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Supporting Information

Nucleus-selective co delivery of proteins and drugs for synergistic antitumor therapy

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General

Materials and Reagents

All chemical reagents were used as supplied without further purification unless otherwise specified. Deoxyribonuclease I (DNase I) was obtained from Shanghai Sangon Biotech Co., Ltd. (Shanghai, China). Dithiothreitol (DTT), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium (MTT) and 4, 6-diamidino-2-phenylindole (DAPI) were purchased from Shanghai Sangon Biotech Co., Ltd. (Shanghai, China). Doxorubicin hydrochloride (DOX•HCl) and 2-Hydroxyethyl disulfide were purchased from Macklin Biological Co., Ltd. (Shanghai, China). Amantadine was purchased from Meryer Chemical Technology Co., Ltd. (Shanghai, China). Sodium azide (NaN_3), methyl-β-cyclodextrin (M-β-CD), genistein were purchased from Bide Pharmatech Ltd. (Shanghai, China). Ivermectin was obtained from Aladdin Reagent Co., Ltd. (Shanghai, China).

Instruments

NMR data were recorded on Agilent 400MR-DD2, DLS, Zeta was measured by NanoBrook Omni. Fluorescence microscopy images were observed by Leica DM6.Tissue sections of H&E were imaged by Leica DMi8. Absorbance was recorded by Agilent Cary60. Cell flow cytometry was performed on CytoFLEX. In vivo fluorescence was analyzed with an IVIS Lumina imaging system (PerkinElmer). Mass spectra were performed on Waters Acquity SQD. Transmission electron microscope (TEM) was conducted by Hitachi H-7500 electron microscope

Experimental Section.

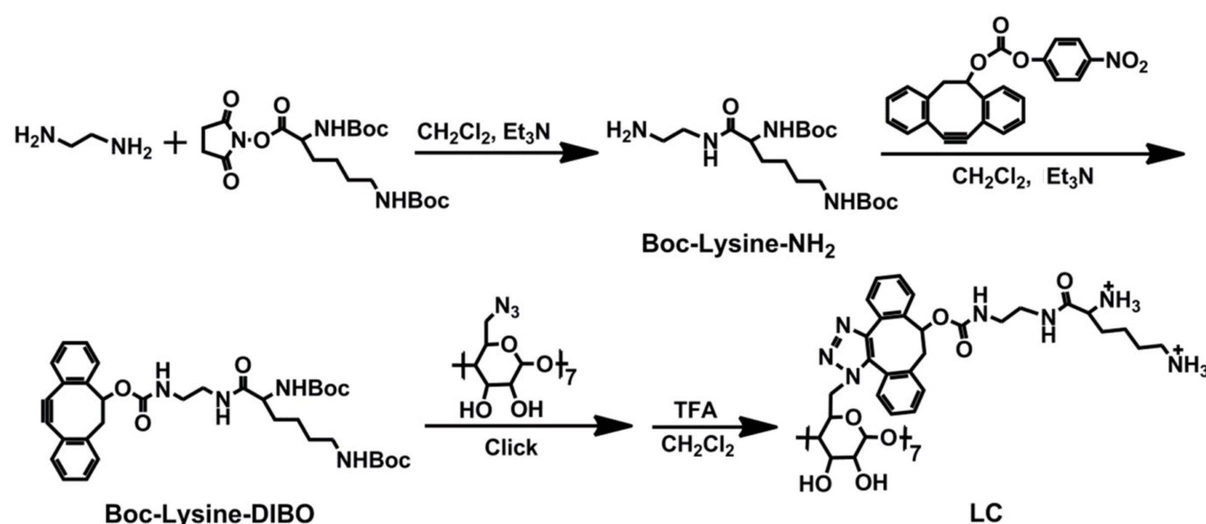

Figure S1. Synthetic routes of LC.

Synthesis of Compound Boc-Lysine-NH_2

Ethylenediamine (670 mg, 11.16 mmol) was added to a solution of N, N'-Di-Boc-L-lysine (500 mg, 1.13 mmol) in DCM (40 mL). After stirring overnight at room temperature, the solvent was evaporated and the residue was purified by column chromatography with dichloromethane/methanol (v/v = 20:1) as eluent to afford compound Boc-Lysine-NH_2 as a white solid.

Synthesis of Compound Boc-Lysine-DIBO

Compound Boc-Lysine-NH_2 (201 mg, 0.52 mmol) was added to a solution of 4-nitrophenyl chloroformate-substituted DIBO (100 mg, 0.26 mmol) in 10 mL DCM, then trimethylamine (56 μL, 0.40 mmol) was added. The reaction mixture was stirred overnight at room temperature and the solvent was removed in vacuum. The residue was dissolved in EtOAc, washed with water (2 x 50 mL), filtration and solvent removal to afford the crude product, which was further purified by column chromatography with petroleum ether/ethyl acetate (v/v = 1:1) as eluent to get compound Boc-Lysine-DIBO as white solid. ^1H-NMR (400 MHz, $CDCl_3$): δ 7.2-7.6 (m, 8H), δ 6.61 (t, 1H), δ 5.75 (t, 1H), δ 5.46 (t, 1H), δ 5.19 (t, 1H), δ 4.63 (t, 1H), δ 3.98 (t, 1H), δ 3.2-3.6 (m, 4H), δ 3.06 (m, 2H), δ 2.86 (m, 2H), δ 1.73 (m, 4H), δ 1.42 (m, 18H), δ 1.31 (m, 2H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 173.2, 156.5, 156.4, 156.1, 152.1 ,151.1, 130.0, 128.1, 127.2, 126.3, 126.0, 123.9, 121.3, 113.0, 110.0, 80.3, 79.3, 54.9, 46.2, 41.1, 39.8, 38.7, 29.7, 28.5, 28.3, 22.6. LC-MS (m/z): $[M+H]^+$ calcd for $C_{35}H_{46}N_4O_7$, 635.77; found, 635.60.

Synthesis of Compound LC

To a solution of compound Boc-Lysine-DIBO (67 mg) in DMF (5 mL) was added Heptakis-(6-azido-6-deoxy)-β-cyclodextrin (20 mg). After stirring at room temperature for 2 h, the reaction solution was concentrated and poured into ether. The precipitates were further reacted with trifluoroacetic acid to remove Boc to get the final product LC as solid. ^1H-NMR (400 MHz, CD_3OH): δ 8.0 (s, 7H), δ 7.7-7.0 (m, 63H), δ 6.1-5.9 (m, 7H), δ 5.6-5.8 (m, 7H), δ 4.1-3.7 (m, 35H), δ 3.6-3.3 (t, 28H), δ 3.01-2.75 (m, 28H), δ 2.0-1.2 (m, 70H), HR-MS(m/z): $[M+H]^+$ calcd for $C_{217}H_{273}N_{49}O_{49}$, 4352.977; found, 4353.026.

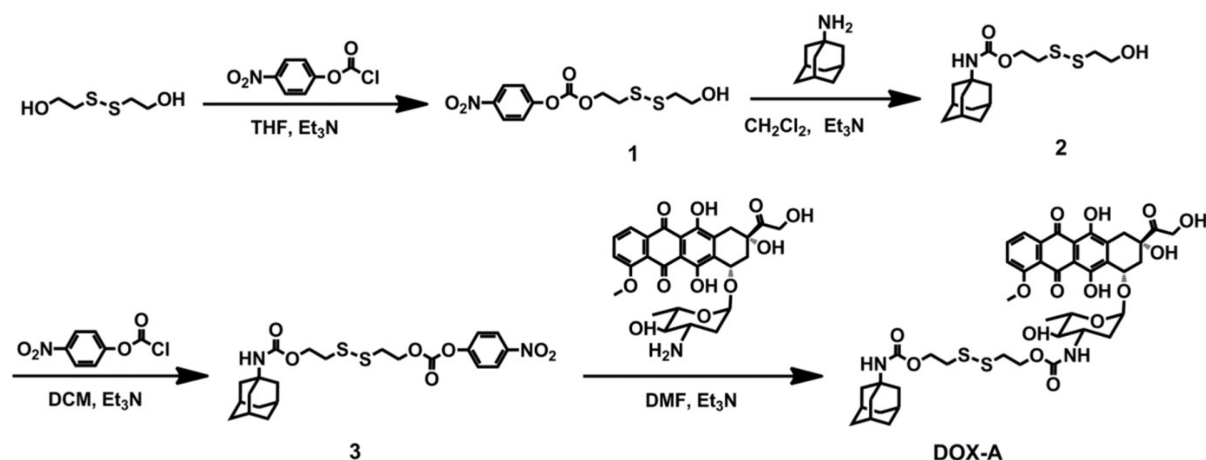

Figure S2. The synthetic route of DOX-A.

Synthesis of Compound 1

2-Hydroxyethyl disulfide (1g, 3.2 mmol) and triethylamine (323 mg, 3.2 mmol) were dissolved in THF (50 mL) and cooled to 0 °C. A solution of p-nitrophenyl chloroformate (522 mg, 2.5 mmol) in 40 mL THF was added dropwise to the solution. The mixture was stirred for another 2 h, then the solvent was removed in vacuum. The residue was dissolved in EtOAc, washed with water (2 x 50 mL), filtration and solvent removal to afford the crude product, which was further purified by column chromatography with petroleum ether/ethyl acetate (v/v = 3:1) as eluent to get compound 1 as light yellow liquid with a yield of 50%. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.26 (d, 2H), δ 7.37 (d, 2H), δ 4.55(t, 2H), δ 3.89 (s, 2H), δ 3.03 (t, 2H), δ 2.90 (t, 2H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 155.68, 152.88, 145.38, 125.33, 121.77, 66.87, 60.23, 41.48, 36.45.

Synthesis of Compound 2

Compound 1 (500 mg, 1.6 mmol) and Amantadine (355 mg, 2.4 mmol) were dissolved in DCM, then triethylamine (237 mg, 2.4 mmol) was added into mix solution. The reaction mixture was stirred overnight at room temperature and the solvent was removed in vacuum. The residue was dissolved in DCM, washed with saturated $NaHCO_3$ solution (2 x 50 mL) and brine (2 x 50 mL), filtration and solvent removal to afford the crude product, which was further purified by column chromatography with petroleum ether/ethyl acetate (v/v = 2:1) as eluent to get Compound 2 as white solid with a yield of 89.2%. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 4.23 (s, 2H), δ 3.85 (d, 2H), δ 2.84-2.92 (m, 4H), δ 1.85-2.0 (m, 9H), δ 1.63 (s, 6H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 154.10, 60.18, 50.81, 41.71, 37.64, 36.21, 29.36. LC-MS (m/z): $[M+H]^+$, calcd for 332.49, found: 332.52.

Synthesis of compound 3

Compound 2 (446 mg, 1.24 mmol) and triethylamine (251 mg, 2.4 mmol) were dissolved in DCM (50 mL). Then p-nitrophenyl chloroformate (500 mg, 2.4 mmol) was added into the mixture, which was stirred overnight at room temperature. The mixture was washed with water (2 x 50 mL) and organic layer was collected. For further purified by column chromatography with petroleum ether/ethyl acetate (v/v = 5:1) as eluent to get Compound 3 as light yellow solid with a yield of 72.5%. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.25 (d, 2H), δ 7.37 (d, 2H), δ 4.53 (t, 2H), δ 4.25 (s, 2H), δ 3.02 (t, 2H), δ 2.14 (t, 2H), δ 1.89-2.04 (m, 9H), δ 1.63 (s, 6H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 155.35, 155.95, 152.30, 145.41, 125.31, 121.81, 66.86, 61.56, 50.81, 41.69, 38.07, 36.12, 29.36. LC-MS (m/z): $[M+H]^+$, calcd for 497.59, found: 497.46.

Synthesis of compound DOX-A

DOX·HCl (54 mg, 0.1 mmol) were dissolved in DMF, triethylamine (20 mg, 0.2 mmol) was added into and stirred for 1h. Then Compound 3 (50 mg, 0.1 mmol) was added into the mixture and stirred overnight at room temperature. After removing the solvent, the resulted crude product was purified by column chromatography with ethyl acetate/methanol (5:1) to achieve DOX-A as red solid with a yield of 56.2%. ^{1}H-NMR (400 MHz, $CDCl_3$): δ 8.00 (d, 1H), δ 7.76 (t, 1H), δ 7.38 (d, 1H), δ 4.62-4.76 (m, 3H), δ 4.62 (s, 1H), δ 4.06-4.20 (m, 7H), δ 3.68-3.83 (m, 3H), δ 3.08-3.21 (m, 2H), δ 2.93-2.96 (m, 2H), δ 2.85-2.90 (m, 4H), δ 1.82-1.95 (m, 11H), δ 1.63 (s, 6H), δ 1.28 (t, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ 213.89, 186.98, 186.59, 160.97, 156.16, 155.57, 155.35, 135.75, 135.38, 133.64, 133.56, 120.73, 119.79, 118.42, 111.48, 111.31, 100.74, 69.51, 69.30, 67.40, 65.534, 56.64, 47.05, 41.68, 36.20, 33,91, 29.35, 16.90. FT-MS (m/z): $[M-H]^-$, calcd for 899.28, found: 899.27.

Preparation and characterization of D/LC nanoparticles

For the preparation of D/LC nanocomplexes, LC aqueous solution was simply mixed with DNase I solution with the mass ratio of DNase to LC was 1:0, 1:1, 1:2, 1:3 and 1:4, respectively. The whole preparation process was simple and easy to operate without additional separation and purification steps. The resulting complexes were electrophoresed on the 1.5% (W/V) agarose gel for 20 min, following stained by Coomassie Blue to verify the encapsulation of DNase I. The loading efficiency of DNase I was calculated by the following equation:

Loading Efficiency of DNase I = (weight of protein in the nanoformulations/weight of nanoformulations) x 100%

DOX release study

For DOX release experiment, DD/LC nanoparticles (Equivalent to 1 mg for DOX and 1 mg for DNase I) were loaded into a dialysis bag (MWCO: 2000) and the dialysis bag was placed into a mixture solution of 60% PBS and 40% ethanol (containing 0 mM DTT, 5 mM DTT, or 10 mM DTT). The volume of solution was kept constant by adding mixture solution after each sampling at prescribed time intervals. The release of DOX was estimated by a UV-Vis absorption spectrum detected at 488 nm.

Loading Efficiency of DOX = (weight of DOX in the nanoformulations/weight of DD/LC nanoformulations) x 100%

Dye Labeling of DNase I

For the cellular uptake and biodistribution studies, DNase I was labeled with fluorescein isothiocyanate (FITC) and Cy7 respectively. Briefly, DNase I (1mg) was dissolved in sodium bicarbonate solution (0.1M, pH=9), and mixed with 10 times excessive FITC-NHS and Cy7-NHS solution respectively. The reaction mixture was sheltered from light and stirred overnight at 4 °C. The resulting dye labeled DNase I was purified by dialysis and kept at 4°C for further use.

Cell culture

4T1 cells was purchased from national infrastructure of cell line resource (China) and cultured in complete 1640 (10% FBS, 1% penicillin/streptomycin) at 37 °C in the presence of 5% CO_2.

Cellular uptake

To investigate the cellular internalization of DD/LC, breast cancer cells 4T1 cells cells were seeded in 96-well plates and incubated 12 h at 37 °C under 5% CO_2. 100 μL fresh medium containing DOX, DNase I-FITC, D/LC or DD/LC (10 μg/mL for DOX and 10 μg/mL for DNase I-FITC) was then added. After 2 h incubation, the cells were stained with DAPI and imaged with fluorescent microscopy.

Endocytosis mechanism

4T1 cells (density of 1×10^4 cells/well) were seeded in 96-well plates and incubated 12 h at 37 °C under 5% CO_2. The cells were incubated with different endocytosis inhibitor (10 mM NaN_3, 10 mM M-β-CD or 200 μM genistein) or placed at 4 °C for 1 h, and then DD/LC was added and incubated at 37 °C or 4 °C for another 2 h. After that cells were trypsinsized and collected for flow cytometry analysis.

Escape of lysosome and time-lapsed cell imaging

4T1 cells were seeded in 96-well plates and incubated overnight. D/LC was added into the plate. After 2 h incubation, cells were washed with PBS and incubated with the mixture of Lyso-Tracker red and Hoechst 33342 for 20 min. Then, the cells were observed by fluorescent microscopy. The same method, 4T1 cells were incubated with DD/LC and observed at 0, 5, 10, 20, 40 min.

Nucleus transport mechanism

To explore the nuclear transport mechanism of DD/LC nanoparticles, 4T1 cells were preincubated with ivermectin (15 μM) for 2 h and then DD/LC was added for further incubating another 2 h. After that, the cells were stained with DAPI and imaged with fluorescent microscopy.

In vitro cytotoxicity

4T1 cells (density of 1×10^4 cells/well) were seeded in 96-well plates and incubated for overnight. Then the medium was replaced with 100 uL fresh medium containing free DOX, DNase I, D/LC, DD/LC (1μg/mL for DOX, 50-300 nM for DNase I). After 24 h incubation, the cell viability was measured by performing MTT assays.

The coefficient of drug interaction (CDI)

Drug interactions in vitro were analyzed according to CDI. CDI was calculated according to the formula: CDI = $V_{AB} / (V_A \times V_B)$, where V_A and V_B are the survival values of respective single agent; V_{AB} is the survival values of two drugs combination.

$$\mathrm{CDI} = \frac{V_{(DD/LC)}}{V_{(DOX)} \times V_{(D/LC)}} = \frac{0.5896}{0.9082 \times 0.8228} = 0.79$$

Animal Model

All animal experiments were carried out in compliance with the requirements of the National Act on the use of Experimental Animals (People's Republic of China) and were approved by the Experimental Animal Ethical Committee of Chongqing University Cancer Hospital. Female BALB/c-nude mice (6-8 weeks) were supplied by the Animal Center of Chongqing Medical University (Chongqing, China).

Biodistribution

4T1 tumor-bearing mice were intravenously injected with free DNase I-Cy7, D/LC or DD/LC (1 mg/kg for DNase I-Cy7, 1 mg/kg for DOX). The mice were imaged at different time points (30 min, 2, 4, 6, 8, 12, 24 h postinjection) by small animal imaging system (IVIS Lumina III, USA). 24 h after administration, the mice were sacrificed. Major organs (heart, liver, spleen, lung and kidney) and tumors were imaged and analyzed by an IVIS Lumina imaging system.

In vivo antitumor activity

For anti-tumor therapy studies, breast cancer 4T1 cells (2×10^6) were injected subcutaneously into female BALB/c-nude mice. When the tumor volume grew to approximately 100 mm^3, the mice were randomly divided into 6 groups (n=10): (1) PBS group, (2) DNase I group, (3) DOX group, (4) DNase I + DOX group, (5) D/LC group and (6) DD/LC group. 150 μL of different samples with equal amount of DNase I (3 mg/kg) and DOX (2 mg/kg) were intravenously injected into the mice every three days for a total of four times. The tumor sizes and survival rate of the mice were measured every other day during the experiment. The tumor volumes value was calculated as the following equation: tumor volumes = width $(mm)^2 \times$ length (mm) $\times 1/2$. At the end of experiment, the tumor was excised and weighed. For histological examination, tumor tissues and major organs (heart, liver, spleen, lung and kidney) were collected for hematoxylin and eosin (H&E) staining.

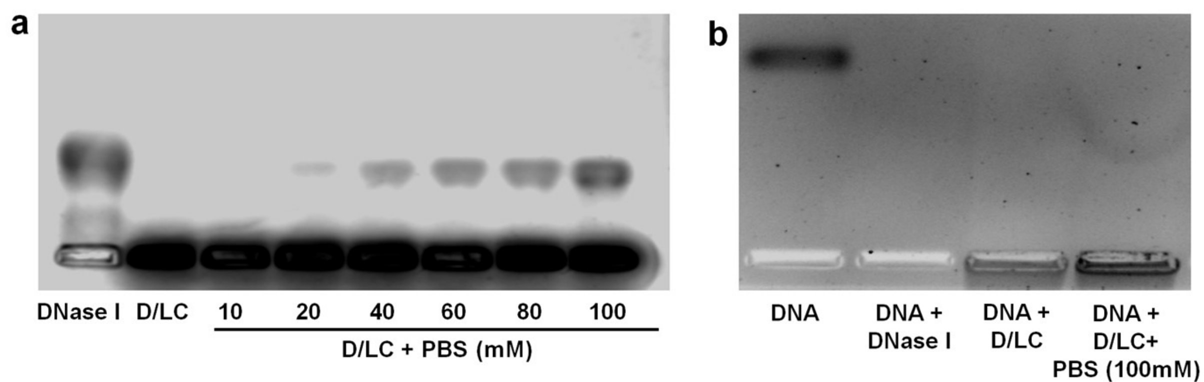

Figure S3. Agarose gel assay for analyzing (a) the intact DNase I release from D/LC nanocomplex in PBS with high ionic strength, (b) the DNA digestion ability of free DNase I , D /LC nanocomplex and released DNase I from D/LC nanocomplex.

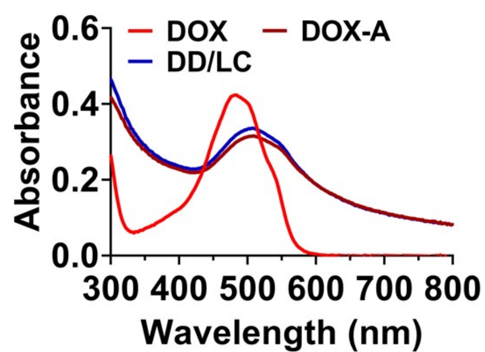

Figure S4. UV-Vis absorption of free DOX (64 μg, 0.32 μg/μL), DOX-A (106 μg, 0. 535 μg/μL) and DD/LC (415 μg, 2 μg/uL).

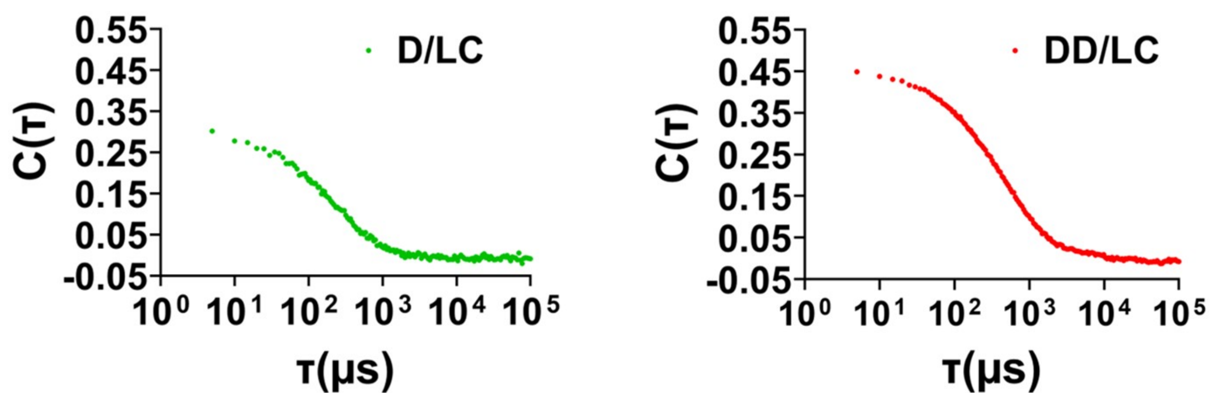

Figure S5. Correlation functions from DLS measurements.

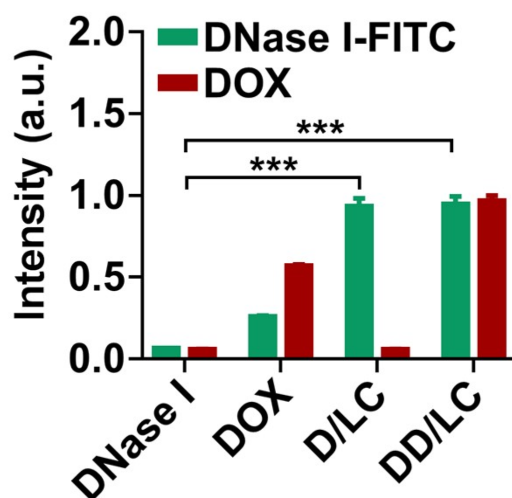

Figure S6. Quantitative analysis of the uptake efficiency by flow cytometry.

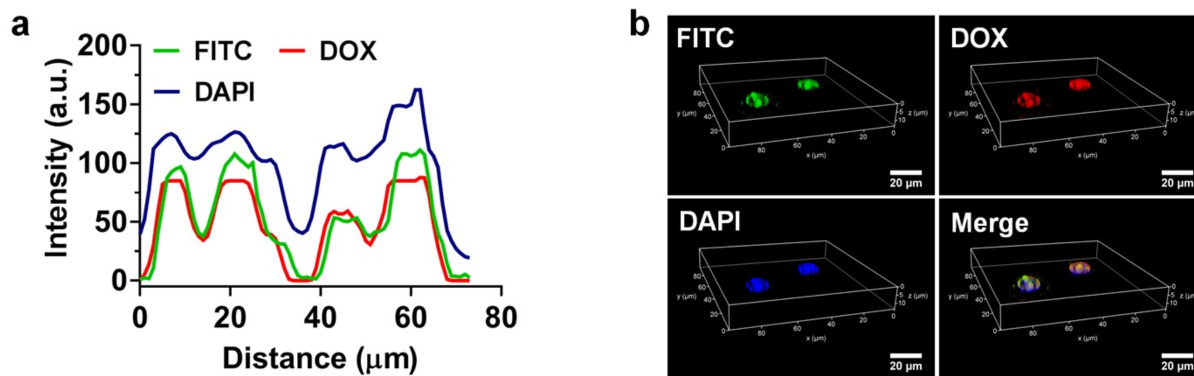

Figure S7. (a) Corresponding fluorescence intensity profiles of cells treated with DD/LC nanoparticles. (b) 3D reconstruction of confocal z-stack images showed well co-localized DNase I and DOX in the nuclei. DNase I was labeled with FITC, and nuclei were stained by DAPI. Scale bars: 20 μm.

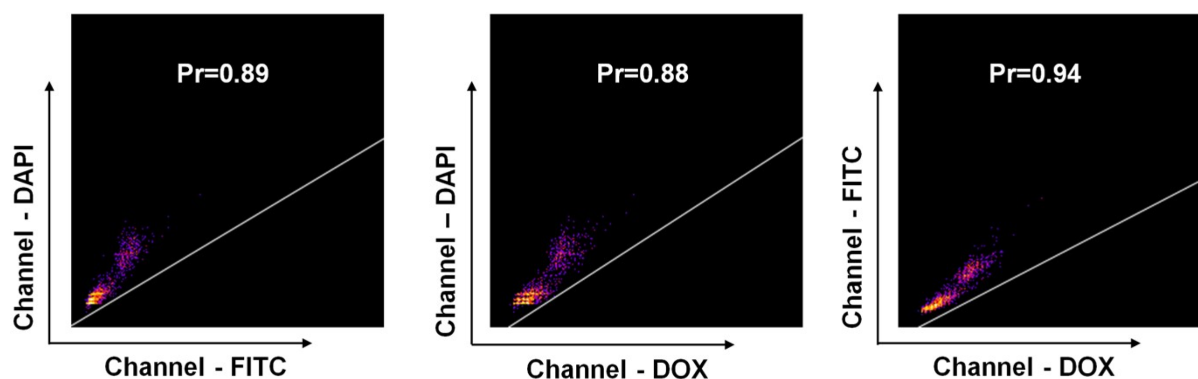

Figure S8. The Pearson's correlation coefficient illustrating the co-localization of DNase I and DOX in the nucleus.

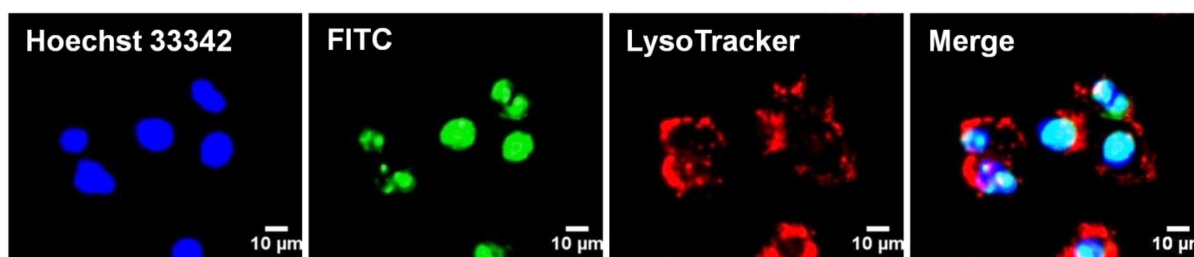

Figure S9. 4T1 cells were incubated with D/LC and then stained with LysoTracker Red for imaging lysosome escape. DNase I was labeled with FITC and nuclei were stained by hoechst33342. Scale bars: 10 μm

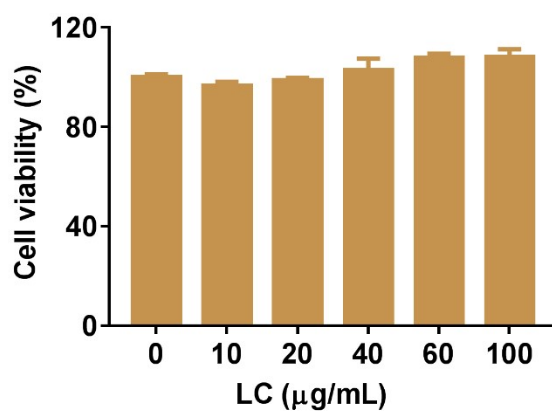

Figure S10. Cell viabilities of 4T1 cells after incubation with different concentrations of LC.

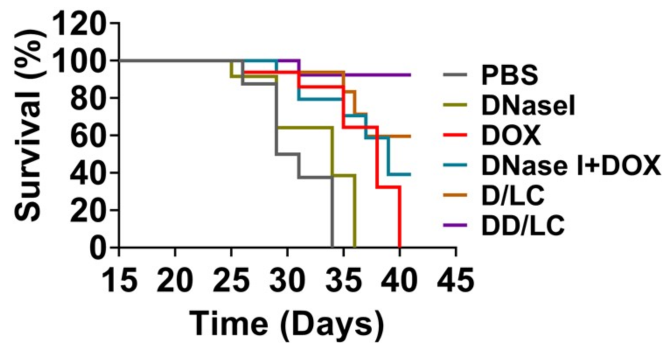

Figure S11. Percentage of survival rate of mice after different treatments.

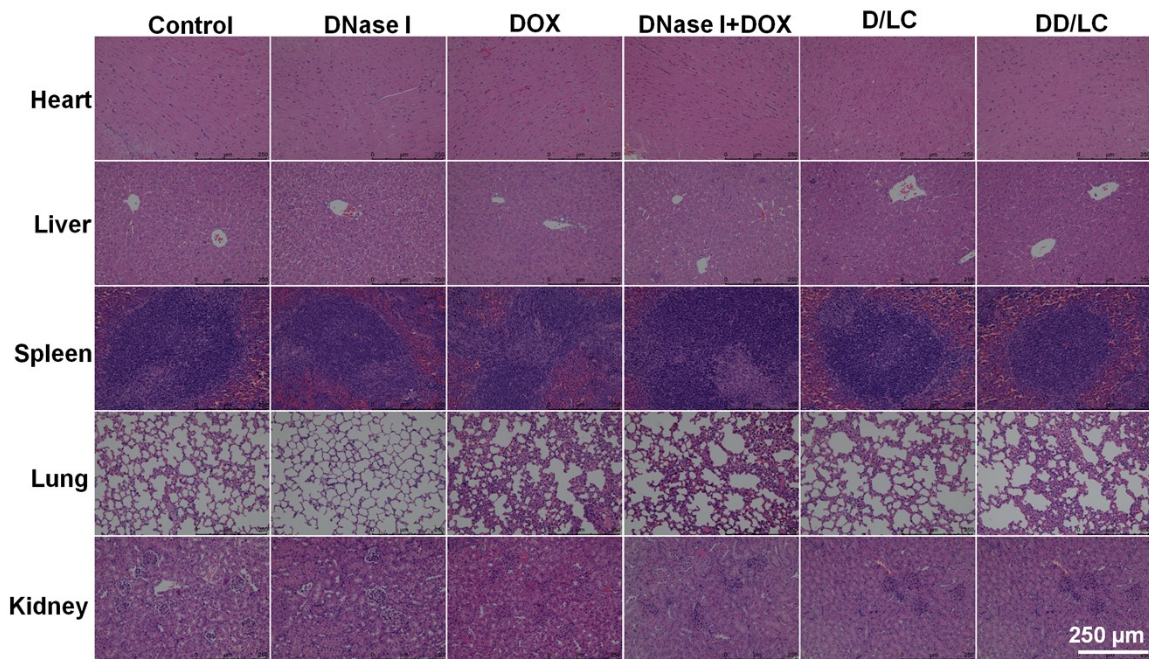

Figure S12. H&E staining of the main organs after different treatments. Scale bars: 250 μm