Supplementary information

Hydroxyl-Modified Fullerene $C_{70}(OH)_8$ Induces Pyroptosis for Cancer Therapy

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Materials and reagents.

Fullerene (C_{70}) was purchased from Henan Fullerene Nano New Materials Technology Co., Ltd. Acetic acid (CH_3COOH), $AgClO_4$, o-dichlorobenzene (o-DCB), common solvent (chloroform, acetone, chloroform, tetrahydrofuran etc.) were all obtained from commercial sources.1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(amino-(polyethyleneglycol)-2000) (DSPE-PEG-2000) were purchased from Sigma-Aldrich. 1,3-Diphenylisobenzofuran (DPBF) and Dihydroethidium (DHE) were provided by Thermo Fisher Scientific Co., Ltd. Cell Counting Kit-8 (CCK8), BCA kit and 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, 97%) were provided by Beyotime Biotechnology. Human cervical carcinoma cells (HeLa) cells was obtained from the American Type Culture Collection (ATCC, USA). DMEM and fetal bovine serum media were obtained from Adamas life. Primary antibodies against Caspase-3/cleaved Caspase-3, cleaved PARP, GASDME and β-actin were purchased from Cell Signaling Technology.

Characterizations.

^{1}HNMR and ^{13}CNMR were measured on Bruker 600 MHz spectrometers. Transmission electron microscopy (TEM) images were captured on a JEM-2100 transmission electron microscope (Hitachi Co. Ltd., Japan). Dynamic light scattering (DLS) was measured on a Zetasizer nano ZS instrument (DLS, Zetasizer nano zs90). Ultraviolet-visible (UV-vis) absorption spectra were collected using a UH4150 Spectrophotometer (Hitachi Co. Ltd., Japan). Fluorescence spectra were measured using a FLS980 fluorescence spectrometer (Edinburgh Co. Ltd., Edinburgh UK). Cells were

imaged using an A1R-si confocal laser scanning microscope (Nikon, Tokyo, Japan). A Super Max3100 microplate reader was used in this study (Shanpu, Shanghai, China) to measure the cells absorption after cck8 assay. Cellular Photocytotoxicity Irradiators (LED Model, PR-CPC2-white) and Small Animal Phototherapy Irradiators (Mix Model, PR-PDT-MIX) were provided by Shenzhen PURI Materials Technologies Co., LTD.

Synthesis of $C_{70}(OH)_8$.

Dissolve compound $C_{70}Cl_8$ (200 mg, 0.182 mmol) in a 100 mL round-bottom flask. Add 30 mL of o-dichlorobenzene, 5 mL of acetic acid, and 1 mL of water. Begin stirring, and after thirty minutes, a suspension will form. Introduce $AgClO_4$ (1 g, 4.9 mmol) and stir at room temperature for 20 hours. As the reaction progresses, the mixture will gradually become clear. Carefully pour off the supernatant, and then gradually add a tetrachloroethane/methanol mixture (10:1) to dissolve the precipitate. For separation, use a tetrachloroethane/methanol mixture (7:3) as the mobile phase on a silica gel preparation plate to isolate compound 27 (41.4 mg, 0.046 mmol, 25%). ^{1}H NMR (600 MHz, DMSO-d_6) δ 7.78 (s, 2H, -OH), 7.65 (s, 2H, -OH), 7.60 (s, 2H, -OH), 7.47 (s, 2H, -OH). ^{13}C NMR (151 MHz, DMSO-d_6) δ 155.56, 155.18, 154.56, 153.60 (1C), 152.26, 152.23, 151.71, 151.37 (1C), 150.78 (3C), 150.44, 150.19, 149.07, 148.99, 148.94 (4C), 147.98, 146.15, 145.94, 145.57, 145.26, 144.41, 144.19, 143.72 (1C), 143.37, 143.08, 142.73, 141.66, 138.49, 137.84, 136.87, 134.67, 131.78, 74.93, 74.74, 74.46, 73.39. ^{13}C NMR (151 MHz, CD_3OD) δ 155.67, 154.97, 154.95 (1C), 154.84, 153.36, 152.47 (1C), 152.40, 152.09 (1C), 151.87, 151.10, 150.68, 150.41, 150.28, 149.90, 149.82, 149.56, 149.27,

147.27, 147.10, 146.80, 146.03, 145.86, 145.72, 144.85 (1C), 144.53, 144.46, 143.98, 143.10, 138.32, 137.73, 136.73, 135.60, 132.61, 75.51, 75.35, 75.07, 74.03. ESI-FT-ICR-HRMS-Positive $C_{70}H_7O_7$ (M + H^+ - H_2O) calculated 959.0186, found 959.0185; $C_{70}H_8NaO_8$ (M + Na^+) calculated 999.0111, found 999.0117.

Preparation of $C_{70}(OH)_8$@NP.

Briefly, 1 mg $C_{70}(OH)_8$ was dissolved in 1.0 mL DMSO, 10 mg DSPE-PEG (2000) was dissolved in 4 mL water. Then, DMSO mixed solution was dropped into water (DSPE-PEG (2000)) and stir for 1 h at room temperature to prepare a homogeneous nanoplatform. Then dialysis in deionized water for 48 h, change water every 6 h. The unassembled compounds were extracted by centrifugation at 4000 rpm for 10 min. Finally, the solution was frozen dried into powder.

The generation of singlet oxygen (DPBF Assay).

$C_{70}(OH)_8$@NP or $C_{70}(OH)_8$ and DPBF was mixed in water (50% DMSO), then exposed to laser for 10 second, the change of UV-Vis absorption wavelength in 416 nm (DPBF) was detected every 10 second. (White light, 2.5 mW/cm^2).

DHE Assay for superoxide anion (O_2^-)

$C_{70}(OH)_8$@NP or $C_{70}(OH)_8$ and DHE was mixed in water (50% DMSO), then exposed to laser for 10 second, the fluorescence of DHE ($\lambda_{Ex}/\lambda_{Em}$:535/610 nm) was detected every 10 second. (White light, 2.5 mW/cm^2)**.**

Cell Culture

HeLa cells were cultured in high-glucose DMEM medium supplemented with 10% FBS and 1% penicillin-streptomycin. All cells were incubated in a humidified chamber containing 5% CO_2 at 37 ℃.

CCK8 assay for cell toxicity.

Cells were seeded in 96-well plates (10^4 cells/well), cultured at 37 °C in an atmosphere containing 5% CO_2 for 24 h. Then cells were treated by drugs ($C_{70}(OH)_8$@NP or $C_{70}(OH)_8$.) with different concentration and incubated for 24 h, the cells were then washed with PBS, and then irradiated for 10 min or 20 min with a white light LED (30 $mW \cdot cm^{-2}$). The PBS was then replaced with fresh culture media (100 μL) and the cells were cultured for another 24 h. CCK8 solution (10 μL in 90 μL DMEM) was added to each well after refreshing the medium, incubated for another 1 h. Finally, the OD_{450} was measured using a microplate reader. The IC_{50} value is calculated according to the experimental results by Prism GraphPad.

Intracellular ROS

The ROS generation was detected by DCFH-DA and DHE assay kit according to Manufacturer's manual. Typically, HeLa cells were inoculated in confocal dish, after cultured for 24 h, $C_{70}(OH)_8$@NP or $C_{70}(OH)_8$ was added into cells dish, after another 24 h, exposed to white light for 10 min (30 mW/cm^2) after replaced the cultured medium with fresh medium contained with DCFH-DA or DHE. Finally, ROS production observed by intracellular fluorescence under confocal microscopy.

Flow cytometry (Annexin V-FITC/PI staining)

HeLa cells were seeded into a 6-well plate (1 × 10^6 cells per well). After 24 h, cells treated with $C_{70}(OH)_8$@NP for another 24 h. Exposed to white light for 10 min (30 mW/cm^2) after replaced the cultured medium with fresh medium. Then digest and collect cells using trypsin without EDTA. Subsequently, determine the apoptosis rate using Annexin V-FITC/PI dual staining kit and flow cytometry. The experimental operation is carried out according to the instructions of the kit.

Western blotting.

Firstly, HeLa cells were inoculated in 10 cm Petri dishes and grew to over 80%, washed with pre-cooled PBS and centrifuged for 5 min at 2000 rpm after treated with $C_{70}(OH)_8$@NP and laser (10 min, 30 mW/cm^2). Then lysed in 100 μL of Lysis Buffer-about 30 min at 4 °C, the cells subsequently centrifuged for 10 min at 11000 rpm, protein concentrations were detected by BCA protein assay kit. Secondly, protein samples (40 μg/lane) extracted were separated by 10% or 12% SDS-PAGE and transferred onto 0.45 μm polyvinylidene difluoride (PVDF) membranes. After they were blocked with PBST containing 5% bovine serum albumin (BSA) for 2 h at room temperature, incubated with primary antibody overnight at 4 °C, followed by washes with PBST (x 4) and incubated with diluted the enzyme horseradish peroxidase (HRP)-conjugated secondary antibodies (1:5000) for 1 h at room temperature. After washed with PBST (x 4), the signals were detected by ECL reagent and quantified. β-actin was used as the loading control.

The antitumor effect *in vivo*.

This study was performed in strict accordance with the guidelines for the welfare and use of animals in cancer research (British Journal of Cancer (2010) 102, 1555 – 1577) and was approved by Institutional Animal Care and Use Committee of Sino research (China，Beijing) Biotechnology Co., Ltd. (Protocol number: ZYZC2024080008S). 5-6 weeks-old female mice (Balb/c-nude) were provided by SiPeiFU (Beijing) Biotechnology Co., Ltd.

First, 2 × 10^6 HeLa cells suspended in 0.1 mL DMEM were subcutaneously injected into each mouse. When tumor volume reached approximately 100 mm^3, all mice were randomized into several groups (n = 4), and $C_{70}(OH)_8$@NP were performed intratumoral injection. After 24 h, all mice exposed to white light laser 10 min (0.2 $W \cdot cm^{-2}$), tumor sizes were measured every 2 days, and the actual size of tumors calculated by the formula: length×$width^2$/2. After 15 days, all mice were sacrificed, tumor tissues and other organs were isolated for further study.

Statistical analysis. Statistical significance was determined by *t* tests (two-tailed unpaired) using Prism 7 (GraphPad) and origin pro 8 software. **P* ≤ 0.05, ***P*≤ 0.01, ****P*≤ 0.001 and *****P*≤ 0.0001.

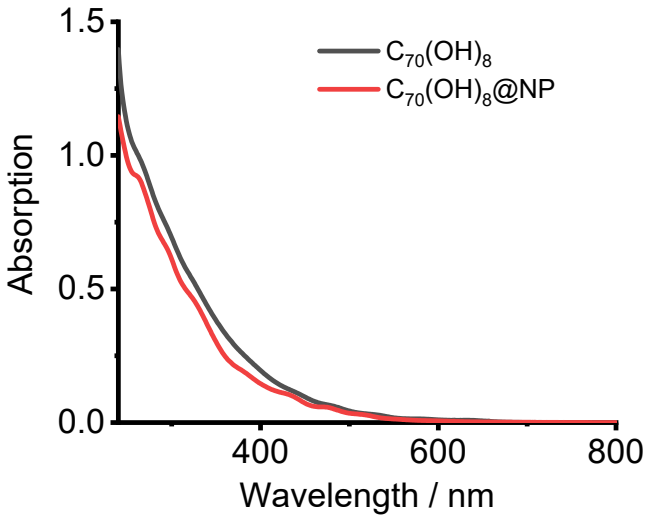

Fig. S1 UV–vis spectra of $C_{70}(OH)_8$@NP and $C_{70}(OH)_8$ in water.

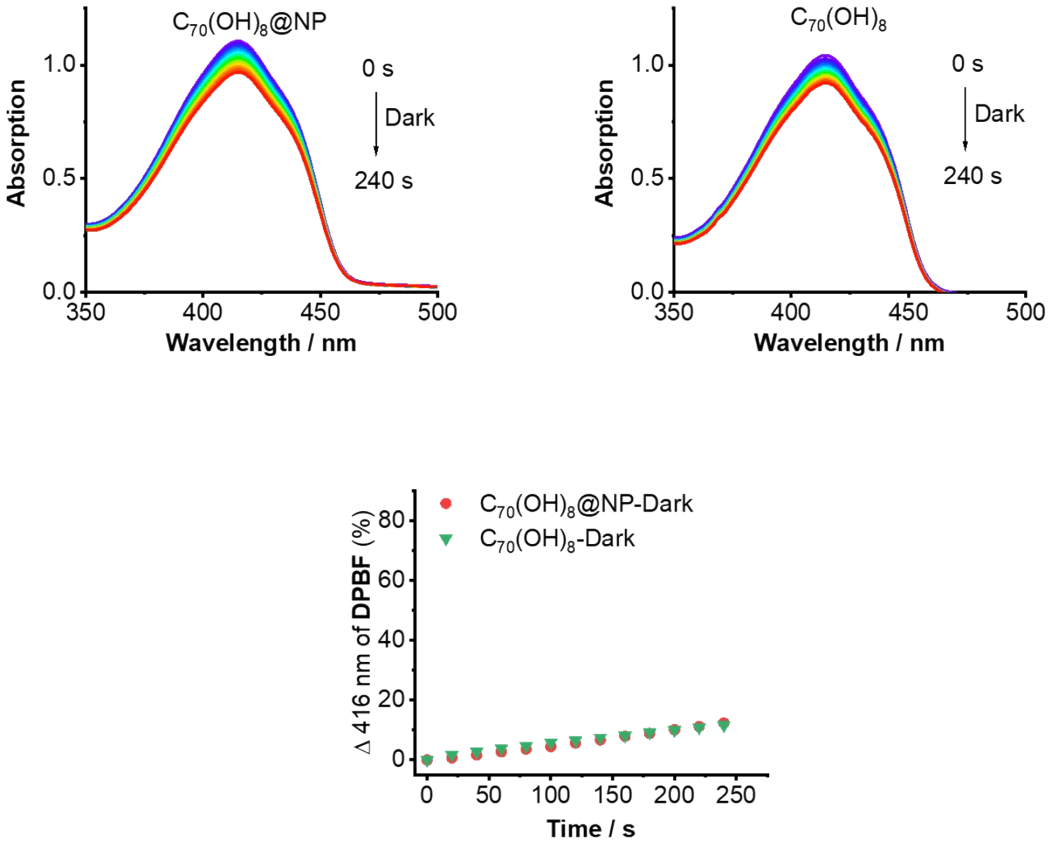

Fig. S2 UV–vis spectra of DPBF after oxidation by $C_{70}(OH)_8$@NP and $C_{70}(OH)_8$ under dark condition, and the absorption curve of DPBF at 416 nm was showed.

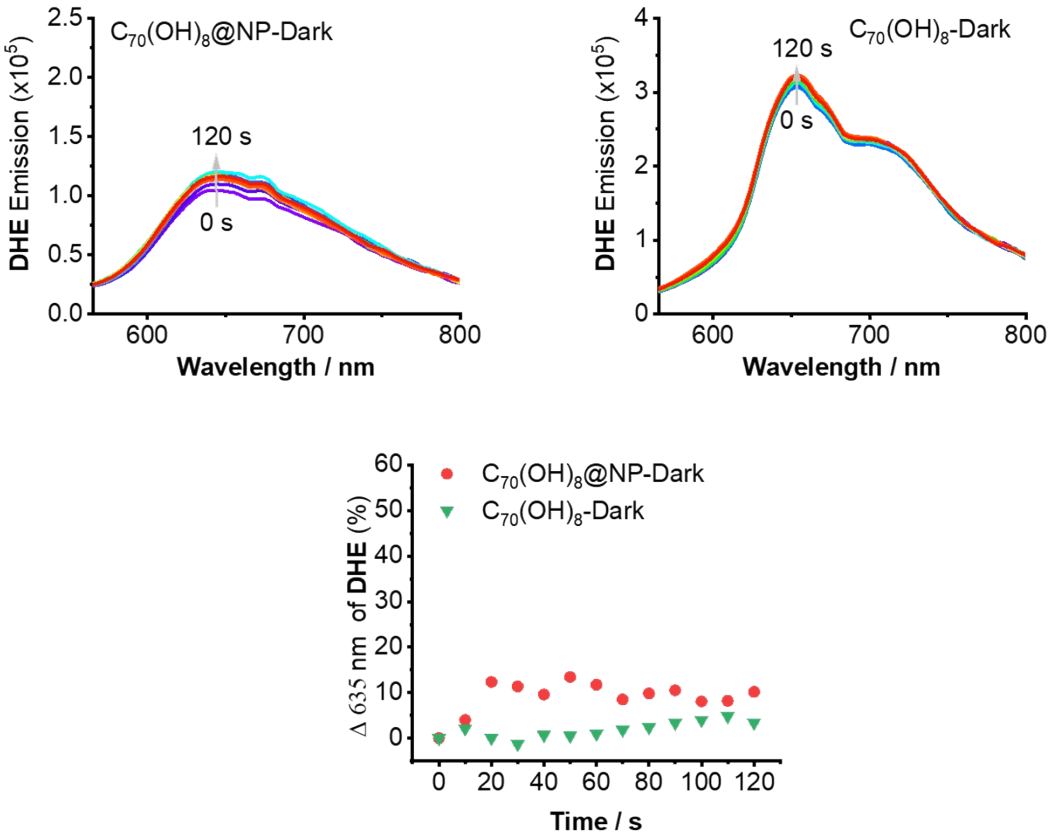

Fig. S3 Emission spectra of DHE after oxidation by $C_{70}(OH)_8$@NP and $C_{70}(OH)_8$ under dark condition and the emission curve of DHE at 635 nm are showed.

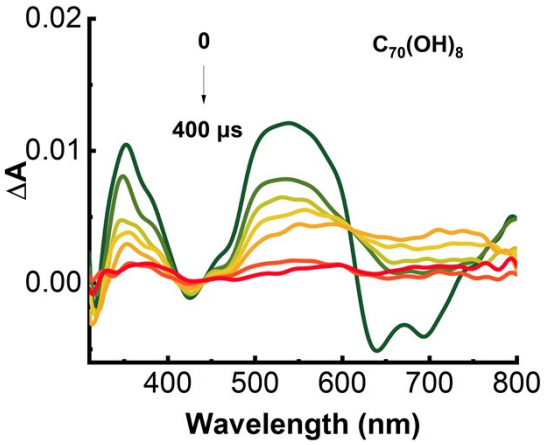

Fig. S4 Transient absorption difference spectra of $C_{70}(OH)_8$ measured at selected times in water (0 μs - 400 μs) under N_2 atmosphere.

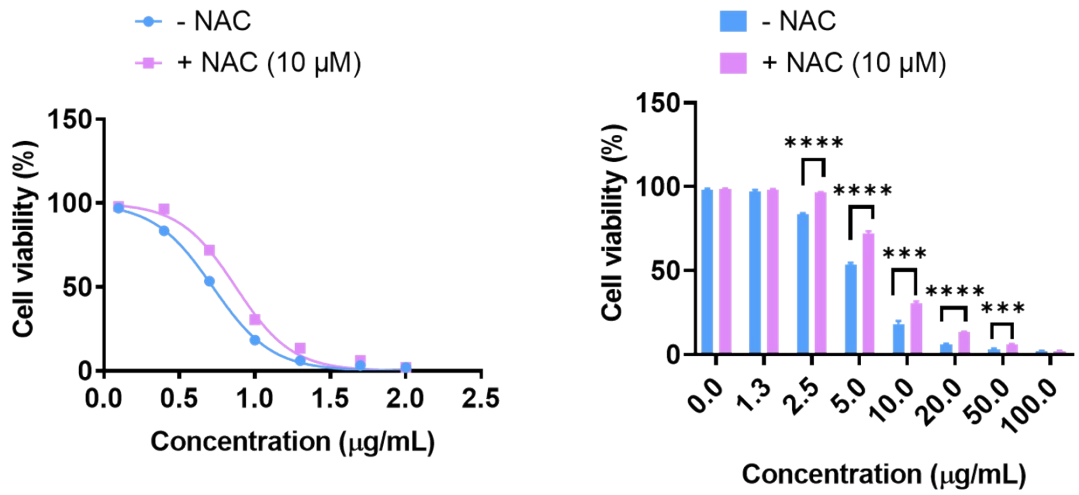

Fig. S5 The cytotoxicity of $C_{70}(OH)_8$@NP to HeLa cells under white light conditions (30 mW/cm^2, 10 min) with or without NAC (10 μM). (mean ± SD. ***p < 0.001, ****p < 0.0001)

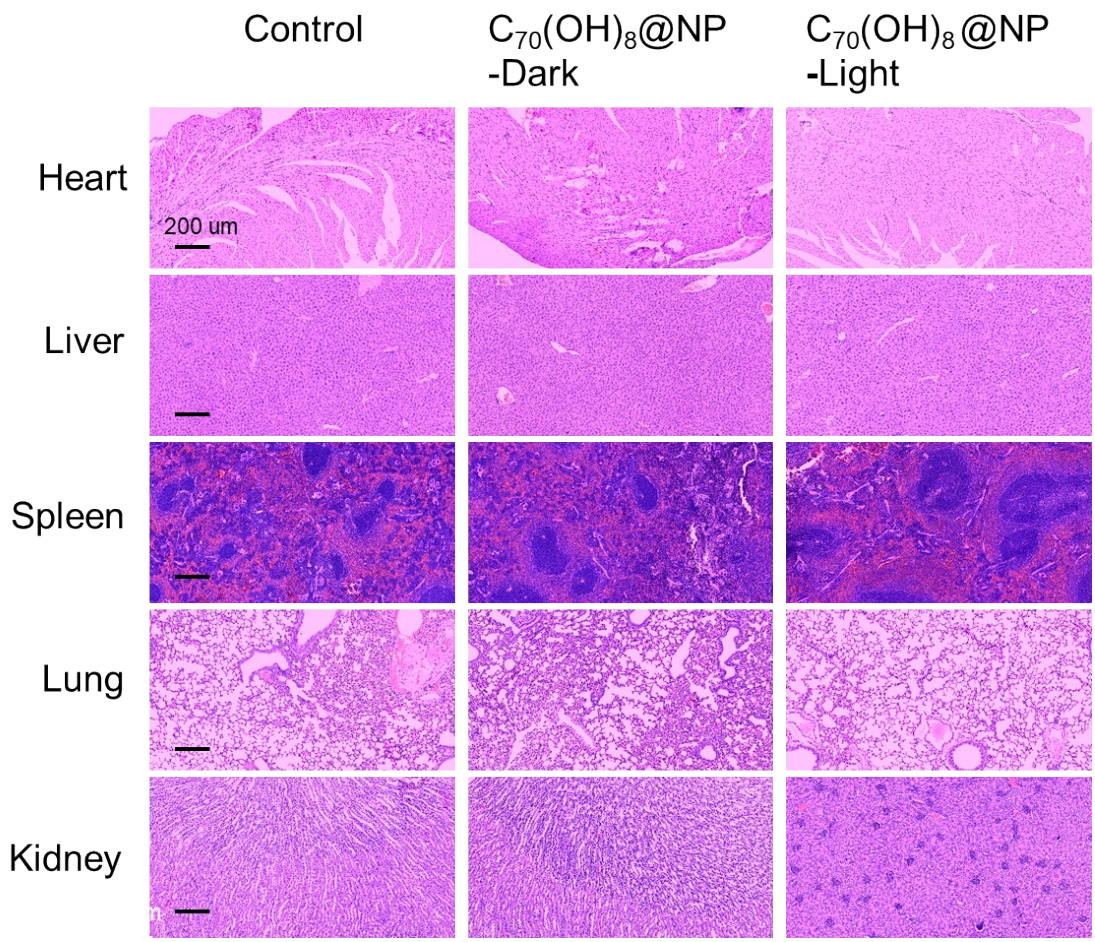

Fig. S6 Hematoxylin and Eosin (H&E) stained images of major organs from mice injected with different drugs (Control, $C_{70}(OH)_8$@NP-Dark, $C_{70}(OH)_8$@NP-Light) after 15 days. Scale bar: 200 μm.