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Cell-Specific Internalization Study of an Aptamer from Whole Cell Selection

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Abstract: Nucleic acid aptamers have been shown many unique applications as excellent probes in molecular recognition. However, few examples are reported which show that aptamers can be internalized inside living cells for aptamer functional studies and for targeted intracellular delivery. This is mainly due to the limited number of aptamers available for cell-specific recognition, and the lack of research on their extra- and intracellular functions. One of the major difficulties in aptamers' in vivo application is that most of aptamers, unlike small molecules, cannot be directly taken up by cells without external assistance. In this work, we have studied a newly developed and cell-specific DNA aptamer, sgc8. This aptamer has been selected through a novel cell selection process (cell-SELEX), in which whole intact cells are used as targets while another related cell line is used as a negative control. The cell-SELEX enables generation of multiple aptamers for molecular recognition of the target cells and has significant advantages in discovering cell surface binding molecules for the selected aptamers. We have studied the cellular internalization of one of the selected aptamers. Our results show that sgc8 is internalized efficiently and specifically to the lymphoblastic leukemia cells. The internalized sgc8 aptamers are located inside the endosome. Comparison studies are done with the antibody for the binding protein of sgc8, PTK7 (Human protein tyrosine kinase-7) on cell surface. We also studied the internalization kinetics of both the aptamer and the antibody for the same protein on the living cell surface. We have further evaluated the effects of sgc8 on cell viability, and no cytotoxicity is observed. This study indicates that sgc8 is a promising agent for cell-type specific intracellular delivery.

Keywords: cell recognition · DNA recognition · DNA · drug delivery · internalization

Introduction

Aptamers, derived from an evolution process called SELEX (Systematic Evolution of Ligands by Exponential enrichment), are single strand nucleic acids capable of binding to their target molecules with high affinity and specificity.[1,2] Unlike monoclonal antibodies, aptamers have low molecular weights and can be chemically synthesized and readily modified.[3–6] These features make aptamers promising probes for molecular recognition and also as diagnostic and therapeutic reagents.[7–10] Although aptamers have been very effective in vitro, one of the limitations for an in vivo application is that most of aptamers, unlike small molecules, cannot be directly taken up by cells without external assistance.[11] Several inherent factors such as nucleic acid's charge and size present a potent barrier to cellular uptake. The negatively charged phosphate backbone of the nucleic acid molecule is the primary cause for its inadequate and inefficient cellular association, owing to electrostatic repulsion from the negatively charged cell surface. Moreover, oligonucleotides longer than 25 bases are inherently difficult to import into cells because of their size and tending to self-hybridize, thereby affecting their uptake.[12] Though efforts have been made to incorporate aptamers into liposome vesicles.[13,14] or other delivery vector systems,[15,16] it is highly desirable to generate new aptamers that can be internalized directly into cells and explore their in vivo applications. Up to now, the only successful example is anti-PSMA (prostate-specific membrane antigen) aptamer. A previous study has shown that PSMA is constitutively endocytosed into LNCaP cells as an internalizing protein and the application of anti-PSMA monoclonal antibody increases its internalization rate.[17] After the iden-

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tification of the anti-PSMA aptamer that binds to prostate cancer cells with nanomolar affinity,[18] Farokhzad et al. also reported that anti-PSMA aptamers can be internalized. They further prepared nanoparticle–PSMA aptamer conjugate and proved that it could directly enter the target cells in a receptor-dependent way.[19] Based on this study, an increasing interest in using PSMA aptamer to deliver siRNAs,[20,21] toxins,[22] and anticancer drugs[23] into cells have been shown in recent years. Therefore, a great potential is expected in the discovery of new internalizing aptamers and their use as delivery agents.

The difficulty in the discovery of internalizing aptamers is mainly due to the limited number of aptamer candidates available for cell-specific recognition. We have recently developed the cell-SELEX method to generate a panel of aptamers successfully.[24] These aptamers are selected using whole intact cells as targets while a related cell line is used as a negative control for the selection. Cell-SELEX enables the generation of multiple aptamers for molecular recognition of the target cells, and these aptamers could be used as excellent molecular recognition probes. The cell-SELEX provides at least four significant advantages: 1) Multiple aptamers are generated from a single SELEX process; 2) the selected aptamers are effective for recognition as the selection is done while the target molecules such as cell-membrane receptors are in their native state in a living cell; 3) the selected aptamers can be directly used for cell identification and binding studies as the work reported herein; 4) the selected aptamers enable the identification of the binding sites on cell membrane surface which could be recognized as potential biomarkers. Using cell-SELEX, we have generated many interesting aptamers for a variety of cells. sgc8 is the most studied ssDNA aptamer from our newly developed cell-SELEX technology.[24] It binds to acute lymphoblastic leukemia (ALL) T-cells with high affinity (K_d = 0.8 nM). Moreover, it is able to specifically recognize the target leukemia cells that are mixed with normal human bone marrow aspirates, and identify the cancer cells closely related to the ALL cell line in real clinical specimens. Human protein tyrosine kinase-7 (PTK7), the target protein of sgc8 on the cell membrane, has been recently identified as a potential new biomarker for leukemia.[25] Nonetheless, only limited information about properties of sgc8 and PTK7[26–28] has been reported, and further investigation of the functions of sgc8 on target cells is necessary.

In this work, we study the internalization of dye-labeled sgc8 by fluorescence imaging and flow cytometry assays. The results showed that the aptamer was specifically internalized to the target ALL T-cells without influencing the cell viability. Those internalized sgc8 aptamers were located in the endosome, a key intracellular compartment where the delivery agents release their cargos.[29,30] In addition, we examine the internalization of anti-PTK7 monoclonal antibody and found the antibody was also internalized to the same location with a constant speed as sgc8. Besides anti-PSMA aptamer, this study shows another example for internalizing aptamers. By easily modification sgc8 holds a great promise to be a potential lymphoblastic leukemia T-cell specific delivery agent.

Results and Discussion

Trypsin treatment for internalization study: Here we developed trypsin treatment in internalization study. Both flow cytometry and confocal results illustrated that this method provided an easy way to remove the outside fluorescence signal. Since dye-labeled sgc8 (sgc8-TAMRA (Tetramethyl-6-Carboxyrhodamine)) binds with the membrane protein PTK7 and generates fluorescence signals which interfere with the detection of the intracellular aptamer, especially in flow cytometry assay, we applied trypsin treatment to remove cell-surface bound aptamer for the internalization study. We found that trypsin treatment could successfully remove specific binding of sgc8 with target cells. As shown in Figure 1A and B, when the internalization was inhibited at 4 °C, the fluorescence signal caused by the binding of sgc8-TAMRA to the membrane of CCRF-CEM (a type of ALL cells) was entirely eliminated after trypsin treatment. The flow cytometry assay confirmed our observations. Initially, the intensity profile of the cells with alexa488 labeled sgc8 showed a large shift compared to that with the labeled TDO5, a control ssDNA which did not recognize CCRF-CEM cells (Figure 2A). However, after trypsin treatment, the intensity profile of sgc8-alexa488 shifted left and overlapped with the TDO5-alexa488 profile, indicating specific binding of the aptamer to cell membrane was completely removed. Therefore, any fluorescence signals detected from sgc8-TAMRA treated cells after trypsin treatment should come from aptamers internalized into the cells. This method was also used for the study of antibody internalization. Figure 2C showed that trypsin was able to remove specific binding of anti-PTK7 antibody to CCRF-CEM cell surface.

Cell uptake of aptamer sgc8: The aptamer internalization study was carried out at 37 °C. After a 2-h incubation with sgc8-TAMRA, we observed fluorescence signal both inside and on the cell membrane (Figure 1C). By treating the cells with trypsin to remove the surface binding, the internalized sgc8-TAMRA was shown clearly (Figure 1D). We also changed the focusing plane from the middle section of the cell (Figure 1E) to the cell membrane (Figure 1F) to ensure the observation of intracellular fluorescence signals. The flow cytometry results further confirmed the aptamer internalization. Although the cells had been treated with trypsin after aptamer application at 37 °C, an evident up-shift of cellular fluorescence intensity was shown in the profile compared with that from the experiments carried out at 4 °C, where endocytosis was inhibited and cellular uptake was completely eliminated (Figure 2B). On the other hand, no shift was detected for TDO5-alexa488 in flow cytometry assay (Figure 2B) and no fluorescence was observed inside the cells incubated with TDO5-TAMRA using confocal imaging (Figure 1H) under the same conditions as sgc8-

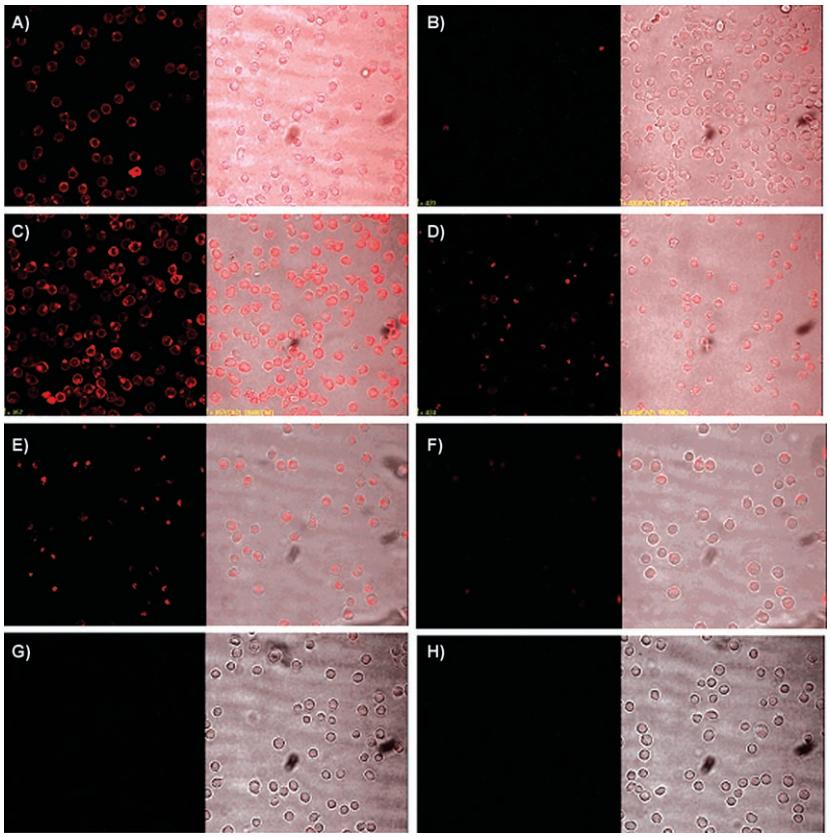

Figure 1. Confocal images of sgc8-TAMRA binding and internalization in CCRF-CEM cells. In each picture, left side is the fluorescence image, right side is the overlay of optical and fluorescence images. Both the fluorescence image and optical image show the scale bar of 5 μm. A) Sgc8-TAMRA can specifically bind with CCRF-CEM cells before trypsin treatment at 4 °C. B) Trypsin treatment removed specific binding of sgc8-TAMRA outside the cell membrane at 4 °C. C) Without using trypsin, fluorescence signals were seen both on the cell membrane and inside the cell after two hours′ incubation of sgc8-TAMRA with cells at 37 °C. D) With trypsin removing the binding of sgc8 on the cell membrane after 2 h′ incubation at 37 °C, internalized sgc8-TAMRA is clearly shown inside the cell. To make sure the fluorescence was inside the cell, focus plane was changed from inside the cell (E) to the cell membrane (F). TDO5-TAMRA did not bind with CCRF-CEM cells at 4 °C (G), and could not be internalized into cells at 37 °C even after two-hour incubation (H).

alexa488/sgc8-TAMRA. This excluded the possibility that the internalization was caused by alexa488/TAMRA dye itself and/or the permeability change of the cell membrane during the aptamer incubation. The results provided strong evidences that sgc8 could be internalized by the CCRF-CEM cells.

To investigate the fate of the sgc8 aptamer inside the cell, we examined the intracellular distribution of sgc8 by incubating the cells with sgc8-TAMRA- and alexa633-labeled transferrin (transferrin–alexa633) simultaneously. Transferrin is an 80 kDa serum glycoprotein that has been reported to be taken up by cell via receptor-mediated endocytosis; alexa633-labeled transferrin is commonly used to identify the location of endosome.[30] The co-localization of the aptamer with transferrin indicated most of internalized sgc8 were inside endosomes (Figure 3). Due to the action of a proton-pumping ATPase of the endosomal membrane, the endosomal compartment is usually acidic, where many internalized extracellular molecules change their structures at low pH to release their bound substance.[29,30] Similarly, the internalized aptamers could potentially take advantage of it and release its molecular cargo to fulfil targeted delivery. However, the endosomal trafficking also included the pathway of proteosomal or lysosomal degradation or recycling to the plasma membrane, therefore modifications of aptamer and its cargo are needed for the purpose of controlled release at interest location. Ted et al. designed a disulfide linkage between the anti-PSMA aptamer and siRNA, so that the disulfide linkage should be cleaved upon entering the endosome and releasing the siRNA for processing or function.[20] Similar or related methods are need for our future development of the sgc8 aptamer as a delivery. Another concern is that DNA molecules can be cleaved by intracellular nuclease; therefore it remains possible that the dye-labeled aptamer might have been cut after internalization and it was the dye rather than the aptamer that entered the endosome. However, we have excluded this possibility based on our observations and the following reasons: First, free dye cannot enter the cells. Even if it is transported into cells with external assistance in a non-receptor dependent way, it will diffuse into the entire intracellular compartment including the nucleus and cytoplasm,[31] instead of confined regions inside a small part of cytoplasm as observed by us. Second, we tried different dye (alexa488 and TAMRA) labeled sgc8 to observe the localization. As shown in Figure 3, both alexa488- and TAMRA-labeled sgc8 are located in the endosome, which excluded the possibility that endosome is the preference place for a certain dye. Third, internalization of sgc8 occured after the binding with its target protein, and we show that this process is receptor-dependent, in which a small region of the plasma membrane invaginates to form a new intracellular vesicle containing the complex of receptor and dye-labeled aptamer, therefore protecting the aptamer from being cleaved until inside the endosome,[29] though it's quite likely that the aptamers are degraded by the lysosomal enzymes once they inside the endosome, however, it has been inside the endosome and fulfilled its responsibility as a delivery. It's the time to release its cargo.

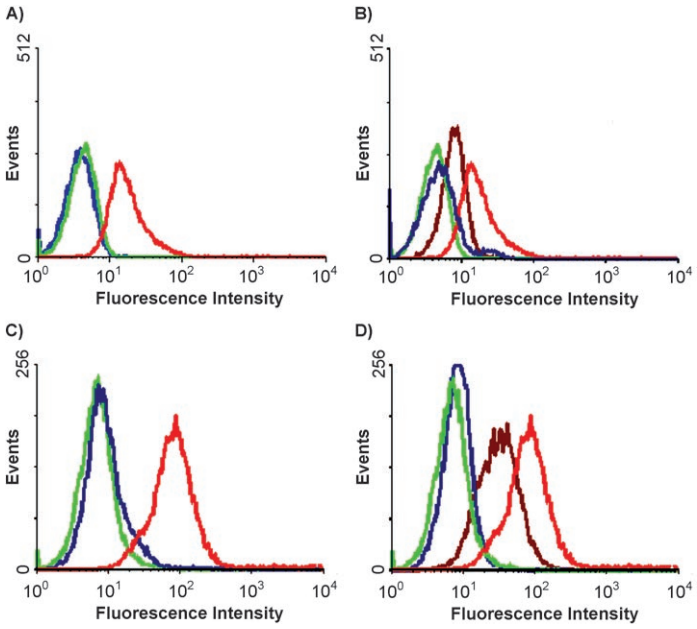

Figure 2. Flow cytometry results of aptamer and antibody binding with CCRF-CEM cell under different conditions. All the aptamers and antibodies were labeled with alexa488. A) Trypsin removed specific binding of sgc8. Blue line: TDO5 (control aptamer) incubated with target cells without trypsin treatment at 4°C. Red line: sgc8 incubated with target cells without trypsin treatment at 4°C. Green line: sgc8-target cell incubation after trypsin treatment at 4°C. B) After incubation for two hours at 37°C, trypsin was applied to remove the membrane-bound aptamers, fluorescence shift existed with sgc8 incubated cells (brown line) but not with TDO5 incubated (control aptamer, blue line) cells. Green line and red line show the same meanings as those in Figure 2A. C) Binding of anti-PTK7 antibody without (red line) and with (green line) trypsin treatment at 4°C. Blue line shows the nonspecific binding of IgG at 4°C. D) Internalization of anti-PTK7 antibody. Cells were incubated with anti-PTK7 antibody (brown line) or IgG (control antibody, blue line) for 30 min at 37°C. Trypsin was added before observation. Green line: anti-PTK7 incubated target cells with trypsin treatment at 4°C. Red line: anti-PTK7 incubated target cells before trypsin treatment at 4°C.

To monitor the sgc8 internalization kinetic process, we studied the time dependence of internalization by flow cytometry. The intensity at each time interval was normalized to the fluorescence signal from the cell surface at 4°C. As shown in Figure 4A, the internalization of sgc8 into the cell showed a constant rate during the two hour monitoring, which is similar to a previous report that studied the kinetics of EGF (epidermal growth factor) ligand internalization into its target cell.[32]

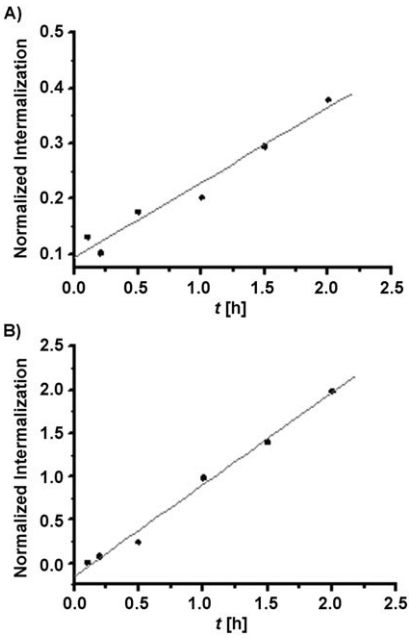

Figure 4. Quantitative analysis of sgc8 (A) and anti-PTK7 antibody internalization (B) with time. Both were labeled with alexa488. The signal is normalized against the fluorescence intensity at 4°C.

Internalization of anti-PTK7 monoclonal antibody: Since PTK7 is the target protein of sgc8,[25] we further investigated the internalization of anti-PTK7 antibody. Figure 2D indicated that anti-PTK antibody can also be internalized into the cells. Moreover, the antibody of PTK7 showed the same intracellular distribution in endosome (Figure 5) and the same linear relationship between the amount of internalization and incubation time (Figure 4). A faster internalization speed of anti-PTK7 antibody was also shown compared with sgc8, which consisted with the observation in anti-PSMA antibody and PSMA aptamer.[17] This study demonstrated that the property of sgc8 internalization was

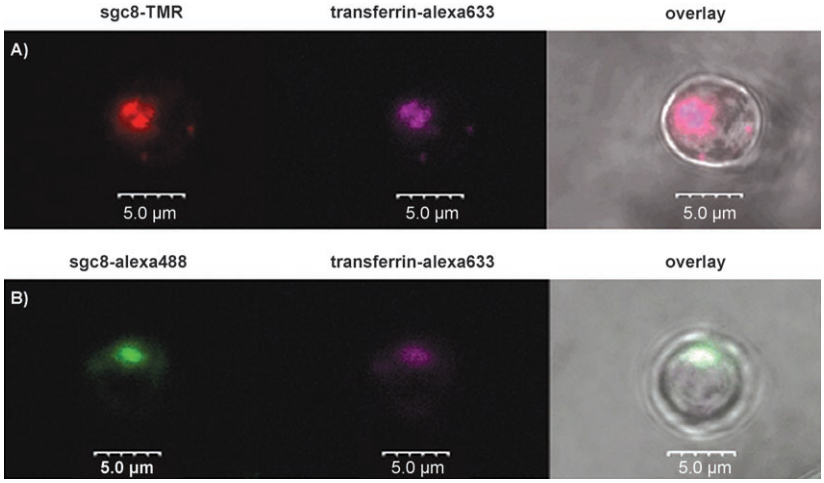

Figure 3. Co-localization of sgc8 and transferrin in endosomes. A) Sgc8 labeled with TAMRA. B) Sgc8 labeled with alexa488. From left to right: Images for sgc8-TAMRA/sgc8-alexa488, transferring-alexa633, overlay of the fluorescence channels and bright field channel, respectively. Scale bar: 5 μm.

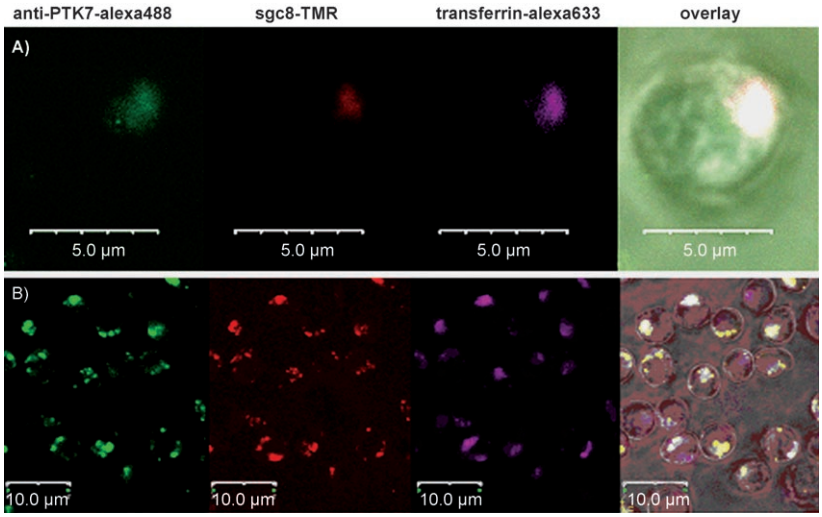

Figure 5. Co-localization of anti-PTK7 antibody, sgc8 and transferrin in endosomes after two hour incubation at 37 °C with pretreatment of trypsin. From left to right: Images for anti-PTK7-alexa488, sgc8-TAMRA, transferring-alexa633, overlay of these three channels and bright field channel, respectively. Scale bar: 5 μm for A, and 10 μm for B.

related to the internalization property of its target protein PTK7. Compared with PTK7 antibody, the sgc8 aptamer can be easily synthesized chemically and thus more attractive as delivery reagents.

Cell-specificity of sgc8 internalization: For in vivo application, it is important for the therapeutic reagents to target only particular cell types, thereby limiting side effects that result from nonspecific delivery. We investigated the specificity of sgc8 internalization with different types of cells. We incubated the dye-labeled sgc8 with NB4 cells, which have been shown not to bind sgc8 in previous research,[24] for 2 h at 37 °C. Both flow cytometry assay (Figure S1A, Supporting Information) and confocal imaging (Figure S1B, Supporting Information) showed no difference in fluorescence signal compared with results obtained at 4 °C. The delivery specificity was further confirmed by testing other targeted lymphoblastic leukemia cells including Jurkat (TIB-152, human acute T cell leukemia), and other control B-cells such as Ramos (CRL-1596, human Burkitt's lymphoma) (data not shown). Therefore, sgc8 can only be internalized into its target cells, which also indicates that sgc8 internalization is in a receptor-specific way rather than due to passive diffusion.[11]

Cell viability study with sgc8 aptamer: From the internalization study of sgc8, the influence of sgc8 on cell viability has not been observed for two hours of incubation. We further tested long-term (chronic) toxicity of sgc8 aptamer to CCRF-CEM cells. The cells were incubated with sgc8 for 48 h and tested by commonly used MTT (3-(4,5)-dimethylthiahiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Very little cytotoxic effect was observed even at high aptamer dosage (Figure S2, Supporting Information). This finding indicated sgc8 could be a promising delivery agent with minimum cytotoxicity.

Conclusion

In summary, we have shown a cell-specific aptamer, sgc8, itself can be internalized into the T-cell lymphoblastic leukemia cells without external assistance. The aptamer is taken up by the cells and delivered to the endosome with minimum cytotoxicity. These observations have demonstrated that sgc8 is a promising reagent for T-cell leukemia-specific delivery. It is also confirmed that the anti-PTK7 antibody, which targets the same protein as the aptamer, is internalized to the cells in a similar way. The internalization kinetics of both the aptamer and the antibody for the same protein on a living cell surface has been studied and compared. The internalization study of sgc8 and anti-PTK7 antibody could provide valuable information for the understanding of the cellular internalization functions of PTK7. Using the easy modification and easy synthesis features of an aptamer as molecular recognition probe, we will be able to conjugate sgc8 with other molecules of interest (siRNA, toxin, anti-cancer drug) for specific intracellular delivery.

Experimental Section

Cell lines, antibodies and reagents: CCRF-CEM human acute lymphoblastic leukemia cell (CCL-119, T-cell line), and NB-4 (acute promyelocytic leukemia), gifts from Professor Ying Li (Department of Pathology, University of Florida), were grown in pH 7.4 RPMI 1640 medium (ATCC) supplemented with 10 % FBS (Fetal Bovine Serum) (heat inactivated, GIBCO) and penicillin/streptomycin (100 IU mL^{-1}) (Cellgro). Cells were washed before and after incubation with the washing buffer (4.5 gL^{-1} glucose and 5 mM $MgCl_2$ in Dulbecco's phosphate buffered saline with calcium chloride and magnesium chloride (Sigma)). The binding buffer used for aptamer selection was prepared by adding yeast tRNA (0.1 mg mL^{-1}) (Sigma) and BSA (1 mg mL^{-1}) (Fisher) into the washing buffer to reduce nonspecific binding.

The monoclonal anti-PTK7 antibody, which recognizes human protein tyrosine kinase-7, was purchased from Miltenyi Biotec Company. Transferrin-alexa633, chicken anti-rat IgG-alexa488 antibody (anti-IgG-alexa488) were both from Molecular Probes. Labeling anti-PTK7 antibody with alexa488 was done using Alexa488 Monoclonal Antibody Labeling Kit (Molecular Probes), following the manufacturer's protocol (Invitrogen).

DNA synthesis and labeling: All DNA synthesis reagents were from Glen Research (Sterling, VA). All probes and DNAs were synthesized with an ABI3400 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA). TAMRA CPG and normal phosphoramidites were used for TAMRA (Tetramethyl-6-Carboxyrhodamine) labeled sgc8 synthesis. dA-

CPG, normal phosphoramidites and 5′-amino-modified C6 were used for alexa488-labeled sgc8 synthesis. Deprotection was done by treating the oligonucleotides with *tert*-butylamine/methanol/water 1:1:2 for 3 h at 65 °C. The solutions resulted from deprotection were precipitated in ethanol. The precipitates were then dissolved in triethylammonium acetate (0.5 mL, 0.1 M, pH 7.0) for further purification with high-pressure liquid chromatography (HPLC). The HPLC purification was performed on a ProStar HPLC Station (Varian, CA) equipped with fluorescence and a photodioarray detector. A C-18 reverse phase column (Alltech, C18, 5 μM, 250x4.6 mm) was used. We conjugated the 5′-amine-modified sgc8 with alexa488 using the Alexa488 Succinimidyl Esters (Molecular Probes), according to the manufacturer's protocol (Invitrogen).

Trypsin treatment of cells: Cells were first washed with washing buffer (500 μL) to remove the FBS (Fetal Bovine Serum) in the medium or the binding buffer which might quench the function of trypsin, then incubated with Trypsin (500 μL, 0.05 %)/EDTA (0.53 mM) in HBSS at 37 °C for 10 min. After the incubation, 50 μL FBS was added and the cells were washed with the washing buffer (500 μL) once again and suspended in binding buffer (200 μL with 0.1 % NaN_2) for the detection.

FACS-based internalization assay: Flow cytometry analysis was performed on a FACScan (Becton Dickinson Immunocytometry Systems, San Jose, CA) by counting 30000 events, and the data were analyzed with WinMDI software. Internalization of sgc8-alexa488 and anti-PTK7-alexa488 is normalized as (MI@37 °C trypsin–MI@4 °C trypsin)/(MI@4 °C–MI@4 °C trypsin), where MI@4 °C trypsin and MI@37 °C trypsin are the mean fluorescent intensity with trypsin treatment (see the above procedures for details) before and after internalization, separately. MI@4 °C stands for the mean fluorescent intensity without trypsin treatment before internalization. To acquire all these data, the cells were grown at a concentration of 2×10^6 mL^{-1} before the experiments. For the sgc8-alexa488 and anti-PTK7-alexa488 binding experiment, cells (5×10^5) were first washed with washing buffer (500 μL) at 4 °C, then stained on ice with alexa488-labeled anti-PTK7 or sgc8 (200 nM) in binding buffer (200 μL) containing 10 % FBS for 30 min. Cells were washed with washing buffer (500 μL) three times and suspended in binding buffer (200 μL, with 0.1 % NaN_3) for the detection of MI@4 °C. The same binding experiments were performed and followed by the trypsin treatment procedure as above to get MI@4 °C trypsin. To detect sgc8 and anti-PTK7 antibody internalization, cells (5×10^5) were first washed with washing buffer (500 μL) at 4 °C once, then incubated with alexa488-labeled anti-PTK7 or sgc8 (200 nM) in RPMI-1640 medium at 37 °C for 10, 20, 30 min, 1, 1.5, 2 h, respectively. Internalization was stopped by placing the cells on ice immediately after the incubation and washing buffer (500 μL) at 4 °C was added. Complying with trypsin treatment method described above, MI@37 °C trypsin was obtained. The chicken anti-rat IgG-alexa488 antibody (anti-IgG-alexa488) and alexa488 labeled TDO5 (TDO5-alexa488) were used as negative controls. All these experiments were repeated three times.

Confocal imaging: All cellular fluorescent images were collected on the FV500-IX81 confocal microscope (Olympus America Inc., Melville, NY) with 60x oil immersion objective (NA = 1.40, Olympus, Melville, NY). Excitation wavelength and emission filters were as following: alexa488, 488 nm laser line excitation, emission BP520 ± 12 nm filter; alexa633, 633 nm laser line excitation, emission LP650 filter; TAMRA, 543 nm laser line excitation, emission BP580 ± 20 nm filter. The cells were treated with sgc8 labeled with TAMRA (sgc8-TAMRA) or anti-PTK7 antibody labeled with alexa488 (anti-PTK7-alexa488) following the same steps as that described in the flow cytometry section. For the co-localization experiment, cells (5×10^5) were first washed with washing buffer (500 μL) at 4 °C once, and then incubated with sgc8-TAMRA (200 nM) and anti-PTK7-alexa488 (200 nM) in RPMI-1640 medium at 37 °C for 2 h, and transferrin-alexa633 (60 nM) was added 30 minutes before the termination of incubation. Internalization was stopped by placing the cells on ice immediately after the incubation and washing buffer (500 μL) at 4 °C was added, followed by trypsin treatment mentioned above. Before the imaging, a volume of cell suspension (100 μL) was dropped on the poly-D-lysine coated 35 mm glass bottom dishes (Mat Tek Corp) then waiting for 3 min to make the cells settle down. We repeated these experiments three times and analyzed using the Fluoview analysis software.

Cytotoxicity assays: Cellular toxicity of sgc8 aptamer was assessed using the CellTiter 96 Cell Proliferation Assay (Promega, Madison WI) according to the manufacture's instructions. This assay is based upon the conversion of a tetrazolium substrate to a formazan product by viable cells.[33] Cells (1×10^4 cells per well) were cultured in 96-well microtiter plates in complete growth medium with various sgc8 concentrations. For the chronic toxicity, cells were incubated with the sgc8 aptamer for 48 h prior to counting the number of viable cells.

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