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Molecular Assembly of an Aptamer–Drug Conjugate for Targeted Drug Delivery to Tumor Cells

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The conjugation of antitumor drugs to targeting reagents such as antibodies is a promising method that can increase the efficacy of chemotherapy and reduce the overall toxicity of the drugs. In this study, we covalently link the antitumor agent doxorubicin (Dox) to the DNA aptamer sgc8c, which was selected by the cell-SELEX method. In doing so, we expected that this sgc8c–Dox conjugate would specifically kill the target CCRF-CEM (T-cell acute lymphoblastic leukemia, T-cell ALL) cells, but with minimal toxicity towards nontarget cells. The results demonstrated that the sgc8c–Dox conjugate possesses many of the properties of the sgc8c aptamer, including high binding affinity ($K_d = 2.0 \pm 0.2$ nM) and the capability to be efficiently internalized by target cells. Moreover, due to the specific conjugation method, the acid-labile linkage connecting the sgc8c–Dox conjugate can be cleaved inside the acidic endosomal environment. Cell viability tests demonstrate that the sgc8c–Dox conjugates not only possess potency similar to unconjugated Dox, but also have the required molecular specificity that is lacking in most current targeted drug delivery strategies. Furthermore, we found that nonspecific uptake of membrane-permeable Dox to nontarget cell lines could also be inhibited by linking the drug with the aptamer; thus, the conjugates are selective for cells that express higher amounts of target proteins. Compared to the less effective Dox-immunoconjugates, these sgc8c–Dox conjugates make targeted chemotherapy more feasible with drugs having various potencies. When combined with the large number of recently created DNA aptamers that specifically target a wide variety of cancer cells, this drug-aptoconjugation method will have broad implications for targeted drug delivery.

Introduction

The lack of drugs with tumor cell specificity often results in life-threatening toxic effects for patients undergoing traditional chemotherapy for cancer. To overcome this problem and improve the selectivity of cancer therapy, cytotoxic drugs should be delivered to tumor-specific sites. Biochemical synthesis of ligand-linked drug conjugates plays an important role in accomplishing this goal. For instance, monoclonal antibodies (mAb) that bind to specific markers on the surface of tumor cells have been covalently linked to drugs for such targeted cancer therapy. These mAb-immunoconjugates can selectively deliver drugs to tumors and, hence, improve antitumor efficacy while reducing the systemic toxicity of otherwise beneficial therapies. In addition, immunoconjugates have been created using drugs that have a wide range of functions and potencies. So far, only the immunoconjugates that incorporate drugs with much higher potencies demonstrated impressive results in preclinical models, and they are currently being evaluated in clinical trials.[1,2] An example of one of these advanced agents is the humanized anti-CD33 antibody–alicheamicin conjugate Mylotarg, which has already been approved for the treatment of acute myeloid leukemia (AML).

In recent years, new of single-stranded oligonucleotides called aptamers have emerged as a novel class of molecules that rival antibodies in both therapeutic and diagnostic applications.[3–6] Aptamers not only combine the advantages of antibodies, such as high affinity, excellent specificity and low toxicity or immunogenicity, but they are also stable and easy to synthesize, modify and manipulate. Aptamers which can bind their specific targets are selected from a process called SELEX (systematic evolution of ligands by exponential enrichment).[7,8] Following several selection cycles, aptamers from a DNA or RNA pool can be selected and enriched by repetitive binding of their target molecules. Recently, cell-SELEX has been developed for the generation of aptamers for specific recognition of target tumor cells such as T-cell acute lymphoblastic leukemia (T-cell ALL), small-cell lung cancers and liver cancers.[9–13] These aptamers can be generated relatively easily. They are highly specific for different types of tumor cells and have excellent affinity. Since they can provide specificity at the molecular level, we believe that these aptamers can be used to enhance the

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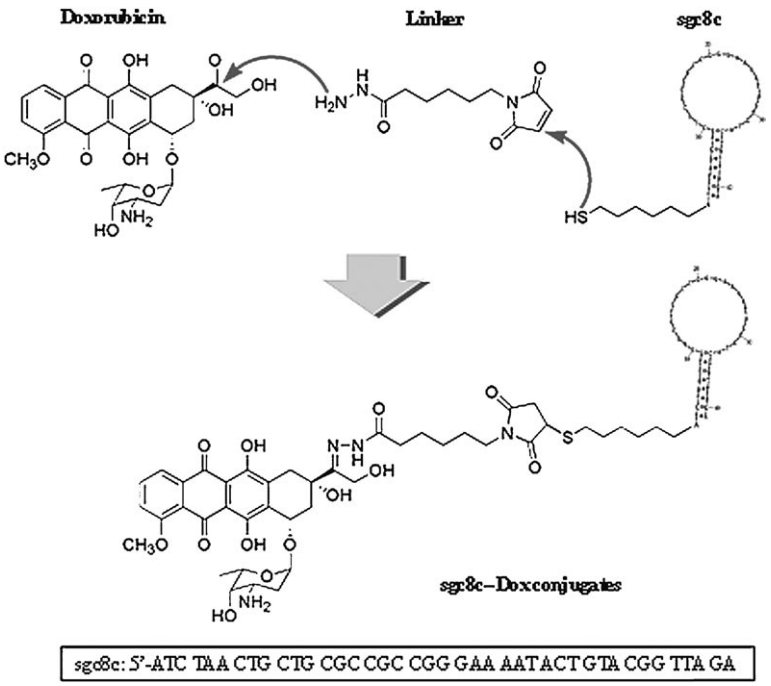
efficacy of experimental and/or commercial drugs in clinical applications. Molecular engineering is thus needed to create molecular conjugates that have specificity and drug potency.

In this report, we explored the usage of DNA-based aptamers, selected from cell-SELEX, for the molecular engineering of a ligand–drug conjugate for targeted drug therapy applications. Specifically, we used an aptamer, which was selected for human T-cell ALL CCRF-CEM cell lines,[11] as a drug carrier for targeting specific tumor cells. Sgc8c can recognize the protein tyrosine kinase 7 (PTK7), a transmembrane receptor highly expressed on CCRF-CEM cells[14] with high binding affinity ($K_d \sim 1$ nM). Its high specificity and well-characterized DNA structure[15] give sgc8c the capacity to distinguish between target leukemia cells and normal human bone marrow aspirate, as well as identify cancer cells closely related to the target cell line in clinical specimens.[11,16] Recently, we also demonstrated that sgc8c can internalize into the target cells after the binding to its target protein.[17] For these reasons, sgc8c is considered a good candidate for proof-of-concept. Doxorubicin (Dox) is the most utilized anticancer drug against a range of neoplasms, including acute lymphoblastic and myeloblastic leukemias, as well as malignant lymphomas.[18] However, its efficacy in cancer treatment is impeded by such toxic effects as myelosuppression, mucositis, alopecia, and, most concerning, cumulative cardiac damage.[19] Therefore, we have molecularly assembled Dox into our aptamer probe through a simple conjugation method in order to demonstrate the feasibility of this target-specific approach in intracellular drug delivery.



Scheme 1. Conjugation of the drug doxorubicin (Dox) to aptamer sgc8c for targeted delivery to cancer cells.

Results and Discussion

Aptamer–Dox conjugates are easily prepared

The synthesis procedure for producing the sgc8c–Dox conjugate is shown in Scheme 1. In order to release the chemotherapeutic agent from the conjugate after internalization, we chose a hydrazone linker to conjugate sgc8c with Dox. Several studies have already demonstrated that Dox C-13 hydrazone derivatives possess a cytotoxic effect comparable to unconjugated Dox[20] and allow the release of Dox at pH 4.5–5.5.[21] After conjugation, the sgc8c–Dox was purified by HPLC (see Figure S1 in the Supporting Information) and dialysis against buffer (10 mM Tris-HCl, pH 7.4) for 12 h to remove the physically conjugated Dox.[4] The amount of Dox was then determined by UV absorption at 495 nm ($\varepsilon_{495} = 12\,000\ cm^{-1}\,M^{-1}$), while that of sgc8c was calculated from the absorption at 260 nm according to the formula [Eq. (1)]:

$$\text{sgc8c(M)} = \frac{A_{260} - (\varepsilon_{260} \times A_{495}/\varepsilon_{495})}{397\,600} \quad (1)$$

(A is the observed absorbance at noted wavelength), which includes a correction for the absorbance of Dox at the same wavelength ($\varepsilon_{260} = 24\,000\ cm^{-1}\,M^{-1}$). The Dox:sgc8c ratio we achieved was around 0.5; this indicates that the unconjugated sgc8c not been well-separated from conjugated sgc8c. Oligonucleotide-based molecular probes are usually considered stable and easy to synthesize, manipulate, and handle. They can tolerate harsh conditions, such as high temperature and extreme pH environments and can be stored for a long time without stabilization reagents. Using the molecular engineering strategy described here, we are able to easily assemble a single Dox into the sgc8 aptamer. Following this method, we also believe that it is feasible to incorporate multiple Dox molecules in a similar, but controllable, manner within one aptamer. This maneuver is relatively hard to achieve in current mAb-based immunoconjugation methodology.

Aptamer–Dox conjugates maintain specific binding and high affinity to target cancer cells

Although most oligonucleotide-based molecular probes can be modified at the 3′ or 5′ ends without interfering with their biological properties, it is necessary to confirm the specificity of the newly synthesized sgc8c–Dox conjugate. In order to assess the binding of the conjugate to target cells, a simple competition assay was performed by first incubating the conjugate and then incubating a fluorescein-labeled sgc8c. The

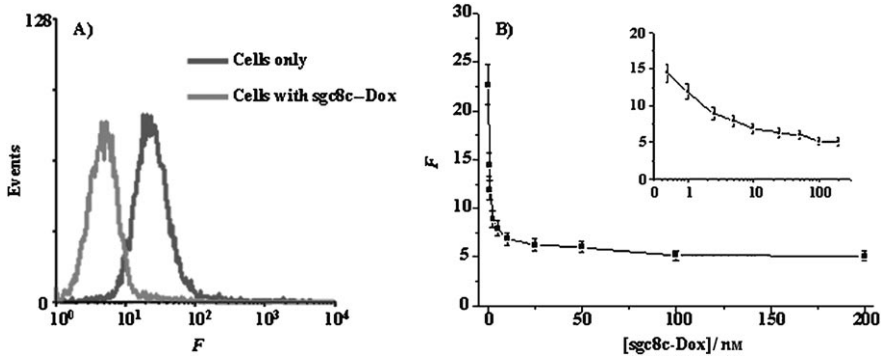


Figure 1. Binding assay of sgc8c–Dox conjugates to CCRF-CEM cells. A) Flow cytometry assay for the binding of sgc8c–Dox conjugates with CCRF-CEM cells. The curves represent the fluorescence from fluorescein-sgc8c incubated with pure cells and cells labeled with sgc8c–Dox (200 nM). B) Flow cytometry to determine the binding affinity of sgc8c–Dox conjugates to CCRF-CEM cells. The fluorescence is derived from the second stain of cells by fluorescein-labeled sgc8c. Inset is the plot of mean fluorescence [a.u.] versus sgc8c–Dox concentration [nM] in log scale.

fluorescence intensity was then determined by flow cytometry. The observation that less fluorescence was detected from the CCRF-CEM cells after the binding sites had already been saturated with sgc8c–Dox (Figure 1 A), confirms that the binding between sgc8c–Dox conjugates toward CCRF-CEM cells is through the specific binding of sgc8c to its target. It should also be noted that the conjugation of Dox to sgc8c by hydrazone linker has no effect on the ability of the aptamer–Dox conjugate to bind to the CCRF-CEM cells. Specifically, the binding affinity (K_d) of sgc8c–Dox was determined to be 2.0 ± 0.2 nM through an indirect fluorescence measurement (Figure 1 B. This result is quite comparable to the unconjugated sgc8c.

Aptamer–Dox conjugates possess drug efficacy similar to unconjugated parent Dox

In order to evaluate the cytotoxic effect of Dox after molecular assembly, the human leukemia CCRF-CEM cell line was chosen for anticancer drug testing. The relative viability of cells treated with sgc8c–Dox in the concentration range of 0 to 2.5 μM was measured by MTT (3-(4,5)-dimethylthiahiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, and the results shown in Figure 2 A demonstrate that the inhibition concentration (IC_{50}) of sgc8c–Dox is 0.32 ± 0.04 μM which is exactly the same as free Dox ($IC_{50} = 0.32 \pm 0.03$ μM) (Figure 2 B. The cytotoxic effect of free sgc8c and its inherent toxicity toward CCRF-CEM cells was observed to be less than 20 ± 5% (Figure 2 C. Based on these results, we can conclude that the pharmacological component of our aptamer sgc8c conjugate still possesses high potency in cancer therapy. From the differences in the cytoxic effects of sgc8c–Dox and free sgc8c, we can also rule out any possible toxicity from the aptamer itself.

Dox plays an important role in cancer treatment and is considered to be the most utilized anticancer drug worldwide. However, only it is only able to stop cell proliferation through intercalation of DNA in the cell's nucleus. Several reports have demonstrated that free Dox is membrane permeable and can be uptaken by cells through a passive diffusion mechanism, rapidly transported to the nuclei, and readily bound to the chromosomal DNA.[22] Within 15 min, cells treated with Dox already show an intense red fluorescence in the nuclear region; this suggests that most of the Dox is predominately accumulated there.[23] Our previous internalization study[17] showed that sgc8c can be specifically uptaken by the CCRF-CEM cells after receptor binding; however, it has been shown that sgc8c tends to accumulate inside the endosomal compartment. Therefore, in

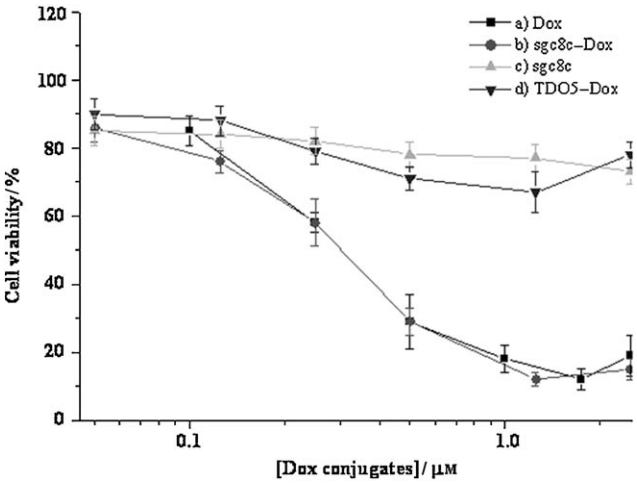


Figure 2. Cytotoxicity assays of A) sgc8c–Dox conjugates, B) free Dox, C) free sgc8c, and D) TDO5–Dox conjugates with the CCRF-CEM cell line. The cells (2×10^4 cells/well) were incubated with Dox or aptamer–Dox conjugates (0 to 2.5 μM) in culture medium without FBS at 37 °C, 5% CO_2 for 2 h. After drug treatment, cells were subsequently grown in fresh medium (10% FBS) for 48 h. The cytotoxicity was then measured by an MTT assay.

order to prove that the similar cytotoxicities of sgc8c–Dox conjugates compared to unconjugated Dox is the result of the release of free Dox from the conjugates inside the endosomal environment, we tracked the intracellular distribution of sgc8c–Dox conjugates by monitoring the red fluorescence of Dox by using confocal microscopy (Olympus America Inc., Melville, NY). By simultaneously incubating the cells with sgc8c–Dox and Alexa633-labeled transferrin (transferrin–Alexa633), we were able to track the location of both sgc8c–Dox and the endosomal compartment. After 30 min incubation, most of the sgc8c–Dox was colocalized with transferrin; this indicates that the conjugates were still internalized inside the endosomes (Figure 3). However, after 1 h, some of the red dots (Dox signal) had spread out into the cytosol, and at 2 h they were finally evenly distributed inside the cells. We believe that it is the acidic environment of the endosomal compartment[24] that

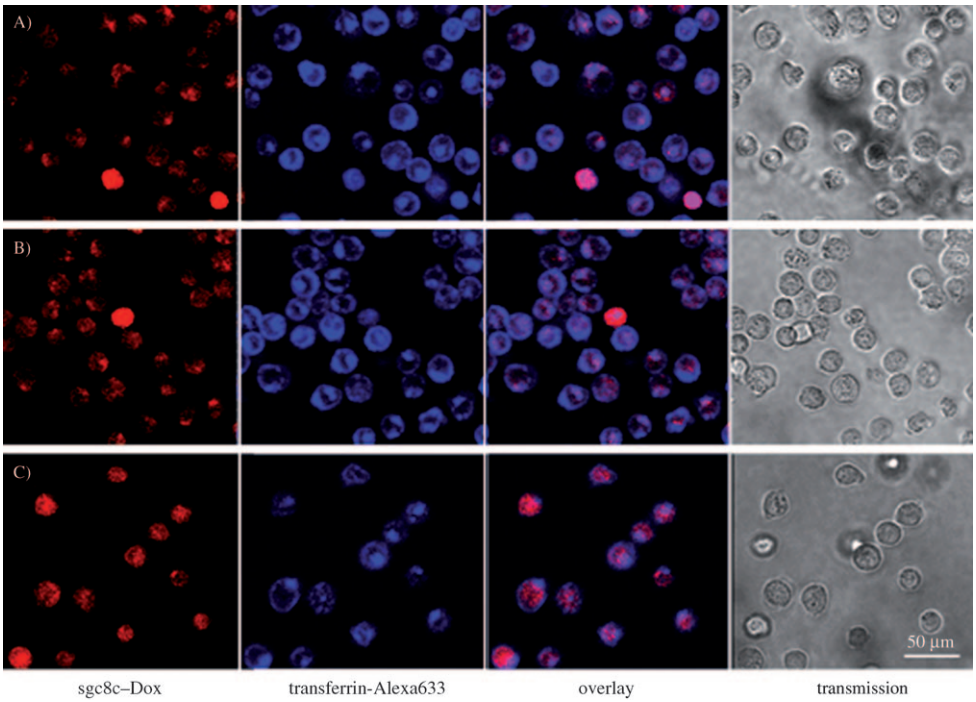


Figure 3. Confocal images display the distribution of sgc8c–Dox conjugates inside CCRF-CEM cells at different time points: A) 30 min, B) 1 h, and C) 2 h. The cells (10^6) were incubated with sgc8c–Dox (0.5 μM) and transferrin-Alexa633 (60 nM) in culture medium without FBS at 37 °C, 5% CO2 for 2 h. From left to right, the fluorescence images were monitored for sgc8c–Dox, transferrin-alexa633, overlay of these two channels, and bright field channel, respectively. Transferrin-Alexa633 will both bind to the surface and internalize to the endosomal compartment of CCRF-CEM cells.

specifically cleaves the acid-labile linker and allows the Dox to be rapidly transported to and function in the nucleus. This is supported by our published results[17] that show no accumulation of fluorophore-conjugated sgsc8c within the cytosol at the same time point, since the fluorescent conjugate is not acid-labile.

According to the literature, several in vitro studies demonstrated that the immunoconjugates of Dox were only moderately potent and often less active than the parent drugs.[2,25] Therefore, highly potent drugs with IC_{50} values in the range of 0.01–0.1 nM are usually required for immunoconjugates to achieve successful cancer treatment toward tumor cell lines.[2] On the other hand, our sgc8c–Dox conjugate is as potent as free Dox in target cells; this means that it will not be necessary to use highly potent drugs in future conjugates. This improved potency compared to immunoconjugates can be traced to the high affinity of the aptamer conjugate as well as the efficient internalization of this aptamer by its target cell.

Aptamer–Dox conjugates show excellent specificity for killing target cancer cells

We also examined the specificity of sgc8c–Dox by comparing the cytotoxic effect of a different aptamer–Dox conjugate to CCRF-CEM cells. The TDO5 aptamer binds specifically to the human Burkitt's lymphoma cell line, Ramos, and can therefore be used as a negative control to show specificity of the sgc8c–Dox conjugate. After incubation of cells with 0 to 2.5 μM TDO5–Dox, an MTT assay (Figure 2D demonstrated that the cellular toxicity (less than 21±10%) was similar to that of free sgc8c. Moreover, less fluorescence was observed inside the cells with confocal microscopy when applying TDO5–Dox in 0.5 μM for 2 h incubation (Figure S2), indicating that the non-specific uptake of Dox is minimized by linking with the control DNA, TDO5. This comparative study provides strong evidence that while TDO5–Dox shows no internalization, our sgc8c–Dox can recognize and be uptaken by its target cells specifically. Therefore, it is clear that only the aptamer conjugated drug, which can bind and be internalized by the target cells, can deliver drugs inside the cells and inhibit cell growth.

Sgc8c–Dox internalization occurs through receptor-mediated uptake

We further made use of the fluorescence of Dox for cellular uptake studies. Even though the fluorescence of Dox decreased about five-fold after linking with our aptamer (data not shown), we were still able to monitor its fluorescence by flow cytometry after incubating cells with free and aptamer-conjugated Dox. Figure 4A shows the fluorescence intensity from Dox-treated CCRF-CEM cells at different drug concentrations (dashed line) after three wash cycles to remove unbound Dox. It should be noted that the fluorescence signal comes from both the signal inside and on the cell membrane. Since only the drug inside the cells can have an effect, we used trypsin to remove the surface-bound drug. After trypsin treatment, the fluorescence signal showed a linear relationship with different Dox concentrations (solid line in Figure 4A). This result agrees well with the passive diffusion phenomenon of transporting Dox inside the cells since the mass transportation by

simple diffusion is directly proportion to the concentration gradient. However, after subtracting the fluorescence intensity from nonspecifically interacted TDO5–Dox from the sgc8c–Dox signal, we observed a different curve upon cellular uptake (Figure 4B. The fluorescence intensity of sgc8c–Dox increases linearly and finally saturates after 0.5 μM. The saturation of this curve proves that the internalization of sgc8c–Dox occurs in a receptor-dependent manner which is different from the passive diffusion of free Dox. At high conjugate concentrations, all binding sites become occupied and there can be no more internalization.

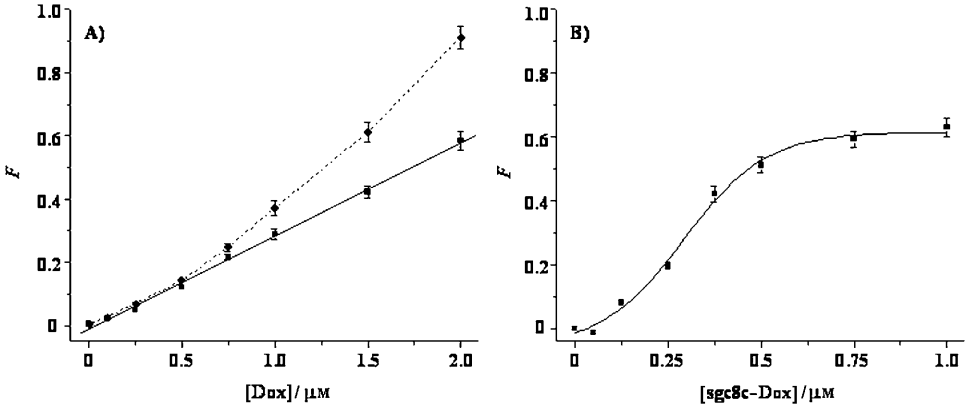


Figure 4. Quantitative analysis of A) Dox and B) sgc8c–Dox inside CCRF-CEM cells. The cells (105 cells) were incubated with Dox or aptamer–Dox conjugates in culture medium without FBS at 37 °C, 5% CO_2 for 2 h. A) Dashed and solid lines represent the fluorescence signal from Dox before and after trypsin treatment, respectively. Trypsin was applied to remove the membrane-bound Dox. B) Fluorescence signal from sgc8c–Dox inside cells determined by flow cytometry. All the signals have been subtracted by the fluorescence intensity from TDO5–Dox nonspecifically uptaken by CCRF-CEM cells.

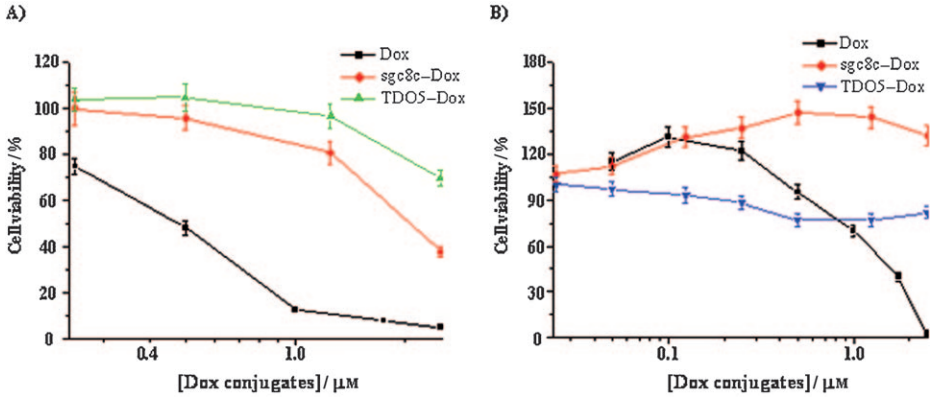


Figure 5. Cytotoxicity assays of free Dox, sgc8c–Dox, and TDO5–Dox conjugates with two additional cell lines: A) NB-4 and B) Ramos cells. All the conditions are the same as those in Figure 2.

The fluorescence signal quantified by flow cytometry in these internalization assays coincided well with the cytotoxicity results. For example, sgc8c–Dox shows stronger potency compared to TDO5–Dox, at concentrations higher than 125 nM. Similarly, the fluorescence difference between sgc8c–Dox and TDO5–Dox starts to increase at 125 nM. Furthermore, at the concentration above 0.5 μM, both the cytotoxicity of sgc8c–Dox to the cells as well as the fluorescence difference between sgc8c–Dox and TDO5–Dox achieve their maximum capacity.

By comparing the either fluorescence or cytotoxic effects among different aptamer-(sgc8c and TDO5)-conjugated drugs, our investigation has thus far shown that sgc8c can be useful for targeted delivery of membrane-permeable drugs. Compared to the reported prostate-specific membrane antigen (PSMA) RNA aptamer, which can promote the uptake of gelonin (a ribosomal toxin that cannot penetrate the cell membrane by itself) even at significantly high concentrations,[3] our conjugation strategy with the DNA aptamer, specifically, sgc8c, can be more useful for delivering universal toxic payloads to tumor cells, such as membrane-permeable anticancer drugs that show no specificity to target cells.

Sgc8c–Dox conjugates show selective killing efficiency for different cancer cells

To further prove that the linkage with an aptamer can be useful for targeted drug delivery, we tested the cytotoxicity of our sgc8c–Dox conjugates with two more cell lines, NB-4 and Ramos (Figure 5). For CCRF-CEM, NB-4 and Ramos cells, the IC_{50} of free Dox is about 0.32 ± 0.03 μM, 0.49 ± 0.07 μM and 1.46 ± 0.22 μM, respectively. However, our sgc8c–Dox conjugates show a 6.7-fold increase in toxicity to their target CCRF-CEM cells (Figure 2), when compared to that of NB-4 cells (Figure 5). The selectivity is even better when compared with the Ramos cells, since the toxicity in Ramos cells essentially leveled off at the highest concentrations tested with no indication that further increases would lead to additional toxicity. We also conducted additional experiments by treating these three cell lines with TDO5–Dox (Figure 5). Compared to the cellular fluorescence of cells labeled with (R-phycoerythrin)–gc8c and (R-phycoerythrin)–TDO5 (20 min incubation) by flow cytometry (Figure 6), we determined that the cytotoxicity is well correlated to the different expression levels of protein receptors on the membrane surface of these cell lines. It should be noted that both the binding test and MTT assay were conducted at 37 °C, during which TDO5 lost its binding capability to the target Ramos cells. These results further confirmed the high specificity of our aptamer–-drug conjugate and its potential in targeted drug delivery. The reason TDO5 does not have strong

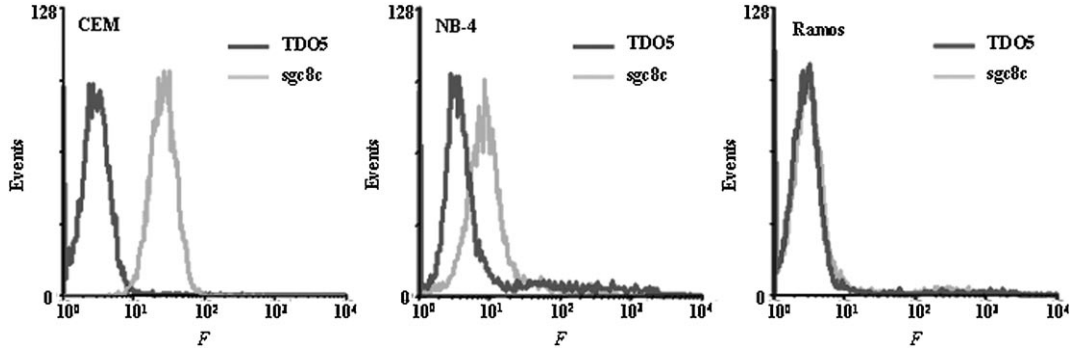


Figure 6. Flow cytometry assay for the binding of biotin-labeled TDO5 and sgc8c with three different cell lines: CCRF-CEM, NB-4, and Ramos cells. Cells (10^5) were incubated with biotin-labeled TDO5 and sgc8c at 37 °C for 20 min in 100 μL culture medium without FBS. After washing twice, cells were mixed with streptavidin–(R-phycoerythrin) (20 min on ice), and the fluorescence was determined by flow cytometry.

binding at 37 °C is under investigation. Preliminary results by NMR show that the second structures of TDO5 at different temperatures are quite different.

When treating Ramos cells with Dox in low concentration (less than 0.25 μM), it is interesting to note that higher MTT signals, which can be interpreted as cell growth, are comparable to those observed for untreated cells. This result is specific to Ramos cells since no similar effect is seen for most leukemia cell lines (data not shown). In terms of morphological changes, the size of cells with 0.25 μM-Dox treatment increases about 1.4-fold, while the cell numbers remain equal (with 20% deviation) in comparison to the untreated Ramos cells (data not shown). Higher metabolic activity of proliferating cells may be attributed to an increasing MTT signal. However, the detailed mechanism that underlies the drug effect is still under investigation.

DNA-based aptamers are usually considered stable and easily modified with different drug molecules and nanomaterials. Therefore, we also tried to synthesize a multiple-Dox–aptamer conjugate through a bifunctional linker (see more experimental details in the Supporting Information). The ratio of Dox to aptamer of our final product (sgc8c–3Dox) is about 2.9 and each Dox molecule can be cleaved from the acid-labile linkage independently. However, the IC_{50} of sgc8c–3Dox, compared to that of sgc8c–Dox with CCRF-CEM cells, shows no significant difference (with 14% deviation) based on the same concentration of sgc8c. The toxicity was, therefore, less than expected. Our binding assay demonstrates that this resulted from the comparatively reduced binding capability of sgc8c–3Dox to the target cells ($Kd = 20 \pm 3$ nM).

Conclusions

In summary, we applied effective molecular engineering schemes to link aptamers with drug molecules for targeted delivery. We confirmed that linking the universal antitumor agent doxorubicin with our DNA-based, cell-SELEX-selected aptamer, sgc8c, is feasible for targeted drug delivery. The aptamer prevents the nonspecific uptake of Dox and decreases cellular toxicity to the nontarget cells. It also efficiently internalizes into cells and selectively delivers toxic payloads to specific cells which abundantly express the target proteins. We also took advantage of the fluorescent properties of these drug conjugates to confirm their uptake by target cells occur after binding to the target proteins. Release of drugs from the acid-labile linkages in endosomes, as well as transport to nuclei, could also be monitored by confocal microscopy at different time points. In the future, we plan to further investigate the antitumor activity of our aptamer–Dox conjugates in human xenograft models inside nude mice. This effort will lay the groundwork for continued development of target-specific drugs for efficient therapy having reduced side effects. When implemented with recently created aptamers for various cancer cell lines, this simple drug–aptamer conjugation method will have wide-ranging implications in targeted drug delivery of many different cancers.

Experimental Section

Chemicals: Doxorubicin hydrochloride (Dox) was purchased from Fisher Scientific (Houston, TX, USA). *N*-(Epsilon-maleimidocaproic acid) hydrazide (EMCH) was purchased from Pierce Biotechnology (Rockford, IL, USA). Tris(2-carboxyethyl)phosphine (TCEP) was obtained from Sigma–Aldrich. Deoxyribonucleotides and 5′-thiol modifiers were purchased from Glen Research (Sterling, VA, USA). Unless otherwise noted, starting material, reactants and solvents were obtained commercially from Fisher Scientific.

Aptamers: Aptamer sgc8c (5′-ATC TAA CTG CTG CGC CGC CGG GAA AAT ACT GTA CGG TTA GA-3′) and a control sequence TDO5 (5′-CAC CGG GAG GAT AGT TCG GTG GCT GTT CAG GGT CTC CTC CCG GTG-3′) with a 5′-disulfide group were synthesized on ABI3400 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA, USA). The completed sequences were then deprotected in AMA (ammonium hydroxide/40% aqueous methylamine 1:1) at 65 °C for 20 min and further purified with reversed-phase HPLC (ProStar, Varian, Walnut Creek, CA, USA) on a C-18 column.

Synthesis of aptamer–Dox conjugates: The synthesis of aptamer–Dox conjugates was similar to that of immunoconjugate BR96–Dox.[21] Briefly, Dox hydrochloride (5 mg, 8.62 μmol) and EMCH (10 mg, 44.4 μmol) were dissolved in methanol (4 mL). Trifluoroacetic acid (3 μL) was added, and the solution was stirred at room temperature for 24 h while being protected from light. The methanolic solution was concentrated under reduced pressure at room

temperature to a volume of 0.25 mL. Acetonitrile (2.5 mL) was added, and the resulting suspension was allowed to stand at 4 °C for 48 h for crystallization of the product. The red solid hydrazone was isolated by centrifugation, washed with fresh methanol/acetonitrile (1:10), and dried under vacuum to yield the (6-maleimidocaproy1) hydrazone of Dox (2.9 mg, 3.85 μmol, 44.6% yield).

A disulfided DNA (300 nmol) was reduced with TCEP in PBS (pH 7.4) for 2 h at room temperature. TCEP was removed by G-25 Sephadex size-exclusion column (NAP™-5, Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with PBS. The eluate was added to the (6-maleimidocaproy1) hydrazone of Dox (2 μmol) dissolved in dimethylformamide (DMF, 50 μL) and incubated on ice for 12 h. The product conjugates were purified by HPLC with Tris-HCl buffer (10 mM, pH 7.4) and acetonitrile as mobile phase and monitored with UV at 260 and 495 nm. After concentration under vacuum to 0.1 mL, the amount of conjugate was measured by UV scan. 140 nmol of conjugate was obtained (47.7% yield).

Cell lines: Human T-cell ALL (CCRF-CEM) and human B-cell Burkitt's lymphoma (Ramos) cell lines were obtained from the American Type Culture Collection (Manassas, VA). The acute promyelocytic leukemia cell line NB-4 was obtained from the Department of Pathology at the University of Florida. All of the cells were grown in RPMI-1640 containing fetal bovine serum (FBS, 10%) and penicillin–streptomycin (100 IU mL^{-1}) at 37 °C in a humid atmosphere with 5% CO_2.

Flow cytometric analysis: The binding affinity of sgc8c–Dox conjugate was determined by incubating CCRF-CEM cells (10^5) on ice for 20 min with a serial concentration of sgc8c–Dox conjugates in culture medium (100 μL) without FBS. Cells were then washed twice with washing buffer (0.5 mL) and suspended in fluorescein-labeled sgc8c (25 nM, 0.1 mL) for further incubation (20 min on ice). Before flow cytometric analysis, cells were washed with washing buffer once more and suspended in washing buffer (0.2 mL). The mean fluorescence intensity of cells labeled with fluorescein–sgc8c was used to calculate the equilibrium dissociation constant (K_d) of sgc8c–Dox and CCRF-CEM cell interaction by fitting the dependence of fluorescence intensity (F) on the concentration of the sgc8c–Dox (L) to the equation $F = B_{max}[L]/(K_d + [L])$. The binding assay experiments were repeated at least three times.

To monitor the uptake of Dox and aptamer–Dox by CCRF-CEM cells, cells (5×10^5) were incubated with Dox or aptamer–Dox conjugates in 500 μL culture medium without FBS at 37 °C, 5% CO_2. After 2 h, cells were then washed twice with washing buffer (500 μL) and suspended in washing buffer (100 μL) for flow cytometric analysis. For trypsin treatment, cells were first washed twice with washing buffer (500 μL), and then incubated with trypsin (500 μL, 0.05%)/EDTA (0.53 mm) in HBSS at 37 °C for 10 min. After the incubation, FBS (50 μL) was added and the cells were washed with the washing buffer (500 μL) once again and suspended in washing buffer for the fluorescence measurement.

Cytotoxicity assay: Chemosensitivity of cell lines to Dox or aptamer–Dox conjugates was determined using the CellTiter 96 cell proliferation assay (Promega, Madison, WI, USA). The cells (2×10^4 cells/well) were incubated with Dox or aptamer–Dox conjugates (0 to 2.5 μM) in culture medium without FBS at 37 °C, 5% CO_2. After 2 h, 75% of media was removed and fresh media (10% FBS) were added for further cell growth (48 h). For cytotoxicity measurement, CellTiter reagent (20 μL) was added to each well and incubated for 2 h. Using a plate reader (Tecan Safire microplate reader, AG, Switzerland), the absorption was recorded at 490 nm. The percentage of cell viability was determined by comparing Dox and aptamer–Dox conjugate-treated cells with the untreated control.

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