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Thiomers: Influence of molecular mass and thiol group content of poly (acrylic acid) on efflux pump inhibition

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ABSTRACT

The aim of the present study was to investigate the influence of molecular mass and thiol group content of poly(acrylic acid)–cysteine conjugates on the permeation of sulforhodamine 101 and penicillin G. acting as substrates for multidrug resistance-associated protein 2 efflux pump. Poly(acrylic acids) of 2 kDa, 100 kDa, 250 kDa, 450 kDa and 3000 kDa were conjugated with cysteine. The thiol group content of all these polymers was in the range from 343.3 ± 48.4 μmol/g to 450.3 ± 76.1 μmol/g. Transport studies were performed on rat small intestine mounted in Ussing-type chambers. Since 250 kDa poly(acrylic acid) showed the highest permeation enhancing effect, additionally thiolated 250 kDa polyacrylates displaying 157.2 μmol/g, 223.0 ± 18.1 and 355.9 μmol/g thiol groups were synthesized in order to investigate the influence of thiol group content on the permeation enhancement. The permeation of sulforhodamine was 3.93- and 3.85-fold improved using 250 kDa poly(acrylic acid)–cysteine conjugate exhibiting 355.9 ± 39.5 μmol/g and 223.0 ± 18.1 μmol/g thiol groups. Using the same conjugates the permeation of penicillin G was 1.70- and 1.59-fold improved, respectively. The study demonstrates that thiolated poly(acrylic acid) inhibits Mrp2 mediated transport and that the extent of inhibition depends on the molecular mass and degree of thiolation of the polymer.

1. Introduction

Oral administration of drugs is the most popular route of administration since it provides more convenient dosing for patients. The oral bioavailability of many drugs is, however, quite poor due to the presence of intestinal epithelium representing a biochemical barrier to the drug delivery into systemic circulation. The extent to which a compound is absorbed by the intestinal epithelium is regulated, in large part, by specific membrane transport systems located to the apical membrane of the cell. Drugs crossing the apical membrane of the intestinal epithelial cell may show the affinity for one or more efflux pump transporters, which extrude compounds back to the intestinal lumen, limiting their absorption into blood (Chan et al., 2004). These apical efflux transporters are principally ABC proteins such as *P*-glycoprotein (P-gp) and Multi drug resistance proteins (Mrp). Recently Mrp2 was identified as a 1545-amino acid, 190 kDa ATP-dependent protein efflux pump that preferably transports organic anions such as furosemide, indometacin, daunomycin and methotrexate (Takano et al., 2006). In contrast to Mrp2, *P*-glycoprotein substrates commonly include neutral or positively charged hydrophobic drugs. In humans Mrp2 is mainly expressed in liver, kidney tubules and intestine showing high abundance in duodenum, becoming lowest toward the terminal ileum and colon (Dietrich et al., 2003). The use of polymers as pharmaceutical excipients in oral drug delivery systems as permeation enhancers has been encouraged by results of numerous studies. So far, polymers have been established as vehicles for drug delivery through paracellular route. Very recently it was shown that thiolated chitosan, chitosan-4-thiobutylamidine, acts as an absorption enhancer for cationic fluorichrome, rhodamine 123, by inhibiting *P*-glycoprotein efflux pump (Föger et al., 2006). Since high molecular mass thiolated polymers are macromolecular structures that due to their high molecular mass cannot be absorbed from the gastrointestinal tract, occurrence of adverse events and pharmacokinetic interactions with co-administered drugs can be excluded. The aim of this study was to investigate whether thiolated polyacrylic acid can inhibit Mrp2 mediated efflux and to what extent molecular mass and thiolation degree of the thiolated polymer influence the inhibition. In order to determine the influence of molecular mass of the thiolated polymer on the absorption enhancing effect, poly(acylic acids) of five different molecular mass ranges were tested. Based

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on results of previous works of our research group, it was possible to determine the outcome of immobilisation of thiol groups on of the poly(acrylic acids) during the synthesis (Bernkop-Schnürch and Thaler, 2000). Such controlled synthesis enabled close insight into factors that may influence the inhibition. Experiments were performed on the freshly excised rat jejunum, using organic anionic compounds sulforhodamine 101 (Miller et al., 2002) and penicillin G (Bakos et al., 2000), as Mrp2 model substrates.

2. Materials and methods

2.1. Materials

Poly(acrylic acid) (PAA, MM 2 kDa; 100 kDa; 250 kDa; 450 kDa), cysteine hydrochloride, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDAC), *N*-(2-hydroxyethyl) piperazine-*N*′-(2-ethane-sulfonic acid) (HEPES), Ellman's reagent [DTNB, 5,5′-dithiobis(2-nitrobenzoic acid), penicillin G sodium salt (1,000,000 units) and sulforodhamine 101 were purchased by Sigma, Austria. Polycarbophil (PCP, MM 3000 kDa) was purchased by Noveon.

2.2. Methods

2.2.1. Synthesis of poly(acrylic acid)–cysteine conjugates used for the determination of influence of the molecular mass on inhibition of Mrp2

The covalent attachment of cysteine to PAAs and PCP was achieved by the formation of amide bonds between primary amino group of the amino acid and carboxylic acid groups of the polymer. First, polymer was hydrated in demineralized water and the pH value of the polymer solution was adjusted to 6 by the addition of 5 M NaOH. Then, EDAC in final concentration of 200 mM was added in order to activate the carboxylic acid moieties of the hydrated polymer. After 20 min of incubation under stirring at room temperature, L-cysteine was added and the pH was readjusted to 6. Reaction mixtures were incubated for 3 h at room temperature under stirring. The resulting conjugates were isolated by dialysis according to the method described previously (Bernkop-Schnürch and Steininger, 2000). After dialysis, the pH of all samples was readjusted to 6 and frozen. Frozen aqueous polymer solutions were freeze-dried at −30 °C and 0.01 mbar.

2.2.2. Synthesis of conjugates with various degrees of thiolation

In order to investigate the influence of the thiol group content of the polymer on its inhibitory effect to Mrp2, PAA of molecular mass 250 kDa was modified with cysteine to different degrees. A degree of modification of the polymer was pre-determined by adding different final concentrations of EDAC to activate a certain number of carboxylic moieties of the polymer as shown in Table 1.

2.2.3. Determination of the thiol group content

The degree of modification was determined by measuring the amount of thiol groups of the polymer–cysteine conjugates and the corresponding controls using Ellman's reagent as described previously (Bernkop-Schnürch et al., 1999).

2.2.4. Transport studies

Ussing-type chamber with a surface area of 0.64 cm^2 was used to perform permeation studies with sulforhodamine 101 and penicillin G sodium salt. The freshly prepared incubation medium containing 250 mM NaCl, 2.6 mM MgSO4, 10 mM KCl, 40 mM glucose and 50 mM $NaHCO_3$ was buffered with 40 mM HEPES. The small intestine of Sprague Dawley male rats weighting 240–260 g was excised immediately after sacrificing the animal and mounted in the Ussing-type chamber, without stripping off the underlying muscle layer. The donor and acceptor compartments of the Ussing-type chamber were filled with 1.0 ml of the incubation medium of pH 6.8 and 7.4, respectively. Permeation studies were performed in an atmosphere of 95% O_2 and 5% CO_2 at 37 °C and were started 15–20 min after the mounting of the tissue. The solution in the donor chamber was replaced with incubation medium containing test polymers at concentrations of 0.5% (m/v) with either 0.001% (m/v) sulforhodamine 101 or 0.1% (m/v) penicillin G. In control studies, sulforhodamine 101 and penicillin G in the same final concentrations as above, were added to the apical compartment for absorptive (AP to BL) transport or to the basolateral compartment for secretory (BL to AP) transport. During 180 min of incubation period 100 μl samples were taken from the acceptor chamber every 30 min and the volume was replaced by the same medium equilibrated at 37 °C. Cumulative corrections were made for the previously removed samples.

2.2.5. Quantification of permeated sulforhodamine 101 and penicillin G

The permeation enhancing effect of PAAs/PCP–cysteine conjugates was evaluated by measuring the amount of permeated sulforhodamine 101 in the acceptor chamber using fluorimeter (Fluostar Galaxy spectrometer, BMG Labtechnologies, Offenburg, Germany) and of permeated penicillin G sodium salt using HPLC-UV (La Chrom, Elite, MERCK Hitachi, Japan). Isocratic elution was performed using 73% eluent A (25 mM KH2PO4 buffer pH 3) and 27% eluent B (acetonitrile-HPLC grade, Sigma, Austria) for 20 min. The column used was Nucleosil 100-5C18 (250 × 4 mm). The flow rate was kept constant at 1 ml/min. Samples of 20 μl were injected and detected at 325 nm at a retention time of 25 min. As control served unmodified poly(acrylic acids).

2.2.6. Calculation of apparent permeability coefficient

The apparent permeability coefficients (Papp) for sulforhodamine 101 and penicillin G were calculated according to the following equation:

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

where Papp is the apparent permeability coefficient (cm/s), Q is the total amount permeated within the incubation time (μg), A is the diffusion area of the Ussing chamber (cm^2), c is the initial

Table 1
Concentration of reagents used for synthesis of polyacrylate–cysteine conjugates and obtained degree of modification.

Conjugate	Aqueous polymer solution (g/80 ml)	EDAC (final conc. mM)	Cysteine (g)	Thiol groups (μmol/g polymer mean ± SD; $n = 3$)
PCP-cys	1.0	200	1.0	386.1 ± 23.6
PAA-450-cys	1.0	200	1.0	450.3 ± 76.1
PAA-250-cys-I	1.0	200	1.0	355.9 ± 39.5
PAA-250-cys-II	1.0	100	1.0	223.0 ± 18.1
PAA-250-cys-III	1.0	50	1.0	157.7 ± 27.2
PAA-100-cys	1.0	200	1.0	343.3 ± 48.4
PAA-2-cys	1.0	200	1.0	354.9 ± 15