic SalB, which is charged negatively. Caco-2 cell culture model is used to investigate drug absorption and is recognized by Food and Drug Administration (FDA) as viable models of human intestinal absorption. The tight-junctions of Caco-2 monolayer formed at the apical side of the monolayer can discriminate the transcellular and paracellular transport of drugs across the epithelial layer. The drug permeability to Caco-2 monolayer is expected to correlate well with that of the intestinal membrane in vivo. Some reports have shown the possibility that the oral absorption of drugs in human body can from their permeability to Caco-2 monolayer. Hence, Caco-2 cell model, which measures the permeability, is used to indicate the absorption in

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Em-C3, Ottawa, Canada) at 200 bar and 25°C to obtain an opalescent suspension by passing three cycles through a high-pressure homogenizer (Avestin, Canada). The suspension was then mixed with sodium tetraphosphate (TPP) in a molar ratio of 2:100, followed by mechanical stirring of 1000 rpm at room temperature. After homogenization, the mixture was added to the CS solution. A nano suspension was obtained upon the addition of sodium tetraphosphate to the CS solution via the complex formation. The positively charged copolymers and the negatively charged TPP under mild conditions formed NPs as complex electrostatic interactions between the positively charged copolymers and the negatively charged TPP.

Instruments and materials
Rosmarinic acid (99.8%) and SalB (99.5%) were purchased from Sigma-Aldrich (St. Louis, MO). Cloned Caco-2 TC7 cells were a generous gift from Dr. Ming Hu of INSERM U178 (Houston, TX). Sodium TPP was purchased from Merck (Darmstadt, Germany). 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and Hank's balanced salt solution (HBSS; Merck) were used for transport studies. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) and Hank’s balanced salt solution (HBSS; Merck) were used for the transport assay. MTT assay
For the transport assay, Caco-2 cells (passage 38) were seeded on Transwell® (Biotechnology Co., Ltd. Shanghai Brilliance Cup) inserts and cultured in six well Transwell plates, which have a surface area of 4.2 cm². For the transport assay, Caco-2 cell monolayers grown in Transwell® (6-well) plates were used for the transport studies between days 21 and 23.

Permeability studies in the Caco-2 cell culture model
For the transport assay, Caco-2 cell monolayers grown on Transwell® inserts were used for the transport studies between days 21 and 23. The TEER values of the cell monolayer measured were more than 350 Ω·cm².

Preparation and characterization
NPs were formed as complex electrostatic interactions between the positively charged copolymers and the negatively charged TPP under mild conditions. Therefore, CS was dissolved in acetic acid solution (1.0% volume/volume). The concentration of acetic acid in aqueous solution was 1.5 times greater than of CS. Then, 20 ml sodium TPP solution (1.0 mg/mL) was added to CS solution. A nano suspension was obtained upon the addition of sodium TPP aqueous basic solution to the CS aqueous solution via mechanical stirring of 1000 rpm at room temperature. And homogenized by passing three cycles through a high-pressure homogenizer (Avestin Em-C3, Ottawa, Canada) at 200 bar and 25°C to obtain an opalescent dispersion of the NPs. Particle size distribution (Z-average), polydispersity index (PDI), and zeta potential of the dispersions were determined by photon correlation spectroscopy (Zetasizer Nano-ZS Malvern Instruments, Worcestershire, UK). Measurements were performed at 25°C, and the results were shown as the mean of three successive measurements of at least three independent samples. Each sample was diluted with distilled water to adjust the signal level. Measurements were carried out in dilute 1% acetic acid medium, and each sample was measured in triplicate. Then the morphological evaluation was performed using transmission electron microscopy (TEM; JEOL-1200EX, Japan).

MTT assay
The cytotoxic effects on Caco-2 cells were determined with MTT assay. Caco-2 cells used for MTT assay were plated in 96 well cell culture plates at a density 2 × 10⁴ cells/well and preincubated for 24 h before treatment. Then the cells were treated with samples (10 μmol SalB, in the presence CS or NPs with different MW) in serum-free medium (pH 5.5) for 24 h. The solutions were removed and fresh cell culture medium was added after treatment. Then the cells were incubated for 4 h to stabilize them. After stabilization, the cells were incubated with MTT (0.1 mg/mL MTT in serum-free medium) for 4 h. Finally, the medium was removed, and the resulting formazan crystal was dissolved with 100 μL DMSO per well. The cell relative viability (%) was calculated by using a microplate reader (Universal Microplate Analyzer, Model AOPUS01 and AI53601, Packard BioScience, CT, USA). Viability of nontreated control cells was arbitrarily defined as 100%.

Relative cell viability = \( \frac{OD_{\text{sample}550} - OD_{\text{blank}550}}{OD_{\text{control}550} - OD_{\text{blank}550}} \times 100 \)  

Where OD₅₅₀, sample is the absorption values of culture plates without cells at 550 nm, OD₅₅₀, control is the absorption values of samples that were treated blank HBSS at 550 nm, OD₅₅₀, sample is the absorption values of samples that were treated experimental samples at 550 nm.

Animal experiments
Male Sprague-Dawley rats weighing 200–250 g were obtained from the SLAC Laboratory Animal Center of Shanghai (Shanghai, China). The animals were housed under standard conditions of temperature, humidity, and light. Food and water were provided ad libitum. The rats were fasted overnight before the day of the experiment. All animal experiments procedures were conducted according to the Guiding Principles in the Use of Animals in Toxicology.
SalB, and the samples were collected at 0, 1, 2, 3, and 4 h after incubation. To each transport sample (400 μL), the addition of rosmarinic acid (20 μM) was as a preservative and internal standard. The resulting mixtures were swirled for 30 s and centrifuged at 15,000 rpm for 15 min. The supernatant was analyzed by UPLC within 24 h. The permeability of SalB was calculated using the following equation:

\[ P_{app} = \frac{V}{S \times C dt} \times \frac{1}{dC dt} \times \frac{dm dt}{S \times C} \]

Equation (2)

Where \( V \) is the volume of the receiver (typical volume 2.5 mL), \( S \) is the surface area of the cell monolayer (typical surface area 4.2 cm²), \( C \) is the initial concentration, \( \frac{dC}{dt} \) is the rate of concentration change on the receiver side, and \( \frac{dm}{dt} \) is the rate of drug transport. The rate of drug transport was obtained by linear regression analysis.

**TEER experiments**

Caco-2 cell layers revealing TEER ≥350 Ω·cm²⁻¹ were used in these experiments. Cell medium was removed and replaced with HBSS (buffered at pH 5.5 with HEPES on the apical and basolateral sides of the cell layers) before sample application. Cells were equilibrated in HBSS (incubated at 37°C, 5% CO₂) for 1 h, then TEER was measured, which was treated as the baseline TEER. NPs and CS (visually transparent in HBSS at pH 5.5) were then applied to the apical side of the cell layers, following which cells were incubated with the samples for 4 h. TEER was measured at times 0, 1, 2, 3, and 4 h (in the presence of the tested samples, in HBSS) following the sample application. Background TEER of the filter (100–110 Ω·cm²⁻¹) was subtracted from the measurements under all conditions. All samples were measured in triplicate. The change on TEER meaning of the cell monolayer was measured with a Millicell-Electrical Resistance System (Millipore Corp., Bedford, MA).

**Pharmacokinetic studies**

Male rats were divided randomly into nine groups for administration of a single dose of SalB or SalB CS of different MW or SalB NPs of different MW. The nine groups of rats were administered oral doses equivalent to 500 mg/kg of SalB. To determine the serum drug concentrations and calculate the pharmacokinetic parameters, blood samples were collected at 0, 10, 15, 20, 30, 45, 60, 90, 120, and 240 min after dosing. The blood samples were centrifuged at 3000 rpm for 10 min, and then the supernatants were collected into tightly sealed plastic tubes (containing a heparin sodium anticoagulant solution). We added 200 μL of an internal standard working solution (5 μg/mL rosmarinic acid in acetonitrile) to 200 μL of the plasma sample, and 800 μL of acetonitrile was then added to the mixture and vortexed for 30 s to precipitate the protein. The resulting mixture was centrifuged at 13,000 rpm for 15 min at 4°C. One milliliter of the upper organic phase was transferred to the receiver side, and the supernatant was analyzed by UPLC within 24 h. The permeability of SalB was studied in the Caco-2 model. The effect of CS MW on the permeability of SalB through the differentiated Caco-2 cell layer grown on a permeable filter support was examined. The apical to basolateral transepithelial passage of SalB is presented in Table 2, from which we can see that the permeation of SalB through the Caco-2 model. The conditions for UPLC analysis of SalB in the transport samples were as follows: System, Waters Acquity UPLC with a photodiode array detector and Empower software (Waters, Milford, MA); column, Acquity UPLC BEH C18, 1.7 μm, 2.1 × 50 mm (Waters). The UPLC elution condition was optimized as follows: 0–4 min (acetonitrile: Water containing 0.1% AcH = 40:60); flow rate 0.4 ml/min; column temperature 35°C; wavelength 286 nm; and injection volume 8 μL.

**Analytical methods**

UPLC was used to detect SalB in the transport samples obtained in the Caco-2 model. The conditions for UPLC analysis of SalB in the transport samples were as follows: System, Waters Acquity UPLC with a photodiode array detector and Empower software (Waters, Milford, MA); column, Acquity UPLC BEH C18, 1.7 μm, 2.1 × 50 mm (Waters). The UPLC elution condition was optimized as follows: 0–4 min (acetonitrile: Water containing 0.1% AcH = 40:60); flow rate 0.4 ml/min; column temperature 35°C; wavelength 286 nm; and injection volume 8 μL. The retention time for SalB was 1.69 min. The retention time for rosmarinic acid (the internal standard) was 2.61 min.

**Statistical analysis**

All experiments were performed at least in triplicate. Data were presented as the mean ± standard deviation. Student’s t-test was used to analyze the data. A two-tailed t-test (Microsoft Excel®) was used to identify significant differences (P < 0.05) compared with the controls.

**RESULT AND DISCUSSION**

**Characterization of the NPs**

The surface morphology and shape of the NPs were observed by scanning electron microscopy. The NPs were almost spherical, with a regular shape [Figure 2]. The particle size, size distribution, and zeta potentials of the NPs were also detected, as shown in Table 1. The NPs ranged from 100 to 300 nm in size with different MWs, and the PDI was less than 0.2. The particles had a relatively narrow size distribution and all of them had strong positive charge. The size measured in hydrated state is proposed to be a little higher than the size measured by TEM method. In this study, the results were based on the depiction of the size of dried state (actual diameter) by TEM versus hydrated state (hydrodynamic diameter) measured by light scattering.

**Cytotoxicity test**

The cytotoxicity of SalB, in the presence CS or NPs was assessed by MTT assay on the Caco-2 cell line. SalB, in the presence CS or NPs have no toxic effect on applied cells, and the effect was observed, as in Figure 3.

**The effect on in vitro absorption**

The in vitro stability of NPs at different environments of the gastrointestinal tract was different. According to reference, the morphology of the NPs at pH range of 2.5–6.6 was spherical in shape with a smooth surface; otherwise they became unstable and subsequently broken apart. Therefore pH 5.5 was used in vitro absorption, which was available in the intestinal environment. The effect of CS MW on the permeability of SalB through the differentiated Caco-2 cell layer grown on a permeable filter support was examined. The apical to basolateral transepithelial passage of SalB is presented in Table 2, from which we can see that the permeation of SalB through the...
Caco-2 cell layer was changed and depends on the CS MW at 4 μg/ml, w/v concentrations. CS achieved good penetration on the Caco-2 cell layer in a MW-dependent manner. The different transport phenomena caused the different values of apparent permeability coefficient (Papp). The relatively high Papp value of 2.39 × 10⁻⁶ cm/s was calculated from the CS 100 kDa penetration data. By decreasing CS MW, decreased Papp values of 1.92 × 10⁻⁶ cm/s, 1.45 × 10⁻⁶ cm/s, and 1.26 × 10⁻⁶ cm/s were obtained by CS 50 kDa, CS 30 kDa, and CS 3 kDa treatment, respectively. The fastest transport was inspected with CS 100 kDa. The penetration rates enhanced as the MW increased. There was more than 4.43-times enhanced transport observed with CS 100 kDa by 4 h treatment when compared with free SalB transport. Meanwhile the different transport phenomena of NPs may result from different mechanisms. As the MW increased at concentrations (4 μg/ml, w/v), average size of NPs was increased may conduct the decreasing penetration rates. The relatively high Papp value of 10.1 × 10⁻⁶ cm/s was calculated from the NPs 3 kDa permeability data. By increasing CS MW, decreased Papp values of 8.35 × 10⁻⁶ cm/s, 7.73 × 10⁻⁶ cm/s, and 6.92 × 10⁻⁶ cm/s were obtained by NPs 30 kDa, NPs 50 kDa, and NPs 100 kDa treatment, respectively. The fastest transport was inspected with CS 100 kDa by 4 h treatment when compared with free SalB transport. Meanwhile the different transport phenomena of NPs may result from different mechanisms. As the MW increased at concentrations (4 μg/ml, w/v), average size of NPs was increased may conduct the decreasing penetration rates. The relatively high Papp value of 10.1 × 10⁻⁶ cm/s was calculated from the NPs 3 kDa permeability data. By increasing CS MW, decreased Papp values of 8.35 × 10⁻⁶ cm/s, 7.73 × 10⁻⁶ cm/s, and 6.92 × 10⁻⁶ cm/s were obtained by NPs 30 kDa, NPs 50 kDa, and NPs 100 kDa treatment, respectively. The permeability of NPs 3 kDa was 18.70 and 4.23 times enhanced, while that of free SalB and CS 100 kDa.

**TEER experiments**

Evaluation of the CS and NPs in opening tight junctions was conducted in vitro in Caco-2 cell monolayer. Transport experiments were performed using the HBSS (at pH 5.5) medium. And the penetration of compact junction in the Caco-2 cell monolayer was monitored by TEER measurements. The records of TEER were calculated as a percentage relative to baseline TEER (measured just prior to experiment initiation) and plotted versus exposure time. The effect of CS or NPs at concentrations (4 μg/ml, w/v) on the TEER of the Caco-2 layers are presented in Figure 4 (Figure 4a and b for CS and NPs, respectively). Both profiles exhibited a typical pattern of a steep decrease in TEER for all the samples applied. It might be that the CS and NPs with a positive surface charge could reduce the TEER of Caco-2 cell monolayer effectively. After removal of the CS or NPs, which was incubated, a slow increase in TEER was observed. This observation was
obviously more remarkable. In contrast, such results were not observed for the control group without incubation with CS or NPs.

On one hand, the CS samples led to MW-dependent decreases in TEER with 1 h of exposure, with TEER falling to 76.11%, 73.12%, 68.23%, and 59.35% at exposure CS MW of 3 kDa, 30 kDa, 50 kDa, and 100 kDa, respectively [Figure 4a]. The effect stabilized after 4 h with the TEER values recorded at 44.69%, 38.64%, 34.34%, and 28.46%, for the respective MW at 4 h. And the effect of NPs was the same as CS.

On the other hand, the continuous decrease in the TEER values reached around 30% of initial values at 4 h after treatment. And the same phenomenon was observed on the NPs. After the removal of CS or NPs and supply of fresh culture medium, the TEER values started to increase. At 4 h after treatment of fresh culture medium, the TEER values were completely recovered to around 50% of initial levels. Therefore, both CS and NPs had reversibility of opening tight junctions.

Pharmacokinetic studies

We assessed the oral bioavailability of the SalB in rats and compared it with the SalB-CS and SalB-NPs. The mean SalB plasma concentration versus time plots for nine samples equivalent to 500 mg/kg doses of SalB orally administered to rats (n = 6) are shown in Figure 5. A summary of the statistical analysis is shown in Table 3. The average values for maximum concentration and time to maximum concentration after oral administration of SalB were 2.73 μg/mL and 28.14 min, respectively, while those after oral administration of the SalB-3 kDa NP were 49.78 μg/mL and 59.14 min, respectively. The average AUC<sub>0-∞</sub> of the SalB-3 kDa NP in rats was 184.51 μg·min/L, which was significantly higher than that of the free SalB. The AUC<sub>0-∞</sub> of the SalB-3 kDa NP was 16.03 times greater than that of free SalB. The size of SalB-NP decreased as the MW of CS. And the relative bioavailability of SalB increased with an increase in the MW of SalB-CS. Furthermore, the reference reported that the absorption enhancing effect of CS on the improvement of the intestinal absorption was affected by their concentrations, which was observed at relatively higher concentration.[29] However, in this article, the low concentration of CS (4 μg/mL, w/v) was found to have a great absorption enhancing effect on SalB. The reasons that the absorption enhancing effects of CS might be because of interactions with negatively charged groups of glycocalix and the low viscosity of CS at 4 μg/mL.[30]

Meanwhile, CS-NPs have been developed by the ionotropic gelation method using TPP anions.[31] The purpose of CS-NPs was to provide a nanoscale system adsorbent SalB (negatively charged substrates), for its application. However, the trends in TEER and PapP values develop are not in parallel between CS and NPs as they reflected the different functional properties. NPs have the same effect as CS on opening the tight junctions. But the absorption enhancing effect by NPs was significantly larger than CS. In addition, it was proposed that aside the mechanisms possesses by CS, there was another possible and crucial mechanism responsible for NPs improving drug absorption is endocytosis.[32] NPs showed different mechanisms of distribution and cellular uptake when compared with CS. The strong positive charge of NPs makes it possible to bind negatively charged substrates, such as lipids, in intestinal epithelial cells. It was reported that adhesion of NPs to the cell surface is a prerequisite to generate the interaction of cells with NPs. After adhesion, the internalization of NPs in cells may occur by fluid phase endocytosis or phagocytosis. Cellular uptake of NPs is a significant concern in absorption enhancement.

NPs can promote the drug delivery across cell surface barriers and result in opening of the tight junctions between epithelial cells transiently. Then, CS just has the function of opening of tight junction. Based on the above results, we drew the schematic diagram of intestinal SalB transport in presence of CS or NPs [Figure 6].

Moreover, the average AUC<sub>0-∞</sub> of the SalB-100 kDa CS in rats was 48.86 μg·min/L, which was significantly higher than that of the free SalB. The AUC<sub>0-∞</sub> of the SalB-100 kDa CS was 4.25 times greater than that of free SalB. The relative bioavailability of SalB increased with an increase in the MW of CS. Interestingly, the time to maximum concentration values of SalB-CS were also decreased. And there is also no significant difference between SalB-CS.

The tight gastrointestinal epithelium represents major barriers to drug delivery. Therefore, various oral delivery strategies have been examined to overcome these difficulties. This study described the existence of a range of CS-based delivery systems with considerable potential for oral application. Several proprietary properties such as biocompatibility, biodegradability, and nontoxicity offer CS boundless potentials for pharmaceutical applications.[27] In the biopharmaceutical opinion, CS has an enhancement of paracellular drug transport via transient opening of compact junction between epithelial cells.

Pharmacokinetik parameters of SalB, SalB-CS, and SalB-NPs orally in rats (n=6)

The data are presented as the mean±SD. AUC<sub>0-∞</sub>: Area under concentration-time curve; T<sub>max</sub>: Time to maximum plasma concentration; C<sub>max</sub>: Maximum plasma concentration; SalB: Salvianolic acid B; CS: Chitosans; NPs: Nanoparticles; SD: Standard deviation

Table 3: Pharmacokinetic parameters of SalB, SalB-CS, and SalB-NPs orally in rats (n=6)

| Parameters | SalB | 3 kDa CS | 30 kDa CS | 50 kDa CS | 100 kDa CS | 3 kDa NPs | 30 kDa NPs | 50 kDa NPs | 100 kDa NPs |
|------------|------|---------|---------|---------|---------|---------|---------|---------|---------|
| AUC<sub>0-∞</sub> (μg·min/L) | 10.23±1.01 | 22.57±2.14 | 28.61±2.41 | 37.57±3.71 | 45.38±3.97 | 180.23±20.01 | 167.23±18.56 | 145.73±15.46 | 130.43±13.01 |
| AUC<sub>0-∞</sub> (μg·min/L) | 11.51±1.49 | 23.47±2.49 | 29.07±2.82 | 39.22±3.47 | 48.86±4.16 | 184.51±24.45 | 172.33±19.26 | 150.51±20.11 | 135.81±16.38 |
| T<sub>max</sub> (min) | 28.14±4.13 | 43.14±5.01 | 44.26±4.33 | 46.32±2.98 | 44.78±4.36 | 59.14±5.71 | 58.29±4.56 | 60.45±5.51 | 61.96±4.99 |
| C<sub>max</sub> (μg/mL) | 2.73±0.22 | 6.27±0.79 | 7.64±0.93 | 10.42±1.41 | 12.56±2.05 | 49.78±6.77 | 44.23±5.29 | 42.31±5.21 | 36.58±4.27 |
CONCLUSION

It was concerned that CS NPs had a more positive effect of absorption enhancement on Caco-2 cell model when compared with CS. And the penetration rates on the Caco-2 cell layer enhanced as the CS’s MW increased. In addition, our results indicate that NPs of a smaller size improve the oral absorption of salB. However, the mechanism that CS NPs had an absorption enhancement needs to be further investigated.

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Conflicts of interest

There are no conflicts of interest.

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ABOUT AUTHORS

Ke Liang, is PhD for urology, attending doctor in the First Affiliated Hospital of Soochow University, China. He is interested in oral formulation design and investigations in pharmaceutical field.

Zeng-yong Jia, is an Professor at the Department of Pharmacy, The Suzian First Hospital, China. His research interest is in the area of Nano Pharmaceutics and its evaluation. Additionally, he is also a clinical pharmacist and interested in proper use of clinical medicine.