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Supplementary Information

Multifunctional Nano-Delivery System Enhances the Chemo-*co*-Photo Therapy of Tumor Multidrug Resistance via Mitochondrial-Targeting and Inhibiting P-glycoprotein-Mediated Efflux

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Methods and Experiments

1. Materials

N-hydroxysuccinimide (NHS), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC·HCl), IR-780 iodidend, and (4-carboxybutyl) triphenylphosphonium bromide (TPP-COOH), 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Aladdin Reagent Co., Ltd. (Shanghai, China). Methoxy poly (ethylene glycol)-amino ($mPEG_{2k}$-NH_2, Mw=2000) were obtained from Hunan HuaTeng Pharmaceutical Co., Ltd. 3-Mercaptopropionic acid, D-α-Tocopherol polyethylene glycol 1000 succinate (TPGS), DAPI and RPMI 1640 were supplied by Sigma Aldrich. Doxorubicin hydrochloride (DOX·HCl) was purchased from Beijing Hvsf United Chemical Materials Co., Ltd. (Beijing, China). PEG-TK was synthesized according to the literature.[1] MCF-7 and MCF-7/ADR cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/mL), and streptomycin (100 U/mL) in a humidified atmosphere of 5% CO_2 at 37°C. BALB/c nude mice (female, the weight of 16-18 g) were purchased from Beijing HFK Bioscience Co., Ltd. (Beijing, China). All animal experiments were performed in compliance with the guidelines of the Care and Use of Laboratory Animals and approved by the Experiment Animal Administrative Committee of the Institute of Radiation Medicine (Tianjin, China).

2. Preparation and characterization of ROS-responsive polymeric micelle

2.1 Synthesis of PEG-TK-DOX prodrug (PTD)

$mPEG_{2k}$-TK (0.05 mmol), EDC·HCl (0.1 mmol) and NHS (0.1 mmol) were dissolved in 2 mL of anhydrous DMSO. The reaction was performed under nitrogen atmosphere for 3 h at

room temperature to activate one end carboxyl group of thioketal (activated $mPEG_{2k}$-TK). DOX·HCl (0.05 mmol) were dissolved in 1mL of anhydrous DMSO, and 3 times the molar amount of triethylamine was added. The mixture was stirred at room temperature to remove hydrochloric acid of DOX·HCl for 3 h.

Transfer the DOX solution to the DMSO solution containing activated $mPEG_{2k}$-TK, and continue to stir and react for 48 h in the dark at room temperature. The resulted PEG-TK-DOX was purified by dialysis using 2000 D cutoff dialysis membrane against DMSO and distilled water, respectively, and freeze-dried.

2.2 Synthesis of TPP-TPGS

TPGS (0.33 mmol), (4-carboxybutyl) triphenylphosphonium bromide (TPP-COOH, 1 mmol) were dissolved in 5 mL of anhydrous dichloromethane, stir to complete dissolve. Subsequently, DCC (1.33 mmol) and DMAP (0.17 mmol) were added to the above reaction system, and the reaction was stirred for 24 h at room temperature. After the reaction, the insoluble DCU was removed by filtration, and dichloromethane was removed using a rotary evaporator. After the obtained solid product was re-dissolved in water, the solid insoluble in water was removed by filtration. The filtrate was transferred to a regenerated cellulose dialysis bag for 24 h, and freeze-dried to obtain TPP-TPGS.

2.3 Preparation of IR780 loaded drug delivery system (PTD/T/IR780) and mitochondria-targeted drug delivery system (PTD/TT/IR780)

PTD/TT/IR780 was prepared through solvent evaporation method. PTD (8 mg), TPP-TPGS (2 mg), and IR780 (2 mg) were dissolve in 1mL of acetone, stir to complete dissolve. Subsequently, under vigorous stirring, the mixture solution was added dropwise to 10 mL of

distilled water. After stirring for 30 min at room temperature, the acetone in the system was removed by rotary evaporation. The resulted solution was further purified by membrane (0.22 μm) and freeze-dried.

PTD/T/IR780 was prepared with similar procedure as mentioned above, where TPP-TPGS was replaced by TPGS.

Drug loading content (DL%) and was calculated by the following equations:

$$DL\% = \frac{weight\ of\ loaded\ drug}{weight\ of\ PTD/TT/IR780} \times 100\%$$

2.4 Characterizations

The structures of the synthetic compounds were characterized by ^{1}H NMR spectra using an ^{1}NMR spectrometer (Mercury Vx-300, USA). The size distribution and morphology of particles was characterized by dynamic light scattering (DLS, Zetasizer Nano-ZS, Malvern 3600, Worcestershire, U.K.), and transmission electron microscope (TEM, HT7700, Hitachi, Japan), respectively

3. Density functional theory calculation

In order to study the possible non-covalent interactions between the molecules of IR780, DOX, and tocopheryl succinate (TOS), we carried out the density functional theory (DFT) calculations using the Gaussian 16 suite of programs. Molecular optimization is performed at the B3LYP-D3 (BJ)/6-31G* level.[2-5] Harmonic vibration frequency calculation is implemented by the same method for optimized structures to ensure them as the local minimum structures. The structure of the IR780, DOX, and TOS molecule, and the complexes of them were optimized, and the independent gradient model (IGM) analysis was employer to make the weak interaction visible. Since the flexible alkane chain in the TOS

molecule will increase the complexity of optimization, the calculation time and the unknown uncertainty, and it has little effect to the weak interaction between the molecules when simulation calculated. The alkane chain is simplified to a methyl group when TOS is optimized.

4. *In vitro* cytotoxicity assay

MTT assay was performed to evaluate the cytotoxicity of micelles against tumor cells. Briefly, the MCF-7/ADR cells were seeded at a density of 8×10^3 cells/well in 96-well plate, respectively, and cultured for 24 h to allow cell attachment and stabilization. The cells were then treated with DOX, IR780, PTD/T/IR780 or PTD/TT/IR780, respectively, at equivalent DOX concentrations ranging from 0.31 to 5 μg/mL with or without laser irradiation (1 W cm^{-2}, 5 min, at the incubation time of 6th hour) for 24h. After that, each well was added with 10 μL of MTT solution (5 mg/mL) and incubated for 4 h. Then the medium was replaced with 150 μL DMSO to dissolve the formed purple formazan crystals. The absorbance at 570 nm of each well was determined and cell viability was calculated by the following formula:

$$Cell\ viability = \frac{Abs(sample) - Abs(blank)}{Abs(control) - Abs(blank)} \times 100\%$$

5. Apoptosis assay

MCF-7/ADR cells (5×10^5cells/well) were seeded in 6-well plate, respectively, and cultured for 24h to allow cell attachment and stabilization. The cells were then incubated with DOX, IR780, PTD/T/IR780 or PTD/TT/IR780, respectively, at DOX equivalent concentrations to 5 μg/mL with or without laser irradiation (1 W cm^{-2}, 5 min, at the incubation time of 4th hour). After another 12 h of incubation, the cells were collected and stained using the Annexin V-FITC/7-ADD kit. Flow cytometry and confocal laser scanning

microscope (CLSM) was utilized to analyze the cell apoptosis.

The photodamage of cells caused by the micelles was visually observed by fluorescence microscopy. MCF-7/ADR cells (5×10^5) were seeded in 6-well cell culture plate and cultured for 24 h to allow cell attachment and stabilization. The cells were incubated with DOX, IR780, PTD/T/IR780, and PTD/TT/IR780 micelles at DOX equivalent concentrations to 5 μg/mL were separately added for incubation for 4 h and treated with or without laser irradiation (1 W cm^{-2}, 5 min). After another 8 h of incubation, the cells were collected and stained with a mixture of Calcein-AM /PI and then observed by fluorescence microscopy.

6. *In vitro* cellular ROS generation

The intracellular ROS generation was studied via DCFH-DA probe with laser irradiation. MCF-7/ADR cells were cultured in 6-well plates and then incubated for 24 h at 37 °C. The culture medium was substituted by RPMI-1640 medium with free DOX, IR780, PTD/T/IR780 or PTD/TT/IR780 micelles, and the cells received incubation of 4 h. The cells treated with or without laser irradiation (1 W cm^{-2}, 5 min). Finally, each well was provided with 2×10^{-5}m DCFH-DA for incubation of 20 min. The fluorescence intensity derived from DCF was immediately measured by a CLSM and a flow cytometer for qualitative and quantitative detection, respectively.

7. *In vitro* cellular mitochondria-targeting uptake and distribution

The distribution of the micelles within the cell was observed by a CLSM. The method is as follows: select cells growing in the logarithmic phase and inoculate them into a laser confocal cell culture dish, the cell inoculation density is 1×10^5 cells/well. Place the confocal culture dish at 37°C and culture in a constant temperature incubator with a carbon dioxide

concentration of 5% for 24 h to make the cells stable and fully adherent. Aspirate the original medium and add fresh medium containing free DOX, IR780, PTD/T/IR780 or PTD/TT/IR780 micelles for incubation of 4 h. The corresponding concentration of DOX is set to 5 μg/mL. Subsequently, the cells treated with or without laser irradiation (1 W cm^{-2}, 5 min). After incubating for 18 h in the cell incubator, aspirate the medicated medium and rinse with PBS preheated at 37°C twice. Add 2 mL of pre-warmed Mito-Tracker Green to each well and incubate at 37°C for 30 min. Finally, PBS rinsed twice to reduce the interference of green background. Observe under a CLSM.

8. *In vitro* cellular mitochondria function

The cationic dye 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethyl-imidacarbocyanine (JC-1) assay was performed to evaluate the integrity of mitochondrial functions. MCF-7/ADR cells (5×10^5cells) were cultured in 12-well plates for 24h to allow cells attachment and stabilization. The cells were incubated with DOX, IR780, PTD/T/IR780 or PTD/TT/IR780 micelles at DOX equivalent concentrations to 5 μg/mL was separately added for incubation for 4 h, and treated with laser irradiation (1 W cm^{-2}, 5 min). After another 18 h of incubation, a solution of JC-1 reagent (10 μg/mL) was added and incubated for 10 min at 37°C. Subsequently, the cells were washed for 3 times with PBS, and centrifugation. The resulting was analyzed by flow cytometry.

9. *In vivo* Antitumor Study and histochemistry Analysis

The *in vivo* anti-tumor effect of different micelles was performed on MCF-7/ADR tumor-bearing nude mice model. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All procedures performed in studies involving

animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted. This article does not contain any studies with human participants performed by any of the authors. MCF-7/ADR cells (1×10^7 cells) were subcutaneously injected into the right armpit of BALB/c nude mice (female) to generate tumor bearing mice. When the tumor volume reached ~100 mm^3, tumor bearing mice were randomly divided into four groups (n=5) and intravenously injected with saline, DOX, IR780, PTD/T/IR780 or PTD/TT/IR780 micelles solutions at 2 μmol kg^{-1} for DOX and 1.4 μmol kg^{-1} for IR780 every 3 days. The body weight and tumor volume were measured every 2 days. Tumor volume was calculated according to the following formula: $V=Lc\times W^2/2$, where L and W represent longest and shortest diameter respectively. After 18-days treatment, all mice were sacrificed, and main organs (heart, liver, spleen, lung, and kidney) with tumors were collected for immunohistochemistry analysis.

10. *In vivo* Imaging and biodistribution Analysis

MCF-7/ADR cells (1×10^7cells) were subcutaneously injected into the right armpit of BALB/c nude mice (female) to generate tumor bearing mice. The mice were separated into two groups randomly after the tumor volumes were ~200 mm^3. The mice in the two groups were injected with IR780 or PTD/TT/IR780 micelles (2 μmol kg^{-1}) via the tail vein, respectively. The fluorescence signals were determined at 1, 3, 6, 12, 24 and 48 h to show the biodistribution using the live animal in vivo imaging system (IVIS Kinetic).

11. Statistical Analyses

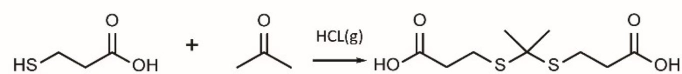
Values were expressed as mean ± SD. Comparisons between and within groups were conducted with unpaired Student's tests and repeated-measures ANOVA using GraphPad

Prism 7.0 (San Diego, CA, USA), respectively. Probability (p) values of <0.05 were considered statistically significant.

Table S1 Physicochemical Characterization of formulations

Formulations	Size (nm)	Zeta Potential (mV)	DL(DOX) %	DL(IR780) %
PTD	210	-12.98	17.4	/
PTD/TPGS	173	-12.66	15.8	/
PTD/T/IR780	155	-10.27	14.4	6.0
PTD/TT/IR780	151	10.62	12.5	6.3
PTD/TT/IR780(+H_2O_2)	/	44.47	/	/

A



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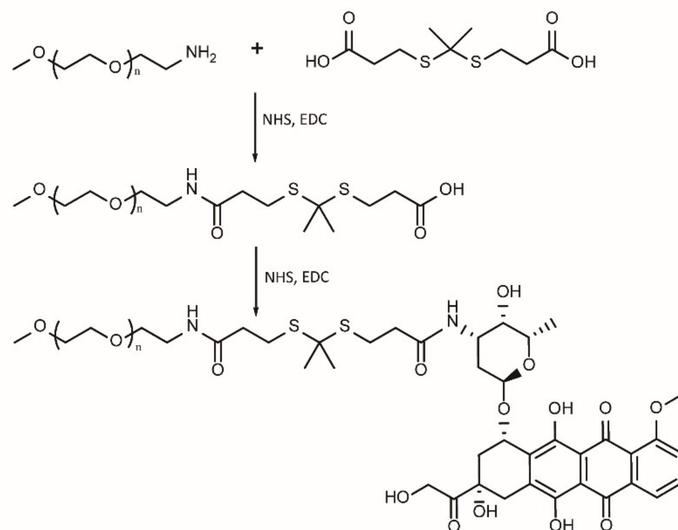

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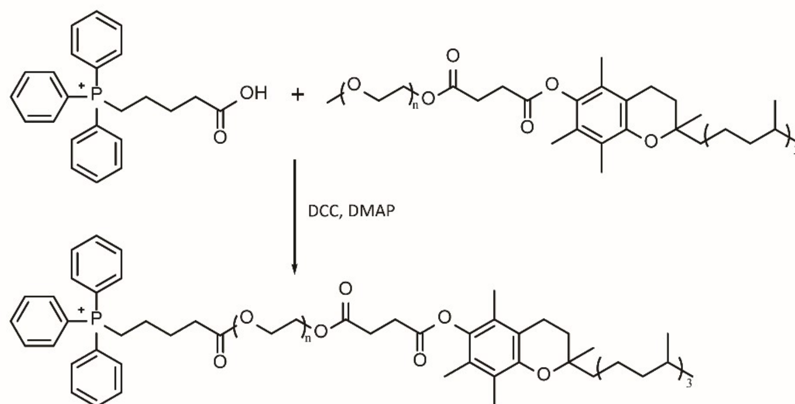

Scheme S1 Synthetic routes of TK (A), PTD (B), and TPP-TPGS (C).

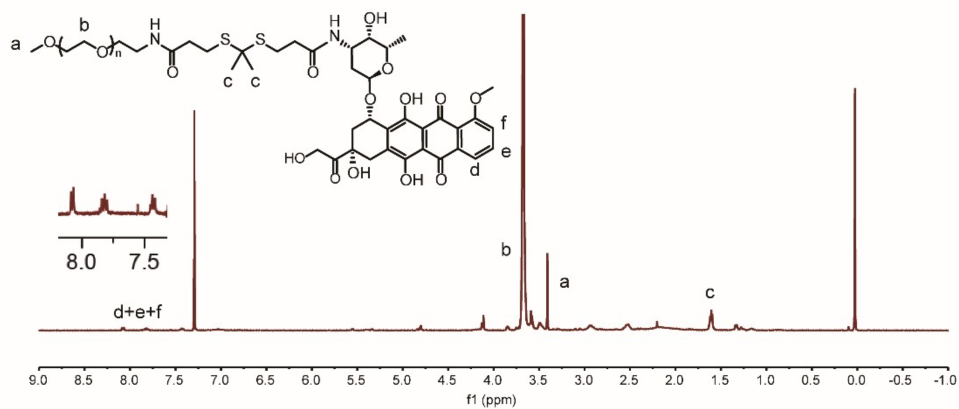

Fig. S1 ^{1}H NMR spectra of PTD.

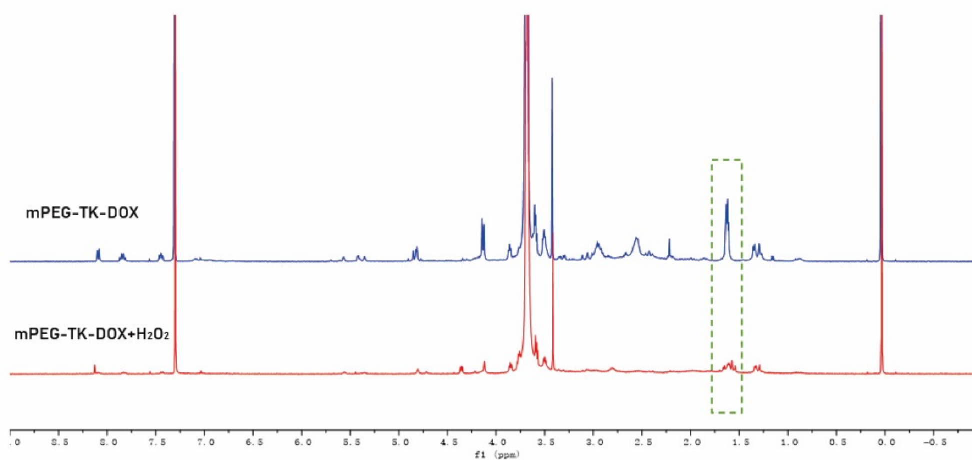

Fig. S2 ^{1}H NMR spectra of PTD with and without H_2O_2.

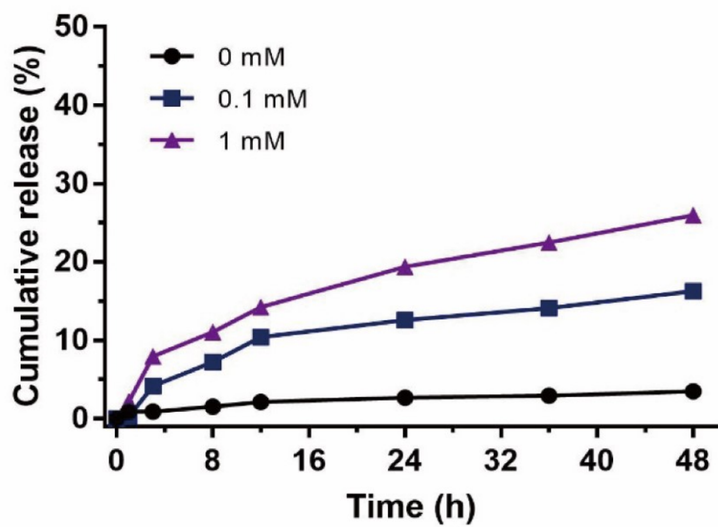

Fig. S3 Cumulative PTD release profile of DOX in the presence of different concentrations of H_2O_2.

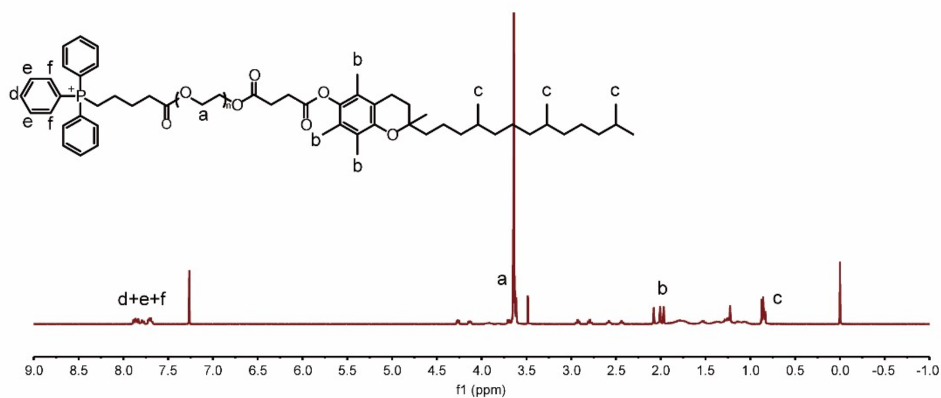

Fig. S4 ^{1}H NMR spectra of TPP-TPGS.

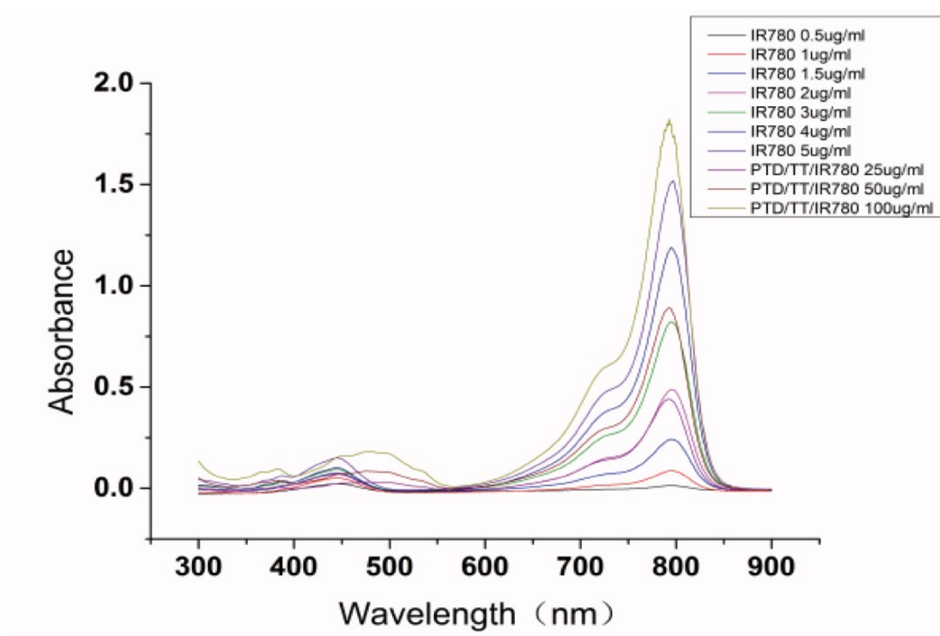

Fig. S5 UV/Vis absorption spectra of IR780 and PTD/TT/IR780 micelles.

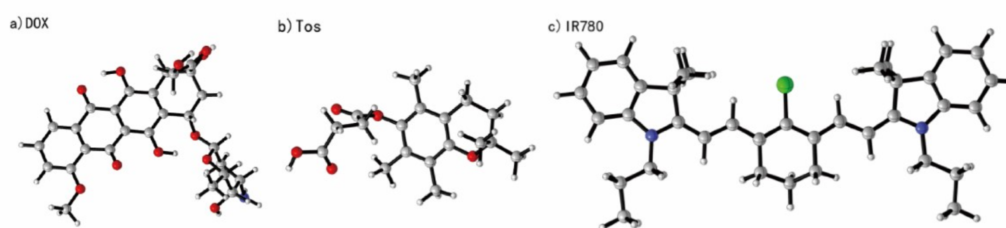

Fig. S6 The molecular structures of DOX (A), TOS (B), and IR780 (C).

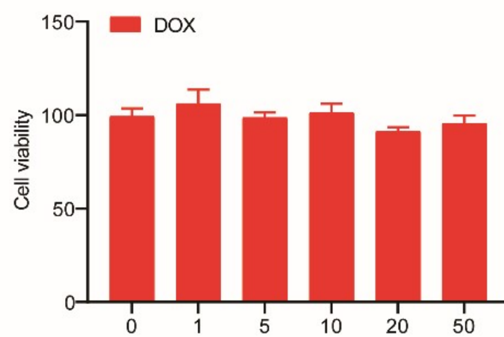

Fig. S7 *In vitro* anticancer activity of DOX on MCF-7/ADR.

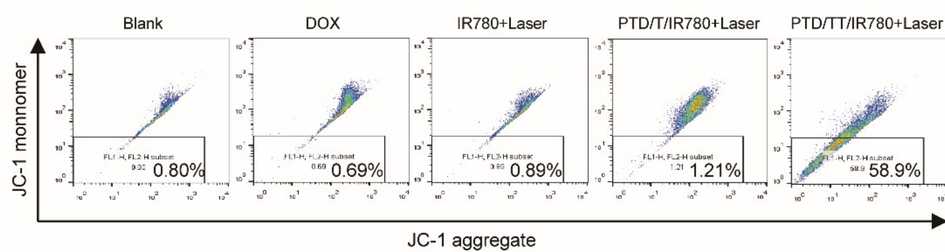

Fig. S8 Flow cytometry analyses of mitochondrial function of MCF-7/ADR cells after treated with DOX, IR780, PTD/T/IR780, and PTD/TT/IR780 micelles with 808 nm laser irradiated (1 W cm^{-2}, 5 min) by JC-1 assay.

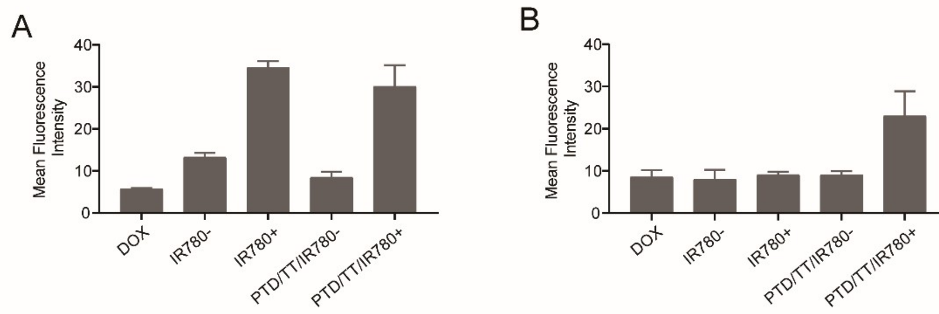

Fig. S9 Quantitative analysis of the intracellular ROS level in MCF-7 cells (A) and MCF-7/ADR cells (B).

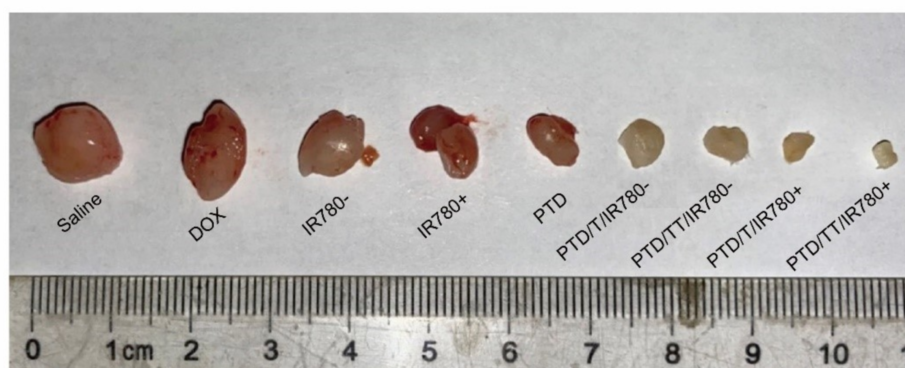

Fig. S10 Photograph of representative tumors taken out of different treatment groups after 18-days treatment.

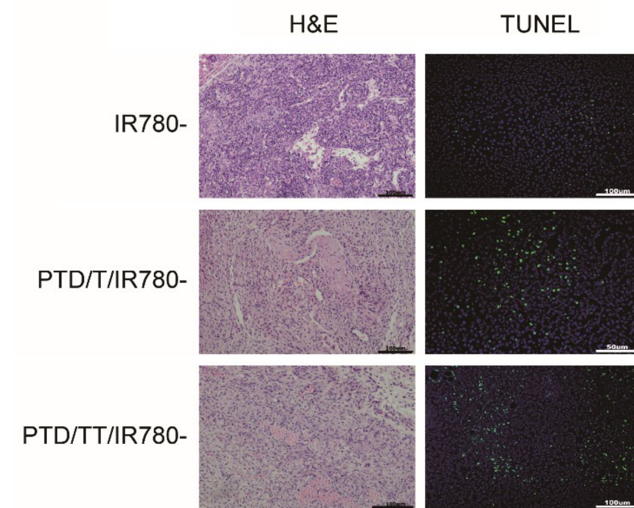

Fig. S11 H&E and TUNEL staining images of tumor slices after different treatment. Scale bar=100 μm.

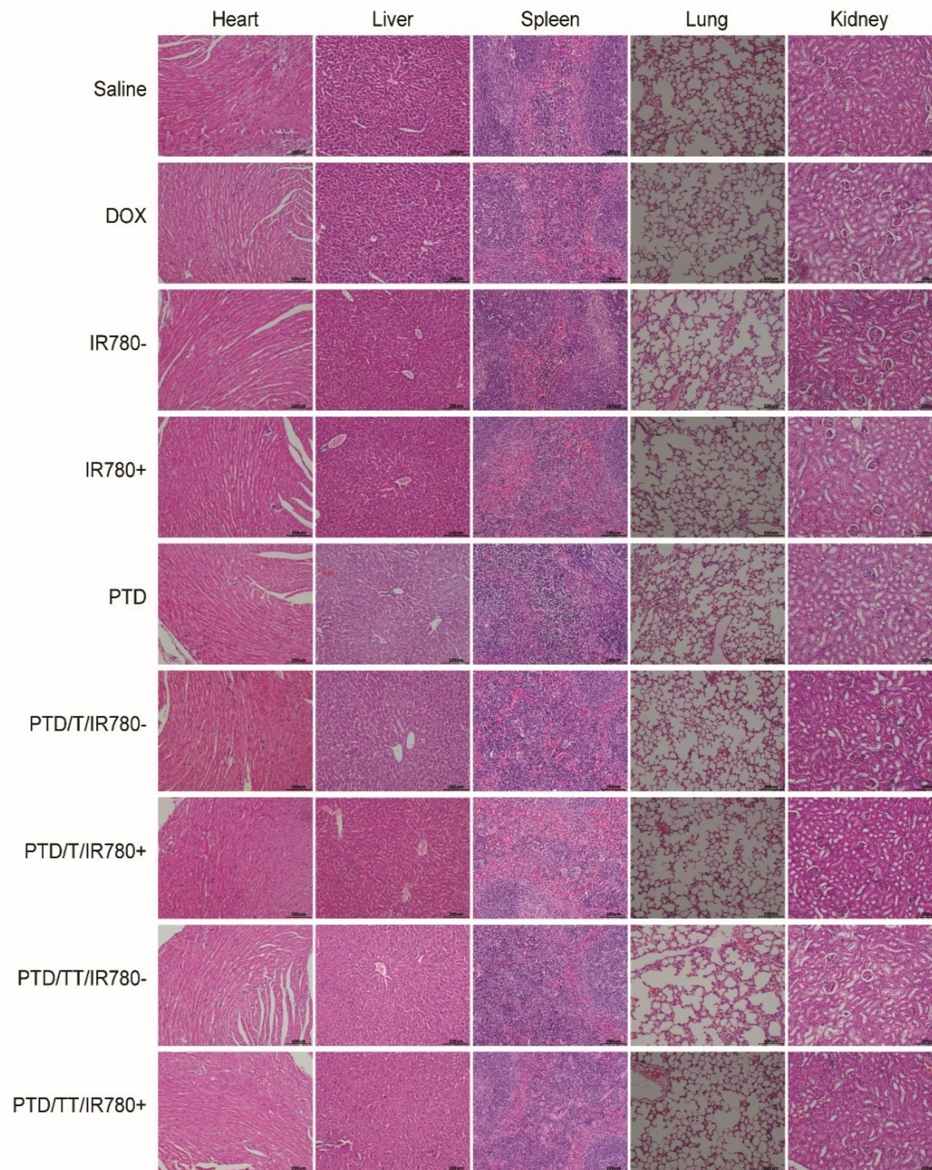

Fig. S12 H&E staining assay of normal organs of different treatment groups after 18-days treatment.

References

1. X. Lv, Y. Zhu, H. Ghandehari, A. Yu and Y. Wang, *Chem Commun (Camb)*, 2019, 55, 3383-3386.
2. S. Grimme, J. Antony, S. Ehrlich and H. Krieg, *J Chem Phys*, 2010, **132**, 154104.
3. C. Lee, W. Yang and R. G. Parr, *Phys Rev B Condens Matter*, 1988, **37**, 785-789.
4. A. D. Becke, *The Journal of Chemical Physics*, 1993, **98**, 1372-1377.
5. A. D. Becke, *The Journal of Chemical Physics*, 1993, **98**, 5648-5652.