Supporting Information

Zhu et al. 10.1073/pnas.1220817110

SI Materials and Methods

DNA Synthesis, Labeling, Purification, and Quantification. All DNA probes were synthesized on an ABI3400 DNA/RNA synthesizer (Applied Biosystems). Coupled on the 5′-end of these DNA probes was fluorescein (FITC) or biotin, 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) on a C-18 column using 0.1 M triethylamine acetate (TEAA) (Glen Research) and acetonitrile (Sigma-Aldrich) as the eluent. The collected DNA products were dried and detritylated by dissolving and incubating DNA products in 200 μL of 80% acetic acid for 20 min. 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.

Materials. Washing buffer contained 4.5 g/L glucose and 5 mM $MgCl_2$ 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) to the washing buffer to reduce background binding. Doxorubicin hydrochloride (Dox) was purchased from Fisher Scientific. Transferrin–Alexa 633 conjugate was purchased from Molecular Probes.

Cell Lines and Cell Culture. Cell lines CCRF-CEM (human T-cell ALL) and Ramos (human B-cell Burkitt's lymphoma) were obtained from the American Type Culture Collection. Cells were cultured in RPMI medium 1640 supplemented with 10% FBS (heat-inactivated; Gibco) and 100 IU/mL penicillin–streptomycin (Cellgro) at 37 °C in a humid atmosphere with 5% CO_2. The cell density was determined before each experiment using a hemocytometer.

Agarose Gel Electrophoresis. Formation of the resultant aptamer-tethered nanotrains (aptNTrs) was confirmed by agarose gel (3%) electrophoresis (90 V, 60 min), followed by UV imaging and fluorescent imaging using a Typhoon 9410 variable mode imager.

Atomic Force Microscopy. An atomic force microscopy (AFM) study was performed on a Nanoscope IIIa (Veeco) using tapping mode in ambient air. The unpolymerized M1 and M2, preformed sgc8–NTrs, and sgc8–NTr–Dox were diluted 50×, deposited on aminopropyl silatrane-mica surfaces for 3 min, rinsed with double-distilled H_2O, and dried using argon gas. The radius of curvature of the silicone tip was about 10 nm. Topographic images were obtained with 512×512 pixels^2 at a scan rate of 2 Hz. To calculate the frequency distributions, the lengths of nanotrains were measured using the ImageJ software.

Transmission Electron Microscopy Study of Gold nanoparticle-Loaded Nanotrains. Samples were prepared by incubating gold nanoparticles (13 nm) with preformed aptNTrs, in which M1 and M2 were labeled with thiol, for 1 h. The resultant sample was placed on a copper grid and dried at room temperature. Transmission electron microscopy (TEM) images were obtained on a Hitachi H-7000 NAR transmission electron microscope. Afterward, the samples were imaged by using TEM at a working voltage of 100 kV.

Drug Loading Study by Fluorescence Spectrometry. The drug loading was monitored by fluorescence spectrometry [excitation (Ex): 480 nm], using a Fluorolog-Tau-3 spectrofluorometer (Jobin Yvon).

Stability of sgc8–NTr–Dox by a Drug Diffusion Assay. Free Dox (30 μM, 300 μL) and sgc8–NTr–Dox (30 μM Dox equivalent, 300 μL) were prepared and transferred into MINI Dialysis Units [3.5 molecular weight cut off (MWKO); Thermo Scientific]. Each unit bottom was immersed in 3 mL of PBS buffer (supplemented with 5 mM Mg^{2+}) in an individual well of a 12-well plate, with a magnetic rod at the bottom of each well. The plate was placed on a magnetic stirrer (130 rpm). At the indicated time points, a 120-μL aliquot was collected from each well for Dox fluorescence measurement [Ex: 480 nm; emission (Em): 590 nm] using a Fluorolog-Tau-3 spectrofluorometer (Jobin Yvon). The collected samples were then returned to the corresponding wells. Data points for each sample were fit by nonlinear regression using Origin 8 software to a first-order release model as follows:

$$F_{\text{released}} = \alpha\left[1 - \exp\left(-\ln(2)t/t_{1/2}\right)\right], \qquad \textbf{[S1]}$$

where t is the time (in hours), $t_{1/2}$ is the diffusion half-time, and α is the maximum fluorescence intensity of the released drug.

Laser-Scanning Confocal Microscopy Imaging. All cell fluorescent images were collected on a Leica TCS SP5 confocal microscope (Leica Microsystems) with a 100× oil immersion objective and Leica confocal software. Cells were observed in differential interference contrast mode. Ar 488-nm, He-Ne 543-nm, and He-Ne 633-nm lasers were used for excitations of Dox, tetramethylrhodamine (TAMRA), and Alexa 633 or Cy5, respectively. Cells (2×10^5 in 200 μL of medium) were incubated with sgc8–NTrs, free Dox, or sgc8–NTr–Dox for 2 h. Cells were washed with washing buffer, suspended in medium (200 μL), treated with transferrin–Alexa 633 (60 nM), and incubated for 0.5 h. The resultant cells were washed and resuspended in binding buffer for microscopic observation.

Internalization Assay Using Flow Cytometry. The internalization of TAMRA-labeled aptNTrs into cells was studied by incubating sgc8–NTrs (120 nM aptamer equivalents) with cells (2×10^5) in binding buffer (200 μL) at 37 °C. The internalization was terminated by putting cells on ice. Cells were washed with washing buffer, trypsinized (if applicable) with trypsin EDTA (1×, 500 μL; Cellgro) for 15 min, washed twice with washing buffer, and suspended in binding buffer (200 mL). The TAMRA fluorescence intensities of cells were then determined by flow cytometric analysis on a FACSAria II system (BD Biosciences). Data were analyzed with FCS Express 4 software (De Novo Software).

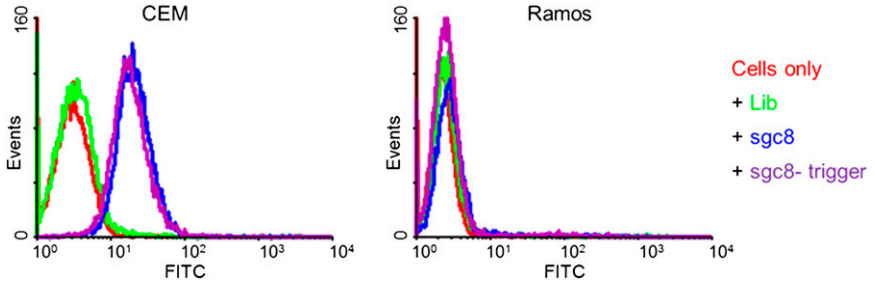

Fig. S1. Flow-cytometric results indicating that sgc8–trigger maintained binding abilities to target CEM cells, but not to nontarget Ramos cells. Lib (random sequences), sgc8, and sgc8–trigger were labeled with FITC.

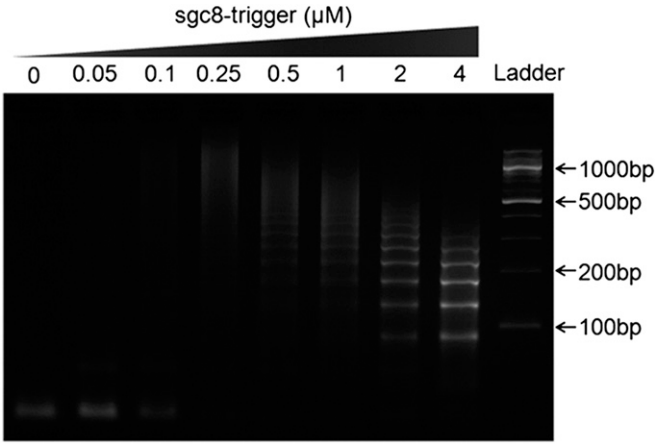

Fig. S2. Optimization of the self-assembly of aptNTrs by agarose gel electrophoresis. A series of increasing concentrations of sgc8–trigger (0–4 μM, as marked above each lane) were added to mixtures of M1 and M2 (5 μM each), followed by agarose gel electrophoresis. The smallest initial ratio of sgc8–trigger to each monomer with the largest amount of monomers consumed is 1:10, which was used in subsequent studies.

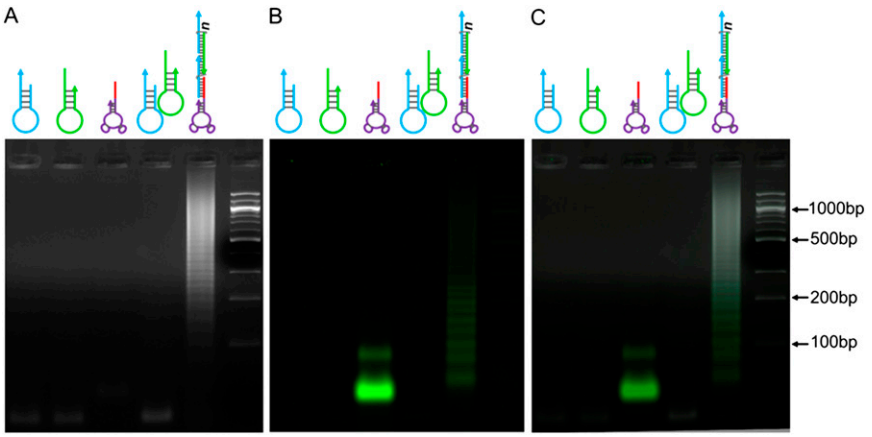

Fig. S3. Verification of aptNTr formation. Images of fluorescent native agarose gel electrophoresis (same as those shown in Fig. 2*A*) scanned at two wavelengths showing the self-assembly of sgc8-tethered DNA nanotrains initiated by sgc8–trigger and the incorporation of FITC-labeled aptamer (sgc8) moiety in nanotrains. The samples were depicted above the corresponding lanes, and the upper band for sgc8–trigger resulted from its homodimers. [(*A*) All DNA species stained by ethidium bromide (EB); (*B*) FITC on sgc8–trigger; (*C*) merged signals.]

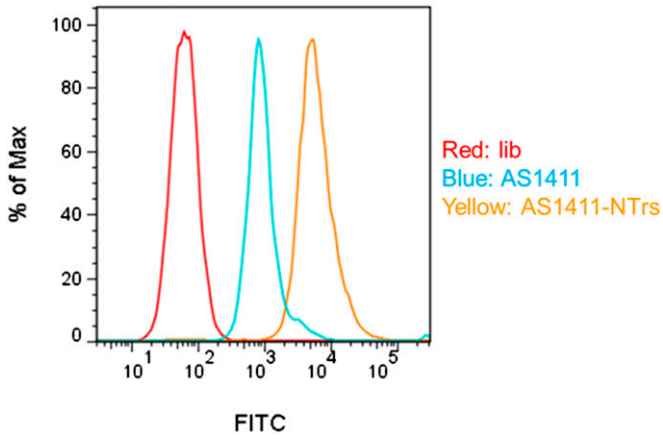

Fig. S4. Flow-cytometric analysis indicating the specific recognition of AS1411 and AS1411–NTrs to target Huh7 cells (human hepatoma cells). AS1411, M1, and M2 in AS1411–NTrs were labeled with FITC. Data were analyzed using the FlowJo software.

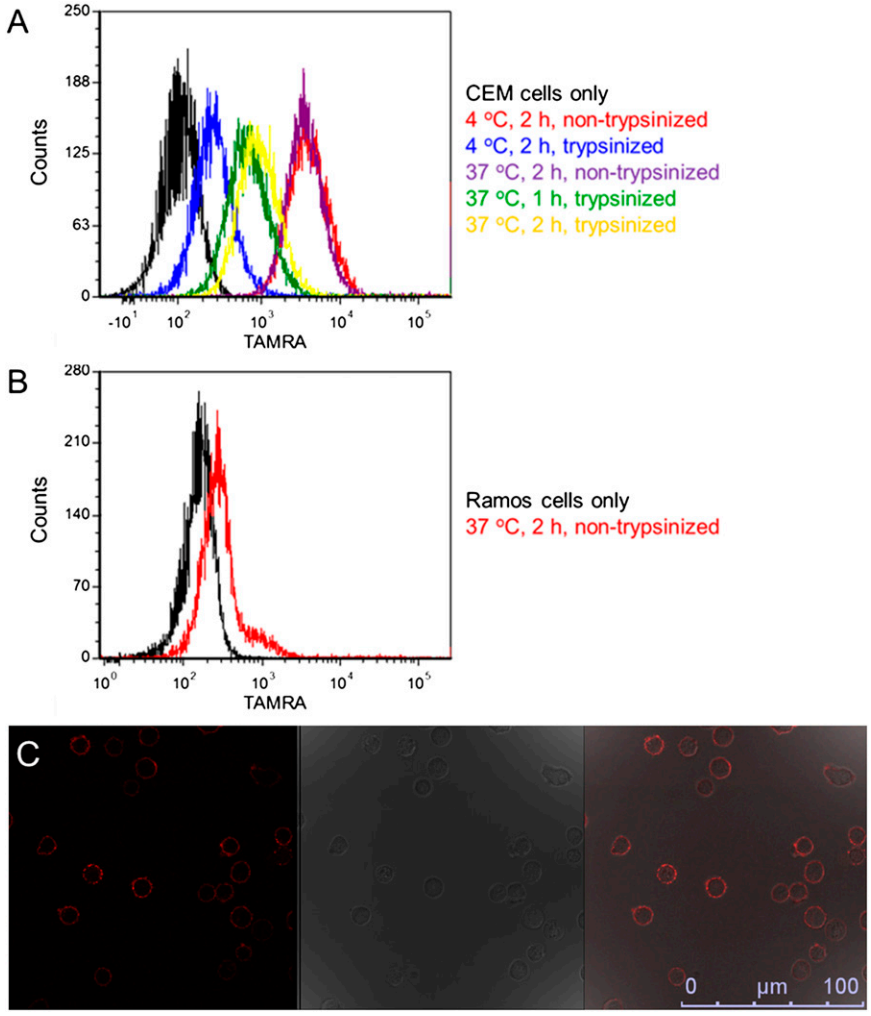

Fig. S5. (*A* and *B*) Flow-cytometric analysis indicating the selective binding and internalization of sgc8–NTrs into target CEM cells (*A*), but not to nontarget Ramos cells (*B*). sgc8–NTrs were prepared from sgc8–trigger, M1, and TAMRA-labeled M2. Cells were incubated with sgc8–NTrs (100 nM in terms of sgc8–trigger) at 4 °C or 37 °C for 1 or 2 h as denoted, trypsinized, if applicable, and subjected to flow-cytometric analysis. Because trypsinization digested cell surface protein, cell surface-bound sgc8–NTrs were removed. After trypsinization, the enhanced fluorescence intensities of CEM cells incubated with sgc8–NTrs at 37 °C, compared with those at 4 °C, indicated that these nanotrains were internalized (*A*). In contrast, Ramos cells incubated with sgc8–NTrs at 37 °C showed very little signal enhancement (*B*). This demonstrated the selectivity of the binding and internalization of sgc8–NTrs. Data were analyzed using the FCS Express software. (*C*) Confocal laser-scanning microscopy images displaying the binding of sgc8–NTrs (100 nM sgc8–trigger equivalents) on target CEM cells at 4 °C for 2 h, followed by transferrin–Alexa 633 staining. sgc8–NTrs stayed on cell membrane (M2: labeled with TAMRA). (Scale bars: 100 μm.)

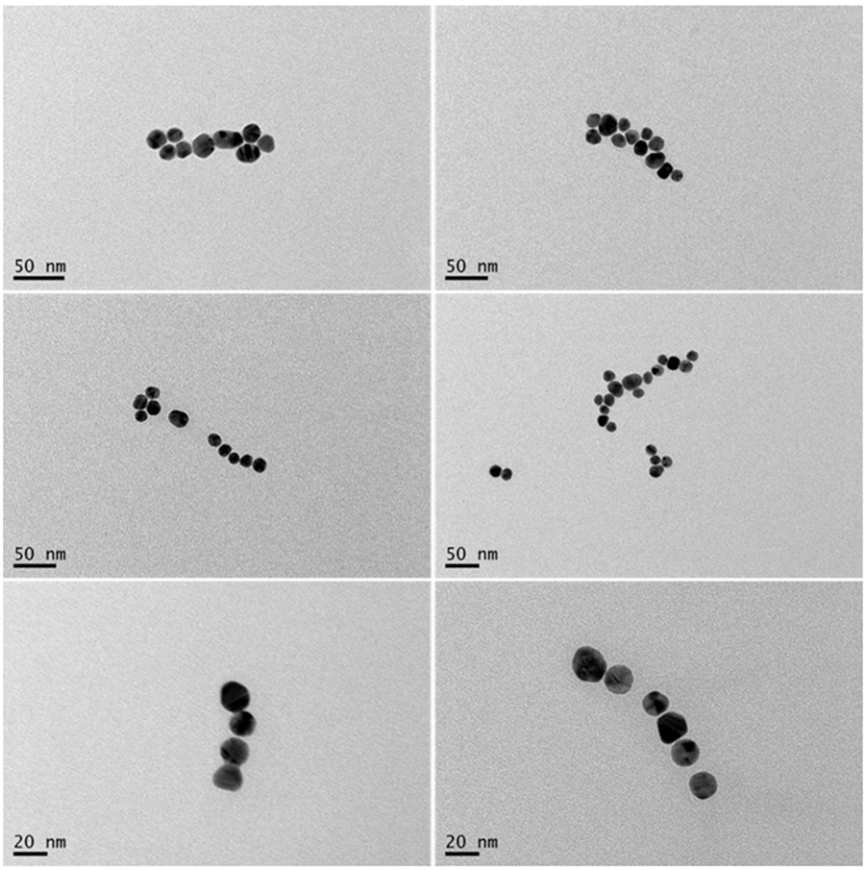

Fig. S6. TEM images of 13-nm gold nanoparticles loaded on sgc8–NTrs. Scale bars were as denoted.

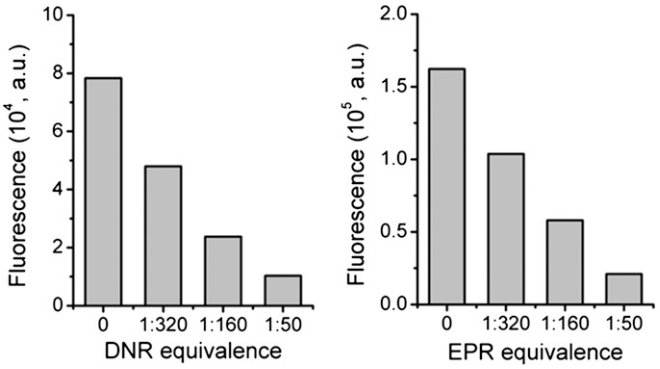

Fig. S7. Fluorescence intensities of molecular drugs (2 μM) with increasing molar equivalents of sgc8–NTrs. The fluorescence quenching indicated that drugs were loaded into sgc8–NTrs. (Ex: 480 nm; Em: 590 nm.)

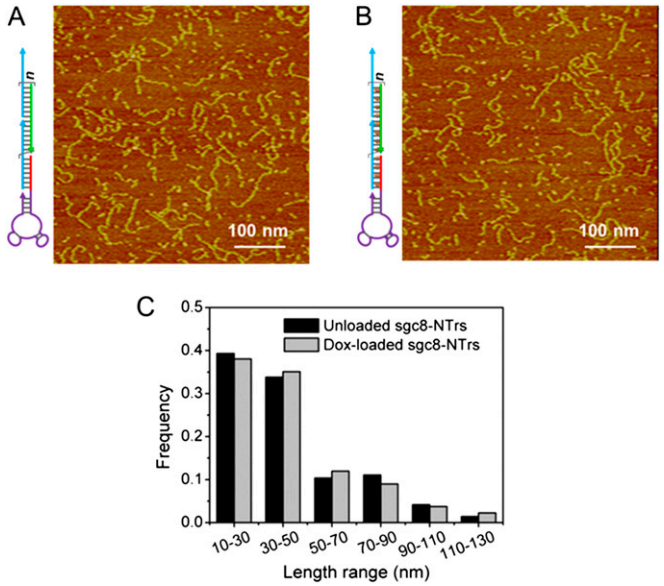

Fig. S8. Stability and integrity of Dox-loaded with sgc8–NTrs. (*A* and *B*) AFM images (same as those shown in Fig. 1*A*) depicting the morphologies of unloaded sgc8–NTrs and sgc8–NTrs loaded with Dox. (*C*) Frequency distributions of length range of nanotrains shown in *A* and *B*. The comparable morphologies and length frequency distributions of unloaded nanotrains and nanotrains loaded with Dox again demonstrated the integrity and stability of sgc8–NTr–Dox. Nanotrain lengths were analyzed using ImageJ software.

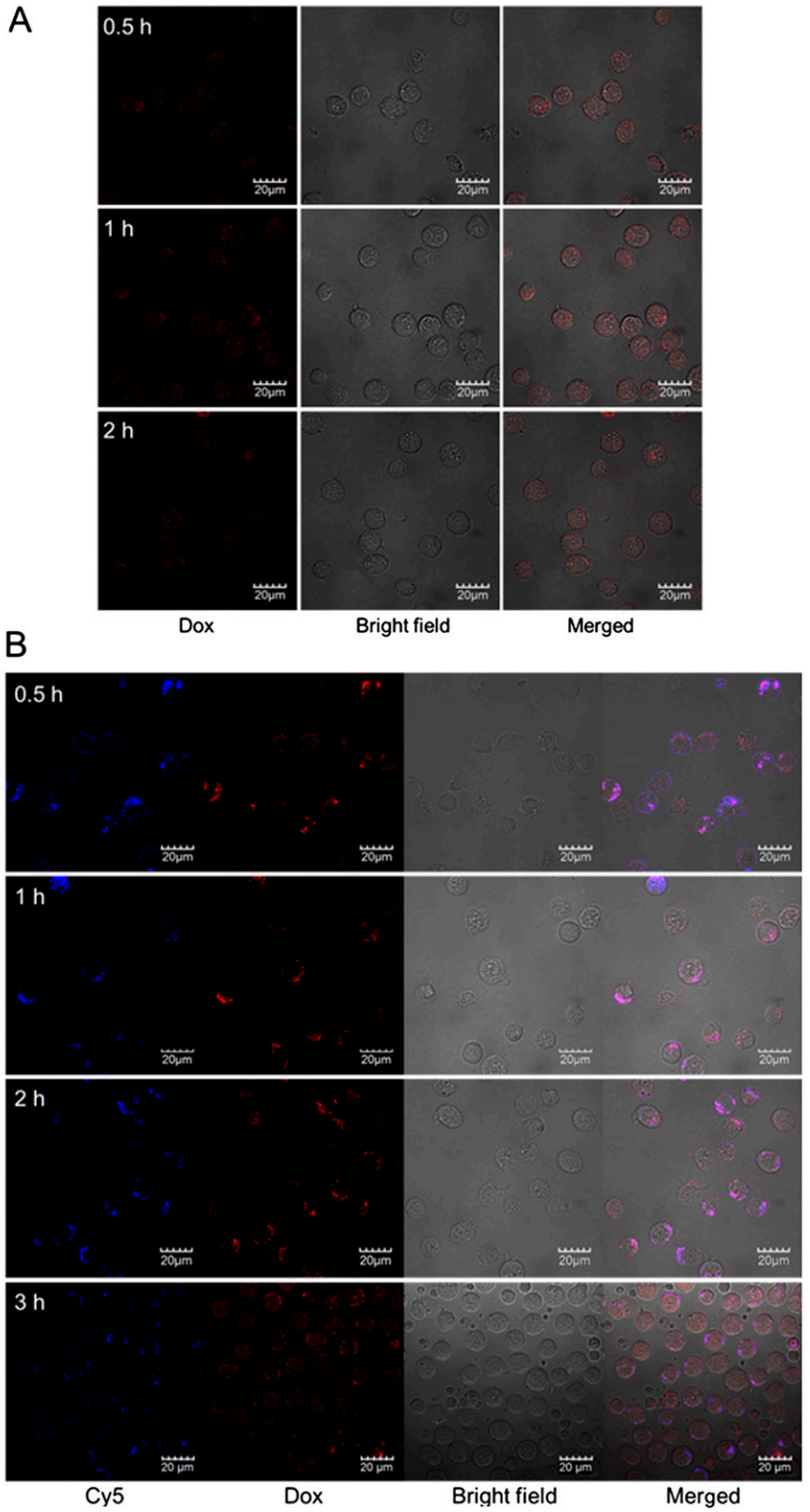

Fig. S9. Confocal laser-scanning microscopy images displaying the time-dependent intracellular behaviors of free Dox (*A*), and Dox and aptNTrs delivered by sgc8–NTr–Dox (*B*) in CEM cells. (*A*) CEM cells were treated with free Dox (Dox: 2 μM) for different time lengths, as denoted on the *Left*. The Dox fluorescence intensity kept constant within the time length studied, and the drug was distributed evenly within a short period. (*B*) CEM cells were treated with sgc8–NTr–Dox (2 μM Dox equivalents) for 0.5, 1, 2, and 3 h. The intracellular Dox fluorescence intensity of cells was gradually enhanced, indicating gradual Dox unloading from sgc8–NTrs. Dox was initially colocalized with nanotrains and then gradually distributed in other cytoplasmic areas (M1 and M2: labeled with Cy5; [Dox]/[sgc8–NTr] = 50:1). (Scale bar: 20 μm.)

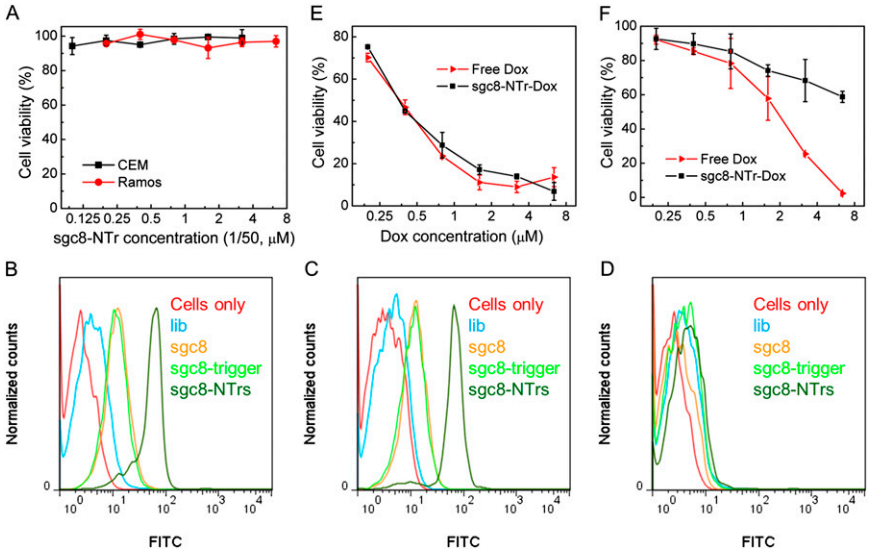

Fig. S10. Selective cancer cell recognition ability of aptNTrs and selective cytotoxicity induced by Dox delivered via biocompatible aptNTrs, under simulated physiological environment. (*A*) MTS assay results suggesting no apparent cytotoxicity induced by sgc8–NTrs in CEM cells and Ramos cells, indicating good biocompatibility of DNA nanotrain-based drug transporters. (*B–D*) Flow-cytometric analysis indicating the selective recognition of sgc8, sgc8–trigger, and sgc8–NTrs to target CEM cells (*B* and *C*), but not to nontarget Ramos cells (*D*), both in binding buffer (*B*) and in FBS (10%)-containing cell culture medium (*C* and *D*), at 37 °C. lib: random sequences; lib, sgc8, sgc8–trigger, and M1 and M2 in sgc8–NTrs were labeled with FITC. Data were analyzed using the FlowJo software. (*E* and *F*) MTS assay results showing, in FBS (10%)-containing cell culture medium, the selective cytotoxicity of Dox transported by sgc8–NTrs in target CEM cells (*E*), but much less cytotoxicity in nontarget Ramos cells (*F*), compared with nonselective cytotoxicity induced by free Dox in both CEM cells and Ramos cells. Compared with the same assay using cell culture medium without FBS (Fig. 4*B*), the loss of some viability of Ramos cells treated with sgc8–NTr–Dox is presumably due to the nuclease cleavage of sgc8–NTrs during 2-h incubation.

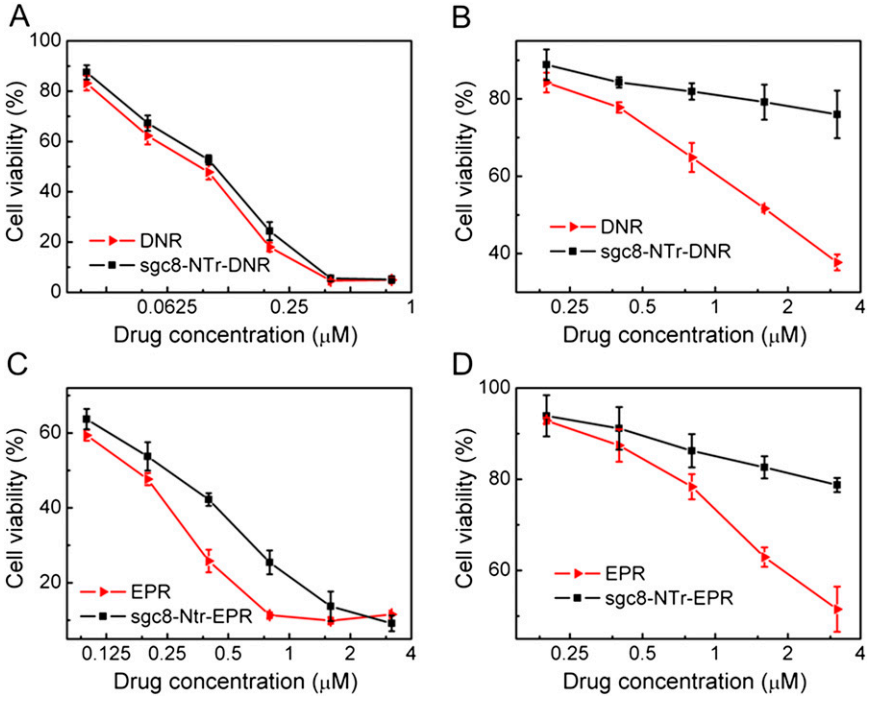

Fig. S11. MTS assay results showing the targeted cytotoxicities of molecular drugs, DNR (*A* and *B*) and EPR (*C* and *D*) transported by sgc8–NTrs, compared with that of the corresponding free drugs, using target CEM cells (*A* and *C*) and nontarget Ramos cells (*B* and *D*).

Table S1. Sequences of DNA probes

Probes	Sequences (5′–3′)
1. M1	CGTCGTGCAGCAGCAGCAGCAACGGCTTGCTGCTGCTGCTGCTGC
2. M2	TGCTGCTGCTGCTGCTGCACGACGGCAGCAGCAGCAGCAAGCCGT
3. Sgc8–trigger	TGCTGCTGCTGCTGCTGCACGACGTTTATCTAACTGCTGCGCCGCCGGG
4. Sgc8	ATCTAACTGCTGCGCCGCCGGGAAAATACTGTACGGTTAGA

FITC, TAMRA, or Cy5, if applicable, were labeled at the 5′-ends of M1 and M2; thiol group was labeled at the 3′-ends of M1 and M2. (In sgc8–trigger, the red sequence indicates the trigger probe, black indicates the linker, and purple indicates the aptamer. Sequences in the same colors in M1 and M2 are complementary.)