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Supporting Information

for *Small*, DOI 10.1002/smll.202305440

Mechanoregulatory Cholesterol Oxidase-Functionalized Nanoscale Metal–Organic Framework Stimulates Pyroptosis and Reinvigorates T Cells

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Keywords: Cholesterol depletion, pyroptosis, tumor microenvironment remodeling, nanoscale metal-organic framework, immune checkpoint inhibitor

Materials

All starting chemicals including for the synthesis of Hf-TBP were purchased from Sigma-Aldrich (USA) or ThermoFisher (USA) and used directly without purification unless otherwise specified. Cholesterol oxidase was purchased from Milipore Corp (USA). CyQUANT™ lactate dehydrogenase (LDH) assay kit, primary caspase 1 antibody, ACK buffer, fixable viability dye eFluor 506, anti-CD16/32, protein quantitative reagent kit-BCA, Fluo 3-AM, IL-1 beta mouse uncoated ELISA kit and collagenase I, goat anti-rabbit lgG secondart antibody were purchased from ThermoFisher Scientific (USA). Cleaved Gasdermin D Rabbit mAb (GSDMD-N ASP276), beta-actin mouse mAb were purchased from Cell Signaling Technology (USA). Phosphate buffered saline (PBS) for cell culture, cell strainers (40 μm), RPMI-1640 medium and DMEM medium (4.5 g/L glucose) were purchased from Corning (USA). Trypsin-EDTA solution was purchased from Cytiva (USA). 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA), dead cell apoptosis kit with annexin V Alexa Fluor 488 & propidium iodide (PI) were purchased from Invitrogen. Fetal bovine serum (FBS) was purchased from VWR (USA). HyClone penicillin-streptomycin 100X solution was purchased from Cytiva (USA). Sodium pyruvate solution (100 mM stock), Hepes solution (1 M), and Pen/Strep 100X solution were purchased from Cytiva (USA). Filipin III solution, DNase I, was purchased from Sigma-Aldrich (USA). FAM-FLICA® caspase-1 (YVAD) Assay Kit was purchased from ImmunoChemistry Technologies. Flipper-TR, a live cell fluorescent membrane tension probe, and Acti-stain fluorescent phalloidins were purchased from Cytoskeleton, Inc. (USA). Anti-CRT Alexa Fluor 488 was purchased from NOVUS Biologicals (USA). CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) was purchased from Promega (USA). APC anti-mouse CD86 antibody, PE-Cyanine 7 anti-mouse CD80 antibody, PE-Dazzle CD 11c antibody, granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin-4 (IL-4) were purchased from PeproTech. IL-6， IFN-γ, and TNF-α ELISA kits were purchased from Invitrogen. DNase I was purchased from Roche (USA). CD45-AlexaFluor488 (30-F11), CD11b-SuperBright600 (M1/70), F4/80-PerCP/Cy5.5 (BM8), CD206-APC (MR6F3), CD11c-SuperBright436 (N418), MHCII-PE-Cy7 (M5/114.15.2), CD45-PacificBlue (30-F11), CD3Ɛ-SuperBright600 (145-2C11), CD4-AlexaFluor488 (GK1.5), CD8α-APC-eFluor780 (53-6.7), CD244.2-PE (m2B4 (B6)458.1), PD1-APC (RMP1-30), TIM3-PE (RMT3-23), LAG3-APC (C9B7W). CD244.2-PE, PD1-APC, TIM3-PE, and LAG3-APC were purchased from BioLegend. All other antibody conjugates were purchased from Thermal Fisher Scientific (USA). MC38 and 4T1 cells were obtained from Dr. Weichselbaum and Prof. Kron at the University of Chicago, respectively.

Methods

Transmission electron microscopy (TEM) was carried out on an FEI Spirit 120kV LaB6 Electron Microscope. Powder X-ray diffraction (PXRD) data were collected on a Bruker D8 Venture diffractometer using a Cu Kα radiation source (λ = 1.54178 Å) and processed with PowderX software. BioTek Synergy HTX microplate reader was used to detect the absorption or FL intensity of the 96-well plate. FT-IR spectra were collected by using Thermo NEXUS 670 Near-, Far-, and Mid-FTIR with attenuated total reflectance (ATR) accessory (for powder sample). UV-Vis spectra were collected using a Shimadzu UV-2600 UV-Vis spectrophotometer. Dynamic light scattering (DLS) and ζ-potential measurements were performed on a Malvern Zetasizer Nano ZS instrument. Inductively coupled plasma-mass spectrometry (ICP-MS) data were collected using an Agilent 7700x ICP-MS and analyzed using an ICP-MS Mass Hunter version 4.6 C.01.06. Samples were diluted in a 2% HNO_3 matrix and analyzed with ^{159}Tb as internal standards against a 10-point standard curve between 1 ppb and 500 ppb ($R>0.999$ for ^{178}Hf). Data collection was performed in Spectrum Mode with triplicates per sample and 100 sweeps per replicate. Flow cytometry data were collected on an LSR-Fortessa 4-15 (BD Biosciences, USA) at the Cytometry and Antibody Technology Facility at the University of Chicago and analyzed by FlowJo software (Tree Star, USA). Confocal laser scanning microscopy (CLSM) images and fluorescence-lifetime imaging microscopy (FLIM) were collected on a Leica Stellaris 8 laser scanning microscope at the Integrated Light Microscopy Core at the University of Chicago, and analysis was done with Image J software (NIH, USA). The wound healing experiment was recorded and analyzed by IncuCyte S3 (Essen BioScience) at Cellular Screening Center at the University of Chicago. Tissue sections were prepared with an ultramicrotome (Reichert/Leica Ultracut E, German). The histological slides were scanned on a CRi Pannoramic SCAN 40x whole slide scanner by Integrated Light Microscopy Core at the University of Chicago and analyzed with the QuPath-0.2.3 software.[1] The absorbance and fluorescence from well plates were read by a BioTek Synergy HTX microplate reader.

Synthesis of Hf-TBP

H_4TBP was synthesized according to the previous report.[2] Hf-TBP was prepared based on the previous report with slight modifications.[3] In a one-dram glass vial, 1 mL of 2 mg/mL $HfCl_4$ in DMF was mixed with 60 uL 88% formic acid and briefly sonicated. Afterward, 1 mL of 1.9 mg/mL H_4TBP in DMF was added to the vial and mixed well. The vial was then kept in a 125 ℃ oven for 2 days before the mixture was cooled to room temperature. Sponge-like purple precipitate and colorless supernatant should be observed at this time. The purple precipitates were collected by centrifugation at 14,000 g and washed by DMF, ethanol with 1% triethylamine, and ethanol sequentially to afford a final Hf-TBP suspension in ethanol. The Hf-TBP stock suspension was then characterized by DLS, PXRD, TEM, UV-Vis, and ICP-MS, and stored in dark at 4 ℃ until further use.

Synthesis of Hf-TBP/COD

1 mL of COD (30 unit mL^{-1}) aqueous solution was dropwise added to 10 mL of Hf-TBP aqueous solution (Hf = 0.5 mM) under vigorous stirring at room temperature. Six hours later, the precipitates were collected by centrifugation at 14,000 *g* and washed with water 3 times. The concentration of COD in the supernatant was detected by standard protein quantitative reagent kit BCA to calculate the loading efficiency of COD on the prepared Hf-TBP/COD. The loading efficiency was ~56.12%.

$$\text{Loading efficiency } \% = \text{unit(loaded COD)} \div \text{unit(added COD)} \times 100\%$$

ROS generation

The content of total ROS was detected by the DCFH assay. 0.5 mL DCFH-DA (1 mM) in DMSO was hydrolyzed by 2 mL NaOH (10 mM) aqueous solution in the dark for 30 minutes under violent stirring, and the hydrolytic process was stopped by adding 10 mL PBS (25 mM, pH 7.4) to afford DCFH stock as the detection probe of total ROS in test tube. Hf-TBP/COD (TBP = 5 μM) in PBS solution (pH 7.4, 10 mM) was added to the freshly prepared DCFH solution with a final DCF concentration of 10 μM. The PBS solution with the same DCFH concentration served as a blank control. 200 μL of each suspension was added to 96-well plates (n = 3), and the whole plate was illuminated by light (660 nm, 100 mW/cm^2) for different periods of time (0, 2, 5, 10, and 15 min). The fluorescence signal (Ex. 485/20 nm; Em. 520/20 nm) was then collected with an HTX microplate reader immediately after light irradiation.

COD release

1 mL of Hf-TBP/COD (COD: 5 unit mL^{-1}) was dissolved in PBS solution (pH 7.4, 10 mM) with or without the light illumination (660 nm, 100 mW/cm^2) for 15 min, and the solution was stirred at 37 ºC for 0, 24, and 48 h. The precipitates were collected by centrifugation at 14,000 g and washed with water 3 times. The concentration of COD in the supernatant was detected by the standard BCA method to calculate the release of COD from the prepared Hf-TBP/COD.

Cell culture

4T1 cells were cultured in RPMI-1640 medium (Corning, USA) supplemented with 10% fetal bovine serum (VWR, USA, filtered) and 1% HyClone penicillin-streptomycin 100X solution (Cytiva, USA). MC38 cells were cultured in DMEM medium (4.5 g/L glucose, Corning, USA) supplemented with 10% fetal bovine serum (VWR, USA, filtered), 1% HyClone penicillin-streptomycin 100X solution, 1% sodium pyruvate solution (100 mM stock), 1% Hepes solution (1 M), and 1% Pen/Strep 100X solution.

The spleens of C57BL/c mice were harvested to detect the toxicity of prepared nanoparticles to spleen cells. The spleens were harvested and digested by 600 μL of RPMI-1640 + 0.5 mg/mL collagenase I (Gibco) + 50 μg/mL DNase I (Sigma-Aldrich) cocktail at 37 °C for 30 minutes. The digests were neutralized with 6 mL RPMI-1640 medium with 10% FBS and gently ground and filtered through sterile cell strainers (40 μm, Corning) to obtain single cell suspensions (~10^7 cells/mL). The cell pellets were collected by centrifugation in 15 mL ep tubes at 300 g for 10 minutes at 4°C. After that, the red blood cells were then lysed with ACK buffer (ThermoFisher Scientific, 2 mL per sample), and the remaining cells were washed with FACS buffer (0.5% BSA in PBS). The cell pellets were collected by centrifugation with 300 g for 10 minutes at 4 °C. The supernatants were collected to detect the toxicity of COD and Hf-TBP/COD.

All cells were cultured in a humidified atmosphere containing 5% CO_2 at 37°C. Mycoplasma was tested on a regular basis by the MycoAlert detection kit (Lonza Nottingham, Ltd.).

Cell viability

The cytotoxicities of COD, Hf-TBP, and Hf-TBP/COD in 4T1 and MC38 cells were detected by MTS assay. 4T1 or MC38 cells were seeded in 96-well plates at a density of 2500 cells/well and incubated overnight. Different concentrations of COD, Hf-TBP, and Hf-TBP/COD were added to each well and incubated for 48 hours. 100 μL of 10% (v/v) of MTS reagent was added to each well. 60-120 minutes later, the absorbance of each well at 490 nm was read by a Synergy HTX plate reader to calculate cell viability.

The cytotoxicities of Hf-TBP and Hf-TBP/COD in spleen cells were also detected by MTS assay. 100 μL of spleen cells (10^4 cells/well) were seeded in 96 well plates containing different concentrations of COD and Hf-TBP/COD. After 24 hours, 10 μL of MTS reagent was added to each well. 60-120 minutes later, the absorbance of each well at 490 nm was read by a Synergy HTX plate reader to calculate cell viability.

The cytotoxicities of Hf-TBP and Hf-TBP/COD with light irradiation on 4T1 and MC38 cells were also detected through similar procedures as mentioned above. After treatment, cells were irradiated by light (660 nm, 100 mW/cm^2) for 15 min, and the cells were cultured for another 24 hours. The cell viability of cancer cells was detected by the same MTS assay as mentioned above.

Cellular uptake

4T1 cells were seeded in 6-well plates at a density of 2×10^5/well and cultured overnight. Hf-TBP and Hf-TBP/COD were added at an equivalent TBP (10 μM) concentration into the medium. The cells were incubated in a 37 ℃ incubator for 1, 2, 3, 4, and 8 hours. At each time point, the medium was aspirated, and the cells were washed with PBS three times and trypsinized to obtain cell suspensions. The cell pellets were collected by centrifugation (300 *g*, 4 min), and the signal of TBP was detected by flow cytometry (PE-Cy5 channel).

Depletion of cholesterol

4T1 cells were seeded in 6-well plates at a density of 2×10^5/well and cultured overnight. The cells were incubated with Hf-TBP, COD, and Hf-TBP/COD with different concentrations of COD (0, 0.125, 0.25, 0.5, 1 unit mL^{-1}) for another 24 hours. The medium was then aspirated, and the cells were washed with PBS three times, trypsinized to obtain cell suspensions, and collected by centrifugation (300 *g*, 4 min). The cells were fixed with 4% paraformaldehyde at room temperature for 20 minutes. Then, cells were washed with PBS 3 times and stained with Filipin III PBS solution (0.1 mg/mL) for 30 min at 4 °C. Then, the cells were washed with FACS buffer 3 times, and the signal of Filipin III was detected by flow cytometry (BV421 channel). The cholesterol ratio in 4T1 cells was calculated according to the following formula:

$$\text{Cholestrol \%} = [\text{Filipin III signal (S)} \div \text{Filipin III signal (PBS)}] \times 100\%$$

H_2O_2 generation

The generation of H_2O_2 during the biocatalytic process of COD was detected by flow cytometry with the DCFH-DA probe. 4T1 cells were seeded in 6-well plates at a density of 2×10^5/well and cultured overnight. Then, cells were stained with DCFH-DA in FBS-free medium solution (10 μM) for 30 min at 37 °C. The cells were then incubated with Hf-TBP, COD, and Hf-TBP/COD with different concentrations of COD (0, 0.125, 0.25, 0.5 unit mL^{-1}) for another 24 hours. After that, the medium was aspirated, and the cells were rinsed with PBS 3 times, trypsinized to obtain cell suspensions, and collected by centrifugation (300 *g*, 4 min). Then, the cells were suspended in FACS buffer solution, and the signal of ROS was detected by flow cytometry (FITC channel).

Cytoskeletal protein observation

Acti-stain fluorescent phalloidins were used to stain the F-actin in fixed cells. 4T1 cells were seeded in 6-well plates at a density of 2×10^5/well and cultured overnight. PBS, COD (0.06 unit mL^{-1}), or Hf-TBP/COD (COD: 0.06 unit mL^{-1}) was added to each well and incubated for 24 hours. 1×, 0.5×, 0.25× PBS solutions, or 1× ACK medium solutions were added to each well and incubated for 30 min. Cells were washed with PBS twice and fixed in 4% PFA for 20 min at RT. Next, the cells were permeabilized in a permeabilization buffer for 5 min at room temperature. The cells were then washed once with PBS at room temperature for 30 s and 200 µl of 100 nM Acti-stain 488 phalloidin PBS solution was added to the cells which was incubated at RT for 30 min. The

cell nuclei were stained by Hoechst (10 μg mL^{-1}) at RT for 10 min, and the calcium ion contents in the cells were observed by CLSM (Ex: 488 nm).

Evaluation of antioxidant properties

The antioxidant properties of cancer cells were detected by MTS assay. 4T1 cells were seeded in 96-well plates at a density of 2500 cells/well and incubated overnight. PBS, COD (0.06 unit mL^{-1}), or Hf-TBP/COD (COD: 0.06 unit mL^{-1}) was added to each well and incubated for 24 hours. Different concentrations of H_2O_2 medium solution were added to the wells and incubated for 45 min. 100 μL of 10% (v/v) of MTS reagent was added to each well. 60-120 minutes later, the absorbance of each well at 490 nm was read by a Synergy HTX plate reader to calculate cell viability.

Osmotic fragility evaluation

The osmotic fragility of the cancer cell membrane was evaluated by the rupture ratio of 4T1 cells stimulated by isotonic solution or ACK solution by using the LDH assay kit. 4T1 cells were seeded in 96-well plates at a density of 2500 cells/well and incubated overnight. PBS, COD (0.06 unit mL^{-1}), or Hf-TBP/COD (COD: 0.06 unit mL^{-1}) was added to each well and incubated for 24 hours. 1×, 0.5×, 0.25×, 0.125×, and 0.063× PBS solutions; 1×, 0.5×, 0.25×, 0.125× ACK medium solutions were added to the wells and incubated for 30 min. The cells were washed with PBS twice, and the LDH contents in the supernatant of 4T1 cells were detected by LDH assay kit. The cells incubated with lysis buffer for 45 min served as positive control (maximum LDH release and 100% rupture). The rupture ratio was calculated according to the following formula:

$$\text{Rupture } \% = [\text{Abs} \div \text{maximum Abs (10X Lysis Solution)}] \times 100\%$$

Resistance to hypotonic solutions

The resistance properties of cancer cells were detected by MTS assay. 4T1 cells were seeded in 96-well plates at a density of 2500 cells/well and incubated overnight. PBS, COD (0.06 unit mL^{-1}), or Hf-TBP/COD (COD: 0.06 unit mL^{-1}) was added to each well and incubated for 24 hours. 1×, 0.5×, 0.25×, 0.125×, and 0.063× PBS solutions were added to the wells and incubated for 30 min. 100 μL of 10% (v/v) of MTS reagent was added to each well. 60-120 minutes later, the absorbance of each well at 490 nm was read by a Synergy HTX plate reader to calculate cell viability.

Calcium ion influx

The calcium ion influx was detected by Fluo calcium indicator. 4T1 cells were seeded in 6-well plates at a density of 2 × 10^5/well and cultured overnight. Hf-TBP, COD, or Hf-TBP/COD was added at an equivalent TBP concentration of 10 μM or an equivalent COD concentration of 0.11 unit mL^{-1}, and further incubated in a 37 ºC incubator for 24 hours. Then, the cells were irradiated with light (660 nm, 100 mW/cm^2, 15 min). Six hours later, the cells were stained by Fluo-3, AM (5 μM) in PBS without calcium ions solution at 37 ºC for 30 min, and the cells were rinsed by PBS without calcium ions solution for 3 more times. The cell nuclei were stained by Hoechst (10 μg mL^{-1}) at RT for 10 min, and the contents of calcium ions in 4T1 cells were observed by CLSM (Ex: 488 nm). To detect the calcium ion influx stimulated by hypotonic solution, 4T1 cells were seeded in 6-well plates at a density of 2 × 10^5/well and cultured overnight. The cells were pre-incubated with 1 mL of COD or Hf-TBP/COD at an equivalent concentration of COD of 0.11 unit mL^{-1} at 37 ºC for 12 h. Then, 1 × PBS and 0.5 × PBS solutions were added to the cells for 30 additional min. The cells were stained by Fluo-3, AM (5 μM) in calcium-free PBS solution at 37 ºC for 30 min, and the cells were rinsed with PBS without calcium ions solution for 3 more times. Cell nuclei were stained by Hoechst (10 μg mL^{-1}) at RT for 10 min, and the contents of calcium ions in 4T1 cells were observed by CLSM (Ex: 488 nm).

Membrane tension measurement

The cell membrane tension was detected by Flipper-TR Kit using FLIM. 4T1 cells were seeded in 6-well plates at a density of 2 × 10^5/well and cultured overnight. PBS, Hf-TBP, COD, or Hf-TBP/COD was added at an equivalent TBP concentration of 20 μM or an equivalent COD concentration of 0.22 unit mL^{-1}. 3.75 h later, the cells were incubated with 0.5×PBS solution for 15 min. Then, the medium was replaced with the same medium containing 2 μM of Flipper-TR probe and incubated for 30 min.

Next, FLIM imaging was performed on a FLIM system equipped with a time-correlated single-photon counting module. Excitation was performed using a pulsed 488 nm laser, and the emission signal was collected through a 600/50-nm bandpass filter. FLIM software (PicoQuant) was used to generate the conventional color-coded average lifetime maps and fit the fluorescence decay data (from full images) to a dual exponential model using the fitting tool.

ROS generation upon irradiation of light

4T1 cells were seeded in the cell culture dishes at a density of 1.5×10^5 and cultured overnight. Hf-TBP, COD, or Hf-TBP/COD was added at an equivalent TBP concentration of 10 μM or an equivalent COD concentration of 0.11 unit mL^{-1}, and further incubated in a 37℃ incubator for 24 hours. The cells were washed with PBS 3 times, and 1 mL FBS-free culture medium containing 30 μM DCFH-DA was added and incubated for another 30 min. Then the cells were irradiated with light (660 nm, 100 mW/cm^2, 15 min), washed with PBS 3 times, collected by trypsinization and centrifugation, and resuspended in FACS buffer for flow cytometry (FITC channel). The cells without light irradiation served as control.

Caspase-1 expression

The activation of caspase-1 was detected by the FAM-FLICA® caspase-1 (YVAD) assay kit following the manufacturer's protocol. Caspase 1 FLICA was reconstituted with 50 μL DMSO, and 200 μL PBS was then added to dilute the FLICA solution. 4T1 cells were seeded in cell culture dishes at a density of 1.5×10^5 and cultured overnight. Hf-TBP, COD, or Hf-TBP/COD was added at an equivalent TBP concentration of 10 μM or equivalent COD concentration of 0.11 unit mL^{-1}, and further incubated in a 37 ℃ incubator for 24 hours. The cells were then irradiated with light (660 nm, 100 mW/cm^2) for 15 min and kept in the incubator for another 12 h. The cells were then washed with the washing buffer in the assay kit 3 times, and 300 μL staining medium (added 10 μL of diluted FLICA to 290 μL of RPMI 1640 medium solution) was added to the culture dishes and cultured for 1 hour at 37°C. The cells were rinsed by PBS 3 times and trypsinized to obtain cell suspensions. Then, the cells were washed with PBS 3 times, and the fluorescence signal was measured by flow cytometry (FITC channel).

LDH release

The release of LDH was detected by CyQUANT™ LDH assay kit. 4T1 cells were seeded into the 96-well plates (2.5×10^3 cells per well) for 24 h, and 100 μL of Hf-TBP and Hf-TBP/COD with different concentrations of TBP solutions (0, 1.56, 3.13, 6.25, 12.5 and 25 μM) were added to each well and incubated for 24 h. 10 μL of 10X lysis buffer was added to the control group and the cells were cultured in an incubator at 37°C for 45 minutes (maximum Abs and 100% LDH release). The cells were then irradiated with light (660 nm, 100 mW/cm^2) for 15 min and cultured for another 12 h. Then, 50 μL of the reaction mixture was added to each sample well, then mixed by gentle tapping, and the mixture was kept at room temperature for 30 min at dark. At last, 50 μL of stop solution was added to each sample well and mixed by gentle tapping. The absorbance at 490 nm and 680 nm of each well was measured within 2 hours. The background signals were subtracted as specified by the manufacturer's protocol (subtract the 680-nm absorbance value (background signal from instrument) from the 490-nm absorbance value). The LDH release ratio was calculated according to the following formula:

$$\text{LDH release } \% = [\text{Abs} \div \text{maximum Abs (10X Lysis Solution)}] \times 100\%$$

Observation of cells morphology

Morphology changes of 4T1 cells were monitored by taking bright-field images. 4T1 cells were seeded into cell culture dishes overnight (1.0×10^5 cells per dish). The cells were then incubated with Hf-TBP, COD, and Hf-TBP/COD at an equivalent concentration of TBP of 20 μM for 24 hours. The cells were irradiated with light (660 nm, 100 mW/cm^2) for 15 min and cultured for another 12 h. The bright-field images of cancer cells were captured to show the morphology changes. To observe a real-time morphology change, we also performed an in situ PDT with the laser embedded in CLSM. The laser at 660 nm from Leica Stellaris 8 confocal microscope was used as an *in situ* excitation source for Hf-TBP, and the laser power was calibrated to 100 mW/cm^2 by a photometer. The cells were imaged in the sequence mode with continuous 660 nm laser irradiation at room temperature.

A time-lapse video was generated using the bright-field images to show the morphology changes. 4T1 cells were seeded into cell culture dishes overnight (1.0×10^5 cells per dish), and the cells were incubated with Hf-TBP, COD, and Hf-TBP/COD at an equivalent concentration of TBP of 20 μM for 24 hours. The cells were washed with PBS three times and stained with Calcein-AM (5 μM, green) in RPMI-1640 medium for 60 min, and then washed with PBS once and cultured in RPMI-1640 medium containing 10% FBS for 15 min. The cells were washed with PBS three times and cultured in PI (2 μg mL^{-1}, red) containing RPMI-1640 medium. Then, the laser (630 nm) of CLSM was used to irradiate the cells, and a time-lapse video was generated to record cell morphology changes. As the membrane of cancer cell was destroyed, the green fluorescence from Calcein-AM disappeared from the cytoplasm while PI entered and stained the nuclei with red color.

GSDMD cleavage

4T1 cells were seeded in 6-well plates at a density of 2×10^5/well and cultured overnight. The cells were treated with 2 mL of Hf-TBP (TBP: 10 μM), COD (0.11 unit mL^{-1}), and Hf-TBP/COD (TBP: 10 μM; COD: 0.11 unit mL^{-1}) and cultured for 24 h. The cells were then irradiated with light (660 nm, 100 mW/cm^2) for 15 min. Then, the cells were rinsed with PBS 3 times and trypsinized to obtain cell suspensions, collected by centrifugation (300 *g*, 4 min). The cells were washed with cold PBS twice and subjected to RIPA lysis buffer on ice. After grinding, the cell lysates were collected and centrifuged (14000 g, 15 min) at 4 °C. The protein in the supernatants was separated on SDS-PAGE and transferred to a PVDF membrane. The membrane was blocked with TBST buffer containing 5% non-fat powdered milk for 1 h. Thereafter, the membrane was incubated with diluted primary antibodies to GSDMD-N (1:1000) and Beta-actin (1:1000) at 4 °C overnight. After extensive washing with TBST buffer, the membrane was incubated with horseradish peroxidase-coupled secondary antibody (goat anti-rabbit IgG, 1:2000) and (anti-mouse IgG, 1:2000) at RT for 1 h. After washing away the secondary antibodies, the membrane was incubated with Pierce ECL Plus Western Blotting Substrate (Thermo Fisher Scientific, USA) and visualized using a FluorChem R system (ProteinSimple, USA).

IL-1β secretion

The secretion of IL-1β was detected by IL-1β ELISA kit. 4T1 cells were seeded in 6-well plates at a density of 1.5×10^5 cells per well with 2 mL medium and cultured overnight. The cells were treated with 0.8 mL of Hf-TBP (TBP: 10 μM), COD (0.11 unit mL^{-1}), and Hf-TBP/COD (TBP: 10 μM; COD: 0.11 unit mL^{-1}) and cultured for 24 additional hours. The cells were then irradiated with light (660 nm, 100 mW/cm^2) for 15 min. The cells without treatment served as control. The supernatant of the cells was collected, and the concentration of IL-1β was detected by the ELISA kit.

CRT expression

The surface translocation of CRT was evaluated via flow cytometry after different treatments. 4T1 cells were seeded in 6-well plates at a density of 1.5×10^5 cells per well with 2 mL medium and cultured overnight. The cells were treated with 2 mL of Hf-TBP (TBP: 10 μM), COD (0.11 unit mL^{-1}), and Hf-TBP/COD (TBP: 10 μM; COD: 0.11 unit mL^{-1}) and cultured for another 24 h. The cells were then irradiated by light (660 nm, 100 mW/cm^2) for 15 min. Then, the cells were rinsed by PBS 3 times and trypsinized to obtain cell suspensions. The cells were stained with anti-CRT Alexa Fluor 488 (1:150 dilution in FACS buffer) on ice at dark for 30 minutes, washed with PBS once, and resuspended in FACS buffer for flow cytometry analysis (FITC channel). The cells without treatment served as control.

Wound healing experiments

The wound healing assay was performed to evaluate the invasion and migration ability of 4T1 cells after COD, Hf-TBP, Hf-TBP/COD, PBS(+), COD(+), Hf-TBP(+), and Hf-TBP/COD(+) treatments. The cells were first seeded on an Incucyte Imagelock 96-well plate (Sartorius) at a density of 5×10^5 cells/mL with 100 μL medium per well and further cultured overnight. The wound was first created with an Incucyte 96-well wound maker tool. The cells were washed with PBS twice, and COD, Hf-TBP or Hf-TBP/COD was added to the wells at an equivalent TBP concentration of 10 μM and COD of 37.3 μM. The cells were incubated for 4 additonal hours (n = 3) followed by light irradiation (100 mW/cm^2, 15 minutes, 90 J/cm^2 as total dose). Then, the cells were put into IncuCyte S3 for live imaging for up to 24 hours and analyzed with a scratch wound analysis module.

Phosphatidylserine expression

4T1 cells were seeded in 6-well plates at a density of 1.5×10^5/well and cultured overnight. Hf-TBP, COD, or Hf-TBP/COD was added at an equivalent TBP concentration of 6.25 μM for 24 hours and irradiated with light (660 nm, 100 mW/cm^2) for 15 min. 12 hours later, and the cells were washed with PBS, trypsinized to afford single cell suspensions, and stained with annexin V Alexa Fluor 488 & PI kit following the manufacturer's protocol. The cells were then resuspended in the binding buffer for flow cytometric analysis (Annexin-V in the FITC channel, PI in the PE-dazzle 594 channel).

***In vitro* stimulation of DCs**

8-week-old female C57BL/c mice were used to isolate the bone marrow-derived immature DCs (BMDCs). Specifically, the bone marrow was obtained by flushing the femur and tibia with PBS. After the lysis of red blood cells with ACK buffer, the bone marrow cells were seeded in culture dishes with complete RPMI 1640 containing an additional 20 ng mL^{-1} of granulocyte-macrophage colony-stimulating factor (GM-CSF) and 10 ng mL^{-1} of interleukin-4 (IL-4). Fresh medium was added to the culture dishes every two days. On the 6th day, the non-adherent and loosely adherent cells were collected as BMDC for experiments. 3×10^6 non-adherent and loosely adherent cells in 1 mL medium were seeded in 6-well and co-incubated with 0.5 mL of the residual 4T1 cancer cells after different treatments (PBS, Hf-TBP/COD, COD(+), Hf-TBP(+), and Hf-TBP/COD(+)) were added for 24 hours. DCs were then obtained via centrifugation at 300 *g*, blocked by anti-CD16/32 antibodies (1:100) at 4ºC for 20 min, and then stained with APC anti-mouse CD86 antibody (1:100), PE-Cyanine 7 anti-mouse CD80 Antibody (1:50) and PE-Dazzle CD 11c (1:100) in 100 μL PBS containing 1% BSA for 30 min at room temperature. The cells were then rinsed with FACS buffer and analyzed by flow cytometry.

Anti-tumor efficacy

BALB/c mice (6-8 weeks) were obtained from Charles River Laboratories, Inc (USA) and bred in-house at the animal facility at the University of Chicago. The study protocol (ACUP72408) was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Chicago. To evaluate the *in vivo* therapeutic efficacy, subcutaneous 4T1 tumor models were established in BALB/c mice by inoculating 2×10^6 cells/mouse subcutaneously onto the right flanks at day 0. When 4T1 tumors reached 110~130 mm^3, the mice were randomized for irradiation treatment. PBS, COD, Hf-TBP, or Hf-TBP/COD was intratumorally injected with an equivalent TBP dose of 0.2 μmol and COD dose of ~2.24 units in 20 μL PBS. 24 hours later, and the mice were anesthetized with 2.5% (v/v) isoflurane/O_2 and the 4T1 tumors were irradiated with light (660 nm, 100 mW/cm^2) for 15 min. The therapeutic effect of the combination treatment was also evaluated according to the same method. Hf-TBP and COD were intratumorally injected into 4T1 tumors sequentially with an TBP dose of 0.2 μmol and COD dose of ~2.24 units. 24 hours later, and the mice were anesthetized with 2.5% (v/v) isoflurane/O_2 and the 4T1 tumors were irradiated with light (660 nm, 100 mW/cm^2) for 15 min. Subcutaneous MC38 tumor models were established in C57BL/6 mice by inoculating 2×10^6 MC38 cells/mouse subcutaneously onto the right flanks. MC38 tumors were also treated according to the same methods as mentioned above when MC38 tumors reached 100~120 mm^3.

The length and width of tumor tissues were measured with an electronic caliper (tumor volume = length × width^2/2), and body weights were monitored with an electronic scale. At the endpoint of the experiments, the mice were euthanized, and the tumors and major organs were sectioned for hematoxylin-eosin (H&E) staining to evaluate general toxicity. The tumor growth inhibition index (TGI) was defined as the equation below:

$$TGI = (1 - \frac{Me}{Mc}) \times 100\%$$

where Me and M_C represent average tumor weights of treated mice and average tumor weights in the control group at endpoints, respectively.

Synergistic effects

To determine whether there is a synergistic effect existing in the combination of Hf-TBP mediated PDT and cholesterol depletion, the percent tumor volume (relative to PBS control) after the combination treatment (Hf-TBP/COD(+)) was compared with a purely additive result from Hf-TBP/COD(-) and Hf-TBP(+). The additive index was calculated by the equation:

$$f_{\text{additive}} = f_{\text{Hf-TBP(+)}} \times f_{\text{Hf-TBP/COD(-)}}$$

$f_{\mathrm{Hf-TBP(+)}}$ and $f_{\mathrm{Hf-TBP/COD(-)}}$ are the percent tumor volumes in the Hf-TBP(+) and Hf-TBP/COD(-) group. $f_{combination}$ is the percent tumor volume in Hf-TBP/COD(+) group. If $f_{\mathrm{additive}} > f_{combination}$, there is a synergistic effect; If $f_{\mathrm{additive}} = f_{combination}$, there is an additive effect; If $f_{\mathrm{additive}} < f_{combination}$, there is an antagonistic effect.[4]

Immune profiling and cytokine quantification

MC38 tumor-bearing C57BL/6 mice (n = 5) were treated with PBS, Hf-TBP/COD, Hf-TBP (+), COD (+), and Hf-TBP/COD(+), and the tumors were harvested at day 14 for immune cell profiling by flow cytometry. The tumors were weighed and digested with 600 μL of RPMI-1640 + 0.5 mg/mL collagenase I (Gibco) + 50 μg/mL DNase I (Sigma-Aldrich) cocktail at 37 °C for 45 minutes. The digests were neutralized with 6 mL RPMI-1640 medium with 10% FBS and gently ground and filtered through sterile cell strainers (40 μm, Corning) to obtain single cell suspensions (~10^7 cells/mL). The cell pellets were collected by centrifugation in 15 mL ep tubes at 300 g for 10 minutes at 4°C, while the supernatants were collected for cytokine testing by IL-6, IFN-γ, and TNF-α ELISA kits. The concentration of IL-6 or TNF-α in the tumor tissue normalized based on the tumor mass. The red blood cells were then lysed with ACK buffer (2 mL per sample), and the remaining cells were washed with FACS buffer, transferred to a round-bottom 96-well plate, and stained first with Fixable Viability Dye eFluor 506 (ThermoFisher Scientific, 1:1000 in FACS buffer). The cells were then washed with FACS buffer, blocked by anti-CD16/32 antibody (ThermoFisher Scientific, clone 93, 1:100) at 4 °C for 15 minutes, and stained with the fluorochrome-conjugated rat anti-mouse antibodies 1:150 at 4 °C for 45 minutes. The antibodies, conjugated dyes, and clone numbers were listed as follows: CD45-AlexaFluor488 (30-F11), CD11b-SuperBright600 (M1/70), F4/80-PerCP/Cy5.5 (BM8), CD11c-SuperBright436 (N418), MHCII-PE-Cy7 (M5/114.15.2), CD45-PacificBlue (30-F11), CD3Ɛ-SuperBright600 (145-2C11), CD4-AlexaFluor488 (GK1.5), CD8α-APC-eFluor780 (53-6.7), CD244.2-PE (m2B4 (B6)458.1), PD1-APC (RMP1-30), TIM3-PE (RMT3-23), LAG3-APC (C9B7W). All other antibody conjugates were purchased from ThermoFisher Scientific. The cells were finally washed and resuspended in FACS buffer and analyzed on an LSR Fortessa 4-15 flow cytometer.

Depletion of cholesterol in tumor tissues

4T1 tumors after different treatments were harvested to make single-cell suspensions according to the same method mentioned above. The cells were fixed with 4% paraformaldehyde at room temperature for 20 minutes. Then, the cells were washed with PBS 3 times and stained with Filipin III PBS solution (0.1 mg/mL) for 30 min at 4°C. The signal of cholesterol was detected by flow cytometry (BV421 channel).

Hemolysis assay

The red blood cells (RBCs) were collected from BALB/c mice for hemolysis assay. 0.5 mL of blood was diluted with 15 mL of PBS, and RBCs were separated from the serum via centrifugation at 1,200 rpm for 10 min. After washing six times, the RBC suspension was diluted with 15 mL of PBS. 1 mL of RBCs suspension was centrifuged and added with 1 mL of PBS (negative control), deionized water (positive control), or PBS containing Hf-TBP/COD (TBP: 0, 6.25, 12.5, 25, 50, 100, and 200 μM). After 2-hour incubation at 37 °C, the mixture was centrifuged at 14,500 rpm for 10 min, and 100 μL of supernatant was transferred to a 96-well plate. The absorbance at 540 nm of supernatant was detected by the HTX microplate reader. The hemolysis ratios were calculated based on the equation: Hemolysis (%) = (sample absorbance − negative control absorbance)/(positive control absorbance − negative control absorbance) × 100.

Immunohistochemistry analysis

A group of subcutaneous 4T1-bearing mice were established and treated in the same way as described above but euthanized on the day of the endpoint. The tumors were excised and fixed in 4% PFA for 48 h and 70% ethanol for 1 day. The tissues were embedded in paraffin, sectioned, and stained for H&E and TUNEL by the Human Tissue Resource Center at the University of Chicago. The slides were scanned on a CRi Pannoramic SCAN 40× whole slide scanner by Integrated Light Microscopy Core at the University of Chicago. The expressions of CD3 and caspase-1 in the tumor tissues were also measured by CLSM.

Statistical analysis

All the statistical analysis was performed on Origin Lab software. The experimental results were presented with average values, and expressed as the mean ± SD. P value was calculated with Microsoft Excel software by student's two-tailed t-test, *, $p<0.05$, **, $p<0.01$, ***, $p<0.001$, and ****, $p<0.0001$ were used in all figures to show the statistical significance.

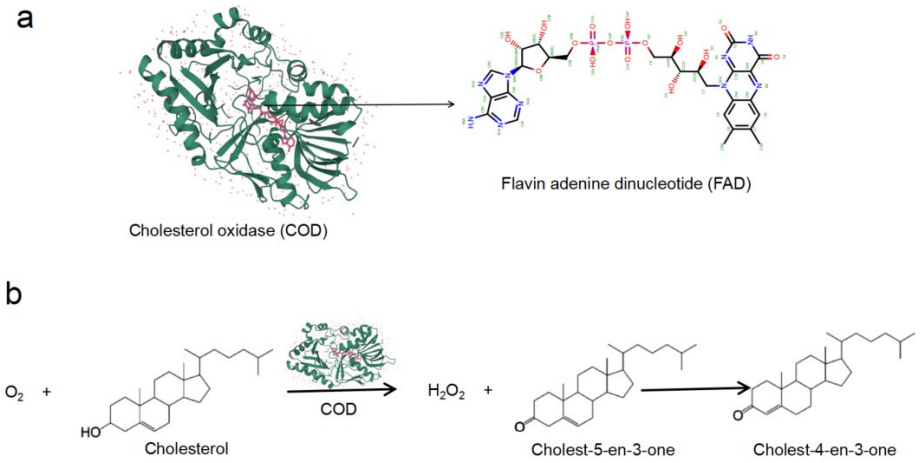

Figure S1. (a) Structure of COD. (b) Biocatalytic process of cholesterol oxidase. The enzyme COD, containing flavin adenine dinucleotide (FAD), can catalyze the conversion of cholesterol and oxygen into cholest-5-en-3-one, which is then isomerized into cholest-4-en-3-one (as shown in Figure S1b). Upon binding to the surface of the lipid bilayer, COD undergoes a conformational change and forms a lid with a length of approximately 20 Å in the active site. [5] This lid acts as a hydrophobic channel that can extract cholesterol from the membrane bilayers and transport it to the enzyme's active site while the lid's outer surface remains hydrophilic.[6]

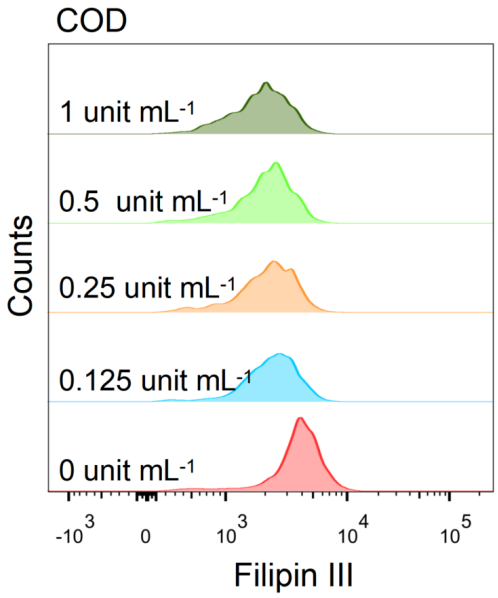

Figure S2. Depletion of cholesterol in 4T1 cells after incubation with COD at different concentrations.

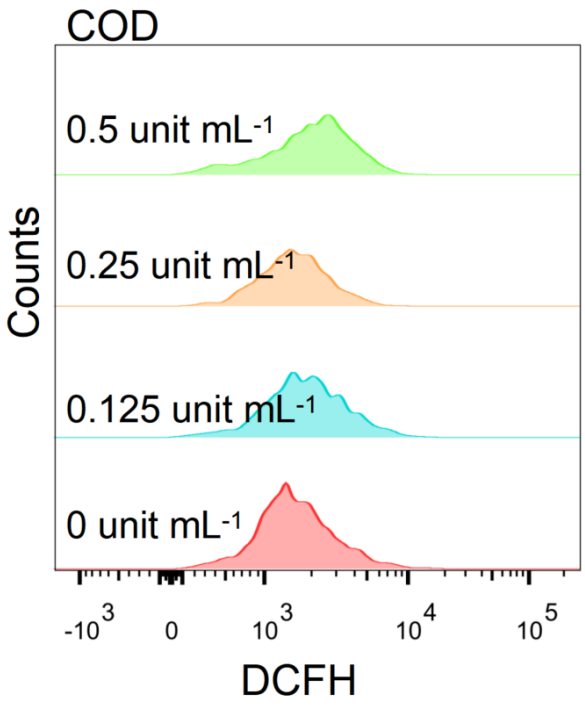

Figure S3. Generation of ROS in 4T1 cells after incubation with COD at different concentrations.

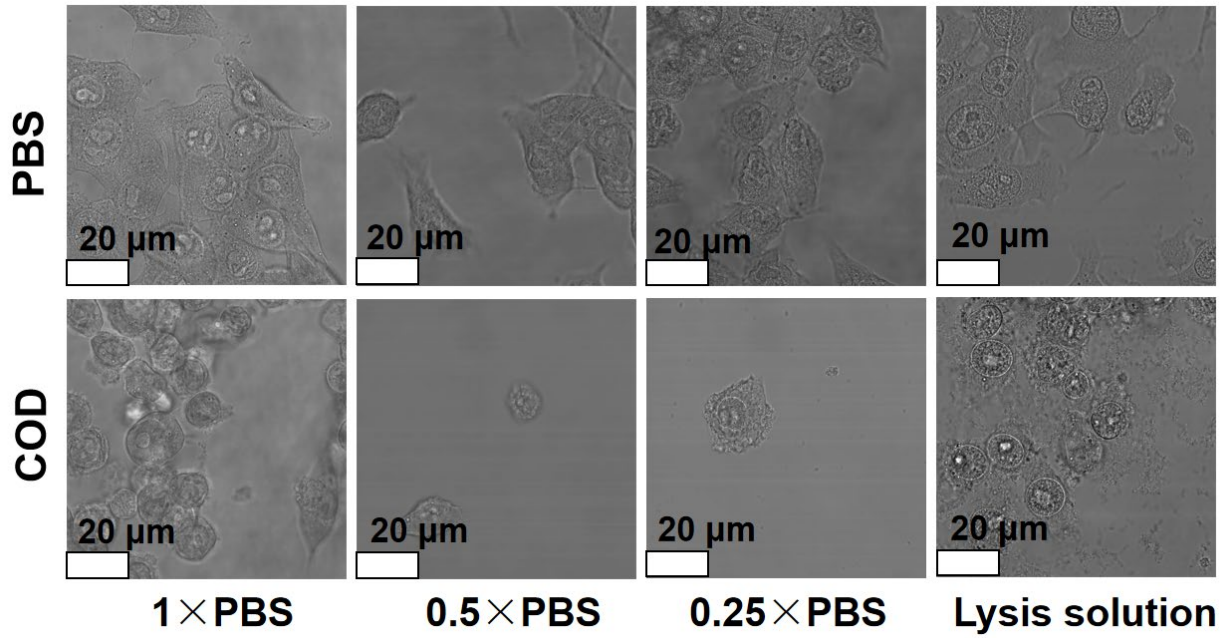

Figure S4. Morphology changes of PBS- and COD-treated 4T1 cells (0.06 unit mL^{-1}, for 12 h) after incubation with hypotonic solutions (0.5×, 0.25×PBS) for 30 min or lysis solution for 1 min.

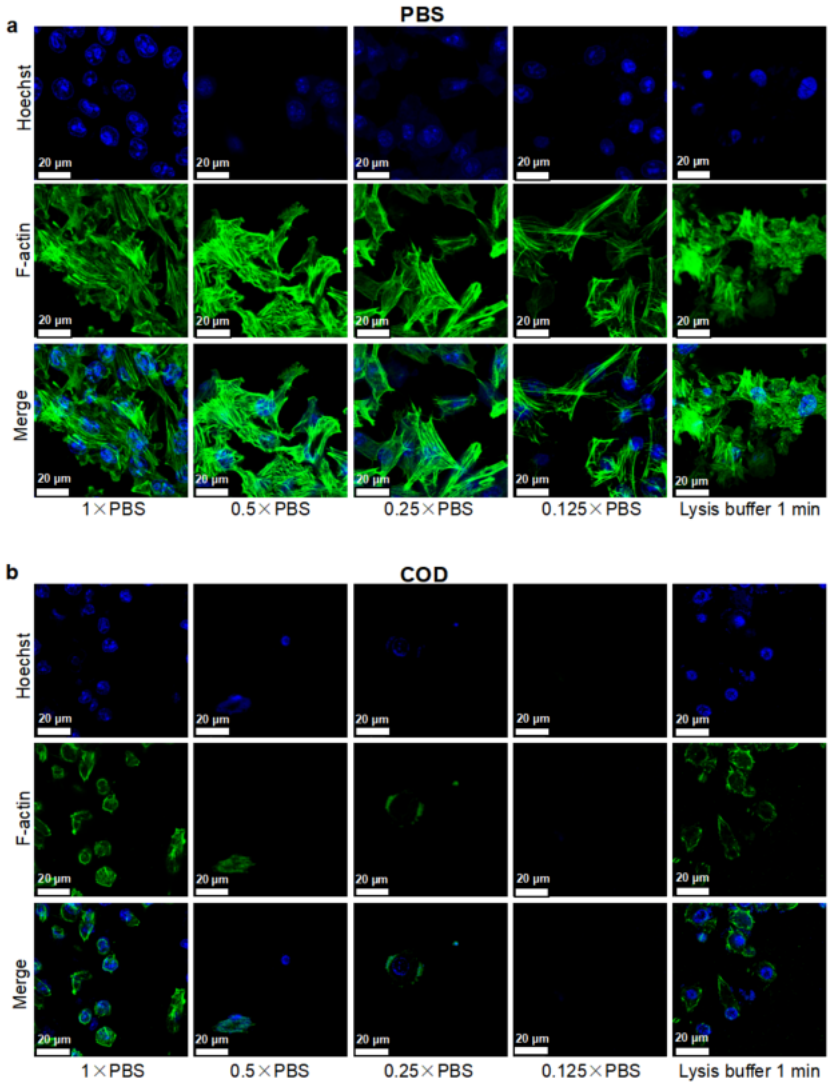

Figure S5. (a) F-actin cytoskeleton (green) in 4T1 cells incubated with hypotonic solutions (0.5 ×, 0.25 ×, and 0.125 × PBS) for 30 min or lysis solution for 1 min. (b) F-actin cytoskeleton (green) in 4T1 cells pre-incubated with COD (0.06 unit mL^{-1}) for 12 h and further incubated with hypotonic solutions (0.5 ×, 0.25 ×, and 0.125 × PBS) for 30 min or lysis solution for 1 min.

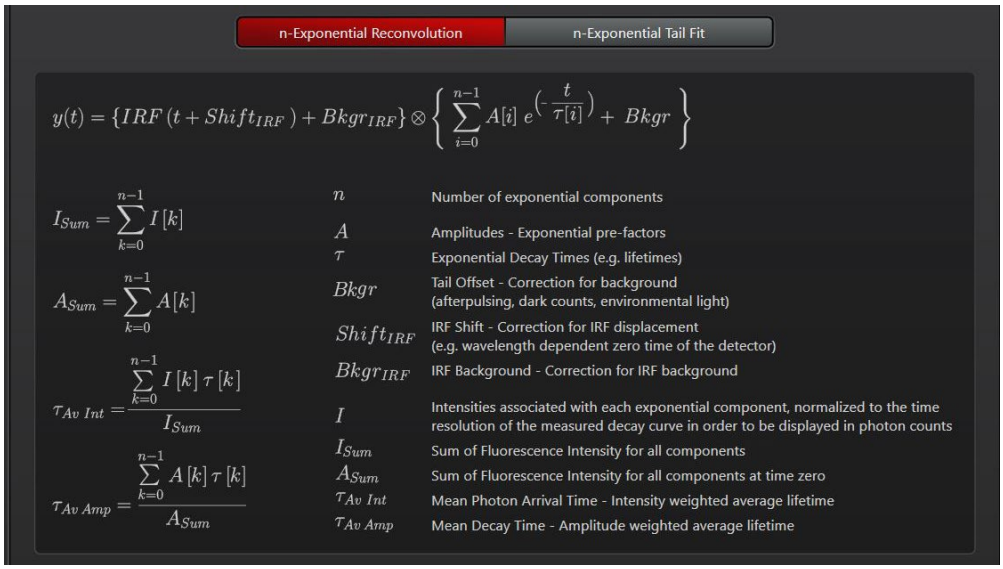

Figure S6. Selected fit model for determining the variation of the lifetime of Flipper-TR for the evaluation of plasma membrane mechanical tensions after different treatments.

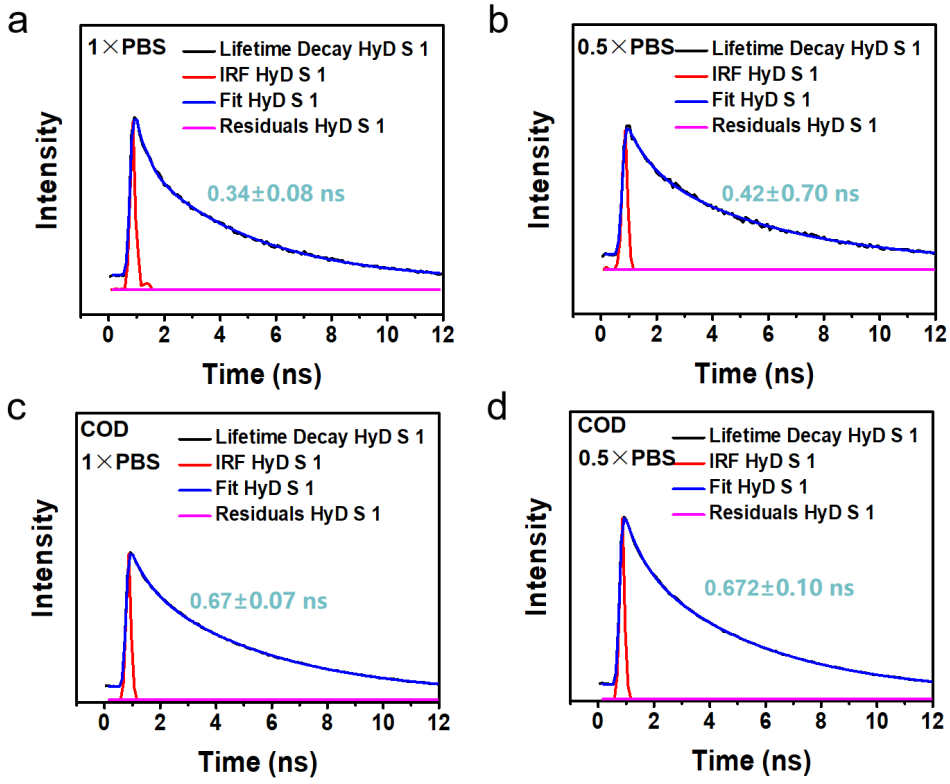

Figure S7. Fitted τ_1 lifetime curves of Flipper-TR in 4T1 cells after PBS (a), 0.5×PBS (b), COD (c), and COD + 0.5×PBS treatments (d).

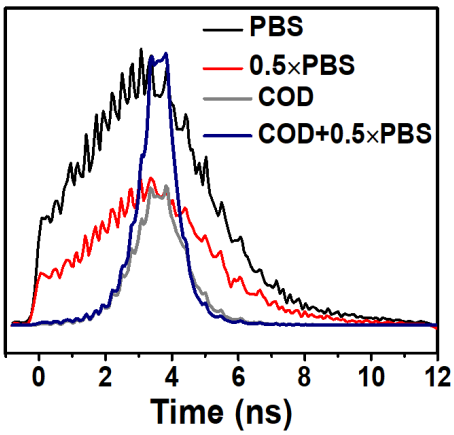

Figure S8. Fitted τ_2 lifetime curves of Flipper-TR in 4T1 cells after PBS, 0.5×PBS, COD, and COD + 0.5×PBS treatments.

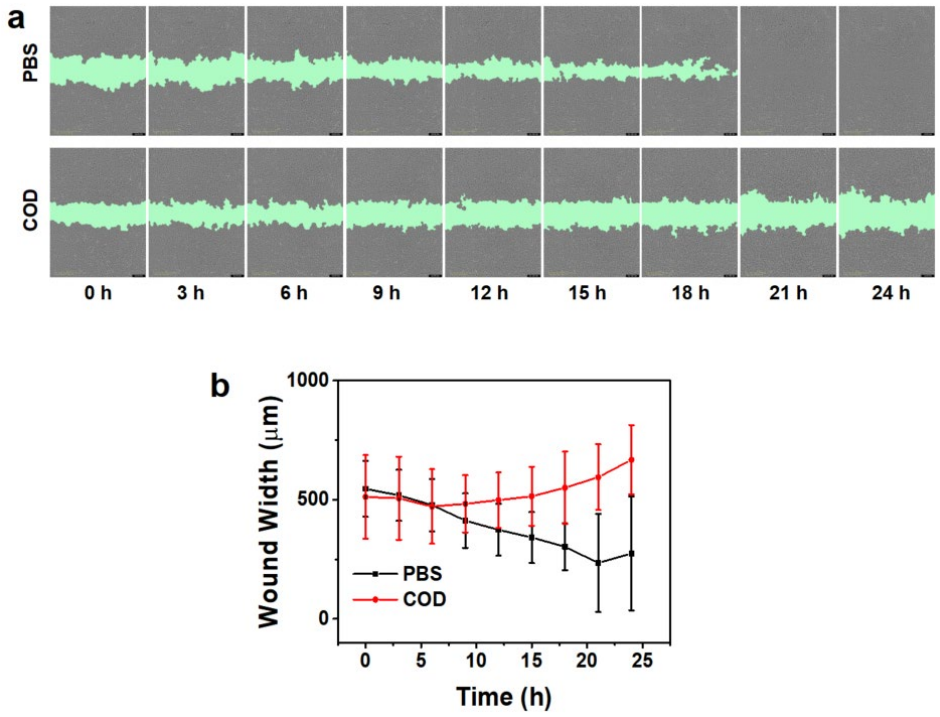

Figure S9. (a) Scratch wound assays of PBS and COD treatments on 4T1 cells. (b) Time-dependent average wound width after PBS or COD treatment on 4T1 cells (n = 3).

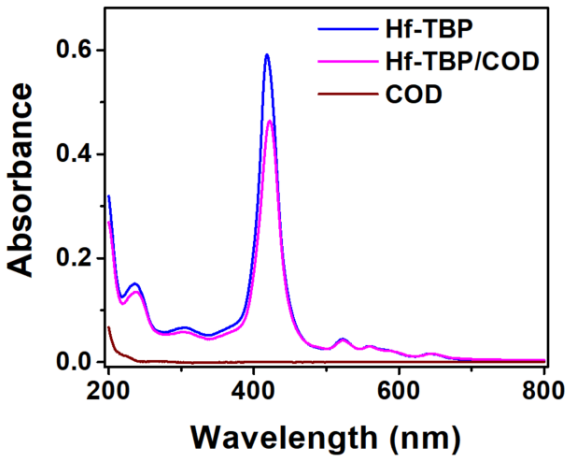

Figure S10. Ultraviolet-visible (UV-vis) spectra of COD, Hf-TBP, and Hf-TBP/COD in water.

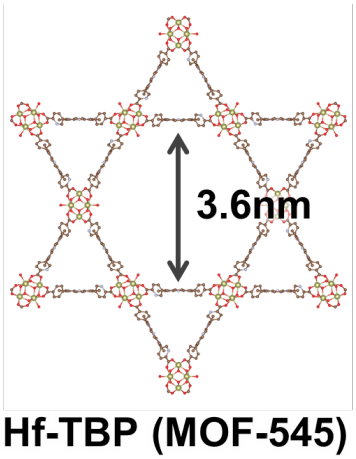

Figure S11. Structure of Hf-TBP nMOF.

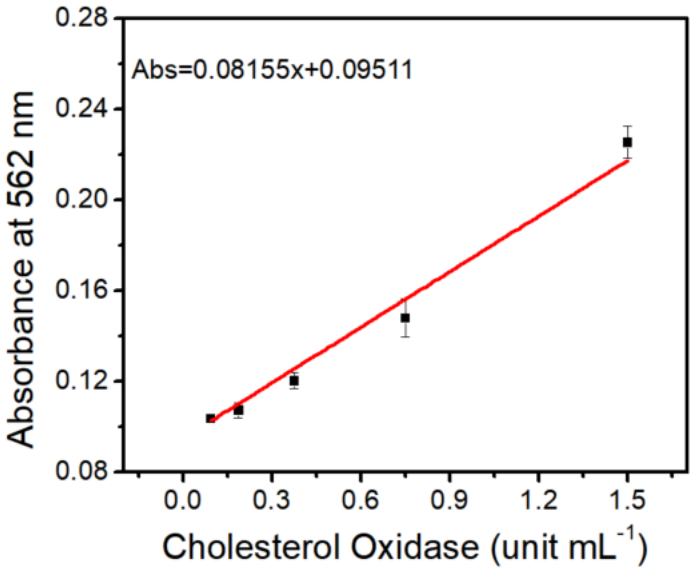

Figure S12. Standard curve of COD determined by BCA assay.

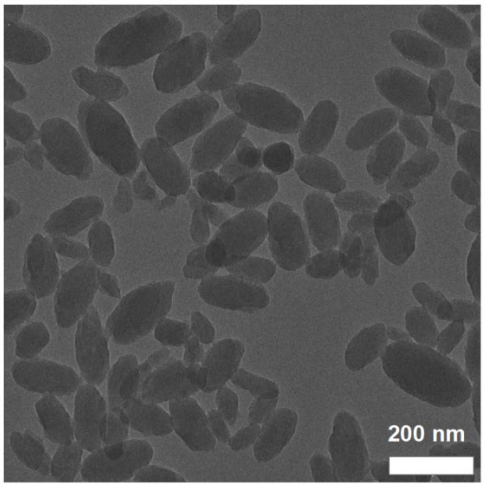

Figure S13. TEM image of Hf-TBP.

```
            16          26          36          46          56
DNGGYVPAVVIGTGYGAAVSALRLGEAGVQTLMLEMGQLWNQPGPDGNIFCGML
        66          76          86          96          106
NPDKRSSWFKNRTEAPLGSFLWLDVVNRNIDPYAGVLDRVNYDQMSVYVGRGVG
   116         126         136         146         156         166
GGSLVNGGMAVEPKRSYFEEILPRVDSSEMYDRYFPRANSMLRVNHIDTKWFED
          176         186         196         206         216
TEWYKFARVSREQAGKAGLGTVFVPNVYDFGYMQREAAGEVPKSALATEVIYGN
     226         236         246         256         266         27
NHGKQSLDKTYLAAALGTGKVTIQTLHQVKTIRQTKDGGYALTVEQKDTDGKLL
            286         296         306         316         326
ATKEISCRYLFLGAGSLGSTELLVRARDTGTLPNLNSEVGAGWGPNGNIMTARA
        336         346         356         366         376
NHMWNPTGAHQSSIPALGIDAWDNSDSSVFAEIAPMPAGLETWVSLYLAITKNP
   386         396         406         416         426         436
QRGTFVYDAATDRAKLNWTRDQNAPAVNAAKALFDRINKANGTIYRYDLFGTQL
          446         456         466         476         486
KAFADDFCYHPLGGCVLGKATDDYGRVAGYKNLYVTDGSLIPGSVGVNPFVTIT
     496         506
ALAERNVERIIKQDVTAS
```

Figure S14. Amino acid sequence of COD.

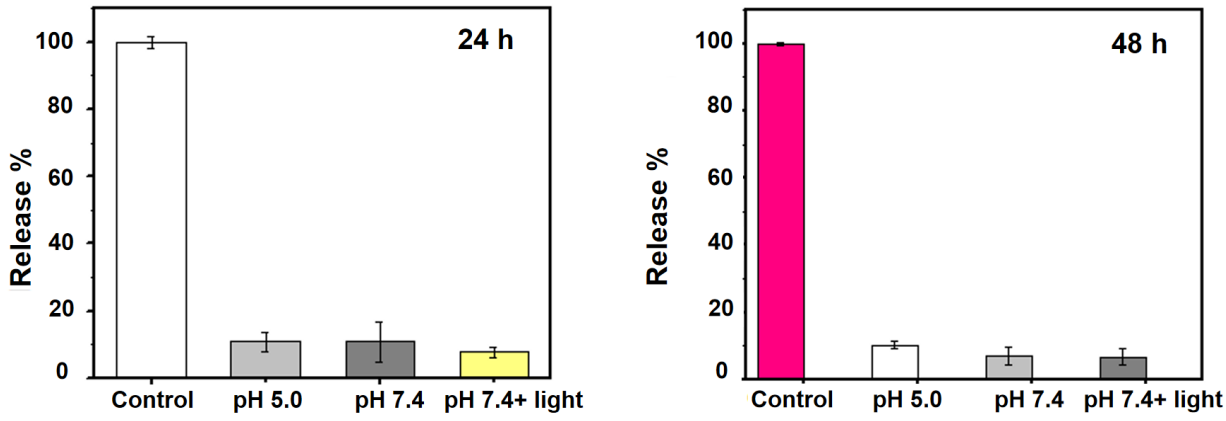

Figure S15. Release of COD under different conditions (free COD was served as positive control), n = 3.

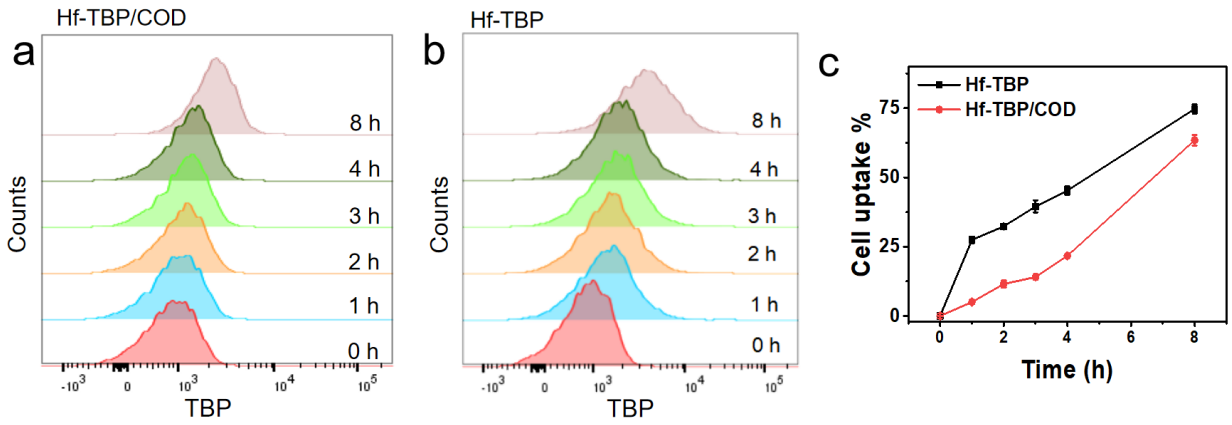

Figure S16. (a) Time-dependent cellular uptake of Hf-TBP/COD (TBP: 5 μM) at different time points (0, 1, 2, 3, 4, and 8 h) detected by flow cytometry. (b) Time-dependent cellular uptake of Hf-TBP (TBP: 5 μM) at different time points (0, 1, 2, 3, 4, and 8 h) detected by flow cytometry. (c) Cellular uptake of Hf-TBP and Hf-TBP/COD (TBP: 5 μM) at different time points, n = 3.

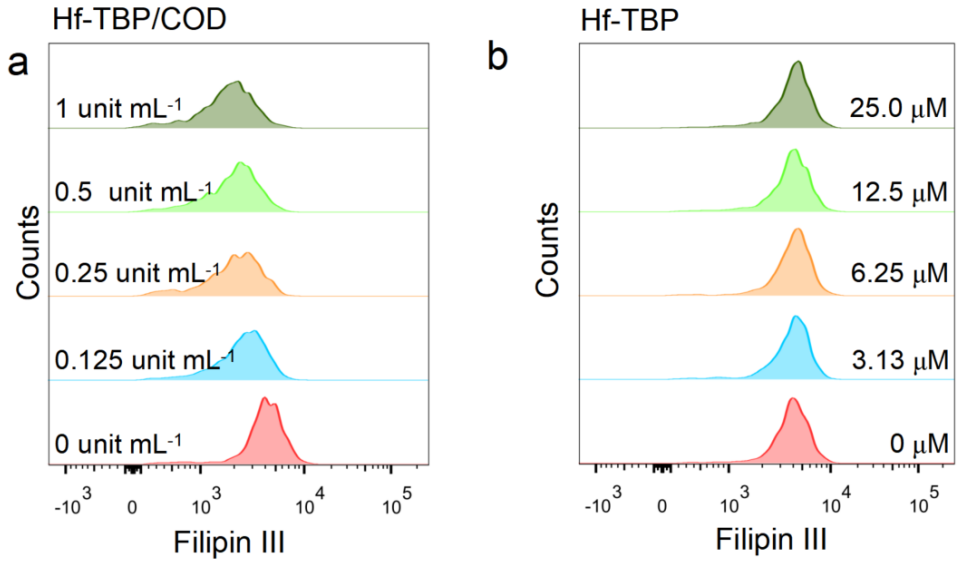

Figure S17. Depletion of cholesterol in 4T1 cells after incubation with Hf-TBP/COD (a) or Hf-TBP (c) at different concentrations.

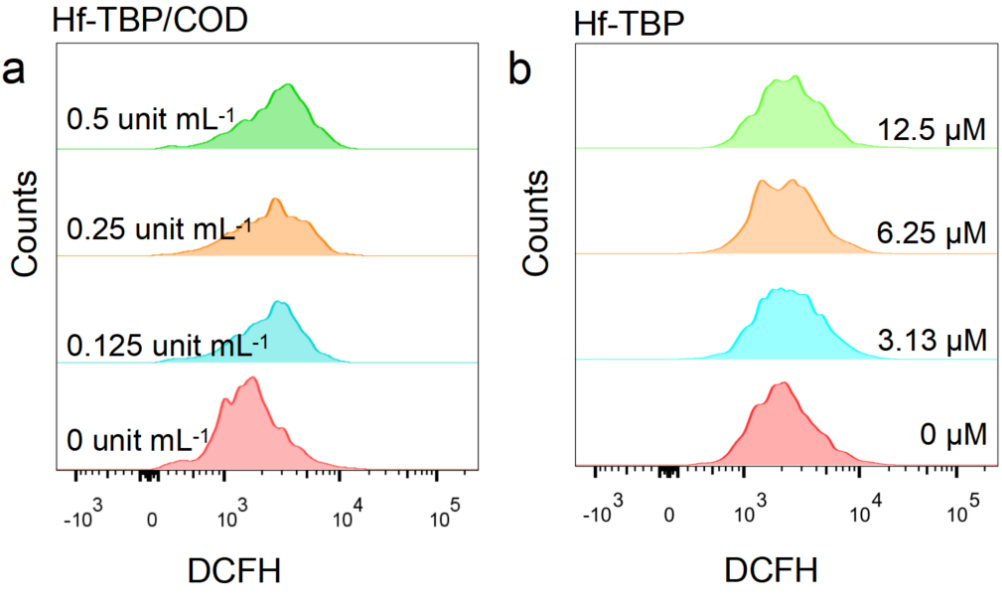

Figure S18. Generation of ROS in 4T1 cells after incubation with Hf-TBP/COD (a) or Hf-TBP (b) at different concentrations. Hf-TBP showed a slight effect on ROS generation, which might result from the oxidative burst.[7] Cells may recognize nanoparticles as foreign pathogenic microorganisms, and promote the destruction of these antigens after phagocytosis by producing ROS, during a complex process known as the oxidative burst.[8] Slight increase of ROS content in cancer cells during the complex oxidative burst process did not cause obvious toxicity (**Figure S18**).

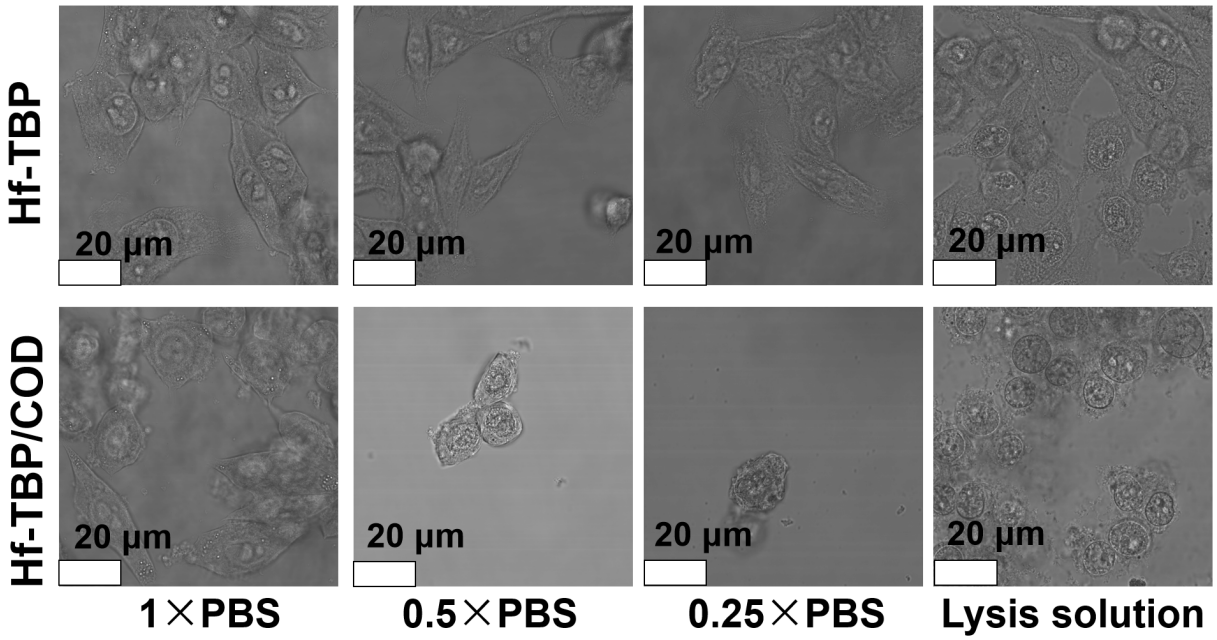

Figure S19. Morphology changes of 4T1 cells pre-incubated with Hf-TBP or Hf-TBP/COD (COD: 0.06 unit mL^{-1}) for 12 h followed by incubation with hypotonic solutions (0.5×, 0.25×PBS) for 30 min or lysis solution for 1 min.

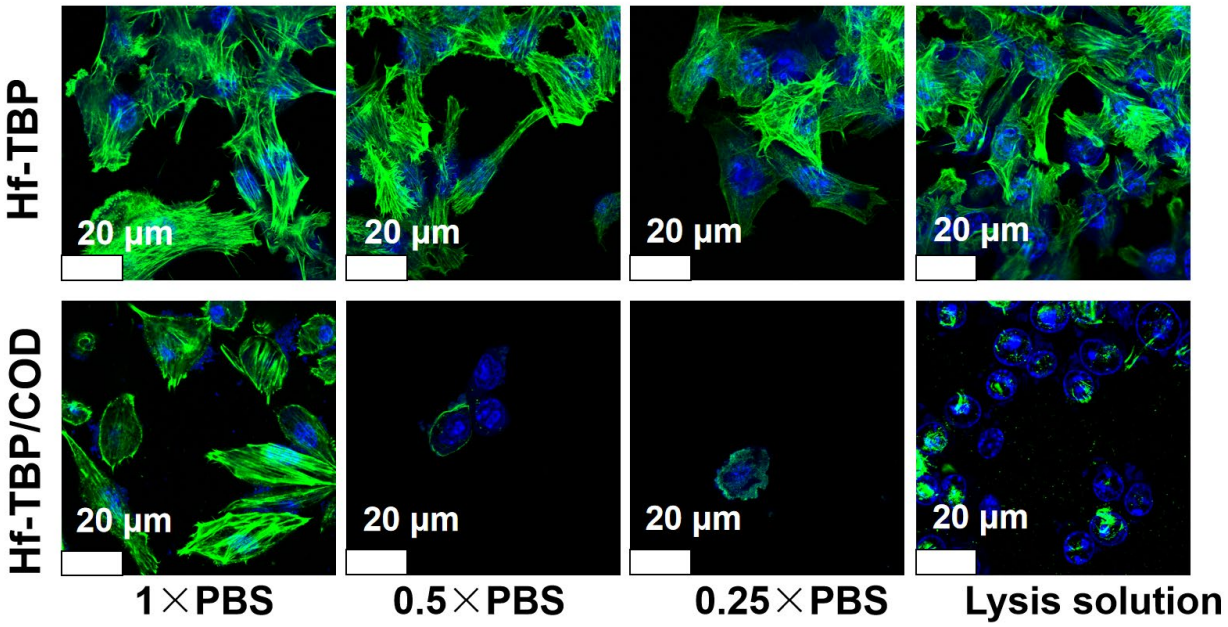

Figure S20. F-actin cytoskeleton assemblies of 4T1 cells pre-incubated with Hf-TBP or Hf-TBP/COD (COD: 0.06 unit mL^{-1}) for 12 h followed by incubation with hypotonic solutions (0.5×, 0.25×PBS) for 30 min or lysis solution for 1 min.

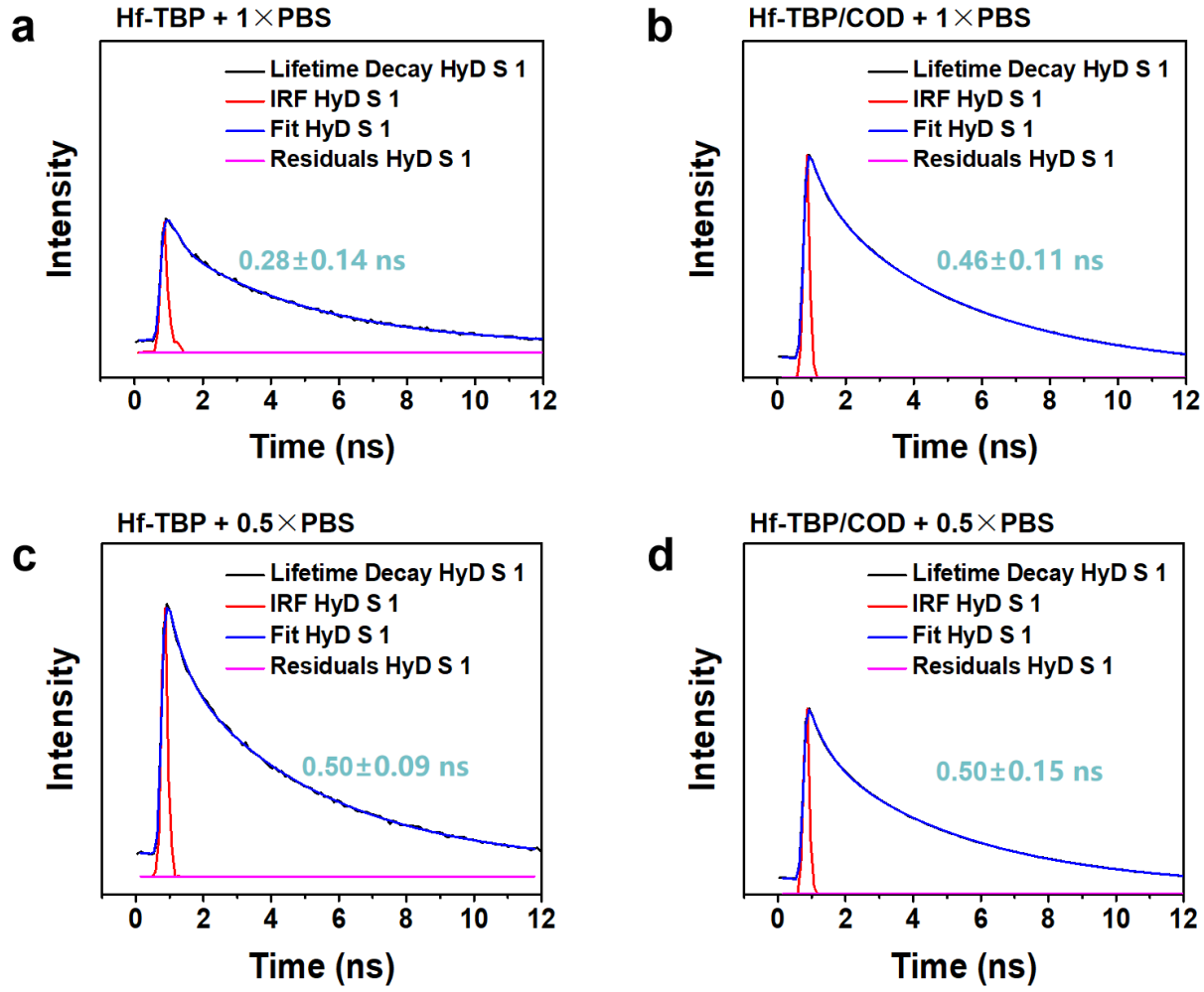

Figure S21. Fitted τ_1 lifetime curves of Flipper-TR in 4T1 cells after Hf-TBP (a) and Hf-TBP/COD treatments (b). Fitted τ_1 lifetime curves of Flipper-TR in 4T1 cells after Hf-TBP + 0.5×PBS (c) and Hf-TBP/COD + 0.5×PBS treatments (d).

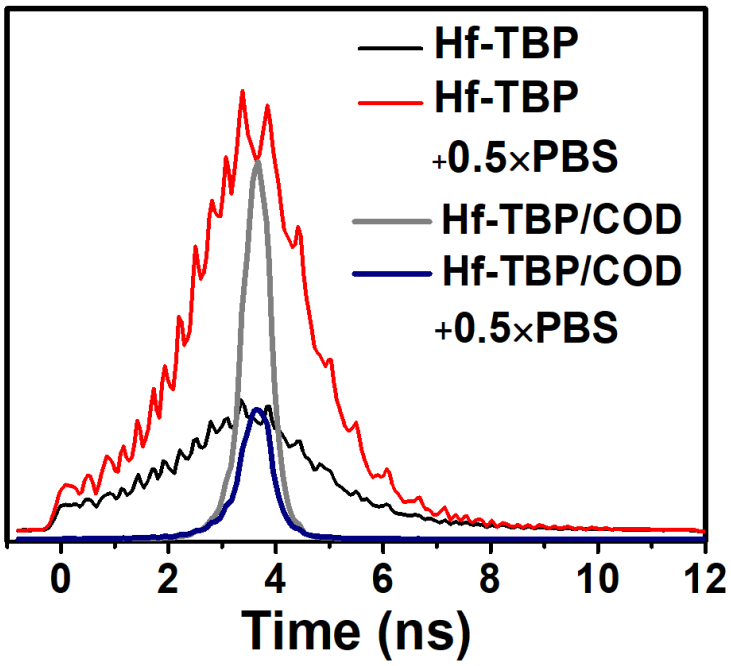

Figure S22. Fitted τ_2 lifetime of Flipper-TR in 4T1 cells after Hf-TBP, Hf-TBP/COD, Hf-TBP + 0.5×PBS, or Hf-TBP/COD + 0.5×PBS treatment.

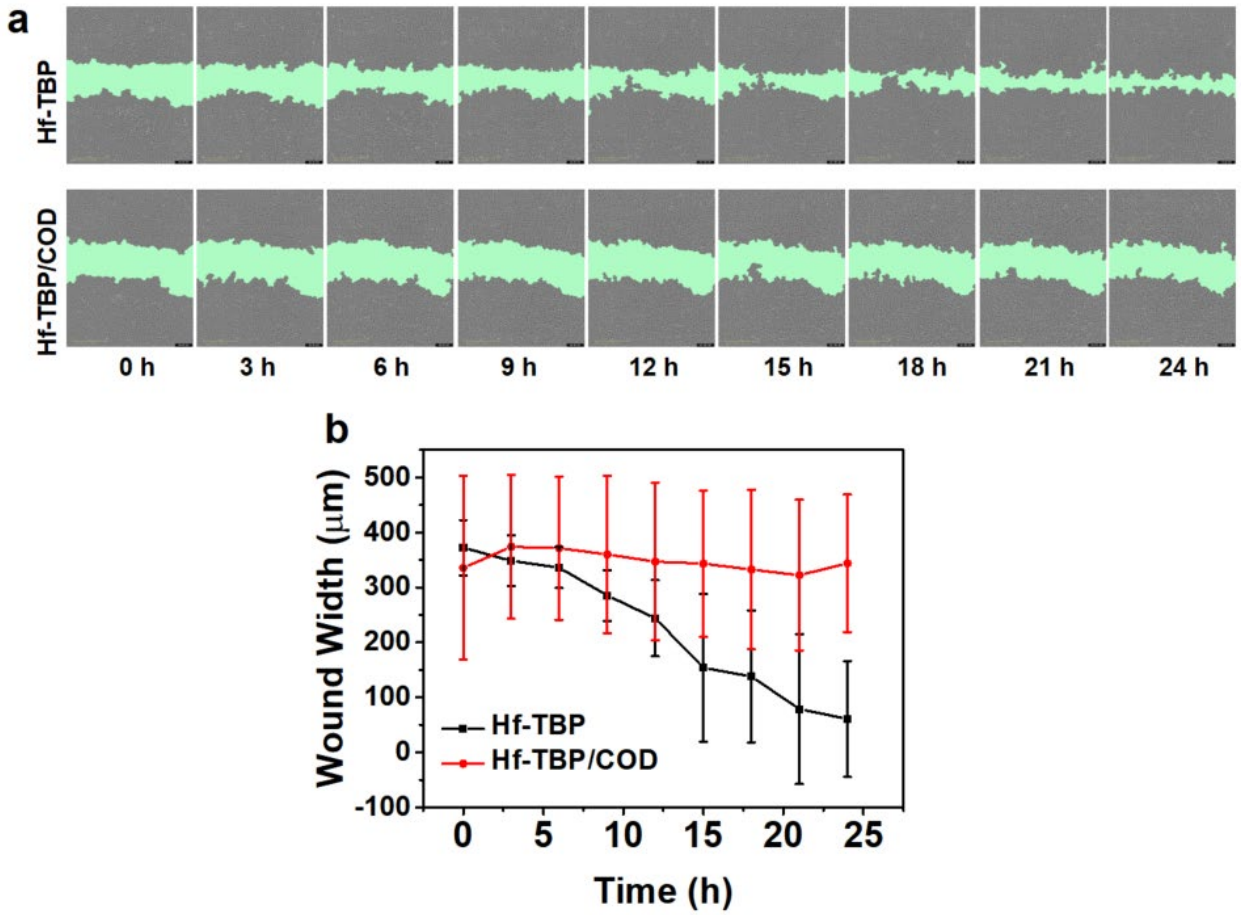

Figure S23. (a) Scratch wound assays of Hf-TBP and Hf-TBP/COD treatments on 4T1 cells. (b) Time-dependent average wound width after Hf-TBP or Hf-TBP/COD treatment on 4T1 cells (n = 3).

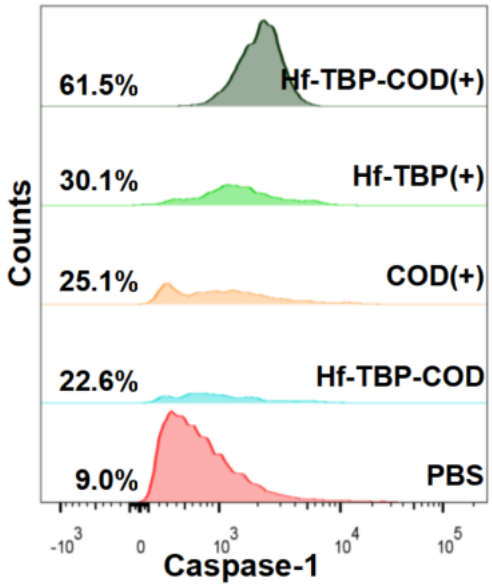

Figure S24. Expression levels of caspase-1 in 4T1 cells after 660 nm light irradiation (90 J/cm^2).

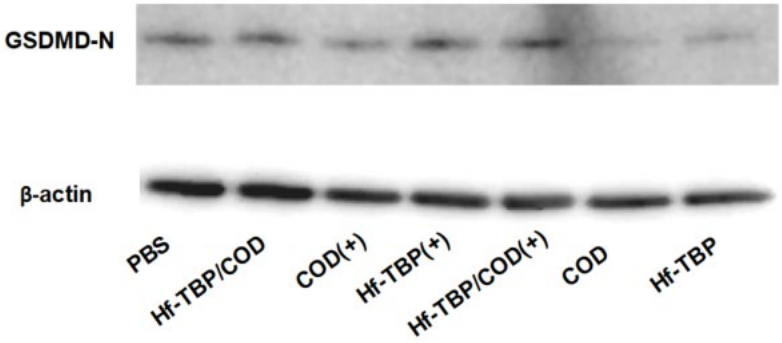

Figure S25. Western Blot of cleaved GSDMD in 4T1 cells after different treatments.

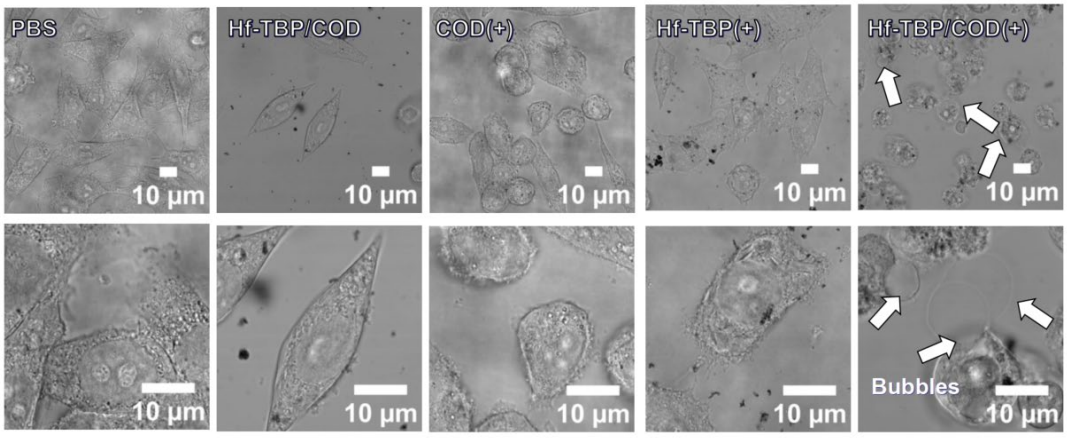

Figure S26. Morphology changes of 4T1 cells induced by pyroptosis after PDT treatment (90 J/cm^2).

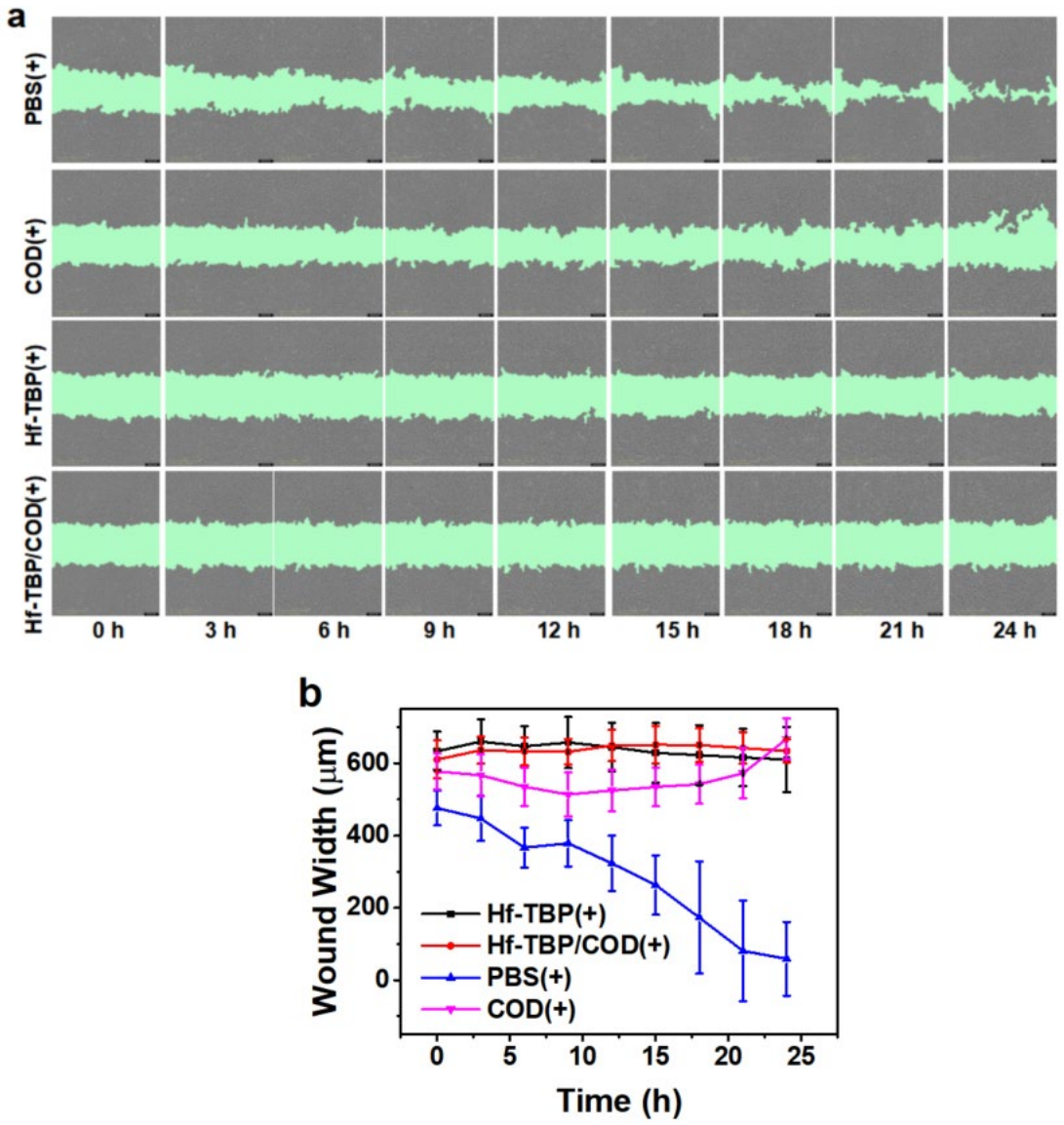

Figure S27. (a) Scratch wound assays of PBS(+), COD(+), Hf-TBP(+) and Hf-TBP/COD(+) treatments on 4T1 cells. (b) Time dependent average wound width after PBS(+), COD(+), Hf-TBP(+) or Hf-TBP/COD(+) treatment on 4T1 cells (n = 3).

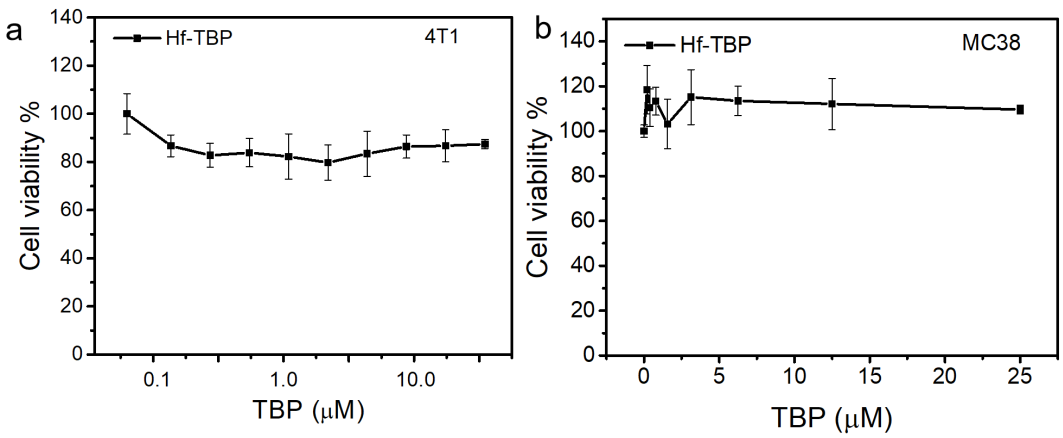

Figure S28. Cell viability of 4T1 (a) and MC38 cells (b) after Hf-TBP treatment (n = 3).

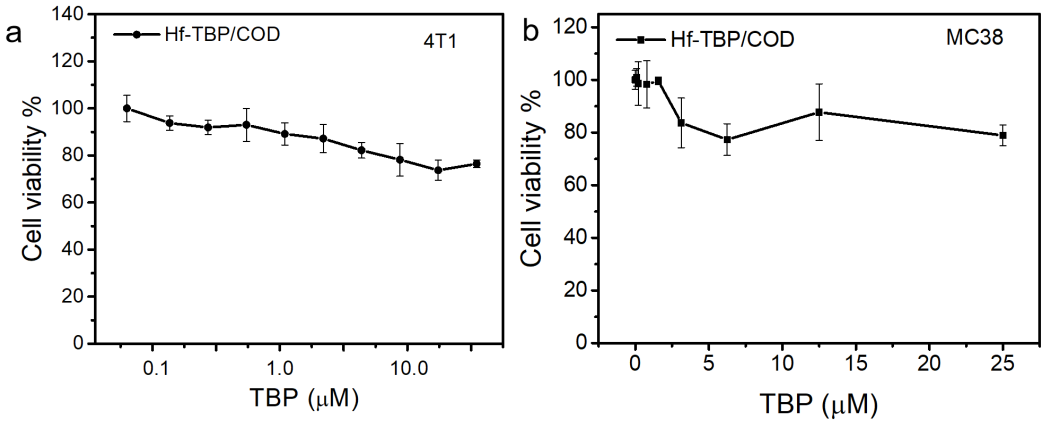

Figure S29. Cell viability of 4T1 (a) and MC38 cells (b) after Hf-TBP/COD treatment (n = 3).

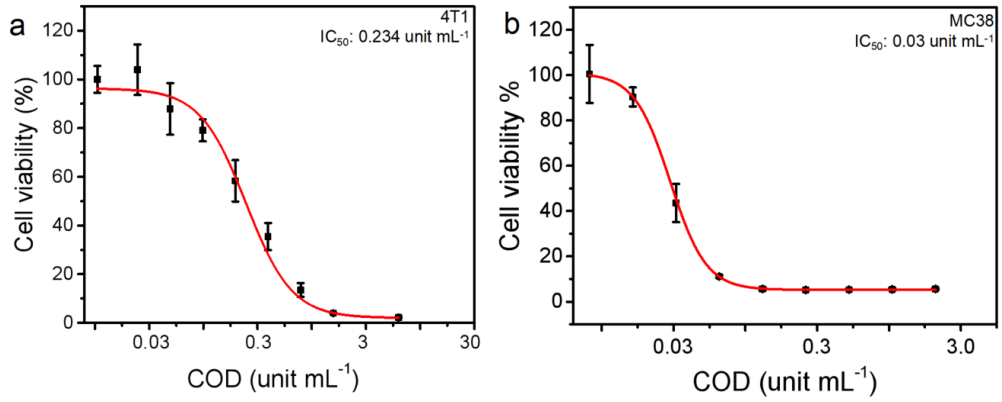

Figure S30. Cell viability of 4T1 (a) and MC38 cells (b) after COD treatment (n = 3).

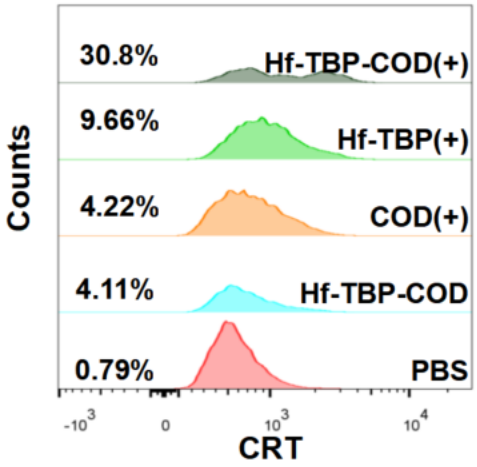

Figure S31. Expression levels of CRT in 4T1 cells after different treatments.

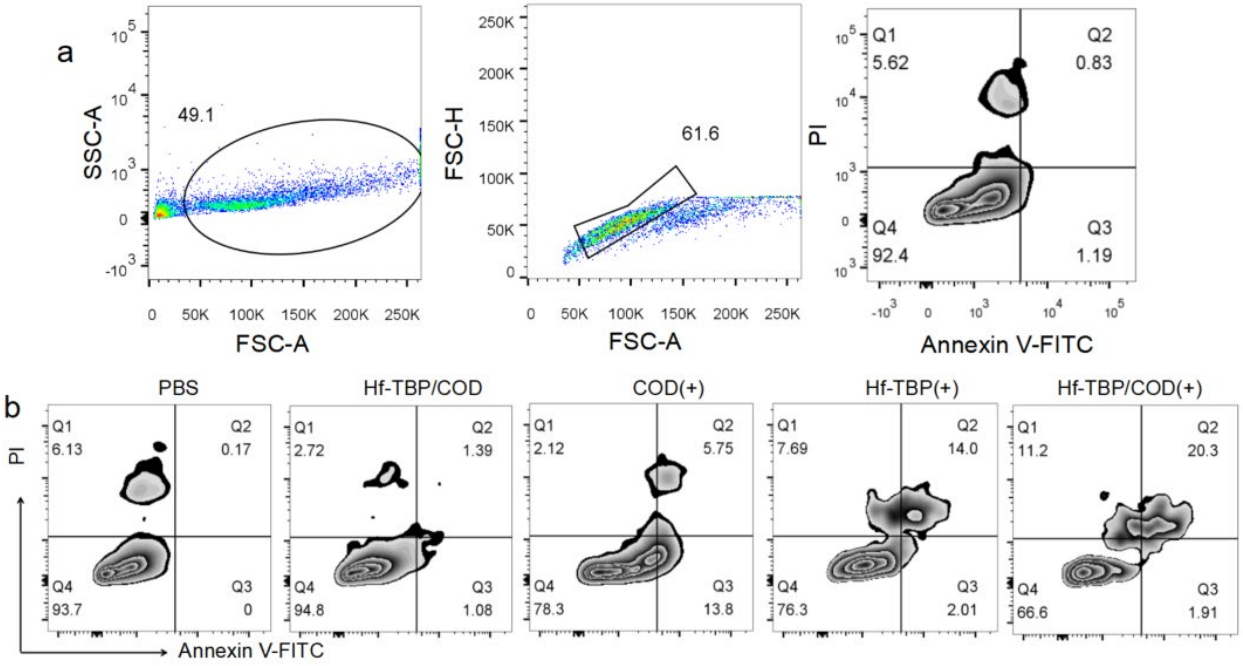

Figure S32. (a) Gating strategy in the Annexin-V-PI assay. (b) Staining of ICD with Annexin-V and PI in 4T1 cells by flow cytometry.

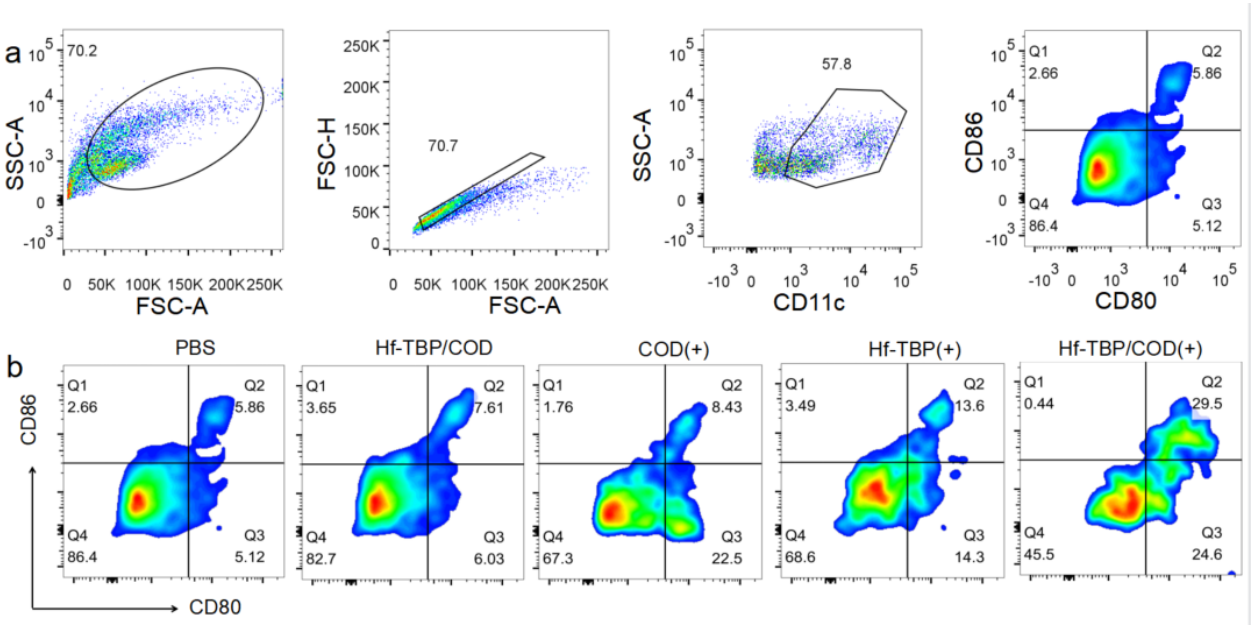

Figure S33. (a) Gating strategy for DC maturation by flow cytometry. (b) Maturation of BMDCs ($CD80^+$ $CD86^+$) stimulated by treated 4T1 cells.

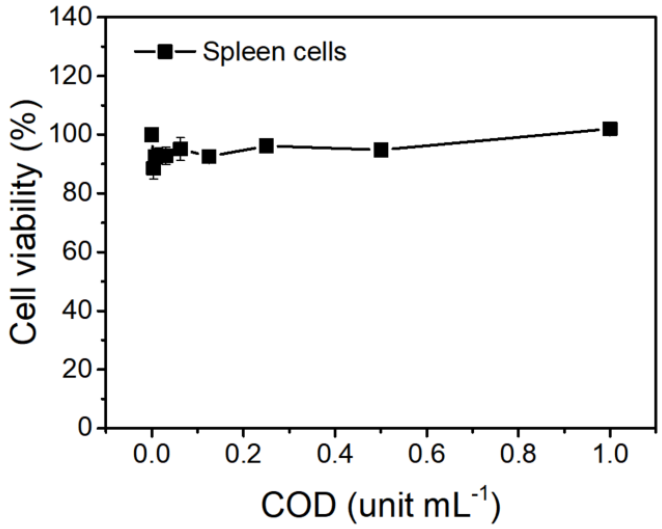

Figure S34. Cell viability of spleen cells after incubation with COD for 24 h (n = 3).

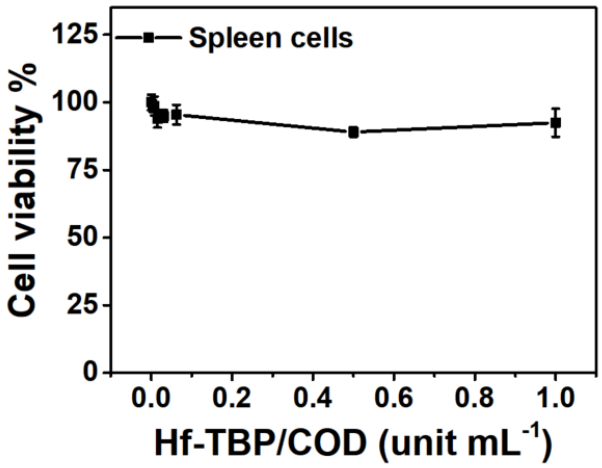

Figure S35. Cell viability of spleen cells after incubation with Hf-TBP/COD for 24 h (n = 3).

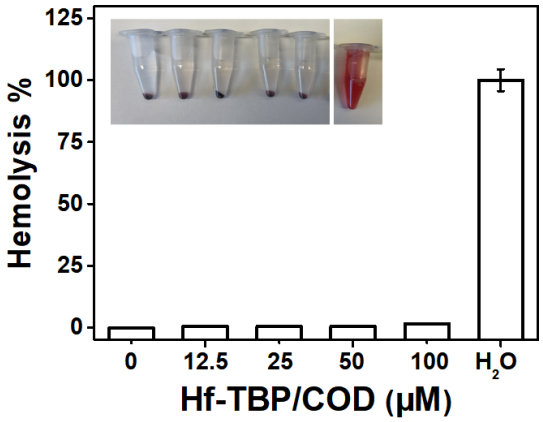

Figure S36. Hemolysis assay of Hf-TBP/COD (n = 4).

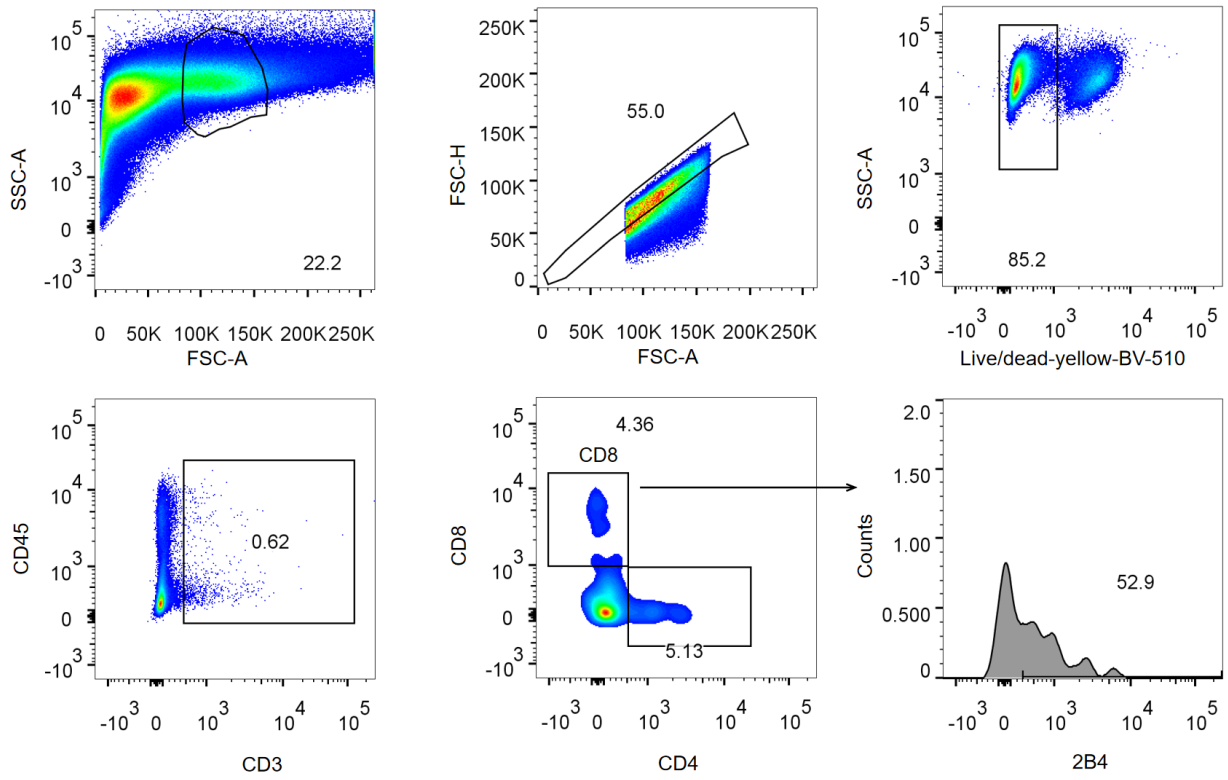

Figure S37. Gating strategy for 2B4 expression in $CD8^+$ T cells.

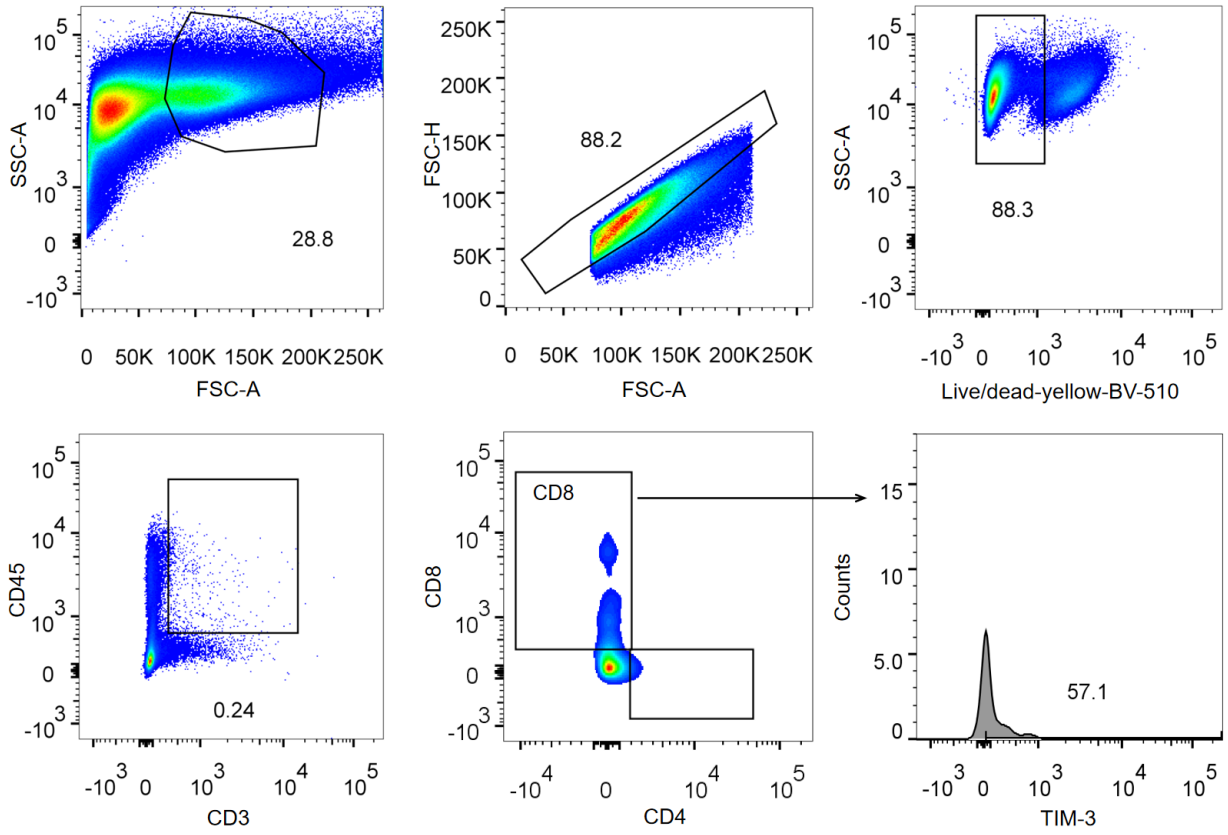

Figure S38. Gating strategy for TIM-3 expression in $CD8^+$ T cells.

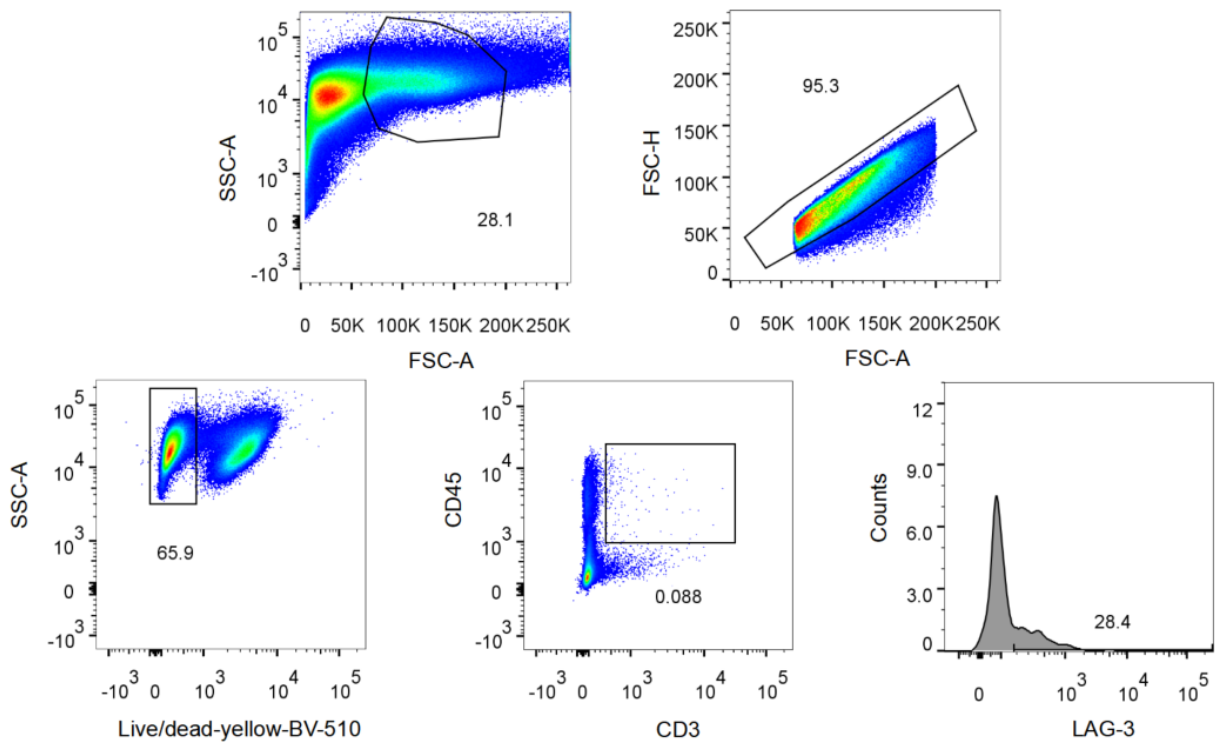

Figure S39. Gating strategy for LAG-3 expression in $CD3^+$ T cells.

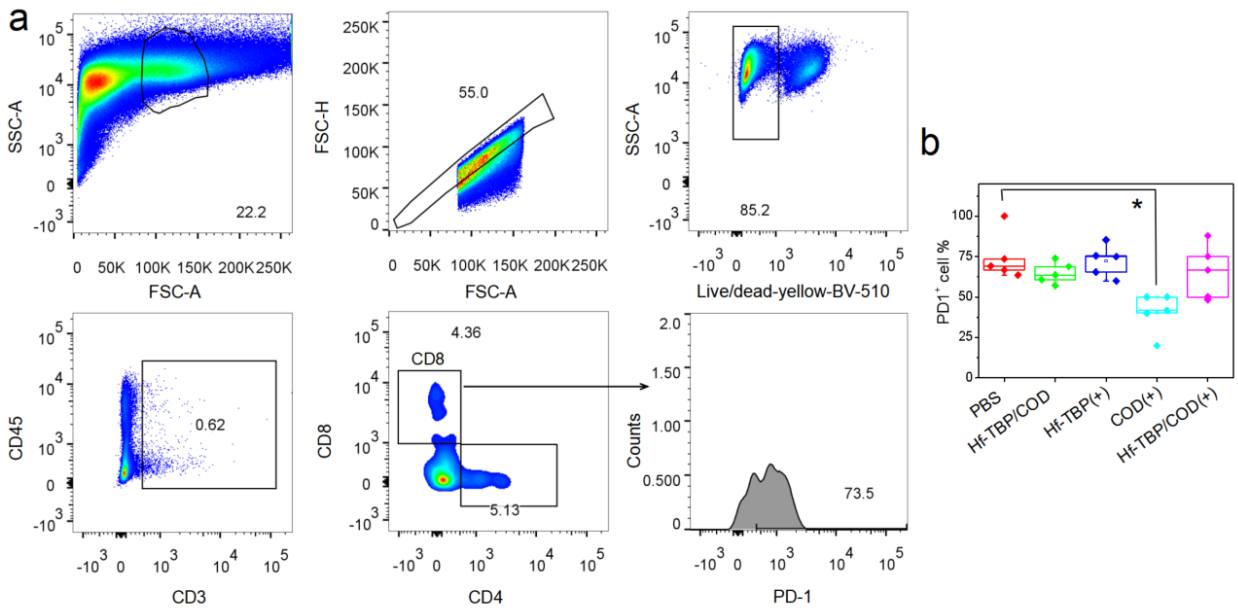

Figure S40. (a) Gating strategy for PD-1 expression in $CD8^+$ T cells. (b) Expression levels of PD-1 in MC38 tumor tissues after PDT treatment.

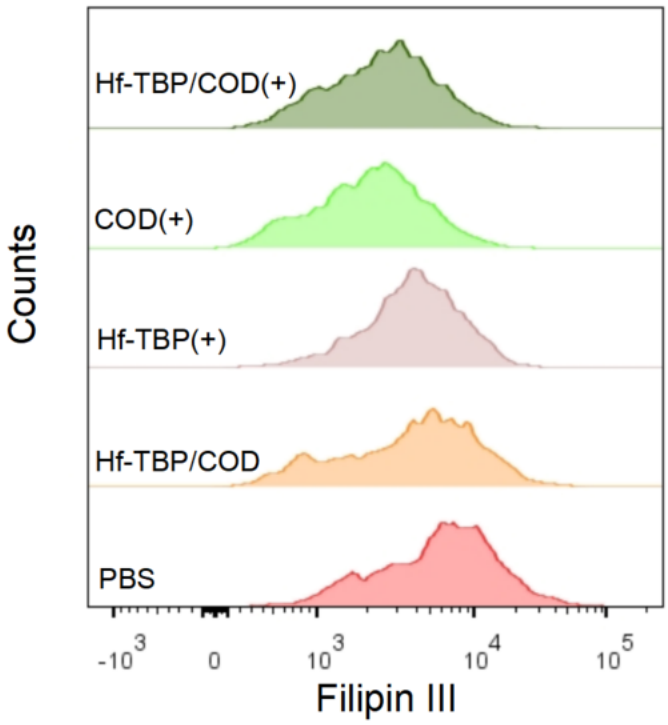

Figure S41. Cholesterol levels in the tumor tissues probed by Filipin III detected by flow cytometry.

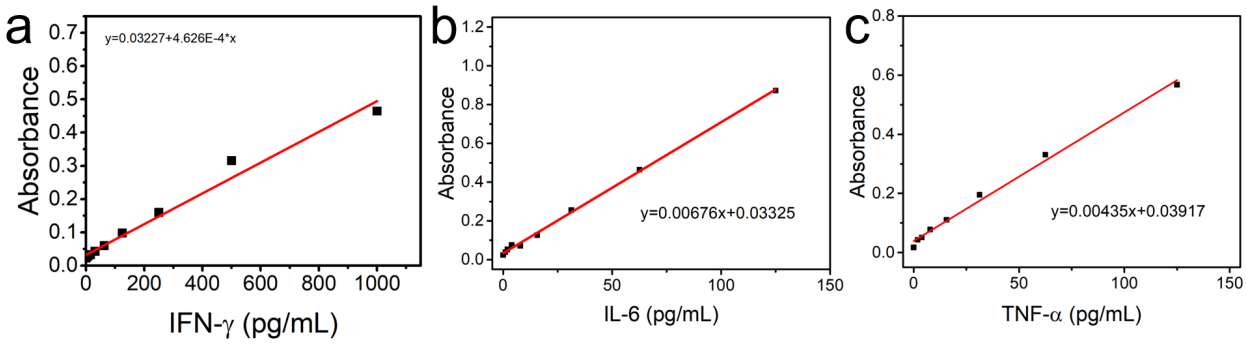

Figure S42. (a) Standard curve of IFN-γ. (b) Standard curve of IL-6. (c) Standard curve of TNF-α.

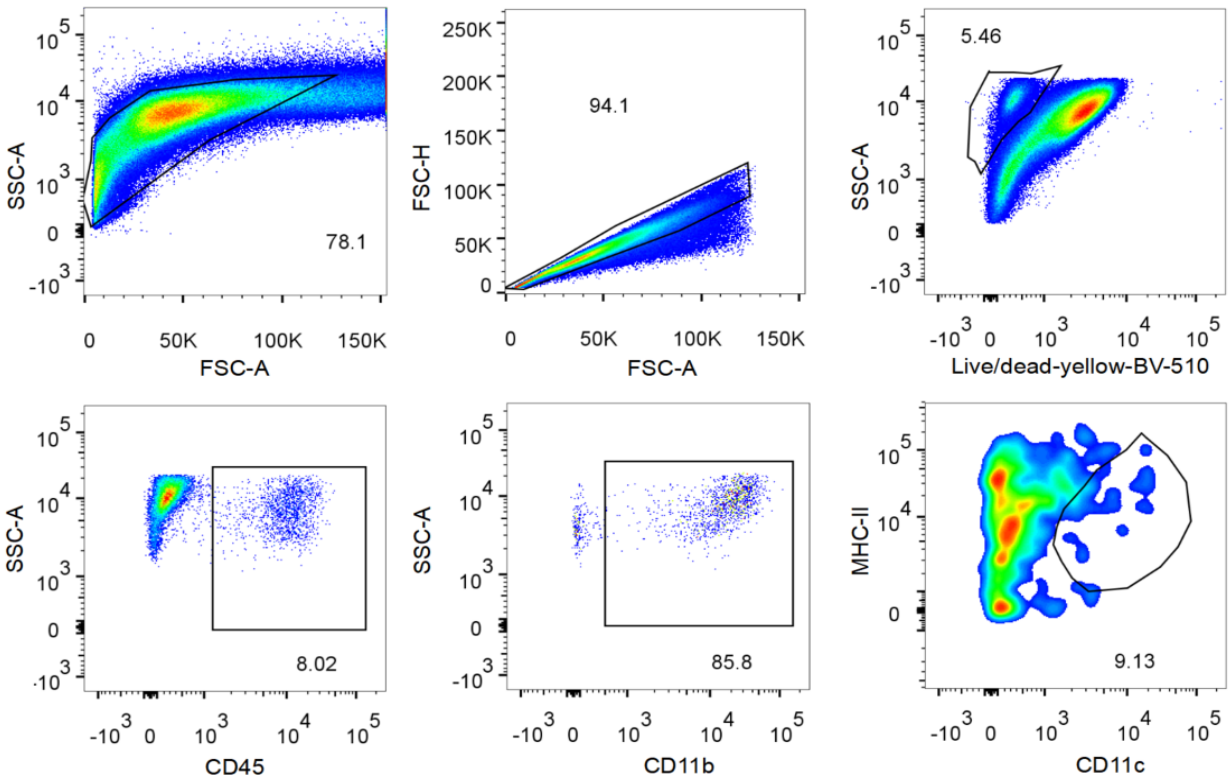

Figure S43. Gating strategy for DCs ($CD45^+$ $CD11b^+$ $MHCII^+$ $CD11c^+$).

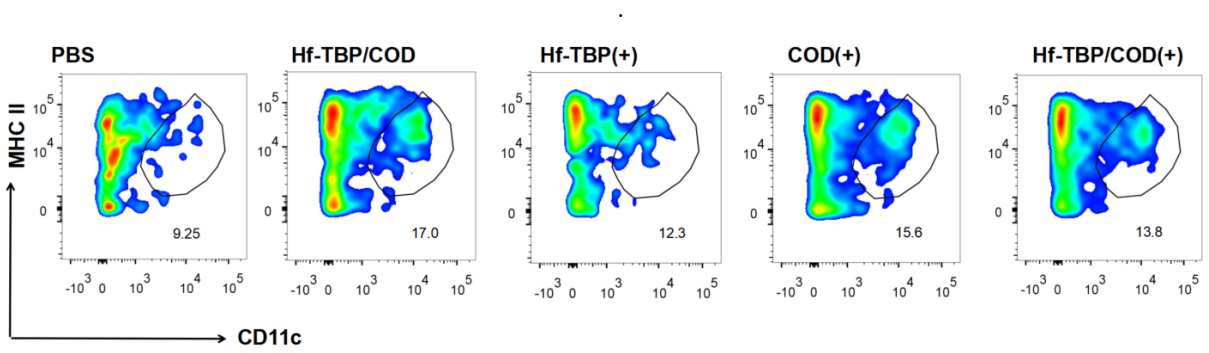

Figure S44. Representative gating strategies for DCs ($CD45^+$ $CD11b^+$ $MHCII^+$ $CD11c^+$) major histocompatibility complex class II molecules.

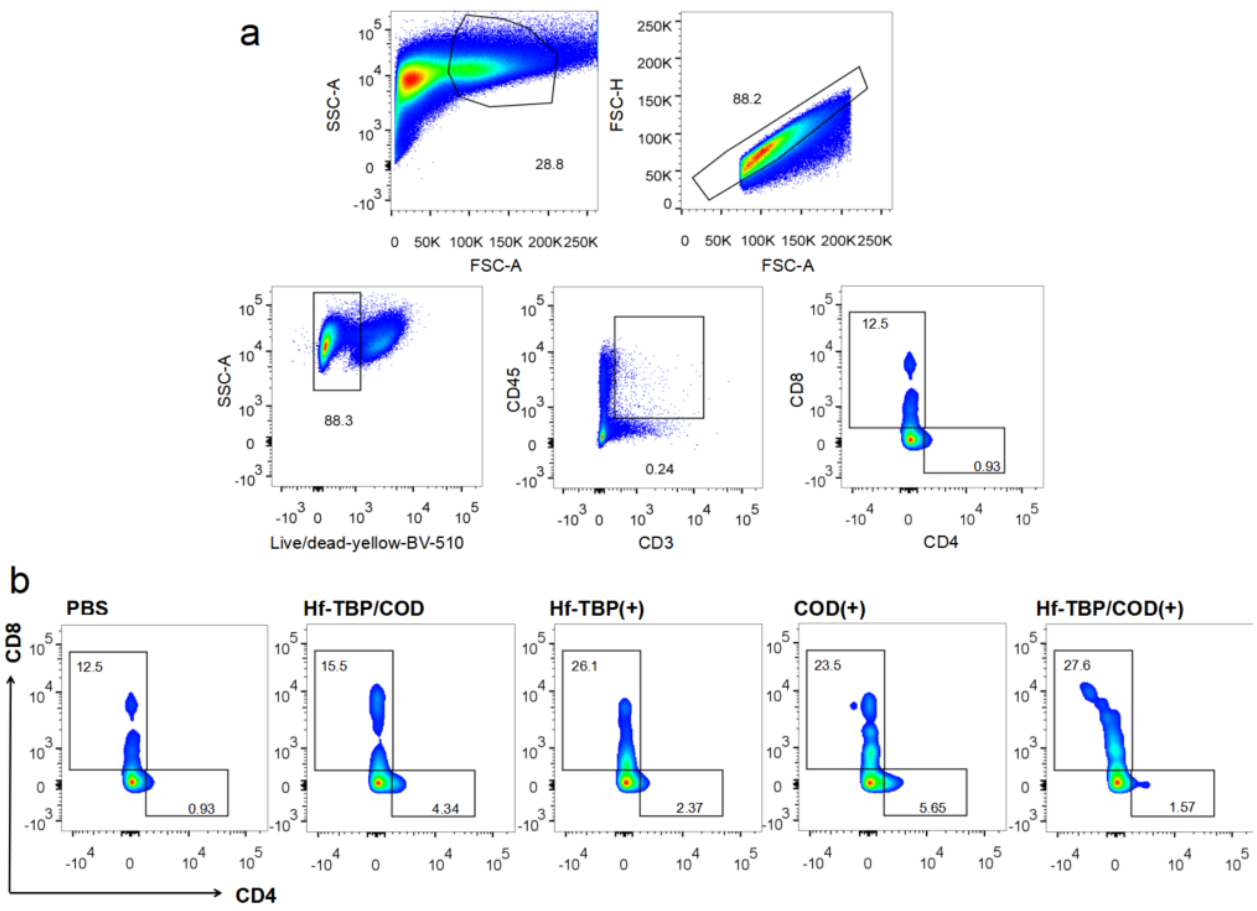

Figure S45. (a) Gating strategy for cytotoxic ($CD45^+$ $CD3^+$ $CD8^+$) and helper T cells ($CD45^+$ $CD3^+$ $CD4^+$). **(b)** Representative gating strategies for cytotoxic and helper T cells in ($CD45^+$ $CD3^+$ $CD8^+$ for cytotoxic T cells, $CD45^+$ $CD3^+$ $CD4^+$ for helper T cells).

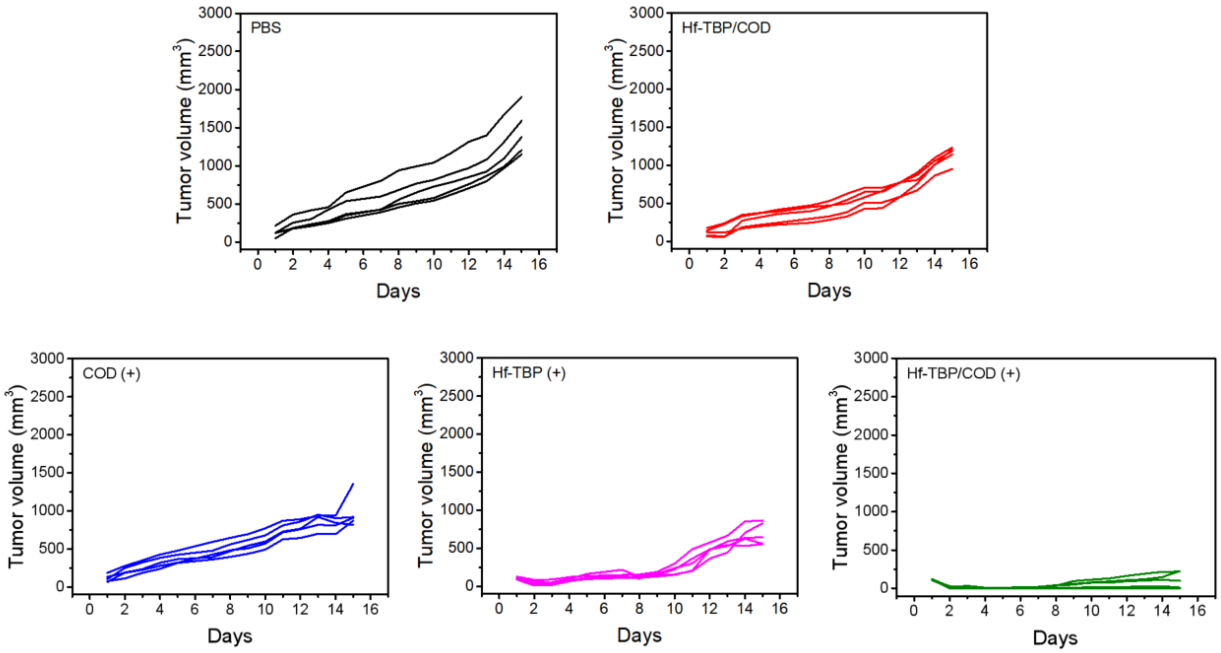

Figure S46. The individual growth curve of 4T1 tumors after different treatments.

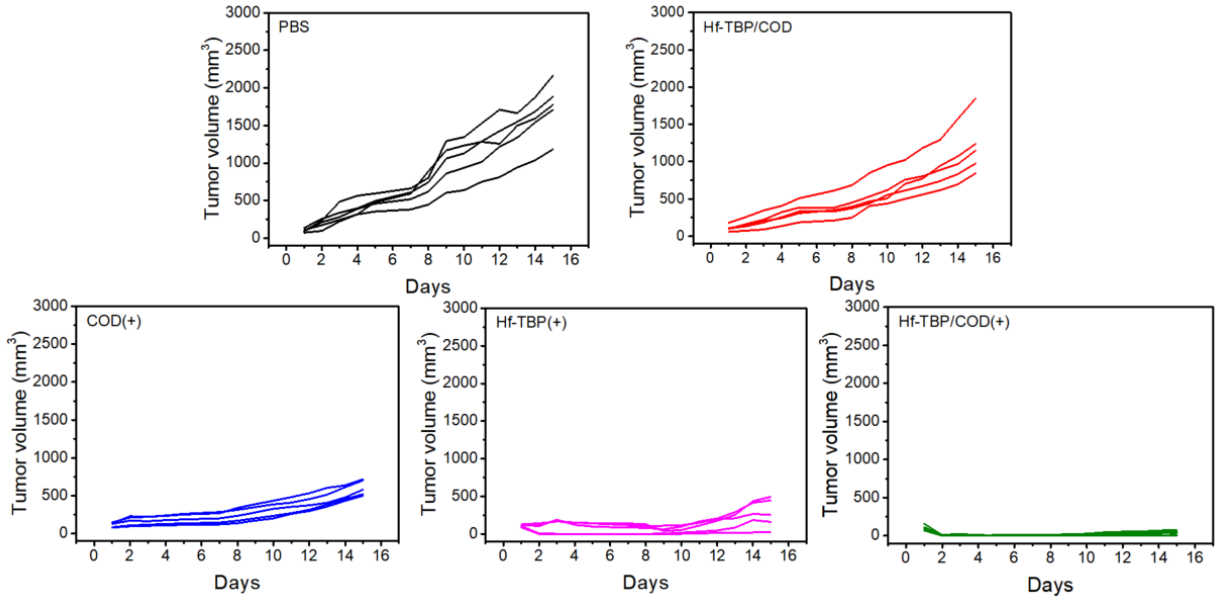

Figure S47. Individual growth curves of MC38 tumors after different treatments.

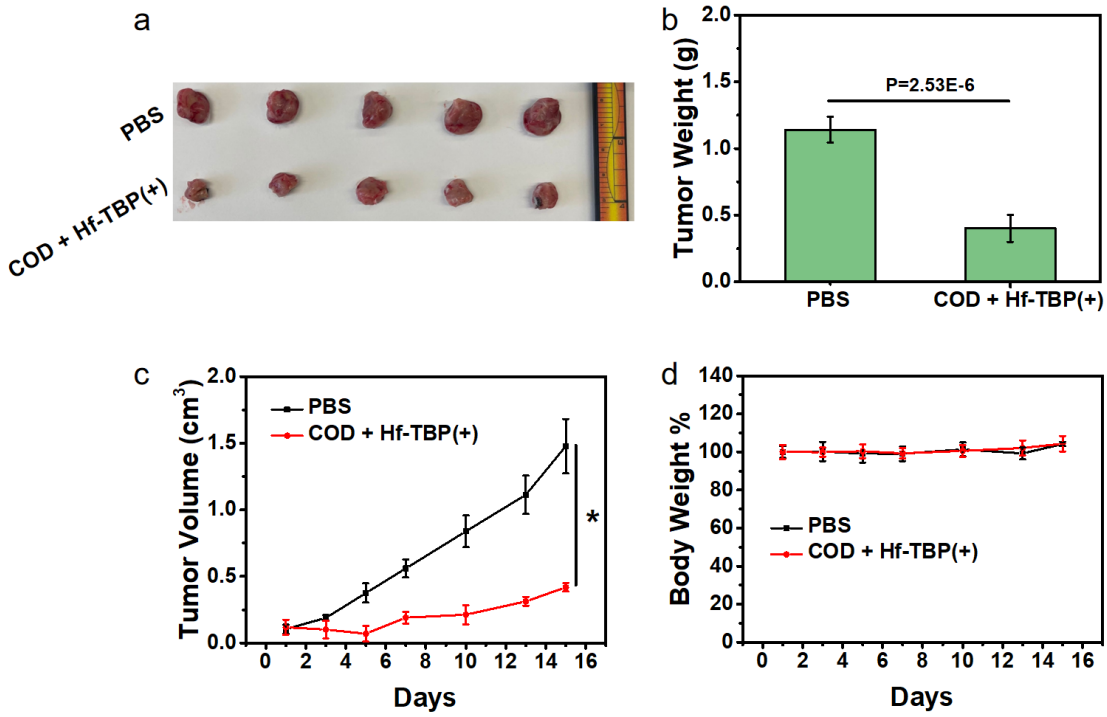

Figure S48. Photographs (a), weights (b), growth curves (c) of 4T1 tumors after treatment with PBS or sequential injection of COD and Hf-TBP followed by light irradiation. The TGI value was calculated to be 0.648. (d) Relative body weights of 4T1 tumor-bearing BALB/c mice. n = 5, *, $p < 0.05$.

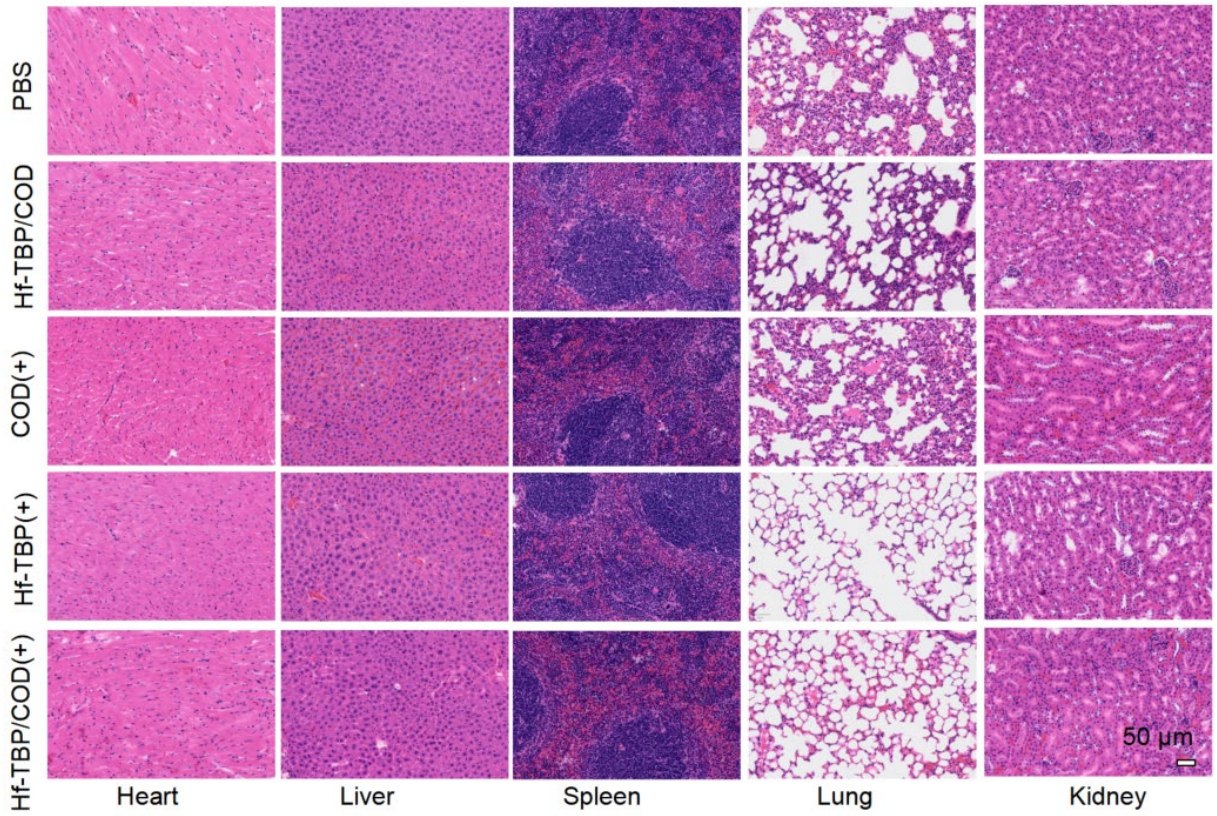

Figure S49. H&E staining of different normal organs (heart, liver, spleen, lung, and kidney) in different treatment groups of 4T1-tumor bearing BALB/c mice.

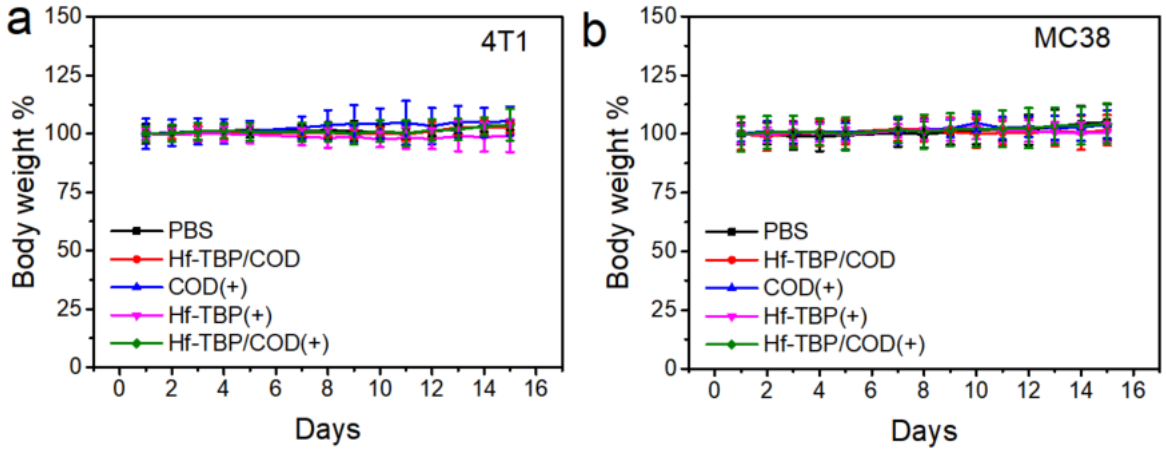

Figure S50. Relative body weights of 4T1 tumor-bearing BALB/c mice (a) and MC38 tumor-bearing C57BL/6 mice (b), n = 5.

Table S1. Dissociation constants and isoelectric points (pI) of amino acids.

Amino acids	pK_1(-COOH)	pK_2(-NH_3^+)	pK_R	pI
Glycine Gly G	2.34	9.60		5.97
Alanine Ala A	2.34	9.60		6.02
Valine Val V	2.32	9.62		5.97
Leucine Leu L	2.36	9.60		5.98
Isoleucine Ile I	2.36	9.68		6.02
Serine Ser S	2.21	9.15		5.68
Threonine Thr T	2.63	10.43		6.53
Asparticacid Asp D	2.09	9.82	3.86	2.97
Asparagine Asn N	2.02	8.8		5.41
Glutamic acid, Glu	2.19	9.67	4.25	3.22
Glutarnine Gln Q	2.17	9.13		5.65
Arginine Arg R	2.17	9.04	12.48	10.76
Lysine Lys K	2.18	8.95	10.50	9.74
Histidine His H	1.82	9.17	6.00	7.59
Cystine Cys C	1.71	8.33	10.78	5.02
Methionine Met -M	2.28	9.21		5.75
Phenylalanine Phe F	1.83	9.13		5.48
Tyrosine Tyr Y	2.20	9.11	10.07	5.66
Tyrosine Tyr Y	2.38	9.39		5.89
Proline Pro P	1.99	10.60		6.30

Table S2. Uptake percentage of Hf-TBP and Hf-TBP/COD (TBP: 5 μM) at different time points (0, 1, 2, 3, 4, and 8 h).

Time (h)	Hf-TBP %	Hf-TBP/COD %
1	27.5	5.0
2	32.3	11.5
3	39.5	14.0
4	45.3	21.7
8	74.8	63.5

Table S3. TGI values of 4T1-bearing BALB/c and MC38-bearing C57BL/6 mice at day 15.

Treatment	TGI (4T1)	TGI (MC38)
Hf-TBP/COD	0.366	0.267
COD(+)	0.458	0.583
Hf-TBP(+)	0.543	0.798
Hf-TBP/COD(+)	0.917	0.950

Table S4. f_{additive} and $f_{combination}$ values of 4T1 tumor-bearing BALB/c and MC38 tumor-bearing C57BL/6 mice at day 15.

Treatment	TGI (4T1)	TGI (MC38)
f_{additive}	0.290	0.148
$f_{combination}$	0.083	0.05

Since f_{additive} is greater than $f_{combination}$, there is a synergistic effect.[4]

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