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Controlling miRNA-like Off-target Effects of an siRNA with Nucleobase Modifications

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Abstract

SiRNAs can cause unintended gene silencing due to miRNA-like effects because of the similarity in function of an siRNA guide strand and a miRNA. Here we evaluate the effect on miRNA-like off targeting of introducing the adenosine derivative 7-EAA and triazoles prepared from 7-EAA at different positions in an siRNA guide strand. We find that a sterically demanding triazole placed in the RNA duplex major groove at position six of the guide strand dramatically reduces miRNA-like off targeting potency. A high-resolution structure of an RNA duplex bearing a novel, major-groove localized triazole is reported, which suggests that modified triazoles could be disrupting the hAgo2-guide-target RNA ternary complex. Five different triazole modifications were tested at the guide strand 6-position for effects on on-target and miRNA-like off target knockdown potency. A 7-EAA triazole bearing a benzylamine substituent displayed on-target knockdown activity as potent as the native siRNA, while having an IC₅₀ against a miRNA-like off target > 100-fold higher. Melting temperature studies revealed no obvious correlation between potency in knockdown assays and a modification's effect on duplex stability. These results, along with known structures of hAgo2-guide-target ternary complexes, are used to rationalize the effect of 7-EAA triazoles on miRNA-like off target effects.

Graphical Abstract

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“Structural information provides important insight to how major groove-modified triazole-containing siRNAs selectively knockout their intended target.

Introduction

Short interfering RNAs (siRNAs) function as triggers for the RNA interference (RNAi) pathway leading to the targeted cleavage of mRNAs complementary to the siRNA guide strand¹. In humans, this is the result of loading the guide strand into the Argonaute 2 (hAgo2) nuclease followed by binding of the hAgo2-guide complex to a site in an mRNA target with Watson-Crick complementarity to the guide. A fully complementary guide-target complex results in cleavage of the target RNA by hAgo2.^{1,2} The potency of easily obtained siRNAs and the programmable nature of the approach allowing for any gene product to be targeted simply by changing the siRNA sequence stimulated efforts worldwide to advance siRNAs into the clinic.^{1–6} However, because of the similarity in function of an siRNA guide strand and a miRNA, siRNAs can lead to unintended gene silencing due to miRNA-like effects.^{7–9} Indeed, a published screening study using single siRNAs targeting 6000 different human genes showed that all the hits in the screen arose from miRNA-like off-target effects instead of the intended target knockdown.¹⁰ A challenge in the optimization of siRNAs is to develop guide strand modifications that will reduce miRNA-like off target effects.^{8,9} Furthermore, since siRNAs are universally used as gene knockdown tools in biology, a complete understanding of what controls their specificity is essential for the proper use of the knockdown data.

Previous reports have identified strategies for sugar modification that modulate miRNA-like off-target effects, however less information is available on the effect of modifications to the nucleobases.^{11,12} Structures of hAgo2 bound to guide and target RNAs suggest nucleobase modification could influence both protein-RNA and RNA-RNA recognition events involving the siRNA guide strand.^{13,14} Previously, we described an adenosine analog, 7-ethynyl-8-aza-7-deazaadenosine (7-EAA, Figure 1), that pairs normally with uridine in duplex RNA and causes minimal perturbation to the normal A-form helix.¹⁵ Furthermore, 7-EAA is efficiently converted to triazole analogs via copper-catalyzed azide/alkyne cycloaddition (CuAAC) reactions allowing exploration of different modifications at specific adenosine positions projecting into the duplex RNA major groove. Here we evaluate the effect on miRNA-like off targeting of introducing 7-EAA and triazoles derived from 7-EAA at different positions in an siRNA guide strand. We find that introducing a sterically demanding triazole into the RNA duplex major groove at position six of the guide strand dramatically reduces miRNA-like off targeting potency. We also report the crystal structure of a duplex RNA bearing a novel triazole analog derived from 7-EAA. This structure was used to rationalize the effect of 7-EAA triazoles on miRNA-like off target effects, and how different triazole modifications can tune the potency of a siRNA-like on-target versus a miRNA-like off-target.

Results and Discussion

We chose to analyze the effects of nucleobase modifications in the guide strand of an siRNA targeting the human PIK3CB message.⁸ This siRNA has been shown to knockdown its intended target in HeLa cells as well as other endogenous transcripts with complementarity to the guide strand seed region (nucleotides 2–8), including the YY1 and FADD mRNAs.^{8,16} To facilitate our analysis, we used siRNA off-target activity reporter plasmids with copies of the YY1 and FADD sequences inserted into the 3' UTR of the *Renilla* luciferase gene. These plasmids also encode firefly luciferase as a transfection control. In earlier work, we demonstrated the effect of 7-EAA and the 7-EAA triazole NEtPip (Figure 1B) at each of the adenosine positions in the PIK3CB siRNA guide on on-target knockdown.¹⁷ These adenosine analogs project substituents into the duplex RNA major groove without substantial alteration of base pairing or duplex conformation and were tolerated at most positions tested in the guide strand except positions 1, 3 and 10.¹⁷ To examine the effect of these modifications on off-target knockdown activity, we singly modified each adenosine position in the PIK3CB siRNA guide strand and measured knockdown of the *Renilla* luciferase message containing either the YY1 or FADD sequence in the 3' UTR (Figure 2).

For both off-target sequences we found that placement of 7-EAA at positions 3 or 20 was poorly tolerated as was the 7-EAA triazole NEtPip at position 6 (Figure 2). Interestingly, placement of both 7-EAA and 7-EAA triazole NEtPip at the guide strand position 1 resulted in significant off-target knockdown. In addition, both modifications at position 10 seem to have little effect or even increase potency for off-target knockdown. We plotted the difference between off-target activity (YY1 or FADD, from Figure 2)¹⁶ and on-target activity (PIK3CB) as previously reported^{16,17} to help identify guide strand positions where these modifications alter selectivity (Figure 3). While many of the modifications made to the 3' end of the siRNA do not appear to change siRNA selectivity (Figure 3), modifications in the 5' half of the siRNA and 7-EAA at position 20 do alter the on-target vs off-target selectivity.

At position 1 and 10 of the siRNA guide, both 7-EAA and 7-EAA triazole NEtPip increase selectivity for the off-target sequences. Analogs placed at these positions inhibit knockdown activity for the on-target sequence, likely because these major groove modifications either reduce hAgo2 binding or block cleavage of the target RNA. Position 10 resides outside the guide strand seed region where Watson-Crick complementarity is generally required for on-target cleavage, but not required for miRNA-like knockdown.¹⁸

Conversely, our adenosine analogs increase on-target selectivity when placed within the seed region (positions 3 or 6) or at the 3'-end of the siRNA (position 20) (Figure 3). At guide position 3, adenosine replacement with either 7-EAA or 7-EAA triazole NEtPip results in discrimination against the YY1 sequence. Use of 7-EAA at guide position 20 increases selectivity over both YY1 and FADD off-target sequences. Importantly, placement of 7-EAA triazole NEtPip at guide position 6 imparts the greatest increase in selectivity against both off-targets. In addition, triazole formation at this position has a beneficial effect on target selectivity since the parent alkyne (7-EAA) at this position inhibited on-target and off-target

knockdown similarly (Figure 3). These results indicate that a triazole modification directed toward the major groove at guide position 6 can reduce miRNA-like off target activity.

In complexes of hAgo2 bound to guide and target RNA, the nucleotide at guide position 6 base pairs with the complementary target strand within the protein's central cleft.¹⁴ Modifications that destabilize duplex formation within the seed region, including at guide position 6, are known to reduce off-target transcript knockdown.^{11,12,19} Indeed, a recent report showed that introduction of an abasic residue at position 6 of the guide strand reduced miRNA-like off-target effects.²⁰ However, T_M studies with model RNA duplexes indicated that 7-EAA triazole NEtPip does not substantially destabilize the duplex structure ($T_M = 1.5^\circ\text{C}$ replacing one A-U pair with one 7-EAA triazole NEtPip-U base pair in a 12 bp duplex where a mismatch destabilizes this duplex $T_M = 8.5^\circ\text{C}$) and a crystal structure of a 7-EAA triazole NEtPip-containing RNA duplex showed typical A-form geometry.¹⁵ Thus the influence of 7-EAA triazole NEtPip on miRNA-like off targeting appears to arise from effects not previously observed with sugar modified guide strands.²¹ Modeling 7-EAA triazole modifications at guide position 6 in the reported hAgo2 ternary complexes could advance our understanding of how these analogs affect siRNA selectivity. However, our structural analysis of duplex RNA containing this triazole did not allow us to unambiguously position the N-ethylpiperidine in the major groove.¹⁵ Therefore, we prepared a new analog expected to have lower conformational flexibility in the triazole substituent, which could lead to more order in modified RNA crystals. This analog (7-EAA triazole BrPh) is a product of a CuAAC reaction with 7-EAA-containing RNA and 1-azido-4-bromobenzene. The new triazole modification was introduced into a self-complementary 16 nt RNA strand used by us previously for crystallography of 7-EAA-modified duplex RNA (Figure 4). The modified RNA was synthesized, purified and crystallized as reported previously.¹⁵ A structure for the RNA bearing the BrPh triazole modification was solved at 1.65 Å resolution using the bromine atom to facilitate phasing. An examination of the electron density map clearly reveals the location of the bromine in the structure but the phenyl ring electron density is not as well defined. Nevertheless, the location of the bromine atom is consistent with the conformation shown in (Figure 4) with an intramolecular hydrogen bond between the modified triazole and the N6 amino group of 7-EAA. The analog engages in Watson-Crick base pairing with uridine in a typical A-form helix, while simultaneously extending the bromophenyl substituent into the duplex major groove (Figure 4).

Importantly, the new structure provides additional insight on how 7-EAA triazoles at guide position 6 might influence siRNA selectivity. The recently reported crystal structures of hAgo2-target-guide ternary complexes reveal the environment of a guide position 6 adenosine in a complex with a miRNA-like target RNA (Figure 5B). The Hoogsteen edge of the nucleobase is directed toward a cavity in the protein-RNA complex capable of accommodating the 7-ethyne group of 7-EAA (Figure 5B). However, using the data from our 7-EAA BrPh-containing RNA crystal structure, we can generate a model of this triazole at guide position 6 in the ternary complex that suggests sterically demanding triazole modifications would clash with Y804 and potentially destabilize a miRNA-like targeting complex (Figure 5C). Importantly, the narrow central cleft in this complex will not accommodate pairing beyond guide nucleotide 9. This requires a conformational change in hAgo2 that widens the central cleft¹⁴. We suggest here that the conformational change that

widens the central cleft and allows a fully paired duplex to bind (like with an siRNA on-target sequence) may also relieve a 7-EAA triazole/Y804 clash. This model explains how the 7-EAA triazole modifications might reduce miRNA-like effects but allow for efficient on-target knockdown whereas the smaller parent alkyne (7-EAA) is less effective.

To explore the effect of modification at position 6 further, we measured IC₅₀ values for on-target and off-target knockdown for several different guide position 6 modified siRNAs (Table 1). In addition to the two 7-EAA triazoles already described above (NEtPip, BrPh), three new analogs were also tested; aminobenzyl (NH₂Bn), pyridinyl benzyl (PyrBn) and 3-pyridinyl (Pyr) (see Figure 1 for structures). Since a positive charge in the duplex RNA major groove is likely stabilizing,^{22,23} each of these new modifications had the potential to stabilize the guide-target duplex with a positive charge. Triazoles NH₂Bn and PyrBn each have positively charged substituents of different sizes. The Pyr analog was designed, based on the BrPh-containing RNA crystal structure, to stabilize an RNA duplex by forming a hydrogen bond (in its protonated form) with the phosphodiester backbone.

We found that modifying guide position 6 with NEtPip resulted in a three-fold increase in the measured IC₅₀ for on-target knockdown (IC₅₀ = 15 ± 5 pM (A at g6) vs IC₅₀ = 44 ± 18 pM (NEtPip at g6)) (Table 1). However, this modification caused a much more substantial reduction in off-target knockdown activity for the YY1 off target sequence. Indeed, while the unmodified siRNA efficiently reduced luciferase expression for the YY1 sequence (IC₅₀ = 15 ± 5 pM), little off-target knockdown could be measured with the siRNA bearing 7-EAA triazole NEtPip at position 6 up to 5 nM siRNA concentration (Table 1). The BrPh analog also did not knockdown the YY1 off target up to 5 nM siRNA concentration. However, this modification reduced on-target knockdown potency by five-fold (IC₅₀ = 15 ± 5 pM (A at g6) vs IC₅₀ = 82 ± 8 pM (BrPh at g6)). Off-target knockdown activity for the remaining triazoles (Pyr, NH₂Bn and PyrBn) correlated with the size of the modification with the largest (PyrBn) showing no knockdown up to 5 nM siRNA and the smallest (Pyr) knocking down the YY1 off target with an IC₅₀ = 308 ± 77 pM. This is consistent with modeling these analogs into guide position 6 of the hAgo2-guide-target ternary complex representative of a miRNA recognition complex. The Pyr analog nearly fits the existing cavity whereas the NH₂Bn and PyrBn modifications, like BrPh, would clash with Y804 (Figure 5D,E,F).

Interestingly, the NH₂Bn triazole at guide position 6 led to on-target knockdown potency similar to the unmodified siRNA (IC₅₀ = 13 ± 5 pM) whereas the other triazoles (Pyr, NEtPip, BrPh and PyrBn) inhibited on target activity (Table 1).

Taken together with the off-target activity data for the NH₂Bn-modified RNA (IC₅₀ = 1358 ± 177 pM), this analog imparts a >100-fold selectivity for the on-target sequence without loss of potency. As indicated above, the NEtPip triazole at guide position 6 also substantially improves on-target selectivity but with a reduction in on-target potency. Since no structure of an hAgo2-guide-target complex corresponding to an siRNA-on target site has been reported, on-target activity cannot be rationalized based on known structures of protein-RNA complexes.

To gain further insight into the effect of these novel modifications on RNA duplex stability, we measured T_M values for model 12 bp duplexes bearing the 7-EAA triazoles opposite different base-pairing partners. These measurements showed each new 7-EAA triazole (BrPh, Pyr, NH_2Bn and PyrBn) paired with U destabilizes an RNA duplex similar to an A–G mismatch (Figure 6). This destabilizing effect may arise from the close approach to the phosphodiester backbone of the aromatic ring appended to the triazole (Figure 4), which is common to all these analogs (Figure 1). The presence of a positive charge (PyrBn, NH_2Bn) or site of possible protonation and H-bonding to the RNA (Pyr) did not overcome this destabilizing effect. Nevertheless, each of these triazoles showed selectivity for pairing with U over A, G or C (Figure 6). Importantly, the effect on RNA duplex stability alone cannot explain the superior on-target activity of NH_2Bn compared to the other 7-EAA triazoles.

In summary, these studies have identified a position in an siRNA guide strand (position 6) where sterically demanding modifications of the nucleobase can reduce off-target knockdown potency. We also identified a guide position 6 modification (7-EAA triazole NH_2Bn) that maintained full on-target knockdown potency of the unmodified siRNA while decreasing off target potency by > 100 fold. Characterization by X-ray crystallography of a related modification (7-EAA triazole BrPh) in RNA suggests a possible mechanism for the reduction in off-target potency. While the PIK3CB siRNA sequence used here required an A analog at guide position 6, it is possible that other major groove modified nucleobases could yield similar results. For instance, triazoles formed from 5-ethynyl pyrimidines could potentially alter on-target specificity. This work advances our understanding of the effects of nucleoside analogs on siRNA properties and informs the design of new RNAi reagents for more selective knockdown.

Materials and Methods

All RNA oligos except for the modified 7-EAA strands were ordered from GE Dharmacon Inc., and were purified by polyacrylamide gel electrophoresis and desalted by C18 Sep-Pak cartridges (Waters), and incorporated by procedures described below. The concentration of all RNAs were determined by UV absorbance at 260nm on NanoDrop™. HeLa cells were purchased from ATCC. Reagents for cell passaging were products of Gibco. Dual luciferase reported assays were accomplished using the Dual-Luciferase® Reporter Assay System by Promega. NeoFX siPort reagent for transfection was purchased from ThermoFisher. Reagents used in synthesis were either purchased from ThermoFisher, Sigma and Synthonix.

RNA synthesis and purification

Synthesis and purification of 7-EAA containing strands were synthesized using the 7-EAA phosphoramidite synthesis as previously described.¹⁵ The automated RNA synthesis, the 5' phosphorylated PIK3CB guide strand and RNA purification of the final products were carried out as previously described.^{16,17,24} The general procedure for the copper catalyzed azide/alkyne cycloaddition (CuAAC) reaction was carried out as previously described for all modifications, except the PhBr modification, for each sequence.^{15–17,24} Mass spectrometry data was acquired using a Bruker UltraFlex™ MALDI-TOF/TOF. Identities of the oligonucleotide triazoles were confirmed by MALDI-TOF mass spectroscopy using a

saturated solution of 3-hydroxypicolinic acid in 0.1 M aqueous dibasic ammonium citrate as a matrix. Mass spectra were recorded in the negative ionization mode and calibrated to an external DNA standard of 7352.1 Daltons. Samples and standards were desalted using ethanol precipitation and C18 Sep-Pak cartridges (Waters) for MALDI-TOF sample preparation.

4-aminobenzylazide and 3-azido pyridine

Synthesis of the 3-azido pyridine and 4-aminobenzylazide were carried out as previously described.^{25,26}

Synthesis of 1-(4-azidobenzyl) pyridin-1-ium bromide

To a flame dried 50 mL round bottom flask, 1-azido-4-(bromomethyl) benzene, which was synthesized as previously reported,²⁶ was added to the flask under an argon atmosphere (200 mg, 0.95 mmols). Anhydrous pyridine was added to the flask (5 mL) and the reaction was stirred for 24 h. After 24 h, the reaction mixture showed complete conversion by TLC, was evaporated to a residue and dissolved in anhydrous acetonitrile three times. The final product was isolated after drying overnight *in vacuo* as a red-brown solid (288 mg, 99%). ¹H NMR (DMSO, 300 MHz): δ (ppm) 9.21(d, J=5.9 Hz, 2H), 8.60(m, 1H), 8.17 (t, J=7.7 Hz 2H), 7.61 (d, J=8.2 Hz, 2H), 7.2 (d, J=8.8 Hz, 2H), 5.85 (s, 2H). ¹³C NMR (DMSO, 150 MHz): δ (ppm) 146.44, 145.16, 141.07, 131.28, 128.92, 120.32. ESIHRMS (m/z): calcd for C₁₀H₁₃N₄O₅ [M+]⁺ calcd 211.0978, obsd 211.0977.

General CuAAC procedure for the BrPh triazole modifications for the 12mer, 16mer and 21mer sequences

A solution of crude 7-EAA containing RNA (3.5 nmol) HEPES buffer (pH 7, 12.8 μ L, final concentration 100 mM) was treated with tris-[1-(3-hydroxypropyl)-1H-[1,2,3]triazol-4-yl)methyl]-amine ligand (100 mM solution in H₂O, 4.2 μ L, 420 nmol), 4-bromophenylazide (100mM solution in DMSO, 15.1 μ L, 1512 nmol) and CuBr (100 mM solution in DMSO, 2.1 μ L, 214 nmol). The solution was incubated for 12 h at room temperature. The reaction mixture was then diluted with PAGE loading buffer (80% formamide containing 10 mM EDTA) and PAGE-purified (19% denaturing, 20 W, 6–8 h). RNA was extracted, purified and analyzed as previously reported.^{15–17,24}

SiRNA duplex formation and RNAi activity assay

Duplex formation and assays were carried out as previously described. Briefly, reverse transfection of HeLa cells was accomplished using siPORT NeoFX (Ambion) in Opti-MEM media (GIBCO) to deliver the psiCHECK-2-PIK3CB plasmid and siRNA duplexes or hybridization buffer as a negative control. The reverse transfection was performed in 96-well plates, where each well contained 0.5 μ L siPORT, 20 ng psiCHECK-2-PIK3CB (or psiCHECK-2-YY1 or psiCHECK-2-FADD)^{16,17,24} and 30 pM (for PIK3CB or 0.5 nM for YY1 or 0.25 nM for FADD) siRNA for the NetPip walkthrough assay. IC₅₀ analyses were done ranging between 1 pM to 10nM, for the NetPip activity assays. IC₅₀ analyses for the other modifications were accomplished between 1pM to 5000 pM. All siRNAs referenced in

Figure 1 and Figure 2 were tested side-by-side for each different plasmid in the same 96-well plate assay.

Crystallization of RNA Duplex

RNA bearing the BrPh triazole modification at a concentration of 300 μ M was mixed with an equal volume of Hampton Nucleic Acid Mini Screen (NAM) reagent 14 (10% (v/v) 2-methyl-2,4-pentanediol (MPD), 12 mM spermine, 80 mM KCl, 20 mM MgCl₂, 40 mM sodium cacodylate pH 7.0) and equilibrated against a reservoir solution of 35% (v/v) MPD, similar to our previous work.¹⁵ Crystals, which appeared after 13 days, were flash cooled in liquid nitrogen for X-ray diffraction data collection.

Data Collection and Structure Determination

X-ray diffraction data were collected on beam line 8.3.1 at the Advanced Light Source (ALS) using the Dectris Pilatus3 S 6m detector at 100K. A multiwavelength fluorescence scan was taken near the bromide absorption edge to determine the optimal X-ray energy at the bromide peak. Two data sets were collected from one crystal using inverse beam method at energies of 13,473 eV (peak) and 13,601 eV (remote) to 1.75 Å resolution for phase determination. A subsequent crystal diffracted X-rays to 1.65 Å, which was used for structure refinement. Diffraction intensities were processed and scaled with the software packages XDS²⁷ and AIMLESS²⁸, respectively. Like our previous RNA crystal structure, the crystals crystallize in space group *C2* with pseudo *R32* symmetry^{15,29}, with unit cell parameters; $a = 74.7 \text{ Å}$, $b = 43.2 \text{ Å}$, $c = 48.9 \text{ Å}$, $\beta = 120.6^\circ$. There are three RNA 16mers (1.5 duplexes) in the crystallographic unit ($V_M = 2.0 \text{ Å}^3/\text{Da}$, solvent content ~60%). Strands A and B generated one duplex, and strand C was situated at the crystal 2-fold to generate an C-C duplex. The phases were determined using multi-wavelength anomalous dispersion (MAD) techniques. The peak and remote datasets were used in the AutoSol algorithm in PHENIX.³⁰ Initial phases with a Figure of Merit (FOM) of 0.16 were density modified in PHENIX to a final FOM of 0.86. The resulting electron density map clearly defined all nucleotides in the asymmetric unit. The model was built using COOT³¹ incorporating the bromine-modified nucleotide in position 5. The structure was refined using PHENIX to 1.65 Å resolution to a final *R*-factor and *R*-free of 0.2167 and 0.2558 respectively. Data collection and refinement statistics are presented in Table S1 in the Supporting Information. Coordinates and Structure Factors are deposited in the Protein Data Bank, PDBID 6BGB.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Sequences and modifications used. (A) PIK3CB siRNA guide strand paired with its on-target sequence or two miRNA-like off target sequences (YY1, FADD) Watson-Crick mismatches are shown in red. (B) Structures of 7-EAA and 7-EAA triazoles formed from CuAAC reactions with different azides.

Figure 2.

RNAi activity of the PIK3CB siRNAs against either the YY1 (left) or FADD (right) off target sequences. RNAi activity was measured in HeLa cells and expressed as a normalized ratio of a control luciferase (firefly) to a luciferase containing the off-target sequence in the 3' UTR (*Renilla*). Graphs detail the RNAi activity of the unmodified siRNA (red), 7-EAA (blue) and 7-EAA triazole NtPip (green). The activity for 7-EAA and 7-EAA triazole NtPip was measured at eight positions along the siRNA guide strand (1, 3, 6, 10, 12, 15, 18 and 20) in triplicate at a single concentration (0.5 nM siRNA against YY1 and 0.25 nM siRNA against FADD).

Figure 3.

Effects of modifications on siRNA selectivity. Graphs represent the difference in off-target and on-target activity by modification and guide strand position. Modification patterns that produce values above zero are considered to be more selective for the on-target sequence, as the activity against the on-target sequence is greater than the activity for the off-target sequence. Conversely, those points below zero represent a decrease in selectivity for the on-target sequence.

Figure 4.

Crystal structure of 7-EAA BrPh triazole-modified RNA. (A) The sequence of the self-complementary 16 nt RNA used for crystallization. (B) RNA duplex structure solved to 1.65 Å resolution. (C) The 7-EAA BrPh triazole hydrogen bonds to the exocyclic amine while it maintains traditional Watson-Crick base pairing with U.

Figure 5. Models of the 7-EAA modified triazoles in hAgo2-guide-target complex.¹⁴ Each picture highlights the steric clash which could occur in the closed-form miRNA-like structure with Y804. Notably, the Pyr modification is best accommodated compared to all modifications, and is the only modification that was observed to have a measurable IC₅₀ for the YY1 off-target sequence. (A) hAgo2 in complex with guide and miRNA-like target showing guide position 6 (red).¹⁴ (B) Environment of g6-t6 base pair. (C-F) Models of 7-EAA triazoles at g6.

Figure 6.

Melting temperatures for the triazolyl modifications in this study. The letter “X” designates the position where triazole modifications were incorporated into the 12 bp duplex design, while “Y” designates any of the nucleobases U, G, C or A.

Table 1

Potency and Selectivity of siRNAs with guide position 6 modifications (X)

X	On-Target IC ₅₀ pM, (PIK3CB)	Off-Target IC ₅₀ pM, (YY1)	Off/On ratio (YY1/PIK3CB)
A	15 ± 5	15 ± 5	1.0
NEtPip	44 ± 18	< 5,000	> 113
BrPh	82 ± 8	< 5,000	> 61
Pyr	35 ± 1	308 ± 77	8.8
NH ₂ Bn	13 ± 5	1358 ± 177	104
PyrBn	108 ± 22	< 5,000	> 46