Supporting Information

An orally active bradykinin B_1 receptor antagonist engineered as a bifunctional chimera of sunflower trypsin inhibitor

Yibo Qiu, Misako Taichi, Na Wei, Huan Yang, Kathy Qian Luo, and James P. Tam*

Contents

Experimental Section

Heat and pH stability assay

For the heat stability assay, all six peptides TIBA, SLBA, TIBA-L, SLBA-L, SFTI-1 and DALK were dissolved in a pH 7 phosphate buffer (0.1 M) at a final concentration of 0.1 mM. Each solution was subjected to 100 °C treatment in a PCR machine with heat-cap for 6 h. The same peptides were kept at 25 °C for the same duration as controls. At various time intervals, aliquots were taken and analyzed by Shimadzu Nexera UPLC. Fmoc-Gly-OH was used as an internal standard. The retention time and MS of the peptide were compared before and after heat treatment. Reactions were repeated 3 times. The peptide integrity was calculated based on the UPLC profile using the Lab Solution software.

For the pH stability assay, all six peptides TIBA, SLBA, TIBA-L, SLBA-L, SFTI-1 and DALK were subjected to 0.2 M aqueous HCl at a final concentration of 0.1 mM at 25 °C. The same peptides were dissolved in pH 7 phosphate buffer (0.1 M) for the same duration as controls. At various time intervals, aliquots were taken and analyzed by Shimadzu Nexera UPLC. Fmoc-Gly-OH was used as an internal standard. The retention time and MS of the peptide was compared before and after heat treatment. Reactions were repeated 3 times. The peptide integrity was calculated based on the UPLC profile using the Lab Solution software.

Pepsin and trypsin stability assay

Peptides (0.2 mg) together with pepsin or trypsin (8 µl, 0.5µg/µl in *Milli-Q* H_2O) were dissolved in 1 ml 100 mM sodium citrate buffer (pH 2.5) or 1 ml 100 mM ammonium bicarbonate (NH_4HCO_3) buffer (pH 7.8) with an enzyme peptide ratio of 1:50 and incubated at 37 °C. At various time intervals, aliquots were taken and analyzed by Shimadzu Nexera UPLC. Fmoc-Gly-OH was used as an internal standard. The retention time and MS of the peptide were compared before and after heat treatment. Reactions were repeated 3 times.

Serum stability assay

We used 25% human serum (Sigma Aldrich) in Roswell Park Memorial Institute (RPMI) medium to perform the serum stability assay. To remove the lipid components, serum was centrifuged at an rcf of 18,000 g, and then 1 ml supernatant was allocated to a 1.5 ml Eppendorf tube and incubated at 37 °C for 15 min before use. Peptide stocks were diluted in serum to make a final concentration of 1 mg/ml. At various time intervals, 100 µl aliquots were removed and added to 200 µl 90% ethanol to precipitate the serum proteins. Then the mixture was cooled at 4 °C then centrifuged at an rcf of 18,000 g for 10 min. The pellet was discarded and the

supernatant was analyzed by Shimadzu Nexera UPLC. Fmoc-Gly-OH was used as an internal standard. The retention time and MS of the peptide were compared before and after heat treatment. Reactions were repeated 3 times. The peptide integrity was calculated based on the UPLC profile using the Lab Solution software.

Trypsin inhibition assay and inhibition constant determination

To test the trypsin inhibition effect of peptides, we conducted a trypsin inhibition assay in 96-well plate with a total volume of 100 µl per well. Each well contained 94 µl of 0.05 M Tris-HCl containing 0.025 M $CaCl_2$ (pH 8) and 1 µl 0.5 µg/µl bovine β-trypsin (T1426, Sigma). First, peptides were diluted to each well to reach a concentration of 0-100 µM (0, 0.5, 1.0, 10.0, 50.0 and 100.0 µM). Then peptide and trypsin was incubated at 25 °C for 15 min. Just before monitoring by the spectrometer, 5 µl of 80 mM N_α-Benzoyl-DL-arginine 4-nitroanilide hydrochloride (BAPNA) was added to each well by a multi-channel pipette and the mixture was mixed well quickly. The process of the reaction was monitored by an ELISA microplate reader (Tecan-2) with an excitation wavelength of 405 nm. The reaction kinetic was measured at 37 °C over 60 min. The absorbance of each well at 405 nm was recorded and plotted against the time and concentration of each peptide. Negative control contained 94 µl Tris-HCl buffer, 1 µl 0.5 µg/µl trypsin and 5 µl substrate without addition of peptides. In blank, the mixture contained 95 µl Tris-HCl buffer, 5 µl substrate and trypsin was not added. All the reactions were repeated 3 times. The inhibition constant for SFTI-1 and TIBA was determined using the same reaction as mentioned above. Instead, SFTI-1 and TIBA were seriously diluted to a concentration of 0-31.25 nM (0, 1.95, 3.91, 7.81, 15.63 and 31.25 nM). Colorimetric trypsin substrate BAPNA was added using two different concentration of 40 mM and 60 mM. Each reaction was carried out as triplicates. The reciprocal of reaction velocity was expressed as $min*A405^{-1}$ and plotted against different concentration of inhibitors. Single regression line for each substrate was obtained and the Ki was calculated from the intersection of the two lines using Dixon plot.

Cytotoxicity

Hela cells were seeded into a 96-well plate with 15,000 cells per well. TIBA and SLBA were each diluted in Dulbecco's Modified Eagle Medium (DMEM) and 6% Fetal Bovine Serum (FBS), and 100 µl were transferred to each well. The positive control was loaded with 100 µl of 0.2% Triton X-100 from Sigma Aldrich in 100% Phosphate Buffered Saline (PBS). The negative control contained 100 µl DMEM with 6% FBS without peptide. The 96-well plate was then incubated at 37°C for 24 h. After 24 h, 10 µl of PrestoBlue™ Cell Viability Reagent was added to each well for 2 h. The 96- wells plate was then loaded into a fluorescent microplate reader (Tecan-2) to record its fluorescence with an excitation wavelength of 560 nm and an emission

wavelength of 590 nm at 10 nm bandwidth each. The fluorescence values were used to calculate the total viability of Hela cells.

Fluo-4 Direct calcium assay

Hela cells were seeded in 4-cm dishes for overnight. A dye loading buffer was prepared by adding 10 ml of Hanks' balanced salt solution with 20 mM HEPES buffer solution and 100 μl of 250 mM probenecid solution. DMEM was removed and 1 ml dye fluo-4 Direct^{TM} calcium dye (from Invitrogen) was added into each dish. Cells were incubated at 37 °C from 30 min. Then various concentrations of peptides (0.078, 0.156, 0.3125, 0.625, 1.25, 2.5, 5, 10 μM) were added and the reaction was incubated at 37 °C. Subsequently, the fluo-4 Direct^{TM} calcium dye was removed, and cells were washed by dye-loading buffer. Then 100 nM Des-Arg^{9}-BK as the B1 agonist which contained the antagonist peptides at corresponding concentrations was added into each dish. Upon addition of the agonist, 200 sequential images were immediately captured at an excitation wavelength of 480 nm and an emission wavelength of 535 nm. Measurements were given in increase folds of fluorescence intensity as the maximum response minus the minimum response divided by the minimum response.

Hot plate assay

Female ICR (Institute of Cancer Research) mice (18-22 g) were obtained from Laboratory Animal Services Center of Jiangsu University. All procedures complied with Regulations for the Administration of Affairs Concerning Experimental Animals and were approved by the Chinese Universities Animal Experimentation Ethics Committee (SCXK 2013-0011). Prior to the hot plate assay, mice were brought to the test room and allowed to acclimate to the environment for 30 min. Then they were placed into a transparent cylinder on a hot plate apparatus (model: YLS-6A, diameter: 200 mm) and their latencies to the pain response (licking hind paws) were recorded. Mice with long response time (more than 60 s) or extremely sensitive ones (less than 10 s) were excluded from the assay. Mice with normal latencies were then randomly divided into several groups with 6-8 mice each. Their body masses and basal latencies were recorded. They were then subjected to an *i. p.* injection of 100 μl either engineered peptide TIBA, positive control (DALK) or the vehicle (phosphate buffered saline, PBS, pH 7.4) at various doses (0.03 mg/kg, 0.1 mg/kg, 0.3 mg/kg and 1 mg/kg). After 10 min, mice were placed on a hot plate apparatus with the temperature set at 55 ± 0.1 °C. The time for each mouse to lick its hind paw was recorded and a cut-off time of 60 s was set to avoid tissue damage of mice. For oral administration, engineered peptide TIBA, positive control (DALK) or the vehicle (PBS) at various doses (0.3 mg/kg, 1 mg/kg, 3 mg/kg and 10 mg/kg) were administered by oral gavage (0.2 ml) and allowed to be absorbed for 30 min. Then,

the reaction time of each mouse was recorded in the same way as in the *i.p.* injection. The results were presented as the increment of latency (%), which was calculated as the following equation:

$$Increment\ of\ latency = \frac{tested\ latency\ (s) - basal\ latency\ (s)}{basal\ latency\ (s)} * 100\%$$

Each mouse has its own baseline latency, which is used as vehicle. Data (p values) were analyzed using a two-way ANOVA method by comparing groups treated with peptides and the vehicle. Results were expressed as mean ± S.E.M. (n=6-8)

NMR determination of structures for SLBA and TIBA

To determine the structures of SLBA and TIBA, 2D ^{1}H-^{1}H TOCSY and NOESY spectra were recorded for each of them using a Bruker 600 MHz NMR spectrometer (Bruker, IL, USA) equipped with a cryogenic probe. The concentration of peptide was around 1.2 mM. The temperature for the NMR experiments was 298K. For both of SLBA and TIBA, the lyophilized peptide was dissolved in miliQ water with 5% D2O (pH around 3.5). The mixing times were 80 and 200 ms for TOCSY and NOESY experiments respectively. The spectrum width was 12 ppm with the center of the spectrum at 4.735 ppm. All the spectra were processed using the software NMRpipe.[1]

The assignment of NOESY spectrum was done using the software Sparky 3.115.[2] For each amide proton HN_i, it should have cross peaks with the side chain protons of the previous residue i-1 in NOESY spectrum. In this way, the peaks in the NOESY spectrum were assigned. The distance restraints were extracted based on the intensities of the cross peaks in NOESY spectrum. The distance restraints were classified to three types according to the peak intensity: strong, $1 \leq d < 1.8$; medium, $1.8 \leq d < 3.4$; weak, $3.4 \leq d \leq 5$. The dihedral angle restraints were extracted based on the $^{3}J_{HN\text{-}H\alpha}$ splitting in 1D spectrum. If the splitting was larger than 8 Hz, the Φ angle was defined between -100° to -160°. Structure calculation was done using the software CNSsolve 1.3.[3] The structures generated were uploaded to the online server PDBsum (http://www.ebi.ac.uk/thornton-srv/databases/pdbsum/Generate.html) to generate Ramachandran plot. The structure was displayed by the software Chimera 1.6.2.[4]

Supporting data:

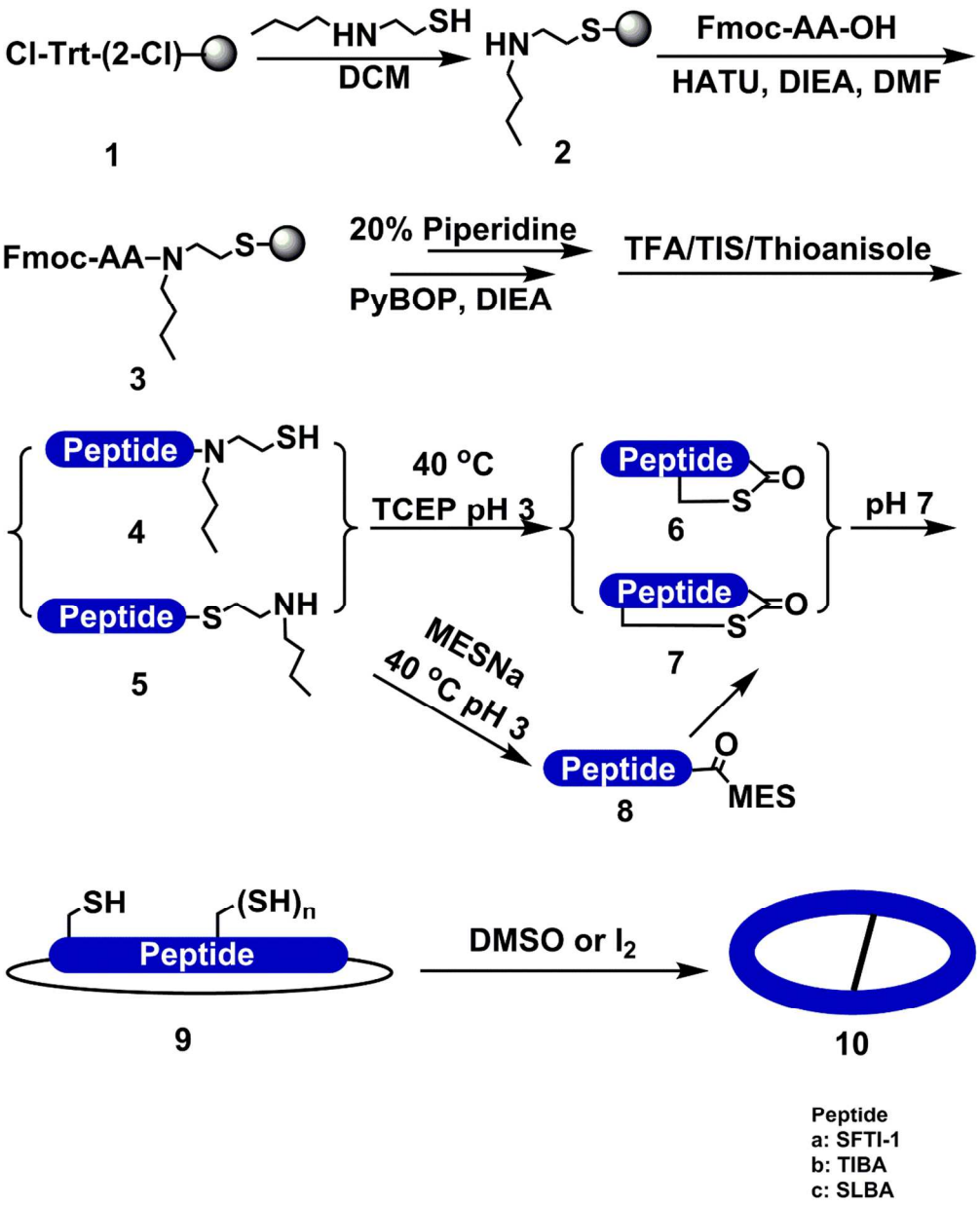

Figure S1. A scheme demonstrating the chemical synthesis of SFTI-1. Synthesis of TIBA and SLBA followed the same procedure. 2-(butylamino)ethanethiol (TEBA) was added to Cl-Trt(2-Cl) resin **1** to afford resin **2**. Then using HATU/DIEA, the first amino acid was introduced to the linker and followed by standard coupling using PyBOP/DIEA. After TFA cleavage, a crude peptide precipitation containing N and S form peptide-TEBA **4** and **5** was obtained and further transformed into thioester **8** or thiolactone **6, 7** in presence or absence of MESNa at pH 3. After the pH was adjusted to 7, S-N acyl shift reaction occurred spontaneously to afford cyclic reduced peptide **9**, which was further oxidized by DMSO to give cyclic peptide **10** with one disulfide bond.

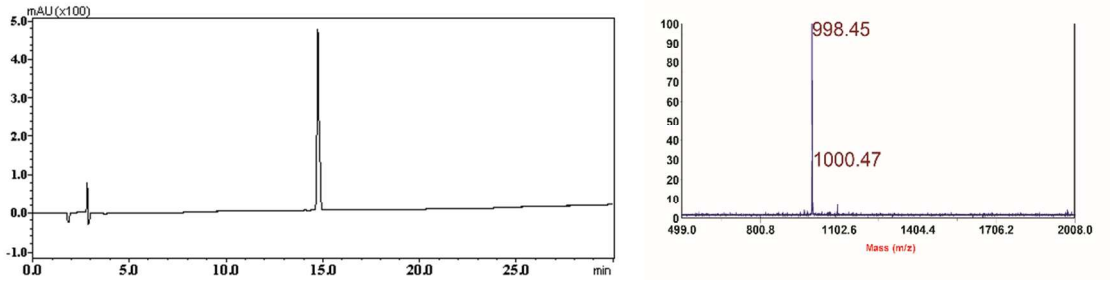

Figure S2. HPLC and MS profile of DALK. HPLC condition: 10-60% buffer B over 30 min at 1 ml/min (buffer A, 0.1% TFA in water; buffer B, 0.1% TFA in acetonitrile). C18 column (Phenomenex Aeris PEPTIDE XB-C18, 4.6 mm x 250 mm, 3.6 µm).

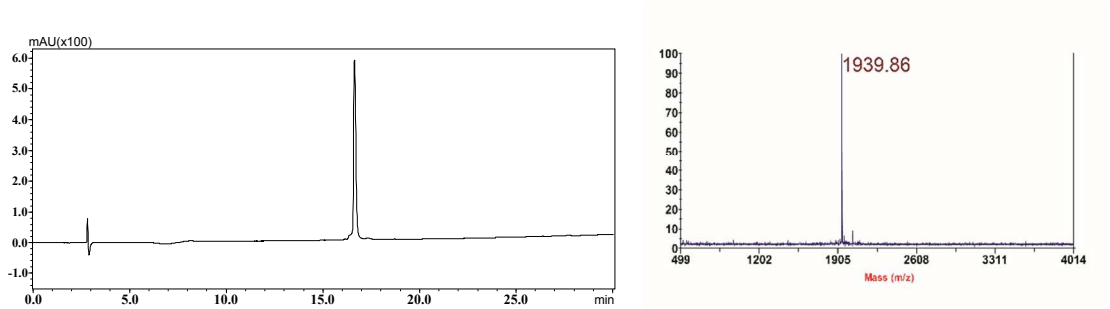

Figure S3. HPLC and MS profile of TIBA-L. HPLC condition: 10-60% buffer B over 30 min at 1 ml/min (buffer A, 0.1% TFA in water; buffer B, 0.1% TFA in acetonitrile). C18 column (Phenomenex Aeris PEPTIDE XB-C18, 4.6 mm x 250 mm, 3.6 µm).

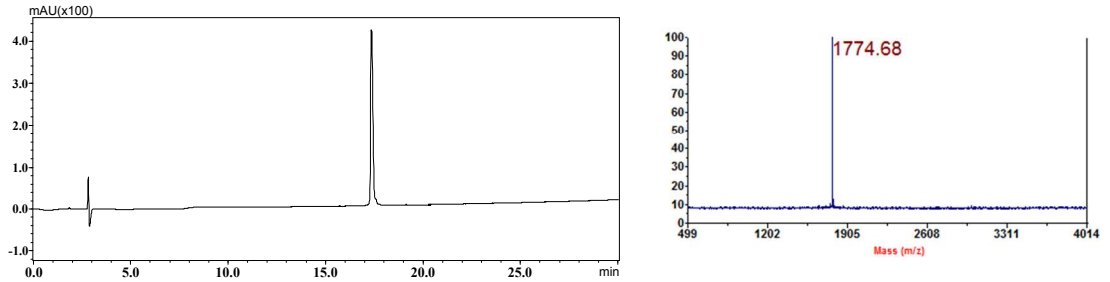

Figure S4. HPLC and MS profile of SLBA-L. HPLC condition: 10-60% buffer B over 30 min at 1 ml/min (buffer A, 0.1% TFA in water; buffer B, 0.1% TFA in acetonitrile). C18 column (Phenomenex Aeris PEPTIDE XB-C18, 4.6 mm x 250 mm, 3.6 µm).

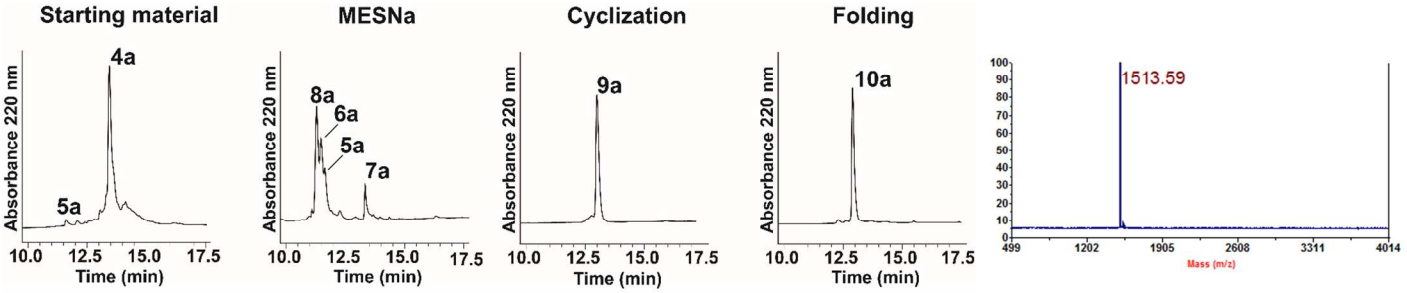

Figure S5. HPLC and MS profiles of synthetic intermediates in SFTI-1 synthesis. HPLC condition: 10-60% buffer B over 17.5 min at 0.3 ml/min (buffer A, 0.1% TFA in water; buffer B, 0.1% TFA in acetonitrile). C18 column (GRACE, VisionHT, C18, 100 mm x 2 mm, 1.5 µm).

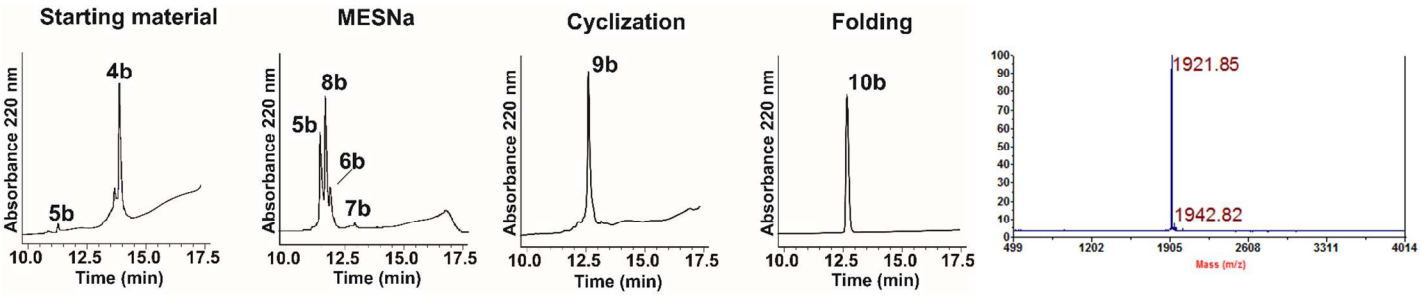

Figure S6. HPLC and MS profiles of synthetic intermediates in TIBA synthesis. HPLC condition: 10-60% buffer B over 17.5 min at 0.3 ml/min (buffer A, 0.1% TFA in water; buffer B, 0.1% TFA in acetonitrile). C18 column (GRACE, VisionHT, C18, 100 mm x 2 mm, 1.5 µm).

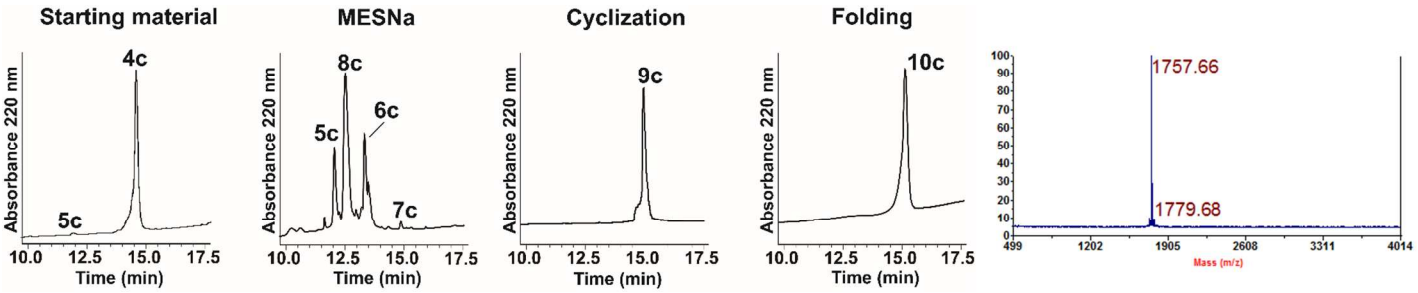

Figure S7. HPLC and MS profiles of synthetic intermediates in SLBA synthesis. HPLC condition: 10-60% buffer B over 17.5 min at 0.3 ml/min (buffer A, 0.1% TFA in water; buffer B, 0.1% TFA in acetonitrile). C18 column (GRACE, VisionHT, C18, 100 mm x 2 mm, 1.5 µm).

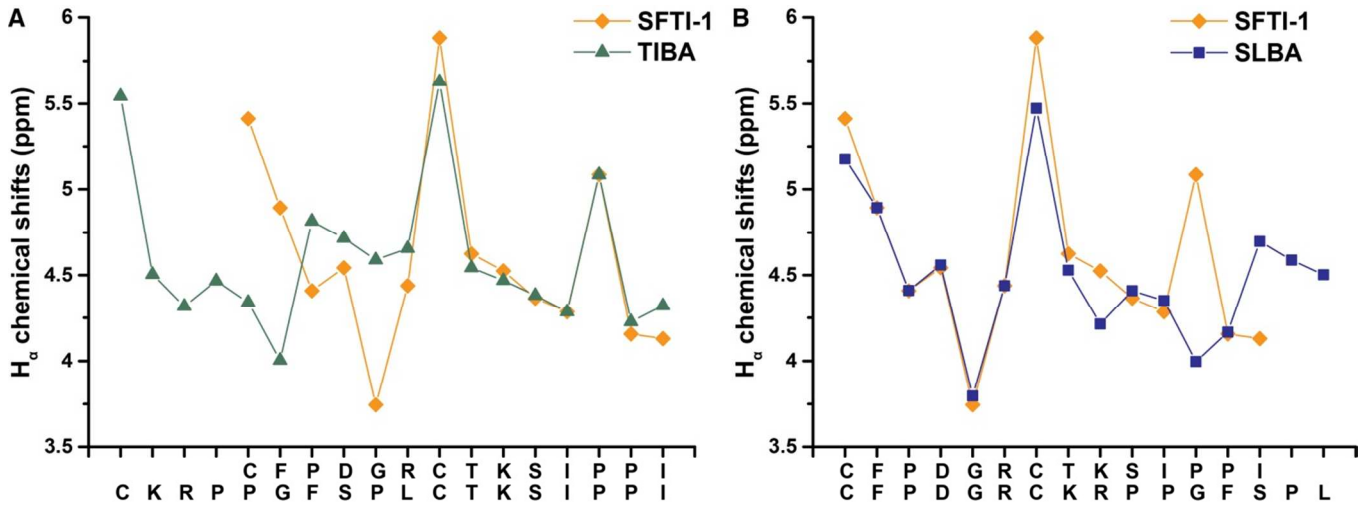

Figure S8. Chemical shift analysis of SFTI-1 (orange ♦), TIBA (green ▲) and SLBA (navy ■). Sequences for each peptide are aligned. Results showed that TI loop in TIBA and SL loop in SLBA overlapped nicely with corresponding part in SFTI-1.

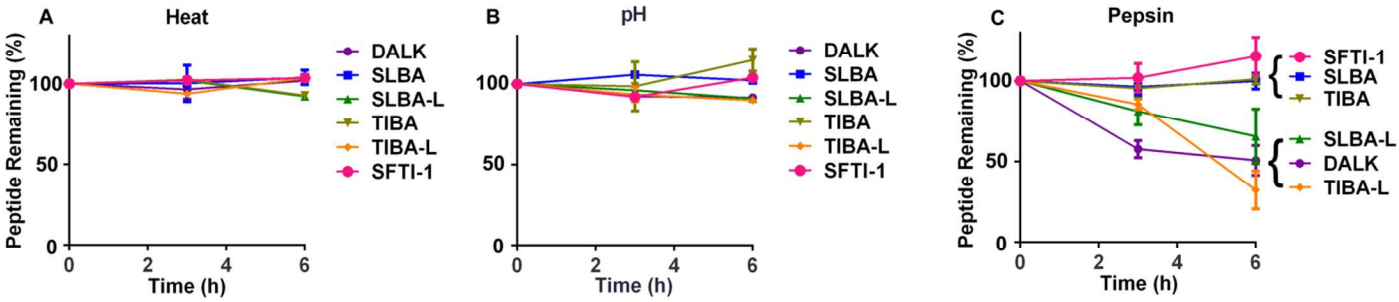

Figure S9. Heat, low pH, pepsin and trypsin stability of engineered peptides. Peptide stability of DALK (●), SLBA (■), SLBA-L (▲), TIBA (▼), TIBA-L (♦) and SFTI-1 (●) under A) heat (100 °C), B) low pH (0.2 M HCl), and C) pepsin digestion conditions. The Y axis represents the relative amount of peptides remaining at each time points compared to the starting time point (0 h). Experiments are repeated in triplicates and results are shown as mean ± SD.

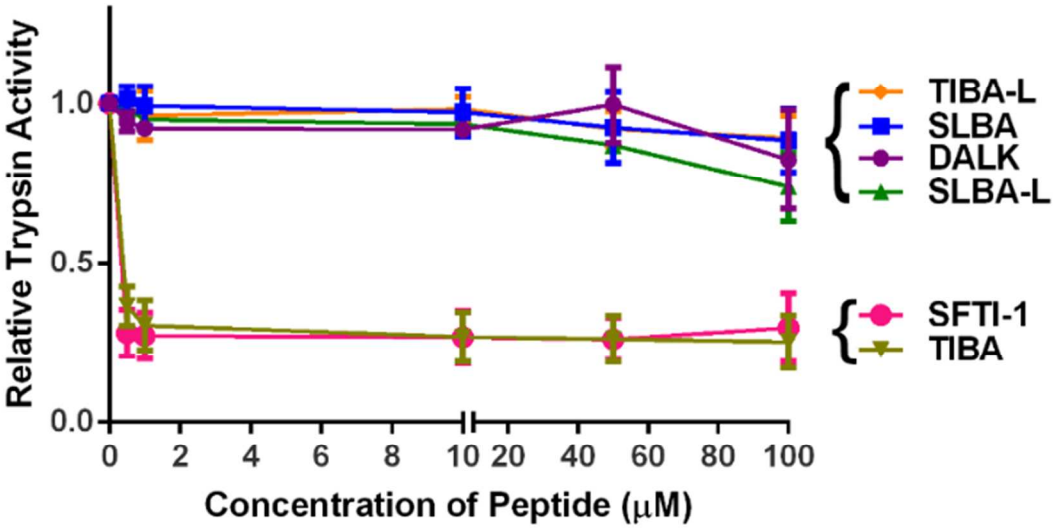

Figure S10. Trypsin inhibition activities of engineered peptides. Trypsin inhibitory assay was performed on DALK (●), SLBA (■), SLBA-L (▲), TIBA (▼), TIBA-L (♦) and SFTI-1 (●).Experiments are repeated in triplicates and results are shown as mean ± SD.

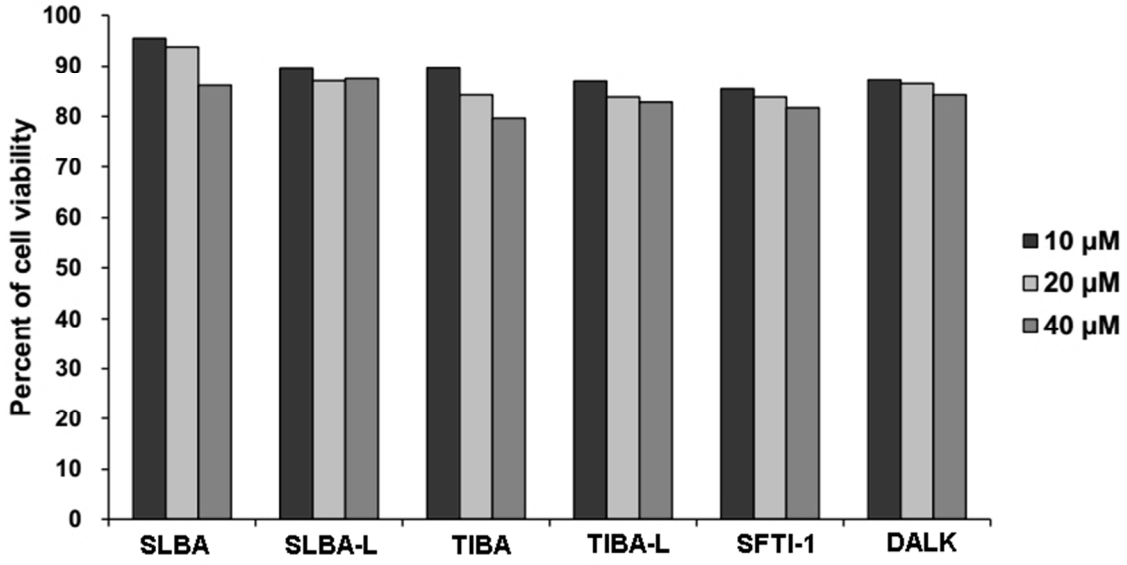

Figure S11. Cytotoxicity of SLBA, SLBA-L, TIBA, TIBA-L, SFTI-1 and DALK against HeLa cell. The cell amount in the control group without adding in any peptides was determined as 100% cell viability.

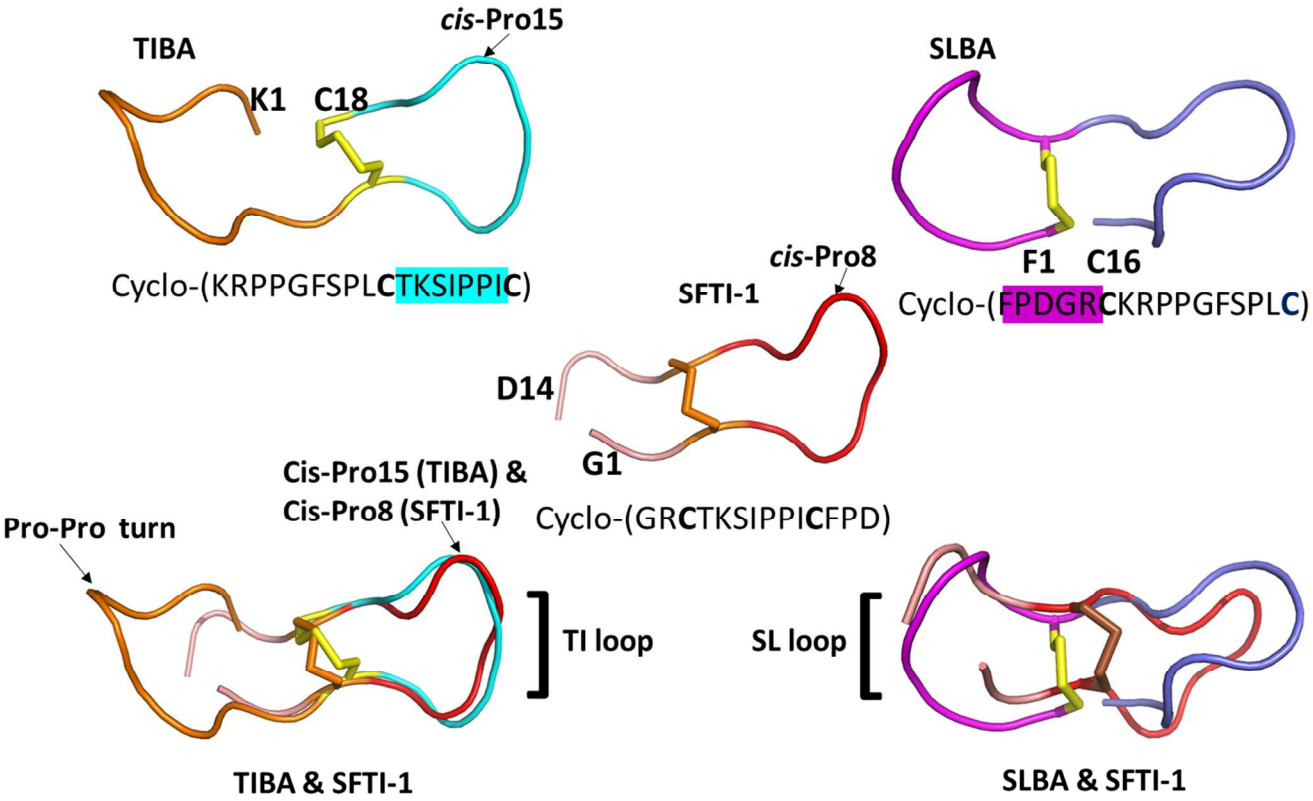

Figure S12. Overlay structures of TIBA, SLBA and SFTI-1 shown as cartoon. Cyclic backbones are not graphically closed in all structures. The TI loop of TIBA is highlighted in cyan and the SL loop of SLBA is highlighted in magenta. The overlay structures of TIBA and SFTI-1 clearly showed the overlapping of TI loop in each peptide. The *cis*-pro15 in TIBA and *cis*-pro8 in SFTI-1 overlap perfectly. The Pro^4-Pro^5 dipeptide in the DALK region of TIBA (indicated by the arrow) exists in a turned structure

Table S1. Parameters and restrains of structure calculation of TIBA and SLBA.

Experimental Restraints and Structural Statistics of 20 Lowest-Energy Structures of SLBA and TIBA among the 100 Structures Generated by CNSsolve 1.3		
	SLBA	*TIBA*
NMR Distance Restraints	*64*	*95*
Intra-Residue NOE (|i-j|=0)	*34*	*48*
Sequential NOE (|i-j|=1)	*21*	*32*
Medium-Range NOE (1<|i-j|≤5)	*3*	*3*
Long-Range NOE (|i-j|>5)	*8*	*12*
Dihedral Angle Restraints	*4*	*8*
Structural Statistics (16 residues, C^1-L^{16})		
Violations per Structure		
NOE Violation (Å)	*0.035±0.006*	*0.039±0.003*
Diheral Angle Violation (°)	*0.454±0.113*	*0.235±0.119*
Ramachandran Plot Region (41 residues)		
Residues in Most Favored Regions	*5 62.5%*	*5 50%*
Residues in Additional Allowed Regions	*3 37.5%*	*5 50%*
Residues in Generously Allowed Regions	*0 0%*	*0 0%*
Residues in Disallowed Regions	*0 0%*	*0 0%*
Number of End-Residues (excl. Gly and Pro)	*2*	*2*
Number of Glycine Residues	*2*	*1*
Number of Proline Residues	*4*	*5*
Mean RMSD from the Average Coordinates (C^1-L^{16} of SLBA, C^1-L^{18} of TIBA)		
Backbone Atoms(Å)	*2.00±0.61*	*2.61±1.00*
Heavy Atoms(Å)	*3.38±0.82*	*3.90±1.23*

Reference:

(1) Delaglio, F.; Grzesiek, S.; Vuister, G. W.; Zhu, G.; Pfeifer, J.; Bax, A. NMRPipe: a multidimensional spectral processing system based on UNIX pipes. *J Biomol NMR* **1995,** *6(3)*, 277-293.
(2) Goddard, T. D.; Kneller, D. G. Sparky 3. In University of California, San Francisco.
(3) Brunger, A. T.; Adams, P. D.; Clore, G. M.; Delano, W. L.; Gros, P.; Grosse-Kunstleve, R. W.; Jiang, J. S.; Kuszewski, J.; Nilges, M.; Pannu, N. S.; Read, R. J.; Rice, L. M.; Simonson, T.; Warren, G. L. Crystallography & NMR system: A new software suite for macromolecular structure determination. *Acta Crystallogr* **1998,** *54*, 905-921.
(4) Huang, C. C.; Couch, G. S.; Pettersen, E. F.; Ferrin, T. E. Chimera: An Extensible Molecular Modeling Application Constructed Using Standard Components. In *Pac Symp Biocomput*, 1996; Vol. 1, p 724.