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Biaryl modification of the 5′-terminus of one strand of a microRNA duplex induces strand specificity

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ABSTRACT

MicroRNAs (miRNAs) are single-stranded non-coding RNAs composed of 20–23 nucleotides. They are initially transcribed in the nucleus as pri-miRNAs. After processing, one strand from the miRNA duplex (miR-5p/miR-3p duplex) is loaded onto the RNA-induced silencing complex (RISC) to produce a functional, mature miRNA that inhibits the expression of multiple target genes. In the case of some miRNAs, both strands can be equally incorporated into the RISC as single strands, and both strands can function as mature miRNAs. Thus, a technique for selective expression of miR-5p and miR-3p strands is required to identify distinct targets of miRNAs. In this Letter, we report the synthesis and properties of miRNA duplexes carrying biaryl units at the 5′-terminus of one strand. We found that incorporation of biaryl units at the 5′-terminus of one strand of miRNA duplexes induced strand specificity in these duplexes. Further, we succeeded in identifying endogenous mRNA targets for each strand of the duplex by using the biaryl-modified miRNA duplexes.

MicroRNAs (miRNAs) are short non-coding RNAs that suppress gene expression at the post-transcriptional level in animals and plants.[1] In mammals, miRNAs target mRNAs through translational repression and degradation. miRNA genes are mainly transcribed by RNA polymerase II to produce primary miRNA transcripts (pri-miRNAs) containing a 5′-cap structure and a poly(A) tail. The pri-miRNA is processed into a precursor stem-loop structure called pre-miRNA by the microprocessor complex Drosha-DGCR8. The pre-miRNA is then exported from the nucleus to the cytoplasm where the terminal loop is excised from the stem by the Dicer enzyme to create an miRNA duplex of 20–23 bp. One of the strands from the miRNA duplex is loaded onto the RNA-induced silencing complex (RISC) to become a functional mature miRNA strand that inhibits the expression of multiple target genes.[2] Over 700 miRNAs have been identified in humans, and some have been found to play important roles in development, differentiation, and proliferation.[3] Among them, miR-199a has been known to exhibit aberrant expression patterns in human cancer and infection. It has been reported that miR-199a expression is impaired in different types of cancer such as hepatic cancer, ovarian cancer, and cervical cancer.[4–6] Interesting insights about the role of miR-199a in skeletal development and hepatitis C virus (HCV) replication have also been published.[7,8]

It is believed that RISC preferentially selects and incorporates one of the strands of the miRNA duplex, depending on its thermodynamic features. The strand with lower thermodynamic stability at its 5′-terminus (the guide strand) preferentially binds to the RISC and becomes functional, whereas the other strand (the passenger strand) is degraded.[9,10] However, recent studies show that strand selection does not always follow this rule. In the case of some miRNA duplexes, both strands can be incorporated into the RISC as single strands, and each strand can function as a mature miRNA, despite the thermodynamic asymmetry between the strands.[11] Among human miRNAs registered in the online microRNA database miRBase <http://www.mirbase.org/>, 36% originate from the 5′-strand (miRNA-5p), 37% originate from the 3′-strand (miRNA-3p), and 27% are miRNA duplexes, each producing two miRNAs from the 5′- and 3′-strands.[12,13] miR-199a is a type of miRNA duplex that produces two mature miRNAs, miR-199a-5p and miR-199a-3p. It is believed that the members of an miRNA duplex can target two distinct sets of genes according to the nucleotide sequence.

A technique for selective expression of miR-199a-5p and miR-199a-3p is required to identify the distinct targets of miRNAs generated from pre-miRNA generated in this manner. Pre-miRNA expression vectors or double-stranded miRNAs are commonly used for over-expression, but these methods do not specify which strand is expressed. It was recently reported in siRNA studies that

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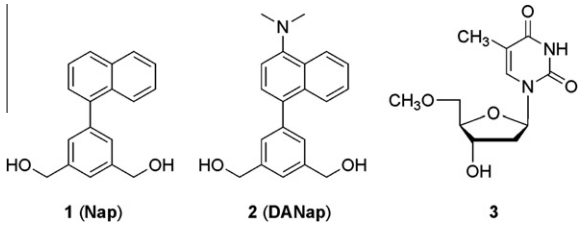


Figure 1. Structures of biaryl units and nucleoside analogs.

5′-*O*-methyl-modification of the 5′-terminus of one strand efficiently allows its antisense strand to be loaded onto the RISC, despite thermodynamic instability.[13] This is because phosphorylation of the 5′-terminus—an important factor for RISC loading—is inhibited by the 5′-*O*-methyl group. However, this modification can be applied to only the sequence with uridine at the 5′-terminus of the passenger strand, since only 5′-*O*-methylthymidine (**3**) (Fig. 1) is now commercially available. Recently, we also found that the incorporation of naphthalene-type biaryl unit **1** (**Nap**) into the 5′-termini of the passenger strands of siRNAs can suppress the silencing activities induced by the passenger strands (off-target effect) without reducing the silencing activities of the guide strands.[14,15] The **Nap**-modification was more effective than the 5′-*O*-methyl-modification in inhibition of function of a passenger strand. From these results and existing information, we deduced that the addition of biaryl units to the 5′-terminus of one strand of an miRNA duplex could induce better strand specificity of the miRNA duplexes than with the addition of a methyl group, since biaryl units are sterically bulkier than methyl groups, and increase the thermal stability of duplexes by hydrophobic interactions. Further, the **Nap**-modification can be applied to all sequences including the sequence without uridine at the 5′-terminus of a passenger strand.

In this Letter, we report the synthesis and properties of miR-199a duplexes carrying biaryl units at the 5′-terminus of one strand of each duplex (Figs. 1 and 2). We examined gene silencing activities of the modified miRNA duplexes by the dual-luciferase reporter assay. In addition, we tried to identify endogenous mRNA targets for miR-199a-5p and miR-199a-3p strands by using the modified miR-199a duplexes.

We selected two types of biaryl compounds, naphthalene-type **1** (**Nap**) and dimethylnaphthalene type **2** (**DANap**), as the monomer units to be incorporated in the miRNA duplexes. **DANap** is sterically bulkier than **Nap**. The synthesis of the phosphoramidite unit of **DANap** is depicted in Scheme 1. An arylboronic acid derivative **4** was coupled with 1-bromo-4-(dimethylamino)naphthalene (**5**) in the presence of $PdCl_2$(dppf) (dppf = 1,1′-bis(diphenylphosphino)-ferrocene) at 65 °C to afford the biaryl derivative **6** with a yield of 81%. Subsequently, **6** was desilylated by tetra-*n*-butylammonium fluoride (TBAF) treatment to afford biaryl unit **2** with a yield of 66%. One of the two hydroxy groups of **2** was protected by a 4,4′-dimethoxytrityl (DMTr) group to give a mono-DMTr derivative **7**, with 44% yield. Compound **7** was phosphitylated by the standard procedure to afford the corresponding phosphoramidite **9** with 76% yield. All oligonucleotides (ONs) were synthesized using the phosphoramidites of **Nap** and **DANap** with a DNA/RNA synthesizer.

The ability of modified miRNAs to suppress gene expression was determined by the dual-luciferase reporter assay using a psi-CHECK-2 vector (Promega) with *Renilla* and firefly luciferase genes as reporter genes. We constructed psi-199a-5p and psi-199a-3p vectors by inserting sequences complementary to miR-199a-5p and miR-199a-3p strands in the *Luc* 3′-UTR region of the psiCHECK-2 vectors. Reporter vectors and synthesized miRNA duplexes were co-transfected into HeLa or HeLa S3 cells with TransFast (Promega) or Lipofectamine2000 (Invitrogen) as transfection reagents, and luciferase activities were measured after 24 h. The signals of *Renilla* luciferase were normalized to those of firefly luciferase (Fig. 3).

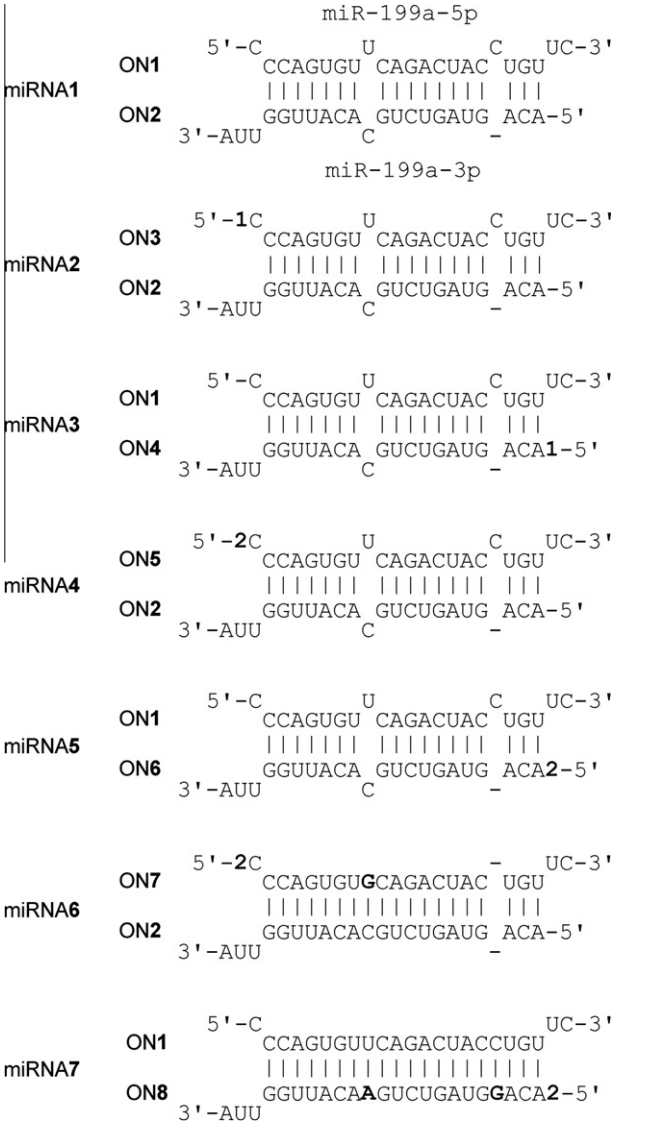
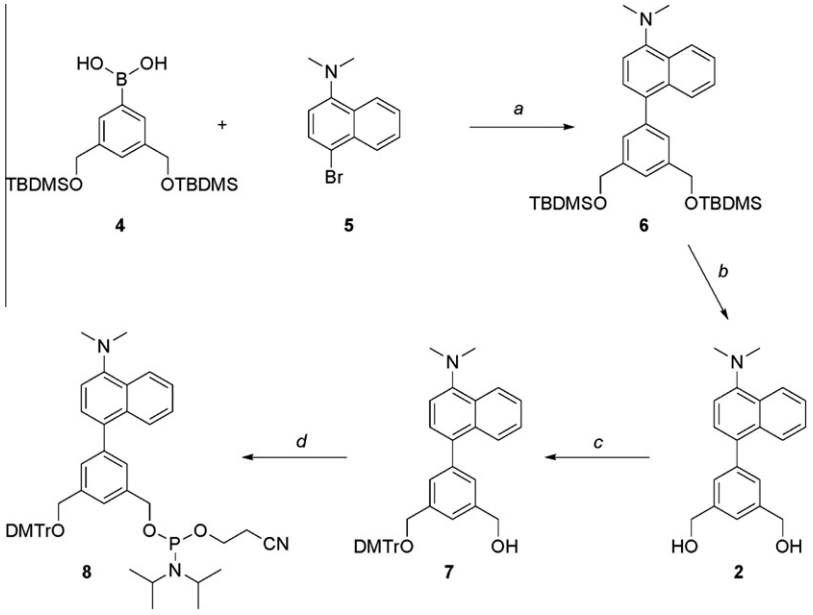


Figure 2. Sequences of oligonucleotides (ONs) and miRNAs used in this study. Structures of **1** (**Nap**) and **2** (**DANap**) are indicated in Fig. 1. Mutated bases are shown in bold letters.

An unmodified miR-199a duplex, miRNA**1**, effectively reduced luciferase activity in both vectors in a dose-dependent manner; luciferase activity of the psi-199a-5p vector was reduced from 72% to 41% and that of the psi-199a-3p vector, from 61% to 22%. Differences in the reduction levels between vectors may reflect the asymmetry of RISC loading between natural miR-199a-5p and miR-199a-3p. When miRNA**2** and miRNA**4**—modified with **Nap** or **DANap** at the 5′-termini of miR-199a-5p strands—were transfected, luciferase activity of the psi-199a-5p vector was higher than that of the psi-199a-3p vector with both the modifications (Fig. 3a). Thus, it was found that the **Nap** and **DANap** modifications at the 5′-termini of miR-199a-5p strands could induce miR-199a-3p strand specificity of miR-199a-5p/miR-199a-3p duplexes.

On the other hand, when miRNA**3** and miRNA**5**—modified with **Nap** or **DANap** at the 5′-termini of miR-199a-3p strands—were transfected, luciferase activity of the psi-199a-3p vector was higher than that of the psi-199a-5p vector with the **DANap** modification



Scheme 1. Reagents and conditions: (a) $PdCl_2(dppf)\cdot CH_2Cl_2$, NaOH, THF/$H_2O$ (5:1 v/v), 65 °C, 24 h, yield of biaryl derivative **6**: 81%; (b) TBAF, THF, room temperature, 12 h, yield of biaryl unit **2**: 66%; (c) DMTrCl, pyridine, room temperature, 12 h, yield of mono-DMTr derivative **7**: 44%; (d) chloro(2-cyanoethoxy)(*N*,*N*-diisopropylamino)phosphane, *i*-Pr_2NEt, THF, room temperature, 1 h, yield of corresponding phosphoramidite **9**: 76%.

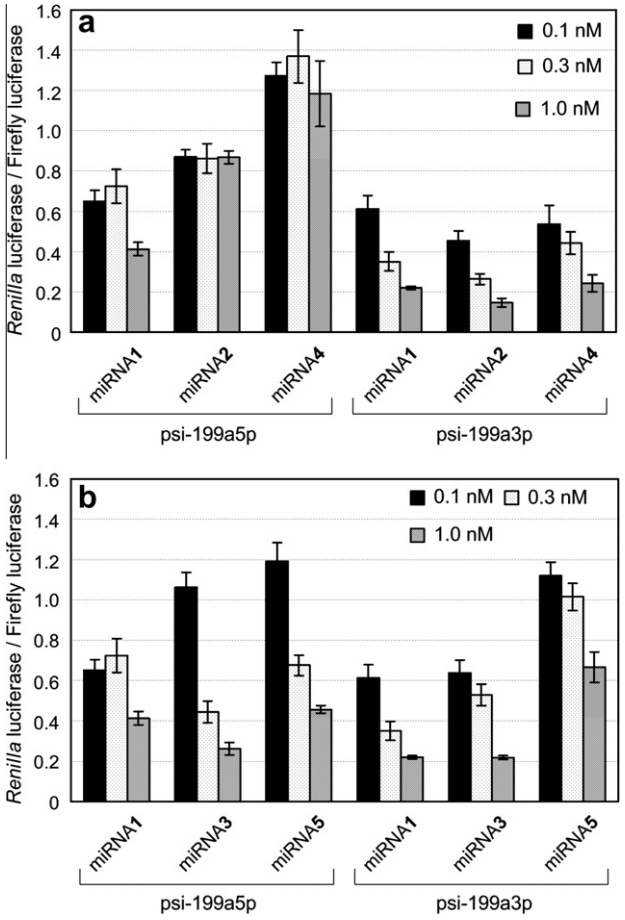


Figure 3. Dual-luciferase reporter assay (**1**). Reporter vectors and synthesized miRNA duplexes (final concentrations: 0.1, 0.3, or 1.0 nM) were transfected into the cells (HeLa cells) using TransFast (Promega). Firefly and *Renilla* lu-ciferase activities were measured consecutively using Dual-Luciferase Reporter Assay System (Promega). *Renilla* lu-ciferase expression was normalized to that of Firefly lu-ciferase. Normalized expression in mock-transfected cells was set as 1.0. The results were confirmed by at least four independent transfection experiments and are expressed as the average from four experiments as mean ± SD.

while that of the psi-199a-3p vector with the **Nap**-modification was similar to that of the psi-199a-5p vector at concentrations of 0.3 nM and 1.0 nM (Fig. 3b). These results indicate that the **DANap** modification is the most promising in eliciting miR-199a-5p and miR-199a-3p strand specificities of miR-199a-5p/miR-199a-3p duplexes.

We succeeded in inducing the expression of the miR-199a-5p and miR-199a-3p strands individually, using the **DANap**-modified miR-199a duplexes. However, small amounts of unanticipated changes in expression can still occur in these modified miRNAs when injected into cells. In order to eliminate this off-target effect, we further artificially changed the sequence of the opposite strands to form complete double-stranded RNAs as depicted in Fig. 2. We evaluated the strand specificity of these duplexes by

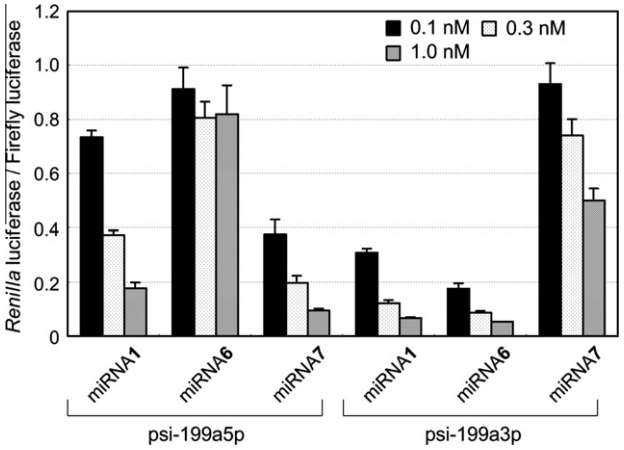


Figure 4. Dual-luciferase reporter assay (2). Reporter vectors and synthesized miRNA duplexes (final concentrations: 0.1, 0.3, or 1.0 nM) were transfected into the cells (HeLa S3 cells) using Lipofectamine2000 (Invitrogen). Firefly and *Renilla* lu-ciferase activities were measured consecutively using Dual-Luciferase Reporter Assay System (Promega). *Renilla* lu-ciferase expression was normalized to that of Firefly lu-ciferase. Normalized expression in mock-transfected cells was set as 1.0. The results were confirmed by at least four independent transfection experiments and are expressed as the average from four experiments as mean ± SD.

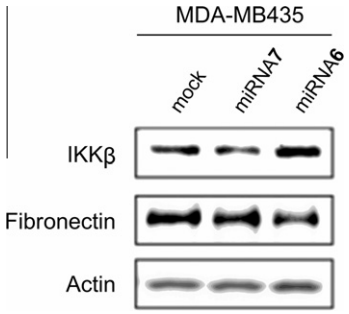


Figure 5. Detection of target proteins by high-level expression of miR-199a-5p and miR-199a-3p. MDA-MB-435 cells were transfected with 30 nM **DANap**-modified miRNA duplexes, and after 48 h, protein extracts were prepared. IKKβ, fibronectin, and β-actin proteins were analyzed by western blotting.

using the same luciferase assay system (Fig. 4). For **DANap**-modified duplexes, miRNA**6** modified at the 5′-terminus of the miR-199a-5p strand reduced luciferase activity of psi-199a-3p from 18% to 5%, but not of psi-199a-5p. In contrast, miRNA**7** modified at the 5′-terminus of the miR-199a-3p strand reduced luciferase activity of psi-199a-5p from 38% to 9%, but not of psi-199a-3p. It is noteworthy that the 5p and 3p strands of miRNA**6** and miRNA**7** did not suppress luciferase activity efficiently due to the applied mutations, and the anticipated effects of the synthesized duplexes were enhanced as compared to duplexes with the natural miRNA sequence, thus resulting in better strand selectivity.

It was previously reported that IKKβ is a target of miR-199a-5p and fibronectin, a target of miR-199a-3p.[16,17] Therefore, we tried to determine endogenous miR-199a target genes by using the strand-specific over-expression method that we devised. MDA-MB-435 cells (expressing low levels of miR-199a) were transfected with **DANap**-modified miR-199a duplexes for over-expression. Western blotting showed that in MDA-MB-435 cells, IKKβ expression was suppressed by selective expression of miR-199a-5p originating from miRNA**7** duplexes, and not miRNA**6** (Fig. 5). It was also shown that fibronectin expression was specifically suppressed by miRNA**6**, and not miRNA**7**. Our method involving selective over-expression of miR-199a-5p and miR-199a-3p could be used for the regulation of endogenous target gene expression and that it could be applied for the identification of new target genes.

In conclusion, we have demonstrated the synthesis of miRNA duplexes modified with biaryl compounds. We found that the **DANap** modification was the most effective in eliciting strand specificity of miR-199a-5p/miR-199a-3p duplexes. Further, we succeeded in identifying endogenous mRNA targets for miR-199a-5p and miR-199a-3p strands by using the **DANap**-modified miR-199a-5p/miR-199a-3p duplex. Thus, **DANap** modification would be useful to identify endogenous mRNA targets for other miRNAs.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.10.077.

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