Electronic Supplementary Information

**Furin substrate as a novel cell-penetrating peptide: Combining delivery vector and inducer of cargo release**

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1. Experimental section

1.1 Materials

RVRR, (RVRR)_3, (RVRR)_2-KLAKLAKLAKLAK and (RVRR)_3-KLAKLAKLAKLAK were customized from China Peptides Co., Ltd. (Hefei, China). Fluorescein isothiocyanate-(FITC) modified peptides such as FITC-RVRR, FITC-R_6, FITC-(RVRR)_9, FITC-(RVRR)_3, FITC-KLAKLAKLAKLAK, FITC-(RVRR)_2-KLAKLAKLAKLAK and FITC-(RVRR)_3-KLAKLAKLAKLAK were customized by Guo Ping Co., Ltd. (Hefei, China). Fmoc-Arg(Pbf)-OH, Fmoc-Val-OH were purchased from GL Biochem (Shanghai, China) Ltd., Roswell Park Memorial Institute (RPMI-1640), Dulbecco's Modified Eagle Medium (DMEM) and Fetal Bovine Serum (FBS). Antibiotics (Penicillin-Streptomycin) were purchased from Macgene Biotech Co., Ltd. (Beijing, China). Golgi-Tracker Red, caspase-3 activity kit were purchased from Beyotime Biotechnology (Shanghai, China) and Lysosome tracker deep were purchased from Thermo Fisher Scientific (Shanghai, China).

1.2 Synthesis of FITC labeled peptides

All peptides were prepared using standard solid-phase fluorenylmethoxycarbonyl (Fmoc) chemistry with 1-Hydroxybenzotriazole (HOBT) and O-Benzotriazole-N,N,N',N'-tetramethyl-uronium-hexafluorophosphate (HBTU) as the peptide coupling reagents. The fluorescein moiety FITC was attached by an aminohexanoic acid spacer by treating a resin-bound peptide (0.5 mmol) with FITC (1.0 mmol) and diisopropyl ethyl amine DIEA (5 mmol) in DMF (6 mL) for overnight. Cleavage from the resin was achieved by using 90:10 TFA/CH_2Cl_2 and stirred the solution for 3 hours at room temperature. Removal of the solvent and precipitated with cold ether. The crude was centrifuged, the ether was removed, and the resulting orange solid was purified by HPLC (H_2O/CH_3CN in 0.1% TFA). The products were characterized by ESI-mass. The purity of the peptides was >95% as determined by analytical RP-HPLC (H_2O/CH_3CN in 0.1% TFA). The HPLC purification methods of each peptides and the HPLC spectrum are listed in supporting figure.

1.3 Cell culture

The MDA-MB-231 (human breast cancer cells), LoVo (human colorectal cancer cells), C_2C_12 (myoblast cells) were purchased from the Procell Life Science & Technology Co., Ltd. (Wuhan, China). LoVo and C_2C_12 were cultured in RPMI-1640 supplemented with 10% (v/v) (FBS). MDA-MB-231 cell were cultured in DMEM medium supplemented with 10% (v/v) fetal bovine serum (FBS).

1.3.1 Flow cytometry analysis

Flow cytometry analysis was used to evaluate the cellular uptake ability of CPPs using MDA-MB-231, LoVo and C_2C_12. Three cell lines were plated at 500,000 cells/well in 6-well plates and incubated at 37°C for overnight until completely adherence. Cells were washed twice with serum-free DMEM prior to treatment. FITC labeled peptides were diluted from 1 mM stock solutions in PBS to 10 μM solution in
serum-free DMEM. 2mL of diluted solution was added to 6-well plate. Cells were incubated at 37°C for designed time. After incubation, the media was removed and washed twice with PBS. 1 mL of Trypsin with EDTA was added and incubated for 3 min. The solution of each well was transferred to 1.5 mL EP tube and centrifuged at 1000 rpm for 5 min. After removal of the supernatant, cells were re-suspended in 500 μL of PBS and transferred to FACS tubes. The fluorescence intensity of each well was analyzed on a flow cytometry analyzer. Results were analyzed by FCS express 6 Reader software. Data presented is the mean fluorescence intensity from 10,000 cells analyzed. The excitation wavelength was 488nm. The detection wavelength was 525 nm.

1.3.2 Fluorescence cellular imaging

Confocal laser scanning microscopy (CLSM) analysis was used to evaluate the cellular uptake and distribution of fluorescein isothiocyanate (FITC) labeled peptides. In general, cells were cultured in 35-mm dishes covered with three coverslips and incubated at 37°C overnight until totally adherent. The cells were incubated with FITC labeled peptide (10 μM) and diluted with serum-free medium at 37°C. After incubation, the cells were washed with PBS twice and analyzed using CLSM (with a FV1000 Olympus instrument). The excitation wavelength was 488 nm and the detection region from 495 to 545 nm. Intracellular localization was investigated using Golgi-Tracker Red (Beyotime) and Lysosome-Tracker Red (Thermo-Fisher Scientific) according to their instruction books. The excitation wavelength of Golgi-Tracker Red is 543 nm and its detection wavelength from 595 to 645 nm.

1.3.3 In vitro assay for cell viability

The MDA-MB-231 cells were cultured in DMEM supplemented with 10% serum in 96-well plates. The concentration of (RVRR)_2, (RVRR)_3, KLA, (RVRR)_2-KLA, and (RVRR)_3-KLA, R_6, R_6-KLA, R_9, R_9-KLA, chlorambucil (CHL), CHL-RV3 ranged appropriately, and were diluted with serum-free DMEM and incubated for 24 h at 37°C. Subsequently, the incubating solutions were removed, and 100 μL of MTT (0.5 mg/mL) diluted with serum-free DMEM was added into each well. After 4 h of incubation, the MTT solution was aspirated, and the formazan crystals were dissolved using 100 μL of DMSO. The 96-well plate was shaken for 5 min. Absorbance was detected at 490 nm by a micro-plate reader.

1.3.4 Furin incubation and HPLC analysis

FITC-RV3-KLA (50 μM) was incubated with pure furin enzyme (2 units) for overnight at 37°C in 0.1 mL furin buffer. The assay buffer contains 100 mM HEPES, pH 7.4, 0.5 % Triton X-100, 1 mM CaCl_2, and 1 mM GSH. After incubation, HPLC analysis of products and enzyme hydrolysis is a linear 25 min gradient from 5% to 75% acetonitrile in water with 0.1% TFA.

1.3.5 Caspase-3 Assay

Caspase-3 activity was measured by following the manufacturer’s instructions. Briefly, MDA-MB-231 cells were incubated with 10 μM (RVRR)_3-KLA for 4 and 8 h or with 20 μM (RVRR)_3-KLA for 4 and 8 h. The cells were then collected and washed with PBS. The cells were lysed using lysis buffer, after which the lysates were centrifuged at 12,000 g at 4°C for 15 min. For caspase-3 activity assay, cell lysate (50 μL) and caspase-3 detecting buffer (40 μL) were incubated with 10 μL of caspase-3
substrate (2 mM Ac-DEVD-pNA) in a total volume of 100 µL at 37°C for 2 h. The colorimetric release of p-nitroaniline from the Ac-DEVD-pNA substrate was recorded at a wavelength of 405 nm.

1.4 In vivo therapeutic efficacy and systemic toxicity studies

Animal procedures were in agreement with the guidelines of the Institutional Animal Care and Use Committee. Female nude mice C57 BL were subcutaneously injected with $4 \times 10^6$ MDA-MB-231 cells into the right armpit. When the average tumor volume reached approximately 150 mm$^3$, the mice were randomly divided into four groups (3 mice per group). Each mice was administered with 25 µL of PBS, KLA, RV3-KLA and R9-KLA intratumorally at a concentration of 2 mM every other day. Tumor volumes and body weights were measured every other day. Tumor volumes were calculated using the following formula: volume = (length × width$^2$)/2. After observation for 16 days, the mice were taken photos first and then sacrificed to excise the tumors. The tumors were weighed, measured and taken photo.
2. Additional figures and tables

Scheme S1 Chemical structure of chlorambucil (CHL)

Fig. S1 Flow cytometry curves of MDA-MB-231 cells after incubated with FITC-RV3 at 37°C for 5- or 10 min.
**Fig. S2** CLSM images of MDA-MB-231 cells incubated with 10 μM FITC-RV2 at 37 °C for 1 hour, 5 hours and 20 hours. Green channel: $\lambda_{ex} = 488$ nm, $\lambda_{em} = 495 - 545$ nm. scale bar = 20 μm.

**Fig. S3** Circular Dichroism (CD) spectra of R9 and RV3 (20 μM) in PBS buffer.
Fig. S4 HPLC traces of FITC-RV3-KLA (lower), FITC-RV3-KLA with furin (middle) and FITC-RV3-KLA incubated with the inhibitor before addition of furin for 1 h (upper).

Fig. S5 ESI-Mass of HPLC peaks at 13.6 minutes (a) and 15.2 minutes (b).
**Fig. S6** Cell viability of R9-KLA and R9 against MDA-MB-231 cells after 24 hour incubation. Data represent the mean ± SD (n = 6).

**Fig. S7** Cell viability of RV2-KLA, RV2 and KLA against MDA-MB-231 cells after 24 hours incubation. Data represent the mean ± SD (n = 6).
**Fig. S8** Cell viability of R6-KLA, R6 against MDA-MB-231 cells after 24 hours incubation. Data represent the mean ± SD (n = 6).

**Fig. S9** CLSM images of MDA-MB-231 cells incubated with 10 μM FITC-KLA at 37 °C for 2 hours and 4 hours. Green channel: $\lambda_{ex} = 488$ nm, $\lambda_{em} = 495 - 545$ nm. Scale bar = 20 μm.
**Fig. S10** (a) Standard line of detecting caspase-3 activity. (b) The images of samples from left to right: control, MDA-MB-231 cell lysate incubate with 0 µM, 10 µM RV3-KLA for 4 h, 10 µM RV3-KLA for 8 h, 20 µM RV3-KLA for 4 h, 20 µM RV3-KLA for 8 h.

**Table S1.** The absorption values of samples in Figure S5b at 405 nm.

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Fig. S11 CLSM images of MDA-MB-231 cells co-incubated with 10 μM FITC-RV3-KLA and Golgi Tracker (33 μg/ml) for 0.5-, 1-, 3-, 5 h at 37°C. Green channel: $\lambda_{ex}=488$ nm, $\lambda_{em}=495-545$ nm. Red channel: $\lambda_{ex}=543$ nm, $\lambda_{em}=595-645$ nm. Scale bar=20 μm.

Fig. S12 CLSM images of MDA-MB-231 cells co-incubated with 10 μM FITC-RV3-KLA and Mito-Tracker for 3 hours at 37 °C. Green channel: $\lambda_{ex} = 488$ nm, $\lambda_{em} = 495 - 545$ nm. Red channel: $\lambda_{ex} = 543$ nm, $\lambda_{em} = 620 - 670$ nm. scale bar = 20 μm.
Fig. S13 CLSM images of MDA-MB-231 cells co-incubated with 10 μM FITC-RV3-KLA and Lyso-Tracker for 3 hours at 37 °C. Green channel: \( \lambda_{\text{ex}} = 488 \) nm, \( \lambda_{\text{em}} = 495 \) - 545 nm. Red channel: \( \lambda_{\text{ex}} = 543 \) nm, \( \lambda_{\text{em}} = 575 \) - 625 nm. scale bar = 20 μm.

Fig. S14 The original body weight of different groups
Fig. S15 Photographs of different groups showing the size of tumor during the treatment with PBS, KLA and RV3-KLA and R9-KLA.

Fig. S16 Body weight changes of mice during treatment.
Fig. S17 HPLC trace of RVRRRVRR.
Fig. S18 ESI-Mass spectrometry of RVRRVRR.
Fig. S19 HPLC trace of FITC-RVERRRRKLAKKLAKLAKLAK.
Fig. S20 The ESI-Mass spectrometry of FITC-RVRRRVRRKLAKLAKKLAKLAKLAK.
Sample Information

Order ID : Syn-66238
Name : SYNS611-1
Sequence : FITC-RVRRRVRVRRVRRKAKLAKKLAKLAKLAK
Lot No. : JT-66238
Pump A : 0.1% Trifluoroacetic in 100% Water
Pump B : 0.1% Trifluoroacetic in 100% Acetonitrile
Total Flow : 1ml/min
Wavelength : 214nm
Analytical column type : SHIMADZU Inertsil ODS-SP (4.6*250mm*5um)
Dissolution method : 15%ACN+85%H2O
Inj. Volume : 20ul

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Address: 320 Lvlin Road, Biceai, Pudong new area, Shanghai, China 201204
Tel: +86-21-50630980  Fax: +86-21-50630980  FAX: 201204  http://www.synpeptide.com

Fig. S21 HPLC trace of FITC-RVRRRVRVRRVRRKAKLAKKLAKLAKLAK.
Fig. S22 ESI-Mass spectrometry of FITC-RVERRRVRVRKLA. KLAKLAKLAK.
Fig. S23 HPLC trace of FITC-RRRRRKLAKLAKKLAKLAK.
Fig. S24 ESI-mass of FITC-RRRRRRKLAKLAKKLAKLAKLAK.
Sample Information

Order ID: Syn-78823
Name: SYNS868-2
Sequence: FITC-ACP-RRRRRRRRRKLAKLAK
Lot No: JT-78823
Pump A: 0.1% Trifluoroacetic in 100% Water
Pump B: 0.1% Trifluoroacetic in 100% Acetonitrile
Total Flow: 1 ml/min
Wavelength: 220 nm
Analytical column type: SHIMADZU Inertsil ODS-SP (4.6*250mm*5um)
Inj. Volume: 30 ul

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Fig. S25 HPLC trace of FITC-RRRRRRRRRKLAKLAKLAKLAK.
Fig. S26 ESI-Mass spectrometry of FITC-RRRRRRRRKLAKLAKKLAKLKAKLAK.
Fig. S27 HPLC trace of RVRRRVRRVRRGVGGKLALAKKLAKLAK.
**Fig. S28** ESI-Mass spectrometry of RVRRVRRRRVGGKLAKKLAKKLAK.
Fig. S29 HPLC trace of RRRRRRRRKLAKLAKKLAKLAK.
**Fig. S30** ESI-Mass spectrometry of RRRRRRRRKLAKLAKKLAKLAK.
Fig. S31 HPLC trace of KLAKLAKKLAKLAK.
Fig. S32 ESI-mass spectrometry of KLAKLAKKLAKLAK.