Electronic Supplementary Material (ESI) for ChemComm.

Two-photon responsive Napthyl tagged p-hydroxyphenacyl based drug delivery system: uncaging of anti-cancer drug in the phototherapeutic window with real-time monitoring

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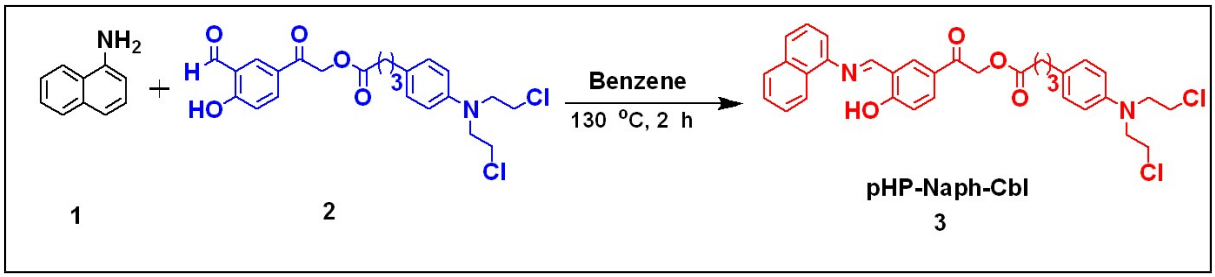
1. General Information:

All commercially available anhydrous solvents dimethylformamide (DMF), dichloromethane (DCM), petroleum ether (PE) and ethyl acetate (EA) and other chemicals were used without further purification. Acetonitrile and dichloromethane were distilled from CaH_2 before use. NMR spectra were recorded on a 500 and 400 MHz instrument. ^1H NMR chemical shifts were referenced to the tetramethylsilane signal (0 ppm), ^{13}C NMR chemical shifts were referenced to the solvent resonance (77.23 ppm, Chloroform-d). Chemical shifts (δ) are reported in ppm, and spin−spin coupling constants (J) are given in Hz. The following abbreviations were used to explain multiplicities: s = singlet, d = doublet, t = triplet, q = quadruplet, m = multiplet. UV/vis absorption spectra were recorded on UV/vis spectrophotometer and fluorescence spectra were recorded on fluorescence spectrophotometer. High-resolution mass spectra (HRMS) were recorded on ESI-TOF (electrospray ionization-time-of-flight). Photolysis of drug release was carried out using a 125 W medium pressure mercury lamp. RP-HPLC was taken using mobile phase acetonitrile/water (90:10), at a flow rate of 1mL/min (detection limit: UV 254 nm). Chromatographic purification was done with 60–120 mesh silica gel. For reaction monitoring, precoated silica gel 60 F254 TLC sheets were used. Fluorescence emission spectra were recorded on Hitachi F-7000 fluorescence spectrophotometer and Shimadzu RF- S5 6000 spectrofluorophotometer. HRMS spectra were recorded on a JEOL-AccuTOF JMS-T100L mass spectrometer. Photolysis was carried out using 125 W medium pressure mercury lamp supplied by SAIC (India).

2. Synthesis of pHP-Naph-Cbl:

We have synthesized our DDS, (**pHP-Naph-Cbl**) (**Scheme S1**) followed by reported procedure up to compound **2** and then compound **2** was condensed with commercially available 1-Napthylamine using Dean-Stark Apparatus and benzene as a solvent gave our desired DDS **pHP-Naph-Cbl.**



Scheme S1. Synthesis of photoresponsive DDS **pHP-Naph-Cbl**

2.1. Experimental procedure and Spectroscopic data:

2-oxo-2-oxoethyl-4-(4-(bis (2-chloroethyl) amino) phenyl) butanoate (126 mg, 0.27 mmol) was dissolved in 5 mL of benzene and 1-aminonaphthalene (34 mg, 0.27 mmol) was added to the mixture refluxed using Dean–Stark apparatus to remove the water and kept for refluxing at 130 ^{0}C for 2 h. After two hours, the reaction mixture is taken and cooled down to room temperature and evaporates the benzene using rotavap. Pure product was obtained by separation in column chromatography using 20 % ethyl acetate in pet ether to furnished the DDS **pHP-Naph-Cbl** 79 % yield as a yellow coloured gummy solid.

^{1}H NMR (400 MHz, $CDCl_3$) δ 14.26 (s, 1H), 8.78 (s, 1H), 8.21 (d, *J* = 5.0 Hz, 1H), 8.13 (s, 1H), 7.99 (d, *J* = 8.7 Hz, 1H), 7.90 (s, 1H), 7.84 (d, *J* = 8.2 Hz, 1H), 7.63 – 7.47 (m, 3H), 7.32 – 7.22 (m, 1H), 7.16 (d, *J* = 8.7 Hz, 1H), 7.11 (d, *J* = 7.9 Hz, 2H), 6.63 (d, *J* = 7.9 Hz, 2H), 5.34 (s, 2H), 3.73 – 3.67 (m, 4H), 3.65 – 3.60 (m, 4H), 2.64 (t, *J* = 7.3 Hz, 2H), 2.52 (t, *J* = 7.2 Hz, 2H), 2.06 – 1.97 (m, 2H). **^{13}C NMR** (101 MHz, $CDCl_3$) δ 190.17, 173.11, 166.03, 162.67, 145.17, 144.36, 134.03, 133.11, 132.82, 130.60, 129.80, 128.08, 127.69, 126.84, 125.98, 122.91, 119.22, 118.00, 114.27, 112.23, 65.55, 53.63, 40.55, 33.87, 33.20, 26.76. **HRMS** (ESI-TOF) m/z: $[M + H]^+$ Calcd for $C_{33}H_{33}Cl_2N_2O_4$ 591.1817; Found 591.1823.

3. ^{1}H, ^{13}C spectra and HRMS of pHP-Naph-Cbl:

3.1 ^{1}H NMR spectrum of compound pHP-Naph-Cbl

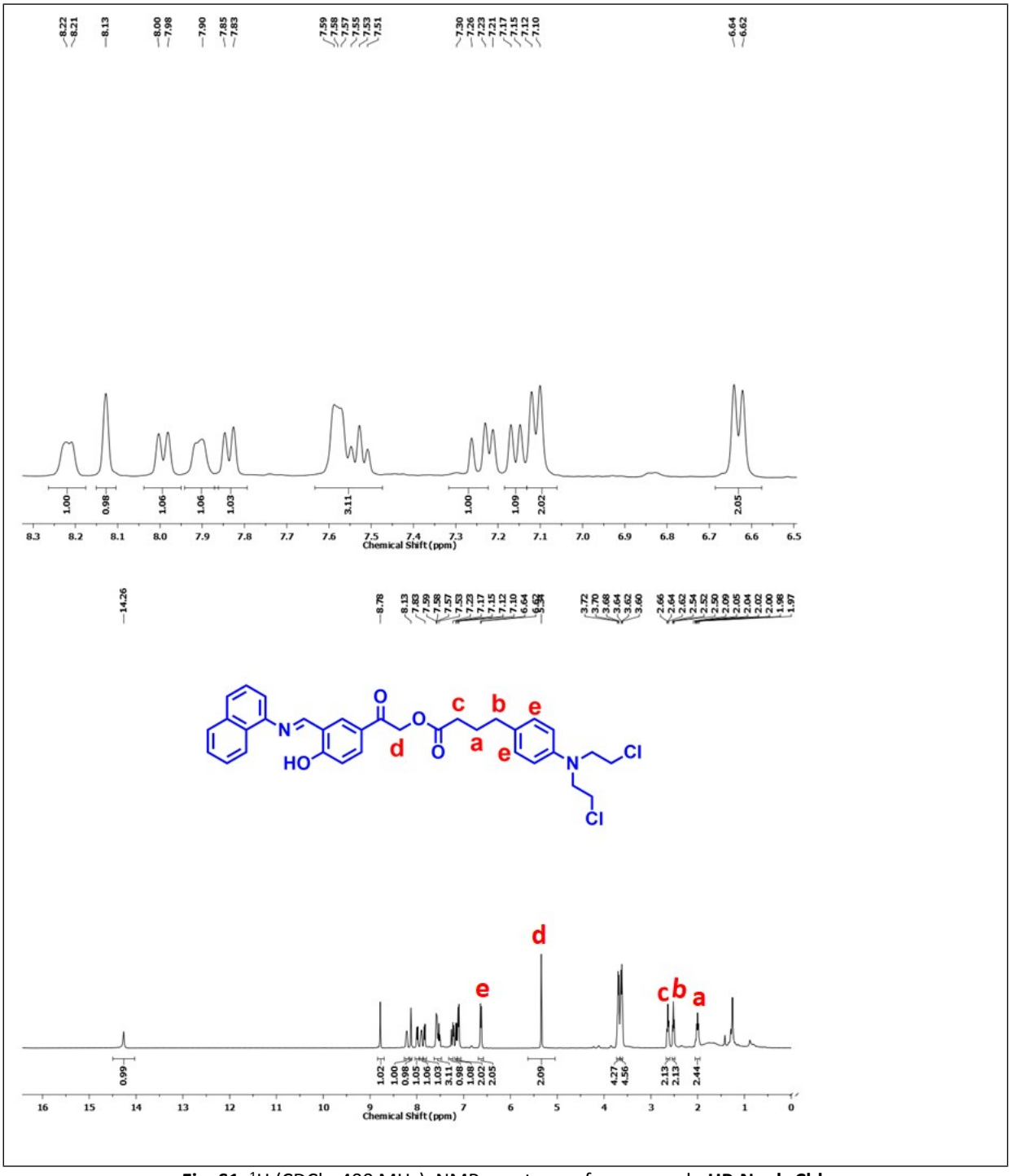


Fig. S1 ^{1}H ($CDCl_3$, 400 MHz) NMR spectrum of compound **pHP-Naph-Cbl.**

3.2 ^{13}C NMR spectrum of compound pHP-Naph-Cbl

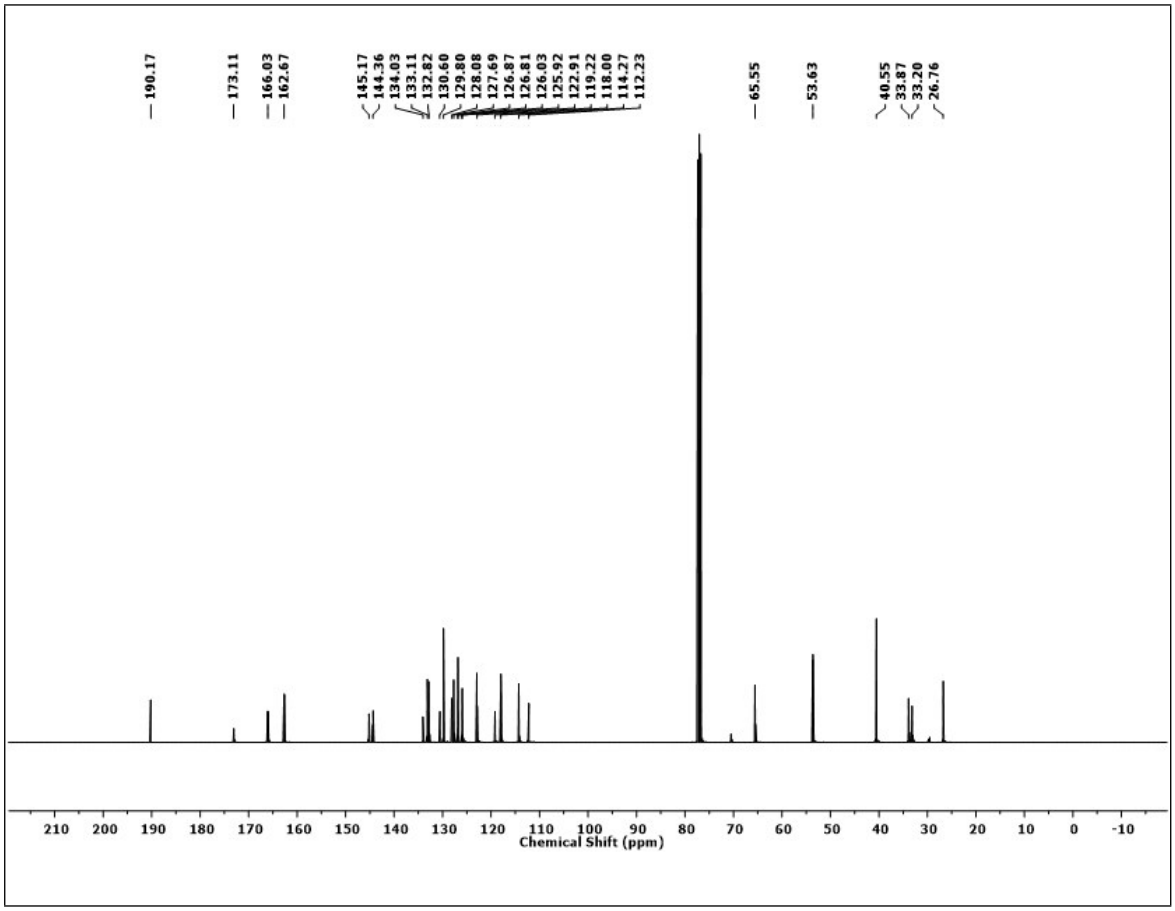


Fig. S2 ^{13}C ($CDCl_3$, 101 MHz) NMR spectrum of compound **pHP-Naph-Cbl**.

3.3 HRMS of compound pHP-Naph-Cbl

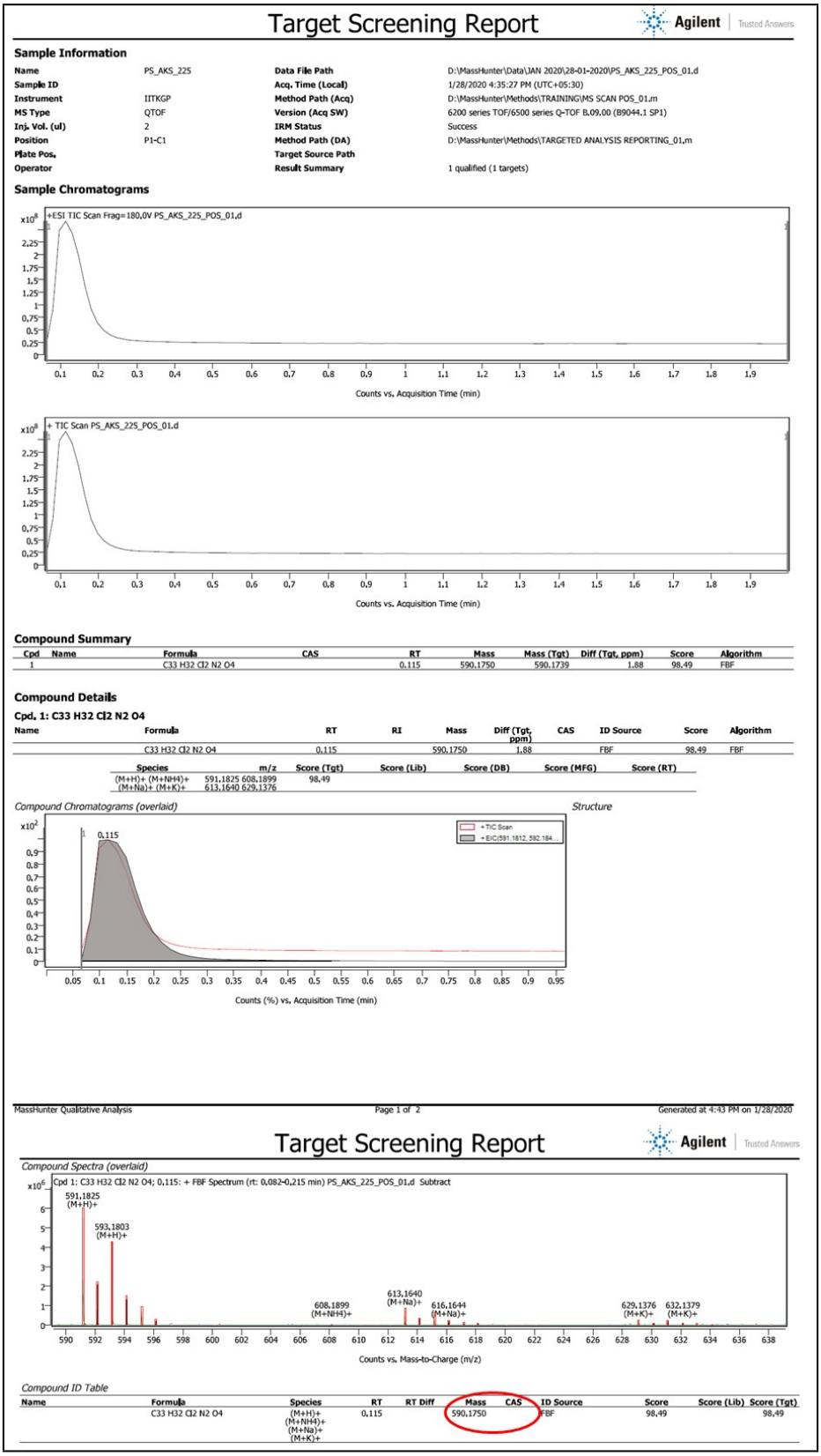


Fig. S3 HRMS of compound **pHP-Naph-Cbl.**

4. Photophysical properties of pHP-Naph-Cbl:

The photophysical properties, absorption and emission spectra of photoresponsive DDS **pHP-Naph-Cbl** and were recorded for different solvents using UV-Vis and Fluorescence spectrophotometer respectively. In each case, DDS of **pHP-Naph-Cbl** (1×10^{-4} M) solution was prepared with different solvent and after degasification of solution, the readings were taken.[1]

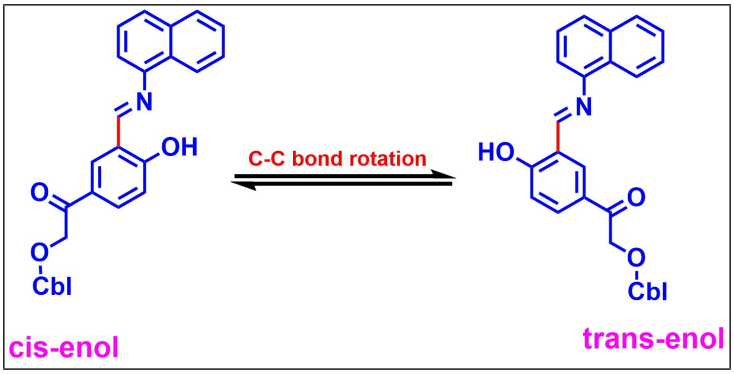


Fig S4: Existence of cis-trans enolic form of DDS **pHP-Naph-Cbl.**

5. Measurement of fluorescence quantum yields:

The fluorescence quantum yield (QY) of **pHP-Naph-Cbl** was determined by the reference point method. Quinine sulfate in 0.1 M H_2SO_4 (literature quantum yield: 0.547) by using a standard sample to calculate the QY.[2] **pHP-Naph-Cbl** was dissolved in CH_3CN/PBS buffer of pH 7.4 (1:9). The absorbance values of the solutions at the excitation wavelength were measured with UV–Vis spectrophotometer. Photoluminescence (PL) emission spectra were recorded by Hitachi F-7000 fluorescence spectrophotometer at an excitation wavelength of 350 nm and 320 for Quinine sulphate and **pHP-Naph-Cbl** respectively.

$$\frac{(\Phi)_s}{(\Phi)_r} = \frac{(A)_s}{(A)_r} \times \frac{(Abs)_s}{(Abs)_r} \times \frac{(\eta)_r}{(\eta)_s}$$

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

Table S1: Fluorescent quantum yield (φ_f) of **pHP-Naph-Cbl.**

Compound	**Fluorescent quantum yield (φ_f)[a]**
pHP-Naph-Cbl	**0.22**

[a]Fluorescent quantum yield (error limit within ±5%)

6. Stability of DDS pHP-Naph-Cbl:

To check the stability of our DDS in biological medium, **pHP-Naph-Cbl** (1 x 10^{-4} M, 1:9 v/v) in ACN was prepared and to this aqueous 10 % fetal bovine serum was added maintaining pH at 7.4. The above solution was kept in dark conditions for seven days. After seven days aliquots of 25 µL were injected into the RP-HPLC, and the percentage of decomposition was quantified from the RP-HPLC peak area and compared with the injected original sample. The experiments were repeated three times and the results are presented in **Table S2**. Similarly, we also checked the stability of our DDS at pH 5.5 and 8. For this case, we prepared a binary mixture solution of our DDS in ACN/Water (1 X 10^{-4} M, 1:9, V/V), and kept it in dark conditions for seven days. After seven days aliquots of 25 µL were injected into the HPLC, and the percentage of decomposition was quantified from the RP-HPLC peak area and compared with the injected original sample. These results are given in T**able S2**.

The above results show that our DDS in both the conditions (biological medium and different pH) showed good stability.

Table S2: Percentage of decomposition under the dark condition of **pHP-Naph-Cbl** in biological medium and at different pH at 37 °C.[a]

		Biological medium	**Different pH**	
DDS	**Time**	**10 % fetal bovine serum pH = 7.4**	**pH = 5.5**	**pH = 8**
pHP-Naph-Cbl	**7** days	**4 %**	**7 %**	**9 %**

[a] As examined by RP-HPLC.

7. Photolysis of pHP-Naph-Cbl:

To check the ability of **pHP-Naph-Cbl** as a photoresponsive drug delivery system, 20 ml solution of **3** (1×10^{-4} M) in CH_3CN/PBS buffer (1:9 V/V) of pH 7.4 and was irradiated with UV light (≥ 365 nm) using medium pressure mercury lamp (125 W) and UV cut-off filter (1M $CuSO_4$ solution). To demonstrate the precise control over the drug delivery, the solution was exposed to light and dark conditions, periodically. Aliquots of 25 µL were injected in each case into the HPLC. The released drug was quantified from the RP-HPLC peak area and compared with the injected original sample.

8. HRMS of photoproduct Cbl and pHP-Naph-COOH:

8.1 HRMS of photoproduct Cbl

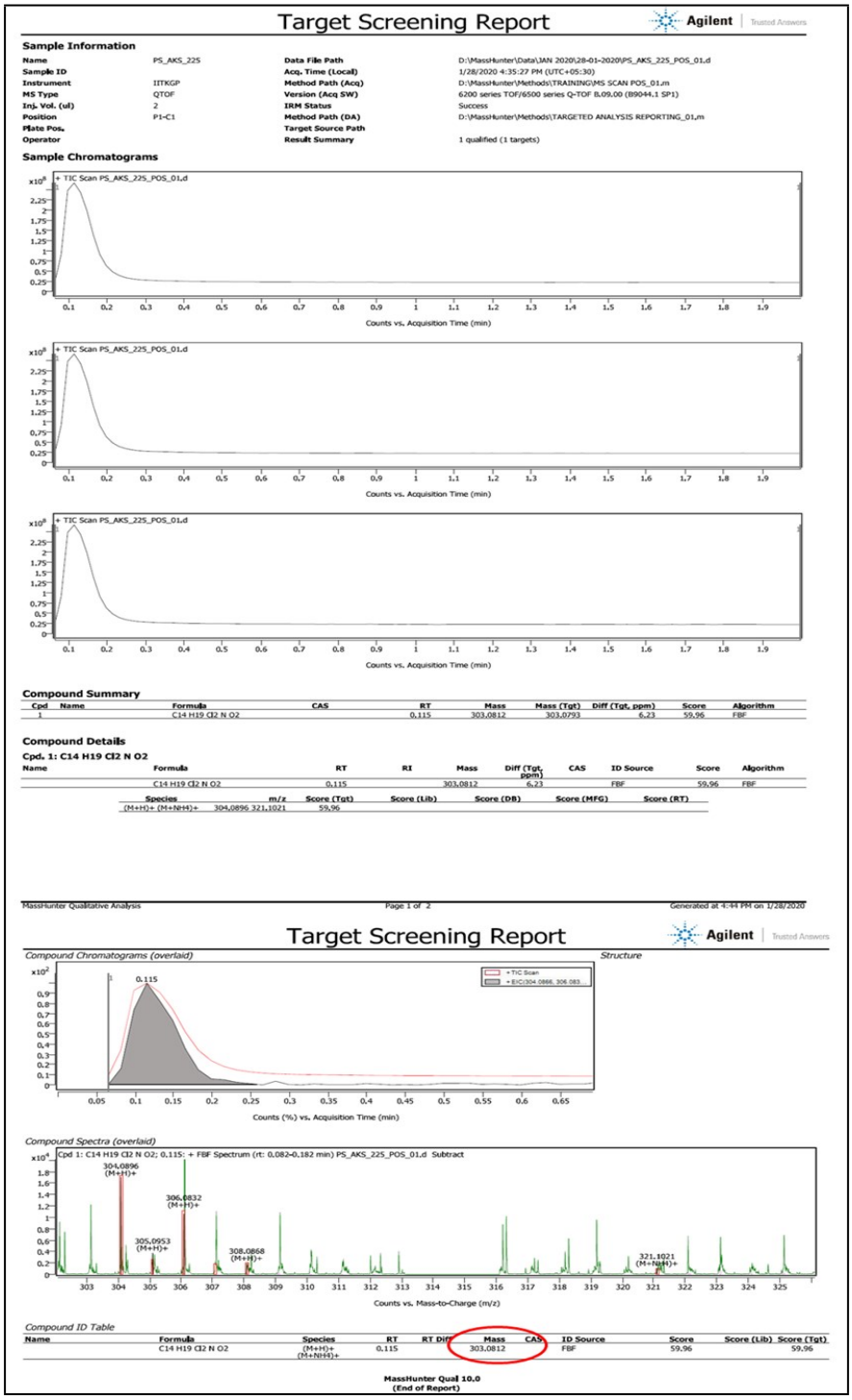


Fig. S5 HRMS of anti-cancer drug **Cbl** (after 15 min of photoirradiation).

8.2 HRMS of photoproduct pHP-Naph-COOH

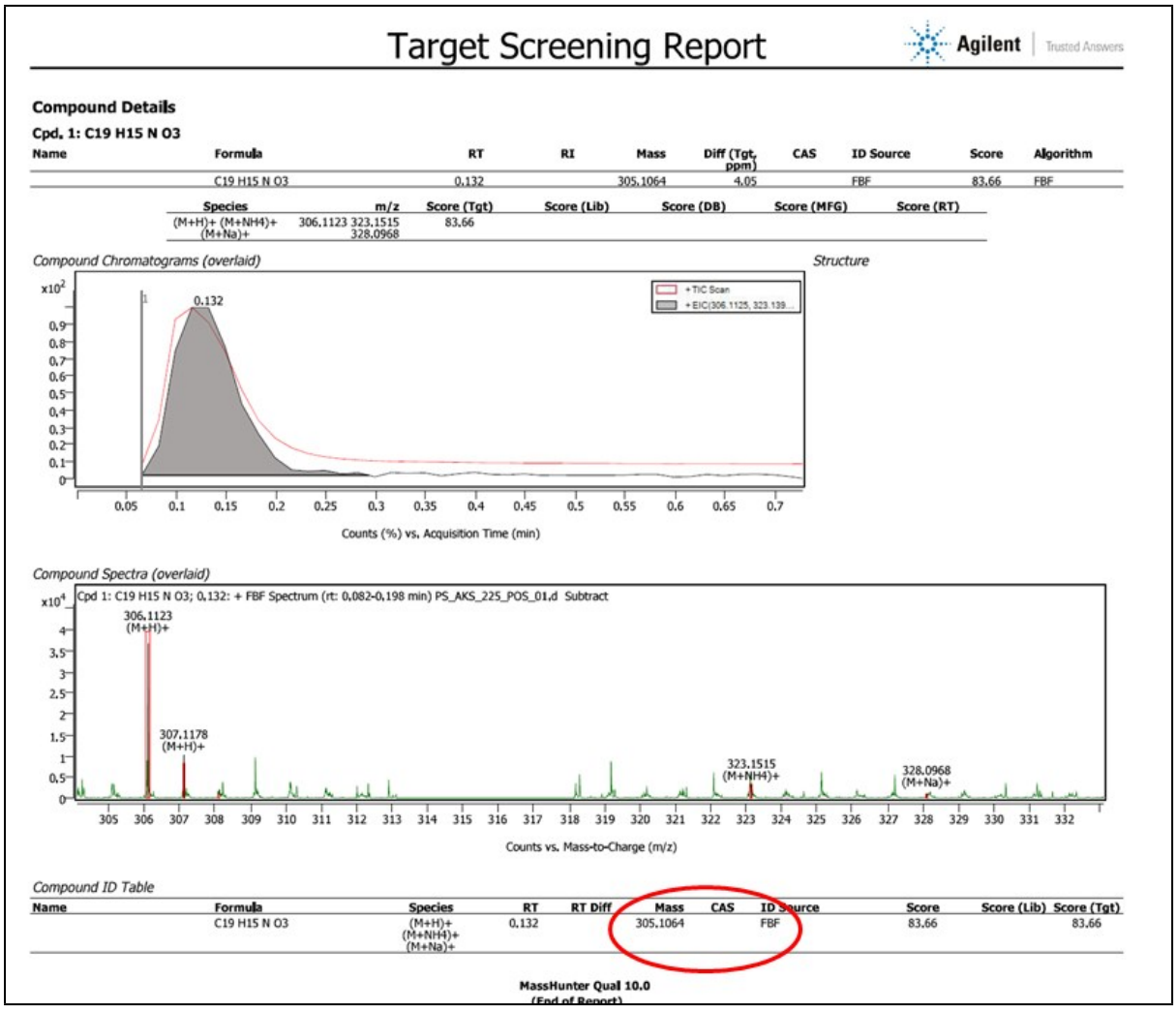


Fig. S6 HRMS of photoproduct **pHP-Naph-COOH** (after 15 min of photoirradiation).

9. Procedure for the quantification of the released drug from pHP-Naph-Cbl:

The quantification of the released drug from **pHP-Naph-Cbl** concerning time was carried out by exposing the solution to the UV light at regular intervals of time (**Fig. S6 a**). Aliquots of 25 µL were injected into HPLC after each interval. The released drug was quantified from the HPLC peak area in comparison with the injected original sample.

To demonstrate the precise control over the drug delivery, the solution was exposed to light and dark conditions periodically (**Fig. S6 b**). Aliquots of 25 µL were injected in each case into the HPLC. The released drug was quantified from the HPLC peak area in comparison with the injected original sample.

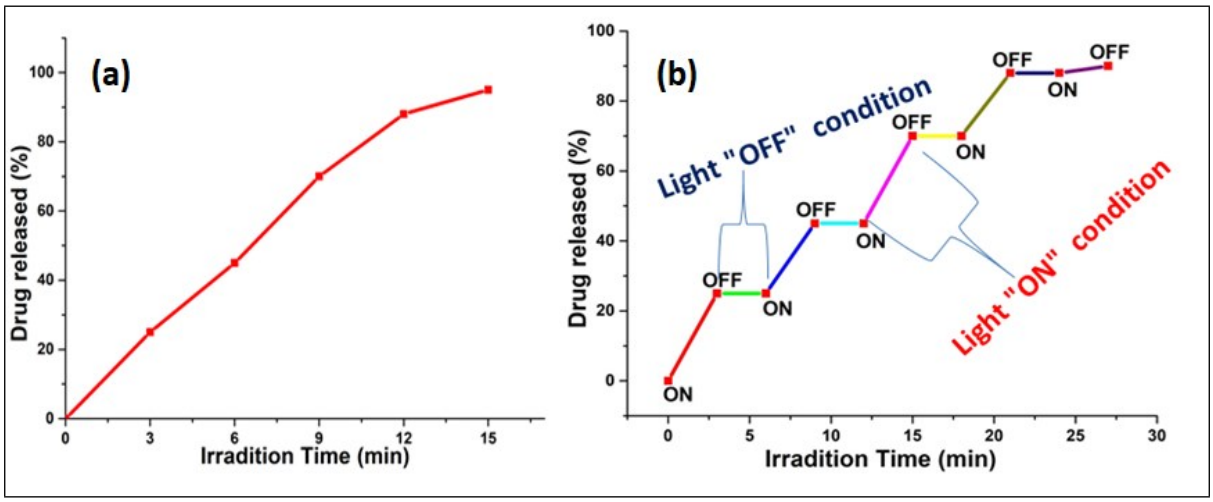


Fig. S7 (a) The amount of drug released from **pHP-Naph-Cbl on** photolysis (λ ≥ 365 nm) within different intervals of time. (b) Release of the drug under light and dark conditions. "ON" and "OFF" implies the switching on and off of the light source, respectively.

10. Triplet quencher study:

Quenching studies in presence of triplet state quencher[3] (potassium sorbate) was conducted to ensure whether the photo-release occurs from singlet or triplet state, in which photolysis of **pHP-Naph-Cbl** (1×10^{-4} M) was carried out in presence 200 µM concentration of triplet quencher potassium sorbate (PoS). At different times of irradiation (0, 3, 6, 9, 12 and 15 min) aliquots were collected and analyzed by RP-HPLC using mobile phase acetonitrile and water (90:10 v/v), at a flow rate of 1mL/min (detection: UV 250 nm). Peak

areas were determined by RP-HPLC. HPLC peaks areas of caged compound (from HPLC peak area) versus irradiation time were plotted. The result showed that at a concentration of 200 μM of PoS, the drug release by **pHP-Naph-Cbl** was completely arrested; indicating that photorelease occurs from the triplet excited state (**Fig. S7**).

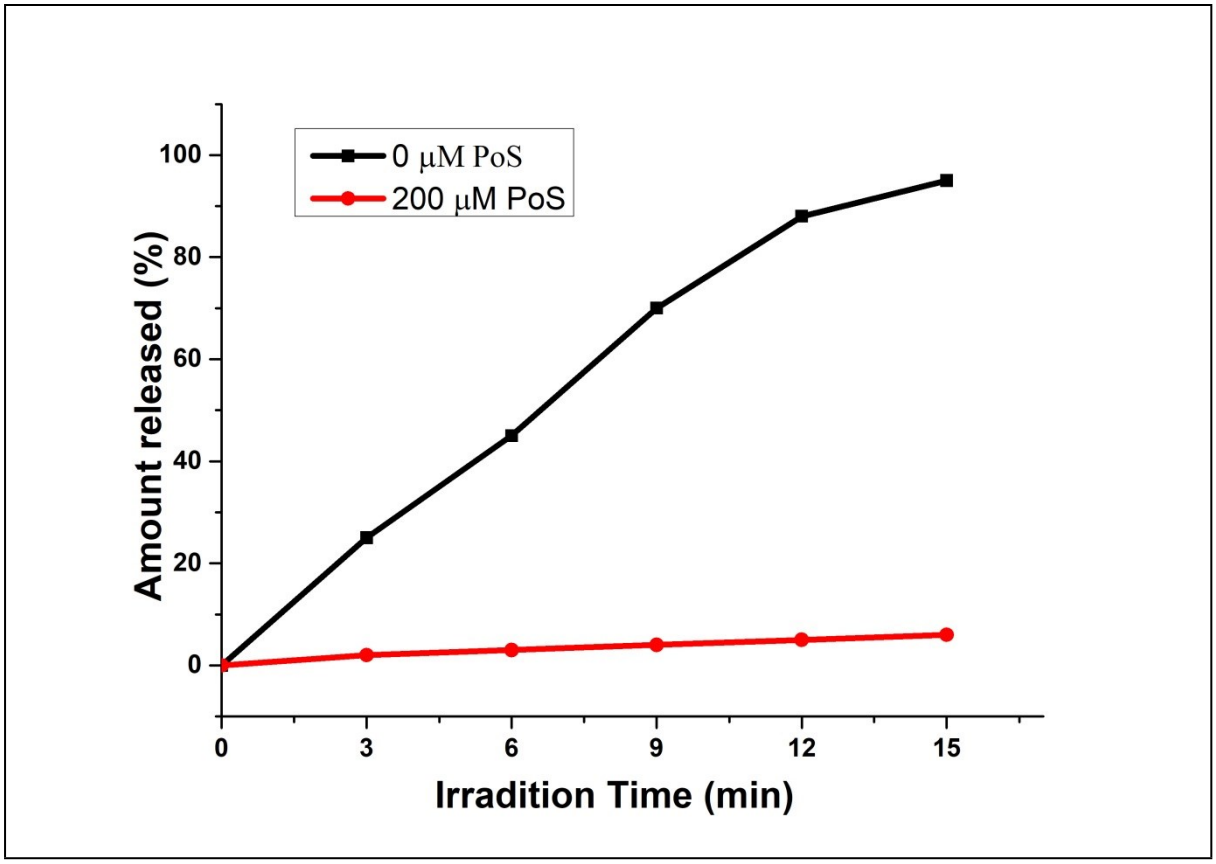


Fig. S8 Time course of photolysis of **pHP-Naph-Cbl** under irradiation of UV light (λ ≥ 365 nm) in the presence of 200 μM triplet quencher potassium sorbate (PoS).

11. 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. A solution of potassium ferrioxalate, 1, 10-phenanthroline and the buffer solution were prepared following the literature procedure.[4] A Solution (0.006 M) of potassium ferrioxalate was irradiated using 125W medium pressure Hg lamp as a UV light source (λ ≥365 nm) and 1 M $CuSO_4$ solution as a UV cut-off filter. At regular intervals of time (3 min), 1mL 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 the formed red phenanthroline-ferrous complex 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 the phenanthroline-ferrous complex at several known concentrations of Fe^{2+} ion in the dark. From the slope of the graph, the molar absorptivity of the phenanthroline-ferrous complex was calculated at 510 nm, which was found to be similar to the reported value. Using the known quantum yield for potassium ferrioxalate actinometer at 363.8 nm, the number of Fe^{2+} ions formed during photolysis and the fraction of light absorbed by the actinometer, the incident photon flux (I_0) at 365 nm of the 125W Hg lamp was determined as 1.55 x 10^{17} photons s^{-1} cm^{-2}.

12. Photochemical quantum yield determination for pHP-Naph-Cbl:

A 20 mL solution of **pHP-Naph-Cbl** (1 × 10^{-4} M) was prepared in CH_3CN/PBS buffer (1:9 V/V) of pH 7.4 and solution was made inert by passing nitrogen gas through the sample for 15 min. and irradiated using 125 W medium pressure Hg lamp as light source (≥ 365 nm) and 1 M $CuSO_4$ solution as UV cut-off filter. At regular intervals of time, 20 μl aliquots of the sample were taken and analysed by RP-HPLC using mobile phase acetonitrile, at a flow rate of 1mL/min (detection: UV 254 nm). Peak areas were determined by RP-HPLC, which indicated a gradual decrease of the caged compound with time, and the average of three runs. The reaction continued until the consumption of the ester is less than 10 % of the initial area. Based on HPLC data for the caged compound, the natural logarithm of the concentration of the 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 the caged compound was calculated using equation (1)

$$(\Phi)_{CG} = (\Phi)_{act} \times [(kp)CG/(kp)act] \times [Fact/FCG] \text{ ---------- } (1)$$

Where, the subscript 'CG' and 'act' denote caged compound and actinometer, respectively. Φ is the relative photolysis quantum yield, kp is the photolysis rate constant and F is the fraction of light absorbed. Potassium ferrioxalate was used as an actinometer.[4]

13. Determination of photochemical quantum yield for pHP-Naph-Cbl at different water fraction and in pure acetonitrile:

Photolysis of our DDS **pHP-Naph-Cbl** (1×10^{-4} M) carried out in different volume of water fractions and in pure acetonitrile. and the solution was made inert by passing nitrogen gas through the sample for 10 min. and irradiated using 125 W medium pressure Hg lamp as light source (≥ 365 nm) and 1M $CuSO_4$ solution as UV cut-off filter for 15 min., then 20µl aliquots of the sample were taken and analysed by RP-HPLC using mobile phase acetonitrile, at a flow rate of 1mL/min (detection: UV 254 nm). We observed that there was no anti-cancer drug was released in pure acetonitrile and f_w upto 80 %.

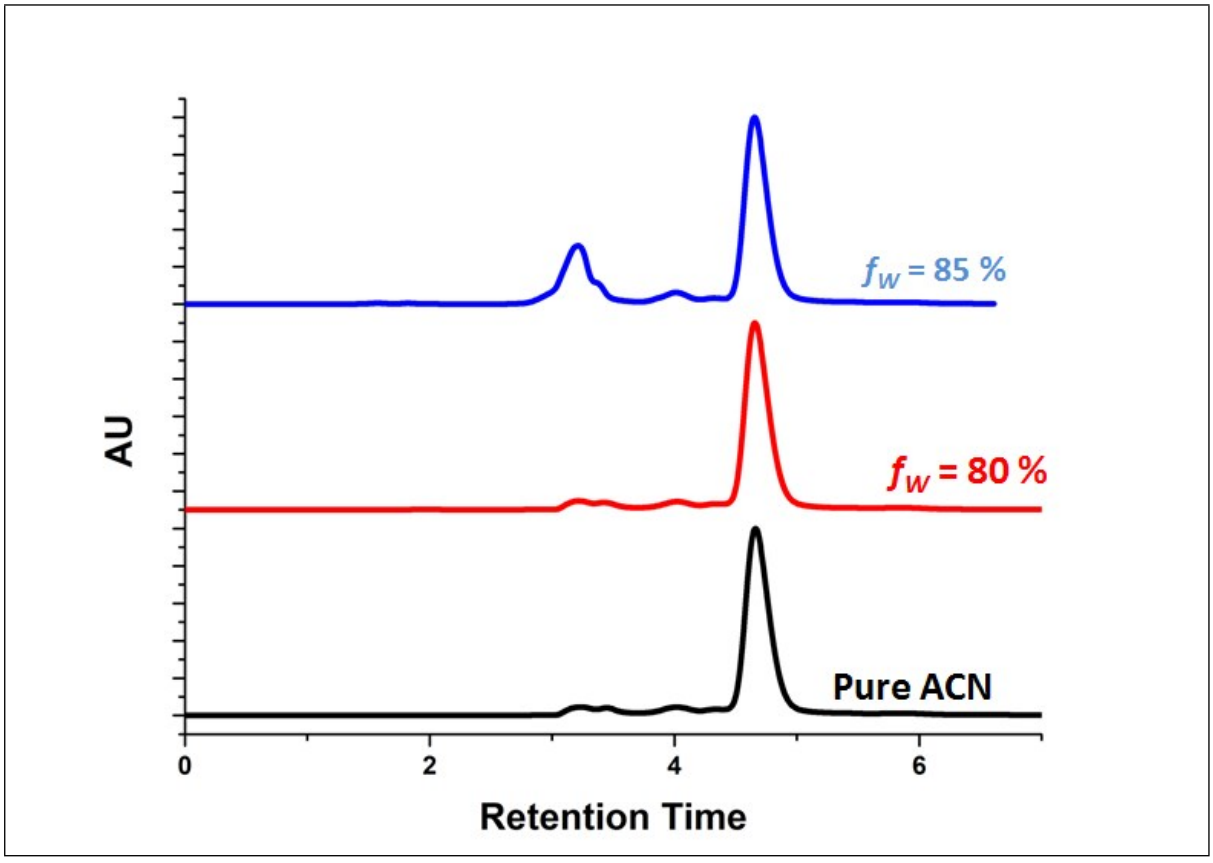


Fig. S9 HPLC chromatogram of **pHP-Naph-Cbl** in pure **ACN** and at different volume of water fractions (f_w) after 15 min of photoirradiation.

Table S3: Photochemical quantum yields of **pHP-Naph-Cbl** in different water fractions (f_w) and in pure acetonitrile

Water fractions (f_w)	**Quantum yield[a]**	**Quantum yield[a] (In pure ACN)**
80 %	0.03	0.01
85 %	0.23	
≥ 90 %	0.49	

[a]Photochemical quantum yield (error limit within ±5%)

14. Quantum yield of photolysis of pHP-Naph-Cbl at different pH:

Photolysis of **pHP-Naph-Cbl** (1×10^{-4} M) in CH_3CN/PBS buffer (1:9 v/v) of different pH (5.5, 7.4, and 8) was carried out at different intervals of irradiation (0, 3, 6, 9, 12, and 15min). After each interval 25 µL aliquots were collected and analyzed by RP-HPLC using mobile phase acetonitrile and water (90:10), at a flow rate of 1mL/min (detection: UV 250 nm). Peak areas were determined by RP HPLC. HPLC peak areas of caged compound versus irradiation time were plotted. The quantum yield of **pHP-Naph-Cbl** was calculated at different pH (**Table S4**) by using potassium ferrioxalate as an actinometer.

Table S4. Quantum yield of photolysis of **pHP-Naph-Cbl** at different pH.

pHP-Naph-Cbl in different pH	**Time taken for 95 % release**	**Quantum Yield[a]**
5.5	10	0.32
7.4	15	0.49
8	34	0.56

[a]Photochemical quantum yield (error limit within ±5%)

We performed the photolysis in different pH medium to demonstrate the interdependence of ESIPT process and the photorelease. The pH study indicated that the phenolic **–OH** of the **pHP** group remains intact in acidic pH. In neutral and basic pH, the **pHP** group remains partial and completely deprotonated, respectively. Since deprotonation is the key step for

the release for **pHP** group, it is expected that the quantum yield of photorelease will be more in basic pH than in neutral and acidic pH. The results of the pH study as provided in the **Table S4** indicated that the photochemical quantum yield for **pHP-Naph-Cbl** is quite similar to that obtained in neutral pH 7.4 (quantum yield = 0.49) and basic pH 8 (quantum yield= 0.56). This is only possible if the ESIPT phenomenon occurs between **-C=N** group of the naphthalene moiety and the **–OH** group of **pHP,** resulting in deprotonation of **–OH** group of **pHP** which then assists the photorelease.[5]

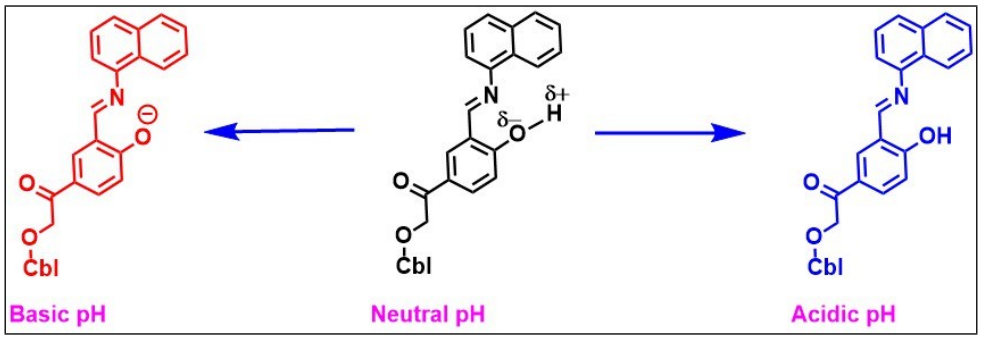


Fig. S10 Different forms of existence of phenolic **–OH** in **pHP-Naph-Cbl** at different pH.

15. Z-Scan Measurement:

We performed the open aperture Z-scan with **pHP-Naph-Cbl** (1 x 10^{-4} M) in DMSO/Water, (1:9 v/v) with pulsed laser (Pulse width 100 fs, repetition rate 80 MHz at 700 nm with 1.0-Watt power).

The normalized transmittance (after diving with reference voltage) through a nonlinear medium where TPA occurs is fitted with the equation (2).

$$T(z) = \sum_{n=0}^{\infty} \frac{(-q_0)^n}{(n+1)^{\frac{3}{2}}(1+x^2)^n} \quad \ldots\ldots(2)$$

Where $x = z/z_R$, $q_0 = \beta L_{eff} I_0$; I_0 is the maximum on-axis intensity in the focus, β is TPA coefficient and z_R is the Rayleigh length.

With the value β extracted from the Eq. (1) TPA cross-section has been determined by equation (3).

$$\sigma = \sum_{n=0}^{\infty} \frac{h\beta}{N_A \rho \lambda \times 10^{-3}} \ldots\ldots(3)$$

Where h is the Planck constant, N_A is Avogadro's number, λ is the wavelength of the laser and ρ is the concentration in mole per litre. σ is commonly given in an SI unit (GM) defined as 1 GM=10^{-50} cm^4 s $photon^{-1}$ $molecule^{-1}$.[6]

Two-photon absorption cross-section value of this sample is **19.72 GM.**

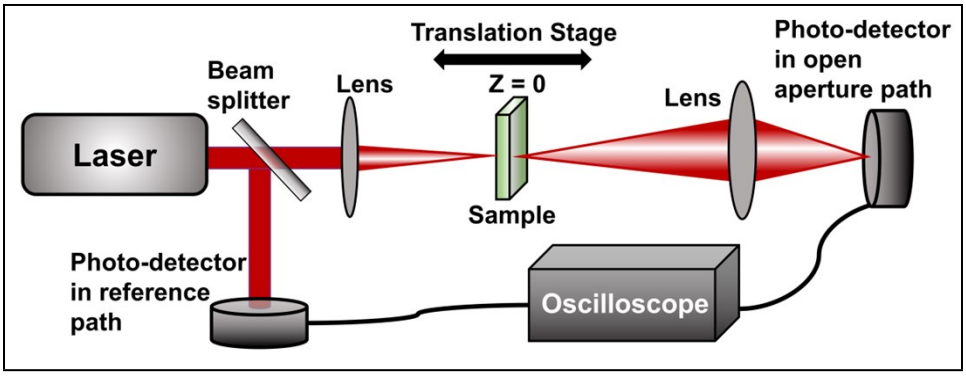


Fig. S11 Schematic representation of open aperture Z-scan experimental setup

16. Two-photon photolysis:

1 mL solution of **pHP-Naph-Cbl** (1 x 10^{-4} M) in DMSO/PBS buffer of pH 7.4 (1:9 v/v) was taken in a 1 mm quartz cuvette and irradiated with a laser of wavelength 700 nm with 100 fs pulses at 85 MHz rate with a 60 µm diameter beam spot. During the photolysis at regular time intervals, small aliquots (25 µL) were taken out from the solution for the HPLC study. The released drug was quantified from the HPLC peak area in comparison with the injected original sample. We have found that after 3 h of photolysis 25 % of the drug got released.

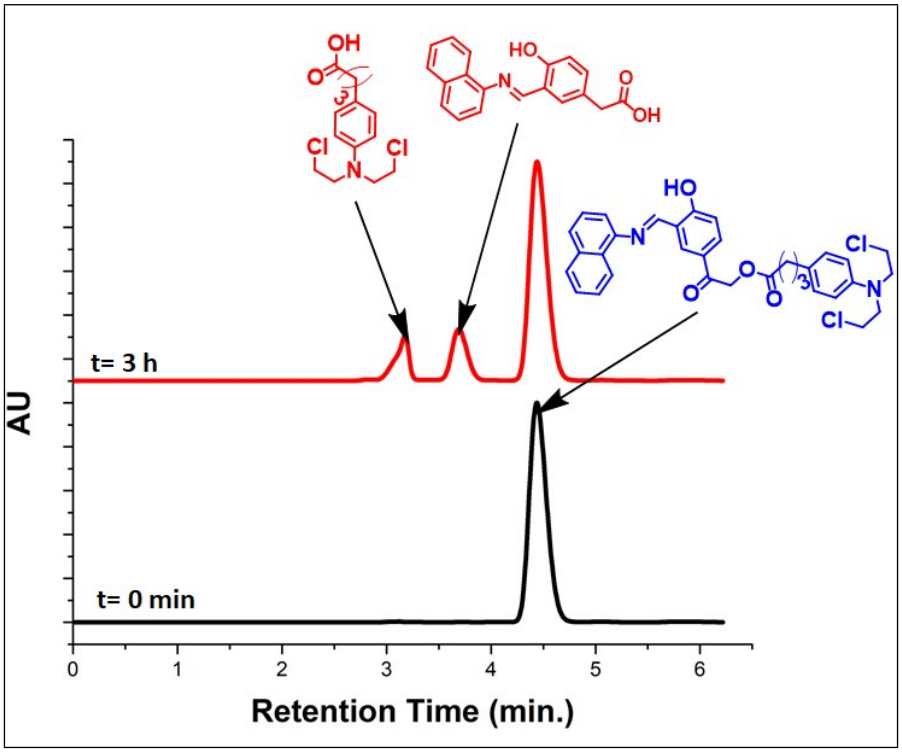


Fig. S12 HPLC overlay chromatogram of **pHP-Naph-Cbl** at different time intervals of light irradiated with a laser of wavelength 700 nm with 100 fs pulses at an 85 MHz rate.

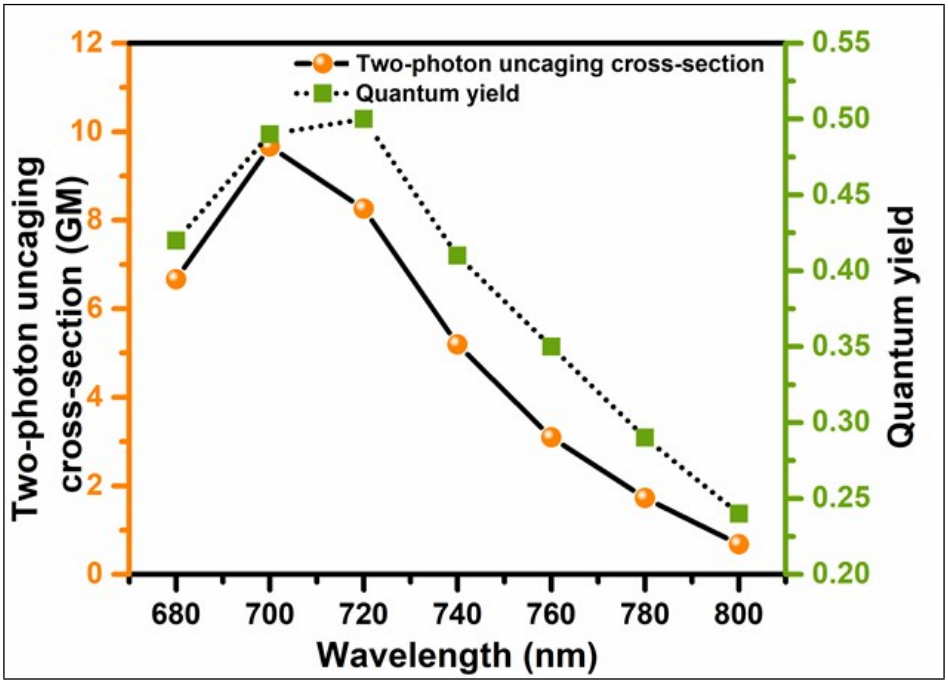


Fig. S13 Two-photon uncaging cross-section of DDS **pHP-Naph-Cbl** at different wavelengths.

17. Antiproliferative activity assays:

17.1. Cell lines:

MCF-7 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 % foetal bovine serum. Cells were cultured at 37 ^{0}C in a CO_2 incubator (Thermo Fisher Scientific, USA).

17.2 In Vitro Cytotoxicity assay:

Cancer cell line (1 × 10^5 cells/well of a 96-well plate) was treated with different concentrations of **pHP-Naph-Cbl** and **Cbl** (4–500 μM) and incubated for 4 h at 37 ^{0}C in a CO_2 incubator. Thereafter, the cells were irradiated by UV light, λ ≥ 365 nm for 15 min (keeping the culture plate 6 cm away from the light source) using UV-visible lamp (Bangalore Genei Pvt. 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 below. Cell viability was calculated using the formula, Viability (%) = 100 × A2/A1; [Where A2 = Absorbance of the treated cell; A1 = Absorbance of the control cells].

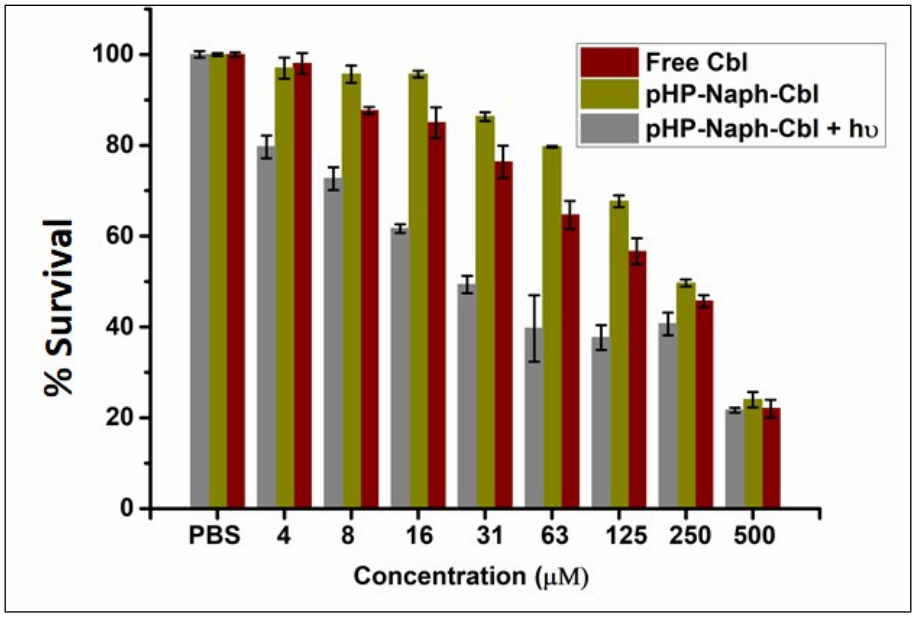


Fig. S14 Percentage of cell survival in cell line MCF-7, before and after, at different time intervals of photoirradiation.

17.3 Fluorescence microscopy:

MCF-7 cell line (1 × 10^5 cells/6-well) were treated with (500 μM) of our DDS **pHP-Naph-Cbl** and put aside for 4 h at 37 °C. Then, the cells were irradiated with UV light (λ ≥ 365 nm) for 0–15 min. Thereafter, the plate was incubated for 48 h at 37 ^{0}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 was carried by Image J software. The captured cellular images were processed in the Image J software and the fluorescence intensity values for the selected regions were compared.

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