Spiropyran-Coumarin Platform: An Environment Sensitive Photoresponsive Drug Delivery System

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Supporting Information

1) General Experimental Techniques:

All reagents were purchased from Sigma Aldrich and were used without further purification. ^{1}H NMR spectra were recorded on a BRUKER-AC 400-MHz spectrophotometer. Chemical shifts are reported in ppm from tetramethylsilane with the solvent resonance as the internal standard ($CDCl_3$: 7.26 ppm, *Acetone-d_6*: 2.0 ppm). Data are reported as follows: chemical shifts, multiplicity (s = singlet, d = doublet, dd = doublet of 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 ppm from tetramethylsilane with the solvent resonance as the internal standard ($CDCl_3$: 77.0 ppm, Acetone-d_6: 39.5 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; HRMS spectra were recorded on a JEOL–AccuTOF JMS–T100L mass spectrometer. Photolysis of the SP-Cou-Cbl conjugate was carried out using 125-W medium-pressure Hg 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) were used. RP–HPLC was recorded using acetonitrile and water in mobile phase, at a flow rate of 1mL/min. Imaging was done using an Olympus FV1000 confocal microscope with objective lens magnification of 20X, numerical aperture of 0.45 and zoom: ×2.4 ×8.26.

2) Synthesis of Spiropyran-Coumarin-Chlorambucil (SP-Cou-Cbl) Conjugate:

Step (i): Indoline (1 mmol) and methyliodide (5 mmol) were heated to 110°C and stirred for 4 h. After cooling to room temperature, the precipitated material was isolated by vacuum filtration and washed with cold toluene followed by Et_2O to obtain product **4** (97%) as a light pink salt. ^{1}H NMR (400 MHz, *CDCl$_3$*): δ = 1.35 (s, 6H), 3.05 (s, 3H), 3.85 (s, 2H), 6.54-6.56 (d, J = 4 Hz, 1H), 6.75-6.79 (t, J = 4 Hz, 1H), 7.09-7.12 (t, J = 4 Hz,1H), 7.14-7.16 (d, *J* = 4 Hz, 1H). RMS (ES+) m/z calcd for $C1_2H_{16}N$: 174.1204 $[M+H]^+$; found: 174.1200.

8-formyl-7-hydroxyl-4-(hydroxymethyl)coumarin (3):

Step (ii): 7-hydroxy-4-(hydroxymethyl)-coumarin (**1**) (1 equiv) and hexamine (4 equiv) in trifluoroacetic acid (TFA) were refluxed for 2 h. After cooling the reaction mixture was extracted with dichloromethane (DCM), and the combined organic layers were dried over $MgSO_4$ and evaporated under reduced pressure, pale yellow solid of 8-formyl-7-hydroxy-4-(hydroxymethyl)-coumarin (**2**) was obtained. The crude material was purified by column chromatography through silica gel using EtOAc/pet ether (2:8) to obtain a light yellow powder (yield 20%); mp:180-182 ^{0}C; ^{1}H NMR ($CDCl_3$, 200 MHz): δ = 12.27 (s, 1H), 10.63 (s, 1H), 8.24 (s, 1H), 7.68-7.63 (d, J = 9.2 Hz, 1H), 6.96-6.92 (d, J = 9.2 Hz, 1H), 6.45 (s, 1H), 5.35 (s, 1H), ^{13}C NMR ($CDCl_3$, 50 MHz): δ = 193.1, 165.5, 159.7, 158.6, 148.3, 131.7, 130.6, 114.8, 110.7, 108.9, 60.4. HRMS cal. For $C_{11}H_9O_5$ $[MH^+]$: 221.0450, found: 221.0441.

Step (iii): Chlorambucil (1 equiv) was dissolved in 1 mL oxalyl chloride and was stirred for 1 h at 60 ^{0}C. Then oxalyl chloride was removed under vacuum to afford the acid chloride of chlorambucil as brown oil. Then the acid chloride was dissolved in dry DMF (5 mL) and the esterification reaction was carried out without further purification. To the solution of the acid chloride in DMF (5 mL) compound **2** (1.2 equiv) was added followed by triethylamine (0.45 mmole). The mixture was stirred at 55 ^{0}C temperature for 8 h. Then the reaction mixture was extracted with ethyl acetate (3 × 30 ml) and the solvent was evaporated. The crude reaction mixture was purified by silica gel column chromatography using 20 % ethyl acetate in pet ether to afford the caged conjugate **3** in 96 % yield as a brown colored solid. ^{1}H NMR (400 MHz, *CDCl$_3$*): δ = 0.86-1.24 (m, 2H), 1.25-1.27 (t, J = 2 Hz, 2H), 1.40-1.44 (t, J = 2Hz, 2H), 3.64-3.74 (m, 8H), 5.25 (s, 2H), 6.40 (s, 1H), 6.72-6.75 (t, J = 3.5 Hz, 1H), 6.93-6.94 (d, J = 2H, 1H), 7.09-7.13 (m, 3H), 7.64-7.66 (d, J = 4 Hz, 1H), 10.64 (s, 1H), 12.27 (s, 1H); ^{13}C NMR (100 MHz, *CDCl$_3$*): δ = 26.4, 33.9, 40.5, 53.6, 60.8, 110.3, 112.3,

112.9, 114.6, 129.6, 131.6, 144.5, 149.3, 158.7, 165.4, 172.5, 173.4, 193.1. HRMS cal. For $C_{25}H_{26}Cl_2NO_6$ [MH^+]: 506.1059, found: 506.1041.

Step (iv): 3 (1 equ.) and **4** (1 equ.) were refluxed in dry methanol (5 mL) for 3 h and cooled to room temperature. Water (100 mL) was added to the mixture. The formed purple solid was recovered by filtration and dried in vacuo, afforded 5. 1H NMR (400 MHz, acetone-d_6): δ = 7.79-7.78 (d, J = 4 Hz, 1H), 7.70- 7.69 (d, J = 4 Hz, 1H), 7.55- 7.46 (t, J = 8 Hz, 2H), 7.48-7.46 (d, J = 8 Hz, 2H), 7.19- 7.16 (t, J = 4Hz , 1H), 7.12- 7.10 (d, J = 8 Hz, 3H), 6.89-6.85 (t, J = 8 Hz, 1H), 6.77-6.72 (t, J = 8 Hz, 1H), 6.66- 6.64 (d, J = 8 Hz, 1H), 6.32 (s, 1H), 6.04-6.01 (d, J = 12 Hz, 1H), 5.36 (s, 2H), 3.80- 3.75 (m, 8H), 2.79 (s, 3H), 2.63-2.60 (t, J = 8 Hz,), 2.55-2.52 (t, J = 4 Hz, 2H), 1.99-1.97 (t, J = 4 Hz , 2H), 1.34 (s, 3H), 1.33 (s, 3H). ^{13}C NMR (100 MHz, *acetone-d_6*): δ = 172.1, 159.3, 157.4, 150.4, 150.0, 147.2, 144.7, 136.2, 130.0, 129.5, 127.6, 125.2, 122.0, 121.4, 120.1, 119.4, 112.2, 111.6, 109.1, 107.1, 106.9, 105.7, 61.0, 53.0, 51.8, 40.7, 33.6, 32.8, 26.7, 25.2, 19.3, 13.4. HRMS cal. For $C_{35}H_{34}Cl_2N_2O_5$ [MH^+]: 632.1845, found: 632.1815.

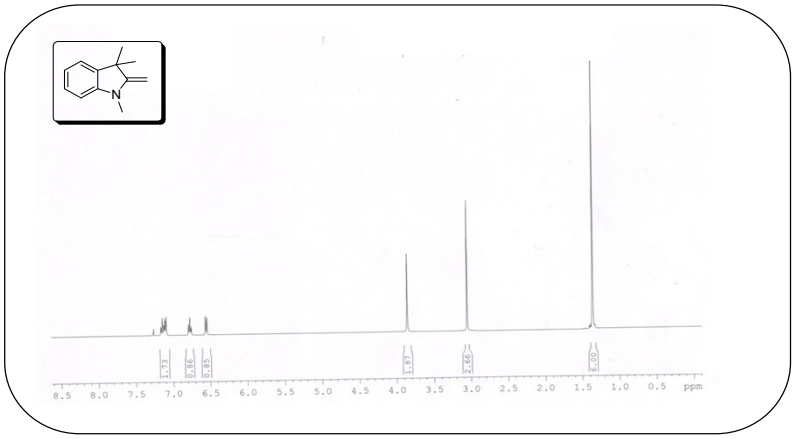

Figure S1: ^1H NMR of compounfd 4.

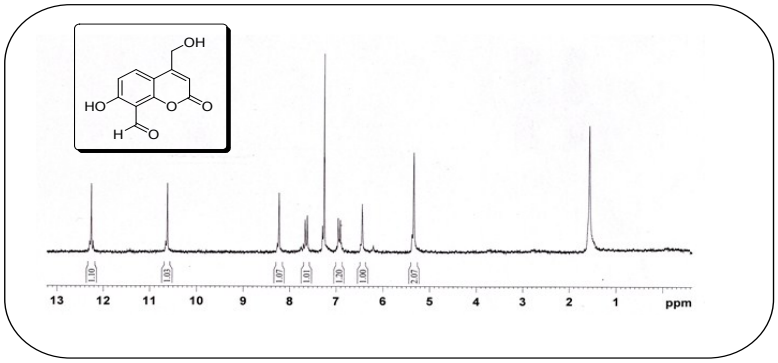

Figure S2: ^{1}H NMR of 8-formyl-7-hydroxyl-4-(hydroxymethyl)coumarin (2).

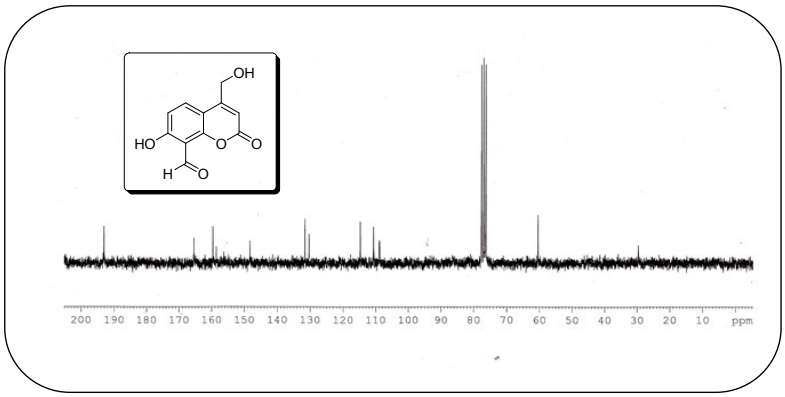

Figure S3: ^{13}C NMR of 8-formyl-7-hydroxyl-4-(hydroxymethyl)coumarin (2).

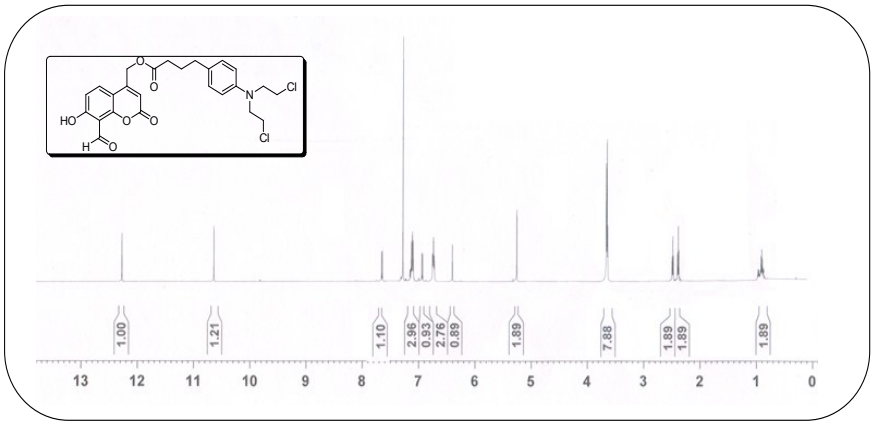

Figure S4: ^{1}H NMR of compound 4.

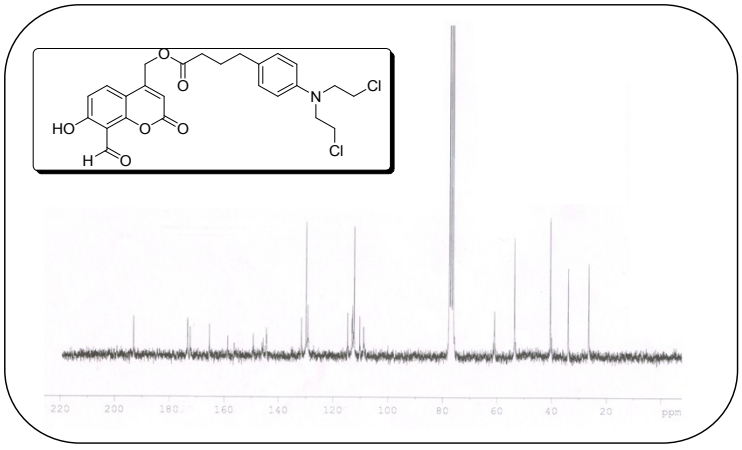

Figure S5: ^{13}C NMR of compound 4.

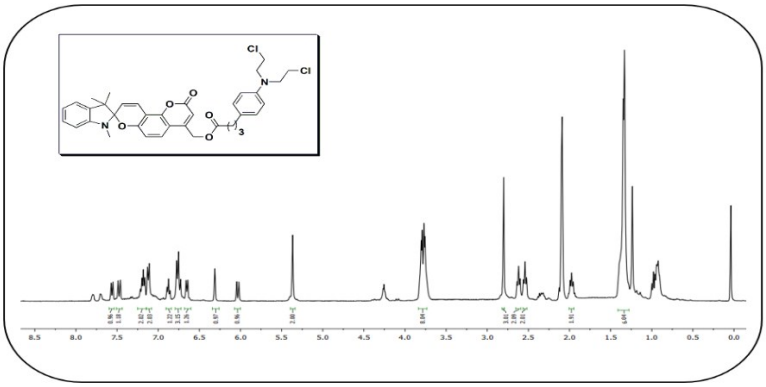

Figure S6: ^{1}H NMR of compound 5.

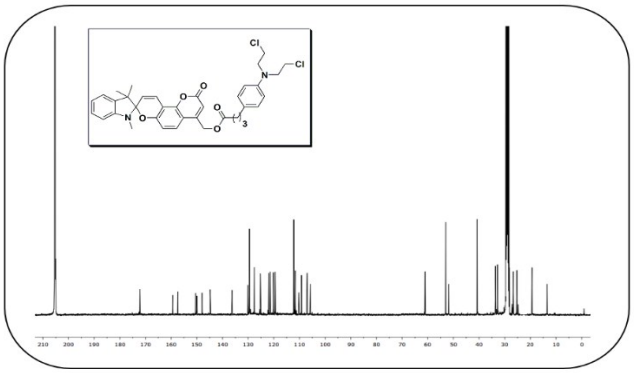

Figure S7: ^{13}C NMR of compound 5.

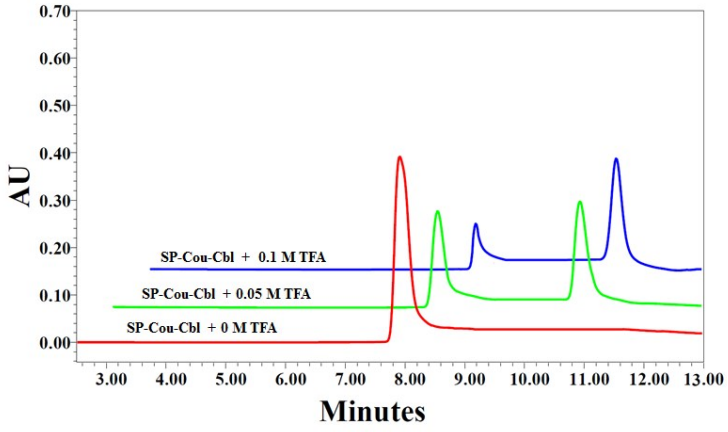

Figure S8: HPLC chromatogram of **SP-Cou-Cbl** upon addition of TFA.

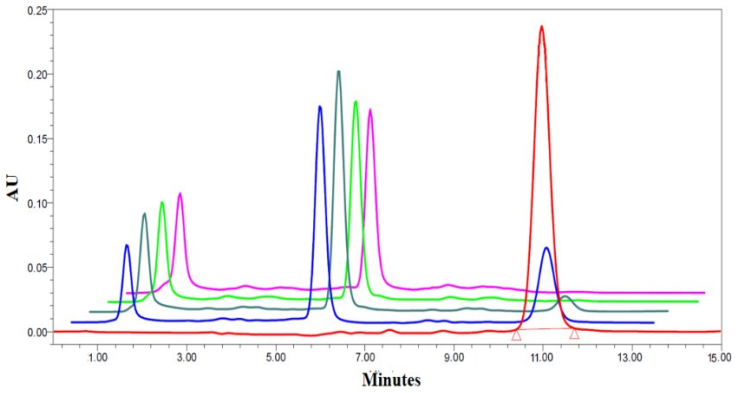

Figure S9: HPLC chromatogram of (**SP-Cou-Cbl** + TFA) at different time interval of visible light irradiation.

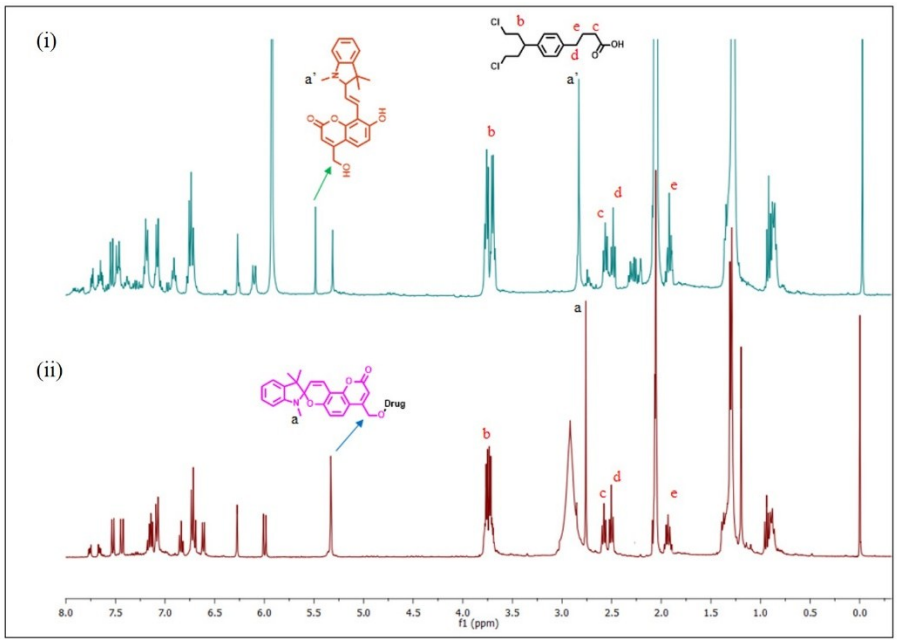

Figure S10: (i) ^{1}H NMR spectra of SP-Cou-Cbl (2×10^{-4} M) in *acetone-d_6* in presence of TFA (0.08 M) upon photoirradiation (≥ 410 nm) for 20 min. (ii) ^{1}H NMR spectra of SP-Cou-Cbl (2×10^{-4} M) in *acetone-d_6*.

Photophysical properties of SP-Cou-Cbl:

The absorption and emission spectra of a degassed solution of SP-Cou-Cbl (2×10^{-5} M) in different solvents were recorded on a UV-vis spectrophotometer and fluorescence spectrophotometer respectively.

NMR study:

For NMR experiments stock solutions of SP-Cou-Cbl (2×10^{-4} M) and 0.1 M TFA were prepared in *acetone-d_6*. The stock solution of SP-Cou-Cbl was prepared in NMR tube and the stock solution of 0.1 M TFA was in made up in a 5 mL in under inert condition and in absence of light.

Photochemical rate constant determination for SP-Cou-Cbl:

A 20 mL solution of SP-Cou-Cbl (1×10^{-4} M) was prepared in acetonitrile water (7:3). 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, 1 mL of the aliquots was taken and analyzed by RP-HPLC using mobile phase acetronitrile , at a flow rate of 1mL/min (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 each caged compounds, we plotted normalized [A] (HPLC peak area) versus irradiation time. We observed an exponential correlation for the disappearance of the caged compounds which suggested a first order reaction. The rate constant k_p was determined from the slope of the kinetic plot.

Further, the quantum yield for the photolysis of caged compounds was calculated using equation (1)

$$(\Phi p)_{CG} = \frac{(k_p)_{CG}}{I_0 \quad (F_{CG})} \qquad \text{---------- (1)}$$

Where, the subscript 'CG' denotes caged compound. Φ_p is the photolysis quantum yield, k_p is the photolysis rate constant and I_0 is the incident photon flux 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 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 2.886×10^{16} quanta s^{-1}.

Materials and Methods

Cell line procurement and maintenance Human breast carcinoma (MDA-MB-231) was obtained from the National Centre for Cell Science (NCCS), Pune, India and routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium supplemented with 10% fetal bovine serum, 100 U/ml streptomycin and 100 U/ml penicillin in a humidified cell incubator with an atmosphere of 5% CO_2 at 37 ºC.

In vitro **cellular uptake and localization studies** To study the cellular uptake and localization of SP-Cou-Cbl, MDA-MB-231 cells were first seeded at a density of 1×10^5 cells per well in a 6 well plate, left to adhere and then incubated with 10 µg/mL of SP-Cou-Cbl for 4 h at 37 ºC in a CO_2 incubator. After incubation, the cells were irradiated with visible light (≥ 410 nm)

using a UV lamp (Bangalore Genei Pvt.Ltd) for 0, 10, 20 and 30 min. Thereafter, the cells were fixed using 4% paraformaldehyde for 10 min and washed twice with PBS. Imaging was done using a confocal microscope (CLSM; Olympus FV 1000 attached to an inverted microscope IX81, Japan).

In vitro cytotoxicity assay

Before irradiation: The cytotoxicity of PP, SP-Cou-Cbl and chlorambucil (Cbl) were determined without light irradiation on MDA-MB-231 cells using an MTT assay according to the method by Mosmann et al. The breast cancer cells (1 X 10^4 cells per ml in a 96 well plate) were treated for 48 h with different concentrations (1.25, 2.5, 5, 10, and 20 μg/ ml) and the cell viability was determined by an MTT assay (Mosmann, 1983), measuring the absorbance at 595 nm using a micro plate reader (Thermo Scientific,USA). The cell viability was calculated using the formula: viability (%) = 100 A2/A1, where A2 is the absorbance of the treated cells and A1 is the absorbance of the control cells.

After irradiation: The breast cancer cells (1 X 10^4 cells per ml in a 96 well plate) were treated with different concentrations of PP, SP-Cou-Cbl and chlorambucil (Cbl) (1.25, 2.5, 5, 10, and 20 μg/ ml) and incubated for 4 h at 37 ºC in a CO_2 incubator. Thereafter, the cells were irradiated with visible light (≥ 410 nm) for 30 min (keeping the culture plate 6 cm from the light source) using as a light source (Bangalore Genei Pvt. Ltd) under aseptic conditions. After UV irradiation, the cells were again incubated for 48 h and the cell viability was measured by an MTT assay (Mosmann, 1983). The cell viability was calculated as described above.

Confocal microscopy

The cytoxicity of SP-Cou-Cbl after UV-irradiation (≥365nm) for different lengths of time was also studied by confocal microscopy. MDA-MB-231 cells (1 X10^5 cells per well) were treated with 10 μg/mL of SP-Cou-Cbl and put aside for 4 h at 37 1C. Then, the drug treated cells were irradiated with visible light (≥ 410 nm) for 0–30 min. Thereafter, the plate was incubated for 48 h at 37 ºC in a CO_2 incubator. The effect of the released drug on the cell was visually observed on a confocal microscope (IX81, Olympus).

Cell cycle analysis

The cell cycle distributions of the cells (with and without drug treatment) were analyzed by propidium iodide (PI) DNA staining, according to the method by Devi et al, 2014. Briefly, the MDA-MB-231 cells ($1X10^6$) after SP-Cou-Cbl (10 µg/ ml) treatment, UV irradiation (30 min) and incubation for 24 h were harvested for cell cycle analysis by flow cytometry. The cells were washed twice with Phosphate Buffered Saline (PBS, pH 7.4) and fixed overnight in 70% ethanol at 20 ºC. Then, the cells were washed with PBS, followed by incubation with 250 µg/ml RNase A and 50 µg/mL propidium iodide (a DNA intercalating dye) at room temperature. Ten thousand cells were counted per analysis. PI fluorescence was detected in the pulse-processed FL2 channel. The distributions of cells in the different cell cycle phases were analyzed on a Becton-Dickinson FACS Aria flow cytometer and CellQuest pro software. The percentage of cells corresponding to sub G0/G1, G0/G1, S and G2/M phases of the cell cycle were analyzed from the DNA histogram. A sub-G0/G1 fraction of cells was identified as an apoptosis population.