

Construction of an Aptamer Modified Liposomal System Targeted to Tumor Endothelial Cells

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We describe herein the development of a high affinity and specific DNA aptamer as a new ligand for use in liposomal nanoparticles to target cultured mouse tumor endothelial cells (mTECs). Active targeted nanotechnology based drug delivery systems are currently of great interest, due to their potential for reducing side effects and facilitating the delivery of cytotoxic drugs or genes in a site specific manner. In this study, we report on a promising aptamer candidate AraHH036 that shows selective binding towards mTECs. The aptamer does not bind to normal cells, normal endothelial cells or tumor cells. Therefore, we synthesized an aptamer–polyethylene glycol (PEG) lipid conjugate and prepared aptamer based liposomes (ALPs) by the standard lipid hydration method. First, we quantified the higher capacity of ALPs to internalize into mTECs by incubating ALPs containing 1 mol%, 5 mol% and 10 mol% aptamer of total lipids and compared the results to those for unmodified PEGylated liposomes (PLPs). A confocal laser scanning microscope (CLSM) uptake study indicated that the ALPs were taken up more efficiently than PLPs. The measured K_d value of the ALPs was 142 nM. An intracellular trafficking study confirmed that most of the rhodamine labeled ALPs were taken up and co-localized with the green lysotracker, thus confirming that they were located in lysosomes. Finally, using an aptamer based proteomics approach, the molecular target protein of the aptamer was identified as heat shock protein 70 (HSP70). The results suggest that these ALPs offer promise as a new carrier molecule for delivering anti-angiogenesis drugs to tumor vasculature.

Key words aptamer based liposome; targeted drug delivery; tumor endothelial cell; heat shock protein 70 (HSP70); cell-based systematic evolution of ligands by exponential enrichment

Inhibiting angiogenesis is a promising strategy for the treatment of cancer and other disorders related to metastasis.¹⁾ Metastases are the cause of 90% of all human cancer deaths. Chemotherapy of cancer metastases, which can be effective in some patients, is often associated with significant toxicity, poor bio-distribution, an unusual pharmacokinetic profile, resistance and non-specificity.^{2,3)} Major progress in the treatment of cancer has been achieved over the past decades. In this context, the first angiogenesis inhibitors were reported in the 1980s from Folkman Laboratories. In 2004, the Food and Drug Administration (FDA) approved first aptamer drugs and Pegaptinib arrived on the market for the treatment of the wet (neovascular) form of age related macular degeneration (AMD). Many angiogenesis inhibitors are currently in the advanced clinical level research, and continuous efforts are needed to improve cancer therapy with regard to patient safety.^{1,4)} Our goal in this study was to evaluate an aptamer ligand for targeting our recently isolated tumor endothelial cells, with the objective of developing a new active targeted drug delivery system.

Among the known drug delivery systems, liposomes are the most successful nanotechnology-based drug delivery system. Twelve liposomal drug products have already been launched in the world and many liposomal drugs are currently in the clinical trial stage.⁵⁾ Liposome nanoparticles have been extensively studied as a tool or carrier for encapsulating drugs or genes. The addition of a conjugate of polyethylene

glycol (PEG) linked to a lipid anchor (distearoylphosphatidyl-ethanolamine) to the liposomal formulation was shown to significantly prolong liposome circulation time. PEG-coating contributes to stabilizing stress of the vesicles and provides important protection against opsonisation, resulting in delayed hepatic reticuloendothelial system (RES) clearance and greatly extending circulation time.⁶⁾ Indeed, a hallmark of the long-circulating PEG-modified liposomal drug carriers are their enhanced accumulation in tumors.^{7,8)} Ligand based liposomes are the most recent addition and technically well accepted vehicle for successful drug delivery.

Aptamers, which are short oligonucleotides either ssDNA or RNA, were first independently screened by a method involving the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) by the two research groups Ellington, Szostak and Tuerk, Gold in 1990.^{9,10)} The recently developed cell-based aptamer selection, termed cell-SELEX, uses intact living cancer cells as the target and closely related cells as controls to produce aptamers capable of identifying molecular differences between cancer cells.¹¹⁾ Over the last several years, aptamers have been recognized as an excellent, superior, fastest growing and promising new class of targeting ligand, especially in the nanotechnology platform for drug delivery in the area of cancer research. Since they possess several advantages as a targeting ligand, even over other ligands that are used in drug delivery, such as antibodies which were described by Cullen and Greene.¹²⁾ The binding of an aptamer to a target is very selective and occurs with a high affinity. Aptamers can be chemically modified to enhance their stability in biological

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fluids, to have a low or non immunogenicity, versatile synthetic accessibility, thermal stability, because of their small size, they can easily and rapidly diffuse into tissues and organs, thus permitting faster targeting in drug delivery.^{13,14} To date, a variety of aptamers against different cancer cells have been isolated based on whole cell-SELEX, but only a very few of them have been tested for use in drug delivery.^{15,16}

The objective of the present study was to target the tumor vasculature, which is an attractive and challenging target for the delivery of anticancer agents. When nanoparticles are injected intravenously to target cancer cells, they must penetrate cross the endothelial cells lining against oncotic pressure that is generated from inside the tumor microenvironment. At the same time, for *in vivo* applications, it is important for the therapeutic reagents to target only a particular cell type, thereby limiting side effects that result from nonspecific delivery. It would be possible to solve this problem by targeting the vasculature.^{17,18} Considering this, we examined a newly identified aptamer candidate AraHH036 that selectively binds to cultured tumor endothelial cells and implemented these aptamer conjugated liposomal nano particles as a carrier to target the tumor vasculature. We evaluated the uptake capacity, the binding affinity and the intracellular distribution of such a system to evaluate whether it could be used to deliver anticancer agents.

MATERIALS AND METHODS

Isolation of Mouse Tumor Endothelial Cells (mTECs)

All experiments involving animals and their care were carried out consistent with Hokkaido University guidelines, and protocols approved by the Institutional Animal Care and Use Committee. Endothelial cells were isolated as previously described.^{19–23} Briefly, normal skin endothelial cells (skin-ECs) were isolated from the dermis as controls. mTECs were isolated by magnetic bead cell sorting using an IMag cell separating system (BD Biosciences, San Jose, CA, U.S.A.). CD31-Positive cells were sorted and plated on 1.5% gelatin-coated culture plates and grown in EGM-2 MV (Clonetics, Walkers, MD, U.S.A.) and 15% fetal bovine serum (FBS). Diphtheria toxin (DT) (500 ng/mL, Calbiochem, San Diego, CA, U.S.A.) was added to the TEC subcultures to kill any remaining human tumor cells. Human cells express heparin-binding epidermal growth factor (EGF)-like growth factor (hHB-EGF), a DT-receptor. However, DT does not interact with mouse HB-EGF and murine ECs therefore survive this treatment.

Cells and Cell Lines We used primary cultured skin-ECs that were isolated from normal mice skin as a negative control. Primary cultured mTECs as target for selection, were isolated from human tumor xenografts of melanoma tumor cells (A375) into nude mice. A375, a super-metastatic human malignant melanoma cell, was a kind gift from Dr. Isaiah J. Fidler (University of Texas, M.D. Anderson Cancer Centre, Houston). OS-RC-2, human renal clear carcinoma cells were purchased from the RIKEN Cell Bank (Tsukuba, Japan). NIH3T3 cells were obtained from the American Type Culture Collection (Manassas, VA, U.S.A.).

Maintenance of Cell Cultures Primary cultured mTECs and normal skin-ECs were cultured using a special medium, namely EGM-2 MV (Lonza). Human renal cell carcinoma, OS-RC-2 cells were cultured in RPMI-1640, containing 10%

fetal bovine serum. NIH3T3 and A375 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum. To prevent microbial growth, penicillin (100 unit/mL) and streptomycin (100 μ g/mL) were added to all cell culture media. Cell cultures were maintained at 37°C in a 5% CO₂ incubator at 95% humidity. For regular cell cultures a 0.1% trypsin solution was used to dissociate the cells from the surface of the culture dish. However, RepCell was used (Cell Seed Inc., Tokyo, Japan) during the entire selection of a DNA aptamer, flow cytometry assay and during aptamer targeted protein purification.

Selection of Aptamers The cell-SELEX method was used to isolate aptamers specific for primary cultured mTECs. The method for selection has been described previously.⁹ Briefly, 200 pmol of an 82mer random ssDNA library with fluorescein isothiocyanate (FITC) tagged at the 5'-end and a biotin tag at the 3'-end in 1 \times selection buffer (500 μ L), was used to start the selection. The random library was first heated in a thermo-block at 80°C for 10 min, and then cooled slowly to form a secondary structure, which was then incubated with the target cells on ice for 45 min. Only the target cell bound library was collected for the next cycle selection. A total of 12 rounds of selection was performed with 2 rounds of negative selection at the 11th and 12th rounds. Finally, the aptamers that were bound to the surface of the cells were eluted by heating at 95°C for 5 min. The bound DNA was purified by phenol-chloroform extraction followed by ethanol precipitation. Regular polymerase chain reaction (PCR) was optimized and used to amplify the bound libraries after each cycle of selection. Asymmetric PCR was used to generate ssDNA for the next round selection. Eventually, cloning and sequencing was performed to identify the desired aptamer candidates.

Binding Assay by Fluorescence Activated Cell Sorting (FACS) A FACS experiment was performed to check the binding capacity of the AraHH036 (5'-ATGGGGGGCAGTGTACGGCGCGCCGGTGT-3') aptamer against primary cultured tumor endothelial cells, normal endothelial cells, normal cells and tumor cells. To perform the experiment, 200 pmol of aptamer and 200 pmol of zero cycle libraries was heated 80°C for 10 min, and cooled slowly to permit secondary structures to be formed. A five molar excess of yeast tRNA and bovine serum albumin (BSA) used as a nonspecific binding agent. The RepCell dishes that were used permit cells to be detached without the need for using trypsin that can damage cell surface proteins. 1.0 \times 10⁶ cells were incubated with aptamer and the control zero-cycle ssDNA library on ice for 45 min, respectively. The cells were spun down (5000 \times g, 5 min 4°C) to remove the supernatant that contained unbound DNA. The cells were washed three times with 1 \times selection buffer. Finally, we performed a fluorescence analysis of the sample on a FACSCalibur flow cytometer (BD Biosciences) in which 10000 events were counted.

Synthesis of Aptamer–Maleimide–PEG₂₀₀₀–1,2-Distearoyl-*sn*-glycero-3-phosphoethanolamine (DSPE) Conjugates The aptamer (100 nmol) was conjugated by reacting it with a 5 fold excess of maleimide–PEG₂₀₀₀–DSPE by gentle overnight soaking in a Bio-shaker at room temperature. The aptamer was purchased from Sigma-Genosys. For the conjugation reaction, the disulphide (S–S) bonds of the aptamer were first cleaved by the treatment with an excess TCEP solution on ice for 30–40 min. After the conjugation reaction, the excess

maleimide-PEG₂₀₀₀-DSPE was removed by dialysis (MWCO 4000) against 1% sodium dodecyl sulfate (SDS), 50 mM phosphate buffer at pH 7 with the solvent being changed three times at 4 h intervals. Further dialysis (MWCO 3500–5000) was performed in 50 mM ammonium hydrogen carbonate buffer at pH 8.0 by changing the solvent three times at every 4 h intervals. The purified aptamer–lipid conjugate was ion-exchanged with Zip-Tip C18 and examined by agarose-gel electrophoresis and matrix assisted laser desorption/ionization-time of flight (MALDI-TOF) MS spectroscopy.

Preparation of Liposomes Liposome (LP) formulations were prepared by the standard lipid hydration method. The molar ratio of egg phosphatidylcholine (EPC), cholesterol (Chol) and rhodamine-1,3-dioleoyl-*sn*-glycerophosphoethanolamine (DOPE) was 70:30:1. About 1, 5 and 10 mol% of PEG₂₀₀₀-DSPE or Aptamer-PEG₂₀₀₀-DSPE of the total lipid was added to the lipid solutions during the preparation of the PLPs or ALPs, respectively. All lipids were dissolved in chloroform-ethanol solutions, and, a lipid film was prepared by evaporating all of the solvents under a stream of nitrogen gas. The dried lipid film was hydrated by adding *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer (10 mM, pH=7.4) for 10 min at room temperature, followed to the sonication for approximately 30 s to a min in a bath type sonicator (AU-25C, Aiwa, Tokyo, Japan). The average size and diameter of liposomes were measured by using a Zetasizer Nano ZS ZEN3600 (Malvern Instrument, Worcester-shire, U.K.).

Quantitative Cellular Uptake of ALPs in mTECs by Spectrofluorometry To perform a standard quantitative cellular uptake analysis, 4.0×10^4 cells were seeded per cm² in 24-well plates (Corning Incorporated, Corning, NY, U.S.A.), followed by incubating the plates overnight at 37°C in an atmosphere of 5% CO₂, and 95% humidity. On the next experimental day, medium from cells cultured in 24 well-plates was removed by aspiration and the cells then washed once with warm 1× phosphate buffered saline (PBS). Next, rhodamine labeled liposomal solution at different concentration was added to the cells, followed by incubation for 1 h at 37°C in an atmosphere of 5% CO₂, and 95% humidity. After 1 h of incubation, the cells were washed twice with 1× warm PBS supplemented with 100 nM cholic acid and the cells were then incubated with 1× reporter lysis buffer at -80°C for 20 min to achieve lysis. The lysed cells were placed on ice to melt. Finally, the lysed solution was centrifuged at 12000 rpm for 5 min at 4°C to remove cell debris. The efficiency of cellular uptake in terms of the Fluorescence intensity of rhodamine in the supernatant solution was measured using a FP-750 Spectrofluorometer (JASCO, Tokyo, Japan) at the excitation and emission range (550–590 nm).

Measurement of K_d of ALPs To measure the binding affinity of aptamer based liposomes (ALPs), 4.0×10^4 cells were seeded per cm² in 24-well plates (Corning Incorporated) and incubated overnight at 37°C in an atmosphere of 5% CO₂ and 95% humidity. On the next experimental day, different concentrations of rhodamine labeled liposomes (0–1875 nM) incubated with cells for 1 h. After incubation, the cells were washed twice with 1× PBS supplemented with 100 nM cholic acid. Finally, the mean fluorescence intensity of the ALPs to the target cells was used to calculate specific binding. PEGylated liposomes at same concentrations were used as a

control. The equilibrium dissociation constant K_d was measured by fitting the dependence of the fluorescence intensity of specific binding to the concentration of the ligands to the equation $Y = B_{\max} X / (K_d + X)$ using the Sigma Plot 12 (Systat Software Inc., U.S.A.).

Qualitative Cellular Uptake and Intracellular Distribution of ALPs in mTECs Confocal laser scanning microscopy was used to visualize the uptake of the ALPs and their intracellular distribution. The mTECs were first seeded in a 35 mm glass bottom dish with 2 mL of medium and then incubated for 24 h. The cell density was 2.0×10^5 cells/glass bottom dish. On the next experimental day, the cells were incubated with 5 mol% of the total lipid of the ALPs and PLPs in Krebs buffer for 1 h at 37°C under an atmosphere of 5% CO₂ at 95% humidity. To confirm the intracellular distribution, the cells were stained with LysoTracker green (DND-26) 1 (μg/mL) for 30 min at 37°C. After 2–3 washings with 1× PBS supplemented with 100 nM cholic acid, the cells were viewed and imaged by confocal laser scanning microscopy.

RESULTS

Confirmation of the High Affinity Binding Capacity of the Aptamer Ligand To check whether the isolated aptamer candidate AraHH036 has the capacity to bind to mTECs, we carried out a FACS experiment and the findings showed that the aptamer treated mTECs exhibited a high shift compared to the control zero cycle library (Fig. 1). It was very important to check the selectivity of this aptamer as to whether it binds to other cells or not. To answer this question, we carried out a FACS experiment under the same condition for this aptamer as above, but against normal endothelial cells, *i.e.*, a normal NIH3T3 cell line, A375 metastatic tumor cell lines and OS-RC-2 human renal cancer cell lines. This aptamer had no capacity to bind against any of the above cells, except against A375, where a slight binding was detected (Fig. 2). This result confirmed the selective binding and high binding affinity of this promising candidate with respect to mTECs and also indicates that the molecular target to which this aptamer bound is expressed on the mTECs but not on the other cell lines tested.

Synthesis of a DNA Aptamer Conjugate with Maleimide-PEG₂₀₀₀-DSPE The aptamer-PEG₂₀₀₀-DSPE was successfully synthesized by conjugating a 5'-thiol-modified

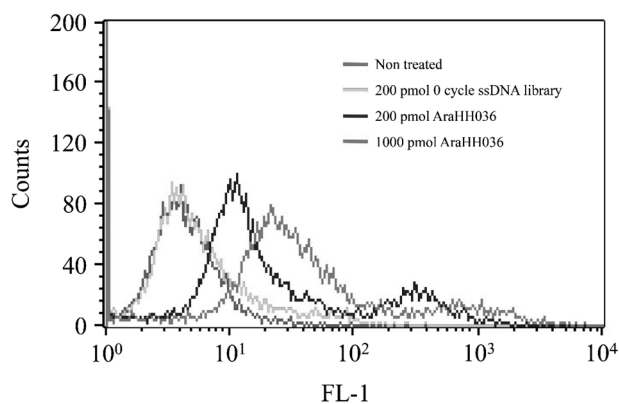


Fig. 1. Binding Assay of Aptamer AraHH036 against Target mTECs

A flow cytometry binding assay was performed, where the high affinity binding of FITC-aptamer at doses of 200 pmol and 100 pmol was observed. The zero cycle library at 200 pmol was used as a control.

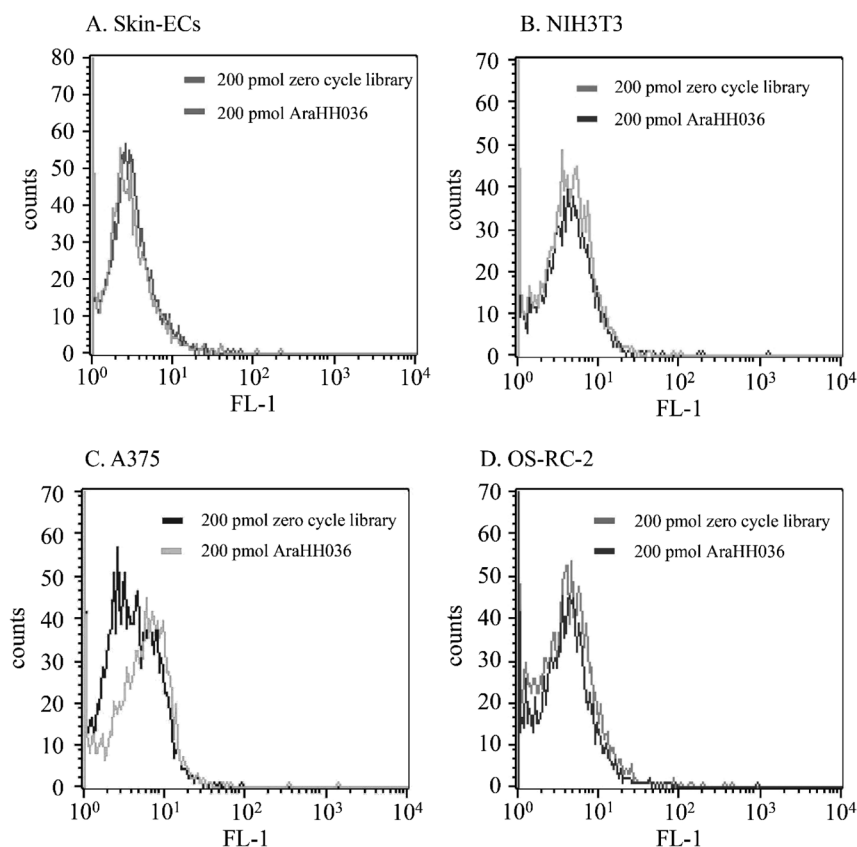


Fig. 2. Binding Assay of Aptamer AraHH036 to Normal Cells and Tumor Cells

Flow cytometry binding assay of aptamer at the dose 200 pmol. A. Normal skin-ECs, B. Normal NIH3T3, C. A375, D. OS-RC-2. In all cases, our aptamer show no binding. The 200 pmol zero cycle library was used as a control.

aptamer and 5 equimolar amounts of maleimide-PEG₂₀₀₀-DSPE. We confirmed the conjugation as well as the molecular weight of the product by MALDI-TOF MS (Fig. 3). By applying dialysis using a 3500–5000 MWCO membrane, excess free lipid was successfully removed overnight. The final quantification of aptamer-PEG₂₀₀₀-DSPE was done by UV-visible spectroscopy at 260 nm and the conjugated material was ready for preparing liposomes.

Preparation of Liposomes The standard lipid hydration method was successfully employed to prepare ALP. Zeta-sizer was used to analysis the particle size of ALP (Table 1). PEGylated liposomes without conjugating ligand was prepared as a control.

Quantitative Cellular Uptake Study of the ALP in mTECs Spectrofluorometry was used to perform the quantitative uptake assay. To evaluate the function of our constructed ALPs against primary cultured tumor endothelial cells, we first carried out an *in vitro* quantitative cellular uptake experiment using Rhodamine labeled 1, 5 and 10 mol% of the total lipid of ALP and PLP mTECs. The fluorescence intensity of the ALPs was found to be higher than that for PLP for three different doses compared to the control (Fig. 4). The enhanced cellular uptake in terms of fluorescence intensity was statistically significant compared to control PEG-LPs.

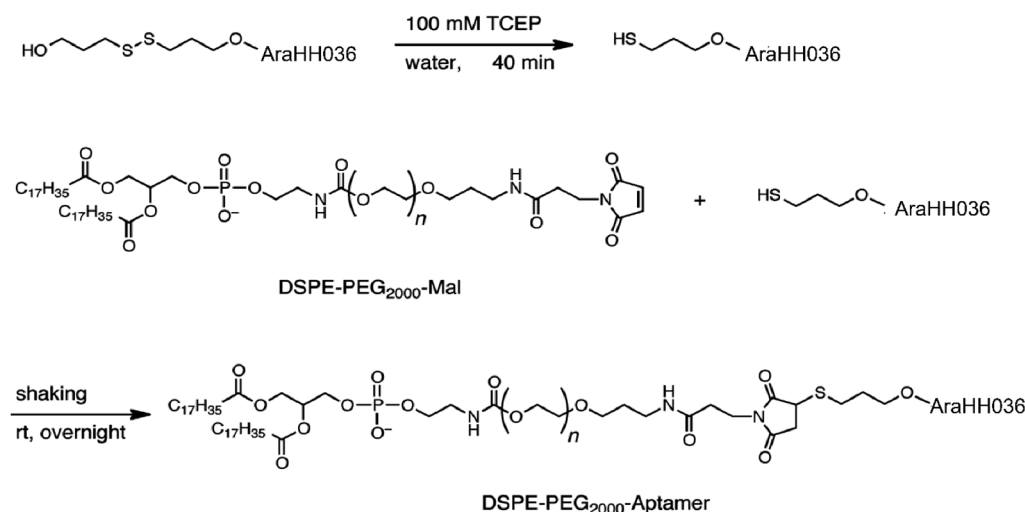
Measurement of the Binding Affinity (K_d) of the ALPs The mean fluorescence intensity for various concentrations of the rhodamine labeled ALP was measured. The experiments were repeated three times. The K_d value was 142 nM (Fig. 5). We also attempted to measure the K_d PLPs, but no binding

with the mTECs was detected.

Qualitative Cellular Uptake and Intracellular Distribution of ALPs in mTECs To demonstrate the actual localization of the internalized ALP nano-carrier system, rhodamine labeled ALPs and PLPs were incubated for 1 h with mTECs. The mTECs were stained with green lysotracker. A confocal laser scanning microscope (CLSM) study showed that most of the internalized ALPs were merged with lysotracker, indicating that they were located in the lysosomal compartment; neither did PLPs show any affinity towards for mTECs nor were they merged or colocalized in the lysosomal compartment (Fig. 6).

DISCUSSION

To better understand the effects of the tumor microenvironment on the properties of endothelial cells and to understand how they are different from normal endothelial cells, our collaborative group isolated very pure tumor endothelial cells. Isolating and culturing tumor endothelial cells is a challenging task, because (i) endothelial cells are usually enmeshed in a complex type of tissue, consisting of vessel wall components, stromal cells, and tumor cells; and (ii) only a small fraction of cells within these tissues are actually endothelial cells. It is assumed that a single tumor endothelial cell can support many tumor cells.^{19–23} Thus, to develop an anti-angiogenic therapy, for targeting endothelial cells might be a much more effective strategy than targeting the actual tumor cells themselves. In order to achieve vascular targeting, our strategy was to con-



TCEP = tris(2-carboxyethyl)phosphine hydrochloride, efficient reducing agent for disulfide linkage

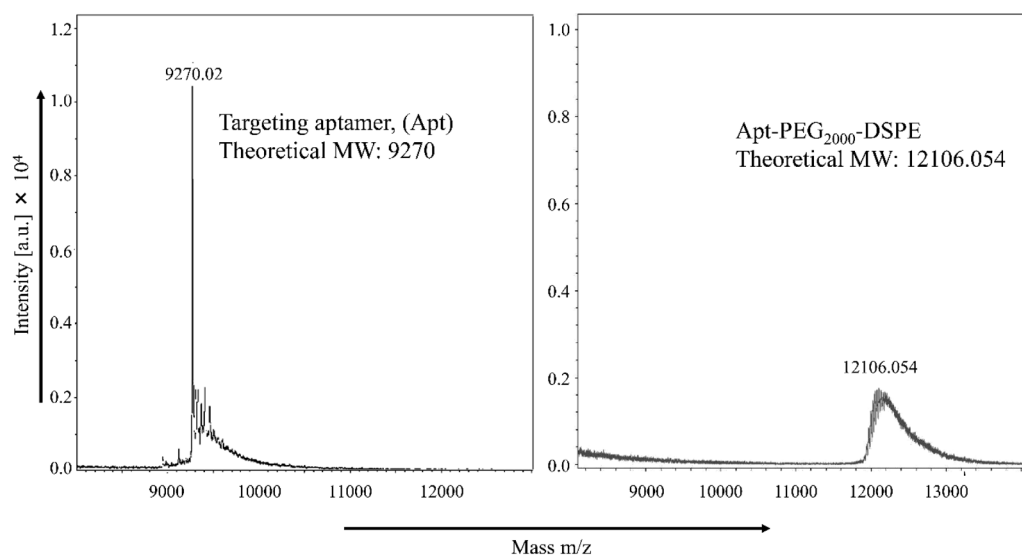


Fig. 3. Conjugation of the Aptamer-PEG₂₀₀₀-DSPE

(A) Synthesis of the thiol modified aptamer AraHH036 with maleimide-PEG₂₀₀₀-DSPE. Reduced aptamer and excess maleimide-PEG₂₀₀₀-DSPE were reacted in water overnight at 37°C. (B) MALDI-TOF MS spectrometry was employed to confirm the conjugation.

Table 1. Physical Properties of Liposomes

Name of liposome	Particle data	
	Size (nm)	ZP (mV)
PLPs	99±10	-19±6
ALPs	100±8	-28±7

struct an active targeting liposomal nano-carrier system.

It was recognized that a technique for increasing the selectivity of the interaction of the liposomes with diseased cells was desirable. Ligand based active targeting is the most powerful and a better option for achieving the selective and highest therapeutic efficacy with minimal adverse effects *via* receptor mediated endocytosis.²⁴⁾ In this present study, we attempted to utilize our recently screened DNA aptamer ligand AraHH036 for the first time, to construct a new ligand based targeted PEGylated liposomal nano-carrier system and to

carry out a functional analysis. In cell-based selection one common phenomena reflects many sequences for a single target with maximum similarity and, or, many sequences for many different targets due to an affinity and different binding moiety towards the target. In our case, the similarity within the different sequences was minimal, which may be due to the binding of aptamer candidates to the different targets expressed on the surface of the primary cultured tumor endothelial cells. At the same time, it should be noted that, in an earlier study, we found one very promising candidate AraHH001 for targeting to primary cultured tumor endothelial cells.²⁵⁾ However, we also identified some other promising candidates with different sequences and minimal similarity, therefore, we continued our analysis and only recently concentrated on this promising aptamer ligand AraHH036 in an attempt to identify a new liposomal nanocarrier for vascular targeting. We first investigated the binding capacity of the FITC-labeled aptamer for cultured tumor endothelial cells using a standard flow cy-

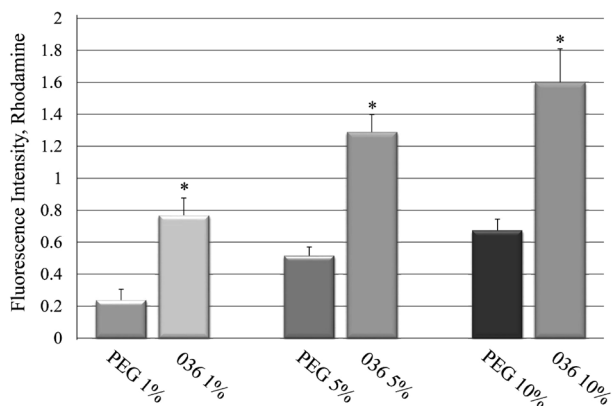


Fig. 4. Quantitative Cellular Uptake Assay for ALPs & PLPs

SM-ECs, 4.0×10^4 /24-well were treated with 5 mol% of the total lipid of ALPs or PLPs for 1 h at 37°C. The cellular uptake is expressed as mean \pm S.D. The statistical analyses performed in a pair of cellular uptake of PEG- and aptamer-modified liposomes at each concentration by unpaired Student's *t*-test ($p < 0.05$ vs. PEGylated liposome, $n=3$).

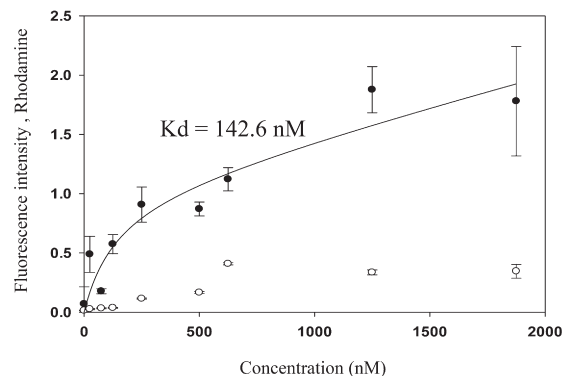


Fig. 5. Determination of Binding Affinity, the K_d Value of ALPs

mTECs were treated with various concentrations of ALPs or PLPs labeled with rhodamine DOPE. The average rhodamine intensity of the ALPs or PLPs versus varying concentrations were plotted to determine the dissociation constant, K_d . The experiment was repeated three times.

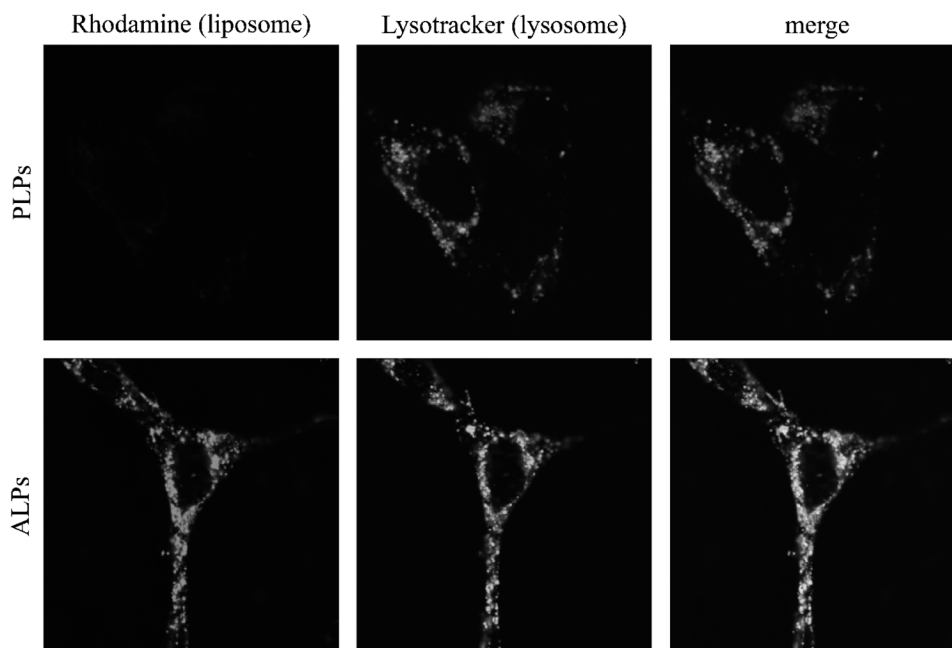


Fig. 6. A CLSM Uptake Assay and Intracellular Trafficking of ALPs

SM-ECs, 2.0×10^5 10/35 mm glass bottom was treated with 5 mol% of the total lipid of ALPs or PLPs for 1 h at 37°C. PEG-LPs and Apt-PEG-LPs containing rhodamine incubated with SM-ECs for 1 h at 37°C. Cells were stained with Green Lysotracker for 30 min.

ometry assay. We observed a very high binding affinity for the mTECs (Fig. 1). We tested the selectivity of this ligand using the same FACS experiment using four different normal and tumor cells. No binding to these cells was detected (Fig. 2).

Liposomes are the most advanced and effective drug carrier for therapeutics. Therefore, we next prepared an aptamer modified PEGylated nano-carrier system by conjugating the 5'-thiol aptamer ligand at the maleimide-PEG terminus on the liposomes. First, we cleaved the aptamer-S-S bond to produce an aptamer-SH bond by treatment with a reducing agent TCEP. A NAP-column was used to purify the aptamer-SH which was then conjugated with maleimide-PEG₂₀₀₀-DSPE. A purified aptamer-lipid conjugation was obtained by dialysis (MWCO 3500-5000). The molecular weight and purity of the conjugates was confirmed by MALDI-TOF spectroscopy (Fig.

3). Finally, the aptamer-lipid concentration was determined by UV-visible spectroscopy.

PEG chains interfere with both the coupling of ligands to the lipid bilayer and the interaction of these ligands with the intended biological targets. To improve the selectivity and cellular uptake, attaching the aptamer ligands to the surface of the PEG chains is a potentially valuable approach. These ligands, when coupled to the PEG terminus, do not interfere with the binding of ligands to their respective recognition molecules.²⁶⁾ Although EPR effects are based on the structural features of the neovasculature, long circulating liposomes can passively accumulate in tumor tissue, but PEGylation inhibits cellular uptake. Therefore, active targeting is a very promising and effective route to solving this problem and, at the same time, to take advantage of the long retaining properties of PEG. Finally, liposomes were prepared by the lipid hydra-

tion method and their physicochemical properties were confirmed by a Zetasizer (Table 1). Interestingly, as an amount of PEGylation increased, the cellular uptake parallelly increased. We observed similar increase in cellular uptake by PEGylation with liver endothelial cells.²⁷⁾ This may be because of a unknown property of endothelial cells.

We also carried out cellular uptake studies, quantitatively and qualitatively by utilizing the ALPs and the PLPs nano carrier in primary cultured tumor endothelial cells. A one h uptake study using different concentrations of total lipids, such as 1, 5 and 10 mol% in the ALPs and PLPs were carried out using the mTECs. The uptake of the ALPs nano-carrier system by mTECs was significant, compared to that for the unmodified PEGylated nano-carrier system. The uptake of the ALPs was dose dependent. This result also indicates that the targeted aptamer first recognized the cellular surface of the target molecule and was then internalized. Next, to visualize the extent of enhanced cellular uptake we carried out an *in-vitro* qualitative CLSM uptake study (Fig. 4). The rhodamine labeled 5 mol% of total lipids of the ALPs was found to have a much higher internalization capacity in mTECs compared to unmodified PLPs. Therefore, the above results suggest that modifying the PEGylated liposomes with the targeting ligand is critical for the association of and enhanced internalization of the nano-carrier system by mTECs. At the same time, due to the steric repulsion of the PEG polymer in unmodified PEGylated liposomes, the extent of association to the target mTECs was decreased, and, as a result, the uptake efficacy was lower. We also determined the binding affinity, K_d value of our ALPs. The K_d value was in the nano molar range, 142 nM (Fig. 5).

We next carried out an intracellular trafficking experiment in which the uptake of rhodamine labeled ALPs was evaluated using lysotracker green as an intracellular marker. A CLSM study of intracellular trafficking showed that most of the ALPs were co-localized with lysotracker green, appearing as yellow (Fig. 6). However, some remaining ALPs that were not colocalized (red) remained intact inside the cytoplasm. PLPs were not taken up substantially and therefore, it was difficult to determine whether or not they were colocalized.

Finally, we attempted to identify the binding target of the aptamer ligand that was expressed selectively on the surface of the mTECs. An aptamer based proteomic approach was successfully used to isolate the molecular target. MALDI-TOF MS analysis confirmed that the molecular target of this aptamer ligand is heat shock protein HSP70 (Suppl. Fig. 1). HSP70 is heat shock proteins or heat stress proteins consist of chaperons of approximately 70 kDa in size. HSP70 is cytotoxic to tumor cells but not in normal cells. It plays a very important role in cancer relevant pathways.²⁸⁾ This protein has been shown to be released from cells as the result of acute stress as well as being secreted after exposure to a number of stimuli. This protein inhibits apoptosis and also plays a role in resistance to cancer chemotherapy. The intracellular expression of HSP70 in different tumor cells are well established. Among researchers, still contradictory matter when tumor cell surface expression issue comes.^{29–31)} The exact reason of its expression on the surface of cultured mTECs is not currently known. More experimental studies need to be done and remains to be clarified in the future.

CONCLUSION

We developed a high affinity and selective DNA aptamer AraHH036 by means of the cell-SELEX method. Utilizing this aptamer we successfully prepared an aptamer based PEGylated liposomal delivery system that targets cultured tumor endothelial cells. The results of the study indicate that active targeting is a potentially useful tool for enhancing cellular uptake, compared to unmodified PEGylated liposomes. The binding affinity of this newly designed ALP was found to be in the nanomolar range. This study also reports on the nature of the molecular target of this aptamer ligand, which is referred to as HSP70. Therefore, we conclude that this system is potentially very promising and could be useful for the active targeting and delivery of drugs and or, gene therapy in the future. At the same time, it also represents a good example of using an aptamer ligand and the identification of biomarkers in terms of understanding such events at the molecular level.

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