Modulation of Tetraplex Formation by Chemical Modifications of a G_4-Containing Phosphorothioate Oligonucleotide

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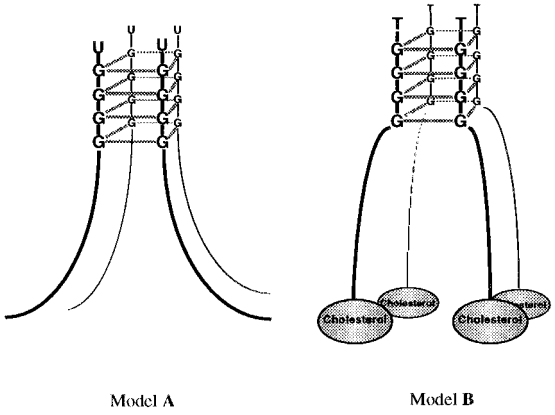
Antisense oligodeoxynucleotides can inhibit cellular and viral gene expression in a sequence specific manner. Their specificity, stemming from Watson−Crick base pairing with targeted RNA, provides opportunities for the development of therapeutics for human diseases including cancer and viral infections.[1] It has been previously reported that a 20 residue phosphorothioate oligonucleotide 5′-d(TGGGGCTTACCTTGCGAACA)-3′ (PS, in Table 1) is a potent antisense inhibitor of human cytomegalovirus (HCMV).[2]



Model **A** Model **B**

PS contains four contiguous guanosine residues (G_4) near its 5′-end. Such runs of consecutive guanine bases in RNA or DNA can self-assemble into four-stranded tetraplexes *via* guanine−guanine Hoogsteen base pairs.[3] These tetraplexes contain stacks of guanine quartet (G-quartet) planes with the phosphate backbones running in either parallel or antiparallel orientation; both have been observed by X-ray and NMR analysis.[4]

In addition to antisense inhibition,[5,2b] G_4-containing oligonucleotides can show other biological activity. A G-quartet structure made of phosphorothioate oligonucleotides inhibited cell fusion of HIV*via* interactions with a virus envelope protein,[6] and a non-antisense effect of G_4 was responsible for the antiproliferative activity of *c*-myb and *c*-myc "antisense" oligonucleotides.[7] Oligonucleotides capable of G-quartet formation might possess special biological properties *in vivo*, as implicated by discoveries of proteins that promote the formation of tetraplex structures[8] and elicit specific cleavages in their vicinity.[9] The antisense and/or antiviral efficacy of a G_4-containing oligonucleotide could therefore be influenced by its tendency to form tetraplexes.

Table 1. Oligonucleotides Used in This Study[a]

oligonucleotides[b]	sequences and modifications[c]
Sense RNA	3′-GCUGCACCCCGAAUGGAACGCUUGUCUGCC-5′
PS	5′-TGGGGCTTACCTTGCGAACA-3′
4x4 OMe	5′-**UGGG**GCTTACCTTGCG**AACA**-3′
2x4 OMe	5′-**UG**GGGCTTACCTTGCG**AACA**-3′
1x4 OMe	5′-**U**GGGGCTTACCTTGCG**AACA**-3′
0x4 OMe	5′-TGGGGCTTACCTTGCG**AACA**-3′
4x0 OMe	5′-**UGGG**GCTTACCTTGCGAACA-3′
4x4 OMe (I)	5′-**UGGG**ICTTACCTTGCG**AACA**-3′
2x4 OMe (I)	5′-**UG**GIGCTTACCTTGCG**AACA**-3′
5′-Chol	5′-T*GGGGCTTACCTTGCGAACA-3′
3′-Chol	5′-TGGGGCTTACCTTGCGAAC*A-3′
4xOMe 3′-Chol	5′-**UGGG**GCTTACCTTGCGAAC*A-3′
10mer	5′-**UGGG**GCTTAC-3′
4Rx0	5′-UGGGGCTTACCTTGCGAACA-3′

[a] These synthetic oligonucleotides were purified by either HPLC or denaturing PAGE, then precipitated from solutions in 0.3 and 0.1 M NaCl using ethanol, and quantified by UV absorbance at 260 nm. [b] Sense RNA has phosphodiester backbones, and the rest contain phosphorothioate backbones unless otherwise indicated. [c] Underlined bases represent unmethylated RNA residues, bold faced bases represent 2′-*O*-methyl RNA residues, I represents inosine, and * represents a phosphoramidate linkage P−NH$(CH_2)_6$NH−CO−O−cholesteryl.[11]

2′-*O*-Methylated oligonucleotides have been shown to hybridize to complementary RNA with increased affinity, possibly resulting from stabilization of its A-form conformation.[10] A cholesteryl substituent in oligonucleotides has been shown to stabilize duplexes and triplexes by interstrand hydrophobic interactions.[11,12] The present study was designed to analyze how these modifications on PS would influence the formation of G-quartets and affect their ability to form duplexes with complementary RNA.[13] The oligonucleotides used are listed in Table 1.

Analyzed by denaturing polyacrylamide gel electrophoresis (PAGE), all the oligonucleotides ran as a single band with the expected mobility (data not shown). When analyzed by nondenaturing PAGE, however, 4×4 OMe showed formation of a much lower mobility species (lane 8 of Figure 1), whose regeneration following heat denaturation was favored more in

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(13) Nondenaturing PAGE analysis was applied throughout this study. To each sample (20 μL) was added 4 μL of 20% glycerol before loading onto a 20% (19:1 acrylamide:bis(acrylamide)) gel containing 0.5 × TBE (45 mM Tris-Borate pH 8, 1mM EDTA) and 50 mM NaCl. The gel was run in 0.5xTBE/50 mM NaCl buffer at constant voltage of 75 V so that the temperature of the gel remained below 30 °C. After orange G dye in a separate lane reached bottom, the gel was placed on a fluorescent TLC plate and photographed under UV illumination (254 nm).

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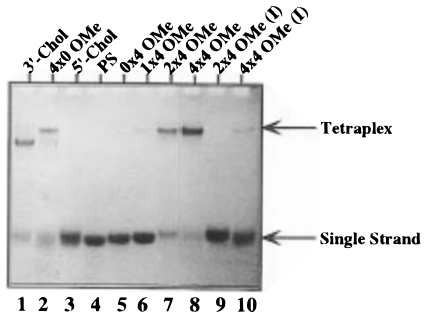

Figure 1. Effect of chemical modifications on the relative amounts of tetra-stranded vs single-stranded oligonucleotides. Each sample contained 0.1 mM oligonucleotide, 10 mM Tris pH 7, 1 mM EDTA, and 50 mM NaCl and was incubated at room temperature for 1 h before analysis.[13]

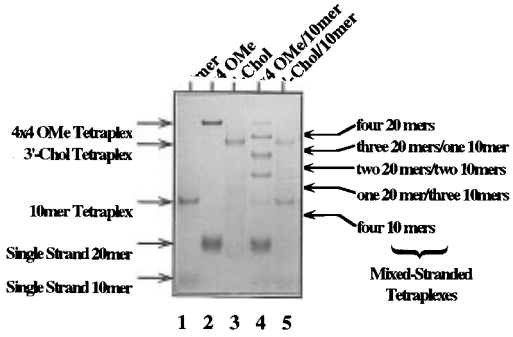

Figure 2. Formation of mixed-stranded tetraplexes. Aqueous solutions (10 μL) containing either 2 nmol of one oligonucleotide or a mixture of two oligonucleotides (2 nmol each) were heated at 90 °C for 3 min and placed on ice immediately thereafter. Samples were brought to 20 μL containing 10 mM Tris pH 7, 1 mM EDTA, and 200 mM KCl, reheated at 90 °C for 3 min, and cooled to 30 °C over 2−3 h before analysis.[13]

the presence of KCl than NaCl of same concentration (data not shown), typical for tetraplex structures.[14]

To unambiguously demonstrate its tetraplex identity, 4×4 OMe was mixed with a shorter, partially 2′-*O*-methylated 10mer and then heat denatured and annealed in the presence of K^+. Nondenaturing PAGE analysis showed expected mixed-stranded tetraplexes with five bands of varying intensity (lane 4 of Figure 2). Similar results were observed for 4×0 OMe/10mer and 4R×0/10mer (data not shown), but 3′-Chol/10mer showed only two distinct bands corresponding to individual tetraplexes of 3′-Chol and 10mer (lane 5 of Figure 2).

The degree of tetraplex formation depended on the extent of 2′-*O*-methylation at the 5′-end, whereas PS and 0×4 OMe did not afford any detectable tetraplexes; the intensity of tetraplex bands increased in the order of 4×4 OMe > 2×4 OMe > 1×4 OMe (lanes 4−8 of Figure 1). As expected for Hoogsteen base paired G-quartet structures, replacing a guanosine by inosine dramatically reduced tetraplex formation (compare lane 7,8 with 9,10 in Figure 1). It has been demonstrated that a RNA tetraplex $(UGGGGU)_4$ is much more stable than its DNA counterpart[15] and that RNA forms more stable duplexes with 2′-*O*-methylated RNA than with DNA.[10] The present study indicates that 2′-*O*-methylation of guanine also promotes the formation of G-quartet structures, with either 2′-*O*-methylated or unmodified guanines (as in 4R×0/10mer). Taken together, C3′-endo sugur pucker might have contributed to stablize tetraplex structures.

3′-Chol ran predominantly as a tetraplex whereas 5′-Chol ran exclusively single stranded (lanes 1,3 of Figure 1). In 3′-Chol, the interstrand hydrophobic interaction of cholesteryl groups

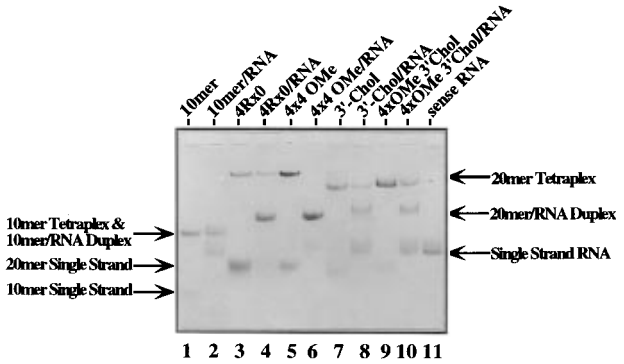

Figure 3. Hybridization of various oligonucleotides with sense RNA. Each sample contained 0.1 mM oligoncleotide(s) (antisense:sense = 1:1 where applicable), 10 mM Tris pH 7, 1 mM EDTA, and 100 mM KCl and was incubated at 37 °C for 1 h before analysis.[13]

could help assemble the tetraplex (model **B**). In the case of 5′-Chol, steric effects might impede G-quartet formation due to the proximity of cholesteryl groups to the G_4 motif.

The tetraplex of 3′-Chol migrated slightly faster than that of 2′-*O*-methylated oligonucleotides, although the mobility of the single-strand oligomers is similar (compare lane 1 with others in Figure 1). An oligonucleotide possessing both modifications, 4×OMe 3′-Chol, showed mobility similar to 3′-Chol (lanes 7,9 of Figure 3). These findings are consistent with the parallel-stranded tetraplex models depicted as **A** and **B**. **A** represents the tetraplex structure formed by 4×4 OMe, with negatively charged phosphate backbones separated as far as possible. **B** illustrates that the hydrophobic interactions amongst cholesteryl groups might overcome the charge−charge repulsion and stabilize the tetraplex structure. The observed mobility differences support this proposal: the more compact complex **B** should migrate faster than **A**. Circular dichroism spectra of these oligonucleotides are similar (data not shown), with single maxima at ∼265−270 nm, consistent with characteristic spectrum reported for parallel-stranded tetraplexes[16] that are generally more stable than corresponding antiparallel structures.[4a]

To probe the effect of tetraplex formation on their ability to hybridize with complementary RNA, these oligonucleotides were incubated with sense RNA in buffered aqueous solutions with NaCl or KCl. PAGE analysis revealed that tetraplexes of 1×4 OMe, 2×4 OMe, and 4×4 OMe readily dissociated to form duplexes (examples shown in lanes 5, 6 of Figure 3), whereas about half of 3′-Chol and 4 × OMe 3′-Chol remained as tetraplexes (lanes 7−10). Prolonged incubation increased duplex formation (data not shown), indicating a slow exchange amongst monomers, duplexes, and tetraplexes. The increased tetraplex stability of 3′-Chol and 4×OMe 3′-Chol probably resulted from decreased accessibility of 3′-ends for duplex formation.

In conclusion, we have demonstrated that two strategies can be utilized to promote G-quartet formation: 2′-*O*-methylation of the contiguous guanine residues and covalent attachment of hydrophobic groups at positions distant from them. These oligonucleotides retain the ability to hybridize with a complementary RNA sequence, a crucial property for antisense applications. Since the extent of G-quartet formation might influence the biological properties and functions of antisense oligonucleotides in cells, this structural motif might result in different, hopefully favorable pharmacokinetics properties such as cellular uptake, distribution, and metabolism.

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