Electronic Supplementary Material (ESI) for ChemComm.

Redox-responsive Xanthene-coumarin-chlorambucil-based FRET-guided theranostics for "activatable" combination therapy with real-time monitoring

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Supporting Information

1. General information

Materials: All reagents were purchased from Sigma Aldrich and used without further purification. Acetonitrile and dichloromethane were distilled from CaH_2 before use. ^1H NMR spectra were recorded on a BRUKER-AC 200 MHz spectrometer. Chemical shifts are reported in ppm from tetramethylsilane with the solvent resonance as the internal standard (deuterochloroform: 7.26 ppm). Data are reported as follows: chemical shifts, multiplicity (s = singlet, d = doublet, t = triplet, m = multiplet), coupling constant (Hz). ^{13}C NMR (50 MHz) spectra were recorded on a BRUKER-AC 200 MHz Spectrometer with complete proton decoupling. Chemical shifts are reported in ppm from tetramethylsilane with the solvent resonance as 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, FT-IR spectra were recorded on a Perkin Elmer RXI spectrometer and HRMS spectra were recorded on a JEOL-AccuTOF JMS-T100L mass spectrometer. Photolysis of fluo-mor nanoparticles were carried out using 125 W medium pressure Hg lamp supplied by SAIC (India) for one photon (1PE) and 740 nm diode laser for two-photon (2PE). Chromatographic purification was done with 60-120 mesh silica gel (Merck). For reaction monitoring, precoated silica gel 60 F254 TLC sheets (Merck) were used. Cell culture media and all the other materials required for culturing were obtained from Gibco, USA.

2.1 Synthesis of Xan-SS-Cou-Cbl (6)

i. Synthesis of 6-Hydroxy-9-methyl-3H-xanthen-3-one (Xan) (5)[1]

2.4 mL (20.4 mmol) aqueous hydrobromic acid (46 %) was added to 10.1 mL acetic anhydride (106.89 mmol) at 0 °C in drop wise manner under constant stirring. Thereafter, resorcinol (4 g, 36.36 mmol) was introduced into the reaction mixture in one portion. The resulting mixture was then heated for 1 h at 90 °C until a dense red precipitate formed. After 1 h, the reaction mixture was cooled to room temperature at the precipitate was collected by filtration under suction. The precipitate was washed with glacial acetic acid (3 × 5 mL) and ice-cold propan-2-ol (5 mL). The precipitate was obtained in 40 % yield.

2 g. of as-obtained precipitate was then treated with dry pyridine (40 mL) under vigorous stirring at room temperature for 1.5 h. Finally, red precipitate of 6-Hydroxy-9-methyl-3H-xanthen-3-one **(5)** was afforded by adding water into the pyridine-containing reaction mixture. The precipitate was collected under suction, washed with cold water and dried under vacuum. Yield: 85 %. UV-vis (EtOH): λ_{max} (log ε): 451 (1.45), 478 (1.75). FTIR (KBr, cm^{-1}): 1310, 1560, 3270. ^{1}H NMR (DMSO-d_6, 400 MHz): δ = 9.89 (s, 2H), 7.64-7.61 (d, J = 8.8 Hz, 2H), 6.59-6.57 (d, J = 6.8 Hz, 2H), 6.47 (s, 2H), 5.23 (s, 2H). ^{13}C NMR (DMSO-d_6, 100 MHz): δ = 159.29 (2C), 152.87 (2C), 125.65 (2C), 112.57 (2C, 112.10 (2C), 102.84 (2C), 95.36 (1C).

ii. Synthesis of 7-hydroxy-4-bromomethyl coumarin (2)[2]

To 4 g resorcinol (36.33 mmol) was added 5.1 mL bromoethylacetoacetate (36.33 mmol) at 0 °C under constant stirring. After that, the reaction mixture was treated with catalytic amount (1.8 mL) of 70 % H_2SO_4. The reaction was continued for 8 h at room temperature. Finally, compound **3** was afforded as white precipitate upon addition of excess water to the reaction mixture. The resulting precipitate was filtered, washed with water and dried under vacuum. Yield: 90 %.

iii. Synthesis of coumarin-chlorambucil conjugate (Cou-Cbl) (3)[3]

Anticancer drug chlorambucil (0.24 g, 0.78 mmol) was treated with K_2CO_3 (0.13 g, 0.94 mmol) in presence of dimethylformamide (DMF) at room temperature. Thereafter, compound **3** (0.2 g, 0.78 mmol) was added to the reaction mixture and reaction was continued for 6 h at room temperature under dark condition. Cou-Cbl (**3**) was obtained as light yellow solid and purified using column chromatography with 35 % ethyl acetate in pet ether as the eluent system. Yield: 75 %. UV-vis (EtOH): λ_{max} (log ε): 330 (1.45). FTIR (KBr, cm^{-1}): 1720, 1622, 3210. ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 7.41-7.40 (d, J = 9 Hz, 2H,), 7.08-7.07 (d, J = 8.4 Hz, 2H), 7.018 (s, 1H), 6.888-6.869 (d, J = 9 Hz, 1H), 6.65-6.64 (d, J = 8.4 Hz, 2H), 6.34 (s, 1H),

5.27 (s, 2H), 3.73-3.62 (m, 8H), 2.62-2.60 (t, J = 7.5 Hz, 2H), 2.50-2.47 (t, J = 7.5 Hz), 2.01-1.98 (m, 2H). ^{13}C NMR (DMSO-d_6, 150 MHz): δ = 172.87, 161.73, 160.22, 155.30, 150.06 (2C), 144.50, 130.07, 129.72 (2C), 113.60, 112.26 (2C), 110.48, 109.38, 103.72, 61.06, 53.61 (2C), 40.55 (2C), 33.88, 33.31, 26.51.

iv. Synthesis of coumarin-chlorambucil-thiol conjugate (4)[4]

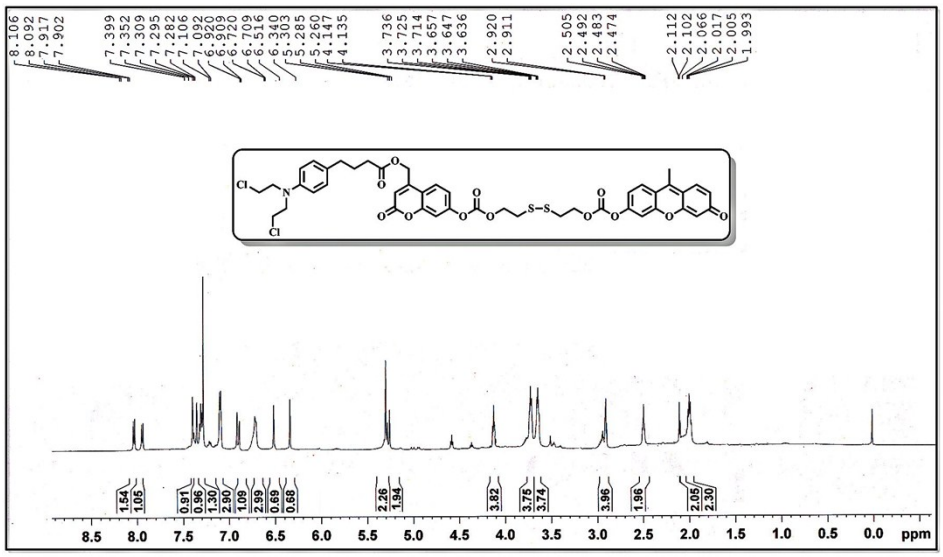
To a solution of Cou-Cbl **(4)** (0.33 g, 0.68 mmol) in DCM (5 mL) was added N,N-dimethylaminopyridine (DMAP) (0.21 g, 1.70 mmol) at 0 °C under constant stirring. Thereafter, 0.25 g (0.86 mmol) triphosgene was introduced in several portions. The reaction was continued at room temperature for 4 h. Afterwards, the solvent was evaporated under vacuum. Residual was dissolved into further 5 mL of DCM to which another DCM solution of 2,2'-hydroxyethyldisulfide (0.08 mL, 0.69 mmol) containing catalytic amount of DMAP was reacted. After stirring for 16 h at room temperature, the mixture was concentrated under vacuum. Pure compound **4** was obtained upon column chromatography using 30 % ethyl acetate/pet ether as eluent. Yield: 45 %. m. p 210 °C. UV-vis (EtOH): λ_{max} (log ε): 330 (1.45). FTIR (KBr, cm^{-1}): 1720, 1622, 3210. ^{1}H NMR ($CDCl_3$, 600 MHz): δ = 7.70-7.69 (d, J = 8.4 Hz, 1H), 7.61-7.60 (d, J = 9 Hz, 1H), 7.39 (s, 1H), 7.10-7.09 (d, J = 8.4 Hz, 2H), 6.72-6.70 (d, J = 6.6 Hz, 2H), 6.34 (s, 1H), 5.30 (s, 2H), 3.94-3.93 (t, J = 6 Hz, 2H), 3.73-3.63 (m, 8H), 3.05 (t, J = 5 Hz, 2H), 2.92-2.91 (t, J = 5 Hz, 2H), 2.63-2.61 (t, J = 7.2 Hz, 2H), 2.50-2.48 (t, J = 6.6 Hz, 2H), 2.11-2.06 (t, J = 6 Hz, 2H), 2.01-1.99 (m, 2H). ^{13}C NMR (DMSO-d_6, 150 MHz): δ = 172.63, 160.30, 152.96, 152.67, 151.78, 150.55, 148.60 (2C), 129.81 (2C), 125.81, 118.54, 117.46, 115.00, 112.99 (2C), 110.45, 66.88, 61.12, 60.46, 53.88 (2C), 40.27 (2C), 33.90, 33.24, 29.74 (2C), 26.45. HRMS calcd for $C_{29}H_{33}Cl_2NO_8S_2$ [MH+], 658.1103; found, 658.1105.

v. Synthesis of xanthene-coumarin-chlorambucil-thiol conjugate (Xan-SS-Cou-Cbl) (6)[4]

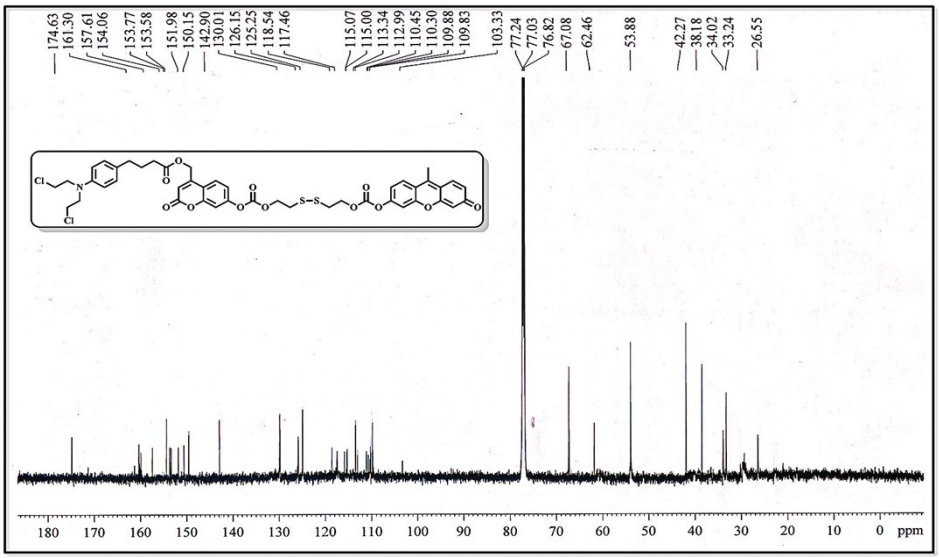
To a solution of thiolated Cou-Cbl **(5)** (60 mg, 0.09 mmol) in DCM (5 mL) was added N,N-dimethylaminopyridine (DMAP) (10 mg, 0.1 mmol) at 0 °C under constant stirring. Thereafter, 70 mg (0.24 mmol) triphosgene was introduced in several portions. The reaction was continued at room temperature for 4 h. Afterwards, the solvent was evaporated under vacuum. Residual was dissolved into 3 mL of DMF to which another DMF solution of xanthenes (**2**) (24 mg, 0.11 mmol) containing catalytic amount of DMAP was added. After stirring for 16 h at room temperature, the mixture was concentrated under vacuum. Pure compound **6** was obtained upon column chromatography using 40 % ethyl acetate/pet ether as eluent. Yield: 35 %. m. p. 220 °C. UV-vis (EtOH): λ_{max} (log ε): 330 (1.45). FTIR (KBr, cm^{-1}): 1725, 1620, 3010. ^{1}H

NMR ($CDCl_3$, 600 MHz): δ = 8.10-8.09 (d, J = 8.4 Hz, 1H), 7.917-7.902 (d, J = 9 Hz, 1H), 7.39 (s 1H), 7.35 (s, 1H), 7.31-7.29 (d, J = 8.4 Hz, 1H), 7.11-7.09 (d, J = 8.4 Hz, 3H), 6.92-6.90 (d, J = 6.6 Hz, 1H), 6.72-6.71 (d, J = 6.6 Hz, 3H), 6.51 (s, 1H), 6.34 (s, 1H), 5.30 (s, 2H), 5.26 (s, 2H), 4.14-4.13 (t, J = 7.2 Hz, 4H), 3.73-3.63 (m, 8H), 2.92-2.91 (t, J = 5.4 Hz, 4H), 2.50-2.48 (t, J = 6.6 Hz, 2H), 2.11-2.06 (d, J = 6 Hz, 2H), 2.01-1.99 (m, 2H). ^{13}C NMR ($CDCl_3$, 150 MHz): δ = 174.63, 161.30, 157.61, 154.06 (2C), 153.77, 153.58, 151.98 (3C), 150.15 (2C), 142.90 (2C), 130.81 (2C), 126.15, 125.25 (2C), 118.54, 117.46, 115.07, 115.00, 113.34 (2C), 112.99, 110.45, 110.30, 109.88, 109.83 (2C), 103.33, 67.08 (2C), 62.60, 53.08 (2C), 42.27 (2C), 38.18 (2C), 34.02, 33.24, 26.55. HRMS calcd for $C_{44}H_{41}Cl_2NO_{12}S_2$ [MH+], 910.1525; found, 910.1529.

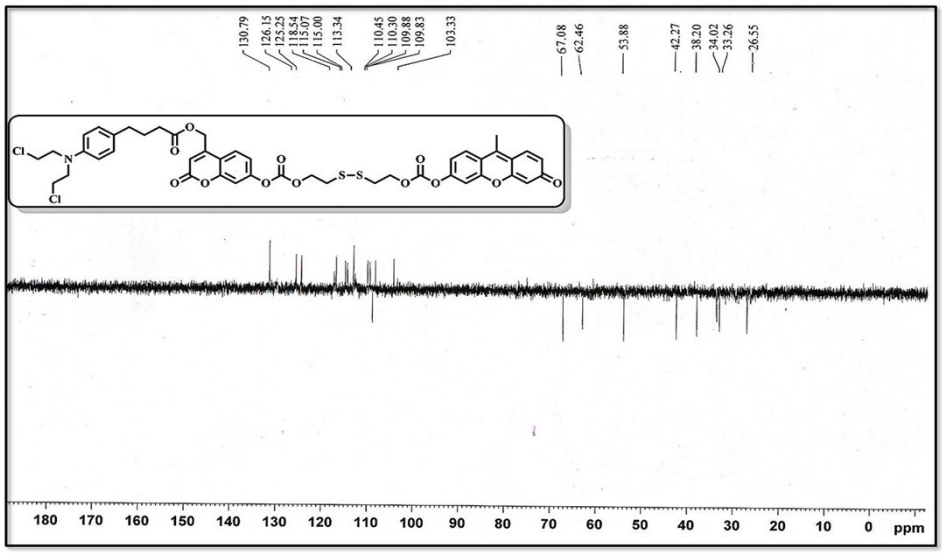
2.2 Characterization of Xan-SS-Cou-Cbl Conjugate



^1H NMR spectrum of Xan-SS-Cou-Cbl (**6)** conjugate ($CDCl_3$, 600 MHz)



^{13}C NMR spectrum of Xan-SS-Cou-Cbl (**6)** conjugate ($CDCl_3$, 150 MHz)



DEPT 135 NMR spectrum of Xan-SS-Cou-Cbl (**6)** conjugate ($CDCl_3$, 150 MHz)

3. Redox-responsive photophysical properties of Xan-SS-Cou-Cbl (6)

3.1 Steady state UV-vis and fluorescence spectral study

The UV-vis spectrum of Xan-SS-Cou-Cbl (**6**) was recorded in a HEPES buffer solution containing 10 % ethanol (pH 7.2) at ambient temperature. Xan-SS-Cou-Cbl (**6**) showed three distinct absorption peaks at 328 nm, 451 nm and 478 nm showing the presence of both fluorophores viz. coumarin-chlorambucil conjugate (Cou-Cbl) and xanthene on the same molecule. Clearly, the absorption peak at 328 nm corresponds to the λ_{max} of Cou-Cbl moiety due to n-π^* transition; however, other two wavelengths (λ_{max} = 451 and 478 nm) were characteristics of the xanthene moiety (**fig. S1a**). It is evident from the UV-vis spectra in **figure 6.1a** that the coumarin moiety could be excited by light of 330 nm while emits in the range 400-500 nm (as shown in **fig. S1b**). Interestingly, the absorption range of xanthene moiety falls in the same region of coumarin emission (**fig. S1b**). Thus, in the presence of disulfide bridge, coumarin moiety upon excitation with 330 nm light transfers its energy to xanthene moiety serving as a FRET donor. Xanthene, on the other hand, exhibited its characteristic emission maxima at 516 nm upon energy transfer from coumarin, which confirmed the FRET process occurring from coumarin to xanthene. Thus, strong overlap between emission of Cou-Cbl and the excitation band of xanthene supported their FRET donor-acceptor relation (**fig. S1b**).

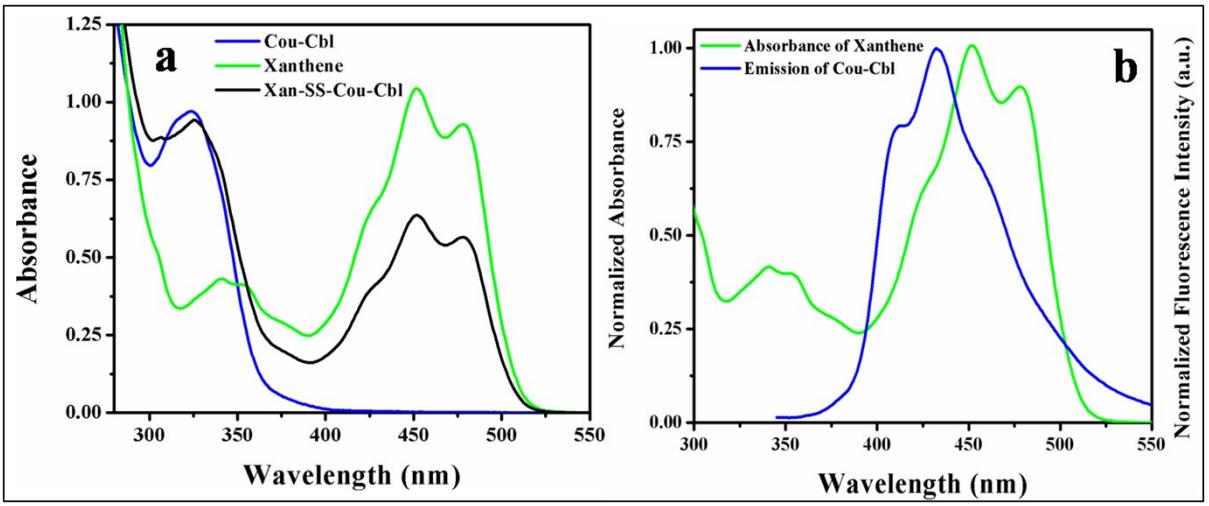


Figure S1 (a) Comparative UV-vis spectra of Xan-SS-Cou-Cbl, xanthene and Cou-Cbl**.** Each spectrum was measured at same concentration of 15 μM in HEPES buffer solution containing 10 % ethanol (pH 7.2), and **(b)** overlapping between emission of donor (Cou-Cbl) and absorption band of acceptor (xanthene) for prominent FRET process.

To further validate the FRET process in between coumarin and xanthene moiety, the fluorescence property of compound **6** at an excitation wavelength 350 nm was compared with the emission spectra of coumarin and xanthene at the same excitation wavelength. As expected, we observed an emission maximum at 516 nm for compound **6**. This can be attributed to the energy transfer process from coumarin moiety to xanthene upon excitation at 350 nm leading to the characteristic emission of the FRET-acceptor xanthene. However, free Cou-Cbl exhibited its characteristic emission maximum at 408 nm upon excitation at 350 nm owing to absence of any FRET process. Similarly, xanthene showed only negligible fluorescence at 516 nm upon excitation at 350 nm. This comparison further verified the ongoing FRET from coumarin to xanthene moiety in compound **6**.

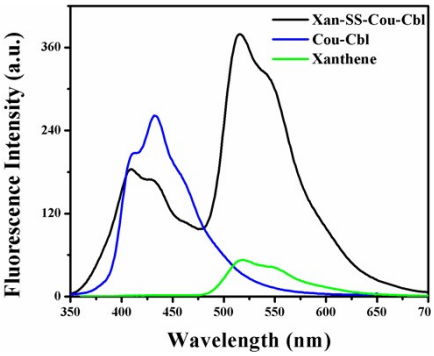


Figure S2 Comparative fluorescence spectra [excitation wavelength = 350 nm] of compound **6**, xanthene and Cou-Cbl**.** Each spectrum was measured at same concentration of 15 μM in HEPES buffer solution containing 10 % ethanol (pH 7.2).

3.2 Competitive fluorescence study of Xan-SS-Cou-Cbl (6)

To estimate the utility of Xan-SS-Cou-Cbl as a prodrug system under physiological condition, we monitored the change in fluorescence property of Xan-SS-Cou-Cbl in presence of various biologically relevant amino acids (**fig. S3a)** and metal ions viz. Na^{+}, K^{+}, Ca^{2+}, Mg^{2+}, Fe^{2+}, Cu^{2+} and Zn^{2+}(**fig. S3b)**. As seen in **fig. S3b,** the change in fluorescence property took place only for thiol containing biological reducing agents viz. GSH, cysteine (Cys), homo-cysteine (Hcy) and dithiothreotol (DTT). However interference by Cys, Hcy and DTT can be neglected owing to their relatively low concentration in biological medium as compared to GSH.[5] For all other non-thiol amino acids and metal ions, Xan-SS-Cou-Cbl did not show any remarkable change in fluorescence property. This can be visualized by the colors of the vials seen under fluorescence lamp (**fig. S4**).

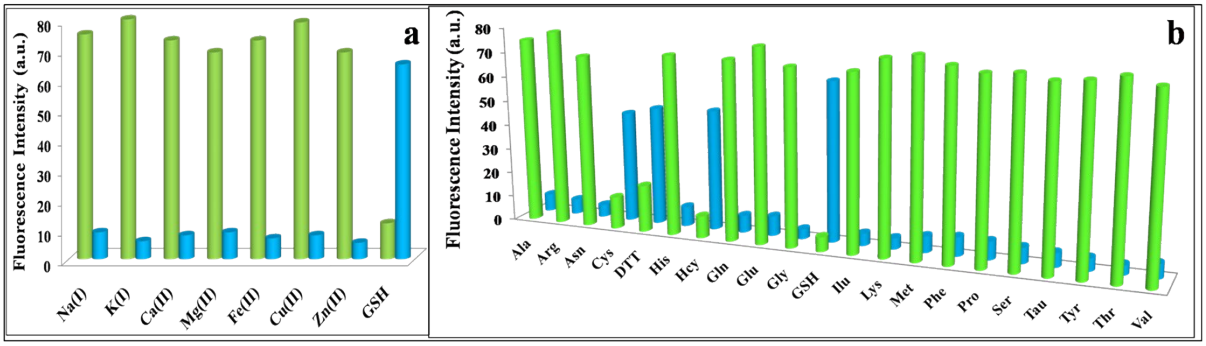


Figure S3 Change in fluorescence of Xan-SS-Cou-Cbl (15.0 μM in HEPES buffer with 10 % ethanol) recorded in the presence of **(a)** various metal ions (20 mM), and **(b)** biologically relevant thiol, non-thiol-based amino acids (20 mM), respectively. Excitation wavelength = 330 nm. [green bar = emission at 516 nm; blue bar = emission at 408 nm]

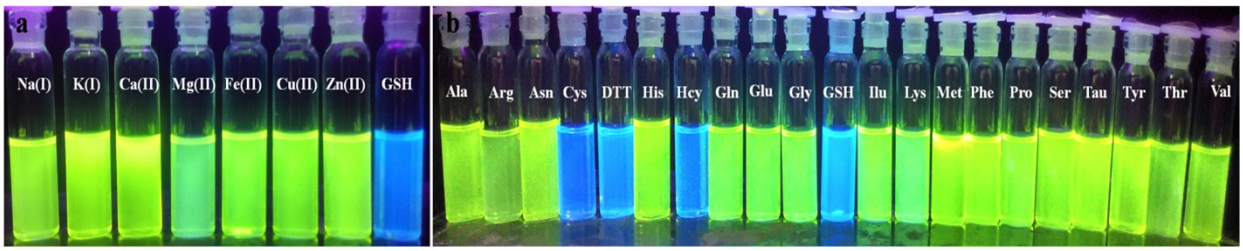


Figure S4 Images of vials containing Xan-SS-Cou-Cbl with **(a)** metal ions, and **(b)** amino acids under laboratory fluorescence lamp.

3.3 Effect of pH on fluorescence property of Xan-SS-Cou-Cbl (6)

Effect of pH on the fluorescent nature of Xan-SS-Cou-Cbl was also studied. For this purpose, 1 M NaOH or 1 M HCl was added gradually into a solution of Xan-SS-Cou-Cbl in ethanol:HEPES buffer (pH 7.2). It was observed that, in absence of GSH, Xan-SS-Cou-Cbl showed sufficient stability with respect to the pH of the medium indicated by the persistent green fluorescence (λ_{em} = 516 nm) through the pH range 3 to 8 (**fig. S5**). On the other hand, in

presence of GSH, steady decrease in green fluorescence (λ_{em} = 516 nm) and simultaneous increase in intensity at λ_{em} = 408 nm was observed upon excitation at 330 nm in the pH range 6 to 8. Physiological pH ranges from 5-8 during which Xan-SS-Cou-Cbl showed sufficient stability in absence of GSH.

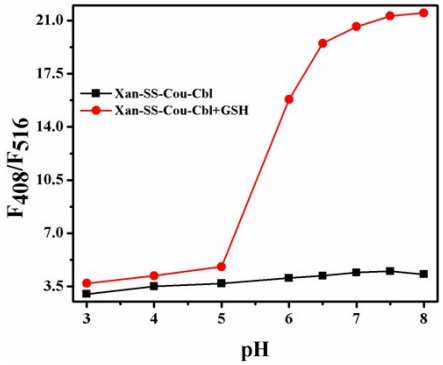


Figure S5 Relative change in fluorescence intensity (F_{408}/F_{516}) of Xan-SS-Cou-Cbl (15 μM) in HEPES buffer solution containing 10 % ethanol (pH 7.2) recorded in presence or absence of 5 mM GSH at different pH values. F_{408} = emission at 408 nm and F_{516} = emission at 516 nm. λ_{ex} = 330 nm. pH values were adjusted by successive addition of 1 M HCl or NaOH.

3.4 Mechanism of redox-responsive disruption of Xan-SS-Cou-Cbl

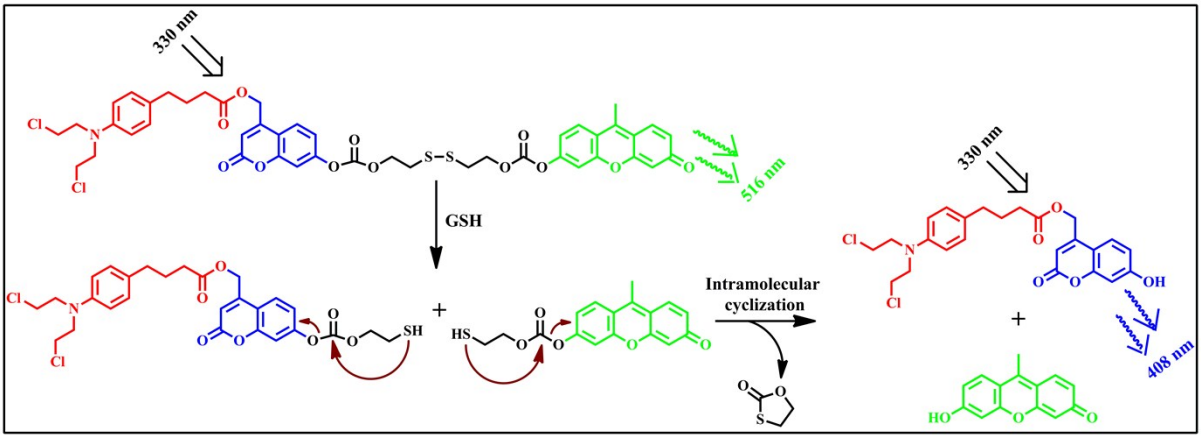


Figure S6 Mechanism of reaction between Xan-SS-Cou-Cbl **(6)** with GSH.

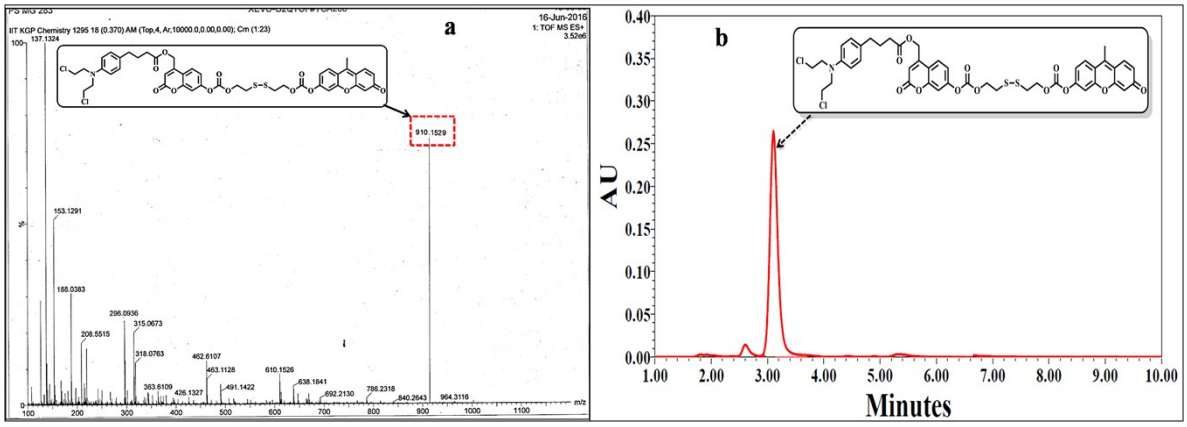


Figure S7 (a) Mass spectrum (TOF MS ES+), and **(b)** HPLC chromatogram of Xan-SS-Cou-Cbl (**6**) before addition of GSH.

3.5 Measurement of fluorescence quantum yield

The quantum yield of the Xan-SS-Cou-Cbl was determined by reference point method following **equation 1**.[6,7] Quinine sulfate in 0.1 M H_2SO_4 (literature quantum yield: 54 %) was used as a standard sample to calculate the Q.Y. of Xan-SS-Cou-Cbl. Xan-SS-Cou-Cbl was dissolved in HEPES buffer containing 10 % ethanol. The absorbance values of the solutions at the excitation wavelength were measured with UV–vis spectrophotometer. Photoluminescence (PL) emission spectra of all the sample solutions were recorded by Hitachi F-7000 fluorescence spectrophotometer at an excitation wavelength of 330 nm.

$$\frac{\Phi_S}{\Phi_R} = \frac{A_S}{A_R}\frac{(Abs)_R}{(Abs)_S}\frac{\eta_S^{\,2}}{\eta_R^{\,2}} \qquad \text{..... (1)}$$

Where **Φ** represents quantum yield, **Abs** represents absorbance, **A** represents area under the fluorescence curve, and **η** is refractive index of the medium. The subscripts **S** and **R** denote the corresponding parameters for the sample and reference, respectively.

4 Redox-responsive photochemical properties of Xan-SS-Cou-Cbl

4.1 Redox-responsive singlet oxygen generation from Xan-SS-Cou-Cbl

In order to confirm the selective singlet oxygen generation by **Xan-SS-Cou-Cbl** after treatment with GSH, we recorded the photodegradation rate of 1,3-diphenylisobenzofuran (DPBF, Aldrich).[8] The degradation study was carried out with Xan-SS-Cou-Cbl both before and after reacting with GSH, separately. The singlet oxygen quantum yield (Φ_Δ) was determined using Rose Bengal as the reference, having a singlet-oxygen quantum yield of 0.74 in water. An equimolar solution of Xan-SS-Cou-Cbl (34 μM) and DPBF (35 μM) was prepared in HEPES buffer with 10 % ethanol. In the solution, the photosensitizer concentration was adjusted to possess the same absorbance (typically 0.1) at 473 nm and the initial concentration of DPBF was 1.26×10^{-4} M. During the experiment, the solutions were stirred vigorously in the presence of O_2 gas. The solutions were further irradiated with light of wavelength ≥ 350 nm; the bleaching of the absorption band of DPBF at 425 nm was monitored. The solution of DPBF alone was also irradiated and the obtained result was subtracted to diminish the errors originating from the photo-activity of DPBF. The Φ_Δ of each solution was calculated by the following **equation 2**,

$$\Phi_\Delta = (K_S/K_R) \times \Phi_R \qquad \text{…….(2)}$$

Where K is the slope of the photodegradation plot of DPBF against time (s), the subscripts S and R denote the sample and the reference, respectively, and Φ_R is the singlet-oxygen quantum yield of the reference (Rose Bengal).

Singlet oxygen generation abilities of the released xanthene and coumarin were compared with the GSH-treated Xan-SS-Cou-Cbl solution. It was found that released coumarin (free Cou-Cbl) and the xanthene dye exhibited singlet oxygen quantum yield values of 0.24 and 0.15 respectively. However, the singlet oxygen quantum yield of Xan-SS-Cou-Cbl after treatment with GSH was found to be around 0.38. This can be attributed to the dual contribution from both Cou-Cbl and xanthene present in the solution. As observed from the quantum yield values, Cou-Cbl has better singlet oxygen generation ability than xanthene dyes.

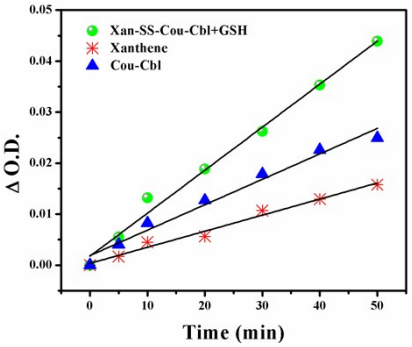


Figure S8 Comparative study of photodegradation of DPBF at 425 nm by Xan-SS-Cou-Cbl upon treatment with GSH, free xanthene, and free Cou-Cbl.

4.2 Monitoring of drug release by RP-HPLC[9]

The redox-responsive photoinduced drug release from Xan-SS-Cou-Cbl was examined after treating the compound with GSH for 1 h. Upon reaction with GSH, Cou-Cbl was released (as confirmed by mass spectrum **fig. 2**). The reaction mixture was then subjected to light irradiation and the release of anticancer drug chlorambucil with respect to time was monitored by reverse phase HPLC. A 1 X 10^{-4} M solution of Cou-Cbl in a 10 mL mixture of 70:30 acetonitrile–water was exposed to UV-vis light (≥ 350 nm) using a 1 M $CuSO_4$ solution as a filter under N_2 atmosphere for 60 min. A medium pressure Hg lamp of 125 W powers was used as the light source. A 50 μL aliquot of the solution was collected after each 10 min. The clear supernatant solutions, obtained after centrifuging, were then passed through reversed-phase HPLC using acetonitrile:water (7: 3) as the mobile phase. The flow rate was maintained at 1 mL min^{-1}.

4.3 Determination of photochemical quantum yield[9]

These experiments were carried out using a previously described method. 1 X 10^{-4} M solution of Xan-SS-Cou-Cbl in acetonitrile/water (7:3 v/v) mixture was taken in a quartz

cuvette. It was irradiated under UV light by 125 W medium pressure Hg vapor lamp using a suitable filter 1 M $CuSO_4$ (the transmittance for the above filter = 350 to 410 nm) after treating with GSH for 1 h. At a regular interval of time, 20 µL of the aliquots was taken and analyzed by RP-HPLC using mobile phase acetonitrile/water (7:3), at a flow rate of 1 mL min^{-1} (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 caged compound is less than 5 % of the initial area (**fig. S7**). 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. Further, the quantum yield for the photolysis of caged compounds was calculated using below **equation 3,**

$$\phi_p = \frac{(k_p)_{CP}}{I_0 (F_{CP})} \qquad \text{.....(3)}$$

Where, the subscript 'CP' denotes caged compound. Φp is the photolysis quantum yield, kp 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.

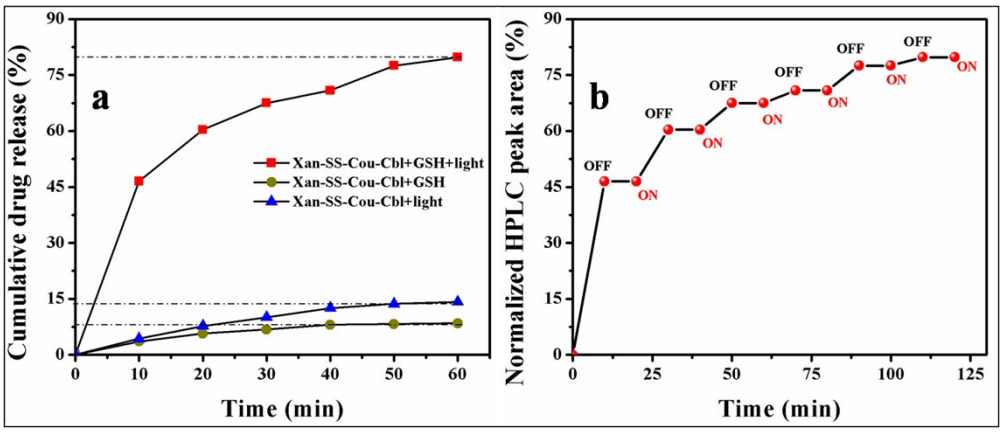


Figure S9 (a) Percentage of chlorambucil released from Xan-SS-Cou-Cbl, and **(b)** controlled light triggered release of chlorambucil under bright and dark conditions. "ON" indicates the beginning of light irradiation; "OFF" indicates the ending of light irradiation.

5. *In vitro* application of Xan-SS-Cou-Cbl

5.1 Redox-responsive photoinduced cytotoxicity study of Xan-SS-Cou-Cbl in HeLa and HEK 293 cell line[1]

The cytotoxicities of Xan-SS-Cou-Cbl in cancerous HeLa and non-cancerous HEK 293 cells were determined with and without UV irradiation using MTT assay.

Before Irradiation

Both HeLa and HEK 293 cells (1×10^4 cells per well of a 96-well plate) were incubated for 24 h at 37 °C in 5 % CO_2 with different concentrations of Xan-SS-Cou-Cbl (0–9 μM in HEPES buffer containing 10 % ethanol). Thereafter, fresh media containing MTT (0.40 mg/mL) were added to the 96-well plate and incubated at 37 °C in 5 % CO_2 for an additional 4 h. Formazan crystals formed were dissolved in DMSO after decanting the media, and then absorbance was recorded at 595 nm.

After Irradiation

Cells growing in log phase were seeded into a 96-well cell culture plate at 1×10^4 cells/mL. Different concentrations of Xan-SS-Cou-Cbl (0–9 μM in HEPES buffer containing 10 % ethanol) were added into the wells with an equal volume of PBS in the control wells. The cells were then incubated at 37 °C in 5 % CO_2 for 24 h. Thereafter, fresh media containing MTT (0.40 mg/mL) were added to the 96-well plate and incubated at 37 °C in 5 % CO_2 for an additional 4 h. Thereafter, the cells were irradiated at wavelengths ≥ 410 nm for 30 min (keeping the culture plate 6 cm from the light source) under aseptic conditions. After UV irradiation, the cells were again incubated for 24 h and the cell viability was measured by MTT assay. The cell viability was calculated using the following **equation 4**.

$$\text{Inhibition (\%)} = \frac{\text{Absorbance (untreated cells) - Absorbance (treated cells)}}{\text{Absorbance (untreated cells)}} \quad \ldots\ldots\ldots \textbf{(4)}$$

5.2 Cellular Internalization Study of Xan-SS-Cou-Cbl

The HeLa and HEK 293 cells were grown in their log phase. Cells were seeded in 96-well plates in Dulbecco's modified Eagle's (DMEM) medium containing 10 % fetal bovine serum (FBS) for 8 h. Different concentrations of Xan-SS-Cou-Cbl in HEPES buffer containing 10 % ethanol were added and incubated at 37 °C in 5 % CO_2 for 4 h. Then the media was discarded and cells were fixed using 4 % paraformaldehyde followed by washing twice with phosphate-buffered saline (PBS). Imaging was done using confocal laser scanning microscopy (CLSM) with suitable filters.

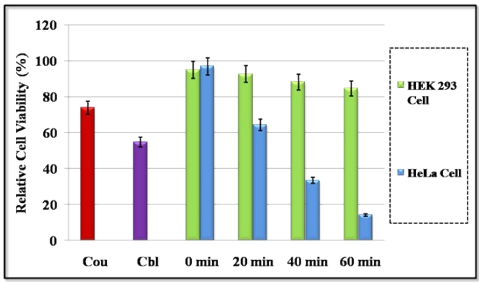


Figure S10 Comparative cell viability study of compound **6**, free coumarin (Cou) and free chlorambucil (Cbl) after irradiating with UV-vis light (≥ 350 nm wavelength) at different time

intervals. blue bar = HeLa and green bar = HEK 293 cell lines**.** Concentration of the samples = 25 μM. Values are presented as means ± standard deviations of three different observations.

6. References

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