Oligonucleotides Containing a Covalent Conformationally Restricted Phosphodiester Analog for High-Affinity Triple Helix Formation: The Riboacetal Internucleotide Linkage

Robert J. Jones,* S. Swaminathan, John F. Milligan, Shalini Wadwani, Brian C. Froehler, and Mark D. Matteucci*

Gilead Sciences, 353 Lakeside Drive Foster City, California 94404

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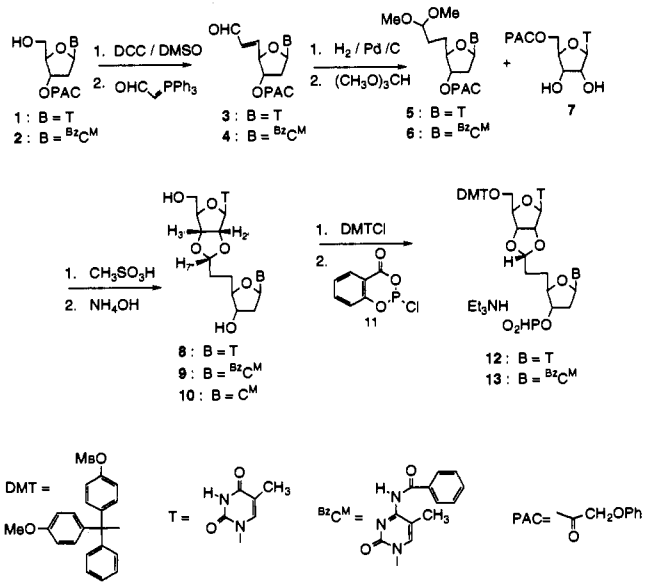
The high-affinity, sequence-specific recognition of duplex (ds) DNA is an area of biological and chemical interest.[1] Oligonucleotides (ONs) have been shown to recognize polypurine tracts of dsDNA in a sequence-specific manner via triple helix formation.[2] Optimization of this ligand (ON)–receptor (dsDNA) interaction can be conceptually achieved by restricting the conformational freedom of unbound ON to resemble its bound conformation. The enhancement of ligand–receptor interactions through conformational restriction has been elegantly demonstrated in the field of crown ethers[3] and cryptands[4] and has been extended to ligands binding to hormone receptors and enzyme inhibitors.[5] We report the application of this concept to the ON–dsDNA ligand–receptor interaction by replacing the phosphodiester internucleotide connection with a conformationally restricted acetal linkage which we have termed "riboacetal."[6]

Riboacetal dimers were incorporated into ONs as thymine–thymine (T·T) and thymine–5-methylcytosine[7] (T·C^M) dimer blocks. Specifically for the T·T dimer (Scheme I), Moffatt oxidation and Wittig homologation[8] of 3′-protected thymidine **1** afforded the corresponding unsaturated aldehyde **3**, which was catalytically reduced and converted to the dimethylacetal **5**. Coupling of this acetal with diol **7**[9] under acidic catalysis and removal of the ester protecting groups yielded dimer **8** as a single diastereomer. This compound was determined to be in the endo conformation from analysis of the NOESY crosspeaks of the $H_{7''}$ of **8** with the $H_{2'}$ and $H_{3'}$.[10] Sequential dimethoxytritylation and phosphitylation[11] afforded the H-phosphonate **12**.

Scheme I. Synthesis of Riboacetal Backbone Linkages



The T·T and T·^{Bz}C^M riboacetal dimer blocks **12** and **13** were incorporated into 15mer ONs by solid-phase DNA chemistry using an H-phosphonate protocol.[12] Chimeric ONs **16–20** (Table I) were synthesized where riboacetal linkages (·) were incorporated two, four, or seven times, alternating with phosphodiester bonds (p). ON **15**, which contained all phosphodiester linkages, served as the control for binding studies. The ONs were purified by PAGE, and nucleoside composition analysis afforded the expected ratios of riboacetal dimers and nucleosides.[13]

Triple helix binding affinities were determined by thermal denaturation (T_m) and DNase I footprint analyses. The T_m values of ONs **15–20** were measured against the hairpin DNA target **14**,[14] and the denaturations resulted in two transitions. The first transition corresponded to the melting of the triple helix and the second to the hairpin target.[15] As shown in Table I, the triplex T_ms increased with increasing riboacetal incorporations from a ΔT_m of 8 °C for the two linkages of ON **16** to a ΔT_m of 26 °C for the seven linkages of ON **20**.[16] These data demonstrate that the ONs containing riboacetal linkages recognize dsDNA with an enhanced affinity over the phosphodiester control.

The affinity and specificity of triple helix formation by ONs **15** and **20** were also determined by DNase I footprint analysis.[17,18] The autoradiogram derived from the DNase I footprint analysis (Figure 2, supplementary material) showed that the control phosphodiester **15** completely protected the perfectly matched

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(6) (a) The chemical name for the T–T riboacetal **8** is 2′,3′-O-[1,2,5,6-tetradeoxy-1-(thymin-1-yl)-β-D-*erythro*-heptofuranuron-(7*R*)-ylidene]-5-methyluridine. Molecular modeling suggested that the riboacetal scaffold could favorably position the heterocycles in space for either A or B form helix formation. (b) After completion of this manuscript, a conformationally constrained nucleoside analog was reported: Tarkoy, M.; Bolli, M.; Schweizer, B.; Leumann, C. *Helv. Chim. Acta* **1993**, *76*, 481.

(7) 5-Methyl-2′-deoxycytidine (C^M) was used in place of 2′-deoxycytidine since it has previously shown improved triple helix binding properties. Lee, J. S.; Woodsworth, M. L.; Latimer, L. J. P.; Morgan, A. R. *Nucleic Acids Res.* **1984**, *12*, 6603. Maher, L. J., III; Wold, B.; Dervan, P. B. *Science* **1989**, *245*, 725. Povsic, T. J.; Dervan, P. B. *J. Am. Chem. Soc.* **1989**, *111*, 3059. Xodo, L. E.; Manzini, G.; Quadrifoglio, F.; van der Marel, G.; van Boom, J. H. *Nucleic Acids Res.* **1991**, *19*, 5625.

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(15) The Tm of the hairpin duplex was 75 ± 1 °C.

(16) The concentration of all ONs was 2.8 μ*M*, and the following buffer was used: 140 mM KCl, 5 mM Na_2HPO_4 (10 m*M* Na^+), 1 mM $MgCl_2$, and pH 7.2. These salt conditions were chosen to approximate the intracellular cationic environment. See: Alberts, B.; *et al. Molecular Biology of the Cell*; Garland: New York, 1989; p 301.

(17) The target sequence was incorporated into a restriction fragment to provide information on specificity as well as binding affinity. The restriction fragment, shown schematically in Figure 1, contains four cassettes which differ only in the base at the fifth position, which was varied from guanine to adenine, thymine, or cytosine in cassettes 1–4, respectively. The ONs **15** and **20** described above (Table I) target cassette 2 of this plasmid, while cassettes 1, 3, and 4 each contain a one-base mismatch.

(18) Jones, R. J.; Lin, K.-Y.; Milligan, J. F.; Wadwani, S.; Matteucci, M. D. *J. Org. Chem.* **1993**, *58*, 2983.

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Table I. Triplex T_m (±0.5 °C) Analyses of Riboacetal ONs[a]

DNA Duplex Target: **14**: 5' d(AGAGAGAGAGAAAAAGGA T T
3' (TCTCTCTCTCTTTTTCCT T T

Sequences		# RA	Tm(°C)	ΔTm(°C)
15:	5'd(TpCpTpCpTpCpTpCpTpCpTpTpTpTpT)	0	34	-
16:	5'd(TpCpTpCpTpCpTpCpTpCpT•TpT•TpT)	2	42	+8
17:	5'd(T•CpT•CpTpCpTpCpTpCpTpTpTpTpT)	2	41	+7
18:	5'd(TpCpTpCpTpCpT•CpT•CpT•TpT•TpT)	4	50	+16
19:	5'd(T•CpT•CpT•CpT•CpTpCpTpTpTpTpT)	4	52	+18
20:	5'd(T•CpT•CpT•CpT•CpT•CpT•TpT•TpT)	7	60	+26

[a] #RA = number of riboacetal linkages. In compounds **15–20**, C designates 5-methyl-2'-deoxycytosine. • = riboacetal linkage. p = phosphodiester bond.

cassette 2 of the restriction fragment against DNase I digestion at 10 μM and afforded partial protection at 1 μM. The riboacetal ON **20** showed complete protection at 0.01 μM, the lowest concentration tested. Cassettes 1, 3, and 4, which each contain a different single base mismatch, were not protected from DNase I cleavage using ON **15**. ON **20** displayed binding to the single base mismatched targets at 1 μM, which is a 100-fold higher concentration than required to bind to the exact target, cassette 2. These data demonstrate that the riboacetal moiety in this context substantially increased the binding affinity of an ON for dsDNA while maintaining the ability to discriminate a single nucleoside mismatch.

The stability of the riboacetal ON–RNA duplex was determined by Tm analysis. The duplexes were derived from ONs **15** and **20** and complementary single-stranded (ss) RNA **21** or the corresponding ssRNA sequence containing a G–T mismatch (**22**).[19] The data (Table II) showed the Tm of the riboacetal ON **20** to be lower than the Tm of the phosphodiester control **15** by 7.5 °C when hybridized to the exact complement. The binding affinity with the one-base mismatch target **22** showed the phosphodiester control **15** to decrease by 2 °C, while the Tm of the riboacetal ON decreased 5.0 °C when compared with the T_ms with the exact complement **21**. This greater specificity toward a mismatch in duplex binding might be due to the rigidity of the riboacetal, which may have less tolerance for the wobble structure involved in the T–G mismatch base pair.[20]

Dimers containing the riboacetal linkage were stereospecifically synthesized and incorporated into ONs. A 15mer chimeric ON which contained seven riboacetal linkages alternating with seven phosphodiester linkages displayed a 100-fold increase in affinity for a dsDNA target when compared to the control phosphodiester ON but a decrease in affinity for ssRNA. The enhanced binding of the riboacetal containing ONs to a dsDNA target could be simply due to the replacement of anionic phosphodiesters with neutral surrogates and the consequent elimination of electrostatic

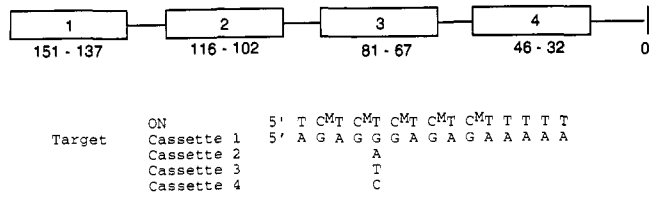

Figure 1. *Pvu*II–*Sal*I restriction fragment used in triple helix footprint assay. Four polypurine tracts were cloned into pUC19 to create p412. Each polypurine cassette is identical except for the base at the fifth position. The specificity of triple helix formation is judged by the relative affinity of an ON for its targeted cassette compared with its affinity for the other cassettes. C^M = 5-methyl-2'-deoxycytidine.

Table II. Duplex T_m (±0.5 °C) Analyses of Riboacetal ONs

RNA Targets complement **21**: 3' AGAGAGAGAGAAAAA
mismatch **22**: 3' AGAGGGAGAGAAAAA

	T_m (°C)	
sequences[a]	**21**	**22**
15 (diester)	62.5	60.5
20 (7 RA)	55.0	50.0

[a] Sequences are as shown in Table I. In compounds **15** and **20**, C designates 5-methyl-2'-deoxycytosine.

repulsion.[21] This is not likely the major effect in that related neutral phosphate mimics such as formacetal (–OCH_2O–) and 3'-thioformacetal (–SCH_2O–) do not show enhanced binding to dsDNA relative to phosphodiesters when tested under the same conditions in the same sequences.[18] These formacetal and 3'-thioformacetal analogs are isosteric to the phosphodiester linkage[22] but do not bear the covalent conformational restriction of the riboacetal. Therefore, the covalent conformational restriction of the riboacetal linkage along with the perturbation of the ribose sugar pucker[23] could be responsible for the enhanced triple helix formation. The specific affinity observed in this study suggests that the riboacetal linkage is a promising phosphodiester analog for sequence-specific ON agents targeting genomic DNA, which may afford the potential to modulate gene expression *in vivo*.

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Note Added in Proof: An extension of ref 6b has been published: Egli, M.; Lubini, P.; Bolli, M.; Dobler, M.; Leumann, C. *J. Am. Chem. Soc.* **1993**, *115*, 5855.

Supplementary Material Available: Figure 2 and experimental details and characterization data for the compounds described herein (12 pages). Ordering information is given on any current masthead page.

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(21) We thank a referee for pointing out this possibility.

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