Supporting Information

Liposomal cyanine dyes with enhanced nonradiative transition for synergistic phototherapy of tumor

Yuan Wang,[‡a] Di Liu,[‡a] Meng You,[a] Hong Yang,*[a] and Hengte Ke*[a]

[a] Jiangsu Key Laboratory of Neuropsychiatric Diseases, College of Pharmaceutical Sciences, Soochow University, Suzhou 215123, China.

*Corresponding authors.

E-mail: yanghong@suda.edu.cn (H. Yang); htke@suda.edu.cn (H. Ke).

‡These authors contributed equally.

Experimental Section

Materials

DSPC (1,2-dioctadecanoyl-sn-glycero-3-phophocholine) was obtained from Aikang Fine Chemical Company (Shanghai, China). Cholesterol was purchased from Yuanju Bioscience Technology Limited Company (Shanghai, China). Cypate was synthesized as reported in the references.[1, 2] Hoechst 33342 and Lysotracker Green DND-26 were purchased from Invitrogen. AO was purchased from Amresco. Decylamine were obtained from Sigma-Aldrich.

Preparation and Characterization

20 mg lipid, 5 mg cholesterol and 2 mg Cypate were mixed at the ratio of 10:2.5:1 in 0.4 mL organic solvent consisted of 0.3 mL absolute ethyl alcohol and 0.1 mL ethyl acetate. Then, the mixture was stirred with the speed of 1250 rpm for 5 min at 50°C, making lipid and drug fully dissolved. Under the condition of stirring and ultra-sonication, the mixture was dispersed into 10 mL distilled water for emulsification. Subsequently, liposomes loading Cypate (Lipo-Cy) were prepared by volatilizing organic solvent with the speed of 1250 rpm for 24 h at 37°C. The solution of free Cypate was prepared by dissolving Cypate in 5% DMSO.

The entrapment efficiency of Lipo-Cy was determined by ultrafiltration method. 1.0 mL Lipo-Cy was centrifuged with 5000 r min^{-1} at 4°C for 1 h by an ultrafiltration tube (Mw = 100000). Then, the upper liquid in ultrafiltration tube was collected and diluted with methanol to measure the 785 nm absorbance (A) using UV-vis Spectrophotometer (UV2600, Shimadzu). Another Lipo-Cy of the same volume was directly diluted with

methanol to measure the 785 nm absorbance (A_0) using UV-vis Spectrophotometer (UV2600, Shimadzu). Then, the encapsulation efficiency (EE) was calculated using the formula EE (%) = $A/A_0 \times 100\%$.

The hydrodynamic diameter and zeta potential of Lipo-Cy were measured using dynamic light scattering (DLS, NICOMP 380zls, UK) at 25°C. The morphology of Lipo-Cy was observed using Transmission electron microscope (TEM, Tecnai-G20). Next, the entrapment efficiency of Cypate within liposomes was evaluated using ultrafilter centrifuge with 100 kDa membrane filters. The absorbance spectrums of Lipo-Cy and Cypate were measured using UV-vis Spectrophotometer (UV2600, Shimadzu).

Stability

To investigating the chemical stability, the Lipo-Cy and free Cypate (0.75 $mg \cdot mL^{-1}$ Cypate, each 20 μL) were dispersed respectively into 3 mL pH5.0 buffer, pH7.4 buffer, media and serum, in which methanol was used to help Cypate to disperse in aqueous solvent. The absorbance was measured using UV-vis spectrophotometer (UV2600, Shimadzu) at 0, 4, 8, 24, and 48 h, respectively.

To investigating the physicochemical stability, the Lipo-Cy and free Cypate at 10 μg mL^{-1} Cypate were irradiated at 785 nm (1.5 W cm^{-2}) for different time, followed by the measurement of absorption using UV-vis spectrophotometer (UV2600, Shimadzu).

To investigating the thermal stability, the Lipo-Cy and free Cypate at 10 μg mL^{-1} Cypate were irradiated at 785 nm (1.5 W cm^{-2}) for 5 min, followed by the removal of light exposure for cooling down to room temperature. Subsequently, additional four

irradiation/cooling procedures were repeated. The temperature was detected using a thermometer.

Photothermal Effect and Singlet Oxygen Quantum Yield

To explore the photothermal effect, Lipo-Cy and free Cypate at the concentration of 2.0, 5.0 and 10.0 μg·mL^{-1} Cypate were separately irradiated at 785 nm (1.5 W·cm^{-2}, FS-Optics, China) for 300 s. Meanwhile, the temperature of the solution was measured using a thermometer at an interval of 25 s during irradiation.

For singlet oxygen quantum yield (Φ_Δ) measurement, different formulations including free Cypate and Lipo-Cy were evaluated using 1,3-disphenylisobenzofuran (DPBF) as a chemical quencher and free ICG as a reference compound (Φ_Δ^{ICG}=0.14). The solutions of ICG (1.0 μg mL^{-1}), Cypate (1.0 μg mL^{-1}) and Lipo-Cy (1.0 μg mL^{-1}) containing DPBF (30 μM) were irradiated using 785 nm laser at 1.5 W cm^{-2}. Each sample was irradiated for 300 s and the absorbance of DPBF at 415 nm was measured every 20 s. The values of Φ_Δ were calculated using the following relationship $\Phi_\Delta=\Phi_\Delta^{ICG}\cdot W\cdot I^{ICG}/(W^{ICG}\cdot I)$, where W and W^{ICG} are the DPBF photobleaching rates in the presence of Cypate and ICG, respectively. I and I^{ICG} are the rates of light absorption by Cypate and ICG, respectively.

Fluorescent Emission Spectra and Quantum Yield

Fluorescent spectrum of Cypate in DMSO and Lipo-Cy in water was collected by a LS 55 fluorescence spectrophotometer (Perkin Elmer) under 785 nm excitation. Fluorescent quantum yield (Φ_F) was measured using indocyanine green (ICG) in DMSO as reference (Φ_F = 10.6%). Cypate in DMSO and Lipo-Cy in water were excited

at 785 nm, respectively, and then their fluorescent emission spectra were obtained, followed with integration analysis of emission intensity for fluorescence quantum yield calculation.

Cellular Uptake and Endocytic Pathways

4T1 cells were seeded on the six-well plates (10^6 cells/well) and incubated overnight in RPMI 1640 containing 10% FBS. Lipo-Cy and free Cypate at the dose of 10.0 μg·mL^{-1} were added into the wells, respectively. After 6 and 24 h incubation, the medium was removed and the cells were washed three times with PBS. The cells were then incubated with 0.5 mL trypsin (Sigma-Aldrich, USA) for 3 min at 37°C, and collected for cell counting through the centrifuge, followed by ultrasonication. Then, Cypate from the cells was extracted using methanol. Finally, the concentrations of Cypate were measured using UV-vis assay.

To explore endocytic pathways, the 4T1 cells were seeded in 6-well plates (10^6 cells/well) for 24 h incubation. Then, the cells were treated with PBS (control) and various inhibitors including chlorpromazine (inhibitor of clathrin-mediated uptake, 10 μg·mL^{-1}), nystatin (inhibitor of caveolae-mediated uptake, 5 μg·mL^{-1}), and amiloride (inhibitor of macropinocytosis, 100 μg·mL^{-1}) in serum-free RPMI 1640 medium for 2 h at 37°C or 4°C, respectively. Then, Lipo-Cy (10 μg·mL^{-1} Cypate) were further added for 1 h incubation. Subsequently, the cells were washed 3 times using PBS, treated with trypsin for 3 min at 37°C, centrifuged at 4°C, and finally suspended in 0.5 mL PBS. The amount of Cypate was measured by using UV-vis assay after cell counting and extraction, respectively.

Subcellular Distribution

Confocal laser scanning microscopy (CLSM) was applied to observe the intracellular distribution of Lipo-Cy after cellular uptake by 4T1 cells. Firstly, 4T1 cells were seeded on glass dishes (10^6 cells/well) for 12 h cultivation. Then, the cells were treated with Lipo-Cy (30 μg·mL^{-1} Cypate) for 2 h incubation at 37°C, and further subjected to the presence or absence of 3 min irradiation (785 nm, 1.5 W cm^{-2}). Afterwards, the cells were washed using PBS, followed by incubating with 1.5 mL Hoechst 33342 (10 μg·mL^{-1}) for 10 min and 1.5 mL Lysotracker Green DND-26 (100 nM) for another 3 min incubation at 37°C. Finally, the cells were rinsed with PBS for 3 times and observed using CLSM (Zeiss LSM650).

Intracellular Singlet Oxygen and Lysosomal Disruption

Dihydroethidium (DHE) and acridine orange (AO) were employed as intracellular indicator of singlet oxygen and acidic lysosomes, respectively. Firstly, 4T1 cells were seeded on glass dishes overnight and treated with PBS (control) and Lipo-Cy at the doses of 0.5, 1.0 and 2.0 μg·mL^{-1} for 6 h, respectively. Then, the cells were incubated in fresh medium, followed by 3 min irradiation (785 nm, 1.5 W cm^{-2}) or not. After 1 h incubation at 37°C, the cells were washed with PBS and further incubated with DHE (5.0 μM) for 30 min or AO (100 μg·mL^{-1}) for 20 min. Finally, the cells were observed through CLSM (Zeiss LSM650) after washing.

Cellular Cytotoxicity

To evaluate cellular cytotoxicity, 4T1 cells were incubated with Lipo-Cy or free Cypate at the doses of 1.0, 2.0, 5.0, 10.0 and 25.0 μg·mL^{-1} for 24 h, respectively. Then, the

cells were washed using PBS, followed by irradiation for 3 min (785nm, 1.5 W cm^{-2}) or not. After 24 h, the cell viability was evaluated using MTT assay.

Biodistribution, *in vivo* NIRF Imaging and Infrared Thermography

Balb/c mice bearing subcutaneous 4T1 tumors were intravenously injected with Lipo-Cy or free Cypate at the dose of 7.5 mg·kg^{-1} Cypate. 24 h latter, the major tissues including heart, liver, spleen, lung, kidney and tumor were extracted from the mice and imaged using IVIS Lumina II with the excitation wavelength of 745 nm. The NIRF signals of Cypate in the tissues were quantified to describe the *ex vivo* biodistribution at 24 h post-injection.

To study *in vivo* NIRF imaging, Lipo-Cy and free Cypate were intravenously injected into Balb/c nude mice bearing 4T1 tumors at the dose of 5.0 mg kg^{-1}, respectively. Then, the mice were imaged using IVIS Lumina II under 745 nm excitation at 24, 48, 72, and 96 h post-injection. The average NIRF intensity at tumor was calculated at different time.

To monitor the *in vivo* photothermal capacity, Lipo-Cy was administrated to the tumor-bearing mice via intravenous injection at various dose including 0.5, 1.5, 3.0, 4.0 and 5.0 mg kg^{-1}. And then the tumors were irradiated for 5 min (785 nm, 1.5 W cm^{-2}) at 24 h post-injection. Simultaneously, the infrared camera (FLIR E50) was applied to observe the temperature at the tumor during 5 min irradiation.

Anticancer Efficacy and Histological Staining

To investigate the antitumor efficacy, the tumor-bearing mice were intravenously injected at the doses of 0.5, 1.5, 3.0, 4.0 and 5.0 mg kg^{-1} Cypate or Lipo-Cy when the

tumor volume reached ~75 mm^3. Subsequently, the tumors were irradiated at 1.5 W cm^{-2} (785 nm) for 3 min after 24 h. Meanwhile, the tumor volumes were monitored during 22 days post-irradiation according to the equation of $V = L \times W^2/2$, where W is the tumor measurement at the widest point, and L is the tumor dimension at the longest point. The relative tumor volume was applied to present the antitumor efficacy.

To further validate the photothermal damage of Lipo-Cy *in vivo*, their histological staining of tumors was carried out. The female Balb/c mice bearing 4T1 tumors were injected intravenously with PBS, free Cypate and Lipo-Cy at the dose of 5.0 mg kg^{-1}, respectively, followed by irradiation at the tumors for 3 min at 24 h post-injection (785 nm, 1.5W cm^{-2}). Then the major tissues including heart, liver, spleen, lung, kidney and tumor were harvested at 6 h after PTT treatment. The various tissues were fixed with 4% formaldehyde solution overnight, and further frozen and sectioned with the thickness of 10 μm. Finally, haematoxylin & eosin (H&E) staining was conducted and the sections were observed using an IX73 bright field microscopy (Olympus).

Figures and Tables

Table S1. Encapsulation efficiency of Lipo-Cy with different drug-to-lipid ratio.

m_{Cypate}: m_{Lipid}	EE (%)
1:5	61.75 ± 7.54
1:10	98.11 ± 1.13
1:20	83.24 ± 3.41

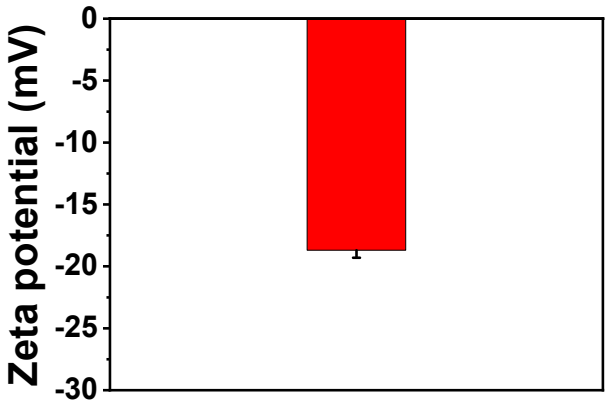

Figure S1. The zeta potential of Lipo-Cy.

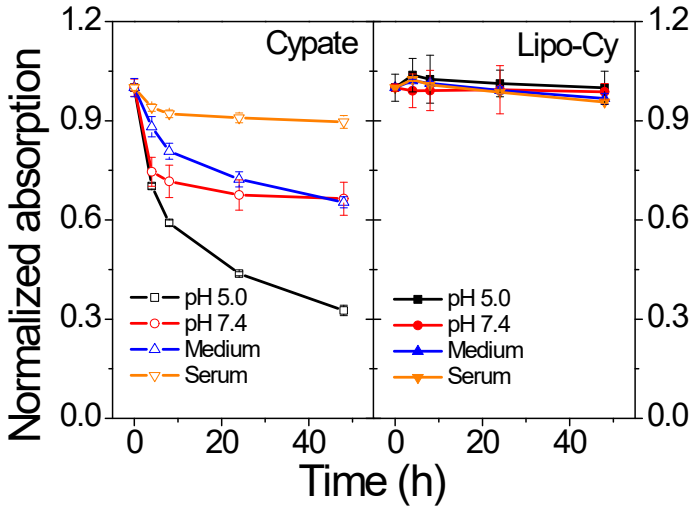

Figure S2. Normalized absorbance of Cypate and Lipo-Cy in aqueous solution (pH5.0 or pH7.4), medium, or serum at different time.

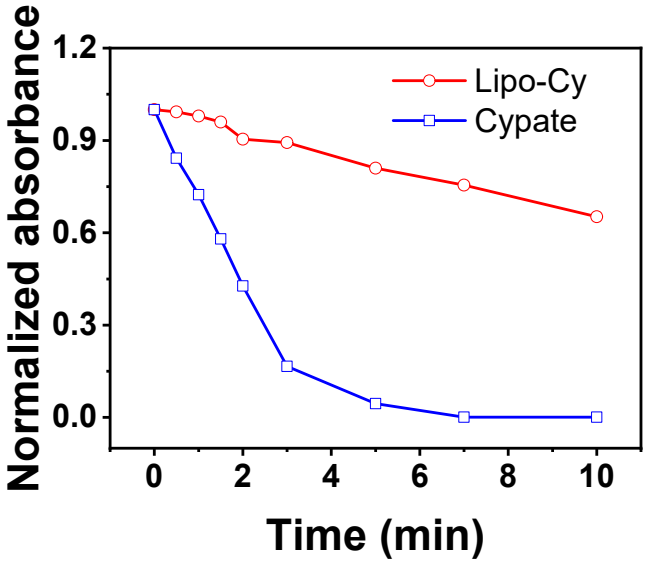

Figure S3. UV-vis absorption of Cypate and Lipo-Cy in water under 785 nm irradiation (1.5 W cm^{-2}) for 10 min.

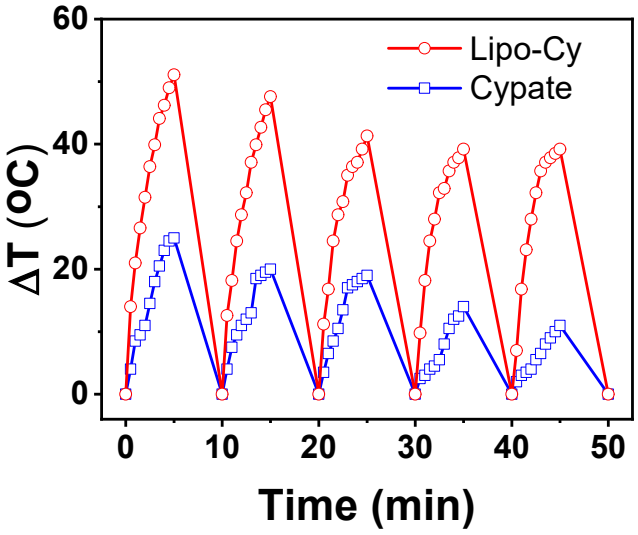

Figure S4. Temperature elevation of Cypate and Lipo-Cy under five irradiation/cooling cycles.

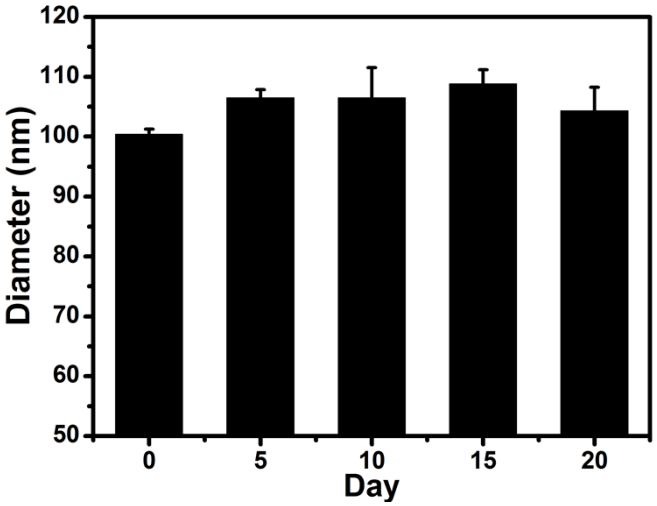

Figure S5. Size change of the Lipo-Cy in aqueous solution at different time.

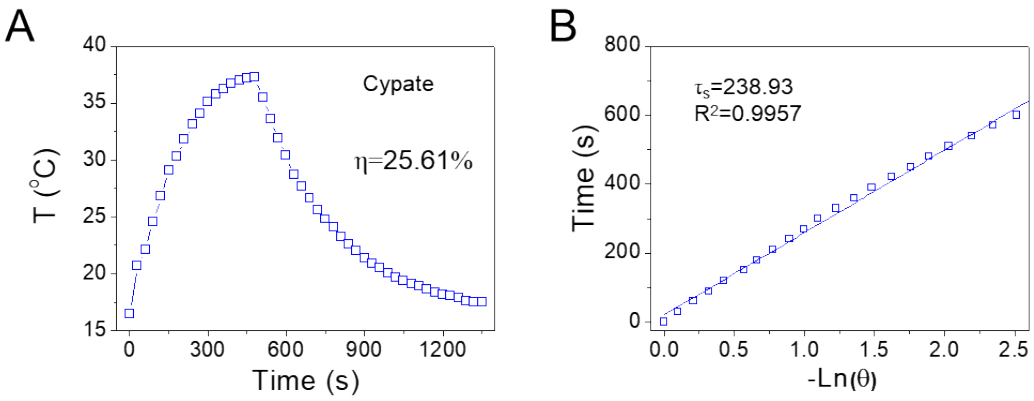

Figure S6. (A) Photothermal conversion efficiency of Cypate under irradiation (785 nm, 1.5 W cm^{-2}), followed by subsequent cooling to room temperature; (B) Time constant (τ_s = 238.93 s) for calculating photothermal conversion efficiency by applying the linear time data from the cooling period versus negative natural logarithm of driving force temperature.

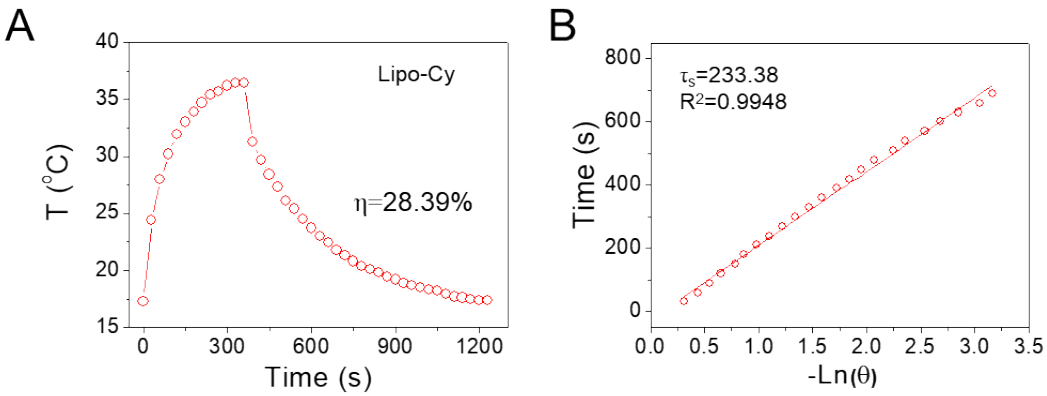

Figure S7. (A) Photothermal conversion efficiency of Lipo-Cy under irradiation (785 nm, 1.5 W cm^{-2}), followed by subsequent cooling to room temperature; (B) Time constant (τ_s = 233.38 s) for calculating photothermal conversion efficiency by applying the linear time data from the cooling period versus negative natural logarithm of driving force temperature.

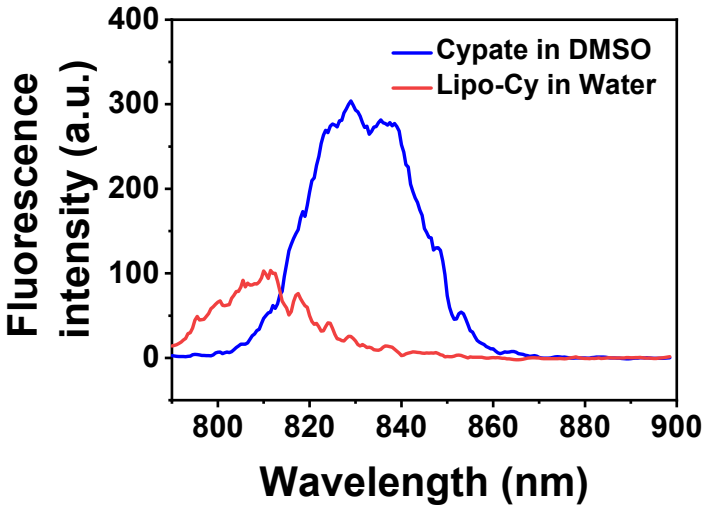

Figure S8. Fluorescent emission spectra of Cypate in DMSO and Lipo-Cy in water under 785 nm irradiation.

Table S2. Fluorescence quantum yield (Φ_F) of Cypate in different formulations

Formulation	Φ_F
Cypate in DMSO	(8.54 ± 1.14) %
Lipo-Cy in water	(0.35 ± 0.02) %

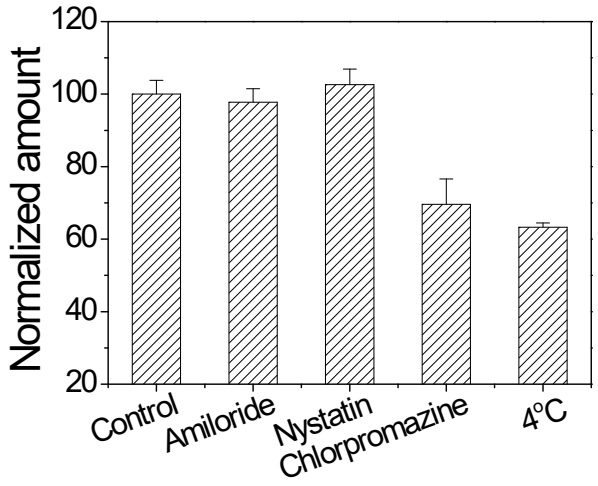

Figure S9. Normalized amount of Lipo-Cy internalized by 4T1 cells treated with PBS, chlorpromazine, nystatin, amitoride at 37°C, and PBS at 4°C, respectively.

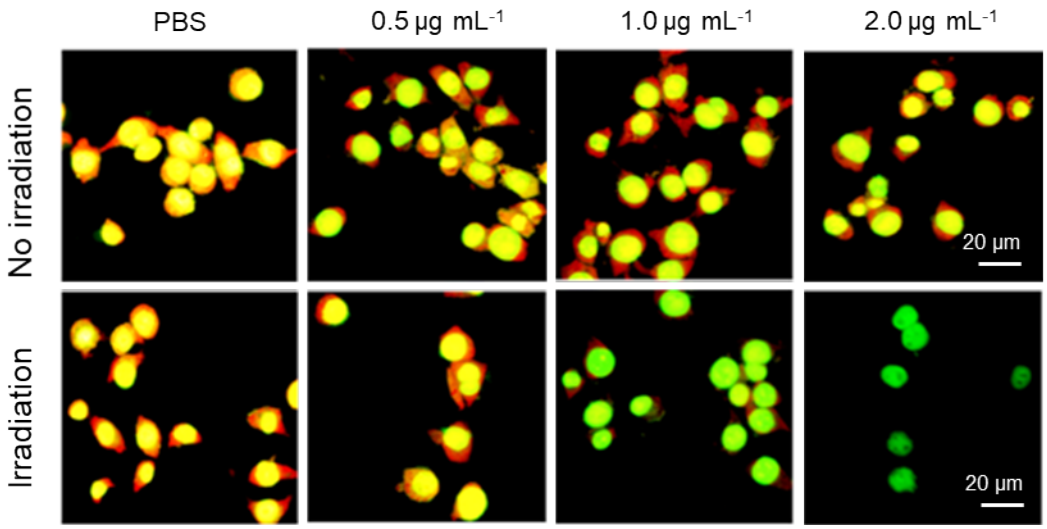

Figure S10. AO staining of 4T1 cells treated with PBS and Lipo-Cy under irradiation or not (785 nm, 1.5 W cm^{-2}, 3 min), respectively.

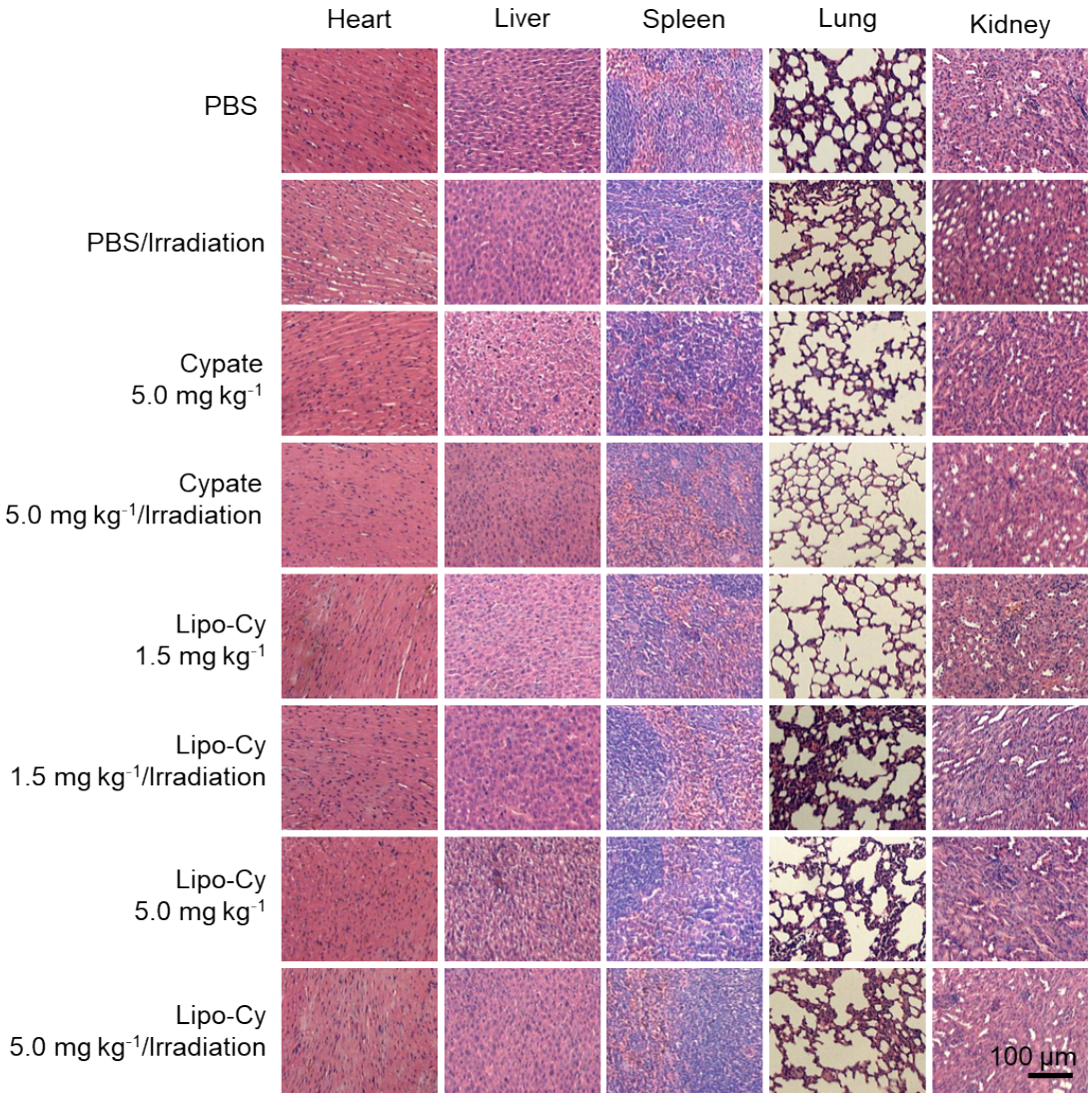

Figure S11. H&E staining of normal organs harvested from mice with different treatments at 6 h post-irradiation of tumors.

References

1. Y. Ye, S. Bloch, J. Kao and S. Achilefu, *Bioconjug Chem*, 2005, **16**, 51-61.

2. Y. Ye, W. P. Li, C. J. Anderson, J. Kao, G. V. Nikiforovich and S. Achilefu, *JACS*, 2003, **125**, 7766-7767.