Supporting Information for

Phospholipid-Mimic Oxaliplatin Prodrug Liposome for Treatment of Metastatic Triple Negative Breast Cancer

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#: Equal contribution to this study

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Experimental Section

Materials

Succinic anhydride, hexadecyl isocyanate, 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT), 1-ethyl-3-(3-dimethyl amino propyl) carbodiimide hydrochloride (EDCI), 4-dimethylaminopyridine (DMAP) were all purchased from Sigma-Aldrich (Shanghai, China). 4',6-diamidino-2-phenylindole (DAPI), Lyso-Tracker green (DND-26), and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindotricarbocyanine iodide (DiR) were obtained from Life Technologies (Shanghai, China). Cholesterol, 1-palmitoyl-2-hydroxysn-glycero-3-phosphocholine and DSPE-PEG_{2k} were purchased from A.V.T. Pharmaceutical Co., Ltd (Shanghai, China). Oxaliplatin (OXA) was purchased from Platinum Energy. Co. Ltd (Shandong, China). The RPMI1640 medium, fetal bovine serum (FBS), penicillin-streptomycin solution and trypsin-EDTA solution were purchased from Gibco (Tulsa, OK). All other chemicals if not mentioned were analytical grade and obtained from SinoPharm Chemical Reagent Co., Ltd. (Shanghai, China).

Synthesis of Oxalipid prodrug

Firstly, OXA (0.25 g, 0.63 mmol) was dissolved in 4 mL of 30% H_2O_2. The reaction was continued at room temperature for 24 h. The solution was condensed through rotary evaporation to obtain raw product. The resulting product was re-dissolved in cold methanol and precipitated in cold ethyl ether, washed twice with cold ethyl ether and dried under vacuum to obtain OXA(IV)-OH as white powder (0.26 g, yield 95%).

Secondly, succinic anhydride (0.050 g, 0.5 mmol) was added into the solution of

OXA(IV)-OH (0.215 g, 0.5 mmol) in 3 mL of DMSO. After 12 h reaction under dark at room temperature, the solution was precipitated in cold ethyl ether, washed twice with cold ethyl ether and dried under vacuum to obtain OXA(IV)-COOH as white powder (0.24 g, yield 90%).

Thirdly, hexadecyl isocyanate (0.12 g, 0.45 mmol) and OXA(IV)-COOH (0.2 g, 0.38 mmol) were co-dissolved in 3 mL of DMF. The reaction was continued under dark at room temperature for 24 h, then the solution was condensed by vacuum evaporation and precipitated in cold ethyl ether. After washed twice with cold ethyl ether and dried under vacuum, fat acid-mimic Hexadecyl-OXA(IV)-COOH was obtained as white powder (0.18 g, yield 60%).

Finally, 1-palmitoyl-2-hydroxysn-glycero-3-phosphocholine (62 mg, 0.125mmol) was added into the solution of Hexadecyl-OXA(IV)-COOH (0.1 g, 0.125 mmol) dissolved in 5 mL of DCM with the addition of catalytic amount DMAP (18.3 mg, 0.15 mmol) and EDCI (28.6 mg, 0.15 mmol). After 48 h reaction under dark at room temperature, the solution was condensed by vacuum evaporation and precipitated in cold ethyl ether. After washed twice with cold ethyl ether and dried under vacuum, phospholipid-mimic OXA(IV) prodrug (Oxalipid) was obtained as white powder (0.13g, yield 80%). The final product was characterized using ^{1}H-NMR and ESI-MS spectrum examination. ESI-MS (negative mode): formula: [$C_{53}H_{102}N_4O_{16}PPt$], Calc. 1276.4, found 1276.5.

Preparation and characterization of Oxalipid liposome (PMOL)

PMOL was prepared using film hydration method. Briefly, 9 mg of Oxalipid, 0.5 mg

of cholesterol and 0.5 mg of DSEP-PEG_{2k} were co-dissolved in 3 mL of chloroform/methanol (V/V = 9:1), the solvent was dried under vacuum to form lipid film and then hydrated with 3.0 mL of phosphate buffer saline (PBS, pH 7.4) solution at 60 ℃ for 20 min. The obtained bigger vesicles were extruded 20 times through 200 nm, 100 nm and 50 nm polycarbonate filter. The preparation of DiR-labeled PMOL (PMOL@DiR) was in an identical way except for the addition of 0.5 mg of DiR before lipid film formation. Unloaded drugs were removed using centrifugal filter device (molecular weight cutoff (MWCO) = 30 kDa). For the preparation of iRGD-targeted PMOL (iPMOL), DSPE-PEG_{2k} was functionalized with a targeting ligand iRGD according to our previous publication.[1]

The hydrodynamic particle size and surface charge of the resulting PMOL were determined using the dynamic light scattering (DLS) examination (Nanosizer, Malvern Instrument). The morphology of liposome was examined using transmission electron microscopy (TEM) measurement operated at 120 kV (JEOL, Japan). The drug loading ratio (DL) and encapsulation efficiency (EE) of drug were measured using inductively coupled plasma mass spectrometry (ICP-MS).

Cell lines and cell culture

Murine breast cancer cell line of 4T1 cells, human colon cancer cell line of HCT116 cells and human non-small cell lung cancer cell line of A549 cells were all obtained from cell bank of Chinese Academy of Sciences (Shanghai, China). 4T1 cells were cultured in complete RPMI 1640 cell culture medium containing 10% fetal bovine serum (FBS), 2.5 g/L of glucose, 0.11 g/L of sodium pyruvate, 100 U/mL of penicillin

G sodium and 100 μg/mL of streptomycin sulfate. HCT 116 and A549 cells were both cultured in complete RPMI 1640 cell culture medium containing 10% fetal bovine serum (FBS), 100 U/mL of penicillin G sodium and 100 μg/mL of streptomycin sulfate. All the cells were maintained at 37 ℃ in 5.0% CO_2 atmosphere.

Cellular uptake of PMOL

The cellular uptake of PMOLwas examined using confocal laser scanning microscope (FluoView 1000, Olympus, Japan). Briefly, 4T1 cells were seeded on a live cell imaging glass bottom dish a density of 2×10^4 cells/well and then treated with PMOL@DiR for 4 h or 24 h. Afterwards, the cells were stained with DAPI and examined by confocal laser scanning microscopic (CLSM).

The cellular uptake mechanism of PMOL was examined through pre-inubating cells with several endocytosis inhibitors. Breifly, 4T1 cells were seeded in a 24 cell plate at a density of 1×10^5 cells per well and then treated with 0.45 M hypertonic sucrose (inhibitor of clathrin-mediated endocytosis) or 500 μg/ml methyl *β*-cyclodextrin (inhibitor of caveolin-mediated endocytosis) or 10 mM amiloride (inhibitor of macropinocytosis-mediated endocytosis) for 30 min and then incubated with DiR-labeleld PMOL for 1 h. The cells were then washed twice with PBS and collected after trypsinisation. The cells were treated with 0.3% trypan blue for 5 min to quench extracellular fluorescence, resuspended in PBS and then analyzed using a FACS Calibur flow cytometric system. The cellular Pt content was futherly measured using ICP-MS.

Cytotoxicity assay *in vitro*

The chemotoxicity of PMOL was measured using MTT assay. Briefly, 4T1 cells, HCT116 cells and A549 cells were seeded in the 96-well plate at a density of 3,500 cells per well in 100 μL of medium, respectively. After 24 h pre-incubation, the cells were incubated with OXA or PMOL for 24 h. The cell viability was expressed as the relative cell viability by normalizing with that of the untreated cells controls.

PMOL-induced cell apoptosis was examined with the Annexin V-FITC Apoptosis Detection kit. Briefly, 4T1 cells seeded in 24-well tissue culture plate were incubated with PMOL or OXA at an indentical Pt concentration of 10 μM for 12 h. Afterwards, the cells were harvested, washed twice with cold PBS, stained with Annexin V-FITC and PI respectively, and analyzed with a FACS Calibur flow cytometric system.

Pharmacokinetics and distribution of PMOL

To investigate the pharmacokinetics of PMOL, Sprague Dawley (SD) rats (female, 4~5 weeks, 180 ± 10 g, Shanghai Experimental Animal Center, Shanghai, China) were i.v. injected with 1 mL of Oxa or PMOL solution at an identical Pt dose of 2 mg/kg. Blood samples were collected at 5 min, 30 min, 1 h, 2 h, 6 h, 12 h and 24 h time intervals postinjection. The blood concentration of platinum was quantitatively examined using ICP-MS.

To invesitgate PMOL distribution *in vivo*, Balb/c nude mice (female, 3~4 weeks, 18 ± 2.0 g, Shanghai Experimental Animal Center, Shanghai, China) were injected with 1 $\times 10^6$ 4T1 cells on the right mammary gland. Seven days later, the mice bearing 4T1 tumors of 200 mm^3 were i.v. injected with DiR-labeled PMOL at an equal Pt

dose of 2.0 mg/kg. Near-infrared fluorescence imaging *in vivo* were carried out with an IVIS imaging system (Xenogen, Alameda, CA). The mice were sacrificed 24 h later and major organs were collected for determining organ distribution of Pt using ICP-MS.

Anti-tumor efficay of PMOL

Balb/c nude mice beraing 4T1 tumors of 50 mm^3 were randomly grouped (n = 5) and i.v. injected with OXA or PMOL at an equal Pt dose of 2.5 mg/kg at the 1st, 7th and 14th day. The tumor volume and the body weight were monitored druing the whole peroid. The tumor volume was calculated by formula $V = L \times W \times W/2$ (L, the longest dimension; W, the shortest dimension). All mice were sacrificed 19 days post the first injection and the tumors were weighted. The lungs were perfused with india ink and collected for counting of the metastasitic nodules in the lungs. All the major organs (e.g,, heart, liver, lung, spleen and kidneys) were harvested, fixed in 4% formalin solution, dehydrated and subjected to H&E staining.

Statistical analysis

Results are given as mean ± S.D. One way analysis of variance (ANOVA) was used to determine the significance of the difference. Statistical significance was set at $p < 0.05$ (* $p < 0.05$, ** $p < 0.01$).

Reference

1. B. Feng, F. Zhou, Z. Xu, T. Wang, D. Wang, J. Liu, Y. Fu, Q. Yin, Z. Zhang, H. Yu, *Adv. Funct. Mater.*, 2016. **26**, 7431–7442.

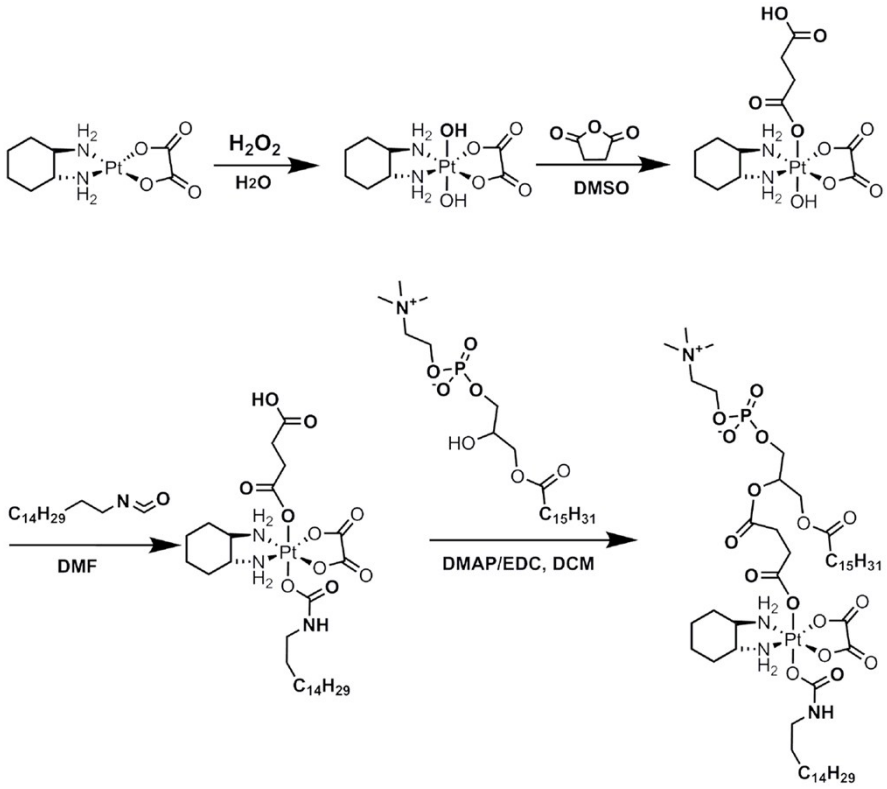

Figure S1. Synthesis process of Oxalipid**.**

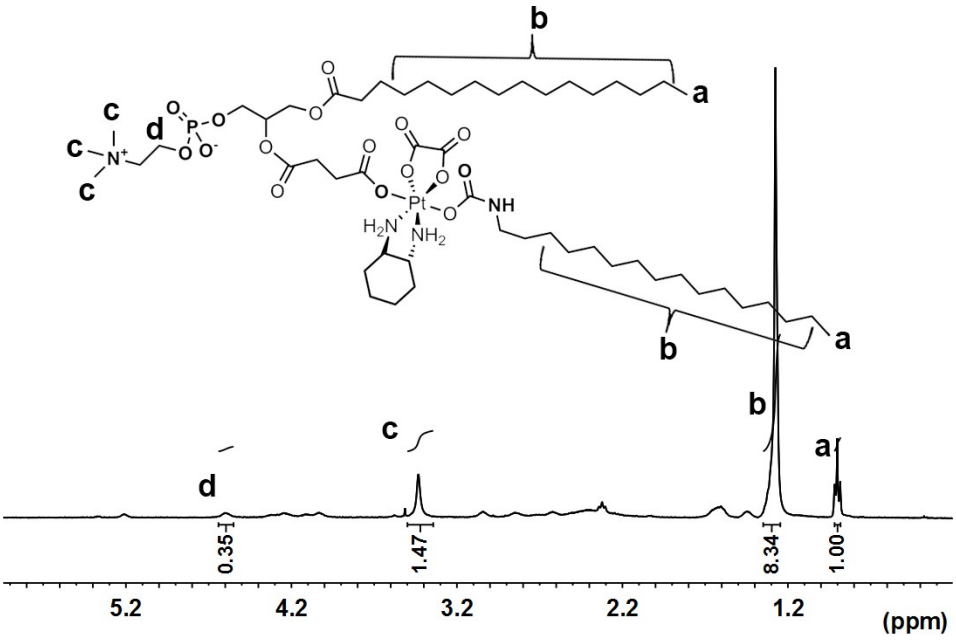

Figure S2. ^{1}H-NMR spectrum of Oxalipid.

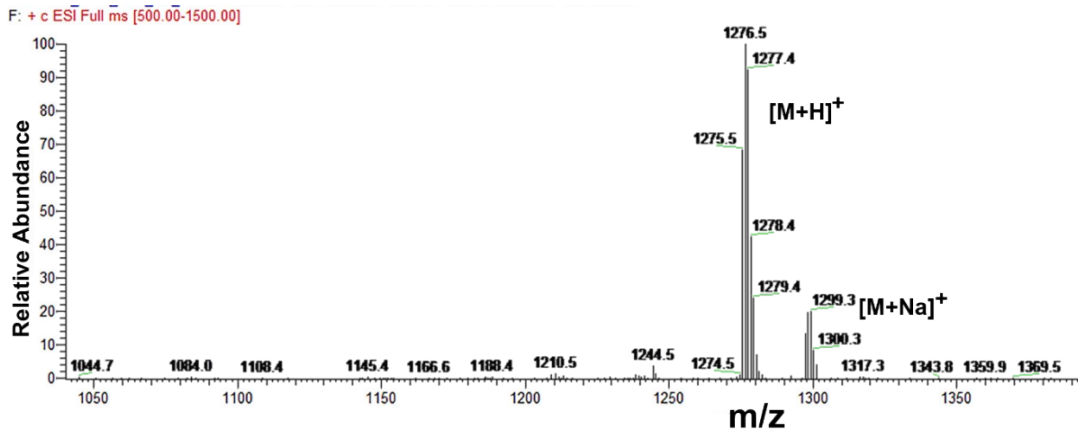

Figure S3. ESI-MS spectrum of Oxalipid.

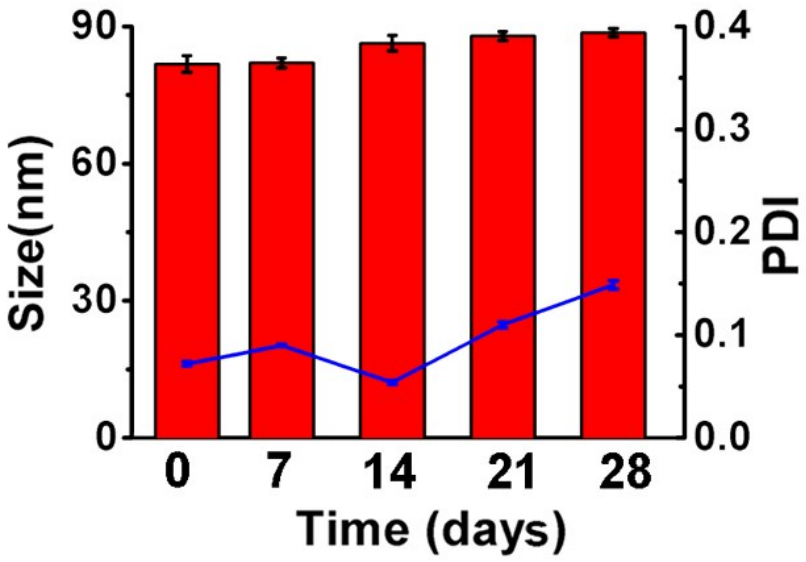

Figure S4. Serum stability of PMOL nanoparticles.

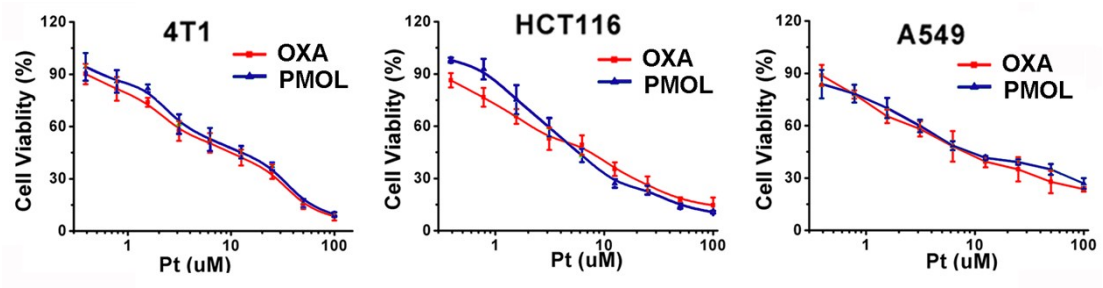

Figure S5. The cytotoxocity assay of PMOL in different cancer cells.

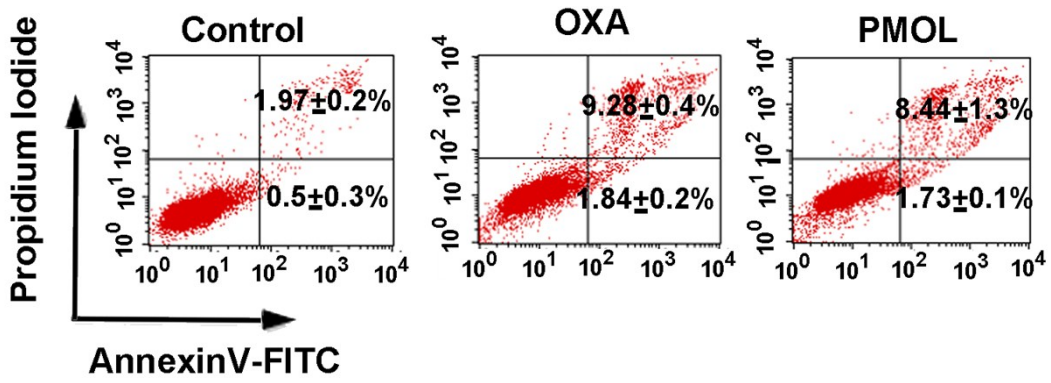

Figure S6. Flow cytometric exminaiton of PMOL-induce appotosis in 4T1 cells.

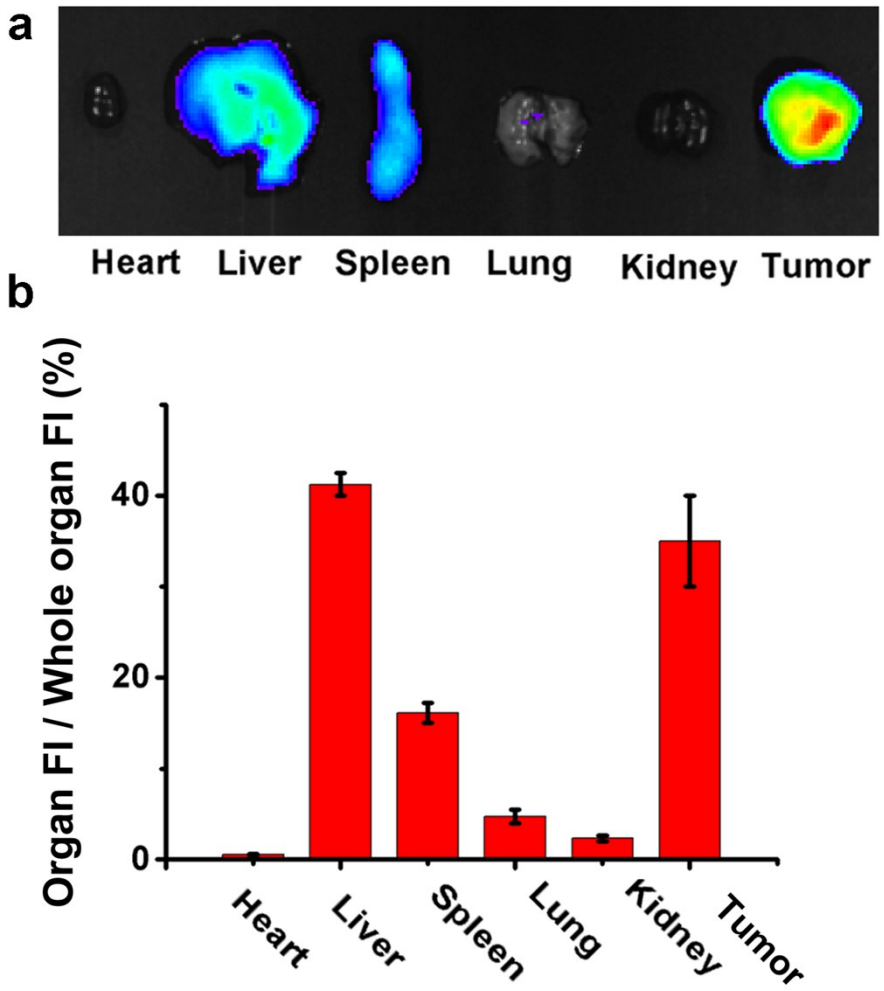

Figure S7. The *ex-vivo* distribution of PMOL examined at 24 h post injection and the semi-quantitative analysis of PMOL distribution in the major organs as determined by normalizing the fluorescence intensity with the whole organ fluorescence intensity.

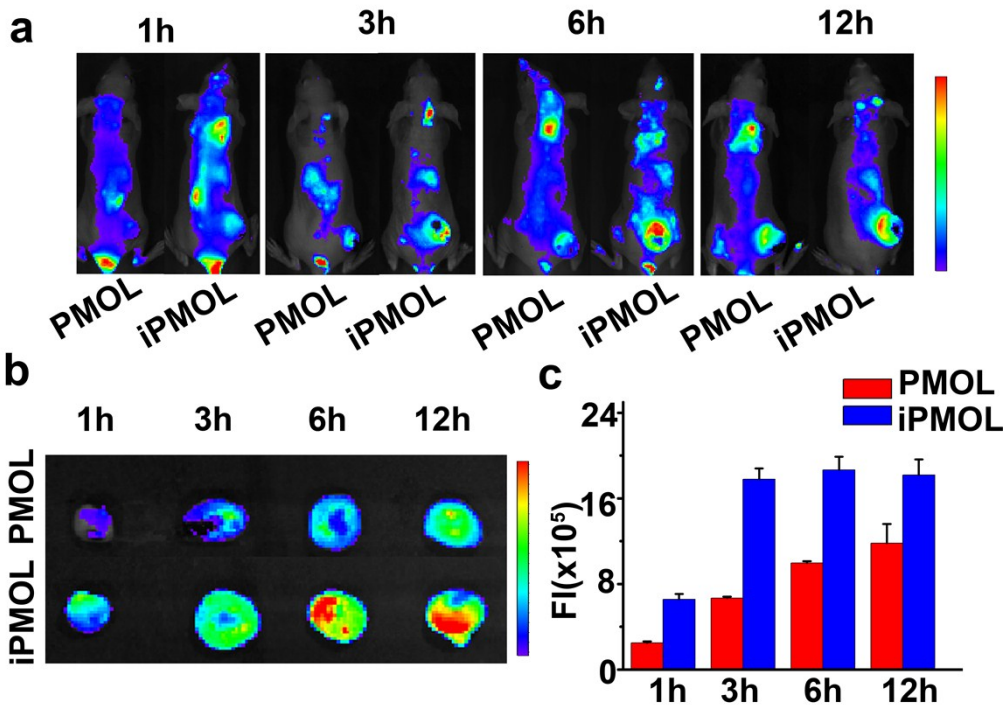

Figure S8. (a) Fluorescence imaging of active tumor targeting effect of iPMOL in 4T1 tumor-bearing mice in vivo; (b) Ex-vivo Tumor distribution of DiR-labeled PMOL and iRGD modifiyed PMOL (iPMOL); (c) Tumoral fluorescence intensity of PMOL and iPMOL at different time point postinjection.

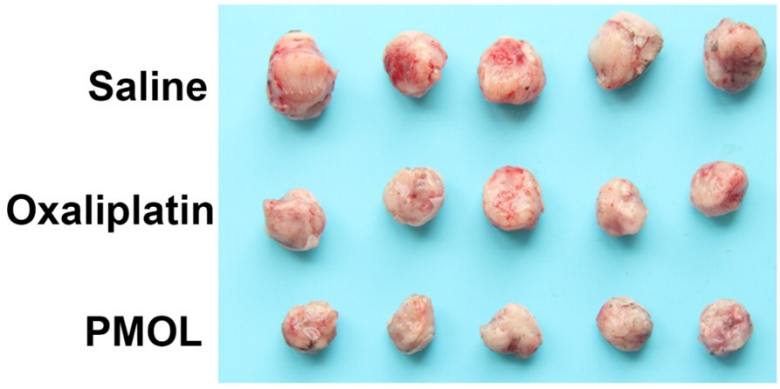

Figure S9. Photograph of tumors in different groups examined at the end of antitumor study.

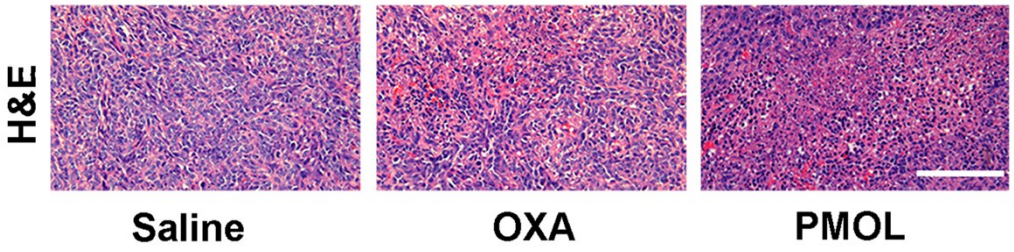

Figure S10. H&E staining of the tumor sections (scale bar 200 μm)

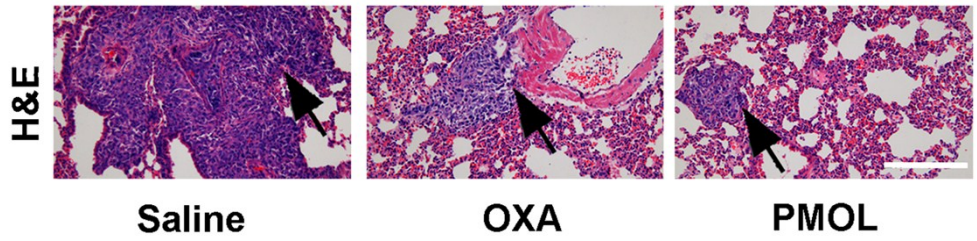

Figure S11. H&E staining of the lung sections (scale bar 200 μm).

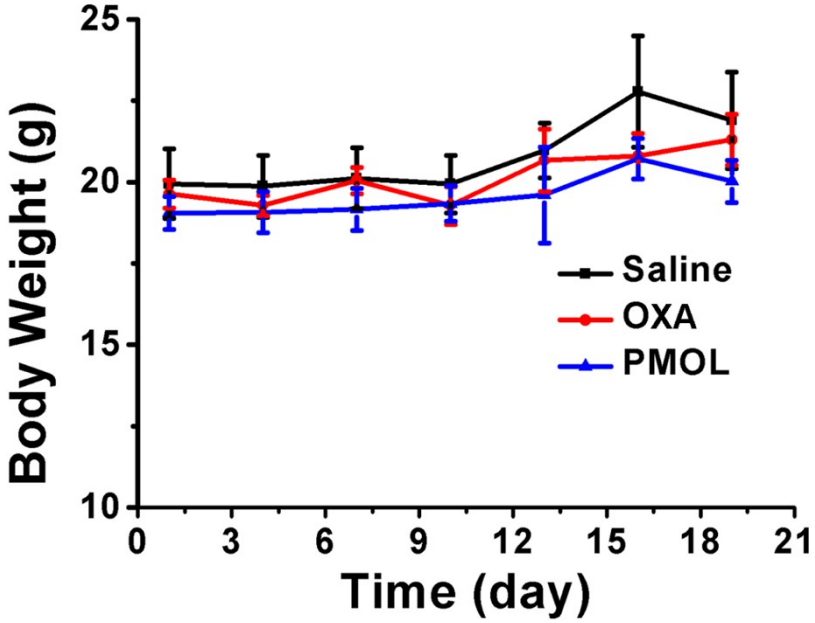

Figure S12. Change of body weight during the whole period of anti-tumor study.

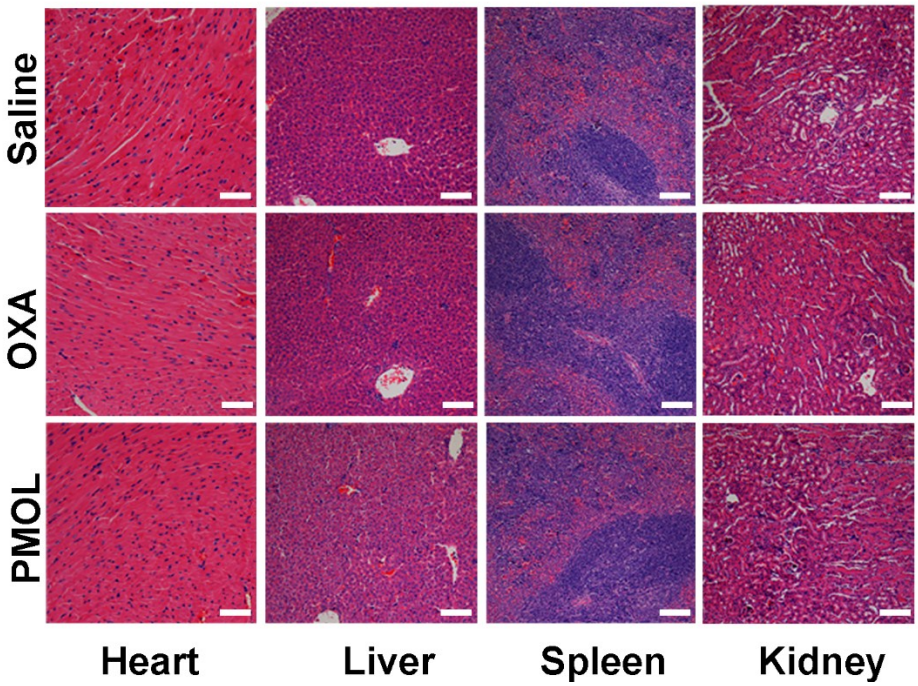

Figure S13. Histopathological examination of the major organs (*e.g.*, liver, spleen and kidney) at the end of the animal studies (scale bar 100 μm).