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Study of phosphorothioate-modified oligonucleotide resistance to 3′-exonuclease using capillary electrophoresis

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Abstract

The effect of phosphorothioate (PS) internucleotide linkages on the stability of phosphodiester oligodeoxyribonucleotides (ODNs) was investigated using 25-mer ODNs containing single or multiple PS backbone modifications. The in vitro stability of the oligomers was measured both in 3′-exonuclease solution and in plasma. For the separation of ODNs, capillary electrophoresis with a replaceable polymer separation matrix was used. As expected, DNA fragments with PS linkages at the 3′-end were found to be more resistant to 3′-exonuclease hydrolysis. Also increasing exonuclease resistance was the non-specific adsorption of phosphorothioate ODNs to enzyme.

Keywords: Phosphorothioate; Oligonucleotides; 3′-Exonuclease

1. Introduction

The concept of antisense oligonucleotide (AON) drugs is based on specific binding to a target molecule (e.g., mRNA, DNA) resulting in the suppression of protein biosynthesis [1,2]. Along with strict hybridization specificity, the biostability of AONs is crucial for successful therapeutic use. The mechanism of antisense action was described and demonstrated by Zamecnik and Stephenson [3]. Zamecnik et al. [4] also reported an antisense approach to inhibit de novo infection by HIV-1 using an unmodified oligodeoxyribonucleotide (ODN). Unmodified ODNs are not stable in vivo; thus, their therapeutic potential is limited. To increase the biostability of AONs, a variety of ODN analogs have been proposed including methylphosphonate [5,6], phosphoramidate [7,8], 2′-*O*-methyl- [9], 2′-*O*-propyl- [10], phosphotriester [11,12] and phosphorothioate [13,14] ODNs. All of these derivatives were shown to possess an increased resistance towards nucleases. A strategic combination of several modifications can also be used to enhance AONs nuclease resistance and improve their pharmacological/toxicological properties [8].

Even though the phosphorothioate (PS) ODN modification is one of the most conservative, the chemical properties of a phosphorothioate molecule are different from its phosphodiester counterpart. For example, a phosphorothioate has a lower pK_a value than a phosphodiester [15] because of differences in charge delocalization. PS-ODN retention behavior on reversed-phase HPLC [16] suggests that they are also more hydrophobic and therefore may exhibit more complex secondary structure. The substitution of a single oxygen to sulfur also creates a chiral center on

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the phosphate. Although PS-ODNs have entered advanced phases of clinical trials [17,18], the impact of phosphorothioate backbone stereogenicity on stability, target binding, and pharmacological properties is still subject of studies [19–21].

Several papers have been published studying in vitro [14] and in vivo [22,23] stability and pharmacokinetics of PS-ODNs. Most of these studies were based on polyacrylamide slab gel electrophoresis using radioisotope labeled ^{32}P, ^{35}S or ^{14}C phosphorothioate oligonucleotides. The ability of this method to separate, identify and quantify the parent oligonucleotide and its metabolites was insufficient and at times very poor. The use of total sample radioactivity counts sums not only parent drug but also shorter metabolites and even free isotopes.

The previously mentioned physico-chemical properties of PS-ODNs make their separation difficult. Consequently, few reports of the successful application of polyacrylamide [14,24] or other polymer matrices [25,26] for capillary electrophoresis (CE) are found in the literature. Applying some of the replaceable polymer solutions designed for the CE separation of ODNs [27,28] did not give us satisfactory results for chemically modified ODNs. Even though the separation selectivity was comparable to ODNs, poor resolution of PS-ODNs was obtained due to extensive peak broadening. Similar peak broadening observed in HPLC leads us speculate that this CE behavior can be explained as a partial separation of PS-ODNs diastereoisomers.

The goal of this work is to study the mechanism of phosphorothioate resistance to 3′-exonuclease hydrolysis. Our investigation focuses on 25-mer oligonucleotides complementary to the gag mRNA region of HIV-1. The influence of single or multiple phosphorothioate modifications on the stability of the 25-mer parent oligomer is studied. Human plasma and 3′-exonuclease solution (bovine mucosa Phosphodiesterase I) were chosen as the reaction media.

Using Bio–Rad's Dynamic Sieving Polymer, we designed a sieving matrix for screening PS-ODN digestion in 3′-exonuclease solution by capillary electrophoresis with UV absorbance detection (CE–UV). Employing an easily replaceable polymer solution as the separation matrix made the data collection amenable to automation.

2. Experimental

2.1. Chemical and reagents

Water and methanol (MeOH) were HPLC grade (JT Baker, Inc., Phillipsburg, NJ, USA). Human plasma/serum (male) was obtained from Sigma Chemical (St. Louis, MO, USA). Uniflo® 13 mm diameter 0.2 μm cellulose triacetate disposable syringe filters were obtained from Schleicher and Schuell (Keene, NH, USA). Oligomer HPLC purification was performed on Nucleopak PA-100, 250×9 mm column (Dionex, Sunnyvale, CA, USA) and polystyrene–divinylbenzene PRP-3, 150×4.1 mm I.D. column (Hamilton, Reno, NV, USA). Lithium bromide and other chemicals were obtained from Fluka Chemical Corp. (Ronkonkoma, NY, USA). Deionized formamide was purchased from American Bioanalytical (Natick, MA, USA). Phosphodiesterase I, a 3′-exonuclease enzyme isolated from bovine intestinal mucosa, was obtained from Sigma (St. Louis, MO, USA).

The ODN, PS-ODN, and mixed ODN/PS-ODNs were synthesized in our lab with an Expedite™ 8909 synthesizer (PerSeptive Biosystems, Framingham, MA, USA), deprotected, purified and reconstituted in deionized water. Table 1 lists the oligomers used in this study.

2.2. Sample preparation and reaction conditions

2.2.1. Human plasma

Plasma samples containing oligomer were left to react at 37°C. The reaction was stopped by freezing. The samples were purified from plasma by solid-phase extraction. A more detailed protocol is presented elsewhere [14].

2.2.2. 3′-exonuclease solution

To evaluate the effect of PS modifications on the stability of ODNs, we used a 3′-exonuclease solution which mimics very well the 3′-exonuclease activity of human plasma [26]. The enzyme solution was prepared to give a rate of 3′-end ODN hydrolysis similar to the observed rate in vitro in human plasma. Phosphodiesterase I (from bovine intestinal mucosa) stock solution contained 25 m*M* Tris-boric

Table 1
Oligomers used in this study

Oligomer	
1	no PS 5′-CTC TCG CAC CCA TCT CTC TCC TTC T-3′
2	PS at position 20 (between bases 19 and 20 from 5′-end) 5-CTC TCG CAC CCA TCT CTC TsCC TTC T-3
3	PS at positions 20 and 23 5-CTC TCG CAC CCA TCT CTC TsCC TsTC T-3
4	PS at position 25 5′-CTC TCG CAC CCA TCT CTC TCC TTCs T-3′
5	9 PS groups from 5′-end 5′-CsTsCs TsCsGs CsAsCs CCA TCT CTC TCC TTC T-3′
6	all PS 5′-CsTsCs TsCsGs CsAsCs CsCsAs TsCsTs CsTsCs TsCsCs TsTsCs T-3′

All molecules are 25-mers with the same sequence, s=phosphorothioate modification

acid buffer (TBA) pH 7.4 and 8.33 n*M* (0.001 unit $(ml)^{-1}$) of enzyme. Diluting the enzyme in 25 m*M* TBA buffer pH 7.4 kept reaction conditions constant. The same concentration of oligomer (1.3 μ*M*) as in the plasma reaction was used.

2.3. Capillary electrophoresis

Automated CE–UV assay: As the CE–UV system, a BioFocus® 2000 Capillary Electrophoresis System (Bio–Rad, Hercules, CA, USA) was used. Samples were injected electrokinetically for 8 s at 13 kV and run at 15 kV. The composition of replaceable polymer solution was designed for PS-ODN separation as follows: To 2.2 g of Bio–Rad Dynamic Sieving Polymer was added deionized formamide (4.8 ml), 1.2 ml of 10× TBA pH 9 (1*M* Tris, 0.3 *M* boric acid), and 1.2 ml of 8.3*M* urea solution in 2× TBA buffer (0.2 *M* Tris, 0.06 *M* boric acid). The separation matrix was stable for a week at 4°C. A high concentration of polymer and formamide in the matrix were essential for the successful separation of oligonucleotides containing multiple PS modifications. The CE column was liquid thermostated. An elevated temperature (35°C) significantly reduces the separation run time and slightly improves peak efficiency. For analysis a 25 cm (20 cm to detection window) coated capillary, 75 μm I.D.×375 μm O.D. (BioCap oligonucleotide from Bio–Rad) was used. Filling of the capillary with polymer solution was automated by applying a high pressure nitrogen pulse for 220 s. After each run the capillary was washed with HPLC water. To facilitate automation, reactions were run in the instrument, and samples were injected at set intervals. Peak shape and resolution were not influenced by the enzyme concentrations that were used.

3. Results and discussion

To study the stability of isolated PS modifications on the ODN backbone, we evaluated several oligomers with single or multiple PS substitutions. An unmodified ODN (Oligomer 1) was used as a control. As expected, we observed rapid digestion of this compound in 3′-exonuclease solution (Fig. 1). If we modify this molecule to include a single PS group (e.g., Oligomer 2), we would expect fragments produced by the exonuclease activity that possess a terminal PS internucleotide to be more resistant to degradation. As seen in Fig. 2, the accumulation of the 20-mer fragment indicates slower degradation of this fragment than other fragments including the parent oligomer which has a terminal internucleotide phosphodiester group.

Similarly, modification of the 25-mer ODN at positions 20 and 23 (Oligomer 3) results in more

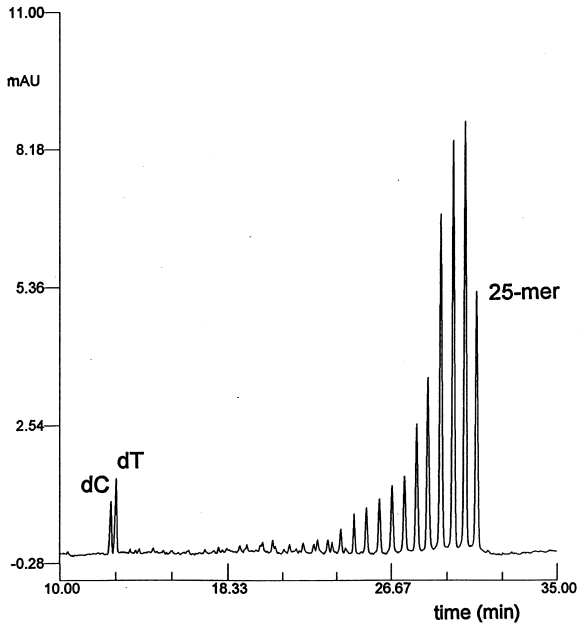

Fig. 1. Separation of ODN (Oligomer 1) metabolites of 3′-enzymatic hydrolysis. Reaction time was 55 min. Peaks dC and dT (12.55 and 12.81 min) correlate with the 5′-mononucleotides, deoxycytidine and deoxythymidine 5′-monophosphate nucleotides, respectively. Migration order was established by spiking the solution with the appropriate mononucleotides. For conditions see the experimental section.

stable 20- and 23-mer fragments. As expected from the previous measurement, the increase of both corresponding peaks (20- and 23-mer) was observed (Fig. 3). The lower susceptibility of the 20- and 23-mer fragments to enzymatic digestion becomes more apparent at longer times. The baseline separation of parent oligomer and shorter metabolites seen in the figures was obtained using a replaceable polymer matrix. This separation is comparable to that obtained by CE with a non-replaceable linear polyacrylamide matrix [14].

Even though fragments with the PS group at the 3′-end are more resistant to 3′-exonuclease cleavage, one or two PS linkages in the backbone of a 25-mer ODN does not significantly influence the stability of other unprotected fragments or parent oligomer (Table 2). Based on these metabolite patterns, the logical way to protect an oligomer is to modify the 3′-terminus with an internucleotide PS linkage (Oligomer 4). The stability of Oligomer 4 was surprisingly high (Table 2), and in contrast to the digestion of Oligomers 2 and 3, only parent compound was prominently present. After cleavage of the protecting internucleotide linkage, subsequent digestion was so quick that *N*-1 and *N*-2 fragments were noticeable only as minor peaks. Even though at least two modified internucleotide linkages are usually attached to the 3′-end for protection against 3′-exonucleases, in our case, the very good stability of Oligomer 4 suggests that protection of the 3′-end of oligomers by a single PS internucleotide linkage is also a reasonable choice.

To investigate the influence of multiple PS modifications on ODN stability, an oligomer was synthesized containing nine PS groups at the 5′-end (Oligomer 5). Phosphodiester groups occupied the remainder of internucleotide linkages affording no protection against exonuclease cleavage at the 3′-end. Surprisingly, the enzymatic hydrolysis of this compound was about six times slower than Oligomer 1 (see Table 2). This finding may be explained by greater adsorption of Oligomer 5 to the enzyme compared to Oligomer 1. Most likely, the greater affinity of the PS part of the molecule and thus the slower dissociation of Oligomer 5 from the enzyme affects the kinetics of hydrolysis.

To test this hypothesis we designed the following experiment: Equimolar concentrations of Oligomer 1 (ODN, no PS) and Oligomer 6 (all PS) were prepared. The 3′-exonuclease solution was spiked with this substrate (0.625 μM of each oligomer) to give a total oligomer concentration of 1.3 μM (equivalent to previous experiments); other reaction conditions were kept constant. The reaction half-life of ODN (Oligomer 1) in the presence of PS-ODN was 15.5 times longer than in the absence of PS-ODN (see Table 2). This finding can be explained by a competition of both oligomers for the enzyme. Since the molar concentration of the enzyme was 154 times lower than the total concentration of oligomer substrate, we speculate that the PS-ODN can effectively block the enzyme and limit the rate of ODN hydrolysis (Fig. 4). This measurement is consistent with literature reports of non-specific adsorption of PS-ODNs to proteins [29,30]. The estimation of the dissociation constant K_d by methods such as mobility shift assay and fluorescence polarization, however, was found to be extremely

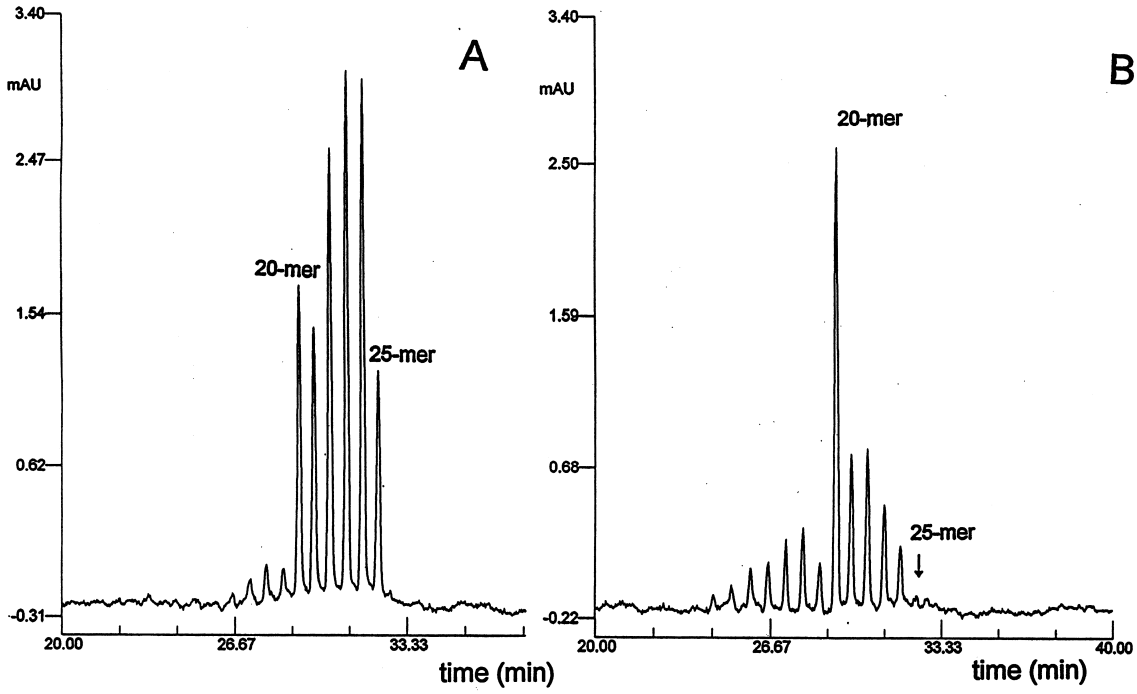

Fig. 2. Digestion of Oligomer 2 in 3′-exonuclease solution. Phosphorothioate modification at position 20 results in an accumulation of the 20-mer peak after (A) 65 min of reaction and (B) 165 min of reaction. For conditions see Section 2.

difficult due to extensive oligomer digestion at higher exonuclease concentration.

The metabolism of Oligomers 1–5 was also studied in human plasma, the data from which were consistent with measurements in the 3′-exonuclease solution. The plasma medium gave us a faster rate of degradation, but no difference was found in the digestion pattern.

The digestion kinetics of an all PS-ODN (oligomer 6) has been previously described [31] as a sum of two parallel processes: a slow and quicker phase of hydrolysis. This behavior was observed in vitro in 3′-exonuclease solution, as well as in plasma. It has been shown that this digestion is related to the stereoselective cleavage of PS internucleotide linkages. Consistent with literature [20,21,32] the *S*-configuration of 3′-end linkages is highly resistant, while the *R*-configuration is more susceptible to hydrolysis. Both half-lives of digestion are shown in Table 2 for the in vitro in plasma reaction; in the case of 3′-exonuclease digestion, only the rate of the 25-mer all-PS-ODN with the *R*-configuration was calculated (the slope from only the first two h of reaction was used in the half-life calculation). It was also found that stereoselective cleavage of the 3′-end of oligomers results in a characteristic right triangle shaped profile for all-PS-ODN metabolites [31].

Digesting Oligomer 4 we had similar expectations, namely, a two exponential kinetics of terminal PS linkage digestion; however, the ln plot of 25-mer concentration versus time was found to be linear over 0–72 h. Our experiments don't give us a clear explanation for this observation.

The experiments described above demonstrate that the PS modification serves as a protection of the oligomer backbone. It agrees with the usual strategy that the most effective modification is to protect the 3′-end of the oligomer. For example, an oligomer with a single PS modification at the 3′-end (Oligomer 4) was found to be approximately 44 timers more stable in 3′-exonuclease solution than an oligomer having the same sequence but without the modification. However, a single PS group doesn't protect the oligomer with the same efficiency as does

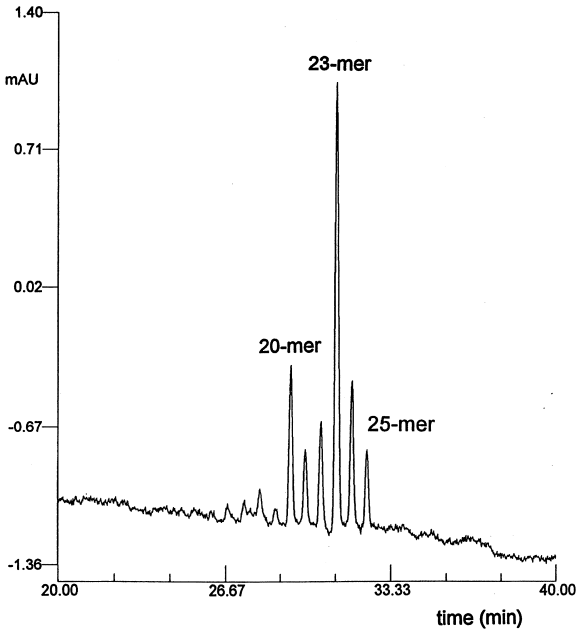

Fig. 3. Digestion of Oligomer 3 in 3′-exonuclease solution. Phosphorothioate backbone modifications at positions 20 and 23 result in the accumulation of 20- and 23-mer fragments. For conditions see Section 2.

multiple PS protection. This fact leads us to speculate that part of the all-PS oligomer resistance can be related to another mechanism of oligomer protection, namely the strong adsorption to 3′-exonuclease. To the best of our knowledge, this mechanism has not yet been reported in the literature. Saturation of the human enzymatic system by PS-ODNs may explain in part why the in vivo measured half-life of phosphorothioate AONs is dose-dependent [14].

4. Conclusions

Despite the extensive development of antisense drugs and the introduction of advanced second generation AONs with combined chemical modifications for better protection against enzymatic cleavage, the mechanism of resistance to nucleases is not clearly understood. This difficulty in understanding the impact of the chemical modifications on their protection against nucleases is partially due to the lack of reliable analytical methods. In this work we developed an automated CE–UV method for screening products of ssDNA metabolism. This method was used to study the kinetics of digestion of ODNs with single or multiple PS modifications on the backbone. The pattern of metabolites obtained during exonuclease digestion suggests that internucleotide PS modifications act as effective protection for the corresponding parent oligomer or metabolite(s). While studying the enzymatic digestion of oligonucleotides containing multiple PS backbone modifications, we found an additional effect most likely caused by strong DNA adsorption to 3′-exonuclease

Table 2
Digestion rate of 25-mer oligomers in a reaction mixture containing 3′-exonuclease (bovine intestinal mucosa phosphodiesterase I)

Oligomer	$t_{1/2}$ of parent oligomer (h)[a]	Note
1	0.269	1.3 μ*M* of Oligomer 1
2	0.38	–
3	0.32	–
4	11.90	–
5	1.55	–
6	30.46 (*R*[b])	–
6	3.85 (*R*); 1155.25 (*S*)	in vitro in plasma
1	4.19	in the presence of Oligomer 6 (0.650 μ*M* both)[c]

[a] Half-life, $t_{1/2}$, based on the first two h of reaction.
[b] *R* or *S* denotes the absolute configuration at 3′-end internucleotide linkage phosphorus.
For details see Fig. 4.

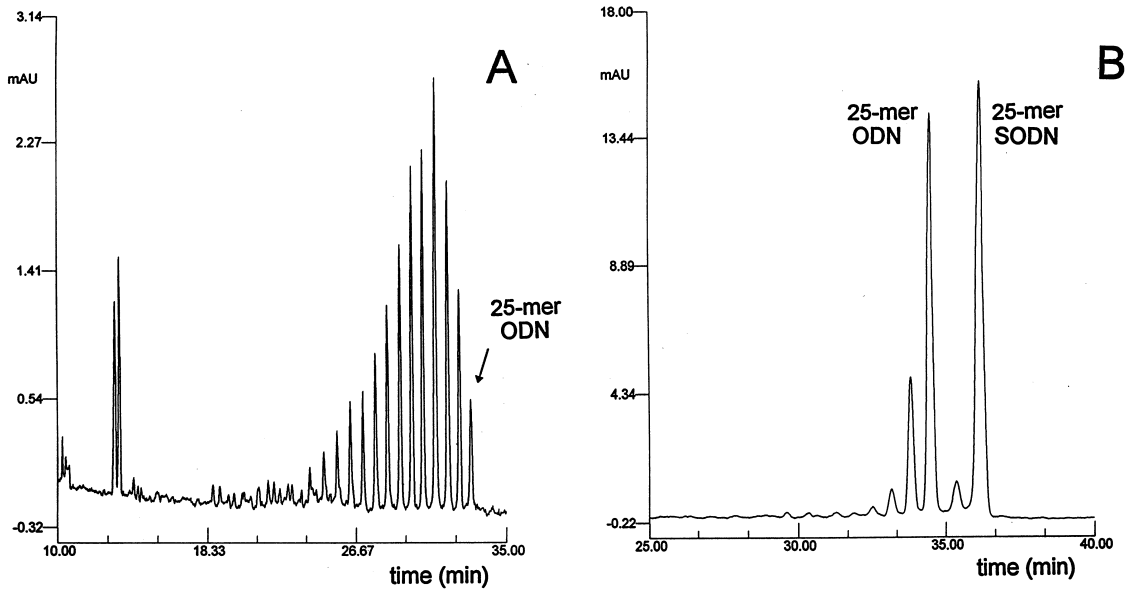

Fig. 4. Digestion of Oligomer 1 in (A) 3′-exonuclease solution and (B) 3′-exonuclease solution in the presence of Oligomer 6. Reaction conditions were identical; the time of reaction in both cases was 115 min. For details see text and Section 2.

which improved the stability of phosphorothioate DNA.

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