Supporting Information for

Minimal C-Terminal Modification Boosts Peptide Self-Assembling Ability for Necroptosis of Cancer Cells

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Content

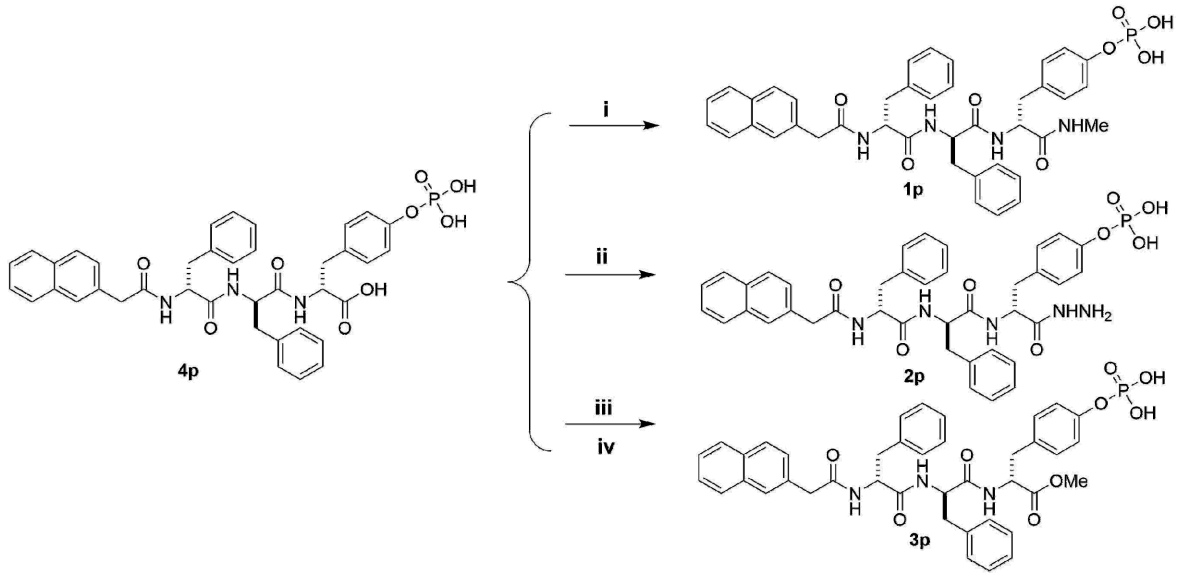
S1. Experiment materials and instruments

All chemical reagents and solvents were used as receiving from commercial sources without further purification. 2-Cl-trityl chloride resin (1.0-1.2 mmol/g), Fmoc-OSu and other Fmoc-amino acids were obtained from GL Biochem (Shanghai, China). Other chemical reagents and solvents were obtained from Fisher Scientific; alkaline phosphatase was purchased from Biomatik. Dulbecco's modified Eagle's medium (DMEM), McCoy's 5a Medium were purchased from ATCC and fetal bovine serum (FBS) and penicillin/streptomycin were purchased from Gibco by life technologies.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from ACROS Organics. All precursors were purified with Water Delta600 HPLC system, equipped with an XTerra C18 RP column and an in-line diode array UV detector. We obtained LC-MS spectrum on Waters Acquity Ultra Performance LC with Waters MICROMASS detector, ultraviolet-visible (UV) spectra on JASCO J-810 spectrophotometer, and ^{1}H-NMR spectra on Varian Unity Inova 400, and TEM images on Morgagni 268 transmission electron microscope. MTT assay for cell toxicity test on DTX880 Multimode Detector.

S2. Synthesis and characterization of the precursors

We synthesized Fmoc-Tyr(PO_3H_2)-OH based on previous work for directly use of solid phase peptide synthesis (SPPS). With SPPS, we synthesized **4p** based on protocols in published paper.[1] The following scheme demonstrate the synthetic route for **1p**, **2p** and **3p** from modification of **4p**. Then all compounds were purified by reverse phase HPLC using acetonitrile (0.1% TFA) and double-distilled water (0.1% TFA) as the eluents.



i: Methylamine, HBTU, DIPEA ; ii: Hydrazine, HBTU, DIPEA; iii: TMSBr; iv:MeOH

Scheme S1. Synthesis route of **1p**, **2p**, **3p**

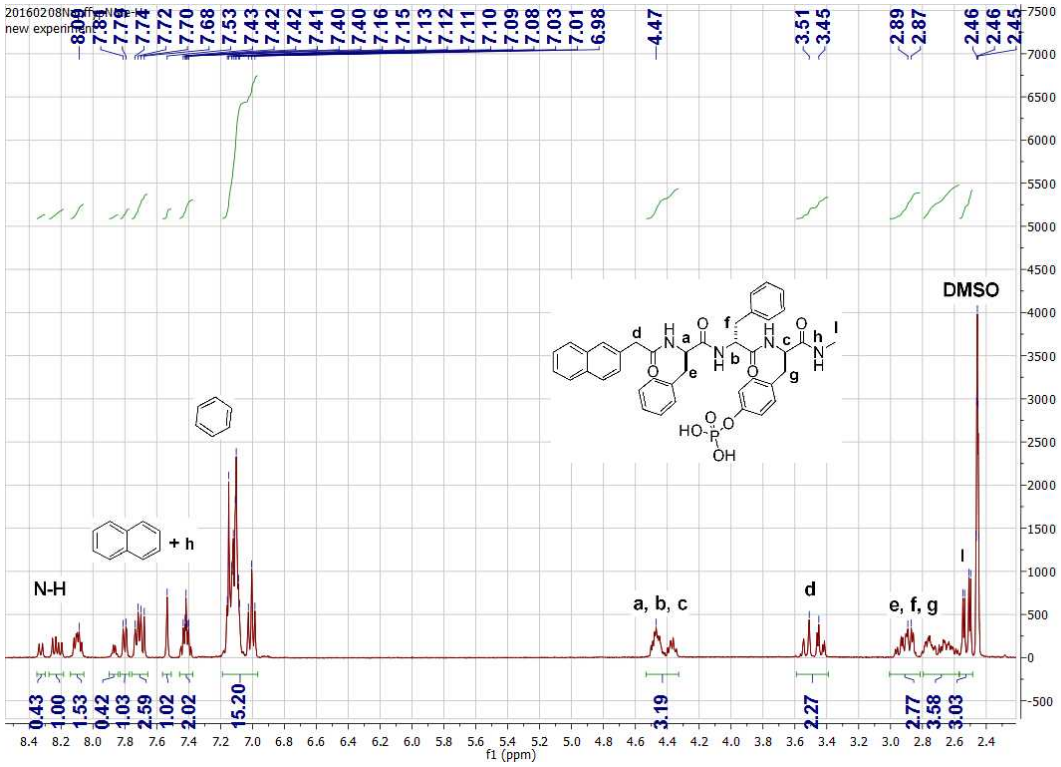


Figure S1. ^{1}H NMR of **1p** in DMSO-d_6.

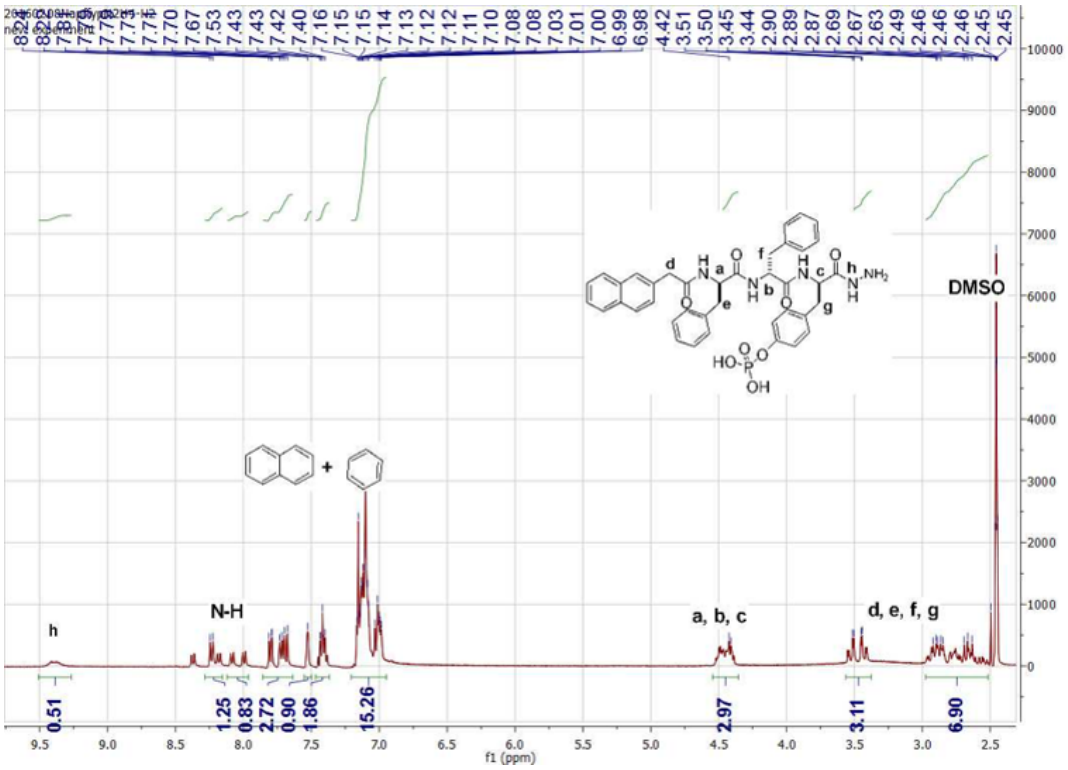


Figure S2. ^{1}H NMR of **2p** in DMSO-d$_6$.

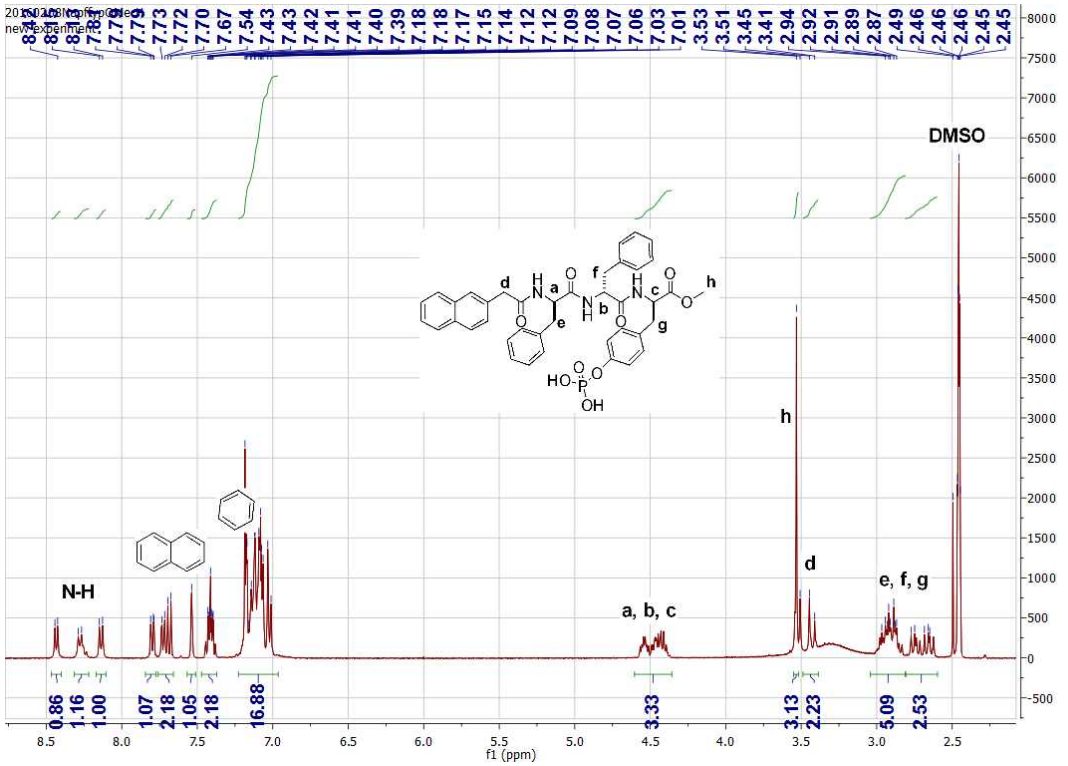


Figure S3. ^{1}H NMR of **3p** in DMSO-d$_6$.

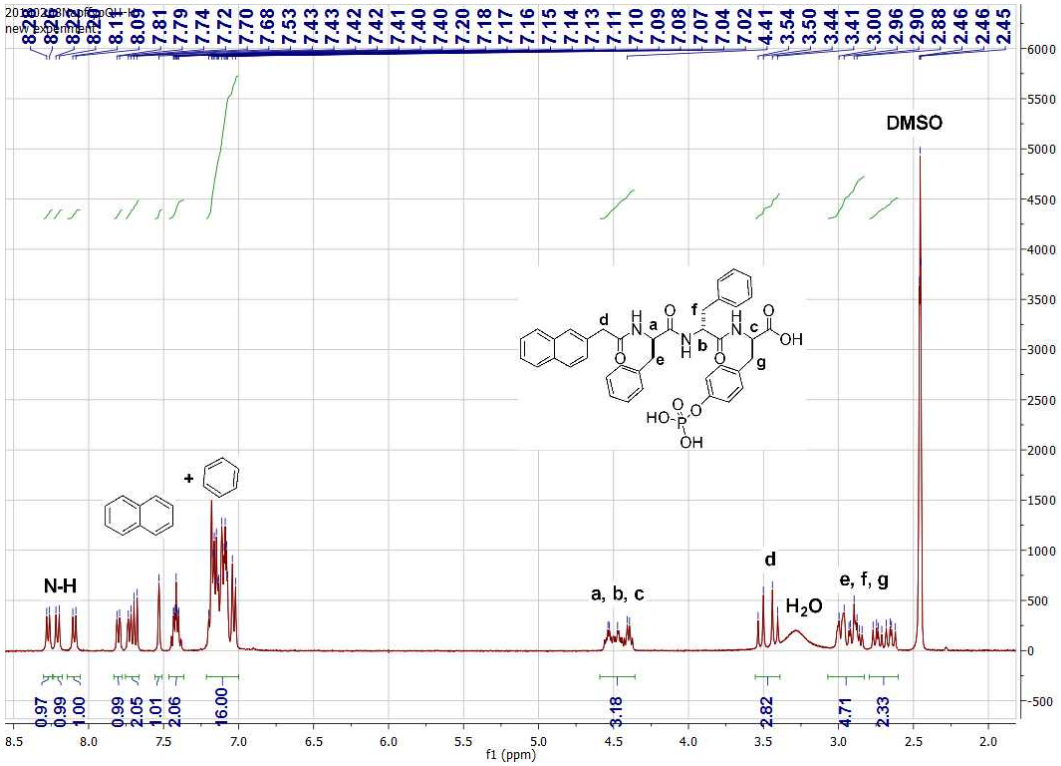


Figure S4. ^{1}H NMR of **4p** in DMSO-d$_6$.

LC-MS (ESI):

1p (m/z): $C_{40}H_{41}N_4O_8P$, calc. 736.27; observed $[M-H]^-$ 735.57.

2p (m/z): $C_{39}H_{40}N_5O_8P$, calc. 737.26; observed $[M-H]^-$736.50.

3p (m/z): $C_{40}H_{40}N_3O_9P$, calc. 737.25; observed $[M-H]^-$736.56.

4p (m/z): $C_{39}H_{38}N_3O_9P$, calc. 723.23; observed $[M-H]^-$722.55.

S3. General procedure for hydrogel preparation

We dissolve lyophilized precursors (**1p**/**2p**/**3p**/**4p**) in distilled water, adjust pH with 1M NaOH to

reach pH 7.4, and make final concentrations all equal to 1 mM, followed by addition of 3 U/mL ALP. Optical images were taken 12 hours after the ALP addition.

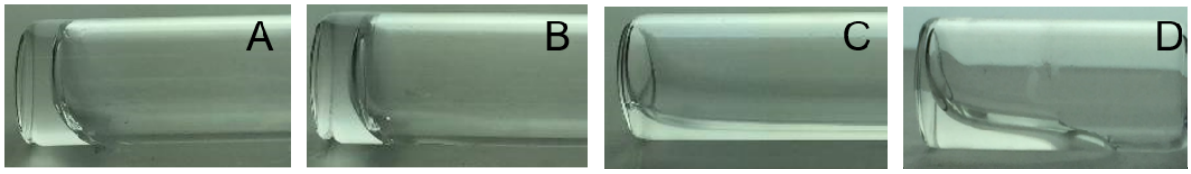


Figure S5. Optical images of 1 mM A) **1p**, B) **2p**, C) **3p** and D) **4p** after addition of ALP (3U/mL).

S4. TEM sample preparation

We first place 5 μL samples (preparation procedure described in S3) on 400 mesh copper grids coated with continuous thick carbon film (~ 35 nm) which is glowed discharged. Then we washed the grid with ddH_2O twice. Finally we stained the sample loaded grid with a large drop of the UA (uranyl acetate) and allow to dry in air.

S5. Rheological measurement

All rheological experiments were performed at 25°C ±0.10°C in dynamic time sweep using TA ARES-G2 rheometer (TA Company, America) by 25 mm parallel plates. To minimize evaporation, a solvent trap was employed and a low viscosity mineral oil was applied around the sample. Time sweep experiments data were collected at 6.28 rad/s frequency and 1.0% strain.

S6. Static light scattering measurement

We performed static light scattering on using an ALV (Langen, Germany) goniometer and correlator system with a 22 mW HeNe (λ = 633 nm) laser and an avalanche photodiode detector.

We prepared the samples of **1p**, **2p**, **3p** or **4p** with the concentration of 12.5 μM, 25 μM and 50 μM in pH7.4 PBS buffer. After addition of 2 U/mL ALP, we incubated the tubes for 24 hours and test the static light scattering at degree of 30, 60, 90, and 120°. The resulting intensity ratios are proportional to the amount of aggregates in the samples.

S7. Cell culture and MTT assay

Cell culture: Sarcoma osteogenic (Saos-2) cells and human marrow stromal cells (HS-5) were purchased from American-type Culture Collection (ATCC, USA). Saos-2 cells were cultured in McCoy's 5a Medium (for Saos-2) supplemented with 15% v/v fetal bovine serum, 100 U/mL penicillin and 100 μg/mL streptomycin; HS-5 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% v fetal bovine serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified atmosphere of 5% CO_2.

MTT assay: The cytotoxicity was determined by the viability of cells with MTT assay. Saos-2 cells lines or HS-5 cell lines were seeded in 96-well plates at 1×10^5 cells/well for 24 followed by culture medium removal and subsequently addition of culture medium containing different concentration (20 μM, 50 μM, 100 μM, 200 μM and 500 μM) of the precursors (immediately diluted from fresh prepared aqueous stock solution of 10 mM or DMSO stock solution of 250 mM). After 24/48/72 hours, 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-HCl solution was added to stop the reduction reaction and dissolve the purple formazan. The absorbance of each well at 595 nm was measured by a multimode microplate reader. The results were expressed as cell viability percentage relative to untreated cells. The

cytotoxicity assay was performed three times and the average value of the three measurements was taken.

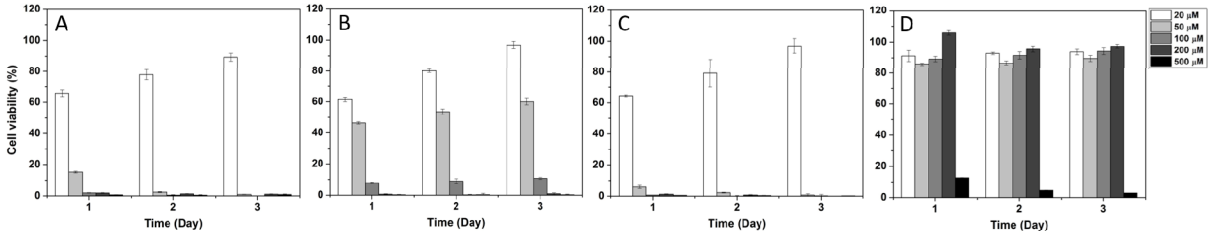


Figure S6. Cell viability of Saos-2 cells incubated with A) **1p**, B) **2p**, C) **3p** and D) **4p** at 24, 48 and 72 hours.

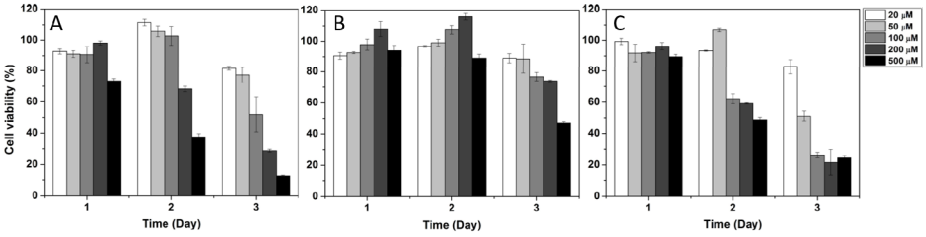


Figure S7. Cell viability of HS-5 cells incubated with A) **1p**, B) **2p**, C) **3p** at 24, 48 and 72 hours.

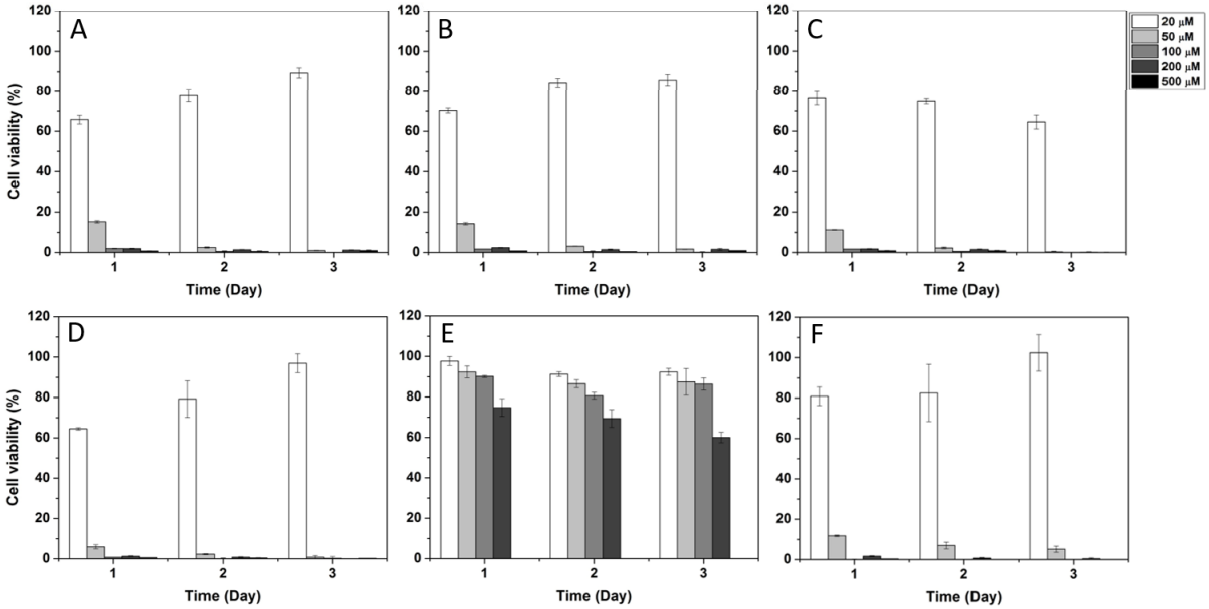


Figure S8. Cell viability of Saos-2 cells incubated with **1p** in A) pH7.4 B) pH8.0 or C) DMSO stock solution and **3p** in D) pH7.4 E) pH8.0 or F) DMSO stock solution.

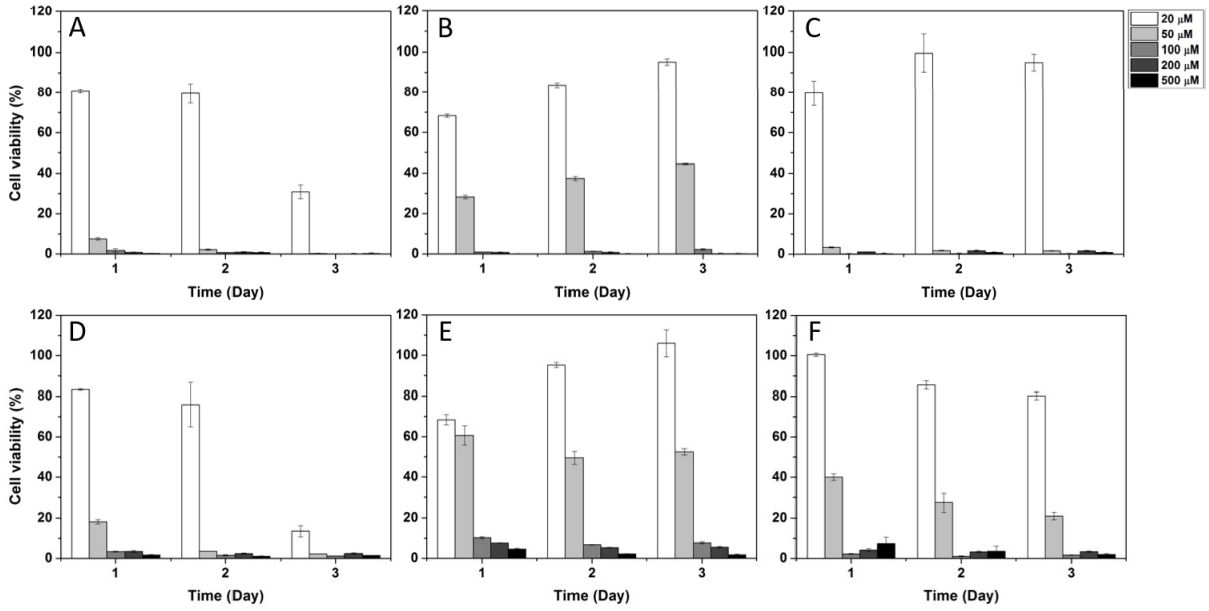


Figure S9. Cell viability of Saos-2 cells incubated with A) **1p**, B) **2p**, or C) **3p** in the presence of PLAP inhibitor L-Phe and D) **1p**, E) **2p**, or F) **3p** in the presence of TNAP inhibitor levamisole. [L-Phe] = 1 mM; [levamisole] = 1 mM

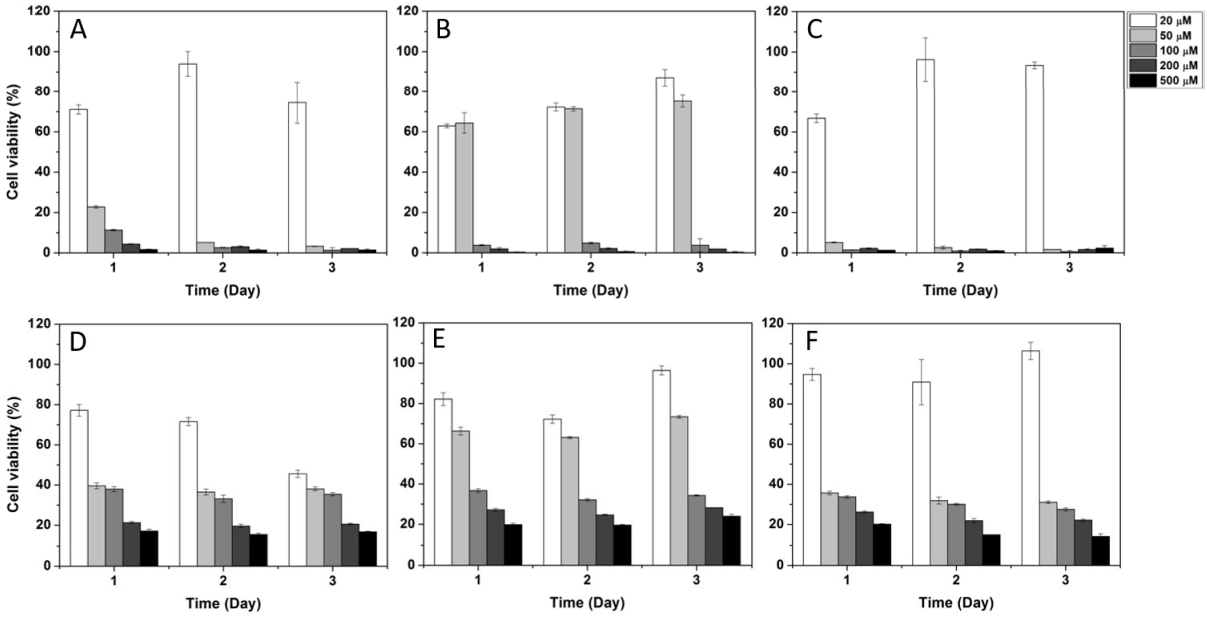


Figure S10. Cell viability of Saos-2 cells incubated with A) **1p**, B) **2p**, or C) **3p** in the presence of a pan-caspase inhibitor (zVAD-fmk) or D) **1p**, E) **2p**, or F) **3p** in the presence of a necroptosis inhibitor (Nec-1). [zVAD-fmk] = 45 μM; [Nec-1] = 50 μM.

S8. Confocal microscopy

Saos-2 cells were seeded in 96-well plates at 1×10^5 cells/well for 24 followed by culture medium removal and subsequently addition of fresh culture medium or culture medium containing 500 μM of the precursors. After 1 h of incubation, cells were washed with PBS buffer for 3 times and stained with 1.0 μg/ml Hochst 33342 for 10 min at 37 °C in dark, followed by three-times wash with PBS buffer and stained with 0.1 mg/mL Congo red at 37 °C in dark. Finally, the cells were rinsed three times in PBS, and then kept in the live cell imaging solution (Invitrogen Life Technologies A14291DJ) for imaging.

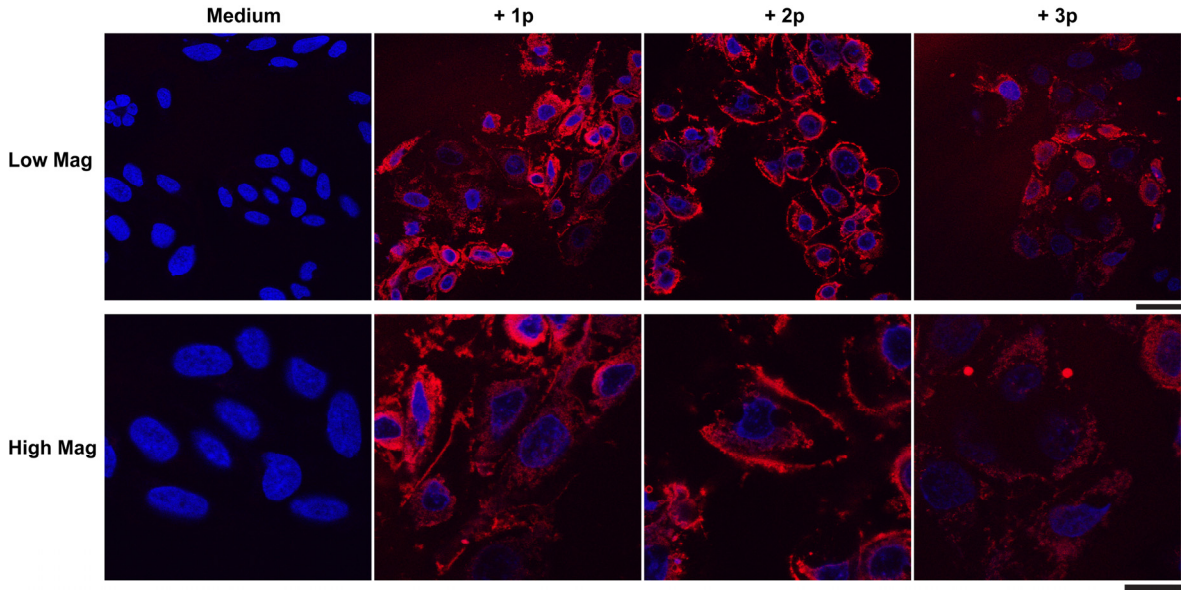


Figure S11. Fluorescence images of Saos-2 cells stained with Congo red (red) and Hoechst (nuclei, blue) after the treatment of culture medium, **1p** (500 μM), **2p** (500 μM) or **3p** (500 μM) for 1 hour. Scale bars: upper=40 μm, bottom=20 μm.

Reference

(1) Shi, J.; Du, X.; Yuan, D.; Zhou, J.; Zhou, N.; Huang, Y.; Xu, B. *Biomacromolecules* **2014**, *15*, 3559; Ottinger, E. A.; Shekels, L. L.; Bernlohr, D. A.; Barany, G. *Biochemistry* **1993**, *32*, 4354.