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3'-3'-LINKED OLIGONUCLEOTIDES: SYNTHESIS AND STABILITY STUDIES

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Abstract: As part of a therapeutic strategy involving multiple targeting, we have synthesized oligonucleotides which are linked *via* 3'-3' phosphoric diester linkages. These 3'-3' analogs showed increased resistance against nuclease-mediated degradation compared to the 5'-3'-linked oligonucleotides. Copyright © 1996 Elsevier Science Ltd

Inhibition of protein synthesis using antisense oligonucleotides represents a new therapeutic approach with promise for the chemotherapy of a number of diseases.[1] Historically, although 5'-3' phosphoric diester (PO) oligonucleotides were among the first analogs to be evaluated for inhibition of gene expression,[2] they have been largely overlooked as potential antisense agents because of their susceptibility to rapid degradation by nucleases present in plasma. Thus, various backbone-modified oligonucleotides, complementary to target messenger RNA, have been studied as modulators of gene expression primarily as antisense agents. Amongst the numerous analogs which have been evaluated to date, the phosphorothioate oligonucleotides targeted against viral genomes and cancer have advanced to various stages of clinical development.[3] These backbone-modified oligonucleotides are of therapeutic interest because they show enhanced stability against nucleases compared to PO oligonucleotides. Notwithstanding this beneficial property of modified oligonucleotides, in certain instances, a duplex of the modified oligonucleotide with a complementary RNA target is found to be less stable than that formed using PO oligonucleotide.[3] Furthermore, duplexes formed with some of the non-ionic oligonucleotides (e.g., methylphosphonate oligonucleotides) were not substrates for RNase H.[4] Thus, although the stability of oligonucleotides against nuclease-mediated degradation may be increased by chemical modifications, the antisense activity of some of the oligonucleotides may be compromised by incorporating chemical modifications. Another important factor which is of concern in oligonucleotide-based therapeutics is the potential for the development of resistance brought about by mutation of the target site in the RNA. A compounding problem which also needs to be addressed is the presence of secondary opportunistic infections caused by different viral and bacterial agents present in an immunocompromised and complex disease such as AIDS.[5] To address these diverse issues we have been studying antisense oligonucleotides, which target multiple m-RNAs of the same or different pathogens or different segments of the same target m-RNA. We reasoned that by using this multiple targeting strategy it would be possible to develop therapeutic agents that can act in a synergistic fashion, thus allowing for potentiation of the therapeutic efficacy of a given oligonucleotide.

With the need to have oligonucleotides with minimal structural modifications and to implement the multiple targeting strategy, we considered the linking together of two PO oligonucleotides by joining their terminal 3'-OH groups. In principle, the 3'-ends of the two oligonucleotides could be linked *via* 3'-5' or 3'-3' linkages. In the present study, we considered the intriguing possibility of linking two oligonucleotides targeted

against m-RNAs of HIV-1, *via* 3'-3' PO-linkage. We envisioned that although PO oligonucleotides, having 5'-3' phosphoric diester linkages, are inherently sensitive to nuclease-mediated degradation, 3'-3'-linked PO oligonucleotides may be sufficiently altered conformationally such that they may not be substrates for nucleolytic enzymes. Thus, the 3'-3' analogs may be anticipated to have improved stability characteristics. These expectations were also based on several lines of experimental evidence obtained from our laboratory and others: (a) the major pathway of degradation of normal and modified oligonucleotides is exonuclease-mediated and occurs from the 3'-end;[6] (b) the rate of degradation of oligonucleotides can be slowed down by "3'-capping";[6] and (c) oligonucleotides with terminal 3'-3' internucleotidic PO linkages[7] and those containing alternating 3'-3'-α and β nucleotides[8] are more resistant to nuclease-mediated degradation. Reported herein are our results on the synthesis, characterization, and comparative stability against nuclease-mediated degradation of 3'-3' and 5'-3'-linked oligonucleotides.

The oligonucleotide sequences **1-5** chosen for the present study are shown in Table 1 and target the *gag* and *tat*-mRNA of HIV-1. The phosphorothioate analog of **1** has been demonstrated to be a potent anti-HIV agent.[3] The sequences **1-5** were synthesized on a 3 μm scale using phosphoramidite chemistry.[9a] The 3'-3'-

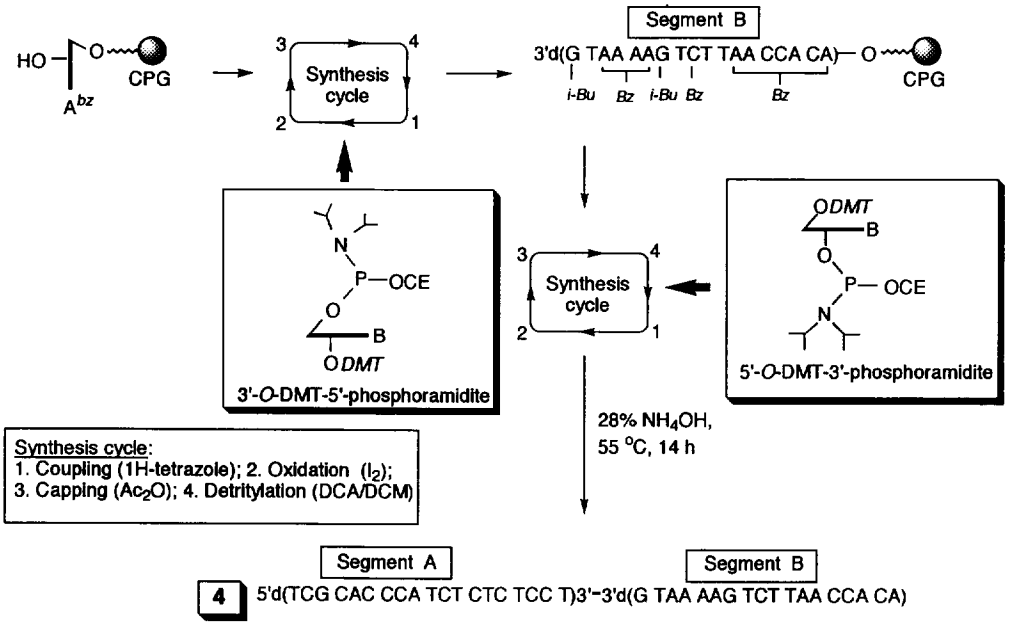
Table 1. Antisense oligonucleotide sequences **1-5**

Seq. #	Sequence
1	5'd(CTC TCG CAC CCA TCT CTC TCC TTC T)3'
2	5'd(CTC TCG CAC CCA TCT CTC TCC TTC T)3'- 3'd(TCT TCC TCT CTC TAC CCA CGC TCT T)5' A B
3	5'd(CTC TCG CAC CCA TCT CTC TCC TTC T)3'- 5'd(CTC TCG CAC CCA TCT CTC TCC TTC T)3' A B
4	5'd(TCG CAC CCA TCT CTC TCC T)3'- 3'd(GTA AAA GTC TTA ACC CAC A)5' A B
5	5'd(TCG CAC CCA TCT CTC TCC T)3' - 5'd(ACA CCC AAT TCT GAA AAT G)3' A B

Oligonucleotides **1-3** are complementary to the *gag* m-RNA of HIV-1. In oligonucleotides **4-5**, the segment **A** is complementary to *gag* m-RNA of HIV-1 and segment **B** is complementary to *tat* m-RNA of HIV-1. In the case of the oligonucleotides **3** and **5**, the two segments **A** and **B** are connected at their backbone by 5'-3' internucleotidic PO linkages while in **2** and **4**, the two segments are connected at their backbone by 3'-3' internucleotidic PO linkages. For the synthesis of the sequences **2** and **4** refer to Scheme 1.

sequences **2** and **4** were prepared starting with the nucleoside whose 5'-terminus was attached to controlled-pore-glass (CPG) support *via* succinyl linkage (Scheme 1). Chain elongation was carried out using the 3'-*O*-(4, 4'-dimethoxy) trityl (DMT) 5'-*O*-phosphoramidite nucleoside monomers. Following the assembly of the

right side segment (segment B, **2** and **4**), the critical 3'-3' POlinkage, to attach the left side segment (segment A, **2** and **4**), was established using 5'-*O*-DMT-3'-*O*-phosphoramidite monomer and the chain assembly continued (Scheme 1).[9b] Extended coupling times were employed in the synthesis of **2** and **4**. Following their synthesis, the deprotection of the oligonucleotides (DMT-on) were carried out using aqueous NH_4OH (28%, 55 °C, 12 h). All the oligonucleotides were purified using reversed-phase (C-18) chromatography and isolated



Scheme 1. Synthesis of the oligonucleotide **4**

using standard protocols. The percentage of the full-length oligonucleotides was adjudged to be ca. 93~94% by capillary gel electrophoresis (data not shown). The oligonucleotides **1**, **3**, and **5** were similarly prepared using standard phosphoramidite chemistry.[9]

Next, the stability of oligonucleotides against nuclease-mediated degradation was evaluated after exposure of the oligonucleotides to *3'-phosphodiesterase*, 10% fetal calf serum (FCS) and human plasma. For the analysis of the degradation pattern, two methods were employed: (a) direct HPLC analysis of the oligonucleotides and (b) polyacrylamide gel electrophoresis (PAGE) of [γ-^{32}P]-labeled oligonucleotides.

The direct HPLC analysis of oligonucleotides (Fig. 1) was performed by employing a modification of a reported procedure.[10] For our analysis, we employed a reversed-phase column connected to an ion-exchange column through a Rheodyne® valve assembly. The elution gradient chosen was such that the reversed-phase (C-18) column retained the oligonucleotide while allowing the passage of plasma proteins and other plasma components from the incubate. By switching the Rheodyne valve system to the inject position followed by change of the elution buffer, the oligonucleotide from the C-18 column was allowed to enter the ion-exchange column where the analysis of the oligonucleotide and its degradation products was carried out using appropriate buffer (Fig. 1). Interestingly, the HPLC profiles of the 5'-3' analogs (**1**,**3** and **5**) exposed to

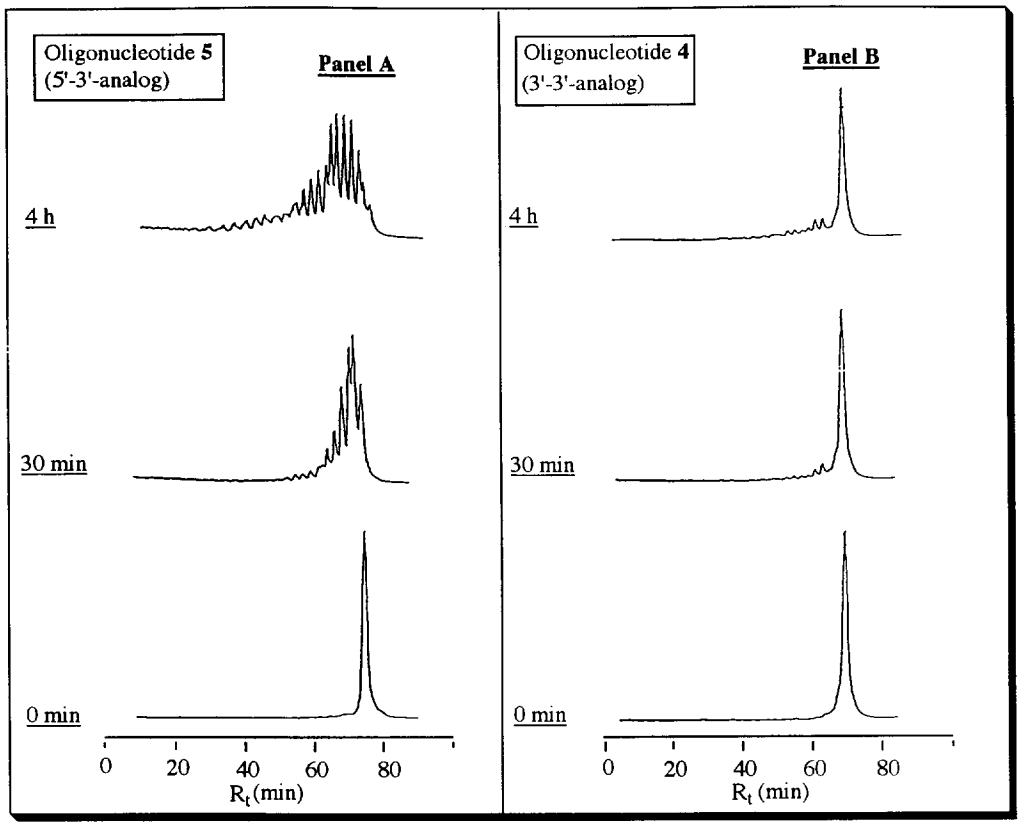


Figure 1. HPLC analysis of the nuclease-mediated degradation of oligonucleotides **4** and **5**. Aliquots (20 μg) of each oligonucleotide was incubated with 60 μL of human plasma at 37 °C. At regular intervals, each aliquot was diluted with 150 μL of Tris.HCl buffer (25 mM, pH 7.0), filtered on a 0.2 μM membrane filter and injected into HPLC. HPLC was performed on a Waters instrument (Waters 600 Controller and a 996 photodiode array UV detector (200 to 320 nm) and the data analyzed using Millenium® software. Two columns, connected in series using Delta-Pak C-18 (300 °A, 5 μm, 3.9 /20 mm) and GEN-PAK FAX ion-exchange (4.6/100 mm), were used. The columns were connected to a Rheodyne® valve system such that at the valve load position, samples could be introduced into the Delta-Pak column and at the inject position, samples entered the ion-exchange column. Elution was carried out in two consecutive steps (i) At the load position, flow rate of 0.6 mL/min and an isocratic gradient of 100% A (25 mM Tris.HCl, 0.07 M LiCl, pH 7.0) for 7 min followed by a gradient of 0 to 100% B (80/20 *v/v*, 25 mM Tris.HCl, 0.07 M LiCl/acetonitrile, pH 7.0) over one min; (ii) The Rheodyne valve was then switched to the inject position and elution continued using a flow rate of 0.5 mL/min, with an isocratic gradient of 100% B for 2 min followed by a gradient of 0 to 55% C (80/20 *v/v*, 25 mM Tris.HCl, 1.0 M LiCl/acetonitrile, pH 7.0) over 65 min.

FCS and human plasma revealed the rapid disappearance of the intact oligonucleotide and progressive appearance of N-1, N-2, and other smaller fragments, which confirmed our earlier findings[6] that in serum, the nuclease-mediated degradation of oligonucleotides occurred from the 3'-end (Fig. 1, Panel A). In case of the 3'-3' analogs **2** and **4**, exposed to FCS, and human plasma, most of the oligonucleotide remained intact and only a small percentage of the N-1 and N-2 fragments resulting from degradation was seen (Fig. 1, Panel B).

These HPLC results were also confirmed by monitoring the degradation kinetics of oligonucleotides by PAGE analysis (data not shown). For the analysis by PAGE, the oligonucleotides were 5'-end labeled using [γ-^{32}P]ATP in the presence of *polynucleotide kinase*. The 5'-end-labeled oligonucleotides were then incubated with *3'-phosphodiesterase*, FCS or human plasma, as appropriate. Aliquots were drawn at different times and subjected to PAGE followed by autoradiography. Computer-derived quantitation of the bands was achieved by phosphorimager analysis (Molecular Dynamics).

Thus, both HPLC and PAGE methods of analyses indicated that, in the case of PO oligonucleotides **1**, **3**, and **5**, incubation with *3'-phosphodiesterase*, FCS, or human plasma, resulted in the rapid degradation of the oligonucleotides with a half-life ($t_{1/2}$) of degradation in the range of 3 to 5 min.[11] In comparison, however, the 3'-3'-linked oligonucleotides **2** and **4**, had half-lives of ca. 4~7 h. Thus, the 3'-3'-linked analogs **2** and **4** were quite resistant to degradation than **1**, **3**, and **5**.

A critical determinant of the therapeutic potential of a given oligonucleotide is its in vivo stability and pharmacokinetic profile. Encouraged by the remarkable in vitro stability demonstrated by the 3'-3'-linked oligonucleotides, the evaluation of their in vivo stability against nucleases was undertaken (Fig. 2). To

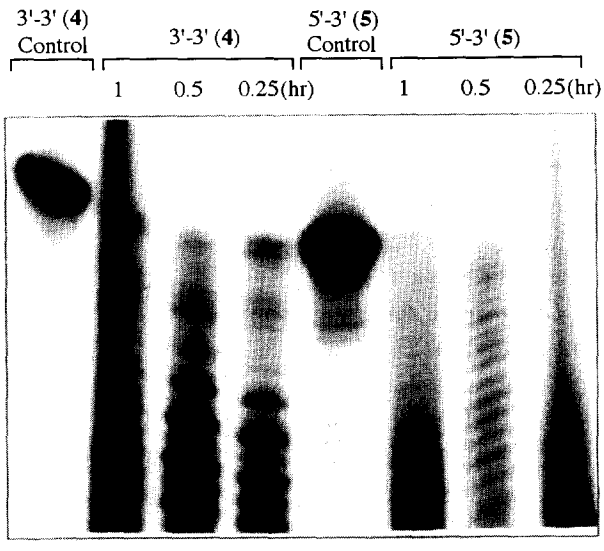


Figure 2. In vivo stability of 3'-3' and 5'-3' oligonucleotide analogs (**4** and **5**) by PAGE. Female CD-1 mice (6 weeks old, 20 g) were injected with the oligonucleotides (dissolved in RPMI media) at 10 mg/Kg dose into the tail vein. At various time points, mice were euthanized and plasma collected. 50 μL of plasma was incubated with DNA extraction buffer (10 μL) and 10 μl of *proteinase K* (10 mg/mL) for 90 min at 37 °C. The samples were then extracted once with phenol/$CHCl_3$ and with $CHCl_3$ and oligonucleotide precipitated with ethanol. The samples were end-labeled with [γ-^{32}P]ATP using *polynucleotide kinase*. After incubation with RNase A (10 mg/mL) for 10 min, the oligonucleotides were precipitated and subjected to PAGE.

determine their in vivo stability, the oligonucleotides were injected at a single dose level of 10 mg/Kg into the tail vein of CD-1 mice. At various time-points, the mice were euthanized and plasma collected. The oligonucleotides were extracted from the plasma as described previously.[12] The crude oligonucleotides were then end-labeled with [γ^{32}-P]ATP and analyzed by PAGE.[12] Examination of the PAGE profile (Fig. 2) revealed that the 5'-3'-linked oligonucleotides were completely degraded within 15 min. The 3'-3'-linked oligonucleotides **2** and **4** were also degraded, *albeit* more slowly, and the presence of significant amounts of full length oligonucleotides could be seen after 1 h following injection. In vivo, the degradation of these oligonucleotides may be due to the presence of 3'- and 5'-exo nucleases as well as endonucleases.

Thus, our studies reveal that in vitro, the 3'-3'-linked PO oligonucleotides show remarkable stability against nuclease-mediated degradation compared to 5'-3' oligonucleotides. In vivo, although both classes of these oligonucleotides suffered degradation, the 3'-3'-oligonucleotides were degraded more slowly. Based on these initial stability studies it would appear that the 3'-3'-linked PO-oligonucleotides are only of limited therapeutic utility in multiple targeting strategy. Since phosphorothioate oligonucleotides are more resistant to nuclease-mediated degradation in vivo, they may be more appropriate candidates for multiple targeting strategy. In this context, we have completed the synthesis and evaluation of 3'-3'-linked phosphorothioate oligonucleotides. The results of these studies will be the subject of a future publication.

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