Supporting Information

Flavonol-based carbon monoxide delivery molecule with endoplasmic reticulum, mitochondria and lysosome localization

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Table of Contents **Page**

Table of Contents **Page**

Experimental Section – Synthesis and Characterization

Chemicals and Reagents. Unless otherwise noted, all chemicals and reagents were obtained from commercial sources and used as received unless otherwise specified. Compounds **1**, **2**, **4** and **5** were prepared as previously reported.[1]

Physical Methods. ^{1}H and ^{13}C{^{1}H} NMR spectra were collected using a Bruker Avance III-HD Ascend-500 (proton at 500 MHz) spectrometer and are referenced to the residual solvent peak in C*D*Cl3 (^{1}H: δ 7.26 ppm; ^{13}C: δ 77.16 ppm); DMSO-d_6 (^{1}H: δ 2.50, 13C: δ 39.52). Multiplicity is reported as follows: s (singlet); d (doublet); t (triplet); m (multiplet); *J* values are given in Hz. UV-vis spectra were recorded at ambient temperature using a Hewlett-Packard 8453A diode array spectrophotometer. Fluorescence emission spectra were collected using a Shimadzu RF-530XPC spectrometer in the range of 400-800 nm, with the excitation wavelength corresponding to the absorption maxima of the molecule. The excitation and emission slit widths were set at 1.5 nm for all the experiments. Mass spectral data were collected at the Mass Spectrometry Facility, University of California, Riverside. ESI/APCI mass spectra were recorded on an Agilent LCTOF (2006) with a Windows XP operating system. A LuzChem photoreactor equipped with LSC-420 lamps was used for all photochemical reactions. An Apogee Instruments MQ-500 full spectrum quantum meter was used to determine the photon flux of the illumination set ups. Quantum yields were determined using ferrioxalate as a standard to measure photon flux (~10^{16}-10^{17} photons/second).[2,3] Carbon monoxide (CO) was quantified as previously described.[4]

Fluorescence Quantum Yields and Lifetime of 4. Steady-state and time-resolved emission data for **4** were collected at room temperature using an Edinburgh FLS980 spectrometer. The deaerated sample was first bubbled with N_2 for 30 minutes before spectra were obtained. The sample was excited using light output from a housed 450 W Xe lamp passed through a single grating (1800 l/mm, 250 nm blaze) Czerny-Turner monochromator. Emission from the sample was passed through a single grating (1800 l/mm, 500 nm blaze) Czerny-Turner monochromator and finally detected by a peltier-cooled Hamamatsu R928 photomultiplier tube. The dynamics of emission decay were monitored by using the FLS980's time-correlated single-photon counting capability (1024 channels; 100 ns window) with data collection for 5,000 counts. Excitation for time-correlated single photon counting (TCSPC) was provided by an Edinburgh EPL-405 ps pulsed light emitting diode (405 ± 10 nm, pulse width 57.6 ps) operated at 10 MHz. Emission was passed through a 420 nm long-pass filter and then a single grating (1800 l/mm, 500 nm blaze) Czerny−Turner monochromator and finally detected by a Peltier-cooled Hamamatsu R928 photomultiplier tube. Time-resolved emission data were fit using an Edinburgh software package with an exponential reconvolution function fit using an IRF obtained from solvent and cuvette only. Quantum yield data was collected at room temperature using a Hamamatsu Quantaurus-QY Spectrometer. The sample was excited

using a 405 nm output from a 150 W Xenon arc lamp and detected by a cooled 1024 channel CCD detector.

RP-HPLC Analysis of 1 and 4. A RP-HPLC system consisting of an Agilent Eclipse XDB-C18 column (150 x 4.6 mm; 5 µm particle size), a LC-2030 pump and a LC-2040 PDA detector was run at 35 °C. Each flavonol was dissolved in HPLC grade acetone to a final concentration of approximately 500 µg/mL. A 5 µL of each solution was injected via autosampler. A mobile phase flow rate of 0.8 mL/min was used with a starting solvent composition of 35:65 vol/vol of water/acetone. Over 10 minutes this ratio was changed to 5:95 water:acetone. The absorption properties of the eluent were monitored at 254 nm.

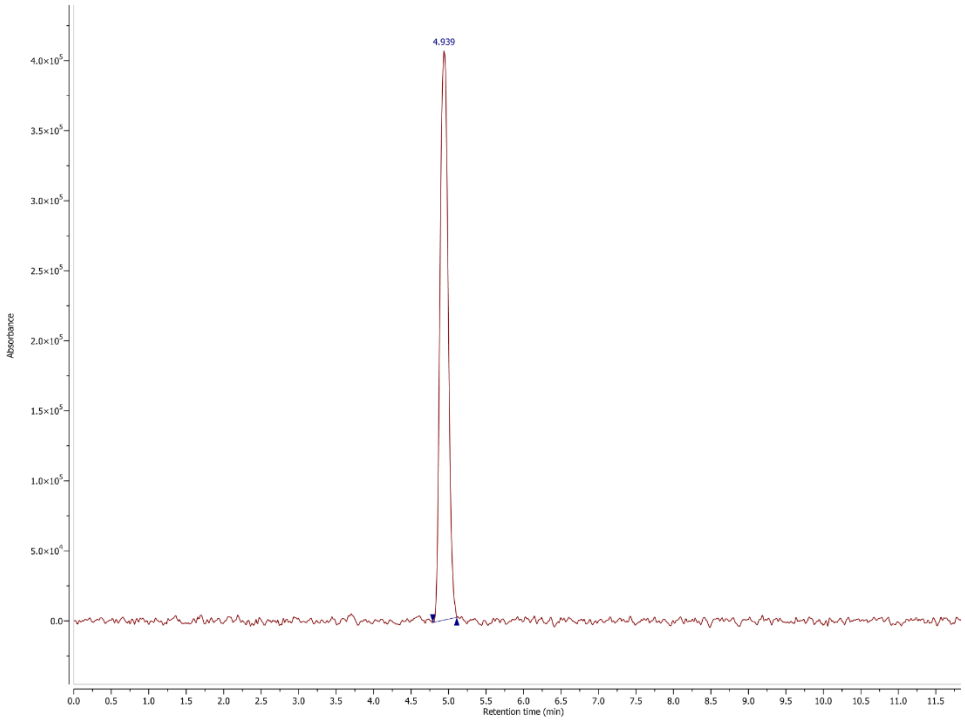

Figure S1. RP-HPLC chromatogram of **1**.

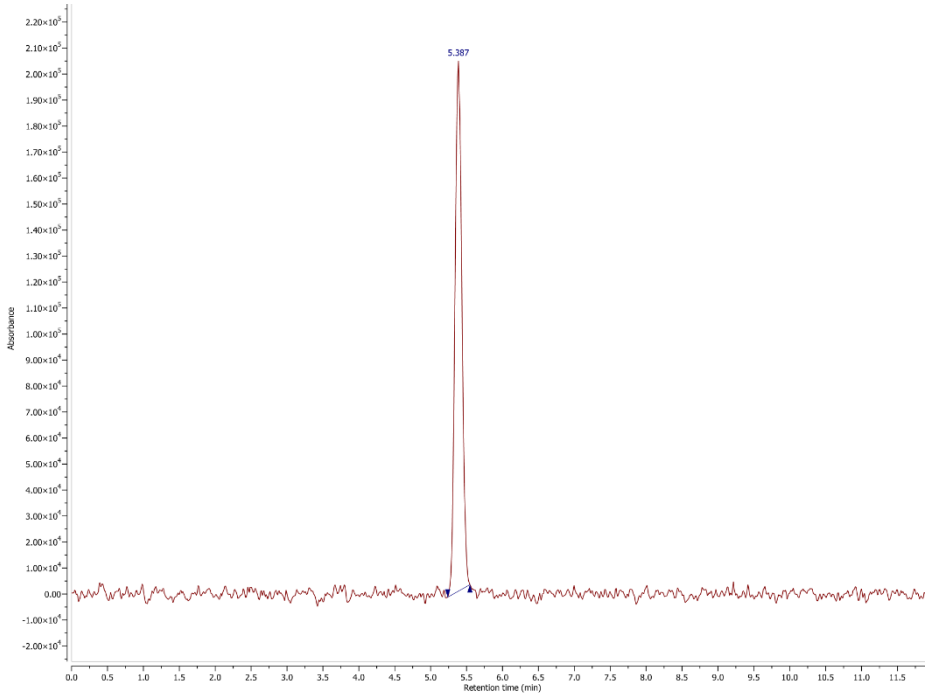

Figure S2. RP-HPLC chromatogram of **4**.

X-ray crystallography. A single crystal of **4** was mounted on glass fiber loop using paratone oil. Data collection was performed using a Rigaku XtaLAB Mini II Diffractometer with MoK_α (λ = 0.71073 Å) radiation at 100 K. An absorption correction was done using a Gaussian grid with a 0.5 mm 1D horizontal Gaussian beam correction for the graphite monochromator. The structure was solved using Olex2[5] with SHELXT[6] using intrinsic phasing method and refined using the SHELXL[7] program with the least square method. All non-hydrogen atoms were refined with anisotropic thermal parameters. The hydrogen atoms were placed in geometrically calculated positions and further refined using a riding model. Isotropic parameters of all hydrogen atoms were fixed to 1.2 times the *U* value of the atoms with the methyl and hydroxyl groups being 1.5 times *U*.

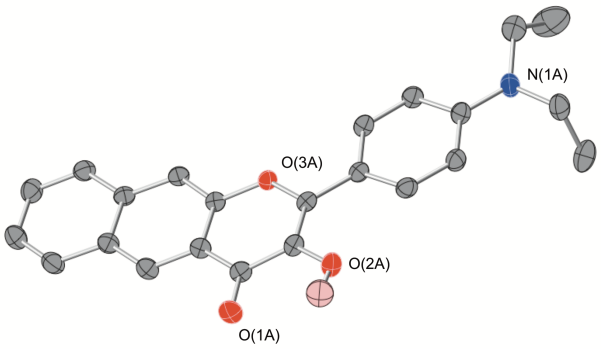

Figure S3. Thermal ellipsoid (50%) representation of one of the two molecules in the asymmetric unit of **4**.

Table S1. Selected bond distances for **4**.

Atom	Distance (Å)
O(1A)-C(2A)	1.251(2)
O(2A)-C(1A)	1.365(2)
O(3A)-C(13A)	1.372(2)
C(1A)-C(13A)	1.372(2)

4: UV-vis (solvent, λ_{max}, nm) (ε, M^{-1} cm^{-1}): DMSO, 445 (32,720); acetonitrile: 439 (36,620); DMEM + 10% FBS: 449 (27,216)

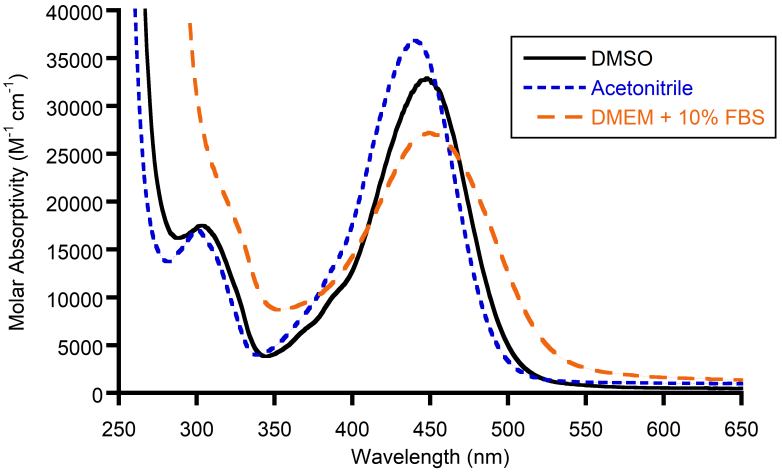

Figure S4. Absorption spectra of **4** in DMSO, acetonitrile and DMEM + 10% FBS (0.04 mM).

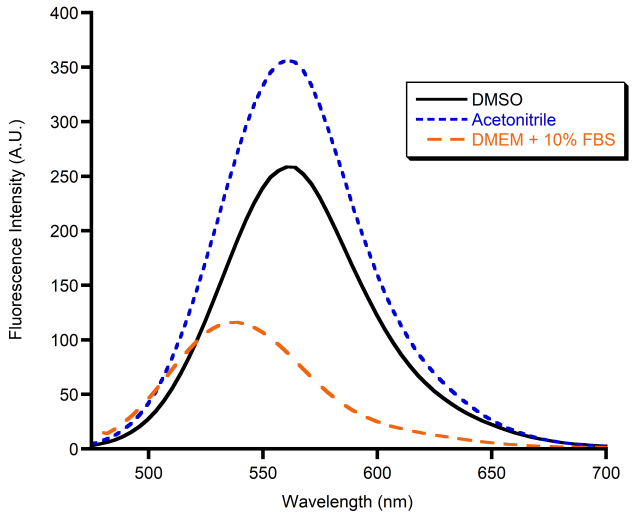

Figure S5. Emission spectra of **4** in DMSO, acetonitrile and DMEM + 10% FBS (0.04 mM).

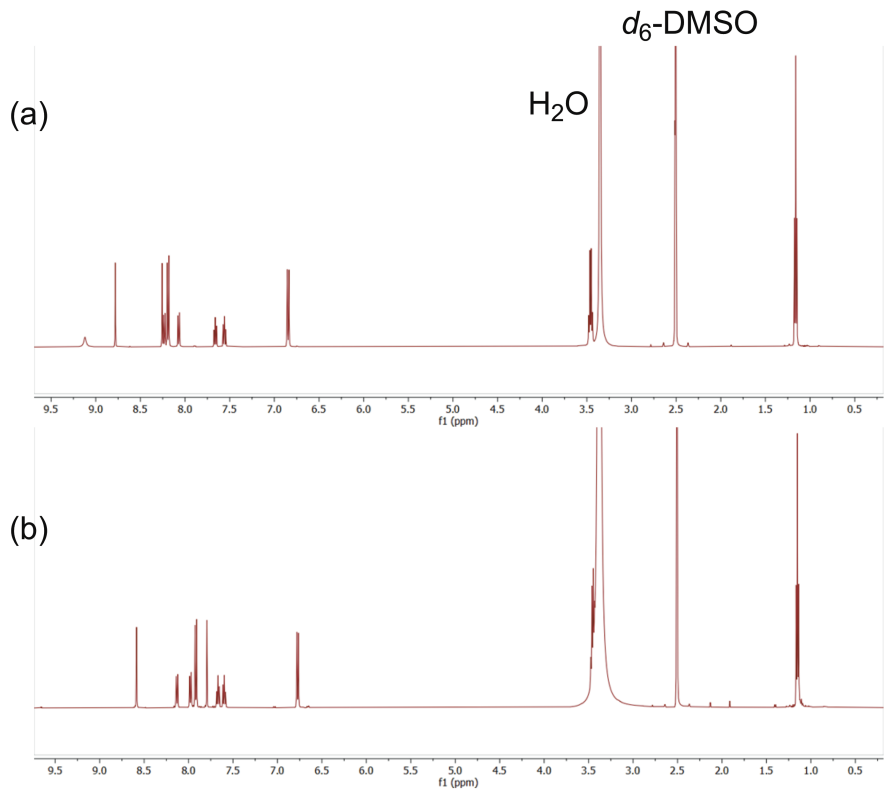

Figure S6. (a) ^{1}H NMR of **4** in d_6-DMSO. (b) ^{1}H NMR of the visible light-induced (λ_{ill} = 419 nm) CO release reaction of **4** in d_6-DMSO. The ^{1}H NMR features in (b) match those of the depside **5** which has been previously reported.[4]

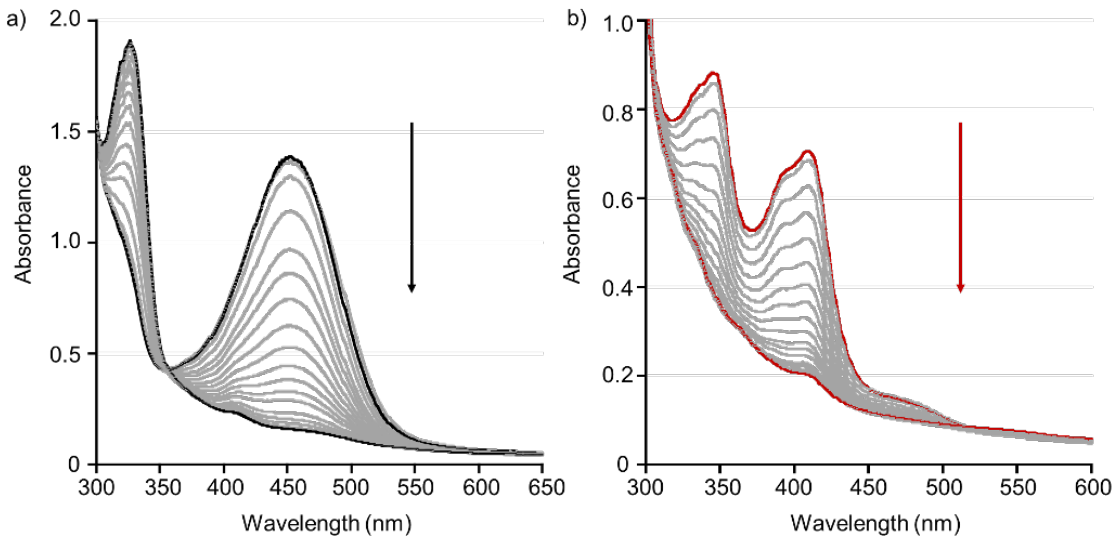

Figure S7. Absorption changes for the reaction of a) **4** and b) **1** upon illumination with visible light (λ_{ill} = 419 nm, intensity = 2,450 lx) under air for 23 minutes (0-1380 sec.) at room temperature in DMEM/F12K media with 10% FBS.

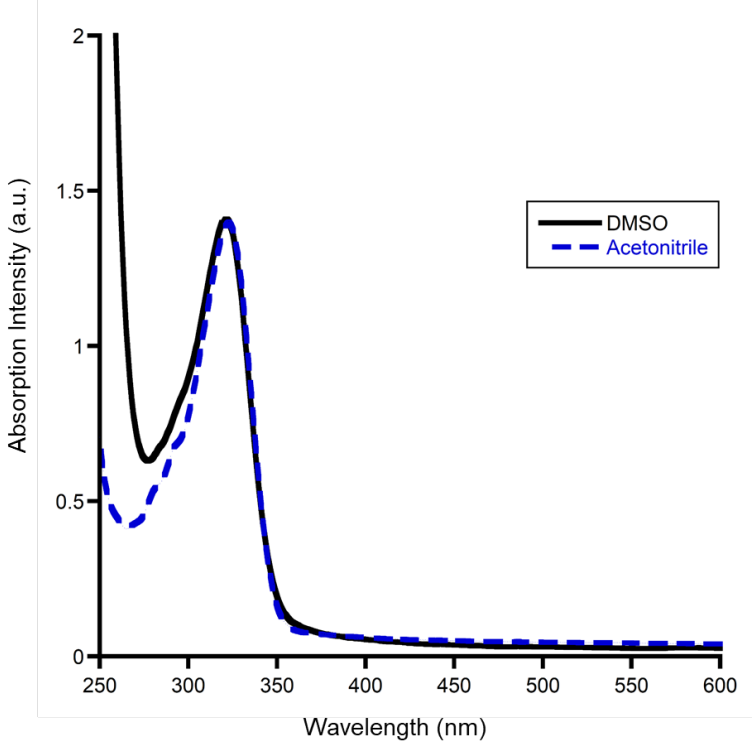

Figure S8. Solutions of **5** in DMSO and acetonitrile after 24 hours of illumination of **4** with visible light.

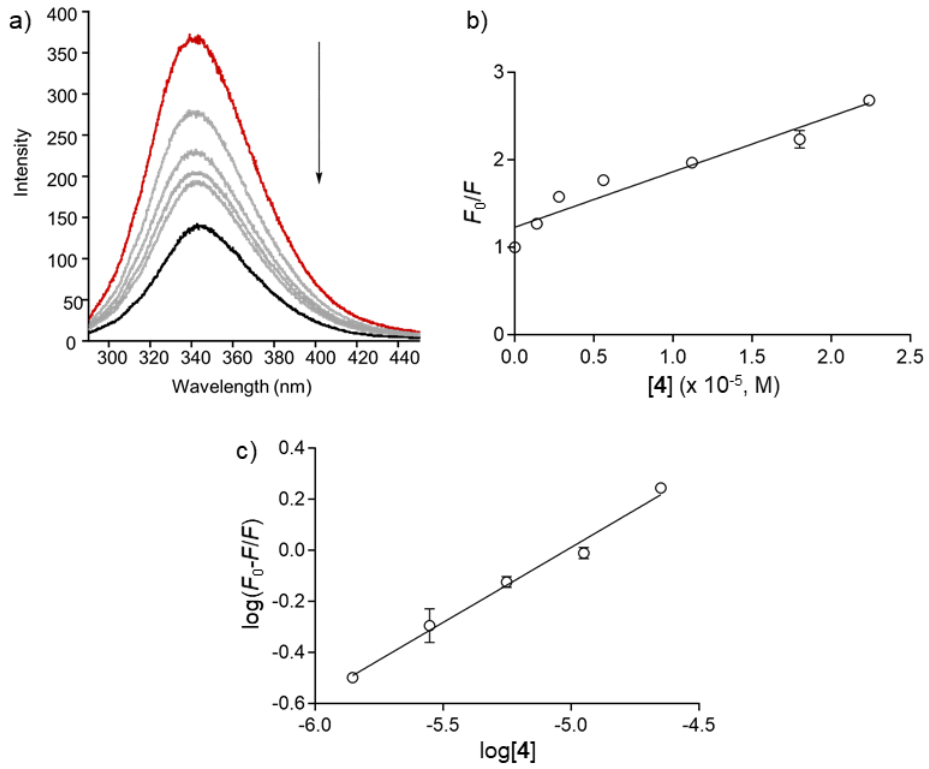

Figure S9. (a) Fluorescence emission spectra of BSA (1.4 μM) in the presence of various concentrations of **4** in TRIS/NaCl:DMSO(96:4% v:v, pH = 7.4); T = 298 K, λ = 282 nm. [**4**] = 0. 1.4, 2.8, 5.6, 8.4, 11.2, 16.8 and 22.4 μM. (b) Stern-Volmer plot of data for titration of BSA with **4**. (c) Modified Stern-Volmer plot of data for titration of BSA with **4**.

Cell Culture Growth Parameters. Human lung adenocarcinoma human alveolar basal epithelial cells (A549 cells) were obtained from ATCC (Manassas, VA) and cultured in phenol-red free DMEM-F12K Media (Caisson Laboratories) supplemented with 10% (v/v) FBS (Caisson Laboratories) and 1% penicillin-streptomycin (Sigma-Aldrich) at 37 °C with 5% CO_2. The A549 cells were fed every 3 days with fresh media and sub-cultured in T-75 flasks at 4000 cells/cm^2. Cells were detached by the addition of TrypsinEDTA 1X solution (3 mL; Caisson Laboratories) for 5 min, then neutralized with fresh media (8 mL). A549 cells were counted using a Cell Countess instrument (ThermoFisher) according to the manufacturer's protocol. Only passages 1−10 of the A549 cells were used in experiments.

Human lung normal fibroblast cells (HFL-1 cells) were obtained from ATCC and cultured in F-12K Medium (Kaighn's modification of Ham's) (ATCC) supplemented with 10% (v/v) FBS (Caisson Laboratories) at 37 °C with 5% CO_2. The media was changed every three days. Cell growth, subculturing, and counting were performed in the same manner as for A549 cells. Only passages 1−5 were used in experiments.

Mouse macrophage cells (RAW 264.7 cells) were obtained from ATCC and cultured in phenol-red free DMEM-F12K media (Caisson Laboratories) supplemented with 10% (v/v) FBS (Caisson Laboratories) and 1% penicillin-streptomycin (Sigma-Aldrich) at 37 °C with 5% CO_2. The media was changed every other day. Cell growth, subculturing, and counting were performed in the same manner as for A549 cells. All experiments were performed within 5 passages of the RAW 264.7 cells.

MTT Assays. Three independent biological experiments, each with three technical replicates, were performed for **4** and **5** under dark and under visible light-induced CO release conditions. For all experiments, MTT (Sigma-Aldrich) was prepared fresh at 5 mg/mL in sterile PBS. This solution was filtered through a 0.22 μm PES filter. The cells were seeded in 96-well plates (Corning, NY) at 1.0 x 10^4 cells/well for 24 h. The cells were then treated with each compound at 0.08-100 μM with a final DMSO concentration that did not exceed 0.4% (v/v). For visible light-triggered *in situ* CO release, the plates were exposed to blue LED array (light density of 66,351 lx) for 1 h and incubated for an additional 23 h. Then, MTT solution (20 μL) was added and the cells were incubated for an additional 4 h. The metabolized formazan pellets were solubilized by adding 200 μL of DMSO and absorption values were measured using a Modulus™ Microplate reader (Turner Biosystems) at 560 and 750 nm (background). The final results were background subtracted and normalized to vehicle control (0.4% DMSO). Data analysis was performed using GraphPad Prism 7 (La Jolla, California), with values reported as mean ± SEM. IC_{50} values were calculated as nonlinear regression with a bottom constraint of zero.

MTT data:

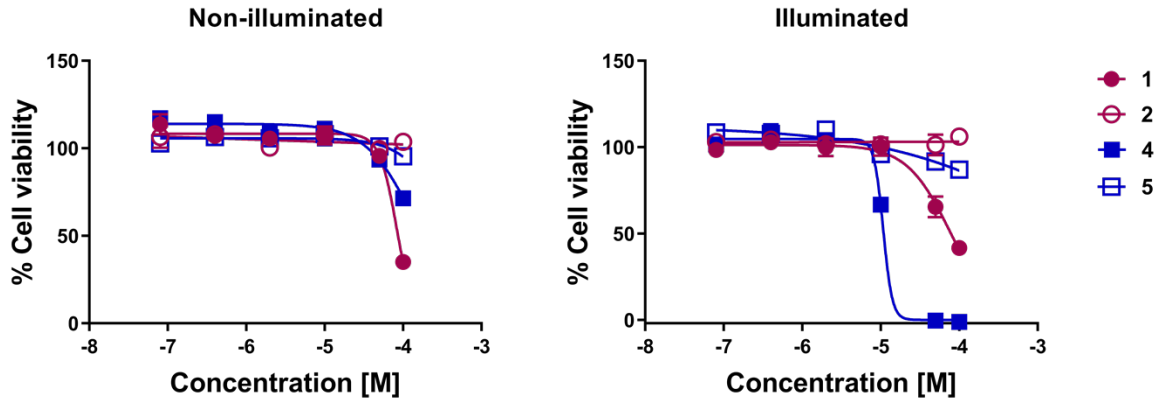

Figure S10. Plot of A549 cell viability expressed in % cell viability vs concentration of compounds. The IC_{50} values were determined using a four-parameter nonlinear regression for assays wherein at least 50% reduction in cell viability was observed. No IC_{50} value was determined for the tested compounds. Values displayed represent the average ± SEM (standard error of the mean) of three independently replicated experiments. Some data points shown in the plot have error bars that are smaller than the size of the symbol, thus, are not seen.

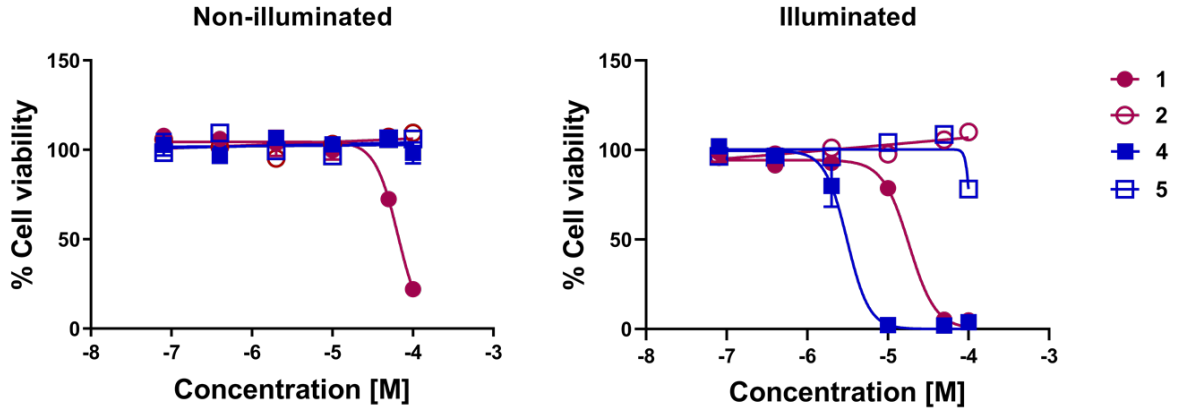

Figure S11. Plot of HFL-1 cell viability expressed in % cell viability vs concentration of compounds. The IC_{50} values were determined using a four-parameter nonlinear regression for assays wherein at least 50% reduction in cell viability was observed. No IC_{50} value was determined for the tested compounds. Values displayed represent the average ± SEM (standard error of the mean) of three independently replicated experiments. Some data points shown in the plot have error bars that are smaller than the size of the symbol, thus, are not seen.

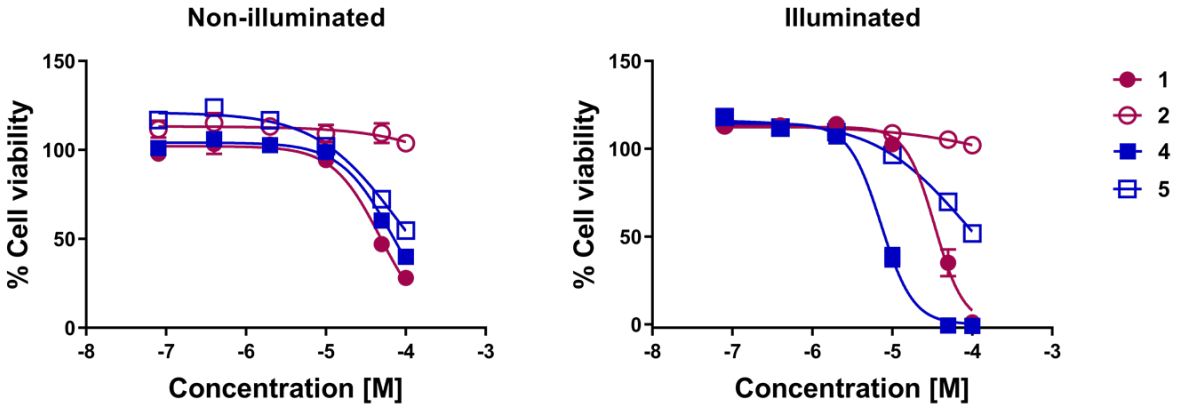

Figure S12. Plot of RAW 264.7 cell viability expressed in % cell viability vs concentration of compounds. The IC_{50} values were determined using a four-parameter nonlinear regression for assays wherein at least 50% reduction in cell viability was observed. No IC_{50} value was determined for the tested compounds. Values displayed represent the average ± SEM (standard error of the mean) of three independently replicated experiments. Some data points shown in the plot have error bars that are smaller than the size of the symbol, thus, are not seen.

Table S2. Calculated IC_{50} values for **1** and **2** in all tested cell lines determined by the MTT assay with and without exposure to light. (ND: IC_{50} above detection limit (100 μM))

Cell line	Without Light		With Light	
	1	**2**	**1**	**2**
A549	83.0±1.2	ND	77.2±3.2	ND
HFL-1	65.1±1.8	ND	18.3±1.6	ND
RAW 264.7	47.7±1.4	ND	33.9±5.4	ND

Table S3. Calculated IC_{50} values for **4** and **5** in all tested cell lines determined by the MTT assay with and without exposure to light. (ND: IC_{50} above detection limit (100 μM))

Cell line	Without Light		With Light	
	4	**5**	**4**	**5**
A549	ND	ND	10.7±1.2	ND
HFL-1	ND	ND	3.1±0.3	ND
RAW 264.7	67.2±2.0	ND	7.2±2.4	79.0±2.0

Cellular Uptake Studies of 1 and 4 using Fluorescence Microscopy. A549, RAW 264.7 or HFL-1 cells were seeded into Millicell E-Z-Slide culture chambers (EMD Millipore, Billerica, MA) at an initial density of 1.0 x 10^4 cells/cm^3 and allowed to adhere to the chamber slides for 24 hours. The cells were then treated with compound **1** or **4** (50 μM) for 4 h. Each compound was prepared as a stock solution (50 mM) in DMSO and then diluted to a final concentration of 50 μM in the culture media, reaching 0.2% of final DMSO concentration. The cells were then incubated for 15 min with nuclear stain (Hoechst 33342 dye) followed by three washes with PBS buffer to remove all residual dye. These steps were performed with minimal light exposure. Cell imaging was performed using a Zeiss Axio Observer inverted microscope (Carl Zeiss Microscopy, Thornwood, NY) equipped with fluorescence detection. Images were acquired at 20x air magnification with the following excitation and emission parameters: blue channel, λ_{ex} = 310-390 nm and a detection wavelength range λ_{em} = 420-470 nm; green channel, λ_{ex} = 450-490 nm and a detection wavelength range λ_{em} = 500-550 nm. Acquired images were processed by universal adjustment to enhance contrast levels (same settings were applied for all acquired images in each detection channel) using ZEISS ZEN 2.3 Lite software.

Concentration Dependent Fluorescence Studies of 4 at 1-10 μM: Concentration dependent studies. A549, RAW 264.7 or HFL-1 cells were seeded into Millicell E-Z-Slide culture chambers (EMD Millipore, Billerica, MA) at an initial density of 1.0×10^4 cells/cm^3 and allowed to adhere to the chamber slides for 24 hours. The cells were then treated with compound **4** (1, 5, 10 μM) for 4 h. The cells were next incubated for 15 min with nuclear stain (Hoechst 33342 dye) followed by three washes with PBS buffer to remove all the residual dye. All the steps described above were performed with minimal light exposure. Cell imaging was performed using a Zeiss Axio Observer inverted microscope (Carl Zeiss Microscopy, Thornwood, NY) equipped with fluorescence detection. Images were acquired at 20x air magnification with the following excitation and emission parameters: blue channel, λ_{ex} = 310-390 nm and a detection wavelength range λ_{em} = 420-470 nm; green channel, λ_{ex} = 450-490 nm and a detection wavelength range λ_{em} = 500-550 nm. Acquired images were processed by universal adjustment to enhance contrast levels (same settings were applied for all acquired images in each detection channel) using ZEISS ZEN 2.3 Lite software.

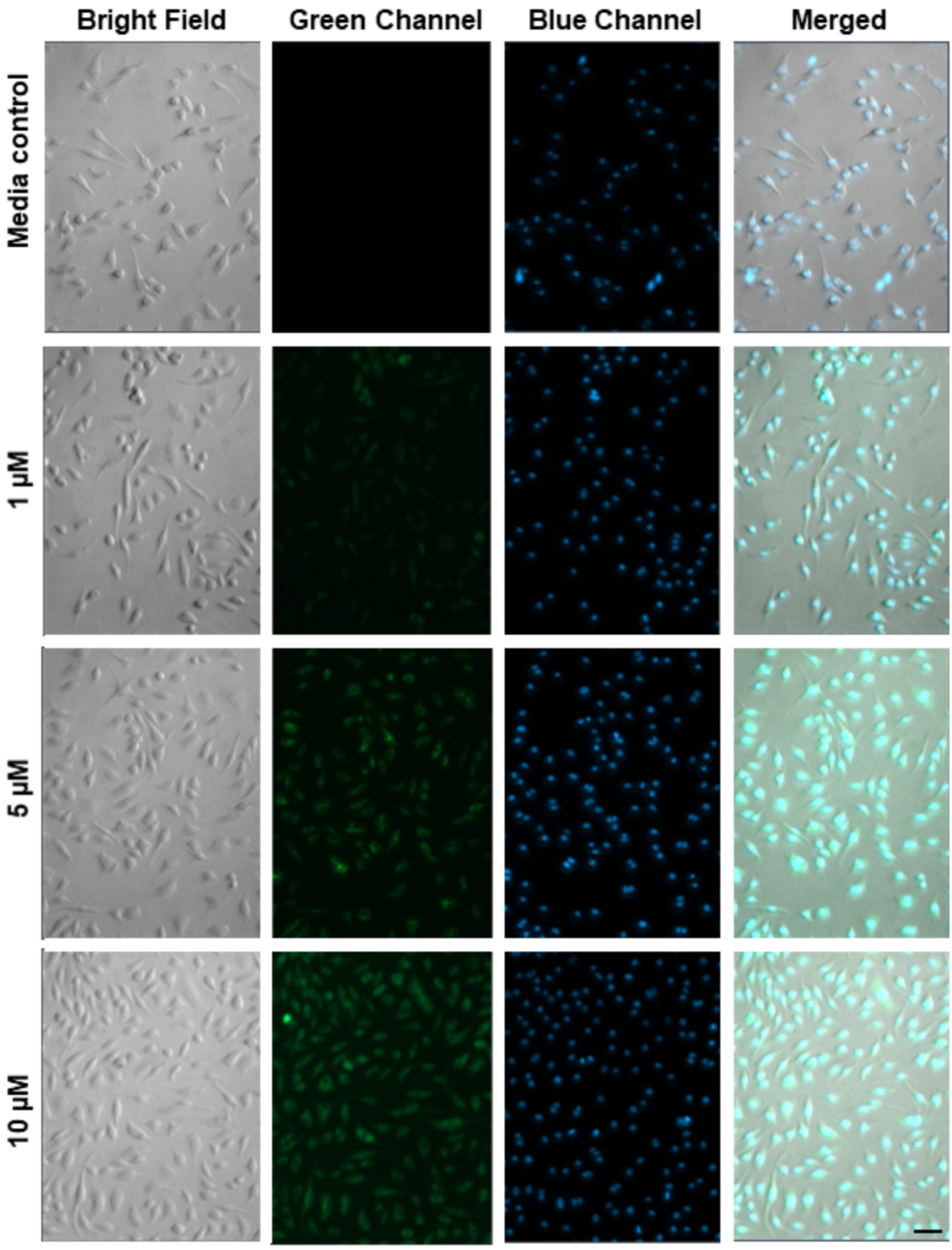

Figure S13. Fluorescence microscopy images of RAW 264.7 cells incubated for 4 h with **4** at 1, 5 and 10 µM followed by washing of the cells prior to imaging. Row 1: Media control for all experiments. The cells were co-stained with Hoechst 33342 nuclear dye (blue) to assess cell integrity. Size of bar = 40 µm.

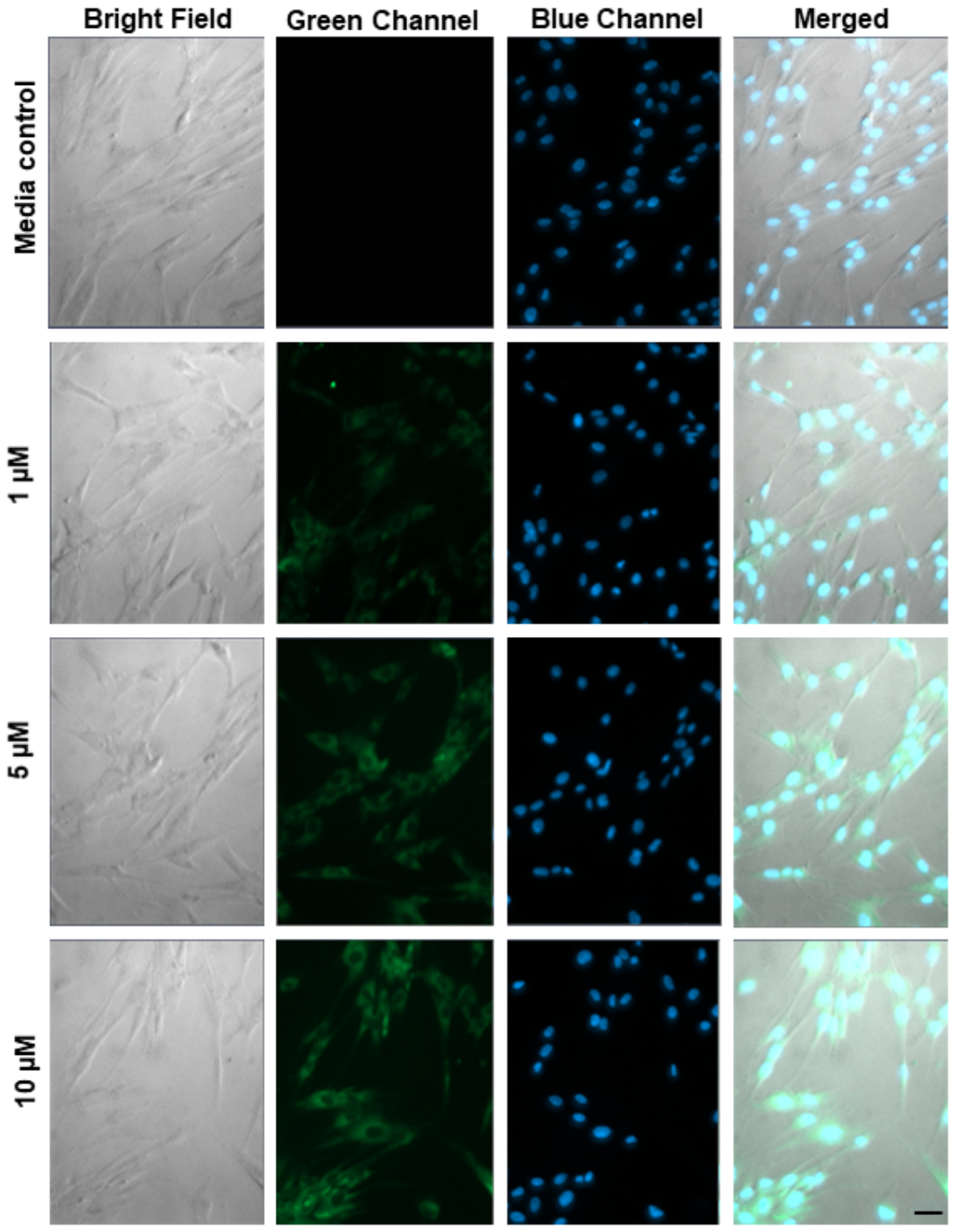

Figure S14. Fluorescence microscopy images of HFL-1 cells incubated for 4 h with **4** at 1, 5 and 10 µM followed by washing of the cells prior to imaging. Row 1: Media control for all experiments. The cells were co-stained with Hoechst 33342 nuclear dye (blue) to assess cell integrity. Size of bar = 40 µm.

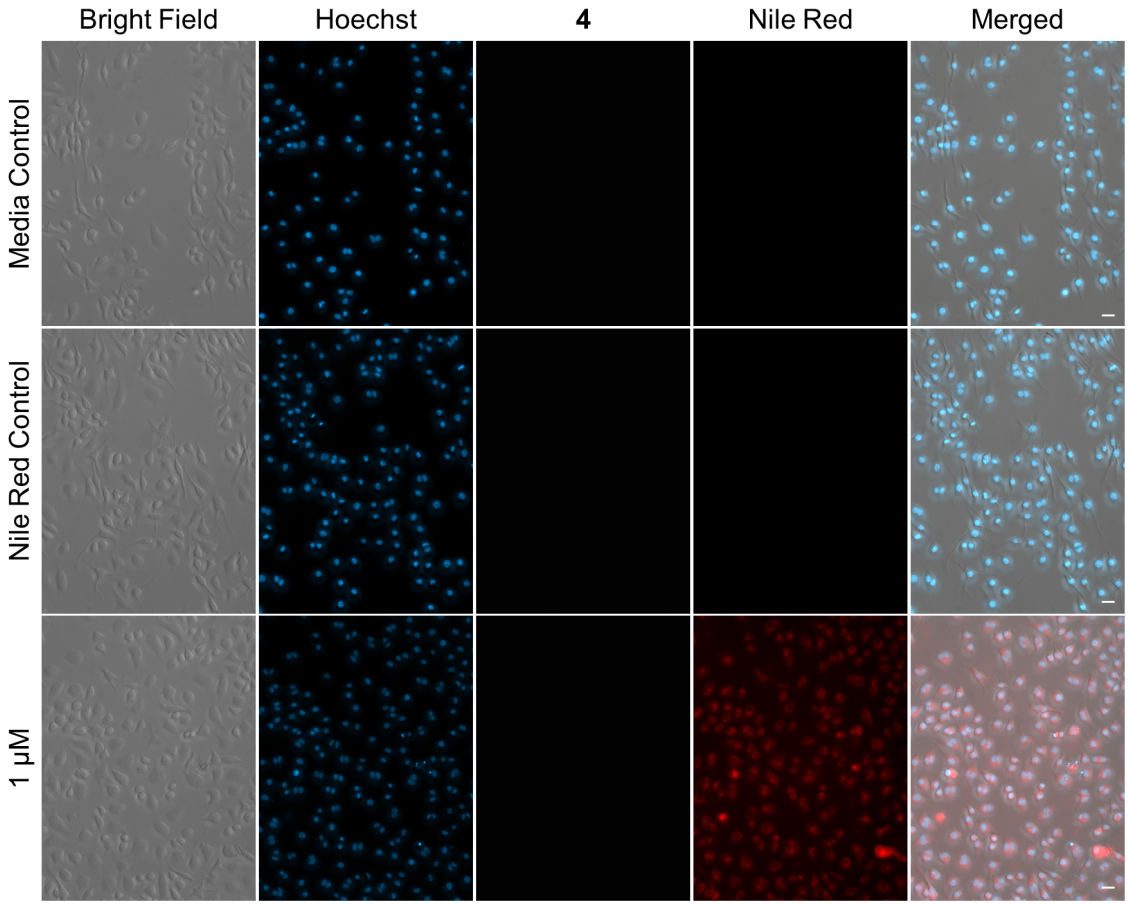

Figure S15. Fluorescence detection of CO release from **4** (1 μM) using a Nile red-based CO sensor (1-Ac) in A549 cells. Row 1: Media control. Row 2: 1-Ac control after illumination for 1 h. Row 3: Compound **4** incubated for 4 h, followed by cell washes with media, introduction of 1-Ac and illumination for 1 h. All cells were co-stained with Hoechst 33342 nuclear dye (blue channel) to assess cell integrity and illuminated with 460 nm LED array. Green channel: Detection of fluorescence emission of **4**. Red channel: Detection of CO sensor. Size of bar = 20 μm**.**

Confocal Imaging of 4. A549 cells were seeded at 200,000 cells/cm^3 in a 4-well Millicell E-Z Slides (Millipore) for 24 h of adherence. Then, 1 μM of **4** in DMSO was added for an additional 1 h. The cells were co-stained with Hoechst (0.5% v/v) for 15 min. Residual compound was washed out via three rinses with fresh media. The cells were imaged using a Zeiss LSM 710 confocal microscope using the following excitation lasers and emission ranges : **4**, λ_{ex} = 488 nm, λ_{em} = 493-570 nm; Hoechst 33342 stain, λ_{ex} = 405 nm, λ_{em} = 410-451 nm. The acquired images were universally adjusted using ZEISS ZEN Lite software v2.3.

Co-localization Studies of 4 Using ER-Tracker (ETR) in A549 Cells. A549 cells were seeded at 200,000 cells/cm^3 in a 4-well Millicell E-Z Slides (Millipore) for 24 h of adherence. Then, 25 μM of **4** in DMSO were added for an additional 1 h. The cells were co-stained with Hoechst (0.5% v/v) and ER-Tracker Red (BODIPY TR Glibenclamide) (ThermoFisher) at 300 nM each for 15 min. Residual stains were washed out three rinses using fresh media. The cells were imaged using a Zeiss LSM 710 confocal microscope using the following excitation lasers and emission ranges : **2** λ_{ex} = 488 nm, λ_{em} = 493-570 nm; Hoechst 33342 stain, λ_{ex} = 405 nm, λ_{em} = 410-451 nm; ER-Tracker, λ_{ex} = 561 nm, λ_{em} = 578-610 nm. The acquired images were universally adjusted using ZEISS ZEN Lite software v2.3.

Co-localization Studies of 4 Using Mito-Tracker (MTR) in A549 Cells. This experiment was performed as described for ER-Tracker with the exception of the co-staining dye. In this case we applied MitoTracker Deep Red (ThermoFisher) at 300 nM final concentration.

Pearson's Coefficient Determination for 4 co-localized with ER-Tracker, MTR or LTR in A549 cells. The coloc 2 plugin (open-source FIji ImageJ 1.51t) was used to determine colocalization of **4** with ETR, MTR, and LTR in A549 cells using background-subtracted (50 pixel radius) confocal image pairs with the Costes threshold regression method. Each cell was isolated as a region of interest to avoid skewing the correlation by the low-intensity background pixels. Pearson r correlation values were obtained for 27 cells from five separate fields of view obtained from three independent experiments. A map of co-localized pixels for a representative field of view was also generated and colorized by fluorescence emission intensity. Also, co-localization of **2** with ETR, MTR and LTR was verified for a subset of cells by plotting light intensity for emission channels for **4** (green), ETR, MTR or LTR (red), and Hoechst 33342 nuclear stain (blue) along a linear cross section of the cell.

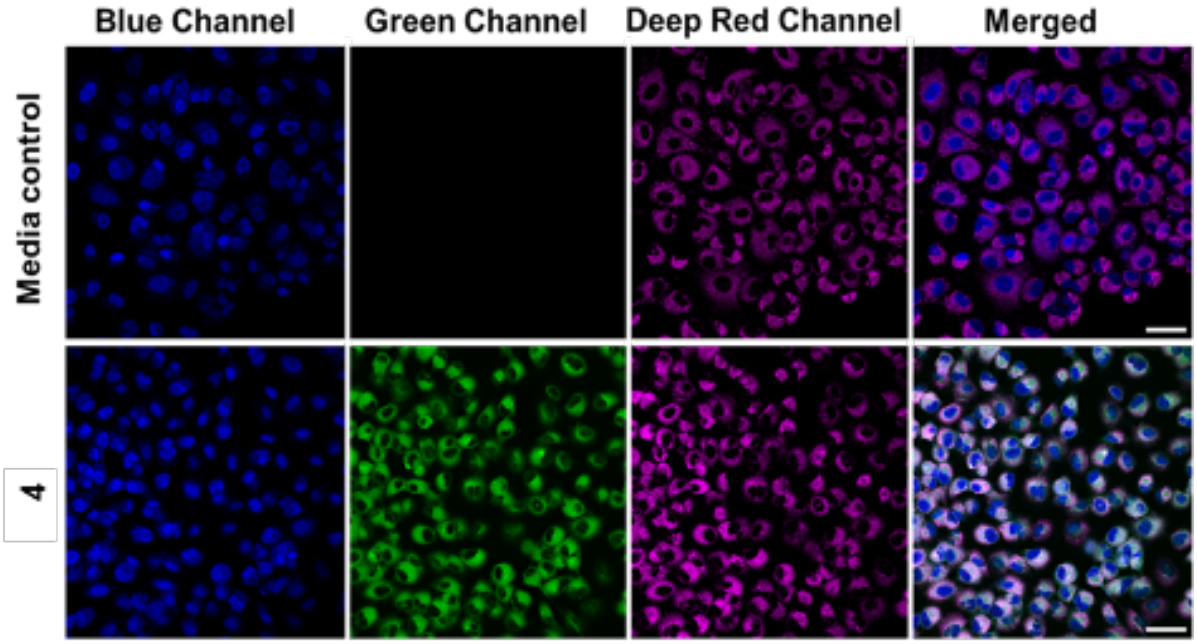

Figure S16. Co-localization of **4** with MitoTracker Deep Red in A549 cells. Cells were treated with vehicle control (0.4 % DMSO) (row 1), or **4** (25 μM, row 2) for 1 h, then counterstained with Hoechst 33342 and MitoTracker Deep Red. These representative images shown depict the Hoechst nuclear stain (blue channel), **4** (green channel), MitoTracker Deep Red (deep red channel), and a merge of the three fluorescence channels. Experiments repeated at least three times, with at least 30 cells visualized for each experiment. Scale bar indicates 50 μm.

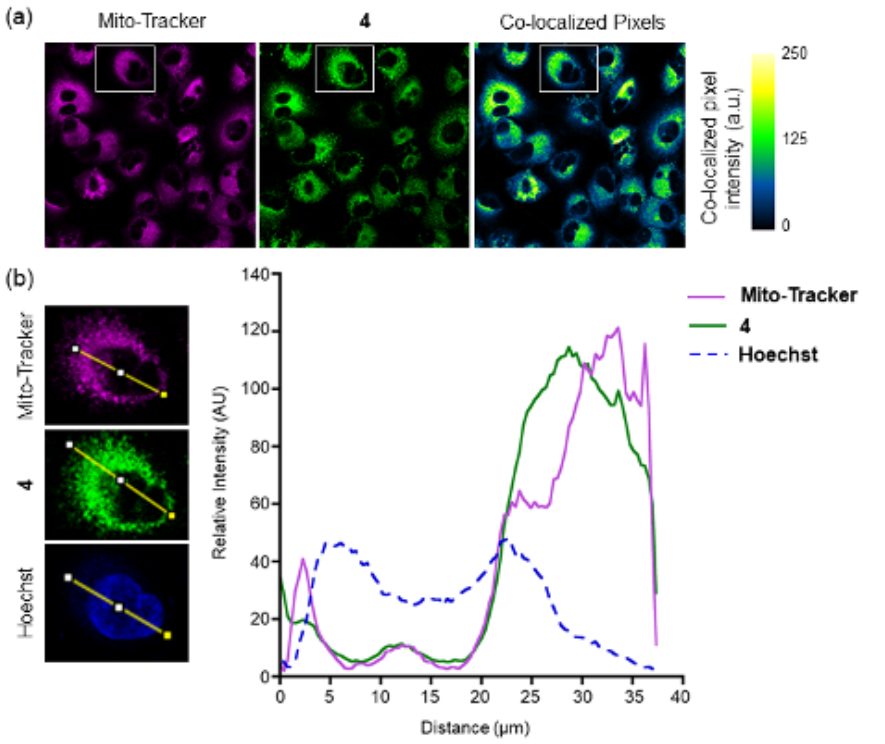

Figure S17. Confocal images of A549 cells co-stained with **4**, MitoTracker Deep Red, and Hoechst 33342. (a) Independent and colocalized pixels of **4** and MitoTracker Deep Red. (b) Overlaid intensity profile of regions of interest (ROIs) in the co-stained A549 cells as indicated by the white arrows.

Co-localization Studies of 4 Using Lyso-Tracker (LTR) in A549 Cells. This experiment was performed as described for ER-Tracker with the exception of the co-staining dye. In this case we applied LysoTracker Red DND-99 (ThermoFisher) at 300 nM final concentration.

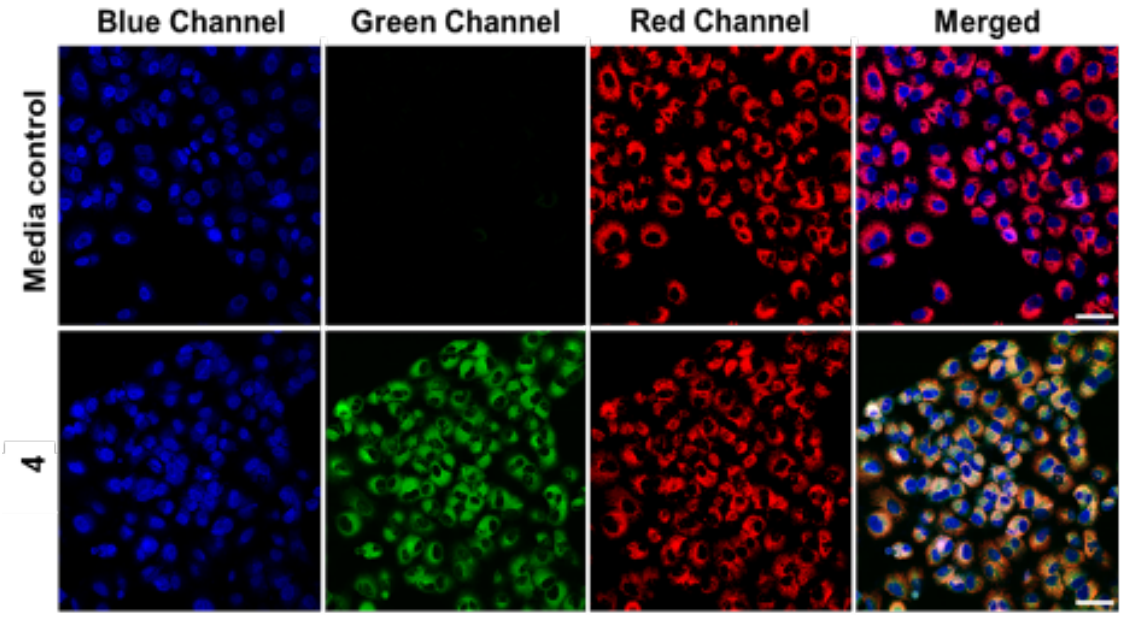

Figure S18. Co-localization of **4** with lysosomes in A549 cells. Cells were treated with vehicle control (0.4 % DMSO) (row 1), or 25 μM **4** (row 2) for 1 h, then counterstained with Hoechst 33342 and Lyso-Tracker Red. Representative images shown depict the Hoechst nuclear stain (blue), the Lyso-Tracker lysosomal stain (red) the CO donor (green) or a merge of the three fluorescence channels. Experiments repeated at least three times, and at least 30 cells were visualized for each experiment. Scale bar indicates 50 μm.

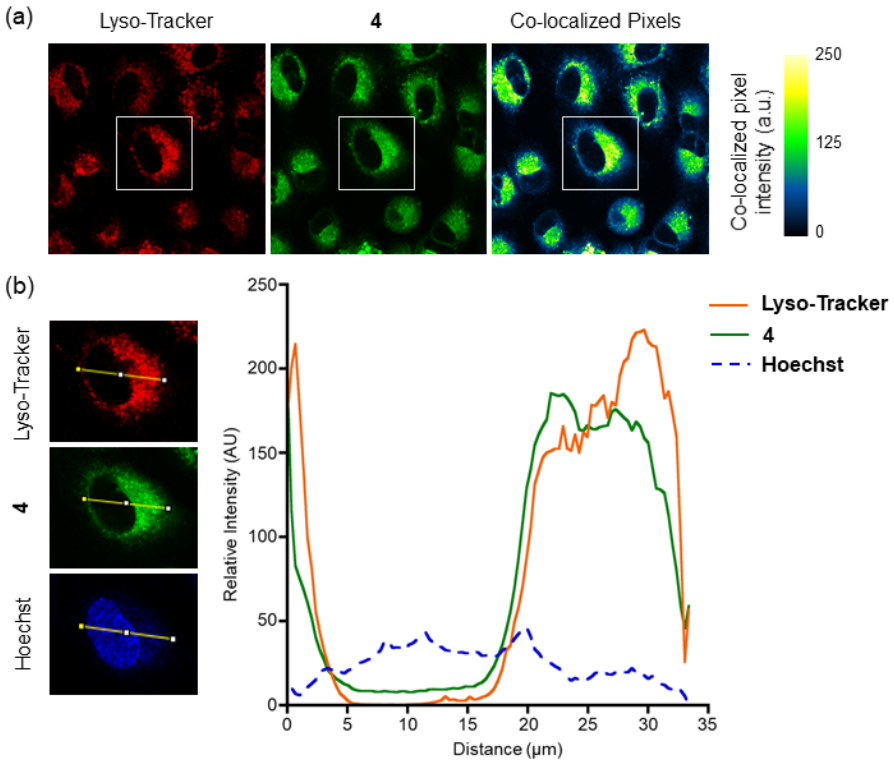

Figure S19. Confocal images of A549 cells co-stained with **4**, Lyso-Tracker, and Hoechst 33342. (a) Independent and colocalized pixels of **4** and Lyso-Tracker. (b) Overlaid intensity profile of regions of interest (ROIs) in the co-stained A549 cells as indicated by the white arrows.

TNF-α quantification: RAW 264.7 cells were seeded in 96-well plates at an initial density of 1.0 x10^4 cells/well 24 h before the experiment. On the day of the experiment, the cells were incubated with vehicle (0.4% DMSO) or **1**, **2, 4** or **5** at a concentration range of 0.04-50 μM for 4 h protected from light. One of the two prepared plates was then subjected to illumination using a blue LED array (66,351 lx, light intensity) for 1 h at 37 °C. Both plates were then incubated for 6 additional hours in the dark at 37 °C. Thereafter, lipopolysaccharides (LPS) from *E. coli* O127:B8 (Sigma Aldrich) were added to the cell culture media to a final concentration of 1 μg/mL per well and the plates were incubated for one more hour. Vehicle control wells without LPS treatment were used as the negative control whereas the ones treated with LPS were used as positive control. Cell culture supernatant (50 μL) was then collected and used for determination of TNF-α concentration in each well/treatment by using a commercial ELISA kit (TNF alpha Mouse Instant ELISATM kit (InvitrogenTM)) following the supplier protocol. The results are presented as means ± SEM (standard error of the mean) from three independent experiments (two technical replicates in each experiment for each compound). Data were analyzed by two-way ANOVA followed by Sidak's multiple comparison posthoc tests to compare effects of all treatments to the LPS positive control or to compare effects of treatment with compounds under illuminated and non-illuminated conditions. A ρ value <0.05 was considered to be statistically significant.

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