Supporting Information

Self-delivery of *N*-hydroxylethyl Peptide Assemblies to the Cytosol Inducing Endoplasmic Reticulum Dilation in Cancer Cells

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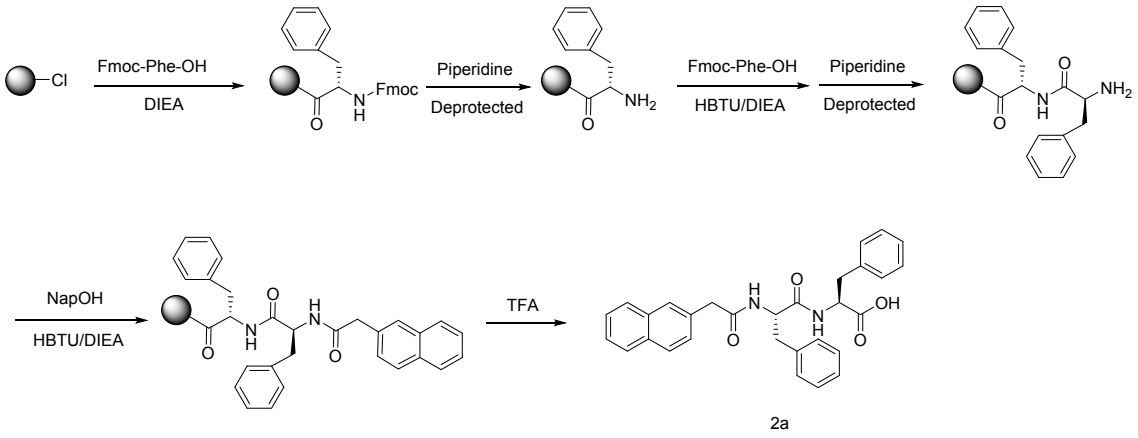
Contents

Synthesis

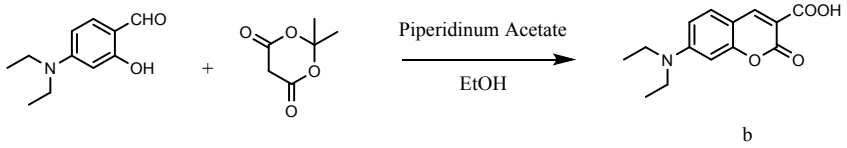
Materials and instruments: Fmoc-Amino acid and 2-chlorotrityl chloride resin were purchased from GL Biochem (Shanghai, China); Dimethylformamide (DMF), Dichloromethane (DCM), Tetrahydrofuran (THF), methanol, ethanol, hexane were purchased from FUJIFILM Wako Pure Chemical Corporation, Japan; *N*,*N*-diisopropylethylamine (DIEA), 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (98.5%), trifluoroacetic acid (TFA) (98%), piperidine, *N*-hydroxysuccinimide (NHS, 98%), N,N'-diisopropylcarbodiimide (DIC, 99%), *N*-(3-dimethylaminopropyl)-*N*'-ethylcarbodiimide hydrochloride (EDC, 98%), 1-Hydroxybenzotriazole hydrate (HOBT, 97%), 4-dimethylaminopyridine (DMAP), ethylene glycol, ethanolamine, *N*-Boc-ethylenediamine (98%), Meldrum's acid (98%) were purchased from Sigma Aldrich. Piperidinum acetate, 4-(diethylamino)salicylaldehyde (98%) were obtained from Tokyo Chemistry Industry (TCI). Organic solvents were dehydrated using solvent purification system. HPLC purification was performed on a Agilent 1260 Infinity Preparative Pump with Agilent 1260 Infinity Diode Array Detector. A 19 mm×150 mm XBridge® Peptide BEH C18 column (Waters) was used for semi-preparative HPLC purification. Mass spectra were recorded using a Thermo LTQ-ETD mass spectrometer (ESI-MS). ^{1}H and ^{13}C NMR spectra were recorded on a JEOL 400 (400 MHz and 101MHz, respectively) spectrometer.



Scheme S1. Synthesis of compound **2a**.

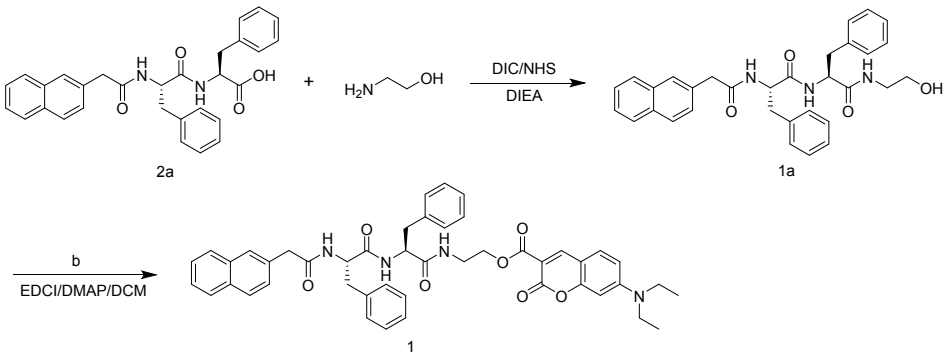
Compound 2a: Compound 2a was obtained via solid phase peptide synthesis (SPPS). 2-Chlorotrityl chloride resin (2.0 g, 2mmol) was swelled in anhydrous DCM for 20 min, L-Fmoc-Phe-OH (1.94g, 5mmol) dissolved in anhydrous DMF was conjugated to resin with DIEA in 30 min. Then washed with anhydrous DMF for 3 times, unreacted sites in the resin were blocked with DCM/MeOH/DIEA (80:15:5) for 20 min and washed by anhydrous DMF for 5 times. Then piperidine solution (20% in DMF) was added into reaction for 30 min to remove the Fmoc protecting group on the amino acid. We carried out the peptide elongation according to standard SPPS protocol to get the desired compound. 2a was cleaved

off the resin using TFA for 2 h. After removing the solvent, anhydrous ether was added into the concentrated product and white precipitation of 2a was obtained.[1]



Scheme S2. Synthesis of compound **b**.

Compound b was synthesized by following the published method.[2]



Scheme S3. Synthesis of compound **1**.

Compound 1a: It was synthesized by following the published method.[3]

^{1}HNMR (400 MHz, DMSO-d_6) δ 7.59 (d, J = 6.7 Hz, 1H), 7.49 (d, J = 6.6 Hz, 1H), 7.29 (t, J = 4.5 Hz, 1H), 7.25 (d, J = 6.4 Hz, 1H), 7.20 (d, J = 6.3 Hz, 1H), 7.17 (d, J = 6.8 Hz, 1H), 7.05 (s, 1H), 6.97 – 6.91 (m, 2H), 6.76 – 6.68 (m, 12H), 4.69 (t, J = 4.3 Hz, 1H), 4.60 – 4.53 (m, 2H), 3.83 (dd, J = 11.2, 3.4 Hz, 1H), 3.79 – 3.76 (m, 1H), 3.48 – 3.43 (m, 2H), 3.36 – 3.31 (m, 2H), 3.22 (dd, J = 11.0, 6.9 Hz, 1H), 3.15 (dd, J = 11.0, 7.9 Hz, 1H).

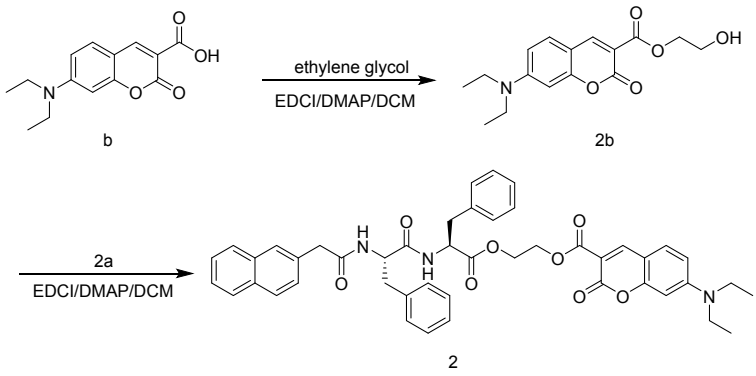
Compound 1: In a 100mL round-bottom flask, compound b (0.35g, 1.4mmol) was dissolved in 30mL anhydrous DCM. After adding EDCI (0.41g, 2.2mmol) and DMAP (17mg, 0.14mmol) in to the flask, the mixture was kept stirring at room temperature for 5 min. Compound 1a (0.7g, 1.3mmol) was transferred into the reaction flask, and kept stirring at room temperature overnight. After removing the solvent, the crude product was purified by silica gel column chromatography to get the target compound (yield: 72%).

^{1}HNMR (400 MHz, DMSO-d_6) δ 7.81 (s, 1H), 7.58 (dd, J = 19.9 Hz, 6.5 Hz, 2H), 7.52 – 7.43 (m, 1H), 7.24 (d, J = 5.9 Hz, 1H), 7.20 – 7.12 (m, 2H), 7.05 (d, J = 4.3 Hz, 1H), 7.01 – 6.89 (m, 3H), 6.78 – 6.63 (m, 11H), 6.36 – 6.28 (m, 1H), 6.16 (d, J = 1.3 Hz, 1H), 4.62 – 4.54 (m, 2H), 4.28 – 4.21 (m, 1H), 3.86 –

3.81 (m, 3H), 3.76 – 3.70 (m, 5H), 3.50 – 3.44 (m, 1H), 3.40 – 3.30 (m, 2H), 3.28 – 3.20 (m, 2H), 3.19 – 3.10 (m, 2H), 1.86 (t, *J* = 5.5 Hz, 6H) ppm.

13CNMR(101 MHz, DMSO-d_6) δ 157.25, 156.98, 156.37, 156.31, 150.71, 146.83, 146.04, 142.68, 140.04, 130.57, 130.50, 130.46, 127.52, 126.73, 125.78, 125.74, 123.80, 123.77, 123.73, 122.85, 122.76, 122.71, 122.48, 122.36, 122.29, 122.20, 121.44, 121.35, 121.30, 121.19, 120.77, 108.25, 105.95, 97.08, 70.69, 68.16, 63.58, 55.90, 54.19, 53.58, 50.35, 49.04, 45.03, 30.27 ppm.

MS: calculated for $C_{46}H_{48}N_4O_7$ is 768.91, found 767.18, $[M - H]^-$.



Scheme S4. Synthesis of compound **2**.

Compound 2b: Compound b (1.0g, 3.8mmol), EDC (0.81g, 4.2mmol), and DMAP (50mg, 0.4 mmol) were transferred into a 100mL round-bottom flask, dissolved by 30mL anhydrous DCM. Then ethylene glycol (2.36g, 38mmol) diluted in 5mL anhydrous DCM was added into the reaction flask. The reaction mixture was stirred at room temperature for 36h. The reaction mixture was diluted by 50mL EtOAc, and washed by 25mL water twice. The organic layer was collected and dried using anhydrous Na_2SO_4 for 2h. Silica gel column chromatography was applied for purification to obtain the target compound (yield: 71%).

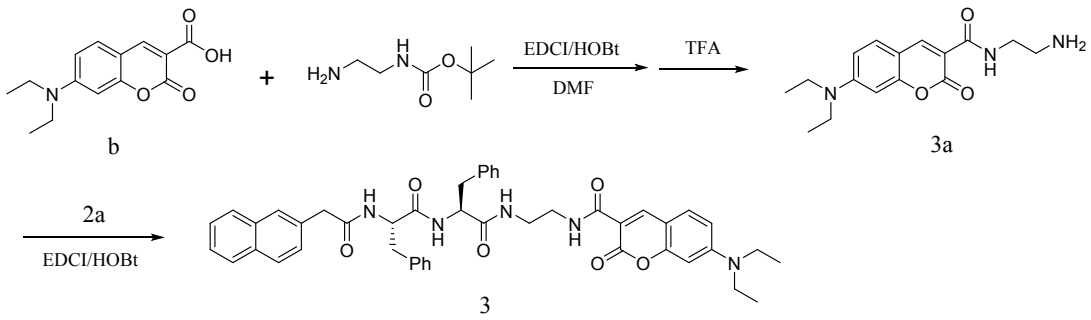
^{1}HNMR (400 MHz, DMSO-d_6) δ 8.55 (s, 1H), 7.58 (d, *J* = 9.0 Hz, 1H), 6.74 (dd, J = 9.0, 2.4 Hz, 1H), 6.51 (d, *J* = 2.3 Hz, 1H), 4.82 (m, 1H), 4.16 – 4.13 (m, 2H), 3.62 (m, 2H), 3.44 (q, *J* = 7.1 Hz, 4H), 1.10 (t, J = 7.1 Hz, 6H) ppm.

Compound 2: In a 100mL round-bottom flask, compound 2a (0.3g, 1.0mmol) was dissolved in 30mL anhydrous DCM. After adding EDC (0.21g, 1.1mmol) and DMAP (15mg, 0.1mmol), the reaction mixture was kept stirring at room temperature for 5 min. Compound 2b (0.57g, 1.2mmol) was transferred into the reaction flask, and kept stirring at room temperature overnight. After removing the solvents, the crude product was purified using silica gel column chromatography to get the target compound as yellow solid (yield: 68%).

^{1}HNMR (400 MHz, DMSO-d_6) δ 7.85 (dd, J = 22.1 Hz, 6.2, 1H), 7.78 (d, J = 12.0 Hz, 1H), 7.59 (dd, J = 17.9 Hz, 6.9, 1H), 7.23 (d, J = 5.7 Hz, 1H), 7.18 – 7.11 (m, 2H), 7.05 – 6.98 (m, 2H), 6.96 – 6.89 (m, 2H), 6.77 – 6.63 (m, 10H), 6.61 – 6.55 (m, 1H), 6.33 (ddd, J =11.7, 7.2, 4.7 Hz, 1H), 6.17 (dd, J = 8.4, 1.7 Hz, 1H), 4.65 – 4.55 (m, 2H), 4.51 – 4.39 (m, 4H), 3.80 (dt, J = 12.5, 6.2 Hz, 1H), 3.77 (m, 5H), 3.63 (s, 2H), 3.49 – 3.39 (m, 1H), 3.36 – 3.22 (m, 2H), 3.16 – 3.06 (m, 1H), 1.87 (t, J = 5.5 Hz, 6H) ppm.

^{13}CNMR (101 MHz, DMSO-d_6) δ 157.69, 157.51, 157.35, 156.18, 150.82, 146.90, 145.97, 142.75, 140.05, 130.55, 130.49, 130.11, 130.00, 127.55, 126.71, 125.90, 125.86, 125.75, 123.80, 123.73, 123.66, 122.99, 122.69, 122.67, 122.46, 122.34, 122.26, 122.17, 121.65, 121.27, 121.17, 120.75, 108.28, 105.98, 97.08, 70.56, 70.16, 63.25, 55.91, 54.13, 50.68, 49.84, 30.28 ppm.

MS: calculated for $C_{46}H_{47}N_3O_8$ is 769.90, found 768.18, $[M - H]^-$.



Scheme S5. Synthesis of compound **3**.

Compound 3a was synthesized by following the published method.[4]

Compound 3: In a 100mL round-bottom flask, compound 2a (0.39g, 0.8mmol), HOBt (0.14g, 1.1mmol) and EDCI (0.2g, 1.2mmol) was dissolved in 30mL anhydrous DMF, and kept stirring at room temperature for 5 min. Compound 3a (0.32g, 1.1mmol) was transferred into the reaction flask, and kept stirring at room temperature for 24h. After removing the solvent, the crude product was purified by silica gel column chromatography to get the target compound (yield: 75%).

^{1}HNMR (400 MHz, DMSO-d_6) δ 7.94 (t, J = 4.6 Hz, 1H), 7.88 (s, 1H), 7.59 (d, J = 6.8 Hz, 1H), 7.44 – 7.37 (m, 1H), 7.27 – 7.23 (m, 1H), 7.21 – 7.13 (m, 2H), 7.10 – 7.01 (m, 2H), 6.98 – 6.89 (m, 2H), 6.75 – 6.63 (m, 11H), 6.38 (dd, J = 7.3, 1.8 Hz, 1H), 6.23 (d, J = 1.8 Hz, 1H), 4.64 – 4.48 (m, 2H), 3.81 - 3.75 (m, 5H), 3.66 – 3.60 (m, 3H), 3.59 – 3.54 (m, 2H), 3.54 – 3.47 (m, 2H), 3.35 – 3.29 (m, 2H), 3.26 – 3.06 (m, 2H), 1.87 (t, J = 5.6 Hz, 6H) ppm.

^{13}CNMR(101 MHz, DMSO-d_6) δ 160.00, 157.14, 156.24, 150.48, 149.66, 149.17, 146.20, 142.36, 138.60, 130.63, 130.53, 127.54, 126.73, 125.78, 125.68, 123.79, 123.73, 122.84, 122.71, 122.48, 122.35, 122.30, 122.20, 121.40, 121.29, 121.17, 120.76, 108.49, 107.91, 106.51, 97.09, 63.65, 63.44, 55.87, 54.18, 50.69, 50.35, 30.27 ppm.

MS: calculated for $C_{46}H_{48}N_4O_7$ is 767.93, found 766.18, $[M - H]^-$.

CES catalysed hydrolysis

In buffer

Esterase (CES) from rabbit liver lyophilized powder was purchased from Sigma-Aldrich (E0887-500UN). Following the instruction, CES stock solution (1u/5μL) was prepared in borate buffer (pH 8.0). Stock solutions of compound **1**, **2**, or **3** (40 mM in DMSO) were diluted in borate buffer to the desired concentration. Proper volume of CES stock solution was added into 200 μL of compound solution reaching final concentration of 1U. HPLC and LCMS were applied to identify the reaction products and monitor the progress of hydrolysis at 37°C.

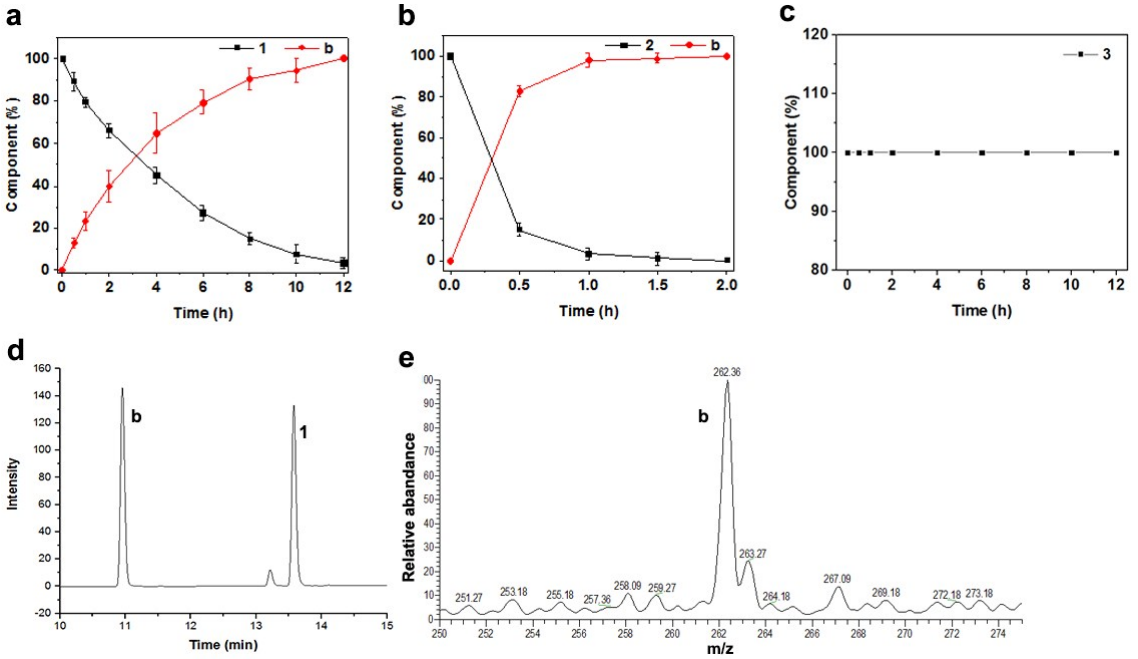


Figure S1. Kinetic profiles of CES catalysed hydrolysis of **1** (a), **2** (b) and **3** (c) in borate buffer at 37°C; (d) HPLC spectrum of HeLa cell lysate after 6h incubation of **1** (50 μM). (e) LCMS spectrum of major product obtained from HeLa cell lysate after 12h incubation of **1** confirming it's molecule **b.**

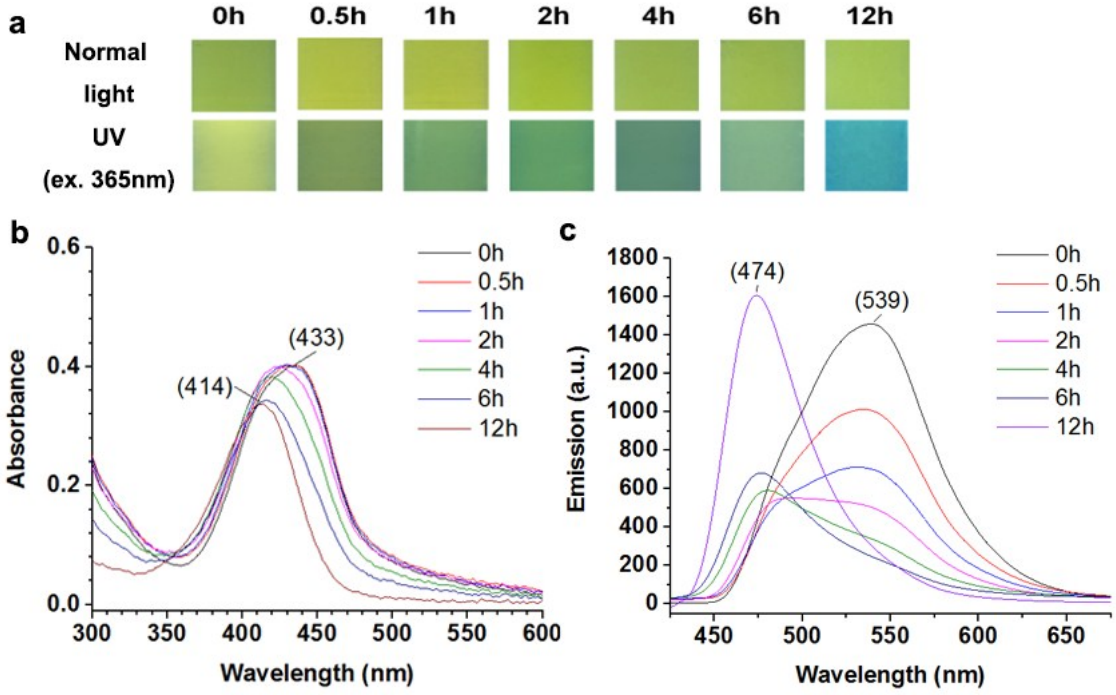
In cell lysate and extracellular culture medium

HeLa cell was cultured in 10 cm polystyrene culture plate. When cells reached approximately 80% confluence in the log phase growth period, aspirated media and gently washed by PBS buffer. Removed the PBS buffer, 2 mL of Trypsin solution was added and incubated at 37°C with a humidified atmosphere of 5% CO_2 for 3 min to detach the cells. 6 mL of cold PBS buffer was added and transferred to a 15 mL tube, centrifuged at 4 °C to obtain the HeLa cells. Cell lysate was obtained by adding 200μL cold CelLytic M solution (Sigma-Aldrich) to the harvested HeLa cells, incubated for 30 min at 0 °C on a shaker. Then centrifuged the lysed cells for 5min at 20000g at 4 °C to collect the supernatant and stored at

-20 °C. The obtained cell lysate was applied to 1, 2 within 3 days to ensure the best enzyme activity. Fresh culture medium and post incubated culture medium that collected from HeLa cell culture after 24h incubation were applied as dilution solution without adding extra CES to study the kinetic profile of extracellular CES catalysed hydrolysis at 37°C, no degradation of the original compound was observed, confirmed by HPLC. Treated HeLa cells were collected after time-dependent incubation with three compounds. Sonication (20s × 5 times) was applied to break the cell membrane, centrifuged at 13000 × g for 5 min, and then collected the supernatant. Further analysed using HPLC-MS to study the intracellular CES catalysed hydrolysis.

UV-Vis Absorption and emission spectroscopy

UV-vis spectra were collected using Thermo Nanodrop 2000C spectrophotometer. The path length of the cuvette was 1 cm. The detection range was set to 250-700 nm and the spectral resolution was set to 1.0 nm. Emission spectra were collected using Hamamatsu fluorescence spectrometer. The excitation wavelength was set to 405 nm and emission collection wavelength was set between 415 and 700 nm.



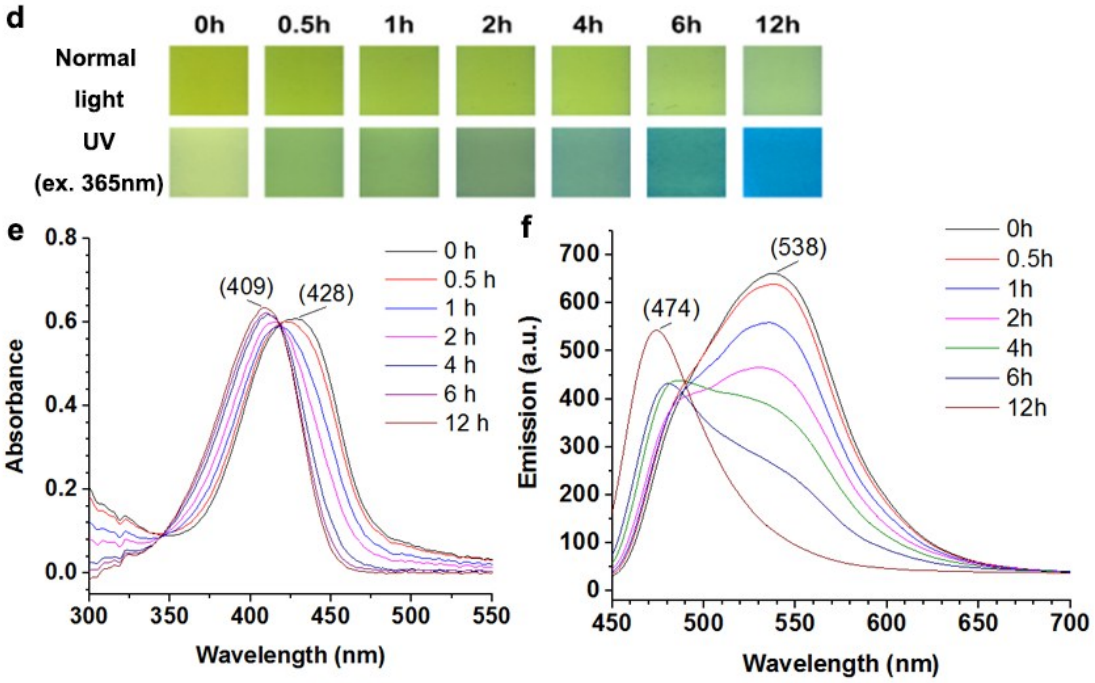


Figure S2. (a) Time-dependent optical images under normal light (upper row) and UV light (bottom row) of **1** (50 μM with 1u of CES) in borate buffer. Time-dependent UV-Vis absorbance (b) and emission spectra (ex. 405nm) (c) of **1** (50 μM with 1u of CES) in borate buffer; Time-dependent optical images under the normal light (upper row) and uv light (bottom row) of 2 (50 μM with 1u of CES) in borate buffer (d). Time-dependent UV-Vis absorbance (e) and emission spectra (ex. 405nm) (f) of 2 (50 μM with 1u of CES) in borate buffer.

1- Octanol/water partition coefficients

Slow-stirring method was applied to determine 1-octanol/water partition coefficients ($P_{Oct/Wat}$). The 1-octanol and Mili-Q water were mutually saturated before using for the experiments. Varied concentrations of 1, 2, and 3 were dissolved in 1-octanol and UV-Vis absorption spectra were measuerd to obtain the working standard curves. Briefly, PO/W was obtained by dissolving 1, 2, 3 in 1-octanol, then mixed with same volume of Mili-Q water. The mixture was vigorously stirred for 24h and left to settle until complete separation of the two phases. UV-Vis absorbance was determined in the 1-octanol layer, and further compared with working standard to obtain $P_{Oct/Wat}$ value.

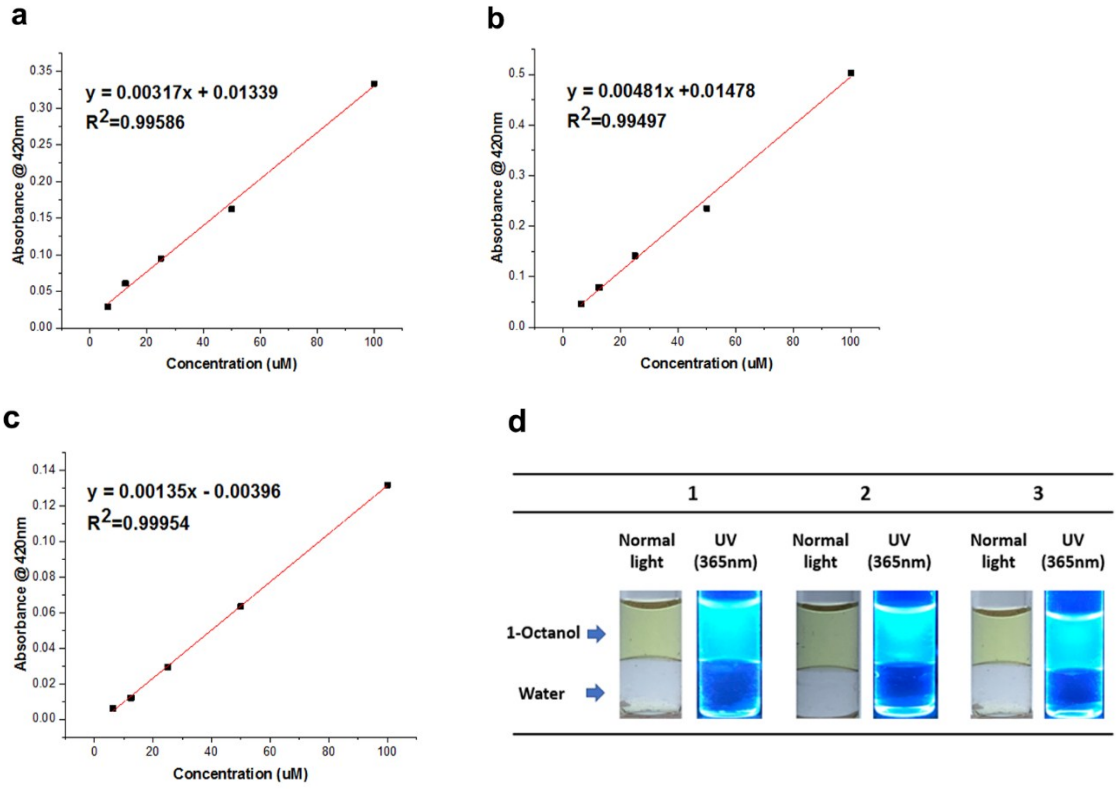


Figure S3. Working standard curve of 1(a), 2(b), and 3(c) obtained from UV-Vis absorption spectra; Normal light and UV images of 1, 2, and 3 after complete separation of the two phases.

TEM of molecular assembly in buffer

Carbon-coated copper grids were glow discharged to enhance hydrophilicity; then 5 μL of sample solution was placed on the grid, leave the sample on the grid around 60s, removed the excess fluid with a filter paper; washed the grid with 5 μL of distilled water 3 times, removed the excess water with filter paper; 5 μL of 1% uranyl acetate was placed on the grid, after 10s, removed the excess solution by touching the edge of grid using a filter paper; dry the grid at room temperature.

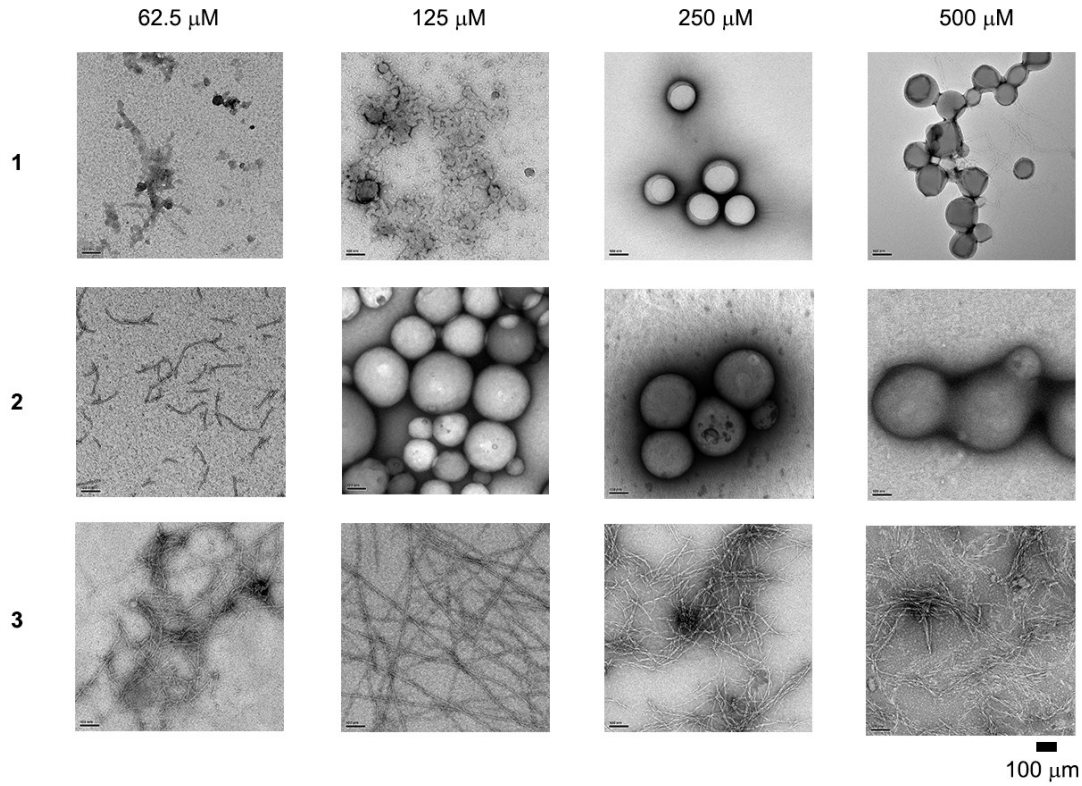


Figure S4. TEM images of concentration-dependent 1, 2 and 3 dissolved in borate buffer/DMSO (v/v = 95:5)

CD spectroscopy

CD spectra were collected on a spectrometer JASCO J-820 with bandwidth of 1.0 nm in the ultraviolet (UV) region (190-350 nm) using a 1 mm quartz cuvette. Theoretical curve was obtained by adding the CD spectra of single components together to get the sum.[5]

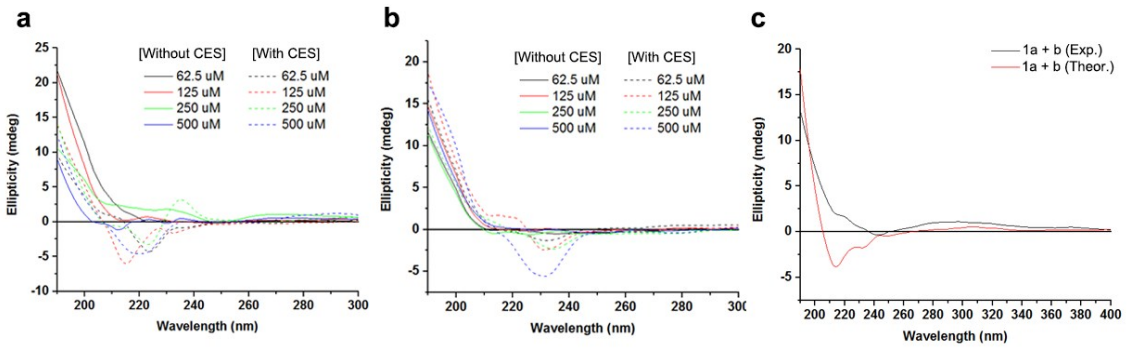


Figure S5. (a-b) CD spectra of 1 and 2 at different concentrations (solid line) and after CES hydrolysis (dash line); (c) CD spectrum of (1a + b) at 125uM in H_2O/DMSO (v/v = 95:5), theoretical spectrum is the simple sum of the spectra obtained from 1a and b in H_2O/DMSO (v/v = 95:5).

Cell culture

HeLa, SKOV3, OVCAR-3, HepG2, PANC-1 cell lines were purchased from American-type Culture Collection (ATCC, USA) and cultured in ATCC recommended media. HeLa and HepG2 cell lines were cultured in DMEM containing 10% FBS, whereas SKOV3 cell line was cultured in McCoy's medium with 10% FBS, OVCAR3 was cultured in RPMI 1640 medium with 20% FBS and PANC1 was cultured in RPMI 1640 with 10% FBS. Incubation was carried out at 37°C with a humidified atmosphere of 5% CO_2. The cells were maintained at 80% confluency and used for the bioassays.

Cell viability assay

Cells in exponential growth phase were seeded in a 96 well plate at a concentration of 1×10^4 cells/well for all cell lines. The cells were allowed to attach to the wells for 12 h at 37ºC, 5% CO_2. The culture medium was removed followed by addition of 100 μL culture medium containing different concentrations (12.5, 25, 50, 100, 200 μM) of compound 1, 2, 3, 4, 5 and 6 (immediately diluted from 80 mM stock solution in DMSO). After the desired time of exposure, 10 μL MTT solution (5 mg/mL) was added to each well and incubated at 37°C for another 4 h, and then 100 μL of SDS solution (10% in Milli-Q water) was added to stop the reduction reaction and dissolve the purple formazan. The absorbance at 570 nm was measured using Nivo3 microplate reader (PerkinElmer). All experiments were performed in triplicate and repeated three times.

Cellular uptake assay

HeLa cells were seeded in a 35 mm culture dish at a concentration of 2×10^5 cells/dish. The cells were allowed to attach to the dish bottom for 12h at 37ºC, 5% CO_2. The culture medium was removed followed by addition of 2 mL culture medium containing a concentration of 10 μM of compound **1**, **1a**, **2**, or **2a**. The concentrations of the remaining compound in the medium were quantified by HPLC at 0, 1, 3, and 6h.

Cellular uptake quantification by flow cytometry

HeLa cells were seeded in a 6 well plate at a concentration of 2×10^5 cells/well. The cells were allowed to attach to the bottom for 12h at 37ºC, 5% CO_2. The culture medium was removed followed by addition of 2 mL culture medium containing a concentration of 10 μM of compound 1 and 2. After treated certain time (0h (ctrl.), 1h, 3h, 6h, 12h and 24h), all cells were harvested and washed with ice PBS twice. 50 μL of ice PBS buffer was adding into the obtained cells, and analysis of fluorescence of individual cells were carried out by flow cytometer (Merck Millipore ImageStream X Mark Ⅱ). 20,000 cells were measured for each experimental condition with excitation wavelength at 405 nm.

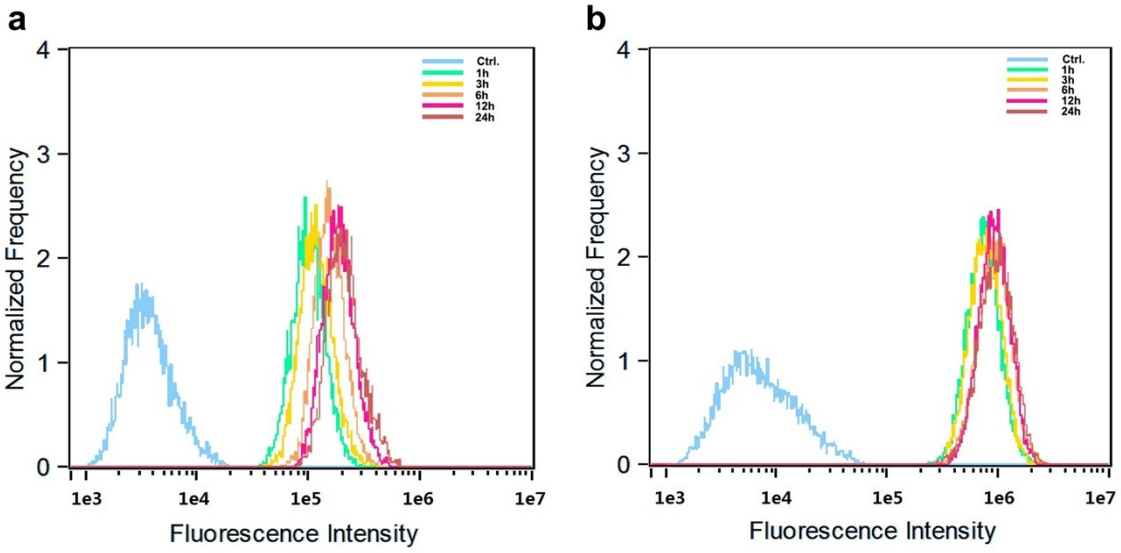


Figure S6. Internalized relative quantification of compound 1 (a) and compound 2 (b) by flow cytometer.

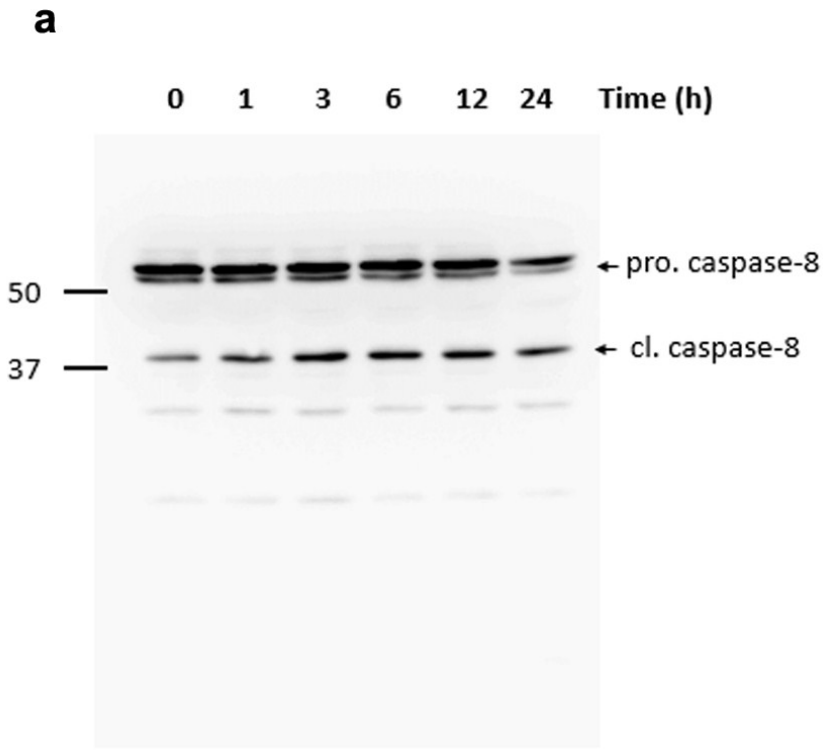
TEM of HeLa cell

HeLa cells were seeded in a 35 mm culture dish at a concentration of 2×10^5 cells/dish. The cells were allowed to attach to the dish bottom for 12h at 37ºC, 5% CO_2. The culture medium was removed followed by addition of 2 mL culture medium containing a concentration of 50 μM of compound 1 and 2. After 6h incubation, the medium was removed and washed twice by warm 1× PBS buffer. Cell resin block preparation followed the published method.[6] The thin cell section (～100nm) was prepared using Leica EM UC6. Following the staining process using uranyl acetate and lead citrate, the cell section was observed under transmission electron microscope (JEOL JEM-1230R, 100kV).

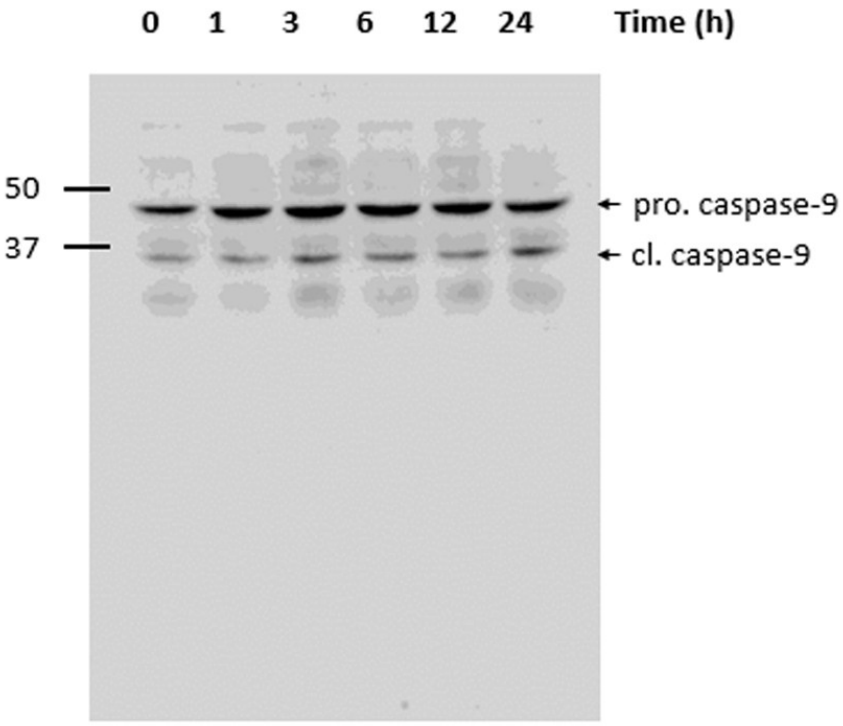
Western blotting

HeLa cells were cultured in 6-well plate to reach 70% confluency and 20 μM of 1 was added. After treated certain time (0h (ctrl.), 1h, 3h, 6h, 12h and 24h), all cells were harvested and washed with ice PBS twice. 50μL CelLytic M solution (Sigma-Aldrich) containing protease inhibitors cocktail was added and incubated for 30 min on a shaker. Then centrifuge the lysed cells for 5min at 20000g to pellet the cellular debris. The concentrations of lysates were determined using the bicinchoninic acid (BCA) method. Proteins were dissolved in SDS sample buffer containing 2% β-mercaptoehtanol. Samples (10μL of each) were loaded and separated by 10% Laemmli-SDS-PAGE. For Western blot analysis, the proteins were transferred onto a polyvinylidene difluoride (PVDF; Bio-Rad, Her-cules, CA, USA) membrane. After blocking with Blocking one-P (Nacalai tesque, Kyoto, Japan), the membrane was incubated with the antibody. Specifically, incubating with 1:1000 dilution for caspase-8 (Abcam, ab32397), caspase-9 (Santa Cruz, sc-56073) and DR-5 (Abcam, ab8416) antibody, 1:200 dilution for caspase-12 (Santa Cruz, sc-

21747), and 1:2000 dilution for beta-actin (Abcam, ab8227) antibody for overnight at 4°C. After washing, the membrane was incubated with 2,000-fold diluted peroxidase-conjugated goat anti-mouse IgG (Bio-Rad, Hercules, CA, USA) or 2,000-fold diluted peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad, Hercules, CA, USA) for 45 min, and then stained using ECLTM prime Western blotting detection reagent (GE Healthcare, Little Chalfont, UK).



b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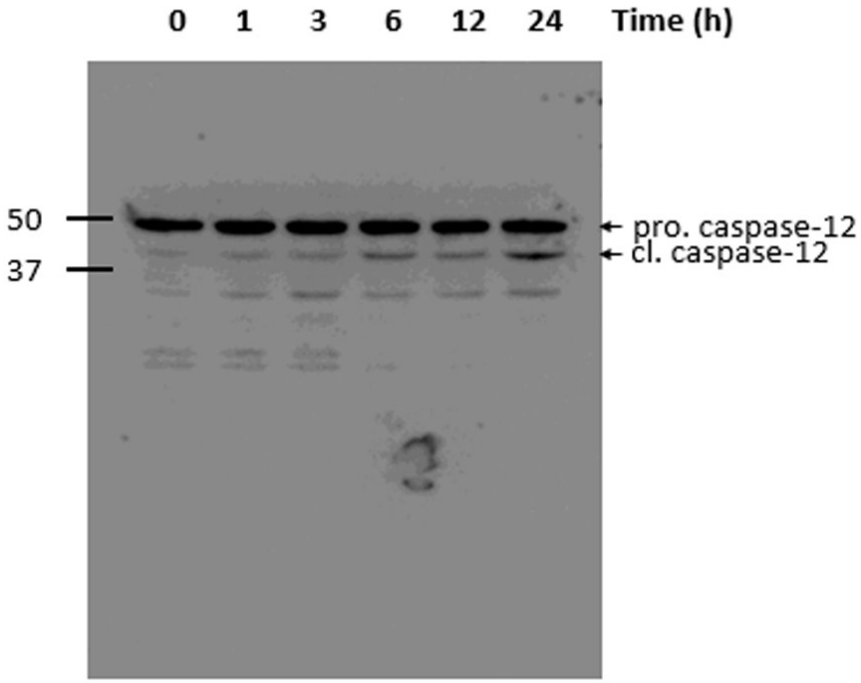
50
37
pro. caspase-9
cl. caspase-9

c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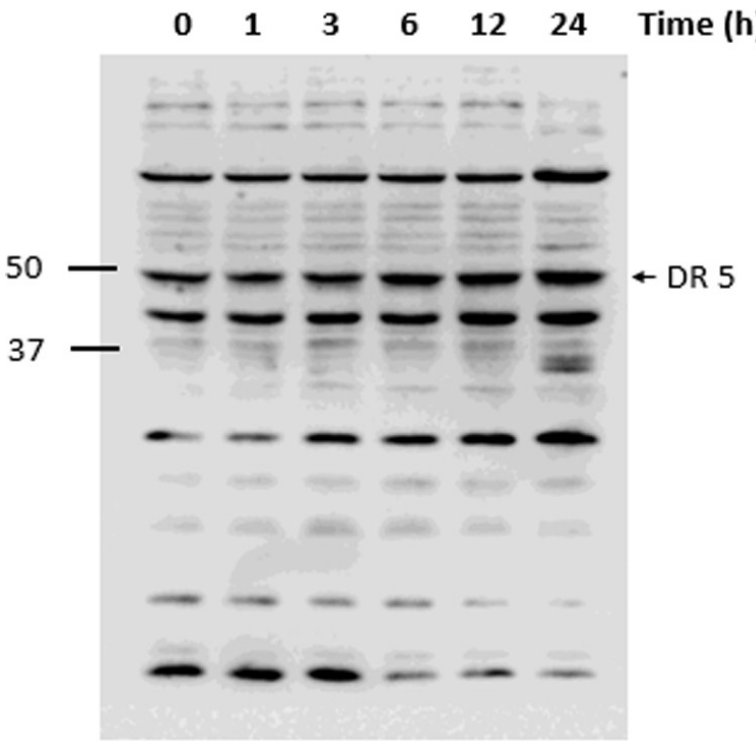
37
pro. caspase-12
cl. caspase-12

d



e

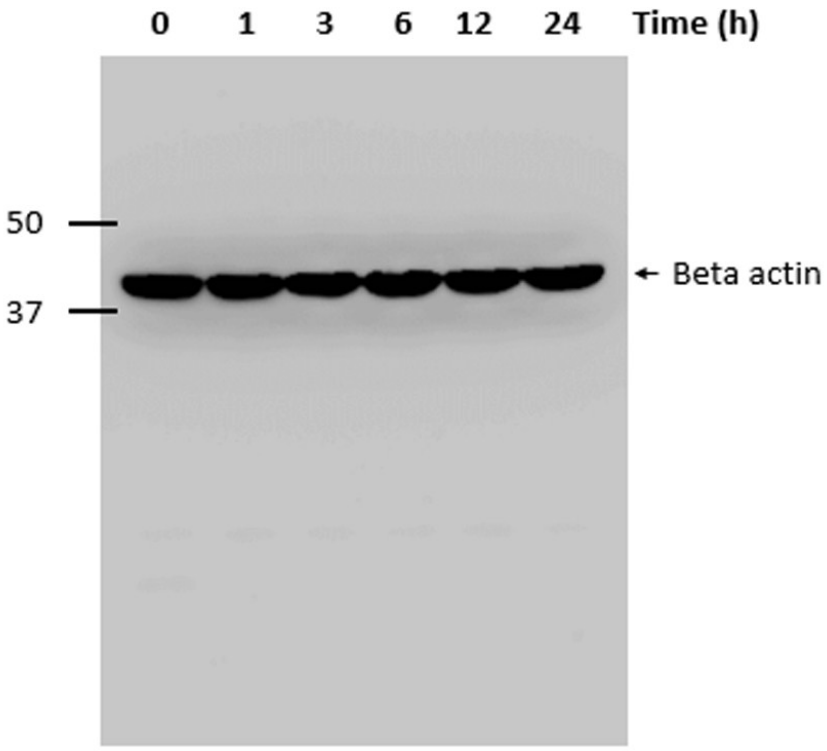


Figure S7. Western blotting images of caspase-8 (a), caspase-9 (b), caspase-12 (c), DR-5 (d) and beta-actin (e).

Cell confocal imaging

1 x 10^5 cells in exponential growth phase were seeded in 35mm glass bottom dish. The cells were allowed to attach for 24 h at 37ºC under 5% CO_2. Then, the culture medium was removed and changed to fresh medium containing **1**, **2** and **3** at 10μM. After incubation for 24h, cells were washed using PBS buffer three times, and stained with commercial fluorescent labels including CellMask™ Green plasma membrane stain, ER-Tracker™ Red (BODIPY™ TR Glibenclamide) and MitoTracker™ Green FM from ThermoFisher. After removing the staining solution, the cells were washed three times using PBS buffer. The cells were observed in Cell Imaging Solution using confocal microscope (LSM 780, Carl Zeiss).

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