SUPPLEMENTARY INFORMATION

Amphiphilic Zinc Phthalocyanine Photosensitizers: Synthesis, Photophysicochemical Properties and *in vitro* Studies for Photodynamic Therapy

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1. Materials

3-Nitrophthalonitrile,[1] 4-nitrophthalonitrile,[2] 2-(2-{2-[3-(dimethylamino)phenoxy] ethoxy}ethoxy)ethanol **1,** 2-(2-{2-[3-(diethylamino)phenoxy] ethoxy}ethoxy)ethanol **4**,[3] 4-[2-(2-{2-[3-(dimethylamino)phenoxy]ethoxy}ethoxy)ethoxy]phthalonitrile **2**,[4] 4-[2-(2-{2-[3-(diethylamino)phenoxy]ethoxy}ethoxy)ethoxy]phthalonitrile **5**[4] were prepared according to procedure giving in the literature. All reagents and solvents were of reagent grade quality and were obtained from commercial suppliers. All solvents were dried and purified as described by Perrin and Armarego.[5]

2. Equipments

The IR spectra were recorded on a Perkin Elmer 1600 FT-IR spectrophotometer using KBr pellets. ^{1}H and ^{13}C-NMR spectra were recorded on a Varian Mercury 200 MHz spectrometer in $CDCl_3$, DMSO-d_6 and chemical shifts were reported (δ) relative to Me_4Si as internal standard. Mass spectra were measured on a Micromass Quatro LC/ULTIMA LC-MS/MS

spectrometer and MALDI-MS of complexes were obtained in dihydroxybenzoic acid as MALDI matrix using nitrogen laser accumulating 50 laser shots using Bruker Microflex LT MALDI-TOF mass spectrometer. Electronic absorption spectra were measured on a Shimadzu 2101 UV-Vis spectrophotometer. Fluorescence excitation and emission spectra were recorded on a Varian Eclipse spectrofluorometer using 1 cm path length cuvette at room temperature. Fluorescence lifetimes were measured by a time correlated single photon counting (TCSPC) method using FLUOROLOG-3 spectrofluorometer (Horiba JobinYvon, Edison, NJ) equipped with a NanoLED and a standard air cooled R928 PMT detector.

3. Photophysical parameters

3.1. Fluorescence quantum yields and lifetimes

Fluorescence quantum yields (Φ_F) are determined in DMSO by the comparative method using by equation 1,[6,7]

$$\Phi_F = \Phi_F(Std)\frac{F \cdot A_{Std} \cdot n^2}{F_{Std} \cdot A \cdot n^2_{Std}} \qquad (1)$$

where F and F_{Std} are the areas under the fluorescence emission curves of the samples (**2a-b**, **3a-b**, **5a-b** and **6a-b**) and the standard, respectively. A and A_{Std} are the respective absorbances of the samples and standard at the excitation wavelengths, respectively. n^2 and n^2_{Std} are the refractive indices of solvents used for the sample and standard, respectively. Unsubstituted ZnPc (Φ_F = 0.20)[8] was employed as the standard in DMSO. The absorbance of the solutions at the excitation wavelength ranged between 0.04 and 0.05.

Fluorescence lifetime measurements were performed with the FLUOROLOG-3 spectrofluorometer instrument using TCSPC technique. A NanoLED was used as a light source, and standard air cooled R928 PMT was used as a detector. The instrumental response

function was measured with the use of Ludox® (Sigma-Aldrich) colloidal solution at excitation wavelengths of samples. Signals were acquired using a Datastation FluoroHub-B single photon counting controller and data analysis was performed using the commercially available DAS 6 decay analysis software package from Horiba JobinYvon. Goodness of fit was assessed by minimizing the reduced chi squared function, each trace contained 10000 points and the reported lifetime values are the result from at least three independent measurements.

4. Photochemical parameters

4.1. Singlet oxygen quantum yields

Singlet oxygen quantum yield (Φ_Δ) determinations are carried out using the experimental set-up described in literature[9,10] in both DMSO and PBS solutions. Typically, a 3 mL portion of the respective unsubstituted or tetra-substituted zinc (II) phthalocyanine solutions (C= $1x10^{-5}$ M) containing the singlet oxygen quencher was irradiated in the Q band region with the photoirradiation set-up described in references.[9, 10] Φ_Δ values were determined in air using the relative method with ZnPc (in DMSO) or $ZnPcS_{mix}$ (in aqueous media) as standards. 1,3-Diphenylisobenzofuran (DPBF) and 9,10-antracenediyl-bis(methylene)dimalonoic acid (ADMA) were used as chemical quenchers for singlet oxygen in DMSO and aqueous media, respectively. Equation 2 was employed for the determination of Φ_Δ values:

$$\Phi_\Delta = \Phi_\Delta^{Std} \frac{R \cdot I_{abs}^{Std}}{R^{Std} \cdot I_{abs}} \qquad (2)$$

where Φ_{Δ}^{Std} is the singlet oxygen quantum yields for the standards ZnPc (Φ_{Δ}^{Std} = 0.67 in DMSO)[11] and $ZnPcS_{mix}$ (Φ_{Δ}^{Std} = 0.45 in aqueous media)[10], R and R_{Std} are the DPBF (or ADMA) photobleaching rates in the presence of the respective samples (**2a-b**, **3a-b**, **5a-b** and **6a-b**) and standards, respectively. I_{abs} and I_{abs}^{Std} are the rates of light absorption by the samples (**2a-b**, **3a-b**, **5a-b** and **6a-b**) and standards, respectively. To avoid chain reactions induced by quenchers (DPBF or ADMA) in the presence of singlet oxygen, the concentration of quenchers (DPBF or ADMA) was lowered to ~$3x10^{-5}$ M.[12] Solutions of sensitizer (C= $1x10^{-5}$ M) containing quencher (DPBF or ADMA) were prepared in the dark and irradiated in the Q band region. DPBF degradation at 417 nm and ADMA degradation at 380 nm were monitored. The light intensity $6.21x10^{15}$ photons s^{-1} cm^{-2} was used for Φ_{Δ} determinations.

4.2. Photodegradation quantum yields

Photodegradation quantum yield (Φ_d) determinations are carried out using the experimental set-up described in literature.[9,10] Photodegradation quantum yields were determined using equation 3 in both DMSO and PBS solutions.

$$\Phi_d = \frac{(C_0 - C_t) \cdot V \cdot N_A}{I_{abs} \cdot S \cdot t} \qquad (3)$$

where C_0 and C_t are the samples concentrations before and after irradiation, respectively. V is the reaction volume, N_A is the Avogadro's constant, S is the irradiated cell area and t is the irradiation time, I_{abs} is the overlap integral of the radiation source light intensity and the absorption of the samples. A light intensity of $2.17x\ 10^{16}$ photons s^{-1} cm^{-2} was employed for Φ_d determinations.

5. Binding of quaternized zinc (II) phthalocyanine derivatives to BSA protein

The binding of the water soluble quaternized zinc (II) phthalocyanine derivatives (**2b**, **3b**, **5b** and **6b**) to BSA were studied by spectrofluorometry at room temperature in PBS solutions. An aqueous solution of BSA (fixed concentration) was titrated with varying concentrations of the respective quaternized zinc (II) phthalocyanine solutions. BSA was excited at 280 nm and fluorescence emission spectra were recorded between 290 nm and 500 nm. The fluorescence intensity for BSA decreased by the addition of the quaternized zinc (II) phthalocyanines (**2b**, **3b**, **5b**, and **6b**) solutions and these reductions were related to quaternized phthalocyanine concentrations by the Stern-Volmer relationship (Equation 4):

$$\frac{F_0^{BSA}}{F^{BSA}} = 1 + K_{SV}^{BSA}[Pc] \qquad (4)$$

and k_{SV}^{BSA} is given by Equation 5:

$$K_{SV}^{BSA} = k_q \tau_{F(BSA)} \qquad (5)$$

where F_0^{BSA} and F^{BSA} are the fluorescence intensities of BSA in the absence and presence of quaternized phthalocyanines (**2b**, **3b**, **5b**, and **6b**) respectively; K_{SV}^{BSA}, the Stern-Volmer quenching constant; k_q, the bimolecular quenching constant; and $\tau_{F(BSA)}$, the fluorescence lifetime of BSA ($\tau_{F(BSA)}$= 10 ns).[13-15] The K_{SV}^{BSA} values were obtained from the plots of F_0^{BSA} / F^{BSA} versus [Pc] and the k_q values can be determined from equation 6.

6. Biological parameters

6.1. Cell Culture

All tissue culture media and reagents were obtained from PAN Biotech. Both human HeLa cells and human HuH-7 cells were cultured in DMEM supplemented with 10% fetal bovine

serum, 1% antibiotics (penicillin-streptomycin) and were incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. The cells were regularly subcultured according to their growth rate.

6.2. Light source

The Lumacare Model LC-122 consists of two main parts: a Quartz halogen lamp light source (100 W) housing with a control panel and power supply, and a fiber-optic probe (FOP) adaptors with filters designed to meet any optical protocol ranging from 380 to 750 nm. The fiber-optic probes of the LC-122 offers output power from 10 $mW.cm^{-2}$ up to 1 $W.cm^{-2}$ at their output tips, depending on filter transmission wavelength. For illumination protocol we used FOP systems with activation wavelength of 690 ± 10 nm. The exposure area was 50 · 75 mm and the distance between FOP tip and the cell plate surface was 20 cm. The light power of FOP systems on the exposure area was measured with a power meter that has silicon detector (Ophir). The exposure energy is controlled from the control panel by a timer.

6.3. Cytotoxicity

The cells were prepared as described above. Exponentially growing cells seeded onto 96-well plates at 4000 cells per well and allowed 24 h to attach. Various concentrations of phthalocyanines were added to exponentially growing cells and after 24 h the cells were irradiated with laser irradiation. For dark cytotoxicity experiments, phthalocyanine was added to triplicate wells in a final concentration of phthalocyanine of 0.5, 1, 2, 5, 10 and 20 μM and the cells were incubated with phthalocyanine for 24 h. The compounds were replaced with fresh medium. And then, cytotoxicity was measured using tetrazolium compound reagent (WST-1, Roche) for the quantification of cell proliferation and cell viability. It is a

colorimetric assay and based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in proliferating cells.

To each well, 20 µL of WST-1 compound was added and the plate was incubated for 4 h before reading. WST-1 tetrazolium compound is metabolized by metabolically active cells into a colored formazan product that can be measured by reading the absorbance at 450 nm with a microplate reader. The average of the triplicate wells for each sample was calculated.

6.4. Phototoxicity studies

For the phototoxicity experiments, the both cell lines were prepared as described above, treated with the same Pc photosensitizer concentrations of 0.5, 1, 2, 5, 10 and 20 µM and incubated for 24 h. After compound loading, the medium was removed and replaced with fresh medium. For phototoxicity, cells were exposed to the light from FOP systems with activation wavelength of 690 nm. The total light dose was approximately 1 and 2 J cm^{-2}. After illumination the cells were incubated for 24 h and then the plates were measured by reading optical density at 450 nm by the Universal Microplate Reader. After incubation, the optical densities were measured as above mentioned.

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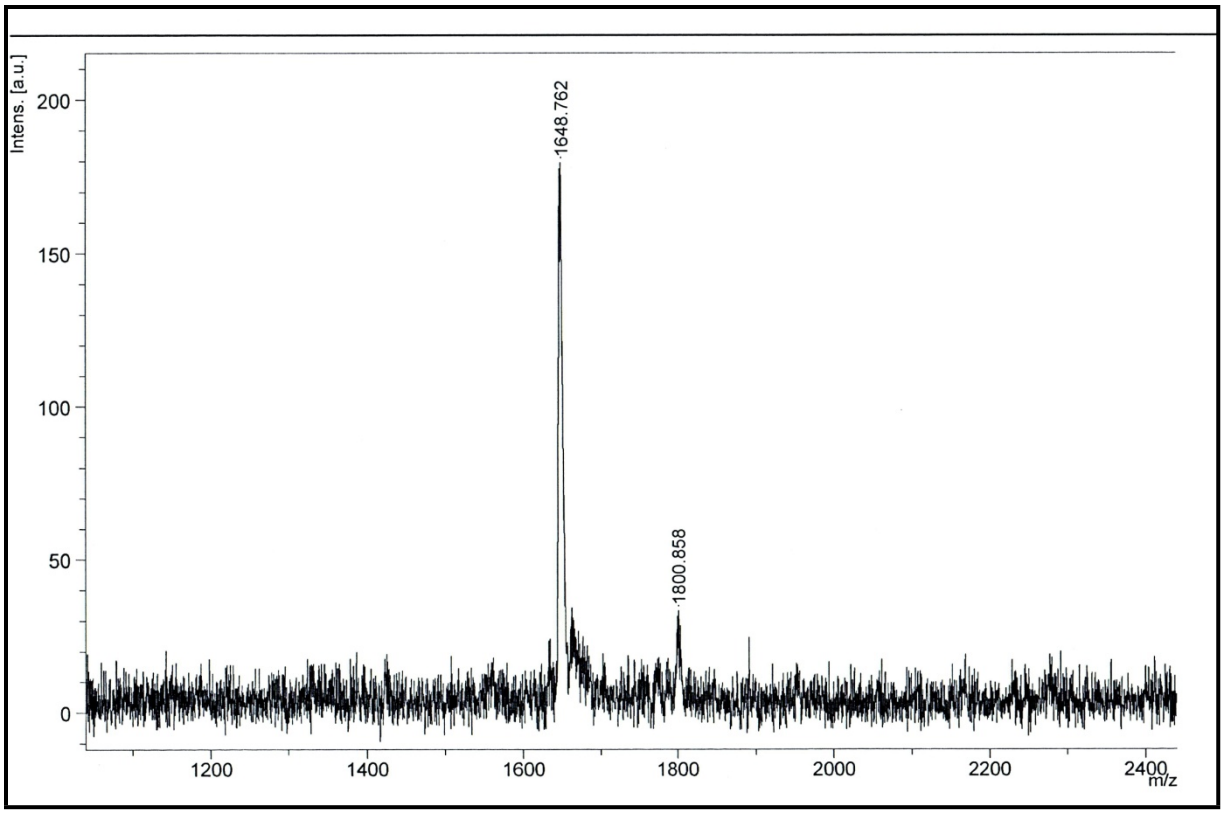

Fig. S1 MALDI-TOF mass spectrum of complex **2a**.

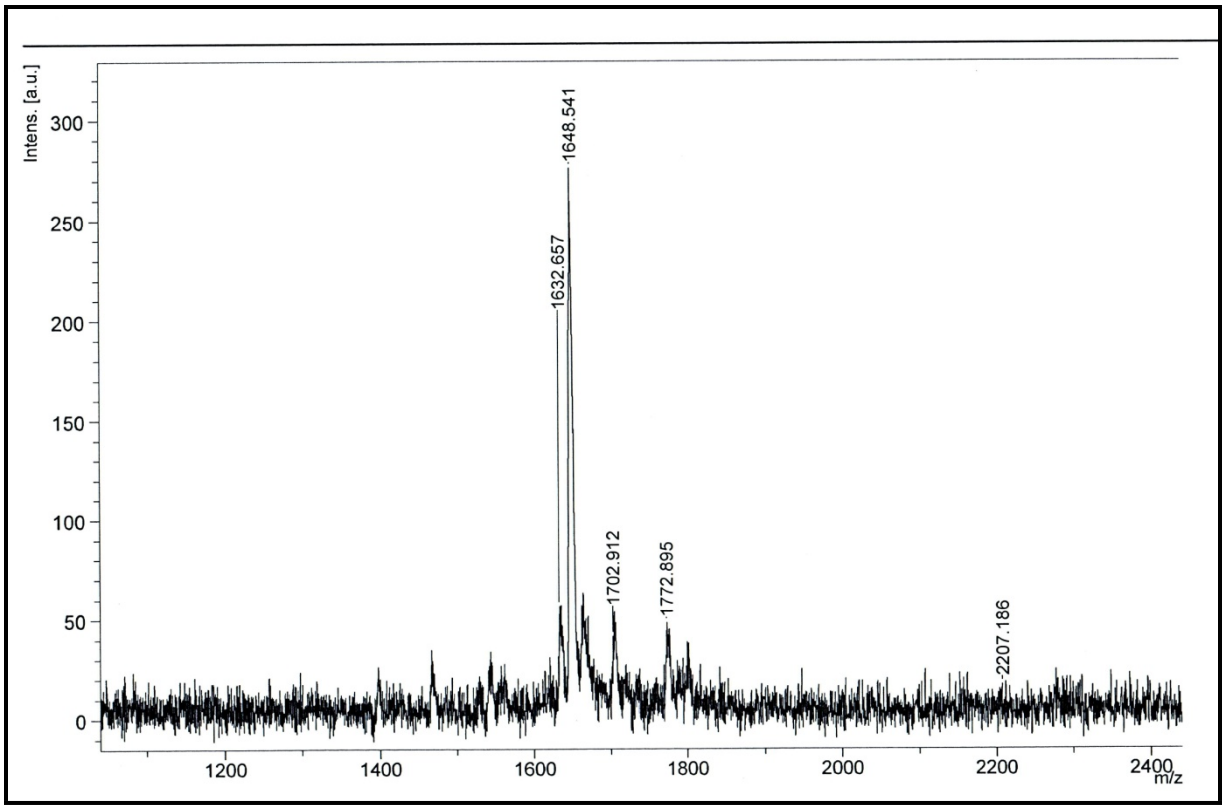

Fig. S2 MALDI-TOF mass spectrum of complex **3a**.

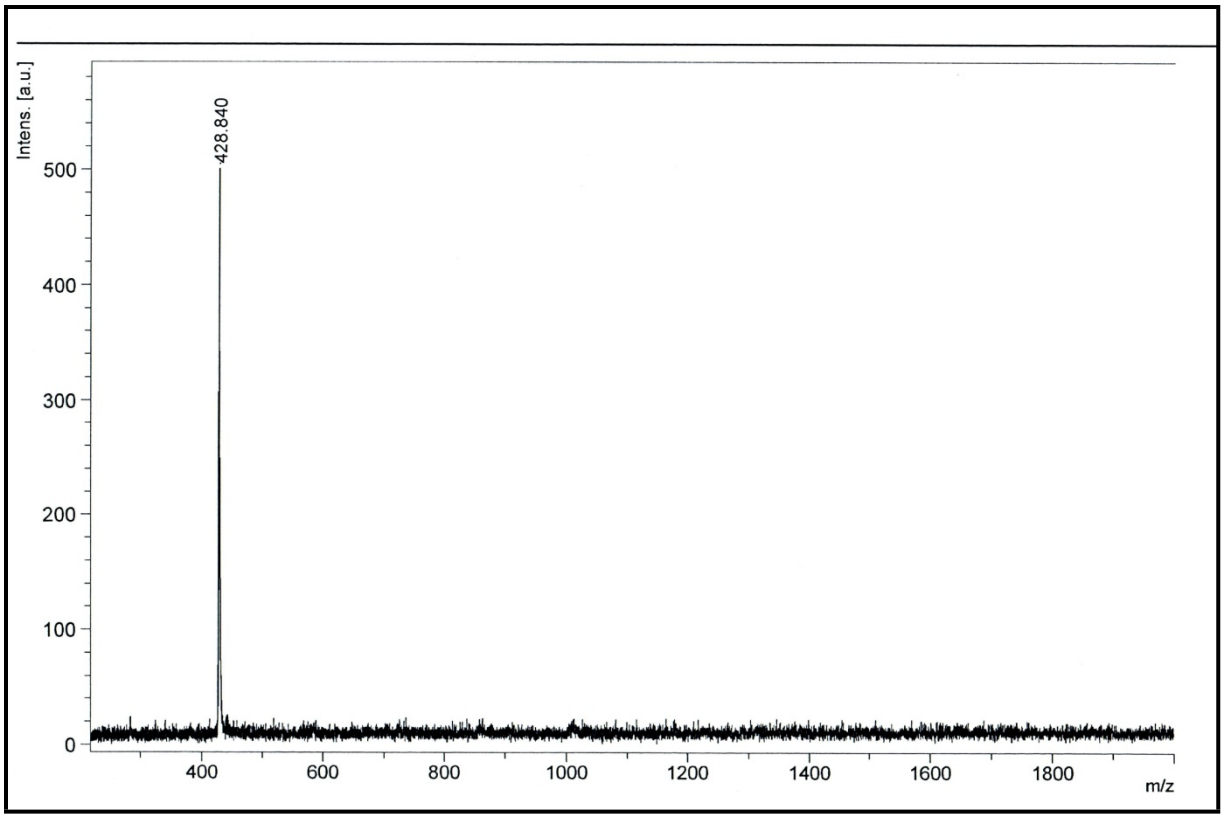

Fig. S3 MALDI-TOF mass spectrum of complex **2b**.

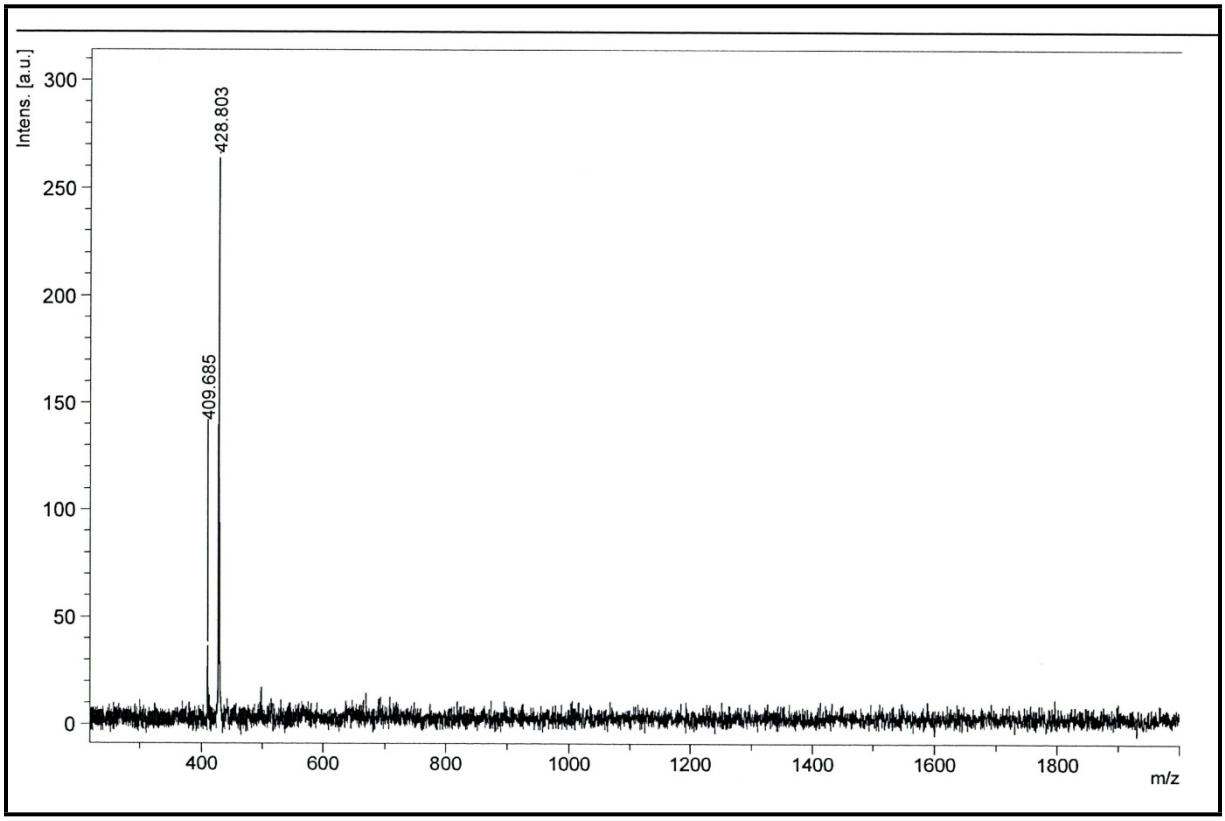

Fig. S4 MALDI-TOF mass spectrum of complex **3b**.

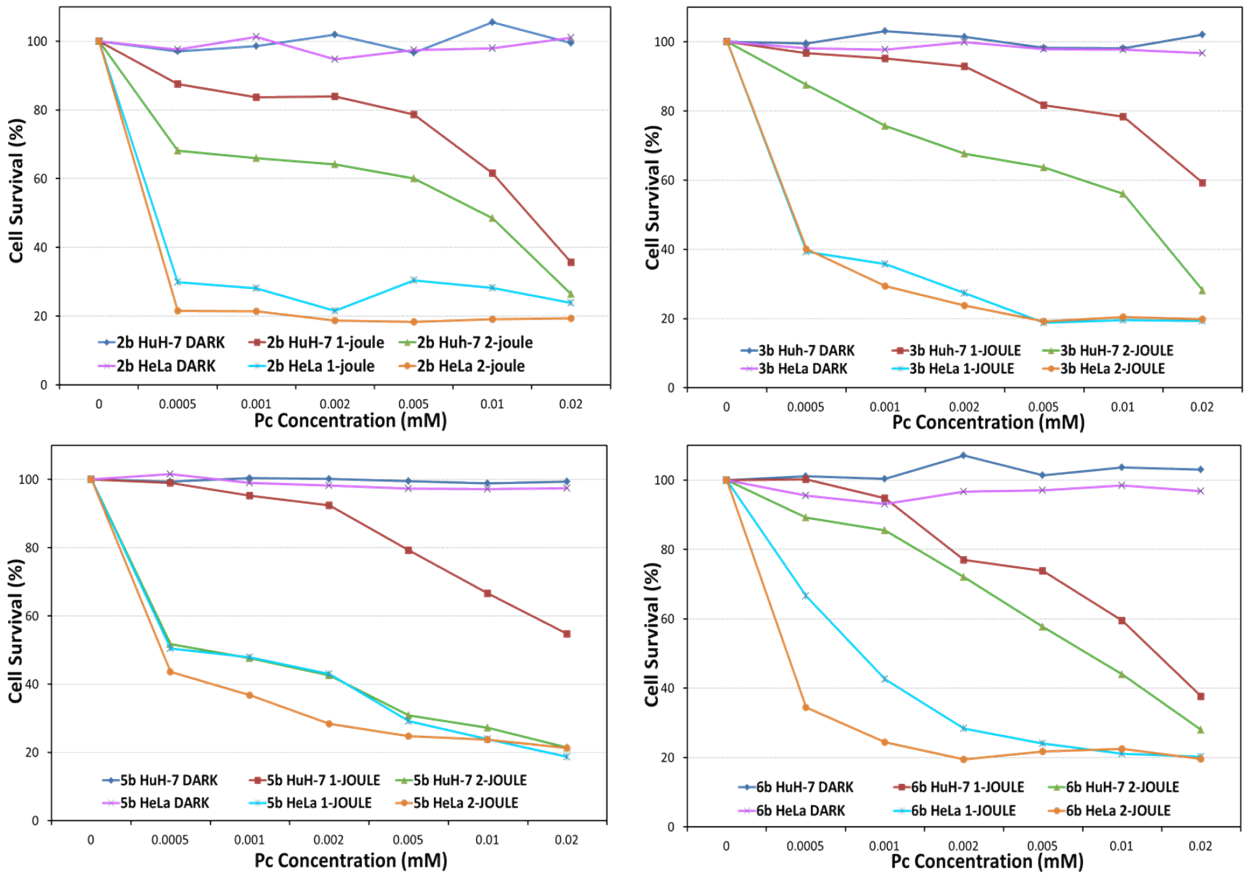

Fig. S5 Survival of HeLa and HuH-7 cells following illumination with 1 J.cm^{-2} and 2 J.cm^{-2} of 690 nm (± 10 nm) light after 24 h with various concentration of phthalocyanine molecules (**2b**, **3b**, **5b** and **6b**) administration. Each data represents the mean ± SD of three experiments.