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Chemically modified oligonucleotides with efficient RNase H response

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Abstract—Ten different chemically modified nucleosides were incorporated into short DNA strands (chimeric oligonucleotides **ON3**–**ON12** and **ON15**–**ON24**) and then tested for their capacity to mediate RNAse H cleavage of the complementary RNA strand. The modifications were placed at two central positions directly in the RNase H cleaving region. The RNA strand of duplexes with **ON3**, **ON5** and **ON12** were cleaved more efficiently than the RNA strand of the DNA:RNA control duplex. There seems to be no correlation between the thermal stability between the duplexes and RNase H cleavage.

In the antisense technology, RNA is targeted by Watson-Crick hybridization of a complementary antisense oligonucleotide (AON). The goal of inhibiting gene expression in a specific way may be accomplished by preventing mRNA maturation, blocking translation or more commonly by the induction of degradation.[1,2] To be effective the AON has to be able to enter the cell, be stable towards nucleases, be non-toxic and show high binding affinity and specificity towards the target mRNA. Considerable progress with respect to stability and binding has been made by use of chemically modified AONs. Introducing nucleotide analogues with constrained North type (*N*-type; C3′-*endo* type) furanose ring conformations has proven successful with respect to obtaining strong binding towards an RNA target, with LNA (locked nucleic acid) being a prominent example.[3] An LNA monomer contains an O2′–C4′ linkage (Fig. 1) that locks the furanose ring in an *N*-type conformation leading to unprecedented binding affinity towards complementary RNA for AONs composed of a mixture of, for example, LNA and DNA nucleotides (LNA/DNA mixmers).[4–9] Incorporation of LNA nucleotides into an AON induces the formation of almost canonical A-form helix structures of the duplexes formed with RNA complements,[6,7,10,11] and LNA can thus be characterized as a structural mimic of RNA. In contrary, the stereoisomeric. α-L-LNA monomer is locked in a conformation that results in AONs that structurally mimic DNA whereby duplexes between DNA/α-L-LNA mixmers and RNA adopt intermediate A/B duplex geometries.[12,13] Remarkably, both LNA and α-L-LNA nucleotides induce very high RNA binding affinities of AONs with increases in thermal denaturation temperatures (T_m values) of ~2–8 °C per modification.[8,9,14]

The efficiency of AONs containing modified nucleotides is often limited by their inability to induce degradation of target mRNA by the ubiquitous RNase H enzyme. Specifically, RNase H is incompatible with substrate duplexes with *N*-type nucleotides like LNA or O2′-alkylated-RNA nucleotides dispersed throughout the AON.[14,15] This limitation has been circumvented by the use of the so-called gapmers, which

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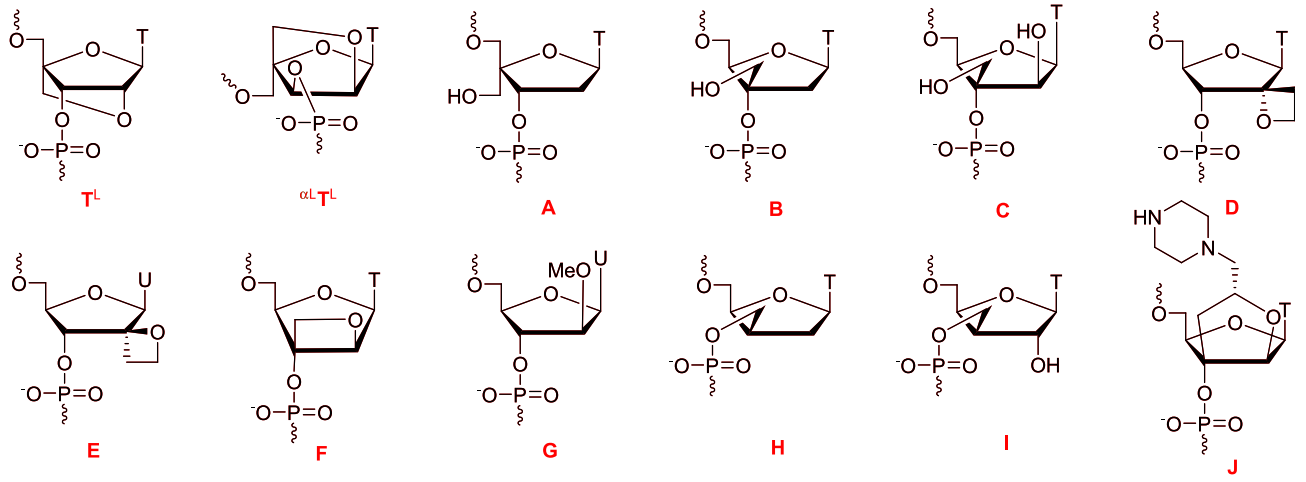

Figure 1. Chemical structures of the modified nucleotides included in the study. See text for references.

are chimeric AONs with a central continuous stretch of RNase H recruiting nucleotides (typically phosphorothioate DNA but alternatively, for example, phosphorothioate FANA nucleotides[16]) flanked by affinity-enhancing modified nucleotides (e.g. LNA, α-L-LNA or O2′-alkylated-RNA nucleotides).[17–20] Noteworthy, it has been found that the optimal gap size is motif-dependent, that a right balance between gap size and affinity is required,[19] and that the presence of one or two DNA-mimicking α-L-LNA monomers within the gap is compatible, at least in part, with RNase H activity.[14,20]

We report herein the effect of twelve chemically modified nucleotides on RNase H cleavage of DNA:RNA duplexes. This study supplements other reports on the effect of other nucleotide modifications on RNase H cleavage.[15] The modifications were incorporated at two central positions in an oligodeoxynucleotide (ON) where RNAse H cleaves the RNA strand of such a small DNA:RNA duplex (Table 1). The structures of the twelve modified nucleotides are shown in Figure 1, and listed below with a short description:

T^{L}: LNA (locked *N*-type furanose conformation; RNA mimic)[4]
^{αL}T^{L}: α-L-LNA (locked 'inverted' *N*-type furanose conformation; DNA mimic)[14]
A: 4′-*C*-Hydroxymethyl-DNA (flexible *S*-type furanose conformation; DNA mimic)[21]
B: 3′-*C*-Hydroxymethyl-DNA (flexible *S*-type furanose conformation; DNA mimic)[22]
C: 3′-*C*-Hydroxymethyl-ANA (flexible *S*-type furanose conformation; *arabino*-configuration; DNA mimic).[23]
D: 2′-spiro-RNA (flexible *N*-type furanose conformation; RNA mimic)[24]
E: 2′-spiro-ANA (flexible *S*-type furanose conformation; *arabino*-configuration; DNA mimic)[24]
F: C3′,O2′-linked-ANA (restricted *E*-type furanose conformation; *arabino*-configuration; DNA mimic)[25,26]
G: 2′-*O*-methyl-ANA (flexible *S*-type furanose conformation; *arabino*-configuration; DNA mimic)[27]
H: C3′-methylene-extended-2′-deoxy-XNA (*S*-type furanose conformation; *xylo*-configuration; DNA mimic)[28]
I: C3′-methylene-extended-XNA (*S*-type furanose conformation; *xylo*-configuration; DNA mimic)[23]
J: Piperazino-functionalized C3′,O2′-linked-ANA (restricted *E*-type furanose conformation; *arabino*-configuration; DNA mimic)[29]

The RNA binding affinities (T_m values) of the chimeric ONs with one modified nucleotide incorporated at distinct central positions in the strand were determined by thermal denaturation studies in a medium salt buffer solution (see Table 1). As expected from earlier reports, significant increases in thermal stability relative to the **ON0**:RNA reference duplex were observed for monomers **T^{L}**, **^{αL}T^{L}** and **F**. Monomers **A**, **B** and **J** induced no significant changes, whereas the remaining monomers exhibited decreased thermal affinities towards RNA complements. These results underlines the difficulties of obtaining strong RNA binding for DNA mimicking monomers, with α-L-LNA,[14] C3′,O2′-linked-ANAs[26] and FANA[30] as the major exceptions.

The compatibility of **ON0**–**ON24** with respect to RNase H cleavage of the corresponding duplexes formed with complementary RNA was investigated using an RNA sequence 5′-AGGUCCAUAGAGAC-3′ that was [^{32}P]-labelled at its 5′-end. The radioactive RNA was mixed with unlabelled RNA (1 pmol/final sample) and a fourfold excess of the ON strand to be studied in a solution containing 20 mM Tris–HCl, pH 7.5 and 100 mM KCl. The reactions were incubated at 65 °C for 2 min followed by slow cooling to 37 °C. An equal volume of a solution containing 20 mM Tris–HCl, pH 8.0, 100 mM KCl, 20 mM $MgCl_2$, 2 mM DTT with 0.2 U of *E. coli* RNase H (Amersham) per final sample was added and incubation was continued at 37 °C. In control samples, RNase H was not added. Aliquots were withdrawn at the time points 2, 10 and 30 min after RNase H addition. A basic hydrolysis of labelled RNA were performed by heating to 90 °C for 15 min in 100 mM Na_2CO_3, pH 9.0, 2 mM

Table 1. Differences in thermal denaturation temperature for the modified ONs towards the RNA complement relative to reference DNA:RNA duplex (ON0:RNA)

5′-GTC TC**X** ATG GAC CT			Monomer X	5′-GTC TCT A**X**G GAC CT		
ODN	$T_m(\Delta T_m)$	Amount cleaved		ODN	$T_m(\Delta T_m)$	Amount cleaved
ON0	48.0	++	**T**	**ON0**	48.0	++
ON1	(+6.5)[a]	++	$\mathbf{T^L}$	**ON13**	(+5.5)[a]	
ON2	(+6.0)[a]	+	$\mathbf{^{\alpha L}T^L}$	**ON14**	(+5.0)[a]	+
ON3	(0.0)	+++	**A**	**ON15**	(+0.5)	++
ON4	(−1.0)	++	**B**	**ON16**	(−0.5)	++
ON5	(−4.0)	+++	**C**	**ON17**	(−5.0)	++
ON6	(−9.0)	+	**D**	**ON18**	(−10.0)	+
ON7	(−11.5)	+	**E**	**ON19**	(−10.5)	+
ON8	(+3.0)	++	**F**	**ON20**	(+3.0)	+
ON9	(−3.5)[a]	+	**G**	**ON21**	(−5.5)[a]	++
ON10	(−2.5)	++	**H**	**ON22**	(−2.5)	+
ON11	(−4.0)	++	**I**	**ON23**	(−6.0)	+
ON12	(−1.0)[a]	+++	**J**	**ON24**	(0.0)[a]	+

The positions of incorporation of modified nucleotides (X in the sequences) are shown at the top. The thermal denaturation studies were performed in 10 mM sodium phosphate, 100 mM sodium chloride, 0.1 mM EDTA, pH 7.0 with 1.5 μM concentrations of the two complementary strands. The absorbance was monitored at 260 nm while raising the temperature at a rate of 1 °C min^{-1}. The melting temperatures (T_m values) were determined as the maxima of the first derivatives of the melting curves obtained. An estimate of the total RNAse H cleavage is also shown with a '+' system; +++ for strong cleavage and + for weak cleavage (taken from Fig. 2).

[a] Reference duplex melted at 52.5 °C.

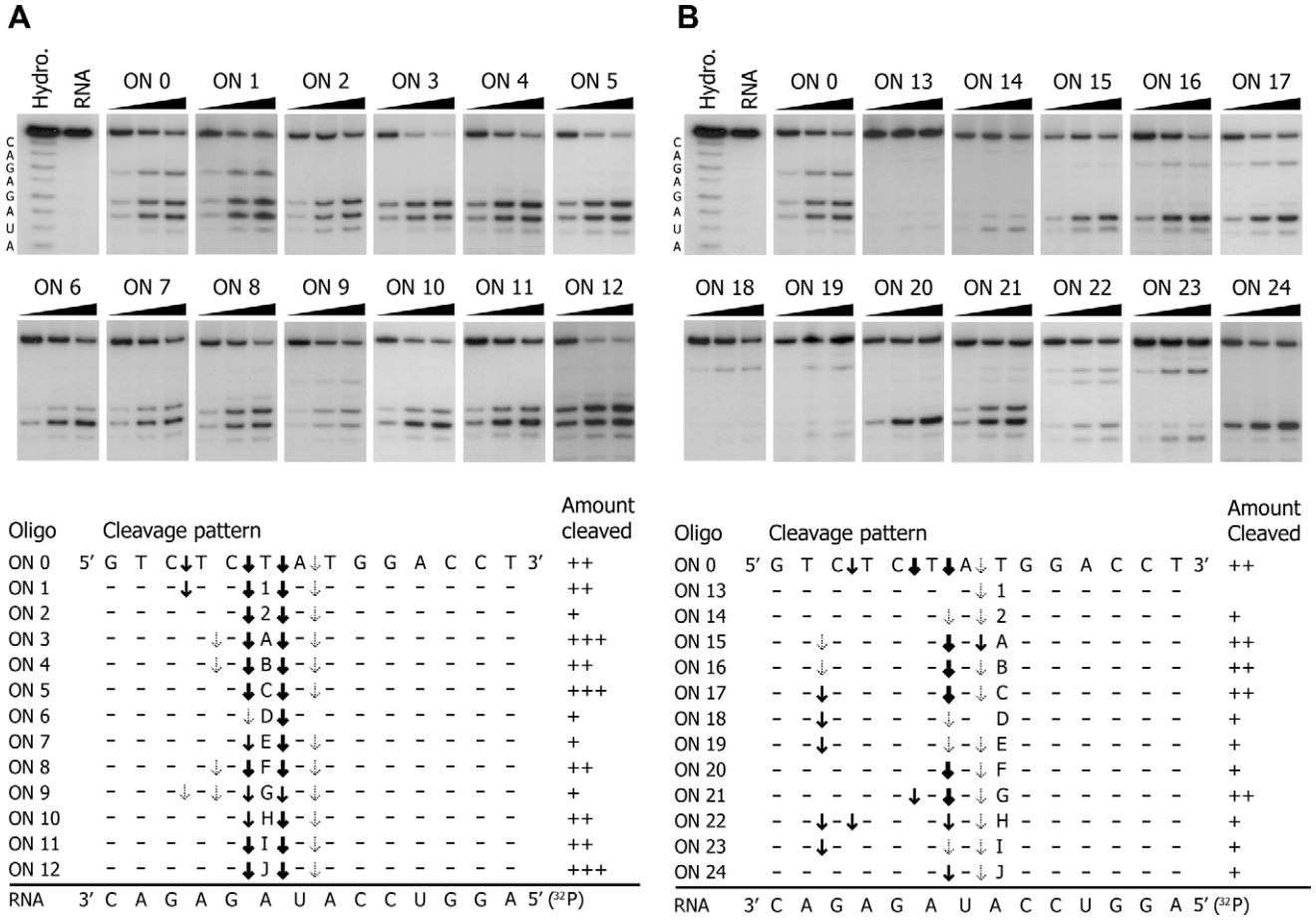

Figure 2. RNAse H cleavage of target RNA analysed by gel electrophoresis. Representative gels for each duplex are shown with a triangle at the top indicating the increase in incubation time (2, 10 and 30 min). A ladder resulting from basic hydrolysis of the RNA sequence is shown in the top left box. All experiments were repeated at least twice. (A) Results for ONs modified at position no. 6; (B) Results for ONs modified at position No. 8. The exact cleavage site and the extent of cleavage are shown with arrows below the gel figures—A bold arrow indicates strong cleavage while a thin arrow indicates weak cleavage. (A) and (B) '1' in the sequence designates a T^L monomer and '2' an $^{\alpha L}T^L$ monomer.

EDTA followed by cooling on ice and the addition of formamide dye. All reactions were analysed by PAGE (20% polyacrylamide containing 8.3 M urea) followed by autoradiography.

The RNase H cleavage patterns are depicted in Figure 2(A and B) and discussed below in relation to the cleavage pattern observed for the unmodified reference duplex **ONO**:RNA. The LNA (**ON1** and **ON13**) and α-L-LNA (**ON2** and **ON14**) containing duplexes are among the worst substrates for RNase H. The enzyme requires a base paired region of approximately 6–7 nucleotides for binding, and likely the incorporation of LNA or α-L-LNA nucleotides in or around this region is incompatible with RNase H binding. This correlates with the preferred gap size of at least 7–8 DNA nucleotides found for LNA–DNA–LNA gapmers.[19,20] In fact all modified duplexes were cleaved to some extent except the LNA containing **ON13**:RNA duplex. The cleavage pattern varies for the different duplexes indicating that there are some alterations in RNAse H selectivity and compatibility. Remarkably, duplexes containing **ON3**, **ON5** or **ON12** show enhanced cleavage even though the cleavages occur directly across of the modified position. As **ON3**, **ON5** and **ON12** do not form the most stable duplexes, the extent of RNase H cleavage is not directly correlated with increasing stability of a duplex. Some of the duplexes show reduced RNase H activity but many support cleavage as well as the unmodified control, although the cleavage pattern varies (Fig. 2). It is noteworthy that especially for the ONs modified at position 8 (Fig. 2B), an additional cleavage site is introduced near the 3′ end of the target RNA. It is furthermore noteworthy that modifications **A**, **C** and **J**, which all are DNA mimics, enable enhanced cleavage despite being incorporated within the initial RNase H binding region (modifications at position No. 6). RNase H has been reported to bind along the minor groove of DNA:RNA duplexes adopting intermediate A/B geometries[31] which provides an explanation for the observed RNase H cleavage induced by the DNA mimics. As RNase H binds in the minor groove it is not surprising that the C3′ substituents of nucleotide monomers **C** and **J** are well tolerated as they are oriented towards the major groove. It is, however, notable that also the C4′ substituent of nucleotide monomer **A** that points towards the minor groove is well tolerated.

In summary, this study has unambiguously demonstrated that it is possible to apply modifications within AONs across RNA cleavage sites, which enable RNase H cleavage of an RNA complement with equal or even improved efficiency compared to unmodified DNA AONs. These results complement ongoing efforts to optimize the chemical composition of the wing segments of gapmer AONs.[32]

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