Electronic supplementary information

Reprogramming of tumor microenvironment by a PCN-224@IrNCs/D-Arg nanoplatform for the synergistic PDT, NO, and radiosensitization therapy of breast cancer and improving anti-tumor immunity

Yi-Ming Zou [a], Rong-Tian Li[a], Lei Yu [c], Ting Huang[a], Jian Peng [a], Wei Meng[a], Bin Sun [a], Wen-Hua Zhang [b], Zhi-Hong Jiang [d], Jun Chen [a,*], Jin-Xiang Chen [a,**]

[a]*NMPA Key Laboratory for Research and Evaluation of Drug Metabolism, Guangdong Provincial Key Laboratory of New Drug Screening, School of Pharmaceutical Sciences, Southern Medical University, Guangzhou 510515, People's Republic of China*
[b] *College of Chemistry, Chemical Engineering and Materials Science, Soochow University, Suzhou 215123, People's Republic of China*
[c]*Department of Dermatology, Zhujiang Hospital of Southern Medical University, No. 253 Gongye Avenue, Guangzhou 510091, People's Republic of China*
[d]*Macau University of Science and Technology, Taipa, Macau 999078, People's Republic of China*

Materials and instruments

Zirconyl chloride octahydrate ($ZrOCl_2 \cdot 8H_2O$), Iridium trichloride hydrate ($IrCl_3 \cdot xH_2O$), 3,3',5,5'-tetramethylbenzidine dihydrochloride hydrate (TMB) and D-arginine (D-arg) was purchased from Shanghai Macklin Biochemical Co., Ltd. Tetrakis(4-carboxyphenyl)porphyrin (TCPP) were obtained from Jilin Chinese Academy of Sciences-Yanshen Technology Co., Ltd. Benzoic acid and ascorbic acid were acquired from Shanghai Aladdin Biochemical Technology Co., Ltd. Sodium borohydride ($NaBH_4$) was obtained from Anhui Zesheng Technology Co., Ltd. Ninhydrin was provided by Shanghai Yuanye Bio-Technology Co., Ltd. 9,10-Anthracenediyl-bis(methylene)dimalonic acid (ABDA) were purchased from Bide Pharmatech Co., Ltd. ATP assay kit and Nitric oxide assay kit were purchased from Shanghai Beyotime Biological Co., Ltd. 3-Amino, 4-aminomethyl-2',7'-difluorofluorescein diacetate (DAF-FM DA) was purchased from AAT Bioquest Inc (AAT Bioquest, USA). 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) and 4',6-diamidino-2-phenylindole (DAPI) solution was obtained from Beijing Solarbio Science & Technology Co., Ltd. Annexin FITC/PI apoptosis detection kit was purchased from Yeasen Biotechnology (Shanghai) Co., Ltd. Giemsa dye was obtained from Beisuo Biotechnology (Zhuhai) Co., Ltd. Gamma-H2A histone family member X (γ-H2AX) antibody (H2AX Rabbit mAb) was obtained from ABclonal Technology Co., Ltd. Calregulin polyclonal antibody (CRT), HMG-1 polyclonal antibody (HMGB1) and Hypoxia-inducible factor-1alpha (HIF-1α) antibody (rabbit mono-IgG) was acquired from Proteintech Group Inc, USA. The secondary antibodies, IgG/Alexa Fluor®594 (goat anti-rabbit) and IgG/FITC (goat anti-rabbit) were obtained from Proteintech Group Inc, USA. Murine interleukin 4 (IL-4) were purchased from Proteintech Group Inc, USA. Antibodies used for flow cytometry were all purchased from Elabscience Biotechnology Co., Ltd.` Collagenase IV, hyaluronidase and Dnase I were purchased from Glpbio Ins, USA.

The morphology and size of the nanoparticles were examined by transmission electron microscopy (TEM) on JEOL JEM-2100F. High-angle annular dark-field scanning transmission electron microscopic (HAADF-STEM) images and elemental mapping images were taken on JEOL JEM-2100F operated at an accelerating voltage of 200 kV. Powder X-ray diffraction (PXRD) patterns of the nanoparticles were collected by a Rigaku Ultima IV diffractometer. Dynamic light scattering (DLS) and zeta potential were analyzed with a Zetasizer Nano ZS from MALVERN Instruments. The UV-Vis absorption spectra were collected with a UV-Vis Spectrometer Lambda 25 from PerkinElmer Instruments. The fluorescence spectra were collected with a fluorescence spectrophotometer WATERS Prep 150 from WATERS Instruments. The fourier transform infrared (FT-IR) spectra were collected with a Bruker EQUINOX55 spectrometer in the KBr particle technique. The inductively coupled plasma–mass spectrometry (ICP-MS) was collected with an ICAP PROI from Thermo Fisher instruments. The laser at 660 nm for PDT was carried on the Stone Laser instruments from Beijing, China. The optical density (OD) values were collected using a TECAN M1000PRO microplate reader. The microscope images were collected using an Olympus FV3000 confocal laser scanning microscope (CLSM). Radiation therapy was performed with a MultiRad 225 irradiator from Faxitron instruments.

Nanoparticle preparation.

Preparation for PCN-224: The PCN-224 nanoparticles were prepared by the following method. Briefly, the powder of $ZrOCl_2 \cdot 8H_2O$ (30 mg, 0.093 mmol) and benzoic acid (280 mg, 2.293 mmol) in DMF (5 mL) was added into a round bottom flask and stirred to give a clear solution. Then, the solution of TCPP (10 mg, 0.013 mmol) in DMF (4 mL) was added. The reaction was stirred under the nitrogen atmosphere at 90 °C for 5 h in the dark. After cooling to room temperature, the reaction solution was centrifuged at 13 000 rpm for 5 min and the precipitates were washed three times with DMF and ultrapure water respectively, and then dispersed in ultrapure water for further characterization and experiments.

Preparation for PCN-224@IrNCs (**PI**): The aqueous mixture of the above synthesized PCN-224 solution (1 mg/mL, 4 mL), the iridium trichloride solution (0.4 mg/mL, 20 mL) and ascorbic acid (AA, 6 mg, 0.034 mmol) were added into a round bottom flask.

After stirring for 20 min at room temperature, the mixture was heated to 95 °C, and the freshly prepared aqueous $NaBH_4$ (1 M, 100 μL) was added dropwise into the mixture under vigorous stirring for 15 min. After cooling to room temperature, the solution was centrifuged at 13 000 rpm for 5 min and the precipitates were washed three times with ultrapure water, and then dispersed in ultrapure water for further characterization and experiments.

Preparation for PCN-224@IrNCs/D-Arg (**PID**): The **PID** nanoparticles could be obtained by mixing D-Arg (1 mg/mL) with PCN-224@IrNCs (1 mg/mL) nanoparticles in an aqueous solution in a 2 : 5 mass ratio. After stirring for 12 h at room temperature, the solution was centrifuged at 13 000 rpm for 5 min, and the precipitates were washed three times with ultrapure water and then dispersed in ultrapure water for further characterization and experiments.

D-Arg loading efficency

The loading of D-Arg was calculated from the standard curve of D-Arg using the ninhydrin colorimetric method. Firstly, a standard solution of 0.3 mM D-Arg was configured, and then 0, 0.2, 0.4, 0.6, 0.8, and 1.0 mL were added to the tubes and made up to 1 mL with water. Secondly, 1 mL of acetate buffer (pH = 5.4, 2 M) was added into each tube, then 1 mL of ninhydrin color development solution was added. The mixture was heated in a water bath at 100 ℃ for 15 min, then cooled with water for 5 min, and 3 mL of 60% ethanol solution was added then OD 570 nm with a UV spectrophotometer was measured. The standard curve was plotted with OD 570 nm as the vertical coordinate and D-Arg content as the horizontal coordinate. The samples were determined as described above in agreement with the method. The amount of D-Arg can be calculated from the standard curve by measuring the OD 570 nm in the supernatant of the sample. Loading efficency was calculated according to the equation:

$$Loading\ efficency\ (\%) = \frac{D-Arg\ (total) - D-Arg\ (supernatant)}{Sample\ amount} \times 100\ \%$$

where D-Arg (total), D-Arg (supernatant) and Sample amount are the total amount of D-Arg, the amount of D-Arg in the supernatant of the sample and the total amount of the sample, respectively.

D-Arg release

To determine the release behavior of D-Arg from nanoparticles, the freshly prepared **PID** (100 μg/ml) incubated at 37 ℃ under a shaker at 100 rpm/min for 0, 0.5, 1, 4, 8, 12, 24, 36, 48 and 72 h. Then the samples were centrifuged, 1 mL of supernatant was taken, then 1 mL of ninhydrin color development solution and 1 mL of acetate buffer (pH = 5.5, 2 M) were added, then boiled in boiling water for 15 min, cooled and 3 mL of 60% ethanol solution was added, and OD 570 nm was measured by UV.

PID long-term stability in vitro.

To verify the long-term stability of the nanoplatform. **PID** (100 μg/mL) was added to PBS and RPMI-1640 + 10% FBS solution for 7 days at 37 ℃, respectively. And the hydrodynamic diameter and polymer dispersion index (PDI) were recorded with a nanoparticle size and zeta potential analyzer on days 0, 1, 3, 5 and 7.

Cell culture

Mouse-derived macrophages RAW 264.7 were cultured in Dulbecco's Modified Eagle's Medium High Glucose (DMEM, Procell™) with 10% fetal bovine serum (FBS, Procell™) and antibiotics (penicillin 100 U/mL and streptomycin 100 μg/mL, Procell™) and murine breast cancer cells 4T1 were cultured in RPMI-1640 containing 10% fetal bovine serum (FBS, Procell™) and antibiotics (penicillin 100 U/mL and streptomycin 100 μg/mL, Procell™). Cells were cultured at 37 ºC and 21% O_2/5% CO_2. For hypoxic cells, incubate at 37 ºC, 5% CO_2, 1% O_2.

Cell uptake

To study the cells uptake of **PID**, 4T1 cells (3×10^6 cells/well) were cultured in 6-well culture plates for 24 h. Then, after incubation with **PID** (100 μg/ml) for 0, 2, 4 and 8 h, the medium was discarded, washed three times with PBS, and observed under an inverted fluorescence microscope. (LSM880, ZEISS, 10 x objective, Ex = 405 nm)

Detection of NO in 4T1 cells

The intracellular NO was evaluated by 3-Amino, 4-aminomethyl-2',7'-difluorofluorescein diacetate (DAF-FM DA) and then imaged by confocal laser scanning microscopy (CLSM; E_X: 488 nm, 10x objective). In a summary, $3 \times 10^{5`}$ 4T1 cells were seeded into six-well cell culture plates and incubated for 24 hours at 37 °C, 5% CO_2/21% O_2, or 5% CO_2/1% O_2 (hypoxic conditions). Cells were then incubated with 100 μM H_2O_2 and 15 μg/mL D-Arg or 100 μg/mL **PID**. After 4 h, The treated cells were rinsed and incubated with 5 μM of DAF-FM DA for 20 min and then treated with 660 nm laser irradiation (0.22 W/cm^2) for 5 min for PDT or X-ray (4 Gy, 160 kV, 25.0 mA) for RT, followed by rinsing the cells with PBS to remove free DAF-FM DA. Cells were imaged by the CLSM, and fluorescence intensity was measured by ImageJ.

MTT assay

The viability of 4T1 cells was assessed by the MTT assay. Briefly, 4T1 cells were seeded in a 96-well plate (5×10^3 cells per well) and cultured for 24 h and then further incubated with different concentrations (0, 25, 100, 200, 300, and 400 μg/mL) of D-Arg, PCN-

Address correspondence to Jin-Xiang Chen, email: jxchen@smu.edu.cn; Jun Chen, email: chenj258@smu.edu.cn

224, **PI**, and **PID** for 24 h. After that, 100 μL of MTT (0.5 mg/mL) was added and the incubation was continued for 4 h. After discarding the medium, 100 μL of DMSO was added to each well and the plate was shaken for 10 min. The OD at 490 nm was measured using a microplate reader. Similarly, the cytotoxicity of H_2O_2 to 4T1 cells was examined at 24 h.

The RAW 264.7 cells were seeded into 96-well plates at a density of 1500 cells per well and cultured at 37 ºC with 5% CO_2/21% O_2 for 24 h. **PID** was added to the cells at different concentrations (0, 50, 100, 150, 200, 250, 300, 350 and 400 μg/mL) and incubated under normoxic conditions for 24 h. The medium was removed and then 100 μL of MTT solution (0.5 mg/mL) was added to each well and incubation was continued for 3 h. The incubation was then continued with 100 μL of DMSO at 37 °C for 10 min on a shaker at 100 rpm/min. The absorbance was measured at 490 nm using a microplate reader.

Cell viability was calculated according to the equation:

$$Cell\ viability\ (\%) = \frac{Oe - Ob}{Oc - Ob} \times 100\ \%$$

where Oe, Oc, and Ob are the OD values of the experimental, control, and blank wells, respectively.

Flow cytometry apoptosis assay

4T1 cells were seeded into six-well cell culture plates (3×10^5 cells each) and incubated for 24 h at 37 ℃, 5% CO_2/21% O_2 (normoxic conditions) or 5% CO_2/1% O_2 (hypoxic conditions). After that, cells were incubated with PBS or 100 μg/mL **PID**. After 4 h, the treated cells were rinsed and incubated with 100 μM H_2O_2 for 20 min. Afterwards, the cells were treated with PDT or RT (PDT: 660 nm laser irradiation (0.22W/cm^2) for 5 min; RT: 4 Gy (160 KV, 25.0 mA). The cells were then cultured with fresh media for another 24 h in the incubator (37 ºC, 5% CO_2, 21% or 1% O_2). Afterward, the cells were washed with PBS buffer, stained with Annexin V-FITC/PI, and analyzed by flow cytometry (FACSCanto II, BD).

Clonogenic assay

4T1 cells were seeded into twelve-well cell culture plates (500 cells each) and incubated for 24 h at 37 ℃, 5% CO_2/21% O_2 (normoxic conditions) or 5% CO_2/1% O_2 (hypoxic conditions). After that, cells were incubated with PBS or 100 μg/mL **PID**. After 4 h, the treated cells were rinsed and incubated with 100 μM H_2O_2 for 20 min. Afterwards, the cells were treated with PDT (660 nm laser irradiation (0.22W/cm^2 for 5 min) or RT (4 Gy, 160 KV, 25.0 mA). The cells were three times washed with PBS before being cultivated in a hypoxic environment until the control cells had grown into substantial colonies (more than 100 cells per colony). The colonies were created, then stained with Giemsa dye and preserved with 4% paraformaldehyde. The effects of different treatments were evaluated by analyzing the survival fraction (SF) of the colonies as follows:

$$Plating\ Efficiency\ (PE) = \frac{Number\ of\ colonies\ formed\ in\ untreated\ control}{Number\ of\ cells\ seeded}$$

$$Survival\ Fraction\ (SF) = \frac{Number\ of\ colonies\ formed\ after\ treatment}{Number\ of\ cells\ seeded\ \times\ PE}$$

In vitro BMDC activation

To investigate the effect of **PID**-mediated antigen release on DC activation, BMDCs obtained from the bone marrow of 7-8 week old C57BL/6 mice were inoculated in 6-well plates (1×10^6 cells/well) and incubated with supernatants of differently treated 4T1 cells for 24 hours. To detect DC stimulation, BMDCs suspensions were incubated with anti-CD11c-FITC, anti-CD80-PE and anti-CD86-APC antibodies. DCs were then analyzed for maturation by flow cytometry.

Hemolysis

The mouse whole blood (1 mL) was collected in an anticoagulation tube by ocular blood sampling and then centrifuged at 2500 rpm for 30 min to obtain the red blood cells, which were then washed three times with 10 mM HEPES buffer. Afterward, the red cell suspension (2 μL) was mixed with an aqueous dispersion of **PID** (10, 20, 50, 80, 100, 200, and 400 μg/mL), using HEPES buffer as a negative control, and 1% Triton as a positive control. After incubation at 37 °C for 2 h, the mixture was centrifuged at 3000 rpm for 10 min, and then the samples were photographed and the supernatant with OD at 540 nm was measured by a microplate reader. The hemolysis rate (Hr) was calculated according to the equation:

$$Hr\ (\%) = \frac{ODs - ODn}{ODp - ODn} \times 100\ \%$$

where ODs, ODp, and ODn are the OD values of the samples, the positive control, and the negative control, respectively.

Establishment of mouse models of subcutaneous breast cancer

Female BALB/c mice (3–4 weeks) were purchased from the Animal Experiment Center of Southern Medical University (animal license code: No.44002100031030). The subcutaneous breast cancer model was prepared according to the following procedure. The hair was removed from the right posterior side back of the mice and 2×10^6 4T1 cells were injected subcutaneously. When the tumor volume reached 100 mm^3, the therapeutic regimens were applied. The tumor volume was calculated by the equation of $V = A*B^2/2$, where A and B represent the longest and shortest diameters of the tumor.

Antitumor effects in mice

After the tumors reached *ca.* 100 mm^3, mice were randomly divided into 8 groups (6 mice each). Mice were injected intratumorally with 100 μL PBS or **PID** at a dose of 10 mg/kg. Then, each group was treated differently with PBS, PBS + PDT, PBS + RT, PBS + PDT + RT, **PID**, **PID** + PDT, **PID** + RT, **PID** + PDT + RT (RT: 4 Gy, 160 kV, 25.0 mA, PDT: 638 nm irradiation, 0.45 W/cm^2, 5 min). After 24 h of treatment, one mouse from each group was randomly selected for execution, and tumors were excised and fixed with 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with TUNEL and H&E. To examine DNA damage and hypoxia in mice, tumors were immunofluorescence-stained with antibodies to γ-H2AX and HIF-1α. The tumor volume and body weight of the remaining mice were monitored daily for a total of 15 days. At the end of treatment, mice were executed and tumors were excised and weighed.

Hematoxylin and eosin (H&E) staining of heart, liver, spleen, lung, kidney and tumor

After the tumors reached *ca.* 100 mm^3, mice were randomly divided into 8 groups (6 mice each). Mice were injected intratumorally with 100 μL PBS or **PID** at a dose of 10 mg/kg. Then, each group was treated differently with PBS, PBS + PDT, PBS + RT, PBS + PDT + RT, **PID**, **PID** + PDT, **PID** + RT, **PID** + PDT + RT (RT: 4 Gy, 160 kV, 25.0 mA, PDT: 638 nm irradiation, 0.45 W/cm^2, 5 min). After 24 h of treatment, one mouse from each group was randomly selected for execution, and tumors were excised and fixed with 4% paraformaldehyde, embedded in paraffin, sectioned, and stained with TUNEL and H&E. To examine DNA damage and hypoxia in mice, tumors were immunofluorescence-stained with antibodies to γ-H2AX and HIF-1α. The tumor volume and body weight of the remaining mice were monitored daily for a total of 15 days. At the end of treatment, mice were executed and tumors were excised and weighed.

Statistical analysis

The statistical analysis was performed with OriginPro 2021 software (OriginLab, Northhampton, MA). Student's t test was used to evaluate the statistical significance. P values < 0.05 were regarded statistically significant (*p < 0.05, **p < 0.01, ***p < 0.001).

Sample	Zr (mg/L)	Ir (mg/L)
PCN-224 (100 mg/L)	19.2392	0
PI (100 mg/L)	15.4320	56.7090
PID (100 mg/L)	14.6113	49.2614

Table S1. ICP-MS of PCN-224, **PI** and **PID**.

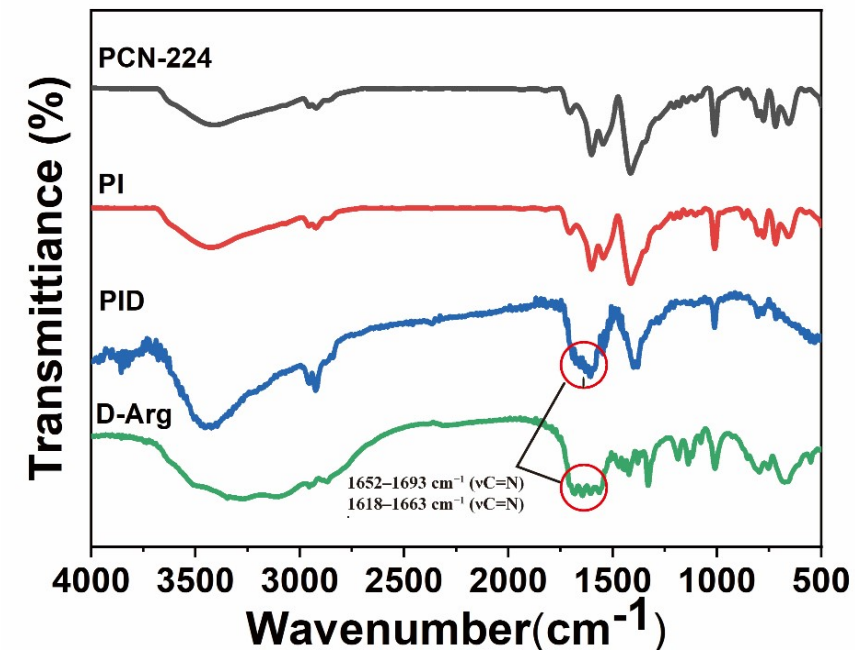

Fig. S1 FT-IR spectrum of TCPP, PCN-224, **PI** and **PID**.

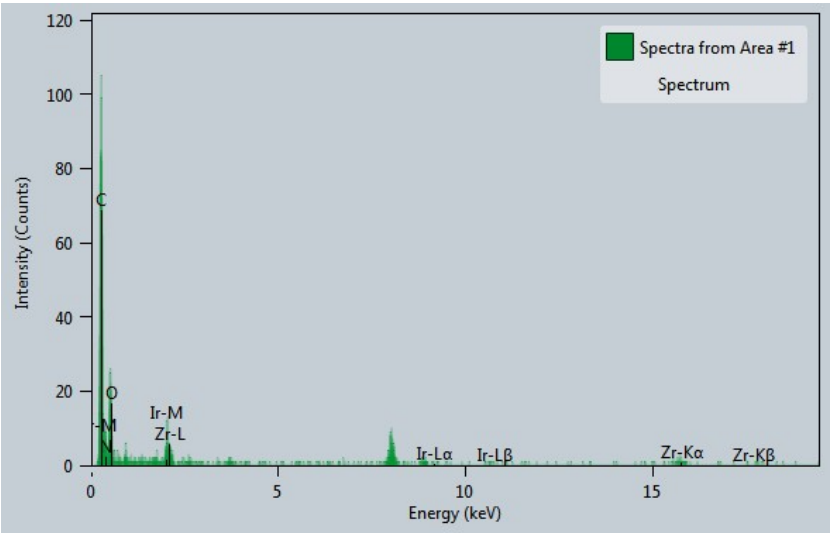

Fig. S2 The energy-dispersive X-ray spectroscopy of **PID**.

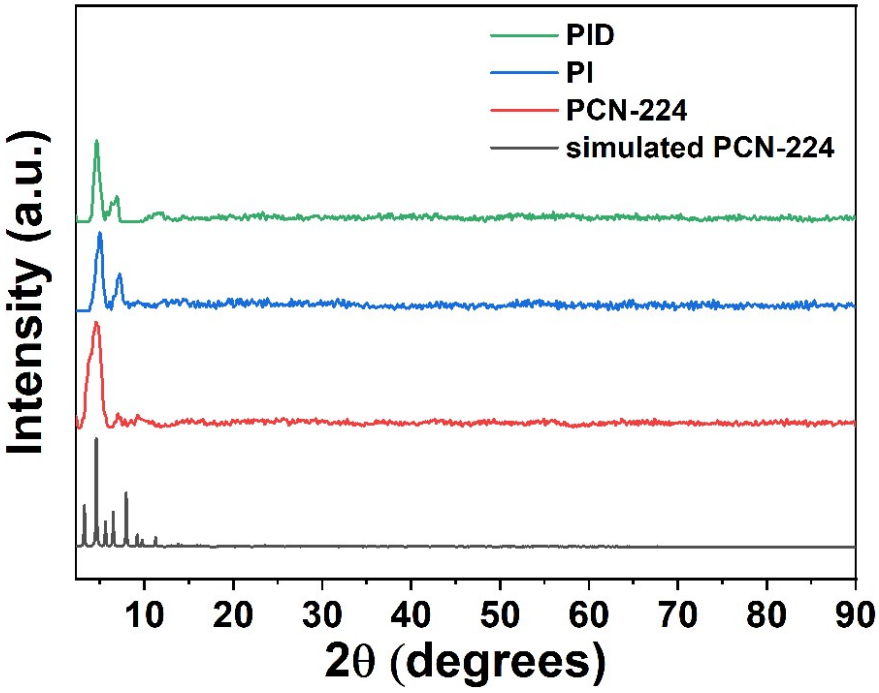

Fig. S3 The PXRD patterns of PCN-224, **PI**, **PID** and simulated PCN-224.

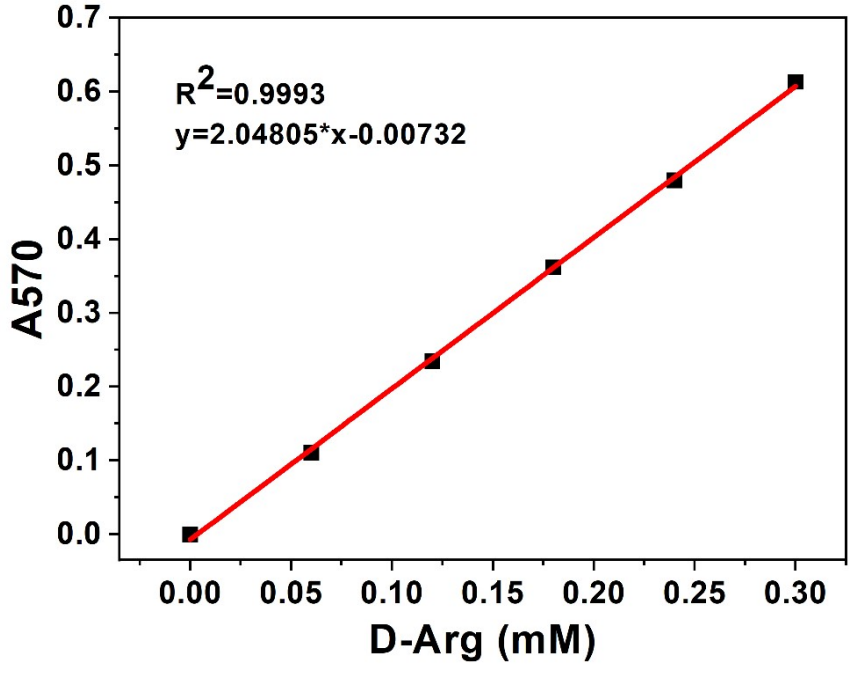

Fig. S4 The standard curve of D-Arg.

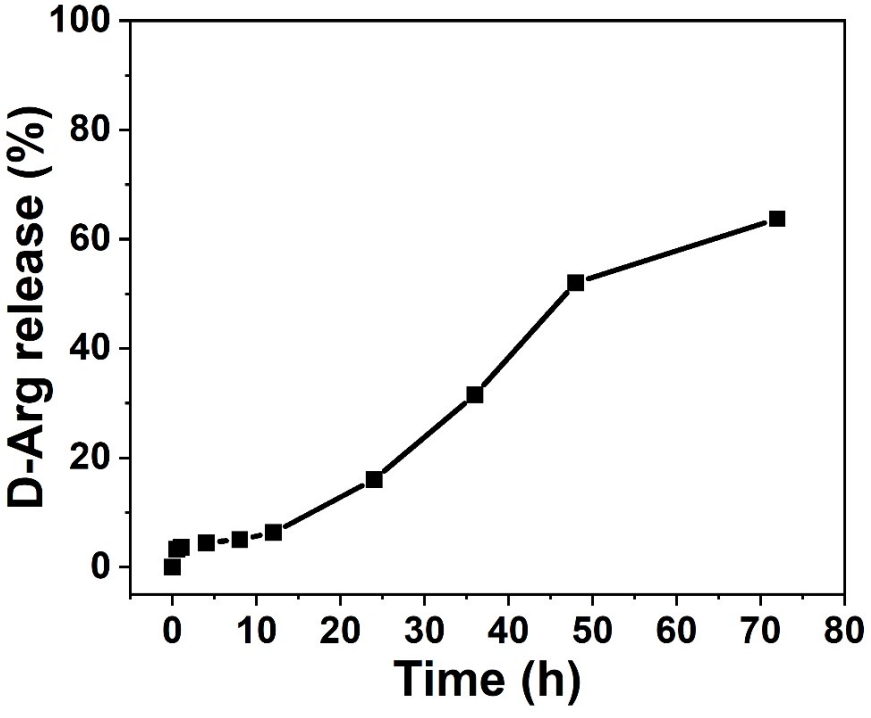

Fig. S5 Release curves of D-Arg at different time points.

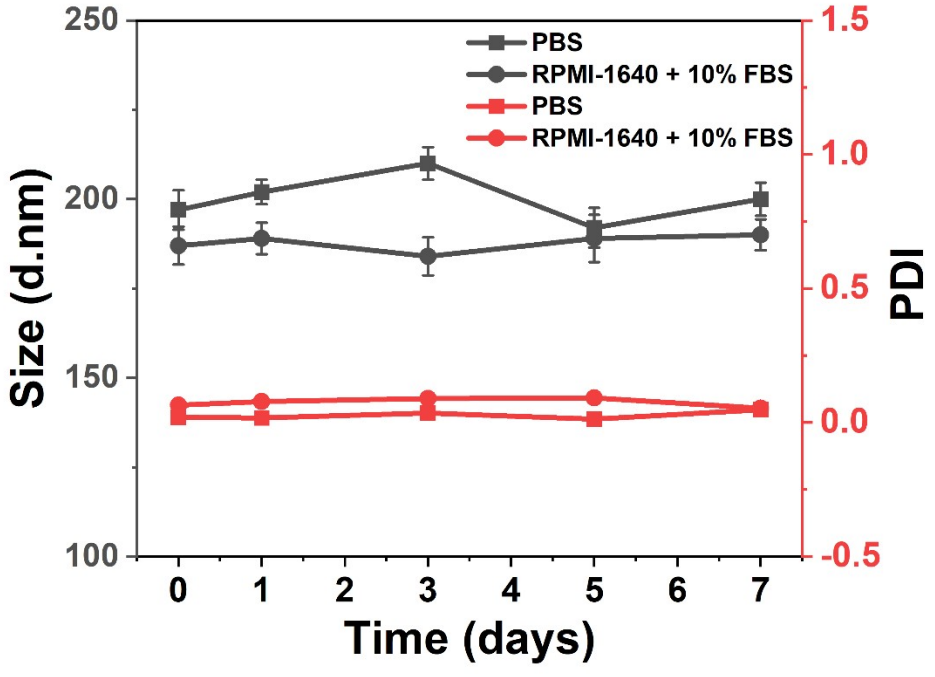

Fig. S6 PID (100 μg/mL) was incubated with PBS and RPMI-1640 + 10% FBS at 37 °C, and the hydrodynamic diameter and PDI were recorded on days 0, 1, 3, 5 and 7, respectively.

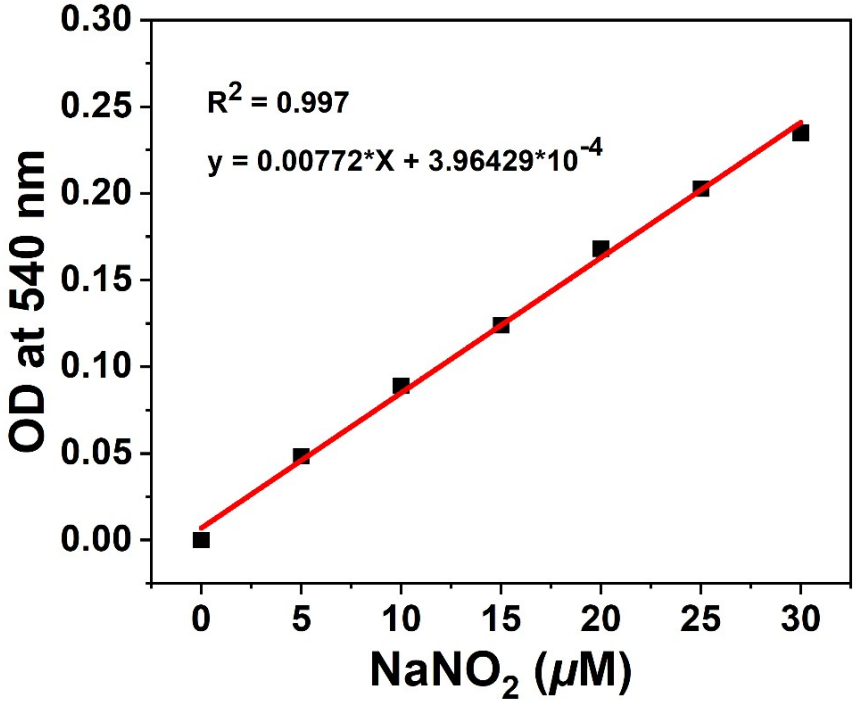

Fig. S7 The standard curves of $NaNO_2$.

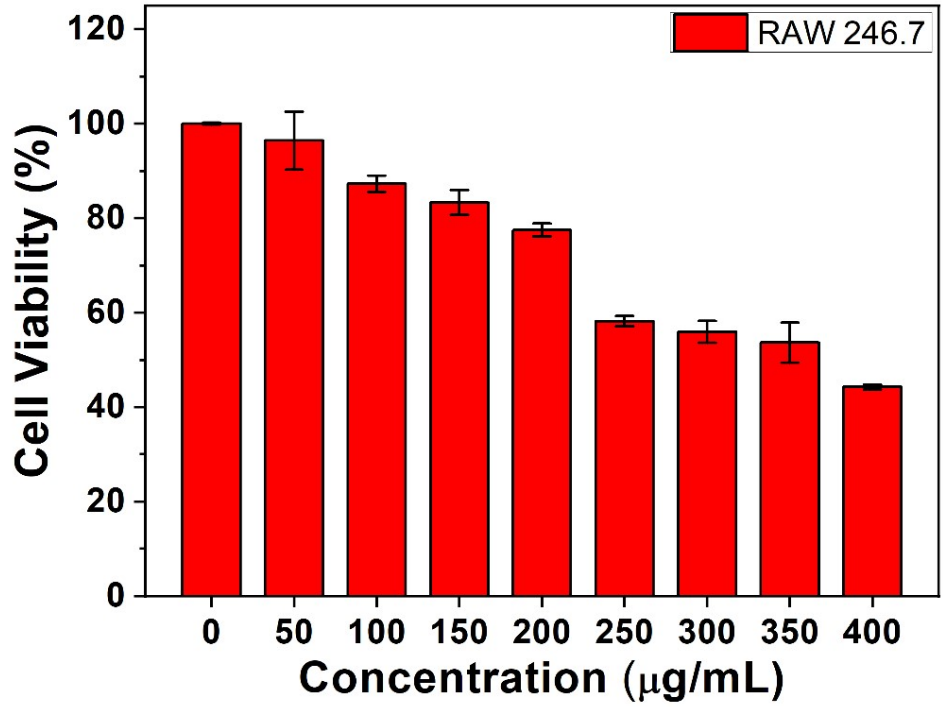

Fig. S8 Cell viability of RAW 246.7 cells with different treatments of **PID** for 24 h.

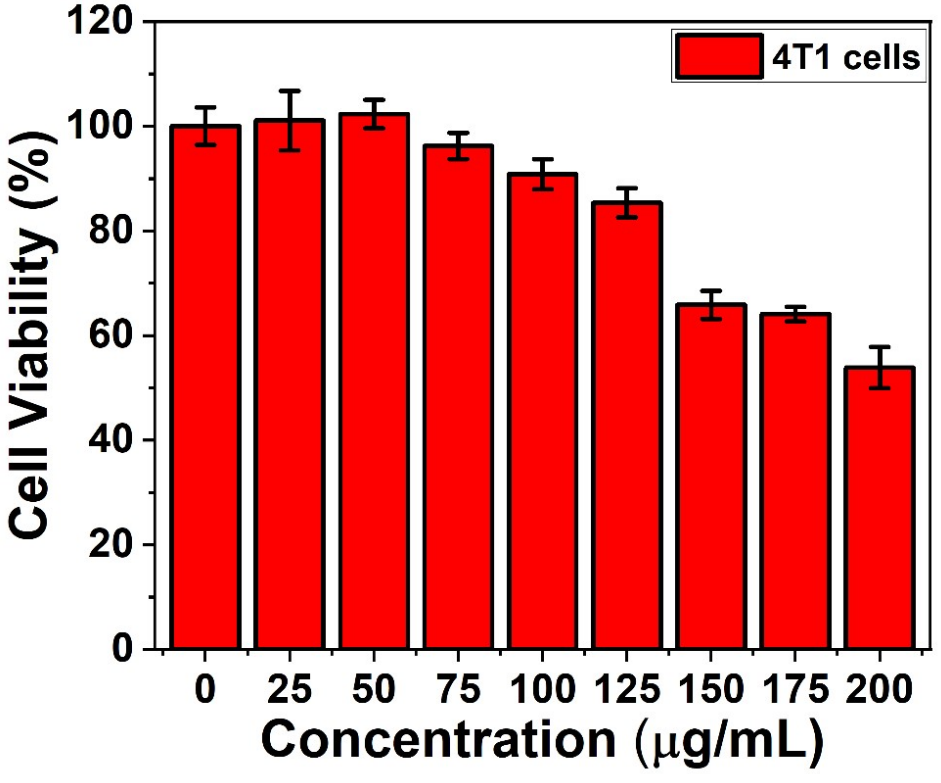

Fig. S9 Cell viability of 4T1 cells with different concentrations of H_2O_2 for 24 h.

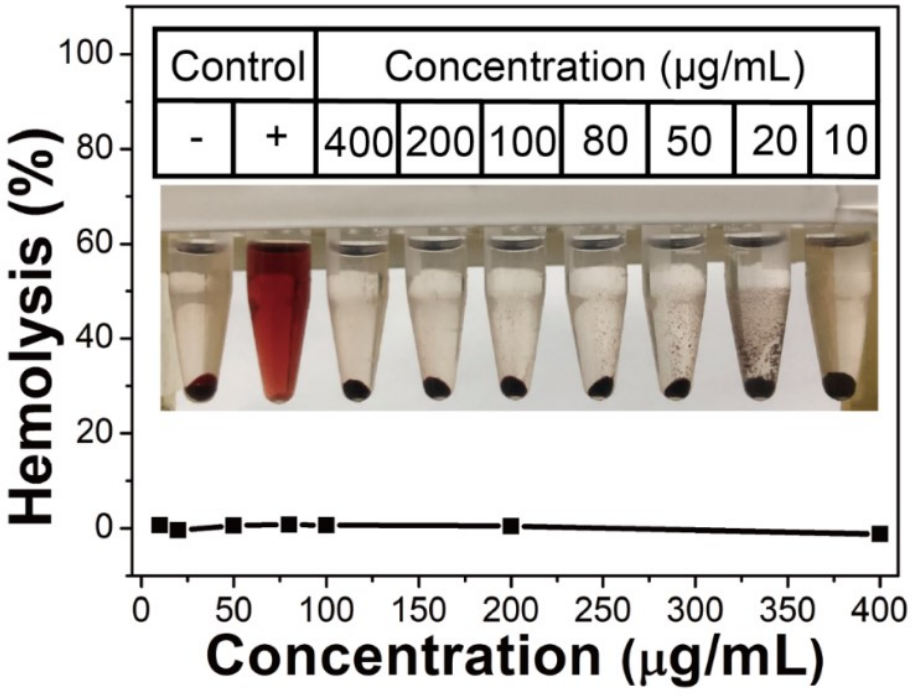

Fig. S10 Hemolytic activity of **PID**.

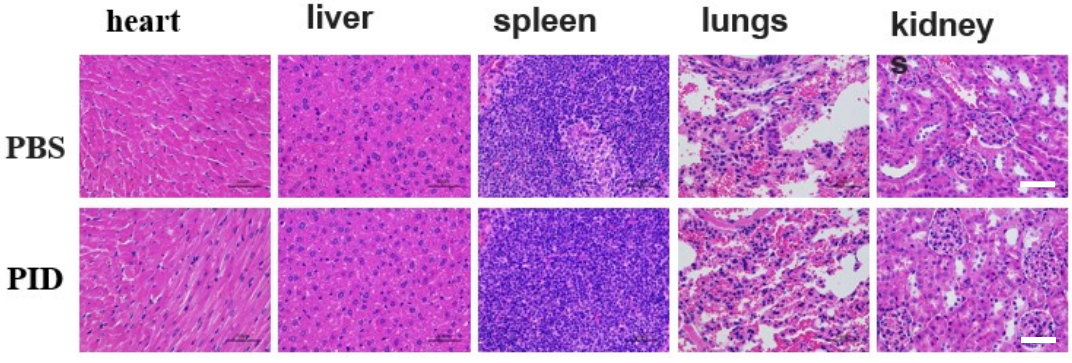

Fig. S11 H&E staining of organ tissue slices after 15 days treatments for **PID** and PBS groups. Scale bar = 20 μm.

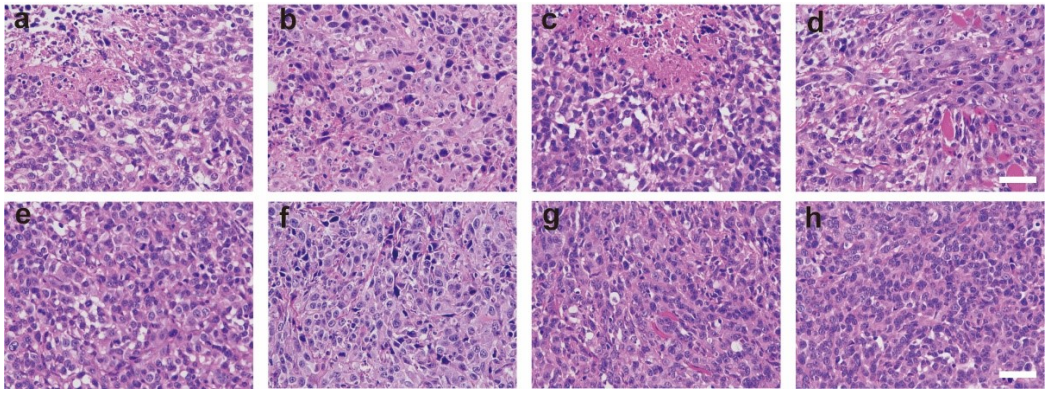

Fig. S12 H&E staining of tumor tissue slices after treatments for different groups. (a: **PID**+PDT+RT; b: **PID**+PDT; c: **PID**+RT; d: **PID**; e: PBS+PDT+RT; f: PBS+PDT; g: PID+RT; h: PBS. Scale bar = 20 μm.

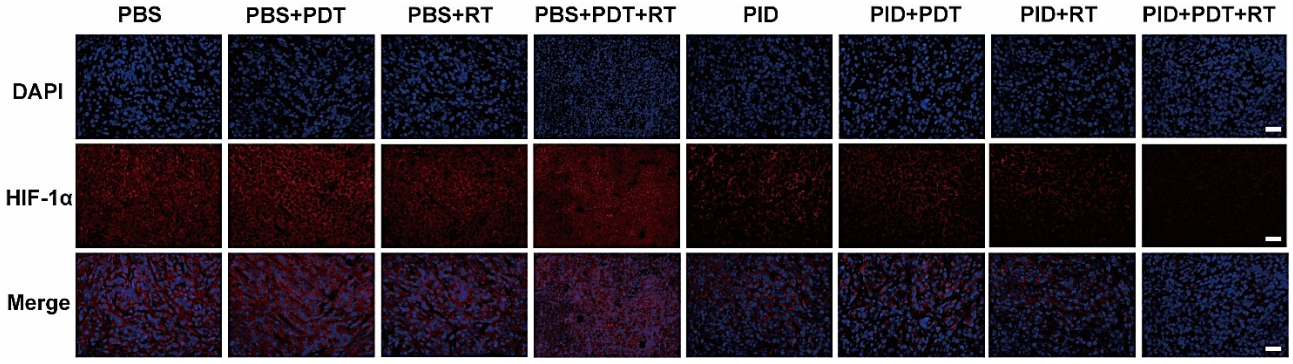

Fig. S13 Expression of HIF-1α in tumors after treatment in different groups. The nucleus was stained by DAPI (blue) and the hypoxia site was stained with HIF-1α antibody (red). Scale bar = 50 μm.

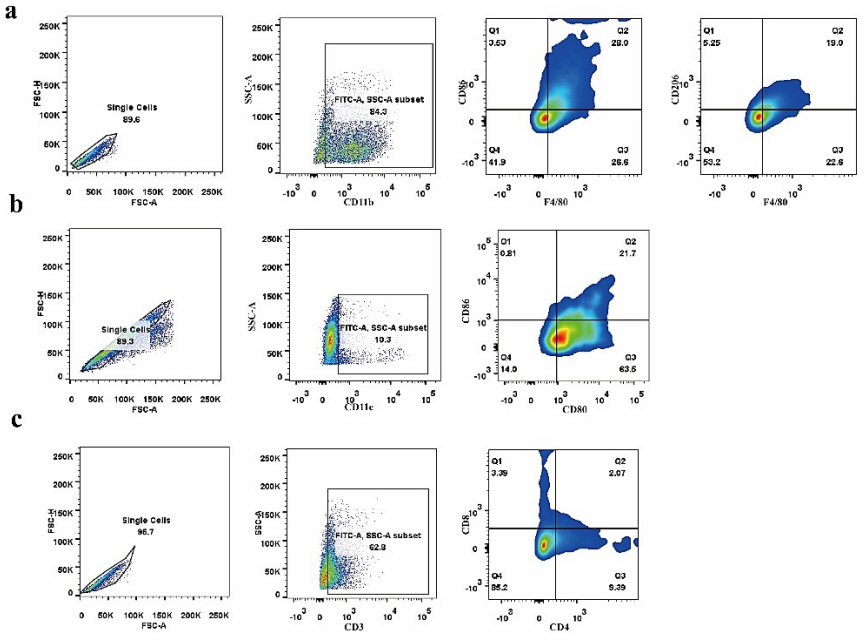

Fig. S14 Gating strategy to sort (a) M1 and M2 macrophages, (b) matured DCs, and (c) $CD4^+$ T cells and $CD8^+$ T cells.

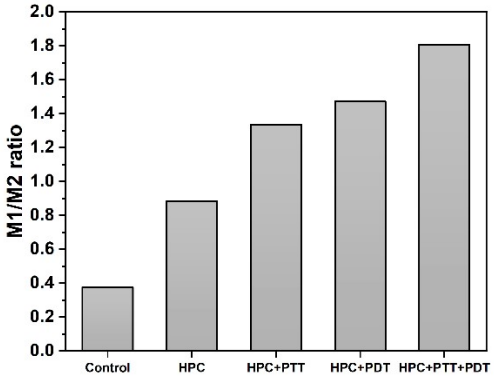

Fig. S15 Ratio of M1 macrophages/M2 macrophages in primary tumors after different treatments.

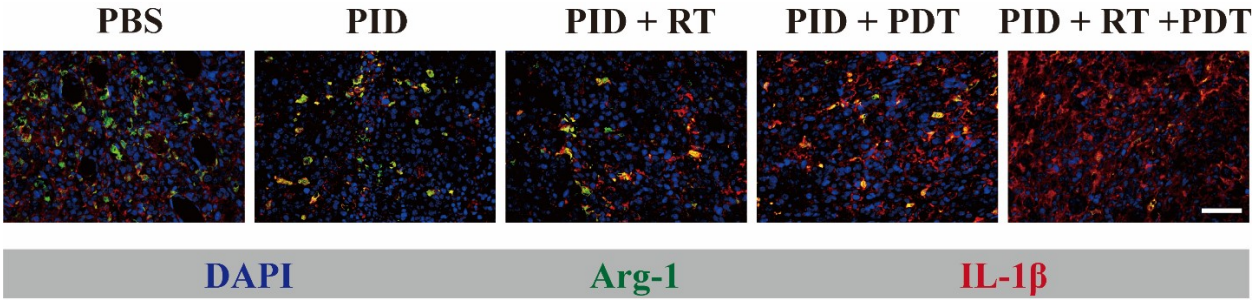

Fig. S16 The Arg-1 (green) and IL-1β (red) immunohistochemically stained excised primary tumors after the different treatments. (Scale bar = 20 μm).

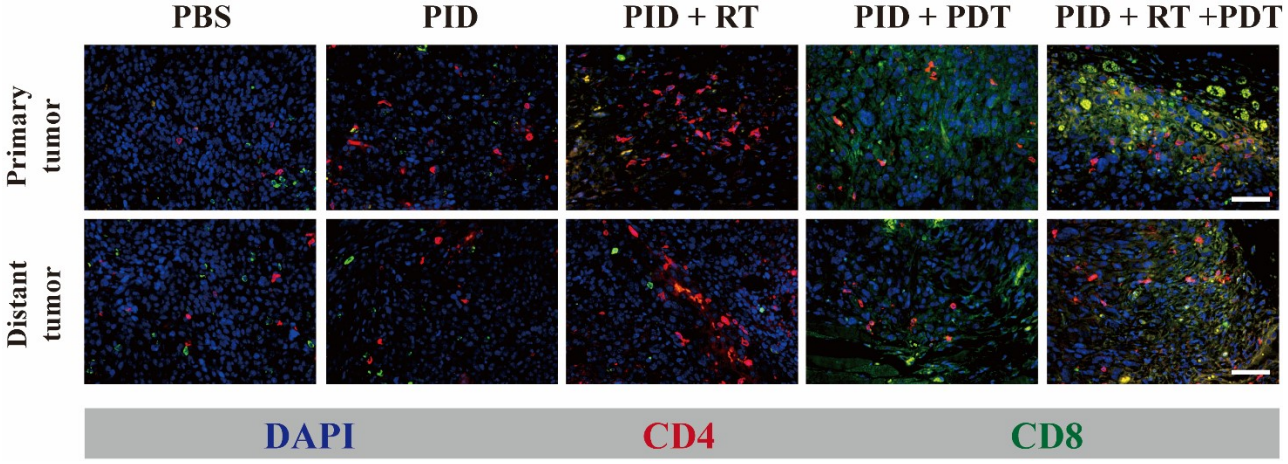

Fig. S17 The $CD4^+$ (red) and $CD8^+$ (green) immunohistochemically stained excised primary and distant tumors after the different treatments. (Scale bar = 20 μm).