Comparison of the RNase H Cleavage Kinetics and Blood Serum Stability of the *North*-Conformationally Constrained and 2′-Alkoxy Modified Oligonucleotides[†]

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ABSTRACT: The RNase H cleavage potential of the RNA strand basepaired with the complementary antisense oligonucleotides (AONs) containing *North−East* conformationally constrained 1′,2′-methylene-bridged (azetidine-T and oxetane-T) nucleosides, *North*-constrained 2′,4′-ethylene-bridged (aza-ENA-T) nucleoside, and 2′-alkoxy modified nucleosides (2′-*O*-Me-T and 2′-*O*-MOE-T modifications) have been evaluated and compared under identical conditions. When compared to the native AON, the aza-ENA-T modified AON/RNA hybrid duplexes showed an increase of melting temperature ($\Delta T_m = 2.5-4$ °C per modification), depending on the positions of the modified residues. The azetidine-T modified AONs showed a drop of $4-5.5$ °C per modification with respect to the native AON/RNA hybrid, whereas the isosequential oxetane-T modified counterpart, showed a drop of $\sim 5-6$ °C per modification. The 2′-*O*-Me-T and 2′-*O*-MOE-T modifications, on the other hand, showed an increased of T_m by 0.5 °C per modification in their AON/RNA hybrids. All of the partially modified AON/RNA hybrid duplexes were found to be good substrates for the RNase H mediated cleavage. The K_m and V_{max} values obtained from the RNA concentration-dependent kinetics of RNase H promoted cleavage reaction for all AON/RNA duplexes with identical modification site were compared with those of the reference native AON/RNA hybrid duplex. The catalytic activities (K_{cat}) of RNase H were found to be greater ($\sim 1.4-2.6$-fold) for all modified AON/RNA hybrids compared to those for the native AON/RNA duplex. However, the RNase H binding affinity ($1/K_m$) showed a decrease ($\sim 1.7-8.3$-fold) for all modified AON/RNA hybrids. This resulted in less effective ($\sim 1.1-3.2$-fold) enzyme activity (K_{cat}/K_m) for all modified AON/RNA duplexes with respect to the native counterpart. A stretch of five to seven nucleotides in the RNA strand (from the site of modifications in the complementary modified AON strand) was found to be resistant to RNase H digestion (giving a footprint) in the modified AON/RNA duplex. Thus, (i) the AON modification with azetidine-T created a resistant region of five to six nucleotides, (ii) modification with 2′-*O*-Me-T created a resistant stretch of six nucleotides, (iii) modification with aza-ENA-T created a resistant region of five to seven nucleotide residues, whereas (iv) modification with 2′-*O*-MOE-T created a resistant stretch of seven nucleotide residues. This shows the variable effect of the microstructure perturbation in the modified AON/RNA heteroduplex depending upon the chemical nature as well as the site of modifications in the AON strand. On the other hand, the enhanced blood serum as well as the 3′-exonuclease stability (using snake venom phosphodiesterase, SVPDE) showed the effect of the tight conformational constraint in the AON with aza-ENA-T modifications in that the 3′-exonuclease preferentially hydrolyzed the 3′-phosphodiester bond one nucleotide away (n + 1) from the modification site (n) compared to all other modified AONs, which were 3′-exonuclease cleaved at the 3′-phosphodiester of the modification site (n). The aza-ENA-T modification in the AONs made the 5′-residual oligonucleotides (including the n + 1 nucleotide) highly resistant in the blood serum (remaining after 48 h) compared to the native AON (fully degraded in 2 h). On the other hand, the 5′-residual oligonucleotides (including the n nucleotide) in azetidine-T, 2′-*O*-Me-T, and 2′-*O*-MOE-T modified AONs were more stable compared to that of the native counterpart but more easily degradable than that of aza-ENA-T containing AONs.

Antisense oligonucleotides (AONs)[1] have been widely used as a tool for down-regulation of gene expression through RNA targeting either by (i) blocking the message transmission by binding to the target mRNA and/or by (ii) catalytic cleavage of the target mRNA in the AON/RNA by the activation of RNase H, a ubiquitous enzyme cleaving the

[1] Abbreviations: AON, antisense oligonucleotide; aza-ENA, 2′-*N*,4′-*C*-ethylene-bridged nucleic acids; 2′-*O*-Me, 2′-*O*-methyl; 2′-*O*-MOE, 2′-*O*-[2-(methoxy)ethyl]; aze, azetidine; oxe, oxetane; 2′-F, 2′-fluoro; LNA, locked nucleic acid; PAGE, polyacrylamide denaturing gel electrophoresis; Tris-HCl, tris(hydroxymethyl)aminoethane hydrochloride; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; ENA, 2′-*O*,4′-*C*-ethylene-bridged nucleic acids.

RNA part of AON/RNA hybrid duplexes (*1*). The second strategy has essential advantages over the steric blocking because it requires only a relatively small amount of AON for catalytic turnovers. The permanent destruction of the target RNA message with catalytic turnover may provide enough lifetime in cells (*2*) for the AON to act as a potential drug provided the AON has sufficient stability in the blood serum and is capable of penetrating in the cell (Delivery). Considerable attention has been directed toward the chemical design of AONs that would enhance the target affinity, specificity, capability to recruit RNase H, and nuclease stability. The first generation of AONs, phosphorothioate DNA analogues, showed an increase of the nucleases resistance, but their therapeutic potential was limited due to low binding affinity to the target RNA, less sequence specificity, and strong affinity to the heparin-binding proteins (*3*, *4*). Reduction of toxicity with enhanced binding affinity was achieved by the second generation of AONs based on a mixed backbone oligonucleotides (*5*), involving 2′-*O*-alkyl modifications, 2′-*O*-alkyl modifications with glycol ether linkages, and 2′-F and 2′-*O*-aminoalkyl modifications (*6*–*8*). The disadvantage of these chimeric oligonucleotides was that they have poor nuclease stability (*9*) as well as low RNase H recruitment and cleavage capability (*7*, *8*). AONs containing boranophosphates (*10*) and methylphosphonates (*11*) enhanced RNase H activity, whereas binding affinity to the target RNA was not significantly improved.

The AONs with one or more conformationally locked nucleoside residues have also been an area of considerable interest (*12*–*19*). Introduction of conformationally constrained nucleotides can make the AON strand locked to mimic the DNA- or RNA-type conformation (*1*, *20*). It is known that for eliciting RNase H activity (*2*, *21*) the AON in the AON/RNA hybrid duplex should retain the B-type DNA conformation with the *South*-type sugar; at the same time the RNA moiety should retain its A-type helix character with the *North*-type sugar (*22*). Incorporation of a conformationally preorganized *North*-type nucleotide into an oligodeoxynucleotide modulates the thermal stability of the AON/RNA duplex depending upon the type of conformational constraint. For example, each locked nucleic acid (LNA) (*23*, *24*) residue increases the T_m by 4−8 °C per modification, each oxetane-T (*25*, *26*) or azetidine-T (*27*) moiety reduce the T_m by 4−6 °C per modification but no change in the T_m for oxetane-A and oxetane-G residues (*28*). The ability of these *North*-type conformationally constrained AONs is that the local conformational change is transmitted to the neighboring nucleotides in the AON (*29*) to transform a small stretch of the AON strand to the RNA-type. This essentially produces an RNA/RNA-type duplex, which is not cleaved by RNase H, thereby showing the footprints in the RNase H cleavage reaction of the heteroduplex (*1*, *25*, *26*, *30*). Thus, LNA-modified AONs showed high affinity to the target RNA, and the gapmer of LNA/RNA heteroduplexes required gaps of 7−8 native deoxynucleotides between LNA moieties to elicit RNase H activity (*31*). In the case of conformationally constrained oxetane-modified AONs (*25*, *26*, *28*–*30*), a gap of only five nucleotides is needed to regain the RNase H eliciting capability compared to seven to eight nucleotides for the LNA.

To improve the design principle of the AON strand, clearly we need to understand how the introduction of various 2′-
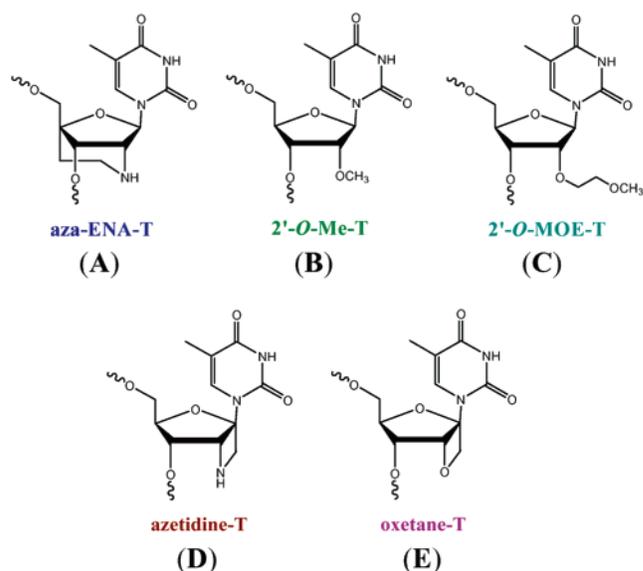

FIGURE 1: Structure of the chemically modified nucleotide monomer moieties: aza-ENA-T, 2′-*N*,4′-*C*-ethylene-bridged nucleic acid thymidine; 2′-*O*-Me-T, 2′-*O*-methylthymidine; 2′-*O*-MOE-T, 2′-*O*-[2-(methoxy)ethyl]thymidine.

substituents modulate the preorganization of the AON strand and the AON/RNA duplex for the recruitment of RNase H, as well as give the AON strand adequate stability toward cellular nucleases. We report here the RNase H cleavage properties and kinetics of AON/RNA duplexes with 2′-*O*-Me-T, 2′-*O*-MOE-T modifications (**B** and **C** in Figure 1) and compare their RNase H cleavage properties with those of the conformationally constrained modifications in the sugar moiety involving 1′,2′-methylene bridged [azetidine-T (**D**) and oxetane-T (**E**) in Figure 1] (*25*, *27*) with a *North-East*-type sugar conformation and 2′,4′-ethylene bridged nucleoside, aza-ENA-T (**A** in Figure 1) (*32*), with a *North*-constrained conformation. We show that each of these modifications shows uniquely different RNase H recruitment and cleavage properties, which potentially can open up new avenues for different applications. We also show here how the blood serum and the 3′-exonuclease (using snake venom phosphodiesterase, SVPDE) stability of the modified AON strand can be steered by effective change of the chemical nature of the substituent as well as its site in the AON strand.

MATERIALS AND METHODS

Materials. *Escherichia coli* RNase H (5 units/μL, specific activity 420 000 units mg^{-1}, molecular weight 21 000 g mol^{-1}), T4 polynucleotide kinase (30 units/μL), and [γ-^{32}P]-ATP were purchased from Amersham Pharmacia Biotech (Sweden). Phosphodiesterase I (*Crotalus Adamanteus* venom) and human serum (AB male) were from Sigma. Target RNA was from IBA BioTAGnology (Germany).

Oligonucleotide Synthesis. Azetidine-T (*27*), aza-ENA-T (*32*) (Scheme S1 and Experimental Section in Supporting Information), 2′-*O*-Me-T (*33*), and 2′-*O*-MOE-T (*34*, *35*) modified nucleosides (Figure 1) were synthesized following the previously reported procedures. Synthesis of modified oligonucleotides **1**−**11** and the complementary DNA (Table 1) was carried out using an Applied Biosystems 392 automated DNA/RNA synthesizer, as previously described

Table 1: Oligonucleotides of Various AONs and the Thermal Denaturation Studies of Their Duplexes with Complementary RNA or DNA Targets[a]

AONs	AON Oligonucleotides	$T_m/°C$ With RNA	T_m	$T_m/°C$ With DNA	T_m*	MALDI-MS of AON 1 - 11: Found/calc [M+H]+
1	5'-d(CTTCATTTTTTCTTC)-3'	44	–	45	–	4448.6/4448.7
2	5'-d(CTTCATTTTTTC**T**-aza-ENATC)-3'	48	+4	44.5	-0.5	4489.7/4491.1[b]
3	5'-d(CTTCATTTT**T**-aza-ENATCTTC)-3'	46.5	+2.5	42.5	-2.5	4489.7/4490.7
4	5'-d(CTTCATT**T**-aza-ENATTTCTTC)-3'	47.5	+3.5	42	-3	4489.7/4490.7
5	5'-d(CTTCA**T**-aza-ENATTTTTCTTC)-3'	48	+4	42	-3	4489.7/4490.8
6	5'-d(CTTCA**T**-O-MeTTTTTCTTC)-3'	44.5	+0.5	41	-4	4479.4/4478.8
7	5'-d(CTTCA**T**-O-MOETTTTTCTTC)-3'	44.5	+0.5	40.5	-4.5	4523.2/4522.8
8	5'-d(CTTCATTTTTTC**T**-azeTC)-3'	38.5	-5.5	41	-4	4475.3/4475.7
9	5'-d(CTTCATTTT**T**-azeTCTTC)-3'	40	-4	39.5	-5.5	4475.6/4475.7
10	5'-d(CTTCATT**T**-azeTTTCTTC)-3'	40	-4	39	-6	4475.4/4475.7
11	5'-d(CTTCA**T**-azeTTTTTCTTC)-3'	40	-4	41	-4	4475.5/4475.7
12	5'-d(CTTCA**T**-oxeTTTTTCTTC)-3'	39	-5	40.5	-4.5	4478.7/4478.0[c]
Target RNA	5'-r(GAAGAAAAAAUGAAG)-3'					
Target DNA	5'-d(GAAGAAAAAATGAAG)-3'					

[a] T_m values measured as the maximum of the first derivative of the melting curve (A_{260} vs temperature) recorded in medium salt buffer (60 mM Tris-HCl at pH 7.5, 60 mM KCl, 0.8 mM MgCl$_2$, and 2 mM DTT) with temperature range 20−70 °C using 1 μM concentrations of the two complementary strands; $\Delta T_m = T_m$ relative to RNA complement; $\Delta T_m* = T_m$ relative to DNA complement. [b] [M + 2H]+. [c] [M − H]−.

(27). AONs were purified by 20% polyacrylamide 7 M urea denaturing gel electrophoresis (PAGE), extracted with 0.3 M NaOAc, and desalted with C18-reverse phase cartridges. Purity of the products was greater than 95% as determined by PAGE.

UV Melting Experiments. Determination of the T_m values of the AON/RNA or AON/DNA hybrid duplexes was carried out in a buffer, containing 60 mM Tris-HCl (pH 7.5), 60 mM KCl, 0.8 mM MgCl$_2$, and 2 mM dithiothreitol (DTT). Absorbance was monitored at 260 nm in the temperature range from 20 to 70 °C using a Lambda 40 UV spectrophotometer equipped with a Peltier temperature programmer with a heating rate of 1 °C/min. Samples (mixture of 1 μM AON and 1 μM RNA or DNA) were denatured at 80 °C for 5 min followed by slow cooling to 17 °C prior to the measurements. T_m values were obtained from the maxima of the first derivatives of the melting curves. All T_m values given are the averages of three independent sets of experiments and are within a ±0.2 °C error range.

[32]*P Labeling of Oligonucleotides.* The oligoribonucleotide and oligodeoxyribonucleotides were 5′-end labeled with ^{32}P using [γ-^{32}P]ATP and T4 polynucleotide kinase by a standard procedure. Labeled RNA and AONs were purified by 20% 7 M urea PAGE, and specific activities were measured using a Beckman LS 3801 counter.

RNase H Digestion Assay. Target 0.1 μM RNA (specific activity 50 000 cpm) and a 25-fold excess of AON were incubated in a buffer containing 20 mM Tris-HCl (pH 8.0), 20 mM KCl, 10 mM MgCl$_2$, 0.1 mM EDTA, and 0.1 mM DTT at 21 °C in the presence of 0.04 U *E. coli* RNase H. Prior to the addition of the enzyme, reaction components were preannealed in the reaction buffer by heating at 80 °C

for 5 min followed by 1.5 h equilibration at 21 °C. Total reaction volume was 30 μL. Aliquots of 3 μL were removed after 7, 15, 35, and 60 min, and the reactions were terminated by mixing with stop solution (7 μL) containing 0.05 M EDTA, 0.05% (w/v) bromophenol blue, and 0.05% (w/v) xylene cyanole in 80% formamide. The samples were subjected to 20% 7 M urea PAGE and visualized by autoradiography.

To obtain the rate constants for RNaseH cleavage, three independent sets of experiments were performed with the target RNA (0.1 μM, specific activity 50 000 cpm) and a 25-fold excess of AON by incubating them in a buffer containing 20 mM Tris-HCl (pH 8.0), 20 mM KCl, 10 mM MgCl$_2$, 0.1 mM EDTA, and 0.1 mM DTT at 21 °C in the presence of 0.03 U *E. coli* RNase H. Aliquots of 3 μL were removed after 5, 10, 15, 20, 25, and 40 min, and the reactions were terminated by mixing with stop solution as described before. The samples were subjected to 20% 7 M urea PAGE and visualized by autoradiography. Quantitation of cleavage products was performed in each set of experiments using a Molecular Dynamics PhosphorImager. All values are averages and standard deviations of three independent experiments and are normalized.

Kinetics of RNase H Hydrolysis

(A) Calibration of RNase H Concentration. The solution of 15mer AON **1**/RNA duplex, [AON] = 2.5 μM, [RNA] = 0.1 μM in a buffer containing 20 mM Tris-HCl (pH 8.0), 20 mM KCl, 10 mM MgCl$_2$, 0.1 mM EDTA, and 0.1 mM DTT at 21 °C in 30 μL of the total reaction volume in the presence of 0.06 U of RNase H has been used as standard substrate to calibrate the amount of RNase H used in kinetics experiments. The percentage of RNA cleavage was moni-
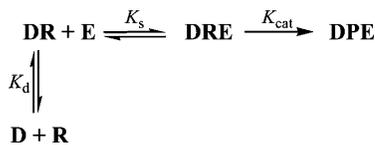
$$\text{DR} + \text{E} \underset{}{\overset{K_s}{\rightleftharpoons}} \text{DRE} \xrightarrow{K_{cat}} \text{DPE}$$

$$K_d \big\updownarrow$$

$$\text{D} + \text{R}$$

FIGURE 2: Kinetic pathway for RNase H hydrolysis of an AON substrate. **D** is the AON; **R** is the target RNA; K_d is the equilibrium constant of dissociation of the heteroduplex **DR**; **E** is RNase H enzyme; K_s is the association constant of the substrate−enzyme complex **DRE**; K_{cat} is the catalytic constant; **DPE** is the AON−product−enzyme complex.

tored by gel electrophoresis as a function of time (2−5 min) to give the initial velocity. Thus, the initial velocity of the RNase H cleavage reaction (0.01158 μM/min) for the above standard substrate corresponds to 0.06 U activity of enzyme in 30 μL of the total reaction mixture. These are based on seven independent experiments. According to the Michaelis−Menten equation, eq 1 (*36*), the initial velocity of the reaction linearly depends on the enzyme concentration. Therefore, using the initial velocity (0.01158 μM/min) for the standard substrate corresponding to 0.06 units/30 μL concentration of the RNase H, the correlation coefficient was found by dividing the observed experimental initial velocity by the standard initial velocity of 0.01158 μM/min. Then using this correlation coefficient the initial velocities of the reactions in each experiment were corrected to the real initial velocities corresponding to the identical enzyme concentration. This correlation coefficient was used to calibrate the initial velocity of the RNase H promoted cleavage reaction for each substrate presented in this work.

(*B*) *RNA Concentration-Dependent Experiments*. ^{32}P-Labeled RNA (0.01, 0.04, 0.08, 0.2, 0.4, or 0.8 μM, specific activity 50000 cpm) and AONs (2.5 μM) were incubated with 0.06 U of RNase H in buffer, containing 20 mM Tris-HCl (pH 8.0), 20 mM KCl, 10 mM MgCl$_2$, and 0.1 mM DTT at 21 °C. Total reaction volume was 30 μL. Prior to the addition of the enzyme, reaction components were preannealed in the reaction buffer by heating at 80 °C for 5 min followed by 1.5 h of equilibration at 21 °C. After 2−5 min, aliquots (3 μL) were mixed with stop solution (7 μL), containing 0.05 M EDTA, 0.05% (w/v) bromophenol blue and 0.05% (w/v) xylene cyanol in 80% formamide, and subjected to 20% 7 M urea PAGE. The concentration of the cleavage products was determined and plotted as a function of time (Supporting Information, Figures S4−S8) to give the initial velocities of the reactions. Values of the kinetic parameters (K_m and V_{max}) in this method were determined directly from v_0 vs [S_0] plots using SigmaPlot Program (version 2000), where the correlation equation was $y = ax/(b + x)$, where $a = V_{max}$ and $b = K_m$. All values for the kinetic of RNA cleavage reactions are averages and standard deviations of three independent experiments and are normalized.

Method for Kinetic Analysis. The kinetic analysis of RNase H promoted cleavage of the AON/RNA duplex involves three distinct steps (Figure 2): (i) the duplex formation, (ii) the duplex (i.e., substrate)−enzyme complex formation, and (iii) cleavage of the RNA substrate in the AON/RNA duplex, and formation of the product−enzyme complex.

According to the Michaelis−Menten equation, eq 1, the initial velocity (v_0) of the cleavage reaction depends on the

concentration of the enzyme (E_0) and the substrate (S_0 = [RNA]):

$$v_0 = \frac{E_0 S_0 K_{cat}}{K_m + S_0} \tag{1}$$

where

$$K_{cat} E_0 = V_{max} \tag{2}$$

Equation 1 can be used for monitoring RNA cleavage kinetics by RNase H only when RNA is saturated by AON. In this case, we can consider that the substrate concentration is equal to the concentration of RNA. Also we assume that the initial velocity (v_0) is the rate of product formation only for the first few percentages when the products have not significantly accumulated and also the substrate concentration has not significantly changed. In this case, changes in RNA concentrations are linear with time.

To avoid the influence of thermal stabilities (T_m's) of the AON/RNA heteroduplexes [to minimize influence of K_d (Figure 2)], all the kinetic experiments were performed at a temperature (21 °C) much lower than the T_m values of the duplexes and in saturated conditions.

From eq 1, initial velocity (v_0) in unsaturated conditions ($S_0 \le K_m$) increases with an increase of substrate concentration (S_0) and has a saturation plateau when $S_0 \rightarrow \infty$. Since we have used a wide range of RNA concentrations or, alternatively, substrate concentrations (S_0) the Michaelis−Menten equation (eq 1) will have different forms under low and high substrate concentrations.

In the case of low substrate concentration ($S_0 \ll K_m$), eq 1 can be rewritten as eq 3:

$$v_0 = \frac{V_{max}}{K_m} S_0 \tag{3}$$

Under these conditions, the initial velocity (v_0) is linearly dependent upon the substrate concentration (S_0) with the linear coefficient V_{max}/K_m.

Under high substrate concentration ($S_0 \gg K_m$), eq 1 takes the form of eq 4:

$$v_0 = V_{max} = K_{cat} E_0 \tag{4}$$

This means that the initial velocity (v_0) is dependent only on V_{max}, or in other words, on K_{cat} and E_0.

3′-Exonuclease Degradation Studies. The stability of the AONs toward 3′-exonucleases was tested using snake venom phosphodiesterase (SVPDE) from *Crotalus adamanteus*. All reactions were performed at 3 μM DNA concentration (5′-end ^{32}P labeled with specific activity 50 000 cpm) in 56 mM Tris-HCl (pH 7.9) and 4.4 mM MgCl$_2$ at 21 °C. Exonuclease concentration of 2.5 ng/μL was used for digestion of oligonucleotides. Total reaction volume was 14 μL. Aliquots of 3 μL were removed at 1/2, 1, 2, and 24 h and quenched with stop solution (0.05 M EDTA in 80% formamide). These samples were subjected to 20% 7 M urea PAGE and visualized by autoradiography. Quantitation of cleavage products was performed using a Molecular Dynamics PhosphorImager.

Stability Studies in Human Serum. 5′-End ^{32}P labeled oligonucleotides with specific activity 90 000 cpm at 2 μM

concentration were incubated in 26 μL of human serum (AB male) at 21 °C. Total reaction volume was 36 μL. Aliquots (3 μL) were removed after 1/4, 1/2, 1, 2, 9 h (in another set of experiments, after 4, 10, 24, 34, 48 h) and quenched with 7 μL of stop solution (0.05 M EDTA in 80% formamide). They were resolved in 20% 7 M urea PAGE and visualized by autoradiography. The modified AON stability studies in the human serum were performed at least twice in each case.

RESULTS

(A) Binding Affinity of the Chimeric Antisense Oligonucleotides to the Target RNA and DNA. Incorporation of the single aza-ENA-T modification (**A** in Figure 1) to AON (entries **2**−**5** in Table 1) significantly enhances its affinity to complementary RNA (ΔT_m +2.5 to +4 °C) depending upon the site of the modification in the AON strand. The different ΔT_m values can be due to the site-dependency of the variable conformational preorganization dictated by the *North*-fused sugar moiety to the single-stranded AON. Single azetidine-T incorporation (**D** in Figure 1) enhances the T_m of the AON/RNA duplex (entries **8**−**11** in Table 1) by ∼1 °C compared to that of the isosequential oxetane-T modified hybrid duplex (*25, 30*); however this leads to a ∼4−5 °C drop in T_m with respect to the unmodified AON/RNA hybrid duplex. Incorporation of 2′-*O*-Me-T or 2′-*O*-MOE-T modifications (**B** and **C** in Figure 1) to AON (entries **6** and **7** in Table 1) did not change the target binding affinity significantly compared to the unmodified hybrid duplex. However, with complementary DNA, all modified AONs showed a significant drop in duplex melting (Table 1).

(B) RNase H Cleavage. All of the aza-ENA-T modified AONs **2**−**5**/RNA duplexes, azetidine-T modified AONs **8**−**11**/RNA, 2′-*O*-Me-T modified AON **6**/RNA, and 2′-*O*-MOE-T modified AON **7**/RNA hybrids (Table 1) were found to be good substrates for the *E. coli* RNase H with varying cleavage potential and patterns. All azetidine-T modified AONs **8**−**11**/RNA duplexes showed a similar cleavage pattern as those of the isosequential oxetane-T modified duplexes (Figure 3, Supporting Information Figure S1) (*25, 29, 30*). The RNase H mediated cleavage pattern of azetidine-T modified AON **8**/RNA was found to be quite similar to the cleavage pattern of the native AON **1**/RNA duplex except that there was no cleavage at the A5 and A6 positions. In the modified AONs **9**−**10**/RNA hybrids with azetidine-T, five nucleotides toward the 3′-end from the site opposite to the modification were found to be insensitive toward RNase H promoted cleavage (Figure 3). On the other hand for the AON **11**/RNA duplex, six nucleotides toward the 3′-end from the site opposite to the modification showed resistance toward the RNase H mediated cleavage. These observations are presumably owing to the local steric and structural alterations brought by the modification, thereby preventing the flexibility and accessibility required for the RNase H cleavage (*1*). However, in the case of aza-ENA-T modified AONs **2**−**5**/RNA hybrid duplexes, the RNA cleavage patterns were found to be different from those of the isosequential azetidine-T modified AONs **8**−**11** and oxetane-T (*25, 30*) modified AONs. Comparison of the cleavage pattern of isosequential AON/RNA hybrids shows the following molecular discrimination by the RNase H: (i) There is no cleavage at the A7 position of the complementary RNA for aza-ENA-T containing AON **2**/RNA hybrid in

contrast to the azetidine-T containing AON **8**/RNA hybrid or isosequential oxetane-T modified AON/RNA hybrid duplex (Figure 3, Supporting Information Figure S1). (ii) For aza-ENA-T modified AON **4**, the main cleavage site was found at A13 position of the complementary RNA unlike the isosequential azetidine-T modified AON **10** and isosequential oxetane-T (*25, 30*) modified AON, which showed additional strong cleavage at A7 and G12 positions. (iii) In the case of aza-ENA-T modified AON **5**, seven nucleotides toward the 3′-end from the site opposite to the modification were found to be insensitive toward RNase H promoted cleavage. This cleavage pattern was also found to be identical to that of the isosequential 2′-*O*-MOE-T modified AON **7** (Figure 3). (iv) Only aza-ENA-T incorporated AON **3** showed a comparable cleavage footprint pattern of a resistant stretch of five nucleotides as for the isosequential azetidine-T AON **9** and isosequential oxetane-T modified AON. (v) The cleavage pattern of the complementary RNA in the AON **6**/RNA hybrid duplex, containing 2′-*O*-Me-T modification, has been found to be similar to that of the isosequential azetidine-T modified AON **11** and oxetane-T modified AON but with less intensive cleavage at the A9 position (Figure 3, Supporting Information, Figure S1).

To evaluate the RNase H cleavage potential of the modified hybrids (Table 1) containing aza-ENA-T, azetidine-T, 2′-*O*-Me-T, and 2′-*O*-MOE-T modifications (Figure 1), we estimated rate constants at 0.1 μM RNA concentration under saturation conditions when the enzyme is completely saturated by the substrate (see Materials and Methods, for gel pictures Supporting Information, Figures S2 and S3). At appropriate time points, aliquots were removed from the sample. The amount of remaining RNA was determined and plotted as a function of time (Figures 4 and 5). Kinetic evaluation with a monoexponential decay function (Tables 2 and 3) showed slightly faster reaction rates for the RNase H cleavage of RNA in AON/RNA hybrid duplexes containing 2′-*O*-Me-T (AON **6**), 2′-*O*-MOE-T (AON **7**), azetidine-T (AONs **8**−**11**), and aza-ENA-T modified AONs **3** and **5** than for the native AON **1**/RNA duplex. The observed reaction rate in the case of aza-ENA-T modified AON **4** was found to be same and in the case of aza-ENA-T modified AON **2** the rate was found to be slightly slower than that of the native AON **1**/RNA hybrid duplex (Tables 2 and 3).

(C) Michaelis−Menten Kinetics of the Aza-ENA-T, Azetidine-T, 2′-O-Methoxy-T and 2′-O-Methoxyethoxy-T Modified AON/RNA Hybrids and Their Comparison with the Oxetane-T and Native AON/RNA Hybrid Duplexes. To explore the impact of chemically modified oligonucleotides containing various conformationally constrained residues as well as 2′-sugar modifications on the binding affinity and the catalytic activity of the RNase H, detailed Michaelis−Menten kinetics (see Method for kinetic analysis) has been carried out for each of the isosequential aza-ENA-T (AON **5**), azetidine-T (AON **11**), 2′-*O*-Me-T (AON **6**), and 2′-*O*-MOE-T (AON **7**) modified AON/RNA duplexes. Kinetic parameters were subsequently compared with those of the native (AON **1**) and oxetane-T (AON **12**) modified AON/RNA hybrids (Table 1).

The extent of the RNA cleavage by RNase H in all AONs (**1**, **5**−**7**, and **11**)/RNA hybrid duplexes was investigated by changing the RNA concentration at a constant effective concentration of RNase H and constant AON concentration,
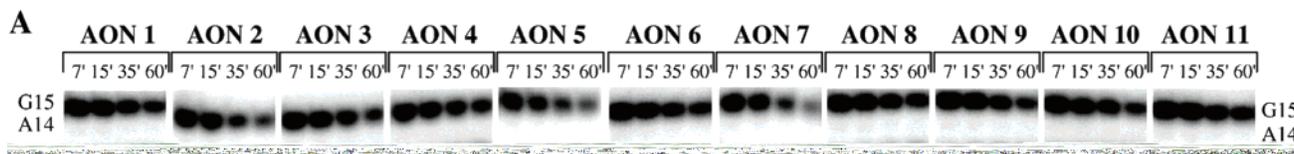
FIGURE 3: (A) Autoradiograms of 20% denaturing PAGE, showing the cleavage pattern of 5′-^{32}P-labeled target RNA by *E. coli* RNase H in native AON **1**/RNA, aza-ENA-T modified AONs **2**−**5**/RNA, 2′-*O*-Me-T modified AON **6**/RNA, 2′-*O*-MOE-T modified AON **7**/RNA and azetidine-T modified AONs **8**−**11**/RNA hybrid duplexes after 7, 15, 35, and 60 min of incubation. Conditions of cleavage reactions: RNA (0.1 μM) and AONs (2.5 μM) in buffer containing 20 mM Tris-HCl (pH 8.0), 20 mM KCl, 10 mM MgCl$_2$, and 0.1 mM DTT at 21 °C; 0.04 U of RNase H. Total reaction volume 30 μL. (B) Pictorial representation of RNase H cleavage pattern of AONs **1**−**11**/RNA hybrid duplexes, where vertical arrows show the RNase H cleavage sites.

which is sufficient for complete saturation of RNA for all varying concentrations. Thus, the initial velocities (v_0) have been obtained at six different RNA concentrations (ranging from 0.01 to 0.8 μM) (Table 4) at saturation conditions for RNA by AONs **1**, **5**−**7**, and **11** (2.5 μM), which were subsequently plotted as a function of substrate (AON/RNA hybrid duplex) concentration ([S_0]) to give the Michaelis−Menten parameters, K_m and V_{max} (Table 5, Figure 6). Since $V_{max} = E_0 K_{cat}$ (E_0 is initial effective enzyme concentration), and for all of our concentration-dependent kinetic experiments the value of E_0 was identical, V_{max} is thus proportional to K_{cat}.

The effective enzyme activity (K_{cat}/K_m) for all chemically modified substrates was without exception lower than that for the native substrate (Table 5, Figure 7). These data showed that the catalytic constant (K_{cat}), which represents the maximum number of substrate molecules converted to products per active site per unit time (*36*), is greater for the modified AON/RNA duplexes [from 1.4- to 1.6-fold for

azetidine-T (AON **11**), 2′-*O*-Me-T (AON **6**) and 2′-*O*-MOE-T (AON **7**), and ∼2.6-fold for aza-ENA-T (AON **5**)] in comparison with the native AON **1**/RNA hybrid. However, the introduction of modified moiety to the AON decreases the enzyme binding affinity (1/K_m) of AON/RNA duplex [from 1.3- to 1.7-fold less for 2′-*O*-Me-T (AON **6**) and oxetane-T (AON **12**), from 3.8- to 4.2-fold less for azetidine-T (AON **11**) and 2′-*O*-MOE-T (AON **7**), and ∼8.3-fold less for aza-ENA-T (AON **5**)]. Therefore, effective enzyme activity (K_{cat}/K_m), which refers to the properties and the reactions of the free enzyme and free substrate decreases for all of the chemically modified substrates [∼1.1-fold less for 2′-*O*-Me-T (AON **6**), ∼1.4-fold less for oxetane-T (AON **12**), ∼2.6-fold less for 2′-*O*-MOE-T (AON **7**), ∼2.7-fold less for azetidine-T (AON **11**), and ∼3.2-fold less for aza-ENA-T (AON **5**)] (Table 5, Figure 7).

(*D*) *3′-Exonuclease Stability of AON Chimeras.* Rapid degradation of unmodified AONs by various nucleases present in the cell media is a serious problem for nucleic
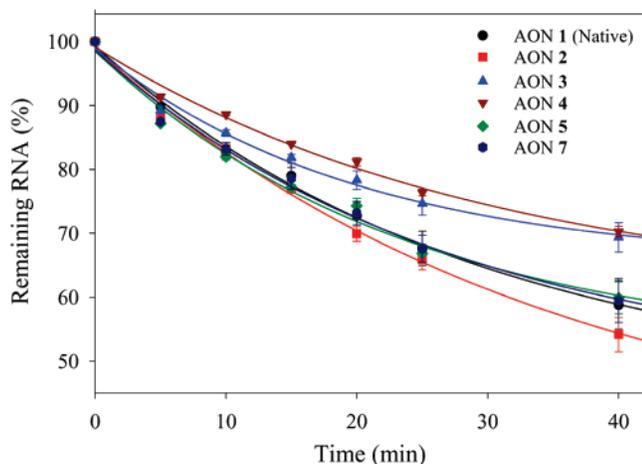
FIGURE 4: Pseudo-first-order cleavage kinetics of 5′-^{32}P-labeled target RNA by RNase H in native AON **1**/RNA, aza-ENA-T modified AONs **2−5**/RNA and 2′-*O*-MOE-T modified AON **7**/RNA hybrid duplexes after 5, 10, 15, 20, 25, and 40 min of incubation. Conditions of cleavage reactions: RNA (0.1 μM) and AONs (2.5 μM) in buffer containing 20 mM Tris-HCl (pH 8.0), 20 mM KCl, 10 mM MgCl$_2$, and 0.1 mM DTT at 21 °C; 0.03 U of RNase H in a total reaction volume of 30 μL. Target RNA remaining was densitometrically evaluated and plotted as a function of time. All values are averages and standard deviations of three independent experiments (see Materials and Methods for full experimental details).

FIGURE 5: Pseudo-first-order cleavage kinetics of 5′-^{32}P-labeled target RNA by RNase H in native AON **1**/RNA, azetidine-T modified AONs **8−11**/RNA and 2′-*O*-Me-T modified AON **6**/RNA hybrid duplexes after 5, 10, 15, 20, 25, and 40 min of incubation. Conditions of cleavage reactions: RNA (0.1 μM) and AONs (2.5 μM) in buffer containing 20 mM Tris-HCl (pH 8.0), 20 mM KCl, 10 mM MgCl$_2$, and 0.1 mM DTT at 21 °C; 0.03 U of RNase H in a total reaction volume of 30 μL. Target RNA remaining was densitometrically evaluated and plotted as a function of time. All values are averages and standard deviations of three independent experiments (see Materials and Methods for full experimental details).
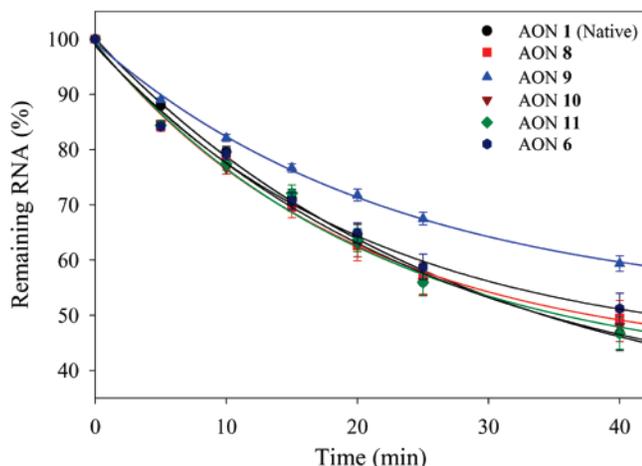
acid based therapeutics. As a result, many chemical modifications of natural DNA have been investigated for improvement of AON stability in vivo (37−40). To examine the effect of 3′-exonuclease on the chemically modified chimeric oligonucleotides, AONs **2−11** (Table 1) were tested against SVPDE. ^{32}P-labeled oligonucleotides were incubated with enzyme at 21 °C for up to 24 h. Aliquots were removed at the appropriate time points to analyze the cleavage pattern (Figure 8). The unmodified AON **1** was completely degraded

Table 2: Observed Reaction Rates and Relative Rates Normalized to the Value for the Control Native Oligonucleotide for RNase H Digestion for the Native AON **1**/RNA, aza-ENA-T Modified AONs **2−5**/RNA and 2′-*O*-MOE-T Modified AON **7**/RNA Hybrid Duplexes[a]

AONs	k (per min)	k_{rel}
1 (native)	0.034 ± 0.005	1
2	0.029 ± 0.006	0.8
3	0.05 ± 0.01	1.5
4	0.033 ± 0.008	1
5	0.042 ± 0.011	1.2
7	0.036 ± 0.009	1.1

[a] All values are averages and standard deviations of three independent experiments.

Table 3: Observed Reaction Rates and Relative Rates Normalized to the Value for the Control Native Oligonucleotide for RNase H Digestion for the Native AON **1**/RNA, Azetidine-T Modified AONs **8−11**/RNA, and 2′-*O*-Me-T Modified AON **6**/RNA Hybrid Duplexes[a]

AONs	k (per min)	k_{rel}
1 (native)	0.036 ± 0.005	1
8	0.050 ± 0.007	1.4
9	0.044 ± 0.004	1.2
10	0.047 ± 0.006	1.3
11	0.038 ± 0.008	1.1
6	0.048 ± 0.008	1.3

[a] All values are averages and standard deviations of three independent experiments.

after 30 min, whereas the full length of aza-ENA-T (AONs **2−5**) and azetidine-T (AON **8**) still remained to certain extent (~41, ~14, ~9, ~6, and ~21%, respectively). For 2′-*O*-Me-T (AON **6**), 2′-*O*-MOE-T (AON **7**), and azetidine-T modified AONs **9−11** degradation went faster with respect to the isosequential aza-ENA-T (AON **2−5**) (Figure 8). Also, all aza-ENA-T modified AONs were cleaved by 3′-exonucleases at the phosphodiester linkage (n + 1) at the 3′-end, which is one nucleotide after the modification site (n) of the oligonucleotide. This is an interesting observation in view of the fact that the identical AON oligonucleotides with *North−East* constrained azetidine and oxetane (*26*) modifications were cleaved at the 3′-phosphodiester of the modification site (n) under the same conditions (Figure 8). It is likely that the above difference in the 3′-exonuclease promoted cleavage sites reflects a tighter conformational constraint for aza-ENA-T residue in the modified AONs, compared to azetidine and oxetane modifications, such that the conformational hyperspace of the adjacent 3′-phosphodiesters (at n) adopts a conformation which prevents hydrolysis of the 3′-phosphodiester at the modification site (n). However, this effect of the tighter conformational constraint from aza-ENA-T at (n) vanishes at the (n + 1) phosphodiester linkage toward the 3′-end and is cleaved by 3′-exonuclease in a normal manner as for the (n) phosphodiester linkage of azetidine and oxetane modifications.

(*E*) *Human Serum Stability of AON Chimeras.* The stability of AONs was also checked against human serum, which contains mainly 3′-exonucleases (*9*). The digestion pattern was similar to that obtained from the digestion with SVPDE. When compared with the native AON **1**, which was completely degraded after 2 h, ~15−22% of full length of aza-ENA-T modified AONs **2−5** was left undegraded after the same period of time (Supporting Information, Figure S9).

Table 4: Dependence of the Initial Velocity (v_o) on the Substrate Concentration[a]

[RNA], μM	v_o, μM min^{-1}				
	AON **1** native	AON **6** 2′-O-Me-T	AON **7** 2′-O-MOE-T	AON **11** azetidine-T	AON **5** aza-ENA-T
0.01	$0.28 \times 10^{-2} \pm 2 \times 10^{-4}$	$0.36 \times 10^{-2} \pm 3 \times 10^{-4}$	$0.14 \times 10^{-2} \pm 1 \times 10^{-4}$	$0.16 \times 10^{-2} \pm 1.5 \times 10^{-4}$	$0.14 \times 10^{-2} \pm 1 \times 10^{-4}$
0.08	$1 \times 10^{-2} \pm 5 \times 10^{-4}$	$1.5 \times 10^{-2} \pm 11 \times 10^{-4}$	$0.86 \times 10^{-2} \pm 5 \times 10^{-4}$	$0.89 \times 10^{-2} \pm 5 \times 10^{-4}$	$0.95 \times 10^{-2} \pm 5 \times 10^{-4}$
0.4	$2 \times 10^{-2} \pm 13 \times 10^{-4}$	$2.3 \times 10^{-2} \pm 13 \times 10^{-4}$	$2.1 \times 10^{-2} \pm 11 \times 10^{-4}$	$1.9 \times 10^{-2} \pm 18 \times 10^{-4}$	$2 \times 10^{-2} \pm 20 \times 10^{-4}$
0.8	$1.9 \times 10^{-2} \pm 17 \times 10^{-4}$	$3.2 \times 10^{-2} \pm 12 \times 10^{-4}$	$2.6 \times 10^{-2} \pm 17 \times 10^{-4}$	$2.2 \times 10^{-2} \pm 20 \times 10^{-4}$	$3.3 \times 10^{-2} \pm 23 \times 10^{-4}$

[a] Initial velocity has been found from the data taken from 2 to 5 min of the reaction time. All values are averages and standard deviations of three independent experiments.

Table 5: Kinetic Characteristics of RNA Cleavage by RNase H in the AON/RNA Hybrid Duplexes[a]

AONs	T_m (°C)	V_{max} 10^{-2} (μM min^{-1})	K_m 10^{-2} (μM)	K_{cat} (min^{-1})	V_{max}/K_m (min^{-1})	K_{cat}/K_m (μM^{-1} min^{-1})	relative K_{cat}/K_m
1 (native)	44	2.1 ± 0.2	6.4 ± 2.0	92.6 ± 8.8	0.36 ± 0.11	1578 ± 482	1
6 (2′-O-Me-T)	44.5	3.3 ± 0.4	12.2 ± 5.0	145.5 ± 17.6	0.32 ± 0.13	1413 ± 574	0.89
7 (2′-O-MOE-T)	44.5	3.4 ± 0.1	24.2 ± 2.1	149.9 ± 4.4	0.14 ± 0.02	619 ± 88	0.39
11 (azetidine-T)	40	2.9 ± 0.3	23.6 ± 6.0	127.9 ± 13.2	0.13 ± 0.03	587 ± 135	0.37
5 (aza-ENA-T)	48	5.4 ± 0.9	53.2 ± 16.9	238.1 ± 39.7	0.11 ± 0.04	493 ± 179	0.31
12 (oxetane-T)[b]	39	2.0 ± 0.1	8.3 ± 2.2	88.2 ± 4.4	0.26 ± 0.07	1151 ± 310	0.73

[a] $K_{cat} = V_{max}/E_0$ ($E_0 = 0.06$ U per 30 μL $= 2 \times 10^{-3}$ U μL$^{-1} = 2.26757 \times 10^{-4}$ μM), enzyme specific activity = 420 000 U/mg or 2.38×10^{-6} mg U$^{-1} = 1.13386 \times 10^{-13}$ mol per unit, MW = 21 000 g mol^{-1}. All values are averages and standard deviations of three independent experiments. [b] V_{max} and K_m were taken from ref 26 for comparison with other modified AONs.
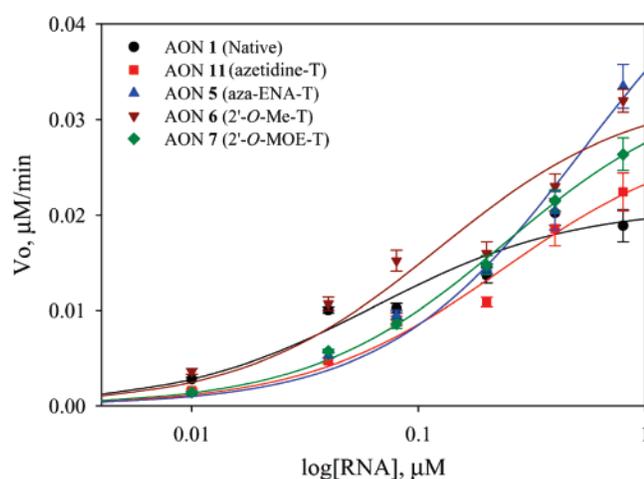

FIGURE 6: Initial velocity of the hydrolysis of the target RNA in the AON/RNA hybrids by RNase H as a function of the RNA concentration. Conditions of the cleavage reaction: RNA (from 0.01 to 0.8 μM) and AONs (2.5 μM) in buffer containing 20 mM Tris-HCl (pH 8.0), 20 mM KCl, 10 mM MgCl$_2$ and 0.1 mM DTT at 21 °C, 0.06 U of RNase H. Total reaction volume was 30 μL. All values are averages and standard deviations of three independent experiments (see Materials and Methods for full experimental details).

For other AONs the behavior of exonuclease tolerance has been found to be similar to that of the digestion with SVPDE. Thus, after 2 h of digestion in human serum, the 2′-O-Me-T modified AON **6**, 2′-O-MOE-T modified AON **7**, and azetidine-T modified AONs **8**−**11** (Supporting Information, Figure S9) were found to be almost fully degraded. It should be noted that, similar to the SVPDE digestion, all aza-ENA-T modified AONs were cleaved by human serum at the phosphodiester linkage, which is one nucleotide after (n + 1) the aza-ENA-T modification site (n) toward the 3′-end. These 5′-residual oligonucleotides (including the n + 1 nucleotide) for aza-ENA-T modified AONs **2**−**5** as well as the 5′-residual oligonucleotide (including the n nucleotide) for azetidine-T modified AON **8** still remained in human
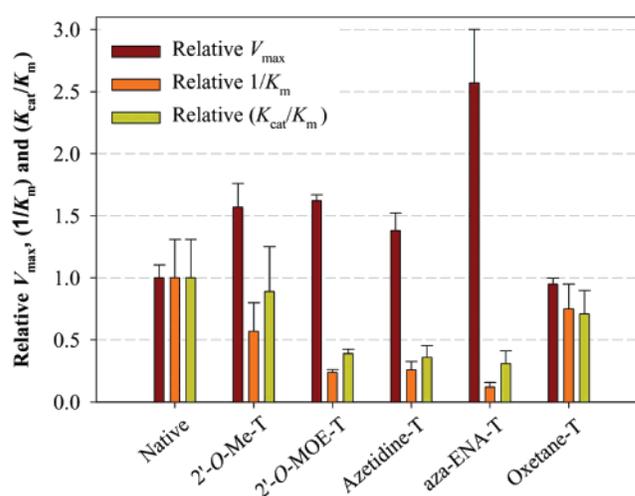

FIGURE 7: Relative (with respect to the native AON) V_{max}, $1/K_m$, and (K_{cat}/K_m) values for the RNase H promoted RNA hydrolysis in AON/RNA hybride duplexes formed by the native AON **1** and modified AONs **5**−**7**, **11**, and **12** (Table 1). All values are averages and standard deviations of three independent experiments (see Materials and Methods for full experimental details).

serum after 48 h at 21 °C (Figure 9). However, the 5′-residual oligonucleotides (including the n nucleotide) of the azetidine-T modified AONs **9** and **10** remained only up to 34 h under the same experimental condition. In contrast, the 5′-residual oligonucleotides (including the n nucleotide) of 2′-O-Me-T modified AON **6**, 2′-O-MOE-T modified AON **7** and one of the azetidine-T modified AONs (AON **11**) have been found to be stable only for 10 h (Figure 9).

DISCUSSION

The structural requirement of oligonucleotide hybrids to be recognized and to be cleaved by RNase H has been well studied. Many of the conformationally constrained nucleotides have been shown to enhance the stability of the modified AON/RNA heteroduplex, but they failed to show any RNase H recruiting capability (1, 6, 20, 23). Increase in
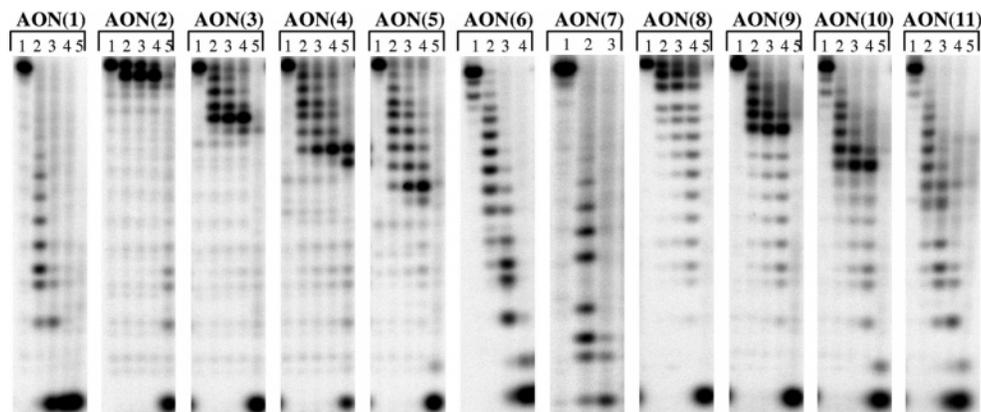
FIGURE 8: PAGE analysis of the SVPDE degradation of AONs **1**−**11**. Lanes 1−5 represent the time points taken after 0, 1/2, 1, 2, and 24 h of incubation with enzyme. The percentage of AON left after 0.5 h of incubation: 0% of AON **1**, ~41% of AON **2**, ~14% of AON **3**, ~9% of AON **4**, ~6% of AON **5**, ~2% of AON **6**, ~2% of AON **7**, ~21% of AON **8**, ~4% of AON **9**, ~3% of AON **10**, and ~2% of AON **11**.
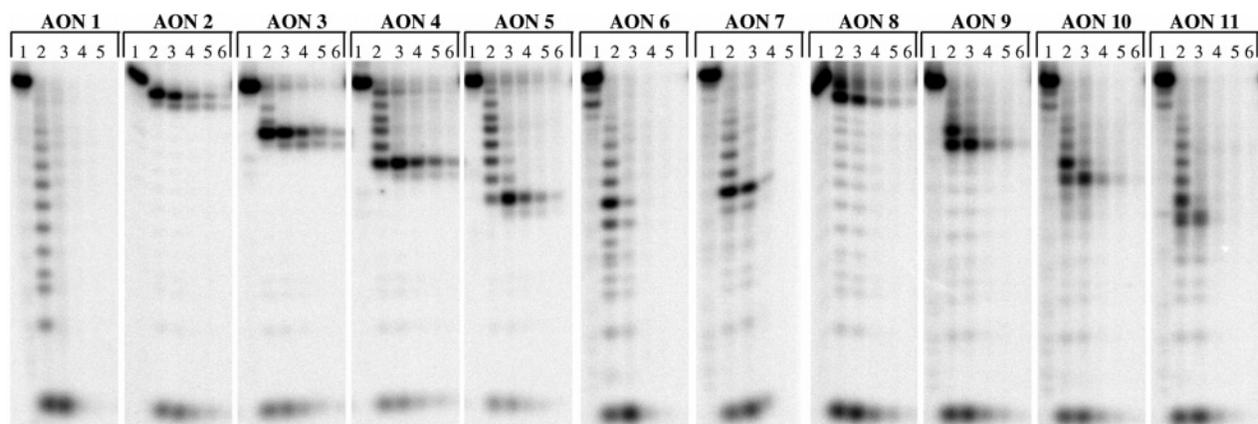

FIGURE 9: Autoradiograms of 20% denaturing PAGE, showing the degradation pattern of 5′-^{32}P-labeled AONs **1**−**11** (see Table 1 for the AON oligonucleotides) in human serum. Lanes 1−6 represent the time points taken after 0, 4, 10, 24, 34, and 48 h of incubation with serum.

the affinity of AON toward target RNA without inducing RNase H activity does not give expected results for developing new antisense drugs (*41*). It has emerged that for a modified AON/RNA heteroduplex to become a substrate for RNase H the functional groups present in the minor groove of the duplex should not inhibit binding of the enzyme. The 2′-OH group of the target RNA should be easily accessible to the enzyme, and the duplex should have enough flexibility to allow optimal RNase H cleavage activity (*1, 42*).

Therefore, we investigated the ability of AON chimeras of different kinds to recruit RNase H. Chimeric oligonucleotides (Table 1) containing conformationally constrained residues [aza-ENA-T (**A** in Figure 1) and azetidine-T (**D** in Figure 1)] and 2′-modifications [2′-*O*-Me-T (**B** in Figure 1) and 2′-*O*-MOE-T (**C** in Figure 1)] at various positions in duplex with the complementary RNA were compared with the native as well as with the isosequential oxetane-T (**E** in Figure 1) (*25, 29, 30*) modified counterpart.

The binding affinity of the chimeric AONs to the target RNA was determined first. An increase of the binding affinity toward RNA in the case of aza-ENA-T modification was expected due to the incorporation of *North*-conformationally constrained sugar as it improves stacking between the nearest-neighbors (*43*) and thus minimizes the entropic energy penalty in the free-energy of stabilization for the duplex formation with RNA. This increase in T_m for aza-

ENA-T modified AONs **2**−**5** (2.5 to 4 °C per modification) could also be attributed to the electrostatic interaction of 2′-amino function of aza-ENA-T with the neighboring phosphate at the physiological pH, which is known to lead to efficient duplex formation (*44, 45*). This particular argument can also account for azetidine-T modified AON **11**/RNA hybrid duplexes ($\Delta T_m = -4$) when compared to the isosequential oxetane-T modified AON **12**/RNA hybrids ($\Delta T_m = -5$) (Table 1). As azetidine or oxetane analogues have *North*−*East* constrained sugars with anomeric carbon participating in the modification, the AON containing these analogues therefore could change the nucleobase orientation only to less efficient stacking between the nearest-neighbors, leading to the drop in T_m toward the target RNA with respect to the unmodified AON/RNA hybrid.

To map the structural perturbation in the microenvironment owing to the modification in the variously modified AON/RNA duplexes (Table 1, Figure 1), we determined the cleavage footprint of the complementary RNA strand by RNase H. Thus, the comparison of the RNase H cleavage pattern of the complementary RNA in AON/RNA heteroduplexes containing different modifications showed that the RNase H indeed can discriminate the local variations of the microstructure of the heteroduplex substrate brought about by incorporation of various modified residues in the AON. However, the global helical conformation of the AON/RNA

hybrid duplexes with respect to the unmodified hybrid duplex was not changed, which was evident from the CD comparison (*29*, *30*, *32*). We conclude that the resistant stretch of five ribonucleotides in the chimeric azetidine-T modified AONs **9**−**10**/RNA and aza-ENA-T modified AON **3**/RNA duplexes is created, as for isosequential oxetane-T AONs chimeras (*30*), as a result of microstructure perturbation in those resistant stretches of the modified heteroduplexes compared to the regular native AON/RNA duplex. However, in the case of 2′-*O*-MOE-T modified AON **7**/RNA and aza-ENA-T modified AON **5**/RNA duplexes, a stretch of resistant seven nucleotides toward the 3′-end from the site opposite to the modification site were found due to the insensitiveness toward the RNase H promoted cleavage. In contrast, in the case of 2′-*O*-Me-T modified AON **6**/RNA and azetidine-T modified AON **11**/RNA duplexes, a resistant stretch of six nucleotides was found to be insensitive toward the RNase H cleavage (Figure 3). These results suggest that the conformational preorganization of the single modified AONs depends on the type as well as on the site of incorporation of the modification in the AON strand, which in the heteroduplex form with the complementary RNA can transmit the conformational changes of the AON to the RNA strand. Interestingly, the resulting microstructure perturbations can be recognized by the enzyme in AON/RNA hybrids in a variable manner depending upon the type and site of modifications in the AON strand.

Observed reaction rates from pseudo-first-order kinetics for the RNase H cleavage of RNA strand in the modified AON/RNA hybrid duplexes (Tables 2 and 3) showed that aza-ENA-T modified AON **3** ($k_{rel} \approx 1.5$) and azetidine-T modified AON **8** ($k_{rel} \approx 1.4$) were the most efficient for activation of RNase H, while aza-ENA-T modified AON **2** ($k_{rel} \approx 0.8$) induced slightly slower cleavage compared with that of the native AON **1**/RNA duplex ($k_{rel} = 1$). The observed reaction rate in the case of aza-ENA-T modified AON **4** ($k_{rel} \approx 1$) was found to be the same as native AON **1**/RNA duplex. AONs containing 2′-*O*-Me-T (AON **6**, $k_{rel} \approx 1.3$), 2′-*O*-MOE-T (AON **7**, $k_{rel} \approx 1.1$), azetidine-T (AONs **9**, $k_{rel} \approx 1.2$; **10**, $k_{rel} \approx 1.3$ and **11**, $k_{rel} \approx 1.1$), and aza-ENA-T (AON **5**, $k_{rel} \approx 1.2$) showed slightly faster relative rates than for the native AON **1**/RNA duplex. Thus, the pseudo-first-order reaction rate constants suggest in general that the incorporation of a single modification to the AON leads to RNase H digestion as good as or better than the native AON except for one, AON **2**.

Therefore, to evaluate the effect of different sugar modifications on the RNase H activity a detailed Michaelis−Menten kinetics was carried out for the isosequential AONs containing aza-ENA-T (AON **5**), azetidine-T (AON **11**), 2′-*O*-Me-T (AON **6**), 2′-*O*-MOE-T (AON **7**) modifications, which are then compared with the native AON **1**/RNA hybrid, as the standard. Kinetic data showed an expected decrease in effective enzyme activity for all modified AON/RNA hybrids (Table 5, Figure 7). The 2′-OH of the RNA which is stereochemically located in the minor groove of the AON/RNA hybrid duplex plays important role in the RNA cleavage reaction (*1*). Therefore, the site for the RNase H catalytic cleavage activity is highly sensitive toward conformational changes as well as toward which substituent is stereochemically oriented at the minor groove. Introduction of conformationally constrained nucleotides or 2′-alkoxy

modification in the AON strand of AON/RNA hybrid duplex made the minor groove irresponsive to the structural adjustment required for recognition and interaction by RNase H. Also, the introduction of a single positively charged 2′-propoxyamine modification into the chimeric AON portion of the heteroduplex substrate resulted in decreased RNase H binding affinity (*8*). Electrostatic interaction between the positively charged modification and the enzyme can be responsible for the reduction in binding affinity. Therefore, the decrease of the enzyme binding affinity and a resulting loss in the effective enzyme activity for azetidine-T modified AON **5**/RNA hybrid in comparison with the oxetane-T (*26*) counterpart AON **12**/RNA can be attributed to the ability of the endocyclic 2′-amino function of azetidine-T to be protonated at the physiological pH (*27*) (pK_a of 6.07). A similar reason may also be responsible in the case of aza-ENA-T modified AON **5**, in comparison with its analogous ENA (*46*) modified AON, as the endocyclic aza-ENA-nitrogen can also be protonated at the physiological pH (*32*), even more efficiently (pK_a of 6.66) than the azetidine-T (pK_a of 6.07).

To check the influence of modification incorporated to the AON on its resistance to nucleolytic degradation, chimeric AONs **2**−**11** were tested with SVPDE and human serum. In both cases, the enzyme digestion pattern for AONs was found to be similar (Figures 8 and 9). Even though the oligomers were not end-blocked, ∼6 to 41% (when digested with SVPDE) and ∼15 to 22% (when digested with human serum) of the full length of AONs **2**−**5** containing aza-ENA-T modification remained after 2 h, depending on the incorporation site of the modification to the AON, whereas native AON **1** was completely degraded (Figure 8, Supporting Information Figure S9). On the other hand, all of the aza-ENA-T modified AONs **2**−**5** were cleaved by 3′-exonucleases only at the phosphodiester linkage at one nucleotide after (n + 1) the modification site (n) toward the 3′-end, and the 5′-residual oligonucleotides (including the n + 1 residue) still remained in human serum after 48 h (Figure 9). In contrast, all azetidine-T modified AONs (AON **8**−**11**), 2′-*O*-Me (AON **6**) and 2′-*O*-MOE (AON **7**) modified AONs were cleaved at the 3′-phosphodiester of the modification site (n) toward the 3′-end. However, these 5′-residual oligonucleotides (including n) showed lower stability toward the human serum compared to those of the aza-ENA-T modified AONs **2**−**5** (including n + 1 residue), except for azetidine-T modified AONs **8** (5′-residual oligonucleotide including n was remaining after 48 h). The 5′-residual oligonucleotides (including n) for the azetidine-T modified AONs **9**−**10** were found to be less stable (remaining after 34 h) in the human serum, whereas 5′-residual oligonucleotides (including n) for the azetidine-T modified AON **11**, 2′-*O*-Me-T modified AON **6**, and 2′-*O*-MOE-T modified AON **7** were found to be the least stable (remaining only until ∼10 h) in it among all modified AONs used in this study (Figure 9).

The above results suggest that conformational effect of the aza-ENA-T modification in the AON chimeras is transmitted toward the 3′-end and recognized by 3′-exonucleases. This influence of the aza-ENA-T when only a single modification at second position from the 3′-end of AON can give significant stability toward 3′-exonucleases can be used for further design of aza-ENA-T modified AONs.

In this study, chimeric oligonucleotides containing different modified units were evaluated for the RNase H cleavage potential of the RNA strand in AON/RNA hybrid duplex, their relative stability in blood serum and in the presence of 3′-exonuclease (SVPDE). To achieve the goal for efficient antisense-based therapeutics we need to find a balance between a high target affinity, better RNase H recruitment, and higher stability in the presence of cellular nucleases.

All of the chimeric AONs, except AON **11**, with conformationally constrained nucleotides showed higher 3′-exonuclease stability compared to the 2′-alkoxy modified AONs. For the recruitment of the RNase H in the AON/RNA hybrid duplexes chimeric AONs with 2′-*O*-Me-T and oxetane-T modifications were found to be better substrates than the AONs containing 2′-*O*-MOE-T, azetidine-T, and aza-ENA-T at the same modification site. If binding to the target RNA is rate limiting (*7*), the increase in affinity to the target RNA with less efficient cleavage would have better results than high enzyme activity and loss in duplex stability. Therefore, the aza-ENA-T modified AONs is a promising antisense agent compared to the other *North−East* constrained and 2′-alkoxy modified counterparts mainly because (i) it shows higher affinity to the complementary target RNA; (ii) it is found to be a good substrate but with less effective enzyme activity for the RNase H; and (iii) it also shows improved resistance toward nucleases, which is one of the most desired requirements for antisense technology.

SUPPORTING INFORMATION AVAILABLE

Experimental methods and experimental conditions for the preparation of aza-ENA-T and its phosphoramidite building block (Scheme S1). Autoradiograms of 20% denaturing PAGE, showing the cleavage kinetics of 5′-^{32}P-labeled target RNA by *E. coli* RNase H in native AON **1**/RNA, oxetane-T modified AONs **12−15**/RNA, aza-ENA-T modified AONs **2−5**/RNA, 2′-*O*-MOE-T modified AON **7**/RNA, azetidine-T modified AONs **8−11**/RNA and 2′-*O*-Me-T modified AON **6**/RNA hybrid duplexes (Figures S1−S3). Cleavage kinetics of target RNA (0.01, 0.04, 0.08, 0.2, 0.4, or 0.8 μM) by RNase H in the native AON **1**/RNA, azetidine-T modified AON **11**/RNA, aza-ENA-T modified AON **5**/RNA, 2′-*O*-Me-T modified AON **6**/RNA and 2′-*O*-MOE-T modified AON **7**/RNA hybrid duplexes (Figures S4−S8). PAGE analysis of the degradation of AONs **1−11** in human serum until 9 h (Figure S9). Pseudo-first-order cleavage kinetics of 5′-^{32}P-labeled target RNA by RNase H in native AON **1**/RNA, aza-ENA-T modified AONs **2−5**/RNA, 2′-*O*-MOE-T modified AON **7**/RNA, azetidine-T modified AONs **8−11**/RNA and 2′-*O*-Me-T modified AON **6**/RNA hybrid duplexes (Figures S10−S11). This material is available free of charge via the Internet at http://pubs.acs.org.

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