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Research paper

An oral oligonucleotide delivery system based on a thiolated polymer: Development and in vitro evaluation

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ABSTRACT

The purpose of this study was to develop and evaluate an oral oligonucleotide delivery system based on a thiolated polymer/reduced glutathione (GSH) system providing a protective effect toward nucleases and permeation enhancement. A polycarbophil–cysteine conjugate (PCP–Cys) was synthesized. Enzymatic degradation of a model oligonucleotide by DNase I and within freshly collected intestinal fluid was investigated in the absence and presence of PCP–Cys. Permeation studies with PCP–Cys/GSH versus control were performed in vitro on Caco-2 cell monolayers and ex vivo on rat intestinal mucosa. PCP–Cys displayed 223 ± 13.8 μmol thiol groups per gram polymer. After 4 h, 61% of the free oligonucleotides were degraded by DNase I and 80% within intestinal fluid. In contrast, less than 41% (DNase I) and 60% (intestinal fluid) were degraded in the presence of 0.02% (m/v) PCP–Cys. Permeation studies revealed an 8-fold (Caco-2) and 10-fold (intestinal mucosa) increase in apparent permeability compared to buffer control. Hence, this PCP–Cys/GSH system might be a promising tool for the oral administration of oligonucleotides as it allows a significant protection toward degrading enzymes and facilitates their transport across intestinal membranes.

1. Introduction

Recent advances in biotechnology have brought up antisense oligonucleotides as new therapeutic agents in order to improve the medical treatment of cancer, viral diseases, or genetic disorders [1]. Administration of oligonucleotides (ODNs) is currently limited to parenteral routes, mainly intravenous and subcutaneous, due to their poor oral bioavailability. Major reasons for this poor oral bioavailability are their rapid degradation in the GI-tract by nucleases, on the one hand, and limited intestinal permeability to hydrophilic macromolecules with a high negative charge density, on the other hand. Thus far, strategies to overcome the problem of degradation by nucleases are mainly based on chemical modifications, such as formation of phosphorothioates [2] or methoxyethyl phosphorothioates [3]. Coadministration of permeation enhancers such as medium chain fatty acids turned out to be a promising strategy to increase ODN uptake across gastrointestinal epithelia [4].

Thiolated polymers with excellent mucoadhesive features have been developed [5]. These so-called thiomers are hydrophilic polymers, such as polycarbophil (PCP), modified with thiol-bearing molecules, like L-cysteine, on their polymer backbone. Thiomers show enzyme inhibitory activity toward metalloenzymes [6]. Hence, it is assumed that they also exhibit inhibitory effects toward nucleases, which are abundantly present in the intestine. Their mechanism of enzyme inhibition is based on the deprivation of divalent metal cations from the enzyme structure. Thiomers also show excellent permeation-enhancing properties in combination with reduced glutathione (GSH) based on a reversible opening of tight junctions, which are mainly responsible for limited paracellular uptake of hydrophilic macromolecules [7]. In combination with their further advantages, including enhanced mucoadhesion and cohesive strengths, thiomers seem to be a promising candidate for oral ODN delivery.

Therefore, the aim of this study was to develop an oral delivery system for ODNs based on PCP–Cys/GSH and to determine its inhibitory activity toward nucleases and its permeation-enhancing effect. Enzyme inhibition studies were performed with DNAse I as well as fresh porcine intestinal fluid. Permeation studies were performed on differentiated Caco-2 cell monolayers and freshly excised rat intestinal mucosa. In these studies, a 30-mer phosphorothioate antisense oligonucleotide was chosen as model ODNs. The utilized ODN was used as a model since it was shown to be able to inhibit the growth of *Plasmodium falciparum* [8]. Therefore, it might be a valuable tool for the therapy of malaria diseases.

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2. Material and methods

Noveon® AA-1 USP Polycarbophil (PCP) was obtained as a kind donation from Lubrizol, Germany. The 30-mer model phosphorothioate oligonucleotide with the sequence 5′-ATG TAA TAT TCT TTT GAA CCA TAC GAT TCT-3′ (molecular mass 9608 Da) was purchased from VBC Biotech, Vienna, Austria. Caco-2 cells were obtained from German Collection of Microorganisms and Cell Cultures, Brunswick, Germany. Fetal calf serum was obtained from PAA Laboratories GmbH, Pasching, Austria. All other chemicals were of analytical grade and were purchased from Sigma Aldrich, Vienna, Austria.

2.1. Synthesis of polycarbophil–cysteine conjugate

The polycarbophil–cysteine conjugate was synthesized according to a method described previously [9]. Polycarbophil (MW 3000 kDa) was neutralized with 5 M NaOH as described previously [10]. First, 2 g of neutralized polymer was hydrated in 500 ml of demineralized water. The carboxylic acid moieties of the polymer were activated by adding 1-ethyl-3-(dimethylaminopropyl) carbodiimide (EDAC) in a final concentration of 50 mM. This mixture was then stirred for 45 min. Afterward, 0.25 g of L-cysteine hydrochloride was added and pH was adjusted to 4.0 by adding 1 M HCl. The reaction mixture was stirred and incubated at room temperature for 3 h. Cysteine was covalently linked to polycarbophil via the formation of amide bonds between the primary amino group of cysteine and a carboxylic group of the polymer. Resulting conjugates were isolated by dialysis against 1 mM HCl containing 2 μM EDTA at 10 °C in the dark to avoid oxidation of the cysteine moieties, two times against the same medium but additionally containing 1% NaCl and one more time against 1 mM HCl. In order to prepare a non-thiolated control, polycarbophil was treated in exactly in the same manner but EDAC was omitted during the reaction. The purified polymers were lyophilized by freeze-drying aqueous polymer solutions at −30 °C and 0.01 mbar (Benchtop 2 K, VirTis, NY, USA). Freeze-dried polymers were stored at 4 °C until further use.

2.2. Determination of immobilized thiol groups

The amount of thiol groups on the polycarbophil–cysteine conjugate was determined via Ellman's reagent (5,5′-dithiobis(nitrobenzoic acid), DTNB) as described previously [5]. For this purpose, 1 mg polymer was hydrated in a 0.5 M phosphate buffer before a 0.03% solution of DTNB in the same buffer was added. Thiol concentrations were derived from a calibration curve that was obtained from solutions of different L-cysteine concentrations.

2.3. Intestinal stability of oligonucleotide

2.3.1. Protection against DNase I degradation

To evaluate the protection of ODNs from DNase degradation by PCP–Cys, mixtures of PCP–Cys and ODNs (1 mg/ml) were incubated with DNase I in artificial intestinal fluid (AIF). AIF is a physiological salt solution containing 20 mM bicarbonate, 139 mM chloride, 5 mM potassium, 140 mM sodium, 4 mM calcium and 3 mM magnesium ions (pH 7.0). Mixtures of 250 μl of PCP–Cys polymer solution (0.04% m/v) adjusted to pH 7.4, 250 μl of AIF containing 1 mg/ml of ODNs, and 3 μl of DNase I (3 Units) were incubated at 37 °C while shaking at 400 rpm (Thermomixer comfort 2 ml, Eppendorf, Germany). After predetermined time intervals of up to 4 h, 10 μl of 0.5 M EDTA solution, pH 8.0, was added to each sample in order to stop the reaction. ODNs were incubated with DNase I in the absence of PCP–Cys as control. After incubation, samples were centrifuged at 29,700*g* and 4 °C for 60 min (Sigma 3–18K centrifuge) and 30 μl of the supernatant was analyzed by a weak–base anion exchange HPLC assay, using a PRP-X600 Anion Exchange 4.6 × 100 HPLC column (Hamilton Co., Reno, Nevada, USA). A two-eluent gradient system was used with eluent A consisting of 80% 100 mM Tris (pH 8.0)/20% acetonitrile and eluent B consisting of 80% 100 mM Tris, 2.5 M LiCl (pH 8.0)/20% acetonitrile. A linear gradient from 100% eluent A to 100% eluent B in 15 min at a flow rate of 2 ml/min was performed. The amounts of ODNs were determined by measuring absorbance at 260 nm [11].

2.3.2. Incubation with intestinal fluid

The intestinal fluid from a freshly slaughtered pig was collected and frozen on dry ice. For this study, samples of 250 μl of ODN solution (1 mg/ml) were incubated with 250 μl of intestinal fluid containing 0.04% PCP–Cys at 37 °C under continuous shaking (400 rpm). At predetermined time points, aliquots of 50 μl were withdrawn and 10 μl of 0.5 M EDTA, pH 8.0, was added in order to stop any further enzymatic degradation. Withdrawn aliquots were substituted by 50 μl of intestinal fluid. ODNs without additional PCP–Cys were incubated with intestinal fluid in the same manner as a control. The degree of ODN degradation was determined via HPLC as described previously.

2.4. Caco-2 cell monolayer permeation studies

Caco-2 cells were employed to evaluate the effect of PCP–Cys on intestinal permeability. These cells originate from human adenocarcinoma and exhibit characteristics of intestinal epithelia, such as microvilli, intercellular tight junctions, enzymes, nutrients, and efflux transporters, and are appropriate models for evaluating the permeation of drug molecules across intestinal epithelia [12]. Cells were seeded in 75-cm^2 flasks and cultured until confluency. Caco-2 cells were maintained in MEM medium supplemented with 20% (v/v) of heat-inactivated fetal calf serum (FCS) and 0.01% (v/v) of penicillin–streptomycin solution. The pH was adjusted to 7.4 using 7.5% (m/v) sodium bicarbonate. Cells were maintained at 37 °C in an atmosphere of 5% CO_2. Cells in flasks were first washed with phosphate-buffered saline (PBS) and then treated with trypsin/EDTA solution (0.05%) at 37 °C for 20 min. The cell suspension was centrifuged at 900 rpm, and the supernatant removed in order to remove trypsin. Cells were resuspended in culture medium and thereafter seeded at 12 wells of a Transwell® plate (Corning Inc., Corning, NY, USA) before being cultivated for 3 weeks. The transepithelial electrical resistance (TEER) across cell monolayers was monitored by means of a volt ohmmeter with a chopstick electrode (World Precision Instruments, Berlin, Germany) during cell culturing and during the permeation experiment. Caco-2 cell monolayers were used at resistance readings between 600–700 Ω cm^2. After 2 h of pre-incubation with fresh medium, the medium in the donor chamber of Transwell® plate was substituted with MEM medium (1 ml) containing 1 mg/ml of ODNs and 0.5% (m/v) of PCP–Cys conjugate with or without 0.25% (m/v) reduced GSH. Permeation experiments with ODNs but without additives served as controls. In order to prevent enzymatic degradation of ODNs, 0.5 mM EDTA was added to each chamber prior to sampling. Samples of 100 μl were withdrawn from the acceptor chamber of Transwell® plates every 30 min over a time period of 2 h. The removed volume was immediately replaced by 100 μl fresh MEM. The EDTA concentration was kept constant in all experiments in order to allow investigation into the effect of PCP–Cys alongside the permeation-enhancing effect of EDTA. The amount of permeated ODNs was determined via HPLC as described previously. The apparent permeability coefficients (P_{app}) for ODNs were calculated according to the equation below. All sample solutions were removed after the permeation