Supporting Information

Development of A Novel Anti-tumor Theranostic Platform: Near-Infrared Molecular Upconversion Sensitizer for Deep-seated Cancer Photodynamic Therapy

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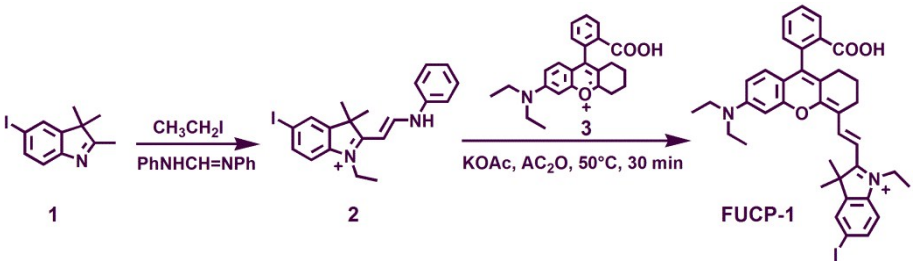
Materials and Methods

Compound 1[1], 3[2] and NRh-1[3] were prepared according to reported procedures. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 1,3-diphenylisobenzofuran (DPBF) were obtained from Energy Chemical Co.. Annexin V-FITC apoptosis detection kit, Singlet oxygen sensor green (SOSG), DCFH-DA, and Calcein-AM/PI Double Stain Kit was purchased from KeyGEN BioTECH. All the other reagents were analytical grade mentioned herein, without special treatment. NMR spectra were obtained using a Bruker Avance III 500 spectrometer. Mass spectral studies were carried out using a LTQ Orbit rap XL instruments. Absorption and emission spectra for were performed on Lambda 35 UV-visible spectrophotometer (PerkinElmer), VARIAN CARY Eclipse fluorescence spectrophotometer (Serial No. FL0812-M018), and FLS1000 Photoluminescence Spectrometer (Edinburgh Instruments) respectively. Absolute photoluminescence quantum yields were obtained from Absolute PL Quantum Yields Spectrometer C11347 (Quantaurus-QY). Confocal laser scanning microscope (CLSM) images were carried by Olympus FV1000-IX81 confocal laser scanning microscope. Small animals' fluorescence imaging were performed on by NightOWL II LB983 living imaging system. Cell apoptosis assay was carried by Attune Nxt Acoustic Focusing Cytometer (Thermo Fisher Scientific).

The tumor-bearing BALB/c mice were originally purchased from SPF experimental Animal Center of Dalian Medical University. This study was 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). The animal protocol was approved by the local research ethics review board of the Animal Ethics Committee of Dalian University of Technology (Certificate number//Ethics approval no. is 2018-043)



Scheme S1. Chemical structure and synthetic route of FUCP-1.

Synthesis of 2: Compound 1 (2.85 g, 10 mmol) and Iodoethane (3.12 g, 20 mmol) was dissolved in toluene (30 mL) and then the mixture was heated under reflux for 12 h. The resulting product was poured into ice-cold diethyl ether (100 mL) and then the resulting precipitate was filtered, and washed with cold diethyl ether (150 mL) for 3 times. The cruder was collected without further purification and then dissolved in a solution of N,N'-bis-phenylformamidine (15 mmol) in acetic acid (30 mL). The mixture was heated to 110 ˚C and kept for 4 h. The mixture was poured into ice-cold diethyl ether and washed with cold diethyl ether (150 mL) for 3 times. The cruder product was purified by silica column chromatography (DCM/MeOH = 50:1, v/v) to obtain compound 3 as a yellow powder (2.81 g, 51.7%). ^{1}H NMR (500 MHz, d_6-DMSO) δ = 12.12 (d, *J*=12.6, 1H), 8.68 (t, *J*=12.7, 1H), 8.10 (s, 1H), 7.81 (d, *J*=8.3, 1H), 7.55 – 7.44 (m, 4H), 7.41 – 7.26 (m, 2H), 6.18 (d, *J*=12.3, 1H), 4.11 (d, *J*=7.1, 2H), 1.70 (s, 6H), 1.31 (t, *J*=7.2, 3H). ^{13}C NMR (126 MHz, d_6-DMSO) δ = 177.50, 153.08, 144.39, 141.89, 139.18, 137.97, 132.22, 130.69, 127.19, 119.30, 114.61, 91.84, 91.01, 50.42, 28.34, 12.58. HRMS (ESI): m/z calcd. for $C_{20}H_{22}IN_2^+$ = 417.0822, found m/z = 417.0816.

Synthesis of FUCP-1: Compound 2 (1.09 g, 2 mmol), compound 3 (0.95 g, 2 mmol) and KOAc (0.19 g, 2 mmol) were dissolved in an Ac_2O solution (20 mL). The mixture was heated at 50 ˚C for 30min. Then, the mixture was poured into 50 mL ice-cold diethyl ether and filtered, washed with cold ethyl ether for 3 times. The cruder product was purified by silica column chromatography using DCM/MeOH (v/v, 30:1) mixture as the eluent. The green fraction was collected (1.02 g, 63.8%). ^{1}H NMR (500 MHz, MeOD) δ = 8.57 (d, *J* = 13.7, 1H), 8.08 – 7.97 (m, 1H), 7.81 (s, 1H),

7.71 (d, *J* = 8.3, 1H), 7.59 – 7.51 (m, 2H), 7.17 – 7.10 (m, 1H), 7.04 – 6.95 (m, 2H), 6.87 (dd, *J* = 9.3, 2.3, 1H), 6.76 (d, *J* = 2.2, 1H), 6.06 (d, *J* = 13.7, 1H), 4.08 (q, *J* = 7.0, 2H), 3.60 (q, *J* = 7.1, 4H), 2.69 (s, 2H), 2.57 – 2.47 (m, 1H), 2.45 – 2.35 (m, 1H), 1.87 (s, 1H), 1.78 (s, 6H), 1.37 (t, *J* = 7.0, 3H), 1.27 (t, *J* = 7.1, 6H). ^{13}C NMR (101 MHz, MeOD) δ = 174.72, 171.30, 166.11, 160.45, 158.84, 154.96, 145.19, 144.48, 141.61, 141.35, 139.54, 136.06, 133.41, 131.82, 131.58, 131.48, 130.88, 130.24, 123.48, 118.94, 116.75, 115.24, 113.64, 99.15, 97.18, 88.28, 46.95, 40.48, 29.58, 29.51, 28.61, 26.19, 22.67, 13.61, 12.80. HRMS (ESI): m/z calcd. for $C_{38}H_{40}IN_2O_3^+$ = 699.2078, found m/z = 699.2076.

Calculation of absolute photoluminescence quantum yields: Fluorescence quantum yields were determined with the integrating sphere. The following equations were used to calculate absolute photoluminescence quantum yields:[4]

$$\Phi_f = \frac{PN(Em)}{PN(Abs)} = \frac{\int \frac{\lambda}{hc}\left[I_{em}^{sample}(\lambda) - I_{em}^{reference}(\lambda)\right]d\lambda}{\int \frac{\lambda}{hc}\left[I_{ex}^{reference}(\lambda) - I_{ex}^{sample}(\lambda)\right]d\lambda}$$

Where, PN(Abs) is the number of photons absorbed by a sample and PN(Em) is the number of photons emitted from a sample, λ is the wavelength, h is Planck's constant, c is the velocity of light, Isample ex and Ireference ex are the integrated intensities of the excitation light with and without a sample respectively, Isample emand Ireference em are the photoluminescence intensities with and without a sample, respectively.

Photodegradation experiments: FUCP-1 was dissolved in dichloromethane at a concentration of 10 μM and the solution was then irradiated by 700 nm (200 mW/cm^2, 800 mW/cm^2) and 808 nm (200 mW/cm^2, 800 mW/cm^2) laser for 20 min at room temperature respectively. The irreversible bleaching of the dye at the absorption peak was monitored as a function of time. Samples were tightly sealed, but not deoxygenated with nitrogen.

Singlet oxygen ($^1O_2$) detection: The singlet oxygen generated by FUCP-1 was evaluated by using singlet oxygen capture agent, 1, 3-diphenlisobenzofuran (DPBF).

Briefly, the absorbance of DPBF at 415 nm was adjusted to about 1.0 in DCM. Samples were added into a cuvette and the absorbance of the photosensitizer (FUCP-1 or NRh-1) was then adjusted to around 0.5. Then, the cuvette was exposed to 808 nm laser (800 mW/cm^2) for different time (30, 60, 90, 120, 150, 180, 210, 240 s), and absorption spectra were measured immediately. To demonstrate the $^1O_2$ generation by FUCP-1 during the one-photon upconversion process is temperature-related, the temperature of samples was adjusted to -20, 0, or 25 °C. Then, the samples was exposed to the 808 nm laser and absorption spectra were measured immediately.

Intracellular singlet oxygen imaging: Singlet oxygen sensor green (SOSG) and 2′, 7′-dichlorofluorescin diacetate (DCFH-DA) was used for intracellular singlet oxygen imaging by FUCP-1. 4T1 cells were incubated with 5 μM FUCP-1 for 4 h, and then stained with 10 μM SOSG for another 2 h (or DCFH-DA for 0.5 h). In $^1O_2$ quenching groups, 100 μM NaN_3 was add to the cells for 1 h before the cells were stained with DCFH-DA. Then, the cells were irradiated with 808 nm (800 mW/cm^2) laser for 10 min. Confocal fluorescence imaging was used to observe the intracellular $^1O_2$ level.

Optical imaging of cells: 4T1 cells were stained with 5 μM FUCP-1 and then incubated at 37°C in a 5% CO_2 humidified atmosphere for various time (10, 30, 60, 120, and 240 min). Fluorescence imaging was then carried out with a spectral confocal microscope (Olympus, FV1000), using a 60 × objective oil lens. Excitation wavelength: 808 nm. Emission wavelength was collected at 680–760 nm.

Cytotoxicity in vitro determined by MTT method: 4T1 cells were plated at 96-well plates (1 × 10^4 cells per well and incubated in 100 μL). After 24 h of cell adhension, 4T1 cells were treated with FUCP-1 at a serial of concentrations (0.63, 1.25, 2.5, and 5.0 μM) for 4 h. All groups were triplicated. After incubation for 24 h, the medium was removed and cells were washed with PBS twice. Then 808 nm (800 mW/cm^2, 10 min) laser was employed for PDT and cells were allowed to continue growing for 24 h. For different optical power PDT experiments, cells treated with 5 μM FUCP-1were exposed to 808 nm laser (10 min) for diferent optical density (0, 200, 400, 800, 1600 mW/cm^2). MTT solution (100 μL of 0.5 mg/ml in PBS) was added to each well, and the cells further incubated at 37 ºC for 4 h. Subsequently, the medium was carefully

removed, and 100 μL DMSO was added to dissolve the formazan crystals. The absorbance value was measured at 490 nm with a Bio-Rad microplate reader and the cell viability was calculated by the following formula:

$$Cell\ viability\ (\%) = \left(OD_{ps} - OD_{blank}/OD_{control} - OD_{blank}\right) \times\ 100\%$$

Cell apoptosis assay: 4T1 cells were plated at 12-well plates (3×10^4 cells/well), then divided into 4 groups: treated with PBS, FUCP-1 (5 μM), 808 nm laser, FUCP-1 + 808 nm laser (800 mW/cm^2, 10 min); After 800 nm laser irradiation, the cells were resuspended in 0.4 ml binding buffer and an Annexin V-FITC Apoptosis Detection Kit (5 μl annexin V-FITC and 5 μl propidium iodide per group) was used before analysis by flow cytometry.

In vivo optical imaging of mice: FUCP-1 (10 μM, 100 μL) was intravenously injected into 4T1 tumor-bearing BALB/c mice and the mice were imaged using a NightOWL II LB983 small animal in vivo imaging system with a 808 nm excitation laser and a 721 ± 32 nm emission filter.

In vivo PDT evaluation: 4T1 tumor-bearing BALB/c mice were divided into 5 groups: group 1, only PBS injection; group 2, PBS injection and irradiation under 808 nm laser (800 mW/cm^2, 15 min); group 3, only FUCP-1 injection (1.2 mg/kg); group 4, FUCP-1 injection and irradiation under 808 nm laser (800 mW/cm^2, 15 min) with 5 mm pork tissue; group 5, FUCP-1 injection and irradiation under 808 nm laser (800 mW/cm^2, 15 min). After 8 h post-injection, tumor region was irradiated with 800 nm laser respectively. The volume of all mice was measured using a vernier caliper for 14 days after different treatments. Tumor volume = width × width × length/2.

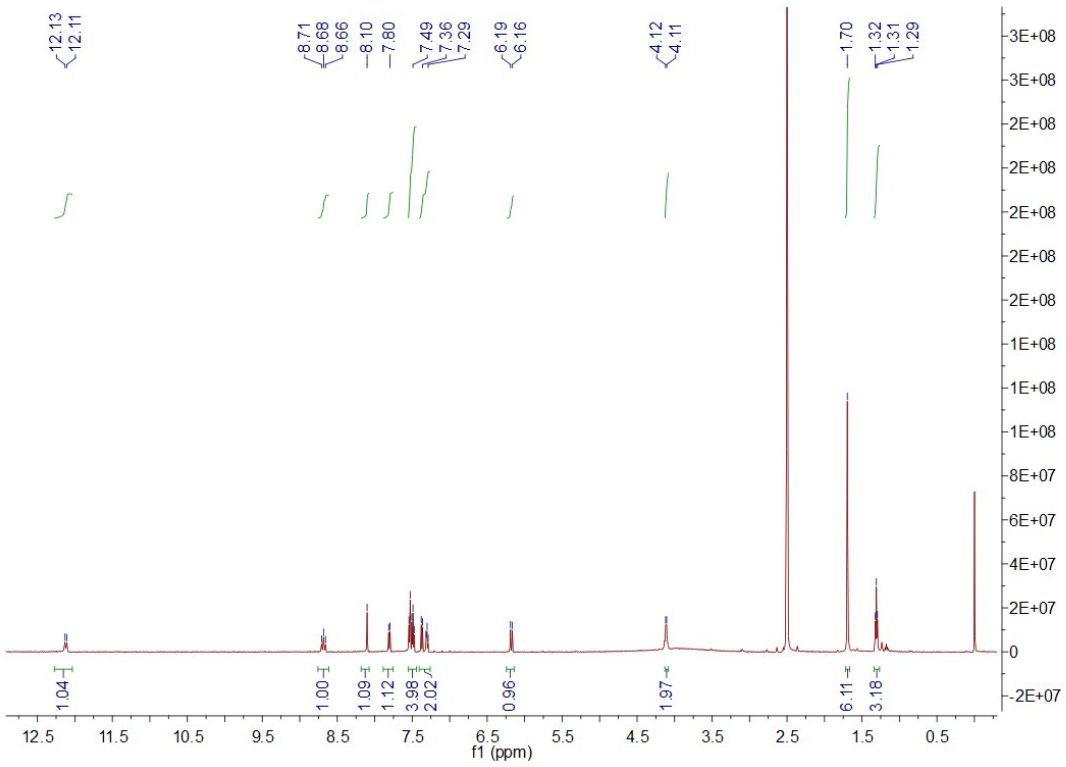


Fig. S1 ^{1}H-NMR spectrum of compound 2.

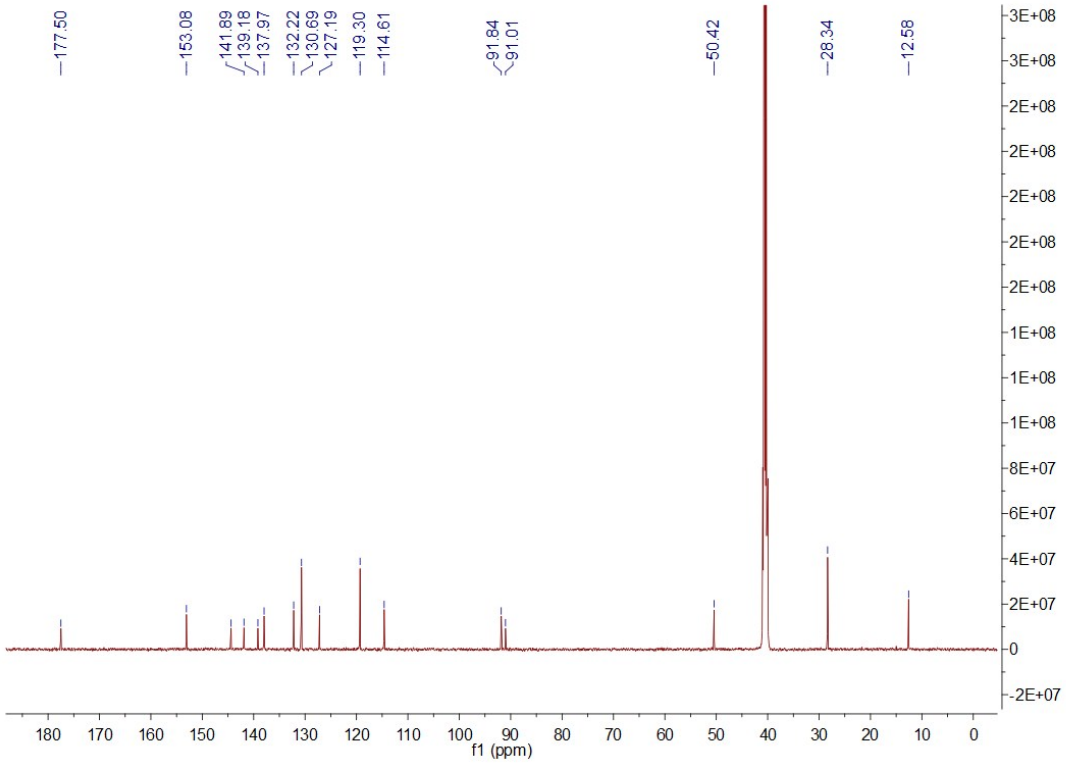


Fig. S2 ^{13}C-NMR spectrum of compound 2.

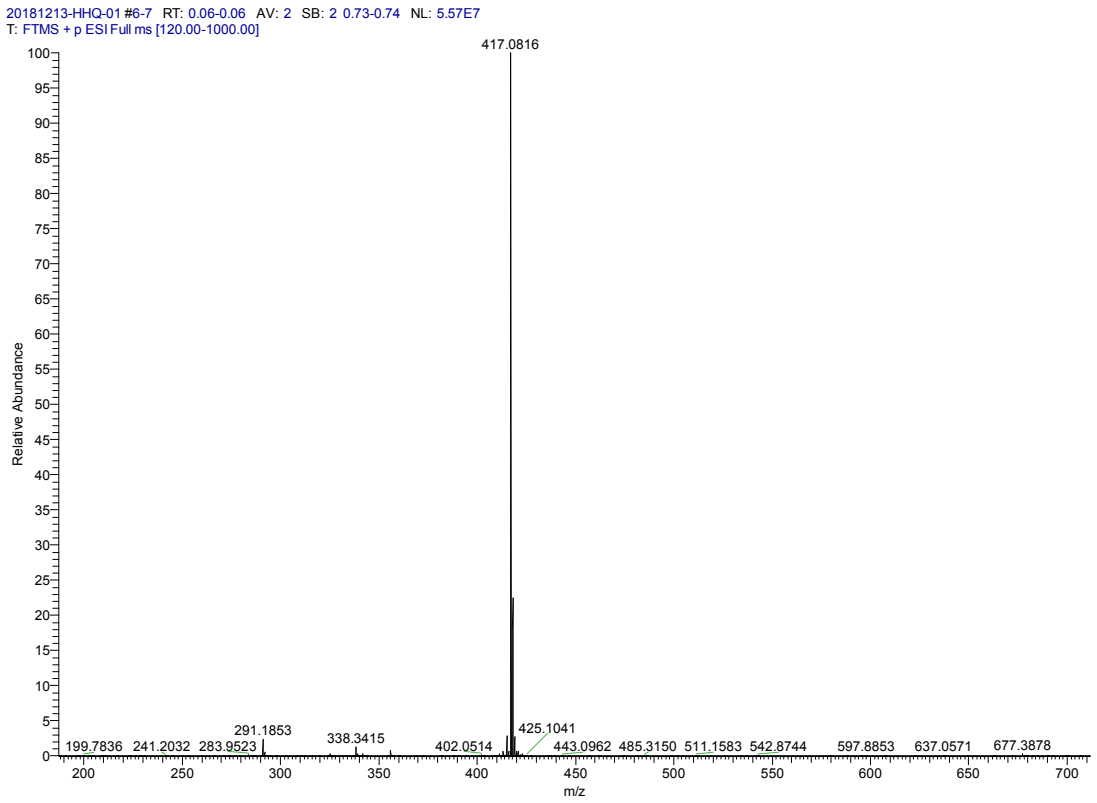


Fig. S3 HRMS of compound 2.

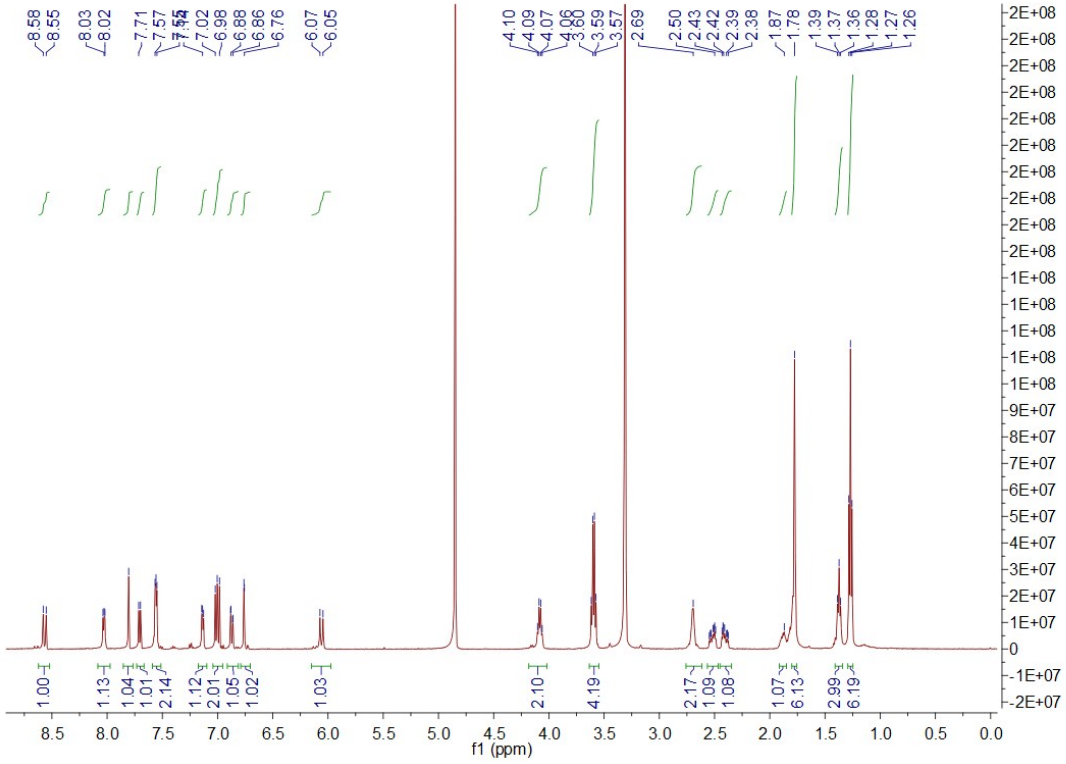


Fig. S4 ^{1}H-NMR spectrum of compound FUCP-1.

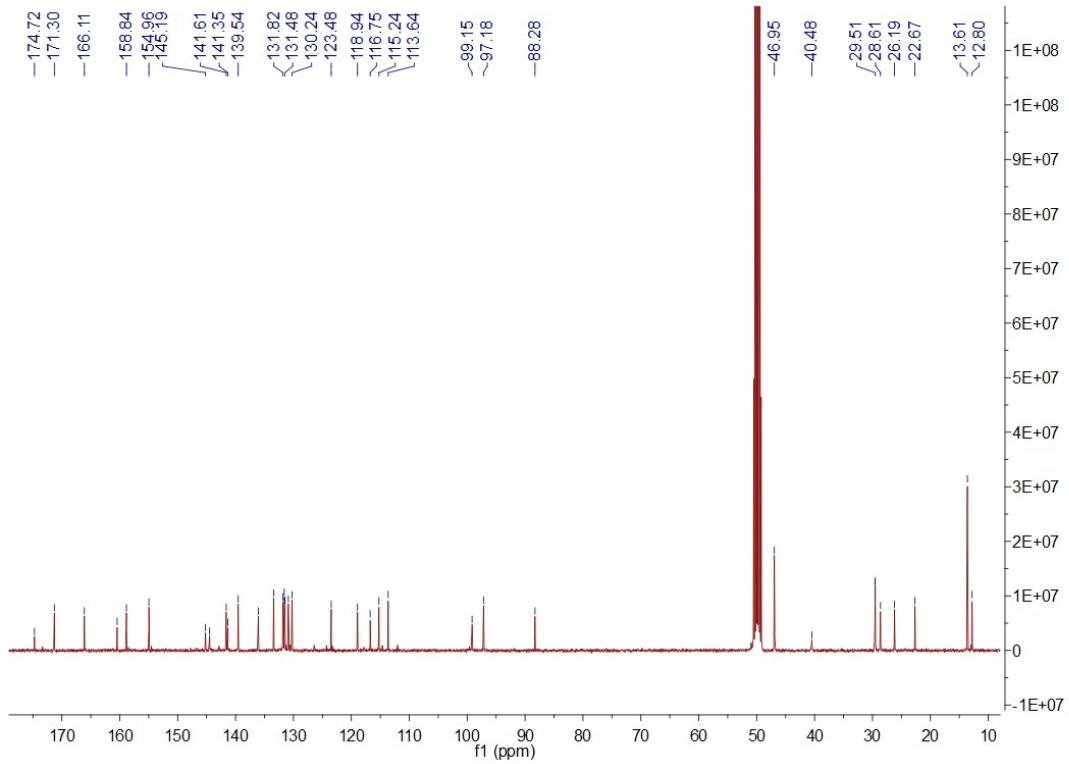


Fig. S5 ^{13}C-NMR spectrum of compound FUCP-1.

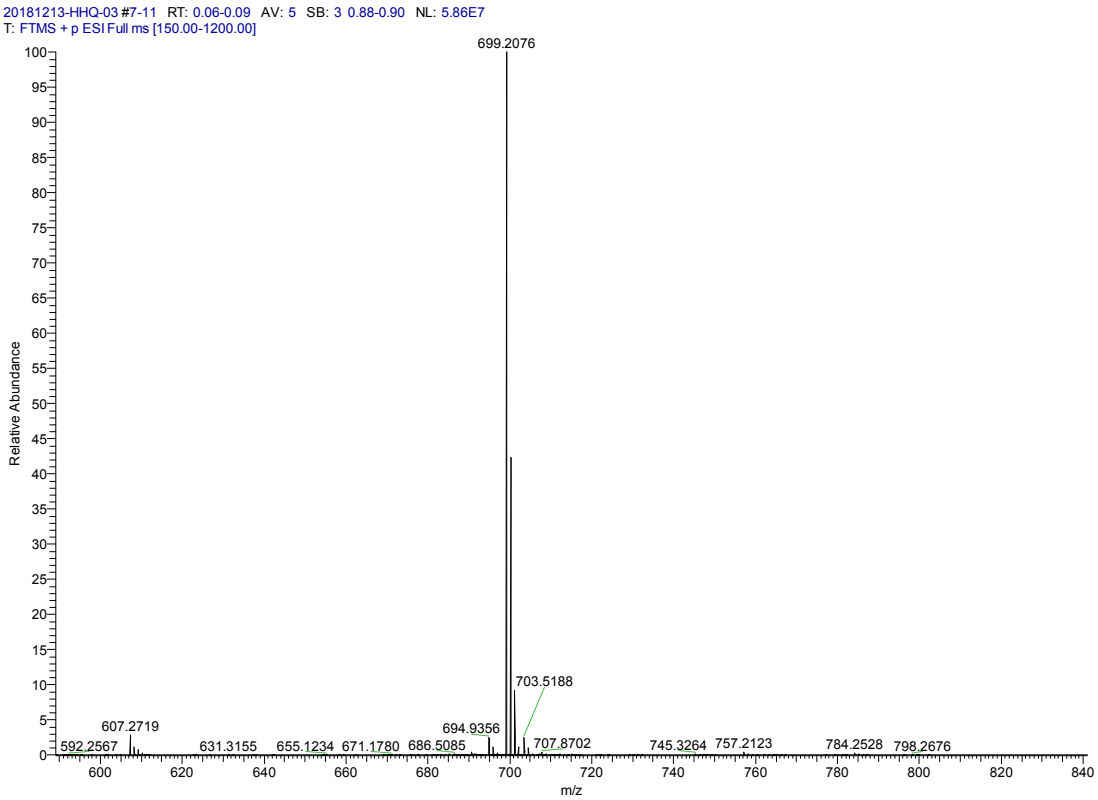
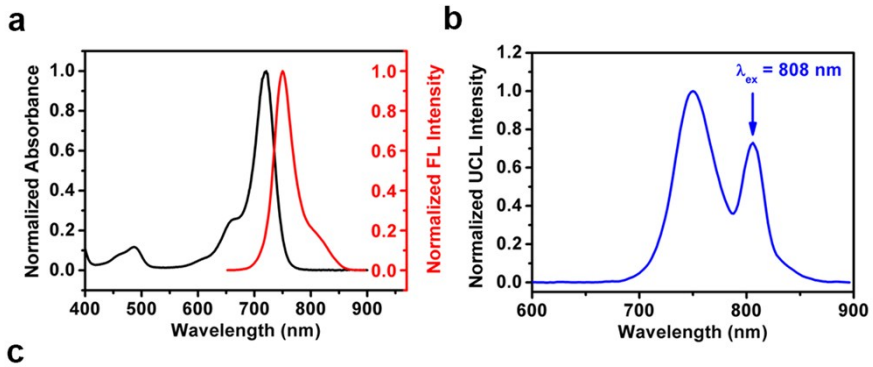


Fig. S6 HRMS of compound FUCP-1.



c

	λ_{ex} (nm)	λ_{em} (nm)	Φ_f
Stokes	700	750	39.5%
Anti-Stokes	808	750	12.6%

Fig. S7 a) Normalized UV-vis (black), fluorescence (red) and b) UCL spectra of FUCP-1 in dichloromethane. c) Photophysical date of FUCP-1 in dichloromethane.

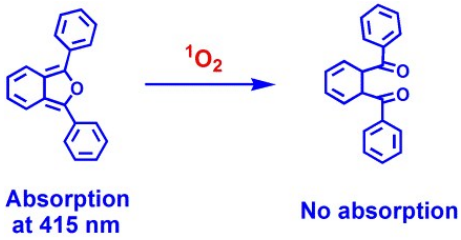


Fig. S8 Schematic illustration of DPBF for detecting $^1O_2$ generation.

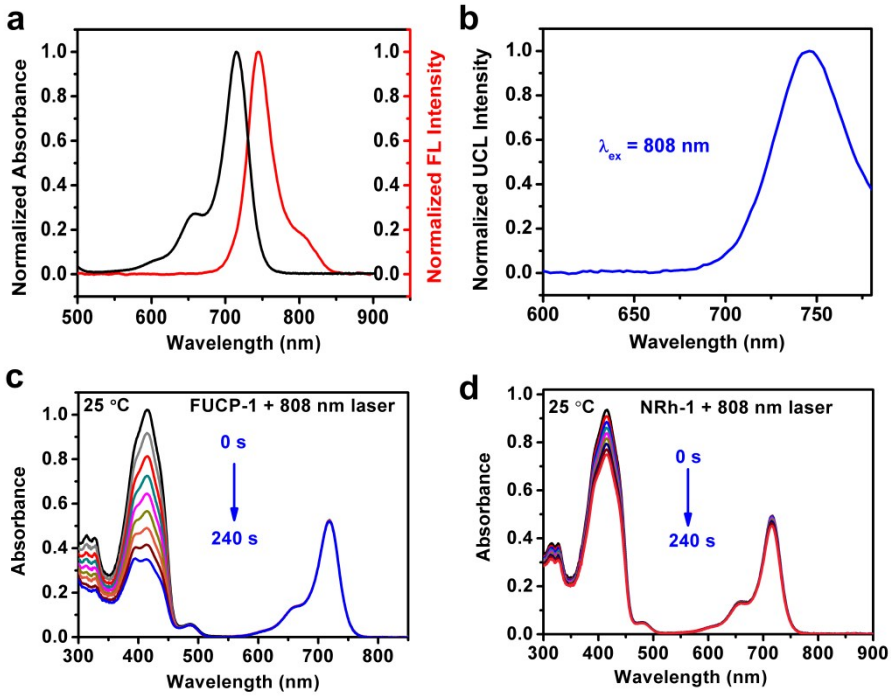


Fig. S9 a) Normalized absorbance (black) and fluorescence (red) spectra of NRh-1. b) Normalized UCL fluorescence spectra of NRh-1. Absorption spectra changes of DPBF in the presence of c) FUCP-1 and d) NRh-1 under 808 nm laser irradiation for 240 s at 25°C.

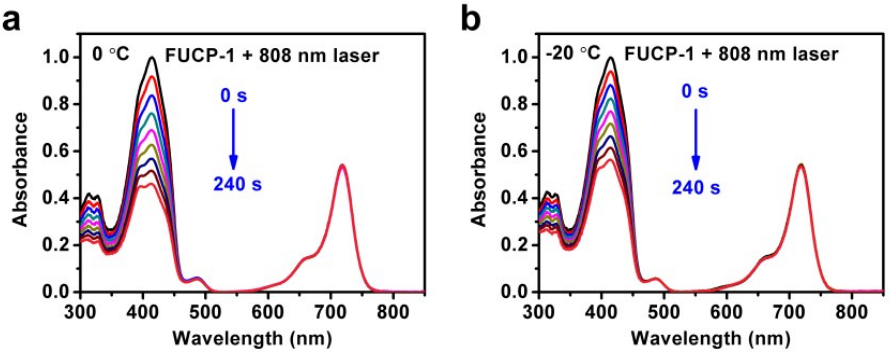


Fig. S10 Absorption spectra changes of DPBF in the presence of FUCP-1 under 808 nm laser irradiation for 240 s a) at 0 °C and b) -20 °C.

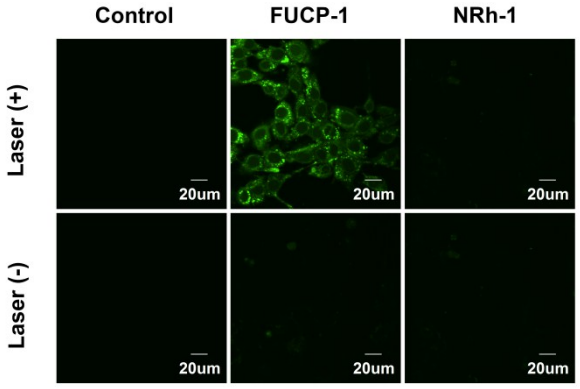


Fig. S11 Confocal fluorescence images of 4T1 cells incubated with SOSG (10 μM) under different treatments

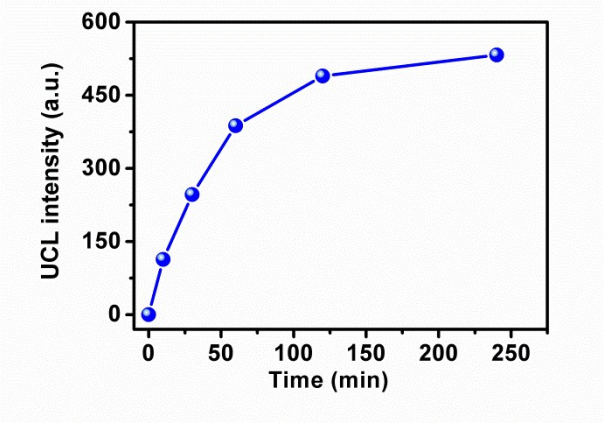


Fig. S12 UCL intensity changes of FUCP-1 (5 μM) in 4T-1 cells after incubation for different times (0, 10, 30, 60, 120 and 120 min).

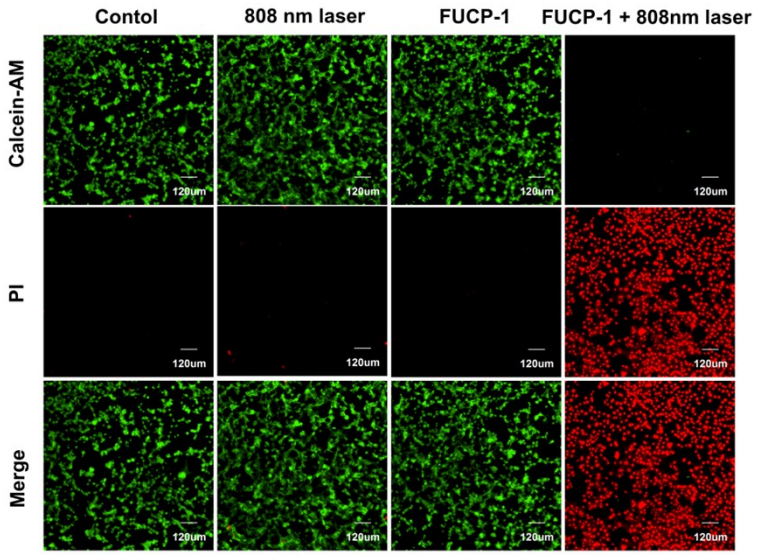


Fig. S13 Multichannel confocal fluorescence images Green channel (λ_{ex} = 488 nm, λ_{em} = 505–555 nm) for Calcein-AM stained live cells. Red channel (λ_{ex} = 559 nm, λ_{em} = 695–745 nm) for stained death cells. The optical density of 800 nm laser was 800 mW/cm^2 in all cases.

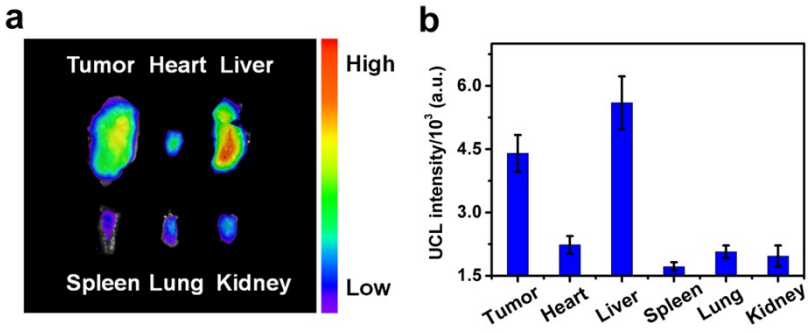


Fig. S14 a) UCL bioimaging signals of FUCP-1 acquired from BALB/c mice. b) UCL imaging of various organs (heart, liver, spleen, lung, kidney) after 8 h injection.

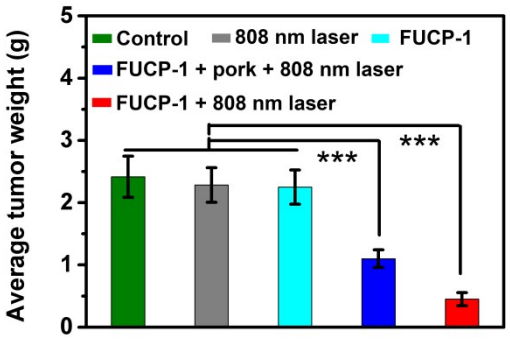


Fig. S15 Average weight of tumors harvested at 14 d post-treatment. (n=6, Data were expressed as mean ± SD, ***p < 0.001 determined by Student's *t* test).

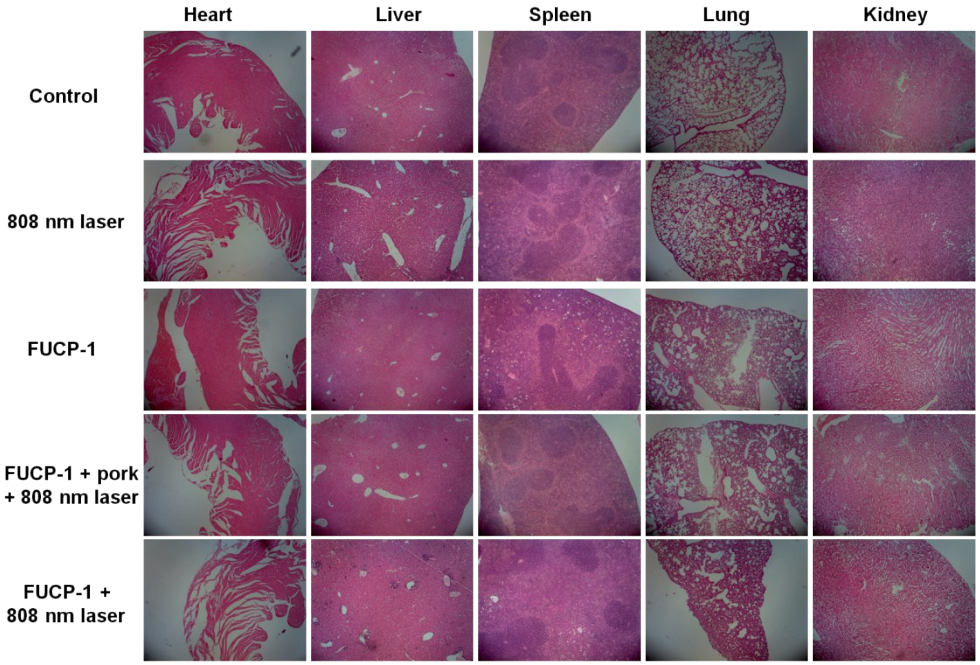


Fig. S16 H&E stained images of heart, liver, spleen, lung, and kidney achieved from various groups after two week treatment.

References:

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