Supporting Information for

"Visualization of Vesicular Transport from the Endoplasmic Reticulum to Lysosome using an Amidine Derived Two-Photon Probe"

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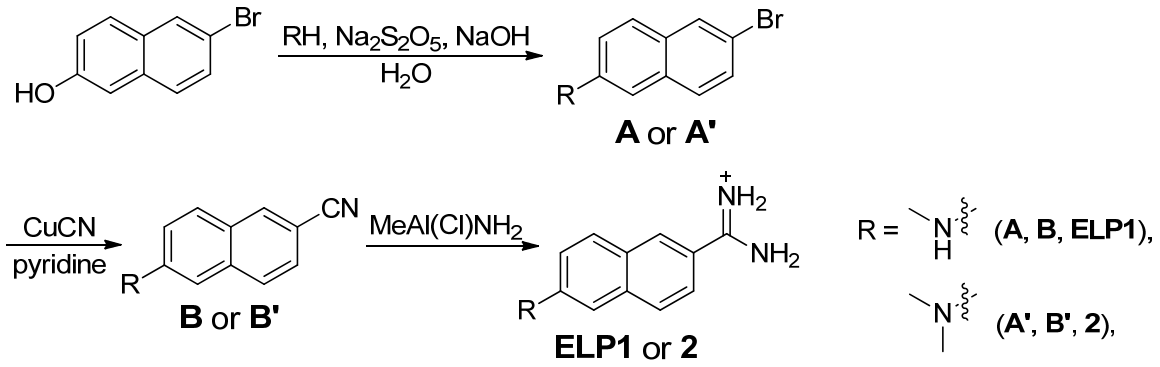
Synthesis of 2 and ELP1. Compound **A**, **B**,[1] and chloro(methyl)aluminum amide[2] were prepared by the literature methods and syntheses of **2** and **ELP1** are described below.



Scheme S1. Syntheses of **2** and **ELP1**.

Synthesis of **2.** To a stirred solution of ammonium chloride (1.0 g, 22.4 mmol) in dry benzene (11.2 mL), trimethylaluminum (2.0 M solution in toluene, 11.2 mL, 22.4 mmol) was slowly added at 0℃ and the reaction mixture was stirred for one hour at room temperature under nitrogen atmosphere. To this solution, **B'** (100 mg, 0.51 mmol) in dry toluene (2.0 mL) was added dropwise to the reaction mixture and heated at 90℃ for 8 h. The reaction mixture was cooled and poured carefully into a stirred slurry of silica gel in chloroform at 0℃. The mixture was stirred for 10 min at RT and the silica gel was filtered. The filter cake was washed with methanol and the filtrate was evaporated. The product was purified by column chromatography using 10% methanol in chloroform as eluent to afford **2** as a yellow solid (47 mg, 43%); ^{1}H NMR (DMSO-d_6, 400 MHz): δ (ppm) 9.19 (brs, 4H), 8.34 (d, J = 1.6 Hz, 1H), 7.86 (d, J = 9.2 Hz, 1H), 7.79 (d, J = 8.8 Hz, 1H), 7.69 (dd, J = 8.8, 1.6 Hz, 1H), 7.34 (dd, J = 9.2, 2.4 Hz, 1H), 7.00 (d, J = 2.4 Hz, 1H), 3.08 (s, 6H); ^{13}C NMR (DMSO-d_6, 100 MHz): δ (ppm) 165.2, 150.0, 137.2, 130.0, 129.2, 126.0, 124.0, 123.5, 119.4, 116.9, 104.3, 39.9; MS (Q-TOF): *m/z* calculated for $[C_{13}H_{16}N_3]^+$: 214.1344, found: 214.1348.

Synthesis of **ELP1.** To a stirred solution of ammonium chloride (1.0 g, 22.4 mmol) in dry benzene (11.2 mL), trimethylaluminum (2.0 M solution in toluene, 11.2 mL, 22.4 mmol) was slowly added at 0℃ and the reaction mixture was stirred for one hour at room temperature under nitrogen atmosphere. To this solution, **B** (100 mg, 0.55 mmol) in dry toluene (2.0 mL) was added dropwise to the reaction mixture and heated at 90℃ for 10 h. The reaction mixture was cooled and poured carefully into a stirred slurry of silica gel in chloroform at 0℃. The mixture was stirred for 10 min at RT and the silica gel was filtered. The filter cake was washed with methanol and the evaporation of the filtrate gave a dark yellow solid. The product was purified by column chromatography using

15% methanol in chloroform as eluent to obtain **ELP1** as a yellow solid (23 mg, 21%); ^{1}H NMR (DMSO-d_6, 400 MHz): δ (ppm) 9.29 (s, 2H), 9.09 (s, 2H), 8.29 (s, 1H), 7.68 (m, 3H), 7.08 (dd, *J* = 8.8, 2.4 Hz, 1H), 6.72 (d, *J* = 2.4 Hz, 1H), 6.69 (d, *J* = 5.0 Hz, 1H), 2.80 (d, *J* = 5.0 Hz, 3H); ^{13}C NMR (DMSO-d_6, 100 MHz): δ (ppm) 165.3, 150.2, 138.0, 129.7, 129.3, 125.5, 124.4, 123.6, 119.4, 118.5, 100.8, 29.4; HRMS (FAB$^+$): *m/z* calculated for $[C_{12}H_{14}N_3]^+$: 200.1182, found: 200.1189.

Spectroscopic measurements. Absorption spectra were recorded on a S-3100 UV-Vis spectrophotometer and fluorescence spectra were obtained with FluoroMate FS-2 fluorescence spectrophotometer with a 1.0 cm standard quartz cell. The fluorescence quantum yield was determined by using 9,10-diphenylanthrancene (Φ = 0.93 in cyclohexane) as the reference by using literature method.[3]

Water solubility. Small amount of **ELP1** was dissolved in DMSO to prepare the stock solutions (1.0×10^{-2} M). The solution was diluted to $1.0 \times 10^{-5} \sim 5.0 \times 10^{-8}$ M and added to a cuvette containing 3.0 mL of PBS buffer (10 mM, pH 7.4) by using a micro syringe. In all cases, the concentration of DMSO in buffer was maintained to be 0.1 %.[4] The plot of fluorescence intensity against the probe concentration were linear at low concentration and showed downward curvature at higher concentration (Figure S1). The maximum concentration in the linear region was taken as the solubility. The solubility of **ELP1** in PBS buffer was ~ 8.0 μM.

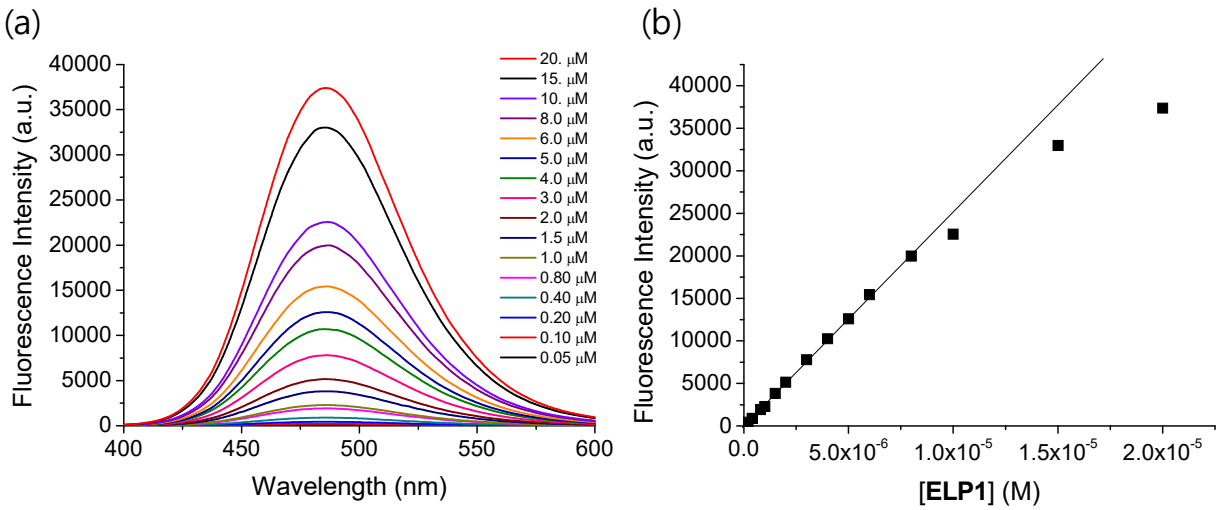


Figure S1. (a) One-photon fluorescence spectra and (b) plot of fluorescence intensity against the concentration of **ELP1** in PBS buffer (10 mM, pH 7.4). The excittion wavelength was 335 nm.

Table S1. Photophysical data for **ELP1** in various solvents and buffer.[a]

	Solvent	$\lambda_{max}^{(1)}$ $(10^{-4}\,\varepsilon)$[b]	λ_{max}^{fl} [c]	Φ[d]	$\lambda_{max}^{(2)}$ [e]	$\Phi\delta_{max}$ [f]
ELP1	1,4-dioxane	333	428	1.00	750	14
	DMF	356	470	0.99	740	32
	EtOH	364	461	1.00	750	28
	H_2O	335 (1.75)	487	0.55	740	19

a) All data for buffer were measured in PBS buffer (10 mM, pH 7.4). b) λ_{max} of the one-photon absorption spectra in nm. The number in parenthesis is molar extinction coefficient in $M^{-1}cm^{-1}$. c) λ_{max} of the one-photon emission spectra in nm. d) Fluorescence quantum yield, ±10%. e) λ_{max} of the two-photon emission spectra in nm. f) The peak two-photon action cross sections in GM (1 GM = 10^{-50} cm^4 s $photon^{-1}$), ±15%.

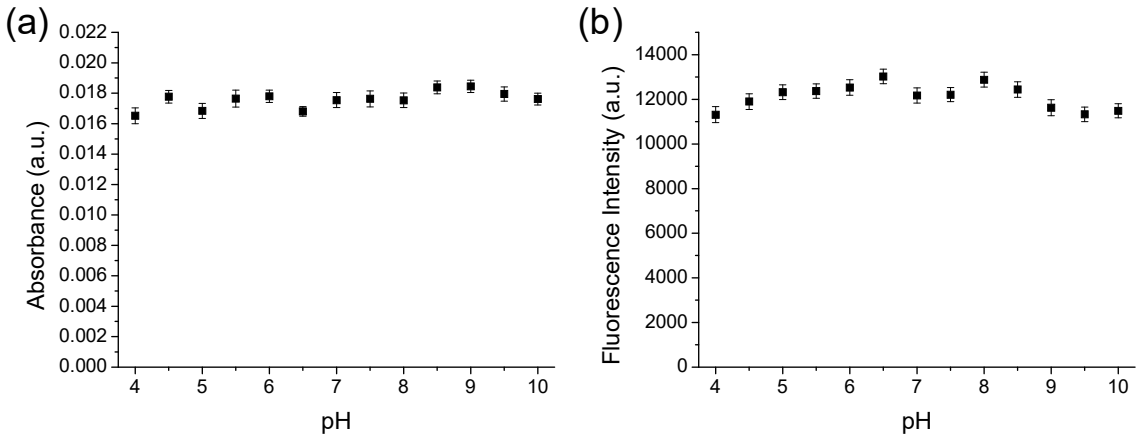


Figure S2. Effect of pH on the (a) UV absorbance and (b) one-photon fluorescence intensity of 1.0 μM **ELP1** in universal buffer (0.1 M citric acid, 0.1 M KH_2PO_4, 0.1 M $Na_2B_4O_7$, 0.1 M Tris, 0.1 M KCl) at 25 °C. The excitation wavelength was 335 nm (n = 3).

Measurement of Two-Photon Cross Section. The two-photon cross section (δ) was determined by using femto second (fs) fluorescence measurement technique as described.[5] **ELP1** (1.0×10^{-6} M) was dissolved in various solvents (1,4-dioxane, EtOH, DMF, PBS buffer (10 mM, pH 7.4)) and the two-photon induced fluorescence intensity was measured at 720−880 nm by using rhodamine 6G as the reference, whose two-photon property has been well characterized in the literature.[6] The intensities of the two-photon induced fluorescence spectra of the reference and sample emitted at the same excitation wavelength were determined. The TPA cross section was calculated by using $\delta = \delta_r(S_s\Phi_r\phi_r c_r)/(S_r\Phi_s\phi_s c_s)$: where the subscripts *s* and *r* stand for the sample and reference molecules. The intensity of the signal collected by a CCD detector was denoted as *S*. Φ is the fluorescence quantum yield. ϕ is the overall fluorescence collection efficiency of the experimental apparatus. The number density of the molecules in solution was denoted as *c*. δ_r is the TPA cross section of the reference molecule.

Cell Culture. All the cells were passed and plated on glass-bottomed dishes (NEST) before imaging for two days. They were maintained in a humidified atmosphere of 5/95 (v/v) of CO_2/air at 37 °C. The cells were treated and incubated with 2 μM of **ELP1** at 37 °C under 5 % CO_2 for 15 min, washed three times with phosphate buffered saline (PBS; Gibco), and then imaged after further incubation in colorless serum-free media for 30 min. The culture mediums for each cell are as below.

HeLa human cervical carcinoma cells (ATCC, Manassas, VA, USA): MEM (WelGene Inc, Seoul, Korea) supplemented with 10 % FBS (WelGene), penicillin (100 units/ml), and streptomycin (100 μg/mL).

MDA-MB231 cells (ATCC, Manassas, VA, USA): RPMI1640 (WelGene Inc, Seoul, Korea) supplemented with 10 % FBS (WelGene), penicillin (100 units/ml), and streptomycin (100 μg/mL).

HT29 cells (ATCC, Manassas, VA, USA): RPMI1640 (WelGene Inc, Seoul, Korea) supplemented with 10 % FBS (WelGene), penicillin (100 units/ml), and streptomycin (100 μg/mL).

Cell viability. To evaluate the cytotoxic effect of **ELP1** in HeLa cells, MTS (cell Titer 96H; Promega, Madison, WI, USA) and CCK-8 kit (Cell Counting Kit-8; Dojindo, Japan) assay were performed according to the manufacture's protocol. The results are shown in Figure 3, which revealed that the **ELP1** has low cytotoxicity at its different concentration in our incubation condition.

Two-Photon Fluorescence Microscopy. Two-photon fluorescence microscopy images of **ELP1**-labeled cells were obtained with spectral confocal and multiphoton microscopes (Leica TCS SP8 MP) with ×10 dry, ×40 oil, and ×100 oil objectives, numerical aperture (NA) = 0.30, 1.30, and 1.30, respectively. The two-photon fluorescence microscopy images were obtained with a DMI6000B Microscope (Leica) by exciting the probes with a mode-locked titanium-sapphire laser source (Mai Tai HP; Spectra Physics, 80 MHz pulse frequency, 100 fs pulse width) set at wavelength 750 nm and output power 2673 mW, which corresponded to approximately 1.7 mW average power in the focal plane. Live cell imaging was performed using the live cell incubator systems (Chamlide IC; Live Cell Instrument) for stable cell environment by maintaining proper temperature, humidity and pH over the long term. To obtain images at 400–600 nm range, internal PMTs were used to collect the signals in an 8 bit unsigned 512×512 and 1024×1024 pixels at 400 and 200 Hz scan speed, respectively.

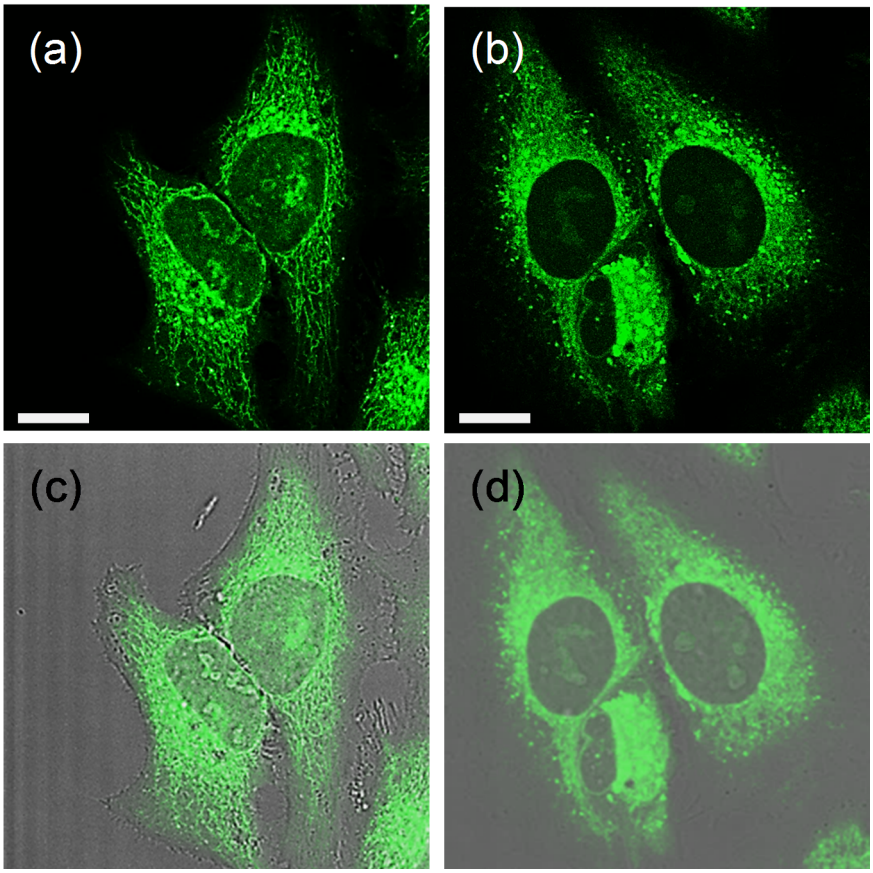


Figure S3. TPM images of HeLa cells labeled with 2.0 μM (a) **ELP1** (b) **2**. (c,d) The images were merged with the corresponding DIC images. The excitation wavelength was 750 nm. Scale bars = 20 μm. Cells shown are representative images from replicate experiments (n = 10).

Colocalization experiment. Co-localization experiments were conducted by co-staining the HeLa, MDA-MB231, HT29 cells with appropriate combinations of **ELP1** (2.0 μM) for 15 min. and each commercial organelle markers (5.0 μM of BODIPY TR ceramide complexed to BSA for Golgi, 1.0 μM of LysoTracker Red DND-99 for Lysosome, 1.0 μM MitoTracker Red FM for Mitochondria, 1.0 μM of ER-Tracker Red for Endoplasmic Reticulum) for 30 min. TPM and OPM images were obtained by collecting the emissions at 400–525 nm (**ELP1**, λ_{ex}= 750 nm)and 600–650 nm (Organelle markers, λ_{ex}= 552 nm), respectively. The background images were corrected, and the distribution of pixels in the TPM and OPM images acquired in the green and red channels, respectively, was compared by using scatter gram. The Pearson's colocalization coefficient (*A*) was calculated by using AutoQuant X3 program.

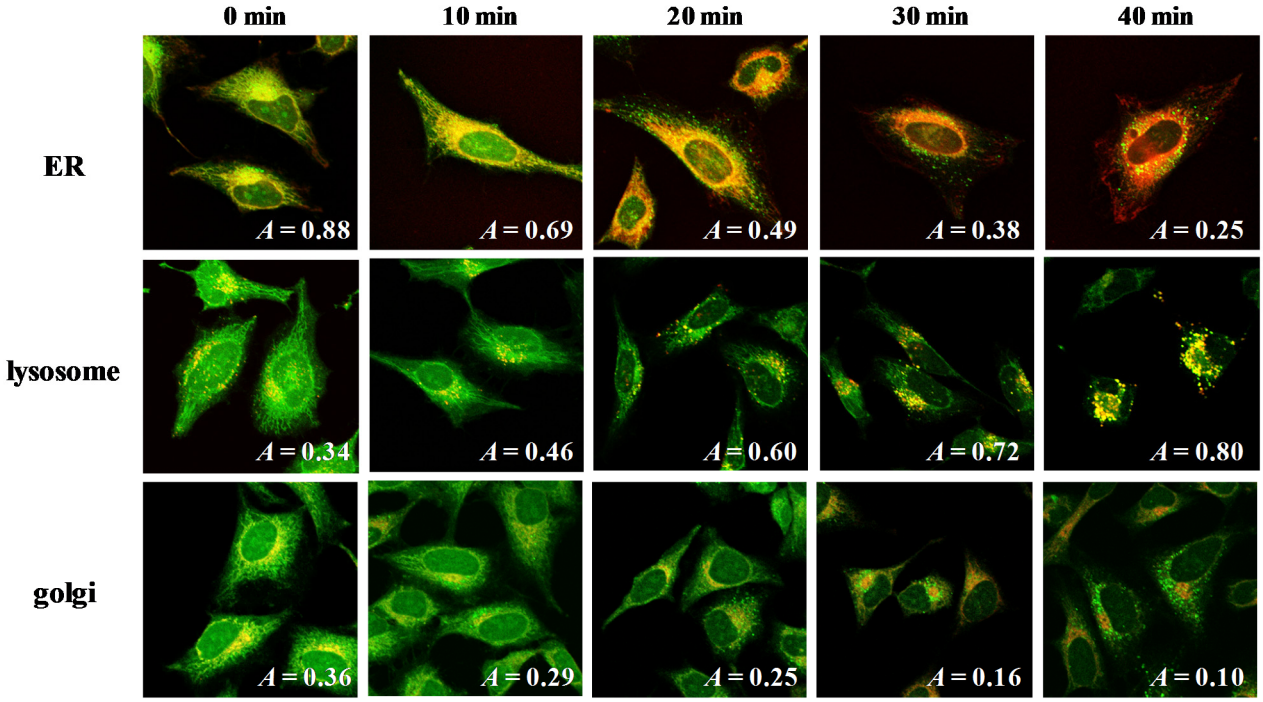


Figure S4. Time course merged images of HeLa cells co-labeled with 2.0 μM **ELP1** (green fluorescence, TPM images) and organelle markers (red fluorescence, OPM images, 1.0 μM of ER-Tracker Red for ER, 1.0 μM of LysoTracker Red DND-99 for lysosome, 5.0 μM of BODIPY TR ceramide complexed to BSA for golgi). The excitation wavelengths for TPM and OPM are 750 and 552 nm, respectively. Cells shown are representative images from replicate experiments (n = 10).

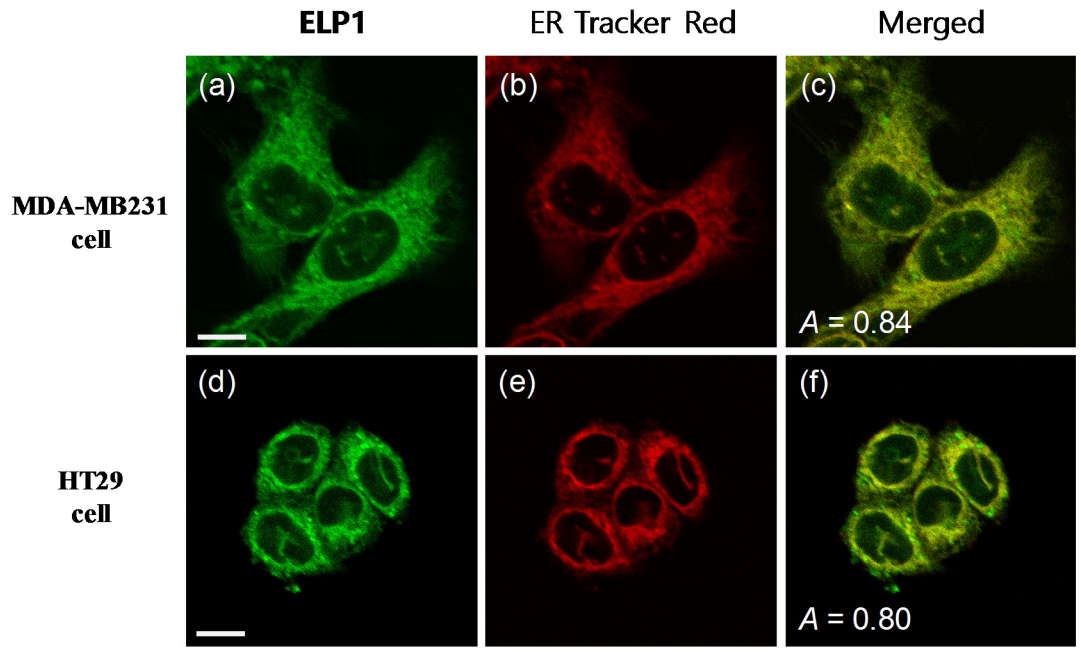


Figure S5. Colocalization between **ELP1** and ER marker in (a,b,c) MDA-MB231 cells and (d,e,f) HT29 cells. (a,d) TPM, (b, e) OPM images of each cell co-labeled with 2.0 μM **ELP1** and 1.0 μM of ER-Tracker Red. (c,f) Merged images. The excitation wavelengths for TPM and OPM are 750 and 552 nm, respectively. Scale bars = (a) 6, (d) 8 μm, respectively. Cells shown are representative images from replicate experiments (n = 10).

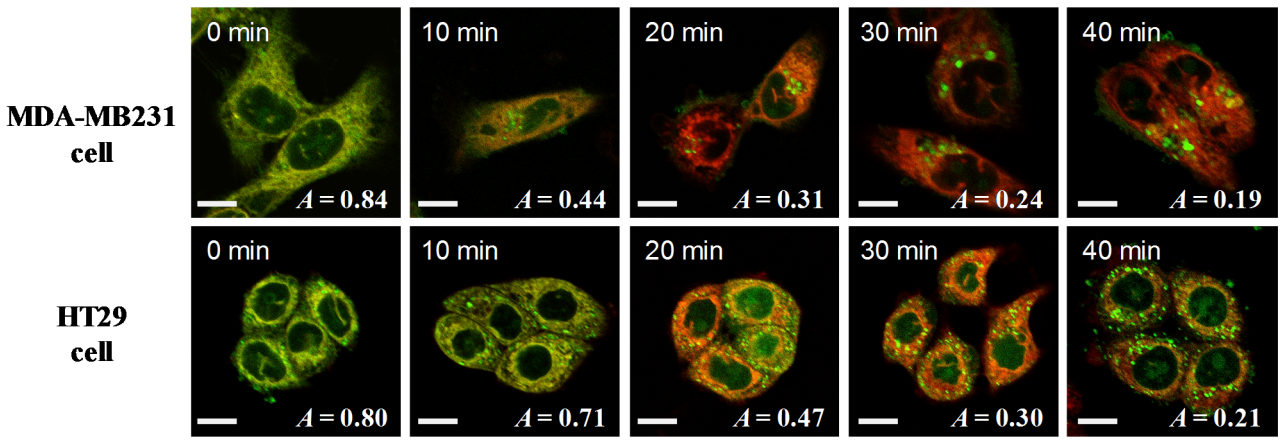


Figure S6. Time course merged images of MDA-MB231 cells and HT29 cells co-labeled with 2.0 μM **ELP1** (green fluorescence, TPM images) and 1.0 μM of ER-Tracker Red (red fluorescence, OPM images). The excitation wavelengths for TPM and OPM are 750 and 552 nm, respectively. Scale bars = 8 μm. Cells shown are representative images from replicate experiments (n = 10).

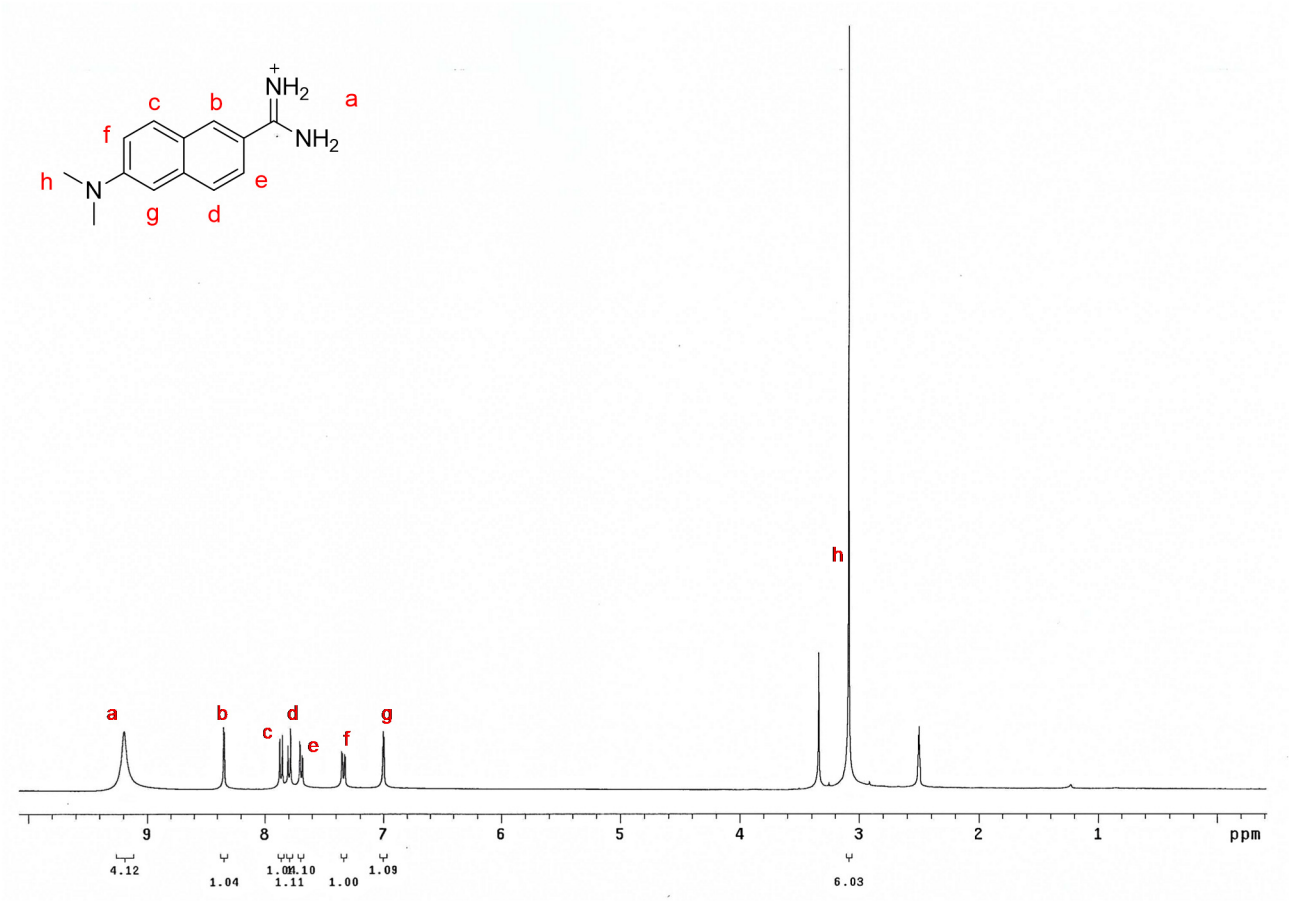


Figure S7. ^{1}H-NMR spectrum (400 MHz) of **2** in DMSO-d$_6$

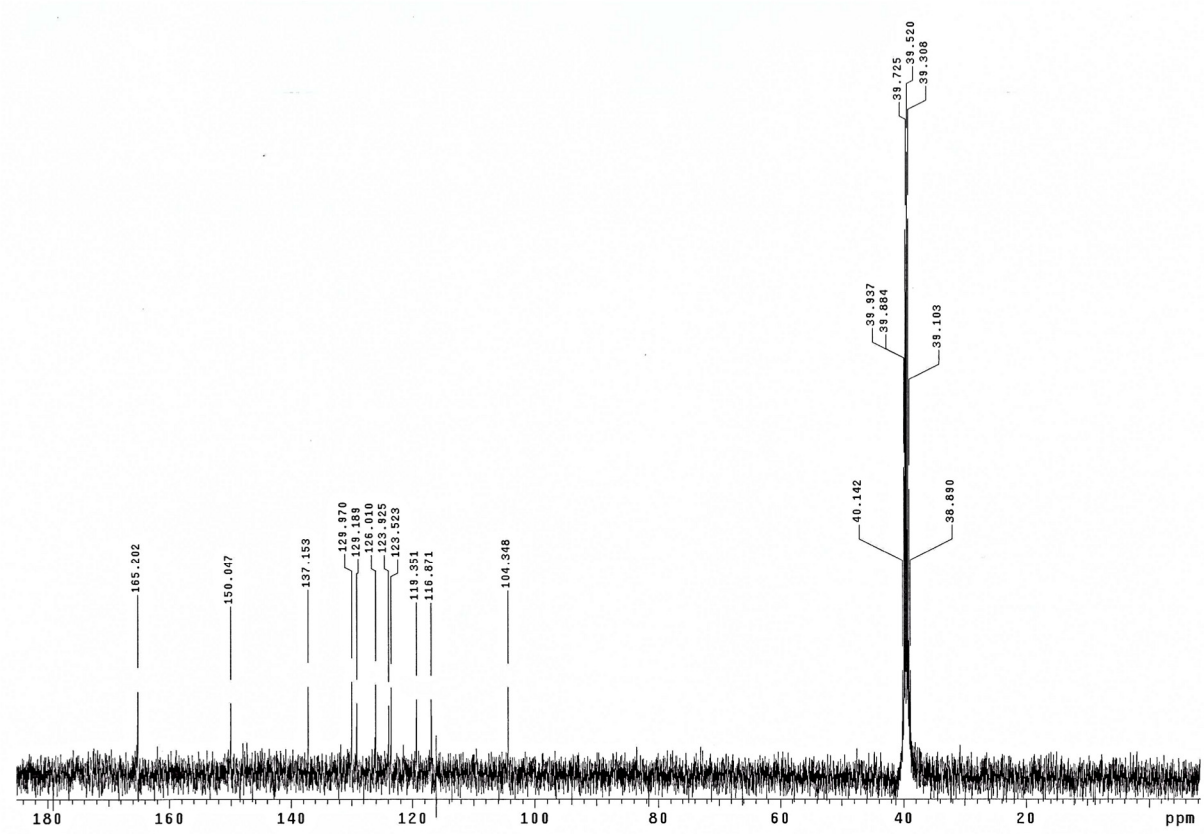
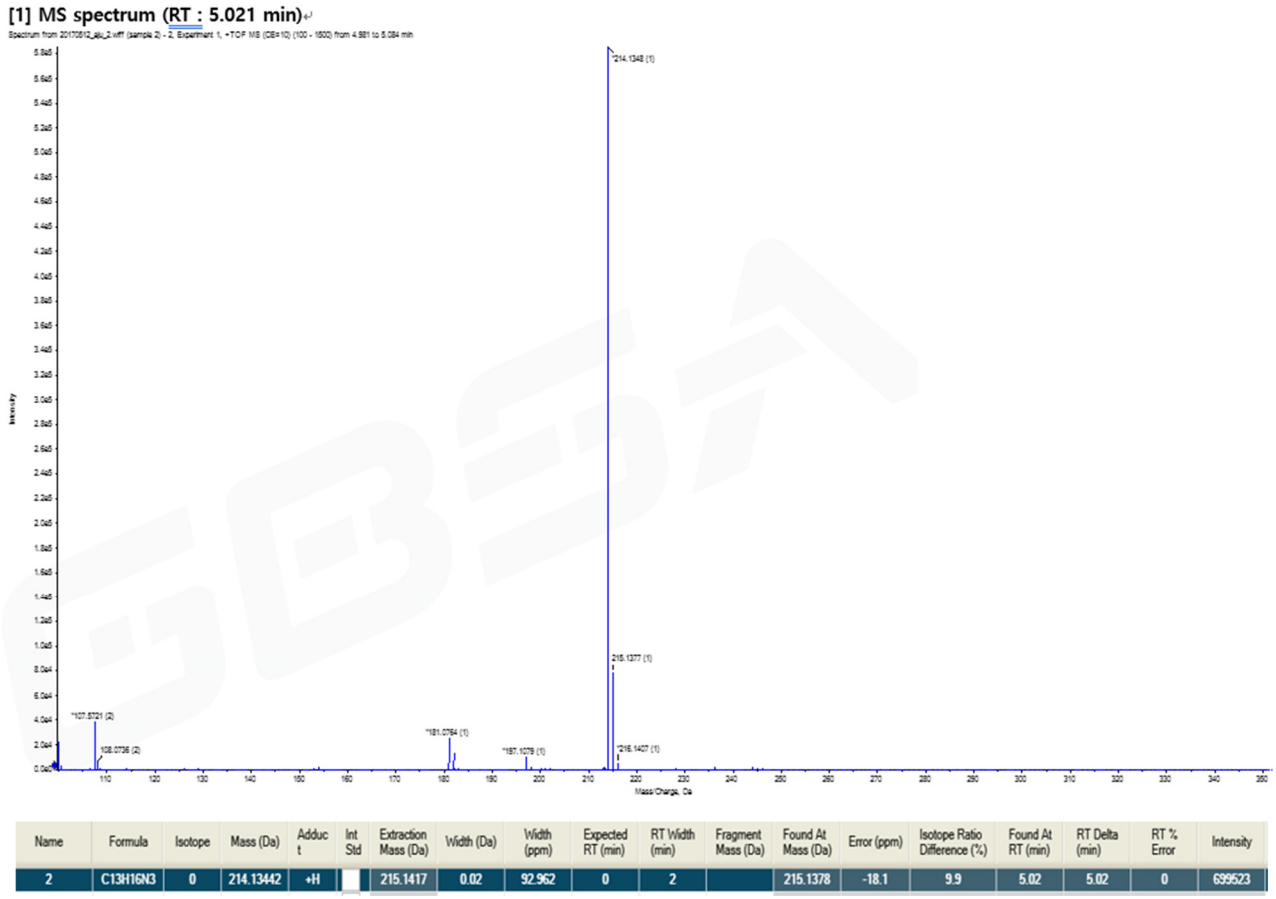


Figure S8. ^{13}C-NMR spectrum (100 MHz) of **2** in DMSO-d$_6$



Name	Formula	Isotope	Mass (Da)	Adduct	Int Std	Extraction Mass (Da)	Width (Da)	Width (ppm)	Expected RT (min)	RT Width (min)	Fragment Mass (Da)	Found At Mass (Da)	Error (ppm)	Isotope Ratio Difference (%)	Found At RT (min)	RT Delta (min)	RT % Error	Intensity
2	C13H16N3	0	214.13442	+H		215.1417	0.02	92.962	0	2		215.1378	-18.1	9.9	5.02	5.02	0	699523

Figure S9. MS(Q-TOF) spectrum of **2**

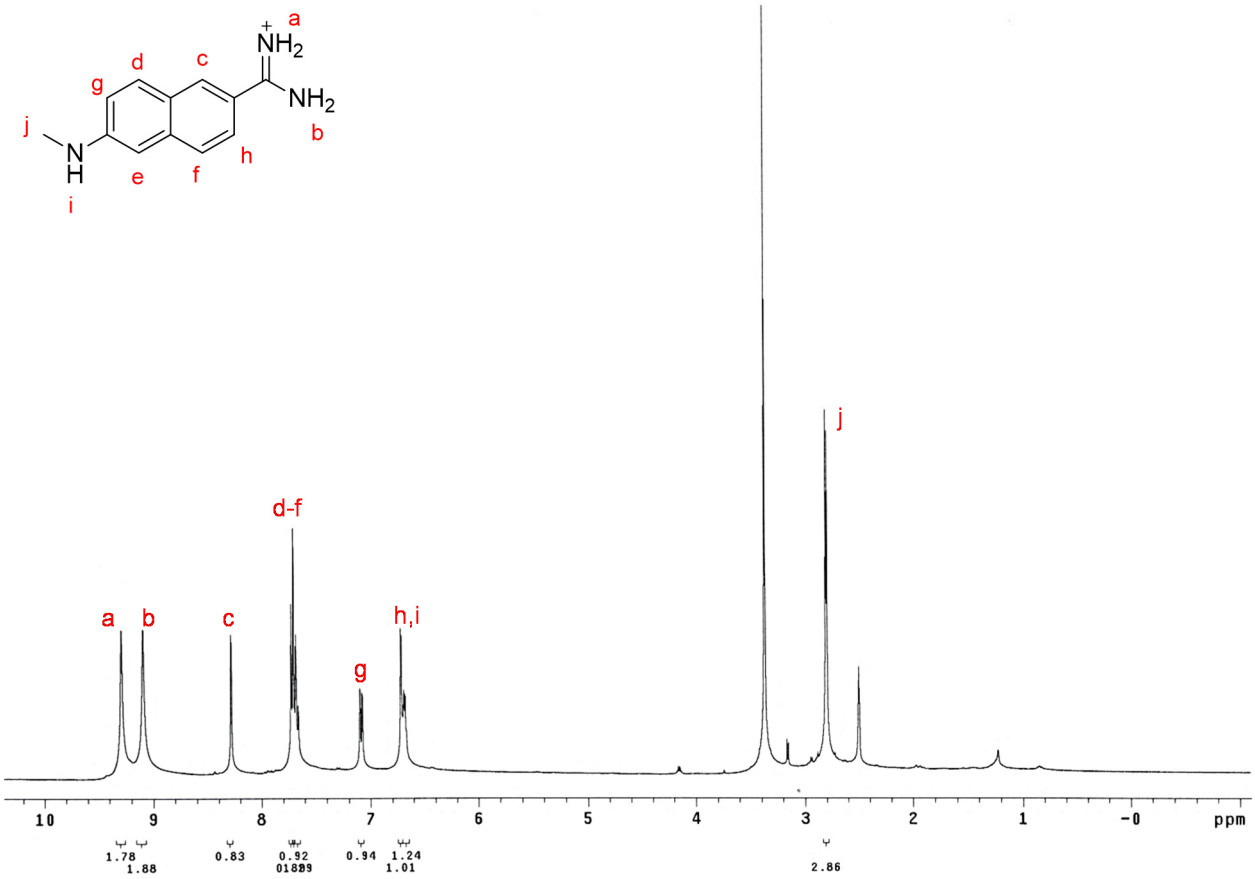


Figure S10. [1]H-NMR spectrum (400 MHz) of **ELP1** in DMSO-d$_6$

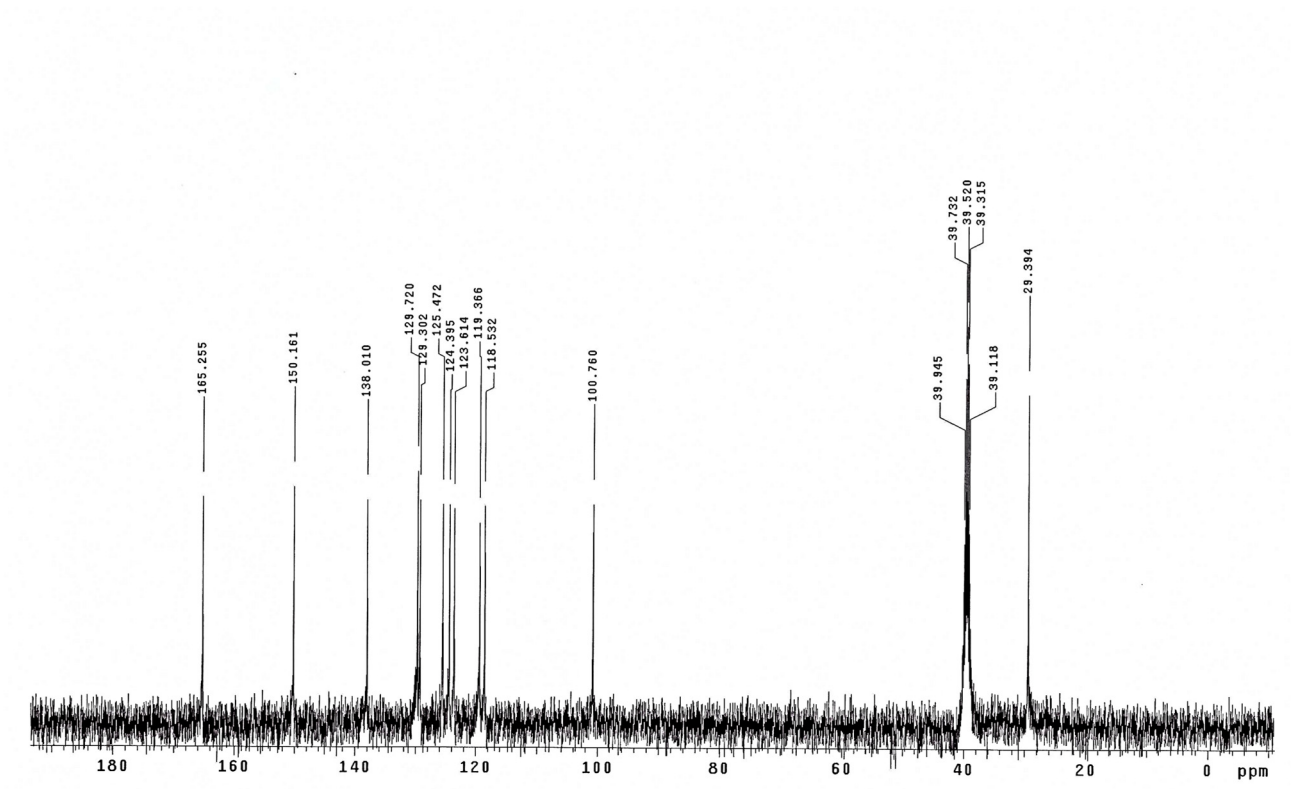


Figure S11. [13]C-NMR spectrum (100 MHz) of **ELP1** in DMSO-d$_6$

2.2. MS spectrum (RT : 3.83 min)

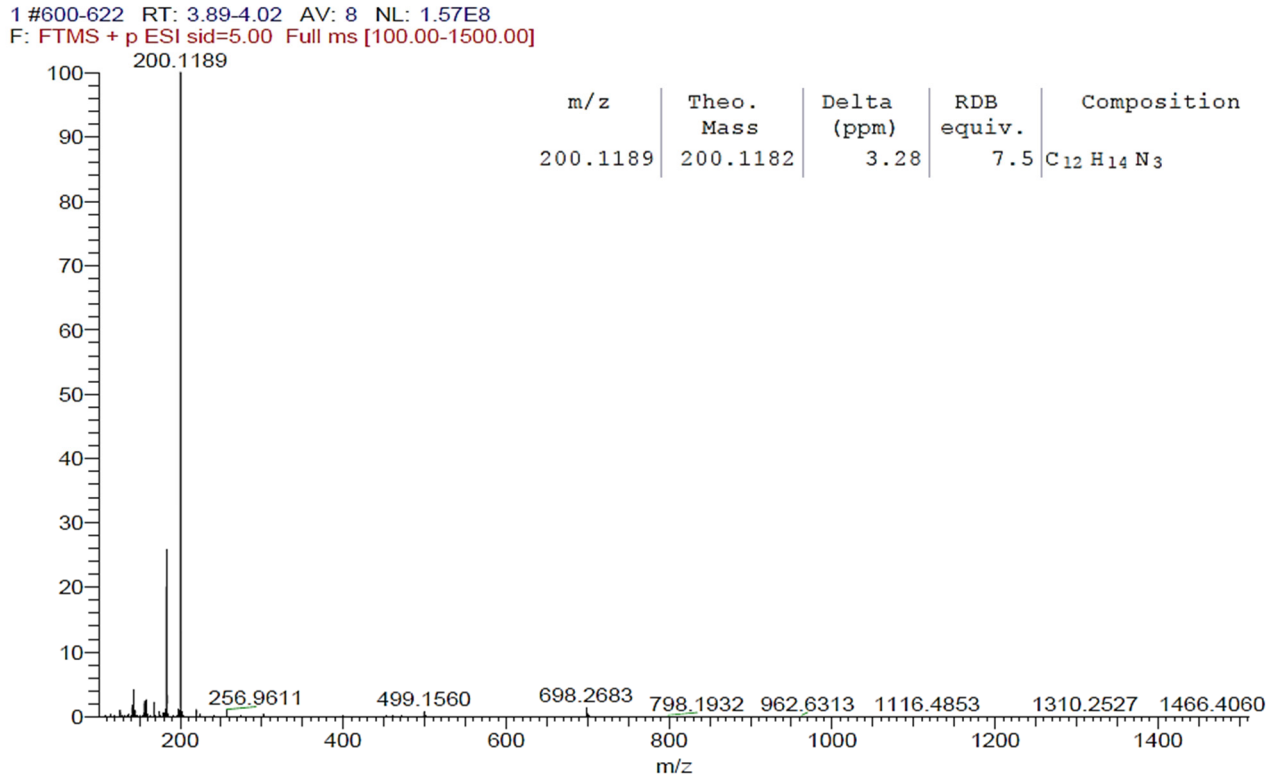


Figure S12. HRMS spectrum of **ELP1**

References

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