Supporting Information for

Assemblies of D-peptides for Targeting Cell Nucleolus

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Content

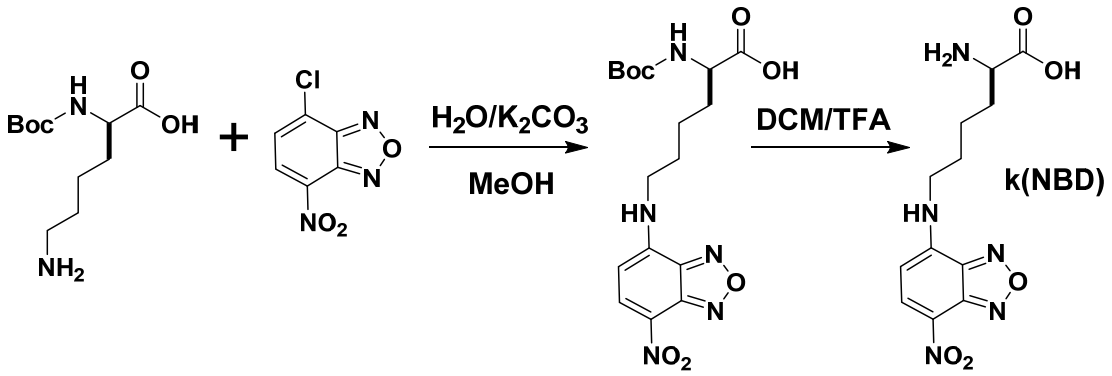
S1. Experiment materials and instruments

2-Cl-trityl chloride resin (0.6 mmol/g), Fmoc protected amino acid, HBTU and Fmoc-OSu were obtained from GL Biochem (Shanghai, China). N, N-diisopropylethylamine (DIPEA) and other chemical reagents and solvents were obtained from Fisher Scientific. All the chemical reagents and solvents were used as received from commercial sources without further purification. Minimum Essential Media (MEM) and McCoy's 5a Medium from ATCC, and fetal bovine serum (FBS) and penicillin/streptomycin from Gibco (Life technologies). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from ACROS Organics. Ethyl-isopropyl-amiloride was purchased from CAYMAN CHEMICAL. Chlorpromazine was obtained from Alfa Aesar. 96-well plates were obtained from CELLTREAT. Filipin III was purchased from Santa Cruz Biotechnology, Inc. M-βCD, Hoechst 33342, ER tracker and LysoTracker were bought from Fisher Scientific. The normal goat serum, the fluorescently labeled secondary antibodies and anti-Fibrillarin antibody were obtained from ABCAM. The RNA extraction kit was obtained from Thermofisher (Cat. nos. 12183018A). MTT assay for cell viability test on DTX880 Multimode Detector (BECKMAN COULTER). CLSM images were obtained using Zeiss LSM 880 confocal microscopy at the lens of 63× with oil. All precursors were purified with Water Delta600 HPLC system, equipped with an XTerra C18 RP column. TEM images on Morgagni 268 transmission electron microscope, and MTT assay for cell viability test on DTX880 Multimode Detector.

S2. Synthesis and characterization of the precursors

Synthesis of k(NBD)

Scheme S1. Synthetic procedure of k(NBD)



To a 10 mL water solution containing Boc-Lys-OH (2.2 mmol, 541.9 mg) and K_2CO_3 (6 mmol, 829.2 mg), 4-Chloro-7-nitrobenzofurazan (NBD-Cl, 2 mmol, 399.1 mg) in 20 mL of methanol was added dropwisely under nitrogen protection. The reaction mixture was stirred at room temperature for about 12 h. Methanol was removed by a rotary evaporator. The obtained aqueous solution was acidified to about pH 3 by HCl (2 N). The acidic aqueous solution was then extracted by ethyl ether for 3 times. The combined organic solution was dried over $MgSO_4$ and then concentrated by a rotary evaporator. We next used DCM/TFA (2:1) to remove the Boc group of Boc-k(NBD) at room temperature for 3 h. After concentrated by a rotary evaporator, the resulted dark powder was directly used for the next step.

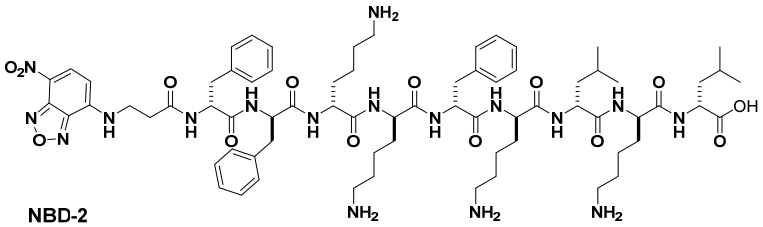
Solid phase peptide synthesis

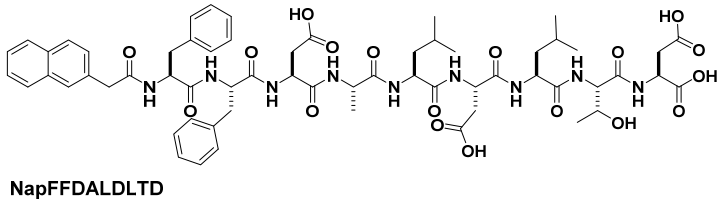
All the peptides were prepared by standard solid-phase peptide synthesis (SPPS) using 2-chlorotrityl chloride resin and the corresponding Fmoc-protected amino acids with side chains properly protected. The first amino acid was loaded onto the resin at about 0.6 mmol/g of resin. After loading the first amino acid to the resin, the capping regent (DCM: MeOH: DIPEA = 17: 2: 1) was used to ensure that all the active sites of the resin were protected. 20% piperidine in DMF was used to remove the Fmoc group, the next Fmoc-protected amino acid was coupled to the free amino group using HBTU as the coupling reagent. The growth of the peptide chain followed the established Fmoc SPPS protocol. The crude peptides were collected using TFE-mediated cleavage method: The peptide derivative was cleaved using 20% of trifluoroethanol (TFE) in DCM for 1 h. After removing the DCM, 20 mL of ice-cold ethyl ether/per gram

of resin was then added to above solution. After discarding most of the supernatant, the resulting precipitate was filtered and dried by a lyophilizer.

Synthesis of D-1

50 mg of peptide with side chain protected was dissolved in 10 mL of N,N-dimethylformamide (DMF), followed by 3 equiv. of N-hydroxysuccinimide (NHS) and 3 equiv. of N, N′-disopropylcarbodiimide (DIC) were added. After being stirred at room temperature for 3 h, 1.2 equiv. of k(NBD) was then added with 3 equiv. N-diisopropylethylamine (DIPEA). After stirring overnight, the solution was evaporated under reduced pressure to yield a viscous solution. We next used 95% of TFA in DCM to remove the protected group on the peptide. After stirring for 3 h, the solution was evaporated under reduced pressure and ethyl ether was then added to above solution. After discarding most of the supernatant, the resulting precipitate was filtered and dried by lyophilizer. The target products were purified by reverse phase HPLC (overall yield is 28%).





Scheme S2. Molecular structure of **NBD-2** and **Nap-FFDALDLTD**

S3. Purification of sample by HPLC

We used Water Delta600 HPLC system, equipped with an XTerra C18 RP column to purify the resulted powder after SPPS. We use acetonitrile (from fisher, HPLC grade) plus 0.1% trifluoroacetic acid (TFA)

and water (from fisher, HPLC grade) plus 0.1% TFA as eluent. We used gradient eluted program for purifying peptides as detailed in Table S1.

S4. Cell culture and MTT assay

T98G, Saos-2, and HeLa cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA), MES-SA/dx5 cells from Sigma-Aldrich Co. T98G and HeLa cells were propagated in Minimum Essential Media (MEM, Invitrogen Life Technologies) with 10% FBS and 1% P/S (100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin, Invitrogen Life Technologies). Saos-2 cells were cultured in McCoy's 5A with 15% FBS and 1% P/S. MES-SA/dx5 cells were cultured in McCoy's 5A with 10% FBS and 1% P/S. The incubation conditions for all the cells are at 37 °C in a humidified atmosphere of 5% CO_2.

MTT assay: T98G cells seeded in a 96-well plate with the density of 1×10^4 cells per-well (total medium volume of 100 μL). 24 hours post seeding, after the removal of the medium, we added the solutions with a serial of concentrations (5 concentrations) of **D-1** to each well. Cells without the treatment of the precursors were used as the control. At designated time (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 cytotoxicity assay was performed three times and the average value of the three measurements was taken.

S5. Live cell imaging

For all live imaging, cells in exponential growth phase were seeded in a confocal dish (3.5 cm) at 1.5×10^5 cells per dish. After putting the dish in an incubator for 24 h, we removed culture medium, and added fresh medium containing the peptides for different time points. After removing the medium and using live cell imaging buffer to wash the cells for one time, we added 1 mL of Hoechst (1 μg mL^{-1}) for 10 minutes. Then, the cells were washed three times with live cell imaging buffer and imaged by Zeiss LSM 880 confocal microscopy at the lens of 63× with oil.

For endocytosis mechanism experiment, we first pre-incubated different endocytosis inhibitors of EIPA (100 μM, ethyl-isopropyl-amiloride), CPZ (30 μM, chlorpromazine), Filipin Ⅲ (5 μg/mL) and M-βCD (5 mM) with T98G cells for 30 minutes, and then added 200 μM of **D-1** to the above confocal dish. After co-incubating the inhibitor with **D-1** for another 1 h, we removed the culture medium, washed the T98G cells by live cell imaging solution for 3 times. After being stained with Hoechst 33342 for 10 minutes, washed by live cell imaging solution for another 3 times, the T98G cells were imaged by CLSM immediately.

For staining lysosome (or ER), T98G cells were treated with **D-1** for 2 h, the medium was removed from the confocal dish. The cells were washed by PBS for 3 times, then the pre-warmed (at 37°C) LysoTracker containing medium or ER tracker containing buffer was added. The T98G cells were incubated under growth conditions for 40 minutes before removing the staining solution. The cells washed by live cell imaging solution 3 times before staining by the nucleus dye Hoechst 33342. Then the cells were washed by live cell imaging solution and imaged immediately by confocal laser scanning microscope.

S6. Immunofluorescence

After incubating T98G cell lines (1.5×10^5) in a 3.5 cm confocal dish for 24 h at 37 °C in a humidified atmosphere of 5% CO_2, we added the **D-1** (200 μM) for 2 h. After three washes with PBS buffer, T98G cells were fixed by 4% formaldehyde for 15 minutes at 37 °C and then incubated in 1% BSA/10% normal goat serum/0.3 M glycine in 0.1% PBS-Tween for 1 h to permeate the cells and block non-specific protein-protein interactions. After another three washes with PBS buffer, the cells were treated with primary antibodies overnight at 4 °C. The secondary antibody was (Alexa Fluor® 647 goat anti-rabbit (or mouse) IgG (H+L) used at the concentration of 2 μg mL^{-1} for 1 h. Finally, we used Hoechst 33342 to stain cell nucleus and imaged on a Zeiss LSM 880.

S7. Transmission electron microscopic

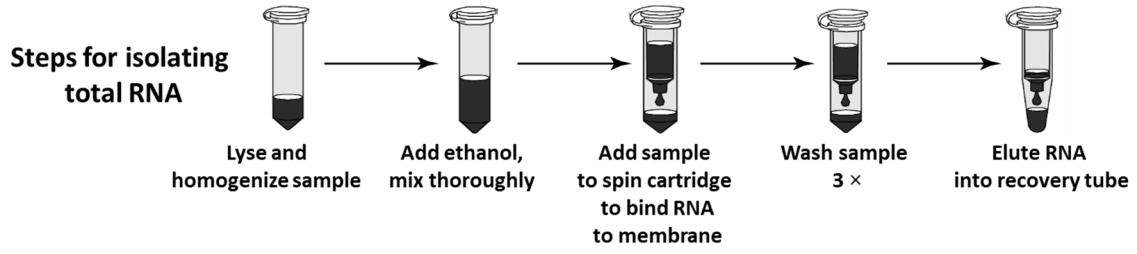
Grids (400 mesh copper grids coated with continuous thick carbon film ~35 nm in thickness, purchased from Pacific Grid Tech Co) were glow discharged for 30 seconds, and then we placed sample solution on

the grid (5 μL, sufficient to cover the grid surface), About 25 s later, we placed three large drops of ddH_2O on parafilm to let the grid touch the water drops (each for 5 seconds) with the sample-loaded surface facing the parafilm, and then tilted the grid and gently let water to be absorbed from the edge of the grid using a filter paper. Immediately after rinsing, we placed three large drops of uranyl acetate (UA) staining solution on parafilm to let the grid touch the staining solution drops with the sample-loaded surface facing the parafilm, and then tilt the grid and gently let the stain solutions to be absorbed from the edge of the grid using a filter paper. After drying the grid, we examined the nanostructure on Morgagni 268 Microscope.

S8. Circular dichroism measurement

CD spectra were recorded (185−230 nm) using a JASCO 810 spectrometer under a nitrogen atmosphere. The peptide was placed evenly on the 1 mm thick quartz cuvette and scanned with 0.1 nm interval for three times. The percentage of secondary structures in different samples was calculated by the programs provided in DichroWeb.

S9. Isolation of RNA from T98G cells



Scheme. S3. Procedure of purifying RNA from T98G cells.

We followed the procedure that supplied by company (Cat. nos. 12183018A).

1. Remove the culture medium from the T98G cells, then add 0.6 mL lysis buffer with 2-mercaptoethanol;
2. Vortex the above suspension until the cell pellet is dispersed and the cells appear lysed;

3. Pass the lysate 5-10 times through an 18-to 21-gauge syringe needle;
4. Add one volume 70% ethanol to each volume of cell homogenate;
5. Vortex to mix thoroughly and to disperse any visible precipitate that may form after adding ethanol;
6. Transfer up to 700 μL of the sample to the spin cartridge and centrifuge at 12,000 g for 15 seconds at room temperature. Discard the flow-through, and reinsert the spin cartridge into the same collection tube;
7. Repeat step 6 until the entire sample has been processed;
8. Add 700 μL wash buffer I to the spin cartridge and centrifuge at 12,000 g for 15 seconds at room temperature. Discard the flow-through and the collection tube, and place the spin cartridge into a new collection tube;
9. Add 500 μL wash buffer II with ethanol to the spin cartridge and centrifuge at 12,000 g for 15 seconds at room temperature. Discard the flow-through;
10. Repeat step 9 once;
11. Centrifuge the spin cartridge at 12,000 g for 1-2 minutes to dry the membrane with bound RNA. Discard the collection tube and insert the spin cartridge into a recovery tube;
12. Add 30-100 μL RNase-free water to the center of the spin cartridge and incubate at room temperature for 1 minute;
13. Centrifuge the spin cartridge for 2 minutes at 12,000 g at room temperature to elute the RNA from the membrane into the recovery tube and store the purified RNA at -80 °C for long-term storage.

S10. Statistical analysis of the diameter of nanoparticles

We used the software of image J for analysis of diameter. The detailed process are shown below:

1) Opening the software and the TEM image;

2) Using the button of ‘magnifying tool’ to magnify the scale bar that existed in the TEM image;

3) Using the button of ‘straight’ and press shift to measure the length of scale bar, press the button of ‘analyze’ and select ‘set scale’, one can see the distance in pixels of the scale bar, add the known distance of scale bar, after add the unit of length, press ok. Thus, one obtains the distance of each pixels in the TEM image;

4) After using the button of ‘straight’ to measure the diameter of nanoparticle, pressing ‘analyze’ and select ‘measure’. One gets the diameter of this nanoparticle;

5) After measuring enough nanoparticles in one TEM image, repeating the process of 1 to 4, one can get much more nanoparticles from other individual TEM images;

6) After measuring, clicking summarize, one can get the mean and sd of the nanoparticles.

S11. Supplemental figures

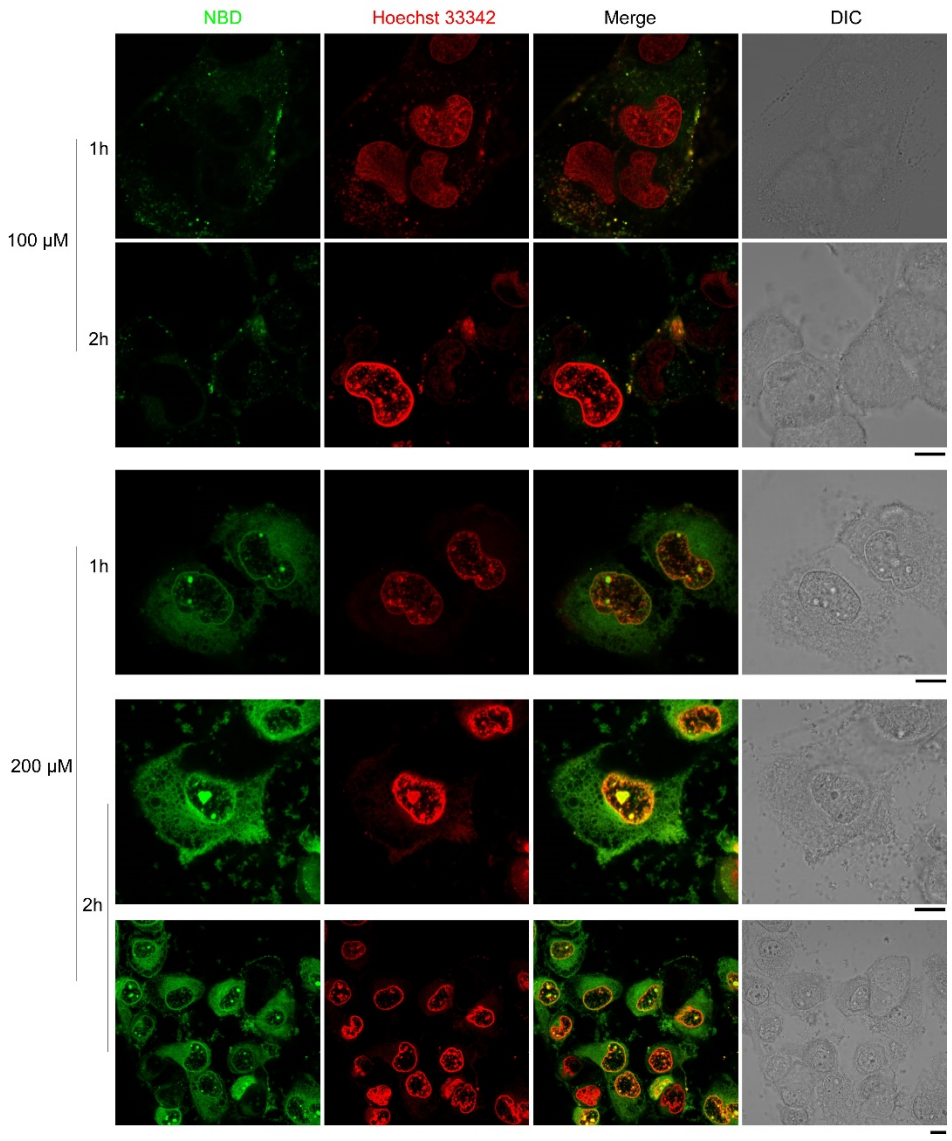


Figure S1. CLSM images of T98G cell lines treated with **D-1** (100 or 200 μM) for 1 and 2 h. Scale bar is 10 μm. For better comparing, we changed the color of the Hoechst 33342 to red.

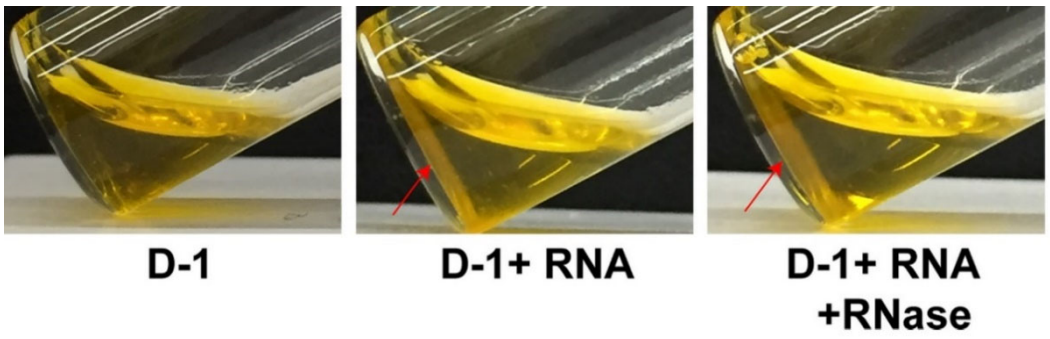


Figure S2. Optical image of 0.2 wt% of **D-1** without or with the treatment of RNA (extracted from T98G cells) and RNase. Arrows indicate precipitate.

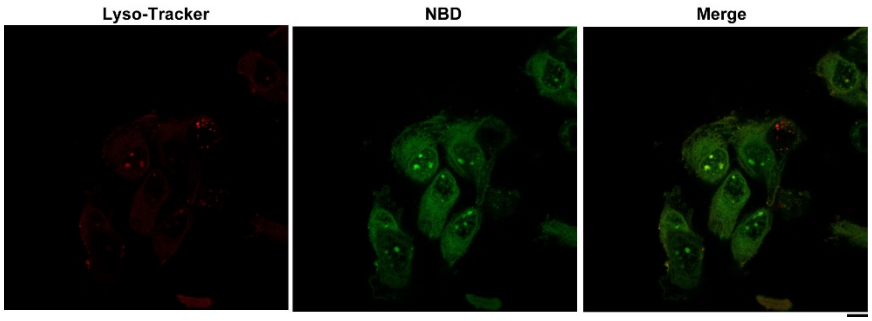


Figure S3. CLSM images of T98G cells treated with **D-1** (200 μM) and then stained with Lyso-Tracker. Scale bar is 10 μm.

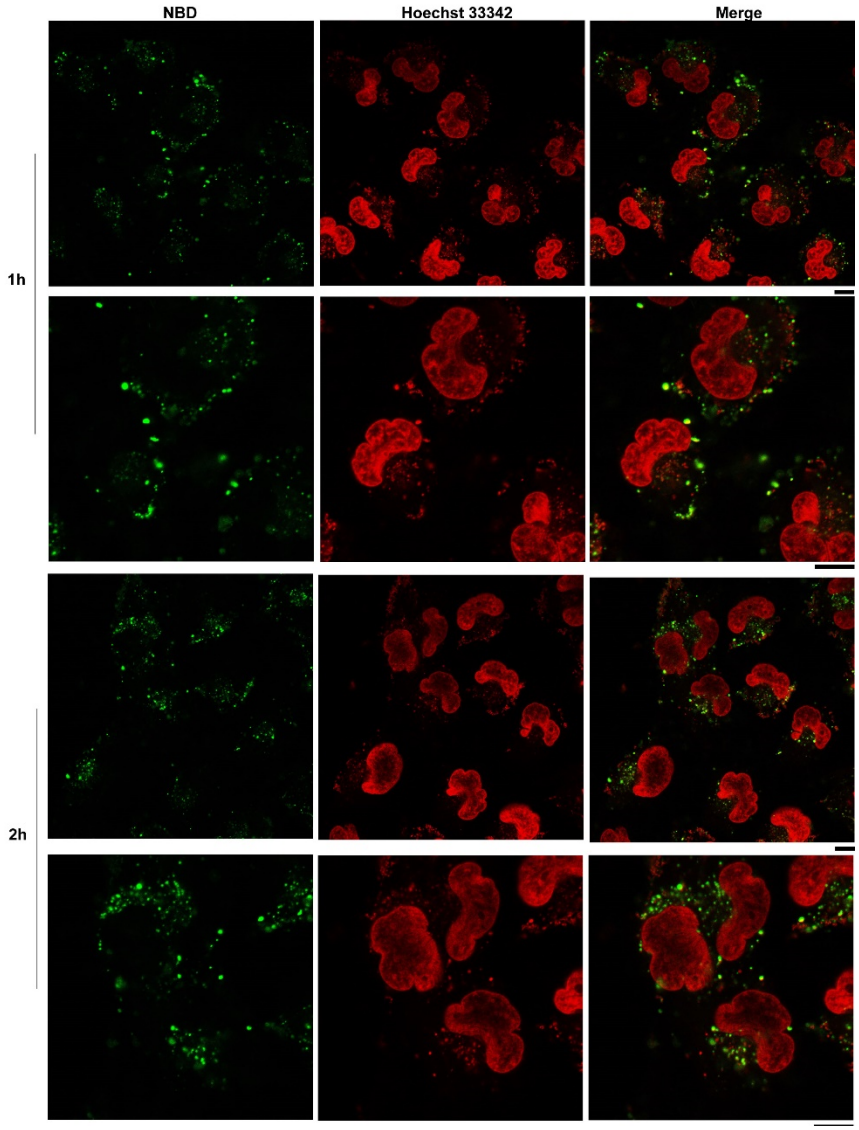


Figure S4. CLSM images of T98G cells treated with **L-1** (200 μM) for 1 h and 2h. Scale bar is 10 μm. For better comparing, we changed the color of the Hoechst 33342 to red.

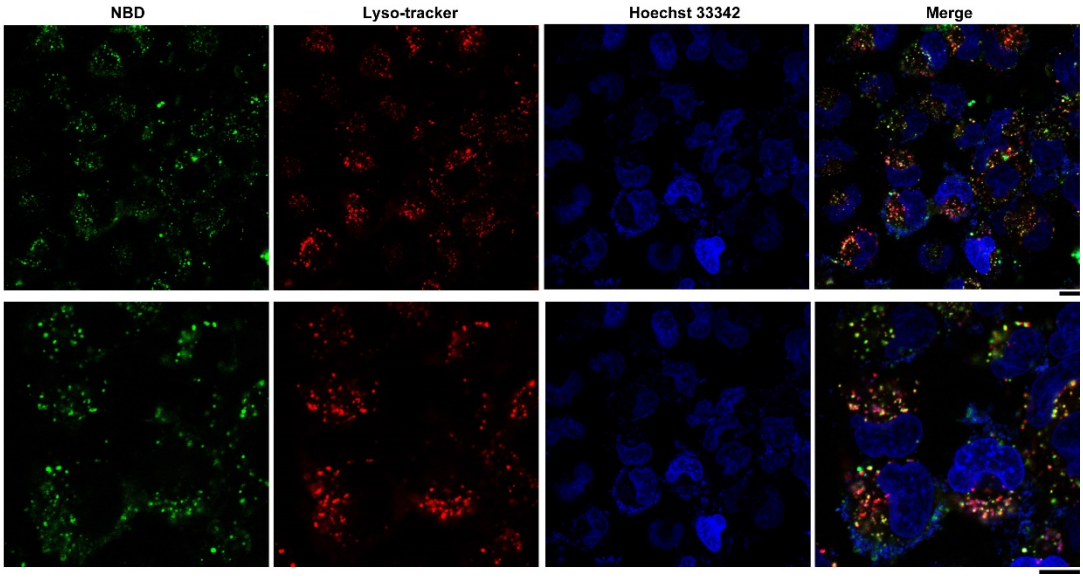


Figure S5. CLSM images of T98G cells treated with **L-1** (200 μM) for 2 h and then stained with Lyso-Tracker. Scale bar is 10 μm.

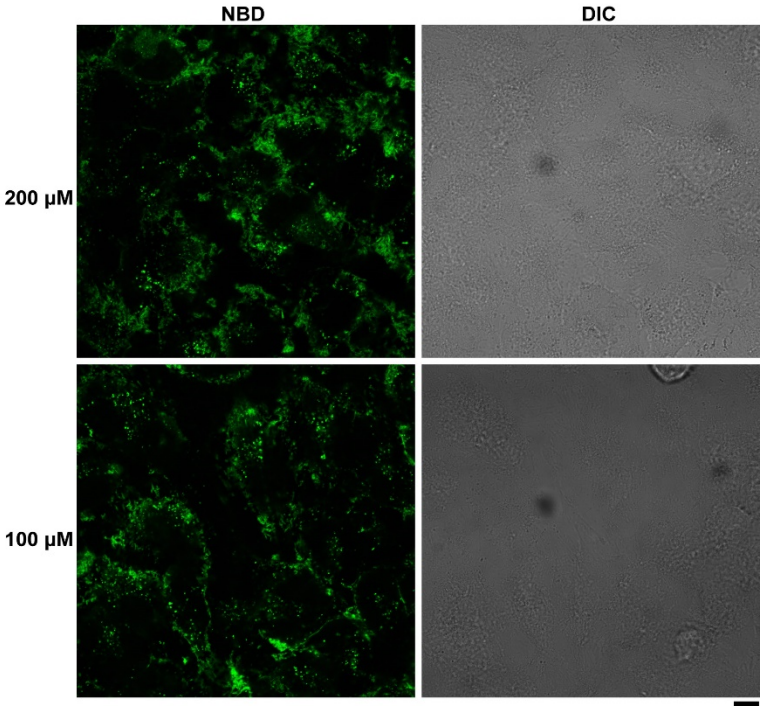


Figure S6. CLSM images of T98G cell lines treated with **NBD-2** (200 or 100 μM) for 2 h. Scale bar is 10 μm.

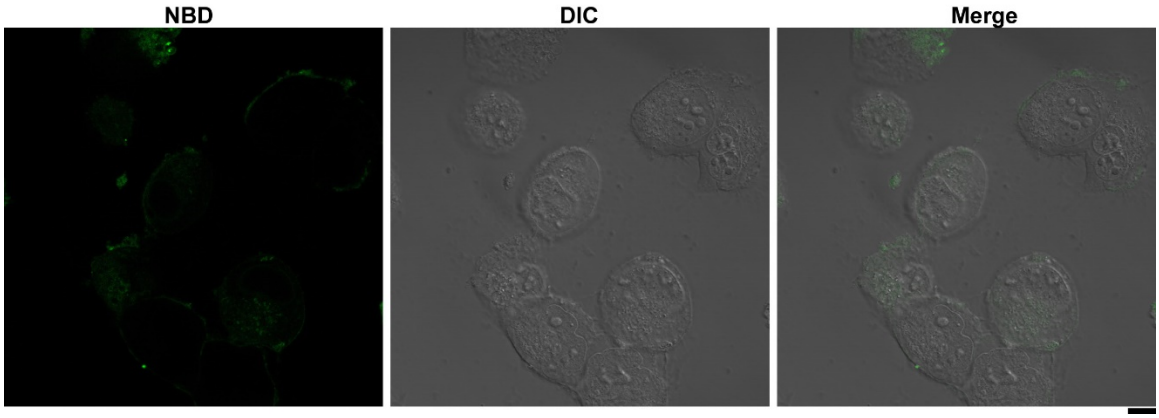


Figure S7. CLSM images of T98G cells treated with **D-1** (200 μM) for 1 h at 4 °C. Scale bar is 10 μm.

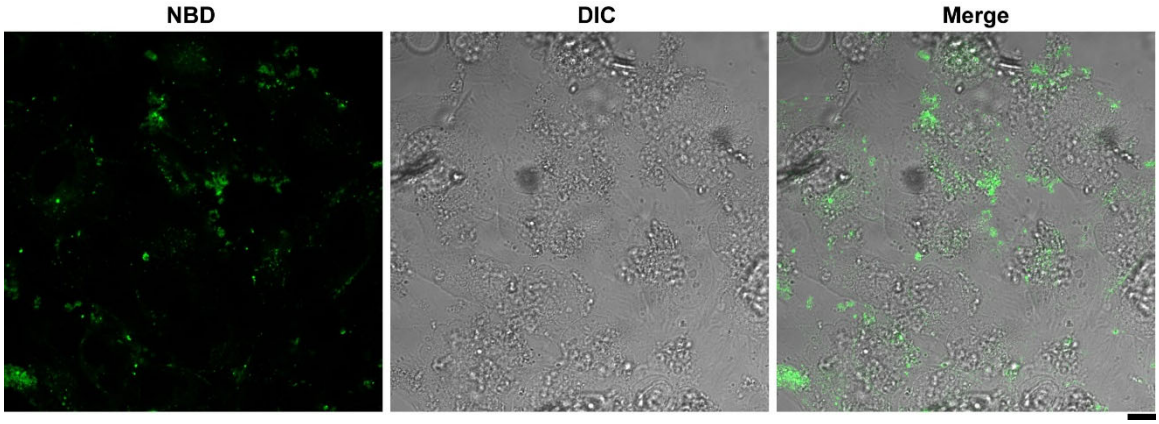


Figure S8. CLSM images of T98G cells treated the mixture of NapFFDALDLTD (200 μM) with **D-1** (200 μM) for 2 h. Scale bar is 10 μm.

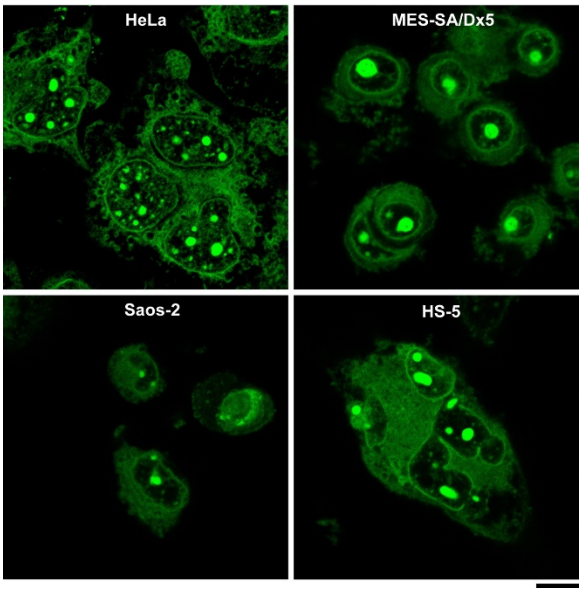


Figure S9. Fluorescent images of HeLa cells, MES-SA/Dx5 cells, Saos-2 cells and HS-5 cells treated with **D-1** (200 μM) for 2 h. Scale bar is 10 μm.

Figure S10. Fluorescent image of T98G cells treated with culture medium and analyzed by immunofluorescence of γH2AX. Scale bar is 20 μm.

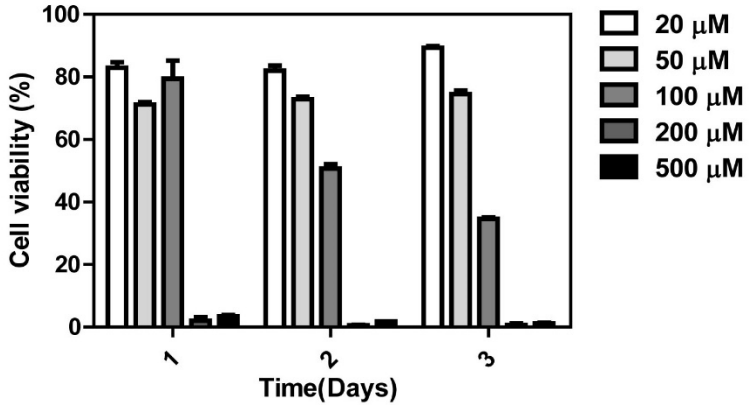


Figure S11. Cytotoxicity of **D-1** against T98G cell lines for 24 h, 48 h and 72 h.

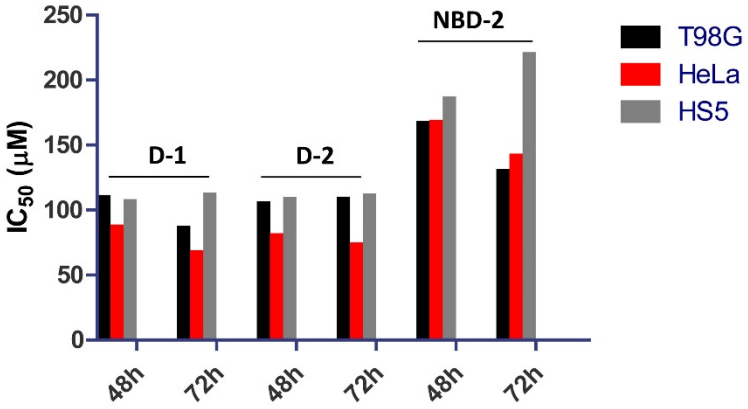


Figure S12. Summary of IC_{50} of **D-1**, **D-2** or **NBD-2** against T98G, HeLa and HS-5 cell lines for 48 h and 72 h.

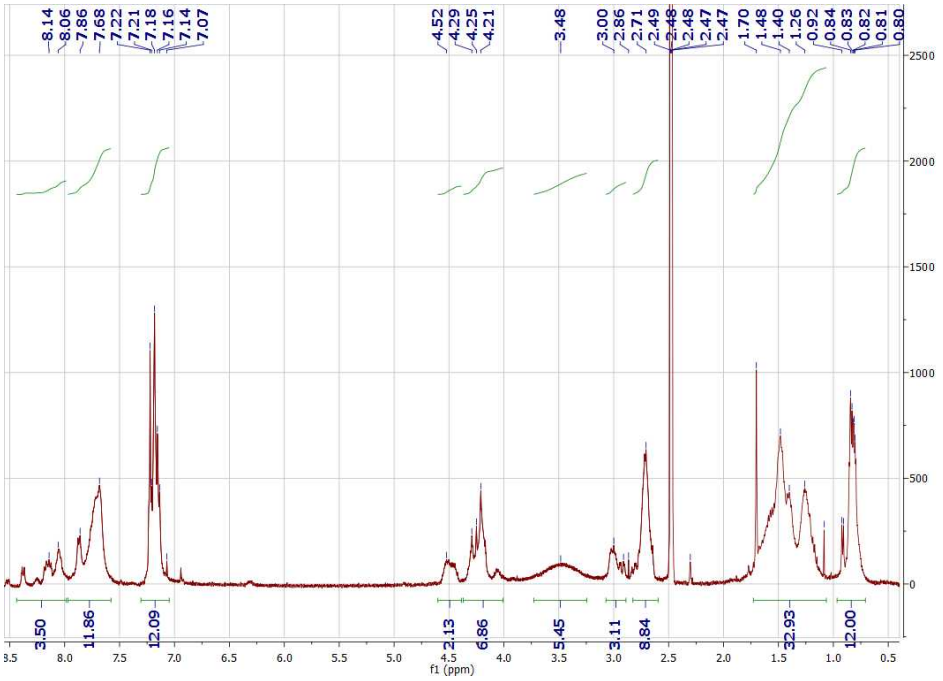


Figure S13. ^{1}H NMR of **D-1** in DMSO-*d6*

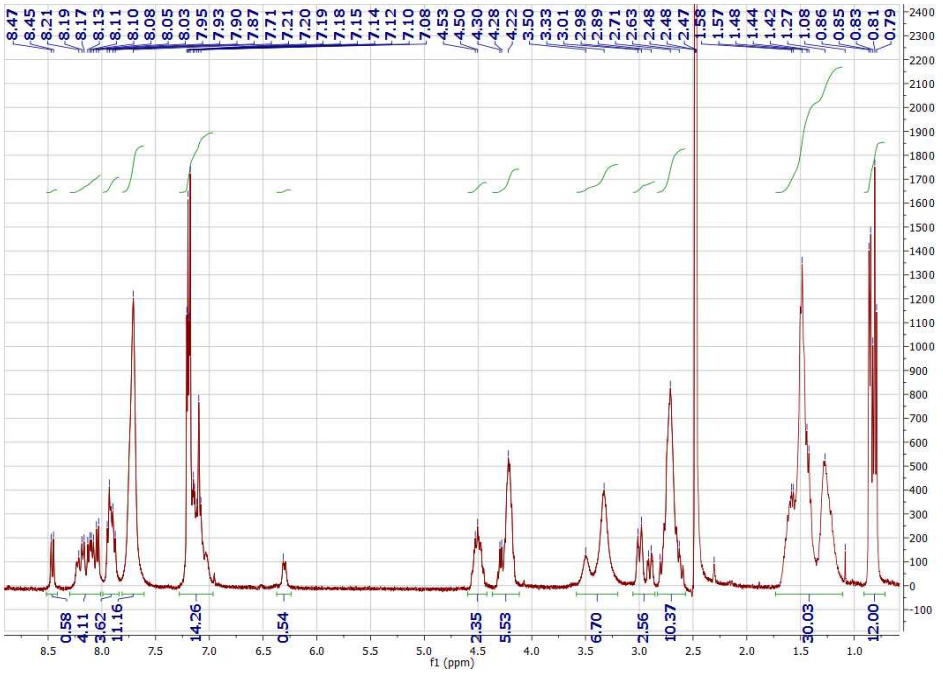


Figure S14. ^{1}H NMR of **NBD-2** in DMSO-*d6*

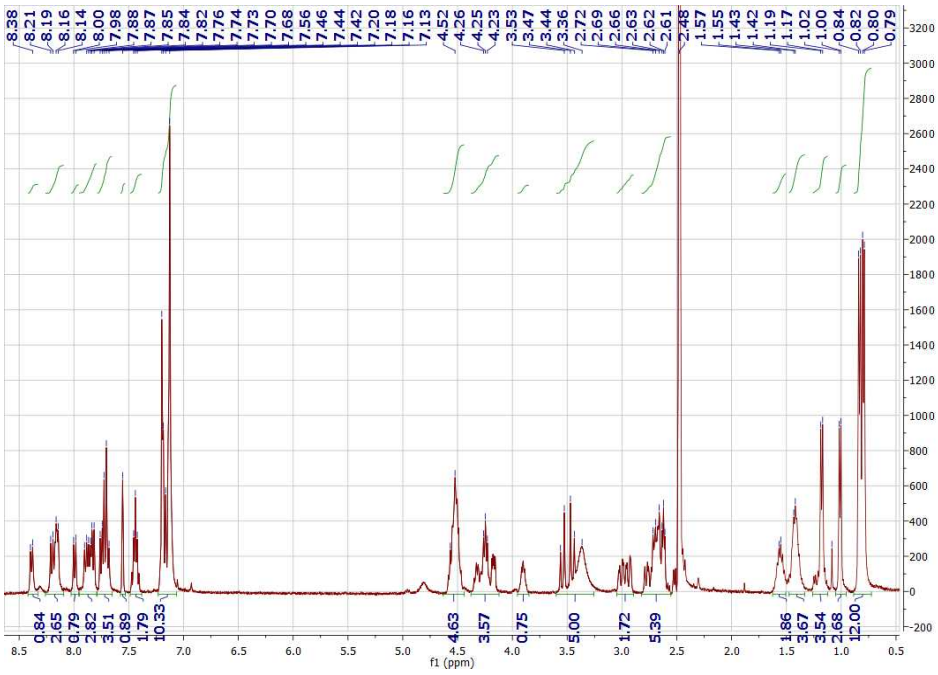


Figure S15. ^{1}H NMR of NapFFDALDLTD in DMSO-*d6*.

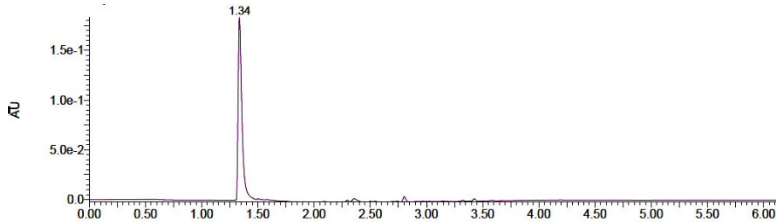


Figure S16. HPLC spectra of **D-1**.

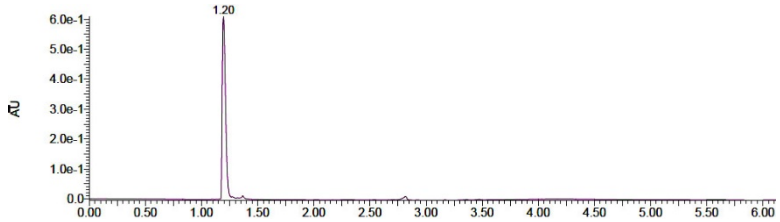


Figure S17. HPLC spectra of **D-2**

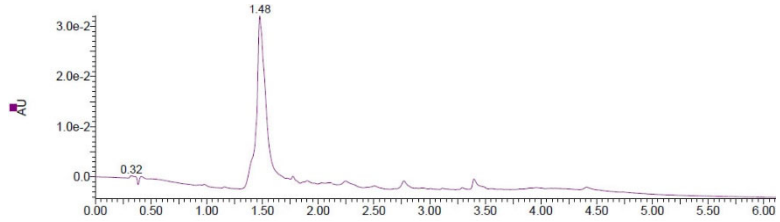


Figure S18. HPLC spectra of **L-1**

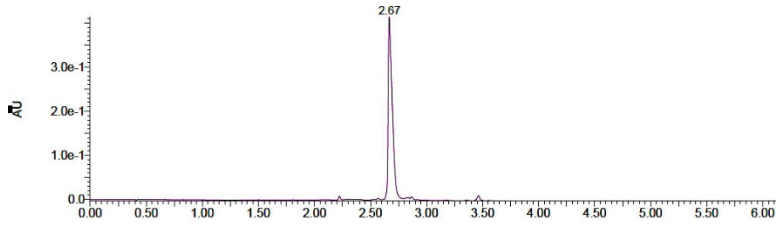


Figure S19. HPLC spectra of **NBD-2**

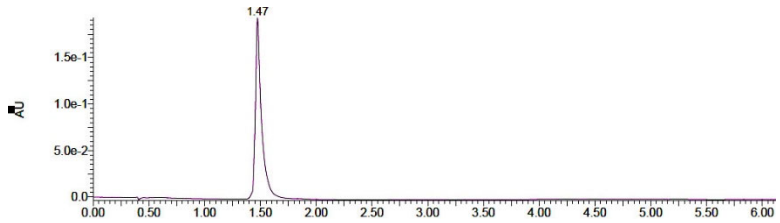


Figure S20. HPLC spectra of **NapFFDALDLTD**

S12. Supplemental tables

Table S1. Eluting gradient for HPLC

Time (minute)	Flow (ml/min.)	H_2O %	Acetonitrile %
0	10	90	10
15	10	50	50
16	10	0	100
18	10	0	100
20	10	90	10
23	10	90	10

Table S2. Summary of MS (ESI) results of compounds.

Name	Calc.	Obsvd. $(M-H)^-$
D-1	1654.90	1653.63
D-2	1530.87	1529.95
L-1	1654.90	1652.52
NBD-2	1431.80	1430.97
NapFFDALDLTD	1223.54	1221.93