Electronic Supplementary Information

A Mitochondrial-Metabolism-Regulatable Carrier-Free Nanodrug to Amplify the Sensitivity of Photothermal Therapy

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Experimental Section

Materials

Atovaquone (ATO) was acquired from Dalian Meilun Biotech Co., Ltd. New Indocyanine Green (IR820) was provided by Beijing J&K Technology Co., Ltd. Hoechst 33342, ATP assay kit, HSP70 Rabbit Monoclonal Antibody and HSP90 Mouse Monoclonal Antibody were bought from Beyotime Biotechnology Co., Ltd. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was provided by Aladdin Industrial Corporation. Annexin V-FITC/PI Apoptosis Detection Kit was supplied by Dalian Meilun Biotech Co., Ltd.

Preparation and characterizations of IR820/ATO NPs

To prepare the self-assembly of IR820/ATO NPs, nanoprecipitation method was used. Briefly, 400 μL of ATO in dimethyl sulfoxide (DMSO) solution (10 mg mL^{-1}) was slowly added into 4 mL of IR820 in deionized water (0.5 mg mL^{-1}) under stirring at 800 rpm for 20 min. Next, the mixture solution was dialyzed with 2 L purified water in the dark to remove DMSO and further filtered by membrane with 0.8 μm pore size. UV-Vis absorption spectra of IR820, ATO and IR820/ATO NPs in mixed solvent (The volume ratio of DMSO and water was 10:1) was obtained using a UV-Vis spectrophotometer (UV-8000S, Metash). A transmission electron microscopy was used to acquire

the TEM photographs of IR820/ATO NPs (TEM, JEM-200CX). Measurements of particle size and potential were achieved on the Malvern Zetasizer Nano ZS.

Photothermal capability in vitro

Solutions of the free IR820 and IR820/ATO NPs were irradiated by near-infrared (NIR) laser to compare the photothermal effect of them. In brief, 1 mL solution of IR820/ATO NPs containing IR820 of different concentrations (100, 50, 25, 10, 0 μg mL^{-1}) was illuminated by 808 nm laser with 1 W cm^{-2} for 5 min (MW-GX-808/10W). During irradiation, solution temperature was monitored by the infrared thermal imaging camera (Testo 869). For comparison, the photothermal effect of free IR820 (100 μg mL^{-1}) was measured simultaneously.

Cell culture

Murine mammary carcinoma cells (4T1) were cultured in RPMI 1640 medium containing 1% penicillin−streptomycin and 10% fetal bovine serum (FBS) in a humid atmosphere containing 5% CO_2 at 37 °C.

Cellular uptake

The cellular uptake behavior of IR820 and IR820/ATO NPs was evaluated by flow cytometry. 2×10^5 4T1 cells were seeded in each well of 6-well plates and cultivated for 24 h. Afterwards, the liquid in each well was replaced with the new medium containing IR820 or IR820/ATO NPs (IR820: 20 μg mL^{-1}) and the cells were continued to incubate for 1, 2, 4, 6 h. After incubation, used PBS to washed cells, collected by centrifugation. Finally, the cellular uptake was evaluated by flow cytometer (BD Accuri C6 Plus).

In vitro cytotoxicity

To evaluate cytotoxicity of IR820/ATO NPs, MTT assay was performed. Briefly, 8×10^3 4T1 cells were seeded in each well of 96-well plates and cultivated for 24 h. Then, the liquid in each

well were replaced by the new medium containing various concentrations of ATO, IR820 or IR820/ATO NPs. The concentrations of ATO ranged from 1.5 to 30.2 μg mL^{-1} and free IR820 varied from 1 to 20 μg mL^{-1}, respectively. At 6 h after incubation, these cells were washed by PBS, incubated with new medium. And then, the laser groups cells were illuminated by 808 nm laser (1 W cm^{-2}, 1 min). At 20 h after further incubation, 10 μL MTT solution (5 mg mL^{-1}) was added into each well and cells were continued to incubate for 4 h. Then, the solution in the well was discarded. To dissolve the formazan, 200 μL DMSO was added to each well. Finally, each well absorbance was evaluated at 490 nm by the microplate reader (RS-232C). The cell viability rate was evaluated by the formula:

$$\text{Cell viability rate (\%)} = \frac{A_{sample} - A_{blank}}{A_{negative} - A_{blank}} \times 100\%$$

$A_{negative}$ represented the absorbance of only cells without any treatments, A_{blank} represented the absorbance of only culture medium without cells and drugs, A_{sample} represented the absorbance of cells with various treatments.

Cell apoptosis assay

To measure cell apoptosis, flow cytometry of Annexin-FITC was used. 2×10^5 4T1 cells were seeded in each well of 6-well plates and cultivated for 24 h. Afterwards, the cells were incubated with the fresh medium containing ATO, IR820 or IR820/ATO NPs (IR820: 20 μg mL^{-1}). After 6 h incubation, we discarded culture liquid and substituted with new medium. The laser groups cells were irradiated by 808 nm laser (1 W cm^{-2}, 1 min). Finally, the cells were washed, collected, resuspended, and used Annexin V-FITC/PI kit to measure the apoptosis analysis of cells.

Assessment of intracellular ATP level

2×10^5 4T1 cells were seeded in each well of 12-well plates and cultivated for 24 h. Afterward,

the 4T1 cells were treated with the fresh medium containing ATO or IR820/ATO NPs (IR820: 20 μg mL^{-1}) in darkness for 24 h. 4T1 cells administrated with no treatments were considered as control. Finally, ATP assay kit was used to evaluate the intracellular ATP level on the basis of the protocol.

In vitro expression of HSP70 and HSP90

2×10^5 4T1 cells were seeded in each well of 6-well plates and cultivated for 24 h. And then, these cells were cultured with the new medium containing different drugs of ATO, IR820 or IR820/ATO NPs (IR820: 20 μg mL^{-1}) for 6 h. After culture medium replaced with new medium, the laser groups cells were illuminated by 808 nm laser at 1 W cm^{-2} for 1 min. After different treatments, we discarded the culture medium and washed cells with PBS. Next, each well cells were lysed with 100 μL radio immunoprecipitation assay (RIPA) lysis buffer containing the phenylmethanesulfonyl fluoride (PMSF) for 20 min on the ice, and we collected the supernatant by centrifugation for 12000 rpm at 4 °C and determined the protein count using BCA protein assay kit. Next, proteins were boiled with 10% sodium dodecyl sulfate polyacrylamide (SDS-PAGE) for 5 min. Then, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes. After blocking in 5% skim milk in Tris buffer saline with Tween 20 (TBST), the membranes were washed by TBST. Thereafter, the membranes were incubated with the primary antibody of HSP70, HSP90 and β-actin at 4 °C overnight. Afterwards, the membranes were washed by TBST and further kept with the secondary antibody for another 1 h. Finally, proteins were imaged by ECL detection reagent and the chemiluminescence signals of proteins were characterized to evaluate the expression of protein.

Establishment of tumor model

Healthy BALB/c mice (18-22 g, 6-8 weeks old, female) were obtained from the Shandong University laboratory animal center (Jinan, China). All animal experiments were conducted under

the approval of Shandong University Animal Experiment Ethics Review and the Health Guide for the Care and Use of Laboratory Animals of National Institutes. 0.1 mL PBS suspension containing 8×10^5 4T1 cells was subcutaneously inoculated to each BALB/c mouse into the right anterior armpit to establish tumor model. The longest diameter (L) and the shortest diameter (W) of tumor were evaluated by vernier caliper, and the volume of tumor was calculated by the following formula $V = L \times W^2/2$.

In vivo fluorescence imaging

When tumors reached nearly 100 mm^3, the mice bearing 4T1 tumors were intravenously injected with IR820 (3 mg kg^{-1}) or IR820/ATO NPs (containing 3 mg kg^{-1} IR820). Then, mice were anesthetized and imaged by the NIRF imaging living system (Xenogeny IVIS Kinetic System) at 1, 4, 6, 8, 24 h after injection to investigate the tumor accumulation in vivo. After, the mice were sacrificed and the relative tumors and the main organs (spleen, liver, kidney, heart and lung) were collected at 24 h after post-injection for further testing fluorescence intensities.

In vivo photothermal performance

When tumors reached nearly 150 mm^3, normal saline, IR820 (3 mg kg^{-1}) or IR820/ATO NPs (containing 3 mg kg^{-1} IR820) were intravenously injected into the mice bearing 4T1 tumors. 6 h after the post-injection, the tumor regions were exposed to 808 nm laser with 1 W cm^{-2} for 5 min. During the process of irradiation, the photothermal photographs was recorded by infrared thermal imaging camera to measure the temperature of the tumor site.

In vivo antitumor efficacy

When the size of tumor became around 80 mm^3, the BALB/c mice bearing 4T1 tumor were grouped at random into 4 groups (n=5), including normal saline (NS), ATO, IR820 with laser, IR820/ATO NPs with laser. The solution of drugs was intravenous injected every two days and the

corresponding IR820 dosage was 3 mg kg^{-1}. After 6 h post-injection, tumors of mice were irradiated (808nm, 1 W cm^{-2}, 5 min). Volume of tumors and weight of mice were recorded every two days. When the last treatment was completed, the mice were sacrificed. Afterwards, the tumors as well as major organs of mice were collected. After photographing of excised tumors, the tumors were weighed. We calculated the tumor inhibition rate by the following formula:

$$\text{Tumor inhibition rate } (\%) = \frac{W_{NS} - W_T}{W_{NS}} \times 100\%$$

where W_{NS} represented the average weight of NS group tumor, while W_T was the weight of the treated group tumor.

Histology analysis

The collected main organs (spleen, kidney, liver, heart and lung) and the tumors which mentioned above were incubated in 4% paraformaldehyde solution for further histology analysis. The related tissues and tumors were stained with hematoxylin and eosin (H&E) to observe pathological feature changes of them. The tumors were also stained by HSP70 and HSP90 immunofluorescence to evaluate the antitumor mechanism.

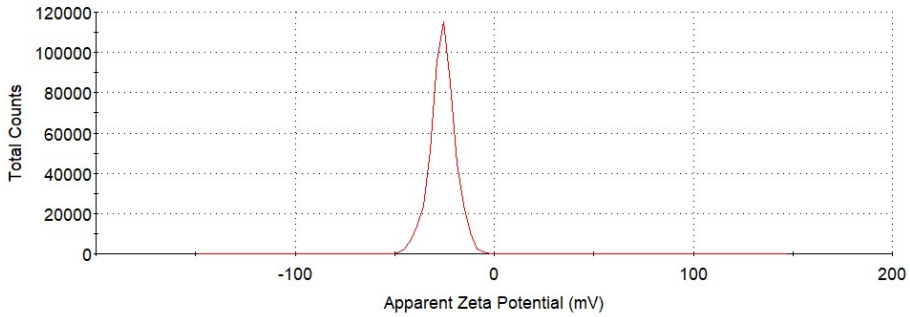

Fig. S1 The zeta potential of IR820/ATO NPs.

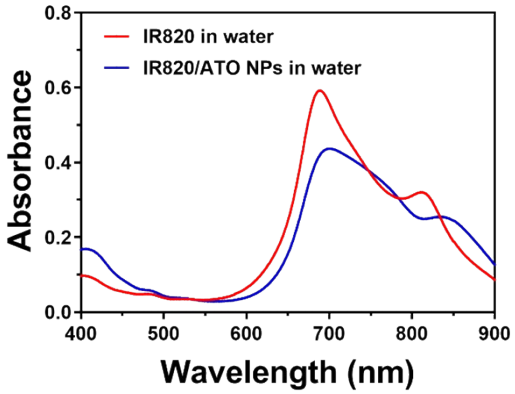

Fig. S2 UV-Vis spectra of IR820/ATO NPs and IR820 in water.

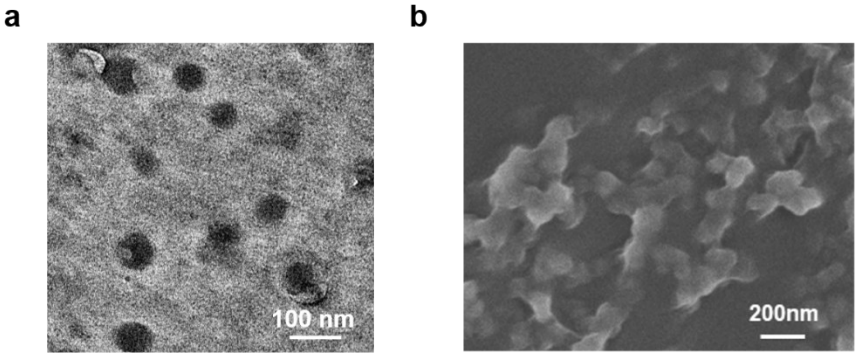

Fig. S3 (a) TEM image and (b) SEM image of IR820/ATO NPs after storing 7 days.

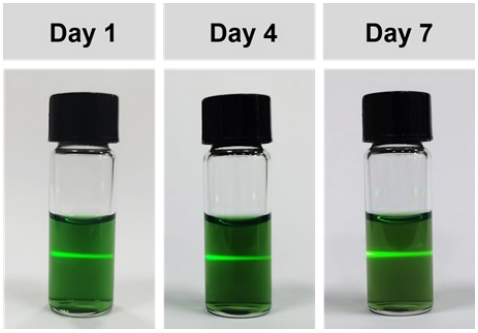

Fig. S4 Appearances of IR820/ATO NPs after being stored in dark for different time.

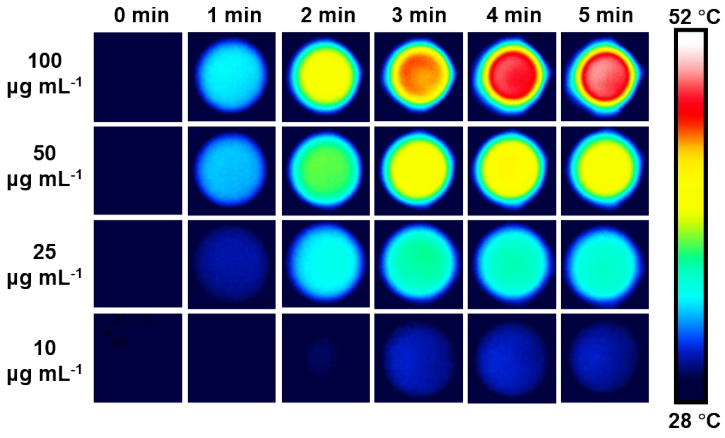

Fig. S5 Thermographic images of IR820/ATO NPs by the 808 nm laser irradiation for 5 min.

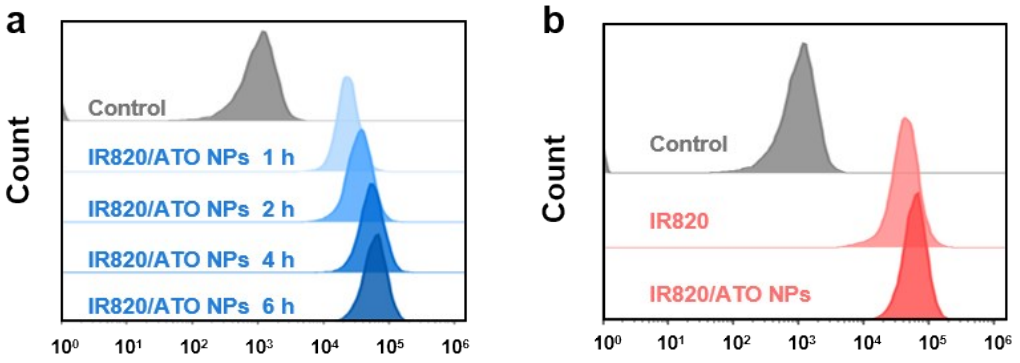

Fig. S6 (a) Flow cytometric results of cellular uptake of IR820/ATO NPs in 4T1 cells after incubation of different time and (b) Cellular uptake of 4T1 cells after incubation with control, IR820, IR820/ATO NPs for 6 h.

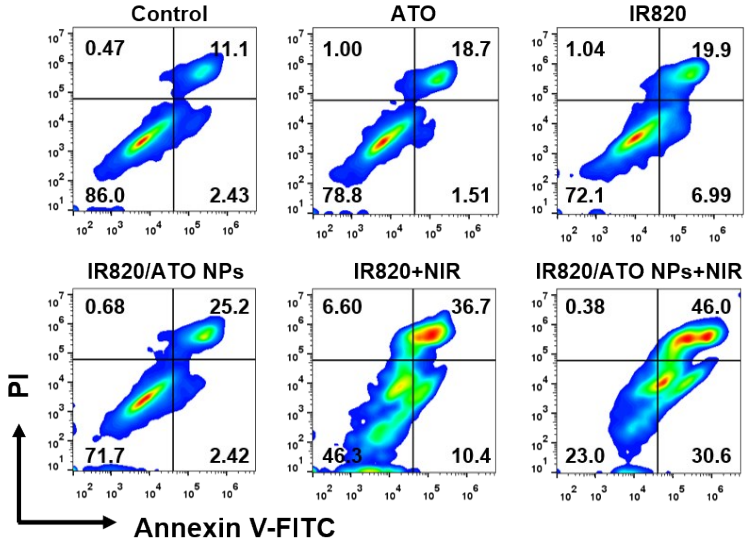

Fig. S7 Flow cytometric results of apoptosis of 4T1 cells after different treatments.

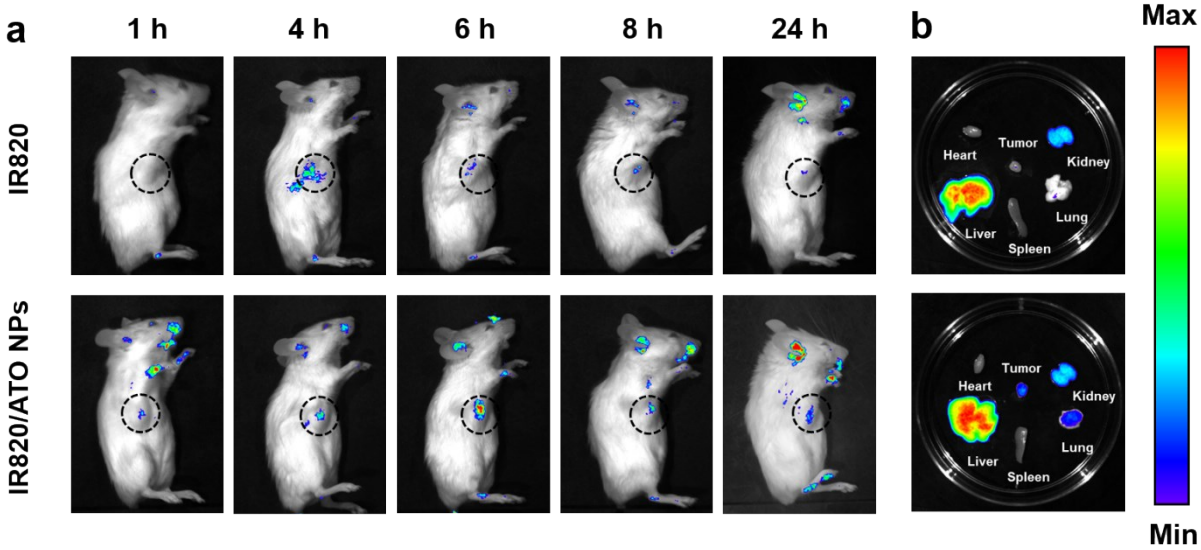

Fig. S8 (a) Fluorescence imaging and biodistribution of IR820 and IR820/ATO NPs in the mice

bearing 4T1 tumors at different times after post-injection. The black circles represent tumor sites. (b) Fluorescence images of the relative tumors as well as major organs after 24 h post-injection of the IR820 and the IR820/ATO NPs.

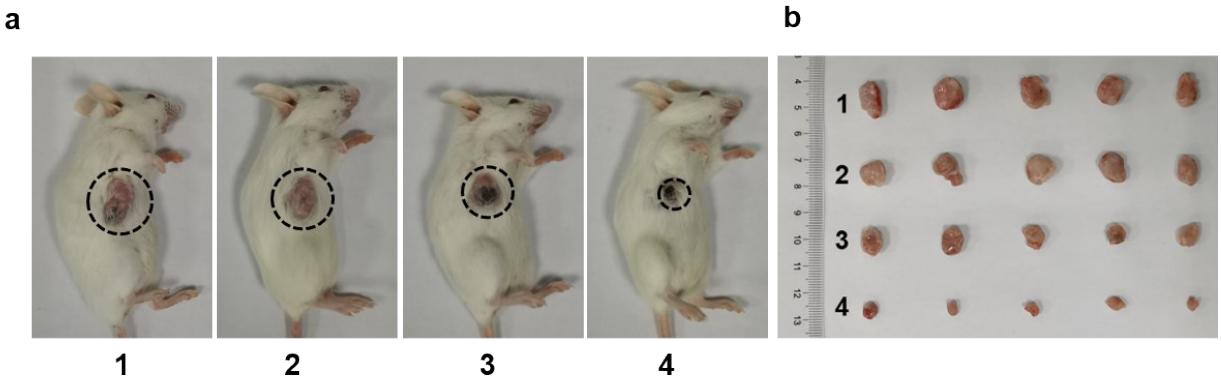

Fig. S9 (a) Photographs of the mice bearing 4T1 tumors after different treatments. (b) Photographs of tumors of different groups. (1 normal saline, 2 ATO, 3 IR820+NIR, 4 IR820/ATO NPs+NIR)

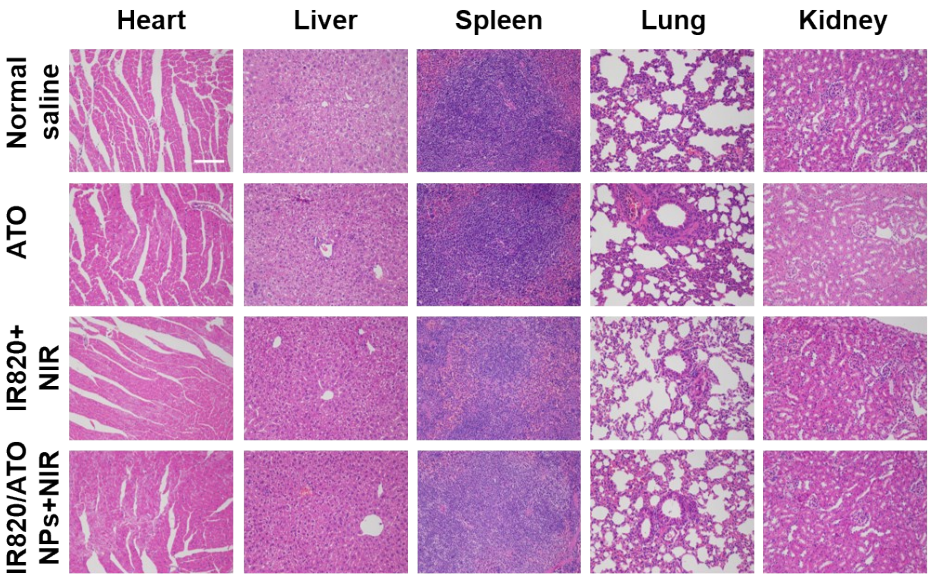

Fig. S10 H&E staining images of main organs with various treatment for 4T1 tumor-bearing mice. Scale bar: 100 μm.