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Understanding the Effect of Locked Nucleic Acid and 2′-*O*-Methyl Modification on the Hybridization Thermodynamics of a miRNA−mRNA Pair in the Presence and Absence of AfPiwi Protein

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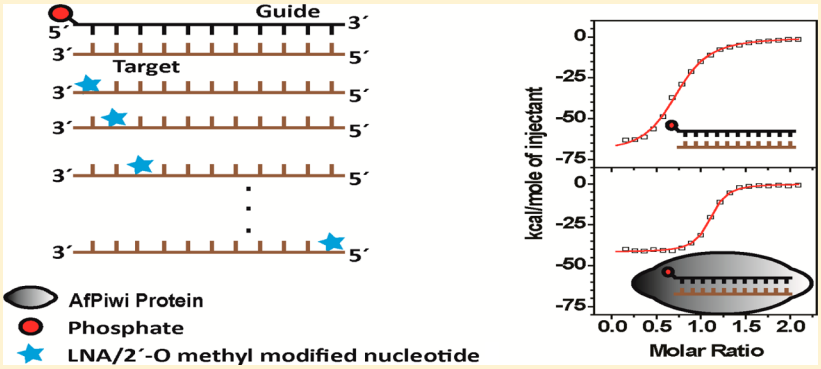
Supporting Information



ABSTRACT: miRNAs are some of the key epigenetic regulators of gene expression. They act through hybridization with their target mRNA and modulate the level of respective proteins via different mechanisms. Various cancer conditions are known to be associated with up- and downregulation of the oncogenic and tumor suppressor miRNAs, respectively. The levels of aberrantly expressed oncogenic miRNAs can be downregulated in different ways. Similarly, restoration of tumor suppressor miRNAs to their normal levels can be achieved using miRNA mimics. However, the use of miRNA mimics is limited by their reduced biostability and function. We have studied the hybridization thermodynamics of the miRNA 26a (11-mer, including the seed sequence) guide strand with the mRNA (11-mer) target strand in the absence and presence of AfPiwi protein. We have also inserted locked nucleic acids (LNAs) and 2′-*O*-methyl-modified nucleotides into the guide strand, in a walk-through manner, to assess their effect on the binding efficiency between guide and target RNA. Insertion of LNA and 2′-*O*-methyl-modified nucleotides into the guide strand helped to strengthen the binding affinity irrespective of the position of insertion. However, in the presence of AfPiwi protein, these modifications reduced the binding affinity to different extents depending on the position of insertion. Insertion of a modification leads to an increase in the enthalpic contribution with an increased unfavorable entropic contribution, which negatively compensates for the higher favorable enthalpy.

RNA silencing is one of the important mechanisms for the regulation of gene expression at the post-transcriptional level.[1,2] This process is strongly mediated by small, single-stranded RNA (miRNA), which modulates the levels of their target protein by mRNA degradation or translational inhibition.[3−5] Several diseases have been found to be associated with deregulation of the miRNA levels.[6,7] A change in the intracellular levels of these miRNAs led to the aberrant production of proteins and eventually a disease condition.[8−12]

Depending on their role in tumorigenesis or tumor suppression, miRNAs are divided into two types, oncogenic miRNAs (oncomirs) and tumor suppressive miRNAs. The oncomir family of miRNAs includes those miRNAs that can regulate cancer-related processes such as cell growth and tissue differentiation, and their upregulation leads to a cancer condition.[13] Aberrant levels of miRNA-26 have been shown to occur in many tumors and are known to perform specific functions.[14−16] On the other hand, tumor suppressive miRNAs regulate the expression level of tumor suppressor genes, and their downregulation causes various problems. In colorectal cancer, the level of tumor suppressive miRNAs miRNA-143 and -145 is reduced significantly.[17] More importantly, only the levels of mature miRNA were found to be depleted, and the precursor levels remained unaffected in normal versus tumor tissues, thus indicating interruption in the miRNA processing pathway.[18]

miRNAs are not the only players involved in this whole process; it requires many accessory proteins to perform these functions. One of the important families of proteins, Argonaute,

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is essential for the recognition of target mRNA.[19,20] These proteins trigger nucleation of the mRNA binding event through the RISC complex guided by the seed sequence of miRNA. Human Argonaute proteins contain three domains, PAZ, Middle, and PIWI, each assigned to a different function during miRNA recognition and RISC assembly. The 3′-end of miRNA interacts with the PAZ domain, with the last two nucleotides sitting in the cleft of the PAZ domain, whereas the Middle domain confers the 5′-phosphate recognition of miRNA with a specific nucleotide preference.[21−23] On the other hand, the PIWI domain contains an RNase H-like pocket that catalyzes the cleavage of the nucleic acid.[24] Recently, the crystal structure of human Argonaute protein showed that miRNA makes contact with every domain of the protein, including the linkers. These contacts are primarily mediated through the phosphate backbone of the miRNA.[25,26] This study also shows that the three nonbridging oxygen atoms present at the 5′-phosphate are extensively bonded to different conserved amino acids of *Homo sapiens* Argonaute2 (Hs-Ago2) protein. Of eight amino acids that are bonded to the 5′-phosphate group, four are highly conserved (Hs-Ago2-Q545, Hs-Ago2-K570, Hs-Ago2-Y529, and Hs-Ago2-K533) among different subfamilies of PIWI and Ago protein. However, other interactions of the seed sequence with Hs-Ago2 are neither very specific nor as strong as that of the 5′-phosphate.[26]

Analyses of different Ago family proteins reveal a high degree of structural similarity at the domain level with almost identical active sites.[26] We have chosen PIWI/MID domain protein AfPiwi from *Archaeoglobus fulgidus*, which is an archeal homologue of Ago protein, as a model protein for our study.[27,28] As shown earlier, AfPiwi can be used to study the interaction of the 5′-end of the miRNA with its target mRNA.[29]

There are many studies showing the importance of miRNA mimics as therapeutics against various disease conditions at the cellular[30] and systemic level.[31] These mimics can be used in the form of a single strand and a double strand.[32,33] Although these mimics have been successfully used against many diseases, their biostability and functionality are still major challenges. Various nucleotide modifications have been known to enhance the biostability and affinity of the oligonucleotide for their target *in cellulo* and *in vivo*. Insertion of LNA and 2′-*O*-methyl-modified nucleotides is known to enhance the hybridization efficiency and *in vivo* stability of the oligonucleotides.[34,35] These modifications have been shown to enhance the resistance of the DNA−RNA oligonucleotides against various nucleases in a position-dependent manner.[36,37] The chemistries of both modifications are entirely different from each other and those of unmodified nucleotides, so these modifications could either enhance the binding through positive interactions with the target strand or hamper formation of the ternary complex by interfering with the binding of AfPiwi to the miRNA. As seen from the model of PIWI−RNA interaction, generated on the basis of the crystal structure of the PIWI domain of AfPiwi and the crystal structure of a 21-nucleotide RNA duplex bound to p19 protein,[38] AfPiwi protein makes differential contacts with every residue of the mature miRNA, so it is possible that different modifications would exhibit a diverse range of effects on ternary complex formation. These modified nucleotides can also be used in miRNA mimics to enhance their biostability and binding. However, these nucleotides cannot be inserted randomly into any position in the miRNA mimics.

Here we have taken an 11-nucleotide sequence from the 5′-end of miRNA-26a, including the seed sequence for mRNA recognition. The complete complementary sequence was taken as the target RNA (11-mer). This length was chosen on the basis of the previous studies in which AfPiwi protein was shown to enhance the binding affinity of the miRNA seed sequence to target RNA in a position-dependent manner and the protein spans 11 nucleotides of the miRNA.[29] We have inserted LNA and 2′-*O*-methyl-modified nucleotides (molecular structures shown in Figure 1) in a walk-through manner, i.e., one modification at a time in the guide sequence. The terminal nucleotide at the 5′-end was phosphorylated in the guide sequence and all the sequences containing a modified nucleotide. Isothermal titration calorimetry (ITC) experiments were performed to understand the effect of insertion of LNA and 2′-*O*-methyl-modified nucleotides on the binding affinity and thermodynamics of binding of the guide strand of miRNA and its target RNA. ITC experiments were performed in the absence and presence of AfPiwi protein to assess the positions at which these modifications can be tolerated.

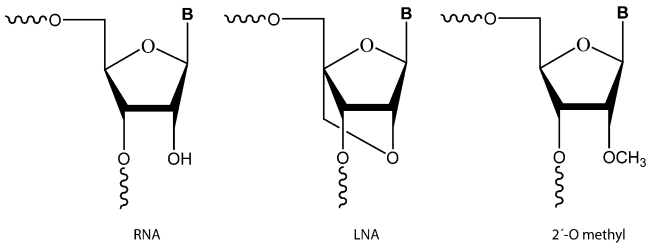

Figure 1. Chemical structures of the RNA, LNA, and 2′-*O*-methyl monomers. RNA contains a -OH group 2′-carbon atom. In LNA, the 2′-OH is attached to the 5′-carbon atom through the CH_2 group. In the case of 2′-*O*-methyl modification, the 2′-hydroxyl group is methylated.

MATERIALS AND METHODS

The guide strand of miRNA [5′-UUCAAGUAAUC-3′ (5′-end phosphorylated)] and target mRNA (5′-GAUUACUUGAA-3′) were purchased from Eurogentec at high-performance liquid chromatography (HPLC) purity. All HPLC-purified LNA and 2′-*O*-methyl-modified oligonucleotides (containing the 5′-end phosphate) were also purchased from Eurogentec. These modified nucleotides were inserted into the guide sequence in a walk-through manner, i.e., one modified nucleotide in one sequence. Their concentration was measured by collecting the absorbance at 260 nm using a Cary 100 UV−vis spectrophotometer. All the melting experiments were also performed on the same instrument by monitoring the absorbance change at 260 nm and keeping the heating rate at 0.5 °C/min. The extinction coefficients of the guide and target strand, including the oligonucleotides containing LNA and 2′-*O*-methyl-modified nucleotides, are listed in Table 1. AfPiwi cloned in modified pET 17b (Novagen) containing a His_6 tag at the N-terminus with a PreScission protease recognition site was a kind gift from D. Barford's lab (The Institute of Cancer Research, Surrey, U.K.).

Protein Expression and Purification. The plasmid containing the AfPiwi open reading frame (ORF) was transformed into *Escherichia coli* strain BL21(DE3), and expression was conducted for 4 h at 37 °C. Protein expression was induced by addition of 1 mM isopropyl β-D-1-thiogalactopyranoside (purchased from Sigma-Aldrich) to the growing amount of broth in the presence of 100 μg/mL

Table 1. Sequences of Guide RNA and Different Modified Guide Strands and Their Molar Extinction Coefficients

oligonucleotide	sequence	extinction coefficient (ε) (M^{-1} cm^{-1}) at 25 °C	
target	5′-GAUUACUUGAA	118300	
guide (0)	5′-UUCAAGUAAUC	118300	
		ε for LNA oligos	ε for 2′-*O*-methyl oligos
1	5′-**U**UCAAGUAAUC	113200	114600
2	5′-U**U**CAAGUAAUC	113900	114600
3	5′-UU**C**AAGUAAUC	114000	114600
4	5′-UUC**A**AGUAAUC	114600	114600
5	5′-UUCA**A**GUAAUC	114600	114600
6	5′-UUCAA**G**UAAUC	114000	114600
7	5′-UUCAAG**U**AAUC	114600	114600
8	5′-UUCAAGU**A**AUC	114600	114600
9	5′-UUCAAGUA**A**UC	114700	114600
10	5′-UUCAAGUAA**U**C	114600	114600
11	5′-UUCAAGUAAU**C**	118300	114600

ampicillin (purchased from Sigma-Aldrich) when the absorbance of the growth medium reached 0.6 OD unit. The protein was purified using Ni-NTA beads (purchased from Sigma-Aldrich) and finally running it on a Superdex 200 10/300 GL column (GE-healthcare life sciences). Additionally, benzonase nuclease (purchased from Sigma-Aldrich) was added to the lysis buffer and incubated at 70 °C for 30 min. We have used the absorbance value at 280 nm to calculate the molar concentration of AfPiwi using a value of 82740 M^{-1} cm^{-1} as the molar extinction coefficient (at 280 nm) (http://web.expasy.org/protparam/). All the measurements were taken using a Cary 100 (Varian) spectrophotometer.

Isothermal Titration Calorimetry. ITC experiments were performed using the 11-mer guide strand of the miRNA at a concentration of 1 μM in an ITC cell, and a 25 μM solution of 11-mer target mRNA was added via syringe. All the ITC experiments were performed using a Microcal VP-ITC (Microcal, Inc., Northampton, MA) instrument. The temperature of the cell was kept at 37 °C, and the reference power of the cell was fixed at either 5 or 10 μcal/s. The volume of each injection was 6 μL, except for the first injection, which had a volume of 3 μL, and the stirring speed was kept constant at 307 rpm. The spacing between each injection was kept at 180 s, which is typical for binding experiments in the presence of the AfPiwi protein, and the preformed complex of AfPiwi with the guide strand was kept in the ITC cell and target RNA added via syringe. The protein concentration was taken in a 5-fold excess versus that of guide strand RNA to ensure the full association of RNA with protein. Each experiment of this type was accompanied by a control experiment in which target RNA was titrated only in buffer and the heat of dilution was measured. All the experiments were performed in 10 mM Tris buffer containing 150 mM KCl, 1 mM DTT, and 5% glycerol (pH 7.5). Each heat burst was integrated using Origin version 7.0 to calculate the area under the curve that gives the heat changes associated with each injection. The binding isotherm obtained from interaction was then fit using a model of one set of binding sites[39] to give binding stoichiometry n along with binding affinity K_a and enthalpy change ΔH.

RESULTS

Binding of the Guide Strand to Target RNA in the Absence of AfPiwi Protein. In this study, we have shown the effects of incorporation of modified nucleotides on the parameters for binding of the miRNA seed sequence to the mRNA target sequence in the presence and absence of AfPiwi protein. We chose LNA and 2′-*O*-methyl-modified nucleotides (structures shown in Figure 1) and introduced them into the guide sequence in a walk-through manner, one modification per sequence. The sequences of the modified nucleotide containing the guide strand along with their positions and molar extinction coefficient are listed in Table 1. The 11-mer seed sequence of miRNA-26a was titrated with its complementary target sequence, and the binding affinities along with thermodynamic parameters were calculated. We performed UV melting experiments with the guide and target RNA duplex in 10 mM Tris buffer containing 150 mM KCl, 1 mM DTT, and 5% glycerol (pH 7.5). The melting temperature (T_m) of the unmodified guide strand with target RNA was determined to be 46.5 °C. Similarly, melting experiments were performed to determine the effect of incorporation of LNA and 2′-*O*-methyl-modified nucleotides on the stability of the duplex. They showed variable stabilization ranging from 0.1 to 4 °C (data not shown). All the ITC titration experiments were performed at 37 °C, as the miRNA−mRNA pair was able to form duplex at this temperature [shown in the UV melting profile (Figure 2)].

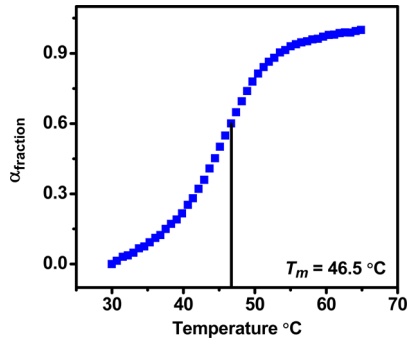

Figure 2. UV melting profile of the 11-mer guide strand with its complementary sequence 11-mer target RNA strand. The buffer consisted of 10 mM Tris containing 150 mM KCl, 1 mM DTT, and 5% glycerol (pH 7.5).

We also tried to conduct ITC titrations at 25 °C. However, at this temperature, the binding affinity exceeded the limit of the ITC instrument at which it can measure accurately. Figure 3a represents the ITC titration profile of the guide strand with the target RNA strand in the absence of AfPiwi protein. The binding isotherm was fit with the model of one set of binding sites,[39] to calculate the binding parameters. The binding of the guide strand with target RNA showed a binding stoichiometry n of 0.9 with a binding affinity K_a of 2.0×10^7 M^{-1}. The binding enthalpy of the guide and target hybridization was highly exothermic with a ΔH of −70.4 kcal/mol (Table 2). However, this enthalpy change seems to be significantly different from the enthalpy change calculated using nearest neighbor parameter ΔH of approximately −87.0 kcal/mol. The plausible explanation for this difference could be the stacking interactions among single strands of the guide itself, which need to be broken before duplex formation. Although this binding was enthalpically favorable, the entropic changes were found to be highly unfavorable with a $T\Delta S$ of −60.1 kcal/mol. RNA−RNA

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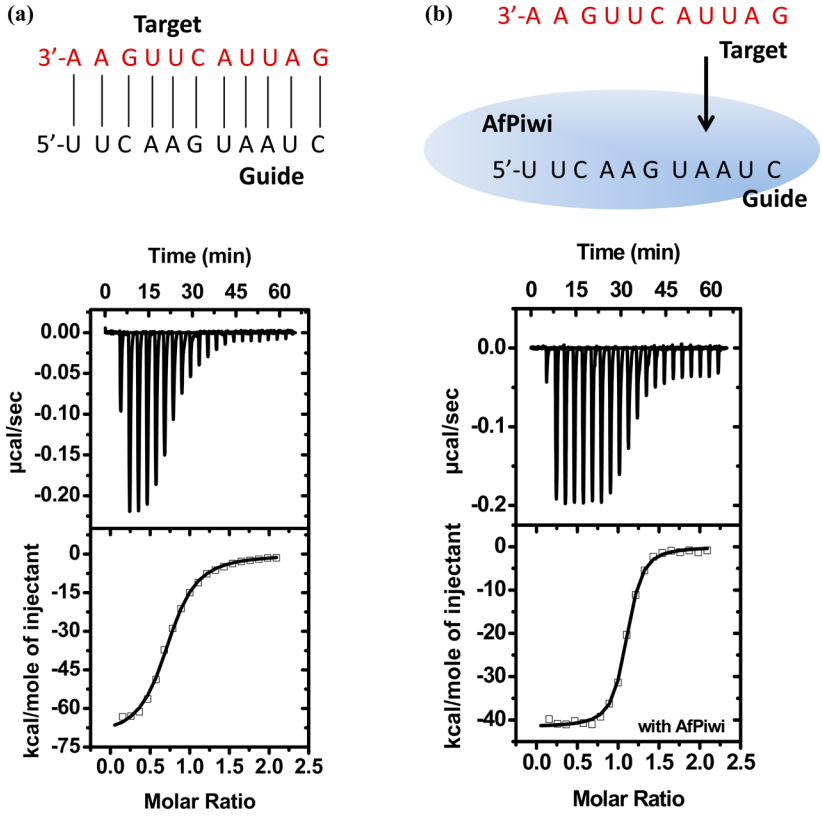

Figure 3. ITC profile of binding of the guide strand to the target mRNA strand in the absence (left) and presence (right) of AfPiwi protein.

Table 2. Effects of AfPiwi Protein on the Thermodynamics of Binding of the Guide to Target RNA

oligonucleotide	n	K_a ($\times 10^7$ M^{-1})	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)	ΔG (kcal/mol)
guide	0.9	2.0 ± 0.1	−70.4 ± 0.3	−60.1 ± 0.1	−10.3
guide and AfPIWI	1.0	12.0 ± 0.2	−42.3 ± 0.2	−30.8 ± 0.2	−11.5

hybridization mostly exhibits a large range of unfavorable entropy changes because of the loss of degrees of freedom of two RNA strands. The exothermic enthalpy change can be attributed mostly to stacking interactions and the formation of new hydrogen bonds during hybridization.[40]

Effect of AfPiwi on the Binding of the Guide Strand to Target RNA. AfPiwi is an archaeal homologue of Argonaute protein. It contains a positively charged channel required for RNA binding and is able to form a complex with the siRNA-like duplex.[27] As AfPiwi does not contain two conserved aspartic acids present in the adjacent β-strands, it does not exhibit slicer activity. These aspartic acid residues are essential for coordination with a divalent cation, thereby imparting catalytic RNase H function.[28,41] Parker et al. have previously shown that AfPiwi enhances the binding affinity of the guide RNA for the target in the seed sequence in a position-dependent manner.[29] We have taken 11-mer guide RNA and measured the effect of AfPiwi on its binding to target RNA. The binding profile in the right panel of Figure 3b shows the ITC titration profile of the guide with the target in the presence of AfPiwi protein. The analysis of this binding profile using one set of binding sites[39] showed that the presence of protein does not change the binding stoichiometry of the two RNAs but increases the binding affinity 6-fold with a K_a of 12.0×10^7 M^{-1}. The enthalpy change (ΔH) was decreased to −42.3 kcal/mol (Table 2) as compared to the enthalpy change in the absence of the protein (−70.4 kcal/mol). Although the relative change in enthalpy ($\Delta\Delta H$ = −28.1 kcal/mol) of binding is unfavorable, it is favorably compensated by a large decrease in negative unfavorable entropy. The entropy change for binding ($T\Delta S$) become less unfavorable (−30.6 kcal/mol) compared to that in the absence of the protein (−60.1 kcal/mol). This decrease in unfavorable negative entropy results in a relative entropy change ($T\Delta S$) of −29.5 kcal/mol which in turn establishes better binding. It is evident from these results that the presence of AfPiwi increases the binding affinity significantly by decreasing the large unfavorable entropic component. Although there is a significant decrease in the binding enthalpy in the presence of the protein, this could be attributed to the energy required for the binding of the guide strand to the AfPiwi protein.[29] The reduction in unfavorable entropy could be a result of preorganization of the guide strand to the protein, thus leading to the loss of degrees of freedom of free guide RNA that in turn results in a reduction of the overall loss of entropy upon duplex formation.[29] Another reason behind the entropic change being more favorable upon binding of the protein could be the release of water molecules, especially if the protein makes van der Waals contacts with the RNA bases or results in significant deformation of the RNA.

Effect of LNA Incorporation on the Binding of the Guide and Target Strand. LNAs are the nucleotide analogues that lock the ribose sugar in a conformation closely similar to the one that RNA adopts after hybridization.[42,43] LNA antisense oligonucleotides (ASOs) have been proven to

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be more successful than unmodified ASOs as therapeutics against a xenographed cancer model.[44] Apart from these, LNA-modified nucleotides have also been shown to enhance the biostability and functionality of the small interfering RNA.[45] Here, ITC titrations were performed to see the effect of LNA incorporation at different positions of the guide strand RNA, and all the binding profiles are shown in the left panels of Figures S1−S11 of the Supporting Information. We have incorporated LNA-modified nucleotides in the guide strand in a walk-through manner and shown their effect on the binding affinity and thermodynamic parameters of binding with the target RNA strand. The histogram shown in Figure 4a

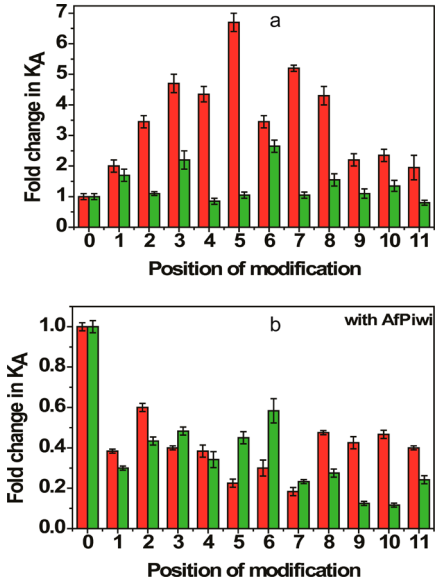

Figure 4. Change in the binding affinity of the guide strand binding to the target mRNA strand due to the presence of LNA and 2′-*O*-methyl modifications at different positions. 0 represents the guide strand without modification, whereas other numbers represent the position of the modification of the guide strand. Red bars represent data for the LNA modification and green bars data for the 2′-*O*-methyl modification (a) in the absence of AfPiwi protein and (b) in the presence of AfPiwi protein.

represents the relative change in binding affinity of the LNA-containing guide strand to target RNA in the absence of AfPiwi protein. The binding affinity of different sequences containing LNAs (1−11) was normalized relative to the binding affinity of the guide for the target without modification (0). Incorporation of LNA-modified nucleotides increased the binding affinity of the guide strand for target RNA in the absence of AfPiwi to different extents, irrespective of the position. Inspection of Table 3 reveals that the increase in binding affinity was mostly attributed to the increase in the favorable enthalpic change with a small change in the entropic component. LNA insertion resulted in better hybridization, leading to the enhancement of binding affinity of the guide for target RNA in the absence of AfPiwi protein. This increase in binding affinity ranged from 2- to 6.5-fold depending on the position of the LNA in the guide strand. The maximal enhancement in binding affinity (>6.5-fold) was observed in the case of LNA5, with the least favorable effect seen at LNA1 and LNA11 positions. This increased binding affinity resulted from the increased favorable negative enthalpic component at all positions except LNA3 and LNA11. Compared to that of unmodified guide−target binding (ΔH = −70.4 kcal/mol), the negative enthalpy was reduced to −65.7 kcal/mol at LNA3 and −58.2 kcal/mol at LNA11. However, this reduction in enthalpy was positively compensated by the similar reduction in unfavorable component from a $T\Delta S$ of −60.1 kcal/mol (unmodified guide−target binding) to a $T\Delta S$ of −54.5 kcal/mol at LNA3 and a $T\Delta S$ of −47.5 kcal/mol at LNA11. This enthalpy−entropy compensation resulted in more than 4.5- and ∼2-fold enhancement of the binding affinity of LNA3 and LNA11, respectively, for the target RNA compared to that of unmodified guide−target binding. On the other hand, LNA insertion resulted in an increased unfavorable negative entropic component, compensated by an increased negative enthalpy and leading to a mild increase in binding affinities at positions LNA1−LNA10.

However, in the presence of AfPiwi protein, in the binding profiles shown in the right panels of Figures S1−S11 of the Supporting Information, the binding affinity of the guide strand for target RNA was decreased by >50% at all the positions when compared to the binding affinity of the guide for target RNA without any modification. Thus, in the presence of LNA-modified nucleotides, the positive effect of AfPiwi protein on the binding of miRNA to target RNA was compromised. Table 4 lists the binding affinity and thermodynamic parameters of the binding of guide RNA containing LNAs to the target RNA in the presence of AfPiwi protein. Insertion of LNA reduces the positive effect of AfPiwi protein to different extents, from a 40% reduction at the LNA2 position to an 82% reduction in binding affinity enhancement. However, comparison of binding

Table 3. Thermodynamic Parameters for the Binding of the Guide RNA Strand to the Target Strand without and with LNA-Modified Nucleotides at Specified Positions

oligonucleotide	n	K_a ($\times 10^7$ M^{-1})	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)	ΔG (kcal/mol)
LNA1	1.2	4.0 ± 0.2	−71.8 ± 0.5	−61.1 ± 0.1	−10.7
LNA2	1.2	6.9 ± 0.2	−76.6 ± 0.2	−65.6 ± 0.2	−11.0
LNA3	1.1	9.4 ± 1.0	−65.7 ± 0.5	−54.5 ± 0.2	−11.2
LNA4	1.1	8.7 ± 0.9	−74.7 ± 0.7	−63.5 ± 0.1	−11.2
LNA5	0.8	13.4 ± 1.0	−71.8 ± 0.5	−60.3 ± 0.3	−11.5
LNA6	1.2	6.9 ± 0.8	−74.4 ± 0.9	−63.4 ± 0.2	−11.0
LNA7	0.7	10.4 ± 0.1	−78.8 ± 0.5	−67.5 ± 0.1	−11.3
LNA8	1.2	8.6 ± 0.9	−80.8 ± 0.8	−69.6 ± 0.4	−11.2
LNA9	0.9	4.4 ± 0.2	−78.2 ± 0.2	−67.4 ± 0.2	−10.8
LNA10	1.1	4.7 ± 0.2	−85.8 ± 0.4	−75.0 ± 0.3	−10.8
LNA11	1.0	3.9 ± 0.4	−58.2 ± 0.6	−47.5 ± 0.1	−10.7
guide	0.9	2.0 ± 0.1	−70.4 ± 0.4	−60.1 ± 0.2	−10.3

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Table 4. Thermodynamic Parameters for the Binding of the Guide RNA Strand for the Target Strand without and with LNA-Modified Nucleotides at Specified Positions in the Presence of AfPiwi Protein

oligonucleotide	n	K_a ($\times 10^7$ M^{-1})	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)	ΔG (kcal/mol)
LNA1	0.8	4.8 ± 0.1	−76.8 ± 0.8	−66.0 ± 0.2	−10.8
LNA2	1.1	7.3 ± 0.2	−69.0 ± 0.2	−57.9 ± 0.3	−11.1
LNA3	1.2	4.8 ± 0.2	−76.9 ± 0.3	−66.1 ± 0.3	−10.8
LNA4	1.2	5.1 ± 0.3	−49.1 ± 0.2	−38.3 ± 0.2	−10.8
LNA5	1.2	3.8 ± 0.1	−53.9 ± 0.3	−43.2 ± 0.4	−10.7
LNA6	1.3	2.7 ± 0.2	−57.5 ± 0.4	−47.0 ± 0.2	−10.5
LNA7	1.2	2.2 ± 0.1	−83.5 ± 0.3	−73.1 ± 0.3	−10.4
LNA8	1.2	5.7 ± 0.3	−71.6 ± 0.5	−60.7 ± 0.4	−10.9
LNA9	0.9	5.1 ± 0.1	−74.7 ± 0.4	−63.9 ± 0.3	−10.8
LNA10	1.0	5.6 ± 0.2	−71.8 ± 0.3	−60.9 ± 0.2	−10.9
LNA11	1.1	4.8 ± 0.1	−75.8 ± 0.2	−65.0 ± 0.5	−10.8
guide	1.0	12.0 ± 0.2	−42.3 ± 0.1	−30.9 ± 0.3	−11.4

Table 5. Thermodynamic Parameters for the Binding of the Guide RNA Strand for the Target Strand without and with 2′-*O*-Methyl-Modified Nucleotides at Specified Positions

oligonucleotide	n	K_a ($\times 10^7$ M^{-1})	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)	ΔG (kcal/mol)
2′-*O*-methyl 1	0.9	3.4 ± 0.4	−68.2 ± 1.6	−57.66 ± 0.2	−10.6
2′-*O*-methyl 2	1.3	2.2 ± 0.1	−79.4 ± 0.5	−69.1 ± 0.4	−10.3
2′-*O*-methyl 3	1.1	4.4 ± 0.4	−67.4 ± 1.9	−56.6 ± 0.2	−10.8
2′-*O*-methyl 4	0.9	1.7 ± 0.1	−82.0 ± 0.9	−71.8 ± 0.3	−10.2
2′-*O*-methyl 5	1.3	2.1 ± 0.2	−83.7 ± 0.8	−73.4 ± 0.1	−10.3
2′-*O*-methyl 6	0.9	5.3 ± 0.8	−62.1 ± 0.2	−51.2 ± 0.4	−10.9
2′-*O*-methyl 7	1.1	2.1 ± 0.1	−80.9 ± 0.6	−70.6 ± 0.3	−10.3
2′-*O*-methyl 8	1.0	3.1 ± 0.3	−70.2 ± 0.8	−59.7 ± 0.3	−10.5
2′-*O*-methyl 9	1.1	2.2 ± 0.1	−72.7 ± 0.4	−62.3 ± 0.5	−10.4
2′-*O*-methyl 10	1.3	2.7 ± 0.1	−73.8 ± 0.8	−63.3 ± 0.2	−10.5
2′-*O*-methyl 11	1.0	1.6 ± 0.1	−70.9 ± 0.6	−60.8 ± 0.3	−10.1
guide	0.9	2.0 ± 0.1	−70.4 ± 0.4	−60.1 ± 0.2	−10.3

Table 6. Thermodynamic Parameters for the Binding of the Guide RNA Strand to the Target Strand without and with 2′-*O*-Methyl-Modified Nucleotides at Specified Positions in the Presence of AfPiwi Protein

oligonucleotide	n	K_a ($\times 10^7$ M^{-1})	ΔH (kcal/mol)	$T\Delta S$ (kcal/mol)	ΔG (kcal/mol)
2′-*O*-methyl 1	0.8	3.6 ± 0.3	−60.6 ± 0.8	−49.9 ± 0.3	−10.7
2′-*O*-methyl 2	1.2	5.2 ± 0.4	−62.8 ± 0.4	−52.0 ± 0.2	−10.8
2′-*O*-methyl 3	0.9	5.8 ± 0.7	−58.0 ± 0.7	−47.1 ± 0.4	−10.9
2′-*O*-methyl 4	1.1	4.1 ± 0.3	−70.0 ± 0.6	−59.2 ± 0.1	−10.8
2′-*O*-methyl 5	1.3	5.4 ± 0.2	−68.5 ± 0.2	−57.6 ± 0.2	−10.9
2′-*O*-methyl 6	0.9	7.0 ± 0.6	−40.2 ± 0.3	−29.1 ± 0.3	−11.1
2′-*O*-methyl 7	0.9	2.8 ± 0.3	−78.6 ± 0.6	−68.1 ± 0.2	−10.5
2′-*O*-methyl 8	1.0	3.3 ± 0.2	−73.0 ± 0.5	−62.4 ± 0.3	−10.6
2′-*O*-methyl 9	1.3	1.5 ± 0.1	−65.4 ± 0.8	−55.3 ± 0.4	−10.1
2′-*O*-methyl 10	1.5	1.4 ± 0.1	−29.6 ± 0.2	−19.5 ± 0.3	−10.1
2′-*O*-methyl 11	1.2	2.9 ± 0.3	−31.1 ± 0.9	−20.6 ± 0.2	−10.5
guide	1.0	12.0 ± 0.2	−42.3 ± 0.1	−30.9 ± 0.3	−11.4

affinities in Tables 3 with 4 represents the effect of LNA on the binding affinity in the absence and presence of AfPiwi protein at different positions. The presence of an LNA at positions 1, 2, and 9−11 showed a mild change in the binding affinity, whereas the presence of an LNA at positions 3−8 decreases the binding affinity in the presence of AfPiwi protein. Comparison of the thermodynamic parameters of binding in the absence and presence of AfPiwi protein suggested that there is an increase in the favorable binding enthalpy when an LNA is present at positions 1, 3, 7, and 11 (Figure 4b, red bars, and Table 4). However, this increased favorable enthalpy at these positions was negatively compensated by a similar increase in the unfavorable entropy. On the other hand, LNAs when present at positions 2, 4−6, 8, and 9 lead to a decrease in the favorable enthalpy, which was again positively compensated by the decrease in unfavorable entropy. Thus, incorporation of LNA at different positions compromises the effect of the AfPiwi protein through large enthalpy−entropy compensation.

Effect of 2′-*O*-Methyl Modification on the Binding of the Guide and Target Strand. One of the ways to increase the biostability of the RNA is to add modified nucleotides at different positions. 2′-*O*-Methylation is one such modification that is well-known to enhance the biostability of the oligos against the nucleases, inside the cell.[46,47] Here we have studied the effect of 2′-*O*-methyl insertion in a walk-through manner in the guide strand RNA on its binding to the target RNA. The

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green bars in Figure 4a show the binding affinity of the guide (0) and the sequences containing a 2′-*O*-methyl group at different positions for the target RNA. The binding profiles are shown in the left panels of Figures S12−S22 of the Supporting Information, and the result obtained after analysis is summarized in Table 5. Contrary to LNA modification, 2′-O-methyl modification shows a position-dependent effect on the binding affinity of the guide for target RNA. This is in agreement with our earlier studies in which we noticed that the net effect of modification on thermodynamic parameters might be positional and that the neighboring bases flanking the modification might influence the favorable or unfavorable formation of the modified duplexes.[48,49] A similar observation was also made by another group.[50] It enhances the binding affinity at positions 1, 3, 6, 8, and 10, whereas it does not exhibit any effect on the binding affinity at other positions (Figure 4a, green bars). However, in the presence of AfPiwi protein (right panels of Figures S12−S22 of the Supporting Information), these modifications show a drastic decrease in the binding affinity of the guide for target RNA (Figure 4b, green bars, and Table 6). Thus, 2′-*O*-methyl modification also nullifies the effect of AfPiwi protein on the hybridization efficiency of the guide with target RNA. However, comparison of the effect of 2′-*O*-methyl modification in the presence (Table 6) and absence (Table 5) of AfPiwi protein shows an increase in the binding affinity in the presence of the protein. This increase in binding affinity was observed at all the positions because of the reduced unfavorable entropy.

DISCUSSION

Synthetic miRNAs are one of the potential therapeutics for many cancers and other diseases. Although miRNA mimics have been used successfully against different diseases, their biostability and functionality are major concerns. The use of modified nucleotides can resolve this problem to a great extent. However, these modified nucleotides cannot be inserted randomly in the sequence because of the involvement of RISC complex proteins. Here, the results describe the positions at which modified nucleotides can be inserted, including their effect on the hybridization efficiency of miRNA−mRNA in the presence and absence of AfPiwi protein. The presence of AfPiwi protein enhances the binding affinity of 11-mer miRNA for the 11-mer target RNA sequence by 6-fold. This binding affinity enhancement is primarily mediated through the reduction of the unfavorable entropy component as shown earlier by Parker et al.[29] Although there is colossal amount of reduction in the unfavorable entropy component, there is also a large amount of negative compensation by reduction in the favorable enthalpic component, thus leading to an only 6-fold increase in the binding affinity of the two RNA molecules. This reduction in the favorable enthalpy of miRNA−target hybridization in the presence of AfPiwi protein could result from the transfer of binding energy of guide strand RNA to AfPiwi to guide target strand complex formation.[29] Furthermore, the effect of incorporation of an LNA into the guide strand at various positions was assessed in the absence and presence of AfPiwi protein. UV melting experiments of the RNA oligos containing LNA and 2′-*O*-methyl modifications were performed to assess their stability in the absence of protein (data not shown). As is evident from many earlier studies, incorporation of LNA-modified nucleotides can enhance the stability of the heteroduplex up to 2−10 °C per LNA moiety, and it can enhance the biostability of the small interfering RNA (siRNA) greatly.[51] Thus, we chose to insert a single LNA nucleotide in a walk-through manner into the guide strand and examined its effect on the binding of the guide strand to the target RNA strand. ITC results summarized in Table 3 reveal that LNA insertion leads to an increase in the binding affinity of the guide strand for target RNA in the absence of AfPiwi protein, independent of the position at which they were inserted. This tighter binding is attributed to the increase in favorable negative enthalpy at all positions, because of the better stacking, except at LNA3 and LNA11, where preorganization leads to a decrease in the unfavorable entropy of binding of two RNA strands. Thus, our results support the hypothesis that LNA increases the binding affinity either by preorganization or better stacking but not simultaneously.[50] However, in the presence of AfPiwi protein, LNA insertion negatively affects the formation of the AfPiwi−guide−target ternary complex. This weakening of the binding of the guide to target RNA in the presence of AfPiwi protein is primarily conferred through an increase in the unfavorable entropy. Thus, the presence of LNA interferes with preorganization of guide RNA with AfPiwi protein. However, there is a significant increase in the exothermic enthalpy change because of the stronger hybridization of the guide to target RNA. These results show that the presence of LNA increases the affinity of the strands, especially when the modification is toward the center of the sequence. In the presence of the protein, the pattern is not so clear but the modifications seem to decrease the affinity of the strands more toward the center (this is clearer if we compare the binding affinity of a given LNA duplex in the presence and absence of the protein). This suggests that the more stable LNA duplexes change conformation less readily (are less likely to adopt the conformation induced by the protein). This would also be consistent with the more favorable enthalpy in the presence of the protein (compared to the unmodified duplex) observed when the modifications are in the center because with less van der Waals contacts between the protein and the duplex or with less distorted RNA duplexes, there will be a lower rate of release of water molecules and a more favorable enthalpy. The crystal structure of AfPiwi protein and the model of AfPiwi in a complex with a 21-nucleotide RNA duplex shows that the RNA sits in the positively charged channel made by basic and polar residues.[27] The side chains of these amino acids interact with the negatively charged phosphate sugar backbone of the RNA.[27] A further crystal structure of human Ago2 protein in complex with miR-20a suggests that the 5′-terminal phosphate forms an extensive network of contacts with conserved amino acids present in both Hs-Ago2 and AfPiwi protein. Other conserved amino acids that are involved in binding include Hs-Ago2-N562 and Hs-Ago2-R792. Both of these make contact with the phosphate backbone of the second nucleotide; Hs-Ago2-R792 also interacts with the third nucleotide. Thus, the presence of an LNA-modified nucleotide could be sterically hindering the preorganization of guide RNA to AfPiwi.

Another modification, the 2′-*O*-methyl-modified nucleotide, is primarily used to enhance the biostability of the oligonucleotides. The presence of a 2′-*O*-methyl-modified nucleotide in the guide strand sequence increased the binding affinity of the guide strand for the target RNA strand in a position specific manner. It strengthened the binding at positions 1, 3, 6, 8, and 10, leaving the binding affinity unaltered at other positions in the absence of AfPiwi protein. In the case of 2′-*O*-methyl modification, the increase in binding

affinity was mostly driven by the more favorable entropy; however, the exothermic−enthalpic component was also reduced, leading to a mild increase in binding affinity. On the other hand, the binding affinity at other positions remained unaltered because of the increased exothermic−enthalpic change, which was negatively compensated by an increase in the unfavorable entropy change. In the presence of AfPiwi protein, the 2′-*O*-methyl-modified nucleotide exerts a negative effect and decreases the binding affinity at every position, with the smallest effect at position 6. However, if we compare the effect of the 2′-*O*-methyl-modified nucleotide on binding, in the presence and absence of AfPiwi protein we observe an increase in binding affinity in the presence of protein. Also, the 2′-*O*-methyl-modified nucleotide show an enhanced effect toward the center of the sequences as compared to the ends both in the presence and in the absence of AfPiwi protein. Although in the presence of protein, the 2′-*O*-methyl-modified nucleotide seems to have better preorganization (lower unfavorable entropy) at positions 10 and 11, there is no significant increase in the binding affinity as compared to that of the unmodified duplex. One possible explanation for this observation could be the less efficient hybridization caused by the 2′-*O*-methyl-modified nucleotide, when present at the 3′-ends of the sequence.

In recent years, several LNA- and 2′-*O*-methyl-containing oligonucleotide-based therapeutics have been used successfully against different diseases.[52−55] LNA-containing oligonucleotides are well-known for their biostability and better binding efficiency. Similarly, 2′-*O*-methyl modifications are also used to enhance the biostability of the oligonucleotides inside the cells. Thus, this study provides a better understanding of the positions at which these modified nucleotides can be inserted without affecting the binding parameters drastically.

CONCLUSIONS

This study provides a thorough understanding of the effect of LNA and 2′-*O*-methyl modifications on the thermodynamics of binding of the guide to the target RNA strand. Here we have shown that in the absence of AfPiwi protein, LNA modifications enhance the binding affinity by increasing the favorable enthalpic component of binding; however, the 2′-*O*-methyl modification increases the binding affinity through a reduction in the unfavorable entropic component. However, when present with AfPiwi protein, these modifications tend to reduce the binding affinity possibly because of steric interference with the preorganization of the guide strand to AfPiwi protein. Thus, these modifications nullify the positive effect of AfPiwi protein on the hybridization of the guide strand to target RNA. Although these modifications reduce the binding affinity of guide RNA for target RNA, position 2 for LNA and position 6 for 2′-*O*-methyl modifications can be selected to have the least unfavorable effect on the binding of two RNAs. Thus, there seems to be a trade-off between the binding affinity and the biostability of the guide strand with respect to target mRNA.

ASSOCIATED CONTENT

Supporting Information

Figures illustrating the ITC profiles of the guide strand (containing an LNA or a 2′-*O*-methyl-modified nucleotide at various positions) binding to target RNA in the absence and presence of AfPiwi protein (Figures S1−S22). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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REFERENCES

(1) Almeida, R., and Allshire, R. C. (2005) RNA silencing and genome regulation. *Trends Cell Biol. 15*, 251−258.
(2) Voinnet, O. (2002) RNA silencing: Small RNAs as ubiquitous regulators of gene expression. *Curr. Opin. Plant Biol. 5*, 444−451.
(3) Brodersen, P., and Voinnet, O. (2009) Revisiting the principles of microRNA target recognition and mode of action. *Nat. Rev. Mol. Cell Biol. 10*, 141−148.
(4) Pillai, R. S. (2005) MicroRNA function: Multiple mechanisms for a tiny RNA? *RNA 11*, 1753−1761.
(5) Tetreault, N., and De Guire, V. (2013) miRNAs: Their discovery, biogenesis and mechanism of action. *Clin. Biochem. 46*, 842−845.
(6) Soifer, H. S., Rossi, J. J., and Saetrom, P. (2007) MicroRNAs in disease and potential therapeutic applications. *Mol. Ther. 15*, 2070−2079.
(7) Lu, M., Zhang, Q., Deng, M., Miao, J., Guo, Y., Gao, W., and Cui, Q. (2008) An analysis of human microRNA and disease associations. *PLoS One 3*, e3420.
(8) Gascon, E., and Gao, F. B. (2012) Cause or Effect: Misregulation of microRNA Pathways in Neurodegeneration. *Front. Neurosci. 6*, 48.
(9) Reinhardt, A., Feuillette, S., Cassar, M., Callens, C., Thomassin, H., Birman, S., Lecourtois, M., Antoniewski, C., and Tricoire, H. (2012) Lack of miRNA Misregulation at Early Pathological Stages in *Drosophila* Neurodegenerative Disease Models. *Front. Genet. 3*, 226.
(10) Croce, C. M. (2009) Causes and consequences of microRNA dysregulation in cancer. *Nat. Rev. Genet. 10*, 704−714.
(11) Iorio, M. V., and Croce, C. M. (2012) MicroRNA dysregulation in cancer: Diagnostics, monitoring and therapeutics. A comprehensive review. *EMBO Mol. Med. 4*, 143−159.
(12) Rau, F., Freyermuth, F., Fugier, C., Villemin, J. P., Fischer, M. C., Jost, B., Dembele, D., Gourdon, G., Nicole, A., Duboc, D., Wahbi, K., Day, J. W., Fujimura, H., Takahashi, M. P., Auboeuf, D., Dreumont, N., Furling, D., and Charlet-Berguerand, N. (2011) Misregulation of miR-1 processing is associated with heart defects in myotonic dystrophy. *Nat. Struct. Mol. Biol. 18*, 840−845.
(13) Esquela-Kerscher, A., and Slack, F. J. (2006) Oncomirs: MicroRNAs with a role in cancer. *Nat. Rev. Cancer 6*, 259−269.
(14) Liu, X. X., Li, X. J., Zhang, B., Liang, Y. J., Zhou, C. X., Cao, D. X., He, M., Chen, G. Q., He, J. R., and Zhao, Q. (2011) MicroRNA-26b is underexpressed in human breast cancer and induces cell apoptosis by targeting SLC7A11. *FEBS Lett. 585*, 1363−1367.
(15) Leeper, N. J., Raiesdana, A., Kojima, Y., Chun, H. J., Azuma, J., Maegdefessel, L., Kundu, R. K., Quertermous, T., Tsao, P. S., and Spin, J. M. (2011) MicroRNA-26a is a novel regulator of vascular smooth muscle cell function. *J. Cell. Physiol. 226*, 1035−1043.
(16) Gao, J., and Liu, Q. G. (2011) The role of miR-26 in tumors and normal tissues (Review). *Oncol. Lett. 2*, 1019−1023.
(17) Li, J. M., Zhao, R. H., Li, S. T., Xie, C. X., Jiang, H. H., Ding, W. J., Du, P., Chen, W., Yang, M., and Cui, L. (2012) Down-regulation of

fecal miR-143 and miR-145 as potential markers for colorectal cancer. *Saudi Medical Journal 33*, 24−29.
(18) Thomson, J. M., Newman, M., Parker, J. S., Morin-Kensicki, E. M., Wright, T., and Hammond, S. M. (2006) Extensive post-transcriptional regulation of microRNAs and its implications for cancer. *Genes Dev. 20*, 2202−2207.
(19) Hutvagner, G., and Simard, M. J. (2008) Argonaute proteins: Key players in RNA silencing. *Nat. Rev. Mol. Cell Biol. 9*, 22−32.
(20) Meister, G. (2013) Argonaute proteins: Functional insights and emerging roles. *Nat. Rev. Genet. 14*, 447−459.
(21) Landthaler, M., Gaidatzis, D., Rothballer, A., Chen, P. Y., Soll, S. J., Dinic, L., Ojo, T., Hafner, M., Zavolan, M., and Tuschl, T. (2008) Molecular characterization of human Argonaute-containing ribonucleoprotein complexes and their bound target mRNAs. *RNA 14*, 2580−2596.
(22) Hu, H. Y., Yan, Z., Xu, Y., Hu, H., Menzel, C., Zhou, Y. H., Chen, W., and Khaitovich, P. (2009) Sequence features associated with microRNA strand selection in humans and flies. *BMC Genomics 10*, 413.
(23) Ghildiyal, M., Xu, J., Seitz, H., Weng, Z., and Zamore, P. D. (2010) Sorting of *Drosophila* small silencing RNAs partitions microRNA* strands into the RNA interference pathway. *RNA 16*, 43−56.
(24) Song, J. J., Smith, S. K., Hannon, G. J., and Joshua-Tor, L. (2004) Crystal structure of Argonaute and its implications for RISC slicer activity. *Science 305*, 1434−1437.
(25) Schirle, N. T., and MacRae, I. J. (2012) The crystal structure of human Argonaute2. *Science 336*, 1037−1040.
(26) Elkayam, E., Kuhn, C. D., Tocilj, A., Haase, A. D., Greene, E. M., Hannon, G. J., and Joshua-Tor, L. (2012) The structure of human argonaute-2 in complex with miR-20a. *Cell 150*, 100−110.
(27) Parker, J. S., Roe, S. M., and Barford, D. (2004) Crystal structure of a PIWI protein suggests mechanisms for siRNA recognition and slicer activity. *EMBO J. 23*, 4727−4737.
(28) Parker, J. S., Roe, S. M., and Barford, D. (2005) Structural insights into mRNA recognition from a PIWI domain-siRNA guide complex. *Nature 434*, 663−666.
(29) Parker, J. S., Parizotto, E. A., Wang, M., Roe, S. M., and Barford, D. (2009) Enhancement of the seed-target recognition step in RNA silencing by a PIWI/MID domain protein. *Mol. Cell 33*, 204−214.
(30) Uhlmann, S., Mannsperger, H., Zhang, J. D., Horvat, E. A., Schmidt, C., Kublbeck, M., Henjes, F., Ward, A., Tschulena, U., Zweig, K., Korf, U., Wiemann, S., and Sahin, O. (2012) Global microRNA level regulation of EGFR-driven cell-cycle protein network in breast cancer. *Mol. Syst. Biol. 8*, 570.
(31) Trang, P., Wiggins, J. F., Daige, C. L., Cho, C., Omotola, M., Brown, D., Weidhaas, J. B., Bader, A. G., and Slack, F. J. (2011) Systemic delivery of tumor suppressor microRNA mimics using a neutral lipid emulsion inhibits lung tumors in mice. *Mol. Ther. 19*, 1116−1122.
(32) Wang, Z. (2011) The guideline of the design and validation of MiRNA mimics. *Methods Mol. Biol. 676*, 211−223.
(33) Chorn, G., Klein-McDowell, M., Zhao, L., Saunders, M. A., Flanagan, W. M., Willingham, A. T., and Lim, L. P. (2012) Single-stranded microRNA mimics. *RNA 18*, 1796−1804.
(34) Grunweller, A., Wyszko, E., Bieber, B., Jahnel, R., Erdmann, V. A., and Kurreck, J. (2003) Comparison of different antisense strategies in mammalian cells using locked nucleic acids, 2′-O-methyl RNA, phosphorothioates and small interfering RNA. *Nucleic Acids Res. 31*, 3185−3193.
(35) Doessing, H., and Vester, B. (2011) Locked and unlocked nucleosides in functional nucleic acids. *Molecules 16*, 4511−4526.
(36) Cummins, L. L., Owens, S. R., Risen, L. M., Lesnik, E. A., Freier, S. M., McGee, D., Guinosso, C. J., and Cook, P. D. (1995) Characterization of fully 2′-modified oligoribonucleotide hetero- and homoduplex hybridization and nuclease sensitivity. *Nucleic Acids Res. 23*, 2019−2024.
(37) Vester, B., and Wengel, J. (2004) LNA (locked nucleic acid): High-affinity targeting of complementary RNA and DNA. *Biochemistry 43*, 13233−13241.
(38) Ye, K., Malinina, L., and Patel, D. J. (2003) Recognition of small interfering RNA by a viral suppressor of RNA silencing. *Nature 426*, 874−878.
(39) Pierce, M. M., Raman, C. S., and Nall, B. T. (1999) Isothermal titration calorimetry of protein-protein interactions. *Methods 19*, 213−221.
(40) Yakovchuk, P., Protozanova, E., and Frank-Kamenetskii, M. D. (2006) Base-stacking and base-pairing contributions into thermal stability of the DNA double helix. *Nucleic Acids Res. 34*, 564−574.
(41) Song, J. J., and Joshua-Tor, L. (2006) Argonaute and RNA: Getting into the groove. *Curr. Opin. Struct. Biol. 16*, 5−11.
(42) Corey, D. R. (2007) Chemical modification: The key to clinical application of RNA interference? *J. Clin. Invest. 117*, 3615−3622.
(43) Kaur, H., Babu, B. R., and Maiti, S. (2007) Perspectives on chemistry and therapeutic applications of locked nucleic acid (LNA). *Chem. Rev. 107*, 4672−4697.
(44) Mook, O. R., Baas, F., de Wissel, M. B., and Fluiter, K. (2007) Evaluation of locked nucleic acid-modified small interfering RNA in vitro and in vivo. *Mol. Cancer Ther. 6*, 833−843.
(45) Elmen, J., Thonberg, H., Ljungberg, K., Frieden, M., Westergaard, M., Xu, Y., Wahren, B., Liang, Z., Orum, H., Koch, T., and Wahlestedt, C. (2005) Locked nucleic acid (LNA) mediated improvements in siRNA stability and functionality. *Nucleic Acids Res. 33*, 439−447.
(46) Monia, B. P., Lesnik, E. A., Gonzalez, C., Lima, W. F., McGee, D., Guinosso, C. J., Kawasaki, A. M., Cook, P. D., and Freier, S. M. (1993) Evaluation of 2′-modified oligonucleotides containing 2′-deoxy gaps as antisense inhibitors of gene expression. *J. Biol. Chem. 268*, 14514−14522.
(47) Yoo, B. H., Bochkareva, E., Bochkarev, A., Mou, T. C., and Gray, D. M. (2004) 2′-O-Methyl-modified phosphorothioate antisense oligonucleotides have reduced non-specific effects in vitro. *Nucleic Acids Res. 32*, 2008−2016.
(48) Kaur, H., Wengel, J., and Maiti, S. (2008) Thermodynamics of DNA-RNA heteroduplex formation: Effects of locked nucleic acid nucleotides incorporated into the DNA strand. *Biochemistry 47*, 1218−1227.
(49) Kaur, H., Arora, A., Wengel, J., and Maiti, S. (2006) Thermodynamic, counterion, and hydration effects for the incorporation of locked nucleic acid nucleotides into DNA duplexes. *Biochemistry 45*, 7347−7355.
(50) McTigue, P. M., Peterson, R. J., and Kahn, J. D. (2004) Sequence-dependent thermodynamic parameters for locked nucleic acid (LNA)-DNA duplex formation. *Biochemistry 43*, 5388−5405.
(51) Braasch, D. A., and Corey, D. R. (2001) Locked nucleic acid (LNA): Fine-tuning the recognition of DNA and RNA. *Chem. Biol. 8*, 1−7.
(52) Straarup, E. M., Fisker, N., Hedtjarn, M., Lindholm, M. W., Rosenbohm, C., Aarup, V., Hansen, H. F., Orum, H., Hansen, J. B., and Koch, T. (2010) Short locked nucleic acid antisense oligonucleotides potently reduce apolipoprotein B mRNA and serum cholesterol in mice and non-human primates. *Nucleic Acids Res. 38*, 7100−7111.
(53) Aboul-Fadl, T. (2005) Antisense oligonucleotides: The state of the art. *Curr. Med. Chem. 12*, 2193−2214.
(54) Dias, N., and Stein, C. A. (2002) Antisense oligonucleotides: Basic concepts and mechanisms. *Mol. Cancer Ther. 1*, 347−355.
(55) Stein, D., Foster, E., Huang, S. B., Weller, D., and Summerton, J. (1997) A specificity comparison of four antisense types: Morpholino, 2′-O-methyl RNA, DNA, and phosphorothioate DNA. *Antisense Nucleic Acid Drug Dev. 7*, 151−157.