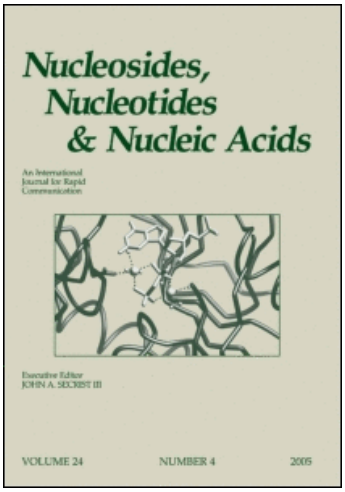

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Stabilization of DNA Double and Triple Helices by Conjugation of Minor Groove Binders to Oligonucleotides

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ABSTRACT

New conjugates containing two parallel or antiparallel carboxamide minor groove binders (MGB) attached to the same terminal phosphate of one oligonucleotide strand were synthesized. The conjugates interact with their target DNA stronger than the individual components. Effect of conjugated MGB on DNA duplex and triplex stability and their sequence specificity was demonstrated on the short oligonucleotide duplexes and on the triplex formed by model 16-mer oligonucleotide with HIV polypurine tract.

Key Words: Oligonucleotides; Minor groove binders; Oligocarboxamides; Conjugation; Duplex; Triple helix.

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Two classes of ligands recognizing double-stranded DNA in a sequence-specific manner are known: N-methylpyrrole/N-methylimidazole oligocarboxamide minor groove binders (MGB)[1,2] and triple helix-forming oligonucleotides (TFO).[3] Being covalently linked together, these ligands are able to bind simultaneously to the major and the minor grooves of the target dsDNA[4,5] and thus potentially interfere with key cellular processes such as replication and transcription.

The conjugation of oligonucleotides to N-terminal amino group of oligocarboxamide minor groove binders was carried out by activation of oligonucleotide terminal phosphate with triphenylphosphine/dipyridyldisulfide (Fig. 1).[6,7] After incubation with activators in organic media the second activation of the same terminal phosphate group could take place and the second residue of oligocarboxamide MGB could be attached via prosphorodiamidate formation.

Using this method, we obtained a series of short DNA duplexes where one oligonucleotide strand was attached to two parallel (N → C) or antiparallel (N → C, C → N) oligocarboxamide residues (Table 1). The conjugates were purified by HPLC on reverse phase (C18) and analyzed by denaturing gel electrophoresis and UV-spectrophotometry.[7] Phosphorodiamidate structure of 1:2 conjugates was confirmed by mass-spectrometry and ^{31}P-NMR (161.95 MHz).

Sequence-specific stabilization of DNA duplexes by one or two MGB residues conjugated to one duplex strand has been shown by thermal denaturation method. The results are shown in Table 1. The most important conclusion is that two parallel or antiparallel tetrapyrrole ligands attached to the same strand of a duplex A have similar stabilizing effect on the double-stranded DNA with a complementary A:T strands ($\Delta Tm = 40° \pm 4°C$) as a hairpin ligand $(Py)_4$-γ-$(Py)_4$ described by Dervan.[1,8,9]

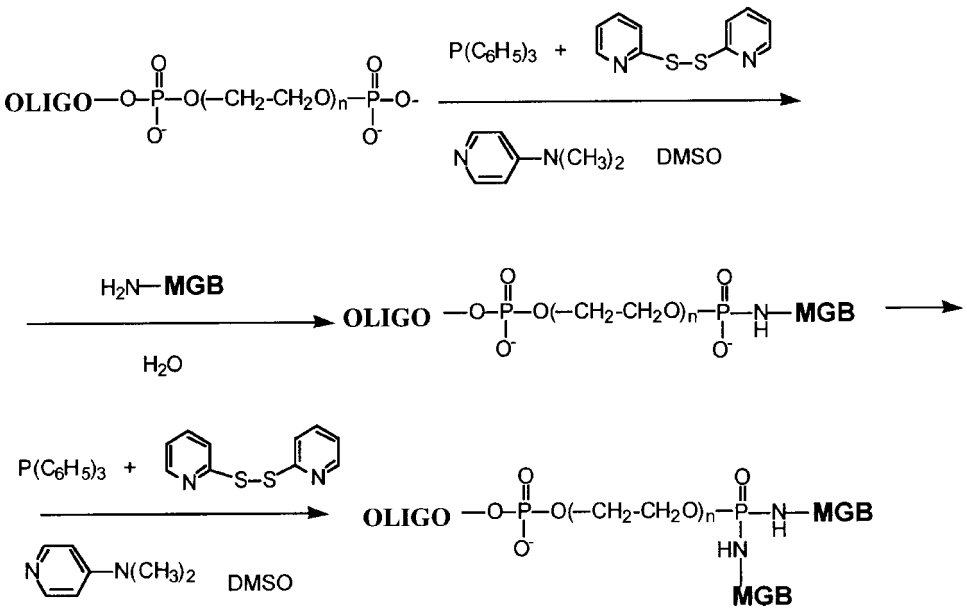

Figure 1. Synthesis of oligonucleotide conjugates with carboxamide minor groove binders.

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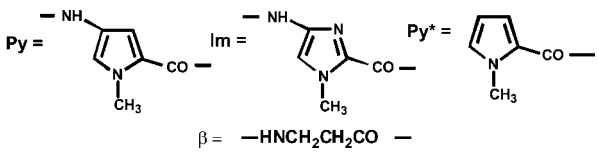
Table 1. Thermal denaturation temperatures of duplexes containing one strand conjugated to one or two minor groove binders. Conditions of thermal denaturation experiments: 0.01 M phosphate buffer 0.1 M NaCl-0.001 M EDTA, oligonucleotide concentrations 1.3–3 μM, temperature change rate 0.2°C/min, detection at 260 and 330 nm.

Expt n°	Duplex	X_1	X_2	T_m, °C
1	A	$\sim O^-$	$\sim O^-$	20
2	A	$\sim O^-$	$\sim NH(CH_2)_5CO\mathbf{(Py)_4}NH(CH_2)_3NEt_2$	46
3	A	$\sim NH(CH_2)_5CO\mathbf{(Py)_4}NH(CH_2)_3NEt_2$	$\sim NH(CH_2)_5CO\mathbf{(Py)_4}NH(CH_2)_3NEt_2$	60
4	A	$\sim O^-$	$\sim NH(CH_2)_5CO\mathbf{(Py)_4}$-$NH(CH_2)_3CO\mathbf{(Py)_4}NH(CH_2)_3NET_2$	58
5	A	$\sim NH(CH_2)_5CO\mathbf{(Py)_4}OEt$	$^*\mathbf{Py(Py)_3}NH(CH_2)_3NH\sim$	56
6	B	$\sim O^-$	$\sim O^-$	26
7	B	$\sim O^-$	$\sim NH(CH_2)_5CO\mathbf{ImβImPy}$-$NH(CH_2)_3CO\mathbf{ImβImPy}NH(CH_2)_3NMe_2$	56
8	B	$\sim NH(CH_2)_5CO\mathbf{ImβImPy}NH(CH_2)_3NMe_2$	$BocNH(CH_2)_5CO\mathbf{ImβImPy}NH(CH_2)_3NH\sim$	52
9	B	$\sim NH(CH_2)_5CO\mathbf{ImβImPy}NH(CH_2)_3NMe_2$	$\sim NH(CH_2)_5CO\mathbf{PyImβIm}NH(CH_2)_3NMe_2$	46

A: 5'-CGTTTATT-pX_1X_2
3'-GCAAATAA
B: 5'-TTGCGC-pX_1X_2
3'-AACGCG



∼: place of attachment to the terminal phosphate (p) of oligonucleotide strand.

In order to confirm sequence specificity of dsDNA:2(MGB) interaction we have synthesized a duplex B with alternating G:C pairs. We constructed and attached to one strand three sequence-specific MGB combinations that must recognize this sequence: a hairpin octacarboxamide and two linear tetracarboxamides in parallel or antiparallel orientation (Table 1). According to results reported earlier,[10,11] we replaced one methylpyrrole residue in every tetracarboxamide chain by more flexible β-alanyl unit. All the three combinations strongly stabilized the target duplex (Table 1, $\Delta Tm = 20–30°C$). This stabilization was observed only when both carboxamide chains were designed according to Dervan rules[1,8,9] and covalently attached to oligonucleotide strand. No stabilizing effect of free MGBs was detected (data not shown).

These results demonstrate a strong and a sequence specific affinity of the two linked oligocarboxamide chains for double-stranded DNA that is comparable (if not better) with classic hairpin MGB conjugates. In order to combine dsDNA-binding properties of both components in one molecule, we attached one and two hairpin hexa- or octamethylpyrroles to 16-mer oligopyrimidine DNA or 2′-O-methyl RNA oligonucleotide $T_4CT_4C_6T$ (designated as HIV-T) via triethyleneglycolphosphate linker (Fig. 1). This linker is long enough to circumvent one of the duplex strands and to connect MGB in the minor groove with TFO in the major groove.[4] The oligonucleotide forms a triple helix with a conservative polypurine tract of HIV proviral DNA which is located in genes *pol* and *nef* of the provirus (artificial target in the form of double-stranded hairpin HIV-Loop is shown on Fig. 2). The remarkable feature of this sequence is that its 5′-adjacent region contains a large A:T tract, an ideal target for oligopyrrole MGB.

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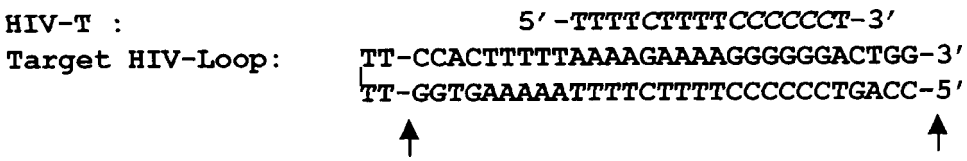

Figure 2. Sequence of the target HIV-Loop DNA and of the triple-helix forming oligonucleotide HIV-T. Original sequence of the HIV proviral polypurine tract fragment from genes *pol* and *nef* is indicated by arrows. For technical reasons, the target sequence was synthesized as a hairpin with two complementary strands connected by tetrathymidylate linker. Two versions of HIV-T were used: with i) DNA backbone and ii) 2′-O-methyl-RNA backbone. All the cytosines in DNA version (in italics) were methylated at position 5.

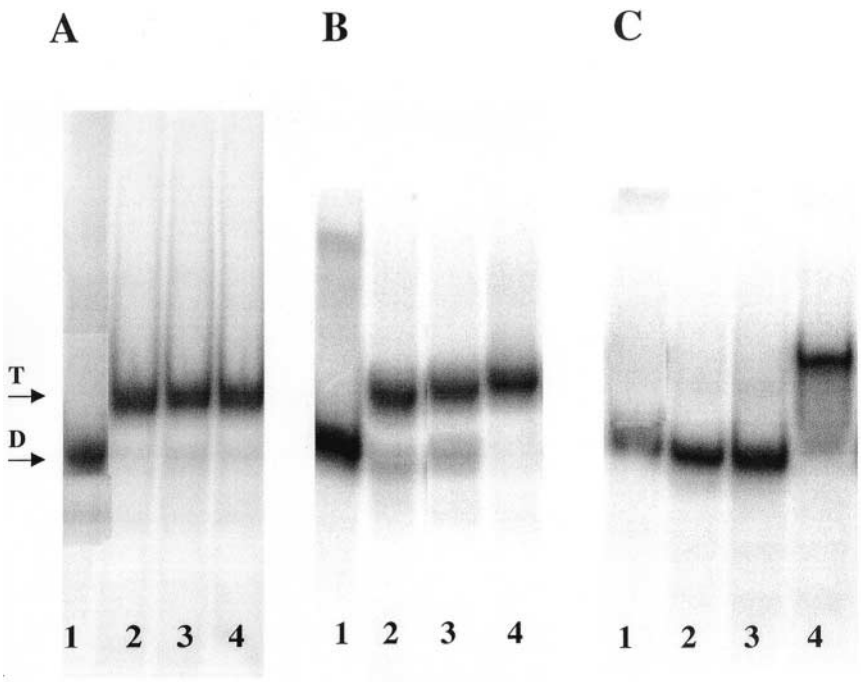

Figure 3. Gel retardation experiments at 10°C (non-denaturating polyacrylamide gel; 20% in **A**) 50 mM MES buffer, 0.05 M NaCl, 0.005 M $MgCl_2$, pH 6.0; **B**) 50 mM HEPES buffer, 0.05 M NaCl, 0.005 M $MgCl_2$, pH 7.0; **C**) 50 mM TBE buffer, 0.05 M NaCl, 0.005 M $MgCl_2$, pH 8.3. **1**. Oligonucleotide HIV-Loop (5′-^{32}P), **2**. HIV-Loop with non-modified HIV-T; **3**. HIV-Loop with the conjugate HIV-T-$(Py)_3$-γ-$(Py)_3$; **4**. HIV-Loop with the conjugate HIV-T-[$(Py)_3$-γ-$(Py)_3$]$_2$. Concentration of HIV-Loop is 50 nM, HIV-T-20 μM. Positions of duplex (D) and triplex (T) are indicated by árrows.

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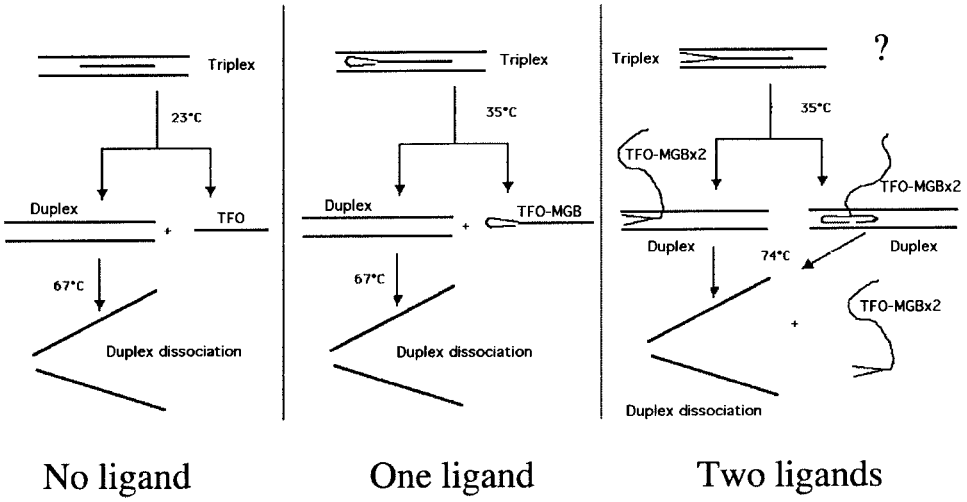

Figure 4. Hypothesis of complex formation and dissociation mechanisms between oligonucleotide-TFO conjugates and target double stranded DNA.

Formation of the complex between conjugates and the target was demonstrated by electrophoresis on a non-denaturing polyacrylamide gel with ^{32}P-labeled double-stranded target HIV-Loop (gel retardation). As is seen from Fig. 3, all conjugates form stable triplexes in standard conditions (MES buffer, pH 6 and 7), independently of the backbone structure (DNA or 2′-O-methyl RNA).

TFO with only one attached hairpin hexapyrrole formed slightly more stable triplex (37°C compared to 24°C for non-modified oligonucleotide) that dissociated at pH > 6. When mismatched oligonucleotide was used, no interaction with a duplex was detected in the case of 1:1 TFO:MGB conjugate. However, conjugates with two parallel MGB residues form stable complexes with double-stranded target even at pH $= 8.3$ and at the temperature > 55°C(Fig. 3). Moreover, the complex of the target with 1:2 conjugates was formed under these extreme conditions even when TFO was substituted by a short non-complementary oligonucleotide C_4T. (data not shown). It means that, in contrast to 1:1 TFO:MGB conjugates, interaction of 1:2 conjugates with double-stranded target is mainly determined by MGB component. Taking into account the size of A:T-rich region of the target DNA and possibility to have different conformations for two attached MGB residues, we proposed a hypothesis of complex formation mechanism that is shown on Fig. 4.

Strong and sequence-specific dsDNA-binders can serve as the base for potential therapeutic agents acting on the level of the genomic DNA.

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