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Nuclease resistant methylphosphonate-DNA/LNA chimeric oligonucleotides

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ABSTRACT

Synthesis of chimeric 9-mer oligonucleotides containing methylphosphonate-linkages and locked nucleic acid (LNA) monomers, their binding affinity towards complementary DNA and RNA, and their 3′-exonucleolytic stability are described. The obtained methylphosphonate-DNA/LNA chimeric oligonucleotides display similarly high RNA affinity and RNA selectivity as a corresponding 9-mer DNA/LNA chimeric oligonucleotide, but much higher resistance towards 3′-exonucleolytic degradation.

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Antisense oligonucleotides (AONs) targeting RNA have been widely used to evaluate gene function in vitro and in vivo,[1–4] and several antisense therapeutics are in clinical trials.[5,6] In order to be effective and practically useful in the above technologies, AONs should feature specific characteristics including high-target RNA binding affinity, sequence specificity and nuclease resistance. Phosphorothioate DNA oligonucleotides, which have been the most widely used AONs, exhibit nuclease resistance, but have a number of limitations, such as sequence independent toxicity, immune stimulation and low RNA affinity.[7–9] Numerous modified oligonucleotides (ONs) have been developed in the past few decades to improve the properties of AONs.[10–15] Locked nucleic acid (LNA, Fig. 1), which is a conformationally restricted RNA mimic displaying unprecedented binding affinities towards complementary single stranded RNA or DNA,[16–19] is a promising modification for AON applications,[20–22] and is currently being utilized in various genomic technologies.[23–26] Introduction of consecutive LNA monomers from the 3′-end of an AON efficiently protects against 3′-exonucleolytic degradation,[16] whereas isolated LNA monomers in DNA/LNA chimeric AONs offer no significant protection.[21] To ensure sufficient biostability of DNA/LNA chimeric AONs, the use of phosphorothioate linkages throughout the AON has proven very efficient.[27,28]

Methylphosphonate-DNA (MP-DNA, Fig. 1) is another class of potential AONs which contain non-ionic internucleoside linkages.[29–31] MP-DNA displays stabilization towards nucleolytic degradation but unfortunately also significantly reduced binding affinity towards complementary RNA.[32–34] In a first attempt of combining the desirable properties of methylphosphate linkages and LNA monomers, we reported synthesis, satisfactory RNA binding properties and increased 3′-exonucleolytic stability of ONs containing a single methylphosphonate-LNA (MP-LNA, Fig. 1) monomer.[35] However, the fact that MP-LNA monomers are not commercially available has hampered further exploration of this class of potential AONs. Herein, we describe solid phase synthesis of chimeric 9-mer ONs containing MP-DNA and LNA monomers (MP-DNA/LNA) together with their DNA/RNA binding properties and stability against 3′-exonucleolytic degradation. Our aim is to develop AONs devoid of phosphorothioate linkages which combine stability under physiological conditions and high RNA binding affinity with easy availability.

MP-DNA/LNA ONs (**ON4** and **ON6**, Table 1) and MP-DNA/DNA ONs (**ON3** and **ON5**, Table 1) containing different number of MP-linkages (monomers $\mathbf{G_{Me}}$ or $\mathbf{A_{Me}}$) were synthesized in 1.0 μmol scale by automated solid phase DNA synthesis using commercially available MP-DNA and LNA-T phosphoramidites. The step-wise coupling efficiencies, based on the absorbance of the dimethoxytrityl cation released after each coupling step, of all monomers were >98% when applying standard conditions as recommended by suppliers. The chimeric ONs were, as O5′-DMT-ON derivatives, deprotected and simultaneously cleaved off the solid support using a mixture of ethylenediamine and ethanol (1/1, v/v) at 55 °C for 55 min.[36] Subsequent purification by RP-HPLC followed by detritylation provided the fully deprotected ONs. After purification, the purities (>80%) and composition of **ON3**–**ON6** were characterized by RP-HPLC and MALDI-TOF mass spectrometry, respectively.[37]

The ability of the MP-DNA/LNA ONs to form duplexes with complementary RNA and DNA in medium salt buffer (120 mM [Na^+])

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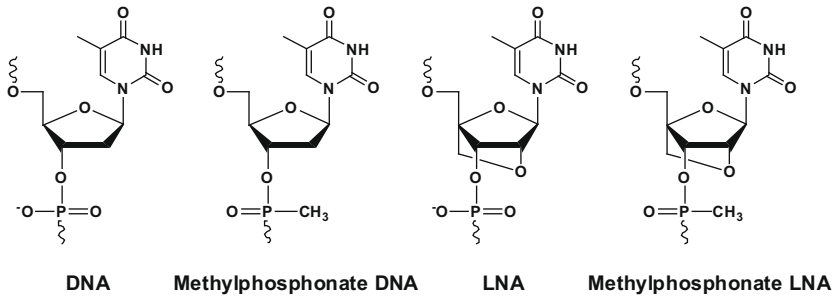

Figure 1. Structures of the thymine nucleotide monomers of DNA, methylphosphonate DNA, LNA and methylphosphonate LNA.

Table 1
T_m values (°C) of the oligonucleotides used in this study towards complementary single-stranded DNA and RNA[a]

Sequence	T_m with DNA (°C)		T_m with RNA (°C)	
	120 mM [Na⁺][b] (ΔT_m)	30 mM [Na⁺][c] (ΔT_m)	120 mM [Na⁺][b] (ΔT_m)	30 mM [Na⁺][c] (ΔT_m)
5′-d(GTGATATGC) (**ON1**)	29 (±0)	14 (±0)	27 (±0)	16 (±0)
5′-d(G**T**^{L}GA**T**^{L}A**T**^{L}GC) (**ON2**)	44 (+15)	28 (+14)	53 (+26)	36 (+20)
5′-d(GTG$_{Me}$ATA$_{Me}$TGC) (**ON3**)	27 (−2.0)	15 (+1.0)	22 (−5)	<10[d] (n.d.[e])
5′-d(G**T**^{L}G$_{Me}$A**T**^{L}A$_{Me}$**T**^{L}GC) (**ON4**)	39 (+10)	27 (+13)	47 (+20)	39 (+23)
5′-d(G$_{Me}$TG$_{Me}$A$_{Me}$TA$_{Me}$TG$_{Me}$C) (**ON5**)	25 (−4.0)	18 (+4.0)	<10[d] (n.d.[e])	<10[d] (n.d.[e])
5′-d(G$_{Me}$**T**^{L}G$_{Me}$A$_{Me}$**T**^{L}A$_{Me}$**T**^{L}G$_{Me}$C) (**ON6**)	34 (+5.0)	28 (+14)	40 (+13)	33 (+17)

[a] T_m values were obtained as the maximum of the first derivative of the melting curve (A_{260} vs temperature) in buffer using 1.0 μM concentrations of the two components. ΔT_m values calculated relative to the all-DNA reference **ON1** are shown in parentheses. **T**^{L} = thymin-1-yl LNA monomer, A_{Me} = adenine-9-yl DNA methylphosphonate monomer, G_{Me} = guanin-9-yl DNA methylphosphonate monomer.
[b] T_m values measured in medium salt buffer (100 mM NaCl, 10 mM NaH_2PO_4, 5 mM Na_2HPO_4, 0.1 mM EDTA, pH 7.0).
[c] T_m values measured in low salt buffer (10 mM NaCl, 10 mM NaH_2PO_4, 5 mM Na_2HPO_4, 0.1 mM EDTA, pH 7.0).
[d] No cooperative transition above 10 °C was observed.
[e] Not determined.

was evaluated by UV melting experiments (Table 1). Reference duplex melting temperatures (T_m values) were obtained for the corresponding all-DNA (**ON1**), DNA/LNA (**ON2**) and MP-DNA/DNA (**ON3** and **ON5**) ONs. As reported earlier[33,34] introduction of MP-DNA monomers into DNA (**ON3** and **ON5**) induced decreased T_m values. We furthermore note an additive affinity-decreasing effect of MP-DNA monomers, especially towards complementary RNA. As reported earlier, introduction of three LNA monomers into a DNA ON results in significantly increased T_m values (**ON2** relative to **ON1**). The data obtained for the MP-DNA/LNA chimeras **ON4** and **ON6** reflect high-affinity targeting of DNA/RNA complements also for these constructs, and the affinity-decreasing effect on the MP-DNA monomers is more than counterbalanced by the affinity-enhancing effect of LNA monomers. Furthermore encouraging in light of AON development is the fact that the MP-DNA/LNA chimeras display RNA targeting selectivity in line with the parent DNA/LNA chimeras.

Next, the effects of sodium ion concentration on the T_m values of the chimeric ONs were investigated (Table 1). As expected, decreasing salt concentration (from 120 to 30 mM NaCl) destabilized the duplexes, even for **ON3**–**ON6** containing non-ionic methylphosphonate linkages. Although the affinity-decreasing effect was also observed for the MP-DNA/LNA chimeric ONs, **ON4** containing two MP-linkages and three LNA monomers showed remarkably higher T_m value ($\Delta T_m > 30$ °C) towards RNA relative to **ON3** containing two MP-linkages without LNA modification. A similar trend was observed for **ON6** relative to **ON5**.

Stability towards nucleolytic degradation is an important factor when applying AONs in biological systems, and it has been reported that 3′-exonucleolytic activity is the primary cause of AON degradation. The susceptibility of **ON1**–**ON6** towards the 3′-exonuclease snake venom phosphodiesterase (SVPDE)[38] was evaluated (Fig. 2). The ONs were 5′-^{32}P-labelled using ^{32}P-ATP and T4 polynucleotide kinase and mixed with the corresponding unla-

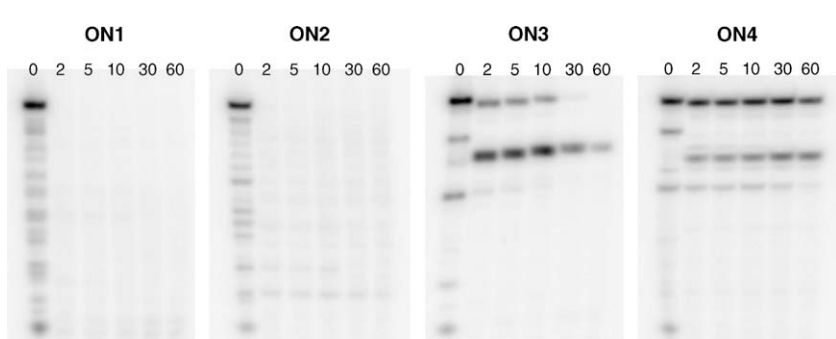

Figure 2. Snake venom phosphordiesterase degradation of [^{32}P]-labelled DNA (**ON1**), DNA/LNA (**ON2**), MP-DNA/DNA (**ON3**) and MP-DNA/LNA (**ON4**) oligomers (9-mer). The numbers above the lanes indicate the time of incubation (in minutes).

belled ON prior to digest reactions. These were performed at 25 °C in 8 μL of the following buffer: 0.1 M Tris–HCl pH 8.0, 0.1 M NaCl, 14 mM $MgCl_2$. In each reaction, 5 pmol of the ON was digested with 0.002 U of SVPDE (*Crotalus adamanteus*). The reactions were stopped by addition of 1 μL of a solution containing formamide and EDTA to a 1 μL sample of the reaction mixture, and the reaction products were analyzed by denaturing PAGE (20%). Although improvement in the stability towards 3′-exonuclease degradation by introduction of LNA into a DNA strand has been reported,[16,39,40] we here observed complete and rapid degradation of all-DNA **ON1** and LNA-modified **ON2** (Fig. 2). MP-DNA/DNA **ON3** showed significantly improved resistance but complete degradation was seen after 60 min of incubation. In contrast, MP-DNA/LNA **ON4** was remarkably stable with more than 50% full-length compound remaining after 60 min of incubation. The resistance against 3′-exonucleolytic degradation induced by a MP-DNA monomer is furthermore visualized by the emergence of an intermediate length band during degradation of **ON3** and **ON4**. We assign these bands as 6-mer ONs resulting from cleavage of three nucleotides from **ON3** and **ON4**. These results demonstrate that MP-DNA/DNA/LNA chimeric ONs having MP-DNA and LNA monomers dispersed with standard DNA monomers are significantly more stable against nucleolytic degradation than all-DNA or DNA/LNA chimeric ONs. The high resistance of MP-DNA/LNA chimeric ONs is resulting from combined effects of the MP-linkages and the LNA moieties.

In summary, we herein have introduced chimeric methylphosphonate-DNA/LNA oligomers as a new class of potential AONs. These constructs display high-affinity RNA targeting, and the combination of methylphosphonate-DNA with DNA and LNA monomers efficiently inhibits 3′-exonucleolytic degradation. Chimeric methylphosphonate-DNA/LNA oligonucleotides therefore constitute an alternative to phosphorothioate-DNA/LNA oligonucleotides for antisense applications, and the neutral methylphosphonate linkages can be foreseen to alter the biodistribution relative to currently applied antisense molecules. All nucleotide building blocks necessary to synthesize these novel constructs are commercially available and no significant synthetic hurdles prevent their evaluation as antisense agents in cell culture and in vivo. We thus believe that methylphosphonate-DNA/LNA oligonucleotides have potential for practical antisense applications aiming at mRNA and miRNA targeting.

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