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Efficient activation of visible light-activatable CA4 prodrug through intermolecular photo-unclick chemistry in mitochondria

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1. General Experimental Section

All solvents and reagents were used as obtained from Sigma Aldrich, Thermo Fisher Scientific, and Pharmco-AAPER, unless otherwise stated. All reactions were monitored using TLC (silica gel matrix on aluminum plate, Sigma-Aldrich, cat # Z193291). Column chromatography was performed using 40-63 μm silica gel from Sorbent Technologies. NMR spectra were recorded at 25 ºC with a 300 MHz spectrometer (Varian Mercury). NMR solvents with residual solvent signals were used as internal standards. High-resolution mass spectra (HRMS) were collected using an Agilent 6538 UHD Accurate Mass QTOF (Santa Clara, CA) equipped with an electrospray ionization source in positive (and/or) negative ion mode at the Mass Spectrometry Facility at the University of Oklahoma. Analytical HPLC using HP Agilent 1100 was used for the purity evaluation. Mobile phase was pumped at a flow-rate of 0.5 or 0.6 mL/min. Bondapak C (5 μM) column (250 X 4.6 mm I.D. 12109949TS) was used for the HPLC, which was preceded by a guard column containing C/Corasil Bondpak (particle size 37−50 μM). Detection was effected at 254 nm under isocratic conditions. A 531 nm diode laser (vendor Changchun New Industries Optoelectronics Tech. Co. Ltd and cat # MGL-H-532-1W) was used to illuminate samples or cells. The power density of illumination was measured by thermal sensor (S302C, Thorlabs, Inc., Newton NJ) and a power meter (PM100D, Thorlabs, Inc.).

Synthesis. Compound **BDP**[1] and compound **3**[2] were synthesized as reported previously. The purity of the biologically evaluated compounds **Rh-L-CA4** and **Rh-L-BDP** was confirmed to be >95% by HPLC (Figs. S5 and S6).

BDP. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.44 (s, 6H), 2.56 (s, 6H), 5.98 (s, 2H), 6.95 (d, *J* = 8.1 Hz, 2H), 7.13 (d, *J* = 8.1 Hz, 2H). HRMS ESI (m/z): $[M+H]^+$ calculated for $C_{19}H_{19}BF_2N_2O$, 341.1559; found, 341.1660

Compound 4. Compound **4** was prepared according to the method described for compound **3** employing BDP (450 mg, 1.32 mmol) and propynoic acid (469 mg, 6.62 mmol), DCC (1365 mg, 6.62 mmol), DMAP (16 mg, 0.13 mmol), and dry THF (20 mL) to yield the dark red solid compound **4** (404 mg, 78%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.41 (s, 6H), 2.56 (s, 6H), 3.11 (s, 1H), 5.98 (s, 2H), 7.32 (d, *J* = 8.1 Hz, 2H). HRMS ESI (m/z): $[M+H]^+$ calculated for $C_{22}H_{19}BF_2N_2O_2$, 393.1508; found, 393.1740.

Compound 1. RhB (741 mg, 1.55 mmol) and DCC (638 mg, 3.09 mmol) in CH_2Cl_2 (12 mL) was stirred at 0^{o}C for 30 min under argon atmosphere. To the solution, tert-butyl 4-(hydroxymethyl)piperidine-1-carboxylate (500 mg, 2.32 mmol) was added. The mixture was then stirred at room temperature for 24 h. The reaction mixture was dissolved with water and extracted with diluted with CH_2Cl_2 (30 mL). The combined organic layer was dried over anhydrous Na_2SO_4, filtered, and the solvents were removed by evaporation. The crude product was purified by column chromatography using DCM–methanol (9:1) to give compound **1** as a red solid (774 mg, 74%). ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.85 (s, 3H), 1.25 (s, 4H), 3.33 (s, 6H), 1.44 (s, 9H), 1.61 (m, 6H), 2.58 (m, 2H), 3.65 (d, *J* = 6.1 Hz, 1H), 3.89 (m, 4H), 4.01 (m, 4H), 4.06 (d, *J* = 6.0 Hz, 2H), 5.29 (s, 2H), 6.84 (s, 1H), 6.87 (s, 1H), 6.99 (s, 1H), 7.03 (s, 1H), 7.26 (d, *J* =7.5 Hz, 1H), 7.65-7.79 (m, 2H), 8.22 (d, *J* = 7.8 Hz, 1H). MS ESI (m/z): Calculated for $C_{39}H_{50}ClN_3O_5$ ($[M-Cl]^+$): 640.3745; found: 640.3701.

Compound 2. Compound **1** (600 mg, 0.94 mmol) was dissolved in dry dichloromethane (10 mL). After trifluoroacetic acid (0.46 mL) was added to the solution at 0˚C, it was stirred under

nitrogen for 1 h. The reaction mixture was then concentrated under a vacuum and was immediately used in the next step.

Rh-L-CA4. Compound **2** (69 mg, 0.14 mmol) and compound **3** (50 mg, 0.14 mmol) were dissolved in dry DCM (30 mL), and the solution was stirred at room temperature for 1 h. The solvent was removed under reduced pressure to give the crude product, which was then purified by column chromatography using DCM:methanol (9:1) as an eluent to give **Rh-L-CA4** (93 mg, 81%). ^{1}H NMR (300 MHz, CD_2Cl_2) δ 1.32 (t, *J* = 7.2 Hz, 9H), 2.79 (br s, 3H), 3.12 (br s, 4H), 3.64 (m, 6H), 3.67 (s, 6H), 3.74 (s, 3H), 3.78 (s, 3H), 3.92 (d, *J* = 5.7 Hz, 1H), 4.75 (d, *J* = 12.9 Hz, 1H), 6.53 (s 2H), 6.83 (m, 2H), 6.87 (m, 2H), 6.99 (d, *J* = 2.4 Hz, 1H), 7.10 (m, 1H), 7.13 (m, 1H), 7.33 (m, 1H), 7.43 (d, *J* = 12.9 Hz, 1H), 7.82 (m, 2H), 8.34 (m, 1H). HRMS ESI (m/z): Calculated for $C_{55}H_{62}N_3O_9S_iCl$ ($[M-Cl]^+$): 908.4481; found: 908.4241.

Rh-L-BDP. Compound **2** (84 mg, 0.16 mmol) and compound **4** (65 mg, 0.16 mmol) were dissolved in dry DCM (30 mL), and the solution was stirred at room temperature for 1 h. The solvent was removed under reduced pressure to give the crude product, which was then purified by column chromatography using DCM:methanol (9:1) as an eluent to give **Rh-L-BDP** (126 mg, 84%). ^{1}H NMR (300 MHz, CD_2Cl_2) δ 1.61 (m, 9H), 1.66 (s, 6H), 1.70 (m, 4H), 2.55 (s, 2H), 3.65 (m, 10H), 3.95 (m, 2H), 4.78 (d, *J* = 12.6 Hz, 1H), 5.98 (s, 1H), 6.87 (s, 2H), 6.95 (m, 2H), 7.10 (m, 2H), 7.30 (m, 5H), 7.53 (d, *J* = 12.7 Hz, 1H), 7.79 (m, 2H), 8.28 (m, 1H). HRMS ESI (m/z): Calculated for $C_{56}H_{61}BClF_2N_5O_5S_i$ ($[M+H]^+$): 932.4585; found: 932.4728.

Mp-L-CA4. To a stirred solution of **3** (20.0 mg, 0.054 mmol) in THF (3 mL), morpholine (7.09 mg, 0.081 mmol) diluted in THF (0.5 mL) was added at 0 °C under N_2 gas. The reaction mixture was stirred for 45 min at room temperature. After that, the reaction mixture was concentrated to obtain an off-white solid, which was purified by silica gel column chromatography using ethyl

acetate and hexane (E : H = 1 : 1 v/v) as eluents to get 22 mg (0.048 mmol, 89%) of compound **2** as an off-white solid.

^{1}H NMR (CD_2Cl_2, 400 MH_Z) δ 7.47 (d, J = 12.8 Hz, 1H), 7.11 (dd, J = 8.4, 1.6 Hz, 1H), 6.97 (d, J = 1.6 Hz, 1H), 6.85 (d, J = 8.4 Hz, 1H), 6.52 (s, 2H), 6.47 (d, J = 12.0 Hz, 1H), 7.43 (d, J = 12.0 Hz, 1H), 4.83 (d, J = 12.8 Hz, 1H), 3.78 (s, 3H), 3.74 (s, 3H), 3.70 (t, J = 4.8 Hz, 4H), 3.67 (s, 6H), 3.27 (t, J = 4.8 Hz, 4H). HRMS ESI (m/z): Calculated for $[C_{25}H_{30}NO_7]^+$: 456.2022 $[M+H]^+$; found: 456.2021.

Dark- and photo-toxicity. Phototoxicity and dark-toxicity of a fixed concentration of **HAL** plus various concentrations of **Rh-L-CA4,** and individual fixed concentrations (0.5 mM) of **HAL** were determined, with or without illumination. AY-27 cells were maintained in minimum essential medium (α-MEM) supplemented with 10% bovine growth serum, 2 mM L-glutamine, 50 units/mL penicillin G, 50 μg/mL streptomycin, and 1.0 μg/mL fungizone. AY-27 cells (10,000 cells/well) were seeded on 96-well plates in complete medium (200 μL) and were then incubated for 24 h at 37 °C in 5% CO_2. **Rh-L-CA4** stock solution (2 mM) was prepared in DMSO. The stock solution was further diluted with complete medium to obtain appropriate final concentrations. **HAL** (1 mM) was prepared in the complete medium. The medium in each well (200 μL) was replaced with the **HAL** solution (100 μL) and the diluted **Rh-L-CA4** (100 μL). The plates were incubated for 2 h and were then removed from the incubator. The medium in each well was removed, and fresh complete medium (200 μL) was added to each well. *For the phototoxicity study*: The plate, without a cover, was placed on an orbital shaker (Lab-line, Barnstead International) and was illuminated with a diode laser (531 nm, 10 mW/cm^2) for 30 min. To ensure the uniformity of the light during the illumination, each plate was shaken gently on an orbital shaker. *For the dark toxicity study:* Plates were kept in the dark for 30 min, and

were then returned to the incubator. After 24 h, cell viability was determined with the MTT assay.[3] Briefly, a 10-μL solution of MTT (10 mg in 1 mL PBS buffer) was added to each well, and the plate was incubated for 4 h. Then, the MTT solution was removed and the cells were dissolved in 200 μL of DMSO. The absorbance of each well was measured at 570 nm, with background subtraction at 650 nm. The cell viability was then quantified by measuring the absorbance of the treated wells, compared with that of the untreated wells (controls) and expressed as a percentage.

Procedure for monitoring the cleavage of the linker (BDP-L-Rh) by FRET. Stock solutions of PpIX (2 mM) and **BDP-L-Rh** (2 mM) were prepared in DMSO. The stock solutions (10 μL) were then diluted with Dulbecco's Modified Eagle Medium **(**1990 μL) with 5% fetal bovine serum to give 10 μM of PpIX and **BDP-L-Rh** solutions, respectively. The diluted PpIX and **BDP-L-Rh** solutions (1 mL each) were taken and combined. The resulting solution was illuminated with a diode laser (531 nm, 10 mW/cm^2). Forty μL was taken at time interval and diluted with 3960 μL methanol. The solution was excited at 470 nm and the fluorescence measured from 490 nm to 700 nm.

Cleavage of Rh-L-BDP in cells. AY-27 cells were maintained in minimum essential medium (α-MEM) supplemented with 10% bovine growth serum, 2 mM L-glutamine, 50 units/mL penicillin G, 50 μg/mL streptomycin, and 1.0 μg/mL fungizone. AY-27 cells (100,000 cells/well) were seeded on 24-well plates in the medium and were then incubated for 24 h at 37°C in 5% CO_2. HAL (500 μL, 1 mM) and Rh-L-BDP (500 μL, 2 μM) were prepared in the complete medium and were added to each well. The plates were then incubated for 2 h, after which they were removed from the incubator. The medium was removed from the wells. The plate, without a cover, was placed on an orbital shaker (Lab-line, Barnstead International) and

illuminated with a diode laser (531 nm, 10 mW/cm^2) for 30 min. After the illumination, the cells were digested with DMSO (200 μL). Half of the cell lysate (100 μL) was diluted with methanol (3900 μL). The solution was excited at 470 nm and the fluorescence was measured from 490 nm to 700 nm. To determine the BDP fluorescence intensity at 100% cleavage of Rh-L-BDP in the cell lysate, 1 μL of 2 mM PpIX in DMSO was added to the well containing medium (200 μL) after the illumination, and the well was again illuminated for 30 min.

Tubulin Polymerization Assay. The fluorescence-based tubulin polymerization was determined using a kit supplied by Cytoskeleton, Inc. (cat # BK011P). The basic principle is that fluorescence increases as a fluorescence reporter is incorporated into microtubules during the course of polymerization. The assay was performed following the experimental procedure as reported previously.[4]

Procedure for understanding solvents' effects on the fluorescence of Rh-L-BDP. Stock solutions of Rh-L-BDP (2 mM in DMSO) were diluted as follows: A 10-μL aliquot of stock was diluted with 4 mL of complete RPMI 1640 media. Then, a 10-μL aliquot was taken from this solution and was further diluted with 1 mL of the complete medium. To obtain fluorescence spectra, 200 μL of this solution was diluted with 4 mL of medium. For the chloroform and methanol solutions, the same procedure was followed, with the respective solvents. The excitation wavelength was 470 nm with a slit width of 5 nm; emission measured from 490 to 700 nm (Fig. S13).

Procedure for understanding the fluorescence spectra of various concentrations of Rh-L-BDP. First, stock solutions of Rh-L-BDP (2 mM) were prepared in DMSO. A 10-μL aliquot of stock was diluted with 4 mL of methanol to give a concentration of 5 μM. Fluorescence spectra were obtained from a concentration from 5 μM to 2.5 nM and from 1 nM to 0.1 nM (Fig. S14).

Procedure for identifying the source of the middle peak in the lower graph in Fig. S11. To understand the peak at 540 nm, we obtained fluorescence spectra of neat solvents (Fig. S15). The DMSO and methanol mixture was prepared in the same way as previously described, without any Rh-L-BDP dissolved. No solvent indicates an empty cuvette. The peak was detected from the neat solvents.

2. NMR Spectral Data

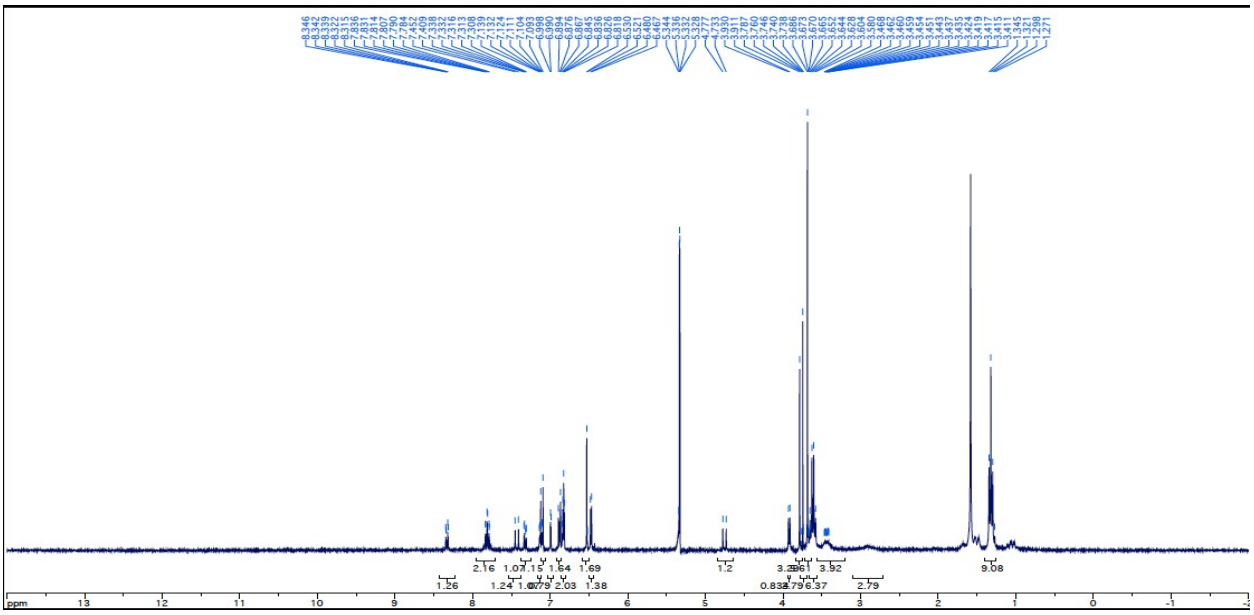


Figure S1. ^{1}H-NMR spectrum (300 MHz) of **Rh-L-CA4** in CD_2Cl_2.

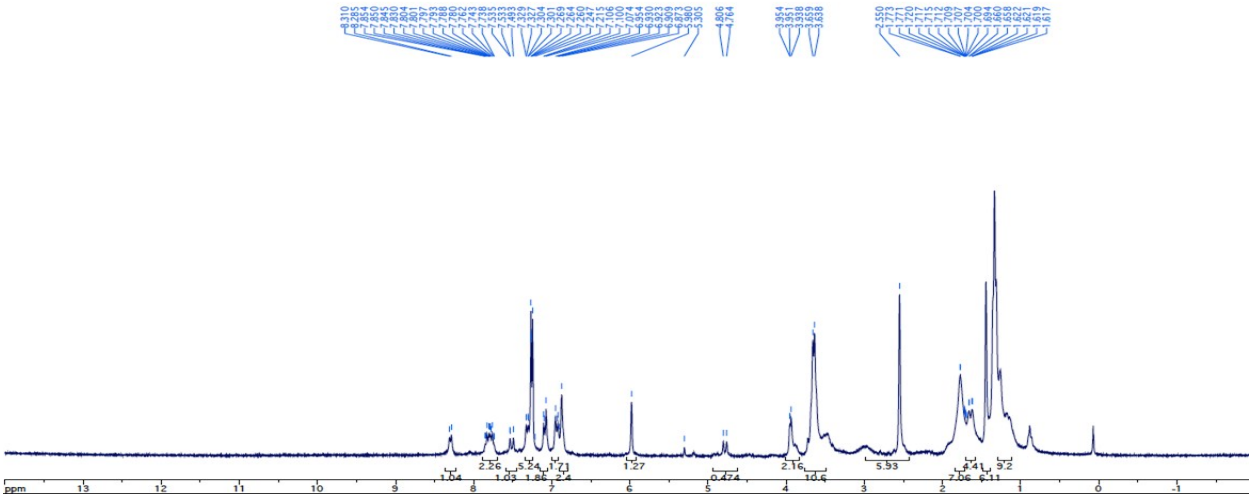


Figure S2. ^{1}H-NMR spectrum (300 MHz) of **Rh-L-BDP** in CD_2Cl_2.

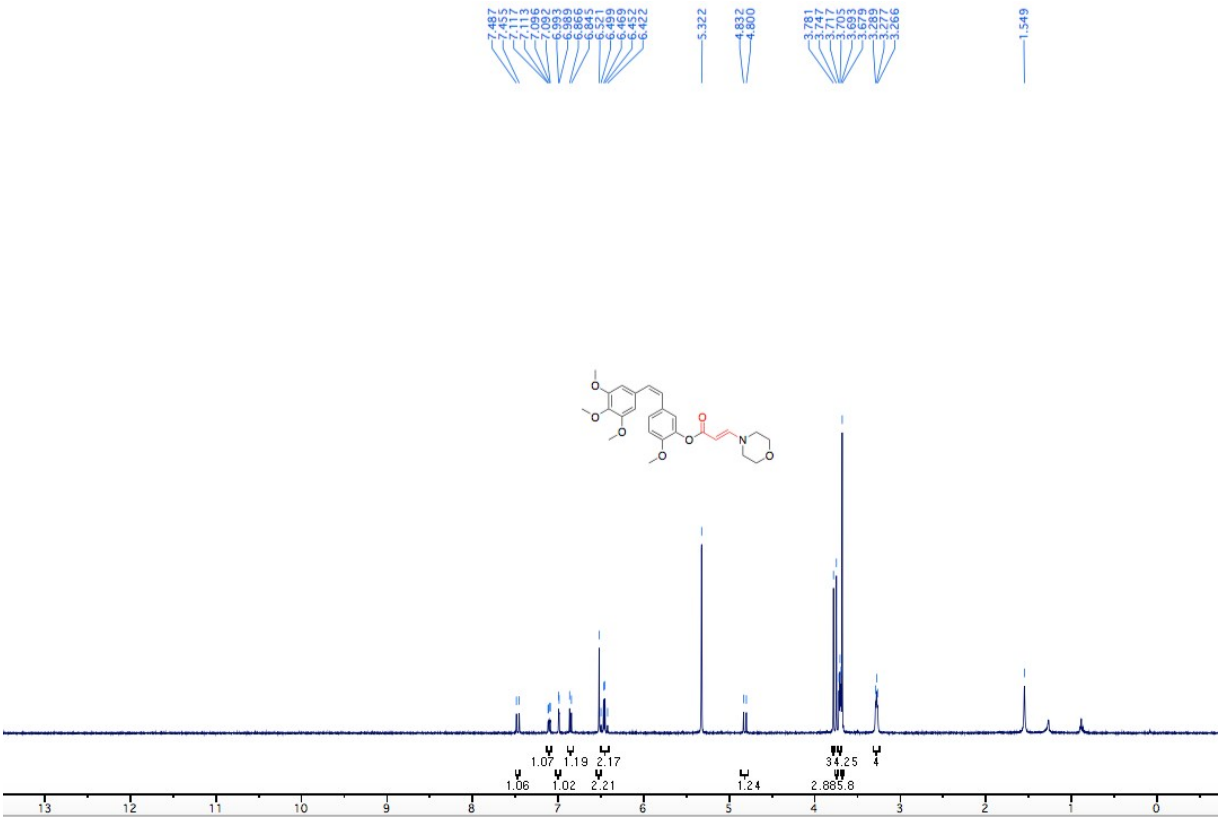


Figure S3. ^{1}H-NMR spectrum (300 MHz) of **Mp-L-CA4** in CD_2Cl_2.

3. Mass Spectra of BDP, Rh-L-CA4, Rh-L-BDP, and Mp-L-CA4

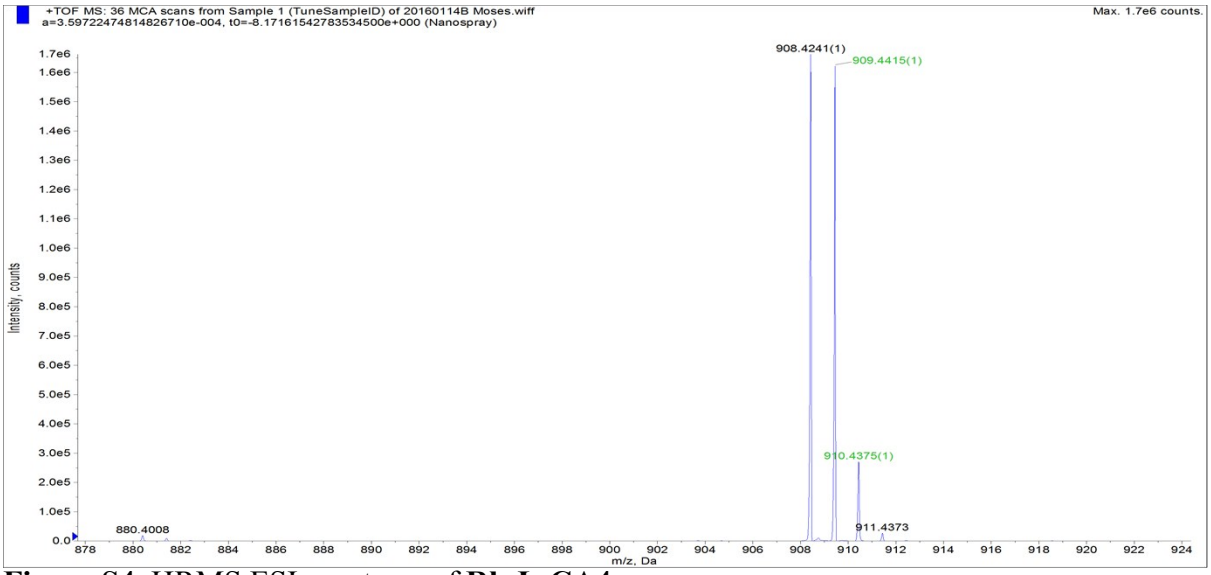


Figure S4. HRMS ESI spectrum of **Rh-L-CA4.**

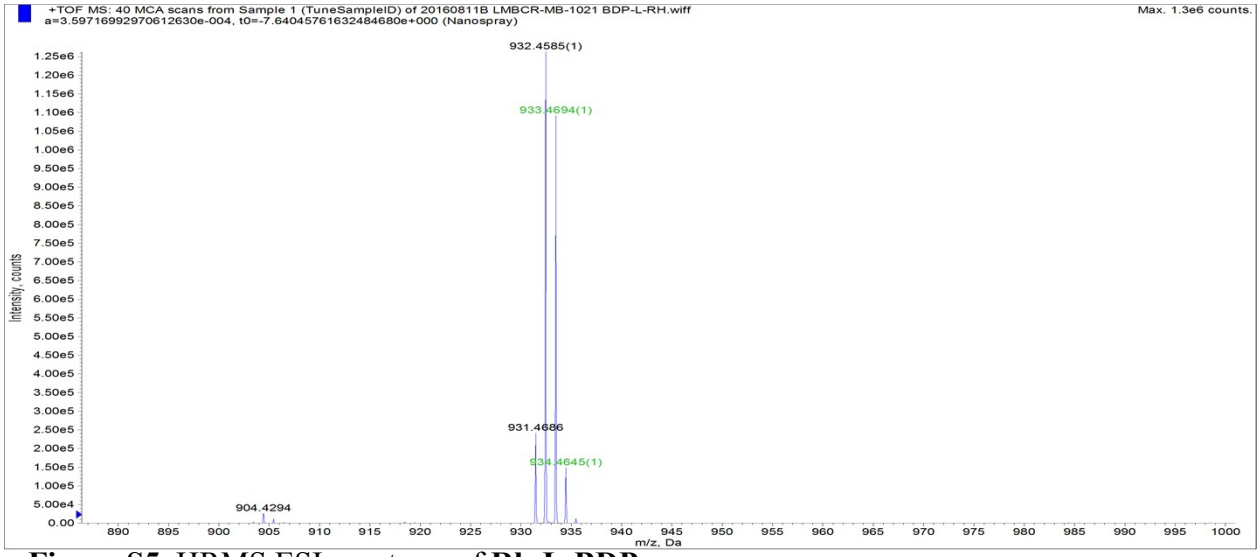


Figure S5. HRMS ESI spectrum of **Rh-L-BDP**.

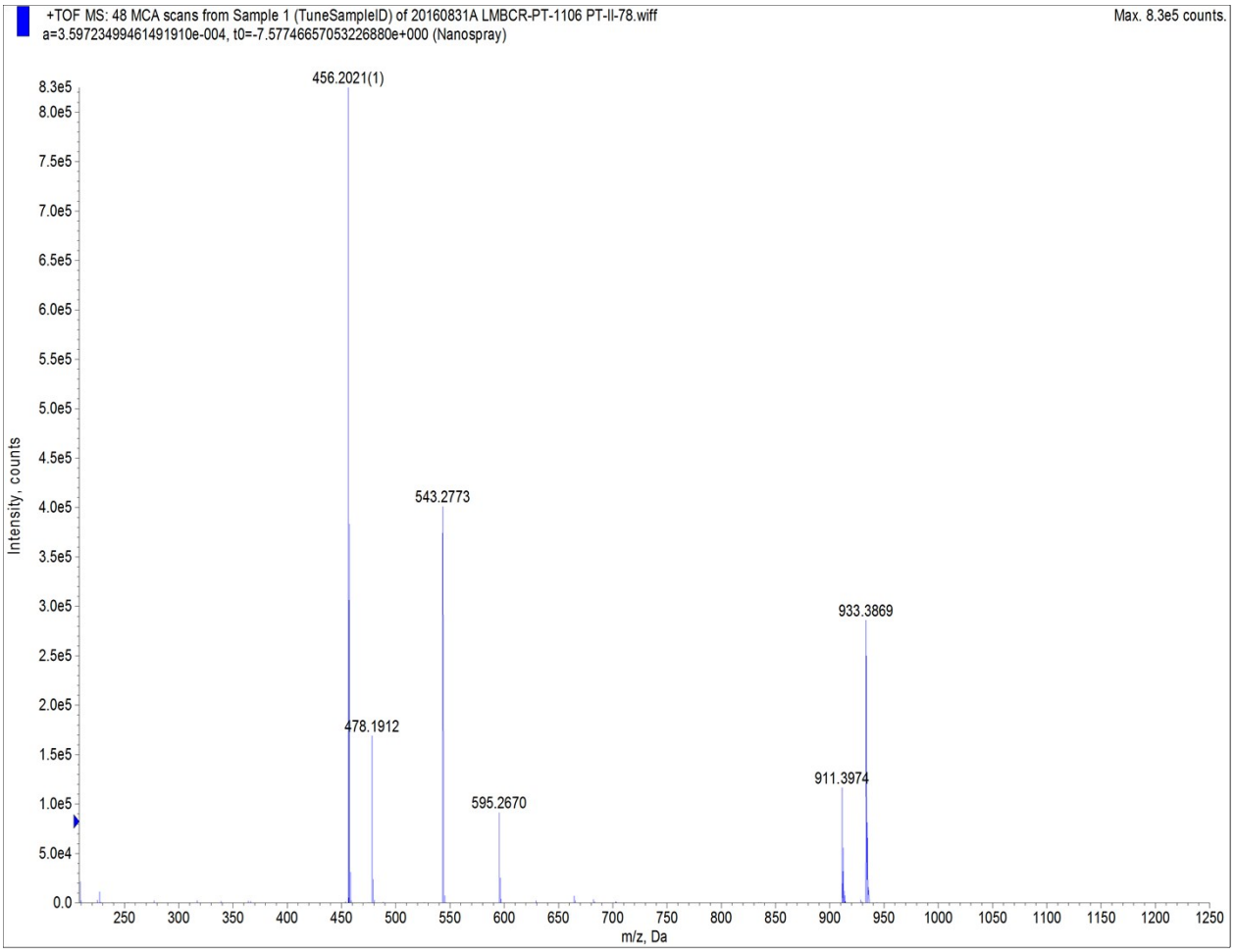


Figure S6. HRMS ESI spectrum of **Mp-L-CA4.**

4. HPLC Chromatograms of Rh-L-CA4, Rh-L-BDP, and Mp-L-CA4.

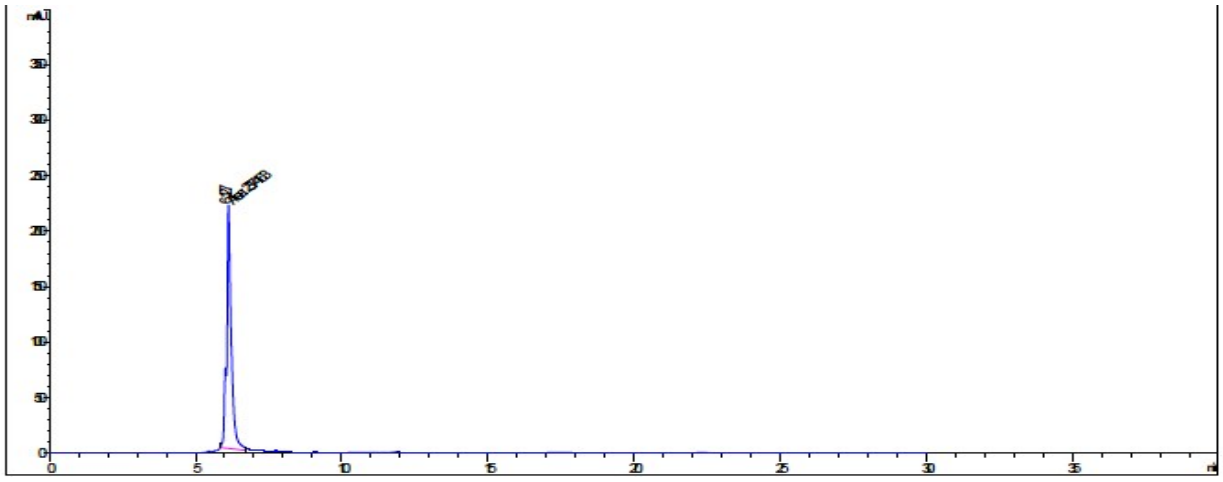


Figure S7. HPLC chromatogram of **Rh-L-CA4**: mobile phase = 95% acetonitrile: 5% methanol flow rate = 0.5 mL/min, detection at 254 nm = tR (retention time) = 6.13 min; purity = 98%.

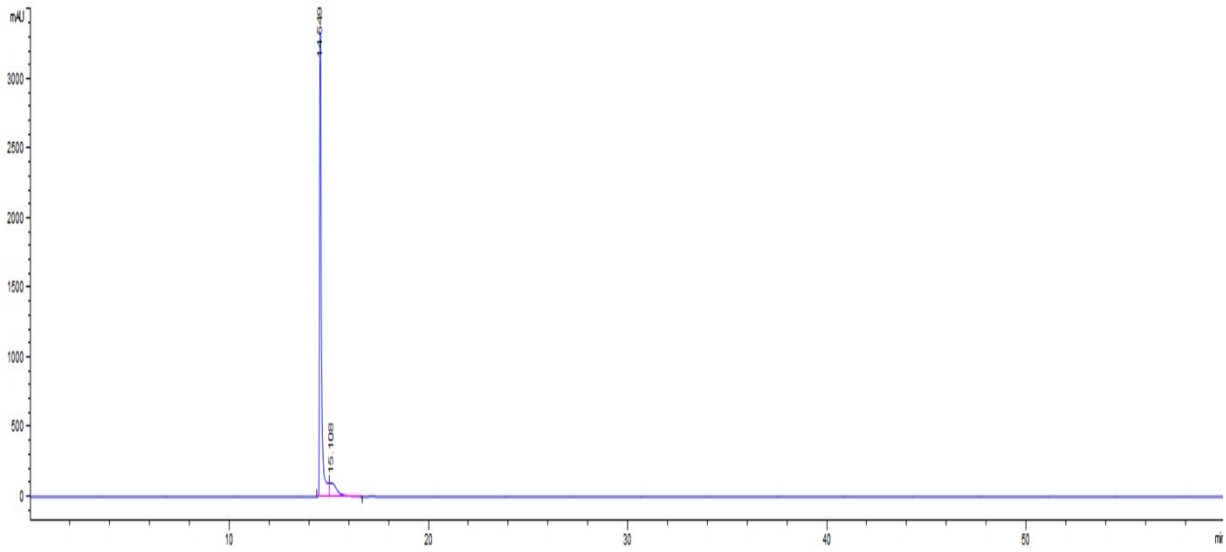


Figure S8. HPLC chromatogram of **Rh-L-BDP**: mobile phase = 95% acetonitrile: 5% methanol flow rate = 0.5 mL/min, detection at 254 nm = tR (retention time) = 14.64 min; purity = 97%.

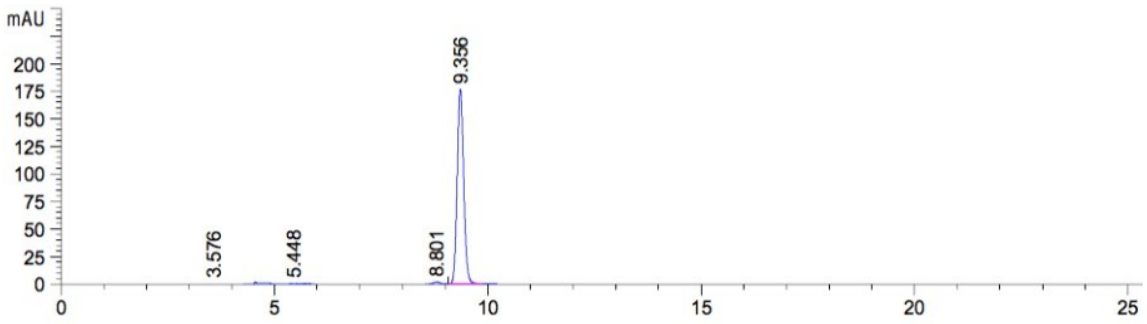


Figure S9. HPLC chromatogram of **Mp-L-CA4**: mobile phase = 70% acetonitrile: 30% dH_2O flow rate = 0.5 mL/min, detection at 254 nm = tR (retention time) = 9.36 min; purity = 97%.

5. UV-vis and Fluorescence Spectra of a Mixture of RhB and BDP, and Rh-L-BDP

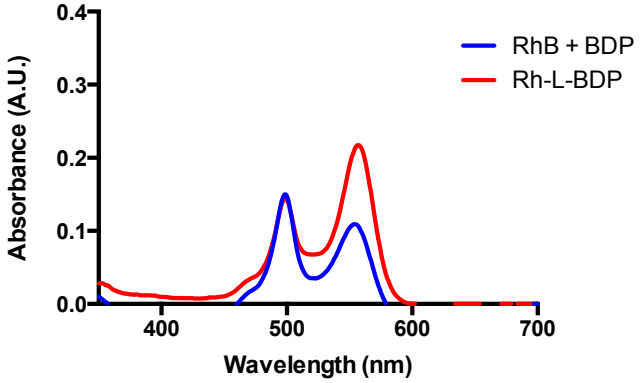


Figure S10. UV-vis spectra of an equimolar mixture of RhB + DBP, and Rh-L-BDP (2 μM) in methanol.

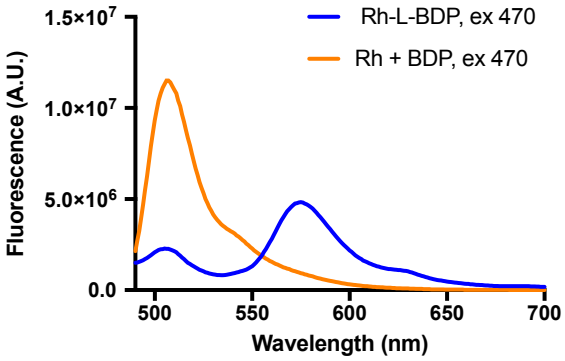


Figure S11. Fluorescence spectra of an equimolar mixture of RhB + BDP, and Rh-L-BDP (50 nM) in methanol: excitation at 470 nm. In the Rh-L-BDP spectrum, the BDP peak was smaller and the Rh peak was much larger than that of the mixture, demonstrating efficient FRET from BDP to Rh.

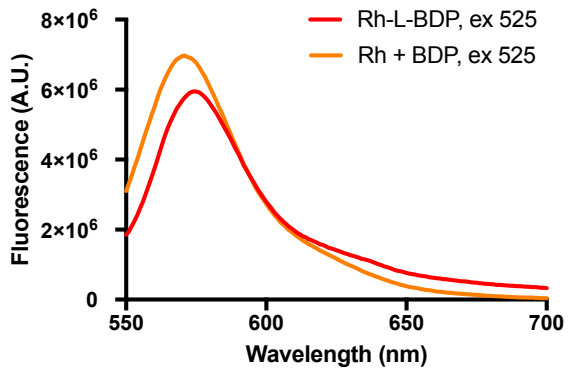


Figure S12. Fluorescence spectra of an equimolar mixture of RhB + BDP and Rh-L-BDP (50 nM) in methanol: excitation at 525 nm. Rh peaks of the mixture and Rh-L-BDP were similar, meaning no significant energy transfer via FRET from Rh to BDP.

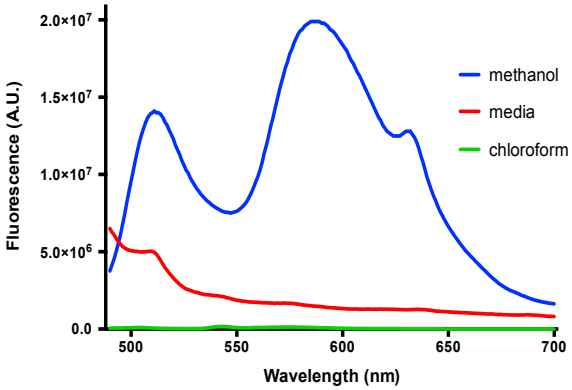
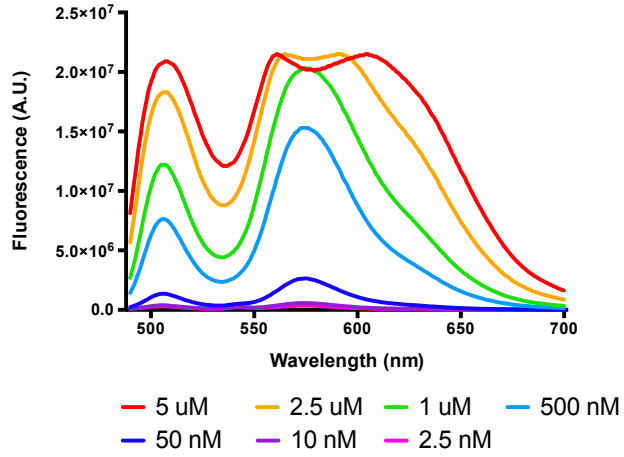


Figure S13. Fluorescence spectra of Rh-L-BDP in three different solvents: excitation at 470 nm. Methanol showed the greatest fluorescence emission among the three solvents.

A)



B)

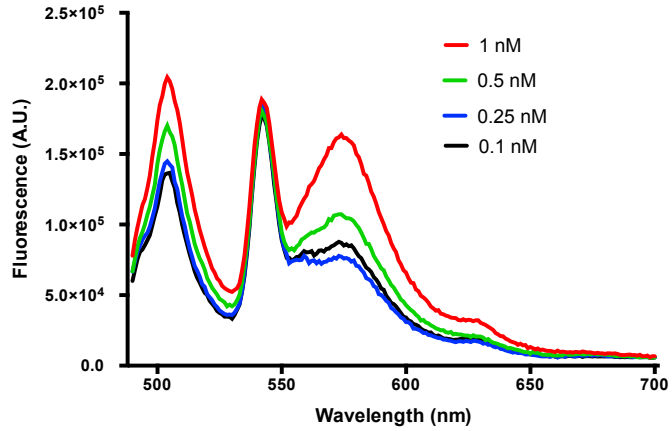


Figure S14. Fluorescence spectra of various concentrations of Rh-L-BDP in methanol: excitation at 470 nm: A) 2.5 nM-5 μM and B) 0.1 nM-1 nM. The middle peak in the lower spectra came from solvents, as shown in Fig. S15.

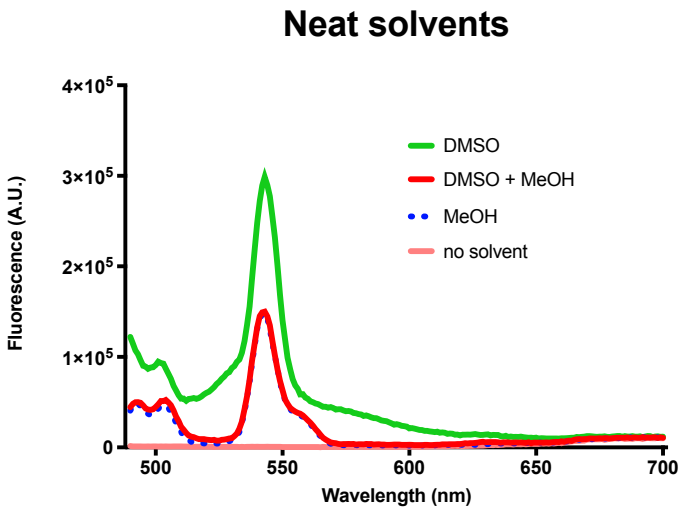


Figure S15. Fluorescence spectra of blank solvents: excitation at 470 nm. Fluorescence spectra of MeOH and a mixture of DMSO and MeOH overlapped.

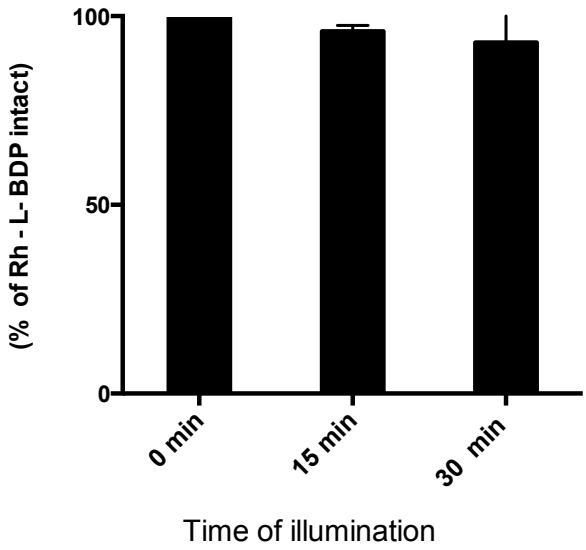


Figure S16. Stability of Rh-L-BDP in cells with illumination (531 nm at 10 mW/cm^2). Rh-L-BDP was stable in the cells, even with illumination for 30 min.

6. Dark and Phototoxicity Data *In Vitro*.

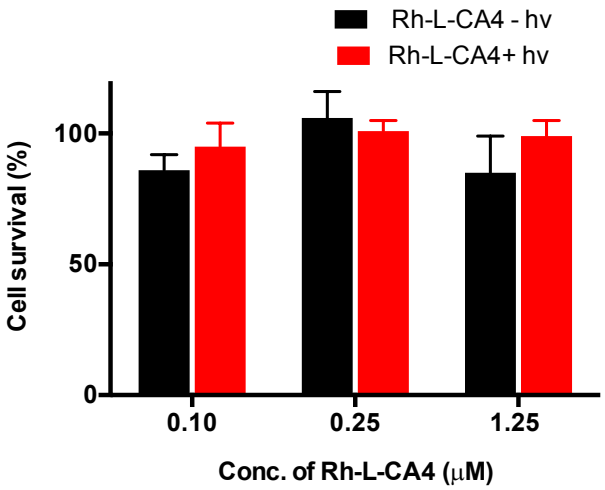


Figure S17. Dark and Photo-toxicity of Rh-L-CA4 alone (without HAL). Rh-L-CA4 alone did not show any meaningful cell kill both with or without illumination (531 nm at 10 mW/cm^2).

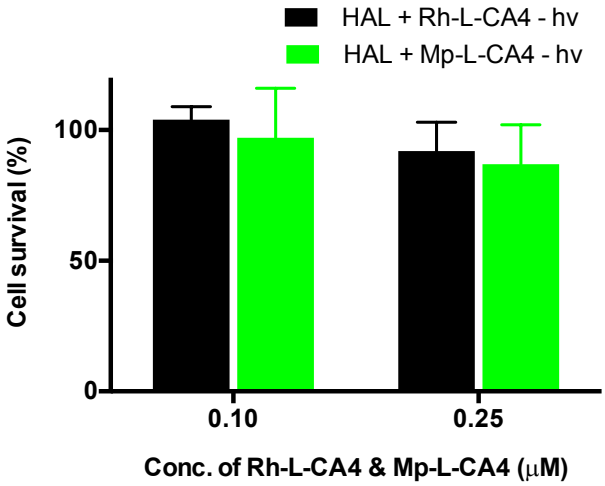


Figure S18. Dark toxicity of [HAL + Rh-L-CA4] vs. [HAL + Mp-L-CA4]. Without illumination, neither [HAL (0.5 mM) + Rh-L-CA4] nor [HAL (0.5 mM) + Mp-L-CA4] showed significant dark toxicity at 0.1 and 0.25 prodrug concentrations.

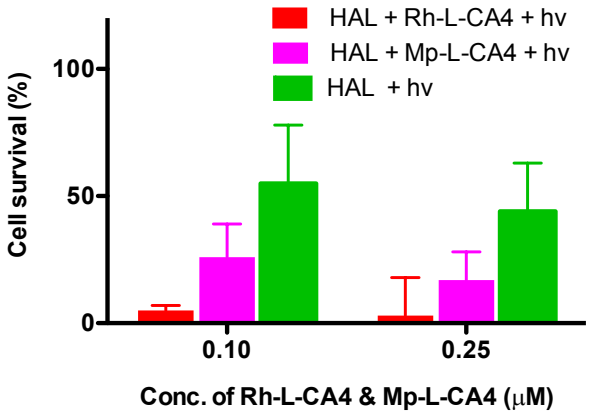


Figure S19. Phototoxicity of [HAL + Rh-L-CA4], [HAL + Mp-L-CA4], and [HAL (0.5 mM)]. With illumination (531 nm at 10 mW/cm^2), both [HAL (0.5 mM) + Rh-L-CA4] and [HAL (0.5 mM) + Mp-L-CA4] showed large dark toxicity at 0.1 and 0.25 prodrug concentrations. Rh-L-CA4 showed higher cell kill than Mp-L-CA4.

References

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(2) Hossion, A. M. L.; Bio, M.; Nkepang, G.; Awuah, S. G.; You, Y. *ACS Med. Chem. Lett.,* **2013**, *4*, 124.
(3) Mosmann, T. *J. Immunol. Methods* **1983**, *65*, 55.
(4) Bio, M.; Rajaputra, P.; Nkepang, G.; Awuah, S. G.; Hossion, A. M. L.; You, Y. *J. Med. Chem.,* **2013**, *56*, 3936.