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A H_2O_2-responsive theranostic platform for chemiluminescence detection and synergistic therapy of tumor

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Experimental Procedures

Materials. 4-hydroxyybenzyl alcohol, 1,8-octanediol, oxayl chloride, Rubrene, lapachone, hydrogen peroxide (H_2O_2), tetrahydrofuran (THF), ethylacetate, dichloromethane, hexane, Na2SO4, NaCl, DMSO, dimethylsulfoxide (DMSO) were purchased from Shanghai Chemical Co. (China). Fetal bovine serum (FBS), trypsin, antibiotics (penicillin-streptomycin) penicillin-streptomycin, Dulbecco's phosphate buffered saline (PBS), Roswell Park Memorial Institute-1640 (RMPI-1640), Hoechst 33342, MitoTracker Green, LysoTracker Green, Propidium Iodide (PI) and Annexin V-FITC were purchased from Invitrogen Corp. Anti-CD3-FITC, anti-CD4-APC and anti-CD8a-PE antibodies were purchased from Dakewe Biotech Co., Ltd.

Characterization. The scanning electron microscopy (SEM) and transmission electron microscopy (TEM) images were observed by scanning electron microscope (Sigma) and transmission electron microscope JEM-2100. The particle size was detected by nano-ZS ZEN3600 (Malvern Instruments). The cell viability was measured by microplate reader (Bio-Rad, Model 550, USA). Flow cytometry of cell uptake behaviors and cell death was analyzed by BD FACSAria TM III. The chemiluminescence imaging results were obtained by IVIS imaging systems (Perkin-Elmer). Fluorescence microscopy images were observed by a confocal laser scanning microscopy (CLSM, DI8, Leica, Germany).

Cell Culture. 4T1 cells were cultured in RPMI 1640 media at 37 ^oC with 5% CO_2. The media contained 10% fetal bovine serum.

Synthesis of L-HPOX. The POE was synthesized by reacting 4-ydroxybenzyl alcohol and octanediol with oxalyl chloride according to literature.[1] Briefly, 4-ydroxybenzyl alchol (1.984 g, 16mmol) and 1,8-octanediol (2.34 g, 16mmol) was dissolve in 20 mL THF, the solution was The solution is placed in a 4 ℃ ice water bath and triethylamine (8.34 mL, 60 mmol) was added under the protection of N_2. After stirring for 5 minutes, a THF solution (25 mL) containing oxalyl chloride (2.71 mL, 32mmol) was added dropwise to the solution under the protection of N_2. After 20 min, the reaction was stirred at room temperature overnight. Then saturated NaCl

solution was added to quench the reaction. The solution was extracted three times with dichloromethane and dried over anhydrous Na_2SO_4. After the solvent was removed by rotary evaporation, the remaining product was recrystallized with cold n-hexane to obtain POE. ^1H NMR (400 MHz, $CDCl_3$, δ): 7.52 (m, 2H, Ar), 7.2 (m, 2H, Ar), 5.30 (m, 2H, -OCH_2-PhO), 4.28 (m, 1.5H, $COOCH_2CH_2$), 1.72 (m, 1.5H, -OCH_2CH_2), 1.40 (m, 3H, -$OCH_2CH_2CH_2CH_2CH_2CH_2CH_2CH_2O$-).

For synthesizing of L-HPOX, 10 mg POE, 100 mg Pluronic F-127 (PF-127), 2 mg Reb and 3 mg Lapachone were dissolved in 3 mL trichloromethane, and the solution was evaporated. After adding 10 mL ultra-pure water, the mixture was sonicated for 30 min, washed three times with water in the ultrafiltration tube to remove unencapsulated Lapa and Reb and then filter with a 2 μm filter to obtain the H_2O_2-responsive nanoparticles loading rubrene and lapachone (L-HPOX). L-HPOX was washed three times through an ultrafiltration tube before use.

Chemiluminescence ability of L-HPOX. The chemiluminescence performance of L-HPOX solution (1 mg/mL) was detected by IVIS spectrum imaging system. The L-HPOX solution in the test tube was detected first as the blank. Then 100 μM H_2O_2 was added in the L-HPOX solution. At 0.5 h, 1 h, 1.5 h, 2 h, 3 h, 4 h, 5 h and 6 h after adding H_2O_2, the tube was detected by IVIS spectrum imaging system and the pictures were collected. In the experiment to study the relationship between chemiluminescence intensity and H_2O_2 concentration, a 96-well plate was prepared. Each well was added the same concentration of L-HPOX. And the corresponding different concentrations of H_2O_2 were added each well. The chemiluminescence of 96-well plate was detected by IVIS spectrum imaging system. The correspondence between the average chemiluminescence intensity and the concentratio of H_2O_2 was analyzed.

The chemiluminescence of L-HPOX *in vitro* and *in vivo.* All animal studies were approved by the Institutional Animal Care and Use Committee (IACUC) of the Animal Experiment Center of Wuhan University (Wuhan, China). All mouse experimental procedures were performed in accordance with the Regulations for the Administration of Affairs Concerning

Experimental Animals approved by the State Council of People's Republic of China.

Female Balb/c mice were used and the hair on the inner thighs was shaved. We injected PBS, PBS+L-HPOX, L-HPOX + 1 μM H_2O_2, L-HPOX + 10 μM H_2O_2 separately into their thighs and the chemiluminescence intensity of mice were detected by IVIS spectrum imaging system.

For inflammation induced H_2O_2 detection, Female Balb/c mice were chose and the hair on the abdomen was shaved. 100 μL LPS (500 μg/mL) was injected in the abdomen of the mouse. After 4 h, 100 μL L-HPOX (1 mg/mL) solution was injected into the same area and chemiluminescence of the mouse was detected by IVIS spectrum imaging system.

For cellular level H_2O_2 detection, RAW 264.7 were seeded and cultured for 24 h. Then the cells were co-cultured with 1 mg/mL LPS for 1 h. Next, the medium containing LPS was collected and L-HPOX was added for chemiluminescence detection. At the same time, fresh medium containing L-HPOX was added to the cells. After 30min of co-cultivation, the medium was removed and replaced with PBS. The cell culture dish were detected by IVIS spectrum imaging system. Cells without LPS cultured was used as control.

Drug release ability of L-HPOX. 1 mL of prepared L-HPOX (5mg/mL) was taken and put in a dialysis bag (Mw=3500). We prepared three sets of such dialysis bag. And the dialysis bags were separately put into the tubes with different concentration (1 μM, 10 μM, 100 μM) of H_2O_2. Then these tubes were placed in the 37 ℃ constant temperature oscillator. At the time point of 2h, 4h, 6h, 8h, 10h, 12h and 24h, some of the solution outside the dialysis bag was taken out and the content of Lapachone was detected by HPLC.

Cytotoxicity of L-HPOX. The cytotoxicity of L-HPOX was researched by MTT assay. 4T1 cells were cultured in 96 well plates and incubated for 24 h. Then gradient concentrations of Lapachone and L-HPOX were added into the cells. After co-cultivation for 24 h, a solution of MTT (20 μL, 5 mg/mL) was added into each well. The 96 well plates were incubated for further 4 h. Then the culture medium with MTT was removed and the residue was dissolved in 150 μL

DMSO. The absorbance was measured and cell viabilities (%) were calculated according to follows: $100 \times OD_{(samples)}/OD_{(control)}$, in which $OD_{(control)}$ and $OD_{(samples)}$ represented the absorbance in the absence and presence of samples at 570 nm. The cytotoxicity of L-HPOX was also evaluated by flow cytometry. 4T1 cells were cultured in 6-well plates and incubated for 24h. Then HPOX, Lapachone, L-HPOX (50 μg/mL) were separately added into each well. After co-cultivation for 24 h, the cells in each well were collected and stained by Annexin V-FITC/PI, and then analyzed by flow cytometry.

Tumor imaging ability of L-HPOX +DMXAA. The enhanced cancer targeting ability of L-HPOX combinating with DMXAA were observed by IVIS spectrum imaging system. Female BALB/c mice bearing 4T1 tumor on the right hind leg were injected with DMXAA intravenously. After 4 h, the mice were injected with L-HPOX intravenously and the chemiluminescence images of the mice were detected by IVIS spectrum imaging system at different times. Mice injected with only L-HPOX were used as the control group.

Antitumor effects of L-HPOX +DMXAA. Female BALB/c mice were subcutaneously injected 4T1 cells on the right hind leg. When the volume of tumor was about 100 mm^3, the mice were divided into six groups (PBS, HPOX, DMXAA, Lapa, L-HPOX, L-HPOX + DMXAA). There are 10 mice in each group. Then, PBS (200 μL), HPOX (200 μL), DMXAA (200 μL), Lapa (200 μL), L-HPOX (200 μL) and L-HPOX + DMXAA (200 μL) were separately injected intravenously from day 1. In L-HPOX + DMXAA group, mice were injected with DMXAA firstly and then injected witn L-HPOX 4 h later. The concentration of each group corresponds to 15 mg/mL Lapachone and 15 mg/mL DMXAA. On day 1, 4, 7, each droup of the mice were injected with corresponding preparations. The cancer volume and body weight of every mouse were recorded every two days. The cancer volume was calculated following: $V = W^2 \times L/2$, in which W and L standed for minor and major length.

Antitumor ability and anti-metastatic ability of L-HPOX + DMXAA. In order to prove the immune activation and anti-metastasis effect in vivo, mice were injected with luciferase-4T1

(4T1-luc) cells through the tail vein on day 8, and the growth of lung metastases tumor was monitored by bioluminescence imaging.

Tumor infiltrating $CD8^+$ T cells were evaluated by flow cytometry. After the mouse was sacrificed, the tumor tissue was peeled off and was treated with digestive enzymes. The cells were collected and treated with anti-CD4-FITC, anti-CD11c-PE and anti-CD8-APC. Then the cells were evaluated by flow cytometry.

1. D. Lee, S. Khaja, J. C. Velasquez-Castano, M. Dasari, C. Sun, J. Petros, W. R. Taylor, N. Murthy, *Nat. Mater.* **2007**, 6, 765.

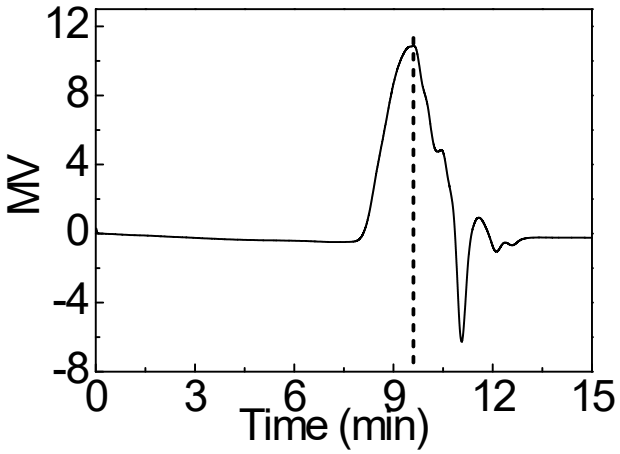

Fig. S1. Gel permeation chromatograph (GPC) result of polymer POE.

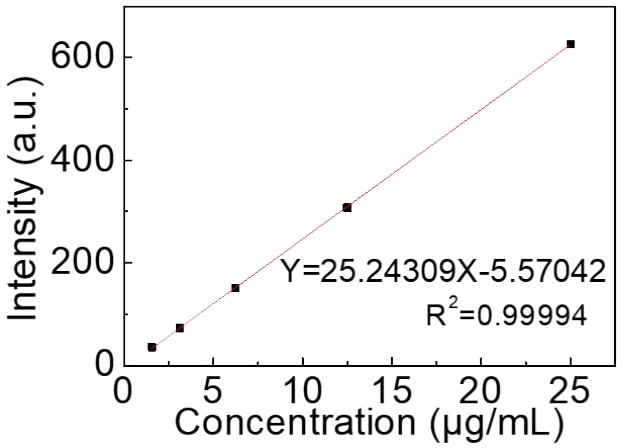

Fig. S2. Standard curve of fluorescence emission intensity of Reb as a function of concentration.

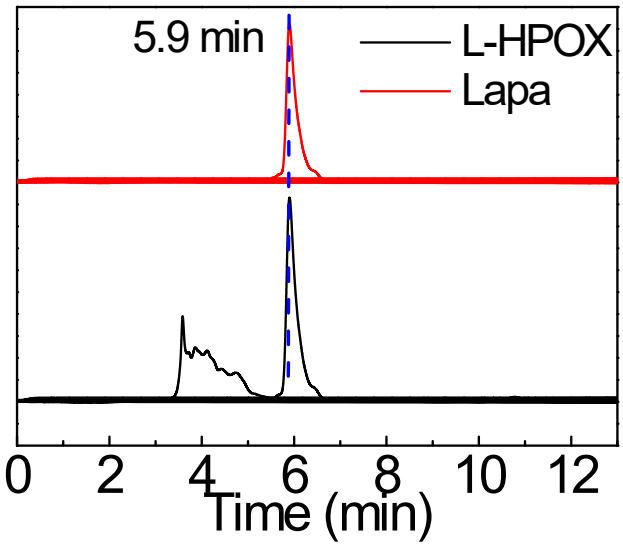

Fig. S3. High Performance Liquid Chromatography (HPLC) curve of Lapa and L-HPOX.

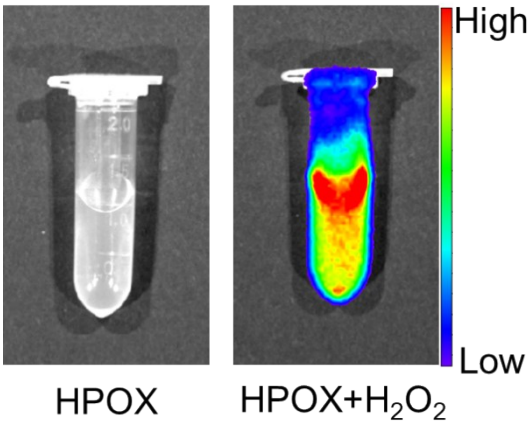

Fig. S4. The chemiluminescence of HPOX before (left) and after (right) adding H_2O_2.

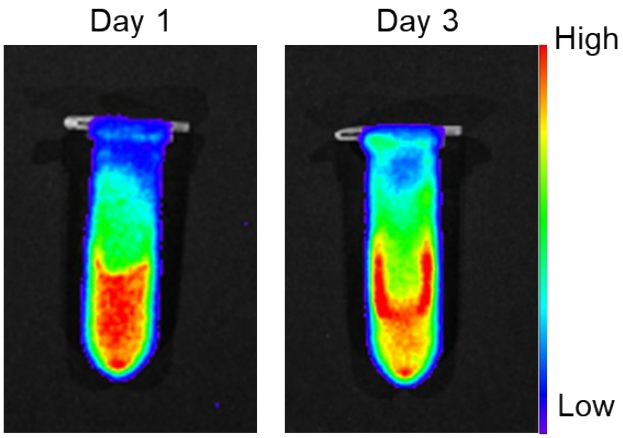

Fig. S5. Chemiluminescence intensity of L-HPOX at Day 1 and Day 3.

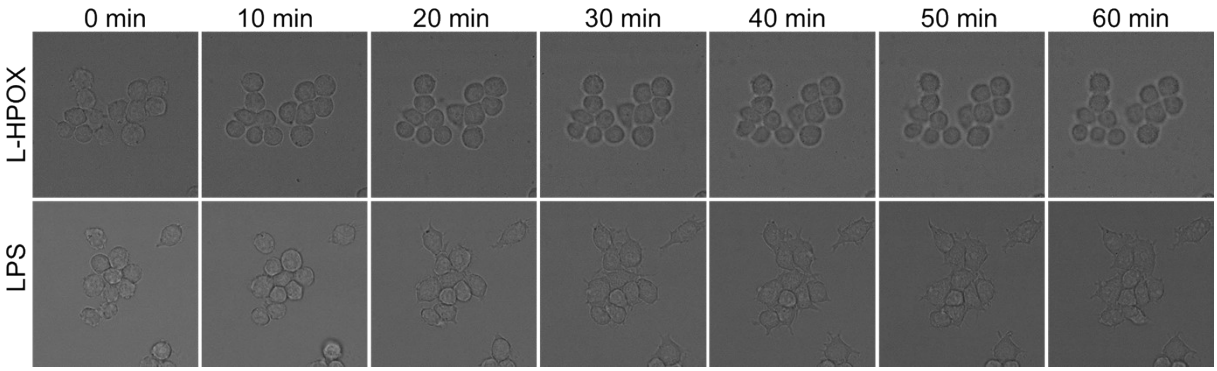

Fig. S6. The cell morphology of RAW 264.7 at different time (0, 10, 20, 30, 40, 50 and 60 min) after being treated with L-HPOX or LPS.

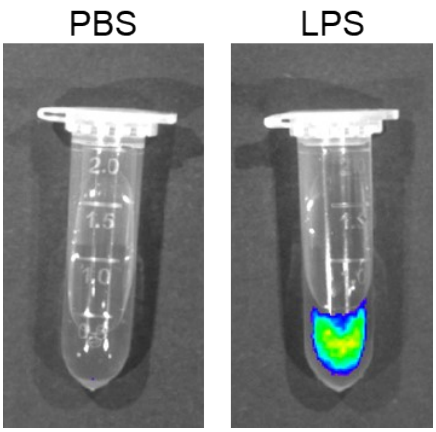

Fig. S7. RAW264.7 were treated with PBS or LPS. After 1 h, the cell culture medium was collected separately and added with L-HPOX. Then, the chemiluminescence of cell culture medium of PBS-treated RAW 264.7 (left) and LPS-treated RAW 264.7 (right) were detected.

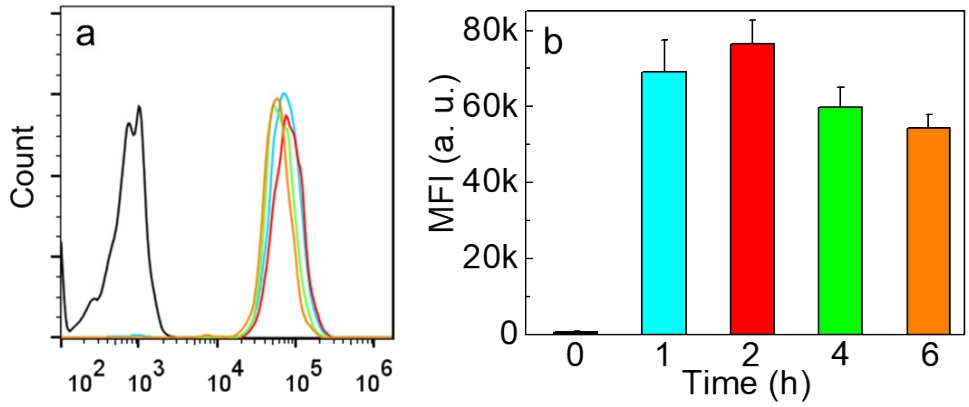

Fig. S8. a) Flow cytometry analysis of the cellular uptake behaviors of L-HPOX at 0h (black line), 1h (blue line), 2h (red line), 4h (green line) and 6h (orange line). b) And the corresponding mean fluorescence intensity (MFI) at different time.

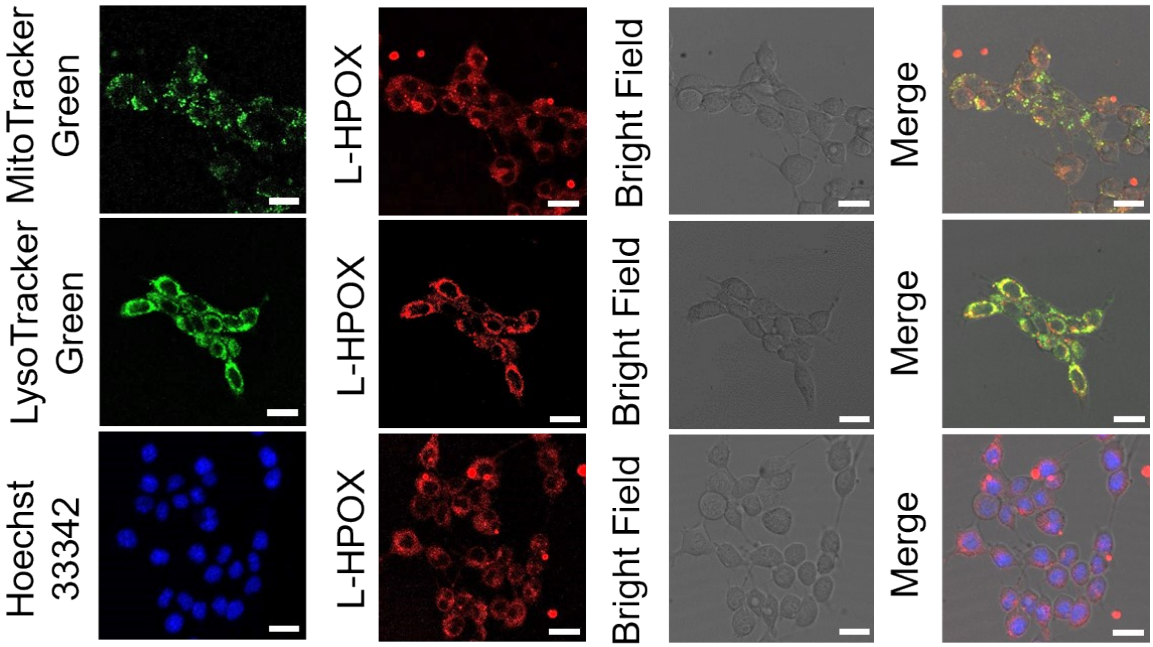

Fig. S9. CLSM images of 4T1 cells after treatment with L-HPOX for 4 h and stained by Hoechst 33342, Lysotracker Green or Mitotracker Green. Scale bar: 20 μm.

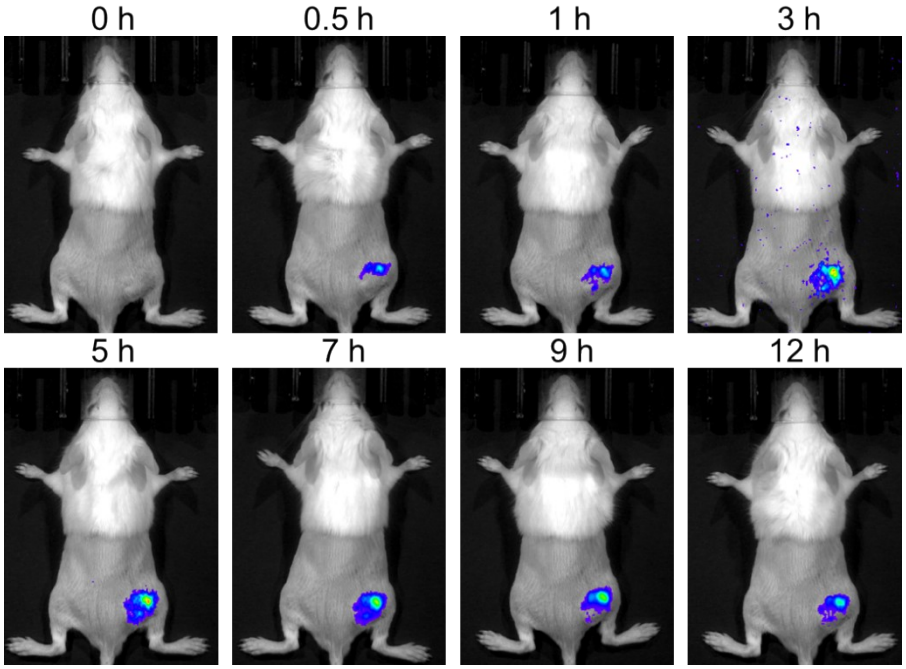

Fig. S10. The chemiluminescence imaging of mice at different time (0h, 0.5h, 1h, 3h, 5h, 7h, 9h, 12h) after being injected with L-HPOX by tail vein.

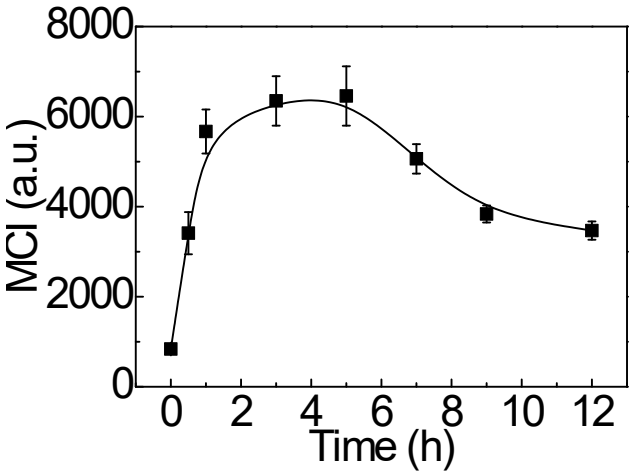

Fig. S11. The mean chemiluminescence intensity (MCI) of mice at different time after being treated with DMXAA and L-HPOX.

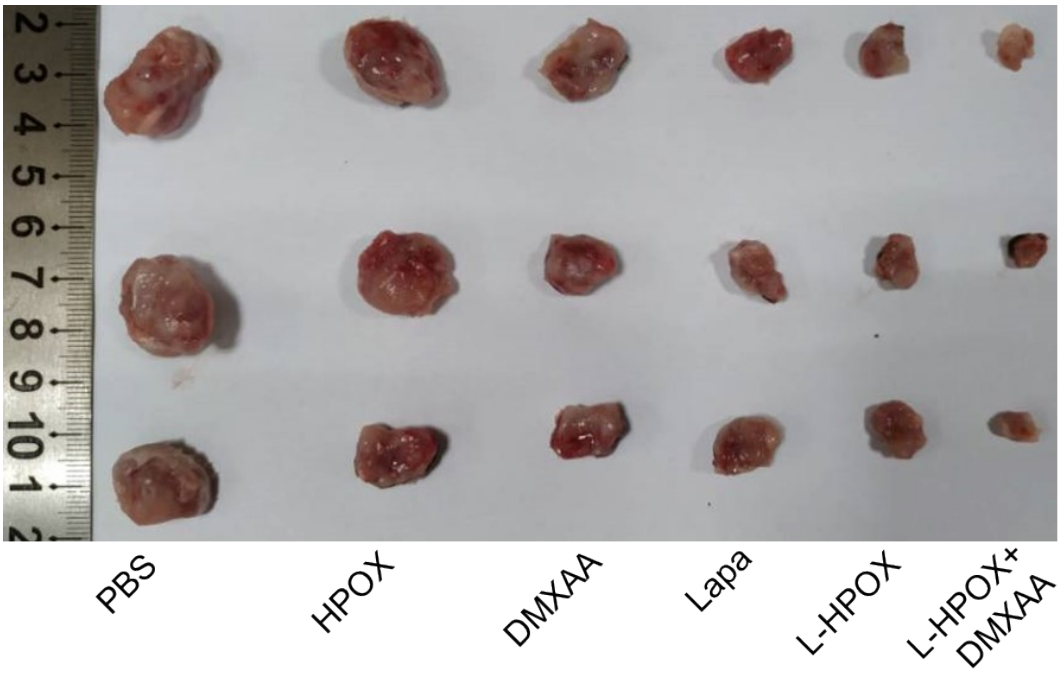

Fig. S12. Isolated tumor tissue of the subcutaneous 4T1-tumor-bearing mice after treatment with PBS, HPOX, DMXAA, Lapa, L-HPOX and L-HPOX+DMXAA.

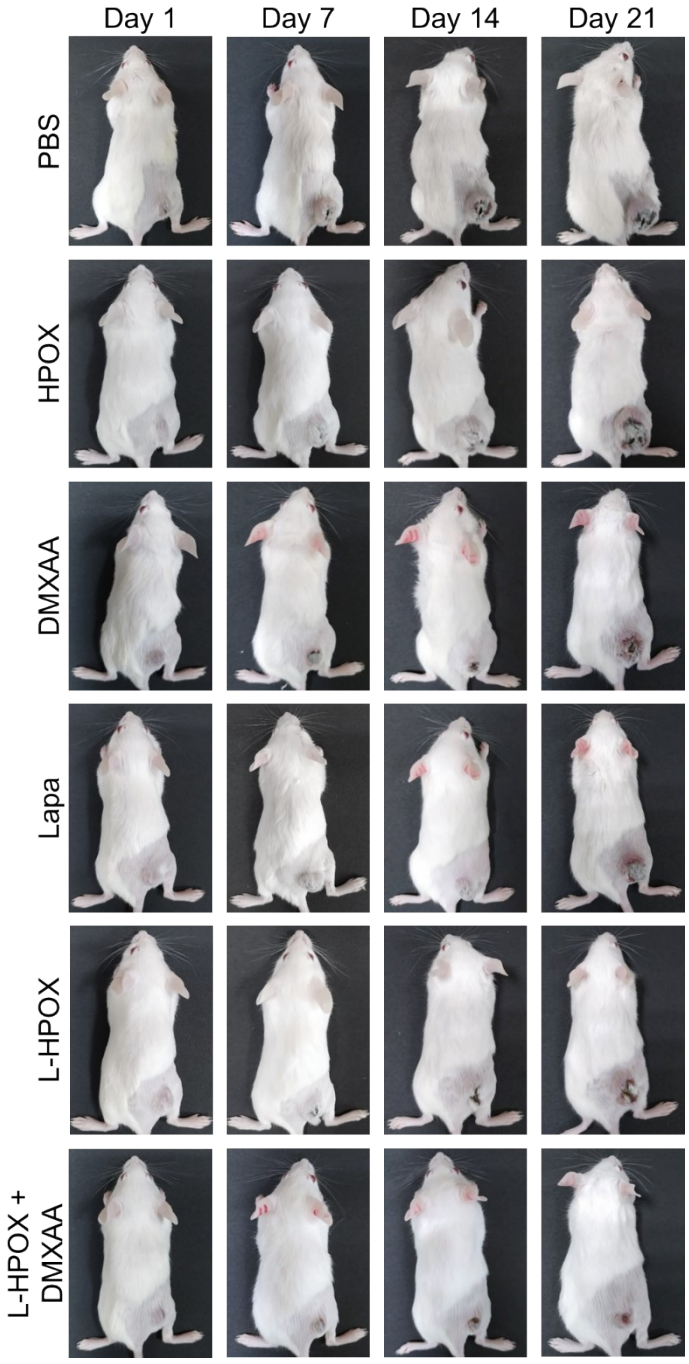

Fig. S13. The photographs of the mice at Day1, Day7, Day14 and Day21 after treatment with PBS, HPOX, DMXAA, Lapa, L-HPOX and L-HPOX+DMXAA.

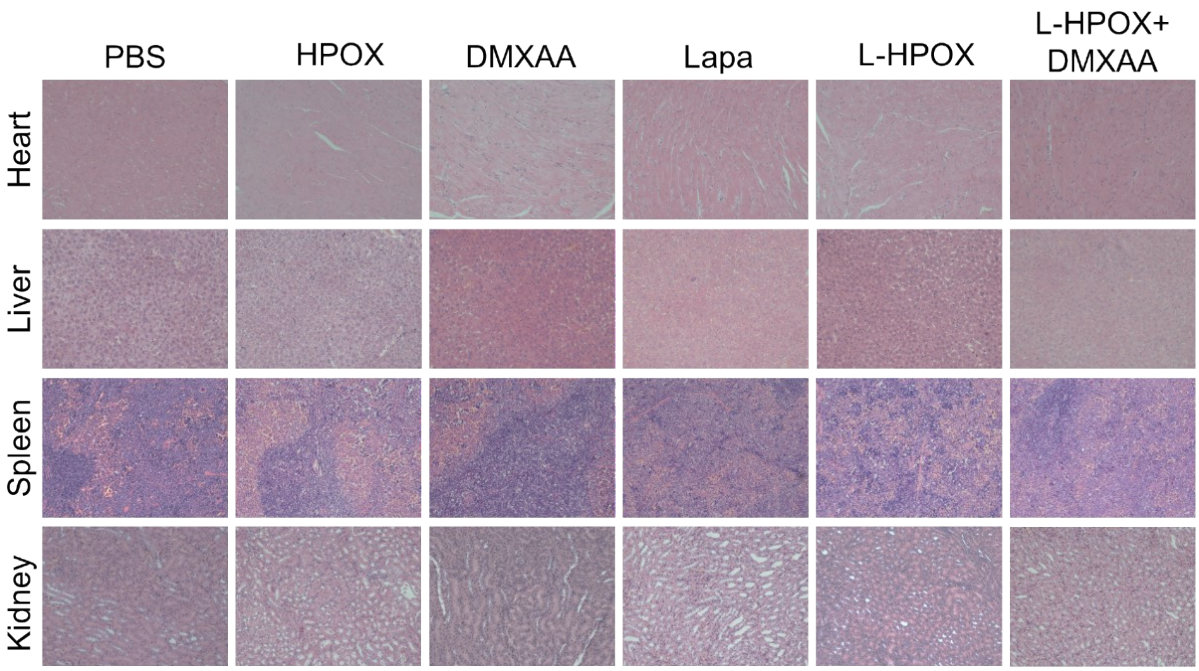

Fig. S14. H&E staining analysis of the collected organs. H&E staining: 200×magnification.

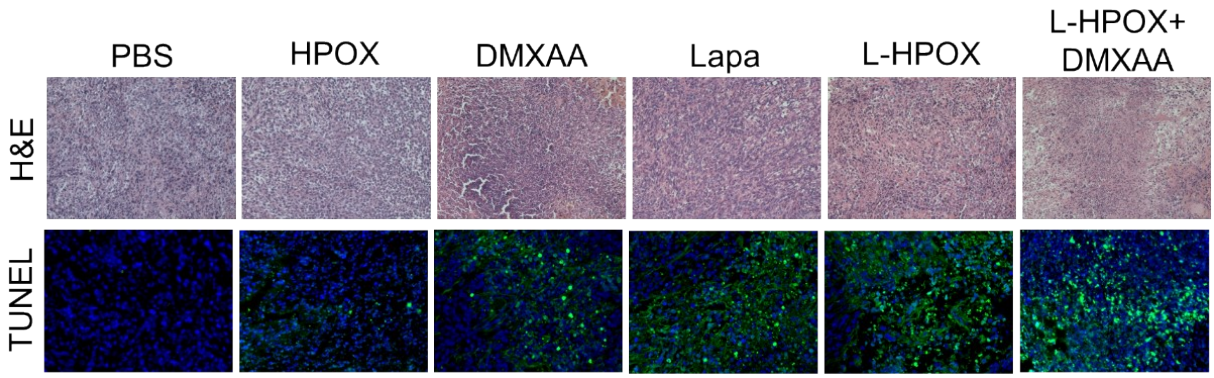

Fig. S15. The H&E staining (top) and TUNEL labeling images (bottom) of tumor tissues, 400×magnification.