Supporting Information

Biosynthetic cell membrane vesicles to enhance TRAIL-mediated apoptosis driven by photo-triggered oxidative stress

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Materials and methods

1. Materials

Poly (D, L-lactide-co-glycolide) (PLGA, lactide/glycolide ratio: 75/25) was purchased from Jinan Daigang Biomaterial Co., Ltd (Jinan, China). 4',6-diamidino-2-phenylindole (DAPI), 2',7'-dichlorodihydr fluorescein diacetate (DCFH-DA, ≥97%), 9,10-anthracenediylbis (methylene) dimalonic acid (ABDA, ≥90%) and glutathione (GSH) were purchased from Sigma-Aldrich (St.Louis, MO, USA). Chlorin e6 (Ce6, 93-98%) was obtained from J&K Chemical Ltd (Beijing, China). Dimethyl sulfoxide (DMSO) was obtained from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Coomassie brilliant blue R-250 was purchased from Beijing Solarbio Science & Technology Co., Ltd. (Beijing, China). Polybrene was purchased from Santa Cruz biotechnology Inc (Texas, USA). DiI was purchased from Us Everbright Inc (Suzhou, China). Lipofectamine 3000 Transfection Kit (lot: 2098843), SuperSignal™ West Pico PLUS Chemiluminescent Substrate and anti-DR5 (MA5-35224) were bought from Thermo Fisher Scientific (Waltham, MA, USA). Anti-TRAIL (ab10516), anti-FADD (ab124812) and b-actin (ab49900) were purchased from Abcam (Cambridge, UK). Anti-Ki67 (GB11141) was obtained from Servicebio Technology Co., Ltd. (Wuhan, China). Anti-cleaved-caspase 8 (#8592), anti-cleaved-caspase 3 (#9661S) and anti-cytochrome c (#11940) were purchased from Cell Signaling Technology (CST, Boston, USA). Mouse TRAIL ELISA Kit (DY1121) was supplied by R&D SYSTEMS (Minneapolis, USA). Cell Counting Kit-8 (CCK-8) was provided by Dojindo Labratories (Kumamoto, Japan). LIVE/DEAD Viability/Cytotoxicity Kit was bought from Invitrogen (Eugene, OR, USA). Annexin V-allophycocyanin (APC)/propidium iodide (PI) Apoptosis Detection Kit was obtained from KeyGEM Biotech (Jiangsu, China). One step TUNEL Apoptosis Assay Kit (Green Fluorescence, C1088) was purchased from Beyotime Biotech Inc (Shanghai, China). If not specifically mentioned, the involved reagents were analytically pure and used as received. Ultrapure Water was provided by a Milli-Q Gradient System (18.2MΩ resistivity, Millipore Corporation, Bedford, MA, USA).

2. Cell culture

CHO-S cells (Chinese hamster ovary cells), hepa1-6 cells (murine hepatoma cells), 4T1 cells (murine breast cancer cells) and NIH 3T3 cells (murine embryonic fibroblasts cells) were employed in the current work. All these cells were cultured in Dulbecco's Modified Eagle Medium (DMEM)

supplemented with 10% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA, USA) and 1% penicillin-streptomycin (Gibco BRL, Grand Island, NY, USA) in a humidified incubator containing 5% CO_2 at 37 ℃.

3. Preparation and quantification

3.1 Establishment of stable TRAIL-expressing cell line

The plasmid encoding murine TRAIL with an enhanced green fluorescent protein (EGFP)-tag was firstly constructed (pCDH-CMV-TRAIL-EGFP-EF1α-Blasticidin) based on our previously reported work.[1] Afterwards, the obtained plasmid was packaged into lentivirus with Lipofectamine 3000 Transfection Kit. Thereafter, the viral supernatants were harvested at 48 h post transfection and then concentrated by ultracentrifugation at 25, 000 rpm for 90 min at 4 ℃. Subsequently, the CHO-S cells were infected with the lentivirus in the cell medium containing 5 μg/mL polybrene. After two days of infection, the transfected CHO-S cells were selected with blasticidin (10 μg/mL) to obtain stable-expressing murine TRAIL cells.

3.2 Extraction of engineered membranes

The cell membranes of CHO-S cells with or without genetic modification were extracted using the approaches described previously.[2, 3] The two types of cells were specifically scraped with scrapers and then rinsed with PBS thrice by centrifugation at 800 g for 10 min. Afterwards, the cell membranes were extracted by the Membrane and Cytosol Protein Extraction Kit (Beyotime, P0033) and then washed with PBS containing protease inhibitor cocktail and PMSF, and eventually stored at -80 ℃ for the future use.

3.3 Fabrication of CP NP

Ce6 loaded PLGA nanoparticle (CP NP) was fabricated *via* the nano-precipitation method described earlier.[4, 5] Typically, 100 mg of PLGA and 1 mg of Ce6 dissolved in 2 mL of DMSO were added dropwise into 10 mL of ultrapure water under magnetic stirring. Then, the product was poured into a dialysis bag (MWCO 3500, Beijing Solarbio Science□Technology Co., Ltd, China) for purification. The resultant CP NP was collected using a centrifugal filter device (MWCO 10 kDa) by centrifugation at 13000 rpm for 60 min to remove free Ce6.

3.4 Preparation of CPT MV and CPF MV

The CP NP@TRAIL membrane vehicle (CPT MV) was prepared using the reported procedures.[1, 6] 0.3 mg of the cell membranes after ultrasonic disruption and 0.11 mg of the above obtained CP NP were mixed into 1 mL of ultrapure water and successively extruded through 1 μm, 0.4 μm and 0.2 μm nuclepore track-etched membranes to obtain the final product of CPT MV. The CP NP@TRAIL-free membrane vesicle (CPF MV) was also prepared using the same procedures except that the feeding cell membranes were without TRAIL modifications.

3.5 Quantitative analysis of the components

The amounts of encapsulated Ce6 in CP NP and CPT MV/CPF MV were determined using a Ce6 concentration standard curve measured by UV-Vis absorption measurement (Spectra 206 Max M5, Molecular Devices).[7] In addition, the content of TRAIL protein in the final product was analyzed by Mouse TRAIL ELISA Kit.

4. Characterization

4.1 Identification of the modified TRAIL on the engineered cell membrane

The stable cell line expressing TRAIL protein was seeded in the 20 mm confocal dishes at the density of 2×10^5 per dish overnight. After that, the cells in each dish were rinsed with PBS twice and added with 1 mL of 4% formaldehyde for 15 min and then stained with DAPI (5 μM per dish) and DiI (5 μM per dish). After 15 min of staining, the cells were examined by a confocal laser scanning microscopy CLSM (Zeiss LSM780). To quantitatively analyze the modified TRAIL protein, the genetically engineered cell line and normal CHO-S cell line were digested and resuspended in PBS and finally examined by flow cytometry (BD, FACSVerse, USA) to analyze the percentage of the EGFP-positive cells. The TRAIL expression of the genetically engineered cell line was further verified by western blot analysis, using the procedures reported earlier.[7] Briefly, the cells were fully lysed and the proteins were separated on an SDS-polyacrylamide gel and then transferred from the gel onto a nitrocellulose membrane. After 1 h of blocking with 5% BSA PBS solution, the membrane was incubated with the primary antibody of TRAIL (1 μg/mL) overnight and then incubated with the secondary antibody for 1 h. Finally, the results were revealed by a SuperSignal™ West Pico PLUS Chemiluminescent Substrate and recorded by a ChemiDoc™ MP imaging system (BIO-RAD, USA).

The Hepa1-6 cells cultured in 20 mm confocal dishes were transfected with DR5-mcherry plasmid (pCDH-CMV-DR5-mCherry-EF1α-Blasticidin) using Lipofectamine 3000 Transfection Kit for 36 h. Then, the cells in one dish were incubated with TRAIL MVs (50 μg/mL based on protein content) for 1 h at 37 ℃, while the cells in the other dish were incubated with TRAIL MVs that were pretreated by anti-TRAIL (10 μg/mL, 2 h) as control. Finally, the cells were stained by DAPI and then observed by CLSM.

4.2 Characterization of CP NP and CPT MV

The morphologies of the products were observed by a transmission electron microscope (TEM; FEI Company, TALOS F2000) performed at 200 kV accelerating voltage. The hydrodynamic size, zeta potential, dispersity and stability of the formulations were evaluated by dynamic light scattering (DLS) analysis using a Zeta Sizer (Nano ZS, Malvern, UK). The FT-IR spectra were confirmed by a Fourier Transform Infrared Spectrometer (PerkinElmer, Spectrum-2000). The UV-Vis absorption measurement was conducted on a microplate reader (Spectra 206 Max M5, Molecular Devices). The laser-triggered ROS generation abilities of CP NP and CPT MV were analyzed using ABDA (2 mM) as the indicator measured by UV-Vis spectrometer. 670 nm laser was employed as exciting light for analyzing the laser-triggered ROS (light source: K660E06M-2.000W, BWT Beijing Ltd.).

The total proteins of CPF MV and CPT MV were analyzed by Coomassie blue staining according to the procedures reported previously.[8] The formulations were diluted with loading buffer, boiled and loaded onto an SDS-PAGE gel and stained by Coomassie blue solution. The gel was washed and ultimately exposed with a ChemiDoc™ MP imaging system.

5. *In vitro* experiments

5.1 *In vitro* PDT effects of CP NP

The CCK-8 viability assay was employed to evaluate the PDT effects of CP NP in both hepa1-6 cell line and 4T1 cell line. The cells seeded in 96-well plates at the density of 8×10^3 per well were treated with CP NPs with serial concentrations for 8 h and then cultured with fresh medium, followed by laser irradiation for 5 min (670 nm, 30 mW/cm^2). After another 24 h of incubation, the cells were washed with PBS and then cultured in fresh medium containing 10 μL of CCK-8 solution and 90 μL of culture medium per well for 45 min. The absorption intensity at 450 nm was recorded

at a Spectra Max M5 microplate reader. The relative cell viabilities (%) were measured from four wells in parallel and expressed as the means ± SD, calculated from the formula cell viability (%) = OD (sample)/OD (control) × 100%.

To analyze DR5 expressions after the CP NP-mediated PDT, the cells seeded in 6-well plates at the density of 2×10^5 were incubated with CP NP (at Ce6 concentration of 1.25 μg/mL in hepa 1-6 cells and 1.5 μg/mL in 4T1 cells) for 8 h and then cultured with fresh medium and exposed to laser as mentioned above. After 24 h of laser irradiation, the cells were harvested and the DR5 expressions of the cells were determined by western blot analysis using the abovementioned procedures.

5.2 Cellular uptake of CPT MV

To evaluate the cellular uptake of CPT MV, hepa1-6 cells and 4T1 cells were respectively seeded in 20 mm confocal dishes (at the density of 2×10^5 per dish) for 24 h and then added with CPT MVs (at Ce6 concentration of 1.25 μg/mL in hepa 1-6 cells and 1.5 μg/mL in 4T1 cells respectively). After 12 h of incubation, the cells were washed with PBS and then fixed with 4 % paraformaldehyde and finally stained with DAPI and observed by CLSM.

5.3 *In vitro* cytotoxicity and synergistic anticancer effects of CPT MV

The cytotoxicity of CPT MV in hepa 1-6 cell line, 4T1 cell line and NIH 3T3 cell line was examined using CCK-8 assay described above. The cells were co-incubated with CPT MV for 24 h. The applied formulations were measured by Ce6 concentration (from 0.75 to 1.75 μg/mL, TRAIL concentration from 0.60-1.40 μg/mL).

In order to investigate the synergistic anticancer effects of CPT MV, CCK-8 viability assay was firstly carried out. Hepa 1-6 cells and 4T1 cells were seeded in 96-well plates for 24 h (at the density of 8×10^3 per well). Subsequently, the cells were incubated with CPT MV (at Ce6 concentration of 1.25 μg/mL in hepa 1-6 cells and 1.5 μg/mL in 4T1 cells respectively) for 12 h and then replaced with fresh culture medium. After that, the cells were irradiated by 670 nm laser (30 mW/cm^2) for 5 min and sequentially cultured with CPT MV for another 24 h. Finally, the cell viability was determined by CCK-8 using the same processes mentioned above. The CPF MV was employed as control, which was applied to the cells at Ce6 concentration equal to that of CPT MV.

The anticancer effects of CPT MV were also evaluated by live/dead staining and apoptosis

assays. The cells were treated by CPT MV and laser irradiation using the same methods described above. After 24 h of the indicated treatment procedures, in live/dead staining assay, the cells seeded in a 96-well plate were stained using a LIVE/DEAD Viability/Cytotoxicity Kit and then observed using a fluorescence microscope (Zeiss Axio Vert.A1). For apoptosis assay, the treated cells seeded in a 24-well plate were stained using Annexin V-APC/PI Apoptosis Detection Kit and finally detected by flow cytometry.

5.4 The mechanism of CPT MV-mediated synergistic therapies

To explore the mechanisms of CPT MV-mediated synergistic anticancer effects, intracellular ROS generation, DR5 up-regulation, FADD recruitment, cytochrome c (Cyt c) release, caspase 8 and caspase 3 activations were carefully evaluated. The CPF MV was employed to evaluate the pure PDT effects as control. Hepa 1-6 cells were used in the following studies. The cells were treated by CPT MV and laser irradiation using the same methods described above. For detecting intracellular ROS productions, after 12 h of co-incubation with CPT MV, the cells seeded in 96-well plates were washed with PBS and then cultured with fresh medium containing 0.1 mM of DCFH-DA probe (per well) for 1 h. Thereafter, the medium was replaced and then the cells were exposed to laser irradiation (670 nm, 30 mW/cm^2) for 5 min and eventually observed by a fluorescence microscope.[7] The DR5 up-regulations after CPT MV-mediated PDT were verified by western blot analysis. For observing FADD recruitments, after receiving CPT MV-mediated PDT, the cells seeded in confocal dishes were washed, fixed and then added with 0.1 % Triton-100 to break cell membranes for 15 min. Subsequently, the cells were cultured with 5 % BSA PBS solution for 1 h and then consecutively incubated with anti-FADD and relevant secondary antibody according to the manufacturer's protocol. Ultimately, the cells were washed and stained with DAPI before observation under the CLSM. For analyzing the protein expressions (cleaved caspase 8/3, Cyt c), western blot analysis was performed using the procedures mentioned above. For ROS, DR5 and FADD analysis assays, GSH was used to neutralize the generated ROS as control. For protein evaluation assays, the CPT MV was pretreated with anti-TRAIL as control to further conform the TRAIL functions.

6. *In vivo* experiments

All animal experiments were operated strictly according to the "National animal management

regulations of China" and the guidelines approved by the Animal Ethics Committee of Mengchao Hepatobiliary Hospital of Fujian Medical University.

6.1 Tumor model

To establish the tumor model, 1×10^7 hepa 1-6 cells suspended in 100 μL of PBS were subcutaneously injected into the right flank of hind leg region of male C57BL/6 mice (Wushi Laboratory Animal Co. Ltd., China). 7 days later (on Day 0), when the tumor size reached approximately 70 mm^3, the tumor-bearing mice were randomly divided into different groups.

6.2 Hemolysis property analysis

The hemolysis assay was performed according to previously reported methods.[9] Red blood cells were isolated and purified from whole blood of healthy C57BL/6 mice. The CPT MV PBS solution (Ce6 concentration: 1-100 μg/mL) mixed with red blood cells was incubated at 37 ℃ for 4 h, and then the mixture was centrifugated at 4000 rpm for 10 min. The sample was photographed and the absorbance of supernatant at 542 nm was determined by UV-Vis absorption measurement. Distilled water and PBS were used as positive and negative controls.

6.3 *In vivo* tumor accumulation ability of CPT MV

To investigate the tumor accumulation effects of CPT MV after systemic administration, four tumor-bearing mice were intravenously injected with CPT MV ((Ce6: 2 mg/kg) in 200 μL of PBS). After 2-24 h of intravenous injection, the tumor-bearing mice were sacrificed. Thereafter, the major organs and tumors were dissected from the mice and finally imaged by a ChemiDoc™ MP imaging system.

6.4 *In vivo* antitumor effects mediated by CPT MV

To evaluate the CPT MV-mediated antitumor effects *in vivo*, the tumor-bearing mice were randomly allocated into five groups: (1) PBS; (2) Laser; (3) CPT MV; (4) CPF MV + Laser; (5) CPT MV + Laser. The details of each group were explained as follows:

(1) PBS: PBS as control;

(2) Laser: pure laser irradiation;

(3) CPT MV: pure TRAIL effects;

(4) CPF MV + Laser: pure PDT effects;

(5) CPT MV + Laser: synergistic antitumor effects.

All formulations in 200 μL of PBS (at an equivalent Ce6 dosage: 2 mg/kg) were intravenously injected into the mice on day 0 and day 2. After 12 h of each injection, the tumor site was exposed to 670 nm laser irradiation at the power density of 0.4 W/cm^2 for 5 min. The therapeutic effects were examined by monitoring tumor volume in each group every 2 days, up to 16 days. The tumor size was measured using an electronic caliper. The tumor volume (V) was calculated using the equation tumor length × (tumor width)^2/2. Relative volume V/V_0 (V_0 as the initial tumor volume before therapy) was used to evaluate the relative tumor growth rate. Simultaneously, the body weights of the treated mice were surveyed at the same time to evaluate the side effects. All the tumors were harvested at the end of the studies for the following photographing and weighing.

6.5 Histological change and protein expression evaluation of treated tumors

To evaluate the histological changes of tumors and protein expressions, one tumor-bearing mouse was sacrificed in each group on day 3. The harvested tumors from the treated mice were fixed in formalin, embedded by paraffin, and further sectioned into slices with thickness of 4 mm. The slices were stained with hematoxylin and eosin (H&E) for histopathology, and respectively stained with anti-Ki67, anti-cytochrome c and anti-cleaved caspase 3 for immunohistochemical analysis, and finally observed by a Zeiss microscope (Axio Lab.A1). The slice stained with anti-DR5, anti-cleaved caspase 8 and TUNEL were imaged by CLSM for DR5, cleaved-caspase 8 and TUNEL immunofluorescence analysis.

To demonstrate the ROS generation after CPT MV-mediated PDT *in vivo*, the mice after 10 h of CPT MV injection (on day 2) were intratumorally injected with DCFH-DA probe (100 mM, in 30 μL of PBS). After 2 h of DCFH-DA injection, the tumor sites were exposed to laser irradiation. Subsequently, the treated tumors were extracted and made into frozen sections and observed by CLSM.

6.6 Systemic toxicity of CPT MV analysis

To analyze the potential systemic toxicity of CPT MV, at the end of indicated treatments, the major organs of one treated mouse in each group were harvested, fixed in formalin, embedded by paraffin, sectioned into slices, stained with H&E and finally observed by a Zeiss microscope. In addition, the serum biochemistry analyses were also performed to evaluate the biosafety of CPT MV. The blood of the mice after indicated treatments was collected in EDTA2K spray-coated tubes

and analyzed using an automated hematology analyzer.

Statistical analysis

All quantitative data were presented as the mean ± standard deviation (SD). Statistical analysis was performed using GraphPad Prism 8. Statistical significance (*$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$) was evaluated using two-tailed Student t-test for comparison between two groups and one-way analysis of variance (ANOVA) for comparison among diverse groups. When $p < 0.05$, there is statistical significance between the compared groups.

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Supplementary Figures

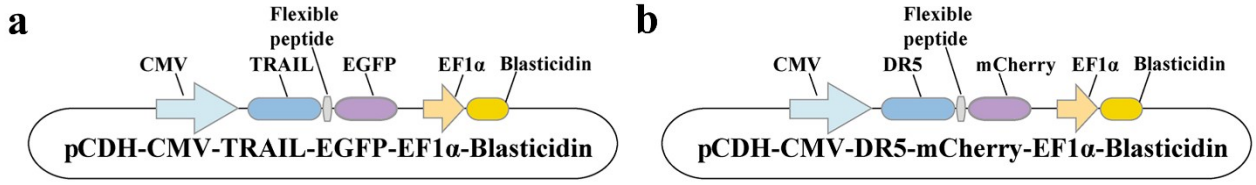

Figure S1. Information of the plasmids employed in the current work: a) the plasmid encoding murine TRAIL with EGFP-tag (pCDH-CMV-TRAIL-EGFP-EF1α-Blasticidin) and b) the plasmid encoding DR5 with mCherry-tag (pCDH-CMV-DR5-mCherry-EF1α-Blasticidin).

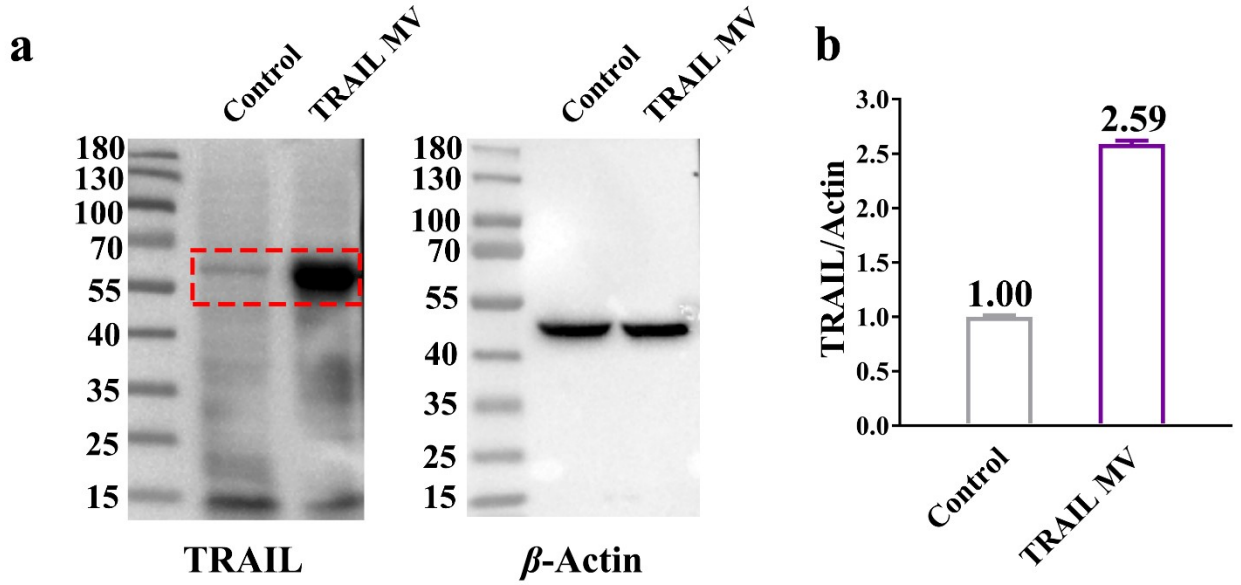

Figure S2. (a) Full gel scan images. (b) The gray value ratios of TRAIL/Actin. Related to Figure 2d.

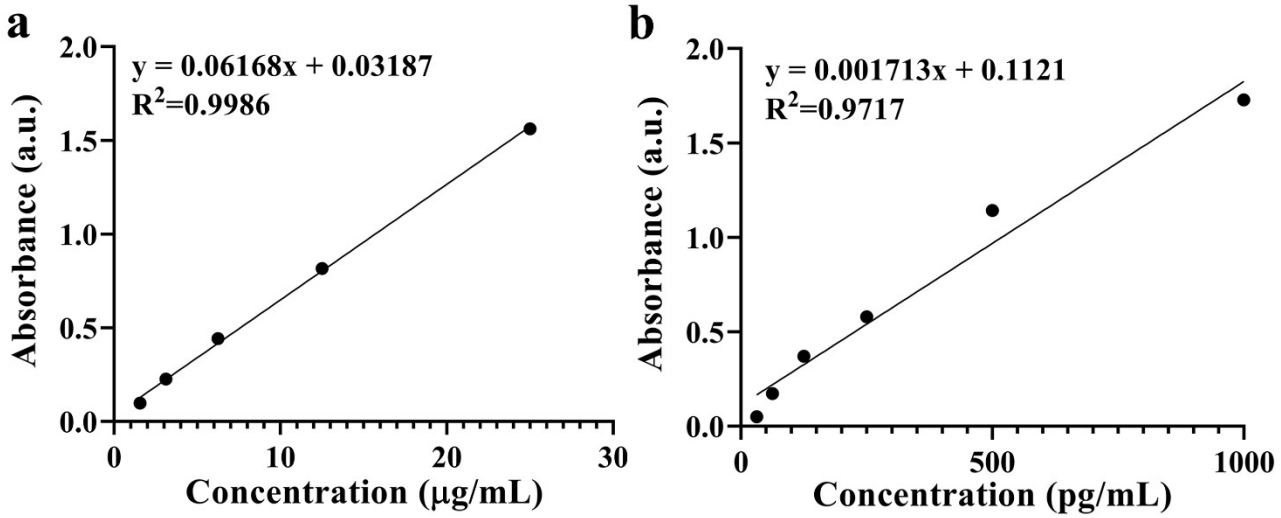

Figure S3. (a) The standard curve of Ce6 (absorbance at 670 nm) measured by UV-Vis absorption analysis. (b) The standard curve of TRAIL measured by ELISA analysis.

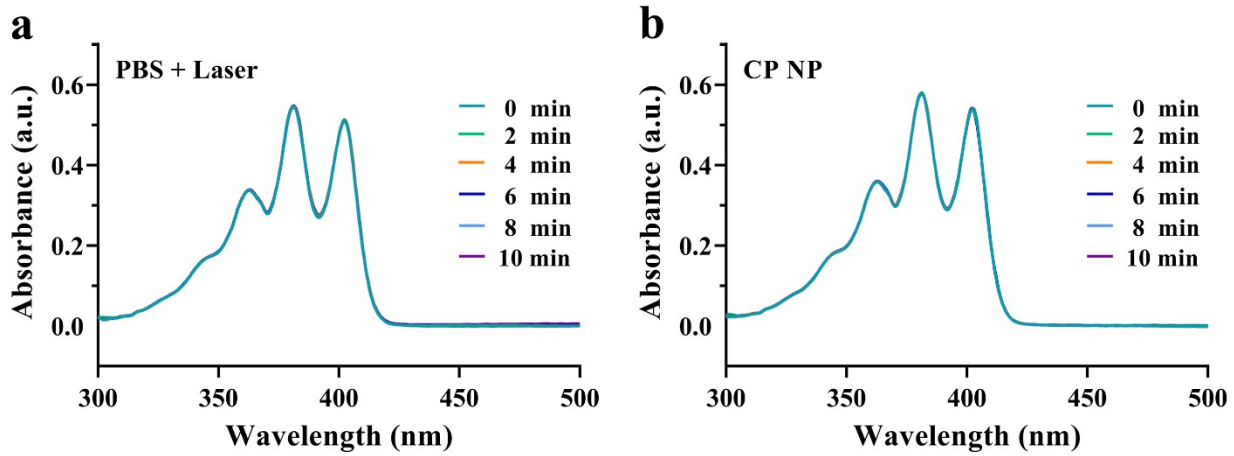

Figure S4. (a) The UV-Vis spectra of ABDA treated with PBS under different times of laser irradiation. (b) The UV-Vis spectra of ABDA treated with CP NP under different times of room light. (670 nm laser, 30 mW/cm^2).

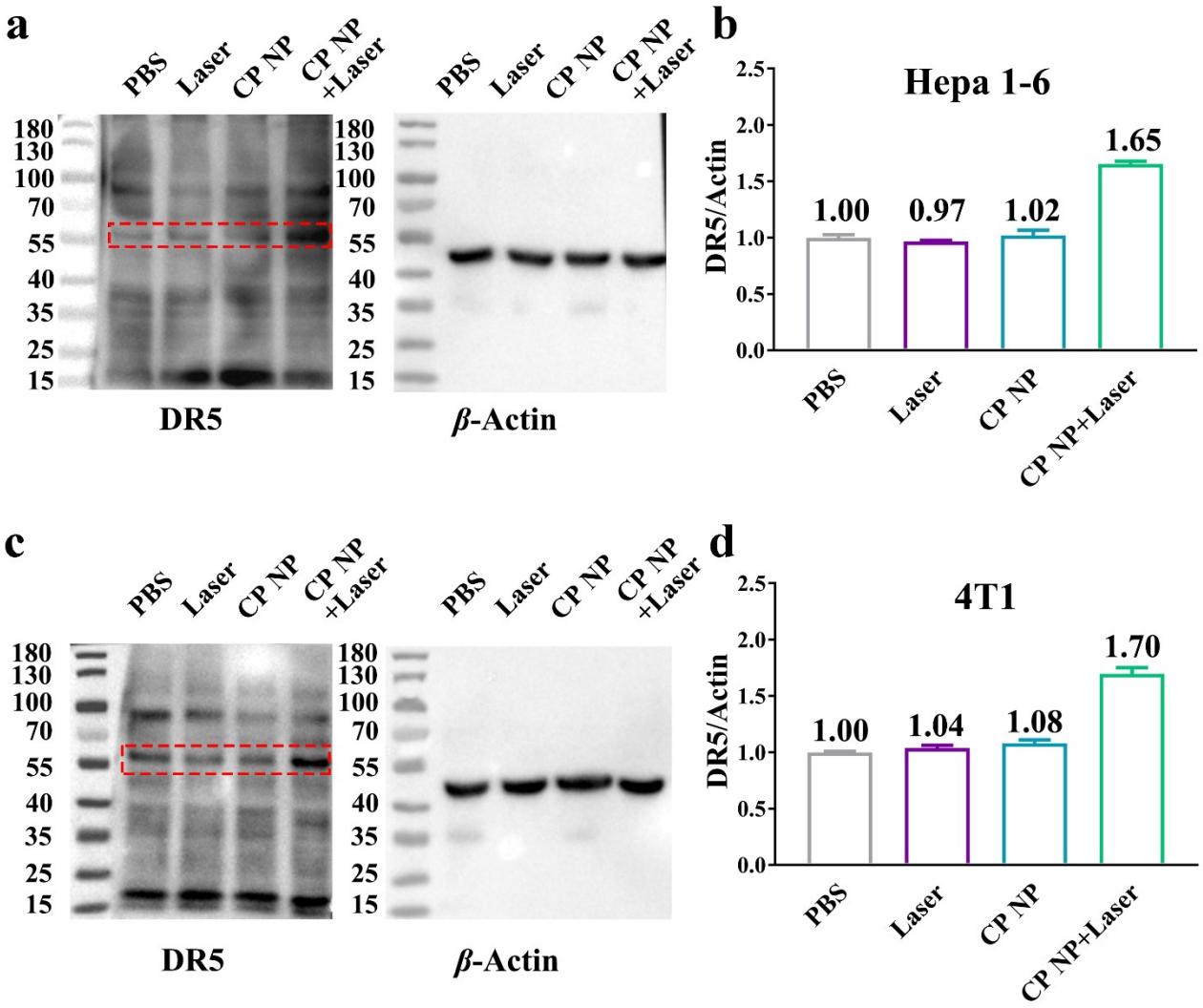

Figure S5. (a) Full gel scan images related to Figure 3h. (b) The gray value ratios of DR5/Actin related to Figure 3h. (c) Full gel scan images related to Figure 3i. (d) The gray value ratios of DR5/Actin related to Figure 3i.

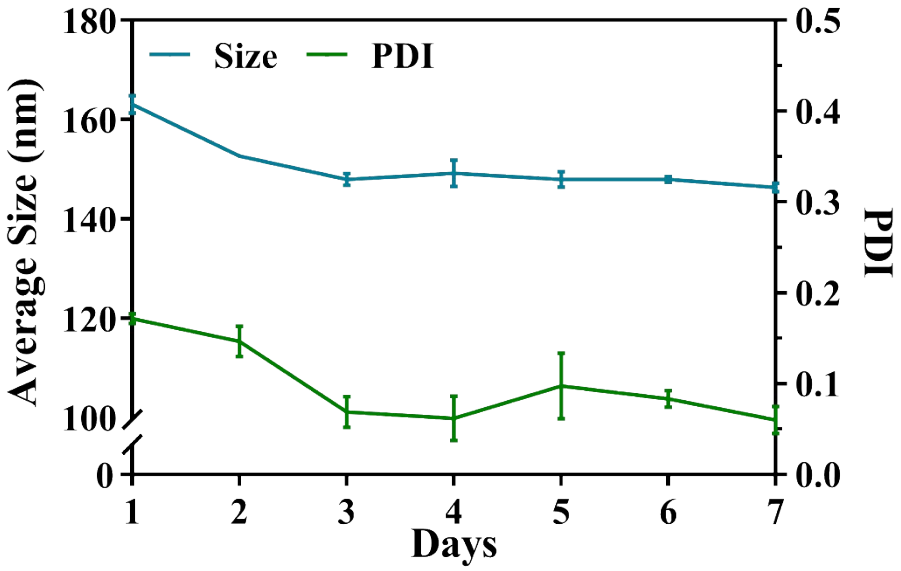

Figure S6. The hydrodynamic size and the corresponding polydispersity index (PDI) of CPT MV in PBS stored for 7 days measured by DLS.

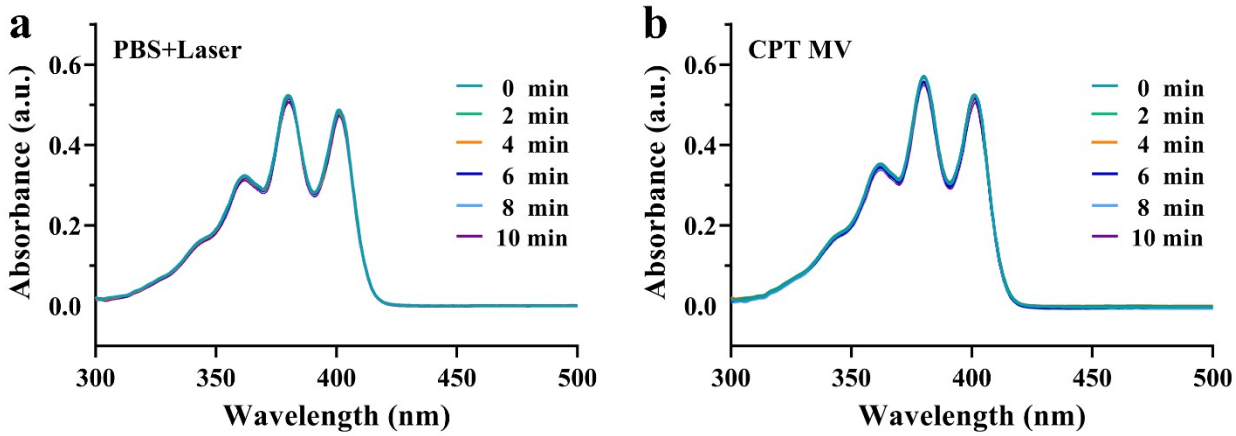

Figure S7. (a) The UV-Vis spectra of ABDA treated with PBS under different times of laser irradiation. (b) The UV-Vis spectra of ABDA treated with CPT MV under different times of room light. (670 nm laser, 30 mW/cm^2).

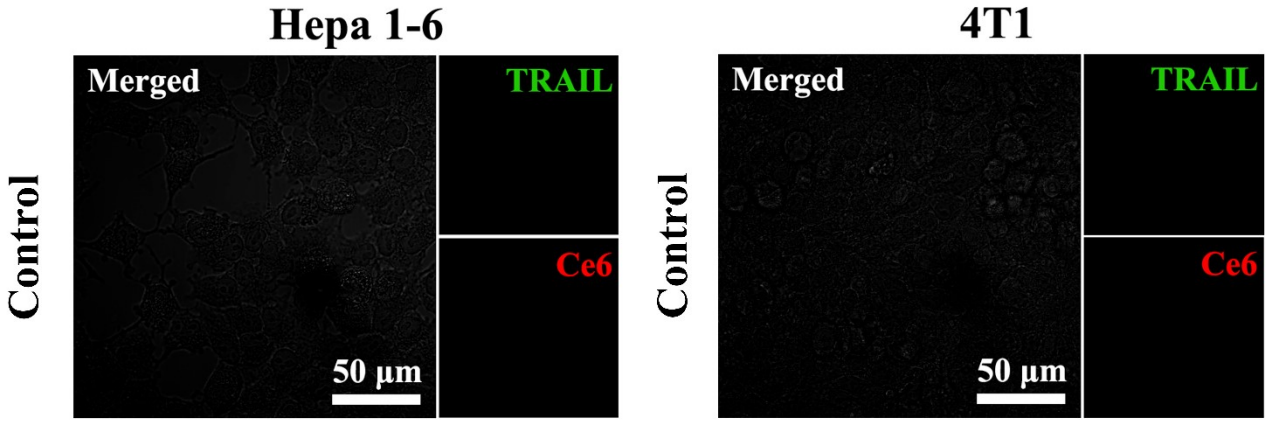

Figure S8. CLSM images of hepa 1–6 cells and 4T1 cells without CPT MV treatment as control.

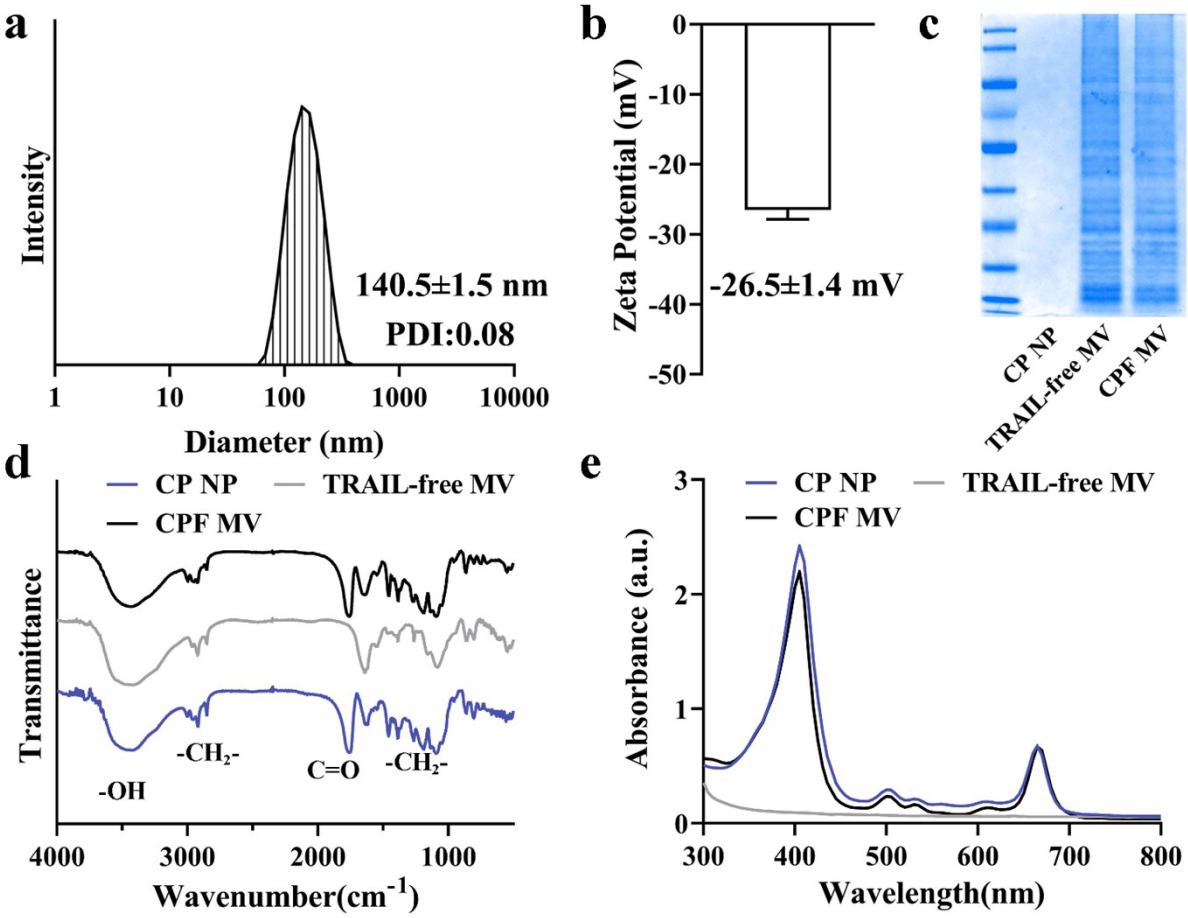

Figure S9. (a) Size distribution and (b) zeta potential of CPF MV determined by DLS. (c) Coomassie blue staining, (d) FT-IR spectra and (e) UV-Vis absorption spectra of CP NP, TRAI-free MV and CPF MV. The hydrodynamic size and zeta potential of CPF MV were similar to those of CPT MV. Coomassie blue staining revealed that TRAIL-free MV and CPF MV contained the same proteins. The results of FT-IR and UV-Vis absorption measurements demonstrated that CPF MV consisted of CP NP and TRAIL-free membrane. All these results indicated the successful fabrication of CPF MV.

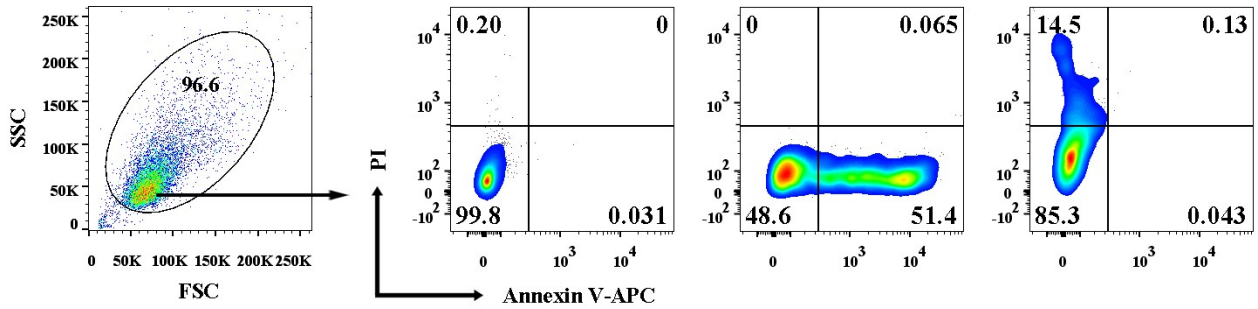

Figure S10. Representative FACS gating strategy for detecting the apoptotic and necrotic cells. Ralated to Figure 5d.

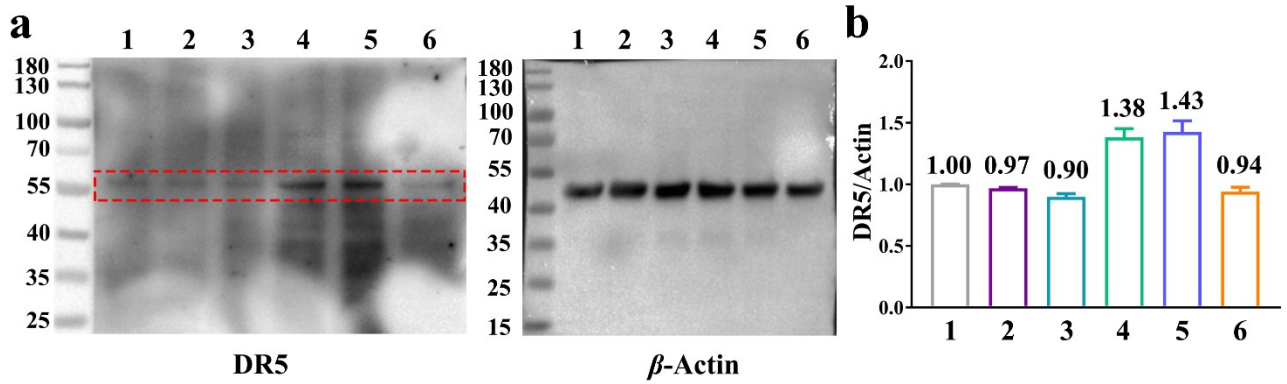

Figure S11. (a) Full gel scan images. (b) The gray value ratios of DR5/Actin. Related to Figure 6c. (Group1: PBS, Group2: Laser, Group3: CPT MV, Group4: CPF MV + Laser, Group5: CPT MV + Laser, Group6: CPT MV + Laser + GSH)

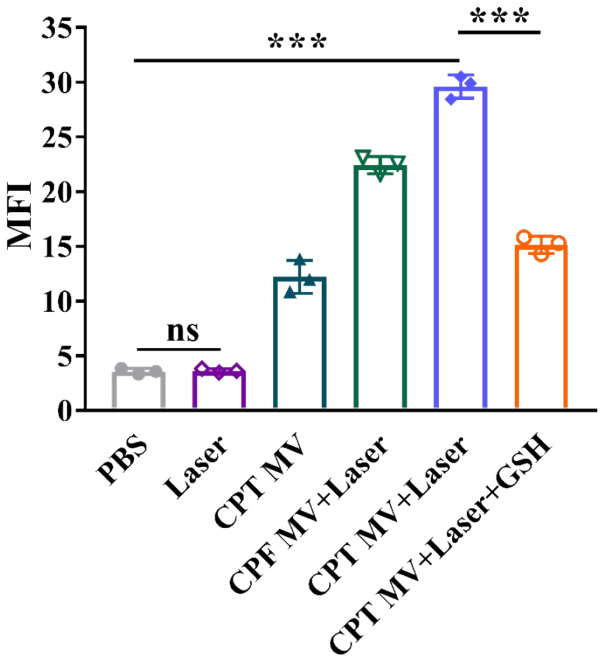

Figure S12. Mean florescence intensity of FADD related to Figure 6d (mean ± SD, n = 3, *p < 0.05, **p < 0.01, ***p < 0.001).

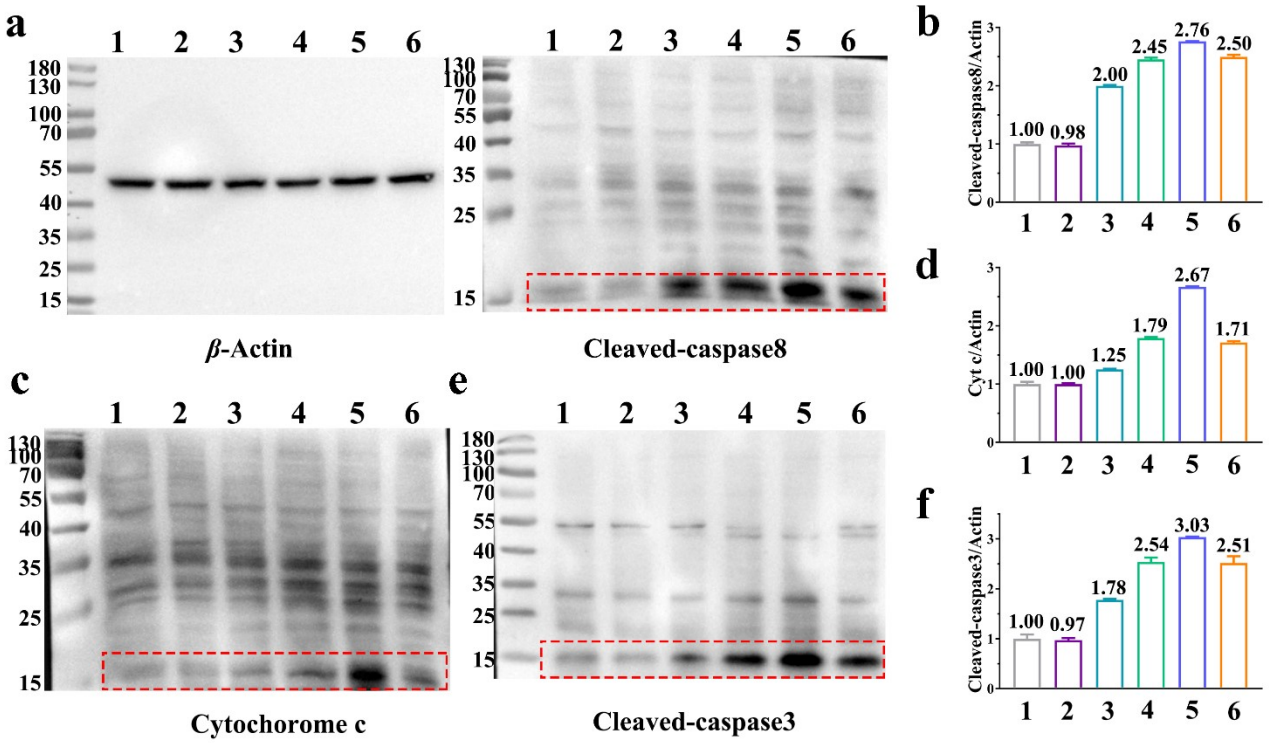

Figure S13. (a) Full gel scan images of cleaved-caspase 8 western blot analysis. (b) The gray value ratios of Cleaved-caspase 8/Actin. (c) Full gel scan images of Cyt c western blot analysis. (d) The gray value ratios of Cyt c/Actin. (e) Full gel scan images of cleaved-caspase 3 western blot analysis. (f) The gray value ratios of Cleaved-caspase 3/Actin. (Group1: PBS, Group2: Laser, Group3: CPT MV, Group4: CPF MV + Laser, Group5: CPT MV + Laser, Group6: CPT MV + Laser + anti-TRAIL)

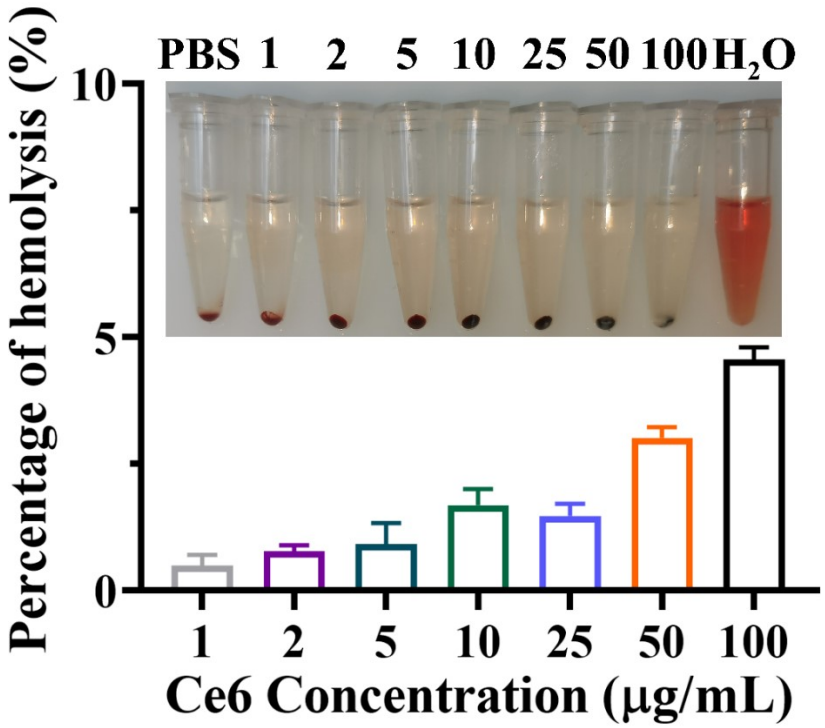

Figure S14. Hemolysis assay of red cells treated with different concentrations of CPT MV.

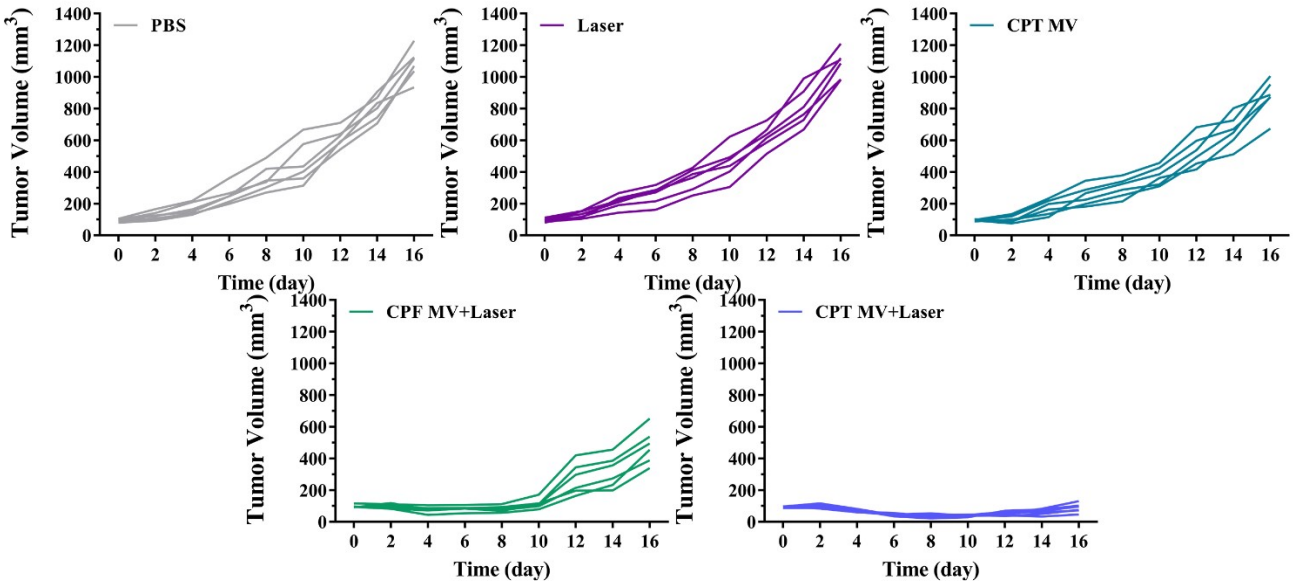

Figure S15. Tumor volume changes of each mouse in different groups. Related to Figure 7f.

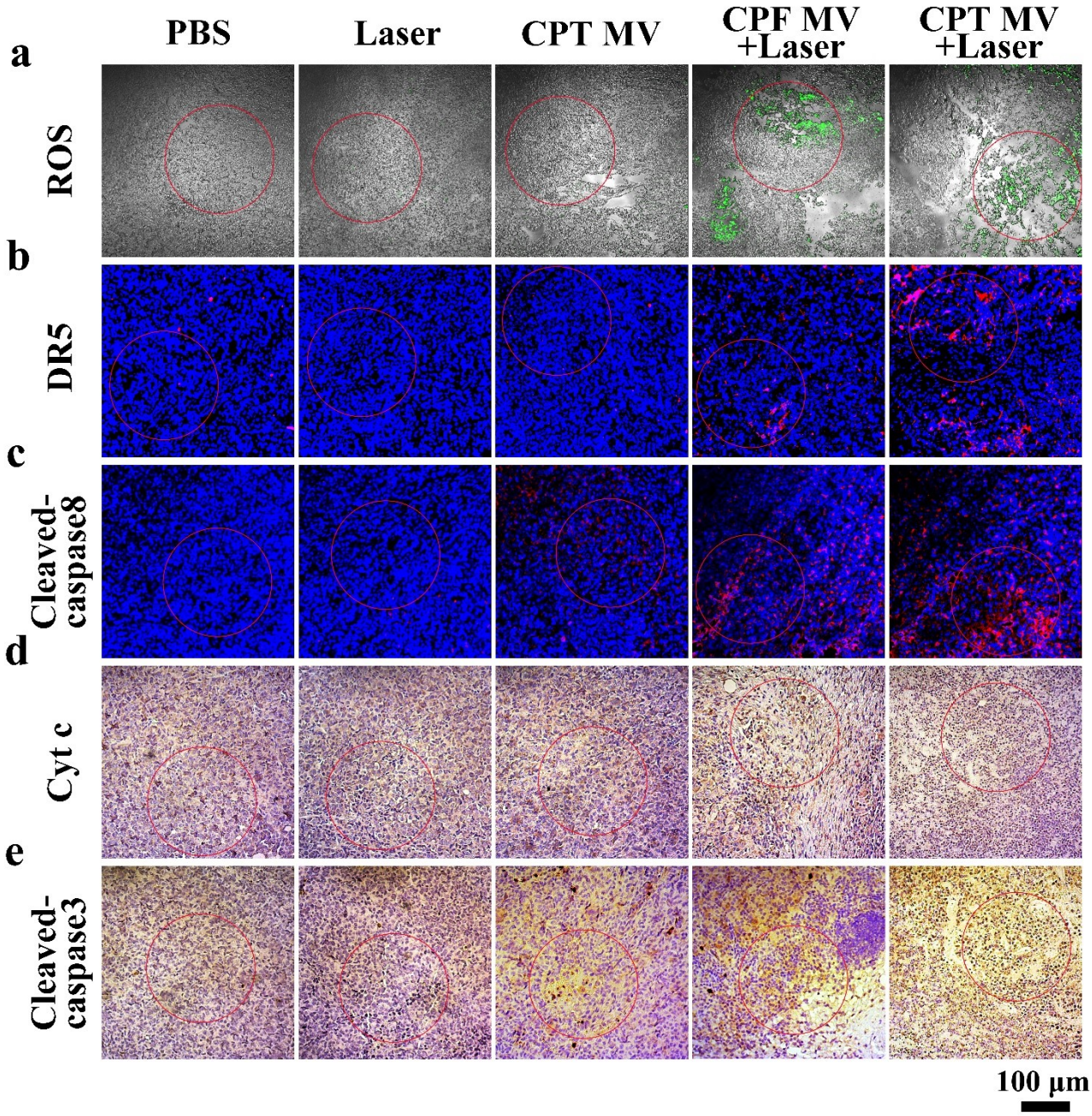

Figure S16. (a) CLSM images of the frozen sections of the extracted tumors receiving intratumoral DCFH-DA injection and different treatments. (b) DR5 immunofluorescence staining, (c) Cleaved-caspase 8 immunofluorescence staining, (d) Cyt c immunohistochemical staining and (e) Cleaved-caspase 3 immunohistochemical staining of the dissected tumors on day 3. Original images without cropping related to Figure 8.

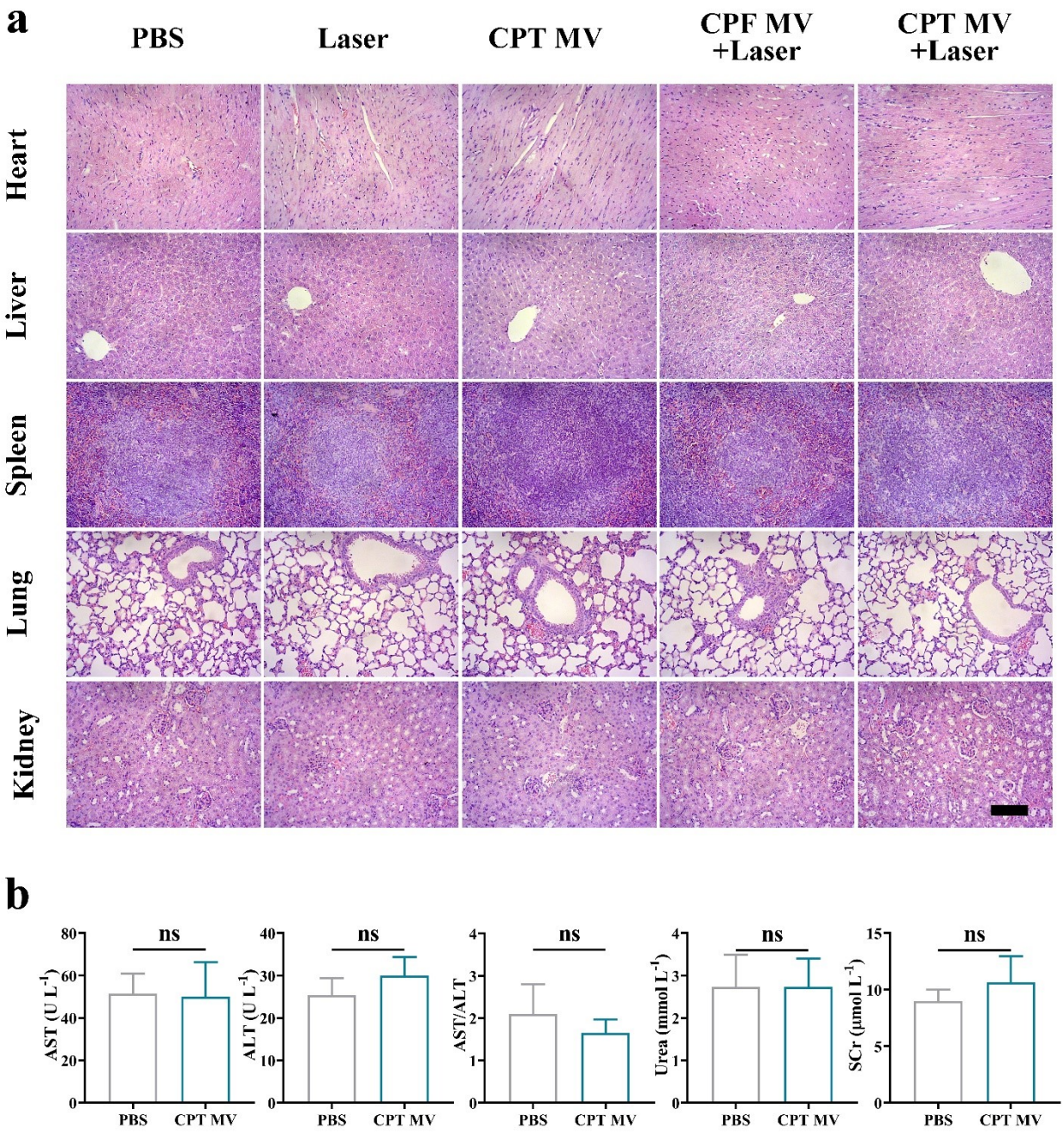

Figure S17. (a) H&E staining of the harvested organs at the end of the indicated treatments. Scale bar: 100 μm. (b) The serum biochemical indicators (hepatic and renal functions) of the mice after intravenous injection of different formulations.