Supporting Information

Helix Stabilized, Thermostable, and Protease-Resistant Self-Assembled Peptide Nanostructures as Potential Inhibitors of Protein-Protein Interactions

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(1) Monomeric $p53_{17\text{-}28}$ peptide

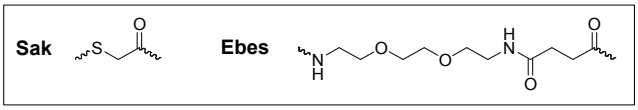
H-GETFSDLWKLLPEC-NH_2

(2) Fluorescein-$p53_{17\text{-}28}$

FAM-GETFSDLWKLLPEC-NH_2

(3) A model peptide with a linker equivalent to the length of ~6 amino acids

cyclo[-(Sak)-(Ebes)-WKWEWYWKWEW-(Ebes)-SAEAAAKEAAAKAC-]



(4) A model peptide with a linker equivalent to the length of ~5 amino acids

cyclo[-(Sak)-GSGWKWEWYWKWEW-(Ebes)-AEAAAKEAAAKAC-]

(5) A model peptide with a linker equivalent to the length of ~4 amino acids

cyclo[-(Sak)-SGWKWEWYWKWEWSGSGAEAAAKEAAAKAC-]

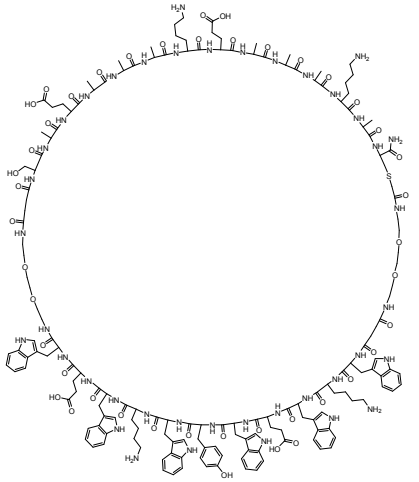
(6) p53 *g*-peptide

cyclo[-(Sak)-SGWKWEWYWKWEWSGSGETFSDLWKLLPEC-]

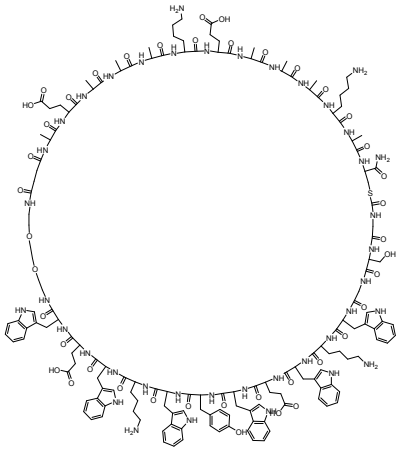
(7) Oligo(EG)-p53 *g*-peptide

cyclo[-(Sak)-SGWKWEWYWKWEW-{ε-K[α-$(Ebes)_3$]}-SGETFSDLWKLLPEC-]

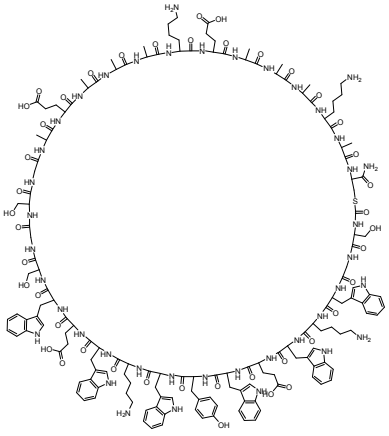
Figure S1. Sequence of peptides.



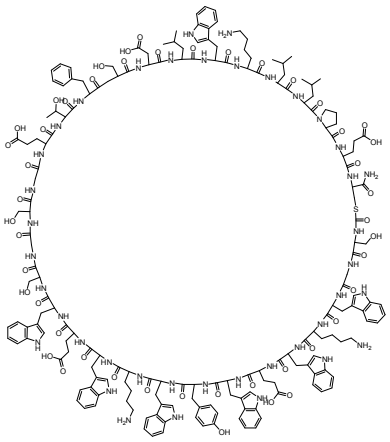
(3) A model peptide with a linker equivalent to the length of ~6 amino acids



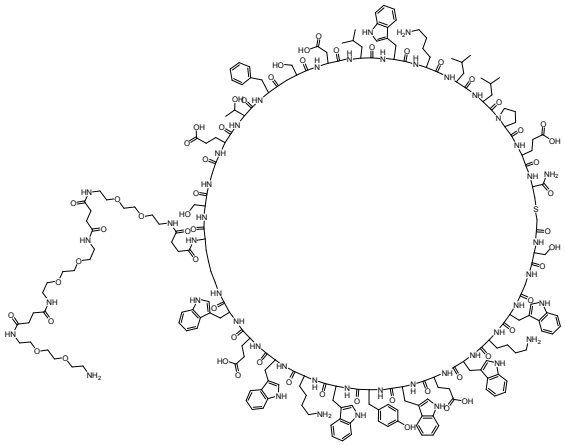
(4) A model peptide with a linker equivalent to the length of ~5 amino acids



(5) A model peptide with a linker equivalent to the length of ~4 amino acids



(6) p53 *g*-peptide



(7) Oligo(EG)-p53 *g*-peptide

Figure S2. Chemical structures of cyclic peptides.

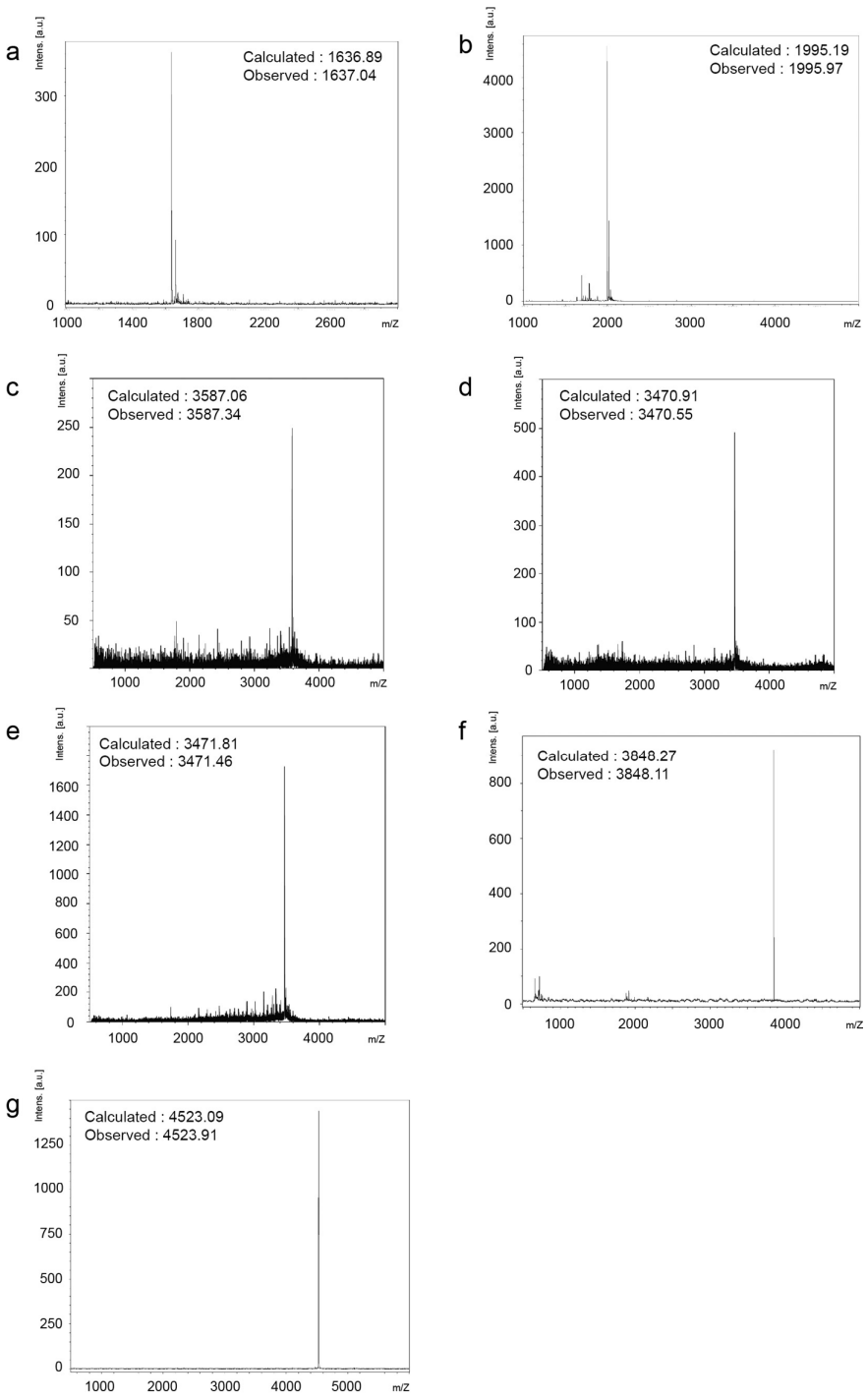


***Figure S3*.** MALDI-TOF MS spectra. **a**, Monomeric $p53_{17\text{-}28}$ peptide. **b**, Fluorescein-$p53_{17\text{-}28}$. **c**, A model peptide with a linker equivalent to the length of ~6 amino acids. **d**, A model peptide with a linker equivalent to the length of ~5 amino acids. **e**, A model peptide with a linker equivalent to the length of ~4 amino acids. **f**, p53 *g*-peptide. **g**, Oligo(EG)-p53 *g*-peptide.

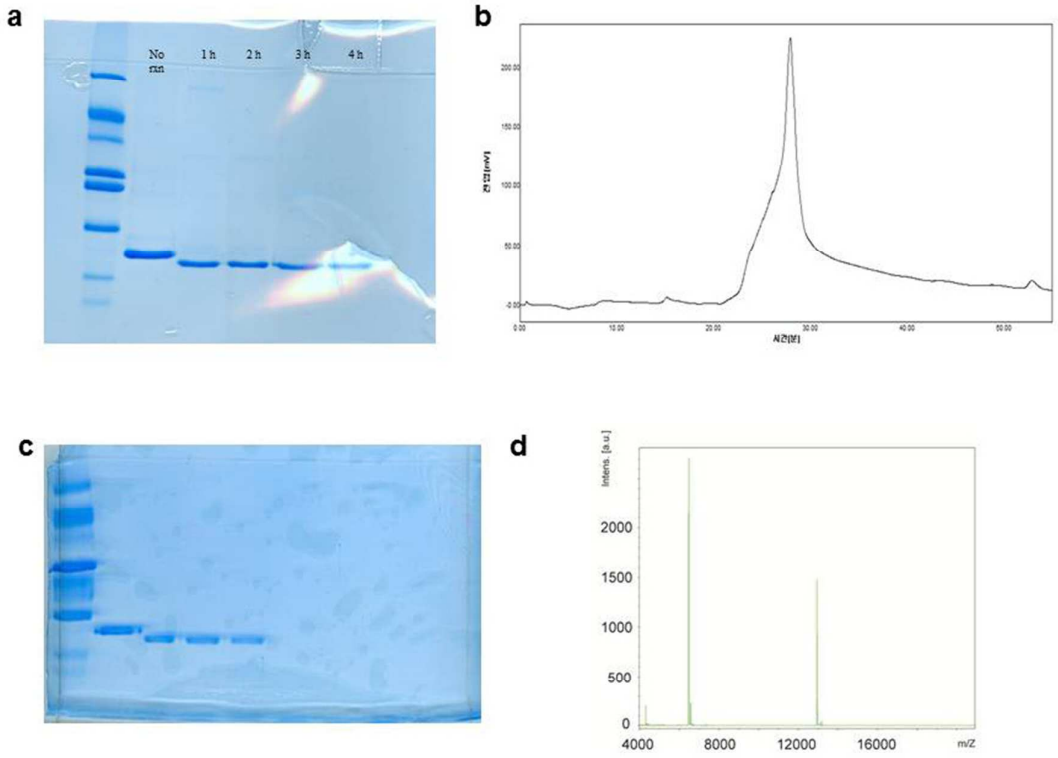


Figure S4. His_6-tagged MDM2 protein expression and purification of tag-free MDM2. **a**, Thrombin cleavage reaction to remove His_6-tag from the expressed protein. **b**, Purification of MDM2 protein from the thrombin cleavage reaction mixture by gel filtration chromatography. **c**, SDS-PAGE of purified MDM2 protein. **d**, MALDI-TOF MS spectrum of purified MDM2 protein.

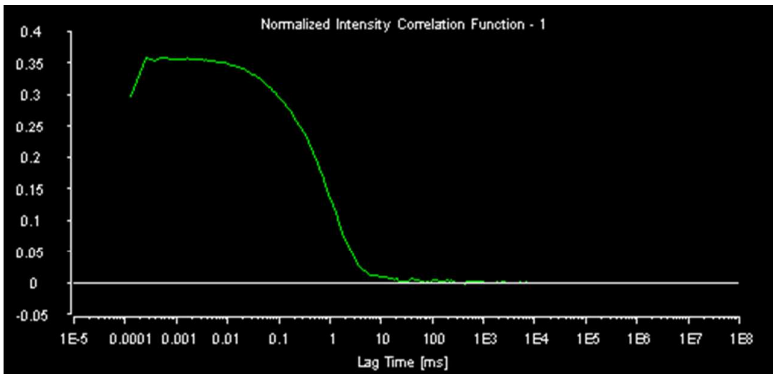


Figure S5. DLS autocorrelation function for p53 αSSPN.

EXPERIMENTAL SECTION

Peptide synthesis and cyclization procedures

Peptides were synthesized on a Rink Amide MBHA resin LL (Novabiochem) using standard Fmoc protocols on a Tribute™ peptide synthesizer (Protein Technologies, Inc). Standard amino acid protecting groups were employed except Cys(Mmt) and Lys(Dde), in which an acid-labile methoxytrityl (Mmt) and *N*-[1-(4,4-dimethyl-2,6-dioxocyclohex-1-ylidene)ethyl] (Dde) groups were used, respectively. The oligoethylene glycol-based linker, *N*-(Fmoc-8-amino-3,6-dioxaoctyl)succinamic acid (Fmoc-PEG2-Suc-OH or Fmoc-Ebes-OH), was purchased from Anaspec.

For cyclization, the peptide-attached resin (20 μmol of N-terminal amine groups) was swollen in *N*-methyl-2-pyrrolidone (NMP) for 30 min. Then, bromoacetic acid was first coupled to the N-terminal part of the resin-bound peptide. Before addition to the resin, a mixture of bromoacetic acid (28 mg, 200 μmol) and *N*,*N*′-diisopropylcarbodiimide (15.5 μL, 100 μmol) in NMP was incubated for 10 min for carboxyl activation. Following addition of the mixture to the resin, the reaction was continued for 1 h with shaking at room temperature, in a 6 mL polypropylene tube with a frit (Restek). The resin was then washed successively with NMP and dichloromethane (DCM). For orthogonal deprotection of the Mmt group from the cysteine, the resin was treated with 1% trifluoroacetic acid (TFA) in DCM several times (1 min × ~7). Intramolecular cyclization reaction was performed in 3 mL of 1% diisopropylethylamine (DIPEA) in NMP overnight with shaking at room temperature. The resin was then successively washed with NMP and DMF.

Deprotection of Dde from Lys(Dde) was performed in 2% hydrazine in DMF. Then coupling of the solubility enhancer, Fmoc-Ebes-OH, was followed using standard Fmoc protocols.

For cleavage and final deprotection, the resin was treated with cleavage cocktail (TFA:1,2-ethanedithiol:thioanisole; 95:2.5:2.5) for 3 h, and was triturated with *tert*-

butyl methyl ether (TBME). The peptides were purified by reverse-phase HPLC (water–acetonitrile with 0.1% TFA). The molecular weight was confirmed by MALDI-TOF mass spectrometry. The purity of the peptides was >95% as determined by analytical HPLC. The peptide concentration was determined spectrophotometrically in 8 M urea using a molar extinction coefficient of tryptophan (5,500 $M^{-1}cm^{-1}$) at 280 nm.

Circular dichroism (CD) spectroscopy

CD spectra were measured using a Chirascan™ circular dichroism spectrometer equipped with a Peltier temperature controller (Applied Photophysics). The spectra were recorded from 260 to 190nm using a 2 mm path-length cuvette. Scans were repeated five times and averaged. Molar ellipticity was calculated per amino acid residue.

Dynamic light scattering

DLS experiments were performed at room temperature using an ALV/CGS-3 compact goniometer system equipped with a He-Ne laser operating at 632.8 nm. The detector optics employed optical fibers coupled to an ALV/SO-SIPD/DUAL detection unit, which employed an EMI PM-28B power supply and an ALV/PM-PD preamplifier/discriminator. The signal analyzer was an ALV-5000/E/WIN multiple-tau digital correlator with 288 exponentially spaced channels. The scattering angle was 90º. The size distribution was determined using a constrained-regularization method.

Fourier transform infrared spectroscopy (FT-IR)

For FT-IR measurement, 100 µL of the sample (50 µM in water) was cast from the solution onto ZnSe window. Three thousand scans were acquired on a Bruker VERTEX 70 FT-IR spectrometer.

Atomic force microscopy (AFM)

For AFM, typically 2 μL of the sample in water was deposited onto a freshly cleaved mica surface and dried in air for overnight. The images were obtained in tapping mode with a Nanoscope IV instrument (Digital Instruments). AFM scans were taken at setpoint of 0.8-1 V and scanning speed was 1-2 Hz.

Transmission electron microscopy (TEM)

Two μL samples were placed onto a carbon-coated copper grid and dried completely. Then 2 μL of 2 % (w/v) uranyl acetate solution was added for 1 min and excess solution was wicked off by filter paper. Sample concentrations were typically 5-20 μM. The specimens were observed using a JEOL-JEM 2010 instrument operating at 120 kV. The data were analyzed using DigitalMicrograph™ software.

Protein expression and purification

The six His (His_6)-tagged, recombinant human MDM2 (hDM2) protein (N-terminal domain spanning amino acids 17-125) was overexpressed in *E. coli*. The plasmid pET28a-hMDM2(17-125) [Kanamycin-resistance (Kan^R), kindly provided by Dr. Gregory Verdine, Harvard.] expressing the truncated human hDM2 (a.a. 17-125) with N-terminal six His (His_6)-tag, together with thrombin cleavage site was introduced into *E. coli* strain BL-21-Codon Plus (DE3)-RIPL [Chloramphenicol-resistance (Cm^R), Agilent Technologies] using standard chemical transformation method. The transformed single colony was cultured in LB media with the drugs Cm (25 μg/mL) and Kan (20 μg/mL) at 30 °C until optical density (OD) at 600 nm reaches ~0.5, then treated with isopropyl β-D-thiogalactoside (IPTG; 0.1 mM) at 16 °C for 18 h to induce the recombinant protein. The *E. coli* was collected by spinning the culture at 6000 rpm (Sorvall) for 10 min at 4 °C.

All the purification steps were performed at 4 °C, unless specified otherwise. The *E. coli* cell pellet was resuspended in the lysis buffer [20 mM Tris·HCl pH 8.0, 500 mM NaCl, 10% glycerol, 20 mM imidazole, 0.1% (v/v) NP-40, 5 mM β-mercaptoethanol] with protease inhibitor cocktail (1 mM PMSF, 10 μg/ml aprotinin, 5 μg/ml pepstatin and 5 μg/ml leupeptin) and treated with lysozyme (1 mg/ml) for 45 min to lyse the bacterial cell wall. Then the resuspended cells were sonicated to break the cells and the viscous genomic DNA until the cell lysate is not viscous any more. After removing cell debris by centrifugation at 14, 000 × g for 10 min, the recombinant protein from the cell lysate was purified in the lysis buffer using Ni-NTA agarose beads following the standard purification protocol provided by the vendor (Qiagen), except the elution which was performed in the lysis buffer with 250 mM imidazole.

For the removal of His_6-tag from the recombinant human MDM2 protein, 0.5 U of

thrombin was mixed with 20 μg of the hDM2 protein in thrombin cleavage buffer (50 mM Tris·HCl pH 7.5, 150 mM NaCl, 2.5 mM $CaCl_2$). The sample was incubated at 4 °C overnight. Progress of the cleavage reaction was monitored by SDS-PAGE. The sample was subjected to gel filtration chromatography using a Superdex 75 column at 4 °C. Elution buffer was 50 mM Tris pH 8.0, 140 mM NaCl. The sample purity and identity were confirmed by SDS-PAGE and MALDI-TOF mass spectrometry (Supplementary Fig. 4). The concentration of purified MDM2 protein was determined by Micro BCA protein assay (Pierce).

Interaction and competition assay

The protein-protein interaction assays were performed using a fluorescence polarization (FP) assay in a buffer containing 50 mM Tris pH 8.0, 140 mM NaCl at room temperature. Fluorescence anisotropy measurements were done in a 384-well plate using a Victor X5 multilabel plate reader (Perkin Elmer).

The dissociation constant (K_d) was calculated by fitting experimental data to the following explicit equation, which is based on single-site binding model[1-3].

$$A = A_0 + (A_i - A_0)\left[\frac{([\text{MDM2}] + [\text{ligand}] + K_d) - \sqrt{([\text{MDM2}] + [\text{ligand}] + K_d)^2 - 4[\text{MDM2}][\text{ligand}]}}{2[\text{ligand}]}\right]$$

A = measured fluorescence anisotropy

A_i = fluorescence anisotropy of ligand in the presence of an infinite concentration of MDM2

A_0 = fluorescence anisotropy of in the absence of MDM2

[MDM2] = concentration of MDM2 protein

[ligand] = concentration of ligand, Fluorescein-$p53_{17\text{-}28}$

[MDM2] increased progressively, while [ligand] held constant. [Fluorescein-$p53_{17\text{-}28}$] was 2.4 nM.

Competition data were fitted to a single-site binding model with variable Hill slope.

$$A = A_0 + (A_i - A_0)/(1 + 10^{\{(\log EC_{50} - [\text{ligand}]) \times \text{Hill Slope}\}})$$

For FP competition assay, FAM-p53_{17-28}/MDM2 complexes (2.4 nM/3000 nM; the MDM2 concentration was more than 3-fold greater than K_d) was titrated with an unlabeled competitor. The data were analyzed using GraphPad Prism and SigmaPlot software.

Protease resistance experiment

For the peptide digestion[4], Chymotrypsin was added to the samples (final peptide:chymotrypsin mass ratio was 5:1) and incubated at 37 °C. Aliquots were taken from the samples at 0, 1, 5, 15, 30, 60, and 120 min, and were immediately treated with TFA to a final concentration of 0.1 % (v/v) in order to quench the digestion reaction. Before analysis, self-assembled peptide nanostructures were disassembled by treating with 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP), a strong β-breaker[5, 6], to a final concentration of 50% (v/v) for 30 min. The samples were then monitored chromatographically using a reversed phase HPLC (water/acetonitrile with 0.1% TFA) and the recovery was calculated by integration of the 280 nm signal. The molecular weight was confirmed by MALDI-TOF mass spectrometry.

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