Electronic Supplementary Materials

Cyclization of a G4-specific peptide enhances its stability and G-quadruplex binding affinity

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Experimental Methods

Cyclic peptide synthesis

The linear *Rhau23* peptide was synthesized by Chempeptide (Shanghai, China) with >95% purity. The cyclization reaction was performed in potassium phosphate buffer (20 mM KPi, pH 6.0) containing 1 µM OaAEP1 and 50 µM linear *Rhau23* peptide at 37°C for 2 hours. The reaction was stopped by adding 10 µl of 1 M HCl and purified by HPLC system (Shimadzu) using a reversed-phase C18 column using the gradient of acetonitrile according to 10-50% for 40 minutes or 5-25% in 20 minutes following by 25-30% for another 10 minutes. The elution peaks were collected and analysed by MALDI-TOF Mass Spectrometry (ABI 4800 MALDI TOF/TOF). The correct fraction containing cyclic peptide was lyophilized and diluted to the desired concentration in de-ionized water.

Circular dichroism (CD) spectroscopy

The samples were prepared in 400 µl solution containing 40-50 µM peptide in 90% TFE. The mixtures were incubated at room temperature for 60 minutes before starting the measurements. The CD spectra were recorded by J-815 CD spectrometer (Jasco) in 1 mm path-length cuvette at 20^{o}C. The CD signal was scanned from 180 nm to 280 nm with 200 nm/min scanning speed and 2 nm bandwidth. The baseline was taken from 400-µl of 90% TFE before each measurement. The spectra were accumulated 10 times and analysed by Spectra Analysis (Jasco). The CD signal was normalized according to the peptide concentration calculated using its extinction coefficient of 6990 $M^{-1}.cm^{-1}$ at 280 nm (https://web.expasy.org/cgi-bin/protparam/protparam) and displayed in molar ellipticity.

Isothermal titration calorimetry (ITC)

All peptides and DNA samples were prepared in the same buffer containing 20 mM KPi, pH 6.8. The DNA sample was annealed by heating at 95^{o}C and slowly cooling down to room temperature in the water bath. The ITC measurements were performed by MicroCal iTC200 (Malvern). Each titration experiment was performed by adding 2 µl of 250 µM peptide into the sample cell containing 15 µM DNA *T95-2T*. Sample temperature was maintained at 25^{o}C. Each titration includes 19 injections. The control experiment was done by titration of 250 µM peptide into buffer containing 20 mM KPi pH 6.8. The data were analysed to obtain the binding affinities using the MicroCal PEAQ-ITC Analysis Software (Malvern).

Exopeptidase stability assay

The stability of the linear and cyclic peptides against exopeptidase were performed in the reaction buffer (25 mM Tris-HCl, 0.5 M NaCl, pH 7.5) containing 25 µM of the tested peptides and 0.5 µM carboxypeptidase A (Sigma Aldrich, USA). The mixtures were incubated at 37°C. After 1, 2, 5, 10, 20, 40, and 60 minutes, 50 µl of the reaction was taken and quenched by adding 5 µl HCl 1 M. The reactions were monitored by HPLC at 280 nm using the gradient of acetonitrile according to 5-25% in 20 minutes and 25-30% in 10 minutes. The integration of elution peaks was calculated and converted into percentage.

Electrophoretic mobility shift assay

The DNA samples were prepared in desired buffer (Table S2) and annealed as described in ITC experiment. The DNA and peptide were mixed at final concentration 100 μM and 200 μM, respectively, and incubated for 30-60 minutes at room temperature. Sucrose was added to the samples at 8 % final concentration before loading into 10-20 % native gels (37.5:1 acrylamide/bis-acrylamide) containing 1X TBE supplement with 100 mM KCl or 100 mM NaCl. The gels were run in 1X TBE, supplemented with 100 mM KCl or 100 mM NaCl at 20°C or 4°C, 50 V, for 60-400 minutes. DNA was visualized by UV shadowing (AlphaImager HP).

Cytotoxicity assays

The A549 (lung cancer) cell line was used in the cytotoxicity test. The cell was first seeded at $3x10^3$ cells onto 96-well plate and grown for 24 hours, 37°C, 5% CO_2. Then, peptides were added at 100 μM final concentration and incubated for another 24 hours. The cell viability was tested by Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc) following the procedure from the manufacturer. 0.5% SDS was used as a positive control for the cytotoxicity[1]. Cell viability in percentage was calculated according to the viability of untreated cells.

Supplementary Tables

Table S1 Thermodynamic profile of the binding of linear *Rhau23* and cyclic *Rhau23* with *T95-2T*.

Name	N (sites)	K_D (nM)	ΔH (kJ/mol)	ΔG (kJ/mol)	-TΔS (kJ/mol)
Rhau23	1.79	678 ± 271	-82	-35.2	46.7
c-*Rhau23*	1.97	129 ± 48	-55.8	-39.4	16.4

Table S2 DNA sequences were used in this study.

Name	Sequence (5'–3')	Annealing buffer	Structure
T95-2T	TTGGGTGGGTGGGTGGGT	20 mM KPi, 100 mM KCl, pH 7	parallel G4[2]
Human telomeric	TTGGGTTAGGGTTAGGGTTAGGGA	20 mM KPi, 100 mM KCl, pH 7	(3 + 1) G4[3]
Oxytricha telomeric	GGGGTTTTGGGG	20 mM NaPi, 100 mM NaCl, pH 7	(2 + 2) G4[4]
$d(CG)_6$	CGCGCGCGCGCG	20 mM NaPi, 100 mM NaCl, pH 7	duplex[5]

Supplementary Figures

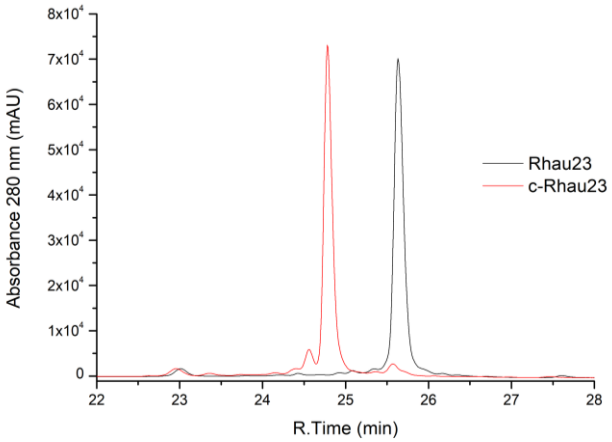

Fig. S1 HPLC profile of *Rhau23* cyclization by OaAEP1. The 50 μM linear *Rhau23* peptide was incubated at 37°C for 2 hours in absence (black line) and presence (red line) of 1 μM OaAEP1 enzyme. The HPLC results showed, in presence of OaAEP1, more than 95% precursor peak has been shifted.

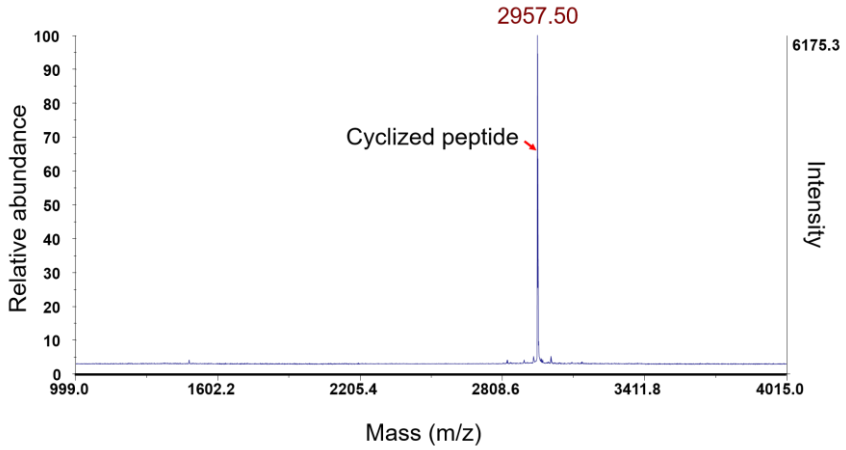

Fig. S2 Mass spectroscopy profile of cyclized product.

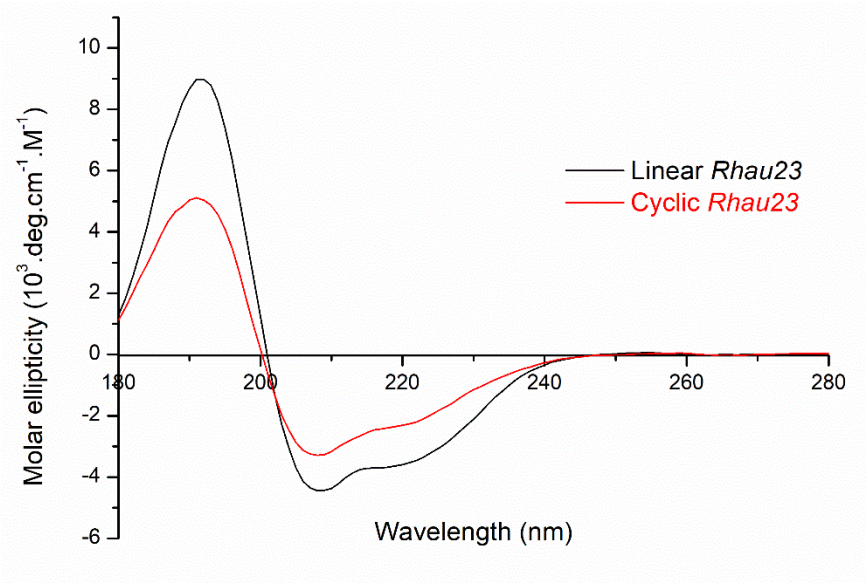

Fig. S3 Circular Dichroism spectra of linear and cyclic *Rhau23* peptides.

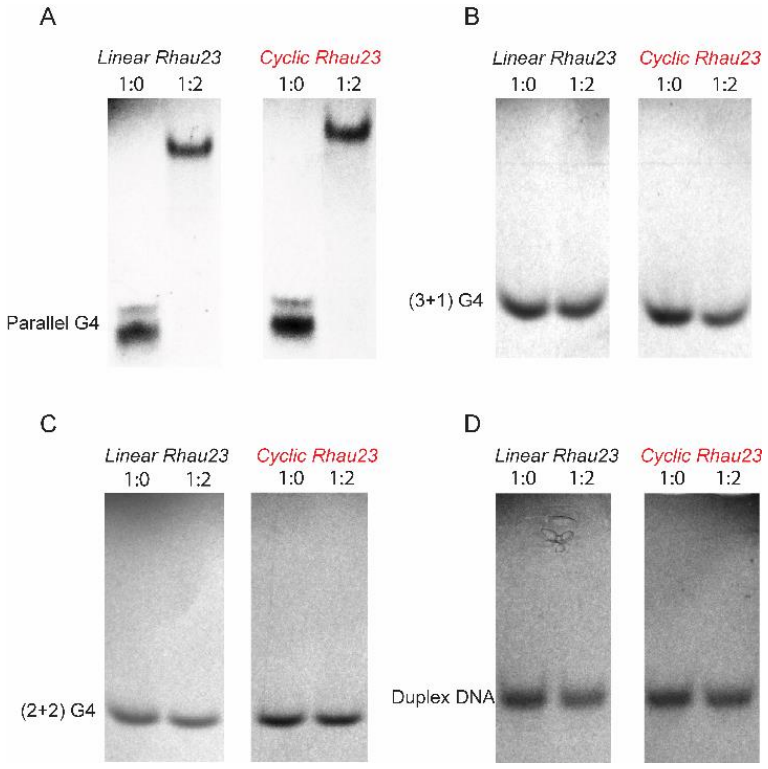

Fig. S4 EMSA for binding of linear and cyclic *Rhau23* peptides with different DNA structures: (A) Parallel G4, (B) (3 + 1) hybrid G4, (C) (2 + 2) antiparallel G4, (D) DNA duplex. DNA concentration was fixed at 100 μM. Peptides were mixed with DNA at the DNA:peptide molar ratio of 1:2. The parallel, (3 + 1), (2 + 2) G4s, and duplex DNA were formed by *T95-2T*, human telomeric, *Oxytricha* telomeric, and $d(CG)_6$ sequences, respectively. DNA sequences are shown in Table S2.

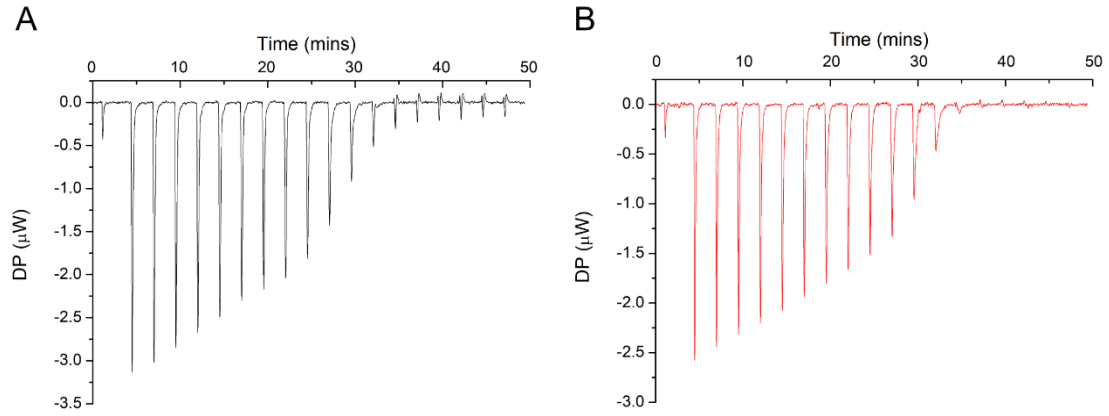

Fig. S5 Heat data of ITC titration experiments. A-B Titration of *Rhau23* and *c-Rhau23* with *T95-2T*, respectively. The heat changes in peptide-to-DNA was subtracted by peptide-to-buffer titration.

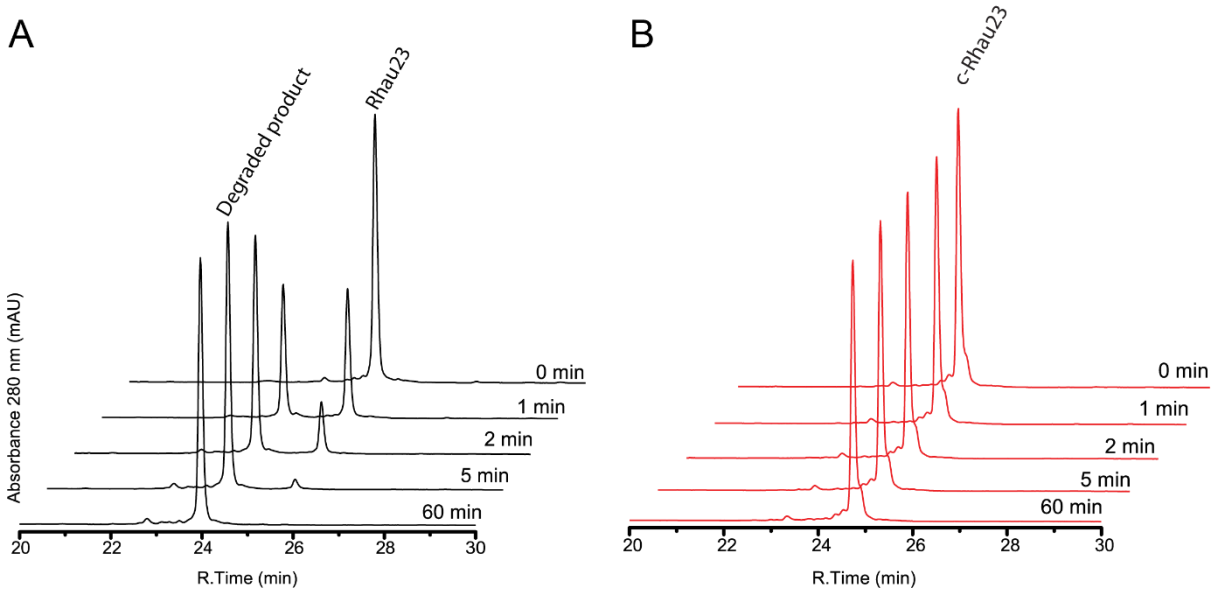

Fig. S6 Enzymatic stability of cyclic *Rhau23* against carboxylpeptidase A. HPLC analysis stability of *Rhau23* (A) and *c-Rhau23* peptides (B) against carboxypeptidase A at 0, 1, 2, 5 and 60 minutes. The degradation of linear peptide was stopped at Pro25, due to very little or no activity of carboxylpeptidase A with Proline, producing an in-completed degraded product. The enzymatic stability assay was tested within pH range from 4.5-7.5.

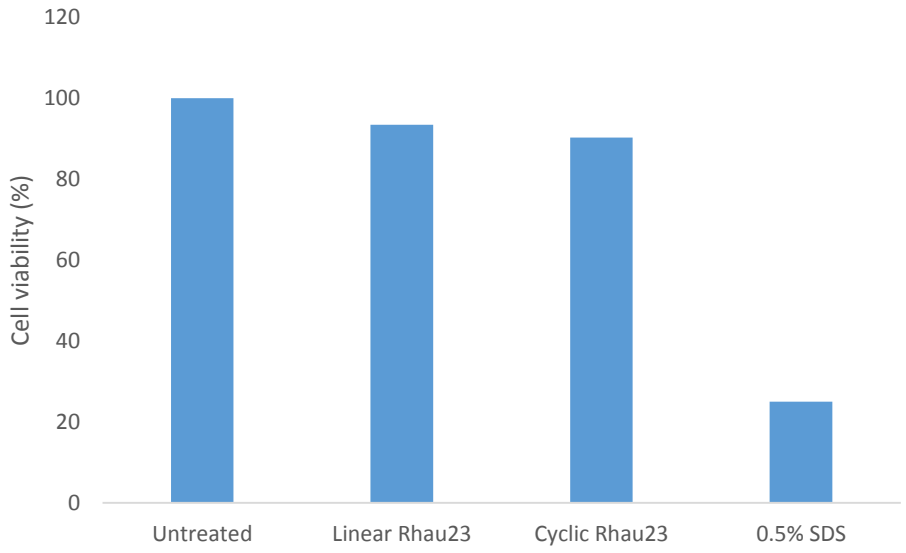

Fig. S7 Cytotoxicity of linear and cyclic *Rhau23* peptides on the A549 cell line. Cells were treated with 100 µM linear and cyclic peptides for 24 hours before cytotoxicity test was taken. Untreated and 0.5% SDS treated cells were used as the negative and positive control, respectively.

References

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