Near infrared band responsive ROS regulator selectively inhibits breast cancer cells by programming combination phototherapy

Yanan Liu[a,*], Guanglong Yuang[b], Xu Chen [b,c,*], Jie Liu[b,*]

[a].Shenzhen Longhua Maternity and Child Healthcare Hospital, Shenzhen, 518110, China.

[b].Department of Chemistry, College of Chemistry and Materials Science, Jinan University, Guangzhou 511436, China.

[c]. Department of Rheumatology and Immunology, Guangdong Second Provincial General Hospital, Guangzhou 510317, P. R. China

*Corresponding author, Jie Liu, E-mail: tliuliu@jnu.edu.cn;

Yanan Liu, E-mail: yananliu0321@163.com; Xu Chen, E-mail: 15521370063@163.com.

Experimental Section

Materials: Ascorbic acid (AA), KBr, K_4PdCl_4, $Na_3IrCl_6 \cdot 6H_2O$ and ethylene glycol (EG) were purchased from Aladdin Reagent (Shanghai, China). polyvinylpyrrolidone (PVP, Mw = 48000) was purchased from Macklin. 5,5-dimethyl-1-pyrroline N-Oxide (DMPO) and 2,2,6,6-Tetramethylpiperidinooxy（TEMPO）were purchased from Sigma Aldrich Chemical Co. Cell culture medium DMEM/1640 and fetal bovine serum (FBS) were purchased from Gibco (Life Technologies AG, Switzerland). The Reactive oxygen species assay kit（DCFH-DA）, 4', 6-diamidino-2-phenylindole (DAPI) and mitochondrial membrane potential assay kit were purchased from beyotime,Shanghai. The CCK-8 kit was purchased from Dongren Chemical Technology Co., Ltd (Shanghai). The AV-FITC/PI Apoptosis Detection Kit was purchased from Beijing 4A Biotech Co., Ltd. Calcein-AM/PI live/dead cell double staining kit was purchased from BestBio Co., Ltd (Shanghai). All reagents and solvents were commercially available and were used without further purification unless

otherwise statement. Milli-Q water (18.2 MΩ cm) was used in all experiments.

Preparation of PIH@R:

Pd NCs： 250 mg AA, 278 mg PVP and 1500 mg KBr were dissolved in 20 mL water and then added 5 mL K_4PdCl_4 aqueous solution (32 mg/mL)after preheating in an oil bath at 85 ° C for 5 min。Finally, the reaction was continued at 85 ° C for 3 h under condensation reflux. After the reaction completed, the reaction solution was cooled to room temperature, and the product was separated and collected by centrifugation, and then purified with water and acetone.

Pd-Ir NCs：10mg Pd NCs, 40 mg AA, 200 mg PVP, 160 mg KBr and 7.0 mL EG were mixed in 20mL vials and then preheated to 110 ℃. After preheating 20min, the reaction temperature was raised to 180 ° C, and then 6mL EG solution contained 0.6mg $Na_3IrCl_6 \cdot 6H_2O$ is injected to reaction mixture at the rate of 2.0 mL h^{-1}. After the injection, the reaction mixture was kept at 180 ° C for 1h. The solid products are collected by centrifugation, washed with acetone once, washed with water twice, and finally freeze-dried for collection.

PIH@R: 10 mL 3 mg/mL Pd-Ir NCs solution was added to a small conical flask. Then H_2 is injected (1 ml / s) from the bottom of the Pd-Ir NCs solution for 24 h to obtain PdH0.2-Ir (PIH). Then PIH was collected by centrifugation. 5 mg PIH and 1 mg RBT ($[Ru(bpy)_2(tip)]^{2+}$) is dissolved in 5 mL water (containing 0.5 mL DMSO) for 24 h stir. Finally, the product was collected by centrifugation to obtained PIH@R.

Characterization: The transmission electron microscopy (TEM) images and element mapping were obtained by a JEOL microscope (JEM-2100F, JEOL, Japan) at an accelerating voltage of 200 kV. Nitrogen sorption isotherms and pore size distribution were measured at 77K with a surface area analyzer (TriStar II 3020, Micromeritcs, USA). Dynamic hydrodynamic diameter (DLS) and zeta

potential were analyzed by a nano Zetasizer (Malvern ZEN3600 Zetasizer Nano-ZS, Britain). FT-IR spectra were obtained by NIC0LET iS50+ iN10(USA).

Cell culture: The mouse breast cancer cells 4T1, Human breast cancer cells MDA-MB-231, Human Normal Liver cells Lo2 were purchased from American Type Culture Collection (ATCC). They were cultured in Dulbecco's modified Eagle's medium (DMEM)or Roswell Park Memorial Institute-1640 (RPMI-1640) with 10% (v:v) foetal bovine serum, 100 U mL^{-1}penicillin, and 100 μg mL^{-1}streptomycin in an incubator (Thermo Scientific) at 37 °C under the atmosphere of 5% $CO_2$and 90% relative humidity. To digest cells and subculture, 0.25% (w:v) trypsin was used.

Cytotoxicity measurement: 4T1/MDA-MB-231/Lo2 cells were seeded into 96-well plates at 6×10^3 cells per well and incubated for 24h. Then the cells were treated with Pd, Pd-Ir, PIH, PIH@R at various concentrations (10–40 μg mL^{-1}, n=5). The control group was treated with PBS only. After 4 h, the medium was replaced with fresh medium, and then the cells were exposed to an 808/1064 nm laser. After incubation for another 16 h, the cellular viability was tested by the CCK-8 method.

Cell uptake assay: 4T1 cells were seeded in glass-bottom culture dishes at a density of 1.5×10^5 cells per dish and incubated for 24 h. Afterwards, the previous medium was removed, and 1 mL of fresh medium containing 20 μg mL^{-1} PIH@R was added. After incubation at various time intervals (1, 2, 4, 6h), the cells the cells were washed three times with PBS and then fixed with paraformaldehyde (4%) for 25 min and stained by DAPI for 5 min. Then, the images of the cells were recorded by a CLSM (Zeiss LSM880 Airyscan, German).

Measurement of intracellular hydrogen release: 4T1/MDA-MB-231/Lo2 cells were seeded into 6-well plates at 2×10^5 cells per well and incubated for 24h. Afterwards, the previous medium was removed, and 1 mL of fresh medium containing 100 μg mL^{-1} MB-Pt probe was added. After incubation

for 2 h, the cells were washed with PBS three times and then incubated with 1 mL of fresh medium containing 20 μg mL^{-1} PIH@R. After 4h incubation, the cells were exposed to 808/1604 nm lase and then digested to be observed and photographed under a microscope.

Intracellular ROS analysis: 4T1/MDA-MB-231/Lo2 cells were seeded into 6-well plates at 2×10^5 cells per well and incubated for 24 h. Then the cells were treated with PSB/ PIH@R (20 μg mL^{-1}) respectively. After 4 h, the medium was replaced with 1 mL fresh DCFH-DA working fluid for another 30 min incubation, and then the cells were exposed to an 808/1064 nm laser. After laser treatment, the cells were washed with PBS and immediately analyzed by an inverted fluorescence microscope. For the flow cytometry analysis, the cells were treated as mentioned above. After NIR treatment, the cells were suspended in PBS and then analyzed using a flow cytometry.

Mitochondrial membrane potential detection: 4T1/MDA-MB-231 cells were seeded into glass-bottom culture dishes at a density of 1.5×10^5 cells per dish and incubated for 24 h. After incubation with Pd, Pd-Ir, PIH, PIH@R (20 μg mL^{-1}) for 4 h, corresponding cells were exposed to 808/1064 nm laser and then stained with 1 mL JC-1 dye working solution for 30 min. The red fluorescence (the aggregated JC-1) and green fluorescence (the monomeric JC-1) were observed by using CLSM (Zeiss LSM880 Airyscan, German), and the rate of monomeric JC-1 was quantified by using a flow cytometer (BD FACSCanto, USA) at 37°C.

Live/dead stain of HeLa cells: MDA-MB-231/4T1 cells were seeded into dish at 1.5×10^5 cells per well and incubated for 24 h. Then the cells were treated with Pd, Pd-Ir, PIH, PIH@R (20 μg mL^{-1}). The NIR treatments were carry out at 4h. After NIR treatment, the cells were washed with PBS, stained with Calcein-AM/PI for 20 min, and imaged by CLSM.

In vivo therapeutic evaluation: Female BALB/c mice (5 weeks old) were purchased from Beijing

Huakang Biotechnology Co., Ltd., China. All animal procedures were performed in accordance with the protocol approved by the Laboratory Animal Science Department of Jinan University. All animal experiments were in accordance with the guidelines of the Animal Care and Use Committee of the Laboratory Animal Science Department of Jinan University. For in vivo therapeutic evaluation, all BALB/c were randomly assigned (n = 3) into 8 groups: (1) PBS; (2) Pd-Ir; (3) PIH NCs; (4) PIH@R; (5) PIH@R+ NII; (6) PIH@R+ NI (7) PIH@R+ NI/NII (8) PIH@R+ NII / NI. They were subcutaneously transplanted with 4T1 mammary cancer cell (1×10^6 cells per site) when they were 6 weeks old. When the tumor volume reached approximately 100 mm3, mice were injected with different formulations via the tail vein (50 μL, 6 mg kg^{-1}). NIR treatments were conducted after 8 h injection. The first day of treatment was defined as day 1 and the same treatments were repeated on the seventh day. Each mouse's body weight and tumor volume were recorded every two days. The formula for calculating tumor volume was: volume = $W^2 \times L/2$, in which “W" and “L" represented the width and length of the tumor respectively. All mice were sacrificed 24 days later and the tumor was completely stripped and then fixed in PBS containing 4% formaldehyde. Tumor tissues were then stained with TUNEL, H&E, and Ki-67, and pathologically analyzed under a microscope. Other major organs (heart, liver, spleen, lung, and kidney) were also removed for H&E staining to assess acute toxicity.

Results and Discussion

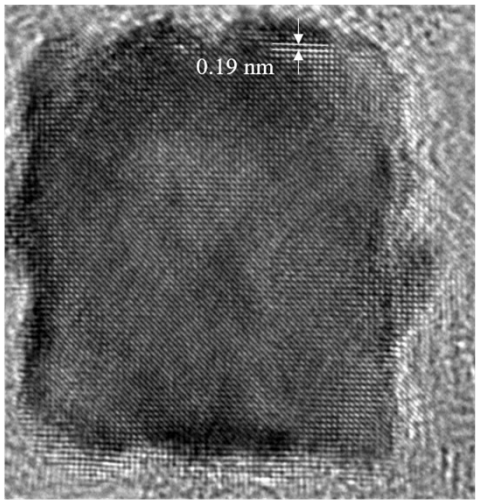


Figure S1. Lattice spacing of Pd-Ir NCS surface layer.

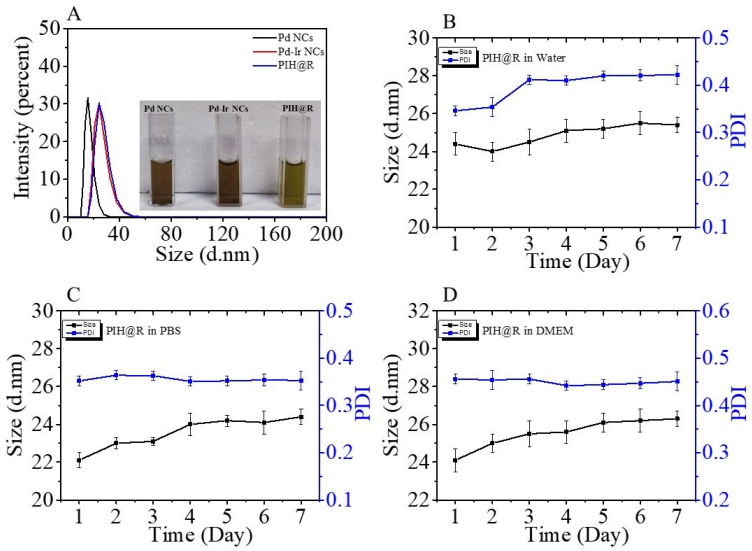
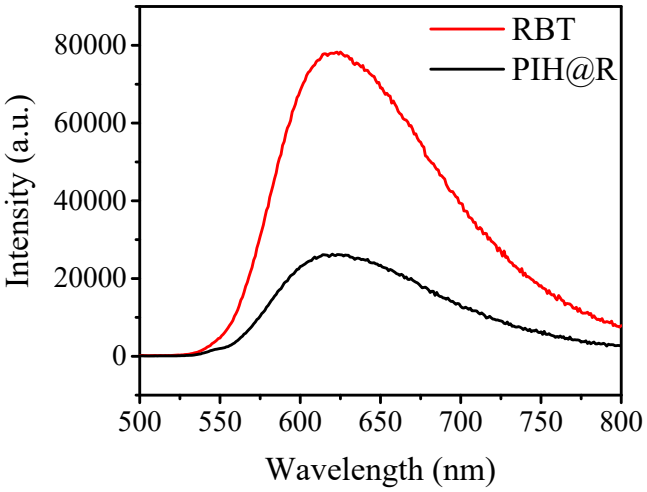


Figure S2. (A) The dispersibility data of Pd NCs, Pd-Ir NCs and PIH@R in aqueous solution. (B) The size and PDI stability of PIH@R in aqueous solution within 7 days. (C) The size and PDI stability of PIH@R in PBS within 7 days. (D) The size and PDI stability of PIH@R in DMEM within 7 days.



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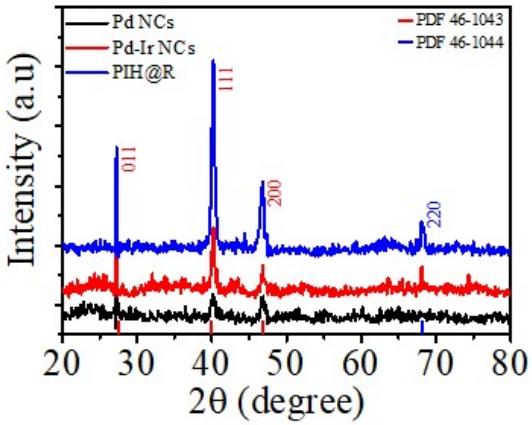
2 **Figure S3**. Emission spectrum of RBT and PIH@R (Excitation wavelength=488 nm).



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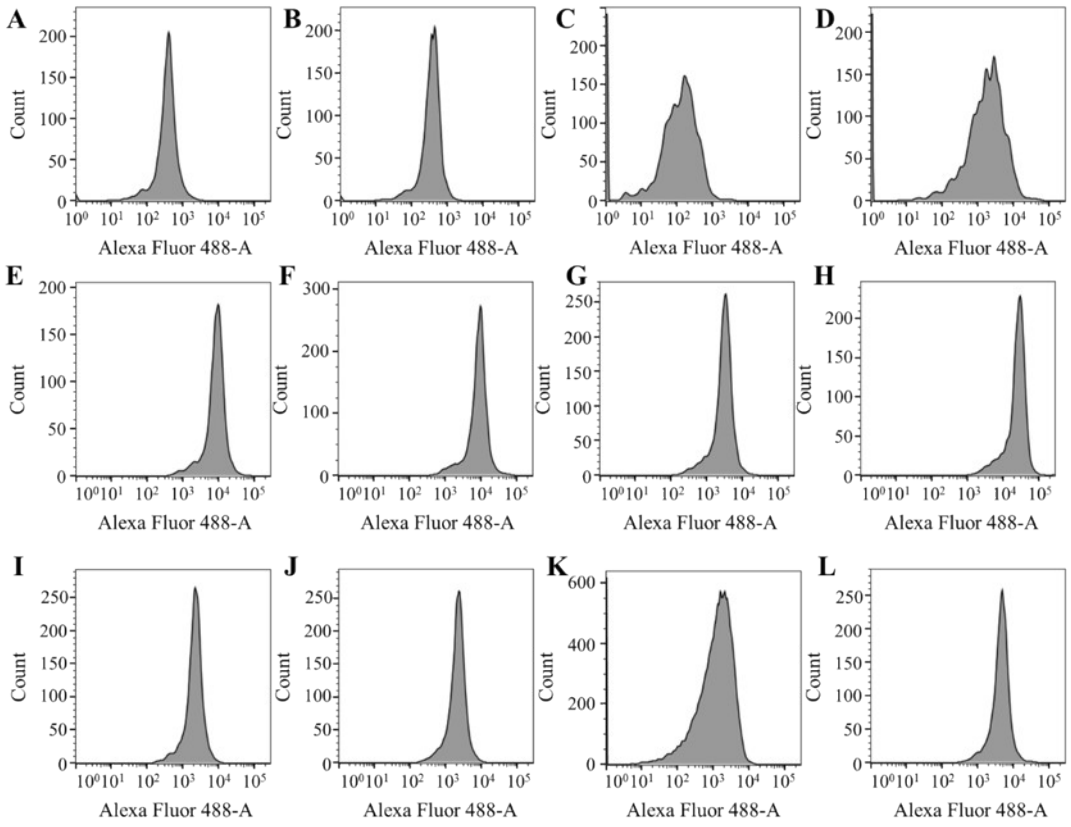
4 **Figure S4**. XRD patterns of Pd NCs, Pd-Ir NCs and PIHR.



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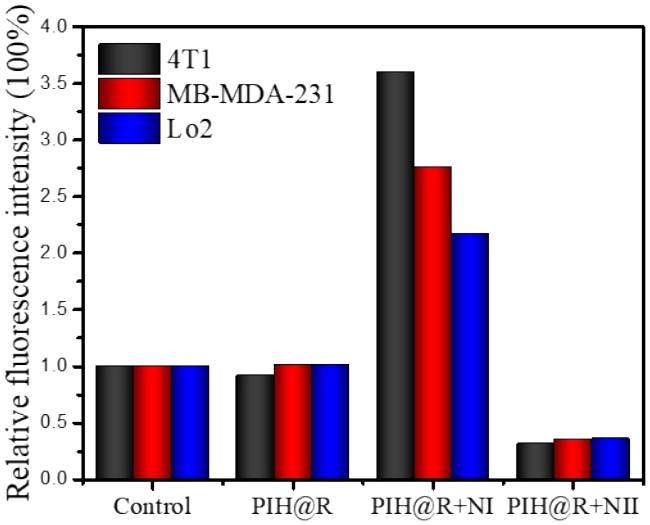
2 **Figure S5**. 4T1(A-D), MDA-MB-231(E-H) and Lo2(I-L) cells flow cytometry assay of ROS. PBS

3 groups(A,E,I)，PIH@R groups(B,F,J), PIH@R +NI groups(C,G,K)，PIH@R+NII groups(D,H,L).



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5 **Figure S6**. Corresponding quantization analyses to flow cytometry assay of ROS.

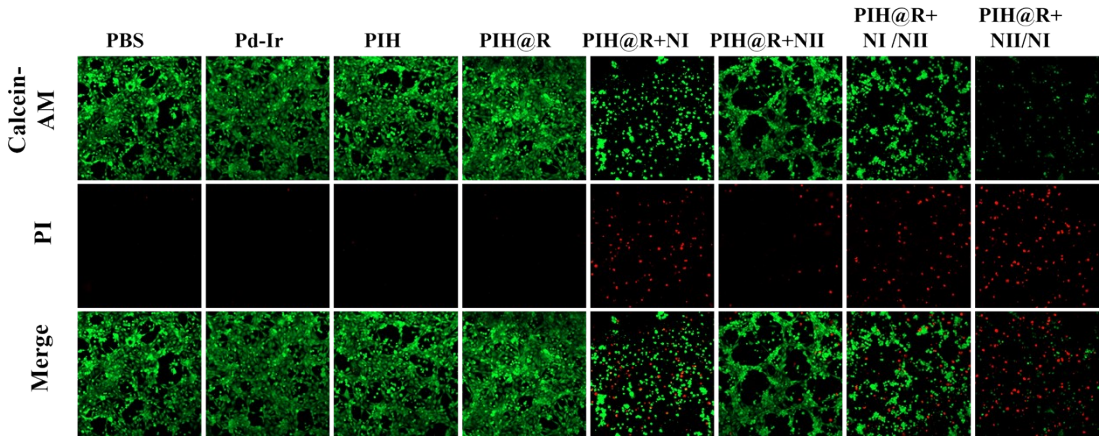

Figure S7. Living / dead staining of 4T1 cells after different treatments.

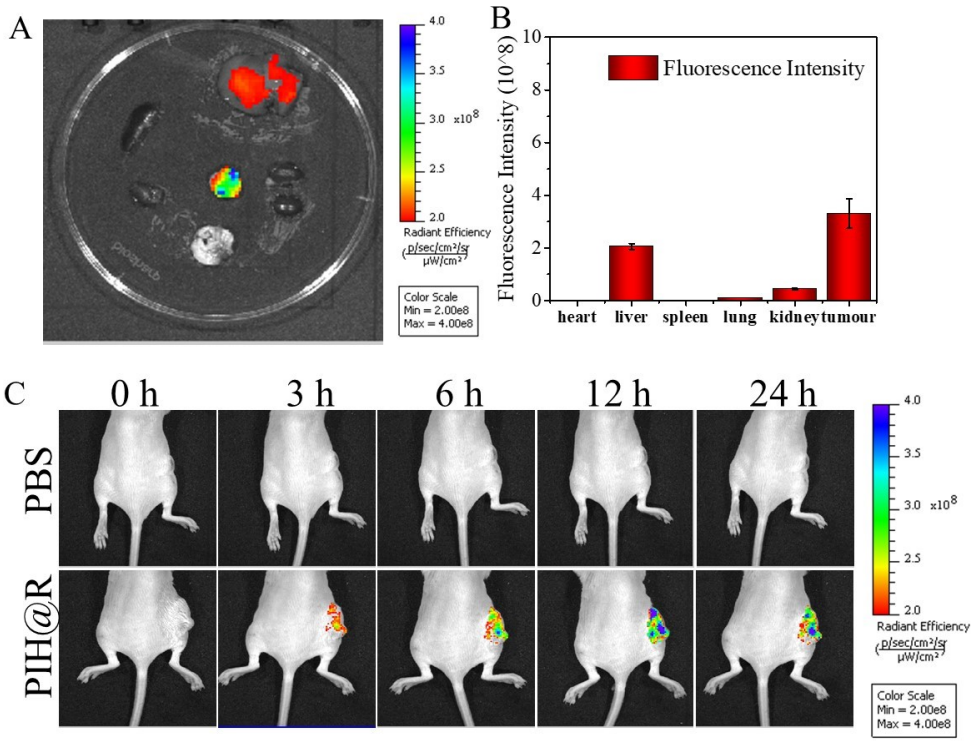

Figure S8. A) Fluorescence imaging of mice mainly organs at 12 h after intravenous injection **PIH@R**. B) The quantification of corresponding fluorescence intensity at 12 h after intravenous injection **PIH@R**. C) The fluorescence imaging of mice at different time after treated with PBS and **PIH@R** (Excitation wavelength=488 nm, Emission wavelength=615 nm).

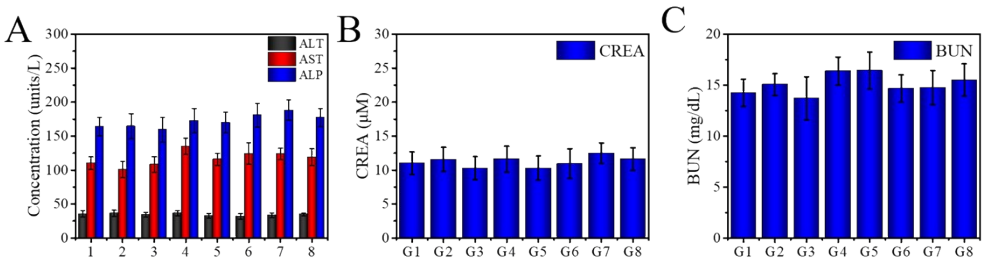

Figure S9. Analysis of liver and renal function in mice with different treatments. (G1-G8 represent PBS，Pd-Ir，PIH，PIH@R，PIH@R+NI，PIH@R+NII，PIH@R+NI/NII，PIH@R+ NII/NI groups respectively).

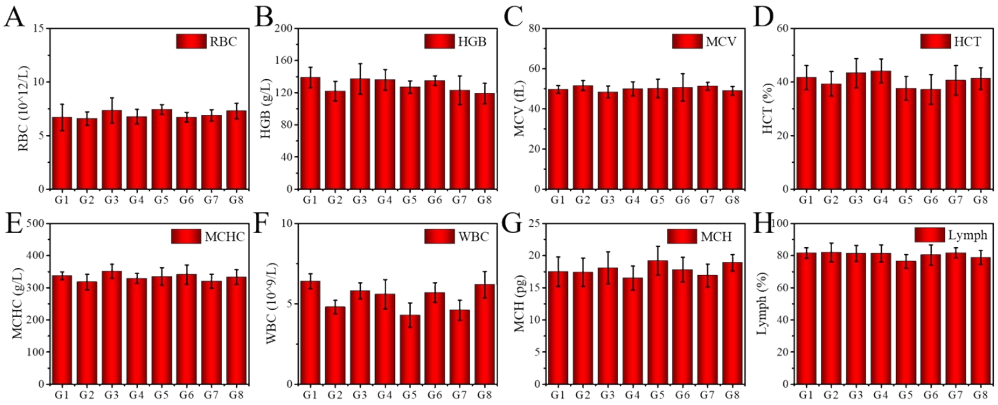

Figure S10. Assay of complete blood panel. A) red blood cells, B) hemoglobin, C) mean corpuscular volume, D) hematocrit, E) mean corpuscular hemoglobin concentration, F) white blood cells G) mean corpuscular hemoglobin and H) lymph. (G1-G8 represent PBS, Pd-Ir, PIH, PIH@R, PIH@R+NI, PIH@R+NII, PIH@R+NI/NII, PIH@R+ NII/NI groups respectively).

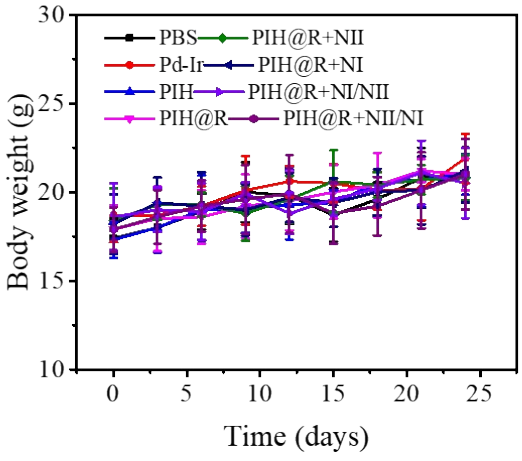

Figure S11. Weight changes of mice during treatments.