Mixed backbone antisense oligonucleotides: design, biochemical and biological properties of oligonucleotides containing 2′-5′-ribo- and 3′-5′-deoxyribonucleotide segments

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ABSTRACT

We have designed and synthesized mixed backbone oligonucleotides (MBOs) containing 2′-5′-ribo- and 3′-5′-deoxyribonucleotide segments. Thermal melting studies of the phosphodiester MBOs (three 2′-5′ linkages at each end) with the complementary 3′-5′-DNA and -RNA target strands suggest that 2′-5′-ribonucleoside incorporation into 3′-5′-oligodeoxyribonucleotides reduces binding to the target strands compared with an all 3′-5′-oligodeoxyribonucleotide of the same sequence and length. Increasing the number of 2′-5′ linkages (from six to nine) further reduces binding to the DNA target strand more than the RNA target strand [Kandimalla,E.R. and Agrawal,S. (1996) *Nucleic Acids Symp. Ser.,* 35, 125–126]. Phosphorothioate (PS) analogs of MBOs destabilize the duplex with the DNA target strand more than the duplex with the RNA target strand. Circular dichroism studies indicate that the duplexes of MBOs with the DNA and RNA target strands have spectral characteristics of both A- and B-type conformations. Compared with the control oligonucleotide, MBOs exhibit moderately higher stability against snake venom phosphodiesterase, S1 nuclease and in fetal calf serum. Although 2′-5′ modification does not evoke RNase H activity, this modification does not effect the RNase H activation property of the 3′-5′-deoxyribonucleotide segment adjacent to the modification. *In vitro* studies with MBOs suggest that they have lesser effects on cell proliferation, clotting prolongation and hemolytic complement lysis than do control PS oligodeoxyribonucleotides. PS analogs of MBOs show HIV-1 inhibition comparable with that of a control PS oligodeoxyribonucleotide with all 3′-5′ linkages. The current results suggest that a limited number of 2′-5′ linkages could be used in conjunction with PS oligonucleotides to further modulate the properties of antisense oligonucleotides as therapeutic agents.

INTRODUCTION

Oligonucleotide analogs are extremely interesting because they can be used as diagnostic agents and molecular biological tools (1). The possible therapeutic use of oligonucleotides as effective gene regulatory agents in antisense and antigene approaches has kindled further interest in the development of oligonucleotide analogs in recent years (2–4). Rapid degradation of 'natural' phosphodiester (PO) backbone oligonucleotides by cellular nucleases (5,6) necessitated chemical modification of the PO backbone. Several chemically modified oligonucleotides, such as methylphosphonate (7,8), phosphorothioate (PS) (9,10) and phosphoramidate (11) oligonucleotides, are more stable against nucleases. Many of these modifications have been tested against several disease targets *in vitro* and *in vivo* (12). The PS oligonucleotides advanced to human clinical trials (13–15) because of their desirable pharmacokinetic and safety profiles observed *in vitro* and *in vivo* (5,6). In order to improve the pharmacokinetic and safety profiles of antisense PS oligodeoxyribonucleotides, mixed backbone oligonucleotides (MBOs) have been designed that contain at least two different chemical modifications. Recent studies on MBOs, such as hybrids, chimeras, etc., suggest that MBOs are more stable *in vivo* and exhibit fewer charge- and immune-related side effects while retaining the biological activity of PS oligodeoxyribonucleotides (16,17).

Most of the modifications currently explored for antisense purposes use the commonly occurring 3′-5′ linkage. In addition

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to the predominant 3′-5′ internucleotide linkage, a less abundant 2′-5′ internucleotide linkage is also formed in interferon-treated cells (18,19) and during intron splicing (20). Although formation of the 2′-5′ linkage is preferred over a 3′-5′ linkage under simulated prebiotic conditions (21,22), nature's selection of the 3′-5′ linkage over 2′-5′ linkage to preserve genetic material is not clear to date (23–25). A recent report described selective binding of 2′-5′-RNA or mixed backbone oligonucleotides (MBOs) of 2′-5′- and 3′-5′-RNA to natural (3′-5′) RNA targets over DNA based on thermal melting studies (26). The utility of 2′-5′-linked oligonucleotides, however, for antisense uses has not been explored extensively (26,27).

The 'natural' 3′-5′-linked oligonucleotides exist predominantly in the C2′-*endo* and C3′-*endo* sugar conformations (28). The C2′-*endo* sugar conformation exists exclusively in DNA, giving an extended B-type duplex structure, while the C3′-*endo* sugar conformation occurs in both RNA and DNA nucleotides giving a compact A-type structure in RNA and DNA duplexes (28). Recent molecular modeling (29) and NMR (30) studies showed that the C2′-*endo* sugar conformation is predominant in 2′-5′-RNA and the C3′-*endo* sugar conformation exists in 2′-5′-DNA. These conformations are exactly the opposite of what is observed with 3′-5′-ribo- and deoxyribonucleotides. We predicted that MBOs with a limited number of 2′-5′-ribonucleosides within a 3′-5′-deoxyribonucleotide core might bind efficiently to the 'natural' DNA and RNA complementary strands, since such MBOs possess a uniform intranucleotide phosphate distance throughout the oligonucleotide chain.

We chose a 25 base sequence (5′-AGAAGGAGAGAGAUG-GGUGCGAGAG-3′) from the initiation codon region of the HIV-1 *gag* mRNA as the target sequence for the present studies. A PS oligodeoxyribonucleotide complementary to this site has been studied extensively for its pharmacokinetic and safety profiles (14) and is currently in human clinical trials. We synthesized MBOs with different numbers of 2′-5′ linkages and in different locations within the 25mer sequence (Fig. 1). We studied the duplex forming ability of the MBOs with both the DNA and RNA complementary strands by UV thermal melting and gel mobility shift assays. The conformations of the duplexes of MBOs with the DNA and RNA target strands were characterized by circular dichroism (CD) spectroscopy. RNase H activation, nuclease stability and biological properties of the MBOs, including *in vitro* lymphocytic proliferation, coagulation and complement activation, were examined.

MATERIALS AND METHODS

Oligonucleotide synthesis and purification

Oligonucleotides were synthesized on a Milligen 8700 DNA synthesizer on a 1–2 μM scale using phosphoramidite chemistry (31). β-Cyanoethyl phosphoramidites were obtained from Millipore or Pharmacia. 3′-t-Butyldimethylsilyl-2′-β-cyanoethyl phosphor-amidites and 2′-t-butyldimethylsilyl-3′-β-cyanoethyl phosphor-amidites for 2′-5′- and 3′-5′-RNA synthesis respectively were purchased from Chemgenes. Either iodine oxidant or Beaucage reagent (32) was used, as required, for the synthesis of PO and PS oligonucleotides respectively. After synthesizing the oligo-nucleotides, CPG was treated with concentrated ammonium hydroxide at room temperature for 2 h and then the supernatant was heated at 55°C for 6 h for oligonucleotides **7** and **8**.
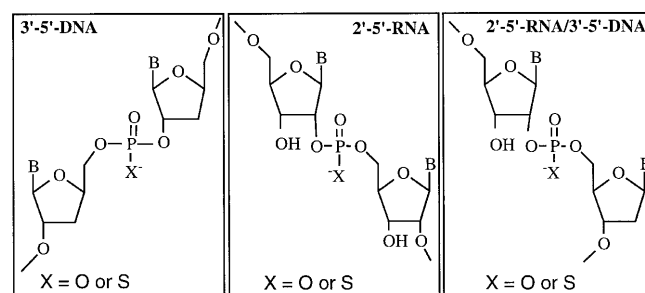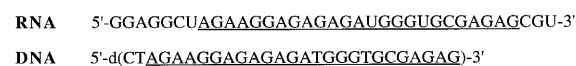

Figure 1. Oligonucleotide sequences used in the study. Bold letters represent 2′-5′-ribonucleotides. *2′-5′ linkage. Blank and filled rectangles represent PO and PS segments respectively. Structures of 3′-5′-DNA, 2′-5′-RNA and 2′-5′-RNA/3′-5′-DNA are shown in boxes.

Oligonucleotides with a 5′-DMT group were purified on a Waters 650 HPLC system using a 0–50% gradient of 0.1 M ammonium acetate and 80% acetonitrile containing 0.1 M ammonium acetate on a C_{18} reverse phase column. The appropriate peak was collected, concentrated and treated with 80% acetic acid at room temperature for 1 h to remove the 5′-DMT group. The oligo-nucleotides were desalted on Waters C_{18} Sep-pack cartridges and quantified by measuring absorbance at 260 nm using extinction coefficients calculated by the nearest neighbor method (33) after ascertaining the purity by PAGE.

MBOs (**1–6**) and the target oligoribonucleotide (RNA) were deprotected with a 3:1 mixture of ammonium hydroxide and ethanol at 55°C for ~15 h and then with 1 M tetrabutylammonium fluoride at room temperature for another 15 h. MBOs and normal RNA were then purified on 20% denaturing PAGE, eluted from the gel and desalted using C_{18} Sep-pack cartridges (Waters).

UV thermal denaturation studies

Thermal denaturation studies were performed by mixing MBOs with the DNA or RNA target strands in equimolar ratios in 10 mM disodium hydrogen phosphate, pH 7.5 ± 0.1, 100 mM sodium chloride buffer. The solutions were heated to 95°C for 10 min and allowed to come to room temperature slowly before being stored at 4°C overnight. The final total concentration of the oligonucleotide

strands was 2.0 μM. Spectrophotometric measurements were performed at 260 nm on a Perkin-Elmer Lambda 2 Spectrophotometer attached to a thermal controller and a personal computer using 1 cm path length quartz cuvettes at a heating rate of 0.5°C/min. Melting temperatures (T_m) were taken as the temperature of half-dissociation and were obtained from first derivative plots. Precision in T_m values, estimated from variance in two or three repeated experiments, was ±0.5°C.

CD experiments

The same oligonucleotide sample solutions used for UV thermal melting studies were used for CD experiments. The CD spectra were recorded on a JASCO J-710 Spectropolarimeter with a 0.5 cm quartz cell attached to a Peltier thermal controller. The samples were equilibrated at the required temperature for 15 min before recording the spectra. Each spectrum was an average of eight scans with the buffer blank subtracted, which was also an average of eight scans and obtained at the same scan speed (100 nm/min). All the spectra were noise reduced using the software supplied by Jasco Inc. and the molar ellipticities were calculated using the same software.

Electrophoretic mobility shift assay

The DNA target strand was labeled at the 5′-end with [32P] using [γ-[32P]]ATP (Amersham) and T4 polynucleotide kinase (Promega) (34). The RNA target strand was labeled at the 3′-end using T4 RNA ligase (New England Biolabs) and [[32P]]pCp (New England Nuclear) using standard protocols (34). A small amount of DNA or RNA target strand (~3000 c.p.m. labeled and 1 nM cold) was mixed with different ratios of MBOs in 10 mM disodium hydrogen phosphate, pH 7.4–7.6, 100 mM sodium chloride buffer. The samples were heated at 95°C for 15 min and allowed to come to room temperature before being stored at 4°C overnight. The samples were loaded on a non-denaturing 10% polyacrylamide gel with glycerol dye. The gel was run at room temperature using 50 mM Tris, 50 mM glycine buffer, pH 7.5. After electrophoresis the autoradiogram was developed by exposing the dried gel to Kodak X-Omat AR film at –70°C with an intensifying screen.

RNase H assay

The RNase H assay was performed as described earlier (35). Briefly, a small amount of the 3′-end-labeled RNA, 90 pmol yeast tRNA and the oligonucleotides under study were mixed in 30 μl 20 mM Tris–HCl, pH 7.5, 10 mM MgCl$_2$, 10 mM KCl, 0.1 mM DTT, 5% w/v sucrose and 40 U RNasin (Promega) and incubated at room temperature for 30 min. An aliquot (7 μl) was removed as a control and 0.8 U *Escherichia coli* RNase H (Boehringer-Mannheim) was added to the remaining reaction mixture. The reaction mixture with RNase H was incubated at room temperature and aliquots (7 μl) were removed at different time intervals. The samples were analyzed on a 7 M urea–20% polyacrylamide gel. After electrophoresis the autoradiogram was developed by exposing the dried gel to Kodak X-Omat AR film at –70°C with an intensifying screen.

Nuclease stability assays

Oligonucleotides were labeled at the 5′-end with [32P] using [γ-[32P]]ATP (Amersham) and T4 polynucleotide kinase (Promega) (34). The stability of the oligonucleotides in cell culture medium containing 10% fetal calf serum was tested by incubating a small amount of labeled oligonucleotide together with 100 ng cold oligonucleotide in DMEM cell culture medium (Gibco BRL) containing 10% non-heat-inactivated fetal calf serum (Gibco BRL) at 37°C in a final volume of 40 μl. Aliquots were removed at different time points.

For the snake venom phosphodiesterase assay, labeled oligonucleotide and cold oligonucleotide in buffer (10 mM Tris, pH 8.0, 100 mM sodium chloride, 10 mM MgCl$_2$) were incubated with 0.01 U snake venom phosphodiesterase (Boehringer-Mannheim) at 21°C (final volume 40 μl). Aliquots were removed at different time intervals for electrophoretic gel analysis. For the S1 nuclease assay, reactions were carried out as above but in 100 mM sodium acetate, pH 5.0, 10 mM zinc acetate buffer and with 1.4 U S1 nuclease (Gibco BRL) incubated at 37°C in a final volume of 50 μl. Aliquots were removed at different time intervals for electrophoretic gel analysis. Nuclease reactions were stopped by adding 5 μl formamide gel loading buffer to each sample and heating at 90°C for 5 min. All samples were then run on 20% polyacrylamide, 7 M urea gels and visualized by autoradiography.

In vitro cell proliferation assay

The cell proliferation assay was carried out as described earlier (36). Spleen cell (4–5-week-old male CD1 mouse, 20–22 g; Charles River, Wilmington, MA) suspensions were prepared and plated in 96-well dishes at a density of 10^6 cells/ml in a final volume of 100 μl. The cells were incubated at 37°C after adding 10 μl oligonucleotide solution. After 44 h incubation, 1 μCi [[3H]]thymidine (Amersham) was added and the cells were pulse labeled for another 4 h. The cells were harvested by an automatic cell harvester and the filters were counted using a scintillation counter. All experiments were carried out in triplicate.

Clotting assay

The activated partial thromboplastin time (aPTT) assay was performed with citrated normal human donor plasma in duplicate on an ST4 coagulation instrument (American Bioproducts, Tarsippany, NJ) according to recommended procedures using Actin FSL (Baxter Dade, Miami, FL) and 25 mM calcium to initiate clot formation, which was measured photometrically. Normal plasma aPTT values ranged from 27 to 39 s. Data were calculated as percent prolongation of clotting time compared with the saline control.

Hemolytic complement assay

A fresh normal human serum was mixed with oligonucleotides and then assayed for complement lysis of sheep red blood cells (Colorado Serum Co.) sensitized with anti-sheep red cell antibody (hemolysin; Diamedix, Miami, FL) as previously described (37–39) using 1:200 serum dilutions in triplicate. Hemoglobin release into cell-free supernatants was measured

spectrophotometrically at 541 nm. Data were calculated as 50% inhibition of lysis compared with the saline control.

HIV-1 inhibition assay

The HIV-1 inhibition assay was carried out as previously described (35). Briefly, serial dilutions of antisense oligonucleotides were prepared in 50 µl volumes of complete medium (RPMI-1640, 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin) in triplicate in 96-well plates. Virus, diluted to contain a 90% CPE dose of virus in 50 µl, was added, followed by 100 µl 4×10^5 MT-4 cells/ml in complete medium. The plates were incubated at 37°C in 5% CO_2 for 6 days. MTT dye was added and quantitated at A_{540}–A_{690} as described. Percent inhibition was calculated by the formula (experimental – virus control)/(medium control – virus control) × 100.

RESULTS AND DISCUSSION

Design and synthesis of mixed backbone oligonucleotides (MBOs)

Oligonucleotide sequences synthesized are shown in Figure 1. Oligonucleotides **1**–**3** contain three 2′-5′ linkages at each of the 5′- and 3′-ends. Oligonucleotides **4**–**6** contain three 2′-5′ linkages at each of the ends and an additional three 2′-5′ linkages in the middle (Fig. 1). Oligonucleotides **7** and **8** are control oligonucleotides containing all 3′-5′ linkages. An RNA synthesis cycle was used for coupling of 2′-5′-ribomonomers. Iodine or sulfurizing (3H-1,2-benzodithiol-3-one 1,1-dioxide; Beaucage reagent) oxidizing agent was used, as required, to synthesize PO or PS oligonucleotides respectively. After the synthesis, standard RNA (3′-5′) deprotection and purification protocols were followed. Incorporation of 2′-5′ linkages and base composition were confirmed by nuclease digestion of oligonucleotides and HPLC identification of the hydrolysis products (27).

UV thermal melting study

We studied thermal stability of the duplexes of MBOs with the DNA and RNA target strands in 10 mM disodium hydrogen
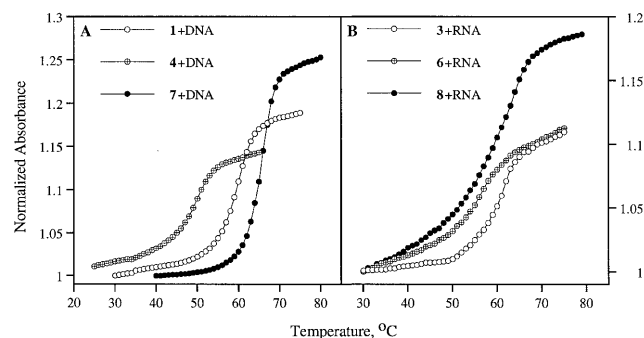

Figure 2. Thermal melting curves in 10 mM disodium hydrogen phosphate, pH 7.5, 100 mM sodium chloride for (**A**) duplexes of oligonucleotides **1**, **4** and **7** with the DNA target strand and (**B**) duplexes of oligonucleotides **3**, **6** and **8** with the RNA target strand. See Materials and Methods for oligonucleotide concentrations and experimental conditions.

phosphate, pH 7.5, and 100 mM sodium chloride. The T_m values determined for each oligonucleotide duplex with the RNA and DNA target strands are shown in Table 1. In general, sharp, cooperative and single transition melting curves were observed for all the oligonucleotides (Fig. 2A). Melting transitions were slightly broader with PS analogs than with PO analogs (Fig. 2B). The duplexes of MBO **1** with the DNA (Fig. 2A) and RNA target strands showed lower T_m values (~2.9 and 2.7°C respectively) than the duplexes of the control oligonucleotide **7** with the same DNA and RNA target strands (Table 1). The presence of 2′-5′ linkages in the middle of the sequence produced a higher destabilizing effect on the duplex with the DNA strand (ΔT_m –10.7°C) than with the RNA strand (ΔT_m –6.0°C) (Table 1). Similar results were observed with the duplexes of oligonucleotides **2** and **5**, which have 2′-5′ PO and 3′-5′ PS linkages (see Table 1 for T_m values). The lower hypochromicity of the duplexes of MBOs compared with the duplexes of control oligonucleotides **7** and **8** could reflect reduced stacking interactions of 2′-5′-nucleotides than 3′-5′-nucleotides (26,30).

Table 1. T_m values of duplexes of MBOs with the DNA and RNA target strands and their effects on cell proliferation, activated partial thromboplastin clotting time (aPTT), hemolytic complement and HIV-1 inhibition

Oligonucleotide	T_m (°C)		HIV-1 inhibition, IC_{50} (nM)	Stimulation index (lymphocyte proliferation) (at 10 µg/ml)	50% prolongation of clotting (aPTT) (µg/ml)	50% inhibition of complement lysis (µg/ml)
	DNA	RNA				
1	60.6	67.8	nd	0.67 ± 0.11	>>100.0	nd
2	52.5	63.3	99.9	2.82 ± 0.37	58.0	95.2
3	51.9	61.1	29.8	4.93 ± 0.15	51.1	59.1
4	52.8	64.5	nd	nd	>>100.0	nd
5	43.2	61.2	812.5	1.09 ± 0.05	115.0	nd
6	39.4	56.4	nd	0.23 ± 0.04	98.3	>500.0
7	63.5	70.5	nd	1.10 ± 0.13	>>100.0	>500.0
8	56.6	63.5	24.9	6.16 ± 0.12	23.2	34.2

nd, not determined.

Figure 4. Gel mobility shift assay for the binding of control oligonucleotides and MBOs to the DNA target strand. Lanes 1–5 contain the DNA target strand and the antisense oligonucleotides in the ratios 1:0, 1:0.1, 1:0.2, 1:1 and 1:2 respectively.
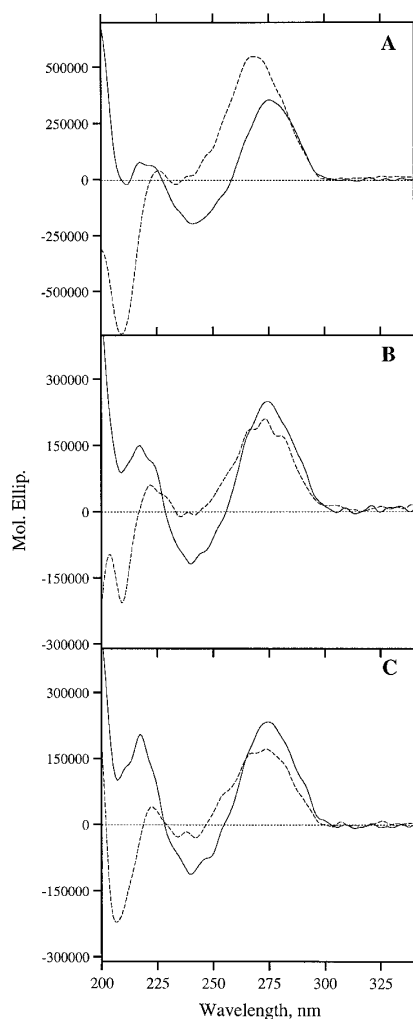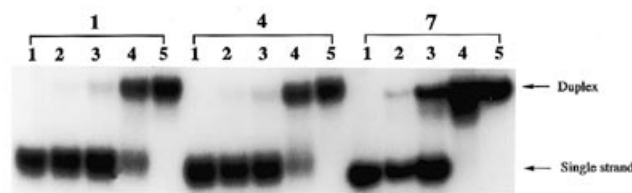

Figure 3, CD spectra of duplexes of oligonucleotides **8** (A), **3** (B) and **6** (C) with the DNA (——) and RNA (- - -) target strands. See Materials and Methods for oligonucleotide concentrations and experimental conditions.

Oligonucleotides **3** and **6** are PS analogs of **1** and **4** respectively. PS analogs showed interesting hybridization properties with the DNA and RNA strands. The duplexes of oligonucleotide **3** with the DNA and RNA (Fig. 2B) target strands had T_m values ~4.7 and 2.4°C lower respectively than the duplexes of control PS oligonucleotide **8** with the same DNA and RNA (Fig. 2B) target strands (Table 1). Similarly, oligonucleotide **6**, with nine 2′-5′ linkages, had T_m values 17.2 and 7.1°C lower than the duplexes of oligonucleotide **8** with the DNA and RNA target strands respectively (Table 1).

Comparison of the T_m values of the duplexes of PO and PS oligonucleotides with the DNA and RNA strands suggest that the 2′-5′ PS linkage has a greater destabilizing effect on the duplex with the DNA strand than the duplex with the RNA strand (Table 1), whereas PS modification of the 3′-5′ linkage has similar destabilizing effects on the duplex formed with the DNA and the RNA target strands.

CD experiments

CD spectra of the DNA and RNA duplexes with MBOs are recorded. A representative set of CD spectra for PS analogs (**3**, **6** and **8**) are shown in Figure 3. The duplexes of MBOs exhibit CD spectral characteristics similar to those of the duplexes of control oligonucleotides containing all 3′-5′ linkages. The CD spectra of the duplexes of MBOs with the DNA target strand suggest both B- and A-type (mixed) conformations (40). The duplexes of MBOs with the RNA target strand exhibit A-type CD spectral characteristics similar to those of the control oligonucleotide **8** (Fig. 3). The higher wavelength positive band of the RNA duplexes of MBOs is centered around 274 nm however, unlike that of the control oligonucleotide duplex with the RNA strand (268 nm). The CD experimental results confirm that MBOs **1**–**6** form ordered right-handed double helical structures with both the DNA and RNA complementary strands, like the control PO (**7**) and PS (**8**) oligonucleotides.

Electrophoretic mobility shift assay

Duplex formation by MBOs with both the DNA and RNA target strands is further confirmed by the electrophoretic mobility shift assay. A representative gel for the PO analogs (**1**, **4** and **7**) with the DNA target strand is shown in Figure 4. The appearance of a slow moving band with increasing concentrations of oligonucleotides suggests formation of duplex structures with the DNA target strand. The absence of any other bands except the duplex band at higher ratios (1:2) suggests that the new oligonucleotides form complexes with 1:1 stoichiometry, i.e. duplex structures only. These gel mobility shift experiments also suggest that the control oligonucleotide **7** has a higher affinity for the target strand than the two MBOs **1** and **4**. Similar results were obtained with other oligonucleotides with both the DNA and RNA target strands (data not shown).

RNase H hydrolysis

RNase H is an enzyme that selectively recognizes a 3′-5′-DNA–RNA heteroduplex and hydrolyzes the RNA strand of the heteroduplex (41). RNase H possesses both endo- and 3′→5′ exonuclease activities (42). RNase H requires a 4–6 bp hybrid duplex to elicit its activity on the target RNA strand (43). We investigated the RNase H activation properties of MBOs using the same 35mer RNA target strand used for the spectroscopic studies.
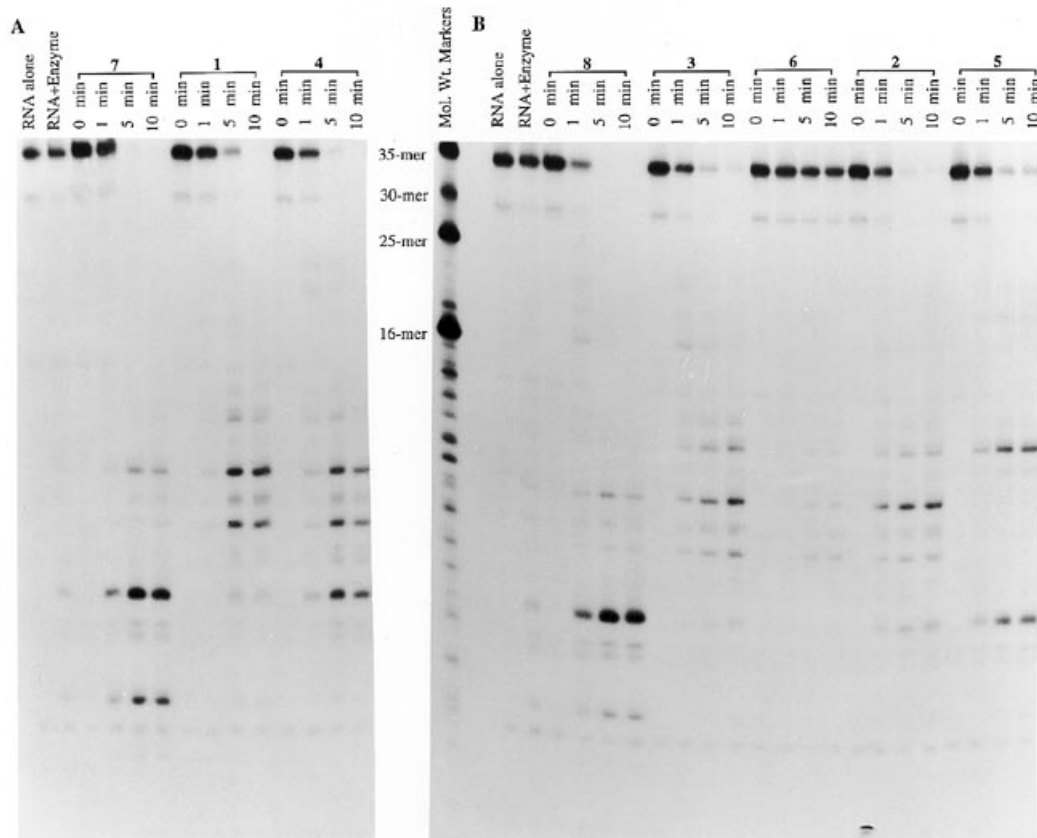
Figure 5. RNase H hydrolysis pattern of the RNA target strand in the absence and presence of (**A**) phosphodiester analogs of the control oligonucleotide (**7**) and MBOs (**1** and **4**) and (**B**) phosphorothioate analogs of the control oligonucleotide (**8**) and MBOs (**2**, **3**, **5** and **6**) at the indicated time points. Lanes labeled RNA + enzyme are the control lanes in the absence of antisense oligonucleotide. See Materials and Methods for experimental conditions.

Figure 5 shows the RNase H hydrolysis pattern of the target RNA in the presence of control oligonucleotides and MBOs. Both PO and PS analogs gave similar hydrolysis patterns. The rates of RNase H hydrolysis, however, were different for PO and PS analogs (43). The RNA hydrolysis pattern is different in the presence of MBOs than in the presence of control oligonucleotides. The absence of intense RNA hydrolysis bands in the lower half of the gel in the presence of MBOs **1–3** (Figs 5 and 6) compared with oligonucleotides **7** and **8** suggests that RNase H does not recognize the duplex region of 2′-5′-RNA with the RNA target strand. This result has been verified by synthesizing an all 2′-5′-oligoribonucleotide and studying its RNase H activation properties (data not shown). The RNA hydrolysis pattern in the presence of **1–3** also suggests that, as a result of the presence of 2′-5′ linkages at both the ends of the oligonucleotides, RNase H hydrolysis is confined to the middle of the RNA target strand, the portion that hybridizes with the 3′-5′-oligodeoxyribonucleotide segment of the MBOs.

Hybridization of MBOs **4–6** to the RNA target resulted in a slightly different RNase H hydrolysis pattern than in the case of MBOs **1–3**, but RNase H hydrolysis is confined to the heteroduplex region in this case also. Note that the lighter bands seen in the middle of the gel located around the 16mer marker in the lanes with control oligonucleotide and MBOs **1–3** were absent in the lanes containing MBOs **4–6**. This is the location where the central

RNA 2′-5′ linkages are present in the MBOs. These results suggest that 2′-5′-RNA does not evoke RNase H activity.

The 5′-phosphorylated trimer and higher oligomers of 2′-5′-adenosine activate an endonuclease, RNase L, (44) that degrades RNA and inhibits protein synthesis (45). This is an established mechanism for the action of interferon in virus-infected cells (45). It is not known whether 2′-5′-linked sequences such as the ones studied here would evoke RNase L activity.

Nuclease stability

Natural PO backbone oligonucleotides are digested in <5 min *in vivo*, making them unsuitable for therapeutic uses (5,6). PS analogs are considerably more stable *in vivo* (6,46). Any modified oligonucleotide that could be useful as an antisense agent should show reasonable stability against nucleases as well as acceptable hybridization properties with the target RNA. We have studied the stability of oligonucleotides **1**, **4** and **7** in DMEM cell culture medium containing 10% non-heat-inactivated FCS. Figure 6 shows the stability of the PO analogs of MBOs and the control oligonucleotide. Oligonucleotide **7** was digested quickly in serum with a short half-life (<30 min; Fig. 6). This result is consistent with the reported data on PO oligonucleotides (5). At the 2 h time point only a faint band of intact oligonucleotide **7** was present. MBOs **1** and **4** were intact up to 4 h. Both of the MBOs
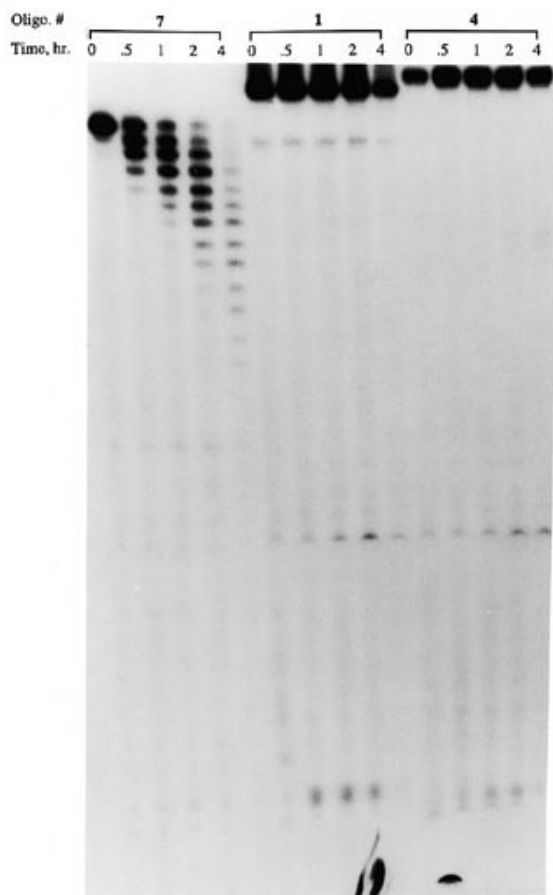
Figure 6. Stability of phosphodiester analogs of control oligonucleotide **7** and MBOs **1** and **4** in DMEM cell culture medium containing 10% fetal calf serum at different time points.

showed negligible digestion in this time period. These *in vitro* results suggest that 2′-5′ linkages are more stable to serum nucleases than 3′-5′ linkages.

The studies with snake venom phosphodiesterase (a 3′-exonuclease) suggest that oligonucleotides **1** and **4** have slightly higher stability than oligonucleotide **7** (Fig. 7A). Digestion of **1** and **4** in the presence of snake venom phosphodiesterase was mainly due to slow endonucleolytic activity rather than exonucleolytic activity. In studies with S1 nuclease (an endonuclease) both **1** and **4** were quickly digested, as in the case of **7** (Fig. 7B). Based on these results, we presume that the PS analogs of MBOs **1** and **4** may be more stable against nucleases than are PO analogs. We could not study the stability of PS analogs of MBOs **3** and **6** because of end-labeling problems.

HIV-1 inhibition studies

We studied the activity of MBOs compared with the control PS oligonucleotide **8** in inhibiting HIV-1 replication. The results are shown in Table 1 as the concentration required to inhibit viral replication by 50% (IC_{50}). All the oligonucleotides tested showed dose-dependent inhibition of viral replication but with different IC_{50} values. The control PS oligonucleotide **8** had an IC_{50} of 24.9 nM. A 4-fold higher concentration of MBO **2** was required to

achieve the IC_{50}. The PS MBO **3** had an IC_{50} of 29.8 nM, comparable with that of the control PS oligonucleotide IC_{50}. Although the PO analogs of MBOs showed greater stability against exo- and endonucleases and a greater affinity for target RNA *in vitro*, they did not show significant activity against HIV-1. The lack of HIV-1 inhibition by PO MBOs could result from their susceptibility to endonucleases. MBO **5**, which has nine 2′-5′ linkages, showed insignificant HIV-1 inhibition (IC_{50} > 800 nM). We have not included scrambled or random or mismatched oligonucleotides to demonstrate sequence specificity of **3**, as it is not a new sequence or not targeting a new site that has not been studied earlier. The current focus of the modification is to improve the pharmacokinetic properties and reduce side effects that are associated with first generation PS oligonucleotides, such as **8**.

Effect of MBOs on cell proliferation

Oligonucleotides induce spleen cell proliferation and antibody production *in vitro* and *in vivo* (36,47). These effects are sequence and chemical modification dependent (36). Recent studies suggest that unmethylated CpG dinucleotide motifs could be responsible for these effects (47). We have studied whether the MBOs induce cell proliferation *in vitro* and compared the results with those of the control oligonucleotides (**7** and **8**). The results are shown in Table 1 as proliferation index at 10 µg/ml concentration of the oligonucleotides. These results show that oligonucleotides with the PS modification have a greater effect on cell proliferation than those with the PO backbone. Comparison of the data for oligonucleotides **3** (4.9), **6** (0.23) and **8** (6.16) further suggest that 2′-5′-RNA motifs have a lower cell proliferation effect than does the control PS oligonucleotide (**8**).

Effect of MBOs on coagulation and complement

PS oligonucleotides show dose-dependent prolongation of coagulation and activation of complement *in vitro* (38,39) and *in vivo* (48). These effects are sequence independent but length dependent (48). Recent studies suggest that these effects could be modulated by backbone modifications (38,39). We studied the effects of MBOs on both coagulation and complement activities. The results are presented in Table 1 as the oligonucleotide dose that prolonged the aPTT by 50% and inhibited complement lysis by 50%.

In the coagulation assay, PO oligonucleotides **1**, **4** and **7** had negligible effects up to 100 µg/ml compared with a saline control. Control PS oligonucleotide **8** produced 50% prolongation of the clotting time at ~23 µg/ml. In the case of PS MBOs, a 2- to 3.5-fold higher concentration was required to attain 50% prolongation compared with that of oligonucleotide **8** (Table 1). These results suggest that the 2′-5′-RNA PS modification has less of an effect than the 3′-5′-DNA PS oligonucleotides in the aPTT coagulation assay.

The PO oligonucleotides of both control oligonucleotides and MBOs showed minimal effects on complement hemolytic activity, whereas the control PS oligonucleotide **8** inhibited serum complement hemolytic activity. PS MBOs also produced inhibition, but at higher concentrations than control oligonucleotide **8** (Table 1). These results suggest that PS 2′-5′-RNA oligonucleotides have a lower complement activation activity than normal DNA phosphorothioates.
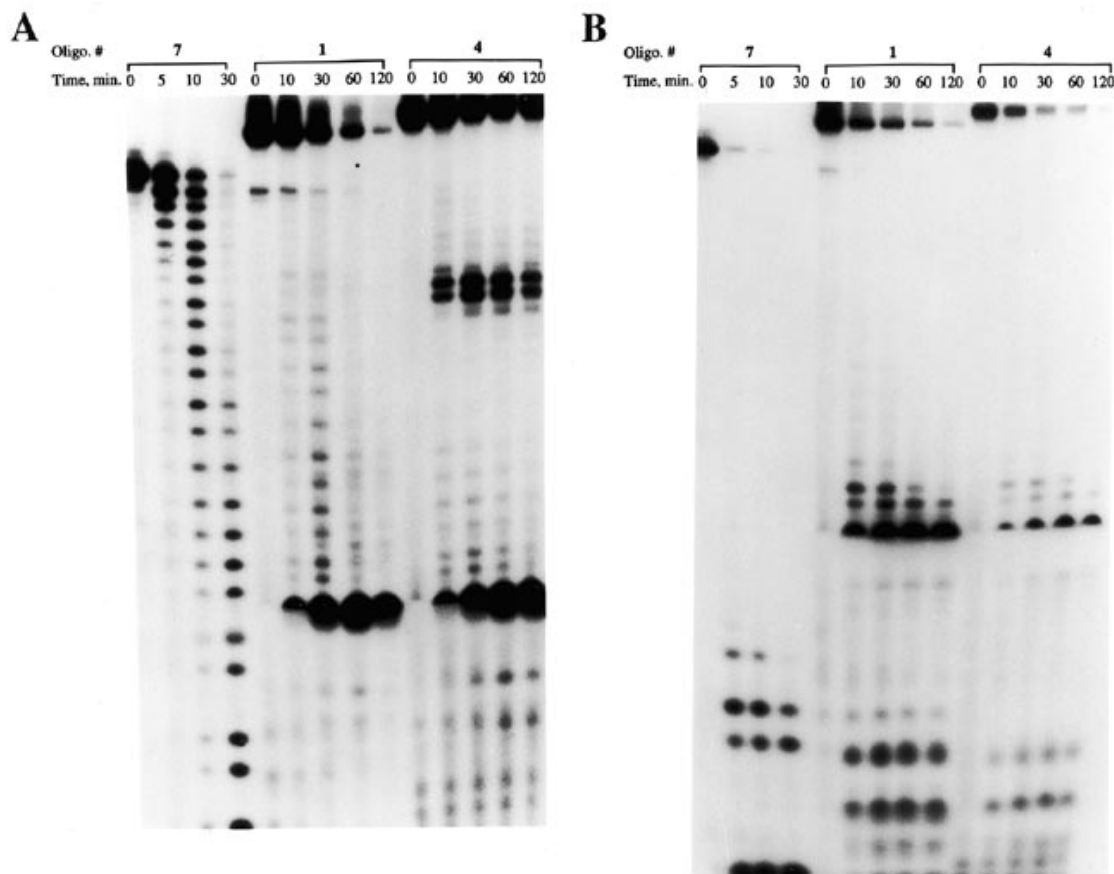
Figure 7. Stability of phosphodiester analogs of control oligonucleotide **7** and MBOs **1** and **4** against (**A**) snake venom phosphodiesterase and (**B**) S1 nuclease at different time points.

Conclusions

MBOs containing 2′-5′-ribonucleotides and 3′-5′-deoxyribonu-cleotides bind to both DNA and RNA target strands. The affinity of the MBOs for the DNA complementary strand decreases with increasing 2′-5′-ribonucleotide linkages. Interestingly, PS modification further reduces the binding affinity of MBOs for the DNA target strand. Higher thermal stability of MBO duplexes with RNA suggest that 2′-5′-RNA binds to the RNA target with higher affinity than the DNA target (26). Although *in vitro* studies show that PO MBOs have moderately greater stability against nucleases, insignificant activity in an HIV-1 inhibition assay in cell culture suggests that they are susceptible to nucleases. 2′-5′-Ribonucleotides do not evoke RNase H activity, as is the case with 2′-5′-deoxyribonucleotides (49). Control PS oligo-nucleotide **8** inhibits HIV-1 replication sequence specifically. We have shown that MBO **3** with the same sequence has activity comparable with that of control PS oligonucleotide **8**.

The phosphate (PS) group disposition is different in 2′-5′ linkages than in 3′-5′ linkages (unpublished modeling results) and hence we predicted that PS oligonucleotides containing 2′-5′ linkages might exhibit lower binding to plasma and cellular proteins than 3′-5′-linked PS oligonucleotides. Our preliminary results suggest that the MBOs with 2′-5′ linkages have different protein binding properties than the PS 3′-5′-oligonucleotides (data not shown). In addition, the MBOs are less immune

stimulatory and have significantly reduced effects on both complement and coagulation than control oligonucleotide **8**. These results correlate with the lower protein binding affinity of MBOs containing 2′-5′-ribonucleotide segments. The pharma-cokinetic and toxicological properties of PS MBOs are currently under evaluation.

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