Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 4, No. 8, pp. 1041-1046, 1994

0960-894X/94 $6.00+0.00

0960-894X(94)E0098-Y

OPTIMIZATION OF HYBRIDIZING ABILITIES AND NUCLEASE RESISTANCE IN THE DESIGN OF CHIMERIC α–ANOMERIC OLIGODEOXYNUCLEOTIDES CONTAINING β–ANOMERIC GAPS

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Abstract: Various chimeric oligonucleotides consisting of α- and β-anomeric deoxynucleotide units have been synthesized and their hybridizing abilities to complementary DNA or RNA single strands, and their stability in cell culture medium or cell extracts, were studied. The results provide guide lines for optimization of antisense chimeric oligodeoxynucleotides.

Chimeric oligonucleotides consisting of a stretch of deoxynucleotide units with several phosphate-modified nucleotides at both 5'- and 3'-ends have proven to be more efficient antisense agents than the corresponding fully-unmodified oligodeoxynucleotides in both acellular[1,2] and cellular[3,4] systems. These modifications have included methylphosphonate analogues[1,2] and phosphoramidates[3,4]. More recently, it has been shown that chimeric phosphorothioate oligodeoxynucleotides containing various 2'-sugar modifications at both ends displayed enhanced antisense potencies in inhibiting Ha-*ras* gene expression compared with the unmodified uniform phosphorothioate analogue[5]. Upon hybridization to the complementary RNA target of these "sandwich" analogues, the central part of the oligomer was able to elicit RNase H-mediated hydrolysis of the RNA moiety in the resulting RNA•DNA duplex[6], while the nuclease-resistant flanks contributed to the specificity for the recognition of the target and to the stability of the hybrid. When methylphosphonate flanks were used, the observed higher specificity was attributed to a weaker tendency to tolerate mismatches due to reduced affinity for their complementary sequence when compared with phosphodiester ones[2,7,8], and to the inability of "sandwich" oligos bound to partially complementary RNA sites through base-pairing involving modified nucleotides to elicit RNase H activity[9]. Moreover, their reduced affinity for complementary RNA strands was invoked to explain enhanced RNase H activity through a faster dissociation of the heteroduplex-enzyme complex, thereby allowing the chimeric molecules to act in a more "catalytic" manner[2].

α-Oligodeoxynucleotides consisting exclusively of α-anomeric deoxynucleotide units were reported by us and others as nuclease-resistant sugar-modified analogues[10,11,12] able to form stable duplexes with either complementary DNA or RNA strands. In the resulting heteroduplexes, the two strands were found in a parallel orientation[13,14,15] and α-oligodeoxynucleotide•RNA duplexes were not substrates of RNase H[15]. More recently, alternating α,β-oligothymidylates were introduced[16] and optimization of their hybridizing

abilities with poly rA or dA_{28} was achieved by introducing non-natural phosphodiester linkages. As a result of the opposite mode of binding of α– and β–anomeric oligonucleotides, it was shown that introduction of 3'→3' or 5'→5' linkages at the junctions between two consecutive thymidylates of opposite anomeric configuration was able to partly restore the hybridizing capacity observed with unmodified oligothymidylate and to enhance nuclease resistance. Here we report that chimeric mixed-sequence oligonucleotides consisting of a stretch of natural deoxy-β-nucleotide units with several deoxy-α-nucleotide units at both 5'- and 3'-ends are able to form stable and fully base-paired duplexes with complementary DNA or RNA strands. Thermal stability of these heteroduplexes decreases with the number of unnatural junctions between α- and β-nucleotides, and with the ratio of purine α-nucleotides to total α-nucleotides within the chimeric oligonucleotide.

As an extension of our previous work[17] related to the comparative evaluation of the antisense properties of various oligonucleotide analogues directed against the splice acceptor site of the HIV-1 *tat* RNA and in order to make relevant comparisons, the same target (**Table 1**) was used in the present study. Chimeric α,β-oligonucleotides complementary to the target and corresponding to different combinations were synthesized[18] (**Table 1**).

Table 1. Sequences of HIV-1 *tat* target and oligonucleotide analogues complementary to the target

Oligomers	Sequences	Comments
1	βA βG βA βA βT βT βG βG βG βT βG βT	single-stranded DNA target
2	βA βG βA βA βU βU βG βG βG βU βG βU	single-stranded RNA target
3	βT βC βT βT βA βA βC βC βC βA βC βA	unmodified β-strand antiparallel to the target
4	αT αC αT αT αA αA αC αC αC αA αC αA	all-α-strand parallel to the target
5	αT αC αT αT βA βA βC βC αC αA αC αA	α,β,α stretches parallel to the target
6	αT αC αT αT βA βA βC βC αC αA αC αA	α,β,α stretches antiparallel to the target
7	αT αC αT αT βA βA βC βC αC αA αC αA	central β-stretch with external α-stretches
8	βT αC βT αT βA βA βC βC αC βA αC βA	central β-stretch with external alternating α,β-stretches
9	βT αC βT αT βA αA βC αC βC αA βC αA	fully alternating α,β strand with two 3'-OH termini
10	αT βC αT βT αA βA αC βC αC βA αC βA	fully alternating α,β strand with two 5'-OH termini

Greek type letter preceeding each upper case letter refers to the anomeric configuration of the corresponding individual nucleotide. Arrows indicate the 5'→ 3' orientation. A single arrow covering several upper case letters indicates a homogeneous orientation of the corresponding nucleotides. Head to head arrows and tail to tail arrows depict the presence of unnatural 3'→ 3'- and 5'→ 5'-phosphodiester links respectively

Oligonucleotide analogues **5-7** contain a central stretch of four deoxy-β-nucleotides with two terminal stretches of four deoxy-α-nucleotides each. In oligos **5** and **6** the three constitutive stretches are uniformly oriented and natural phosphodiester linkages were exclusively present. In contrast, oligo **7** possesses the correct orientation of the three stretches for optimal hybridization to the target; i.e. anti parallel for the β-stretch and parallel for the two α-stretches. Consequently, chimeric oligo **7** contains two unnatural inter nucleotide linkages (3'→3' and 5'→5') at the boundaries of the different stretches. In oligo **8**, the external stretches consist of alternating α, β-nucleotides in order to evaluate their contribution to resistance to exonucleases. Finally, fully alternating α,β-oligos **9** and **10** possess different proportions of purine and pyrimidine nucleotides in either α- or β-anomeric configuration and have, respectively, two 3'-ends and two 5'-ends which should provide them with different behaviour towards 3' or 5'-exonucleases[19].

First, hybridization properties of oligos **5-10** to the DNA target **1** were evaluated by determining Tm values derived from melting curves recorded at 260nm. Data are shown in **Table 2.**

Table 2. Thermal stability of hybrids formed between antisense oligomers and DNA or RNA targets. **n**: number of unnatural phosphodiesters links; **r**: ratio of purine-α-nucleotides to total α-nucleotides

			vs DNA		vs RNA	
Oligos	n	r	Tm °(C)	ΔTm/n	Tm °(C)	ΔTm/n
3	0		47.6*		46.1*	
4	0	0.33	42.8*		43.1*	
5			13.2		ND	
6			<0		ND	
7	2	0.25	44	-1.8	41.2	-2.4
8	8	0	41.5	-0.6	36.8	-1.2
9	11	0.5	25.4	-2	19.6	-2.4
10	11	0.17	36	-1	27.3	-1.7

Melting temperature experiments were performed with equimolar mixtures of complementary oligonucleotides each at a concentration of 3 μM in 0.1 M NaCl, 10 mM sodium cacodylate, pH 7. An Uvikon 810 spectrophotometer (Kontron) fitted with a thermostated cell holder and interfaced with an IBM PC compatible was used. Heating rate was 0.5°C min^{-1}.
* from reference 17.

Worth noting is the relatively high Tm value obtained with oligo **7** which was only 3.6°C lower than that corresponding to the unmodified β–oligo **3** and slightly higher than the Tm value of the hybrid formed with the all α-anomeric oligo **4**. In contrast, a sharp drop in hybrid stability was observed with oligos **5** and **6** which is in agreement with a partial hybridization (if any) with the DNA target involving either the two terminal α-stretches or the central β-stretch of **5** and **6** respectively. These results confirmed that the orientation found previously[13,14,15] with α-oligodeoxynucleotides for duplex formation with either natural DNA or RNA complementary strands were also valid for chimeric oligos consisting of α- and β-stretches. Whether this result could be extended to alternating α,β-stretches or even to fully alternating α,β-oligos was addressed with compounds **8-10**. Weaker hybrids were obtained with Tm values decreasing with an increasing number of unnatural 3'→3' or 5'→5' phosphodiesters linkages. Surprisingly, fully alternating

α,β-oligos **9** and **10** which have the same number of unnatural links (n 11) exhibited very different Tm values. The corresponding values for average variation in melting temperature per unnatural link (ΔTm/n) were consequently different (-2°C for oligo **9** and -1°C for oligo **10**). When considering all four chimeric oligos **7-10**, ΔTm/n values varied from -0.6°C up to -2°C and varied directly with the ratio (r) of purine-α-nucleotides to total α-nucleotides. The lowest destabilizing effect (ΔTm/n -0.6°C per modified linkage) was found for oligo **8** which had no purine-α-nucleotide (r 0) and was identical to the value calculated from Beaucage's data[16] relative to fully alternating α,β-dT_{28} / β-dA_{28} duplex under comparable buffer conditions. The highest destabilizing effect (ΔTm/n -2°C per modified linkage) appeared with oligo **9** which exhibited the highest r value (r 0.5). The pertinence of factor r to correlate the variations in ΔTm/n values is supported by previous results. It was shown that for a given sequence of A.T and C.G base pairs, a heteroduplex formed between an α-hexadeoxynucleotide and its complementary β-DNA strand was less stable when the α-strand was purine-rich than when it was pyrimidine-rich[20]. Interestingly, similar results were obtained when chimeric oligos **7-10** were hybridized with the complementary RNA target **2** (**Table 2**), although larger destabilizing effects were observed (-1.2°C > ΔTm/n > -2.4°C per modified linkage).

Data relative to the stability of chimeric oligos **7-10** in culture medium or total cell extracts are reported in **Table 3**.

Table 3. A: Half-life of chimeric oligonucleotides in RPMI 1640 medium supplemented with 10% inactivated fetal calf serum incubated at 37°C[17]; **B:** Percentage of degradation of oligomers in CEM-SS cell extracts at 37°C[21]. Analyses were performed by HPLC[22].

	A RPMI 1640 medium + 10% FCS	**B** CEM-SS cell extracts
Oligos	$t_{1/2}$ of 12-mer	% degradation of 12-mer at t=24 hours
3	11 min.	100% ($t_{1/2}$=24 min.)
4	25 min.*	34%
7	32 min.*	23%
8	16 min.*	13%
9	15 min.**	4%
10	5.5 days	ND

* Formation of a stable shorter fragment was observed which was tentatively attributed to a 11-mer.
** Formation of a stable fragment was observed which was tentatively attributed to a 10-mer.

In RPMI 1640 medium with 10% heat-deactivated fetal calf serum, where predominant 3'-exonuclease activity has been reported[23], all listed compounds except oligo **10** were half-degraded within half an hour. However, whereas β-oligo **3** exhibited a typical pattern of degradation products resulting from progressive hydrolysis from 3'-end[21], each chimeric oligo **7**, **8** and **9** was degraded into one shorter species which was then virtually stable for several hours. These results were tentatively assigned to the removal of one (or two for oligo **9**) nucleotide unit(s) at the 3'-termini, thus generating a 3'-exonuclease-resistant 11-mer (or 10-mer)

with two 5'-termini. This hypothesis was supported by the considerably higher resistance observed with oligo **10** ($t_{1/2}$ 5.5 days), and by similar results obtained with a β-oligodeoxynucleotide having a terminally inverted polarity with a 3'→ 3' phosphodiester linkage[19,24].

When compared to unmodified β-oligo **3**, oligos **4**, **7-9** were highly resistant to hydrolysis in total CEM-SS cell extracts. In addition to the stability induced by replacement of β-nucleotide units by α-nucleotide units (compare oligos **3** and **4**), substitution of natural 3'→5' phosphodiester linkages by unnatural 3'→ 3' or 5'→ 5' links also contributed significantly to the enhancement of nuclease resistance of the chimeric oligos (compare oligos **4** and **9**).

In conclusion, these results provide guide lines for the design of chimeric α,β-oligonucleotides. Optimal stability against exonucleases present in sera and cellular extracts can be attained by introduction of terminally inverted polarity whatever the anomeric configuration of the terminal nucleotide is. The advantage of using properly oriented α- and β-nucleotide units is that every base of the chimeric oligos participates in the specificity in the recognition of the target, and in the thermal stability of the resulting hybrid. Additional resistance against endonuclease hydrolysis is available by introducing, as much as possible, unnatural 3'→ 3' and 5'→ 5' phosphodiester linkages by alternating α- and β-nucleotide units within the sequence except, of course, in the central stretch of β-nucleotides designed to elicit RNase H activity. It is possible to modulate affinity of chimeric α,β-oligonucleotides for DNA or RNA targets by varying the number of modified internucleotidic linkages and the ratio of purine α-nucleotides to total α-nucleotides present in a given sequence. Higher affinity requires that this ratio be kept as low as possible.

A compromise between these requirements should provide optimized antisense oligonucleotides and work is in progress along this line.

Acknowledgment. We thank S. Vichier-Guerre and A. Pompon for their technical support in the set up of the On-line ISRP cleaning HPLC technique[22] and P. Scheiner for careful reading of the manuscript. This work was supported by grants from the "Association pour la Recherche contre le Cancer" (A.R.C.) and from the "Agence Nationale de Recherche sur le Sida" (A.N.R.S.).

References and Notes.

1. Larrouy, B.; Blonski, C.; Boiziau, C.; Stuer, M.; Moreau, S.; Shire, D.; Toulmé, J.-J. *Gene* **1992**, *121*, 189.
2. Giles, R. V.; Tidd, D. M. *Anti-Cancer Drug Design* **1992**, *7*, 37.
3. Dagle, J. M.; Walder, J. A.; Weeks, D. L. *Nucl. Acids Res.* **1990**, *18*, 4751.
4. Potts, J. D.; Dagle, J. M.; Walder, J. A.; Weeks, D. L.; Runyan, R. B. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 1516.
5. Monia, B. P.; Lesnik, E. A.; Gonzalez, C.; Lima, W. F.; McGee, D.; Guinosso, C . J.; Kawasaki, A. M.; Cook, P. D.; Freier, S. M. *J. Biol. Chem.* **1993**, *268*, 14514.
6. Crouch, R. J.; Dirksen, M. L. *Ribonucleases H*; Linn, S. M.; Roberts, R. J., Eds; Nucleases. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, **1982**, 211.
7. Quartin, R. S.; Wetmur, J. G. *Biochemistry* **1989**, *28*, 1040.
8. Maher III, L. J.; Dolnick, B. J. *Nucl. Acids Res.* **1988**, *16*, 3341.

9. Furdon, P. J.; Dominski, Z.; Kole, R. *Nucl. Acids Res.* **1989**, *17*, 9193.
10. Morvan, F.; Rayner, B.; Imbach, J.-L.; Thenet, S.; Bertrand, J.-R.; Paoletti, J.; Malvy, C.; Paoletti, C. *Nucl. Acids Res.* **1987**, *15*, 3421.
11. Bacon, T. A.; Morvan, F.; Rayner, B.; Imbach, J.-L.; Wickstrom, E. *J. Biochem. Biophys. Methods* **1988**, *16*, 311.
12. Thuong, N. T.; Asseline, U.; Roig, V.; Takasugi, M.; Hélène, C. *Proc. Natl. Acad. Sci. USA* **1987**, *84*, 5129.
13. Morvan, F.; Rayner, B.; Imbach, J.-L.; Lee, M.; Hartley, J. A.; Chang, D. K.; Lown, J. W. *Nucl. Acids Res.* **1987**, *15*, 7027.
14. Gagnor, C.; Bertrand, J.-R., Thenet, S.; Lemaître, M.; Morvan, F.; Rayner, B.; Lebleu, B.; Imbach, J.-L.; Paoletti, C. *Nucl. Acids Res.* **1987**, *15*, 10419.
15. Gagnor, C.; Rayner, B.; Leonetti, J.-P.; Imbach, J.-L.; Lebleu, B. *Nucl. Acids Res.* **1989**, *17*, 5107.
16. Koga, M.; Moore, M. F.; Beaucage, S. L. *J. Org. Chem.* **1991**, *56*, 3757.
17. Morvan, F.; Porumb, H.; Degols, G.; Lefebvre, I.; Pompon, A.; Sproat, B. S.; Rayner, B.; Malvy, C.; Lebleu, B.; Imbach, J.-L. *J. Med. Chem.* **1993**, *36*, 280.
18. Oligonucleotides **4-10** were prepared on an ABI 381 A DNA synthesizer via the phosphoramidite route following the standard procedure and using deoxy-α- and β-nucleosides 3'- or 5'-phosphoramidites. Deoxy-α-nucleoside 5'-phosphoramidites were obtained with an overall yield ranging from 25% to 68% starting from corresponding base-protected α-nucleosides. First, the starting compound was sequentially protected in 5'-position with a *tert*-butyldimethylsilyl (TBDMS) group and in 3'-position with a 4,4'-dimethoxytrityl (DMTr) group. 5'-hydroxyl of the resulting 5'-O-TBDMS-3'-O-DMTr-2'-deoxy-α-nucleoside was selectively deprotected by treatment with tetrabutylammonium fluoride and subsequently phosphitylated to give the corresponding α-nucleoside 5'-cyanoethyl-phosphoramidite.
^{31}P-NMR (CD_3CN) of α-nucleoside 5'-phosphoramidites, δ in ppm relative to external 85% H_3PO_4. α-dC^{Bz} δ 149.90 and 149.79; α-dA^{Bz} δ 150.11 and 149.92; α-dG^{ibu} δ 150.12 and 149.84.
19. Ramalho Ortigão, J. F.; Rosh, H.; Selter, H.; Fröhlich, A.; Lorenz, A.; Montenarh, M.; Seliger, H. *Antisense Res. Dev.* **1992**, *2*, 129.
20. Paoletti, J.; Bazile, D.; Morvan, F.; Imbach, J.-L.; Paoletti, C. *Nucl. Acids Res.* **1989**, *7*, 2693.
21. Vichier-Guerre, S.; Pompon, A.; Lefebvre, I.; Imbach, J.- L. *Antisense Res. Dev.* in press.
22. Pompon, A.; Lefebvre, I.; Imbach, J.-L. *Biochem. Pharmacol.* **1992**, *43*, 1769.
23. Tidd, D. M.; Warenius, H. M. *Br. J. Cancer* **1989**, *60*, 343.
24. Shaw, J.-P.; Kent, K.; Bird, J.; Fishback, J.; Froelher, B. *Nucl. Acids Res.* **1991**, *19*, 747.

(*Received* 11 *October* 1993)