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Glutathione and thiolated chitosan inhibit multidrug resistance P-glycoprotein activity in excised small intestine

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Abstract

The aim of the present study was to evaluate the influence of glutathione (GSH), the thiomer chitosan-4-thiobutylamidine (chitosan-TBA) and a combination of both compounds on P-glycoprotein (P-gp) activity. Permeation studies were performed with freshly excised guinea pig ileum mounted in Ussing chambers using the fluorescent dye rhodamine-123 (Rho-123) as P-gp substrate. Apparent permeability coefficients (P_{app}) as well as efflux ratios (secretory P_{app}/absorptive P_{app}) were calculated and compared with values gained from experiments with the well-established P-gp inhibitors terfenadine and verapamil.

In the presence of terfenadine, verapamil as well as GSH, the absorptive transport of Rho-123 across intestinal tissue increased, while the secretory decreased with efflux ratios around 1.0. Chitosan-TBA and especially chitosan-TBA/GSH not only enhanced absorption of Rho-123, but also reduced the basolateral to apical secretion of Rho-123 resulting in efflux ratios of 1.1, 0.8 and 0.5. The study indicates that chitosan-TBA/GSH is a potentially valuable tool for inhibiting the ATPase activity of P-gp in the intestine.

Keywords: Thiomers; Glutathione; P-glycoprotein inhibitor; Rhodamine-123

1. Introduction

Oral administration is the most popular route for drug administration, since dosing is convenient, non-invasive and many drugs are well absorbed by the gastrointestinal tract.

However, the gastrointestinal mucosa represents a major physical and biochemical barrier to the systemic availability of orally ingested, pharmacologically active molecules [1]. The function of the biochemical barrier depends largely upon intracellular metabolizing enzymes and specific membrane transport systems. Among these transport systems, P-glycoprotein (P-gp) is the most widely studied and probably the most important efflux pump in controlling the disposition of drugs [2]. P-gp, a 170 kDa transmembrane protein polarized to the apical membrane is widely expressed throughout the body, particularly in the intestinal mucosa, canalicular membranes of the liver, proximal tubule of the kidney and endothelial cells of the blood–brain barrier [3]. Drugs that are substrates for P-gp bind to the transporter and are transported back to the apical side of intestinal mucosal cells via an ATP-dependent process that greatly reduces their overall permeability and oral bioavailability. Overall P-gp plays a major physiological role as a barrier for entry of xenobiotics as well as a mechanism to eliminate xenobiotics from systemic circulation. Structurally diverse compounds have been identified as substrates for P-gp, including anticancer agents, antibiotics, antivirals, calcium channel blockers and immunosuppressive agents. In order to increase the therapeutic efficacy of these compounds, auxiliary agents being capable of inhibiting P-gp are highly on demand. A great number of compounds was found to be capable of suppressing the P-gp functioning, for example poloxamers [4], polyethyleneglycol and -derivatives [5], (R-) verapamil and cyclosporine [6], however only few were proven effective in various clinical studies [7].

Ten years ago, Al-Shawi and co-workers [8] reported that sulfhydryl-substituted purines gave substantial inhibition of P-gp ATPase activity, which was dithiothreitol reversible suggesting a covalent reaction with one or more cysteine residues of P-gp.

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The objective of this study was to test the hypothesis that toxicologically harmless compounds may inhibit P-gp activity. Reduced glutathione (GSH) and the thiolated polymer chitosan-4-thio-butylamidine (chitosan-TBA, Fig. 1) [9] were chosen as test compounds as they can be regarded as safe [10] and are not absorbed from the GI-tract in significant quantities remaining consequently concentrated on the membrane [11]. Permeation studies using freshly excised guinea pig ileum were performed in order to evaluate the influence of these sulfhydryl compounds on P-gp functioning in comparison to the well-established P-gp inhibitors terfenadine [12] and verapamil [13]. As P-gp substrate the fluorescent dye rhodamine-123 (Rho-123) was utilized, which has been extensively used as an index of P-gp mediated transport in rodent and tissue culture models [14].

2. Materials and methods

2.1. Materials

Chitosan (medium molecular mass: 400 kDa) was obtained from Fluka, Buchs, Switzerland; 2-iminothiolane HCl (Traut's reagent) from Pierce, Oud Beijerland, Netherlands. Rhodamine 123 (Rho-123) was purchased from Acros Organics, Geel, Belgium. All other compounds and reagents were obtained from Sigma, St. Louis, MO.

2.2. Synthesis and purification of the chitosan-TBA conjugate

Initially, to obtain a 1% (m/v) solution 500 mg of chitosan were dissolved in 50 ml of 1% acetic acid by stirring the mixture for 1 h. After adjusting the pH to 6 with 5 M NaOH, 400 mg of 2-iminothiolane HCl were added. The coupling reaction was allowed to proceed for 14 h at room temperature under continuous stirring. For purification, the resulting chitosan-TBA conjugate was dialyzed against 5 mM HCl, two times against 5 mM HCl containing 1% NaCl, against 5 mM HCl and finally against 0.4 mM HCl. NaCl was added to reduce ionic interactions between the conjugate and unlinked 2-iminothiolane. Samples prepared in the same way but omitting the addition of 2-iminothiolane HCl served as control. Polymers were adjusted to pH 4, freeze-dried at −30 °C/0.01 mbar (Christ Beta 1-8K; Germany) and stored at 4 °C until further use [9].

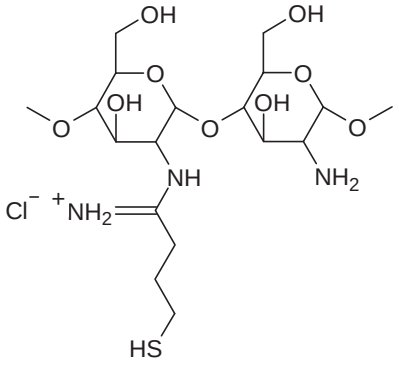

Fig. 1. Schematic presentation of the presumptive chemical substructure of chitosan-TBA (chitosan-4-thiobutylamidine) conjugate.

2.3. Determination of the thiol group content

The amount of free sulfhydryl groups on modified chitosan was determined photometrically using Ellman's reagent as described previously by our research group [15]. The total amount of sulfhydryl groups fixed on the polymer is represented by the summation of reduced thiol groups and of oxidized thiol moieties in form of disulphide bonds.

To determine this total amount of sulfhydryl groups the reaction with Ellman's reagent was performed after reducing disulfides with sodium borohydride: To 0.5 mg of the polymers 0.35 ml demineralized water were added. After a hydration time of 30 min 0.65 ml 0.05 M Tris buffer pH 6.8 and 1.0 ml of a freshly prepared 4% (w/v) sodium borohydride solution were added. The samples were incubated for 1 h in an oscillating waterbath at 37±0.5 °C. Thereafter, remaining sodium borohydride was inactivated by addition of 200 µl 5 M HCl and agitating for 10 min. The pH of the reaction mixture was adjusted to 8.0 with 1 ml of 1 M phosphate buffer pH 8.0. After addition of 100 µl Ellman's reagent (40 mg 5,5′-dithiobis(2-nitrobenzoic acid) in 10 ml of 0.5 M phosphate buffer pH 8.0) the samples were incubated for 15 min at room temperature. Aliquots of 200 µl were transferred to a 96-well microtitration plate and the absorbency was measured at a wavelength of 450/620 nm with a microplate reader (Anthos reader 2001, Salzburg, Austria). The quantity of iminothiolane immobilized on the polymer was calculated using L-cysteine standards. The amount of disulfide bonds was calculated by subtracting the quantity of free thiol groups from the totality of thiol moieties present on the polymer [16].

2.4. Permeability studies

For the studies, nonfasting guinea pigs weighing 400–450 g were used. After sacrificing by cervical dislocation, the distal ileum (20 cm) was immediately removed. The tissue was cut into strips of 1.5 to 2 cm, rinsed free of luminal contents and mounted in modified Ussing chambers (0.64 cm^2 surface area) without stripping off the underlying muscle layer. Preheated (37 °C) incubation 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 Bis–Tris (bis[2-hydroxyethyl]imino-tris[hydroxymethyl]methane) pH 6.0 was added to the apical (AP) and basolateral (BL) chambers (1 ml). To ensure oxygenation and agitation, a mixture of 95% O_2 and 5% CO_2 was bubbled through each compartment. The temperature within the chambers was maintained at 37±1 °C. A 30 min equilibration period was allowed before the beginning of permeability measurements. In control studies, Rho-123 in a final concentration of 0.001% (w/v) was added to the apical compartment for absorptive (AP to BL) transport or to the basolateral compartment for secretory (BL to AP) transport. Over a time period of 3 h