Electronic Supplementary Information

Light Uncages a Copper Complex to Induce Nonapoptotic Cell Death

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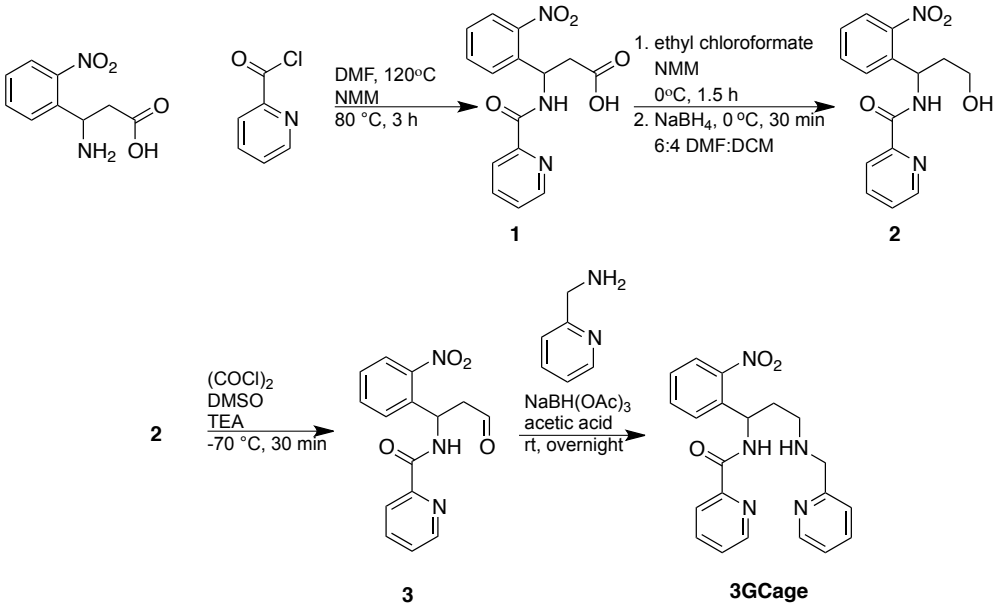
Experimental

General. All chemicals were purchased commercially and used as received unless otherwise noted. All reactions were carried out in the dark and glassware was covered with foil. ^{1}H NMR spectra were recorded on a Varian Inova 400 spectrometer and chemical shifts are reported in ppm with J values in Hz. Routine liquid chromatography/mass spectrometry (LC/MS) was performed using an Agilent 1100 series apparatus with an LC/MSD trap and a Daly conversion dynode detector. A Varian Polaris C18 (150 × 1 mm) column was used and peaks were detected by UV absorption at 256 nm. A linear gradient from 2% A in B to 75 % A in B was run from 1 to 22 min with a total run time of 24 min, where A is MeCN /2% formic acid and B is water/2% formic acid. High-resolution mass spectra (HRMS) were recorded on an Agilent LCMS-TOF model 6224 spectrometer. A Supelco Ascentis 2 × 50 mm, 2.6 μ C-18 column was used with the Agilent 1200 series LC. UV-Vis spectra were recorded on a Cary 50 UV-Vis spectrophotometer. All photolysis experiments were conducted in a Rayonet RPR-100 Photochemical Reactor containing 16 bulbs (14 Watts each) with a maximum intensity output centered at 350 nm.

Synthesis

Scheme S1



3-(2-nitrophenyl)-3-(picolinamido)propanoic acid (1). In a clean, dry round-bottom flask equipped with a stir bar, 3-amino-3-(2-nitrophenyl)propanoic acid (0.105 g, 0.5 mmol) was dissolved in hot DMF (20 mL) and 2 equiv. of NMM (0.110 mL, 1mmol) were added with constant stirring. After 5 min, 1 equiv. of picolinoyl chloride hydrochloride (0.089 g, 0.5 mmol) was added and the reaction mixture was stirred for another 3 h at 80 °C. The solvent was reduced on a rotary evaporator and the reaction mixture was purified by silica gel chromatography using EtOAc-MeOH (7:3 v/v) as eluent. The collected fractions were subjected to LCMS and the fractions containing product (m/z = 316) were combined and the solvent was removed to leave a reddish oil. Yield (92%). ^{1}H NMR: (400 MHz, $CDCl_3$) δ ppm 9.32 (d, *J* = 8.17 Hz, 1H), 8.58 (dd, *J* = 4.79, 0.80 Hz, 1H), 8.09 (dd, *J* = 7.83, 0.96 Hz, 1H), 7.97 (d, *J* = 8.19 Hz, 1H), 7.81 (tt, *J* = 7.75, 7.75, 1.46, 1.46 Hz, 1H), 7.71 (d, *J* = 7.91 Hz, 1H), 7.55 (t, *J* = 7.63, 7.63 Hz, 1H), 7.45-7.36 (mult, 2H), 6.07 (dd, *J* = 13.43, 6.46 Hz, 1H), 3.13 (d, *J* = 6.02 Hz, 1H)

N-(3-hydroxy-1-(2-nitrophenyl)propyl)picolinamide (2). A portion of **1** (0.158 g, 0.5 mmol) was dissolved in DMF-DCM (6:4, v/v) in a round-bottom flask equipped with a stir bar. One equiv. each of NMM (0.055 ml, 0.5 mmol) and ethyl chloroformate (0.048 ml, 0.5 mmol) were added and the reaction mixture was stirred at -15 °C for 90 min. A solution of sodium borohydride (1.5 mmol, 3 equiv.) in 5 mL of deionized water was added in one portion and the solution continued stirring at r.t. for another 3 h, after which time the solvent was removed by rotary evaporator. The resulting oil was dissolved in DCM (20 mL) and extracted with saturated Na_2CO_3 (3 × 20 mL). The organic layers were combined, washed with deionized water (2 × 20 mL), dried over anhydrous $MgSO_4$, filtered, and the solvent was removed under vacuum to yield a yellow oil that was purified by silica gel chromatography using an EtOAc : hexane (8:2 v/v) solvent system. The collected fractions were subjected to LCMS and fractions containing product (m/z = 302) were combined and the solvent was removed in vacuo to leave a yellow oil. Yield (88 %). ^{1}H NMR (400 MHz, $CDCl_3$) δ ppm 9.07 (1H, d, J=8 Hz), 9.07 (d, J=7.6 Hz, 1H) 8.56 (d, J=4 Hz, 1H), 8.11 (d, J=8 Hz, 1H), 7.91 (d, J=8 Hz, 1H), 7.81 (dt, J=2 Hz, 8Hz, 1H), 7.65 (d, J = 8 Hz, 1H), 7.58 (t, J=8 Hz, 1H), 7.44-7.39 (mult, 2H), 5.83 (dt, J=8.85, 8.82, 4.25 Hz, 1H), 3.83-3.72 (mult, 2H), 2.31-2.08 (mult, 2H)

N-(2-formyl-1-(2-nitrophenyl)ethyl)picolinamide (3). In a clean, dry 25 mL round-bottom flask equipped with stir bar, septum and N_2 inlet, oxalyl chloride (0.232 ml, 2.66 mmol, 4 equiv.) was dissolved in dry DCM (4 mL) and cooled to -78 °C. To it dry DMSO (0.378 ml, 5.31 mmol, 8 equiv.) was added and stirred for another 10 min. A 1.0-mL solution of **2** (0.200 g, 0.664 mmol) in dry DCM was added to the cooled reaction vessel and stirred for 15 min followed by addition of triethylamine (0.740 mL, 5.31 mmol, 8 equiv.). After 15 min the reaction mixture was warmed to 0 °C and then brought to r.t. prior to addition of deionized water (80 mL). After stirring for 10 min, the solution was extracted with DCM (3 × 10 mL) and the organic layers were combined, washed with deionized water and brine (1 × 10 mL), dried over $MgSO_4$, filtered and evaporated to get an oil, which was purified by silica gel column chromatography using EtOAc: DCM (50:50 v/v). Yield (30%). ^{1}H NMR: (400 MHz, $CDCl_3$) δ ppm 9.80 (s, 1H), 8.95 (d, *J* = 8 Hz, 1H), 8.54 (d, *J* = 4 Hz, 1H), 8.08 (d, *J* = 8 Hz, 1H), 7.97 (d, *J* = 9 Hz, 1H), 7.80 (t, *J* = 8 Hz, 1H), 7.71 (d, *J* = 8 Hz, 1H), 7.59 (t, *J* = 8 Hz, 1H), 7.45-7.39 (mult, 2H), 6.18 (dd, *J*=14, 7 Hz, 1H), 3.21 (d, *J*=6 Hz, 1H)

3Gcage (Pyridine-2-carboxylic acid {1-(2-nitrophenyl)-3-[(pyridine-2-ylmethyl)-amino]-propyl}-amide). A portion of **3** (0.066 g, 0.220 mmol) was dissolved in 6 mL of dry THF. To it, 2 equiv. of 2-aminomethyl pyridine (0.046 ml, 0.44 mmol) and 2 drops of glacial acetic acid were added followed by sodium triacetoxy borohydride (0.551 g, 2.60 mmol, 3.5 equiv.). The reaction mixture was stirred overnight at r.t., after which was added 9 mL of a saturated sodium bicarbonate aqueous solution. After stirring for 15 min, the solution was extracted with ethyl acetate, organic fractions were collected, washed with deionized water, dried over $MgSO_4$ and filtered. The solvent was removed on a rotary evaporator to get a yellow oil that was purified by silica gel column chromatography using DCM:MeOH gradient going from 0 to 20% MeOH. The collected fractions were subjected to LCMS and fractions containing 3Gcage (m/z = 392) were combined and evaporated to dryness to give a yellow product. Yield (70%). The ^{1}H NMR spectrum matched the reported values.[1]

[CuCl(3Gcage)], (Cu3G). 3GCage (0.063 g, 0.160 mmol) was dissolved in EtOH (5 mL). To the yellow solution, a portion of solid $CuCl_2 \cdot 2\ H_2O$ (0.033 g, 0.190 mmol, 1.2 equiv.) was added. The mixture was refluxed for 1.5 h and then the solvent was removed in vacuo. The crude product was purified by column chromatography (alumina, 100% MeOH) to obtain 0.053 g (0.110 mmol) of blue powder. Yield (67%). For experiments where high purity was required, smaller amounts of the complex were recrystallized from hot EtOH. HRMS, found: 453.0850 (M^+ calcd

[1] Ciesienski, K. L.; Haas, K. L.; Franz, K. J., *Dalton Trans*. **2010,** *39*, 9538-9546.

453.0857 for $C_{21}H_{20}CuN_5O_3$)

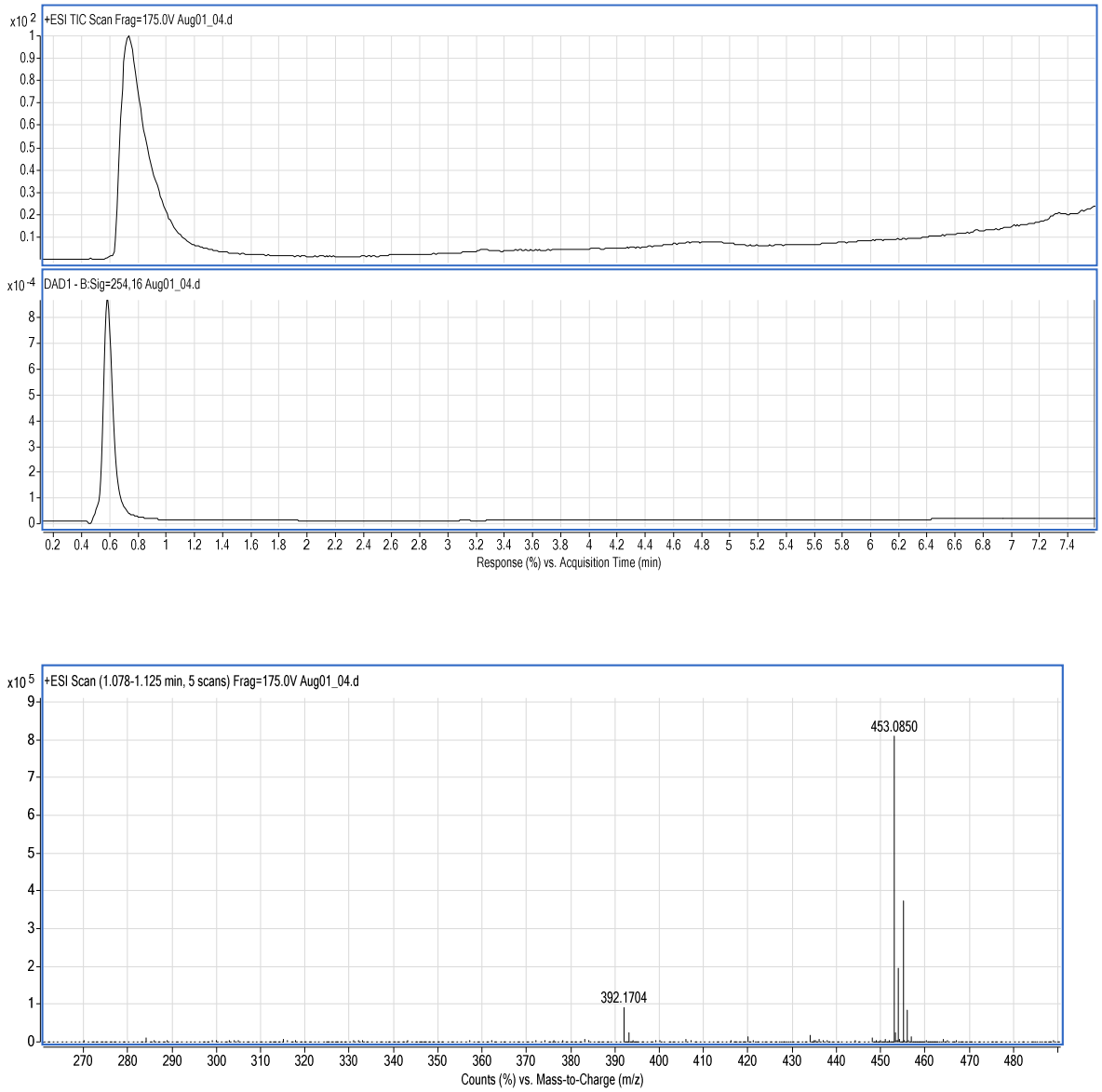


Figure S1. High-resolution LCMS of [CuCl(3Gcage)] (**Cu3G**) to show bulk purity. **Top panel:** total ion chromatogram (TIC), showing the presence of only one ionizable species in the sample. **Middle panel:** LC chromatogram, with detection set at 254 nm, showing the presence of one absorbing species in the sample. **Bottom panel:** Mass spectrum extracted from the TIC between 1–1.12 min. The major ion detected at 453.0850 corresponds to the parent M^+ signal of $[Cu(3Gcage)]^+$, where Cl^- has dissociated. The isotopic pattern matches the predicted pattern for this Cu complex. The second ion peak at 392.1704 corresponds to the $(M+H)^+$ ion for the free ligand, with a calculated value of 392.1717 for $[C_{21}H_{22}N_5O_3]^+$. This species is a fragment of the Cu complex, since the free ligand, which elutes at a different time under these conditions, is not detected in this sample. Elution conditions: 0.25 mL/min for 0–2 min at 40% B, then linear gradient over 8 min to 80% B, where A is 98/2 H_2O/acetonitrile with 5 μM ammonium formate and B is 2/98 H_2O/acetonitrile with 5 μM ammonium formate.

Stability of Cu3G: The stability of Cu3G was assessed in the presence of bathocuproine disulfonate (BCS), ascorbic acid, cysteine, glutathione, and histidine. In each experiment, 1 mL of a 200-μM Cu3G solution in PBS buffer (pH = 7.4, 154 mM NaCl, 1.06 mM KH_2PO_4, 5.6 mM Na_2HPO_4) was made from a 100% DMSO stock $[DMSO]_{final}$ = 4%). The solution was transferred to a quartz cuvette and the UV-Visible absorption spectrum was collected. The challenge component(s) was added to a final concentration of 1 mM, without changing the total volume by more than 1%. Experiments with BCS were conducted with 50 μM Cu3G and 100 μM BCS. Spectra were collected every 15 min for 1 h, and then hourly for at least an additional 3 h. A final spectrum was collected 18 h after initial mixing. The absorption band corresponding to Cu(II) d-d transition centered at λ_{max}= 584 nm was followed to determine complex stability.

Electrochemistry: Cyclic voltammograms were recorded on a Cypress system 2R cyclic voltammeter, model BASi-CGME. A 10^{-3} M solution of **Cu3G** was prepared in 0.1 M tetraethylammonium perchlorate (TEAP) in acetonitrile. Platinum wire, platinum electrode and silver (Ag/AgCl in 3.5 M KCl) electrodes were used as an auxiliary electrode, working electrode and reference electrode, respectively. Midpoint potential was calculated as $E_{1/2}^{'} = (E^{red} + E^{ox})/2$, where E^{red} and E^{ox} are the reduction and oxidation peak potentials, respectively. Where indicated, corrected potentials are reported relative to the saturated calomel electrode (SCE) or normal hydrogen electrode (NHE) according to Ag/AgCl = -0.039 vs. SCE or 0.205 vs. NHE.

Cell lines and culture: All cell culture media including Eagle's Minimum Essential Medium (MEM), Opti-MEM (a reduced serum medium that is a modification of Eagle's MEM buffered with HEPES and sodium bicarbonate and supplemented with hypoxanthine, thymidine, sodium pyruvate, L-glutamine, trace elements and growth factors) Iscove's Modified Dulbecco's Medium (IMDM), Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), nonessential amino acids, pyruvate, trypsin-EDTA (0.25%) were purchased from Gibco. Human cancer cell lines MCF-7 (breast cancer; MEM with 10% FBS), HeLa (cervical carcinoma; DMEM with 10% FBS) and HL-60 (leukemia; IMDM with 20% FBS) were purchased from Duke University Cell Culture Facility. The cells were incubated in respective media supplemented with FBS, 100 U/mL penicillin and 100 U/mL streptomycin in fully humidified atmosphere containing 5% CO_2 maintained at 37 °C.

Cell viability assay: About 1.2×10^4 cells/100 μL/well were seeded in a complete growth medium in 96-well plates (Corning, NY) 24 h before treatment. Stock solutions of compounds to be tested were freshly prepared in DMSO and dilutions were made in Opti-MEM (final DMSO conc < 1%). The growth medium was substituted with test compounds prepared in fresh complete medium at selected concentrations and the cells were incubated for 24 and 48 h in the dark. The plates that received UV light were first incubated with the test compounds in the dark for 1h before photoirradiation in a Rayonet photoreactor; 60 sec (3Gcage) and 90 sec (**Cu3G**) respectively. After 24 h and 48 h, 40 μL of CellTitre Blue (Promega) were added to each well and the plates were incubated for 4 h in the dark, after which the fluorescence was measured at 572 nm on a plate reader (Wallac 1420 Victor 2 Multilabel Counter, Perkin Elmer). % Viability was expressed by the equation,

$$\% \text{ Viability} = [(Em(test)-Em(Triton\ x\text{-}100)) / (Em(control)-Em(Triton\ X\text{-}100))]*100$$

Where 1% triton X-100 was used as a negative control (0% cell viability). All measurements were done in triplicate under identical conditions with results presented as mean of the measurements ± standard deviation.

Cell Imaging: The bright-field images of HeLa cells treated with 3Gcage and Cu3G over 24 h in the dark or after photoirradiation were taken on a Zeiss Axio Observer widefield fluorescence microscope under 40 × magnification. For samples exposed to UV light, cells were first treated with test compound for 1 h, then the entire plate was exposed to UV light in a Rayonet photoreactor for 60 s or 90 s prior to 24 h incubation in the dark. Samples that received 50 μM H_2O_2 with or without test compound and with or without UV exposure were imaged after 18 h to show the vacuolar structures at a timepoint prior to complete rounding and lifting of the cells. Transmission density, contrast, brightness and scan speed were held constant. Image processing was carried out with MetaMorph.

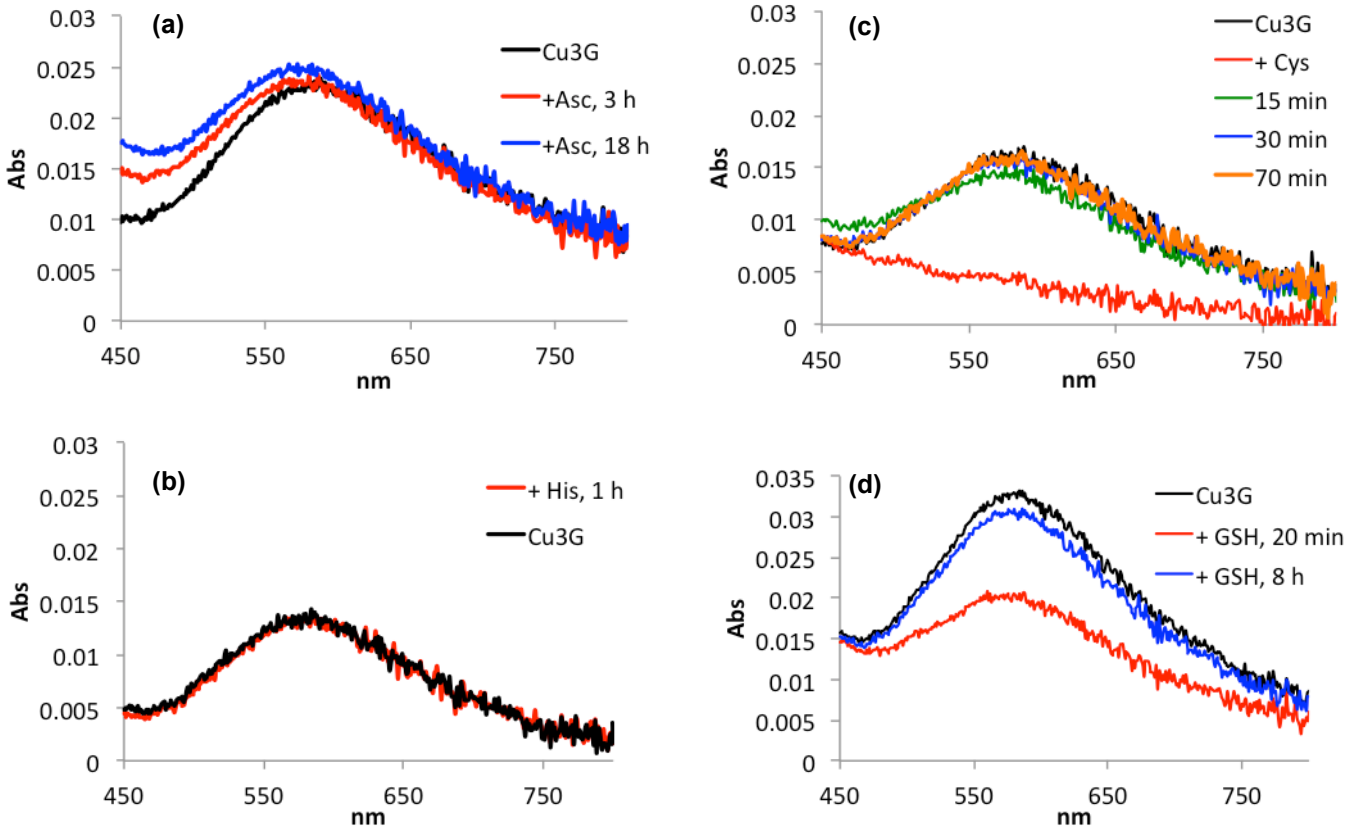


Figure S2. Visible absorption spectra showing the d-d band of 200 μM Cu3G in PBS buffer in the presence of 1 mM of **(a)** ascorbate, **(b)** histidine **(c)** cysteine, and **(d)** glutathione. The persistence of the absorbance feature centered at 584 nm indicates that ascorbate does not reduce Cu3G even after 18 h. Cu3G is also stable in the presence of the chelating amino acid histidine (spectrum was unchanged after 18 h). Both cysteine and glutathione induce an initial loss of the feature at 584, which is fully restored over time. The results in **(c)** and **(d)** suggest that cysteine and glutathione can induce reduction of Cu3G, but are not strong enough to prevent reoxidation and Cu(II) is ultimately retained by the 3Gcage ligand.

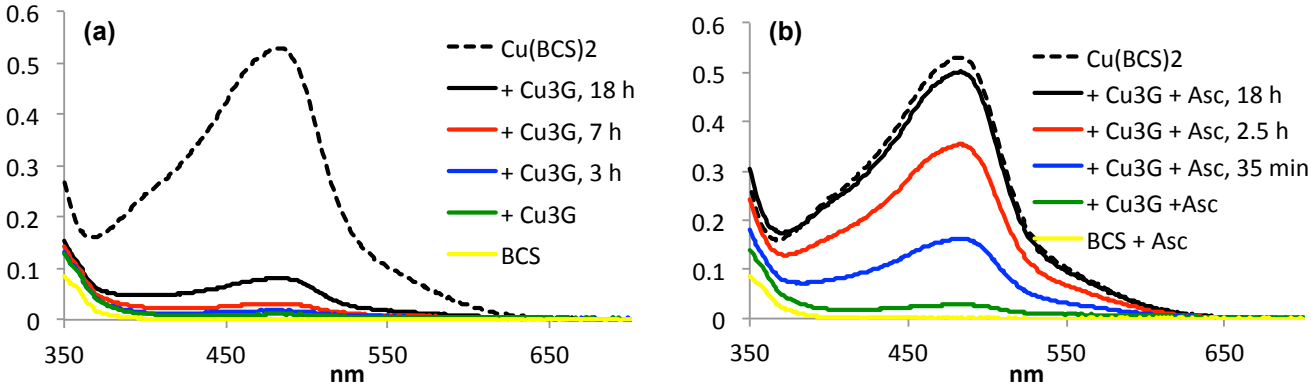


Fig S3. **(a) Cu3G** (50 μM) retains its bound metal in the presence of bathocuproine disulfonate (BCS, 100 μM). **(b)** The addition of 1 mM ascorbic acid to the solution containing both **Cu3G** and BCS results in transfer of Cu(I) to $[Cu(BCS)_2]^{3-}$ over the course of several hours. Both experiments conducted in PBS buffer, 4% DMSO)

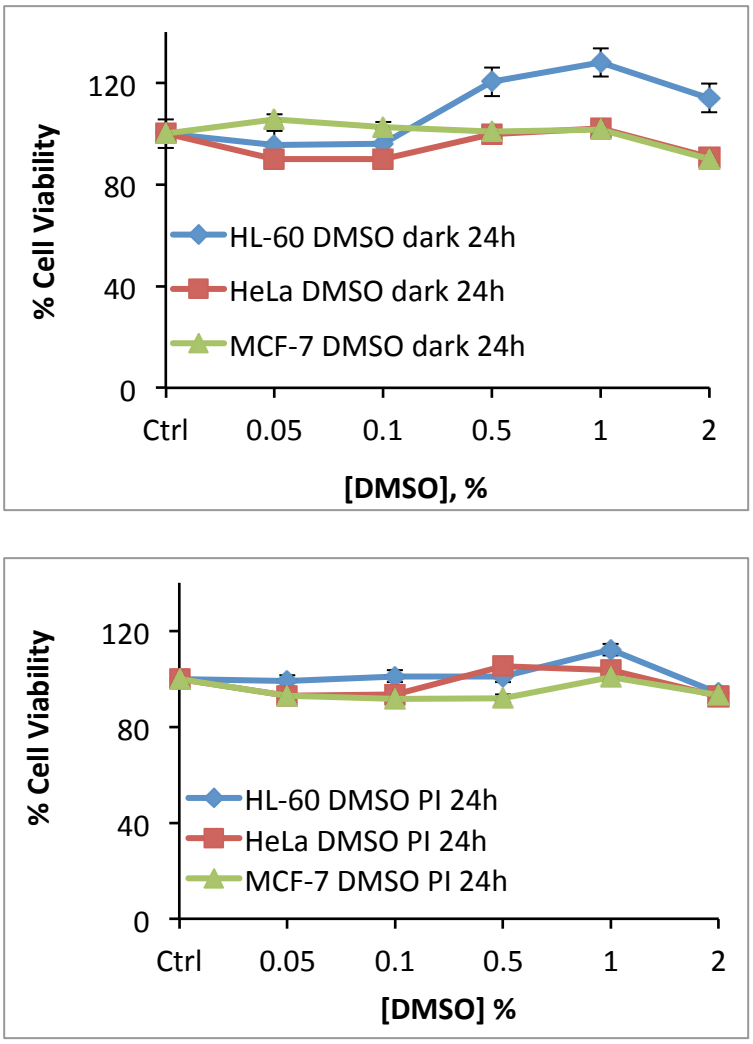


Figure S4. Vehicle control experiments. HL-60, HeLa, and MCF-7 cells were treated with increasing concentrations of DMSO then incubated for 24 h in the dark (top panel), or irradiated with 350-nm UV light for 60 s followed by overnight incubation in the dark (bottom panel). Cell viability was assayed by CellTitre Blue. As indicated by these control experiments, neither 2% DMSO nor 60 s photoirradiation causes a decrease in cell viability (same results observed for 90 s irradiation).

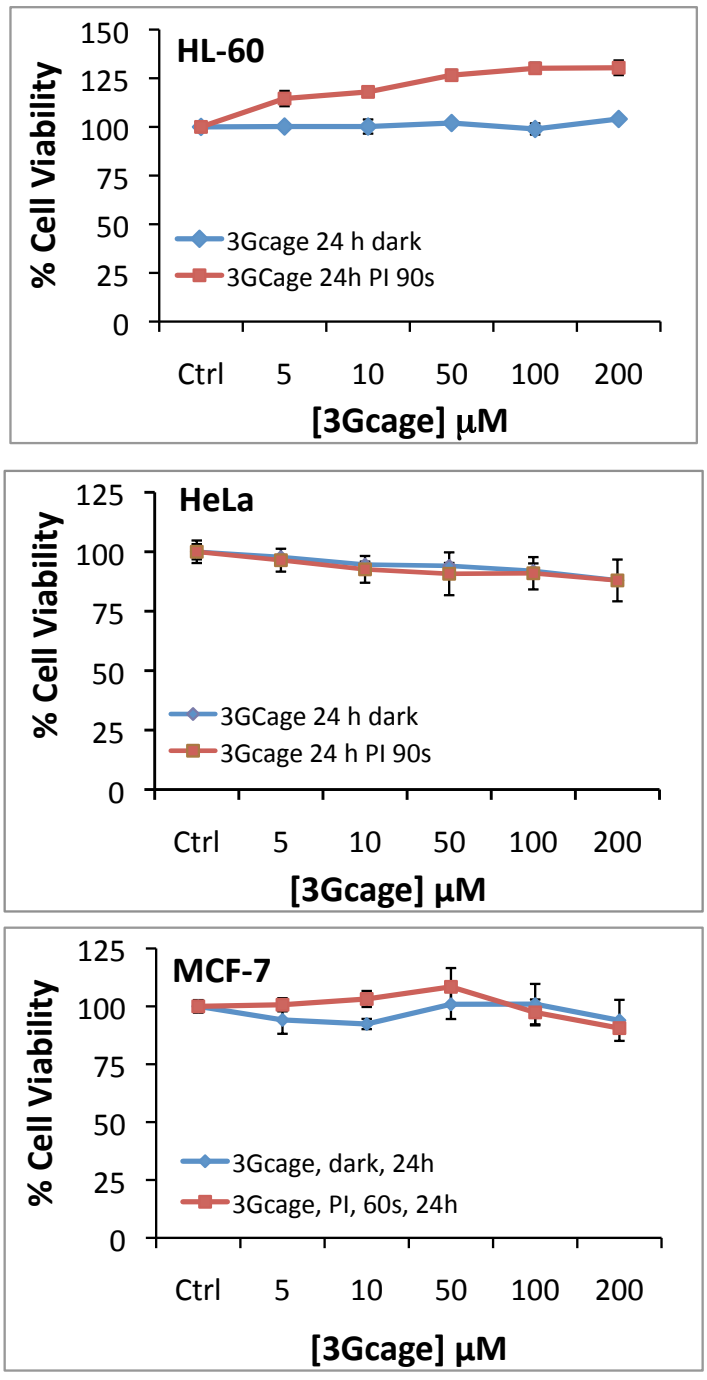


Figure S5. Effect of free ligand 3GCage on cell viability of (top) HL-60, (middle) HeLa, and (bottom) MCF-7 cancer cell lines. Blue diamonds indicate that cells were incubated with the test compound in the dark, while red squares indicate that cells were treated with the test compound for 1 h, irradiated with 350-nm UV light for 60 s, then further incubated overnight in the dark. Cell viability was assayed by CellTitre Blue. Error bars represent standard deviation from experiments done in triplicate. The 3Gcage ligand, with or without photoirradiation, does not induce cell death in the absence of copper.

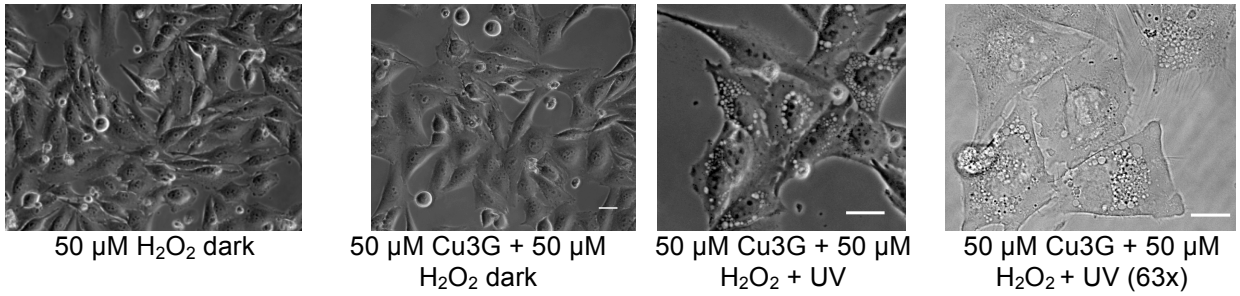


Figure S6. Representative phase contrast images of HeLa cells incubated for 18 h with treatments as indicated, where UV specifies 90 s photoirradiation prior to incubation. Images taken on a Zeiss Axio Observer widefield microscope with 40x (or 63x) magnification. Scale bars represent 10 μM.

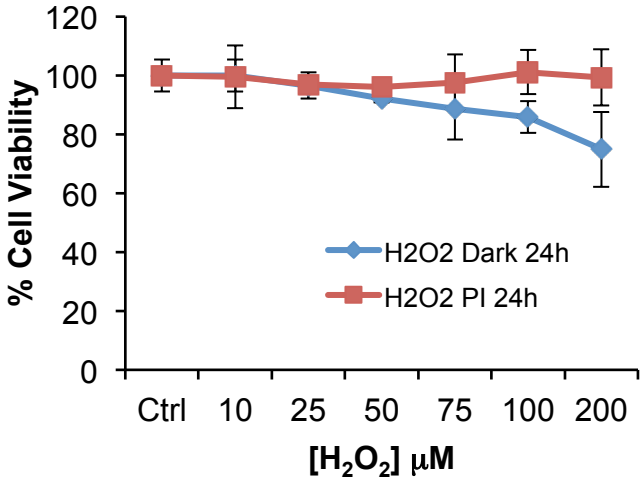


Figure S7. Effect of H_2O_2 on HeLa cell viability. Blue diamonds indicate that cells were incubated with indicated concentration of H_2O_2 in the dark, while red squares indicate that cells were treated with H_2O_2 and irradiated with 350-nm UV light for 60 s, then further incubated overnight in the dark. Cell viability was assayed by CellTitre Blue. Error bars represent standard deviation from experiments done in triplicate. (PI = photoirradiated)