Supporting Information

pH and Thermal Dual-Sensitive Nanoparticle-Mediated Synergistic Anti-Tumor Effect of Immunotherapy and Microwave Thermotherapy

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Materials and methods

Materials. Acrylonitrile (AN) was purchased from Beijing Qinghongfu Technology Co. Ltd. (Beijing, China) and purified by atmospheric distillation before use. Acrylamide (AAm), azodiisobutyronitrile (AIBN), and dimethyl sulfoxide (DMSO) were provided by Aladdin (Shanghai, China). The methoxy polyethylene glycolsuccinimidyl carbonate (mPEG-SC) was purchased from Seebio BiotechCo., Ltd (Shanghai, China). The LfcinB (Mw=3.1kDa) was synthesized by Qiangyao Biological Co. Ltd (Shanghai, China). Hyaluronic acid (HA) (Mw = 4 kDa) was obtained from Bloomage Freda Biopharma Co. Ltd (Tianjin, China). Fluorescein isothiocyanate isomer I (FITC) and indocyanine green (ICG) were from Aladdin (Shanghai, China). Rhodamine B isothiocyanate (RITC) was provided by Yuanye Biological Technology Co. Ltd (Shanghai, China). RPMI 1640 medium and fetal bovine serum (FBS) obtained from Sigma (St. Louis, MO, USA) and Sijiqing Biological Engineering Materials Co. Ltd (Hangzhou, China), respectively. The ELISA kits were all purchased from Meimian industrial Co., Ltd (Jiangsu, China).

Synthesis and characterization of P-(AAm-co-AN)-PEG. Firstly, P-(AAm-*co*-AN) with a UCST of 43°C was synthesized by solution copolymerization of AN and AAm initiated by AIBN. Briefly, 11.218g (158mmol) of AAm was weighed into a 500-mL three-necked flask and dissolved in 167mL of anhydrous DMSO. Subsequently, 2.226g (42mmol) of AN was added. Nitrogen was pumped for 1 h to remove the oxygen from the system. After that, 33mL of separately degassed anhydrous DMSO containing 0.5165g (3.145mmol) of AIBN was dropped into the system through a constant pressure dropping funnel. Then placed the flask into a water bath which had been preheated to 60 °C. The reaction mixture was subsequently polymerized for 8h under nitrogen protection and rapidly cooled to room temperature in an ice bath. The product was precipitated in 10-fold excess volume of methanol. The precipitate was then washed thrice with methanol and dried in a vacuum oven at 70°C for 24h. Next the PEG was introduced to P-(AAm-*co*-AN) to improve the properties of the polymer for later *in vivo* application. Briefly, 140mg of P-(AAm-*co*-AN) was weighed into a 50-mL flask and dissolved in 10mL of DMSO, to which 340mg of mPEG-SC (Mw = 5kDa) was added. The flask was placed into a water bath that had been preheated to 50°C, and the reaction mixture was stirred for 48h. The reaction mixture was then dialysis against deionized water with a dialysis membrane (MWCO: 7kDa) for 48h, followed by lyophilization and the P-(AAm-*co*-AN)-PEG was obtained.

The ^{1}H-NMR spectra of the polymers were obtained using an NMR spectrometer (AC-80, BrukerBioSpin, Germany). P-(AAm-*co*-AN), mPEG-SC, and P-(AAm-*co*-AN)-PEG were dissolved in DMSO-*d6* at concentrations of 20mg/mL. The molecular weights of the copolymers were analyzed using gel permeation chromatography (GPC) with DMSO as an eluent. PLgel MIXED-C columns (particle size: 5μm; dimensions: 7.5mm × 300mm) that had been calibrated with narrow dextran monodisperse standards were employed with a differential refractive index detector. The flow rate was 0.6mL/min. Dispersed the polymers in water at a concentration of 1mg/mL to facilitate the determination of UCST value, the optical transmittance of polymer solutions at different temperature was measured at 637nm using an ultraviolet-visible spectrophotometer (UV-2401, Shimadzu, Japan). The UCST value of P-(AAm-*co*-AN) and P-(AAm-*co*-AN)-PEG were determined at the temperature when the optical transmittance became constant.

Preparation and characterization of LfcinB/HA/P-(AAm-co-AN)-PEG Nanoparticles (LHPN).

Due to the large number of amino groups, P -(AAm-*co*-AN)-PEG exhibits electro positivity in aqueous solution and the higher temperature of the solution, the more positive charge is on the polymer. As a cationic peptide, LfcinB is also positively charged in aqueous solution. Here, we combined P-(AAm-*co*-AN)-PEG, LfcinB and HA, which is electro negative, to form a ternary composite nanoparticle through electrostatic interaction. Briefly, 10mg of HA was charged in a vial bottle and dissolved in 0.5mL deionized water. 1mg of LfcinB was dissolved in 0.5mL deionized water and added to the vial bottle dropwise at room temperature. After stirring for a while, the binary composite nanoparticles (LHN) formed by LfcinB and HA can be obtained. Subsequently, 0.5mg of P-(AAm-*co*-AN)-PEG dissolved in 50μL hot water (50℃) was added to the LHN solution which was preheated to 50℃ and stirred for 30min. Cooling to room temperature and the ternary composite nanoparticles (LHPN) composed of LfcinB, HA and P-(AAm-*co*-AN)-PEG was obtained. The LHPN solution was then analyzed by dynamic light scattering (DLS) and TEM. The encapsulation efficiency (EE) and drug loading (DL) were determined by assaying the filtrate of ultrafiltration centrifugation by HPLC (Mobile phase: acetonitrile: water was 74: 26, containing 1‰ of trifluoroacetate; Detection wavelength: 220nm). EE% and DL% were calculated by the following formulas:

$$\mathrm{EE\%} = \frac{total\ \ amount\ \ of\ \ drug\ \ added - the\ \ amount\ \ of\ \ drug\ \ in\ \ the\ \ filtrate}{total\ \ amount\ \ of\ \ drug\ \ added} \times 100\%$$

$$\mathrm{DL\%} = \frac{total\ \ amount\ \ of\ \ drug\ \ added - the\ \ amount\ \ of\ \ drug\ \ in\ \ the\ \ filtrate}{weight\ \ of\ \ NPs} \times 100\%$$

The blank nanoparticles were prepared in the same manner as LHPN except that LfcinB was not added.

pH and thermal-triggered size changes of LHPN. The size changes of LHPN in response to pH and thermal were monitored by DLS. LHPN solution was divided into five aliquots and diluted 10 times with PBS and treated as follows for 30min: (i) PBS (pH7.4), 25℃, (ii) PBS (pH7.4), 37℃, (iii) PBS (pH7.4), 43℃, (iv) PBS (pH5.5), 37℃, (v)PBS (pH5.5), 43℃. Subsequently, the particle sizes were measured by DLS. There are three repeat groups for each sample.

The pH and thermal dual-sensitive drug release behavior of LHPN. The release profiles of LfcinB-loaded LHPN in distinct environments were tested by dialysis method. The dialysis bags containing 0.5mL of LfcinB and LHPN (concentration of LfcinB was 1mg/mL) were immersed into falcon tubes containing 4.5mL PBS with pH 5.5 and 7.4, respectively. The tubes were put into incubator shakers (37℃ and 43℃, respectively) and horizontally shaken at 100rpm/min. At each pre-set time point, 200μL of release medium was withdrawn and measured by HPLC. Each time point was performed trice.

To further verify that LHPN has a stimuli-responsive drug release behavior, another release experiment was performed. LHPN was first released at 37℃ (pH7.4) for 6h, then adjusted the pH of release medium to 5.5 and shaken at 43℃ for 30min to destroy the LHPN. After that, the pH was adjusted back to 7.4 and continued to release for anther 8h at 37℃. 200μL of release medium was withdrawn at each pre-set time point and measured by HPLC. Each time point was performed trice.

Cell culture. CT-26 cells in RPMI 1640 medium supplemented with 10% (v/v) FBS and penicillin/streptomycin (100 U/mL of each) were maintained in the cell incubator (37℃ and 5% CO_2). The cells were regularly split using trypsin/EDTA. For the hyperthermia treated groups, the cells were placed in the cell incubator (43℃ and 5% CO_2, 30min) at 6h post-exposure to the test agents, followed

by incubation at 37℃ for pre-set time period.

Cellular uptake. For ease of detection and observation, LfcinB was labeled with FITC to form FITC-LfcinB. Briefly, 10mg of LfcinB was charged into a vial bottle containing 4mL deionized water, then 0.65mg of FITC was weighed into this solution and the resulting mixture was stirred overnight at room temperature. The reaction system was dialyzed against de-ionized water for 24h and the obtained solution was stored in fridge for following use. LHPN prepared by FITC-LfcinB can be used to determine the cellular uptake.

CT-26 cells were suspended in serum-free medium and seeded in 24-well plate at a density of 5×10^4 cells per well and allowed to attach overnight. Subsequently, the cells were treated with FITC labeled LfcinB and LHPN (at a final LfcinB concentration of 100μg/mL) respectively for 1, 2, 4, 6, 10 and 24 hours. For qualitative investigation, the cells were washed trice with PBS and fixed, the nuclei were stained with DAPI and observed using fluorescence microscope (Axio Observer A1, Zeiss, Germany). For quantitative investigation, the cells were resuspended in PBS and fluorescence intensity was determined by flow cytometry (FC 500 MCL; Beckman Coulter, USA), blanked by untreated cells. In addition, the intra- and extracellular amount of LfcinB was determined directly by HPLC. The cells were treated with free LfcinB or LHPN (at a final LfcinB concentration of 100μg/mL) for 6h or 24h. The medium was collected and the cells were harvested and lysed. To test the free LfcinB treated group, the medium was directly examined by HPLC, while the cell lysates were dissolved in methanol overnight to extract LfcinB, followed by HPLC detection. As for the LHPN treated group, both of the medium and cell lysates were adjusted to pH5.5 and incubated at 43℃ for 30min to stimulate LfcinB released from LHPN, then the test method was the same as above.

Intracellular drug release. The intracellular drug release was determined by fluorescence resonance energy transfer (FRET) and conducted with two probes, FITC and RITC, as donor and acceptor, respectively. FITC was conjugated on LfcinB and the protocol referred to the section of **Cellular uptake**. P-(AAm-*co*-AN)-PEG was modified by RITC. For details, 10mg of P-(AAm-*co*-AN)-PEG was charged into a vial bottle containing 5mL DMSO, then 0.8mg of RITC was weighed into this solution and the resulting mixture was stirred overnight at room temperature. The reaction system was dialyzed against de-ionized water for 24h and lyophilized. First, the FRET phenomenon was verified in the LHPN solution. LHPN prepared by FITC-LfcinB, HA and RITC-P-(AAm-*co*-AN)-PEG was used for detection and the damaged LHPN was destroyed under the acidic and thermal conditions (pH5.5, 43℃) for 30min. The emission spectra of FITC-LfcinB, RITC-P-(AAm-*co*-AN)-PEG, completed LHPN and damaged LHPN were recorded at room temperature with a fluorescence spectrometer (F-2500, Hitachi High-Technologies Co., Japan) with 450nm as the excitation wavelength. The split widths for the excitation and emission were 20 and 2.5nm, respectively.

To further confirm the intracellular LfcinB released from LHPN with hyperthermia treatment, confocal laser scanning microscope (CLSM) observations were performed. Briefly, CT-26 cells were suspended in serum-free medium and seeded in 12-well plate at a density of 1×10^5 cells per well and allowed to attach overnight. Subsequently, the cells were treated with LHPN (composed of FITC-LfcinB, HA and PITC-P-(AAm-*co*-AN)-PEG, at a final LfcinB concentration of 100μg/mL) for 6 hours. Then the hyperthermia treated group was placed in the cell incubator which had pre-set to 43℃ for 30min, followed by incubation at 37℃ for 30min. After washed trice with PBS and fixed, the cells were

observed using CLSM (IX83-FV3000-OSR, Olympus, Japan).

Cytotoxicity and apoptosis. Firstly, the cytotoxicity of blank nanoparticles was measured by MTT assay. CT-26 cells were suspended in serum-free medium and seeded in 96-well plate at a density of 1×10^4 cells per well and allowed to attach overnight. Then the cells were exposed to blank nanoparticles at a series of concentrations for 48 hours. Subsequently, 20μL of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution (5mg/mL) was added to each well for an additional 4 hours incubation at 37℃. After that, the medium was replaced with 100μL of DMSO to dissolve the purple formazan crystals in the bottom of the well. The plate was shaken for 30min, and the absorbance of the solution in each well was measured by microplate reader at 570nm. Cell viability was calculated in reference to negative cells without exposure to test agents. All of experiments were repeated thrice.

Subsequently, the anti-tumor effects of free LfcinB and LHPN combined with or without hyperthermia were determined by MTT assay. CT-26 cells were suspended in serum-free medium and seeded in 96-well plate at a density of 1×10^4 cells per well and allowed to attach overnight. Then the cells were exposed to free LfcinB or LHPN at different concentrations (at a final LfcinB concentration of 0, 10, 20, 50, 80, 120, 160, 200, 250 and 300μg/mL) for 48hours. The hyperthermia treated groups were placed in the cell incubator which had pre-set to 43℃ for 30min at 6h post-exposure to the test agents, followed by incubation at 37℃. Cell viability was measured as described above. In addition, the cytotoxicity of LHPN to the Human Umbilical Vein Endothelial Cells (HUVECs) was examined according the protocol mentioned above.

Cell apoptosis induced by free LfcinB and LHPN combined with or without hyperthermia were investigated by western blot and flow cytometry. For western blot, CT-26 cells were suspended in serum-free medium and seeded in 6-well plate at a density of 5×10^5 cells per well and allowed to attach overnight. The cells were exposed to LfcinB and LHPN (at a final LfcinB concentration of 200μg/mL) treated with or without hyperthermia for 8h. Then cells were lysed and subjected to SDS-PAGE, immunoblotted with caspase-3 antibodies and β-actin antibodies (1:10000, Abcam, UK). Finally, the proteins were visualized by Chemiluminescence imager (ChemiDoc XRS+, Bio-Rad, USA). For flow cytometry, the apoptosis effect was evaluated by Annexin V-FITC/PI apoptosis detection kit (Beyotime Biotech, China). CT-26 cells were treated as depicted in the western blot assay. Subsequently, the cells were harvested and stained according to the manufacturer's instructions. Finally, the cells were analyzed by flow cytometer.

The exposure of DAMPs (CRT, HMGB1 and ATP) of tumor cells after different treatment were detected. Briefly, CT-26 cells were treated with free LfcinB or LHPN (at a final LfcinB concentration of 200μg/mL) with or without hyperthermia. The expression of CRT was observed by their immunofluorescence *via* CLSM at the time of 8h (Calreticulin Rabbit Monoclonal Antibody, 1:500, Beyotime, China). Semi-quantitative analysis was performed using Image J software. After incubating for 48h, the cell culture supernatant was collected and the contents of ATP and HMGB1 were detected by corresponding ELISA kits.

Mitochondrial targeting and mitochondrial membrane potential (Δψm) measurement. CT-26 cells were suspended in serum-free medium and seeded in 12-well plate at a density of 1×10^5 cells per well and allowed to attach overnight. Then the cells were treated with FITC labeled LfcinB and LHPN (at a final LfcinB concentration of 100μg/mL) respectively for 2, 4 and 8 hours. Afterwards, the medium was

removed and mitochondria was stained by MitoTracker Deep Red FM dye (Thermo Fisher Scientific, USA). After washed trice with PBS and fixed, the nuclei were stained with DAPI and observed using CLSM.

The mitochondrial membrane potential (Δψm) were determined with mitochondrial membrane potential assay kit with JC-1 (Beyotime, China). CT-26 cells were seeded in 12-well plate at a density of 1×10^5 cells per well. After attached, the cells were treated with free LfcinB or LHPN with or without hyperthermia. At 8h, the cells were processed according to the product description. Finally, the cells were observed by fluorescence microscope.

Immune response of spleen lymphocytes *in vitro*. Spleen lymphocytes were extracted from the spleens of BALB/c mice using lymphocyte density gradient centrifugation with Ficoll-paque PREMIUM. Firstly, the proliferation rate of lymphocytes after different treatments was determined by flow cytometer. Lymphocytes were suspended in PBS and stained by CFDA-SE (Beyotime, China) at a final concentration of 5μM, incubated at 37℃ for 10min and washed trice immediately with pre-cooled PBS containing 10% serum. Then the CFDA-SE-labeled lymphocytes were resuspended in 1640 medium and plated at a density of 1×10^6 per well in six-well plate, and cocultured with CT-26 cells at a ratio of 10:1 for 48 hours in the presence of PBS, free LfcinB or LHPN (at a final LfcinB concentration of 200μg/mL). The hyperthermia groups were subjected to heat treatment at 6 hours of cultivation. Lymphocytes treated with LfcinB or LHPN alone was performed as control. Cells were collected and analyzed by flow cytometry.

Next, the amount of $CD3^+$ $CD4^+$ and $CD3^+$ $CD8^+$ T cells were analyzed by flow cytometry after different treatments. Briefly, lymphocytes were cocultured with CT-26 cells at a ratio of 10:1 for 48 hours in the presence of PBS, free LfcinB or LHPN (at a final LfcinB concentration of 200μg/mL). The hyperthermia groups were subjected to heat treatment at 6 hours of cultivation. After incubation, cells were collected and stained with the indicated antibodies including FITC-CD3, PE-CD4 and APC-CD8 (BioLegend, USA), analyzed by flow cytometry. In addition, the cytokine levels in the supernatant of the co-incubation system including IL-2, TNF-α and IFN-γ were detected using ELISA kits.

Biodistribution of LHPN and free LfcinB. BALA/c mice (male, 6 to 8 weeks old, 18−20 g) were purchased from Slack Laboratory Animal Co., Ltd (Shanghai, China). All animal experiments were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals with the approval of the Scientific Investigation Board of Zhejiang University, Hangzhou, China. The xenografted tumor models were established by subcutaneous injection of CT-26 cells (1×10^6) dispersed in serum-free RPMI 1640 medium into the right flanks of BALB/c mice. Treatment began when the tumor volume reached 500 mm^3. For ease of observation, LfcinB was subjected to indocyanine green (ICG) labeling to form ICG-LfcinB. Briefly, 1mg of ICG was weighted into a vial bottle containing 2mL deionized water, then 1.5mg of EDC, 1mg of NHS was added and dissolved. The mixture solution was stirred at room temperature for 4h to activate the sulfonic acid groups of ICG. Subsequently, 2mg of LfcinB was charged into this solution and stirred overnight at room temperature. The reaction system was dialyzed against de-ionized water for 24h and the obtained solution was stored in fridge for following use. 200μL of free ICG-LfcinB or ICG-LfcinB loaded LHPN was injected into the mice via the tail vein and at 1, 6, 12, 24 and 48h after injection, the treated mice were anesthetized and the fluorescence images were acquired by Maestro *in vivo* imaging system. 48h after injection, the

mice were sacrificed to harvest the main organs (heart, liver, spleen, lung, kidneys, and tumor). Fluorescence images were acquired, and the fluorescence intensity of these organs was measured *ex vivo* using an *in vivo* imaging system.

***In vivo* antitumor efficacy and immune response.** After one week of CT-26 cells injection subcutaneously, the mice were randomly sorted into six groups (6 mice per group) to respectively receive one of the following test agents once the other day: Saline, Saline+MW, LfcinB, LfcinB+MW, LHPN, LHPN+MW. 10mg/kg LfcinB per dose was used in the treatment and at 24h post-*i.v.* injection of the test agents, the mild microwave (MW) was applied locally for 30min. The microwave probe was positioned 1cm away from the fixed animal and oriented towards the tumor. The body weight and tumor volume were calculated every 2 days. The tumor volume was calculated using the formula: $a^2 \times b/2$, in which a and b represent the smallest and largest diameters of the corresponding tumor, respectively.

After receiving seven times of injection and microwave hyperthermia in 14 days, the mice were monitored for another 11days and sacrificed. The main organs (heart, liver, spleen, lung, kidney, and tumor) were harvested and fixed in 4% paraformaldehyde, embedded in paraffin, cut into 5μm slices and stained with H&E, then examined under a light microscope. To investigate the number of neovascularization in tumor tissue, CD31 levels were examined by immunohistochemistry. To demonstrate the ICD of tumor tissues, CRT and HMGB1 levels were studied by immunohistochemistry. To examine the immune response, $CD8^+$ CTL cells and IFN-γ levels in the tumors were analyzed using immunofluorescence, while the infiltration of mature DCs (CD86), active NK cells (CD69), Tregs (Foxp3) and perforin were studied by immunohistochemistry. T cells ($CD3^+$, $CD4^+$, and $CD8^+$) in spleen and tumor were isolated and analyzed using flow cytometry. Levels of TNF-α, IL-2 and IFN-γ in serum, spleen and tumor were examined using the ELISA kits.

Besides, the melanoma-bearing mice model was established and the antitumor efficacy was studied. Briefly, the xenografted tumor models were established by subcutaneous injection of B16 cells (1×10^6) dispersed in serum-free DMEM medium into the right flanks of C57BL/6 mice. One week later, the mice were randomly sorted into seven groups (6 mice per group) to respectively receive one of the following test agents: Saline, Saline+MW, LfcinB, LfcinB+MW, LHPN, LHPN+MW, LHPN+MW+anti CD8. The dose of drug and the application of MW were consistent with the CT-26-bearing mice. The anti-CD8 antibody (BioXcell, USA) was intraperitoneal (i.p.) injected to deplete the $CD8^+$ T cells at the days of -3, 0, 3 and 6. The body weight and tumor volume were calculated every 2 days and the survival time was monitored. The levels of CRT and HMGB1 in tumor tissues were studied by immunohistochemistry. The infiltration of $CD8^+$ CTL cells, active NK cells (CD69), Tregs (Foxp3) and perforin were studied by immunohistochemistry.

Statistical Analysis. Statistical calculations were performed using Prism 7 software (GraphPad). Data were expressed as means and SD. Differences were statistically evaluated by one-way ANOVA test or Student's *t* test. The differences were considered to be statistically significant for a p value of <0.05. To analyze the survival time of mice, Kaplan-Meier survival curves were generated, and Log-rank Mantel–Cox tests were performed. P values of < 0.05 were considered significant.

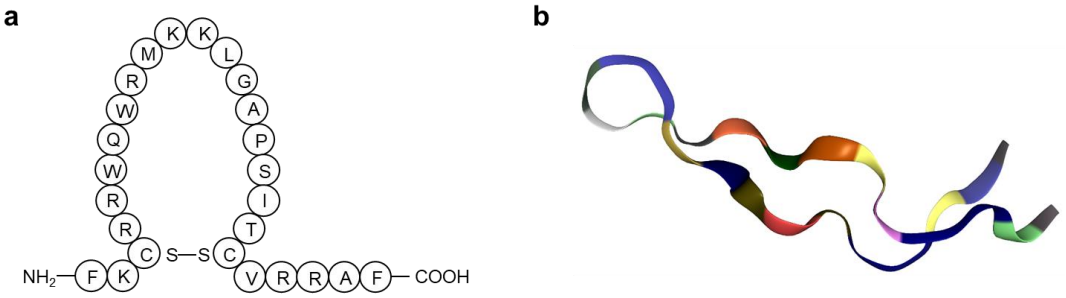
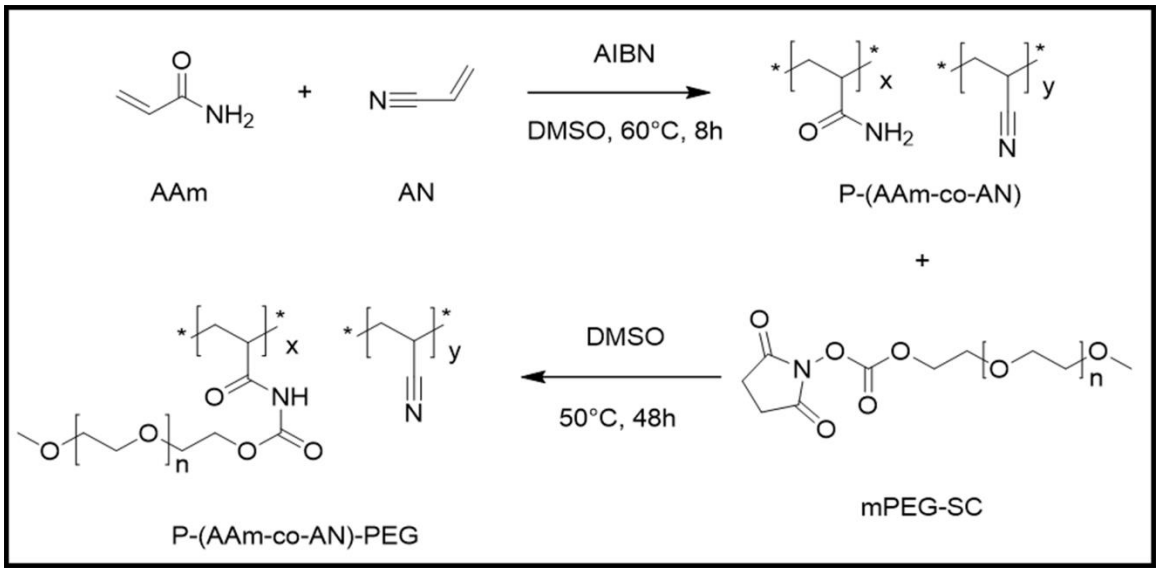


Figure S1. The structure of LfcinB. **(a)** The primary structure of LfcinB referenced to the previous report.[1, 2] **(b)** The conformation of LfcinB in solution downloaded from Protein Data Bank.



Scheme S1. Synthesis route of P-(AAm-*co*-AN)-PEG.

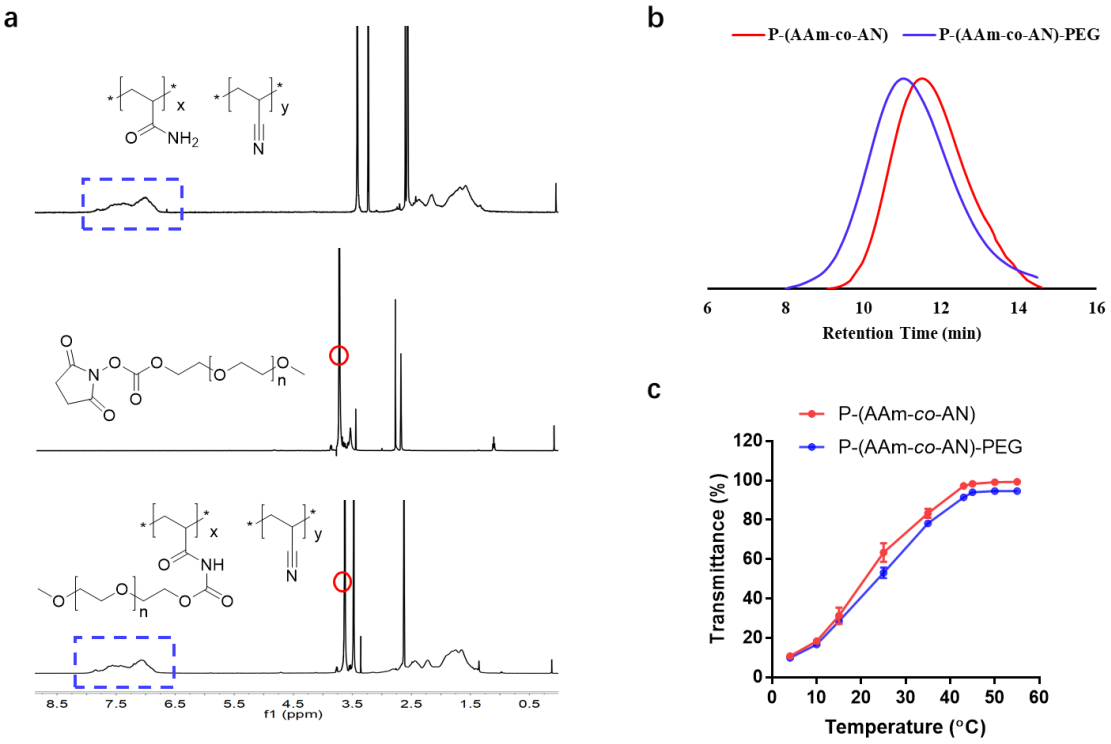


Figure S2. Characterization of P-(AAm-*co*-AN)-PEG. **(a)** The ^{1}H-NMR spectra of P-(AAm-*co*-AN), PEG and P-(AAm-*co*-AN)-PEG. **(b)** The standard GPC traces and **(c)** the optical transmittances of P-(AAm-*co*-AN) and P-(AAm-*co*-AN)-PEG.

a

Recovery rate

	Medium	Cell lysate
Free LfcinB	90.20%	61.40%
LHPN	85.30%	52.30%

b

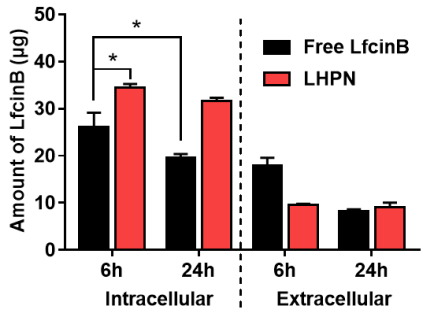


Figure S3. Quantitatively determination the amount of LfcinB in the cell after incubated with free LfcinB or LHPN for 6h and 24h. (a) Recovery rate of the detection method. (b) The amount of LfcinB intra- and extracellular after incubating with free LfcinB or LHPN for 6h and 24h.

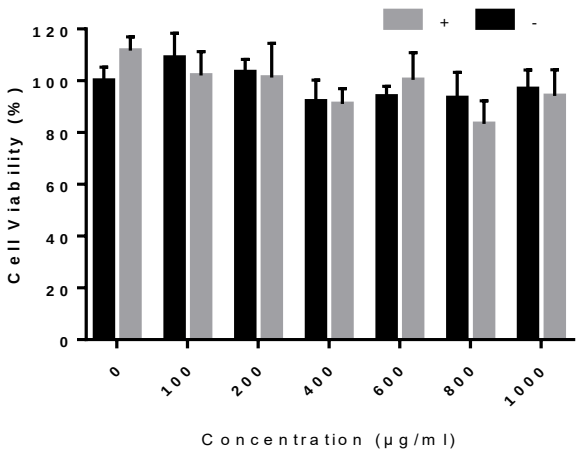


Figure S4. Biocompatibility of blank nanoparticles. CT-26 cell viabilities after 48h incubated with a series of concentrations of blank nanoparticles in the presence (+) or absence (-) of hyperthermia.

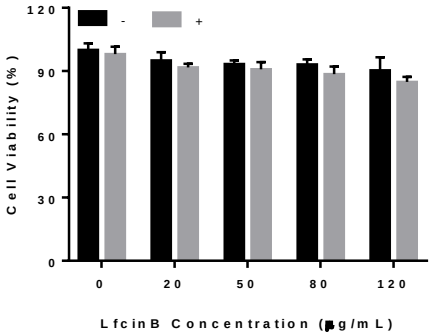


Figure S5. Cytotoxicity of LHPN to HUVECs with (+) or without (-) hyperthermia after 48h incubation as a function of the concentration of LfcinB.

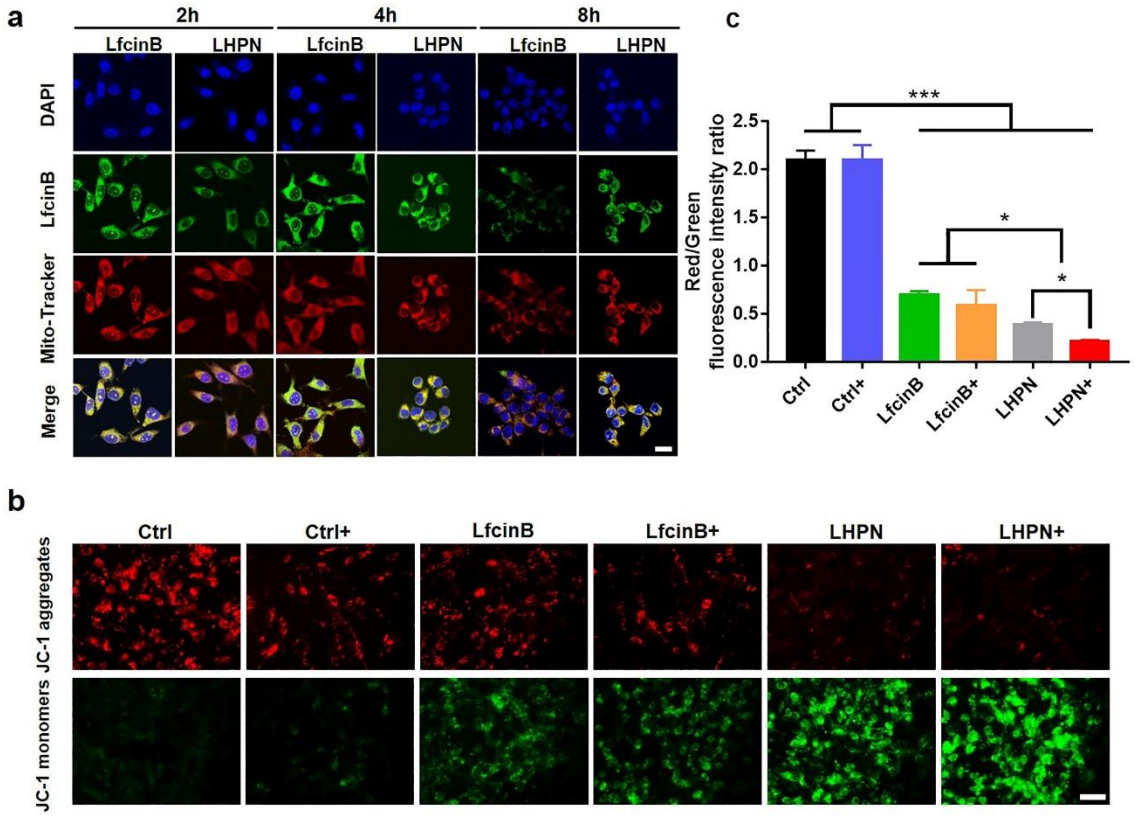


Figure S6. Ability of LfcinB and LHPN targeting mitochondria and disrupting mitochondrial membrane potential (Δψm). **(a)** Confocal microscope images of CT-26 cells incubated with free LfcinB or LHPN for different times, LfcinB was labeled by FITC and the mitochondria was stained by MitoTracker Deep Red FM (Red). Scale bar = 20μm. **(b)** Fluorescent microscope images of CT-26 cells stain by JC-1 after incubated with free LfcinB or LHPN for 8 hours with or without hyperthermia. Scale bar = 50μm. **(c)** The Δψm of mitochondria was calculated based on **b)**. The results are expressed as mean and SD. *p<0.05, **p<0.01, ***p<0.001, n=3. (ANOVA)

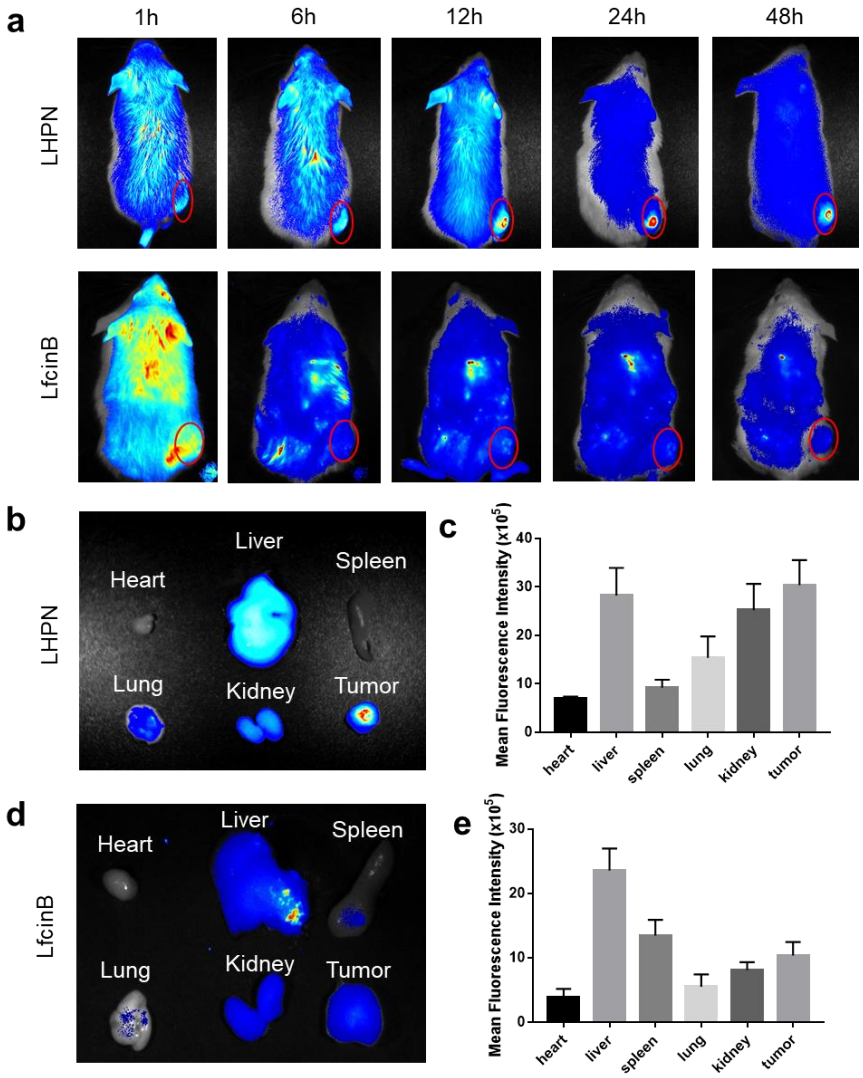


Figure S7. Biodistribution of LHPN and free LfcinB. **(a)** Tumor imaging of CT26-bearing mice after intravenous injection of ICG-LfcinB loaded LHPN or free ICG-LfcinB at different times. The red circle marks the tumor site. **(b)** Fluorescence images and **c)** mean fluorescence intensity of ICG-LfcinB loaded LHPN in tumor and other major organs 48 hours after intravenous injection. **(d)** Fluorescence images and **e)** mean fluorescence intensity of ICG-LfcinB in tumor and other major organs 48 hours after intravenous injection.

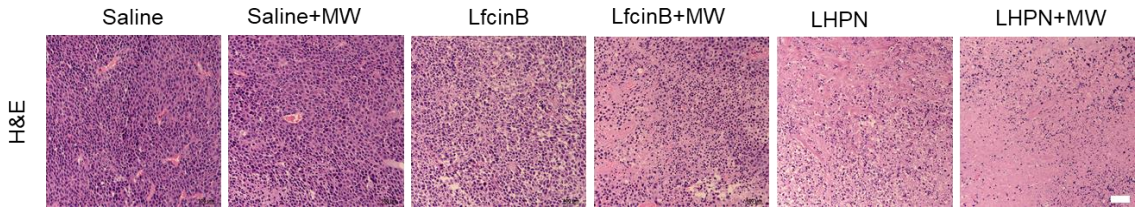


Figure S8. Microscopic images of H&E-stained cross-sections of the tumors at the end of observation. The scale bar=100μm.

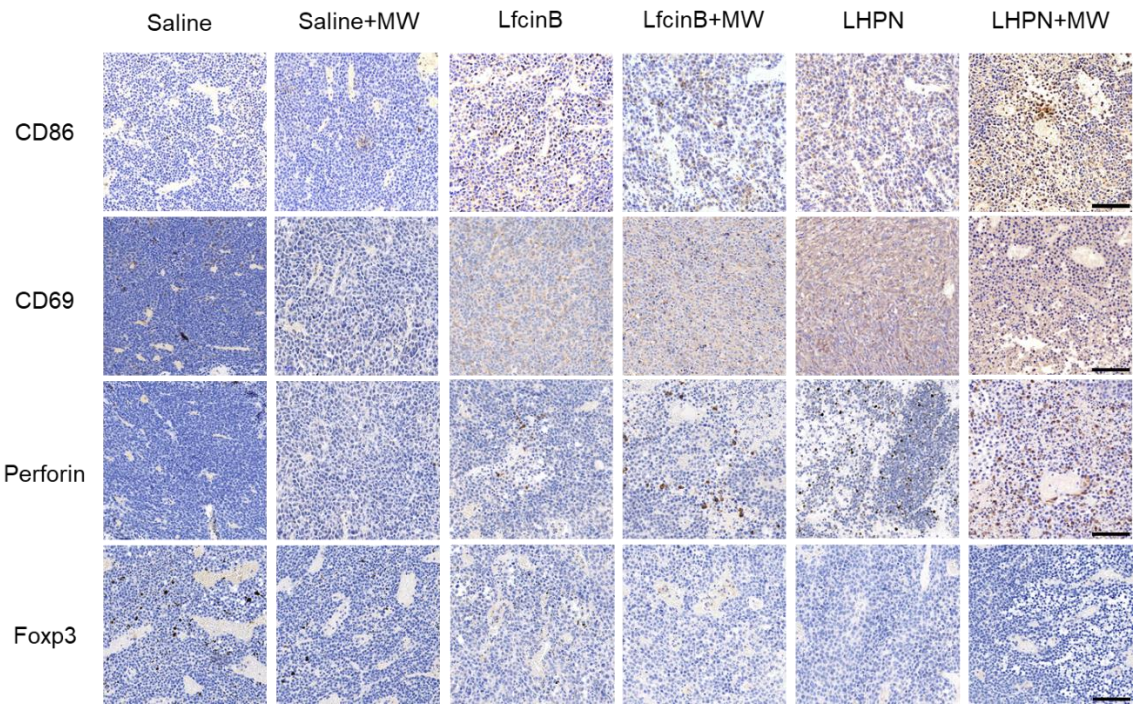


Figure S9. Immunohistochemistry was used to examine the levels of CD86, CD69, Perforin and Foxp3 in CT-26 tumor sections. Scale bar=100μm.

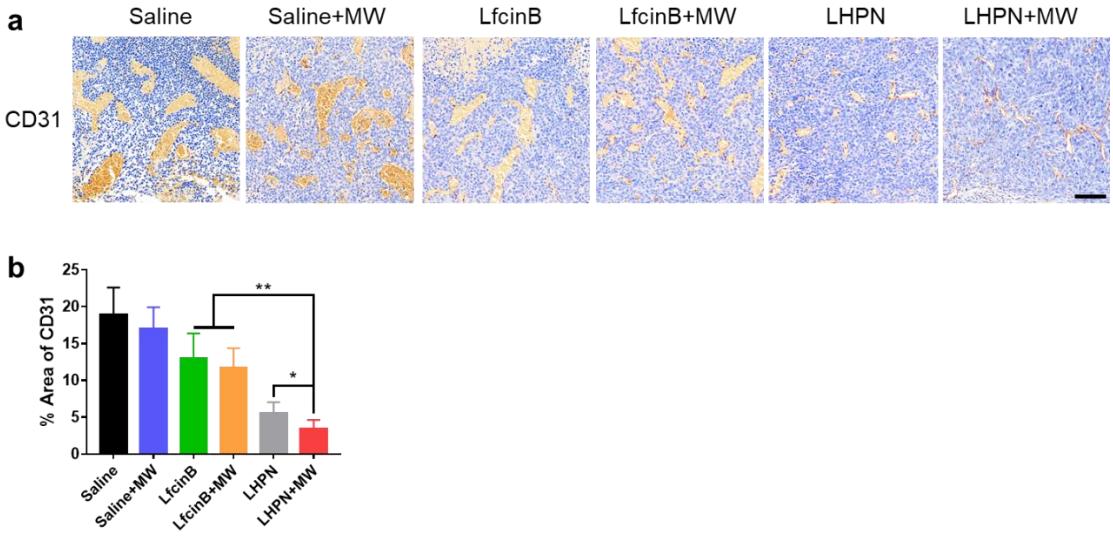


Figure S10. CD31 expression levels in tumors at the end of observation. **(a)** Immunohistochemistry images indicating the expression of CD31 in tumor sections at the end of the observation. Scale bar =100μm. **(b)** Semi-quantitative analysis of CD31 expression using Image J software based on **a).** The results are expressed as mean and SD. *p<0.05, **p<0.01, n=3. (ANOVA)

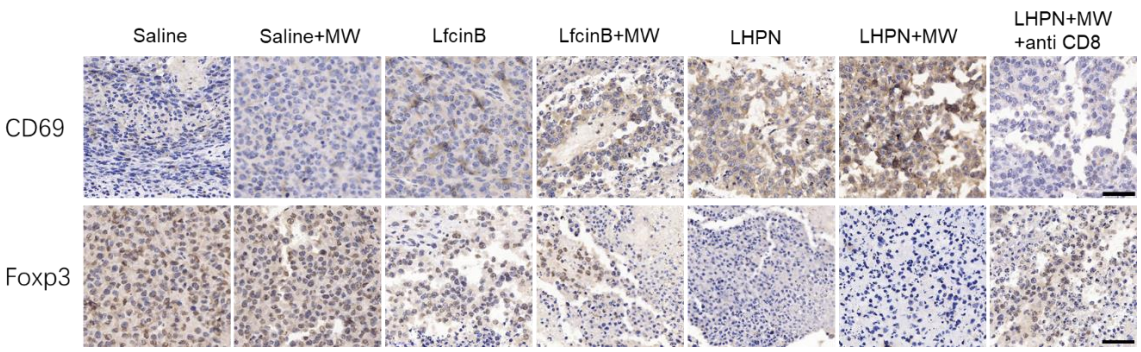


Figure S11. Immunohistochemistry was used to examine the levels of CD69 and Foxp3 in melanoma tumor sections. Scale bar=50μm.

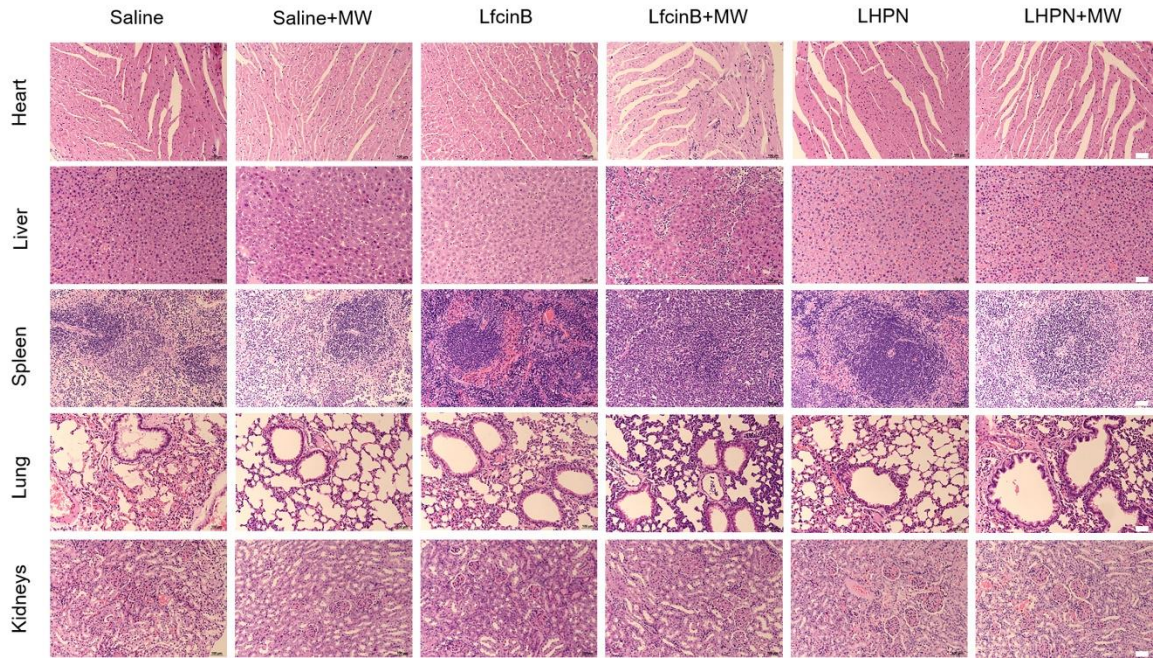


Figure S12. Microscopic images of H&E-stained cross-sections of major organs (heart, liver, spleen, lung and kidneys) at the end of observation. The scale bar = 100μm.

Reference

(1) Hwang, P. M.; Zhou, N.; Shan, X.; Arrowsmith, C. H.; Vogel, H. J. *Biochemistry* **1998,** 37, (12), 4288-4298.

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