Supporting Information For

Targeting Breast Cancer Stem Cells by Self-Assembled, Aptamer-Conjugated DNA Nanotrain with Preloading Doxorubicin

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NT-AKTin-DOX and TA6NT-AKTin-DOX.

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Table S1. Sequences of DNA.

DNA	Sequences (5'-3')
M1	CGTCGTGCAGCAGCAGCAGCAACGGCTTGCTGCTGCTGCTGCTGC
M2	TGCTGCTGCTGCTGCTGCACGACGGCAGCAGCAGCAGCAGCAAGCCGT
TA6	GAGATTCATCACGCGCATAGTCTTGGGACGGTGTTAAACGAAAGGGGACGACCGACTATGCGATGATGTCTTC
TA6-tethered trigger	TGCTGCTGCTGCTGCTGCACGACGTTTGAGATTCATCACGCGCATAGTCTTGGGACGGTGTTAAACGAAAGGGGACGACCGACTATGCGATGATGTCTTC

Table S2. Sensitivity of BCSCs to the combination of AKTin and DOX. A theoretical curve was calculated for combined inhibition using the equation $E_{bliss} = E_A + E_B - E_A \times E_B$, where E_A and E_B are the fractional inhibitions obtained by AKTin alone at 0.3 μM and free DOX alone at 1 μM. Here, E_{bliss} is the fractional inhibition that would be expected if the combination of the two drugs was exactly additive. If the experimentally measured fractional inhibition is greater than E_{bliss}, the combination was said to be synergistic. If the experimentally measured fractional inhibition is less than E_{bliss}, the combination was said to be antagonistic.

Cell Line	E_{AKTin}	E_{DOX}	E_{bliss}	
			Predicted	Observed
BCSCs	0.11	0.37	0.44	0.54

Supplementary Figures

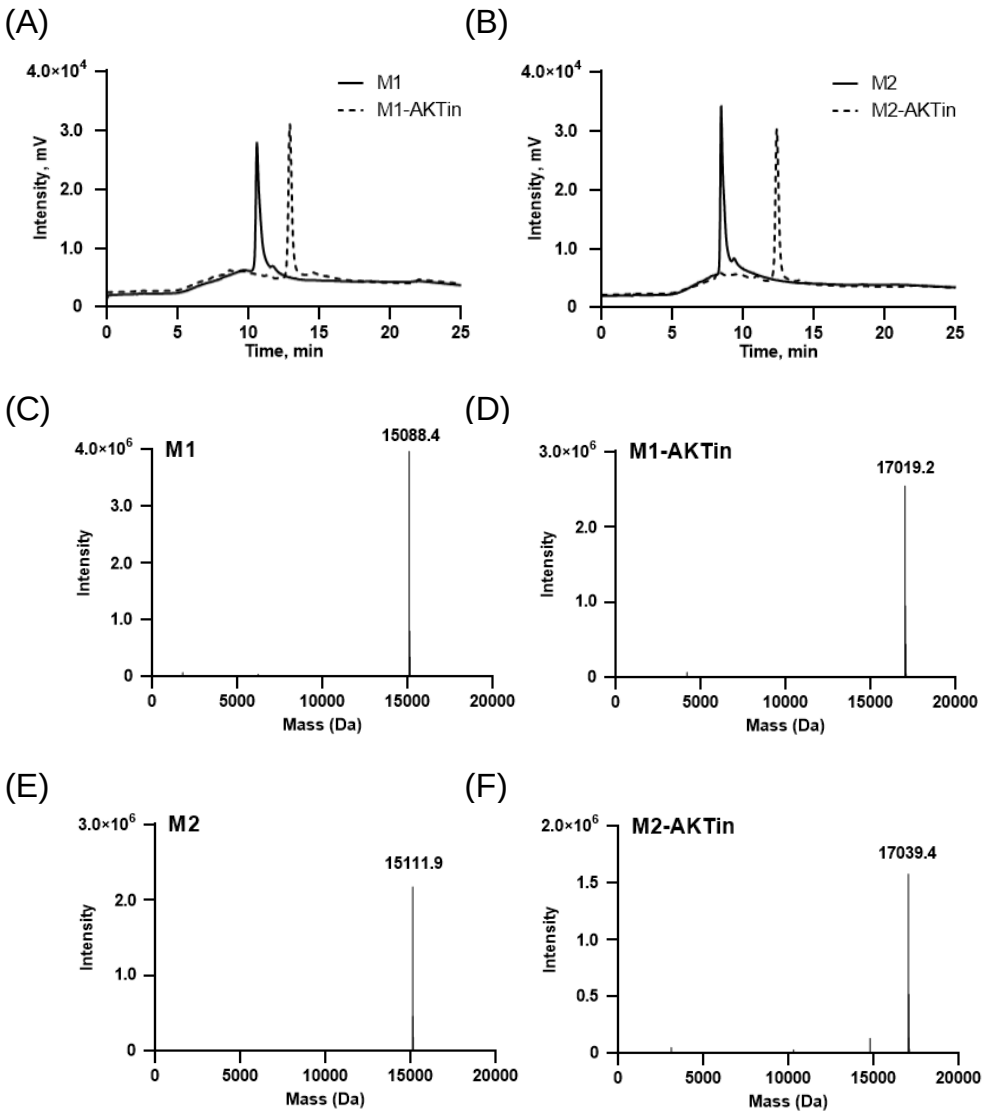


Figure S1. Comprehensive characterization of DNA building blocks. (A) HPLC chromatograms of M1 and M1-AKTin at wavelength of DNA (260 nm). (B) HPLC chromatograms of M2 and M2-AKTin at wavelength of DNA (260 nm). ESI mass spectra of (C) M1, (D) M1-AKTin, (E) M2 and (F) M2-AKTin. Mass spectrometry detection was performed using a Thermo LCQ Deca XP Plus ion trap MS, which was operated with electrospray ionization in the positive mode.

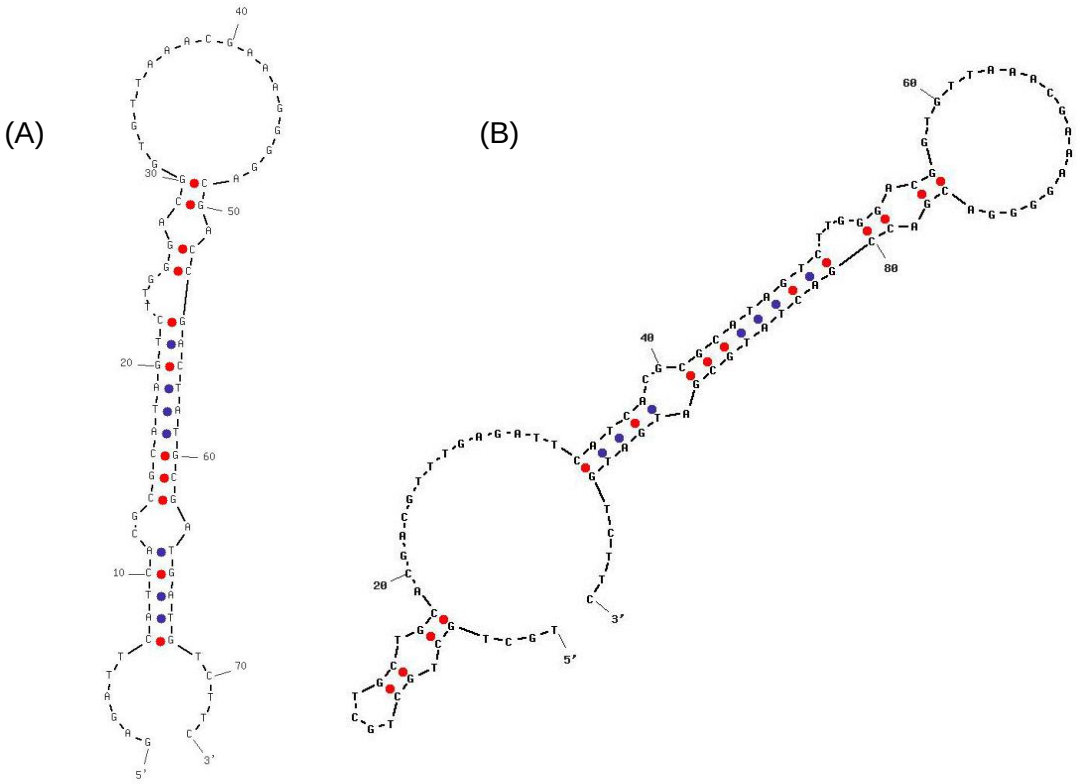


Figure S2. Predicted secondary structure of (A) TA6 and (B) TA6-tethered trigger.

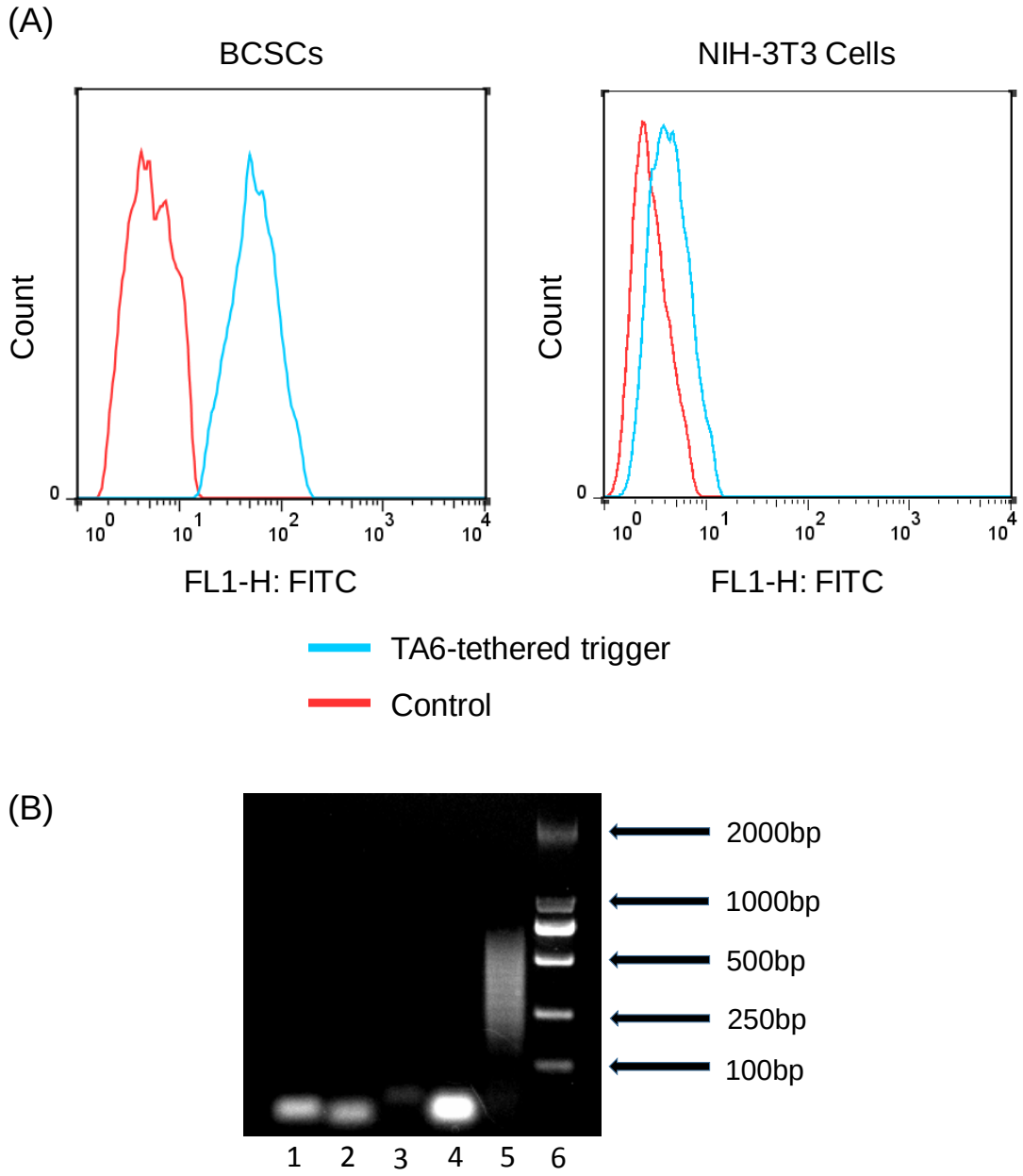
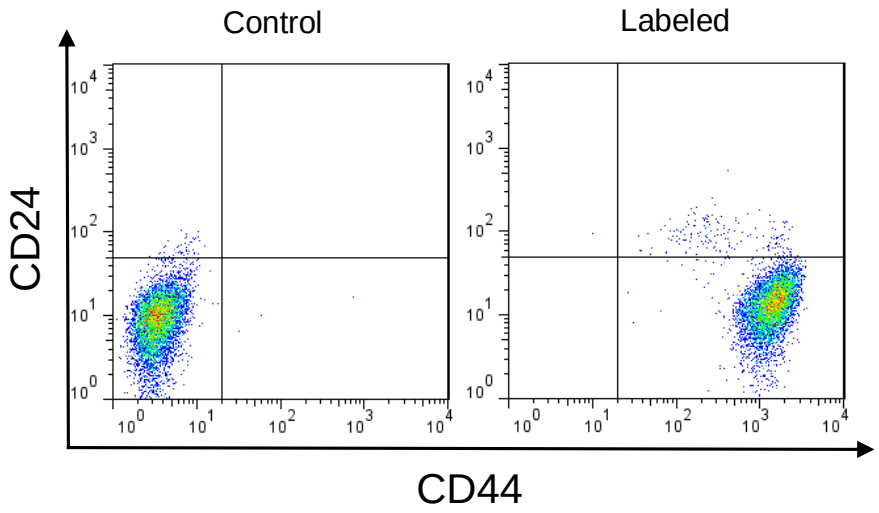


Figure S3. (A) Specificity of TA6-tethered trigger verified by flow cytometry. (B) Agarose gel electrophoresis indicates the self-assembly of TA6NT-AKTin initiated by TA6-tethered trigger. Lane 1: M1-AKTin. Lane 2: M2-AKTin. Lane 3: TA6-tethered trigger. Lane 4: M1-AKTin and M2-AKTin. Lane 5: TA6-tethered trigger, M1-AKTin and M2-AKTin. Lanes 6: DL2000 DNA marker.

(A)



(B)

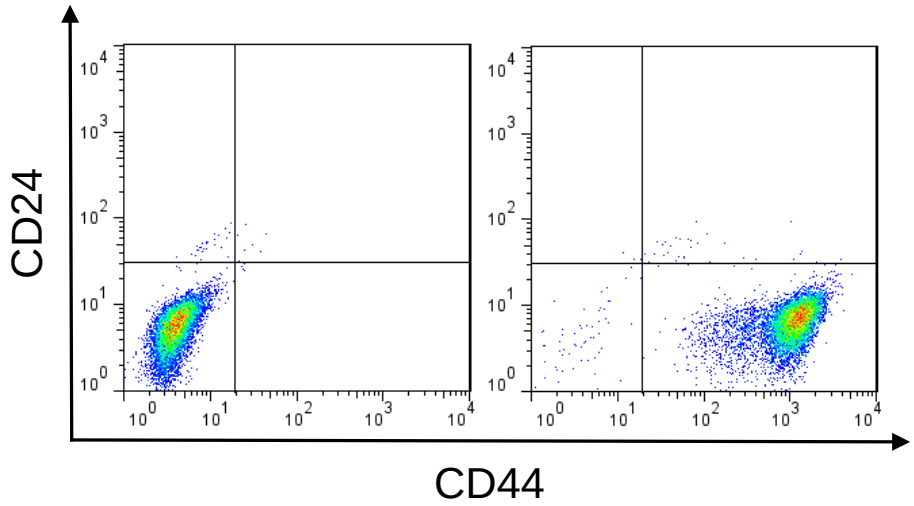
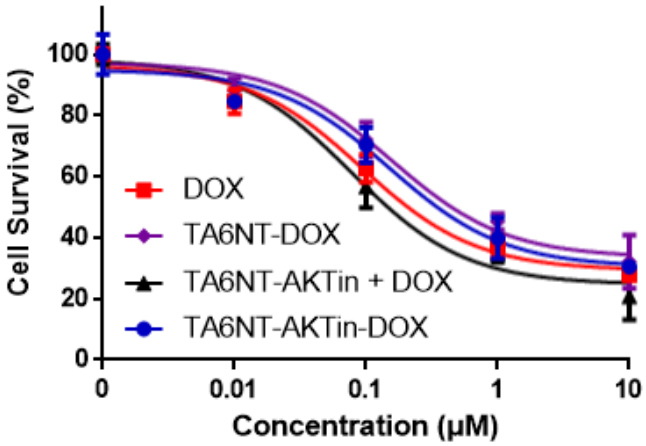


Figure S4. Flow cytometry of anti-CD24-APC and anti-CD44-PE antibodies-labeled BCSCs and the control at early and last passages of BCSCs. The conditions of flow cytometry were described in supplementary methods.

(A)



(B)

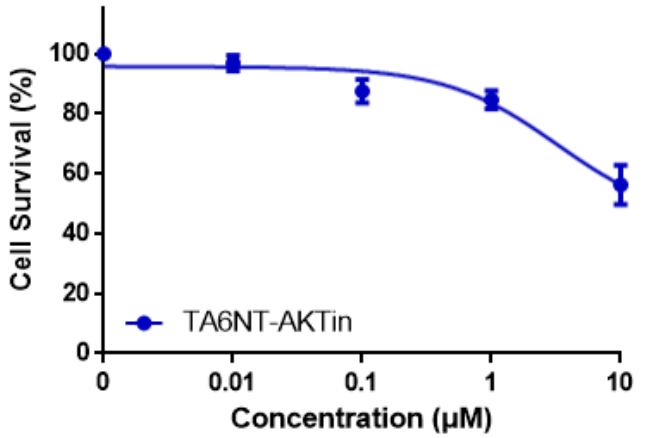


Figure S5. Cytotoxicity profiles of (A) free DOX, TA6NT-DOX, TA6NT-AKTin together with DOX and TA6NT-AKTin-DOX for MCF-7 cells and (B) TA6NT-AKTin for BCSCs (n = 3). Their IC_{50} values were 434.7 ± 52.6 nM, 993.6 ± 124.9 nM, 279.9 ± 34.9 nM, 678.2 ± 85.0 nM in (A) and 19.4 ± 2.7 μM in (B), respectively.

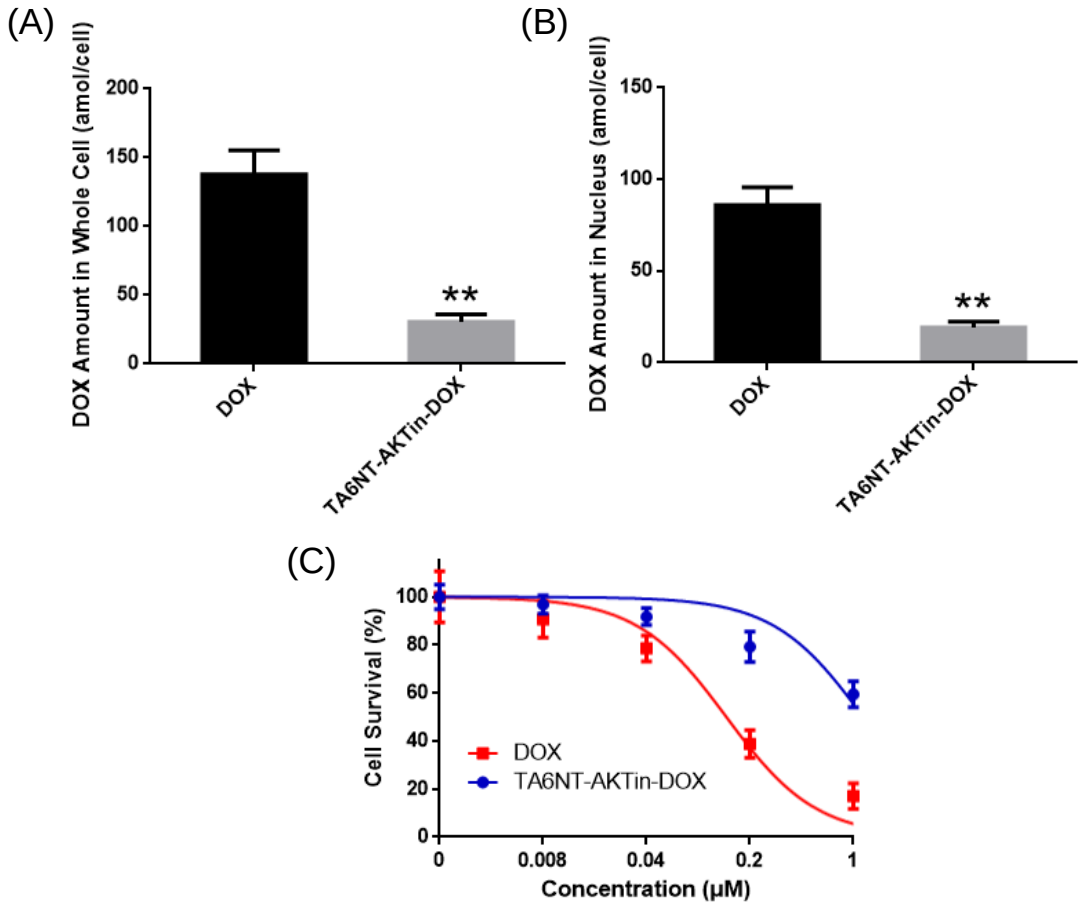
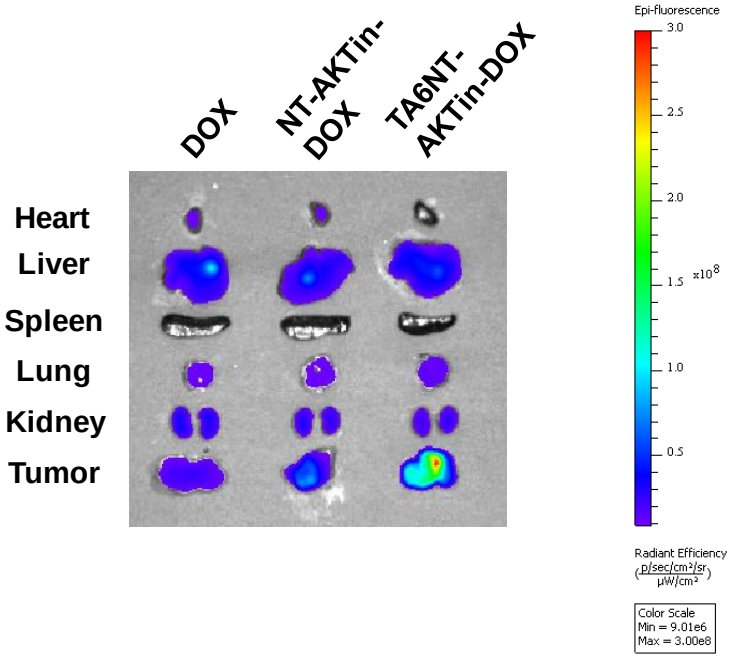


Figure S6. The measured DOX amounts in (A) the whole cell and (B) nucleus isolated from NIH-3T3 cells (n = 3). (C) Cytotoxicity profiles of free DOX and TA6NT-AKTin-DOX for NIH-3T3 cells (n = 3). Two-tailed Student's t-test was used. ** $p < 0.01$.



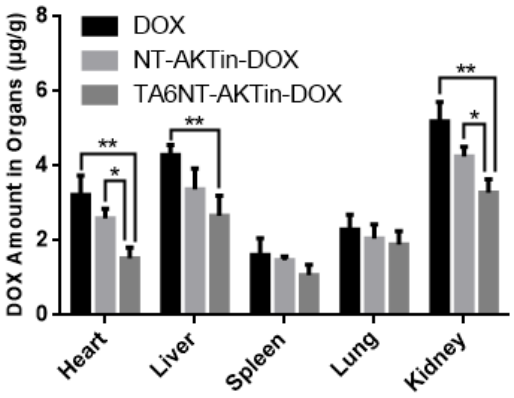


Figure S7. The measured DOX amounts in the organs of mice treated with DOX, NT-AKTin-DOX and TA6NT-AKTin-DOX. Two-tailed Student's t-test was used. * $p < 0.05$, ** $p < 0.01$.

Supplementary Methods

S1. Chemicals and Reagents

Maleimide modified peptide AVTDHPDRLWAWEKF (AKTin) was developed by ChinaPeptides Co., Ltd. (Shanghai, China). TA6-tethered trigger, M1, M2 and fluorescein isothiocyanate-labeled TA6-tethered trigger were synthesized by Sangon Biotechnology Co., Ltd. (Shanghai, China). Doxorubicin hydrochloride (DOX·HCl) was purchased from Hisun Pharmaceutical Co., Ltd. (Zhejiang, China). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT), D-(+)-mannitol and *N*-(2-hydroxyethyl) piperazine-*N'*-(2-ethanesulfonicacid) (HEPES) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Trypan blue was obtained from Generay Biotech Co., Ltd (Shanghai, China). MammoCult™ Human Medium Kit, Hydrocortisone Stock Solution and Heparin Solution were purchased from STEMCELL Technologies Inc. (Vancouver, BC, Canada). Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were obtained from Invitrogen (Burlington, ON, Canada). Penicillin/streptomycin solution was supplied by HyClone Laboratories, Inc. (Logan, Utah, USA). Ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) was obtained from Sinopharm Chemical Reagent Company (Shanghai, China). Phosphate buffered saline (PBS) was purchased from Beyotime Institute of Biotechnology (Jiangsu, China). Acetonitrile (ACN) and methanol were HPLC grade and were purchased from ROE Inc. (Newark, NJ, USA). Formic acid (FA) was purchased by Xilong Chemical Industrial Factory Co., Ltd (Shantou, China). Water was purified and deionized using a Milli-Q system from

Millipore (Bedford, MA, USA).

S2. Cell Culture

$CD44^+/CD24^-$ cells sorted from MCF-7 cells (MCF-7 BCSCs) were kindly donated by Dr. Yongmei Yin (Department of Oncology, the First Affiliated Hospital of Nanjing Medical University, China). MCF-7 BCSCs were maintained routinely in complete mammosphere culture medium supplemented with 4 μg/mL heparin solution and 0.48 μg/mL hydrocortisone stock solution at 37°C under 5% CO_2 atmosphere. NIH-3T3 cells (ATTC, Manassas, VA) were cultured in DMEM supplemented with 10% FBS. Cells were counted with a hemocytometer (Qiujing, Shanghai, China). Cell viability was assessed by trypan blue (0.4%) exclusion, which was completed by mixing cell suspension, trypan blue and 1 × PBS in a ratio of 2:5:3 and the percentage of viable cells was counted following 5 min incubation at 37°C. Notably, the cells were kept up to 5 passages to ensure the presence of the cell stem properties.[1]

S3. Flow Cytometry

To characterize BCSCs, 1×10^6 of BCSCs were stained with 1 μL anti-CD44-PE antibody (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) and 1 μL anti-CD24-APC antibody (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) in PBS at 4°C for 25 min. Cells were then washed three times with PBS and analyzed on a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). The

data were analyzed using the Cell Quest software (BD Biosciences, San Jose, CA).

To validate the recognition ability of TA6-tethered trigger, fluorescein isothiocyanate-labeled TA6-trigger (200 nM) was separately incubated with 1×10^6 of BCSCs or NIH-3T3 cells at 37°C for 2 h. Cells were then washed three times with PBS and analyzed on the FACScan flow cytometer. The data were analyzed using the Cell Quest software.

S4. LC-MS/MS

We used an Agilent Series 1290 UPLC system (Agilent Technologies, Waldbronn, Germany) and a 6460 Triple Quad LC-MS mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) for LC-MS/MS analysis. The liquid chromatography separation was performed on a hypersil gold column (3 μm, 20 mm × 2.1 mm; Thermo Fisher Scientific, USA) with an injection volume of 10 μL at room temperature. The mobile phase consisted of solvent A (water with 0.1% FA) and solvent B (ACN) at a ratio of 75:25. The flow rate was 0.3 mL/min.

The mass spectrometer was interfaced with an electrospray ion source and positive MRM mode was operated. Q1 and Q3 were both set at unit resolution. The temperature of drying gas was held at 350°C and its flow rate was 10 L/min. Nebulizer pressure was optimized to 35 psi, while electrospray capillary voltage was set to 4000 V. Data were collected and analyzed using Agilent MassHunter Workstation Software (version B.01.04).

S5. Bliss Synergy Calculations

The Bliss independence (BI) model is used to define the effect of two drugs assumed to act through independent mechanisms. BI is described by the equation $E_i = (E_A + E_B) - (E_A \times E_B)$, where Ei is the predicted effect (percentage of inhibition in this study) by the combination of drugs A and B if they were to act additively and independently, and E_A and E_B are the observed effects of each drug alone, respectively. When observed inhibition exceeds predicted inhibition, the two compounds are considered to act synergistically.

Reference

1 Lin X, Chen W, Wei F, et al. POMC maintains tumor-initiating properties of tumor tissue-derived long-term-cultured breast cancer stem cells. *Int J Cancer.* 2017; 140(11): 2517-2525.