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, $(RVRR)_3$, $(RVRR)_3$, $(RVRR)_2$-KLAKLAKKLAKLAK and $(RVRR)_3$-KLAKLAKKLAKLAK were customized from China Peptides Co., Ltd. (Hefei, China). Fluorescein isothiocyanate-(FITC) modified peptides such as FITC-RVRR, FITC-R_6, FITC-R_9, FITC-$(RVRR)_2$, FITC-$(RVRR)_3$, FITC-KLAKLAKKLAKLAK, FITC-$(RVRR)_2$-KLAKLAKKLAKLAK and FITC-$(RVRR)_3$-KLAKLAKKLAKLAK 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 flowcytometry 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 ℃. 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

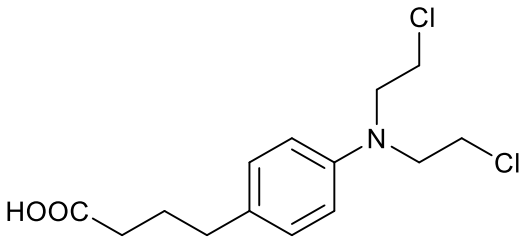
Caspase-3 activity was measured by following the manufacturer's instructions. Briefly, MDA-MB-231 cells were incubated with 10 μM $(RVRR)_3$-**KLA** for 4 and 8 h or with 20 μM $(RVRR)_3$-**KLA** for 4 and 8 h. The cells were then collected and washed with PBS. The cells were lysed using lysis buffer, after which the lysates were centrifuged at 12,000 g at 4 ℃ 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 ℃ 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 4×10^6 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**)

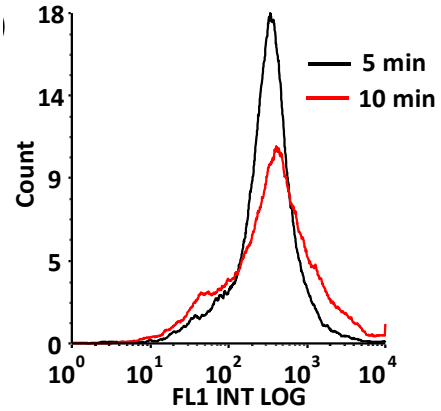


Fig. S1 Flow cytometry curves of MDA-MB-231 cells after incubated with FITC-RV3 at 37°C for 5- or 10 min.

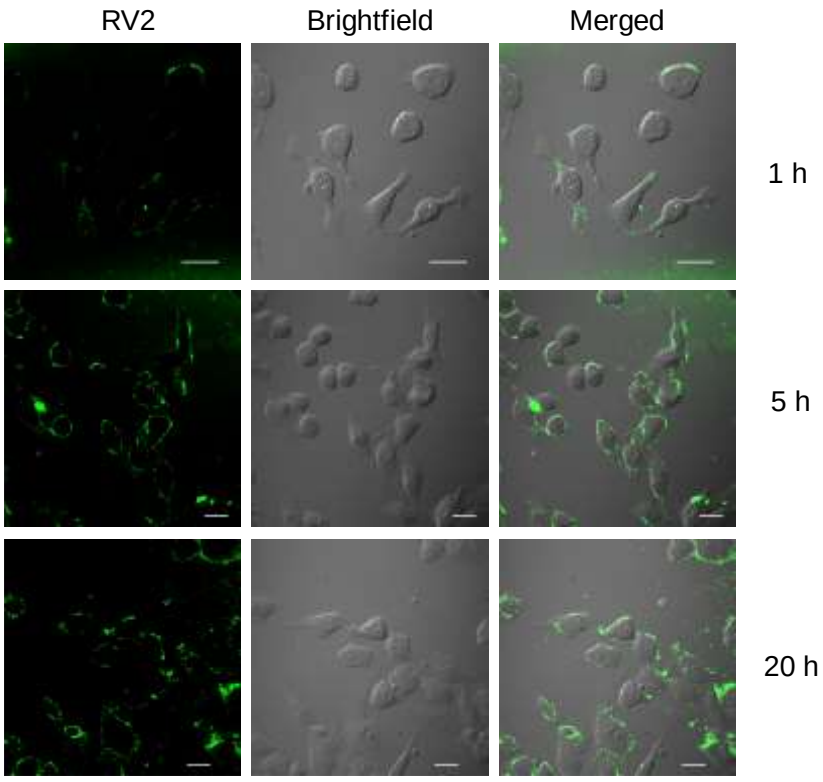


Fig. S2 CLSM images of MDA-MB-231 cells incubated with 10 μM FITC-RV2 at 37 °C for 1 hour, 5 hours and 20 hours. Green channel: λ_{ex} = 488 nm, λ_{em} = 495 - 545 nm. scale bar = 20 μm.

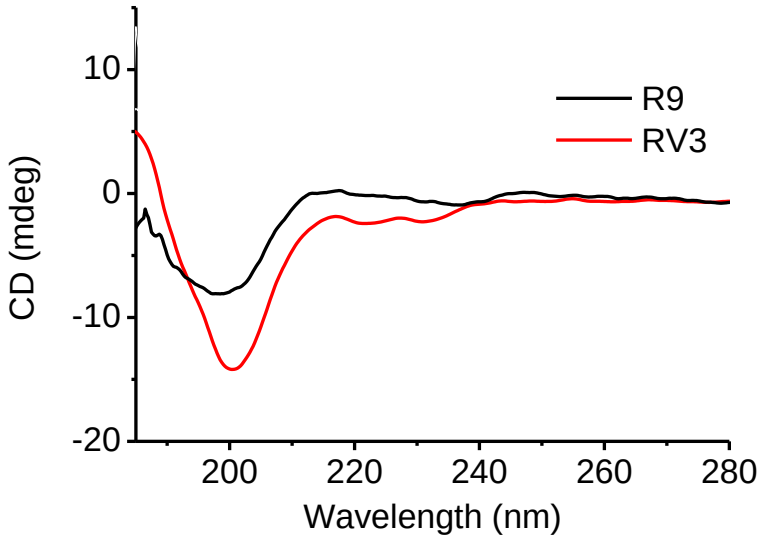


Fig. S3 Circular Dichroism (CD) spectra of R9 and RV3 (20 μM) in PBS buffer.

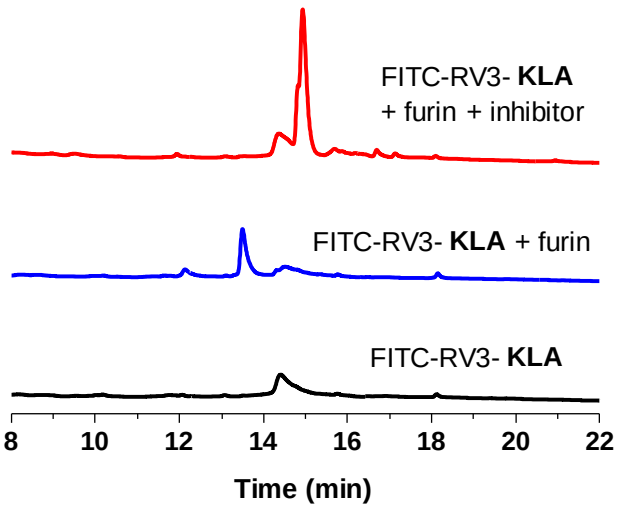


Fig. S4 HPLC traces of FITC-RV3-**KLA** (lower), FITC-RV3-**KLA** with furin (middle) and FITC-RV3-**KLA** incubated with the inhibitor before addition of furin for 1 h (upper).

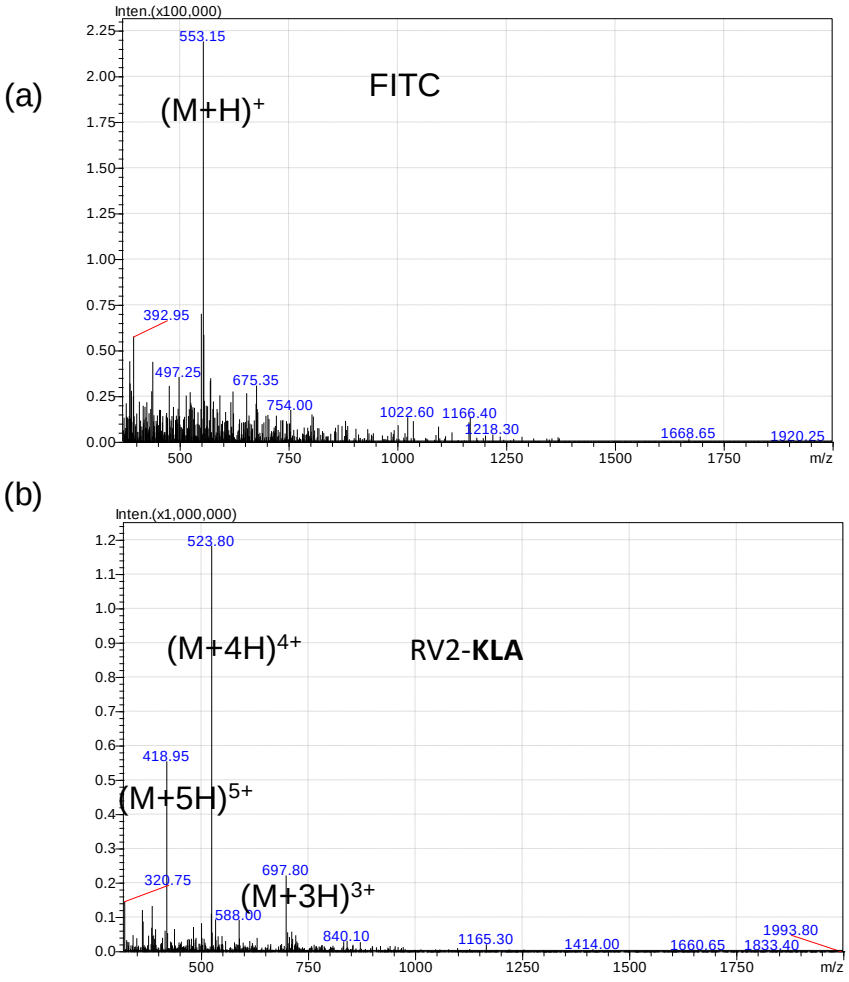


Fig. S5 ESI-Mass of HPLC peaks at 13.6 minutes (a) and 15.2 minutes (b).

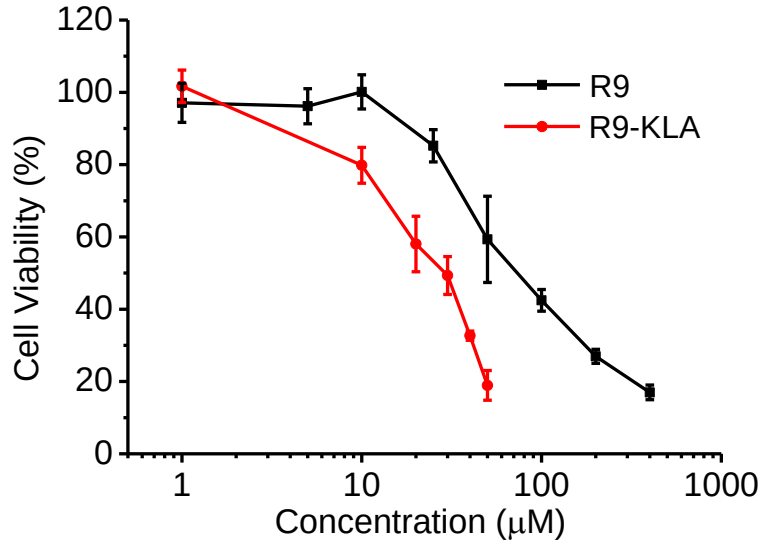


Fig. S6 Cell viability of R9-**KLA** and R9 against MDA-MB-231 cells after 24 hour incubation. Data represent the mean ± SD (n = 6).

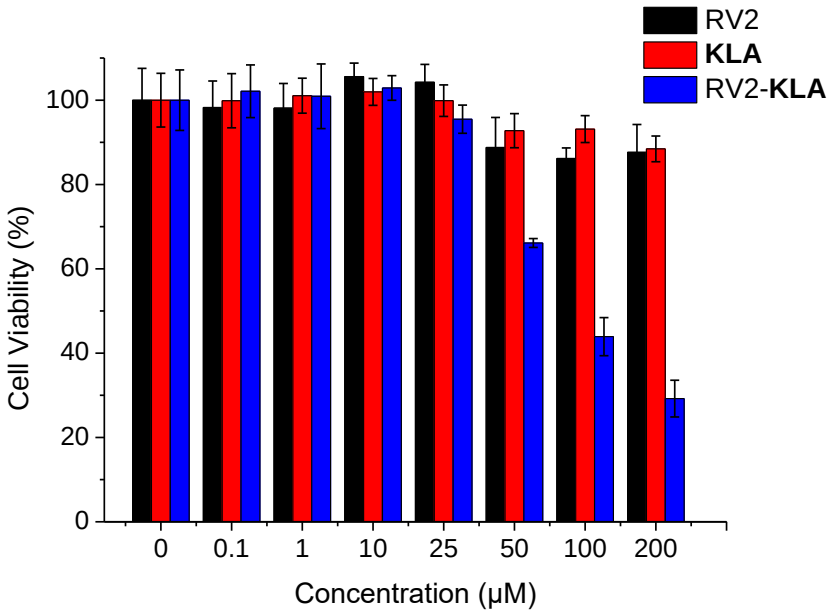


Fig. S7 Cell viability of RV2-**KLA**, RV2 and **KLA** against MDA-MB-231 cells after 24 hours incubation. Data represent the mean ± SD (n = 6).

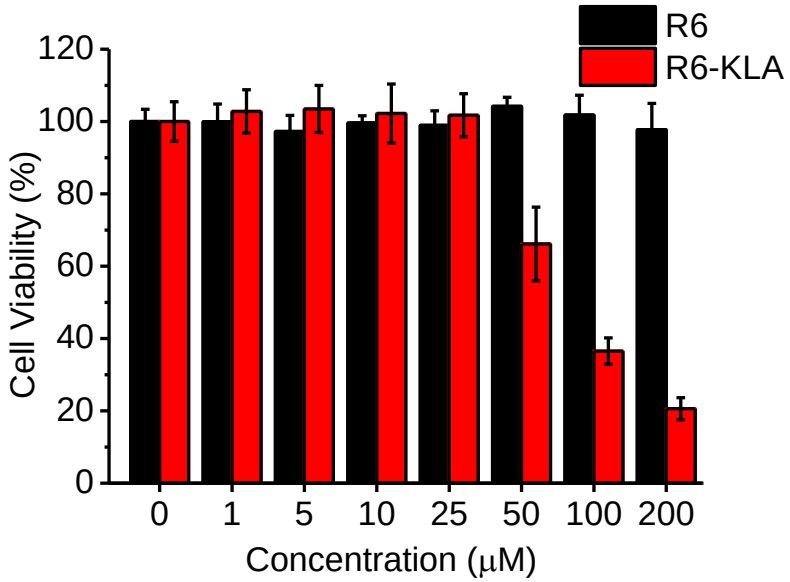


Fig. S8 Cell viability of R6-**KLA**, R6 against MDA-MB-231 cells after 24 hours incubation. Data represent the mean ± SD (n = 6).

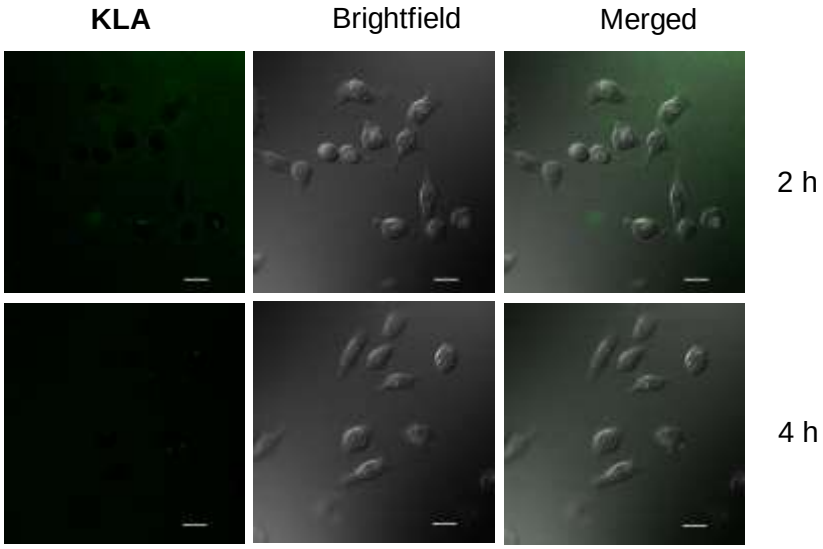


Fig. S9 CLSM images of MDA-MB-231 cells incubated with 10 μM FITC-**KLA** at 37 °C for 2 hours and 4 hours. Green channel: λ_{ex} = 488 nm, λ_{em} = 495 - 545 nm. scale bar = 20 μm.

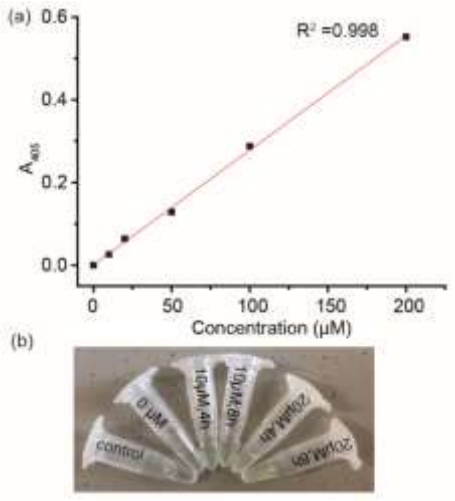


Fig. S10 (a) Standard line of detecting caspase-3 activity. (b) The images of samples from left to right: control, MDA-MB-231 cell lysate incubate with 0 μM, 10 μM RV3-**KLA** for 4 h, 10 μM RV3-**KLA** for 8 h, 20 μM RV3-**KLA** for 4 h, 20 μM RV3-**KLA** for 8 h.

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

control	control	0.075
	0 μM	0.158
10 μM	4 h	0.220
	8 h	0.266
20 μM	4 h	0.352
	8 h	0.416

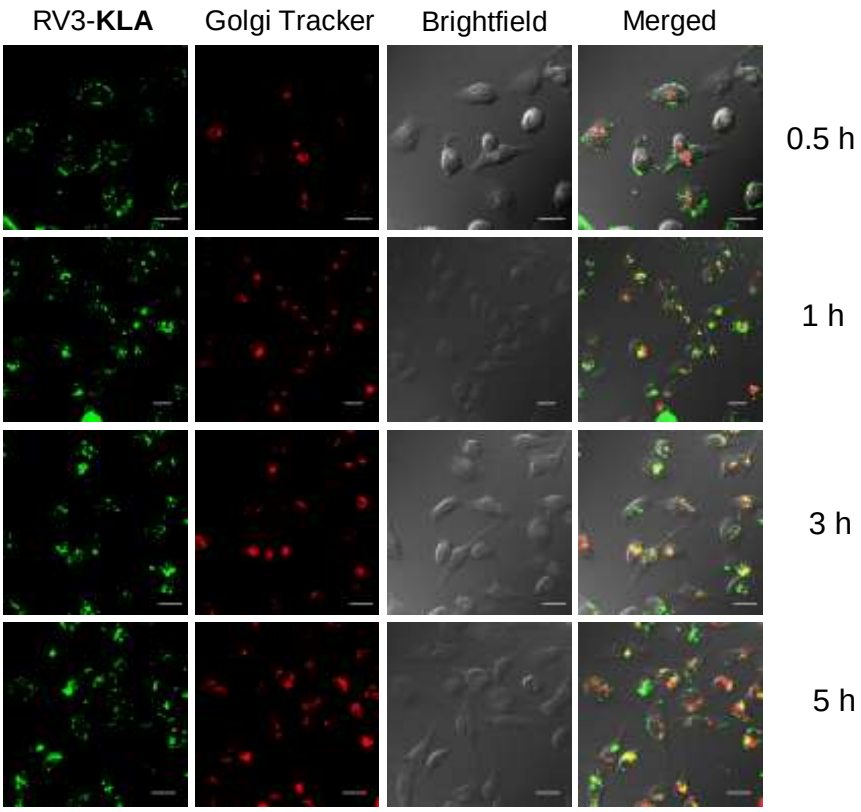


Fig. S11 CLSM images of MDA-MB-231 cells co-incubated with 10 μM FITC-RV3-KLA and Golgi Tracker (33 μg/ml) for 0.5-, 1-, 3-, 5 h at 37°C. Green channel: λ_{ex}=488 nm, λ_{em}=495-545 nm. Red channel: λ_{ex}=543 nm, λ_{em}=595-645 nm. Scale bar=20 μm.

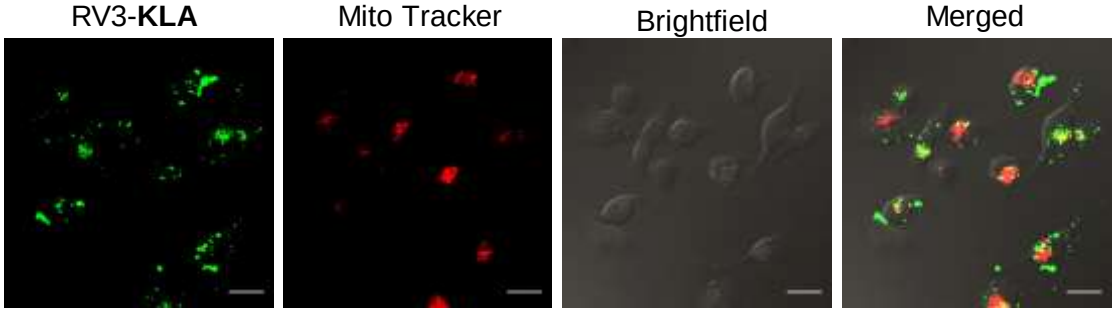


Fig. S12 CLSM images of MDA-MB-231 cells co-incubated with 10 μM FITC-RV3-**KLA** and Mito-Tracker for 3 hours at 37 °C. Green channel: λ_{ex} = 488 nm, λ_{em} = 495 - 545 nm. Red channel: λ_{ex} = 543 nm, λ_{em} = 620 - 670 nm. scale bar = 20 μm.

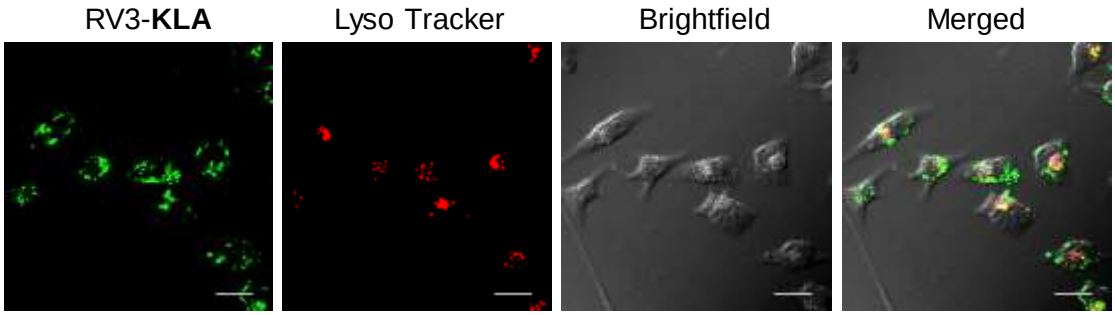


Fig. S13 CLSM images of MDA-MB-231 cells co-incubated with 10 μM FITC-RV3-**KLA** and Lyso-Tracker for 3 hours at 37 °C. Green channel: λ_{ex} = 488 nm, λ_{em} = 495 - 545 nm. Red channel: λ_{ex} = 543 nm, λ_{em} = 575 - 625 nm. scale bar = 20 μm.

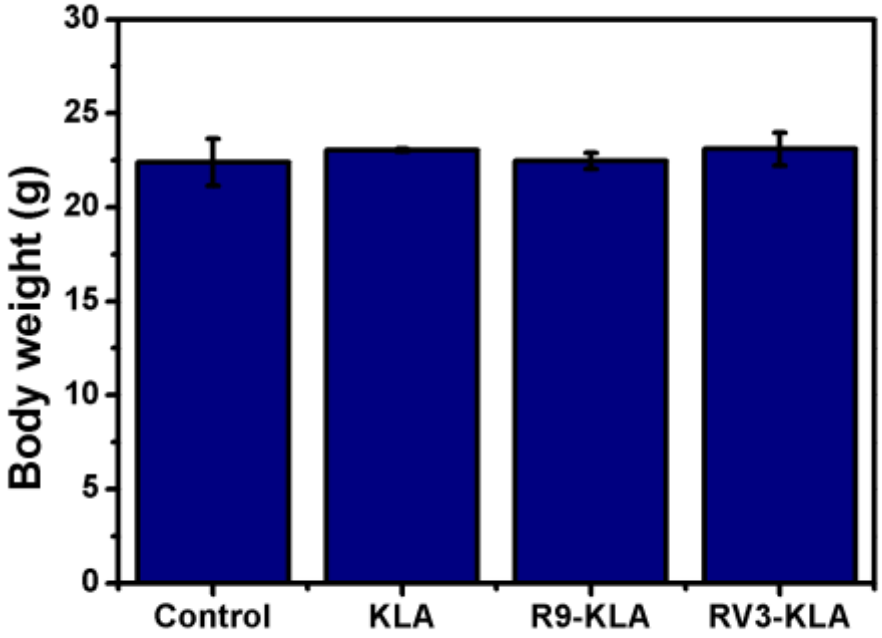


Fig. S14 The original body weight of different groups

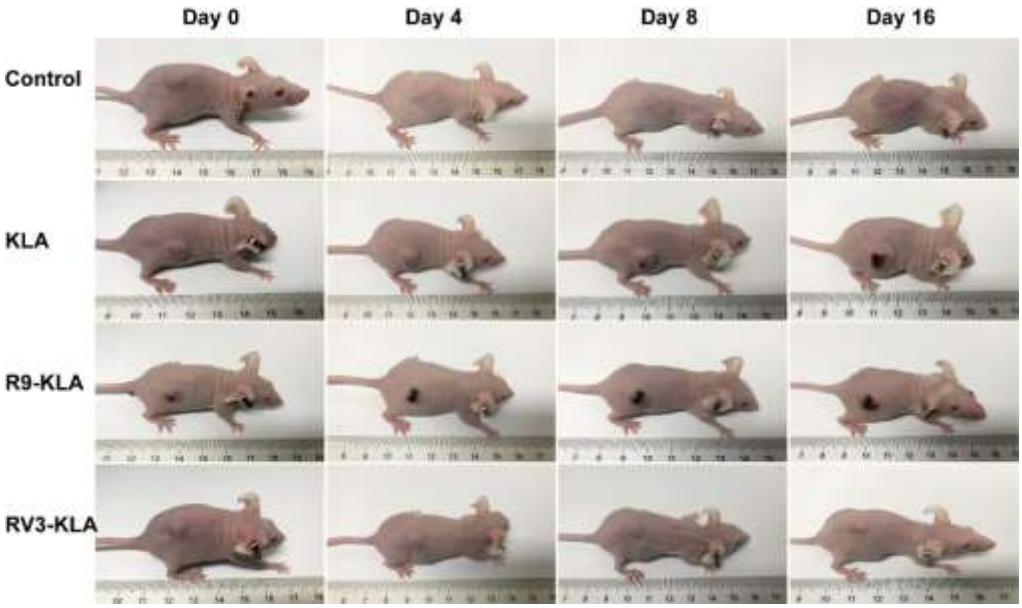


Fig. S15 Photographs of different groups showing the size of tumor during the treatment with PBS, **KLA** and RV3-**KLA** and R9-**KLA**.

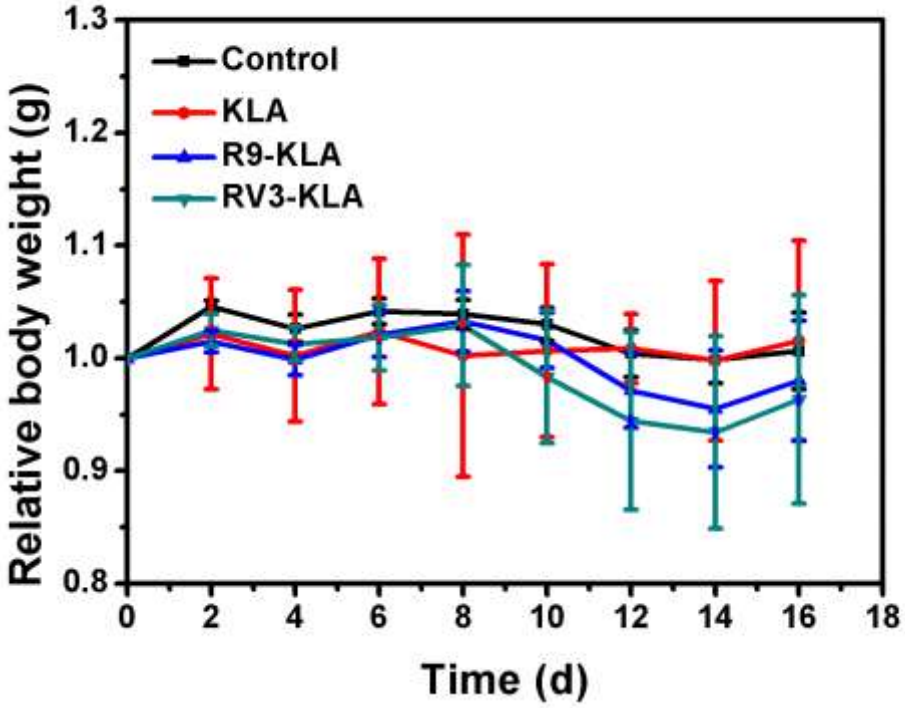


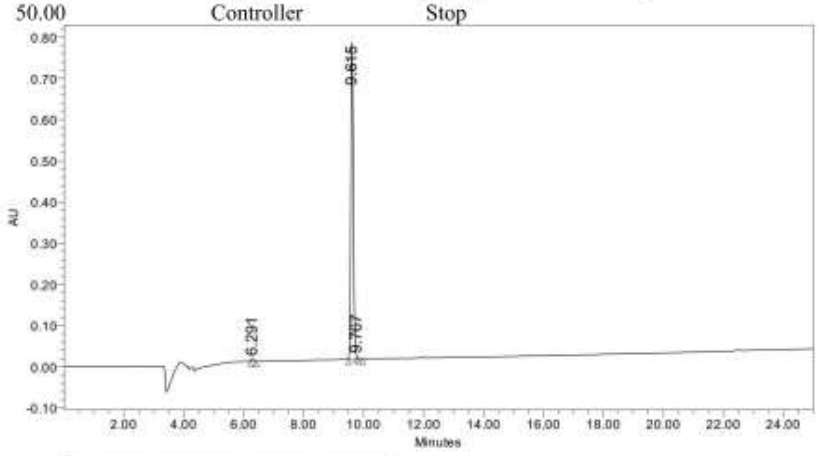
Fig. S16 Body weight changes of mice during treatment.

High Quality Peptide

Sample Information

Order ID	:	Syn-64497
Name	:	SYNS560-2
Sequence	:	RVRRRVRR
Lot No.	:	JT-64497
Pump A	:	0.1% Trifluoroacetic in 100% Water
Pump B	:	0.1% Trifluoroacetic in 100% Acetonrtrile
Total Flow	:	1ml/min
Wavelength	:	214nm
Analytial column type	:	SHIMADZU Inertsil ODS-SP（4.6*250mm*5um）
Dissolution method	:	15%ACN+85%H2O
Inj. Volume	:	30ul

Time	Module	Action	Value
0.01	Pumps	B.Conc	1.0
25.00	Pumps	B.Conc	50
30.00	Pumps	B.Conc	100
36.00	Pumps	B.Conc	100
40.00	Pumps	B.Conc	2
50.00	Controller	Stop	



	RT	Area	% Area	Height
1	6.291	26430	0.60	4777
2	9.615	4369892	99.11	761321
3	9.767	12726	0.29	5892

Synpeptide Co., Ltd
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Fig. S17 HPLC trace of RVRRRVRR.

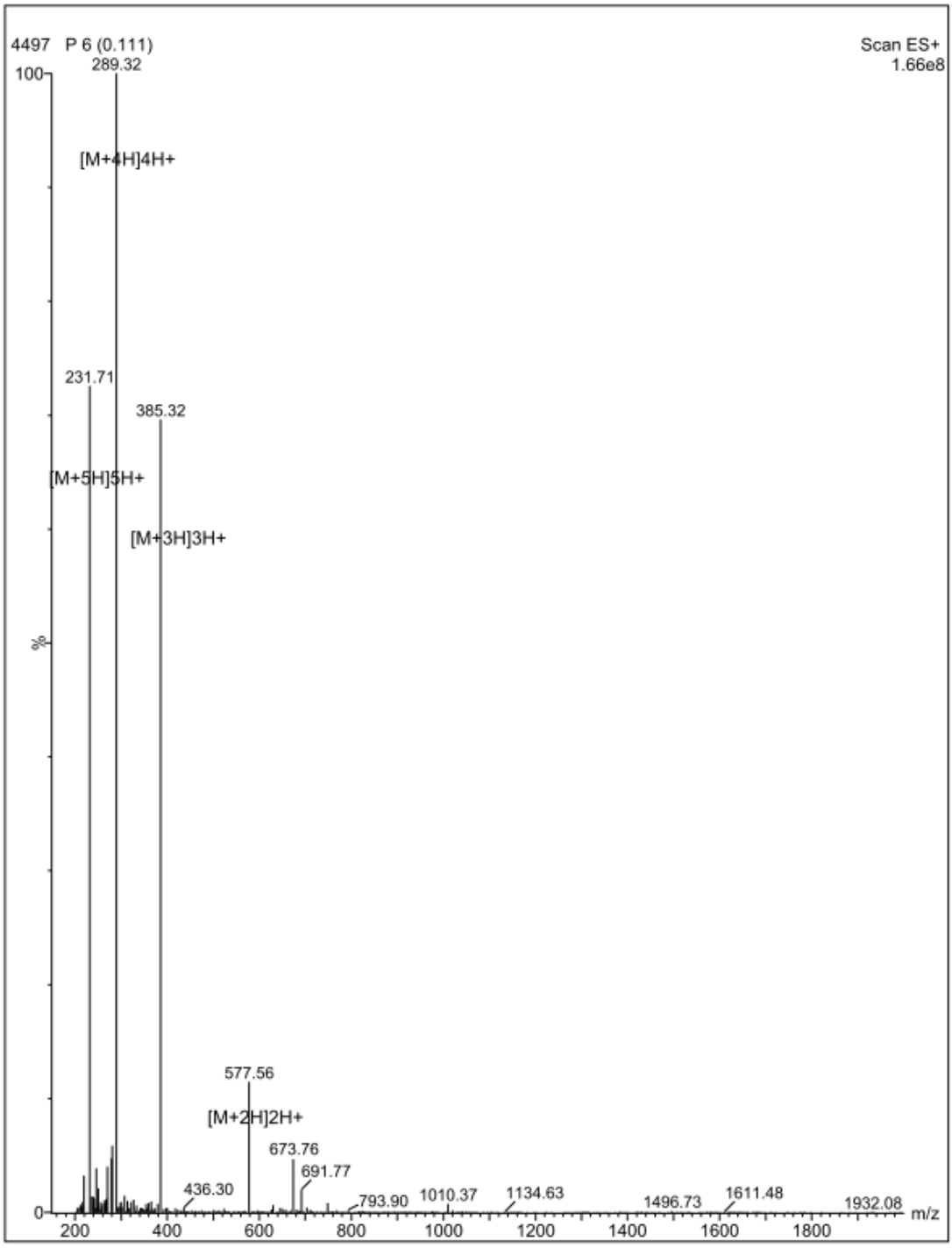
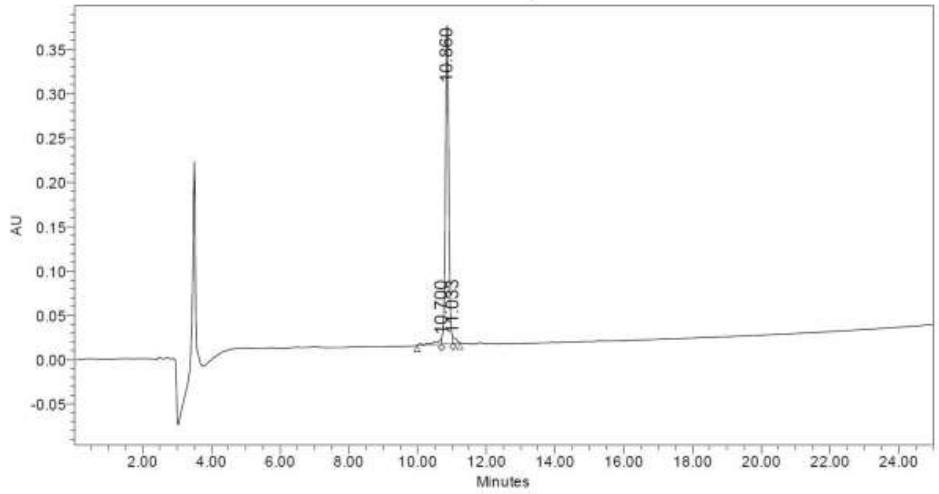


Fig. S18 ESI-Mass spectrometry of RVRRRVRR.

Sample Information

Order ID	:	Syn-66232
Name	:	SYNS611
Sequence	:	FITC-RVRRRVRRKLAKLAKKLAKLAK
Lot No.	:	JT-66232
Pump A	:	0.1% Trifluoroacetic in 100% Water
Pump B	:	0.1% Trifluoroacetic in 100% Acetonrtrile
Total Flow	:	1ml/min
Wavelength	:	214nm
Analytial column type	:	SHIMADZU Inertsil ODS-SP（4.6*250mm*5um）
Dissolution method	:	15%ACN+85%H2O
Inj. Volume	:	20ul

Time	Module	Action	Value
0.01	Pumps	B.Conc	15
25.00	Pumps	B.Conc	75
30.00	Pumps	B.Conc	100
36.00	Pumps	B.Conc	100
40.00	Pumps	B.Conc	2
50.00	Controller	Stop	



	RT	Area	% Area	Height
1	10.700	82004	3.24	6751
2	10.860	2407079	95.04	360751
3	11.033	43513	1.72	6576

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Fig. S19 HPLC trace of FITC-RVRRRVRRKLAKLAKKLAKLAK.

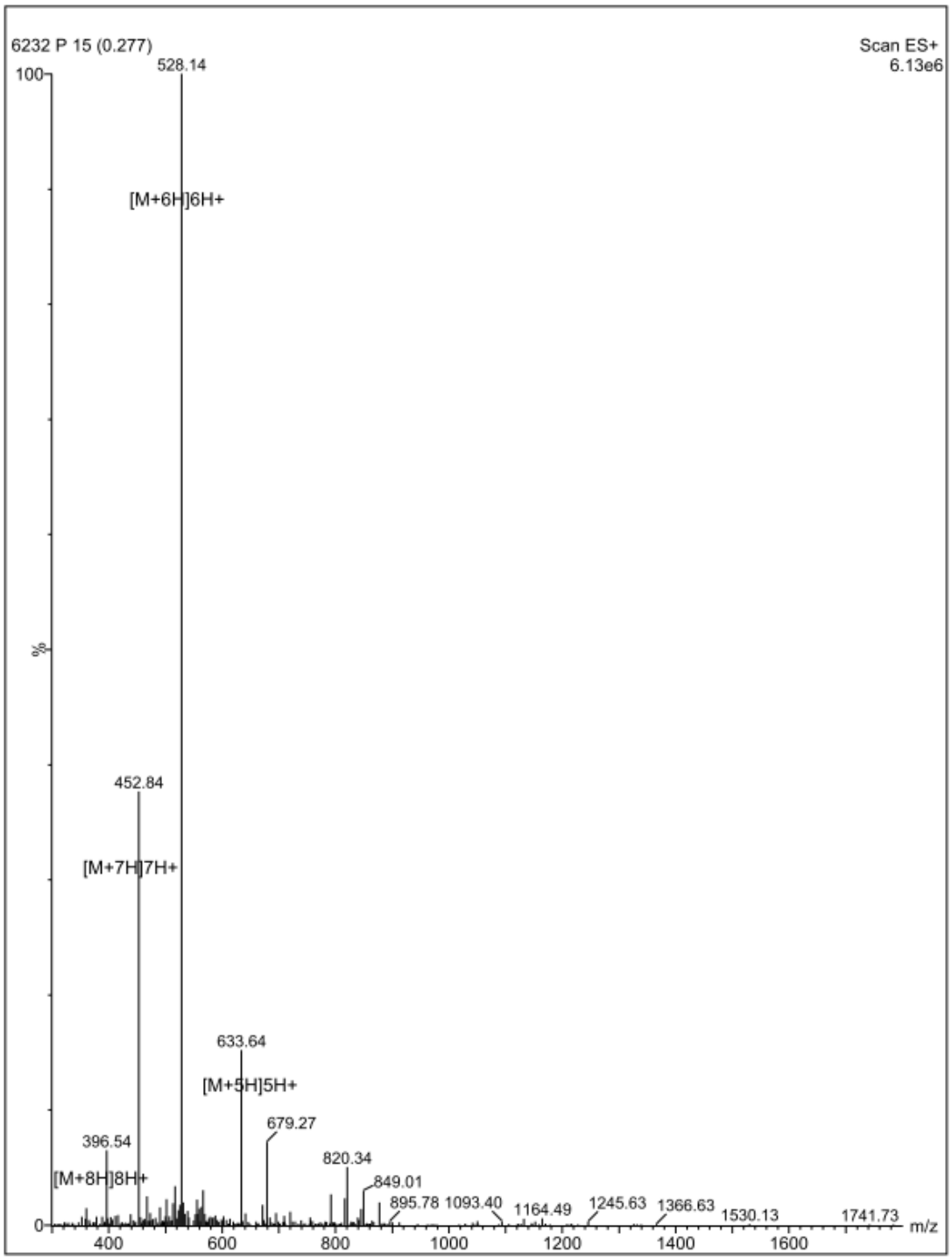
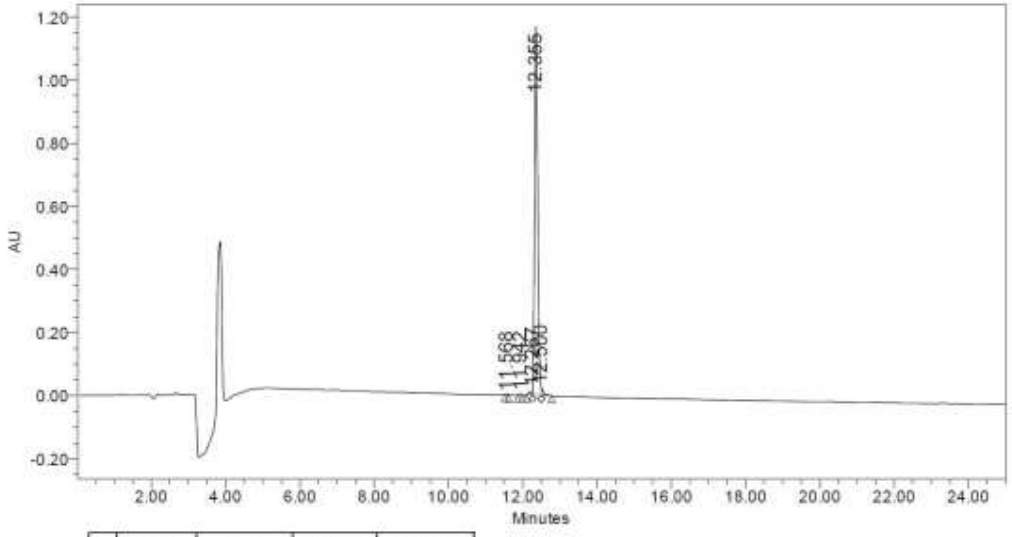


Fig. S20 The ESI-Mass spectrometry of FITC-RVRRRVRRKLAKLAKKLAKLAK.

Sample Information

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

Time	Module	Action	Value
0.01	Pumps	B.Conc	10
25.00	Pumps	B.Conc	70
30.00	Pumps	B.Conc	100
36.00	Pumps	B.Conc	100
40.00	Pumps	B.Conc	2
50.00	Controller	Stop	



	RT	Area	% Area	Height
1	11.568	7744	0.11	2220
2	11.942	14594	0.21	3467
3	12.267	59453	0.84	16328
4	12.355	6927025	97.46	1169486
5	12.500	98531	1.39	25944

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Fig. S21 HPLC trace of FITC-RVRRRVRRRVRRKLAKLAKKLAKLAK.

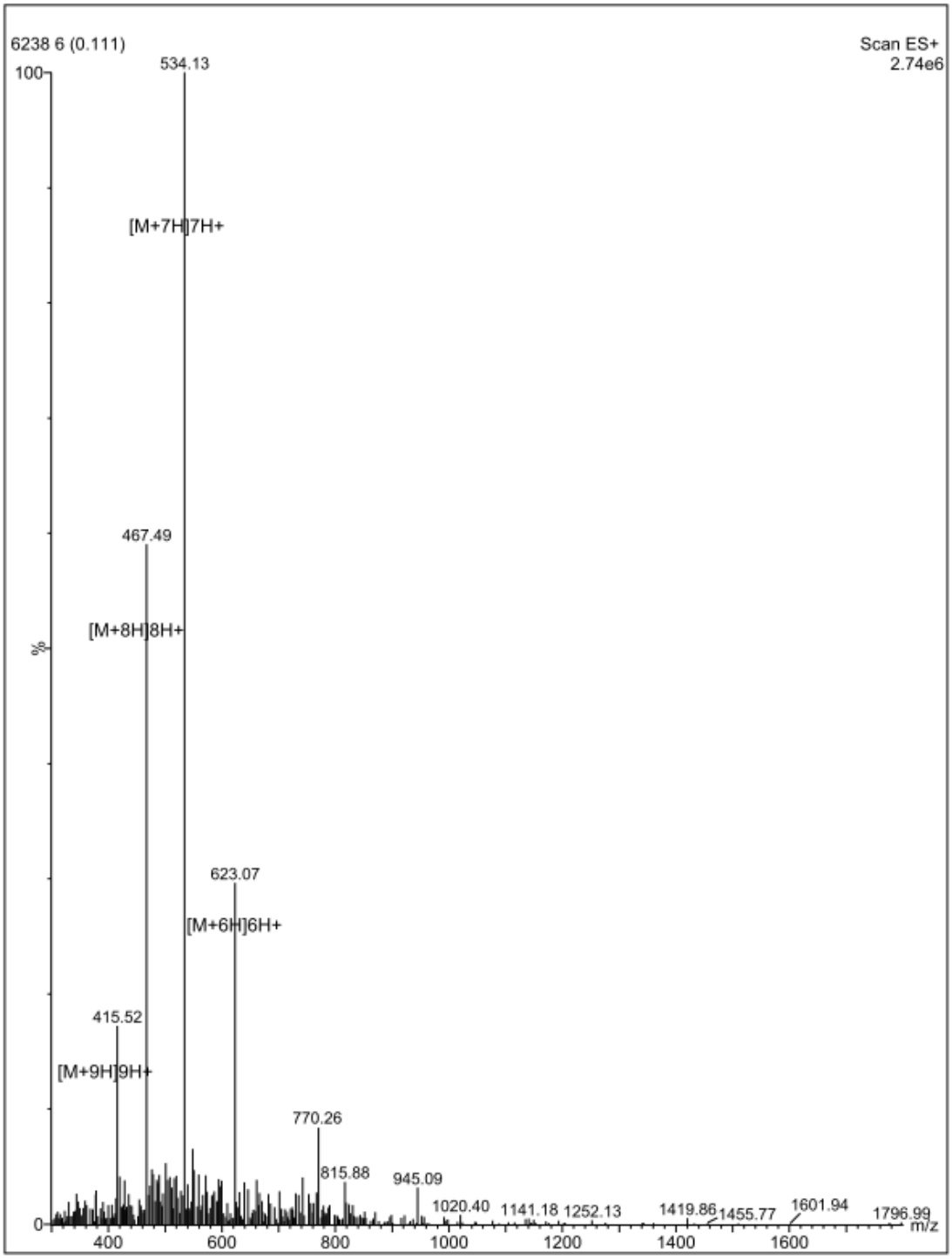


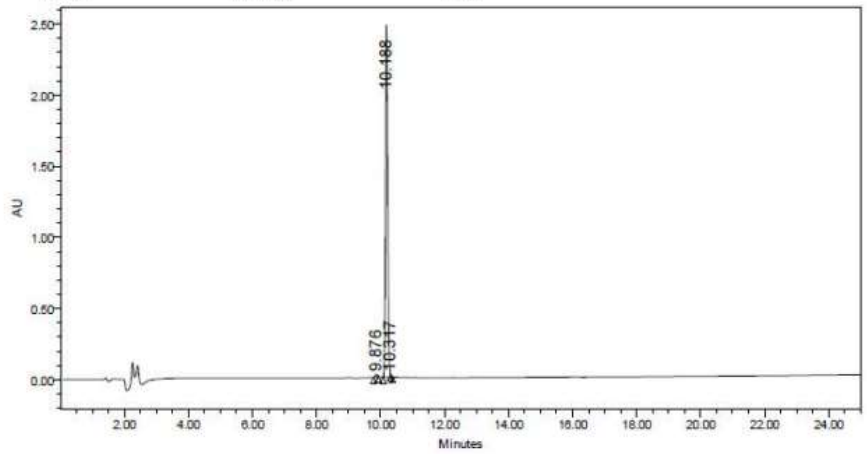
Fig. S22 ESI-Mass spectrometry of FITC-RVRRRVRRRVRRKLAKLAKKLAKLAK.

High Quality Peptide

Sample Information

Order ID	: Syn-78822
Name	: SYNS868-1
Sequence	: FITC-ACP-RRRRRRKLAKLAKKLAKLAK
Lot No	: JT-78822
Pump A	: 0.1% Trifluoroacetic in 100% Water
Pump B	: 0.1% Trifluoroacetic in 100% Acetonrtrile
Total Flow	: 1ml/min
Wavelength	: 220nm
Analytial column type	: SHIMADZU Inertsil ODS-SP（4.6*250mm*5um）
Inj. Volume	: 30ul

Time	Module	Action	Value
0.00	Pumps	B.Conc	10
25.00	Pumps	B.Conc	70
25.01	Pumps	B.Conc	100
30.00	Pumps	B.Conc	100
30.01	Pumps	Stop	



	RT	Area	% Area	Height
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2	10.188	11633409	99.09	2446931
3	10.317	29923	0.25	21902

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Fig. S23 HPLC trace of FITC-RRRRRRKLAKLAKKLAKLAK.

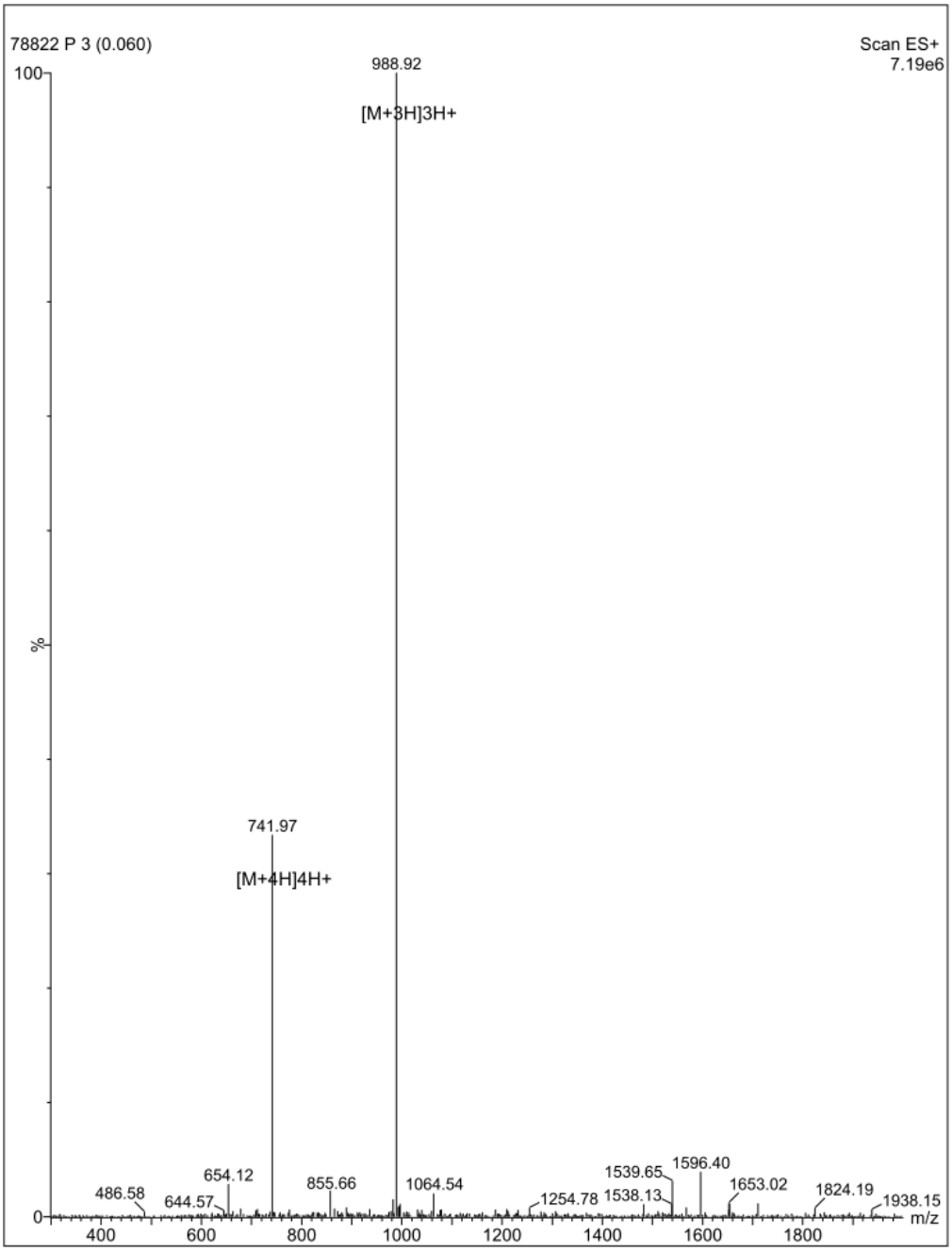


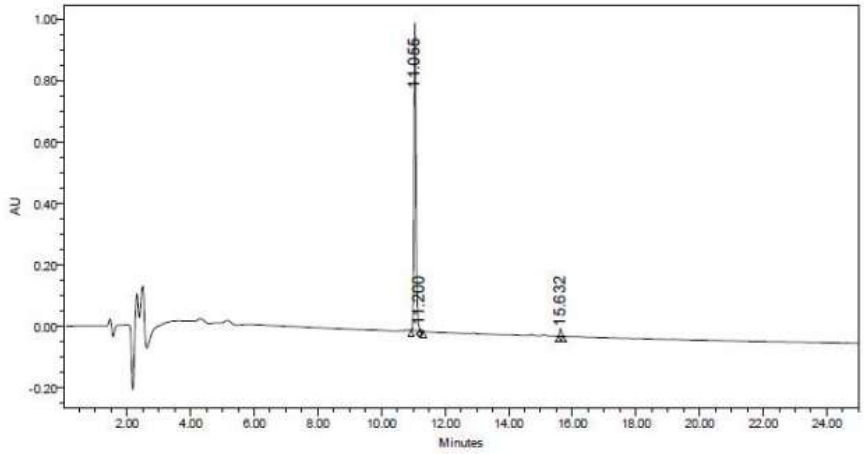
Fig. S24 ESI-mass of FITC-RRRRRRKLAKLAKKLAKLAK.

High Quality Peptide

Sample Information

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

Time	Module	Action	Value
0.00	Pumps	B.Conc	10
25.00	Pumps	B.Conc	70
25.01	Pumps	B.Conc	100
30.00	Pumps	B.Conc	100
30.01	Pumps	Stop	



	RT	Area	% Area	Height
1	11.055	4498840	97.53	983992
2	11.200	23460	0.51	5885
3	15.632	90346	1.96	22480

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Fig. S25 HPLC trace of FITC-RRRRRRRRRKLAKLAKKLAKLAK.

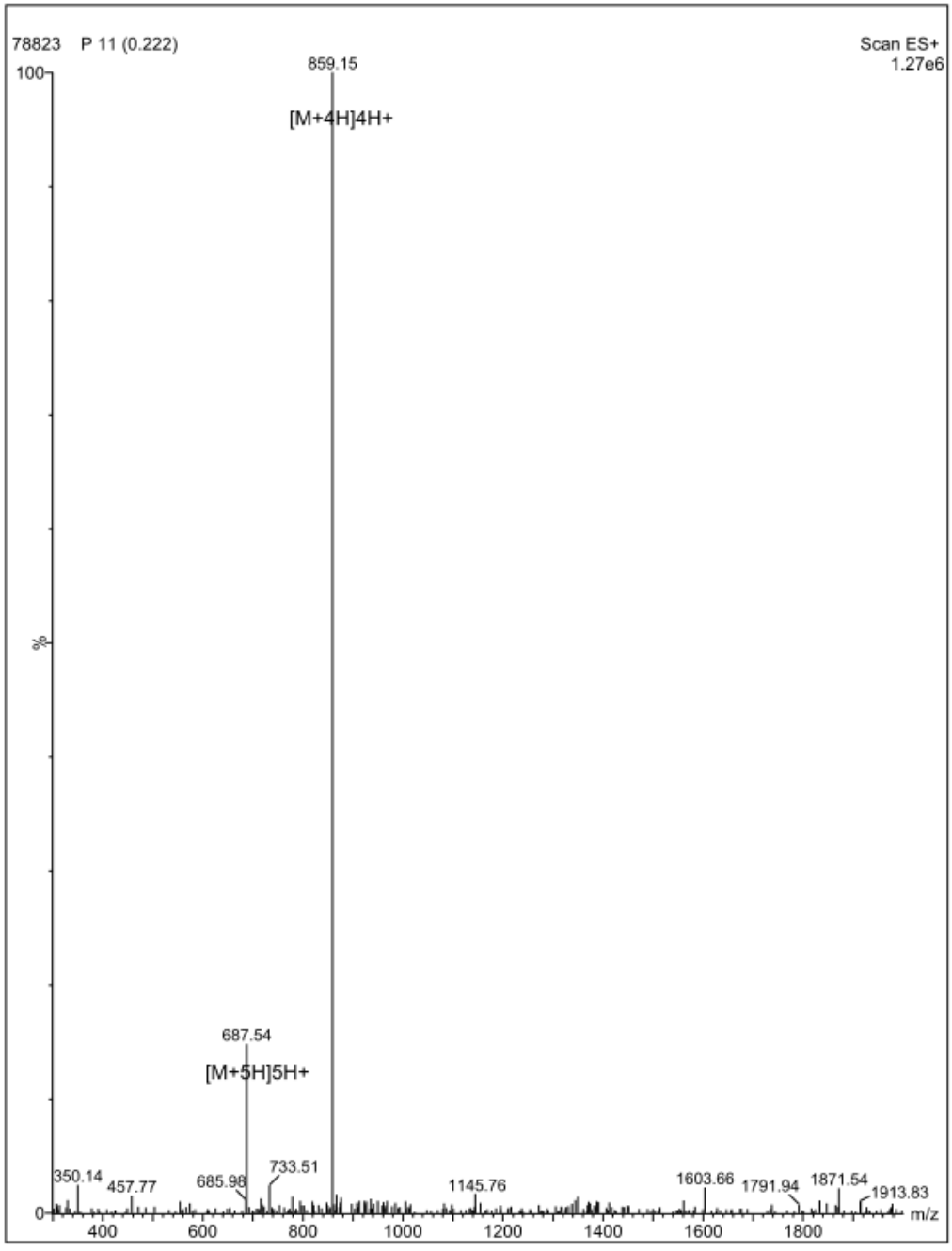


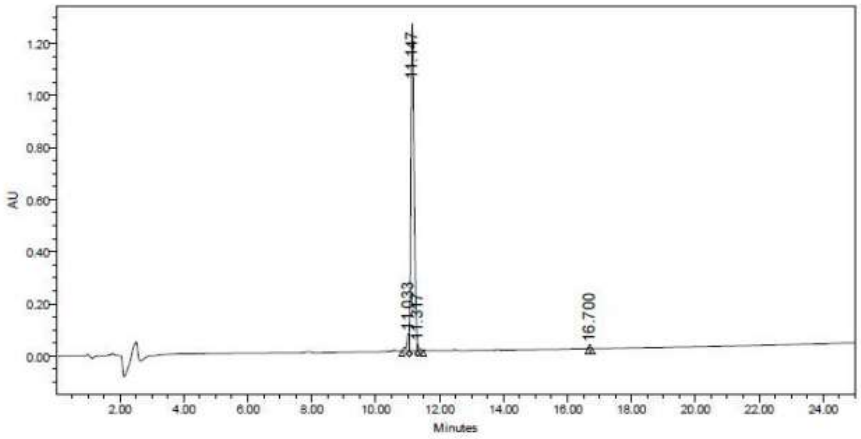
Fig. S26 ESI-Mass spectrometry of FITC-RRRRRRRRRKLAKLAKKLAKLAK.

High Quality Peptide

Sample Information

Order ID	: Syn-78824
Name	: SYNS868-3
Sequence	: RVRRRVRRRVRRGGKLAKLAKKLAKLAK
Lot No	: JT-78824
Pump A	: 0.1% Trifluoroacetic in 100% Water
Pump B	: 0.1% Trifluoroacetic in 100% Acetonrtrile
Total Flow	: 1ml/min
Wavelength	: 220nm
Analytial column type	: SHIMADZU Inertsil ODS-SP（4.6*250mm*5um）
Inj. Volume	: 30ul

Time	Module	Action	Value
0.00	Pumps	B.Conc	5
25.00	Pumps	B.Conc	65
25.01	Pumps	B.Conc	100
30.00	Pumps	B.Conc	100
30.01	Pumps	Stop	



	RT	Area	% Area	Height
1	11.033	324079	3.51	66392
2	11.147	8795384	95.27	1256117
3	11.317	65951	0.71	26058
4	16.700	46604	0.50	12417

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Fig. S27 HPLC trace of RVRRRVRRRVRRGGKLAKLAKKLAKLAK.

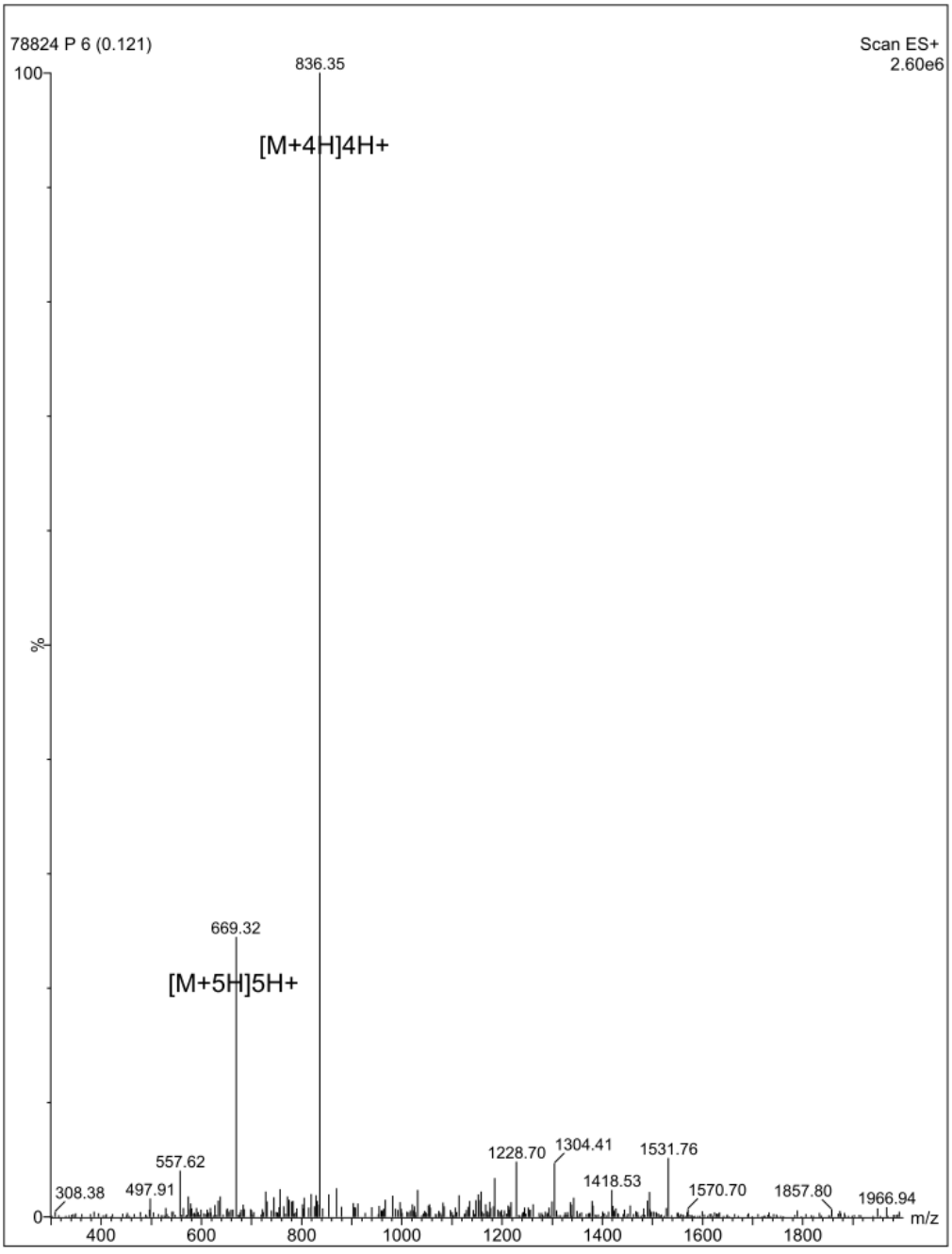


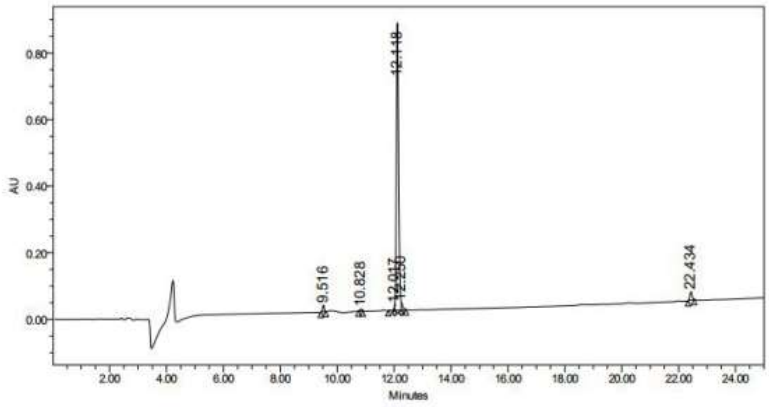
Fig. S28 ESI-Mass spectrometry of RVRRRVRRRVRRGGKLAKLAKKLAKLAK.

High Quality Peptide

Sample Information

Order ID	: Syn-82020
Name	: SYNS963-1
Sequence	: RRRRRRRRRKLAKLAKKLAKLAK
Lot No	: JT-82020
Pump A	: 0.1% Trifluoroacetic in 100% Water
Pump B	: 0.1% Trifluoroacetic in 100% Acetonrtrile
Total Flow	: 1ml/min
Wavelength	: 220nm
Analytial column type	: SHIMADZU Inertsil ODS-SP（4.6*250mm*5um）
Inj. Volume	: 30ul

Time	Module	Action	Value
0.00	Pumps	B.Conc	10
25.00	Pumps	B.Conc	70
25.01	Pumps	B.Conc	100
30.00	Pumps	B.Conc	100
30.01	Pumps	Stop	



	RT	Area	% Area	Height
1	9.516	81441	1.62	18795
2	10.828	24467	0.49	5200
3	12.017	23919	0.48	15917
4	12.118	4659724	92.89	853831
5	12.250	81808	1.63	21485
6	22.434	145229	2.89	24689

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Fig. S29 HPLC trace of RRRRRRRRRKLAKLAKKLAKLAK.

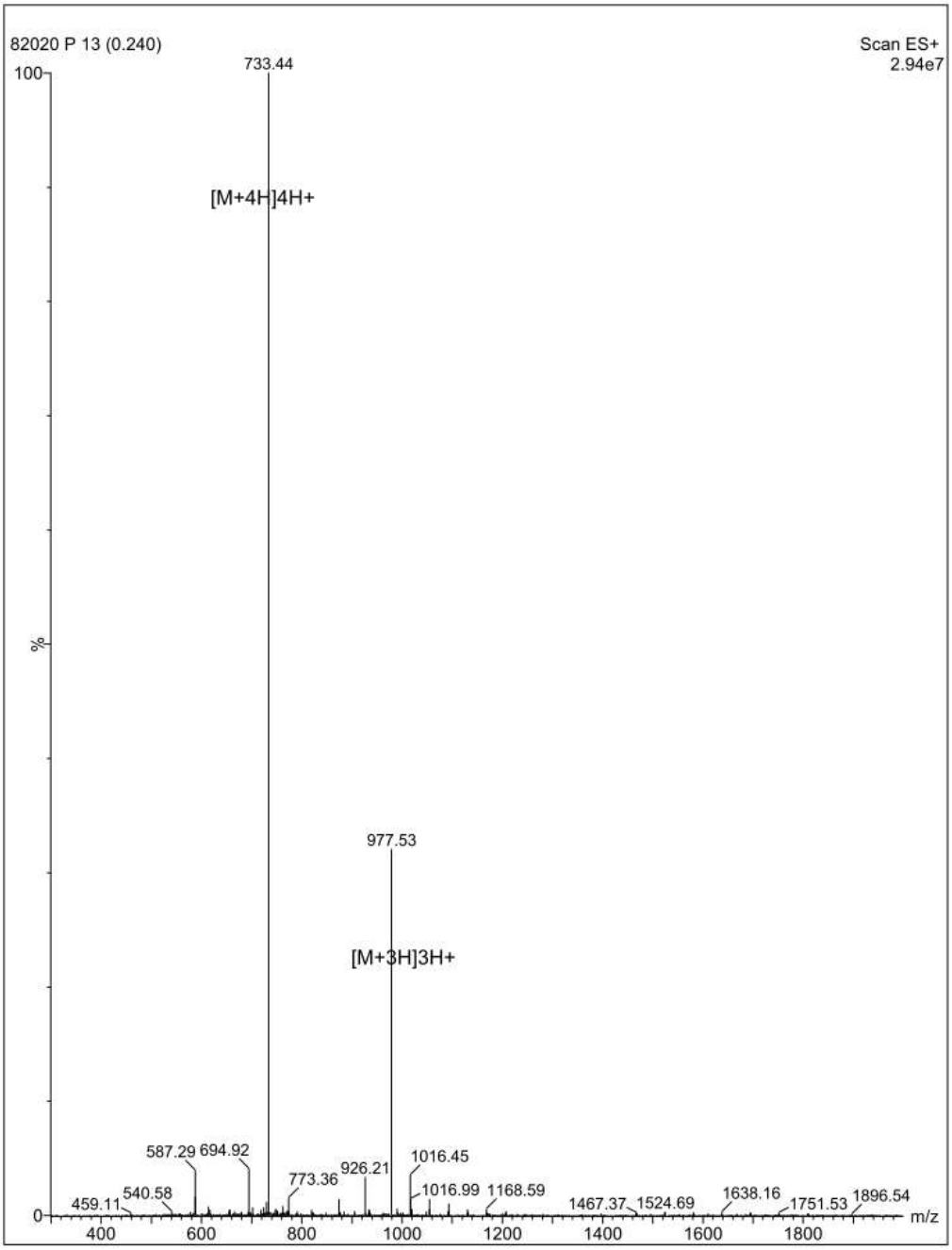


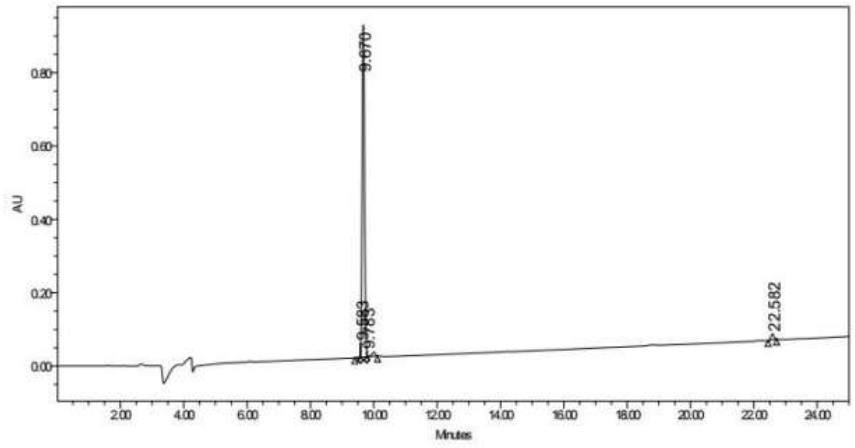
Fig. S30 ESI-Mass spectrometry of RRRRRRRRRKLAKLAKKLAKLAK.

High Quality Peptide

Sample Information

Order ID	: Syn-82022
Name	: SYNS963-3
Sequence	: KLAKLAKKLAKLAK
Lot No	: JT-82022
Pump A	: 0.1% Trifluoroacetic in 100% Water
Pump B	: 0.1% Trifluoroacetic in 100% Acetonrtrile
Total Flow	: 1ml/min
Wavelength	: 220nm
Analytial column type	: SHIMADZU Inertsil ODS-SP（4.6*250mm*5um）
Inj. Volume	: 30ul

Time	Module	Action	Value
0.00	Pumps	B.Conc	10
25.00	Pumps	B.Conc	70
25.01	Pumps	B.Conc	100
30.00	Pumps	B.Conc	100
30.01	Pumps	Stop	



	RT	Area	%Area	Height
1	9.583	31603	0.62	37518
2	9.670	4851581	95.35	899401
3	9.783	103770	2.04	14065
4	22.582	100983	1.98	15304

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Fig. S31 HPLC trace of KLAKLAKKLAKLAK.

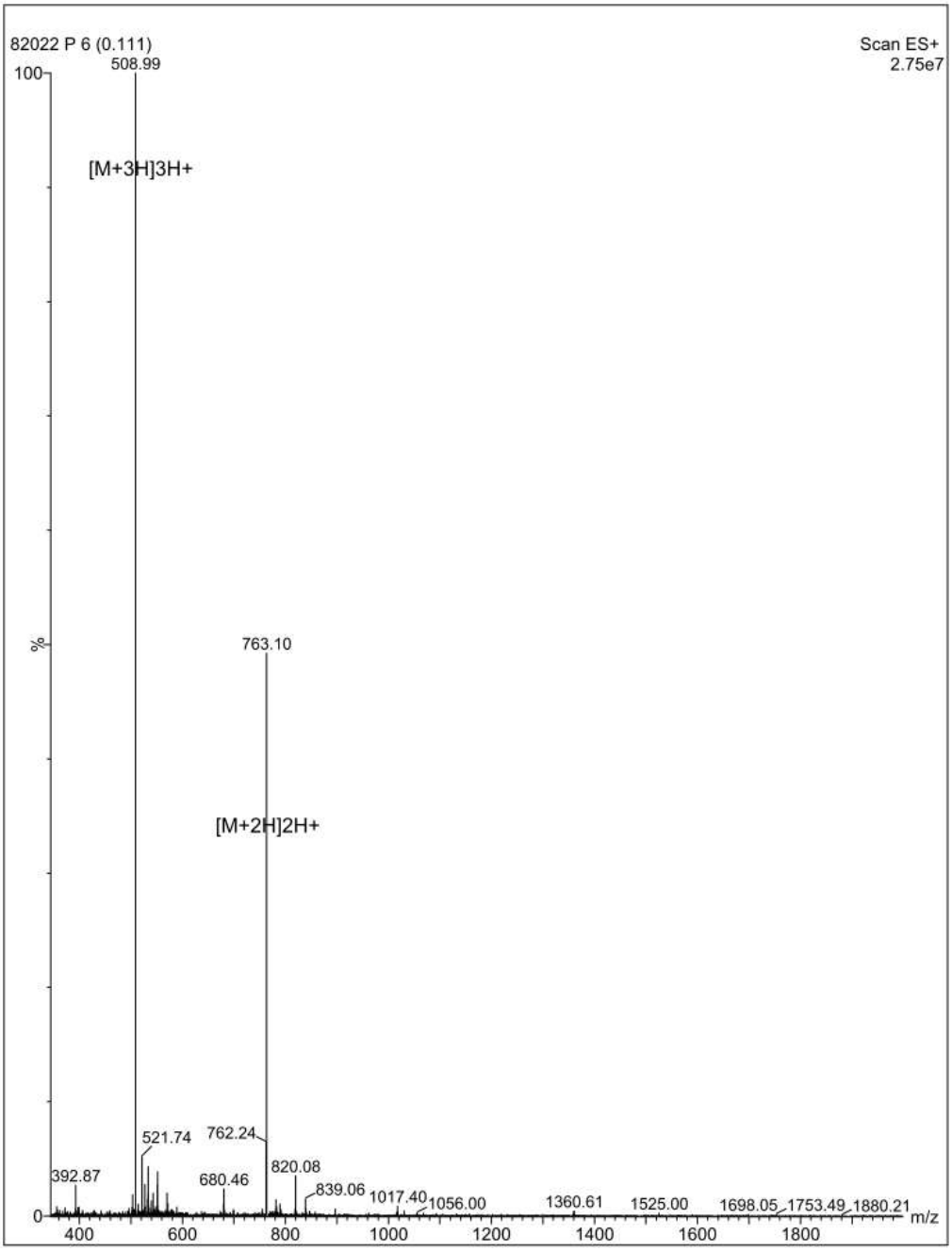

Fig. S32 ESI-mass spectrometry of KLAKLAKKLAKLAK.