Supporting Information for

Intranuclear Assembly of Leucine-Rich Peptides for Selective Death of Osteosarcoma Cells

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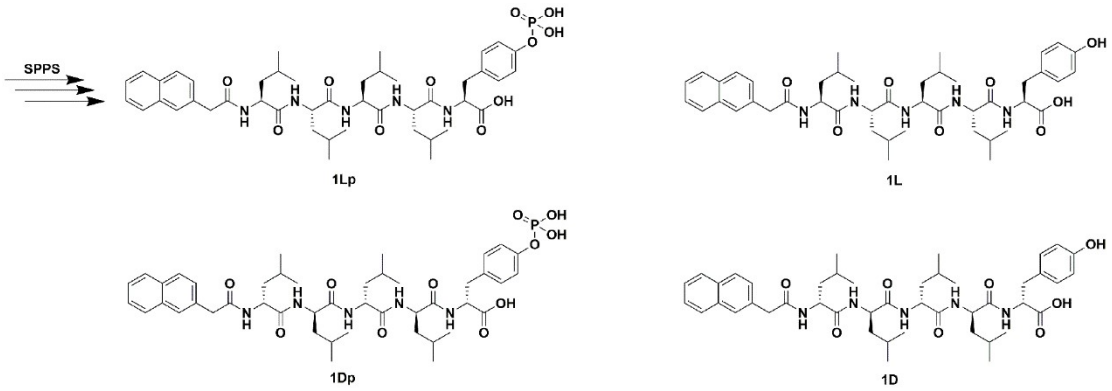
Content

S1. Materials and instruments

2-Cl-trityl chloride resin (1.0-1.2 mmol/g), HBTU, Fmoc-OSu, and other Fmoc-amino acids were purchased from GL Biochem (Shanghai, China). Other chemical reagents and solvents were purchased from Fisher Scientific. Alkaline phosphatase was purchased from Biomatik (Cat. No. A1130, Alkaline Phosphatase [ALP], 30000 U/mL, in 50% Glycerol.), Fetal bovine serum (FBS) and penicillin-streptomycin from Gibco by Life Technologies. All precursors were purified with Agilent 1100 Series Liquid Chromatograph system, equipped with an XTerra C18 RP column and Variable Wavelength Detector. The LC-MS spectra were obtained with a Waters Acquity Ultra Performance LC with Waters MICROMASS detector, and ^{1}HNMR spectra on Varian Unity Inova 400. Circular dichroism (CD) spectra were obtained with a Jasco J-810 Spectropolarimeter.

S2. Synthesis and characterization of the precursors



Scheme S1. Synthetic route of **1Lp, 1L**, **1Dp** and **1D**.

Synthesis of Fmoc-$_L$-Tyr(PO_3H_2)-OH and Fmoc-$_D$-Tyr(PO_3H_2)-OH

The mixture of P_2O_5 (35 mmol, 10 g), H_3PO_4 (133 mmol, 13 g) and H-$_L$-Tyr-OH (18 mmol, 3.22 g) was stirred for 24 h at 80ºC in N_2 atmosphere. After adding 30 mL H_2O and stirred for 30 min at 80ºC, the reaction mixture was cool to room temperature. The reaction mixture was added to butanol (650 mL) dropwise and recrystallized at 4ºC overnight, filtration provided H-$_L$-Tyr(PO_3H_2)-OH as white power. To the solution of H-$_L$-Tyr(PO_3H_2)-OH (2 mmol, 522 mg) in H_2O (20 mL), the solution of Fmoc-OSu (2.4 mmol, 808 mg) in MeCN (20 mL) was added. After adjusting pH to ~8 by triethylamine (TEA), the solution was stirred at room temperature for 2 h. After removal of MeCN by evaporation, 60 mL H_2O was added and the pH of the solution was adjusted to ~3 by 1 M HCl. After extraction by ethyl acetate (100 mL × 3), the organic part was washed by 1 M HCl

(100 mL ×2) and brine (100 mL ×1). After being dried by Na_2SO_4, filtered and concentrated by evaporation, Fmoc-$_L$-Tyr(PO_3H_2)-OH was provided as white powder. By the same method, Fmoc-$_D$-Tyr(PO_3H_2)-OH was provided as white powder.

Synthesis of 1Lp, 1L, 1Dp, 1D, and 1DLp

1Lp, **1L**, **1Dp**and **1D**were synthesized by solid phase peptide synthesis with 2-Cl-trityl chloride resin, Fmoc-$_L$-Tyr(PO_3H_2)-OH, Fmoc-$_D$-Tyr(PO_3H_2)-OH, Fmoc-$_L$-Tyr(tBu)-OH, Fmoc-$_D$-Tyr(tBu)-OH, Fmoc-$_L$-Leu-OH, Fmoc-$_D$-Leu-OH, 2-naphthylacetic acid, HOBt and HBTU. Purification with HPLC provided **1Lp**, **1L**, **1Dp** and **1D** as white power.

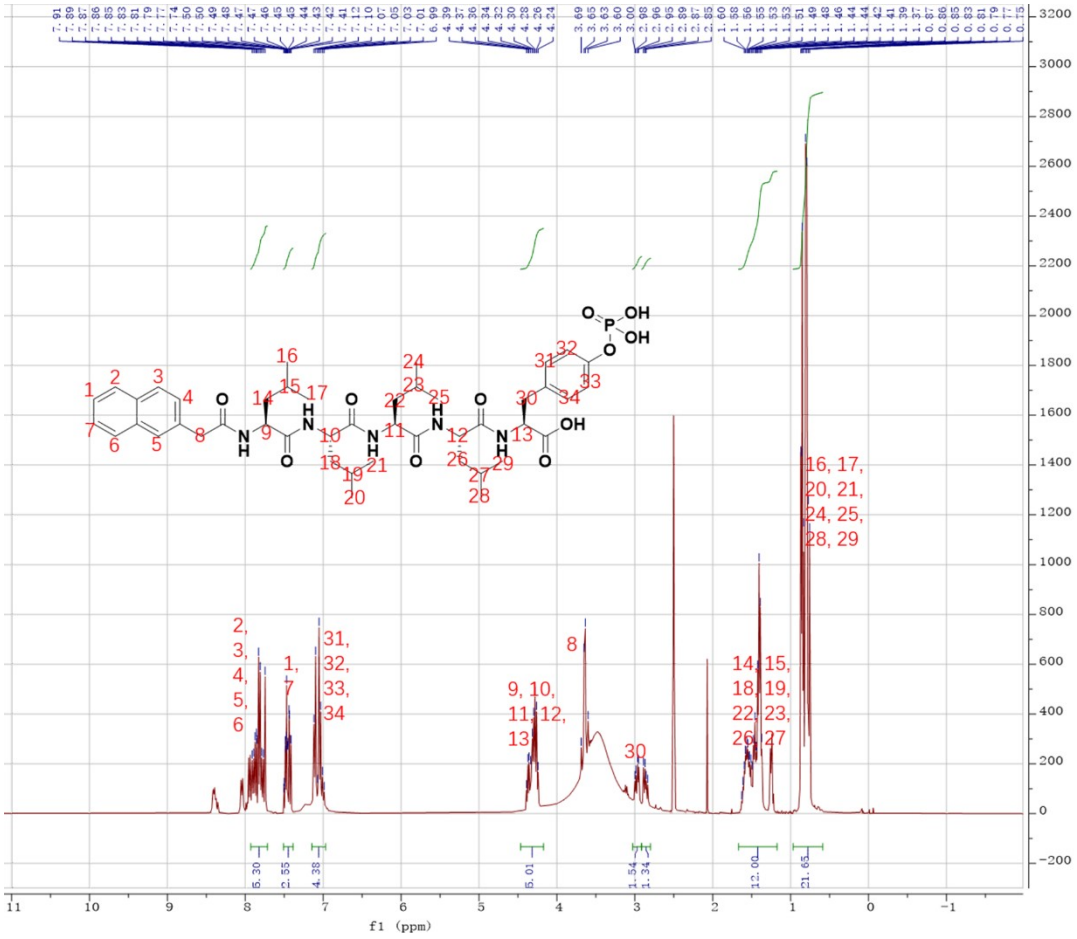


Figure S1. ^{1}H NMR spectrum of **1Lp**.

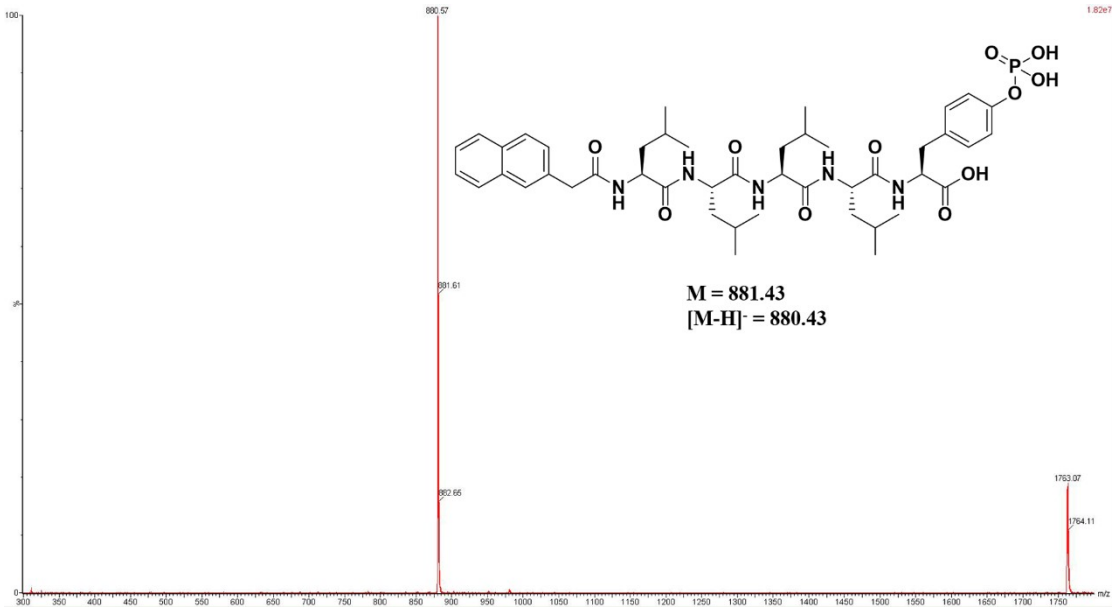


Figure S2. Mass spectrum of **1Lp** (M/Z = 880.43).

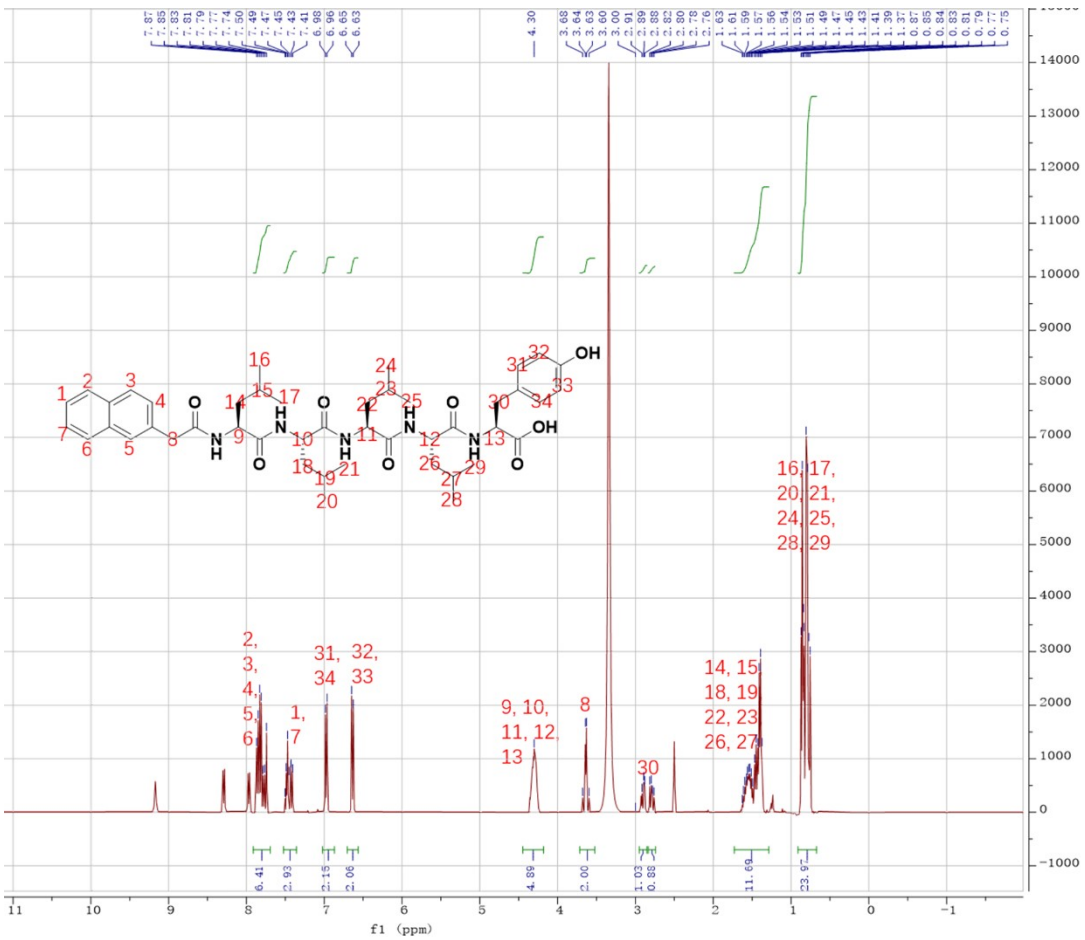


Figure S3. ^{1}H NMR spectrum of **1L**.

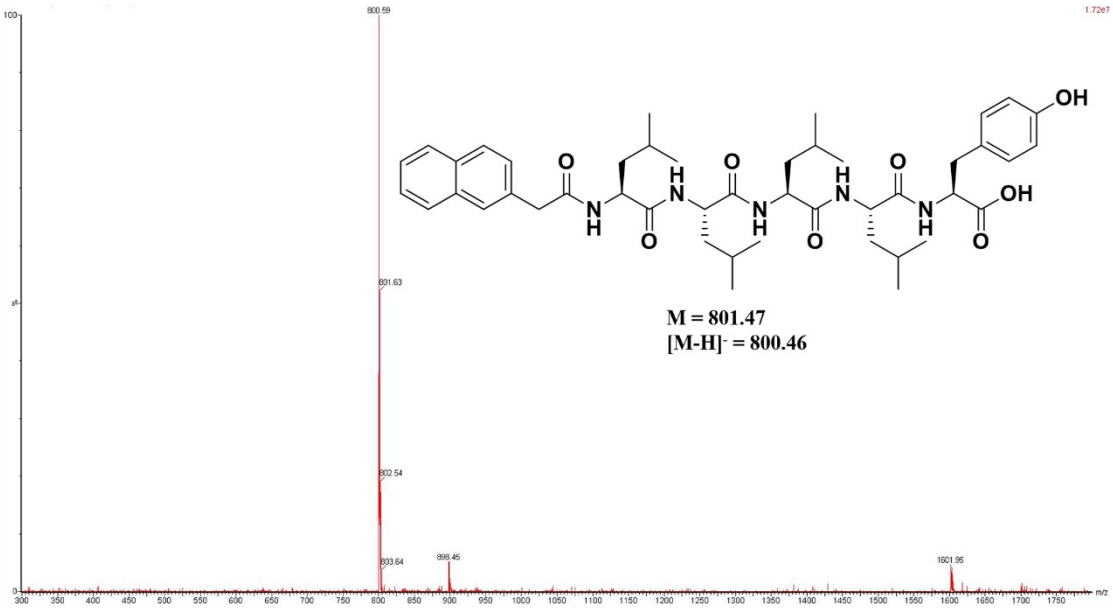


Figure S4. Mass spectrum of **1L** (M/Z = 800.46).

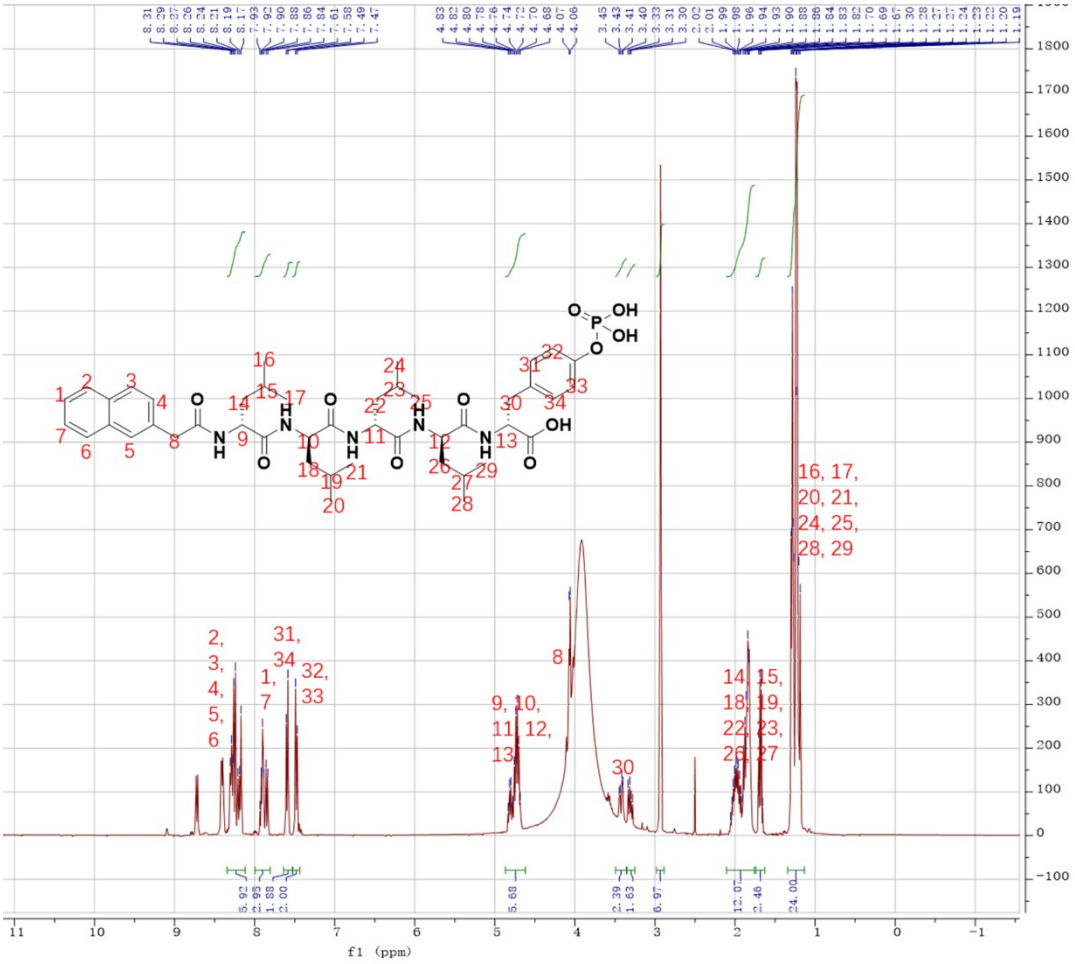


Figure S5. ^{1}H NMR spectrum of **1Dp**.

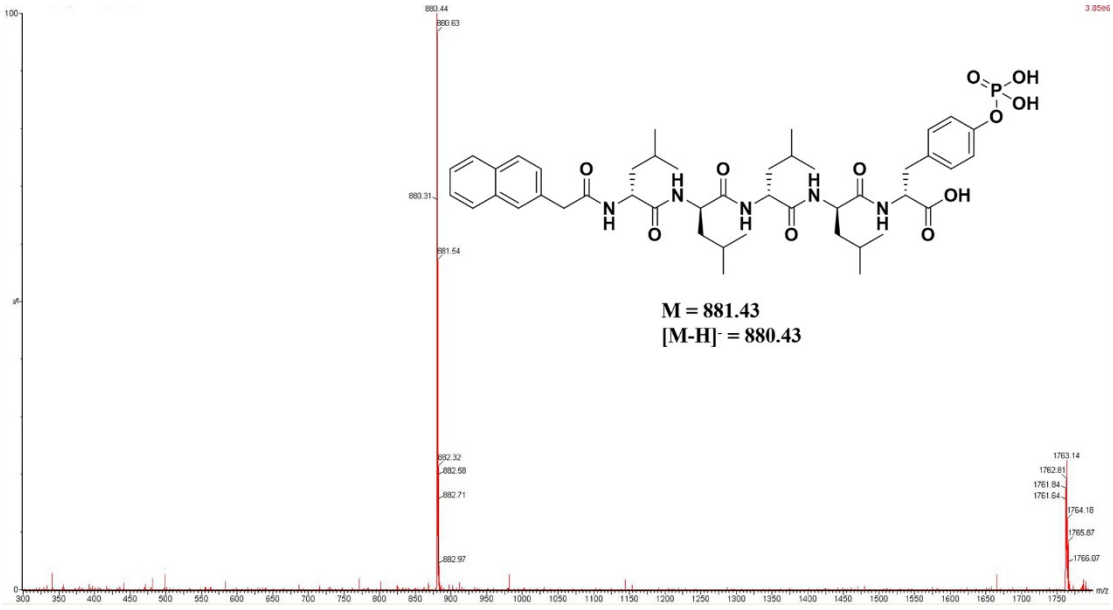


Figure S6. Mass spectrum of **1Dp** (M/Z = 880.43).

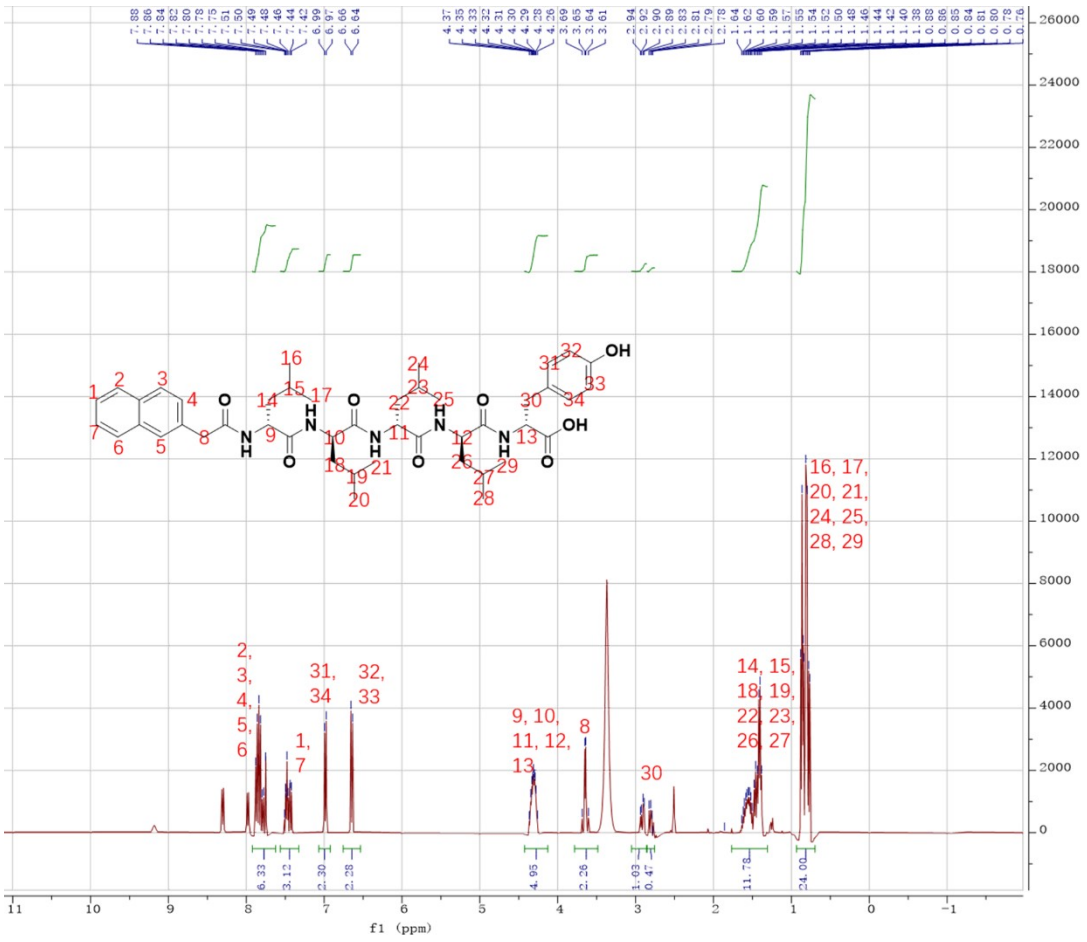


Figure S7. ^{1}H NMR spectrum of **1D**.

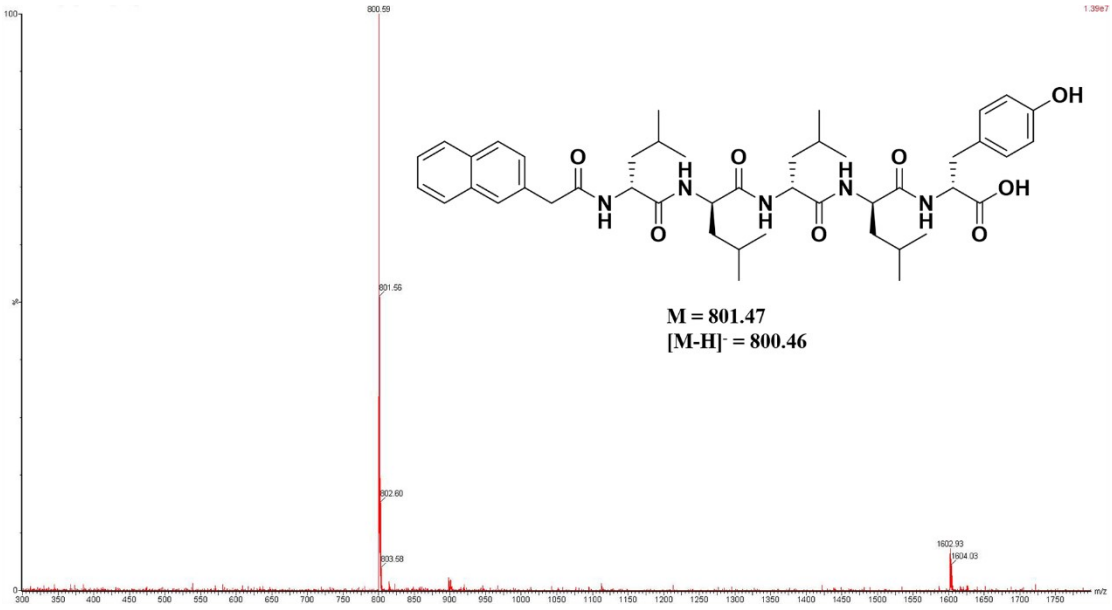


Figure S8. Mass spectrum of **1D** (M/Z = 866.59).

S3. TEM sample preparation

After placing 5 μL samples on 400 mesh copper grids coated with continuous thick carbon film (~35 nm) which is glowed discharged, we washed the grid with ddH_2O and UA (uranyl acetate). The sample loaded grid was stained with the UA. The residual UA was removed by filter paper and then dried in air.TEM images were obtained with Morgagni 268transmission electron microscope.

S4. Critical micelle concentration (CMC) measurement

The CMCs were determined using pyrene as the fluorescent probe. Different concentrations of compounds were prepared in pyrene-saturate solutions. The fluorescence spectra of pyrene solutions with different concentration compounds were obtained. The intensity ratio of 378 nm/393 nm (I_{378}/I_{393}) was determined by a Synergy H1 hybrid multi-mode microplate reader. Plot I_{378}/I_{393} against the concentrations of compounds. The concentration at the turning point is the CMC.

S5. Cell culture

SJSA-1 cell line, Saos2 cell line, HeLa cell line, HepG2 cell line, OVSAHO cell line, A2780 cell line, A2780cis cell line, HS-5 cell line and HEK293 cell line were purchased from American Type

Culture Collection (ATCC, USA). SJSA-1 cells, OVSAHO cells, A2780 cells, and A2780cis cells were cultured in RPMI-1640 Medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. Saos2 cells were cultured in McCoy's Medium supplemented with 15% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. HeLa cells, HepG2, and HEK293 cells were cultured in Eagle's Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. HS-5 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. All cells were maintained at 37°C in a humidified atmosphere of 5% CO_2.

S6. Cell viability

We determined the cytotoxicity against cells by using MTT assay. Cells were seeded in 96-well plates at 1×10^5 cells/well for 24 hours followed by culture medium removal and subsequently addition of culture medium containing different concentration of **5** (immediately diluted from fresh prepared 10 mM stock solution). After 1/2/3 hours, the culture medium with **5** was replaced by fresh culture medium and 10 μL MTT solution (5 mg/mL) was added to each well and incubated at 37°C for another 4 h. Then 100 μL of SDS-HCl solution was added to stop the reduction reaction and dissolve the formazan. The absorbance of each well at 595 nm was measured by a DTX880 Multimode Detector. The results were calculated as cell viability percentage relative to untreated cells. Data were obtained by from three independent wells (n = 3).

S7. Co-culture

0.8 million RFP-SJSA-1 and 0.8 million HS-5 are co-cultured overnight to adhere. The cell mixtures are treated with **1Lp** or **1Dp** for 4 h. After refreshing the culture media, the cells are cultured overnight and then seeded into CLSM dish. After culture overnight to adhere, the cells are stained with Hoechst, count the cells by using CLSM.

S8. LDH assay

LDH release (%) was measured using Pierce™ LDH Cytotoxicity Assay Kit purchased from Thermo Scientific™, using the protocols as following:

1. Seed SJSA-1 cells at 1×10^5 cells/well in triplicate wells in a 96-well plate for 24 hours to allow attachment.

2. Remove culture medium and incubate the cells with the precursors (**1Lp**, **1Dp**) at different concentrations. Add 10 μL Lysis Buffer (10 ×).

3. After incubation with precursors at different time, transfer 50 μL of each sample medium to a 96-well plate.

4. Add 50 μL Reaction Mixture to each sample well and mix gently.

5. After incubating the plate at room temperature for 30 minutes protected from light, add 50 μL of Stop Solution to each sample well and mix by gentle tapping.

6. Measure the absorbance at 490 and 680 nm using a Biotek Synergy 4 hybrid multi-mode microplate reader. Before calculation of LDH release, subtract the 680 nm absorbance value (background) from the 490 nm absorbance [(LDH at 490 nm) - (LDH at 680 nm)]. The LDH release (%) was calculated as percentage relative to maximum LDH activity controls.

S9. Dephosphorylation rate

The peptide **1Lp** or **1Dp** was incubated with ALP (0.5 U/mL) at 37ºC. At several certain time point, we quenched the dephosphorylation by adding methanol. The mixtures were injected into HPLC to measure the dephosphorylation ratios at different time points.

S10. Confocal laser scanning microscopy (CLSM) imaging

Cells in exponential growth phase were seeded in a confocal dish (3.5 cm) at 1.0×10^5 cells per dish and then incubated in incubator for 24 h. We removed culture medium, and added fresh medium containing precursors for different time points. After removing the medium and washing the cells with PBS (2 mL × 2), the cells were stained in PBS containing Congo red (0.1mg/mL) for 10 min at room temperature. After washing the cells with PBS (2 mL × 2), the cells were used for CLSM imaging in live cell imaging solution.

S11. Drug resistance test

SJSA-1 cells were seeded in three 40 mm culture dishes. The cells in the dishes were treated with (1) **1Lp**, (2) **1Dp**, or (3) culture medium for 5 weeks, respectively. The concentration of **1Lp** and

1Dp increase slowly from 20 μM to 100 μM. Then the cells were seeded into 96-well plates and test the cytotoxicity of the peptides (**1Lp** and **1Dp**) against stimulated cells and unstimulated cells by using MTT assay.

S12. Video list

Video 1: Time-lapse CLSM of RFP expressing SJSA-1 cells being incubated with **1Lp** (200uM)

Video 2: Time-lapse CLSM of RFP expressing SJSA-1 cells being incubated with **1Dp** (200uM)

S13. Supporting data

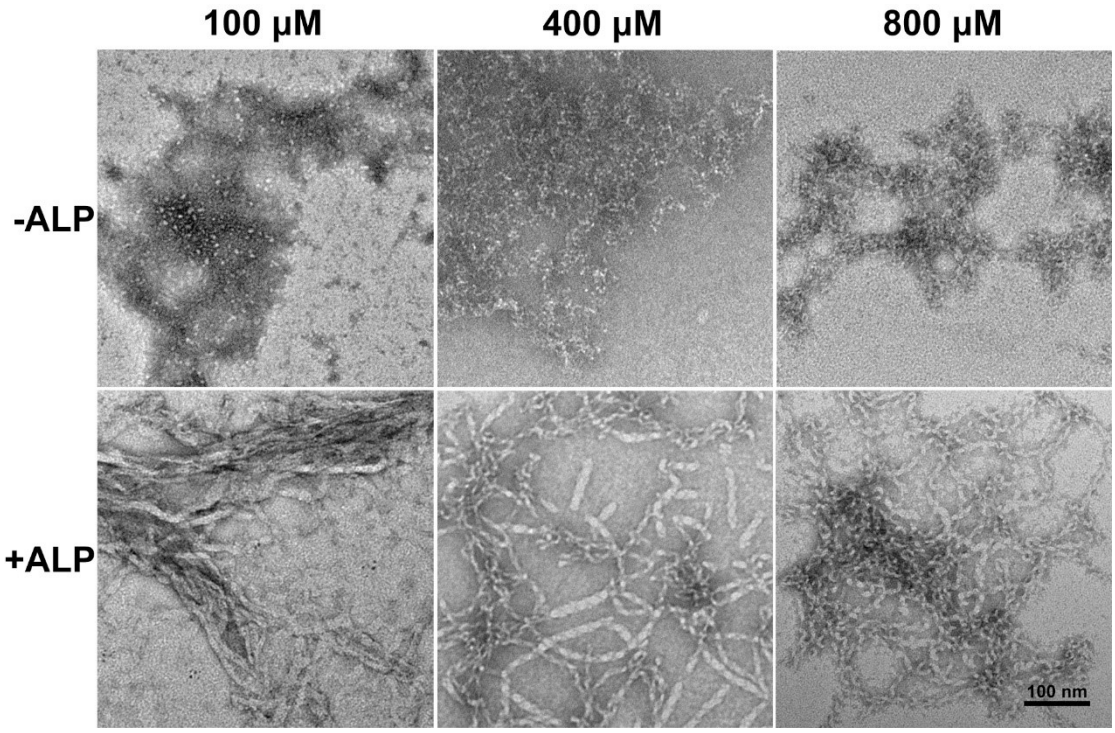


Figure S9. Transmission electron microscopes (TEM) images of **1Lp** (100 μM, 400 μM, 800 μM, PBS) before and after dephosphorylation by ALP (0.5 U/mL) for 24 h.

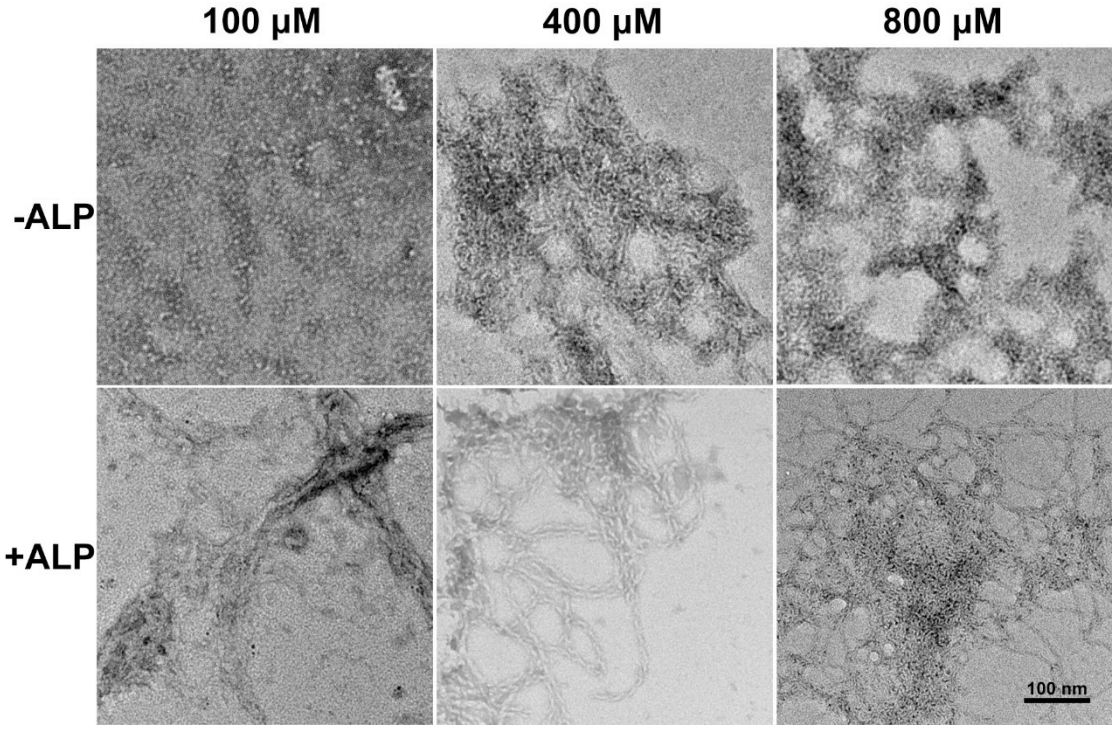


Figure S10. Transmission electron microscopes (TEM) images of **1Dp** (100 μM, 400 μM, 800 μM, PBS) before and after dephosphorylation by ALP (0.5 U/mL) for 24 h.

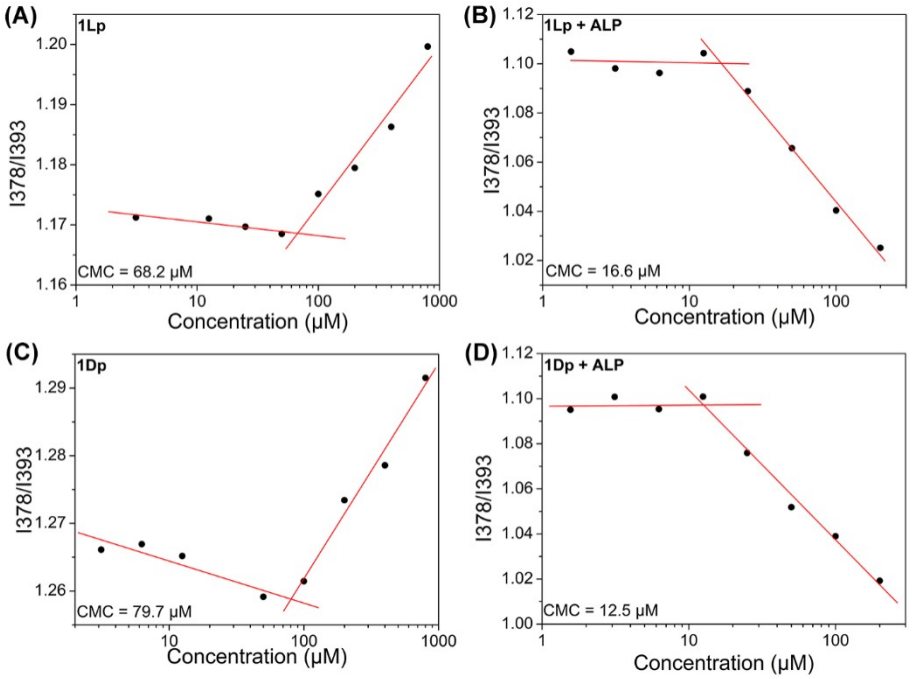


Figure S11. Critical micelle concentration (CMC) of (A) **1Lp** (PBS), (B) **1Lp** + ALP (PBS, 37°C, 24 h), (C) **1Dp** (PBS) and (D) **1Dp** + ALP (PBS, 37°C, 24 h).

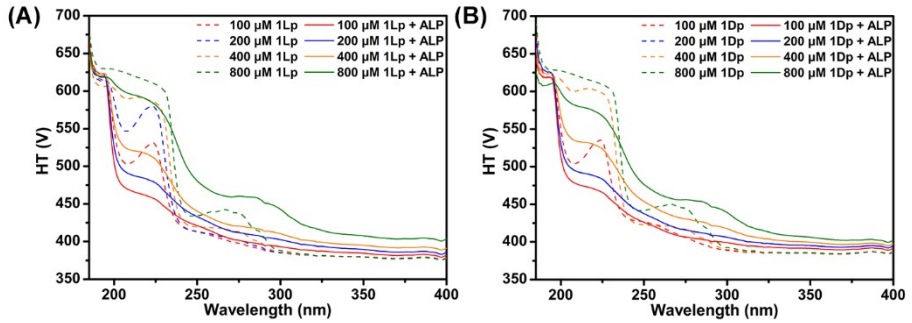


Figure S12. HT voltages of the CD spectra in Fig. 3.

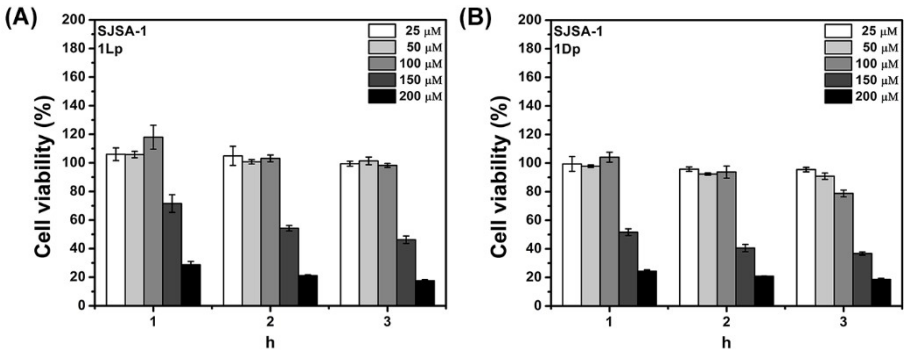


Figure S13. Cell viability of SJSA-1 cells incubated with **1Lp** or **1Dp** for 1 h, 2 h or 3 h.

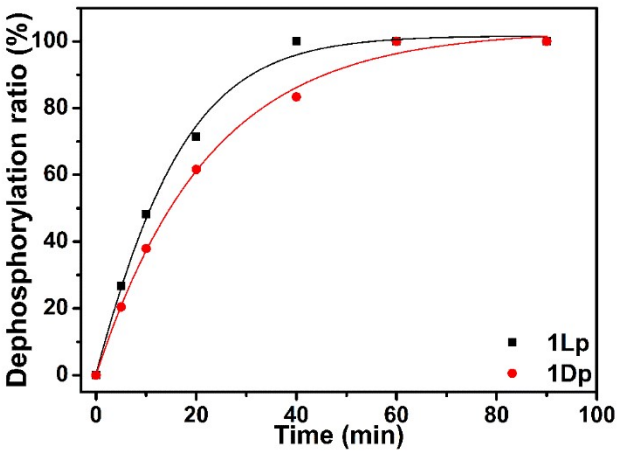


Figure S14. Dephosphorylation rate of **1Lp** and **1Dp** by ALP (0.5 U/mL, 37ºC).

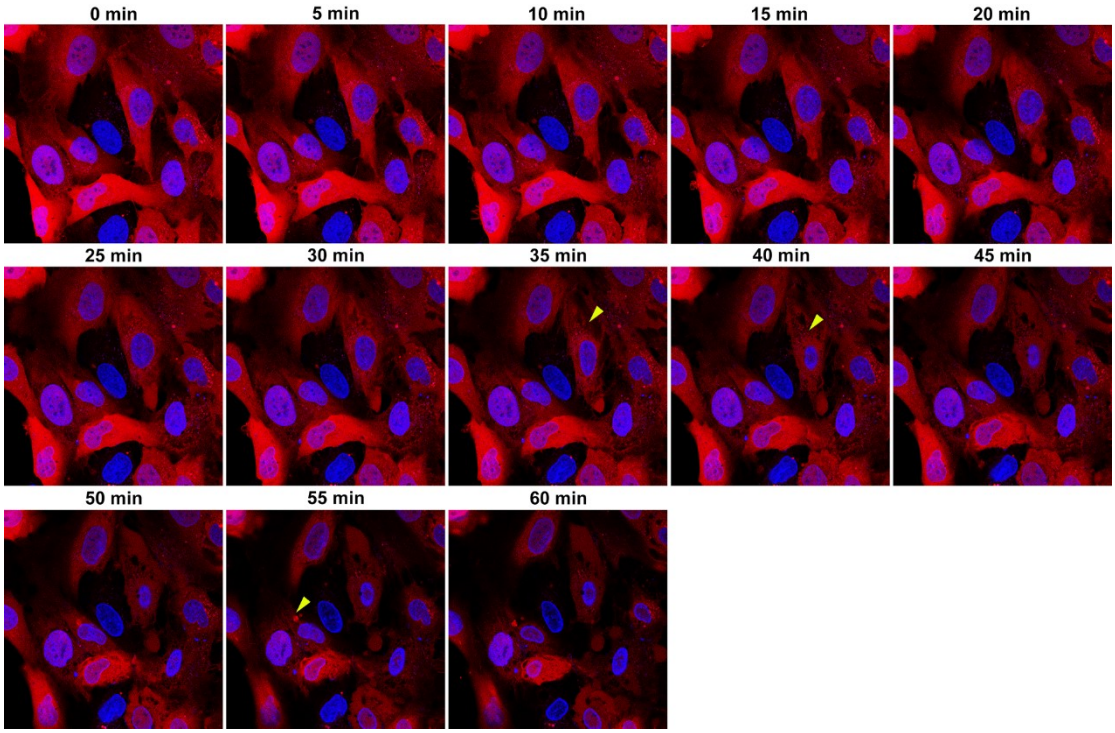


Figure S15. CLSM images of RFP-expressing SJSA-1 after incubating with **1Lp** (200 μM) for different time (Video 1). The yellow arrows show the disruption of cell membranes and release of cytoplasm.

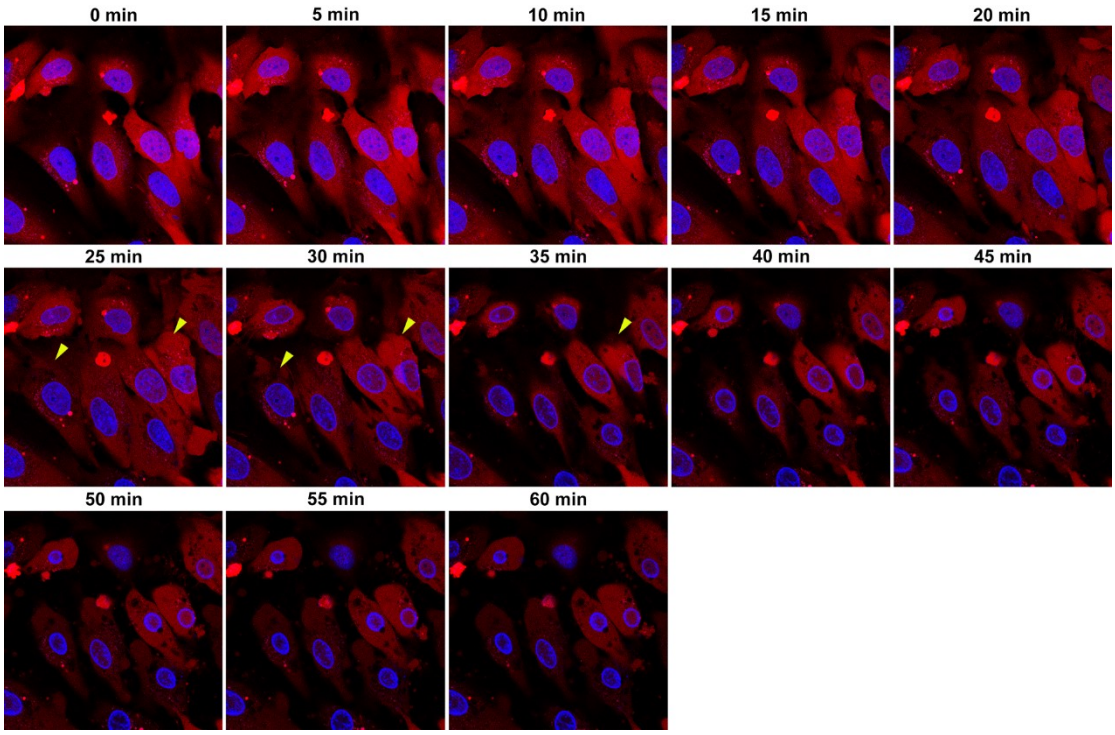


Figure S16. CLSM images of RFP-expressing SJSA-1 after incubating with **1Dp** (200 μM) for different time (Video 2). The yellow arrows show the disruption of cell membranes and release of cytoplasm.

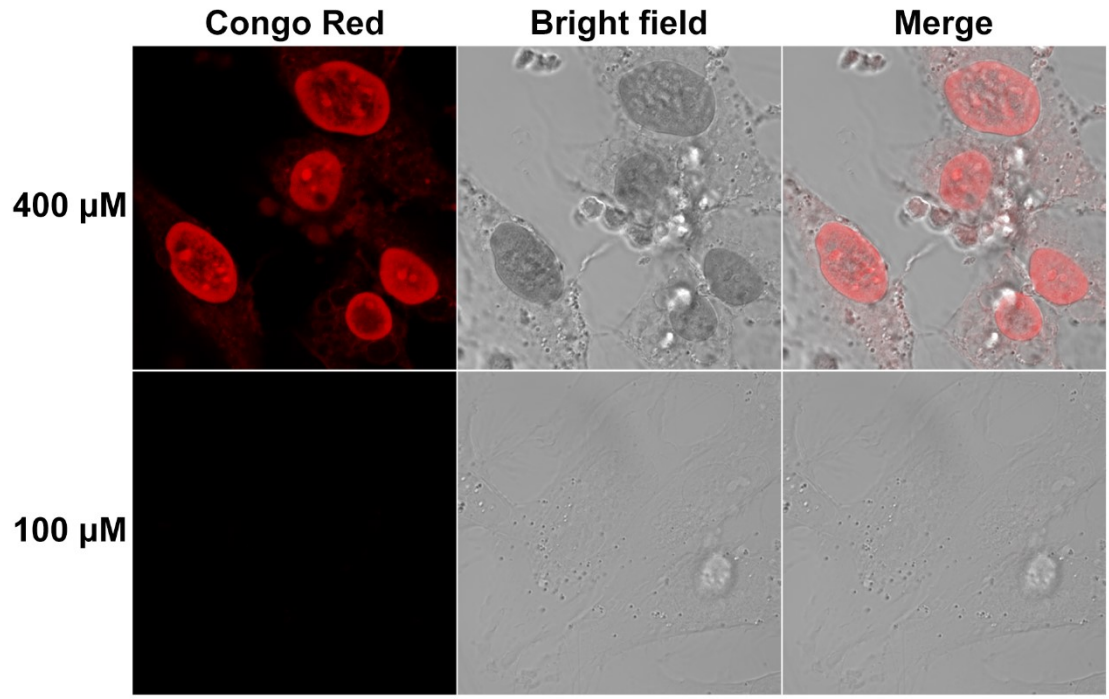


Figure S17. Congo red staining of SJSA-1 cells after incubated with **1Lp** (100 μM and 400 μM) for 4 h.

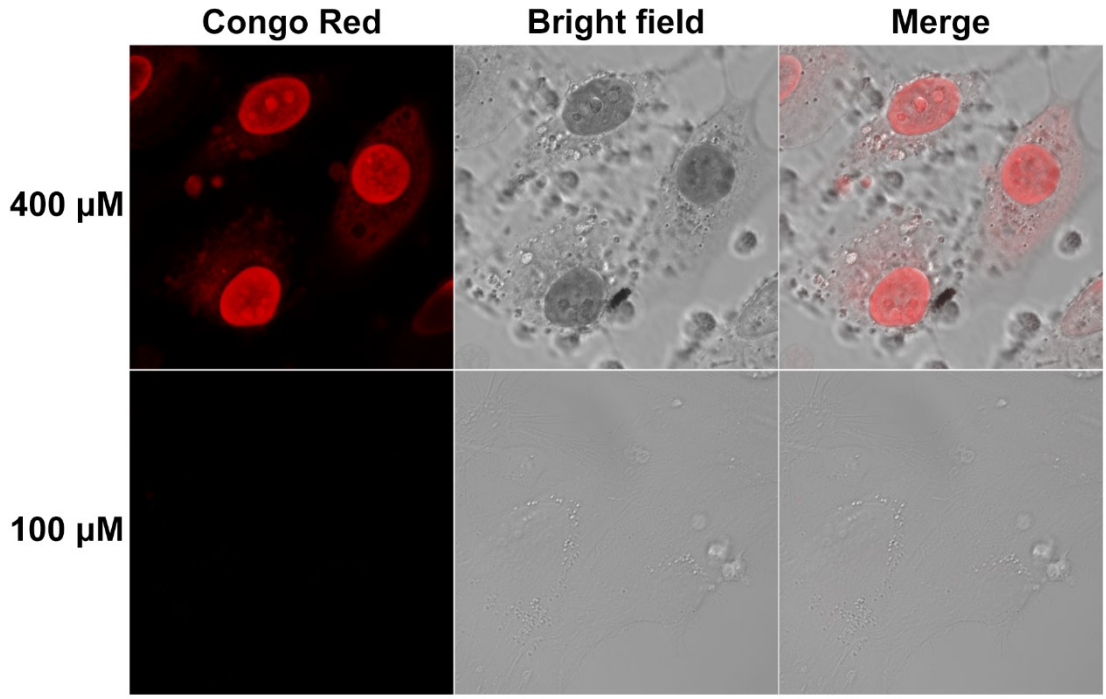


Figure S18. Congo red staining of SJSA-1 cells after incubated with **1Dp** (100 μM and 400 μM) for 4 h.