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Supporting Information

Dual-Responsive Self-Assembly in Lysosomes Enables Cell Cycle Arrest for Locking Glioma Cells Growth

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The authors' contributions in this work as follows:

Dr. Y. Cai, and Dr. W. Huang supervised the project. Dr. Y. Cai and Dr. Z. Yu designed the study protocols, Dr. J. Zhan, Miss S. Ma, and Miss W. Ma performed the synthesis and characterization of materials. Dr. J. Zhan, Miss S. Ma, Miss W. Ma performed the cell experiments. Miss J. Zhong, Miss W. Ma, Miss S. Ma, and Miss Y. Wang performed the animal experiments. Dr. Y. Cai and Dr. J. Zhan collected data and performed all the data analysis. Dr. Y. Cai, and Dr. J. Zhan co-wrote the initial manuscript, designed the supplementary experiments and modified the revised manuscript.

Contents

S1 Synthesis and characterization

S1.1 Materials and methods

Materials: 2-Chlorotrityl Chloride resin (100-200 mesh, loading: 0.939 mmol/g), N-fluorenyl-9-methoxycarbonyl (Fmoc)-protected L-amino acids and o-benzotriazole-N, N, N', N'-tetramethyluroniumhexafluorophosphate (HBTU) were obtained from GL Biochem Ltd. (Shanghai, China). N, N'-dimethylformamide (DMF), dichloromethane (DCM), acetic anhydride, methanol, piperidine, diisopropylethylamine (DIEPA), trifluoroacetic acid (TFA), triisopropylsilane (TIS) and anhydrous ether were purchased from Shanghai Chemical Co. (China). 4-Chloro-7-nitrobenzo-2-oxa-1, 3-diazole (NBD-Cl), β-Alanine and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), Chlorpromazine, β-CD, filipin Ⅲ, amiloride and pyrene were purchased from Aladdin Reagent Co. Ltd. (Shanghai, China). Cisplatin was obtained from ThermoFisher Scientific Co. Ltd. (China). Recombinant Human Legumain/Asparaginyl Endopeptidase (250 pmol/min/μg) was purchased from R&D systems (USA). Cell cycle and Apoptosis Analysis Kit, Lyso-Tracker Red and Hoechst 33342 were purchased from Beyotime Biotechnology Co., Ltd. (China). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM), trypsin, and Penicillin-streptomycin were purchased by Biological Industries (Israel). Anti-HIF-1α, anti-cyclin-D1 and anti-β-actin were purchased from Abcam Co. Ltd (Hong Kong, China). Immobilon Western HRP substrate was purchased from Millipore Co., Ltd. (USA). All other solvents and reagents were commercially available, used directly without further purification.

Cell lines: The murine glioma (C6) cells and murine cardiomyocytes (H9C2) cells were continuously cultured in our laboratory.

General methods: All peptides were purified by HPLC (Agilent 1200) system using a C18 RP column. Mass was operated on a Prelude SPLC + TSQ Quantiva LC-MS/MS (ThermoFisher Scientific). The size of nanoparticles was performed on a Malvern Zetasizer Nano. The fluorescence spectra were recorded on Fluorescent meter (FluoroMax-4, HORIBA, France). The morphology of nanoparticles was carried out on a JEM-1400Flash transmission electron microscope (JEOL, Japan). The optical density (OD) values were recorded on an Infinite M1000 Pro (Tecan, Switzerland). The confocal images were obtained by a confocal laser scanning microscopy (LSM 880 with Airyscan, Carl Zeiss). The cell cycle analysis was carried out on the flow cytometer (BD LSRFortessa X-20). The protein expression signal was detected by using a Bio-Rad Chemioc MP system (Bio-Red Laboratories, USA).

S1.2 Peptide synthesis

Comp.1, ***Comp.2***, ***Comp.3*** and ***Comp.4*** were synthesized by standard phase peptide synthesis (SPPS). All the peptides were purified by HPLC. ESI-MS (positive mode) for ***Comp.1***: Calculated MS: 1681.65, Found: $[M+H^+]$ = 1682.95; ***Comp.2***: Calculated MS: 1552.54, Found: $[M+H^+]$ = 1553.21; ***Comp.3***: Calculated MS: 1423.42, Found: $[M+H^+]$ = 1424.57; ***Comp.4***: Calculated MS: 1695.68, Found: $[M+H^+]$ = 1696.95.

S1.3 Characterization of nanostructures

The hydrodynamic size of ***Comp.1*** and ***Comp.4*** nanoparticles in PBS (pH 7.4, 0.03 wt.%) was measured by Malvern Zetasizer Nano at room temperature. The peptide solutions in pH 7.4 or 5.0 were texted continuously for 14 days. The morphology of ***Comp.1*** and ***Comp.4*** in pH 7.4 or 5.0 were obtained by a JEM-1400Flash transmission electron microscope (TEM) and scanning electron microscope (SEM) (JEOL, Japan).

S1.4 The CMC determination of *Comp.1* and *Comp.4*

Firstly, 100 μL of pyrene acetone solution (1.2×10^{-5} mol L^{-1} in acetone) was placed in a 5 mL container, after acetone was volatilized at room temperature in the dark, and 2 mL peptide PBS solution (pH 7.4 or 5.0) with a series of concentration was added. The mixture was vortexed and allowed to stand for 24 hours under dark at room temperature. Then, the excitation spectra of sample solutions were obtained by a fluorophotometer (Ex = 300-360 nm, Em = 393 nm, slit widths at 10 nm). The CMC values of the ***Comp.1*** and ***Comp.4*** nanoparticles were determined to be the intersection of the horizontal tangent line and the tangent line of the inflection point curve.

S1.5 Fluorescence measurement

S1.5.1 pH related fluorescence intensity change study of *Comp.1*

The 0.1 wt.% ***Comp1*** was dispersed in 1.0 mL PBS buffer with different pH values (pH = 5.0、5.5、6.8、7.4), the fluorescence emission spectrum was recorded by Fluorescent meter (Ex = 475 nm, Em = 495-700 nm).

S1.5.2 pH related fluorescence intensity change study of *Comp.1* + legumain

The 0.1 wt.% Comp1 was dispersed in 1.0 mL PBS buffer with different pH values (pH = 5.0、5.5、6.8、7.4), after adding 1 ng μL^{-1} legumain in the different solutions above the fluorescence emission spectrum was recorded by Fluorescent meter (Ex = 475 nm, Em = 495-700 nm).

S1.6 Conversion ratio

Comp.1 (0.1 wt. %) was dispersed in 2.0 mL of assay buffer solution (pH = 5.0). And the preprocessed legumain (1 ng μL^{-1}) was then added to the above solution at the preset time point at 37 ºC. The conversion ratio was then analyzed by HPLC. The conversion ratio was obtained by integrating the area of the peak.

S2 Cell experiments

S2.1 Cell culture

The murine glioma (C6) cells and murine cardiomyocytes (H9C2) cells were continuously cultured in our laboratory. They were cultured in DMEM medium (10% fetal bovine serum (FBS), 1% penicillin-streptomycin (10000 U mL^{-1})). All cells were cultured in a humidified atmosphere containing 5% CO_2 at 37 ºC.

S2.2 Cell proliferation study

C6 cells or H9C2 cells in logarithmic growth phase were seeded on 96-well plates (5×10^3 cells per well) and further cultured for 24 h, then they were incubated with DMEM medium containing a series concentration of ***Comp.1*** or ***Comp.4*** for 24 h, 48 h, 72h, respectively. Finally, 10 μL of MTT solutions (5 mg mL^{-1}) were added to each well, and the cells were incubated with MTT for 4 h, then the supernatant were aspirated and 150 μL of DMSO was added to each well. The optical density (OD) value of each well was recorded by a microplate reader.

S2.3 Cellular Internalization

S2.3.1 Co-localization in lysosomes

C6 cells or H9C2 cells were seeded in petri dishes (2×10^4 cells per well) and cultured in DMEM and further cultured for 24 h. Then the cells were incubated with DMEM medium containing 200 μM ***Comp.1*** or ***Comp.4*** for 4 h. After removing the drug-containing medium and washing the cells thrice by PBS, cells were further cultured for predetermined times (0 h, 24 h, 48 h). The Lyso-Tracker Red (1 μM) was added into petri dishes and further incubated for 60 min, then the cells were washed thrice by PBS. And then stained with Hoechst 33342 (1 μg mL^{-1}) for 15 min. The cells were washed thrice by PBS and observed by CLSM (Ex = 405 nm, Em = 430-460 nm; Ex = 488 nm, Em = 500-550 nm; Ex = 543 nm, Em = 560-700 nm).

S2.3.2 Intracellular fluorescence quantification

C6 cells or H9C2 cells were seeded in 6-well plates (3×10^5 cells per well) and cultured in DMEM and further cultured for 24 h. Then the cells were incubated with DMEM medium containing 200 μM ***Comp.1*** or ***Comp.4*** for 4 h. After removing the drug-containing medium and washing the cells thrice by PBS, cells were further cultured for predetermined times (0 h, 24 h, 48 h). The cells were digested by trypsin-EDTA, and collected into centrifuge tubes. After being washed with PBS, cells were analyzed the NBD fluorescence by flow cytometry.

S2.3.3 The mechanism of the uptake of *Comp.1*

We used four inhibitors of different endocytotic processes to identify the possible pathways of cellular uptake, methyl-b-cyclodextrin (M-β-CD) against lipid raft-dependent endocytosis, chlorpromazine (CPZ) against clathrin-mediated endocytosis, ethylisopropylamiloride (EIPA) against macropinocytosis, and Filipin III against caveolae-mediated endocytosis.

C6 cells or H9C2 cells were seeded in petri dishes (2×10^4 cells per well) and cultured in DMEM and further cultured for 24 h. The cells upon treatment of ***Comp.1*** (200 μM) at 37 ºC and 4 ºC for 4 h, and upon treatment of ***Comp.1*** plus β-cyclodextrin crystalline (β-CD, 5 mM), chlopromazine (CPZ, 30μM), amiloride (EIPA, 50 μM), and filipin III (5 μg mL^{-1}) at 37 ºC for 1 h and further treatment of ***Comp.1*** (200 μM) at 37 ºC for 4 h. The cells were washed thrice by PBS and observed by CLSM (Ex = 488 nm, Em = 500-550 nm).

S2.4 Cell cycle analysis

The population of C6 cells in different phases of the cell cycle was detected by flow cytometry. Cell cycle distribution was determined by staining DNA with PI. C6 cells were seeded in 6-well plates (3×10^5 cells per well) and cultured for 24 h. After treatment with or without ***Comp.1*** or ***Comp.4*** for 4 h, removing the drug-containing medium and washing the cells thrice by PBS, cells were further cultured for 24, 48, 72 h, respectively. Cells were incubated with fresh culture medium for 72 h as control group. Cells were collected, washed and re-suspended in ice-cold PBS buffer (pH 7.4) for two cycles. After fixed with 70% alcohol at 4 ºC overnight, the cells were stained with 0.5 mL propidium iodide (PI) assay buffer for 30 min at room temperature. The percentages of cells in specific phases of the cell cycle were determined by flow cytometry.

S2.5 DNA synthesis analysis

Cells were plated at 4×10^4 cells/well in 24-well plates allowed to adhere. After treatment with or without ***Comp.1*** or ***Comp.4*** for 4 h, removing the drug-containing medium and washing the cells thrice by PBS, cells were further cultured for 72 h. Then cells were washed with PBS, and incubated in serum-free DMEM containing 10 μmol/L 5-ethynyl-2'-deoxyuridine (EdU, a BrDU substitute; Guangzhou RiboBio Co., Ltd, Guangzhou, China) for 2 h. The cells were washed with PBS, fixed, and permeabilized in PBS containing 2% formaldehyde, 0.5% Triton X-100, and 300 mmol/L sucrose for 15 min. After washing with PBS, the cells were blocked using 10% FBS in PBS for 30 min, and incorporated EdU was detected by incubation with the fluorescent-azide coupling solution (Apollo®, Guangzhou RiboBio Co., Ltd) for 30 min. The cells were washed three times with PBS containing 0.05% Tween-20 (PBST), incubated with the DNA-staining dye Hoechst 33342 for 30 min, and washed in PBS. Images were captured using a fluorescent microscope, and the average nuclear fluorescent intensity was calculated from at least 50 non-S-phase cells randomly selected in 5 different fields of view.

S2.6 Western blotting

The protein levels of HIF-1α, cyclin-D1 in C6 cells were detected by performing immunoblotting assays. C6 cells were seeded in 6-well plates (3×10^5 cells per well) and cultured for 24 h. After treated with or without ***Comp.1*** or ***Comp.4*** for 4 h, removing the drug-containing medium and washing the cells thrice by PBS, cells were further cultured for 24 h, 48 h, 72 h, respectively. Cells were harvested and lysed in the cell lysis buffer containing proteinase inhibitors. After quantitative analysis by BCA, equal amount (30 μg) of samples were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was blocked in 5% skim milk. Membranes were incubated with HIF-1α antibody, cyclin-D1 antibody and β-actin antibody at 4 ºC overnight followed incubation with HRP-labeled secondary antibody. The membranes were finally detected by Immobilon Western HRP substrate using a Bio-Rad Chemioc MP system.

S3 Animal experiment

S3.1 Animal Model

BALB/c nude mice (4-6 weeks old, about 18 g) were purchased from the Southern Medical University Animal Center. To prepare C6 tumor-bearing mice, C6 cells (1×10^7 cells/200 μL) in PBS were subcutaneously injected in right hind leg of BALB/c mice. All animal experiments were carried out under the guidelines evaluated and approved by the ethics committee of Southern Medical University (Resolution No. L2017084).

S3.2 *In vivo* antitumor study

When the tumor volume increased to about 100 mm^3, randomly, C6 tumor-bearing mice were divided into four groups (Control group, ***Comp.1*** group, ***Comp.4*** group, and Cisplatin group, n = 5). Mice were injected with PBS, ***Comp.1*** (2 mM in PBS, 100 μL), ***Comp.4*** (2 mM in PBS, 100 μL), Cisplatin (2.2 mg/kg) solutions respectively in tail vein every other day. The body weights of mice and tumor volumes were recorded every other day (the following is the tumor volume calculation formula: $V = a \times (b)^2/2$, a = tumor length, b = tumor width). After 14 days, the mice were sacrificed and the tumors were weighted and photoed.

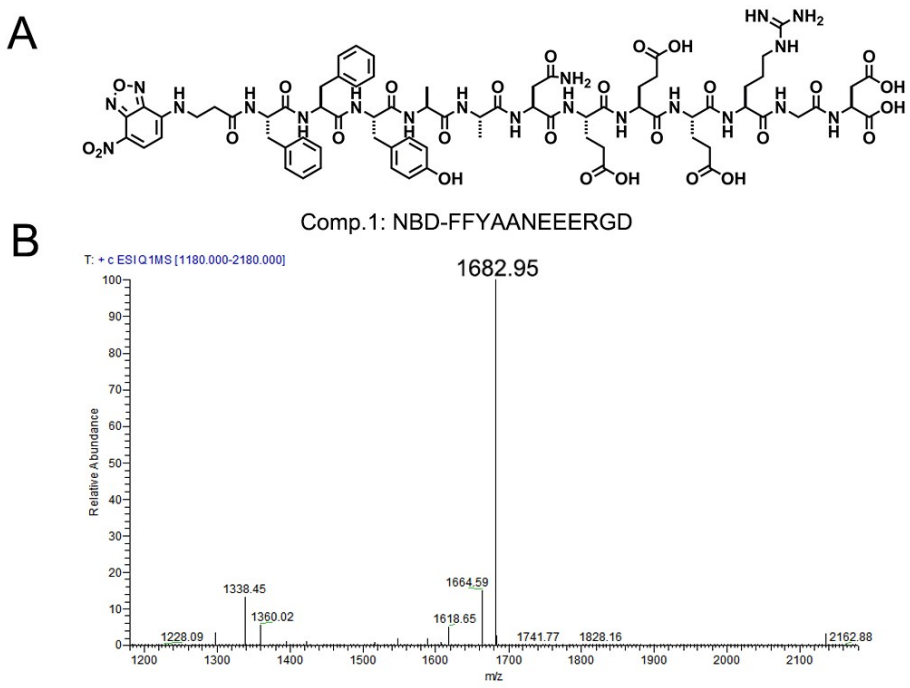


Fig. S1 The characterization of ***Comp.1*** by ESI-MS. Calc. M = 1681.65, obsvd. [M+H$^+$] = 1682.95.

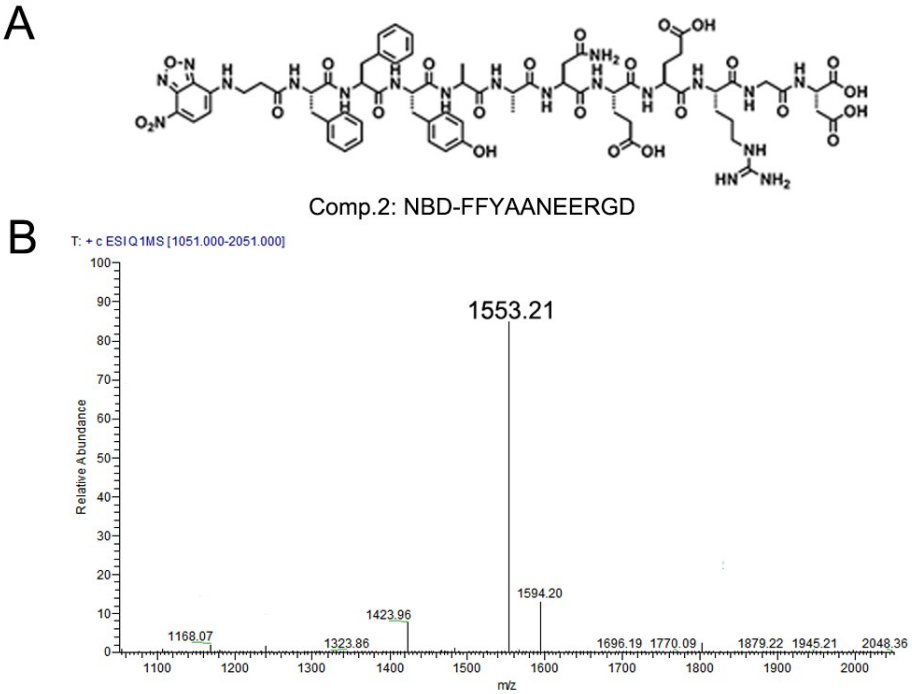


Fig. S2 The characterization of ***Comp.2*** by ESI-MS. Calc. M = 1552.54, obsvd. $[M+H^+]$ = 1553.21.

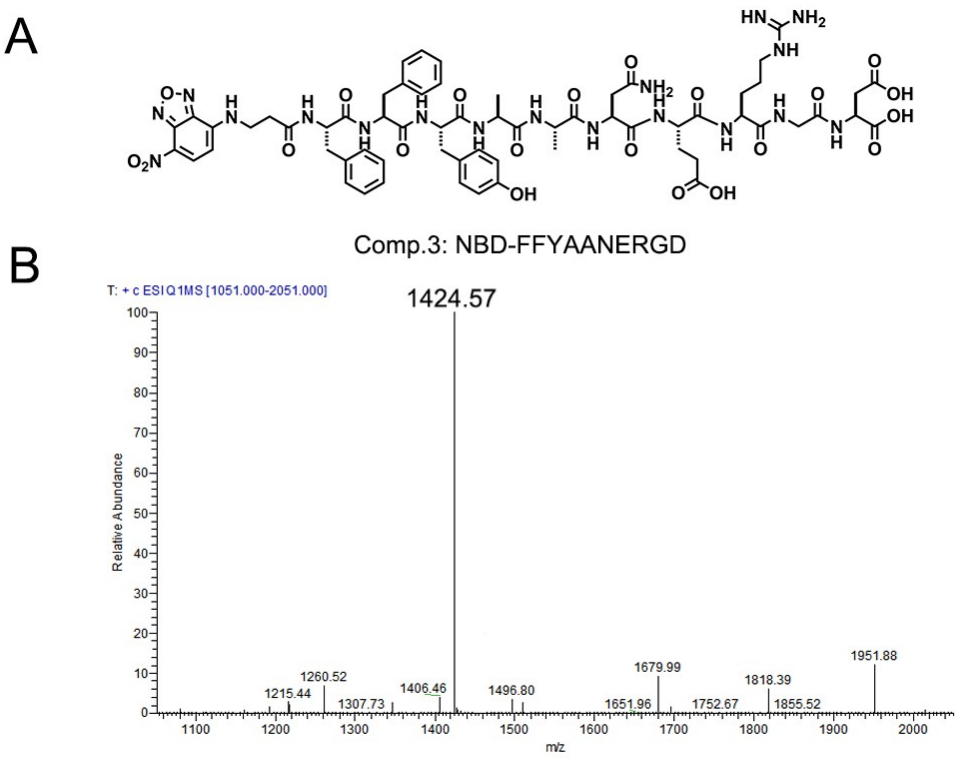
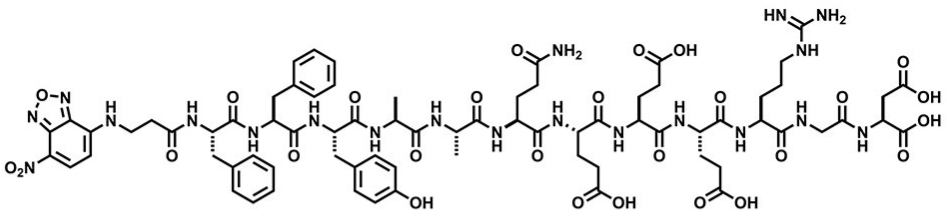


Fig. S3 The characterization of ***Comp.3*** by ESI-MS. Calc. M = 1423.42, obsvd. [M+H$^+$] = 1424.57.

A



Comp.4: NBD-FFYAAQEEERGD

B

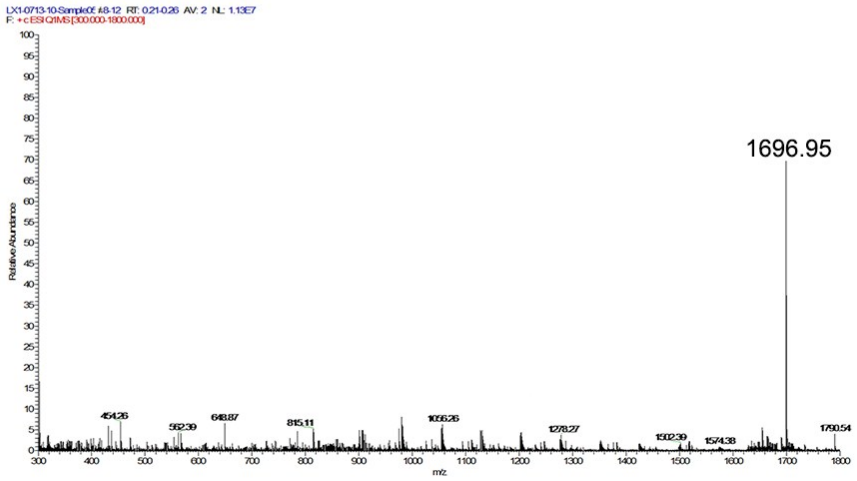


Fig. S4 The characterization of ***Comp.4*** by ESI-MS. Calc. M = 1695.68, obsvd. $[M+H^+]$ = 1696.95.

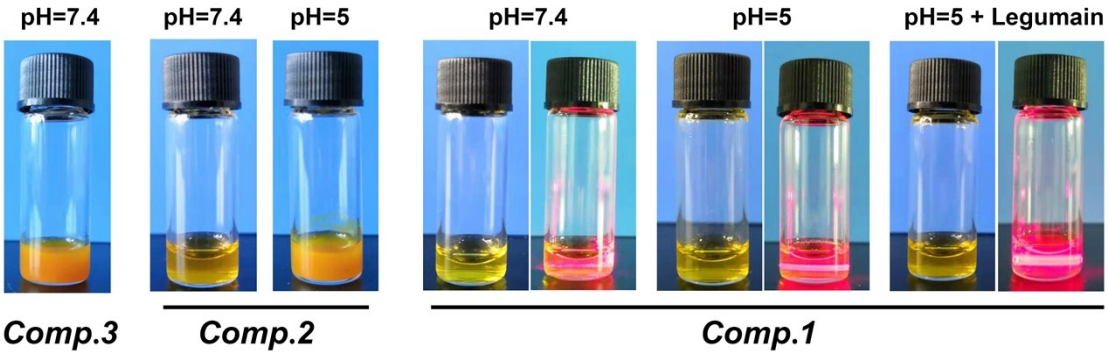


Fig. S5 Optical images of solutions of ***Comp.1***, ***Comp.2***, or ***Comp.3*** (0.1 wt. %) in different conditions.

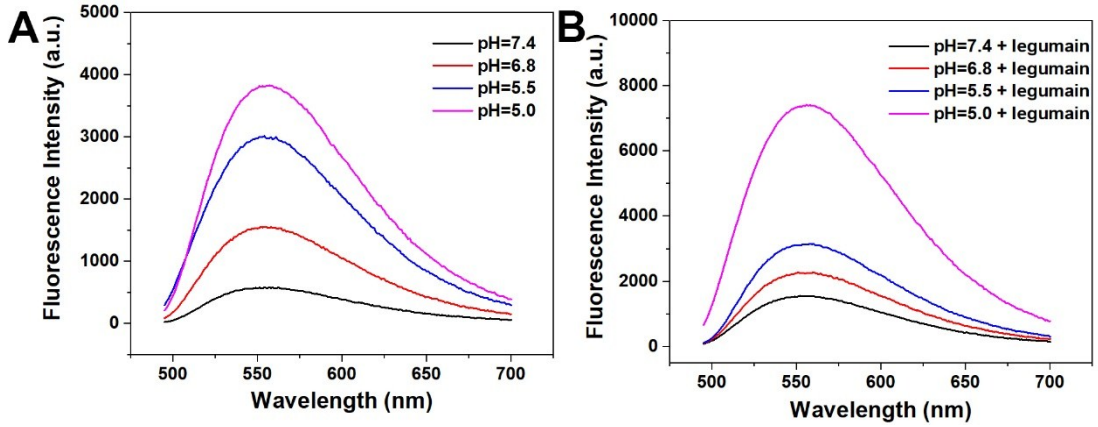


Fig. S6 Fluorescence intensity of ***Comp.1*** (0.1 wt. %) at different pH value (A) and then added legumain for 12 h (B).

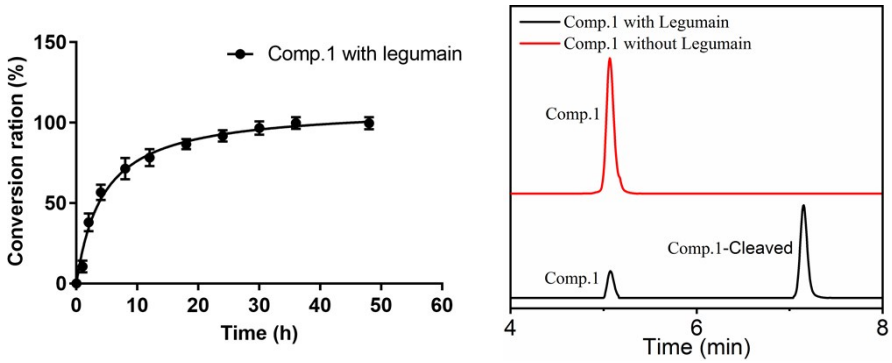


Fig. S7 Conversion ratio of molecular self-assembly by HPLC. The data are shown as mean ± SD (n = 6).

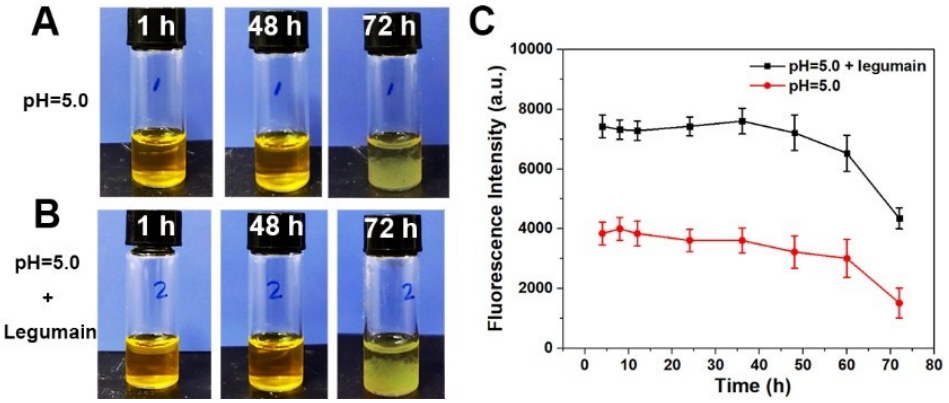


Fig. S8 Optical images of ***Comp.1***(0.1 wt.%) at pH=5 (A) and added legumain (B) for 1 h, 48 h, and 72 h. (C) Fluorescence intensity at 550 nm of ***Comp.1*** at different time points at pH=5 and after adding legumain (pH = 5, 1 ng μL^{-1}).

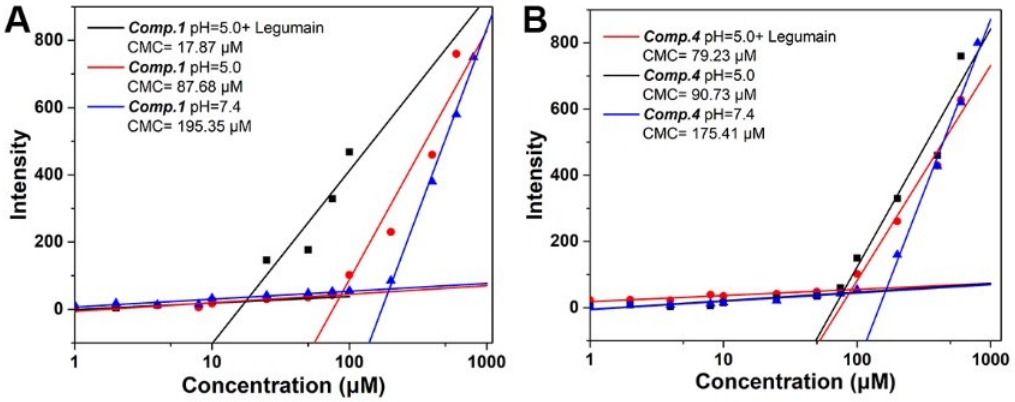


Fig. S9 The critical micelle concentrations of ***Comp.1*** and ***Comp.4*** at pH=7.4, pH=5 and pH=5 + legumain.

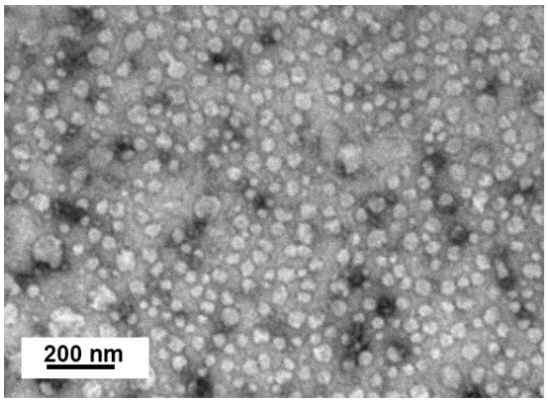


Fig. S10 TEM image of ***Comp.1*** (200 μM) at pH=7.4.

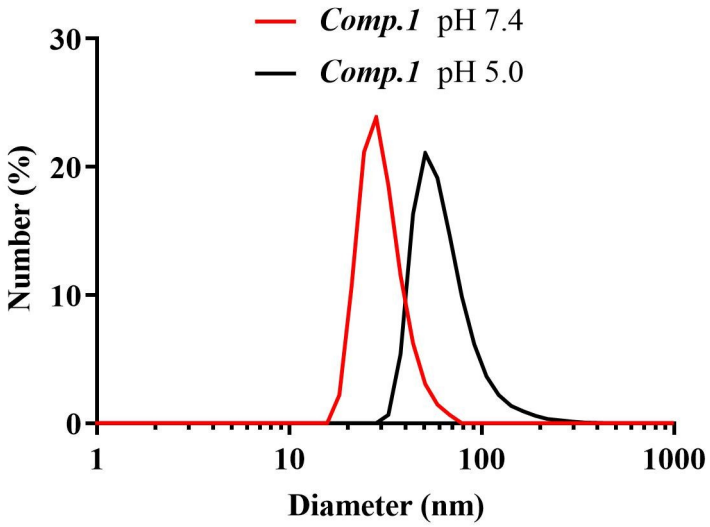


Fig. S11 Size distribution of ***Comp.1*** (200 μM) determined by DLS at pH=7.4 and pH=5.0.

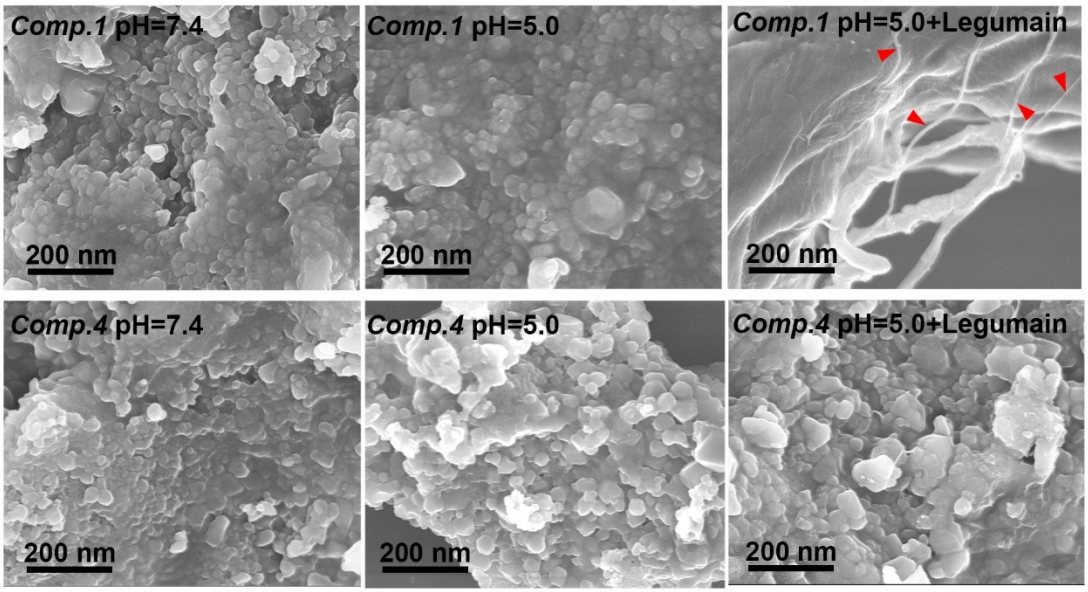


Fig. S12 SEM images of ***Comp.1*** and ***Comp.4*** (200 μM) at different conditions, red arrows represented the formation of nanofibers.

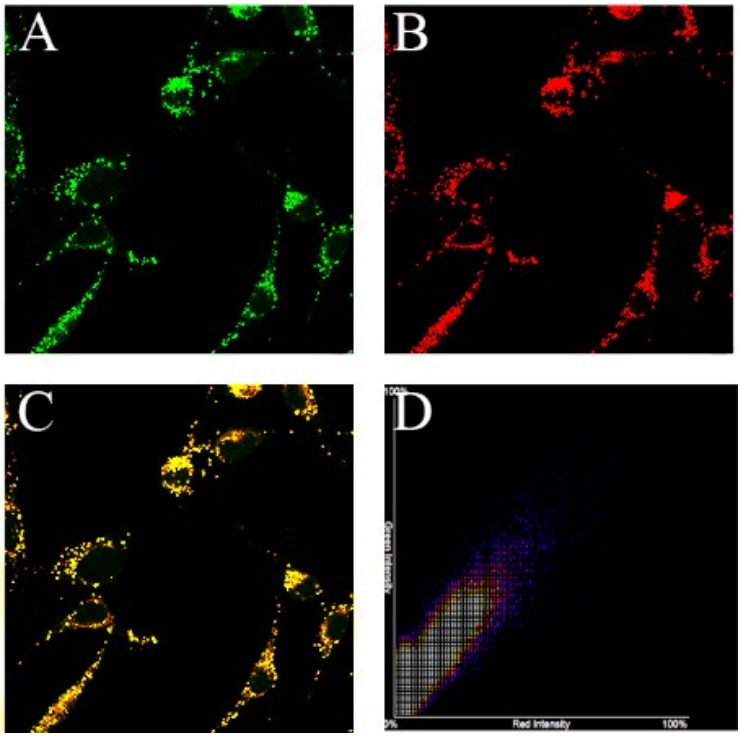


Fig. S13 Quantification of the overlap of green and red fluorescence signals in Fig. 2 (4 h + 0 h in C6 cells) by Pearson's and Mander's coefficients.

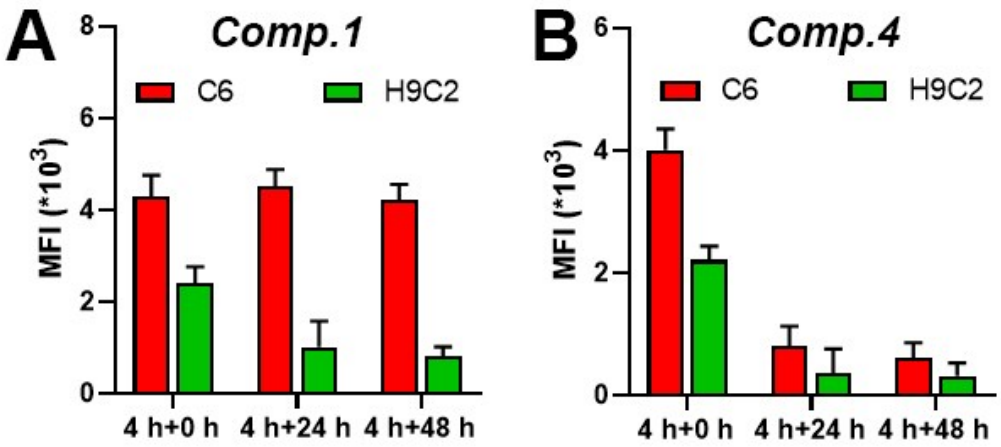


Fig. S14 Mean NBD fluorescence intensity of C6 cells and H9C2 cells after treatment with A) ***Comp.1*** (200 μM) and B) ***Comp.4*** (200 μM) for 4 h at preset time points were detected by flow cytometry. The data are shown as mean ± SD (n = 3), ns means no significant difference, p*** < 0.001, the statistical differences were analyzed by one-way ANOVA.

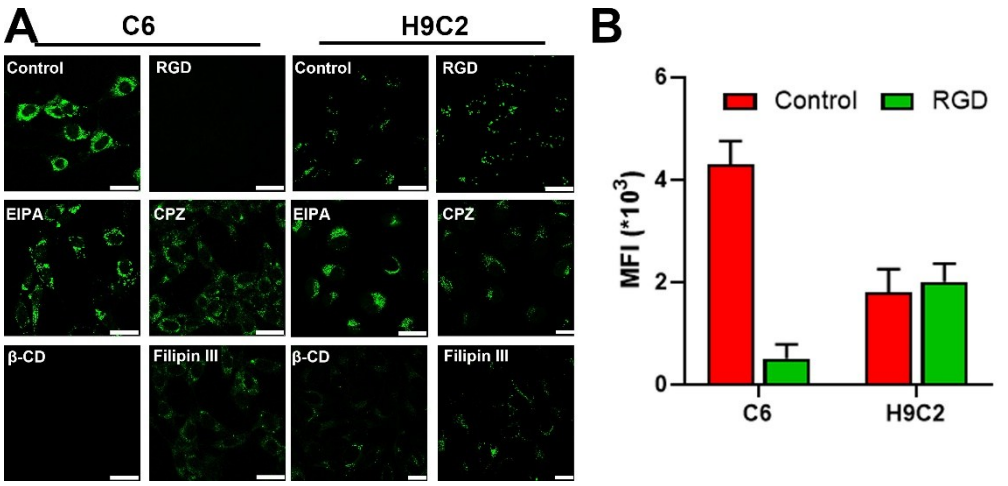


Fig. S15 Endocytosis mechanism of ***Comp.1*** (200 μM) in C6 cells and H9C2 cells. (A) Confocal images in the absence (control) or presence of the inhibitors EIPA (100 μM), CPZ (30 μM), β-CD (5 mM), Filipin III (5 μg/mL), and RGD (1 mM), Scale bar=25 μm. (B) Fluorescence intensity of ***Comp.1*** (200 μM) in C6 cells and H9C2 cells with or without RGD pretreatment by flow cytometry.

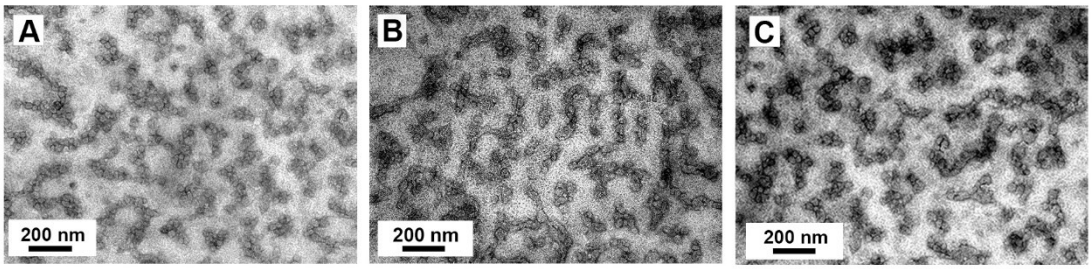


Fig. S16 TEM images of ***Comp.4*** (200 μM) at pH=7.4, pH = 5 and pH=5 + legumain (1 ng μL^{-1}).

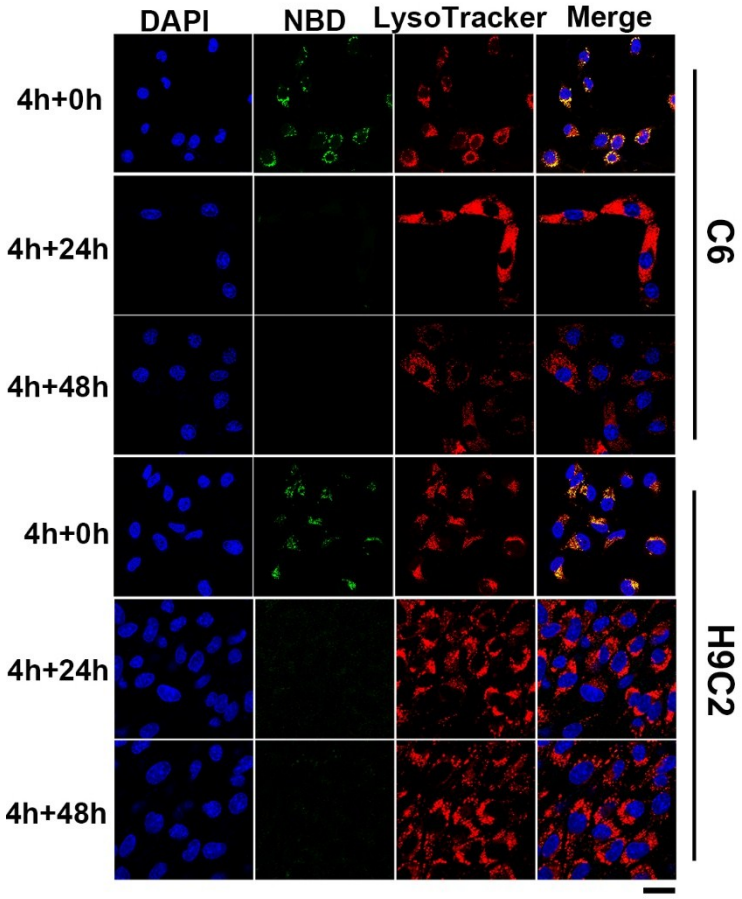


Fig. S17 Confocal images of C6 cells (tumor cell) and H9C2 cells (normal cell) treated with ***Comp.4*** (200 μM) for 4 h and further incubated for 0 h, 24 h, 48 h. Merge indicated the merge field of NBD (green, λexc. = 488 nm), DAPI (blue, λexc. = 405 nm) and Lyso-Tracker Red (red, λexc. = 543 nm). Scale bar: 20 μm.

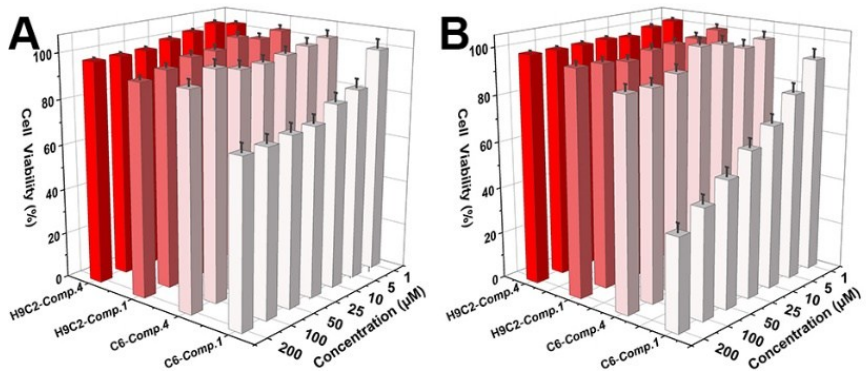


Fig. S18 The proliferation of C6 cells or H9C2 cells treated with ***Comp.1*** or ***Comp.4*** for 4 h firstly, then cells further cultured with fresh medium for A) 24 h; B) 48 h.

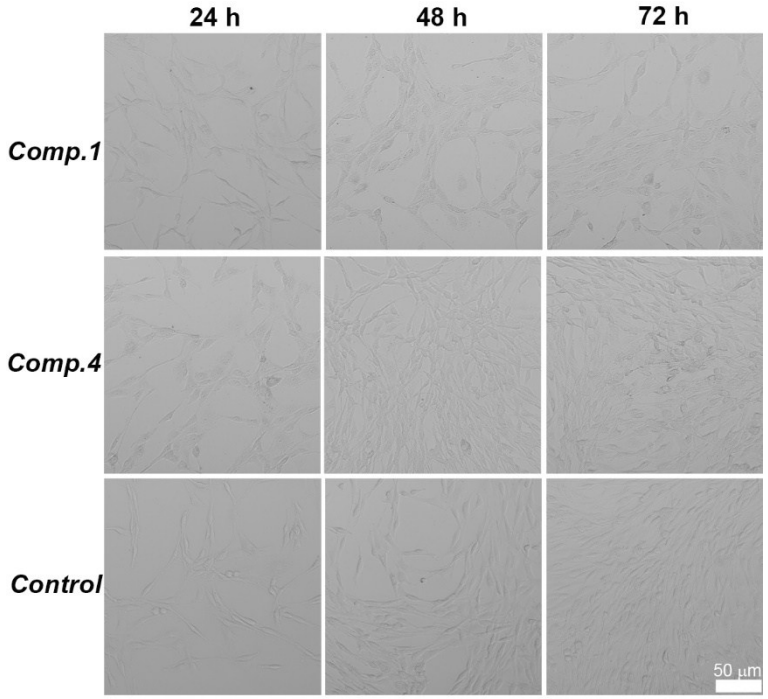


Fig. S19 Optical images of C6 Cells treated with ***Comp.1*** and ***Comp.4*** (200 μM) for 4 h firstly, then cells further cultured with fresh medium for 24 h, 48 h, and 72 h.

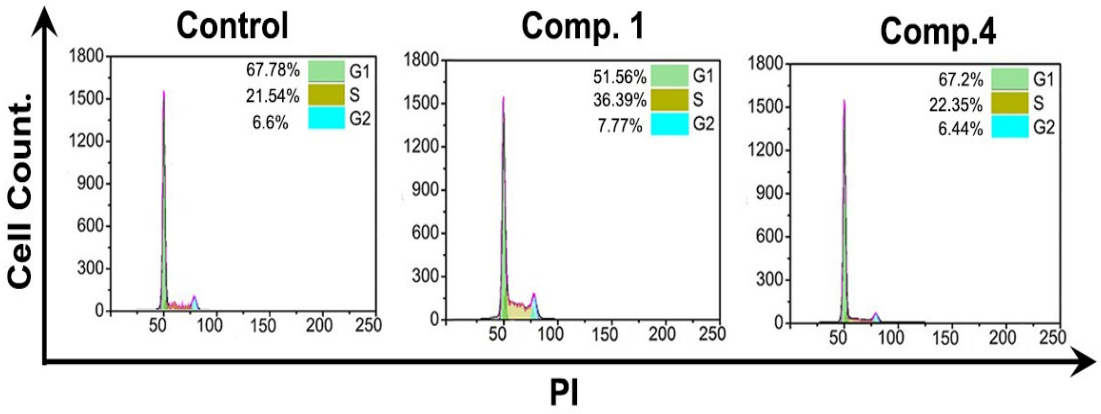


Fig. S20 Cell cycle analysis of C6 cells by flow cytometry in different groups at 72 h (200 μM).

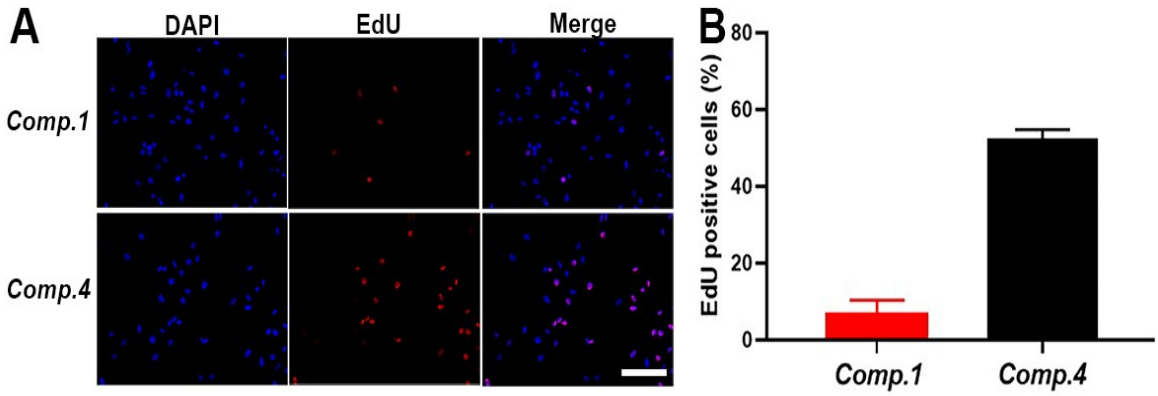


Fig. S21 (A) DNA replication determined by EdU staining of C6 cells treated with ***Comp.1*** and ***Comp.4*** (200 μM, Scale bar: 100 μm); (B) the EdU positive cell ratio quantified by Image J.

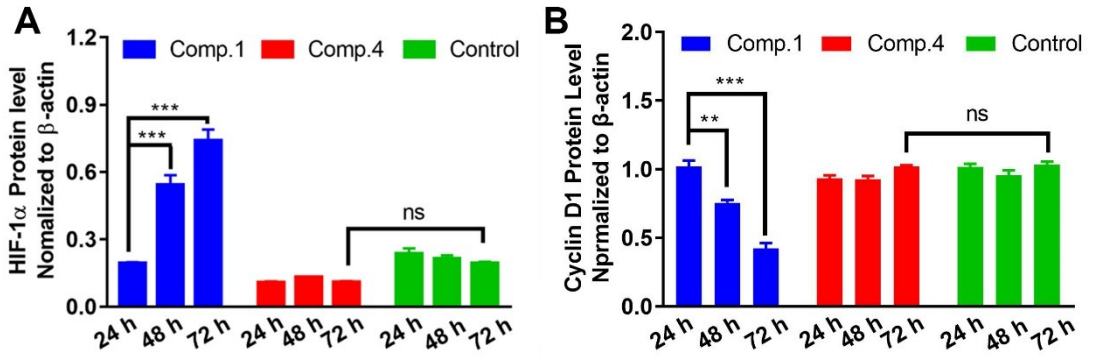


Fig. S22 Western blot analysis values of HIF-1α (A) and cyclin-D1(B) expression in C6 cells. The data were shown as mean ± SD (n = 3), ns means no significant difference, $p^{**} < 0.01$, $p^{***} < 0.001$, the statistical differences were analyzed by one-way analysis of variance (ANOVA).

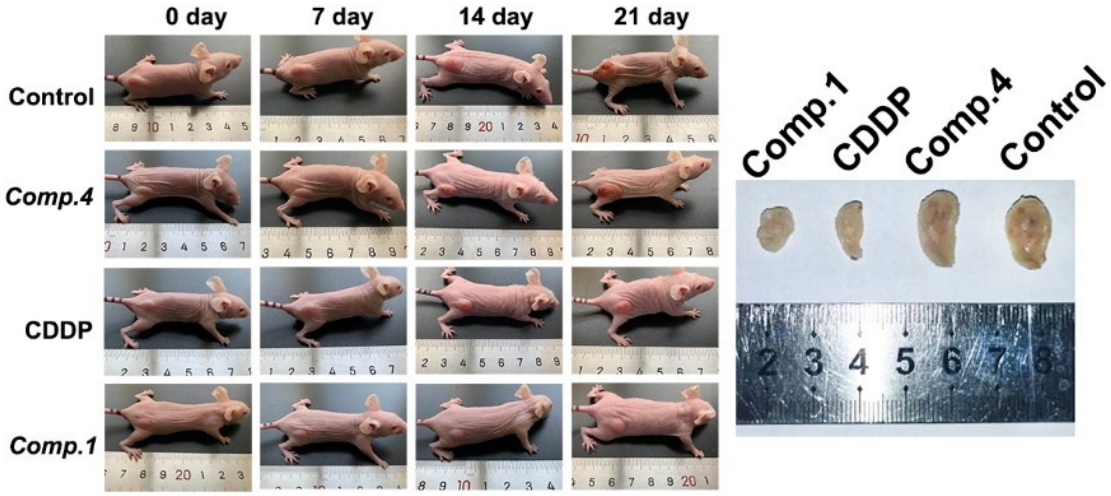


Fig. S23 Optical images of the change in tumors size *in vivo* and the shape of tumors *ex vivo*.