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Hybrid Oligonucleotides: Synthesis, Biophysical Properties, Stability Studies, and Biological Activity

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Abstract—We have designed and synthesized hybrid oligonucleotides **2–5**, as analogues of oligodeoxynucleoside phosphorothioates, in an effort to have agents with improved 'antisense activity' with reduced phosphorothioate content. The hybrid oligonucleotides contain segments of 2′-*O*-methyl ribonucleoside phosphoric diesters and oligodeoxynucleoside phosphorothioates. Thus, compared with the 'all' phosphorothioate analogues **1** and **6**, the analogues **2–5** showed significantly reduced effect on complement activation. In addition, thermal denaturation studies with complementary RNA revealed that the analogues **2–5** had higher T_m compared with that with oligodeoxynucleoside phosphorothioates. Additionally, the RNA component of the oligo/RNA duplex is efficiently cleaved by RNase H, the site of endonucleolytic cleavage being dictated by the length of the oligodeoxynucleoside phosphorothioate segment. Copyright © 1996 Elsevier Science Ltd

Introduction

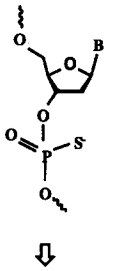
Oligonucleotides complementary to a target messenger RNA have shown promise as therapeutic agents for 'antisense-mediated inhibition' of protein synthesis.[1–3] To derive therapeutic benefit using antisense-based technology, 'active' oligonucleotides must have the following additional characteristics: (a) they should be resistant to degradation by cellular nucleases; (b) they must be efficiently internalized; (c) they should hybridize in a sequence-specific manner with the target RNA; (d) although not essential, they should activate RNase H towards cleavage of the RNA/DNA duplex; (e) in vivo, they should have adequate bioavailability, with a favorable pharmacokinetic profile (absorption, distribution and excretion); (f) they should be nontoxic.

Oligodeoxyribonucleoside phosphorothioates (PS oligos; **1**, Fig. 1) have been extensively studied from a therapeutic perspective, and several aspects of its in vitro and in vivo biological activity,[1–3] pharmacokinetics, and metabolic fate[4,5] are known or are currently under investigation. Pharmacokinetic studies of PS oligos in mice,[5,6] rats[7–10] and monkeys[4] following iv bolus administration, show that PS oligos are distributed in most tissues. We and others[11,12] have reported certain hemodynamic changes (e.g., hypotension), in monkeys, upon iv bolus administration of PS oligos. These changes were related to complement activation, and could be easily minimized by administering the oligonucleotides by slow iv infusion.

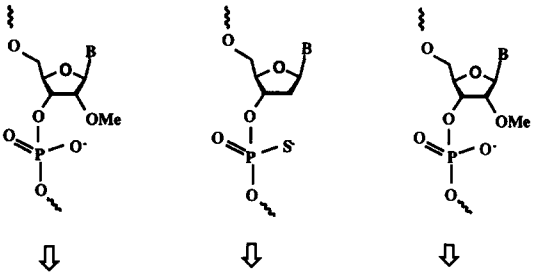
In order to further improve the properties of PS-oligonucleotides, we had evaluated 'hybrid' oligonucleoside phosphorothioates (H-oligos) as anti-HIV agents. The H-oligos are deoxyribonucleoside phosphorothioates flanked by segments of contiguous 2′-*O*-methyl ribonucleoside phosphorothioate linkages at the 3′- and 5′-ends[13,14] (Fig. 1). Using an in vitro complement activation assay,[15] we had recently found that the phosphoric diester deoxyribonucleosides (PO oligo) and H-oligo **6** have much reduced propensity to activate complement in blood compared with the PS oligo **1**. Our studies with hybrid oligonucleotides demonstrated that (a) they had greater anti-HIV activity compared with the PS-oligo **1**, (b) they were more resistant to nuclease-mediated degradation, (c) they formed duplex with complementary RNA that have higher T_ms than that with PS-oligo, and (d) the duplexes were substrates for RNase H. Other reports[16] comparing H-oligos with PS-oligos indicate similar patterns of biophysical and biochemical properties. All these results are suggestive that biological and biochemical properties of oligonucleotides are strongly influenced by subtle structural alterations.

Prompted by the above results, we have now synthesized various modified antisense 25-mer hybrid oligonucleotide constructs **2–5** (Fig. 1) that are deoxyribonucleoside phosphorothioates flanked by stretches of 2′-*O*-methyl ribonucleoside phosphoric diester linkages (HO-oligos) and compared them with the H-oligo **6** and the PS-oligo **1**, in respect of their

biophysical properties, biochemical properties, biological activity and their potential to activate complement. Reported herein are our findings.



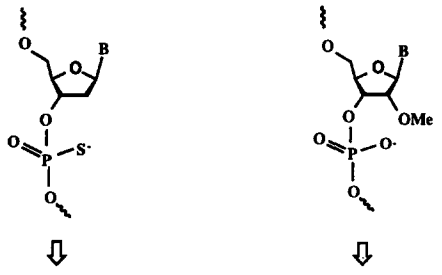
1 5'-CTC-TCG-CAC-CCA-TCT-CTC-TCC-TTC-T-3'



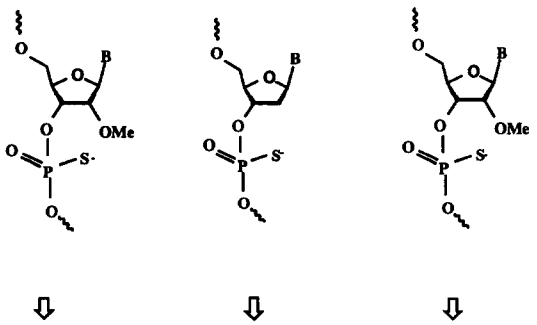
2 5'-CUC-UCG-CAC-CCA-TCT-CTC-TCC-UUC-U-3'

3 5'-CUC-UCG-CAC-CCA-TCT-CTC-TCC-UUC-U-3'

4 5'-CUC-UCG-CAC-CCA-TCT-CTC-TCC-UUC-U-3'



5 5'-CTC-TCG-CAC-CCA-TCT-CUC-UCC-UUC-U-3'



6 5'-CUC-UCG-CAC-CCA-TCT-CTC-TCC-UUC-U-3'

Figure 1. Structures of PS-oligo, HO-oligos, and H-oligo. B represents the nucleobase. The shadowed section indicate the 2′-*O*-methyl ribonucleotide segments.

Results

Synthesis and characterization of the oligonucleotides

The oligonucleotides were synthesized and characterized by ion-exchange chromatography, capillary gel electrophoresis (Fig. 2), PAGE and by ^{31}P NMR. Quantitative analysis by capillary gel electrophoresis revealed that all the oligos contained greater than 94% of the desired 'N-mer' and less than 1–6% of truncated sequences. In the ^{31}P NMR, the expected resonances for PS (δ, 54 ppm) and PO (δ, −4 ppm) were observed in the correct integral ratio for each HO analogue.

Hemolytic complement assay

One of the goals of the present study was to determine whether the substitution of PS in the flanking sequences of the H-oligo **6** (at its 3′- and 5′-ends) by PO, will help reduce complement activation while substantially retaining most of the other desirable characteristics of an antisense oligonucleotide. Using an in vitro assay,[15] we found no significant changes in serum hemolytic complement levels as measured by CH50 values following treatment with **1**, at doses of 10 μg/mL or less. However, at high dose levels of 1000 μg/mL (Fig. 3, Panel A), the PS-oligo **1** induced a 100% reduction in CH50 values. At a dose level of 1000 μg/mL **6** produced a 70% decrease in CH50 levels where as the HO-oligo **2** produced only a 40% reduction in CH50. In a study comparing the oligos **1–8**, at a dose level of 200 μg/mL (Fig. 3, Panel B), the HO-oligos **2–5**, **7**, and **8** were much less active than PS-oligo **1** and H-oligo **6** in causing a reduction in hemolytic complement. Thus our studies reveal that, in this regard, HO-oligos are superior to the H-oligos and PS-oligos as they have less potential to activate complement.

Nuclease-stability of HO-oligos

To determine the stability of oligonucleotides against nucleases, 5′-^{32}P-labeled oligonucleotides were incubated with fetal bovine serum (FBS). As expected, the analogues **2–5** which contain 2′-OMe PO segments were less stable to serum-mediated hydrolysis compared to the H-analogue **6** (data not shown) and the PS-analogue **1** (Fig. 4). Thus, in the case of serum-mediated hydrolysis of the analogues **2–5**, the hydrolysis was initiated in a stepwise mode from 3′-end and progressed rapidly to the first deoxyribonucleoside phosphorothioate linkage, wherefrom further degradation seemed to have slowed down (Fig. 4). This is evident from the presence of major bands corresponding to 22-mer, 21-mer, and 20-mer, even after 2 h, following incubation of **2–5** with serum. Interestingly, the rate of degradation of **5** was much slower with serum compared with **2–4**. Again, under identical conditions, PS-oligo **1** was resistant to degradation as indicated by the presence of mostly intact oligo following exposure to serum.

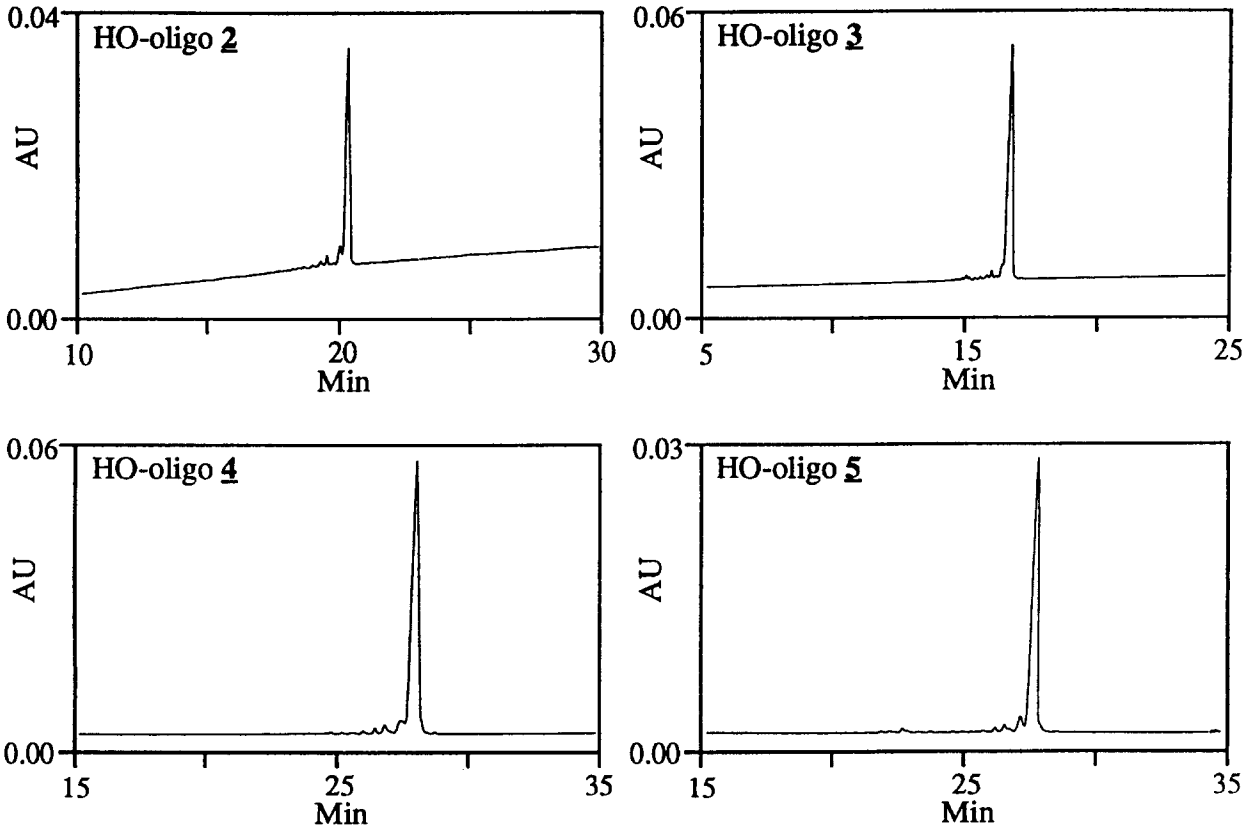


Figure 2. Capillary gel electrophoretic profiles of the purified HO-oligos **2–5**.

RNase H-mediated cleavage of the RNA-oligo duplex

RNase H is an endonuclease which degrades the RNA strand of the RNA/DNA duplex producing oligoribonucleotides with 5′-phosphate and 3′-OH termini and single-stranded DNA. For our study, a 3′-end labeled synthetic RNA corresponding to codon 764–801 of the HIV-1 *gag* gene, was incubated with excess of each PS-oligo **1**, HO-oligo **2–7** in the presence of RNase H. The samples were then subjected to PAGE followed by autoradiography (Fig. 5). Autoradiography revealed a single prominent site of endonucleolytic cleavage in the RNA with minor multiple sites in the case of duplexes formed between RNA and the HO-oligos **2–5**. In the case of the duplexes between **2–4** and RNA, the predominant endocleavage site seemed to be the RNA phosphoric diester linkage adjacent to the flanking 2′-OMe sequence at the 3′-end in the HO-oligo whereas with the HO-oligo **5** and PS-oligo **1**, the RNA cutting site was moved further downstream. The H-oligo **6** induced two prominent sites of endocleavage of the RNA (data not shown).

These observations are consistent with other reports[16–18a] that 2′-*O*-methyl oligoribonucleotides are incapable of activating RNase H-mediated cleavage within the RNA/2′-*O*-methyl RNA duplex domain, but can *direct* the cleavage to an adjacent site in the duplex formed between RNA and the H-oligo. Our results also illustrate the versatility of RNase H in performing endonucleolytic cleavage with duplexes formed between RNA and various oligo constructs **1–6**. The HO-oligo **7** (with a mismatch sequence) did not trigger the cleavage of RNA which would be consistent with its inability to form a duplex with the RNA. Other control experiments confirmed that the endonucleolytic cleavage of the oligos was RNase H-mediated.

Thermal denaturation studies

T_m determinations of oligos **1–8** carried out against complementary 35-mer DNA and 25-mer RNA are shown in Table 1. With DNA as the complementary strand, only modest gain in T_m, of 0.9 °C for **2** to 1.7 °C for **5**, is realized in comparison to the deoxy analogue **1**. However with RNA as the complementary strand, significant increase in T_m is noted with the maximum gain observed with analogue **4** (+11.1 °C) to a minimum of +5.8 °C for analogue **2** when compared to **1**, presumably reflecting an increased duplex stability with the HO-oligos. Quite clearly, this gain in duplex stability and binding affinity to the complementary targets (as indicated by T_m) is contributed both by the 2′-*O*-methyl ribonucleoside component as well as by the PO-linkages. Circular dichroism (CD) spectra also reveal that **2–5** form duplex like structures with RNA (data not shown).

Anti-HIV activity

The antisense oligonucleotides **1–8**, which are 25-mer oligonucleotides complementary to the *gag* initiation

codon of HIV-1, were evaluated as anti-HIV agents. The inhibitory effect of the oligonucleotides on HIV-1 replication was evaluated in a $CD4^+$ T-cell line (MOLT-3) infected with low multiplicity of infection of HIV-1 type III_B. After 7 days of culturing, the viral p24 levels (ng/10^6 cells, average of two experiments) were determined on days 7, 10, and 14. For comparison, both **1** and **6** were evaluated for anti-HIV activity. A sustained dose-dependent reduction of viral p24 levels and virtual abolition of p24 was seen at 0.5 μM dose of **1** and **6** (IC_{50}, 0.1–0.3 μM).

We also undertook a comparative dose–response study of the antiviral efficacy of **2–5** relative to **1** and **6**, (Table 2). The IC_{50} values for **2, 3, 4**, and **5** were 0.36, 0.42, 1.50, and 0.76 μM, respectively, compared with the IC_{50} of 0.1–0.3 μM. All the analogues **2–5** were less potent than **1** and **6**. Within the HO analogue series, **2** and **3** were more potent than **4** and **5**. In the antiviral assay described above, the random oligos **7** and **8** produced significantly less antiviral effect (0–30%) at various doses. The apparent slight antiviral effect due to random oligos might represent a nonsequence-

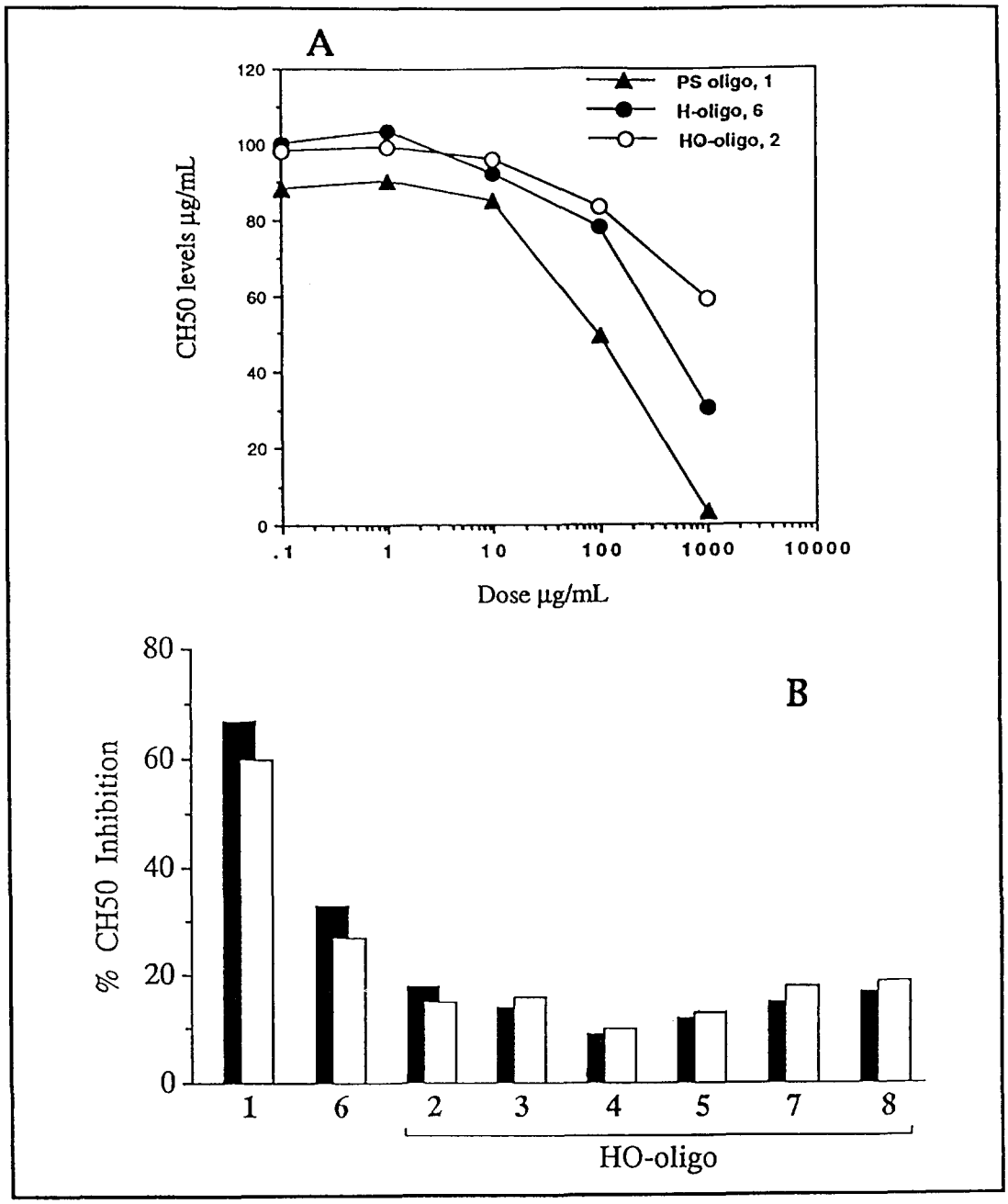


Figure 3. Panel A: Complement activation as a measure of residual CH50 levels following incubation with oligonucleotides **1, 6**, and **2**. The concentration of oligos used were 0.1, 1, 10, 100, and 1000 μg/mL. Panel B: Comparative % reduction in hemolytic complement levels following incubation with oligos **1–8** at a dose level of 200 μg/mL. Filled versus open bars represent results from two independent experiments using different donor serum.

specific activity due to its polyanionic backbone.[18b,c] Such nonsequence-specific antiviral effects are usually observed in short-term assays and are not seen in long-term studies.[19] In summary, it can be stated that **2–5** produced a dose-dependent inhibition of HIV-1 replication, with the analogue **2** being the most active in this series and may be related to the presence of larger fragments (17 base-pair) which may retain substantial antisense activity even after the 3′- and 5′-ends have been digested. However, more studies are required to understand the correlation between stability, binding affinity to the target, and antiviral activity of these analogues.

Discussion

In the present study, we have shown that the replacement of PS-linkages by PO-linkages in the 2′-OMe ribonucleoside series, produced analogues which retain their anti-HIV activity. More importantly, complement activation studies reveal that the HO-oligos are much less active than the H-oligos and PS-oligos at high doses. However, the molecular mechanisms underlying these results are not known. None the less, we would predict a significant reduction in adverse hemodynamic response in monkeys, upon iv administration of the herein described HO-oligos. However, it is pertinent to mention that a Phase I clinical study involving the safety and pharmacokinetic evaluation of PS-oligo **1**, revealed that single dose of 1 mg/kg infused over a 2-h period of PS-oligo **1**, in asymptomatic HIV-positive patients, was very well tolerated.[20,21] We have also recently initiated the Phase II clinical trial of PS-oligo **1**, against AIDS. Other clinical trials in humans are also under way to test the efficacy of PS-oligos against viral infections and cancer.[22,23]

Our studies reveal that the HO-oligo **2** is a potent anti-HIV agent ($IC_{50} = 0.36$ μM) with much reduced adverse hemodynamic effects. However, in achieving this goal, their stability against cellular nucleases, and to some degree the anti-HIV activity, seem to have been compromised. With these results in hand, we recently evaluated bioreversible analogues[24a,b] derived from **2** and found them to be substantially more stable towards nucleases compared with **2**. For example, the analogues of HO-oligos which are functionalized as the S-acyloxyalkyl esters are converted back to HO-oligos upon hydrolysis with cellular esterases. Also these bioreversible analogues were devoid of cytotoxicity and did not

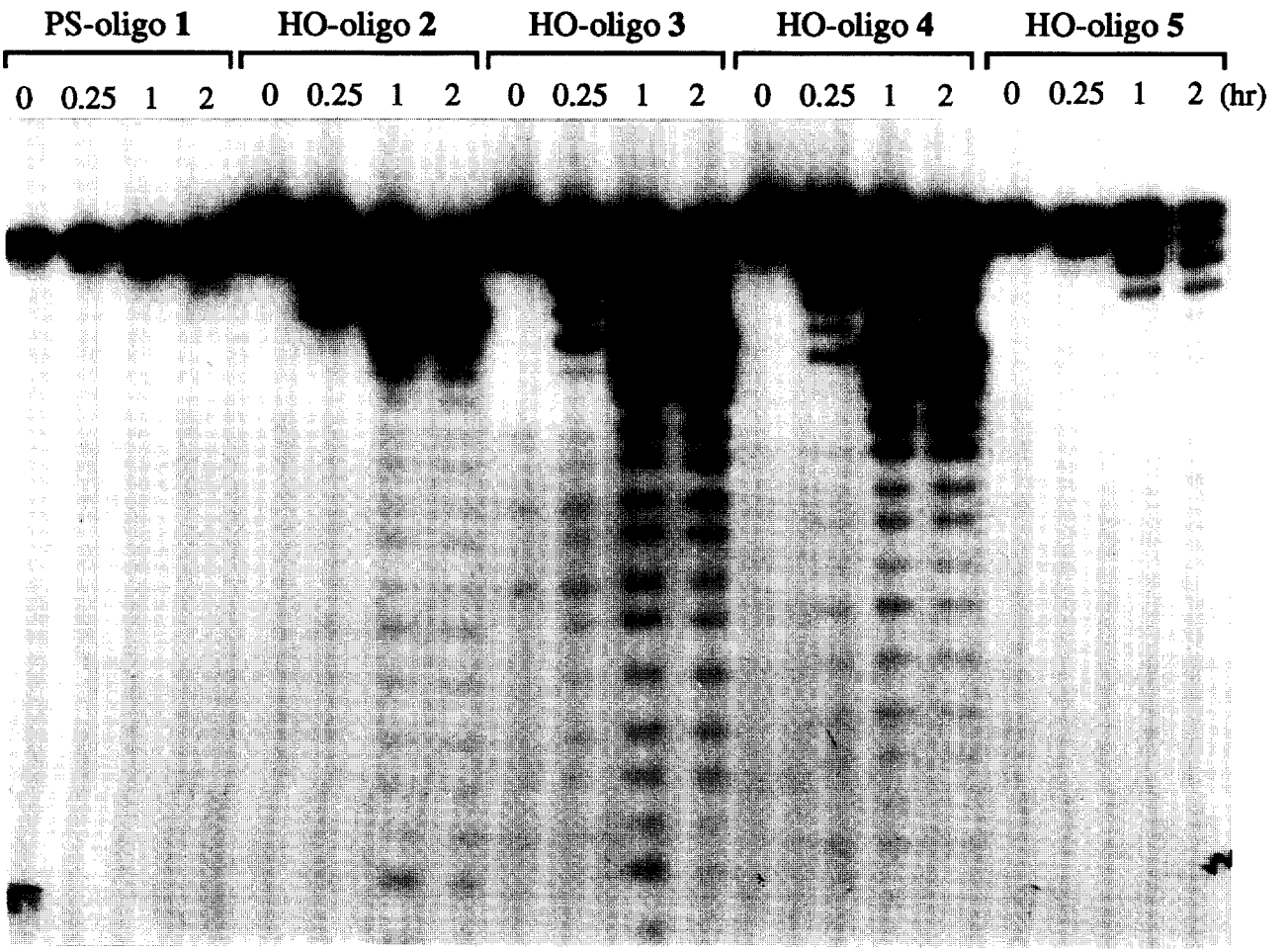


Figure 4. Fetal bovine serum-mediated degradation of the purified HO-oligos 2–5. For comparison, the PS-oligo **1** was used as a control.

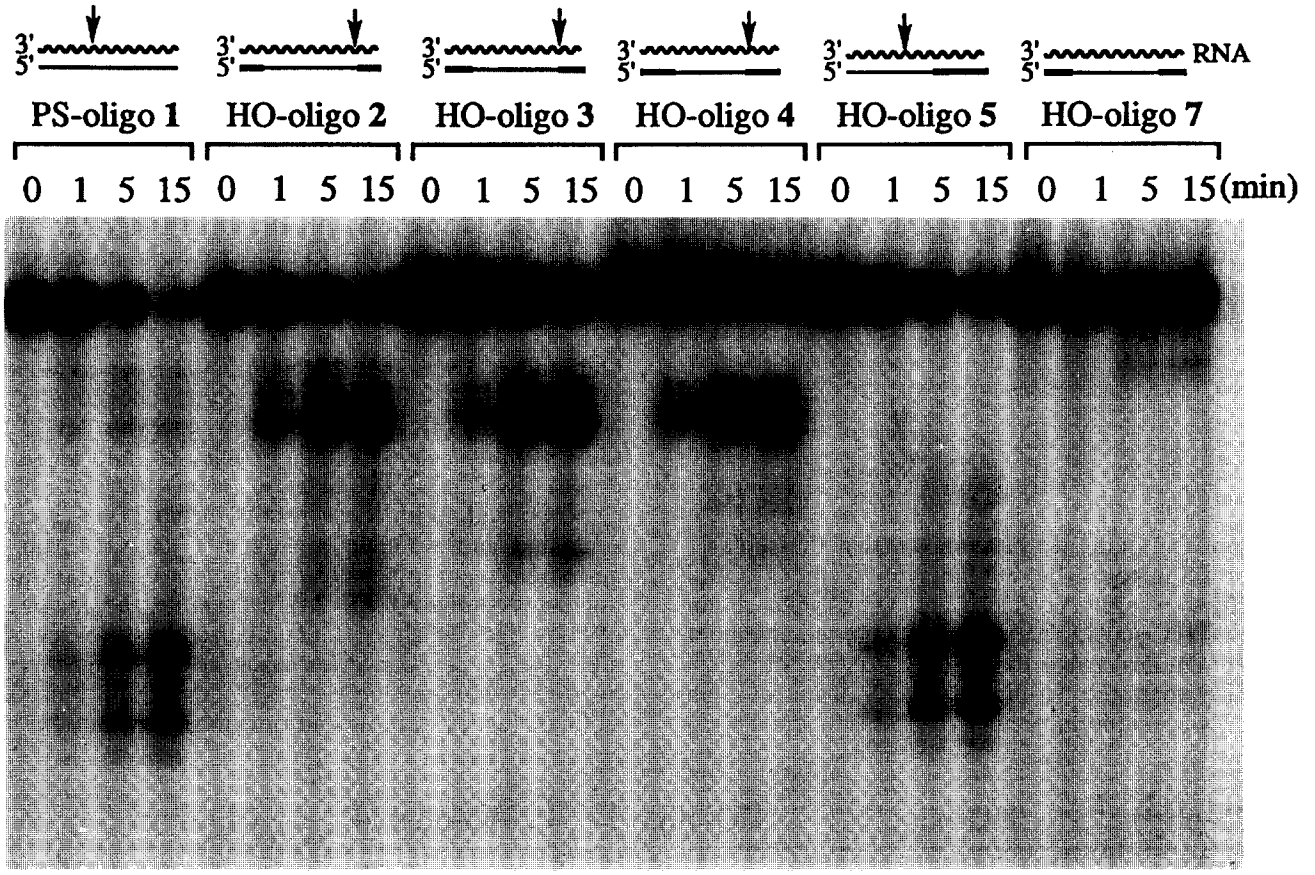


Figure 5. Cleavage of the RNA/DNA duplex derived from purified HO-oligos **2–7** and PS-oligo **1** by RNase H. Arrows depict the approximate site of endonucleolytic cleavage in the RNA.

Table 1. Thermal denaturation studies on oligonucleotides

Oligo no.	Sequence	T_m (°C) (with DNA)	T_m (°C) (with RNA)
1	5' CTC TCG CAC CCA TCT CTC TCC TTC T 3' (PO) (PO)	51.9	3.2
2	5' [CUCU] CG CAC CCA TCT CTC TCC [UUCU] 3' (PO) (PO)	52.8	69.0
3	5' [CUCUC] G CAC CCA TCT CTC TC [CUUCU] 3' (PO) (PO)	53.1	71.0
4	5' [CUCUCG] CAC CCA TCT CTC T[CCUUCU] 3' (PO)	53.1	74.3
5	5' CTC TCG CAC CCA TCT [CUCUCCUUCU] 3'	53.6	71.8
6	5' [CUCU] CG CAC CCA TCT CTC TCC [UUCU] 3' (PO) (PO)	51.3	67.0
7	5' [CUAU] CT CAA CCC TCG CTA TCA [UUAU] 3' (PO)	<20	—
8	5' CTAT CT CAA CCC TCG [CUAUCAUUAU] 3'	<20	—
9	5' GAG GCT AGA AGG AGA GAG ATG GGT GCG AGA GCG TC 3' (sense DNA)		
10	5' AG AAG GAG AGA GAU GGG UGC GAG AG 3' (sense RNA)		

Boxes represent 2′-*O*-methyl ribonucleotides; **1**, PS-oligo; **2–5**, HO-oligos; **6**, H-oligo. The oligos **1–6** are complementary to the sense DNA **9** and sense RNA **10**. The HO-oligos **7–8** represent mismatch sequences and is included for comparison.

Table 2. Comparative HIV-1 IC_{50} of oligos **1–6**

Oligos	IC_{50} μM (at day 10)
1	0.13
2	0.36
3	0.42
4	1.50
5	0.76
6	0.15

[a]Viral p24 levels were measured at 7, 10, and 14 days following treatment with oligos **1–6** at various concentrations. (See Materials and Methods section for detail.)

cause complement activation at various dose levels. In an alternate approach, we have found that analogues, where the 2′-OMe oligonucleotide forms the central core which is extended on both 3′- and 5′-ends by phosphorothioate segments ('inverted hybrids'), showed improved antisense properties.[24c]

In conclusion, our studies demonstrate that it is possible to perform therapeutic optimizations of the antisense oligonucleotides by subtle structural changes in the nucleoside sugar residue and internucleotidic phosphate linkages of the active molecule. Knowledge gained from these studies should help guide the development of future generation of antisense oligonucleotides as therapeutic agents.

Materials and Methods

All solvents and chemicals (analytical reagent grade) were purchased from manufacturers and used as such. Aminopropylated controlled pore glass support (80–130 μm) was purchased from Schott Gerae, (Hofheim a. Ts.). Oligonucleotides were synthesized on a 10 μmol scale on a Milligen/Biosearch 8700 DNA synthesizer. For convenience, two synthesizers were sequentially employed. ^{31}P NMR spectra were recorded on a Bruker-AM 300 spectrometer using trimethyl phosphate in D_2O as the external reference. Capillary gel electrophoresis was performed on a Beckman Pace instrument operating at 14.1 kv as described before.[25,26] CPG-linked 5′-*O*-DMT-2′-*O*-methyl-uridine was synthesized by standard protocols.

Oligonucleotide synthesis

H-Oligo **6** and HO-oligos **2–8** (Fig. 1, Table 1) were synthesized using phosphoramidite chemistry[27] on a Biosearch 8700 DNA synthesizer. When using 2′-*O*-methyl, 3′-*O*-phosphoramidite 5′-*O*-DMT ribonucleoside monomers, an extended coupling program *viz.*, 'RNA coupling' was used to form the internucleotidic phosphite linkage. For the synthesis of phosphorothioates, oxidative sulfurization reaction was carried out using 3*H*-1,2-benzodithiole-3-one-1,1-dioxide.[28] After synthesis, further processing, purification and analysis were carried out as described.[25] The PS-oligo **1** was prepared as described before.[25]

Oligodeoxyribonucleotide **9** and oligoribonucleotides (RNA) **10** and **11** were prepared on a 1 μmol scale by phosphoramidite chemistry using standard synthesis programs and purified by PAGE.

Hemolytic complement activation assay

Normal donor blood was allowed to clot at room temperature for 30 min, cooled on ice for 15 min, and then centrifuged at 4 °C to recover the serum. Aliquots of the serum were mixed with the oligos at different concentrations (10–1000 μg/mL). After incubation at 37 °C for 15 min, the mixtures were chilled in ice-bath and diluted 1:50 in ice-cold buffer and CH50 analysis carried out as described.[15] A similar protocol was employed for CH_{50} determination at a single dose of 200 μg/mL of the oligonucleotide.

Labeling of oligonucleotide

The oligonucleotides were ^{32}P-labeled at the 5′-end using [γ-^{32}P]ATP as reported earlier.[29] For RNase H studies (vide infra), oligoribonucleotides were 3′-end-labeled using oligonucleotide 3′-end labeling kit (Boehringer Mannheim).[29]

Stability studies in 10% fetal bovine serum

These studies were carried out as described previously.[29] Briefly, ^{32}P-labeled oligonucleotides (100 ng, 10^6 cpm) were incubated with 100 μL of media containing 10% fetal bovine serum (FBS). At 0, 15 min, 1 h and 2 h, 25 μL samples were removed diluted with water (100 μL) and extracted with phenol saturated with chloroform (100 μL) and ethanol precipitated. The samples were subjected to PAGE (20%, 8 M urea) followed by autoradiography.

RNase-H mediated degradation of RNA/DNA duplex

Experiments on RNase H-mediated degradation of RNA/DNA duplex were performed, as described[29] using a chemically synthesized 36-mer phosphoric diester oligoribonucleotide **11** (sequence 5′-AGA AGG AGA GAG AUG GGU GCG AGA GCG UCA GUA UUA) corresponding to the codon 777–812 of the HIV *gag* gene.

Thermal denaturation studies

These studies were carried out using both **9** (sense DNA) and **10** (sense RNA). The sense DNA **9** was a 35-mer oligodeoxyribonucleotide corresponding to the codon 770–804 (with four extra bases in the 5′-direction and six extra bases extending in the 3′-direction of the 25-mer antisense sequence). The RNA **10** was a 25-mer sense RNA strand complementary to the antisense sequence. Oligonucleotide mixtures (sense and antisense) (0.2 $Å_{260}$ units of each) in 1 mL of buffer (100 mM NaCl, 10 mM NaH_2PO_4, pH 7.4) were heated to 95 °C for 5 min and gradually cooled to room temperature. The samples were heated at the rate of

0.5 °C/min prior to measurements of absorbance. Thermal transitions in absorbance were recorded at λ_{260} nm using a GBC 920 double beam UV–vis spectrophotometer.

Anti-HIV activity of oligonucleotides

Anti-HIV activity evaluation of oligonucleotides were carried out as previously described.[19,30] Briefly, $CD4^+$ T-cell line (MOLT-3. 5×10^5 per mL) were infected with HIV-1 type III_B. After 2 h, cells were washed and treated with oligonucleotides **1–8** at various concentrations. Cells were incubated for 7 days, split, fresh media and oligonucleotides were added and allowed to grow. Cells were further split at 10 days and treated with fresh media and oligonucleotides and cultured for further 4 days. The viral replication was measured by quantitative p24 ELISA and viable cell number was determined as previously described.[19]

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