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Development of Novel Antisense Oligonucleotides for the Functional Regulation of RNA-Induced Silencing Complex (RISC) by Promoting the Release of microRNA from RISC

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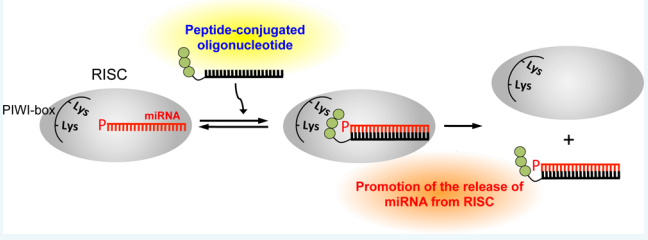
Supporting Information

ABSTRACT: MicroRNAs (miRNAs) are known to be important post-transcription regulators of gene expression. Aberrant miRNA expression is associated with pathological disease processes, including carcinogenesis. Therefore, miRNAs are considered significant therapeutic targets for cancer therapy. MiRNAs do not act alone, but exhibit their functions by forming RNA-induced silencing complex (RISC). Thus, the regulation of RISC activity is a promising approach for cancer therapy. MiRNA is a core component of RISC and is an essential to RISC for recognizing target mRNA. Thereby, it is expected that development of the method to promote the release of miRNA from RISC would be an effective approach for inhibition of RISC activity. In this study, we synthesized novel peptide-conjugated oligonucleotides (RINDA-as) to promote the release of miRNA from RISC. RINDA-as showed a high rate of miRNA release from RISC and high level of inhibitory effect on RISC activity.



INTRODUCTION

MicroRNAs (miRNAs) are a type of noncoding RNA that induce post-transcriptional gene silencing of their target genes and regulate a wide range of biological processes, including apoptosis, differentiation, metabolism, and cell proliferation.[1−6] According to recent reports, the aberrant expression of miRNAs is associated with most pathological disease processes, including carcinogenesis.[7−12] In fact, a vast number of studies have reported that miRNA expression is significantly different between normal and tumor cells in humans.[13] Therefore, miRNAs are considered significant therapeutic targets for cancer therapy.

The primary transcripts of miRNA genes are transcribed in the nucleus.[14] The precursor miRNAs are exported and processed to an approximately 22-nucleotide miRNA duplex[15,16] by some enzymes, such as Drosha, Exportin-5, and Dicer.[17−19] The miRNA duplex is loaded into Argonaute protein (Ago) and rapidly unwound in a ribonucleoprotein complex called RNA-induced silencing complex (RISC).[20−23] During this process, one strand of the miRNA duplex is retained in RISC, while the other strand is released from Ago.[20,24] MiRNAs do not act alone but exhibit their function by forming RISC. Therefore, to regulate miRNA functions, RISC activity must be controlled.

The most conventional approach for the inhibition of RISC functions is an antisense oligonucleotide (ASO) with the complementary sequence of the miRNA in RISC.[25−27] In fact, several studies have demonstrated the effectiveness of ASO as an anticancer drug targeting miRNA.[28] ASOs have been optimized for increased binding affinity, improved nuclease resistance, and in vivo delivery. To achieve the purpose, a variety of chemical modifications of ASO have been developed, including modifications of the nucleobase, the sugar, and the internucleotide linkages.[29] These modifications have improved the inhibitory effects of ASO on miRNA functions and have been adapted for nucleic acid drugs.[28,29] For instance, an miRNA inhibitor termed "Miravirsen", which is a β-D-oxy-locked nucleic acid-modified phosphorothioate antisense oligonucleotide targeting the liver-specific miRNA-122 (miR-122), has demonstrated broad antiviral activity and has shown promising results in phase II clinical trials.[30,31] Now, ASOs are considered promising molecules for the regulation of RISC functions; therefore, ASOs should be improved further as with other regulation pathways for miRNA. The purpose of this

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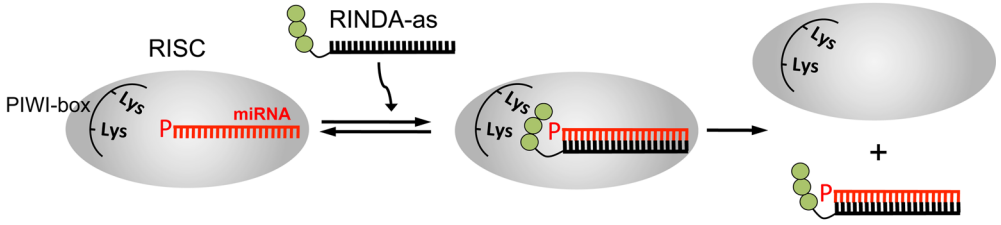
(A)



(B)

Sequences of antisense oligonucleotides

		Seed region *								Cleavage site **							3′ region								Tm (°C)
		N1	N2	N3	N4	N5	N6	N7	N8	N9	N10	N11	N12	N13	N14	N15	N16	N17	N18	N19	N20	N21			
miRNA (mir-Luc)	5′–	a	u	u	g	a	a	u	c	u	u	a	u	a	g	u	c	u	u	g	c	a	–3′		
ASO	3′–	A	A	C	U	U	A	G	A	A	U	A	U	C	A	G	A	A	C	G	U	–5′			76 ± 0.5
ASO-23	3′– U U U	A	A	C	U	U	A	G	A	A	U	A	U	C	A	G	A	A	C	G	U	–5′			76 ± 0.4
RINDA(K3)-as	^{N}Lys-Lys-Lys^{C}– 3′–	A	A	C	U	U	A	G	A	A	U	A	U	C	A	G	A	A	C	G	U	–5′			76 ± 0.4
RINDA(E1)-as	^{N}Glu^{C}– 3′–	A	A	C	U	U	A	G	A	A	U	A	U	C	A	G	A	A	C	G	U	–5′			76 ± 0.9
RINDA(E2)-as	^{N}Glu-Glu^{C}– 3′–	A	A	C	U	U	A	G	A	A	U	A	U	C	A	G	A	A	C	G	U	–5′			76 ± 1.2
RINDA(E3)-as	^{N}Glu-Glu-Glu^{C}– 3′–	A	A	C	U	U	A	G	A	A	U	A	U	C	A	G	A	A	C	G	U	–5′			76 ± 0.4
RINDA(E4)-as	^{N}Glu-Glu-Glu-Glu^{C}– 3′–	A	A	C	U	U	A	G	A	A	U	A	U	C	A	G	A	A	C	G	U	–5′			76 ± 1.8
RINDA(E5)-as	^{N}Glu-Glu-Glu-Glu-Glu^{C}– 3′–	A	A	C	U	U	A	G	A	A	U	A	U	C	A	G	A	A	C	G	U	–5′			77 ± 1.1
RINDA(E6)-as	^{N}Glu-Glu-Glu-Glu-Glu-Glu^{C}– 3′–	A	A	C	U	U	A	G	A	A	U	A	U	C	A	G	A	A	C	G	U	–5′			77 ± 1.9
Control ASO	3′–	U	A	U	G	G	A	G	U	C	A	A	U	G	U	U	A	A	A	U	A	–5′			–

augc:RNA , AUGC: 2′-OMe RNA, G : ENA,
Tm : [mir-Luc]=[ASO]=[RINDA-as]= 2 μM in 10 mM phosphate buffer (pH 7.0), 100 mM NaCl
* : Target recognition in RNA silencing is governed by seed region of guide strand in RISC
** : Target RNA is cleaved at this site in RISC

Figure 1. (A) Schematic illustrations of release of miRNA from RISC by using RINDA-as. (B) Sequences of antisense oligonucleotides.

study was to develop novel ASOs to further improve their inhibitory effects on RISC functions.

Recently, MacRae and co-workers reported interesting phenomena in which highly complementary wild-type RNA significantly accelerates the release of miRNA from Ago in RISC.[32] MiRNA is a core component of RISC and essential to RISC for recognizing target mRNAs and regulating gene expression. From these findings, we expected that the development of a method to promote the release of miRNA from RISC would be an effective approach for inhibition of RISC activity. In the present study, we intended to increase the inhibitory effects of ASO on RISC activity by releasing miRNA from RISC. Here we designed an antagonistic peptide called "RINDA" (RISC-inhibitor disturbing active-site of RISC) to promote the release of miRNA from RISC and synthesized novel peptide-conjugated oligonucleotides (RINDA-as). In this paper, the applicability of the RINDA-as to inhibit RISC functions is presented and interesting findings are disclosed.

RESULTS AND DISCUSSION

Design and Synthesis of Peptide-Conjugated Oligonucleotides. Among the proteins of the RISC components, Argonaute2 protein (Ago2) is an essential component of RISC for the cleavage of specific target mRNA. The Ago2 family has a highly conserved basic motif called PIWI-box. The phosphorylated 5′-end of miRNA is anchored in the PIWI-box and interacts with the lysine residues of the PIWI-box. The mutations of the Lys that interact with the 5′-phosphate of miRNA in the PIWI-box resulted in an attenuated holding ability of miRNA in Ago2.[33,34] Therefore, by disturbing the interaction between the PIWI-box and 5′-end of miRNA, it is expected that the release of miRNA from Ago2 would be induced. We designed an antagonist called "RINDA" (RISC-inhibitor disturbing active site of RISC) to disturb the interaction between the PIWI-box and 5′-end phosphate of miRNA (Figure 1A). For this purpose, we selected two kinds of peptides as RINDA. To interact with 5′-phosphate of miRNA entrapped in cationic PIWI-box, a lysine trimer was designed as RINDA(K3) to mimic the interaction between the 5′-phosphate of miRNA and PIWI-box. On the other hand, to interact with the lysine residues in PIWI-box interacting 5′-phosphate of miRNA, a glutamic acid trimer was also designed as RINDA(E3). According to a simulation of spatial structure by MacroModel, we predicted that the length of the peptide would need to be at least 3 residues long to reach the PIWI-box.

We conjugated RINDA peptides to the 3′-end of ASOs targeting miRNA (Figure 1B). These oligonucleotides have the 2′-*O*,4′-C-ethylene-bridged nucleosides (ENA) in the sequences.[35,36] Oligonucleotides containing ENA show very high affinity toward their complementary RNA and have higher nuclease resistance than that of wild-type RNA. Therefore, we selected ENA as modification on RINDA-as.

As the target of ASOs, miRNA targeting firefly luciferase mRNA (mir-Luc) was selected. The synthesis of RINDA-conjugated oligonucleotide (RINDA-as) was conducted by a combination of Fmoc solid-phase chemistry and the phosphoramidite method on 3′-Amino-Modifier CPG (GLEN RESERCH) (Figure S1). First, we evaluated the thermodynamic stability of duplexes between RINDA-as and the miRNA of mir-Luc. The results are summarized in Figure 1B. The T_m values of the duplex between RINDA-as and miRNA were approximately equal to those of ASO. These results indicate that RINDA did not affect the thermodynamic stability of the ASO-miRNA duplex.

RINDA(E3)-as Promoted the Release of miRNA from RISC. Next, we evaluated the effects of RINDA-as on the release of miRNA from RISC by the unloading assay. For this

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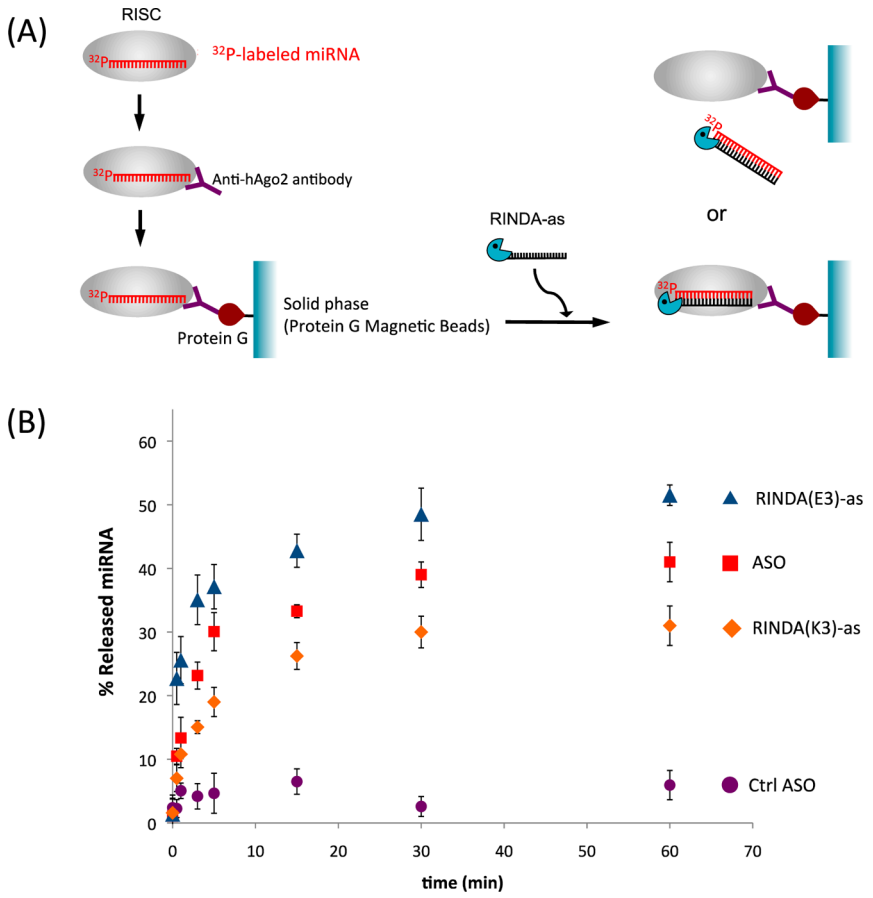

Figure 2. (A) Schematic illustrations of the unloading assay. Ago2 was immobilized on Protein G Magnetic beads using anti-Ago2 antibody. After the unloading reaction, the beads were washed and the supernatant were isolated from the beads. The dissociation ratio of miRNA from RISC was evaluated by measuring the radioactivity. (B) Time course analysis of the percent of released miRNA from RISC. RISC on beads = 10 fmol. [RINDA-as] = [ASO] = [Ctrl ASO] = 1 μM. In the case of RINDA(E3)-as, it was found that the rate of released miRNA from RISC was faster than the case of ASO. In contrast, in the case of RINDA(K3)-as, we found that the rate of released miRNA from RISC was slower than the case of the other oligonucleotides.

experiment, we prepared immobilized RISC magnetic beads as described in Materials and Methods (Figure 2A). The dissociation ratio of miRNA from RISC was evaluated by measuring the radioactivity (Figure 2B). Surprisingly, we found that miRNA was released from RISC in a time-dependent manner using ASO, which did not have any peptides on the 3′-end. As described in the Introduction, MacRae's group revealed that highly complementary wild-type RNA significantly accelerates the release of miRNA from Ago in RISC;[32] however, there is no report to elucidate whether the release of miRNA from RISC is induced by binding to chemical-modified ASOs which do not accept cleavage by RISC. To the best of our knowledge, our result is the first example that the chemically modified ASOs induce the release of miRNA from RISC and suggests the presence of the inhibitory pathway in RISC activity by releasing miRNA form RISC using ASO. We think this finding will bring a new mechanistic interpretation of the function of ASO targeting miRNA.

Furthermore, we also found that the releasing effect was enhanced in the case of RINDA(E3)-as. In contrast, in the case of RINDA(K3)-as, the ratio of released miRNA from RISC was lower than in the case of ASO. In the case of control ASO (ctrl ASO), whose sequence was not complementary to the miRNA of mir-Luc, the release of miRNA was not observed. These results show that miRNA is released from RISC by binding to ASO, and an anionic peptide promotes the releasing effect of miRNA by ASO.

In the case of RINDA(K3)-as, the amount of released miRNA from RISC was the lowest compared with that of other oligonucleotides. At first, we expected that RINDA(K3)-as would interact with the 5′-phosphate of miRNA anchored in the PIWI-box and enhance the release of miRNA from RISC. However, contrary to our expectations, the amount of released miRNA from RISC was the lowest among the case of other oligonucleotides. For this reason, we speculate that the electrostatic repulsion between RINDA(K3) and basic amino acid residues in the PIWI-box might not be conducive to RINDA(K3)-as entering the PIWI-box in RISC.

In contrast, in the case of RINDA(E3)-as, the ratio of released miRNA was the highest compared with that of other oligonucleotides. These results suggest that the interaction between the lysine residues in the PIWI-box and RINDA(E3) is important for the release of miRNA from RISC. This result also has implications for design of ASOs targeting miRNA. The conventional ASOs can act as sequence specific inhibitors of miRNA function and RINDA(E3)-as can promote the releasing effect of miRNA from RISC, which might be more effective than the conventional ASOs.

To evaluate the effect of the number of glutamic acid residues in RINDA-as on the release of miRNA from RISC, we

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synthesized a series of RINDA(En)-as, RINDA(E1)-as, to RINDA(E6)-as (Figure 1B). The results are shown in Figure 3.

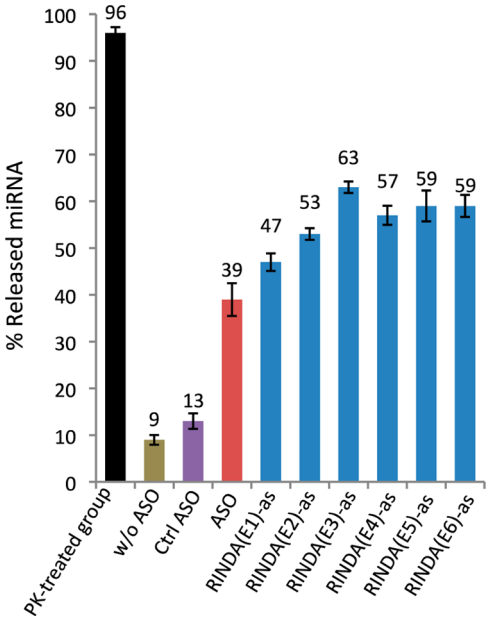

Figure 3. Percent of the released miRNA from RISC by RINDA(En)-as. RISC on beads = 10 fmol. [RINDA-as] = [ASO] = [Ctrl ASO] = 1 μM. PK-treated group: Proteinase K treated group. RINDA(E3)-as showed the highest ratio of released miRNA from RISC than that of the other RINDA-as.

The ratio of released miRNA proportionally increased to the number of glutamic acids in RINDA-as, and the released ratio showed a maximum at RINDA(E3)-as, which has three glutamic acid residues, suggesting that the ratio of released miRNA was the highest when using RINDA(E3). From the above findings, it is suggested that the optimal length of RINDA(En) to interact with the lysine residues in the PIWI-box is three.

To examine whether the releasing effect is enhancing by anionic moieties of RINDA-as or not, we prepared a 23-mer antisense oligonucleotide (ASO-23) with the same negative charges as RINDA(E3)-as in their phosphate groups. Then, we evaluated the ratio of released miRNA from RISC when using ASO-23 (Figure 4). In the case of ASO-23, the ratio of released miRNA from RISC was almost equal to that of ASO but lower than that of RINDA(E3)-as. This observation indicates that the number of anionic moieties in the phosphate groups of an oligonucleotide is not important for enhancing the release of miRNA from RISC; however, the structure of glutamic acids has some essential factors for interacting with basic amino acid residues in the PIWI-box.

Furthermore, it was also found that the dissociation ratio of miRNA from RISC is plateauing at ~40% in either case. We also evaluated the releasing effect of miRNA from RISC by oligonucleotides for a prolonged time (~24 h) and found that the experimental results did not change (data not shown). Therefore, the reaction is certainly equilibrated at 1 h. We used an excess of antisense oligonucleotides (ASO) in either case, so the above results suggest that about 60% of RISC did not release the miRNA by binding to ASO. These findings indicate that there are several kinds of RISC complexes and their miRNA-holding abilities in RISC are different depending on the complex formation of RISC. It is known that RISC is composed of Argonaute−miRNA complex which is a core component of RISC, and subcomponent proteins including Dicer, TRBP, and TRNC6A/GW182.[37,38] RISC exhibits its various functions by associating such proteins and it is predicted that the functions of RISC are different on the complex of RISC. We speculate that the difference of the ability to hold miRNA in RISC is attributed to subcomponents of RISC.

Release of miRNA from RISC Is Effective for the Inhibition of RISC Activity. As mentioned above, the release of miRNA using ASO was enhanced by the conjugation of RINDA(E3). To investigate the relationship between the releasing and inhibitory effects on RISC activity, we evaluated the inhibitory effects of RINDA-as on RISC function in HeLa

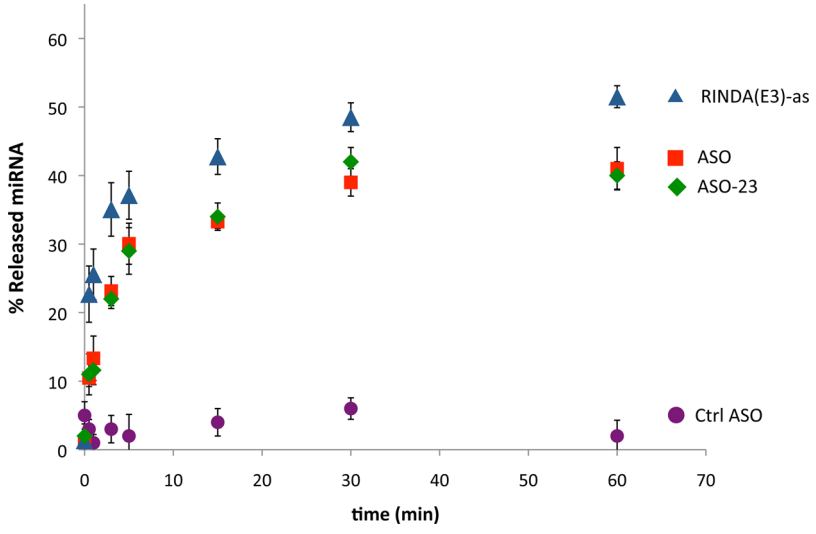

Figure 4. Time course analysis of the percent of released miRNA from RISC. In the case of ASO-23, the rate of released miRNA from RISC was equal to that of ASO, but lower than the case of RINDA(E3)-as. From these results, the number of anionic moieties in the phosphate groups of an oligonucleotide is not important for enhancing the release of miRNA from RISC. RISC on beads = 10 fmol. [RINDA-as] = [ASO] = [ASO-23] = [Ctrl ASO] = 1 μM.

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cells. As a target of RINDA-as, luciferase mRNA was selected. In this system, luminescence intensity of firefly luciferase is already down-regulated by RISC derived from anti-luciferase miRNA. If RINDA-as inhibits the RISC function, it is expected that luminescence intensity would be recovered. The results are shown in Figure 5. Luminescence intensity was substantially

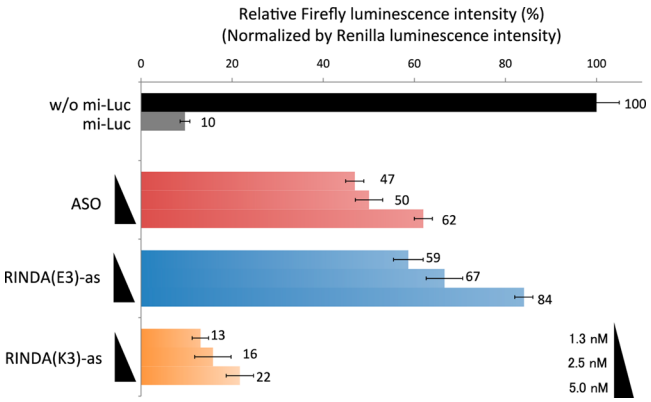

Figure 5. Inhibitory effects of RINDA-as on RISC function in HeLa cell. HeLa cells were transfected with 20 nM mir-Luc duplex. The luminescence intensity of RINDA(E3)-as-treated cells was greater than that of ASO. On the other hand, the luminescence intensity of RINDA(K3)-as-treated cells was down-regulated compared with that of ASO-treated cell.

recovered in the case of ASO and RINDA(E3)-as. In particular, the luminescence intensity of RINDA(E3)-as-treated cells was greater (+10−20%) than that of ASO-treated cells. These results show that the inhibitory effect on the RISC activity of RINDA(E3)-as is higher than that of ASO. On the other hand, the luminescence intensity of RINDA(K3)-as treated cells was down-regulated (−30−40%) compared with that of ASO-treated cells, suggesting that the inhibitory effect of RINDA-(K3)-as was lower than that of ASO. Good relationships between inhibitory effects on the RISC activity and release of miRNA from RISC were observed. Taking these results into consideration, enhancing the release of miRNA from RISC is a promising way for inhibition of RISC activity.

CONCLUSIONS

In this study, we successfully demonstrated the specific regulation of RISC activity using RINDA-as. We found that ASO, which did not have any peptides, induced the release of miRNA from RISC. This significant finding implies new mechanisms for inhibition of RISC activity. Furthermore, we succeeded in enhancing the release of miRNA from RISC and the inhibitory effects on RISC activity by conjugating RINDA(E3) to the 3′-end of ASO. These observations suggest that our strategy for inhibition of RISC activity will be a promising method for controlling miRNA activity in cancer cells. The details of the inhibitory mechanisms of RINDA(E3)-as on the RISC activity are still being unveiled. To investigate the details of the underlying mechanisms of the releasing effect introduced by RINDA(E3)-as, we will try to prepare several kinds of RINDA-as that have other peptide sequences or other anionic moieties and will consider the effect of peptide sequence on the releasing effect. Furthermore, in the near future, we will try to investigate X-ray crystallography as a means to reveal the mechanisms of the releasing effect on the RISC activity.

MATERIALS AND METHODS

Oligonucleotide Preparation. Each RINDA-as was synthesized by phosphoramidite chemistry and solid-phase peptide synthesis. The controlled-pore glass supports were treated with 0.4 M NaOH and 80% methanol at 55 °C for 8 h. RINDA-as was purified by RP-HPLC on a CAPCELL PAK C-18 column (4.6 Å 150 mm, Shiseido, Co. Ltd., Tokyo, Japan) with a linear gradient of acetonitrile in 0.1 M triethylammonium acetate (TEAA, pH 7.0) at a flow rate of 0.8 mL min^{-1} (acetonitrile gradient: 0−50% for 50 min). The purity of each oligonucleotide is shown in Figure S2. The mass spectra of each oligonucleotide purified by RP-HPLC were obtained using ESI-MS (Bruker Daltonics K.K., Bremen, Germany). These data are shown in Figure S2.

Measurements of UV-Melting Profiles of Duplexes of RINDA-as with the miRNA of mir-Luc. UV melting profiles of the duplex between RINDA-as and the miRNA of mir-Luc were obtained by a UV spectrophotometer equipped with a programmed thermal controller at an increase rate of 1.0 °C/min. The sample solutions were prepared in 10 mM phosphate buffer (pH 7.0) containing 0.1 M NaCl, and the concentration of oligonucleotides was fixed at 1.0 μM.

Luciferase Reporter Assays. HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FBS, 100 units per mL penicillin, and 100 μg/mL streptomycin at 37 °C in 5% CO_2. The cells were plated onto 96-well plates (32 mm^2/well) at a density of 4.5 × 10^4 cells/mL in an antibiotic-free medium, transfected, and harvested for 24 h. On the next day, cells were transfected with 20 nM of mir-Luc using Lipofectamine RNAiMAX (GIBCO, CA, United States) according to the manufacturer's protocol. On the following day, the cells were cotransfected with pGL4.13 (0.1 μg/well) and pGL4.73 (0.25 μg/well) plasmids and ASOs using Lipofectamine 2000 (GIBCO). After 24 h incubation, the cells were lysed and the luminescence activity of lysate was measured with a Dual Luciferase assay kit (Promega, WI, United States).

In Vitro Unloading Assays. *Immobilizing RISC on Beads.* HEK293T cell lysate expressing hAgo2 was prepared, according to a published method. For RISC assembly, 4 μL of the cell lysate was added to 3 μL of 40 × reaction mix, and 2 μL of mir-Luc duplex (^{32}P-labeled, stock concentration 100 nM), and incubated at 37 °C for 30 min. After incubation, the reaction mix was added to 2 μL anti-hAgo2 antibody and stirred at 4 °C for 30 min. Then, 12 μL reaction mix was added to 20 μL Dynabeads Protein G for immunoprecipitation (Thermo Fisher Scientific Inc., MA, United States) and stirred at 4 °C for 2 h. To remove unbound material, the beads were washed with a washing buffer (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM $MgCl_2$, 0.5 mM TCEP).

Unloading Assay. For this assay, 20 μL RISC-bound beads was suspended in a 1.5 mL centrifuge tube, and was preincubated at 37 °C for 5 min. The unloading reaction was initiated by the addition of 1 μM (final concentration) of antisense oligonucleotide and incubation at 37 °C. After the incubation, the beads were isolated and the supernatant was carefully removed as the "unloaded miRNA" fraction. The beads were washed immediately with 40 μL washing buffer (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM $MgCl_2$, 0.5 mM TCEP) to remove any supernatant. The washed beads were saved as the "RISC (including miRNA)" fraction. The radioactivity of each fraction was measured using a radiation

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analyzer. The dissociation ratio of miRNA from RISC was evaluated by measuring the radioactivity.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.bioconjchem.5b00501.

Additional information about RINDA-as including the synthetic scheme, the experimental results of purification and identification (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Ambros, V. (2004) The functions of animal microRNAs. *Nature 431*, 350−355.

(2) Kloosterman, W. P., and Plasterk, R. H. (2006) The Diverse Functions of MicroRNAs in Animal Development and Disease. *Dev. Cell 11*, 441−450.

(3) Bushati, N., and Cohen, S. M. (2007) microRNA functions. *Annu. Rev. Cell Dev. Biol. 23*, 175−205.

(4) Lima, R. T., Busacca, S., Almedia, G. M., Gaudino, G., Fennell, D. A., and Vasconcelos, M. H. (2011) MicroRNA regulation of core apoptosis pathways in cancer. *Eur. J. Cancer 47*, 163−174.

(5) Braun, T., and Gautel, M. (2011) Transcriptional mechanisms regulating skeletal muscle differentiation, growth and homeostasis. *Nat. Rev. Mol. Cell Biol. 12*, 349−361.

(6) Ambros, V. (2011) MicroRNAs and developmental timing. *Curr. Opin. Genet. Dev. 21*, 511−517.

(7) Krol, J., Leodige, I., and Filipowicz, W. (2010) The widespread regulation of microRNA biogenesis, function and decay. *Nat. Rev. Genet. 11*, 597−610.

(8) Ma, L. (2010) Role of miR-10b in breast cancer metastasis. *Breast Cancer Res. 12*, 210.

(9) Osaki, M., Takeshita, F., and Ochiya, T. (2008) MicroRNAs as biomarkers and therapeutic drugs in human cancer. *Biomarkers 13*, 658−670.

(10) Takamizawa, J., Konishi, H., Yanagisawa, K., Tomida, S., Osada, H., Endoh, H., Harano, T., Yatabe, Y., Nagino, M., Takahashi, T., et al. (2004) Reduced expression of the let-7 microRNAs in human lung cancers in association with shortened postoperative survival. *Cancer Res. 64*, 3753−3756.

(11) Iorio, M. V., Ferracin, M., Liu, C. G., Veronese, A., Spizzo, R., Sabbioni, S., Magri, E., Pedriali, M., Fabbri, M., Croce, C. M., et al. (2005) MicroRNA gene expression deregulation in human breast cancer. *Cancer Res. 65*, 7065−7070.

(12) Guo, J., Miao, Y., Xiano, B., Huan, R., Jiang, Z., Meng, D., and Wang, T. J. (2009) Differential expression of microRNA species in human gastric cancer versus non-tumorous tissues. *J. Gastroenterol. Hepatol. 24*, 652−657.

(13) Ohtsuka, M., Ling, H., Doki, Y., Mori, M., and Calin, G. A. (2015) MicroRNA Processing and Human Cancer. *J. Clin. Med. 4*, 1651−1667.

(14) Lee, T., Kim, M., Han, J., Yeom, K. H., Lee, S., Baek, S. H., and Kim, V. N. (2004) MicroRNA genes are transcribed by RNA polymerase II. *EMBO J. 23*, 4051−4060.

(15) Yi, R., Qin, Y., Macara, I. G., and Cullen, B. R. (2003) Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev. 17*, 3011−3016.

(16) Bohnsack, M. T., Czaplinski, K., and Gorlich, D. (2004) Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. *RNA 10*, 185−191.

(17) Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Radmark, O., Kim, V. N., et al. (2003) The nuclear RNase III Drosha initiates microRNA processing. *Nature 425*, 415−419.

(18) Gregory, R. I., Yan, K. P., Amuthan, G., Chendrimada, T., Doratotaj, B., Cooch, N., and Shiekhattar, R. (2004) The Microprocessor complex mediates the genesis of microRNAs. *Nature 432*, 235−240.

(19) Denli, A. M., Tops, B. B., Plasterk, R. H., Ketting, R. F., and Hannon, G. J. (2004) Processing of primary microRNAs by the Microprocessor complex. *Nature 432*, 231−235.

(20) Carthew, R. W., and Sontheimer, E. J. (2009) Origins and Mechanisms of miRNAs and siRNAs. *Cell 136*, 642−655.

(21) Krol, J., Loedige, I., and Filipowicz, W. (2010) The widespread regulation of microRNA biogenesis, function and decay. *Nat. Rev. Genet. 11*, 597−610.

(22) Huntzinger, E., and Izaurralde, E. (2011) Gene silencing by microRNAs: contributions of translational repression and mRNA decay. *Nat. Rev. Genet. 12*, 99−110.

(23) Lin, S. L., Chang, D. C., Chang-Lin, S., Lin, C. H., Wu, D. T., Chen, D. T., and Ying, S. Y. (2008) Mir-302 reprograms human skin cancer cells into a pluripotent ES-cell-like state. *RNA 14*, 2115−2124.

(24) Krol, J., Loedige, I., and Filipowicz, W. (2010) The widespread regulation of microRNA biogenesis, function and decay. *Nat. Rev. Genet. 11*, 597−610.

(25) Orom, U. A., Kauppinen, S., and Lund, A. H. (2006) LNA-modified oligonucleotides mediate specific inhibition of microRNA function. *Gene 372*, 137−141.

(26) Davis, S., Lollo, B., Freier, S., and Esau, C. (2006) Improved targeting of miRNA with antisense oligonucleotides. *Nucleic Acids Res. 34*, 2294−2304.

(27) Hutvagner, G., Simard, M. J., Mello, C. C., and Zamore, P. D. (2004) Sequence-specific inhibition of small RNA function. *PLoS Biol. 2*, 465−475.

(28) Morenno, P. M., and Pêgo, A. P. (2014) Therapeutic antisense oligonucleotides against cancer: hurdling to the clinic. *Front. Chem. 2*, 87.

(29) Stenvang, J., Petri, A., Lindow, M., Obad, S., and Kauppinen, S. (2012) Inhibition of microRNA function by antimiR oligonucleotides. *Silence 3*, 1.

(30) Lindow, M., and Kauppinen, S. (2012) Discovering the first microRNA-targeted drug. *J. Cell Biol. 199*, 407−412.

(31) Gebert, L. F., Rebhan, M. A., Crivelli, S. E., Denzler, R., Stoffel, M., and Hall, J. (2014) Miravirsen (SPC3649) can inhibit the biogenesis of miR-122. *Nucleic Acids Res. 42*, 609−621.

(32) De, N., Young, L., Lau, P. W., Meisner, N. C., Morrissey, D. V., and MacRae, I. J. (2013) Highly complementary target RNAs promote release of guide RNAs from human Argonaute2. *Mol. Cell 50*, 344−355.

(33) Ma, J. B., Yuan, Y. R., Meister, G., Pei, Y., Tuschl, T., and Patel, D. J. (2005) Structural basis for 5′-end-specific recognition of guide RNA by the A. fulgidus Piwi protein. *Nature 434*, 666−670.

(34) Wang, Y., Sheng, G., Juranek, S., Tuschl, T., and Patel, D. J. (2008) Structure of the guide-strand-containing argonaute silencing complex. *Nature 456*, 209−213.

(35) Morita, K., Hasegawa, C., Kaneko, M., Tsutsumi, S., Sone, J., Ishikawa, T., and Koizumi, M. (2001) 2′-O,4′-C-ethylene-bridged

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Bioconjugate Chem. 2015, 26, 2454−2460

nucleic acids (ENA) with nuclease-resistance and high affinity for RNA. *Nucleic Acids Res. Suppl. 1*, 241−242.
(36) Morita, K., Takagi, K., Hasegawa, C., Kaneko, M., Tsutsumi, S., Sone, J., Ishikawa, T., and Koizumi, M. (2003) Synthesis and properties of 2′-O,4′-C-ethylene-bridged nucleic acids (ENA) as effective antisense oligonucleotides. *Bioorg. Med. Chem. 11*, 2211−2226.
(37) Gagnon, K. T., Li, L., Chu, Y., Janowski, B. A., and Corey, D. R. (2014) Analysis of Nuclear RNA Interference (RNAi) in Human Cells by Subcellular Fractionation and Argonaute Loading. *Cell Rep. 6*, 211−221.
(38) Nishi, K., Nishi, A., Nagasawa, T., and Ui-Tei, K. (2013) Human TNRC6A is an Argonaute-navigator protein for microRNA-mediated gene silencing in the nucleus. *RNA 19*, 17−35.

DOI: 10.1021/acs.bioconjchem.5b00501
Bioconjugate Chem. 2015, 26, 2454−2460