Supporting Information

Metabolically Engineered Bacteria as Light-Controlled Living Therapeutics for Anti-angiogenesis Tumor Therapy

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Experimental section

Materials and characterization

4-Methyl-*N*-phenyl-*N*-(p-tolyl)aniline, *N*-bromosuccinimide, 5-formyl-2-thienylboronic acid, [1,1′-bis(diphenylphosphino)ferrocene]dichloropalladium(II), piperidine, propargyl bromide solution, D-alanine, copper (I) bromide, K_2CO_3, 4-methylpyridine, dichloromethane (DCM), anhydrous dimethyl sulfoxide (DMSO), toluene, methanol, ethanol, chloroform, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), anhydrous *N*,*N*-dimethylformamide (DMF) and all other materials for organic synthesis were purchased from Sigma-Aldrich and used without further purification. Live-or-Dye™ fixable viability staining kits was purchased from Biotium. TNF-α, IL-12, and IFN-γ ELISA kits were purchased from Life technologies, USA. Anti-CD3-APC, anti-CD4-FITC, anti-CD8-PE and anti-VEGFR2-FITC antibody were purchased from Abcam. The murine vascular endothelial growth factor receptor 2 (VEGFR2) was cloned into pcDNA3.1 vector to construct the VEGFR2 plasmid

transduced attenuated S. typhimurium (strain VNP20009), which was provided by College of Animal Sciences, Zhejiang University, P. R. China. 4T1 cell was purchased from American Type Culture Collection (ATCC, Manassas, VA). Female BALB/C mice (6-8 weeks old) were purchased from the Zhejiang Chinese Medical University and maintained in a pathogen-free environment under controlled humidity and temperature. The animal experiments were performed in accordance with the CAPN (China Animal Protection Law).

^{1}H and ^{13}C NMR spectra were measured on a Bruker Avance 600 (or 400) spectrometer using tetramethylsilane (TMS; $\delta = 0$ ppm) as an internal standard. UV-*vis* and photoluminescence spectra were recorded using Shimadzu UV-1700 and Perkin-Elmer LS 55 spectrometer, respectively. All UV and PL spectra were obtained at room temperature. The SEM images were acquired from the high-resolution Jeol JSM 6340-F Field Emission Scanning Electron Microscopy at 2 kV with a cold stage. Confocal images were taken using confocal microscope (Leica SP8, Germany). Flow cytometric was analyzed by flow cytometry (Beckman Coulter, Life Science, USA). *In vivo* PL images were acquired from *in vivo* imaging system (IVIS Lumina XR system, PerkinElmer).

Synthesis of compound 1: To a solution of 4-methyl-*N*-phenyl-*N*-(p-tolyl)aniline (2.00 g, 7.32 mmol) in dry dichloromethane (40 mL) at 0 °C, NBS (1.43 g, 8.03 mmol) was added in portions. The reaction was slowly warming to r.t and stirred in the dark. The reaction was monitored by TLC. After the reaction, saturated sodium bicarbonate (10 mL) was added to quench the reaction. The aqueous layer was separated and extracted with dichloromethane (10 mL × 3). The combined organic phase was dried over anhydrous sodium sulfate and concentrated under reduced pressure to give a residue which was purified by column chromatography (ethyl acetate / hexane = 1/20, V/V) to give the desired product compound **1** (2.37 g, yield: 92 %) as white solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.28 - 7.26 (m, 2H), 7.08 - 7.04 (m, 4H), 6.99 - 6.93 (m, 4H), 6.90 - 6.85 (s, 2H), 2.31 (s, 6H).

Synthesis of compound 2: A mixture of compound **1** (528 mg, 1.50 mmol), 5-formyl-2-thienylboronic acid (285 mg, 1.80 mmol), $Pd(dppf)Cl_2$ (10 mol%) and K_2CO_3 (2.10 g, 15.00 mmol) were added in mixed solvent (MeOH : toluene = 1 : 1). The reaction mixture was heated to 75 ^{0}C for 16 h. After 16 h, the reaction mixture was filtered and the solvent was removed. The residue was dissolved in DCM and washed by water. The combined organic phase was dried over anhydrous sodium sulfate and concentrated under reduced pressure to give a residue which was purified by column chromatography (ethyl acetate / hexane = 1/10,

V/V) to give the desired product compound **2** (408 mg, yield: 71 %) as yellow solid. ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.84 (s, 1H), 7.71 - 7.68 (d, 1H), 7.50 - 7.46 (m, 2H), 7.29 - 7.26 (m, 1H), 7.13 - 7.08 (d, 4H), 7.06 - 6.96 (m, 6H), 2.33 (s, 6H).

Synthesis of compound 3: 4-Methylpyridine (465 mg, 5.0 mmol) was dissolved in 15 mL MeCN, propargyl bromide solution (1.98 g, 15.0 mmol) was added and the reaction mixture was heated to reflux overnight. After completion, the solvent was evaporated and the crude product was precipitated from EtO_2. The solid was filtered to give compound **3** (731.40 mg, 69%) as a brown solid. ^{1}H NMR (400 MHz, DMSO) δ 8.99 - 8.97 (d, 2H), 8.05 - 8.03 (d, 2H), 5.57 (d, 2H), 4.05 (m, 1H), 2.64 (s, 3H).

Synthesis of compound 4: A solution of compound **2** (110.00 mg, 0.29 mmol) and compound **3** (53.01 mg, 0.25 mmol) was refluxed under nitrogen in dry ethanol catalyzed by a few drops of piperidine overnight. After cooling to room temperature, the mixture was poured into diethyl ether. The residue was dissolved in DCM and washed by water. The combined organic phase was dried over anhydrous sodium sulfate and concentrated under reduced pressure to give a residue which was purified by preparative thin layer chromatography (methanol / dichloromethane = 1/20, V/V) to give the desired product compound **4** (51.93 mg, yield: 36 %) as red solid. ^{1}H NMR (400 MHz, DMSO) δ 8.79 - 8.77 (d, 2H), 8.20 - 8.14 (m, 3H), 7.59 - 7.57 (d, 2H), 7.48 (s, 2H), 7.18 - 7.12 (m, 5H), 7.02 - 6.96 (d, 4H), 6.93 - 6.87 (d, 2H), 4.21 (s, 2H) , 2.55 (m, 1H) , 2.29 (s, 6H).

Synthesis of MeTTPy-D-Ala (MA) : Compound **4** (28 mg, 0.047 mmol) and 3-azido-D-alaninehydrochloride (6.5 mg, 0.050 mmol) were dissolved in argon degassing DMSO (1.5 mL) in a 5 mL round bottom flask. Copper (I) bromide (4.7 mg, 0.033 mmol) was dispersed in 0.1 mL trimethylamine and injected into the solution. The reaction was stirred at room temperature overnight. Then the mixture was filtrated by polyethersulfone 0.45 μm membrane filters and subjected to HPLC for purification. HPLC conditions: column: Agilent ZORBAX SB-C18 (9.4×150 mm); gradient: 0-6-13-17 min, 25%-80%-100%-100% B (A: H_2O containing 0.1% TFA, B: acetonitrile containing 0.1% TFA); flow rate is 2.0 mL min^{-1}; UV-*Vis* detector: 254 nm. **MA** was obtained as red powder (10.3 mg, yield: 32 %). ^{1}H NMR (400 MHz, DMSO) δ 8.60 (d, 2H), 8.04 (m, 3H), 7.53 (d, 2H), 7.43 (d, 1H), 7.34 (d, 1H), 7.17 - 7.04 (m, 6H), 7.02 - 6.89 (m, 7H), 4.26 (s, 2H), 3.73 (m, 2H), 2.66 (s, 1H), 2.32 (s, 6H). HR ESI-MS, m/z: $[M]^+$ calcd 706.1726, found 706.0505.

Preparation of the MA-labeled *Salmonella*： The *Salmonella* cells were routinely grown at 37 °C and 220 rpm in LB medium. After overnight culture, the bacteria were collected and the optical density at 600 nm (OD_{600}) was adjusted to 1.0 in PBS. The bacterial suspension was added into **MA** (0 ~ 40) μM solutions with the same volume, mixed and co-cultured at 37 °C for 30 min. The mixtures were centrifuged at 6000 rpm for 4 min and the supernatants were discarded, washed twice and the **MA**-labeled bacteria were obtained.

***In vitro* ROS detection by ABDA:** The ROS was detected using ABDA as the ROS indicator. 5 μL of ABDA stock solution was added into 1 mL of sample solution (10 μM), and the mixtures were irradiated by white light (400 - 800 nm) at a power density of 30 mW cm^{-2}. The absorbance of ABDA was monitored at different exposure time at 378 nm to obtain the decay rate of the photosensitizing process.

VEGFR2 DNA release induced under light radiation: To quantify the amount of DNA released from the supernatant of **MA**-treated bacterial samples, 10 mL LB cultures were inoculated with 2.5% (v/v) of an overnight starter culture, incubated at 37 °C. Next, bacterial solutions were divided into four copies (0.25 mL) and added to 0.25 mL PBS, then radiated (30 mW cm^{-2} white light) for 10 min, and incubated at 30 °C for an additional 6 hour. The bacterial culture in dark was used as the control. Treated bacteria cells were centrifuged at 14000g for 2 min and supernatants were measured at absorbance of 260 nm for DNA quantification.

Intracellular trafficking of MA-labeled *Salmonella*: The cellular uptake of **MA**-labeled *Salmonella* in 4T1 cell lines was evaluated. 4T1 were plated on 8-well plates at a density of 2×10^4/well and grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific Inc., USA) and 10 μg/mL gentamicin (Thermo Fisher Scientific Inc, USA) for 20 h at 37 °C at a humidified 5% CO_2 incubator. The medium was replaced with 300 μL of fresh medium and 100 μL of **MA**-labeled *Salmonella* at bacteria/cell ratio of 20 were added to each well. After 2 h incubation, the medium was discarded . After 2 hours, the medium was replaced by media containing the 2×penicillinstreptomycin solution for another 2 h incubation to eliminate extracellular bacteria. The cells were then washed twice with PBS and then treated with 10 μg/mL of Hoechst 33342 in PBS for 10 min. The images were taken on a confocal scanning laser microscope.

For TEM observation of the cell sections, the 4T1 cells were seeded on a 6-well plate at a certain density (2×10^6) cells per well and cultured for 20 h. The medium was replaced with fresh medium containg **MA**-labeled *Salmonella* at bacteria/cell ratio of 20. After transfection for 2 h, the medium was replaced by culture medium containing 2×penicillin-streptomycin solution for 2 h to eliminate extracellular bacteria. The cells were washed twice with PBS, trypsizined with 2.5% trypsin, centrifuged at room temperature, and fixed with 2.5% glutaraldehyde at 4 °C for 4 h. The samples were collected and washed with PBS and post-fixed with 1% osmium tetroxide for 2 h before dehydration in a graded series (25%, 40%, 60%, 80%, 90% and 100%) of ethanol. All the samples were embedded, sliced to a thickness of 50 to 70 nm, and collected on 200-mesh copper grids. The samples were stained with uranyl acetate and lead citrate before TEM observation.

Fluorescent live/dead cells staining experiment: *Salmonellas* were co-incubated with **MA** (0 μM, 5 μM, 10 μM, 20 μM) at 37 °C for 0.5 h. After washing twice to remove the free probes, the cells were re-suspended in PBS. 200 μL of the bacteria cells were put into 96-well plate and irradiated under white light with a power of 30 mW/cm^2 for 10 min. Then the culture media were removed and replaced with 100 μL of calcein-AM and 100 μL of PI solution for 15 min. Finally, the cells were observed by fluorescence microscope, where live cells were stained in green and dead cells in red, respectively.

***In vivo* biodistribution of bacteria:** To study the *in vivo* biodistribution of bacteria after various treatments, specific bacterial growth in hearts, livers, spleens, lungs, and kidneys from mice was determined on days 1 and day 7, tumors was determined at day 1, 3, 7, 22. After homogenizing these tissues in 0.1% Triton X-100, the obtained homogenates were serially diluted and plated onto solid LB agar plates. Then bacterial colonies were counted after 24 h for the accurate bacterial CFUs.

***In vivo* fluorescence imaging:** 100 μL SV@**MA** solutions (20 μM **MA** labeled, 10^6 CFU per mouse) were injected into 4T1 model mice through the tail vein. *In vivo* fluorescence imaging was performed at different time points post-injection using an *in vivo* imaging system (IVIS Lumina XR system, PerkinElmer). The mice were anesthetized with isoflurane and imaged at 4 h. The excitation wavelength was set at 480 nm, and the emission wavelength was chosen from 650 to 800 nm. At 4 h post-injection, the mice were sacrificed. The tumor and normal

tissues (heart, lung, kidney, spleen, and liver) were excised and washed with 0.9% NaCl for the *ex vivo* fluorescence imaging.

The analysis of T cells and cytokine secretion *in vivo*: The female BALB/c mice harboring 4T1 tumor model was used to evaluated for the influence of SV@**MA** on the frequency, proliferation of $CD4^+$, $CD8^+$ T cells, macrophage and cytokines. Briefly, after various treatment, three mice per group were sacrificed and the tumors, spleens and bloods were harvested for detection. The tissues were digested with collagenase IV (175 U/mL), hyaluronidase (100 U/mL), and DNase (30 U/mL) at 37 °C for 60 min. The tumorinfiltrating lymphocytes (TILs) were isolated by passing 75 μm filters and enriched for following analysis. For the T cells frequency detection, the TTLs were labeled with anti-CD3-APC, anti-CD4-FITC, and anti-CD8-PE. The stained TILs were then washed thrice with PBS and analyzed *via* flow cytometry.

For cytokine quantification, the harvested spleen cells were suspended in 5 mL of the RPMI 1640 culture medium supplemented with 10 % FBS for 24 h. The supernatants were collected and cytokines were quantified using mouse TNF-α, IL-12, and IFN-γ ELISA kits (Life technologies, USA).

***In vivo* tumor growth study :** Briefly, 4T1 cells suspended in 200 μL were injected subcutaneously (2.0×10^5 cells) at the right abdominal of 6~8 weeks old female BALB/c mice. When the tumor grew to a volume of 100 mm^3, the mice were randomly assigned to 7 groups (n = 5): control PBS, SV(-L) (Unlabeled *Salmonella* encoding VEGFR2 plasmid without light irradiation, 10^6 CFU per mouse), SE@**MA**(-L) (**MA**-labeled *Salmonella* transformed with the empty vector lacking VEGFR2 plasmid, without light irradiation, 70 μg/kg), SV@**MA**(-L) (**MA**-labeled *Salmonella* encoding VEGFR2 plasmid, without light irradiation, 70 μg/kg, 10^6 CFU per mouse), SV(+L) (Unlabeled *Salmonella* encoding VEGFR2 plasmid with light irradiation, 10^6 CFU per mouse), SE@**MA**(+L) (**MA**-labeled *Salmonella* transformed with the empty vector lacking VEGFR2 plasmid, with light irradiation, 70 μg/kg) and SV@**MA**(+L) (**MA**-labeled *Salmonella* encoding VEGFR2 plasmid, with light irradiation, 70 μg/kg, 10^6 CFU per mouse), 100 μL of each group was injected into 4T1 model mice through the tail vein. White light irradiation (40 mW/cm^2, 20 min) was performed at day 3 and day 6. The tumor growths were monitored by measuring the tumor length and width with a calliper and the tumor volume was calculated as follows: tumor volume V (mm^3) = π/6×length

(mm)×width (mm)^2. Three mice from each group were sacrificed at day 22. Their tumors were dissected, weighed, and then imaged.

In vivo **blood biochemistry analysis:** To evaluate the effects in physiology caused by SV@**MA**, blood biochemical indexes and blood routine indexes were tested respectively. 3 female Balb/c mice were *i.v.* injected with SV@**MA** (1×10^6 CFU per mouse) or PBS, blood samples collected from the serum at day 22 were separated by centrifugation at 4500 rpm for 5 min at the temperature of 4 °C and used for the determination. The blood biochemistry analysis was measured by biochemical auto analyzer (Cobas c311, Roche, Switzerland).

Immunohistochemical analysis of H&E, Ki67 and TUNEL assay: For the histological assay, the tissues were fixed in 4% paraformaldehyde for 24 h. The specimens were dehydrated in graded ethanol, embedded in paraffin, and cut into 5 mm thick sections. The fixed sections were deparaffinized and hydrated according to a standard protocol and stained with hematoxylin and eosin (HE) for microscopic observation.

Apoptosis of the tumor cells in the mice after treatments was determined by the TUNEL method according to the manufacturer's instructions. The proliferation of tumor cells were labeled with Ki67 antibody and all the sections were observed on a Leica (DMLB&DMIL) microscope.

Statistical analysis. All the experiments were repeated at least three times. The data were presented as means ± standard deviations. The statistical significance (*$p < 0.05$ or **$p < 0.01$) was evaluated by the Student's t-test when two groups were compared.

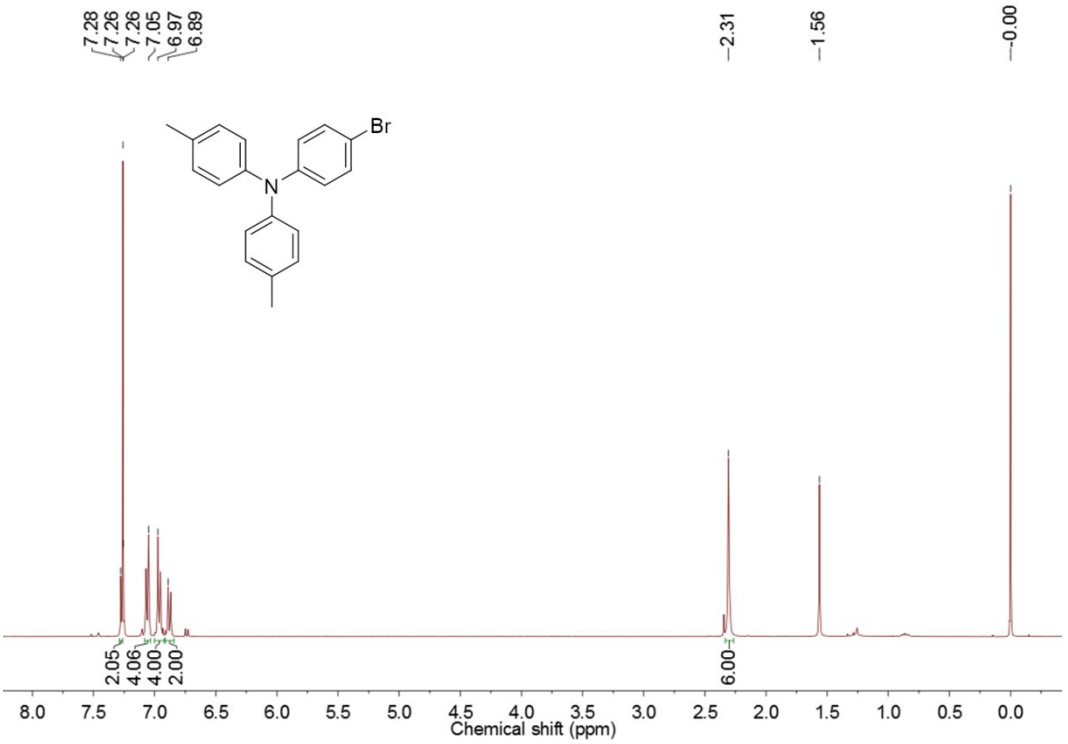

Figure S1. [1]H NMR spectrum of compound **1** in chloroform-*d*.

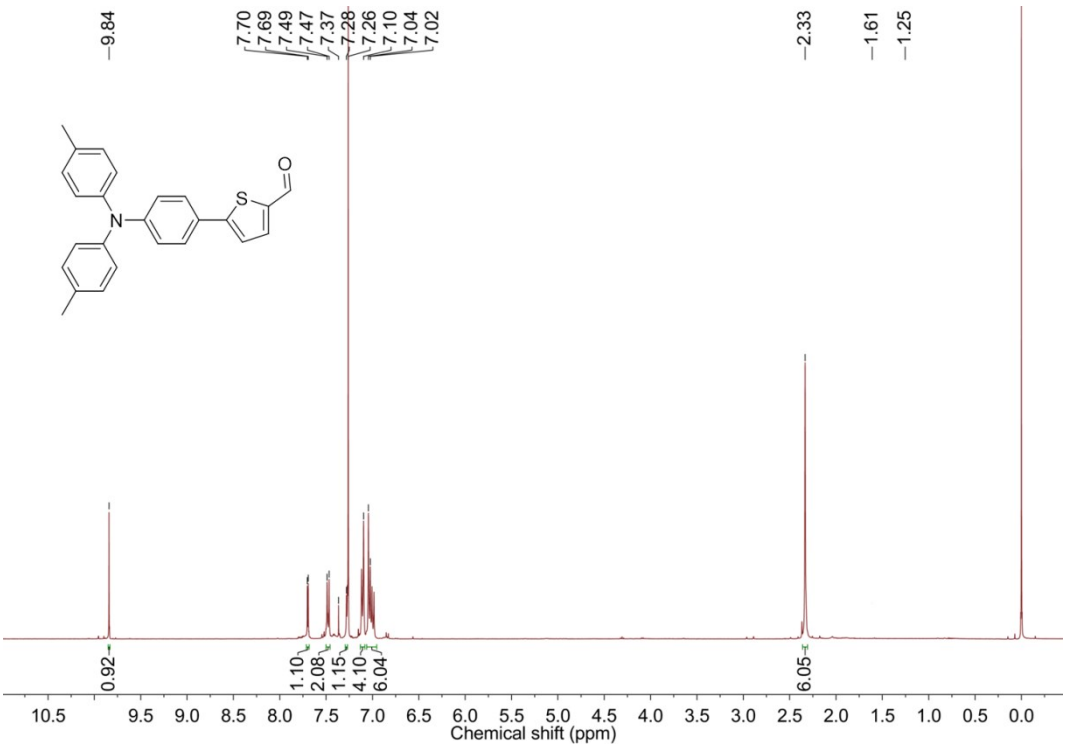

Figure S2. [1]H NMR spectrum of compound **2** in chloroform-*d*.

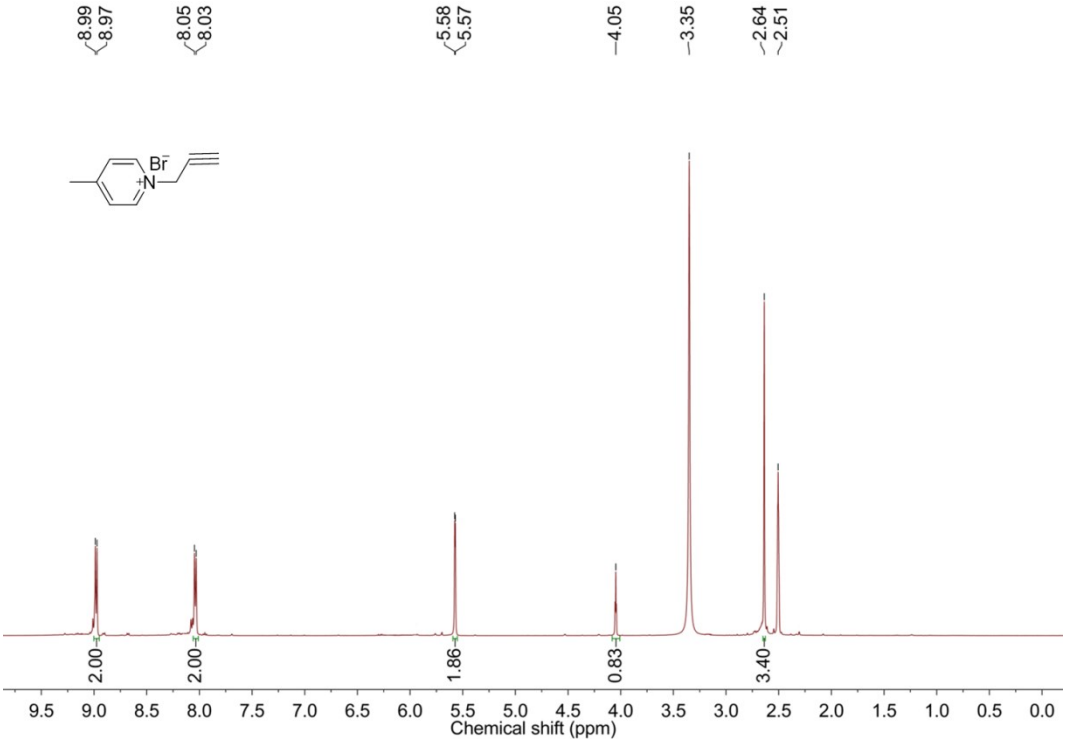

Figure S3. [1]H NMR spectrum of compound **3** in DMSO-*d*.

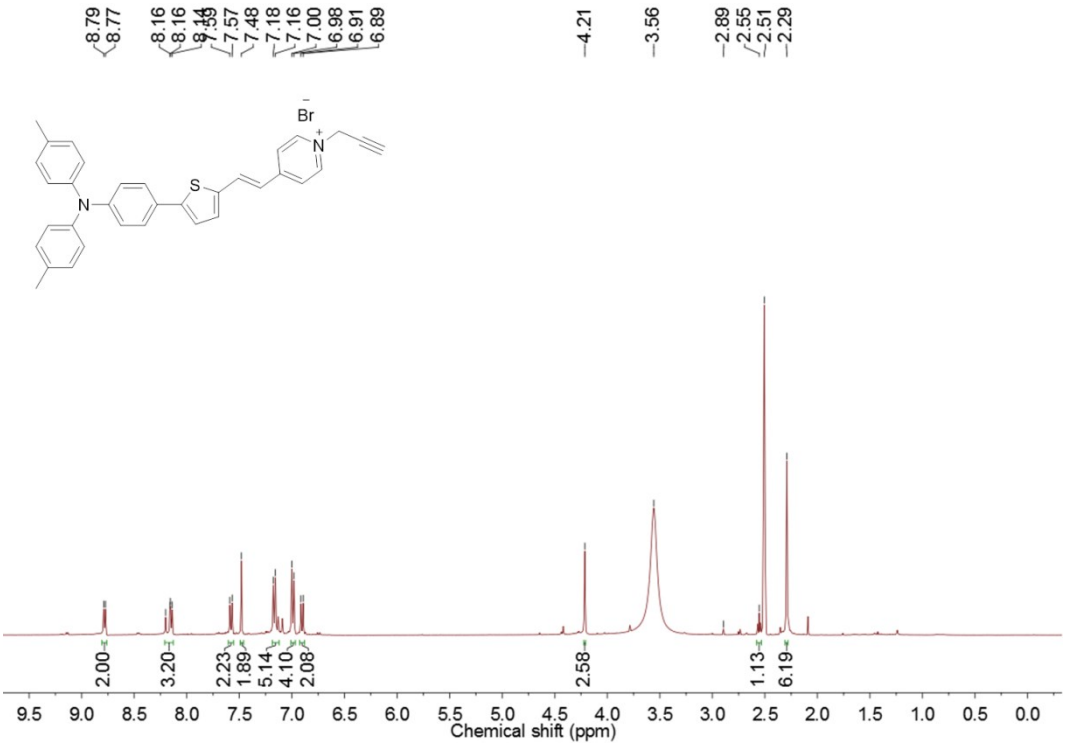

Figure S4. [1]H NMR spectrum of compound **4** in DMSO-*d*.

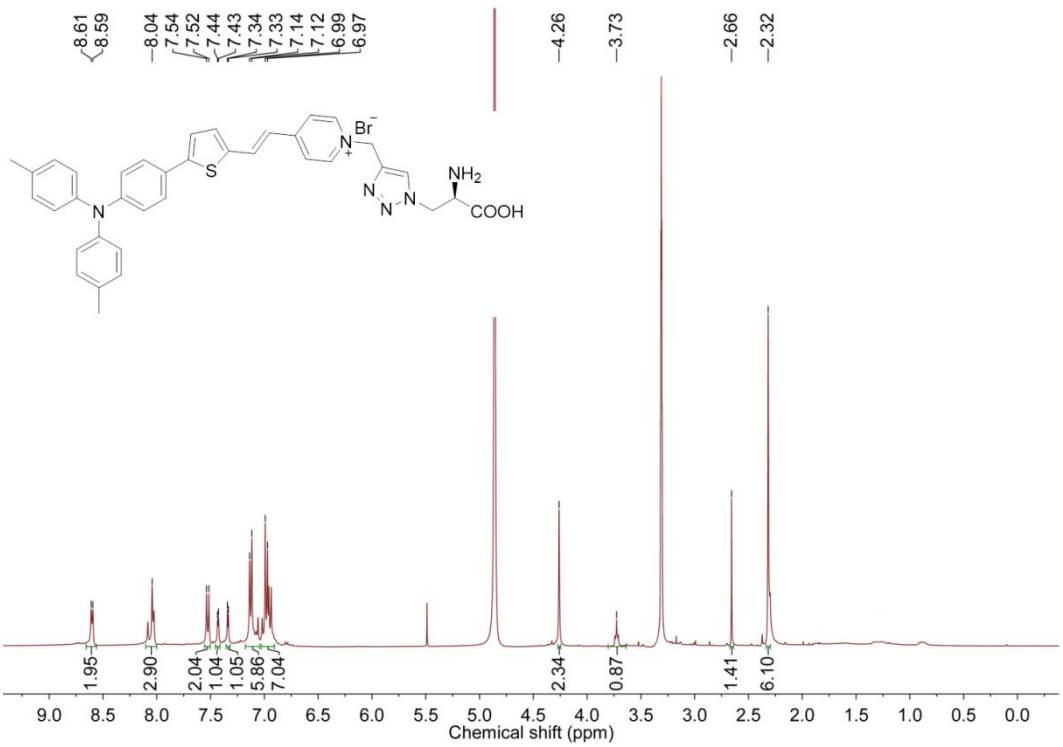

Figure S5. [1]H NMR spectrum of compound **MA** in DMSO-*d*.

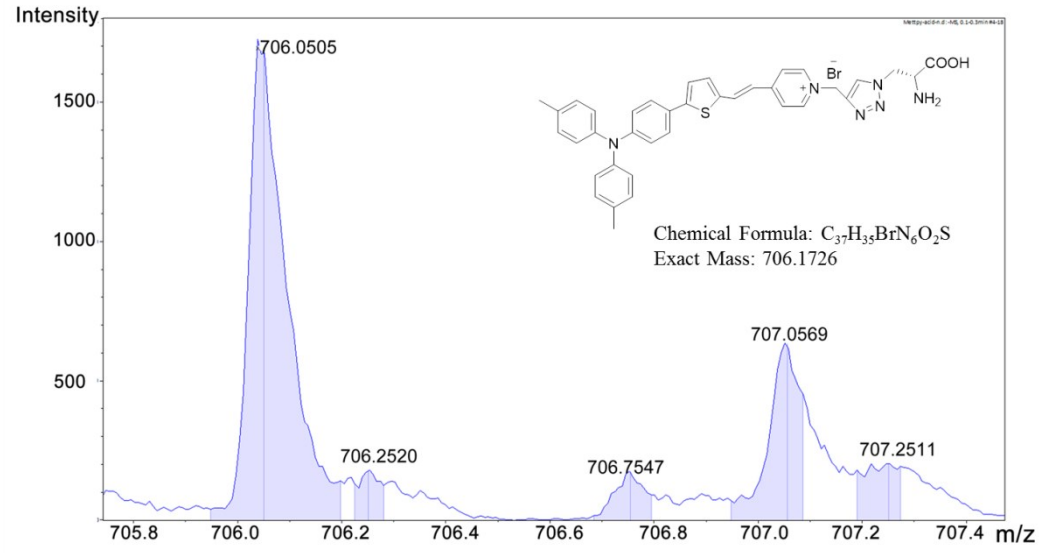

Figure S6. ESI-Mass spectrum of compound **MA**.

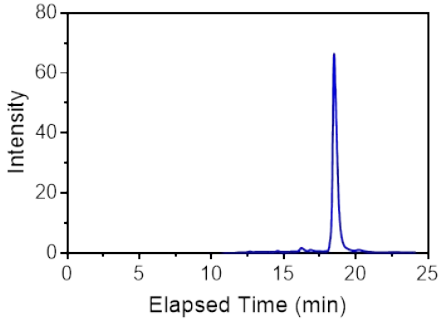

Figure S7. HPLC trace of **MA** monitored at 254 nm. HPLC conditions: column: Agilent ZORBAX SB-C18 (9.4×150 mm); gradient: 0-6-13-17 min, 25%-80%-100%-100% B (A: H_2O containing 0.1% TFA, B: acetonitrile containing 0.1% TFA); flow rate is 2.0 mL min^{-1}.

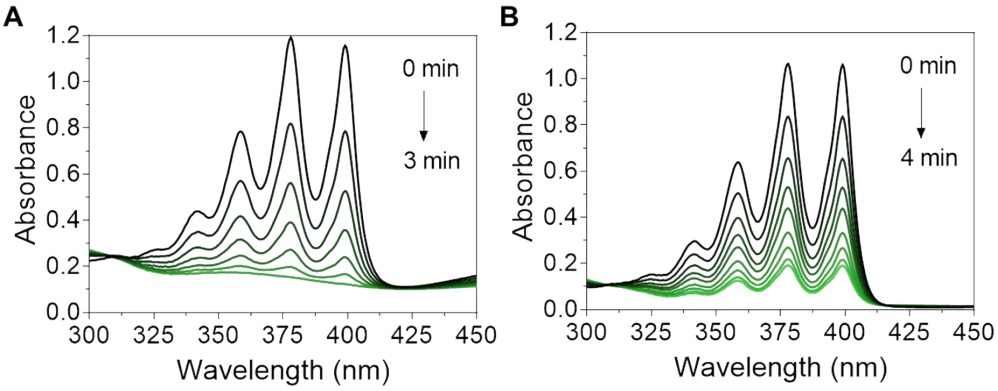

Figure S8. UV-*vis* spectra of ABDA in the presence of (A) **MA** (10 μM) or (B) Rose bengal (10 μM) under white light irradiation 30 mW/cm^2 in DMSO/water (V/V) = 1/99. [ABDA] = 5×10^{-5} M, time interval for recording the UV-*vis* spectra: 30 s.

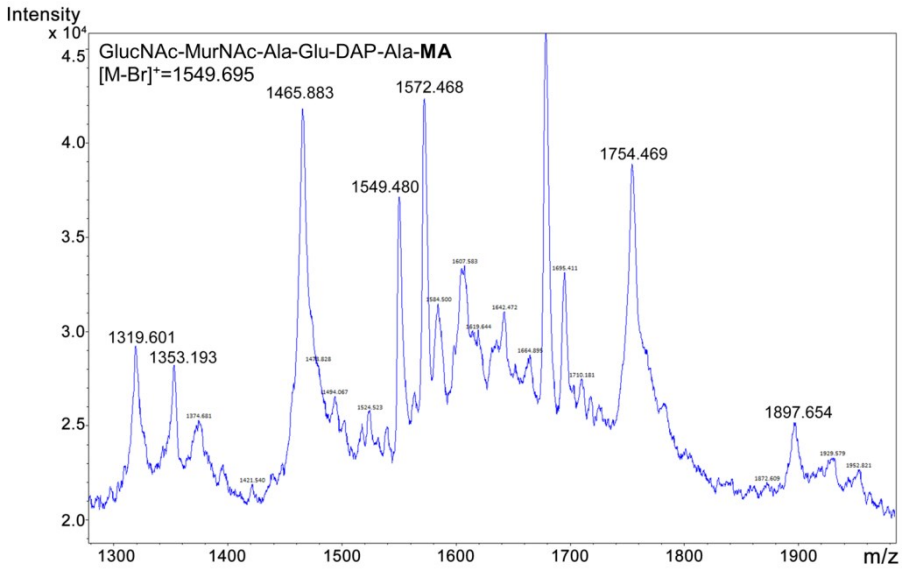

Figure S9. MALDI-TOF MS of **MA** treated *Salmonella* peptidoglycan fragments. M/Z range is 1300-2000.

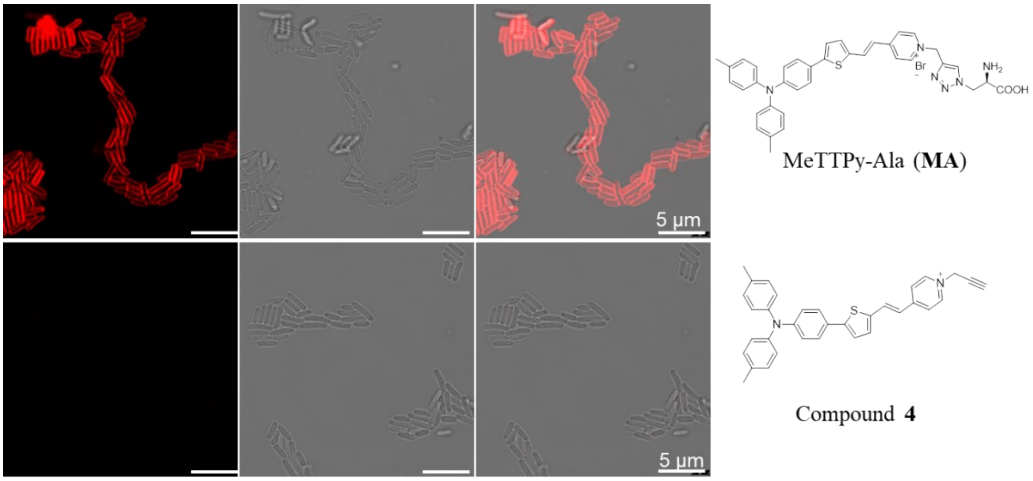

Figure S10. Confocal microscopy images of **MA** (20 μM) and compound **4** (20 μM) labeled *Salmonella* for 30 min.

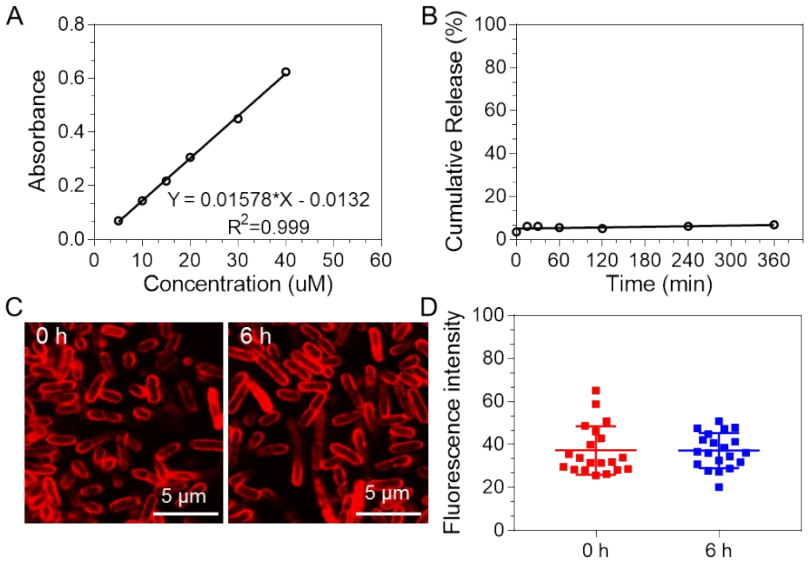

Figure S11. A) The standard absorbance vs concentration curves of **MA** in PBS. B) *In vitro* **MA** release profile at 37 ºC from **MA**@SV (20 μM **MA**) in PBS. C) Confocal microscopy images and D) fluorescence quantitative analysis with *Image J* software of **MA**@SV (20 μM **MA**) after **MA** release assay for 0 h and 6 h in PBS.

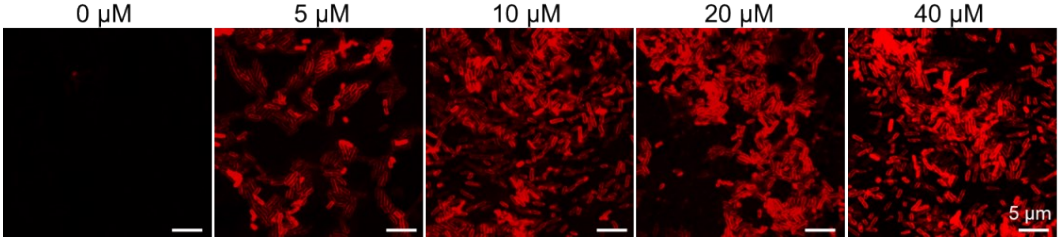

Figure S12. Confocal microscopy images of *Salmonella* labeled with various concentrations of **MA** for 30 min.

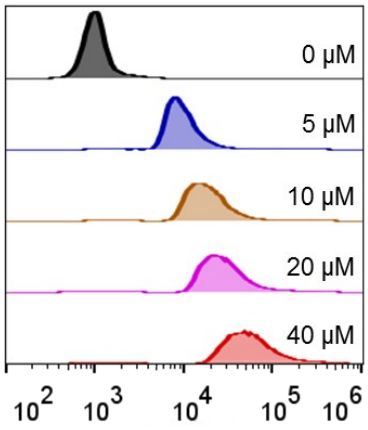

Figure S13. FACS histograms obtained from *Salmonella* labeled with different concentration of **MA** for 30 min.

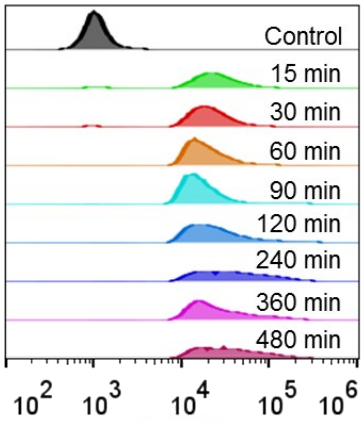

Figure S14. FACS histograms obtained from *Salmonella* labeled with **MA** (20 μM) for different time.

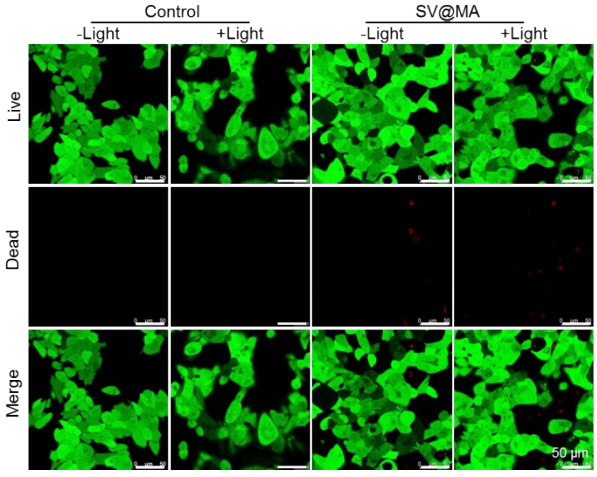

Figure S15. Live and dead assays of 4T1 cells treated with SV@**MA** (20 μM **MA**) for 4 h and then upon light irradiation at 30 mW/cm^2 for 10 min. The cells without SV@**MA** treatment were set as control group. The concentrations of FDA and PI were 10 μg/mL.

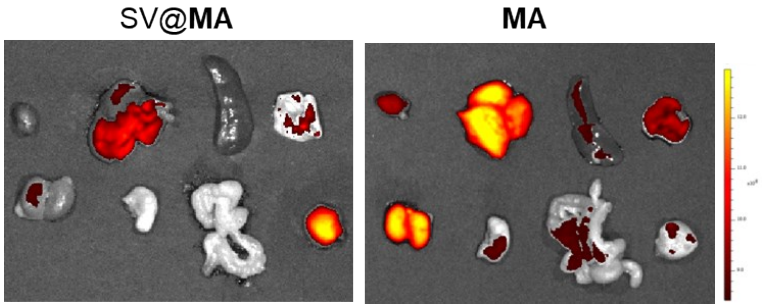

Figure S16. *Ex vivo* images of the main organs (heart, liver, spleen, lung, kidney, tumor, and intestine) harvested at 4 h after BALB/c nude mice with 4T1 xenografts injected with 200 μL of SV@**MA** (20 μM **MA** labeled) or **MA** (20 μM) *via* the tail vein.

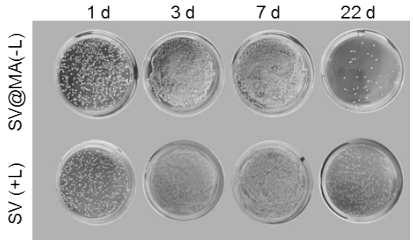

Figure S17. Selective *Salmonella* growth in tumor tissues. Homogenates tumor tissues after various treatments at different days were cultured on solid LB agar at 37 °C for 24 h.

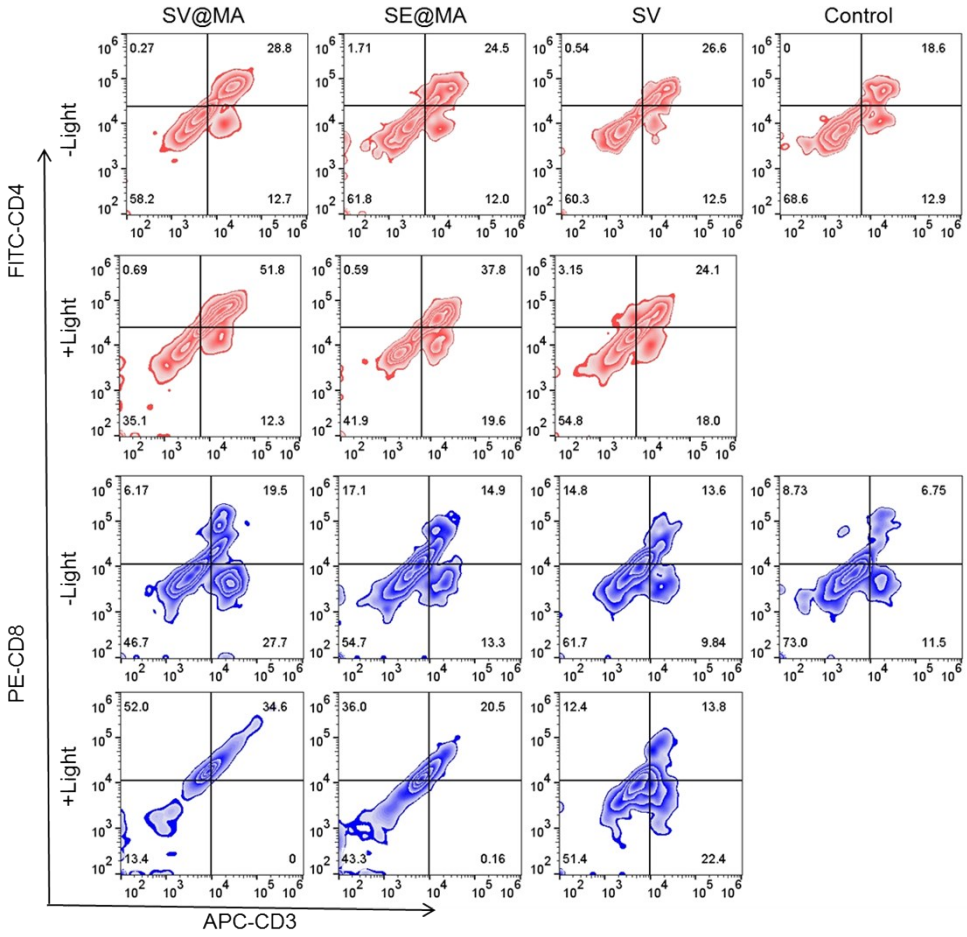

Figure S18. Flow cytometric analysis of $CD4^+$ and $CD8^+$ T cells in BALB/c mice immunized with PBS, SV, SE@**MA**, and SV@**MA** on the 4T1 tumor model with or without white light irradiation. The cells were isolated from the blood and stained with APC-CD3, FITC-CD4 and PE-CD8 antibodies.

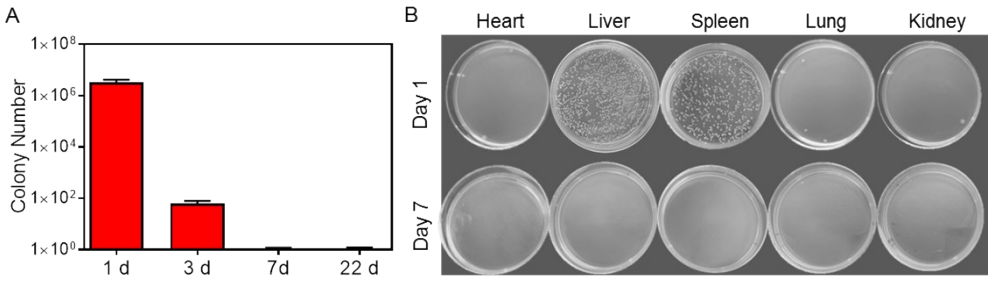

Figure S19. A) Colony numbers of *Salmonellae* from mice blood on day 1, 3, 7 and 22 after cultured on solid LB agar at 37 °C for 24 h (n =3). B) Homogenates of five main organs (heart, liver, spleen, lung, and kidney) on day 1 after injection of SV@**MA** and day 7 after SV@**MA**(+L) treatment were cultured on solid LB agar at 37 °C for 24 h.

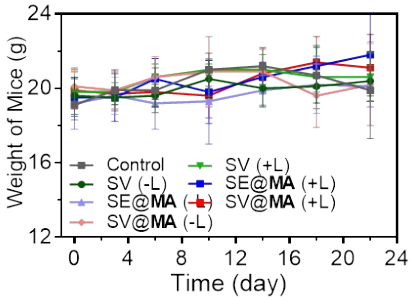

Figure S20. Body weight changes of BALB/c mice with 4T1 xenografts after treated with the different formulations.

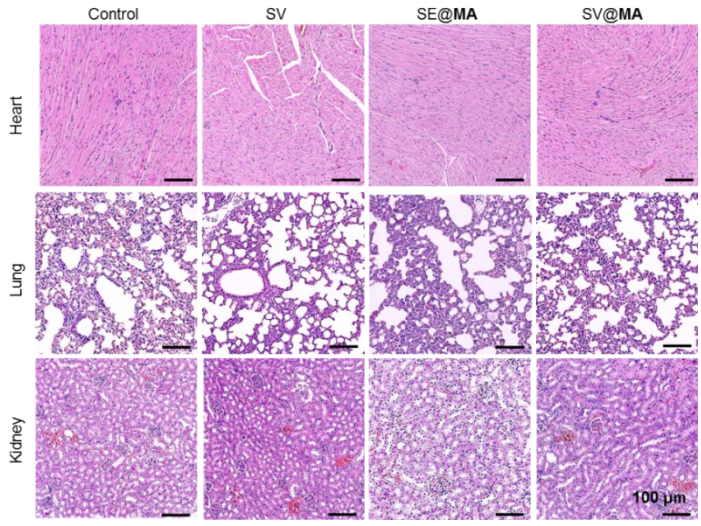

Figure S21. HE analyses of the main organs of BALB/c nude mice bearing 4T1 tumor after the indicated treatment.

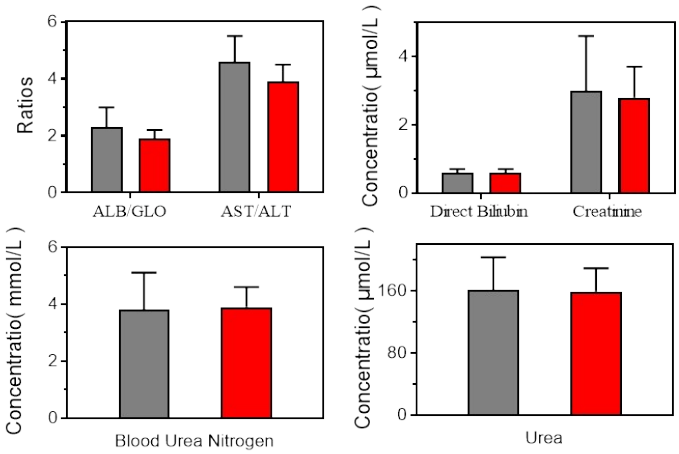

Figure S22. Liver function and renal function results of mice administered with SV@**MA** (Red) or PBS (gray). Data were shown as mean ± SD (n = 3).