Drug Development and Industrial Pharmacy, 2011; 37(6): 648–655

ISSN 0363-9045 print/ISSN 1520-5762 online
DOI: 10.3109/03639045.2010.534484

informa
healthcare

ORIGINAL ARTICLE

Thiolated chitosans: influence of various sulfhydryl ligands on permeation-enhancing and P-gp inhibitory properties

Duangkamon Sakloetsakun[1],[2], Javed Iqbal[1], Gioconda Millotti[1], Anja Vetter[1], and Andreas Bernkop-Schnürch[1],

[1]*Department of Pharmaceutical Technology, Institute of Pharmacy, University of Innsbruck, Innsbruck, Austria, and* [2]*Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, Thailand*

Abstract

Purpose: The influence of various sulfhydryl ligands on permeation-enhancing and P-glycoprotein (P-gp) inhibitory properties of the six established thiolated chitosan conjugates was investigated using Rhodamine-123 (Rho-123) and fluorescein isothiocyanate-dextran 4 (FD4) as model compounds. Methods: Permeation of these compounds was tested on freshly excised rat intestine in Ussing-type chambers. Apparent permeability coefficients (Papp) were calculated and compared to values obtained from the buffer only control. Results: The lyophilized polymers had a thiol group content in the range of 230–520 μmol/g. Results of this study led to the following rank order in permeation enhancement: chitosan-6-mercaptonicotinic acid (chitosan-6MNA) > chitosan-cysteine (chitosan-Cys) > chitosan-glutathione (chitosan-GSH) > chitosan-4-thiobutylamidine (chitosan-TBA) > chitosan-thioglycolic acid (chitosan-TGA) > chitosan-N-acetyl cysteine (chitosan-NAC). In P-gp inhibition studies, 0.5% (m/v) chitosan-NAC showed the highest inhibitory effect on P-gp, where the Papp was determined to be 3.78-fold increased compared with the buffer control. Among these thiolated chitosans, chitosan-NAC and chitosan-6MNA are the most effective polymers being responsible for P-gp inhibition and permeation enhancement, respectively. Conclusion: These thiolated chitosans would therefore be advantageous tools for enhancing the noninvasive bioavailability of active pharmaceutical ingredients.

Keywords: Drug absorption, efflux pump inhibition, permeation enhancing effect, thiolated chitosans, thiomers

Introduction

Chitosan obtained by alkaline deacetylation of chitin is biocompatible, biodegradable, pH-dependent, and non-toxic cationic polymer[1],[2]. Due to mucoadhesive properties of chitosan, an enhancement in the mucosal uptake of poorly absorbable drugs by forming ionic interactions between positively charged group of chitosan and negatively charged substructure of cell surfaces could be achieved[3]. However, due to electrostatic interaction and unstable nature of chitosan in the harsh conditions such as in the stomach, the absorption of drugs using unmodified chitosan as a carrier has been limited. Some approaches have been extensively studied to improve the properties, particularly modification of chitosan as a multifunctional polymer.

By the immobilization of thiol groups on chitosan, its permeation-enhancing and P-glycoprotein (P-gp) inhibitory properties can be further improved significantly. Chitosan-4-thiobutylamidine (chitosan-TBA) conjugate in a final concentration of 0.5% (m/v), for instance, showed permeation-enhancing and P-gp inhibitory 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 2.2- to 3-fold improved compared with the buffer control[4–7]. Furthermore, the Rho-123 transport was improved by the addition of 0.5%

Address for correspondence: Dr. Andreas Bernkop-Schnürch, Department of Pharmaceutical Technology, Institute of Pharmacy, University of Innsbruck, Innrain 52, Josef Möller Haus, A-6020 Innsbruck, Austria. Tel: +43 512 507 5371, Fax: +43 512 507 2933. E-mail: andreas.bernkop@uibk.ac.at

(Received 16 August 2010; accepted 20 October 2010)

(m/v) chitosan-glutathione (chitosan-GSH) conjugate, corresponding to 1.99 μmol thiol moieties/mL with 5% (m/v) reduced 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 using the system 0.5% (m/v) chitosan-TBA and 5% (m/v) GSH. Results showed a 3-fold higher permeation-enhancing effect of the system compared with the unmodified chitosan. When 0.5% (m/v) chitosan-TBA was administered into rats, the plasma concentration–time curve showed significantly higher plasma levels of Rho-123 compared with the control solution[4]. Although thiolated chitosans have been extensively studied in permeation-enhancing and P-gp inhibitory characteristics, the insufficient comparability may be not only due to the individual interpretation of results but also due to the type of intestinal mucosa, a variety of the used concentrations of either thiolated chitosans or GSH, and a different amount of thiol groups immobilized on the chitosan. It was, therefore, the aim of this study to investigate an influence of various sulfhydryl ligands on permeation-enhancing and P-gp inhibitory properties of the six established thiolated chitosan conjugates. The permeation of hydrophilic model compounds, particularly, Rho-123 and fluorescein isothiocyanate-dextran 4 (FD4) across freshly excised rat intestinal mucosa was evaluated in Ussing-type diffusion chambers in vitro.

Materials and methods

Materials

Chitosan (low molecular mass, degree of deacetylation: 83–85%, as specified by the supplier), Rho-123, 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide hydrochloride (EDAC), thioglycolic acid, 2-imminothiolane HCl, 6-mercaptonicotinic acid (6MNA), *N*-acetyl cysteine (NAC), reduced GSH, L-cysteine HCl (Cys), dioxane, sodium borohydride ($NaBH_4$), minimum essential medium eagle modified (MEM) without phenol red, sodium bicarbonate, 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, Vienna, Austria. MEM with phenol red was purchased from PAA Laboratories, Linz, Austria.

Methods

Synthesis of thiolated chitosan conjugates

Chitosan-TGA, chitosan-TBA, chitosan-NAC, chitosan-GSH, chitosan-Cys, and chitosan-6MNA were generated by the covalent attachment of sulfhydryl ligands to the primary amino groups of the cationic polymer chitosan as described previously[9–11]). To activate the carboxylic acid moieties, thiol ligands were chemically treated with EDAC and then transferred to chitosan solutions. The reaction mixtures were incubated at room temperature under continuous stirring for 3 hours. In the case of chitosan-TBA, 2-imminothiolane HCl was added to the chitosan solution without the addition of EDAC. Unbound compounds were isolated by dialysis for 5 times against 5 mM HCl at 10°C and the mixtures were lyophilized and kept at –20°C until further use. The control was prepared in the same manner but omitting EDAC during the coupling reaction.

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[12]. 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 the form of disulfide bonds[7]. L-Cysteine HCl was used to establish a standard curve.

Permeation studies

After killing male Wistar rats weighting 240–250 g, the first 15 cm of the lower part of the intestine was removed. The excised 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. Aliquots (1 mL) of 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 were added to the apical (AP) and basolateral (BL) sides. The Ussing chambers were then placed in a water bath maintaining the temperature of 37°C. After 30 minutes of equilibration period, FD4 in a final concentration of 0.1% (m/v) was placed on the AP side for an absorptive (AP to BL) transport. Over a 3-hour incubation period, 100 μL aliquots were withdrawn from the acceptor chamber at 30-minute intervals and replaced by the same volume of the medium. The transport of FD4 was evaluated in the absence and presence of the test compounds. The amount of permeated marker was analyzed using fluorescence measurements (λ_{ex} = 485 nm and λ_{em} = 535 nm) by a microplate reader (Tecan Austria GmbH, Groedig, Austria). Each test was performed in triplicate.

P-gp inhibition studies

The studies were performed in the AP to BL direction using Rho-123 as the fluorescence marker. Prior studies, an expression of P-gp in rat intestinal mucosa was tested by placing the Ussing chambers in a water bath (37°C) or refrigerator (4°C), respectively. At 4°C, P-gp is inhibited. Hence, an increased transport of Rho-123 at 4°C indicates the presence of P-gp in the rat intestine. The intestine was therefore used to evaluate the P-gp inhibitory properties of thiolated chitosans. In the studies, the polymers in a final concentration of 0.5% (m/v) were added to donor chambers and 100 μL of samples was taken from the acceptor chambers every 30 minutes for 3 hours duration. The transport of Rho-123 served as control. Furthermore, the inhibitory activities of six

established thiolated chitosans in the presence of GSH were determined. The amount of permeated Rho-123 was quantified fluorometrically (λ_{ex} = 485 nm and λ_{em} = 520 nm). Each test was performed in triplicate.

Determination of the TEER

EVOM® (World Precision Instrument Inc., Sarsota, FL, USA) connected to a pair of side by side electrodes was used to monitor the transepithelial electrical resistance (TEER) of the freshly excised rat intestinal mucosa. Measurements were determined at the beginning and at the completion of the experiment to ensure that tissue integrity and viability had not been adversely affected by the experimental conditions.

Statistics and data analysis

Apparent permeability coefficients (P_{app}) for Rho-123 and FD4 were calculated according to the following equation: $P_{app} = Q/Act$, where P_{app} is the apparent permeability coefficient (cm/s), Q the total amount permeated over the incubation period (μg), A the diffusion area of the Ussing-type chamber (0.64 cm^2), c the initial concentration of the model drugs in the donor compartment (μg/cm^3), and t the whole time of experiments (s).

Improvement ratios were calculated from the ratio between the absorptive P_{app} of the tested compounds over the absorptive P_{app} of the buffer control. Data are expressed as mean ± SD. The differences between groups were tested by Student's t-test with $P < 0.05$ as the minimal level of significant difference.

Results

Characterization of thiolated chitosans

By the formation of amide bonds, the sulfhydryl-bearing ligands thioglycolic acid, *N*-acetyl cysteine, reduced GSH, cysteine, and 6MNA were covalently attached to the primary amino group of chitosan yielding chitosan-TGA, chitosan-NAC, chitosan-GSH, chitosan-Cys, and chitosan-6MNA, respectively, as shown in Figure 1. In contrast, chitosan-TBA was generated by amidine bond formation between chitosan and 2-iminothiolane HCl.

The lyophilized polymers were white, odorless, and fibrous-like structures. Only in the case of chitosan-6MNA, the product had a yellow fibrous structure. To compare the permeation-enhancing and P-gp inhibitory properties of thiolated chitosans, all modified chitosans contained an amount of thiol groups in the range of 230–520 μmol/g polymers, as listed in Table 1

TEER measurement

Parallel to the permeation studies, the TEER of rat intestines was measured. A range of initial TEER values of rat intestine was between 50 and 90 Ω cm^2. In the absence of thiolated chitosans, TEER values of the intestine did not change during the experiment. TEER values of the rat intestine treated with the thiolated chitosans slightly decreased by about 10–30% compared with the initial values (Figure 2a)

Permeation studies

The absorptive transport of FD4 on freshly excised rat intestinal mucosa was investigated in the presence of 0.5% (m/v) of thiolated chitosans. The resulting P_{app} values of FD4 are shown in Table 2. Due to the addition of 0.5% (m/v) unmodified chitosan to the buffer, FD4 transport was 1.48-fold improved, compared with the buffer control. In the presence of 0.5% (m/v) thiolated chitosans, corresponding to 0.58–1.15 μmol thiol groups/mL, FD4 transport was 2.57- to 3.95-fold improved. Among all the tested compounds, chitosan-6MNA led to the highest increase in FD4 transport. At 180 minutes, the P_{app} value of chitosan-6MNA was determined to be 4.02 ± 0.56 (×10^{-6} cm/s), which was significantly increased as compared to the values of the control (P = 0.021). The improvement ratios of chitosan-Cys, chitosan-GSH, chitosan-TBA, chitosan-TGA, and chitosan-NAC were approximately 3.4-, 2.8-, 2.7-, 2.7-, and 2.6-fold increased compared to the buffer control, respectively (Figure 2b).

P-gp inhibition studies

As an orientating experiment, the absorptive transport of 0.001% (m/v) Rho-123 across freshly excised rat intestine was determined at 4°C and 37°C. At 4°C, the absorptive P_{app} was 4.76 ± 0.40 (×10^{-6} cm/s), whereas that at 37°C was 2.44 ± 1.41 (×10^{-6} cm/s). The higher drug permeation at 4°C is due to the type of transport process. At 37°C, the absorptive transport is a combination of active efflux pump-mediated transport and passive diffusion[13]. On the contrary, at 4°C, the activity of ATP hydrolysis was low. Consequently, the mechanism of drug efflux is reduced. Therefore, the amount of permeated Rho-123 was higher than at 37°C due to passive transport.

Results obtained by P-gp inhibition studies are summarized in Table 2. In the presence of unmodified chitosan, P_{app} of Rho-123 transport was 4.80 ± 0.19 (×10^{-6}cm/s), whereas in the presence of 0.5% (m/v) chitosan-NAC, P_{app} was 9.21 ± 0.66 (×10^{-6}cm/s), which was 3.78-fold improved (P = 0.042). In contrast, due to the addition of chitosan-TBA, chitosan-GSH, chitosan-TGA, chitosan-6MNA and chitosan-Cys, the Rho-123 transport was approximately 3.03-, 2.85-, 2.21-, 2.12-, and 1.58-fold increased, respectively. The cumulative Rho-123 transport (%) is illustrated in Figure 3. The Rho-123 transport could be further improved by the addition of 0.5% (m/v) GSH, corresponding to 16.25 μmol thiol moieties/mL. A 4.68-fold higher uptake of Rho-123 was achieved in the presence of 0.5% (m/v) chitosan-NAC in combination with 0.5% (m/v) GSH (Figure 4). These results showed a significantly improved permeation-enhancing effect of thiolated chitosans and of the combination of thiolated chitosans and GSH compared with the control. In the presence of thiolated chitosans, the oxidation of GSH on the surface of the mucosa could be prevented, leading to an increase in tight junction permeability[14]

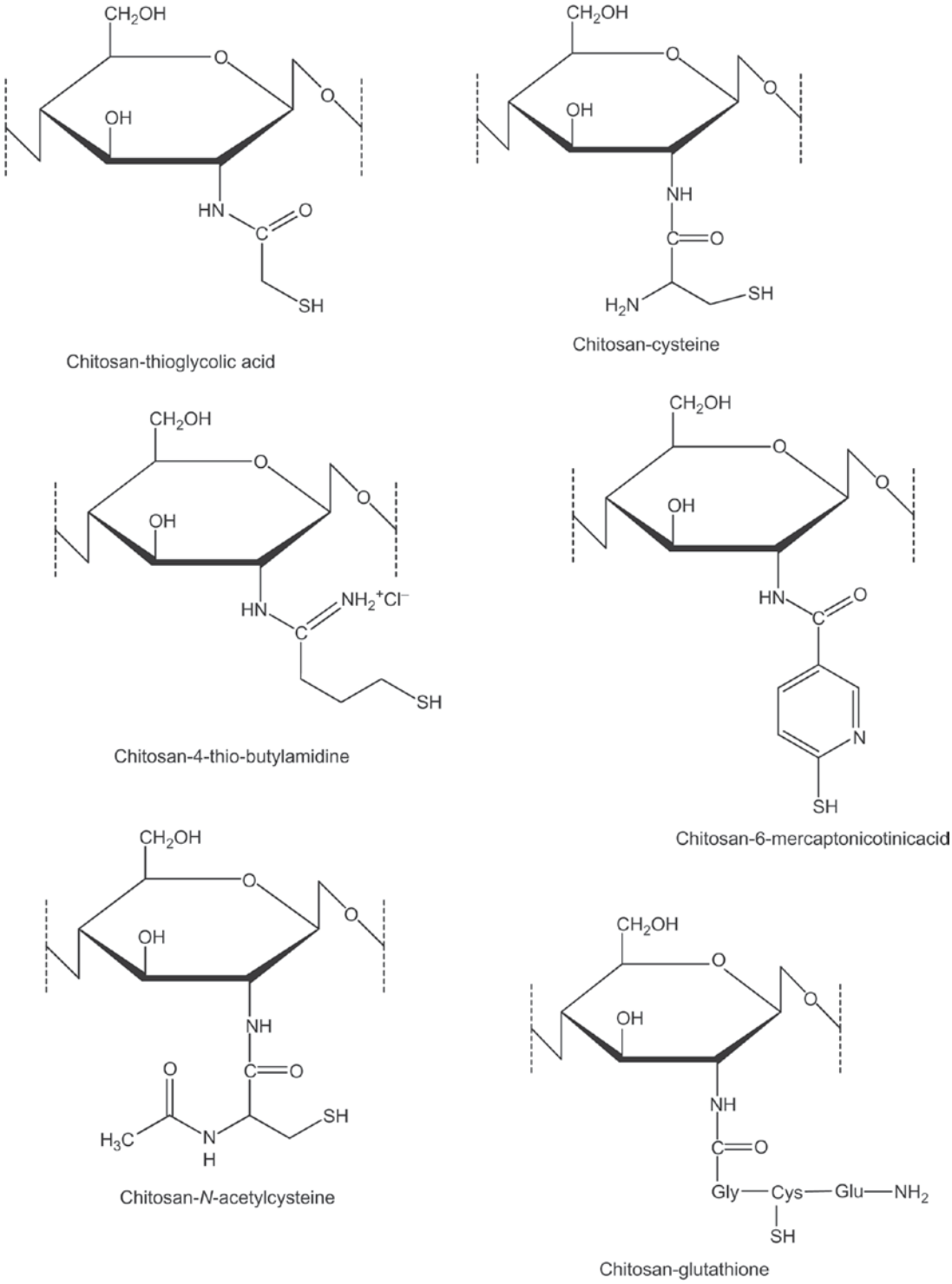

Figure 1. Schematic structures of thiolated chitosans.

Discussion

Chitosan is known as permeation enhancer and this effect of chitosan was more pronounced in the presence of thiol-bearing moieties[15]. In this study, thiolated chitosans were used in a final concentration of 0.5% (m/v) to avoid a too high viscosity of the test solution in the donor compartment. In the presence of 0.5% (m/v) unmodified chitosan, FD4 transport was 1.48-fold improved, which was consistent with previous studies[16,17]. An improvement of FD4 transport by unmodified chitosan is based on the enhancing properties of chitosan. Generally, chitosan binds to the epithelial membrane through a charge-dependent mechanism, leading to F-actin depolymerization and disbandment of the tight junction protein ZO-1[3]. Partly, FD4 could therefore be uptaken paracellularly through the opening of tight junctions.

The permeation-enhancing effect of thiolated chitosans was determined using a paracellular marker. The

Table 1. Comparison of different sulfhydryl reagents utilized for modification of thiolated chitosans

Compounds	Coupling reagents	pK_a	Reduced thiol groups (μmol/g) (mean ± SD)	Total amount of thiol groups (μmol/g) (mean ± SD)
Chitosan-TBA	2-Imminothiolane HCl	9.9	217.59 ± 38.10	279.09 ± 20.83
Chitosan-GSH	Reduced glutathione	8.6	115.56 ± 6.26	276.30 ± 17.47
Chitosan-TGA	TGA	8.5	213.58 ± 24.40	409.64 ± 6.42
Chitosan-NAC	*N*-Acetyl cysteine	8.5	229.69 ± 16.01	315.06 ± 106.39
Chitosan-Cys	Cysteine	8.3	119.60 ± 50.98	230.30 ± 4.12
Chitosan-6MNA	6MNA	—	130.45 ± 11.64	521.71 ± 40.82

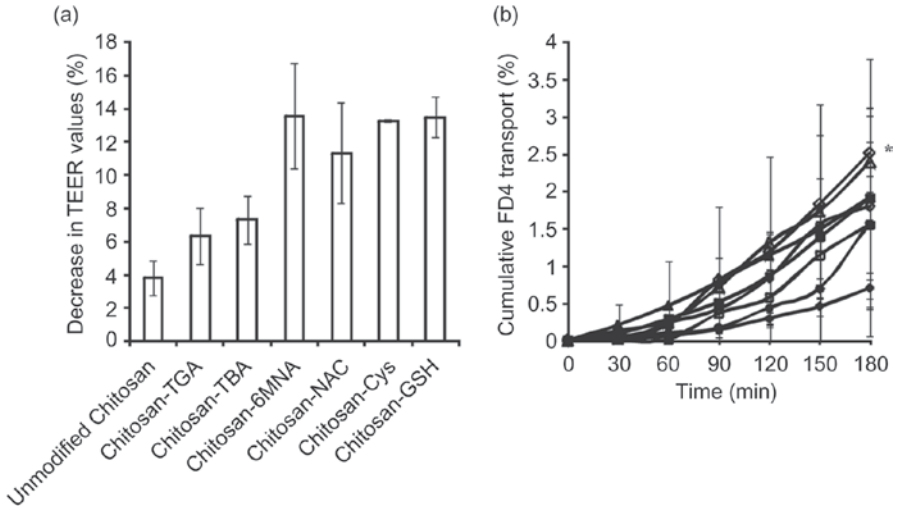

Figure 2. (a) Effect of 0.5% (m/v) thiolated chitosans on a decrease in TEER of rat intestinal mucosa over 3 hours incubation compared with the initial values. (b) The absorptive permeation studies of FD4 across rat intestine. Effect of 0.5% (m/v) unmodified chitosan (●), 0.5% (m/v) chitosan-TGA (■), 0.5% (m/v) chitosan-TBA (▲), 0.5% (m/v) chitosan-NAC (○), 0.5% (m/v) chitosan-6MNA (◇), 0.5% (m/v) chitosan-GSH (□), and 0.5% (m/v) chitosan-Cys (△) compared with buffer only (◆). Indicated values are the mean ± SD of three experiments. *P = 0.021 compared to the buffer control after 180 minutes of incubation.

improved permeation across the intestinal mucosa was associated with a decrease in the TEER, suggesting a loosening of the tightness of paracellular route (Figure 2a). At the tight junctions, several important transmembrane proteins such as claudins, the junctional adhesion molecule (JAM), and occludin being responsible for various functions are present[18]. In the case of occludin, an expression of two extracellular loops from amino acid is considered to provide the cohesiveness of the junctional barrier. Although tyrosine residues being expressed at these loops are phosphorylated by protein tyrosine kinases (PTPase) from the extracellular matrix, the tight junctions will be loosened[19,20]. Moreover, the dephosphorylation of the residues by protein tyrosine phosphatase (PTP) leads to malfunction of the tight junctions, which in turn leads to a closing of the junctions[18]. Accordingly, inhibition of PTP by compounds such as pervanadate, phenylarsine oxide, or reduced GSH leads consequently to more phosphorylated occludin and to more open tight junctions[14]. The mechanism being responsible for an improved permeation enhancement by thiolated chitosans is likely based on the inhibition of PTP as well. It was found that the modified chitosans might be able to form a mixed disulfide bond with the Cys 215 of the PTPase and accelerated GSH concentration by shifting oxidized GSH to GSH[14]. Hence, an increase in tight junction permeability is achieved. Results of permeation studies led to the following rank order in permeation enhancement: chitosan-6MNA > chitosan-Cys > chitosan-GSH > chitosan-TBA > chitosan-TGA > chitosan-NAC. The impact of ligands on the FD4 permeation could be explained in terms of the structure of ligands and the ionization of ligands on chitosan according to Henderson–Hasselbalch equation. As reported by Millotti et al.,[11] 6MNA has two tautomeric forms, which allow chitosan-6MNA to maintain its properties over a broad pH range. When chitosan-6MNA is presented in the intestinal lumen, the thiol groups are ready to be ionized and form disulfide bond with the Cys 215 of the PTPase, resulting in the opening of tight junctions. Furthermore, cysteine and GSH subunits were reported to have a potential in enhancing drug transport due to disulfide bond formation between the polymer and the Cys 215 of the PTPase as well[14]. The increase in permeability of fluorescence marker also depends on the pK_a values of the ligands on chitosan[21]. Generally, the active form of these polymers is the thiolate anion (S^-) and mostly in this form when pH values are slightly above the physiological conditions due to the pK_a of the thiomers. The pK_a values of 2-imminothiolane HCl, reduced GSH,

Table 2. Comparison of the absorptive apparent permeability coefficients (P_{app}) of Rho-123 and FD4 across freshly excised rat intestine in the presence of indicated test compounds. Each point represents the mean ± SD of three experiments

Substrate	Test compounds	P_{app} ($\times 10^{-6}$ cm/s)	Improvement ratio
Rho-123	Buffer (37°C)	2.44 ± 1.41	—
	Buffer (4°C)	4.76 ± 0.40	1.96
	0.5% (m/v) unmodified chitosan	4.80 ± 0.19	1.97
	0.5% (m/v) chitosan-NAC	9.21 ± 0.66	3.78*
	0.5% (m/v) chitosan-TBA	7.39 ± 1.68	3.03
	0.5% (m/v) chitosan-GSH	6.94 ± 0.36	2.85
	0.5% (m/v) chitosan-TGA	5.37 ± 1.84	2.21
	0.5% (m/v) chitosan-6MNA	5.16 ± 0.81	2.12
	0.5% (m/v) chitosan-Cys	3.85 ± 0.02	1.58
FD4	Buffer (37°C)	1.01 ± 0.13	—
	0.5% (m/v) unmodified chitosan	1.58 ± 0.14	1.48
	0.5% (m/v) chitosan-6MNA	4.02 ± 0.56	3.95**
	0.5% (m/v) chitosan-Cys	3.47 ± 1.14	3.42
	0.5% (m/v) chitosan-GSH	2.12 ± 0.16	2.82
	0.5% (m/v) chitosan-TBA	2.77 ± 0.30	2.74
	0.5% (m/v) chitosan-TGA	2.75 ± 0.06	2.72
	0.5% (m/v) chitosan-NAC	2.78 ± 0.24	2.57

*P = 0.042 compared to the buffer control, **P = 0.021 compared to the buffer control.

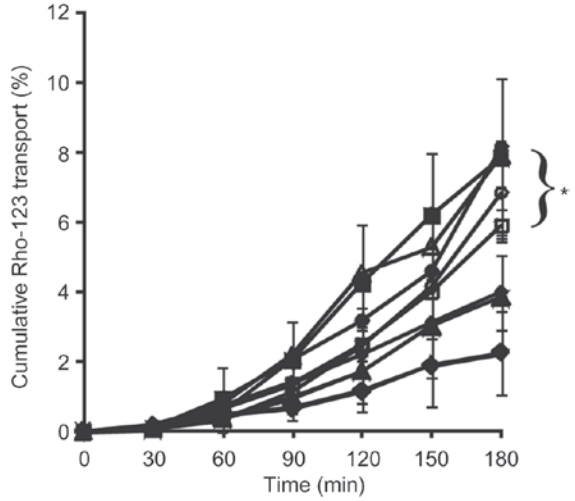

Figure 3. The absorptive permeation studies of Rho-123 across rat intestine. Effect of 0.5% (m/v) unmodified chitosan (▲), 0.5% (m/v) chitosan-TGA (□), 0.5% (m/v) chitosan-TBA (■), 0.5% (m/v) chitosan-GSH (○), 0.5% (m/v) chitosan-6MNA (●), 0.5% (m/v) chitosan-NAC ((), and 0.5% (m/v) chitosan-Cys (◇) compared with buffer only (◆). Indicated values are the mean ± SD of three experiments. *P < 0.05 compared to the buffer control after 180 minutes of incubation.

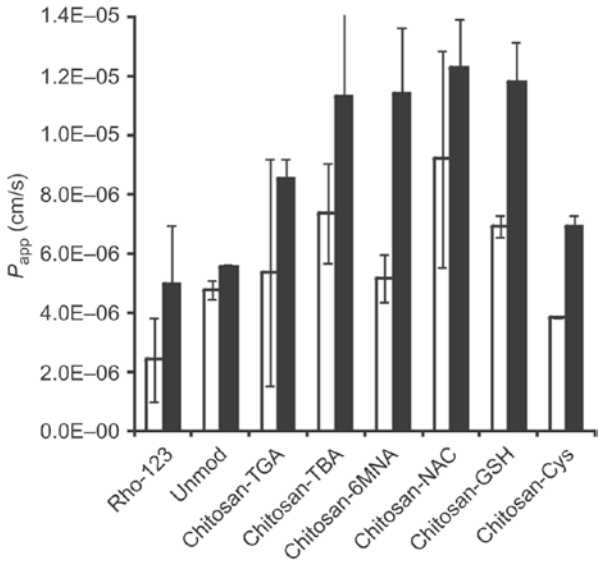

Figure 4. P_{app} values of 0.5% (m/v) thiolated chitosans across rat intestinal mucosa in the absence (white bars) or presence of GSH (black bars). Indicated values are the mean ± SD of three experiments.

TGA, *N*-acetyl cysteine, and cysteine are 9.9, 8.6, 8.5, 8.5, and 8.3, respectively. It is likely that these polymers show a pH-dependent reactivity. Thus, the permeation of FD4 is not significantly altered. Under physiological conditions, chitosan-TBA should be to a higher extent unionized, which leads to a lower degree of disulfide bond formation. Consequently, the permeation of the fluorescence marker could not be shifted. However, chitosan-TBA could show an increase in the permeation of the marker. This phenomenon can be explained by the mucoadhesive properties of chitosan-TBA. Apparently, chitosan-TBA offers the strong mucoadhesion due to additional ionic interactions between the cationic amidine substructure of the thiolated conjugate and anionic substructures of mucus glycoproteins. Thereafter, chitosan-TBA, being attached onto the mucosa as a result of its high mucoadhesive properties, can shift the balance between oxidized and reduced GSH on the membrane to the side of reduced GSH. The permeation of the fluorescence marker was therefore higher compared to those polymers.

A classical indication of efflux pump-mediated involvement in transport kinetics is the difference in permeation rates of substrates in the AP to BL and BL to AP directions. In a previous study performed by our research group, it

was found that the Rho-123 efflux ratio (secretory P_{app}/absorptive P_{app}) was about 2.8-fold. However, the process of drug absorption is relatively complex and several factors such as carrier-mediated transport and both efflux and influx pumps being located on the AP and BL sites might be involved in the permeation of drugs across the intestinal epithelium. Within this study, only the absorptive transport of Rho-123 was investigated to evaluate P-gp inhibitory properties of the thiolated chitosan. A significant improvement of the absorptive transport of Rho-123 across rat intestinal mucosa was observed in the presence of well-established P-gp inhibitors and 0.5% (m/v) thiolated chitosans. Basically, transmembrane efflux proteins such as P-gp translocate substrate from the inner side to the outer side of cells and the mechanism is based on ATP hydrolysis[22]. The inhibitory effect of the well-established low-molecular-weight P-gp inhibitors terfenadine (50 μM) and verapamil (100 μM), for example, was tested on intestinal mucosa[7]. Results of these experiments showed that in the presence of terfenadine and verapamil Rho-123 transport was 1.66- and 1.70-fold improved, respectively. Furthermore, inhibition of P-gp by the block polymer Pluronic P85 (56.67%) has been shown in rat intestinal mucosa. Pluronic P85 increased Rho-123 transport 1.9-fold compared with buffer control.

When the thiolated chitosans have been added to the buffer, chitosan-NAC showed the highest Rho-123 permeation, which was approximately 3.8-fold increased. The postulated mechanism of the efflux pump inhibition is based on an interaction of thiolated chitosans with the channel forming transmembrane region of P-gp. It is likely that thiolated chitosans enter into the channel of P-gp forming subsequently one or two disulfide bonds with one or both cysteine subunits located within the channel[23]. The amount of thiol groups on chitosan-NAC was 1.15 μmol thiol moieties/mL, whereas those of chitosan-Cys and chitosan-6MNA were 0.60 and 0.65 μmol thiol moieties/mL, respectively. It is likely that the P-gp inhibitory effect of thiolated chitosans is based on an amount of thiol groups immobilized on the polymer backbone. The more free thiol moieties are immobilized on thiolated chitosans, the more active is the polymer.

Conclusions

Thiolated chitosans described herein were found to have dramatic effects on the permeability of the small intestine. The most pronounced permeation of FD4 and Rho-123 were found in the presence of chitosan-6MNA and chitosan-NAC, respectively. The amount of free thiol groups immobilized on chitosan has a significant impact on the P-gp inhibitory properties of the applied thiomer, whereas the substructure and the ionization of the ligands are important for the paracellular uptake. Therapeutic use of these compounds as excipients would be an effective tool for improving the oral bioavailability of drugs.

Acknowledgments

The Nano-Health project (No. 0200) as part of the Austrian Nano-Initiative was financed by the Austrian FFG (Forschungsförderungsgesellschaft mbH) (Project No. 819721).

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

References

1. Chandy T, Sharma C. (1990). Chitosan–as a biomaterial. Biomater Artif Cells Artif Organs, 18:1–24.
2. Hejazi R, Amiji M. (2003). Chitosan-based gastrointestinal delivery systems. J Control Release, 89:151–65.
3. Schipper N, Olsson S, Hoogstraate J, deBoer A, Varum K, Artursson P. (1997). Chitosans as absorption enhancers for poorly absorbable drugs 2: Mechanism of absorption enhancement. Pharm Res, 14:923–29.
4. Föger F, Hoyer H, Kafedjiiski K, Thaurer M, Bernkop-Schnürch A. (2006b). In vivo comparison of various polymeric and low molecular mass inhibitors of intestinal P-glycoprotein. Biomaterials, 27:5855–60.
5. Föger F, Schmitz T, Bernkop-Schnürch A. (2006a). In vivo evaluation of an oral delivery system for P-gp substrates based on thiolated chitosan. Biomaterials, 27:4250–55.
6. Föger F, Kafedjiiski K, Hoyer H, Loretzz B, Bernkop-Schnürch A. (2007). Enhanced transport of P-glycoprotein substrate saquinavir in presence of thiolated chitosan. J Drug Target, 15:132–39.
7. Werle M, Hoffer M. (2006). Glutathione and thiolated chitosan inhibit multidrug resistance P-glycoprotein activity in excised small intestine. J Control Release, 111:41–46.
8. Kafedjiiski K, Föger F, Werle M, Bernkop-Schnürch A. (2005). Synthesis and in vitro evaluation of a novel chitosan-glutathione conjugate. Pharm Res, 22:1480–88.
9. Greindl M. (2008). Oral drug delivery: Design and in vitro/in vivo evaluation of novel permeation enhancers and efflux pump inhibitors. Innsbruck: University of Innsbruck.
10. Millotti G, Samberger C, Eleonore F, Sakloetsakun D, Bernkop-Schnürch A. (2010). Chitosan-4-mercaptobenzoic acid: Synthesis and characterization of a novel thiolated chitosan. J Mater Chem, 20:9–18.
11. Millotti G, Samberger C, Fröhlich E, Bernkop-Schnürch A. (2009). Chitosan-graft-6-mercaptonicotinic acid: Synthesis, characterization, and biocompatibility. Biomacromolecules, 10:3023–27.
12. Bernkop-Schnürch A, Schwarz V, Steininger S. (1999). Polymers with thiol groups: A new generation of mucoadhesive polymers? Pharm Res, 16:876–81.
13. Varma M, Ashokraj Y, Dey C, Panchagnula R. (2003). P-glycoprotein inhibitors and their screening: A perspective from bioavailability enhancement. Pharmacol Res, 48:347–59.
14. Clausen A, Kast C, Bernkop-Schnürch A. (2002). The role of glutathione in the permeation enhancing effect of thiolated polymers. Pharm Res, 19:602–8.
15. Bernkop-Schnürch A, Guggi D, Pinter Y. (2004b). Thiolated chitosans: Development and in vitro evaluation of a mucoadhesive, permeation enhancing oral drug delivery system. J Control Release, 94:177–86.
16. Hombach J, Bernkop-Schnürch A. (2009). Chitosan solutions and particles: Evaluation of their permeation enhancing potential

on MDCK cells used as blood brain barrier model. Int J Pharm, 376:104–9.
17. Moghaddam F, Atyabi F, Dinarvand R. (2009). Preparation and in vitro evaluation of mucoadhesion and permeation enhancement of thiolated chitosan-pHEMA core-shell nanoparticles. Nanomedicine, 5(2):14–20.
18. Bernkop-Schnürch A, Kast C, Guggi D. (2003). Permeation enhancingpolymersinoraldeliveryofhydrophilicmacromolecules: Thiomer/GSH systems. J Control Release, 93:95–103.
19. Clausen A, Bernkop-Schnürch A. (2000). In vitro evaluation of the permeation-enhancing effect of thiolated polycarbophil. J Pharm Sci, 89:1253–61.
20. Collares-Buzato C, McEwan G, Jepson SN, Hirst B. (1994). Paracellular barrier and junctional protein distribution depend on basolateral extracellular Ca^{2+} in cultured epithelia. Biochim Biophys Acta, 1222:147–58.
21. Madsen F, Eberth K, Smart J. (1998). A rheological examination of the mucoadhesive/mucus interaction: The effect of mucoadhesive type and concentration. J Control Release, 50:167–78.
22. Szakacs G, Paterson J, Ludwig J, Booth-Genthe C, Gottesman M. (2006). Targeting multidrug resistance in cancer. Nat Rev Drug Discov, 5:219–34.
23. Bernkop-Schnürch A, Hornof M, Guggi D. (2004a). Thiolated chitosans. Eur J Pharm Biopharm, 57:9–17.