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Self-stabilized antisense oligodeoxynucleotide phosphorothioates: properties and anti-HIV activity

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ABSTRACT

A new class of oligodeoxyribonucleotides has been designed, referred to here as 'self-stabilized' oligonucleotides. These oligonucleotides have hairpin loop structures at their 3′ ends, and show increased resistance to degradation by snake venom phosphodiesterase, DNA polymerase I and fetal bovine serum. The self-stabilized region of the oligonucleotide does not interfere in hybridization with complementary nucleic acids as shown by melting temperature, mobility-shift and RNase H cleavage studies. Various self-stabilized oligonucleotides containing increasingly stable hairpin loop regions were studied for their anti-HIV activity. Pharmacokinetic and stability studies in mice showed increased *in vivo* persistence of self-stabilized oligonucleotides with respect to their linear counterparts.

INTRODUCTION

Antisense oligonucleotides and their modified analogs have been used to regulate the expression of genes including ones implicated in cancer or in the replication of viruses and parasites (1–4). A major limitation in the use of antisense oligonucleotides as drugs is their nuclease susceptibility. Unmodified oligodeoxynucleotides in particular have limited survival *in vivo* (5). Oligodeoxynucleotides containing a phosphorothioate backbone have increased nuclease resistance but are eventually degraded (6,7). Degradation of unmodified and phosphorothioate oligodeoxynucleotides is primarily from the 3′ end. Various modifications of oligonucleotides at the 3′ ends avoid this problem (8–12). These include incorporation of a few nuclease resistant internucleotide linkages at a few specific sites at or near the 3′ end (2, 8,12, 19), incorporation of various chemical substituents at the 3′-hydroxyl (9,10,11) and circularization of the oligonucleotide by joining the 3′ and the 5′ ends (13).

Here we have designed and studied 'self-stabilized' oligonucleotides that have hairpin loop structures at the 3′ end. These compounds have increased nuclease resistance, hybridize effectively with the complementary nucleic acids and show increased anti-HIV activity. Self-stabilized oligonucleotide phosphorothioates also show increased *in vivo* stability compared to their linear counterparts.

METHODS AND MATERIALS

Synthesis and purification of oligonucleotides

Oligonucleotides both unmodified (PO) and phosphorothioate (PS) were synthesized on a 10 μmol scale by β-cyanoethyl phosphoramidite chemistry using an automated synthesizer (Millipore 8700, Bedford, MA). For oligodeoxynucleotides containing phosphorothioate internucleotide linkages, the iodine oxidation step was replaced by oxidation with ^{3}H-1,2-benzodithiol-3-one-1,1-dioxide (14). Deprotection and purification of oligonucleotides was carried out as described (15). ^{35}S-labeled phosphorothioate oligonucleotides were synthesized and purified by the same procedure as described earlier (6). The specific activities obtained for oligonucleotides **1**(PS), **2**(PS), **3**(PS), **4**(PS) and **5**(PS) were 1.4×10^{6} cpm/μg, 9.5×10^{5} cpm/μg, 7.6×10^{5} cpm/μg, 2.8×10^{6} cpm/μg and 1×10^{6} cpm/μg respectively. Oligoribonucleotides were synthesized as reported (16).

Exonuclease resistance of self-stabilized oligonucleotides

Sensitivity to 3′-exonuclease degradation was measured by (a) hyperchromicity at 260 nm in the presence of snake venom phosphodiesterase (SVPD) (b) digestion with 3′ exonucleolytic activity of DNA polymerase I (Pol I), and (c) incubation with fetal bovine serum. For studying the rate of digestion by hyperchromicity, 0.2 A_{260} unit of oligonucleotide was dissolved in 0.5 ml buffer (10 mM Tris, pH 8.5, 10 mM $MgCl_2$, 100 mM NaCl) and incubated with SVPD (0.025 μg, 6 units/mg) at 37°C in a thermally regulated cell of a UV spectrophotometer, and A_{260} was recorded against time (Figure 1). To study the resistance of self-stabilized oligonucleotides to the 3′ exonucleolytic activity of Pol I, 5′-^{32}P labeled oligonucleotides (40 pmole) were dissolved in 20 μl buffer (50 mM Tris, pH 7.2, 10 mM $MgSO_4$, 0.1 mM DTT, 0.5 mg/ml BSA) and incubated with *E.coli* Pol I (0.5 μl, 5 units), at 37°C. Aliquots were removed at 0, 30, 60, 120 and 240 minutes and analyzed by PAGE (20% polyacrylamide containing 8.3 M urea) followed by autoradiography (Figure 2 and 3).

For studying nuclease sensitivity of oligonucleotides against fetal bovine serum, 3.5 μg of each oligonucleotide (^{35}S labeled at each internucleotide linkage, specific activity 6×10^{5} cpm/μg) was incubated with 2 ml of culture medium containing 10% fetal bovine serum for 16 hours at 37°C. An aliquot (10 μl) was removed, extracted with phenol-chloroform and precipitated with

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ethanol. The samples were analyzed by electrophoresis on 20% polyacrylamide gel containing 8.3 M urea. The gel was fixed in acetic acid/methanol/water (10:10:80 v/v/v) solution and dried for 3 hours before autoradiography (Figure 4).

Binding of self-stabilized oligonucleotides to the complementary nucleic acids

Duplex stability. Melting temperatures were determined for the duplexes of unmodified or phosphorothioate self-stabilized oligonucleotides with the complementary DNA or RNA. Each oligonucleotide (0.2 A_{260} units) and its complementary nucleic acid were annealed in 1 ml buffer (10 mM Na_2HPO_4, pH 7.4, 100 mM NaCl) by heating to 80°C and then cooling to 40°C at 2°C/minute. The mixture was then reheated to 80°C at a rate of 1°C/minute and the A_{260} was continuously recorded. Melting profiles were obtained for oligonucleotides **1** and **5** (Table 1) containing phosphodiester [**1** (PO), **5** (PO)] or phosphorothioate [**1** (PS), **5** (PS)] linkages hybridized to the complementary oligoribonucleotides (Figure 5).

Binding assay. Hybridization of oligonucleotides **1** (PS) and **5** (PS) (Table 1) with a 39-mer HIV-1 *gag* RNA (5'AGAAGG-AGAGAGAUGGGUGCGAGAGCGUCAGUAUUAAGC3', corresponding to the *gag* region of HIV-1, 324−362) was studied by a gel mobility shift assay. ^{32}P labelled oligonucleotides (10 pmole, ~ 0.3 μCi) were mixed with 39-mer gag RNA (1 pmole) in 10 μl of 20 mM Tris−HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM KCl, 0.1 mM DTT containing 5% sucrose (w/v). The mixture was heated at 60°C for 3 minutes and then cooled to room temperature. RNasin (40 units Promega) and *E.coli* RNase H (0.5 μl, 0.4 units, Promega) were added to the mixture and incubated for 1 hour at 37°C. It was then analyzed on a native 15% polyacrylamide gel (Figure 6).

Site-specific cleavage of complementary RNA by self-stabilized oligodeoxynucleotides

Hybridization of oligonucleotides **1** (PS) and **5** (PS) (Table 1), with 39-mer *gag* RNA was studied using RNase H cleavage. Oligonucleotides (10 pmole) were mixed with 5'-^{32}P labeled 39-mer *gag* RNA (1 pmole) in 30 μl of 20 mM Tris−HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM KCl, 0.1 mM DTT and 5% sucrose (w/v) in the presence of RNasin (40 units, Promega). 7 μl of the mixture was removed as control and to the remaining mixture, 0.5 μl (0.4 units) of *E.coli* RNase H (Promega) was added. The mixture was incubated at 37°C and aliquots of 7 μl were removed at 1 minute, 15 minutes and 30 minutes. All aliquots were mixed with 10 μl formamide. The reaction products were then analyzed by PAGE (20% polyacrylamide gel containing 8.3M urea) and autoradiographed (Figure 7).

Assay for HIV-1 inhibition

H9 lymphocytes were infected with HIV-1 (~0.1 $TCID_{50}$/cell) for one hour at 37°C. Unabsorbed virions were then removed by washing, and infected cells were divided among wells of 24 well plates in fresh medium. Oligonucleotides at various concentrations were added and the cells were cultured for three days. At the end of this time, supernatants from infected cultures were collected and the levels of HIV-1 p24 expression were measured by a monoclonal antibody-based p24 antigen capture assay (17). The level of p24 expression was then compared with that of untreated infected cells. Four concentrations of each oligonucleotide were tested and ED_{50} (a dose to cause 50% suppression of HIV-1 replication) was determined by a dose v/s inhibition plot.

In vivo stability of self-stabilized phosphorothioate oligonucleotides

The oligonucleotides **1**(PS), **2** (PS), **3**(PS) and **4**(PS) and **5**(PS) were mixed with respective unlabeled oligonucleotides to obtain 15 μCi/0.2 mg. Each oligonucleotide, at a dose of 5 mg/kg in 0.25 ml of physiological saline, was administered intravenously in mice through the tail vein. At 24 hour post dosing, the animals were sacrificed and organs were removed. The organs were weighed, homogenized in 5 ml of water and radioactivity was measured.

Oligonucleotides were extracted from the homogenized tissues (200 μl) by the same procedure as described earlier, (6) and analyzed for their stability by electrophoresis on 20%

Table 1. Oligonucleotides and their self-stabilized analogs

Oligonucleotide Number	Sequence (5'-3')	Chain Length
1	CTCTCGCACCCATCTCTCTCCTTCT	25
2	CTCTCGCACCCATCTCTCTCC$^{T}_{T}$ GAGG$_{T}$C	29
3	CTCTCGCACCCATCTCTCTCC$^{T}_{T}$ GAGAGG$_{T}$C	31
4	CTCTCGCACCCATCTCTCTCC$^{T}_{T}$ GAGAGAGG$_{T}$C	33
5	CTCTCGCACCCATCTCTCTCC$^{T}_{T}$ TAGAGAGAGG$_{T}$C	35
6	CTCTCGCACCCA^{T}CTC$_{T}$C GAGAGCGTGGGT$_{C}$TTC^{T}	36
7	CTCTCGCACCCATCTCTCTCCTTCTAGCCTCCGCT	35
8	CTCTCGCACCCATCTCTCTCCTTCT(T)$_{10}$	35

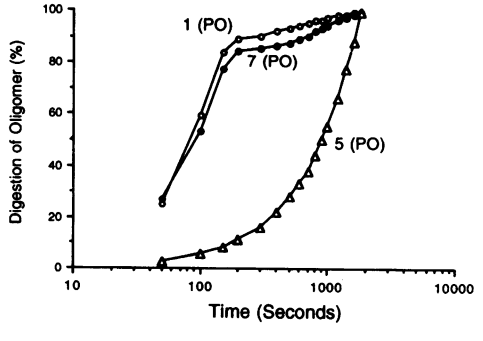

Figure 1. Digestion of phosphodiester-backbone oligonucleotides 1(PO), 5(PO) and 7(PO) with snake venom phosphodiesterase.

polyacrylamide−8.3 M urea gels. The gel was fixed in acetic acid/methanol/water (10:10:80, v/v/v), dried and autoradiographed.

RESULTS

Nuclease sensitivity of self-stabilized oligonucleotides

The nuclease sensitivity of self-stabilized oligonucleotides containing phosphodiester or phosphorothioate internucleotide linkages was studied. Two enzymes—SVPD and Pol I were used for comparative digestion studies of self-stabilized oligonucleotides. SVPD degrades oligonucleotides more readily than Pol I. Oligonucleotides **1** (PO), **5** (PO) and **7** (PO) (Table 1) were digested with SVPD and the rate of digestion was monitored by hyperchromicity. The $t_{1/2}$ of digestion of oligonucleotides **1** (PO), **5** (PO) and **7** (PO) were 88, >1000 and 96 seconds respectively (Figure 1). Thus oligonucleotides **1** (PO) and **7** (PO) containing no self-complementary regions had the same $t_{1/2}$ of digestion, whereas the partially base paired oligonucleotide **5** (PO) was more stable. The increase in stability depended on the number of base pairs in the hairpin loop region. The 3′ exonuclease activity of Pol I digested unmodified oligonucleotide **1** (PO) to mononucleotides in 30 minutes, whereas oligonucleotide **5** (PO) was digested very slowly, and at 120 minutes the enzyme treated mixture contained 35, 34 and 33 mers only (Figure 2).

Similarly, phosphorothioate oligonucleotides **1** (PS), **2** (PS), **3** (PS), **5** (PS) and **8** (PS) were studied for their sensitivity to digestion by 3′ exonuclease using Pol I (Figure 3). Oligonucleotides **1** (PS) and **8** (PS) were digested extensively by 3′ exonuclease at 240 minutes whereas oligonucleotide **5** (PS) was more resistant. Oligonucleotide **2**(PS) and **3**(PS) showed increased stability compared to oligonucleotide **1**(PS). However, they were digested at 240 minutes. The stability was dependent on the number of base pairs in the self-stabilizing region. Thus oligonucleotide **5** (PS) was considerably more stable than oligonucleotides **2** (PS) and **3** (PS). Similar results were obtained

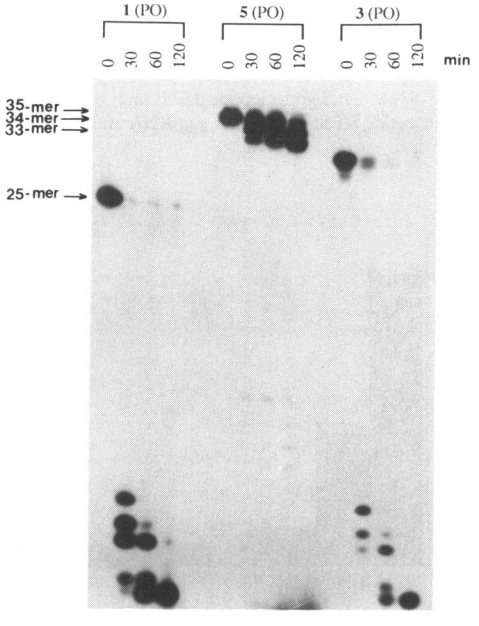

Figure 2. Digestion of oligonucleotides **1**(PO), **3**(PO) and **5**(PO) by 3′-exonucleolytic activity of *E.coli* DNA polymerase I.

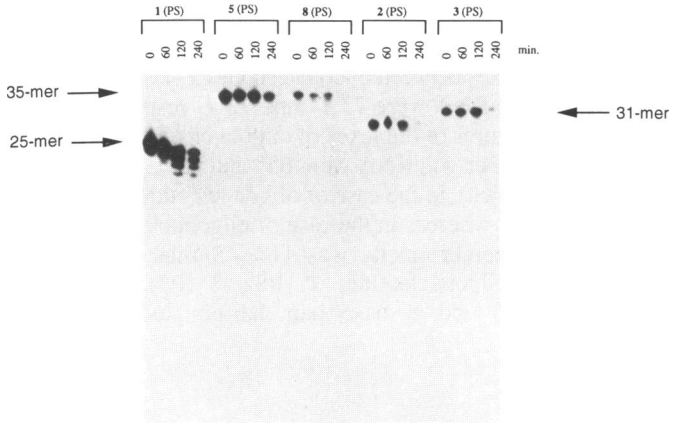

Figure 3. Digestion of oligonucleotide phosphorothioates **1**(PS), **2**(PS), **3**(PS), **5**(PS) and **8**(PS) by 3′-exonucleolytic activity of *E.coli* DNA polymerase I.

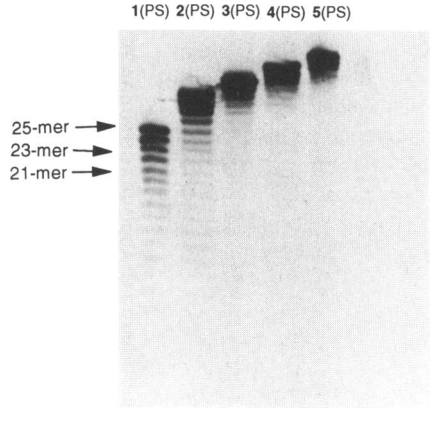

Figure 4. Digestion of oligonucleotide phosphorothioates **1**(PS), **2**(PS), **3**(PS), **4**(PS), and **5**(PS) in presence of 10% calf serum at 37°C for 16 hours.

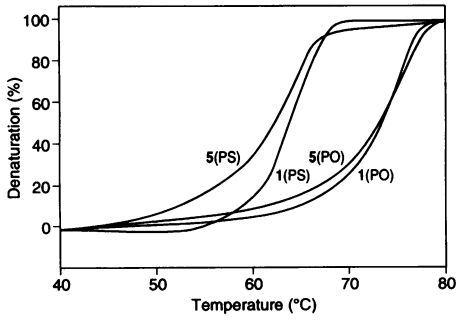

Figure 5. Melting temperature profiles of oligonucleotides to its complementary 39 mer *gag* RNA. A) Oligonucleotide phosphorothioate **5**(PS), B) oligonucleotide phosphorothioate **1**(PS), C) oligonucleotide phosphodiester **5**(PO) and D) oligonucleotide phosphodiester **1**(PO).

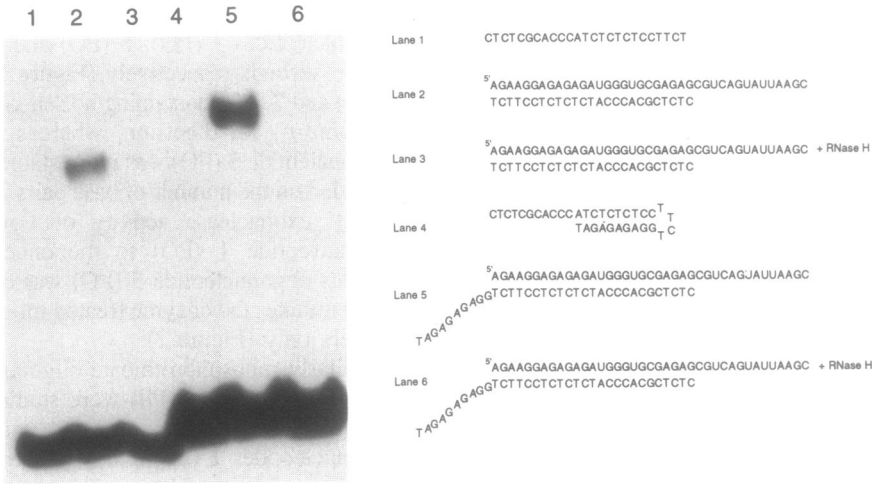

Figure 6. Binding assay—^{32}P labelled oligonucleotide phosphorothioates **1**(PS) and **5**(PS) were incubated with the complementary 39-mer gag RNA either in the presence or absence of RNase H. 1) Oligonucleotide **1**(PS); 2) oligonucleotide **1**(PS) and 39 mer *gag* RNA; 3) oligonucleotide **1**(PS) and 39 mer *gag* RNA after RNase H treatment; 4) oligonucleotide **5**(PS); 5) oligonucleotide **5**(PS) and 39 mer *gag* RNA; 6) oligonucleotide **5**(PS) and 39 mer *gag* RNA after RNase H treatment.

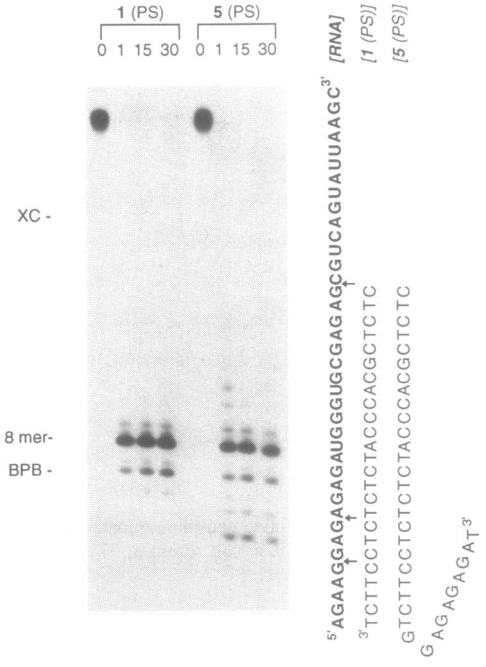

Figure 7. Specific cleavage of 39 mer *gag* RNA in presence of oligonucleotide phosphorothioates **1**(PS) and **5**(PS) by RNase H. Arrows show the major cleavage sites. If the hairpin loop region is intact, RNase H cleavage will yield 16-mer or higher length of RNA.

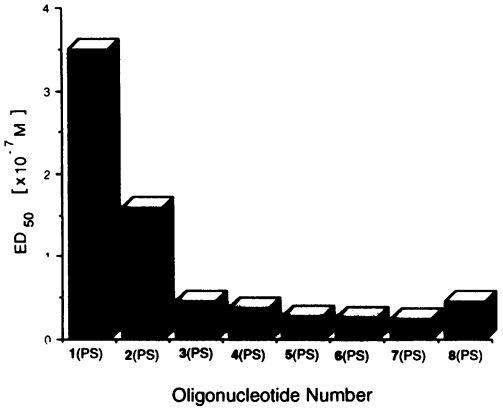

Figure 8. Anti-HIV activity of oligonucleotide phosphorothioates **1**(PS) to **8**(PS). All oligonucleotides showed dose-dependent activity. From the dose response curve, the inhibitory dose to suppress 50% HIV replication (ED_{50}) was determined.

when oligonucleotides **1** (PS), **2** (PS), **3** (PS),**4** (PS) and **5** (PS) were studied for their sensitivity in fetal bovine serum (Figure 4). Oligonucleotides **1** (PS) and **2** (PS) were digested extensively, whereas oligonucleotides **3** (PS), **4** (PS) and particularly **5** (PS) were digested slowly, and most of the oligonucleotides were found to be intact.

Melting temperatures (T_m)

Melting temperatures of oligonucleotides **1** and **5** containing phosphodiester or phosphorothioate linkages with the complementary 39-mer *gag* RNA were determined (Figure 5). The Tm of duplexes between oligonucleotides **1** (PO) and **5** (PO), with 39-mer *gag* RNA were 73.1° and 72°C respectively. The melting temperatures of duplexes of oligonucleotides **1** (PS) and **5** (PS), with 39-mer *gag* RNA were 65° and 63°C respectively. The hyperchromicity in the case of oligonucleotides **1** (PO) and **5** (PO) was 16% whereas in the case of oligonucleotides **1** (PS) and **5** (PS), hyperchromicity was 11%. Similar results were obtained with oligonucleotides **2** (PS), **3** (PS) and **4** (PS) containing 4, 6 and 8 base pair hairpin loop structures respectively.

Binding assay

The binding of the two oligonucleotide phosphorothioates, **1** (PS) and **5** (PS) with 39-mer *gag* RNA was studied by mobility shift on a native 15% polyacrylamide gel (Figure 6). When the mixture of 39-mer *gag* RNA and oligonucleotide **1** (PS) and **5** (PS) was analyzed, the mixture showed an expected shift in mobility attributable to duplex formation. When the mixture of 39-mer

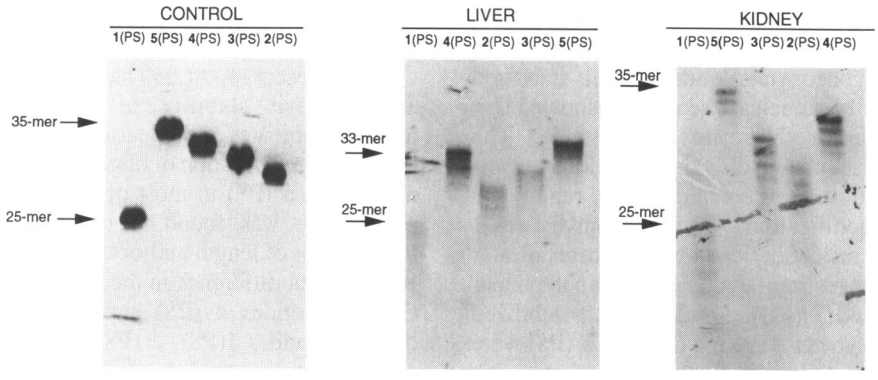

Figure 9. Stability of oligonucleotides phosphorothioate **1**(PS) to **5**(PS) in kidney and liver after intravenous administration to mice. Left hand side (control) is autoradiograph of ^{35}S labeled oligonucleotides before administration and right hand side shows the oligonucleotides phosphorothioate extracted from liver and kidney tissues at 24 hour post dosing.

gag RNA and oligonucleotides **1** (PS) and **5** (PS) was first treated with RNase H, no shift in mobility was observed.

RNase H cleavage

The binding of oligonucleotides and their self-stabilized analogs to 39-mer *gag* RNA was also confirmed by RNase H cleavage. Oligonucleotide **1** (PS) and its self-stabilized analog **5** (PS), both containing phosphorothioate linkages, were studied. Figure 7 shows that specific cleavages of 39-mer *gag* RNA by RNase H in presence of oligonucleotide **1** (PS) and its self-stabilized analog **5** (PS) were at the same sites. The major cleavage sites are shown by arrows in figure 7. This indicates that the hairpin loop structure in oligonucleotide **5** (PS) does not interfere with hybridization and that it opens when oligonucleotide **5** (PS) is hydrogen bonded to complementary RNA. In the absence of complementary oligodeoxynucleotides, no cleavage of 39-mer gag RNA by RNase H was observed (data not shown).

Antiviral activity of oligonucleotide phosphorothioates

The effects of self-stabilized oligonucleotide phosphorothioates on the replication of HIV-1 are presented in Figure 8. The oligonucleotide phosphorothioate **1** (PS), a 25-mer, complementary to the translational initiation region of *gag* mRNA showed a dose dependent activity. Its inhibitory dose to cause 50% suppression (ED_{50}) of HIV-1 replication, as assayed by p24 expression, was 3.5×10^{-7}M. Oligonucleotide phosphorothioate **7** (PS), a 35-mer, fully complementary to the same region of HIV-1, showed an ED_{50} of 0.25×10^{-7}M. This is in agreement with earlier findings that oligonucleotide phosphorothioates show length dependent inhibition of HIV-1 replication (16).

Oligonucleotide phosphorothioates **2** (PS), **3** (PS), **4** (PS), **5** (PS) and **6** (PS), which have hairpin loop structures showed increased antiviral activity compared to oligonucleotide phosphorothioate **1** (PS). The ED_{50} for oligonucleotides **2** (PS), **3** (PS), **4** (PS), **5** (PS) and **6** (PS) were 1.6, 0.45, 0.37, 0.27, and 0.26×10^{-7}M respectively. Oligonucleotide phosphorothioate **8** (PS), which has an additional ten bases at the 3′ end compared to oligonucleotide **1** (PS), but no hairpin loop structure, had an ED_{50} of 0.45×10^{-7} M.

In vivo stability of oligonucleotide phosphorothioates

The *in vivo* stability studies of oligonucleotides phosphorothioate were carried out after intravenous administration in mice. Biodistribution of oligonucleotides **1** (PS), **2** (PS) **3** (PS), **4** (PS) and **5** (PS) in liver and kidney showed no significant difference (data not shown). The excretion rate in urine was similar, an overall 30% of the administered dose of oligonucleotides were excreted at 24 hour post-dosing. However, when these oligonucleotides were analyzed for their stability, a significant difference was observed. (figure 9). Oligonucleotide **1** (PS) was degraded to almost 80% in liver at 24 hour post-dosing. All self-stabilized oligonucleotides **2** (PS), **3** (PS), **4** (PS) and **5** (PS) showed increased stability over their linear counterpart−oligonucleotide **1** (PS). Oligonucleotide **4** (PS) and **5** (PS) were the most stable and less than 20% degradation was observed. Similar increase in stability of self-stabilized oligonucleotides was observed in kidney tissue. The rate of degradation, however, was faster.

These results of *in vivo* stability suggest that the stability of oligonucleotides increases with the stability of the hairpin loop region. These results also corroborate with the *in vitro* study described in figure 4.

DISCUSSION

Antisense oligonucleotide phosphorothioates have been shown to be effective inhibitors of human immunodeficiency virus (18−26), influenza virus (27), human papilloma virus (29) and Herpes simplex virus (30) in tissue culture studies. Preliminary toxicity (18) and pharmacokinetic (6,7) studies in mice, rats and monkeys have been carried out, and the results indicate that oligonucleotide phosphorothioates can be envisioned as potential pharmaceuticals.

In pharmacokinetic studies, we and others have shown that oligonucleotide phosphorothioates are degraded mainly from the 3′ end, and have a half-life of about 24 hours in numerous mouse and rat tissues, following intravenous or intraperitoneal administration. (6,7). In previous studies we increased the half-life of oligonucleotide phosphorothioates (a) by blocking the 3′ hydroxyl with various chemical moieties (10) and (b) by incorporating at the 3′ end a few contiguous nuclease-resistant internucleotide linkages such as methylphosphonate, methylphosphonothioate or phosphoramidates (8).

In the present study, oligodeoxynucleotides have been stabilized by creating hairpin structures at the 3′ end. Oligonucleotides containing increasing numbers of base pairs in the stem region of the hairpins (Table 1) have been compared for their synthesis,

biochemical properties, antiviral activity, pharmacokinetics and *in vivo* stability with their linear counterparts.

Comparative study of oligodeoxynucleotides and self-stabilized oligodeoxynucleotides for their nuclease sensitivity showed that self-stabilized oligodeoxynucleotides are more stable. The nuclease stability of self-stabilized oligodeoxynucleotide was dependent on the stability of the self complementary region and nature of the phosphate modification. As clear from figures 1 and 2, self-stabilized oligonucleotides containing phosphodiester linkages, **5** (PO) were more resistant to nucleases than the linear oligonucleotides **1** (PO) and **7** (PO). Similarly, self-stabilized oligonucleotide phosphorothioates **3** (PS), **4** (PS) and **5** (PS) were found to be more stable, compared to their linear counterparts against nucleases at 37°C, at which temperature the self-complementary region of oligodeoxynucleotide phosphorothioate remained intact (figures 3 and 4). The melting temperatures of oligodeoxynucleotide phosphorothioates **3** (PS), **4**(PS) and **5**(PS) for their hairpin loops (in absence of complementary RNA/DNA) were 39°, 42° and 68°C respectively. The stability of oligodeoxynucleotides also increased with the increased number of bases pairing in the stem region of the oligodeoxynucleotide. In general, self-stabilized oligodeoxynucleotide phosphorothioate showed more resistance to nucleases than their counterparts containing phosphate linkages.

The self-stabilized region of the oligonucleotides does not interfere in binding to complementary nucleic acids. Melting temperature studies of oligonucleotides **1** (PO) and **1** (PS) and their self-stabilized analogs **5** (PO) and **5** (PS) with complementary RNA showed that all four oligonucleotides gave cooperative melting. Similar results of Tm's were obtained for other self-stabilized oligonucleotides when studied using complementary RNA or DNA (data not shown). In the case of self-stabilized oligonucleotides, Tms were not significantly lower compared to their linear counterparts. Binding of self-stabilized oligonucleotides to complementary nucleic acids was also confirmed by cleavage of RNA at specific sites in presence of RNase H and mobility shift assay.

The effect of self-stabilization of an oligonucleotide phosphorothioate on its antiviral activity against HIV was studied in tissue culture. We showed previously that oligonucleotide phosphorothioates (20-mer, 24-mer, 25-mer and 28-mer) complementary to the initiation codon of HIV-1 gag gene are effective in inhibiting HIV-1 replication in acute infection, chronic infection and long term infection assays (24–26, 31). In the present study, oligonucleotides **1** (PS) and **7** (PS), 25-mer and 35-mer respectively, are fully complementary to the initiation codon of gag gene of HIV-1 and oligonucleotides **2** (PS), **3** (PS), **4** (PS), **5** (PS) and **6** (PS) are self-stabilized analogs of oligonucleotide **1** (PS). Oligonucleotide **8** (PS), a 35-mer, which has ten more bases at the 3'-end than oligonucleotide **1** (PS) but is not self-stabilized, was synthesized as a control.

Oligonucleotides **2** (PS), **3** (PS), **4** (PS), **5** (PS) and **6** (PS) showed increased anti-HIV activity, which is probably due to the increased nuclease resistance. Oligonucleotide **5** (PS) and **6** (PS) with ten and twelve base pairs respectively in the stem region were the most effective. The anti-HIV activity of oligonucleotide **7** (PS), which forms 35-base pairs with HIV RNA and self-stabilized analogs **5** (PS) and **6** (PS) had similar anti-HIV potency. Oligonucleotide **8**(PS), a 35-mer containing no self-stabilized region showed almost similar anti-HIV activity as oligonucleotides **5** (PS) and **6** (PS) containing self-hybridized region. It can not be ruled out that increase in anti-HIV activity seen here could also be related to increased length of oligonucleotides, as we have shown in our previous studies (31).

In the present report, we have compared the pharmacokinetics and *in vivo* stability of self-stabilized oligonucleotide phosphorothioates and linear oligonucleotide phosphorothioates in mice. Biodistribution of oligonucleotides **1** (PS), **2** (PS), **3** (PS), **4** (PS) and **5** (PS) in most of the major organs including liver and kidney was found to be similar, suggesting that it is independent of length and/or the hairpin region. However, there is significant difference in the stability of these oligonucleotides. Oligonucleotides **4** (PS) and **5** (PS) were more stable than oligonucleotides **1**(PS), **2** (PS) and **3** (PS). The stability of the oligonucleotides was dependent on the stability of the hairpin loop region. Increased *in vivo* persistence of the self-stabilized oligonucleotide phosphorothioates would provide a longer duration of action *in vivo*, compared to their linear counterparts, thereby improving their potential as pharmaceutical agents.

The approach to self-stabilized oligonucleotides described here is applicable to both oligodeoxyribonucleotides and to oligoribonucleotides containing phosphodiester, phosphorothioate or other nuclease susceptible internucleotide linkages. As self-stabilized oligonucleotides are only partially single stranded they are expected to reduce undesired interaction with sequences in non-targeted nucleic acids.

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