A DNA and Mitochondria Duel-targeted Photosensitizer for Two-Photon Excited Bioimaging and Photodynamic Therapy

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Experimental Section

Materials

Compound S2 and 1-propyl-4-methylpyridinium iodide were synthesized according to the reported literature[1,2]. The nuclear extracts of 4T1 Cells were obtained according to the reported literature[3]. 4,4'-dibromotriphenylamine, and 5-formyl-2-thiopheneboronic acid were purchased from Energy Chemical Co. 1,1'-bis(diphenylphosphino)ferrocene dichloropalladium(II), 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABDA), and piperidine were obtained from Sigma-Aldrich. The widely used commercial photosensitizer (Ce6) was supplied by Shanghai Yuanye Bio-Technology Co., Ltd. The nucleus stained dye (4',6-diamidino-2-phenylindole dihydrochloride, DAPI), and probe for mitochondria (Mito-Tracker Green) were obtained from Beyotime Biotechnology. The calf thymus DNA (ctDNA), trypsin, double antibody, fetal bovine serum (FBS), phosphate buffer saline, mediuM RPMI 1640, and 4% paraformaldehyde fix solutions were purchased from Invitrogen Corporation. The reactive oxygen species Assay kit, Annexin V-FITC/PI apoptosis kit, one step tunel apoptosis assay kit, hematoxylin-eosin staining kit, and antifade solutions were obtained from Solarbio Technology Co., Ltd. The solvent used in this manuscript, including DMSO, DMF, DCM, hexane, toluene, and ethyl acetate were supplied by Adamas. All organic reagents were received without further purification. Bablc female mice (5 weeks old, about 18g) were provided by the animal center of Southern Medical University. The 4T1 cancer cells were presented by the Cell Biology Department of School of Basic

Medical College, Southern Medical University.

Instruments

The ^{1}H-NMR and ^{13}C-NMR spectra of all compounds were recorded on a Bruker Avance Ⅲspectrometer (600 MHz). The IR spectrum of TPA-2PI was performed with Thermo Fisher Nicolet iS500 spectrometer. The UV-vis absorption spectra were acquired on a Thermo Fisher Evolution 300 spectrophotometer. The fluorescence spectra of TPA-2PI were detected with a Thermo Scientific Lumina spectrophotometer. Mass spectroscopy was Mass spectroscopy an LTQ Orbit rap XL instrument. The OD values of cell activity wert determined by a Bioek Elx80 enzyme-linked immunosorbent assay. The confocal laser scanning microscopy (CLSM) images were obtained on an Olympus FV1000-IX81 confocal laser scanning microscope. The two-photon imaging graph were collected by a FV1200MPE multi-photon laser scanning microscopy. The flow cytometry data were obtained from a BD FACSCanto II/BD LSRFortessa X-20 flow cytometry. *In vivo* imaging and *in vitro* organ fluorescence imaging graph were detected by a Bruker FX Pro multimodal small animal *in vivo* imaging system. The mitochondrial morphology was studied by a biological transmission electron microscopy (Bio-TEM).

Synthesis of compound S2

Compound S2 was prepared according to the previously reported method [1]. 4,4'-dibromotriphenylamine (6.05 g, 15 mmol), 5-formyl-2-thiopheneboronic acid (7.02 g, 45 mmol), K_2CO_3 (21.10 g, 150 mmol), and $PdCl_2$(dppf) (1.2 g, 1.6 mmol) were sequentially added to a mixture of toluene and methanol (1 : 1, 180 mL), and heated to

90 °C for 6 h. After cooled down to room temperature and removed the solvent by rotary evaporation, the residue was dissolved in CH_2Cl_2, washed with water, and purified by column chromatography over a silica gel to yield the middle product, the compound S2 as a yellow solid (5.24 g, 75%). ^{1}HNMR ($CDCl_3$, 600 MHz, ppm): δ 9.87 (s, 2H); 7.73-7.72 (d, 2H); 7.58-7.56 (d, 4H); 7.36-7.33(m, 4H); 7.18-7.16 (m, 3H); 7.14-7.13 (d, 4H).

Synthesis of TPA-2PI

A mixture of 1-propyl-4-methylpyridinium iodide (1.32 g, 5 mmol), compound S2 (0.93 g, 2 mmol), and piperidine (4 drops) were dissolved in 30 mL ethanol. This mixture was refluxed for 8 h under an atmosphere of nitrogen. After cooling down to room temperature, the resulting mixture was filtered, and the collected solid was washed with DCM and Ethanol several times to afford the product **TPA-2PI** as a dark red solid (2.29 g, yield: 43%).^{1}H NMR (600 MHz, DMSO-d_6) δ (ppm): 8.91~8.90 (d, 4H), 8.26~8.20 (m, 6H), 7.71~7.69 (d, 4H), 7.57~7.52 (dd, 4H), 7.44~7.40 (t, 2H), 7.20~7.15 (m, 5H), 7.12~7.10 (d, 4H), 4.46~4.43 (t, 4H), 1.96~1.91(dd, 4H), 0.92~0.89(t, 6H). ^{13}C NMR (150 MHz, DMSO-d_6) δ (ppm): 153.48, 147.35, 147.23, 146.52, 143.95, 139.19, 138.25, 134.33, 130.75, 130.16, 129.80, 129.46, 129.14, 127.16, 125.49, 125.06, 124.32, 59.36, 26.30, 10.26. IR (KBr, cm^{-1}): 3427, 3027, 1640, 1596, 1558, 1515, 1494, 1325, 1292, 1202, 1173, 1057. HRMS (ESI, m/z), calculated for $C_{46}H_{43}N_3S_2^{2+}$/2, 355.6428, found 355.6447.

Molecular docking

TPA-2PI was built by Chem3D software and optimized by Gauss to achieve stable

formation conformations in model dynamics. The crystal structure of ctDNA sequence dodecamer d(CGCGAATTCGCG)$_2$ (PDB ID: 3u2n) used for docking studies was obtained from Protein Data Bank[4]. Program suite AutoDock 4.2.0 (http://www.scripps.edu/mb/olson/doc/autodock) and AutoDock Vina were utilized for docking modeling between **TPA-2PI** and ctDNA based on Lamarckian Genetic Algorithm[5].

Photostability of TPA-2PI

To investigate the photostability of **TPA-2PI**, the parameters and procedures are as follows: firstly, the **TPA-2PI** (1 μM) solutions was irradiated by light (300 mW/cm^2) last for 10 mins, the change of absorbance and fluorescence intensity was collected by the spectrometer before and after continuous irradiation. Secondly, the imaging mode was time lapse, and the 4T1 cells stained upon incubation with **TPA-2PI** were analyzed every 2 min while under continuous laser irradiation. Then, ImageJ software was used to assess the FL intensity of each image. The photostability of **TPA-2PI** was expressed by the ratio of the fluorescence intensity at different time intervals during continuous laser irradiation to the initial value as a function of the exposure time[2].

Measurement $^1O_2$ detection

9,10-anthracenediyl-bis(methylene)dimalonic acid (ABDA) was used as an active oxygen indicator to detect the $^1O_2$ production performance of **TPA-2PI**. PBS solution of **TPA-2PI** (1 μM, 20 μL), and ABDA (10 μM, 20 μL) were mixed. The mixed system was then irradiated under white light (400-750 nm, 60 MW/cm^2) and record the change of absorption intensity at 380 nm to detect the UV absorption value of the mixed

solution. The widely used commercial PS (Ce6, 1 μM), and the ABDA solution in absence of **TPA-2PI** were chosen as the control groups.

Cell culturing

4T1 cells were cultured in RPMI 1640 medium. The culture media contained 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (double antibody). All cells were cultured in an incubator at 37 °C in a humidified environment containing 5% CO_2.

Cellular uptake and cytotoxicity

4T1 cells were seeded in confocal cell dishes and cultured overnight to a confluence of around 80%. TPA-2PI was added to the cell dishes. After incubation for 2 h, the cells were washed with 1× PBS and used for confocal imaging. The fluorescence was excited with a 488 nm laser and collected within 585–750 nm. CCK-8 kit was used to detect the cytotoxicity of **TPA-2PI**. 4T1 cells in the logarithmic growth stage were seeded in 96 well plates (1 * 10^4 4T1 cells per well), cultured for 24 hours, sucked out the medium, and added with different concentrations of **TPA-2PI** (0, 0.1, 0.3, 0.5, 1, 2 μM). After incubation for 12 h, removed the solution in the well, added the medium containing CCK-8 (V: V= 1:10), incubated for 2 h, and used the enzyme labeling instrument to detect the absorbance of each well at 450 nm. The relative cell survival rate was calculated as follows: cell survival rate (%) = (OD experiment od blank) / OD control × 100%.

Phototoxicity of TPA-2PI *in vitro*

The cytotoxicity of **TPA-2PI** in the presence of white light irradiation was assessed by CCK-8 assays. 4T1 cells were seeded in 96-well plates and cultured in standard medium

containing 10% FBS and 1% penicillin–streptomycin (double antibody) for 24 h (37 °C, 5% CO_2). The cells were then incubated with **TPA-2PI** solutions at various concentrations (0, 0.1, 0.3, 0.5, 1, 2 μM) in the dark for 24 h. The mixtures were discarded and added with fresh standard DMEM before exposure to white light with different power density (40 mW/cm^2, 60 mW/cm^2, 80 mW/cm^2) for 10 min. The absorbance of CCK-8 at 450 nm was measured by a Bioek Elx80 enzyme-linked immunosorbent assay. Cell viability was expressed by the ratio of the absorbance of the cells incubated with TPA-2PI to that of the cells incubated with culture normal medium. Each experiment was repeated at least four times.

Cellular colocalization

4T1 cells were incubated with **TPA-2PI** (1 μM) in a culture medium for 1 h at 37 °C, then the stained cells were washed with PBS three times. Mito-Tracker Green was added and co-incubated for another 30 min, and cell imaging was then carried out after washing the cells with PBS three times. **TPA-2PI** has collected from 585 to 750 nm (λ_{ex} = 488 nm), which was marked with a red channel. Emission from Mito-Tracker Green was collected based on the manufacturer's instruction.

Two-photon imaging *in vitro*

4T1 cells in the logarithmic growth stage were inoculated in a confocal dish. When the cells grew to 80%, the medium was removed and **TPA-2PI** (1 μM) was added. After co-cultured for another 4-6 h, sucked out the culture medium, fixed it with 4% paraformaldehyde for 10min, added anti fluorescence quenching sealing agent to protect the sample, and observed by multiphoton laser scanning microscope (λ_{ex} = 960

nm).

Flow cytometry

4T1 cells in the logarithmic growth stage were inoculated into 6-well plates ($2*10^5$ cells for each well). When the cells grew to 80%, removed the medium and added **TPA-2PI** (1 μM) medium. After incubated for 6 h, sucked out the medium, washed the residual drugs with PBS twice times. Subsequently, the basic medium containing reactive oxygen species detection kit (DCFH-DA V: V = 1:1000) was added and incubated in dark for 20 minutes. The experimental groups were exposed to white light (80 mW/ cm^2) for 4 min. By contrast, the control group was not exposed to light. After that, the cells in each group were digested and centrifugated after treatment. The intracellular fluorescence was analyzed quantitatively by flow cytometry.

Intracellular $^1O_2$ detection

Prior to intracellular ROS estimation, 4T1 cells were incubated with **TPA-2PI** (1 μM) for 6 h. After this treatment, cells were washed three times with fresh culture medium to remove the residual organic dots, and then incubated with DCFH-DA (2 μM) for another 30 minutes. After washing three times with fresh medium, the cells were processed for two-photon excitation (λ_{ex} = 960 nm) and intracellular ROS estimation.

Cell apoptosis

The 4T1 cells were divided into four groups, including PBS-light group, PBS+light group, **TPA-2PI**-light group, **TPA-2PI**+light group. 4T1 cells in the logarithmic growth stage were inoculated in 6-well plates ($2*10^5$ cells per well), cultured at 37 ^oC and 5% CO_2 for 24 hours, sucked out the medium, and cocultured with **TPA-2PI** (1

μM) for 6 h. The experimental groups were exposed to the white light for 8 minutes (80 mW/cm^2), the solution in the well was removed, the cells were digested with trypsin, the cells were collected by centrifugation. Subsequently, 500 μL FITC working solution (FITC: buffer: water = 3:200:800), and 200 μL DAPI staining solution were added to each group. After staining for 5 min, the cell apoptosis was detected by flow cytometry (Channel: FITC / DAPI).

Mitochondria targeted PDT evidenced by Bio-TEM

For Bio-TEM studies, 4T1 cells were washed with PBS and subsequently fixed with 2.5% glutaraldehyde in 0.1 M PBS buffer for 1 h at 4°C. Then, the 4T1 cells were washed with 0.1M PB for four times. Sections were postfixed with 2% osmium, rinsed, dehydrated and embedded in Durcupan resin (Fluka, Sigma-Aldrich, St. Louis, USA). Semithin sections (1.5 μm) were cut with an Ultracut UC-6 (Leica, Heidelberg, Germany) and stained lightly with 1% toluidine blue. Finally, ultrathin sections (0.08 μm) were cut with a diamond knife, stained with lead citrate (Reynolds solution) and examined under a 200 kV transmission electron microscope FEI Tecnai S-4TF20 (FEI Europe, Eindhoven, Netherlands).

***In vitro* PDT efficiency estimation**

Prior to in vitro PDT treatment, 4T1 cells were incubated with **TPA-2PI** (1 μM) for 6 h. After the treatment, cells were washed three times with fresh culture medium to remove the residual organic dots, and ready for laser scan. After the PDT treatment, cells were incubated either with Annexin V-FITC for 30 minutes, or with fresh medium for 4 hours first and then with DAPI for another 30 minutes. Finally, the cells were

washed three times with fresh culture medium before imaging.

Breast cancer mouse model

All the animal experiments involved in this study were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (8th edition, 2011), and approved by the local research ethics review board of the Animal Ethics Committee of Southern Medical University. Specific female Balb/c mice, 5 weeks of age, originally obtained from the animal center of Southern Medical University, were used to establish breast cancer mouse model. Briefly, $1*10^6$ 4T1 cells were injected subcutaneously into the selected positions to establish the breast tumor model of Balb/c mice. Tumors were allowed to grow to about 100-150 mm^3 in volume before used for in vivo imaging and phototherapy.

***In vivo* abdominal blood vessels imaging**

TPA-2PI working solution (3 mg/kg) was injected into mice through caudal vein. After 10% pentobarbital was injected intraperitoneally to anesthetize mice, the peritoneum was cut open, the organs were adsorbed and fixed with small animal organ fixed adsorber, the fluorescence imaging of blood vessels at various parts was observed with an upright Multiphoton laser scanning microscope, and two-photon imaging pictures were taken (λ_{ex} = 960 nm; λ_{em} = 600-800 nm). Observe different organs at different time points. Scan and image the time series and depth series to test real-time imaging and depth imaging.

***In vivo* imaging in 4T1 tumor-bearing mice by i.v. injection**

To study the tumor targeting ability, the 4T1 tumor-bearing Balb/c mice were

intravenously injected with **TPA-2PI** (5 mg/kg), and the in vivo fluorescence signals were monitored at different post-injection time (3, 12, 24, 48, 72, 96 h) by using the small animals imaging system.

***Ex vivo* fluorescence imaging**

Ex vivo fluorescence imaging was conducted using an imaging instrument *in vivo*. The samples were injected with 0.5 ml of normal saline NS. After exposure, the fluorescence images of regions of interest (ROI) in the organs were collected on the IVISH Spectrum Imaging System and then the FL intensities of each sample were statistically analyzed.

***In vivo* PDT evaluation**

4T1 tumor-bearing mice model was established to evaluate the antitumor efficacy of **TPA-2PI**, $1*10^6$ 4T1 cells in 100 μL DMEM free of FBS injected subcutaneously into the right flank of each mouse. Six days after inoculation, mice were divided into four groups (n =5), including PBS-light group, PBS+light group; **TPA-2PI**-light group, **TPA-2PI**+light group, respectively. The mice were treated by intratumoral injection. The mice were exposed to white light (80 mW/cm^2) for 10 min every day for 2 days. After different treatments, the tumor sizes and body weights were measured every two days. The tumor size was measured every two days using a caliper, and the tumor volume was calculated according to the following formula: volume = $width^2 \times length/2$. For survival study, animals were euthanized when exhibiting the signs of impaired health or when the volume of the tumor exceeded 2000 mm^3.

Immunofluorescence staining

Some of each group's 4T1 tumor-bearing mice were sacrificed and tumor tissues were collected after 18 days of treatment for histological examinations. Hematoxylin and eosin staining, and TUNEL staining assay were performed to assess the treatment efficacy on the tumors. The TUNEL images were observed with a confocal microscope. Hematoxylin and eosin staining were employed to stain the heart, liver, spleen, lung, and kidney slices, while the TUNEL staining were used to stain the tumor slices.

Blood examination

After the treatments, the mice were anesthetized and blood was collected into an anticoagulant tube for routine examinations.

Statistical analysis

Data were expressed as mean ± standard deviation. Two-tailed Student's t-test was used to evaluate the statistical significance. P values < 0.05 were regarded statistically significant.

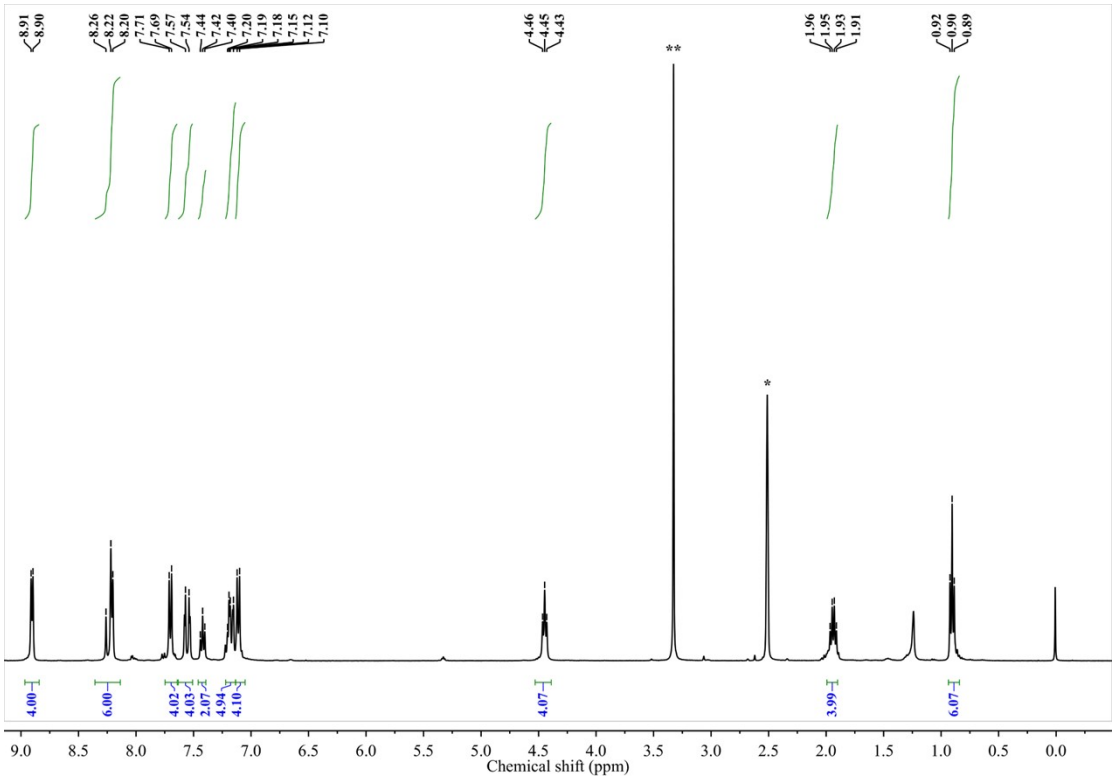

Figure S1. ^{1}H NMR spectrum of **TPA-2PI**.

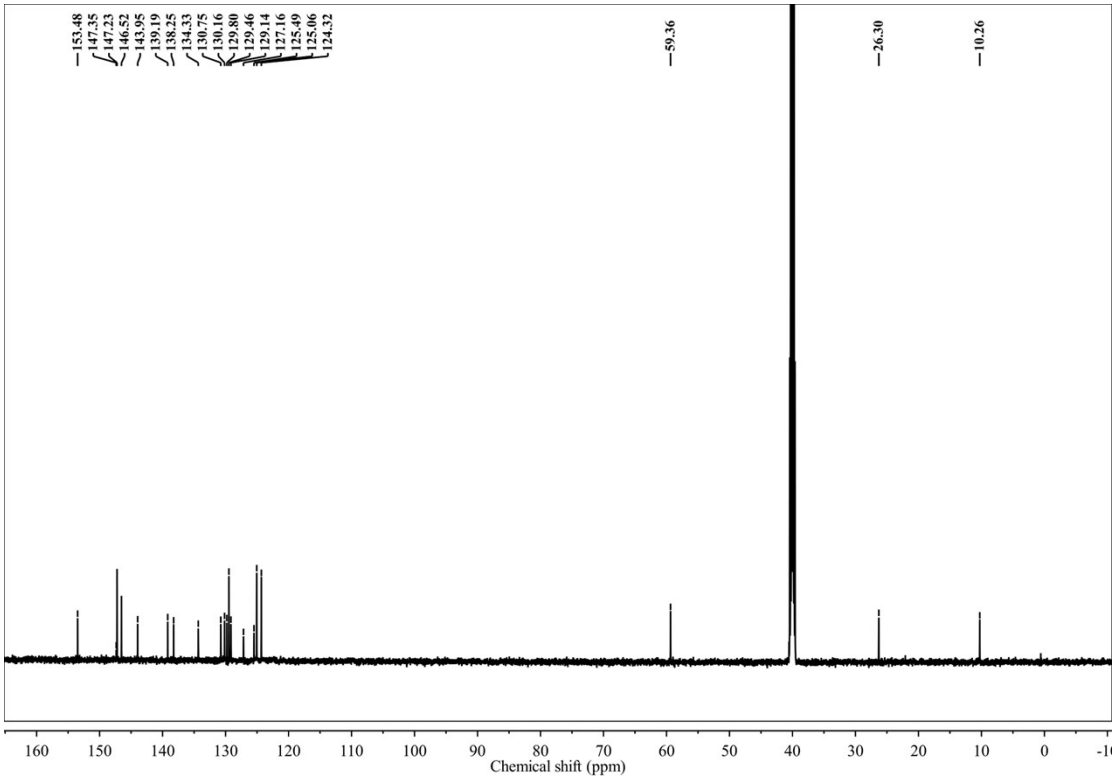

Figure S2. ^{13}C NMR spectrum of **TPA-2PI**.

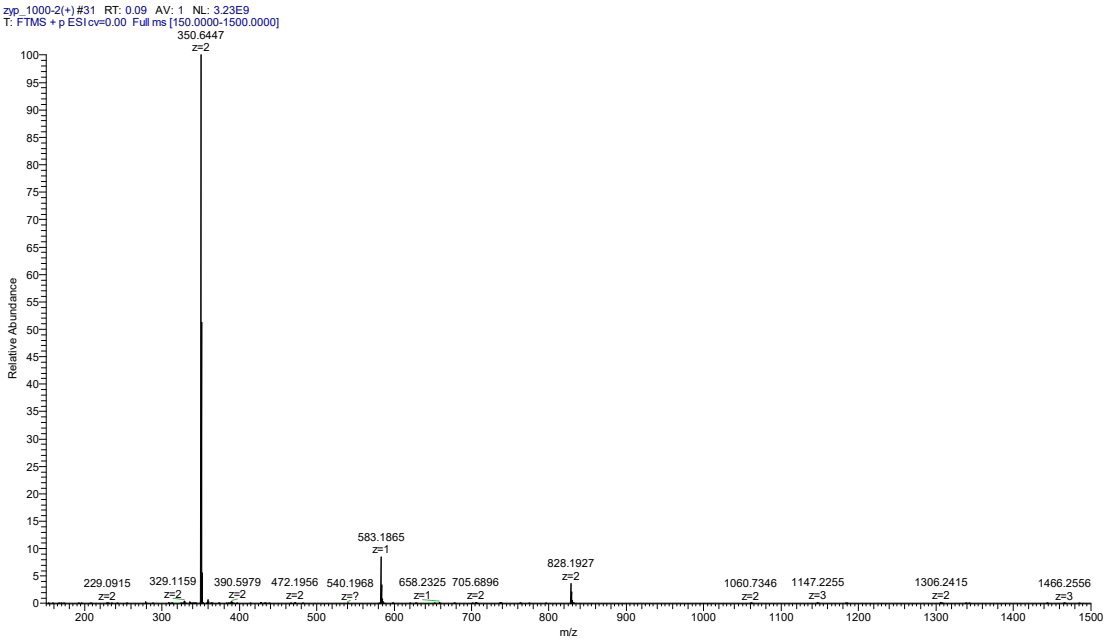

Figure S3. HRMS spectrum of **TPA-2PI**.

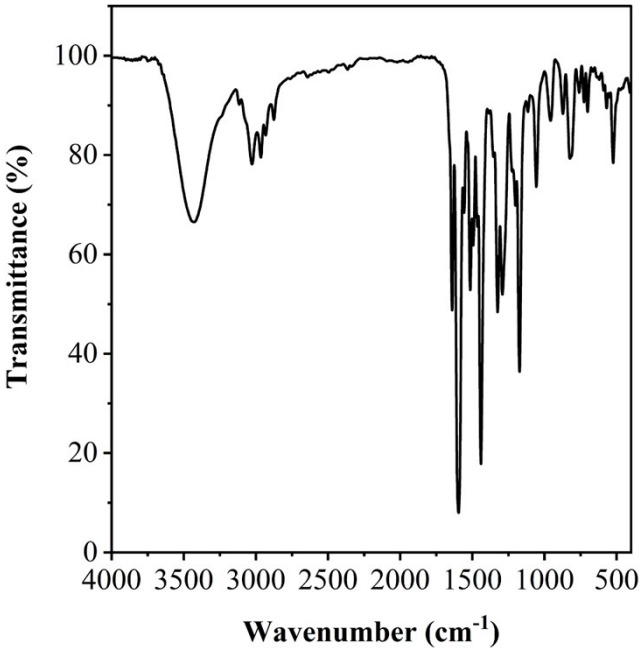

Figure S4. IR spectrum of **TPA-2PI**.

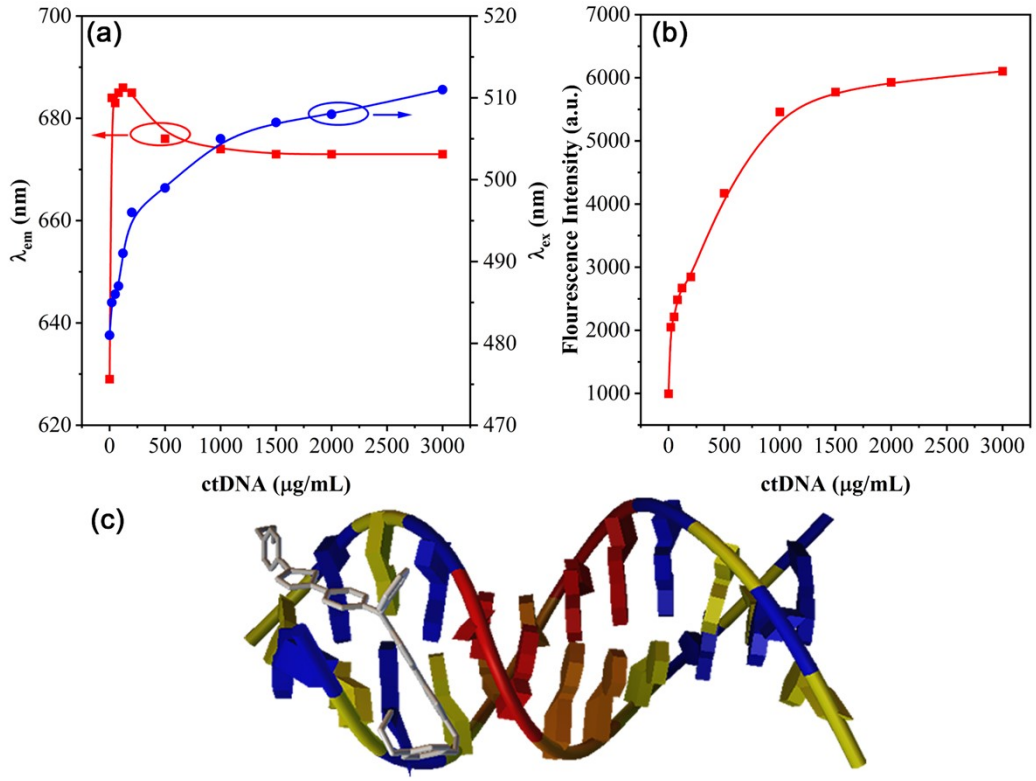

Figure S5. (a) The UV-vis absorption and fluorescence peak wavelength of **TPA-2PI** (1 μM) with different ctDNA concentrations range from 0 μg/mL to 3000 μg/mL. (b) The plot of fluorescence intensity of **TPA-2PI** (1 μM) with different ctDNA concentrations range from 0 μg/mL to 3000 μg/mL (c) Molecular docking model between **TPA-2PI** and ctDNA.

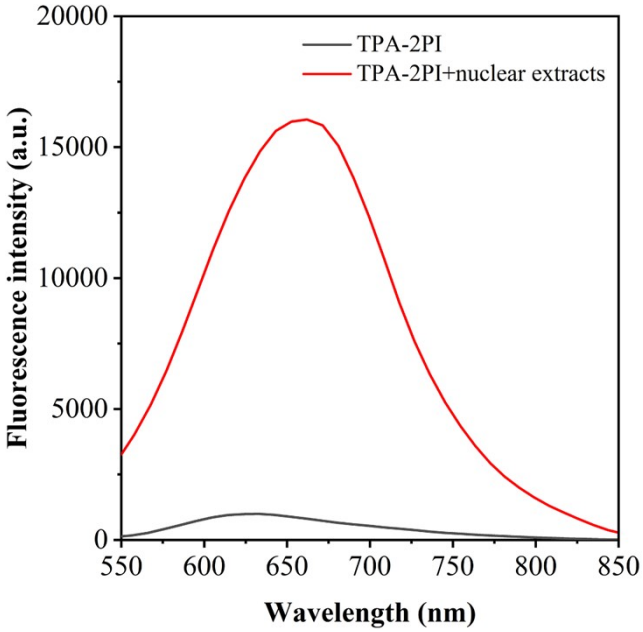

Figure S6. The fluorescence spectrum of **TPA-2PI** (1 μM) in absence and presence of the nuclear extracts of 4T1 cells.

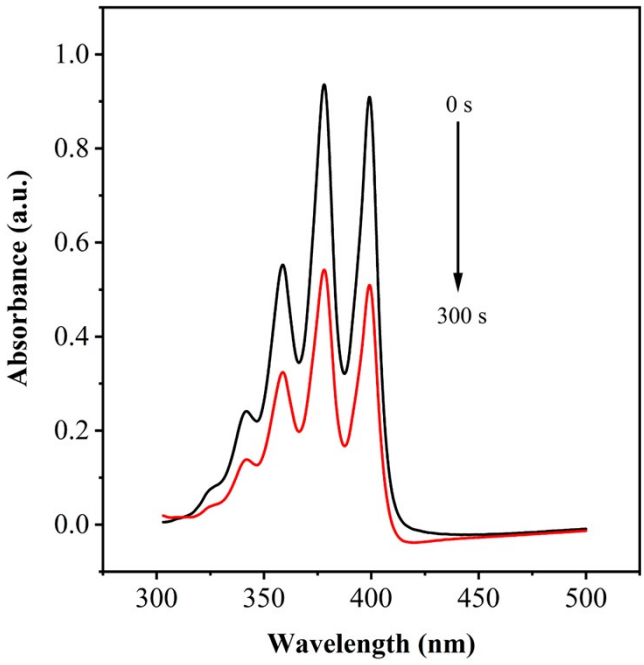

Figure S7. The UV-vis absorption of ABDA (10 μM) in presence of **TPA-2PI** (1 μM) and ctDNA (500 μg/mL) upon xenon lamp last for 300s.

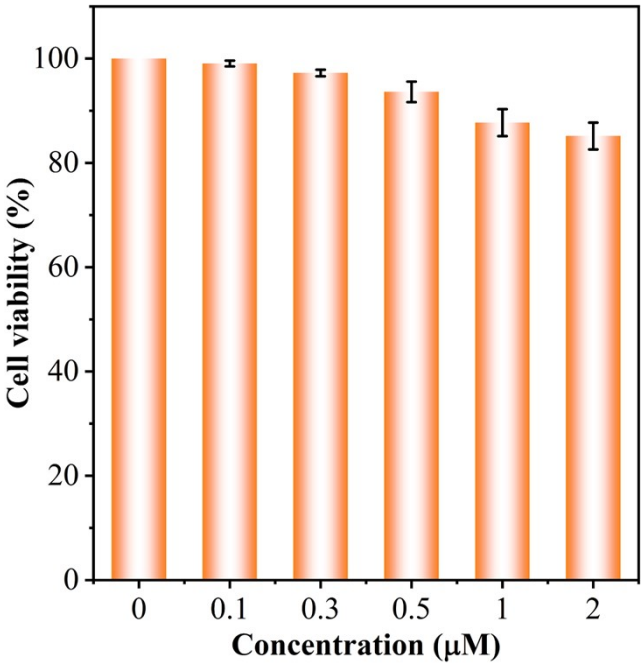

Figure S8. 4T1 cells viability with different **TPA-2PI** concentrations range from 0 μM to 2 μM in absence of white light irradiation.

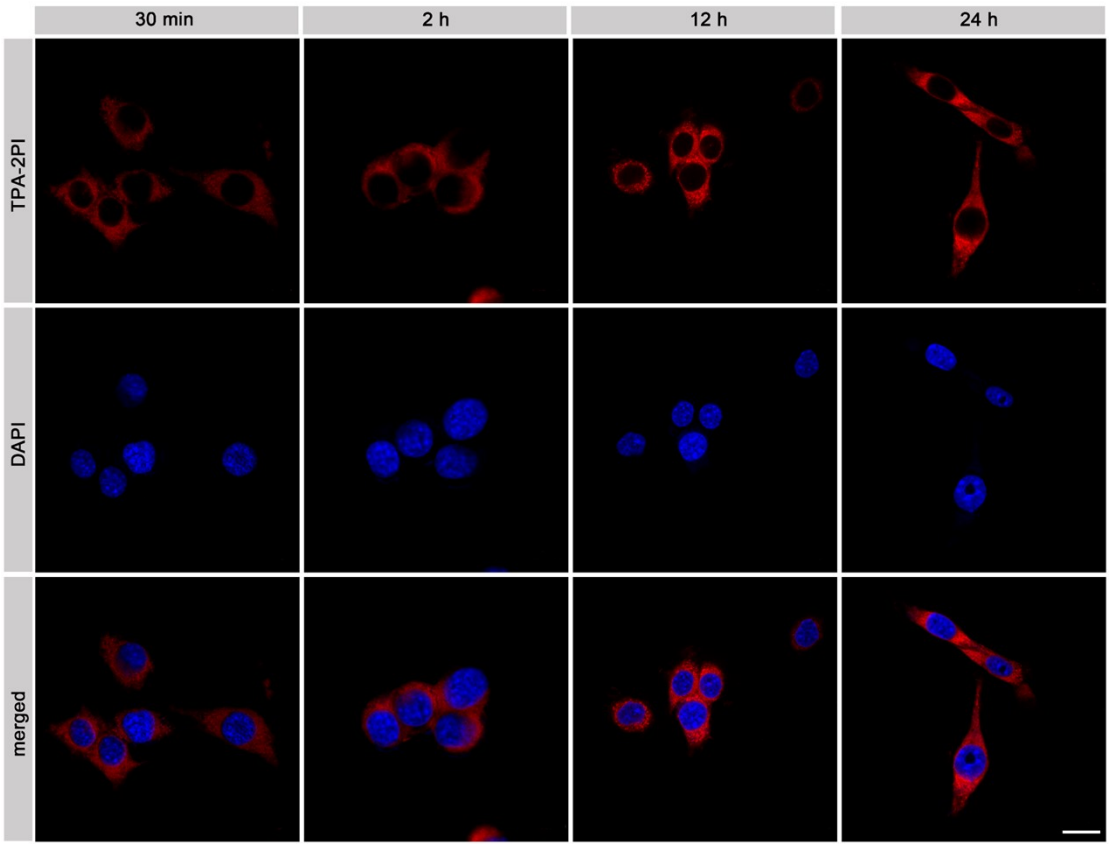

Figure S9. CLSM images of 4T1 cells in presence of **TPA-2PI** (1 μM) during different endocytosis time range from 30 min to 24 h. Scale bar: 20 μm.

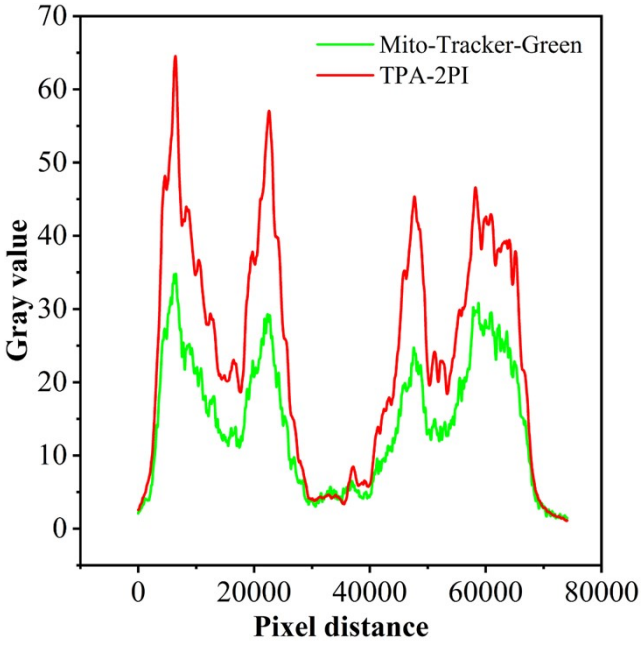

Figure S10. The normalized line intensity profiles of images at colocalization CLSM images

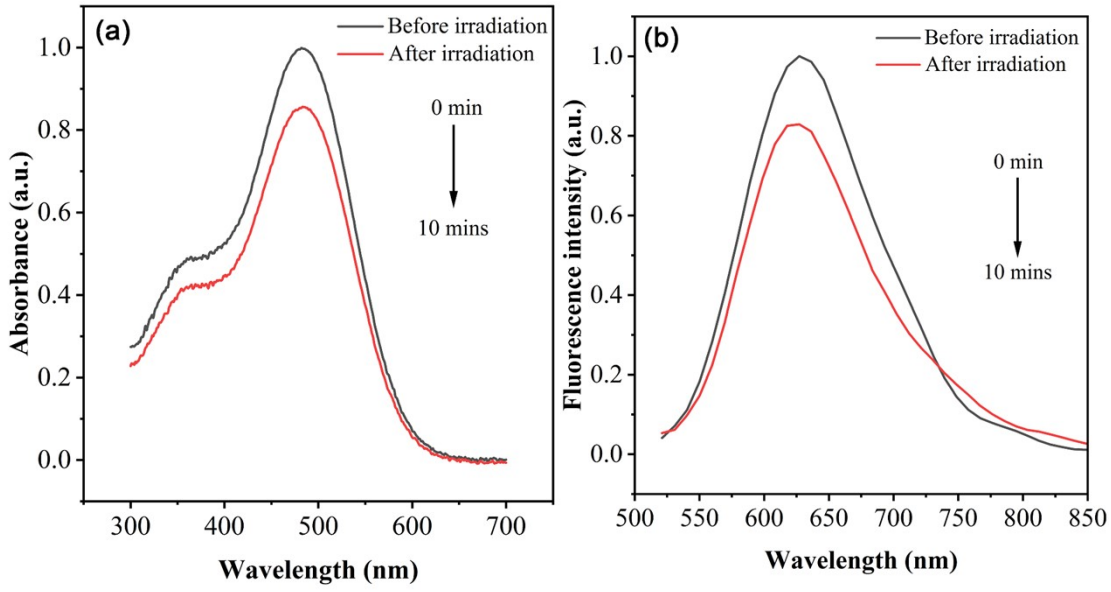

Figure S11. (a) the UV-vis spectrum of **TPA-2PI** (1 μM) before and after irradiation. (b) the FL spectrum of **TPA-2PI** (1 μM) before and after irradiation.

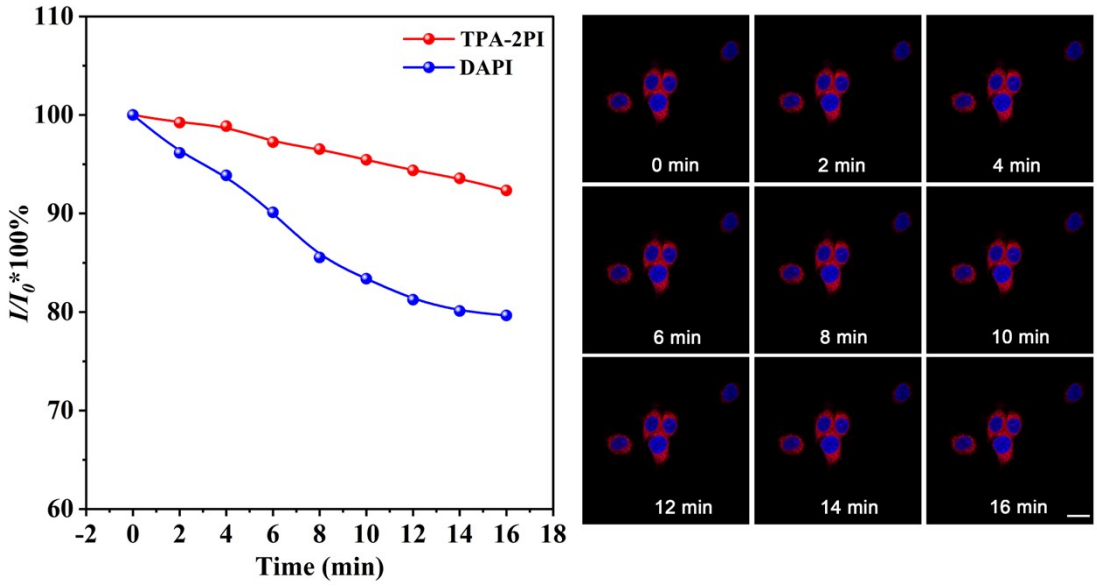

Figure S12. The photostability and merged CLSM images in 4T1 Cells of **TPA-2PI** (1 μM) and DAPI (1 μM) under continuous scanning at 488 nm and 405 nm (2% laser power) for 16 mins, respectively. I_0 is the initial fluorescence intensity and I is the fluorescence intensity of each sample at various time points. Scale bar: 20 μm.

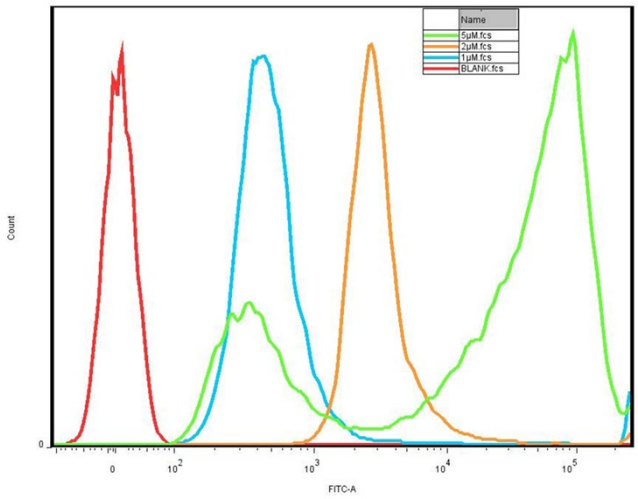

Figure S13. Flow cytometry profiles of 4T1 cells treated by **TPA-2PI** with different concentrations range from 0 μM to 5 μM in presence of white light irradiation (80 mW/cm^2).

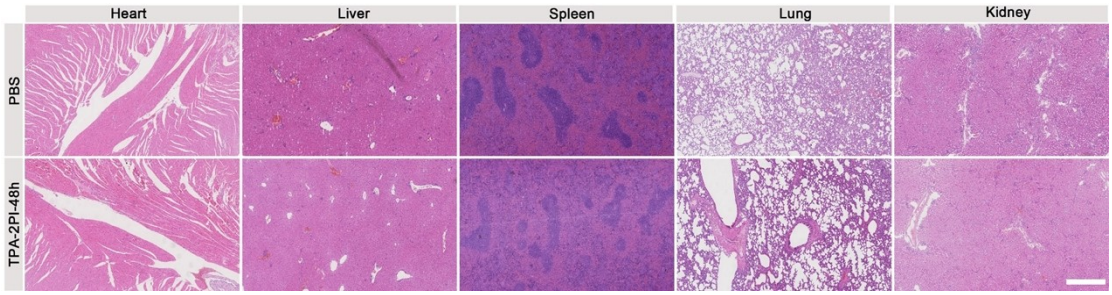

Figure S14. H&E staining of major organs tissues after *in vivo* bioimaging in absence or presence of **TPA-2PI** (1 μM). Scale bar: 200 μm.

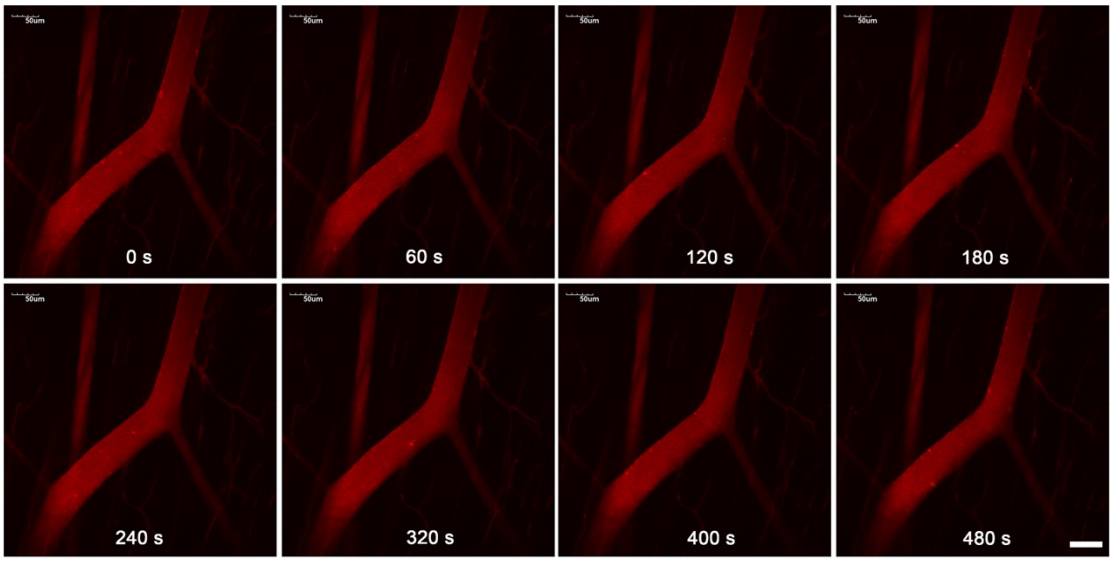

Figure S15. Real-time tracing the blood flow in mice in presence of **TPA-2PI** (1 μM) upon two-photon excitation (λ_{ex} = 960 nm). Scale bar: 50 μm.

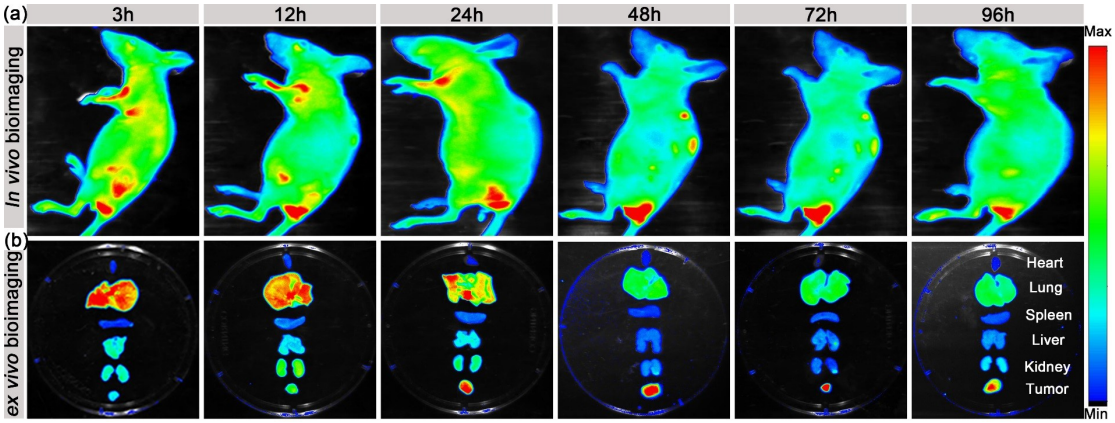

Figure S16. (a) *In vivo* real-time fluorescence imaging of 4T1 tumor-bearing mice after i.v. injection of **TPA-2PI**. (b) *Ex vivo* real-time fluorescence imaging of 4T1 tumor-bearing mice after i.v. injection of **TPA-2PI**.

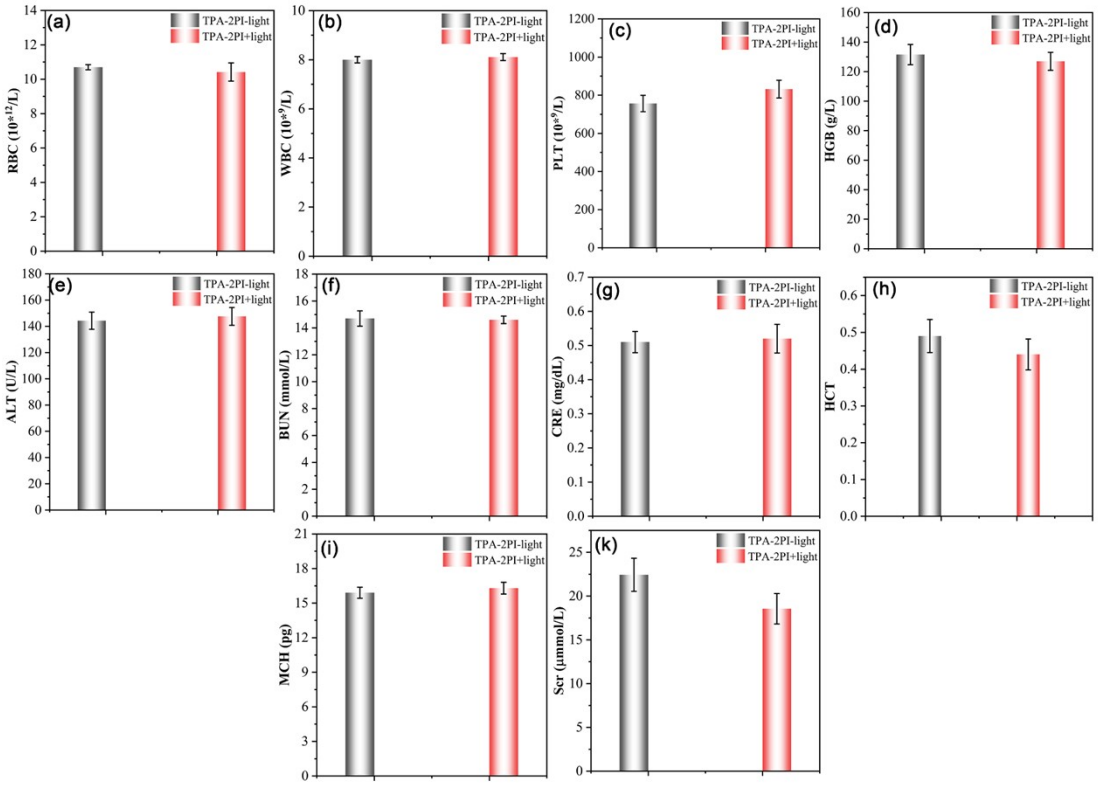

Figure S17. *In vivo* toxicity evaluation on the hematopoietic system (RBC, WBC, PLT, HGB, ALT, BUN, CRE, HCT, MCH, and Scr) of mice in the presence of **TPA-2PI** (1 μM) before and after treatment.

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