Supporting Information for

Original article

Multifunctional polymeric micelle-based chemo-immunotherapy with immune checkpoint blockade for efficient treatment of orthotopic and metastatic breast cancer

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Received 26 December 2018; received in revised form 11 January 2018; 21 January 2018

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Running title: Multifunctional polymeric micelle-based chemo-immunotherapy for treatment of orthotopic and metastatic breast cancer

1. Experimental procedures

1.1. Cell lines and tumor models

The 4T1 murine breast carcinoma cell line was kindly obtained from State Key Laboratory of Biotherapy (Sichuan University, Chengdu, China) and cultured in RPMI-1640 medium (GIBCO) supplemented with 10% FBS (GIBCO), 100 μg/mL streptomycin and 100 U/mL penicillin at 37 ℃ in a humidified atmosphere of 5% CO_2 in air. BALB/c mice (about 5-week-old, 18–21g) were purchased from Dashuo Experimental Animal Company (Chengdu, China). For the establishment of orthotopic tumor model, 1×10^6 4T1 cells were orthotopically implanted into the mammary fat pad of female BALB/c mice. Tumor growth was calculated from caliper measurements with the following formula, $(L\times W^2)/2$ (L, length; W, width). 2×10^5 4T1 cells were injected via the tail vein into BALB/c mice to set up 4T1 metastasis model. All animal experiments were performed according to the rules of Experimental Animals Administrative Committee of Sichuan University.

1.2. Preparation and characterizations of drug-loaded micelles

DOX-loaded micelles were prepared by the ultrasonic emulsification method. First, doxorubicin hydrochloride (10 mg) and trimethylamine (7.2 μL) were added together to 5 mL of dichloromethane and stirred overnight in the dark. Afterwards, LMWH-TOS (100 mg) was directly mixed with the reaction mixture, and stirred at 30 ℃ for 3 h. Then the mixture was added into deionized water (50 mL) under ultrasonic condition (100 W, 5s/5s, 7 min). The resulting emulsion was vaporized by rotary evaporator at 37 ℃ to remove the dichloromethane. Later, the red solution was dialyzed against deionized water for 6 h and lyophilized after being filtrated through a 0.22 μm filter. IMQ-loaded micelles were prepared using the diafiltration method. Briefly, IMQ (4 mg) and polymer (44 mg) were separately dissolved in 8 mL of DMSO, then combined and mixed with stirring at 30 ℃ for 3 h. The mixed solution was dialyzed (MW cut-off 1000) against deionized water for 24 h to remove DMSO and free drug. The deionized water was changed every 2 h during the first 6 h and then replaced once every 6 h. After 24 h of dialysis, the micelles were filtered by a membrane filter (0.22 μm pore) to remove any aggregated particles and drugs, collected by freeze-drying as white powder, and stored at –20 ℃ until reconstitution into aqueous buffers. Blank micelles were prepared by dissolving LT powder in water. The zeta potential and mean size of the micelles were measured by Malvern Zetasizer Nano ZS90 (Malvern Instruments Ltd., U.K). And the morphology of micelles was observed under a transmission electron microscope (TEM; JEM-100CX, JEOL, Japan).

To measure the drug loading capacity of micelles, the micelle dispersion was disrupted by DMSO (100 times volume), and the encapsulation efficiency of DOX was analyzed by RF-5301 PC spectrofluorophotometer (Shimadzu, Japan) at E_x=488 nm, E_m=555 nm while IMQ was determined by a HPLC (Agilent 1200, USA) with a ultraviolet–visible detector at 325 nm. Drug loading capacity (%) = (quality of DOX/IMQ in micelles)/(quality of DOX/IMQ-loaded micelles) ×100.

1.3. Stability of micelles

Turbidity variations of micelles incubated with fetal bovine serum (FBS) were monitored. Briefly, micelles were mixed with equal volume of FBS under 37 ℃ with gentle shaking. At each pre-set time points, 200 μL of the sample was pipetted out and onto a 96-well plate to measure the transmittance at 750 nm by a microplate reader (Thermo Scientific Varioskan Flash). Meanwhile, Dynamic light scattering

(DLS) was used to investigate the aggregation behavior of the three micelles by measuring their sizes were in 50% FBS.

1.4. *In vitro* drug release

Spectrofluorophotometer (Shimadzu, Japan) and HPLC (Agilent 1200, USA) were used to test the *in vitro* release of DOX and IMQ from LT-DOX and LT-IMQ. LT-DOX and LT-IMQ were added into dialysis tubes (MWCO=8000). Then the dialysis tubes were placed into PBS (pH 5.0 and pH 7.4), and allowed to incubate under 37 °C for 48 h. At predetermined time points (1, 2, 4, 6, 8, 12, 24 and 48 h), 0.4 mL release media was collected as samples and replaced with 0.4 mL fresh media.

1.5. *In vitro* cytotoxicity assays and apoptosis assay

4T1 cells were seeded onto a 96-well plate at a density of 2×10^3 cells/well and cultured for 24 h. The culture media was replaced with 200 μL micelle-containing culture medium for 24 h. Then, 20 μL MTT solution (5 mg/mL in PBS) was added into each well and incubated for another 4 h. Finally, the culture media was removed and the residue was dissolved with 150 μL dimethyl sulfoxide (DMSO). The absorbance of each group was measured by Varioskan Flash Multimode Reader (Thermo, USA) at 490 nm. Cell viability (%) = $[A_{test}–A_{PBS}]/[A_{control}–A_{PBS}]\times100$.

The analysis of apoptosis induced by different preparations was determined after annexin V-FITC/PI double staining according to the manufacturer's protocols (Annexin V-FITC Apoptosis Detection Kit, KeyGen biotech, China). Briefly, by the end of the treatment, cells were harvested, washed with cold PBS, suspended in 500 μL binding buffer and stained by 5 μL annexin V-FITC and 5 μL PI. The cells were incubated in the dark for 15 min and then measured by flow cytometer (Cytomics™ FC 500, Beckman Coulter, Miami, FL, USA).

1.6. *In vitro* inhibitory effect on cell migration and invasion

A wound healing assay was utilized to evaluate the ability of cells to migrate. Briefly, 4T1 cells were cultured to near confluence (90%) in 6-well plates. Scratch wounds were generated with a sterile pipet tip, and cells were washed. After treatment with different preparations for 24 h, the wound closure was monitored by optical microscopy. Images were captured at 0 and 24 h.

For the Transwell invasion assay, 1×10^5 cells were plated in the top chamber pre-coated with 60 μL of Matrigel (24-well insert, pore size: 8 μm) in medium containing 2% serum. Medium containing 20% FBS served as a chemoattractant in

the lower chamber. After incubation with different preparations for 48 h, cells that did not invade through the pores were gently removed with a cotton swab. The cells that invade through the pores were fixed with paraformaldehyde, stained with crystal violet, and washed with PBS, finally, monitored by optical microscopy. After that, the upper chamber of each experimental group was decolorized in 33% acetic acid and shaken well. Each group was set up with three duplicate wells. The absorbance of decolorization liquid was analyzed at 570 nm by a microplate reader.

1.7. *In vivo* bio-distribution study

Bio-distribution study was carried out when volumes of the 4T1 tumors reached around 100 mm^3. Mice were injected with free DiD or LT-DiD. In each treatment group, mice were then anesthetized and the fluorescence imaging was monitored by IVIS Spectrum system (Caliper, Hopkington, MA) at 2, 4, 8, 10 and 24 h after injection. Mice were sacrificed after heart perfusion with saline and paraformaldehyde. Hearts, livers, spleens, lungs, kidneys, and tumors were collected at 10 and 24 h. All the organs were imaged with IVIS instrument (Caliper, Hopkington, MA, USA).

1.8. Cellular uptake assay of DiD-labeled micelles

DC2.4 cells were seeded in 6-well plates at a density of 5×10^5 cells per well respectively. When the coverage scale of cells was 80%–90% in per well, free DiD and DiD-labeled micelles were added into the plates for 4 h incubation and final DiD concentration was 1 μg/mL. Then, the cells were collected, suspended and finally analyzed using the flow cytometer (Cytomics FC 500, Beckman Coulter, USA).

1.9. BMDC maturation *in vitro*

Bone marrow cells obtained from the femurs of female BALB/c (4–6 weeks old) were depleted of red cells with lyses buffer solution. Approximately 10^7/mL cells were grown in 12-well plates containing RPMI 1640 medium enriched with 10% FBS, 10 ng/mL IL-4, 20 ng/mL recombinant murine GM-CSF and 100 units/mL penicillin and streptomycin. Cells were gently washed on days 2 and 4 to remove granulocytes. On day 6 of culture, Hepes, Free DOX&IMQ, LT-DOX, LT-IMQ and LT-DOX+LT-IMQ were added into the plates for 24 h incubation. Finally, the cells were stained with anti-CD11c-PE, anti-CD86-FITC and anti-CD80-FITC, and then detected by flow cytometry (Cytomics FC 500, Beckman Coulter, USA).

Supplementary Tables

Table S1 Characterization of different micelles .

Micelle	Size (nm)	PDI	Zeta	Loading capacity (%)
LT-DOX	133.9 ±2.8	0.104 ±0.038	–32.5 ±3.8	8.1 ±1.7
LT-IMQ	112.7 ±1.5	0.192 ±0.014	–35.7 ±4.1	5.2 ±1.3

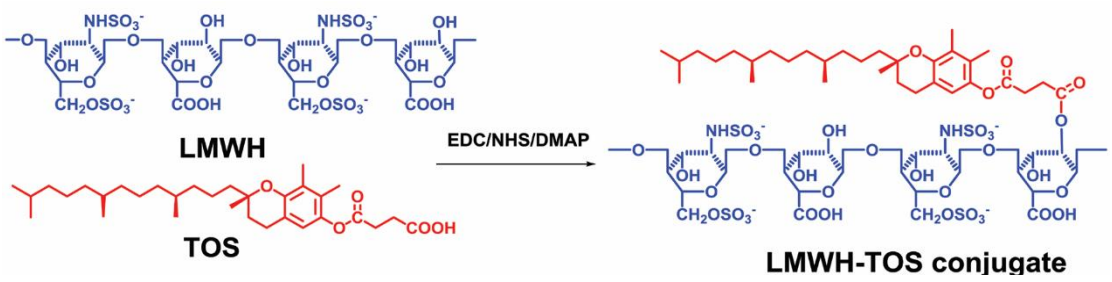
Data represent mean ±SD, *n*=3

Table S2 Pharmacokinetic parameters of DOX and IMQ after intravenous administration of free DOX&IMQ, LT-DOX+LT-IMQ at a dose of 3 mg/kg DOX and 0.75mg/kg IMQ.

Group	CL (L/h/kg)	AUC_{0-t} (μg/L·h)	$AUC_{0-\infty}$ (μg/L·h)	Cmax (μg/L)
Free DOX	6.022 ±0.742	443.226 ±63.537	506.379 ±66.609	1231.394 ±122.977
LT-DOX	2.026 ±0.218	1361.121 ±153.543	1499.792 ±174.521	1914.874 ±159.448
Free IMQ	13.734 ±1.474	71.618 ±19.426	78.412 ±20.552	84.438 ±12.122
LT-IMQ	6.348 ±1.237	154.757 ±35.388	164.560 ±36.229	185.303 ±17.794

Data represent mean ±SD, *n*=3.

Supplementary Figures



Scheme S1 The synthesis procedures and chemical structures of LMWH–TOS conjugates.

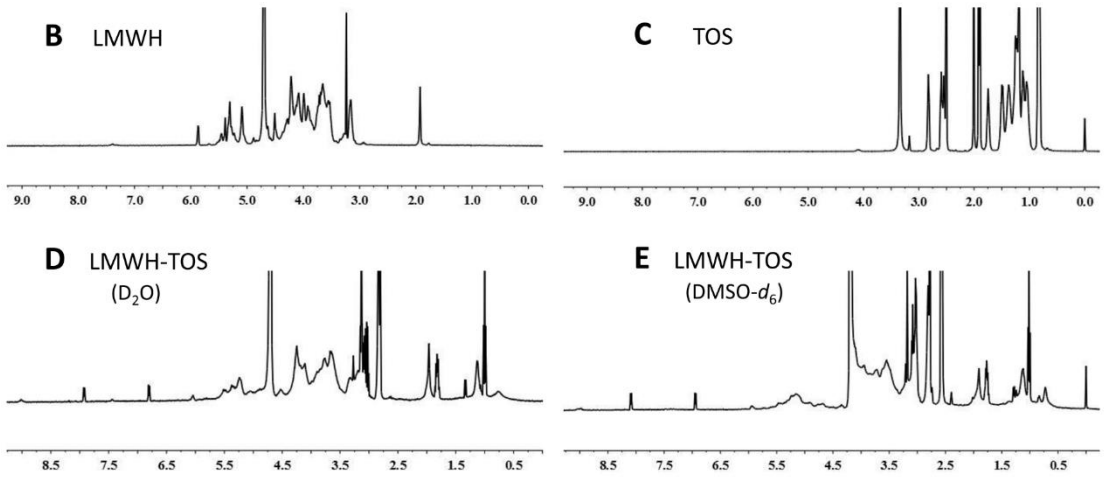


Figure S1 ^{1}H NMR spectra of (A) LMWH in D_2O, (B) TOS in DMSO-d_6, LT in D_2O (C) and in DMSO-d_6 (D).

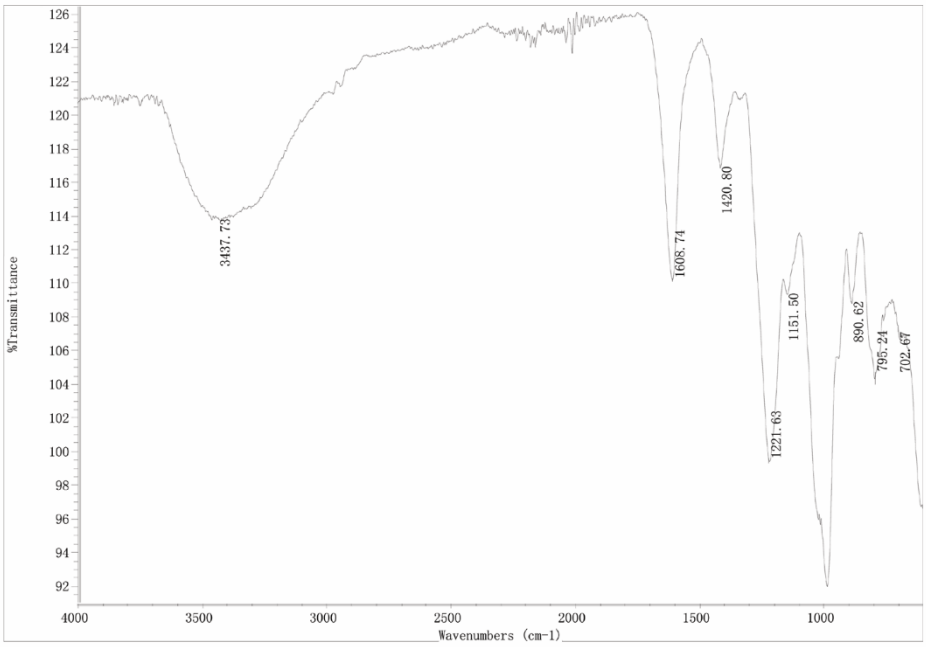


Figure S2 Infrared spectrum of LMWH.

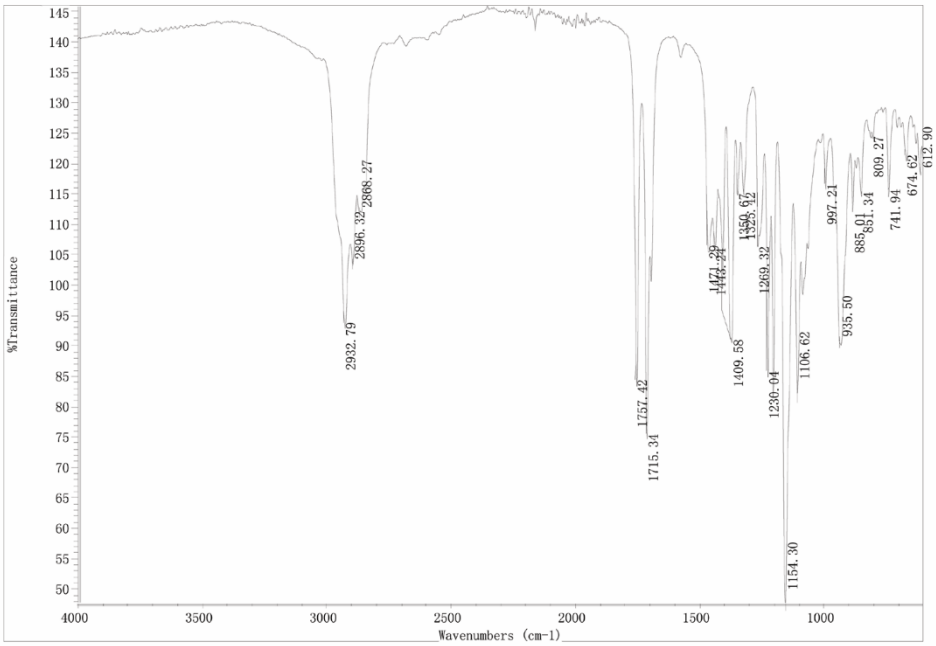


Figure S3 Infrared spectrum of TOS.

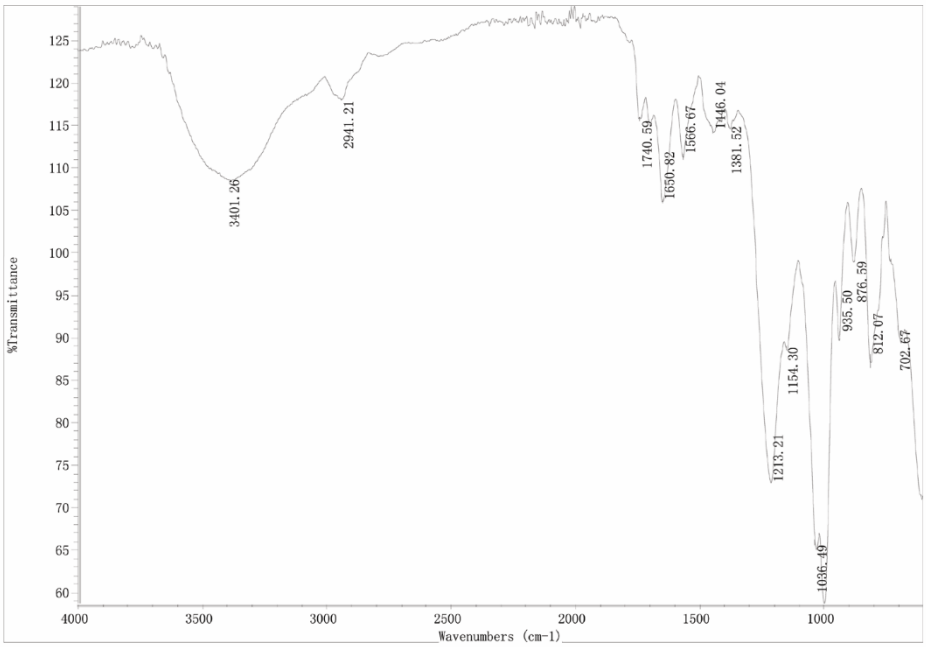


Figure S4 Infrared spectrum of LMWH-TOS.

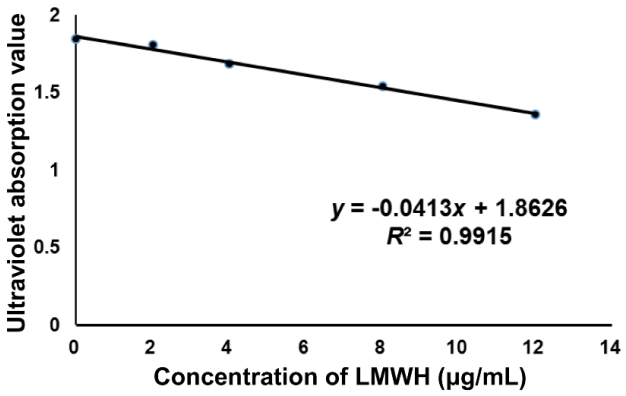


Figure S5 The standard curve of LMWH determined with toluidine blue.

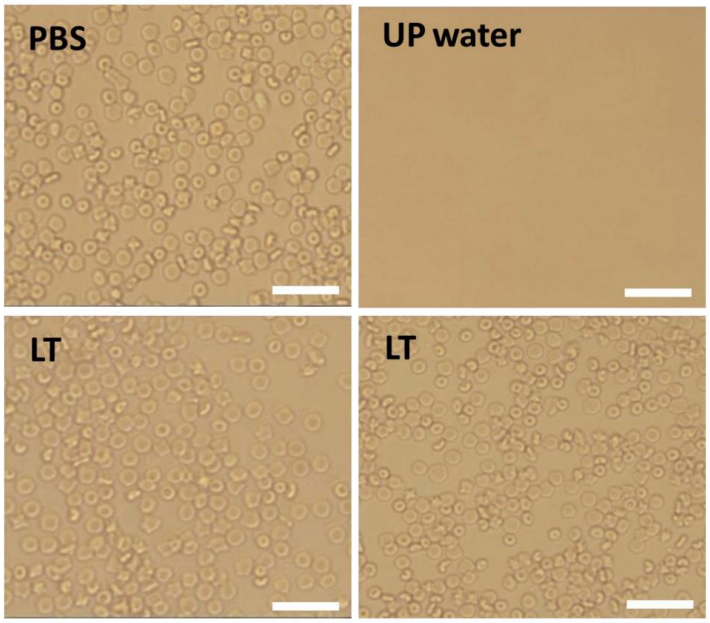


Figure S6 Photos images of erythrocytes treated with PBS, water and LT at the concentration of 900 μg/mL. The scale bar represents 50 μm.

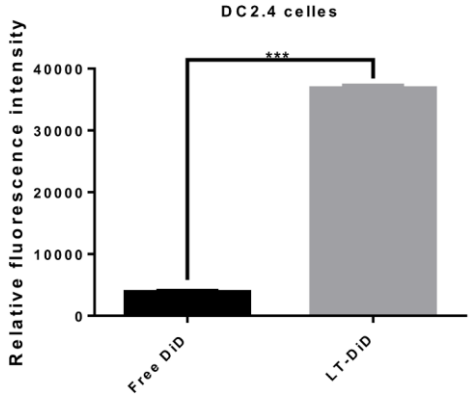


Figure S7 Cellular uptake of DiD-labeled micelles in DC2.4 cells determined by the flow cytometer after 4 h treatment (Data are mean ± SD, n = 3).

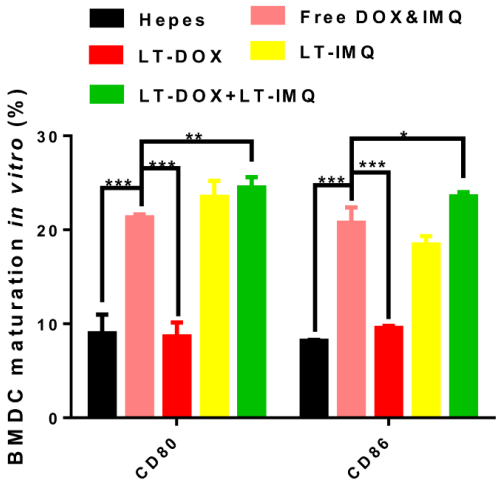


Figure S8 *In vitro* BMDC maturation ($CD11c^{+}CD80^{+}$, $CD11c^{+}CD86^{+}$) after incubating with different preparations (Data are mean ± SD, n = 3).

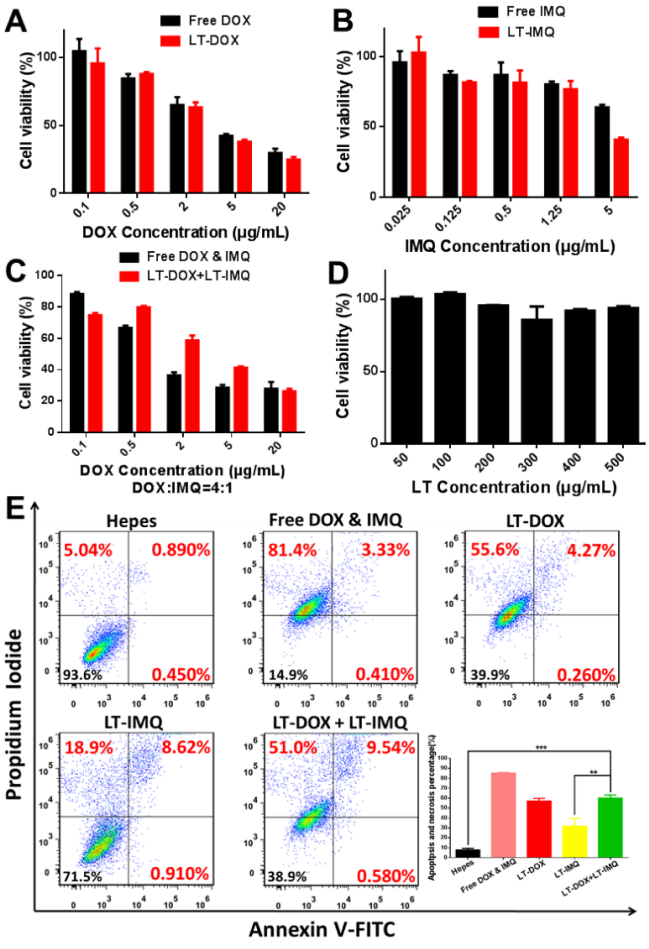


Figure S9 Growth inhibitory effect of (A) free DOX and LT-DOX (B) free IMQ and LT-IMQ (C) free DOX&IMQ and LT-DOX+LT-IMQ (D) LT on 4T1 cells. (E) The apoptosis study of 4T1 cells after incubation with different formations. (Data are mean ± SD, n = 3; $^{*}P$<0.01; and $^{***}P$<0.001).

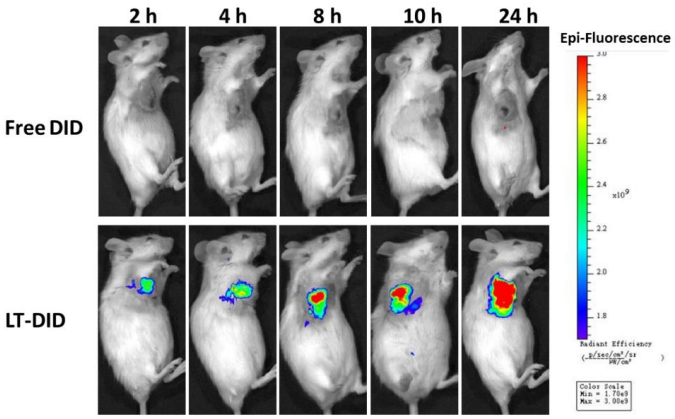


Figure S10 *In vivo* fluorescence images of 4T1-tumor-bearing mice taken at different time points post i.v. injection of free DiD and LT-DiD.

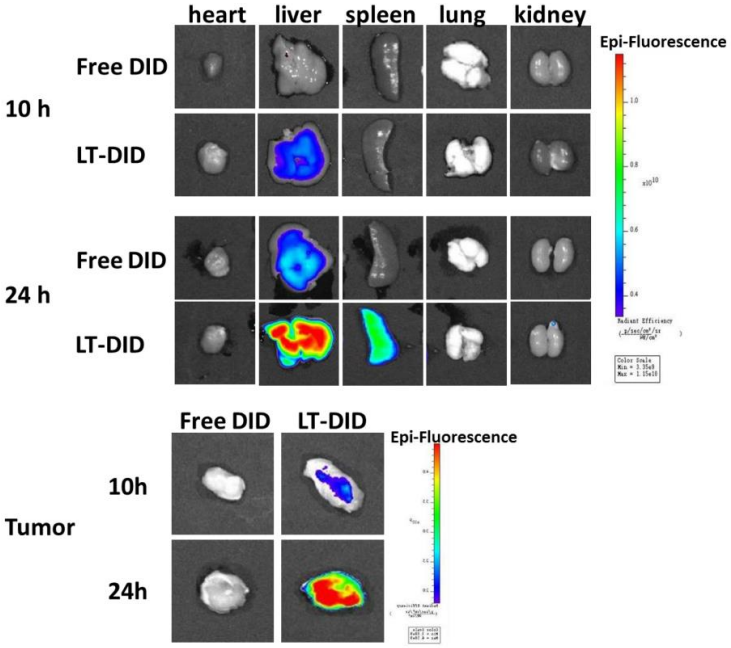


Figure S11 An *ex vivo* fluorescence image of major organs and tumor dissected from the mouse 10 and 24 h post injection.

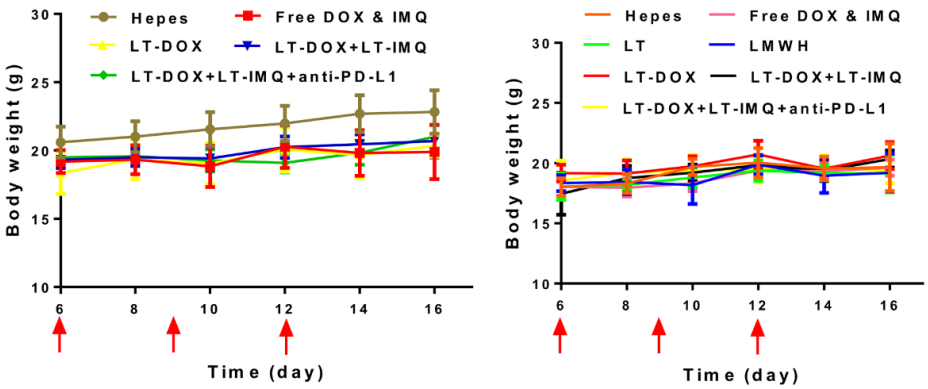


Figure S12 Body weight of 4T1 orthotopic and metastasis tumor-bearing mice during treatment (Data are mean ±SD, *n*=6).

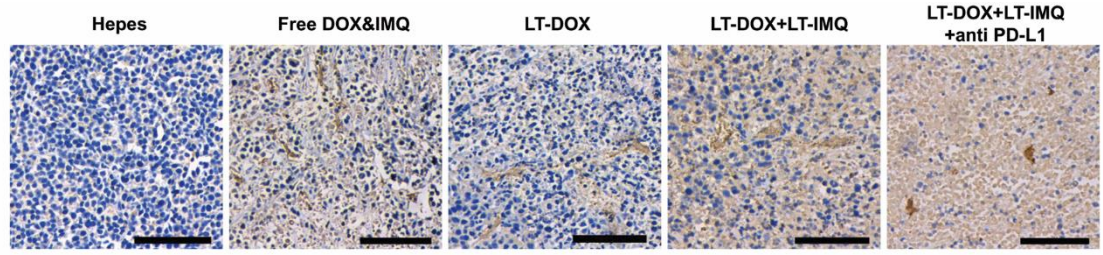


Figure S13 Immunohistochemistry to detect PD-L1 in tumors at the end of therapy. The scale bar represents 100 μm (shown in brown).

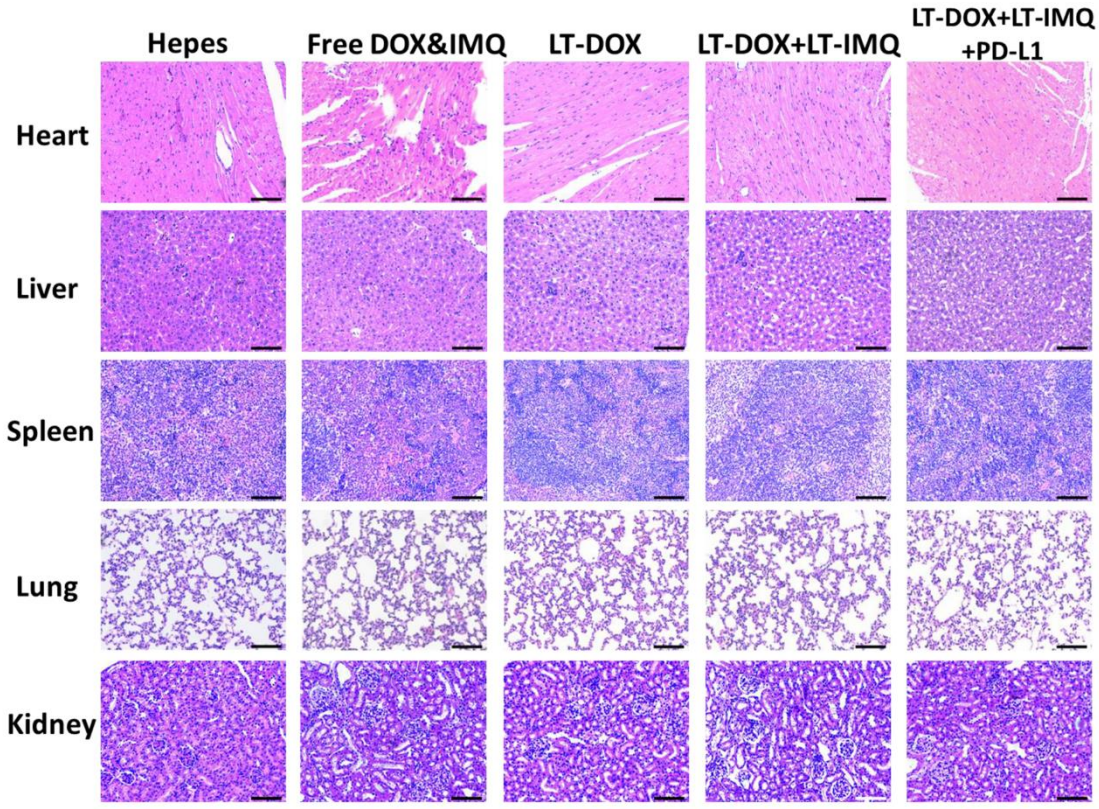


Figure S14 HE staining of major organs after different treatments. The scale bar represents 100 μm.

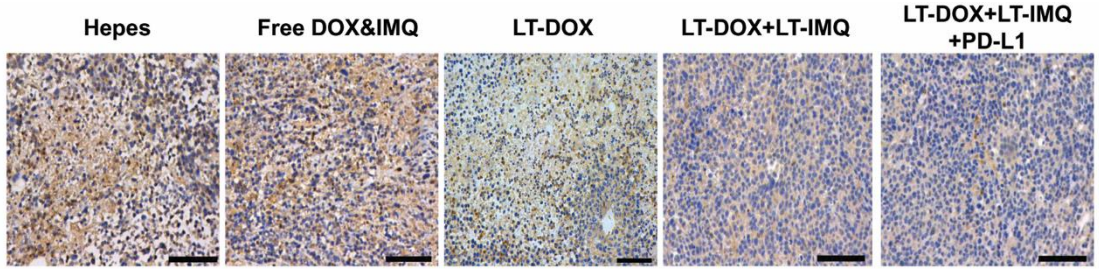


Figure S15 Immunohistochemistry to detect MMP9 in tumors at the end of therapy. The scale bar represents 100 μm (shown in brown).