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# Effect of Aliphatic and Aromatic Thiolated Chitosan Conjugates on Permeability of Ritonavir across Caco-2 Cell Monolayers and Rat Intestinal Membrane

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## Abstract

It was the aim of this study to determine the potential of two aliphatic and two aromatic thiolated chitosan conjugates: chitosan-thioglycolic acid (chitosan-TGA), chitosan-4-thiobutylamidine (chitosan-TBA), chitosan-6-mercaptonicotinic acid (chitosan-6MNA) and chitosan-4-mercaptobenzoic acid (chitosan-4MBA), respectively, as permeation enhancing agents. Ritonavir was employed as model compound. Permeation of the compound was tested both on Caco-2 cells and freshly excised rat small intestine and apparent permeability coefficients ($P_{app}$) were calculated. The lyophilized polymers had a thiol group content in the range of 120-250 µmol/g. The 0.5% (w/v) chitosan-6MNA exhibited a more pronounced effect on the absorptive transport of ritonavir across Caco-2 cells ($P_{app} = 5.90 \pm 0.40 \times 10^{-6}$ cm/s, respectively) in comparison to the control and significantly increased the transport of ritonavir up to 2.2-fold. Moreover, in the presence of 0.5% (w/v) chitosan-4MBA in Ussing-type chambers, the absorptive $P_{app}$ ritonavir was $4.58 \pm 0.32 \times 10^{-6}$ cm/s. According to these permeation results, aromatic thiolated chitosans showed a comparatively higher transport of ritonavir than aliphatic thiolated chitosans. These thiolated chitosans would therefore be an advantageous tool for enhancing the oral bioavailability of poorly absorbable pharmaceutical ingredients.

**Keywords:** permeation enhancing effect, thiolated chitosans, aliphatic-/aromatic thiolated chitosan conjugates, ritonavir

# 1. Introduction

Chitosan is biocompatible, biodegradable pH-dependent and non-toxic cationic polymer obtaining by alkaline deacetylation of chitin (1, 2). Its outstanding ability is to enhance the mucosal uptake of poorly absorbable drugs. The underlying mechanism is likely based on interactions between positively charged groups of chitosan and negatively charged sub structure of cell surfaces (3). So far, however, the absorption of drug using chitosan as permeation enhancer has not been fully augmented. Some approaches have been extensively studied to improve the properties, particularly, a modification of chitosan to become multifunctional polymers.

By the immobilization of thiol groups on chitosan, the permeation enhancing properties of chitosan can be significantly further increased. For example, chitosan-4-thiobutylamidine conjugate (chitosan-TBA) in a final concentration of 0.5% (w/v) showed permeation enhancing properties both *in vitro* and *in vivo* (4, 5). The permeation of Rhodamine-123 (Rho-123) across freshly excised rat and guinea pig intestinal mucosa was in the range of 2.2- to 3-fold improved in comparison to the buffer control (4-7). Furthermore, the Rho-123 transport was augmented by the addition of 0.5% (w/v) chitosan-glutathione conjugate (chitosan-GSH), corresponding to 1.99 µmol thiol moieties/g with 5% (w/v) reduced glutathione (GSH). The transport enhancement ratio in the presence of GSH was calculated to be 4.9 (8). In another study, a similar enhancement of Rho-123 uptake was reached by using the system 0.5% (w/v) chitosan-TBA and 5% (w/v) GSH. Results showed a 3-fold higher permeation-enhancing effect of the system compared to the unmodified chitosan. When 0.5% (w/v) chitosan-TBA was administered to rats, the plasma concentration time curve showed significantly higher plasma levels of Rho-123 compared to the control solution (4).

Nevertheless, the use of compounds such as anti-HIV agents drugs as model compounds for the permeability is not promoted because of their low solubility. Orally administered anti-HIV must be sufficient and consistently absorbed for the successful treatment. Hence, they must have properties that are able to pass through a series of barriers, which act to reduce oral bioavailability. Examples of the barriers are poor aqueous solubility, instability in stomach acid, poor permeation, etc. (9). In the study, ritonavir, the biopharmaceutics classification system (BCS) class 4 (low solubility and low permeability) was chosen as the model compound (Figure 1). Because of the poor aqueous solubility of ritonavir (5 µg/ml at pH 7.4, and 7 µg/ml at pH 4), it is necessary to optimize a delivery system to liberate the drug in order to achieve the therapeutic use (9). It was therefore the aim of this study to investigate an influence of various sulfhydryl ligands on permeation enhancing of the four established thiolated chitosan conjugates. The permeation of hydrophilic model compound, ritonavir across freshly excised rat small intestinal mucosa was evaluated in Ussing-type diffusion chambers and on Caco-2 cells.

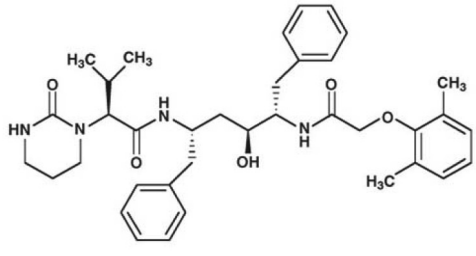


**Figure 1.** Schematic of Ritonavir.

## 2. Materials and Methods

### 2.1 Materials

Chitosan (low molecular weight chitosan: 150 kDa, degree of deacetylation: 83-85%, as specified by the supplier), 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride (EDAC), thioglycolic acid (TGA), 2-imminothiolane HCl, 6-mercaptonicotinic acid (6-MNA), 4-mercaptobenzoic acid (4-MBA), dioxane, sodium borohydride ($NaBH_4$), sodium bicarbonate, phosphate buffered saline (PBS), penicillin-streptomycin, N-([2-hydroxyethyl]piperazine-N´-[2-ethanesulfonic acid]) (HEPES), glucose, sodium chloride (NaCl), potassium chloride (KCl), magnesium sulfate ($MgSO_4$) and sodium hydrogen carbonate ($NaHCO_3$) were obtained from Sigma-Aldrich, Austria. Minimum essential medium eagle modified (MEM), MEM without phenol red fetal calf serum (FCS) were obtained from PAA, Austria. Ritonavir was purchased from Chemos GmbH, Germany. Methanol and water were from VWR International S.A.S., France. Acetonitrile was obtained from Merck KGaA, Germany.

### 2.2 Methods

*2.2.1 Synthesis of chitosan-TGA, chitosan-TBA, chitosan-6MNA and chitosan-4MBA*

Chitosan-TGA, chitosan-TBA, chitosan-6MNA and chitosan-4MBA were generated by the covalent attachment of sulfhydryl ligands to the primary amino groups of chitosan as described previously (10-12). In brief, chitosans were hydrated in 1 % v/v acetic acid to obtain final concentration of 1% w/v. Subsequently, thioglycolic acid, 6-MNA and 4-MBA were chemically treated with EDAC in order to activate the carboxylic acid moieties and then added to chitosan solutions. The reaction mixtures were incubated at room temperature under vigorous stirring for 3 hours. In case of chitosan-TBA, 2-imminothiolane HCl was added to the chitosan solution without being activated by EDAC. Unbound compounds were isolated by dialysis for five times against 5 mM HCl at 10 $^{o}$C and the mixtures were lyophilized (VirTis benchtop 6 K freeze dryer; NY, USA) and kept at -20 $^{0}$C until further use. The control was prepared in the same manner but omitting EDAC during the coupling reaction.

*2.2.2 Determination of the content of thiol group and disulfide bond*

The degree of modification as the amount of thiol groups immobilized on the polymers was quantified by Ellman's method using spectrophotometry (Tecan infinite M200 spectrophotometer, Grödig, Austria) (13). Disulfide contents were evaluated after reduction with $NaBH_4$ and determined by Ellman's reagent. The total amount of these moieties is represented by the summation of reduced thiol groups and oxidized thiol groups in form of disulfide bonds (7). L-cysteine was used to establish a standard curve.

*2.2.3 Chromatographic conditions for ritonavir*

The chromatographic analysis was performed at 30 $^{o}$C, using a Gracesmart C 18 reversed phase column (5µm, 150 mm x 4.6 mm). Ritonavir was dissolved in a mixture of 50% ethanol, 25% propylene glycol and 25% saline solution. The mobile phase consisted of a mixture of acetonitrile:water:methanol (53:43:4, v/v/v). The flow-rate was 1.0 mL $min^{-1}$ and the injection volume was 40 µl. Absorption was determined at 210 nm single diode array detector. A calibration curve was established with ritonavir used in a concentration range of 10-100 µg/mL. Retention time was determined to be 6.2 min.

*2.2.4 Permeation studies on Caco-2 cells*

Caco-2 cells were used at passage number 6-18. Cells were seeded onto 12 wells Transwell polyester membranes (Transwell®, COSTAR, 12 mm

diameter, 0.4 μm pore size). The culture medium was MEM, supplemented with L-glutamine, penicillin-streptomycin and fetal calf serum. The medium was changed every second day and the cells were maintained in an atmosphere of 5% $CO_2$ at 95% relative humidity and 37 °C. Permeation studies were performed after 15 days of seeding. Each experiment was repeated at least three times.

Prior to the studies, each well was washed with phosphate buffer saline (PBS) pH 7.4. Subsequently, 1 ml of MEM without phenol red at pH of 7.4 was added to apical chamber (AP) and 2 ml to basal chamber (BL). After 30 minutes equilibration period, the media of the donor chamber (AP chamber) was replaced by either 0.5% (w/v) thiolated chitosan conjugates with 0.025% (w/v) of the ritonavir or the corresponding unmodified polymers with 0.025% (w/v) of ritonavir. Over a 3 hours incubation period at 37 °C, 150 μL aliquots were withdrawn from the acceptor chamber at 30 minutes intervals and replaced by the same amount of fresh medium. The samples were transferred into a test tube containing 150 μL of methanol. The mixtures were then centrifuged at 13,400 rpm for 10 minutes. The amount of the drug was quantified by HPLC as described previously. The apparent permeability coefficients ($P_{app}$) of ritonavir was calculated according to the following equation

$$P_{app} = Q/(Act)$$

where $P_{app}$ is the apparent permeability coefficient (cm/s), Q is the total amount permeated over the incubation period (μg), A is the diffusion area of the Ussing-type chamber (0.64 $cm^2$) or of transwell® system (1.13 $cm^2$), c is the initial concentration of the model drugs in the donor compartment (μg/$cm^3$), and t is the whole time of experiments (seconds; s).

Improvement ratios were calculated from the ratio between the absorptive $P_{app}$ of tested compounds over the absorptive $P_{app}$ of the buffer control.

*2.2.5 Permeation studies on freshly excised rat small intestinal mucosa*

After sacrificing 240-250 g male Wister rats, the first 15 cm of the lower part of the intestine was removed. The excised small intestine was cut into strips of 1.5 cm, washed free off luminal contents and mounted in Ussing-type chambers without stripping off the underlying muscle layer. The freshly prepared medium containing 250 mM NaCl, 2.6 mM $MgSO_4$, 10 mM KCl, 40 mM glucose and 50 mM $NaHCO_3$ buffered with 50 mM HEPES pH 7.0 in a final volume of 1 mL was added to the apical and basolateral side. The Ussing chambers were then placed in a water bath at 37 °C. After 30 minutes of equilibration period, ritonavir in a final concentration of 0.05% (w/v) in the presence of 0.5% (w/v) thiolated chitosan conjugates or the unmodified chitosan were then placed to the apical side for an absorptive (AP to BL) transport. Aliquots of 150 μl were withdrawn from the BL chamber every 30 minutes over a period of 3 hours and immediately replaced by an equal volume of the transport medium pre-equilibrated at 37 °C. The amount of permeated ritonavir was analyzed using HPLC. Each sample was tested in triplicate.

*2.2.6 Determination of the TEER*

The integrity of cells at the beginning and the end of the studies was measured using EVOM® instrument (World Precision Instrument Inc., Sarasota, USA).

*2.2.7 Statistical and data analysis*

Data are expressed as mean ± S.D. The differences between groups were tested by ANOVA assay with p-value $< 0.05$ as the minimal level of significant difference.

# 3. Results

### 3.1 Characterization of thiolated chitosans

By the formation of amide bonds the sulfhydryl-bearing ligands, TGA, 6-MNA and 4-MBA were covalently attached to the primary amino group of chitosan yielding chitosan-TGA, chitosan-6MNA and chitosan-4MBA, respectively, as shown in Figure 2.(10-12) In contrast, chitosan-TBA was generated by amidine bond formation between chitosan and 2-iminothiolane HCl (5).

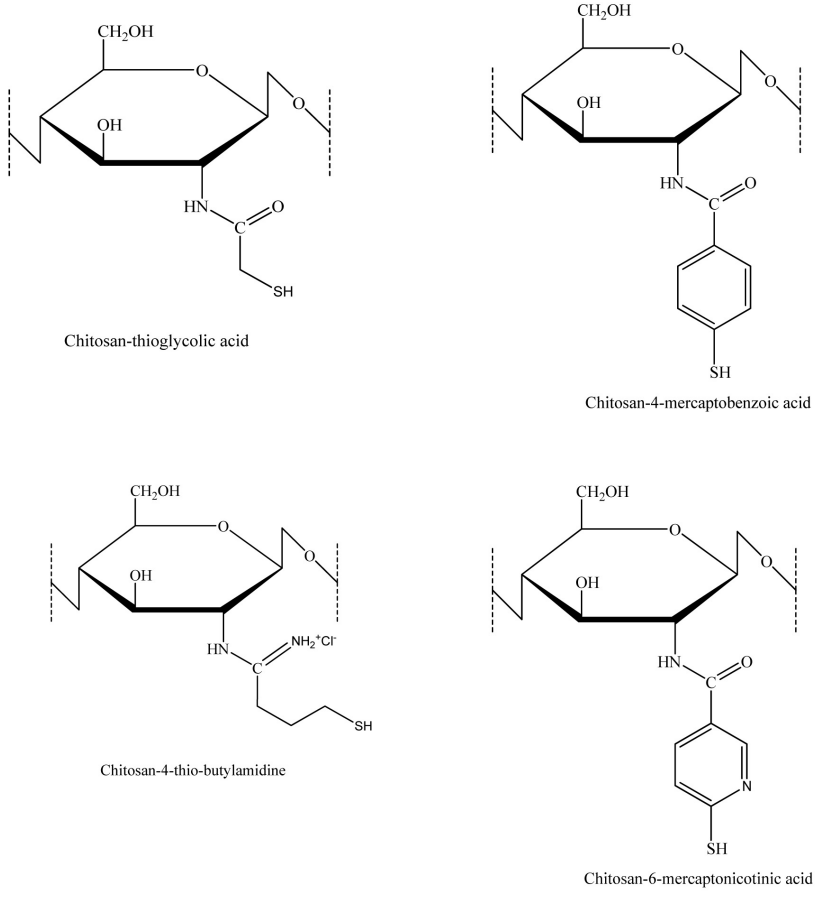


**Figure 2.** Schematic structure of alkyl thiolated chitosans (chitosan-TGA and chitosan-TBA) and aryl thiolated chitosans (chitosan-4MBA and chitosan-6MNA)

The lyophilized polymers were white, odorless and of fibrous-like structure. In case of chitosan-6MNA, the product appeared as a yellow fibrous structure. In order to compare the permeation enhancing effects of the thiolated chitosans, all of the modified chitosans contained an amount of thiol groups in the range of 120-250 µmol/g polymers as listed in Table 1.

**Table 1.** Comparison of different reaction conditions utilized for modification of thiolated chitosans.

| Compounds | Aqueous polymer solution (g/70mL) | Coupling reagents | Reduced thiol groups (μmol/g) (Mean ± SD.) | Total amount of thiol groups (μmol/g) (Mean ± SD.) |
|---|---|---|---|---|
| Chitosan-TGA | 1.0 | TGA | 133.5 ± 18.65 | 195.46 ± 15.18 |
| Chitosan-TBA | 1.0 | 2-imminothiolane HCl | 178.07 ± 26.94 | 181.35 ± 12.17 |
| Chitosan-6MNA | 1.0 | 6-MNA | 123.5 ± 50.23 | 164.2 ± 10.03 |
| Chitosan-4MBA | 1.0 | 4-MBA | 98.48 ± 2.20 | 143.81 ± 3.25 |

**3.2 TEER measurement**

A range of initial TEER values of Caco-2 cells and rat intestine were between 500-800 and 60-90 Ω $cm^2$, respectively. After 3 hours of the experiment, the TEER values slightly decreased of about 20-30% in comparison to the initial values. The decrease of TEER values might be from a loosening of the tightness of paracellular pathway. The decrease of the TEER values, however, was recovered when the cells were continually incubated in an incubator for 24 hours (data not shown). The result is in good agreement with Föger *et al.*(4-6) showing that the decrease of TEER was fully recovered after incubation of the cells with fresh culture medium overnight (6).

**3.3 Permeation studies**

*3.3.1 Permeation studies on Caco-2 cells*

The absorptive transport of ritonavir across Caco-2 cells was not significantly affected by the addition of 0.5% (w/v) unmodified chitosan (p-value = 0.205) as shown in Table 2. However, an increase in the ritonavir permeability and the improvement ratio was found when 0.5% (w/v) aliphatic-/aromatic thiolated chitosans dissolving in the media were applied to the chamber. The improvement ratio of ritonavir transport across the monolayer cells in the presence of chitosan-4MBA, chitosan-TGA and chitosan-TBA was 1.61-, 1.25- and 1.32-fold, respectively, compared to the control (Table 2 and Figure 3). By the addition of 0.5% (w/v) chitosan-6MNA, the ritonavir permeation was significantly 2.20-fold improved in Caco-2 cells (p-value =0.001). Furthermore, the absorptive transport of ritonavir across the cells was investigated at 4 $^{o}$C and 37 $^{o}$C, respectively. At 4 $^{o}$C, the absorptive $P_{app}$ was 2.87 ± 0.33 x$10^{-6}$ cm/s whereas at 37 $^{o}$C it was 2.52 ± 0.42 x$10^{-6}$ cm/s. The increase in drug permeation at 4 $^{o}$C might be due to the possible involvement of active transport mechanisms. (14). At 37 $^{o}$C the absorptive transport is a combination of active efflux pump-mediated transport and passive diffusion, whereas the activity of ATP hydrolysis was low at a cool environment, resulting in a reduction in drug efflux.

**Table 2.** Comparison of the absorptive apparent permeability coefficients ($P_{app}$) of ritonavir across Caco-2 cells in the presence of indicated test compounds. Each point represents the mean ± S.D. of three experiments.

| Substrate | Test compounds | $P_{app}$ x $10^{-6}$ (cm/s) | Improvement ratio |
|---|---|---|---|
| **Ritonavir** | Buffer (37 °C) | 2.52 ± 0.42 | - |
| | Buffer (4 °C) | 2.87 ± 0.33 | 1.14 |
| | 0.5% (w/v) unmodified chitosan | 2.78 ± 0.47 | 1.04 |
| | 0.5% (w/v) chitosan-TGA | 3.35 ± 0.98 | 1.25 |
| | 0.5% (w/v) chitosan-TBA | 3.54 ± 0.66 | 1.32 |
| | 0.5% (w/v) chitosan-6MNA | 5.90 ± 0.40 | 2.20 |
| | 0.5% (w/v) chitosan-4MBA | 4.30 ± 0.52 | 1.61 |

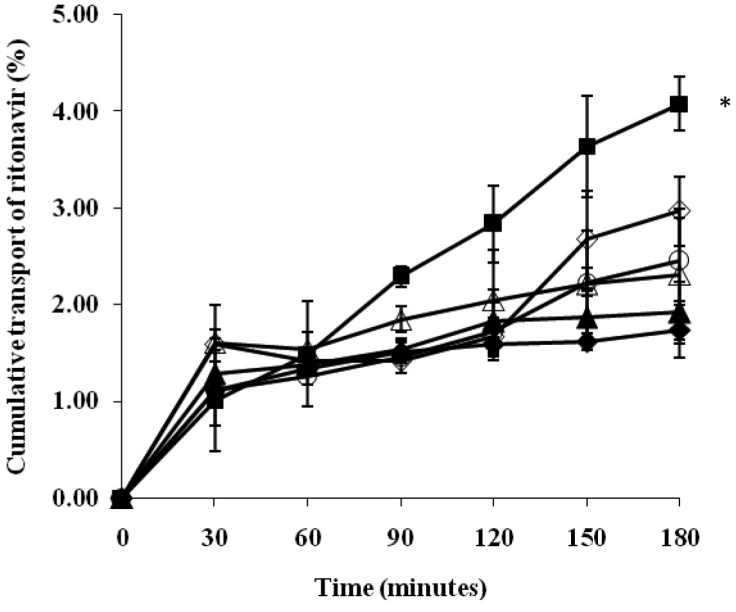


**Figure 3.** *In vitro* transport data of ritonvir (0.025% w/v) across Caco-2 cells in the absorptive direction in absence of polymers (◆), with 0.5% (w/v) unmodified chitosan (▲), 0.5% (w/v) chitosan-TGA (△), 0.5% (w/v) chitosan-4MBA (◇), 0.5% (w/v) chitosan-TBA (○) and 0.5% (w/v) chitosan-6MNA (■). Transport data are mean ± S.D. of at least 3 experiments.
*difference from buffer control p-value = 0.001

*3.3.2 Permeation studies on freshly excised rat intestine*

Results obtained by permeation studies are summarized in Table 3. For ritonavir permeation, a significant difference in ritonavir transport was observed, when 0.5% (w/v) chitosan-6MNA was added to the apical chamber (Figure 4). It was found that the improvement ratio is 1.72-fold improved in rat intestinal mucosa in comparison to the buffer control after 180 minutes of incubation.

**Table 3.** Comparison of the absorptive apparent permeability coefficients ($P_{app}$) of ritonavir across rat intestine in the presence of indicated test compounds. Each point represents the mean ± S.D. of at least three experiments.

| Substrate | Test compounds | $P_{app}$ x $10^{-6}$ (cm/s) | Improvement ratio |
|---|---|---|---|
| **Ritonavir** | Buffer (37 °C) | 3.25 ± 0.87 | - |
| | 0.5% (w/v) unmodified chitosan | 3.18 ± 0.32 | 0.98 |
| | 0.5% (w/v) chitosan-TGA | 3.67 ± 0.18 | 1.13 |
| | 0.5% (w/v) chitosan-TBA | 3.90 ± 0.57 | 1.20 |
| | 0.5% (w/v) chitosan-6MNA | 5.59 ± 1.10 | 1.72 |
| | 0.5% (w/v) chitosan-4MBA | 4.58 ± 0.32 | 1.41 |

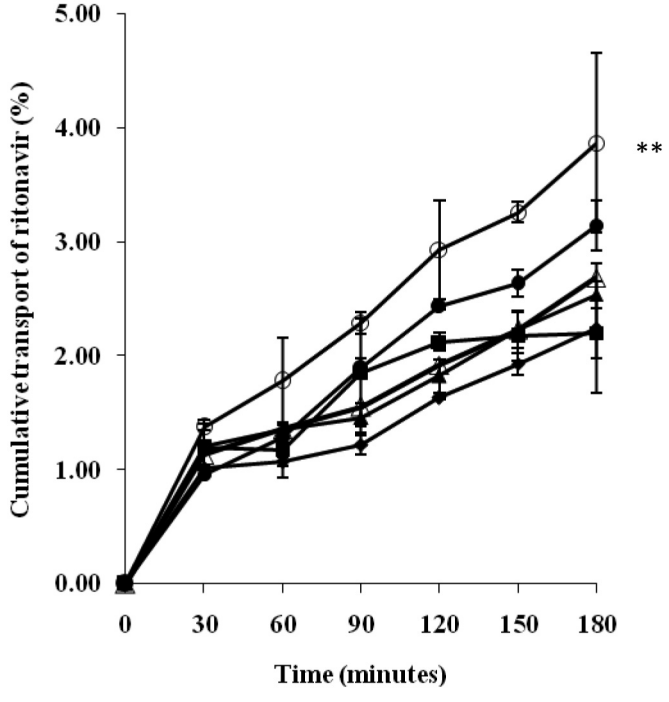


**Figure 4.** *In vitro* transport data of ritonvir (0.05% w/v) across freshly excised rat small intestinal mucosa in the absorptive direction in absence of polymers (◆), with 0.5% (w/v) unmodified chitosan (■), 0.5%(w/v)chitosan-TGA(▲),0.5%(w/v)chitosan-4MBA(●),0.5%(w/v)chitosan-TBA(△)and0.5% (w/v) chitosan-6MNA (○). Transport data are mean ± S.D. of at least 3 experiments.
**difference from buffer control p-value = 0.011

## 4. Discussion

Typically, the transport of compounds across Caco-2 cells was evaluated in terms of $P_{app,}$ which low $P_{app}$ (<1 x $10^{-6}$ cm/s), moderate $P_{app}$ (=1-10 x $10^{-6}$ cm/s), or high $P_{app}$ (>10 x $10^{-6}$ cm/s), correspond to prediction of poor, moderate and well-absorbed compounds, respectively (15). Within this study, the absorptive $P_{app}$ of ritonavir across Caco-2 cells was 2.68 ± 0.26 x $10^{-6}$ cm/s , which is in the rank of moderately absorbed compounds.

Basically, chitosan is used as permeation enhancer and this effect of chitosan was more pronounced in the presence of thiol bearing moieties (16). As shown in Table 2, the absorptive transport of ritonavir across the Caco-2 cells was comparatively high in the presence of 0.5% (w/v) thiolated chitosans compared to the buffer control. The improvement ratios of 0.5% (w/v) chitosan-TGA, chitosan-TBA, chitosan-6MNA and chitosan-4MBA were 1.25, 1.32, 2.20 and 1.61, respectively. The transportations of ritonavir across Caco-2 monolayers achieved withing the current study are in good correlation with rat intestinal mucosa. The greater pronounced of permeation of the drug was found in the presence of aromatic thiolated chitosan, which is not the case for aliphatic modified polymers. The absorptive $P_{app}$ of ritonavir across the intestine in the presence of 0.5% (w/v) chitosan-TGA, for instance, was approximately 1.1-fold compared to the buffer control (17). An increase in the absorptive transport could be explained by the permeation enhancing properties of thiolated chitosans. The improved permeation across the intestinal mucosa and the monolayer cells was associated with a decrease in the TEER, suggesting a loosening of the tightness of paracellular route. At the tight junctions, several important transmembrane proteins such as claudins, the junctional adhesion molecule (JAM) and occludin are presented being responsible for various functions (17-19). In addition, the dephosphorylation of the residues by protein tyrosine phosphatase (PTP) are capable to malfunction of the tight junctions, leading to a closing of the junctions (17). It is assumed that thiolated chitosans influence the structure and function of ATPase of P-gp by the formation of disulfide bond between cysteine and the nucleotide-binding domains (NBDs)-512's binding cassette of P-gp.(19, 20) As demonstrated previously, the combination of chitosan-TBA and GSH system showed *in vivo* significantly higher Rho-123 plasma levels in comparison to all other compounds. The $AUC_{0-12}$ was 4.32-fold improved compared to the control (5). In the presence of thiolated polymers, the oxidation of GSH on the mucosa surface could be prevented (20).

Generally, the permeation enhancing properties of aromatic thiolated chitosans (chitosan-6MNA and chitosan-4MBA) were comparatively higher than that of aliphatic thiolated chitosan both on the monolayer cells and rat intestinal mucosa. Nevertheless, the permeation enhancing properties of chitosan-6MNA and chitosan-4MBA was not significantly different. An increase in ritonavir permeation with addition of aromatic thiomers can be attributed to the reactive thiol group (6-MNA and 4-MBA) bound on the chitosan. As reported by Millotti *et al.*, (11-12) the p*Ka* value of the reactive thiol group is approximately 6.8 and when they are presented in the intestinal lumen, theirs thiol groups are ready to be ionized (11, 12). Thereafter, thiolated polymers, being attached on the mucosa as a result of their high mucoadhesive properties (11) can shift the balance between oxidized glutathione and GSH on the membrane to the side of GSH. Moreover, the reactive thiol group of 4-MNA was reported to show a pH independent reactivity (12). This means that chitosan-4MBA is in the reactive form over the physiological conditions. In contrary, aliphatic thiolated chitosans (chitosan-TGA

and chitosan-TBA) showed a pH dependent reactivity. Basically, the active form of aliphatic thiomers is the thiolate anion ($S^-$) and mostly in this form when pH values are slightly above the physiological conditions due to the p*Ka* of the thiomers. Subsequently, they are not capable to reach the full potential in cases where the pH is lower than the physiological conditions. Thus, the permeation of ritonavir is not negligible. Under the similar amount of thiol groups, chitosan-TBA enhanced the transport of the test compounds more than chitosan-TGA, owing to the additional cationic amidine substructure of the conjugate.

To be concluded, thiolated chitosans described herein were found to have effects on the permeation of compounds across membranes. The most pronounced permeation of ritonavir was found in the presence of chitosan-6MNA. The substructure and the ionization of the ligands are more important for the paracellular uptake rather than the amount of free thiol groups immobilized on chitosan. Therapeutic use of these compounds as excipinets would be an effective tool for improving the oral bioavailability of drugs.

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## References

1. Chandy T, Sharma C. Chitosan-as a biomaterial. Biomater Artif Cells Artif Organs. 1990;18(1):1-24.
2. Hejazi R, Amiji M. Chitosan-based gastrointestinal delivery systems. J Control Release. 2003;89(2):151-65.
3. Schipper N, Olsson S, Hoogstraate J, deBoer A, Vårum K, Artursson P. Chitosans as absorption enhancers for poorly absorbable drugs 2: mechanism of absorption enhancement. Pharm Res. 1997;14(7):923-9.
4. Föger F, Schmitz T, Bernkop-Schnürch A. *In vivo* evaluation of an oral delivery system for P-gp substrates based on thiolated chitosan. Biomaterials. 2006;27(23):4250-5.
5. Föger F, Hoyer H, Kafedjiiski K, Thaurer M, Bernkop-Schnürch A. *In vivo* comparison of various polymeric and low molecular mass inhibitors of intestinal P-glycoprotein. Biomaterials. 2006;27(34):5855-60.
6. Föger F, Kafedjiiski K, Hoyer H, Loretz B, Bernkop-Schnürch A. Enhanced transport of P-glycoprotein substrate saquinavir in presence of thiolated chitosan. J Drug Target. 2007; 15(2):132-9.
7. Werle M, Hoffer M. Glutathione and thiolated chitosan inhibit multidrug resistance P-glycoprotein activity in excised small intestine. J Control Release. 2006;111(1-2):41-6.
8. Kafedjiiski K, Föger F, Werle M, Bernkop-Schnürch A. Synthesis and *in vitro* evaluation of a novel chitosan-glutathione conjugate. Pharm Res. 2005;22(9):1480-8.
9. Aungst BJ. P-glycoprotein, secretory transport, and other barriers to the oral delivery of anti-HIV

drugs. Adv Drug Deliv Rev. 1999;39(1-3):105-16.

10. Greindl M. Oral Drug Delivery: Design and *in vitro / in vivo* evaluation of novel permeation enhancers and efflux pump inhibitors. Innsbruck: University of Innsbruck; 2008.
11. Millotti G, Samberger C, Fröhlich E, Bernkop-Schnürch A. Chitosan-graft-6-mercaptonicotinic acid: synthesis, characterization, and biocompatibility. Biomacromolecules. 2009;10(11):3023-7.
12. Millotti G, Samberger C, Eleonore F, Sakloetsakun D, Bernkop-Schnürch A. Chitosan-4-mercaptobenzoic acid: synthesis and characterization of a novel thiolated chitosan. J Mater Chem Chem. 2010;20:9.
13. Bernkop-Schnürch A, Schwarz V, Steininger S. Polymers with thiol groups: a new generation of mucoadhesive polymers? Pharm Res. 1999;16(6):876-81.
14. Sakloetsakun D, Iqbal J, Millotti G, Vetter A, Bernkop-Schnürch A. Thiolated chitosans: influence of various sulfhydryl ligands on permeation-enhancing and P-gp inhibitory properties. Drug Dev Ind Pharm. 2011;37(6):648-55.
15. Yee S. In vitro permeability across Caco-2 cells (colonic) can predict *in vivo* (small intestinal) absorption in man--fact or myth. Pharm Res. 1997;14(6):763-6.
16. Bernkop-Schnürch A, Guggi D, Pinter Y. Thiolated chitosans: development and *in vitro* evaluation of a mucoadhesive, permeation enhancing oral drug delivery system. J Control Release. 2004;94(1):177-86.
17. Bernkop-Schnürch A, Kast C, Guggi D. Permeation enhancing polymers in oral delivery of hydrophilic macromolecules: thiomer/GSH systems. J Control Release. 2003;93(2):95-103.
18. Collares-Buzato C, McEwan G, Jepson M, Simmons N, Hirst B. Paracellular barrier and junctional protein distribution depend on basolateral extracellular $Ca^{2+}$ in cultured epithelia. Biochim Biophys Acta. 1994;1222(2):147-58.
19. Clausen A, Bernkop-Schnürch A. *In vitro* evaluation of the permeation-enhancing effect of thiolated polycarbophil. J Pharm Sci. 2000;89(10):1253-61.
20. Clausen A, Kast C, Bernkop-Schnürch A. The role of glutathione in the permeation enhancing effect of thiolated polymers. Pharm Res. 2002;19(5):602-8.