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ARTICLE

The P-glycoprotein inhibitory effect and related mechanisms of thiolated chitosan and its S-protected derivative

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Thiolated polymers have attracted more and more attention for its prominent efflux pump inhibitory properties. The aim of this study was to unravel the P-glycoprotein (P-gp) inhibitory mechanisms mediated by new synthetic thiolated chitosan, chitosan-thioglycolic acid (CS-TGA) and its S-protected derivative (CS-TGA-6MNA). P-gp inhibitory activity was first assessed by determining Rhodamine-123 (Rho-123) accumulation in Caco-2 cells, transportation across Caco-2 cell monolayers and across freshly excised rat intestine after exposure to CS-TGA or CS-TGA-6MNA. The results showed that after incubation with CS-TGA or CS-TGA-6MNA, a significant increase in intracellular Rho-123 level was observed in Caco-2 cells, a more transportation in the absorptive (AP→BL) direction and a less transportation in the secretory (BL→AP) direction of Rho-123 was observed in Caco-2 cell monolayers compared with DMEM solution control. In freshly excised rat intestine transportation, more Rho-123 transported in a concentration-dependent manner. Then the P-gp inhibitory mechanisms were evaluated from four aspects: P-gp expression level, P-gp ATPase activity, intracellular ATP level and plasma membrane fluidity. No obvious changes in P-gp expression or intracellular ATP level were detected after exposure. However, the plasma membrane fluidization decreased and the P-gp ATPase activity reduced, which might contribute to the P-gp inhibitory effect. These features approved this novel biomaterial might contribute to enhance the oral bioavailability of P-gp substrate drugs, such as cyclosporine A and paclitaxel.

1. Introduction

P-glycoprotein (P-gp), which is encoded by the multidrug resistance gene 1 (MDR1), has been generally considered as the important reason for multidrug resistance (MDR) in the chemotherapy of cancer.[1,2] It has also been reported that P-gp is constitutively expressed in the apical membrane of enterocytes, acting as a transmembrane transporter protein to decrease drug absorption through the small intestinal mucosa and alter drug pharmacokinetics.[3] Thus this protein may impair the therapeutic efficacy and affect bioavailability of multiple drugs in oral administration.

In recent years, numerous researches are focusing on the development of P-gp inhibitors. In general, P-gp inhibitors can be divided into two main kinds. One kind is small-molecule inhibitors, such as verapamil, cyclosporine A, and their derivatives.[4] Though comparing with the previous inhibitors, newly-developed small-molecule inhibitors have stronger inhibitory effect and lower side effect,[5] there are still some shortcomings hard to overcome: (1) Unwelcome pharmacological activities cause side effects; (2) Interactions with drugs in combination medication reduce the drug efficacy; (3) The ability to exert inhibitory effect at various sites where P-gp is expressed and influence the drug distribution in human body.[6,7] In order to avoid the disadvantages of these small-molecule inhibitors, more and more researchers have paid attention to the polymers with P-gp inhibitory effect and achieved great progress.[8] According to the literature, polymeric pharmaceutical excipients such as Tweens, Pluronics, polysaccharides, polyethylene glycols and derivatives, amphiphilic block copolymers, dendrimers and thiolated polymers can inhibit efflux pumps. In general, the P-gp inhibitory mechanisms of these polymeric inhibitors above are different, they mainly include: change the membrane fluidity; inhibit the P-gp ATPase activity; reduce the intracellular ATP level, interfere ATP-binding sites, block drug binding sites or other sites within the trans-membrane domains of P-gp, etc.[8]

Recently, thiolated polymers have attracted more and more attention for its prominent efflux pump (such as Multi drug resistance proteins (Mrp) and P-gp) inhibitory properties. These thiomers include anionic thiolated polymers, such as poly(acrylic acid)-cysteine (PAA-cys), pectin-cysteine (pect-cys),

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carboxymethylcellulose-cysteine (CMC-cys) and alginate–cysteine (alg-cys),[9,10,11] and cationic thiolated chitosan, such as chitosan-4-thiobutylamidine (CS-TBA), chitosan-thioglycolic acid (CS-TGA), chitosan-N-acetyl cysteine (CS-NAC).[12-15] For P-gp inhibitory effect, Bernkop-Schnürch et al. reported that CS-TBA enhanced the apical compartment to basolateral compartment (AP→BL) transport of Rhodamine-123 (Rho-123, a P-gp substrate) across Caco-2 cell monolayers. Under the same concentration, the ability of CS-TBA to increase Rho-123 absorption is stronger than that of Myrj52 and P85.[13,16] In another study, six established thiolated chitosan conjugates was investigated using Rho-123 as the model compound, and 0.5% (m/v) chitosan-NAC showed the highest inhibitory effect on P-gp, where the Papp was 3.78-fold compared with the buffer control.[17] Though there are some researches focusing on how thiolated chitosan inhibits P-gp,[18] to date, the P-gp inhibitory mechanism study of thiolated chitosan remains incomplete. Most of the mechanism issues were at the state of speculation. At the same time, to overcome the poor stability of conventional thiolated chitosan, S-protected thiolated chitosan has been developed through conjugating protecting group to thiolated chitosan and their P-gp inhibitory function has been proved.[19-21] But no systematic research on the P-gp inhibitory mechanisms of these S-protected thiolated chitosan has been reported.

In the present study, we synthesized chitosan-thioglycolic acid (CS-TGA) and its S-protected derivative chitosan-thioglycolic acid-6-mercaptonicotinamide (CS-TGA-6MNA), and characterized the products by Ellman's assay, UV-spectrophotometry and Fourier transform infrared spectroscopy (FT-IR). Subsequently, *in vitro* and *ex vivo* evaluation on their P-gp inhibitory effect was conducted, including the Rho-123 intracellular accumulation, Rho-123 transportation across Caco-2 cell monolayers and across freshly excised rat intestines. To elucidate the possible inhibitory mechanisms on P-gp efflux in detail, we investigated the influence of CS-TGA and CS-TGA-6MNA on P-gp expression level, P-gp ATPase activity, intracellular ATP amount and plasma membrane fluidity.

2. Results

2.1. Characterization of CS-TGA and CS-TGA-6MNA

The synthetic routes are shown in Fig. 1. A two-step reaction was used to modify chitosan into CS-TGA. The synthetic product CS-TGA looked like a white, odorless fibrous structure after lyophilization. The structure was confirmed by FT-IR (Fig. 2A). Comparing to chitosan, new peaks appeared in the product, which were1155cm^{-1}, 1500cm^{-1}, 2500cm^{-1}, they were amide bonds and thiol groups (S-C bond), C=O double bonds of the amide bond, and H-S bond, respectively. The amount of conjugated free thiol groups was determined to be 814±31μmol/g.

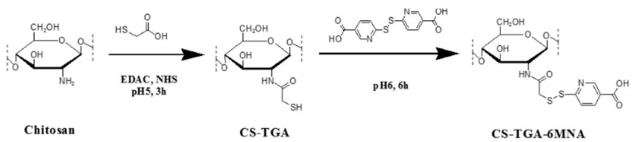

Figure 1 Synthetic routes, reagents, and conditions of CS-TGA and CS-TGA-6MNA.

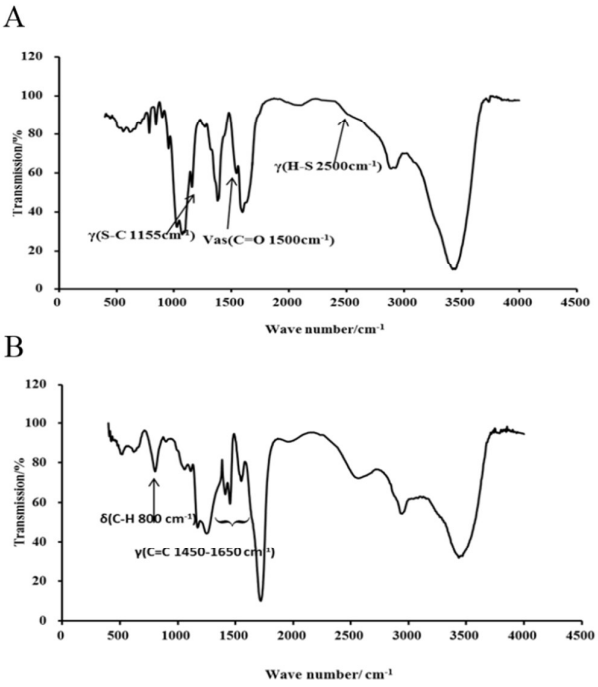

Figure 2 FT-IR spectrogram of CS-TGA (A) and CS-TGA-6MNA (B)

The synthetic product CS-TGA-6MNA appeared as light yellow fibrous structure, and its solubility is much lower than that of CS-TGA. FT-IR determination proved the coupling between CS-TGA and 6MNA (Fig. 2B). The absorption peak at 800cm^{-1} could be assigned to C-H deformation vibration of 6MNA, which owned 1, 4-substituted aromatic ring. Moreover, the peaks between 1450 and 1650cm^{-1} assigned to the C-C stretching vibration represented the existence of aromatic compound. The amount of conjugated 6MNA on CS-TGA-6MNA was 474±11μmol/g.

2.2. P-gp inhibition effect evaluation

2.2.1. *In vitro* biosafety study by SRB assay

As shown in Fig. 3A and 3B, CS-TGA and CS-TGA-6MNA did not have significant inhibition on Caco-2 cells after 24h or 48h below the concentration of 0.25% (w/v). When the concentration reached 0.5% (w/v), both CS-TGA and CS-TGA-6MNA showed some survival inhibition. The survival rates of cells treated by CS-TGA were 84.7% (24h) and 76.7% (48h), and the survival rates of cells treated by CS-TGA-6MNA were 86.0% and 93.2%, respectively. The inhibition was concentration-dependent, but there was no significant difference between 24h incubation and 48h incubation.

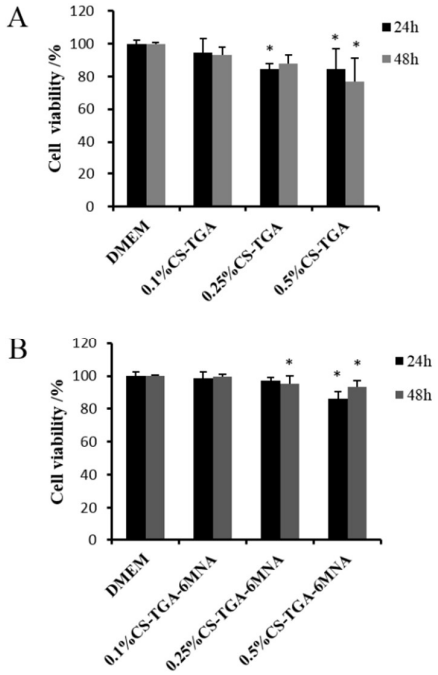

Figure 3 Cell viability of Caco-2 cells treated with CS-TGA or CS-TGA-6MNA. Three different concentration levels of CS-TGA (A) and CS-TGA-6MNA (B) were incubated with Caco-2 cells for 24h (black) and 48h (grey). DMEM medium treated cells served as control. Result was representative means±SD, n=4. *, P<0.05 vs. control.

2.2.2 Intracellular accumulation of Rho-123

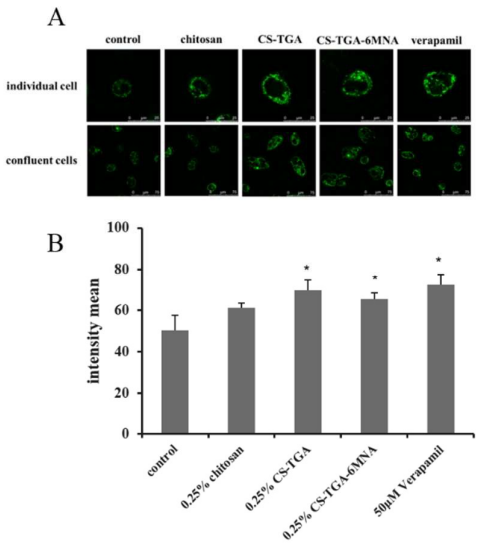

Figure 4 A. Laser scanning confocal microscope images of the cellular uptake of Rho-123 after 30min incubation with different polymers, blank control (DMEM) and positive control (verapamil); B. The flow cytometry analysis results of the cellular uptake of Rho-123 after 30min incubation with different polymers and controls. Indicated values are the means±SD, n=3. *, P<0.05 compared to blank control (DMEM).

After incubation of 30min, Caco-2 cells treated by CS-TGA (0.25%, w/v) or CS-TGA-6MNA (0.25%, w/v) had stronger florescent intensity than control and chitosan group, indicating that there were more Rho-123 accumulation in these groups (Fig. 4A). The results of flow cytometry analysis further confirmed that CS-TGA and CS-TGA-6MNA enhanced the amount of intracellular Rho-123 and they showed similar accumulation as 50μM verapamil group. Unmodified chitosan did not significantly increase the Rho-123 accumulation in cells (Fig. 4B).

2.2.3 *In vitro* evaluation of P-gp inhibition effect on Caco-2 cell monolayers

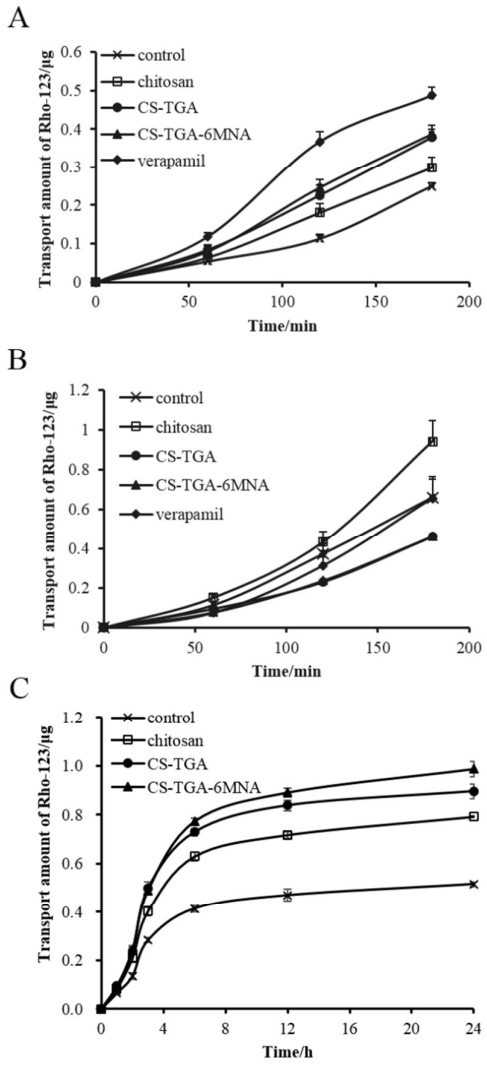

Figure 5 Effects of polymers on the cumulative transport of Rho-123 across Caco-2 monolayer. A, AP→BL transport for 3h; B, BL→AP transport for 3h; C, AP→BL transport for 24h. □, 0.25% (w/v) chitosan; ●, 0.25% (w/v) CS-TGA; ▲, 0.25% (w/v) CS-TGA-6MNA; ◆, 50μM verapamil; ×, control. Indicated values are the means±SD, n=3.

Table 1 Comparison of Rho-123 Papp Values (AP→BL and BL→AP) of chitosan, CS-TGA, CS-TGA-6MNA and verapamil across Caco-2 Cells monolayers.

Permeation enhancer	AP→BL Papp ($\times10^{-6}$cm/s)	BL→AP Papp ($\times10^{-6}$cm/s)	BL→AP Papp/ AP→BL Papp
	Mean±SD	Mean±SD	
DMEM solution	2.04±0.15	5.4±0.85	2.65
Chitosan (0.25%,w/v)	2.45±0.08	7.71±0.54 *	3.15
CS-TGA (0.25%,w/v)	3.09±0.22 *	3.79±0.14 *	1.23
CS-TGA-6MNA (0.25%,w/v)	3.16±0.19 *	3.78±0.08 *	1.20
Verapamil 50μM	3.99±0.17 *	5.37±0.78	1.34

*, P<0.05 compared to blank control (DMEM).

Effects of CS-TGA and CS-TGA-6MNA on bidirectional transport of Rho-123 across Caco-2 cell monolayer were determined at the concentration of 0.25% (w/v). The cumulative transport amount of Rho-123 are shown in Fig. 5 and the calculated Papp of Rho-123 are shown in Table 1. In the AP→BL direction (Fig. 5A and Table 1), Papp of CS-TGA and CS-TGA-6MNA groups were 1.51 and 1.55 fold enhanced comparing to control group, a little lower than that of verapamil (1.96 fold). In the BL→AP direction (Fig 5B and Table 1), CS-TGA and CS-TGA-6MNA showed similar inhibition on Rho-123 transport (both 0.7 fold). Chitosan increased the transport amount of Rho-123 in both AP→BL direction (1.20 fold) and BL→AP direction (1.43 fold). In the long time experiment (Fig. 5C), CS-TGA-6MNA group showed more transported amount than CS-TGA group from 6h. At the end point of the experiment, 24h, they showed significant difference with a p value 0.022.

2.2.4 *Ex vivo* evaluation of P-gp inhibition effect on freshly excised rat intestine

To better mimic the intact absorption of drug in small intestine, we used freshly excised rat intestine to evaluate the effect of thiolated polymers on Rho-123 transport. As indicated in Fig. 6A, during 3h incubation, 0.25% (w/v) of CS-TGA (Papp 5.36×10^{-6}cm/s) and CS-TGA-6MNA (Papp 4.92×10^{-6}cm/s) significantly increased the mucosa side to serosal side (M→S) transport of Rho-123 compared with control (Papp 2.56×10^{-6}cm/s). For unmodified chitosan, there was no significant difference between its Papp of Rho-123 transport (Papp 3.27×10^{-6}cm/s) and the control group. The effect of CS-TGA-6MNA on Rho-123 Papp was concentration-dependent over the range of 0.1%-0.5% (w/v, Fig.6B). The results were consistent with transport across Caco-2 cell monolayers (AP→BL).

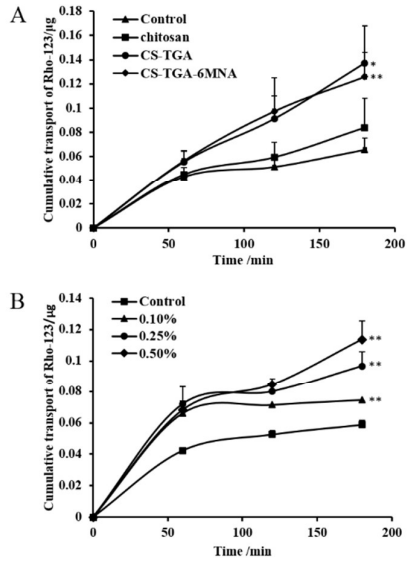

Figure 6. Effects of polymers on the cumulative transport of Rho-123 across rat intestines. A. Effect of 0.25% (w/v) chitosan (■), 0.25% (w/v) CS-TGA (●), 0.25% (w/v) CS-TGA-6MNA (◆); B. Effect of 0.10% (w/v) CS-TGA-6MNA (▲), 0.25% (w/v) CS-TGA-6MNA (●), 0.50% (w/v) CS-TGA-6MNA (◆). Indicated values are the means ± SD, n=3. *, P<0.05, **, P<0.01 compared to the Rho-123 control solution.

2.3. P-gp inhibition mechanisms study

2.3.1. Effect on P-gp expression of Caco-2 cells

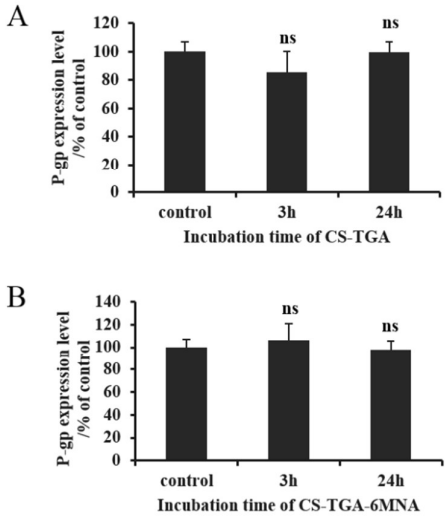

Figure 7 Effect of CS-TGA (A) and CS-TGA-6MNA (B) on the P-gp surface level in Caco-2 cells. Cells were incubated with 0.25% (w/v) CS-TGA for 3h and 24h, measured for P-gp by flow cytometry. Indicated values are the means±SD, n=3. ns, P>0.05 compared to the control in the absence of any polymer.

In the experiments of P-gp expression level determination using flow cytometry, we used the isotype control of FITC-conjugated mouse antihuman monoclonal antibody against P-gp to avoid the interference of non-specific binding on the cell membrane. From the results (Fig. 7A and 7B), we could see that, after 3 or 24h treatment with CS-TGA or CS-TGA-6MNA, the P-gp expression level of Caco-2 cells was not significantly different from the control.

2.3.2. Effect on P-gp ATPase activity

Vanadate, the positive control for the inhibition of P-gp ATPase activity, was used to completely inhibit the ATP depletion by P-gp. As shown in Fig. 8A, when P-gp ATPase was completely inhibited, the luminescence of ATP was more than 500000RLU. The luminescence of ATP in chitosan group was not significantly different from the control group, whose value was approximately 300000RLU. Both of CS-TGA and CS-TGA-6MNA induced significant increase in the remaining unmetabolized ATP level than control, with the luminescence of 350000RLU and 320000RLU. These results indicated that thiolated polymers could reduce the P-gp ATPase activity of Caco-2 cells. Furthermore, the inhibitory effect of CS-TGA-6MNA was in a concentration-dependent manner (Fig. 8B).

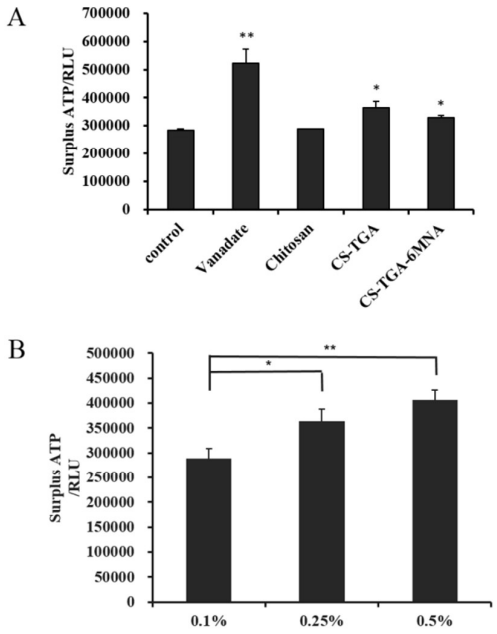

Figure 8 A. The influence of polymers on P-gp ATPase activity in human P-gp membranes. Vanadate was the positive control. *, P<0.05, **, P<0.01 compared to the control solution. B. The influence of CS-TGA-6MNA at various concentrations on P-gp ATPase activity in human P-gp membranes. *, P<0.05, **, P<0.01 compared to CS-TGA-6MNA at a concentration of 0.1% (w/v). Indicated values are the means±SD, n=3.

2.3.3. Effect on plasma membrane fluidity of Caco-2 cells

Fig. 9 showed the membrane microviscosity in Caco-2 cells after treatment with polymers. For unmodified chitosan, the membrane microviscosity of Caco-2 cells didn't show marked change. For CS-TGA and CS-TGA-6MNA, they improved the microviscosity

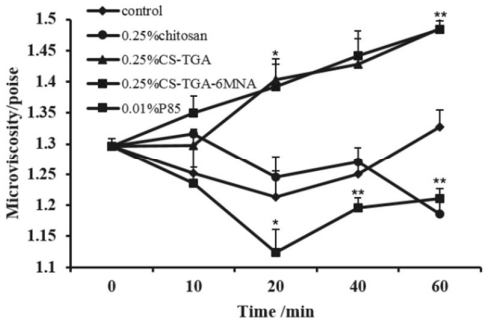

Figure 9 Kinetic effects of chitosan, CS-TGA, CS-TGA-6MNA on the plasma membrane Microviscosity in Caco-2 cells using TMA-DPH as a probe. DMEM-treated cells served as controls. The results are given as the mean±SD, n=5. *, p<0.05， **, p<0.01 compared with the control.

(decrease the membrane fluidity) since 0min, then the microviscosity increased throughout the experiment and reached a maximum at 60min. At the time point of 20min and 60min, statistically significant values for the microviscosity improving effects of CS-TGA and CS-TGA-6MNA were detected. Similar to previous reports, P85, which has already been proved to fluidize membrane of bovine brain microvessel endothelial cells (BBMEC) as an important contributor for P-gp inhibition, induced a decrease in membrane microviscosity (increase in membrane fluidity) at 10min, then the microviscosity continued to go down in the next 10min. Though in rest period of the experiment, the microviscosity of P85 group got a slight recover, its value was still significantly lower than in the beginning.

2.3.4. Effect on intracellular ATP level of Caco-2 cells.

Intracellular ATP levels were quantified using luciferin/luciferase reaction and the results are presented in Fig. 10. Significantly lowered ATP intracellular levels were found in P85-treated cells after 1h (1.15±0.02nmol vs. 1.38±0.07nmol ATP/mg protein in control cells; p<0.05). CS-TGA and CS-TGA-6MNA induced a slight intracellular ATP decrease, but no significant differences were found after the incubation time.

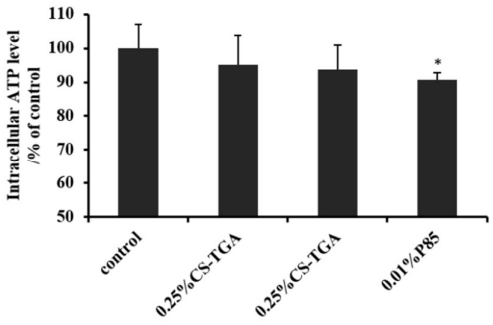

Figure 10 Effect on intracellular ATP levels in Caco-2 cells after cells incubated with polymers and P85 for 60 min. Values are the means±SD, n=3. *, p<0.05 compared to DMEM.

3. Discussion

This work describes a synthesized thiolated chitosan, CS-TGA, and its S-protected derivative, CS-TGA-6MNA, which exhibits P-gp inhibitory effect by decreasing membrane fluidity and inhibiting P-gp ATPase activity at a non-cytotoxic concentration.

In the part of synthesis, we used a two-step reaction to synthesize CS-TGA. DMF was chosen as solvent rather than water, in which the reaction among NHS, EDAC and TGA obtained the product NHS-ester, and then the NHS-ester reacted with chitosan to form CS-TGA. The advantage of the two step synthesis is that NHS-ester is more stable than O-acylisourea ester generated in the conventional one-step synthesis[20], so the hydrolysis is avoided to some extent and with same feed ratio of chitosan, EDAC and TGA, we could achieve higher TGA conjugating rate in chitosan. For the product of CS-TGA-6MNA, it was confirmed by FT-IR and UV scan (data not shown). UV scan first detected the ultraviolet absorption peak of 6MNA monomer at 344nm, providing the basis for quantitative characterization of CS-TGA-6MNA after being reduced by glutathione. The determined amount of conjugated 6MNA, 474±11μmol/g, indicated that half of the free thiol groups in CS-TGA was protected by forming disulfide bonds with 6MNA, and the more stable product CS-TGA-6MNA could be used in the function evaluation and mechanism study.

Before the P-gp inhibitory effect evaluation, we conducted *in vitro* cytotoxicity to determine the biosafety of the synthetic products on Caco-2 cells. The results showed that when the concentration was below 0.5% (w/v), neither CS-TGA nor CS-TGA-6MNA exhibited significant inhibition on Caco-2 cell survival and proliferation after incubating for 24h or 48h, which meant the two biomaterials had low toxicity toward cells, an essential requirement for any material intended for biomedical applications. For the following cellular uptake assay, transport across Caco-2 monolayers and related mechanism study, we chose the relatively safe concentration of 0.25% (w/v) as the incubating concentration.

Subsequently, the P-gp inhibitory effect of CS-TGA and CS-TGA-6MNA was validated from three aspects: Rho-123 cellular uptake assays, Rho-123 transportation across Caco-2 cell monolayers and Rho-123 transportation across freshly excised rat intestine. Rho-123 is a typical P-gp substrate widely used as fluorescent probe in P-gp activity evaluation study. When treated P-gp high expressed cells with P-gp inhibitors, Rho-123 accumulates in cells because of its decreased efflux. Verapamil was chosen as positive control in our study, for it has been well documented that verapamil reduces the efflux of P-gp substrate by a competitive inhibition mechanism.[22] In the Rho-123 cellular uptake assays, CS-TGA and CS-TGA-6MNA (0.25%, w/v) group showed similar accumulation of Rho-123 in cells as verapamil (50μM), indicating their similar P-gp inhibitory effect. Unmodified chitosan at the same concentration did not obviously increase Rho-123 accumulation indicated that the introduction of thiol groups was the reason for the P-gp inhibitory effect of thiolated chitosan, consistent with the previous reported.[23]

Then we developed Caco-2 cell monolayers and conducted the bi-directional transport study. In Caco-2 cell monolayers, cells are highly differentiated with P-gp expressed abundantly on the apical side, so it's an idea model for evaluating the P-gp inhibition of pharmaceutic adjuvants used in oral administration.[24] In general, P-gp substrate (Rho-123, digoxin, etc.) transport study mainly includes two parts, AP→BL transport and BL→AP transport, the former represents drug absorption and can also interpreted as the comprehensive result of drug permeation and efflux, while the latter just focuses on drug efflux. Different kinds of P-gp inhibitors owned different effects on the transport in these two directions, PEG300 can enhance paclitaxel transport from AP to BL and inhibit the transport in the opposite direction in a concentrate-dependent manner, while another P-gp inhibitor, TPGS, only decreases the amount of Rho-123 in BL→AP transport.[24,25] In our study on Rho-123 transportation, the slight increase of Rho-123 on both AP→BL and BL→AP direction in chitosan group can be explained by its ability to open tight junction[26] because of its poor increase in cellular accumulation and no directionality. As for CS-TGA and CS-TGA-6MNA group, they enhanced Rho-123 transport from AP to BL and inhibit the transport in the opposite direction. The more increased part of Rho-123 in AP→BL transportation compared with control and chitosan could be attributed to the P-gp inhibitory effect of the polymers. That is to say, the P-gp inhibitory effect decreased the efflux of Rho-123 to apical side. The decreased transport amounts in BL→AP direction further proved our hypothesis. When CS-TGA or CS-TGA-6MNA added in the AP chamber, the function of P-gp on the apical side was inhibited, resulting the decreased efflux of Rho-123.

There was no significant difference between CS-TGA and CS-TGA-6MNA in the amount of intracellular Rho-123, probably because at the pH of 7.2 (DMEM), alkyl thiols and S-protected thiolated chitosan had similar reaction activity and CS-TGA-6MNA cannot highlight its advantage of pH-independent reactivity from easy disulfide exchange reaction. On the other hand, the time for uptake is too short for CS-TGA-6MNA to exhibit its protection of free thiol groups. That is to say, during the incubation process, there were little thiol groups to be oxidized for CS-TGA to show its stability drawback. In order to prove this issue, we conducted a long time experiment for 24h. From this study, it can be observed that after 6h of incubation, more Rho-123 passed through Caco-2 monolayers in group CS-TGA-6MNA than that of CS-TGA. This indicated that the protected material had a higher effect of P-gp inhibition in relatively longer time period, which might because of oxidization of the unprotected material, CS-TGA. In our previous study on the influence of oxidation of free thiol on permeation function of PAA-Cys and PAA-Cys-6MNA, we have also observed the protecting effect of the 6MNA on its permeation function[27].

In freshly excised rat intestine transportation, more Rho-123 transported after adding CS-TGA or CS-TGA-6MNA and showed a concentration-dependent manner. We noticed that the accumulative amount of rhodamine 123 showed a sharp increase first and then reached a plateau. We try to explain the result as follows: Rho123 transported was the sum of paracellular transport

resulted from the tight junctions opening and transcellular transport. In fact, The TEER of freshly excised rat intestine was not so large as that of Caco-2 monolayer. Furthermore, all the polymers could open the tight junctions. So when the polymers contacted with the intestine, a sharp increase was observed. With the time extension, the changes of tight junctions became weak or disappeared[20], then the transcellular transport became main effector to be reflected on the data. Maybe the transcellular transport is not so obviously as the sudden influence by tight junction opening, especially for chitosan, so the increase became slight. As for the data for 0.10% CS-TGA-6MNA group reaching a plateau, I think maybe the concentration of the polymer was a little low. Its function for P-gp inhibition was limited.

The key point of our research is to unravel the related mechanism of the P-gp inhibitory effect of thiolated chitosan. Towards this question, there are mainly two hypothesises. One is that the interaction of thiolated chitosan with cysteines in the channel forming transmembrane region of P-gp. In the structure of P-gp, channel 2 and chanel 11 exhibit, a cysteine subunit on position 137 and 956, respectively. It has been postulated that thiomers might enter in the channel and form disulfide bonds with one or both of the cysteine subunits, which leads to allosteric change of the transporter blocking the drug efflux.[28,29] The other is that thiolated chitosan exhibit P-gp inhibition by covalent interactions between thiol groups and cell surface exposed cysteine residues of P-gp, changes the conformation of P-gp, and interferes the efflux process. Unfortunately, because of technical limitations, this hypothesis has not been directly proved. We focused on the four common mechanisms in P-gp inhibition of polymeric materials, (1) down-regulated P-gp expression, (2) P-gp ATPase inhibition, (3) ATP-depletion and (4) plasma membrane perturbation, to investigate the P-gp inhibitory mechanisms of thiomers.

The first is the study on P-gp expression changes on cell surface after treatment by thiomers. According to literature, lipophilic material Peceol was found to increase the absorption of amphotericin B by lowering the MDR1 mRNA content and P-gp expression without cell membrane damage or paracellular transport.[30] We applied the same incubation time and a more convenient semi-quantitative method, flow cytometry, to determine P-gp expression level before and after incubation. The results showed that after 24h, there was no change in the P-gp surface level, which rules out down-regulated P-gp expression as a possible mechanism and narrows it to P-gp activity impair.

P-gp itself has ATP hydrolysis activity, and the enzyme activity is directly related to the substrate efflux. For instance, Myrj 52, Cremophor EL, Tween 80, P85 and TPGS 1000 have been reported to inhibit P-gp through this mechanism.[31-33] CS-TGA and CS-TGA-6MNA showed the similar effect and for CS-TGA-6MNA, the P-gp ATPase inhibition increased with concentration rising. It is generally acknowledged that the P-gp ATPase inhibition results from the change of plasma membrane. Given that polymeric materials often are not substrate of P-gp which can compete the membrane binding site, it is more possible that they perturb the plasma membrane to influence P-gp allosteric transition or interact with chemical group of P-gp, lowering the capability to excrete drug.

For prove this hypothesis, we designed study on the time-dependent changes in membrane fluidity using TMA-DPH as probe. The positive control in the study, P85, was documented to increase the membrane fluidity and reduced the combination of P-gp and ATP. However, not all polymeric materials exhibit efflux inhibition by increasing membrane fluidity. When comparing related mechanism of Tween80, Cremophor EL and TPGS 1000, Rege et al. found that Tween80 and Cremophor EL increased membrane fluidity, while TPGS 1000 decreased membrane fluidity to realize the similar effect.[26] Our synthetic products share the same mechanism as TPGS 1000, in 1h incubation, the decrease in membrane fluidity became more and more pronounced. At the same time, the results that chitosan did not show similar effect indicates that the influence of CS-TGA and CS-TGA-6MNA on membrane fluidity is not caused by insertion into lipid bilayer like some surfactants, and thiol groups play an important role in it, maybe the thiol groups of chitosan forming disulfide bonds with cysteine residues from related molecules on the cell membrane lead to membrane movement disorders and decrease the membrane fluidity.

P-gp is a typical energy-dependent transporter, so it's crucial to maintain enough ATP level for the normal function. If the polymeric material has mitochondrial toxicity in cells, the ATP depletion might be the key factor for P-gp inhibition. Batrakova et al. elucidated the specific process that Pluronic series reduced intracellular ATP level by affecting energy metabolism, including their ability to serve as K^+ ionophores and uncouple oxidative phosphorylation, ability to inhibit NADH dehydrogenase, and ability to reduce the activity of the electron transport chains in mitochondria.[34] In our results, CS-TGA and CS-TGA-6MNA can't decrease intracellular ATP significantly, which is in good accordance with *in vitro* cytotoxicity evaluation. If the materials affect metabolism severely in 1h, then it will cause significant impair in cell survival and proliferation after 24h or 48h.

All in all, according to our results, we think thiolated chitosan inhibits P-gp activity mainly through two mechanisms: (1) decreasing membrane fluidity, probably by forming disulfide bonds with molecules on the cell membrane; (2) weakening P-gp capability to hydrolyze ATP, might result from the membrane fluidity change, might due to the interaction of thiol groups with cysteine residues of P-gp. We also notice that the protection of thiol groups with 6MNA does not influence the P-gp inhibitory effect of thiolated chitosan or its mechanism. Considering the better biomucoadhesion and permeation enhancing properties proven by previous researches, the S-protected derivative has a promising prospect in oral administration.

4. Experimental Section

4.1. Materials

Chitosan (MW 110-150 KDa), N-(3-(dimethylamino)propyl)-N'-ethylcarbodiimide hydrochloride (EDAC·HCl), 1-hydroxypyrrolidine-

2,5-dione (NHS), 5,5'-Dithiobis-(2-nitrobenzoic acid) (DTNB), 6,6-Dithiobis(3-Pyridinecarboxylic acid), 3-Pyridinecarboxylic acid (6MNA), rhodamine 123 (Rho-123), verapamil, 1,6-Diphenyl-1,3,5-hexatriene (DPH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2-Mercaptoacetic acid (TGA) was purchased from J&K Scientific Ltd. (Beijing, China). Pluronic 85 were obtained from BASF SE Ltd. (Ludwigshafen, Germany). P-gp Glo™ Assay Systems was obtained from Promega (Madison, WI, USA). BCA Protein Assay Kit was obtained from Applygen Technologies Inc. (Beijing, China). ATP Assay Kit was from Beyotime Institute of Biotechnology (Haimen, China). FITC-conjugated mouse antihuman monoclonal antibody against P-gp and its isotype control were purchased from BD Pharmingen (Sandiego, CA, USA). All other reagents and solvents were analytical or HPLC grade.

Caco-2 intestinal epithelial cell line (from American Type Culture Collection, Rockville, MD) was cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Macgene Biotech, Beijing, China) containing 10% fetal calf serum (Gibco, Carlsbad, USA), 1% penicillin-streptomycin-amphotericin B solution and 1% non-essential amino acids. The cells were cultured in an incubator at 37°C in an atmosphere of 5% CO2/95% air and 99% relative humidity and passaged twice a week.

Male SD rats (about 200g) were obtained from Peking University Animal Center, Beijing, China. All of the animal experiments were performed in compliance with the institutional ethics committee regulations and guidelines on animal welfare (Animal Care and Use Program Guidelines of Peking University).

4.2. Synthesis and characterization of thiolated chitosan and S-protected thiolated chitosan

4.2.1. Synthesis of chitosan-thioglycolic acid (CS-TGA)

Scheme for CS-TGA and CS-TGA-6MNA synthesis is shown in Fig. 1. A modified two-step method was used to synthesize CS-TGA.[35] Briefly, 7g of EDAC, 4g of NHS and 2ml of TGA were dissolved in 40ml of dimethylformamide (DMF). The mixture was stirred overnight to activate the carboxylic acid moieties of TGA. 1g of chitosan was hydrated by 8ml of hydrochloric acid (1M) and dissolved in 100ml of deionized water. Then, the pre-activated TGA solution was added to chitosan solution dropwise, and the pH of the mixture was adjusted to 5. After incubation for 3h under vigorous stirring, the reaction mixture was dialyzed to remove the unreacted compounds for 6 times under 4°C and dark condition. The dialysis solutions were hydrochloric acid (5mM), hydrochloric acid (5mM) / NaCl (1%), and hydrochloric acid (1mM). The thiomer solution was adjusted to pH 4, lyophilized, and kept at 4 °C.

4.2.2. Synthesis of chitosan-thioglycolic acid-(6-mercaptonicotinamide) (CS-TGA-6MNA)

CS-TGA-6MNA was synthesized as previously reported.[20] In brief, 500mg of lyophilized CS-TGA was dissolved in a mixture of water and DMSO (1:1). 120mg of dimer, 6, 6-Dithiobis (3-Pyridinecarboxylic acid), was dissolved in proper amount of mixture of water and DMSO and added to the CS-TGA solution dropwise. After the solutions were fully mixed, its pH was adjusted to 6. After 6h reaction under vigorous stirring at room temperature, the product was purified by dialysis again DMSO (20%, v/v) for 3 times and by dialysis against distilled water for 5 times. After that, the conjugate solution was lyophilized, and kept at 4 °C.

4.2.3. Characterization of CS-TGA and CS-TGA-6MNA

The FT-IR spectra of CS-TGA and CS-TGA-6MNA were determined on a Nicolet avatar 330 FT-IR using the KBr (potassium bromide) method. CS-TGA was confirmed by the presence of the characteristic peaks of newly formed amide bonds and thiol groups. CS-TGA-6MNA was confirmed by the new peaks of aromatic rings.

4.2.4. Quantification of conjugated thiol groups

The degree of thiol group modification in CS-TGA was determined photometrically using Ellman's reagent.[20] To determine the amount of free thiol groups in CS-TGA, a 2mg/ml solution of conjugate and control were prepared. Then 250µl of 0.5M phosphate buffer (pH 8.0) and 500µl of Ellman's reagent (4mg of DTNB dissolved in 0.5M phosphate buffer, pH 8.0) were added to 250µl of conjugate solution. The samples were mixed fully, incubated at room temperature and dark place for 3h, and centrifuged at 12,000× g for 10 min. After that, 300µl of the supernatant was added to a microtitration plate and the absorbance was measured at a wavelength of 450 nm with a Multimode Reader. A TGA calibration curve between 0.1 and 1µmol/ml was established to determine the amount of free thiol group in CS-TGA.

4.2.5. Quantification of conjugated 6MNA

We used glutathione reducing method to determine the amount of conjugated 6MNA in CS-TGA-6MNA.[20] Briefly, 5mg of CS-TGA-6MNA was dissolved in 5ml of reduced glutathione (2%, w/v) and incubated at room temperature and dark condition for 2h. Then, the absorbance of solution at 344 nm was measured using UV–spectrophotometer. A 6MNA calibration curve was established to calculate the amount of 6MNA in CS-TGA-6MNA.

4.3. Evaluation of P-gp inhibition effect

4.3.1. Preliminary *in vitro* biosafety evaluation

To evaluate the possible toxic effect of the synthetic product on cell proliferation, we utilized sulforhodamine B (SRB) assay method.[36] Caco-2 cells were seeded into 96-well plates at a density of 1×10^4 cells per well and cultured for 24h. Then, the cells were treated with CS-TGA and CS-TGA-6MNA at the concentration of 0.1%, 0.25%, 0.5% (w/v) at 37°C for 24h or 48h, followed by fixing cells with cold trichloroacetic acid, washing by deionized water and drying in the air. Cells were then stained with SRB (4%, w/v) for 15min and the excess dye was washed by acetic acid (1%, v/v). After the bound dye dissolved in 10mM Tris buffer, the absorbance was measured using a Multimode Reader at the wavelength of 540nm. The data were expressed as the percentages of viable cells compared to the survival rates of control group (medium treated cells).

4.3.2. Cellular uptake assays of Rho-123

Rho-123 uptake assays were used to assess the effect of CS-TGA and CS-TGA-6MNA on P-gp activity of Caco-2 cells. Caco-2 cells were seeded in confocal dishes or 6-well culture plates at an initial density of 2×10^5 cells per well. After 24h of culture, the cells were

treated with Rho-123 (0.00025%, w/v), in the presence or absence of CS-TGA and CS-TGA-6MNA, at 37°C for 0.5h. The cells were observed under a confocal laser scanning microscope (CLSM, LEICA TCS SP5, Germany) or analyzed with flow cytometry system (FACS Calibur BD, USA);

4.3.3. *In vitro* evaluation of P-gp inhibition effect on Caco-2 monolayers

The Caco-2 cell monolayer was established according to the protocol reported previously.[37] Briefly, Caco-2 cells were seeded in a density of 1×10^5 cells/ml on 12-well Transwell inserts. Cells were allowed to grow and differentiate for 21 days. Only monolayers with a TEER > 500Ω/cm^2 were used for experiment. The transport of Rho-123 was investigated in both absorptive (AP→BL) and secretory (BL→AP) directions. After 0.5h of pre-incubation, Rho-123 (0.001%, w/v) in either blank DMEM medium or conjugates (chitosan, CS-TGA, CS-TGA-6MNA) (0.25%, w/v) containing DMEM medium was added to the donor compartment and blank DMEM medium was added to the receiver compartment. Besides, verapamil (50μM) was selected as the positive control, which was also added to the donor compartment in both AP→BL and BL→AP transport study. 100μl of samples were taken from the receiver compartment and replaced with equal volume of DMEM at 60, 120, and 180min during incubation at 37°C. To investigate the integrity of Caco-2 cell monolayers, TEER values were measured each time after the samples were taken. The samples were transferred to 96-well plate and Rho-123 transported across the monolayers was quantified by using Flex Station 3 microplate reader at excitation wavelength of 485nm and emission wavelength of 525nm. Apparent permeability coefficients (Papp, cm/s) for each group were calculated by the following equation:

$$\text{Papp} = \frac{Q}{A \times c \times t}$$

Where Q is the total amount of Rho-123 permeated within the appointed time (μg), A is the diffusion area of the Transwell inserts (1.13cm^2), c is the initial concentration of materials in the donor compartment (μg/cm^3), and t is the time of the experiment (s). The enhancement ratios were the ratio between Papp of polymer treated groups over Papp of the blank DMEM group.

In order to prove the advantage of CS-TGA-6MNA over CS-TGA in stability, long time AP→BL transport experiment were conducted. All the steps were same as above described excepting the sampling time points at 1, 2, 3, 6, 12 and 24h in this study.

4.3.4 *Ex vivo* evaluation of P-gp inhibition effect on freshly excised rat intestine

The ileum segment of SD rats, which is reported to have high expression of P-gp, was chosen to conduct the ex vivo evaluation of P-gp inhibition effect using Franz diffuser. Briefly, ileum segments of male Sprague-Dawley rats were removed immediately after sacrificing the rats and cleaned the intestinal content. Then, 1cm of ileum segments was cut along the mesentery and mounted in Franz diffusers. 1ml and 3ml of freshly prepared artificial intestinal fluid were added to the donor compartment and receptor compartment, respectively. After all of the Franz diffusers were equilibrated in the 37°C water for 30min, the solution in donor compartments were replaced by Rho-123 (0.001%, w/v) in either blank artificial intestinal fluid or conjugates (chitosan, CS-TGA, CS-TGA-6MNA) (0.25%, w/v) containing artificial intestinal fluid. Similarly, verapamil solution (50μM) was used as the positive control. 100μl of samples were taken from the receiver compartment and replaced with equal volume of artificial intestinal fluid at 60, 120, and 180min during incubation at 37°C. The samples were transferred to 96-well plate and Rho-123 transported across the ileum segments was quantified by using Flex Station 3 microplate reader (excitation wavelength: 485nm; emission wavelength: 525nm). Papp values were calculated as above described. To study the relationship between the concentration of CS-TGA-6MNA and its P-gp inhibition effect, we also designed the experiment with CS-TGA-6MNA at various concentrations (0.1%, 0.25%, 0.5%, w/v).

4.4. P-gp inhibitory mechanism investigation

4.4.1. Effect on P-gp expression of Caco-2 cells

The potential effect of CS-TGA and CS-TGA-6MNA on the P-gp expression of Caco-2 cells was investigated using flow cytometry system. Briefly, Caco-2 cells were seeded in 12-well culture plates at an initial density of 2×10^5 cells per well. After 48h of culture, the cells were treated with CS-TGA or CS-TGA-6MNA (0.25%, w/v), at 37°C for 3h and 24h. Then, the cells were washed three times with PBS, incubated with FITC-conjugated mouse antihuman monoclonal antibody against P-gp according to the previous protocol. Meanwhile, we used the isotype control of the monoclonal antibody against P-gp to the nonspecific labeling. The fluorescent intensity of Caco-2 cells was determined using flow cytometry.

4.4.2. Effect on P-gp ATPase activity

Effects of CS-TGA and CS-TGA-6MNA on P-gp ATPase activity were evaluated using the P-gp-Glo™ Assay (Promega, Germany) with recombinant human P-gp membranes according to the manufacturer's instruction. CS-TGA (0.25%, w/v) or CS-TGA-6MNA (0.25%, w/v) or sodium vanadate at 250μM (positive control) in the provided buffer solution were incubated with 25μg of P-gp-enriched membranes and 5mM Mg-ATP at 37°C for 40min, and the remaining ATP was detected after a 20min signal developing period at room temperature, as a luciferase-generated luminescent signal. The luminescence (RLU) represented the remaining ATP, the more ATP remained, the more significantly P-gp ATPase activity was inhibited. To investigate the relationship between the concentration of CS-TGA-6MNA and its effect on P-gp ATPase activity, we conducted the experiment with CS-TGA-6MNA at various concentrations (0.1%, 0.25%, 0.5%, w/v).

4.4.3. Effect on plasma membrane fluidity of Caco-2 cells

To investigate whether CS-TGA and CS-TGA-6MNA inhibited P-gp activity by influencing cell membrane fluidity, we analyzed the cell membrane fluidity by DPH assay.[38] DPH was often used as a probe to examine the fluidity properties of the hydrocarbon region of the cell membranes. A suspension of Caco-2 cells with a density of 2×10^5 cells/ml was washed twice with PBS and incubated with the DPH (2μM) for 2min at 37°C. Following the initial labeling, cells

were washed twice with PBS to remove extracellular DPH and resuspended in an appropriate volume of PBS at a density of 2×10^5 cells/ml. To evaluate the kinetic effects of CS-TGA and CS-TGA-6MNA on membrane fluidity of Caco-2 Cells, 500μl of 0.25% (w/v) CS-TGA or CS-TGA-6MNA was added to 1.5ml of cell suspension. Subsequently, the fluorescent polarization (P) was determined with Flex Station 3 microplate reader (excitation wavelength: 365nm; emission wavelength: 425nm) at the appointed time. The microviscosity η, which is the reciprocal of the membrane fluidity, could be calculated according to the equation:

$$\eta = \frac{2P}{0.46 - P}$$

We also evaluated the effect of unmodified chitosan (0.25%, w/v) on the membrane fluidity of Caco-2 cells. Blank PBS buffer serve as control and P85 (0.01%, w/v), reported to increase membrane fluidity, was chosen as positive control.

4.4.4. Effect on intracellular ATP level of Caco-2 cells

A luciferin-luciferase method was utilized to determine the intracellular ATP level of Caco-2 cells. Caco-2 cells were seeded in 6-well culture plates at an initial density of 2×10^5 cells per well and incubated for 48h, then the medium was substituted with CS-TGA (0.25%, w/v) or CS-TGA-6MNA (0.25%, w/v). Pluronic85 (P85), documented to decrease intracellular ATP level of cells was selected as positive control. After 1h of treatment at 37°C, the cells were washed twice with PBS, lysed with 200μl lysis buffer from the ATP detection kit, and centrifuged at 12,000×g for 5 min at 4°C. The supernatant was collected for ATP test. The luminescence from a 100μl sample was assayed in a luminometer after 100μl ATP detection buffer from the ATP detection kit was added to it. The standard curve of ATP concentration was prepared from a known amount (1nM-10μM). The relative ATP level was calculated according to the equation:

$$\text{relative ATP level} = \frac{ATP\ value}{protein\ value}$$

The protein value of the sample was measured at 560nm with a Multimode Reader.

4.5. Statistical methods

All data were presented as mean ± standard deviation (SD) unless particularly outlined. The statistical comparisons were performed by one-way analysis of variance (ANOVA). The differences were considered to be significant if $p<0.05$.

5. Conclusion

This study synthesized CS-TGA and its S-protected derivative CS-TGA-6MNA and assessed their P-gp-modulation ability and related mechanisms. The results indicated that both CS-TGA and CS-TGA-6MNA had P-gp inhibitory effects. The P-gp inhibitory mechanisms mainly lies in the decreasing membrane fluidity and inhibiting P-gp ATPase activity, while not influence the expression of P-gp and decrease ATP level at the investigation concentration. This novel biomaterial may contribute to enhance the oral bioavailability of P-gp substrate drugs, such as cyclosporine A and paclitaxel.

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The Table of Contents Entry

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P-glycoprotein (P-gp) inhibitory mechanisms mediated by chitosan-thioglycolic acid (CS-TGA) and its S-protected derivative (CS-TGA-6MNA) lies in the decreasing membrane fluidity and inhibiting P-gp ATPase activity, while not influence the expression of P-gp and decrease ATP level at the investigation concentration.

Keywords: thiolated chitosan, S-protected thiolated chitosan, P-glycoprotein inhibitory activity, P-glycoprotein inhibitory mechanisms