Supporting Information

Mitochondrial NIR imaging probe mitigating oxidative damage by targeting HDAC6

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1. Materials and Methods

Materials and methods

1.1 Materials

We purchased the chemical reagents from Acros Organics (Geel, Flanders, Belgium), Alfa Aesar (Haverhill, MA, USA), Sigma-Aldrich (St. Louis, MO, USA), and TCI (Tokyo, Japan). Dimethyl sulfoxide (DMSO, anhydrous, product no. 1.02952.1000). Cell counting Kit-8 (CCK-8, product no. CK04-13, Dojindo Molecular Tech. Inc, Tokyo, Japan) was used to confirm the cellular toxicity. Additionally, phosphate-buffered saline [PBS pH 7.4 (10×), product no. 70011044], penicillin-streptomycin (product no. 15140-122), and trypsin-EDTA (product no. 25200-056) were purchased from Gibco (Carlsbad, CA, USA).

1.2 Instrumentations

^{1}H NMR spectra were measured with a JNM-ECZ500R (500 MHz, Tokyo, Japan). Additionally, we observed images using a confocal laser scanning microscope (LSM 800, Carl Zeiss, Jena, Germany). Fourier transform infrared spectroscopy was analyzed using Thermo Scientific Nicolet™ iS™ 5 FT-IR spectrometer instrument (32 scans, Waltham, MA, USA). Moreover, 55 mM tert-butyl hydrogen peroxide (product no. ab113851) was purchased from (Abcam, Cambridge, UK), and ESI-MS spectra were measured at the Korea Basic Science Institute (western Seoul, Republic of Korea).

1.3.1 UV/Vis absorption and spectroscopic emission methods

UV/Vis absorption spectra were detected using a spectrophotometer (Agilent, California, USA). Additionally, fluorescence spectra were obtained using a spectro-fluorophotometer (SHIMADZU

CORP. RF-6000, Kyoto, Japan). Next, the materials were dissolved in DMSO to prepare the stock solution (100 μM), which was diluted with PBS (pH 7.4) to 10 μM. Lastly, T2 was dissolved in PBS (10 μM, pH 7.4) and transferred to a 1 cm standard quartz cuvette to obtain UV/Vis absorption and emission spectra.

1.3.2. Fluorescence quantum yield measurements

For fluorescence quantum yield measurements, methylene blue (MB) was used as a reference (Φ_s = 0.02 in water), and the quantum yield of **T2** was calculated using the following equation.

$$\Phi_x = \Phi_s(F_x/F_s)(A_s/A_x)(\lambda_{exs}/\lambda_{exx})(n_x/n_s)^2$$

Where Φ represents the quantum yield; F is the integrated area under the corrected emission spectrum; A stands for the absorbance at the excitation wavelength; λ_{ex} is the excitation wavelength; and the subscripts x and s represent the unknown and the reference, respectively, and n is the refractive index of the solvent.

1.4. Cell culture and primary cortical neuron culture

Cortical neurons were obtained from the cerebral cortex of Sprague Dawley rat embryos (E17). Next, the dissociated neurons were plated on poly-L-lysine-coated coverslips and maintained in minimal essential medium containing 5.5 g/L glucose, 1X Glutamax, and supplemented with 10% fetal bovine serum (FBS).[1] All experiments were initiated 24 h after plating. Experiments with HDAC6 inhibitors were performed 24 h after cell plating by treating the cells with a single dose of the HDAC6 inhibitors treatment for 24 h. Tubastatin A (Tub, Sigma-Aldrich, # SML0044) and T2 were dissolved in DMSO at the indicated concentrations. Control cells were treated with DMSO only.

HeLa cell lines were cultured in a humidified incubator in Roswell Park Memorial Institute 1640 Medium (RPMI 1640 Medium, Cat. No. LM011-01, Welgene, Gyongsan-si, Korea) supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C under 5% CO_2 and 95% air. For the experiment, HeLa cells were cultured in a tissue culture dish (SPL Life Sciences, 11035). After the cells reached 70% confluency, each sample was treated with the prepared compound, T1 or T2. Before mixing with the culture media, each compound was diluted to a 5 μM working solution in DMSO; the final volume contained 1% DMSO in the culture media. Next, the mixture was vortexed to aid uniform dispersion throughout the media before treatment to cells. Each sample was then treated with the mixture for the determined time point, including 0.5 h, 4 h, 8 h, and 24 h

1.5. Neuron viability assay

Oxidative stress was induced by adding 5 mM homocysteic acid (HCA, Sigma-Aldrich, # 219746) to the medium after mixing the cells with PBS.[2] Notably, 500 mM HCA stock solutions were adjusted to pH 7.5. Next, Tub or T2 was added at the time of HCA treatment. After 24 h, the cells were stained with acridine orange/propidium iodide, and images were taken under fluorescence microscopy. Lastly, the ImageJ software was used to count live and dead cells.

1.6. Immunostaining

Tub or T2 was added to cortical neurons grown on glass coverslips in a 24-well plate. After 24 h, the cells were fixed with 4% paraformaldehyde for 15 min at room temperature (RT), washed thrice with PBS, incubated with 0.2% Triton X-100 for 10 min, and washed once with PBS buffer. Next, the cells were blocked with 3% BSA diluted with PBS at RT for 30 min, incubated with 3% BSA containing the

primary antibody [anti-beta-III tubulin (Novus Biologicals, NB100-1612), anti-Tomm20 (Abcam, ab209606), and anti-Cytochrome C (Abcam, ab270249)] overnight at 4°C, and washed once with PBS for 5 min. Afterward, the cells were incubated with 3% BSA containing secondary antibody (Invitrogen) and Hoechst 33342 (Invitrogen, H3570) for 30 min at RT and washed once with PBS. Lastly, the coverslips were mounted and imaged using a Zeiss LSM 800 confocal microscopy.

For NIR imaging, after treating the cells with the compound, the cells were fixed in 4% paraformaldehyde for 15 min at room temperature. Next, the samples were washed thrice with PBS and permeabilized using 0.5% Triton-X for 10 min. After that, the samples were washed thrice with PBS and blocked with 3% bovine serum albumin for 20 min. Subsequently, the cells were incubated with the primary antibody [TOM20 (11802-1-AP, 1:200)] overnight at 4°C. Lastly, secondary antibodies (Alexa Fluor 555, Invitrogen A31572, 1:200) and DAPI were added for 1 h at 37°C, and the samples were rewashed with PBS thrice before imaging.

1.7. Imaging and analysis

For NIR imaging, the samples were analyzed using a custom-built video-rate laser-scanning confocal microscopy system. Three continuous laser modules (Wavelength at 488 nm [MLD 488; Cobolt, Solna, Sweden], 561 nm [Jive; Cobolt], and 640 nm [MLD640; Cobolt]) were employed as excitation light sources for multi-color fluorescence imaging. Additionally, a commercial objective lens (Nikon CFI Plan Fluor, 60X, NA 0.85) was used for imaging the samples. Lastly, three bandpass filters (BPF1; FF03-510/20, BPF2; FF01-600/37, BPF3; FF01-835/70-25, Semrock, Rochester, NY, USA) and photomultiplier tubes (R9110; Hamamatsu Photonics, Hamamatsu, Japan) were used to detect fluorescence signals simultaneously.

1.8. Live imaging and analysis

Oxidative stress was induced by the addition of 5 mM homocysteic acid (HCA) to the medium after cells with PBS. 5 μM Tubastatin A (Tub), 5 μM T1, or 5 μM T2 were added at the time of HCA treatment. After 12 h, the cells were stained with 50 nM Mitotracker™ Green FM (Invitrogen, M7514) and 50 nM tetramethylrhodamine methyl ester (TMRM) (Invitrogen, T668) in phenol red-free DMEM containing 10% FBS at 37°C with 5% CO_2 for 15 min and examined using Olympus fluorescence microscopy. Lastly, the fluorescence intensity of TMRM in each mitochondrion was measured using the ImageJ software.

2. Synthetic procedures

Compounds **2** [3], **7** [4], and **8** [5] were synthesized according to the reported procedure.

2.1. Synthesis of compound 1

A mixture of phenylhydrazine (2 g, 18.49 mmol) and 1-boc-4-piperidone (3.68, 18.49 mmol) was dissolved in EtOH and saturated with concentrated HCl (30 mL) under stirring. Next, the mixture was heated at reflux for 3 h under stirring (TLC control). Afterward, the solvent was evaporated, and the residue was treated with $NaHCO_3$ solution (50 mL) and extracted with CH_2Cl_2 (4 × 100 mL). The combined organic layers were dried on Na_2SO_4 and concentrated to obtain the pure compound **1** (2.45 g, yield: 77%). ^{1}H NMR (500 MHz, DMSO) δ 10.70 (s, 1H), 7.29 (d, *J* = 7.7 Hz, 1H), 7.25 (d, *J* = 8.0 Hz, 1H), 6.98 (td, *J* = 7.5, 1.0 Hz, 1H), 6.90 (td, *J* = 7.5, 1.0 Hz, 1H), 3.84 (s, 2H), 3.01 (t, *J* = 5.7 Hz, 2H), 2.66 (t, *J* = 5.6 Hz, 2H). ^{13}C NMR (125 MHz, DMSO) δ 135.37, 133.24, 125.65, 120.02, 118.09, 117.02, 110.66, 108.10, 42.97, 41.63, 24.04.

2.2. Synthesis of compound 3

Compound **3** was synthesized from compound **2** using a known procedure with modification.[1] Briefly, compound **2** (2.2 g, 8.08 mmol) in anhydrous DMF (5 mL) was added to a stirred solution of 60 mol% NaH (0.65 g, 16.16 mmol) in anhydrous DMF (10 mL) under argon. After 10 min, methyl 4-(bromomethyl) benzoate (2.78 g, 12.12 mmol) in anhydrous DMF (5 mL) was added to the reaction mixture and stirred at room temperature for 4 h. After completion of the reaction, the mixture was poured into cold water (30 mL), and the organic products were extracted with EtOAc (3 × 30 mL), washed with water (3 × 20 mL) and brine (20 mL), dried with Na_2SO_4, filtered, and concentrated *in vacuo*. Lastly, purification using column chromatography (SiO_2, 0−80% EtOAc/hexane) produced compound **3** (2.31 g, 68%)—a dark orange oil. The characterization data were exactly matched with the reported data.

2.3. Synthesis of compound 4

Compound **3** (1 g, 2.38 mmol) was dissolved in CH_2Cl_2 (10 mL), trifluoroacetic acid (TFA, 2 mL) was added, and the reaction mixture was stirred for 2 h at room temperature. Next, the volatiles was removed *in vacuo*, and the residue was concentrated *in vacuo* and used as such for the next reaction without further purification.

The obtained TFA salt was dissolved in anhydrous MeCN (5 mL), and Et_3N (0.66 mL, 4.76 mmol) was added at room temperature. Next, 4-bromo-1-butyne (0.36 mL, 3.57 mmol) in anhydrous MeCN (2 mL) was added, and the reaction mixture was heated to 60°C. Afterward, the reaction mixture was stirred for 2 h at 60 °C and poured into a 1:1 mixture of EtOAc/H_2O (20 mL). Subsequently, the organic layer was isolated, and the aqueous layer was further extracted with EtOAc (2 × 15 mL). After, the

combined organic layers were washed with brine (10 mL), dried on Na_2SO_4, filtered, and concentrated in *vacuo*. Lastly, the resulting crude was purified by silica gel column chromatography using 20–50% EtOAc in hexane to obtain pure compound **5** (0.48 g, yield: 54%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.90 (d, *J* = 8.2 Hz, 2H), 7.46–7.41 (m, 1H), 7.15–7.05 (m, 3H), 7.03 (d, *J* = 8.1 Hz, 2H), 5.22 (s, 2H), 3.85 (s, 3H), 3.80 (s, 2H), 2.90 (t, *J* = 5.6 Hz, 2H), 2.89 – 2.85 (m, 2H), 2.71 (brs, 2H), 2.51 (td, *J* = 7.6, 2.5 Hz, 2H), 2.01 (brs, 1H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 166.51, 136.58, 133.28, 129.93, 129.17, 126.03, 125.77, 121.07, 119.23, 117.60, 108.94, 108.26, 82.57, 69.16, 56.19, 50.16, 49.15, 46.03, 17.33.

2.4. Synthesis of compound 5

Compound **4** (0.20 g, 0.54 mmol) was dissolved in methanol (5 mL), and hydroxylamine 50% aqueous solution (1 mL), hydroxylamine hydrochloride ($NH_2OH.HCl$) (0.19 g, 2.70 mmol), and KOH (0.60 g, 10.8 mmol) were added sequentially and stirred at room temperature for 1 h. After completion of the reaction, the mixture was concentrated under reduced pressure to approximately 2–3 mL, and saturated $NaHCO_3$ (1–2 mL) was added and stirred. The solid product was filtered, washed with water, and dried *in vacuo* to obtain compound **5**—a pale-yellow solid (0.13 g, 64%). ^{1}H NMR (500 MHz, DMSO) δ 11.11 (s, 1H), 9.06 (s, 1H), 7.65 (d, *J* = 8.3 Hz, 2H), 7.37 (dd, *J* = 15.5, 7.8 Hz, 2H), 7.06 (d, *J* = 8.3 Hz, 2H), 7.04 – 6.96 (m, 2H), 5.37 (s, 2H), 3.66 (s, 2H), 2.83 (t, *J* = 5.6 Hz, 2H), 2.79 (t, *J* = 2.6 Hz, 1H), 2.76 – 2.69 (m, 4H), 2.45 (td, *J* = 7.5, 2.6 Hz, 2H). ^{13}C NMR (125 MHz, DMSO) δ 163.80, 141.58, 136.28, 133.93, 131.82, 127.20, 126.33, 125.35, 120.62, 118.82, 117.40, 109.52, 107.72, 83.32, 71.71, 55.98, 49.86, 48.72, 45.38, 22.44, 16.74.

2.5. Synthesis of compound T1

To a stirred solution of compounds **7** (0.20 g, 0.27 mmol) and 6-azidohexan-1-amine (0.15 g, 1.08 mmol) in anhydrous MeCN (5 mL), diisopropylethylamine (0.05 mL, 0.27 mmol) was added and stirred at 80°C for 1 h. Next, the reaction mixture was cooled to room temperature and concentrated *in vacuo*. The resulting crude was purified by silica gel column chromatography using 0.5–1.5% CH_2Cl_2 in methanol to obtain compound **T1** (0.096 g, 42%)—a blue foam-type solid. ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.15 (brd, *J* = 8.6 Hz, 1H), 8.09 (d, *J* = 8.5 Hz, 2H), 7.88 – 7.81 (m, 6H), 7.54 (t, *J* = 7.7 Hz, 2H), 7.36 (t, *J* = 7.5 Hz, 2H), 7.19 (d, *J* = 8.7 Hz, 2H), 5.64 (d, *J* = 12.9 Hz, 2H), 4.08–3.88 (m, 6H), 3.27 (t, *J* = 6.8 Hz, 2H), 2.52 (t, *J* = 6.3 Hz, 4H), 2.00 (s, 12H), 1.88–1.81 (m, 2H), 1.67–1.57 (m, 4H), 1.49–1.44 (m, 4H), 1.41 (brs, 6H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 168.67, 168.37, 137.74, 130.72, 129.90, 129.74, 128.60, 127.29, 123.68, 121.99, 109.37, 93.18, 53.41, 51.33, 49.87, 49.66, 31.31, 28.74, 28.48, 26.34, 26.27, 25.23, 21.62, 11.79.

2.6. Synthesis of compound T2

To the stirred mixture of compounds **5** (22 mg, 0.059 mmol) and **T1**(50 mg, 0.059 mmol) in CH_2Cl_2/MeOH solution (2:1, total = 10 mL), sodium ascorbate (10 mol%) was added. Next, the reaction mixture was degassed for 30 min by purging Argon gas with continuous stirring. Additionally, $CuSO_4.5H_2O$ (5 mol%) was dissolved in MeOH (0.5 mL) in an Eppendorf tube and degassed by purging Argon gas. After 30 min, the degassed $CuSO_4.5H_2O$ solution was added to the reaction mixture and stirred continuously for 6 h. After completion of the reaction (indicated by TLC), the solvent was evaporated *in vacuo,* and the resulting crude was passed through a short silica gel column chromatography (MeOH/CH_2Cl_2 = 2:98 to 12:88) to obtain **T2** (92 mg, 32%)—a blue solid. ^{1}H

NMR (500 MHz, DMSO) δ 11.14 (s, 1H), 9.01 (s, 1H), 8.44 (s, 1H), 8.16 (d, *J* = 8.5 Hz, 2H), 7.99 – 7.94 (m, 4H), 7.89 (s, 1H), 7.73 (d, *J* = 13.0 Hz, 2H), 7.65 (d, *J* = 8.2 Hz, 2H), 7.61–7.53 (m, 4H), 7.36 (dt, *J* = 20.8, 7.1 Hz, 4H), 7.11–6.94 (m, 4H), 5.83 (d, *J* = 12.8 Hz, 2H), 5.36 (s, 2H), 4.32 (t, *J* = 6.7 Hz, 2H), 4.17 – 4.08 (m, 4H), 3.71 (brs, 2H), 3.61 (s, 2H), 3.48 (s, 2H), 2.85–2.80 (m, 2H), 2.79–2.71 (m, 4H), 2.66 (brs, 2H), 2.55–2.52 (m, 2H), 1.87 (s, 12H), 1.80–1.73 (m, 4H), 1.44–1.38 (m, 2H), 1.30–1.26 (m, 6H), 1.23 (s, 2H), 1.18 (brs, 2H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 168.51, 168.01, 162.41, 145.03,141.61, 140.12, 137.74, 136.24, 133.91, 131.75, 130.83, 130.37, 129.95, 129.80, 129.65, 127.96, 127.33, 127.19, 126.44, 126.31, 125.28, 123.51, 122.01, 121.72, 120.61, 119.75, 118.79, 117.35, 110.57, 109.52, 107.62, 93.60, 56.94, 54.90, 49.95, 49.81, 49.65, 49.09, 48.98, 48.89, 45.35, 30.50, 29.70, 27.63, 25.69, 25.63, 24.70, 23.48, 22.37, 21.40, 13.89, 11.67.

3. Supplementary Figures

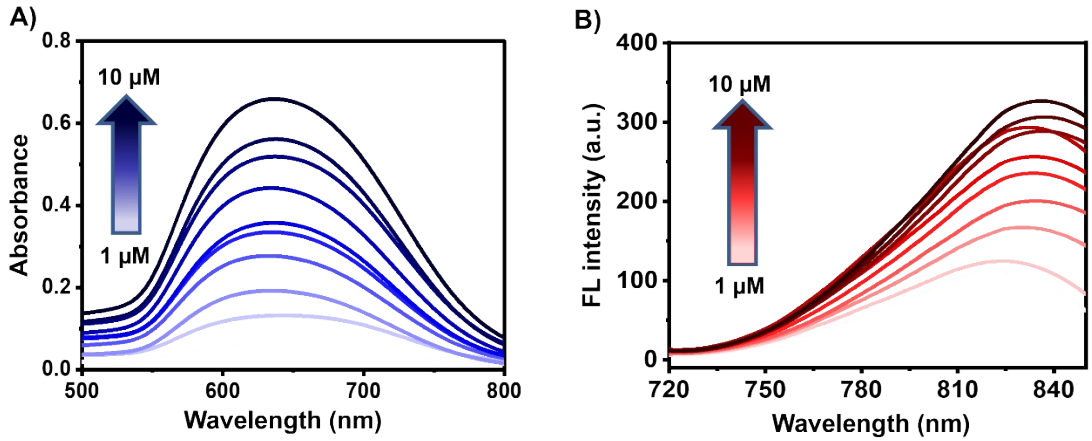

Figure S1. A) Absorbance and B) fluorescence spectrum (Ex: 650 nm) of different concentrations of **T1** (1–10 μM) in PBS solution (1% DMSO) [pH = 7.4, 37°C].

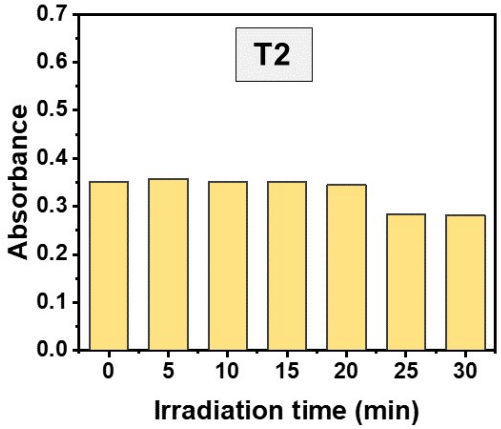

Figure S2. Photostability test of **T2** (10 μM) in PBS (0.01 M, pH = 7.4). White LED light; power density: 20 mW/cm^2.

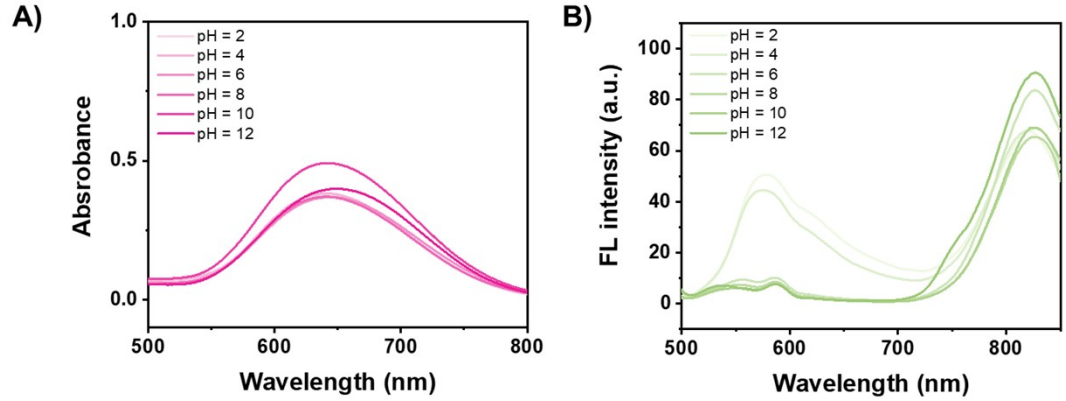

Figure S3. A) Absorption and B) fluorescence spectra of **T2** (10 μM) in various pH condition solvents. pH was adjusted by hydrochloric acid or sodium hydroxide to the pH 2, 4, 6, 8, 10, 12 in distilled water.

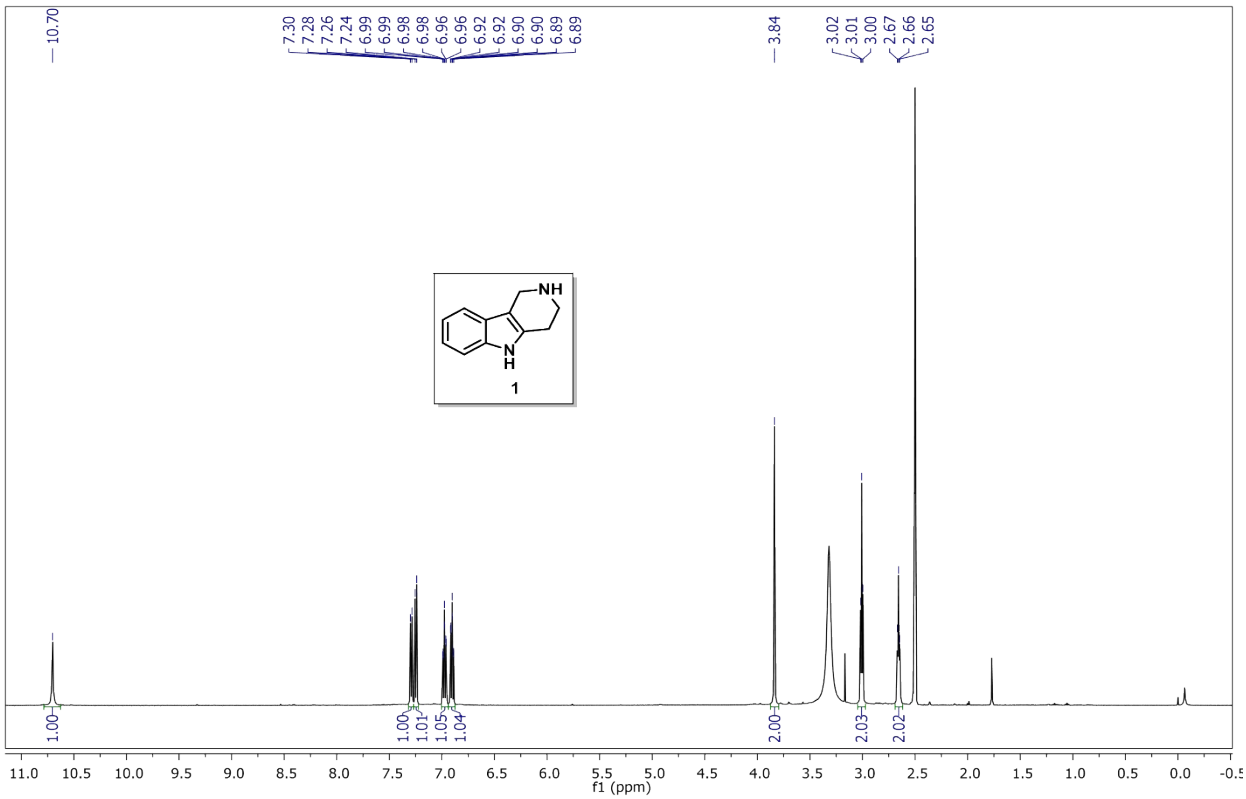

Figure S4. ^{1}H NMR spectrum of **1**

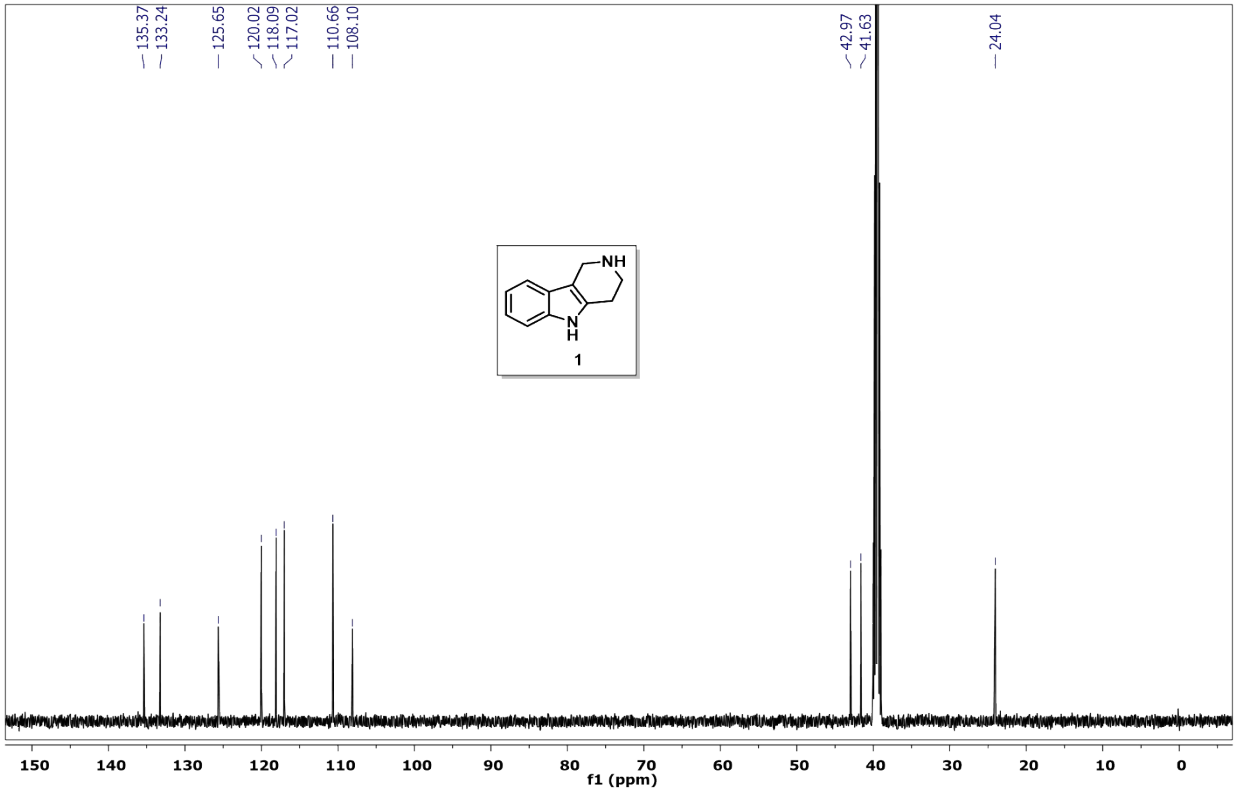

Figure S5. ^{13}C NMR spectrum of **1**

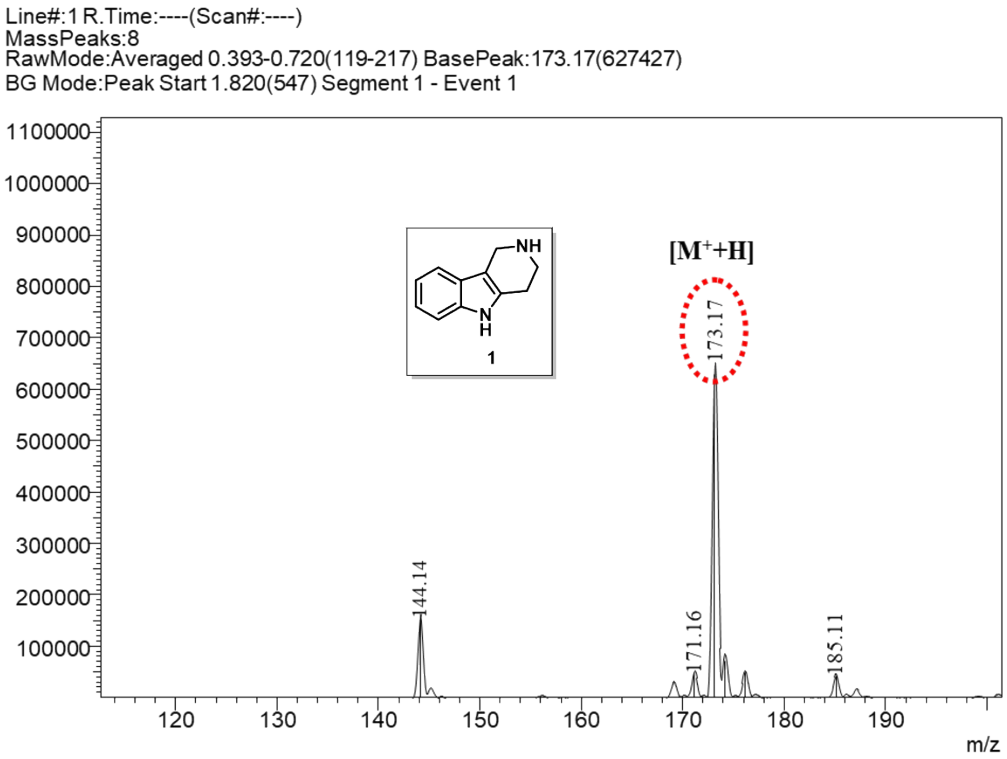

Figure S6. ESI-MS spectrum of **1**

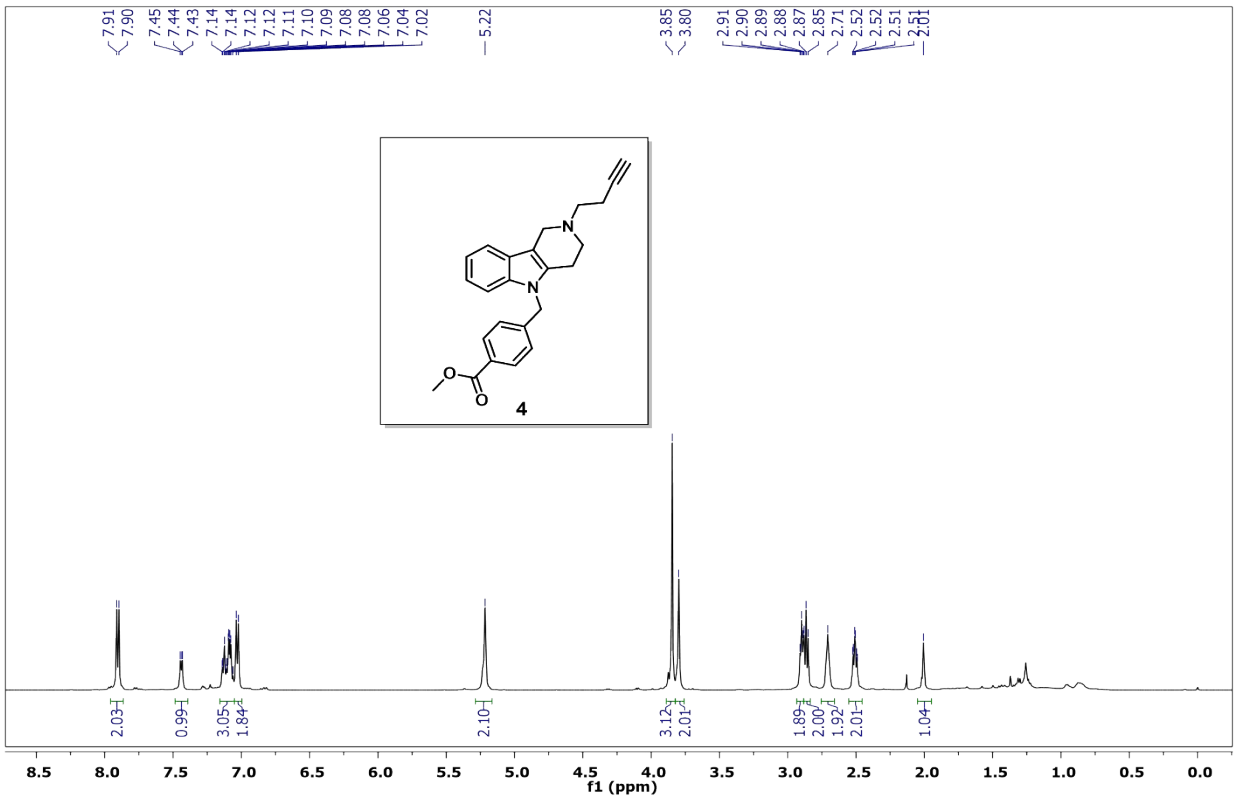

Figure S7. ^{1}H NMR spectrum of **4**

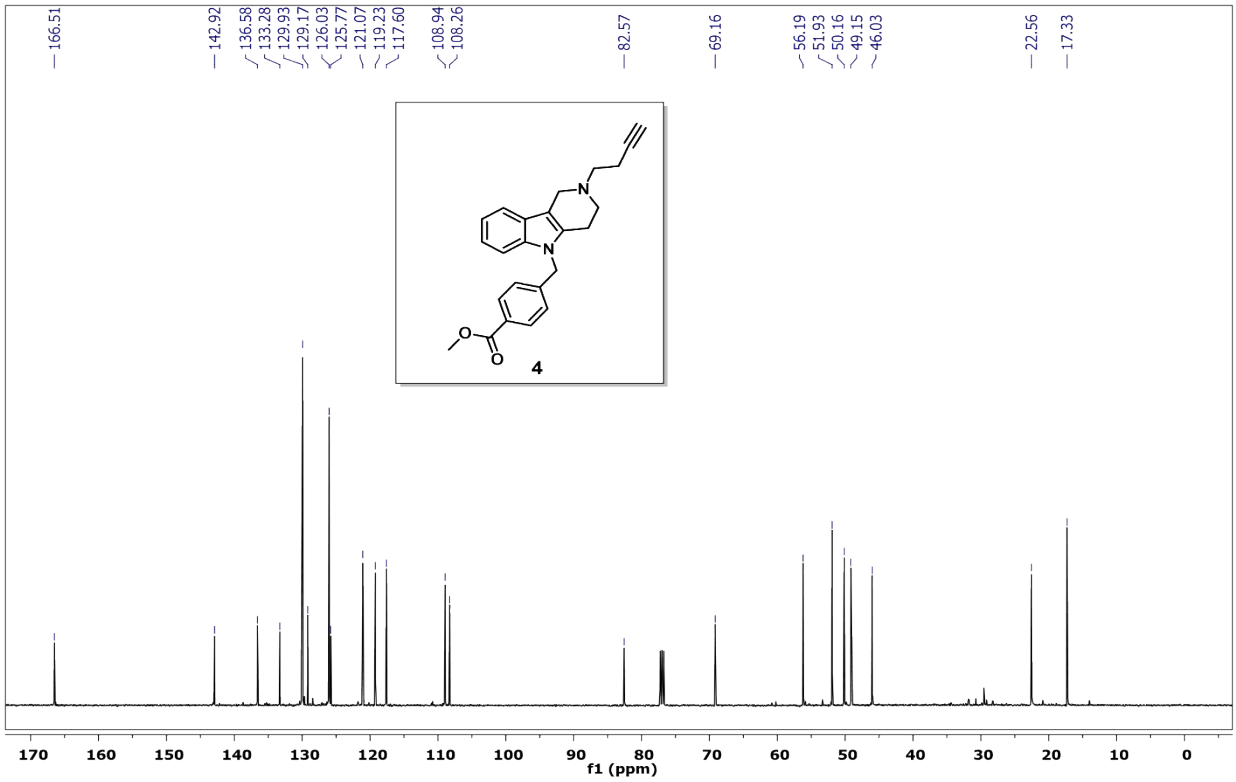

Figure S8. ^{13}C NMR spectrum of **4**

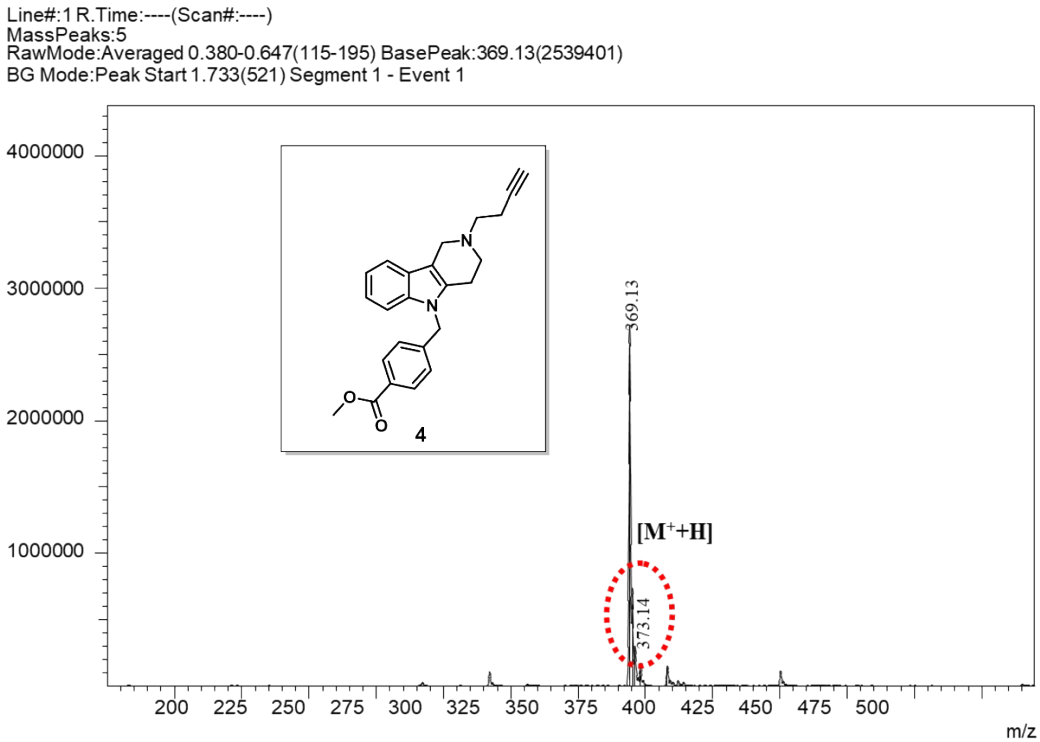

Figure S9. ESI-MS spectrum of **4**

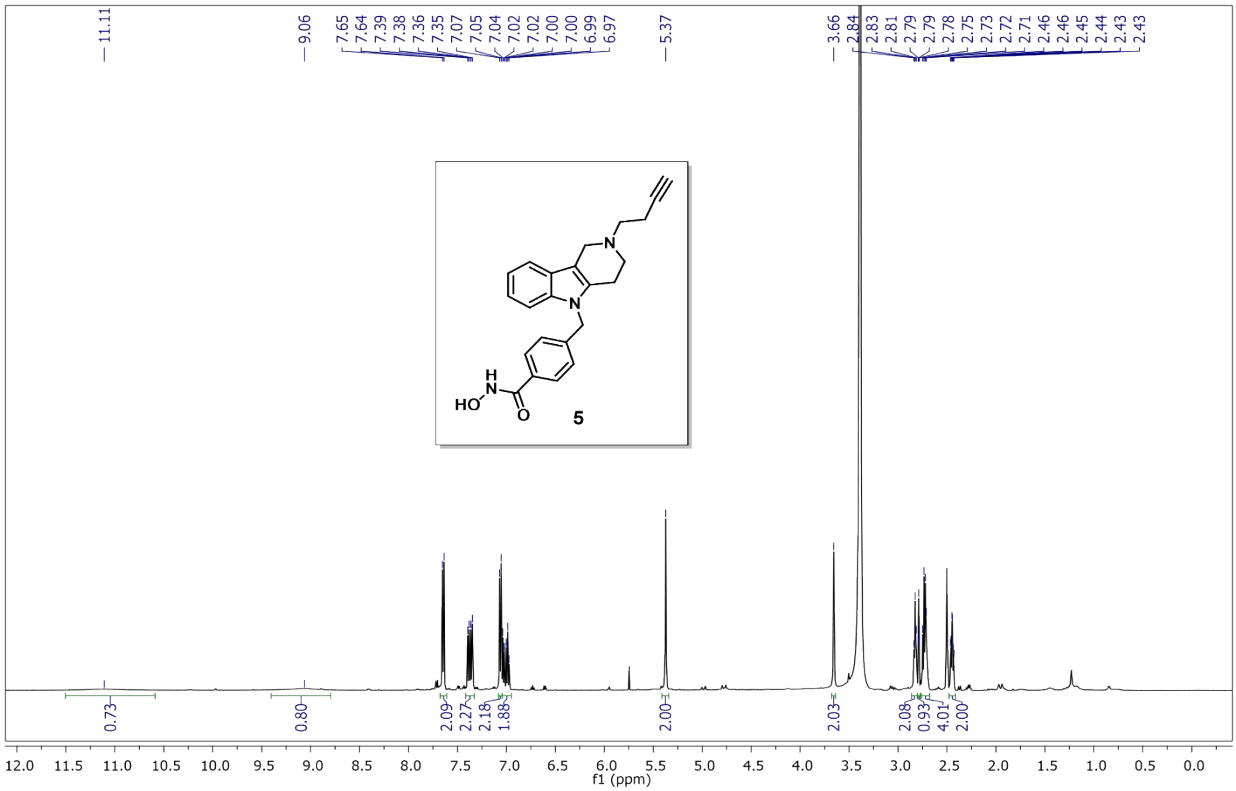

Figure S10. ^{1}H NMR spectrum of **5**

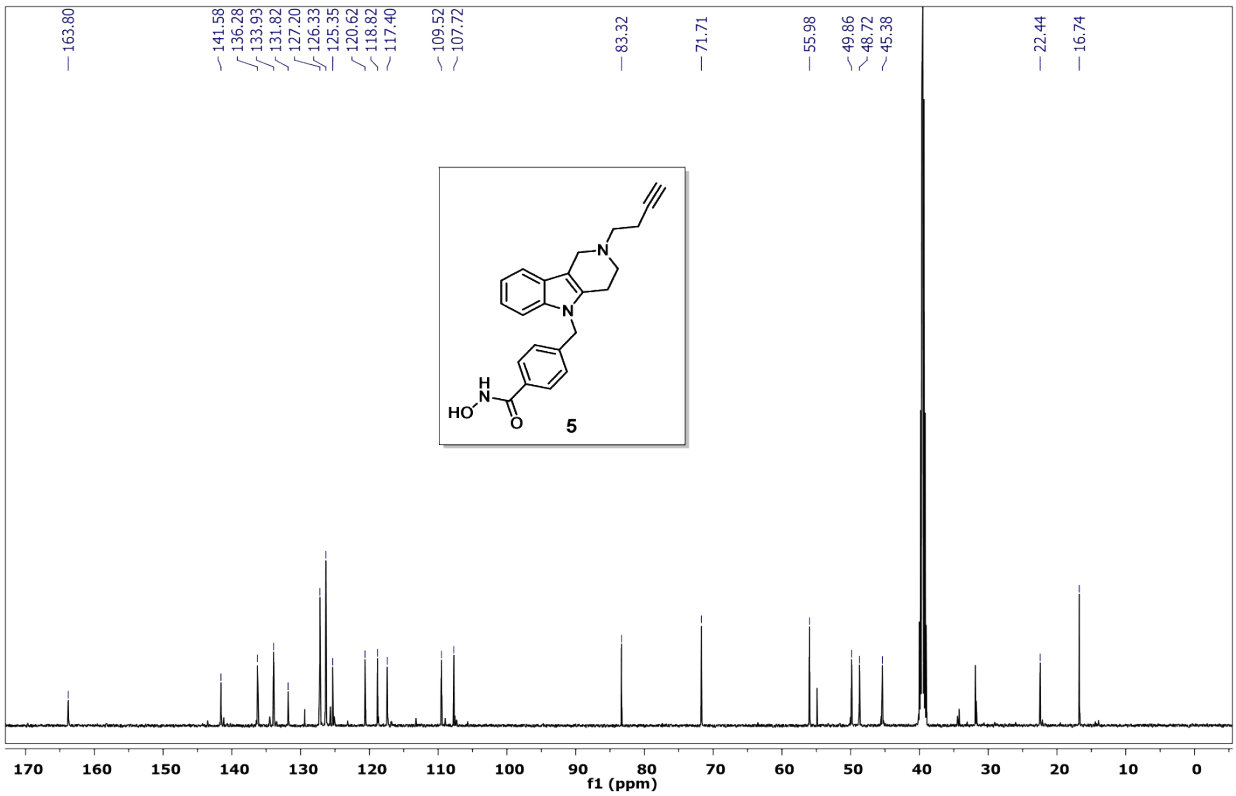

Figure S11. ^{13}C NMR spectrum of **5**

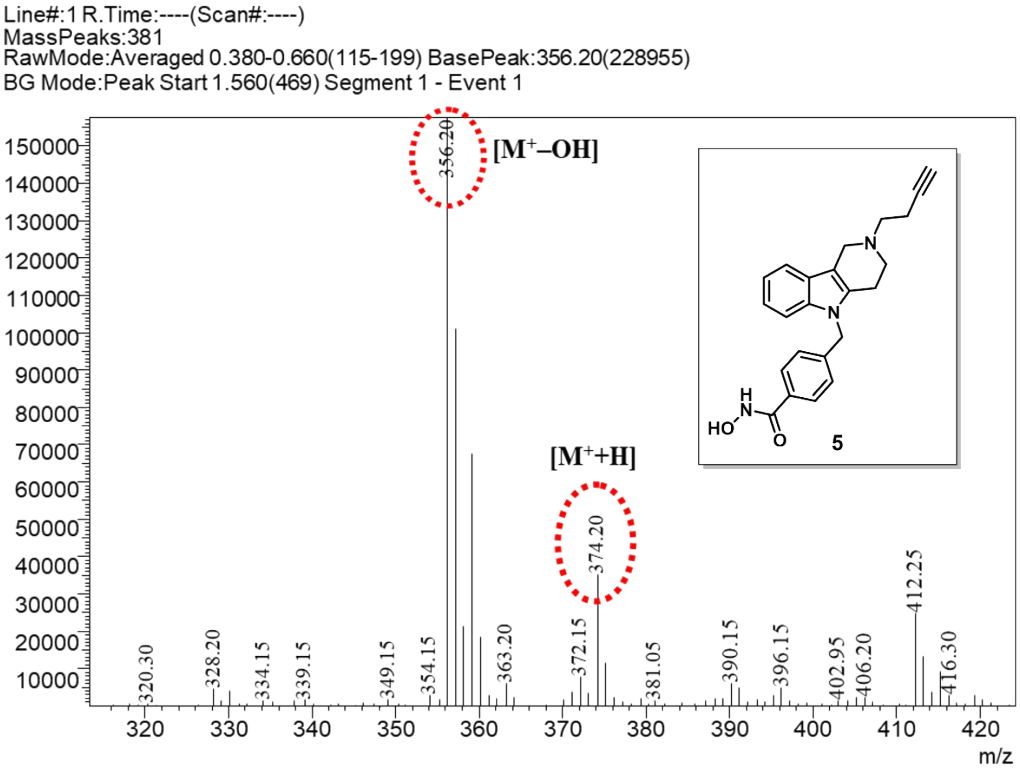

Figure S12. ESI-MS spectrum of **5**

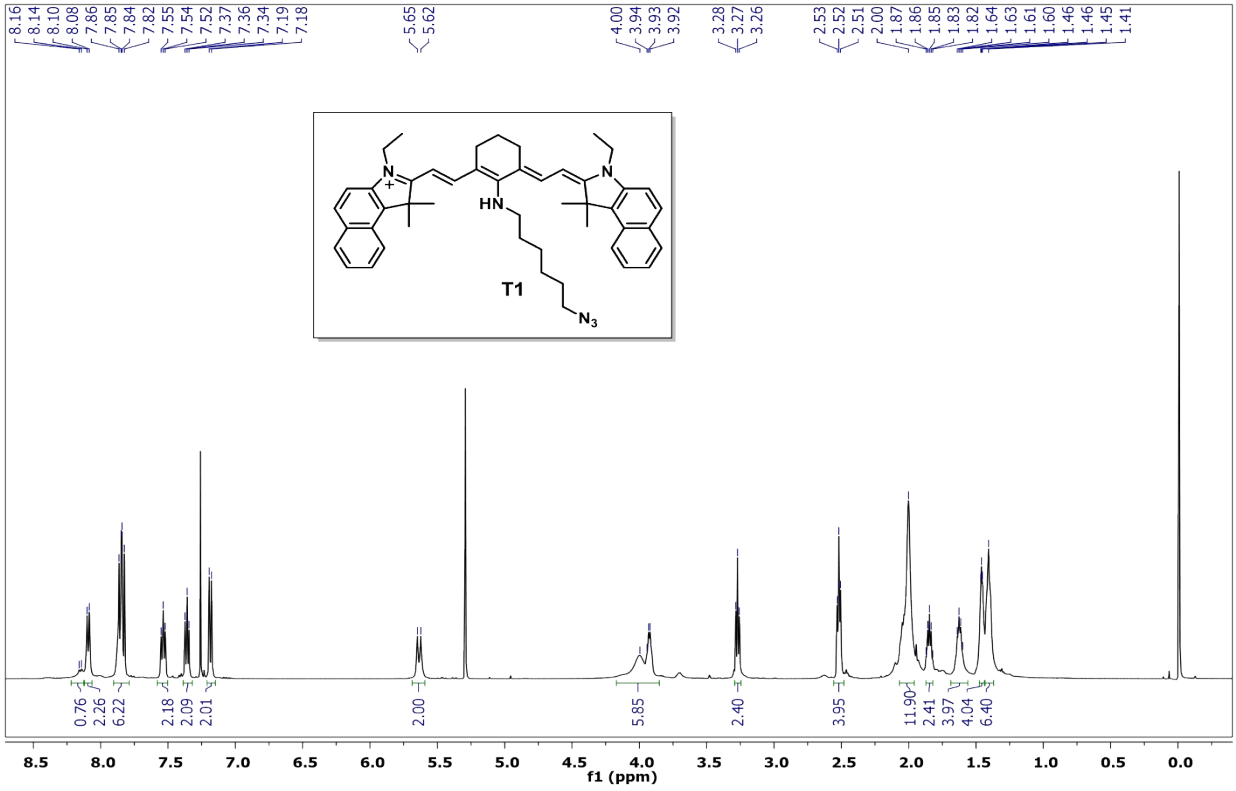

Figure S13. ^{1}H NMR spectrum of **T1**

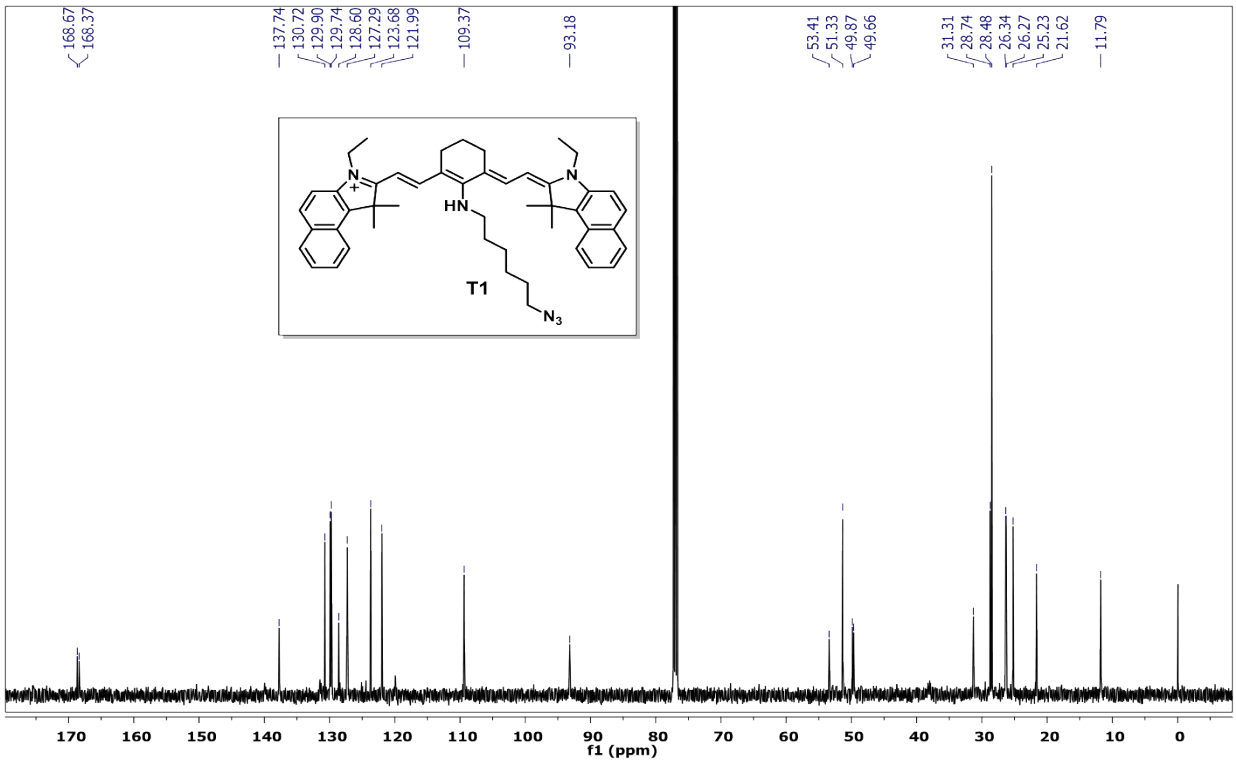

Figure S14. ^{13}C NMR spectrum of **T1**

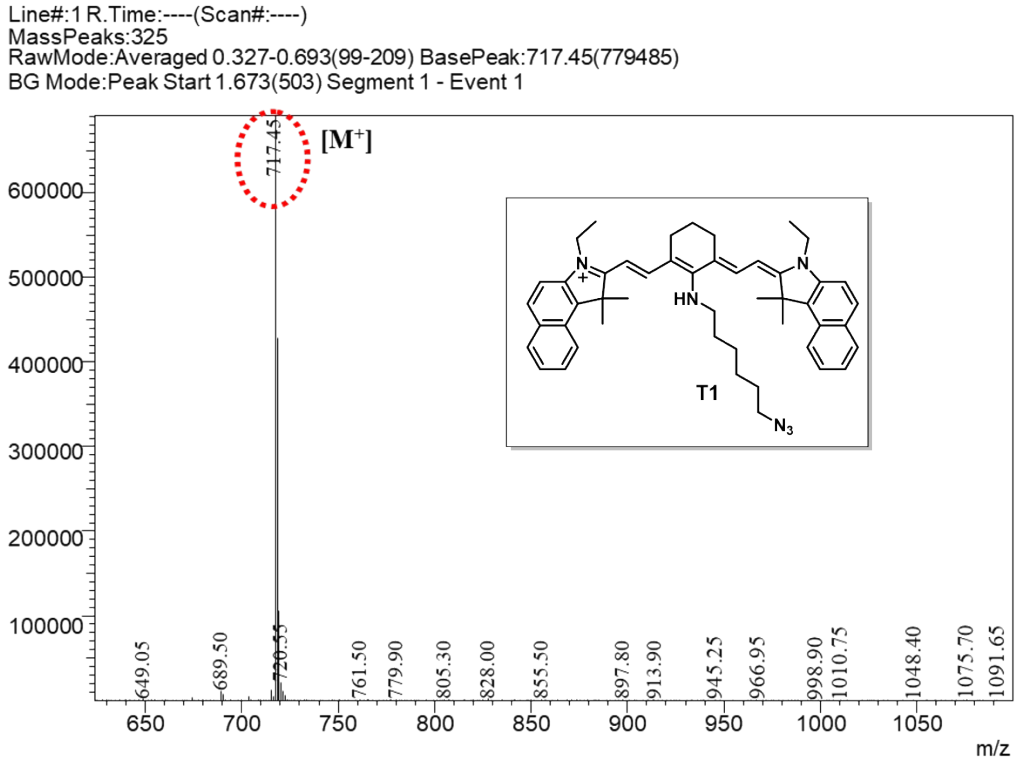

Figure S15. ESI-MS spectrum of **T1**

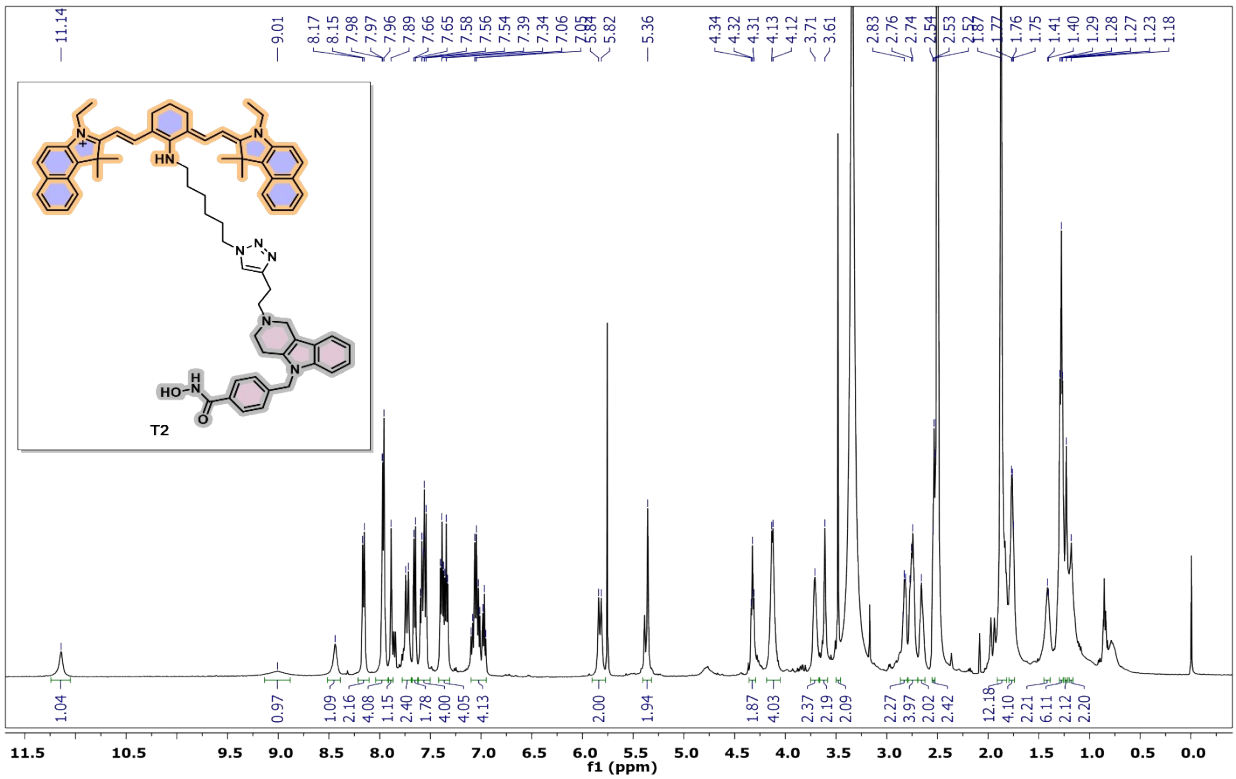

Figure S16. ^{1}H NMR spectrum of **T2**

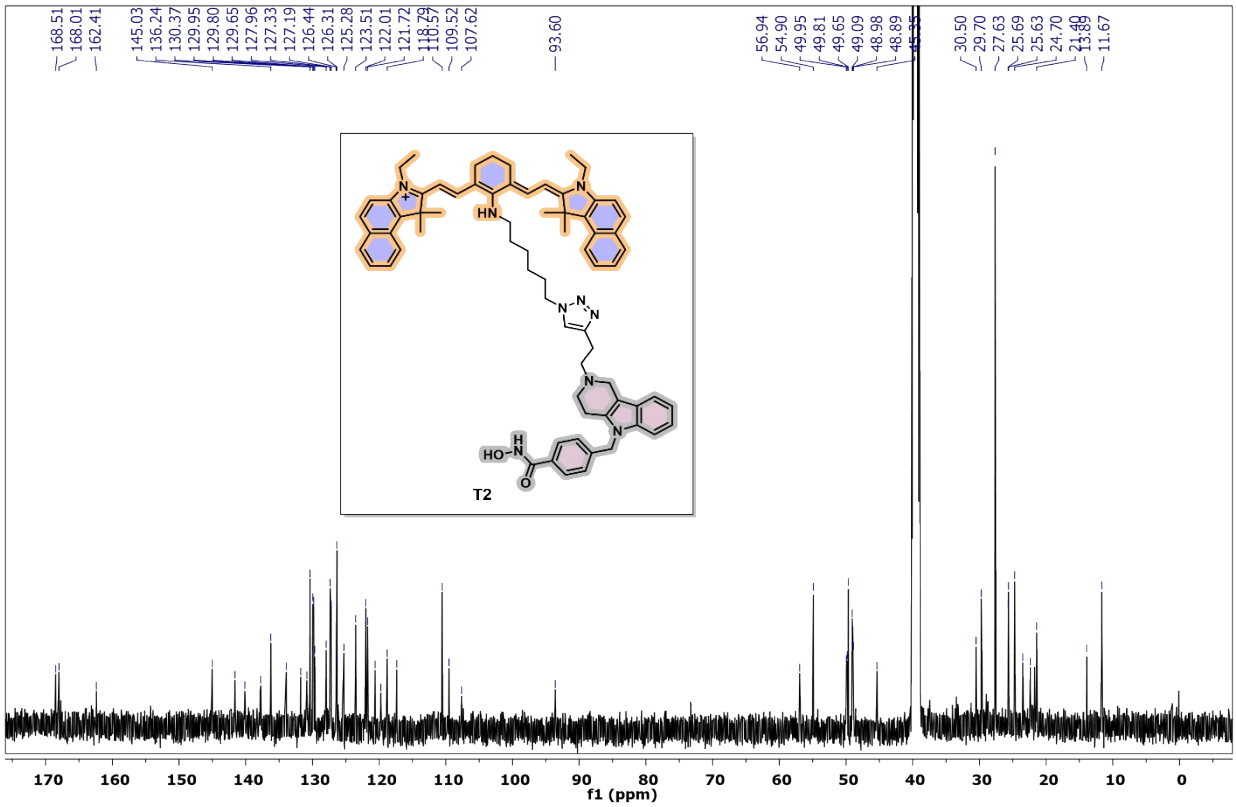

Figure S17. ^{13}C NMR spectrum of **T2**

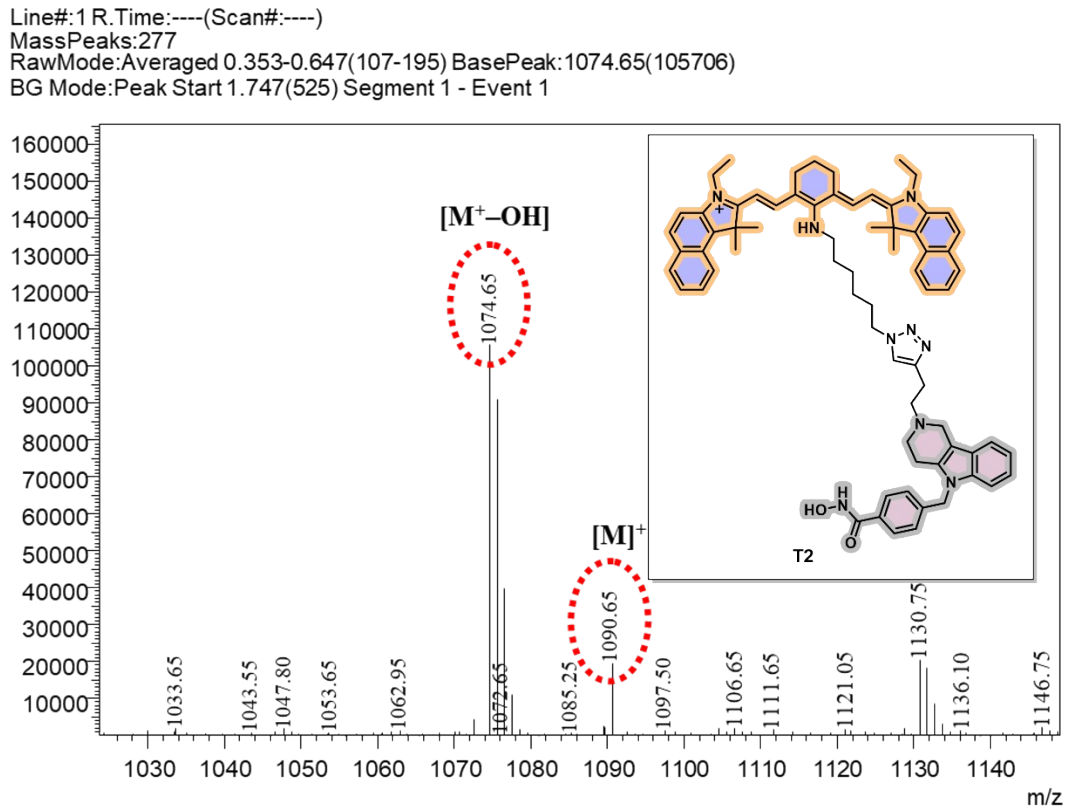

Figure S18. ESI-MS spectrum of **T2**

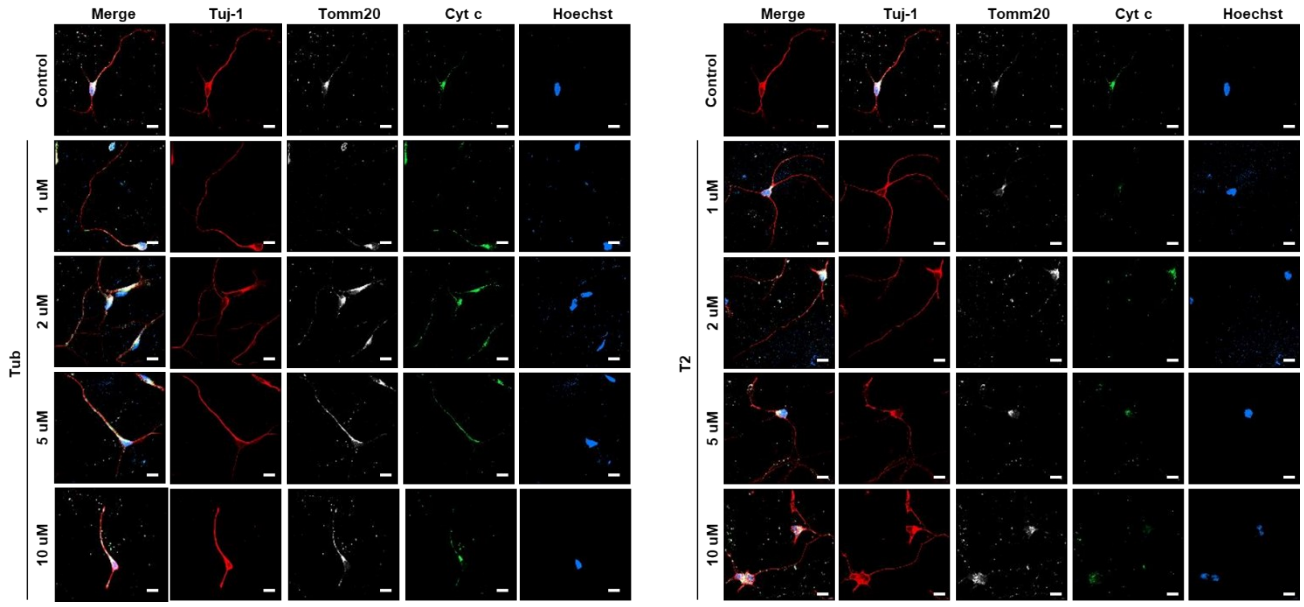

Figure S19. Representative images from immunofluorescence staining of Tuj-1, Tomm20, cytochrome C, and Hoechst in cortical neurons treated with Tuba or Tuba-Cy. Scale bar = 10 μm.

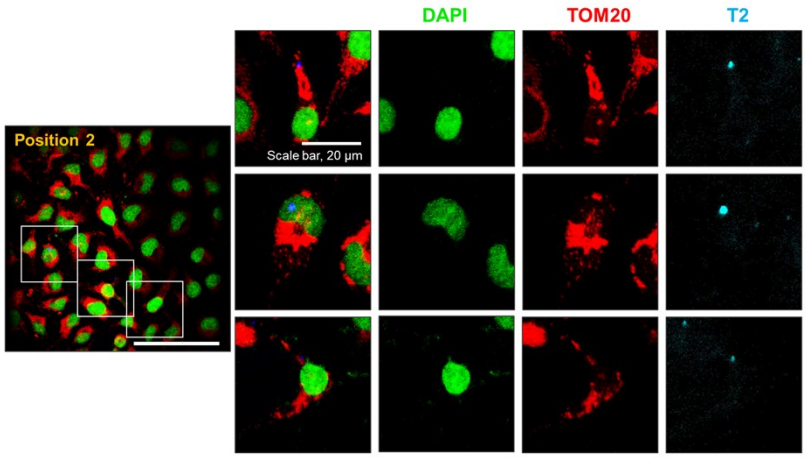

Figure S20. Confocal microscopy images of HeLa cells treated with **T2** (5 mM) for 24h. Scale bar: 20 μm**.**

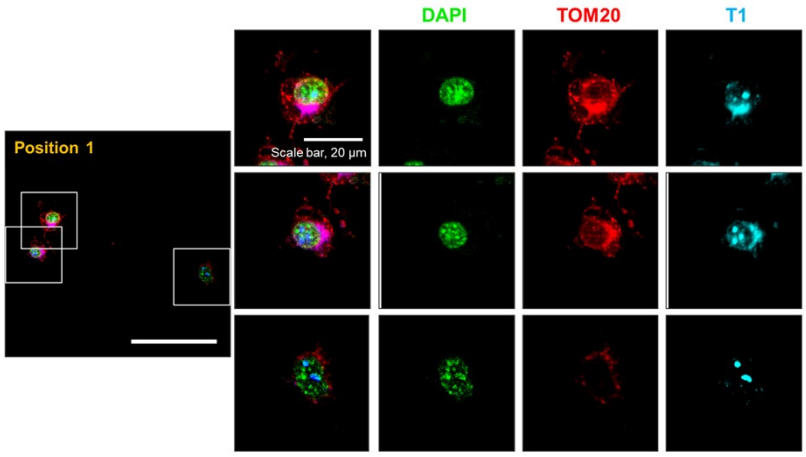

Figure S21. Confocal microscopy images of HeLa cells treated with **T2** (5 mM) for 24h. Scale bar: 20 μm

4. References

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