Supporting Information

A Dual-Analyte Probe: Hypoxia Activated Nitric Oxide Detection with Phototriggered Drug Release Ability

Sandipan Biswas,[a] Y. Rajesh,[b] Shrabani Barman,[a] Manoranjan Bera[a], Amrita Paul[a], Mahitosh Mandal,[b] N.D. Pradeep Singh*[a]

[a]Department of Chemistry, Indian Institute of Technology
Kharagpur 721302, West Bengal, India. E-mail: ndpradeep@chem.iitkgp.ernet.in

[b]School of Medical Science and Technology, Indian Institute of Technology (IIT) Kharagpur, Kharagpur-721302, India

General Experimental Information:

^{1}H NMR (400 MHz) spectra was recorded on a BRUKER-AC 400 MHz spectrometer. Chemical shifts are reported in parts per million from tetramethylsilane with the solvent resonance as the internal standard (deuterochloroform: 7.26 ppm). Data is reported as follows: chemical shifts, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet), coupling constant (Hz). ^{13}C NMR (100 MHz) spectra were recorded on a BRUKER-AC 400 MHz Spectrometer with complete proton decoupling. Chemical shifts are reported in parts per million from tetramethylsilane with the solvent resonance the internal standard (deuterochloroform: 77.0 ppm). UV/vis absorption spectra were recorded on a Shimadzu UV-2450 UV/vis spectrophotometer, fluorescence emission spectra were recorded on a Hitachi F-7000 fluorescence spectrophotometer and HRMS spectra were recorded on a JEOL-AccuTOF JMS-T100L mass spectrometer. Imaging was done using a fluorescence microscope (IX 51, Olympus) high-performance CCD camera with the appropriate filter using Image-Pro discovery 5.1 software. Photolysis of the ester conjugate were carried out using 125 W medium pressure mercury lamp supplied by SAIC (India). Chromatographic purification was done with 60-120 mesh silica gel (Merck). For reaction monitoring, precoated silica gel 60 F254 TLC sheets (Merck) was used. Chromatographic purification was done with 60-120 mesh silica gel (Merck). RP-HPLC was taken using acetonitrile/water as mobile phase (detection: UV 300 nm).

Dithiothreitol (DTT), glutathione (GSH), cysteine (Cys), homocysteine (Hcy), β-Nicotinamide adenine dinucleotide (NADH), DEA•NONOate, Dehydroascorbic acid (DHA) and ascorbic acid (AA) were purchased from Sigma Aldrich and used without further purification. Deionized water and spectroscopic grade MeCN were used for spectroscopic studies. Superoxide solution ($O2^{-}$) was prepared by adding KO_2 (1 mg) to dry dimethyl sulfoxide (1 mL) and stirring vigorously for 10 min. Hydroxyl radical (OH•) was generated *in situ* by the Fenton reaction. Singlet oxygen ($^1O_2$) was generated from ClO^- and H_2O_2. Hypochlorite and hydrogen peroxide solution was prepared by dilution of commercial NaClO solution and H2O2 solution in deionized water. The aqueous solutions of $NaNO_2$ were freshly prepared and used as nitrite (NO_2^-) sources, respectively. A stock solution of DEA·NONOate was prepared in 0.01M NaOH solution. Lyophilized powder of nitroreductase enzyme expressed in Escherichia coli was purchased from Sigma Aldrich.

Synthesis procedure of Compound 6:

Compound 3 was synthesized according to the procedure reported in our earlier work.

Step (iii): Aluminium nitrate nonahydrate [$Al(NO_3)_3.9H_2O$] (1.725g, 4.6 mmol) was added portion wise to compound 3 (1g, 3.067 mmol) in acetic anhydride (Ac_2O, 19 ml) over a 10 min period and stirred at room temperature for overnight. After 12 h the reaction mixture was poured into ice-cold water (100 ml). The resulting yellow precipitate was filtered and washed with water. The solid was further purified by silica gel column chromatography using hexane : ethyl acetate (9:1 v/v) to get compound **4**. Yield = 45 %. ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 10.1 (s, 1H), 8.65 (s, 2H), 6.53 (s, 1H), 4.49 (s, 1H), 4.35-4.29 (q, J = 8 Hz, 2H), 1.39-135 (t, J = 8 Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ = 158.7 (1C), 158.2 (1C), 152.6 (1C), 148.8 (1C), 138.6 (1C), 132.1 (1C), 123.6 (1C), 116 (1C), 111.6 (1C), 108 (1C), 63.6 (1C), 29.6 (1C), 14.3 (1C). Calculated mass [MH^+]: 370.9878 and found mass [MH^+]: 370.9872.

Step (iv): Compound **4** (500 mg, 1.35 mmol) was deprotected by refluxing for 4 h with a mixture of conc. H_2SO_4 (2.5 ml) acetic acid (2.5 ml). After cooling the mixture was poured into water (50 ml) and let stand overnight. The resulting suspension made slightly basic with 50% NaOH with constant cooling. The yellow precipitate was filtered and washed with ice water to afford compound **5**. Yield = 86 %. ^{1}H NMR (DMSO-d_6, 400 MHz) δ = 8.72 (s, 1H),8.13 (s,

1H),6.66(s, 1H),4.92 (s, 2H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ = 158 (1C),156.7 (1C), 150.5 (1C),140 (1C),130 (1C), 127 (1C), 126.2 (1C), 108.6 (1C),106.4 (1C), 60 (1C),31.2 (1C). Calculated mass [MH^+]: 297.9667 and found mass [MH^+]: 298.9673.

Step (v): Compound 5, chlorambucil and potassium carbonate (K_2CO_3) was mixed in 5 ml dry DMF and warmed upto 60 ℃ for 6 h. After cooling, crushed ice was added to the reaction mixture. Then it was extracted with EtOAc (50 ml × 3) and washed with brine. The combined organic layer was dried over sodium sulphate (Na_2SO_4) and concentrated in vaccum. Then compound **6** was purified by silica gel column chromatography using hexane: EtOAc (9 : 1). Yield = 82 %. ^{1}H NMR ($CDCl_3$, 400 MHz) δ = 8.39 (s, 1H), 7.07 (d, J = 8.5 Hz, 2H), 6.72 (s, 1H), 6.64 (d, J = 8.6 Hz, 2H), 6.50 (s, 2H), 6.32 (s, 1H), 5.24 (s, 2H), 3.74 – 3.61 (m, 8H), 2.62-2.58 (t, J = 8 Hz, 2H), 2.49-2.45 (t, J = 8 Hz, 2H), 2.02-1.92 (m, 2H). ^{13}C NMR ($CDCl_3$, 100 MHz) δ = 172.5 (1C), 159.4 (1C), 157.8 (1C), 148.9 (1C), 147 (1C), 144.5 (1C), 130 (1C), 129.6 (2C), 129.5 (1C), 123 (1C),112.2 (1C), 110.8 (2C), 108.3 (1C), 104.1 (1C), 60.7 (1C), 53.6 (2C), 40.5 (2C), 33.8 (1C), 33.2 (1C), 26.4 (1C). Calculated mass [MH^+]: 522.1198 and found mass [MH^+]: 522.1203.

Photophysical studies:

The absorption and emission spectra of a degassed solution of **6** (1 × 10^{-5} M) in MeOH-PBS buffer (30:70) were recorded on a UV-Vis and fluorescence spectrophotometer, respectively. The fluorescence quantum yields (Φ_f) were measured using equation 1.

$$(\phi_f)_{CG} = (\phi_f)_{ST} \frac{(Grad_{CG})}{(Grad_{ST})} \frac{\eta_{CG}^2}{\eta_{ST}^2} \qquad (1)$$

Where the subscripts CG and ST denote caged compound and standard, respectively. Quinine sulfate in 0.1 (N) H_2SO_4 solutions was taken as standard. Φ_f is fluorescence quantum yield, Grad is the gradient from the plot of integrated fluorescence intensity vs absorbance, and η the refractive index of the solvent.

Nitroreductase assay:

Nitroreductase activity experiments were performed employing recombinant forms of Nitroreductase (Lyophilized powder) expressed in *Escherichia coli*. Stock solutions of **6** were prepared in pure DMSO (1 mM) and diluted by PBS buffer solution (0.01 mM NADH, phosphate buffered saline, pH 7.0) to a final concentration (1×10^{-5} M) containing 1% DMSO. Nitroreductase was added to the compound solution to a final enzyme concentration of 10 μg/mL at 37 ºC. The fluorescence intensities were recorded at 3 min intervals from 0 to 10 min.

Photolysis of 6:

To check the ability of **6** as a photoresponsive drug delivery system, 20 ml solution of **6** in ACN-water (30: 70) mixture (1×10^{-4} M) of **6** was treated with 10 eqv $Na_2S_2O_4$ and excess amount of nitric oxide (NO) and exposed to visible-light (≥ 410 nm) by medium pressure mercury lamp (125 W) using UV cut-off filter (1 M $NaNO_2$ solution).

To demonstrate the precise control over the drug delivery, the solution was exposed to light and dark condition, periodically. Aliquots of 25 μL were injected in each case in the HPLC. The released drug was quantified from the HPLC peak area in comparison with injected authentic sample.

Photochemical quantum yield determination of T6:

A 20 mL solution of **T6** (1×10^{-4} M) was prepared in acetonitrile-HEPES buffer (30:70). Nitrogen was passed throughout the solution for 30 min and irradiated using 125 W medium pressure Hg lamp as light source (≥ 410 nm) and 1M $NaNO_2$ solution as UV cut-off filter. At regular interval of time, 20μl of the aliquots was taken and analyzed by RP-HPLC (detection: UV 254 nm). Peak areas were determined by RP-HPLC, which indicated gradual decrease of the caged compound with time, and the average of three runs. The reaction was followed until the consumption of the ester is less than 5% of the initial area. Based on HPLC data for caged compound, the natural logarithm of the concentration of caged compound (lnC) (from HPLC peak area) versus irradiation time was plotted. We observed an exponential correlation for the disappearance of the caged compounds which suggested a first order reaction. Further, the quantum yield for the photolysis of caged compound was calculated using equation (2)

$$(\Phi)_{CG} = (\Phi)_{act} \times [(k_p)_{CG}/(k_p)_{act}] \times [F_{act}/F_{CG}] \text{ ---------- (2)}$$

Where, the subscript 'CG' and 'act' denote caged compound and actinometer, respectively. Φ is the relative photolysis quantum yield, k_p is the photolysis rate constant and F is the fraction of light absorbed. Potassium ferrioxalate was used as an actinometer.

Determination of incident photon flux (I_0) of the UV lamp by potassium ferrioxalate actinometry:

Potassium ferrioxalate actinometry was used for the determination of incident photon flux (I_0) of the UV lamp used for irradiation. Solution of potassium ferrioxalate, 1,10-phenanthroline and the buffer solution were prepared following the literature procedure.[1]

0.006 M solution of potassium ferrioxalate was irradiated using 125 W medium pressure Hg lamp as visible light source (≥ 410 nm) and 1M $NaNO_2$ solution as UV cut–off filter. At regular interval of time (3 min), 1 mL of the aliquots was taken out and to it 3 mL of 1,10-phenanthroline solution and 2 mL of the buffer solution were added and the whole solution was kept in dark for 30 min. The absorbance of red phenanthroline-ferrous complex formed was then measured spectrophotometrically at 510 nm. The amount of Fe^{2+} ion was determined from the calibration graph. The calibration graph was plotted by measuring the absorbance of phenanthroline-ferrous complex at several known concentration of Fe^{2+} ion in dark. From the slope of the graph the molar absorptivity of the phenanthroline-ferrous complex was calculated to be 1.10×10^4 M^{-1} cm^{-1} at 510 nm which is found to be similar to reported value. Using the known quantum yield (1.188 ± 0.012) for potassium ferrioxalate actinometer at 406.7 nm, the number of Fe^{2+} ion formed during photolysis and the fraction of light absorbed by the actinometer, the incident intensity (I_0) of the 125 W Hg lamp was determined as 2.886 x 10^{16} quanta s^{-1} (in fluence rate: 20 mW cm^{-2}).

Antiproliferative activity assays

Cell lines:

HeLa cell line was obtained from the National Centre for Cell Science (NCCS), Pune, India, and maintained in dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Cells were cultured at 37 °C in a CO_2 incubator (Thermo Fisher Scientific, USA).

In Vitro Cytotoxicity assay:

Before Irradiation:

The cytotoxicities of **6**, **A6**, **T6** and chlorambucil (Cbl) were determined without UV irradiation on HeLa cells using MTT assay.[2] cells (1×10^5 cells/well of a 96-well plate) were treated for 48 h with different concentrations (0.1–10 μg mL^{-1}) and cell viability was determined by MTT assay (Mossman, 1983), measuring absorbance at 595 nm using a micro plate reader (Bio-Rad 550). Cell viability was calculated using the formula, Viability (%) = 100 × A2/A1; [Where A2 = Absorbance of the treated cell; A1 = Absorbance of the control cells]).

After Irradiation:

Cancer cell line (1×10^5 cells/well of a 96-well plate) was treated with different concentrations of T6 and Cbl (0.1–10 μg mL^{-1}) and incubated for 4 h at 37 °C in CO_2 incubator. Thereafter, the cells were irradiated by UV-visible light, $\lambda \geq 410$ nm for 45 min (keeping the culture plate 6 cm away from the light source) using UV-visible lamp (Bangalore GeneiPvt. Ltd.) under aseptic condition. After irradiation, the cells were again incubated for 48 h and cell viability was measured by MTT assay (Mossman, 1983). Cell viability was calculated as described above.

Real-time in-vitro drug release and cytotoxicity:

Cytotoxicity of time-dependent control-released drug with irradiation on HeLa cell line was determined by conventional MTT assay (Mossman, 1983). Briefly, cells in exponential growth phase were trypsinised and seeded in 96-well culture plates (1×10^5 cells/well). After 12 h of cell seeding, the medium was replaced with a fresh complete medium (DMEM) containing 10 μg mL^{-1} of **T6** and was kept for 4 h at 37 °C in a CO_2 incubator. Cells were irradiated with visible light (≥ 410 nm) for 0–45 min. The assay was performed in triplicate for each time frame (0, 15, 30, and 45 min). Then the plate was further incubated for 48 h at 37 °C in a CO_2 incubator. Real-time in-vitro drug release was measured spectrophotometrically in accordance with measuring cell viability by MTT assay.

Fluorescence microscopy:

HeLa cells (1×10^5 cells/6-well) were treated with 10 μg mL^{-1} of **6** and put aside for 4 h at 37 °C. The oxygen concentration was reduced to 1% from 20% by introduction of nitrogen gas (N_2) to make hypoxic condition.[3] After that, the cells were incubated with NO donor DEA·NONOate. Then, cells were irradiated with UV-visible light (≥ 410 nm) for 0–45 min. Thereafter, the plate was incubated for 48 h at 37 °C in a CO_2 incubator. The effect of each step and the released drug on the cell was visually observed by fluorescence microscopy (1X51, Olympus) and a high-performance charge-coupled device (CCD) camera with an appropriate filter using Image-Pro Discovery 5.1 software.

The quantification of the cellular fluorescence intensities were carried by ImageJ software. The captured cellular images were processed in the ImageJ software and the fluorescence intensity values for the selected regions were compared.

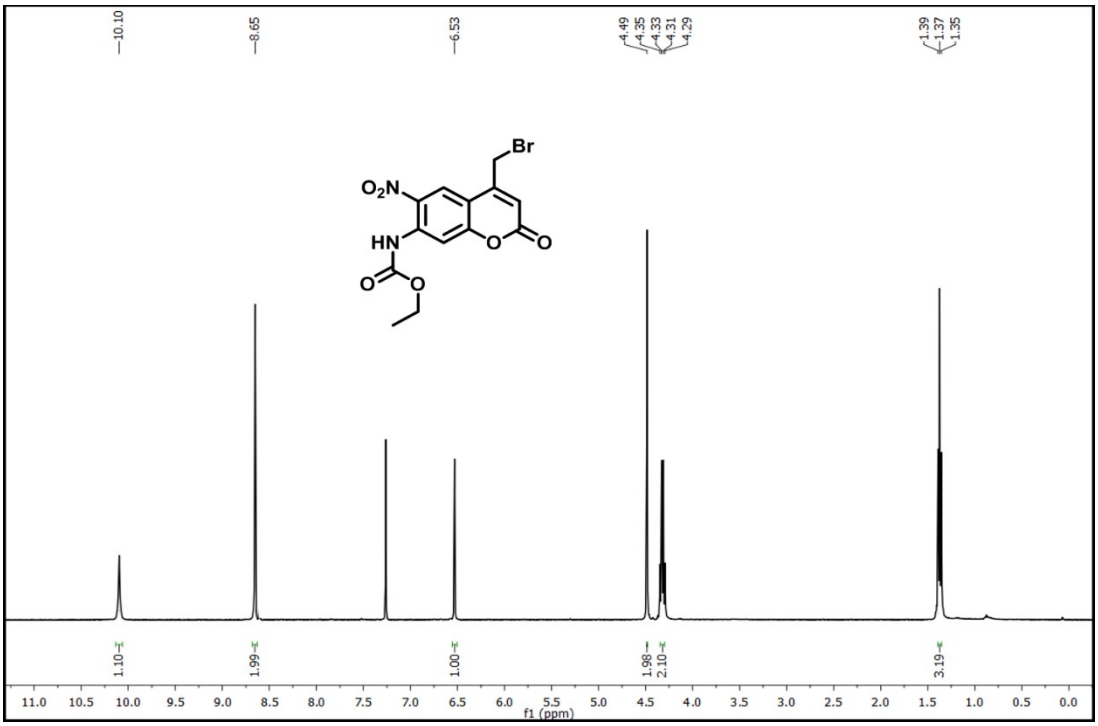

Figure S1: ^{1}H nmr spectrum of **4**.

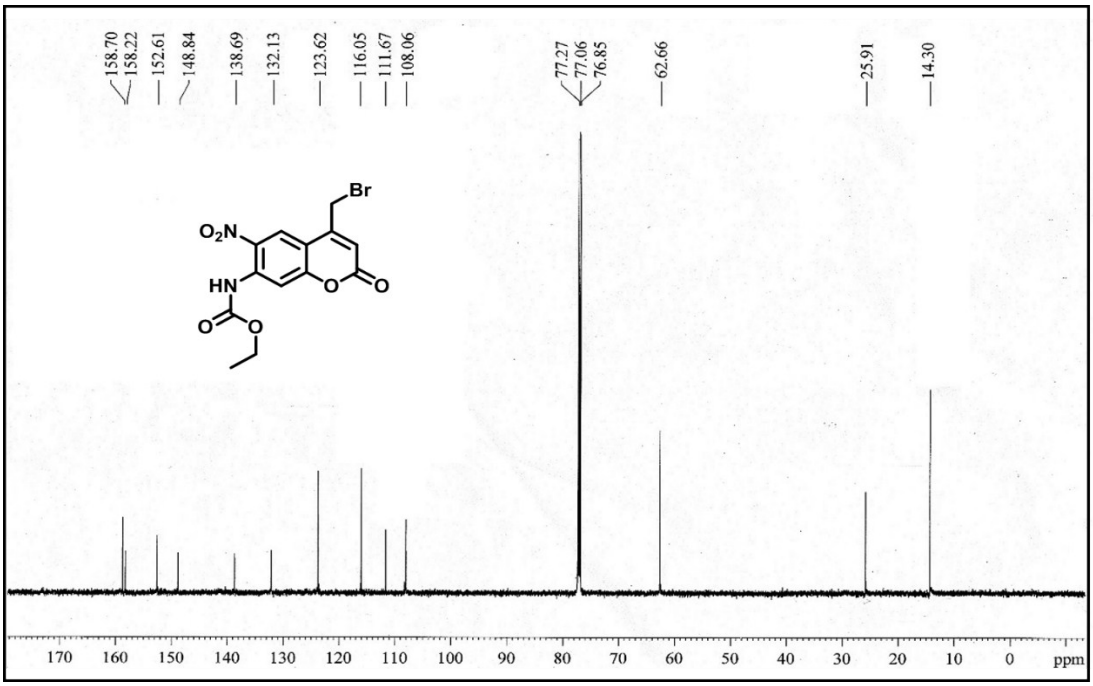

Figure S2: ^{13}C nmr spectrum of **4**.

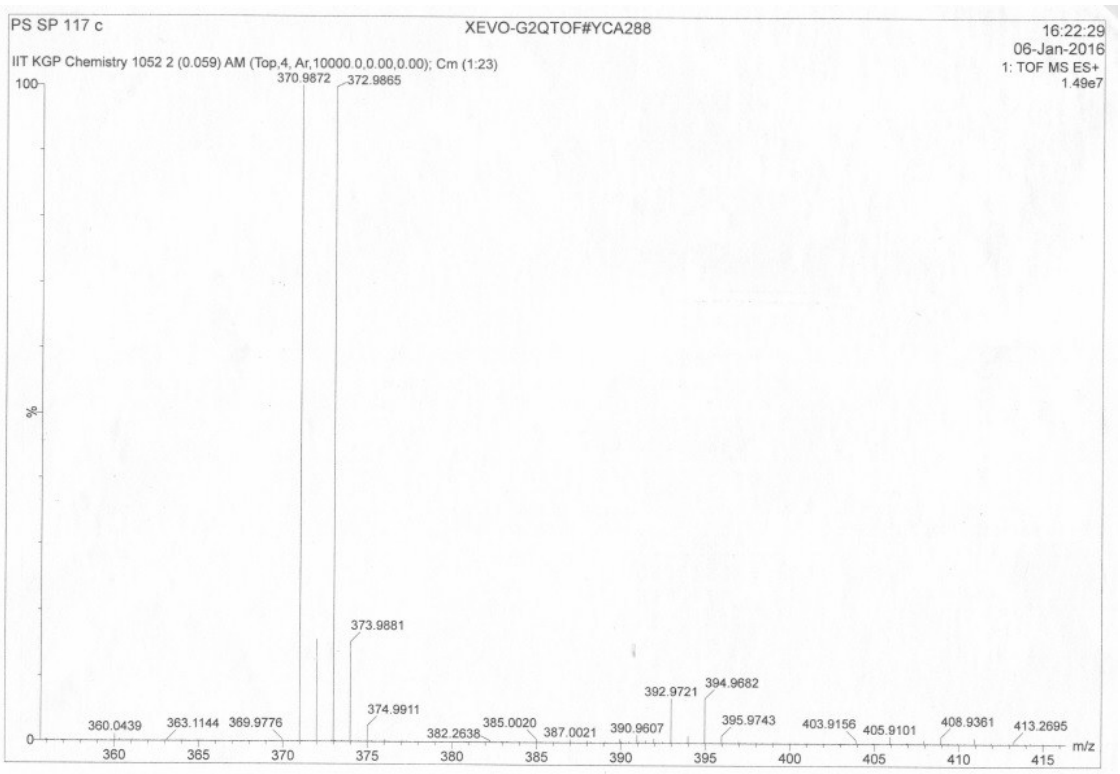

Figure S3: HRMS of **4**.

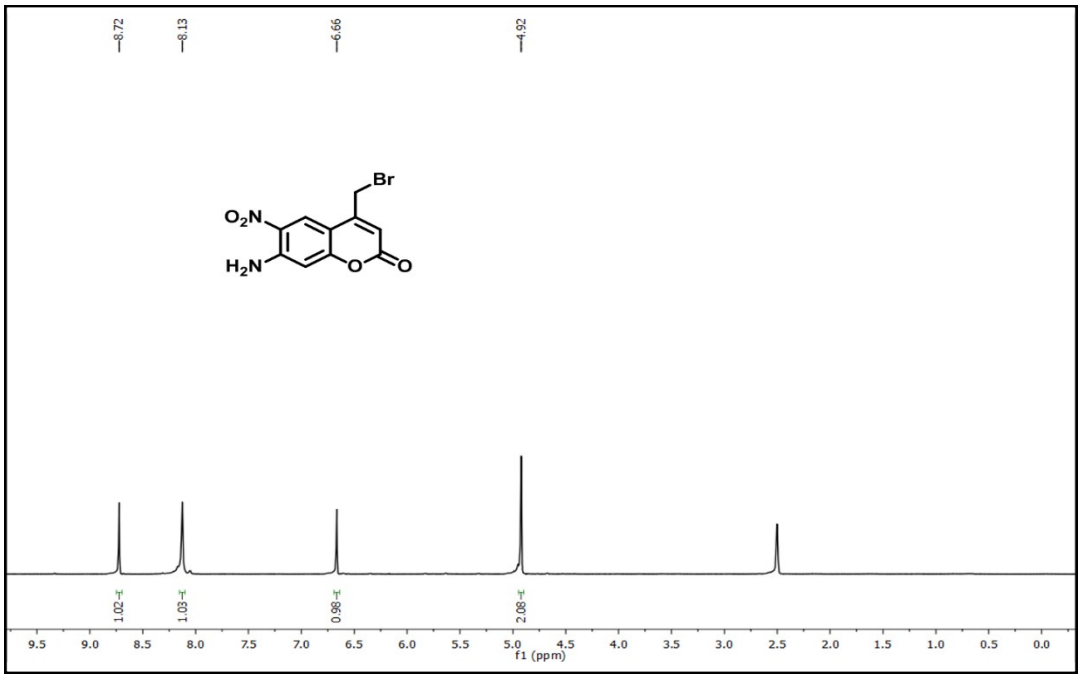

Figure S4: ^{1}H nmr spectrum of **5**.

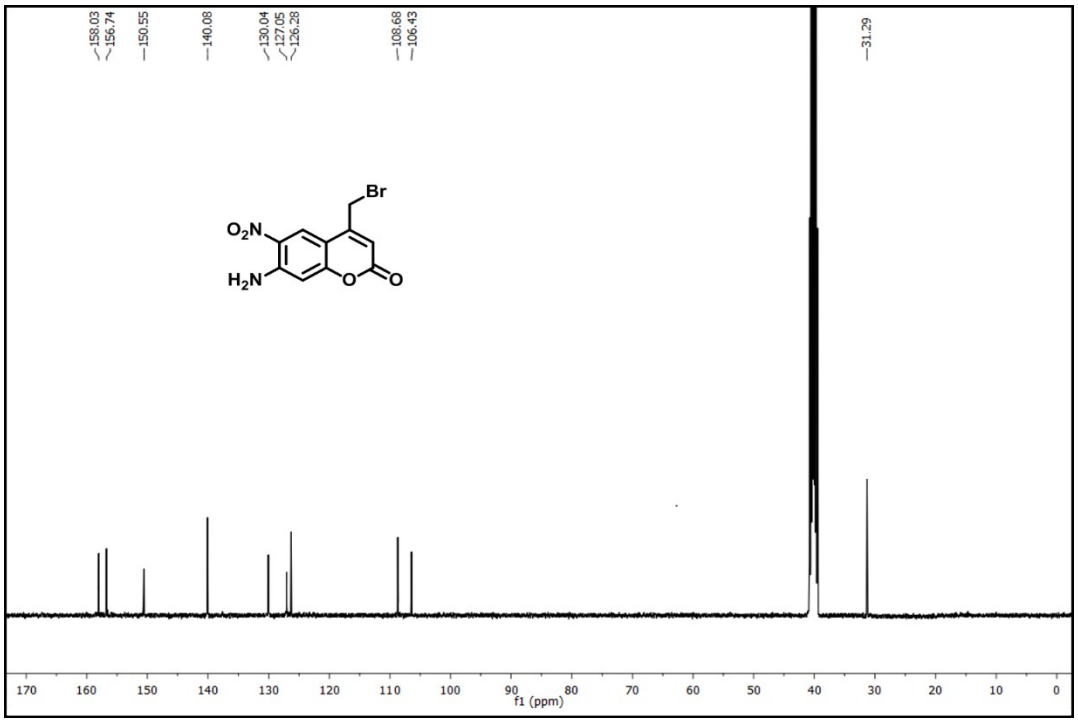

Figure S5: ^{13}C nmr spectrum of **5**.

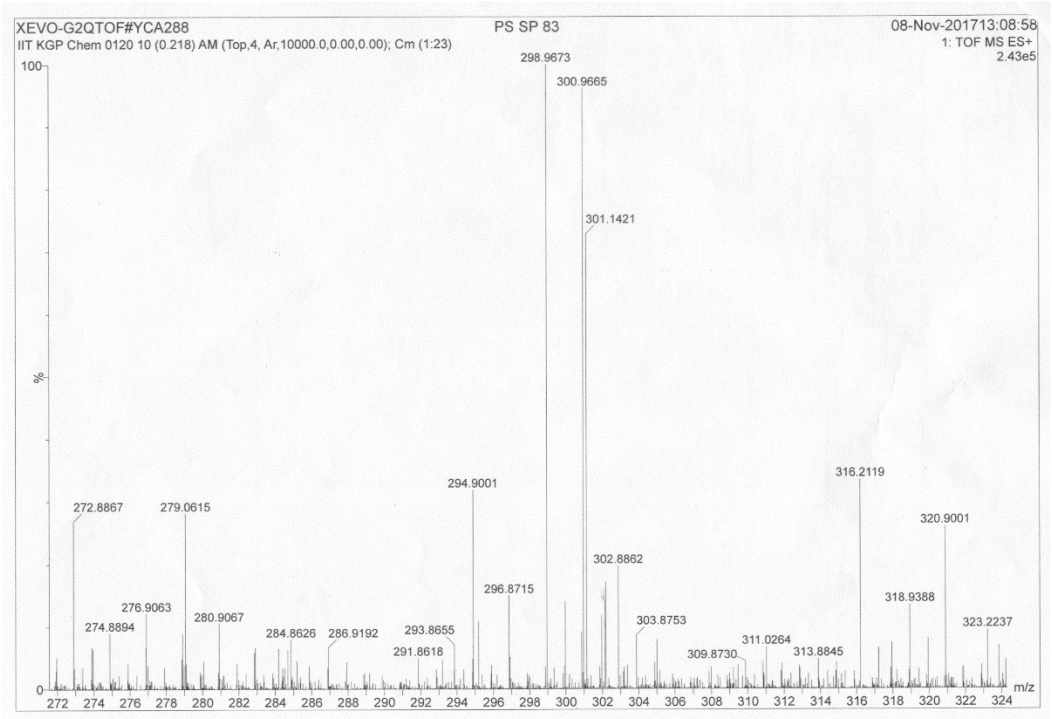

Figure S6: HRMS of **5**.

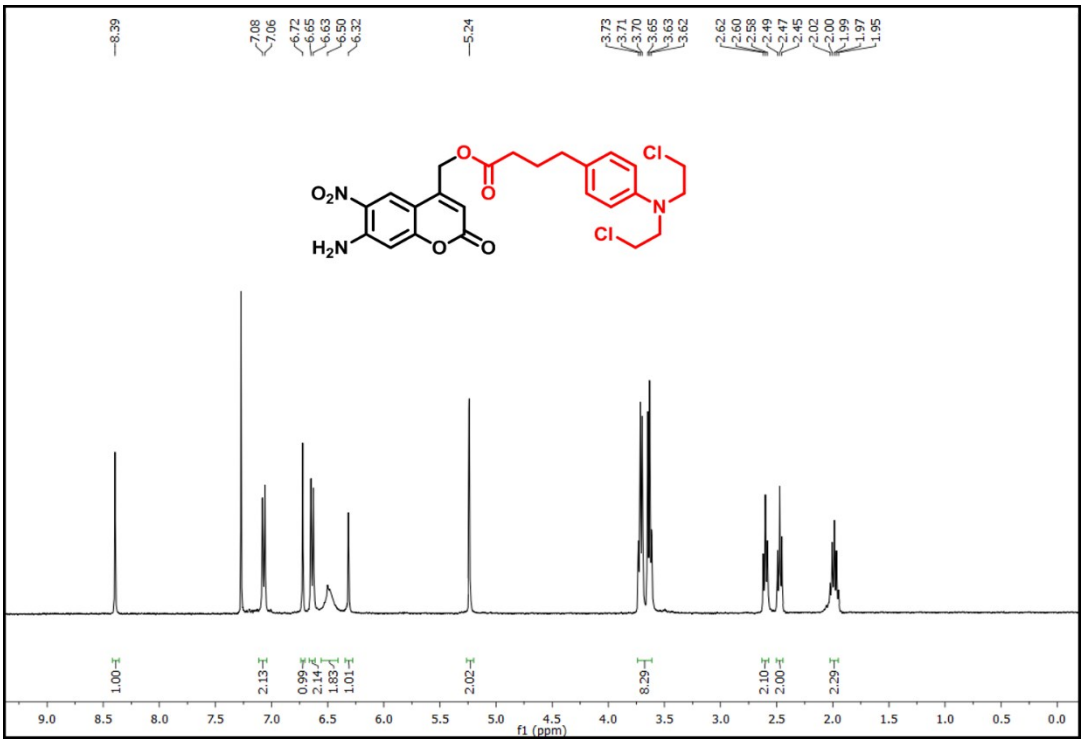

Figure S7: ^{1}H nmr spectrum of **6**.

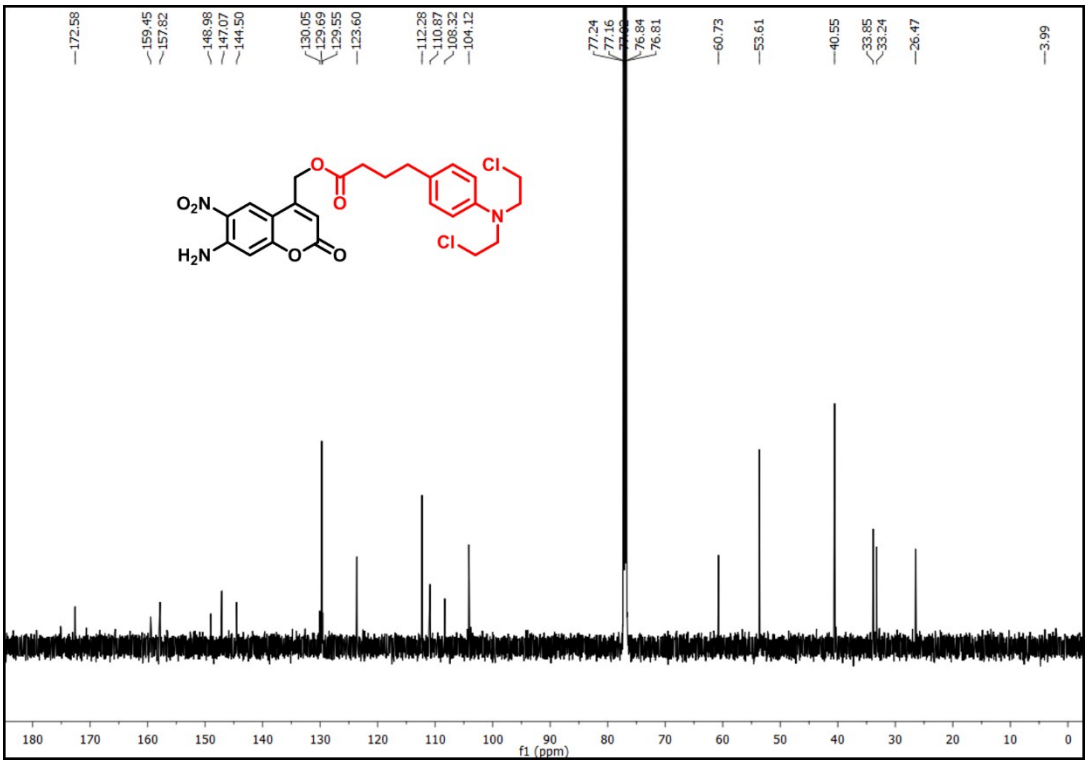

Figure S8: ^{13}C nmr spectrum of **6**.

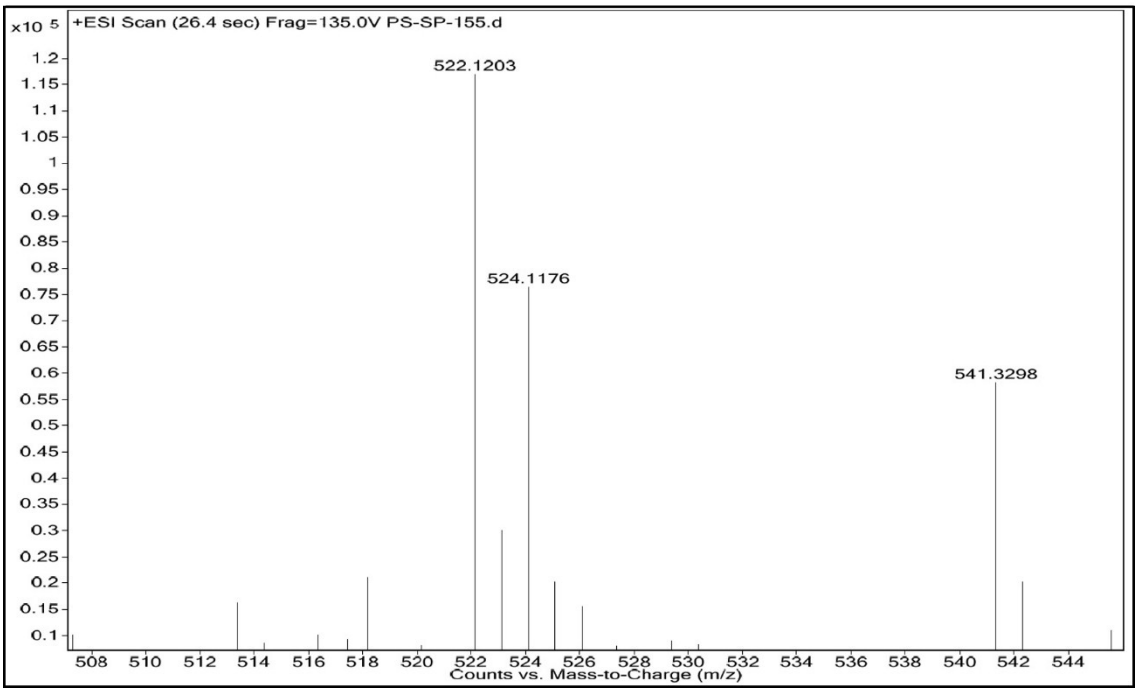

Figure S9: HRMS of **6**.

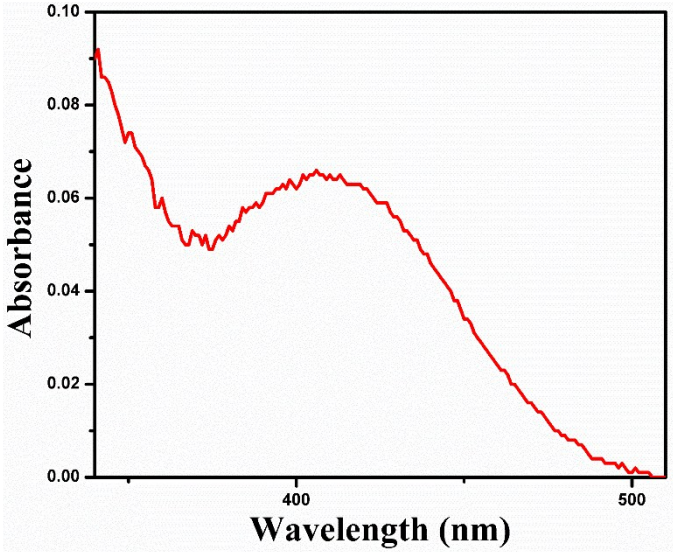

Figure S10: UV absorption spectrum of **6** (1×10^{-5} M) in MeOH-PBS buffer (30:70).

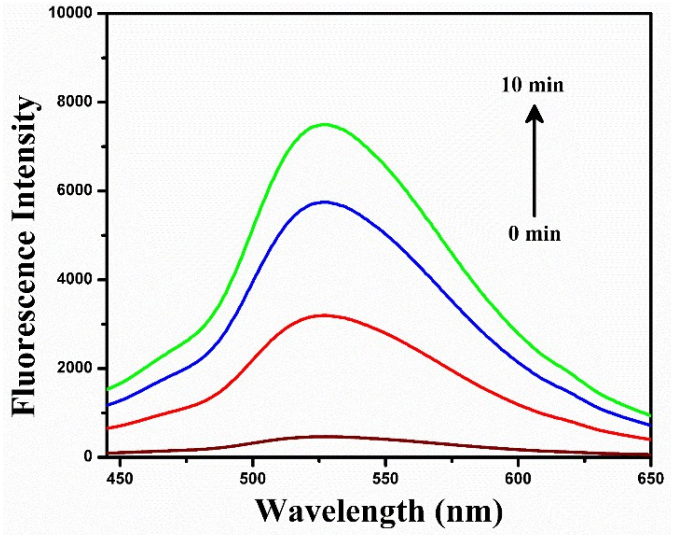

Figure S11: Fluorescent spectra of **6** (1×10^{-5} M in PBS buffer (pH = 7.4) with 1% DMSO, 0.01 mM NADPH) treated with nitroreductase (10 µg/ml).

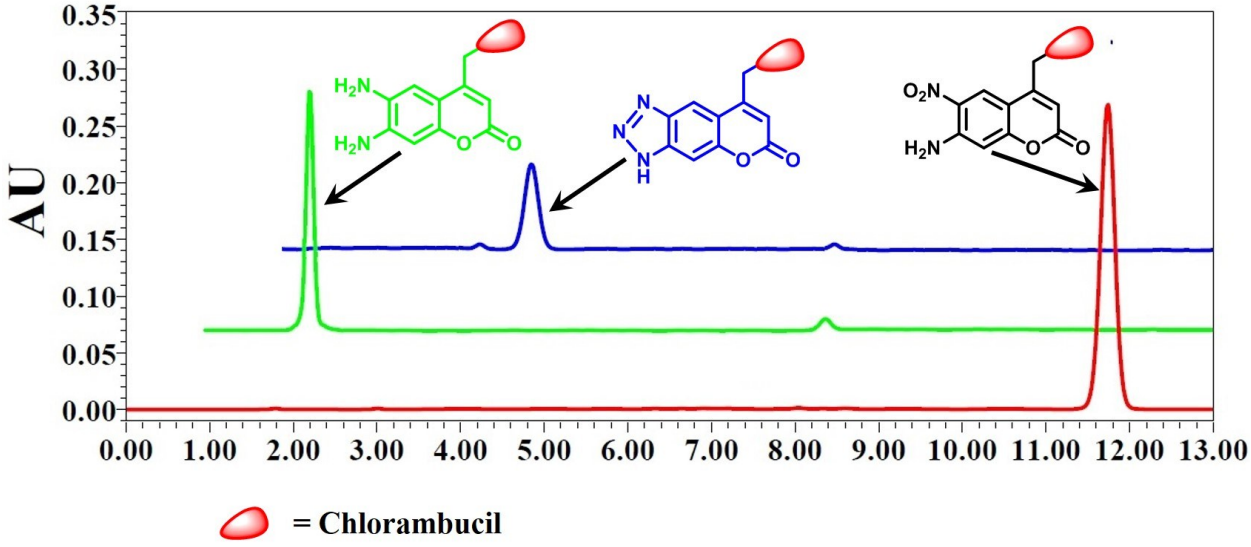

Figure S12: HPLC overlay chromatogram of **6**, 6 after treatment with $Na_2S_2O_4$ (10 eqv) and **6** after treatment with both $Na_2S_2O_4$ (10 eqv) and excess amount of NO.

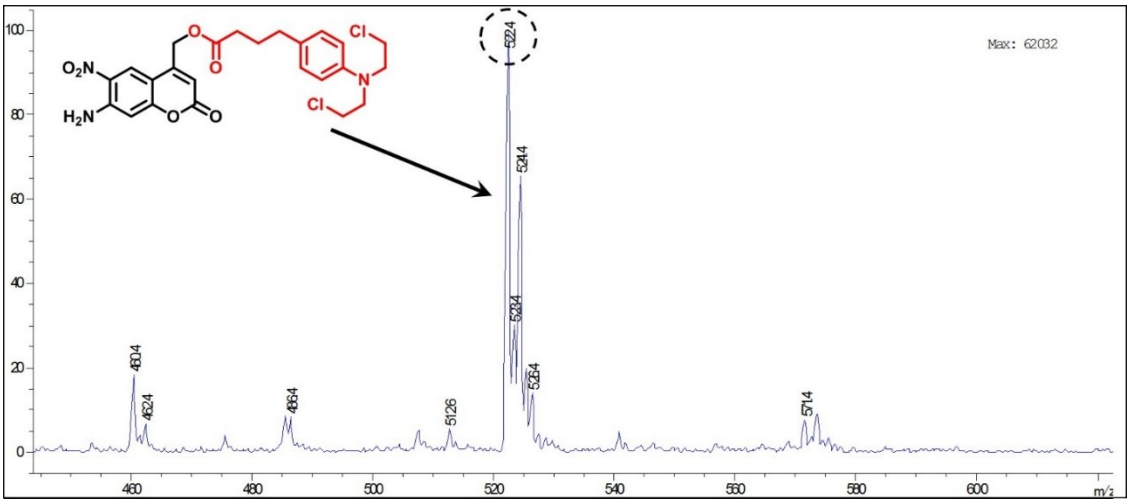

Figure S13: ESI-MS spectrum of **6** collected (t_R 11.66 min) in the HPLC analysis.

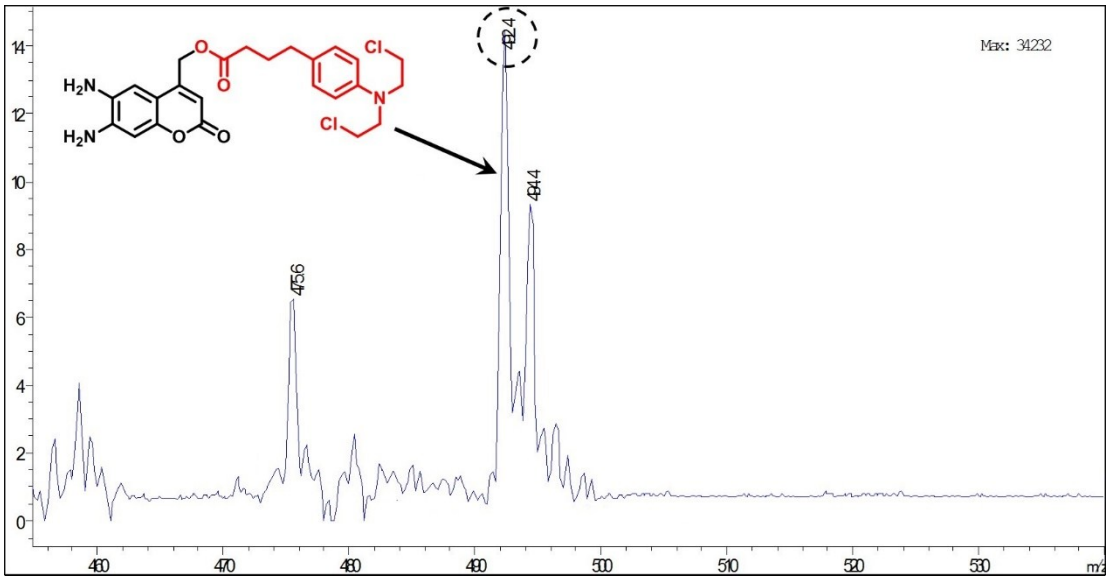

Figure S14: ESI-MS spectrum of A**6** collected (t_R 2.1 min) in the HPLC analysis (after treatment with $Na_2S_2O_4$).

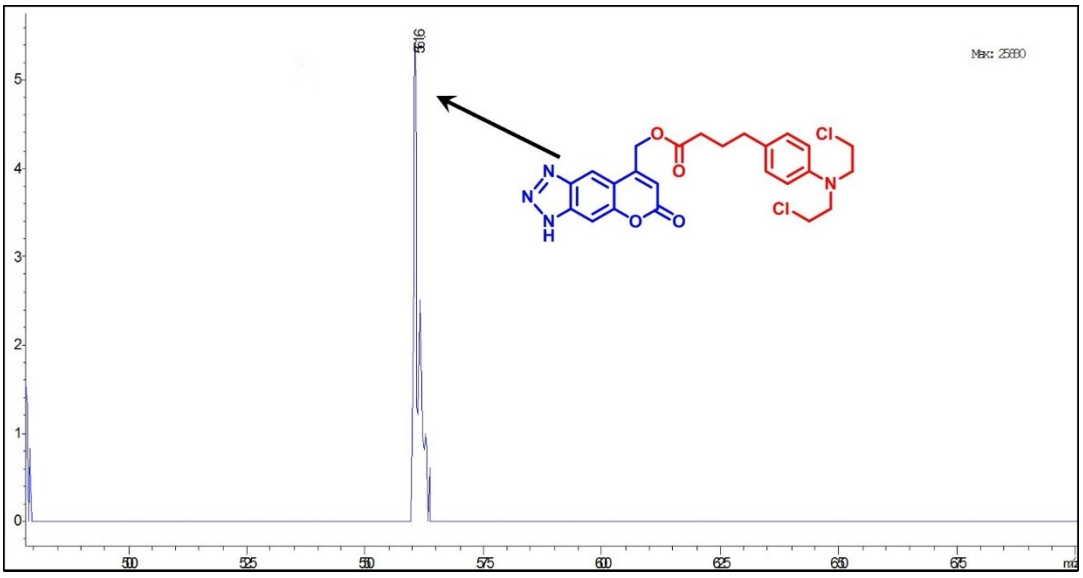

Figure S15: ESI-MS spectrum of **T6** collected (t_R 4.92 min) in the HPLC analysis (after treatment with NO).

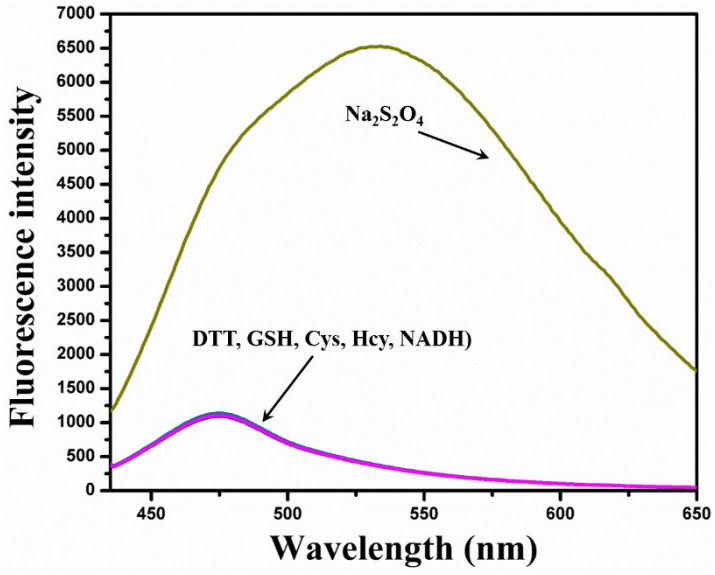

Figure S16: Fluorescence spectra of **6** recorded in the presence of various biological reductants (10 eqv) like dithiothreitol (DTT), glutathione (GSH), cysteine (Cys), homocysteine (Hcy), and β-Nicotinamide adenine dinucleotide (NADH).

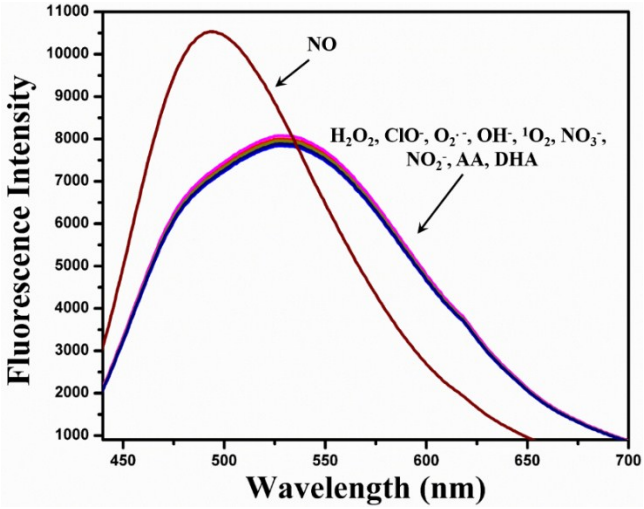

Figure S17: Fluorescence spectra of **A6** recorded in the presence of various reactive oxygen or nitrogen species (10 eqv) including H_2O_2, ClO^-, $O_2^{\cdot -}$, OH^-, $^1O_2$, NO_3^-, NO_2^-, ascorbic acid (AA), and dehydroascorbic acid (DHA).

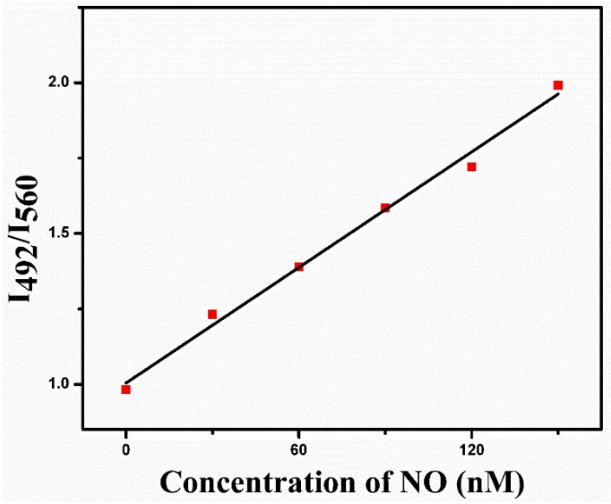

Figure S18: Fluorescent intensity ratio (I_{492}/I_{560}) of **A6** as a function of NO concentration at pH 7.4 (PBS buffer).

The detection limit (DL) of **4** was determined by the following equation[4,5] DL = K*Sb1/S

Where K = 2 or 3; Sb1 = standard deviation of the blank solution; S = slope of the calibration curve.

The detection limit was found to be 24 nM.

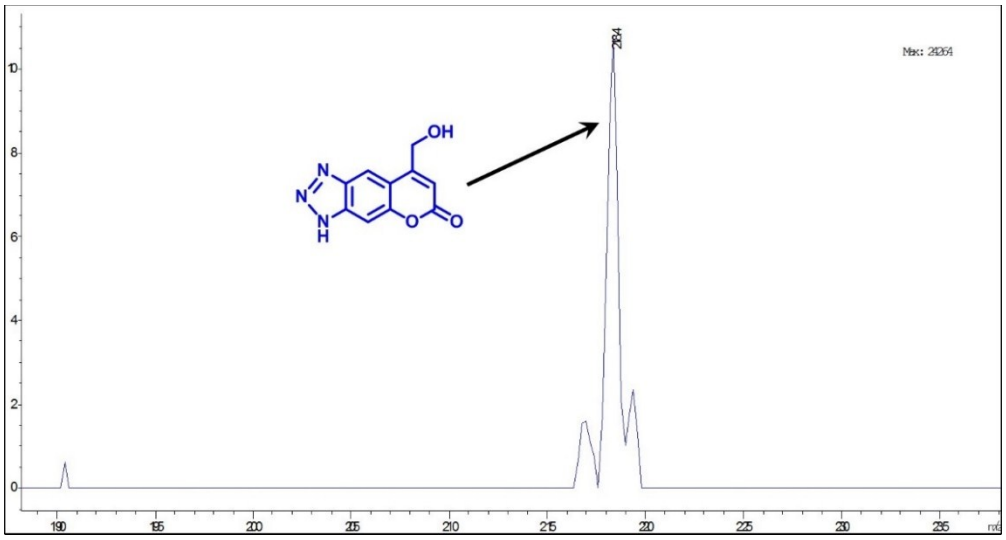

Figure S19: ESI-MS spectrum of **PP** collected (t_R 4.48 min) in the HPLC analysis (after photolysis).

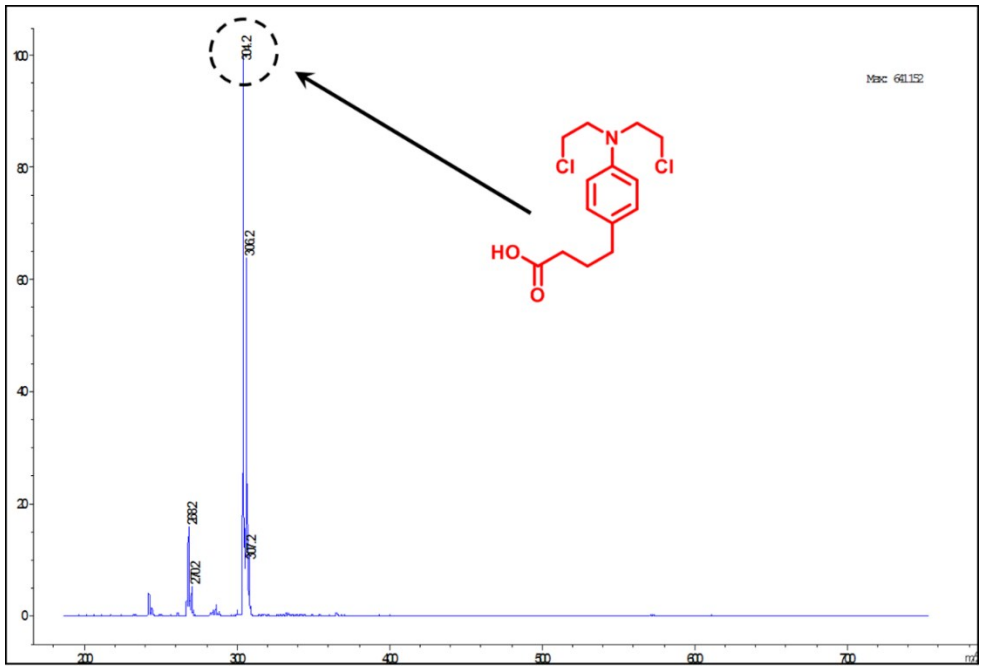

Figure S20: ESI-MS spectrum of chlorambucil collected (t_R 2.65 min) in the HPLC analysis (after photolysis).

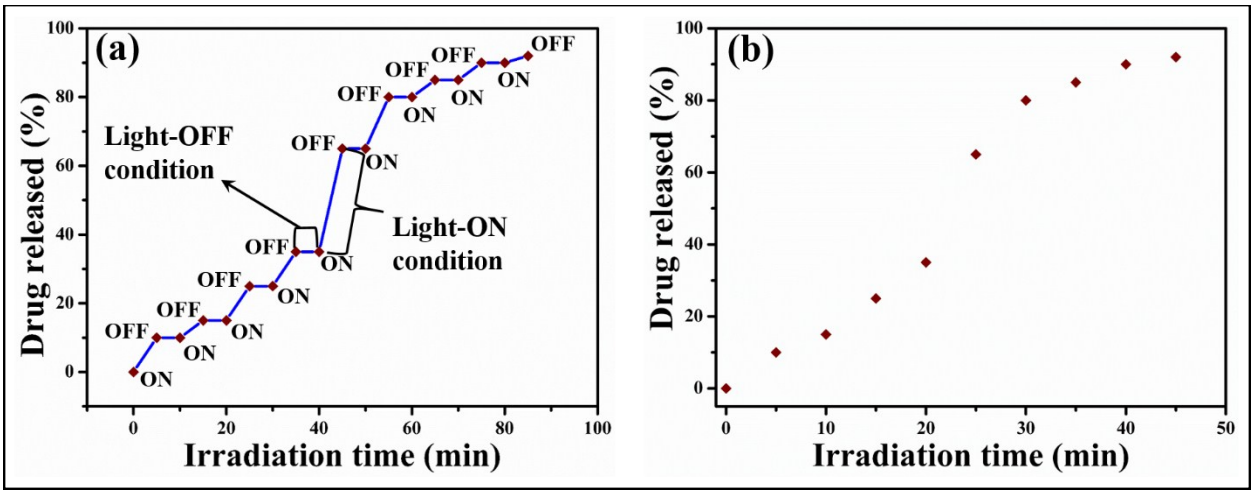

Figure S21: (a) Release of chlorambucil under bright and dark conditions. "On" and "Off" implies the switching on and off of the visible light source, respectively. Blue lines connecting the data points are there purely for guidance. (b) The amount of chlorambucil released from **T6** on photolysis (≥ 410 nm) at different time intervals.

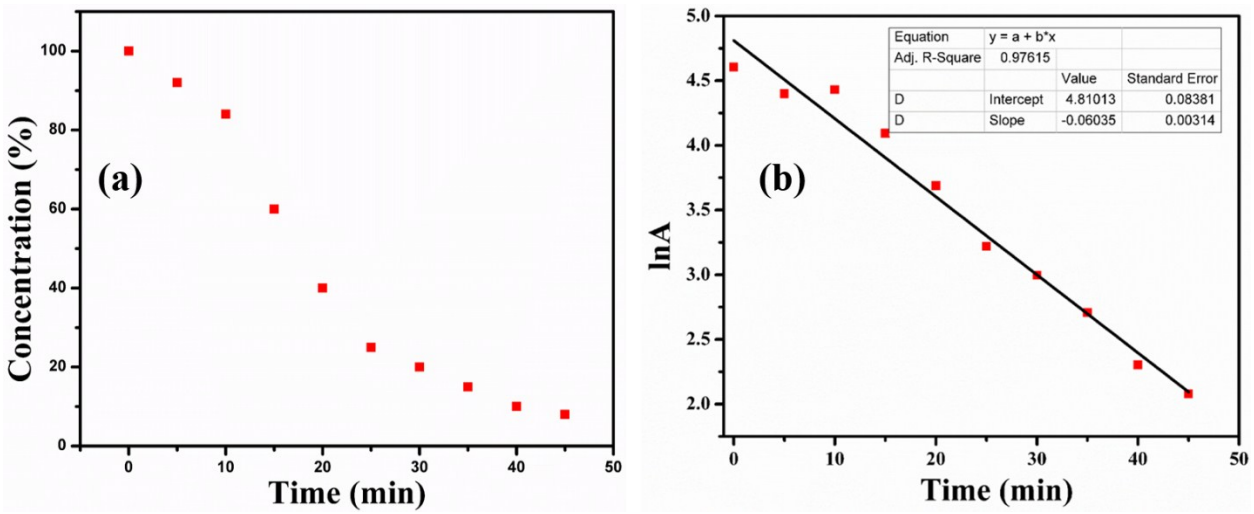

Figure S22: (a) Decomposition of **T6** at different irradiation time, (b) Photolysis rate constant measurement of **T6**. Here, lnA = natural logarithm of HPLC peak area at different time intervals of **T6**.

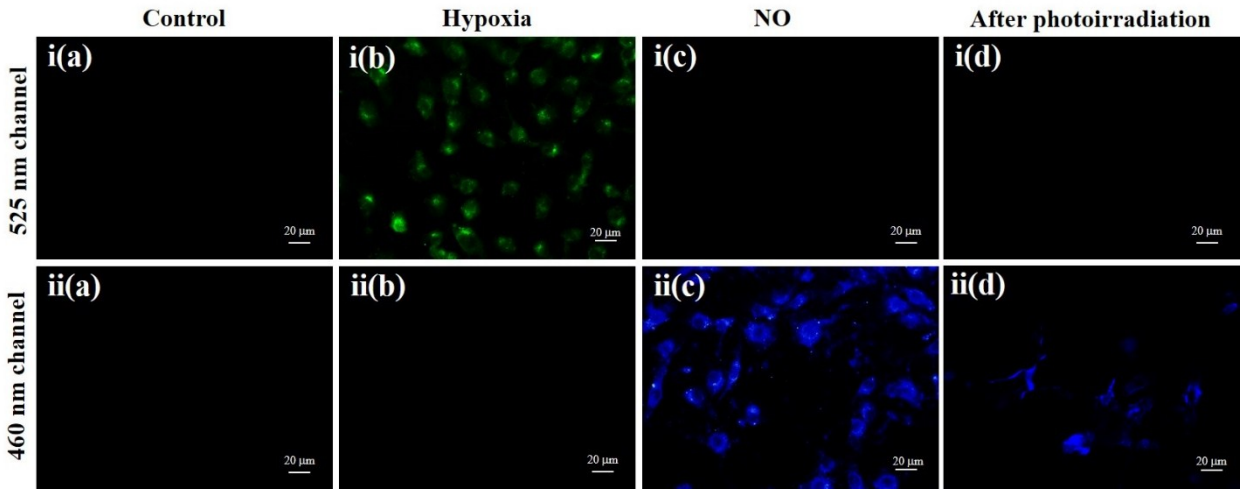

Figure S23: Cellular fluorescence image of HeLa cells after incubated with **6**. (a) Control cell, (b) cells pretreated with **6** were kept in hypoxic condition, (c) hypoxic cells pretreated with **6** were incubated with NO and (d) after visible light (≥ 410 nm) irradiation. 460 nm channel was used as blue emission channel and 525 nm channel was used as green emission channel. Samples were excited at 400 nm during capturing the cellular fluorescence images.

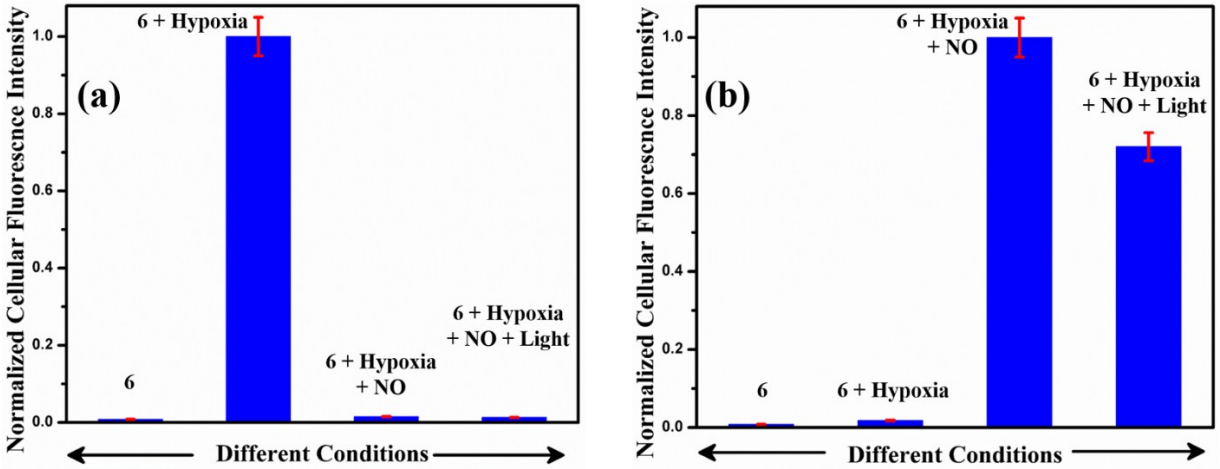

Figure S24: Comparison of cellular fluorescence intensities in (a) green channel (525 nm channel) and (b) blue channel (460 nm channel) at different conditions: (cells treated with **6**, **6** + hypoxia, **6** + hypoxia + light irradiation). Fluorescent intensity of control cell has been deducted during the plot.

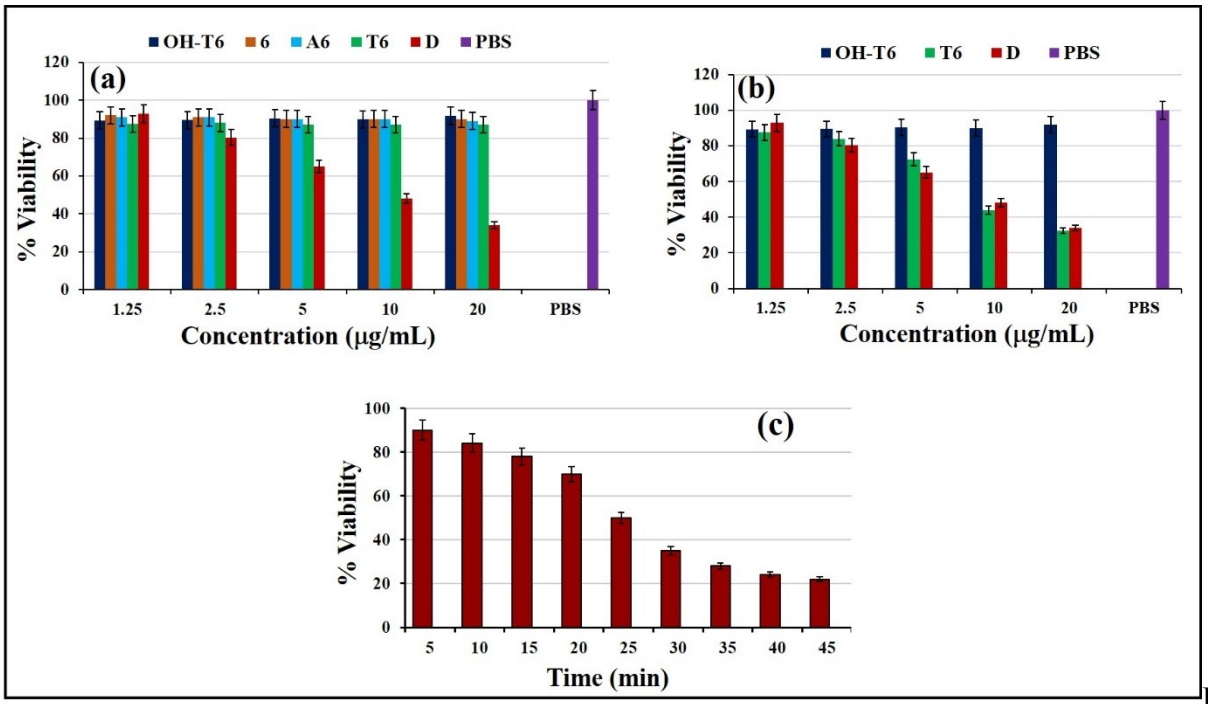

Figure S25: (a) Cell cytotoxicity analysis for HeLa cells: treated with **6**, **A6**, **T6**, photoproduct (**HO-T6**) and chlorambucil (**D**). (b) **T6**, photoproduct (**HO-T6**) and chlorambucil (**D**) after photolysis and (c) **T6** (10 μg mL^{-1}) at different time intervals of light irradiation.

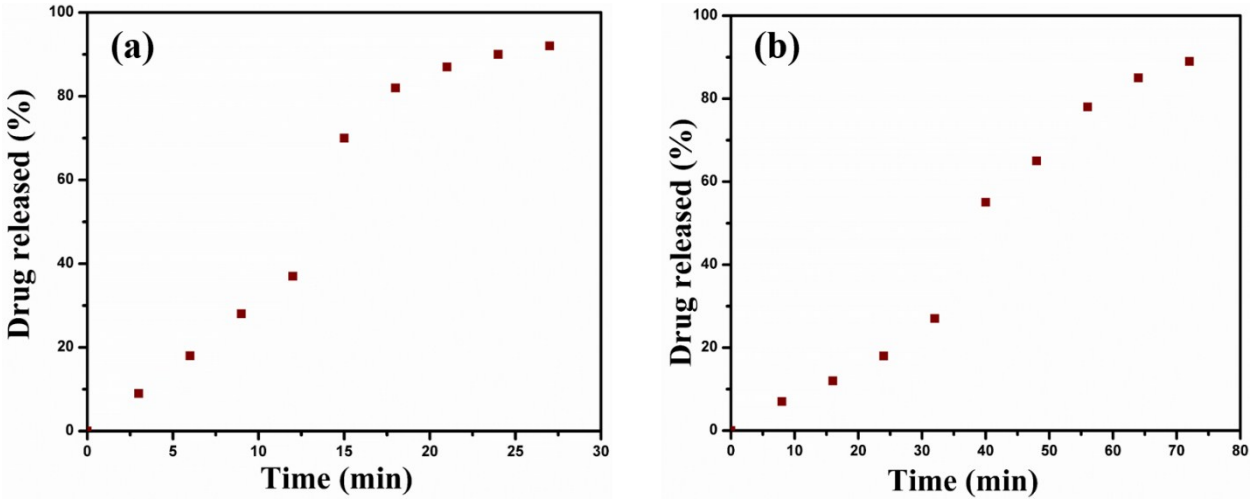

Figure S26: (a) The percent of chlorambucil released from **6**, and (b) the percent of chlorambucil released from **A6** on photolysis (≥ 410 nm) at different time intervals.

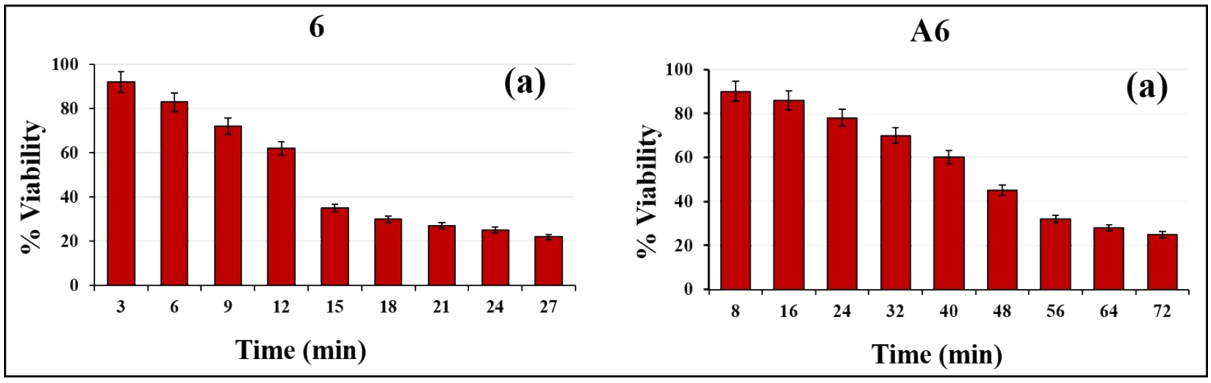

Figure S27: Cell cytotoxicity analysis for HeLa cells: (a) treated with **6** and (b) **A6** after photolysis (10 μg mL^{-1}) at different time intervals of light irradiation.

References:

1. 1. Ryan, E. T.; Xiang, T.; Johnston, K. P.; Fox, M. A., *J. Phys. Chem. A*, 1997, ***101*** **(10)**, 1827–1835.
2. Mosmann, T., *J Immunol Methods*, 1983, ***65*** (1-2), 55-63.
3. Q. N. Lin, C. Y. Bao, Y. L. Yang, Q. N. Liang, D. S. Zhang, S. Y. Cheng and L. Y. Zhu, *Adv Mater*, 2013, ***25***, 1981-1986.
4. M. Zhu, M. Yuan, X. Liu, J. Xu, J. Lv, C. Huang, H. Liu, Y. Li, S. Wang and D. Zhu, *Org Lett*, 2008, ***10***, 1481-1484.
5. S. Biswas, M. Gangopadhyay, S. Barman, J. Sarkar and N. D. P. Singh, *Sensors and Actuators B: Chemical*, 2016, ***222***, 823-828.