

Electronic Supporting Information

**Selective photothermal inactivation of cells labeled with near-infrared
croconaine dye**

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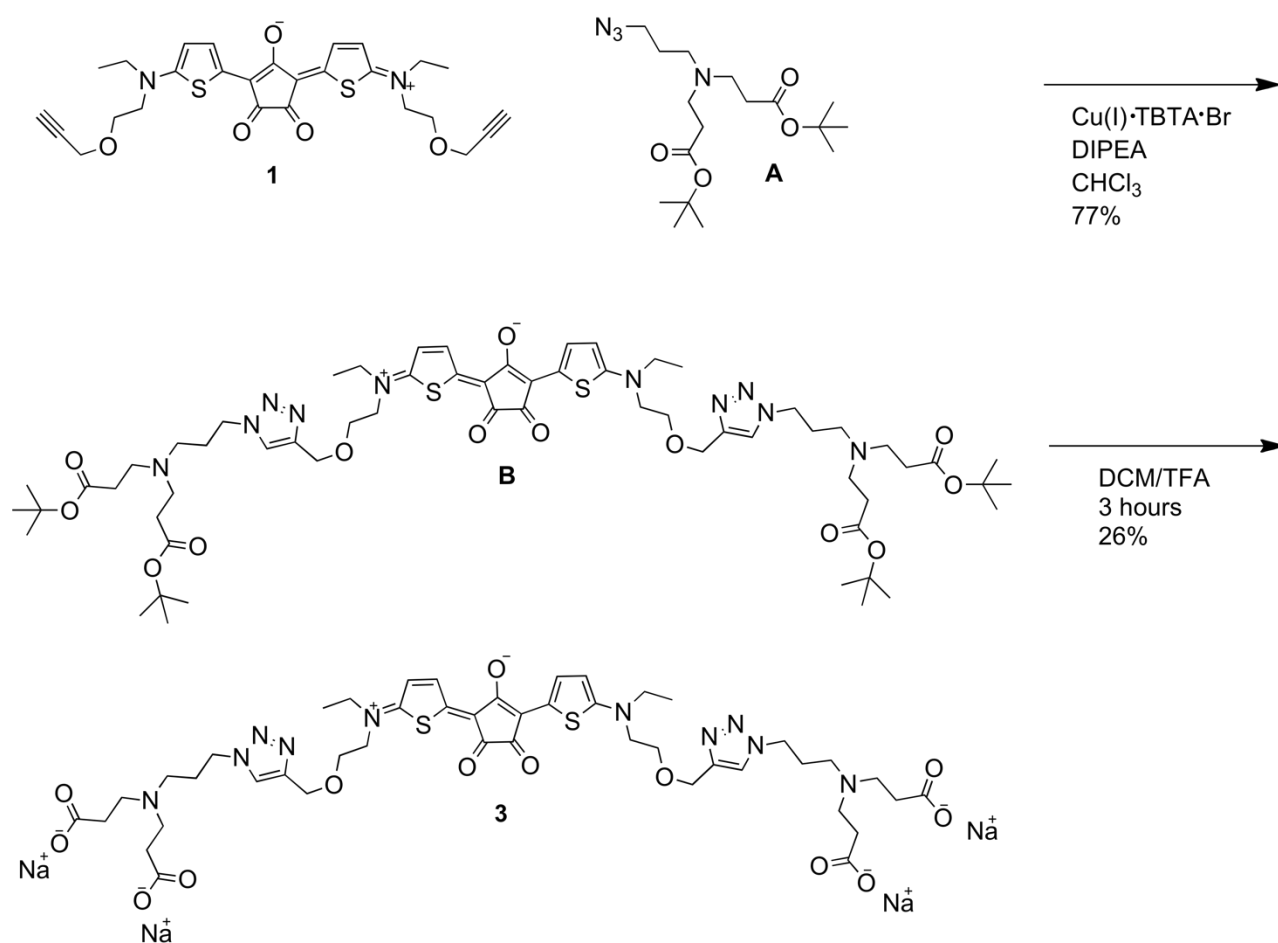
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1. Materials:

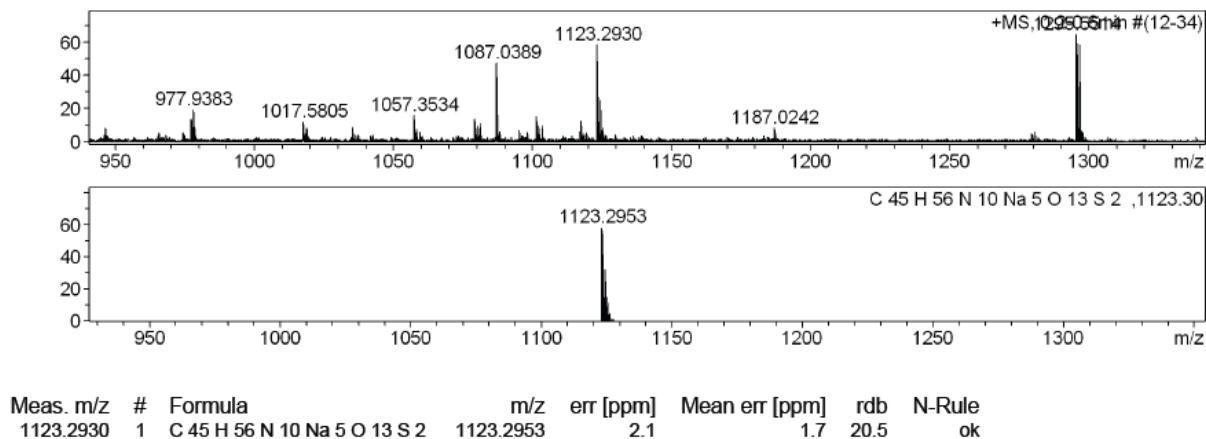
The following materials were purchased; Calcein AM (Invitrogen), PSVue643 (Molecular Targeting Technologies Incorporated), Fetal Bovine Serum (Atlanta Biologicals), Penicillin Streptomycin (Corning), Culture Media and Cells (American Tissue Culture Collection, ATCC). The media was Kaighn's Modification of Ham's F-12 Medium (F-12K; ATCC-30-2004) and the cell lines were CHO-K1 (ATCC: CCL-61) and Jurkat (ATCC: TIB-152). Croconaine dyes **1**^{S1} and **2**^{S2} were prepared during previous studies and compound purity was confirmed by NMR.

Synthesis of Croconaine Dye 3.

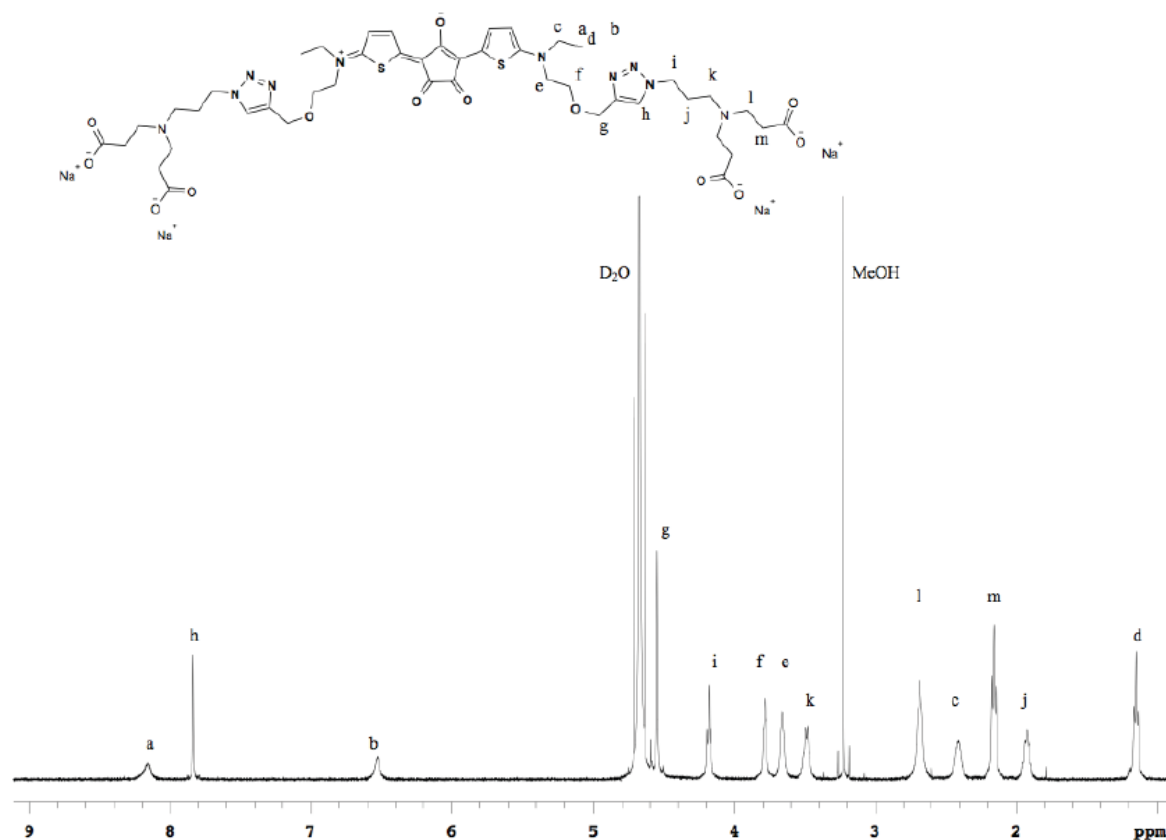


Croconaine dye **1**^{S1} (20 mg, 0.038 mmol), azide **A**^{S3} (4 mg, 0.114 mmol), Cu(I)TBTA·Br (8 mg, 30 mol%) and DIPEA (15 μL , 0.084 mmol) were dissolved in CHCl_3 (4 mL) and stirred at room

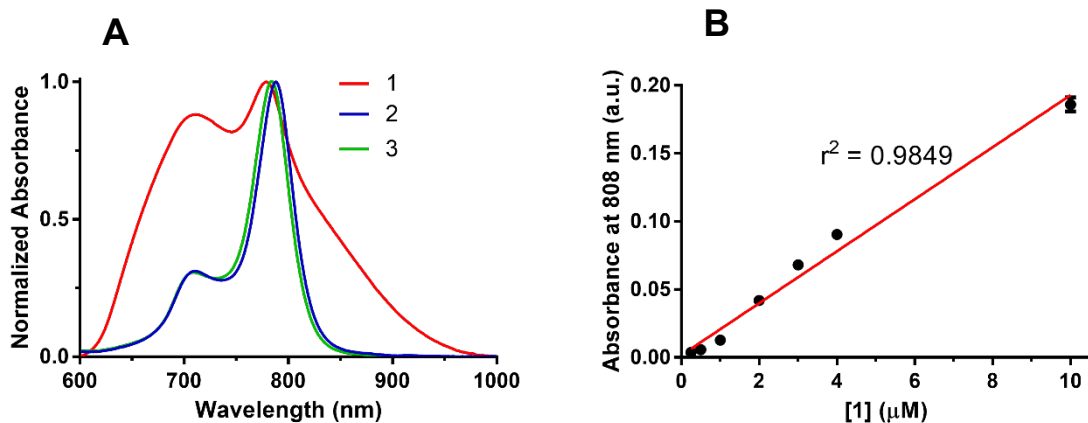
temperature for 24 hours. The solvent was removed *in vacuo* and the residue purified by gradient silica gel column chromatography using 3% MeOH/DCM to elute the product **B** as a black solid (36.4 mg, 77%). ¹H NMR (500 MHz, CDCl₃) δ (ppm) 8.16 (2H, broad s), 7.68 (2H, s), 6.80 (2H, broad s), 4.62 (4H, s), 4.36 (4H, t, *J* = 8 Hz), 3.84 (16H, m), 2.72 (8H, t, *J* = 8 Hz), 2.42 (8H, t, *J* = 8 Hz), 2.05 (4H, m), 1.32 (6H, t, *J* = 8 Hz), 1.40 (36H, s). MS-ESI *m/z* 1237.6392 ([M + H]⁺, calc. for C₆₁H₉₃N₁₀O₁₃S₂ 1237.6360). Croconaine **B** was dissolved in a mixture of 1:1 DCM:TFA and stirred for 3 hours. The solvent and side product were removed *in vacuo* and the resulting residue was dissolved in water. The pH of the solution was adjusted to a pH 7-8 using saturated Na₂CO₃ solution. The aqueous solution was extracted using CHCl₃ to remove any organic contaminants. The resulting aqueous layer was freeze dried then passed through a reverse phase column using 1% MeOH/H₂O to remove any excess salts and elute the product **3** as a dark green solid (4.2 mg, 26%). ¹H NMR (500 MHz, CDCl₃) δ (ppm) 8.18 (2H, broad s), 7.82 (2H, s), 6.56 (2H, broad s), 4.56 (4H, s), 4.21 (4H, t, *J* = 8 Hz), 3.78 (4H, br s), 3.63 (4H, br s), 3.44 (4H, br s), 2.62 (8H, m), 2.41 (8H, m), 2.18 (8H, t, *J* = 8 Hz), 1.92 (4H, m), 1.18 (6H, t, *J* = 8 Hz). MS-ESI *m/z* 1123.2930 ([M + H]⁺, calc. for C₄₅H₅₆N₁₀Na₅O₁₃S₂ 1123.2953). λ_{abs, max} (water) 782 nm. ε (water, 5.0 μM) 1.55 x 10⁵ M⁻¹cm⁻¹.



Electrospray mass spectrum of croconaine **3**.



^1H NMR spectrum (D_2O , 500MHz, 295K) of croconaine **3**.



(A) Normalized absorbance spectrum of croconaine dyes ($5\ \mu\text{M}$) in PBS with 0.5 % DMSO. (B) Beer-Lambert plot of absorbance of **1** at 808 nm ($N = 3$) in PBS with 0.5 % DMSO. The absorbance for **1** is broadened due to self-aggregation and ϵ_{abs} is $2 \times 10^4\ \text{M}^{-1}\text{cm}^{-1}$ at 808 nm.

2. Cell Experiments

Stock solutions of croconaines **2** and **3** were prepared in phosphate buffered saline, pH 7.4 (PBS). Stock solutions containing low concentrations of croconaine **1** were prepared in PBS with 0.5% DMSO. Stock solutions containing high concentrations of croconaine **1** were prepared in PBS with 1-5 % DMSO.

Uptake of Croconaine Dyes into CHO-K1 Cells

CHO-K1 cells were seeded and grown on a 6-well plate to near confluency in F-12K media (supplemented with 10 % FBS and 1 % penicillin streptomycin) at 37 °C and 5 % CO₂. The microwell plate was placed in a heating chamber and thermally equilibrated to 37 °C. Media was removed and croconaine dyes **1** – **3** (1 mL) were added to separate wells (in each well the croconaine absorbance was adjusted to 0.100 at 808 nm) and incubated for 15 min (N = 3, longer incubation times did not change the amount of dye taken up the cells). The supernatant was removed and centrifuged at 5000 rpm for 5 min to remove any cellular debris. The supernatant absorbance at 785 nm was compared to the absorbance of dye alone in PBS. The decrease in dye absorbance was attributed to uptake by the cells.

The 6-well plate contained $\sim 1.2 \times 10^6$ cells in a 1 mL solution of **1** (5 μ M). The cells took up 45% of the dye (2.25×10^{-9} moles) which corresponds to 1.88×10^{-15} mol/cell or 1.1×10^9 dye molecules per cell.

MTT Cell Viability Assay

Cell viability was measured using the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. CHO-K1 cells were seeded into 96-microwell plates, and grown to confluency using F-12K medium (supplemented with 10 % FBS and 1 % penicillin streptomycin) in a humidified incubator (37 °C; 5 % CO₂). The Vybrant MTT cell proliferation assay kit (Invitrogen) was performed according to the manufacturer's protocol. The cells were treated with croconaine dye and incubated in the dark for 18 h at 37 °C. The medium was removed and replaced with 110 μ L of F-12K medium containing MTT (1.2 mM) and the sample incubated for 4 h at 37 °C and 5 % CO₂. An SDS–HCl detergent solution was added and incubated for an additional 12 h. The absorbance of each well was measured at 570 nm (N = 4), and the data were normalized to untreated cells. Only active reductase enzymes in living cells can reduce MTT; therefore, high levels of cell viability are indicated by high absorbance at 570 nm.

Photothermal-Induced Cell Death Monitored by MTT Cell Viability Assay

CHO-K1 cells were grown to near-confluency on a 96-well plate in F-12K media (supplemented with 10 % FBS and 1 % penicillin streptomycin) 37 °C and 5 % CO₂. The microwell plate was placed in a heating chamber and thermally equilibrated to 37 °C. To determine the most effective photothermal croconaine dye, separate wells containing equal numbers of cells were treated with one of the croconaine dyes **1 - 3** (200 µL per well in PBS) such that the dye absorbance in each well was the same (0.025 optical density at 808 nm which corresponds to 1.25 µM of **1**). Each of the wells were irradiated separately from above plate with a continuous wavelength diode laser (ThorLabs Inc, 808 nm, 2 W/cm²). The temperature inside the well was monitored in real time using an infrared camera, and laser-irradiation was stopped once the temperature reached 41.5 °C (about 125 seconds). The supernatant was replaced with 200 µL of fresh media, and the wells incubated overnight in the dark at 37 °C. Cell viability was evaluated by the MTT cell viability assay described above. Cells in control groups were incubated under the same experimental conditions excluding laser irradiation.

Imaging Photothermal-Induced Cell Death Using Fluorescent Live and Dead Cell Stains

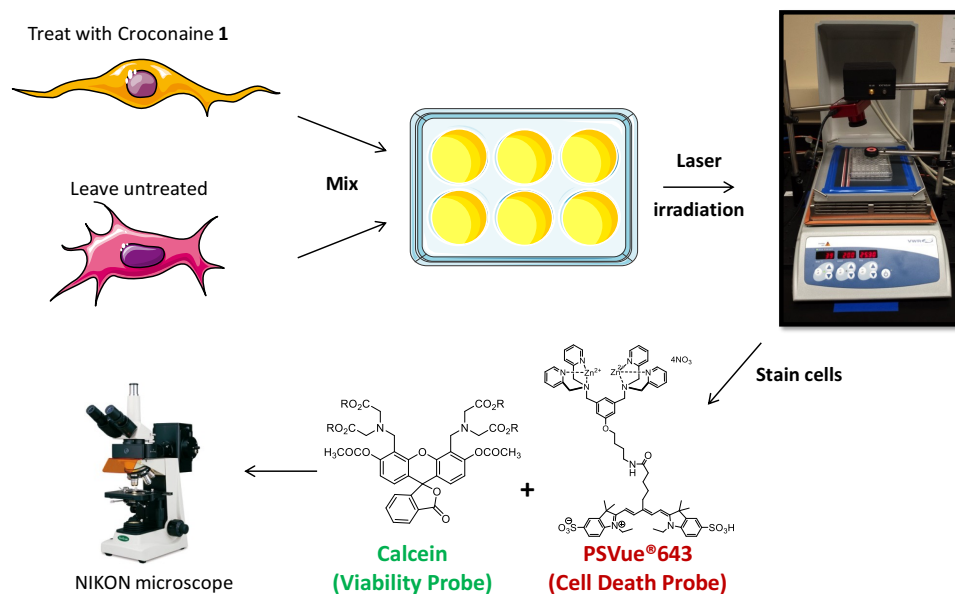
CHO-K1 cells were grown on an 8-chambered well plate to near-confluency in F-12K media (supplemented with 10 % FBS and 1 % penicillin streptomycin) and each well was treated with **1** (5 µM) for 45 minutes. The supernatant was removed and the cells washed with buffer. Each well was irradiated with a narrow laser beam (3 W/cm²) that was focused for 10 minutes on a sub-section that did not cover the entire microwell area. The cells in each well were washed once with PBS, then the supernatant replaced with fresh media and the well kept overnight in a humidified incubator (37 °C; 5 % CO₂). The following day, each well was treated with a binary mixture of green fluorescent live-cell stain Calcein AM (3 µg/mL) and red fluorescent dead-cell stain PSVue643 (10 µM) and allowed to stand for 20 minutes. The cells were washed once and resuspended in PBS. Brightfield and fluorescence microscopy was performed using a Nikon TE-2000U epi-fluorescence microscope equipped with a GFP (ex. 450/90 nm, em. 500/50 nm) filter and Cy5 (ex. 620/60 nm; em. 700/75 nm) filter. Fluorescence images of the border between the dead and living cells were captured using Metamorph software (Universal) and analyzed using ImageJ 1.40g software.

Jurkat cells were incubated with either 10 µM or 50 µM of **1** for 15 minutes. The cells were pelleted by centrifugation at 1000 rpm for 5 minutes and resuspended in fresh F-12K media (supplemented with 10 % FBS and 1 % penicillin streptomycin). Cells were irradiated with an 808 nm diode laser at 2 W/cm² for 0, 5 or 10 min then kept overnight in a humidified incubator (37 °C; 5 %

CO₂). The following day, the cells were treated with the green fluorescent live-cell stain Calcein AM and microscopy was performed as described above.

Selective Photothermal Inactivation of Croconaine Labeled Cells in a Mixed Population of Adherent CHO-K1 and Non-Adherent Jurkat Cells

The work flow is summarized in Scheme S1.



Scheme S1. Work flow for selective photothermal inactivation of cells containing croconaine **1** followed by treatment with green fluorescent live-cell stain Calcein AM and red fluorescent dead-cell stain PSVue643.

Photothermal Inactivation of Croconaine Labeled CHO-K1 Cells

Adherent CHO-K1 cells were grown on an 8-chambered well plate to near confluency in F-12K media (supplemented with 10 % FBS and 1 % penicillin streptomycin). The cells were labeled with dye by incubating with 5 μ M of croconaine **1** for 45 minutes. The extracellular solution was removed, and the labeled cells washed once with PBS buffer. Unlabeled Jurkat cells in F-12K media (1.5×10^5) were added to the wells containing the labeled CHO-K1 cells and each well was irradiated with an 808 nm diode laser at 2 W/cm² until the average temperature of the well reached 41.5 °C (about 120 seconds). The cells were kept overnight in a humidified incubator (37 °C; 5 % CO₂), then Calcein

AM/PSVue643 staining and microscopy was performed as described above. Cells in control groups were incubated with **1** without laser irradiation or incubated with PBS containing no dye and laser-irradiated (2 W/cm^2) for 5 minutes.

Photothermal Inactivation of Croconaine Labeled Jurkat Cells

Non-adherent Jurkat cells were labeled by incubating with $50 \mu\text{M}$ of croconaine **1** for 15 minutes, then mixed with unlabeled CHO-K1 cells and the mixed cells irradiated with an 808 nm laser (2 W/cm^2) for 10 minutes. The irradiated cells were kept overnight in a humidified incubator ($37 \text{ }^\circ\text{C}$; $5 \text{ } \%$ CO_2), then Calcein AM/PSVue643 staining and microscopy was performed as described above.

Statistical Analysis

Thermal curves are presented as mean \pm standard deviation (SD). MTT cell viability data are presented as mean \pm standard error of the mean (SEM).

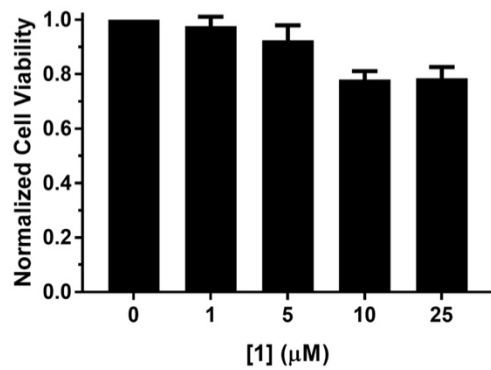


Figure S1. Viability of CHO-K1 cells after incubation with **1** for 18 h in the dark, using an MTT cell viability assay. Data is displayed as mean \pm standard error of the mean (N = 4).

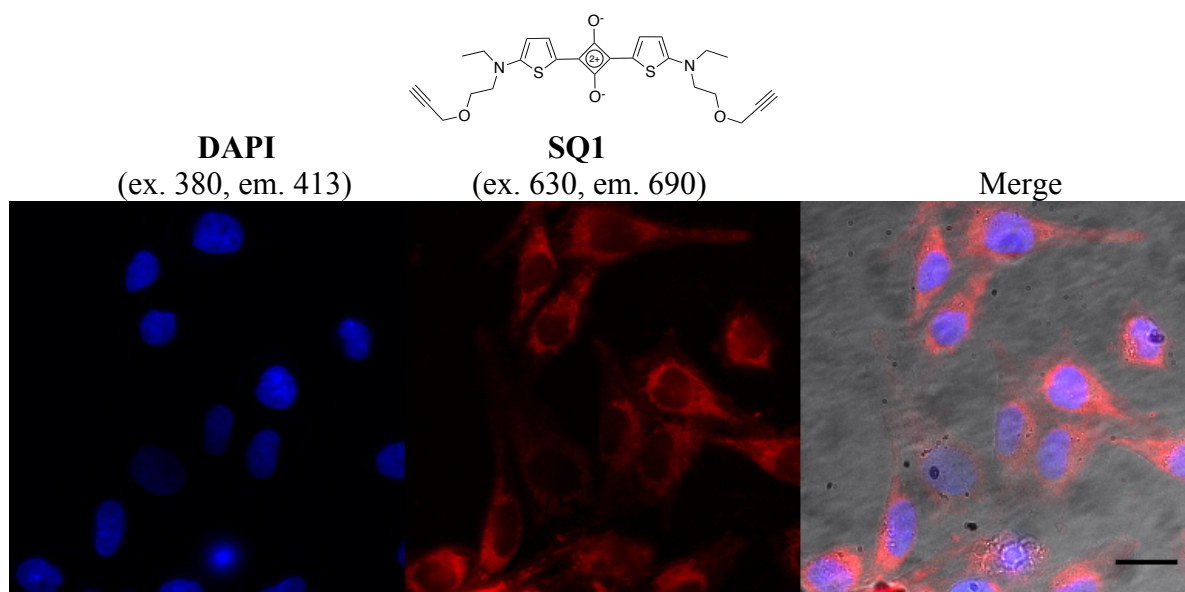


Figure S2. Fluorescence micrographs of CHO-K1 cells after treatment with **SQ1** (5 μ M), a deep-red fluorescent structural analogue of **1**, and **DAPI** a blue fluorescent nuclear stain. Scale bar = 10 μ m.

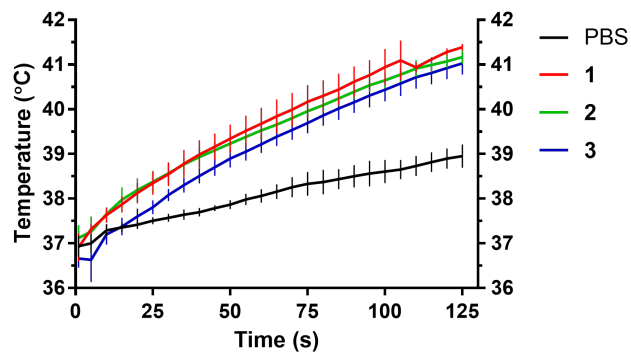


Figure S3. Change in temperature for microwells containing CHO-K1 cells treated with 1.25 μM of croconaine dye in PBS. The microwells were then illuminated with a diode laser operating (808 nm, 2 W/cm^2) and the temperature in all wells (measured using a near-infrared camera) increased steadily over 125 seconds from 37 °C to 41.5 °C.

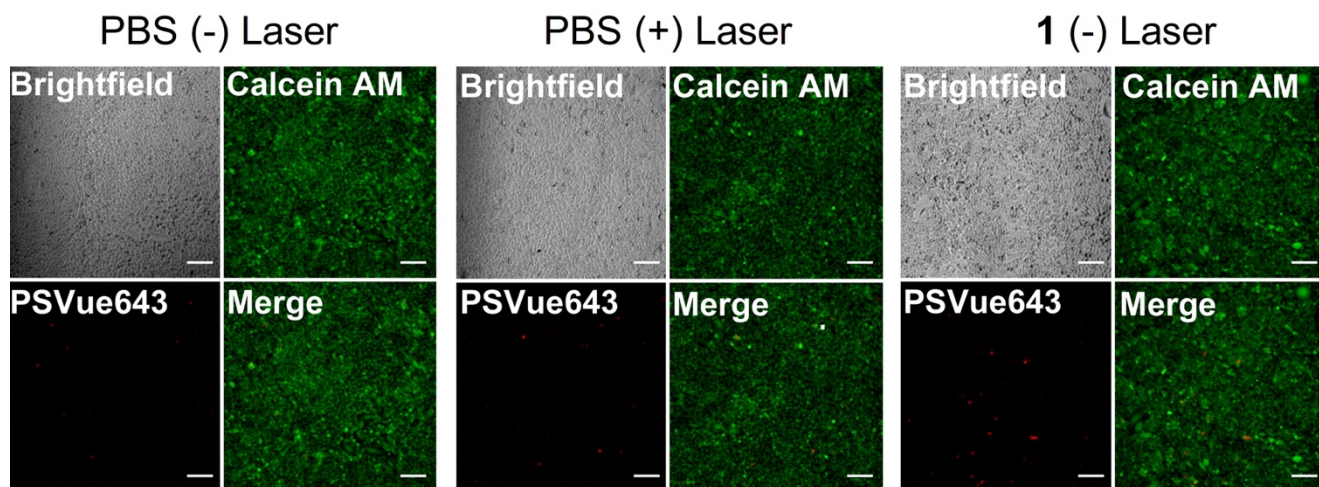


Figure S4. Control experiments for photothermal cell inactivation. Micrographs of CHO-K1 cells that were either laser irradiated (3 W/cm^2) for 10 minutes or not in the presence of PBS or $5 \mu\text{M}$ **1** in PBS at $37 \text{ }^\circ\text{C}$. After irradiation, the cells were incubated overnight in fresh media at $37 \text{ }^\circ\text{C}$, then treated with a binary mixture of the green fluorescent live-cell stain Calcein AM ($3 \mu\text{g/mL}$) and red fluorescent dead-cell stain PSVue643 ($10 \mu\text{M}$) for 20 minutes with a PBS buffer wash. Scale bar = $100 \mu\text{m}$.

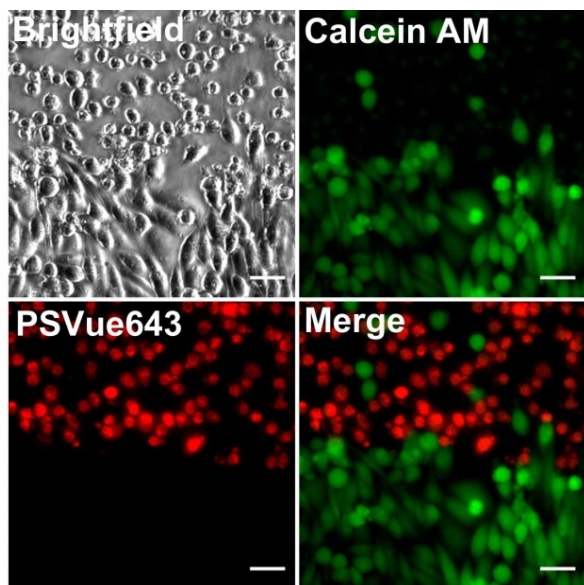


Figure S5. Magnified view of Figure 2. Spatially localized photothermal inactivation of CHO-K1 cells that were labeled with **1**. Micrographs showing cells after a sub-section of the microwell area was irradiated with a narrow laser beam (808 nm, 3 W/cm²) for 10 minutes. After irradiation the cells were incubated overnight in fresh media at 37 °C, then treated with a mixture of red fluorescent live-cell stain Calcein AM (3 µg/mL) and red fluorescent dead-cell stain PSVue643 (10 µM; red) for 20 minutes with a PBS buffer wash. The images clearly show a border between living cells and laser-induced dead cells. Scale bar = 25 µm.

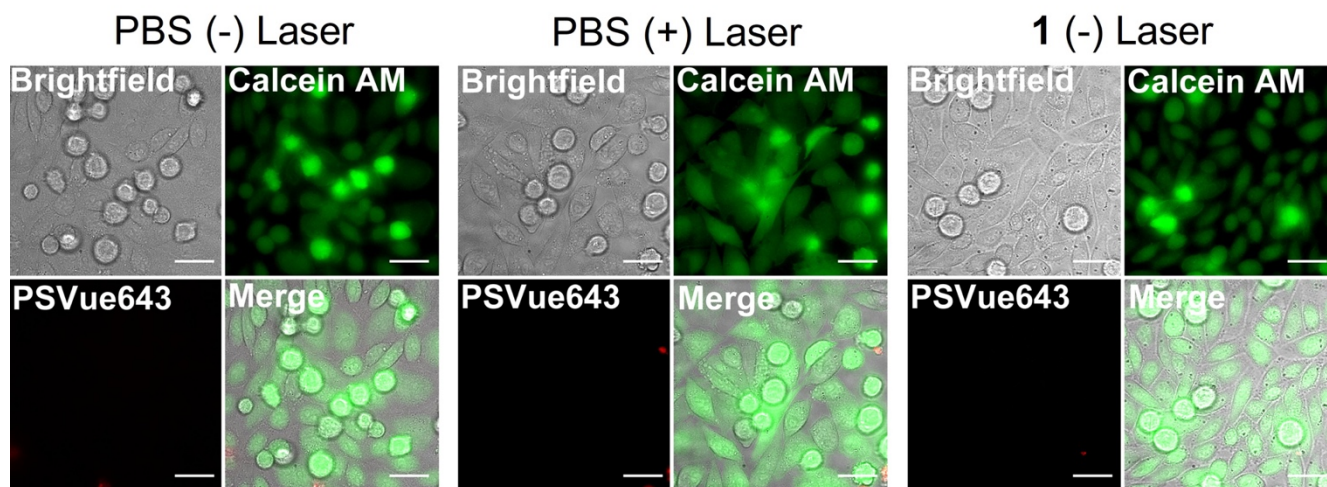


Figure S6. Control experiments for selective photothermal cell inactivation. Micrographs of a mixture of adherent CHO-K1 cells and non-adherent Jurkat cells that were treated with PBS and either; (middle) laser irradiated (2 W/cm^2) for 5 minutes, (left) not irradiated, or (right) CHO-K1 cells pre-labeled with **1** ($5 \mu\text{M}$) for 45 minutes but not laser irradiated. After treatment cells were incubated overnight in fresh media at $37 \text{ }^\circ\text{C}$, then stained with a live/dead cell mixture of Calcein AM ($3 \mu\text{g/mL}$; green) and PSVue643 ($10 \mu\text{M}$; red) for 20 min. Scale bar = $10 \mu\text{m}$.

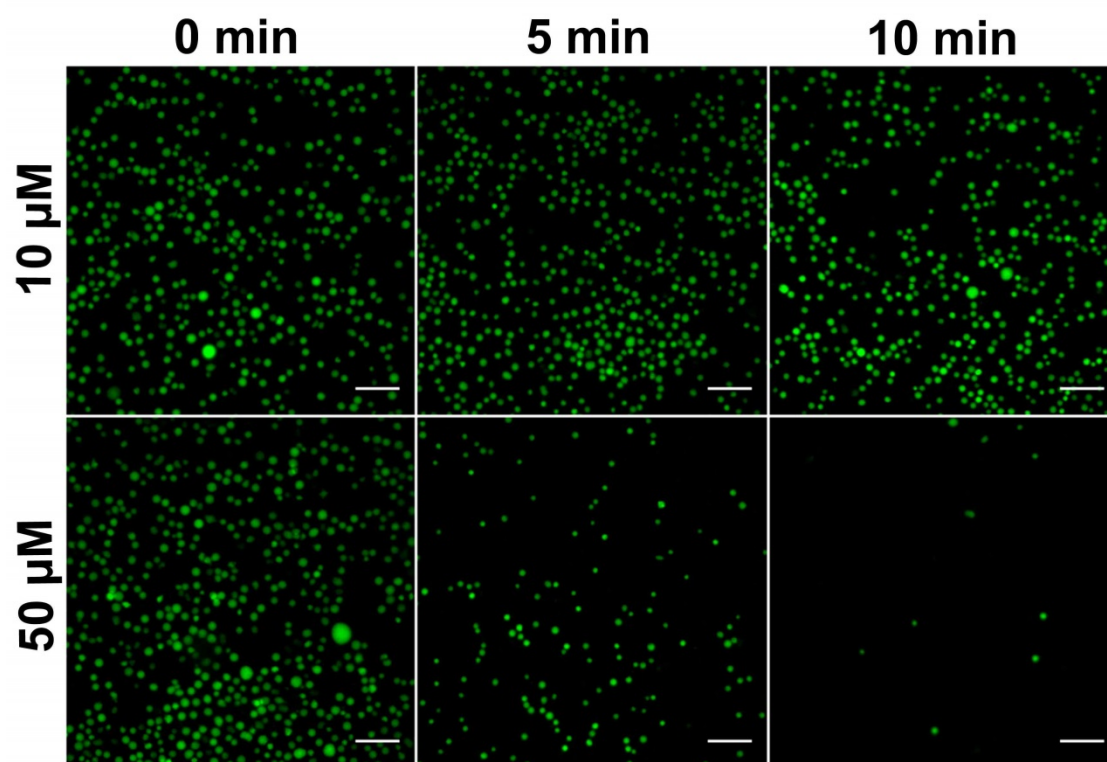


Figure S7. Photothermal inactivation of Jurkat cells that were labeled with **1** (10 or 50 μM) and then laser irradiated ($2 \text{ W}/\text{cm}^2$) for 0, 5 or 10 min. After irradiation cells were washed, incubated overnight at $37 \text{ }^\circ\text{C}$ and treated with green fluorescent Calcein AM ($3 \text{ }\mu\text{g}/\text{mL}$; 20 min) as a stain for cell viability. Scale bar = 100 μM .

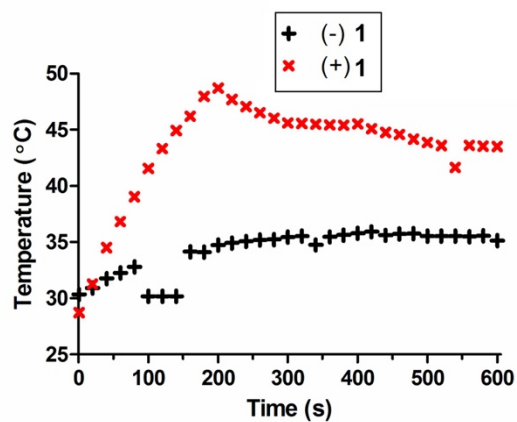


Figure S8. A microwell containing a mixed population of Jurkat cells labeled with **1** (50 μM) and unlabeled CHO-K1 cells was irradiated with the 808 nm laser (2 W/cm^2) for 10 minutes which raised the well temperature close to a steady state of 45 $^{\circ}\text{C}$.

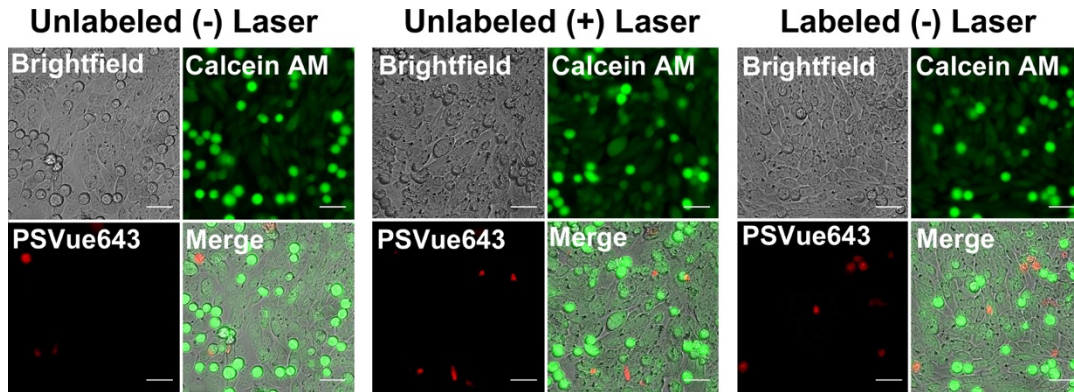


Figure S9. Control experiments. Micrographs of a mixture of adherent CHO-K1 cells and non-adherent Jurkat cells that were either laser irradiated (2 W/cm^2) for 10 minutes, or not irradiated. The Jurkat cells were either labeled with **1** ($50 \mu\text{M}$; 15 min) or unlabeled. After irradiation, the cells were incubated overnight in fresh media at $37 \text{ }^\circ\text{C}$, then treated with green fluorescent live-cell stain Calcein AM ($3 \mu\text{g/mL}$; green) and red fluorescent dead-cell stain PSVue643 ($10 \mu\text{M}$; red) for 20 minutes. Scale bar = $25 \mu\text{m}$.

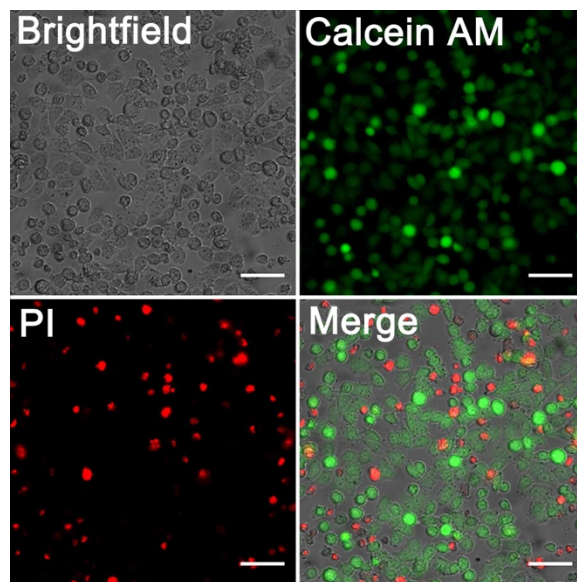


Figure S10. Selective photothermal inactivation of labeled Jurkat cells. Micrographs of a mixture of adherent CHO-K1 cells and non-adherent Jurkat cells that were laser irradiated (2 W/cm^2) for 10 minutes in cell media at $37 \text{ }^\circ\text{C}$. The Jurkat cells were pre-labeled with **1** ($50 \text{ }\mu\text{M}$; 15 min), washed with fresh media then combined with unlabeled CHO-K1 cells. After irradiation, the cells were incubated overnight in fresh media at $37 \text{ }^\circ\text{C}$, then treated with binary mixture of green fluorescent live-cell stain Calcein AM ($3 \text{ }\mu\text{g/mL}$) and red fluorescent dead-cell (necrosis) stain propidium iodide (PI; $3 \text{ }\mu\text{g/mL}$) for 20 minutes. Scale bar = $25 \text{ }\mu\text{m}$.

3. Cellular Expression of Heat Shock Protein

Many cancer cells have higher basal levels of heat shock proteins (HSP) which makes them more tolerant to hyperthermia.^{S4} A report that a Jurkat cell variant expresses high levels of HSP70^{S5} prompted us to quantify the levels of HSP70 in our CHO-K1 and Jurkat cell-lines. The total protein was extracted from these cells and Western Blot was used to compare the expression levels, using antibodies against HSP70 and β -actin as control protein. The results in Figure S10 show that Jurkat cells express much higher basal levels of HSP70, compared to CHO-K1 cells, which may be a reason for the increased tolerance to photothermal inactivation.

Western Blot Analysis.

Cells were rinsed 3x using ice-cold PBS and lysed in 1X RIPA Lysis buffer (Thermofisher) containing 1X Protease inhibitor cocktail (AMRESCO) and sonicated at 30 % amplitude for 1 minute on ice. The collected protein concentration was determined using a BCA Protein Assay Kit (Pierce) and stored in aliquots at -20 °C until use. The cell lysates were separated using SDS-PAGE by loading 50 μ g of total protein onto a 10 % tris-glycine mini gel and applying 80V for 30 minutes and 125V for ~1 hour. Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane (Azure) using a XCellIII Blot Module (Thermofisher) at 25V for 1.5 hours. The membrane was blocked using 5 % nonfat dried milk overnight at 4 °C and rinsed 3x using Tris Buffered Saline + Tween (TBST) before incubation with the primary anti-HSP70/72 (1:1000, ENZO-ADI-SPA-810) for 1 hour at room temperature. The immunoblots were subsequently rinsed 3x with TBST and incubated with the secondary goat anti-mouse antibody conjugated to horseradish peroxidase (HRP) for 1 hour at room temperature. β -actin was probed using the HRP-conjugated β -actin monoclonal antibody (Thermofisher MA5-15739-HRP). Proteins were detected via chemiluminescence imaging using a Bio-Rad ChemiDoc™ MP Imaging System to image the immunoblot after incubation with ECL Plus Western Blotting Substrate (Pierce).

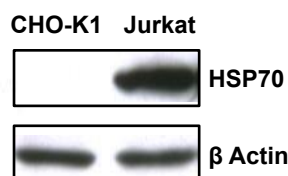


Figure S11. Immunoblots of extracts from CHO-K1 and Jurkat cells indicating basal levels of HSP70 expression. Also shown is the β -actin from both extracts to demonstrate equal loading of total protein.

4. References

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