Supporting Information

A near-infrared aggregation-induced emission photosensitizer with mitochondria specificity enhances radiotherapy for cancer stem cells ablation

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Experimental Section

Materials

All the chemicals used in the chemical reaction were purchased from TCI or Meryer. Cholesterol and 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) were purchased from A.V.T (Shanghai, China). DCFH-DA was purchased from Sigma-Aldrich (USA). All of the aqueous solutions were prepared using purified deionized (DI) water purified with a purification system (Direct-Q3, Millipore, USA). The other solvents used in this work were purchased from Sinopharm Chemical Reagent (China) and Shanghai Macklin Biochemical Technology Co., Ltd. (China).

Synthesis of DACNPy+

4-(cyanomethyl)-1-methylpyridin-1-ium iodide (135 mg, 0.5 mmol) and 4-dimethylaminocinnamaldehyde (90 mg, 0.5 mmol) were dissolved in 10 mL of ethanol into a 50 mL round-bottom flask, followed by 50 uL of piperidine addition into the reaction mixture and then kept at 80 °C to reflux for 6 h. The solution was cooled to room temperature, and the solvent was removed by rotary evaporation. The crude product was purified by flash column chromatography (DCM/MeOH = 10: 1, V/V) to obtain a dark blue solid (80% yield). ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.77 (d, J = 6.9 Hz, 2H), 8.52 (d, J = 11.6 Hz, 1H), 8.08 (d, J = 7.0 Hz, 2H), 7.66 – 7.56 (m, 2H), 7.50 (d, J = 14.6 Hz, 1H), 7.10 (dd, J = 14.6, 11.6 Hz, 1H), 6.76 (d, J = 8.9 Hz, 2H), 4.19 (s, 3H), 3.03 (s, 6H). ^{13}C NMR (400 MHz, DMSO-d_6) δ 153.16, 152.72, 151.85, 148.89, 144.83, 131.45, 121.97, 120.91, 118.45, 115.72, 111.99, 99.97, 45.58. HRMS (ESI) *m*/*z*: $[M-I]^+$ calcd for DACNPy+: 290.1652 Found 290.1666.

Computational details

All structures were optimized using the M06-2X functional and the 6-31G* basis set for all atoms, including Grimme's DFT-D3 empirical dispersion correction with the original damping function. Analytical frequency calculations at the same level of theory identified all stationary points as intermediates (no imaginary frequency). The energy of the excited states were obtained from single-point TD-DFT calculations on the ground-state structures at the M06-2X/6-31G* level. All DFT geometry optimizations and DFT/TD-DFT single-point calculations were performed using the Gaussian 16 program.

Preparation and characterization of DL and DFL

The platelet membranes (PM) were prepared following the procedure described in previous work. Subsequently, 27 mg of 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 2.8 mg of cholesterol, and 2.5 mg of DACNPy+ were dissolved in chloroform. The solution was then evaporated at 40 °C for 80 min using a rotary evaporator to form a thin film. The film was hydrated with phosphate-buffered saline (PBS) solution containing PM (3 mg of protein) at 37 °C for 5 min. The resulting mixture was subjected to ultrasonication and subsequently extruded repeatedly through 200 nm polycarbonate membranes. The resultant DACNPy+-loaded liposomes (DL) with encapsulated PM were designated as DFL particles. These particles were dialyzed overnight against water using a dialysis bag with a molecular weight cutoff (MWCO) of 300 kDa to

remove any unencapsulated DACNPy+. The DACNPy+ loading capacity was determined by UV-vis spectroscopy using a Lambda 35 spectrophotometer (Perkin-Elmer) and calculated using the formula:

Loading Capacity = M_{drug}/M_{DFL}, where M refers to the mass.

For comparison, liposomes loaded with DACNPy+ (DL) but without PM were prepared using the same method. The particle size and zeta potential of the particles were measured using dynamic light scattering (DLS) with a Malvern Zetasizer Nano ZS90 (UK). The morphology of the synthesized materials was examined using a field-emission transmission electron microscope (JEM-F200, Japan). The photoluminescence spectrum was recorded using a spectrometer (FLS1000, UK). Electron paramagnetic resonance (EPR) experiments were conducted on a Bruker EMXplus EPR spectrometer.

DACNPy+ release study

The *in vitro* release profile of DACNPy+ from DFL was evaluated using the dialysis method. 1 mL of DFL containing 0.05 mg of DACNPy+ was added to 1× PBS (pH = 7.4) and subjected to horizontal shaking at 100 rpm for 3 h. To explore the influence of laser irradiation as a stimulus on the release behavior, the release experiment of DFL was initially conducted under two conditions: In the absence or presence of 660 nm laser exposure (0.1 W/cm², 5 min). At specific time points, the solution was centrifuged at 9000 rpm for 10 min. Subsequently, 100 μL of the supernatant was collected, and the concentration of the released DACNPy+ was monitored using a UV–vis spectrophotometer.

$^1O_2$-Generation Detection.

9,10-Anthracenediyl-bis(methylene)dimalonic acid (ABDA) was used as the $^1O_2$-monitoring agent. In the experiments, 13 μL of ABDA stock solution (7.5 mM) was added to 2 mL of DACNPy+ or Ce6 suspension (5 μM), and 660 nm laser (0.5 W/cm^2) was employed as the irradiation source. The absorption of ABDA at 378 nm was recorded at various irradiation times to obtain the decay rate of the photosensitizing process.

Cancer cell line

4T1 mouse breast cancer cell line was obtained from the Cell Bank of the Chinese Academy of Sciences and incubated in RPMI-1640 medium supplemented with 10% FBS in a humidified atmosphere at 37 °C.

The cancer stem cells (CSCs) were sorted from 4T1 cells and cultured as guided by the previous report[1]. 4T1 cells suspension (10000 cells/ml) were seeded in ultra-low attachment surface 6-well plates (Corning, USA) with serum-free DMEM-F12 medium, containing B27 (1:50), epidermal growth factor, basic fibroblast growth factor (20 ng/mL), N_2 additives. Primary cultures of colonospheres were harvested after 7 days in culture and collected after centrifugation, dissociated with Trypsin–EDTA.

The non-cancer stem cells (nCSCs) were also sorted from 4T1 cells. Briefly, 4T1 cells were split in Serum Containing Medium (SCM). This is made up of DMEM/Ham's F12 (1:1) (Hyclone), 10 % Foetal Bovine Serum (FBS) (Biological Industries), 1 % penicillin/streptomycin (P/S) (100 U/ml) (Hyclone), and 1 % L-glutamine (LG) (Lonza). The adherent cells obtained from the SCM were harvested by trypsinization to obtain single cells and then expanded in SCM medium to obtain more cells. These cells were scored as nCSCs. The expressions of CD133 and CD44 on CSCs and nCSCs were tested by flow cytometry.

Animal tumor models

Female BALB/c aged 5-6 week were purchased from Vital River Company (Beijing, China). Balb/c mice were subcutaneous injected with 5×10^6 4T1 cells into the right flank to construct tumor model. All animal experiments were carried out according to the protocol approved by the Ministry of Health in People's Republic of PR China and were approved by the Administrative Committee on Animal Research of the Huazhong University of Science and Technology.

***In vitro* CSC internalization study**

4T1 CSCs were seeded in 24-well plates and cultured for 12 h. Then, 100 μL DL or DFL (containing 0.01mg DACNPY+) was added to the medium. Then, the cells were incubated for 2 h at 37 °C and 5% CO_2 and washed with PBS three times. The cells were harvested, stained with DAPI and Lyso-Tracker green and imaged by using CLSM. On the other section of experiments, 4T1 CSCs were seeded in 24-well plates

and cultured for 12 h. Then, 100 μL DFL (containing 0.01 mg DACNPY+) was added to the medium. The cells were incubated for 1h at 37 °C and 5% CO_2 and washed with PBS. Then, the cells were irradiated by a 660 nm laser (0.1 W/cm^2, 5 min), and washed with PBS. Finally, cells were stained with DAPI and Mito-Tracker green and imaged by using CLSM. Cells without light exposure were used as the control group.

Intracellular ROS, apoptosis, and mitochondrial membrane potential detection

4T1 CSCs (1.5×10^5 per well) were seeded in a 12-well plate for 12 h. Then the cells were incubated with 5 different groups: (1) PBS+L (660 nm laser, 0.5 W/cm^2, 10 min); (2) RT (4 Gy); (3) DFL+L; (4) DL+L+RT and (5) DFL+L+RT. The DACNPY+ concentration was 20 μg/mL. PDT and RT were performed after incubation for 2 hours. Then, DCFH-DA or Annexin V-FITC/PI Apoptosis Kit (Elabscience Biotechnology Co.,Ltd., China) were used according to the instruction.

For mitochondrial membrane potential detection, the cells were washed with PBS three times, fixed with 4% PFA and permeabilized with 0.1% Triton X-100 for 10 min. After washing with PBS three times, the cells were treated with Mitochondrial Membrane Potential Assay Kit (Elabscience Biotechnology Co.,Ltd., China) according to the manufacturer's instructions.

Mitochondrial imaging in tumor cells

The 4T1 CSCs were stained with DACNPy+ with or without pre-treatment with 20 μM Carbonyl Cyanide 3-ChloroPhenylhydrazone (CCCP) for 60 min. Subsequently, the excessive dye was removed, and a new medium was perfused. Then, cell imaging was performed under a fluorescent microscope (IX81, Olympus, Japan).

Live/dead staining

To further visualize the cell phototoxicity of each group, 4T1 CSCs were pre-incubated and treated with 5 different groups as mentioned above. Next, cells were washed with PBS for 3 times, treated with FDA and PI according to the manufacturer's protocol, and performed the cell imaging under a fluorescent microscope (IX81, Olympus, Japan).

Detection of Intracellular GSH and ATP

The commercially available GSH and ATP assay kit was used to detect the depletion of GSH and ATP. 4T1 CSCs were incubated with 5 different groups as mentioned above. After 12 hours of incubation, the GSH and ATP content was measured by employing commercial colorimetric GSH and ATP assay kit. The assay was carried out according to the manufacturer's instructions.

Clonogenic survival assay

500 4T1 CSC per flask were seeded in 25 cm^2 flasks and cultured for 24 h. Flasks were treated were incubated with 5 different groups as mentioned above. To allow formation of colonies, after radiation, the cells were incubated for another 10 days. To determine the clonogenic survival rate, cultures were first fixed with paraformaldehyde, and then stained with trypan blue. Colonies with greater than 50 cells were counted under the microscope, and the survival fractions (SF) were calculated using the formula SF = colonies counted/cells seeded.

Cytotoxicity in CSC-enriched 3D tumorsphere cells

Tumorspheres that were developed from 5000 CSC in 24-well ultralow attachment plates were treated with 5 different groups as mentioned above. After 5 days of incubation, take pictures of the tumor sphere and count the number.

***In vivo* tumor targeting study**

Female Balb/c aged 5-6 week were purchased from Vital River Company (Beijing, China). Balb/c mice were subcutaneous injected with 5×10^6 4T1 cells into the right flank (primary tumors). The mice were divided randomly into 2 different groups (each group included 3 mice): (1) DL; (2) DFL. When tumors reached 300 mm^2, tumor bearing mice received an intravenous injection (i.v.) of 100 μL PBS containing DL or DFL (with DACNPY+ dose of 1 mg/kg). At various time points after the injection (i.e. 6, 12 and 24 h), the mice were anesthetized for fluorescence imaging analysis by using IVIS system. 24 h after injection, the tumor tissues and major organs of the mice were dissected for fluorescence imaging analysis by using IVIS system.

***In vivo* anti-tumor therapy**

Female BALB/c aged 5-6 week were purchased from Vital River Company (Beijing, China). Balb/c mice were subcutaneous injected with 5×10^6 4T1 cells into the right

flank on the -14 days. The mice were divided randomly into 5 different groups (Each group included 5 mice): (1) PBS+L (660 nm laser, 0.5 W/cm^2, 10 min); (2) RT (4 Gy); (3) DFL+L; (4) DL+L+RT and (5) DFL+L+RT. The DACNPY+ dose was 5 mg/kg. The PDT and RT were conducted on the 1st and 2nd day. Mice body weight and tumor volume in all groups were monitored every 3 days. A caliper was employed to measure the tumor length and tumor width, and the tumor volume was calculated according to following formula. Tumor volume = tumor length × tumor width^2 / 2. After 21 days of treatment, mice were sacrificed. The tumor tissues were weighed, and fixed in 4% neutral buffered formalin, processed routinely into paraffin, and sectioned at 4 μm. Then the tumor sections were stained with H&E staining, TUNEL, anti-CD133 antibodies and fluorescence labeled secondary antibody and finally examined by using fluorescence microscope (IX81, Olympus, Japan).

Statistical analysis

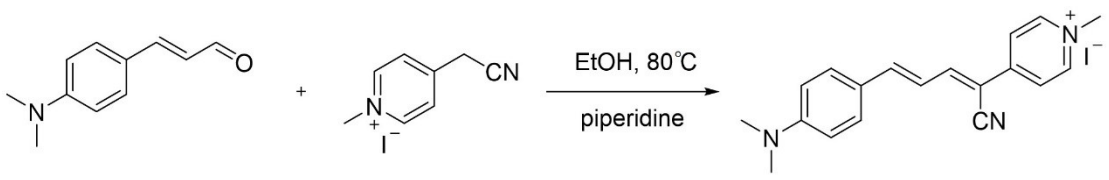
Data analyses were conducted using the GraphPad Prism 5.0 software. For variance analysis, One-way analysis of variance (ANOVA) with Tukey's post hoc test was used. p values of < 0.05 were considered significant. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and ****$p < 0.0001$.

References

[1] X. Xie, K. Jiang, B. Li, S. Hou, H. Tang, B. Shao, Y. Ping, Q. Zhang, *Biomaterials* **2022**, 121598.

[2] D. M. Zhu, W. Xie, Y. S. Xiao, M. Suo, M. H. Zan, Q. Q. Liao, X. J. Hu, L. B. Chen, B. Chen, W. T. Wu, L. W. Ji, H. M. Huang, S. S. Guo, X. Z. Zhao, Q. Y. Liu, W. Liu, *Nanotechnology* **2018**, *29*, 084002; bY. Chen, G. Zhao, S. Wang, Y. He, S. Han, C. Du, S. Li, Z. Fan, C. Wang, J. Wang, *Biomater Sci* **2019**, *7*, 3450-3459.

[3] D. Zhu, M. Lyu, Q. Huang, M. Suo, Y. Liu, W. Jiang, Y. Duo, K. Fan, *ACS applied materials & interfaces* **2020**, *12*, 36928-36937.



Scheme S1. Synthetic route to DACNPy+.

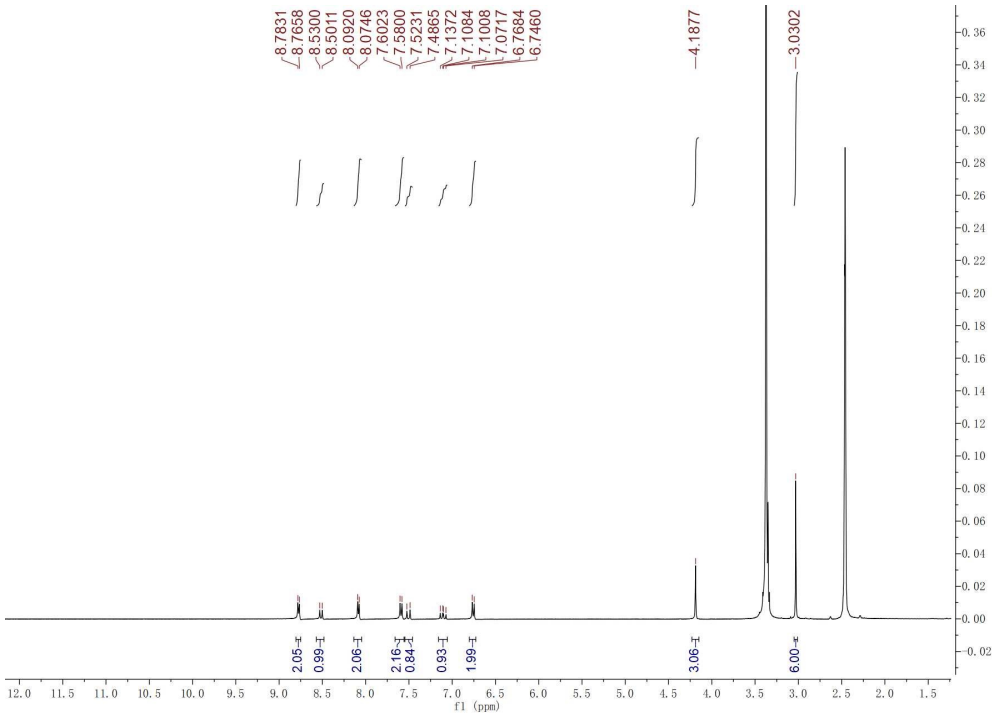


Fig. S1. ^{1}H NMR spectrum of DACNPy+.

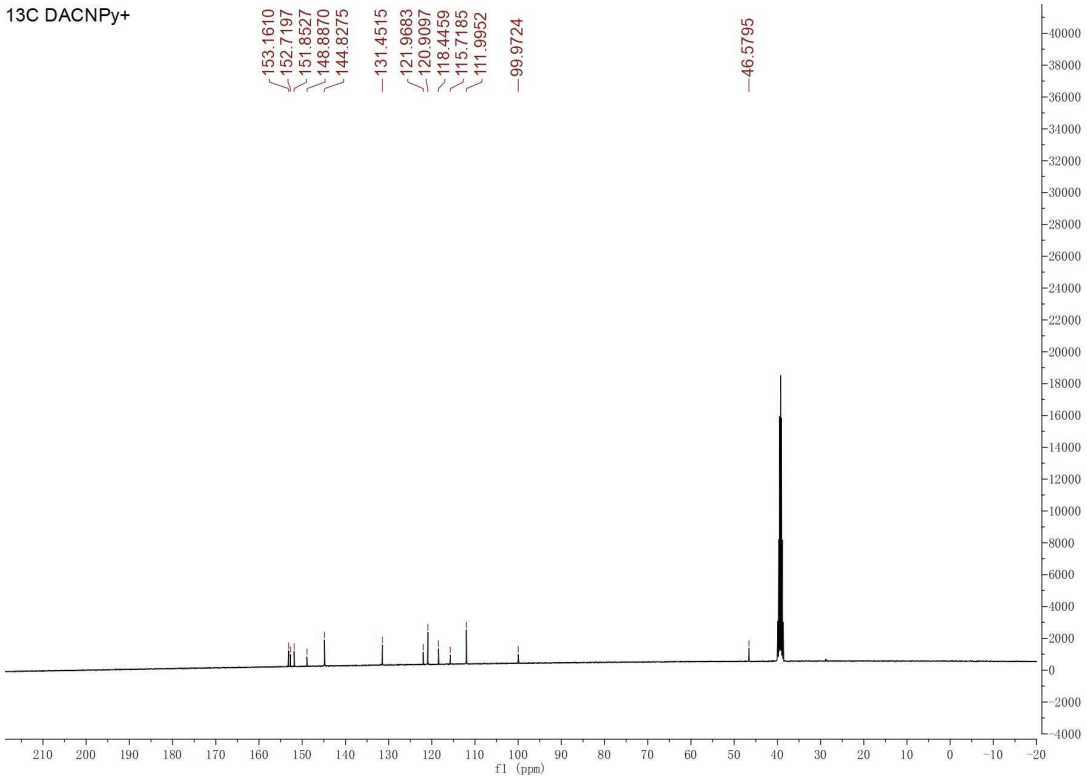


Fig. S2. ^{13}C NMR spectrum of DACNPy+.

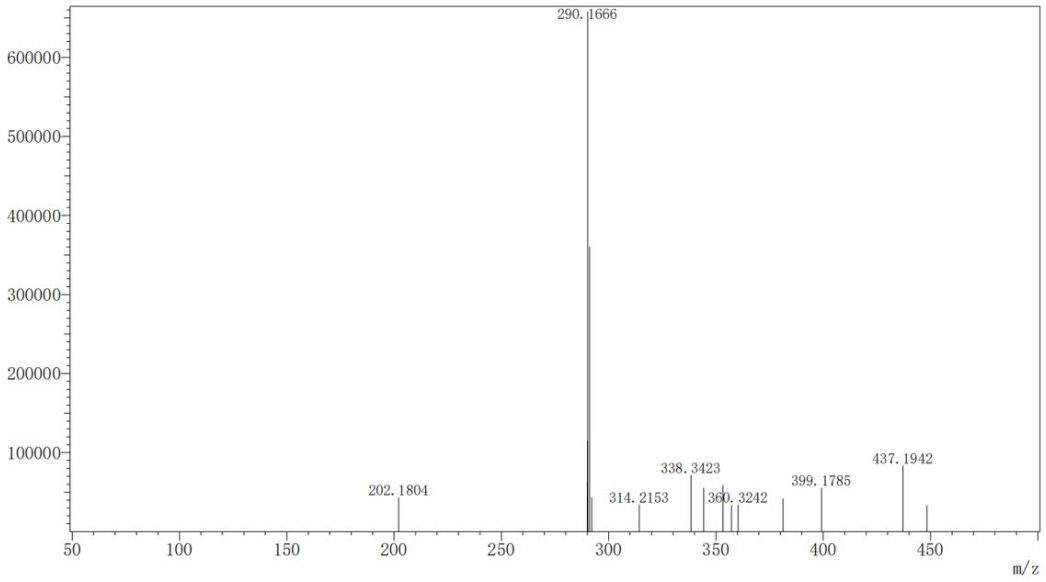


Fig. S3. HRMS spectrum of DACNPy+.

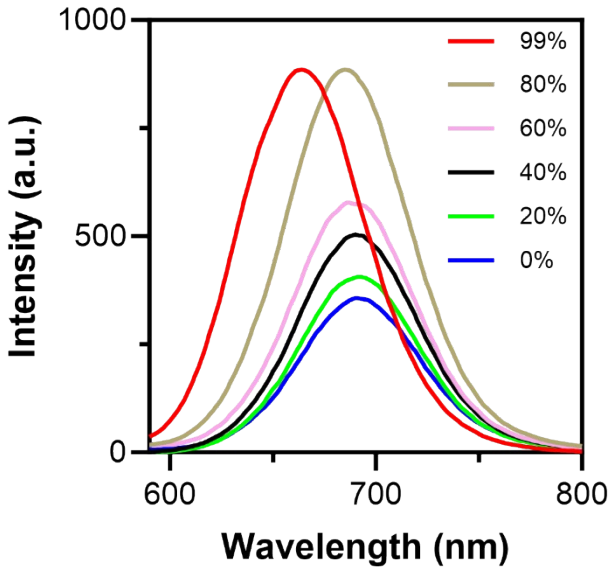


Fig. S4. Fluorescence emission spectra of DACNPy+ (10 μM) with different toluene fractions in DMSO solution (f_t).

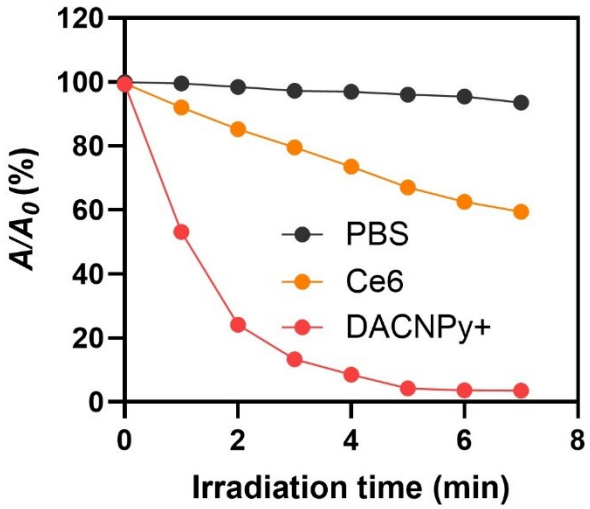


Fig. S5. Plot of A/A_0 against light exposure time, where A_0 and A are the ABDA absorbance (378 nm) before and after irradiation of various photosensitizers, respectively.

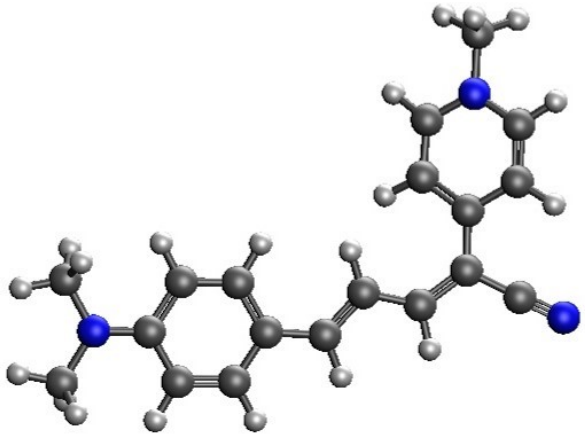


Fig. S6. The optimized configuration of DACNPy+.

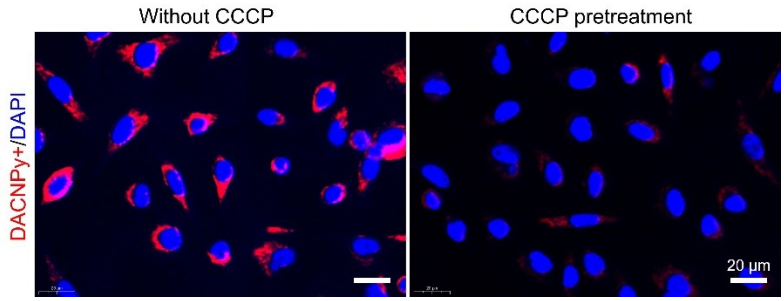


Fig. S7. 4T1 cells stained with DACNPy+ with or without pre-treated with 20 μM Carbonyl Cyanide 3-ChloroPhenylhydrazone (CCCP). Excitation wavelength: 488 nm (for DACNPy+) and 405 nm (for DAPI).

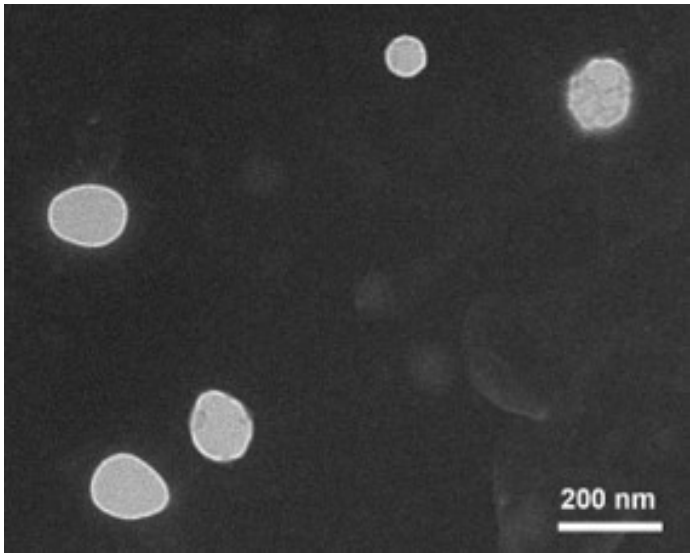


Fig. S8. TEM images of DL nanoparticles. Scale bar = 200 nm.

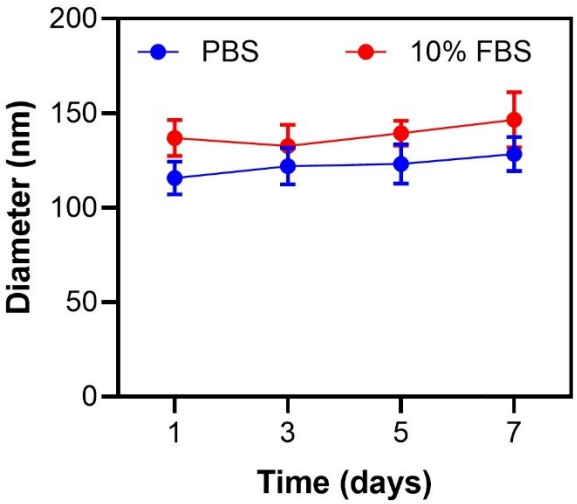


Fig. S9. The hydrated particle sizes of DFL under different days of incubation in PBS or 10% FBS.

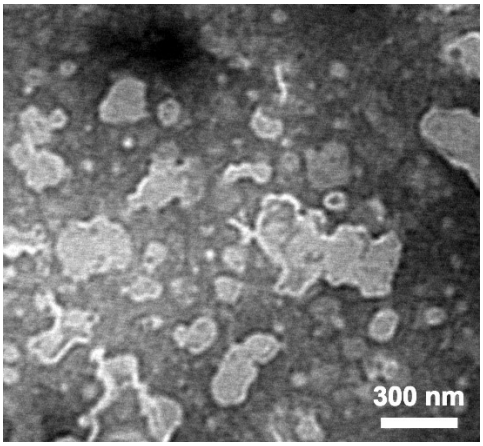


Fig. S10. TEM images of DFL upon 5 min light irradiation.

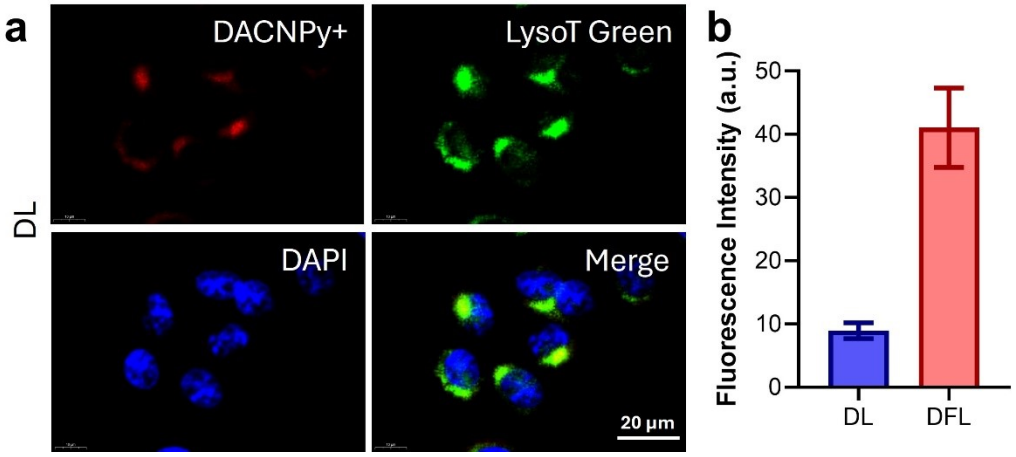


Fig. S11. (a) Confocal images of 4T1 cells co-stained with DL and Lyso-Tracker Green. The nucleus was then stained with DAPI after cell fixation. Scale bar = 20 μm. (b) DACNPy+ Fluorescent intensity in confocal images after incubating 4T1 cells with DL and DFL, respectively.

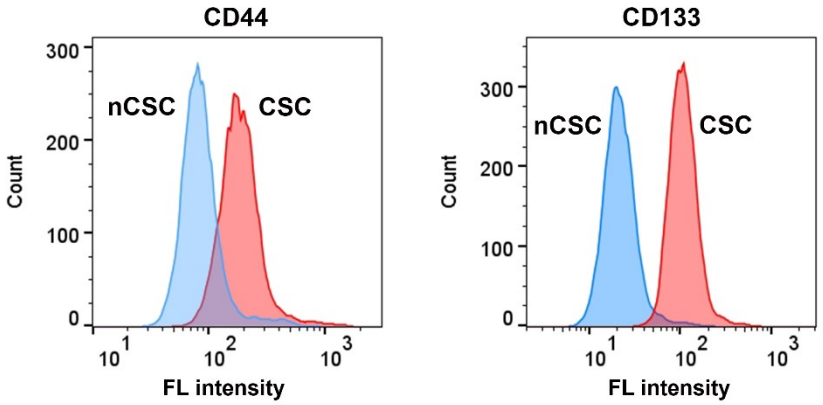


Fig. S12. CD133 and CD44 expression of CSC and non-CSC (nCSC) by flow cytometry.

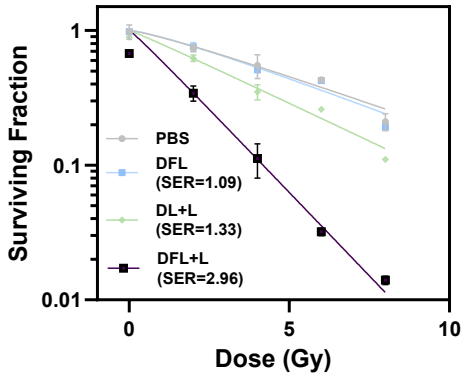


Fig. S13. Survival curves of 4T1 cancer cells pretreated with different radiosensitizers.

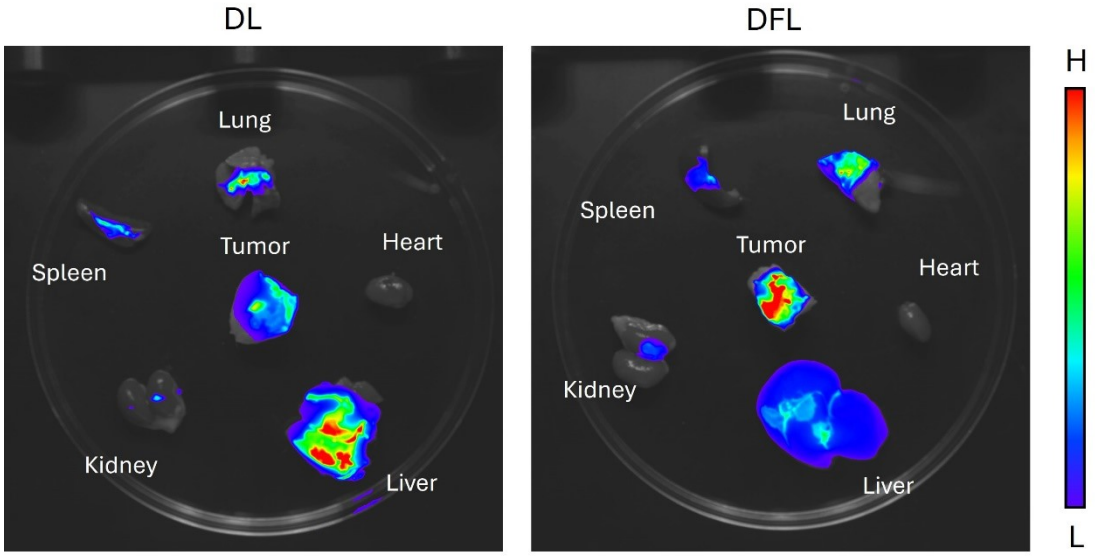


Fig. S14. Fluorescence images of ex vivo tumor and main organs collected from sacrificed mice after injecting DL or DFL for 24 h.

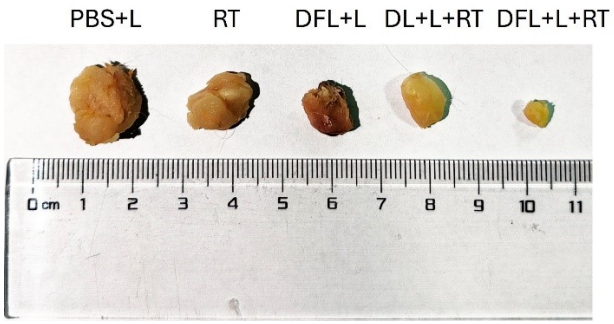


Fig. S15. The representative tumor images after various treatments.

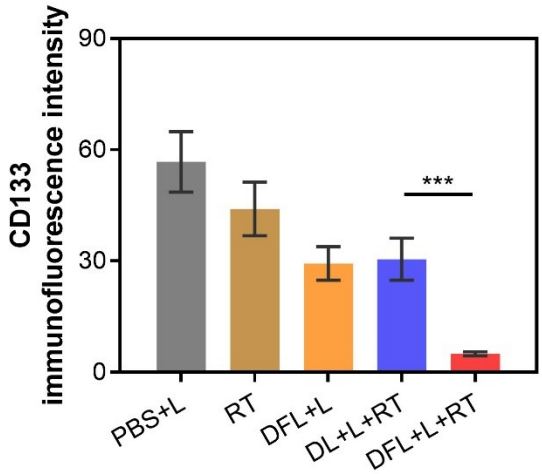


Fig. S16. Quantitative statistics of CD133 immunofluorescence staining results in Fig. 6. ***$p < 0.001$, the data are represented as means ± SD; n = 3.

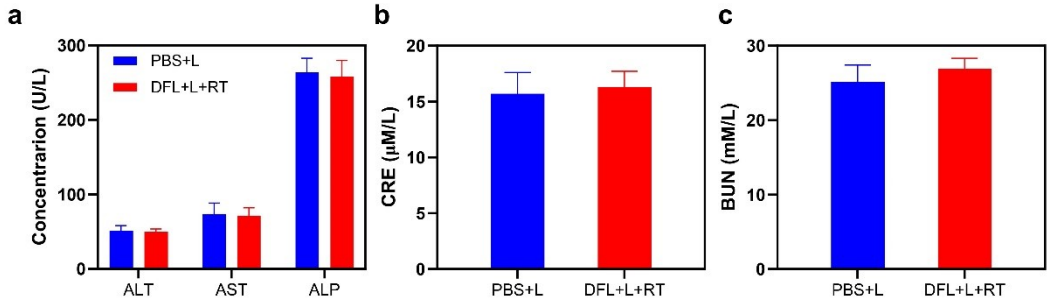


Fig. S17. Routine blood indexes of heathy mice after different treatments.

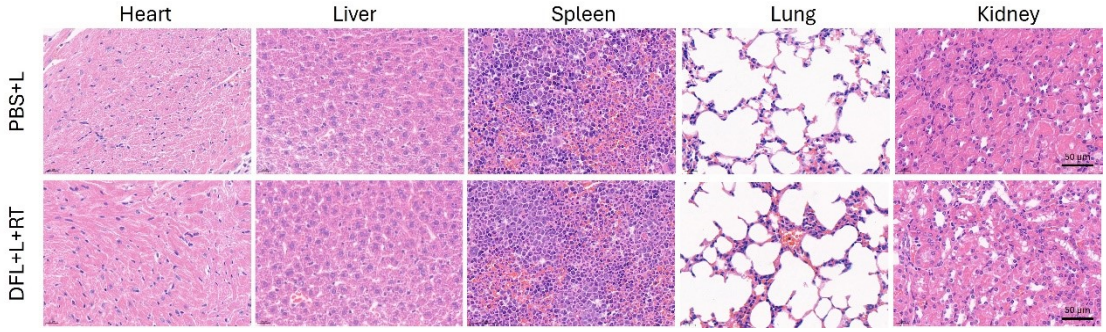


Fig. S18. H&E staining images of major organ slices after different treatments. Scale bar = 50 μm.