Electronic Supplementary Information

C-Phycocyanin as a tumour-associated macrophage-targeted photosensitiser and vehicle of phthalocyanine for enhanced photodynamic therapy

Dong-Hua Wan[1], Bi-Yuan Zheng[1], Mei-Rong Ke[1], Ji-Ying Duan[1], Yun-Quan Zheng[1], Chih-Kuang Yeh[2], Jian-Dong Huang[1]*

[1]College of Chemistry, State Key Laboratory of Photocatalysis on Energy and Environment, FuzhouUniversity, Fuzhou 350108, China.
[2] Department of Biomedical Engineering and Environmental Science, National Tsing Hua University, Hsinchu 30013, Taiwan.

[*] Corresponding author. Tel: +86 591 22866235; Fax: +86 591 22866227; E-mail: jdhuang@fzu.edu.cn

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1. Purification and characterization of CPC

General. C-phycocyanin crude extract was provided by Fuqing King Dnarmsa Spirulina Co., Ltd. (China). DEAE-Sepharose Fast Flow was purchased from Shanghai 3bio technology Co., Ltd. (China). Dialysis (MWCO 3500) was purchased from Shanghai GreenBird Science & Technology Development Co., Ltd. (China). The Thermo Scientific PageRuler^{TM} Low Range Unstained Protein Ladders used in SDS-PAGE are a mixture of seven recombinant proteins ranging from 5K to 100K and a synthetic peptide at 3.4K. The Amersham High Molecular Weight Calibration Kit for Electrophoresis used in Native-PAGE consists of five protein standards (Thyroglobulin, 669K; Ferritin, 440K; Catalase, 232K; Lactate Dehydrogenase, 140K; Bovine Serum Albumin, 67K). Phosphate-buffered saline (PBS) at pH 5.9 contains 5 mM NaH_2PO_4 and 5 mM $Na_2HPO_4{\cdot}3H_2O$. All other reagents and solvents were of analytical pure and used as received.

Characterization. Electronic absorption spectra were obtained on a Shimadzu UV-2450 spectrophotometer (Japan). Fluorescence spectra were recorded using an Edinburgh FL/FS900 spectrofluorometer (England). High-resolution mass spectrum was recorded on Thermo Scientific Exactive Plus mass spectrometer (German). Native PAGE and SDS-PAGE were performed using Bio-Rad vertical slab discontinous gel electrophoresis apparatus (USA).

Purification of CPC. CPC was purified according to previous procedure.[1,2] The PBS (pH 5.9) solution of CPC crude extract (60 mL, 10 mg·mL^{-1}) was loaded on a DEAE-Sepharose Fast Flow resin column (7.5 × 8.5 cm) and was eluted with PBS (pH 5.9) containing three concentration gradients of NaCl at 0.1, 0.2 and 0.5 M. The fractions showing an absorbance ration of A_{620}/A_{280} greater than 4.0 were pooled in the second NaCl gradient and precipitated by adding ammonium sulfate to 50% saturation. After centrifugation, the blue precipitate was dissolved in deionized water and dialyzed overnight at 4°C against deionized water. The purity and homogeneity of

the purified CPC were examined by electrophoresis and spectroscopy. The purified CPC was freeze-dried and kept in a refrigerator until use.

2. Interaction and conjugate of ZnPc with CPC

General. PBS at pH 7.4 contains 5 mM KH_2PO_4 and 5 mM Na_2HPO_4. The unsubstituted zinc phthalocyanine (ZnPc) was purchased from SIGMA (USA). Sephadex G200 was purchased from Beijing RuiDaHengHui Science & Technology Development Co., Ltd. (China). Zeta potential was characterized by Microtrac Nanotrac Wave analyzer (USA).

The interaction of CPC with ZnPc. We investigated the interactions of ZnPc with CPC by a fluorescence quenching method.[3] A solution of CPC in PBS (0.03 mg·mL^{-1}, 2 mL) was titrated with a solution of ZnPc in DMF (1.32 mM, 32 μL). Fluorescence emission spectra was monitored at 600~800 nm upon excitation at 590 nm. With the increasing of ZnPc, the intrinsic emission band of CPC at 648 nm decreases in intensity. The data follow the equation $\lg [(F_0\text{-}F) /F] = \lg K_A + n\lg[Q]$, where F_0 and F are the fluorescence intensities in the absence and presence of the quencher, respectively. $[Q]$ is the concentration of the quencher. K_A is the binding constant, and n is the binding site number. n and K_A can be obtained from the slope and intercept of the linear regression equation.

Preparation of ZnPc-CPC conjugate. The conjugate was prepared by stirring a mixture of ZnPc and CPC in PBS with a molar ratio of 10:1. ZnPC solution in DMF (2 mM, 0.5 mL) was added dropwise to solution of CPC (2 mg·mL^{-1}, 5 mL) in PBS (pH 7.4). The mixture was stirred at ambient temperature for 2 h, then centrifuged at 10000 rpm for 5 min. The supernatant was purified by gel chromatography on a G-200 Sephadex column (2.0×20 cm) using PBS (pH 7.4) as eluent. The concentration of ZnPc in the conjugate was measured in a diluted DMF solution by comparing absorbance with the calibration curve of ZnPc ($\varepsilon_{668} = 2.398\times10^5$ M^{-1} cm^{-1}, see Fig. S7). The protein content in the conjugate was determined with the Bradford protein assay using BSA as standard (see Fig. S8). The molar ratio of ZnPc to CPC in the conjugate can be calculated according to the respective concentrations.

3. *In vitro* test

Organisms and growth conditions. J774A.1 mouse macrophages (from ATCC) and HepG2 human hepatocarcinoma cells (from ATCC) were maintained in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum, penicillin (50 units·mL^{-1}), and streptomycin (50 μg·mL^{-1}) at 37°C in a humidified 5% CO_2 atmosphere.

***In vitro* photocytotoxicity against HepG2 or J774A.1 cells.** The unsubstituted zinc phthalocyanine (ZnPc) was dissolved in DMF to form a 1.0 mM stock solution. CPC and ZnPc-CPC conjugate were dissolved in PBS to form a 20 μM stock solution. These solutions were further diluted with the cellular medium to appropriate concentrations and sterilized with a 0.45 μm filter.

For studies of ZnPc formulated with Cremophor EL (denoted as ZnPc-CEL), the stock solution of ZnPc in DMF (1.0 mM) was diluted to 80 μM with an aqueous solution of Cremophor EL (1%, 1 g in 100 mL of water). The above 80 μM solutions were then diluted with the growth medium to appropriate concentrations and sterilized with a 0.45 μm filter.

The photocytotoxicity assay is the same as the procedure described previously[4] with some modifications. Briefly, HepG2 or J774A.1 cells (about 1×10^4 cells per well) were maintained in 96-well plates overnight at 37°C in a humidified 5% CO_2 atmosphere. The cells were then incubated with 100 μL of the above solutions in the dark for 2 h. After that, the cells were rinsed with PBS and re-fed with 100 μL of the culture medium before being illuminated at ambient temperature. The light source consisted of a 500 W halogen lamp, a water tank for cooling and a colored glass filter with a cut-on wavelength of 500 nm. The fluence rate (λ = 500~800 nm) was 15 mW·cm^{-2}. An illumination of 30 min led to a total fluence of 27 J·cm^{-2}. Then after 24h incubation, cell viability was determined by means of the colorimetric 3-(4,5-dimethyl-2- thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) assay.[5]

Cellular uptake. About 1×10^5 HepG2 or J774A.1 cells in RPMI 1640 medium (0.5

mL) were seeded on confocal dishes and incubated overnight at 37°C under a humidified 5% CO_2 atmosphere. After removing the medium, the cells were incubated with the solutions of ZnPc-CPC conjugate, ZnPc, CPC and ZnPc-CEL (just only for J774A.1 cells) in the medium (0.4 mL) for 2 h under the same condition, respectively. The cells were then rinsed with PBS twice and viewed with Leica laser fluorescent confocal microscope (excited at 635 nm and monitored emission at 640 ~700 nm). The images were then digitized and analyzed by using the SPE ROI Fluorescence Statistics software. The average intracellular fluorescence intensities (a total of 50 cells for each sample) were also determined. For the competitive assay on J774A.1 cells, the procedure was the same as that described above except for the following details. The cells was first incubated with 100 $\mu g \cdot mL^{-1}$ poly I for 0.5 h, and then the solutions of ZnPc-CPC conjugate, ZnPc and CPC in the medium were added for further 2 h co-incubation.

4. *In vivo* test

Hepatoma H22 cells were obtained from the China Center for Type Culture Collection (CCTCC, Wu Han, China); KM mice were purchased from Wushi Animal Co. Ltd. (Fuzhou, China). All animal studies were performed in compliance with guidelines of the Animal Care Committee of Fuzhou University, and also approved by the committee. To build a subcutaneous tumour model, H22 cells (~1×10^7 cells in 200 μL) were inoculated subcutaneously on the axilla of the KM mice (20~25 g). When the tumours had grown to 100~300 mm^3, a CPC or ZnPc-CPC aqueous solution (90 μM, 100 μL) was intravenously injected into the tail vein of the tumour-bearing mice. *In vivo* fluorescence imaging of mice was performed from 650 nm or 690 nm at different time points with AmiX SI imaging system (excited at 605 nm or 640 nm) for CPC and ZnPc-CPC, respectively. After *in vivo* imaging studies, the KM mice were euthanized at 24 h post-injection. Tumours and other organs were harvested and their fluorescence imaging were measured.

5. Reference

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Chromatographia, 2011, **73**, 291-296.

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5. H. Tada, O. Shiho, K. Kuroshima, M. Koyama and K. Tsukamoto. *J Immunol Method*, 1986, **93**, 157-165.

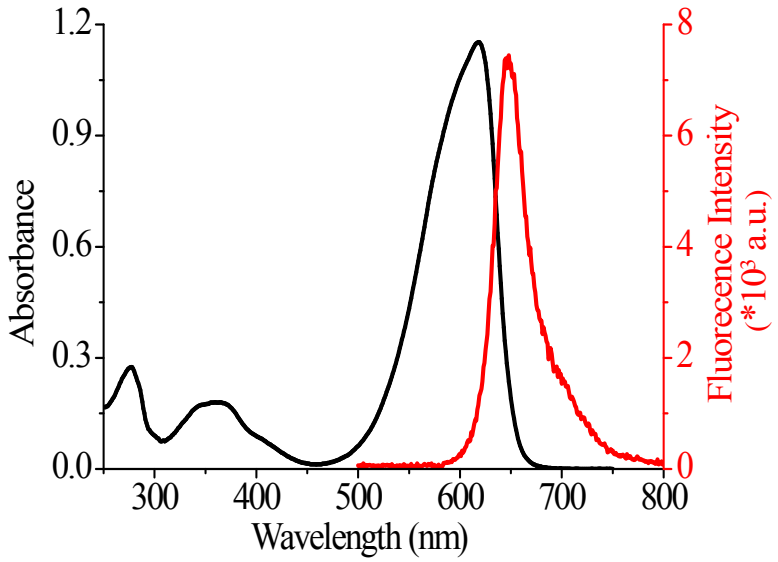

Fig. S1 Electronic absorption (—) and fluorescence emission spectra (—) of CPC. (λ_{ex} = 590 nm)

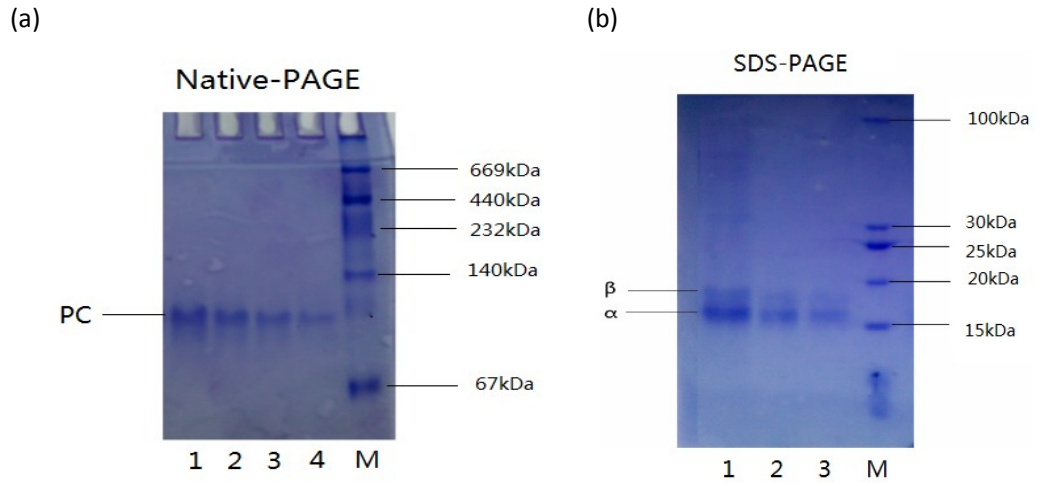

Fig. S2 (a) Native PAGE of CPC run in a 5% separating gel. Lane 1: 3.2 μg; lane 2: 2.4 μg; lane 3: 1.6 μg; lane 4. 1.2 μg; lane M: protein molecular mass standard. (b) SDS-PAGE of of CPC run in a 12% separating gel. Lane 1: 3 μg; lane 2: 2 μg; lane 3: 1 μg; Lane M: low molecular weight markers.

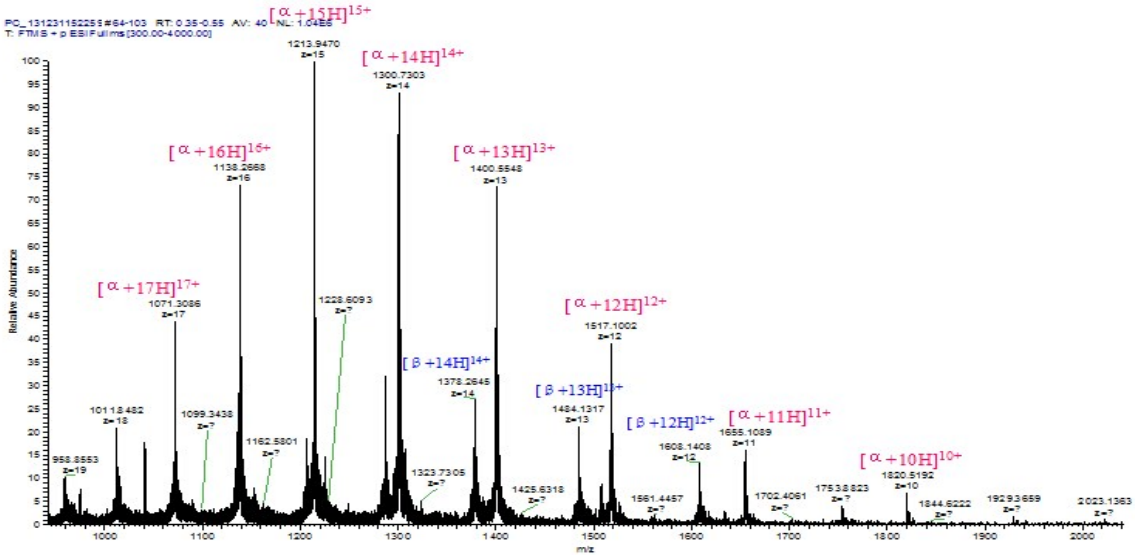

Fig. S3 HRMS spectrum (+ESI scan) of CPC.

Table S1 Mass spectrometry data analysis of α subunit

(m+z）/z	Z	m	SD
1071.309	17	18195.25	0.0000152
1138.267	16	18196.27	0.0000714
1213.947	15	18194.21	0.000042
1300.73	14	18196.22	0.0000690
1400.555	13	18194.21	0.0000416
1517.100	12	18193.20	0.0000971
1655.109	11	18195.20	0.0000126
1820.519	10	18195.19	0.0000123
$\overline{m}$	-	18194.97	-

Table S2 Mass spectrometry data analysis of β subunit

(m+z）/z	Z	m	SD
1378.265	14	19281.70	0.0000389
1484.132	13	19280.71	0.0000903
1608.141	12	19285.69	0.0001679
1753.882	11	19281.71	0.0000388
$\overline{m}$	-	19282.50	-

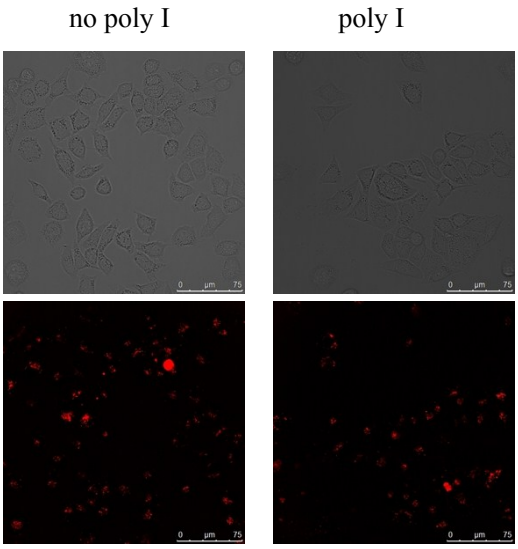

Fig. S4 Bright field (top row) and intracellular fluorescence (bottom row) images of HepG2 cells after incubation with CPC (10 μM) for 2 h, in the absence or presence of poly I.

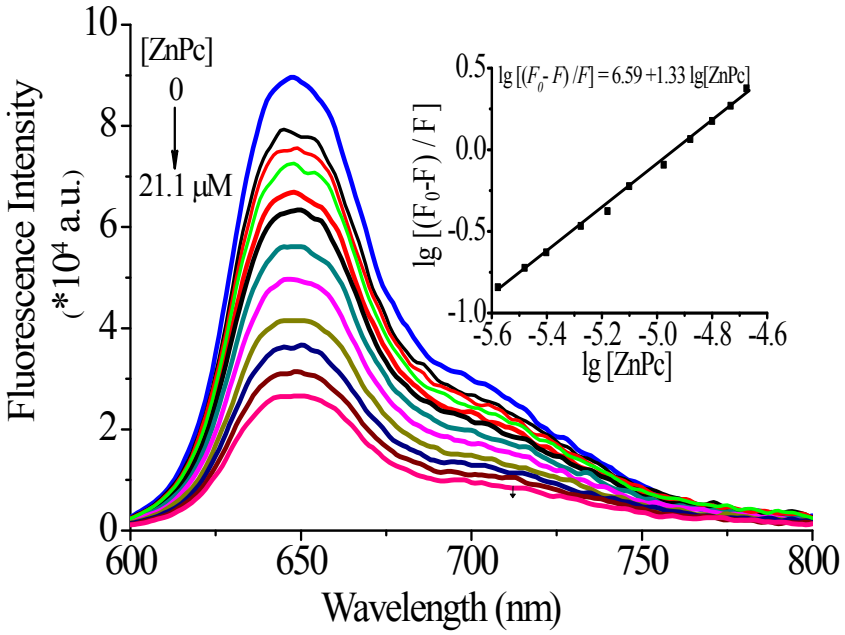

Fig. S5 Changes in fluorescence spectra of CPC in PBS upon titration with ZnPc. The inset shows the corresponding double logarithmic regression curve.

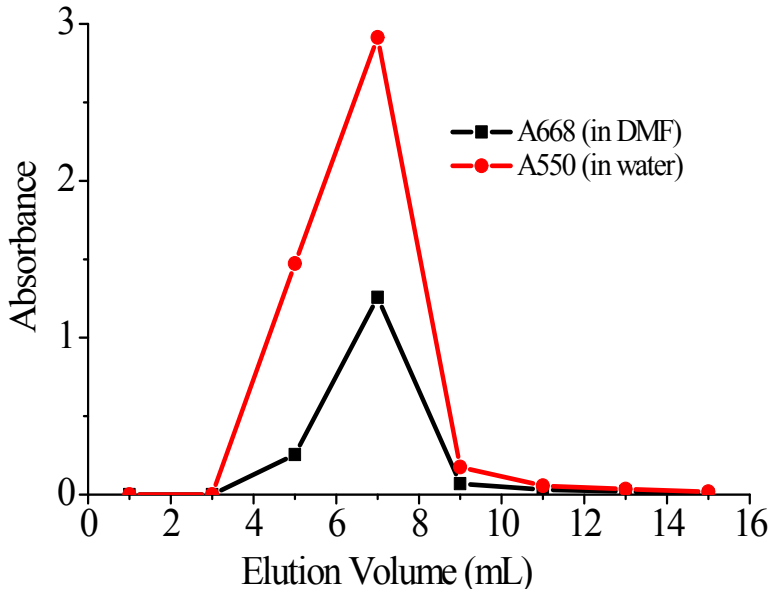

Fig. S6 Elution profiles of the mixture containing ZnPc and CPC as monitored by absorbance at 550 nm and ZnPc absorption in a diluted DMF solution at 668 nm.

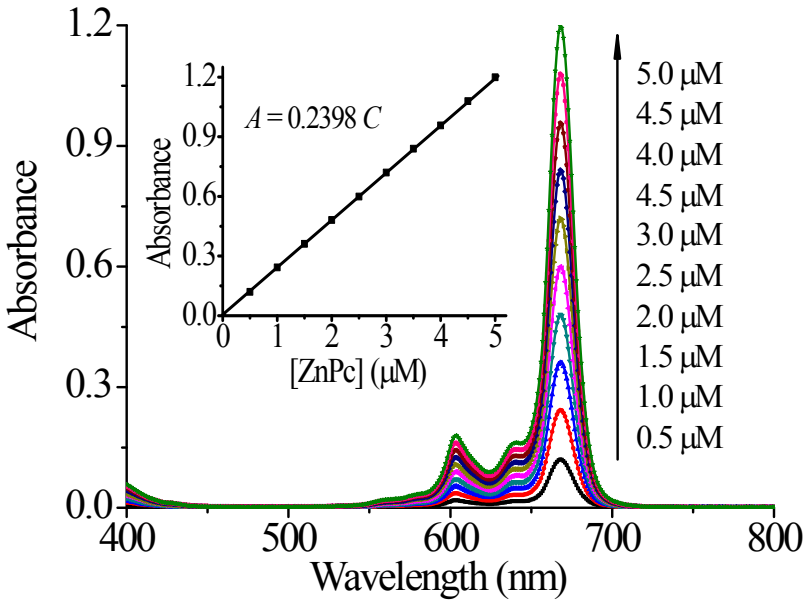

Fig. S7 Electronic absorption spectra of ZnPc at different concentrations in DMF. The inset plots the Q-band absorbance *vs.* the concentration of ZnPc.

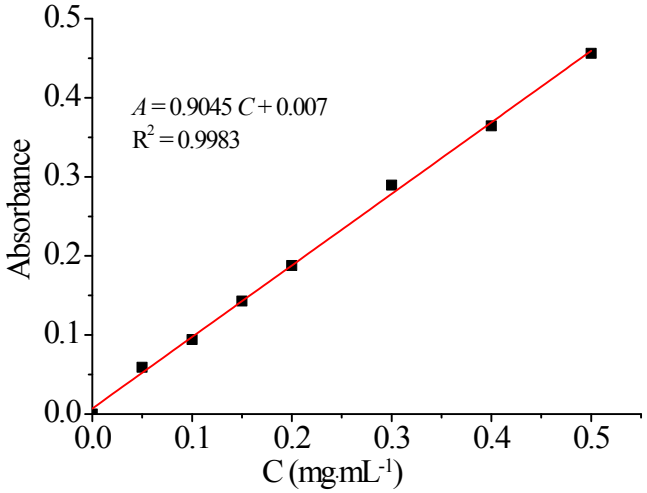

Fig. S8 Standard curve of Bradford protein assay using BSA as standard.

Table S3 Calculation of CI values

	$(D)_{ZnPc}$	$(D)_{CPC}$	$(D_{20})_{ZnPc}$	$(D_{20})_{CPC}$	CI[a]
J774A.1	0.04	0.0067	1.14	10	0.036
HepG2	1.4	0.233	2	20	0.712

[a]CI is determined by the equation $CI= [(D)_{ZnPc}/(D_{20})_{ZnPc}] + [(D)_{CPC}/(D_{20})_{CPC}]$, where $(D_{20})_{ZnPc}$ and $(D_{20})_{CPC}$ are the doses of ZnPc alone and CPC alone that inhibit the cell growth 20%, respectively. $(D)_{ZnPc}$ and $(D)_{CPC}$ are for doses in combination that also inhibit 20%.

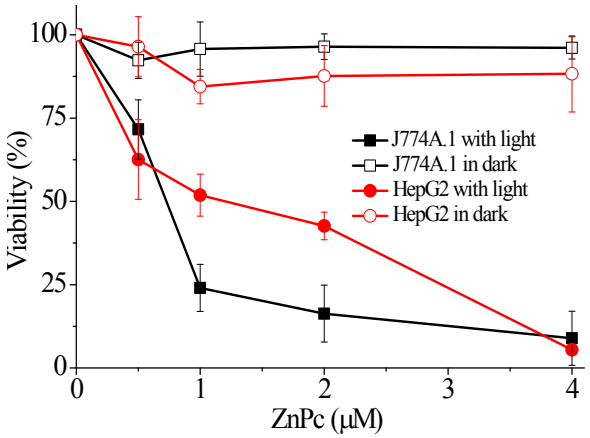

Fig. S9 Cytotoxic effects of ZnPc formulated with Cremophor EL (ZnPc-CEL) on HepG2 and J774A.1 cells in the absence and presence of light. Data are expressed as the means ± standard deviation (SD) (n = 3).

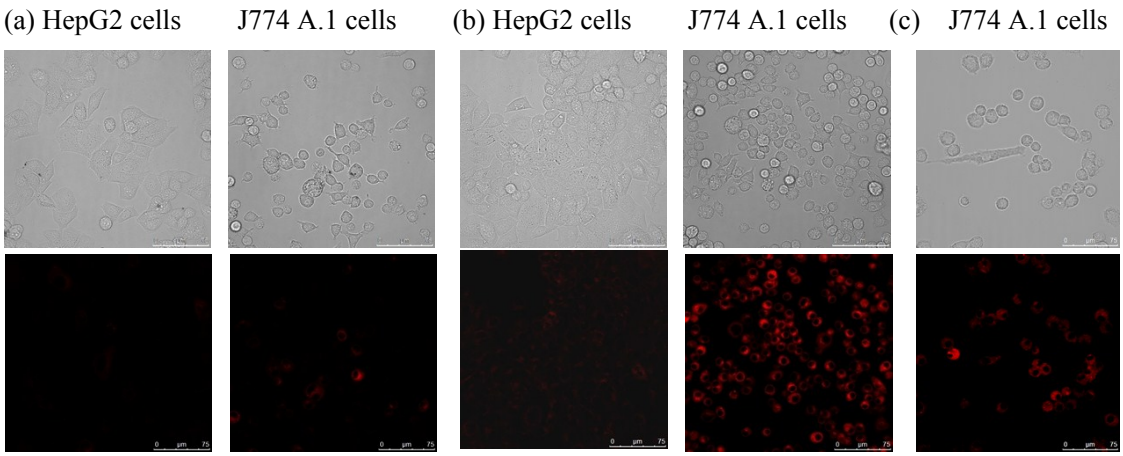

Fig. S10 Bright field (top row) and intracellular fluorescence (bottom row) images of HepG2 and J774A.1 cells after incubation with (a) 2 μM ZnPc; (b) ZnPc-CPC conjugate (2 μM based on ZnPc); (c) ZnPc-CEL (2 μM based on ZnPc) for 2 h.

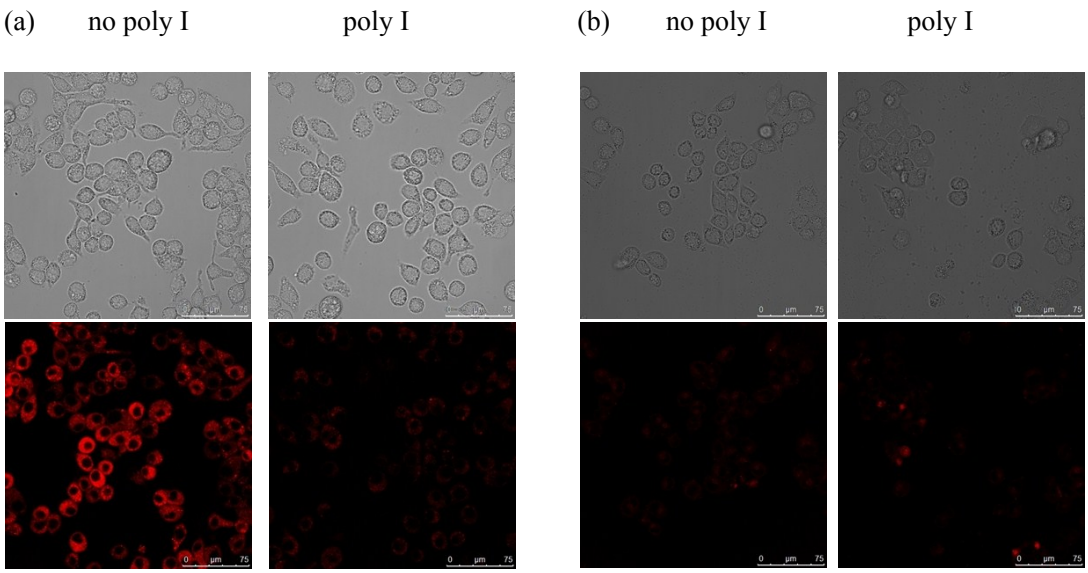

Fig. S11 Bright field (top row) and intracellular fluorescence (bottom row) images of J774A.1 cells after incubation with (a) ZnPc-CPC conjugate (2 μM based on ZnPc); (b) 2 μM ZnPc for 2 h, in the absence or presence of poly I.

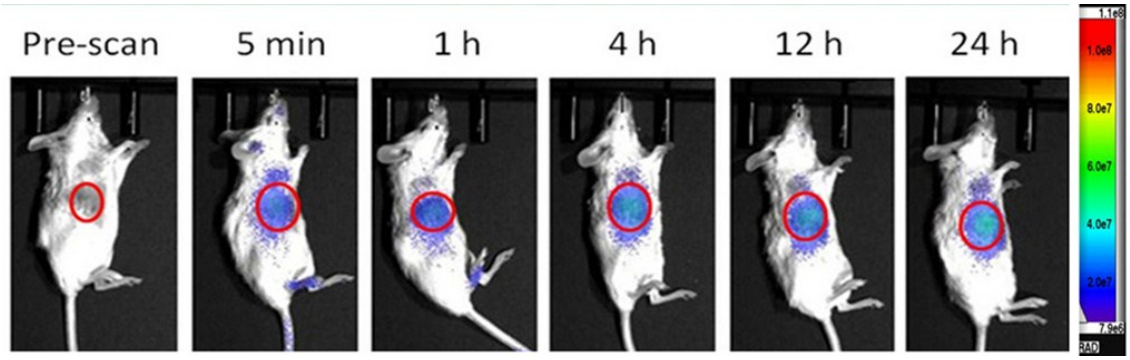

Fig. S12 Fluorescence images of tumour-bearing KM mice before and after intravenous injection of ZnPc-CPC conjugate. The red circles indicate tumour sites.