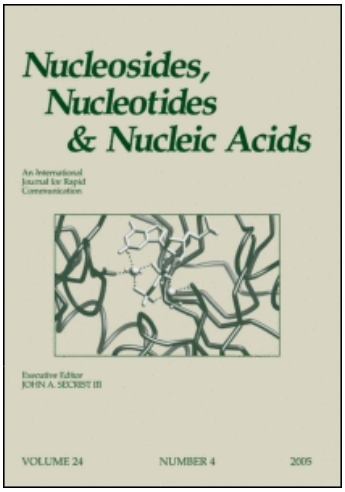

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Binding Properties of the Conjugates of Oligo(2′-O-Methylribonucleotides) with Minor Groove Binders Targeted to Double Stranded DNA

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ABSTRACT

Design, synthesis, physico-chemical and in vitro biological studies of new pyrimidine oligo(2′-O-methylribonucleotide) conjugates with oligocarboxamide minor groove binders (MGB) and benzoindoloquinoline intercalator (BIQ) are described. These conjugates formed stable triple helices with the target double-stranded DNA and inhibited its in vitro transcription upon binding.

Key Words: Oligo(2′-O-methylribonucleotides); Conjugates; Minor groove binders; Triple helix; Transcription inhibition.

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INTRODUCTION

Synthetic oligonucleotides, their analogs and conjugates attract increasing interest as molecular research instruments and as a new class of potential anti-viral and anti-cancer therapeuticals. They can be used for gene expression regulation in a sequence-specific manner by targeting complementary mRNA (antisense oligonucleotides or catalytic nucleic acids), double stranded DNA via triplex formation (anti-gene oligonucleotides) or proteins (aptamers). In order to improve the biological activity of oligonucleotides, significant efforts were applied to the development of oligonucleotide analogs and derivatives with increased resistance to nucleases and increased affinity to nucleic acids targets.[1,2] Oligo(2′-O-methylribonucleotides) as potential anti-gene reagents have several advantages: a simple synthesis procedure, a high stability toward nuclease degradation and an efficient formation of a triple helix with the target DNA.[3] Introduction of terminal 3′-3′-internucleotide linkage completely protects these oligomers toward 3′-exonucleases.[4,5] Both affinity and specificity of the triplex-forming oligo(2′-O-methylribonucleotides) to the target DNA can be increased by conjugation of oligonucleotides to sequence-specific oligocarboxamide minor groove binders (MGB).[6] The introduction of triplex-specific intercalating agent such as benzoindoloquinoline (BIQ) into these conjugates may additionally improve the triplex stability.[7–9]

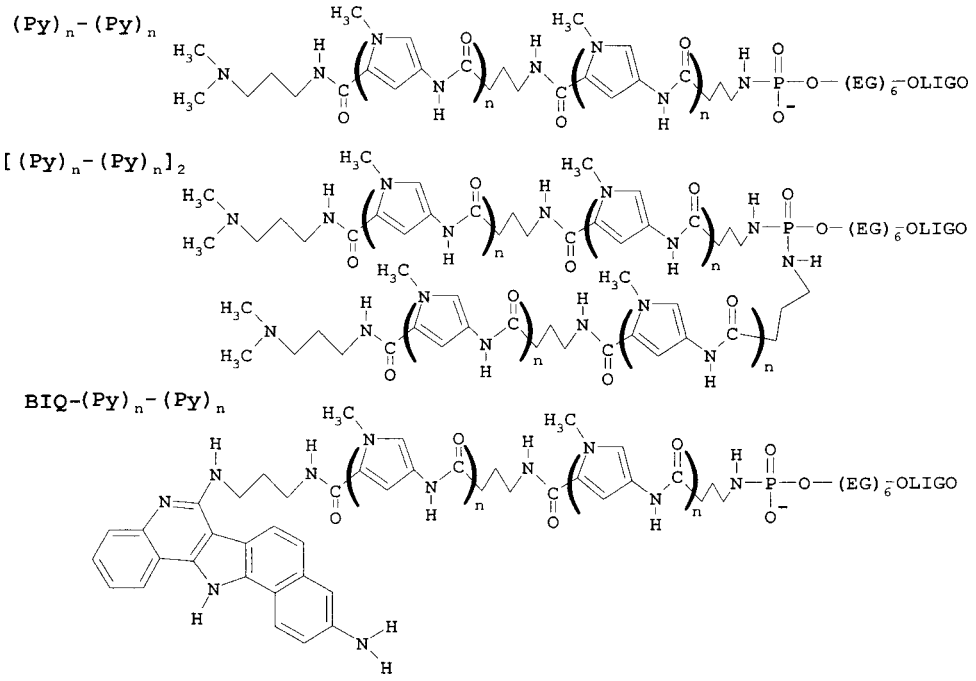

Figure 1. Structure of the synthesized conjugates. OLIGO: oligo(2′-O-methylribonucleotide) with hexa(ethylene glycol) phosphate at the 5′-end; n = 3 or 4; BIQ—6-amino-10-amino-13*H*-benso[6,7]indolo[3,2-c]quinoline; Py—N-methylpyrrole.

Table 1. Dissociation constants of triple complexes of dsDNA with oligo(2′-O-methylribonucleotide) conjugates.[a]

Conjugate	K_d (pH 6.0), nmol	K_d (pH 7.2), nmol
$U^mU^mU^mU^mU^mU^mU^mU^mU^mC^mC^mC^mC^mC^mC^mU^m{}_{3'\text{-}3'}T$ (**I**)	150 ± 70	n/d
$(Py)_3\text{-}(Py)_3pL_6pU^mU^mU^mU^mU^mC^mU^mU^mU^mU^mC^mC^mC^mC^mC^mC^mU^m{}_{3'\text{-}3'}T$ (**II**)	85 ± 40	600 ± 140
$[(Py)_3\text{-}(Py)_3]_2pL_6pU^mU^mU^mU^mC^mU^mU^mU^mU^mC^mC^mC^mC^mC^mC^mU^m{}_{3'\text{-}3'}T$ (**III**)	65 ± 10	70 ± 15
$BIQ\text{-}(Py)_3\text{-}(Py)_3pL_6pU^mU^mU^mU^mU^mU^mU^mU^mU^mC^mC^mC^mC^mC^mC^mU^m{}_{3'\text{-}3'}T$ (**IV**)	150 ± 65	90 ± 17
$(Py)_4\text{-}(Py)_4pL_6pU^mU^mU^mU^mC^mU^mU^mU^mU^mC^mC^mC^mC^mC^mC^mU^m{}_{3'\text{-}3'}T$ (**V**)	160 ± 70	–
$BIQ\text{-}(Py)_4\text{-}(Py)_4pL_6pU^mU^mU^mU^mC^mU^mU^mU^mU^mC^mC^mC^mC^mC^mC^mU^m{}_{3'\text{-}3'}T$ (**VI**)	220 ± 25	–
$BIQ\text{-}(Py)_3\text{-}(Py)_3pL_6pTTTTC^*TTTTC^*C^*C^*C^*C^*T$ (**VII**)	370 ± 70	–
$BIQ\text{-}(Py)_4\text{-}(Py)_4pL_6pTTTTC^*TTTTC^*C^*C^*C^*C^*T$ (**VIII**)	500 ± 120	–

[a]Obtained by gel shift assay at 13°C and pH 6.0 (50 mM MES, 50 mM NaCl, 5 mM $MgCl_2$) or at 37°C and pH 7.2 (50 mM HEPES, 50 mM NaCl, 5 mM $MgCl_2$). Duplex concentration was 60 nM, TFO concentration was between 0,5 and 50 μM. C*—5-methyldeoxycytidine; N^m—2′-O-methylribonucleotide.

RESULTS AND DISCUSSION

A series of pyrimidine oligo(2′-O-methylribonucleotide) conjugates with one or two residues of hairpin MGB (hexa- or octa(N-methylpyrrole)carboxamide) or with MGB residue and intercalator benzoindoloquinoline were synthesized (Fig. 1, Table 1).

For the synthesis of covalent conjugates of oligo(2′-O-methylribonucleotides) and MGB we used the direct coupling of amino-containing MGB residue to the activated 5′-terminal phosphate group of oligonucleotide according to.[9,10] One or two MGB residues were attached to the same terminal phosphate of the oligonucleotide. The triplex-specific intercalator benzoindoloquinoline was eventually attached to the C-terminus of MGB in 1:1 conjugates. The conjugates were analyzed by HPLC, gel electrophoresis, spectrophotometry and their structure was confirmed by electrospray Q-TOF mass spectrometry. Two maximums of absorption were observed in the UV-spectra of oligo(2′-O-methylribonucleotide) conjugated with MGB (Fig. 2), one is characteristic for oligonucleotides and the second is characteristic for MGB attached to oligonucleotide. The A_{260}/A_{310} ratio was measured for each conjugate. It corresponded to 1:1 or 1:2 molar ratios for conjugates with one and two MGB residues, respectively.

The ability of the conjugates for triplex formation with model dsDNA as well as dissociation constants of the complexes were estimated using gel shift and UV thermal denaturation assays.

A synthetic hairpin duplex containing 29 base pairs from a natural polypurine sequence of HIV proviral DNA of genes *nef* and *pol* was used as a target (Fig. 3). Thermal denaturation method showed the same stability of the triplexes formed by non-modified oligo(2′-O-methylribonucleotide) and their deoxyribo-analog containing 5-methyldeoxycytidine with the target dsDNA (T_m 24°C). The 3′-3′-internucleotide linkage at the 3′-terminus of oligo(2′-O-methylribonucleotides) has no influence on the thermal stability of the triplex (data not shown). The separated melting of third strand is

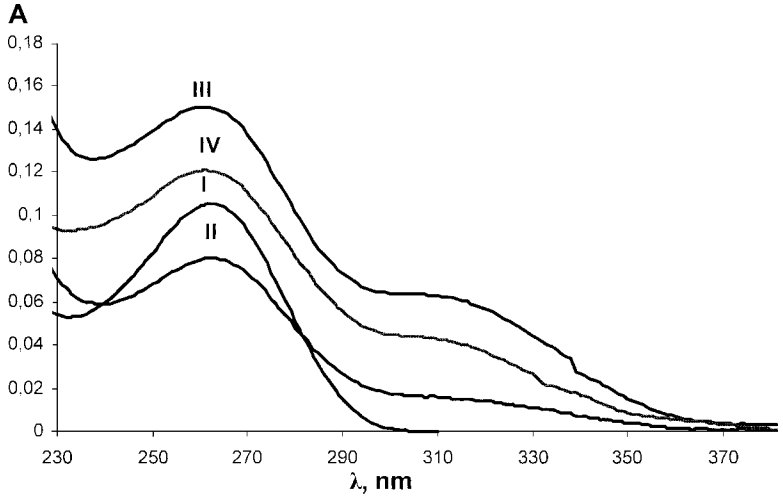

Figure 2. UV-Spectra of oligo(2′-O-methylribonucleotide) (**I**) and the conjugates with one (**II**) or two (**III**) MGB residues or with MGB and BIQ (**IV**).

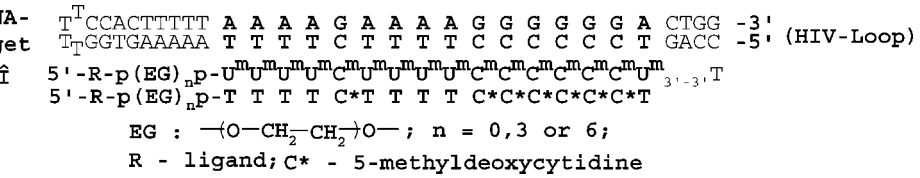

Figure 3. Double stranded hairpin DNA target and oligonucleotide conjugates for the triplex formation studies.

not seen on the melting curves of conjugates despite the triplex was clearly detected by the gel retardation method. However, the simultaneous melting of the duplex and MGB moiety was detected by negative melting peak at 320 nm in the conjugates. Moreover, an increase of duplex melting temperature was shown for the conjugates containing two MGB residues (Fig. 4). It's interesting that two parallel MGB moieties bind to the duplex and stabilize it even at pH 8,3 or in case when oligonucleotide moiety is mutated and is not able to form triplex with a target DNA (data not shown). In addition, for 1:2 conjugates the K_d does not change with pH and temperature (Table 1). It means that two parallel oligocarboxamide ligands play a decisive role in the dsDNA binding.

It was demonstrated that all conjugates form more stable complexes compared to that formed by non-conjugated individual components. The apparent dissociation constants of triple complexes formed by oligonucleotide-MGB conjugates and dsDNA were evaluated from gel retardation experiments at different pH (Table 1). The initial

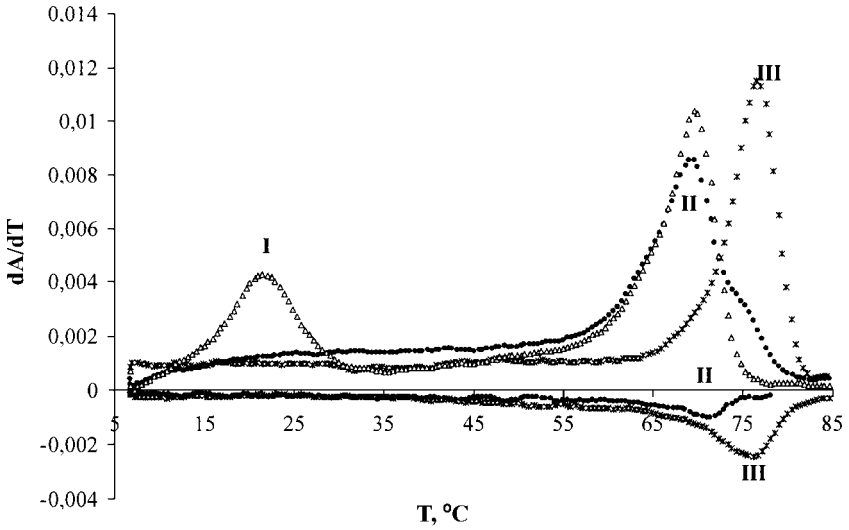

Figure 4. First derivatives of the thermal denaturation curves of the complexes formed by conjugates (**I**, **II** and **III**). Melting conditions: buffer (10 mM cacodylate Na, 100 mM NaCl, 5 mM $MgCl_2$, pH 6.0), [duplex] = $1.3 \cdot 10^{-6}$ M, [TFO] = $1.3 \cdot 10^{-6}$ M. Temperature variation rate was 0.1°C/min. UV-Absorption detection was realized under 260 and 320 nm.

oligo(2′-O-methylribonucleotide) (**I**) formed stable triplex with dsDNA at pH 6.0 but did not form complex at pH 7.2 up to 10 μM concentration. The conjugates of this oligomer with MGB residues (**II-IV**) are capable to form triplexes at pH 6.0 as well as at pH 7.2. The attachment of one or two MGB residues increased the stability of the complex. It was shown that the conjugates with hexa(N-methylpyrrole)carboxamide MGB residues (**II,IV** and **VII**) have better complex formation properties than the conjugates with octa(N-methylpyrrole)carboxamide MGB residues (**V,VI** and **VIII**) due to several structural factors that are under investigation. Control oligodeoxyribonucleotide conjugates (**VII,VIII**) formed less stable triplexes with dsDNA than the analogous conjugates of oligo(2′-O-methylribonucleotides) (**IV,VI**). The conjugates of oligo(2′-O-methylribonucleotides) with two MGB residues or with MGB and BIQ residues have better complex forming properties at pH 7.2 than that with one MGB residue, i.e. at the simulated physiological conditions.

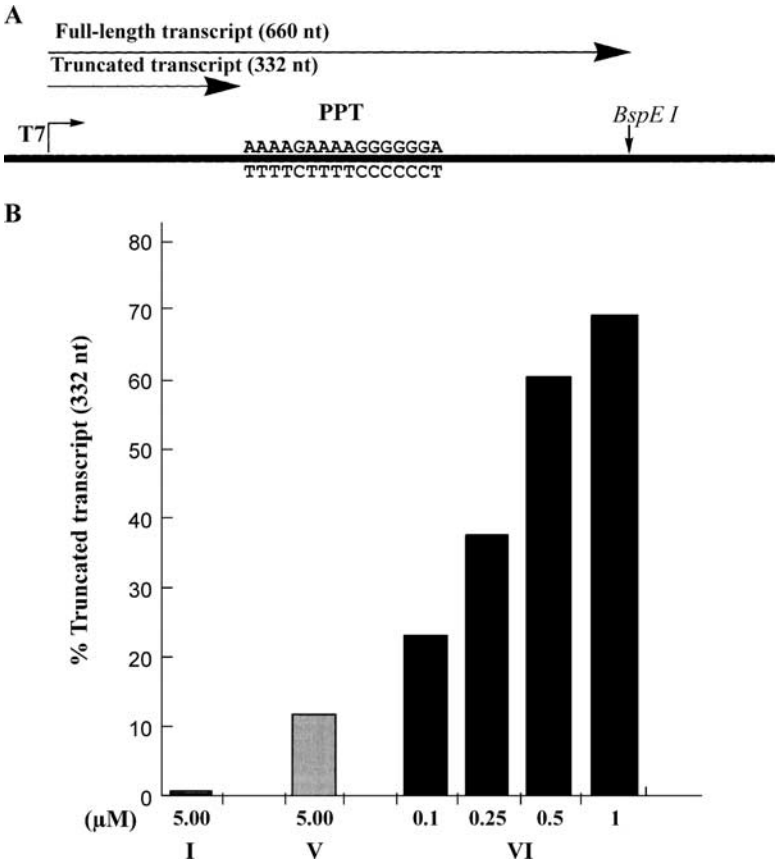

Figure 5. Inhibition of transcription elongation by the conjugates (**V**) and (**VI**). (**A**) Description of transcription system. PPT: the 16-bp HIV polypurine tract. (**B**) Dose-response of transcription inhibition by conjugates at 37°C. The percentage of truncated transcripts is reported as a function of the conjugate concentration.

An in vitro transcription assay was used to determine the ability of oligo(2′-O-methylribonucleotide) conjugates to inhibit transcription. A plasmid containing the polypurine tract (PPT) from HIV fragment under control of T7 promoter was transcribed in vitro after cleavage with *Bsp*EI restriction enzyme (Fig. 5).[11] In transcription assay the oligo(2′-O-methylribonucleotide) conjugate (**VI**) containing both MGB and intercalator residues induced dose-dependent inhibition of transcription at the predicted polypurine site of the dsDNA fragment due to triplex formation and physical block of RNA polymerase T7. The conjugate (**V**) containing only one MGB residue slightly inhibited the transcription at 5 μM and initial oligonucleotide (**I**) have no inhibition effect under the experimental conditions (Fig. 5).

The constructions proposed in this work, especially oligonucleotide-MGB-intercalator conjugates, could be considered as molecular instruments for artificial sequence-specific regulation of gene expression on the level of double-stranded genomic DNA. Inhibition of the gene expression by these conjugates in the cells is currently under investigation.

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