Chimeric siRNAs with chemically modified pentofuranose and hexopyranose nucleotides: altritol-nucleotide (ANA) containing GalNAc–siRNA conjugates: in vitro and in vivo RNAi activity and resistance to 5′-exonuclease

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ABSTRACT

In this report, we investigated the hexopyranose chemical modification Altriol Nucleic Acid (ANA) within small interfering RNA (siRNA) duplexes that were otherwise fully modified with the 2′-deoxy-2′-fluoro and 2′-O-methyl pentofuranose chemical modifications. The siRNAs were designed to silence the transthyretin (Ttr) gene and were conjugated to a trivalent N-acetylgalactosamine (GalNAc) ligand for targeted delivery to hepatocytes. Sense and antisense strands of the parent duplex were synthesized with single ANA residues at each position on the strand, and the resulting siRNAs were evaluated for their ability to inhibit Ttr mRNA expression in vitro. Although ANA residues were detrimental at the 5′ end of the antisense strand, the siRNAs with ANA at position 6 or 7 in the seed region had activity comparable to the parent. The siRNA with ANA at position 7 in the seed region was active in a mouse model. An Oligonucleotide with ANA at the 5′ end was more stable in the presence of 5′-exonuclease than an oligonucleotide of the same sequence and chemical composition without the ANA modification. Modeling studies provide insight into the origins of regiospecific changes in potency of siRNAs and the increased protection against 5′-exonuclease degradation afforded by the ANA modification.

INTRODUCTION

The tremendous potential of small interfering RNAs (siRNAs) in the development of new medicines is demonstrated by the success of patisiran (ONPATTRO®), a first in class drug for the treatment of hereditary transthyretin-mediated amyloidosis and the recent approval (https://www.givlaari.com, https://www.fda.gov/drugs/resources-information-approved-drugs/fda-approves-givosiran-acute-hepatic-porphyria, https://www.fda.gov/news-events/press-announcements/fda-approves-first-treatment-inherited-rare-disease) of the second compound givosiran (GIVLAARI™ for the treatment of acute hepatic porphyria (AHP) (1–6). Many other siRNAs are being evaluated in early to late stage clinical trials (7). siRNAs act through a natural gene regulatory mechanism called RNA interference (RNAi) to silence the expression of a targeted gene (8,9). siRNAs composed of natural RNA nucleotides are rapidly degraded and do not readily reach their target organ when administered systemically. Thus, it is necessary to use chemically modified siRNAs for therapeutic applications (10–14). Typically, siRNAs are modified using terminal phosphorothioate backbone linkages and 2′-deoxy-2′-fluoro (2′-F) (15–21) and 2′-O-methyl (2′-OMe) nucleotide modifications (Figure 1A and B) to increase metabolic stability. Conjugation to a trivalent N-acetylgalactosamine (GalNAc, Figure 1C) ligand, one that allows for the specific recognition and uptake by the asialoglycoprotein receptor (ASGPR) expressed on the surface of hepatocytes, results in targeting to the liver (22). We are investigating additional chemically modified

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nucleotides that can be used in synergy with 2'-F RNA and 2'-OMe RNA for generating potent and safe siRNAs (23–26). For instance, modification of the seed region of the antisense strand with glycol nucleic acid (GNA) can reduce seed-mediated off-target effects (24,27–32). Similarly, placing a 5'-morpholino-2'-OMe RNA nucleotide on the sense strand or a 5'-trans-vinylphosphonate-2'-OMe RNA nucleotide on the antisense strand can improve RNAi activity and specificity (23,33–39). Moreover, incorporation of 4'-C-methoxy-2'-deoxy-2'-fluoro uridine improves metabolic stability without reducing gene silencing activity (26).

Altritol nucleic acids (ANA) have a six-membered sugar with a nucleobase at the 2'(S)-position and a hydroxyl group at the 3'-position (Figure 1B) (40,41). ANA has a pre-organized structure similar to the conformation of an N-type furanose sugar, and the presence of the hydroxyl group at the 3'-position contributes to duplex stabilization via hydration (40). The RNA-like A-type geometry of ANA makes it a candidate for incorporation into siRNAs, as this feature is important for RISC loading. ANA-modified siRNAs have been evaluated and demonstrated to have some promise; (28,42–44) however, the ANA modification has not been tested in the context of other modified nucleotides or in siRNAs conjugated to ligands. More importantly, ANA modified siRNAs have never been tested in vivo. Here we evaluated the in vitro and in vivo gene silencing activity of siRNA duplexes modified with ANA, 2'-F RNA and 2'-OMe RNA nucleotides and conjugated to a trivalent GalNAc moiety (Figure 1A) (14,25). We also evaluated how effectively terminal ANA residues protected the oligonucleotides against nucleolytic degradation. siRNAs modified at single positions were as active as the parent siRNA in RNAi-mediated gene-silencing. Furthermore, it is interesting that two terminal ANA residues imparted resistance to phosphodiesterase II, a 5'-exonuclease. Models of an siRNA guide strand with an ANA modification at position 7 bound to Argonaute 2 (Ago2) and an RNA with two 5'-terminal ANA residues bound to Xrn1, an RNA 5'-exonuclease helped rationalize the favorable RNAi pharmacological activity and metabolic stability, respectively.

MATERIALS AND METHODS

Oligonucleotide synthesis

ANA-modified oligonucleotides were synthesized following the previously published protocol (25) with the exception that 3% trichloroacetic acid in CH2Cl2 was used for detritylation.

In vitro siRNA activity analyses

First, 5 µl of siRNA was placed in each well of a 384-well collagen-coated plate, followed by addition of 4.90 µl of Opti-MEM and 0.1 µl of Lipofectamine RNAiMax (Invitrogen) to each well. Each siRNA was assessed in quadruplicate at final concentrations of 0.1 or 10 nM. Plates were incubated at room temperature for 15 min. Primary mouse hepatocyte cells were suspended in Invitrogen CP rodent medium (BioIVT, #Z990028), and 40 µl of this suspension (containing ~5000 cells) was added to each well. After incubating the cells for 24 h, RNA was isolated using DynaBeads (ThermoFisher). The RNA was then reverse transcribed into cDNA according to the manufacturer’s protocol (Applied Biosystems). Multiplex quantitative polymerase chain reaction (qPCR) reactions were per-
formed using a gene-specific TaqMan assay for Ttr (ThermoFisher Scientific, # Mm00443267_m1) and mouse Gapdh (#4352339E) as an endogenous control. Real-time PCR was performed on a Roche LightCycler 480 using LightCycler 480 Probes Master Mix (Roche). The details of the in vitro analysis has been described in detail in the references 25, 53, 54 and 55: the siRNA concentration required to inhibit relative TTR expression by 50% (IC50) was calculated for a subset of the siRNAs by transfecting as described, eight concentrations ranging from 10 nM to 37.5 fM in a 6-fold dilution series. IC50 values were derived from a four parameter fit model with XLFit.

**Off-target assay**

COS-7 cells were cultured at 37°C, 5% CO2 in Dulbecco’s-modified eagle medium supplemented with 10% fetal bovine serum. Cells were co-transfected in 96-well plates (15 000 cells/well) with 10 ng luciferase reporter plasmid and 0.64 pM to 50 nM siRNA in 5-fold dilutions using 2 μg/ml Lipofectamine 2000 (Thermo Fisher Scientific) according to the manufacturer’s instructions. Cells were harvested 48 h after transfection for the dual luciferase assay (Promega) performed according to the manufacturer’s instructions. The on-target reporter plasmid contained a single perfectly complementary site (5’-AAAAACAGTGTCTTGTGCTCTATAA-3’) to the antisense strand of the Ttr siRNA in the 3’-untranslated (3’ UTR) of Renilla luciferase. The off-target reporter plasmid contained four tandem seed-complementary sites (5’-GCTCTATAA) separated by a 19-nucleotide spacer (5’-AAATATACATAAAATAAA-3’) in the 3’-UTR of Renilla luciferase (27,29–30,45–46). Both plasmids co-expressed firefly luciferase as a transfection control.

**In vivo gene expression silencing**

All studies were conducted using protocols consistent with local, state and federal regulations, as applicable, and were approved by the Institutional Animal Care and Use Committee (IACUC) at Alnylam Pharmaceuticals. Female C56BL/6 mice (Charles River Laboratories) of 6–8 weeks old were dosed subcutaneously with 1 mg/kg siRNA for Ttr (ThermoFisher Scientific, # Mm00443267_m1) and mouse Gapdh (#4352339E) as an endogenous control. Real-time PCR was performed on a Roche LightCycler 480 using LightCycler 480 Probes Master Mix (Roche). The details of the in vitro analysis has been described in detail in the references 25, 53, 54 and 55: the siRNA concentration required to inhibit relative TTR expression by 50% (IC50) was calculated for a subset of the siRNAs by transfecting as described, eight concentrations ranging from 10 nM to 37.5 fM in a 6-fold dilution series. IC50 values were derived from a four parameter fit model with XLFit.

**Nuclease stability assay**

To assess stability in the presence of 3'- or 5'-specific exonucleases, modified oligonucleotides were prepared in a final concentration of 0.1 mg/ml in either 50 mM Tris (pH 7.2) with 10 mM MgCl2 or 50 mM sodium acetate (pH 6.5) with 10 mM MgCl2, respectively. The exonuclease (150 mU/ml SVPE or 500 mU/ml phosphodiesterase II) was added just prior to analysis. Samples were analyzed using IEX HPLC (Dionex DNA-Pac PA200, 4 × 250 mm) using a gradient of 37–52% (mobile phase A: 20 mM sodium phosphate, 15% CH3CN, pH 11; mobile phase B: 1 M NaBr, 20 mM sodium phosphate, pH 11, 15% CH3CN) over 7.5 min with a flow of 1 ml/min. The amount of full-length oligonucleotide was determined as the area under the curve at A360. The percent full-length oligonucleotide was calculated by dividing by the area under the curve at t = 0 and multiplying by 100. The activity of enzyme was compared to 5'- (dT19)U-U-3' or 5'-U, (dT19)-3' for 3'- or 5'-exonuclease activity, respectively. In these sequences, the 's' indicates a phosphorothioate linkage; the other linkages were phosphodiester. Each aliquot of stock enzyme was thawed just prior to the experiment. The half-life was determined by fitting to first order kinetics.

**Molecular modeling**

Coordinates of the crystal structure of the ANA:RNA hybrid duplex (ANA strand: 5’-P-CCGUAUAGCC-3’, where P is a phosphate; RNA strand: 5’-GGCAUUAACGG-3’) with PDB ID: 3OK2 (47) and the crystal structure of microRNA miR-20a bound to human Argonaute 2 (Ago2) with PDB ID: 4F3T (48) were retrieved from the Protein Data Bank www.rcsb.org. The G residue at position 7 of the RNA in the Ago2 complex was excised and replaced by ANA-G3 from the hybrid duplex with the ANA A-strand using the program UCSF Chimera (49). The geometry of the resulting fusion model was locally refined with Amber14 (50), and the glycosidic torsion angle of the ANA purine residue was adjusted slightly to restore stacking on the adjacent residue C8 as seen in the crystal structure of the Ago2 complex with native RNA.

Coordinates of the crystal structure of the complex between the Drosophila melanogaster Xrn1 5'-exoribonuclease and 5’-d(TdCdTdT-T)-3’ with PDB ID: 2Y35 (51) were obtained from the Protein Data Bank. The ANA dimer at positions 9 and 10 was excised from strand A of the crystal structure of the ANA:RNA hybrid duplex and the bases converted to U using the program UCSF Chimera. The dimer was then superimposed on the first two nucleotides from the dT trimer in the Xrn1 complex with the match function in UCSF Chimera using selected backbone and base atoms.

Coordinates of the crystal structure of the complex between the Escherichia coli DNA polymerase I Klenow fragment 3'-exonuclease and 3’-d(GdCdA-P5’ with PDB ID: 1KFS (52) were obtained from the Protein Data Bank. The ANA CG dimer (positions 2 and 3) was excised from strand A of the crystal structure of the ANA:RNA hybrid duplex and the dimer then superimposed on the d(CG) dimer in the Klenow exo complex with the match func-
Figure 2. *In vitro* RNAi activity of siRNAs with ANA in the antisense strand in the presence of a transfecting agent. The nucleotide at the position indicated on the x-axis was substituted with the corresponding ANA nucleotide. siRNAs were tested at a concentration of 10 nM in primary mouse hepatocytes. Plotted is the % Ttr mRNA remaining relative to untreated cells (n = 4, mean ± SD).

Figure 3. *In vitro* RNAi activity of siRNAs with ANA in the sense strand in the presence of a transfecting agent. The nucleotide at the position indicated on the x-axis was substituted with the corresponding ANA nucleotide. siRNAs were tested at a concentration of 10 nM in primary mouse hepatocytes. Plotted is the % Ttr mRNA remaining relative to untreated cells (n = 4, mean ± SD).

Next, coordinates of the ANA and RNA model complexes were stored and separately refined with Amber 14 in the Chimera suite using both steepest descent and conjugate gradient cycles until convergence, i.e. no further change in distances between RNA/ANA 2′-OH groups and atoms from Klenow exo side chains (Tyr-423 C62/Thr-358 Oγ) and main chain (Thr-358 O). At last, all three complexes (DNA, RNA, ANA) were overlaid in UCSF Chimera using only Klenow fragment atoms with the match option.
RESULTS

In vitro activity of ANA-modified siRNAs

The position-dependent impact of the ANA modification on in vitro RNAi activity was assessed by systematically replacing each nucleotide of a previously studied siRNA designed to target rodent (mouse and rat) transthyretin (Ttr) mRNA (Figure 1) with an ANA residue (25,53–55). The parent siRNA is a 21-mer duplex with a two-nucleotide single-overhang on the 3′-end antisense strand modified with 2′-F RNA and 2′-O Me RNA nucleotides and is adapted from our earlier studies (14,25). The ends were protected by introducing two phosphorothioate linkages at each strand extremity, except for the 3′-end of the sense strand, which is conjugated to a trivalent GalNAc ligand for targeted delivery to the liver (Figure 1A) (14,25). The RNAi activity of the resulting siRNAs with single ANA residues was studied in primary mouse hepatocytes (Figure 2). In agreement with a previous study, ANA at the 5′-end of the antisense strand (Figure 2, AS1) reduced potency (42). This loss in activity is probably due to the inability of cellular kinases to introduce a 5′-monophosphate group on the ANA nucleotide. The 5′-monophosphate plays an important role in RISC loading by interacting with the Ago2
MID domain, and the lack of a 5′-monophosphate is known to impair RNAi activity (35,56–57). The RNAi activity was partially restored by installing a phosphate group chemically (Figure 2, AS1P). An ANA modification at position 2 of the antisense strand was also detrimental to RNAi activity, most likely owing to a structural incompatibility (see the ‘Discussion’ section for more details) as seen by the subtle and remarkable difference between 2′-F RNA and 2′-OMe RNA (14).

In the seed region of the siRNA antisense strand (nucleotides 2–8), the ANA modification was better accommodated. The modified duplexes with an ANA nucleotide at position 6 or 7 of the antisense strand had RNAi activity comparable to the parent duplex at 10 nM (Figure 2). In addition, the siRNA with ANA at position 16 of the antisense strand had activity comparable to the parent. However, none of the modified duplexes were more potent than the parent siRNA. Similar results were obtained at a lower concentration of 0.1 nM (Supplementary Figure S1). Single ANA modifications in the sense strand generally appear to lead to a slight loss in activity, although full dose response studies are needed to quantitate such small differences (Figure 3 and Supplementary Figure S2).

The position-dependent impact of the ANA modification on in vitro RNAi activity was also assessed in the absence of a transfecting agent, allowing free uptake of siRNAs (Figures 4 and 5; Supplementary Figures S3 and S4). Overall, the results obtained with free uptake correlates very well with the results when siRNAs were transfected. For instance, loss of activity was observed with ANA at position AS1 or AS2 while siRNAs containing ANA at position AS6 and AS7 maintained their activity.
Based on the initial in vitro screening results, the IC$_{50}$ values for several ANA-modified siRNAs were determined in primary mouse hepatocytes (Figure 6). The siRNA with an ANA at position 2 of the antisense strand was least potent (IC$_{50}$ 1098 pM). The siRNA with ANA at position 7 of the sense strand had an IC$_{50}$ value of 115 pM, which was within experimental error of the IC$_{50}$ of the parent duplex (112 pM). The other ANA-modified siRNAs tested were also of similar potency.

Analysis of on-target and off-target activity in a luciferase reporter assay

Seed-mediated off-target activity has been reported to be a major driver of hepatotoxicity observed in rats for a subset of siRNAs (24). To reduce such off-target effects, thermodynamically duplex-destabilizing, acyclic modifications, such as glycol nucleic acid (GNA) have been incorporated into the seed regions of siRNA duplexes (24,27–32). GNA...
mitigates seed-mediated off-target effects without reducing on-target potency. ANA is well tolerated in the seed region at positions 6 or 7 in the antisense strand and maintains on-target potency. This prompted us to evaluate the effect of ANA on potentially modulating the off-target activity using a luciferase reporter assay in which four regions that are matches to the seed region of the siRNA, but that are not complementary to the rest of the antisense strand, are cloned into the luciferase 3′-UTR region (29,45–46). The siRNAs with ANA at positions 6 and 7 of the antisense strand had on-target activity similar to the parent construct in this assay (Figure 7A), an observation consistent with earlier in vitro screening. The off-target activities for the parent duplex and for the ANA-modified duplexes were also the same within experimental error (Figure 7B). Thus, ANA neither reduces off-target activity nor enhances it. As ANA is not a thermodynamically destabilizing modification like GNA, this result is not surprising (vide infra).

**Silencing of gene expression by ANA-modified GalNAc-siRNAs in mice**

Selected siRNA duplexes were evaluated for their ability to silence the expression of the *Ttr* gene in mice. The animals were dosed at 1 mg/kg subcutaneously, with the control cohort receiving the 1 x PBS. Blood samples were collected just prior to the dosing and 7 and 14 days after dosing to evaluate circulating TTR protein levels in serum. At day 7, siRNAs with ANA at positions 6 or 7 of the antisense strand had potencies comparable to that of the parent duplex. However, at day 14, the siRNA with ANA at position 7 of the antisense strand had potency equivalent to the parent, whereas the siRNA with the ANA at position 6 was less potent (Figure 8A). The reason for this difference may be due to intrinsic metabolic stability differences, but this aspect needs further evaluation. The siRNA with the ANA modification at position 16 of the antisense strand was also significantly less active than the parent duplex (Figure 8A). The siRNA had activity comparable to the parent duplex in vitro, indicative of intrinsic differences between the in vitro and *in vivo* models. The siRNA with the sense strand modified with ANA at position 12 was slightly less active than the parent, but activity was maintained on day 14 (Figure 8A). The siRNA modified with ANA at the 5′ terminus of the antisense strand was not effective in mice (Figure 8A), possibly due to a lack of phosphorylation of the 5′-ANA by endogenous intracellular kinases. Improved RNAi activity in vitro for siRNA duplex carrying a chemically installed phosphate group at the 5′-end of antisense strand supported this hypothesis. However, an impact of the structural difference of ANA from RNA cannot be ruled out completely as an siRNA duplex with a phosphate group at the 5′-end was still less active than the parent duplex. In the in vivo experiments, the animals were sacrificed at day 14, and RNA from liver was extracted, followed by cDNA preparation and multiplexed RT-qPCR analysis to assess the Ttr mRNA levels (Figure 8B). The results were well aligned with those obtained from analysis of TTR protein in serum samples at day 14.

**The conformation of ANA within an RNA strand**

To gain insight into the effects of ANA substitutions, we built a model of an ANA-modified strand bound to Ago2. We used the crystal structure of the Ago2:miR-20a complex (48) as a starting point. The structure of the ANA residue incorporated in the RNA strand in place of G7 was taken from the crystal structure of an ANA:RNA decamer duplex (47). In the ANA:RNA duplex crystal structure, the intra-strand phosphate–phosphate distance for ANA residues is 5.53 Å on average; the distance for RNA residues was an average of 5.93 Å. The closer spacing between phosphates in ANA is due to the δ backbone torsion angle that averages 60° as a result of the chair conformation of the hexose. This angle is around 83° in the RNA strand of the hybrid duplex, a value identical to that in a canonical A-form RNA (58). The spacings between phosphates of residues 6 and 7 and 8 in miR-20a in the complex with Ago2 are shorter than the average distance between adjacent phosphates as a result of a pronounced kink at that site. Thus, an ANA nucleotide with an intrinsically short distance between the phosphate groups is well accommodated at position 7 (Figure 9).

**Nuclease stability**

To evaluate the effect of ANA on resistance of an oligonucleotide to exonuclease cleavage, one or two nucleotides at the 3′ or 5′ end of 5′-UsU(dT19)-3′ or 5′-(dT19)UsU-3′, respectively, where ‘s’ indicates a phosphorothioate linkage in the otherwise phosphodiester-linked oligonucleotide. Stabilities of the ANA-modified strands were compared to the strands with one or two uridines at the appropriate terminus. Oligonucleotides modified at the 3′ end were incubated with snake venom phosphodiesterase (SVPD), a 3′-exonuclease and the degradation of full-length oligonucleotide was monitored using ion-exchange HPLC. Oligonucleotides with two uridines or an ANA had similar stability against SVPD and thus comparable half-lives. The oligonucleotide with two ANA residues had 2-fold longer half-life (Figure 10A).

The 5′-modified oligonucleotides were incubated with phosphodiesterase II (PDEII), a 5′-specific exonuclease.
The oligonucleotide carrying a single ANA modification at the 5′ end was considerably more stable compared to the oligonucleotide carrying uridine. Addition of a second ANA modification at the 5′ end rendered the oligonucleotide inert for a duration of 24 h under the tested conditions (Figure 10B). When an ANA dimer was superimposed on the two 5′-terminal dTs present in the crystal structure of oligo-dT bound to the 5′-exoribonuclease Xrn1 (51), differences were observed in inter-phosphate spacing (shorter for ANA) and phosphate orientation; the increased bulk of the hexose sugar compared to 2′-deoxyribose is also apparent (Figure 11A). Conversely, comparison between the crystal structure of DNA bound to DNA polymerase I Klenow fragment 3′-exonuclease (52) and models of RNA and ANA bound to the same active site reveals only very minor deviations between the orientations of the scissile phosphate group in the three species (Figure 11B). Moreover, the models support the conclusion that additional sugar hydroxyl groups in the RNA and ANA dimers relative to DNA can be accommodated without generating clashes with exonuclease active site residues.

**DISCUSSION**

Altritol nucleic acids (ANA) have a hexopyranose ring system with a preorganized structure similar to the conformation of an N-type furanose sugar, and the presence of the hydroxyl group at the 3′-position contributes to duplex stabilization. The RNA-like A-type geometry of ANA makes it a candidate for incorporation into siRNAs and ANA-modified siRNAs have been evaluated showing therapeutic promise (28,42–44), however the ANA modification had not been tested in the context of fully modified nucleotides or in GalNAc-siRNA conjugates. Here we evaluated in vitro and in vivo gene silencing activity of siRNA duplexes modified with ANA, 2′-F RNA and 2′-OMe RNA nucleotides and conjugated to GalNAc. We also evaluated how ANA nucleotides protected oligonucleotides from nucleolytic degradation.

Placement of a single ANA nucleotide along the siRNA antisense strand (AS) manifested in vitro activities that were comparable to the performance of the parent duplex for
strands with ANA at positions AS6, AS7 or AS16. Further evaluation of ANA-modified duplexes in vivo showed that ANA at AS7 retains favorable potency, whereas siRNAs with ANA at either position AS6 or AS16 displayed lower activities compared to those seen in vitro. The intrinsically shorter phosphate-phosphate spacing in an oligo-ANA backbone compared to RNA appears to render the modification ideal for insertion at position AS7 that is characterized by a kink in the crystal structure of an miRNA:Ago2 complex (48). Indeed, molecular modeling supports this view, whereby replacement of ribonucleotide AS7 by ANA reveals a seamless fit at the site of the kink (Figure 9). Altered backbone torsion angles and the glycosidic angle of the ANA nucleotide were adjusted, without, however, altering the synchiral or antiperiplanar angle ranges. ANA and RNA carbon atoms are colored in cyan and green, respectively, H-bonds are drawn with thin solid lines (panel A), potential clashes are indicated by arrows (panel B), and near overlaps of 5'-3'-oxygen atoms and the 5'-phosphate group are highlighted by solid and dashed circles, respectively.

Figure 12. Models of Ago2 interactions with siRNA guide strand nucleotides (A) AS1 replaced by an ANA residue, and (B) AS2 replaced by an ANA residue. An ANA nucleotide (5',3'-diphosphate) excised from the crystal structure of an ANA:RNA hybrid duplex (PDB ID: 3OK2) (47) was superimposed on either the AS1 or AS2 nucleotide of miR-20a in complex with Ago2 (PDB ID: 4F3T) (48) using the match option in the program UCSF Chimera (49). To improve the fit, selected backbone torsion angles and the glycosidic angle of the ANA nucleotide were adjusted, without, however, altering the synchiral or antiperiplanar angle ranges. ANA and RNA carbon atoms are colored in cyan and green, respectively, H-bonds are drawn with thin solid lines (panel A), potential clashes are indicated by arrows (panel B), and near overlaps of 5'-3'-oxygen atoms and the 5'-phosphate group are highlighted by solid and dashed circles, respectively.

effects is related to the thermal stability of the seed region with potential off-target mRNAs, GNA-based xeno nucleic acid seemed to perform better as ANA is not a thermally destabilizing modification (40).

It is noteworthy that the activity of guide siRNA with ANA at AS1 is only slightly reduced relative to the parent RNA (Figure 2). Conversely, ANA at AS2 results in the most significant loss in activity compared to any position in both the guide and passenger strands (Figures 2 and 3). Unlike the ribose in RNA, the alttritol sugar cannot adopt a different pucker, and its ANA-hydroxyl group is locked in an equatorial orientation and engaged in a H-bond with the side chain of Q548. This results in shorter phosphate–phosphate spacings relative to RNA (δ ≈ 83°, A-form). We previously investigated the effects of glycol nucleic acid (GNA) modification in the guide and passenger strands on siRNA activity (25). GNA features a 4-atom linker that also leads to tighter spacing of adjacent phosphates relative to RNA, and an siRNA with (R)-GNA incorporated at AS7 displayed favorable potency compared to the parent duplex. Therefore, two xeno nucleic acids, one based on a hexose sugar and the other on an acyclic framework, offer similar benefits in terms of enhanced siRNA activity by mimicking a particularly short phosphate-phosphate distance at a kink in the seed region of the guide strand loaded into Ago2. GNA nucleotides when incorporated at position 6 or 7 in the seed region of the antisense strand also reduced the off-target events. However, the luciferase reporter assay indicated that ANA at position 6 or 7 in the seed region of the antisense strand of the tested siRNA against TTR did not affect its off-target activity. While promising, additional siRNA sequences need to be evaluated in this assay to make a generalizable conclusion about the effects of ANA on undesired off-target activity of siRNAs. As the mitigation of off-target effects is related to the thermal stability of the seed region with potential off-target mRNAs, GNA-based xeno nucleic acid seemed to perform better as ANA is not a thermally destabilizing modification (40).
AS2 is not tolerated (14), as it causes short contacts with amino acids from an adjacent α-helical turn. This indicates that the α-helix does not go out of the way to accommodate the methoxy substituent and that the 5′- and 3′-phosphates of AS2 are firmly held in place by interactions with Ago2 side chains. The most likely culprit behind the significantly reduced potency of guide siRNAs with ANA at AS2 is therefore a steric conflict between altritol sugar atoms and Ago2 MID domain residues (Figure 12B). Interestingly, the observed high nuclease resistance of RNAs with dual ANA modification at the 5′-end is most likely also a consequence of steric conflicts in addition to the tighter phosphate–phosphate spacing in ANA (Figure 11).

In summary, we have shown that siRNAs modified with a single altritol six-membered sugar ring induce efficient gene silencing both in vitro and in vivo when judiciously introduced within the siRNA duplex. Specifically, the ANA modification was best accommodated at position 7 of the antisense strand. Previous crystallographic data for an ANA:RNA hybrid revealed that the average distance between the 5′- and 3′-phosphates of an ANA residue is shorter than the average distance between phosphates in RNA. An ANA residue can thus sufficiently mimic a ribonucleotide at position 7 of the antisense strand bound to Ago2, a site at which a kink in the siRNA antisense RNA. An ANA residue can sufficiently mimic a ribonucleotide at position 7 of the antisense strand bound to Ago2, a site at which a kink in the siRNA antisense strand results in local contraction of phosphate–phosphate spacing in ANA (Figure 11).

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REFERENCES


31. Manoharan, M. and Joshua-Tor, L. (2016) siRNA carrying an interfering RNA (sisiRNA) in mice bearing human tumor nucleic acid (UNA) modified siRNA and small internally segmented and off-target effects of locked nucleic acid (LNA) and unlocked nucleic acid:ribonucleic acid duplex.


