Furin-Instructed Molecular Self-Assembly Actuates Endoplasmic Reticulum Stress-Mediated Apoptosis for Cancer Therapy

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Contents

S1. Synthesis and characterization

S1.1 RVRRGFF-OH and KVKKGFF-OH synthesis

Peptide of RVRRGFF-OH and KVKKGFF-OH were prepared by solid phase peptide synthesis (SPPS) using 2-chlorotrityl chloride resin and the corresponding N-Fmoc protected amino acids with side chains properly protected. 20% piperidine in anhydrous N, N'-dimethylformamide (DMF) was used during deprotection of Fmoc group. Then the next Fmoc-protected amino acid was coupled to the free amino group using O-(Benzotriazol-1-yl)-N, N, N', N'-tetramethyluroniumhexafluorophosphate (HBTU) as the coupling reagent. The growth of the peptide chain was according to the established Fmoc SPPS protocol. After the last coupling step, excessive reagents were removed by a single DMF wash for 5 minutes (5 mL per gram of resin), followed by five steps of washing using DCM for 2 min (5 mL per gram of resin). The peptide derivative was cleaved using 95% of trifluoroacetic acid (TFA) with 2.5% of Triisopropylsilane (TIPS) and 2.5% of H_2O for 30 minutes. 20 mL per gram of resin of ice-cold diethylether was then added to cleavage reagent. The resulting precipitate was centrifuged for 20 min at 4 ℃ at 4000 rpm. Afterward the supernatant was decanted and the resulting solid was dried by vacuum pump.

S1.2 RVRRGFF-Capping group and KVKKGFF-Capping group synthesis

RVRRGFF-OH (173.6 mg, 0.1 mmol) and Naphthalen-2-ylmethanamine hydrochloride (21.3 mg, 0.11 mmol) were dissolved in 3 mL DMF with DIPEA (66 μL, 0.4 mmol). The reaction mixture was stirred overnight at room temperature. RVRRGFF-Nap was obtained after HPLC purification. RVRRGFF-Py, KVKKGFF-Nap, and KVKKGFF-Py were obtained according to the same method.

S1.3 1-Nap, 1-Py, 2-Nap and 2-Py synthesis

The Pbf protecting groups of RVRRGFF-Nap (187.5 mg, 0.1 mmol) was removed with 95% of trifluoroacetic acid (TFA), 2.5% of Triisopropylsilane (TIPS) and 2.5% of DCM for 3 hours, then the solvent was removed by rotary evaporation,

40 mL ice-cold diethylether was then added. The resulting precipitate was centrifuged for 20 min at 4 ℃ at 4000 rpm. Afterward the supernatant was decanted and the resulting solid was dried by vacuum pump. Then **1-Nap** was obtained after HPLC purification. **1-Py**, **2-Nap**, and **2-Py** were obtained according to the same method.

S1.4 Furin inhibitor II (H-(D)Arg-Arg-Arg-Arg-Arg-Arg-NH_2, 6R) synthesis

6R was prepared by solid phase peptide synthesis (SPPS) using 2-chlorotrityl chloride resin and Fmoc-D-Arg(pbf)-OH. **6R** was obtained according to the same method as in the **S1.1.**

S1.5 Analytical HPLC trace

DMSO stock solutions of **1-Nap**, **2-Nap** were diluted with furin working buffer (100 mM HEPES, 1 mM $CaCl_2$, pH = 7.4) to a final concentration of 100 μM working solutions, respectively. Next, 20 UmL^{-1} furin were added. The reaction mixtures were incubated at 37°C for 8 h. HPLC analyses were performed on an Agilent 1200 HPLC system equipped with a G1322A pump and in-line diode array UV detector using an Agilent Zorbax 300SB-C18 RP column with CH3CN (0.1% of trifluoroacetic acid (TFA)) and water (0.1% of TFA) as the eluent.

S1.6 Fluorescence spectra

DMSO stock solutions of **1-Py**, **2-Py** were diluted with furin working buffer (100 mM HEPES, 1 mM $CaCl_2$, pH = 7.4) to a final concentration of 100 μM working solutions, respectively. Next, 20 UmL^{-1} furin were added. Fluorescence spectra were recorded on a microplate reader (Bio-RAD iMark™, America) with excitation wavelength set to 342nm.

S1.7 Transmission electron microscope (TEM) and scanning electron microscope (SEM)

DMSO stock solutions of **1-Nap**, **2-Nap**, **1-Py**, **2-Py** were diluted with furin working buffer (100 mM HEPES, 1 mM $CaCl_2$, pH = 7.4) to a final concentration of

100 μM working solutions, respectively. Then, the solution was incubated with 20 UmL^{-1} furin at 37 °C for 8h. Transmission electron micrograph (TEM) images were obtained on a JEM-2100F field emission transmission electron microscope operated at an acceleration voltage of 200 kV. A HITACHI SU8010 (Japan) was used for scanning electron microscopy (SEM) studies.

S1.8 Zeta potential

DMSO stock solutions of **1-Nap**, **2-Nap** were diluted with pure water to a final concentration of 100 μM working solution, respectively. Then the Zeta potential was recorded by a Zeta potential analyzer (Zetasizer Nano, Malvern, UK).

S2. *In vitro* experiments

Cell culture: MDA-MB-435 cells were purchased from Procell Life Science& Technology(Wuhan, China), MDA-MB-468, A549, LO2 cells were maintained in our lab. All cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% v/v fetal bovine serum (FBS), 100 U/mL penicillin and 100 g/mL streptomycin. All cells were at 37 °C in a humidified atmosphere of 5% CO_2.

S2.1 Cell inhibition analysis

The cytotoxicity of the **1-Nap** and **2-Nap** was measured with Cell Counting Kit-8. Cells growing in log phase were seeded into a 96-well cell culture plate at 1×10^4/well. The cells were incubated for 24 h at 37 °C under 5% CO2. After culture medium removal, the cells were incubated with culture medium containing different concentrations of compounds for 24 h and 48 h, respectively. For the furin inhibiting group (**1-Nap** + 500 μM **6R**), cells were pretreated with 6R for 1 h in serum-free medium at 37 °C. After washing with PBS three times, the cells were incubated with culture medium containing **1-Nap**. We then replaced the medium with fresh DMEM medium supplemented with 10 μL CCK-8 solution. After 1 hour, the medium containing CCK-8 was removed and cells were washed three times with PBS. We

applied a microplate reader to measure the optical density (OD) at 450 nm. Cells without the compounds were used as blank control. The cell viability percent was calculated according to the following formula: cell viability (%) = OD (sample) / OD (control) ×100%. The results were calculated as cell viability relative to untreated cells. Data were obtained by three independent wells (n = 3). The concentration of the compounds was recorded as IC_{50} value, when 50% of cell viability.

S2.2 TUNEL

TdT-UTP nick end labeling (TUNEL) assays were performed with the one step TUNEL kit (Beyotime, China) according to the manufacturer's instructions. Cells grown in 6 well cell culture plate were treated as mentioned in **S2.1**. Briefly, the cells were permeabilized with 0.1% Triton X-100 for 2 min on ice followed by TUNEL for 1 h at 37℃. After TUNEL staining, cells were incubated with DAPI for 5 min at room temperature. The FITC-labeled TUNEL-positive cells were imaged under a fluorescent microscope by using 488 nm excitation and 530 nm emission. The DAPI-labeled cells were imaged by using 364 nm excitation and 454 nm emission. The cells with green fluorescence were defined as apoptotic cells.

S2.3 Western blot

MDA-MB-468 cells were collected in RIPA Lysis Buffer (Beyotime, China) with a protease inhibitor cocktail (Beyotime, China). Equal quantities of protein (30 μg/lane) were separated by electrophoresis on 10% SDS-polyacrylamide gels and sequentially electrophoretically transferred to polyvinylidene difluoride membranes (Millipore Corporation, Bedford, MA, USA). Following blocking with 5% BSA for 1 h at room temperature for binding nonspecific sites, membranes were subjected to immunoblotting with primary antibodies (overnight at 4 °C). The following primary antibodies were used: rabbit polyclonal anti-Caspase-12 antibody (1:2000 dilution), rabbit monoclonal anti-GRP78 BIP antibody (1:1000 dilution), rabbit monoclonal anti-DDIT3 antibody (1:1000 dilution), and all the primary antibodies were purchased

from Abcam (Cambridge, UK). Following incubation with corresponding horseradish peroxidase-conjugated goat anti-rabbit secondary polyclonal antibody (1:8,000; cat no. BA1039; Boster Biological Technology, Wuhan, China), blot bands were visualized using an enhanced chemiluminescence (ECL) detection system (Yeasen Biotech Co, Shanghai, China). Densitometric analysis of western blots was performed using Image J software (version 1.44; National Institutes of Health, Bethesda, MD, USA).

S2.4 Cellular uptake

MDA-MB-468 cells in exponential growth phase were seeded in glass bottom cell culture dishes at 4×10^5 cell/dish. The cells were allowed for attachment for 12 h at 37 ºC, 5% CO_2. We incubated MDA-MB-468 cells with **1-Py** at 37℃ and 4℃, respectively. After 1 h, the cells were washed with PBS three times prior to imaging by a fluorescence microscope. Then, **1-Nap** presented in cells was imaged using 405 nm excitation.

S2.5 Endoplasmic reticulum and golgi apparatus targeting

Sample preparation for confocal laser scanning microscopy (CLSM): MDA-MB-468 cells in exponential growth phase were seeded in glass bottom cell culture dishes at 4×10^5 cell/dish. The cells were allowed for attachment for 12 h at 37 ºC, 5% CO_2. The cells were divided into four groups including 100 μM **1-Nap**, 100 μM **2-Nap**, 100 μM **1-Nap** + 500 μM **6R** and blank control. The cells were incubated in serum-free medium containing different compounds at 37 °C for 1 h in a CO_2 incubator, respectively. Then, the cells were washed with HBSS for another three times prior to organelles staining. For the furin-inhibiting group (100μM **1-Nap** + 500μM **6R**), MDA-MB-468 cells were pretreated with furin inhibitor II **6R** (H-(D)Arg-Arg-Arg-Arg-Arg-Arg-NH_2, 500 μM) for 30 min in serum-free medium at 37 °C, washed with PBS for three times, then incubated with 100 μM **1-Nap** in serum-free medium at 37 °C for 1 h, and washed with HBSS for another three times prior to organelles staining.

ER-Tracker Red incubation:

1μM ER-Tracker Red (Yeasen, Shanghai) was added. After incubation for 30 min at 37 ºC, 5% CO_2, cells were rinsed 5 times by PBS buffer, and then kept in PBS buffer for imaging by a Live cell Imaging System.

Golgi-Tracker Green incubation:

5μM Golgi-Tracker Green (Keygen, Jiangsu) was added. After incubation for 30 min at 4ºC, cells were rinsed 5 times by HBSS buffer, and then kept in HBSS buffer for imaging by a Live cell Imaging System.

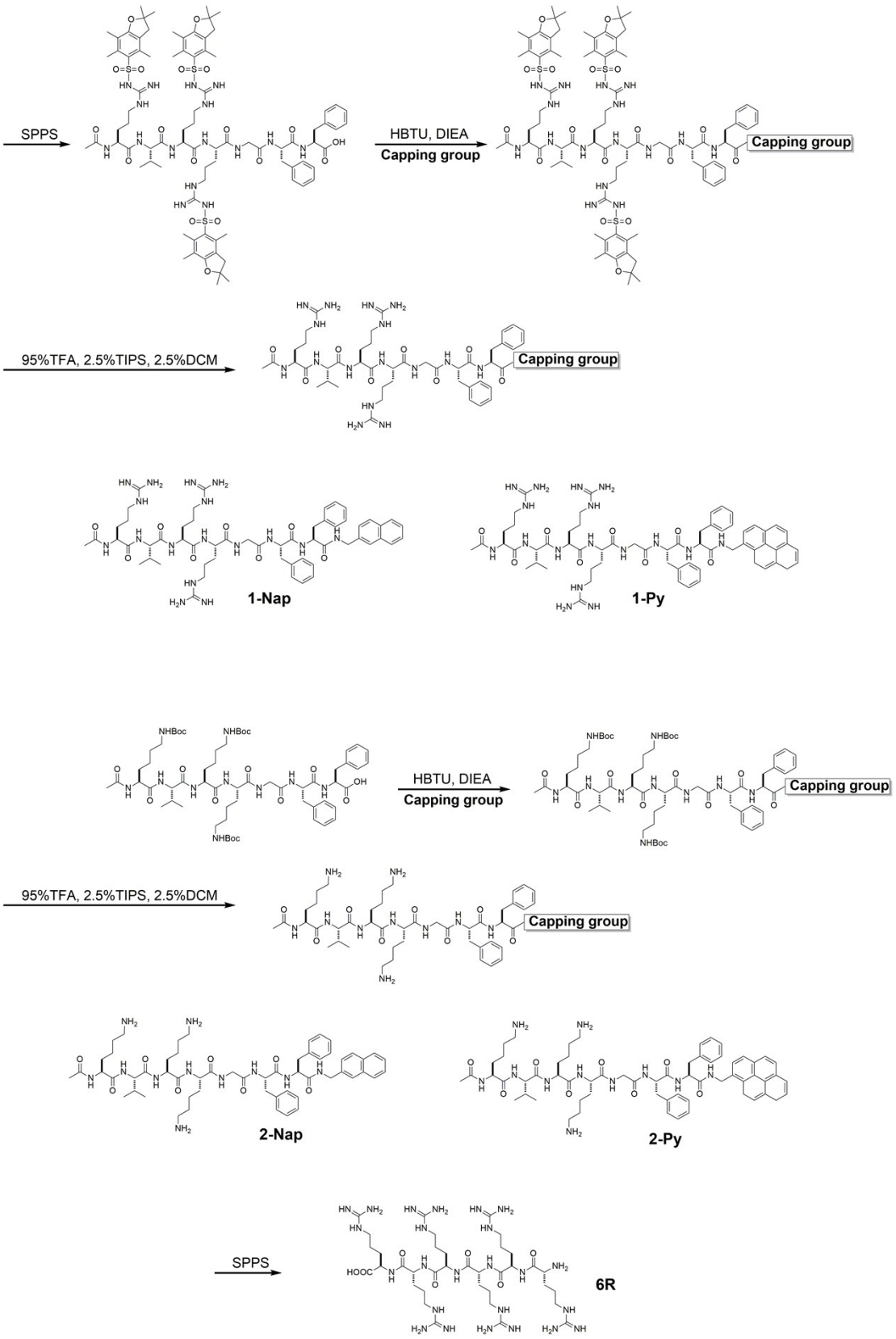
S3. *In vivo* evaluation of antitumor activity

S3.1 Tumor suppression

For breast tumor model, female BALB/c mice received s.c. injection of 4×10^6 MDA-MB-468 cells. Tumor growth was monitored every three days with a caliper. Tumor volume was calculated by the formula: length×width^2/2. When tumors size reached about 60 mm^3, mice were randomly divided into four treatment groups with 5 mice in each group, including: 25 μmol/kg **1-Nap**, 25 μmol/kg **2-Nap**, 25 μmol/kg **1-Nap** + 25 μmol/kg **6R** and vehicle (used as a control). **1-Nap**, **2-Nap** or **1-Nap** with **6R** were dispersed in Cremophor/EtOH/saline (1:1:10). The mice were treated with in situ (the peripheral area of the subcutaneous tumor) injection every three days and the first day injecting compounds was designated as day 0. Mice weight was monitored before receiving treatment.

S3.2 Immunohistochemistry

Paraffin sections of mice tumors were tested using TUNEL kit (Boster, Wuhan, China). In briefly, the sections were incubated with labeling buffer containing TdT and DIG-d-UTP for 120 min at 37°C to incorporate adequately and then incubated with diluted extra avidin-peroxidase for 30 min. DAB was added dropwise to the sections at room temperature for 30 min. All sections were counterstained with hematoxylin. Cells with brown nuclei were recorded as positive ones.



Scheme S1. Chemical structures and synthetic route of **1-Nap, 1-Py, 2-Nap, 2-Py** and **6R (**Furin inhibitor II (H-(D)Arg-Arg-Arg-Arg-Arg-Arg-NH_2).

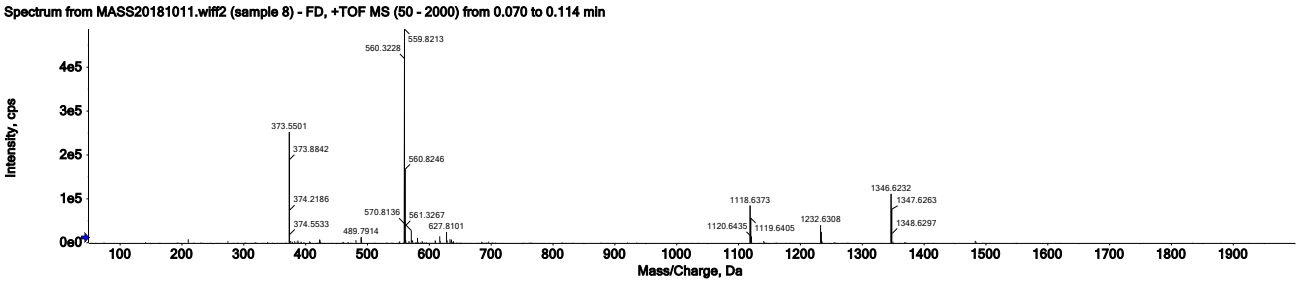


Fig. S1. HR-MS of **1-Nap**.

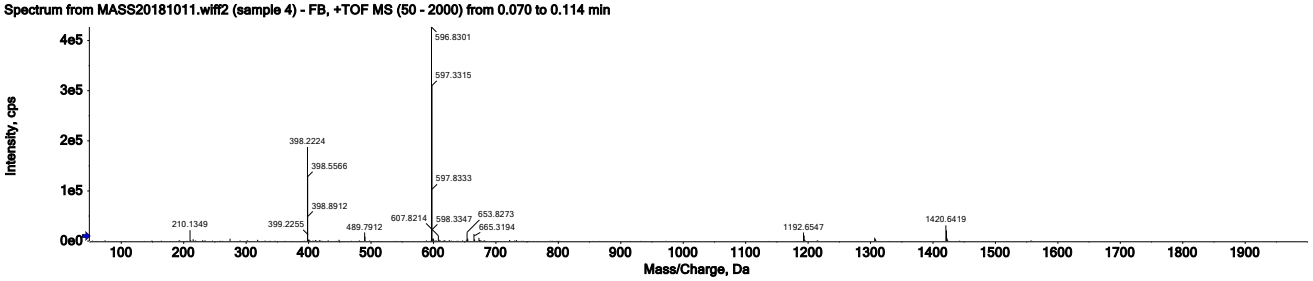


Fig. S2. HR-MS of **1-Py**.

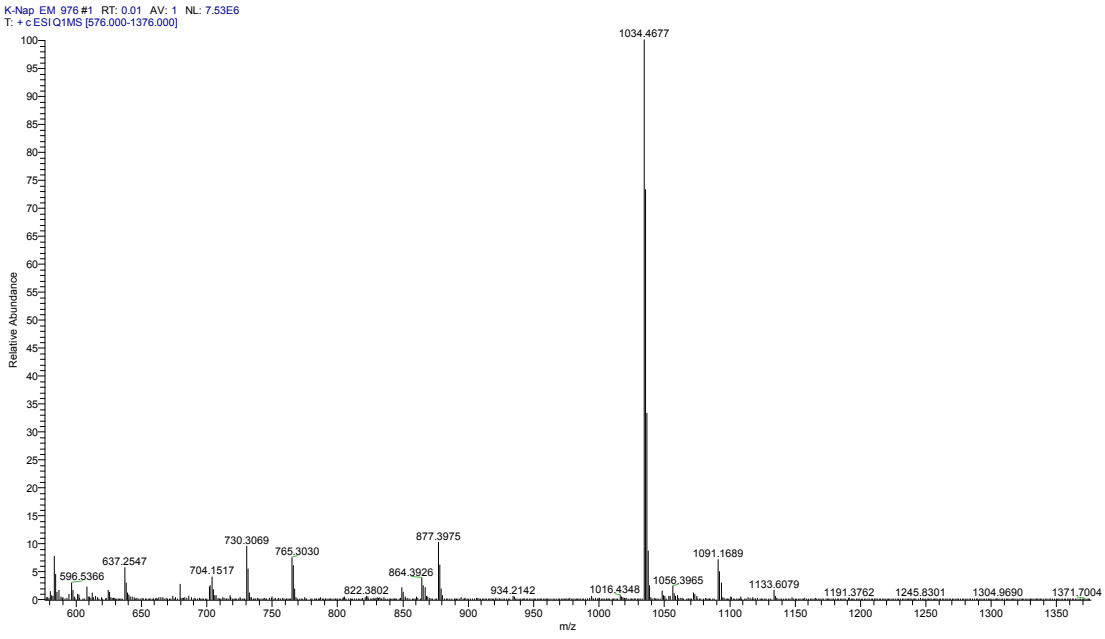


Fig. S3. HR-MS of **2-Nap**.

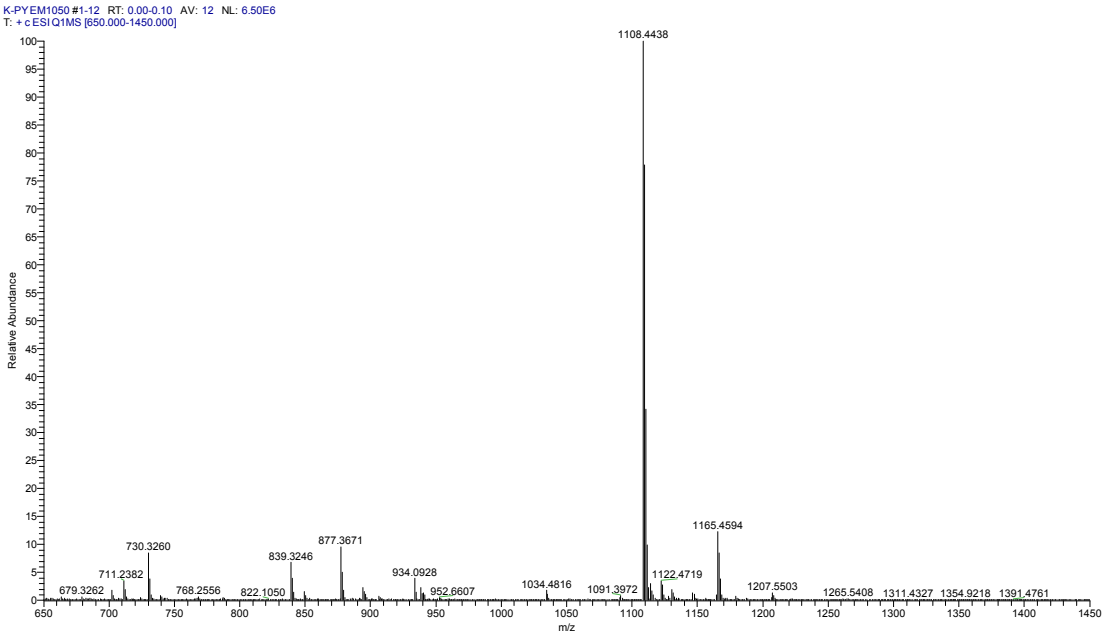


Fig. S4. HR-MS of **2-Py**.

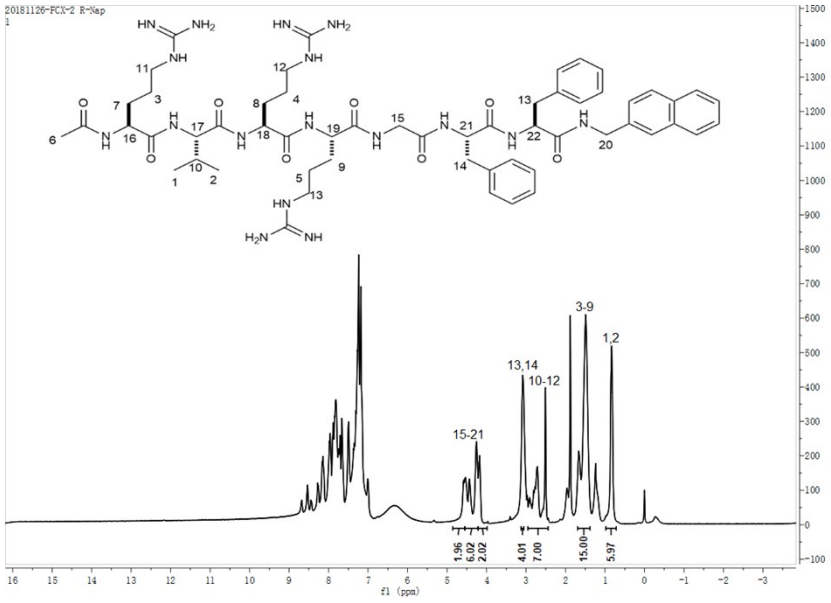


Fig. S5. ^{1}H NMR of **1-Nap**.

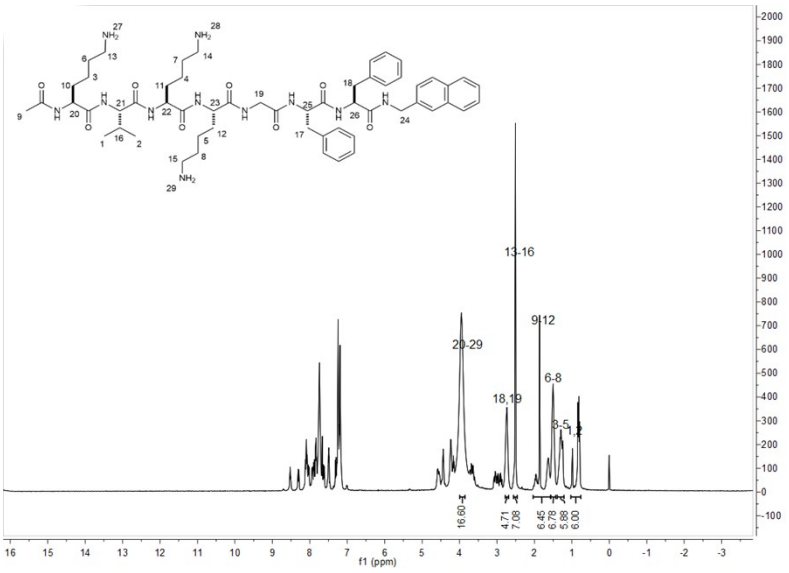


Fig. S6. ^{1}H NMR of **2-Nap**.

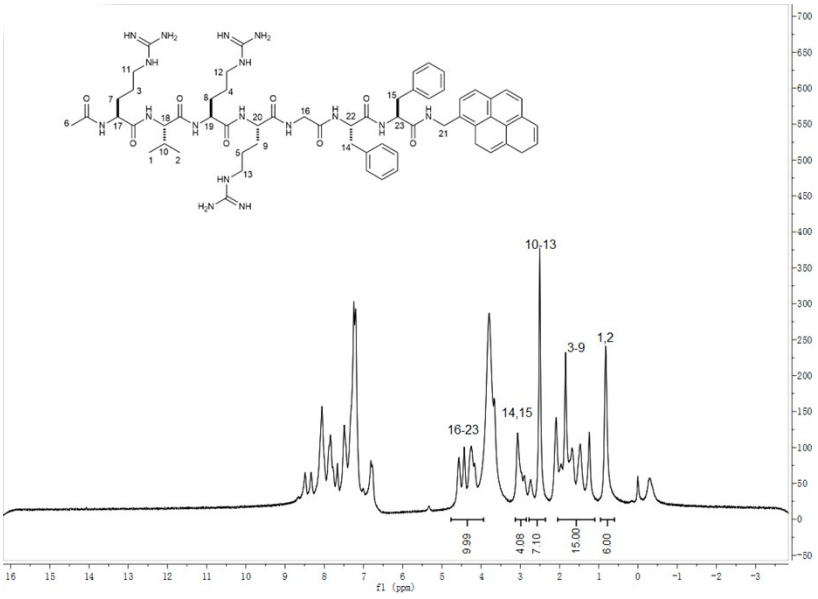


Fig. S7. ^{1}H NMR of **1-Py**.

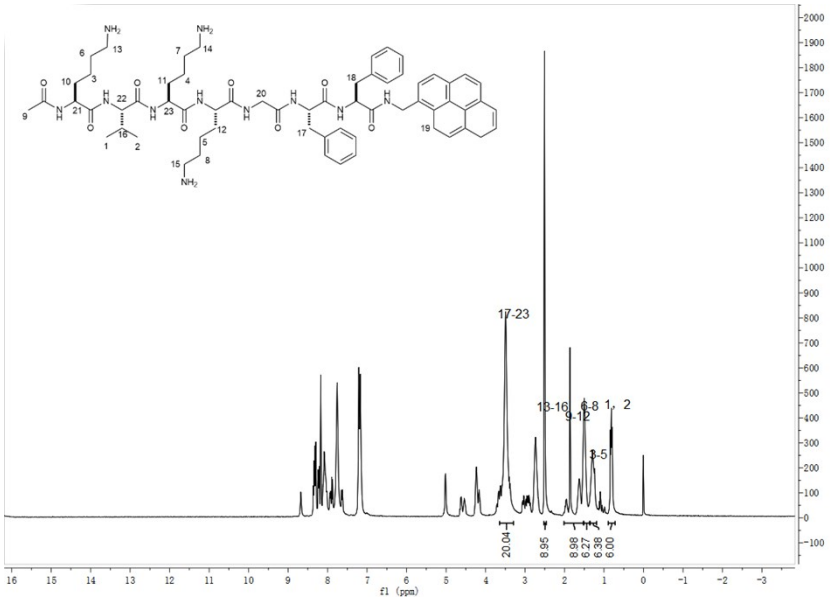


Fig. S8. ^{1}H NMR of **2-Py**.

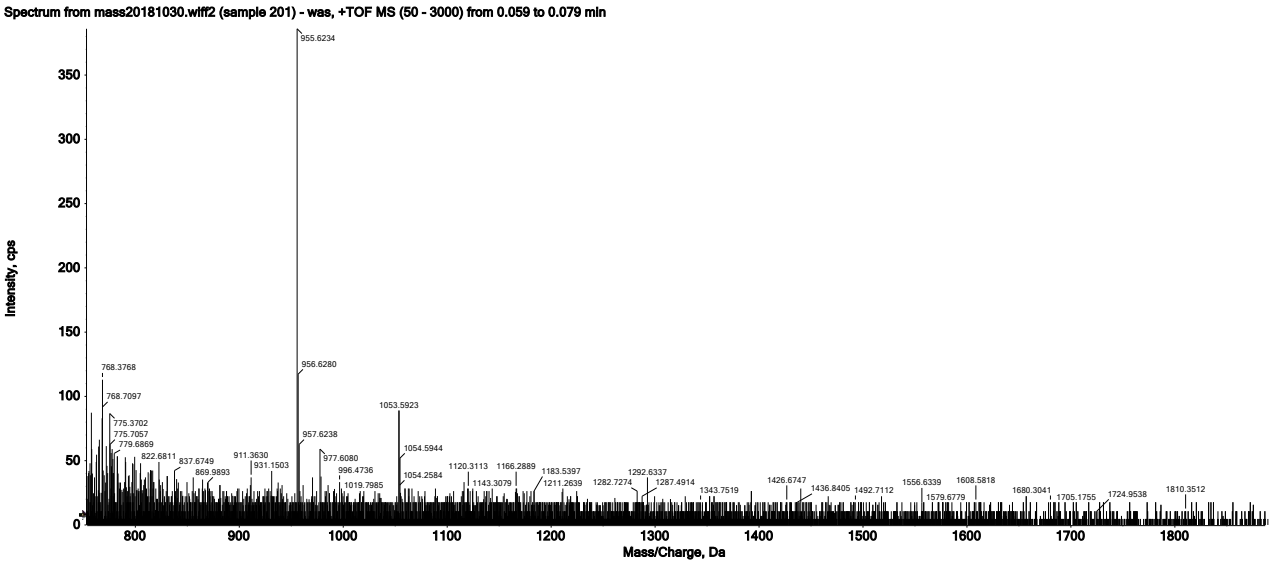


Fig. S9. HR-MS of **6R**.

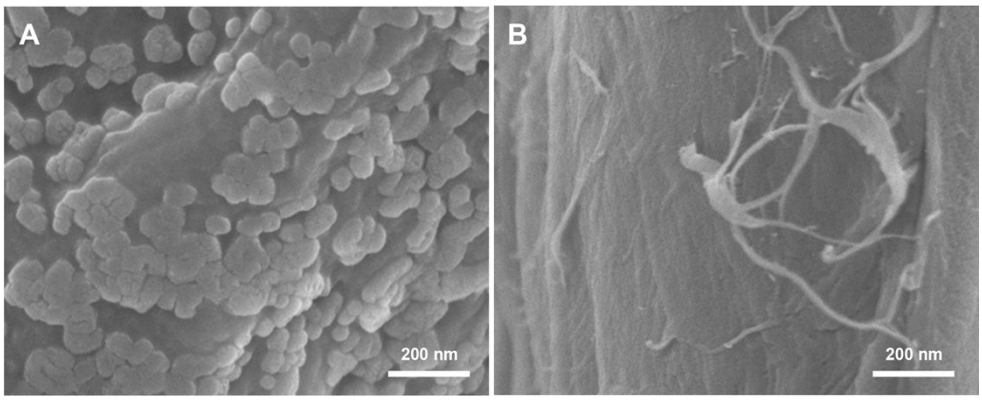


Fig. S10. SEM images of **1-Nap** (A), **1-Nap** + furin (B).

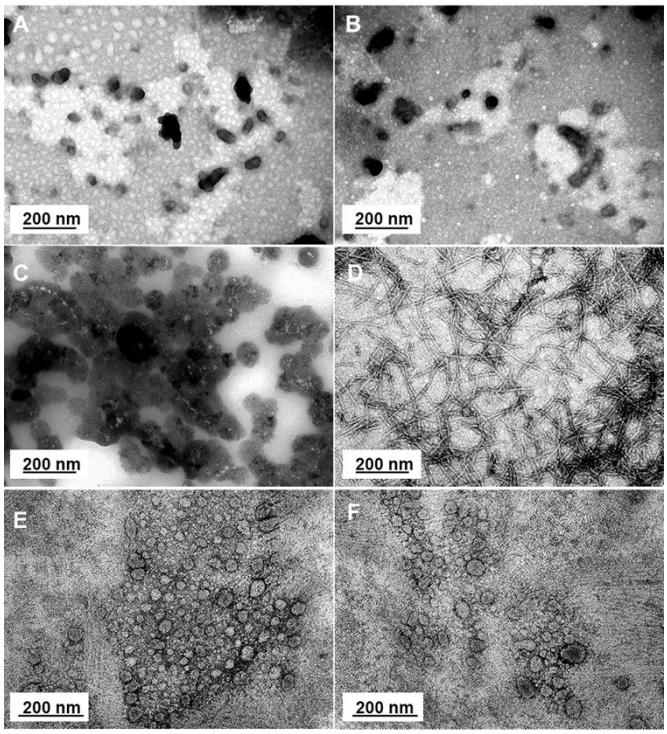


Fig. S11. TEM image of **2-Nap** (A), **2-Nap** + furin (B), **1-Py** (C), **1-Py** + furin (D), **2-Py** (E), **2-Py** + furin (F).

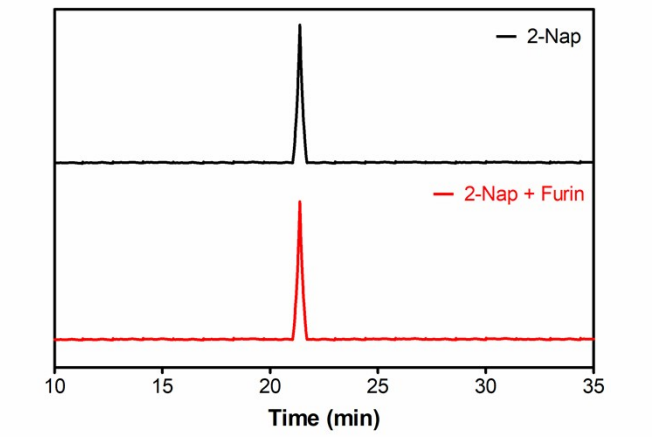


Fig. S12. HPLC traces of **2-Nap** (black), and 100 μM **2-Nap** treated with 20 UmL^{-1} furin in furin buffer at 37 °C for 8 h (red). Absorbance: 280 nm.

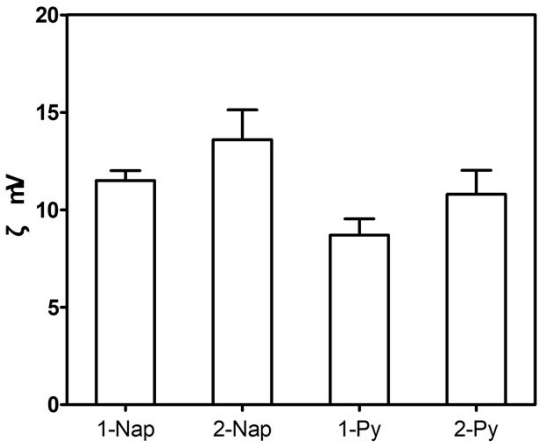


Fig. S13. Zeta potential of **1-Nap** and **2-Nap, 1-Py,** and **2-Py**.

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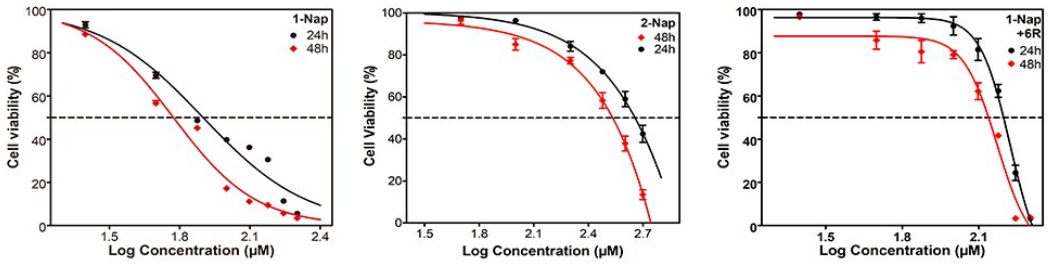

Fig. S14. Cell viability of MDA-MB-468 cells treated with **2-Nap**，**1-Nap**, **1-Nap** + **6R.**

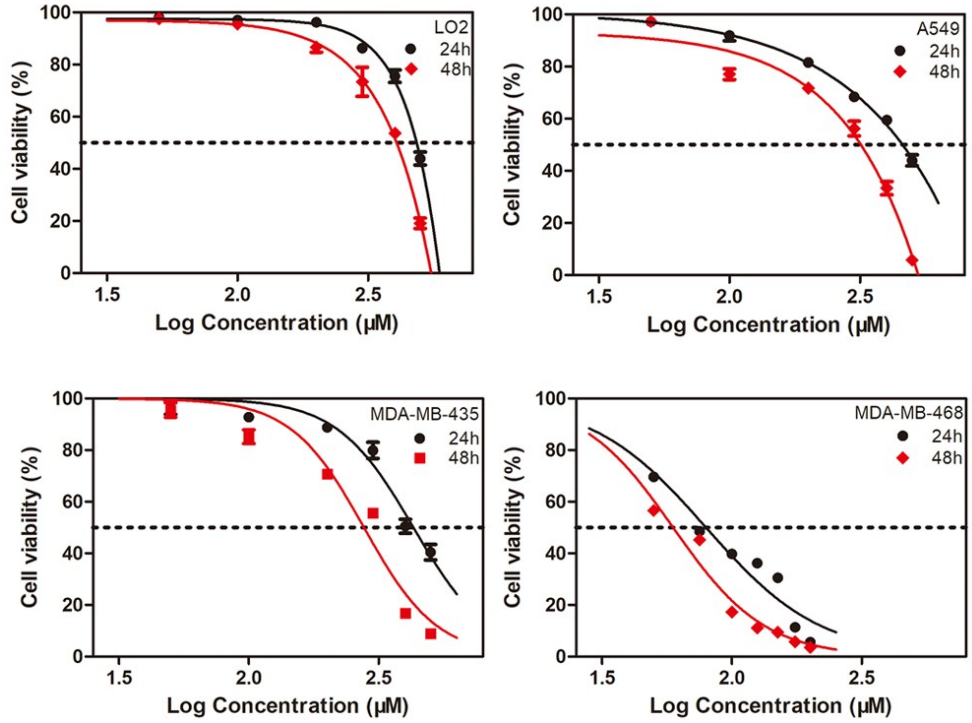

Fig. S15. Cell viability of LO2, A549, MDA-MB-435 or MDA-MB-468 cells treated with **1-Nap**.

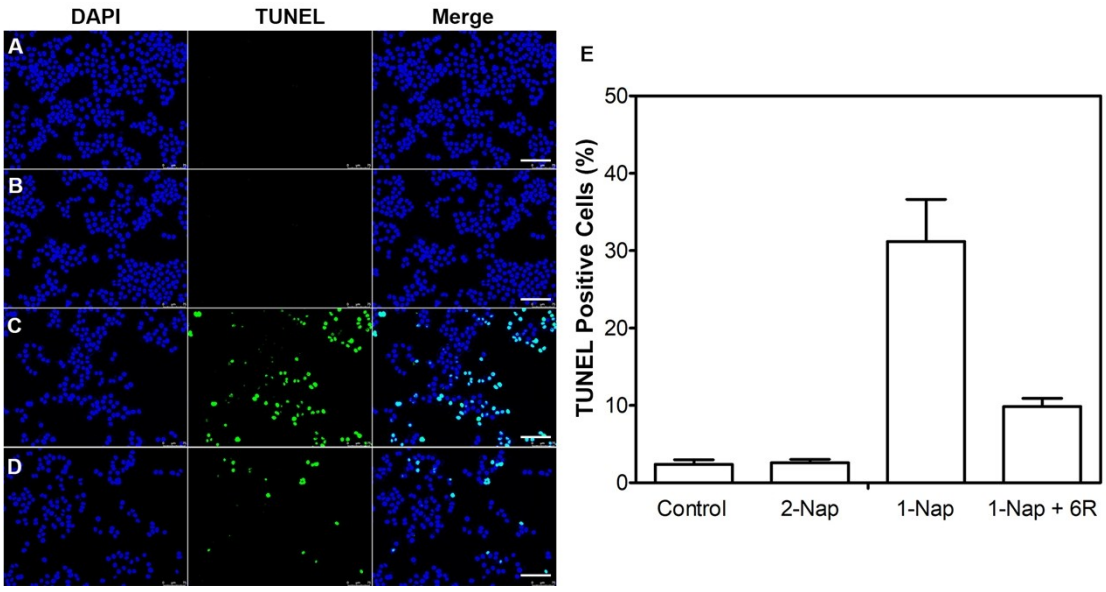

Fig. S16. TUNEL analyses of MDA-MB-468 cells of different treatment of groups, including blank control (A), 80 μM 2-**Nap** (B), 80 μM **1-Nap** (C), and 80 μM **1-Nap** + 500 μM **6R** (D). Blue and green stains indicated cell nucleus and apoptotic cells, respectively, scale bars represent 100 μm. (E) The quantitated TUNEL positive cells in (A-D).

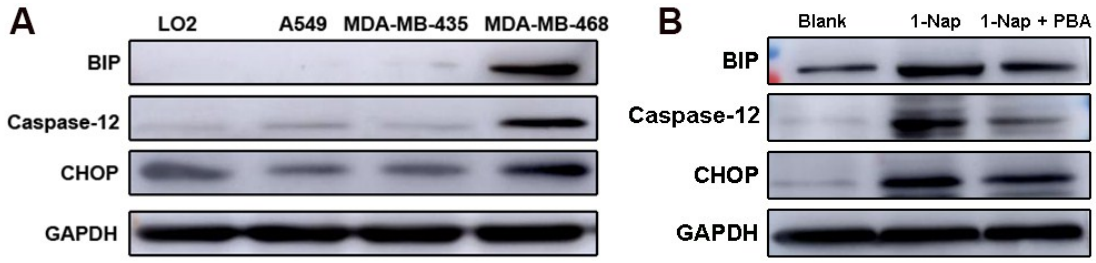

Fig. S17. (A) Western blot analysis of ER-stress markers after treating different cells with **1-Nap** (80 μM). (B) Western blot analysis of ER-stress markers of MDA-MB-468 cells treated with **1-Nap** (80 μM) or **1-Nap** (80 μM) + PBA (5 μM).

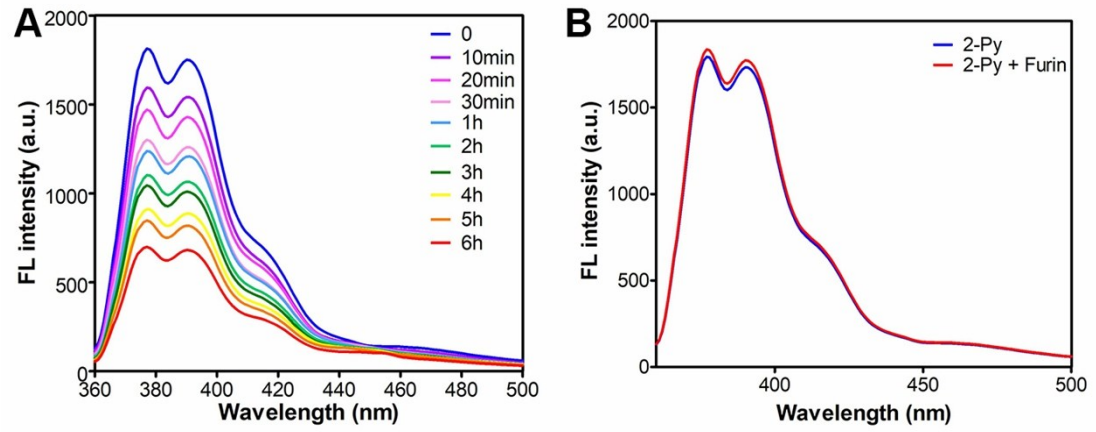

Fig. S18. Fluorescence spectra of **1-Py** (A) and **2-Py** (B) treated with furin (20 UmL^{-1}) in furin working buffer for different time. The concentration of **1-Py** and **2-Py** = 100 μM, excitation: 342 nm.

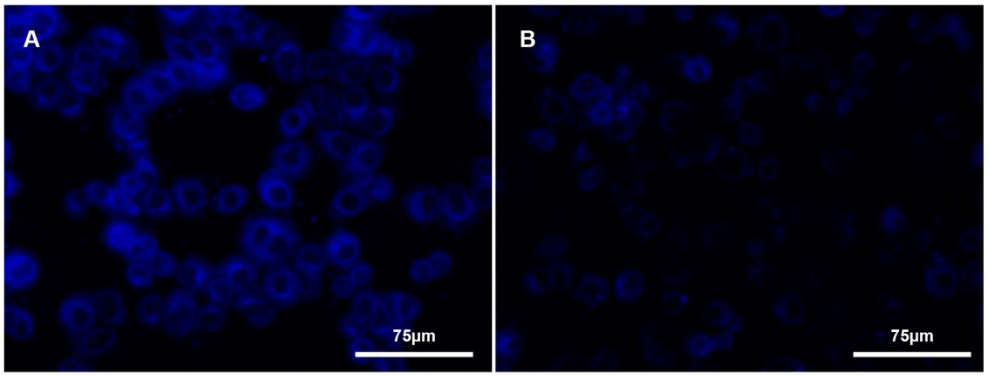

Fig. S19. Fluorescence images of MDA-MB-468 cells treated with 1-**Py** (100 μM) at 37℃(A) and 4℃(B), respectively.

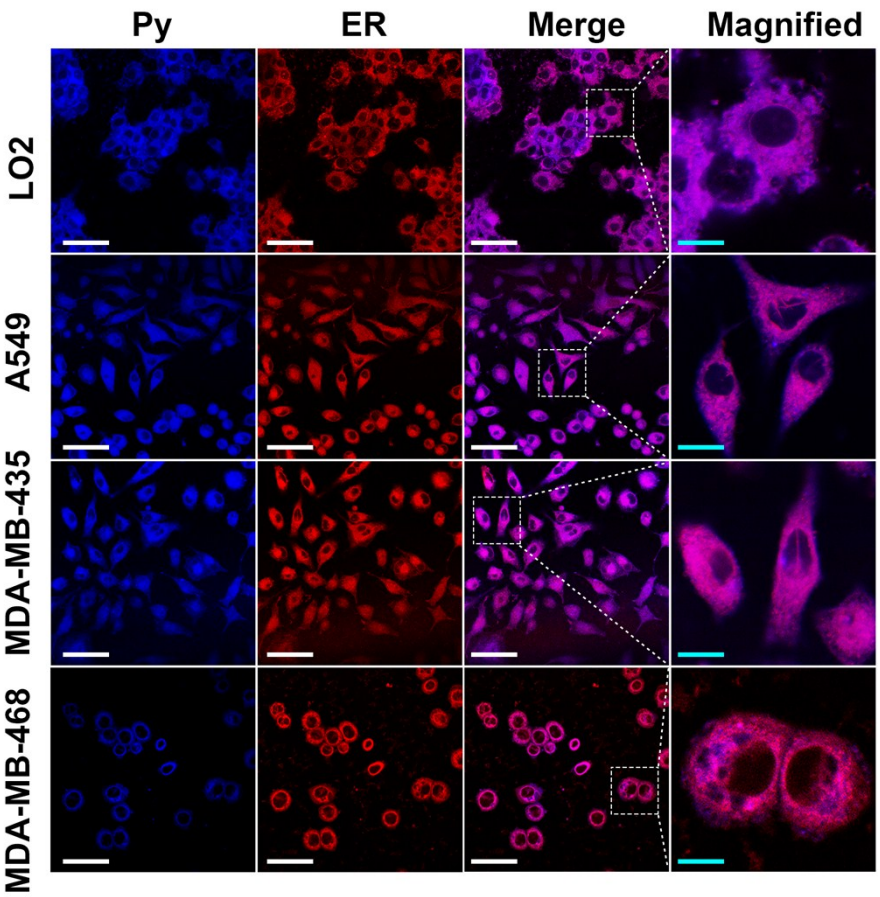

Figure S20. CLSM images of different cells treated with **1-Py** (100 μM), and then stained with ER-Tracker Red. Scale bar represent 50 and 10 μm at low and high magnification images, respectively.

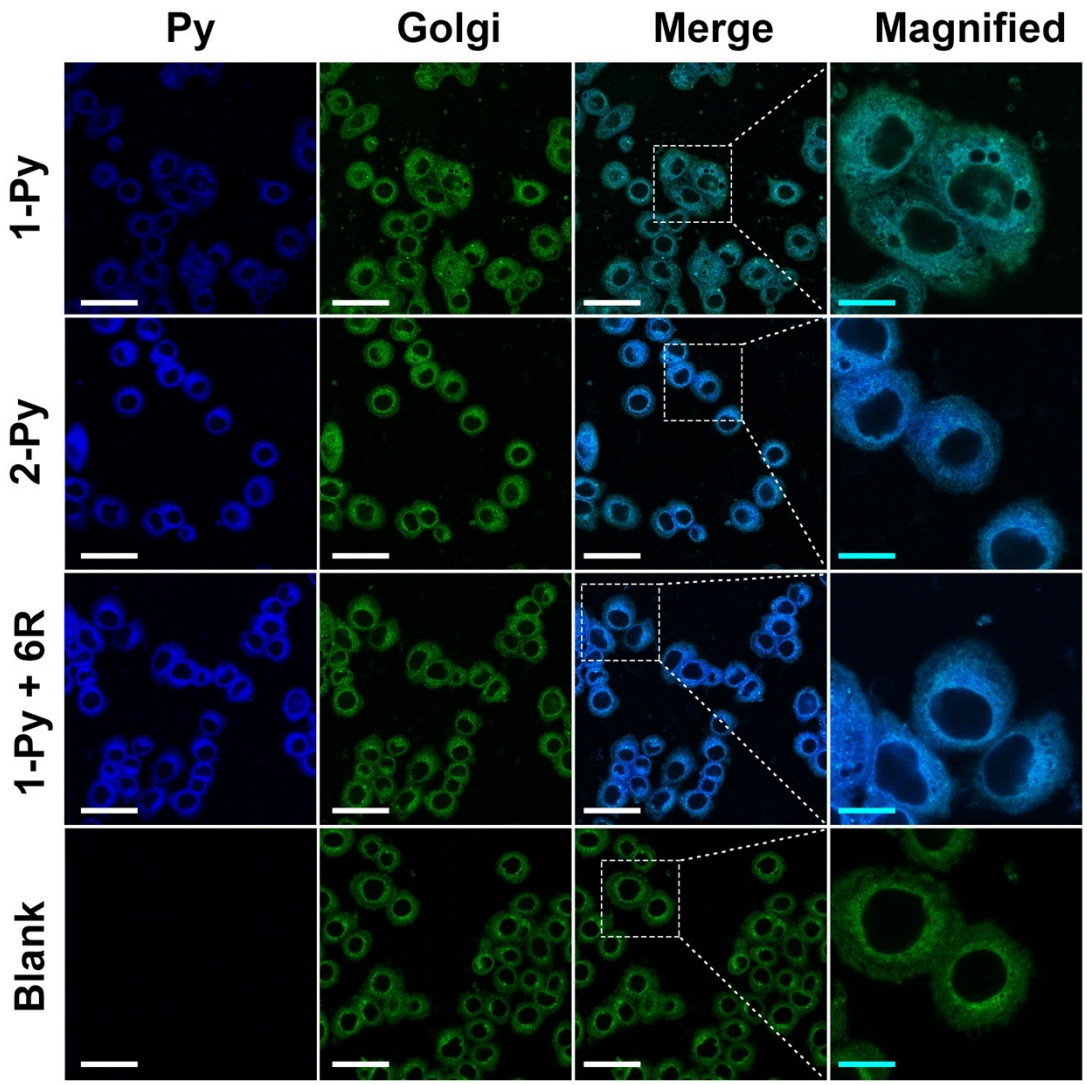

Fig. S21. CLSM images of MDA-MB-468 cells treated with **1-Py** (100 μM), **2-Py** (100 μM), **1-Py** (100 μM) + **6R** (500 μM) and Blank, and then stained with Golgi-Tracker Green. Scale bar represents 50 and 15 μm at low and high magnification images, respectively.

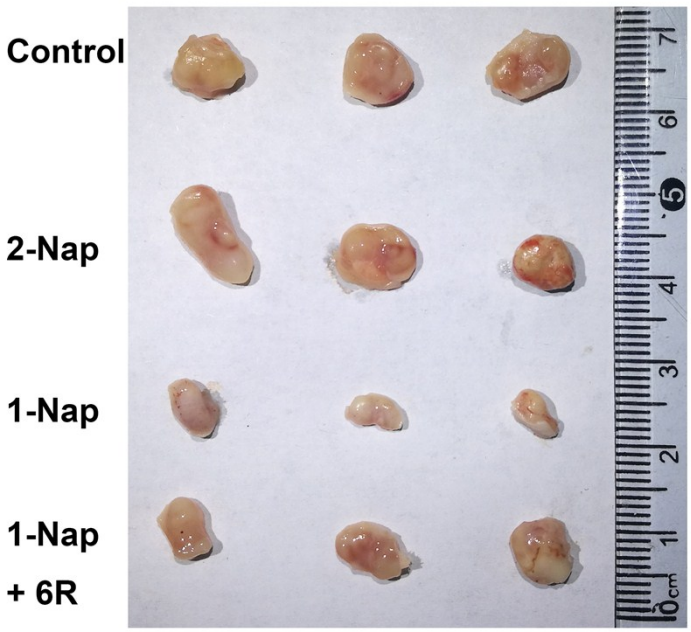

Fig. S22. *Ex vivo* images of tumors extracted from MDA-MB-468 tumor-bearing BALB/c mice at day 19 after being in situ injected with different compounds.