Supporting Information

Enhancing Photodynamic Therapy for Cancer: A Two-Photon Excited Approach with a Novel Mitochondrial-Targeted Photosensitizer

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Experimental section

Materials and Methods

McCoy '5A medium, MEN medium, phosphate-buffered saline (PBS), DMEM medium, Fetal Bovine Serum (FBS), Phosphate-Buffered Saline(PBS), and trypsin (0.25%) were purchased from Nanjing Biohang Biotechnology Co, LTD.Calcein/PI Cell Viability/Cytotoxicity Assay Kit, Lyso-Tracker Green, Mito-Tracker Red CMXRos (Mitochondrial Red fluorescence probe), Mito-Tracker Green (mitochondrial green fluorescence probe) and Reactive Oxygen Species Assay Kit were purchased from Shanghai Biyuntian Biotechnology Co. Cell Counting Kit-8 (CCK-8) was purchased from Beiren Chemical Technology (Beijing) Co, LTD(DOJINDO). Anti-cleaved caspase-3(#9661) was purchased from Cell Signaling.Anti-GSDME-N-terminal, anti-pro caspase-8 and anti-caspase-3 were purchased from abcam.

Characterization

One-photon imaging was performed by ZEISS LSM 980 (Germany), and two-photon imaging was tested by ZEISS LSM 980 laser scanning confocal microscopes. The absorption of CCK-8 was tested by SpectraMax i3x（Molecular Devices）. Centrifuge adopts Hunan Xiangyi L500 table low-speed automatic balancing centrifuge.

Detection of ROS production in aqueous solution

Using DCFH-DA as an indicator, the ROS produced by MeTTPy in aqueous solution was detected with white light (20 mW/ cm^2). First, DCFH-DA was activated to DCFH by general method, and then the indicator in PBS was further diluted to 10μM in MeTTPy solution(10μM). The oxidation of DCF was seen by measuring the increase in emission at 525 nm using an excitation wavelength of 488 nm.

Detection of $O_2^{\bullet-}$ in aqueous solution

Using DHR123 as an indicator, $\mathbf{O_2^{\bullet-}}$ produced by MeTTPy in aqueous solution was detected with white light (20 mW/ cm^2). First, DHR123 was activated to Rhodamine123 by general method, and then the indicator in PBS was further diluted to

10μM in MeTTPy solution(10μM). The emission increase of DHR123 at a wavelength of 525 nm was measured using an excitation wavelength of 495 nm.

Detection of $^1O_2$ in aqueous solution

Using ABDA as an indicator, $^1O_2$ produced by MeTTPy in aqueous solution was detected with white light (20 mW/ cm^2).The indicator in PBS was further diluted to 50 M in MeTTPy solution (10 M), and the indicator's fluorescence signal was monitored within the 330-450 nm range, with 480 nm excitation wavelength. The absorbance drop of ABDA was recorded at 380 nm to represent the $^1O_2$ generation rate.

Colocalization experiment

At 37° C, the U2OS cell line was cultivated in McCoy's 5A complete medium, which contained 10% FBS and 1% P/S, in a cell incubator that was enriched with 5% carbon dioxide. About 1×10^6U2OS cells were inoculated in a confocal dish (20×20 mm), incubated for 48 h, washed 3 times with PBS, and 1.5ml McCoy's 5A complete medium containing MeTTPy(1μM) and Mito Tracker Deep Red FM (50nM) were added. The cells were incubated in the incubator for 30 minutes, cleaned 3 times with PBS, placed in 1.0mlPBS, and immediately observed with LSM980 confocal laser scanning microscope (CLSM).Microscope parameters are set as follows：MeTTPy:λ_{em} = 491−751nm, λ_{ex} =488nm; Mito Tracker Deep Red FM: λ_{em} = 653−756nm, λ_{ex} =639nm

At 37° C, the U2OS cell line was cultivated in McCoy's 5A complete medium, which contained 10% FBS and 1% P/S, in a cell incubator that was enriched with 5% carbon dioxide. About 1×10^6U2OS cells were inoculated in a total of six confocal dishes (20×20 mm), incubated for 48 h, and cleaned three times with PBS. Three of the plates were put into 1.5mlMeTTPy(1μM), Mito-Tracker Green (0.1μM), and Mito-Tracker Red CMXRos (0.1μM)McCoy's, respectively 5A complete culture medium was incubated in a cell incubator for 30min and immediately observed by LSM980 confocal laser scanning microscope (CLSM). The other three dishes were added 1.5ml4% poly-formaldehyde phosphate buffer, fixed for 15 minutes, dried, and cleaned with PBS for

3 times. The three dishes were put into 1.5mlMeTTPy(1μM), Mito-Tracker Green (0.1μM), and Mito-Tracker Red CMXRos (0.1μM)McCoy's, respectively 5A complete culture medium was incubated in a cell incubator for 30min and immediately observed by LSM980 confocal laser scanning microscope (CLSM).Microscope parameters are set as follows:MeTTPy(e, h)：λ_{ex}= 488nm，λ_{em}=491−731nm；Mito-Tracker Green(f, i)：λ_{ex}= 488nm，λ_{em}=491−656nm；Mito-Tracker Red CMXRos(g, j)：λ_{ex}=543nm ，λ_{em}= 547−706nm.

Photostability Test

At 37° C, the U2OS cell line was cultivated in McCoy's 5A complete medium, which contained 10% FBS and 1% P/S, in a cell incubator that was enriched with 5% carbon dioxide. About 1×10^6 U2OS cells were inoculated in four confocal dishes (20×20 mm), incubated for 48 h, cleaned three times with PBS, and 1.5ml McCoy's 5A complete medium containing MeTTPy(1μM) were added to two of the dishes. McCoy's 5A complete culture medium containing Mito-Tracker Green (0.1μM) and Mito-Tracker Red CMXRos (0.1μM) was added to the two other dishes, respectively. The cells were placed in the incubator and left there for a duration of 30 minutes and immediately observed by LSM980 confocal laser scanning microscope (CLSM). Microscope parameters are set as follows: MeTTPy:λ_{em}=491−731nm, λ_{ex} =488nm,λ_{ex} =830nm; Mito-Tracker Green: λ_{em} = 491−656nm, λ_{ex} =488nm; Mito-Tracker Red CMXRos: λ_{em} = 547−706 nm, λ_{ex} =543 nm. The duration of each scanning is 20.13 seconds, and there is no time gap between two consecutive scans. The overall duration of the scanning process is 15 minutes.

One photon confocal microscopy imaging

In situ ROS production was measured in U2OS and K7M2 cells by DCFH-DA. The cells were incubated with MeTTPy(1μM)for 10 min, then DCFH-DA(1μM)was added and incubated for another 20 min. Finally, the cells were rinsed three times with PBS, put in 1ml PBS, and then stimulated for 5 minutes with one-photon Zeiss LSM 980.

Finally, a one-photon Zeiss LSM 980 was used for imaging.Microscope parameters are set as follows:MeTTPy(λ_{ex}=488nm, λ_{em}=490−730nm), DCFH-DA (λ_{ex}=488nm,λ_{em} =510−580nm) .

The biocompatibility test was performed by incubation with U2OS cells, K7M2 cells and 4T1 cells.When the cells had grown to 80% of cell abundance, the medium was removed, The cells were rinsed three times with PBS, and then treated with 0.25% trypsin for 3 minutes. During the experiment, U2OS cells, K7M2 cells or 4T1 cells underwent centrifugation at a speed of 1200 rpm per minute for a duration of 3 minutes, and a suspension of 10,000 cells in fresh culture solution (100 μL) was added to each hole of the 96-well plate. Following an overnight culture, the original medium was substituted with a fresh medium containing varying concentrations of MeTTPy. The mixture was then cultured for an additional 30 minutes. Next, a white light with 28 mW/cm^2 power was illuminated for 30 minutes. The cells were subsequently cultured for an additional 24 hours. Then, each well of the culture medium was substituted with 100 μL of fresh culture medium containing a 10% CCK-8 solution. The measurement of absorbance at a wavelength of 450 nm was taken, and the data were processed and plotted by GraphPad Prism 9.

Live/dead experiments were performed with CA/PI. The U2OS cell lines were grown in McCoy's 5A complete medium that had 10% FBS and 1% P/S added to it. The K7M2 cell lines were grown in DMEM complete medium that had 10% FBS and 1% P/S added to it. At 37° C, the cell cultures were kept in a CO_2 incubator. The cells were placed in a glass bottom confocal dish (20×20 mm) with a density of 10^6, incubated for 48 h, cleaned with PBS 3 times, then added 1.5ml complete medium containing MeTTPy(1μM) and CA (1μM), incubated for 30min, cleaned with PBS 3 times, and added 1ml PBS containing PI (4μM). The single photon Zeiss LSM 980 was then used for continuous imaging.Microscope parameters are set as follows:MeTTPy(λ_{ex}=488nm, λ_{em}=491−731nm), Calcin-AM (λ_{ex}=488nm, λ_{em}=491−656nm) and PI (λ_{ex}=543nm, λ_{em}=547−731nm) .

Two-photon confocal microscopy imaging

In situ ROS production was measured in U2OS and K7M2 cells by DCFH-DA. The cells were incubated with MeTTPy for 10 min, and then DCFH-DA was added and incubated for another 20 min. Finally, it was washed 3 times with PBS, put in 1ml PBS and then received a 15% gain with two-photon Zeiss LSM 980, 830nm, 3730 MW, received 490-730nm, stimulated the cells for 5 minutes, and imaged with two-photon Zeiss LSM 980.

Live/dead experiments were performed with CA/PI. The U2OS cell lines were grown in McCoy's 5A complete medium that had 10% FBS and 1% P/S added to it. The K7M2 cell lines were grown in DMEM complete medium that had 10% FBS and 1% P/S added to it. At 37° C, the cell cultures were kept in a CO_2 incubator. The cells were placed in a glass bottom confocal dish (20×20 mm) with a density of 10^6, incubated for 48 h, cleaned with PBS 3 times, then added 1.5ml complete medium containing MeTTPy(1μM) and CA (1μM), incubated for 30min, cleaned with PBS 3 times, and added 1ml PBS containing PI (4μM). The image was then taken continuously with a two-photon Zeiss LSM 980.Microscope parameters are set as follows:MeTTPy(λ_{ex}=830nm, λ_{em}=546−728nm), Calcin-AM(λ_{em} = 491−561nm, λ_{ex} =488nm), PI (λ_{em} = 550−728nm, λ_{ex} =543 nm).

About 1×10^6U2OS cells or K7M2 cells were inoculated on the tablet and incubated for 48 h. U2OS cells and K4M2 cells were incubated with 1.0 μM of MeTTPy for 40 min. The two-photon imaging properties of MeTTPy at 830 nm were tested using a Zeiss LSM 980 microscope.

In *vivo* fluorescence imaging of mice

In *vivo* fluorescence Imaging of MeTTPy in a Noidscid mice model of K7M2 cell allogeneic tumor was detected using AniView100 Multimode Live Animal Imaging System.4-5 week-old Noidscid mice were purchased from Jiangsu Zhizhaokang Biotechnology Co, LTD. (SYXK2018-0027). The animal experiments were approved by the Chinese Research and Animal Ethics Committee and complied with the laws on

experimental animals. The density of K7M2 cells was 1×10^7 cells were injected subcutaneously and allowed to develop to about 100 mm^3. MeTTPy (4×10^{-3}M, 100 μL/100mm^3tumor) was injected intratumorally and fluorescent images were captured by the AniView100 Multimode Live Animal Imaging System at 0, 0.5, 1, 2, 4,8,24,48,72,96 hours after injections.

In *vivo* PDT and biosafety evaluation

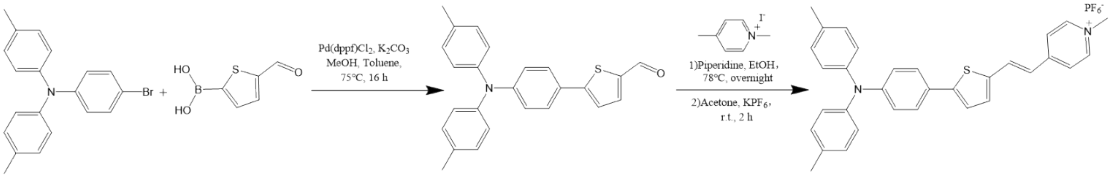
The 8-week-old male BALB/c mice were derived from the Experimental Animal Center of Zhengzhou University. All procedures are in accordance with National Animal Care Institute guidelines. Once the tumor volume reached 80-100 mm^3, K7M2 cells (1×10^7 cells) were injected into the right hind limb. The mice were allocated into 4 groups using a random process, with 4 mice in each group. The treatment group and control group were given intratumoral injections of 100μL MeTTPy (5mg/kg) and normal saline solution, respectively. After 30min, a laser (140 mW) was used to treat the mice for 15 minutes. Before laser irradiation, the mice were consistently sedated with 1% isoflurane by inhalation. After anesthesia, the mice were immobilized in a warm three-axis scaffold. An energy-efficient beam (FIBER ILLUMINATION SYSTEM, CXE-350) is emitted to precisely pinpoint the tumor. The laser's strength is then raised to 140 mW. The control group was subjected to identical irradiation treatment and assessed and recorded their weight and tumor volume every 2 days. Tumor size was assessed every 2 days and calculated using the following technique: volume = (tumor length)$\times$(tumor width)^2/2. On day 14, the tumor was removed and photographed.

Analysis of coke death mechanism induced by MeTTPy

At 37° C, the U2OS cell line was cultivated in McCoy's 5A complete medium, which contained 10% FBS and 1% P/S, in a cell incubator that was enriched with 5% carbon dioxide. Inoculated in 4 petri dishes (10×10 cm), once the cells reached 80% of their total number, the liquid medium was taken out and the cells were carefully rinsed with

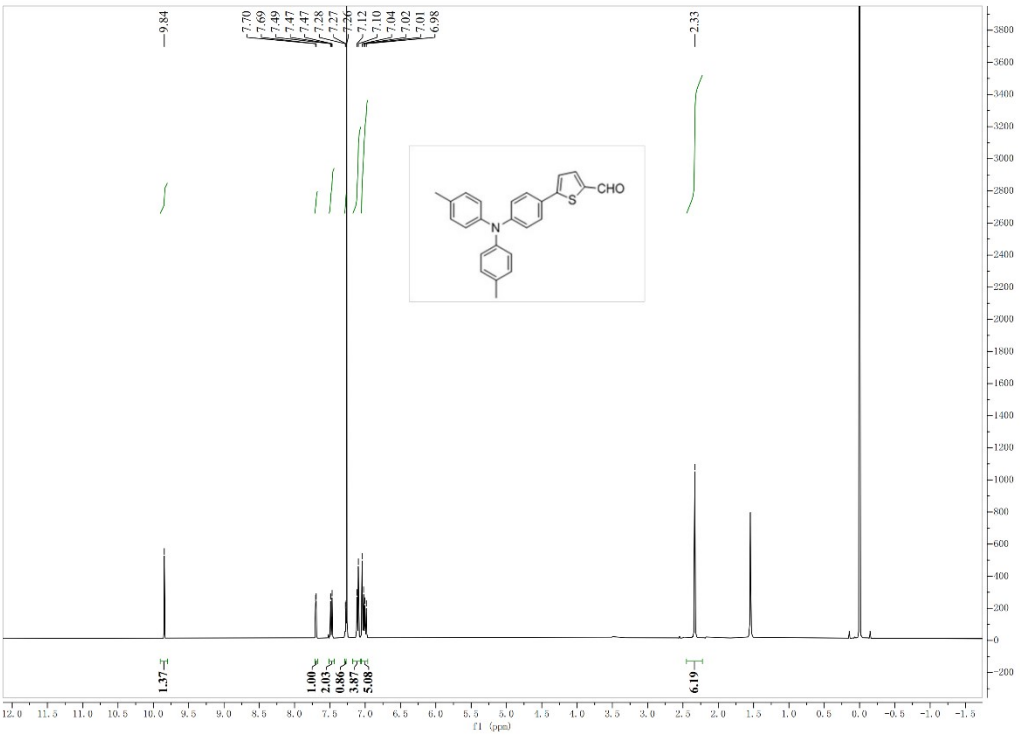
PBS three times. Equal amounts of McCoy's 5A complete culture medium without MeTTPy, McCoy's 5A complete culture medium without MeTTPy, McCoy's 5A complete culture medium with MeTTPy (1μM), McCoy's 5A complete culture medium with MeTTPy (1μM), and McCoy's 5A complete culture medium with MeTTPy (1μm). All were wrapped in tinfoil to avoid light and incubated in the cell incubator for 30min. The four groups were treated as follows respectively: Continue to avoid light, light treatment, continue to avoid light, light treatment. Light treatment is white light with 28 mW/cm^2 power for 15 minutes. After light treatment, The cells were placed in the cell incubator and kept there for a duration of 24 hours, and the medium was sucked out and cleaned with PBS for 3 times. Cell proteins in culture dishes were extracted respectively. Western Blot assay was performed with the extracted protein, gray value analysis was performed with ImageJ, and statistical analysis was performed with GraphPad Prism9.

Synthesis Route of MeTTPy:



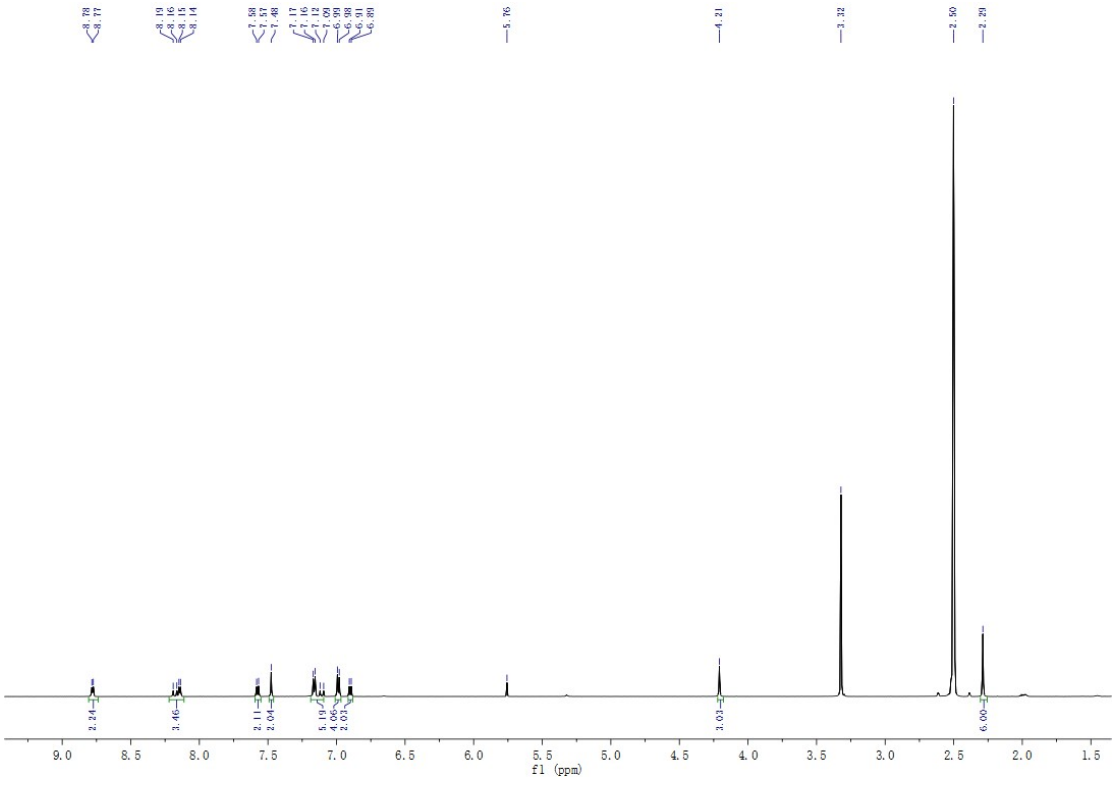
H NMR spectrum of intermediate products:

^{1}H spectrum of 5-(4-(di-p-tolylamino)-phenyl)thiophene-2-carbaldehyde:

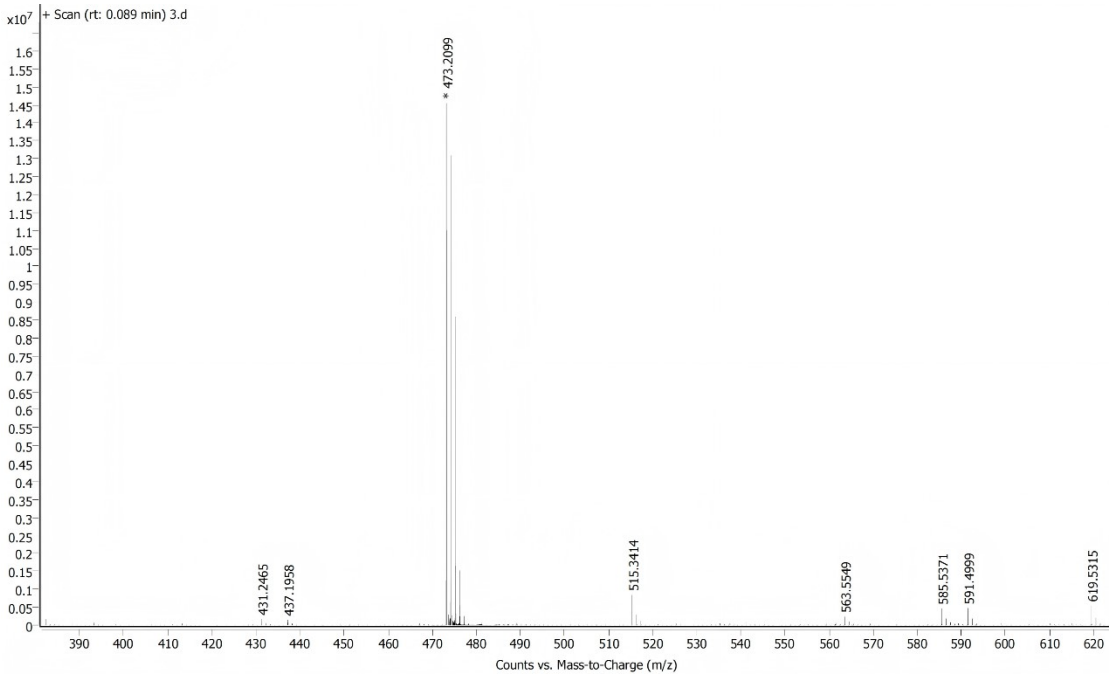


H NMR spectroscopy and mass spectrum of AIEgen:

^{1}H spectrum of MeTTPy:



Mass spectrum of MeTTPy:



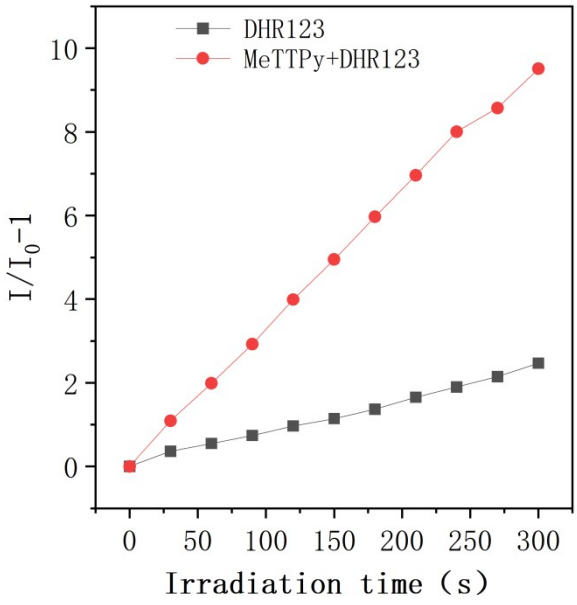


Figure S1. Plots of relative PL intensity (I/I_0-1) of DHR123 (10μM) in the presence of 10μM MeTTPy in PBS, upon white light irradiation with 20 mW/ cm^2 for different times.

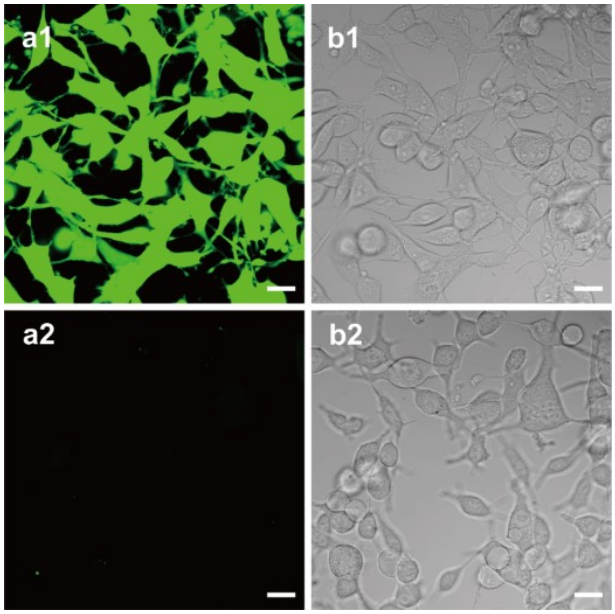


Figure S2. ROS generation in *in situ* test: Single photon CLSM image of K7M2 cells after incubation with 1.0μM MeTTPy and 2μM DCFH-DA for 30 min (a1).K7M2 cells were incubated with 2μM DAFH-DA for 30 min The single photon CLSM image after min (a2). The DCFH-DA image's emission wavelength is 501-544 nm, while its excitation wavelength is 488 nm. b1 and b2 are bright fields. Scale bar: 20μm.

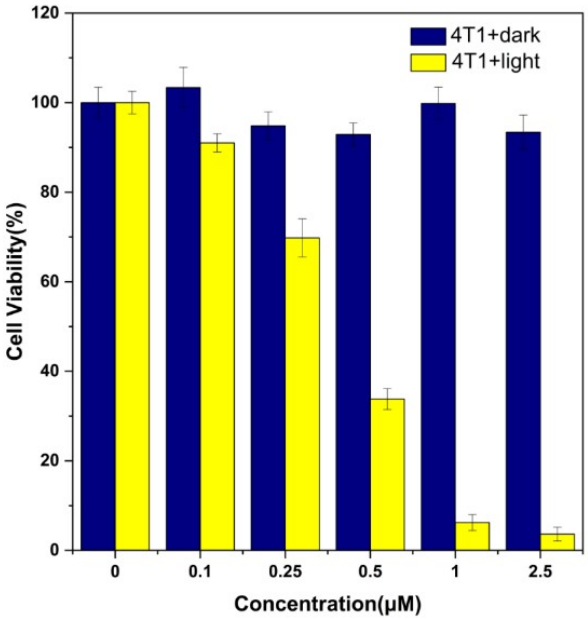


Figure S3. The 4T1 cell viability for MeTTPy with or without white light irradiation at 28 mW/cm^2.

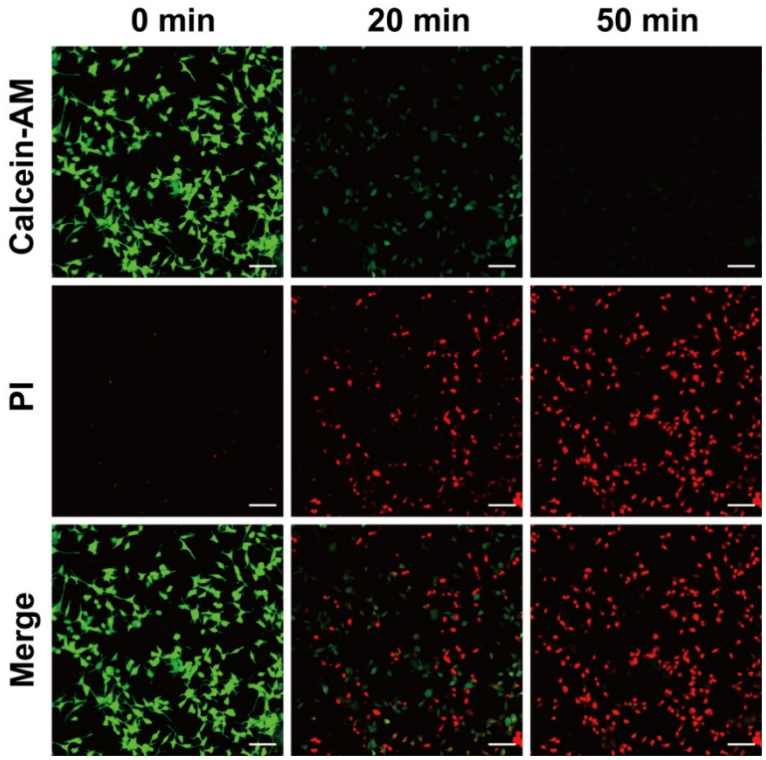


Figure S4. Confocal fluorescence images of K7M2 cells with MeTTPy(λ_{ex}=488nm, λ_{em}=491−731nm), Calcin-AM (λ_{ex}=488nm, λ_{em}=491-656nm) and PI (λ_{ex}=543nm, λ_{em}=547−731nm) under laser irradiation at 488nm or dark. Scale bar: 100μm.CLSM images of K7M2 cells stained with Ca/PI detection kit under different conditions.

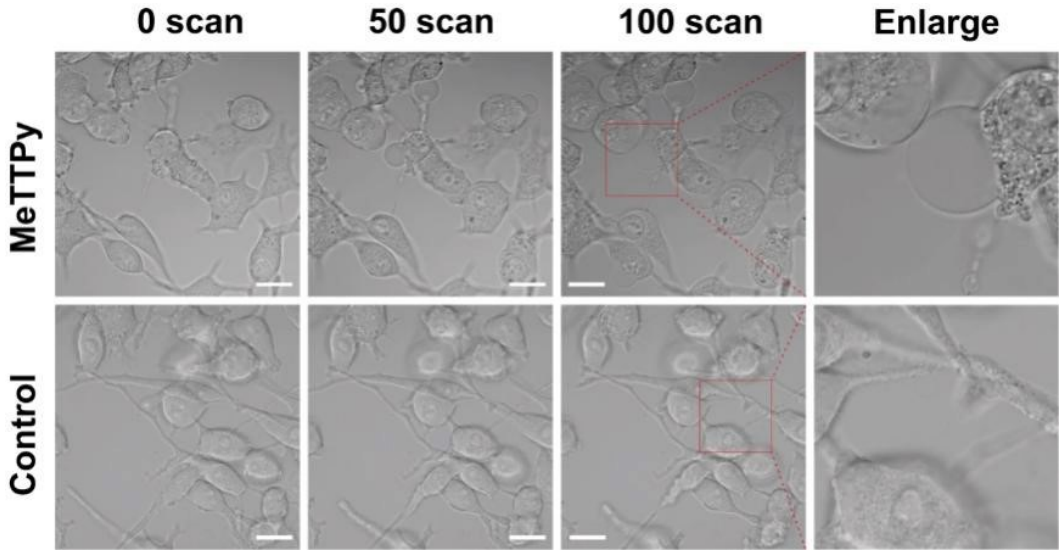


Figure S5. K7M2 cells were incubated with MeTTPy(1uM, λ_{ex}: 488 nm, λ_{em}: 491−731 nm), and confocal fluorescence images were obtained. Only K7M2 cells were seen in λ_{ex}: 488 nm, λ_{em}: 570−740 nm confocal fluorescence images. Scale bar: 20μm.

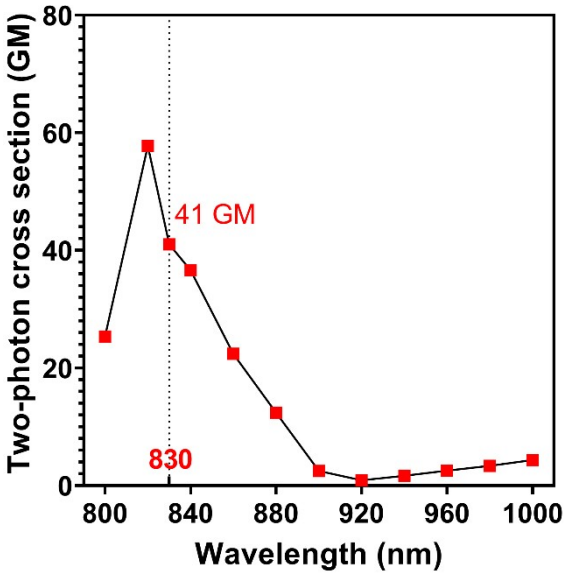


Figure S6. Two-photon absorption cross sections (δ2PA) of MeTTPy versus excitation wavelengths ranging from 800 to 1000 nm. MeTTPy concentration: 50 μM.

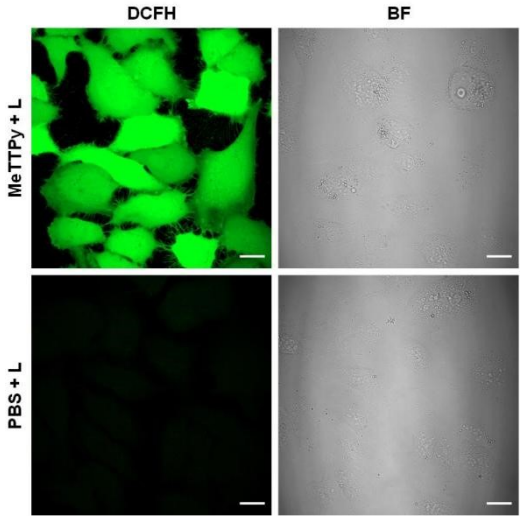


Figure S7.ROS generation in *in situ* test: two-photon CLSM imageof U2OS cells after incubation with 1.0 μM MeTTPy and 2 μM DCFH-DA for 30 min; two-photon CLSM images of U2OS cells were incubated with 2 μM DCFH-DA for 30 min. DCFH-DA:λ_{em} = 500−571 nm, λ_{ex} =488 nm. MeTTPy:λ_{em} = 571−728 nm, λ_{ex} =830 nm. Scale bar: 20 μm.

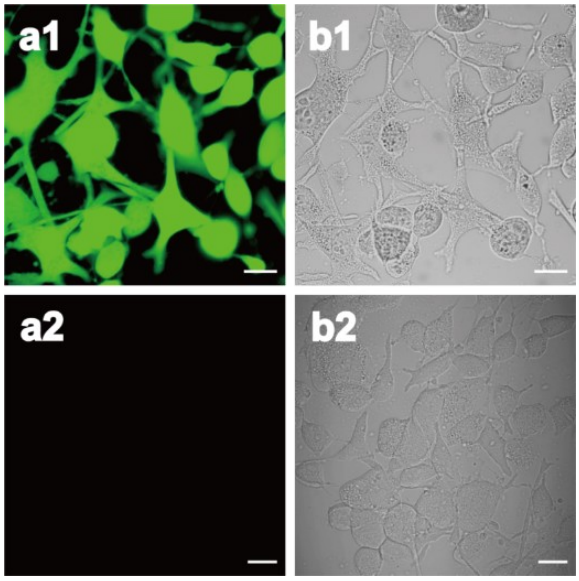


Figure S8.ROS generation in *in situ* test: two-photon CLSM images (a1, b1) of U2OS cells after incubation with 1.0μM MeTTPy and 2μM DCFH-DA for 30min; two-photon CLSM images (a2, b2)of U2OS cells were incubated with 2μM DCFH-DA for 30min. DCFH-DA:λ_{em} = 500−571 nm, λ_{ex} =488 nm. MeTTPy:λ_{em} = 571−728 nm, λ_{ex} =830 nm.b1 and b2 are bright fields. Scale bar: 20μm.

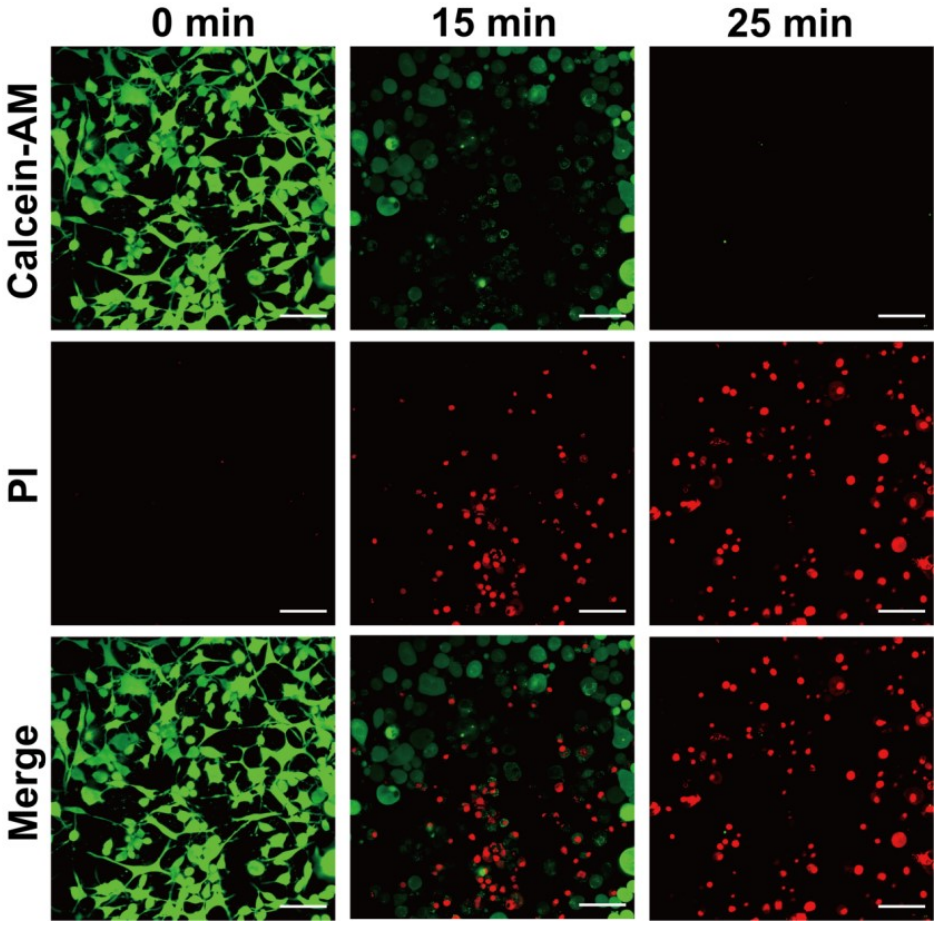


Figure S9. Confocal fluorescence images of Ca/PI and MeTTPy (1μM) under 830 nm femtosecond laser irradiation. Calcin-AM :λ_{em} = 491−561 nm, λ_{ex} =488 nm. PI :λ_{em} = 550−728 nm, λ_{ex} =543 nm. Scale bar: 100μm.

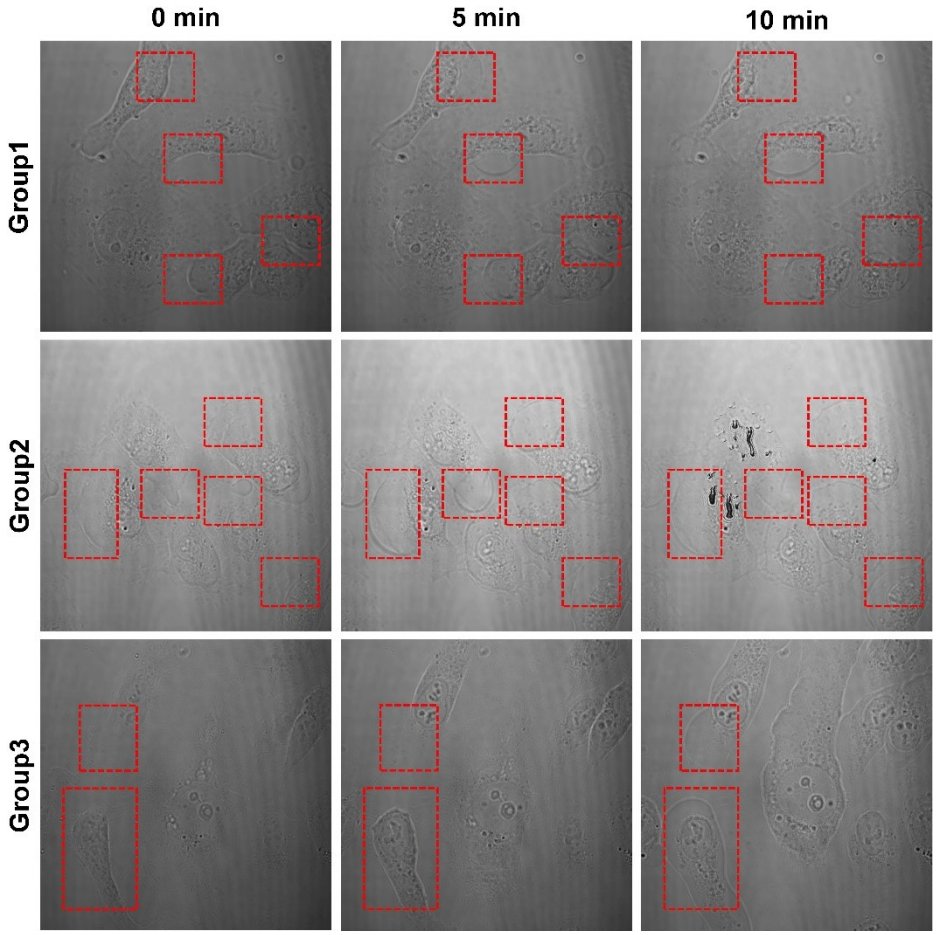


Figure S10. MeTTPy (1 μM, λ_{ex}: 830 nm, λ_{em}: 490−731 nm) was incubated with U2OS cells, and confocal fluorescence images were obtained. Scale bar: 20μm.

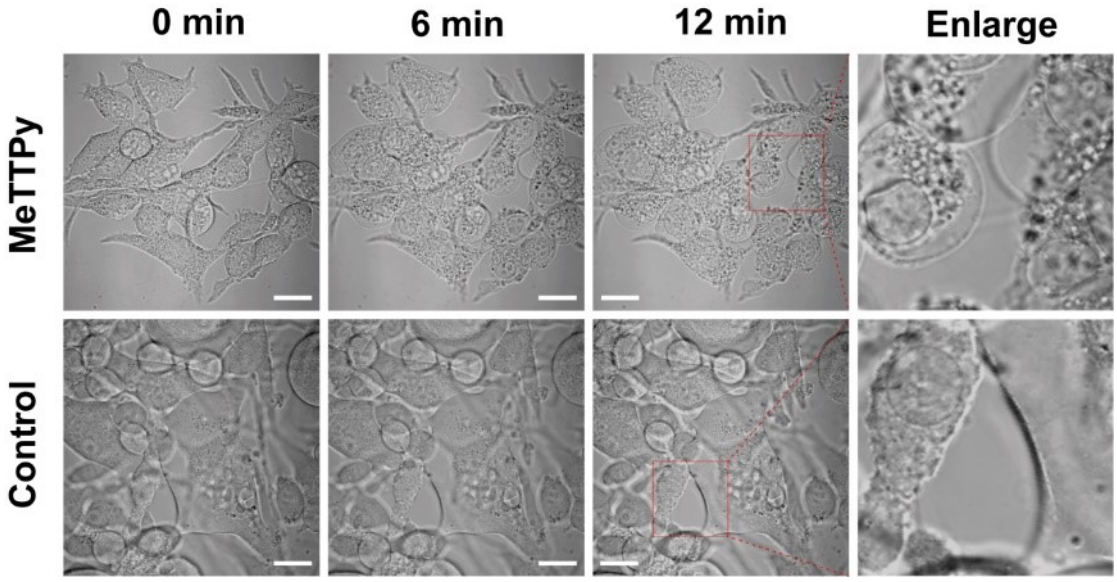


Figure S11. MeTTPy(1uM, λ_{ex}=830 nm, λ_{em}=490-731 nm) was incubated with K7M2 cells, and confocal fluorescence images were obtained. Only K7M2 cells were seen in

confocal fluorescence images(λ_{ex}: 830 nm, λ_{em}: 490-731 nm). Scale bar: 20μm

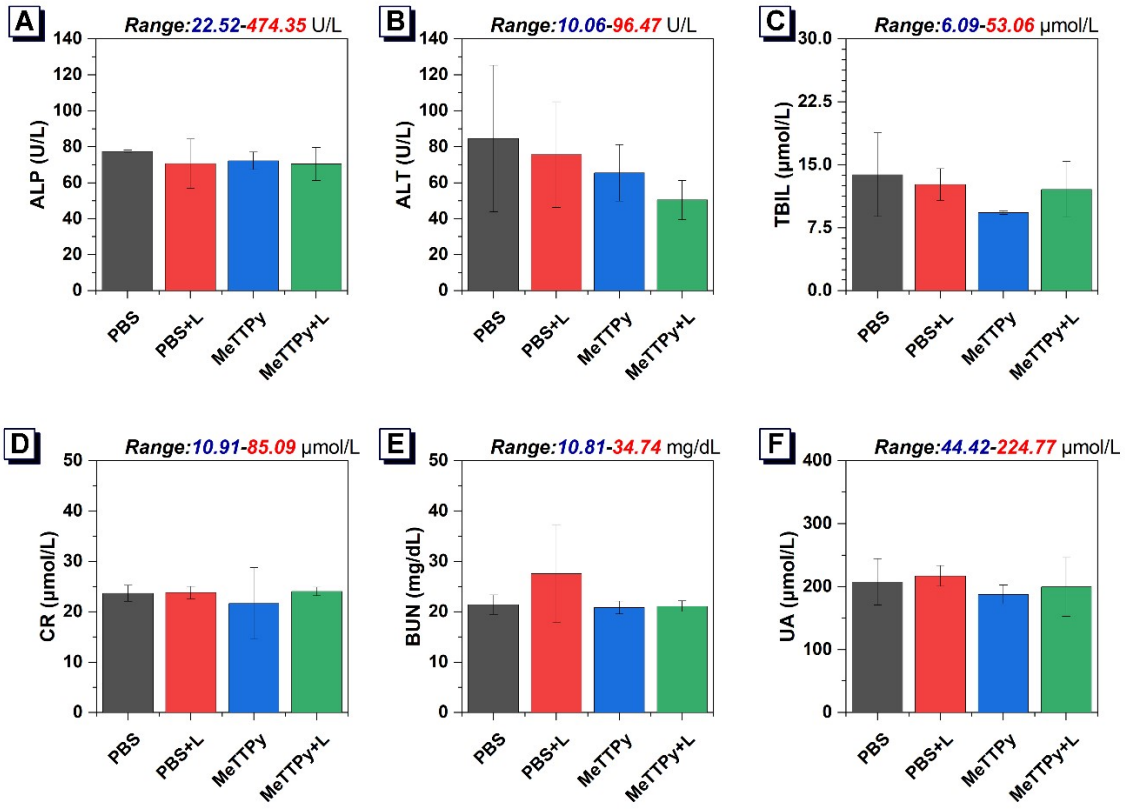


Figure S12. No significant alteration at 14 day postinjection of MeTTPy compared with control group in biochemical blood biomarkers including ALP(A), ALT(B), TBIL(C), CR(D),BUN(E), and UA(F).

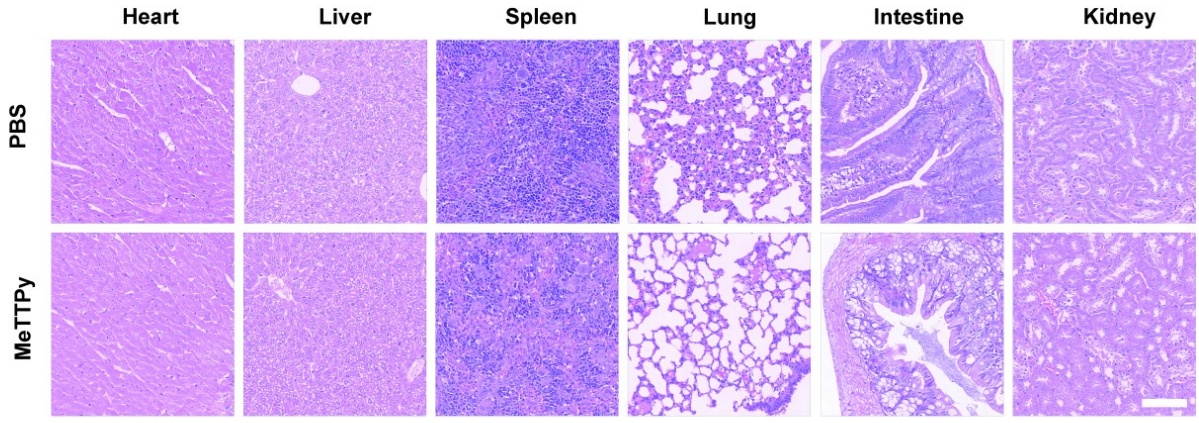


Figure S13. Histological analyses of H&E-stained slices of the main organs of mice. Scale bar: 50 μm.