Supporting Information

An improved tumor-specific therapeutic strategy by the spatio-temporally controlled in situ formation of a Cu(II) complex, leading to prompt cell apoptosis via photoactivation of a prodrug

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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 600 and 400 MHz instrument. ^1H NMR chemical shifts were referenced to the tetramethylsilane signal (0 ppm). 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 a Shimadzu UV-2450 UV/vis spectrophotometer and fluorescence spectra were recorded on a Hitachi F-7000 fluorescence spectrophotometer. High-resolution mass spectra (HRMS) were recorded on ESI-TOF (electrospray ionization-time-of-flight). Photolysis was carried out using a 125 W medium pressure mercury lamp. RP-HPLC was taken using mobile phase acetonitrile/water (1:1), at a flow rate of 1 mL /min (detection: UV 310 nm). Chromatographic purification was done with 60–120 mesh silica gel. For reaction monitoring, precoated silica gel 60 F254 TLC sheets were used. Cytotoxicity were recorded on Synergy™ H1 is a configurable multi-mode microplate reader. Confocal images were recorded on Nikon Ti Eclipse confocal microscope. Cell culture media and all the other materials required for culturing were obtained from Gibco, USA.

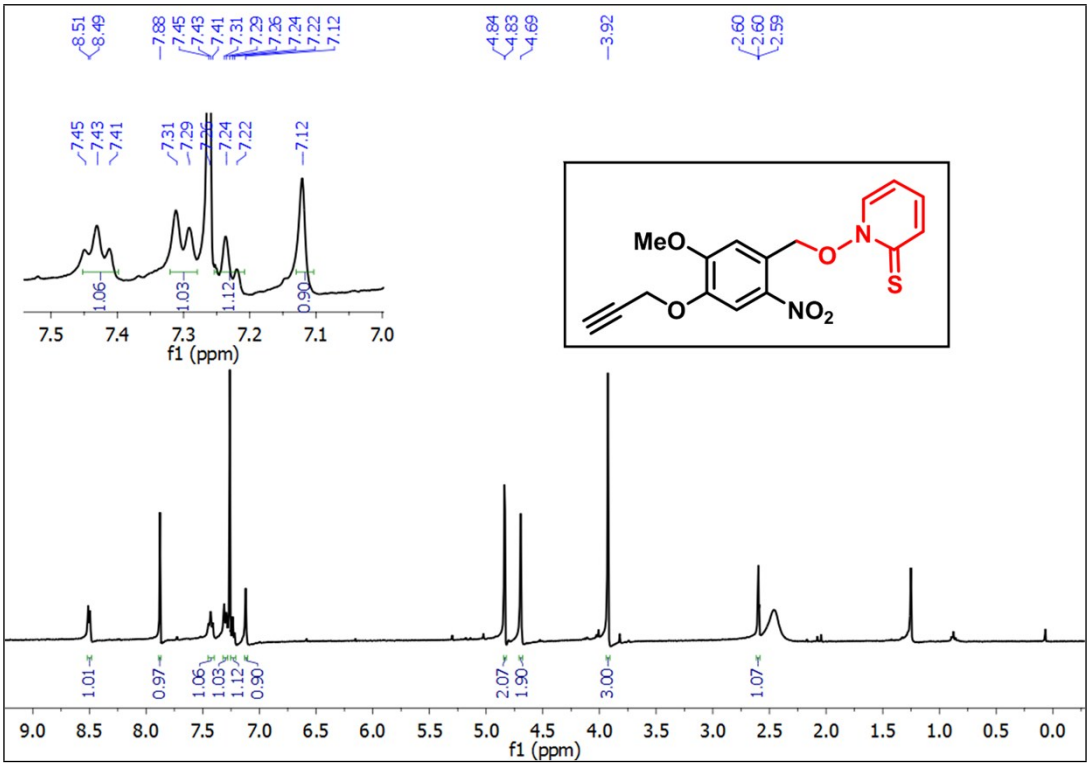
2. Experimental Procedure and spectroscopic data:

1-((5-methoxy-2-nitro-4-(prop-2-yn-1-yloxy)benzyl)oxy)pyridine-2(1H)-thione (2): To a solution of 2-marcapto pyridine-N-oxide (0.25 g, 1.99 mmol) in dry DMF (4 mL), was added potassium carbonate (0.28 g, 1.99 mmol). After 5 min the brominated compound **1** (0.5 g, 1.66 mmol) was added to the reaction mixture and stirred for 1 h. After completion of the reaction, it was quenched with brine solution. Then the aqueous layer was extracted by EtOAc (3×15 mL). The combined organic layer was then dried over Na_2SO_4 and the solvent was removed under vacuum. The residue was purified over silica gel column (30% EtOAc: hexane) to afford compound **2** as a yellow solid (0.507 g, 89%). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.50 (d, *J* = 6.2 Hz, 1H), 7.88 (s, 1H), 7.43 (t, *J* = 7.4 Hz, 1H), 7.30 (d, *J* = 7.6 Hz, 1H), 7.23 (d, *J* = 6.5 Hz, 1H), 7.12 (s, 1H), 4.84 (d, *J* = 2.3 Hz, 2H), 4.69 (s, 2H), 3.92 (s, 3H), 2.60 (t, *J* = 2.3 Hz, 1H). ^{13}C NMR (101 MHz, DMSO-d_6) δ 153.9, 150.4, 146.0, 140.7, 138.7, 127.1, 126.0, 122.9, 121.9, 115.2, 111.2, 79.9, 79.8, 78.8, 56.9, 32.4. HRMS (ESI$^+$) calcd for $C_{16}H_{14}N_2O_5S$ $[M + H]^+$, 347.0696; found: 347.0703.

N-(2-azidoethyl)-5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (Bioazide) (3):[1] A Biotin (100 mg, 0.041 mmol) was placed in 50 ml reaction flask and cooled to 0°C. 2.5 ml $SOCl_2$ was added to the flask and the mixture was stirred for 1 h at room temperature and excess $SOCl_2$ was evaporated. The crude acid chloride was dissolved in 5 ml dry THF to this azido-aminoethane and catalytic amount of triethyl amine was added. The solution was stirred for 4 h at room temperature, and then concentrated in vacuum and the residue was purified by column chromatography using chloroform/methanol (20/1) to give the colourless waxy stuff (80 mg, 60%). ^{1}H NMR (DMSO-d_6, 400 MHz): δ=8.13 (s, 1H), 6.45 (s, 1H), 6.40 (s, 1H), 4.32- 4.29 (m, 1H), 4.12 (m, 1H), 3.38-3.11 (m, 5H), 2.98 (d, 1H, J=12.4 Hz), 2.53 (d, 1H, J=12.9), 2.07 (t , 2H, J=7.4 Hz),1.30-1.20 (m, 6H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 172.9, 163.2, 61.5, 59.7, 55.9, 50.4, 48.5, 38.6, 35.6, 28.6, 25.6, 25.0.

N-(2-(4-((2-methoxy-5-nitro-4-(((2-thioxopyridin-1(2H)-yl)oxy)methyl)phenoxy)methyl)-1H-1,2,3-triazol-1-yl)ethyl)-5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (4): To a THF (2.0 mL) solution of compound **2** (0.100 g, 0.288 mmol) were added sodium ascorbate (10 mol %) and Bio-azide

3 (0.091 g, 0.288 mmol). The reaction mixture as degassed for 15 min by purging argon gas. Then 2.0 mg (0.003 mmol) of $CuSO_4$ in 0.5 mL water was added to the reaction mixture. The reaction was stirred at 30 °C for 1 h. A precipitate appeared after the reaction was completed. The precipitated was filtered off and washed with various solvent (water, DCM, EtOAc and Hexane). The yellow solid obtained after washing was the pure compound **4** (yield: 0.133 g, 70%) yellow solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ 8.36 (d, J = 96.3 Hz, 1H), 8.02 – 7.54 (m, 2H), 7.31 (d, J = 53.7 Hz, 2H), 6.55 – 6.17 (m, 2H), 5.21 (d, J = 83.7 Hz, 2H), 4.90 (s, 2H), 4.50 (d, J = 65.3 Hz, 2H), 4.22 (d, J = 103.2 Hz, 3H), 3.74 (d, J = 156.6 Hz, 3H), 2.86 (d, J = 33.5 Hz, 2H), 2.73 (s, 2H), 2.40 (s, 1H), 2.05 (d, J = 37.3 Hz, 2H), 1.45 (t, J = 94.3 Hz, 5H), 1.28 (s, 3H). ^{13}C NMR (151 MHz, DMSO-d_6) δ 211.0, 163.3, 153.8, 146.2, 145.8, 140.79, 139.9, 139.2, 138.9, 128.5, 126.6, 122.8, 121.9, 121.8, 121.7, 115.3, 115.1, 111.5, 110.9, 61.5, 60.1, 59.7, 57.5, 57.1, 55.9, 32.4, 32.3, 28.6, 23.7. HRMS (ESI$^+$) calcd for $C_{28}H_{34}N_8O_7S_2$ $[M + H]^+$, 659.2065; found: 659.2051.



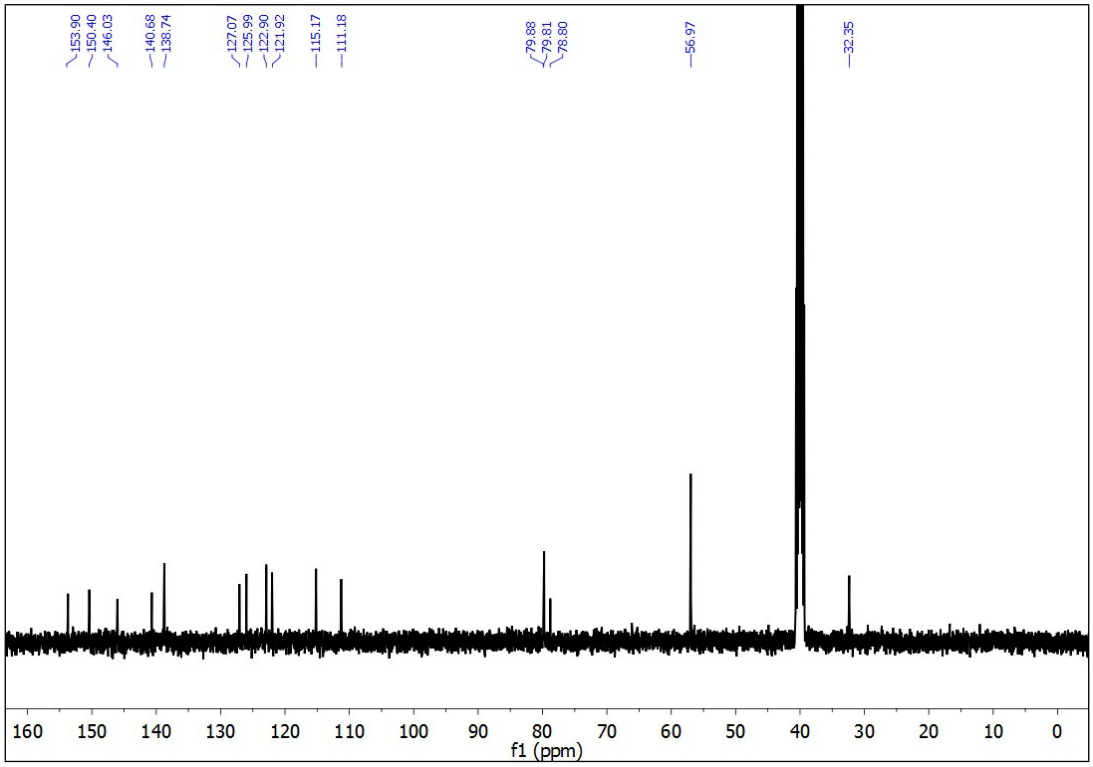
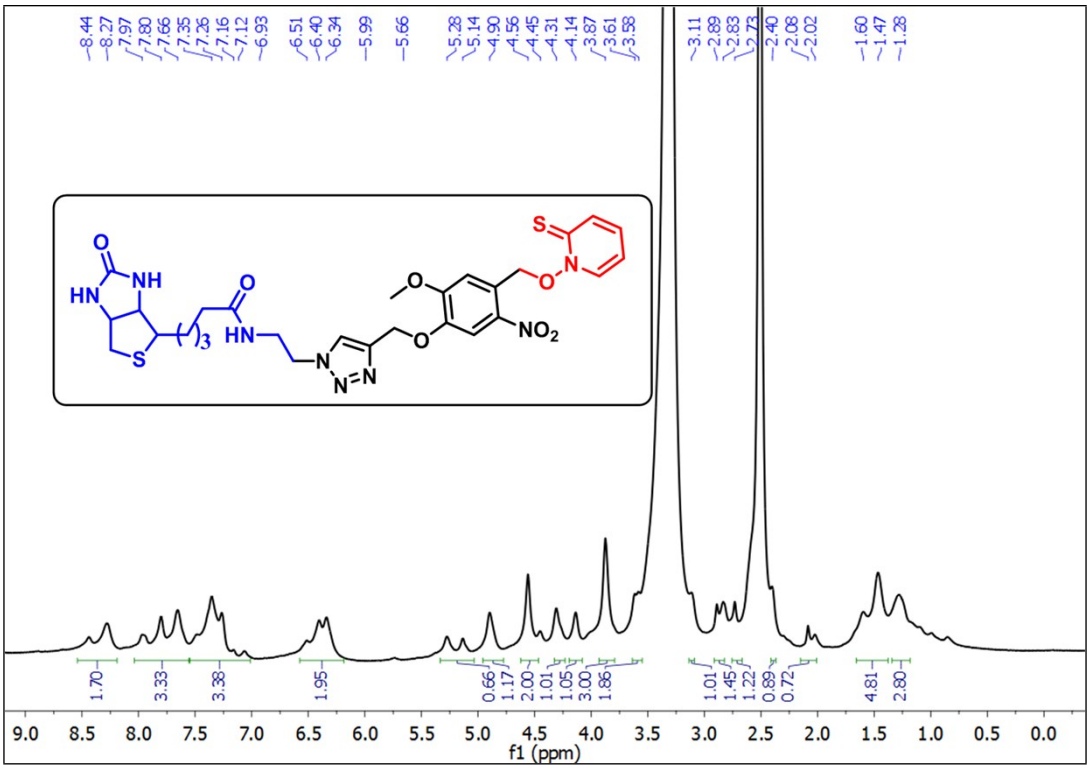


Figure S1. ^{1}H and ^{13}C NMR spectra of **2**



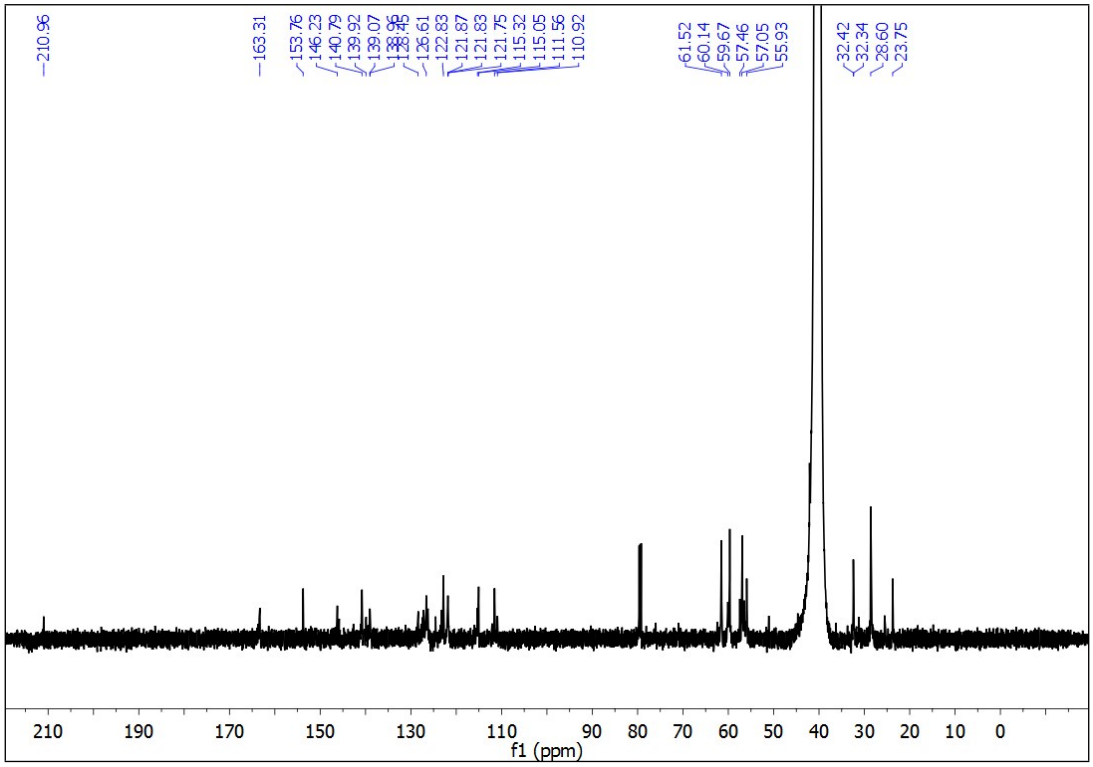


Figure S2. ^{1}H and ^{13}C NMR spectra of **4**

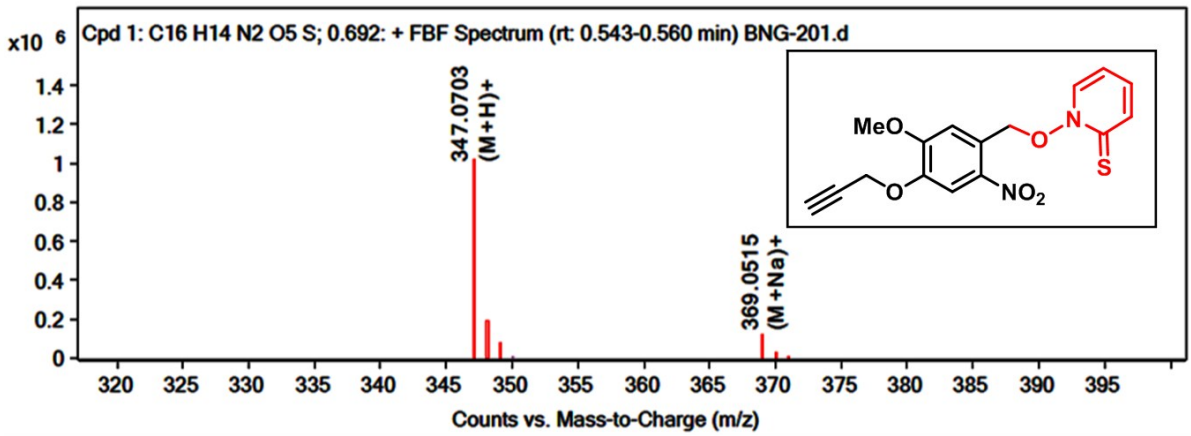


Figure S3. HRMS of **2**

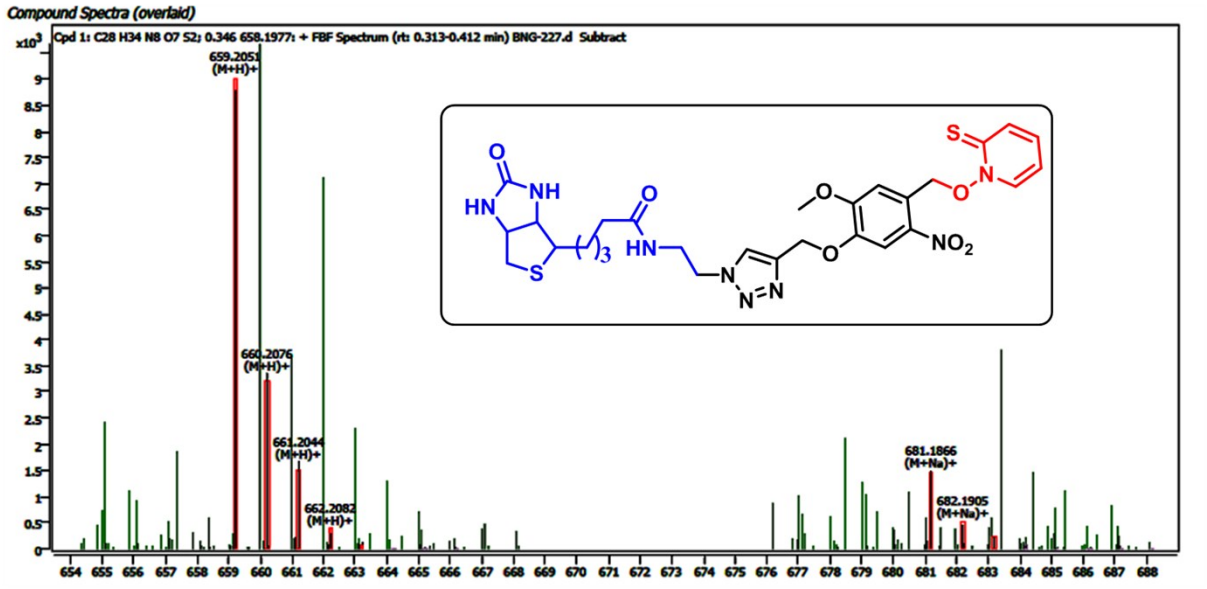


Figure S4. HRMS of **4**

3. Photophysical properties of 2 and 4

The UV absorption spectra of **2** and **4** (100 μM) were taken in DMSO/PBS buffer (0.5:10 v/v). The absorption spectrum of **2** and **4** showed a broad absorption band in the wavelength range of 300-400 nm (**Figure S5**).

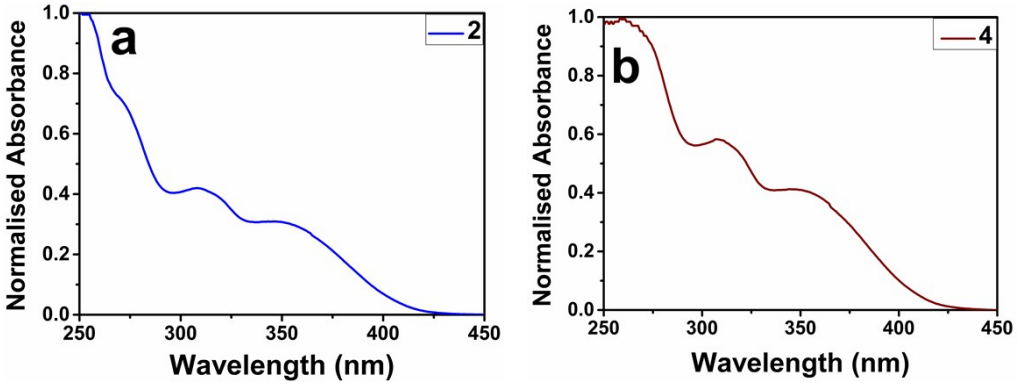


Figure S5. Normalised absorption spectrum of (**a**) **2** and (**b**) **4** in DMSO/PBS buffer (0.5:10 v/v) solvent system.

4. Hydrolytic stability of 2 and 4:

To measure the hydrolytic stability we prepared the solution of **2** and **4** (1×10^{-4} M) in DMSO/PBS buffer (0.5:10 v/v) containing 10 % fetal bovine serum at pH = 7.4 and kept them in dark conditions. The solution was incubated at 30 °C for 15 days. Next, we also

prepared the solution of thiol (GSH; 1 mM), amino acids (glycine; 1 mM), ROS (H_2O_2; 1 mM) added to the previously prepared solution of our prodrug **2** and **4**, separately. The aliquots taken after 15 days were measured by HPLC. The results showed that the decomposition of Cu-chelator prodrug (**2** and **4**) was less than 12% (**Figure S6**) in all solution. Therefore, our designed Cu-chelator donors in solution are sufficiently stable and suitable for biological applications.

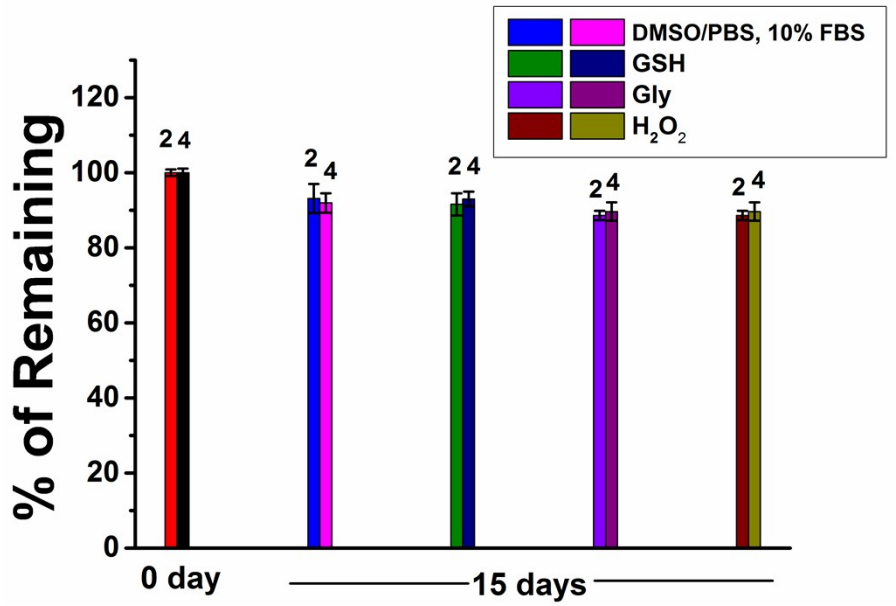


Figure S6: % of remaining of **2** and **4** (1×10^{-4} M), calculated from HPLC peak area during testing the hydrolytic stability at 0 day and 15 days.

5. 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.[2]

Degassed solution (0.006 M) of potassium ferrioxalate was irradiated using 125W medium pressure Hg lamp as UV light source (≥365 nm) and 10% (w/v) $CuSO_4$ solution in 1N H_2SO_4 as UV cut-off filter. At regular interval of time (1 min), 0.25 mL of the aliquots was taken out. To it 0.25 mL 0.018 M of 1,10 phenanthroline solution and 3.5 mL of the sodium acetate 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 511 nm. The amount of Fe^{2+} ion was determined from the calibration graph of $FeSO_4$ and 1,10 phenanthroline. 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 1.11×10^4 M^{-1} cm^{-1} at 511 nm which is found to be similar to reported values.[3b] Using the known quantum yield (1.283 ± 0.023) for potassium ferrioxalate actinometer at 363.8 nm,[3b] the rate of Fe^{2+} ion formation 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 3.7×10^{17} photons $s^{-1}cm^{-2}$.

$$\Phi_p = \frac{k}{(I_0 / N_A)\times F}$$

Where, k is the rate of reaction; I_0 is photon flux; F is fraction of light absorbed; N_A is Avogadro's no.

6. Photolysis of compound 2 and measurement of photochemical quantum yields:

a) Photolysis of 2 using UV irradiation (≥ 365 nm):

A solution of 1×10^{-4} M of the compound **2** was prepared in DMSO/PBS buffer (0.5:10 v/v); pH 7.4. Half of the solution was kept in dark and remaining half was irradiated using 125 W medium pressure Hg lamp as UV light source (λ ≥ 365 nm) and 1M $CuSO_4$ solution as UV cut–off filter. At a regular interval of time, 20 μl of the aliquots were taken and analyzed by RP-HPLC using mobile phase acetonitrile/water (1:1), at a flow rate of 1 mL/min (detection: UV 310 nm). Peak areas were determined by RP-HPLC, which indicated a gradual decrease of the compound **2** with time, and the average of three runs. The reaction was followed until the decomposition of **2** is ~ 90%. Based on HPLC data for decomposition, we plotted [A] (HPLC peak area) versus irradiation time. We observed an exponential correlation for the photolysis of **2** which suggested a first order reaction. Further, the photochemical quantum yields for the decomposition of prodrug **2** was calculated using equation (1). [3]

$$(\Phi_p)_S = (\Phi_p)_{act} \frac{(k_p)_S}{(k_p)_{act}} \frac{(F_{act})}{(F_S)} \qquad (1)$$

Where, the subscript 'S' and 'act' denotes sample and actinometer respectively. Potassium

ferrioxalate was used as an actinometer. Φ_p is the photolysis quantum yield, k_p is the photolysis rate constant and F is the fraction of light absorbed.

b) Two-photon uncaging cross-section (δ_u) of 2 at 730 nm:

The two-photon uncaging cross-section was measured by comparing the photolysis rates of **2** that of 4,5-dimethoxy-2-nitrobenzyl acetate (DMNB-OAc) as a reference (δ_u = 0.035 at 730 nm). Aliquots (100 µL) containing **2** [1 × 10^{-4} M in DMSO/PBS buffer (0.5:10 v/v), pH 7.4] were irradiated varying the time (0-1 h) with the 730 nm Ti: sapphire laser (pulse width 100 fs, 80 MHz) emitting at an average of 0.4 mW. Each sample was monitored by RP-HPLC using mobile phase acetonitrile/water (1:1), at a flow rate of 1 mL/min (detection: UV 310 nm). Similar photolysis experiments were conducted using 100 µL aliquots of DMNB-OAc [1×10^{-4} M in DMSO/PBS buffer (0.5:10 v/v), pH 7.4]. The photolysis solution of DMNB-OAc was also analyzed by RP-HPLC. The compounds were eluted with acetonitrile/water (1:1), at a flow rate of 1 mL / min and monitored by absorbance at 310 nm and the first-order decay constants for the compounds were obtained. To calculate the value of δ_u for **2**, we used the formula $\delta_u\Phi_u$(**2**) = $\delta_u\Phi_u$(reference) × k_{obs}(**2**)/k_{obs}(reference), where $\delta_u\Phi_u$(reference) = 0.035 GM.

In case of single photon photolysis, the formed CuPT complex was isolated from HPLC and analysed by HRMS (**Figure S7**). The result confirmed the formation of CuPT complex during photolysis in presence of Cu(II).

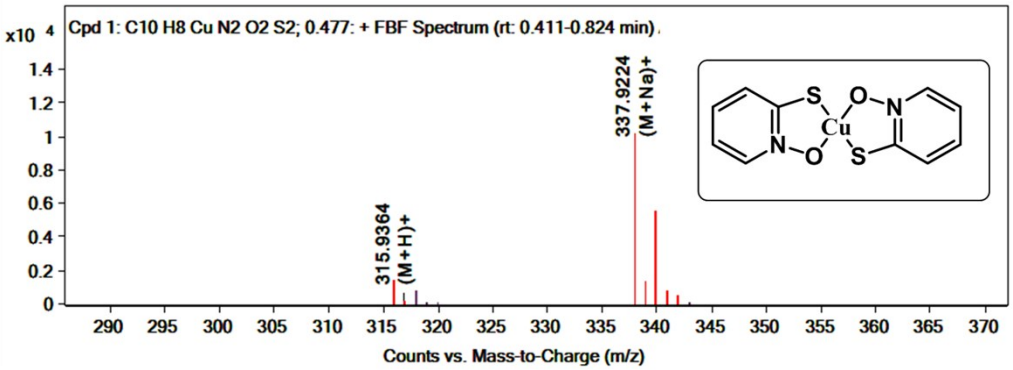


Figure S7: HRMS of isolated complex CuPT, after photolysis in presence of $CuSO_4$ (Calculated exact mass = 315.9396).

Two-photon photolysis: Total 1 mL (10^{-5} M) solution of Cu-chelator donor **2** (containing 0.1 mL 10^{-4} M $CuSO_4$**)** in DMSO/PBS buffer (0.5:10 v/v) of pH 7.4 was taken in a 1mm quartz cuvette and irradiated with a laser of wavelength 730 nm with

0.4 mW power and 60 μm diameter beam spot. During the photolysis after regular time intervals, small aliquots (25 μL) were taken out from the solution for HPLC study. The formed CuPT complex was quantified from the HPLC peak area in comparison with injected authentic sample. We have found that after 1h of photolysis 1.2 μM of the CuPT complex was formed (**Figure S8**).

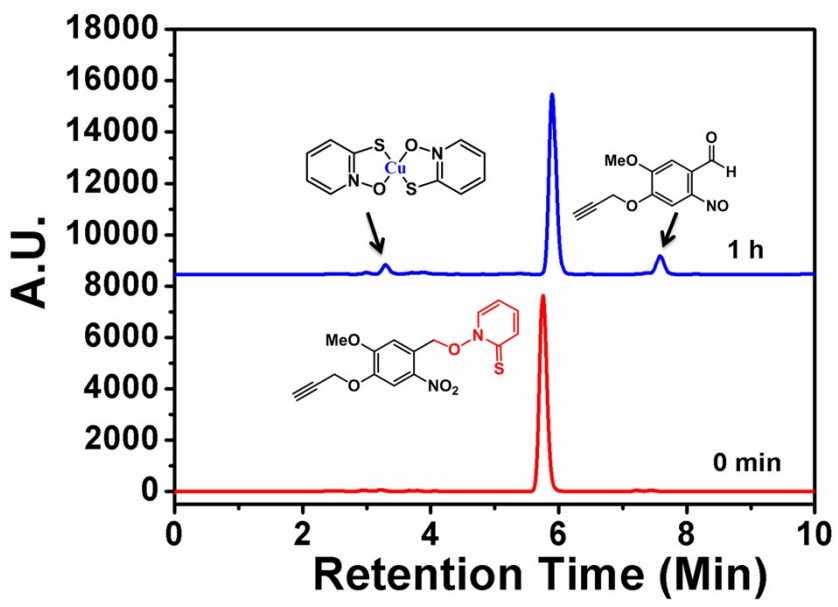
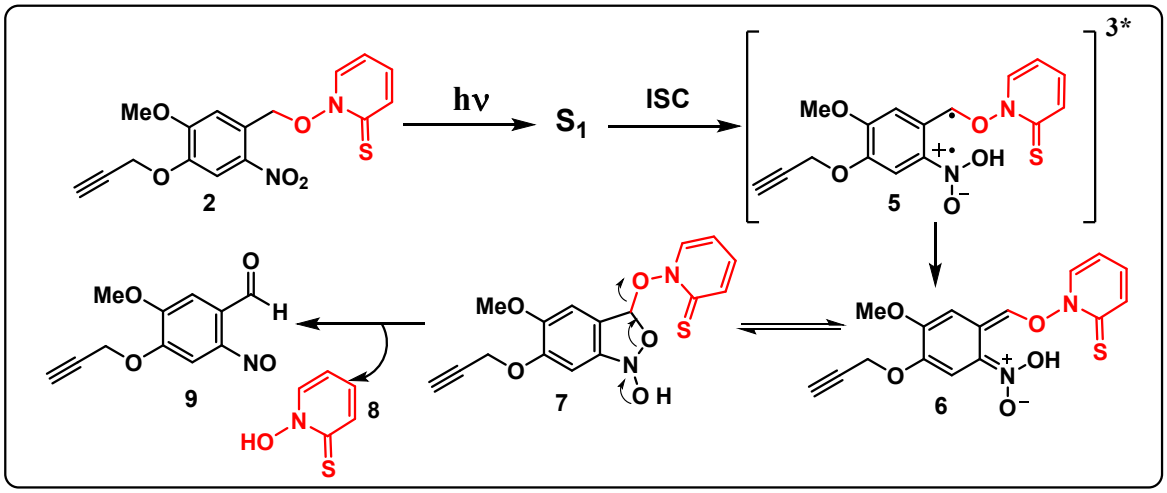


Figure S8: HPLC chromatogram of the Cu-chelator prodrug **2** during two photon photolysis.

7. Possible Photorelease Mechanism:

Based on the literature,[4] we proposed a possible photorelease mechanism for Cu-chelator ligand from **2 (Scheme S1**). It proceeds through the Norrish-type II reaction pathway. After absorbing a photon by *o*-nitro benzyl group it gets excited to singlet state and then undergoes rapid intersystem crossing to its triplet excited state. In the triplet excited state, the oxygen atom of the nitro group abstracts a proton from the methylene carbon of the γ-H position, resulting in intramolecular electron redistribution and formation of the *aci*-nitro tautomer **5**. The aci-nitro intermediate then cyclises to form benzisoxazoline intermediate **6.** Thereafter, subsequent decomposition of the resonance stabilized five-membered ring **6** rapidly generates Cu-prochelator **8** and photoproduct **9**.



Scheme S1. Proposed photorelease mechanism for Cu-chelator prodrug (**PT**).

8. Experimental procedure for biological application study:

(a) *In vitro* cellular uptake and cellular imaging studies of the compound 2 and 4.

The HeLa cells (1×10^4 cells / mL) were seeded on cover slips in DMEM medium. After 24 h,

i) Two sets of cells were treated with 10 μM of biotin tagged compound **4** (with and without light irradiation)

ii) Two sets of cells were treated with 10 μM of non-targeted (without biotin) compound **2** (with and without light irradiation)

The above conditions were further repeated in presence and absence of extracellular Cu(II). These eight sets of cells were incubated for 4 h. Cells were fixed using 3.7% paraformaldehyde. Then, the slides were washed thrice with PBS and prepared with D.P.X mountant. Next, imaging was carried out using a confocal microscope.

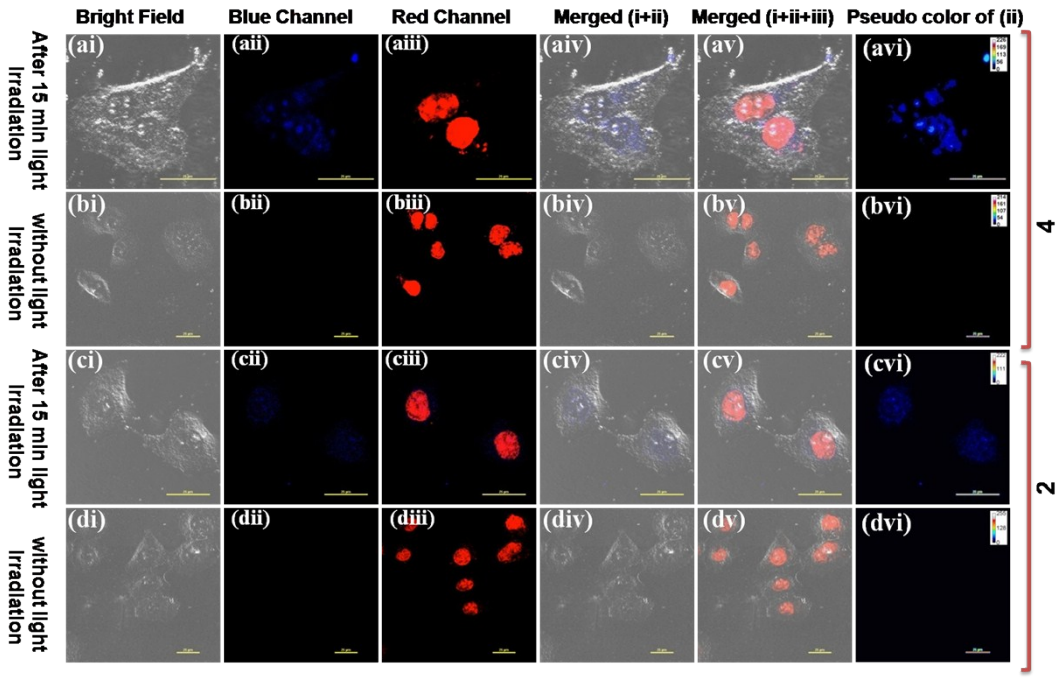


Figure S9. Confocal images of (i) brightfield (ii) Blue channel (iii) Red channel (nucleus were stained with propedium iodide) (iv) Merged images of [i] and [ii] (v) Merged images of [i], [ii] and [iii], (vi) spatial distribution of corresponding pixels in blue channel shown in a pseudocolor scale (ii) (a) compound **4** in absence of extracellular Cu after irradiation (b) compound **4** in absence of extracellular Cu without irradiation (c) compound **2** in absence of extracellular Cu after irradiation (d) compound **2** in absence of extracellular Cu without irradiation on HeLa cells. Scale bar = 25 µm.

From **figure S9**, we observed that, in absence of any extracellular Cu(II) the biotin tagged prodrug **4** predominantly distributed throughout the cell and it formed the CuPT complex inside the cells after l5 min light irradiation. But the cellular distribution of non targeted prodrug **2** (or, concentration of CuPT complex) was comparatively low after light irradiation.

(b) Cell viability assay of the compound 2 and 4 on cancerous HeLa and noncancerous HEK cell line:

The cytotoxicity of the compound **2** and **4** on cancerous HeLa and noncancerous HEK cell line were determined using the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay on HeLa cells before and after irradiation.

i) Cell viability Assay on noncancerous HEK cells:

Before light irradiation:

The cells were seeded into a 96-well cell culture plate at 1 × 10^4 cells / mL. Afterward, different concentrations (2.5, 5, 10 and 20 μM) of compound **2**, **4** and pyrithione (**PT**) were added separately into the wells and an equal volume of PBS buffer was added in the control wells. These three sets are repeated with the different concentration of Cu(II) (2.5, 5, 10 and 20 μM) and with only Cu (II) in to the separate well. The cells were then incubated under 5% CO_2 at 37 °C for 24 h. After that, fresh MTT solution (0.20 mg/mL) in PBS was added to the wells and incubated in the same cellular environment. After 4 h, solution containing MTT was removed and formed Formazan crystals were dissolve in the DMSO. The absorbance was recorded at 570 nm.

After light irradiation:

HEK cells were seeded into a 96-well cell culture plate at 1 × 10^4 cells / mL. Afterward, different concentrations (2.5, 5, 10 and 20 μM) of compound **2**, **4** and pyrithione (**PT**) were added separately into the wells and an equal volume of PBS buffer was added in the control wells. These three sets are repeated with the different concentration of Cu(II) (2.5, 5, 10 and 20 μM) and with only Cu (II) in to the separate well. The cells were then incubated under 5% CO_2 at 37 °C for 24 h. Thereafter, the cells were irradiated under light for 15 min, keeping the cell culture plate 5 cm apart from the light source. After 15 min irradiation, the cells were again incubated for 24 h. Then the cell viability was measured using the MTT assay as described above.

ii) Anticancer activity on cancerous HeLa cells:

Before light irradiation:

The cells were seeded into a 96-well cell culture plate at 1 × 10^4 cells / mL. Afterward, different concentrations (2.5, 5, 10 and 20 μM) of compound **2**, **4** and pyrithione (**PT**) were added separately into the wells and an equal volume of PBS buffer was added in the control wells. These three sets are repeated with the different concentration of Cu(II) (2.5, 5, 10 and 20 μM) and only Cu (II) in to the separate well. The cells were then incubated under 5% CO_2 at 37 °C for 24 h. Then the cell viability was measured using the MTT assay as described above.

After light irradiation:

HeLa cells were seeded into a 96-well cell culture plate at 1 × 10^4 cells / mL. Afterward, different concentrations (2.5, 5, 10 and 20 μM) of compound **2**, **4** and pyrithione (**PT**) were

added separately into the wells and an equal volume of PBS buffer was added in the control wells. These three sets are repeated with the different concentration of Cu(II) (2.5, 5, 10 and 20 μM) and only Cu (II) in to the separate well. The cells were then incubated under 5% CO_2 at 37 °C for 24 h. Thereafter, the cells were irradiated under light for 5 min, keeping the cell culture plate 5 cm apart from the light source. After 15 min irradiation, the cells were again incubated for 24 h. Then the cell viability was measured using the MTT assay as described above.

Further, the cell viability of prodrug **4** (with biotin) and prodrug **2** (without biotin), were analyzed by one way analysis of variance (ANOVA) followed by Tukey test. Changes were identified as significant if p was less than 0.01 (**Figure S10**).

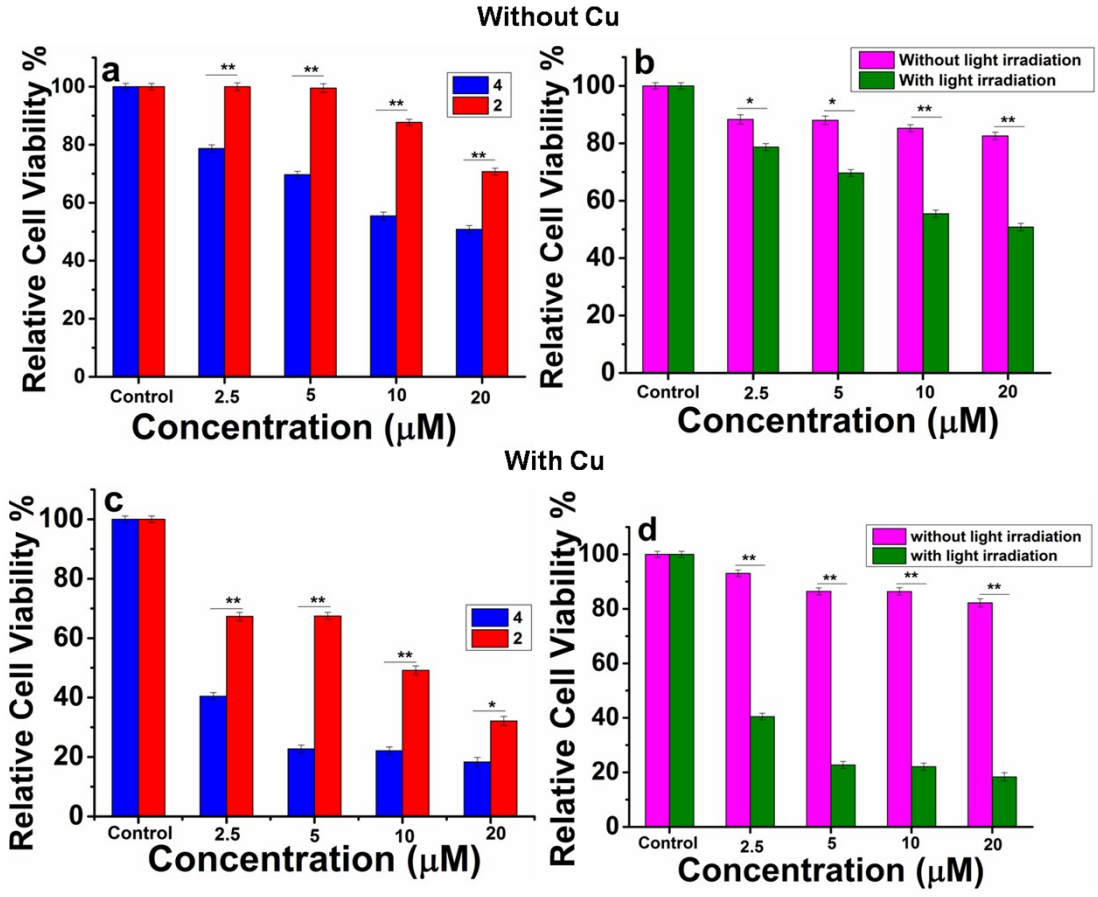


Figure S10: Cell viability assay on HeLa cells (**a**) for prodrug **4** (with biotin) and **2** (without biotin) after 15 min light irradiation (**b**) for prodrug **4** with and without light irradiation [without addition of extracellular Cu(II)]; Cell viability assay on HeLa cells (**c**) for prodrug **4** (with biotin) and **2** (without biotin) after 15 min light irradiation (**d**) prodrug **4** with and without light irradiation [addition of extracellular Cu(II)]. Data presented are the average of

the data obtained in triplicate independent experiments and the standard deviation is within the ± 3% range. Changes were identified as significant *p <0.01, **p <0.001.

iii) Cell viability assay of prodrug 4 on cancerous HeLa cells in presence of biotin:

Hela cells were seeded in 96 well plate for 24 h followed by treated with biotin (100 nm) and incubated for 1 hr then cells were treated with compound **4** in different concentrations (2.5, 5, 10 and 20 μM) and $CuSO_4$ (2 μM) for 6h. The cells were then incubated under 5% CO_2 at 37 °C for 24 h. Finally cells were irradiated with 365 nm light for 15 min and then incubated for 72 h. Thereafter media was discarded and MTT solution was added and cells were further incubated for 4h. After that formazan crystals were formed were dissolved in DMSO : MeOH and absorbance were measured in a microplate reader at 570 nm (**Figure S11**).

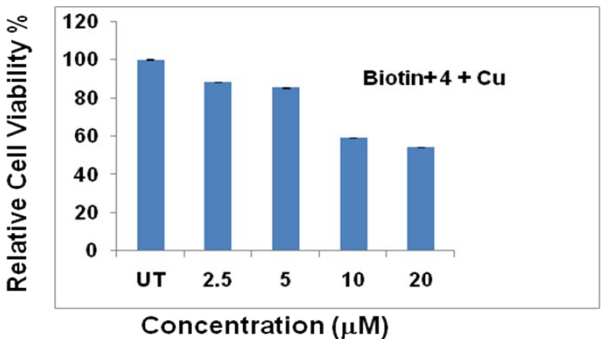


Figure S11: Cell viability assay on HeLa cells in presence of biotin and Cu; treated with prodrug **4** at different prodrug concentration. Data presented are the average of the data obtained in triplicate independent experiments and presented relative to control. The standard deviation is within the ± 3% range. Error bars indicate SDs.

iv) Anticancer activity of prodrug 4 on cancerous HeLa cells at different interval of irradiation time and different concentration of added extracellular Cu(II):

To explore the ability of temporal control of our designed prodrugs over the cell viability, we have conducted the time-dependent cell viability assay using our designed prodrug **4** (Bio-ONB-PT) in the presence of extracellular Cu(II) at different irradiation time (**Fig. S11**, ESI†). The MTT result showed that the cell viability decreased with increasing irradiation time (from 0 min to 15 min) in case of both with and without addition of extracellular Cu(II). Therefore, our designed prodrug exhibited higher anticancer activities with increase in the irradiation time and the concentration of Cu(II) inside the cells (**Figure S12**).

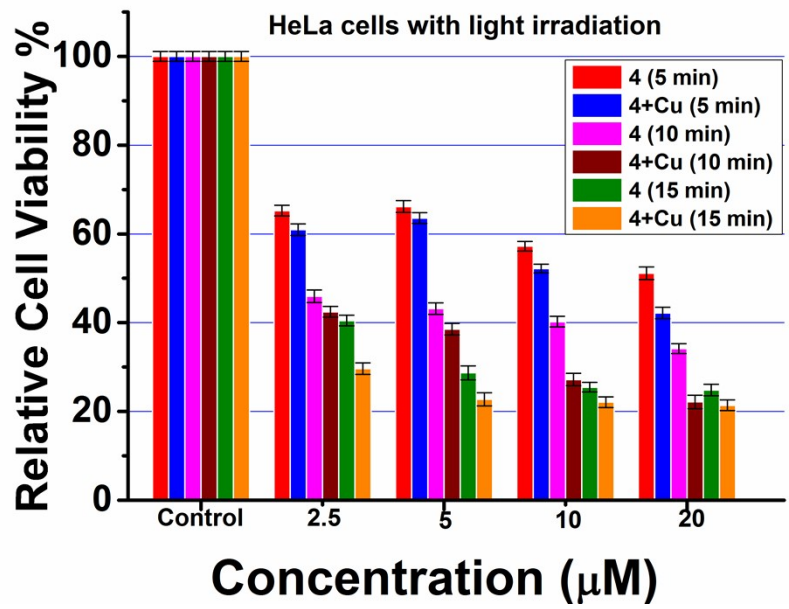


Figure S12: Cell viability assay on HeLa cells treated with prodrug **4** at different interval of irradiation time and different Cu(II) concentration. Data presented are the average of the data obtained in triplicate independent experiments and presented relative to control. The standard deviation is within the ± 3% range. Error bars indicate SDs.

9. References:

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