Supporting Information

Engineering macrophage-derived exosomes for targeted chemotherapy of triple-negative breast cancer

Sha Li,[a, b] Yijing Wu,[c] Fei Ding,[d] Jiapei Yang,[d] Jing Li,[e, f, g] Xihui Gao,*[, h, f] Chuan Zhang*[, d, f] and Jing Feng,*[, e, f, g]

[a.] Anhui University of Science and Technology Affiliated Fengxian Hospital, 6600 Nanfeng Road, Fengxian District, Shanghai, 201499 (China)

[b.] Medical College, Anhui University of Science and Technology, 168 Taifeng Road, Huainan, 232001 (China)

[c.] Zhiyuan College, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai, 200240 (China)

[d.] School of Chemistry and Chemical Engineering, Shanghai Jiao Tong University, 800 Dongchuan Road, Shanghai 200240, People's Republic of China. E-mail: chuanzhang@sjtu.edu.cn

[e.] Department of Laboratory Medicine & Central Laboratory, Southern Medical University Affiliated Fengxian Hospital, 6600 Nanfeng Road, Fengxian District, Shanghai, 201499 (China). E-mail: fengjing8801530@smu.edu.cn

[f.] Joint Research Center for Precision Medicine, Shanghai Jiao Tong University Affiliated Sixth People's Hospital South Campus, 6600 Nanfeng Road, Fengxian District, Shanghai, 201499 (China)

[g.] Shanghai University of Medicine & Health Sciences affiliated Sixth People's Hospital South Campus, 6600 Nanfeng Road, Fengxian District, Shanghai, 201499 (China)

[h.] Key Laboratory of Medical Molecular Virology (MOE/NHC/CAMS), School of Basic Medical Sciences, Fudan University, 131 Dong An Road, Shanghai 200032, China. E-mail: gaoxihui@fudan.edu.cn

1. Materials and Methods

Materials

3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was purchased from Sigma-Aldrich (Shanghai, China). Dimethyl sulfoxide (DMSO) was obtained from Tansoole-reagent (Shanghai, China). The cMET binding peptide (KSLSRHDHIHHHC) was synthesized by China Peptides Co. Ltd (Shanghai, China). The dead cell apoptosis kit with Annexin V Alexa Fluor™ 488 & Propidium Iodide (PI) was purchased from Thermo Fisher Scientific Inc (Shanghai, China). The one-step terminal deoxynucleotidyl transferase dTUP nick labeling (TUNEL) cell apoptosis assay kit was bought from Beyotime Biotechnology (Shanghai, China). LysoTracker™ Green DND-26 were obtained from Thermo Fisher Scientific Inc. (Shanghai, China). Leibovitz's L-15 medium, Dulbecco's Modified Eagle's medium (DMEM), fetal bovine serum (FBS), Trypsin-EDTA (0.25%), and Soybean Trypsin Inhibitor were obtained from Thermo Fisher Scientific Inc. (Shanghai, China). MDA-MB-231 cells (a human breast carcinoma cell line) and RAW264.7 cells (a mouse macrophage cell line) were provided by Stem Cell Bank, Chinese Academy of Science.

Histopathological study.

The tumor tissues excised from triple-negative breast cancer patients were fixed in 10 % neutral buffered formalin and sectioned with a thickness of 10 μm. After incubating with rabbit anti-c-Met (1:100 dilution) primary antibodies at 4 °C for overnight, they were incubated with Alexa Fluor 488-labeled goat anti-rabbit secondary antibody (1:400 dilution) at room temperature for 1 h, followed by nucleus staining by DAPI. The immunofluorescence images were collected using a fluorescence microscope.

Preparation of PLGA nanoparticles.

Doxorubicin hydrochloride was prepared into nanoparticles by the nanoprecipitation method. Briefly, 5 mg of Doxorubicin hydrochloride and 11 μL of triethylamine (TEA) was dissolved in 2 mL of N, N-Dimethylformamide (DMF) and sonicated for ten minutes. 50 mg of Poly (D, L-lactide-co-glycolide) (PLGA, 50:50, Mw 38,000-54,000) was dissolved in 1mL of DMF and sonicated for ten minutes. Mixed the dissolved DOX and PLGA, sonicated for another ten minutes. The polymer solution was then slowly added dropwise to 100mL of ultrapure water containing 2 mg of D-α-tocopherol polyethylene glycol 1000 succinate (TPGS) and stirred for 4 hours. Next, the nanoparticle suspension was filtered through a membrane with a pore size of 0.22 μm and then concentrated by ultrafiltration at 4000 rpm through a 50 kD centrifugal filter unit (Millipore). The obtained PL-D nanoparticles (PL-D) were stored at 4 ℃.

Preparation of exosome membrane coated PLGA nanoparticles.

To collect the exosomes, RAW264.7 cells were grown in T225 flasks with exosome-free fetal

bovine serum (FBS)-containing cell culture medium until the cells reach 80% confluence. The culture medium was collected and centrifuged at 400 g for 5 min to remove cells, and then centrifuged at 16,500 g for 20 min to discard cell debris. The supernatant was then processed by membrane filtration (0.22 μm, Millipore, USA). The exosome pellets were collected by continuous ultracentrifugation at 100,000 g and 4℃ for 2 h. The contents of the exosomes were removed through hypotonic treatment. Resuspended the collected exosomes in a hypotonic buffer (2 mM Tris, 1 mM $MgCl_2$ and 1 mM KCl) with an EDTA-free protease inhibitor cocktail at 4 ℃ overnights. The suspension was further ultracentrifuged at 100,000 g at 4℃ for 4 h using a TLA-100.3 fixed angle rotor in an Optima TL-100 ultracentrifuge (Beckman Coulter). The precipitation was empty exosomes membrane vesicles. Then the exosomes membrane vesicles were re-dispersed in PBS, sonicated and extracted through a microporous membrane (pore size 200 nm) to get a uniform suspension. To prepare exosome membrane coated PLGA nanoparticles, empty exosomes vesicles were mixed with PLGA nanoparticles and then extruded 7 times through a 100-nm polycarbonate porous membrane using an Avanti mini extruder, offering the macrophage exosome coated nanoparticle EP-D. Finally, the c-Met binding peptides (KSLSRHDHIHHHC) were decorated on the exosomal membrane through 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (DSPE-PEG) to obtain the c-Met-targeting nanoparticle MEP-D.

Characterization of nanoparticles

The diameter and zeta potential of the nanoparticles were measured by DLS using a Zetasizer Nano ZS (Malvern Instruments Ltd.). The structure of the exosome-membrane-coated nanoparticles was visualized using a Tecnai G2 Spirit Bio-TWIN electron microscope (FEI) at 120 kV. The coating of exosome membranes on PLGA nanoparticle was verified by confocal microscopy imaging using a Leica TCS SP8 confocal laser scanning microscope (Leica Microsystems). Exosome membrane and PLGA nanoparticle were labelled with 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) and 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO), respectively. DiO was excited at 488 nm and the emission was detected between 500-520 nm, while DiI was excited at 561 nm and the emission was detected between 580-640 nm. To clarify the spectrum interference, we measured the emission spectra of DiO and DiI at the excitation wavelength of 488 nm and 561 nm, respectively. As shown in Figure S12, the fluorescence of DiI was negligible compared with DiO at the excitation wavelength of 488 nm. Besides, the fluorescence of DiO was also negligible compared with DiI at the excitation wavelength of 561 nm, suggesting that the fluorescence of DiO and DiI can be well separated under these conditions.

Cell culture

MDA-MB-231 cells were cultured in Leibovitz's L-15 medium supplemented with 10% FBS in a humidified atmosphere at 37℃ without CO_2. RAW264.7 cells were cultured in Dulbecco's

Modified Eagle's medium containing 10% exosome-free FBS in a humidified atmosphere with 5% CO_2 at 37℃.

Determination of endocytosis pathways.

MDA-MB-231 cells were cultured in 10 mm confocal microscopy dishes with a density of 10,000 cells per dish for 24 hours. Then the cells were treated with drugs with the Dox concentration of 0.5 μg/mL for 1 h and 4 h. Next, cells were washed with PBS, stained with LysoTracker Green and Hoechst according to the product manuals, and observed by using a Leica TCS SP8 confocal laser scanning microscope (Leica Microsystems). The lysotracker was excited at 488 nm and the emission was detected between 500-520 nm, while Dox was excited at 561 nm and the emission was detected between 580-640 nm. Under these conditions, there is almost no fluorescence signal in the cells which were not incubated with Dox and lysotracker, suggesting that the autofluorescence was very low (Figure S13A). After incubated with MEP-D at a Dox concentration of 0.5 μg/mL for 4 h and stained with lysotracker, bright fluorescence was observed in the nuclear region in the red channel (580-640 nm) (Figure S13B). In contrast, there was almost no signal in the nuclear region in the green channel (500-520 nm). Besides, the emission spectra of Dox and lysotracker at the excitation wavelength of 488 nm and 561 nm were also measured. As shown in Figure S13C, the fluorescence of Dox in the wavelength of 500-520 nm was negligible compared with the lysotracker at the excitation wavelength of 488 nm. Also, the fluorescence of lysotracker in the wavelength of 580-640 nm was negligible compared with Dox at the excitation wavelength of 561 nm, indicating that the fluorescence of Dox and lysotracker can be well separated under these conditions.

Flow cytometric analysis of cellular uptake

Flow cytometry was used to analyze the cellular uptake. MBA-MD-231 cells were placed into 6-well culture plates at a density of 1×10^5 cells per well and cultured for 24 h. Then, the original media were replaced with the media containing DOX, PL-D, EP-D, and MEP-D with the same DOX concentration of 0.5 μg/mL. The cells were continued to be cultured for 4 h. Afterward, the cells were washed twice with PBS, digested by trypsin and collected through centrifugation, and finally resuspended in 400 μL of 0.01 M PBS. The fluorescence signals were quantified by counting 10,000 events using a BD LSR Fortessa analyzer (BD Biosciences).

Apoptosis assay.

MDA-MB-231 cells were seeded in 10 mm confocal microscopy dishes (10,000 cells per dish). After 24 h of continuous culture, the culture medium was replaced with drug-free L-15 containing DOX, PL-D, EP-D, and MEP-D, and cells were cultured for another 12 h. Then, cells were washed twice with PBS, stained using 4'6-diamidino-2-phenylindole (DAPI) and the one-step TUNEL apoptosis assay kit in accordance with the manufacturer's protocol. Then apoptosis of MDA-MB-231 cells was observed by confocal fluorescence microscopy (Leica, RCS SP8 STED 3X). The FITC labeled dUTP was excited at 488 nm and the emission was detected between 500-520 nm.

Figure S14 showed the emission spectra of Dox and FITC excited at 488 nm. Although Dox could be excited at 488 nm, its emission mainly located at 540-680 nm. The fluorescence of Dox between 500-520 nm was negligible compared with that of FITC.

MDA-MB-231 cells were seeded in 24-well plates (10,000 cells per well). After 24 h of continuous culture, the culture medium was replaced with drug-free L-15, L-15 containing DOX, PL-D, EP-D, and MEP-D with the same Dox concentration (0.5 μg/mL), and cells were cultured for another 12 h. Then, cells were washed twice with PBS, collected and stained using the Annexin-V/PI cell apoptosis kit according to the manufacture's protocol. The cell apoptosis analysis was performed by flow cytometry (LSRFortessa, Becton Dickinson). MDA-MB-231 cells treated with saline were used as a negative control. Unstained MDA-MB-231 cells, cells stained with PI and cells stained with Alexa Fluor 488 Annexin-V were used as control groups to set gates and voltages.

***In vitro* cytotoxicity.**

The *in vitro* cytotoxicity of drugs was investigated by MTT assay. In particular, the MDA-MB-231 cells were cultured in 96-well-plates with a density of 5,000 cells per well for 24 hours. Then, the original media were replaced with media containing DOX, PL-D, EP-D, and MEP-D with different DOX concentrations ranging from 0 to 2000 ng/mL, and cells were cultured for another 24 h. 20 μL MTT solution (5 mg/mL) was added to the cells in each well and replaced by 150 μL DMSO after 4 h of incubation. In the end, the absorbance of dissolved formazan in each well was measured using a microplate reader (Bio Tek, SynergyH4) at a wavelength of 490 nm. The cytotoxicity of PL, EP and MEP (without Dox) was also evaluated using the same method with different concentrations of the nanoparticles ranging from 0 to 60 μg/mL.

***In vivo* pharmacokinetic study.**

Sprague Dawley rats (SD rats) were randomly divided into 4 groups (4 rats per group). SD rats of the same weight were intravenously injected with an equal volume of free DOX, PD-L, EP-D, and MEP-D containing the same DOX concentration (5 mg/kg), respectively. At the predetermined time points (0.17 h, 0.5 h, 1 h, 2 h, 4 h, 8 h, 12 h, 24 h and 48 h), 500 μL orbital venous blood was collected from each rat, and then the blood was centrifuged to separate the plasma. Finally, the fluorescence intensity of DOX in the plasma was measured at a wavelength of 580 nm.

Orthotopic tumor model

Four-week-old female Balb/c nude mice were bought from Shanghai Slac Lab Animal Ltd (Shanghai, China). All the animal studies were certified by the Animal Ethics Committee of Shanghai Jiao Tong University and performed according to the guidelines for the care and use of

laboratory animals. MDA-MB-231 cells were injected into the right mammary fat pads of the nude mice (1×10^6 cells per pad). The growth of tumors was monitored every week and tumor volumes were calculated in accordance with the formula: V (mm^3) = 1/2 × length (mm) × width (mm) × width (mm).

***In vivo* tumor targeting and biodistribution studies.**

When the volume of tumors reached 400 mm^3, the nude mice were separately injected with Cy5.5-labeled PL-D, EP-D and MEP-D for *in vivo* optical imaging. At the predetermined time points, mice were anesthetized with isoflurane and imaged using an IVIS Lumina II *in vivo* imaging system (Caliper Life Sciences, USA) with 650 nm excitation wavelength and 680-750 nm emission wavelength. After that, the mice were sacrificed and major tissues (heart, liver, spleen, lung, kidneys, tumor) were collected and rinsed by PBS. The tissues were imaged by IVIS Lumina II *in vivo* imaging system. The fluorescence intensities of nanoparticles were analyzed by the Living Image Software. Next, the tumor tissues were fixed with paraformaldehyde, sectioned, and stained with c-Met antibody and Hoechst. The tissues were observed using the Leica TCS SP8 confocal laser scanning microscope (Leica Microsystems).

***In vivo* antitumor efficacy assay.**

When the tumor volumes reached 60 mm^3, tumor-bearing female nude mice were randomly divided into 5 groups (n=4). Thereafter, the mice were intravenously injected with 100 μL of nanoparticles at the same DOX dose of 5 mg/kg through the tail vein every three days for six consecutive doses. The control group was injected with equal volumes of PBS. During the treatment, the mice's weight and tumor length and width were measured every three days, and the tumor sizes were calculated according to the formula V (mm^3) = 1/2 × length (mm) × width (mm) × width (mm). After treatment, the tumor tissues of the mice were stripped. For the TUNEL apoptosis assay, the fixed tumor sections were stained by the one-step TUNEL apoptosis assay kit according to the manufacturer's protocol and observed by fluorescence microscope. To investigate the *in vivo* biosafety, mouse from MEP-D group was dissected after the 9th and 18th days of MEP-D treatment, and the main organs including heart, liver, spleen, lung, kidney, brain and muscle were collected. The main organs were fixed in 4% paraformaldehyde, sliced and stained with H&E, and subsequently observed using an optical microscope.

***In vivo* biocompatibility study.**

To investigate the in vivo biocompatibility of MEP-D, mouse was dissected at 9th and 18th days treatment of MEP-D. The main organs including heart, liver, spleen, lung, kidney, brain and muscle were collected, sectioned and stained with hematoxylin and eosin. The immunogenicity of MEP-D was investigated by evaluating the circulating cytokine levels using enzyme-linked immunosorbent assay. Normal C57BL/6 mice were injected with MEP-D at Dox dose of 5 mg/kg through the tail vein every three days for six consecutive doses. Mice treated with saline and lipopolysaccharide

(0.4 mg/kg, intraperitoneal injection) were included as negative and positive controls, respectively. Serum levels of alanine aminotransferase and aspartate aminotransferase were tested. Normal C57BL/6 mice were injected with saline or MEP-D at the Dox dose of 5 mg/kg through the tail vein every three days for six consecutive doses. Blood samples were collected via eye puncture and serum was harvested by centrifugation at 4°C for biochemical profiling.

Statistics

All statistical data were displayed as means ± standard deviation (SD). Statistical significance between groups was determined by Student's t-test. Differences were considered to be significant when $P < 0.05$.

2. Supplementary Figures

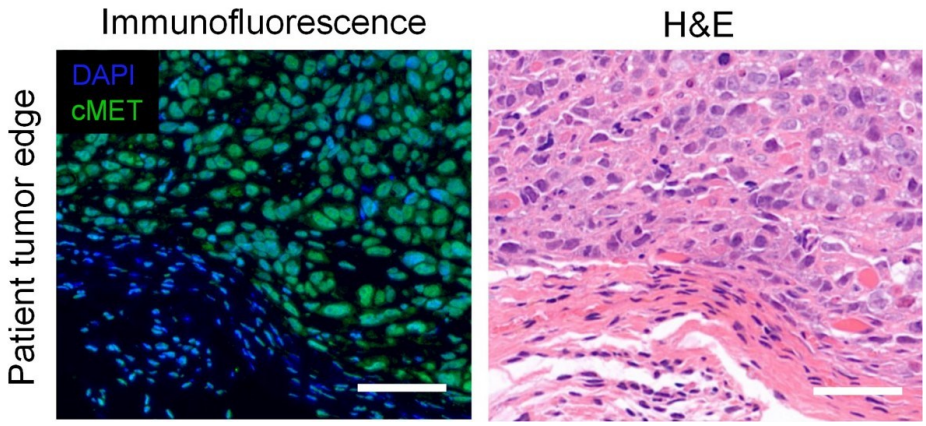

Figure S1. The immunofluorescence image of tumor margins from TNBC patients shows the upregulated expression of c-Met in the tumor tissue. H&E image of the tumor margins from TNBC patients. Bar: 50 μm.

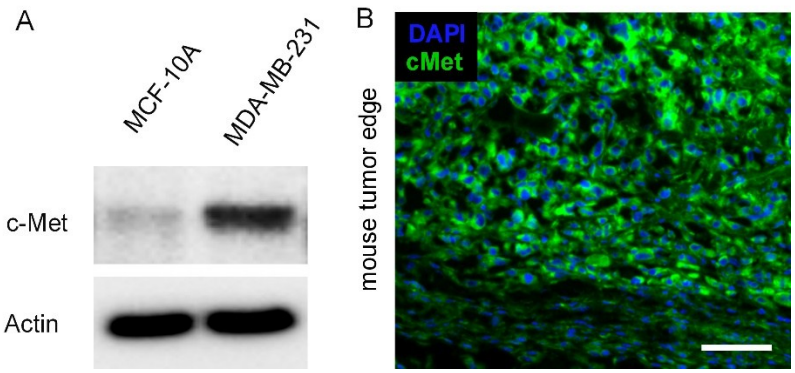

Figure S2. The expression level of c-Met in normal and tumor cells. (A) Western blot analysis of c-Met expression in the MCF-10A and MDA-MB-231 cell lines. (B) Immunofluorescence image of tumor margins from triple-negative breast cancer tumor-bearing mouse. Bar: 50 μm.

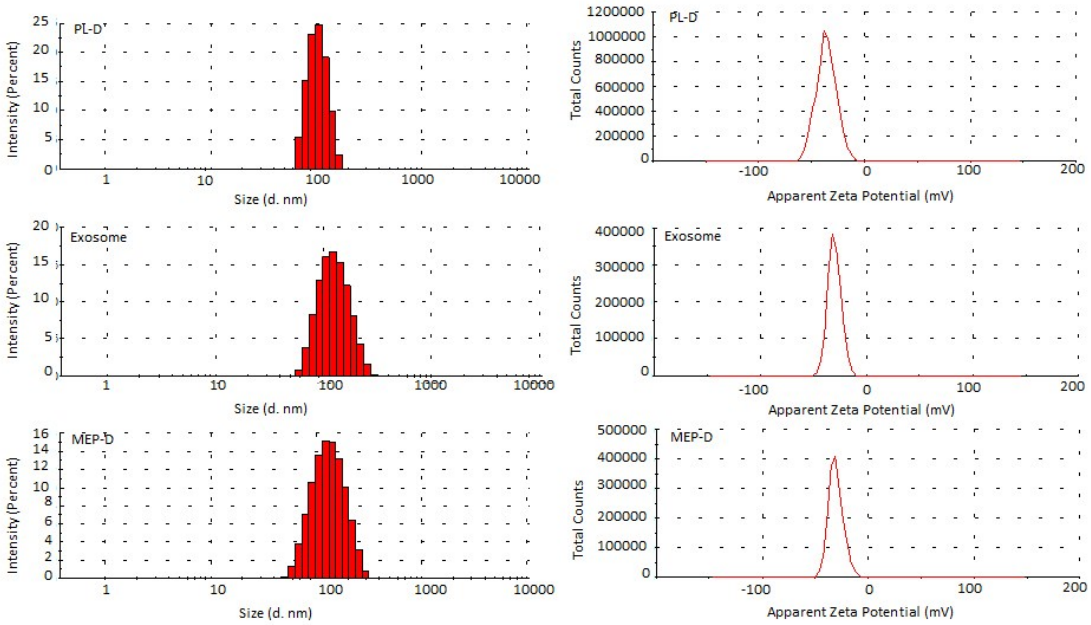

Figure S3. Hydrodynamic diameter and zeta potential of the nanoparticle PL-D, Exosome and MEP-D.

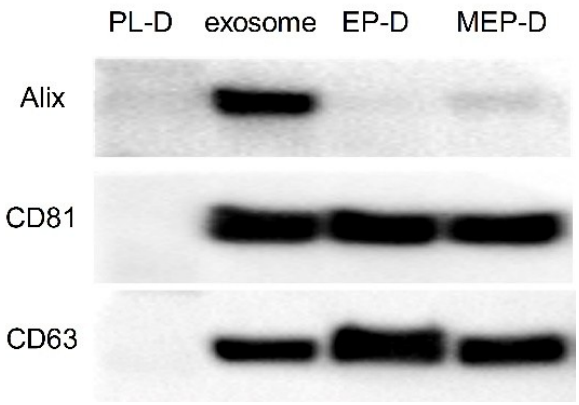

Figure S4. Western blot analysis of the commonly used exosome markers Alix, CD81 and CD63 in PL-D, exosome, EP-D and MEP-D.

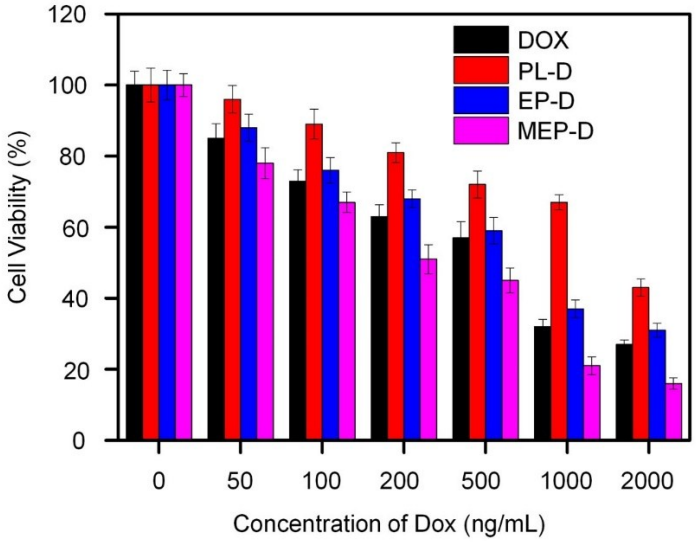

Figure S5. Viability of MDA-MB-231 cells after treatment of DOX, PL-D, EP-D and MEP-D for 24 h with the concentration of DOX ranged from 0 to 2000 ng/mL.

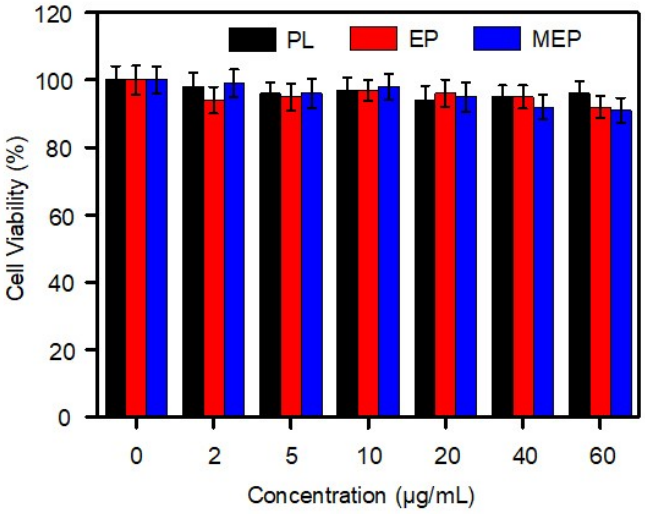

Figure S6. Viability of MDA-MB-231 cells after treatment of PL, EP and MEP for 24 h with concentration ranging from 0 to 60 μg/mL.

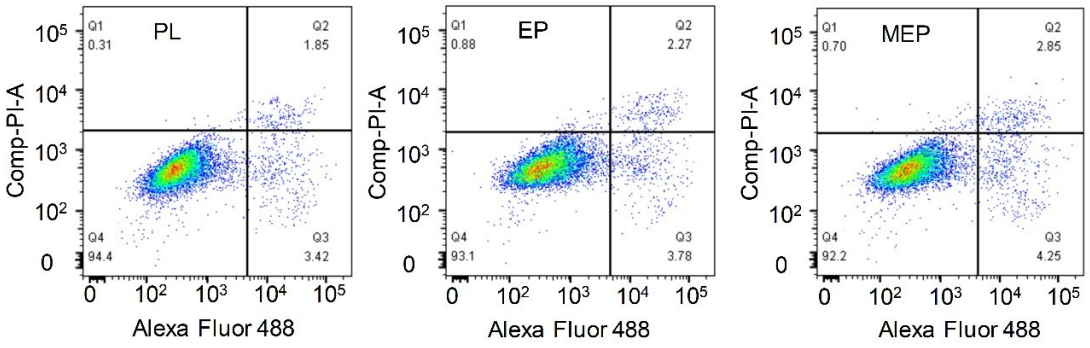

Figure S7. Flow cytometry analysis of MDA-MB-231 cells after staining with Annexin V and PI. The cells were treated with PL, EP and MEP at the same concentration of 15 μg/mL for 12 h.

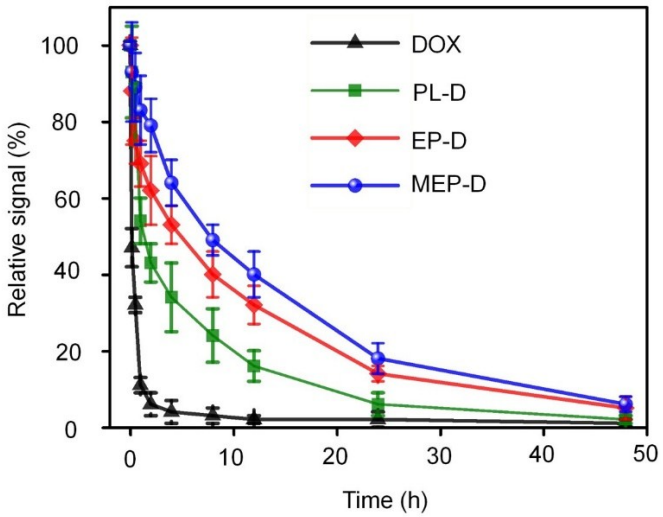

Figure S8. Pharmacokinetics of DOX after intravenous injection of DOX, PL-D, EP-D and MEP-D into mice at DOX dose of 5 mg/kg.

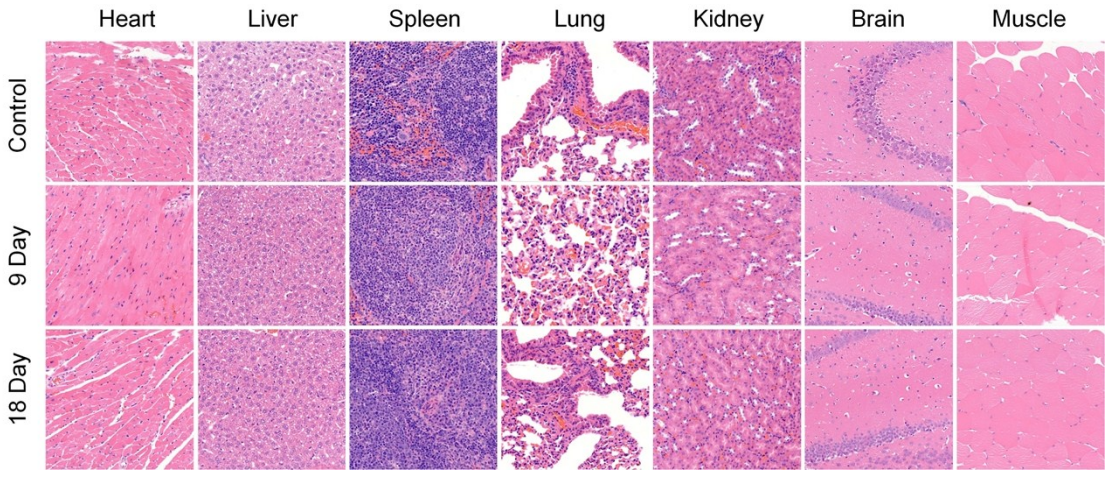

Figure S9. Histological images of the main organs of mice after treatment with PBS or MEP-D every 3 days. Organs were harvested at 9 day or 18 days after treatment and then sectioned for H&E staining.

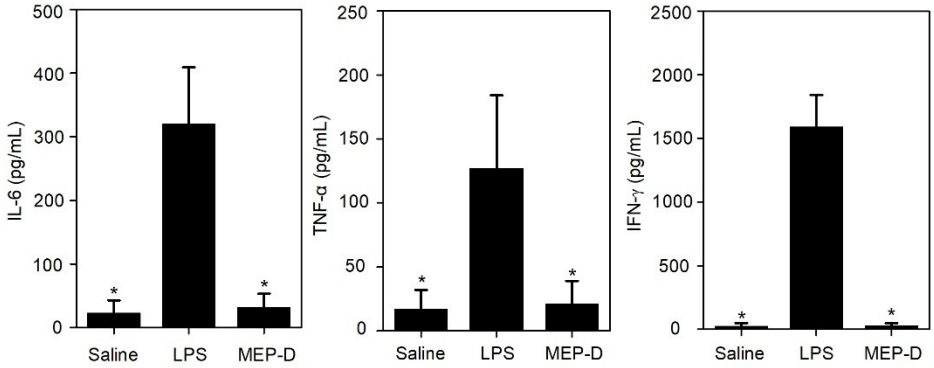

Figure S10. Circulating cytokine levels for C57BL/6 mice treated with saline, lipopolysaccharide and MEP-D (n = 4). *p < 0.05 versus lipopolysaccharide (LPS) group.

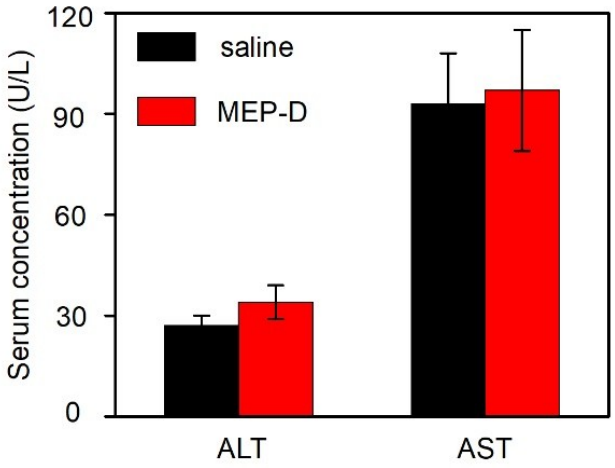

Figure S11. Serum levels of ALT and AST in normal mice after treatment with the saline, or MEP-

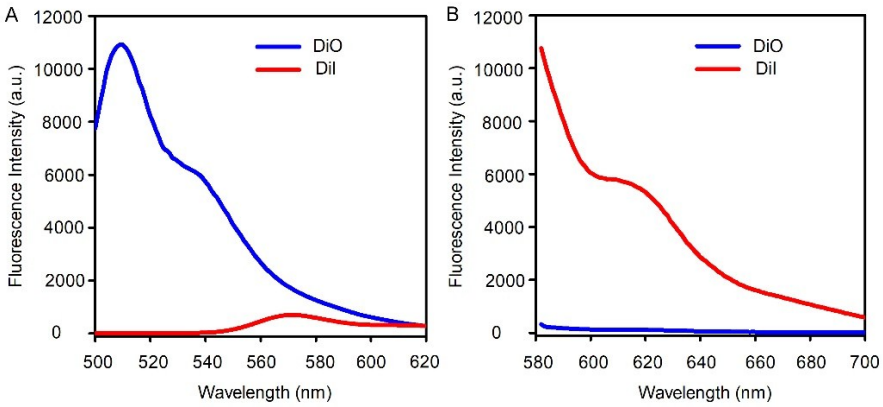

Figure S12. Emission spectra of DiO and DiI at the excitation wavelength of 488 nm (A) and 561 nm (B).

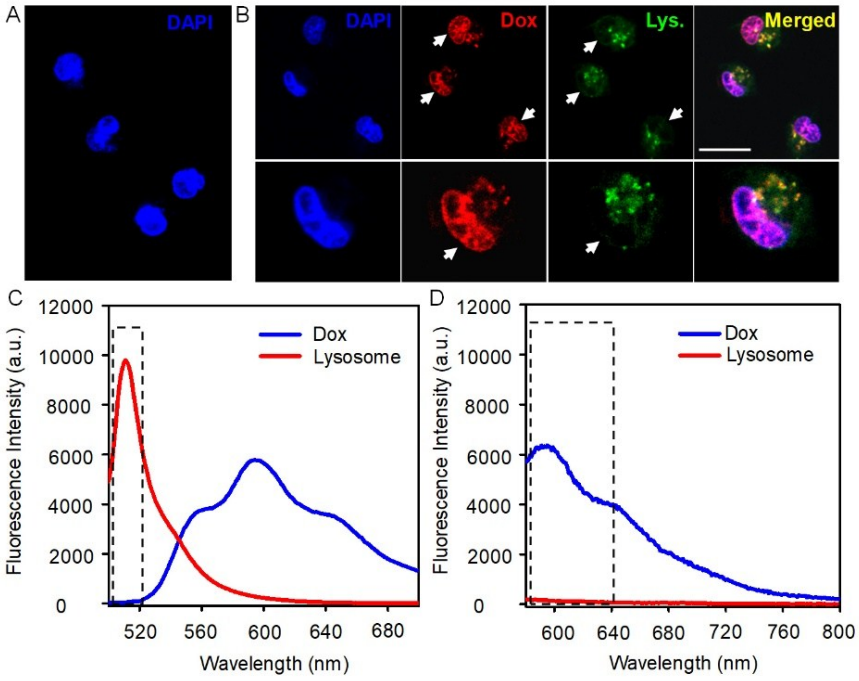

Figure S13. (A) CLSM images of MDA-MB-231 cells without MED-P treatment and lysotracker staining. (B) CLSM images of MDA-MB-231 cells incubated with MED-P for 4 h. The lysosomes were stained by lysotracker Green and the nuclei were stained by DAPI. Scale bar: 20 μm. (C) Emission spectra of Dox and lysotracker at the excitation wavelength of 488 nm and 561 nm.

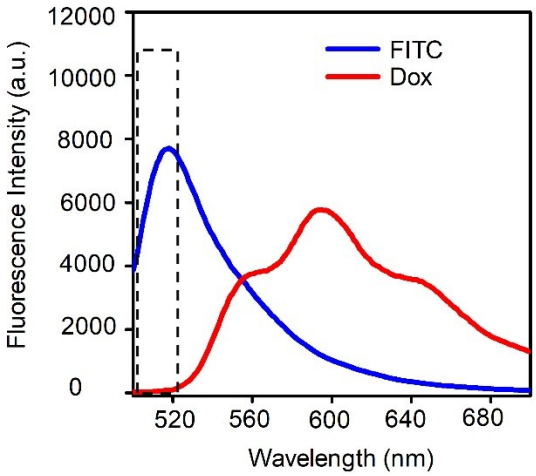

Figure S14. Emission spectra of Dox and FITC at the excitation wavelength of 488 nm.