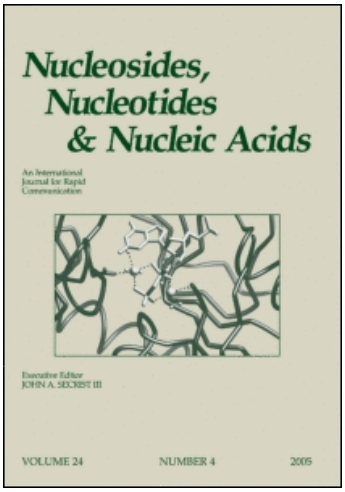

Nucleosides, Nucleotides and Nucleic Acids
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597286

Mixed-Backbone Oligonucleotides Containing Phosphorothioate and Methylphosphonate Linkages as Second Generation Antisense Oligonucleotide

Sudhir Agrawal[a]; Zhiwei Jiang[a]; Qiuyan Zhao[a]; Denise Shaw[b]; Daisy Sun[a]; Carl Saxinger[c]
[a] Hybridon, Inc., Worcester, MA [b] Lurleen B. Wallace Tumor Institute, University of Alabama at Birmingham, South, Birmingham, AL [c] National Cancer Institute-Division of Basic Sciences, Bethesda, MD

To cite this Article Agrawal, Sudhir , Jiang, Zhiwei , Zhao, Qiuyan , Shaw, Denise , Sun, Daisy and Saxinger, Carl(1997) 'Mixed-Backbone Oligonucleotides Containing Phosphorothioate and Methylphosphonate Linkages as Second Generation Antisense Oligonucleotide', Nucleosides, Nucleotides and Nucleic Acids, 16: 7, 927 — 936
To link to this Article: DOI: 10.1080/07328319708006109
URL: http://dx.doi.org/10.1080/07328319708006109

NUCLEOSIDES & NUCLEOTIDES, 16(7-9), 927-936 (1997)

MIXED - BACKBONE OLIGONUCLEOTIDES CONTAINING PHOSPHOROTHIOATE AND METHYLPHOSPHONATE LINKAGES AS SECOND GENERATION ANTISENSE OLIGONUCLEOTIDE

Sudhir Agrawal,*[1] Zhiwei Jiang,[1] Qiuyan Zhao,[1] Denise Shaw,[2] Daisy Sun and Carl Saxinger[3]
[1]Hybridon, Inc., One Innovation Drive, Worcester, MA 01605;[2] Lurleen B. Wallace Tumor Institute, University of Alabama at Birmingham, 1824 6th Ave. South, Birmingham, AL 35233; [3] National Cancer Institute-Division of Basic Sciences, Building 37, Room 6B04, Bethesda, MD 20892

ABSTRACT: Antisense oligonucleotides are being studied as novel therapeutic agents. To further improve the properties of antisense oligonucleotides, we have synthesized phosphorothioate oligonucleotides containing methylphosphonate linkages at the 5'-end, the 3'-end, or in the center, and have evaluated the impact of these linkages on the biophysical properties, biological properties, and some of the safety parameters.

Phosphorothioate oligodeoxynucleotides (PS-oligos) have been shown to be effective in the down regulation of gene expression in various *in vitro* and *in vivo* models and a number of clinical trials are underway.[1]

While PS-oligos continue to show promising results as first generation of antisense oligos, they have certain limitations. Toxicity studies of PS-oligos in rats have shown dose limiting side effects including thrombocytopenia, elevation of liver transaminases, hyperplasia of lymphoid cells and reticuloendothelial cells in various organs[1,2]. Additional side effects have been observed in monkeys following bolus intravenous administration including hemodynamic changes[1,3,4]. Activation of complement and prolongation of activated partial thromboplastin clotting time (aPTT) have been observed as well[4]. Most of these side effects in monkeys can be avoided by slow intravenous infusion[3].

The majority of the side effects observed (e.g. thrombocytopenia, elevation of liver enzymes, complement activation, prolongation of aPTT) have also been observed following administration of dextran sulfate[1] suggesting that these effects may be related to

the polyanionic nature of the oligonucleotides. In our efforts to design second generation antisense oligos that have fewer side effects while retaining the biological potency, we have studied mixed backbone oligonucleotides (MBOs)[1,7-11]. In the MBOs, one segment is a PS - oligo which is a substrate for RNase H and another segment is modified DNA or RNA, which could modulate the biophysical, biochemical, biological, pharmacodynamic and , pharmacokinetic properties and the *in vivo* stability of oligos.

Two classes of MBOs have been studied; one in which modified segments of the oligonucleotides were incorporated at both the 3'- and 5'- ends of PS-oligos (end modified MBOs, Fig. 1) and another in which modified segments of oligonucleotides were incorporated at the 3'-end, the 5'-end, or in the center (Fig. 1). In our earlier studies, we have shown that end modified MBO's have increased biological potency and are significantly more stable *in vivo*[9,10], and produce fewer side effects (e.g., complement activation and prolongation of aPTT)[4] and mitogenic response[11] than unmodified PS - oligos. In the present study, we have designed oligos that contain nonionic methylphosphonate linkages (shaded segment in Table 1) either at the 3'- end , the 5'-end, or in the center of the PS-oligo. The purpose of the study is to understand the impact of these modified oligos on biophysical properties, biological activity, and some safety parameters.

Synthesis of oligonucleotides: The structures of the oligos used in the present study are listed in Table 1. PS-oligo (oligo **1**) was synthesized and purified using standard protocols as described earlier[12]. Oligonucleotides containing phoshorothioate linkages and methylphosphonate linkages were synthesized using the appropriate nucleoside phosphoramidite followed by oxidation with the appropriate reagent. The methylphosphonate segment of the oligo was synthesized using nucleoside methylphosphonamidites followed by oxidation with iodine reagent[13]. The phosphorothioate segment of the oligo was synthesized using standard protocols. MBOs containing both phosphorothioate and methylphosphonate linkages were deprotected in two steps, first with ammonium hydroxide for 2 hours at room temperature and then with mixture of ethylenediamine: ethanol (1:1) for 6 hours at room temp[14]. Purification was carried out as reported earlier[12]. The purity of oligonucleotides was confirmed by capillary gel electrophoresis contained more than 90% N-mer.

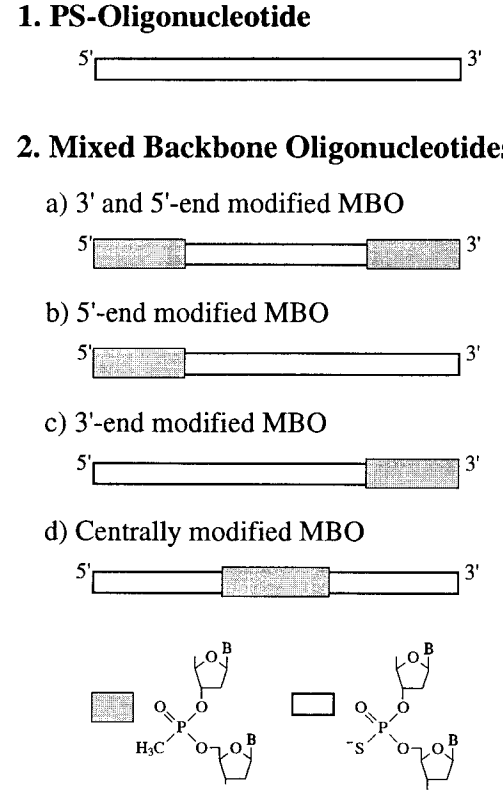

Figure 1. Structure of various mixed - backbone oligonucleotides.

TABLE 1

Oligo #	Sequence	Melting Temperature (°C)	RNase H t ½ (min)	HIV IC_{90} (μM)
1	5'CTCTCGCACCCATCTCTCTCCTTCT3'	63.6	22.4	0.24
2	5'CTCTCGCACCCATCTCTCTCCTTCT3'	61.8	11.5	0.55
3	5'CTCTCGCACCCATCTCTCTCCTTCT3'	61.0	9.7	0.48
4	5'CTCTCGCACCCATCTCTCTCCTTCT3'	60.5	8.1	0.95
5	5'CTCTCGCACCCATCTCTCTCCTTCT3'	57.9	11.5	0.46
6	5'CTCTCGCACCCATCTCTCTCCTTCT3'	57.7	14.4	0.49
7	5'CTCTCGCACCCATCTCTCTCCTTCT3'	56.8	9.3	0.85
8	5'CTCTCGCACCCATCTCTCTCCTTCT3'	60.7	21.2	0.36
9	5'CTCTCGCACCCATCTCTCTCCTTCT3'	60.5	23.0	0.41
10	5'CTCTCGCACCCATCTCTCTCCTTCT3'	59.0	41.8	0.75

Biophysical Studies: The two important parameters for effectiveness of an antisense oligo are its affinity to the target sequence and its ability to cleave RNA in the presence of RNase H. Oligonucleotides listed in Table 1 were studied for their melting temperature (Tm) with complementary oligoribonucleotide (35-mer).Typically the two strands were mixed in a 1:1 ratio to obtain an overall concentration of 0.2 A_{260} units in 10 mM PIPES, 1mM EDTA, and 100 mM NaCl (pH 7). The Tm of oligo **1,** containing phosphorothioate linkages, was higher than other oligos that contained both phosphorothioate and methylphosphonate linkages. In general, centrally modified MBOs (oligos **5**, **6**, and **7**) had lower Tms than 5'-end modified MBOs (oligos **2**, **3**, and **4**) and 3'-end modified MBOs (oligos **8**, **9**, and **10**).

The oligos listed in Table 1 were also studied for their ability to cleave RNA in the presence of RNase H. The comparative rate of cleavage was studied by mixing 0.05 units of oligo with 0.1 A_{260} units of complementary oligoribonucleotide (35-mer) in a buffer containing 20 mM Tris. HCl (pH 7.5), 10 mM $MgCl_2$, 100 mM KCl, 2% glycerol, and 0.1 mM DTT. The mixture was heated and then cooled to room temperature. The sample was then placed in a cuvet of a spectrophotometer, five units of RNase H were added, and the mixture was incubated at 37° C for 180 minutes. Absorbance at 260 nm versus time was recorded and $t_{1/2}$ were calculated from the curve. In general, oligo **1** and 3'-end modified MBOs (oligos **8**,**9**, and **10**) showed similar rates of cleavage ($t_{1/2}$ = 21.2 to 41.8 min), whereas centrally modified MBOs (oligos **5**,**6**, and **7**) and 5'-end modified MBOs (oligos **2**,**3**, and **4**) showed faster cleavage rate ($t_{1/2}$ = 8.1 to 14.4 min) (Table 1). Further studies are in progress to understand the issues which impact the change in the rate of RNA cleavage

Nuclease Stability and degradation profile. Following digestion by *snake venom phosphodiesterase* PS-oligos (oligo **1**) generate a ladder of shorter lengths of oligos both *in vitro* (Fig. 2) and *in vivo* [18]. The experiment was carried out using [^{32}P] end-labeled oligonucleotide in 10 mM Tris. HCl (pH 8) and 10 mM $MgCl_2$ and 0.03 units of *phosphodiesterase* (from *Crotalus durissus*). One of the objectives in designing the various type of MBOs listed in Table 1, and Figure 1, was to (a) understand what type of metabolites are generated following digestion by 3'-*exonuclease*; and (b) if the degradation pattern remains the same *in vivo*, what impact these metabolites may have on

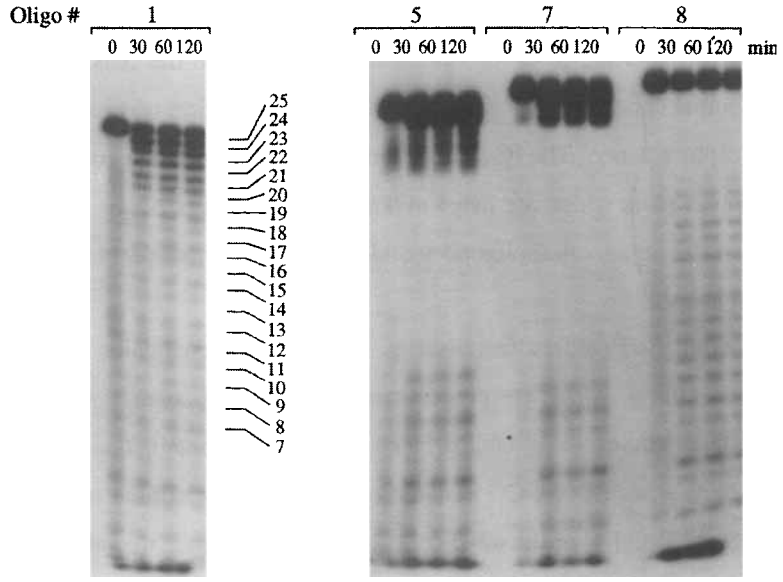

Figure 2. Stability and digestion profile of oligonucleotides against snake venom phosphodiesterase (SVPD). 5'-end labeled oligonucloetides were incubated with SVPD. At the indicated times, an aliquot was analyzed by gel electrophoresis. Oligo **1** following digestion, generated a ladder of shorter oligonucleotides. Oligo **5,** and 7 which contain methylphosphonate likages in the center showed no degradation product in the center. Oligo **8**, which contains six methylphosphonate linkages at the 3'-end , generated a ladder of shorter oligonucleotides starting at 18-19 mers.

the safety profile. With oligo **5** and **6,** which contain methylphosphonate in the center, the phosphorothioate segment of the oligo was digested but further degradation was reduced. If however, the methylphosphonate segment was removed by endonuclease type activity , further digestion of the oligo was observed. In the case of oligo **8**, which contained methylphosphonate linkages at the 3'-end, degradation was slowed down, and following removal of methylphosphonate linkages, stepwise degradation of the phosphorothioate segment of the oligo was observed. Oligos containing methylphosphonate linkages at the 5'-end could not be studied by this procedure as they are not substrates for kinases.

Biological Activity. The biological activity of oligonucleotides against HIV-1 was studied in cell culture assays, using the method reported earlier [15,16]. Molt-3 cells

were infected with HIV-1, and cultured in the presence of oligonucleotides. The cell concentration was adjusted to $4x10^5$/ml on days 3 and 6 and the cells were treated with oligos at the initial concentrations. On day 9, HIV-1 replication was monitored using the p24 antigen capture assay. The IC_{90} value was determined from the concentration versus percent inhibition plots which are listed in Table 1 for various oligos. Oligo **1** was the most potent of the series, other oligonucleotides had two to three fold higher IC_{90} values in the present assay.

Lymophocyte Proliferation Studies. Oligos containing CG dinucleotides have mitogenic effects, which are predominantly B cell response, as shown by both *in vitro* and *in vivo* studies[1,11,17]. Oligos listed in Table 1 contain one CG dinucleotide, however, in certain sequences, CG is modified with internucleotidic methylphosphonate linkages (oligo **2**, **3**, and **4**) or the oligo contains methylphosphonate linkages downstream to CG at the 3'-end (oligos **5**, **6**,and **7**). Comparative studies (Figure 3) of the oligos showed that the lymphocyte stimulation index of oligo **1** was highest; all other oligos had smaller stimulation index. Oligos **8**, **9**, and **10**, which were modified at the 3'-end showed less reduction in the stimulation index than oligos **2** through **7**.

Effect on Coagulation - Prolongation of aPTT. Administration of PS-oligos in monkeys and humans prolongs the aPTT, a clinical parameter measuring blood coagulation. The oligos listed in Table 1 were studied for their ability to prolong aPTT in an *in vitro* assay. The assay was performed using citrated normal donor plasma, according to the procedure reported earlier[4]. The 50% prolongation doses were extrapolated from plots of oligonucleotide concentration versus percent prolongation of the clotting time compared to the appropriate buffer control (Figure 4). All oligonucleotides prolonged aPTT , but the concentration of oligo **1** required to prolong the aPTT by 50% was less than any other oligo tested. Centrally modified MBOs (oligo **5**, **6**, and **7**) showed less effect than 3'- end modified MBOs (oligos **8** ,**9**, and **10**) or 5'-end modified MBOs (oligos **2**, **3**, and **4**). As has been shown earlier, the prolongation of aPTT by PS-oligos results from the polyanionic nature of the oligo and is dependent on the lengths. These MBOs, which contain methylphosphonate linkages, the overall polyanionic nature has been reduced, and consequently had less effect on aPTT.

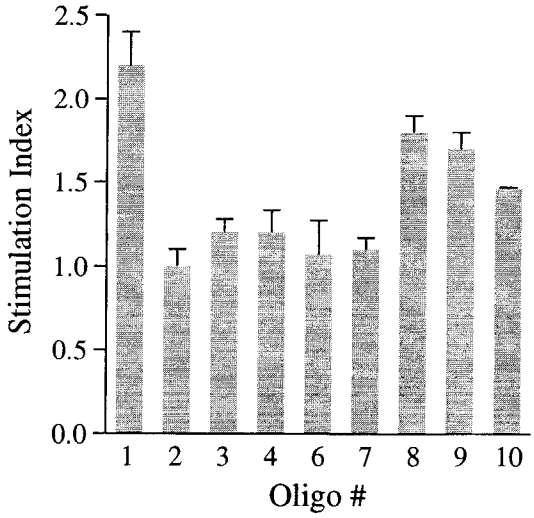

Figure 3. Lymphocyte proliferation index of oligonucleotides.

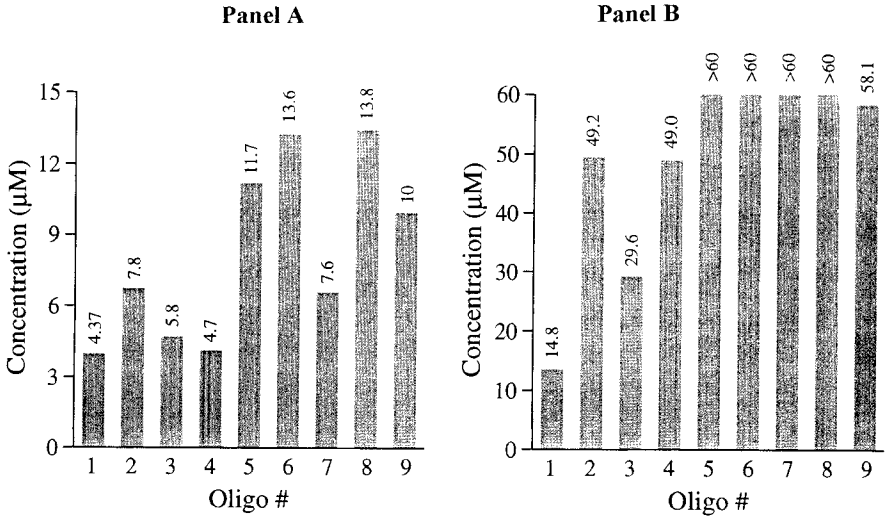

Figure 4. Comparative effect of oligonucleotides on (a) fifty percent prolongation of aPTT (Panel A) and (b) fifty percent of hemolytic complement(Panel B). MBOs had less of an effect on prolongation of aPTT and hemolytic complement activities.

Effect on Complement Activation. In addition to prolongation of aPTT, the other short - term side effects observed in monkeys following administration of PS-oligos were complement activation and reduction of serum hemolytic activity. The oligos listed in Table 1 were studied for their ability to activate complement using fresh normal donor serum according to the procedure described earlier. [4] In Figure 4, data are presented as the oligonucleotide dose producing 50% reduction of hemolytic activity, determined from the plots of oligo concentration versus percentage of control hemolysis. All oligos produced a concentration-dependent reduction in complement induced hemolysis in *in vitro* assays. Oligo **1** at a concentration of about 15 μM reduced hemolysis by 50%; all other oligos required concentrations greater than 15 μM for the same activity.

DISCUSSION

The effectiveness of an antisense oligonucleotide as therapeutic agent depends on many parameters. In *in vitro* studies (cell culture), the biological activity of an oligonucleotide depends on its chemical modification and sequence, the assay system, and the target mRNA. In *in vivo* studies (animal models), the activity of an oligonucleotide also depends on its safety profile, pharmacokinetic tissue disposition, elimination, and *in vivo* stability[1]. To further optimize the properties of PS-oligos as antisense agents, we have made MBOs that have the combined properties of two oligo modifications. These MBOs provide significant increase in *in vivo* stability, an improved safety profile, while retaining biological activity.

In the present study we carried out modifications of PS-oligos by incorporating methylphosphonate linkages at (a) the 5'- end, (b) in the center, and (C) at the 3'- end. The 5'- end modified MBOs have better stability towards 5'- exonucleases but not against 3'- exonucleases compared to PS-oligos. Centrally modified MBOs have no increase in nuclease stability over PS-oligos. The 3'-end modified MBOs have better nuclease stability against 3'- exonuclease, than do PS-oligos , but no increase in stability against 5'-exonuclease. While these MBOs have various degrees of nuclease stability towards exonucleases, the degradation profile and nature of metabolites generated are quite different.

All oligonucleotides showed co-operative Tms with complementary RNA, but the Tms of MBOs were lower (1.8 to 6.8 °C). The other factor that is important for

antisense activity is the ability of oligonucleotides to activate RNase H to cleave complementary RNA. PS-oligos are substrates for RNase H. At higher concentrations, however they inhibit RNase H activity. The 5'-end modified MBOs (oligos **2**, **3**, and **4**) or centrally modified MBOs (oligos **5**, **6**, and 7) showed a higher rate of cleavage of RNA than did PS-oligo (oligo **1**) or 3'-end modified MBOs (oligo **8**, **9**, and **10**). Anti-HIV activity of oligos in a long -term culture assay showed PS-oligo (oligo **1**) to be more potent than other oligos, with an IC_{90} two-to three-fold higher.

The MBOs listed in Table 1 also had less effect on lymphocyte proliferation. This is in agreement with our previous results in which we showed that modifications of oligo containing CG dinucleotide affects the mitogenic response both *in vitro* and *in vivo*. The results of hemolytic complement and coagulation studies with MBOs clearly suggest that reducing the contiguous length of phosphorothioate linkages significantly reduces on prolongation of aPTT and activation of complement effects.

From these studies, it is clear that reducing the polyanionic nature of the PS-oligo significantly reduces the polyanonic-related side effects (prolongation of aPTT and complement activation), and the appropriate placement of methylphosphonate linkages in the PS-oligo provides nuclease stability and improved RNase H activity. Some of the oligonucleotides listed in Table 1 have been studied for pharmacokinetics and safety, and show better safety profile[19].

ACKNOWLEDGEMENTS

Authors are thankful to Dr. Ying Li, Lakshmi Channavajjala, and Allysen Roskey for technical assistance.

REFERENCES

1. Agrawal, S. *Trends in Biotech.* 1996, *14*, 376 and references therein.
2. Sarmiento, U. M.; Perez, J. R.; Becker, J. M.; Narayanan, R. *Antisense Res. and Dev.* **1994**, *4*, 99.
3. Galbraith, W. M.; Hobson, W. C.; Giclas, P. C.; Schechter, P. J.; Agrawal, S. *Antisense Res. and Dev.* **1994**, *4*, 201.
4. Agrawal, S.; Rustagi, P. K.; Shaw, D. *Toxicol. Letters* **1996**, *82/83*, 431.
5. Flexner, C.; Barditch-Crovo, P. A.; Kornhauser, D. M.; Farzadegan, H.;

Nerhood, L. J.; Chaisson, R. E.; Bell, K. M.; Lorensten, K. J.; Hendrix, C. W.; Petty, B. G.; Lietman, P. S. *Antimicrob. Agents and Chemo.* **1991**, *35*, 2544.

6. Cornish, K. G.; Iverson, P.; Smith, L.; Arnerson, M.; Bayever, E. *Pharmacol. Commun.* **1993**, *3*, 239.
7. Agrawal, S.; Mayrand, S. M., Zamecnik, P. C.; and Pederson, T. *Proc. Natl. Acad. Sci USA.* **1990**, *87*, 1401.
8. Metelev, V.; Lisziewicz, J.; Agrawal, S. *Bioorganic & Medicinal Chemistry Letters* **1994**, *4*, 2929.
9. Zhang, R.; Diasio, R. B.; Lu, Z.; Liu, T.; Jiang, Z.; Galbraith, W. M.; Agrawal, S. *Biochem. Pharm.***1995**, *49*, 929.
10. Zhang, R.; Iyer, R.; Tan, W.; Yu, D.; Zhang, Z.; Lu, Z.; Zhao, H.; Agrawal, S. *J. Pharm. Exp. Ther.* 1996, *278*, 971.
11. Zhao, J.; Temsamani, J.; Iadarola, P.; Jiang, Z.; Agrawal, S. *Biochem. Pharm.* 1996, *51*, 173.
12. Padmapriya, A. P.; Tang, J. Y.; Agrawal, S. *Antisense Res. and Dev.* 1994, *4*, 185.
13. Agrawal, S.; Goodchild, J. *Tetrahedron Letters.* **1987**, *28*, 3539.
14. Iyer, R. P.; Yu, D.; Jiang, Z.; Agrawal, S. *Nucleosides and Nucleotides.* 1995, *14*, 1349.
15. Lisziewicz, J.; Sun, D.; Klotman, M.; Agrawal, S.; Zamecnik, P. C.; Gallo, R. *Proc. Natl. Acad. Sci. USA.* 1992, *89*, 11209.
16. Lisziewicz, J.; Sun, D.; Weichold, F. F.; Thierry, A. R.; Lusso, P.; Tang, J.-Y.; Gallo, R. C.; Agrawal, S. *Proc. Natl. Acad. Sci. USA.* **1994**, *91*, 7942.
17. Krieg, A. M.; Yi, A. E.; Matson, S.; Waldschmidt, T. S.; Bishop, G.A.; Teasdale, R.; Koretzky, G. A.; Klinman, D. M.; *Nature.* **1995**, *374*, 546.
18. Agrawal, S.; Temsamani, J.; Tang, J-Y. *Proc. Natl. Acad. Sci. USA.* **1991**, *88*, 7595.
19. Agrawal, S.; Jiang, Z.; Zhao, G.; Shaw, D.; Cai, G.; Roskey, A.; Channavajjala, L.; Saxinger, C.; Zhang, R. (submitted).