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Development of a Novel DNA Oligonucleotide Targeting Low-Density Lipoprotein Receptor

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Low-density lipoprotein receptor (LDL-R) is a cell surface receptor protein expressed in a variety of solid cancers, including lung, colon, breast, brain, and liver, and therefore it opens up opportunities to deliver lysosome-sensitive anticancer agents, especially synthetic nucleic acid-based therapeutic molecules. In this study, we focused on developing novel nucleic acid molecules specific to LDL-R. For this purpose, we performed *in vitro* selection procedure via systematic evolution of ligands by exponential enrichment (SELEX) methodologies using mammalian cell-expressed human recombinant LDL-R protein as a target. After 10 rounds of selections, we identified a novel DNA oligonucleotide aptamer, RNV-L7, that can bind specifically to LDL-R protein with high affinity and specificity (K_D = 19.6 nM). Furthermore, flow cytometry and fluorescence imaging assays demonstrated efficient binding to LDL-R overexpressed human cancer cells, including Huh-7 liver cancer cells and MDA-MB-231 breast cancer cells, with a binding affinity of ∼200 nM. Furthermore, we evaluated the functional potential of the developed LDL-R aptamer RNV-L7 by conjugating with a previously reported miR-21 targeting DNAzyme for inhibiting miR-21 expression. The results showed that the miR-21 DNAzyme-RNV-L7 aptamer chimera efficiently reduced the expression of miR-21 in Huh-7 liver cancer cells. As currently there are no reports on LDL-R aptamer development, we think that RNV-L7 could be beneficial toward the development of targeted cancer therapeutics.

INTRODUCTION

Low-density lipoprotein receptor (LDL-R), a member of the LDL-R gene family, is a cell-surface receptor that recognizes the outer phospholipid layer of cholesterol-rich LDL and mediates the endocytosis of LDL particles.[1] In the physiological condition, LDL-R is most expressed in adrenal gland, cortex, and bronchial epithelial cells.[2] The overexpression of LDL-R has long been associated with increased risk of various neurological and coronary artery diseases, including atherosclerosis, metabolomics syndrome, and steatohepatitis.[3] In recent years, increasing evidence has demonstrated that LDL-R also plays an important role in tumorigenesis and represents an important cell surface marker for a variety of cancers, including lung, liver, breast, brain, and colon cancer.[4] As a result, LDL-R has gained its prominence in cancer research.

In line with previous reports, LDL-R has been utilized for delivering drugs to LDL-R-overexpressed tumor tissues by clathrin-mediated endocytosis.[5,6] Indeed, as an endogenous ligand of the LDL-R, LDL is an ideal natural nanocarrier for loading and delivering cancer therapeutic and diagnostic agents to tumors due to its structural characteristics and lipophilic properties.[7] However, the application of LDL is challenging with current technology, as the large-scale isolation of LDL is very hard due to the rate-limiting step of ApoB purification.[8] Additionally, the natural LDL is normally variable in composition and size, which makes quality control of LDL preparations difficult. Consequently, various LDL analogs and LDL-R antibodies have been developed to exploit the LDL-R-mediated drug delivery approach.[8] However, LDL analogs face problems, including instability, and, in many cases, health concerns caused by the competition of such ligands to endogenous LDL.[8]

Nucleic acid aptamer technology provides a potential strategy to circumvent the obstacles faced by natural LDL and LDL-R antibodies. Aptamers are short single-stranded nucleic acid sequences that can recognize and bind to target molecules with high affinity and specificity in a way similar to antibodies.[9–12] However, differing from traditional antibodies, aptamers possess certain advantages such as easy laboratory production from small to large amounts, no batch-to-batch variation, prolonged shelf life, low/no immunogenicity, low cost, and freedom to incorporate chemical modifications for enhanced binding properties. During the past decades, various aptamer-mediated drug delivery systems for either small molecular chemotherapeutics or oligonucleotide therapeutics molecules (i.e., small interfering RNA [siRNA], antisense oligonucleotide, antimiR) have been developed.[13–15] LDL-R provides an opportunity to deliver lysosome-sensitive drug molecules such as oligonucleotides to cytoplasm or nucleus to meet their endogenous targets.[16]

We envisioned the development of novel nucleic acid aptamers specific to LDL-R, and, in this study, we performed iterative rounds of *in vitro* selection process, known as the systematic evolution of
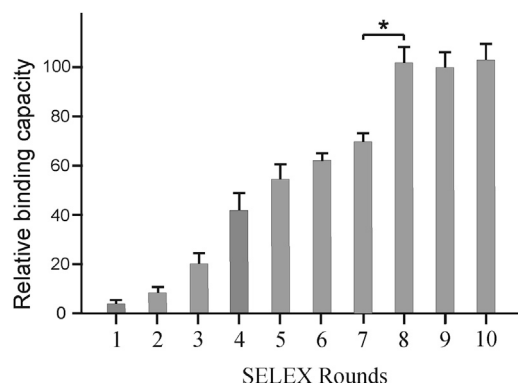
Figure 1. Comparison of the Binding Capacity of Sub-library Pools from 10 Consecutive Rounds of SELEX

Biotin-labeled sub-library pools from iterative rounds of SELEX were incubated with immobilized LDL-R in a nickel-coated 96-well plate at room temperature for 30 min. After incubating with anti-biotin antibody for 1 h, the fluorescence intensity was measured by a plate reader. The binding of each round was calculated after subtracting the mean fluorescence intensity of the binding of the original library (RNV75) to LDL-R protein. *$p \leq 0.05$.

ligands by exponential enrichment (SELEX), to identify DNA aptamers that bind to LDL-R toward delivering therapeutic nucleic acids specifically to LDL-R-overexpressed cancer cells.

RESULTS

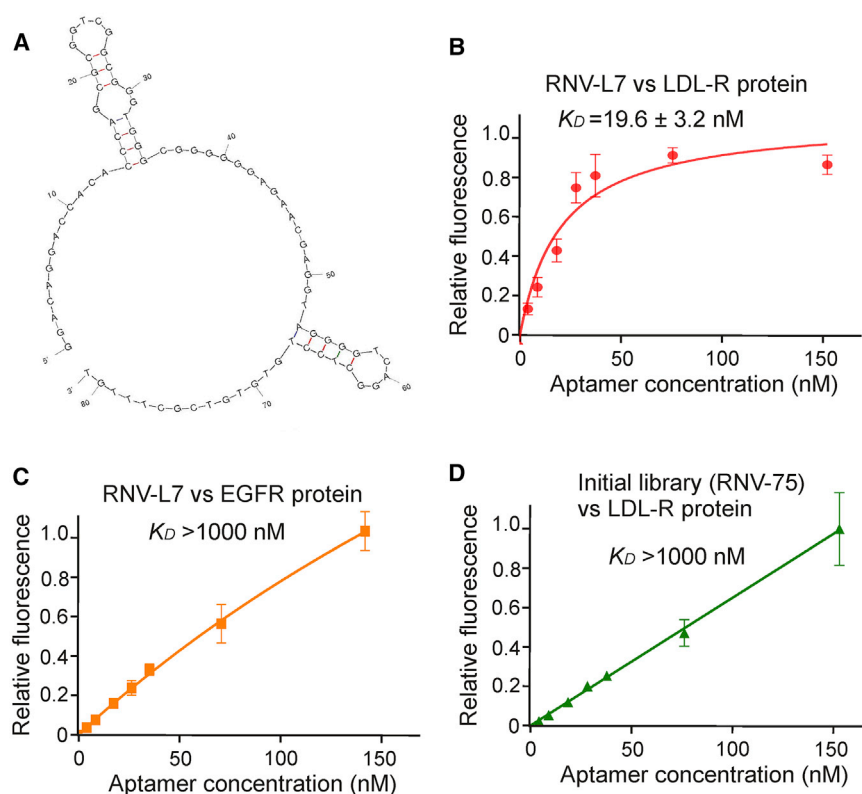### Competitive Elution Facilitates the Selection of Aptamers against LDL-R

Preparation of a protein-immobilized surface using a nickel-coated 96-well plate (histidine-binding plate) ensures an efficient SELEX procedure by allowing easy handling, stringent washing, and quantitative adjustment of the amount of protein target for individual rounds. However, the efficiency of this protein-immobilization method could be affected by two issues. First, LDL-R is a structurally complex cell-surface receptor consisting of a mosaic protein of 839 aa,[17] and the natural structural conformation of certain domains of it could undergo significant changes during the process of immobilization, which ultimately affect the applicability of the developed aptamer to native human LDL-R.[18] Second, during SELEX, the library sequences might randomly bind to the plastic matrices and causes nonspecific selection.[9] In this work, to eliminate the detrimental effect of nonspecific binding and to ensure that the identified aptamers can recognize the LDL-R protein conformation, a competitive elution strategy was introduced. As illustrated in the Materials and Methods section, after target incubation and extensive washes, 50 pmol of free LDL-R protein (more than 10 times that of the immobilized protein) was added to the immobilized LDL-R/single-stranded DNA (ssDNA) library mixture to elute ssDNA sequences that are able to recognize the free LDL-R. With such a competitive elution process, coupled with a stringent negative selection procedure (Table S1), the selection reached a plateau after round 8, with no noticeable improvement in binding capacity being observed from rounds 8 to 10 as assessed via an enzyme-linked oligonucleotide assay (ELONA)

(Figure 1). At the same time, to confirm the specificity of the selection to LDL-R protein, the binding affinity of the original ssDNA library (R0) and the selected sub-ssDNA pools from rounds 6, 8, and10 of the SELEX was tested with both LDL-R protein and a negative control TGpr1 protein. As demonstrated in Figure S1 (Supplemental Information), no notable binding affinity was observed with the negative TGpr1 protein control compared with a significant binding shift observed with the LDL-R protein.

The selection was terminated at round 10, and sub-library pools from rounds 3, 8, and 10 were sequenced using a MiSeq platform, which assisted the screening of aptamer candidates from early to last rounds. After analysis of the sequencing data, potential aptamer candidates (Table S2) were identified based on their counts, and the first 10 sequences for binding capacity and specificity assessments were synthesized. Based on the initial evaluation, an aptamer termed RNV-L7 (5′-GGA CAG GAC CAC ACC CAG CGC GGT CGG CGG GTG GGC GGG GGG AGA ACG AGG TAG GGG TCA GGC TCC TGT GTG TCGCTT TGT-3′) (Figure 2A) was eventually selected for further analysis. As demonstrated by an ELONA assay in Figures 2B–2D, RNV-L7 aptamer showed specific binding to its LDL-R target with a binding affinity value of 19.6 nM. As expected, RNV-L7 aptamer displayed high specificity, as it did not bind to a nonspecific protein, epidermal growth factor receptor (EGFR) (negative control). In addition, the original starting ssDNA library also did not display any binding to LDL-R protein, demonstrating the maturation of a particular sequence. Although the constant primer binding sites normally do not engage in the binding properties of aptamers,[9] in this case, it was apparent that the binding capacity of the RNV-L7 aptamer is also structurally dependent on its primer binding site, as a truncated sequence without primary binding site (5′-CGGTCGGC GGGTGGGCGGGGGGAGAACGAGGTAGGGGTCA-3′) showed complete depletion of binding capacity to LDL-R protein (Figure S2; Supplemental Information).

RNV-L7 Aptamer Is Able to Recognize LDL-R-Positive Cells

In order to achieve targeted cancer treatment, it is imperative for the developed aptamer to recognize the target proteins in their native state on living cells, and to undergo efficient internalization upon aptamer/target binding. We next tested whether the identified RNV-L7 aptamer could recognize LDL-R on the cell membrane. To this end, 6-carboxyfluorescein (FAM)-labeled RNV-L7 aptamer was incubated with both LDL-R-positive cells (Huh-7 liver cancer cells and MDA-MB-231 breast cancer cells) and LDL-R-negative HEK293 cells at 37°C for 30 min followed by flow cytometry assay. As demonstrated in Figure 3A, compared with the LDL-R-negative HEK293 cells, all of the three tested LDL-R-positive cells displayed a noticeable shift in the fluorescence signal, indicating that the identified RNV-L7 aptamer could specifically recognize LDL-R-positive cells. Further kinetic analysis showed that the RNV-L7 aptamer had a medium-high binding capacity to LDL-R-positive cells, with dissociation equilibrium constants of 295 and 252 nM for Huh-7 and MDA-MB-231 cells, respectively (Figure 3B). There was no noticeable fluorescent signal, and specific binding properties were recorded for LDL-R-negative

aptamer and DNAzyme sequences. In principle, if the employed linker does not affect the proper structure formation of either the aptamer or DNAzyme components, this type of linker should be eligible for constructing chimeric molecules. Previously, such linkers with different lengths have been practiced. For example, "AA" linker was used in two previous reports for the design of aptamer-siRNA chimera structures,[14,20] and other reports demonstrated the use of 5-consecutive-nt bridges.[19,21] In addition, we have recently reported the use of d(TTTTT) linker to maintain the structural conformation of the aptamer and the antimiR DNAzyme sections.[19] In line with these previous reports, we also adopted such a 5-consecutive-dT bridge in this study. While the purpose of the aptamer part is to specifically target LDL-R protein and initiate cellular internalization, the antimiR-21 DNAzyme component will recognize and inhibit the expression of endogenous miR-21 target in the cytoplasm. The RNV-L7 aptamer-antimiR-21 DNAzyme chimera was then synthesized and tested in the LDL-R-positive Huh-7 liver cells without using any transfection reagents. As shown in Figure 6, compared with the controls, RNV-L7 could efficiently deliver its antimiR-21 DNAzyme cargo to Huh-7 cells. Further functional assays demonstrated that the RNV-L7 aptamer-antimiR-21 DNAzyme chimera reduced up to 56% of miR-21 expression in Huh-7 cells (Figure 6). Notably, after 48 h of incubation, only the RNV-L7 aptamer-antimiR-21 chimera showed approximately 56% miR-21 inhibition in the tested Huh-7 cells, while other groups, including the untreated group, RNV-L7 only group, antimiR-21 only group, and the negative control aptamer-antimiR-21 chimera group, displayed no difference in terms of miR-21 expression.. These results provide proof-of-principle evidence regarding the rationality of utilizing RNV-L7 aptamer toward targeted drug delivery in liver cancer cells.

HEK293 cells (K_D > 1,000 nM). Importantly, as shown in Figure S3 (Supplemental Information), RNV-L7 aptamer displayed a remarkable binding signal on both LDL-R-positive Huh-7 and MDA-MB-231 cells, whereas no prominent binding signal was observed with the initial SELEX library control. These results, together with the negative binding results from the LDL-R-negative HEK293 cells (Figure 3), suggested the specific binding nature of RNV-L7 to LDL-R-positive cells. Cellular binding potential of RNV-L7 aptamer was further confirmed by fluorescence microscopy imaging. As shown in Figure 4A, following RNV-L7 aptamer incubation, a strong fluorescent signal was observed in all of the three tested LDL-R-positive cells but not in the LDL-R-negative HEK293 cells. Importantly, an efficient internalization of RNV-L7 aptamer to LDL-R-positive cells was recorded (Figures 4B and 4C), after incubating with the RNV-L7 aptamer for 30 min at 37°C, suggesting the possibility of employing RNV-L7 aptamer for targeted cancer treatment.

RNV-L7 Aptamer-Guided DNAzyme Delivery Inhibits miR-21 Expression in LDL-R-Positive Huh-7 Cells

Next, the potential of RNV-L7 as a drug delivery platform was assessed using a previously reported antimiR-21-specific DNAzyme for targeted inhibition of miR-21 in Huh-7 liver cancer cells.[19] As shown in Figure 5, an RNV-L7 aptamer-antimiR-21 DNAzyme chimera structure was designed by conjugating the RNV-L7 aptamer to the antimiR-21 DNAzyme via a 5-consecutive-deoxythymidine (dT) (d-TTTTT-) bridge to ensure the flexibility and the biological function of both the

DISCUSSION

Delivering therapeutic agents precisely to cancer cells via a cell surface marker is one of the primary principles of targeted cancer therapy.[22]
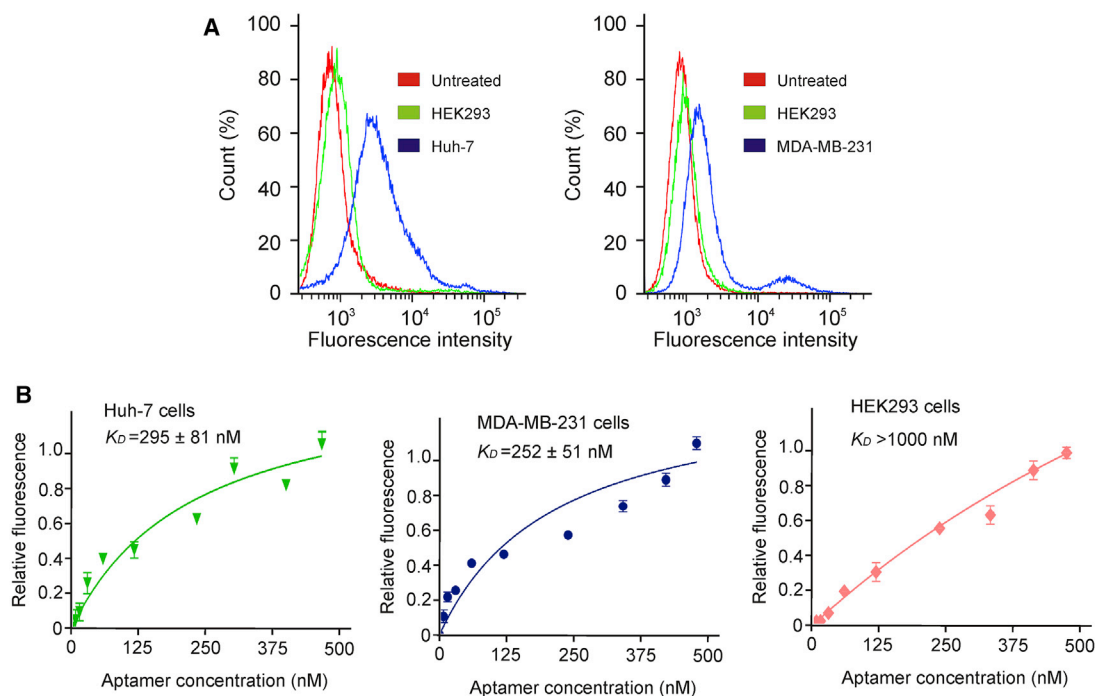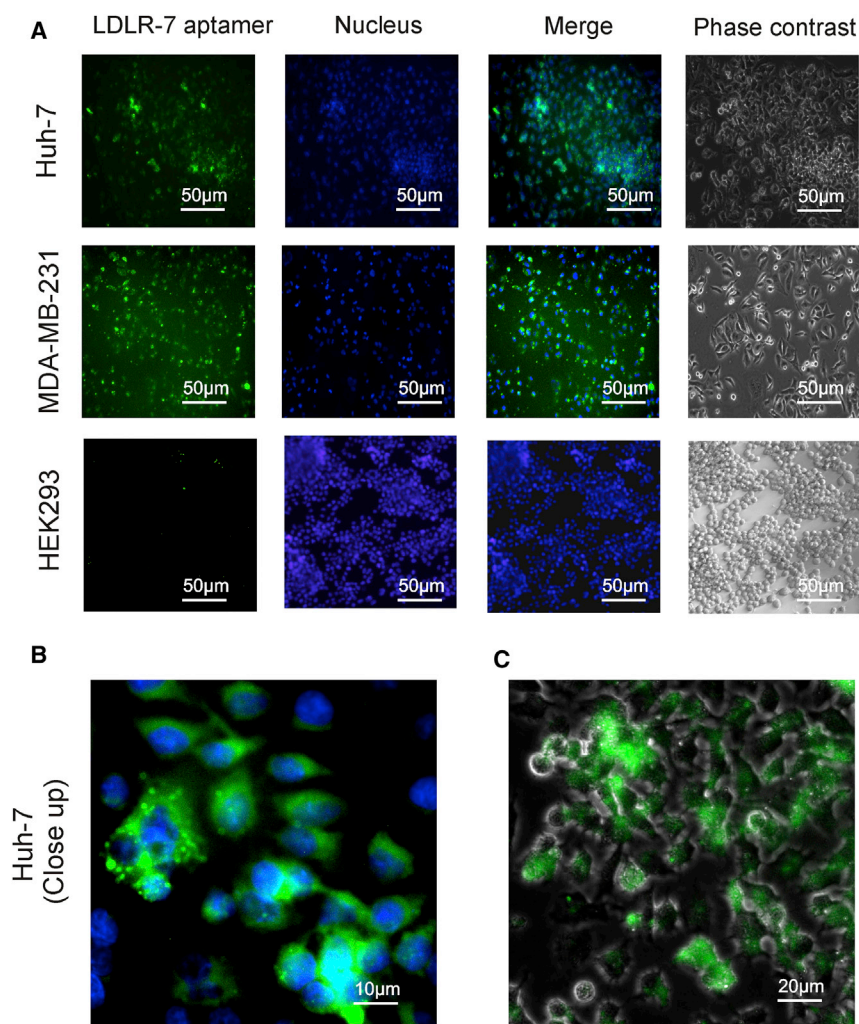
Figure 3. Determination of Specific Binding of RNV-L7 Aptamer to LDL-R-Positive Cells

(A) Quantification of binding of RNV-L7 aptamer to LDL-R-positive Huh-7 and MDA-MB-231 cells versus that to the LDL-R-negative HEK293 cells via flow cytometry analysis. FAM-labeled RNV-L7 aptamer was incubated at a concentration of 200 nM with LDL-R-positive or LDL-R-negative cell lines for 30 min at 37°C. (B) Determination of equilibrium dissociation constants (K_D) of RNV-L7 aptamer to LDL-R-positive cells via flow cytometry by incubating cells at varying concentrations of aptamer (0–480 nM) using an LDL-R-negative HEK293 cell line as a negative control. All results were collected from three independent tests, with excessive (10 μg/test) random tRNA as a competitor to block the potential binding site of protein targets prior to conducting binding assays. K_D was derived using GraphPad Prism program 3.03.

As a key cell membrane protein for receptor-mediated endocytosis, LDL-R is an attractive target for novel cancer therapy, as it is highly expressed in various types of solid cancers and negatively correlated with clinical outcomes of cancer patients.[23] In this work, we investigated the scope of the LDL-R-mediated endocytosis pathway for targeted drug delivery using nucleic acid aptamer technology. Compared to other targeting ligands including antibodies, aptamers possess advantages such as reduced particle size, easy laboratory production in small to large amounts, improved safety profile, low cost, and the ability to incorporate multiple chemical modification for enhanced binding capacity and specificity, which collectively make them ideal ligands for drug delivery. Differing from LDL and LDL analogs, which are eventually delivered to lysosome, LDL-R-specific aptamer may undergo LDL-R-mediated endosomal escape in a way similar to LDL-R binding viruses.[24,25] This is especially advantageous for delivering lysosome-sensitive nucleic acid drug molecules such as siRNA, antimiR, DNAzyme, and antisense oligonucleotide, which need to recognize their endogenous targets in either cytoplasm or nucleus.[14]

Over the past decades, traditional SELEX methodology has undergone dramatic modifications and improvements. However, developing high-performing aptamers is still a major obstacle for aptamer-based research and applications. In order to obtain high-quality aptamers for clinical translation, designing an optimized aptamer selection procedure using the native conformation of LDL-R protein is critical. For this purpose, a competitive elution approach, in line with a previous report,[26] was performed. By using excessive free LDL-R protein to elute ssDNA sequences bound to the immobilized LDL-R protein could potentially eliminate the detrimental effect of any structural changes of LDL-R during target immobilization. This will promote the identification of aptamers binding specifically to LDL-R structure on the cell surface. After selection, the use of a long forward primer containing 20-consecutive dT nt connected to the complementary sequence of the primer-binding region of the library via a triethylene glycol linkage allowed the purification and isolation of selected aptamer sequences. Amplification by PCR will generate a DNA product containing a longer top strand (101 nt) and the shorter aptamer strand (81 nt), and denaturing PAGE readily facilitated an easy separation of this product followed by electroelution and ethanol precipitation. Following next-generation sequencing analysis after 10 rounds of selection and enrichment procedures, a potential aptamer candidate, RNV-L7, was identified. Interestingly, an ELONA assay revealed that RNV-L7 aptamer showed excellent affinity to the recombinant LDL-R protein with an equilibrium dissociation constant value (K_D) of 19.6 nM. However, when tested against LDL-R-overexpressed cancer cells, including Huh-7 liver cancer cells and MDA-MB-231 breast cancer cells, the affinity decreased to 295

A LDLR-7 aptamer | Nucleus | Merge | Phase contrast
Huh-7
MDA-MB-231
HEK293

B Huh-7 (Close up)

C

Figure 4. Specific Binding and Internalization of LDL-R Aptamer to LDL-R-Positive Cells

(A) RNV-L7 aptamer specifically binds to LDL-R-positive Huh-7 and MDA-MB-231 cells but not to LDL-R-negative HEK293 cells. Cells were imaged via fluorescence microscopy after incubation with 200 nM aptamer at 37°C for 30 min. Green, FAM (aptamer); blue, Hoechst 33342 (nuclei). (B and C) Enlarged images to demonstrate the internalization pattern of RNV-L7 aptamer to LDL-R-positive Huh-7 cells. Merge of aptamer with (B) nuclei and (C) phase contrast.
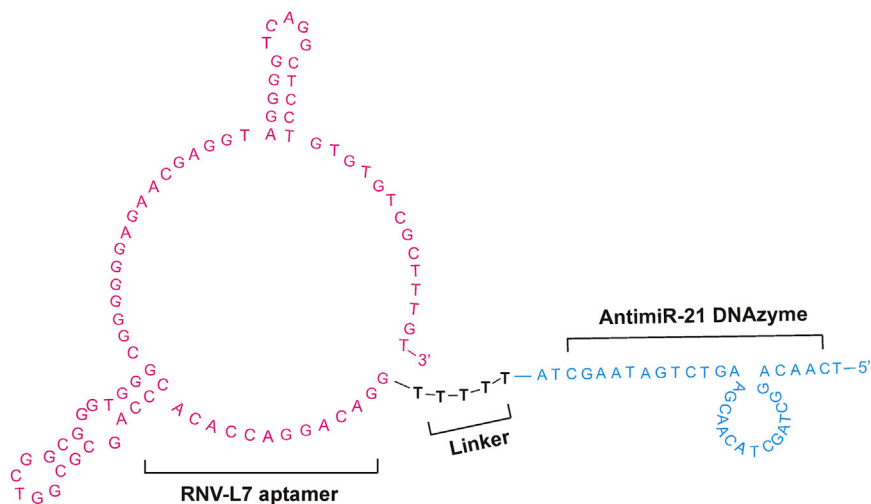
cells.[28] As a result, although the tested high-affinity EpCAM antibody effectively reacts with EpCAM overexpressed cancer cells, it negatively affects normal tissues expressing low levels of EpCAM. This is also true for LDL-R. Although LDL-R is overexpressed in certain cancer cells, it is also expressed with lower levels in a number of normal cells, such as bronchial epithelial cells, adrenal gland cells, and cortex cells.[2] Considering this, the moderately high binding affinity of RNV-L7 ($K_D = \sim$200 nM) to cancer cells could selectively deliver tumor-targeting agent to LDL-R-overexpressed cancer tissues while not significantly affecting normal cells.

To evaluate the drug delivery potential of RNV-L7 aptamer, similar to previously reported aptamer-therapeutic nucleic acid chimeras,[15] a bifunctional aptamer-antimiRzyme chimera was designed using a previously reported miR-21-targeting DNAzyme (antimiR-21 DNAzyme RNV541).[19] A 5-mer-consecutive 2′-d-TTTTT was used to link the RNV-L7 aptamer and the RNV541 antimiRzyme in order to construct the chimera, which might reduce any possible interaction between the aptamer and antimiR-21 DNAzyme, and can also maximize the possibility of retaining the functions of both RNV-L7 aptamer and RNV541 DNAzyme[21] (Figure 5). As indicated in our truncation assays, it was apparent that the binding capacity of the RNV-L7 aptamer is dependent on its primer binding site (Figure S2; Supplemental Information). To confirm this, the miR-21 inhibition assay was initiated to demonstrate that the downregulation of miR-21 expression was in fact by RNV-L7-mediated cellular internalization of the antimiR-21-DNAzyme sequence rather than aptamer sequence-independent nonspecific miR-21 inhibition. For this purpose, we used RNV-L7 aptamer-antimiR-21 DNAzyme chimera, free RNV-L7 aptamer and antimiR-21 DNAzyme sequence controls, and a mock aptamer-DNAzyme chimera structure by employing a mutated RNV-L7 sequence (deleting the 3′ end primer binding sequence, and keeping 80.2% of the original sequence). After confirming the binding capacity of RNV-L7 aptmer-antimiR-21 DNAzyme chimera to LDL-R-positive Huh-7 cells via an imaging assay

and 252 nM, respectively, but it still showed a medium-high binding capacity. However, HEK293 cells that do not express LDL-R did not show any binding, which demonstrated the target specificity of RNV-L7 aptamer. Microscopy imaging further confirmed the binding and internalization properties of RNA-L7 aptamer to LDL-R-overexpressed cells. These results uniquely position RNV-L7 aptamer to deliver various drug payloads to LDL-R-positive cancer cells.

In fact, the medium-high binding affinity of the RNV-L7 aptamer to its cellular targets ($\sim$200 nM) could actually be an advantage over its high-binding-affinity counterparts.[27] A previous clinical trial against solid cancers using an epithelial cell adhesion molecule (EpCAM) monoclonal antibody with a high affinity to EpCAM ($K_D = 0.160$ to 0.190 nM) caused acute pancreatitis in patients, whereas an EpCAM antibody with medium-high affinity ($K_D = 91.0$ nM) showed a better safety profile and was well tolerated in patients.[28] The reason lies in the ubiquitous expression profile of EpCAM proteins.[28] While the targeted EpCAM protein is highly expressed in most human adenocarcinomas, it is also expressed at low levels in normal epithelial

Figure 5. Schematic Illustration of RNV-L7 Aptamer-RNV541 Anti-miRzyme Chimera

The chimeric structure consisted of RNV-L7 aptamer (red) and RNV541 DNAzyme (blue) sections, which were jointed via a d(TTTTT) linker.

(Figure 6A), the potential of this chimera in inhibiting miR-21 expression was tested in the absence of transfection reagents. Interestingly, RNV-L7 aptamer-antimiR-21 DNAzyme chimera yielded 56% miR-21 suppression, which clearly demonstrated the potential of RNV-L7 aptamer-targeted delivery (Figure 6B). Notably, the mock aptamer-DNAzyme chimera control sequence and the free RNV-L7 and anti-miR-21 sequence controls did not show any inhibition of miR-21 (Figure 6B), suggesting the importance of RNV-L7 aptamer for LDL-R-mediated delivery. Unlike other functional nucleic acid-based treatments such as siRNA, which only interrupts and regulates a particular type of gene, antimiRzyme-based miR-21 inhibition can affect post-transcriptional regulation of the miR-21 network by controlling the expression of hundreds of genes implicated in cancer pathology.[19] This suggests that even small-scale suppression of the miR-21 expression could result in a significant anti-cancer effect, and the 56% miR-21 suppression rate observed with the RNV-L7 aptamer-antimiR-21 DNAzyme chimera without using any transfection reagent may therefore be very promising toward targeted cancer treatment.

In summary, we have developed a DNA aptamer that can specifically bind to both free LDL-R protein and LDL-R-expressing cells, and effectively deliver a miR-21-targeting DNAzyme to liver cancer cells. By introducing chemical modifications such as locked nucleic acids and inverted dT,[29-32] we speculate that the efficiency of this aptamer can be improved further. Based on our current results, we think that RNV-L7 aptamer can be used to conjugate with various anti-cancer drug molecules to facilitate targeted cancer therapy.

MATERIALS AND METHODS

Cell Lines and Cell Culture

HEK293 cells were bought from Cell Bank Australia (Sydney, Australia). MDA-MB-231 cells were kindly provided by Prof. Stacey Edward at the Queensland Berghofer Institute for Medical Research and sourced from ATCC, USA. The human hepatocarcinoma cell line, Huh7, was supplied by the JCRB Cell Bank (Osaka, Japan) and purchased from CellBank Australia (Westmead, NSW, Australia). All cells were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) and maintained at 37°C in a humidified incubator under 5% CO_2.

Buffers and Sequence Information

The binding buffer included the following: pH 7.4 PBS containing 4.5 mM $MgCl_2$, 0.005% Tween 20, and 100 μg/mL tRNA (Sigma,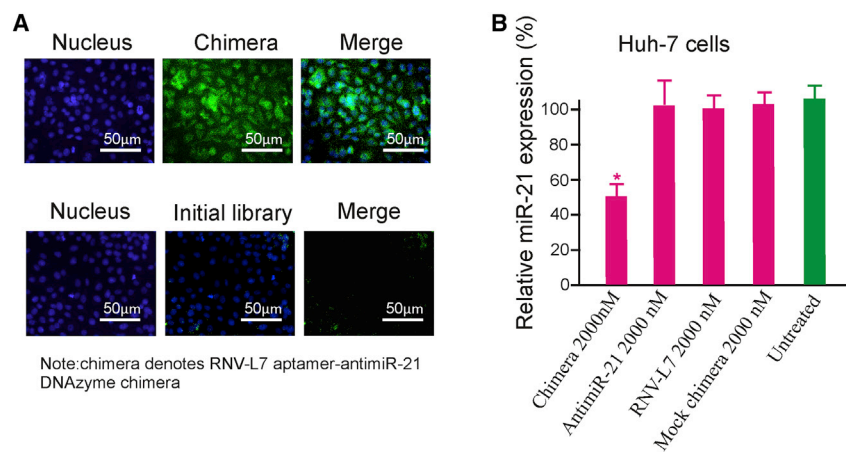 R8759). The wash buffer included the following: pH 7.4 PBS containing 4.5 mM $MgCl_2$ and 0.005% Tween 20. The ssDNA library was as follows: 5′-GGACAGGAC CACACCCAGCG-(N40)-GGCTCCTGTGTGTCGCTTTGT-3′; forward primer, 5′-TTTTTTTTTTTTTTTTTTTTT/iSp9/ACAAAGC GACACACAGGAGCC-3′; reverse primer, 5′-GGACAGGACCA CACCCAGCG-3′. The initial ssDNA library sequence (RNV-75) was synthesized by Integrated DNA Technologies (IDT), with a central 40-nt randomized sequence and primer binding sites on both ends. The reverse primer was conjugated with 20 consecutive dT nt via a triethylene glycol spacer to facilitate denaturing PAGE-based ssDNA separation.[9,33] To prepare biotin-labeled sub-library pools for binding capacity assessment after each round, a forward primer with a 5′-biotin label was synthesized.

LDL-R Protein Preparation

Human recombinant LDL-R protein was expressed in mammalian cells with a sequence (Ala22-Arg 788) of human LDL-R fused with a poly-histidine tag at the C terminus (sourced commercially from Novoprotein, catalog no. CA88). 5 pmol of LDL-R was dissolved in 100 μL of binding buffer and transferred to a well of the Pierce nickel-coated 96-well plates (Thermo Fisher, Australia) to immobilize protein for aptamer selection. As a negative selection control, an equal amount of irrelevant protein, TGpr1, with a comparable molecular weight with LDL-R was used to immobilize another well of the plate using an identical method as for LDL-R.

SELEX Selection

An oligonucleotide library pool (ssDNA, 1000 pmol for initial selection, or 100 pmol for each iterative round) was diluted in 100 μL of binding buffer and denatured at 95°C for 10 min and then cooled on ice for 10 min. After this, the library was added to the plate and incubated with the target protein on a shaker at 700 rpm for 1 h at room temperature. After incubation and extensive washes, 50 pmol of native-state LDL-R (more than 10-fold that of the immobilized protein bait) was added to the immobilized LDL-R/ssDNA library mixture to elute ssDNA sequences and to ensure that the identified

A

Nucleus	Chimera	Merge
50μm	50μm	50μm

Nucleus	Initial library	Merge
50μm	50μm	50μm

Note:chimera denotes RNV-L7 aptamer-antimiR-21 DNAzyme chimera

B

Huh-7 cells

Figure 6. Evaluation of miR-21 Inhibition Using RNV-L7 Aptamer-DNAzyme RNV541 Chimera in Huh-7 Cells

(A) RNV-L7 aptamer-DNAzyme RNV541 chimera specifically binds to LDL-R-positive Huh-7 cells. Cells were imaged via fluorescence microscopy after incubation with 200 nM aptamer at 37°C for 30 min. Green, FAM (chimera); blue, Hoechst 33342 (nuclei). (B) Cells were treated with RNV-L7 aptamer-DNAzyme RNV541 chimera at the indicated concentrations for 24 h in the absence of transfection reagents. The free antimiR-21 DNAzyme, RNV-L7 aptamer-DNAzyme chimera, and the mock aptamer-DNAzyme chimera structure were used as control. The total miRNAs were then isolated and the quantification of miR-21 expression was conducted via quantitative real-time PCR. $**p < 0.01$.

aptamers were capable of recognizing the native-state LDL-R protein. The eluted sequences were enriched by PCR amplification, and the selected aptamer sequences were regenerated by performing denaturing PAGE gel-based ssDNA separation for the next round of SELEX. Counter-selection steps were included from round 6 using an irrelevant protein (TGpr1). The stringency of the selection process was increased to promote the selection of high-affinity aptamers through the adjustment of aptamer concentrations (1 nmol for the first round and 100 pmol afterward), incubation time (1 h for rounds 1–3 and 0.5 h afterward), the volume of washes (from 1.8-mL washes for round 1 to 14 mL gradually for round 10), and the amount of LDL-R targets (5 pmol for rounds 1–3, 3 pmol for rounds 4–7, and 2 pmol afterward). The enrichment of aptamer candidates was monitored using an ELONA assay as detailed below. The selection was stopped when no further improvement was observed in the binding capacity between pools from three successive rounds (rounds 8–10). A detailed procedure about this experiment is demonstrated in Table S1. Enriched candidates were then subjected to next0generation sequencing using a MiSeq platform to identify the bound aptamers.

ELONA Assay

This assay is similar to a quantification method described in a previous publication.[34] Briefly, 1 μg of LDL-R protein was added to 100 μL of PBS and incubated with wells of the nickel-nitrilotriacetic acid (Ni-NTA) plate at room temperature for 1 h. Then, the wells were blocked with 100 μg/mL yeast tRNA (Sigma, R8759) and 1% BSA at room temperature for 1 h. After thorough washing with PBS, wells were incubated with 100 μL of 100 nM aptamers at room temperature for 1 h. Then the wells were washed with wash buffer, followed by the incubation of horseradish peroxidase (HRP)-conjugated anti-biotin antibody (Alpha Diagnostic International, 20361) for 1 h. Then, after thorough washing, the fluorescence intensity was determined with a FLUOstar Omega microplate reader (BMG Labtech) after the addition of QuantaBlu fluorogenic peroxidase substrate (Thermo Fisher, 15169).

Flow Cytometry Assay

Cells were first incubated with blocking buffer for 1 h on ice (binding buffer plus 100 μg/mL tRNA and 1% BSA) followed by two washes

with binding buffer prior to incubation with various concentrations of FAM-labeled aptamer in a 200-μL volume of binding buffer for 1 h at room temperature. The cells were then centrifuged, washed with binding buffer, and resuspended in 200 μL of binding buffer and subjected to flow cytometry analyses.

Fluorescence Microscopy Imaging

Positive and negative control aptamers were prepared in the same manner as described above for flow cytometry. Following the removal of media, cells were incubated in blocking buffer at room temperature for 1 h and washed twice in washing buffer prior to incubation with 200 nM aptamer for 30 min at room temperature. Hoechst 33342 (3 μg/mL; Sigma, B2261) was added to the cells during the final 15 min of incubation. The cells were then washed prior to visualization using a Nikon Eclipse ts100 inverted fluorescence microscope system (Nikon, Japan).

RNV-L7 Aptamer-miR-21 DNAzyme Chimera

The chimeric structure (Figure 5) consisted of LDL-R aptamer RNV-L7 and a previously reported RNV-541 DNAzyme targeting the miR-21 [21] sequences region, which were jointly connected via a d(TTTTT) linker as shown in Figure 5. The chimeric sequence was synthesized in-house on a GE ÄKTA oligopilot plus 10 (GE Healthcare) oligonucleotide synthesizer using standard phosphoramidite chemistry in a 1-μmol scale. A negative control aptamer-DNAzyme chimera sequence was also designed and synthesized: 5′-TCA ACA GGC TAG CTA CAA CGA CAG TCT GAT AAG CTA TTTTTA GGA CAG GAC CAC ACC CAG CGC GGT CGG CGG GTG GGC GGG GGG AGA ACG AGG TAG GG-3′ to evaluate the targeting specificity of the developed RNV-L7 aptamer.

TaqMan qPCR Assay to Measure the Expression of miR-21

Forty-eight hours after RNV-L7-antimiR-21 DNAzyme chimera treatment without using transfection reagents, the total RNA of different treatments was harvested, and cDNA was prepared by a TaqMan microRNA reverse transcription kit (4427975, Thermo Fisher) according to the supplier's specifications. The gene-specific miR-21 primer is designed to match with the hsa-miR-21-5p sequence of 5′-UAGC

UUAUCAGACUGAUGUUGA-3′. Quantitative real-time PCR was performed using TaqMan Universal Master Mix (4440040, Thermo Fisher) in a C1000 thermal cycler, CFX96 real-time system (Bio-Rad, Sydney, Australia) and programmed initially at 95°C for 10 min, 95°C for 15 s, and then 60°C for 1 min and repeated for a total of 40 cycles.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 3.03. An unpaired t test was used for comparisons between two experimental groups. Unless otherwise indicated, all results were averaged from biological triplicates and values are reported as means ± SEM. A p value of less than 0.05 was considered statistically significant.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.omtn.2019.11.004.

AUTHOR CONTRIBUTIONS

R.N.V. conceived the research, T.W. and KR performed the experiments and co-wrote the manuscript, and all authors analyzed the results and proofread and corrected the manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

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