Supporting Information

Autophagy inducing cyclic peptides constructed by methionine alkylation

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Abbreviations

DMEM, dulbecco's modified eagle medium; Et₂O, diethyl ether; FBS, fetal bovine serum; Fmoc, 9-fluorenylmethyloxycarbonyl; HCTU, 2-(1H-6-chlorobenzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate; HPLC, high-performance liquid chromatography; HPLC, high-performance liquid chromatography; MBHA, 4-methyl-benzylhydrylamine; MMP, matrix metalloproteinase; MS, mass spectrometry; OD, optical density; PBS, phosphate buffer saline; SPPS, solid-phase peptide synthesis; TIPS, triisopropylsilane; TFA, trifluoroacetic acid. 5-FAM, 5-Carboxyfluorescein.

Materials

All amino acids and resins used for peptide synthesis were purchased from GL Biochem (Shanghai, China). Other solvents and reagents were purchased from commercial suppliers including Hanhong Chemical (Shanghai, China), Energy Chemical (Shanghai, China) and Tenglong Logistics (Shenzhen, China) and were used without further purification unless otherwise stated.

Peptide synthesis and characterization

Peptides were synthesized on Rink-amide-MBHA resin (loading capacity: 0.34 mmol/g) using manual Fmoc standard solid phase peptide synthesis method. The resin was swelled in DMF and bubbled with N₂ for 30 min. Next, Fmoc deprotection was performed by morpholine (50% in DMF) for 30 min. The resin was washed sequentially with DCM and DMF for 6 times. Then coupling was conducted by adding the mixture of amino acids (5.0 equiv according to initial loading of the resin, unnatural amino acids were used as 3 equivalents), HCTU (4.9 equiv) and DIPEA (10.0 equiv) dissolved in DMF to resin for 1-2 h, followed by washing the resin with DCM and NMP for 6 times sequentially. Repeat the coupling step until all the amino acid residues were assembled. In the end of peptide assembly, peptides were N-terminal capped with 4-pentynoic acid or FAM with a β-Ala linker by reacting 3 equiv with 3 equiv benzotriazol-1-yl-oxytrityrylridino phosphonium hexafluorophosphate (PyBOP), 3 equiv 1-hydroxybenzotriazole (HOBt), and 6 equiv DIPEA. Peptides were cleaved from the resin by treatment of TFA/H₂O/EDT/TIS (94:2.5:2.5:1) for 1 h and dried by nitrogen. The crude peptides were then precipitated with Hexane/Et₂O (1:2 in volume) at 4°C, isolated by centrifugation in 12000 rpm for 5 min and dissolved in water/acetonitrile (2:1 in volume). Purification was performed by RP-HPLC (Shimadzu (Kyoto, Japan) and Agilent (Santa Clara, CA, USA) Zorbax SB-Aq: 4.6 × 250
mm, 220 and 254 nm, flow rate 1.0 mL/min) and confirmed by Shimadzu LC-MS 2020 mass spectrometer equipped with Agilent Zorbax SB-Aq column. LC-MS spectra were carried out on SHIMAZU LC-MS 8030 (ESI-MS) in positive ion mode. Peptides were quantified by their UV absorbance at 280 nm or 494 nm according to amino acid coefficient contribution.

**Preparation of alkylated sulfonium peptides**

Linear peptides were cleaved from the resin and precipitated with Hexane/Et₂O (1:1 in volume). Peptides dried by vacuum were then dissolved in CH₃CN/H₂O (1:2 in volume) to reach a concentration of 10 mM (based on the loading of the resin) followed by adding with 1% (in volume) formic acid to adjust pH to about 3. The alkylated reagents (1.2 equiv) were dissolved by a small amount of DMF. Then the peptides and alkylated reagents were mixed and stirred at room temperature for 24 h. Then the reaction solution was purified by reversed phase HPLC immediately.

**Trypsin stability**

Peptide 3, 3c and 7f were prepared at a concentration of 100 µM in PBS (pH=7.4). To 100 µL of each solution 1 µL trypsin (1 mg/mL) was added and incubated at 37°C. At different time interval, the solution was mixed with 12% trichloroacetic acid (H₂O/CH₃CN: 2:1) to inactivate the trypsin. After removing the inactive trypsin by 12000 rpm centrifuge, the remaining peptide was examined by LC/MS with a grace smart C18 250× 4.6mm column using a 4% per minute linear gradient from 10% to 70% acetonitrile over 15 min. The amount of starting material in each sample was quantified by LC/MS-based peak detection at 254 nm.

**Cell culture and reagents**

Hela cell was obtained from American Type Culture Collection (ATCC) and cultured in Dulbecco modified Eagle medium (Gibco,) supplemented with 10% (v/v) fetal bovine serum (Hyclone, ), in a 5% CO₂ incubator at 37°C. Rapamycin was purchased from Selleckchem (S1039) and used at 5 µM. Hydroxychloroquine (HCQ) was obtained from Selleckchem (S4430) and used at 5 µM. Stock solutions were prepared in DMSO and stored at -20°C and further diluted with the culture media to obtain an optimal range of concentrations for treatments. Protease Inhibitor Cocktail was obtained from Roche (cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail, 04693159001). Primary antibody: LC3B (Sigma, L7543, 1:1000), p62 (Abcam, Ab56416, 1:500), GAPDH (Cell Signaling Technology, 14C10, 1:4000). Anti-rabbit IgG HRP-linked secondary antibody was purchased from Cell Signaling Technology and used at a 1:4000 dilution (Cell signaling technology, 7074s, 1:4000).

**Western blot**

Peptides were dissolved in 5% Dulbecco modified Eagle medium (Gibco) and then cells were treated with peptide in the condition as mentioned in article. Cells were rinsed with phosphate-buffered saline (PBS) and harvested using the lysis buffer (50 mM Tris-HCl pH=6.8, 2% SDS, 6% Glycerol, 1% β-mercaptoethanol, 0.004% bromophenol blue, Roche protease inhibitor cocktail ). Denatured cellular extracts were resolved by 12% SDS-PAGE gels. Protein bands in the gel were then transferred to Nitrocellulose Blotting membranes and blocked in 5% skim milk in TBST (20 mM Tris-HCl pH=8.0, 150 mM NaCl, 0.05% (V/V) Tween 20) for 1 h at room
Membranes were incubated with the primary antibody overnight at 4°C and then washed with TBST buffer. Anti-rabbit IgG HRP-linked secondary antibody diluted with 5% skim milk was used for secondary incubation for 1 hour at room temperature. After washing with TBST, protein bands were visualized with ECL Blotting Detection Reagents (Thermo Scientific, 32106) and ChemiDoc MP imaging system (Bio-Rad).

Flow Cytometry Analysis
Hela cells were seeded overnight in a 24-well culture plate. Then the cells were incubated with FAM-labelled peptides at a concentration of 5 μM in the DMEM with 5% FBS for 4 h at 37°C. After washing with PBS, the cells were exposed to trypsin (0.25%; Gibco) digestion (3 min, 37°C). After washing with PBS and 0.05% Trypan Blue incubation for 5 minutes, the cellular fluorescence was analyzed by a BD FACS Calibur flow cytometer (Becton Dickinson) and CellQuest Pro.

Confocal Microscopy.
mCherry-LC3B-pcDNA3.1 was a gift from David Rubinsztein (Addgene plasmid #40827). Transfection was performed by TransIntro EL Transfection Reagent (Transgen, FT201-01) according to the manufacturer’s protocol. After a 6 h incubation, the transfection medium was replaced with fresh medium. After culturing for 24h, 50 μM peptide was added into culture medium and incubated with cells for 12h at 37°C. After removing the culture medium, the cells were gently washed twice with PBS and then fixed with 4% paraformaldehyde (Sigma, V900894) in PBS for 10 minutes. The cells were washed two times with PBS again and stained with Fluoroshield with DAPI (Sigma, F6057) for 5 minutes. Images were taken on Nikon A1R confocal microscopy under the same parameters.

Peptide sequences, observed masses, and purity following HPLC purification

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Linear Sequence</th>
<th>Calculated MS</th>
<th>Observed MS</th>
<th>Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>pa-VMNAMFHIWH</td>
<td>1467.7</td>
<td>489.9/734.3</td>
<td>95%</td>
</tr>
<tr>
<td>2a</td>
<td>pa-VWMATFMIWH</td>
<td>1503.7</td>
<td>752.3/502.0</td>
<td>96%</td>
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<tr>
<td>3a</td>
<td>pa-VMNATMHIWH</td>
<td>1421.7</td>
<td>711.2/474.6/356.4</td>
<td>92%</td>
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<tr>
<td>3b</td>
<td>pa-VMNATMHIWH</td>
<td>1421.7</td>
<td>711.2/474.6</td>
<td>96%</td>
</tr>
<tr>
<td>3c</td>
<td>pa-VMNATMHIWH</td>
<td>1421.7</td>
<td>711.3/474.7/356.3</td>
<td>95%</td>
</tr>
<tr>
<td>3d</td>
<td>pa-VMNATMHIWH</td>
<td>1497.7</td>
<td>749.9</td>
<td>99%</td>
</tr>
<tr>
<td>3e</td>
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<td>457.9/686.1</td>
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</tr>
<tr>
<td>4a</td>
<td>pa-VmNAmFHIWH</td>
<td>1467.7</td>
<td>490.0/734.1</td>
<td>96%</td>
</tr>
<tr>
<td>5c</td>
<td>pa-VMHAHMTWIN</td>
<td>1421.7</td>
<td>711.5/474.6</td>
<td>99%</td>
</tr>
<tr>
<td>6f</td>
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<td>1622.7</td>
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<tr>
<td>7f</td>
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<td>1665.8</td>
<td>555.7/417.1</td>
<td>93%</td>
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<td>3c-FAM</td>
<td>FAM-βA-VMNATMHIWH</td>
<td>1773.7</td>
<td>885.9/591.2/443.8</td>
<td>94%</td>
</tr>
</tbody>
</table>
Fig. S1

The intensities of LC3 II are normalized to the negative control and quantified as a bar graph corresponding to Fig. 3. The relative LC3 II protein expression levels is quantified using ImageJ software from three independent experiments. Error bars represent SD.

Fig. S2

(A) The autophagy inducing activities of peptides were performed by western blot assay. (B) The intensities of LC3 II are normalized to the negative control and quantified as a bar graph. The relative LC3 II protein expression levels is quantified using ImageJ software from three independent experiments. Error bars represent SD.
Fig. S3 The intensities of LC3 II are normalized to the negative control and quantified as a bar graph corresponding to Fig. 4. The relative LC3 II protein expression levels is quantified using ImageJ software from three independent experiments. Error bars represent SD.

Fig. S4 Flow cytometry comparison of the cellular uptake efficiency of the FAM labelled
peptides 3-FAM, 3c-FAM, 7-FAM, 7f-FAM and FITC-TAT (5 μM, 4 h) in HeLa cells in the medium containing 5% fetal bovine serum. Peptides 3-FAM and 7-FAM are linear peptides corresponding to 3c-FAM and 7f-FAM.

Fig. S5

![Graph showing peptide stability over time](image)

Fig. S5. *In vitro* trypsin (1%) digestion assay. Peptide 3, 3c, and 7f (100 μM) incubated with trypsin (1%) at 37°C for different time intervals. The amounts of the peptides were examined by integration of reverse-phase HPLC.

Fig. S6

![Graph showing peptide stability over time](image)

Fig. S6. *In vitro* cell lysate stability assay. Peptide 3, 3c, and 7f (100 μM) were incubated with Hela cell lysate at 37°C for different time intervals. The amounts of the peptides were examined by integration of LC-MS.
Peptide 5c

Peptide 6f

Peptide 7f