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PROPERTIES OF MIXED BACKBONE OLIGONUCLEOTIDES CONTAINING 3'-O-METHYL RIBONUCLEOSIDES.

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Abstract: Oligonucleotides containing 3'-O-methyl ribonucleosides were synthesized, and their thermal stabilities and global conformations with RNA and DNA have been studied. The duplexes displayed lower T_m values as compared to the unmodified ones, and adopted A-conformations. Furthermore, they are not a substrate for RNase H, are slightly resistant to snake venom phosphodiesterase, and are not cleaved by nuclease S 1

Antisense oligonucleotides have emerged as a potential chemotherapeutic agent in the last decade[1]. The desired properties of antisense oligonucleotides include sequence-specific hybridization to the target mRNA, stability against nucleases, membrane permeability, and selective inhibition of gene expression. Unmodified oligonucleotides (DNA or RNA) under physiological conditions are rapidly degraded by a variety of exo and endonucleases[2,3], which limit their clinical applications. In an effort to overcome these limitation, various modifications have been examined at the heterocyclic bases, the deoxyribo- or ribo- sugars, and the internucleotide phosphodiester linkages[4]. The most commonly employed synthetic modification is the backbone phosphorothioate analog. While this type of modification enhances the stability to nucleolytic hydrolysis, it decreases the stability of duplexes formed with complementary DNA or RNA sequences. In contrast, 2'-modifications are advantageous and alkyl substituents have been incorporated into oligoribonucleotides to improve nuclease resistance[5-10] or affinities for RNA targets[11,12] for therapeutic applications[13,14]. Recently, 2'-5' linked oligoribonucleotides have been the focus of research for antisense therapeutics. The 2'-5' oligoadenylates, $(2'\text{-}5')A_n$, represent an example of naturally occuring RNA isomers suspected to be involved in the regulation of cell growth and in the antiviral mechanism of interferon[15]. The experimental results suggest that an antiparallel duplex is formed by the 2'-5' linked complementary oligoribonucleotides; however, the overall stability is less than that of the corresponding 3'-5' duplexes[16]. In addition, the oligonucleotides possessing 2'-5' linkages exhibit binding selectivity for 3'-5' RNA over 3'-5' DNA, and confer greater resistance to nucleolytic degradation as compared with the natural 3'-5' linked oligomers. Also, 2'-5' / 3'-5' DNA phosphate chimeras have been used to inhibit 5 α reductase expression in cell culture[17]. The single stranded DNA / 2'-5' RNA chimeras and 2'-5' DNA show less non-specific binding to plasma and cellular proteins in comparison with the 3'-5' phosphorothioate oligonucleotides[17,18] and hence produce fewer side effects *in vivo*. Therefore, in order to improve the binding, the RNA specificity, and the nuclease stability, we

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designed mixed backbone oligonucleotides (MBO) containing 2'-5' ribo- and 3'-5' deoxyribonucleosides. In addition, we incorporated a methyl group at the 3'-function of the sugar residue and studied the relative stability, the nuclease sensitivity, and the RNase H susceptibility of these oligonucleotides.

Results and Discussion

The oligonucleotides were synthesized using β-cyanoethylphosphoramidite chemistry on a CPG solid support. The phosphoramidite derivatives (5'-O-dimethoxytrityl-3'-O-methyluridine-2'-O-N, N-diisopropyl-amino (2-cyanoethyl)phosphoramidite and 5'-O-dimethoxytrityl-N^6-benzoyl-3'-O-methyladenosine-2'-O-N, N-diisopropylamino (2-cyanoethyl)phosphoramidite) and the CPG support of 5'-O-dimethoxytrityl-3'-O-methyl-uridine were prepared using standard protocols[19]. The abilities of the modified oligonucleotides to form double helices with their RNA or DNA complements were evaluated by melting temperature studies. The synthetic oligomers, along with the melting temperature (T_m) values for the various duplexes, are listed in Table 1.

Table 1: Thermal dissociation data[a] of modified oligonucleotides

	ODNs	ODN-RNA[b] T_m(℃)	ODN-DNA[c] T_m(℃)
1.	5'-TAT ATT TTT TTT TTT TAT AT-3'	50	43
2.	5'-*UAU AU*T TTT TTT TTT *UAU AU* –2'	45	38
3.	5'-*UAU AU*$T_sT_sT_sT_s$ $T_sT_sT_s$ $T_sT_sT_s$ $T_sT_sT_s$ *UAU AU* –2'	39	32
4.	5'-*UAU AU*T TTT TTT TTT *UAU AU* –2'	48	37
5.	5'-*UAU AU* U UUU UUU UUU*UAU AU*–2'	34	24

[a]T_m s were measured in 10 mM phosphate, 100 mM NaCl, 0.1 mM EDTA, pH 7.0 at a 3 μM duplex concentration. [b]The complementary RNA: 5'-AUA UAA AAA AAA AAA AUA UA-3'. [c]The complementary DNA: 5'-ATA TAA AAA AAA AAA ATA TA-3'. Italics = 2'-5' linkages, underlines = 3'-O-methyl residues, s = phosphorothioate linkages.

The duplexes of sequences **2**, **3**, and **4** with their complementary DNA and RNA had lower thermal stability than the control homoduplexes of DNA. The fully modified 3'-O-methyl oligoribonucleotide (**5**) duplex with its complementary RNA was less stable than the duplex of MBO, **4**. This indicates that the 3'-O-methyl group destabilizes the hybrid of **5** with RNA. This is also evident from the T_m values of duplexes **2** and **4** with RNA, in which the former has a lower value than the latter. We observed a weak and broader transition in duplex of **5** with DNA, which confirmed that the fully 2'-5' linked oligoribonucleotides associate with RNA, but weakly or not at all with DNA[20]. Moreover, the T_m values of the duplexes **4** with RNA and DNA displayed a marked difference, indicative of the higher affinity for RNA than DNA. On the other hand, the BMO, **3** hybridized more weakly with complementary RNA and DNA than the MBO, **2**, because it has phosphorothioate linkages, which exist as diastereomeric mixtures.

The circular dichroism studies of hybrid **6** (5'-UAU AUU UUU UUU UUU UAU AU-3') with its RNA complement displayed a positive band at ~265 nm and a negative band near 208 nm, characteristic of an A-conformation[21] (Fig.1 A). Similar results were observed for **5** regarding the CD bands, but with much lower ellipticities than the corresponding duplex of **6**. These results suggest that fully modified 3'-substituted hybrids adopt an A-form helices, consistent with an RNA:RNA duplex. On the other hand, the hybrids of modified oligonucleotides **2**, **3**, and **4** to RNA complements showed a reduced negative band at 210 nm and positive shoulder at approximately 280~290 nm, which coincides with the positive band of a normal DNA:DNA duplex, as they have central DNA linkages.

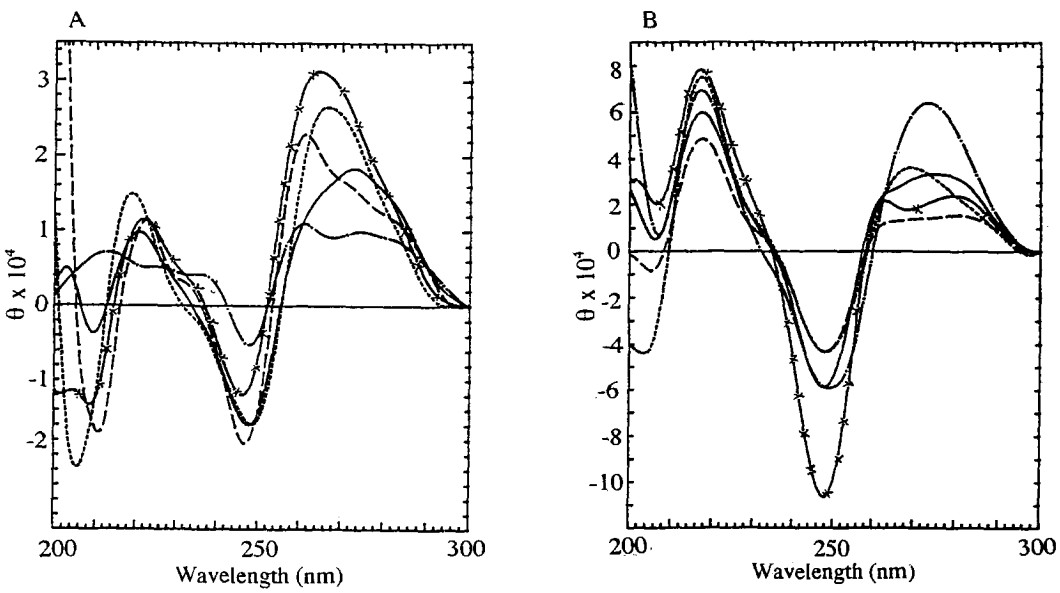

Figure 1. CD spectra of duplexes, **6** or **1** (-×-), **2** (—), **3** (— · —), **4** (— —), and **5** (----) with A) RNA and B) DNA were measured in 10 mM phosphate, 100 mM NaCl, 0.1 mM EDTA, (pH 7.0) at 15 ℃ and 3 μ M duplex concentration

Thus they demonstrate the characteristics of an intermediate or a hybrid between the A and B helices[22,23]. In addition a comparison of the negative bands of duplexes **2** and **4** reveals that the latter had a lower negative band, suggesting that the 3'-O-methyl group was responsible for the hybrid approaching an A-RNA conformation. With the DNA complement, only the modified oligonucleotide **5** exhibits a spectrum like that of the A-form, while the other duplexes resemble the B-DNA conformation (Fig.1 B).

It has been postulated that the antisense activity of antisense oligonucleotides is due, at least in part, to cleavage of the RNA strand of a DNA-RNA duplex by RNase H[1,4]. Therefore, we investigated whether the duplexes of oligonucleotides synthesized with their complementary RNA strands are affected by RNase H activity. As evident from Figure 2, the RNA in duplex **5** was not cleaved at the concentration of *E. coli* RNase H that produced total digestion of the RNA within 20 min, whereas in other duplexes the RNA was easily

hydrolyzed. These results show that the fully modified 3'-O-methyl oligoribonucleotides are not substrates for RNase H. The lack of RNase H activity on the 3'-O-methyl oligoribonucleotides is due to the absence of the nucleophile, 2'-OH, which the enzyme utilizes to attack the adjacent 2'-5' phosphodiester bond.

Figure 2. 20% Polyacrylamide/7M urea gel electrophoresis of 5'-fluorescein isothiocyanate-labeled RNA hydrolyzed by *E. coli* RNase H. The duplexes were incubated in 20 μ L of buffer, containing 20 mM HEPES-KOH (pH 8), 1 mM DTT, 10 mM $MgCl_2$, 50 mM KCl, with 10 units of *E. coli* RNase H for 20 min at 37 ℃. Lane 1, RNA; lane 2, RNA + enzyme; lane 3, RNA + control DNA; lane 4, RNA + control RNA; lane 5, RNA + **2**; lane 6, RNA + **3**; lane 7, RNA + **4**; lane 8, RNA + **5**.

In considering the important factor, nuclease resistance, that determines the effectiveness of antisense oligonucleotides *in vivo* , we examined the 3'-exonuclease sensitivity of the oligonucleotides using snake venom phosphodiesterase (SVPDE). The oligonucleotide / enzyme reaction mixture was monitored as a

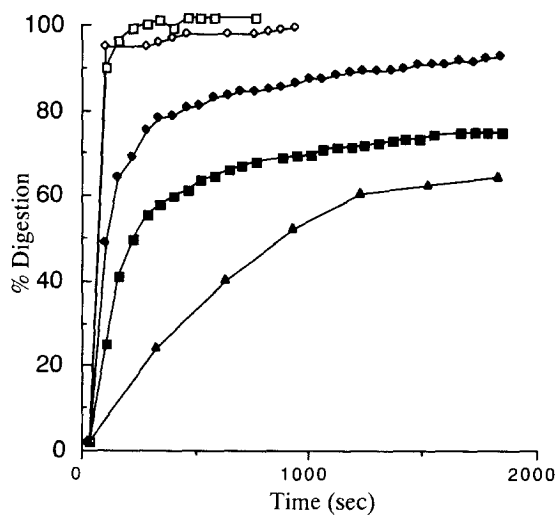

Figure 3. Digestion of oligonucleotides with snake venom phosphodiesterase (SVPDE). 0.3 A_{260} units of oligonucleotides, **1** (◇), **2** (□), **3** (▲), **4** (◆), and **5** (■), were incubated in 800 μ L of buffer, containing 50 mM Tris HCl (pH 8),10 mM $MgCl_2$,100 mM NaCl with 2 μ L of 2 mg/mL SVPDE (0.006 U) at 37 ℃ in a thermally regulated cell of a UV spectrophotometer, and the A_{260} values were recorded against time.

function of time, and the increase in absorbance at λ_{max} (260 nm) was assumed to be related to oligonucleotide degradation (Fig. 3). The percent increase in absorbance during digestion relative to that of t = 0 was converted directly to percent digestion[24]. The 3'-5' linked oligonucleotide (**1**) and MBO (**2**) were almost completely hydrolyzed within 3 min. In contrast, the MBO containing the 3'-O-methyl group, **4**, and oligonucleotide **5** showed resistance to exonuclease degradation. The most stable oligonucleotide appeared to be **3**, as it has phosphorothioate linkages. The relative stability at 10 min digestion is as follows: **3> 5 > 4 > 1** or **2**.

The endonuclease activity was also investigated using the enzyme nuclease S1 (Fig. 4). At the enzyme concentration of 30 U/mL, no degradation of the fully 3'-modified oligoribonucleotides (**5**) was observed within 1 h, whereas oligonucleotides **2** and **4** were almost half-degraded. The oligonucleotide **4** showed only one-fourth degradation within 1 h. Therefore, their stability ranking is **5 > 3 > 4 > 2 > 1**. The variation in the

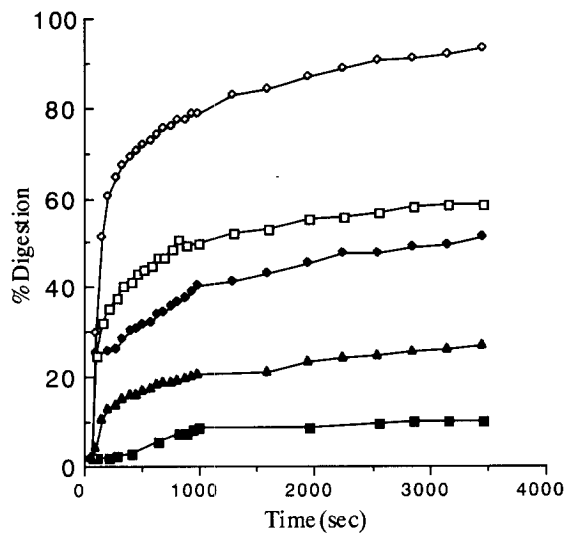

Figure 4. Digestion of oligonucleotides with nuclease S1. 0.3 A_{260} units of oligonucleotides, **1** (◇), **2** (□), **3** (▲), **4** (◆), and **5** (■), were incubated in 800 μL of buffer, containing 50 mM sodium acetate (pH 4.5, at 25 °C), 280 mM NaCl, 4.5 mM $ZnSO_4$ with 30 units of nuclease S1 at 37 °C in a thermally regulated cell of UV spectrophotometer, and the A_{260} values were recorded against time.

abilities of SVPDE and nuclease S1 to cleave the 3'-OMe substrate reflects the differences in the active sites of the individual enzymes, thus leading to varied degrees of steric hindrance by the methyl group on the 3'-position of the ribose.

We conclude that mixed backbone oligonucleotides with a modification at the 3'-function of the sugar residue possess unique properties, such as higher affinity for RNA, adoption of the A-conformation, and resistance to RNase H and nuclease S1. We observed a slight depression in the T_m values of the fully 3'-modified oligoribonucleotide (**5**), so by reducing the number of 3'-O-methyl groups in a sequence a higher T_m value can be achieved. Their weaker resistance towards SVPDE can be increased by introducing few

phosphorothioate linkages at the 3' end of the oligoribonucleotides. These properties make mixed backbone oligonucleotides a potentially advantageous antisense agent.

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