Supplementary Information available

Self-promoted tumor-targeting nanomedicine activates STING-driven antitumor immunity via photodynamic DNA damage and PARP inhibition

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Experimental

Materials

Olaparib (Ola), Niraparib (Nir) and Rucaparib (Ruc) was purchased from MedChemExpress. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), 2',7'-dichlorofluorescein diacetate (DCFH-DA), annexin V-FITC/PI apoptosis kit and DAPI were purchased from Beyotime Biotechnology Ltd. The Cytokine ELISA kit for mouse IFN-β was purchased from Elabscience. The antibody against Phosphorylated histone H_2AX (γ-H_2AX) and PD-L1 were purchased from Abcam. The antibodies against phospho-STING (Ser365) and STING were purchased from Cell Signaling Technology. The antibody against phospho-TBK1 (Ser172) was purchased from Affinity. The antibody against phospho-IRF3 (Ser396) was purchased from ABclonal. Anti-mouse antibody (β-actin, GAPDH, PD-L1) were acquired from Abcam.

Preparation and characterizations of PN and PN-Ola

DMSO solutions of C_{16}-K(PpIX)-WHRSYYTWNLNT (200 mg/mL) and Ola (50 mg/mL) were prepared to formulate PN-Ola. After that 7.5 μL of the peptide and 8 μL of Ola were mixed under ultrasonic conditions, and then 500 μL of ultra-pure water was added. After static ultrasound for 3 min, dialysis was performed for 2 h (MWCO 3500 Da), and the liquid was centrifuged (5000 rpm) for 5 min. The supernatant was collected to obtain PN-Ola for the following studies. Similarly, PN was obtained by ultrasonically mixing C_{16}-K(PpIX)-WHRSYYTWNLNT (7.5 μL) with distilled water (500 μL).

The hydrodynamic size of PN-Ola was measured by the dynamic light scattering method. Its stability in various media (water, PBS, and 10% fetal bovine serum) was investigated by measuring the change in particle size with time. The morphology of PN-Ola nanomedicine was identified by transmission electron microscope (TEM). The contents of Ola and Protoporphyrin IX (PpIX) were determined by high performance liquid chromatography (HPLC) and UV-vis spectroscopy. The UV-vis spectrophotometer determined the UV-vis absorption spectra of PN, PN-Ola, PpIX and Ola in aqueous solution.

***In vitro* drug release behavior**

Briefly, PN-Ola was placed in the dialysis tube (MWCO 3500 Da) and suspended in a dissolution medium (phosphate buffer at pH 7.4) for dialysis. The solution was shaken in a constant temperature shaker (200 rpm, 37 ℃). At predetermined intervals, samples of the release medium were collected, and the accumulated buffer was analyzed using HPLC. The drug release rate of Ola was measured and calculated.

Self-promoting targeting ability of PN-Ola

To test the ability of PN-Ola to target 4T1 cells, 3T3, 4T1 and L929 cells were inoculated in the confocal dish. After 24 h, the cells were taken with PN-Ola (60.6 mg/L) for 2, 4 and 8 h. The cells were treated with Hoechst33342 for CLSM observation. For quantitative analysis, 4T1 cells were planted into 6-well plates, and incubated with the same time gradient as above. The fluorescence intensity of the cells after digestion was detected by flow cytometry.

Further, 4T1 cells were seeded in the confocal dish. After 24 hours of stabilization, the cells were dealt with PN (58.2 mg/L) at 37 °C for 2, 4, and 8 h. As a control, 4T1 cells were pre-incubated with anti-PD-L1 antibody for 24 h to block PD-L1 on the cell surface. Then, the cells were washed with PBS and stained with Hoechst33342 for 15 min. Fluorescence imaging was performed by CLSM. 4T1 cells were inoculated in confocal dishes while the Ola pre-treatment group was co-incubated by adding Ola (10.0 mg/L). Subsequently, PN (58.2 mg/L) was put in and treated for 4 h away from light. Fixation was performed with 4% paraformaldehyde, cells were permeabilized with 0.1% Triton-100, then closed with 20% goat serum and incubated with anti-PD-L1 antibody overnight at 4 ℃. Finally, the cells were stained with goat anti-rabbit IgG H&L and DAPI and visualized by CLSM.

Moreover, 4T1 cells were treated with PN (58.2 mg/L) and PN-Ola (60.6 mg/L) for 4, 8, 12, and 24 hours to assess the self-promoted tumor-targeting ability of PN-Ola. After the treatment by the above methods, 4T1 cells were observed by CLSM and detected by flow cytometry.

Cytotoxicity assay

Live/dead cell staining assay: 4T1 cells were seeded into confocal dishes. Cells were taken with Ola (1.2 mg/L), PN (29.1 mg/L), or PN-Ola (30.3 mg/L) for 4 h. The light groups were exposed to light (638 nm, 0.97 W cm^{-2}) for 10 min. Cells were identified and analyzed by CLSM.

MTT assay: 4T1 cells were incubated with gradient concentrations of Ola, PN or PN-Ola. 4 h later, the cells in the light groups were irradiated with light (638 nm, 0.97 W cm^{-2}) for 15 min. 24 h later, 20 μL of MTT (5 mg/L) was added in each well-protected from light. Then the cells were incubated for 4 h. The medium was discarded and 150 μL DMSO was poured in and completely dissolved to form blue-violet crystals. The absorbance at 570 nm was measured with an enzyme marker to assess cell viability.

Apoptosis assay: 4T1 cells were treated with Ola (1.2 mg/L), PN (29.1 mg/L), or PN-Ola (30.3 mg/L) for 4 h, and then cells in the light group were illuminated by laser (638 nm, 0.97 W cm^{-2}) for 8 min. After washing three times with PBS, all the cells were accumulated and stained for fluorescence using the apoptosis kit in the Annexin-FITC/PI staining for 20 min. Cell fluorescence was analyzed by flow cytometry.

PDT performance of PN-Ola

The singlet oxygen ($^1O_2$) generation was calculated using Singlet Oxygen Sensor Green (SOSG). Specifically, Ola (2.4 mg/L), PN (58.2 mg/L) and PN-Ola (60.6 mg/L) were mixed respectively in PBS containing 10 μL SOSG. Fluorescence spectra were utilized to track changes in fluorescence intensity to assess the production of $^1O_2$ in the presence or absence of light (638 nm, 0.97 W cm^{-2}). In addition, DCFH-DA was used as a probe in CLSM to observe ROS. After 24 h in the confocal dish, 4T1 cells were treated with Ola (1.2 mg/L), PN (29.1 mg/L) and PN-Ola (30.3 mg/L) for 4 h. Next, the cells were processed with DCFH-DA for 30 min. Cell fluorescence was assessed and analyzed with CLSM in the presence or absence of light for 8 min (638 nm, 29.8 mW cm^{-2}).

Western blotting analysis

In vitro PD-L1 protein assay: 4T1 cells were planted in 6-well plates and cultured for 24 h. In the meantime, cells were incubated with Ola (10.0 mg/L), PN (238.5 mg/L)

or PN-Ola (248.5 mg/L) in the dark for 36 h. After incubation, the cells were collected and analyzed by western blotting to verify the expression of PD-L1 protein.

In vitro γ-H_2AX assay: The cells were stimulated with Ola (10.0 mg/L), PN (238.5 mg/L), or PN-Ola (248.5 mg/L) for 24 h in the dark. The light groups were illuminated with light for 1 min (638 nm, 0.97 W cm^{-2}), and the cells in the other groups were incubated in the dark for 12 h. The cells were accumulated for western blotting analysis to verify the expression of γ-H_2AX protein.

In vitro STING pathway assay: The cells were stimulated with Ola (10.0 mg/L), PN (238.5 mg/L) or PN-Ola (248.5 mg/L) for 24 h. The light group was exposed to light for 3 minutes (638 nm, 0.97 W cm^{-2}), whereas the cells in the other groups were left in the dark. DC cells were added to the lower chamber and co-culture for 24 h. DC cells and supernatants were collected. The cells were used for western blotting analysis to detect the expression of STING, p-STING, p-TBK1, and p-IRF3 proteins. Cell supernatants were used for ELISA assay to detect the release of IFN-β.

Immunofluorescence staining of γ-H_2AX

4T1 cells were planted in confocal dishes and cultured for 24 h. Cells were taken with Ola (10.0 mg/L), PN (238.5 mg/L) or PN-Ola (248.5 mg/L). The light groups were illuminated with light for 1 min (638 nm, 0.97 W cm^{-2}). After continuing to incubate for 12 h, the cells were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton-100, closed with 20% goat serum and incubated with anti-γ-H_2AX antibody overnight at 4 °C. Finally, the cells were stained with goat anti-rabbit IgG H&L and DAPI. Intracellular γ-H_2AX expression was noted with CLSM.

***In vivo* biodistribution**

All experiments were performed in accordance with the guidelines for the care and use of laboratory animals provided by the Animal Research Committee of Guangzhou Medical University and approved by the Ethics Committee of Guangzhou Medical University (Approval No.: GY2024-286). Informed consents were obtained from human participants of this study. Animal models were constructed by subcutaneous injection of 4T1 cells into the right hind leg of female BALB/c mice. Subsequently, NP-Mel (32.7 mg/kg) was administered to the tail vein of mice. Fluorescence imaging

was performed at 0.5, 2, 4, 6, 8, 12 and 24 h, respectively. After 24 h of administration, mice were executed and heart, liver, spleen, lung, kidney and tumor tissues were congregated for *in vitro* imaging.

Antitumor Study *in Vivo*

When the average tumor volume reached 200 mm^3, mice were injected subcutaneously with 4T1 cells to construct a solid tumor model. 4T1 cells were injected into mice by tail vein to establish lung metastasis models. Starting from the second day, the drugs were administered via the tail vein every two days at the following doses: Ola was 1.3 mg/kg, PN was 31.4 mg/kg, and PN-Ola was 32.7 mg/kg. The blank group did not receive the drugs. The light group was irradiated with 0.97 W for 6 min after each injection, for a total of three treatments. The body weight and tumor volume of the mice were monitored every 2 days. The mice were executed at the end of the experiment. All tumor tissue and spleen were congregated for weighing and photographing. To check for inhibition of lung metastasis, all lungs were photographed. The heart, liver, spleen, lung, kidney and tumor were also stained for hematoxylin and eosin (H&E). Tumor tissues were subjected to Tunel immunofluorescence staining, Ki67 immunohistochemical staining, and PD-L1 immunofluorescence staining. CD3CD8 immunofluorescence staining was also performed on tumor, spleen and lung tissue. Blood biochemical analyses were performed on serum.

Immune activation measurement

Mice were injected subcutaneously with 4T1 cells to construct a solid tumor model. The dose administered to mice and the frequency of administration was the same as the anti-tumor study *in vivo* described above. After treatment, the tumor and spleen were harvested, processed into single cell suspension, stained and labeled with appropriate antibodies. Stain-labeled immune cells were detected by flow cytometry.

Statistical analysis

All the quantitative results were reported as mean ± standard deviation (SD). The Student's t-test was used to assess the statistical significance of the different groups. The threshold of significance was defined as follows: $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$.

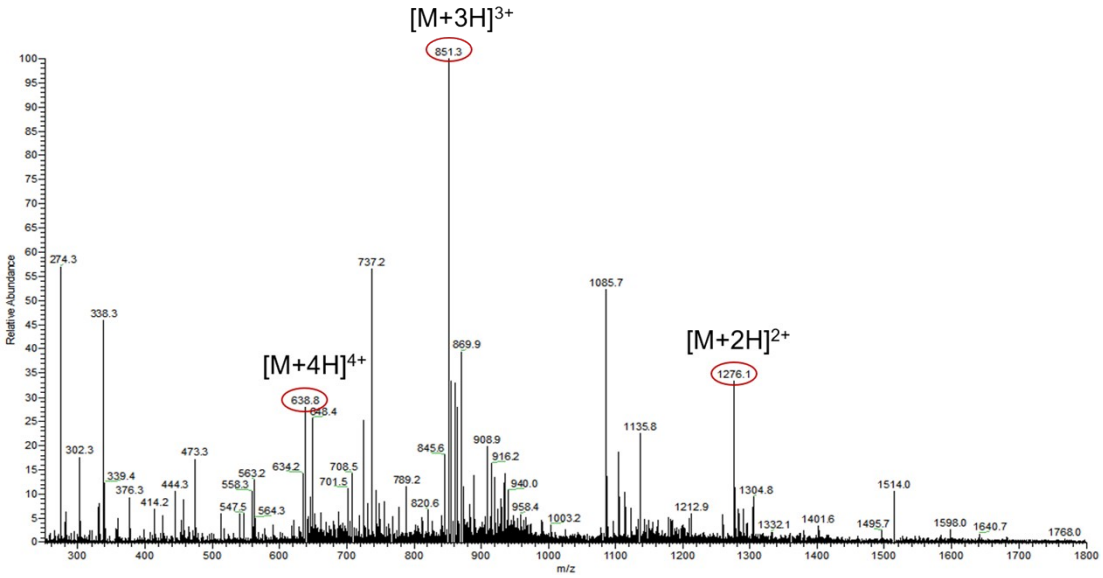

Fig. S1 ESI-MS of the PD-L1 targeted peptide (C_{16}-K(PpIX)-WHRSYYTWNLNT).

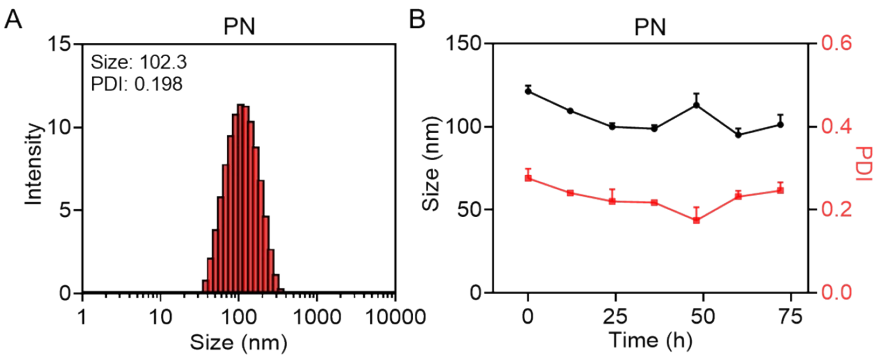

Fig. S2 (A) Particle size image of PN. (B) Variations of hydrodynamic size and PDI of PN in 72 h (n=3).

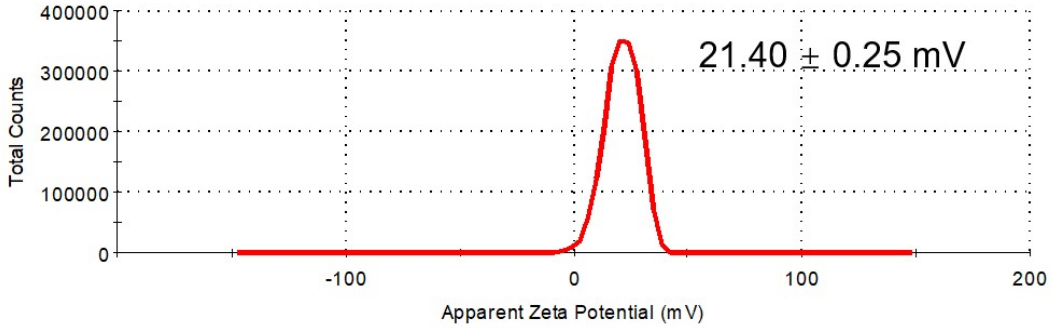

Fig. S3 Potential situation of PN-Ola in water.

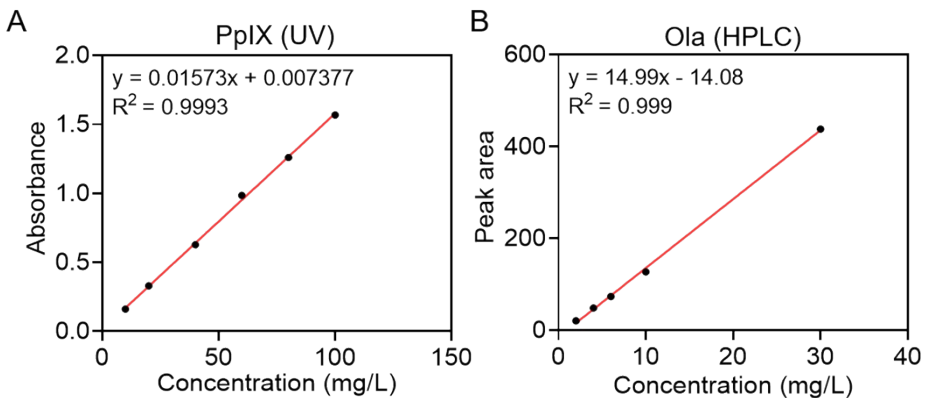

Fig. S4 Standard curves of (A) PpIX and (B) Ola measured by UV-vis and HPLC.

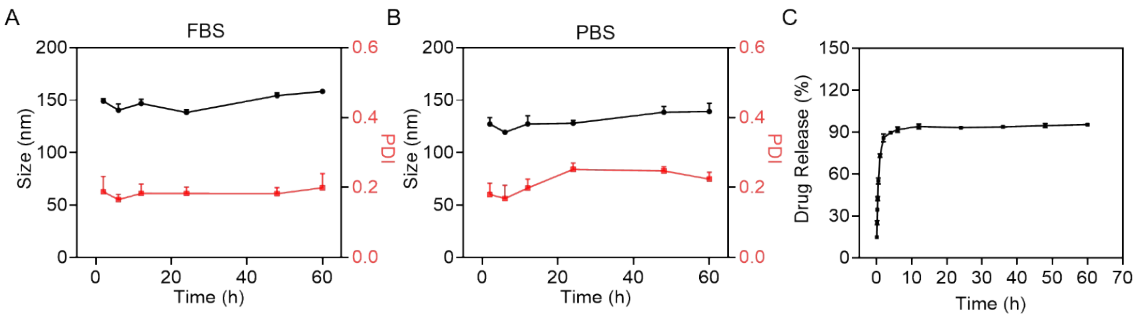

Fig. S5 The changes of particle size of PN-Ola in 60 hours under (A) 10% FBS or (B) PBS (n = 3). (C) The cumulative release of Ola from PN-Ola at PBS (n = 3).

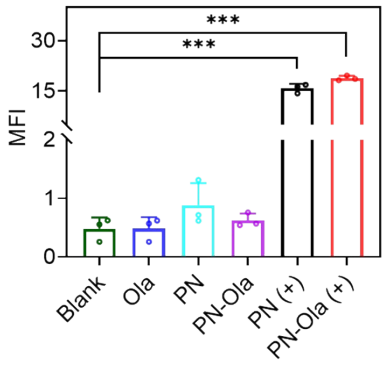

Fig. S6 Quantitative analysis of CLSM images to determine the mean fluorescence intensity (MFI) of the ROS generation in 4T1 cells after different treatments. "+" represented the addition of light irradiation. ***$P < 0.001$ was tested via a Student's t-test.

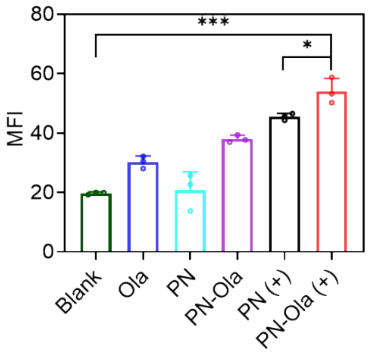

Fig. S7 Quantitative analysis of CLSM images to determine the MFI of the γ-H_2AX expression in 4T1 cells after different treatments. "+" represented the addition of light irradiation. *$P < 0.05$ and ***$P < 0.001$ were tested via a Student's t-test.

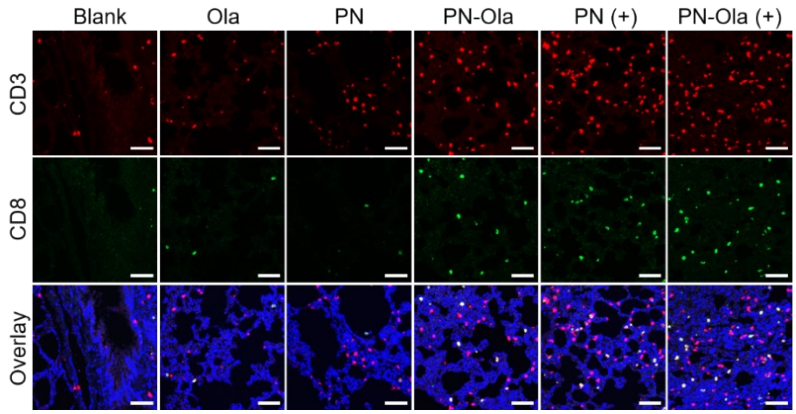

Fig. S8 $CD3^{+}CD8^{+}$ T cells in lung after treatment with Ola, PN and PN-Ola in the presence or absence of light. Scale bar: 50 μm. "+" represented the addition of light irradiation.

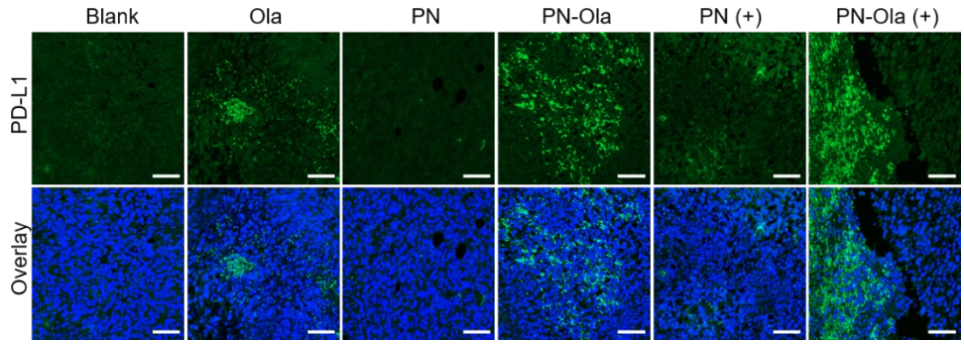

Fig. S9 Immunofluorescence staining of PD-L1 of tumor tissues after treatment with Ola, PN and PN-Ola in the presence or absence of light irradiation. Scale bar: 50 μm. "+" represented the addition of light irradiation.