Electronic Supplementary Information

An active tumor-targeting organic photochemotherapy agent with naproxen for enhanced cancer therapy

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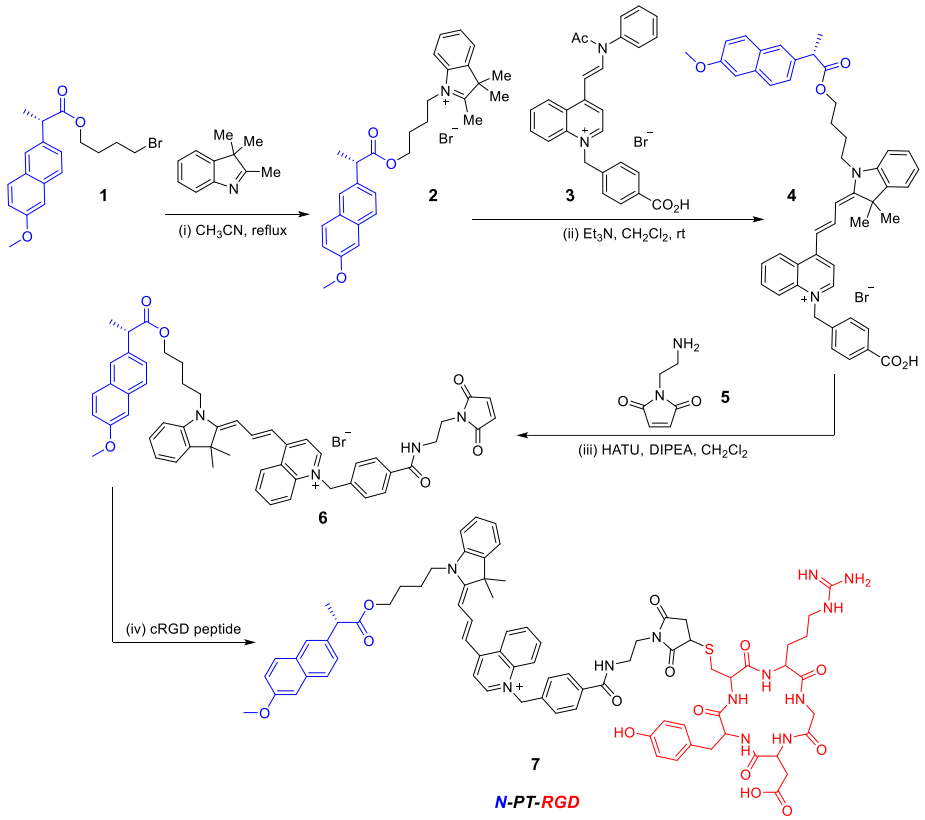
1. Experimental Details:

Materials. 4-Methylquinoline was purchased from Shanghai Macklin Biochemical Co., Ltd. naproxen was obtained from Shanghai Energy Chemical Co., Ltd. 4-(bromomethyl)benzoic acid, Acetylsalicylic acid, 2,3,3-trimethyl-3H-indole, Trifluoroacetic acid (TFA), 2-(Boc-amino)-1-ethanol 2-(7-aza-1H-benzotriazole-1-yl)- 1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) diisopropyl azodicarboxylate (DIAD) were obtained from Tianjin Heowns Biochemical Technology Co., Ltd. *N*,*N*-diisopropylethylamine (DIPEA) was purchased from Adamas Reagent, Ltd. Methanol, acetonitrile, dichloromethane (DCM), toluene, acetic anhydride dimethyl sulfoxide （DMSO） dicyclohexylcarbodiimide (DCC) and triethylamine (TEA) were purchased from China National Pharmaceutical (Shanghai, China). (cRGD) was purchased from Shanghai Dechi Biosciences Co., Ltd. *N*,*N'*-diphenylformamidine was purchased from Alfa Aesar (Tianjin, China). Calcein-AM/PI Double Stain Kit was purchased from Yeasen (Shanghai, China). Mito-Tracker Green (MTG) and the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-digoxigenin nick-end labeling (TUNEL) Apoptosis Detection Kit was obtained from Beyotime Biotechnology (Shanghai, China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2h-tetrazolium bromide (MTT) and dialysis Membranes were obtained from Beijing Solarbio Science & Technology Co., Ltd. Cyclo. The mouse breast cancer cell line 4T1 cell line was purchased from KeyGEN Biotechnology (Nanjing, China). Roswell Park Memorial Institute medium (RPMI 1640) was purchased from KeyGEN Biotechnology (Nanjing, China). Fetal bovine serum (FBS) was purchased from Biological Industries, Israel. Trypsin-EDTA was purchased from Gibco, USA. PBS buffer was purchased from Sangon Biotech Co., Ltd., China. Penicillin/streptomycin was purchased from Gibco. Analytical grade reagents were used with no further purification. The Mouse IL-6 and TNF-α ELISA Kit were purchased from Boster Biological Technology Co., Ltd. Glass Bottom dishes were purchased from Cellvis. 96-well plates were purchased from Hangzhou Xinyou

Biotechnology Co., Ltd. Plastic centrifuge tubes were purchased from GeneBrick Bioscience LLC. All aqueous solutions were prepared using distilled-deionized water of 18.2 MΩ·cm^{-1}.

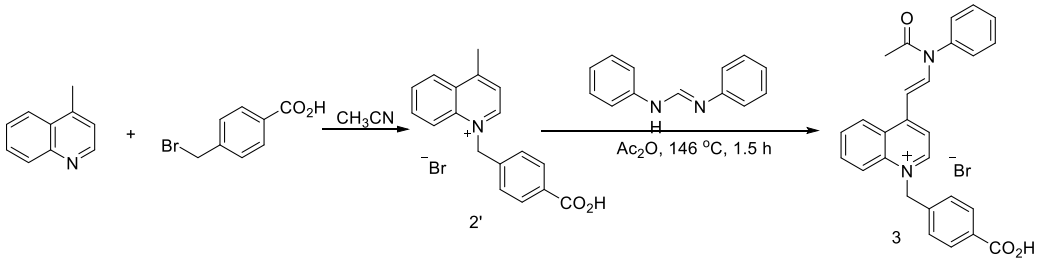
Instruments. MTT assay was performed using a microplate reader (Synergy 2, Biotek, USA). High-resolution transmission electron microscopy (HRTEM) was carried out on a JEM-2100 electron microscope. Fluorescence spectra were recorded using a fluorescence spectrometer (FLS-980, Edinburgh, UK). UV-Vis absorption spectra were measured on a pharmaspec 1700 UV-Visible spectrophotometer (Shimadzu, Japan). Confocal fluorescence images were recorded on a TCS SP8 confocal laser scanning microscopy (Leica, Germany). Zeta potential was monitored using a Malvern Zeta Sizer Nano (Malvern Instruments). Photoacoustic imaging was obtained using an Endra Nexus 128 (Ann Arbor, Michigan). All NMR spectra were recorded on a Bruker NMR spectrometer. High-resolution mass spectra (HRMS) were recorded on a Bruker Daltonics maXis UHR-TOF MS.

2. Synthesis of Organic Photothermal Agent (N-PT-RGD).



Compound 1: (*S*)-(+)-naproxen (690.78 mg, 3.0 mmol), 4-dimethylaminopyridine (36.6 mg, 0.3 mmol), dicyclohexylcarbodiimide (865.2 mg, 4.2 mmol) and 4-bromo-1-butanol (1093 μL, 12 mmol) were mixed in dichloromethane, and the reaction was stirred at 25 ℃ for 12 h. The crude product was evaporated dried and purified using column chromatography over silica gel with the eluent mixture of petroleum ether/ethyl acetate (v/v, 5:1) with a yield of 48%.

Compound 2: Compound **1** (1461.08 mg, 4 mmol) was mixed with liquid 2,3,3-Trimethyl-3H-indole (642 μL, 4mmol) in anhydrous acetonitrile, and the reaction was stirred for 12 h at 80 ℃. The crude product was purified by column chromatography using silica gel with the eluent mixture CH_2Cl_2/MeOH (v/v, 20:1) with a yield of 43%.



Compound 3: 4-methylquinoline (1.59 mL, 12.0 mmol) was mixed with 4-(bromomethyl) benzoic acid (2.15 g, 10.0 mmol) in anhydrous acetonitrile. The reaction was stirred for 6 h under 110 ℃ in an argon atmosphere. The evaporated crude products were dissolved in methanol at which point diethyl ether was added slowly. The precipitate was collected by filtration, washed with diethyl ether, and dried under vacuum to yield compound **2'** as a white solid with a yield of 71%.

Compound **2'** (0.278 g, 1.00 mmol) was mixed with *N*, *N'*-diphenylformamidine (0.392 g, 2.0 mmol) in acetic anhydride. The reaction was stirred under 146 ℃ for 1.5 h, and the mixture was then cooled to room temperature. Diethyl ether was added slowly after the reaction cooled down. The precipitate was collected and washed with diethyl ether. Compound **3** was obtained after vacuum evaporation as a brown solid with a total yield of 78%.

Compound 4: Compound **2** (0.444 g, 1.0 mmol), compound **3** (0.423 g, 1.00 mmol) were mixed with triethylamine (1.391 mL, 10.0 mmol) in dichloromethane (10 mL). The reaction was stirred at 25 ℃ for 24 h. The crude product was obtained after vacuum evaporation. The crude product was purified by column chromatography using silica gel with the eluent mixture CH_2Cl_2/MeOH (v/v, 10:1) with a total yield of 51%.

Compound 5: Maleimide (624 mg, 6.44 mmol) and triphenylphosphine (1658 mg, 6.32 mmol) were mixed with THF (30 mL) in a 100 mL round-bottom flask. *N*-(*tert*-butoxycarbonyl) ethanolamine (5.00 mL, 29.3 mmol) and diisopropyl azodiformate (6.80 mL, 35.1 mmol) were added in succession. The flask was stirred under an inert atmosphere (N_2) overnight and dried over a rotary evaporator. The crude product was filtered through a plug of silica gel using CH_2Cl_2/MeOH (v/v, 40:1) as the eluent a white solid with a yield of 84%.

The last step product was dissolved in 10 mL of trifluoroacetic acid and 20 mL of DCM. The reaction mixture was stirred for 12 h and then dried under vacuum. The crude product was dissolved in 2 mL of methanol and added to anhydrous diethyl ether. The procedure was

collected using filtration and it was dried under a high vacuum to obtain a white solid with a total yield of 80%.

Compound 6: Compound **5** (0.366 g, 0.5 mmol) and *N,N′*-diisopropylethylamine (0.3 mL) were mixed with HATU (0.38 g, 1mmol) in DCM (10 mL). The reaction was stirred at 25 ℃ for 30 min. Compound **6** (0.165 g, 1 mmol) and triethylamine (417 μL, 3.0 mmol) were added to the mixture as follows and the reaction was stirred at 25 ℃ for 24 h. The crude product was dried under vacuum and purified by column chromatography over silica gel using an eluent mixture CH_2Cl_2/MeOH (v/v, 5:1) with a yield of 38%.

Compound 7: compound **6** (42.7 mg, 0.05 mmol) and cRGD (30 mg, 0.05 mmol) was dissolved in 5 mL methanol. The reaction mixture was stirred under room temperature for 12 h. The ethyl acetate precipitated precipitate yield to compound **7**. Compound **7** was further purified by dialysis in water and lyophilized to obtain as a dry blue powder with a yield of 15%. ^{1}H-NMR (400 MHz, D_6-DMSO) δ 8.54-8.50 (t, J = 8 Hz, 1H), 8.47-8.45 (d, J = 8 Hz, 1H), 8.36-8.30 (t, J = 12 Hz, 1H), 7.89-7.76 (m, 5H), 7.66-7.61 (m, 2H), 7.57 (s, 1H), 7.55-7.53 (d, J = 8 Hz, 1H), 7.45-7.43 (d, J = 8 Hz, 1H), 7.41-7.39 (d, J = 8 Hz, 1H), 7.37-7.27 (m, 4H), 7.17-7.13 (t, J = 8 Hz, 1H), 7.03-6.93.(m, 4H), 6.88-6.86 (d, J = 8 Hz, 2H), 6.62-6.59 (dd, J = 8Hz, J = 4 Hz, 1H), 6.03-6.00 (d, J = 12 Hz, 1H), 5.92-5.91 (d, J = 4 Hz, 2H), 4.80-4.73 (m, 2H), 4.52-4.14 (m, 7H), 3.92-3.86 (q, J = 8 Hz, 1H), 3.81-3.65 (m, 8H), 3.59-3.48 (m, 3H), 3.40-3.36 (m, 1H), 3.24-3.05 (m, 5H), 2.90-2.69 (m, 5H), 2.63-2.55 (dt, J = 16 Hz, J = 8 Hz, 1H), 2.38-2.33 (dd, J = 16 Hz, J = 4 Hz, 1H), 1.89 (br, 1H), 1.79 (m, 2H), 1.64 (m, 10 H), 1.53-1.51 (d, J = 8 Hz, 4H), 1.32-1.29 (m, 2H), HRMS (ESI) m/z calcd for $C_{78}H_{87}N_{12}O_{14}S^+$ [M^+]: 1447.6180; found: 1447.6122.

3. Methodology.

Cell lines and animals. 4T1 cells were cultured in RPMI 1640 medium containing 10% FBS and 100 U/mL of 1% antibiotics (penicillin/streptomycin) and were maintained at 37 ℃ in a 5% CO_2/95% air humidified incubator (SANYO). All animal procedures were carried out according to the Principles of Laboratory Animal Care (People's Republic of China) and were approved by the Animal Care and Use Committee of Shandong Normal University (Jinan, China). Balb/c mice (6-8 weeks old, female, ~20 g) were fed with normal conditions. To establish the tumor xenograft models, 4T1 cells (1×10^6) suspended in 50 μL RPMI 1640 were subcutaneously injected into the back of the mice. Photothermal treatments were carried out only once when the tumor volume reaches about 50 mm^3.

IR thermal images. An external laser with a radiation wavelength of 635 nm and a power density of 0.5 W/cm^2 was used to investigate the temperature changes after radiating to water and **N-PT-RGD** aqueous solution. A portable IR thermal camera was used to capture the temperature change.

Co-localization analysis of N-PT-RGD in 4T1 cells by CLSM. 4T1 cells were cultured in RPMI 1640 containing 10% FBS and 100 U/mL antibiotics penicillin/streptomycin under 37 ℃ in a 5% CO_2/95% air incubator. Confocal images were obtained by pressing cells (4×10^3/well) on confocal dishes and followed by incubating for 24 hours. 4T1 cells were washed three times with PBS (10 mM, pH = 7.4), and then incubated with 25 μM **N-PT-RGD** for 4 hours at 37 ℃ before the staining experiments. The petri dish was washed with PBS (10 mM, pH = 7.4) another three times, and followed incubating with Mito-Tracker Green (20 nM) for 15 min at 37 ℃. Finally, each dish was washed with PBS (10 mM, pH =7.4) three times. 4T1 cells were analyzed using a TCS SP8 confocal laser scanning microscopy.

Cytotoxicity Assays. 4T1 cells were plated on sterile 96-well microtiter plates (Beyotime Biotechnology, Jiangsu, China) at an amount of 5×10^3 for 4 h and incubated with 0, 5, 10, 25, 50, and 100 μg/mL **N-PT-RGD** for another 4 h at 37 ℃ under a 5% CO_2 atmosphere. The cells

then were cultured with a fresh complete medium and compared the radiating using a 635 nm laser at a power density of 0.5W/cm^2 for 5 min with blank. Each well added 150 μL MTT solution (0.5 mg/mL) to dissolve the formazan crystals. The absorbance of the 4T1 cells (2.0 ×10^5 per dish) was then measured at 490 nm with an RT 6000 microplate reader in a Triturus microplate reader and plotted. Data were processed using software Gen5 CHS 2.01. In the Calcein-Am and PI co-staining Assays, 4T1 cells incubated with **N-PT-RGD** were either exposed to a 635 nm laser source at 0.5W/cm^2 for 5 min or no treatments. After 4 h of incubation, 4T1 cells were stained with Calcein-AM and PI for 30min to investigate the efficacy of PPT using a fluorescence spectrometer.

In vivo biosafety experiment. Biosafety of materials in 4T1 tumor-bearing mice was tested by using H&E slice analysis of major organs and measurement of mice body weight. The mice of the same age in each treatment group were sacrificed, and their major organs including heart, liver, spleen, lung, kidney, and tumor were carefully removed. The major organs were dissected for H&E staining.

Confocal Imaging. For confocal imaging, the cultured cells were plated on glass-bottom dishes with 20 mm diameter wells. (D29-20-1.5N, Cellvis). Cells were plated in RPMI 1640 supplemented with 10% FBS and 1.0% (w/v) penicillin/streptomycin. Cells were allowed to adhere to the plates overnight before treatments. Following treatment, the cells were washed three times with fresh RPMI 1640 with no FBS or antibiotics and imaged using a Leica TCS SP5 confocal laser-scanning microscope with a 40× objective oil-emersion lens at room temperature.

In vivo antitumor efficacy via injection. For the antitumor efficacy study, when the tumor size reached 50 mm^3, the tumor-bearing mice (n=5) were divided into five groups: only PBS injection; only PT injection; only laser; PT injection, and laser; **N-PT-RGD** injection and laser. Various materials were dissolved in PBS and were injected via the tail vein. After 24 hours, 635 nm laser treatment was performed on only laser; PT injection and laser; S-PT-RGD

injection, and laser group by irradiating the tumor region at the power of 0.5W/cm^2 for 10 min. The effect of different treatment groups was monitored by measuring the tumor size using a Vernier caliper after the treatment. The tumor volume (V) was computed as $V=L\times W^2/2$ by measuring length (L) and width (W).

Apoptosis analysis using TUNEL. To evaluate the apoptotic response in 4T1 cells, we utilized TUNELtechnique. The cultured cells were plated on glass-bottom dishes with 20 mm diameter wells and preincubate with 250 μL of 0 μg/ml, 25 μg/ml, 50 μg/ml, and 100 μg/ml N-PT-RGD for 8 hours. Following incubation, the cells were washed three times with fresh RPMI 1640 and radiated using 635 nm (0.5 W/cm^2) for 10 min. Cells were allowed to adhere to the plates for 4 hours, before testing fluorescence response. The cells were washed with PBS and immersed in the naturally dried cell fixative buffer for 30 minutes. The cells were followed with washing with PBS and treated using 0.3% Triton in PBS solution for 5 min at room temperature. The cell samples were added with 5 μL TdT enzyme and 45 μL fluorescence labeling solution and incubated in the dark for 60 min at 37℃. After incubation, the cells were washed with PBS 3 times, and images were collected using a TCS SP8 confocal laser scanning microscopy (Leica, Germany) with an excitation wavelength of 550 nm (Cy3).

Cytokines TNF-α and IL-6 analysis. The cultured cells were plated on 6-well plates and preincubate with 250 μL of 0 μg/ml, 10 μg/ml, 50 μg/ml, 75 μg/ml, and 100 μg/ml N-PT-RGD for 8 hours. Following incubation, the cells were washed three times with fresh RPMI 1640 and radiated using 635 nm (0.5 W/cm^2) for 10 min. Cells were allowed to adhere to the plates for 4 hours, and the supernatant was collected to simulate Raw 267.4 cells. The supernatant from Raw 267.4 cells was used for TNF-α and IL-6 analysis. The TNF-α and IL-6 (all from Sangon Biotech (Shanghai) Co., Ltd.) were analyzed with ELISA kits according to the vendor's instructions.

4. Supplementary Figures.

Figure S1. Color of **N-PT-RGD** when dissolved in water. (0.50 μg/mL of **N-PT-RGD**)

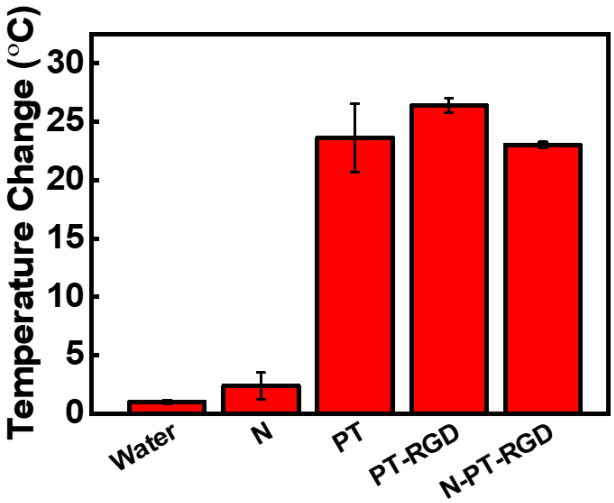


Figure S2. Temperature changes of water, N, PT, PT-RGD, and N-PT-RGD upon radiation at 635 nm for 5 min. (0.50 mg/mL of **N-PT-RGD**)

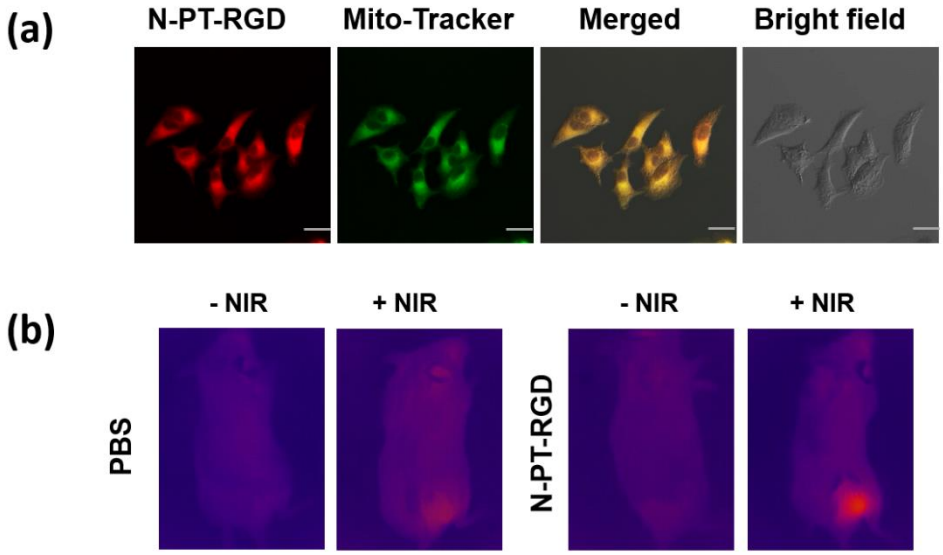


Figure S3. *In vivo* cell colocalization images. a) Localization of N-PT-RGD in cells compare with mitochondria locations. The scale bar is 10 μm. b) Comparison of IR images using PBS injection and N-PT-RGD treatment.

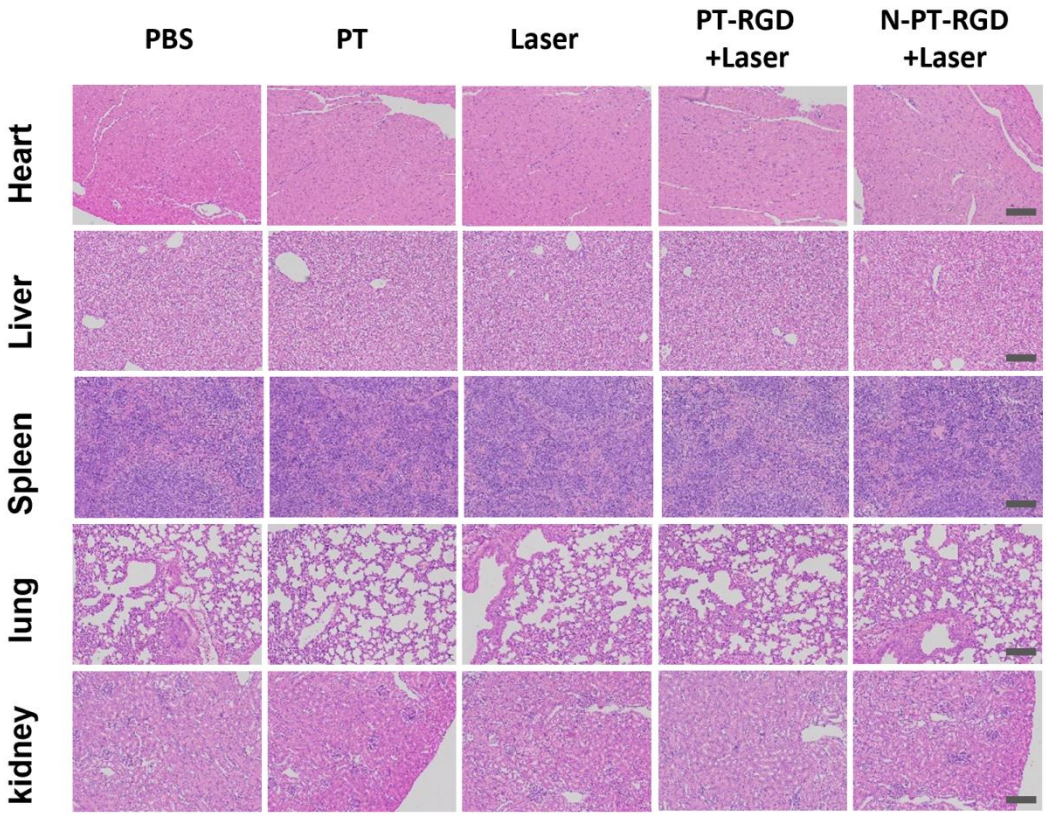


Figure S4. Histology analysis of the major organs (heart, liver, spleen, lungs, and kidneys) of the mice stained with hematoxylin and eosin (H&E) 7 days after various treatments. The scale bar is 200 μm.

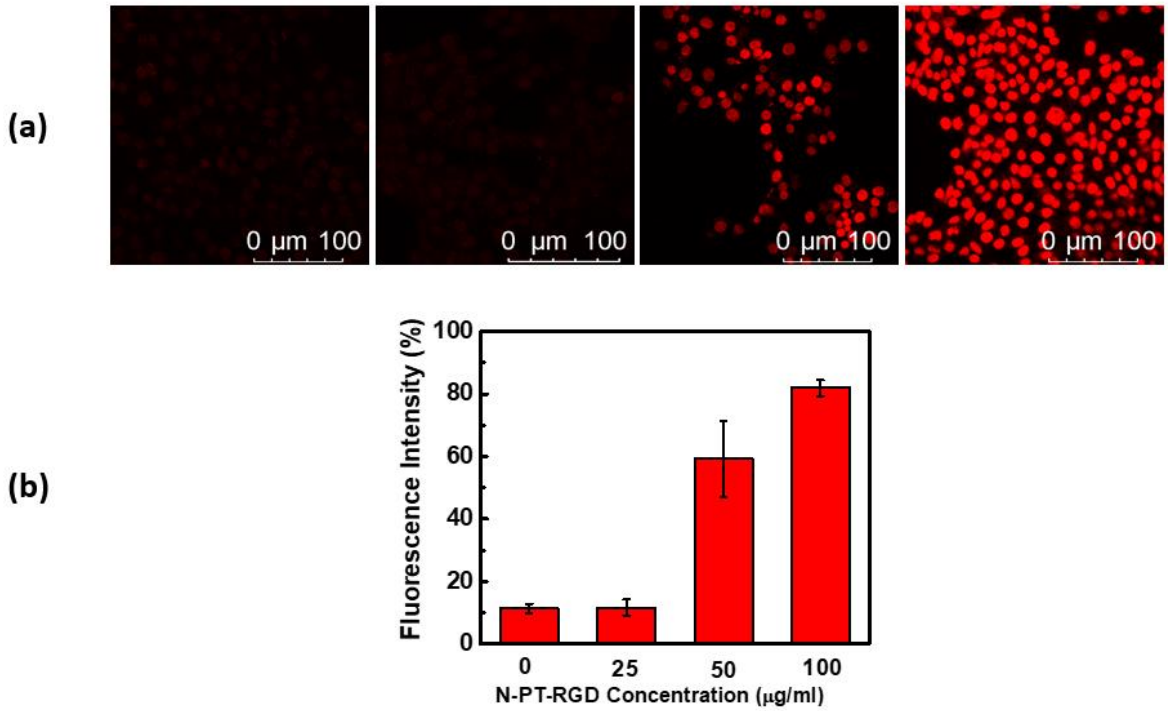


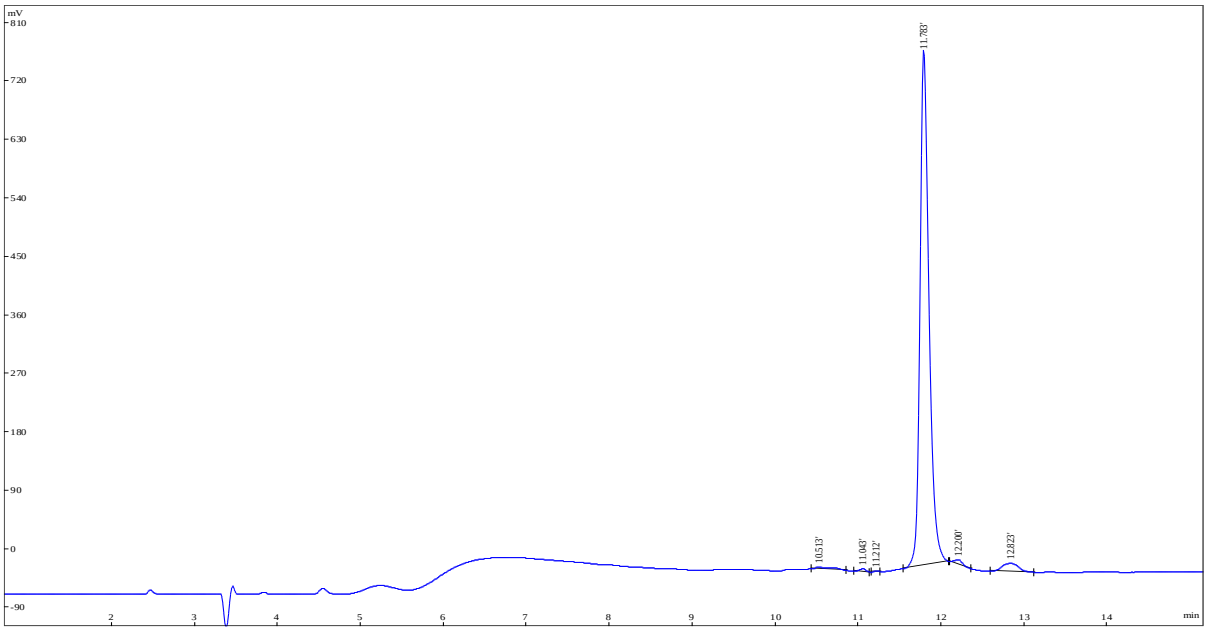
Figure S5. Apoptotic analysis of 4T1 cells. a) Fluorescence response of cell apoptotic level. b) Quantified fluorescence response from a). The scale bar is 100 μm.

5. HPLC, NMR, and MS Analysis Report

Solvent A: 0.1%Trifluoroacetic in 100% Acetonirile
Solvent B: 0.1%Trifluoroacetic in 100% Water

Gradient :	A	B
0.01min	5%	95%
25.0min	70%	30%

Flow rate: 1.0ml/min
Wavelength: 214nm



Rank	Time	Name	Conc.	Area
1	10.513		0.8956	56305
2	11.043		0.362	22760
3	11.212		0.1278	8035
4	11.783		95.12	5979960
5	12.200		0.7279	45760
6	12.823		2.76	173541
Total			100	6286361

Figure S6. HPLC spectrum of N-PT-RGD.

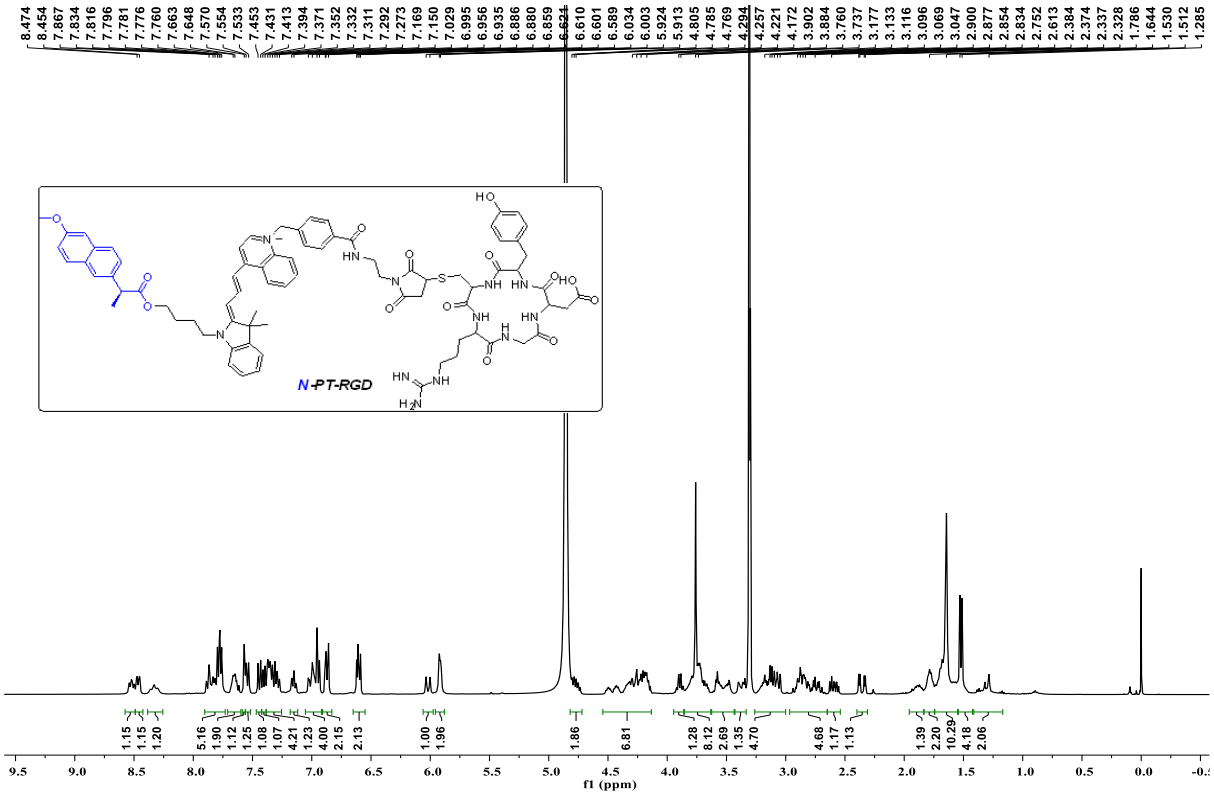


Figure S7. NMR spectrum of **N-PT-RGD**.

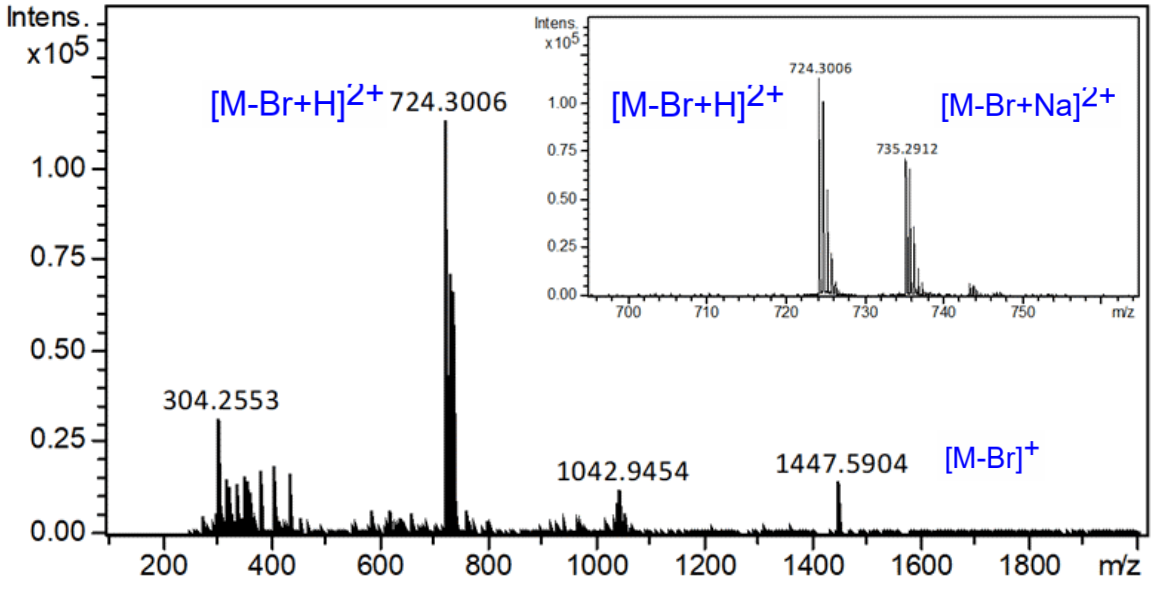


Figure S8. MS spectrum of **N-PT-RGD**.

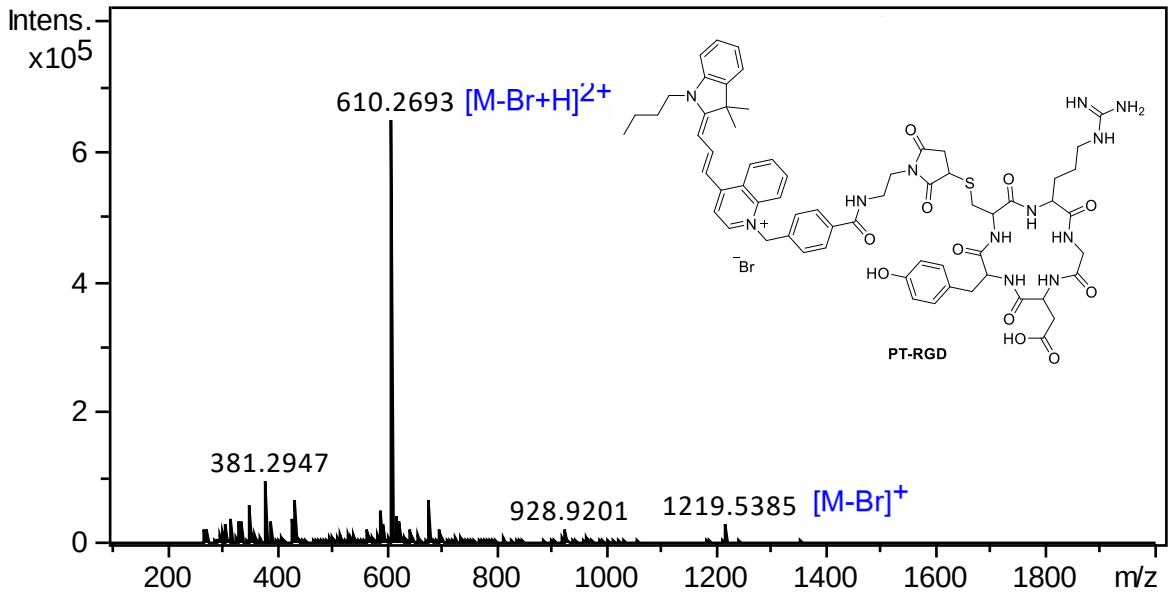


Figure S9. MS spectrum of **PT-RGD**.

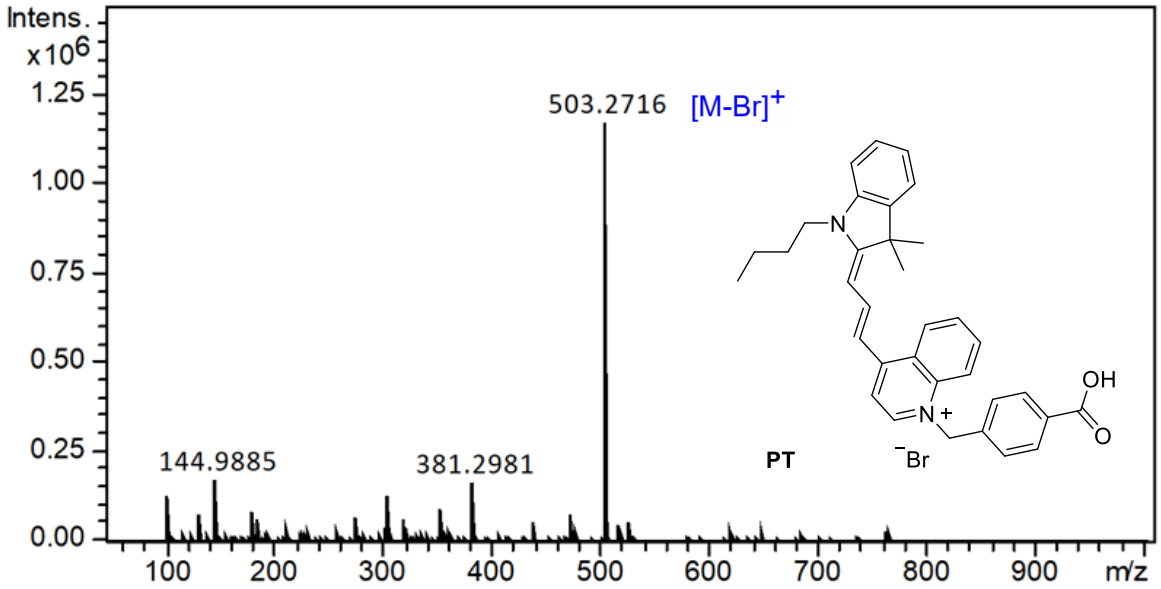


Figure S10. MS spectrum of **PT**.

6. References:

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