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Supporting Information

Enzyme-Mediated Dual-Targeted-Assembly Realizes a Synergistic Anticancer Effect

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Note added after first publication: This supplementary Information file replaces that originally published on 9th May 2019, in which figures S9 and S14 were not included

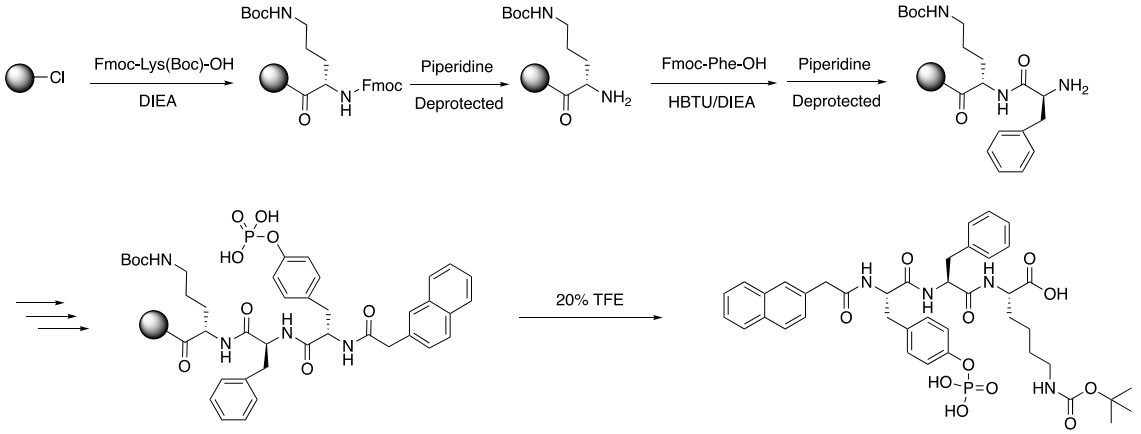
Content

Experimental Section

Materials and Methods:

All chemicals were obtained from commercial sources and were used without further purification. Fmoc-amino acids were purchased from GK Biochem, Shanghai, China. DMF, DCM, methonal, hexane and THF of HPLC grade were purchased from Wako, Japan. N,N-diisopropylethylamine (DIEA), (2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) (98.5%), 2,2,2-trifluoroethanol [1], trifluoroacetic acid (TFA, 98%), piperidine, N-hydroxysuccinimide (NHS, 98%), N,N'-diisopropylcarbodiimide (DIC, 99%), dimethyl terephthalate (99%), acetophenone, sodium hydride (60 % dispersion in mineral oil), lithium hydroxide (98%), triethylamine (99.5%) and boron trifluoride diethyl etherate were purchased from Sigma Aldrich. HPLC purification was performed on a Agilent 1260 Infinity Preparative Pump using a Agilent 1260 Infinity Diode Array Detector VL as the detector. A 19 mm×150 mm XBridge® Peptide BEH C18 column (Waters) was used for semi-preparative HPLC applications. Mass spectra were recorded using a Thermo LTQ-ETD mass spectrometer (ESI-MS). ^{1}H, ^{13}C and ^{31}P NMR spectra were recorded on a Bruker Ascend 400 (400 MHz, 100MHz and 162 MHz, respectively) spectrometer.

Synthesis:

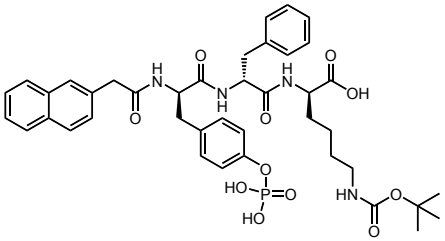


1a

1a: Compound **1a** was synthesized via solid phase peptide synthesis (SPPS) using 2-chlorotrityl chloride resin. 2-Chlorotrityl chloride resin (2.0 g, 2mmol) was swelled in anhydrous DCM for 20 min, L-Fmoc-Lys-[2]-OH (2.33g, 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 5 times. Then 20% piperidine solution (in DMF) was added to react for 30 min to remove the protecting group in the amino acid. We carried out the peptide elongation according to standard SPPS protocol to get the desired compound.

Compound 1a was cleaved off the resin by 20% TFE (in DCM) in 2 h. Ether was added into the concentrated solution; the resulting white precipitate powder was obtained.

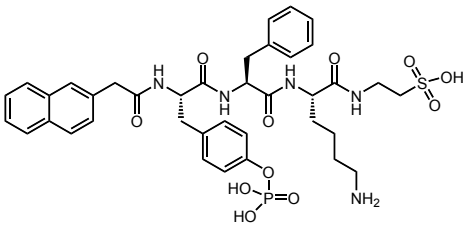
^{1}H NMR (400 MHz, DMSO-d_6) δ = 8.25 – 8.06 (m, 4H), 7.80 – 7.66 (m, 4H), 7.60 (s, 1H), 7.51 – 7.40 (m, 2H), 7.24 – 7.05 (m, 8H), 6.96 (d, J=7.2, 2H), 4.56 - 4.39 (m, 2H), 4.20 - 4.08 (m, 1H), 2.96 – 2.62 (m, 8H), 1.73 – 1.62 (m, 2H), 1.58 – 1.47 (m, 2H), 1.25 (s, 9H), 1.23 – 1.19 (m, 2H) ppm. MS (ESI): calculated for $C_{41}H_{49}N_4O_{11}P$ is 804.31, found 805.00, $[M + H]^+$.



2a

2a: Compound **2a** was synthesized using D-amino acids following the same procedure as compound **1a**.

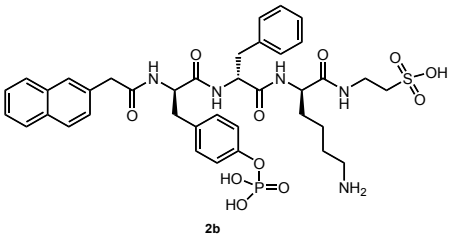
^{1}H NMR (400 MHz, DMSO-d_6) δ = 8.22 – 8.08 (m, 4H), 7.81 – 7.70 (m, 4H), 7.56 – 7.42 (m, 3H), 7.23 – 7.06 (m, 10H), 4.58 - 4.41 (m, 2H), 4.16 - 4.02 (m, 1H), 2.91 – 2.67 (m, 8H), 1.75 – 1.60 (m, 2H), 1.55 – 1.41 (m, 2H), 1.29 (s, 9H), 1.27 – 1.21 (m, 2H) ppm. MS (ESI): calculated for $C_{41}H_{49}N_4O_{11}P$ is 804.31, found 828.73, $[M + Na]^+$.



1b

1b: Dissolve compound **1a** (1.2 mmol, 965.0 mg) and HBTU (2.4 mmol, 910 mg) in 10 mL DMF and stirred at room temperature for 10 min. Then, DIEA (4.8 mmol. 0.84 mL) was added into the solution. The mixture was kept stirring for another 30 min, and taurine (2.4 mmol, 300.0 mg) was added into the solution and the final mixture was stirred at 60 °C for 36 hours. After that, removed the solvent using rotary evaporator. 20% TFA/DCM (10 ml) was added to the remained mixture, and kept stirring for 4 h. After removing the TFA and DCM and the mixture was purified using HPLC and obtained the compound **2b** in a yield of 68.2%.

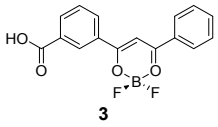
^{1}H NMR (400 MHz, DMSO-d_6) δ = 8.47 – 8.07 (m, 2H), 7.93 – 7.70 (m, 4H), 7.65 – 7.49 (m, 2H), 7.48 – 7.35 (m, 1H), 7.28 – 7.06 (m, 7H), 4.54 - 4.38 (m, 2H), 4.22 - 4.01 (m, 1H), 3.65 – 3.41 (m, 4H), 3.12 – 2.53 (m, 8H), 1.90 – 1.15 (m, 6H) ppm. MS (ESI): calculated for $C_{38}H_{46}N_5O_{11}PS$ is 811.84, found 849.18, $[M + K]^+$.



2b

2b: Compound **2b** was synthesized using **2a** following the same protocol as compound **1b**.

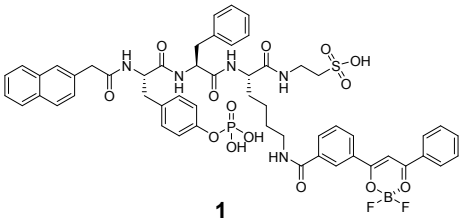
^{1}H NMR (400 MHz, DMSO-d_6) δ = 8.43 – 8.05 (m, 3H), 7.92 – 7.71 (m, 4H), 7.68 – 7.51 (m, 3H), 7.45 – 7.38 (m, 2H), 7.21 – 7.09 (m, 7H), 4.51 - 4.39 (m, 2H), 4.20 – 3.98 (m, 1H), 3.61 – 3.46 (m, 4H), 3.17 – 2.63 (m, 8H), 1.95 – 1.13 (m, 6H) ppm. MS (ESI): calculated for $C_{38}H_{46}N_5O_{11}PS$ is 811.84, found 812.42, $[M + H]^+$.



3

3: Compound **3** was synthesized following the published protocol.[3]

^{1}H NMR (400 MHz, DMSO-d_6) δ = 8.40 (dd, J=8.4, 1.0, 2H), 8.33 (d, J=8.5, 2H), 8.06 (d, J=8.4, 2H), 7.95 (s, 1H), 7.84 (t, J=7.4, 1H), 7.68 (t, J=7.8, 2H) ppm. MS (ESI): calculated for $C_{16}H_{11}BF_2O_4$ is 316.07, found 317.02, $[M + H]^+$.

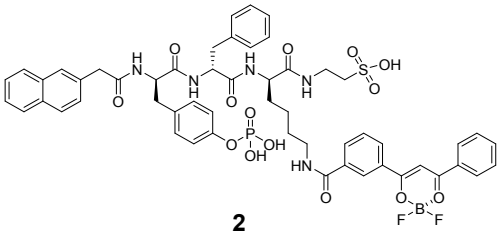


1

1: Dissolve compound **3** (0.32 mmol, 100.0 mg) in 10 ml DMF. HBTU (0.38 mmol, 144.1 mg), DIEA (0.48 mmol, 62.0 mg) and compound **1b** (0.35 mmol, 284.1 mg) were added into the solution, and the mixture was kept stirring at room temperature for 72 h. After remove the solvent, the mixture was purified via HPLC and we obtained the target compound in a yield of 61.5%.

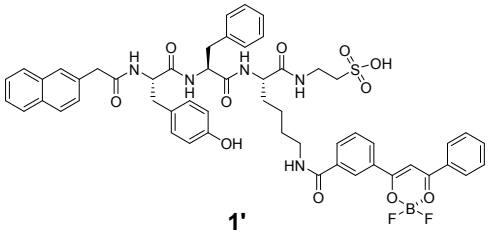
^{1}H NMR (400 MHz, DMSO-d_6) δ = 8.76 (dt, J=24.1, 5.5, 1H), 8.50 – 8.31 (m, 3H), 8.25 (d, J=8.0, 1H), 8.15 – 8.04 (m, 3H), 8.06 (s, 1H), 7.87 (td, J=13.1, 6.2, 3H), 7.72 – 7.64 (m, 3H), 7.64 - 7.41 (m, 3H), 7.18 (m, 9H), 4.48 – 4.40 (m, 1H), 4.18 (m, 1H), 3.63 – 3.52 (m, 2H), 3.42 (d, J=13.6, 1H), 3.37 (m, 2H), 3.32 - 2.93 (m, 3H), 2.91 – 2.78 (m, 2H), 2.75 – 2.57 (m, 2H), 1.67 – 1.51 (m, 2H), 1.48 – 1.31 (m, 2H), 1.25 – 1.11 (m, 2H). ^{13}C NMR (100 MHz, DMSO-d_6) δ = 184.23, 182.65, 171.82, 171.54, 171.32, 165.43, 150.41, 141.25, 138.43, 137.21, 133.20, 132.87, 132.20, 131.78, 131.56, 130.87, 130.56, 130.43, 129.53, 129.43, 128.78, 128.65, 127.32, 126.98, 126.10, 120.06, 95.54, 54.96, 54.37, 53.36, 49.53, 43.59, 39.42,

38.21, 33.46, 29.64, 23.58 ppm. ^{31}P NMR (162 MHz, DMSO-d_6) δ = - 6.25 ppm. MS (ESI): calculated for $C_{54}H_{55}BF_2N_5O_{14}PS$ is 1109.90, found 1109.18, $[M - H]^-$.



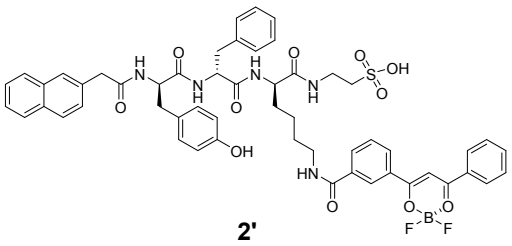
2

2: Compound **2** was synthesized using compound **2b** following the same protocol as compound **1**. ^{1}H NMR (400 MHz, DMSO-d_6) δ = 8.73 – 8.65 (m, 1H), 8.49 – 8.35 (m, 3H), 8.26 (d, J=8.0, 1H), 8.15 (t, J=7.8, 1H), 8.12 – 8.04 (m, 2H), 8.03 (s, 1H), 7.87 – 7.81 (m, 3H), 7.79 – 7.66 (m, 3H), 7.64 – 7.56 (m, 1H), 7.50 – 7.41 (m, 2H), 7.18 – 7.04 (m, 9H), 4.48 – 4.40 (m, 1H), 4.18 – 4.05 (m, 1H), 3.63 – 3.52 (m, 2H), 3.48 (d, J=14.1, 1H), 3.43– 3.36 (m, 2H), 3.32 – 3.17 (m, 2H), 3.02 – 2.93 (m, 1H), 2.93 – 2.82 (m, 2H), 2.78 – 2.67 (m, 2H), 1.69 – 1.53 (m, 2H), 1.51 – 1.30 (m, 2H), 1.23 – 1.09 (m, 2H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ = 183.29, 181.67, 172.62, 171.34, 170.69, 164.13, 151.81, 140.26, 137.93, 137.11, 134.51, 131.98, 130.10, 129.98, 129.56, 128.87, 128.36, 128.13, 127.59, 126.83, 126.17, 125.95, 125.62, 124.88, 124.16, 120.67, 93.59, 53.82, 53.51, 53.14, 49.93, 42.69, 40.12, 39.37, 33.84, 27.84, 25.17 ppm. ^{31}P NMR (162 MHz, DMSO-d_6) δ = - 6.23 ppm. MS (ESI): calculated for $C_{54}H_{55}BF_2N_5O_{14}PS$ is 1109.90, found 1108.27, $[M - H]^-$.



1'

1': Control compound **1'** was synthesized using the same protocol as compound **1**. The difference was changing the starting material from L-Fmoc-Tyr(PO_3H_2)-OH to L-Fmoc-Tyr-OH when doing the SPPS. ^{1}H NMR (400 MHz, DMSO-*d6*) δ = 7.66 – 7.59 (m, 1H), 7.49 – 7.43 (m, 4H), 7.36 – 7.26 (m, 3H), 7.23 – 7.13 (m, 4H), 7.12 – 6.98 (m, 4H), 6.95 – 6.88 (m, 3H), 6.83 – 6.66 (m, 8H), 6.55 – 6.40 (m, 2H), 6.31 – 6.15 (m, 2H), 4.73 – 4.43 (m, 2H), 4.33 (ddd, J=11.4, 7.6, 3.4, 1H), 4.03 – 3.78 (m, 4H), 3.53 – 3.30 (m, 6H), 3.27 – 3.03 (m, 2H), 2.52 – 1.95 (m, 6H) ppm. ^{13}C NMR (100 MHz, DMSO-d_6) δ = 175.28, 174.06, 172.53, 169.26, 168.94, 166.75, 165.92, 161.29, 153.74, 145.23, 144.71, 128.30, 126.89, 126.05, 123.93, 123.56, 123.49, 123.26, 122.82, 122.59, 122.53, 122.17, 121.92, 121.78, 121.74, 121.63, 120.82, 120.61, 120.30, 111.88, 108.08, 94.61, 64.57, 63.96, 59.84, 49.15, 48.30, 38.28, 18.89. MS (ESI): calculated for $C_{54}H_{54}BF_2N_5O_{11}S$ is 1029.36, found 1030.21, $[M + H]^+$.



2'

2': Control compound **2'** was synthesized using the same protocol as compound **2**. The difference was changing the starting material from D-Fmoc-Tyr(PO_3H_2)-OH to D-Fmoc-Tyr-OH when doing the SPPS. ^{1}H NMR (400 MHz, DMSO-*d6*) δ = 7.91 – 7.85 (m, 3H), 7.80 – 7.70 (m, 4H), 7.67 – 7.61 (m, 2H), 7.58 (qd, J=5.7, 3.4, 4H), 7.52 – 7.44 (m, 3H), 7.39 – 7.25 (m, 10H), 7.06 (dt, J=21.0, 10.5, 2H), 6.80 (dd, J=6.7, 1.4, 2H), 5.21 – 5.08 (m, 2H), 4.85 (m, 1H), 4.42 – 4.21 (m, 4H), 4.07 – 4.01 (m, 2H), 3.96 – 3.64 (m, 6H), 3.02 – 2.59 (m, 6H). ^{13}C NMR (100 MHz,) δ = 172.01, 171.22, 167.45, 167.17, 165.80, 165.50, 164.73, 162.02, 155.00, 145.02, 141.52, 141.21, 137.15, 135.00, 130.44, 126.88, 126.79, 126.52, 124.64, 124.39, 124.13, 123.96, 123.53, 123.22, 122.74, 122.59, 122.42, 122.31, 121.32, 120.95, 112.54, 95.32, 64.77, 63.45, 60.71, 54.50, 44.81, 42.93, 39.67, 38.60, 32.11, 26.94, 19.70. MS (ESI): calculated for $C_{54}H_{54}BF_2N_5O_{11}S$ is 1029.36, found 1030.32, $[M + H]^+$.

Absorption and emission spectroscopy: UV-vis spectra were performed 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 to 1.0 nm. All emission spectra were measured using a Hitachi F-7000 fluorescence spectrometer. The excitation wavelength was set to 350 nm and emission collection from 370 to 600 nm.

1 showed maximum absorption at 351 nm. After being hydrolyzed to **1'**, the maximum absorption appeared at 352 nm. **2** had maximum absorption at 352 nm. After hydrolyzed to **2'**, the maximum absorption appeared at 354 nm.

CD spectroscopy: All CD spectra were obtained from a spectrometer JASCO J-820, the bandwidth was set at 1.0 nm with a measurement range from 190 nm to 400 nm, specimen was loaded in a 0.1 mm quartz cuvette.

TEM imaging of molecular assembly in solution: Aliquots (10 μL) of sample solution were added into a glow discharge copper grid (400 mesh) coated with thin carbon film and incubated for 30 s at room temperature. After removing excess solution, the grid was washed with deionized water three times and then stained with 1.0% (w/v) uranyl acetate (UA) by exposing the grid in three drops of UA solution for 30 s. TEM images were captured at high vacuum on transmission electron microscope JEM-1230R (JEOL, Japan).

Cell fractionation: The cell fragmentation procedure was carried out as described previously (Dopp *et al.,* 2008). In brief, 1 x 10^6 cells were seeded in 10 mL culture medium and incubated 12 h for attachment. Then cells were washed twice with PBS, harvested by trypsin treatment, and resuspended in PBS. After the cell number was counted, the cell suspension was centrifuged at 300g for 5 min, and the obtained pellet was resuspended in 10 mL of distilled water for 30 min to lyse the cells. Clumps of unbroken and ruptured cells were removed by centrifugation at 300g for 5 min. Whole cell extract was used to separate cell organelles by differential centrifugation. Plasma membrane and cytosol were collected for further test.

2D cell culture: All cell lines were purchased from American-type Culture Collection (ATCC, USA) and cultured in ATCC recommended medium. HeLa cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin. HS-5 cells were cultured in DMEM containing 10% FBS. Ect1/E6E7 cells were cultured in keratinocyte serum-free medium with 0.1 ng/mL human recombination EGF, 0.05 mg/mL bovine pituitary extract, and 44.1 mg/L CaCl2. Incubation was carried out at 37°C with a humidified atmosphere of 5% CO_2. The cells were maintained 80% confluent and used for the bioassays.

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 24h at 37ºC under 5% CO_2. Then, the culture medium was removed and changed to fresh medium containing **1**, **2** or a mixture of **1** and **2** at different concentrations. After incubation for desired time, cells were washed using 1xPBS buffer three times, and stained with commercial fluorescent labels (LysoTracker Red DND-99, CellMaskTM Deep Red plasma membrane stain from ThermoFisher). After removing the staining solution, the cells were washed three times using 1xPBS buffer. The cells were observed in Live Cell Imaging Solution using confocal microscope (LSM 780, Carl Zeiss).

PLAP inhibition protocol: Cells were seeded in 35 mm glass-bottom culture dishes and allowed for attachment at 37ºC under 5% CO_2 for 24h. Then, culture medium was removed and exchanged to serum-free medium containing PLAP inhibitor, L-phenylalanine (L-Phe) (3mM), for 12h incubation. The mixtures of **1** and **2** at different concentrations were added into the culture medium for another 12h incubation. Cells were washed using 1xPBS buffer three times, and stained with fluorescence labels, LysoTracker Red DND-99, or CellMaskTM Deep Red. After that, the staining solution was removed, and the cells were washed using 1xPBS buffer three times. The cells were observed in Live Cell Imaging Solution using confocal microscope (LSM 780, Carl Zeiss).

SEM imaging: Treated with **1**, the cells were washed using 1xPBS buffer and then cross-linked with 2.5% glutaraldehyde in 0.1 M cacodylate buffer for 10 min. The samples were washed with 0.1 M cacodylate buffer for 5 min (×3) and then further fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer for 30 min, followed by washing with Milli-Q water for 5 min (×3) and then progressive dehydration in a graded series of ethanols (70%, 80%, 90%, 95% and twice in 100%, 3 minutes at each concentration). The cells were rinsed with *t*-butanol for 3 min (×3) and then dried by freeze-dryer overnight. SEM images of the dried samples were captured before platinum coating.

TEM imaging of isolated lysosomes: HeLa cells untreated and treated with **2** were washed using 1xPBS buffer three times. Lysosome Enrichment Kit for Tissue and Cultured Cells (89839, ThermoFisher), and Protease Inhibitor (87785, ThermoFisher) were used for the isolation of lysosomes by following protocol 2 of the instructions. Aliquots (10 μL) of lysosome collections were added onto a glow discharge copper grid (400 mesh) coated with thin carbon film and incubated for 30 s at room temperature. After removing excess solution, the grid was washed with deionized water three times and then stained with 1.0% (w/v) uranyl acetate (UA) by exposing the grid in three drops of UA solution for 30 s. TEM images were captured at high vacuum on transmission electron microscope JEM-1230R (JEOL, Japan).

Apoptosis/necrosis detection assay: Treated with **1 (**200 μM), **2** (200 μM) and mixture of **1** (100 μM) and **2** (100 μM) for 16 hours, HeLa cells were washed using fresh culture medium and stained using apoptosis/necrosis detection kit (ab176749, abcam) by following the standard protocols. The fluorescence image shows cells that ate live in blue (CytoCalcein Violet 450), apoptotic in green (Apopxin Green), and necrotic in red [7-aminoactinomycin D (7-AAD)].

Western blotting: HeLa cells were cultured in 6-well plate to reach 80% confluency and 100 μM of 1 and 2 or mixture (1 + 2) were added. After treated certain time (0h, 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 12.5% 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 Anti-Caspase 8 antibody or Anti-Caspase 9 antibody at 4°C for 12h following the appropriate dilution. After washing, the membrane was incubated with 1,000-fold diluted

peroxidase-conjugated goat anti-mouse IgG (Bio-Rad, Hercules, CA, USA) or 1,000-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).

2D cell viability assay: Cells in exponential growth phase were seeded in a 96-well plate at a concentration of 7×10^3 cells per well and then incubated for 1 day at 37ºC, under 5% CO_2. The culture medium was removed followed by addition of 100 μL culture medium containing different concentrations (50, 100 and 200 μM) of **1**, **2** or a mixture of **1** and **2**. After the desired time of exposure, 10 μL 3-(4,5)-dimethylthiahiazo (-z-y1)-3, 5-diphenytetrazoliumromide (MTT) solution (5 mg/mL) was added into 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. Microplates were then agitated for 5 min, and absorbance was measured using a Tecan microplate reader at the wavelength of 570 nm. All experiments were conducted triplicate. The results were calculated as means, which are expressed as cell viability (%).

Wound healing assay: Cell migration was evaluated by wound healing assay. HeLa cells were seed at 3×10^4 cells/well into each well of a 96-well ImageLock plate (Essen BioScience 4379). After incubating in cell incubator (Panasonic MCO-230AIC) overnight using culture medium containing 10% FBS to form 90% confluent monolayer, cells were serum starved using culture medium containing 2% FBS and incubated for 4h. Homogenous scratch wounds of 700-800 μm wide were made using WoundMaker (Essen BioScience) and washed twice with fresh medium containing 2% FBS to remove unattached cells. Cells were treated with different concentration of 1 or 2 solution with 2% FBS and imaged ever 4h in 10x objective of IncuCyte S3. The confluence mash was defined using IncuCyte analysis software to quantify migrating rate. Data represent the mean ± standard deviation of three independent experiments.

3D spheroid culture: Cells were detached and single-cell suspension in culture medium containing 10% FBS was transferred into a 96-well low attached U plate (Thermo, #174925) at a density of 2.5×10^3 cells per well. The growth of spheroids was imaged every 6h using 4x objective of IncuCyte S3 (Essen BioScience). After 3-day culturing, spheroids of approximately 400 μm in diameter were found.

3D spheroid proliferation/viability assay: When compact spheroids were formed at day 4 after seeding, half of culture media were removed gently using pipette and replaced with flesh media containing different concentration of compounds 1, 2 and mixture of 1 and 2. After 24h, 48h and 72h treatment, 100μL ATP-reacting substrate (Celltiter-glo® 3d cell viability assay; Promega, Madison, USA) was added into each well and mixed thoughtfully using multichannel pipette. After incubating at 37°Cfor 30 min, solutiongs were transferred to black bottom microplate and luminescence was recorded using

Infinite M1000 Pro microplate reader (Tecan). Data represent the mean ± standard deviation of four independent experiments.

3D spheroid migration assay: The compact HeLa spheroids were formed on day 4 after seeding with culture medium containing 10% FBS. Then, the spheroids were serum-starved in culture medium containing 5% FBS for overnight. Then the spheroids were treated with **1**, **2** or a mixture of **1** and **2** at different concentrations in culture medium containing 5% FBS. Images were acquired every 12h in 4x objective of IncuCyte S3. The spheroid area was quantified using IncuCyte analysis software. Data represent the mean ± standard deviation of three independent experiments.

Quantification of PLAP expression: Membrane expression levels of PLAP on HeLa cells were quantified by imaging flow cytometer using PLAP antibody labeling. Single-cell suspensions were harvested by detaching monolayer cells or suspending multi-cellular spheroid cells using 0.5% trypsin and washed with cold DPBS twice. Monoclonal antibodies of PLAP (1:100) were added to incubate single cell suspension on ice for 30min and washed with cold DPBS twice for 10min. Fluorophore AlexaFluor488-conjugated second antibody (1:1000) was added to label on ice for another 30min. After washed with DPBS twice, the cell suspension was loaded into Imaging Flow Cytometer (ImageStream X Mark II, Merck) to count populations. Isotype controls (mouse IgG, abcam) were also performed to exclude non-specific background signals.

Supporting Figures:

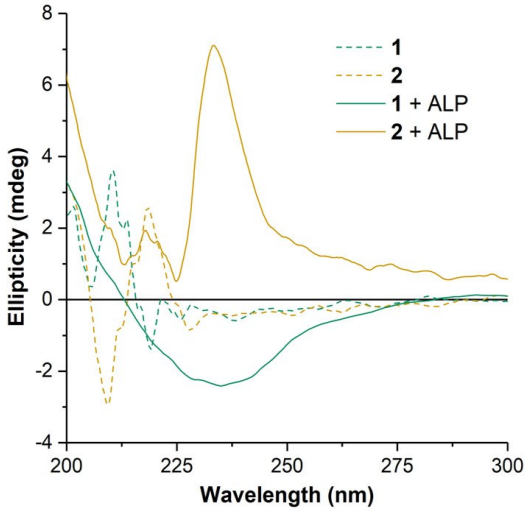


Figure S1. CD spectra of molecules **1** (100 μM) and **2** (100 μM) before and after ALP (1U/mL) catalyzed hydrolysis in 1xPBS buffer.

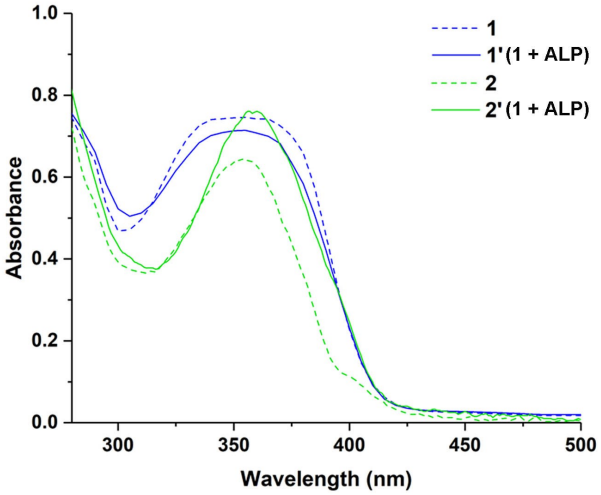


Figure S2. UV-vis absorption of molecules **1** (100 μM) and **2** (100 μM) before and after ALP (1U/mL) catalyzed hydrolysis in 1xPBS buffer.

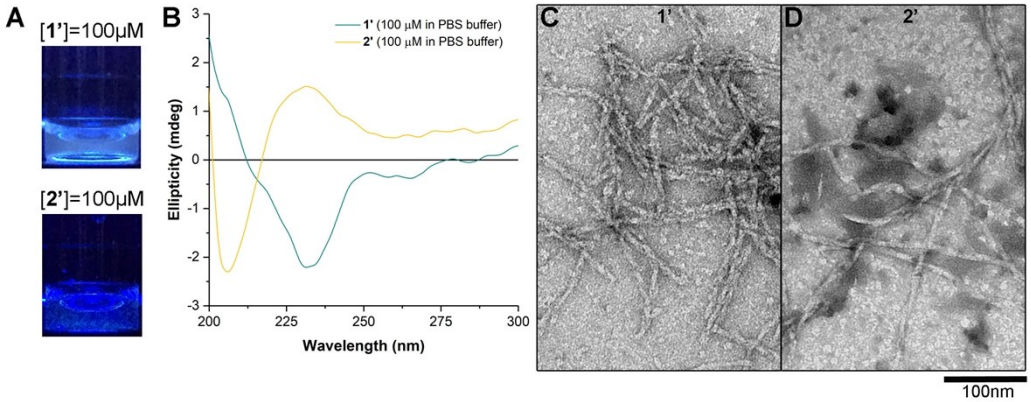


Figure S3. (A) Optical images of **1'** (100 μM) and **2'** (100 μM) in PBS buffer under the UV light. (B) CD spectra of **1'** (100 μM), and **2'** (100 μM) in PBS buffer. TEM images of **1'** (100 μM) (C), **2'** (100 μM) (D) in PBS buffer.

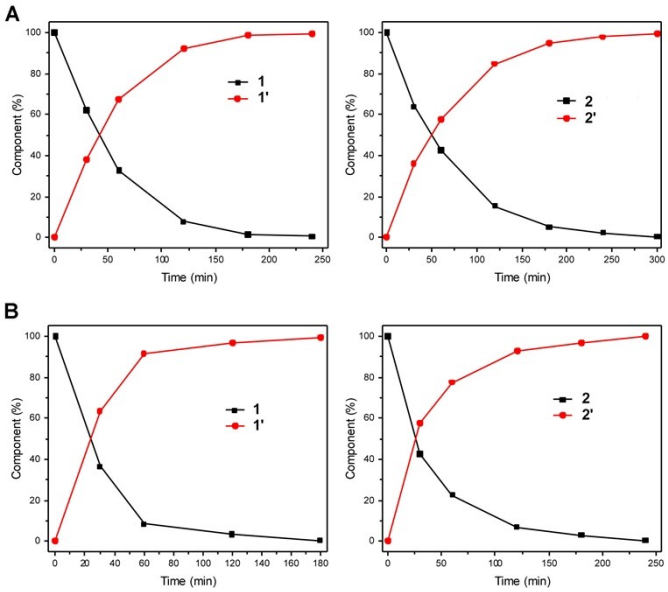


Figure S4. Kinetic profiles of enzyme catalyzed dephosphorylation of **1** (200 μM) and **2** (200 μM) in plasma membrane fraction (A) and in cytoplasmic fraction (B).

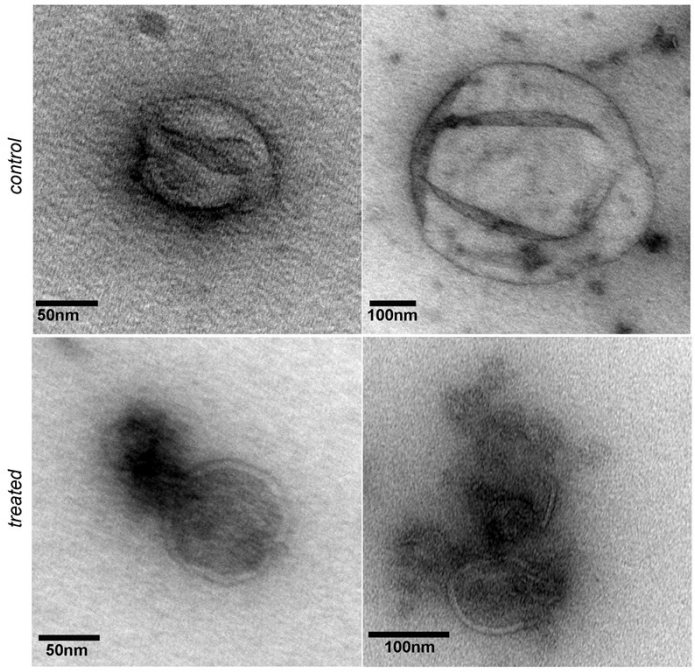


Figure S5. TEM images of isolated HeLa cell lysosomes before (control) and after treated with **2** (100 μM) for 12 hours.

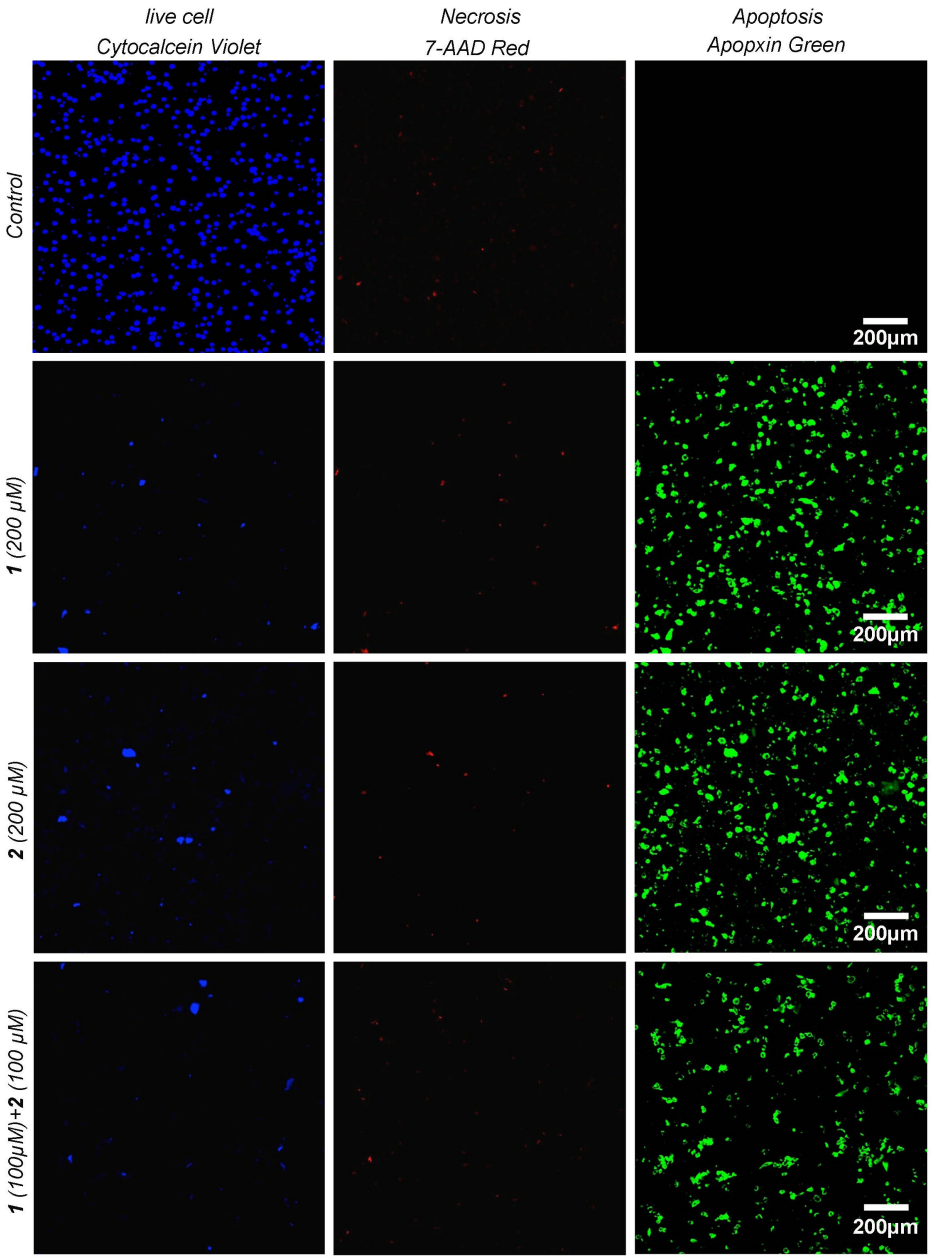


Figure S6. HeLa cells before and after the treatment of **1**, **2**, and the mixture of **1** and **2**, analyzed with Apoptosis/Necrosis Detection Kit (ab176749). Fluorescent analysis shows cells that are live in blue, apoptosis in green and necrotic in red.

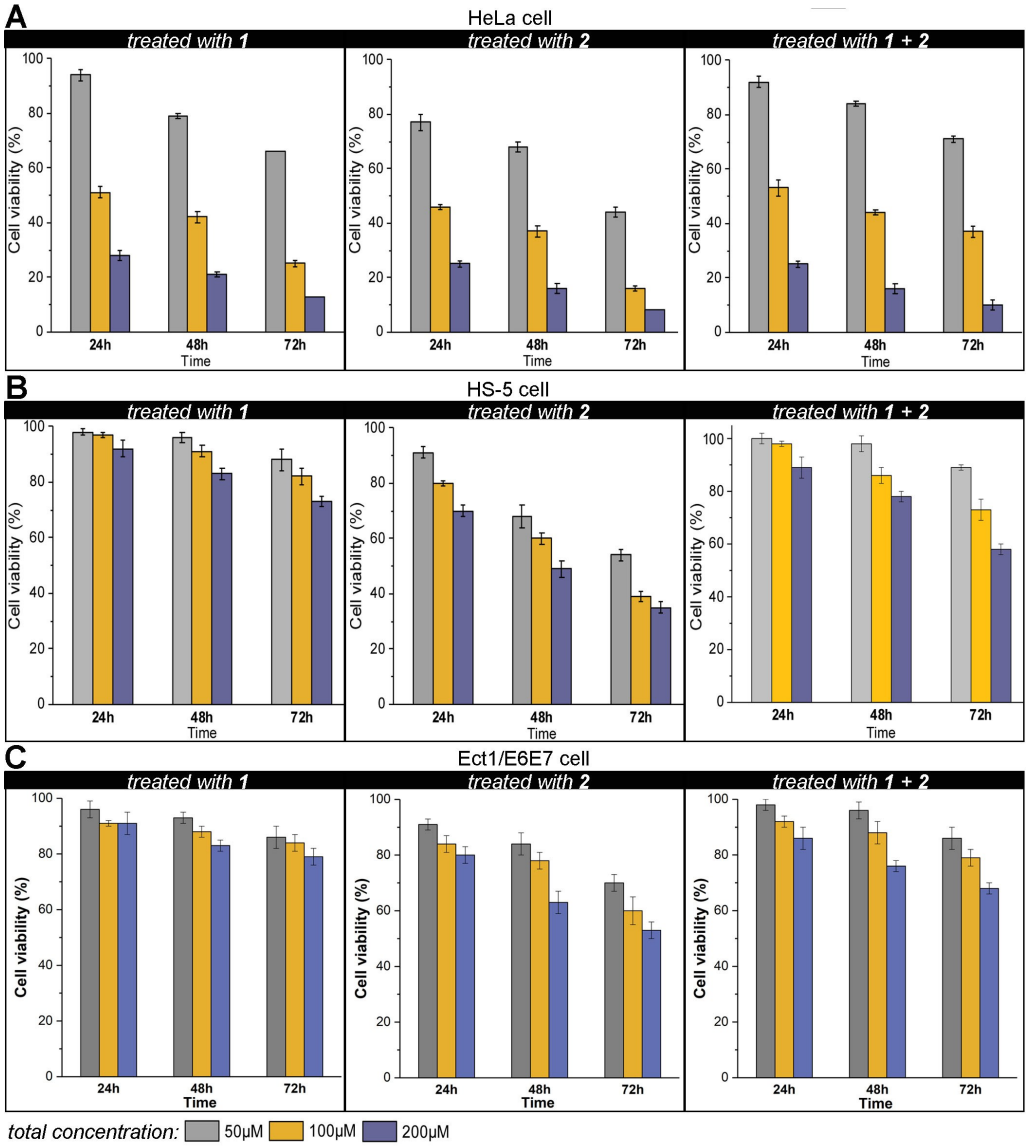


Figure S7. (A) HeLa cell viability over a 3-day period of incubation with **1**, **2**, and mixture of **1** and **2** at 1:1 ratio in 2D cell culture. (B) HS-5 cell viability over a 3-day period of incubation with **1**, **2**, and mixture of **1** and **2** at 1:1 ratio in 2D cell culture. (C) Ect1/E6E7 cell viability over a 3-day period of incubation with **1**, **2**, and mixture of **1** and **2** at 1:1 ratio in 2D cell culture.

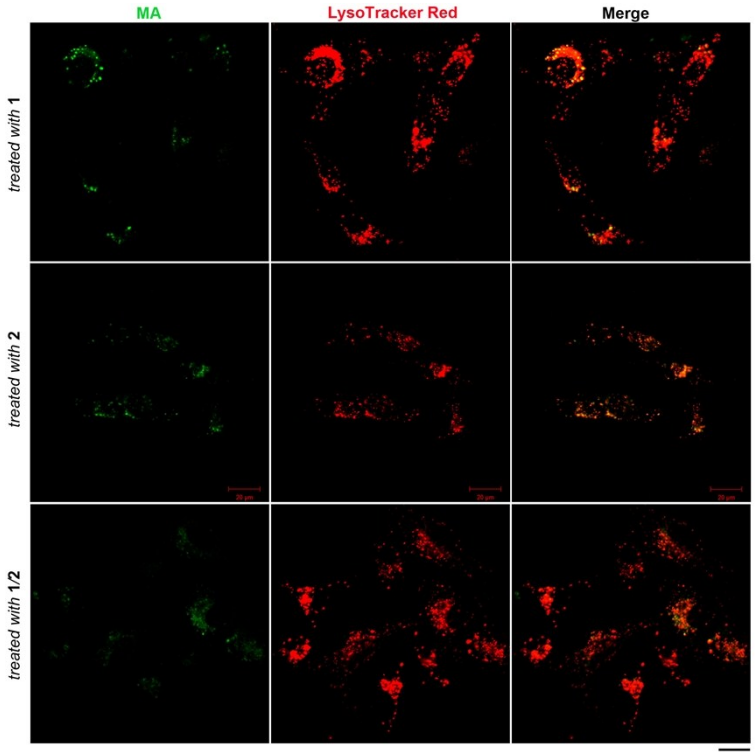


Figure S8. Confocal images of HS-5 cells under the treatment of **1**, **2**, and the mixture of **1** and **2**, co-stained with LysoTracker Red. Scale bar is 20 μm.

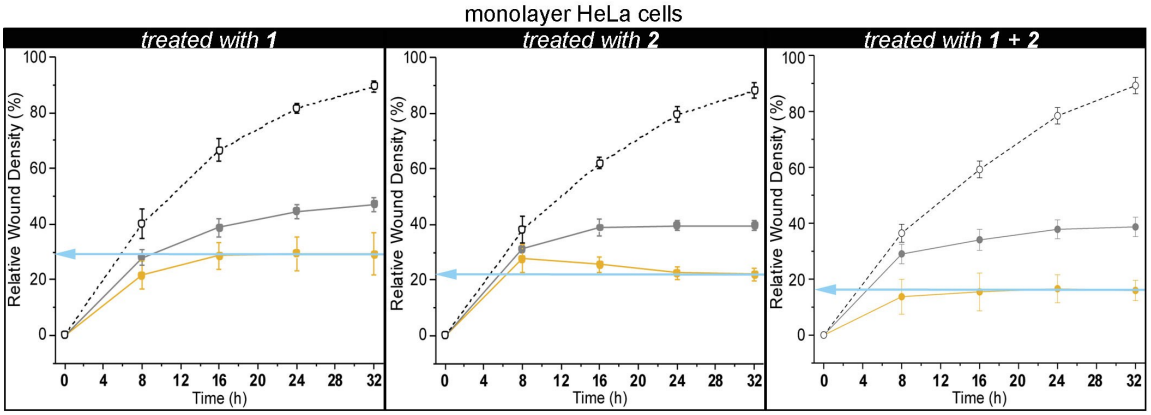


Figure S9. Time-dependent relative wound density of HeLa cells under the treatment of **1**, **2**, and mixture of **1** and **2** at 1:1 ratio.

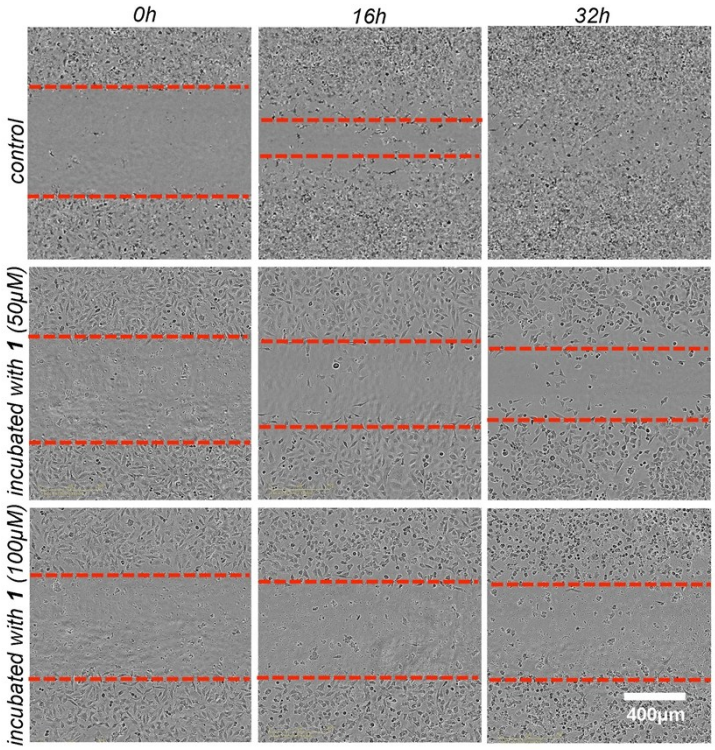


Figure S10. Effect of single administration of **1** on migration potential of HeLa cells was analyzed through wound healing assay. Representative time-lapse DIC images of the wounds.

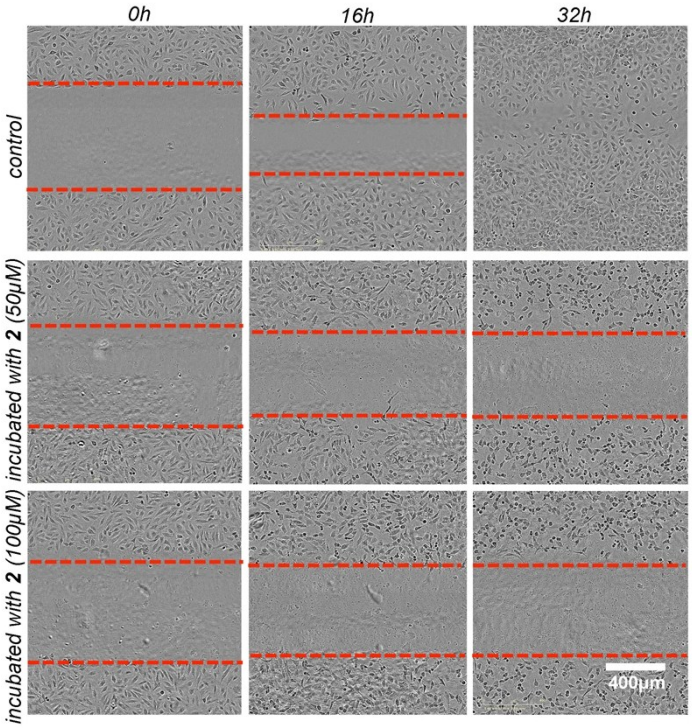


Figure S11. Effect of single administration of **2** on migration potential of HeLa cells was analyzed through wound healing assay. Representative time-lapse DIC images of the wounds.

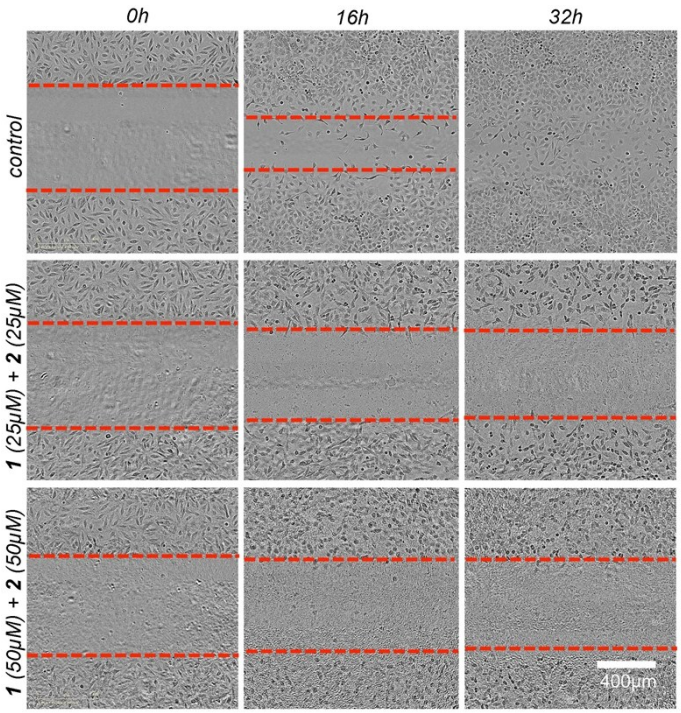


Figure S12. Effect of co-administration of **1** and **2** on migration potential of HeLa cells was analyzed through wound healing assay. Representative time-lapse DIC images of the wounds.

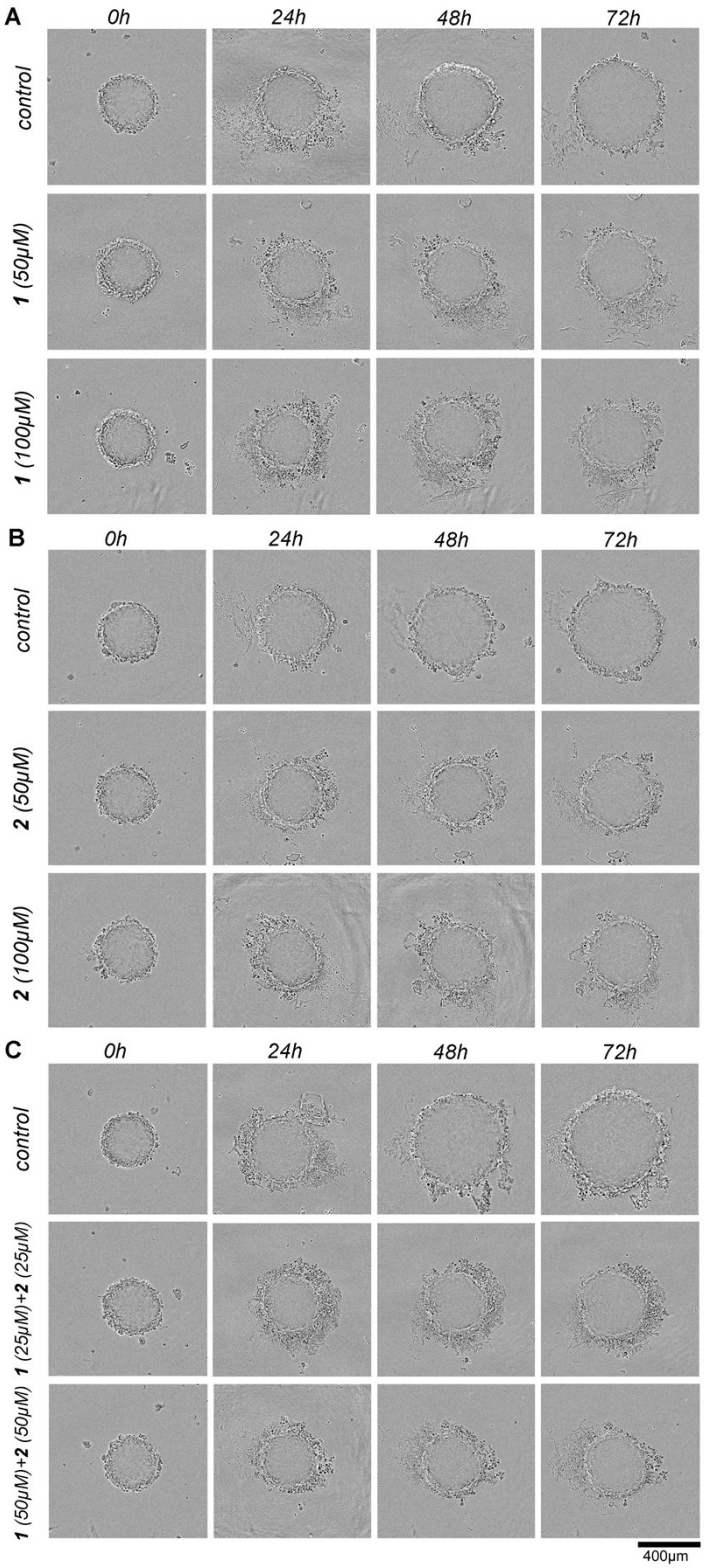


Figure S13. Time-lapse DIC images of 3D HeLa spheroid spreading under the treatment of **1**, **2** and mixture of **1** and **2**.

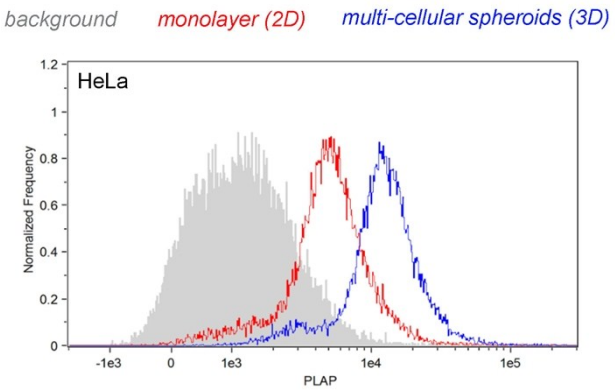


Figure S14. PLAP expression profiles of HeLa cells in 2D and 3D cell cultures. Isotype control (grey) represents background.

Reference

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