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Oligonucleotides Comprised of Alternating 2′-Deoxy-2′-fluoro-β-D-arabinonucleosides and D-2′-deoxyribonucleosides (2′F-ANA/DNA 'Altimers') Induce Efficient RNA Cleavage Mediated by RNase H

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Abstract—Chimeric oligonucleotides comprised of alternating residues of 2′-deoxy-2′-fluoro-D-arabinonucleic acid (2′F-ANA) and DNA were synthesized and evaluated for an important antisense property—the ability to elicit ribonuclease H (RNase H) degradation of complementary RNA. Experiments used both human RNase HII and *Escherichia coli* RNase HI. Mixed backbone oligomers comprising alternating three-nucleotide segments of 2′F-ANA and three-nucleotide segments of DNA were the most efficient at eliciting RNase H degradation of target RNA, and were significantly better than oligonucleotides entirely composed of DNA, suggesting that these mixed backbone oligonucleotides may be potent antisense agents.

Antisense oligonucleotides (AONs) are designed to hybridize to specific regions of a selected mRNA, in order to interfere with the normal processing of that mRNA, thereby leading to reduced expression of the coded protein. AONs are believed to predominantly function by two mechanisms: (1) translational blocking, and (2) destruction of mRNA by ribonuclease H (RNase H).[1] While both of these mechanisms can successfully inhibit the synthesis of the encoded protein by the mRNA, elicitation of RNase H cleavage of target mRNA is considered to be the more efficient of the two mechanisms for suppressing target gene expression.

The first AONs tested in cell culture possessed a phosphodiester backbone.[2] These had poor antisense activity since they were rapidly degraded by nucleases in vitro.[3] Replacement of phosphodiester with phosphorothioate linkages in the AON improved stability to nuclease degradation, but also resulted, unfortunately, in a considerably decreased binding affinity of the oligonucleotide for the target RNA. Subsequently, many AON modifications have been made in an attempt to improve their binding affinity.[4] Currently, the most widely-used AON technology is a mixed-backbone (chimeric) nucleotide consisting of 2′-*O*-alkyl RNA (which cannot elicit RNase H degradation of the target RNA) flanking a central core segment of DNA. This design provides target RNA binding affinity via interaction with the 2′-*O*-alkyl RNA, and elicits RNase H degradation of the target RNA via the DNA core.[5]

We have shown previously that 2′-deoxy-2′-fluoro-D-arabinonucleic acid (2′F-ANA) forms thermally-stable hybrids with RNA, and importantly provide substrates that allow RNase H degradation of the target RNA.[6] Structures of the nucleotide components of these AONs are shown in Figure 1. More recently, we showed that chimeric AONs comprised of 2′F-ANA flanking a DNA core also induced efficient RNase H cleavage of target

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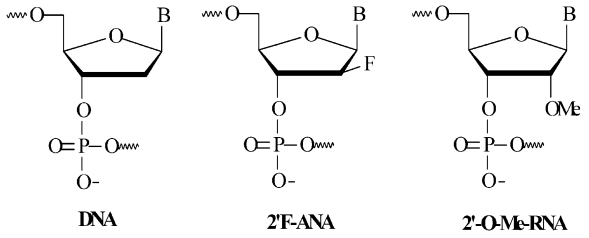

Figure 1. Structure of the nucleotide components of the oligonucleotides studied.

RNA in vitro.[6d] Unlike chimeric AONs comprised of 2′-*O*-alkyl RNA/DNA/2′-*O*-alkyl RNA, the efficiency of RNase H cleavage of target RNA induced by chimeric 2′F-ANA/DNA/2′F-ANA AONs was not limited by the size of the DNA core. In fact, the introduction of a single DNA residue into an otherwise 2′-FANA oligonucleotide provided a significant increase in elicited antisense activity compared to AONs solely comprised of 2′F-ANA.

To further investigate this observation, we prepared AON with alternating segments of 2′F-ANA and DNA ('altimers') in which the contiguous DNA segments ranged from 0 to 6 bases in length, and examined these for their ability to elicit RNase H cleavage of complementary target RNA.

Synthesis of AONs. 2′F-ANA monomers and mixed-backbone AONs comprising 2′F-ANA and DNA were synthesized as previously described.[6b] Oligonucleotides were characterized by anion exchange HPLC, polyacrylamide gel electrophoresis, and MALDI-TOF mass spectrometry. Oligo-rA_{18} RNA and chimeric 2′-*O*-Me RNA/DNA AON were obtained from the University of Calgary DNA Synthesis Laboratory (Calgary, AB, Canada). Sequences of the oligonucleotides used are provided in Table 1.

T_m measurements. AON and complementary target RNA oligonucleotides were mixed in equimolar ratios in 140 mM KCl, 1 mM $MgCl_2$, and 5 mM Na_2HPO_4 buffer, pH 7.2, to provide a total duplex concentration of circa 5 μM. Samples were heated to 90 °C for 15 min, then cooled slowly to room temperature. The AON/RNA duplex solution was then exposed to increasing temperature (0.5 °C/measurement), and the UV absorbance at 260 nm was determined after temperature equilibration. T_m values were calculated from the first derivative of the melt curves[5] and have an uncertainty of ±0.5 °C.

Purification of RNase H. *Escherichia coli* RNase HI was purified as described previously.[7] Human RNase HII was over-expressed and purified following slight modification of the published procedures.[8]

RNase H assay. RNase H assays were carried out at room temperature (≈20 °C). Nucleic acid duplex substrates were prepared by mixing the AON (2 pmol) with 5′-^{32}P-labeled complementary target oligo-rA_{18} RNA (0.5 pmol) in 10 μL of 60 mM Tris–HCl (pH 7.8) containing 60 mM KCl and 2.5 mM $MgCl_2$, followed by heating at 90 °C for 2 min and slow cooling to room temperature. While these conditions may in principle induce triplex formation (T/AT), we excluded this possibility by conducting T_m studies (data not shown) and from the fact that 2′F-ANA (or DNA) does not associate with rPu/dPy duplexes.[9] Duplex substrate solutions were allowed to stand at room temperature for at least 1 h prior to use. Reactions were initiated by the addition of RNase H, and aliquots were removed at various times and quenched by the addition of an equal volume of 98% deionized formamide containing 10 mM EDTA, 1 mg/mL bromophenol blue and 1 mg/mL xylene cyanol. After heating at 100 °C for 5 min, reaction products were resolved by electrophoresis on 16% polyacrylamide sequencing gels containing 7 M urea, visualized by autoradiography, and product formation was quantified by densitometry.

As shown previously,[6b] duplexes of 2′F-ANA/RNA are significantly more stable than the corresponding DNA/RNA duplex. We now find that 2′F-ANA/DNA 'altimer' oligonucleotides, like 2′F-ANA/DNA/2′F-ANA 'gapmer' oligonucleotides, also form similarly stable duplexes with target RNA (Table 1), and that the melting temperature for these AON chimeras directly correlates with the 2′F-ANA content. Previous studies have shown that 2′-*O*-Me RNA AONs also bind to target RNA with a higher affinity than do the corresponding DNA AONs.[10] However, mixed backbone 2′-*O*-Me RNA/DNA 'altimer' AON (**8**–**10**) showed only similar or lower thermal binding affinity for target RNA compared to the all DNA AONs. AON **8** comprised of alternating single residues of 2′-*O*-Me RNA and DNA has a particularly low T_m value. This is consistent with the view that contiguous RNA (north sugar pucker) and DNA (south-east pucker) segments destabilize the double helix by inducing multiple A/B junctions within the AON/RNA duplex.[11] In contrast, 2′F-ANA and DNA exhibit similar conformational preferences (South-East type), thus alternating 2′F-ANA/DNA segments can be accommodated without altering the

Table 1. Antisense oligonucleotide sequences used in this work

AON #	Sequences[a]	T_m (°C)
1	ttt ttt ttt ttt ttt ttt	40
2	**TTT TTT TTT TTT TTT TTT**	53
3	**T**t**T** t**T**t **T**t**T** t**T**t **T**t**T** t**T**t	46
4	**TT**t t**TT** tt**T** **T**tt **TT**t t**TT**	46
5	**TTT** ttt **TTT** ttt **TTT** ttt	47
6	**TTT TTT** ttt ttt **TTT TTT**	48
7	**TTT T**tt t**TT TT**t tt**T TTT**	47
8	U**t**U t**U**t U**t**U t**U**t U**t**U t**U**t	33
9	U̲U̲U̲ ttt U̲U̲U̲ ttt U̲U̲U̲ ttt	42
10	U̲U̲U̲ U̲U̲U̲ ttt ttt U̲U̲U̲ U̲U̲U̲	41
11	tta tat ttt ttc ttt ccc	53
12	**TTA TAT TTT TTC TTT CCC**	67
13	**TTA** tat **TTT** ttc **TTT** ccc	58
14	**TTA TAT** ttt ttc **TTT CCC**	61

[a]Small letters and **bold capital letters** refer to the 2′-deoxyribonucleotide and the 2′-deoxy-2′-fluoro-D-arabino-nucleotide, respectively. U̲ is 2′-*O*-methyl-D-ribouridine nucleotide.

structural features relative to a DNA/RNA (A-like) hybrid (**12**).[12]

Studies with mixed backbone AONs suggest that the ability of these AONs to elicit RNase H-mediated degradation of the target RNA in vitro is predictive of the ability of these AONs to inhibit intracellular gene expression.[6d,5a,13] We therefore evaluated the suitability of various AONs bound to complementary RNA as substrates for *E. coli* RNase HI and human RNase HII.

Homopolymeric altimers.

Figures 2 and 3 show that all homopolymeric 2′F-ANA/DNA chimeras induced target RNA cleavage by human RNase HII and *E. coli* RNase HI. Cleavage efficiency increased as the length of the alternating DNA segments within the 2′F-ANA background was increased. Optimal activity was noted with AON **5**, which comprises alternating trinucleotide segments of 2′F-ANA and DNA. The ability of this 'altimer' AON to elicit human RNase HII degradation of target RNA was significantly better than that of the equivalent all-DNA AON **1**. Furthermore, this characteristic of AON **5** was improved relative to the 2′F-ANA/DNA/2′F-ANA 'gapmer' AON **6** (Fig. 4), a composition that has previously showed excellent inhibitory potency against intracellular gene expression.[6d]

Unlike 'altimer' AON comprised of 2′F-ANA and DNA, similar AON (**8** and **9**) comprised of 2′-*O*-methyl RNA and DNA showed only poor ability to elicit RNase H degradation of target RNA (Fig. 4).

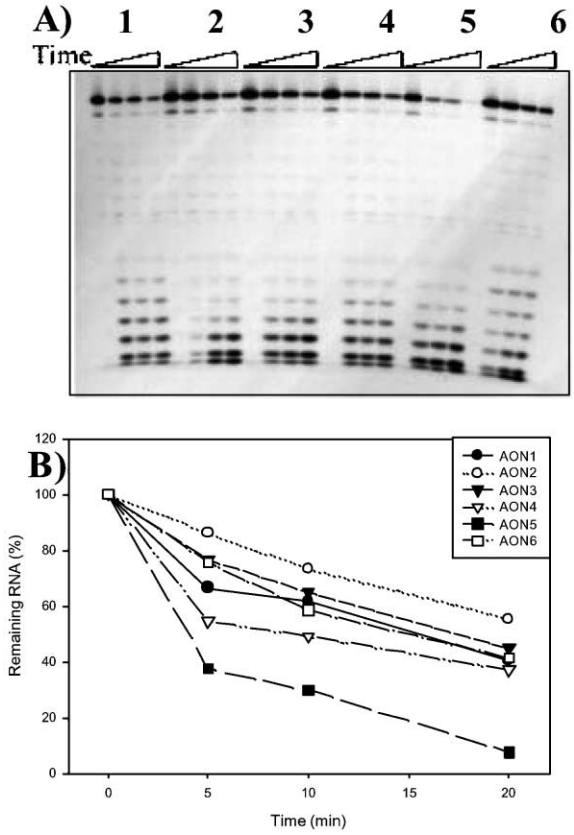

Figure 2. Human RNase HII-mediated cleavage of RNA duplexed with various AON. (**A**) Electrophoretic analysis of ^{32}P-labeled target RNA degradation products. AON/5′-[^{32}P]-RNA duplexes were incubated with human RNase HII at rt. Aliquots were taken at 0, 5, 10, and 20 min, electrophoresed and reaction products visualized by autoradiography. (**B**) Residual full-length 5′-[^{32}P]-target as a function of reaction time. Data were obtained by densitometric analysis of the autoradiogram shown in **A**.

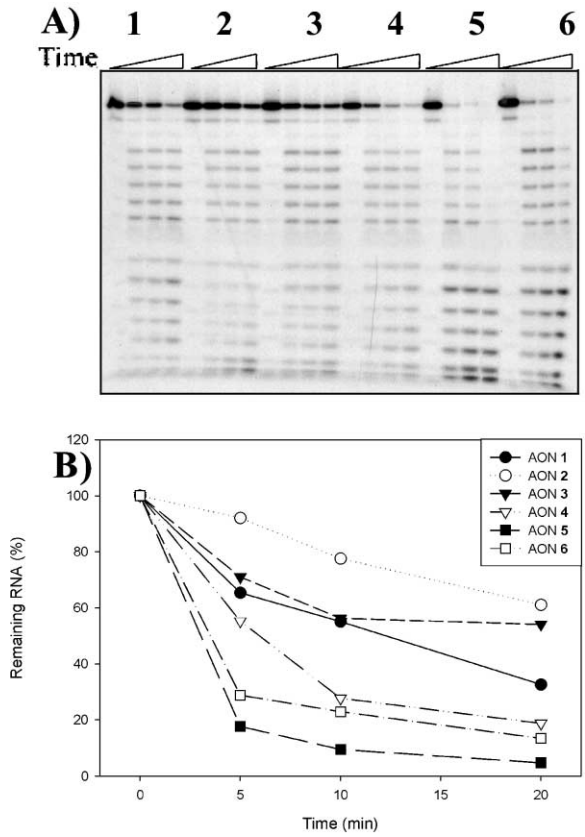

Figure 3. *E. coli* RNase HI-mediated cleavage of RNA duplexed with various AONs. Conditions as described in the legend of Figure 2.

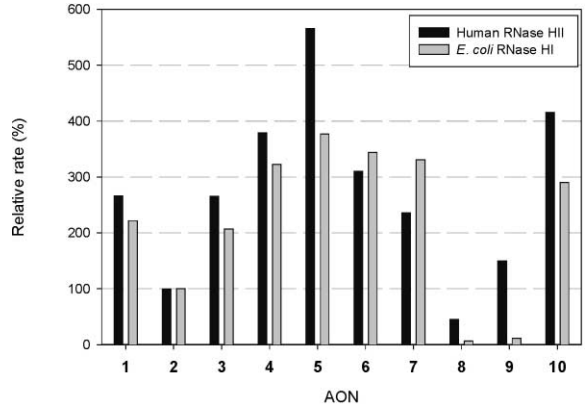

Figure 4. Ability of AONs listed in Table 1 to elicit RNase H degradation of RNA. AON/5′-[^{32}P]-RNA duplexes were incubated with RNase HII (black bars) or *E. coli* RNase HI (shaded bars) for 10 min at rt, then the reaction mixtures were resolved by electrophoresis, visualized by autoradiography, and the loss of intact RNA was quantified by densitometry. Values are normalized to those found for the AON **2** as 100%.

Interestingly, the 2′-*O*-Me RNA/DNA 'gapmer' AON **10** (possessing a relatively long 6 DNA nucleotide core flanked by 2′-OMe RNA) was quite efficient at eliciting RNase H degradation of the target RNA, especially with human RNase HII. This contrasts with our previous findings[6d] in which such AON were poor substrates for *E. coli* RNase HI in vitro, and were virtually inactive at inhibiting intracellular gene expression. The reasons for these differences are not clear, but may be related to the fact that our previous studies targeted heteropolymeric RNA sequences, whereas in this case the target is a homopolymeric RNA target sequence.

Heteropolymeric Altimers

Under a given set of conditions, a homopolymeric altimer may not behave like its heteropolymeric counterpart. To make sure that such scenario is not valid here, we prepared an oligonucleotide of mixed base sequence that is structurally similar to AON **5** (Table 1, AON **13**). AON **13**, upon hybridization with a complementary RNA target, is quite efficient at eliciting RNase H degradation of target RNA, with both *E. coli* RNase H1 and human RNase HII. In fact, the ability of the various AONs to recruit the human and bacterial RNase H activity follows the trend: **13** (altimer) $\geq$**14** (gapmer) $>$**11** (DNA) $>>$**12** (all-2′F-ANA) (data not shown). Studies to assess the nuclease resistance as well as the potency of such 'altimer' AON at inhibiting intracellular specific gene expression are in progress. In conclusion, our data show that AONs comprising alternating small segments of 2′F-ANA and DNA ('altimers') are highly effective at eliciting RNase H degradation of target RNA. Certain 'altimer' AON, namely those possessing alternating trinucleotide segments of 2′F-ANA and DNA, appear to be equivalent or better with respect to this parameter than the 2′F-ANA/DNA/2′F-ANA 'gapmer' AON that were previously shown to possess superior antisense properties compared to the equivalent 2′-*O*-Me RNA/DNA/2′-*O*-Me RNA AON.[6d] In sharp contrast, altimer AONs possessing alternating segments of 2′-*O*-Me RNA and DNA were very poor at eliciting RNase H degradation of target RNA, further highlighting the superior properties of AON based on 2′F-ANA.

Acknowledgements

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