

Re-Engineering RNA Molecules into Therapeutic Agents

Martin Egli^{*,†} and Muthiah Manoharan^{*,‡}

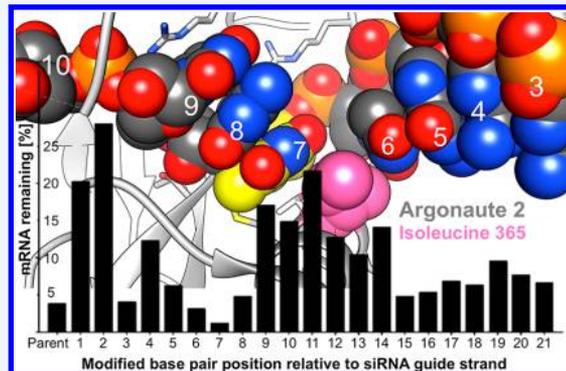
[†]Department of Biochemistry, School of Medicine, Vanderbilt University, Nashville, Tennessee 37232, United States

[‡]Alnylam Pharmaceuticals, 300 Third Street, Cambridge, Massachusetts 02142, United States

CONSPECTUS: Efforts to chemically modify nucleic acids got underway merely a decade after the discovery of the DNA double helix and initially targeted nucleosides and nucleotides. The origins of three analogues that remain staples of modification strategies and figure prominently in FDA-approved nucleic acid therapeutics can be traced to the 1960s: 2'-deoxy-2'-fluoro-RNA (2'-F RNA), 2'-O-methyl-RNA (2'-OMe RNA), and the phosphorothioates (PS-DNA/RNA). Progress in nucleoside phosphoramidite-based solid phase oligonucleotide synthesis has gone hand in hand with the creation of second-generation (e.g., 2'-O-(2-methoxyethyl)-RNA, MOE-RNA) and third-generation (e.g., bicyclic nucleic acids, BNAs) analogues, giving rise to an expanding universe of modified nucleic acids. Thus, beyond site-specifically altered DNAs and RNAs with a modified base, sugar, and/or phosphate backbone moieties, nucleic acid chemists have created a host of conjugated oligonucleotides and artificial genetic polymers (XNAs). The search for oligonucleotides with therapeutic efficacy constitutes a significant driving force for these investigations. However, nanotechnology, diagnostics, synthetic biology and genetics, nucleic acid etiology, and basic research directed at the properties of native and artificial pairing systems have all stimulated the design of ever more diverse modifications.

Modification of nucleic acids can affect pairing and chemical stability, conformation and interactions with a flurry of proteins and enzymes that play important roles in uptake, transport or processing of targets. Enhancement of metabolic stability is a central concern in the design of antisense, siRNA and aptamer oligonucleotides for therapeutic applications. In the antisense approach, uniformly modified oligonucleotides or so-called gapmers are used to target a specific RNA. The former may sterically block transcription or direct alternative splicing, whereas the latter feature a central PS window that elicits RNase H-mediated cleavage of the target. The key enzyme in RNA interference (RNAi) is Argonaute 2 (Ago2), a dynamic multidomain enzyme that binds multiple regions of the guide (antisense) and passenger (sense) siRNAs. The complexity of the individual interactions between Ago2 and the siRNA duplex provides significant challenges for chemical modification. Therefore, a uniform (the same modification throughout, e.g., antisense) or nearly uniform (e.g., aptamer) modification strategy is less useful in the pursuit of siRNA therapeutic leads. Instead, unique structural features and protein interactions of 5'-end (guide/Ago2MID domain), seed region, central region (cleavage site/Ago2 PIWI domain), and 3'-terminal nucleotides (guide/Ago2 PAZ domain) demand a more nuanced approach in the design of chemically modified siRNAs for therapeutic use.

This Account summarizes current siRNA modification strategies with an emphasis on the regio-specific interactions between oligonucleotide and Ago2 and how these affect the choice of modification and optimization of siRNA efficacy. In addition to standard assays applied to measure the effects of modification on the stability of pairing and resistance against nuclease degradation, structural insights based on crystallographic data for modified RNAs alone and in complex with Ago2 from molecular modeling studies are a valuable guide in the design of siRNA therapeutics. Thus, this comprehensive approach is expected to result in accelerated generation of new siRNA-based therapies against various diseases, now that the first siRNA has obtained approval by the US FDA for treatment of hereditary hATTR amyloidosis.



1. INTRODUCTION

Work on chemical modification of nucleosides and nucleotides was initiated in the 1960s. The earliest examples are 2'-deoxy-2'-fluoro-uridine (2'-F U),¹ nucleoside phosphorothioates (PSOs),² and 2'-O-methyl (2'-O-Me) RNA³ (Figure 1). Interestingly, these first-generation modifications figure prominently in the backbones of oligonucleotide drugs approved over the last 20 years and therapeutic candidates currently in phase I to III clinical trials in the US.^{4,5} The antisense oligonucleotide (AON) Vitravene (fomivirsen), approved by the US Food and

Drug Administration (FDA) in 1998 against cytomegalovirus retinitis in AIDS patients, was a fully PS-modified 21mer DNA.^{6–8} Mipomersen (Kynamro) against homozygous familial hypercholesterolemia and approved in 2013 is a 20mer 5-10-5 gapmer with 2'-O-(2-methoxyethyl)-RNA (2'-O-MOE, Figure 1) wings and a central PSO-DNA window.^{9,10} And Macugen (pegaptanib) is an extensively 2'-F/2'-OMe-modified 28mer

Received: December 19, 2018

Published: March 26, 2019

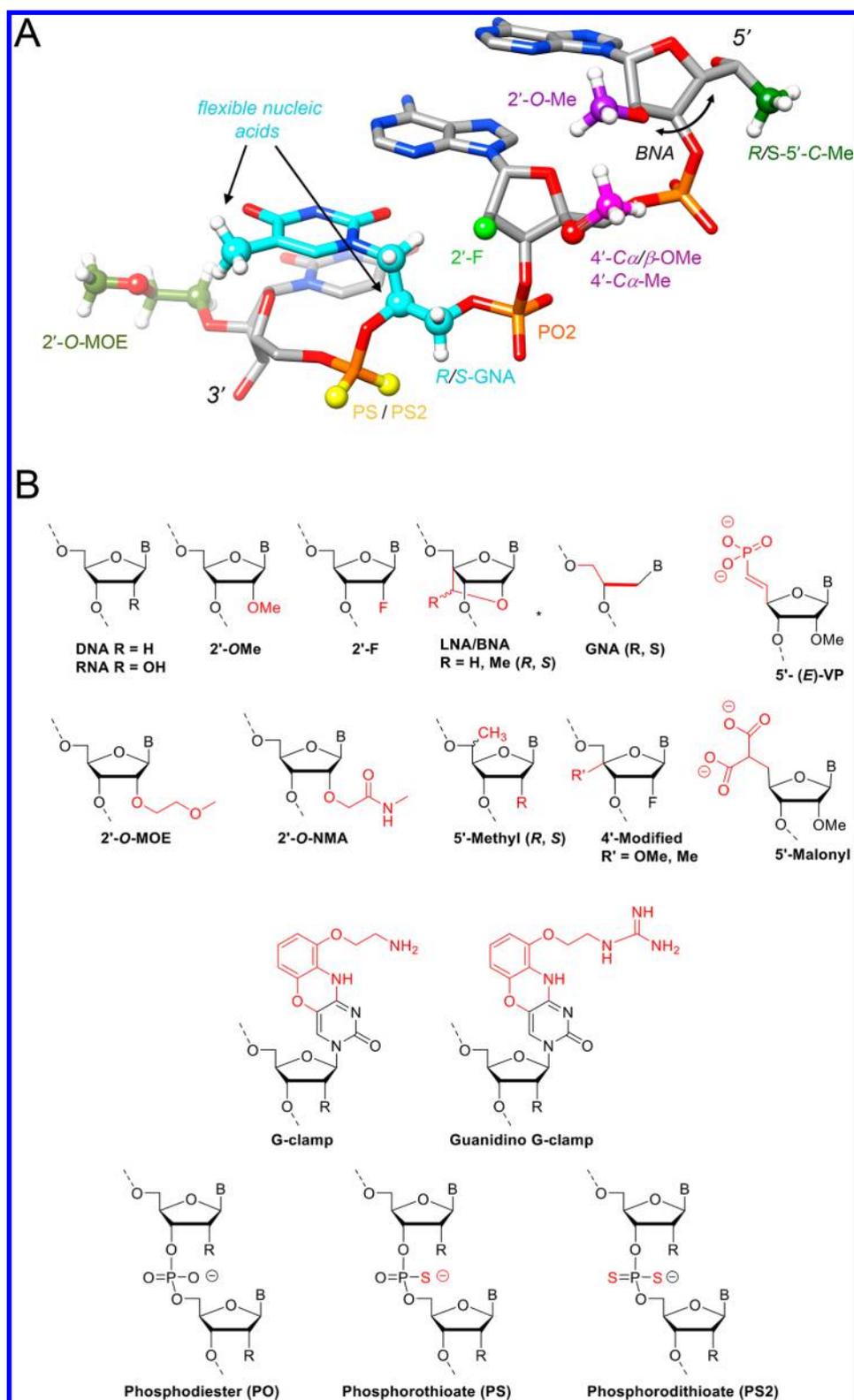


Figure 1. (A) Three-dimensional and (B) chemical structures of selected first, second, and third generation modifications evaluated in nucleic acid therapeutics.

anti-VEGF RNA aptamer that was approved in 2004 for treating wet age-related macular degeneration.^{11–13}

During the past three decades, hundreds of nucleic acid modifications have been synthesized and characterized (Figure 1).^{14–21} Many of these were evaluated *in vitro* and some *in vivo* and in the clinic,^{4,5} and have found use in antisense, siRNA,

aptamer,²² and/or other applications, e.g., Spinraza (nusinersen) that promotes alternative splicing.²³ Concepts in the antisense realm, such as gapmers with central PS-DNA windows (to elicit RNase H-mediated cleavage of the RNA target) but otherwise featuring uniform modifications in the wings, have given way to sophisticated site-specific modification and

conjugation strategies that consider the more complex anatomy and protein interactions of siRNAs and aptamers.²⁴ Thus, modification of siRNAs has to take into consideration both the guide and passenger strands and their regiospecific interactions with RISC Ago2 that minimally concern the 5'-end (Mid domain), central cleavage site (PIWI domain), and 3'-overhang recognition (PAZ domain).^{25,26} Nevertheless, Onpattro (patisiran), an RNAi-based drug for treatment of hereditary transthyretin-mediated ATTR amyloidosis that received FDA approval in August of 2018, also contains the first-generation 2'-OMe modification (<https://www.drugs.com/history/patisiran.html>).²⁷

Second-generation chemistries have moved from the bench to the bedside in several cases. For example, MOE-RNA^{28,29} advanced to FDA approval within about 20 years from its inception: Kynamro (mipomersen) in 2013 and Spinraza (nusinersen) in 2016. The latter drug is an MOE-RNA 18mer for the treatment of spinal muscular atrophy.²³ Tegsedi (inotersen) is also an MOE-DNA-MOE 5-10-5 gapmer like mipomersen in chemical composition but targets the transthyretin gene for the control of transthyretin mediated amyloidosis. This compound was approved in 2018. Our structural analysis demonstrated the unique double gauche interaction which contributes to the binding affinity of MOE-RNA to target RNA.²⁹ The extensive hydration associated with the MOE modification also modulates the protein binding properties of MOE oligonucleotides for favorable biodistribution and tolerability. Synthetic analogues with neutral linkers in place of the negatively charged phosphate, such as amide RNA^{30,31} or peptide nucleic acid,^{32,33} have been investigated in detail. Eteplirsen (Exondys 51), a 30mer oligonucleotide that features a neutral backbone consisting of phosphorodiamidate-bridged methylenemorpholine moieties, was approved for treatment of Duchenne muscular dystrophy in 2016.³⁴

X-ray crystal structures of oligonucleotide analogues and oligos that contain chemically modified residues yield insights into the conformational properties of modifications and how they affect the duplex geometry.^{35,36} Structural data also allow a better understanding of the underlying causes of changes in the pairing stability as a result of modification, e.g., refs 29 and 37–41. For example, we reported a unique high-affinity five-hydrogen-bonded G:modified C pair by making a guanidino G-clamp (Figure 1), but this has never been evaluated *in vitro* or *in vivo* for therapeutic applications. Also, structures of modified oligos in the context of exonuclease active site models often facilitate an interpretation of experimental nuclease resistance data, e.g., refs 40, 42, and 43. Combining insights from crystal structures of Ago2:RNA complexes, modified RNA oligos, and molecular modeling can shed light on the altered activity of chemically modified siRNAs. In turn, such a structural approach helps guide the choice of sites for incorporation of known chemically modified nucleotides into siRNA duplexes and the design of new modifications. This review summarizes recent successes of the structure-based interpretation of siRNA activity data and the creation of new modification chemistries for use in future RNAi therapeutics.

2. ANATOMY AND DYNAMICS OF ARGONAUTE 2

Considering the complexity of protein–siRNA interactions and their consequences for pharmacokinetics and -dynamics, the focus on a single enzyme to gain a better understanding of the relationship between chemical modifications and silencing activity may appear to be a somewhat simplistic endeavor.

Nevertheless, armed with an excellent understanding of the chemistries that afford increased pairing stability and protection against nucleases, one can argue that Ago2 is a key factor in locating and destroying the targeted RNA. Therefore, it stands to reason that a detailed analysis of the architecture of this enzyme and the local interactions with the siRNA duplex will likely provide valuable insights into the effects of a diverse set of modifications on activity and the design of improved modifications for future use in therapeutics.

Human Ago2 is a multidomain protein of 859 amino acids length that binds complementary 21mer guide (5'-phosphorylated) and passenger strands with 3'-terminal dinucleotide overhangs. The latter strand subsequently gets degraded or discarded, and the RNA target loaded into Ago2 opposite the guide strand is cleaved at position 10 by the PIWI domain. PIWI adopts an RNase H-like fold, characterized by a central five-stranded β -sheet flanked by α -helices on both sides (Figure 2A).^{26,44} The 5'-terminal nucleotide of the guide strand is anchored in the MID domain, with the phosphate group tightly bound by multiple lysines, Tyr-529 and Gln-545 (Figure 2B). Between the first and second nucleotides, the guide strand makes a sharp turn before transitioning into the helical seed region (AS2–AS8); this curl is stabilized by Gln-548 and Asn-551 that contact the first bridging phosphate (Figure 2B). The seed region exhibits a kink between AS6 and AS7 that leads to locally contracted phosphate–phosphate distances, whereby the Ile-365 side chain acts as a wedge on the minor groove side (Figure 2C).^{44,45} The central siRNA region includes nucleotides AS9–AS12, including the P10 phosphodiester moiety that is hydrolyzed by PIWI. In the crystal structures of complexes, the RNAs are detached from the active site and several Ago2 loop regions need to shift in order for the active complex to form (Figure 2D). Thus, in the complex with an siRNA duplex bound, the passenger strand only complements the guide strand up to AS9. The latter strand itself is not defined in the electron density beyond AS14 (Figure 2A; dashed lilac line). Therefore, the structural data provide only limited insight into RNA–protein interactions that concern the supplementary siRNA region (AS13–AS17). By comparison, the binding mode of 3'-terminal residues of guide siRNA with the Ago2 PAZ domain is well resolved in crystal structures and reveals a tight grasp particularly of the last nucleotide that includes H-bonds to 2'-OH, 3'-OH, and phosphate oxygens as well as stacking interactions with the nucleobase (Figure 2E).

Ill-defined or absent regions in the structures of complexes attest to the dynamic nature of the multidomain Ago2 protein. The overlay of structures in Figure 2A demonstrates that PAZ domains display deviating orientations in complexes. This mobility is crucial for function and underlies the role of PAZ in peeling off the guide from the passenger strand by grabbing the two 3'-overhanging nucleotides of the former. Importantly, in terms of the use of chemical modifications to confer drug-like properties on RNA, the siRNA guide and passenger strands offer as many as 42 (nucleotide) sites and many more if phosphate, ribose, and base moieties are considered separately for each. In the following chapters, we will travel along the guide siRNA strand in a 5' to 3' direction and discuss the effects on RNAi activity and Ago2 interactions of modifications at selected sites.

3. Ago2MID AND 5'-TERMINAL GUIDE/PASSENGER STRAND MODIFICATIONS

The 5'-phosphate group is important for guide strand recognition and binding, but insufficient metabolic stability *in*

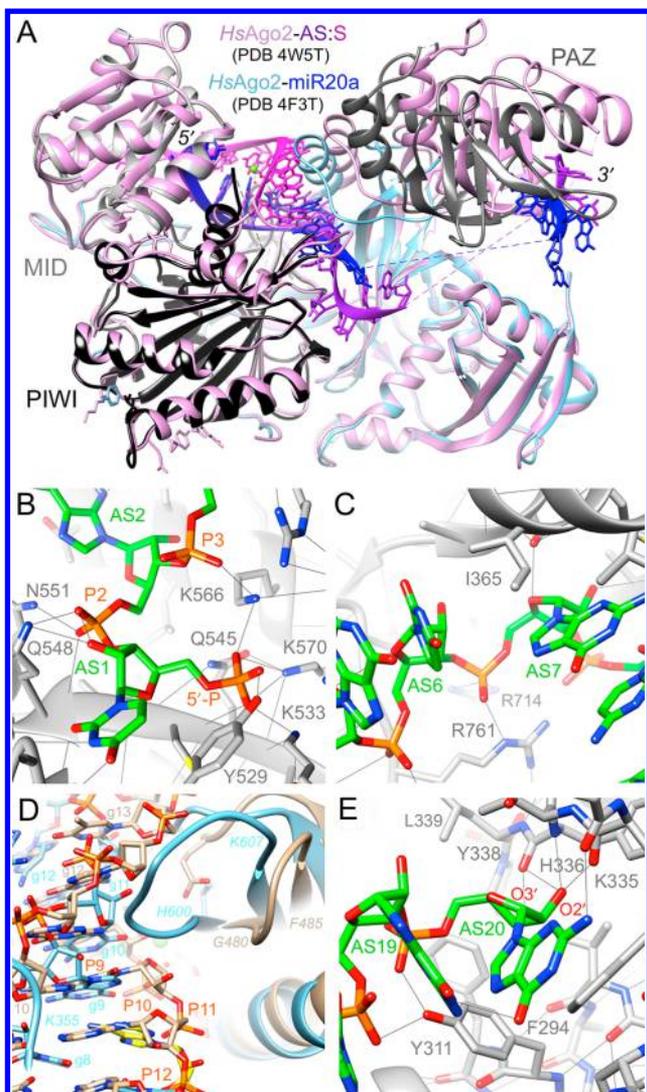


Figure 2. Human Ago2: (A) overall structure with RNAs bound and domains mediating key interactions with siRNA, (B) MID domain and 5'-end (AS1) and sharp turn between guide residues AS1 and AS2, (C) seed region and kink between AS6 and AS7, (D) PIWI domain interactions (*H. sapiens*, light blue; *T. thermophilus*, tan) around the P10 cleavage site, and (E) PAZ domain and 3'-terminal guide siRNA residues. The two complexes in panel A feature partially resolved guide:passenger strand RNAs⁴⁴ and miRNA that takes the position of guide siRNA.⁴⁵

vivo can result in its removal and off-target effects. An elegant way to prevent dephosphorylation and rephosphorylation is the introduction of a nonhydrolyzable phosphate mimic, vinylphosphonate (VP, Figure 1) that leaves unchanged the -2 charge of the phosphate. The *E*-VP stereochemistry provides a better fit relative to the native strand (Figure 3A).⁴⁶ By comparison, in order for phosphate of the *Z*-VP moiety to maintain the various electrostatic interactions, it would require the first nucleoside to adjust its orientation and lead to suboptimal stacking on Tyr-529 (Figure 3B). These structural considerations are in line with the higher activity of modified siRNA duplexes featuring *E*-VP at the 5'-end of the guide strand. We also explored the 5'-C-malonyl group as a bioisostere of the terminal monophosphate (Figure 3C) and found that it enhanced *in vitro* stability, RISC loading, and activity of siRNA.⁴⁷ However, the malonyl moiety did not exhibit sufficient

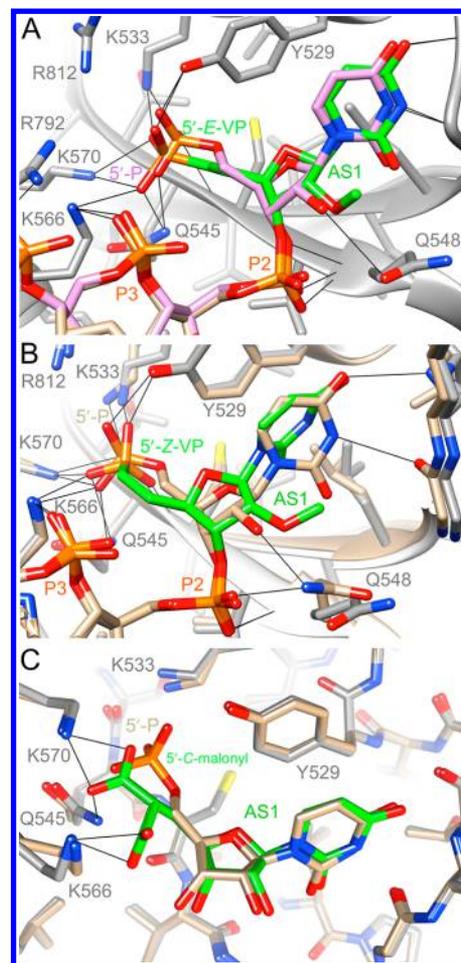


Figure 3. Modifications of the 5'-terminal guide nucleotide (AS1). (A) Crystal structure of 5'-*E*-VP/2'-*O*-Me bound to the Ago2MID domain (PDB ID 5T7B; AS1 and protein carbon atoms/ribbon colored in green and gray, respectively) with the RNA portion of the native complex with a 5'-phosphate overlaid (PDB ID 4F3T; miR20a ribonucleotide carbon atoms colored in pink). (B) 5'-*Z*-VP/2'-*O*-Me molecular mechanics model (AS1 and protein carbon atoms/ribbon colored in green and gray, respectively) overlaid on the native complex with a 5'-phosphate (all carbon atoms colored in tan). (C) Molecular mechanics model of 5'-C-malonyl-modified uridine bound to the separate MID domain (RNA and protein carbon atoms/ribbon colored in green and gray, respectively) and overlaid on the complex with UMP (PDB ID 3LUJ; all carbon atoms colored in tan).

metabolic stability *in vivo*. 5'-Terminal modification is not limited to the guide strand; reduction or prevention of off-target effects via modification of the passenger strand's 5'-end and taking into consideration the spatial and electrostatic properties of the MID domain binding pocket remains an area with room for improvement.⁴⁸

4. AS1/AS2 TURN AND RIBOSE MODIFICATIONS IN THE SEED REGION

RNA is typically associated with the *N*-type C3'-*endo* pucker of the sugar portion. It is therefore noteworthy that the mold provided by the MID domain forces the sugar of the AS1 nucleotide to adopt the DNA-like C2'-*endo* or C1'-*exo* conformations (Figure 4). This constraint prevails irrespective of whether one analyzes the structure of the AS1 nucleotide in the context of a native RNA oligo (Figure 4A,B), in an RNA with a 5'-*E*-VP moiety without (Figure 4C) or with an additional 2'-

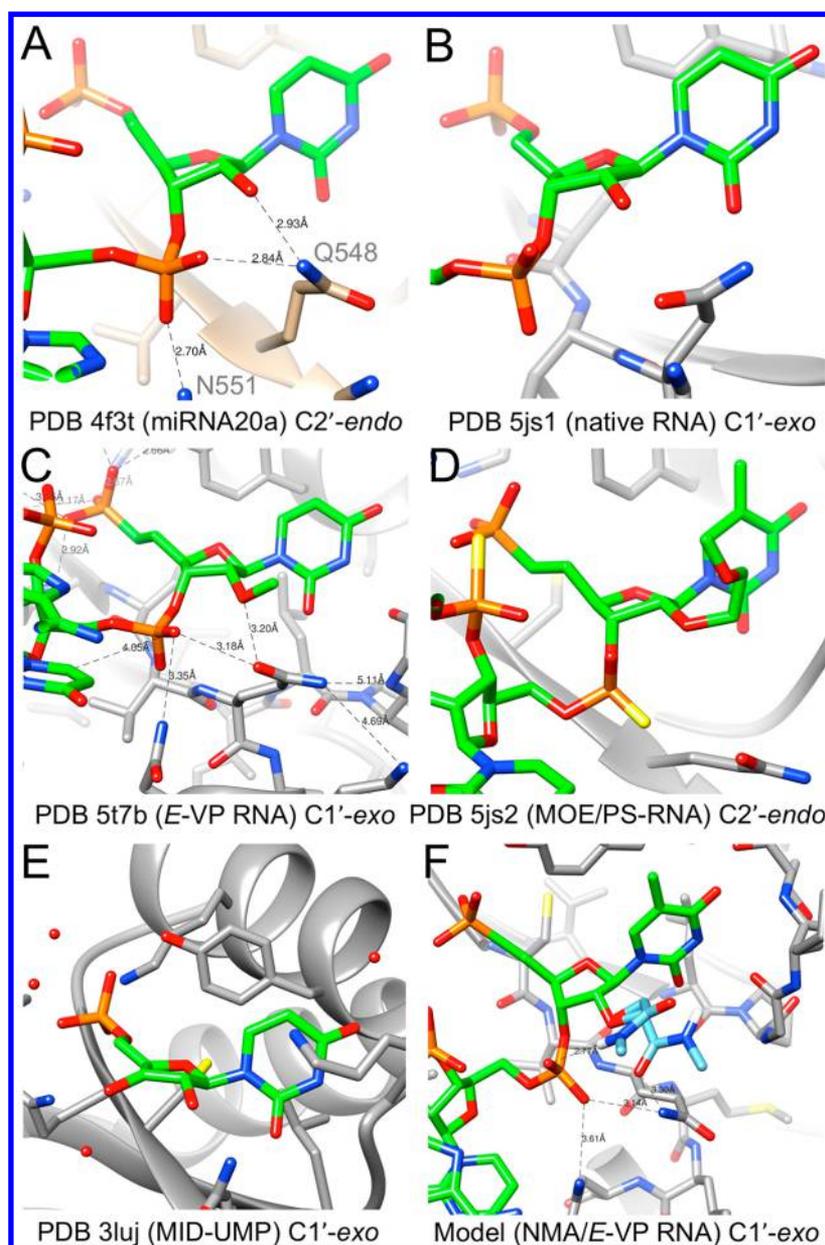


Figure 4. The 5'-terminal siRNA guide strand residue bound to the Ago2 MID domain adopts a South C1'-*exo*/C2'-*endo* DNA-like sugar conformation. Crystal structure of Ago2 bound to (A) miR20a, (B) guide/passenger siRNA strand duplex, (C) 5'-*E*-VP/2'-*O*-Me modified miR20a, and (D) 5'-*E*-VP/2'-*O*-MOE modified miR20a. (E) UMP bound to the Ago2MID domain. (F) Model of 5'-*E*-VP/2'-*O*-NMA modified miR20a bound to Ago2.

O-MOE modification (Figure 4D), or in the monomeric form (UMP, Figure 4E). This observation is consistent with the finding that the first residue of the guide siRNA can be replaced by a deoxy- or an arabinonucleotide as the sugar moieties of both prefer a Southern pucker.⁴⁹ We tested whether a longer 2'-*O*-substituent with the potential of forming H-bonds at the AS1 position yields an activity-enhancing effect. Indeed, the 2'-*O*-[2-(methylamino)-2-oxoethyl]-substituent (2'-*O*-NMA) incorporated into the guide strand in combination with the 5'-*E*-VP moiety improved the activity *in vivo* of an siRNA targeting the *apoB* gene in liver relative to an RNA carrying 5'-*E*-VP alone.⁵⁰ The structural model supports the formation of H-bonds between the NMA substituent and Gln-548 as well as the P2 phosphate, whereby the Southern sugar pucker is once again maintained (Figure 4F). The P2 phosphate located at the tight

turn between AS1 and AS2 became the focus of another investigation into the location-specific consequences of modification for activity. Thus, a neutral amide moiety in place of the negatively charged phosphate is surprisingly well tolerated in the central portion of guide siRNA.³⁰ However, an amide linking AS1 and AS2 triggered a drastic loss in activity and this finding was used to generate a passenger strand with an amide in place of P2 in order to suppress its activity and enhance targeting of the siRNA guide strand.⁵¹

MOE-RNA is an attractive modification that paved the way to the development and FDA approval of the mipomersen and nusinersen therapeutics. The MOE substituent is conformationally preorganized (Figure 5A) and affords enhanced pairing and nuclease stability as well as cellular uptake.²⁹ The substituent adds considerable bulk that does not however pose

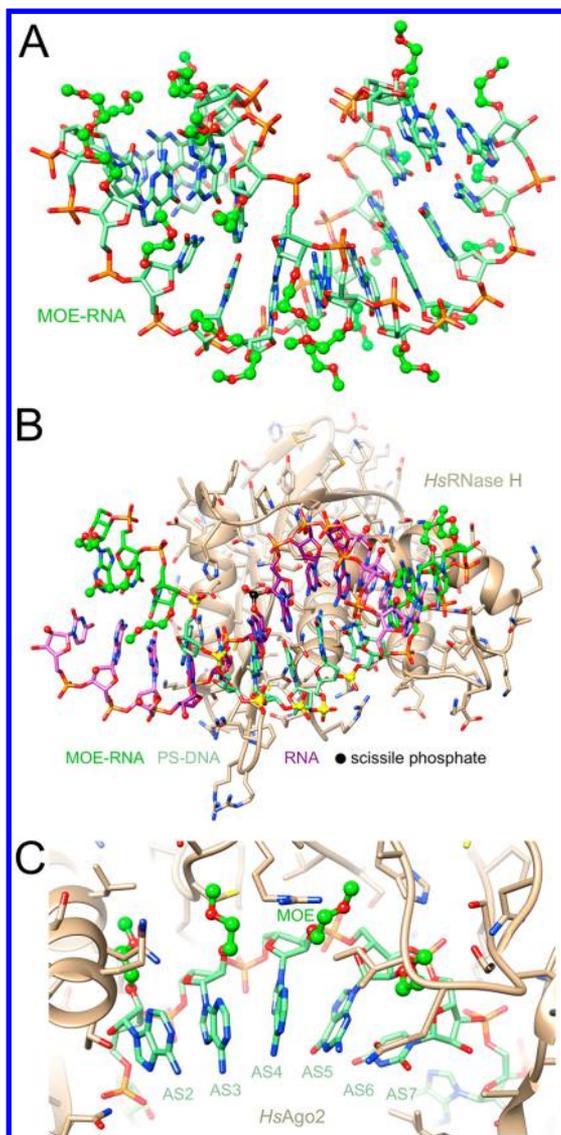


Figure 5. Structure and function of 2'-O-MOE modified RNA. (A) Crystal structure of a MOE-RNA 12mer duplex (PDB ID 468D). (B) Model of a gapmer antisense oligonucleotide with MOE-RNA wings and a central hexamer PS-DNA window opposite RNA bound to RNase H based on the crystal structure of the human enzyme in complex with a 20mer RNA:DNA hybrid (PDB ID 2QKB). 2'-O-MOE substituents are removed from the enzyme active site. (C) Conversely, 2'-O-MOE substituents in the seed region of the siRNA guide strand would clash with Ago2 residues (model with added 2'-O- substituents based on the crystal structure of the complex with miR20a, PDB ID 4F3T).

a problem when the modification is used in the wings of a gapmer with a central PS-DNA window to elicit RNase H (Figure 5B) or in all-MOE oligos that create a steric block to prevent translation or splice modulation. Ago2 tracks siRNAs practically along their entire length, and the space around the majority of riboses appears rather limited. Unlike in the case of RNase H where the spatial constraints are limited to a stretch of six nucleotides around the active site, MOE substituents are too large to be accommodated by Ago2 at most locations in an siRNA. For example, MOE is tolerated at AS1 (Figure 4D) but will cause clashes in the seed region (Figure 5C).

Structural data allow a refined understanding of the consequences for activity of modifications and future predictions as to the usefulness of certain modifications at

specific locations. Ribose substituents need not be as bulky as MOE to strain the spatial constraints set by Ago2. For example, a deoxyribose sugar or a 2'-fluorine substituent is acceptable at AS2 (Figure 6A), but a 2'-O-methyl group in the second nucleotide of the guide strand would cause short contacts with amino acids from an adjacent α -helical turn (Figure 6B). A case in point is the drug patisiran that features a ribonucleotide at AS2 but a 2'-OMe residue at S2.⁵ Similarly, 4'-C α -OMe-modified nucleotides are quite well tolerated at various locations in the guide and passenger strands.^{43,52} However, the methoxy substituent at AS4 clashes with Leu-563 (Figure 6C), whereas the 4'-C α -Me substituent can be well accommodated at the same site (Figure 6D), consistent with a significantly more favorable IC₅₀ value for the latter modification.⁵²

5. AS6/AS7 KINK AND FLEXIBLE AND STERICALLY CHALLENGING MODIFICATIONS

Close inspection of the seed region in Ago2:RNA complexes reveals a roll-bend between AS6 and AS7 in the structure with a bound S:AS duplex⁴⁴ (Figure 7A) and a more pronounced kink in the structure with miR-20a.⁴⁵ Leu-365 acts as a wedge from the minor groove side, and bending results in locally unstacked bases and tight spacing between phosphates. We hypothesized that incorporation of an analogue like glycol nucleic acid (GNA, Figure 1) that features a backbone that is shorter by an atom relative to RNA and lacks a cyclic sugar moiety might be well tolerated at the site of the bend and potentially result in improved activity. Crystal structures of RNA oligos with incorporated (S)- or (R)-GNA-Ts reveal a remarkable range of conformations that the analogue can adopt inside an A-form duplex. Thus, P–P distances vary by over 1 Å and can be as short as 4.8 Å, the sugar of the ribonucleotide preceding GNA can alter its pucker, and the GNA thymine flips and forms a reverse Watson–Crick pair with adenine from the opposite strand (Figure 7B).⁴¹ Also, the structural data explained why (S)-GNA oligos pair with RNA but (R)-GNA oligos cannot. A complete scan of an anti-TTR siRNA duplex by walking an (S)-GNA residue along the entire length of the guide and passenger strands demonstrated favorable activities of guides with GNA at positions AS6 or AS7 compared to the parent strand (Figure 7C). A similar observation was made when the corresponding base pairs were replaced with (S)-GNA (see conspectus graphic). We can rationalize these effects by the intrinsically shorter P–P distances at GNA incorporation sites that match those seen at the AS6/AS7 kink (Figure 7A,D) and the conformational plasticity of GNA that is expected to facilitate bending. Whereas (S)-GNA constitutes a good fit in terms of the kink in the seed region, (R)-GNA is not tolerated and another modification that features a noncanonical backbone, 4'-C β -OMe-U, with an inverted stereochemistry at C4' is not a good match either for the particular conformation adopted by AS6.⁵² Acyclic nucleotide analogues lacking a shorter backbone, so-called unlocked nucleotides (UNA), were previously found to exhibit similar favorable pharmacological properties in the contexts of antisense⁵³ and RNAi⁵⁴ applications.

6. Ago2 PIWI AND MODIFICATIONS ADJACENT TO THE CENTRAL CLEAVAGE SITE

Inspection of human Ago2 complex crystal structures reveals that the central (including the scissile phosphate) and supplementary siRNA regions are less well-defined than the 5'-terminal, seed, and 3'-terminal regions (Figure 2A). An

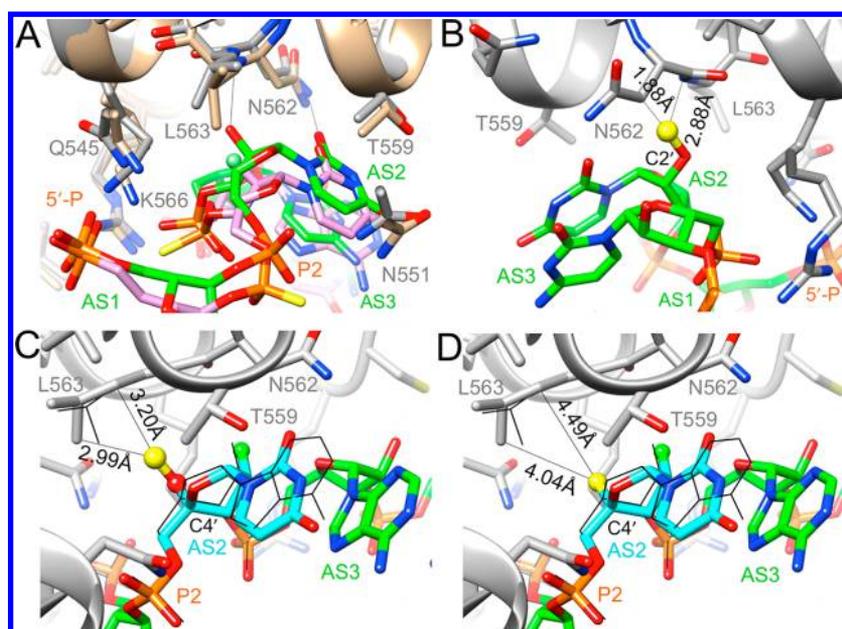


Figure 6. AS2 modifications. (A) Superimposition of the Ago2 complexes with native (green/gray carbon atoms; PDB ID 4WST) and chemically modified guide RNA (pink/tan carbon atoms; PDB ID 5JS2). The 2'-F modification is tolerated at AS2, although it disrupts the H-bond between the 2'-hydroxyl group and the main chain nitrogen of L563. (B) Conversely, the model of a 2'-O-Me modified AS2 shows that the methyl group disrupts the H-bond and causes a clash. (C) The methyl group of 2'-F,4'-C α -OMe U in place of the AS2 residue (thin solid lines) results in short contacts that cannot be removed by energy minimization. (D) Conversely, one can expect the 2'-F,4'-C α -Me modification to be tolerated at AS2.

additional limitation of the structural data is that the RNA strands are not properly engaged at the Ago2 active site and that Mg²⁺ ions are absent (the enzyme uses a dual metal ion mechanism for phosphodiester hydrolysis). Thus, an overlay of the PIWI domains of RNase H (in complex with an RNA:DNA hybrid) and of Ago2 (in complex with an siRNA duplex) reveals markedly different orientations of the two duplexes relative to the respective active sites (Figure 8A). In the RNase H complex, the scissile phosphate is tightly bound between metal ions A and B with a nearby water molecule poised for attack. In the Ago2 complex, only nucleotides S12–S20 of the passenger (target) strand are visible and the supplementary region of the guide strand is essentially missing (Figure 2A). In some complexes with a duplex bound to Ago2 from other organisms, e.g., the enzyme from *T. thermophilus*, the passenger strand sits in close proximity of active site Asp and Glu side chains, but no metal ions are seen (Figure 8B). The overlay of the human and *T. thermophilus* Ago2 complexes displays a good fit of active site residues but deviating orientations of siRNAs, with the portion of the passenger strand defined in the former stopping short of the active site and a loop blocking its way (Figure 8C). These comparisons make clear that the conformations of the human Ago2 enzyme trapped in the structures of complexes have not captured a state that is conducive to cleavage and that multiple loops have to move in order to accommodate guide/target strand duplex in a productive orientation.⁵⁵ Unfortunately, this means that the insights to be gained from the available structural data in regard to a better understanding of the effects on activity of modifications in the central siRNA region as well as clues for the design and placement of new modifications at those sites are rather limited.

What is evident from the structures is that both guide and passenger strands are tracked by Ago2 Arg and Lys residues (e.g., Figure 8B). Therefore, it may initially come as a surprise that siRNA guide strands with neutral amide linkages instead of

phosphate in the central region and opposite the scissile phosphate are well tolerated and actually display higher activity than the native reference RNA.³¹ A comparison of the crystal structure of the native RNase H-DNA:RNA complex with one in which the RNA carries an amide adjacent to the scissile phosphate demonstrated that both amide and phosphate form two H-bonds to the same serine. This study indicated that amide is a useful placeholder of phosphate^{56,57} despite lacking the negative charge of the latter. Among sugar modifications, it appears that 2'-F nucleotides are tolerated at the Ago2 cleavage site.⁵⁸ And in an earlier investigation, we found that the ribodifluorotoluy nucleotide (DFT, T analogue with fluorine instead of O2 and O4) could replace uridine in the guide strand opposite A in the passenger strand, right adjacent to the cleavage site, without affecting the RNAi activity to a significant extent.^{59,60} However, base mismatches including the DFT:G and U:G pairs were not tolerated next to the scissile phosphate. This indicates that there are clear limits to the conformational changes that are tolerated inside the central portion of the siRNA duplex. Another finding that supports this conclusion concerns the relative activities of siRNA duplexes featuring passenger strands with either 2'-F,4'-C α -OMe-U or 2'-F,4'-C β -OMe-U (opposite stereochemistry at C4') at position S11, next to the scissile phosphate. Thus, the siRNA constructs targeting *Ttr* mRNA exhibited IC₅₀ values of 0.06 and 1.83 nM, respectively (parent duplex: 0.08 nM).⁵²

7. Ago2 PAZ AND MODIFICATION OF 3'-TERMINAL GUIDE-STRAND NUCLEOTIDES

The 3'-ends of siRNA duplexes have dinucleotide overhangs, and the guide strand 3'-terminal residues are captured by the PAZ domain (Figure 2A,E). An interaction between PAZ and the 3'-overhanging nucleotides of the passenger strand would result in misincorporation but can be avoided in principle by using an siRNA duplex construct with a blunt end on one side

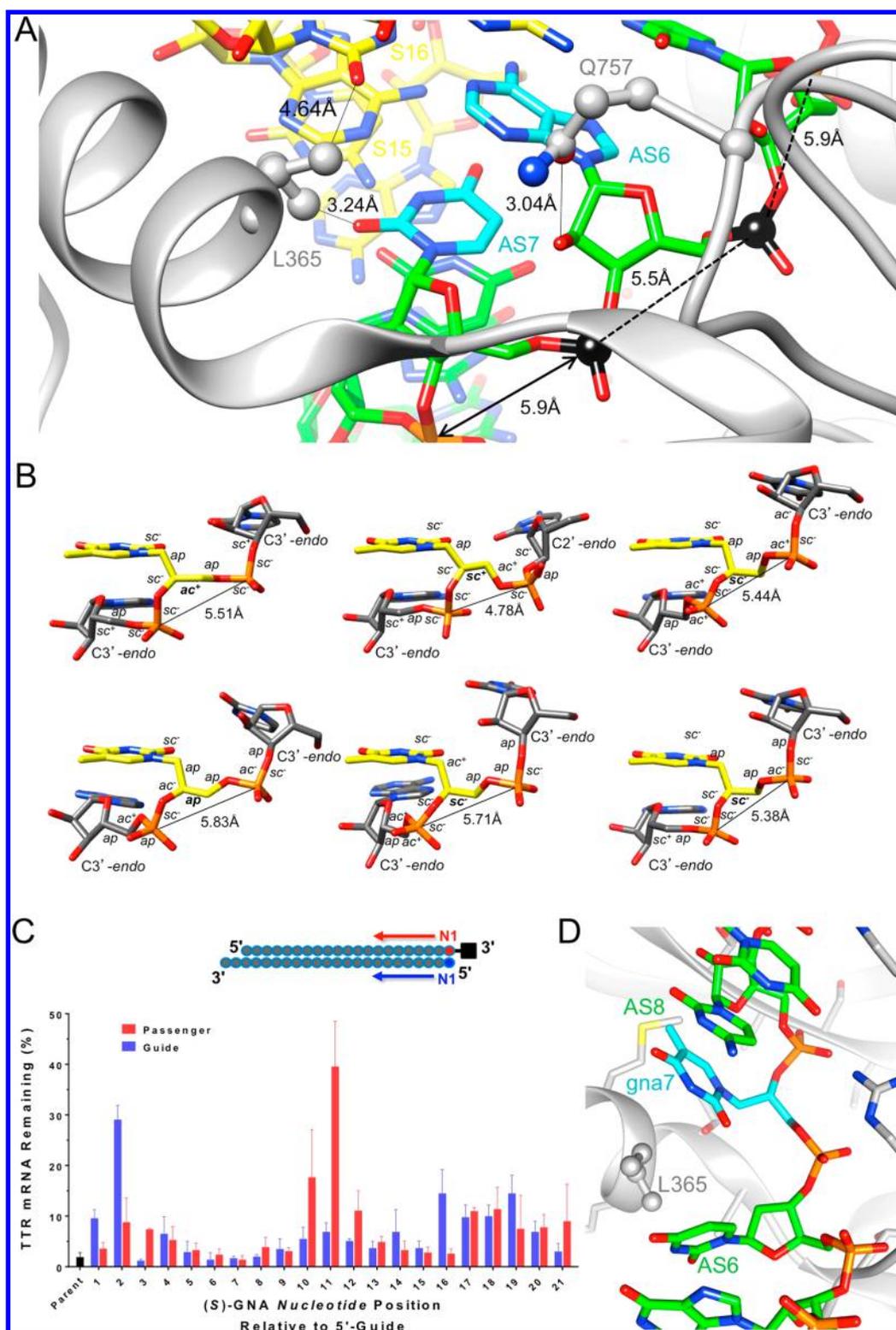


Figure 7. Activity and structure of GNA-modified siRNA. (A) Roll-bend between base pairs AS6:S16 and AS7:S15 of an siRNA duplex bound to Ago2 (PDB ID 4W5T) with concomitant shortening of the local phosphate–phosphate distance. (B) Conformational properties of (S)-GNA-T residues (yellow carbons) in the crystal structure of a modified RNA dodecamer (PDB ID 5VIL). (C) Activity scan using (S)-GNA nucleotides along the entire sense and antisense siRNA strands against mouse TTR mRNA. (D) A model of (S)-GNA-T (cyan carbon atoms) based on the crystal structure of modified RNA incorporated into the AS strand shows a seamless fit at the kink between residues 6 and 7 of siRNA bound to Ago 2 (PDB ID 4F3T).

(Figure 7C). Just like the 5'-end of the guide strand that sits in the MID domain and is well separated from the 3'-terminus of the passenger strand, the 3'-end of the guide is segregated from passenger strand nucleotides. This binding mode clearly serves

the separation of the two once the siRNA duplex is loaded into Ago2 and thus the subsequent accommodation of the target strand opposite guide siRNA. Tight binding of the 3'-terminal overhang of the guide in the PAZ pocket is expected to facilitate

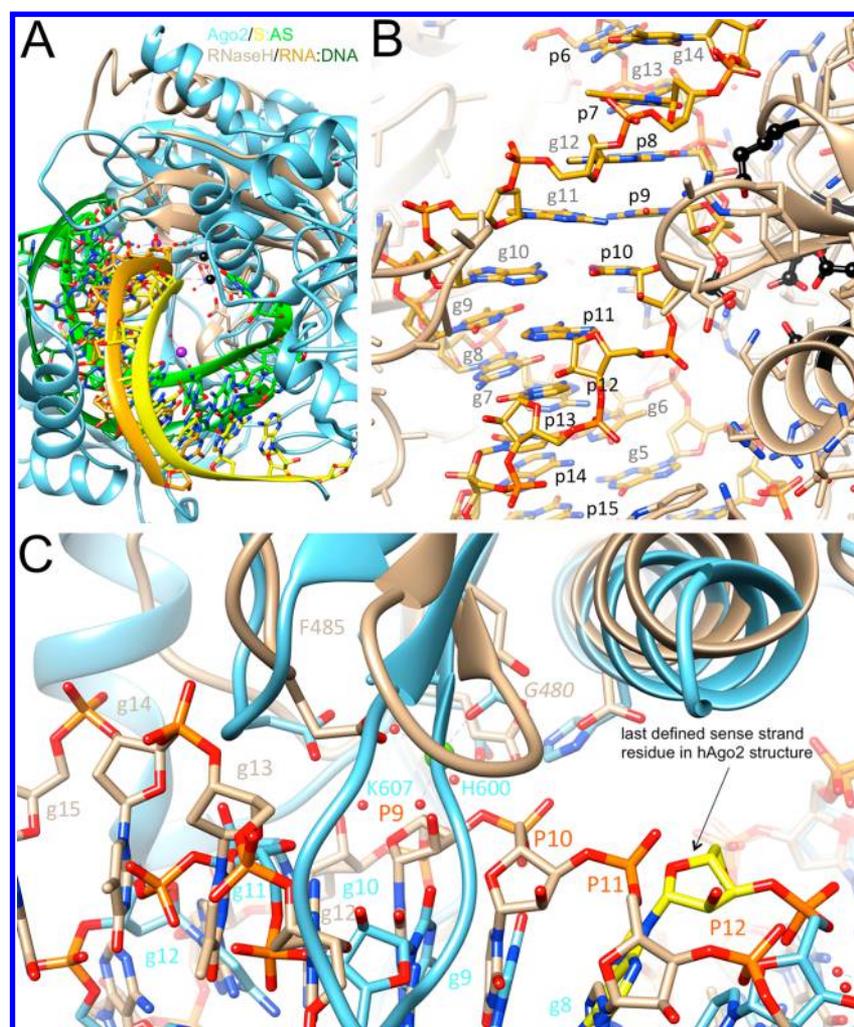


Figure 8. Cleavage region and interactions with the PIWI domain. (A) Superimposition of the PIWI domain of the Ago2:siRNA complex (PDB ID 4WST) with the complex between *B. halodurans* RNase H (PIWI-like fold) bound to a DNA:RNA hybrid (PDB ID 1ZBI). Active site magnesium ions in the latter structure are black spheres. (B) Interactions of *T. thermophilus* Ago2 with a DNA(S=p):RNA(AS=g) duplex (PDB ID 3HJF), revealing extensive interactions between Lys and Arg residues and the sense strand. Scissile phosphate and carbon atoms of active site residues (Asp-Glu-Glu-Asp) are highlighted as black spheres. (C) Superimposition of the hAgo2 (Asp-Glu-Asp-His active site) and TtAgo2 complexes, illustrating the H600–K607 loop in the former blocks the path of the sense strand.

strand separation and exert a favorable influence on activity.⁶¹ Alterations to the sugar moiety at the last two residues and the backbone–base distance are only of moderate influence, but lack of the nucleobase diminishes binding, according to a comparison of IC₅₀ values from an *in vitro* silencing study using various modifications. The binding mode involves the two overhanging nucleotides, and particularly the last phosphate is tightly bound (Figure 9A,B). Beyond the overhang, RNA strands adopt strikingly different orientations in complexes with Ago/Piwi protein PAZ domains (Figure 9A).^{44,45,62,63} Dissociation constants K_d measured for nucleotide monophosphates and dinucleotides range from 10 to 60 μM .⁶⁴ We used electrophoretic mobility shift assays and isothermal titration calorimetry with single and double stranded siRNAs and found K_d values as low as 500 nM.⁶⁵ 2'-O-Methylation (Figure 9B) or the use of deoxyribonucleotides (e.g., patisiran) in the guide strand overhang does not significantly alter binding mode, stability, or activity.

Among the modifications tested thus far for nucleotides at the 3'-end of the guide strand, none appear to significantly enhance binding. Conversely, phosphorodithioate (PS2, Figure 1A)

moieties in place of the third- and second-last phosphates at the 3'-end of the passenger strand led to strong gains in Ago2 affinity (Figure 10).⁶⁶ This effect appears to require a hydrophobic patch on the surface of the protein, sufficient local conformational flexibility of the RNA backbone to allow the PS2 moiety to flex and engage in energetically favorable contacts with the hydrophobic region, aided by an electric field that is generated by adjacent arginine and lysine side chains and polarizes the sulfur atoms. This model is supported by experimental structural data for a PS2-modified RNA-aptamer:thrombin complex and QM and QM/MM model system calculations that confirm the important role of the polarization and dispersion terms in the dramatically increased binding affinity (ref 67 and cited references).

8. CONCLUDING REMARKS

Chemical modification is key to improving pairing affinity, metabolic stability, and cellular uptake of RNA and absolutely essential in the discovery and development of highly active siRNA therapeutic candidates. 3D-structural data of oligonucleotides allow one to visualize the effects of modifications on

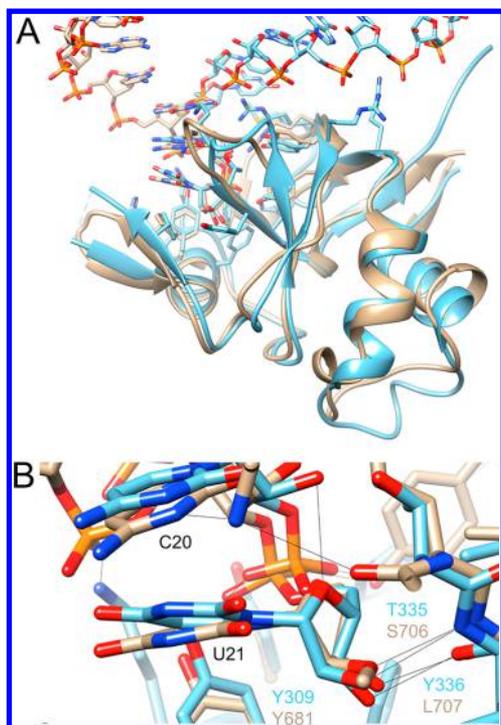


Figure 9. PAZ domain interactions and modifications of the 3'-terminal guide strand nucleotide. (A) Overlay of human Ago1 (PDB ID 1SI3; light blue) and *Drosophila melanogaster* Ago2 (PDB ID 3MJ0; tan) PAZ domain:RNA complexes, illustrating deviating conformations of the RNA strands beyond the 3'-terminal C and U nucleotides (2'-OMe-U in the *Dm*Ago2 PAZ complex). (B) Close-up view of the binding site of the 3'-terminal nucleotide in the two complexes. H-bonds are indicated with thin solid lines and are unaffected by 2'-OMe modification of the last residue.

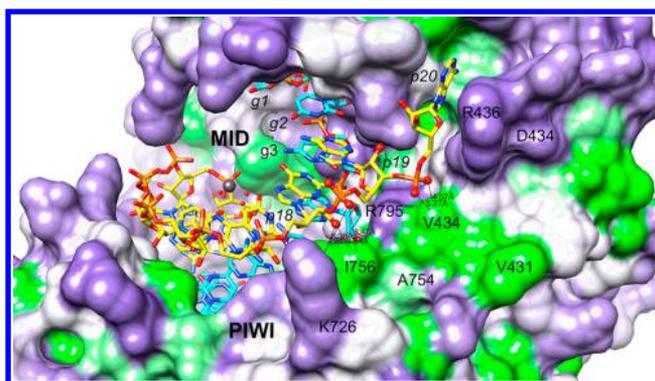


Figure 10. View of the guide (g, cyan carbon atoms) and passenger (p, yellow carbon atoms) siRNA strands lodged in the Ago2MID and PIWI domains based on the crystal structure of the complex with PDB ID 4W5T. The Ago2 surface is colored according to hydrophobicity potential: purple, lowest; white, 0; green, highest. Selected amino acids are labeled, and all distances are in Å. The 3'-penultimate phosphate group in the sense strand (phosphate of p20) and the phosphate of the adjacent residue (p19) are hovering above a hydrophobic patch (Val-431, Val-434, Ala-754, and Ile-756) that is surrounded by basic amino acids (Arg-436, Lys-726, and Arg-795). Replacement of these two phosphates by dithiophosphate groups boosts the binding affinity between the siRNA duplex and Ago2 by more than 4 orders of magnitude from ca. 0.6 μ M to 12.4 pM.⁶⁶

conformation and are helpful for interpreting their role in modulating pairing stability. However, such data are insufficient

to understand how modifications affect RNA–protein interactions. Similarly, structures of proteins bound to native RNA offer only limited insights into the altered activity of chemically modified siRNAs. A case in point is Ago2, an enzyme that is absolutely essential in the RNAi pathway and that we have focused on here to discuss the potential benefits of structural information in conjunction with modeling for gaining insight into the interplay of siRNA modification and activity and harnessing this knowledge for designing future RNAi therapeutics. Inspection of the available Ago2 structures and walking along the siRNA in a 5' to 3' direction from the MID and past the PIWI to the PAZ domain offers a treasure trove of detailed protein–RNA contacts (MID, PAZ) but also lays bare regions (e.g., central and supplementary portions of AS and S siRNAs) that provide no or only limited guidance in interpreting the effects of modifications on affinity and activity or their improved design. Moreover, there currently exists a void in terms of structures of complexes between Ago2 or separate domains and modified RNAs. Because Ago2 is the central player in siRNA duplex unwinding, targeting, and target destruction and local features in Ago2–siRNA complex structures and changes in siRNA activity triggered by (regio-) specific chemical modification can be correlated in many cases (e.g., for the seed region), addressing this void is absolutely critical for the discovery of high-activity RNAi therapeutics.

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: martin.egli@vanderbilt.edu.

*E-mail: mmanoharan@alnylam.com.

ORCID

Martin Egli: 0000-0003-4145-356X

Muthiah Manoharan: 0000-0002-7931-1172

Notes

The authors declare the following competing financial interest(s): M.M. is an employee of Alnylam Pharmaceuticals Inc.

Biographies

Martin Egli is Professor in the Department of Biochemistry at Vanderbilt University, Nashville, Tennessee, USA. He received his diploma and Dr. sc. nat. in chemistry from ETH–Zürich, Switzerland.

Muthiah Manoharan is Senior Vice President of Drug Discovery at Alnylam Pharmaceuticals Inc., Cambridge, Massachusetts, USA. He received his B.Sc. (Special) and M.Sc. in chemistry from American College in Madurai, India, and his Ph.D. in chemistry from the University of North Carolina, Chapel Hill.

■ ACKNOWLEDGMENTS

We dedicate our 25 years of collaborative work to the memory of our mentors, Professors Ernest L. Eliel, Vladimir Prelog, and Alex Rich, for their teachings in the field of structural chemistry. We thank our colleagues M. A. Maier, K. G. Rajeev, K. Charisse, S. Matsuda, I. Zlatev, A. Ke'lin, M. Schlegel, R. G. Parmar, V. Jadhav, P. S. Pallan, L. Lei, J. Harp, T. P. Prakash, X. Yang, P. D. Cook, and M. J. Damha for their valuable contributions and discussions pertaining to the various chemistries described in this account.

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