Supplementary Information for

Enzyme Responsive Rigid-Rod Aromatics Target "Undruggable" Phosphatases to Kill Cancer Cells in Mimetic Bone Microenvironment

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Experiment materials and instruments

Materials

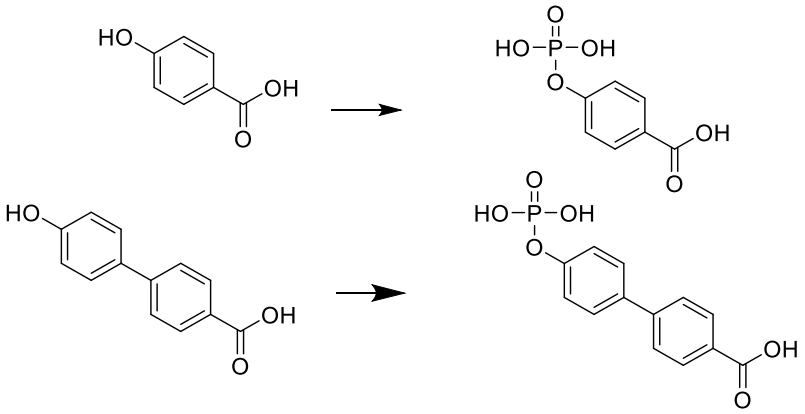
2-Cl-trityl chloride resin (1.0-1.2 mmol/g) and HBTU were obtained from GL Biochem (Shanghai, China). 4'-hydroxy-[1,1'-biphenyl]-4-carboxylic acid was obtained from 1PlusChem. Benzene and phosphorus pentachloride was purchased from Sigma-Aldrich. 4-Chloro-7-nitrobenzofurazan was purchased from Alfa Aesar. Other chemical reagents and solvents were obtained from Fisher Scientific. Alkaline phosphatase was purchased from Biomatik (Cat. No. A1130, alkaline phosphatase [ALP], >1300U/mg, in 50% glycerol.). Minimum Essential Media (MEM), Dulbecco's Modified Eagle Medium (DMEM), McCoy's 5A Medium, and RPMI-1640 Medium were purchased from ATCC. Fetal bovine serum (FBS) and Penicillin-Streptomycin from Gibco by Life Technologies. All chemical reagents and solvents were used as received from commercial sources without further purification.

Instruments

All precursors and compounds were purified by a reverse phase HPLC (Agilent 1100 Series) equipped with an XTerra C18 RP column, and HPLC grade acetonitrile (0.1% TFA) and HPLC grade water (0.1% TFA) were used as the eluents. The LC-MS spectra were obtained with a Waters Acquity Ultra Performance LC with Waters MICROMASS detector. TEM images were taken on a Morgagni 268 transmission electron microscope. The absorbance of each well at 595 nm was measured by a DTX880 Multimode Detector. The confocal images are obtained by using Zeiss LSM 880 confocal microscopy at the lens of 63× with oil.

Chemical synthesis

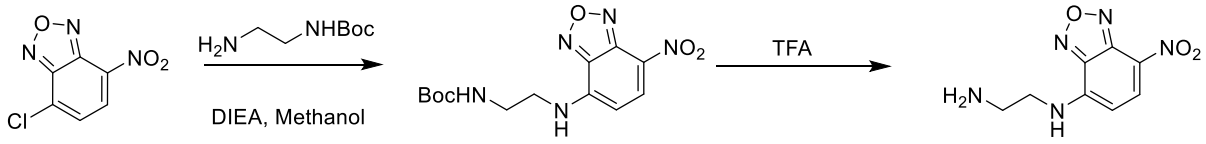
Synthesis of phosphobiphenyl carboxylic acid



Scheme S1. The synthesis of phophophenyl and phosphobiphenyl group.

4-hydroxybenzoic acid (1 eq.) or 4'-hydroxy-[1,1'-biphenyl]-4-carboxylic acid (1 eq.) and PCl_5 (1 eq.) were stirred at room temperature for 45 min. Subsequently, the reaction was brought to completion by sonication at 60 ℃ for 90 min. The ice-cooled reaction mixture was dissolved in 10 mL of acetone and 10 mL of benzene, and 1.4 mL (3 eq.) of distilled water was added dropwise. After stirring at 0 ℃ for 30 min, 20 mL benzene was added. The reaction mixture was stirred at room temperature for 12 h. The precipitate was filtered off, washed with 20 mL benzene, and dried in high vacuum.

Synthesis of NBD-ethylenediamine

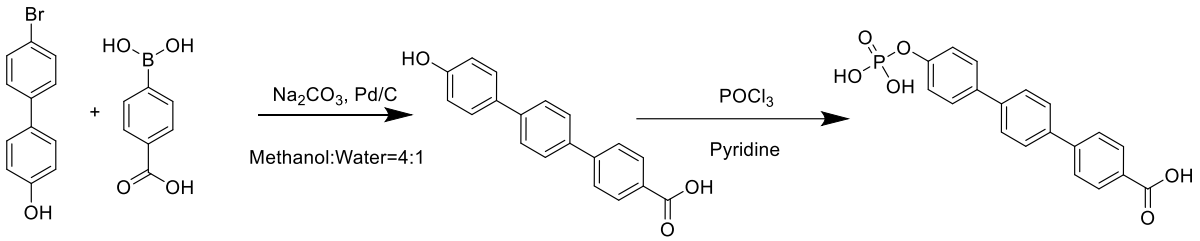


Scheme S2. Synthetic procedure of NBD-ethylenediamine.

NBD-Cl (1 eq.) was dissolved in methanol, and then N-Boc-1,2- diaminoethane (1.2 eq.) was added. DIEA was added dropwise to adjust pH to around 8. After overnight experiment, the organic phase was air-dried, and the obtained crude was purified with

silica gel column chromatography.

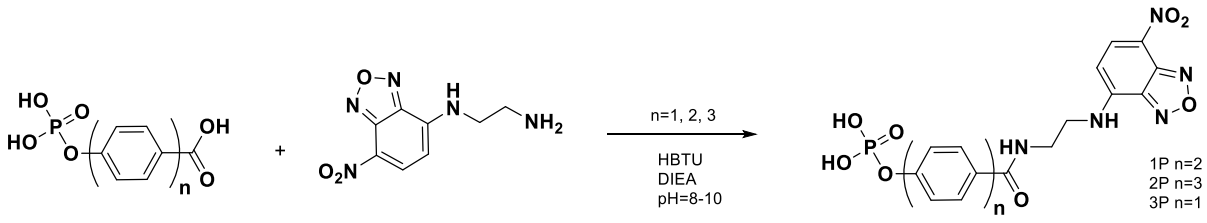
Synthesis of phosphoterphenyl carboxylic acid



Scheme S3. Synthetic procedure of phosphoterphenyl carboxylic acid.

4'-bromo-[1,1'-biphenyl]-4-ol (1 eq), 4-boronobenzoic acid (1.2 eq), Na_2CO_3 (3 eq.), solvent (Methanol:water=4:1), and Pd/C catalyst (0.1 mol%) were added to a round bottom flask equipped with a magnetic stirrer and reflux condenser. The mixture was heated in an oil bath set up at 85℃ for 24 h. Cool the mixture to room temperature. Then the mixture was filtered to remove Pd/C catalyst. In this step, methanol and water were used to dissolve as much as possible obtained compound into the filtered solution. Thereafter, the organic solvent was evaporated under reduced pressure and 80 mL of water was added. The mixture was acidified with 35% HCl and suspended well under stirring. The suspension was filtered and washed with water. The obtained solid was dried.

Synthesis of 1P, 2P, and 3P



Scheme S3. Synthetic procedure of phosphoterphenyl carboxylic acid.

Briefly, 0.1 mmol of phosphophenyl (or phosphobiphenyl, or phosphoterphenyl) group was dissolved in 2 mL dry DMF and 0.12 mmol of HBTU was added directly into the solution. After stirring the mixture for 30 minutes, 0.12 mmol of NBD-ethylenediamine was added into the mixture and DIEA was added dropwise to adjust pH around 8, then keep stirring overnight. After that, the solvent is airdried and the remained oily product was dissolved in methanol and purified by RP-HPLC to obtain the corresponding titled compounds.

TEM sample preparation

a. Place sample solution on the grid (5 μL, sufficient to cover the grid surface).

b. Rinsing: ~10 sec later, place a large drop of the ddH2O on parafilm and let the grid touch the water drop, with the sample-loaded surface facing the parafilm. Tilt the grid and gently absorb water from the edge of the grid using a filter paper sliver. (3 times)

c. Staining (immediately after rinsing): place a large drop of the UA (uranyl acetate) stain solution on parafilm and let the grid touch the stain solution drop, with the sample-loaded surface facing the parafilm. Tilt the grid and gently absorb the stain solution from the edge of the grid using a filter paper sliver.

d. Allow the grid to dry in air and examine the grid as soon as possible.

Cell culture

Six cell lines were used, including Saos2, SJSA1, VCaP, PC3, HepG2 and PNT1A cells. Saos2 and SJSA1 cells are osteosarcoma cells, which were used to mimic osteoblast cells in the tumor microenvironment. VCaP and PC3 cell are both metastatic castration-resistant prostate cancer cells. HepG2 cell is a liver cell line, which was used here to illustrate the liver toxicity. PNT1A cell is a normal prostate cell line, which act as a control for the

prostatic cancer cell lines.

Saos2, SJSA1 and HepG2 cell lines were purchased from American Type Culture Collection (ATCC, USA). VCaP, PC3 and PNT1A cell lines were donated by Dr. JT Hsieh. Saos2 cells were cultured in McCoy's 5A Medium (Gibco, Life Technologies) supplemented with 15% (v/v) fetal bovine serum (FBS) (Gibco, Life Technologies), 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, Life Technologies); SJSA1 and PC3 cell were culture in RPMI1640 (ATCC, USA) Medium supplemented with 10% (v/v) FBS, 100 U/mL penicillin and 100 μg/mL streptomycin; HepG2 cells were cultured in Minimal Essential Medium (MEM) (Gibco, Life Technologies) supplemented with 10% (v/v) FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. VCaP cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. All the cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

MTT assay

Cells were seeded in 96-well plates at 1×10^4 cells/well for 24 hours to allow attachment. After removing the culture medium, fresh culture medium containing different concentration of the precursors were added. After 24/48/72 hours, 10 μL MTT (ACROS Organics) solution (5 mg/mL) was added to each well to incubate at 37 °C for 4 h. 100 μL of SDS-HCl solution was then 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. The MTT assay was performed in triplet (n = 3) and the average value of the three measurements was taken.

For cell death rescue experiment, after cell attachment, we pretreated the cells with ALPL

inhibitors or other cell death inhibitors for 30 min, and then cocultured the cell with the mixture of **1P/2P** with different inhibitors. After 24 hours, same procedures were carried out to get the cell viability percentage relative to untreated cells.

Confocal Laser Scanning microscopy (CLSM)

Confocal dish (35 mm dish with 20 mm bottom well, # 1.5 glass) was used for preparing CLSM samples. All cells were directly seeded on the confocal dish without additional coating.

CLSM imaging of intracellular distribution of 1P and 2P

The cells of interest were seeded on a confocal dish at 1.5×10^5 cells/dish for 24 h. Following the removal of culture medium, fresh culture medium containing 50 μM of 1P or 20 μM of 2P were added. After 4 h of incubation, cells were washed with live cell imaging solution (Life Technologies A14291DJ) for 3 times and stained with 1x ER-Tracker Red, Lysotracker deep red and Mito tracker deep red (Invitrogen) in live cell imaging solution. Then, the samples were brought to the ZEISS LSM 880 confocal laser scanning microscope. The fluorescence images with both channels (488 nm and 561 nm) of the sample stained with ER and Lysosome trackers and the fluorescence images with both channels (488 nm and 633 nm) were taken and saved for further analysis.

CLSM imaging of instant entering of 1P and 2P into single cell type

The cells of interest were seeded on a confocal dish at 1.5×10^5 cells/dish for 24 h. The

samples were washed with 2 mL of cell imaging buffer for 3 times and then the nuclei of cells were stained with Hoechst 33342 for 10 min. After 10 min, the samples were washed with 2 mL of cell imaging buffer for another 3 times to fully remove the extra Hoechst 33342 and the samples were brought to the ZEISS LSM 880 confocal laser scanning microscope. The position of cells and focus plane of the laser beam were determined by the fluorescence of the stained nuclei using laser with the wavelength of 405 nm. After that, the cell imaging buffer in the confocal dish was removed and the compound of interest with certain concentration prepared in the cell imaging buffer was added to the confocal dish. Meanwhile, the CLSM images with both channels (405 nm and 488 nm) of the sample were taken, and the time interval of the time series mode was set to be 1 min. After 30 cycles of imaging, the fluorescence images of both channels were saved for further analysis.

CLSM imaging of instant entering of 1P and 2P into cocultured cells

There is only difference of cell seeding between single cell and cocultured cells. The two types of cells of interest were seeded on a confocal dish at 1.5×10^5 cells/dish with 1 to 1 ratio for 24 h. Then same procedures were followed to get the fluorescence images of both channels and do the further analysis.

Cryo-EM of Compound Nanotubes

Cryo-EM of compound nanotubes

Two samples of compound nanofibers were analyzed using cryo-EM: pBP-NBD (**1P**) at pH 5.6 and BP-NBD (**1**) at pH 7.4. The compound sample (2.5-3 μl) was applied to glow-discharged lacey carbon grids and vitrified using a Leica plunge freezer. Grids were

imaged on a Titan Krios (300 keV, Thermo Fisher) with a K3 camera (Gatan). Micrographs (13,122 for pBP-NBD; 5,609 for BP-NBD) were collected under electron counting mode at 1.08 Å per pixel, using a defocus range of 1–2 μm with ~50 electrons/Å^2 distributed into 40 fractions. Motion correction and CTF estimation were done in cryoSPARC[1-3]. Particles were auto-picked by "Filament Tracer" with a shift of 23 pixels. Next, junk particles and particles in low-resolution averages were removed by multiple rounds of reference-free 2D classifications. A reasonable amount of particles (0.9 million for pBP-NBD; 1.5 million for BP-NBD) having clear 2D average patterns were left for subsequent 3D reconstruction. All possible helical symmetries were calculated from an averaged power spectrum of the raw particles and then were tested by trial and error in cryoSPARC until recognized compound features were observed[4-5]. The final volumes were then sharpened with a negative B-factor automatically estimated in cryoSPARC local filtering, and the statistics are listed in Table S1.

Model building of filament

According to map:map FSC, 3D reconstructions of pBP-NBD and BP-NBD reach 2.4 Å and 2.2 Å resolution, respectively. However, since the nanofiber is made of a small chemical compound, the hand of the cryo-EM map cannot be determined directly. Therefore, we did model building for both hands of the map. First, a geometry restrains of the compound was generated using eLBOW[6]. Then, the model was manually adjusted in Coot [7] and real-space refined in PHENIX[8]. The filament model fits better in the right-handed 1-start map than the left-handed 1-start map, with a much better clash score after refinements (0.8 vs 25.0). Therefore, we suggested the map likely has a right-handed 1-start twist. The refinement statistics of both filaments are shown in Table S1.

Supplementary Figures

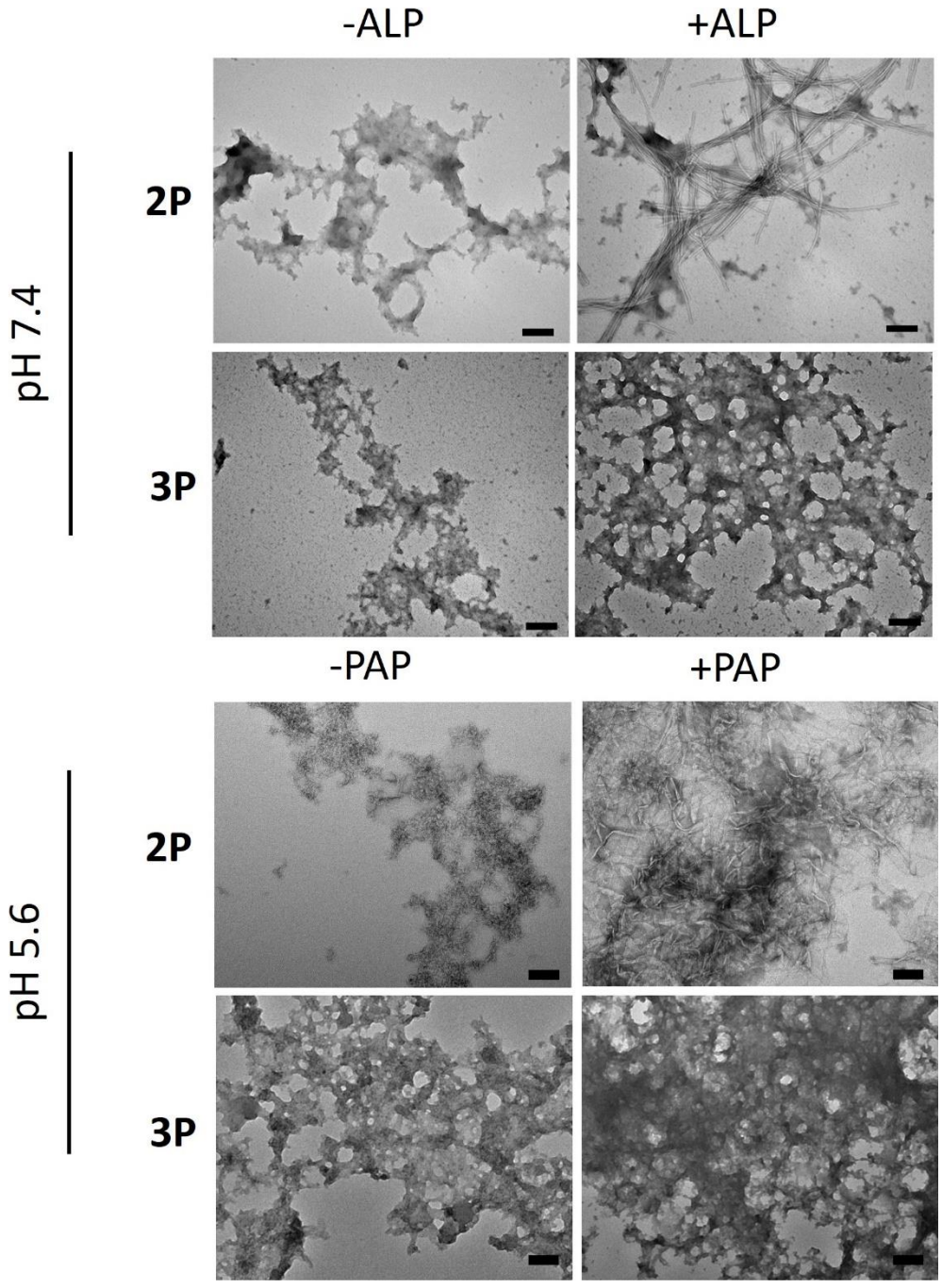


Figure S1. TEM images of **2P** and **3P** (500 μM, pH 7.4) before and after the treatment of ALP (0.1 U/mL) for 24 h and of **2P** (50 μM, pH 5.6) and **3P** (500 μM, pH 5.6) before and after the treatment of PAP (0.1 U/mL) for 24 h. Scale Bar=100 nm.

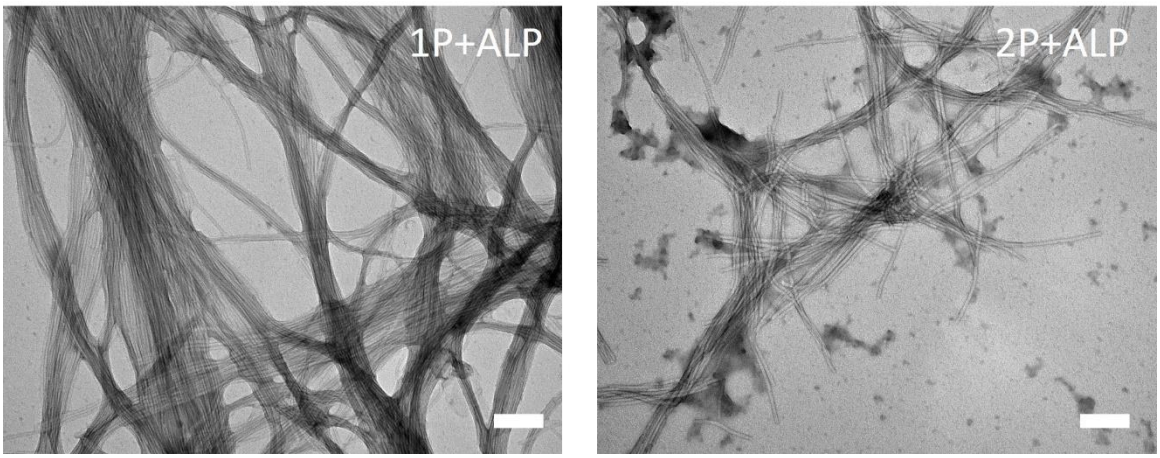


Figure S2. The TEM images of **1P** and **2P** (500 μM) after ALP treatment. [ALP] = 0.1 U/mL. Duration time: 24 h.

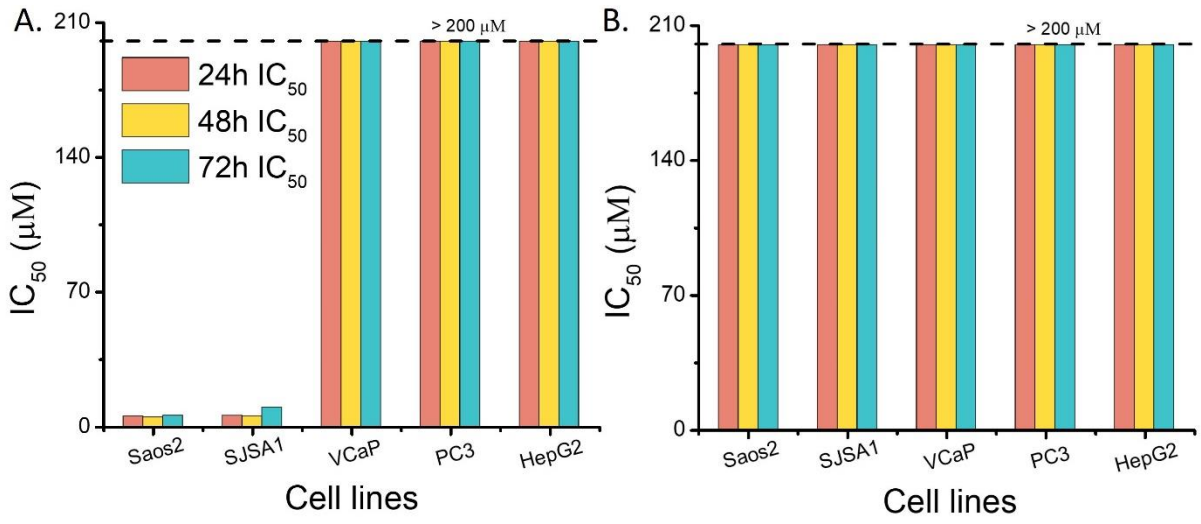


Figure S3. First day IC_{50} of (A) **2P** and (B) **3P** against different cell lines.

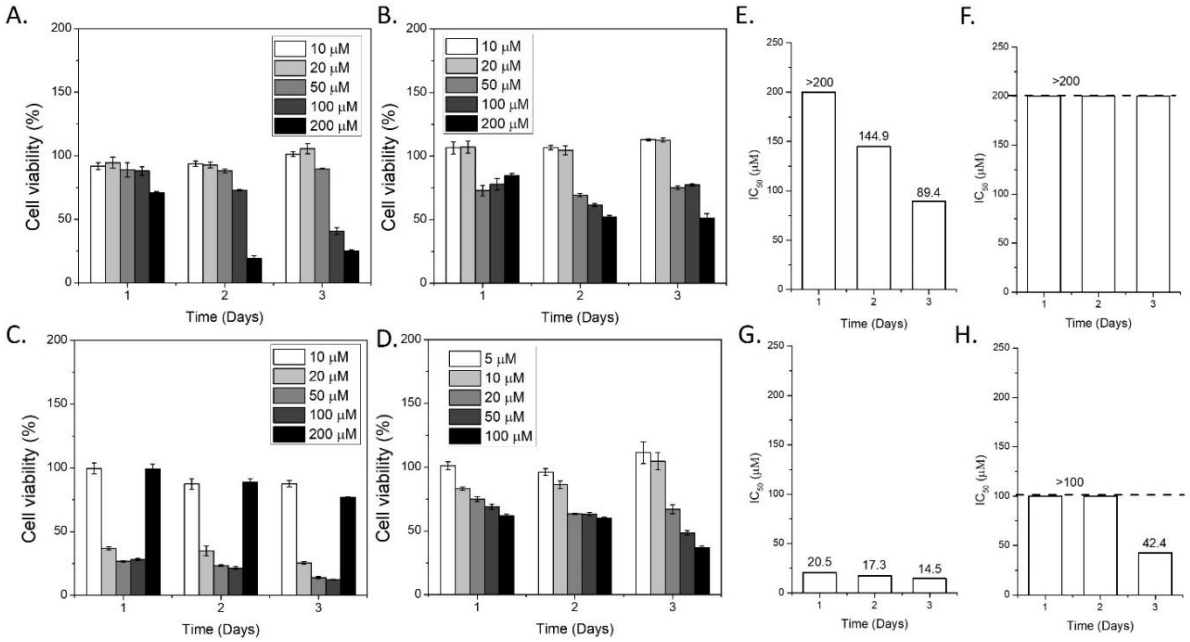


Figure S4. The cell viability of PNT1A treated with **1P** (A), **2P** (C), **1** (C) and **2** (D). The three days IC_{50} of **1P** (E), **2P** (F), **1** (G) and **2** (H) against PNT1A.

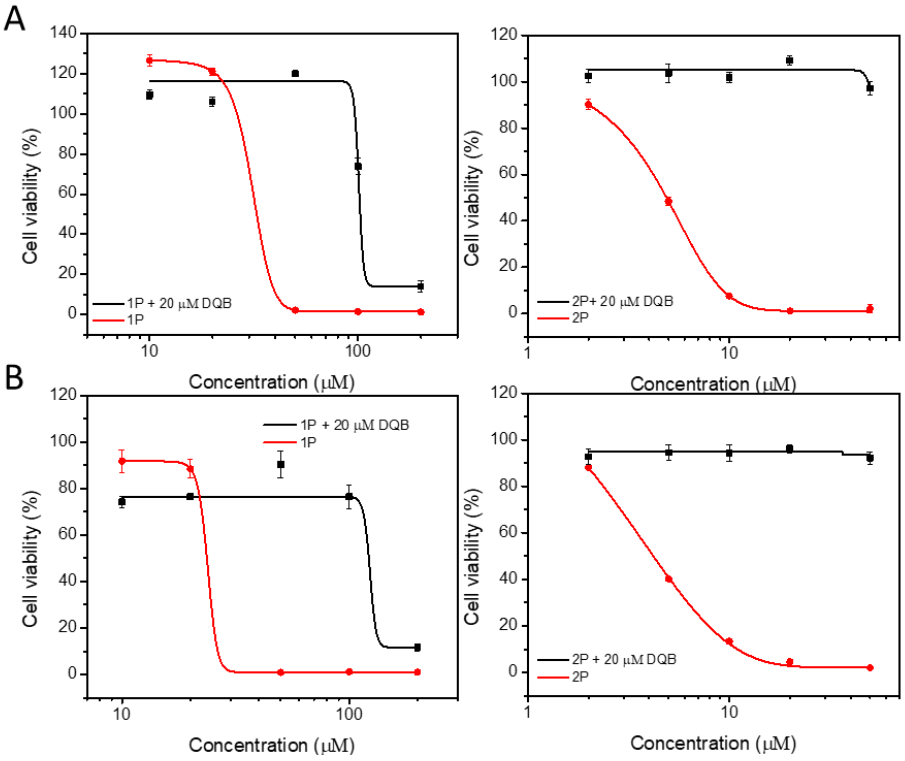


Figure S5. A. The cell viability of Saos-2 treated with **1P** or **2P** for 24 h, and the cell viability of Saos-2 pretreated with 20 μM DQB for 30 min and coculture with DQB and

1P or **2P** for 24 h. B. The cell viability of SJSA1 treated with **1P** or **2P** for 24 h, and the cell viability of SJSA1 pretreated with 20 μM DQB for 30 min and coculture with DQB and **1P** or **2P** for 24 h.

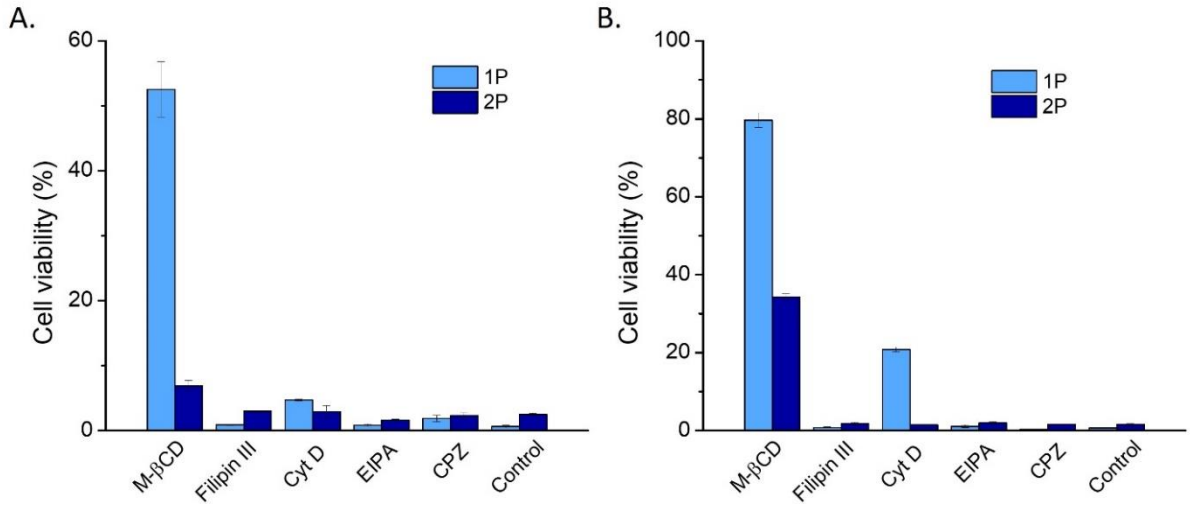


Figure S6. The cell viability of Saos2 (A) and SJSA1 (B) cocultured with different cellular uptake pathway inhibitors and **1P** or **2P** (50 μM). [M-β-CD] =1 mM, [Filipin III] =1 ug/mL, [CytD]=0.1 ug/mL, [EIPA]=0.2 μM, [CPZ]= 1 μM.

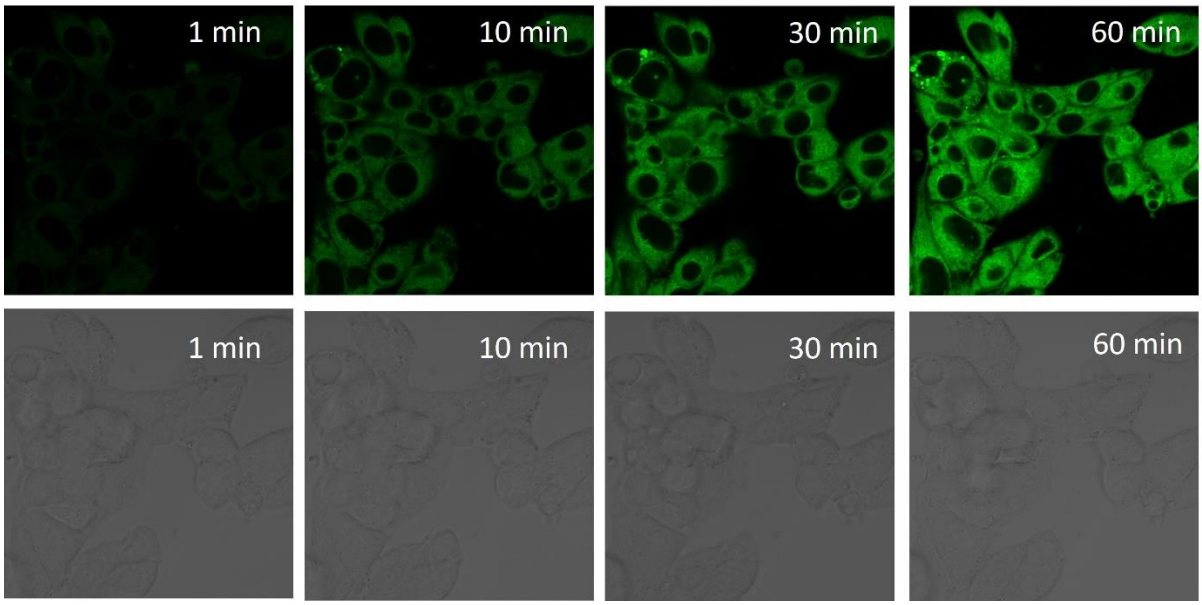


Figure S7. The cellular uptake of **1P** (100 μM) into HepG2 cells.

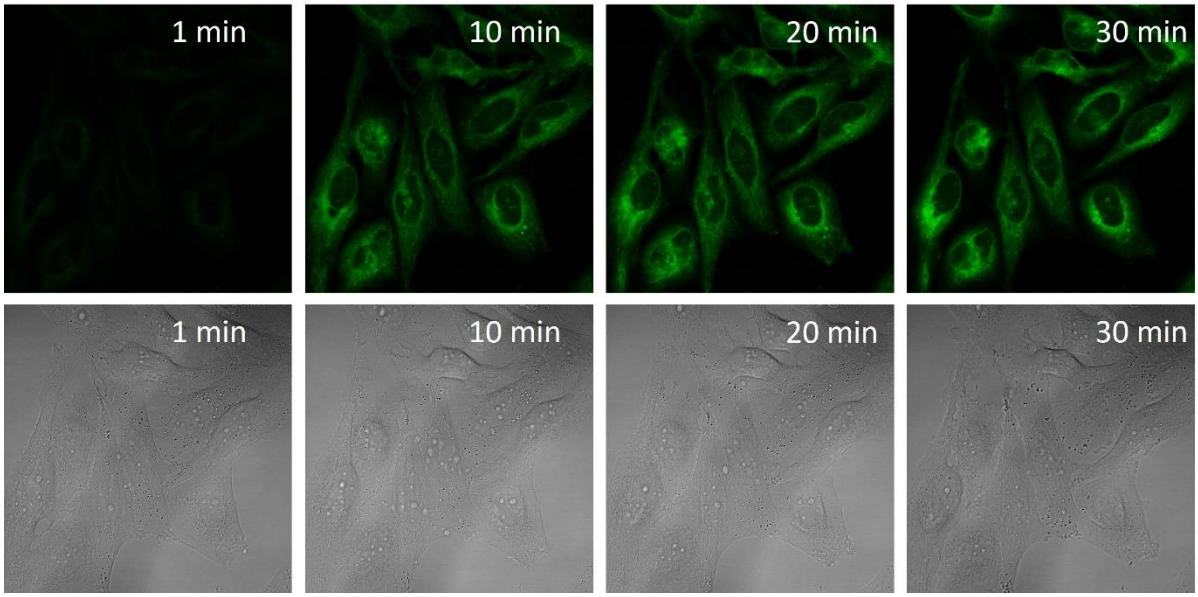


Figure S8. The cellular uptake of **3P** (100 μM) into Saos2 cells.

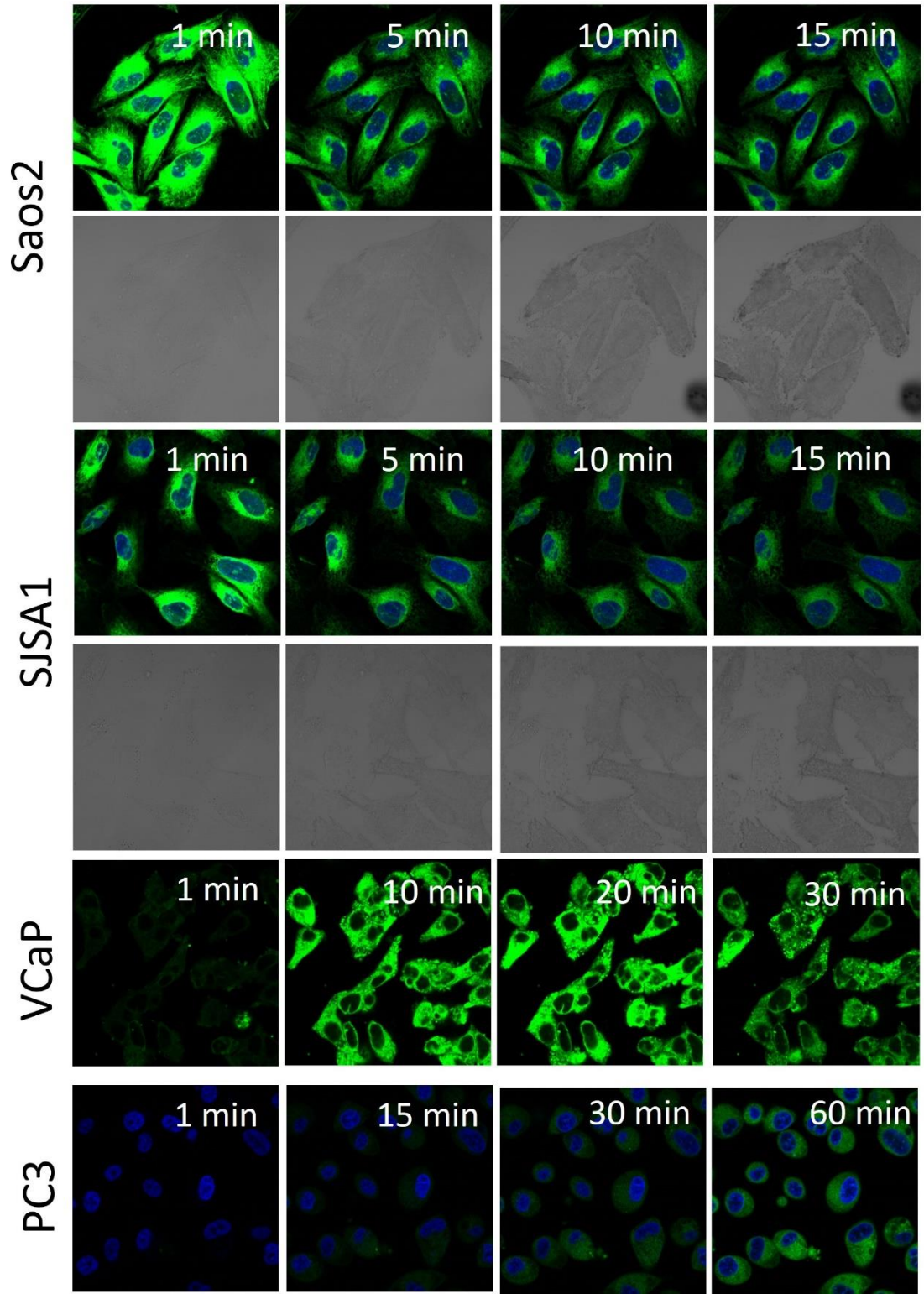


Figure S9. The instant entering of **2P** into different cell lines. [**2P**] =20 μM.

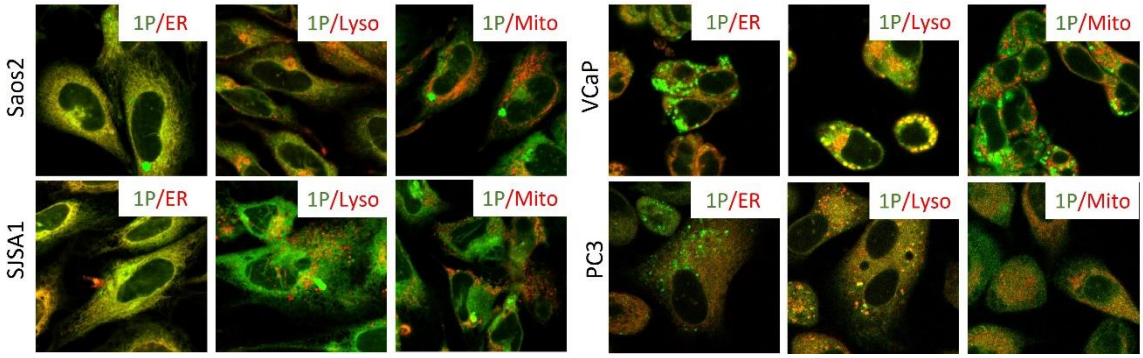


Figure S10. Zoomed in Intracellular distribution of **1P** (50 μM) in Saos2, SJSA1, VCaP and PC3 after 4 h incubation.

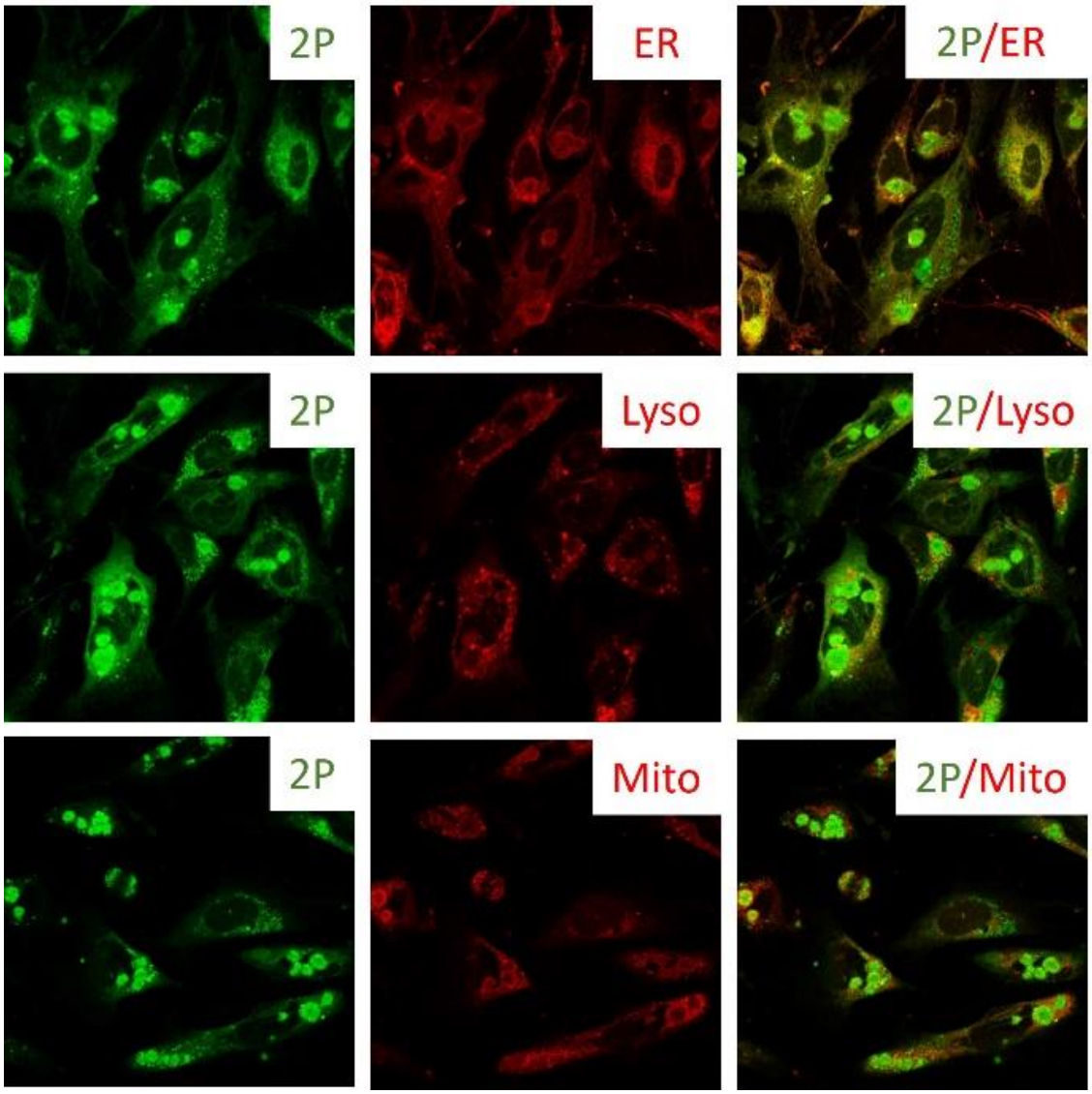


Figure S11. The intracellular distribution of **2P** in SJSA1 cells. [**2P**] =10 μM. Treatment time = 4h.

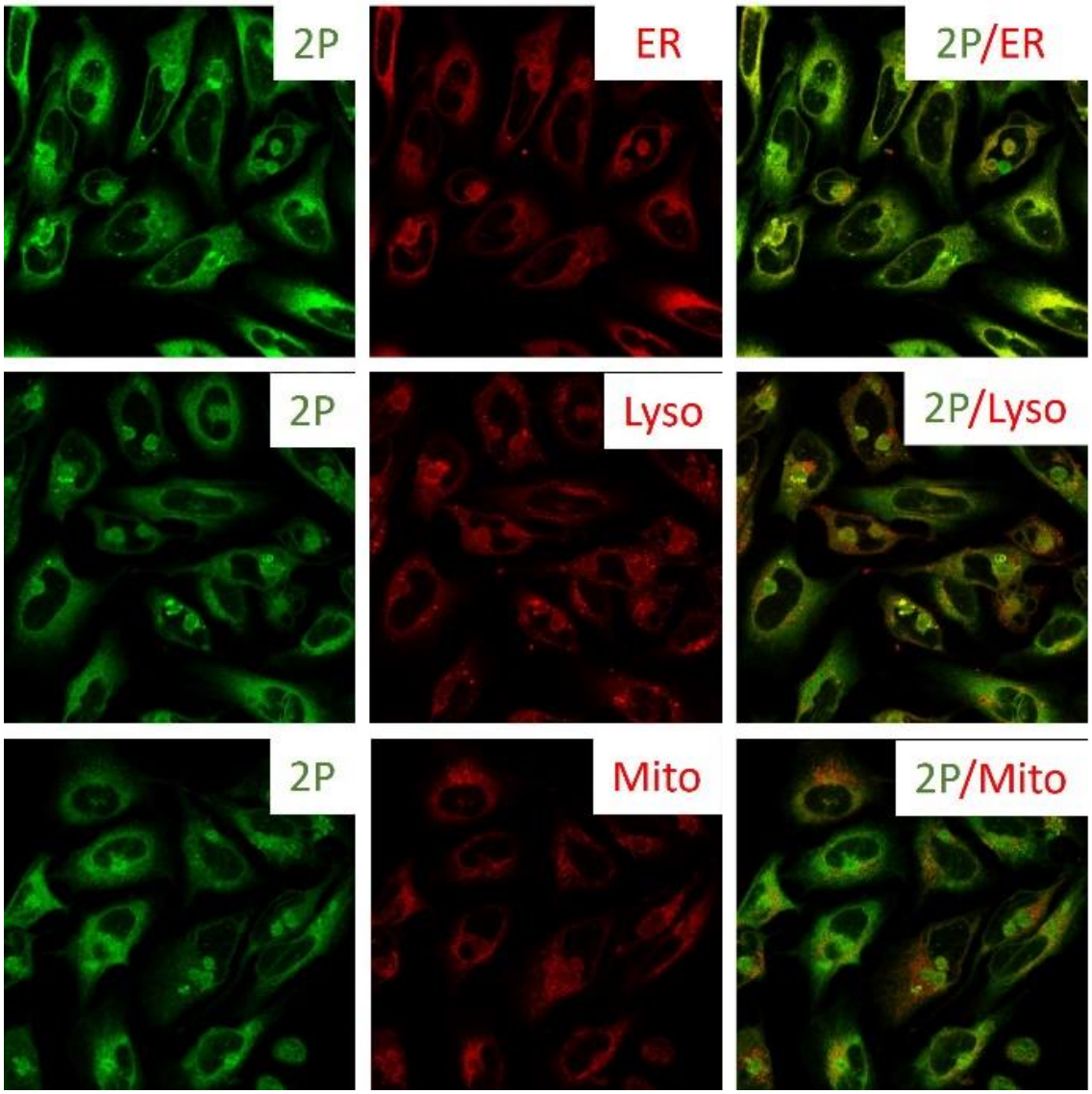


Figure S12. The intracellular distribution of **2P** in Saos2 cells. [**2P**] =10 μM. Treatment time = 4h.

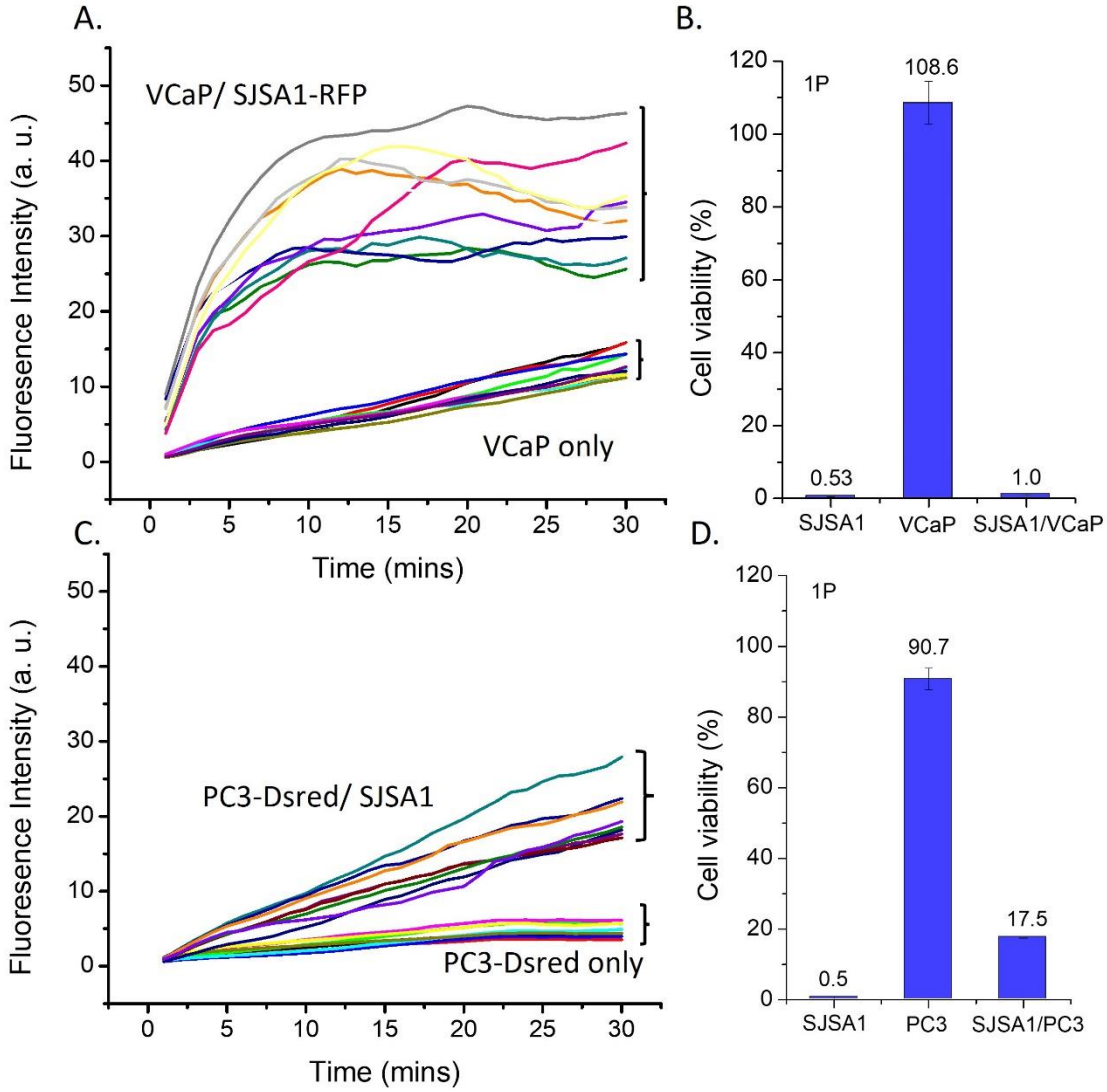


Figure S13. The mean fluorescence intensity of **1P** (50 μM) in the (A) VCaP from VCaP only culture and from the coculture of VCaP and SJSA1-RFP cells and (C) in PC3-DsRed cells from PC3-DsRed only culture and from the coculture of PC3-DsRed and SJSA1 cells. Viabilities of cells treated by **1P** (100 μM) in (B) SJSA1, VCaP, and the coculture of SJSA1 and VCaP and in (D) SJSA1, PC3, and the coculture of SJSA1 and PC3.

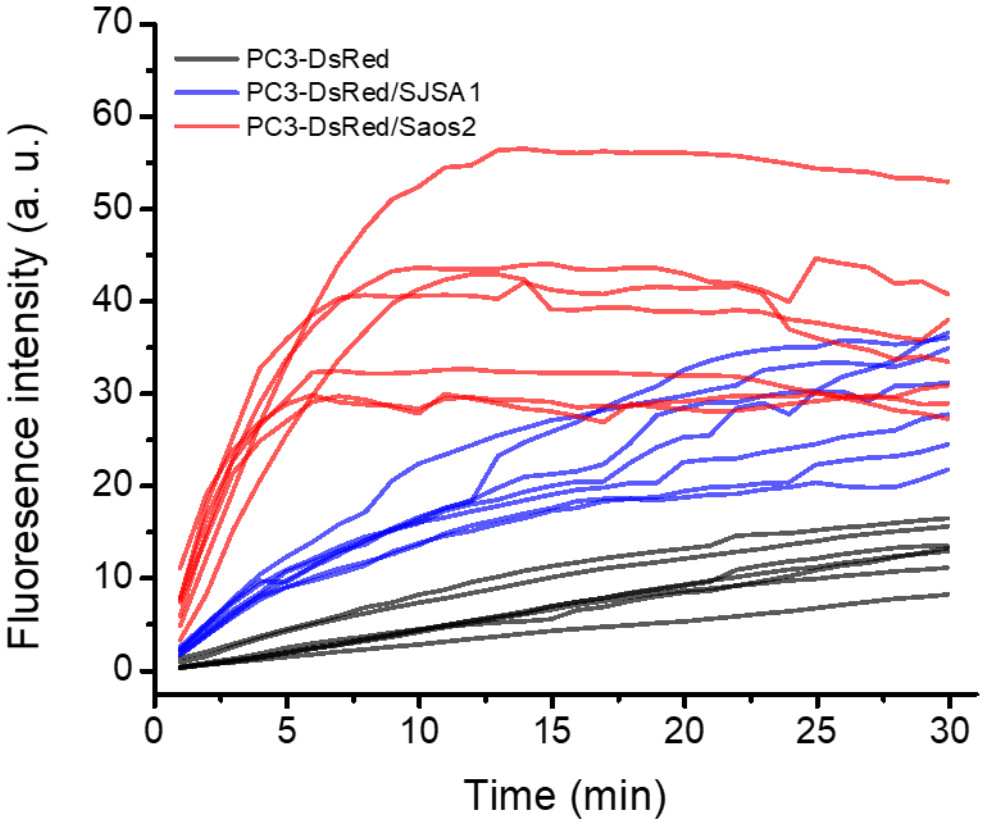


Figure S14. The mean fluorescence intensity of **1P** (50μM) in PC3-DsRed from PC3-DsRed only culture, from the coculture of PC3-DsRed and SJSA1 cells and from the coculture of PC3-DsRed and Saos2 cells.

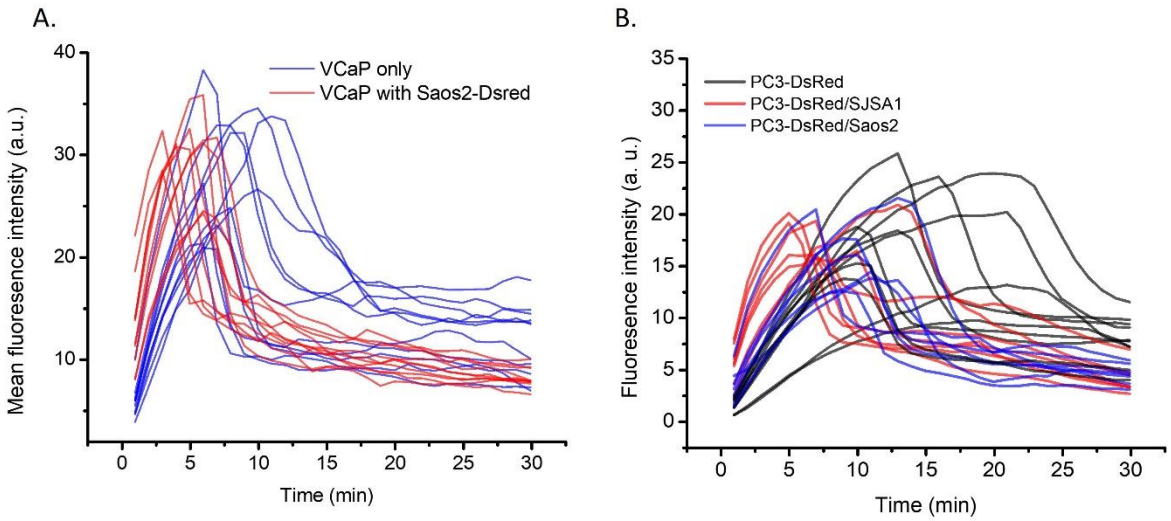


Figure S15. The mean fluorescence intensity of **2P** (20μM) in (A) VCaP from VCaP only culture, and from the coculture of VCaP and Saos2-DsRed cells and (B) in PC3-DsRed from PC3-DsRed only culture, from the coculture of PC3-DsRed and SJSA1 cells and from the coculture of PC3-DsRed and Saos2 cells.

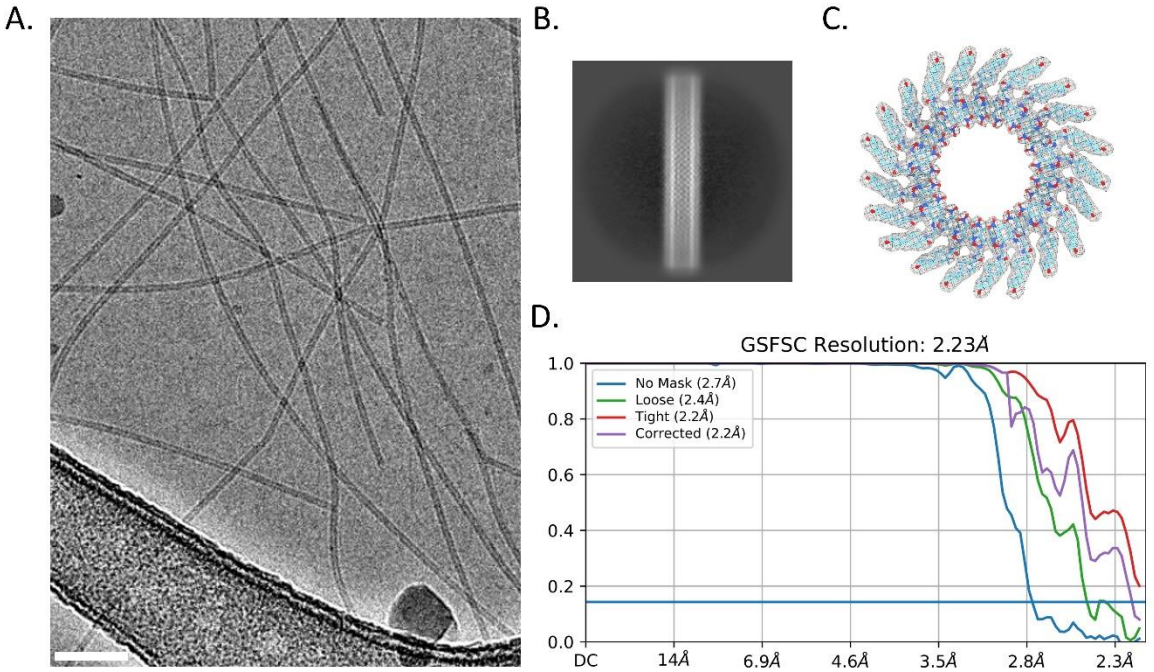


Figure S16. (A) A cryo-EM image of **1** filament. Scale bar 500 Å (B) Average power spectra of **1** tube. (C) 3D reconstruction of **1** filament. (D) map:map FSC calculation.

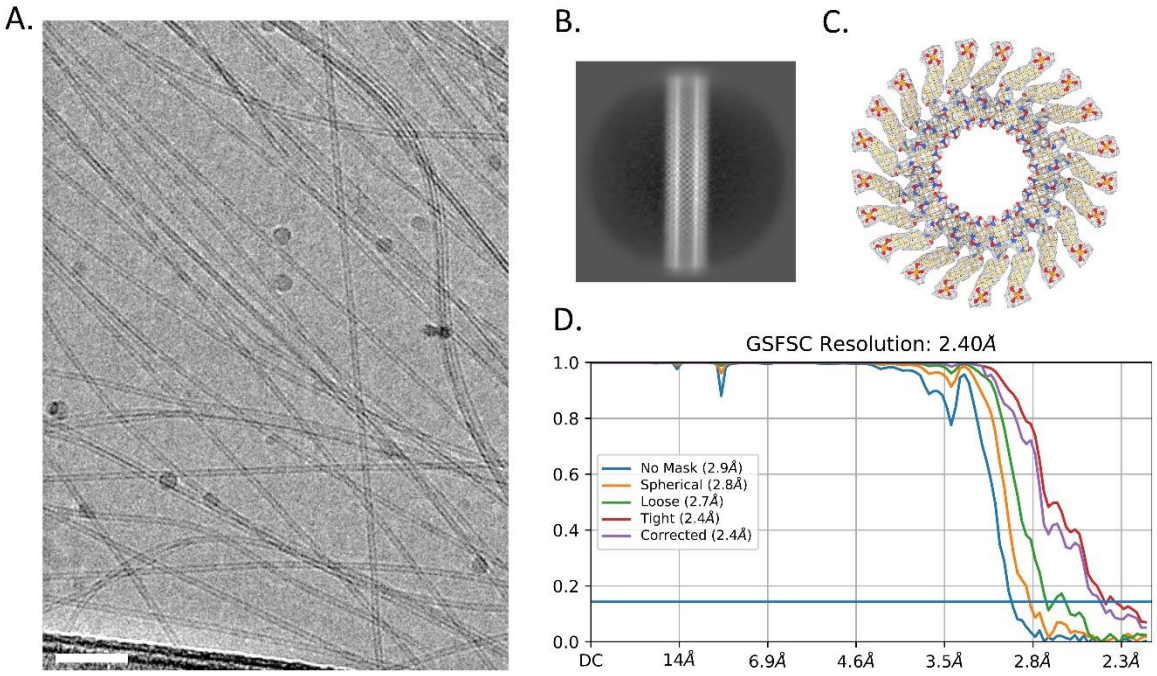


Figure S17. (A) A cryo-EM image of **1P** filament. Scale bar 500 Å (B) Average power spectra of **1P** tube. (C) 3D reconstruction of **1P** filament. (D) map:map FSC calculation.

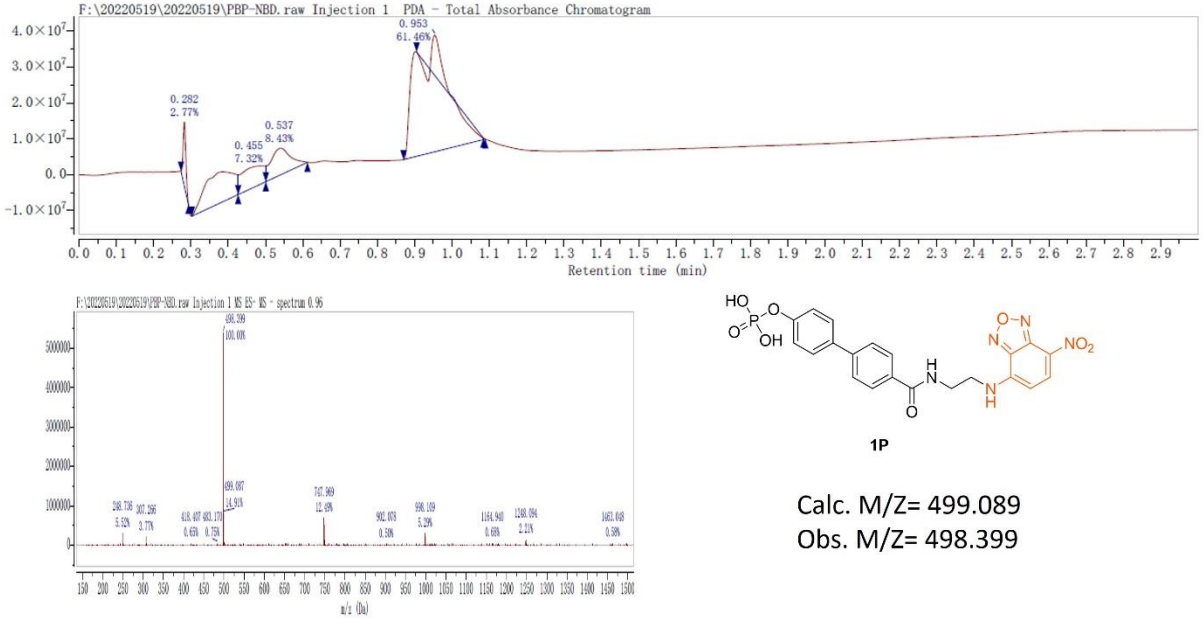


Figure S18. The LC/MS of **1P**.

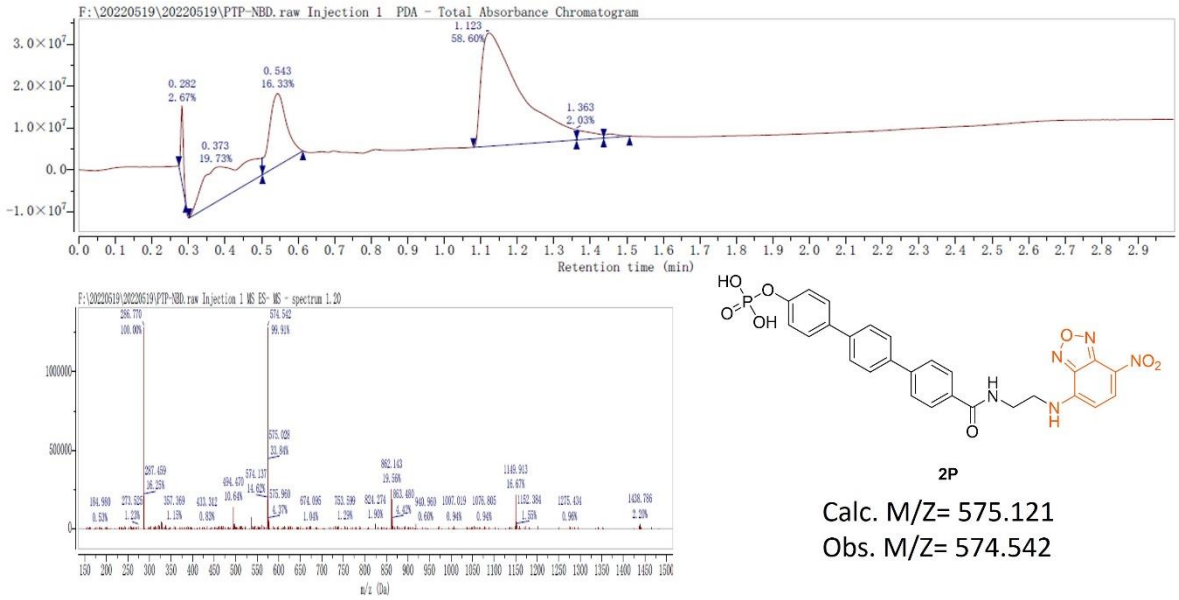


Figure S19. The LC/MS of **2P**.

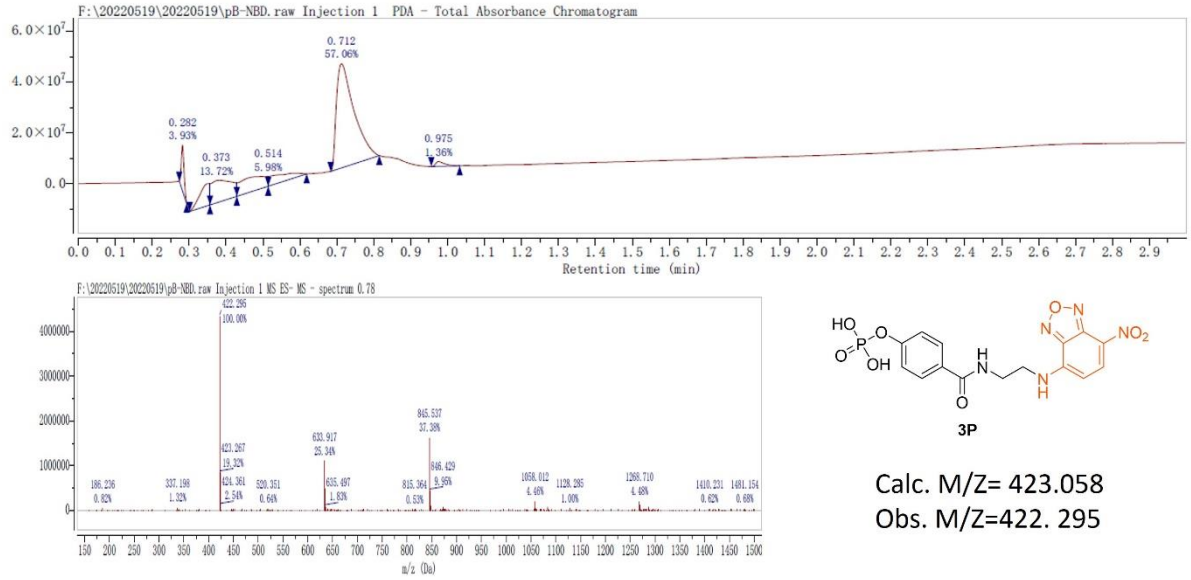


Figure S20. The LC/MS of **3P**.

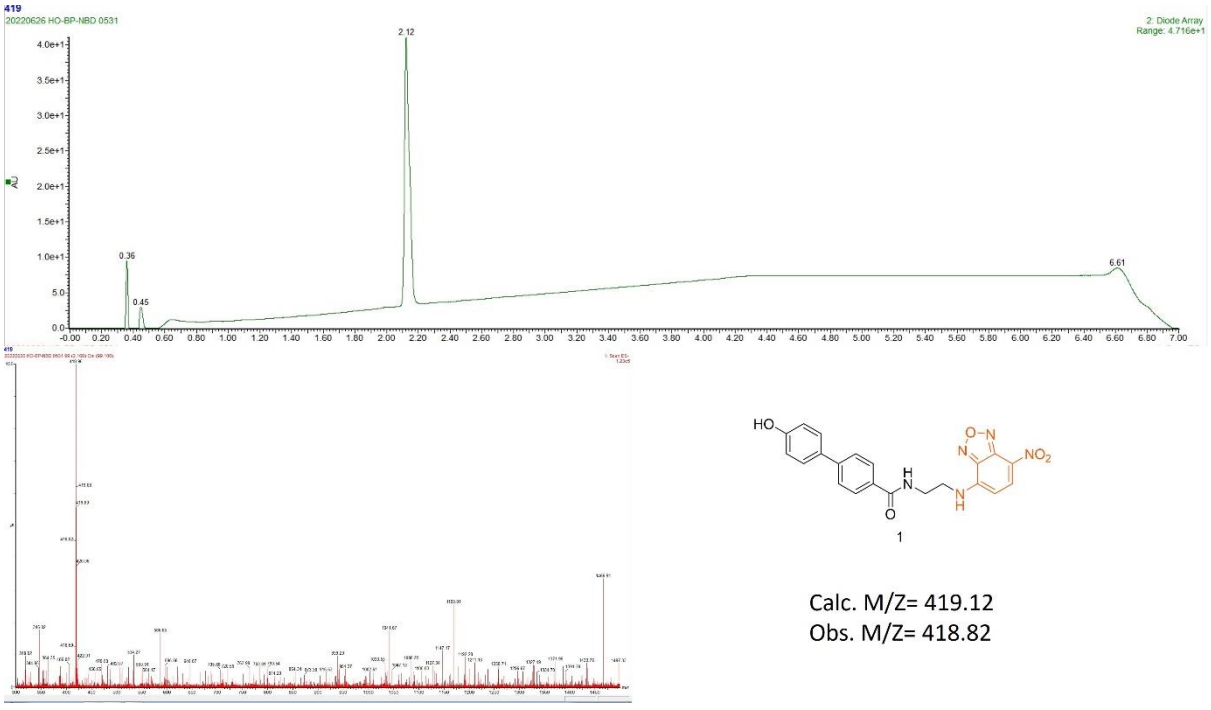


Figure S21. The LC/MS of **1**.

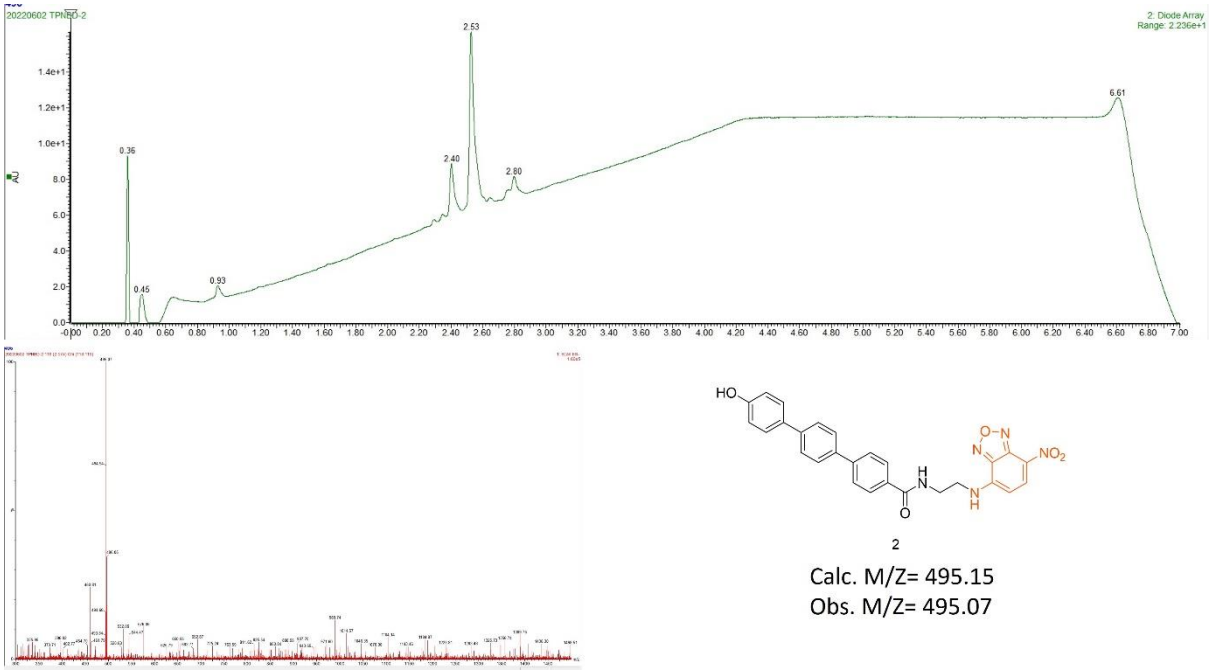


Figure S21. The LC/MS of **2**.

Supplementary Table 1. Cryo-EM and Refinement Statistics of nanotubes 1P and 1

Parameter	**pBP-NBD (1P)**	**BP-NBD (1)**
Data collection and processing		
Voltage (kV)	300	300
Electron exposure (e^{-} Å^{-2})	50	50
Pixel size (Å)	1.08	1.08
Particle images (n)	889,335	1,507,837
Shift (pixel)	23	23
Helical symmetry		
Point group	C7	C7
Helical rise (Å)	2.35	2.34
Helical twist (°)	16.35	16.33
Map resolution (Å)		
Map:map FSC (0.143)	2.4	2.2
Model:map FSC (0.38)	2.7	2.7
d_{99}	3.0	3.0
Refinement and Model validation		
Ramachandran Favored (%)	N.A.	N.A.
RSCC	0.84	0.85
Clashscore	0.8	0.0
Bonds RMSD, length (Å)	0.04	0.08
Bonds RMSD, angles (°)	2.82	1.60
Deposition ID		
PDB (model)	Supplemental data 1	Supplemental data 2
EMDB (map)	EMD-26962	EMD-26963

Reference

1. Zheng, S. Q.; Palovcak, E.; Armache, J.-P.; Verba, K. A.; Cheng, Y.; Agard, D. A., MotionCor2: anisotropic correction of beam-induced motion for improved cryo-electron microscopy. *Nature Methods* **2017,** *14* (4), 331-332.

2. Rohou, A.; Grigorieff, N., CTFFIND4: Fast and accurate defocus estimation from electron micrographs. *Journal of structural biology* **2015,** *192* (2), 216-21.

3. Punjani, A.; Rubinstein, J. L.; Fleet, D. J.; Brubaker, M. A., cryoSPARC: algorithms for rapid unsupervised cryo-EM structure determination. *Nature Methods* **2017,** *14* (3), 290-296.

4. Egelman, E. H., A robust algorithm for the reconstruction of helical filaments using single-particle methods. *Ultramicroscopy* **2000,** *85* (4), 225-234.

5. Wang, F.; Gnewou, O.; Solemanifar, A.; Conticello, V. P.; Egelman, E. H., Cryo-EM of Helical Polymers. *Chemical Reviews* **2022**.

6. Moriarty, N. W.; Grosse-Kunstleve, R. W.; Adams, P. D., electronic Ligand Builder and Optimization Workbench (eLBOW): a tool for ligand coordinate and restraint generation. *Acta Crystallographica Section D* **2009,** *65* (10), 1074-1080.

7. Emsley, P.; Cowtan, K., Coot : model-building tools for molecular graphics. *Acta Crystallographica Section D* **2004,** *60* (12-1).

8. Afonine, P. V.; Poon, B. K.; Read, R. J.; Sobolev, O. V.; Terwilliger, T. C.; Urzhumtsev, A.; Adams, P. D., Real-space refinement in PHENIX for cryo-EM and crystallography. *Acta crystallographica. Section D, Structural biology* **2018,** *74* (Pt 6), 531-544.