Targeted delivery of doxorubicin to breast cancer cells by aptamer functionalized DOTAP/DOPE liposomes

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Abstract. Doxorubicin is used to treat numerous types of tumors including breast cancer, yet dose-associated toxicities limit its clinical application. Here, we demonstrated a novel strategy by which to deliver doxorubicin to breast cancer cells by conjugating cancer cell-specific single-strand DNA aptamers with doxorubicin-encapsulated DOTAP:DOPE nanoparticles (NPs). We utilizing a whole-cell-SELEX strategy, and 4T1 cells with high invasive and metastatic potential were used as target cells, while non-invasive and non-metastatic 67NR cells were used as subtractive cells. Ten potential aptamers were generated after multi-pool selection. Studies on the selected aptamers revealed that SRZ1 had the highest and specific binding affinity to 4T1 cells. Then we developed SRZ1 aptamer-carried DOTAP:DOPE-DOX NPs. *In vitro* uptake results which were conducted by FACS indicated that the aptamer significantly promoted the uptake efficiency of DOTAP:DOPE-DOX NPs by 4T1 cells. ATPlite assay was performed to test 4T1, 67NR and NMuMG cell viability after treatment with free doxorubicin, DOTAP:DOPE-DOX particles and aptamer-loaded DOTAP:DOPE-DOX particles. As expected, the aptamers effectively enhanced accumulation of doxorubicin in the 4T1 tumor tissues as determined by *in vivo* mouse body images and biodistribution analysis. Consistent with the *in vitro* findings, aptamer-conjugated doxorubicin-loaded DOTAP:DOPE particles markedly suppressed tumor growth and significantly increased the survival rate of 4T1 tumor-bearing mice. These studies demonstrated that aptamer SRZ1 could be a promising molecule for chemotherapeutic drug targeting deliver.

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Introduction

Breast cancer is the most common and the leading cause of death from all cancers among women in China (1). Mortality from breast cancer has been rising in recent years. Chemotherapy is still the preferred strategy applied for breast cancer along with surgery and radiotherapy. However, the non-targeted delivery of chemotherapeutic drugs greatly affects normal cells (2), and to a great extent limits the drug dosage to tumor cells altogether compromising the therapeutic effects of the drugs.

Nanoparticles (NPs) are increasingly considered as a powerful tool for chemotherapeutic drug delivery (3-5). Targeted drug delivery using NPs functionalized with targeting ligands is one of the most promising means which augments the selectivity and drug dosage to tumor cells leading to an enhanced therapeutic index of drugs. There are many types of NPs for drug delivery, such as polymeric micelles (6,7), liposomes (8-10), and lipoprotein-based carriers (11,12). The most common vehicle currently used for targeted drug delivery is the liposome. They are biocompatible and biodegradable and can be designed to avoid clearance.

Systematic evolution of ligands by exponential enrichment (SELEX) is an efficient strategy for high throughput *in vitro* selection of bio-library pool (13,14). In recent years, a modified SELEX process using whole living cells as targets was developed and designated as cell-SELEX (15,16), which can select aptamers from unknown complex targets.

In the present study, we aimed to achieve targeted delivery of doxorubicin to 4T1 tumors by conjugating 4T1 tumor cell-specific aptamers which were selected using 4T1 cells (positive selection) and 67NR cells (negative selection). The aptamers with the highest binding affinity and specificity as detected by flow cytometry were carried by doxorubicin-loaded DOTAP:DOPE NPs. Various *in vitro* and *in vivo* studies conducted to date have demonstrated that the AS1411 aptamer enhances the uptake of NPs in 4T1 tumor cells. Hence, these anti-4T1 cell aptamers can be effectively used to target NPs to 4T1 cancer cells.

Materials and methods

Cell culture. Mouse mammary breast cancer 4T1, 4TO7 and 67NR cells were purchased from the American Type Culture Collection (ATCC) and maintained in our laboratory. Normal mouse mammary gland NMuMG cells were purchased from ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10 μg/ml of insulin and 10% fetal bovine serum (FBS). Mouse Lewis lung carcinoma cell line LL/2, mouse hepatoma cell line Hepa 1-6 and mouse B cell lymphoma cell line WEHI-231 were purchased from ATCC and cultured in DMEM with 10% FBS. Mouse colon cancer CT26 cells were cultured in RPMI-1640 medium with 10% FBS.

Random single-strand DNA library and primers. A 60-mer ssDNA library containing molecules with a 30-base random sequence flanked by two primer hybridization sites was chosen as the initial library (5'-CCT GAC AGT CGA GAC-N30-CAC CGG GGT CCT AGG-3'). The FAM-labeled forward primer (5'-FAM-CCT GAC AGT CGA GAC-3'), and biotin-labeled reverse primer (5'-biotin-CCT AGG ACC CCG GTG-3') were used in PCR to obtain the double-labeled DNA and to separate the single-stranded DNA by streptavidin-coated magnetic particles. All sequences were synthesized by Sangon and purified by reverse phase HPLC.

Cell-SELEX. The 4T1 cells with high invasive and metastatic potential were used as target cells, and non-invasive and non-metastatic 67NR cells were used as the negative selection cells in the present study. ssDNA (10 nmol) library was denatured at 100˚C for 5 min and kept on ice for 10 min and dissolved in 500 μl phosphate-buffered saline (PBS) buffer with 1 M $MgCl_2$, 0.1 mg/ml yeast tRNA, 1 mg/ml BSA and 0.1 mg/ml salmon sperm DNA. The 4T1 cells ($5x10^6$) were washed, dissociated and incubated with the ssDNA library on ice for 1 h. After 3 washings, cells were re-suspended in 200 μl DNase-free water. The cell-ssDNA mixture was heated at 100˚C for 5 min, centrifuged at 4,000 rpm for 5 min, and the supernatant containing eluted ssDNA was collected and ssDNA was amplified by PCR using FAM- and biotin-labeled primers. Both positive and negative selections were performed after 5 rounds of selection. The ssDNA pools were initially incubated with 67NR cells, and the supernatant containing unbound ssDNA was collected and incubated with 4T1 cells.

Determination of binding efficiency. To determine the binding efficiency of the selected ssDNA pools with target cells, FAM-labeled ssDNA pools were respectively incubated with $1x10^6$ of the 4T1 target cells or subtractive 67NR cells in 500 μl binding buffer at 4˚C for 30 min. Cells were washed 3 times at 1,000 rpm for 5 min, and the fluorescence intensity was determined by flow cytometry.

Cloning and sequencing of the selected ssDNA pool. Based on the binding efficiency results, the 10th round of enriched ssDNA pool was amplified using unmodified primers and cloned into *Escherichia coli* using the TA cloning kit (Invitrogen). The candidate aptamer sequences were determined by Invitrogen Co., Ltd. (Shanghai, China). Nine aptamers were selected, and the binding affinity was detected by amplifying FAM-labeled ssDNA aptamers using FAM-conjugated primers. In brief, the target cells ($1x10^6$) were incubated with varying concentrations of FITC-labeled aptamers in 500 μl binding buffer on ice for 30 min. Cells were washed twice after incubation, and the fluorescence intensity was determined by flow cytometry. Three aptamers with high binding affinity were sequenced, and the structure of aptamer SRZ1 with best binding affinity was predicted by RNA structure software.

Binding specificity. To study the binding specificity, FAM-conjugated SRZ1 was synthesized by Sangon, Shanghai. To determine the cell specificity of SRZ1, mouse mammary breast cancer 4T1, 4TO7 and 67NR cells, normal mouse mammary gland NMuMG cells, mouse Lewis lung carcinoma cell line LL/2, mouse hepatoma cell line Hepa 1-6 and mouse B cell lymphoma cell line WEHI-231 were used in binding assays by flow cytometry.

Preparation of NPs. The particles employed for the present study were DOTAP:DOPE (molar ratio 50:50) based liposome preparations. DIR or PKH26 dye was added into the liposome formulations at a ratio of 5 mol% if fluorescence indicators were needed. Liposomes were prepared by thin film and hydration method as reported. Briefly, a rotary evaporator was used to remove solvent from a glass tube containing lipid mixed at the appropriate ratios in a water bath at 42˚C until a thin film was deposited. The lipid film was hydrated with ddH_2O, ddH_2O containing doxorubicin or ddH_2O containing both doxorubicin and aptamers in a water bath at 37˚C, and then placed on ice at 15-min intervals for 10 cycles.

Characterization of the NPs. Particle size and ζ-potential of the DOTAP:DOPE liposomes, doxorubicin-loaded DOTAP:DOPE liposomes (DOTAP:DOPE/DOX) or doxorubicin and aptamer-carried DOTAP:DOPE liposomes (DOTAP:DOPE/DOX/aptamer) were determined by Zetasizer.

Uptake of particles. For the uptake study, doxorubicin-loaded DOTAP:DOPE liposomes (DOTAP:DOPE/DOX) or doxorubicin and aptamer-carried DOTAP:DOPE liposomes (DOTAP:DOPE/DOX/aptamer) were respectively incubated with 4T1 cells at 37˚C for 12 h, and then the cells were washed 3 times, and the uptake efficiency was detected by flow cytometry.

Cell viability assay. *In vitro* cytotoxicities of DOTAP:DOPE, free doxorubicin, DOTAP:DOPE/DOX and DOTAP:DOPE/DOX/SRZ1 were analyzed in the 4T1, 67NR and NMuMG cells using the CCK-8 method. Briefly, the cells (100 μl) were cultured in a 96-well culture plate at a density of 10^4/well and were subsequently incubated for 24 h. Then the samples were added to each group (3-wells) for 24 h. The cells were incubated in 110 μl of DMEM containing 10 μl CCK-8 solution for 1 h after removing previous nutrient solution. Absorbance of the suspension was measured at 450 nm on an ELISA reader.

In vitro DOX release. DOTAP:DOPE/DOX, DOTAP:DOPE/DOX/SRZ1 and free doxorubicin (500 μg) were suspended in 2 ml of PBS buffer with a pH of 7.4. The suspensions were placed at 37˚C with constant stirring at 100 rpm. At a desired

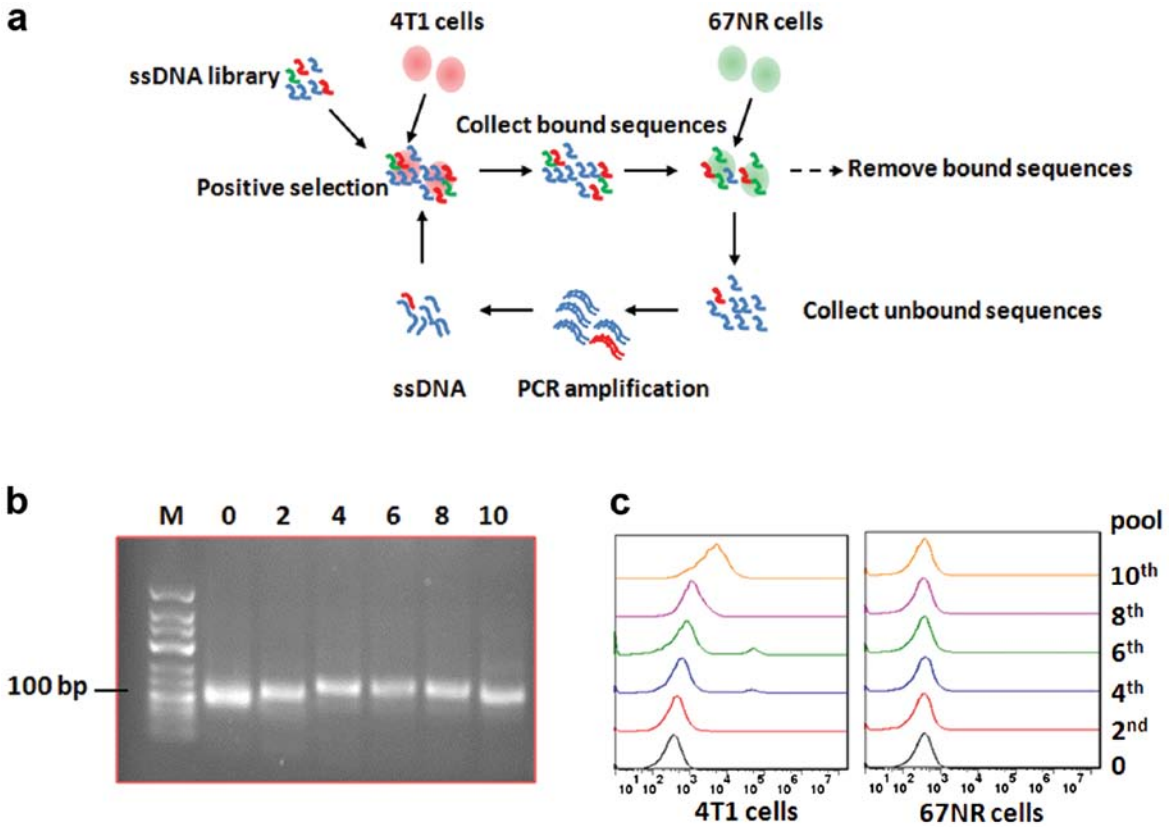


Figure 1. Enrichment of mouse breast cancer 4T1 cell-specific aptamers through subtractive cell-SELEX. (a) Schematic of the selection process of 4T1 cell-specific aptamers using cell-SELEX. The 4T1 cells were used as target cells and 67NR cells were used as negative screen cells. (b) After 10 rounds of enrichment, single-strand DNA bound to 4T1 cells was eluted and amplified to produce double-strand DNA for gel analysis. (c) Flow cytometric assay was used to test the affinity of the selected pools with 4T1 (target cells) and 67NR (subtractive cells).

time point, 1 ml of suspension was removed and centrifuged. The supernatant was collected for UV-vis measurement at a wavelength of 480 nm to determine the release of doxorubicin.

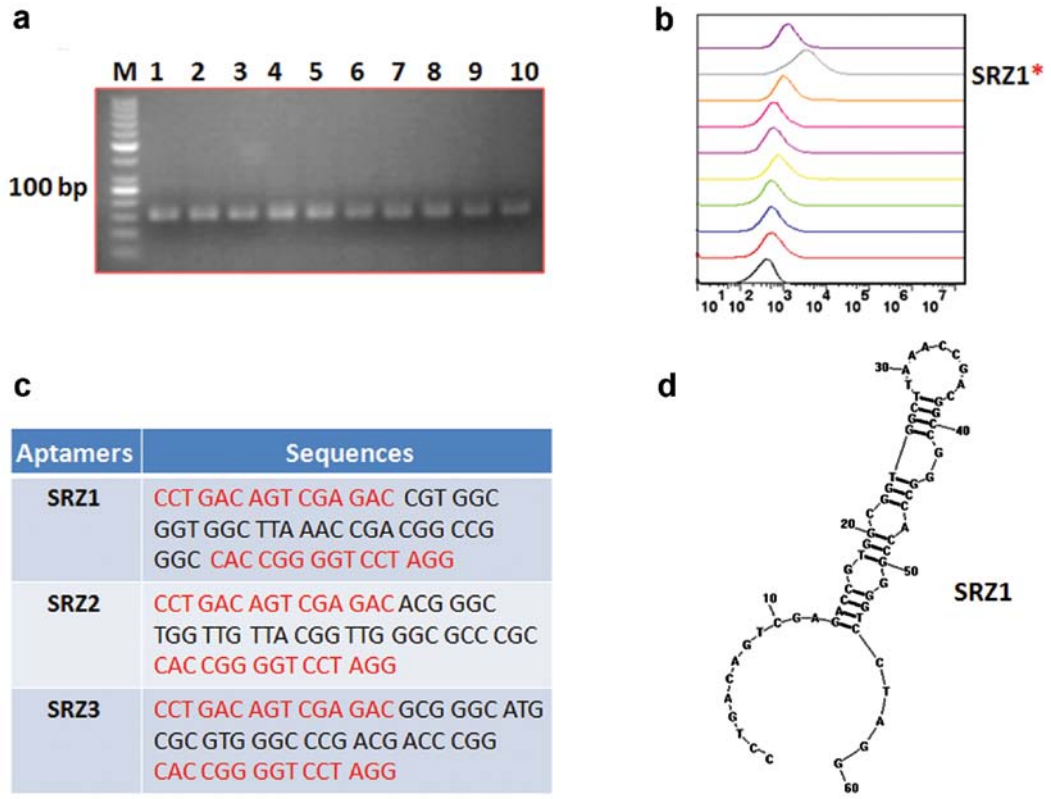
Animals. Female BALB/c mice, 6-8 weeks of age, purchased from the Laboratory Animal Center of Nanjing Medical University were employed in this study. The present study complied with standards for the Care and Use of Laboratory Animals and was approved by the Animal Care and Use Committee of Nanjing Medical University. All of the mice were monitored every day. All surgery was performed under isoflurane anesthesia. All efforts were made to minimize suffering, and all of the mice were finally sacrificed by CO_2 asphyxiation.

In vivo image and biodistribution. To study the *in vivo* targets of DOTAP:DOPE/DOX/aptamer in 4T1 tumor-bearing mice, DIR dye-labeled DOTAP:DOPE/DOX/aptamer liposomes were prepared and intravenously injected into the mice. Twenty-four hours following the injection, mice were anesthetized and mouse bodies were scanned using Kodak image system. The 4T1 tumors, livers, lungs, spleens, kidneys, thymus, heart and gut were removed 48 h following the injection, and the DIR dye signals in each organ were collected using the Kodak image system.

Tumor model. Female BALB/c mice (4 weeks, 18-20 g, 10 mice/group) were used. The 4T1 cells were harvested and resuspended in PBS to a final density of $1x10^7$ cells/ml. Before injection, cells were resuspended in PBS and analyzed by 0.4% trypan blue exclusion assay (viable cells, >90%). For cancer cell injection, ~$5x10^5$ 4T1 cells in 100 μl of PBS were injected into the mammary fat pad of each mouse. Treatment was initiated when the primary tumors reached a mean diameter of 3-4 mm. Tumor-bearing mice were divided into 5 groups and treated with PBS, DOTAP:DOPE, free DOX, DOTAP:DOPE/DOX and DOTAP:DOPE/DOX/SRZ1 every 3 days for 10 times. Tumor volume was measured, and the survival rate of the mice was monitored.

Results

Selection and binding affinity identification of the aptamer pools. Cell-SELEX strategy was applied in the present study to enrich specific anti-4T1 cell aptamers. As shown in the schematic (Fig. 1a), 4T1 cells with high invasive and metastatic potential were used as target cells, while non-invasive and non-metastatic 67NR cells were used as subtracted cells. After 10 rounds of selection, 10 ssDNA pools were enriched, and the original ssDNA pool (0), 2nd, 4th, 6th, 8th and 10th pools were identified by running agarose gel electrophoresis (Fig. 1b). Then the binding affinity of the aptamer pools and 4T1 cells was analyzed by flow cytometry. With increasing rounds of selection, the fluorescence intensity bound on 4T1 cells was significantly increased (Fig. 1c, left), while there was no change in the fluorescence intensity of the 67NR cells (Fig. 1c, right).



Aptamers	Sequences
SRZ1	CCT GAC AGT CGA GAC CGT GGC GGT GGC TTA AAC CGA CGG CCG GGC CAC CGG GGT CCT AGG
SRZ2	CCT GAC AGT CGA GAC ACG GGC TGG TTG TTA CGG TTG GGC GCC CGC CAC CGG GGT CCT AGG
SRZ3	CCT GAC AGT CGA GAC GCG GGC ATG CGC GTG GGC CCG ACG ACC CGG CAC CGG GGT CCT AGG

Figure 2. ssDNA aptamer clones, binding affinity, sequence and secondary structure prediction of the SRZ1 aptamer. (a) Single-strand DNA aptamers were obtained by asymmetric PCR and 10 aptamers were analyzed through 3.8% agrose gel. (b) Binding affinity of 10 aptamers with 4T1 cells was tested by flow cytometry and we named the best affinity aptamer SRZ1. (c) Sequence of the SRZ1 aptamers. (d) Secondary structure prediction of the SRZ1 aptamer.

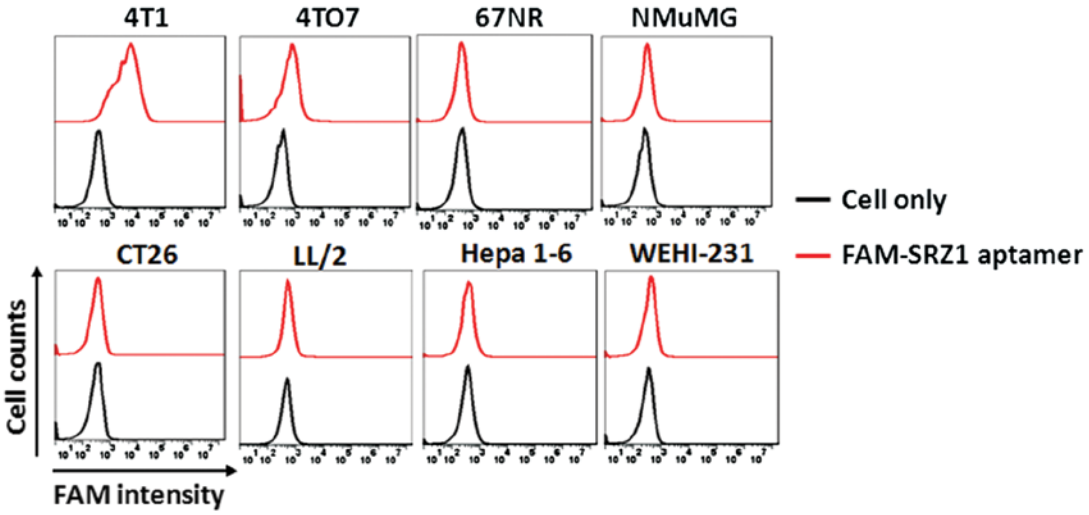


Figure 3. Binding specificity of the SRZ1 aptamer. Flow cytometry was performed to study the specific binding between the SRZ1 aptamer and the different cell lines. Mouse breast cancer cell lines 4T1, 4TO7, 67NR, the mouse normal mammary gland NMuMG cell line, the mouse colon cancer CT26 cell line, the mouse Lewis lung carcinoma cell line LL/2, the mouse hepatoma cell line Hepa 1-6 and the mouse B cell lymphoma cell line WEHI-231 were used in the present study.

Selection, sequencing and structure prediction of the 4T1-specific aptamers. After 10 rounds of enrichment, the selected ssDNA library was PCR-amplified and cloned into *Escherichia coli*. Clones were subjected to sequence analysis, and definite results were obtained from 51 clones, including 12 clones yielding the same sequence named SRZ1, 11 clones yielding the same sequence named SRZ2, 8 clones yielding the same sequence named SRZ3, 6 clones yielding the same sequence named SRZ4, 5 clones yielding the same sequence named SRZ5, 4 clones yielding the same sequence named SRZ6, 3 clones yielding the same sequence named SRZ7, another 2 clones named SRZ8 and SRZ9. ssDNA was obtained by asymmetry PCR from plasmid DNA and identified using agarose gel electrophoresis (Fig. 2a). FAM-labeled SRZ1-9

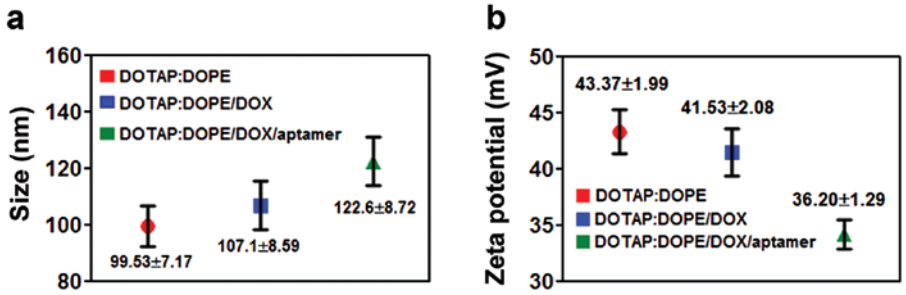


Figure 4. Characterization of the liposomes. Free DOTAP:DOPE, doxorubicin-loaded DOTAP:DOPE (DOTAP:DOPE/DOX) and doxorubicin-loaded SRZ1-conjugated DOTAP:DOPE (DOTAP:DOPE/DOX/aptamer) liposomes were prepared. (a) Size distribution and (b) ζ-potential were characterized using Zetasizer.

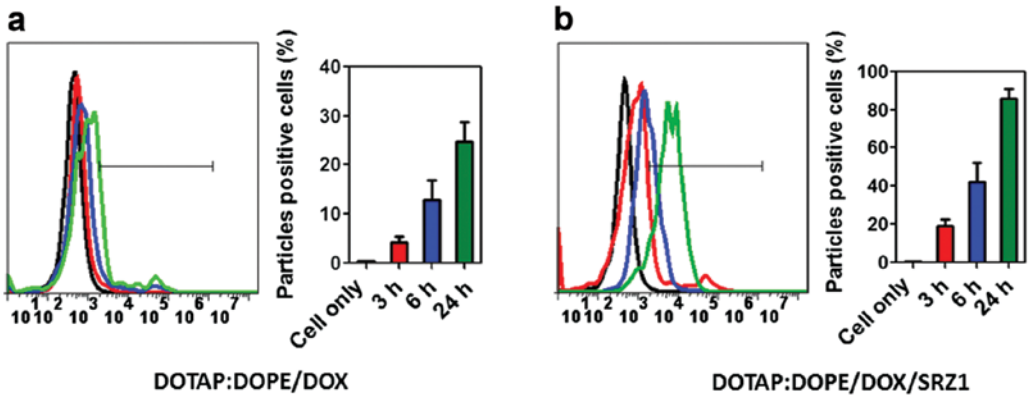


Figure 5. Uptake of DOTAP:DOPE/DOX and DOTAP:DOPE/DOX/SRZ1 by 4T1 cells. Uptake efficiency of DOTAP:DOPE/DOX was detected in 4T1 target cells (a) without or (b) with aptamer SRZ1 conjugation.

aptamers were then synthesized, and the binding affinity was evaluated. Data from the flow cytometry indicated that SRZ1 had the best binding affinity to 4T1 cells (Fig. 2b). The sequences (Fig. 2c) were analyzed, and the structure (Fig. 2d) was predicted by RNA structure software. SRZ1 was selected for detailed study.

SRZ1 specifically binds to 4T1 cells. Mouse mammary breast cancer cell lines (4T1, 4TO7 and 67NR), the mouse normal mammary gland cell line NMuMG, the human colon carcinoma cell line CT26, the mouse Lewis lung carcinoma cell line LL/2, the mouse hepatoma cell line Hepa 1-6 and the mouse B cell lymphoma cell line WEHI-231 were used to test the binding specificity of aptamer SRZ1. As shown in Fig. 3, SRZ1 had highly specific binding capacity to 4T1 cells and low specific binding capacity to 4TO7 cells, yet no or little binding to other cancer cells was noted.

Characterization of DOTAP:DOPE liposomes and liposome-based particles. We successfully selected the 4T1 cell-specific SRZ1 aptamer as indicated above. Thus, we developed a novel 4T1 tumor-targeted chemotherapeutic drug delivery system by conjugating the SRZ1 aptamer on DOTAP:DOPE liposomes. Free DOTAP:DOPE, doxorubicin-loaded DOTAP:DOPE (DOTAP:DOPE/DOX) and doxorubicin-loaded SRZ1-conjugated DOTAP:DOPE (DOTAP:DOPE/DOX/SRZ1) liposomes were prepared by thin film and hydration method. The size distribution (Fig. 4a) and surface ζ-potential (Fig. 4b) were measured using Zetasizer. The size of all of the particles was ~100 nm, and the charge of the particles was positive.

Aptamer SRZ1 promotes uptake of DOTAP:DOPE/DOX by 4T1 cells. We next tested the uptake efficiency of DOTAP:DOPE/DOX and aptamer SRZ1-conjugated DOTAP:DOPE/DOX (DOTAP:DOPE/DOX/SRZ1) by 4T1 cells *in vitro*. DOTAP:DOPE/DOX or DOTAP:DOPE/DOX/SRZ1 was respectively incubated with 4T1 cells for 3, 6 and 24 h, and then the cells were washed and collected by 0.25% Trypsin-EDTA digestion. The doxorubicin signaling in the 4T1 cells was detected by flow cytometry. As shown in Fig. 5a, uptake of DOTAP:DOPE/DOX was markedly increased by carrying 4T1 cell-specific aptamer SRZ1 compared with DOTAP:DOPE/DOX only (Fig. 5b).

Cell toxicity of DOTAP:DOPE/DOX/SRZ1. Cell viability of the 4T1 cells treated with DOTAP:DOPE, free doxorubicin, DOTAP:DOPE/DOX or DOTAP:DOPE/DOX/SRZ1 was analyzed (Fig. 6a). Data from Fig. 3a showed that only the DOTAP:DOPE liposome had no effect on 4T1 cell viability. DOTAP:DOPE/DOX/SRZ1 significantly inhibited cell viability. Further study demonstrated that free doxorubicin markedly suppressed the viability of both 67NR (Fig. 6b) and normal mouse mammary gland NMuMG cells (Fig. 6c), while DOTAP:DOPE/DOX/SRZ1 had less toxicity to 67NR and NMuMG cells. Data from this panel indicated that doxorubicin was targeted delivered to target cells by the conjugated specific aptamers.

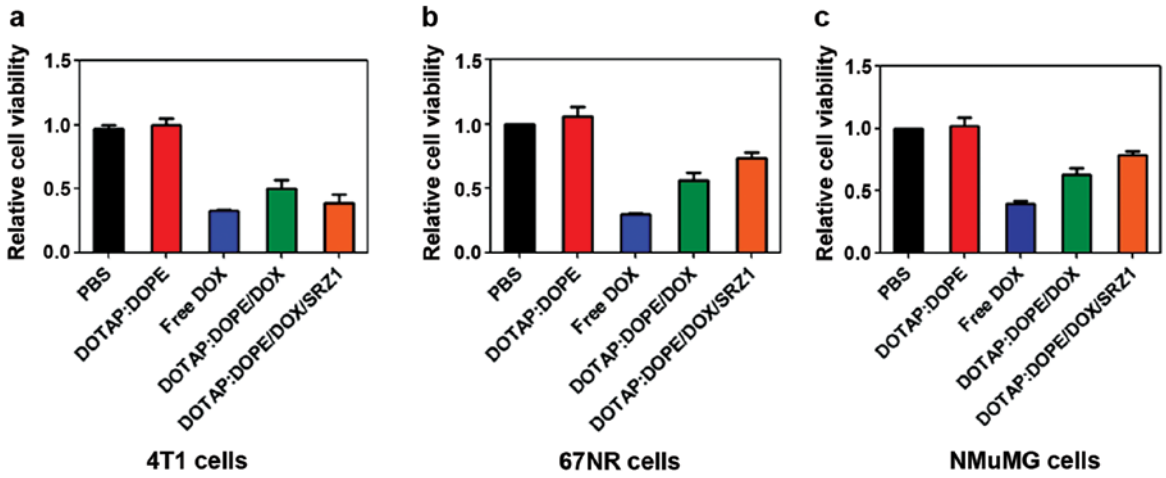


Figure 6. Relative cell viability of the cells treated with DOTAP:DOPE, free doxorubicin, DOTAP:DOPE/DOX or DOTAP:DOPE/DOX/SRZ1. (a) Relative viability of the targeted 4T1 cells. (b) Relative viability of the negative screening 67NR cells. (c) Relative viability of the normal mouse mammary gland NMuMG cells.

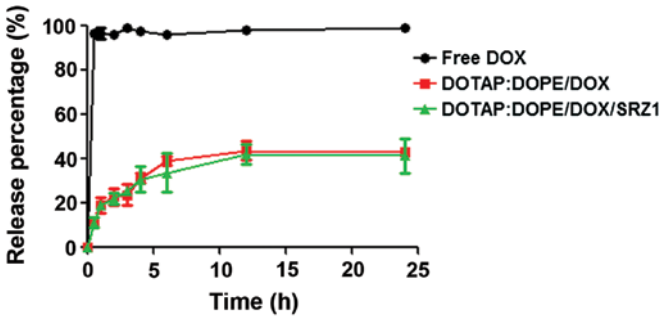


Figure 7. Release profile of doxorubicin from DOTAP:DOPE/DOX and DOTAP:DOPE/DOX/SRZ1. Free doxorubicin, DOTAP:DOPE/DOX or DOTAP:DOPE/DOX/SRZ1 was respectively incubated in PBS with 10% FBS for 0.5, 1, 2, 3, 4, 6, 12 and 24 h, and the release of doxorubicin in buffer was monitored for absorbance at 498 nm.

Release profile of doxorubicin. The release profile of DOX from DOTAP:DOPE/DOX and DOTAP:DOPE/DOX/SRZ1 was investigated in PBS at pH 7.4 (Fig. 7). Both DOTAP:DOPE/DOX and DOTAP:DOPE/DOX/SRZ1 resulted in sustained drug release, and the release of DOX from DOTAP:DOPE/DOX was the same as that from DOTAP:DOPE/DOX/SRZ1.

Targeted delivery and biodistribution in the 4T1 tumor-bearing mice. To study the targeted deliver of DOTAP:DOPE/DOX by aptamer SRZ1 *in vivo*, 4T1 tumor-bearing mice were intravenously administered DIR dye-labeled DOTAP:DOPE/DOX or DOTAP:DOPE/DOX/SRZ1 and body images of the mice were collected 24 h following injection. As shown in Fig. 8a, more DOTAP:DOPE/DOX signals were detected in the 4T1 tumors after conjugating with specific anti-4T1 cell SZR1 aptamer. Seventy-two hours after injection, the mice were sacrificed, and tumors, livers, lungs, spleens, kidneys, thymus and gut were removed and DIR dye signals in each tissue were scanned. Consistent with the body images, aptamer SZR1 significantly increased accumulation of DOTAP:DOPE/DOX in the 4T1 tumor tissues (Fig. 8a, right). Organ scan results indicated that most were DOTAP:DOPE/DOX and DOTAP:DOPE/DOX/SRZ1 distributed in the mouse liver, spleen, kidney and lung (Fig. 8b).

Tumor suppression is effectively enhanced by conjugating SRZ1 aptamer with DOTAP:DOPE/DOX. To test the effects of DOTAP:DOPE/DOX on tumor growth after conjugating with the SRZ1 aptamer, 4T1 tumor-bearing mice were intravenously injected with PBS, free DOTAP:DOPE particles, free DOX, DOTAP:DOPE/DOX or DOTAP:DOPE/DOX/SRZ1 every 3 days. The tumor size was measured every 5 days, and the volume data revealed that DOX only and DOTAP:DOPE/DOX inhibited 4T1 tumor growth, and the suppressive effect of DOTAP:DOPE/DOX was markedly enhanced by the conjugated 4T1-specific aptamer SRZ1 (Fig. 9a). From the survival rate results of the five groups, we determined that the survival time was prolonged after treatment with free DOX and the DOX-loaded DOTAP:DOPE particles, while optimal results were obtained from the aptamer SRZ1-conjugated group (Fig. 9b), which was consistent with the tumor inhibition results.

Discussion

Aptamer-mediated targeted delivery is a promising strategy for improving the therapeutic index of cytotoxic drugs that cause serious systemic toxicities (17,18), such as doxorubicin. In the present study, we identified a novel single-strand DNA aptamer to mouse breast cancer 4T1 cells by using cell-SELEX. Our objective was to establish a more effective chemotherapeutic drug target delivery platform. To our knowledge, SRZ1 is the first aptamer to be identified as specific for mouse mammary breast cancer 4T1 cells. Both *in vitro* and *in vivo* data presented in the present study revealed that SRZ1 was a powerful aptamer for targeted doxorubicin delivery in a mouse breast cancer model therefore providing a novel strategy for human breast cancer treatment.

Doxorubicin is used to treat a variety of tumors including breast cancer. It is considered as the most useful anticancer drug worldwide (19,20). A large number of studies have reported that free doxorubicin can be taken up by both tumor cells and normal cells via a passive diffusion mechanism, and the proliferation of both tumor cells and normal cells was suppressed by doxorubicin through interaction with nuclear

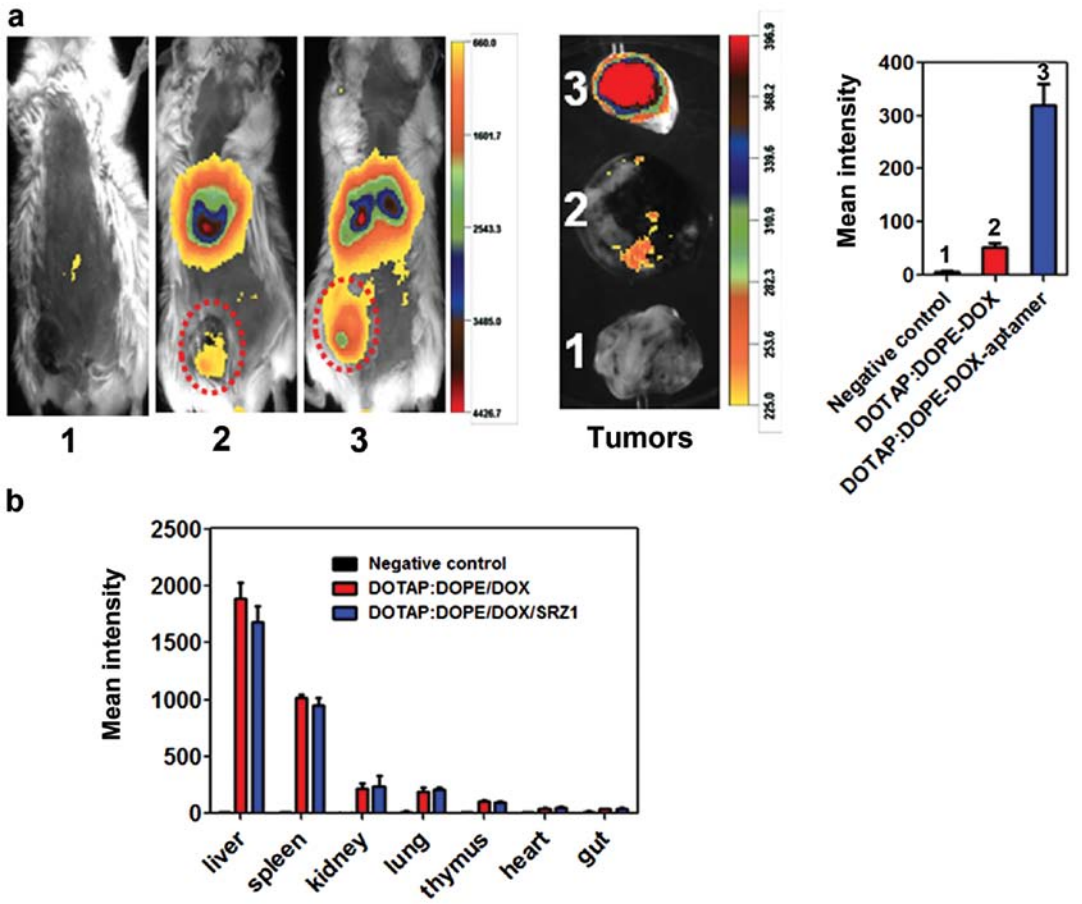


Figure 8. Targeted delivery of DOTAP:DOPE/DOX and biodistribution of doxorubicin. DIO dye-labeled DOTAP:DOPE/DOX and DOTAP:DOPE/DOX/SRZ1 were intravenously injected into 4T1 tumor-bearing mice. DIO signals in mouse bodies (a, left) and 4T1 tumors (a, right) were scanned 24 h following injection. (b) PBS, DOTAP:DOPE/DOX and DOTAP:DOPE/DOX/SRZ1 were injected into 4T1 tumor-bearing mice, and doxorubicin intensity in each organ was determined by a UV spectrometer.

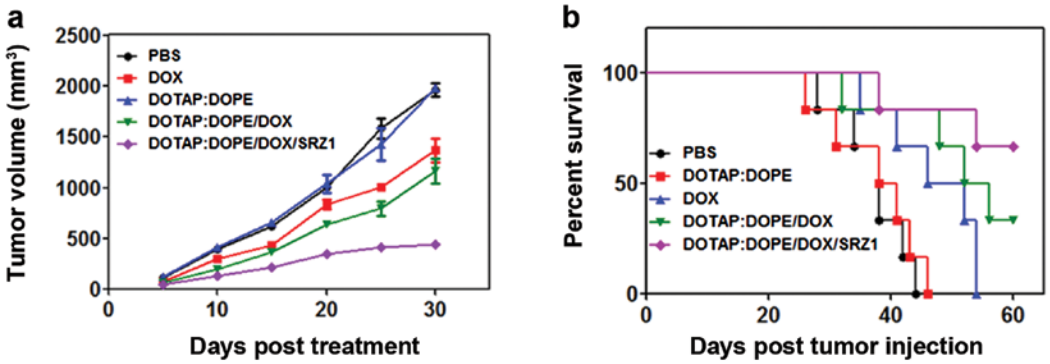


Figure 9. Tumor growth and survival of tumor-bearing mice. (a) Tumor volume and (b) survival rate of mice injected with 4T1 tumor cells. 4T1 tumor-bearing mice respectively were treated with free doxorubicin, DOTAP:DOPE, DOTAP:DOPE/DOX or DOTAP:DOPE/DOX/SRZ1 every 3 days for 10 times.

DNA (21,22). Thus, the acute toxicity of free doxorubicin to normal tissues limits the therapeutic efficacy in clinical use.

In recent years, liposomes have been extensively employed as effective delivery systems to enhance the efficacy of encapsulated chemotherapeutic drugs (23-25). Liposomes provide a better accumulation of drugs in tumor tissues through an enhanced permeability and retention (EPR) effect. Liposomes composed of DOTAP and DOPE have been demonstrated as great potential carriers for the delivery of anticancer agents (26,27). To achieve an optimum therapeutic effect of doxorubicin in a 4T1 breast cancer model, we generated an efficient delivery system for doxorubicin by conjugating 4T1 tumor cell-specific aptamers with doxorubicin-loaded DOTAP:DOPE liposomes. Binding specificity was assessed *in vitro* by FACS and proved that the aptamer SRZ1 was able to bind to mouse 4T1 cells, rather than mouse normal mammary gland cell line NMuMG, human colon carcinoma cell line CT26, mouse Lewis lung carcinoma cell line LL/2, mouse hepatoma cell line Hepa 1-6 and mouse B cell lymphoma cell line WEHI-231. Meanwhile, we demonstrated that the SRZ1

aptamer modified doxorubicin-loaded DOTAP:DOPE had a higher internalization in 4T1 cells than in 67NR cells. This targeting was achieved through the 4T1 cell-specific binding affinity of aptamer SRZ1. Furthermore, we confirmed the specific toxicity of DOTAP:DOPE/DOX/SRZ1 to 4T1 cells, when compared to the negative screening NR67 and normal mouse mammary gland NMuMG cells, thereby limiting the toxicity of doxorubicin to target cells. Additionally, reduced release of doxorubicin in the DOTAP:DOPE/DOX and DOTAP:DOPE/DOX/SRZ1 groups was detected by UV spectrometer. Doxorubicin in free form can be uptaken by cells within 15 min through a passive diffusion mechanism. However, after co-loading doxorubicin and the 4T1-specific SRZ1 aptamer onto DOTAP:DOPE liposomes, internalization of doxorubicin in the target cells was mainly dependent on the interaction between the aptamer and the cell membrane. Thus, less toxicity to 67NR or NMuMG cells was observed in the DOTAP:DOPE/DOX/SRZ1 group than that in the free doxorubicin group. However, due to the target recognition by the SRZ1 aptamer, the local concentration of doxorubicin was markedly increased, compared to the free doxorubicin and DOTAP:DOPE/DOX group. Hence, maximum tumor suppression was achieved in the DOTAP:DOPE/DOX/SRZ1-treated 4T1 tumor-bearing mouse.

Collectively, to maximize the tumor inhibitory function of doxorubicin, we developed a novel delivery system by conjugating a target tumor cell-specific aptamer with doxorubicin-loaded DOTAP:DOPE liposomes. The binding specificity and efficacy of this drug-delivery platform were further investigated *in vitro* and *in vivo*. Nonspecific cellular toxicity to normal cells was significantly reduced after conjugating the target cell-specific aptamer with DOTAP:DOPE/DOX. An aptamer is a type of small molecule and can be designed as a targeting ligand, thus enabling the selective delivery of therapeutic drugs to target cells providing a significant potential for future clinical cancer therapy.

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