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Self-assembled Aptamer-based Drug Carriers for Bi-specific Cytotoxicity to Cancer Cells

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Abstract

Monovalent aptamers can deliver drugs to target cells by specific recognition. However, different cancer subtypes are distinguished by heterogeneous biomarkers, and one single aptamer was unable to recognize all clinical samples from different patients with even the same type of cancers. To address heterogeneity among cancer subtypes for targeted drug delivery, as a model, we developed a drug carrier with broader recognition range of cancer subtypes. This carrier (SD) was self-assembled from two modified monovalent aptamers. It showed bi-specific recognition abilities to target cells in cell mixtures, thus broadening the recognition capabilities of its parent aptamers. The self-assembly of SD simultaneously formed multiple drug loading sites for anticancer drug Doxorubicin (Dox). The Dox-loaded SD (SD-Dox) also showed bi-specific abilities of target cell binding and drug delivery. Most importantly, SD-Dox induced bi-specific cytotoxicity in target cells in cell mixtures. Therefore, by broadening the otherwise limited recognition capabilities of monovalent aptamers, bi-specific aptamer-based drug carriers would facilitate aptamer applications for clinically heterogeneous cancer subtypes which respond to the same cancer therapy.

Keywords

aptamer; drug delivery; self-assembly; cancer heterogeneity; bi-specific

Introduction

Drug delivery systems that specifically recognize cancer cells and induce targeted cytotoxicity will reduce side effects caused by nonspecific drug toxicity. Specific recognition can be realized by using antibodies or aptamers^[1].

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Aptamers, which are selected through Systematic Evolution of Ligands by EXponential enrichment (SELEX), are single-stranded DNA or RNA molecules that can specifically and selectively bind to targets^[1b, 1c]. The targets of aptamers range from metal ions, small molecules, to proteins, and even mammalian cells.^[1b, 2] Recently, our group developed cell-SELEX to select aptamers against whole cells, using target cells for positive selection and non-target cells for negative selection^[1c, 3]. With this technology, aptamers have been selected against cell lines such as CCRF-CEM (human T-cell acute lymphoblastic leukemia (ALL)) and Ramos (human B-cell Burkitt's lymphoma)^[3a]. Compared with antibodies, nucleic acid aptamers have many distinct advantages, such as easy synthesis and modification, reproducible batch-to-batch fabrication, and low cytotoxicity and immunogenicity^[1b, 1c, 4]. As such, aptamers are promising for future biomedical application such as targeted anticancer drug delivery.

However, recent aptamer binding tests with patient samples indicated that a single type of aptamer did not bind all samples from different patients with the same type of cancer^[5], presumably resulting from the heterogeneity of cell surface biomarkers among different patient samples. This suggests that monovalent aptamers selected against cultured cancer cells may not be able to overcome the problem of heterogeneity among different patient samples. Yet, cancer heterogeneity has been widely reported^[6], and more recently, it was further demonstrated by direct single-cell analysis such as genomic sequencing^[7] and dissection of tumor cell transcription^[8]. Therefore, improvement of aptamers for broader range of recognition capabilities would be highly significant for future clinical applications in targeted cancer therapy.

In this context, we propose developing multi-specific, aptamer-based drug carriers that are capable of recognizing and inducing targeted cytotoxicity in different subtypes of cancers. These carriers were designed to be self-assembled from modified monovalent aptamers. The assembly would simultaneously form drug loading sites in the double-stranded linker region. As a model, a bi-specific drug carrier, sgc8c-sgd5a (SD), was developed from monovalent aptamers sgc8c and sgd5a, and evaluated in this study. An anticancer drug Doxorubicin, which is used in chemotherapy of a wide range of cancers, including acute lymphoblastic and myeloblastic leukemias, malignant lymphomas, as well as breast cancer^[9], was chosen in this study. Dox binds preferentially to dsDNA between adjacent GC or CG base pairs through intercalation, and the association of Dox with DNA is reversible.^[10] Dox was loaded into the multiple intercalation sites designed in the dsDNA linker region of SD to study the bi-specific ability of SD for Dox delivery and target cell cytotoxicity. While the recognition abilities of monovalent aptamers are necessarily limited, the broader recognition capability of the bi-specific aptamer-based drug carrier, SD, allowed the cytotoxic effects of Dox to be bi-specifically directed to more types of target cells. Under these conditions, bi-specific aptamer-based drug carriers can sidestep the problem of cancer heterogeneity and, as a consequence, facilitate clinical aptamer applications in targeted therapy of many types and subtypes of cancers that respond to the same therapeutic methods.

Results and Discussion

In order to develop bi- or tri-specific aptamer-based drug carriers, we first constructed bi- and tri-specific aptamers (multi-aptamers) and studied their recognition capabilities. Engineering multi-aptamers is similar to multivalency engineering, which has been previously reported for antibodies^[11] and aptamers^[12] using chemical linkages or nanomaterials for binding affinity improvement, targeted therapy, cell-cell interaction, etc. In this study, chemical linkages were used. As an example shown in Figure. 1, two monovalent aptamers that recognize different cancer subtypes form a bi-specific aptamer via dsDNA linkage. Monovalent aptamers able to recognize different cultured cell lines were

selected as “model building blocks”: sgc8c (S) against CEM cells^[3a, 13], TDO5 (T) against Ramos cells^[14], and sgd5a (D) against Toledo cells^[5a] (sequences in Table S1). To study the generality of linkers, different linkers including a polyT linker, a PEG linker and a dsDNA linker were used to construct S-T20-T, S-PEG-T, S-hyb-T and S-hyb-D (SD), respectively. Bi-specific aptamers with either polyT or PEG linkers were designed to be synthesized on a automated DNA synthesizer, and those with dsDNA linkers were self-assembled by hybridization of two complementary sequences designed to extend from the 3' ends of monovalent aptamers. To further enhance the recognition range of drug carriers, a tri-specific aptamer, sgc8c-sgd5a-TDO5 (SDT), was developed using a Y-shaped dsDNA linker. The formation and purity of multi-aptamers linked by dsDNA were confirmed by agarose gel electrophoresis (Figure. S1).

For drug carriers to realize bi- or tri-specificity, each monovalent aptamer domain must retain its specific binding ability. To test this, an aptamer binding assay was performed with target cells of the parent aptamers. Either FITC or Cy5.5 was used to monitor aptamer binding. Using flow cytometry, specific binding abilities were confirmed at 4°C for bi-specific aptamers S-T20-T (Figure. S2A), S-hyb-T (Figure. S2B), S-PEG-T (Figure. S2C), S-hyb-D (SD) (Figure. 2A), and tri-specific aptamer S-D-T (SDT) (Figure. S3). Furthermore, the dissociation constants (K_d) to target cells were determined for S-T20-T, S-hyb-T and SD. As shown in Table S2, the binding affinities of these bi-specific aptamers to their target cells were comparable to those of the corresponding monovalent aptamers. Therefore, the binding specificity and affinity to both/all target cells were maintained in these multi-aptamers, indicating bi- or tri-specificity.

To develop drug carriers with multi-specific cytotoxicity using multi-aptamers, bi-specific aptamer SD was chosen as a model. It is well known that temperature change can result in aptamer conformation change and, hence, binding ability. Thus, the binding ability of SD was characterized and verified at physiological temperature (37°C) (Figure. S2D).

For future application of SD as a drug carrier in complicated clinical samples, the ability of SD to selectively detect both types of target cells was evaluated in cell mixtures containing non-target cells. To do so, SD binding assays were performed with cell mixtures containing a series of target CEM/Toledo cells of different concentrations into non-target NB4 cells of a fixed concentration. Because the morphology of NB4 cells is distinct from those of either CEM or Toledo cells, the populations of target and non-target cells in flow cytometric results can be easily distinguished and gated for respective fluorescence analysis (Figure. S4A). With as low as 5% target cells in a total mixture of 15,000 cells, both types of target cells were still easily detected, indicating bi-specificity of SD for sensitive cell detection in cell mixtures (Figure. S4).

Next, a quantitative analysis was performed to compare the recognition capabilities of monovalent aptamers sgc8c and sgd5a with that of SD in cell mixtures. Again, 50,000 CEM and/or 50,000 Toledo cells were spiked into 200,000 NB4 cells. Using a binding assay, these cell mixtures were tested with sgc8c, sgd5a and SD, respectively. Similarly as described above, flow cytometric data were analyzed to determine the percentage of cells with fluorescence signal enhancement resulting from aptamer binding. As shown in Figure. 2B, 15.35% and 14.4% cells tested with sgc8c and sgd5a, respectively, showed signal intensities located in M1 (marker for enhanced fluorescence signal range), while the percentage for SD was 26.89%, approximately twice that of either sgc8c or sgd5a alone. Overall, the results showed that SD had broader recognition capability to target cells than either sgc8c or sgd5a parent aptamers alone in the same cell mixtures. The broader recognition capability of SD is, in turn, expected to enhance the recognition range of SD-based drug carrier in targeted drug delivery, thus overcoming the problem of cancer heterogeneity discussed above.

Another key concern integral to intracellular drug delivery is the ability of drug carriers to be internalized. Regarding aptamer-based drug carriers, some aptamers^[15] have already been reported to be specifically internalized into target cells. In our case, it is critical for SD to be internalized into both target cells for successful drug delivery. Therefore, the internalization capability of TAMRA-labeled SD was evaluated through confocal microscopy, and, as shown by the images in Figure. S5, SD was successfully internalized into both CEM and Toledo cells.

To develop a self-assembled drug carrier using SD for bi-specific drug delivery and target cell cytotoxicity, Doxorubicin (Dox) was used in this study. Dox is one of the most utilized chemotherapeutic drugs for a wide spectrum of cancers^[16]. However, lack of specificity leads to many side effects, such as myelosuppression and mucositis^[17]. Interestingly, many anthracycline drugs, including Dox and daunorubicin, can preferentially intercalate into tandem GC or CG sites in dsDNA, resulting in the quenching of Dox fluorescence^[10, 18]. Accordingly, SD was designed with a dsDNA linker having 10 Dox intercalation sites (Table S1). As such, a drug carrier based on SD could be self-assembled through hybridization of the two complementary sequences modified on each aptamer, and the Dox intercalation sites would be simultaneously formed in the dsDNA linker, resulting in further self-assembly of SD-Dox.

The loading of Dox into drug carrier SD was studied using fluorescence spectrometry. As shown in Figure. 3A, Dox fluorescence signal was gradually quenched with increasing fractions of SD. At an SD/Dox molar ratio of 0.1, Dox fluorescence was significantly quenched. To assure the least amount of free Dox in solution, an SD/Dox molar ratio of 0.12 was used in cytotoxicity studies. By fluorescence titration, the overall dissociation constant (K_d) was determined to be 41 ± 5.5 nM (Figure. 3A). By studying Dox intercalation with different SD components (sgc8, sgd5a and dsDNA linker only), we confirmed that the Dox intercalation sites were mostly localized in the linker (Figure. S6B). The loading of Dox into carrier was further confirmed by the enhancement of Dox fluorescence anisotropy with increasing SD fraction (Figure. 3B).

As a consequence of the rapid kinetics of DNA hybridization and Dox intercalation, the self-assembly of SD-Dox complexes is also fairly rapid, and intercalation equilibrium is achieved in less than 10 seconds (Figure. S6A). Moreover, the slow Dox release from SD-Dox in buffer indicated good stability for at least 5 days under our experiment condition (Figure. 3C).

Next, the bi-specificity of the complex SD-Dox was confirmed using flow cytometry, as indicated by the selective binding abilities to target CEM and Toledo cells, but not to NB4 cells (Figure. 4A). Furthermore, the ability of SD-Dox to selectively deliver Dox to target cells was studied by confocal microscopy. In this experiment, cells were incubated with 0.5 μ M free Dox or SD-Dox with the equivalent Dox concentration. After 2-h incubation, cells were washed and observed for Dox fluorescence intensity by confocal microscopy. The uptake of Dox by cells treated with SD-Dox is presumably through two pathways: 1), binding of SD-Dox on target cell surfaces and then internalization of the entire SD-Dox before gradual Dox release inside cells; or 2), uptake of free Dox that diffuses from the highly concentrated SD-Dox on target cell surfaces. As shown in Figure. 4B, the Dox signal intensities in CEM and Toledo cells treated with SD-Dox were more comparable to those of the corresponding cells treated with free Dox, than those of non-target NB4 cells. The slightly lower Dox fluorescence intensities in target cells treated with SD-Dox than those treated with free Dox could be explained by 1), The efficiency of Dox transportation via a macromolecular drug carrier (SD) is lower than uptake of free Dox into cells; 2), The Dox that were not released from SD-Dox yet were still quenched, resulting in lower fluorescence.

Overall, these results indicated that this aptamer-based drug carrier SD bi-specifically delivered Dox into target cells.

Finally, MTS assays were performed to study the specific cytotoxicity of Dox delivery by SD. First, the cytotoxicity of free Dox was investigated for CEM, Toledo and NB4 cells. CEM was reported to show dose-dependent response to free Dox^[19], as did Toledo and NB4 (Figure. S7). Then, the cytotoxicity of SD-Dox to these cells was studied. Because the IC₅₀s of Dox to these three cell types were not exactly the same, they were treated with Dox or SD-Dox at the respective Dox concentrations which could cause about 30% cell viability (CEM: 0.5 μ M, Toledo: 0.7 μ M, NB4: 0.35 μ M). The resultant cell viabilities of each type of cells were then normalized, with 30% cell viability of the corresponding cells treated with free Dox. While free Dox induced 30% normalized cell viabilities, SD-Dox with the equivalent Dox concentrations induced approximately 50%, 50% and 75% normalized cell viability in CEM, Toledo, and NB4 cells, respectively (Figure. 5A). This indicated that Dox delivered by SD induced higher cytotoxicity in both target CEM and Toledo cells than in non-target NB4 cells. However, neither SD alone nor dsDNA linker-Dox showed significant cytotoxicity. We reason that the greater cell viabilities of CEM and Toledo cells treated with SD-Dox than those treated with free Dox were caused by less efficient Dox uptake via SD-Dox in the limited treatment time (2h), which is consistent with the Dox delivery efficiency discussed above.

Furthermore, to mimic clinical situation where target cells were in cell mixtures containing miscellaneous non-target cells, the cytotoxicity specificity of Dox delivered by SD was further examined in cell mixtures containing 50,000 CEM, 50,000 Toledo and 100,000 NB4 cells. Cell mixtures were treated with S-Dox, D-Dox and SD-Dox, respectively, with 0.5 μ M Dox. The treated cell mixtures were stained with Propidium iodide (PI), which accumulated inside dead cells and could be analyzed by flow cytometry. As discussed before, the cell morphologies of CEM/Toledo cells can be easily distinguished from that of NB4 cells by flow cytometry. Again, target cell populations were gated for PI fluorescence intensity analysis. The results indicated that SD-Dox induced approximately the sum of target cell deaths induced by S-Dox and D-Dox, respectively (Figure. 5B).

Overall, these results clearly demonstrated the bi-specific cytotoxicity of Dox delivered by SD, thus broadening the range of targeted cytotoxicity in aptamer-based drug delivery. Previous studies showed that monovalent aptamer-directed drugs induced cytotoxicities only in the corresponding target cells^[19–20]. However, because of the broadened recognition capability of SD, Dox delivered by SD could induce bi-specific cytotoxicities in the cells targeted by both aptamers sgc8c and sgd5a.

Conclusion

In conclusion, we report an facilely self-assembled aptamer-based drug carrier for bi-specificity cytotoxicity. The bi-specificity resulted from the ability of the drug carrier SD to bi-specifically recognize target cells. In particular, SD was able to specifically bind and detect both target cells (CEM and Toledo), but not non-target cells (NB4), in cell mixtures. SD was also shown quantitatively to possess broader recognition capability to target cancer cells than its parent monovalent aptamers. This bivalent solution to the problem of heterogeneity in cancer subtypes is expected to overcome many diagnostic and therapeutic complications. As such, SD was then utilized to deliver Dox, an anticancer drug widely used for cancer chemotherapy, in order for bi-specific cytotoxicity. Through aptamer engineering, this carrier, SD, was designed for easy self-assembly and simultaneously forming multiple Dox intercalation sites on the dsDNA linker. The further drug loading and self-assembly of SD-Dox is rapid, easy to characterize via Dox fluorescence change, and the SD-Dox

complex showed good stability for at least 5 days. The multiple Dox intercalation sites on each SD enabled high drug loading capacity. Furthermore, the Dox-loaded SD, SD-Dox, maintained bi-specific abilities in target cell binding and targeted Dox delivery. Most importantly, Dox delivered by SD induced bi-specific cytotoxicity in both separate and mixed cancer cell solutions, indicating a broad recognition range of targeted therapy. While the recognition abilities of monovalent aptamers are necessarily limited, the broader recognition capability of bi-specific aptamer-based drug carriers allowed drug cytotoxicity to be specifically directed to more subtypes of cancer cells. Under these conditions, bi-specific aptamer-based drug carriers can sidestep the problem of cancer heterogeneity altogether and, as a consequence, facilitate clinical aptamer applications in targeted therapy of many subtypes of cancers that respond to the same therapeutic methods.

Experimental Section

General

Washing buffer contained 4.5 g/L glucose and 5 mM MgCl₂ in Dulbecco's PBS (Sigma Aldrich). Binding buffer was prepared by adding yeast tRNA (0.1 mg/mL) (Sigma Aldrich) and BSA (1 mg/mL) (Fisher Scientific) into the washing buffer to reduce background binding. Doxorubicin hydrochloride (Dox) was purchased from Fisher Scientific (Houston, TX).

Preparation of DNA

All DNA synthesis reagents were purchased from Glen Research, and all DNA probes (Table S1; italicized sequences represent complementary sequence for duplex formation) were synthesized on an ABI3400 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA, USA). Coupled on the 5' end of these DNA probes was Fluorescein (FITC), Biotin, or Cy5, unless otherwise noted. The completed sequences were then deprotected in AMA (ammonium hydroxide/40% aqueous methylamine 1:1) at 65°C for 30 min and further purified by reversed-phase HPLC (ProStar, Varian, Walnut Creek, CA, USA) on a C-18 column using 0.1 M triethylamine acetate (TEAA Glen Research Corp.) and acetonitrile (Sigma Aldrich, St. Louis, MO) as the eluent. The collected DNA products were dried, and detritylation was performed by dissolving and incubating DNA products in 200 µL 80% acetic acid for 20 minutes. The detritylated DNA product was precipitated with NaCl (3 M, 25 µL) and ethanol (600 µL). UV-Vis measurements were performed with a Cary Bio-100 UV/Vis spectrometer (Varian) for probe quantification.

Cell culture

Cell lines CCRF-CEM (Human T-cell ALL), Ramos (human B-cell Burkitt's lymphoma) and Toledo (CRL-2631, B lymphocyte, human diffuse large cell lymphoma) were obtained from the American Type Culture Collection (Manassas, VA). NB-4 (acute promyelocytic leukemia) was obtained from the School of Medicine, Department of Pathology, at the University of Florida. The cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (heat-inactivated, GIBCO) and 100 IU/mL penicillin-streptomycin (Cellgro) at 37 °C in a humid atmosphere with 5% CO₂. The cell density was determined prior to each experiment using a hemocytometer.

Aptamer binding assay

The binding abilities of aptamers were determined by incubating dye-labeled aptamers (400 nM, unless otherwise noted) with cells (2×10^5) on ice for 30 min, followed by washing twice with washing buffer (1 mL) and suspending in binding buffer (200 µL), before flow cytometric analysis. Random sequences (lib) were used as a negative control. The

fluorescence intensities of cells were determined with a FACScan cytometer (BD Immunocytometry Systems). Data were analyzed with WinMDI software.

Binding affinities of aptamers were determined using a series of aptamer concentrations. As negative controls, similar assays were performed using random sequences at the same corresponding concentrations. The increased mean fluorescence intensities of cells bound by dye-labeled aptamers compared with those of random sequences were used to calculate the equilibrium dissociation constant (K_d) by fitting the dependence of fluorescence intensity (F) on aptamer concentration (L) to the equation $F = B_{\max}[L]/(K_d + [L])$ ^[19], where $B_{\max}$ represents binding capacity and reflects the density of binding sites. $B_{\max}/2$ was then used as the binding constant. The binding assay was repeated at least three times.

Detection of target cancer cells in cell mixtures using SD

A series of different concentrations of CEM or Toledo cells was spiked into a fixed concentration of NB4 cells in binding buffer. Cell mixtures were then used for binding assay (as described above). The populations of CEM/Toledo cells were gated based on their distinct sizes and applied to fluorescence intensity analysis.

A similar assay was used to quantitatively compare the recognition capabilities of monovalent aptamers and SD. Specifically, 50,000 CEM/Toledo cells were spiked into 200,000 NB4 cells in binding buffer. Aptamers sgc8c, sgd5a, SD and random sequences were used for the binding assay to determine the percentage of cells that showed fluorescence signal enhancement.

Self-assembly and characterization of SD-Dox

Aptamer-based carrier-drug complex, SD-Dox, was formed by mixing Dox (Fisher Scientific, Houston, TX) and SD. The formation of SD-Dox was monitored (Ex: 480 nm, Em: 590 nm) on a Fluorolog-Tau-3 spectrofluorometer (Jobin Yvon, Edison, NJ). The titration experiment by fluorescence spectrometry was performed by sequential addition of increasing fractions of SD to Dox (1 μ M) in PBS (Sigma). The fluorescence of the resultant solution was recorded to monitor Dox intercalation efficiency. The dissociation constant (K_d) was determined by fitting the recorded fluorescence intensities to $F = B_{\max}[L]/(K_d + [L])$ ^[19], as explained above. Similarly, fluorescence anisotropy was studied with Dox (10 μ M) and increasing SD fraction (Ex: 480 nm, Em: 590 nm).

Dox release study

Free Dox (20 μ M, 200 μ L) and SD-Dox complexes (20 μ M Dox, [SD]: [Dox]=1.2:10, 200 μ L) were prepared and transferred into MINI Dialysis Units (3.5 MWKO, Thermo Scientific, MA). The unit bottoms were immersed in 3 mL PBS buffer in an individual well of a 6-well plate, with a magnetic rod in each well. The plate was placed on a magnetic stirrer (150 rpm). At the indicated time points, a 120 μ L aliquot from each well was collected for Dox fluorescence measurement and then returned to the corresponding well.

Internalization of aptamers and uptake of Dox

All cellular fluorescent images were collected on the FV500-IX81 confocal microscope (Olympus America Inc., Melville, NY) with a 60 $\times$ oil immersion objective (NA=1.40, Olympus, Melville, NY). Excitation wavelength and emission filters: TAMRA, 543 nm laser line excitation, BP 580 $\pm$ 20 nm filter; Dox: 488 nm laser line excitation, emission BP 580 $\pm$ 20 nm filter. Cells (2×10^5 in 200 μ L) were incubated at 37 $^{\circ}$ C with aptamers, Dox, or SD-Dox assembly for 2 h, followed by washing with washing buffer (1 mL) twice at 4 $^{\circ}$ C and suspending in binding buffer (200 μ L) before imaging. Each experiment was repeated three times and analyzed with Fluoview software.

Cytotoxicity assay

The cytotoxicity for each individual type of cells was determined using CellTiter 96 cell proliferation assay (Promega, Madison, WI, USA). Cells (5×10^4 cells/well) were treated with SD, Dox, linker-Dox or SD-Dox in medium without FBS (37°C, 5% CO₂). After incubation for 2 h, cells were precipitated by centrifugation, 80% supernatant medium was removed, and fresh medium (10% FBS, 200 µL) were added for further cell growth (48 h) before removing cell medium. CellTiter reagent (20 µL) diluted in fresh medium (10% FBS, 100 µL) was added to each well and incubated for 1–2 h. The absorbance (490 nm) was recorded using a plate reader (Tecan Safire microplate reader, AG, Switzerland). Cell viability was determined as described by the manufacturer.

The cytotoxicity of aptamer-drug complexes to mixed cells was determined using PI (Invitrogen, Carlsbad, CA). Cell mixtures (50,000 CEM or Toledo cells, 100,000 NB4 cells, in 500 µL FBS-free medium) were treated with aptamer-drug complexes for 1.5 h, before replacing with fresh medium (10% FBS, 500 µL) for further cell growth (24 h). Cells were stained with 1 µg/mL PI at room temperature for 20 min to test target cell death by flow cytometry. Target cell populations were gated in flow cytometric results to analyze PI fluorescence intensity. Cell death percentage was defined as the ratio of dead cell amount (in the range of M1 in Fig. 5B) to total cell amount.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Scheme for a self-assembly of aptamer-based drug carrier SD for bi-specific cytotoxicity. Bi-specific aptamer-based drug carrier, SD, was self-assembled from molecularly-engineered aptamers S and D, simultaneously forming Dox intercalation sites in the dsDNA linker. Subsequently, Dox was loaded into SD through site-specific Dox intercalation, bi-specifically delivered into target cells in cell mixtures, and induce bi-specific cytotoxicity.

Figure 2.

Bi-specific binding ability of drug carrier SD. (A), SD maintained selective binding abilities to target CEM/Toledo cells, but not to non-target NB4 cells. (B), flow cytometric analysis of CEM and Toledo cells in cell mixtures indicated that SD had broader recognition capabilities than sgc8c or sgd5a alone. Cell mixtures contained 50,000 CEM, 50,000 Toledo and 200,000 NB4 cells. (M1: Marker of CEM/Toledo cells with enhanced fluorescence intensities.)

Figure 3.

Characterization of Dox loading into drug carrier SD. (A), The quenching of Dox fluorescence indicated loading of Dox into SD (also shown by the fluorescent image). K_d was determined from $F_{590\text{nm}}$. (B), The enhancement of fluorescence anisotropy with increasing SD fraction also indicated the association of Dox and SD. (C), Compared with free Dox, the slow Dox release from SD-Dox suggested its good stability for up to 5 days.

Figure 4.

Bi-specific binding and Dox-delivery abilities of drug carrier SD. (A), SD-Dox complex maintained bi-specific binding ability to target CEM/Toledo cells, but not to non-target NB4 cells. (B), Confocal microscopy study showed Dox was bi-specifically delivered by SD and released into CEM/Toledo cells, as indicated by the more comparability of Dox fluorescence intensities in Dox- and SD-Dox-treated CEM/Toledo cells, than that of NB4 cells. (Scale bar: 50 μ m).

Figure 5.

Bi-specific cytotoxicity of Dox delivered by SD. (A), MTS assay results showed that SD-Dox complex induced higher normalized cytotoxicity in CEM/Toledo cells than in NB4 cells, indicating bi-specific cytotoxicity in individual cells. (B), In cell mixtures, SD-Dox also showed bi-specific cytotoxicity compared with S-Dox or D-Dox, by PI staining. (M1: dead target cells with enhanced PI fluorescence intensity) Target cell death percentages (percentage of target cells within M1 in total mixed cells) were calculated from the flow cytometric results above.