Electronic Supplementary Material (ESI) for ChemComm.

Supplementary Information

Tumour Enzyme Affinity Mediated Peptide Molecular Crowding for Targeted Disruption of Hyperactivated Glucose Uptake

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Materials and Methods

Materials

Fmoc protected amino acids and hhexafluorophosphate benzotriazole tetramethyl uranium (HBTU) were purchased from GL Biochem (Shanghai) Ltd. N,N-Diisopropylethylamine (DIPEA), Trifluoroacetic acid (TFA), 1-naphthylacetic acid, undecylamine and fluorogenic furin substrate were purchased from Sigma Aldrich. Furin enzyme was purchased from New England Biolabs. 2-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-2-deoxy-D-glucose (2-NBDG) was purchased from AAT Bioquest Inc. Dulbecco's Modified Eagle Medium (DMEM), Minimum Essential Media (MEM), foetal bovine serum (FBS), Antibiotic-Antimycotic, trypsin, propidium iodide, phosphate buffered saline (PBS) were purchased from Thermo Fisher Scientific. All the reagents indicated above were directly used without further purification.

Instruments

NMR spectrum was acquired with the JEOL ECA-400. Liquid chromatography mass spectrums were obtained with the Thermo Finnigan LCQ Fleet MS. Cell viabilities were evaluated with the Tecan Infinite m200 microplate reader. Confocal laser scanning microscope images were taken with the Carl Zeiss LSM 800 confocal laser microscope (Germany). Reverse-phase HPLC was performed with a Shimadzu HPLC system with an Alltima C-18 (250 x 10 mm) column at a flow rate of 3.0 mL / min. TEM images were obtained with the JEOL JEM 1400. Flow cytometry analysis was performed with the BD LSRFortessa X-20 flow cytometer.

1. Synthesis of furin peptide derivative

The compound was synthesized via a standard Fmoc based solid phase peptide synthesis (SPPS) on 2-chlorotrityl chloride resin. The coupling reaction of two amino acids lasted 2-4 hours at room temperature in the presence of HBTU as a coupling reagent and N, N-Diisopropylethylamine (DIPEA) as a basic catalyst respectively. The reaction was allowed to last for 4 hours for coupling with arginine and 2 hours for other amino acids. The deprotection of Fmoc group was performed with 20% piperidine in dimethylformamide (DMF) for 20 min. 1-naphthylacetic acid was coupled to the N-terminus of the peptide using the same SPPS protocol. The product was then cleaved from the resin using 2% trifluoroacetic acid TFA in dichloromethane (DCM) for 30 min and precipitated in cold diethyl ether. The crude peptide was then dissolved in DMF together with undecylamine (1.5 eqv), HBTU (1.5 eqv) and DIPEA (2 eqv) and stirred overnight. The final crude product was immediately purified with HPLC and characterized by MS (ESI): m/z: $[M+H]^+$ calculated: 1201.58; found: 601.77 ($[M+2H]^{2+}$) , 401.64 ($[M+3H]^{3+}$). Negative control peptides Nap-FF and Ac-RVRR-NH$(CH_2)_{10}CH_3$ were similarly synthesized via the above method.

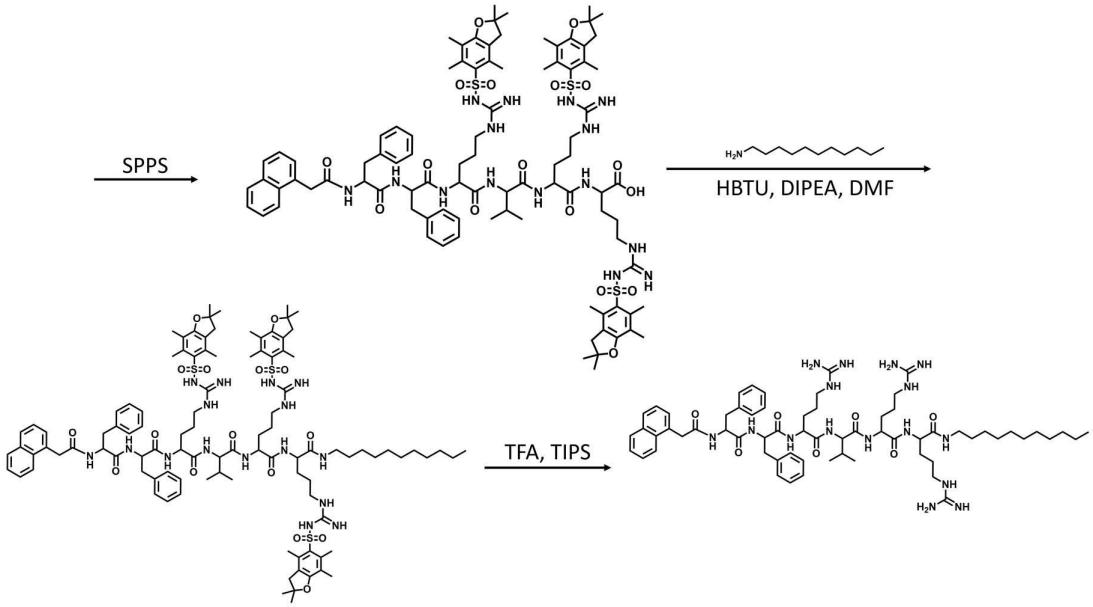

Fig.S1 Synthetic scheme of furin peptide derivative.

2. Transmission electron microscope (TEM) imaging

Furin peptide derivative (100μM) was added into furin working buffer (100 mM HEPES, pH 7.4, 0.5 % Triton X-100, 1 mM $CaCl_2$, and 1mM 2-mercaptoethanol). The sample was then dropped onto copper grids and left to air dry for 24h before TEM observation.

3. Stability Test

Furin peptide derivative (100μM) was incubated in buffer (PBS) or serum solution (FBS) for prolonged periods at 37°C. At different time points, the mixtures were re-suspended in MeOH and then centrifuged. The supernatants were analysed by HPLC showing no obvious degradation in PBS or serum after 48 hours, indicating sufficient stability for living cell experiments.

4. K_i Determination

In brief, pure furin enzyme was pre-incubated with varying concentrations of the test compound in furin working buffer for 30 min at 37°C. Thereafter, 50 mM of furin fluorogenic substrate (Boc-RVRR-AMC) was added with further incubation for 1hr at 37 °C. The fluorescence emission of the solutions was analysed and used to compute the K_i value. All assays were performed in triplicate.

5. General methods for cell culture

Human colon carcinoma, HCT116 was cultured in DMEM supplemented with 10% FBS at 5% CO_2 and 37°C. Human normal colon fibroblast, CCD-18Co was cultured in MEM supplemented with 10% FBS at 5% CO_2 and 37°C. Cell lines were obtained from ATCC.

6. Western blot analysis of furin expression

Cell lysates were collected and centrifuged for 30 min at 4 °C. Protein levels in the supernatants were determined using Nanodrop and normalized to the same concentration. The supernatants were then boiled for 10 min with SDS-PAGE sample loading buffer prior to SDS-PAGE separation and transfer to a PVDF membrane. The membrane was then blocked with 1% BSA-TBST blocking buffer for 2hr at room temperature. Subsequently, anti-furin antibody (1:500) and anti-GAPDH (1:10000) were incubated at 4 °C overnight in 1% BSA−TBST buffer. After washing, goat-anti rabbit IGG (H&L) secondary antibody was added and incubated for 1 h in 1% BSA−TBST. All signals were developed using the Super Signal West Femto kit, visualized with the myECL imager.

7. Immunostaining of GLUT1 expression

Cells were first collected and resuspended in 1% BSA−PBS blocking buffer. Subsequently, the cell suspensions were then treated with anti-GLUT1 antibody (1:500) and incubated on ice for 30 min. After several washes, fluorescent (AlexaFluor488) secondary antibodies were added and incubated for 30 min on ice in the dark. The cells were then collected and resuspended in 1% BSA−PBS, stored on ice and analysed by flow cytometry within 1 hr. Experiments were performed in triplicate and the results were plotted as an average of the median relative fluorescence of AlexaFluor488.

8. Live cell imaging

Cells were seeded at 2 x 10^5 cells/well in 35- mm diameter µ-dish plastic-bottom (ibidi GmbH, Germany) in complete medium for 24hr at 5% CO_2 and 37 °C. The cells were then incubated with 100 µM of test compound for 3hr. Subsequently, excess compound was removed and the cells were washed with PBS 3 times prior to incubation with Congo red (0.1mg/mL) for 30min. Excess Congo Red was removed and cells were washed again with PBS 3 times prior to membrane staining with CellMask Deep Red (2.5µg/mL) for 10min. Live cell imaging was performed immediately.

9. 2-NBDG uptake assays

Cells were seeded in 6 well plates at 2 x 10^5 cells/well for 24hr at 5% CO_2 and 37 °C prior to incubation with 100 µM of test compound for 3hr. Excess compound was removed, and the cells were washed with PBS 3 times before further incubation in glucose free medium for 30min. The medium was subsequently replaced with 100µM of 2-NBDG diluted in glucose free medium and incubated for a final 30min. The 2-NBDG uptake reaction was terminated by removal of the medium and cells were subsequently washed with PBS 3 times. Live cell imaging was performed immediately for confocal samples, while EDTA was used to detach the cells for flow cytometry analysis. Flow cytometry cell samples were resuspended in 1µg/ml propidium iodide solution to exclude dead cells, and were immediately analysed with data from 10000 single cell events collected for each measurement.

10. Cell viability assay

Cells were seeded in 96 well plates at 2 x 10^4 cell/well for 24hr at 5% CO_2 and 37 °C. The culture medium was then replaced with medium containing varying concentrations (0, 25, 50, 100, 200 µM) of test compound for 3hr. Cells were then washed with PBS 3 times prior to further incubation in fresh medium for another 48hr. Thereafter, 10 µL of 3mg/ml TOX8 was added to each well, and the plate was incubated in the dark for another 4hr. Fluorescence at 590 nm was measured by a microplate reader with 560 nm excitation. All experiments were conducted in triplicate and viabilities were expressed in a percentage ratio of treated cells to that of the untreated control.

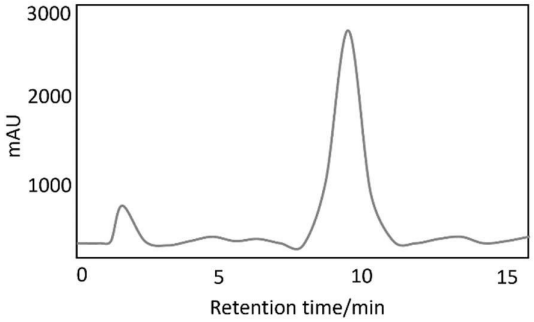

Fig.S2 HPLC analysis of the synthesized furin peptide derivative.

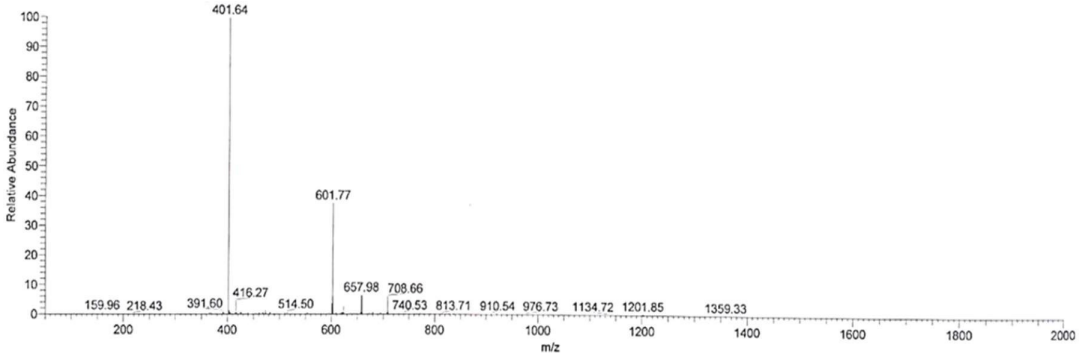

Fig.S3 LC-MS analysis of the synthesized furin peptide derivative

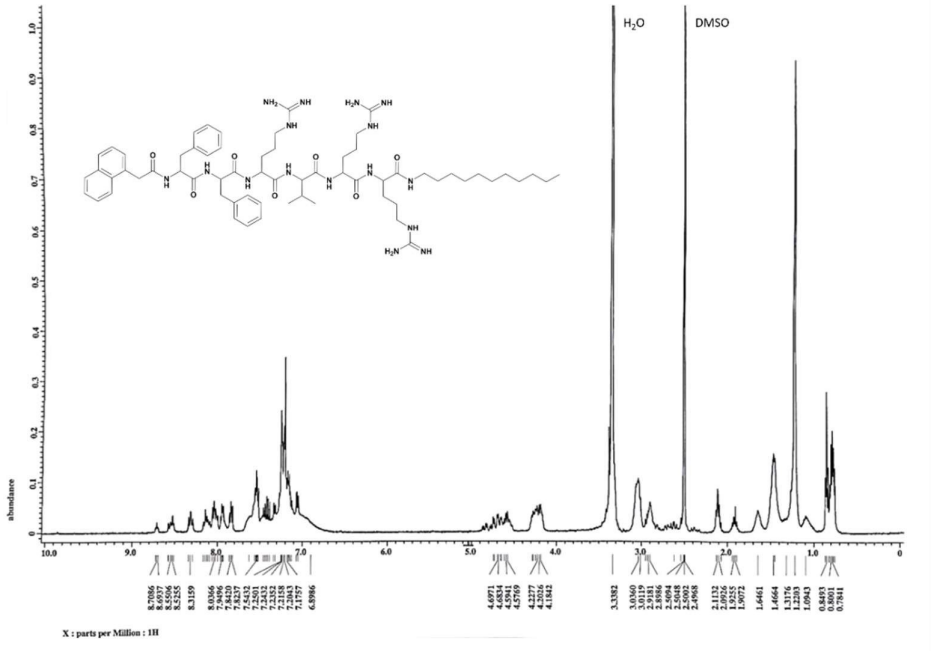

Fig.S4 ^{1}H NMR spectrum of the synthesized furin inhibitor derivative (400MHz, DMSO-d_6)

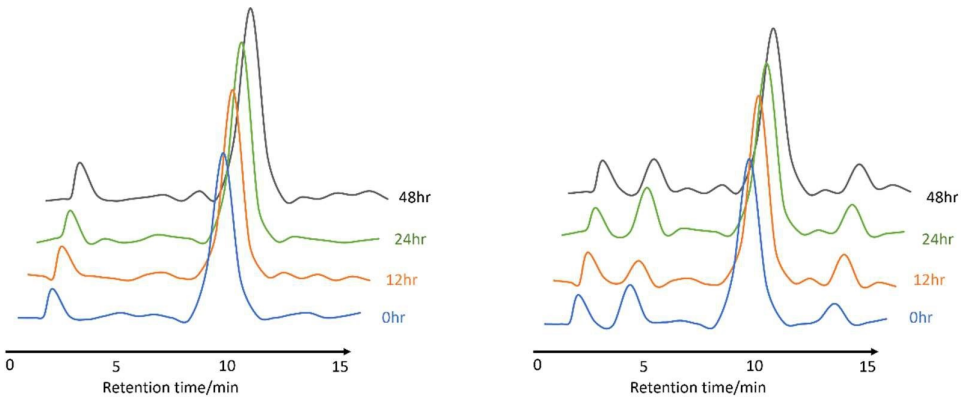

Fig.S5 HPLC analysis of furin peptide inhibitor derivative upon prolonged incubation in buffer (left) and serum (right) at different time points.

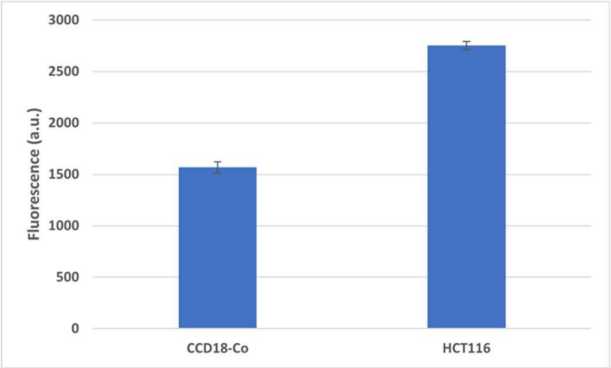

Fig.S6 Analysis of GLUT1 expression in HCT116 and CCD18-Co. Data was plotted as an average of the median fluorescence values obtained via flow cytometry.

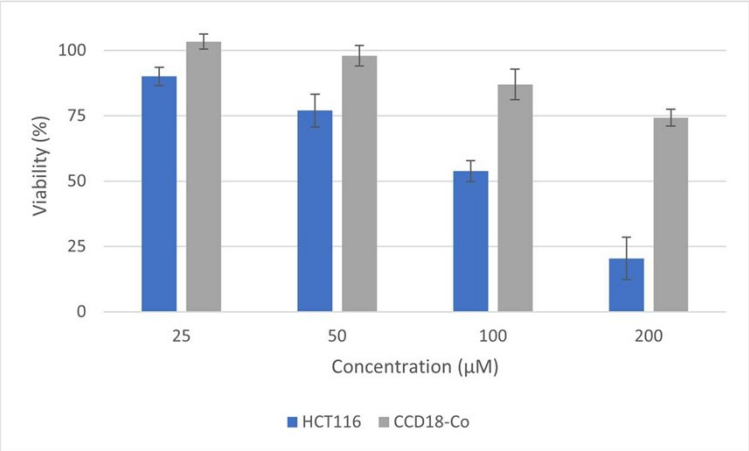

Fig.S7 48hr cell viability of HCT116 and CCD18-Co cells treated with various concentrations of furin peptide derivative relative to untreated control.

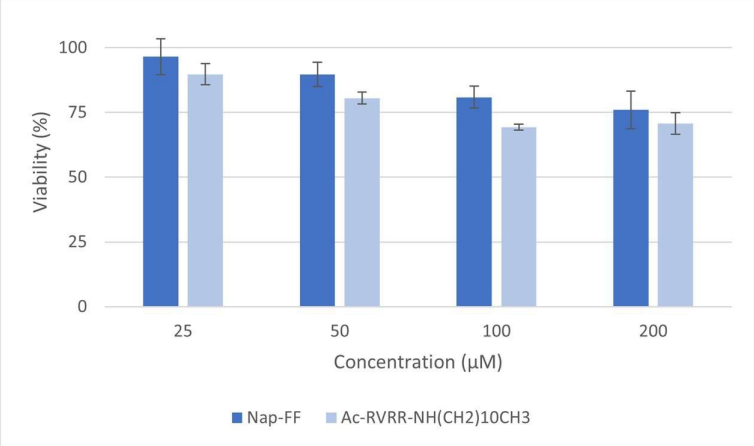

Fig.S8 48hr cell viability of HCT116 cells treated with various concentrations of negative control peptides Nap-FF and Ac-RVRR-NH$(CH_2)_{10}CH_3$ relative to untreated control.

Substrate	K_i
Furin inhibitor derivative	3.0 ± 0.1 μM
Ac-RVRR-NH$(CH_2)_{10}CH_3$	3.7 ± 0.2 μM
Nap-FF	0.11 ± 0.02 mM

Fig.S9 Tabulated K_i values of the corresponding peptide substrate

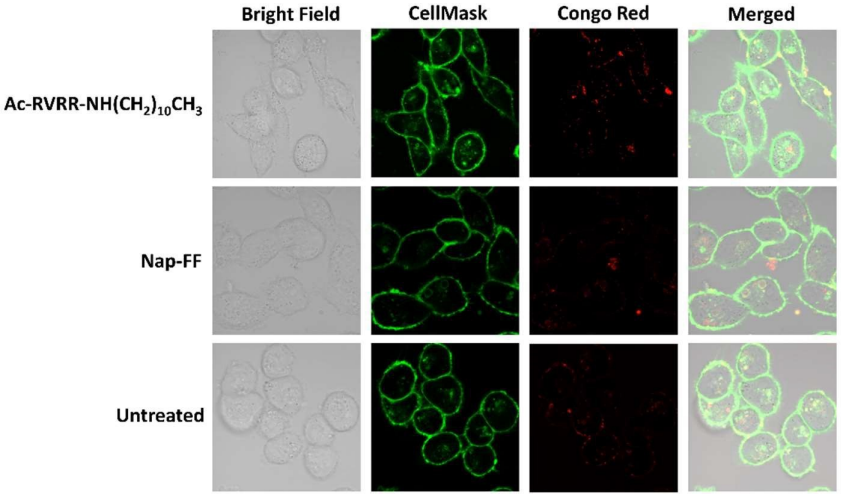

Fig.S10 Confocal images of HCT116 cells treated with negative control peptides (100μM) or solvent (untreated), subsequently stained with Congo red (0.1mg/mL) and CellMask membrane tracker (2.5μg/mL), scale bar: 20μM. Congo red (λex=488nm, λem=614/50nm), Cell Mask (λ_{ex}=640nm, λ_{em}=670/50nm)

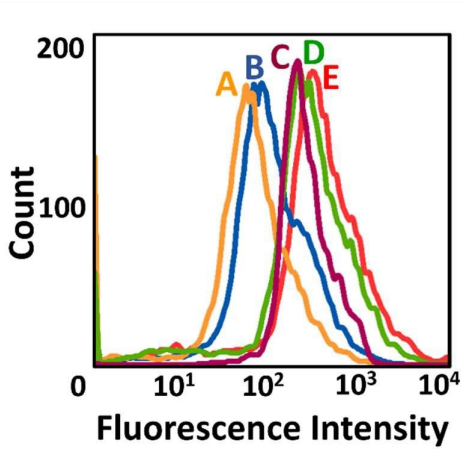

Fig.S11 Flow cytometric analyses of 2-NBDG fluorescence upon corresponding treatments in HCT 116 cells. 2-NBDG (λ_{ex}=488nm, λ_{em}=540/50nm). (A: Phloretin, B: Furin Peptide Derivative, C: Ac-RVRR-NH$(CH_2)_{10}CH_3$, D: Nap-FF, E: Untreated)