Iodinated-Cyanine Dyes for Fast NIR-guided Deep-Tissue Synergistic Phototherapy

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Materials and Methods

Materials

Chemicals were purchased from commercial sources at the highest possible purity and used as received. Cypate (MW 627.32), an ICG derivative, was synthesized in our laboratory. NIR dye Cy7 (MW 682.2), Singlet Oxygen Sensor Green (SOSG), 1,3-diphenylisobenzofuran (DPBF), methylene blue (MB), Methyl thiazolyltetrazolium (MTT), Dulbecco's Modified Eagle Medium (DMEM, Sigma, USA), 2',7'-dichlorodihy drofluorescein diacetate (DCFH-DA), Calcein AM/PI assay kit, Annexin V-FITC/PI apoptosis staining kit, fetal bovine serum, penicillin–streptomycin were purchased from Solarbio (Beijing, China).

Normal cell lines (Osteoblast precursor cells MC3T3; human liver normal cells L-02) and human liver cancer cell lines (HepG2) were purchased from American Type Culture Collection. The Kunming mice and athymic nude mice (nu/nu CD-1) (half male and half female) were obtained from Daren Laboratory Animal Co. Ltd (Qingdao, China).

Synthesis of *methyl 3-iodopropanoate (c)*: Using a modified Finkelstein procedure [1], methyl 3-iodopropionate (c) was prepared from methyl 3-bromopropionate (b) and potassium iodide in acetone. In brief, to a solution of 3-bromopropionic acid (a, 30g, 0.2mol) in MeOH was added $SOCl_2$ (30ml), and the mixture refluxed for 6 h under continuous stirring. The solvent was removed under reduced pressure to give product methyl 3-bromopropanoate (b, 20g, 59.9%). The suspension of b (20g, 0.12 mol), KI (40g, 0.24 mol) in acetone was stirred for 12 h and the solid was removed by filtration and the solution was concentrated in vacuum to afford c (25g, 97.4 %).^{1}HNMR (400 MHz, $CDCl_3$) *d* 3.73 (s, 3H, OCH_3), 3.33 (s, 2H, *J* = 8 Hz, ICH_2), 2.99 (s, 2H, *J* = 4 Hz, CH_2CO_2). ESMS (M+H) found = 214.85, calculated for $C_4H_7IO_2$ = 214.

Synthesis of *5-iodo-2,3,3-trimethyl-3H-indole (f)*: The approach of f was described as previously reported [2]. In brief, 4-iodoaniline (**d**, 20g, 91.3 mmol) was stirred with 15 ml concentrated hydrochloric acid and 15 ml water. The mixture was cooled to -10°C and 45 ml $NaNO_2$ (12.6g, 182.6 mmol) aqueous solution was added with continuous stirring. The suspension stirred for another 30 minutes and then an ice-cold solution of $SnCl_2{\cdot}2H_2O$ (67.99g, 301.3 mmol, in 40 ml of concentrated HCl) was added dropwise. The mixture was stirred at -10°C for 1.5 h and at 5°C overnight. The obtained light brown precipitate was filtered and washed with water, and then stirred with saturated solution of NaOH in water (100 ml) and extracted with ether (200 ml). The ether layer was washed with aqueous solution of NaOH, $Na_2S_2O_3$ and water. After drying with $MgSO_4$

(anhydrous), the ether layer was evaporated to dryness to afford (4-iodophenyl)hydrazine (d, 17.94 g, 83.9 %) as brown powder. Then, a mixture of e (10g, 42.7 mmol) and 3-methyl-2-butanone (6.2 g, 71.98 mmol) was dissolved in 250 ml glacial acetic acid, and refluxed for 8 h under nitrogen. The solvent was evaporated in vacuo. The residue was purified by column chromatography to give product f (6g, 49.3 %) as red oil. ^{1}H NMR ($CDCl_3$): δ 7.60-7.64 (m, 2H, Ar-CH), 7.29 (d, *J* = 8.0 Hz, 1H, Ar-CH), 2.26 (s, 3H, CH_3), 1.30 (s, 6H, CH_3◊2). ESI found = 286.1, calculated for $C_{11}H_{12}IN$ = 285.12.

Synthesis of *5-iodo-1-(3-methoxy-3-oxopropyl)-2,3,3-trimethyl-3H-indol-1-ium (g)*: The mixture of f (3g, 10.5 mmol), c (6g, 28 mmol) in CH_3CN (20ml) was heated to reflux for 48 h. The solvent was evaporated in vacuo. The residue was purified by column chromatography to give product g (2.5g, 64 %).^{1}H NMR (DMSO): δ 8.30 (s, 1H, Ar-CH), 7.99 (d, *J* = 8.0 Hz, 1H, Ar-CH), 7.80 (d, *J* = 8.0 Hz, 1H, Ar-CH), 4.64 (t, J=8.0 Hz, 2H, $COCH_2$), 3.61 (s, 3H, OCH_3), 3.04 (t, J=8.0 Hz, 2H, NCH_2), 2.83 (s, 3H, CH_3), 1.52 (s, 6H, CH_3◊2). ESI found = 372.0, calculated for $C_{15}H_{19}INO_2$ = 372.2.

Synthesis of 1-(2-carboxyethyl)-5-iodo-2,3,3-trimethyl-3H-indol-1-ium*(h)*: To the solution of g (2.5g, 6.7 mmol) in MeOH (25ml), was added the solution of LiOH (1g in 5ml H_2O). The solution was stirred at 40°C for 8h. After neutralization with concentrated HCl to pH=2, the mixture was concentrated in vacuo to give crude product which was purified by column chromatography to afford h (1g, 41.67 %).^{1}H NMR (DMSO): δ 8.30 (s, 1H, Ar-CH), 7.99 (d, *J* = 8.0 Hz, 1H, Ar-CH), 7.80 (d, *J* = 8.0 Hz, 1H, Ar-CH), 4.61 (t, J=8.0 Hz, 2H, CH_2), 2.94 (t, J=8.0 Hz, 2H, NCH_2), 2.83 (s, 3H, CH_3), 1.52 (s, 6H, CH_3◊2). ESI found = 358.03, calculated for $C_{14}H_{17}INO_2$ = 358.2.

Synthesis of *1-(2-carboxyethyl)-2-((1E,3E,5E)-7-((E)-1-(2-carboxyethyl)-5-iodo-3,3-dimethyl-indolin-2-ylidene)hepta-1,3,5-trien-1-yl)-5-iodo-3,3-dimethyl-3H-indol-1-ium (CyI)*: The mixture of glutacondianil hydrochloride (284mg, 1 mmol) and DIEA (500 mg, 3.9 mmol) in DCM (3 ml) was treated with a solution of acetic anhydride (110mg,1.08mmol) in DCM (1 ml) at 0°C. The rection mixture was stirred at room temperature for 3 hours. The solvent was removed by vacuum rotary evaporation and replaced with ethanol (10 ml). Then, a suspension of the h (640mg, 1.79 mmol) and sodium acetate (730 mg, 8.9 mmol) in ethanol (5ml) was added to the above solution, and the mixture was stirred at 80°C overnight. The resulted solution was concentrated by vacuum rotary evaporation, and purified through column chromatography to afford CyI (200mg, 25.7 %) as a green solid. ^{1}H NMR (CD_3OD, 400 MHz) δ:7.92(m, 2H), 7.79(s, 2H), 7.70 (d, J=8.0Hz, 2H), 7.58(s, 1H), 7.13 (d, J=8.0Hz, 2H), 6.58(t, J=14.0Hz, 2H), 6.35(d, J=12.0Hz, 2H), 4.29(s, 4H), 2.65(s, 4H), 1.66(s, 12H).

Characterization

UV-Vis-NIR and fluorescence spectra of CyI were acquired by Beckman Coulter DU 640 spectrophotometers and Fluorolog-3 fluorometer, respectively. The ^{1}H nuclear magnetic resonance (^{1}H NMR) spectra were obtained on Bruker Avance III HD 400MHz instrument (German) at 25.0 ± 1 °C and processed using MestReNova software. Mass spectra were obtained using a 4800 Plus MALDI-TOF/TOF mass spectrometer (AB Sciex, USA). The fluorescence quantum yields of CyI were calculated as Equation 1:

$$\mathrm{QYu} = \mathrm{QYs} \times \frac{As}{Au} \times \frac{Fu}{Fs},$$

where QYu, QYs are the quantum yield of CyI and Cyp, respectively; As, Au represent for absorbance of Cyp and CyI at the excitation wavelength. Fs, Fu denote the area under the curve (AUC) of Cyp's and CyI's fluorescence spectra.

Singlet oxygen quantum yield (Φ_Δ) of CyI were calculated as Equation 2:

$$\Phi_{\Delta(T)} = \Phi_{\Delta(MB)}(\frac{S_T}{S_{MB}})(\frac{F_{MB}}{F_T}), \text{ [3]}$$

where $\Phi_{\Delta(MB)}$ is the singlet oxygen quantum yield for the standard methylene blue (MB, $\Phi_{\Delta(MB)}$=0.52), S is the absorbance of DPBF at 410 nm in the presence of CyI or MB upon irradiation, F is the absorption correction factor of CyI and MB, respectively. $F=1-10^{-OD}$, where OD represents the absorption value of CyI and MB at irradiation wavelength. "T" and "MB" represent CyI and the standard, respectively.

Singlet oxygen $^1O_2$ measurements.

$^1O_2$ production was evaluated using a specific fluorescence probe SOSG, with maximum excitation wavelength at 504 nm and emission wavelength at 525 nm. In brief, 50 μL NIR dyes (cypate, Cy7 or CyI) (50 μg/ml) in PBS (pH=7.4) were mixed separately with 50 μl SOSG (25 μM) and then irradiated by NIR light (808 nm, 0.3, 0.96 or 1.6 W/cm^2) for 5 min. The samples were analyzed with Fluorolog-3 fluorometer.

Photo-to-photothermal efficacy induced by NIR irradiation.

A continuous wave (CW) 808 nm laser device (CL808-20-F, Photons Co Ltd) and a thermocouple thermometer (TES Electrical Electronic Corp, WRNK-104, Taiwan) were applied to monitor the temperature increase induced by CyI: (1) 100 μL NIR dye (Cypate, Cy7 or CyI) aqueous solution (50μg/ml) was irradiated with the laser diode (808 nm, 0.96 W/cm, 10 min); (2) 100 μL of CyI aqueous solution (50μg/ml) was irradiated under the laser diode in different power density (808 nm, 0.3, 0.96 or 1.6 W/cm^2, 10 min).

The photothermal conversion efficiency (η) of CyI was determined according to previous method [4]. The η value was calculated in Equation 3:

$$\eta = \frac{hA\Delta T_{max} - Q_s}{I\,(1 - 10^{-A_\lambda})}$$

where h represents the heat transfer coefficient, A is the surface area of the container, ΔT_{max} is the temperature change of the CyI solution at the maximum steady-state temperature, I is the laser power, A_λ is the absorbance of CyI at 808 nm, Q_s is the heat associated with the light absorbance of the solvent, which is measured independently using pure water. For measuring the photothermal conversion performance of CyI, 200 μL CyI dissolved in water (50 μg/ml) was exposed an 808 nm laser at 0.96 W/cm^2 to steady-state temperature. The solution temperature was measured at intervals of 10 seconds. Then shut off the laser and let the solution naturally cooled down. The temperature was measured every 10 seconds until the temperature was stable. hA can be calculated by applying linear fit to the plot of time vs -lnθ from the cooling period, where θ is the ratio of ΔT to $\Delta Tmax$ (ΔT denotes change of temperature which equals to T-T_{surr}. T and T_{surr} stand for temperature of the solution and surrounding environment respectively).

Safety evaluation of of CyI

MTT assay

To evaluate the cytotoxicity of CyI, MTT assay was conducted at both normal cell lines (MC3T3

and L-02) and human liver cancer cell lines (HepG2) following the standard protocol. In brief, NIR dyes (Cypate, Cy7 or CyI) with particular concentrations (12.5-200 μM) were incubated at the above cell lines for 48 h and the cell viability was calculated. The experiment was carried out in triplicate.

Pharmacokinetics and biodistribution of CyI

Plasma Kinetics Experiment

To detect the CyI concentration in plasma, CyI (3.0 mg/kg) aqueous solution was injected via tail vein in Kunming mice. After that, at specified time (0.5 h, 1 h, 2 h, 4 h, 8 h, 12 h and 24 h), the blood samples were converged in tubes by enucleated mice eyes. These blood samples were centrifuged at 3000 rpm (4 ºC, 15 min). 40 μL of plasma was extracted with 1 mL methyl tert-butyl ether. After centrifuged at 3000 rpm (4 ºC, 15 min), 600 μL of supernatant was converged and dried at room temperature overnight. The residues were dissolved in methanol (200 μL) for HPLC analysis.

Tissue Distribution Experiment

48 Kunming mice were randomly divided into 6 groups, with 8 mice at each time point (1, 2, 4, 8, 12 and 24 h), half male and half female. CyI aqueous solution (3.0 mg/kg) were administered via tail vein injection. The heart, liver, spleen, lung, kidney, intestine and brain were collected according to the predetermined time points. The tissues were dissolved into cold saline solution and tissue homogenates were obtained by auto homogenizer (SCIENTZ-48, Ningbo Scientz Biotechnology, China). After centrifuged at 3000 rpm for 15 min, the supernatants were collected for HPLC analysis.

Dynamics and biodistribution of CyI using NIR imaging system

To non-invasive evaluate the *in vivo* dynamics and biodistribution of CyI, 0.2 mL CyI aqueous solution was injected into normal nude mice (n=5) via the tail vein and monitored for 24 h using NIR imaging system. The detailed protocol was similar as previously reported [5]. Main organs (heart, kidney, lung, spleen, brain, intestines and liver) were excised from mice for fluorescence imaging after intravenous injection of CyI for 2 h and 12 h, respectively. Data are expressed as mean ± SD (n=5).

Evaluation of synergistic PDT/PTT efficacies of CyI in cancer cells

In vitro ROS detection

To validate photodynamic effect from CyI, in vitro ROS production was detected using a fluorescence probe DCFH-DA. In brief, NIR dyes (Cypate, Cy7 or CyI) (50 μg/ml) was incubated in HepG2 cells and irradiated with NIR light (808 nm, 0.96 W cm^{-2}, 1 min). Then the cells were incubated with fresh media containing H_2DCFDA (10 μM) for 30min. In parallel, the cells without NIR dyes were as control. Single oxygen radicals were immediately measured by confocal microscopy (Nikon A1R MP, Japan).

Cell apoptosis

To evaluate *in vitro* synergistic phototherapeutic efficacy of CyI under NIR irradiation, MTT assay was firstly carried out. Test samples (CyI with different concentrations (10-80 μg/ml), or different dyes (Cypate, Cy7 or CyI, 50 μg/ml) were incubated in HepG2 cells for 4 h. After washing with cold PBS, cells were irradiated with NIR light (808 nm) at designed power density for 1 min. To compare ROS/heat production of CyI in deep tissue, we covered the cell dishes with 1-cm pork tissues and then irradiated with NIR light under the same condition as above. After incubation for another 24 h, cell viability rate was calculated. The experiment was carried out in triplicate.

The synergistic PDT/PTT effects of CyI were further evaluated using Calcein AM and propidium iodide (PI) co-staining. HepG2 cells were incubated with PBS or NIR dyes (Cy7 or CyI,

50 μg/ml) and irradiated as stated above. Then the cells were co-stained with calcein AM and PI for confocal imaging (Ex: 490 nm, 535 nm).

Apoptosis of HepG2 cells was further detected by flow cytometry using an Annexin V-FITC/PI apoptosis staining kit. In brief, CyI with different concentrations were incubated in HepG2 cells for 8 h, and the cells were collected to stain with Hoechst and Annexin V-FITC for cell apoptosis analysis by flow cytometer (Accuri C6, BD Biosciences, USA).

***In vivo* evaluation of synergistic PDT/PTT efficacy of CyI**

Evaluation of tumor growth inhibition

30 Nude mice-bearing HepG2 tumors were divided into 6 groups. The mice in each group were intratumoral administered with different solutions (200 μL) and treated with different therapeutic schedules: (A) Saline without laser irradiation (control group); (B) Saline with laser irradiation (0.96 W/cm^2, 1 min, control group); (C) Cypate under NIR irradiation (0.96 W/cm^2, 1 min, 1.5 mg/kg, PTT group); (D) CyI under NIR irradiation (0.3 W/cm^2, 1 min, 1.5 mg/kg, PDT group); (E) CyI under NIR irradiation (0.96 W/cm^2, 1 min, 1.5 mg/kg, synergistic PDT/PTT group); (F) CyI under NIR irradiation and 1cm tissue (0.96 W/cm^2, 1 min, 1.5 mg/kg, synergistic PDT/PTT in deep tissue). Tumor volume, body weight, and survival rate of mice in each group were recorded every other day. After 20 d treatment, tumors and main organs were collected for histological analysis with H&E staining and TUNEL staining.

Evaluation of PDT efficacy

Intratumoral $^1O_2$ generation was evaluated by SOSG. Briefly, mice were divided into 6 groups described above. 200 μL of saline or NIR dyes (Cypate, Cy7 or CyI, 1.5 mg/kg) were mixed separately with 50 μL SOSG (25 μM) and intratumor administered into mice. Then, the tumors were exposed to NIR light (808 nm, 0.3 W/cm^2 or 0.96 W/cm^2, 1 min). After 6 h, tumors were isolated from each mice for confocal imaging.

Statistical Analysis

Data was presented as mean ± SD. Statistical analysis was performed by students' t-test with $P<0.05$ as statistical significance.

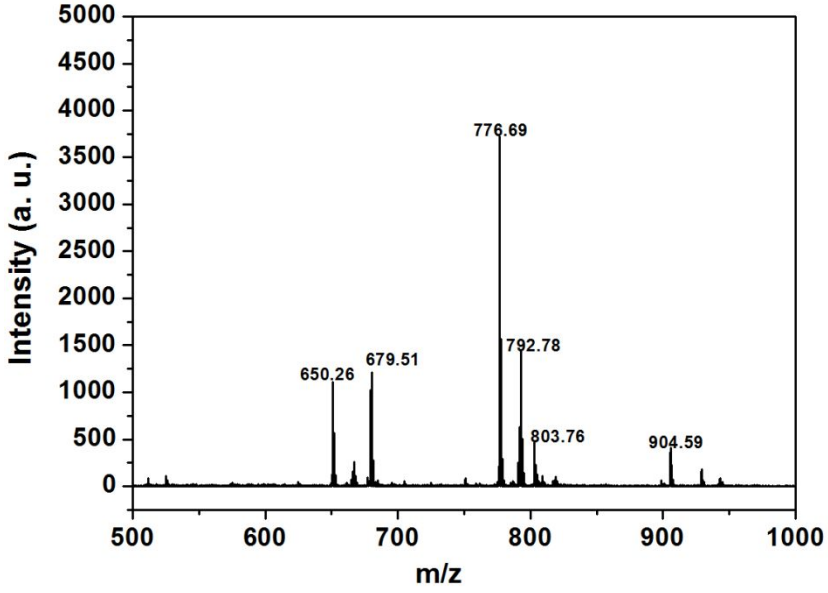
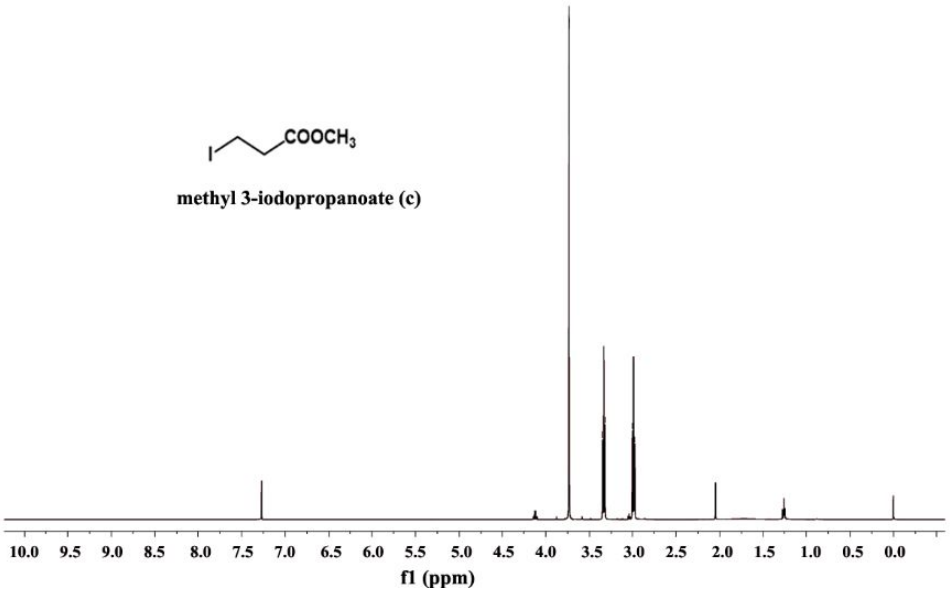
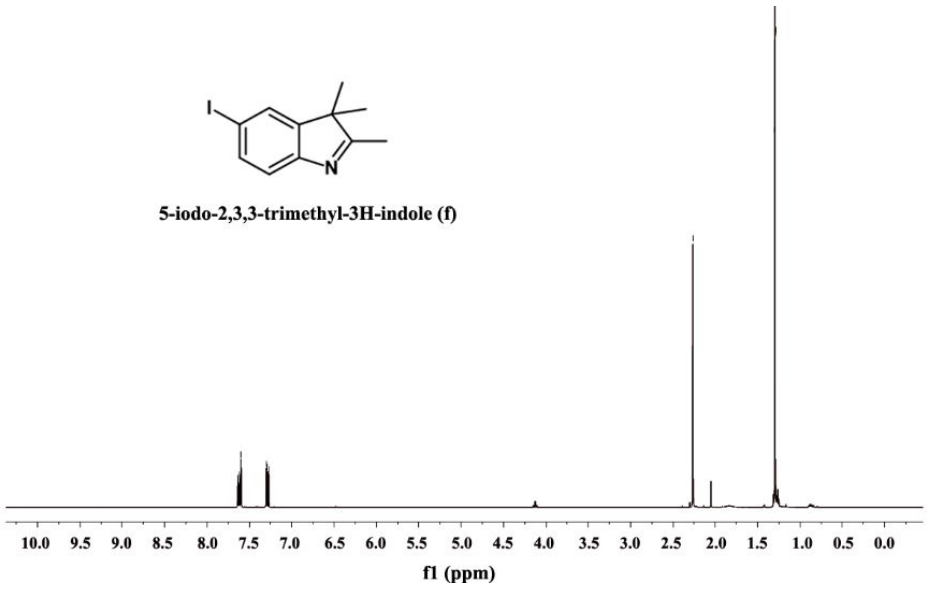


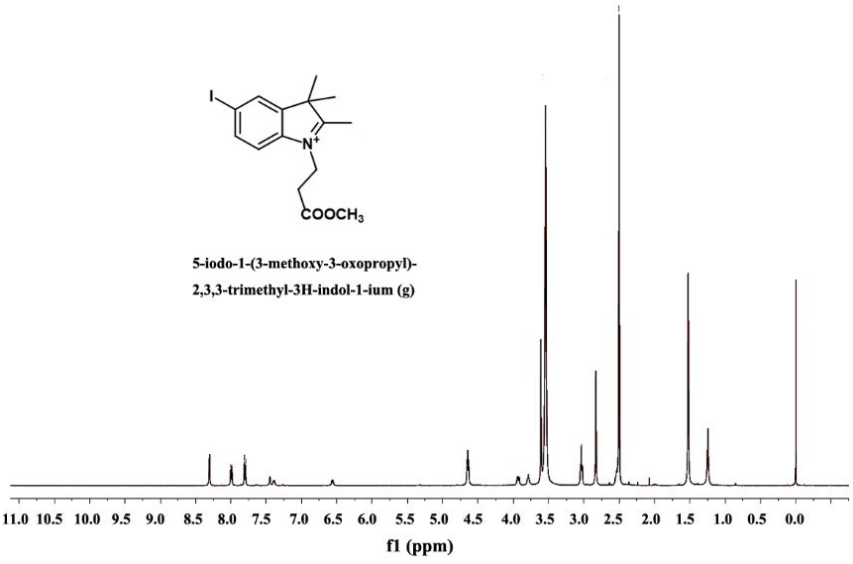
Figure S1. MALDI-TOF-MS profile of CyI by 4800 Plus MALDI-TOF/TOF mass spectrometer (AB Sciex)



methyl 3-iodopropanoate (c)



5-iodo-2,3,3-trimethyl-3H-indole (f)



5-iodo-1-(3-methoxy-3-oxopropyl)-2,3,3-trimethyl-3H-indol-1-ium (g)

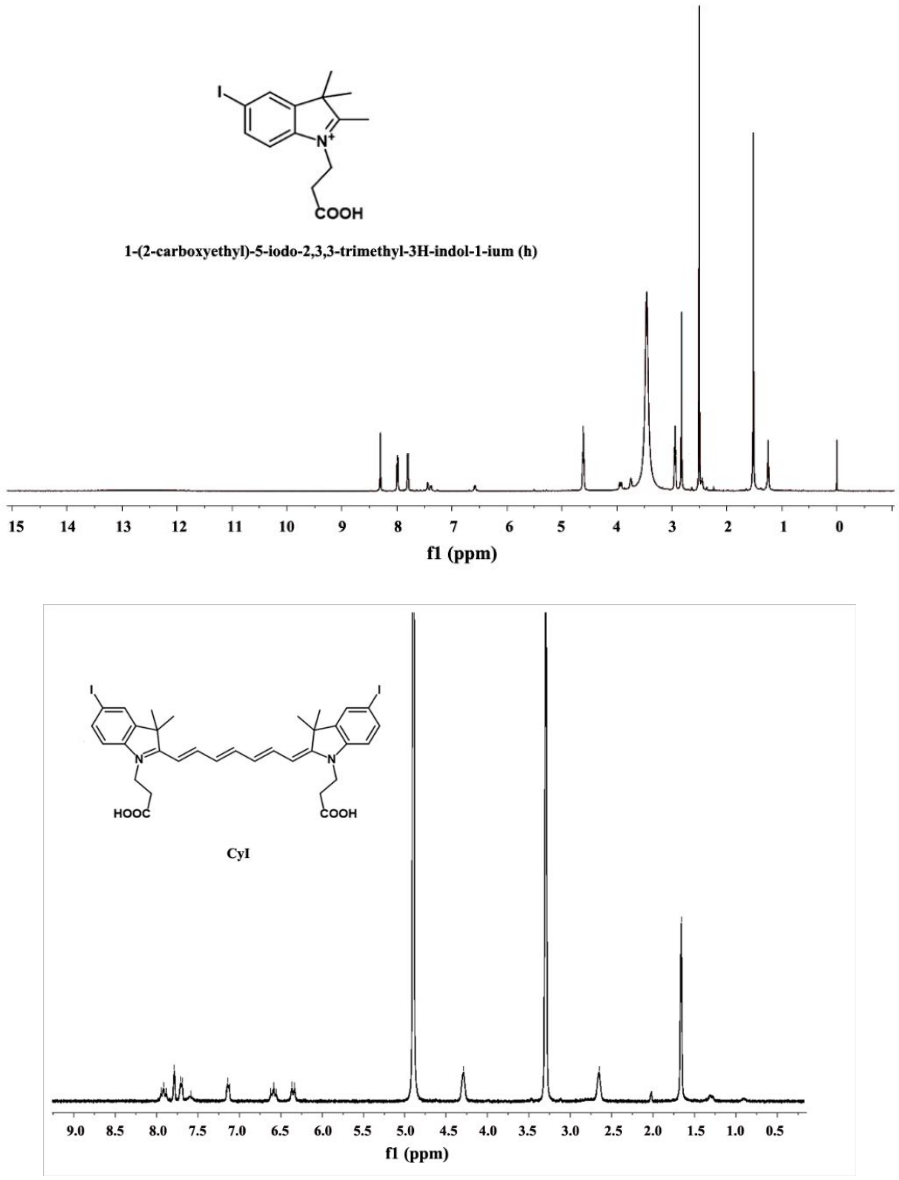


Figure S2. Representative ^{1}H NMR spectrum of methyl 3-iodopropanoate (c), 5-iodo-2,3,3-trimethyl-3H-indole (f), 5-iodo-1-(3-methoxy-3-oxopropyl)-2,3,3-trimethyl-3H-indol-1-ium (g), 1-(2-carboxyethyl)-5-iodo-2,3,3-trimethyl-3H-indol-1-ium(h) and CyI

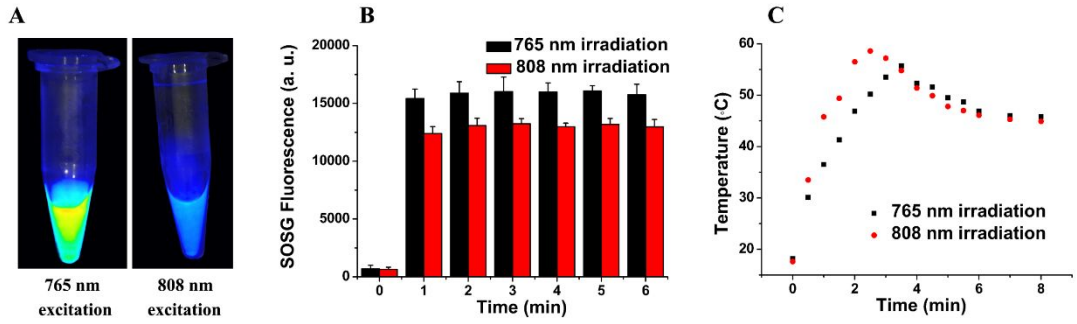


Figure S3 Fluorescence imaging (A), Singlet oxygen generation (B), and temperature change curves of CyI aqueous solution (C) exposed to the 765 nm and 808 NIR laser diode, alternately.

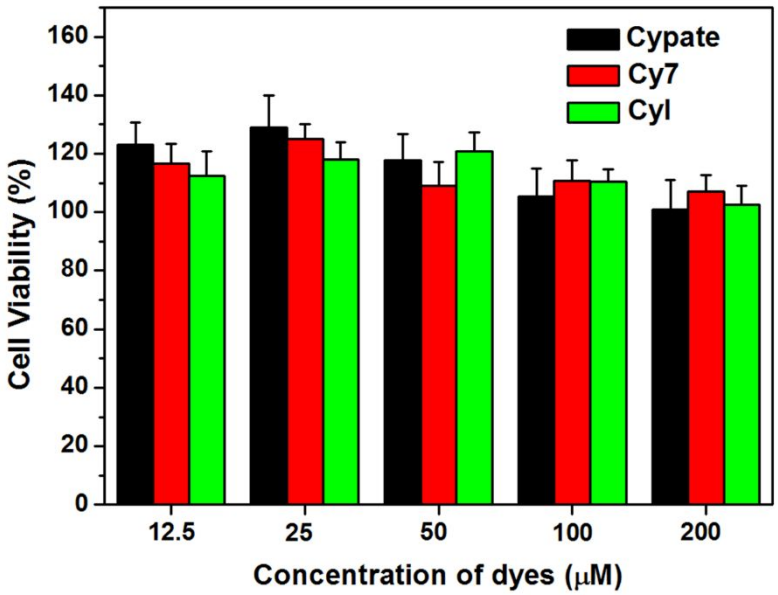


Figure S4. Cell viability of Cypate, Cy7 and CyI in mouse embryo osteoblast precursor MC3T3-E1 cell lines with different concentration (12.5, 25, 50, 100 and 200 μM).

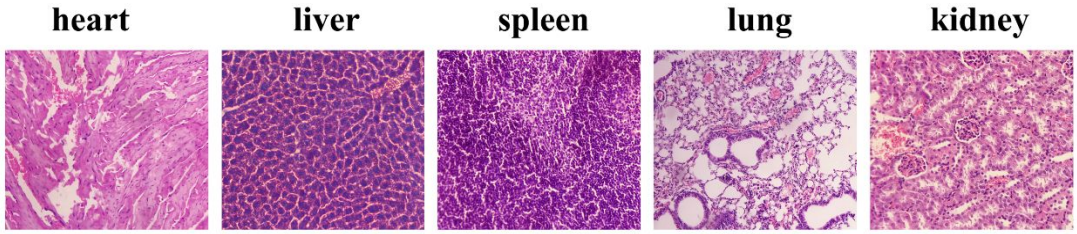


Figure S5. H&E stained images of major organ slices collected from CyI-treated tumors cover with 1-cm pork tissue (808 nm, exposed to 0.96 W/cm^2 light) (image magnification is 200×).

Reference

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