Supplementary Data:

Lectin-mediated pH-sensitive doxorubicin prodrug for pre-targeted chemotherapy of colorectal cancer with enhanced efficacy and reduced side effects

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Supplementary methods:

Materials

Chemicals such as doxorubicin hydrochloride, hydrazine hydrate, D-Luciferin, succinic acid, tricine, and trisodium triphenylphosphine-3, 3', 3''-trisulfonate (TPPTS) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Pierce avidin and hydrazine hydrate was purchased from the Thermo Company (Waltham, MA, USA) and stored at -20 ℃. The (+)-Biotin-PEG_4-hydrazide was purchased from the Biomatrik Company (Zhejiang, China). The $Na^{99m}TcO_4$ was obtained from a commercial $^{99}Mo/^{99m}Tc$ generator (Beijing Atom High Tech Co., Ltd.), and 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC) and N-Hydroxysulfosuccinimide sodium salt (NHS-SO_3^-) were dissolved in distilled water before use. All organic solvents were purchased from the Beijing Chemical Works and used as received. Water with a resistivity of 18.2 mΩ·cm from a Milli-Q reference Water Purification System (Millipore) was used for reactions and solution preparation. Dulbecco's modified Eagle's medium (DMEM), trypsin/ethylenediamine tetra-acetic acid (EDTA), and penicillin/streptomycin were obtained from Hyclone (USA).

High-performance liquid chromatography (HPLC) (Agilent Technologies, Santa Clara, California, United States) was performed on a LabAlliance HPLC system equipped with a UV/Vis detector (λ = 220 nm and 485 nm) and YMC C-18 column (5 μm, 250 × 4.5 mm). The flow rate was 1.0 mL/min. A gradient of H_2O/26 mM NH_4OAc buffer (phase A, pH 7.4) and acetonitrile (phase B) was used. The UV-vis

spectra were obtained using a Varian Cary 300 UV-visible spectrometer, and the fluorescence spectra were obtained using a Cary Eclipse spectrometer. Cell viability was tested using a colorimetric cell-counting kit 8 (CCK-8) assay (Dojindo Laboratories, Kyoto, Japan). Cell uptake and subcellular localization were observed by confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany). Bioluminescence imaging was taken on IVIS Spectrum (IVIS, Waltham, Massachusetts, USA).

Cell culture

HT-29 and LS180 human colorectal cancer cell lines were obtained from American Type Culture Collection (Manassas, VA), and HEK293 human embryonic kidney cell line was obtained from National Infrastructure of Cell Line Resource of China. HT-29 cells and LS180 cells were transcribed for their luciferase genes and were cultivated in DMEM HIGH GLUCOSE medium (Invitrogen, Carlsbad, CA, USA) containing 10% FBS, 100 U/mL penicillin G sodium, and 100 μg/mL streptomycin sulphate. HEK293 cells were cultivated in DMEM HIGH GLUCOSE medium as mentioned above. Cells were incubated at 37 ℃ in a humidified atmosphere containing 5% CO_2.

Animal preparation and tumour model establishment

Female BALB/c nude mice (5 week of age) and female BALB/c mice (5 week of age) were purchased from the Department of Experimental Animals, Peking University Health Science Center. The LS180 tumour model was generated by intraperitoneal injection of 5×10^6 LS180 cells. Animals were fed in a specific pathogen-free (SPF) laboratory animal facility. All operations were according to the

guidelines of the Institutional Animal Care and Use Committee at Peking University.

Radiochemistry of biotin-PEG$_4$-C$_2$H$_4$-NH$_2$

The aqueous solution of biotin-PEG$_4$-C$_2$H$_4$-NH$_2$ (7.19 mg into 100 μL water) and the organic solution of HYNIC-NHS (8.98 mg into 200 μL DMF) were mixed, and DIEA adjusted the pH to 9.0. The reaction was continued for 24 h at room temperature and the solution was purified using HPLC and determined by mass spectra. The product HY-biotin was obtained by freeze drying (10 mg, 67%).

HY-biotin was labeled with ^{99m}Tc using tricine and trisodium triphenylphosphine-3,3',3''-trisulfonate (TPPTS) as chelating agents [1]. The radiochemical purity of ^{99m}Tc-biotin determined by radio-HPLC system was more than 98%.

Affinity detection

The affinity between avidin and bDOX was detected *in vitro*. Avidin solution (7.35 pmol in 100 μL per well) was incubated on a Elisa high adsorption plate overnight at 4 ℃, and then the plate was washed by PBST buffer three times. 1% BSA was incubated followed by for 4 h at 4 ℃ in order to block nonspecific sites. Then washing was operated again. The experiment was divided into four groups (n = 4). In the first group, 1% BSA and superfluous biotin (73.5 pmol in 100 μL) were added; 100 μL 1% BSA was added in group two; 100 μL 1% BSA and superfluous IgG were added in group three; and 100 μL 1% BSA and superfluous vitamin C were added in group four. The block agents were incubated for 0.5 h at 37 ℃. Finally, ullage bDOX (7.35 pmol in 100 μL per well) was added into four groups and incubated for 1 h at 37 ℃. The supernate was taken out and added into a 96-well plant, and OD value of 480/580 nm (excitation/emission) was detected by multiscan spectrum instrument.

Flow Cytometry

The lectin expression levels of different cell lines were compared by flow cytometry according to literature [2]. Briefly, different kinds of cells (HT-29, LS180, HEK293 cell lines) were seeded in 6-well plates, and incubated with FITC-labelled avidin, or FITC-labelled neutravidin for 48 h. Cells of control group were not

incubated with any fluorescent dye. Flow cytometry was performed using a FACSCalibur Flow Cytometer (BD, USA). The percentage of positive cells was analyzed using Flowjo software.

To test which phase of cell cycle that pre-targeted bDOX arrest, cell cycle analysis was performed. HT-29 cells were plated in a six-well plate at a density of 1×10^7 cells per well and pre-targeted bDOX was added. Cells were harvested at 12 h and 24 h and fixed in cold 70% ethyl alcohol. 1 mg/mL RNase I for 0.5 h and 20 μg/mL propidium iodide for 10 min were incubated on cells at room temperature. Then cell cycle was analyzed using a FACSCalibur Flow Cytometer.

Supplementary Data:

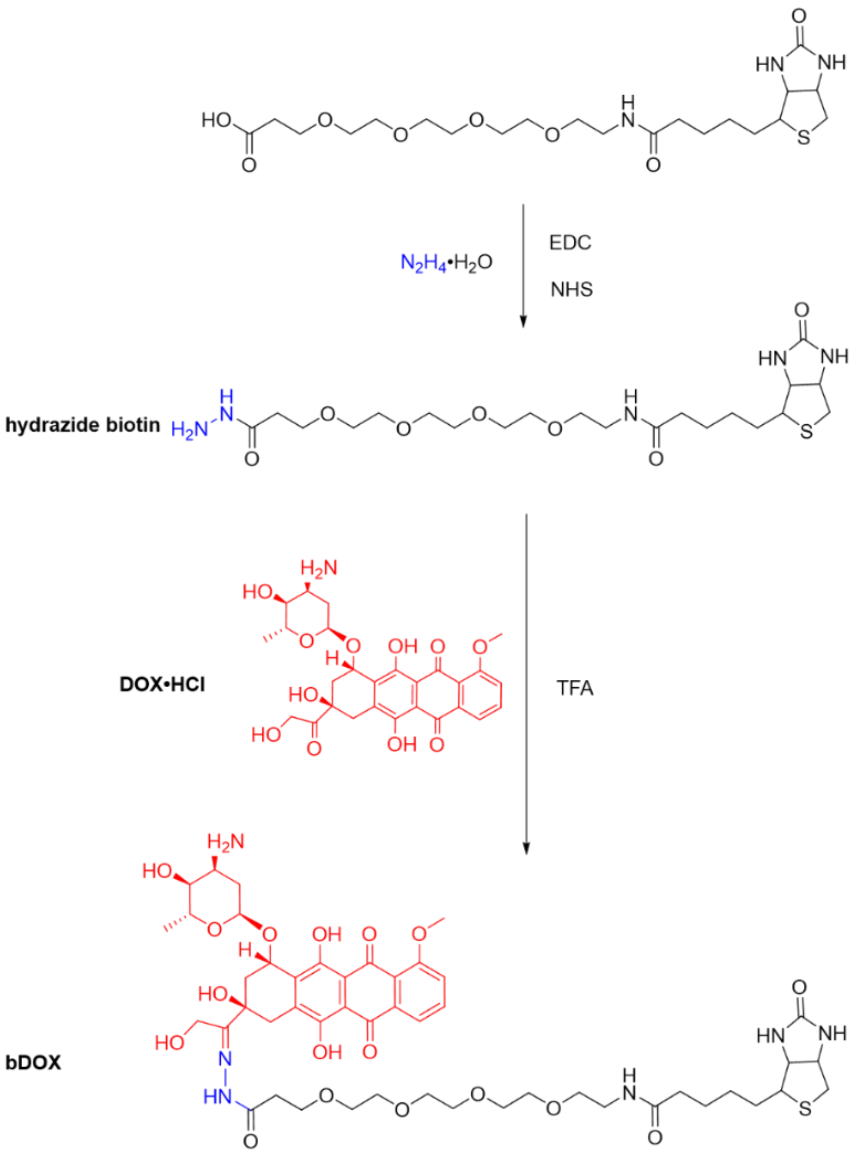

Figure S1. The synthesis route of bDOX.

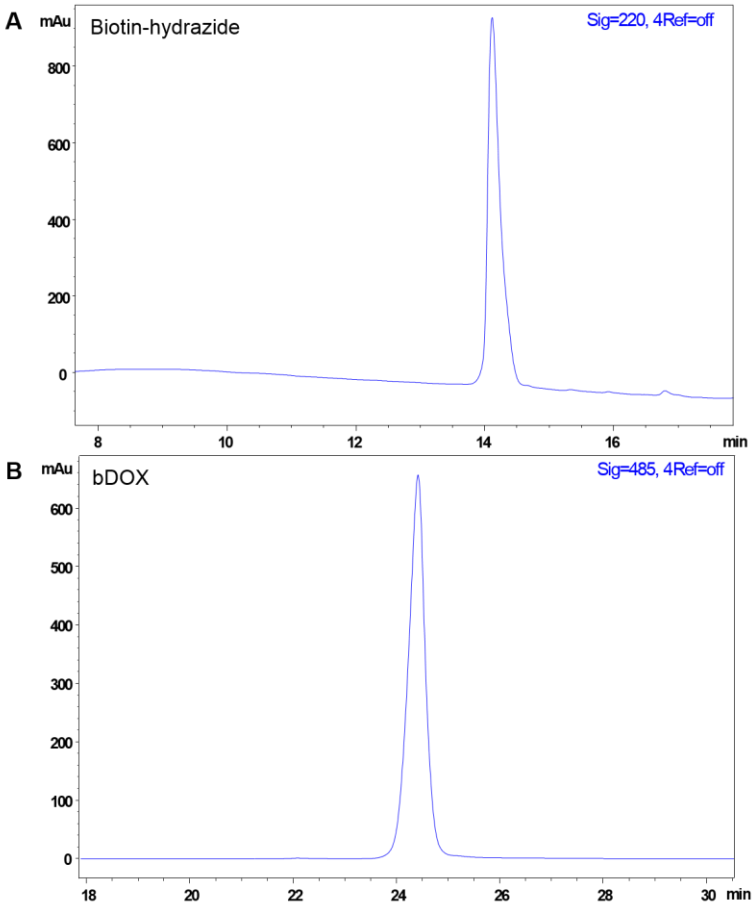

Figure S2. HPLC analysis of (A) Biotin-hydrazide monitored at the wavelength of 220 nm and (B) bDOX at the wavelength of 485 nm on a C18 column.

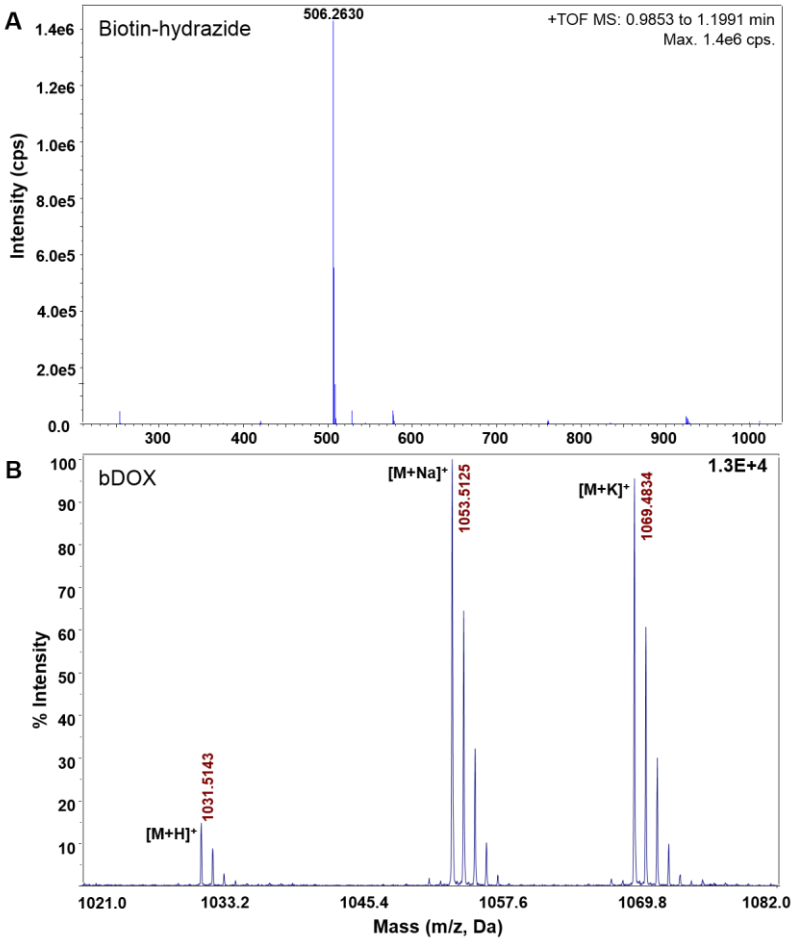

Figure S3. (A) MALDI-TOF mass spectrum of biotin-hydrazide: m/z $[M+H]^+$ = 506.2630. (B) High resolution MALDI-TOF mass spectrum of bDOX: m/z $[M+H]^+$ = 1031.5143, m/z $[M+Na]^+$ = 1053.5125, m/z $[M+K]^+$ = 1069.4534.

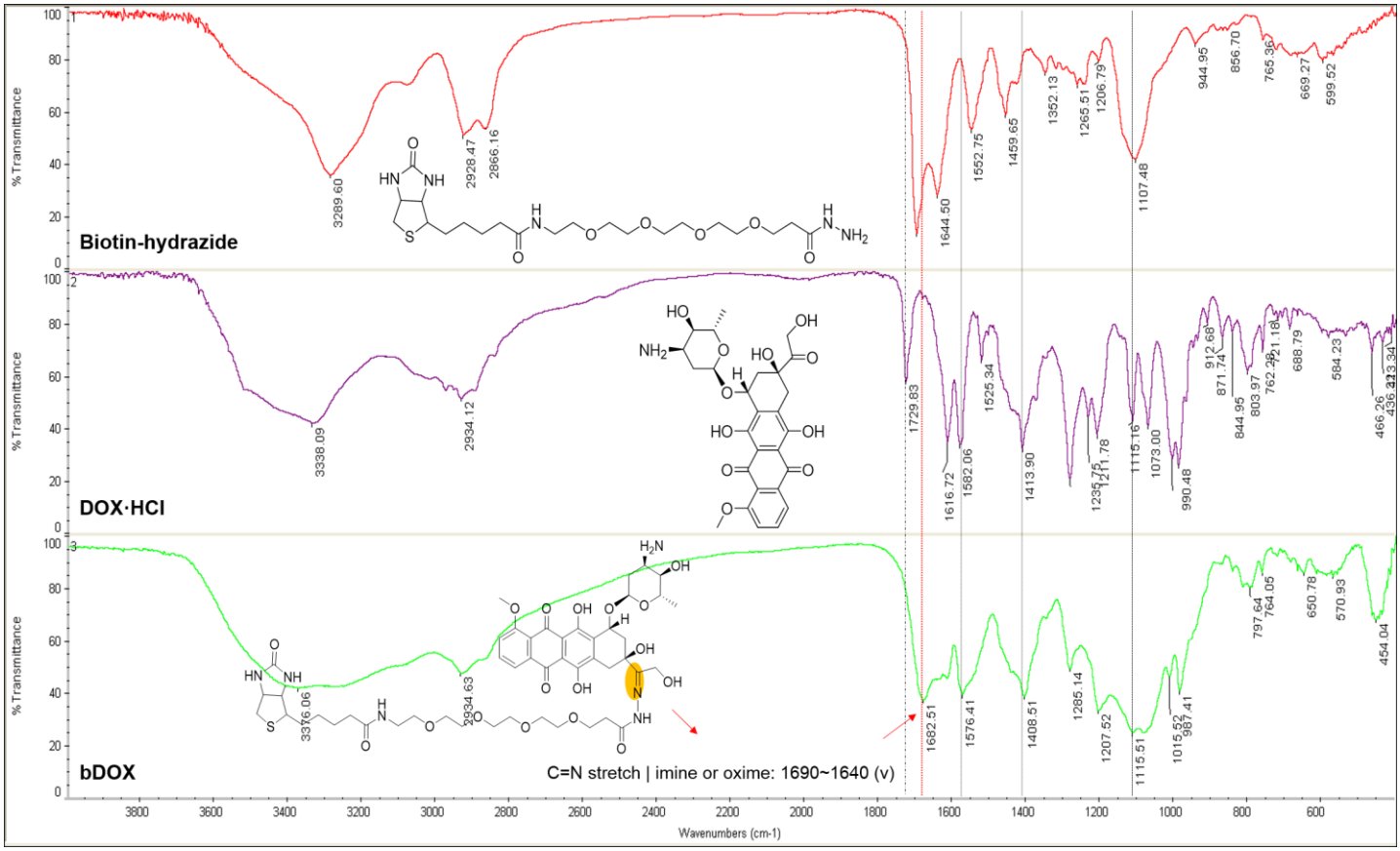

Figure S4. FTIR spectrum of biotin-hydrazide, DOX•HCl and bDOX.

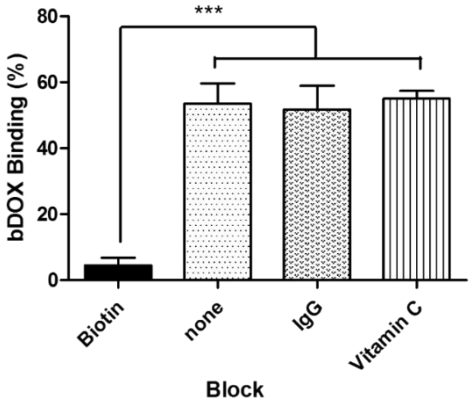

Figure S5. Verification of the affinity between bDOX and avidin. Vertical axis represents the percentage of bDOX binding to avidin after the block of biotin, nothing, IgG or vitamin C. The values represent mean ± SD (n = 3).

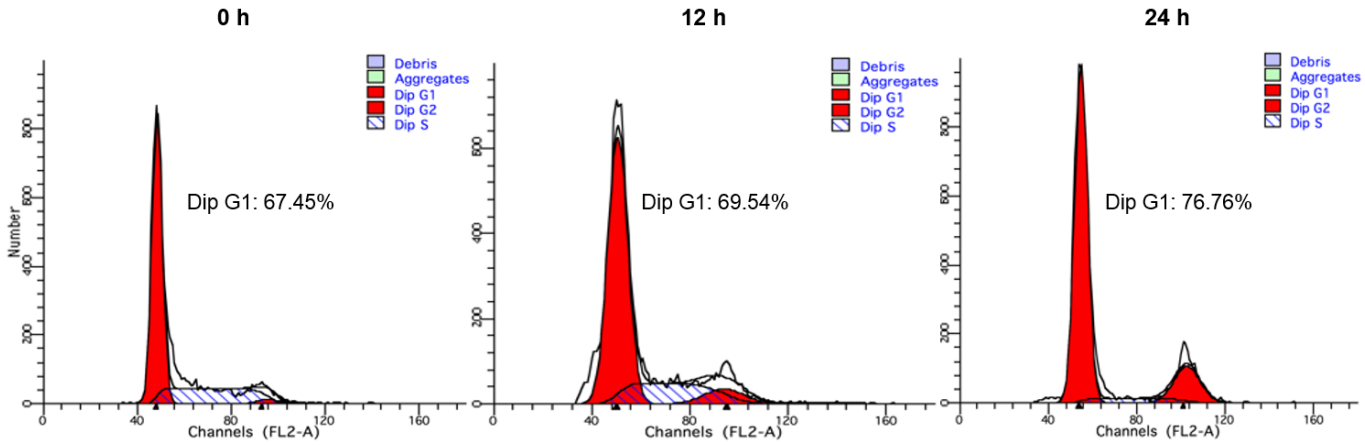

Figure S6. Cell cycle examination of HT-29 cells analyzed by flow cytometry before (A) and 12 h (B), 24 h (C) after treatment with pre-targeted bDOX.

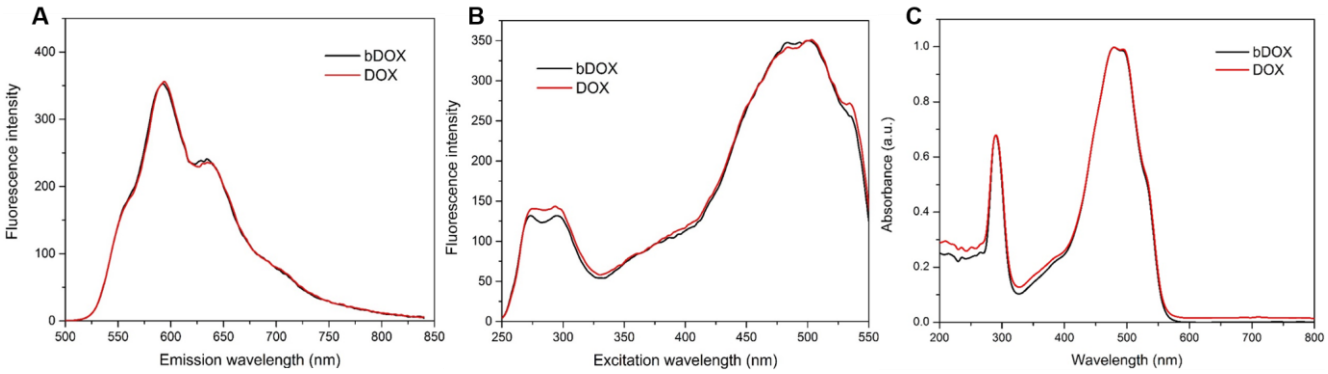

Figure S7. (A) The fluorescence emission of DOX and bDOX, the fluorescence excitation wavelenth was fixed as 480 nm. (B) The fluorescence excitation of DOX and bDOX, the fluorescence emission wavelenth was fixed as 580 nm. (C) The ultraviolet absorption of DOX and bDOX.

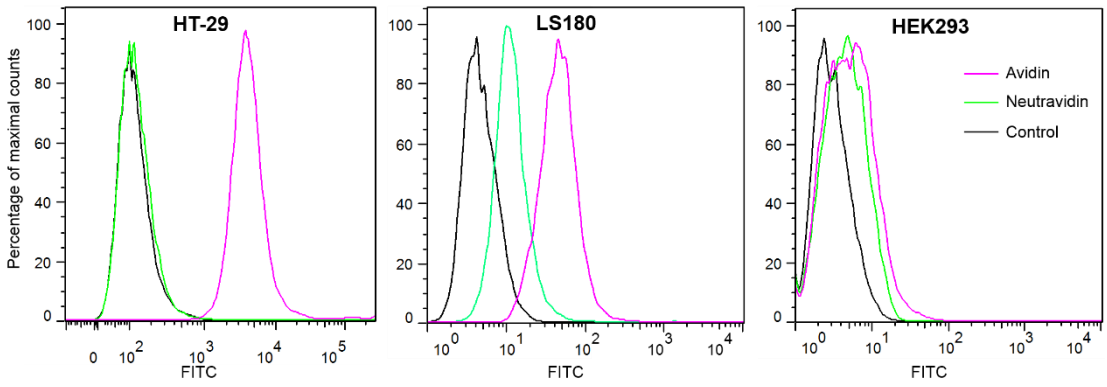

Figure S8. The lectin expression levels of different cell lines (HT-29, LS180, and HEK293 cell lines) examined by flow cytometry. Cells were incubated with FITC-labelled avidin (pink), or FITC-labelled neutravidin (green), or nothing of control group (black). The percentage of avidin-FITC-accumulated cells corresponded to the increase of FITC-labelled avidin's fluorescence, which was 95.4% of HT-29 cells, 39.7% of LS180 cells, and 1.38% of HEK293 cells.

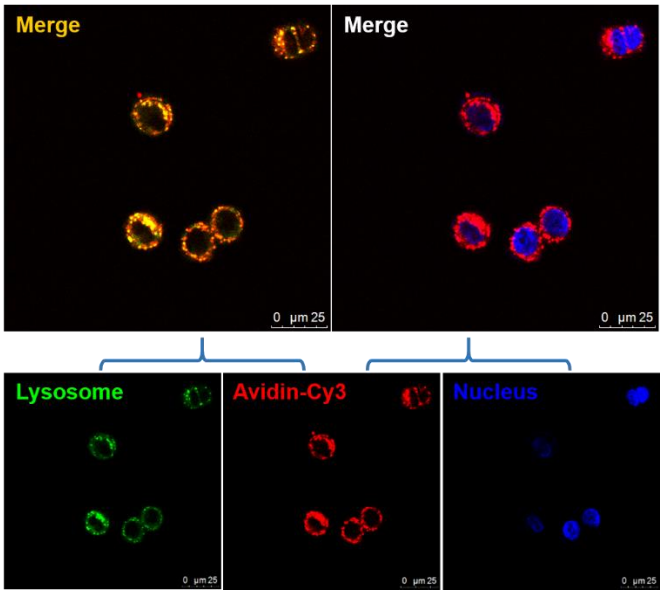

Figure S9. Subcellular distribution of Cy3-labelled avidin. Lysosomes were stained by LysoTracker^{TM} Deep Red and colored in green, nucleus were stained by Hoechst 33342 and colored in blue, and dye Cy3 was colored in red.

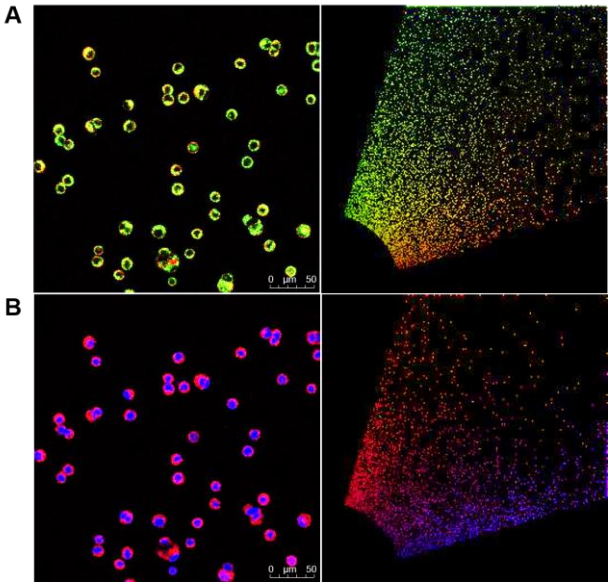

Figure S10. Subcellular distribution of pre-targeted bDOX performed on HT-29 cells. (A) Colocalization rate of bDOX (colored in red) and lysosome (colored in green). (B) Colocalization rate of DOX (hydrolyzed from bDOX) and nucleus (colored in blue). Cells were incubated with avidin 4 h before the addition of bDOX.

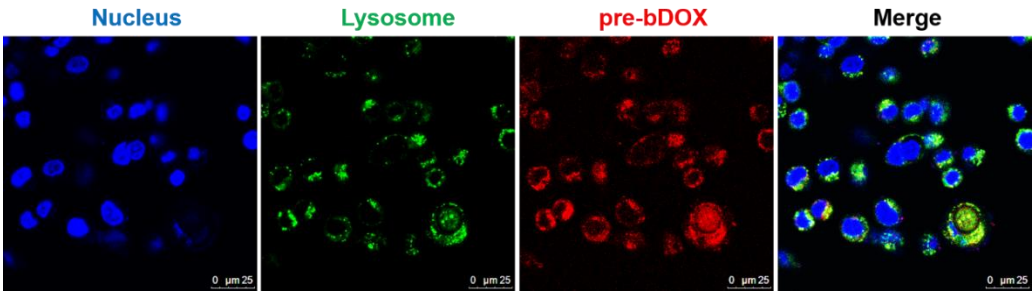

Figure S11. Colocalization of bDOX and lysosome in LS180 cells. Blue color represented DAPI stained nucleus, pseudo-green color for LysoTracker™ Deep Red stained lysosomes, and pseudo-red color for bDOX.

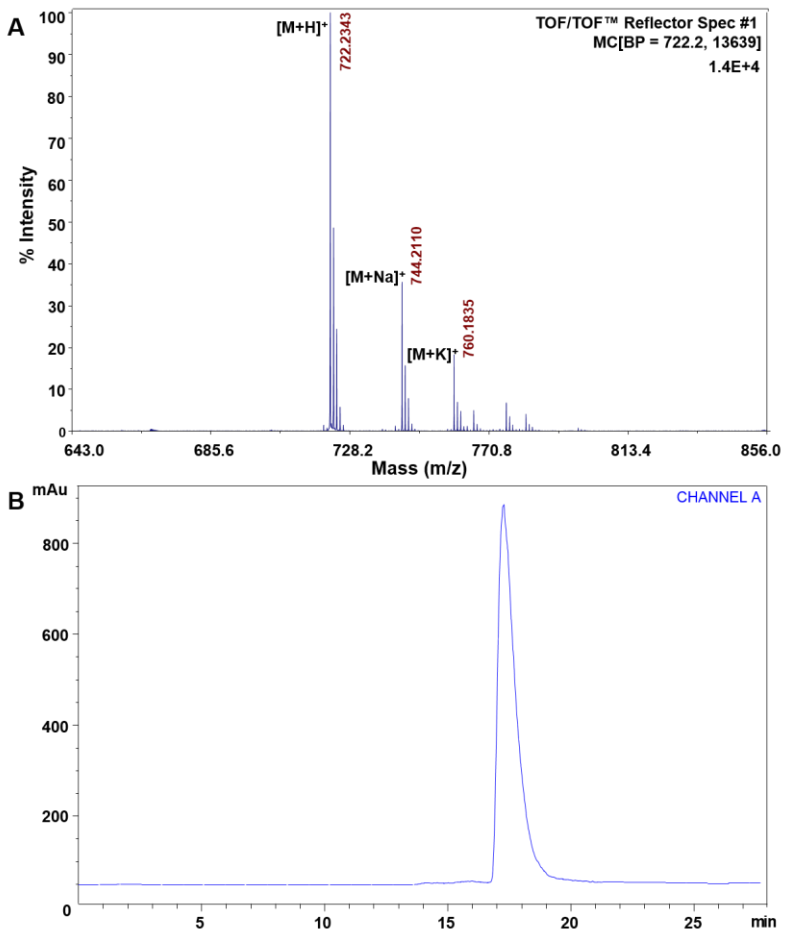

Figure S12. (A) High resolution MALDI-TOF mass spectrum of HY-biotin: m/z $[M+H]^+$ = 722.2343, m/z $[M+Na]^+$ = 744.2110, m/z $[M+K]^+$ = 760.1835. (B) Radio-HPLC analysis of ^{99m}Tc-biotin at channel A on the YMC C-18 column (5 μm, 250 × 4.5 mm) using a 0-80% gradient elution system. The aqueous phase was H_2O (containing 0.05% TFA) and the organic phase was acetonitrile.

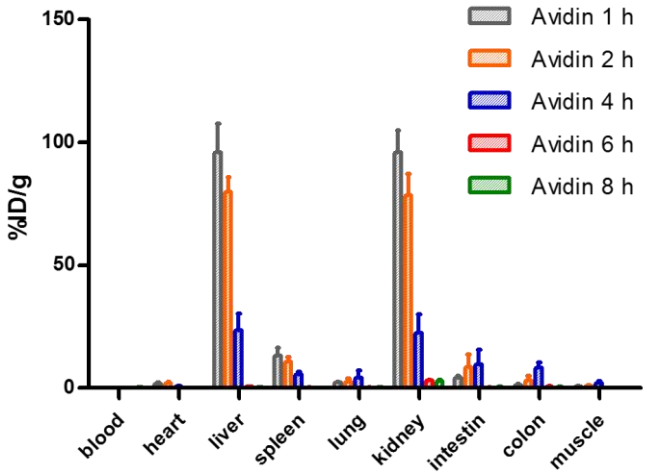

Figure S13. Biodistribution of ^{99m}Tc-labelled biotin in different organs. Avidin was intraperitoneally pre-injected at different time (1, 2, 4, 6, 8 h) before the injection of ^{99m}Tc-biotin, and 2 h followed by the radioactivity measurement. The values represent mean ± SD (n = 4).

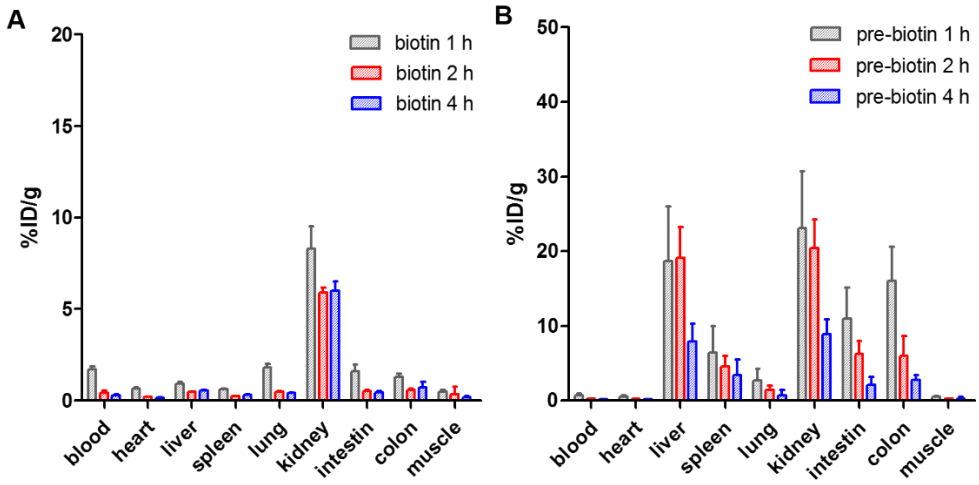

Figure S14. Biodistribution of ^{99m}Tc-biotin measured at different time (1, 2, 4 h) after its injection. PBS (A) or Avidin (B) was intraperitoneally injected 5 h before the injection of ^{99m}Tc-biotin. The values represent mean ± SD (n = 4).

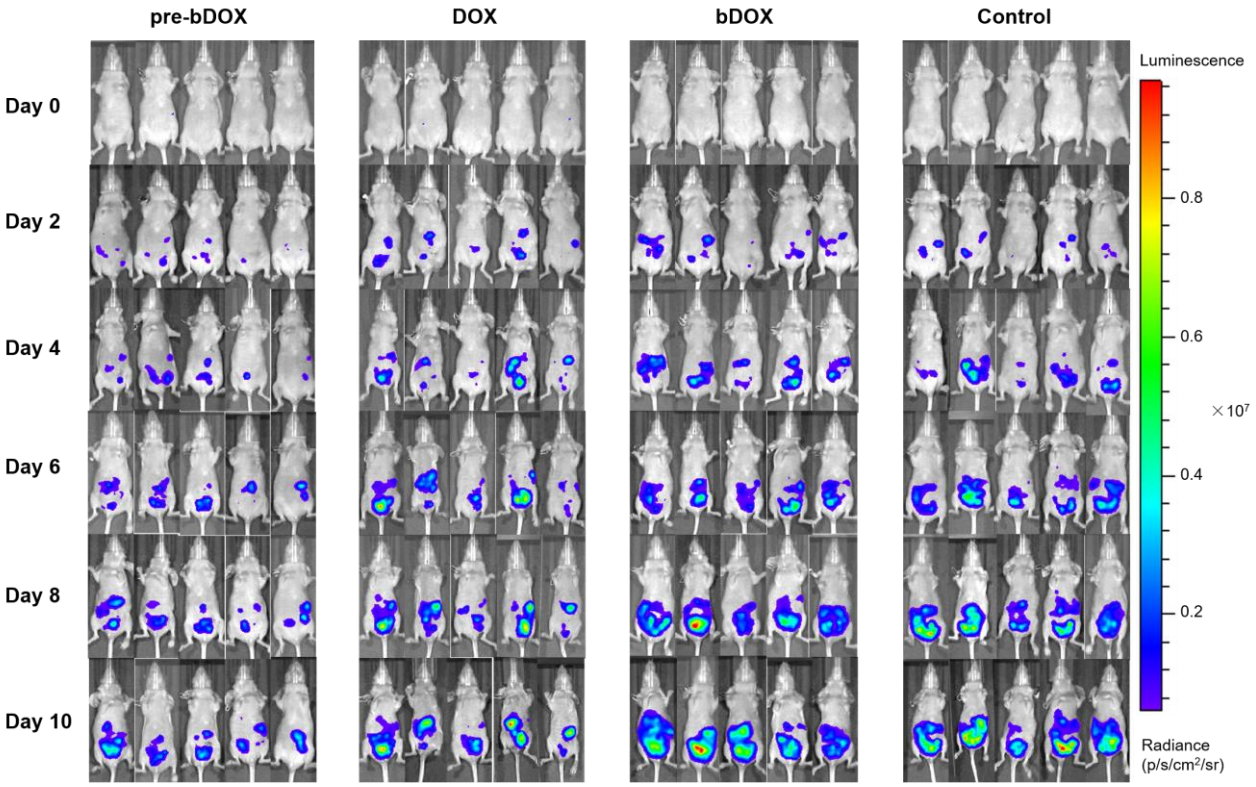

Figure S15. Bioluminescence imaging of all the LS180-bearing nude mice on different days after various treatments, exhibiting their relative tumor growth states.

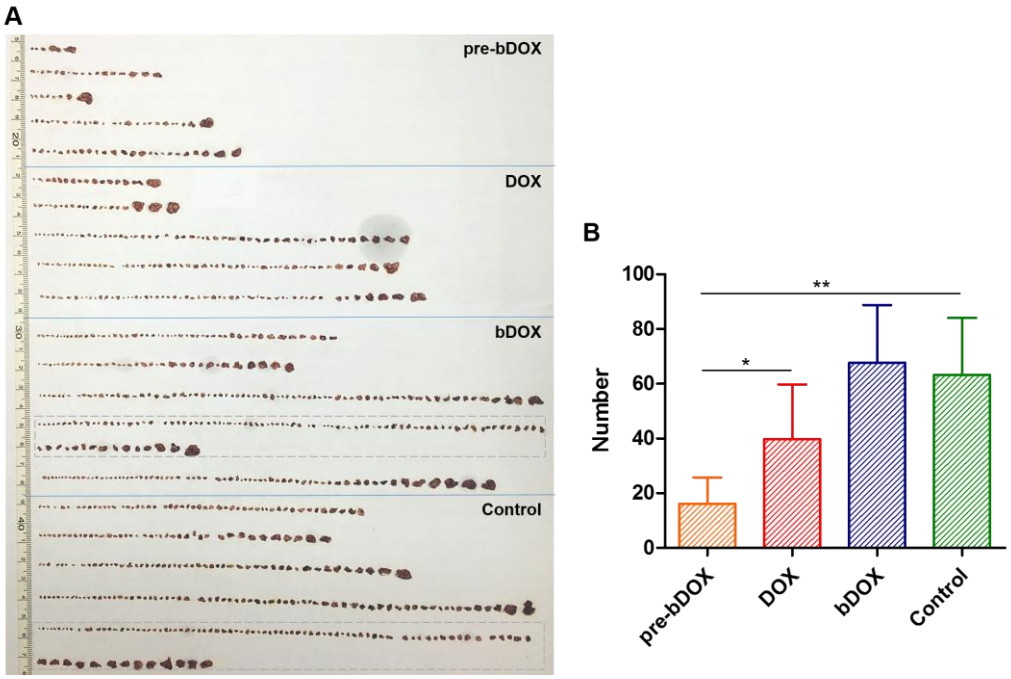

Figure S16. (A) Photograph of every individual tumors from different groups mice with various treatments. Tumor was collected on day 10 after *in vivo* chemotherapeutic evaluation. Each row of tumors were collected from one mouse and

there were five mice in each group. (B) Average tumor number per mouse of different groups calculated from (A). The values represent mean ± SD (n = 5).

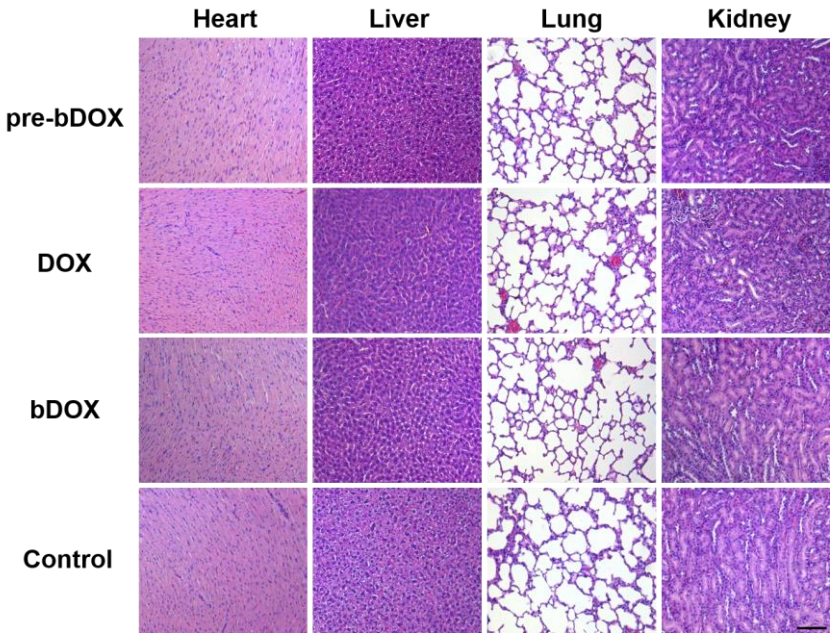

Figure S17. H&E stained tissue sections of major organs after various treatments. Tissue was collected after *in vivo* chemotherapeutic evaluation (day 10). The scale bar represented 200 μm.

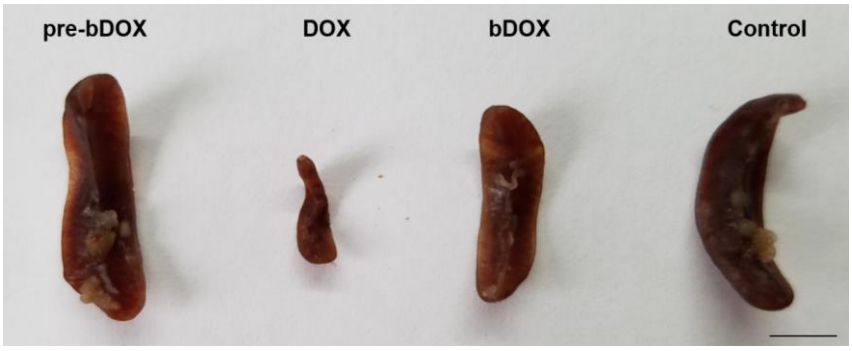

Figure S18. Photograph of representative spleens collected from different groups after therapy (day 10). The scale bar represents 1 cm.

Table S1. IC_{50} values of DOX and pre-targeted bDOX against HT-29, LS180 and HEK293 cells determined by CCK-8 assay.

IC_{50} values (μM)					
HT-29		LS180		HEK293	
pre-bDOX	DOX	pre-bDOX	DOX	pre-bDOX	DOX
0.6679	1.457	10.69	40.96	11.41	2.34

Table S2. (A) Parameters showing the level of colocalization between pre-targeted bDOX and lysosomes. (B) Parameters showing the level of colocalization between pre-targeted bDOX and nuclei.

A

Pearson's Correlation	Overlap Coefficient	Colocalization Rate	Colocalization Area	Area Image	Area Foreground	Area Background
0.7415	0.7530	75.67%	3685.40 μm^2	150156.25 μm^2	4870.52 μm^2	145285.73 μm^2

B

Pearson's Correlation	Overlap Coefficient	Colocalization Rate	Colocalization Area	Area Image	Area Foreground	Area Background
0.2960	0.3342	24.03%	1721.27 μm^2	150156.25 μm^2	7161.73 μm^2	142994.52 μm^2

Movie S1. was seen in the attachment.

Movie S1. 3D image of subcellular distribution of bDOX. Avidin was also pre-added before bDOX. Lysosome and Nucleus were stained by LysoTracker™ Deep Red and Hoechst 33342 respectively.

Reference

1. Zhang X, Yao MN, Chen MH, Li LQ, Dong CY, Hou Y, et al. Hyaluronic Acid-Coated Silver Nanoparticles As a Nanoplatform for in Vivo Imaging Applications. Acs Appl Mater Inter. 2016; 8: 25650-3.
2. Hama Y, Urano Y, Koyama Y, Choyke PL, Kobayashi H. Targeted optical imaging of cancer cells using lectin-binding BODIPY conjugated avidin. Biochem Bioph Res Co. 2006; 348: 807-13.