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Design and implementation of synthetic RNA binders for the inhibition of miR-21 biogenesis

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Abstract

Targeting RNAs using small molecules is an emerging field of medicinal chemistry and holds the promise for the discovery of efficient tools for chemical biology. MicroRNAs are particularly interesting targets since they are involved in a number of pathologies such as cancers. Indeed, overexpressed microRNAs in cancer are oncogenic and various series of inhibitors of microRNAs biogenesis have been developed in recent years. Here, we describe the structure-based design of new efficient inhibitors of microRNA-21. Starting from a previously identified hit, we performed biochemical studies and molecular docking to design a new series of optimized conjugates of neomycin aminoglycoside with artificial nucleobases and amino acids. Detailed investigation about the mode of action and the site of the interaction of the newly synthesized compounds allowed for the description of structure-activity relationships and the identification of the most important parameters for miR-21 inhibition.

Targeting the biogenesis of oncogenic microRNAs using synthetic small molecules recently raised as a promising strategy to affect cancer cells growth.¹⁻³ MicroRNAs (miRNAs or miRs) are short non-coding RNAs that control gene expression upon binding to mRNAs and inducing the inhibition of protein synthesis.⁴ Each miRNA is responsible for the control of the expression of hundreds of proteins and the resulting regulation process is widespread in all eukaryotic cells.⁵ The biogenesis of miRNAs starts with the transcription of a miRNA gene to form a primary miRNA (pri-miRNA) of about 1kb.⁶ Processing of pri-miRNAs by the ribonuclease Drosha leads to shorter precursors of miRNAs (pre-miRNAs) of about 70 nucleotides. Both pri-miRNAs and pre-miRNAs bear a stem-loop structure where double-stranded regions are associated with single-stranded ones thus leading to the formation of particular tridimensional structures offering the possibility for specific targeting using ligands and small molecules.⁷ Pre-miRNAs are then processed by another ribonuclease called Dicer leading to a miRNA duplex of about 22-24 nucleotides. Unwinding of the miRNAs duplex leads to the formation of mature miRNA that forms a multiproteic complex called miRISC and this latter recognizes selectively the target mRNAs leading to inhibition of protein synthesis.⁸ Modifications in miRNAs expression have been recognized as major causes in various pathologies such as cancer.⁹ The miRNAs that are overexpressed in cancer are called oncogenic and inhibit the translation of tumor suppressor proteins, while miRNAs that are underexpressed are tumor suppressors and inhibit the translation of oncogenic proteins. Various approaches have thus been developed to tackle these deregulation processes, such as the use of oligonucleotides that replace for a lacking tumor suppressor miRNA or directly inhibit the function of an oncogenic one.¹⁰ Small-molecule inhibitors have also been developed as inhibitors of the biogenesis of oncogenic miRNAs and they have led to great successes in terms of inhibition activity and specificity.^{3,11-12} Among the most important examples, compounds identified using InfoRNA showed very high specificity toward the miRNA target inhibiting its production upon binding to the corresponding pri-miRNA or pre-miRNA.¹³⁻¹⁶

Recently, we developed efficient pre-miRNAs ligands directed against oncogenic miR-372 biogenesis and able to bind to its precursor pre-miR-372.¹⁷⁻²⁰ miR-372 is overexpressed in various cancers, such as gastric cancers, being directly linked to the proliferation of these cancer cells.²¹⁻²³ To identify new inhibitors of this oncogenic miRNA, we designed conjugates between the aminoglycoside neomycin, artificial nucleobases and amino acids. Aminoglycosides are known RNA ligands employed in the clinic as antimicrobials since they bind to prokaryotic ribosomal RNA thus impairing protein synthesis in bacteria.²⁴ Neomycin belongs to this class and represents a very efficient RNA ligand. Being constituted of aminooligosaccharides, these antimicrobials lack specificity and can bind efficiently other RNA structures.²⁵ Artificial nucleobases are heterocyclic compounds able to form specific hydrogen bonding interactions with DNA and RNA base pairs.²⁶ Finally, amino acids, especially basic ones, are also known to bind to RNA being the main constituent of natural RNA ligands, i.e. peptides. The resulting conjugates bear nucleobase **S** reported to interact with A·U base pair²⁷ as well as three amino acids (Lys, Arg and His) and the aminoglycoside neomycin. The most efficient inhibitor of this series was conjugate

1 containing histidine amino acid (Figure 1A).¹⁷ Compound **1** showed excellent binding and selectivity for pre-miR-372 *in vitro*. Also, it inhibited the proliferation of gastric adenocarcinoma (AGS) cells highly overexpressing miR-372 and this inhibition was specific since other cells that do not overexpress miR-372 were not affected. These studies demonstrated that the antiproliferative effect could be linked to the inhibition of miR-372 expression in AGS cells and they also revealed that this kind of conjugate inhibited in a dose-dependent manner another small set of miRNAs inside cells. Among them, miR-21 was particularly inhibited. miR-21 is a general oncogenic miRNAs in various cancers and is responsible for the repression of several important tumor suppressor proteins thereby representing an interesting anticancer target.²⁸⁻²⁹ Small molecules able to target this oncogenic miRNA have been reported showing the efficiency of this strategy for the development of antiproliferative compounds.³⁰⁻³⁶

In order to assess how compound **1** affects miR-21 biogenesis, we characterized *in vitro* its binding to pre-miR-21. First of all, the dissociation constant (K_D) was evaluated using previously reported fluorescence-based assays.¹⁹ This kind of assay is based on the measurement of fluorescence variation upon binding of increasing concentrations of ligands to a 5'-fluorescein-labeled pre-miR-21. As illustrated in Figure 1B, K_D of compound **1** was 127.6 ± 12 nM, ten times lower than the one of neomycin. Selectivity of **1** for pre-miR-21 in the presence of other nucleic acids competitors was then studied since it is an important parameter for the design of efficient pre-miRNAs inhibitors. We thus measured the K_D in the presence of a large excess (100 eq.) of competitors, such as tRNA and DNA, two intracellular abundant nucleic acid structures. Compound **1** also showed selectivity for pre-miR-21 since the K_D values were maintained in the presence of tRNA or DNA (Table 1, entries 1 and 2). Following these preliminary data, the inhibition of Dicer processing of pre-miR-21 was measured using a FRET-based assay where a 5'-fluorescein-3'-dabcyl-labeled pre-miR-21 was employed as previously reported.¹⁹ Normal cleavage by Dicer enzyme induces an increase in fluorescence, while binding of an efficient ligand that inhibits cleavage blocks fluorescence appearance. Performing this kind of assay over a range of concentrations of ligand allows for the measurement of IC_{50} values. The obtained results for compound **1** showed an IC_{50} of 1.26 ± 0.6 μ M (Figure 1C and Table S1). This promising result prompted us to evaluate the selectivity of the inhibition activity. We thus measured IC_{50} using the same cell-free assay but in the presence of MCF7 cell lysates instead of recombinant Dicer enzyme since a large number of protein and nucleic acid competitors are present in the mixture and could impair the activity of **1** on pre-miR-21 if the compound acts non-selectively. Interestingly, promising selectivity of inhibition could be observed when lysates were employed, since the IC_{50} value was 3.10 ± 0.9 μ M that represents a 2.5 ratio between the two values. Altogether, these results are promising for the development of compounds of that type against miR-21 production. We thus decided to explore the site of interaction of compound **1** on the pre-miR-21 structure using molecular docking. While Figure 1D shows the primary and secondary structure of pre-miR-21 as reported in the miRbase (www.mirbase.org), Figures 1E and 1F illustrate the most stable docking pose obtained using the pre-miR-21 hairpin loop after applying the MC-Fold/MC-Sym pipeline to construct a 3D model. Molecular

docking of **1** using AutoDock showed the possible interactions formed with the target pre-miR-21. We observed that conjugate **1** interacted with residues U27 to G28 and A50 to C52 that are located close to the cleavage site of Dicer enzyme (A29/C46) but in the stem part of the pre-miR-21 structure. It has been demonstrated that the stem region of each pre-miRNA (the so-called ruler) is important for the correct positioning of Dicer and that ligands interacting with this region can be efficient inhibitors of pre-miRNAs processing.³⁷⁻³⁸ More specifically, the interaction of **1** with pre-miR-21 involves the formation of hydrogen bond interactions between the imidazole of histidine and U27 and G28 as well as electrostatic interactions between the neomycin moiety and residues A50-C52. The triazole is also important as it interacts with residue C49. At last, it can be noted that the nucleobase does not seem to be involved illustrating that the spatial distribution of the three binding domains was probably not optimal for efficient binding. We thus decided to explore the possibility to improve inhibition of miR-21 biogenesis upon chemical modifications that could increase and optimize the interactions network formed. Based on the intracellular inhibition of miR-21 expression,¹⁷ on the promising values of K_D and IC_{50} obtained during biochemical assays and on the molecular docking studies described above, we designed compounds bearing a more flexible linker between the neomycin moiety and the nucleobase, as well as a different tridimensional distribution of the RNA binding domains. To this aim, we envisaged to modify the chemical structure of **1** by addition of an aliphatic linker between the triazole and the nucleobase and by placing the amino acid residue on this linker as illustrated in the general structure of Figure 2A. Molecular docking helped us to understand if such compounds could be better pre-miR-21 binders than compound **1**. The docking of a compound of this new series containing histidine as the amino acid (**6c**) is illustrated in Figures 2B and 2C and showed that this compound interacted efficiently with pre-miR-21. In this case, the three moieties histidine, **S** and neomycin participated to the interaction suggesting the possibility for better selectivity and activity compared to **1**. Compound **6c** interacts with residues U15-A17 as well as A50-C51 *via* an increased number of hydrogen bonds and electrostatic interactions compared to compound **1**.

Based on these results, we decided to synthesize a new series of compounds bearing the general structure illustrated in Figure 2A. To this aim, we conjugated neomycin to nucleobase **S** that is the one used in the docking study, but also to **SAr**, a similar artificial nucleobase containing a supplementary aryl substituent that was previously shown to be very promising for better interaction with pre-miRNAs.²⁰ Furthermore, we decided to compare histidine amino acid with alanine to measure the influence of a hydrophobic side chain and with lysine that contain a basic but aliphatic side chain to compare with the histidine imidazole. A control compound containing uracil instead of nucleobase **S** was also prepared to study the influence of the nucleobase on the affinity and biological activity (compound **15**, Scheme S2). The synthesis of conjugated aminoglycosides for better RNA binding is a challenging field and even if a number of synthetic routes have been explored to conjugate various moieties at different positions on the aminoglycoside, new synthetic approaches are always needed.³⁹ Here first, we decided to use a solid-phase synthetic strategy as illustrated in Scheme 1 to avoid purification steps. Rink amide MBHA resin

was first deprotected in the presence of piperidine in DMF and the resulting free amine was charged at 50% with the amino acid *Na*-Fmoc-L-alanine (as in **2a**), *Na*-Fmoc-*Nw*-Boc-L-lysine (as in **2b**) or *Na*-Fmoc-*Nlm*-Boc-L-histidine (as in **2c**) in the presence of HBTU, DIPEA in DMF. After capping the unreacted amines with acetic anhydride in the presence of pyridine in DMF, the added amino acid was deprotected by piperidine in DMF and *Na*-Fmoc-L-propargyl-glycine was coupled to the free amine by HBTU in the presence of DIPEA in DMF. Capping by acetic anhydride in pyridine and DMF led to desired intermediates **3a-c**. The Fmoc group was removed again and the resulting amine was reacted with succinic anhydride in the presence of DIPEA in DMF. Final capping led to desired compounds **4a-c**. This scaffold now contains a resulting free carboxyl group for the introduction of the nucleobase and an alkyne group for the introduction of an azido-substituted neomycin derivative. First, the nucleobases **S** and **SAr**,^{17,20} were coupled to the carboxyl group in the presence of iodomethylpyridinium chloride, DIPEA and DMF leading to compounds **5a/a'-5c/c'**. Then, 1,3 dipolar cycloaddition was performed with Boc-protected 5''-azido-neomycin (Neo(Boc)₆N₃),²⁰ in the presence of CuI and DIPEA and DMF. This step needed 4 equivalents of CuI (2x2eq.) as Cu adsorbs on the solid support. Then, removal of Boc groups by a mixture TFA/TIS led simultaneously to resin cleavage as well as the release of large quantities of copper with desired conjugates **6a/a'-6c/c'** which had to be removed by successive treatments with chelex resin to capture Cu, leading to low overall yields < 20%. These low yields are mainly due to the need to remove copper, released during the cleavage step. To circumvent this constraining purification step, we envisaged to perform the deprotection/cleavage step before the 1,3-dipolar cycloaddition in order to carry out the click reaction in liquid-phase and to avoid copper accumulation on the solid support. This strategy allowed us to obtain compounds **6a/a'** and **6b'** with increased global yields (between 35% and 45%). For comparison, the other compounds (**6b** and **6c/c'**) were prepared through a complete liquid-phase synthesis (Supporting Information Scheme S1) and led to final compounds with overall yields similar to the solid phase methodology but with the need for various purification steps. The synthesis of the desired conjugates was thus successful even if their preparation remains complex.

Compounds **6a/a'-6c/c'** were designed in order to improve affinity and inhibition activity against pre-miR-21 and its biogenesis as previously described for **1**. We thus decided to first study the binding affinity toward pre-miR-21 and compare the obtained values with non-conjugated neomycin and parent compound **1**. As mentioned before, we also prepared compound **15** (Scheme S2) containing natural nucleobase uracil instead of artificial nucleobase **S**. As illustrated in Table 1 (and in Supporting Information Figure S1), all conjugated compounds (entries 3-8) bear a K_D in the low nM range thus showing great improvement in the binding affinity compared to unconjugated neomycin. Compounds containing nucleobase **S** (**1** and **6a-c**) bear better affinity than the ones containing **SAr** nucleobase (**6a'-6c'**) thus suggesting that the addition of a phenyl ring on the nucleobase probably hinders the optimal interaction with the target. Indeed, compounds **6a'**, **6b'** and **6c'** show K_D values of 128.5, 347.7 and 162.2 nM, respectively, thus being weaker binders than the corresponding **S** derivatives **6a**, **6b** and **6c**.

While compound **6a** showed K_D value of 133.3 nM, compounds **6b** and **6c** showed a K_D values of 50.7 and 50.3 nM, respectively, representing a 2.5 fold improvement in comparison with compound **1**. This also illustrates that a positively charged and hydrophilic amino acid side chain is particularly important for interaction since **6b** and **6c** containing lysine and histidine, respectively, have better affinity for the target than **6a** containing alanine. Interestingly, compound **15** containing uracil instead of **S** bears a K_D of 641.3 nM that is a 13-fold higher value compared to **S** conjugates illustrating that the artificial nucleobase also plays an important role in pre-miR-21 binding. This suggests that the spatial distribution of these new compounds could be more favorable for the involvement of the nucleobase in the interaction compared to what observed for compound **1**. Selectivity studies in the presence of tRNA or DNA showed that the ratios between K_D' (dissociation constant in the presence of tRNA) or K_D'' (dissociation constant in the presence of DNA) and K_D are close to 1 (Table 1, entries 3-9). Only compounds **6a'-6b'** and **15** showed slightly higher values. Compounds **6b** and **6c** thus bear the best affinity for the target and all conjugates are selective for pre-miR-21 when compared to other nucleic acid structures.

Based on these binding data, we wondered whether these compounds could be good inhibitors of its processing by Dicer enzyme. We thus performed the FRET-based assay described above for the study of the inhibition activity of **1**. The obtained results (Figure 3, Figure S2 and Table S1) indicate that all conjugates inhibit Dicer processing of pre-miR-21 in the low micromolar range with compounds **6a'**, **6b**, **6c** and **6c'** being the best inhibitors with IC_{50} s below 5 μ M. Selectivity studies performed using MCF7 lysates showed that only compounds **6b** and **6c** bear an optimum selectivity since all other compounds lose two- to four-time inhibition ability. It must be noted that even if the affinity of **6b** and **6c** for pre-miR-21 it was improved compared to the one of compound **1**, the inhibition activity remained in the same range as **1**. However, the inhibition induced by **6b** and **6c** seems to be more specific compared to the inhibition of **1**.

To gain a better understanding of the molecular mechanism of interaction, we thus decided to study more in detail their binding with the target. First, we performed molecular docking studies for compounds **6b** and **6c**. Docking of **6c** with pre-miR-21 was previously illustrated in Figures 2B and 2C while docking of compound **6b** is illustrated in Figures 4A and 4B. Both compounds interact with the same site with similar interactions. More specifically, **6b** interacts with C16-U21 and C49-A53 while **6c** with U15-A17 and A50-C51 upon formation of hydrogen bonds, π - π interactions as well as electrostatic interactions. This binding site is similar to the one of compound **1** and efficient binding and selectivity illustrate that both histidine and lysine are favorable amino acids for the preparation of this type of ligands. Noteworthy, the nucleobases **S** and **SAr** are closely involved in the interaction and the spatial distribution of the three binding domains is more accurate than the one of compound **1**, as demonstrated also experimentally by K_D values. While **6b** and **6c** are the best pre-miR-21 ligands and inhibitors both in terms of affinity and selectivity **6c'** bears a three-time higher K_D value than **6b** and **6c** but very similar inhibition activity. Comparison with **6c'** shows that this latter interacts with another

binding site located at the cleavage site of Dicer, i.e. U27-G28 and C41-A42 (Supporting Information Figure S3) but with a lower number of interactions thus being a less efficient binder. The interactions formed are mainly hydrogen bonds and electrostatic interactions, but further intramolecular interactions are involved probably hindering the correct interaction with the target and causing the observed increase in the K_D value.

To complete these studies, we decided to measure the thermodynamic parameters of the complex formed with pre-miR-21 for all the synthesized compounds in comparison with **1**. Free energies of Gibbs (ΔG°) were first calculated from the dissociation constants ($\Delta G^\circ = -RT \ln K_D$) and found to be very similar (from -36.6 to -41.5 kJ/mol), with **6b** and **6c** forming the most favorable complexes (Figure 4C). To complete the thermodynamic binding profiles, enthalpic (ΔH°) and entropic ($-T\Delta S^\circ$) energy contributions were determined after the determination of ΔG°_T at several temperatures (278–308 K). In all cases, the formation of ligand/pre-miR-21 complexes is driven by entropy indicating a strong desolvation effect during interaction. The ΔG° value can be divided into the $\Delta G^\circ_{\text{nel}}$ value, which reflects the contribution of non-electrostatic interactions to the total free energy, such as non-ionic hydrophobic effects driven by entropy, and specific interactions, including H-bonds, van der Waals interactions, and π -stacking, and the pure electrostatic (polyelectrolyte) contribution, $\Delta G^\circ_{\text{el}}$, which reflects the ionic interactions occurring between two groups of opposite charge and is highly dependent on the salt concentration. We found that the interactions mainly involve non-electrostatic interactions because the $\Delta G^\circ_{\text{nel}}$ component represents 87–99% of the overall free energy (Table S2). Although this is an important parameter that should contribute to the observed selectivity, binding could be probably improved by non-covalent interactions such as H-bonds, π -stacking, π -ions for an increase in the enthalpy component. In this work, we took advantage of an original binder that proved to inhibit miR-21 biogenesis in cancer cells to perform the structure-based design of a new series of pre-miR-21 ligands, based on the tridimensional structure of this RNA. These ligands were synthesized upon conjugation of three RNA binding domains and the *in vitro* study of their affinity, selectivity and inhibition activity allowed for the identification of two compounds, **6b** and **6c**, that are efficient and selective binders. The detailed study of their binding to pre-miR-21 confirmed that the three binding domains, i.e. artificial nucleobase S, neomycin and histidine or lysine, act cooperatively to bind the target and that the binding site is located in the stem part of the pre-miR-21 where two bulges induce the distortion of the double-helix. The obtained results confirm that the structure-based design of efficient binders was successful. Altogether, these results support the idea that the inhibition efficacy of an RNA binder depends upon both the affinity and the binding site on each pre-miRNA. Future works will be devoted to the optimization of these compounds reducing the size of aminoglycoside moiety in the aim to increase selectivity and enthalpic contribution to binding and to confirm their activity in cell-based studies.

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Figures and Schemes

Figure 1. A) Chemical structure of the conjugate **1**. B) Dissociation constant (K_D) evaluation for compound **1** and its affinity for pre-miR-21 sequence. The reported K_D value is expressed in nM and calculated over three independent experiments performed in duplicates. C) IC_{50} evaluation for compound **1** inhibition of pre-miR-21 processing in the presence of *E. coli* RNase III purified enzyme (◆) and in the presence of MCF-7 lysates (●). The reported IC_{50} value is expressed in μ M and calculated over three independent experiments performed in duplicates. D) Structure of pre-miR-21 as reported in the miRbase (www.mirbase.org) with the binding site of **1** highlighted in yellow. E) Docking of **1** with the pre-miR-21 hairpin loop performed using autodock 4, in which the grid boxes were fixed on the entire RNA sequence. F) Compound **1** and direct interacting residues of pre-miR-21 sequence together with the formed interaction in the complex.

Figure 2. A) General structure of the new series of conjugates synthesized in this work. B) Docking of the new conjugate containing neomycin, histidine and nucleobase S (**6c**) with the pre-miR-21 hairpin loop performed using autodock 4. C) Compound **6c** and direct interacting residues of pre-miR-21 sequence together with the interactions in the complex.

Figure 3. IC_{50} values of compounds **6a/a'-6c/c'** against pre-miR-21 72-mer fragment labeled at the 5'-end with fluorescein and at the 3'-end with dabcyI measured upon processing with recombinant enzyme (●). Selectivity of compounds **6a/a'-6c/c'** for pre-miR-21 in the presence of MCF7 cell lysates (●). IC_{50} values were determined from duplicates performed over three independent experiments.

Figure 4. A) Thermodynamic profiles for the high-affinity binding of heterocycle-spermine conjugates **1** and **6a/a'-6c/c'** to the pre-miR-21 at 20 °C: DG° (black bars), ΔH° (dark gray bars), and $-T\Delta S^\circ$ (light gray bars). B) Molecular docking of **6b** in interaction with pre-miR-21 hairpin loop performed using autodock 4 wherein the grid boxes were fixed on the entire RNA sequence. C) Compound **6b** and direct interacting residues of pre-miR-21 sequence together with the interactions in the complex.

Scheme 1. Solid-phase synthetic pathway for the preparation of new conjugates **6a/a'-6c-c'** containing neomycin, artificial nucleobase S and amino acids alanine, lysine and histidine. Final compounds were obtained as TFA salts even if the neutral structure was written for clarity. Reagents: a) piperidine, DMF, r.t., 30 min; b) Fmoc-Ala-OH, Fmoc-Lys-OH or Fmoc-His-OH, HBTU, DIPEA, DMF, r.t., 2h; c) Ac_2O , pyr, DMF, r.t., 10 min; d) piperidine, DMF, r.t., 30 min; e) Fmoc-propargyl-Gly, HBTU, DIPEA, DMF, r.t., 2h; f) Ac_2O , pyr, DMF, r.t., 10 min; g) piperidine, DMF, r.t., 30 min; h) succinic anhydride, DMF, r.t., 1h; i) Ac_2O , pyr, DMF, r.t., 10 min; j) **S** or **SAr**,¹⁸ iodomethylpyridinium iodide, DIPEA, DMF, r.t., 1.5 h; k) Neo(Boc)₆N₃,¹⁷ CuI, DIPEA, DMF, r.t., overnight; l) TFA, TIS, r.t., 2h.

Figure 1.

Figure 2.

Figure 3.

Figure 4.

Scheme 1.

Table 1. Dissociation constants (K_D , nM) measured for pre-miR-21 binding alone, or in the presence of a 100 eq. excess of tRNA (K'_D , nM) or a 100 eq. excess of DNA duplex (K''_D , nM).

Entry	ID	K_D^a [nM] pre-miR-21	K'_D [nM] (tRNA competition)	K'_D/K_D	K''_D [nM] (DNA competition)	K''_D/K_D
1	Neomycin	1390 ± 126	1415 ± 110	1.0	1950 ± 20.2	1.4
2	1	127.6 ± 12.0	134.0 ± 14.2	1.0	171.2 ± 16.5	1.3
3	6a	133.3 ± 20.0	151.2 ± 13.9	1.1	187.3 ± 23.6	1.4
4	6a'	128.5 ± 7.0	169.7 ± 48.5	1.3	260.7 ± 59.6	2.0
5	6b	50.7 ± 7.4	51.0 ± 8.7	1.0	95.7 ± 21.6	1.9
6	6b'	347.7 ± 33.6	404.9 ± 138.3	1.2	876.8 ± 79.2	2.5
7	6c	50.3 ± 11.4	50.4 ± 7	1.0	80.9 ± 3.2	1.6
8	6c'	162.2 ± 39.8	168.1 ± 10.4	1.0	274.2 ± 30.3	1.7
9	15	641.3 ± 165	806.9 ± 270.2	1.3	1459 ± 70.0	2.3

^a Binding studies were performed on 5'-FAM-pre-miR-372 or -373 in buffer A (20 mM Tris-HCl (pH 7.4), 12 mM NaCl, 2.5 mM MgCl₂, and 1 mM DTT).