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MIXED-BACKBONE OLIGONUCLEOTIDES AS SECOND-GENERATION ANTISENSE AGENTS WITH REDUCED PHOSPHOROTHIOATE-RELATED SIDE EFFECTS

Wenqiang Zhou and Sudhir Agrawal*

Hybridon Inc., 155 Fortune Boulevard, Milford, MA 01757, U.S.A.

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Abstract. Mixed-backbone oligonucleotides containing alternative phosphorothioate and phosphodiester linkages in the 2'-O-methylribonucleosides segment show increased affinity with complementary targets, increased stability towards nucleases *in vitro* and *in vivo*, and reduced phosphorothioate-related prolongation of partial thromboplastin time compared to phosphorothioate oligodeoxynucleotides, thereby providing antisense agents with reduced side effects.

Mixed-backbone oligonucleotides (MBOs) provide a handle on modulating the pharmacological, pharmacodynamic, and pharmacokinetic profiles of antisense oligonucleotides. MBOs are currently the best choice as second-generation antisense oligonucleotides over PS-oligos.[1,2] MBOs contain appropriately placed segments of phosphorothioate oligodeoxynucleotide (PS-oligo) and another modified oligodeoxynucleotide[1-3] or oligoribonucleotide.[1,2,4] The advantage of MBOs is that, while they retain the advantages of PS-oligo's stability against nuclease and RNase H activation, the side effects associated with PS-oligos (immune stimulation, complement activation and prolongation of partial thromboplastin time, etc.) can be minimized, depending on the nature of the modified segment incorporated in the MBO.[1,2,5-7] The positioning of the segments of modified oligodeoxynucleotides or oligoribonucleotides in a MBO is critical for its desired properties. In the end-modified MBO,[1] a segment of PS-oligo is placed in the center to provide RNase H activation, and segments of modified oligonucleotide are placed at both the 3'- and 5'-ends to modulate other antisense properties. End-modified MBOs are more effective than PS-oligos as antisense agents,[1,2] and are currently being evaluated in clinical trials as therapeutic agents.

In end-modified MBOs, the nature of the modification at the 2'-position is important in providing increased duplex affinity and stability towards nucleases. The 2'-*O*-methylribonucleoside phosphorothioate[1] and the 2'-*O*-methoxyethoxyribonucleoside phosphodiester[2] are two types of modified nucleotide segments that have been studied most extensively. Incorporation of 2'-*O*-methylribonucleoside in MBOs can increase the duplex stability with the targeted RNA. For an increase in nuclease stability, however, phosphorothioate internucleotide linkages are usually required,[1,4a-b] as the 2'-*O*-methylribonucleoside phosphodiester shows reduced nuclease stability.[4d] Incorporation of 2'-*O*-methoxyethoxyribonucleoside also provides an increase in duplex stability, and also demonstrated, increased nuclease stability *in vitro*, even with phosphodiester internucleotide linkages.[2] Both types of end-modified MBOs have fewer PS-oligo side effects than the PS-oligo.[1,2,6] Differences in the pharmacokinetic and elimination profiles between the two types of end-modified MBOs have been observed, however. MBOs containing 2'-*O*-methylribonucleoside phosphorothioate show tissue distribution profiles similar to those of PS-oligos following intravenous administration,[8] with a

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significant improvement in stability and retention in tissues. MBOs containing 2'-*O*-methoxyethoxyribonucleoside phosphodiester showed rapid elimination in urine and disposition in kidneys.[2]

The aim of this study was to design another type of end-modified MBO, with significantly reduced side effects, without compromising the antisense properties – duplex stability, nuclease stability, RNase H activity, antisense-based biological activity, and tissue disposition. To carry out the studies, we chose a PS-oligo (18-mer, oligo **1**, Table 1) that is complementary to the RIα regulatory subunit of protein kinase A. Oligo **1** has been studied extensively in both *in vitro* and *in vivo* models.[9] In our previous efforts to improve the therapeutic potential of oligo **1**, we studied a MBO (oligo **2**) in which four deoxynucleosides from both the 3'- and 5'-ends were substituted with 2'-*O*-methylribonucleosides. Oligo **2** has antitumor activities similar to those of oligo **1**, but with a significant improvement in pharmacokinetic and toxic profiles observed in mice.[10] Reduction of PS-oligo-related side effects has also been observed.[6] Oligo **2** is presently being evaluated for its therapeutic potential in human clinical trials.

Table 1. Structures of oligos used in this study and their various parameters

Oligo no.	Sequence & Modifications	Tm with RNA (°C)	aPTT 50% conc. (μg/mL)
1	5' GsCsGsTsGsCsCsTsCsCsTsCsAsCsTsGsGsC 3'	62.9	37.1
2	5' GsCsGsUsGsCsCsTsCsCsTsCsAsCsUsGsGsC 3'	72.1	46.6
3	5' GsCsGsUsGsCsCsUsCsCsUsCsAsCsUsGsGsC 3'	84.8	81.9
4	5' GoCoGoUoGoCoCoUoCoCoUoCoAoCoUoGoGoC 3'	87.4	>200
5	5' GsCoGsUoGsCoCsUoCsCoUsCoAsCoUsGoGsC 3'	87.2	>200
6	5' GsCoGsUoGsCsCsTsCsCsTsCsAsCoUsGoGsC 3'	77.3	94.1

o = phosphodiester linkage, s = phosphorothioate linkage, underlined = deoxynucleoside, normal = 2'-*O*-methylribonucleoside.

Based on the design of oligo **2**, our approach to further minimize the prolongation of aPTT was to reduce the number of phosphorothioate linkages in oligo **2** without compromising the stability towards nucleases. To carry out the studies, we first designed and prepared some model oligonucleotides[11] (Table 1) to provide insights into the relationship between the nature of the oligonucleotide (nucleoside sugar and phosphate backbone) and its impact on nuclease stability and thermodynamic stability with target RNA, and most importantly, the PS-oligo-related side effects. These model oligonucleotides included 2'-*O*-methyloligoribonucleoside phosphorothioate (oligo **3**), 2'-*O*-methyloligoribonucleoside phosphodiester (oligo **4**), and 2'-*O*-methyloligoribonucleoside containing alternative phosphorothioate and phosphodiester linkages (oligo **5**). In a study to examine the *in vitro* stability of the oligos towards snake venom phosphodiesterase (SVPD),[12] the stability of oligos **1** to **5** is found to be in the order - oligo **3** ≈ oligo **2** ≈ oligo **5** > oligo **1** >> oligo **4**. These results suggest that substitution of one phosphorothioate linkage with a phosphodiester in the 2'-*O*-methylribonucleoside at alternative sites does not adversely affect the stability of oligo **5** towards SVPD, compared with that of oligo **3**.[13]

Prompted by the above observation and the data described later, we designed and prepared a new type of MBO - oligo **6** (Table 1), which contains a PS-oligo segment (nine deoxynucleosides) in the center flanked by five and four 2'-*O*-methylribonucleosides at both the 3'- and 5'-ends containing alternative phosphorothioate and phosphodiester linkages. Compared with oligo **2**, this newly designed MBO (oligo **6**)

has less phosphorothioate content, thus may have fewer PS-oligo-related side effects. The structural nature of oligo **6** was confirmed by ^{31}P NMR and MALDI-TOF MS analysis (Figure 1).

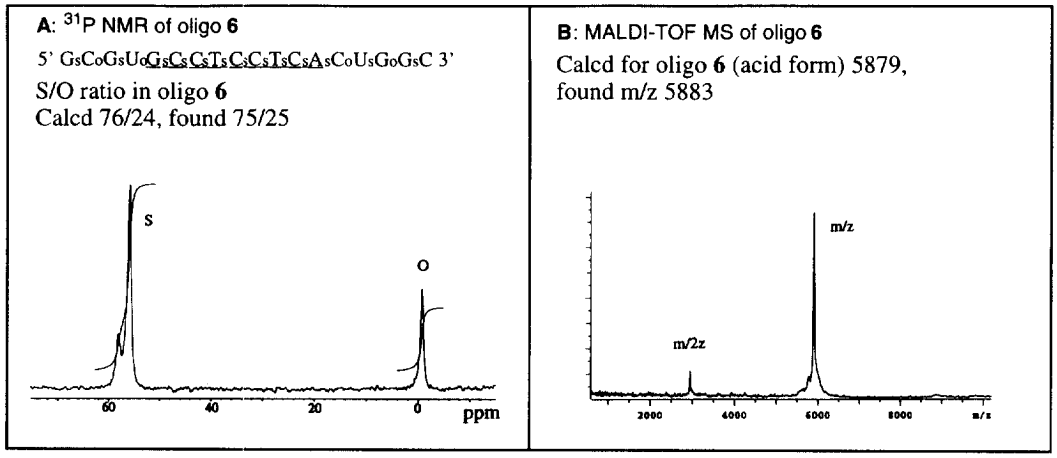

Figure 1. ^{31}P NMR and MALDI-TOF MS spectra of oligo **6**. Underlined letters represent deoxynucleosides; plain letters represent 2'-*O*-methylribonucleosides; S and O represent phosphorothioate and phosphodiester linkages, respectively.

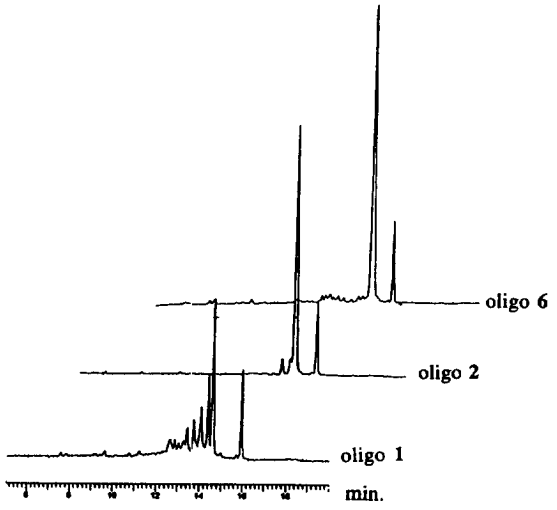

Figure 2. CGE profiles of comparative stability of oligos 1,2 and 6 towards SVPD (0.004 units/50 µL) at 37 °C for 24h. Intact oligo 1 was approximately 34%. Peak at 16 min is of internal standard (PS-oligo 25-mer) added after digestion and before CGE analysis

In the study to compare the *in vitro* stability of the oligos towards SVPD, oligo **6** was found to have stability similar to that of oligo **2** and greater than that of oligo **1** (Figure 2). This indicated the structural design of oligo **6** had no adverse effects on the oligo's nuclease stability *in vitro*.

In the melting temperature (Tm) study to compare the oligos' binding affinity to the complementary RNA phosphodiester,[14] oligo **6** showed an increase of 14.4 °C and 5.2 °C in Tm compared with oligo **1** and oligo **2**, respectively (Table 1). Compared with oligo **2**, the increase of the binding affinity of oligo **6**, as

demonstrated by the increase of Tm, is due to the substitution of four phosphorothioate linkages with phosphodiester linkages and also an additional 2'-*O*-methylribonucleoside.[1]

In the RNase H digestion study,[15] oligos **2** and **6** showed similar cleavage patterns, which differed from that of oligo **1** due to the flanking 2'-*O*-methylribonucleosides in oligos **2** and **6** (Figure 3).[1] This study indicated that the MBO design of oligo **6** had no adverse impact on the oligo's ability to cleave the complementary RNA in presence of RNase H.

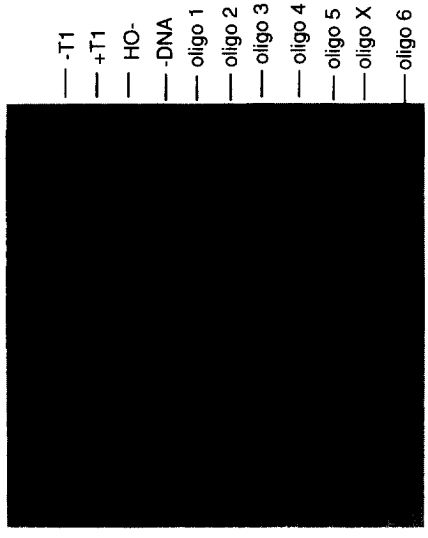

Figure 3. RNase H hydrolysis pattern of the 5'-^{32}P-labeled RNA phosphodiester 30-mer (5'ACCGCCGCCAGUGAGGCACGCAGCCUU3') in the presence of oligos **1** to **6**.
Lane -T1, control lane without RNase T1 added; lane +T1, RNase T1 digestion reaction; lane -OH, alkaline hydrolysis reaction; lane -DNA, control RNA lane without any oligo added; lanes oligos **1** to **6**, in the presence of oligos **1** to **6** respectively and RNA and RNase H. There is no cleavage in the presence of oligos **3**, **4**, and **5** as they are not substrate for RNase H. Lane oligo X is a treatment in the presence of an oligo, which is not included in this report. The structure of the oligos is depicted in Table 1.

The effects of oligos **1** to **6** on prolongation of aPTT were also compared. The study was designed to see if oligo **6**, with a reduced number of phosphorothioate linkages, was indeed able to reduce the PS-oligo-related side effects such as prolongation of aPTT.[16] The results are depicted in Figure 4. All oligos showed concentration-dependent prolongation of aPTT, but with significant differences among the oligos. The clear differences between oligo **1** (PS-oligo) and oligo **3** (2'-*O*-methyloligoribonucleoside phosphorothioate) confirmed our previous observation that the phosphorothioate linkage of the oligodeoxynucleoside (PS-oligo) is more effective in prolonging the aPTT than is the phosphorothioate linkage of the oligoribonucleoside analogs, including 2'-*O*-methylribonucleoside.[17] As expected, oligos **4** and **5** showed the least prolongation of aPTT, due to the dominant content of the 2'-*O*-methylribonucleoside and the least content of phosphorothioate linkages (Table 1). The concentration required for oligos **4** and **5** to prolong aPTT by 50% was > 200 μg/ml (>35 μM). In general, the prolongation of aPTT in presence of oligos **1** to **6** was in the order - oligo **1** > oligo **2** > oligo **3** > oligo **6** > oligo **4** > oligo **5**. To our satisfaction, oligo **6** – the newly-designed MBO in which flanking sequences contain 2'-*O*-methylribonucleosides with alternative phosphorothioate and phosphodiester linkages–showed a significant reduction in its ability to prolong aPTT, compared with oligos **1** and **2**. The concentration required to prolong aPTT by 50% for oligos **1**, **2**, and **6** was 37.1, 46.6, and 94.1 μg/mL, respectively (Table 1).

Prompted by the above *in vitro* results, we extended our study to compare the *in vivo* stability of oligo **6** with that of oligo **1**.[18] Blood samples were drawn 1 h after intravenous administration of these two oligos in mice. The oligo components were then extracted from the plasma[19] and subjected to CGE analysis (with a UV detector). The increased *in vivo* stability of oligo **6**, compared with oligo **1**, was confirmed by the CGE analysis. The CGE profile of oligo **1** showed ~ 55% intact oligo and 45% in degraded form, where as majority of oligo **6** was in intact form (Figure 5).

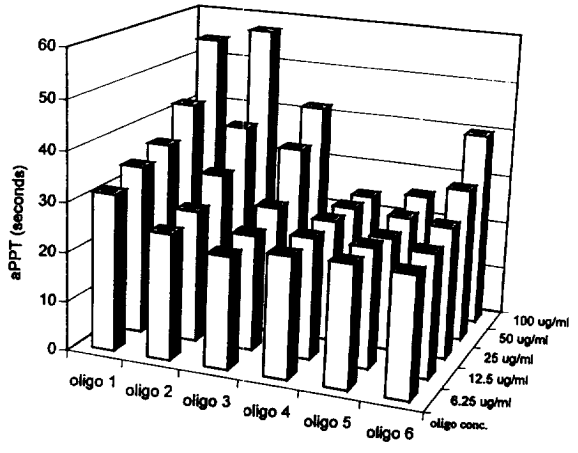

Figure 4. Comparison of the effects of oligos **1** to **6** on prolongation of aPTT using human blood from a healthy volunteer. Each aPTT value is the average of four measurements. The general protocol is described in ref 17.

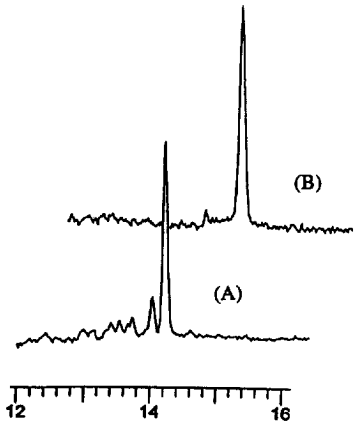

Figure 5. CGE profiles of extracted samples of oligo **1** (A) and oligo **6** (B) from mice plasma 1 h after intravenous administration.

In conclusion, our studies demonstrate that it is possible to optimize the properties of antisense oligos by subtle structural changes in the nucleoside sugar residue and internucleotide linkages, as exemplified by the design of oligo **6**. Our preliminary pharmacokinetic study also showed that the tissue disposition profile of oligo **6** is similar to that of oligo **2**, which suggests that reduction of the phosphorothioate linkages in oligo **6** does not result in significant changes in tissue disposition (data not shown). Other studies are ongoing to fully examine the therapeutic potential of oligo **6**. Similar designs of antisense oligos are being studied in other disease models as well.

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11. The oligonucleotides were synthesized using β-cyanoethyl phosphoramidite chemistry on a 15 μmol scale (Expedite 8909, Perceptive Biosystems, MA) or on a 0.5 mmol scale (Pharmacia OligoPilot II Synthesizer). The 2'-*O*-methyl RNA segments with alternative PS/PO internucleotide linkages in oligos **4**, **5**, and **6** were synthesized by applying the appropriate oxidation reagents in the corresponding synthesis cycles (Beacauge Reagent for PS linkage, and iodine for PO linkage). The oligos were purified by preparative reversed-phase HPLC. The oligo products were characterized by CGE, ^{31}PNMR, and MALDI-TOF MS.
12. For each reaction, oligo (0.5 A_{260} units) was suspended in buffer (50 μL) containing Tris (pH 8.5, 30 mM) and $MgCl_2$ (15 mM). To each solution, 0.004 units of SVPD from *crotallus durissus* (Boehringer Mannheim) was added. The reaction was carried out for 24 h. at 37 °C.
13. In a parallel study, it was found that substitution of the phosphorothioate linkage with a phosphodiester linkage in the PS-oligo (oligo **1**, Table 1) reduced the modified oligos' stability towards SVPD (data not shown).
14. Tm were recorded using a GBC 920 Spectrophotometer (GBC Scientific Equipment, Victoria, Australia). Oligos were mixed with complementary RNA phosphodiester (30-mer, 5'-ACCGCCGCCAGUGAGGA GGCACGCAGCCUU-3') in a buffer containing 10 mM Pipes, 1 mM EDTA, and 100 mM NaCl. The Tm values were obtained from the first derivative plots.
15. For each reaction, the 5'-^{32}P-labeled RNA phosphodiester (30-mer, 0.5 pmol), oligo (5 pmol), and glycogen (50 μmol) were mixed in 12 μL of buffer containing 50 mM $MgCl_2$, 100 mM KCl, 1 mM DTT, 200 mM Tris (pH 7.5), and 5% glycerol. After annealing, 0.078 unit of RNase H (Pharmacia) was added to each solution. The mixture were then incubated at 37 °C for 10 min. The reactions were then quenched by adding 20 μL of gel loading dye to each reaction mixture. The resultant samples were analyzed by 20% PAGE and subjected to autoradiography.
16. General procedure for aPTT: Plasma was obtained from citrated human blood. Serial dilution of the oligos in 0.9% NaCl UPS (saline) were provided in final concs. of 6.25, 12.5, 25, 50 and 100 μg/mL of oligo in plasma. After addition of the oligo samples, the plasma was incubated at 37 °C for 15 min, with gentle agitation. Plasma exposed to vehicle in the same ratio (v/v) as the oligos, and untreated plasma served as negative controls. The assay was conducted in duplicate, providing at least 2 replication for each tube.
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18. Oligo **1** and **6** (1 mg) were administrated intravenously in mice (female, CD-1, 20 -22 g) through the tail vein. Blood samples (200 μL) was collected at 1 h post-dosing. The oligos were extracted from the plasma by the same procedure as ref. 19, and analyzed for their stability by CGE.
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