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Light-Activated Drug Release from Hyaluronic Acid-Targeted Nanoconjugate for Cancer Therapy

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Materials and Methods

Materials and characterization

Hyaluronic acid (Mw = 50 kDa) was purchased from Nanjing Sunlida Biological Technology Co., Ltd. (China). Chlorin e6 (Ce6) was obtained from J&K Chemical Ltd.. Doxorubicin (DOX) was purchased from Wuhan Dahua Co. Ltd.. 1-Ethyl-3(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC·HCl), sulfo-N-hydroxysulfosuccinimide (sulfo-NHS), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma-Aldrich Chemical Co., Ltd. Synthesis of the ROS-cleavable 2,2'-(propane-2,2-diylbis(sulfanediyl))bis(ethan-1-amine) (PDSE) was performed as previously reported.[1] Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Life Technologies Corporation (Gibco, USA). 1,7-Diaminoheptane was purchased from TCI Shanghai Development Co., Ltd. All other reagents and solvents without statement were of analytical grade and used as received.

The proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded in D_2O/ d_6-DMSO cosolvent (1:1 v/v) on a 400 MHz spectrometer (Avance III, Bruker, Germany). The size and zeta potential measurements were carried out in aqueous solution using a Malvern ZS90 dynamic light scattering instrument with a He-Ne laser (633 nm) and 90° collecting optics. The data were analyzed using Malvern Dispersion Technology Software 5.10. Transmission electron microscope (TEM) measurements were performed on a JEOL 2010 high-resolution transmission electron microscope with an accelerating voltage of 200 kV.

Cells Lines and Xenograft Tumor Model

The human breast adenocarcinoma (MDA-MB-231) cells and NIH-3T3 murine fibroblast cells were obtained from the American Type Culture Collection (ATCC, MD, USA). cells were cultured in DMEM medium supplemented with 10% fetal bovine serum at 37 °C using a humidified 5% CO_2 incubator. The xenograft tumor model was generated by injection of 2×10^6 MDA-MB-231 cells (100 μL) with 20% Matrigel® Matrix (Corning, Bedford, MA) into the mammary fat pat of female BALB/c nude mice.

ROS production

The ROS generation upon 660-nm light exposure was detected by dichlorofluorescein diacetate. Ce6, $^{TK}HNCEP_{DOX}$ or $HCENP_{DOX}$ were incubated in phosphate buffer (PB, 20 mM) at pH 7.4. 2.0 mL of dichlorfluorescein diacetate in ethanol was mixed with 8.0 mL of NaOH aqueous solution (10 mM) for 60 min at rt. The nanoparticles were then treated with 660-nm light and the emission fluorescence change at 525 nm was recorded (λ_{ex} = 485 nm). Furthermore, laser-induced ROS generation *in vivo* was examined in MDA-MB-231 tumor-bearing nude mice according to previously reported method. [2]

***In Vivo* Biosafety Evaluation**

Mice were treated daily with various formulation for three days (equivalent DOX dose of 2.5 mg/kg), and then euthanized on the day 4. Serum was collected and mouse alanine aminotransferase (ALT), aspartate transaminase (AST), lactate dehydrogenase (LDH) and blood urea nitrogen (BUN) were measured using quantitative enzyme-

linked immunosorbent assay (ELISA) kits, following validation of each ELISA kit according to the manufacturer's instructions.

For organ damage analysis, mice were euthanized after the ELISA assay and major organs were collected and fixed in 4% paraformaldehyde overnight and finally embedded in paraffin. The paraffin-embedded organ tissues were cut and then stained with H&E and observed by Nikon TE2000U optical microscope.

Statistical analysis

The statistical significance of treatment outcomes was assessed using Student's *t*-test (two-tailed); $p < 0.05$ was considered statistically significant in all analyses (95% confidence level).

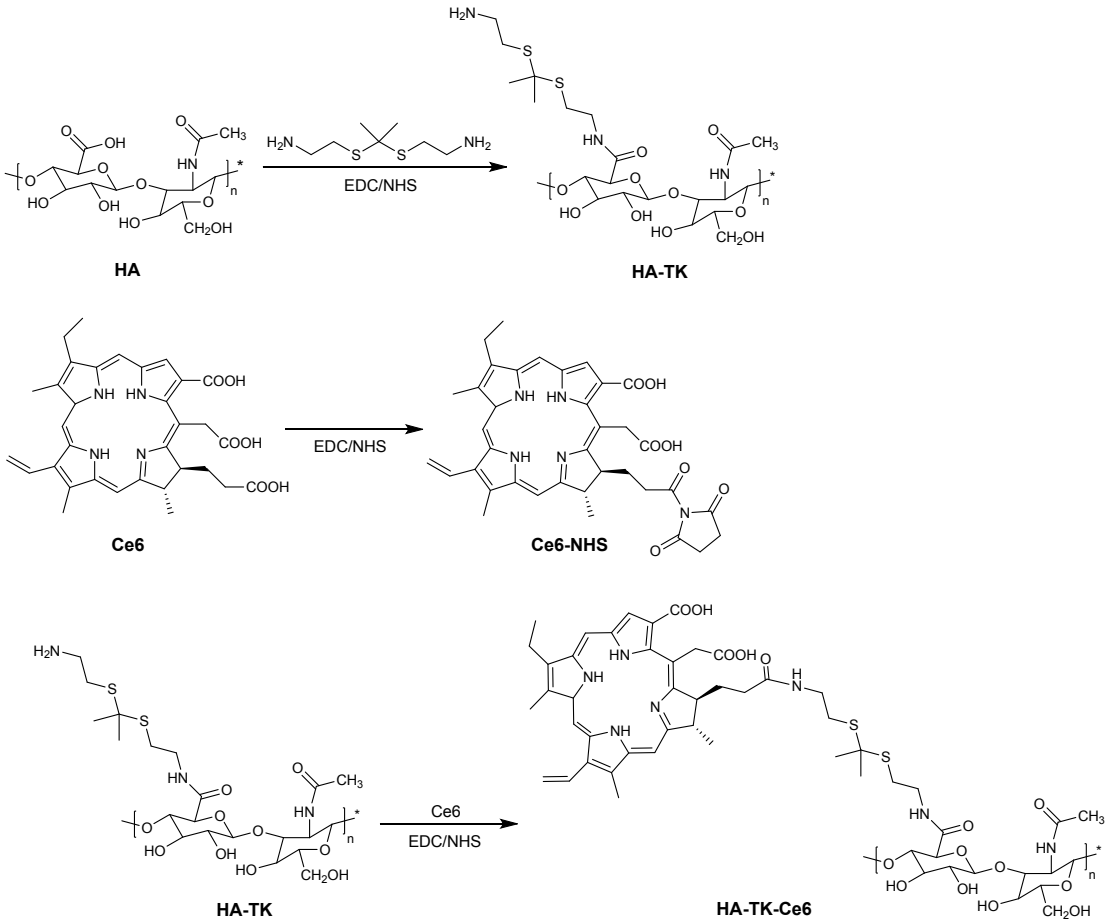

Figure S1. Synthetic route of HA-TK-Ce6 conjugates.

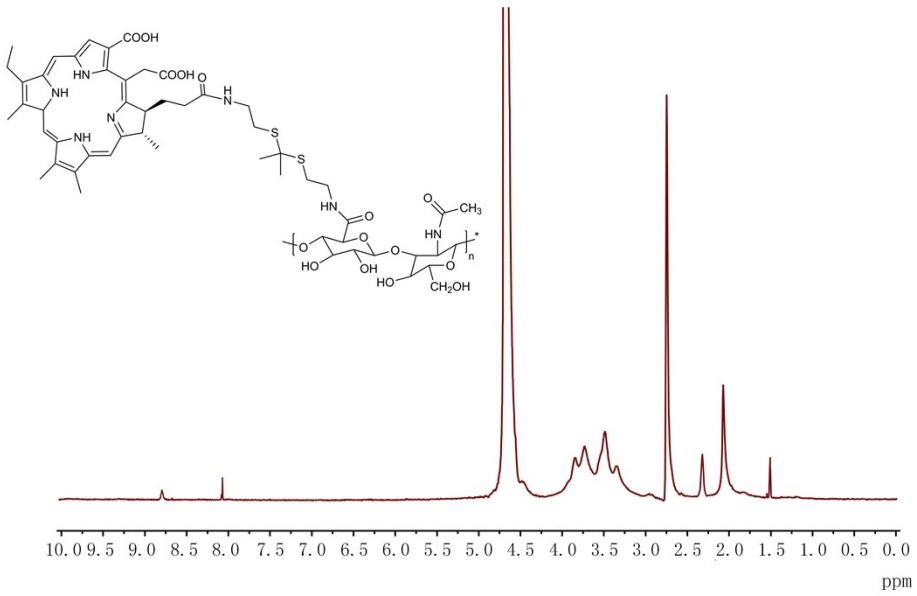

Figure S2. ^{1}H NMR spectrum of HA-TK-Ce6.

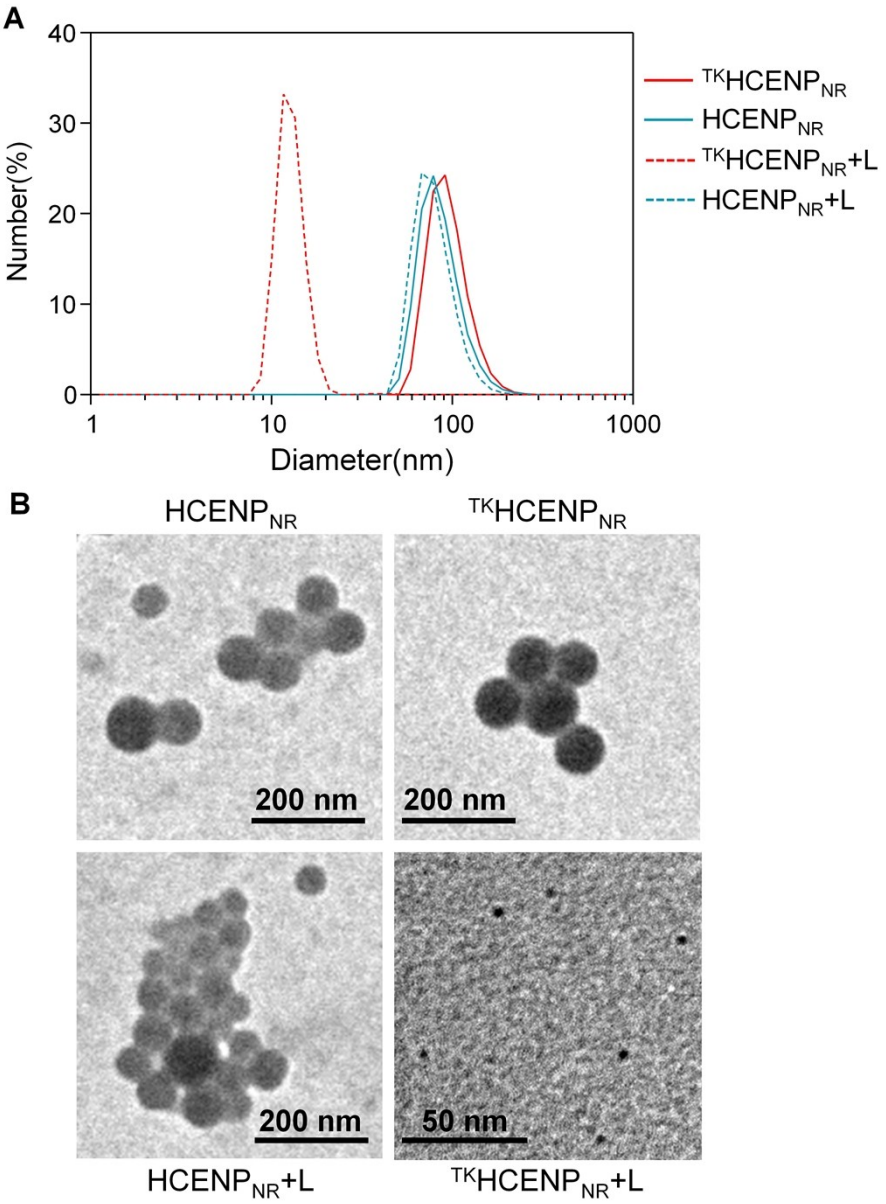

Figure S3. The diameter (A) and morphology (B) changes of $^{TK}HCENP_{NR}$ and $HCENP_{NR}$ with or without 660-nm laser irradiation.

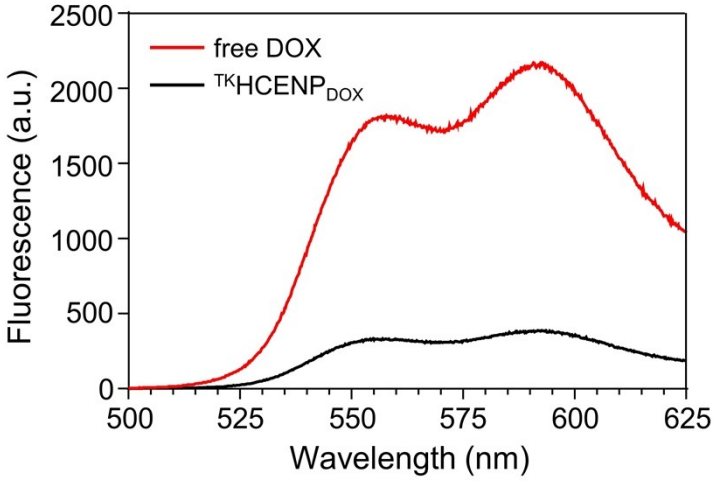

Figure S4. Emission spectra of free DOX or $^{TK}HCENP_{DOX}$ in aqueous solution (Ex=460 nm).

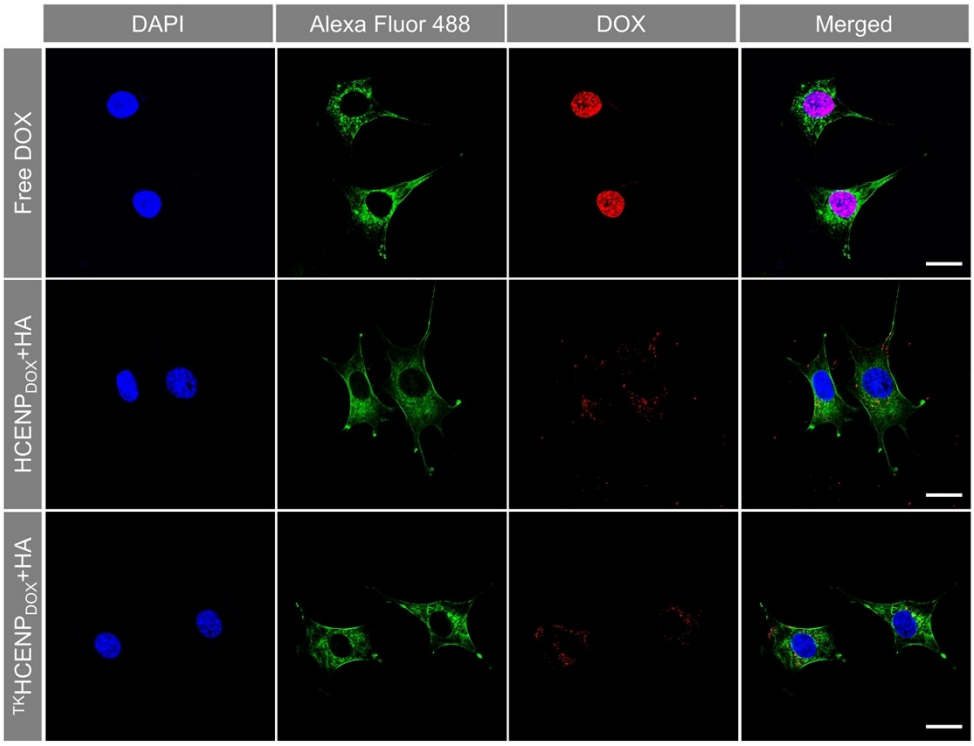

Figure S5. Cellular uptake and subcellular distribution of free DOX, $HCENP_{DOX}$ (with HA pretreatment) and $^{TK}HCENP_{DOX}$ (with HA pretreatment). DAPI (6-diamidino-2-phenylindole, blue) and Alexa Fluor 488 phalloidin (green) were used to stain cell nuclei and F-actin, respectively. The scale bar is 20 μm.

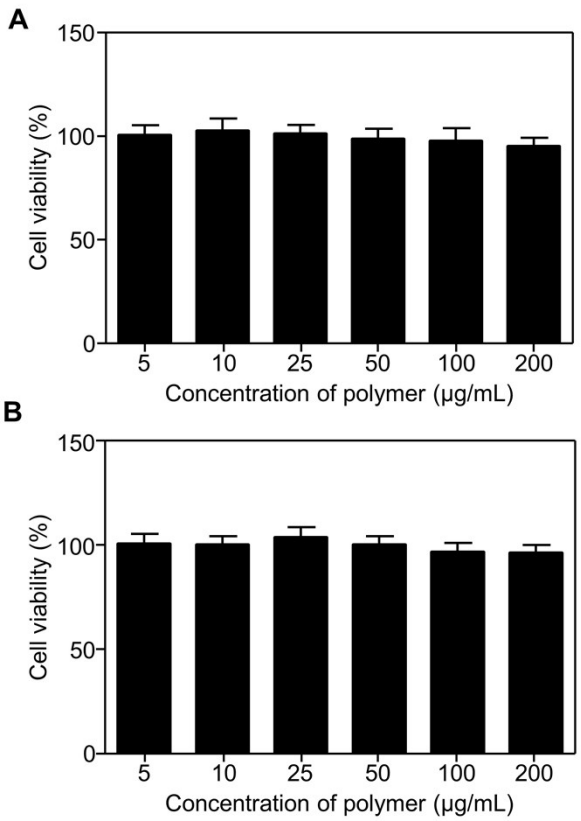

Figure S6. Cytotoxicity of $HCENP_{DOX}$ (A) and $^{TK}HCENP_{DOX}$ (B) against MDA-MB-231 cells for 72 h in dark.

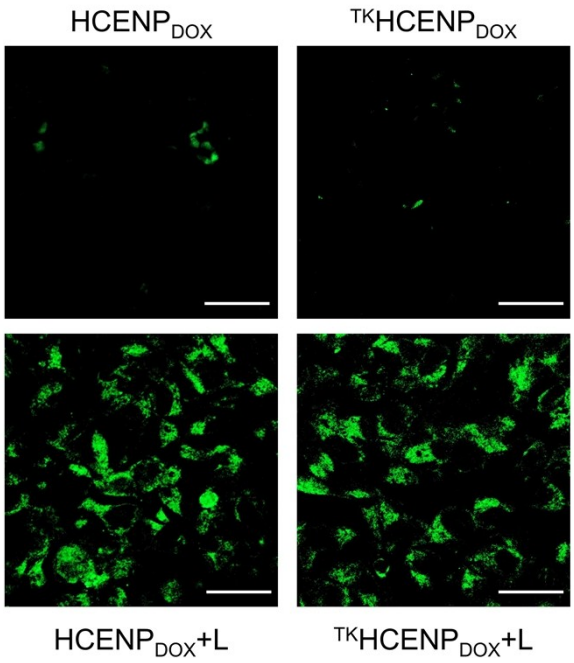

Figure S7. CLSM image of tumor tissues treated with HCENP$_{DOX}$ and ^{TK}HCENP$_{DOX}$ with or without 660 nm laser irradiation. DCF-DA was used as a ROS probe (DCF Ex = 488 nm). The scale bar is 20 μm.

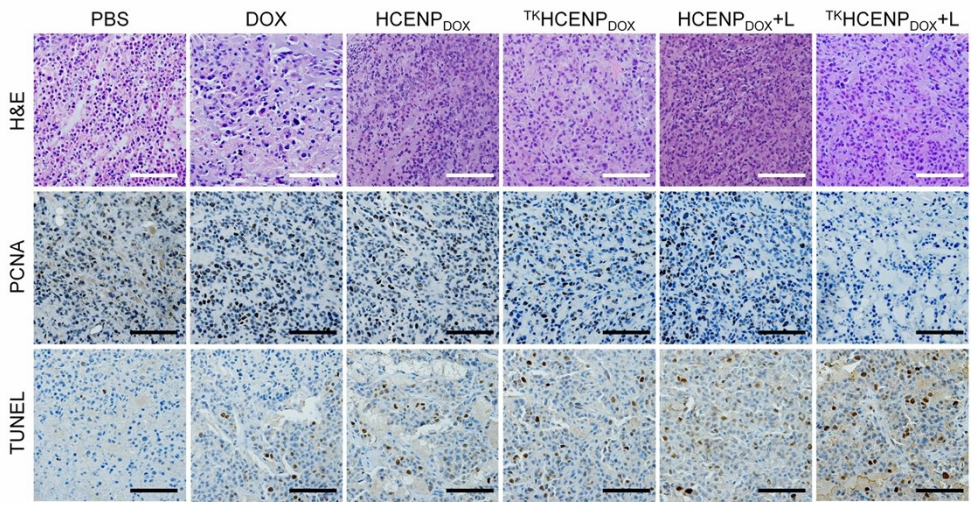

Figure S8. H&E, TUNEL and PCNA analyses of tumor tissues from mice treated with the indicated formulations. TUNEL-positive apoptotic cells and PCNA-positive proliferating cells are stained brown. The scale bar is 200 μm.

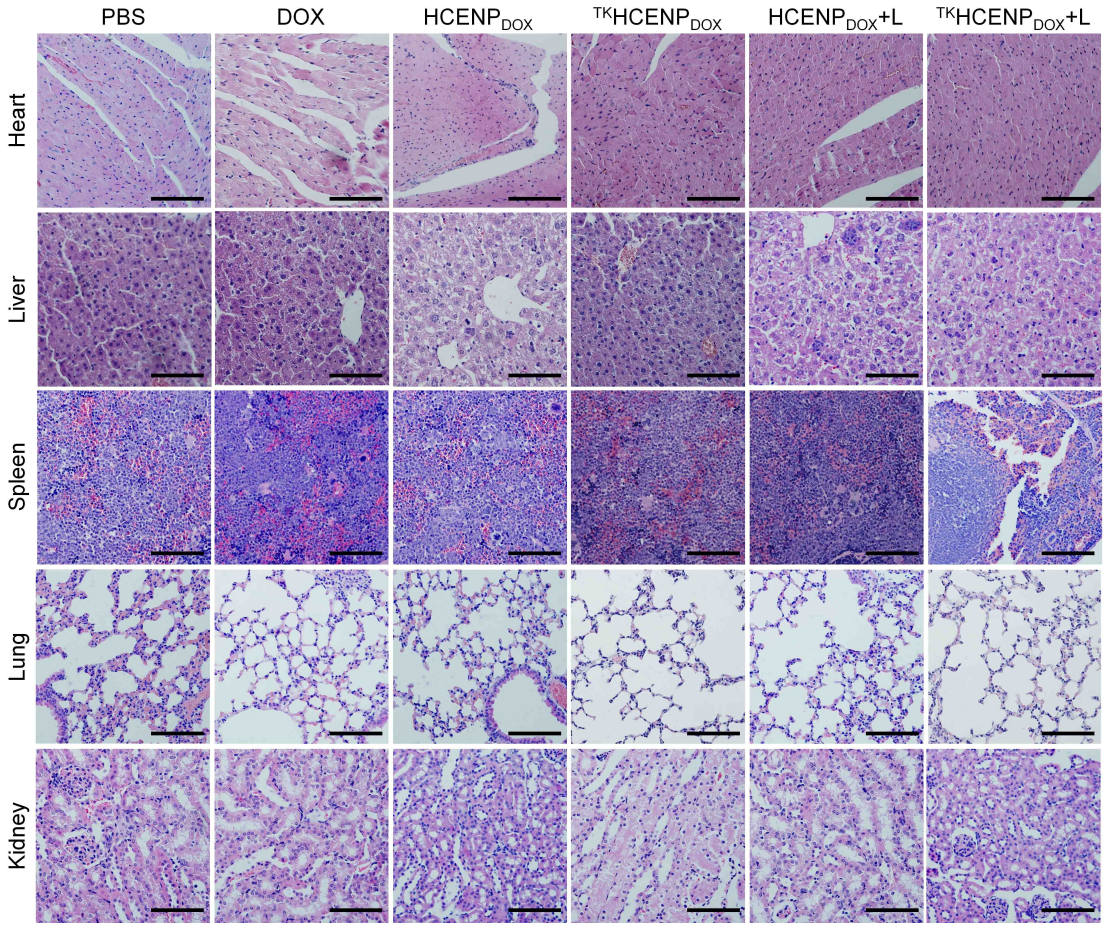

Figure S9. Histopathology analyses of visceral organ sections from MDA-MB-231 xenografted female mice after the tumor growth inhibition experiment. The scale bar is 200 μm.

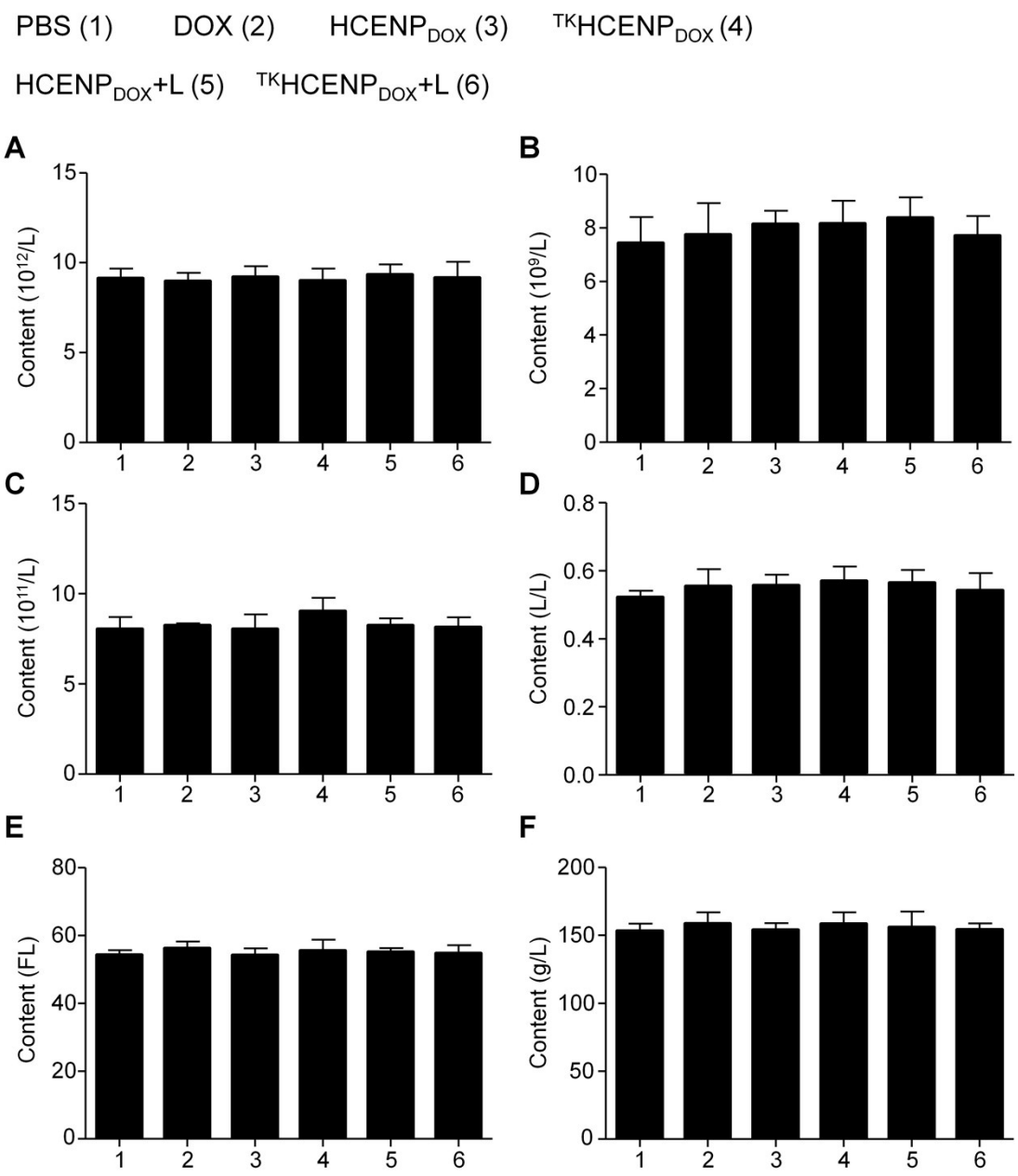

Figure S10. Hematology analysis of the mice after different treatments: (A) red blood cell (RBC), (B) white blood cell (WBC), (C) platelet (PLT), (D) hematocrit (HCT), (E) mean corpuscular volume (MCV), and (F) hemoglobin (HGB), respectively.

Table S1. Drug loading contents (DLCs) of Ce6 and DOX for $^{TK}HCENP_{DOX}$ and $HCENP_{DOX}$.

Parameter	DLC (%)	
	Ce6	DOX
$HCENP_{DOX}$	8.82	5.69
$^{TK}HCENP_{DOX}$	8.56	5.93

References

1 C. Y. Sun, Z. Y. Cao, X. J. Zhang, R. Sun, C. S. Yu, X. Z. Yang, *Theranostics*, 2018, **8**, 2939-2953.

2 T. T. Wang, D. G. Wang, J. P. Liu, B. Feng, F. Y. Zhou, H. W. Zhang, L. Zhou, Q. Yin, Z. W. Zhang, Z. L. Cao, H. J. Yu, Y. P. Li, *Nano Lett.*, 2017, **17**, 5429-5436.