Supporting Information

A mitochondria-targeting heptamethine cyanine-chlorambucil formulated polymeric nanoparticle to potentiate native tumor chemotherapeutic efficacy

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Experimental section

Materials

IR775 chloride and 3-Mercapto-1-propanol were purchased from TCI (Shanghai) Chemical Industry Development Co., Ltd. Chlorambucil (Cbl), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl), triethylamine, anhydrous DMF were purchased from Sarn (Shanghai) Chemical Technology Co., Ltd. 4-dimethylaminopyridine (DMAP) was purchased from Aladdin company. Dichloromethane was dried over calcium hydride and purified by distillation. Dimethyl sulfoxide (DMSO), methanol, petroleum ether and all other reagents were purchased from Sinopharm Chemical Reagent Co. Ltd. and used as received. 3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Sigma-Aldrich. Fetal bovine serum (FBS) penicillin, streptomycin, Dulbecco's modified eagle's medium (DMEM) and Roswell Park Memorial Institute (RPMI 1640) medium were obtained from GIBCO and used as received. Mitotracker Green, JC-1, Rhodamine 123, and ATP Assay Kit were purchased from Beyotime Biotechnology (Shanghai). Anexin V-FITC/PI Assay Kit was purchased from Dojindo Molecular Technologies, Inc. Sulfobromophthalein Sodium (BSP) was purchased from Shanghaiyuanye Bio-TechnologyCo. ,Ltd.

Synthesis of IR775-Cbl

3-Mercapto-1-propanol (70.8 mg) and triethylamine (116.4 mg) were dissolved in anhydrous DMF and stirred at room temperature under nitrogen for 20 min. Then, IR775 chlorine (200 mg) in anhydrous DMF was gently added and the mixture was stirred overnight at room temperature under the protection of nitrogen at dark. After evaporation, the crude product was purified by silica gel column chromatography to afford green solid product, noted as IR775-OH (140 mg, yield: 63%). ^{1}H NMR (DMSO-d_6, 600 MHz, δ, ppm, TMS): 8.80-8.77 (d, 2H), 7.65-7.64 (d, 2H), 7.47-7.46 (m, 4H), 7.32-7.31 (m, 2H), 6.34-6.32 (d, 2H), 4.63 (s, 1H), 3.70 (s, 6H), 3.49 (q, 2H), 2.90-2.87 (t, 2H), 2.71-2.69 (t, 4H), 2.06-2.01 (m, 2H),1.87-1.85 (t, 2H), 1.74 (s, 12H).

After that, IR775-OH (100 mg) and Cbl (52.7 mg) were dissolved in anhydrous DCM, then EDC·HCl (66.4 mg) and DMAP (42.3 mg) were added. The mixture was stirred at room temperature at dark for 24 h. After evaporation, the crude product was purified by silica gel chromatography, affording

IR775-Cbl as green solid product (118 mg, yield: 77%). ^{1}H NMR (DMSO-d_6, 600 MHz, δ, ppm, TMS): 8.75-8.72 (d, 2H), 7.63-7.62 (d, 2H), 7.47-7.45 (m, 4H), 7.32-7.29 (m, 2H), 6.97-6.96 (d, 2H), 6.62-6.60 (d, 2H), 6.33-6.30 (d, 2H), 4.10-4.08 (t, 2H), 3.71-3.67 (m, 14H), 2.90-2.88 (t, 2H), 2.68-2.67 (t, 4H), 2.44-2.42 (t, 2H), 2.26-2.24 (t, 2H), 1.91-1.89 (m, 2H), 1.85-1.83 (m, 2H), 1.74-1.71 (m, 14H).

Aqueous self-assembly of IR775-Cbl to afford Nanoparticles

The IR775-Cbl nanoparticles were prepared using typical solvent exchange method. The powder of IR775-Cbl (2 mg) was dissolved in DMSO (1 mL), then the solution was slowly added to filtered deionized water (9 mL) under intense stirring. After that, the agitation was stopped and DMSO was removed by dialysis to afford the resultant nanoparticle dispersion. Additionally, IR775-Cbl@PF127 also prepared by the similar method, IR775-Cbl (2 mg) and PF127 (10 mg) were dissolved in DMSO (1 mL), followed by the same steps to afford the resultant IR775-Cbl@PF127 nanoparticles for in vivo test.

Cell culture

EMT6 cells (mouse breast tumor cell line) and MCF-7 cells (human breast tumor cell line) were cultured in RPMI 1640 medium, Raw 264.7 cells (mouse mononuclear macrophage) were cultured in DMEM medium, both supplemented with 10% fetal bovine serum (FBS) and 1 % antibiotics in 5% CO_2, 95% air at 37 ºC in a humidified incubator.

Cell uptake and subcellular localization

EMT6 cells were seeded in a six-well plate at a density of $1x10^5$ separately with 2 mL medium and cultured for 24 h. Cells were then incubated with IR775-Cbl at 37 ºC for diverse durations. The cells were then collected and subjected to flow cytometry analysis. The mean fluorescence intensity of each sample was recorded. The data were processed by CytExpert software. EMT6 cells were seeded in a glass bottom petri dish and incubated in culture medium for 12 h, followed by incubation with IR775-Cbl for different durations, respectively, then Mitotracker Green (10 μg/mL) was added to stain the mitochondria for 15 min. The cells were imaged by a commercial laser scanning microscope. The channel of Mitotracker green was recorded at the excitation wavelength of 488 nm, and the channel of IR775-Cbl was recorded at the excitation wavelength of 633 nm.

Mitochondrial membrane potential assay

JC-1 and Rhodamine 123 probes were employed to evaluate the depolarization of mitochondrial membrane potential in EMT6 cells. EMT6 cells were seeded in a glass bottom Petri dish for 12 h and then incubated with Cbl, IR775, IR775/Cbl mixture and IR775-Cbl for 6 h, and finally stained with JC-1 and rhodamine 123 for 20 min, respectively, and observed by confocal microscope. The conditions for JC-1 detection by flow cytometry were similar to those for confocal detection.

Measurement of cellular ATP level

EMT6 cells were seeded at a density of $2x10^5$ cells per well and then incubated for 24 h. Then the cells were treated with diverse samples, Cbl, IR775, IR775/Cbl mixture, IR775-Cbl respectively for 24 h in RPMI 1640 medium at 37 ºC. Cells were then washed with PBS and intracellular ATP was then extracted according to the ATP assay kit instructions; the bioluminescence was measured using a microplate reader.

In vitro cytotoxicity assay

Typical MTT assay was used to determine the cytotoxicity. EMT6 cells were seeded in 96-well plates at an initial density of 5000 cells/well in RPMI 1640 medium. After incubating for 24 h, the cells were treated with the same concentration gradient of Cbl, IR775, IR775/Cbl mixture, and IR775-Cbl, respectively, and incubated at 37 °C for 48 h. After that, MTT reagent (100 μL, 5 mg/mL) was added to each well. The cells were further incubated for another 4 h at 37 °C, and then the medium in each well was removed and replaced by 150 μL DMSO. The 96-well plate was shaken for 20 min to dissolve the formazan crystals, then the absorbance at 490 nm was recorded with a microplate reader.

Apoptosis/necrosis assay

The apoptosis/necrosis was quantified by Annexin V-FITC/PI staining. EMT6 cells were seeded in a six-well plate at an initial density of $1x10^6$ cells/well and cultured for 24 h. Then the cells were treated with Cbl, IR775, IR775/Cbl mixture, IR775-Cbl at 10 μM, respectively. The cells were incubated for 12 h at 37 ℃, then the cells were harvested, washed twice with PBS, and stained with Annexin V-FITC/PI according to the manufacturer's instructions. The cells were incubated for 15 min at dark and then analyzed by flow cytometry. All experiments were performed in triplicate.

Morphological observation of cells and mitochondria by TEM

EMT6 cells were seeded in a six-well plate at a density of $1x10^5$ separately with 2 mL medium and

cultured for 24 h. The cells were treated with the same concentration of Cbl, IR775, IR775/Cbl mixture, and IR775-Cbl, respectively, and incubated at 37 °C for 48 h. Then the cells were quickly scraped off with a cell scraper, centrifuged at 2000 rpm for 8 min, the supernatant was discarded, the cells at the bottom of the tube were collected, and 2.5% glutaraldehyde solution pre-chilled at 4℃ was slowly added along the wall of the tube and fixed at 4℃ for 24h. paraffin-embedded, and stained for further analysis.

Animal and tumor xenografts

Female Balb/c mice (3~4 weeks) were purchased from the Experimental Animal Center of Southern Medical University. All procedures of animal experiments were approved by the Animal Care and Use Committee of South China Normal University (SCNU-BIP-2024-025). $4x10^6$ cells were suspended in 100 μL PBS and were subcutaneously injected into the flanks of each female Balb/c mouse to establish typical EMT6 tumor-bearing mouse model.

In vivo imaging and biodistribution analysis

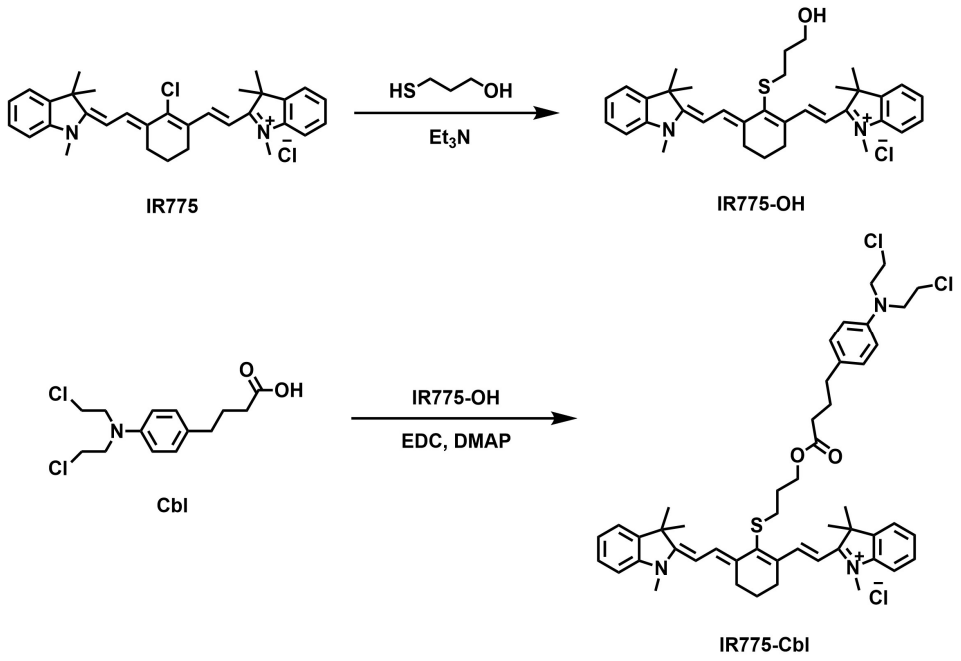
EMT6 tumor-bearing mice were intravenously injected with IR775-Cbl@PF127 at 1 mg/kg, and imaged at the different time points (0, 3, 6, 9, 12 and 24 h) using the *ex/in vivo* imaging system. The mice were sacrificed at 24 h post-injection, then the heart, liver, spleen, lung, kidneys, and tumor tissue were collected for further analysis and imaging. The procedure for in vivo imaging of IR775 was the same as above.

In vivo antitumor efficacy

The EMT6 tumor-bearing mice were randomly divided into 6 groups (n=4): (1) PBS, (2) Cbl, (3) IR775, (4) IR775/Cbl mixture, (5) IR775-Cbl, (6) IR775-Cbl@PF127. The applied dosage was set to be comparable to 0.58 mg/kg Cbl and 1 mg/kg IR775. *In vivo* treatment was performed when the tumor volume was ~80-100 mm^3. The mice in each group were intravenously injected every 3 days for total of 6 times. The tumor volume and body weight were monitored once every 3 days. The tumor volume was calculated as: $V = (L \times W^2)/2$, where L was the long diameter and W was the short diameter determined by a caliper. All mice were sacrificed after the treatments, tumor samples and major organs, including the liver, lung, heart, spleen, and kidney were collected, formalin-fixed, paraffin-embedded, and stained for further analysis.

Characterization

Nuclear magnetic resonance (^{1}H NMR) spectra were recorded on a Bruker Avance DRX-600 (Bruker BioSpin Corporation, Billerica, Massachusetts). The UV-vis absorption spectra were recorded on a spectrophotometer (UV3100, Shimadzu, Japan). Fluorescence spectroscopy measurements were performed on a steady state and transient state fluorescence spectrometer (FL-1000, Edinburgh Instruments Ltd, UK). The size distribution and zeta potentials were determined by a ZetaSizer Nano ZS90 analyzer (Malvern, UK). The nanoparticle morphology was observed on a transmission electron microscope (TEM, JEM-100S, JEOL, Japan). Ex/in vivo imaging system (Odyssey LI-COR, USA) was employed for in vivo fluorescence imaging. Flow cytometry (Cytomics FC 500, Beckman Coulter, USA) was used for flow cytometry analysis.



Scheme S1. Synthetic route employed for the drug conjugate, IR775-Cbl.

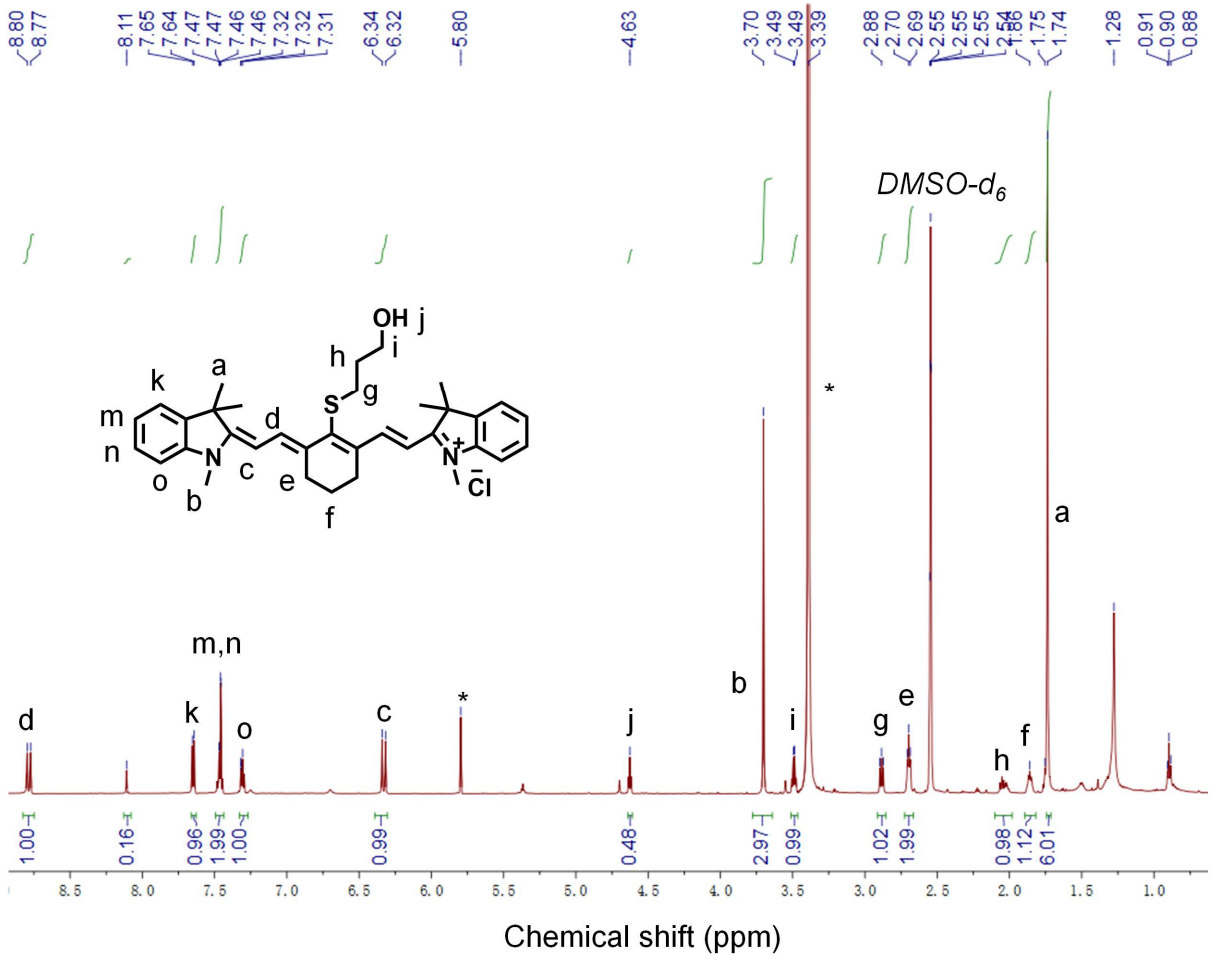


Fig. S1 ^{1}H NMR spectrum recorded for IR775-OH in DMSO-d_6.

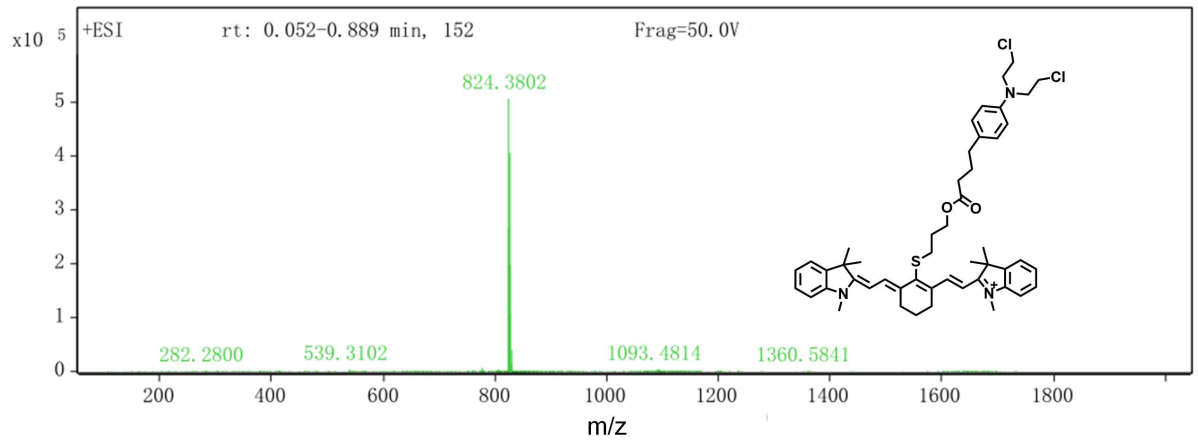


Fig. S2 ESI- HRMS spectrum recorded for IR775-Cbl in MeOH.

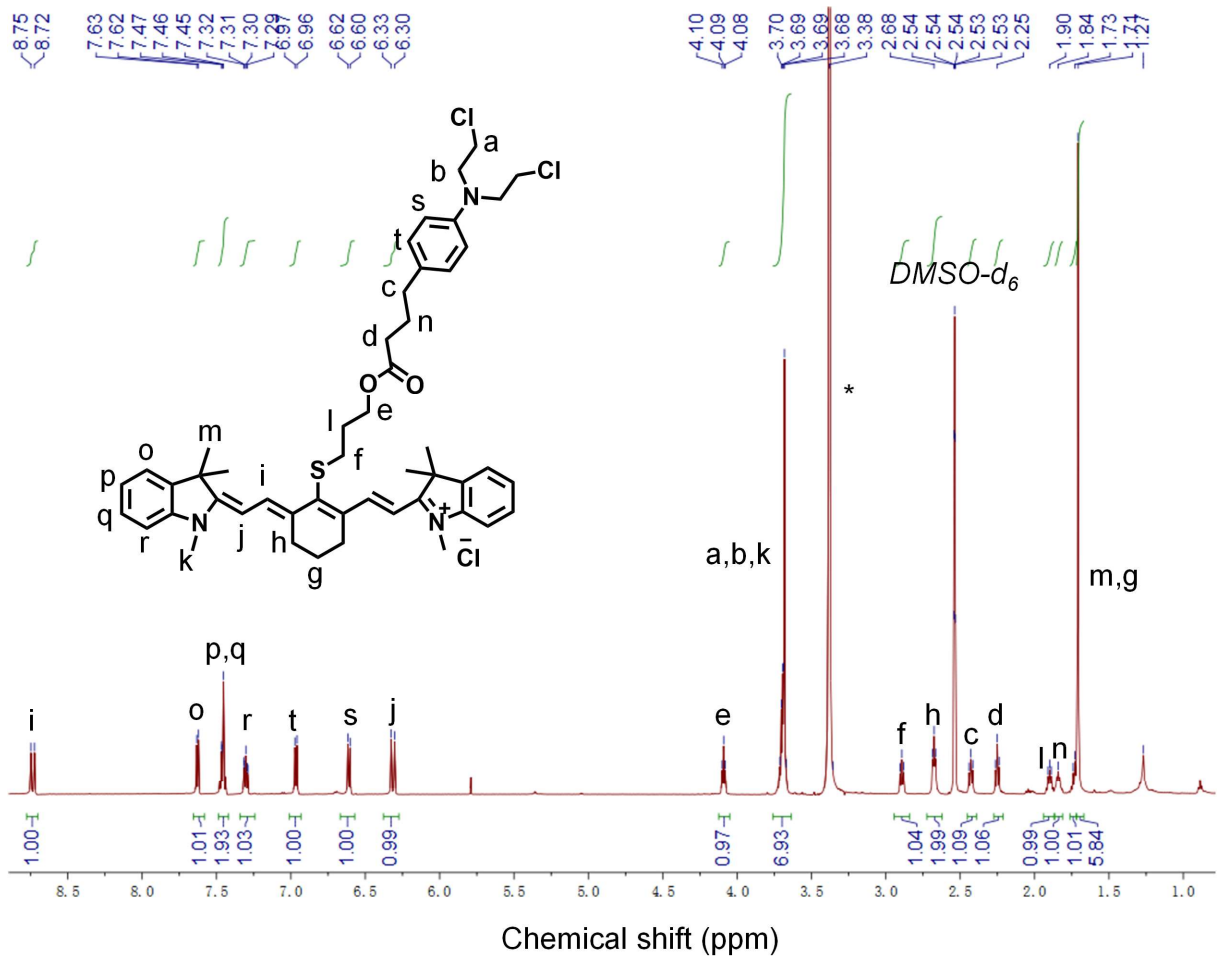


Fig. S3 ^{1}H NMR spectrum recorded for IR775-Cbl in DMSO-d_6.

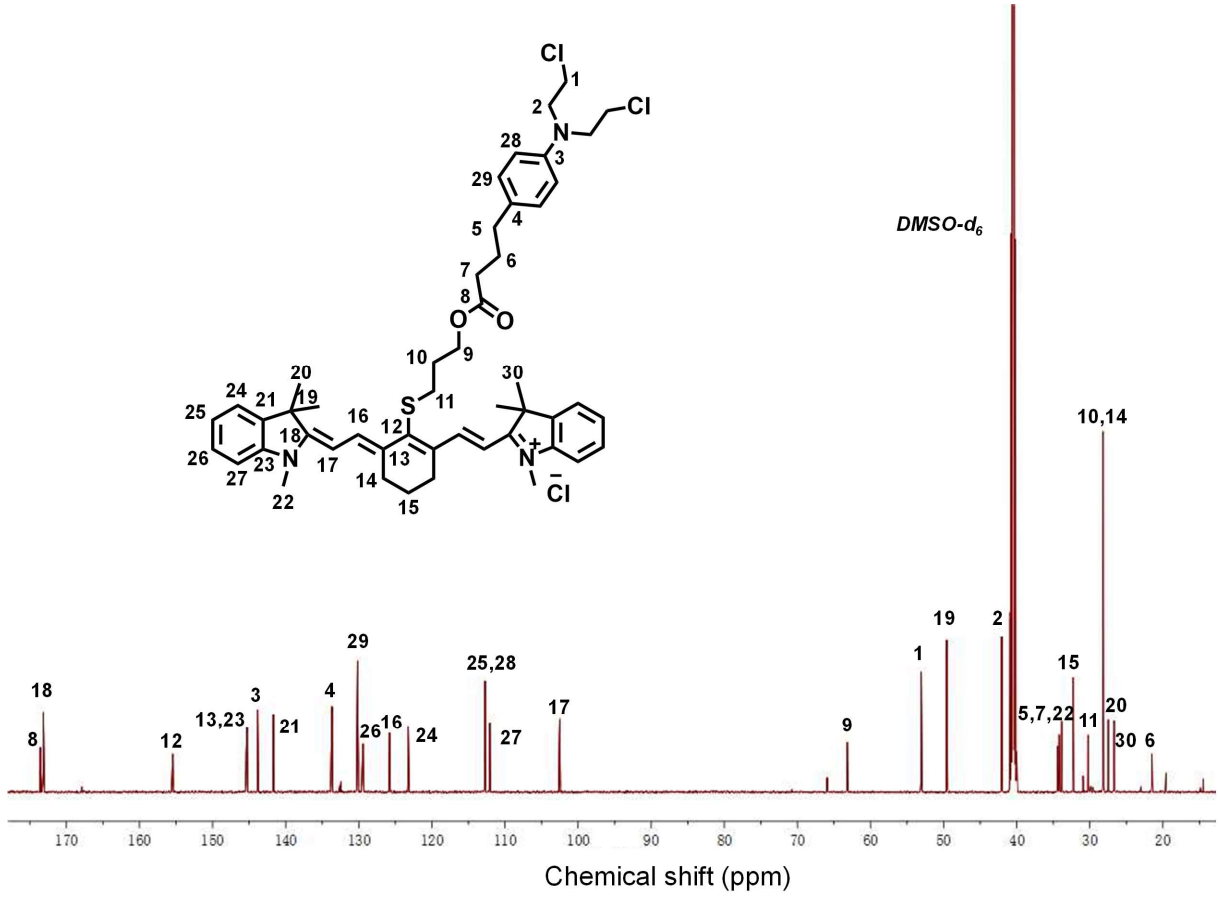


Fig. S4 ^{13}C NMR spectrum recorded for IR775-Cbl in DMSO-d_6.

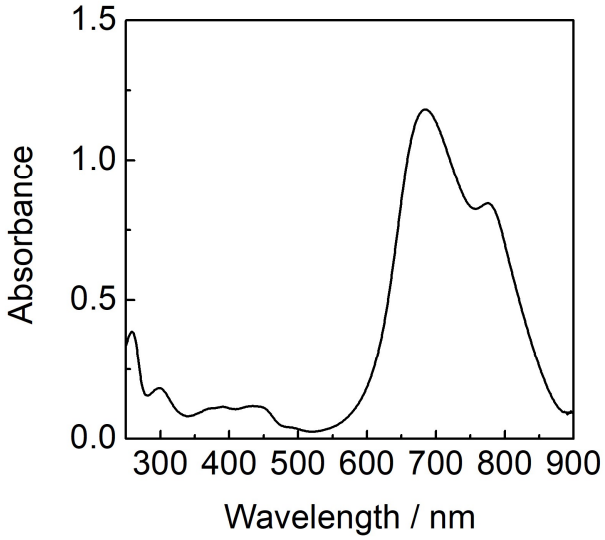


Fig. S5 Absorbance spectrum recorded for the aqueous dispersion of IR775-Cbl.

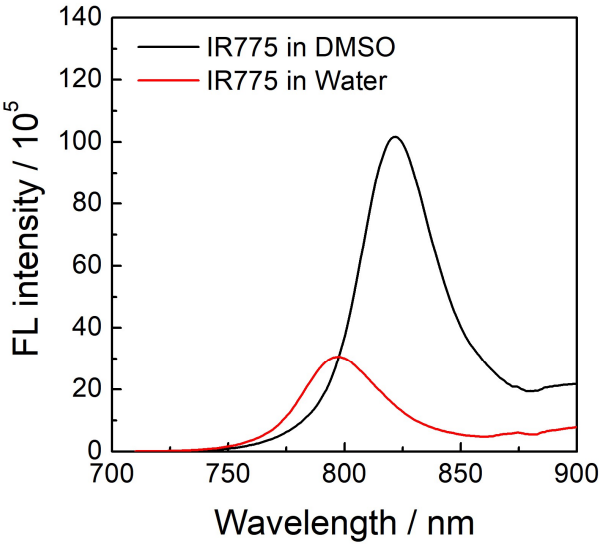


Fig. S6 Fluorescence spectrum recorded for the aqueous dispersion of IR775.

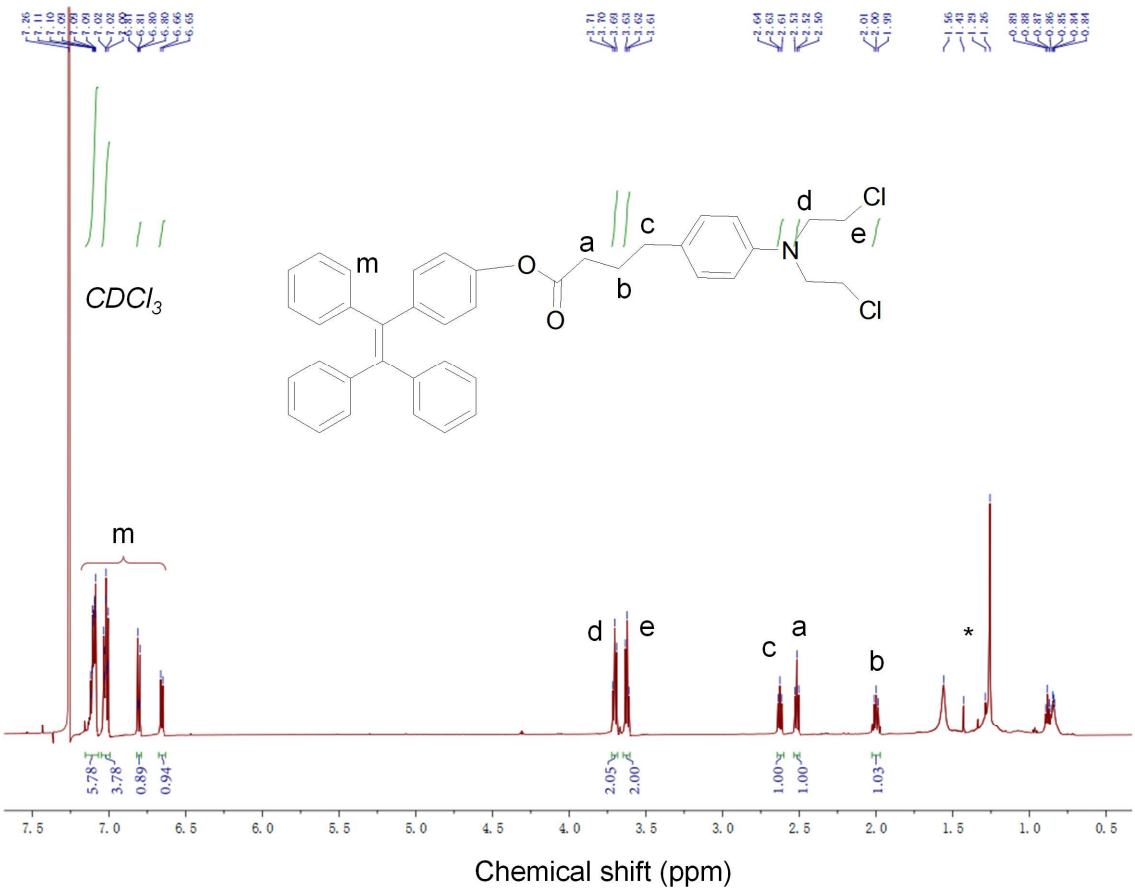


Fig. S7 ^{1}H NMR spectrum recorded for TPE-Cbl in $CDCl_3$.

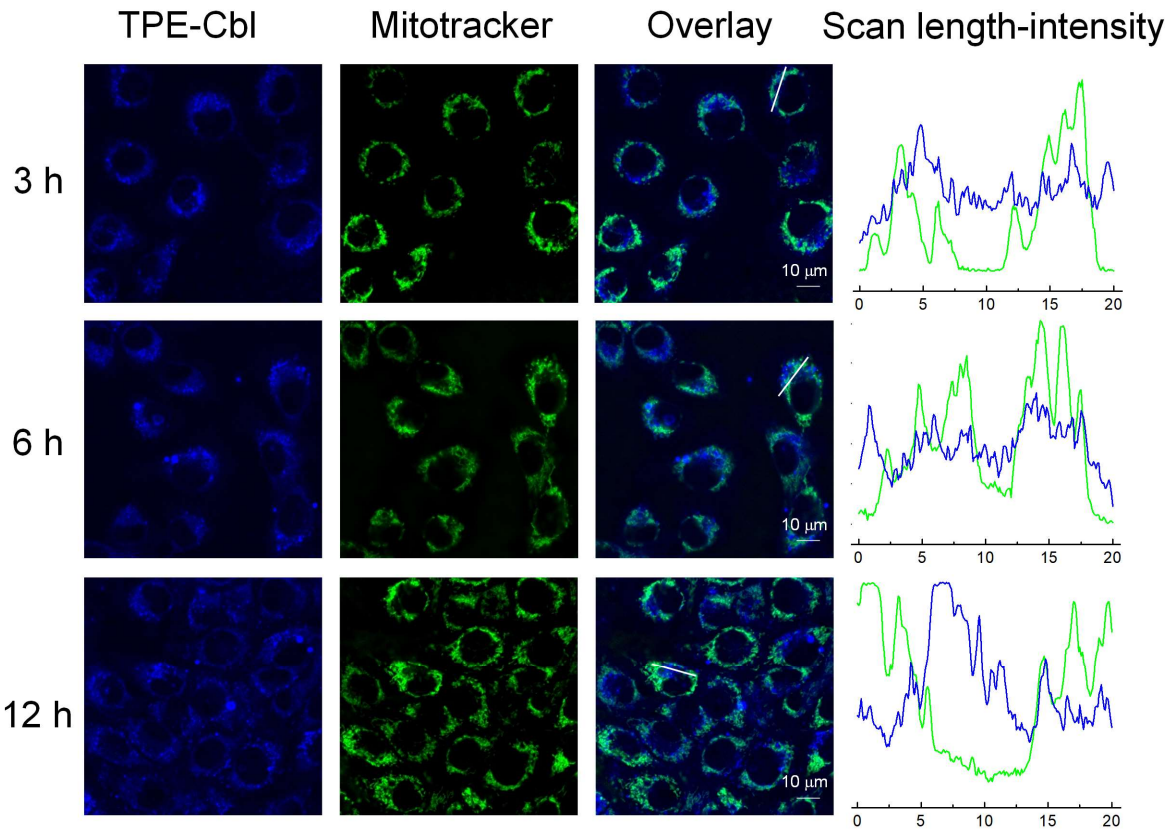


Fig. S8 Intracellular trafficking of TPE-Cbl. Confocal laser scanning microscopy images of EMT6 cells upon incubation with TPE-Cbl. Mitochondria were co-stained with Mitotracker Green (green channel).

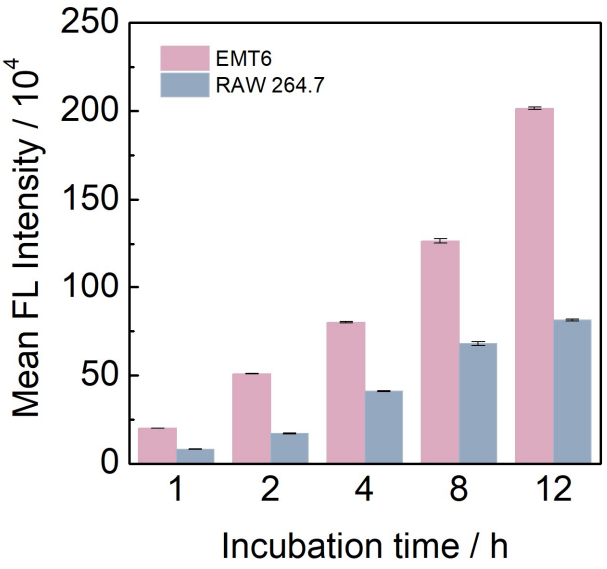


Fig. S9 Flow cytometry analysis for EMT6 cells and Raw 264.7 cells upon incubating with the aqueous dispersion of IR775-Cbl for varying time intervals.

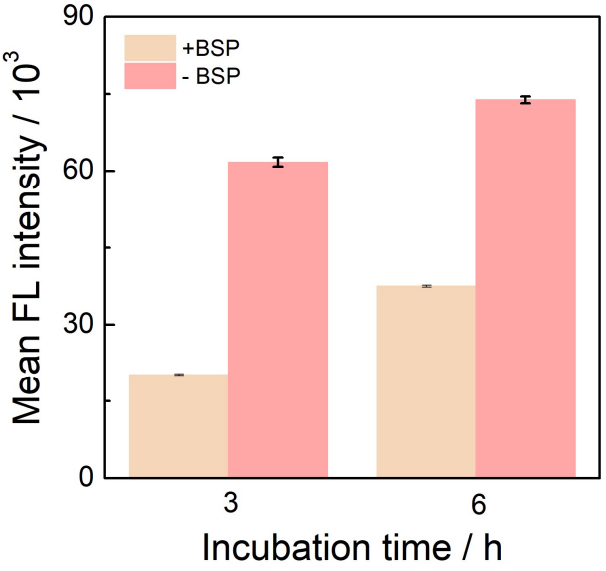


Fig. S10 Flow cytometry analysis for EMT6 cells pretreated with 250 μM BSP for 1h or without BSP upon incubating with the aqueous dispersion of IR775-Cbl for varying time intervals.

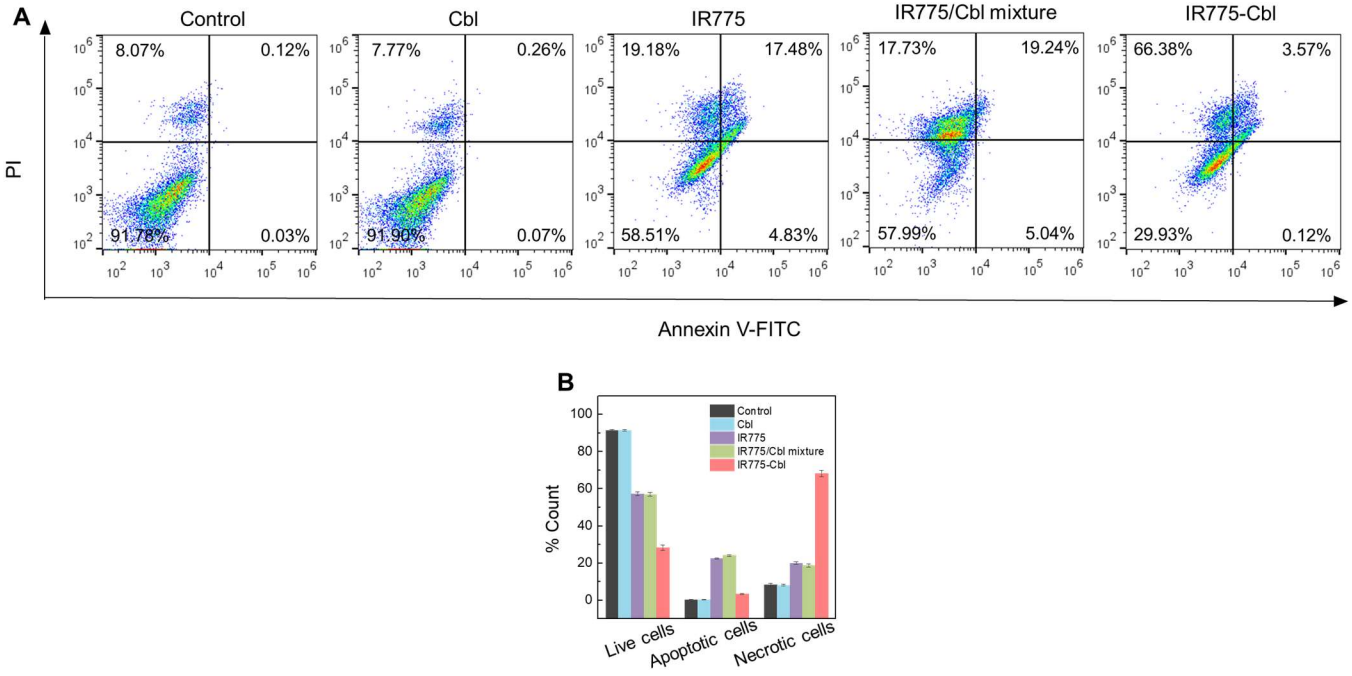


Fig. S11 (A) Cell apoptosis and necrosis analysis of MCF-7 cells by flow cytometry with Annexin V-FITC/PI double staining after different treatments. (B) Statistical analysis of flow cytometry data in (A). (mean± SD, n= 3).

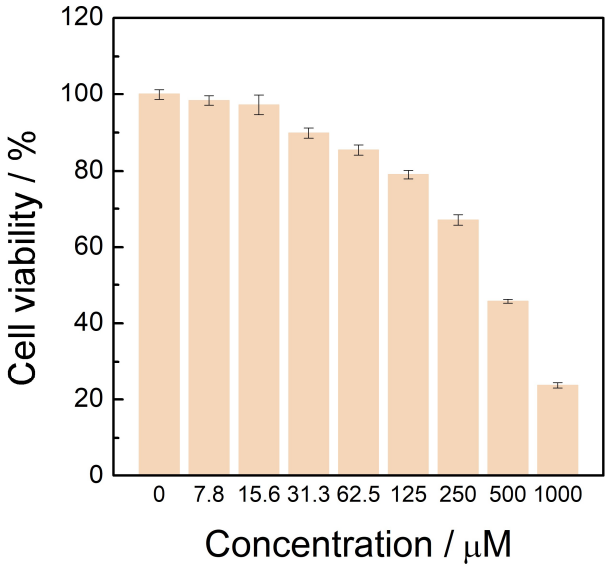


Fig. S12 *In vitro* cytotoxicity of Cbl determined by MTT assay against EMT6 cells upon 48 h treatment. (mean± SD, n= 3).

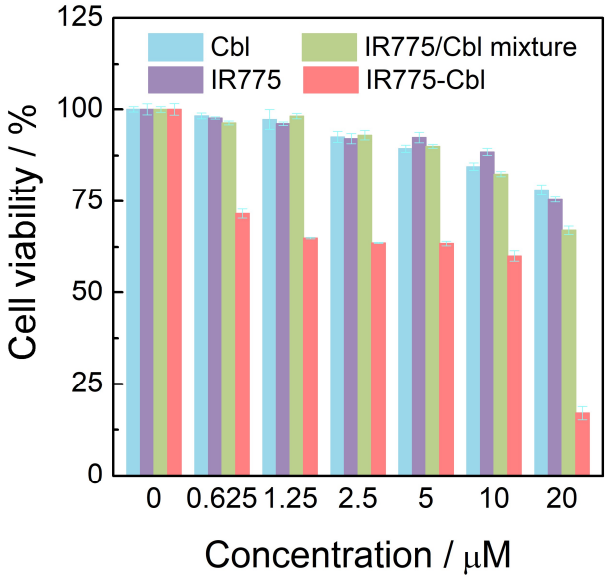


Fig. S13 *In vitro* cytotoxicity determined by MTT assay against MCF-7 cells upon 48 h treatment with four different samples.

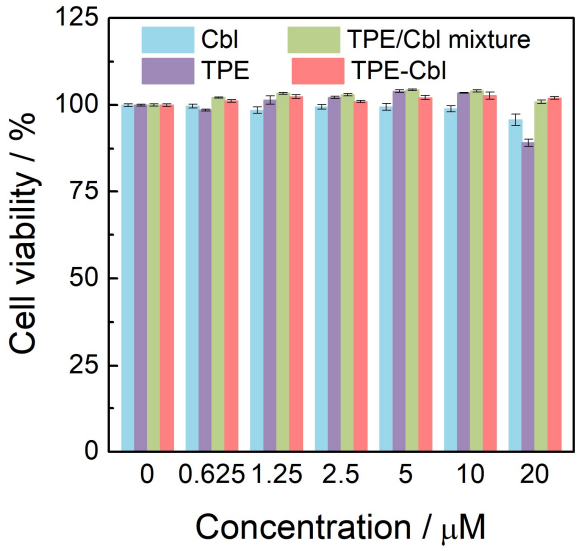


Fig. S14 *In vitro* cytotoxicity of TPE-Cbl determined by MTT assay against EMT6 cells upon 48 h treatment. (mean± SD, n= 3).

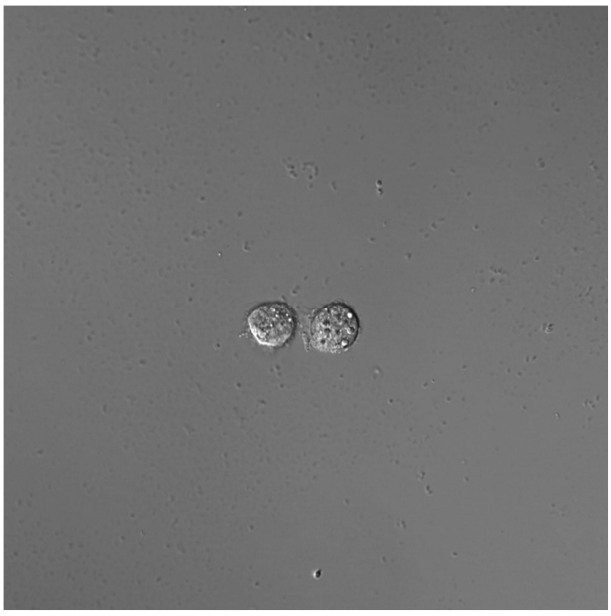


Fig. S15 Bright field image of EMT6 cells upon incubation with IR775-Cbl (~10 μM) for 24 h and gentle washing with PBS. Most of the cells could not adhere to the culture dish.

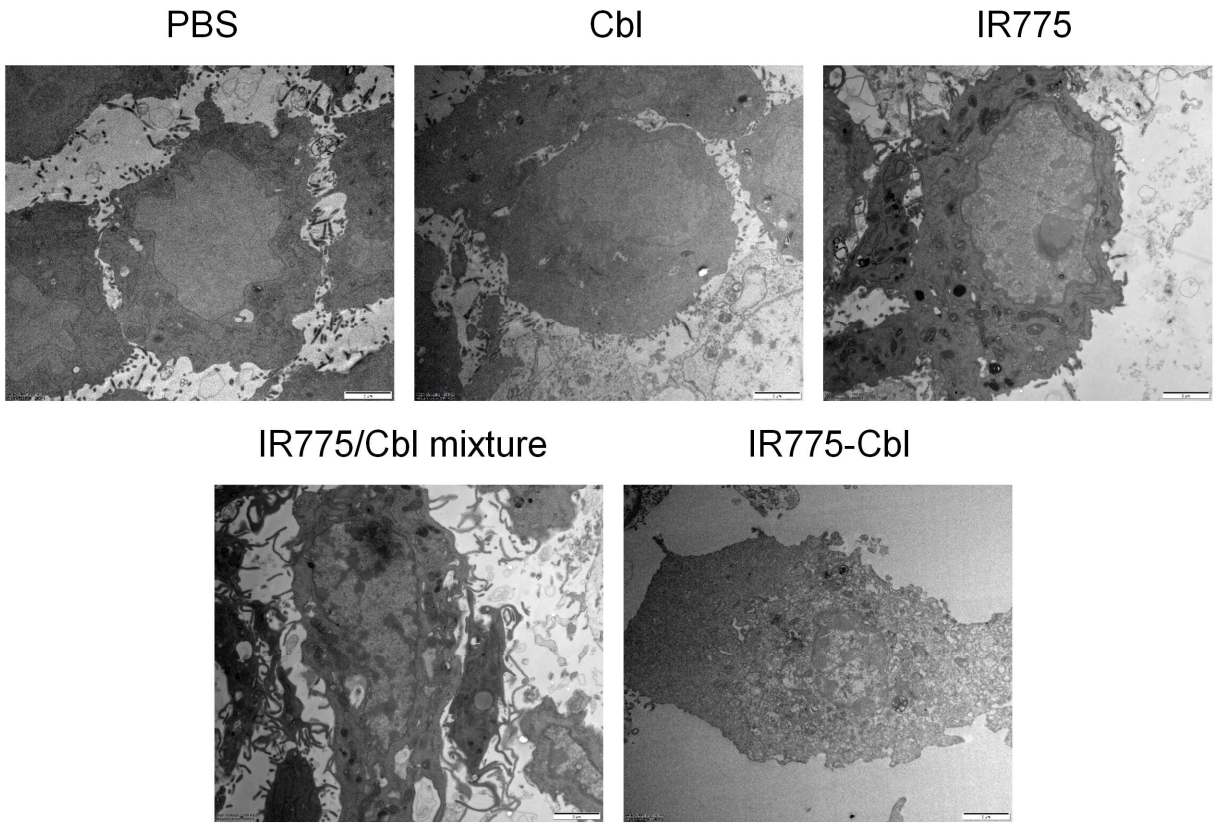


Fig. S16 Transmission Electron Microscopy (TEM) micrographs of EMT6 cells after diverse treatments.

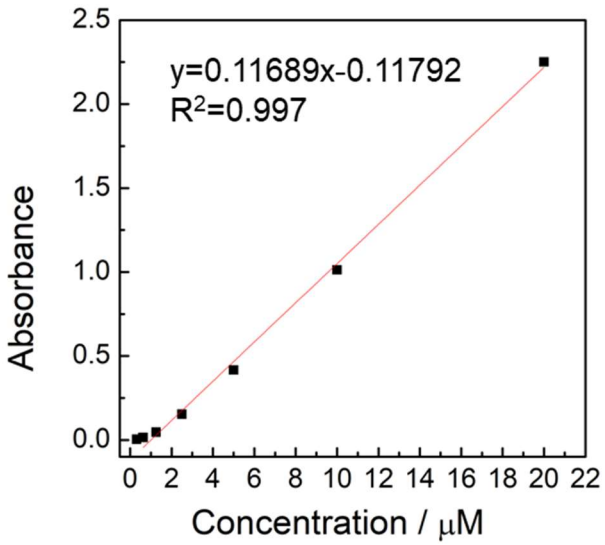


Fig. S17 The standard curve of IR775-Cbl based on the absorbance at 795 nm.

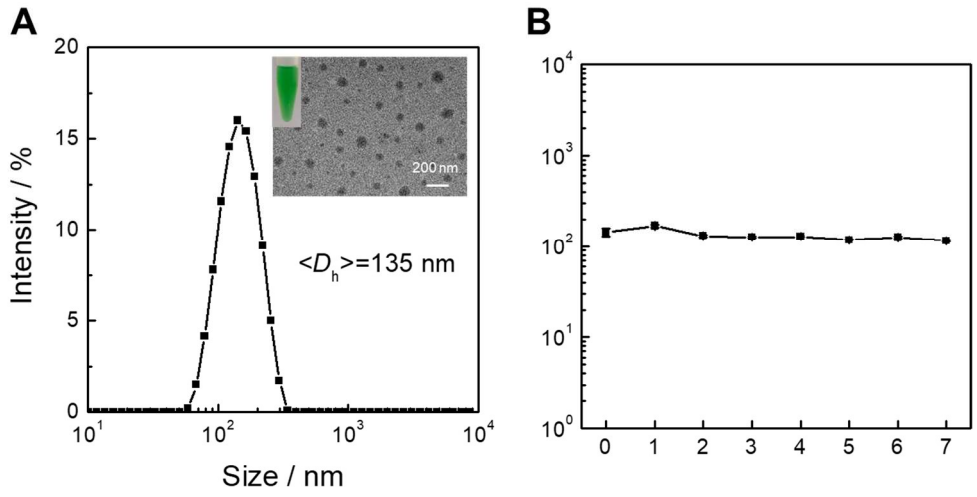


Fig. S18 (A) Hydrodynamic diameter distribution profile of IR775-Cbl@PF127 in aqueous solution. The insets are TEM image and the photograph of IR775-Cbl@PF127 aqueous dispersion. (B) Hydrodynamic diameters recorded for the aqueous dispersion of IR775-Cbl@PF127 upon storage at room temperature.

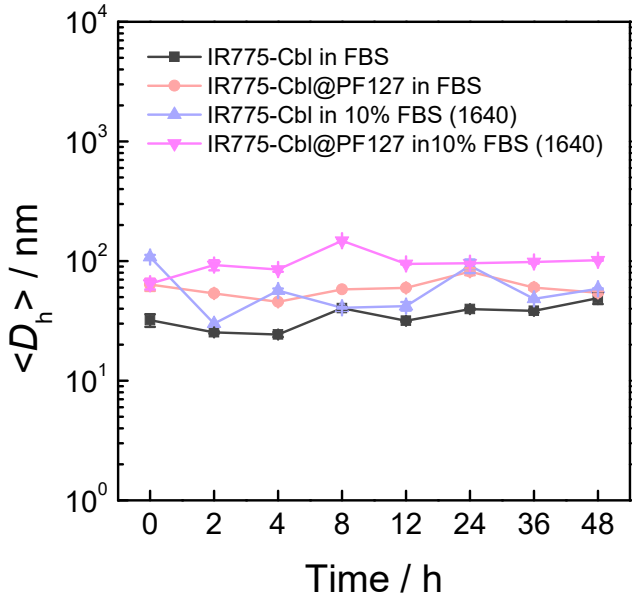


Fig. S19 Hydrodynamic diameters recorded for IR775-Cbl and IR775-Cbl@PF127 in FBS and 10%FBS (1640) at 37 ℃.

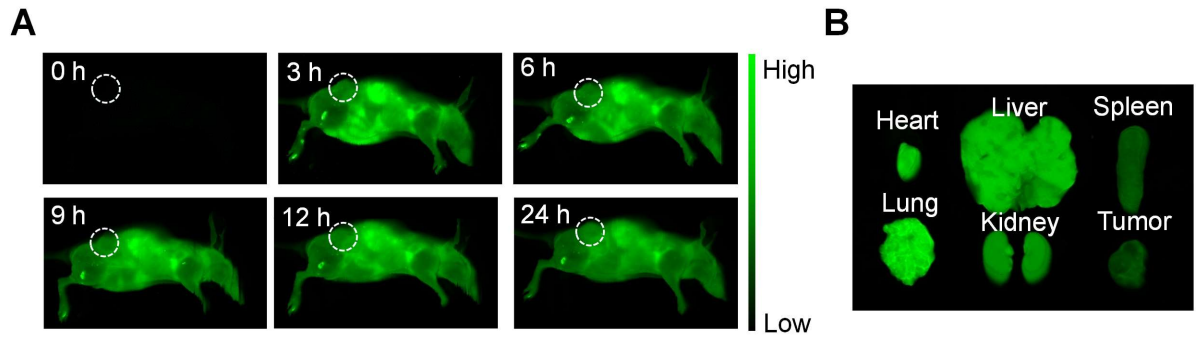


Fig. S20 (A) Fluorescence imaging of EMT6 tumor-bearing mice upon intravenous injection with IR775. (B) Typical fluorescent images of major organs and tumor collected at 24 h post-injection.

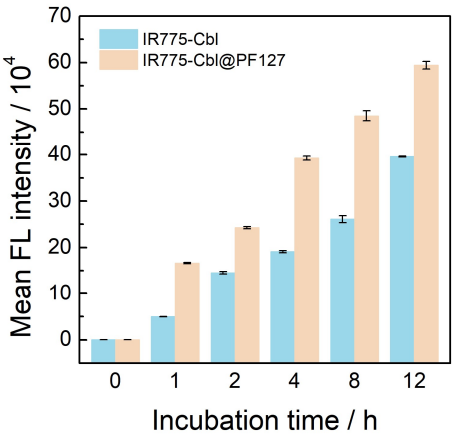


Fig. S21 Flow cytometry analysis for EMT6 cells upon incubating with the aqueous dispersion of IR775-Cbl and IR775-Cbl@PF127 for varying time intervals.

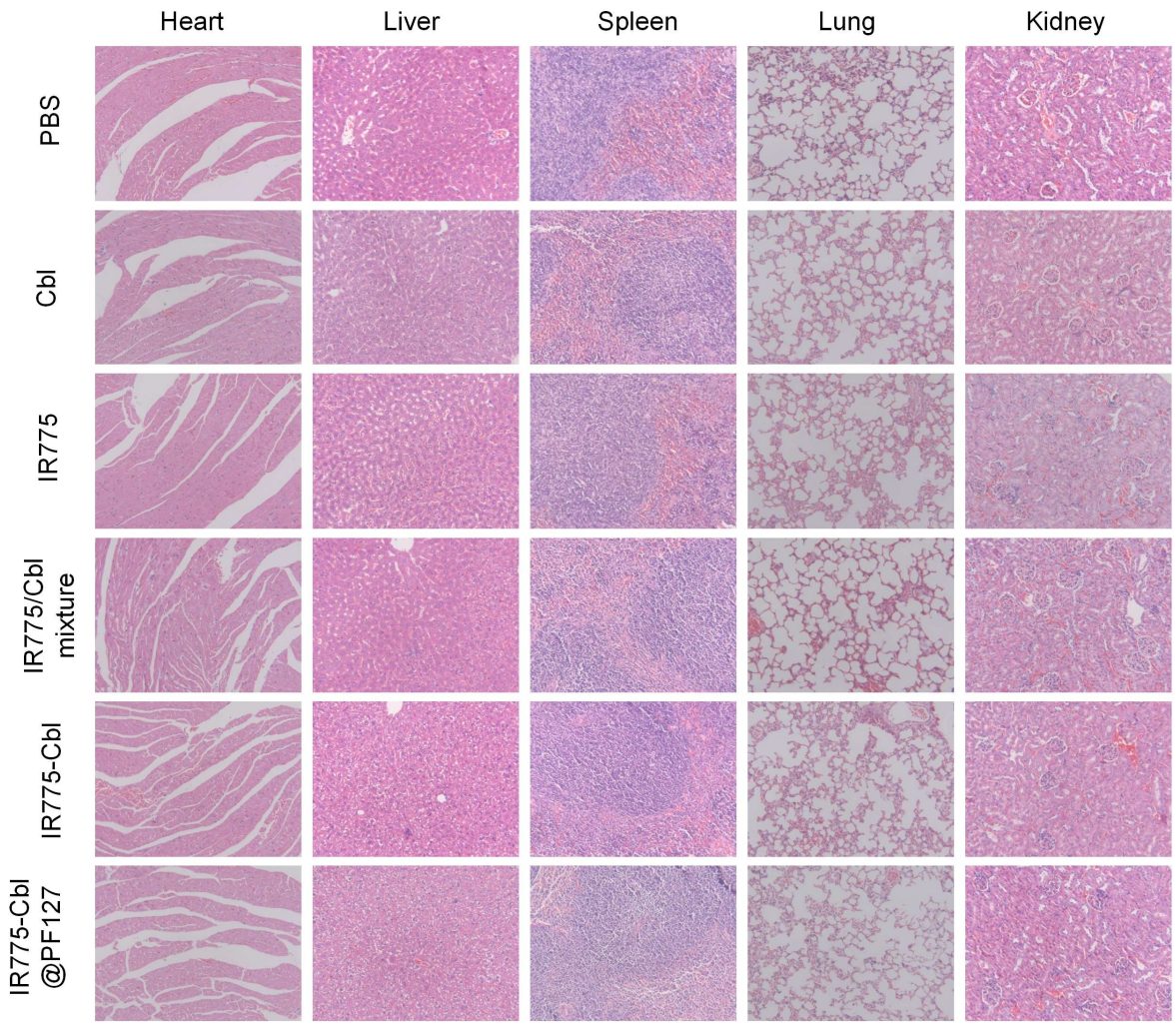


Fig. S22 Typical H&E staining images (20×) of major organs collected from PBS, Cbl, IR775, IR775/Cbl mixture, IR775-Cbl and IR775-Cbl@PF127 treated group, respectively.

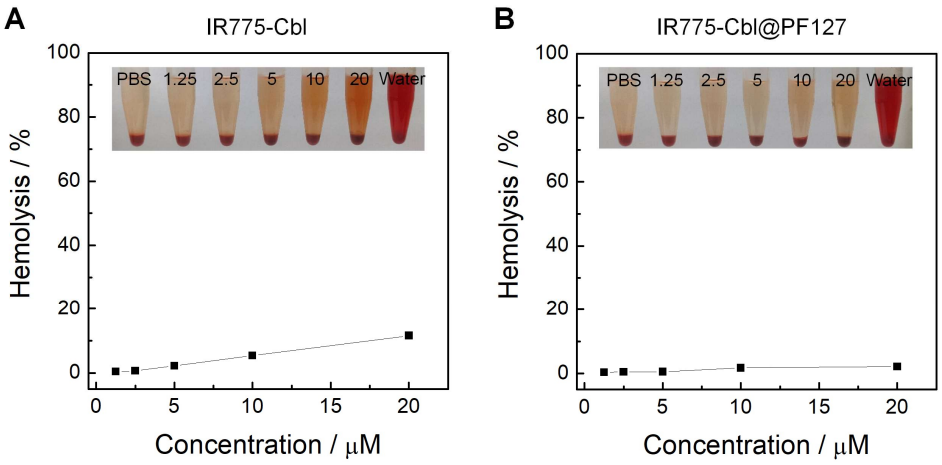


Fig. S23 Hemolytic analysis of red blood cells (RBCs) upon 4 h incubation with (A) IR775-Cbl, and (B) IR775-Cbl@PF127 at diverse IR775-Cbl equivalent contents.