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Development and *In Vitro* Evaluation of a Mucoadhesive Oral Delivery System for Antisense Oligonucleotides

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Abstract

It was the aim of this study to develop an oral phosphorothioate oligodeoxynucleotide (PS-ODN) drug delivery system and to evaluate its properties *in vitro*. Results demonstrated that the 16-mer phosphorothioate oligonucleotide used was completely stable towards enzymatic degradation by secreted and membrane bound intestinal enzymes. Permeation studies with freshly excised intestinal mucosa showed a comparatively high uptake of the PS-ODN with an apparent permeability coefficient (P_{app}) of 8.35 ± 1.24 x 10^{-6} cm/sec. The PS-ODN was incorporated in a poly(acrylic acid)-cysteine carrier matrix system exhibiting high cohesive and mucoadhesive properties. Release studies demonstrated that a controlled and sustained PS-ODN release out of this delivery system could be achieved. Because of these features, the dosage form developed within this study seems to represent a promising novel tool for oral PS-ODN delivery.

Keywords:

antisense oligonucleotides, mucoadhesive polymers, oral drug delivery, thiomers

Introduction

Because of their poor oral bioavailability, which is in the range of a few percent [1, 2, 3], oligodeoxynucleotides (ODNs) are currently administered via invasive

routes, which are complex, painful for the patient and occasionally dangerous. The development of highly efficient oral delivery systems would therefore strongly contribute to increase the possible applications of ODNs as therapeutic agents in the future. Strategies to improve the oral bioavailability of ODNs are based on chemical modifications, such as the generation of phosphorothioates [4] or methoxyethyl phosphorothioates [5] in order to improve the stability of the therapeutic agent also towards gastrointestinal enzymes, and to enhance their absorption from the intestine [6]. The co-administration of permeation enhancers such as medium chain fatty acids turned out to be another promising strategy to increase the gastrointestinal uptake of ODNs [1].

Although mucoadhesive polymers were shown to improve the oral bioavailability of various hydrophilic macromolecules such as therapeutic peptides and heparins [7, 8, 9], their potential for oral ODN delivery has so far not been evaluated. Among mucoadhesive polymers, thiolated polymers or so-called thiomers seem to be of particular interest. The oral uptake of hydrophilic macromolecules incorporated in thiomer matrix systems was shown to be significantly higher than in comparison to corresponding well-established unthiolated mucoadhesive polymers [8, 10, 11].

It was therefore the aim of this study to develop a mucoadhesive oral delivery system for an antisense oligonucleotide comprising a thiolated polymer, and to evaluate the system with regard to drug stability, permeation enhancement and controlled drug release *in vitro*. A 16-mer phosphorothioate oligodeoxynucleotide was chosen as model ODN. As mucoadhesive thiolated polymer a poly(acrylic acid)-cysteine conjugate was chosen, as this thiomer exhibits excellent mucoadhesive and permeation enhancing properties [12, 13].

Experimental

Synthesis of polycarbophil-cysteine conjugate

The poly(acrylic acid)-cysteine conjugate was synthesized according to a method described previously [14]. In brief, cysteine (Sigma; St. Louis, MO) was

covalently linked to poly(acrylic acid) (polycarbophil; Noveon, Raubling, Germany) via the formation of amide bonds between the primary amino group of cysteine and a carboxylic acid group of the polymer. The reaction was mediated by a water-soluble carbodiimide (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride; Sigma, St. Louis, MO). After purification of the conjugate via dialysis the amount of remaining unbound free cysteine was determined with 2,4,6-trinitrobenzenesulfonic acid (TNBS-reagent; Sigma, St. Louis, MO) as described previously [15].

Determination of immobilized thiol groups

The amount of thiol groups on the poly(acrylic acid)-cysteine conjugate was determined via Ellman's reagent (5,5'-dithiobis(nitrobenzoic acid)) as described previously [14].

Stability of the phosphorothioate antisense oligonucleotide towards enzymatic degradation

Intestinal fluid was collected immediately after sacrificing guinea pigs. 180 µl of the intestinal fluid were incubated with 20 µl of an aqueous antisense oligonucleotide solution (0.5 mg/ml; phosphorothioate oligodeoxynucleotide; sequence: 5'-GGC CGC GCT CGA GCA C-3'; VBC-GENOMICS Bioscience Research GmbH; Vienna, Austria) at 37°C. At predetermined time points aliquots of 40 µl were withdrawn and 40 µl of 1% (v/v) trifluoroacetic acid were added to these samples in order to stop any further enzymatic degradation. The degree of enzymatic degradation of the antisense oligonucleotide was then determined via reversed phase HPLC using a Perkin-Elmer (Norwalk, CT, USA) series 200 LC pump, Perkin-Elmer 200 series auto sampler with a 20 µl injection loop and a diode array detector (Perkin-Elmer 235C). Samples were eluted from a Nucleosil 100 – 5 C18 column (250 x 4 mm) with mobile phase A-0.1% TFA and B-acetonitrile/0.1% TFA (9+1) at a flow rate of 1.0 ml min^{-1}; detection was at 220 nm. A linear gradient was applied from 90% A to 40% A over a 14 min period. The amount of

(un)degraded antisense oligonucleotide was quantified utilizing a standard curve in the range from 5 µg/ml to 100 µg/ml established by injecting increasing amounts of undegraded PS-ODN. The detection limit was determined to be 5 µg of PS-ODN per ml.

Permeation studies

Permeation studies were carried out in Ussing type chambers displaying a volume of 1 ml (=1 cm^3) in the donor- and acceptor-chamber and a permeation area of 0.64 cm^2. The incubation medium used as artificial intestinal fluid contained 250 mM NaCl, 2.6 mM $MgSO_4$, 10 mM KCl, 40 mM glucose and 50 mM $NaHCO_3$, buffered with 40 mM HEPES, pH 7.4.

Immediately after sacrificing the guinea pig 15 cm of the small intestine (duodenum) were excised and mounted in the Ussing chamber. All experiments were performed at least three times in an atmosphere of 95% O_2 and 5% CO_2 at 37°C. After 15 - 20 minutes of preincubation with the artificial intestinal fluid, the incubation medium of the donor compartment was substituted by a 0.5% (m/v) solution of poly(acrylic acid)-cysteine conjugate containing 5% (m/v) of reduced glutathione (GSH) and either 0.025% (m/v) phosphorothioate antisense oligonucleotide or 0.1% (m/v) FITC labeled dextran (FD4; molecular mass: 4.3 kDa; Sigma, St. Louis, MO) in artificial intestinal fluid. Permeation experiments without poly(acrylic acid)-cysteine and GSH served as controls. Samples of 200 µl were withdrawn from the acceptor compartment every 30 minutes over a time period of 3 hours. Samples were immediately replaced by 200 µl artificial intestinal fluid equilibrated at 37°C. The amount of permeated phosphorothioate antisense oligonucleotide was determined via HPLC as described above. FITC labeled dextran was quantified using a fluorimeter (SLT, Spectra Fluor; Tecan; Austria). Cumulative corrections were made for the previously removed samples. The apparent permeability coefficients (P_{app}) for phosphorothioate antisense oligonucleotide and FITC labeled dextran were calculated according to the following equation

$$P_{app}=Q/(A*c*t)$$

where P_{app} is the apparent permeability coefficient (cm/sec), 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 concentration of the marker in the donor compartment ($\mu g/cm^3$), and t is the total time of the experiment (sec).

Formulation of matrix tablets

First, 48.5 mg of the poly(acrylic acid)-cysteine conjugate, 1.5 mg of reduced glutathione (Sigma, St. Louis, MO) and 1 mg of the PS-ODN were hydrated/dissolved in 10 ml of demineralised water. The solution was frozen at –20°C and lyophilized (-30°C, 0.1 mbar; Christ Beta 1-8; Germany). This freeze-dried homogenate was compressed (Hanseaten Type EI, Hamburg, Germany) into 2.0 mm diameter flat-faced discs of 5 mg weight. The compaction pressure was kept constant during the preparation of all tablets.

Drug release studies

Matrix tablets as described above were placed in a vessel containing 2 ml of 100 mM phosphate buffer pH 6.8 at 37°C. The vessels were closed up and continuously shaken on an oscillating water bath. At predetermined time points aliquots of 200 µl were withdrawn and replaced with an equal volume of release medium equilibrated at 37°C. Sink conditions were maintained throughout the study. The amount of PS-ODN released was quantified via HPLC as described above. Cumulative corrections were made for previously removed samples.

Statistical data analysis

Statistical data analyses were performed using the Student's *t* test with $p < 0.05$ as the minimal level of significance. Calculations were done using the software XIstat version 5.0 (b8.3).

Results and Discussion

Stability studies

The stability of the antisense oligonucleotide was evaluated in freshly collected intestinal fluid at 37°C. No significant degradation could be determined within an observation period of 3 hours, although other studies reported extensive phosphorothioate oligodeoxynucleotide degradation by intestinal enzymes.[3] The result of this study is shown in Fig. 1.

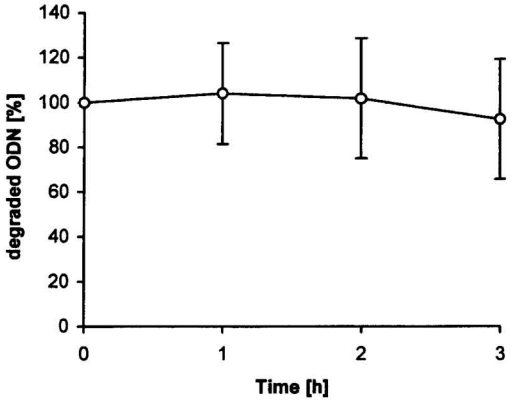

Fig. 1. Stability of the antisense phosphorothioate oligonucleotide towards secreted intestinal enzymes. The ODN was incubated in a concentration of 50 µg/ml in intestinal fluid at 37°C. Indicated values are means of five experiments ±SD.

Apart from this experiment, where the PS-ODN was incubated with secreted enzymes, the degradation of the antisense oligonucleotide by membrane bound intestinal enzymes was evaluated as well. In case of permeation studies the PS-ODN came into direct contact with freshly excised intestinal mucosa. Analysis of the stability of the antisense oligonucleotide in the donor- and acceptor chamber, however, showed no degradation of the PS-ODN at all (data not shown).

An explanation for this inconsistency of results obtained here with previously reported stability studies can be given by the different methods used. In the study performed by Geary et al., for instance, showing a more than 50% degradation of a very similar phosphorothioate antisense oligonucleotide within 3 h of incubation with intestinal enzymes at 37°C, not an intact intestinal tissue but a tissue homogenate was used [3]. In contrast to our study the PS-ODN was thereby also confronted with cytosolic enzymes likely being responsible for this rapid degradation. As the route of intestinal uptake for ODNs is primarily the paracellular and not the transcellular pathway [16], the antisense oligonucleotide will not come into contact with cytosolic enzymes. This theory could be confirmed by results obtained in this study, as no fragments of the PS-ODN could be detected in the donor chamber of the Ussing chamber. Hence, the PS-ODN must have passed the membrane in intact form.

Permeation studies

Thiolated poly(acrylic acid) was shown to exhibit a strong permeation enhancing effect for paracellular drug uptake [17], which can be even further improved by the addition of reduced glutathione (GSH) [13]. According to these studies also the uptake of the model compound FITC labeled dextran, which was used as hydrophilic macromolecular paracellular marker and of the antisense oligonucleotide should be improved in the presence of the thiomer/GSH system. Permeation studies performed with FITC labeled dextran confirmed these previous observations (Fig. 2).

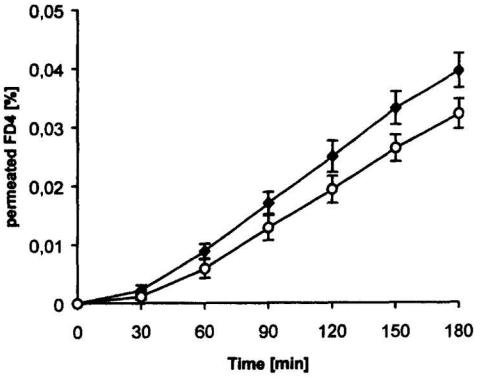

Fig. 2. Permeation enhancing effect of the thiomer/GSH system (♦) in comparison to a buffer only control (o) for FITC labeled dextran. Studies were performed in Ussing-type chambers on freshly excised intestinal mucosa. Indicated values are means of three experiments ±SD.

The P_{app} (apparent permeability coefficient) for the FITC labeled dextran was 0.46 ± 0.04 x 10^{-6} cm/sec, whereas it was 0.57 ± 0.04 x 10^{-6} cm/sec for the same marker compound in the presence of the thiomer/GSH system (means ±SD; n=3-4). The significant improvement in the uptake of this high molecular mass paracellular marker, however, was much less pronounced in comparison to hydrophilic compounds exhibiting a comparatively lower molecular mass (Clausen et al., 2002). In case of the PS-ODN even no significant improvement in its permeation behavior was achieved due to the addition of poly(acrylic acid)-cysteine and GSH (data not shown). The P_{app} for the oligonucleotide without the thiomer/GSH system was determined to be 8.35 ± 1.24 x 10^{-6} cm/sec (means ±SD; n=4) being already more than 10-fold higher than that of FITC labeled dextran. Results of permeation studies with the oligonucleotide are shown in Fig. 3. As the oligonucleotide has a molecular mass of 5.1 kDa and the FITC labeled dextran a molecular mass of 4.3 kDa, the oligonucleotide seems to have a comparatively smaller molecular diameter, which should explain the much higher P_{app} value.

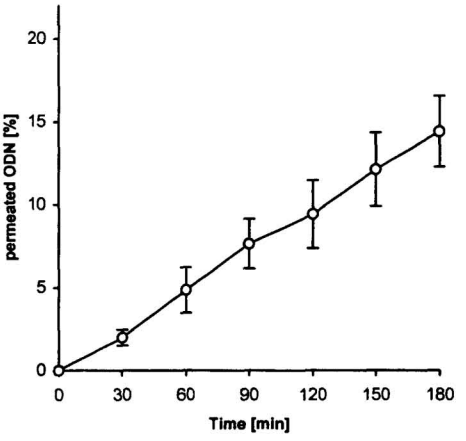

Fig. 3. Permeation of the antisense oligonucleotide across freshly excised intestinal mucosa. Indicated values are means of five experiments ±SD.

Characterization of the mucoadhesive drug delivery system

The PS-ODN was incorporated in the poly(acrylic acid)-cysteine conjugate used as mucoadhesive drug carrier matrix. As various previous studies have shown that the mucoadhesive properties of thiolated poly(acrylic acid) are not significantly reduced by the incorporation of hydrophilic drugs, additional mucoadhesion studies of the delivery system were not performed. Because of the formation of disulfide bonds between poly(acrylic acid)-cysteine and cysteine-rich subdomains of mucus glycoproteins [18], this thiomer shows the so far highest mucoadhesive properties [19]. Due to the adhesion of the delivery system on the intestinal mucosa a comparatively steep concentration gradient of the PS-ODN to the absorption membrane - representing the driving force for passive drug uptake - can be achieved. As shown for other hydrophilic macromolecules (e.g. [7, 8, 9]), this effect should strongly contribute to an improved oral uptake of the PS-ODN. Apart from disulfide bonds between the polymer and the mucus gel layer, the thiomer is also capable of forming disulfide bonds within the polymeric network itself. Because of

this *in situ* cross-linking process the stability of the carrier matrix during the swelling process is strongly improved [20]. Consequently also a controlled and sustained drug release out of the mucoadhesive polymeric network could be achieved. Results of PS-ODN release studies are shown in Fig. 4.

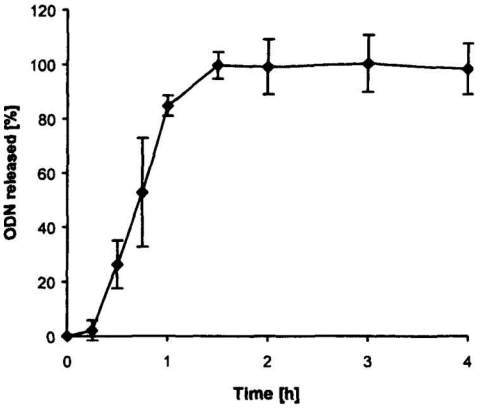

Fig. 4. Release profile of the antisense oligonucleotide. Tablets comprising poly(acrylic acid)-cysteine conjugate, glutathione and the ODN (48.5+1.5+1) were incubated in an artificial intestinal fluid at 37°C. Indicated values are means of three experiments ±SD.

During drug release studies no disintegration of the delivery system was observed within the first 2 hours of incubation in the release medium. Since the tablets can be easily enteric coated as described previously [10], a drug release in the small intestine can be guaranteed. The hydration and swelling of the enteric coating shall take place within an hour in the small intestine at a pH above 5.5. Taking this hydration/swelling process of the enteric coating and the small intestinal transit time for single unit dosage forms of 2.5-3 h into account [21], a sustained drug release for 1.5 hours seems to be appropriate for this novel oral antisense oligonucleotide delivery system.

In conclusion, a mucoadhesive oral delivery system for an antisense oligonucleotide was developed within this study. As the PS-ODN turned out to be very stable towards intestinal enzymes, an additional protection of the oligonucleotide towards these enzymes does not seem essential. Moreover, because of the comparatively high permeability coefficient of the PS-ODN, the addition of permeation enhancers to the delivery system might not be needed. The novel delivery system has the advantage of high mucoadhesive properties - likely providing a steep concentration gradient of the PS-ODN at the absorption membrane - and the capability of a controlled and sustained drug release. Because of these features it represents a promising novel tool for oral PS-ODN delivery.

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