HT-SELEX-based identification of binding pre-miRNA hairpin-motif for small molecules

Sanjukta Mukherjee,1,3 Asako Murata, 1 Ryoga Ishida,2 Ayako Sugai, 1 Chikara Dohno, 1 Michiaki Hamada, 2 Sudhir Krishna, 3 and Kazuhiko Nakatani1

INTRODUCTION

MicroRNAs (miRNAs) are a growing class of non-coding RNAs (ncRNAs) that have emerged as important regulators of gene expression and are predicted to regulate at least half of the human transcriptome at the posttranscriptional level.1-4 miRNAs are generated from their primary transcripts (pri-miRNAs) by two successive enzymatic reactions: digestion of pri-miRNA by enzyme Drosha to give a 3’ overhang hairpin precursor RNA (pre-miRNA) and subsequent digestion of pre-miRNA by enzyme dicer to a mature miRNA of 18–25 nucleotides (nt) in length. miRNAs are assembled into the RNA-induced silencing complexes (RISCs) with argonaute proteins (Ago2), which bind to the 3’-untranslated region (3’-UTR) of target mRNAs and induces translational repression or mRNA degradation.5-9 According to the miRNA database (miRBase: http://www.mirbase.org/), 1,917 miRNA precursors and 2,656 mature miRNAs have been so far identified in humans.7 Misregulation of miRNA expression has been linked to variety of diseases,10-15 such as diabetes,16 cardiovascular,17,18 neurological,19,20 kidney,21 infectious,22 and particularly cancers.23-29

Due to the growing impact of regulatory effects of miRNAs associated with different diseases, it has become a challenge to develop strategies for regulating miRNA function.30-32 There are two major tools to regulate miRNA function: one is antisense oligonucleotides (antagomirs) complementary to the mature miRNA and the other is small molecules that target miRNA biogenesis and/or the formation of RISCs.30-32 The small-molecule modulators33-36 may have advantages over antisense oligonucleotides because they are easily deliverable, their ease of use, and they are cost-effective for chemical alterations and manufacturing. However, the specificity of small molecules to the target sequence and/or secondary structure remains a concern and should be further investigated for application as RNA-targeting drugs. Targeting RNA by small molecules is at an emergent stage and encumbered with challenges due to the lack of fundamental understanding about the chemical motifs within small molecules capable of binding to a specific RNA. Most of the small-molecule modulators of miRNA biogenesis and/or function reported so far were discovered by library screening. Rational design of small molecules37-42 to target and regulate a particular miRNA has been attempted; however, researchers are still far from being able to design novel and potent small-molecule modulators of miRNA function.

Systematic evolution of ligand by exponential enrichment (SELEX)43-49 is a powerful in vitro method for the selection and identification of specific RNA binders for a given molecular target from an RNA library with a randomized sequence. We here describe our high-throughput (HT)-SELEX-based approach to identify hairpin loop sequence motifs

Selective targeting of biologically relevant RNAs with small molecules is a long-standing challenge due to the lack of clear understanding of the binding RNA motifs for small molecules. The standard SELEX procedure allows the identification of specific RNA binders (aptamers) for the target of interest. However, more effort is needed to identify and characterize the sequence-structure motifs in the aptamers important for binding to the target. Herein, we described a strategy integrating high-throughput (HT) sequencing with conventional SELEX followed by bioinformatic analysis to identify aptamers with high binding affinity and target specificity to unravel the sequence-structure motifs of pre-miRNA, which is essential for binding to the recently developed new water-soluble small-molecule CMBL3aL. To confirm the fidelity of this approach, we investigated the binding of CMBL3aL to the identified motifs by surface plasmon resonance (SPR) spectroscopy and its potential regulatory activity on dicer-mediated cleavage of the obtained aptamers and endogenous pre-miRNAs comprising the identified motif in its hairpin loop. This new approach would significantly accelerate the identification process of binding sequence-structure motifs of pre-miRNA for the compound of interest and would contribute to increase the spectrum of biomedical application.
of pre-miRNA serving as the binding site for a small molecule of interest (Figure 1). A pre-miRNA contains a hairpin loop structure, which is relatively close to the dicer cleavage site. Therefore, small molecules binding to the hairpin loop might have potential to inhibit/promote pre-miRNA processing.50 Furthermore, the hairpin loop of each pre-miRNA is comprised of unique sequences with different lengths, which can serve as a selective binding site for a small molecule. Identification of binding hairpin-sequence motifs of pre-miRNA for a small molecular ligand will provide an opportunity for the small molecule to regulate a specific pre-miRNA processing by interfering with the interaction between pre-miRNA and dicer.33-36

Our approach consists of three steps: (1) HT-SELEX against a small molecule using a pre-miRNA library with a randomized hairpin loop by integrating conventional SELEX51 (Scheme S3) with HT sequencing, (2) identification of characteristic sequence-structure motifs for binding to the small molecule by bioinformatic analysis of HT sequencing data, and (3) validation of binding of the small molecule to the identified motifs, and the effect of the ligand’s binding on the dicer cleavage reaction (Figure 1).

CMBLs (cyclic mismatch binding ligands) are recently reported compounds that were designed rationally to recognize an array of guanines (Gs) in nucleic acids by the complementary Watson-Crick hydrogen bonding surface.52,53 Some of CMBLs were shown to bind to specific sequence-structure motifs in DNA/RNA;52-55 however, binding RNA targets for our newly developed CMBL3aL have not been identified yet. The solubility of the drug candidate is an important parameter but also a major challenge for pharmacological researches.56 Addition of a long linker possessing a terminal primary amine in CMBL3aL (Figure 1) makes it highly soluble in water, showing its future potential for any pharmacological evaluation. Herein, we report the synthesis of CMBL3aL (Scheme 1) and demonstrate the potential of our HT-SELEX-based strategy to identify binding hairpin-sequence motifs of pre-miRNA for CMBL3aL.

RESULTS

Motif selection: Identification of enriched sequence motif by SELEX
CMBL3aL has a long linker possessing a terminal primary amine, which was used for immobilization of CMBL3aL on the resin surface (Scheme S2) for SELEX study. Furthermore, for SELEX, two RNA libraries were used, one containing 11 nt (N11 library) and the other containing mixture of 6, 7, 8, 9, 10, and 11 nt (N6–11 library) randomized hairpin loops. Both RNA libraries were designed based on the secondary structure of pre-miR29a (Figure 1). After each selection cycle, eluted RNAs were reverse transcribed to cDNA and amplified by PCR (Figure S1), followed by sequencing using an Ion PGM sequencer.

Careful inspection of the HT sequencing data obtained from both N11 and N6–11 libraries revealed that most of the enriched aptamers at advanced selection cycles comprise consensus sequence motifs in the randomized region (Tables S1 and S2) with the array of Gs, particularly consecutive Gs (GG) flanked by one or two uracils (U). From the aptamers enriched in the N11 pools, we selected five most enriched aptamers (R-Seq1, 2, 3, 5, and 6) in the final selection cycle and one aptamer (R-Seq4) that was dominant in the 8th–10th selection cycles for further analysis (Figures 2A, 2B, and S2). The change in frequency of these aptamers over the selection cycles revealed a gradual enrichment of R-Seq4, which became dominant (frequency = 0.18) at the 9th selection cycle. Prolonged selection cycle resulted in a decrease in the frequency of R-Seq4 and the appearance of elongated aptamers (R-Seq1, 2, 3, 5, and 6) (Figure 2A; Table S1). Formation of such an elongated by-product has already been reported, and several incidences can lead to the formation of an undesired by-product, such as random nature of the library sequences and their mispriming.57,58 However, the underlying reasons for the elongation of aptamers in our study are not clear at this moment. Among these elongated aptamers, R-Seq1 was most significantly enriched after the 10th selection cycle, and its frequency reached 0.82 at the 12th cycle. For SELEX using
the N_{6,11} library, we obtained essentially the same enriched sequences to those obtained from the N_{11} library (Table S2). R-Seq2 was preferentially enriched in the N_{6,11} pools during the 7th–10th selection cycles (frequency = 0.026, 0.26, 0.79, and 0.46). At the 10th selection cycle, R-Seq2 becomes diminished and this was associated with the appearance and gradual enrichment of R-Seq1 during the 8th–10th selection cycles (frequency = 0.003, 0.017, and 0.24) (Figure S2; Table S2).

Motif identification: Binding sequence-structure motifs identification using an in silico method

Next, we performed a more detailed analysis of the obtained sequences to determine sequence-structure motifs important for binding of these aptamers to CMBL3aL. We used recently reported RaptRanker, an in silico method for identifying truly high binding affinity aptamers from HT-SELEX data. This method analyzes HT-SELEX data by evaluating not only the sequence but also the secondary structural motif, and thereby allows for identification of truly important sequence-structure motifs for binding to the target molecule. A brief description of the RaptRanker method mentioned here: RaptRanker generates a set of sub-sequences with a defined window size (k-mer, 10-mer in this study) from the unique sequence of an aptamer, and computes their sub-sequence-structure profiles (SSSPs). According to the similarity of SSSPs, RaptRanker groups sub-sequences into a set of clusters and then calculates the motif frequency for each cluster, which is the sum of the frequency of sub-sequences in a cluster. To evaluate the enrichment of R-Seq1–6 at the sub-sequence level, we calculated the averaged k-mer frequency (AKF) and the averaged motif frequency (AMF) of sub-sequences for each of the six aptamers R-Seq1–6, and plotted them against selection cycles (Figures 2C and 2D). AKF and AMF were obtained by dividing the sum of the frequencies of sequences and motifs, respectively, of sub-sequences obtained from each unique sequence by the number of sub-sequences.

A significant increase in AKF and AMF was observed for R-Seq1, 2, 3, 5, and 6 after the 9th cycle, indicating the enrichment of the sequences and secondary structural motifs of sub-sequences obtained from the unique sequences of these aptamers. Since R-Seq1 was considerably enriched in the pool from the 10th to 12th cycles (Figure 2B), this made it difficult to observe the enrichment of the other aptamers. However, the analysis of SSSPs revealed a substantial enrichment of the other elongated aptamer, especially R-Seq5 and 6, at the sub-sequence level.

We finally visualized the transition of sequence-structure motif of the most enriched sub-sequence obtained from the six aptamers (Figures 3C–3F). The sub-sequences listed in Figure 3B had the highest motif frequency in the final selection cycle among the other sub-sequences obtained from the unique sequence of R-Seq1–6. Note that the five elongated aptamers (R-Seq1, 2, 3, 5, and 6) shared multiple sub-sequences (Figure 2B) and, among them, the sub-sequence “GGUGGUGGUG” of R-Seq1 (positions 12 and 15), R-Seq5 (positions 9 and 11), and R-Seq6 (positions 13 and 16) were grouped into the same cluster, which resulted in having the same motif frequency. As shown in Figure 3C, most of the nucleotide positions in the sub-sequence GGUGGUGGUG belong to “Stem” during the selection cycles. However, the structural context of 3’ end UG changed from Stem to “Hairpin” in the course of selection. Probability changes for some guanines at different positions between Stem and “Internal” were also observed. In the sub-sequences UUGUGGUGG and GUCGGUGGUU, respectively, of R-Seq2 and 3 (Figures 3D and 3E), most of the nucleotides were categorized into Stem and Internal. Unlike the sub-sequences of R-Seq1, 5, and 6, the structural context did not change significantly at all.
nucleotide positions. On the other hand, most of the nucleotide positions in the sub-sequence GGGUGAGCU of R-Seq4 were categorized into Hairpin (Figure 3F) and the change of the structural context was not observed.

Considering the fact that a shift of structural context from Stem to Hairpin was observed in the enriched sub-sequences of R-Seq1, 5, and 6 in later selection cycles, Hairpin might be a more preferable structural feature as a binding site for CMBL3aL. To gain deeper insights into the binding sequence-structure motifs for CMBL3aL, we further investigated the sequence similarity of the hairpin loops formed in the enriched aptamers in advanced selection cycles (Figure 4A). Careful scrutiny of the overall structure of these aptamers revealed that those aptamers harbor one or more hairpin loop with conserved sequences of variable length (AL) (Figures 4A, 4B, S3, and S4). Those encompass characteristic sequence consensus of consecutive guanines flanked by one or two uracils (UGG/GGU/UGGU/UGGUG), indicating the importance of these characteristic hairpin-sequence motifs for CMBL3aL binding.

**Motif validation: Binding assays using SPR spectroscopy**

Next, we selected four aptamers R-Seq1–4 and investigated their binding to CMBL3aL (Figure 4A) by surface plasmon resonance (SPR) spectroscopy. The binding kinetics were determined in single-cycle kinetic mode using a 1:1 fitting model. CMBL3aL showed rapid association and slow dissociation kinetics toward R-Seq1, 3, and 4 immobilized surfaces (Figures 4C, 4E, and 4F), giving an apparent dissociation constant (K_d(app)) values of 218, 682, and 135 nM, respectively (Table S3). To the R-Seq2 immobilized sensor surface (Figure 4D), CMBL3aL showed faster dissociation rate, which resulted in a larger K_d(app) of 1,310 nM. For R-Seq1–3, comparatively higher SPR response were observed than in R-Seq4 (Figures 4C–4F; Table S3). Along with the core binding sequence motif, the flanking sequence context and overall structure of the substrate RNA plays a vital regulatory role on binding affinity as we observed among R-Seq1–4. R-Seq1, 2, and 3 comprise dumbbell-shaped secondary structural motifs, which consist of double-helical stem closed by two hairpin loops. On the other hand, R-Seq4 consists of hairpin loop structures. In R-Seq1, the expected binding sequence motif was present in both hairpins. However, in R-Seq2, 3, and 4 the expected binding sequence motif was present only in one hairpin loop. Furthermore, R-Seq4 differs in its flanking sequence context with respect to R-Seq1, 2, and 3. Next, we evaluated the binding affinity of CMBL3aL toward pre-miR29a, which does not contain a characteristic binding sequence motif. Very weak SPR response and hence low binding affinity was observed for CMBL3aL toward pre-miR29a, comprising the AU-rich hairpin-motif (Figure S5), clearly
demonstrating the importance of the identified sequence motif for CMBL3aL binding.

Functional assays: Effect of CMBL3aL binding on the dicer cleavage reaction of aptamers

Secondary structures in a pre-miRNA and the local structural environment of dicer binding sites have a profound impact on dicer-mediated cleavage and its efficiency.60-67 To evaluate the effect of CMBL3aL on dicer cleavage of these aptamers, we performed an in vitro dicer cleavage reaction in the absence and presence of CMBL3aL using R-Seq1, 2, and 4 as the substrates. Formation of cleaved products (I + P) was observed for R-Seq1, 2, and 4, with a fraction of 15.8%, 16.0%, and 39.3%, respectively, in the absence of CMBL3aL (Figures 4G, 4H, and S6). Reduced formation of the cleaved products for R-Seq1 and 2 compared with R-Seq4 was observed, probably due to the dumbbell-shaped structures of R-Seq1 and 2 (Figure 4A). In the presence of CMBL3aL, no significant change (1.03-fold) in cleaved product formation was observed for R-Seq1 and 2 compared with R-Seq4 was observed, probably due to the dumbbell-shaped structures of R-Seq1 and 2 (Figure 4A). In the presence of CMBL3aL, no significant change (1.03-fold) in cleaved product formation was observed for R-Seq1 and 2 compared with R-Seq4 was observed, probably due to the dumbbell-shaped structures of R-Seq1 and 2 (Figure 4A). In the presence of CMBL3aL, no significant change (1.03-fold) in cleaved product formation was observed for R-Seq1 and 2 compared with R-Seq4 was observed, probably due to the dumbbell-shaped structures of R-Seq1 and 2 (Figure 4A). In the presence of CMBL3aL, no significant change (1.03-fold) in cleaved product formation was observed for R-Seq1 and 2 compared with R-Seq4 was observed, probably due to the dumbbell-shaped structures of R-Seq1 and 2 (Figure 4A).

Application: Effect of CMBL3aL binding on endogenous pre-miRNAs comprising partial binding motifs

To evaluate the scope of this method and expand its applicability on biologically significant endogenous pre-miRNA, we investigated the binding of CMBL3aL using endogenous pre-miR33a and pre-miR24-2 having a UGGU sequence motif in their hairpin loops, which we identified from the miRbase database using BLAST (Figures 5A, S7, and S8). SPR assay revealed significant high SPR response (Figures 5B and S8) of CMBL3aL for pre-miR33a and pre-miR24-2 (Table S4). To demonstrate the importance of the identified motif for CMBL3aL binding, we performed the SPR assay with a pre-miR33a mutant by incorporating point mutations (pre-miR33a-G31A and pre-33a-G32A) in it (Figure S7A–S7D). Decreases in binding response were observed for both pre-miR33a-G31A and pre-33a-G32A with increases in Kd(app) by 1.62- and 1.85-fold (Table S4), respectively. Moreover, in vitro dicer cleavage reaction of pre-miR33a showed dose-dependent decrease in the cleaved products (I + P) formation in the presence of CMBL3aL (Figures 5C and 5D). These data are an indication of the potential of this HT-SELEX-based approach for the identification of binding active hairpin-sequence motifs of pre-miRNA against a molecule of interest.

DISCUSSION

In this study, we uncovered binding sequence-structure motifs of pre-miRNA for our recently designed small-molecule CMBL3aL by utilizing a new HT-SELEX-based approach (Figure 1). Our SELEX study were continued up to 10–12 rounds of selection cycles using the
randomized hairpin loop RNA libraries, followed by sequencing of each selection pool using an Ion PGM next-generation sequencer (Tables S1 and S2). HT sequencing data revealed the presence of a consensus sequence motif (consecutive Gs [GG] flanked by one or two Us) in the randomized region of the enriched aptamers (Figure 2A; Tables S1 and S2). Secondary and tertiary structures of aptamers play a crucial role on their affinity and selectivity. To unveil sequence-structure motifs of these high binding affinity aptamers important for CMBL3aL binding, we applied RaptRanker,59 an in silico method, to our HT sequencing results. RaptRanker generates set of sub-sequences with a structural information and clusters the sub-sequences based on similarity in both nucleotide sequence and secondary structure features, thereby allowing identification of sequence-structure motifs truly significant for binding to the target molecule. This analysis showed most of the nucleotide positions for sub-sequences of R-Seq1, 5, and 6 belong to the stem loop (Figure 3C). For the sub-sequences of R-Seq2 and 3, most of the nucleotides were observed into Stem and Internal loops (Figures 3D and 3E). In contrast, for the sub-sequence of R-Seq4, most of the nucleotide were spotted into Hairpin (Figure 3F). By further inspection we unveiled sequence resemblances within the hairpin loops of most of the enriched aptamers, that embrace with the conserved hairpin-sequence motif comprising consecutive Gs (GG) flanked by one or two Us (UGG/GGU/UGGU) demonstrating its importance for CMBL3aL binding (Figures 4A, 4B, S3, and S4). CMBL analogs encompassed with two 2-amino-1,8-naphthyridine heterocycles, such as CMBL3aL, have been developed using a rational approach to recognize an array of Gs by using their complementary hydrogen bonding surface.52,53 The characteristic binding hairpin-sequence motif obtained from this study indicate that the GG site within it would be the possible binding site for the CMBL3aL. We further elucidated the binding affinity of CMBL3aL toward four selected enriched aptamers (R-Seq1–4) by SPR assay (Figures 4C–4F). Significantly SPR response and high binding affinity were observed for all four aptamers with different binding kinetics. In contrast, much lower binding affinity was observed toward pre-miR29a that comprises an AU-rich hairpin-sequence motif (Figure S5), indicating the importance of the identified hairpin-sequence motif for CMBL3aL binding. We further assessed the effect of CMBL3aL on dicer-mediated cleavage of selected aptamers (R-Seq1, 2, and 4) that differ in their overall secondary structure. No significant change in cleaved product (I + P) formation (Figures S6A and S6B) was observed for R-Seq1, which formed a dumbbell-shaped structure (Figure 4A). This kind of structure has already been reported not to be an appropriate substrate for
the dicer cleavage reaction. In contrast, R-Seq2, which also formed a dumbbell-shaped structure (Figure 4A), showed a 1.86-fold increase in cleaved product (I + P) formation (Figures S6C and S6D), which might be due to the formation of a preferable hairpin loop structure upon CMBL3aL binding. Moreover, a 0.89-fold decrease in cleaved product (I + P) formation (Figures 4G and 4H) was observed for R-Seq4, consisting of a hairpin structure (Figure 4A) favorable for the dicer cleavage reaction.

Several groups have been adopted various strategies for the identification of small molecules that regulate miRNA biogenesis. For example, Davies and Arenz and Bose et al. reported a fluorescence resonance energy transfer (FRET)-based strategy, Tan et al. developed a fluorescence polarization-based screening assay, and Shan et al. and Connelly et al. developed a luciferase-based system that contains complementary sequences of miRNAs in their 3’ UTR of the reporter genes. Furthermore, Disney et al. developed a bioinformatic-based rational approach “Informa” to identify small molecules that bind to Drosha and the dicer binding site of human miRNA precursors by integrating two-dimensional combinatorial screening with HT structure-activity relationships through sequencing (HiT-StARTS). Our group has also reported a fluorescent indicator displacement assay to screen xanthone derivatives against pre-miR29a and identified 21 hit compounds. Furthermore, by utilizing conventional SELEX, as well as by advancement of the standard SELEX method, various approaches have been developed over three decades to identify RNA aptamers, such as cell-SELEX, in vivo-SELEX, immunoprecipitation-coupled (IP)-SELEX, capture-SELEX, microfluidic (M)-SELEX, and so on.

Herein, we described our HT-SELEX-based approach (Figure 1), where we integrated conventional SELEX (Scheme S3) with HT sequencing, followed by bioinformatic analysis. In this study, we were particularly interested in identifying sequence motifs in the pre-miRNA hairpin loop for CMBL3aL binding.

To validate the scope of this method we further evaluated the binding of CMBL3aL toward endogenous pre-miRNAs, which comprises a partial active binding hairpin-sequence motif (Figures 5A and S8), by SPR assay. SPR data revealed significant binding responses for both endogenous pre-miR33a and pre-miR24-2 comprising characteristic binding motifs (Figures 5B and S8B). Further dose-dependent decrease in the cleaved products formation was also observed in the presence of CMBL3aL for the in vitro dicer cleavage reaction of pre-miR33a (Figures 5C and 5D). In conclusion, these data suggest the potential of this new HT-SELEX-based strategy that would accelerate the identification process of the sequence-structure motif of pre-miRNAs for the compound of interest selected from the chemical library and would contribute to increase the spectrum of biomedical application.

**MATERIAL AND METHODS**

**Synthesis of CMBL3aL**

CMBL3aL was synthesized from CMBL3a in two steps (Scheme S1), by reductive amination followed by boc-deprotection. CMBL3a was prepared using a procedure reported previously. Detail synthesis (Scheme S1) and characterization of CMBL3aL are given in the supplementary data.

**In vitro selection (SELEX) procedure**

1. Preparation of CMBL3aL-immobilized resin: CMBL3aL was immobilized on NHS-activated Sepharose 4 Fast Flow resin (GE Healthcare) by amino coupling method according to the manufacturer’s instructions. In brief, to NHS-activated Sepharose 4 Fast Flow resin (0.500 mL) was added a solution of CMBL3aL (50 nmol) in 0.2 M NaHCO3-0.5 M NaCl (pH 8–9) (0.5 mL) containing 5% DMSO. After shaking the suspension at room temperature for several hours, the solution was drained and the resin was further treated with 0.1 M Tris-HCl buffer (1 mL [pH 8.8]) for several hours to block unreacted carboxylic groups on the resin. The resulting resin was sequentially washed with 0.1 M Tris-HCl buffer (pH 8.8) and 0.1 M NaOAc-0.5 M NaCl solution (pH 5.2), which was repeated three times. The resin was washed with 20% aqueous EtOH, and three bed volumes of 20% aqueous EtOH was added to the resin to give a 25% slurry resin suspension. Tris-immobilized resin was also prepared for the preselection to remove RNA species that bind non-specifically to the matrix (Scheme S2).

2. Preparation of DNA and RNA pools: the initial double-stranded DNA pools were prepared by PCR amplification of the oligonucleotide containing the N11 or N6-11 nt randomized region (5’-ACT GAT TTC TTT TGG TGT TCA GAG—randomized region—TTC TAG CAC CAT CAG AAA TCG GTT A-3’) with Platinum Pfx DNA Polymerase (Invitrogen). The primers used in the PCR amplification were: 5’-TAATACGACTCACTATAGGTTGACTTCTTCTTTGAGAG-3’ (forward primer, T7 promoter sequence is underlined) and 5’-TAATACGACTCACTATAGGTTGACTTCTTCTTTGAGAG-3’ (reverse primer). The dsDNA pool was transcribed in vitro with T7 RNA polymerase (MegaShortscript T7 Transcription Kit, Ambion) to yield an RNA pool. The reaction mixture was treated with DNase at 37°C for 30 min to digest the template DNA. The mixture was diluted with RNase-free water, and passed through an NAP-5 column (GE Healthcare) to remove unincorporated NTPs. The RNA was precipitated by adding 7.5 M ammonium acetate and isopropanol, and pelleted by centrifugation. The pellet was dissolved in annealing buffer (10 mM Tris-HCl, 50 mM NaCl, 100 mM KC1 [pH 7.5]). The RNA solution was denatured at 80°C for 3 min, and annealed by slow cooling to room temperature. MgCl2 was added to a final concentration of 5 mM to the annealed RNA, and used for the next binding step.

3. Selection step and regeneration of RNA pool: the RNA solution was preincubated with Tris-resin for 30 min at room temperature to remove non-specific binders to the resin. The flowthrough was applied to CMBL3aL-immobilized resin, and the suspension was incubated at room temperature for 30 min with vortexing every 10 min. The resin was drained and washed three times with washing buffer (10 mM Tris-HCl, 100 mM KC1, 50 mM NaCl, 5 mM MgCl2 [pH 7.5]). Bound RNAs were eluted three times.
with CMBL3aL solution (50 μL, 100 μM) and pooled in a 1.5 mL collection tube. The eluted RNAs were precipitated with 0.3 M sodium acetate and ethanol, and pelleted by centrifugation. The RNA pellet was dissolved in RNAse free water and reverse transcribed with PrimeScript II Reverse Transcriptase (Takara) using the reverse primer (5’-TAAACCAGATCATATAGAGCTTG TAGTTGCTTATGATG-3’/5’-GTTAGTGACTTGGTGAACATG TAATACGACTCATATAGG-3’). The resulting cDNA was PCR-amplified with the forward and the reverse primers as described above. The DNA templates were transcribed in vitro, and the resulting RNAs were subjected to the next round of selection (Scheme S3).

SPR measurement

(1) Immobilization of RNAs to sensor chips: interaction between CMBL3aL and RNA-immobilized surfaces was measured using SPR with a Biacore T200 system (GE Healthcare). For preparation of the RNA-immobilized sensor surface, 5’-biotinylated RNA (R-Seq1, 2, 3, 4, or pre-miR29a) (GeneDesign) was diluted to 0.2 μM in 10 mM HEPES buffer containing 500 mM NaCl and injected over a streptavidin-coated sensor chip (Series S Sensor chip SA, GE Healthcare) at 5 μL/min to obtain an immobilization level of around 500 response unit (RU). The amount of RNA immobilized on sensor surface was 525.3 RU (R-Seq1), 562.1 RU (R-Seq2), 528.7 RU (R-Seq3), 503.4 RU (R-Seq4), and 546.2 RU (pre-miR29a) (used in Figures 4C–4F and S5).

(2) Immobilization of CMBL3aL to sensor chips: (1) for preparation of CMBL3aL-immobilized sensor surface, the carboxy groups of the dextran surface of a sensor chip CM5 (Series S Sensor chip, GE Healthcare) were activated with NHS/EDC (600 s, 100 mM sodium tetraborate, 1 M NaCl [pH 8.5]) was diluted in 10 mM HEPES buffer containing 500 mM NaCl and injected over a streptavidin-coated sensor chip (Series S Sensor chip SA, GE Healthcare) at 5 μL/min to obtain an immobilization level of around 500 response unit (RU). The amount of RNA immobilized on sensor surface was 525.3 RU (R-Seq1), 562.1 RU (R-Seq2), 528.7 RU (R-Seq3), 503.4 RU (R-Seq4), and 546.2 RU (pre-miR29a) (used in Figures 4C–4F and S5).

(3) Preparation of pre-miRNA and its mutants: the dsDNA template was prepared by 3’ fill-in reaction with Klenow fragment using two oligonucleotides overlapping at their 3’ ends. The sequences of oligonucleotides used are listed at the end of this section. The reaction mixture was purified using the Nucleospin Gel and PCR clean up (Machery-Nagel) according to the manufacturer’s instruction. The purified dsDNA template was transcribed in vitro by T7 RNA polymerase (MEGAscript T7 Transcription Kit, Am-bion) to produce the corresponding RNAs. After digestion of the template DNA with DNase I, the RNAs were purified on a 15% denaturing polyacrylamide gel (acylamide:bisacrylamide = 19:1, 6 M urea). Oligonucleotides used to prepare the dsDNA templates for: pre-miR-33a (5’-TAAACCAGATCATATAGAGCTTG TAGTTGCTTATGATG-3’/5’-GTTAGTGACTTGGTGAACATG TAATACGACTCATATAGG-3’), pre-miR-33a_G312A (5’-TAAACCAGATCATATAGAGCTTG TAGTTGCTTATGATG-3’/5’-GTTAGTGACTTGGTGAACATG TAATACGACTCATATAGG-3’), and pre-miR-33a_G312A (5’-TAATACGACTCATATAGTGAC TTAGTTGCTTATGATG-3’/5’-GTTAGTGACTTGGTGAACATG TAATACGACTCATATAGG-3’). The sequences are: (T7 promoter sequence is underlined) (used in Figures 5 and S7).

(4) Binding analysis using RNA-immobilized sensor chip: kinetics of binding of CMBL3aL to the target RNA-immobilized sensor surface was determined by single-cycle kinetics. CMBL3aL in HBS-EP+ buffer (0.01 M HEPES, 0.15 M NaCl, 3.0 mM EDTA [pH 7.4], 0.005% [v/v] surfactant P20) containing 5% DMSO was injected into the RNA-immobilized sensor surface without a regeneration step in between each concentration. Injections were done at 60 μL/min with a contact time of 30 s, followed by dissociation with running buffer for 120 s. All sensorgrams were corrected by subtraction of the blank flow cell response and the buffer injection response (used for Figures 4C–4F and S5).

(5) Binding analysis using CMBL3aL immobilized sensor chip: binding of pre-miR33a, pre-miR24-2 and its mutants to CMBL3aL was also investigated using the CMBL3aL-immobilized sensor chip prepared above. RNA (prepared by in vitro transcription) was diluted to 1 μM in 1× HBS-EP+ buffer, followed by annealing (80°C for 3 min, then slowly cooled down to room temperature. The annealed RNA was diluted in 1× HBS-EP+ buffer at concentrations of 0.01, 0.03, 0.1, 0.3, and 1.0 μM. Each RNA solution was injected over the CMBL3aL-immobilized sensor surface at 30 μL/min with a contact time of 60 s and a dissociation time of 120 s. For pre-miR24-2 contact time of 180 s and a dissociation time of 180 s were used. In between injections, the sensor surface was regenerated by injection of 50 mM NaOH. All sensorgrams were corrected for reference subtraction of the blank flow cell response (used in Figures 5B, S7, and S8).

In vitro dicer reaction

RNA stock solution (100 μM) was diluted in 1× annealing buffer (50 mM Tris-HCl [pH 7], 100 mM NaCl) and to obtain 20 μM RNA solution. The resulting solution was heated at 90°C for 1 min, and kept at 37°C for 1 h to anneal RNA. The annealed RNA can be stored at −80°C until use. For dicer cleavage reaction the annealed RNA solution (20 μM) was incubated with in-house-prepared recombinant human dicer in the presence or absence of 250 nM CMBL3aL at 37°C for 3 h. A typical reaction mixture for dicer cleavage contained 2 μM RNA, 51.3 nM of the recombinant human dicer, 1 mM ATP, 10 mM MgCl2, 0.05 mg/mL BSA, 5% (v/v) DMSO, and
1× Dicer Reaction Buffer (Genlantis) in a 5 μL reaction mixture. After requisite time of incubation, 1 μL of dicer stop solution (Genlantis) or 1 μL of 100 mM EDTA was added to the reaction mixture, followed by the addition of 14 μL of formamide. The resulting sample (20 μL) can be stored at ~80°C; 10 μL of 20 μL of the sample was heated at 90°C for 3 min and cooled immediately on ice followed by analysis using denaturing polyacrylamide gel electrophoresis (PAGE).

Denaturing PAGE conditions: the denatured sample was analyzed by 15% denaturing PAGE (mono-acrylamide/bis-acrylamide = 19:1, 6 M urea) using 1× TBE as running buffer (running condition: 200 V, 60 min). Small RNA II Easy Load (DynaMarker) was used as an RNA marker. Gel was stained with SYBR Gold Nucleic Acid Gel Stain (Thermo Fischer Scientific). RNA bands were visualized using a LAS 4010 system, and quantified using ImageQuant (GE Healthcare) (Figures 4G, 4H, 5C, 5D, and S6).

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2021.11.021.

ACKNOWLEDGMENTS
This work was supported by India-Japan (DST-JSPS) Cooperative Science Programme (IICSP) to promote bilateral scientific collaboration, and JSPS KAKENHI Grant-in-Aid for Scientific Research (A) [19H00924] for KN. Funding for open access charge: JSPS KAKENHI Grant-in-Aid for Scientific Research (A) [19H00924].

AUTHOR CONTRIBUTIONS
K.N., A.M., and S.M. conceptualized the idea. S.M., A.M., R.I., and A.S. performed the experiments and analyzed the data. S.M. and A.M. prepared the figures. S.M. wrote the manuscript draft with the help of A.M. and R.I. K.N., A.M., C.D., M.H., S.K., and K.N. critically reviewed the manuscript. Data contribution, S.M.: Figures 1–5 and S1–S8, Schemes 1–3, Tables S1–S4; A.M. and A.S.: Figures 4G–4F, 5, and S6–S8, Tables S1–S4; R.I. and M.H.: Figures 2B–2D, 3, and S2, Tables S1 and S2. All authors read and approved the final version of the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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Supplemental information

HT-SELEX-based identification of binding pre-miRNA hairpin-motif for small molecules

Sanjukta Mukherjee, Asako Murata, Ryoga Ishida, Ayako Sugai, Chikara Dohno, Michiaki Hamada, Sudhir Krishna, and Kazuhiko Nakatani
Supplementary Information

**HT-SELEX based identification of binding pre-miRNA hairpin-motif for small molecule**

Sanjukta Mukherjee, Asako Murata, Ryoga Ishida, Ayako Sugai, Chikara Dohno, Michiaki Hamada, Sudhir Krishna and Kazuhiko Nakatani
S1. Synthesis and characterization of CMBL3aL

Sodium triacetoxyborohydride (0.0076 g, 0.036 mmol) was added to a solution of CMBL3a (0.012 g, 0.024 mmol) and I (0.0093 g, 0.036 mmol) in dry chloroform (2.5 mL). The reaction mixture was adjusted to pH 6 with acetic acid (2–3 drops), and then stirred at room temperature for 2 h. The reaction mixture was diluted with chloroform (20 ml) and sequentially washed with saturated NaHCO₃ (10 mL), water (10 mL), and brine (10 mL). The organic layer was collected and dried over anhydrous sodium sulfate and evaporated to dryness. The crude product was purified by silica gel column chromatography (2.5–3.0% methanol-chloroform) to give an off-white solid (8.2 mg, 43%) BocCMBL3aL as a pure product. To a stirred solution of compound BocCMBL3aL (7.7 mg, 0.012 mmol) in chloroform (1 mL) was added 4 N HCl-ethylacetate (1 mL) at 0 °C under argon atmosphere. The reaction mixture was then stirred at 0 °C to room temperature for 1 h. After the solvent was evaporated to dryness, the crude product was basified by adding 7 N ammonia in methanol followed by basic silica gel column chromatography (2–5% methanol-chloroform) to obtain pure CMBL3aL as an off-white solid (2.7 mg, 40%) (Scheme S1). ¹H NMR (600 MHz, CD₃OD) δ 8.50 (d, J = 9.0 Hz, 2H), 8.33 (d, J = 7.8 Hz, 2H), 7.77 (d, J = 9.0 Hz, 2H), 7.65 (d, J = 8.4 Hz, 2H), 5.69-5.67 (m, 2H), 5.03 (s, 4H), 4.35 (t, J = 5.4 Hz, 4H), 3.67 (t, J = 7.2 Hz, 2H), 3.37 (t, J = 6.0 Hz, 2H), 3.13 (t, J = 6.6 Hz, 2H), 2.57 (t, J = 6.6 Hz, 2H), 2.47 (td, J = 5.4, 5.25 Hz, 4H), 2.11 (quint, J = 6.75 Hz, 2H), 2.02 (quint, J = 6.45 Hz, 2H); ¹³C NMR (150 MHz, CD₃OD) δ 176.7, 158.3, 154.8, 141.3, 130.4, 123.6, 120.2, 116.1, 71.4, 67.6, 60.3, 56.3, 40.8, 37.1, 34.4, 33.0, 26.3, 24.0; HRMS (ESI) m/z: Calcd. for C₃₃H₄₀N₀₉O₅ [M+H]+ 642.3152; Found: 642.3132; Calcd. for C₃₃H₃₉N₀Na₀₅ [M+Na]+ 664.2972; Found: 664.2949. ¹H NMR and ¹³C NMR spectra available in Figure S9 and S10 respectively.

Scheme S1. Synthetic scheme for preparation of CMBL3aL
Scheme S2. Preparation of CMBL3aL-immobilized resin and Tris-resin

Scheme S3. Schematic representation of the SELEX
Figure S1. Gel images for cycle course PCR of the eluted bound species from after (A) 2\textsuperscript{nd}, (B) 5\textsuperscript{th} and (C) 8\textsuperscript{th} round of selection from the $N_{11}$ library. PCR amplification of the library was monitored at 4, 8, 12, and 16 PCR cycles as denoted in the image. The PCR products were analyzed by 8\% native polyacrylamide (29:1) gel electrophoresis (PAGE) with 1x TBE as running buffer. DNA bands on the gels were visualized by SYBR gold staining.

N.B.: After 8\textsuperscript{th} selection cycle using $N_{11}$ library, we observed the formation of a new PCR product in the early PCR cycles during amplification of the cDNA. This product produced a band around 100 bp (Band ST2 in Figure S1C) that migrated slower than the original DNA pool (Band ST1, Figure S1C). Band ST2 might be due to the formation of elongated by-products, so the amplified 9\textsuperscript{th} DNA pool was purified on an 8\% native polyacrylamide gel, and used for the preparation of 9\textsuperscript{th} RNA pool.

![Gel Images](image.png)

Figure S2. Change in the frequency of R-Seq1~6 in the course of the SELEX. Frequencies of each aptamer from 2\textsuperscript{nd} to 10\textsuperscript{th} cycles of $N_{6,11}$ library were plotted. Frequency was calculated by RaptRanker software.

![Frequency Graph](image.png)
**Figure S3.** The secondary structures of the aptamers enriched in the final selection cycle (12th) of N11 pool (Table S1) as predicted by CentroidFold (http://rtools.cbrc.jp/cgi-bin/index.cgi). The characteristic sequence consensus (UGG/GGU/UGGU/UGGUG) were marked with black arcs.

**Figure S4.** The secondary structures of the aptamers enriched in the final selection cycle (10th) of N6-11 pool (Table S2) as predicted by CentroidFold (http://rtools.cbrc.jp/cgi-bin/index.cgi). The characteristic sequence consensus (UGG/GGU/UGGU/UGGUG) were marked with black arcs.
Figure S5. SPR analysis of CMBL3aL binding to pre-miR29a. CMBL3aL solution was injected onto the pre-miR29a-immobilized sensor surface in increasing concentrations (100-500 nM).

Figure S6. Denaturing PAGE analysis of the dicer-cleaved products from (A) R-Seq1 and (C) R-Seq2 in the absence or the presence (250 µM) of CMBL3aL. (B) and (D) Bar graph representation of the cleaved fraction (I+P, %) determined by densitometric analysis of the corresponding gel image. Single cleavage intermediates and double cleavage products are denoted by ‘I’ and ‘P’, respectively. The bands near 25 nt are corresponding to the band after double cleavage near both the hairpins of R-Se1 and R-Seq2. The band near 30 nt is due to the single cleavage near one hairpin and double cleavage near another hairpin. The band near 50 nt is corresponding to the single cleavage near any one hairpin. *p < 0.01 and **p < 0.05 versus control sample without CMBL3aL addition (paired t-test).
**Figure S7.** (A-B) Secondary structure of human pre-miR33a hairpin mutant pre-miR33a-G31A (A) and pre-miR33a-G32A (B) after incorporation of G to A point mutation at 31 and 32 positions respectively. (C-D) SPR sensorgrams of pre-miR33a-G31A and pre-miR33a-G32A [0.01 (yellow), 0.03 (green), 0.1 (blue), 0.3 (purple), 1.0 (red) µM] binding to the CMBL3aL-immobilized sensor surface (Immobilized amount: 3704 RU).

**Figure S8.** (A) Secondary structure of human pre-miR24-2 (contains UGGU sequence). (B) SPR analysis of pre-miR24-2 [0.01 (yellow), 0.03 (green), 0.1 (blue), 0.3 (purple), 1.0 (red) µM] binding to the CMBL3aL-immobilized sensor surface (Immobilized amount: 2764.8 RU).
Figure S9. $^1$H NMR Spectrum of CMBL3aL (600 MHz, CD$_3$OD)
Figure S10. $^{13}$C NMR Spectrum of CMBL3aL (150 MHz, CD$_3$OD)
Table S1. Sequencing data for 3rd–12th DNA pools obtained from the selection of $N_{11}$ library. The sequencing data obtained by the Ion PGM system were analyzed by RaptRanker software.

(Attached in supporting excel file)

Table S2. Sequencing data for 2nd–12th DNA pools obtained from the selection of $N_{6-11}$ library. The sequencing data obtained by the Ion PGM system were analyzed by RaptRanker software.

(Attached in supporting excel file)

Table S3. Kinetic parameters obtained from SPR analysis for binding of R-Seq1-4 to CMBL3aL using 1:1 fitting model

| RNA    | $k_{on}$ (× 10^3 M⁻¹ s⁻¹) | $k_{off}$ (× 10⁻³ s⁻¹) | $K_{d(app)}$ (× 10⁻⁹ M) (nM) |
|--------|-----------------|-----------------|---------------------------|
| R-Seq1 | 17.5            | 3.809           | 218                       |
| R-Seq2 | 4.626           | 6.067           | 1310                      |
| R-Seq3 | 7.463           | 5.096           | 682                       |
| R-Seq4 | 16.4            | 2.22            | 135                       |

Table S4. Kinetic parameters obtained from SPR analysis for binding of endogenous pre-miRNAs and its mutant to CMBL3aL using 1:1 fitting model

| RNA             | $k_{on}$ (× 10^3 M⁻¹ s⁻¹) | $k_{off}$ (× 10⁻³ s⁻¹) | $K_{d(app)}$ (× 10⁻⁹ M) (nM) |
|-----------------|-----------------|-----------------|---------------------------|
| Pre-miR33a      | 581             | 0.799           | 1.38                       |
| Pre-miR33a-G31A | 644             | 1.444           | 2.24                       |
| Pre-miR33a-G32A | 583             | 1.488           | 2.55                       |
| Pre-miR24-2     | 557             | 1.135           | 2.04                       |

S2. Reference

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