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European Journal of Pharmaceutics and Biopharmaceutics 55 (2003) 19–26

European Journal of Pharmaceutics and Biopharmaceutics

www.elsevier.com/locate/ejphabio

Research paper

Stability of polycationic complexes of an antisense oligonucleotide in rat small intestine homogenates

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Received 19 February 2002; accepted in revised form 26 September 2002

Abstract

Presystemic degradation in the gastrointestinal tract is one of the major problems contributing to the poor oral absorption of antisense oligonucleotides. Complexes between the antisense phosphorothioate oligodeoxynucleotide ISIS 2302 and the polycationic carriers protamine sulfate grade X, protamine chloride grade V, protamine phosphate grade X, poly-L-lysine hydrobromide (PLL), spermidine phosphate salt, spermine diphosphate salt, and Protasan™ G113 and CL113 were formulated in order to increase stability against intestinal nucleolytic degradation. Specific conductivity measurements were carried out to determine the charge ratio of the complex systems. Nuclease stability assays were performed in a rat small intestine homogenate model, which displayed significant exo- and endonuclease activity. Full-length oligonucleotide and metabolites were analyzed by capillary gel electrophoresis with UV detection at 260 nm. Most of the complexes of ISIS 2302 and the polycationic materials, except PLL-based systems, showed a better protection against enzymatic metabolism than free oligonucleotide. Protamine sulfate and protamine chloride considerably enhanced the nuclease stability of the phosphorothioate antisense oligonucleotide. The association of oligonucleotides with several polycationic substances proved to be an alternative to chemical modification in order to stabilize oligonucleotides in the gastrointestinal tract against nucleolytic degradation.

Keywords: Antisense oligonucleotide; Polycationic carriers; Complex; Nuclease metabolism; Tissue homogenates; Capillary gel electrophoresis

1. Introduction

Phosphorothioate oligodeoxynucleotides are widely distributed in multiple species following systemic administration, with major sites of deposition including liver, kidney, spleen and lymph nodes [1–3]. Systemically administered phosphorothioates are currently in several clinical trials and the first antisense drug of that class was approved in 1998 by the FDA [4,5]. The oral delivery of these antisense drugs is, however, problematic. Even though phosphorothioates display greater stability against endogenous nucleases than their phosphodiester analogs, data from multiple ex vivo and in vitro models suggest that these compounds are extensively degraded by the nucleases in the lumen and within the intestinal epithelium, thus hampering oral bioavailability [2,3,6].

New generations of oligonucleotides have been chemically modified at various positions in order to improve hybridization efficiency and enhance resistance against nucleolytic degradation [7]. A promising new chemistry involves modifying the sugar moiety of the antisense oligonucleotides (2′-*O*-(2-methoxyethyl), 2-*O*-MOE). These 2-*O*-MOE modified oligonucleotides not only increase stability, but also display greater pharmacological activity in various systems than their phosphorothioate congeners [7,8].

Association of antisense oligonucleotides and/or DNA with cationic polymers represents an alternative to chemically modifying these drugs in order to enhance stability and, potentially, activity. Numerous studies have shown that complexation of polycations with these novel therapeutic agents improves the control of size, charge, hydrophilic–lipophilic characteristics, the targeting and the pharmacological activity of the transfecting species [9–16]. Polycationic carriers can enhance paracellular absorption of large molecules by tight junction regulation, interact with cell membrane components promoting endocytosis, break endo-

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doi:10.1016/S0939-6411(02)00140-6

somes by 'proton sponge' destabilization and consequently promote DNA delivery to the nucleus [17]. Another advantage of polycationic carriers is that the electrostatic interaction between oppositely charged species is a process which can be reversed under several conditions (i.e. increase in the ionic strength of the environment, pH-change, dilution). Therefore, reversible polycation-oligonucleotide complexation [18,19] may represent an opportunity to protect oligonucleotides against nucleolytic degradation in a reversible way with potentially little impact on RNA binding affinity [20].

In order to determine the impact of oligonucleotide complexation with cationic polymers on intestinal degradation, various polycationic substances were formulated with a 20-mer phosphorothioate oligodeoxynucleotide, ISIS 2302. The stability of free ISIS 2302 was compared to eight ISIS 2302 complexes in an ex vivo rat small intestine homogenate model using modifications of a procedure previously described for rat liver [21]. The major advantage of the model was that metabolism was characterized using the endogenous nucleases present within rat small intestine. Normally, nuclease activity has been studied in solutions of purified bacterial enzymes, nuclease S1, snake venom phosphodiesterase and exonuclease DNase I [18,22–25], which are experimental systems not consistent with the nucleolytic milieu found in the gastrointestinal lumen and epithelium.

2. Materials and methods

2.1. Materials

The polycationic substances were used as received: protamine sulfate grade X, protamine chloride grade V, protamine phosphate grade X, poly-L-lysine hydrobromide (PLL) with MW 9400, spermidine phosphate salt and spermine diphosphate salt (Sigma-Aldrich, Inc., St. Louis, MO), chitosan glutamate and chloride salts (Protasan™ G113 and CL113, Pronova Biomedical a.s, Oslo, Norway).

All chemicals used were reagent grade: ultra pure tris hydrochloride (Tris–HCl) and 1× phosphate-buffered saline (PBS) without calcium or magnesium (Gibco BRL Life Technologies, Inc., Gaithersburg, MD), magnesium acetate tetrahydrate, bis [2-hydroxyethyl] iminotris [hydroxymethyl] methane (bistris) (Sigma-Aldrich, Inc., St. Louis, MO), Bio Rad protein assay reagent (Bio Rad Laboratories, Hercules, CA), proteinase K solution, urea (Boehringer Mannheim GmbH, Germany), Nonidet® P40 (Calbiochem-Novabiochem Corp, La Jolla, CA), acrylamide, and boric acid (Fluka, Neu-Ulm, Switzerland). Ultra pure Milli-Q® water was used throughout the study.

ISIS 2302 (5′-GCC CAA GCT GGC ATC CGT CA-3′), a phosphorothioate oligodeoxynucleotide (PS-ODN) antisense inhibitor of human ICAM-1 (intercellular adhesion molecule) expression [26], ISIS 1049 (5′-GCC GAG GTC CAT GTC GTA CGC-3′), a 21-mer phosphodiester oligodeoxynucleotide (PO-ODN) and poly-thymidine oligonucleotide T27 were synthesized at Isis Pharmaceuticals, Inc. following the phosphoramidite method [27]. All oligonucleotides were reversed-phase HPLC-purified and shown to be greater than 85–90% full-length material by reversed phase HPLC and capillary gel electrophoresis (CGE) analysis.

Male Sprague–Dawley rats (200–300 g) were obtained from Harlan Sprague–Dawley (Madison, WI). The animals were housed in polycarbonate cages and had access to rat chow and water ad libitum in compliance with IUACCAC guidelines.

2.2. Complex formation

Aqueous stock solutions of each of the polycationic substances and the oligonucleotide ISIS 2302 were prepared with ultra pure Milli-Q® water. For the complex formation, 814 μl of the ISIS 2302 stock solution (0.125% (w/v)) was added to different volumes of polycationic stock solutions (0.025% (w/v)) under gentle stirring at room temperature. All systems had a final volume of 15 ml and a constant oligonucleotide concentration of 10 μM. The concentration of the polycationic substance in each of the final systems led to a charge ratio of 1.5 +/− for all formulated complexes, according to charge relationships obtained by conductometric titrations.

2.3. Conductometric titration

A 0.25% (w/v) stock solution of ISIS 2302 in deionized water was added to 45 ml of 0.025% (w/v) aqueous solutions of protamine sulfate grade X, protamine chloride grade V, protamine phosphate grade X, PLL, spermidine phosphate salt, spermine diphosphate salt, and Protasan™ G113 and CL113. Specific conductivity was registered after each titration step at room temperature with a microprocessor Conductivity Meter (LF 537, Wissenschaftlich-Technische Werkstätten, Weilheim, Germany) with a Tetra-Con 96 cell of 0.609 constant. The equivalence point of the titration, which was calculated from the specific conductivity vs. molar fraction of the ISIS 2302 curve, showed the molar ratio at which charge neutralization occurred [28]. All measurements were conducted in triplicate.

2.4. Preparation of rat small intestine homogenates

Fasted naive, male rats were sacrificed and their small intestines removed. Tissue was dissected into 1–2 mm pieces and transferred into 2 ml Fastprep tubes (Bio 101, Inc., Vista, CA) containing 1 ml of ice cold nuclease buffer (100 mM Tris–HCl, 1 mM magnesium acetate, pH 8.0) and 100 μl of Matrix Green lysing beads (Bio 101, Inc., Vista, CA). Homogenates were prepared by placing the tubes into a Bio 101 Fastprep Savant Tissue/Cell disruptor for 25 s at an energy setting of 4.5 [21]. After homogenization, the tubes were placed on ice and the homogenates were pooled

Table 1
Results calculated from conductometric titrations of polycationic carriers with ISIS 2302 (ODN): molar composition at the equivalence point, positive charge equivalents per mol of polycation, carrier capacity (g of ISIS 2302/g of polycationic carrier at the equivalence point) and the molar composition of complexes tested for nuclease stability (charge ratio 1.5 +/−)

Polycationic material	MW	Equivalence point (mol polycation/mol ODN)	Positive charge equivalents	Carrier capacity (g ODN/g polycation)	Complex 1.5 +/− (mol polycation/mol ODN)
Spermine diphosphate	398	6.874	2.76	2.479	10.655
Spermidine phosphate	693	4.405	4.31	2.221	6.710
Protamine sulfate X	4800	1.083	17.58	1.304	1.693
Protamine chloride V	4800	0.818	23.22	1.727	1.281
Protamine phosphate X	4800	1.227	15.48	1.151	1.925
PLL	9400	0.408	46.52	1.768	0.608
Protasan™ CL113	50,000	0.086	221.51	1.577	0.129
Protasan™ G113	80,000	0.068	277.88	1.247	0.103

and passed through a 70 μm mesh nylon cell strainer. The protein concentration was determined using a Bio Rad protein assay kit [29].

2.5. Nuclease stability assays

Solutions of free oligonucleotides ISIS 2302 and ISIS 1049, and complex systems of ISIS 2302 with the polycationic substances were tested for nuclease stability. Small intestine homogenate (450 μl) was added to sterile 2 ml microfuge tubes and placed on ice. Then 50 μl of test systems were added to the tubes resulting in final concentrations of 50 μg/ml protein and 1 μM of either free or complexed oligonucleotide. Reactions were initiated by placing the tubes in a Brunswick gyratory water bath shaker (New Brunswick Scientific Company, Inc., Edison, NJ) at 37 °C. At the end of various incubation times, reactions were terminated by placing the tubes on ice and adding 62.5 μl proteinase K to a final concentration of 100 μg/ml, and 100 μl of a 5× stop buffer solution containing 5% Nonidet® P40, 1.0 M NaCl, 200 mM EDTA and 200 mM Tris–HCl (pH 8.0) [21]. Assays were carried out in triplicate and repeated in a second experiment ($n = 6$).

2.6. Solid phase extraction

Two different solid phase extraction (SPE) columns were required to purify samples prior to CGE analysis. Internal standard (homopolymer T 27-mer phosphorothioate oligodeoxynucleotide) was added to each homogenate sample (100 nM). The first purification step was performed using strong anion exchange columns (Isolute® SAX, 3 ml reservoir, International Sorbent Technology Ltd., Mid-Glamorgan, UK), with running buffer containing 10 mM Tris–HCl, 0.25 M KCl and 20% (v/v) acetonitrile followed by elution buffer with 10 mM Tris–HCl, 0.5 M KCl, 1 M NaBr and 30% (v/v) acetonitrile. The rich anionic environment promoted the dissociation of the oligonucleotide complexes. The second purification step was carried out with non-polar reverse phase columns (Isolute® C18(EC) 100 mg, 10 ml reservoir, International Sorbent Technology Ltd., Mid-Glamorgan, UK), with running buffer consisting of 10 mM Tris–HCl, 0.5 M KCl and 1 M NaBr. Analytes were finally eluted with a 22% (v/v) acetonitrile aqueous solution, transferred to 2 ml microfuge tubes and dried under vacuum (Speed Vac® Plus, SC110A, Savant Instruments, Inc., Farmingdale, NY).

2.7. Capillary gel electrophoretic sample analysis

Samples were resuspended in 40 μl deionized water and floated on 0.025 μm dialysis membranes (Millipore, Bedford, MA) over 10 ml deionized water for 30 min to reduce the concentration of competitive anions that would interfere with the electrophoresis. Samples placed into microvials were analyzed with Beckman P/ACE System Gold 5000 capillary electrophoresis equipment (Fullerton, CA) with UV detection at 260 nm. Capillary columns (Polymicro Technologies, Inc., Phoenix, AZ) were filled with 10% polymerized acrylamide in buffer containing 200 mM bistris, 200 mM boric acid and 8 M urea. Electrophoretic buffer consisted of 200 mM bistris and 200 mM boric acid. Samples were electrokinetically applied using 3–5 kV for 3–5 s, while separations were achieved at a constant voltage of 20 kV at 50 °C in approximately 5 min. Full-length oligonucleotide and metabolites were quantified by comparing sample peak areas relative to the T27 internal standard [30].

3. Results and discussion

3.1. Conductometric titration

The complex formation of ISIS 2302 phosphorothioate with various polycationic substances occurred spontaneously in an aqueous environment. Table 1 shows data obtained from specific conductivity measurements, which defined the molar proportions for neutralization of complexes of ISIS 2302 with spermine diphosphate, sper-

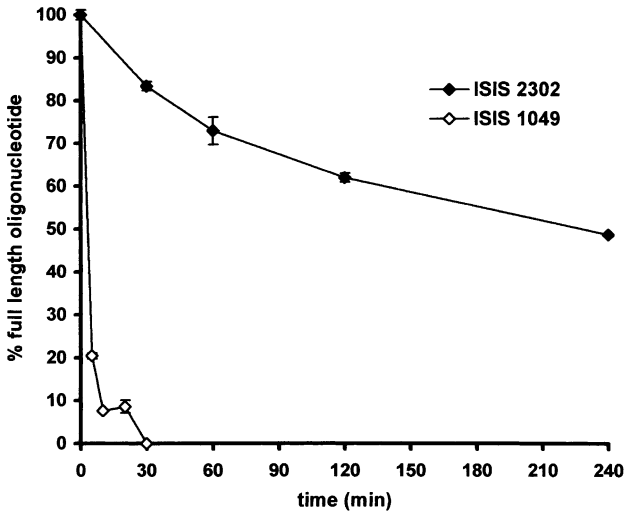

Fig. 1. Stability of 1 μM ISIS 1049 phosphodiester and 1 μM ISIS 2302 phosphorothioate in a fasted rat small intestine homogenate model (50 μg/ml) at 37 °C.

midine phosphate, protamine sulfate, protamine chloride, protamine phosphate, PLL, and Protasan™ CL113 and G113. In a previous study, the validity of this method has been described for PLL-based complexes which displayed good correlation between experimental results and theoretical prevision [19]. It was possible to calculate the relation of the charge ratio (ratio between positive charge equivalents of the polycation and negative charge equivalents of the oligonucleotide) [31] against the molar composition of the complex. Based on conductometric data, all complex systems were formulated at a charge ratio of 1.5 +/− for further nuclease metabolism assays. The carrier capacity, expressed as the amount (in g) of oligonucleotide complexed by 1 g of polycationic material at the equivalence point, showed the following rank order: spermine diphosphate > spermidine phosphate > PLL > protamine chloride > Protasan™ CL113 > protamine sulfate > Protasan™ G113 > protamine phosphate.

3.2. Characterization of nuclease activity in the rat small intestine homogenate model

The nuclease stability of non-complexed and complexed oligonucleotides was studied with an ex vivo fasted small intestine homogenate model methodologically being based on the previously reported ex vivo liver homogenate system [21]. The final assay conditions set for the liver homogenate model mimicked the amount of drug found in rodent tissues from in vivo distribution experiments following systemic administration [2,32].

ISIS 1049, a 21-mer phosphodiester antisense oligodeoxynucleotide, was used as model compound to determine the baseline nuclease activity in the tissue homogenate. ISIS 1049, which exhibits similar chemistry as endogenous nucleic acids, represents a natural substrate for nucleases

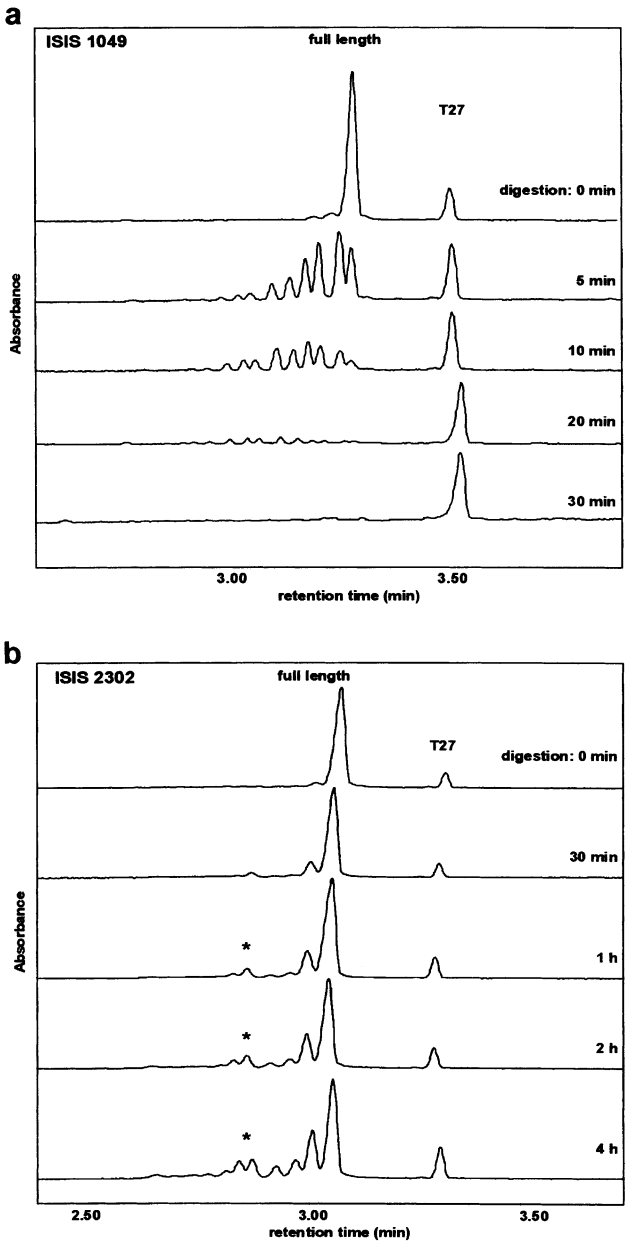

Fig. 2. Electropherograms generated from 50 μg/ml fasted rat small intestine homogenates incubated at 37 °C with (a) 1 μM ISIS 1049 for 30 min and (b) 1 μM ISIS 2302 for 4 h. Internal standard homopolymer polythymidine 27-mer (T27) phosphorothioate oligodeoxynucleotide, * endonucleolytic activity.

found in small intestine homogenate. Fasted rat small intestine homogenates displayed significant nuclease activity. The time to degrade the oligonucleotide ISIS 1049 by 50% ($t_{50\%}$) was 3 min. Within 30 min, all full-length material was degraded (Fig. 1). Electropherograms at different incubation times showed the profile and progression of the metabolism, where peaks migrating earlier than full-length oligonucleotide corresponded to consecutive metabolites n-1 shortmers (Fig. 2a). This processive laddering of smaller length products indicated that the principal enzymatic activity under these conditions was exonucleolytic [21].

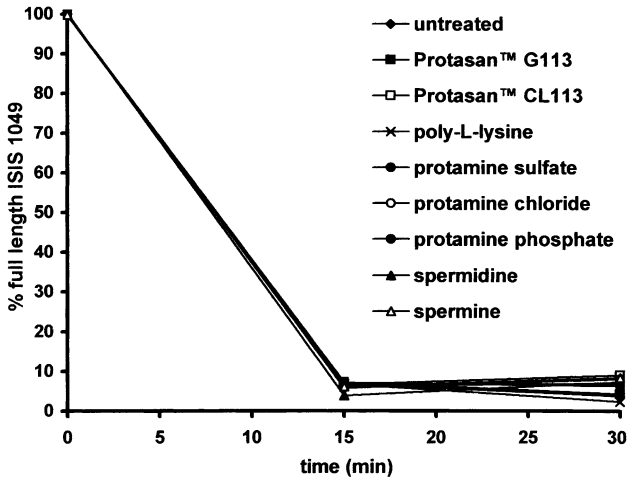

Fig. 3. Stability of 1 μM ISIS 1049 in a fasted rat small intestine homogenate model (50 μg/ml) after 1.5 h pretreatment of the homogenates with several polycationic substances.

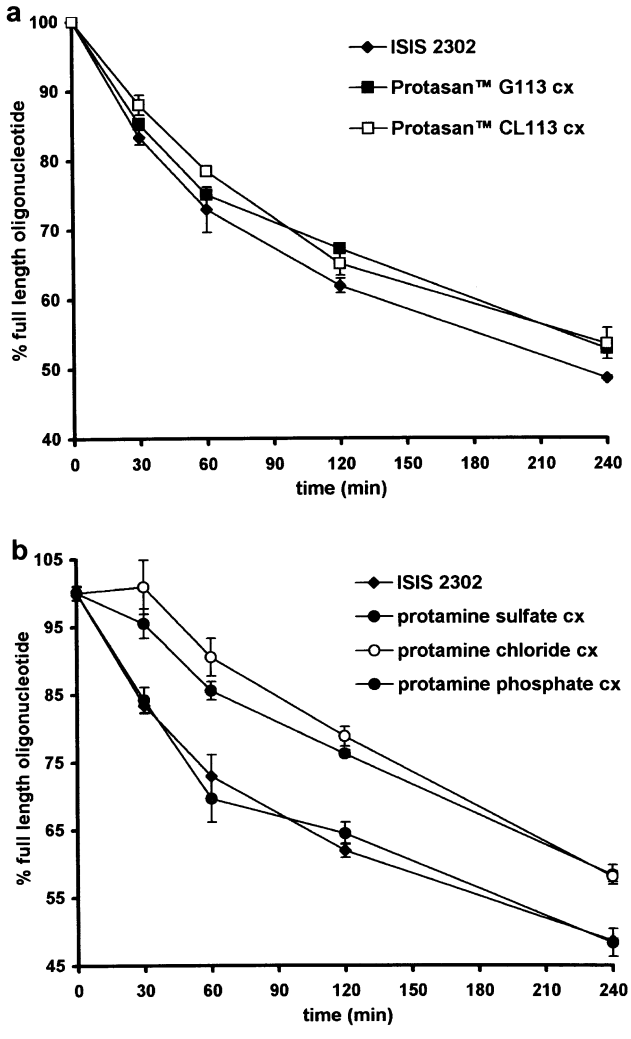

Fig. 4. Comparison of the nuclease stability of 1 μM ISIS 2302 in a fasted rat small intestine homogenate model at 37 °C over 4 h, in free form and complexed at a 1.5 +/− charge ratio with (a) Protasan™ CL113 and G113 and (b) protamine chloride, protamine sulfate and protamine phosphate salts.

The degradation of ISIS 2302, a 20-mer phosphorothioate, was studied under the same experimental conditions. The rate of metabolism was reduced when compared to the phosphodiester oligonucleotide ISIS 1049, with approximately 50% of full-length product remaining after 4 h of incubation (Fig. 1). Other studies have shown that minimal degradation occurs in liver [21] and intestinal homogenates between 4 and 8 h (data not shown). The enhanced resistance to nucleolytic degradation of phosphorothioates compared to their phosphodiester analogs under several experimental conditions has been widely reported [21,23]. Therefore, a reaction time of 4 h was enough to characterize the enzymatic activity on ISIS 2302.

Like ISIS 1049, CGE analysis of experimental tissue homogenates (Fig. 2b) demonstrated that ISIS 2302 was also degraded by exonucleases, as shown by the mainly laddered nature of the metabolite profile. However, the data also indicated the presence of endonucleolytic degradation as shown by the smaller metabolite peaks appearing at approximately 2.8 min that increased over time. Nonetheless, the principal enzymatic activity appears to be exonucleolytic.

3.3. Effect of the pretreatment with polycationic carriers on nuclease activity

The small intestine homogenates were pretreated with each of the polycationic materials used for complex formation in order to determine effects on background nuclease activity (Fig. 3). Test polycationic material was added to the homogenate in the same proportion as required to form an oligonucleotide complex of 1.5 +/− charge ratio. Tissue homogenates were pretreated with the polycationic substances for 1.5 h at 37 °C in a shaker-bath. Afterwards, the model oligonucleotide ISIS 1049 was added to the system and stability over a 30 min period was monitored as described above. The metabolism of ISIS 1049 in untreated and pretreated small intestine homogenates was essentially the same suggesting that the polycationic substances did not inactivate endogenous enzymes under the reported assay conditions.

3.4. Metabolism of complexes of ISIS 2302 with several polycationic materials in rat small intestine tissue homogenates

The metabolism of several complexes of ISIS 2302 with polycationic carriers that might potentially enhance gastrointestinal stability was studied over a 4 h period. The durability of the enzymatic activity in the rat small intestine homogenate was investigated by comparing nuclease activity in freshly prepared homogenate to that incubated in a 37 °C water bath for 4 h. The pattern of degradation of ISIS 1049 was identical in homogenates used immediately after preparation or after 4 h of incubation at 37 °C, demonstrating no loss of enzymatic activity (data not shown). Additionally, homogenates from several animals and different

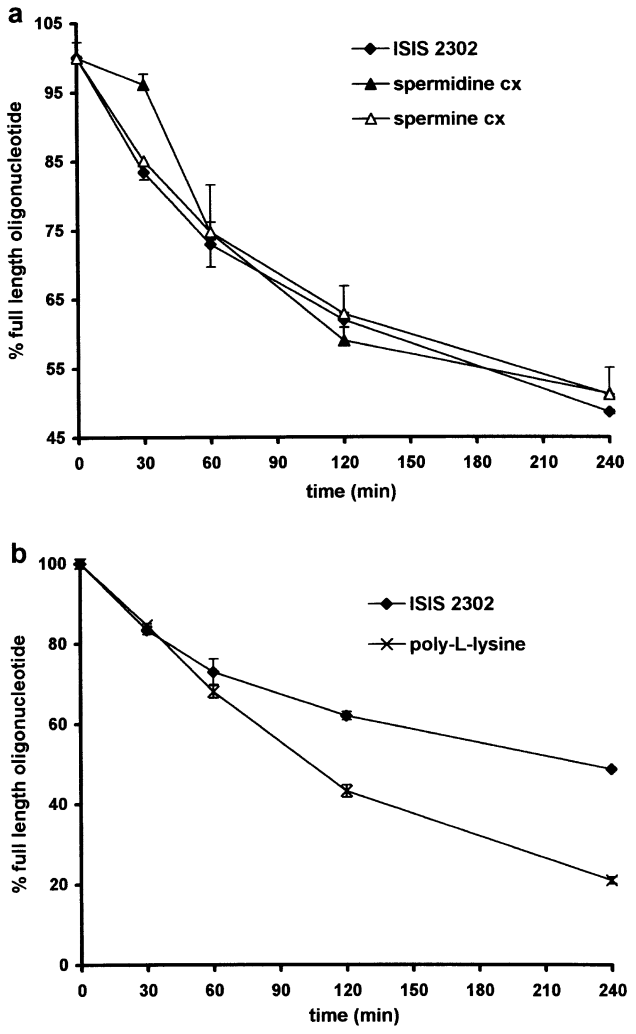

Fig. 5. Comparison of the nuclease stability of 1 μM ISIS 2302 in a fasted rat small intestine homogenate model at 37 °C over 4 h, in free form and complexed at a 1.5 +/− charge ratio with (a) spermidine phosphate and spermine diphosphate and (b) poly-L-lysine.

Table 2
Efficiency of protection displayed by complexes of ISIS 2302 with several polycationic materials after 30 min and 4 h of nuclease stability assay[a]

Polycationic carrier	% efficiency of protection	
	After 30 min	After 4 h
Spermine diphosphate	3.45 (±0.86)	5.12 (±0.02)
Spermidine phosphate	26.15 (±3.25)	5.45 (±2.04)
Protamine sulfate X	28.03 (±1.05)	19.98 (±2.96)
Protamine chloride V	34.64 (±3.07)	19.41 (±1.65)
Protamine phosphate X	2.29 (±3.17)	− 0.60 (±3.29)
PLL	2.47 (±0.92)	− 56.67 (±1.72)
Protasan™ CL113	10.22 (±2.54)	10.10 (±2.63)
Protasan™ G113	3.92 (±0.67)	8.60 (±0.89)

[a] Negative efficiency of protection values represented a negative effect on ISIS 2302 nuclease stability. Values given are the mean and standard deviation from results generated in two separate experiments carried out in triplicate.

experiments performed within several months displayed little variability (data not shown).

Complexes of ISIS 2302 with high molecular weight polycationic carriers, chitosan Protasan™ G113 and CL113, were prepared at a charge ratio of 1.5 +/− as described above. Aliquots of the formulations were added to small intestine homogenates at optimized assay conditions [21]. Complexes with both chitosan salts only slightly improved the oligonucleotide stability (Fig. 4a). During the first hour of incubation, the stability of ISIS 2302 complexed with Protasan™ CL113 was greater than the non-complexed oligonucleotide. At 60 min, the efficiency of protection was about 10% for Protasan™ CL113 complex and 4% for Protasan™ G113 complex. After 4 h of incubation, the efficiency of protection was similar, 10% and 9%, for CL113 and G113 complexes, respectively. (The efficiency of protection (EP) is: % EP = (% FL in complex system − % FL in control)$_{\text{time } x}$ × (% FL start assay/% FL drained assay)$_{\text{control}}$ where FL was the % of full-length product left. The control values were obtained from the degradation of free ISIS 2302. The results were generated from two experiments conducted in triplicate, $n = 6$).

The performance of complexes of ISIS 2302 with several protamine salts was investigated in the same fashion (Fig. 4b). Complex formation with protamine chloride and sulfate considerably improved the nuclease stability of ISIS 2302, displaying an efficiency of protection around 20% at the end of the assay. On the other hand, complexation with protamine phosphate had no impact on oligonucleotide metabolism.

Complexes of ISIS 2302 with small polyamines were also studied (Fig. 5a). At early incubation times, spermidine phosphate salt protected ISIS 2302 from nucleolytic degradation with an efficiency of 26%, but later it displayed just a slight improvement in stability. Spermine diphosphate-based complexes had no effect on the metabolism of ISIS 2302.

Poly-L-lysine-based complexes in this system displayed a behavior contrary to the expected protective effect (Fig. 5b). The extent of degradation of the phosphorothioate oligonucleotide was increased and by the end of the assay only 21% full-length ISIS 2302 was detected. The reasons for this increase in enzymatic activity are unknown and experiments will be performed to fully address this phenomenon. However, the enhancement of enzymatic activity by poly-L-lysine might be attributed to a potential effect on the nucleases themselves. Possibly during incubation the poly-L-lysine dissociated from ISIS 2302 [19] and the free polycation interacted with the natural nuclease inhibitors found in the small intestine, thereby causing an increase in nuclease activity.

The efficiency of protection of the different complexes of ISIS 2302 with the reported polycationic carriers is summarized in Table 2. At 30 min, the polycationic materials, which displayed a good protection were: protamine chloride > protamine sulfate > spermidine phosphate > Protasan™

Table 3
Kinetics of metabolism of free and complexed ISIS 2302 with different polycationic carriers in a fasted rat small intestine homogenate model: half lives ($t_{50\%}$), exponential regressions and correlation coefficients (R^2) of studied systems

Systems	$t_{50\%}$ (min)	Equation	R^2
		y = % full-length, x = time	
Free ISIS 2302	217.50	$y = 91.93\ e^{-0.0028x}$	0.9477
Spermine diphosphate/ISIS 2302	228.06	$y = 92.55\ e^{-0.0027x}$	0.9429
Spermidine phosphate/ISIS 2302	223.97	$y = 95.73\ e^{-0.0029x}$	0.8854
Protamine sulfate X/ISIS 2302	301.85	$y = 100.11\ e^{-0.0023x}$	0.9950
Protamine chloride V/ISIS 2302	305.87	$y = 104.18\ e^{-0.0024x}$	0.9837
Protamine phosphate X/ISIS 2302	216.31	$y = 91.63\ e^{-0.0028x}$	0.9333
PLL/ISIS 2302	105.43	$y = 100.27\ e^{-0.0066x}$	0.9975
Protasan™ CL113/ISIS 2302	254.88	$y = 94.56\ e^{-0.0025x}$	0.9561
Protasan™ G113/ISIS 2302	248.25	$y = 93.00\ e^{-0.0025x}$	0.9515

CL113. At the end of the assay, the rank order was as follows: protamine sulfate > protamine chloride > Protasan™ CL113 > Protasan™ G113.

The half life ($t_{50\%}$) of free and complexed ISIS 2302 was determined by fitting each of the experimental metabolism curves to an exponential regression. Uncomplexed ISIS 2302 showed a $t_{50\%}$ of 217.5 min. This value was improved in all of the reported systems except for the poly-L-lysine-based complex (Table 3). The rank order of polycations which extended the $t_{50\%}$ of ISIS 2302 was: protamine chloride > protamine sulfate > Protasan™ CL113 > Protasan™ G113 > spermine diphosphate > spermidine phosphate. An improvement of the half life predicts a larger fraction of intact ISIS 2302 at the site of intestinal absorption.

Complexation of oligonucleotides to polycations may increase stability against nucleases by potentially forming a steric barrier to enzymes themselves and making oligonucleotide complexes less appropriate as substrates for the endo- and exonucleases found in the small intestine. These data are consistent with experiments investigating the enzymatic degradation of DNA-polyplexes demonstrating that the inactivation of DNase I was attributed to an alteration of the access of the DNA to the appropriate enzymes [16,18,25]. Some in vitro studies with endonuclease DNase I also reported protection from enzyme activity for oligonucleotides loaded onto cationic aminoalkylmethacrylate nanoparticles [24].

The dilution effect introduced by assay conditions (1:10 dilution) in the ex vivo fasted small intestine model could also have had an impact on the dissociation behavior of the complexes. Therefore, the promising results here described were obtained under more extreme characteristics than in other classically used enzymatic models. This study confirmed the potential of complex formation for the oral delivery of antisense oligonucleotides.

4. Conclusions

The oral delivery of phosphorothioate oligonucleotides is problematic due to a variety of factors, including the instability of these compounds in the gastrointestinal tract, as well as the poor permeation characteristics of these drugs [6]. Complex formation with cationic carriers could potentially overcome some of the problems affecting the nuclease stability of oligonucleotides. In this study, a phosphorothioate oligonucleotide, ISIS 2302, has been successfully complexed with different polycations, including chitosan salts, protamine salts, synthetic poly-L-lysine and small polyamines, by spontaneous electrostatic association in aqueous medium. The oligonucleotide formulations were incubated with small intestine homogenates prepared from fasted rats, an experimental situation that mimicked the in vivo small intestine enzymatic milieu. The performances of complexes based on protamine chloride, protamine sulfate, chitosan salts and spermidine were promising [33–35], as they increased the stability of ISIS 2302 against enzymatic degradation. Oligonucleotide complexation could be an interesting tool for the pharmaceutical development of orally available antisense therapeutics.

Acknowledgements

The authors thank P. Flores Méndez, Dr E. Viroonchatapan and M.J. Graham of Isis Pharmaceuticals, Inc. for their valuable contribution.

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