Electronic Supplemental Information for

Achieving Cell Penetration with Distance-Matching Cysteine Cross-Linkers: A Facile Route to Cell-Permeable Peptide Dual Inhibitors of Mdm2/Mdmx

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Materials and General Procedures

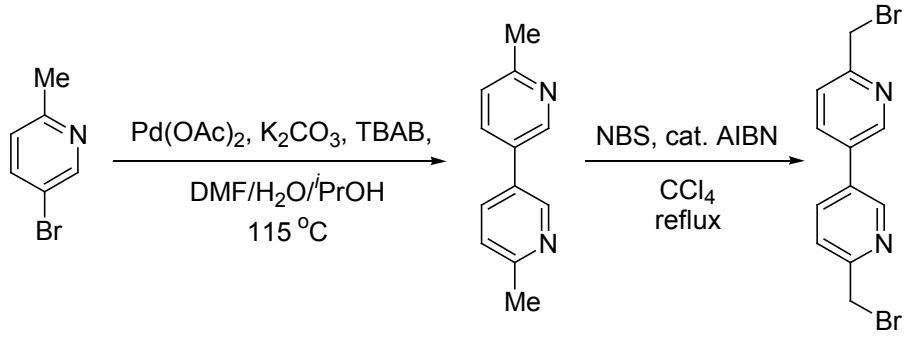
4,4'-Bis-bromomethyl-biphenyl (Bph) was purchased from TCI America and used directly in the cross-linking reactions. All other chemicals and solvents were obtained from commercial sources and used without further purification. Analytical thin layer chromatography (TLC) was performed on Whatman AL SIL G/UV254 flexible plates. Flash chromatography was performed with SiliCycle silica gel 60 Å (40-63 μm). All reactions were carried out under argon using oven-dried glassware. ^{1}H- and ^{13}C-NMR spectra were recorded with either Inova-500 or Gemini-300 MHz NMR spectrometers. Electrospray LC-MS analysis was performed using a Finnigan LCQ Advantage IonTrap mass spectrometry coupled with a Surveyor HPLC system. Peptides were purified using a Gilson semi-preparative reverse-phase HPLC system equipped with a Phenomenex C18 column with a flow rate of 5 mL/min and a gradient of 10-90% ACN/H_2O while monitoring at 220 nm and 254 nm. Analytical HPLC were performed using Phenomenex Luna C18 or Kinetex C18 column (250 × 4.6 mm) with the flow rate set at 1.0 mL/min and UV detection set at 220 and 254 nm. Live cell microscopy was performed on a Zeiss LSM-510 meta-NLO system equipped with a Coherent Chameleon Ultra II Ti/Sapphire laser and external non-descanned detectors. Fixed cell fluorescent microscopy was performed on a Zeiss LSM-710 Confocal Microscope equipped with a continuous laser and fluorescence lifetime (FLIM) detectors.

Table S1. ESI-MS characterization of PDI analogs.

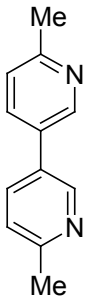
Peptide	Sequence	Mass calculated (M^+, m/z)	Mass found (m/z)
PDI	LTFEHYWAQLTS	1536.7	1536.6 $[M+H]^+$
1	LTFCHYWAQLCS	1512.7	1513.2 $[M+H]^+$
1a	LTFC'HYWAQLC'S [a]	1690.8	1691.5 $[M+H]^+$
1b	LTFC"HYWAQLC"S [b]	1692.6	1693.2 $[M+H]^+$
2	LTFC**R**YWA**R**LCS	1559.8	1560.2 $[M+H]^+$
2a	LTFC'**R**YWA**R**LC'S	1736.8	1737.5 $[M+H]^+$
2b	LTFC"**R**YWA**R**LC"S	1738.8	1739.4 $[M+H]^+$
3	LTFcHYWAQLCS [c]	1512.7	1513.5 $[M+H]^+$
3a	LTFc'HYWAQLC'S	1690.8	1691.4 $[M+H]^+$
3b	LTFc"HYWAQLC"S	1692.6	1693.5 $[M+H]^+$
4a	LTFc'**R**YWA**R**LC'S	1736.8	1737.2 $[M+H]^+$
4b	LTFc"**R**YWA**R**LC"S	1738.8	1739.9 $[M+H]^+$
Fluo-PDI	Fluorescein-Ahx-LTFEHYWAQLTS [d]	1995.6	998.5 $[M+2H]^{2+}$
Fluo-**1**	Fluorescein-Ahx-LTFCHYWAQLCS	1971.7	987.4 $[M+2H]^{2+}$
Fluo-**1b**	Fluorescein-Ahx-LTFC"HYWAQLC"S	2151.0	1076.9 $[M+2H]^{2+}$
Fluo-**2**	Fluorescein-Ahx-LTFC**R**YWA**R**LCS	2018.8	1010.5 $[M+2H]^{2+}$
Fluo-**2b**	Fluorescein-Ahx-LTFC"**R**YWA**R**LC"S	2198.0	1100.2 $[M+2H]^{2+}$
Fluo-**3b**	Fluorescein-Ahx-LTF c"**R**YWA**R**LC"S	2151.0	1076.2 $[M+2H]^{2+}$
Fluo-**4b**	Fluorescein-Ahx-LTF c"**R**YWA**R**LC"S	2198.0	1110.8 $[M+2H]^{2+}$

[a] C' denotes the Bph-linked cysteine; [b] C" denotes the Bpy-linked cysteine; [c] c (lower case) = *D*-cysteine.
[d] Ahx= 6-aminohexanoic acid.

Scheme S1. Synthesis of 6,6'-bis(bromomethyl)-3,3'-bipyridine (**Bpy**).

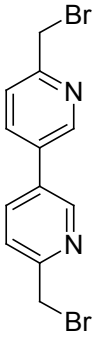


6,6'-Dimethyl-3,3'-bipyridine:[S1]



A mixture of 5-bromo-2-methyl-pyridine (1.37 g, 8 mmol), $Pd(OAc)_2$ (90 mg, 0.4 mmol), K_2CO_3 (1.10 g, 8 mmol), tetra-*n*-butylammonium bromide (1.29 g, 4 mmol) in DMF (0.9 mL)/H_2O (0.35 mL) was stirred at 115 °C under argon for 2 minutes. Isopropanol (480 mg, 8 mmol) was added. After stirring for 48 h, the reaction mixture was allowed to cool down to room temperature. The mixture was added water and ether, and the organic phase was separated, washed with brine, and dried over anhydrous Na_2SO_4. The solvent was then evaporated under reduced pressure and the residue was purified by silica gel column chromatography to afford the desired product as white crystals (0.377 g, 51%): ^{1}H NMR (500 MHz, C_6D_6) δ 8.71 (d, J = 1.5 Hz, 2H), 7.18 (dd, J = 8.0 Hz, 2.5 Hz, 2H), 6.66 (d, J = 8.0 Hz, 2H), 2.45 (s, 6H).

6,6'-Bis(bromomethyl)-3,3'-bipyridine (Bpy):



6, 6'-dimethyl-3, 3'-bipyridine (0.368 g, 2 mmol) was dissolved in 20 mL anhydrous CCl_4. *N*-bromosuccinimide (0.712 g, 4 mmol) and AIBN (20 mg, catalytic amount) were added to the solution. The mixture was refluxed for 5 hours before CCl_4 was removed under vacuum. Recrystallization of the crude from DCM afforded the titled compound as a white solid (90 mg, 14% yield): ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.80 (d, J = 2.1 Hz, 2H), 7.89 (dd, J = 8.1 Hz, 2.4 Hz, 2H), 7.56 (d, J = 8.1 Hz, 1H), 4.61 (s, 4H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 156.76, 147.96, 135.44, 132.26, 123.62, 33.29; HRMS (EI) calcd for $C_{12}H_{10}Br_2N_2$ 339.9205 [M^+], found 339.9206.

Solid Phase Peptide Synthesis: The linear peptides were synthesized by following the standard Fmoc peptide synthesis protocol with Rink amide resin (substitution = 0.66 mmol/g) on a Tribute peptide synthesizer (Protein Technologies, Tuson, AZ). For each coupling reaction, 5 equiv of Fmoc-amino acid, 5 equiv of HBTU, and 10 equiv of *N*-methymorpholine (NMP) were used. The coupling reaction was allowed to proceed for 45 min. For coupling of cysteine, 1 M of trimethylpyridine in DCM/DMF (1:1) was employed along with a reduced preactivation time (0.5 min) in order to minimize racemization.[S2]

The Fmoc deprotection was accomplished by treating the peptide-bound resin with 20% piperidine/DMF (3×, 8 min each). After the amino acids were assembled, the *N*-terminal amine was acylated using 10 equiv of acetic anhydride and 10 equiv of DIEA. The peptides were then cleaved by treating the resin with a cleavage cocktail containing 95% TFA, 2.5% ethane dithiol, 1.5% triisopropylsilane and 1% water for a period of 1.5 hr (extended to 2.5 hr for peptides containing Pbf protecting groups). The peptides were precipitated in diethyl ether, collected by centrifugation, and washed with diethyl ether prior to drying in high vacuum. The crude peptides were purified by Gilson reverse-phase HPLC equipped with a semi-prep Phenomenex C18 column running at a flow rate of 5 mL/min and a gradient of 10-90% acetonitrile/H_2O containing 0.1% TFA. Fractions with greater than 90% purity were pooled and lyopholized to give the desired peptides. For the synthesis of fluorescein-labeled peptides, chain elongation was allowed to continue with Fmoc-Ahx-OH. After removal of Fmoc, the *N*-terminus of the peptide was capped by incubating the peptide with 1.5 equiv of FITC overnight in presence of 2 equiv of DIEA.

Peptide Cross-Linking by Bph or Bpy: Cross-linking reactions were carried out by incubating the purified dicysteine-containing peptides with 1.5 equiv of 4,4'-bis(bromomethyl)-1,1'-biphenyl (Bph, **a**) or 6,6'-bis(bromomethyl)-3,3'-bipyridine (Bpy, **b**) in a mixed solvent of acetonitrile/30 mM NH_4HCO_3 buffer, pH 8.5 (1:4 to 2:3 depending on peptide solubility) to obtain a final peptide concentration of 1 mM. The mixture was stirred at room temperature for 1.5-2 hours. Afterwards, the solvents were evaporated and excess amount of cross-linker was removed by washing the residue with diethyl ether. The cross-linked peptides were then purified by preparative HPLC.

Langevin Dynamics: The distance distributions of Bph and Bpy cross-linkers were calculated using Langevin dynamics simulations. The *S*-methylated cross-linkers were used in this simulation and their structures were shown on the top. The distance between the two sulfur atoms was monitored throughout the simulation. The langevin dynamics simulation was carried out with Hyperchem 8.0. The structures were minimized using Amber 99 force field prior to dynamics simulation. The simulation wsa set up to last 1000 ps with 1-ps timestep at 300 K. A unimodal distributions were observed when the number of conformations was plotted to the *S*-*S* distance. The median *S*-*S* distances for both cross-linkers stand around 11.6 Å.

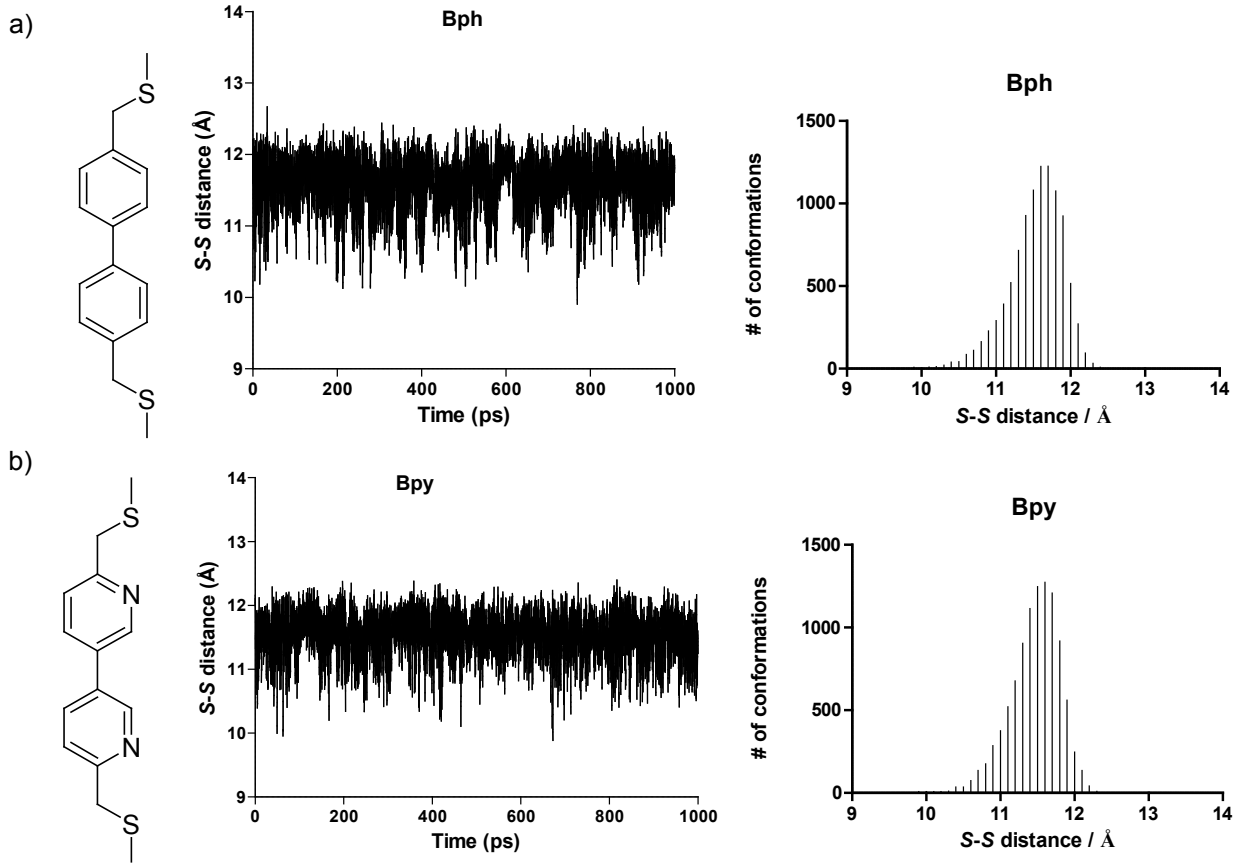


Fig. S1. Distance distributions of *S*-methyl Bph (a) and Bpy (b) cross-linkers calculated using Langevin dynamics simulations.

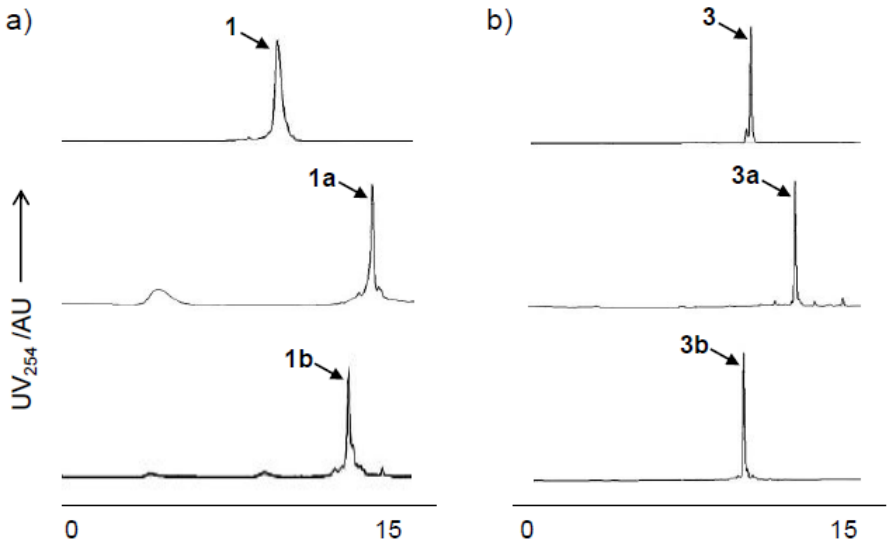


Fig. S2. HPLC traces of crude products after the cross-linking reaction of PDI analogs **1** (a) and **3** (b) with either Bph or Bpy. The identities of each individual peaks were confirmed by electrospray mass spectrometry (see Table S1).

ELISA Assay: GST-Mdm2 and GST-Mdmx containing full-length human Mdm2 and Mdmx and His_6-tagged human p53 were expressed in *E. coli* and purified by glutathione agarose and Ni^{2+}-nitrilotriacetic acid beads, respectively, under non-denaturing conditions. Microtiter plates were coated with 2.5 μg/ml His_6-p53 in PBS buffer for 16 hr. After washing with PBS containing 0.1% Tween-20 (PBST), the plates were blocked with PBST containing 5% nonfat dry milk (PBSTM) for 30 min. Peptides were dissolved in DMSO. GST-Mdm2 and -Mdmx (5 μg/ml) were mixed with peptides in PBSMT containing 10% glycerol and 10 mM dithiolthreitol prior to their additions to the wells at specified concentrations. The incubation was allowed to proceed for 1 hr at room temperature before the plates were washed with PBST. The plates were then incubated with either anti-Mdm2 antibody 5B10 or anti-Mdmx antibody 8C6 in PBSTM for 1 hr, followed by washing with PBST and incubation with horseradish peroxidase-conjugated rabbit anti-mouse Ig antibody for 1 hr. The plates were developed by incubation with TMB peroxidase substrate (KPL, Gaithersburg, MD) and measured the absorbance at 450 nm. The data was analyzed with the Origin 6.0 software (Fig. S3). The sigmodial plots were fitted to Hill equation for one site competitive binding to derive IC_{50} values. The IC_{50} values were reported as averages and standard deviations of three independent experiments.

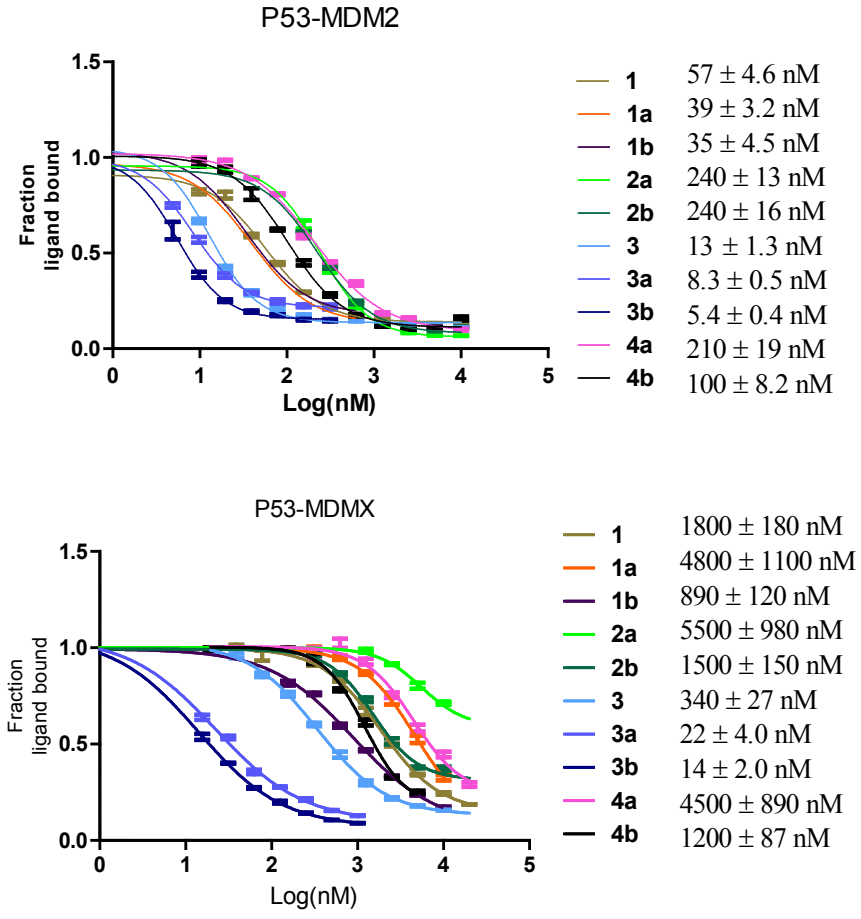


Fig. S3. ELISA data of the cross-linkedd PDI analogs.

Molecular Modeling Studies of 3b: The cordinates for PDI complexed with MDMX was obtained from the PDB Code: 3FDO.[S3] Two *L*-Cys substitutions were introduced to replace Glu-4 and Thr11 of PDI and the resulting peptide **1** was energy-minimized using the Amber 99 force field in Hyperchem 8 to produce a model of MDMX-bound **1** (a). Similarly, *L*-cys at position 4 of peptide **1** was replaced with *D*-cys, and the resulting peptide **3** was energy-minimized to generate MDM-bound **3** (c). Separately, Bpy cross-linker was constructed and energy-minimized initially before connecting to sulfhydryl groups of the cystienes to generate peptides **1b** and **3b**. Then, the cross-linked peptide–MDMX complexes were subjected to complete energy minimization in Hyperchem 8. The optimized complex structural models were shown in (b) and (d).

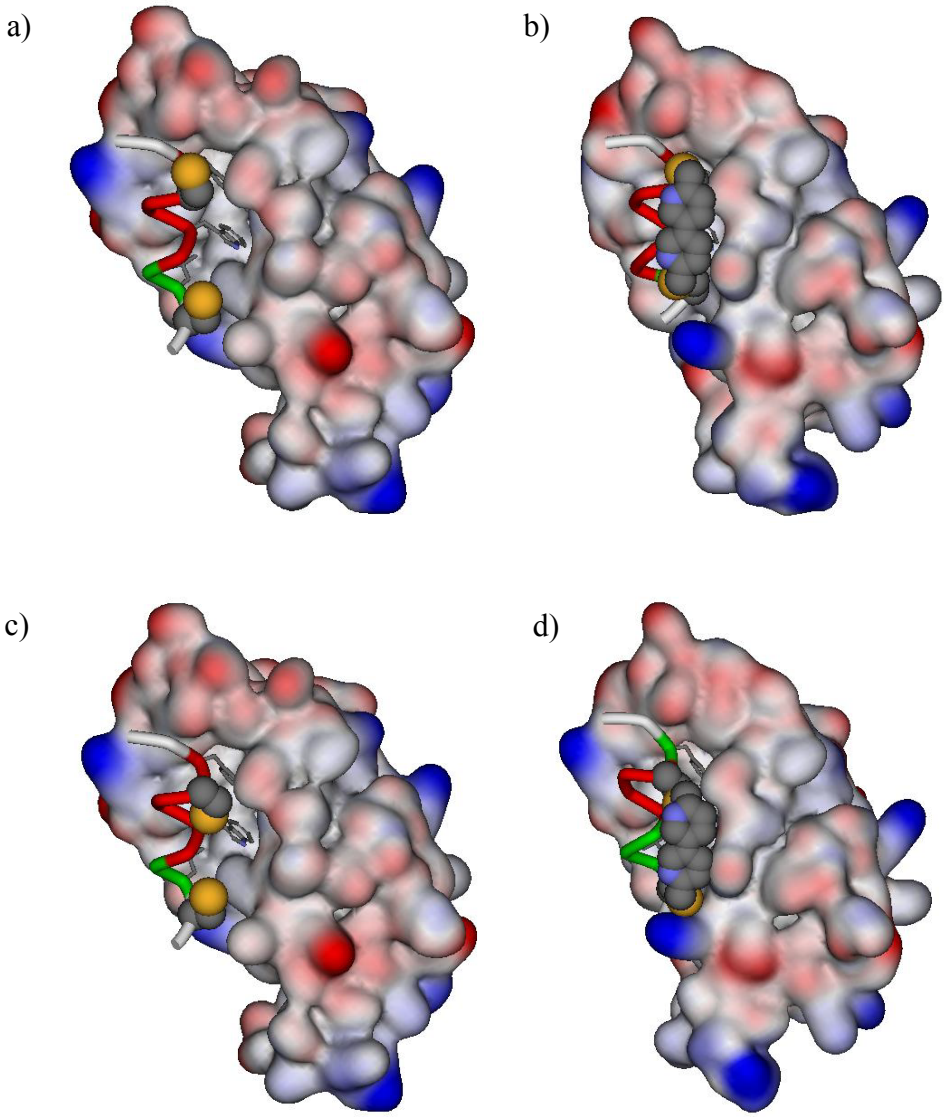


Fig. S4. Possible binding modes of **1** (a), **1b** (b), **3** (c), and **3b** (d) toward Mdmx. The cysteine side chains and the Bpy cross-linkers were shown in CPK model and the helical peptides were shown in tube model.

FACS Analysis: HeLa Cells were cultured in DMEM medium supplemented with 10% fetal bovine serum in 6-well plate in a 37 ºC, 5% CO_2 incubator to reach confluency of around 70%. Cells were then washed twice with PBS before switching to serum-free DMEM medium. After standing in the incubator for 30 min, appropriate amounts of peptides (1 mM in DMSO) were added to the culture plate to obtain a final peptide concentration of 10 μM. Cells were incubated at 37ºC for additional 2 hours. Then medium was removed and cells were washed with PBS (3×) followed by treatment with trypsin to detach the cells. After a brief low-speed centrifugation, HeLa cells were collected and re-suspended in PBS for the FACS analysis.

Circular Dichroism Study: Circular dichroism spectra were recorded with JASCO J-715 CD spectrometer at room temperature using 0.1-cm path length cuvettes. The spectra were recorded in the wavelength range of 190-250 nm and averaged over 3 scans with a resolution of 0.5 nm, a bandwith of 1.0 nm and a response time of 4 s. The sensitivity and the speed of the spectrometer were set to 100 mdeg and 50 nm/min, respectively. All peptides were dissolved in 20% acetonitrile/H_2O to reach final concentration of 50 μM. The mean residue ellipticity was plotted to wavelength, and the helical content of each peptide at 222 nm was derived. The percent helicity was calculated based on $[\theta]_{222}/[\theta]_{max}$. $[\theta]_{max}$ was calculated to be -30384 according to the formula: $[\theta]_{max} = -39500 \times (1-3/n)$ where n is number of bonds.[S4]

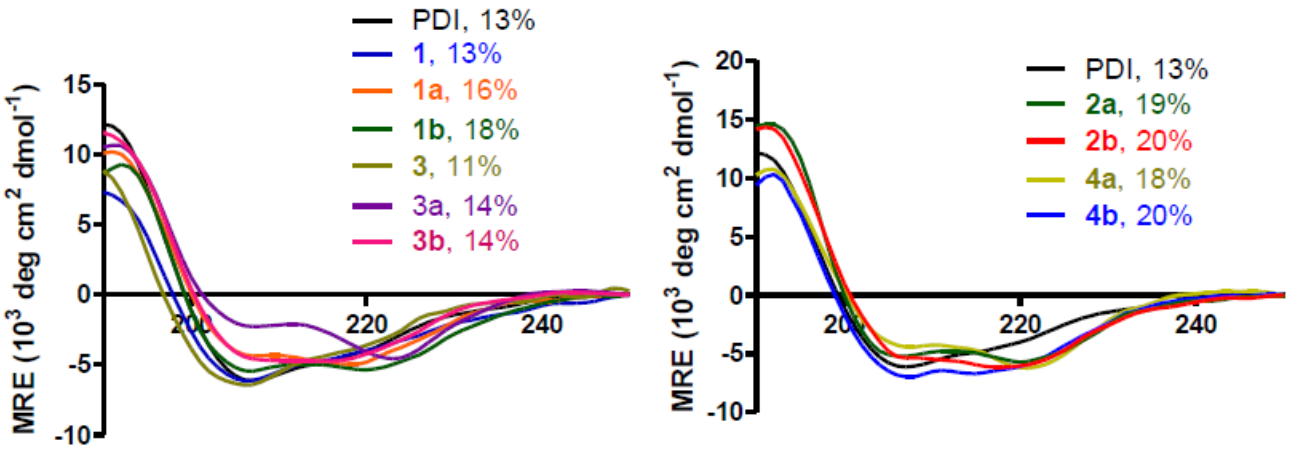


Fig. S5. CD spectra of the linear and cross-linked PDI analogs and their respective percent helicity values. The peptides were dissolved in 20% ACN/H_2O for a final concentration of 50 μM. The percent helicity was calculated based on the $[\theta]_{222}$ values.

Confocal Microscopy: HeLa Cells were seeded into 35 mm glass bottom microwell dishes (MatTek, Ashland, MA) at a density of 3×10^5 in DMEM medium supplemented with 10% fetal bovine serum, and the cells were allowed to grow in a 37ºC 5% CO_2 incubator for 48 hours. After removing medium, cells were washed twice with PBS and then incubated in serum-free DMEM medium for 30 min. Appropriate amounts of peptides (1 mM in DMSO) were then added to obtain a final concentration of 10 μM and cells were incubated at 37ºC for 2 hours. After PBS wash, cells were treated with 4% formaldehyde in PBS for 20 min at room temperature. The fixation medium was removed and the cells were washed with PBS (3×) before placed into the low florescent mounting medium for fluorescent imaging acquisition.

Live Cell Confocal Microscopy: HeLa cells were cultured in a 35-mm glass bottom microwell dish as described previously. The cells were incubated with peptide **3b** for 2 hours in a serum-free medium followed by addition of a 40 μL solution containing 500 μg/mL of Alexa Fluor 586-labeled transferrin (Invitrogen, Carlsbad, CA) and 1.5 μM Hoescht 33342 dye and additional incubation for 30 min. Afterwards, cells were washed with PBS and placed into Earle's balanced salt solution supplemented with MEM essential and non-essential amino acids. All images were taken with a Zeiss LSM-510 meta-NLO system equipped with a Coherent Chameleon Ultra II Ti/Sapphire laser and external non-descanned detectors.

Luciferase Reporter Assay: PA1 cells stably expressing the p53-responsive reporter BP100-Luc were maintained in DMEM medium supplemented with 10% FBS. PA1-BP100 cells (~50,000) were seeded into 24-well plates and treated with 0 - 200 μM peptide 3, 3b, or 0 - 10 μM nutlin-3 in DMEM medium supplemented with 10% FBS for 16 hours. Cells were lysed and luciferase activity was determined.

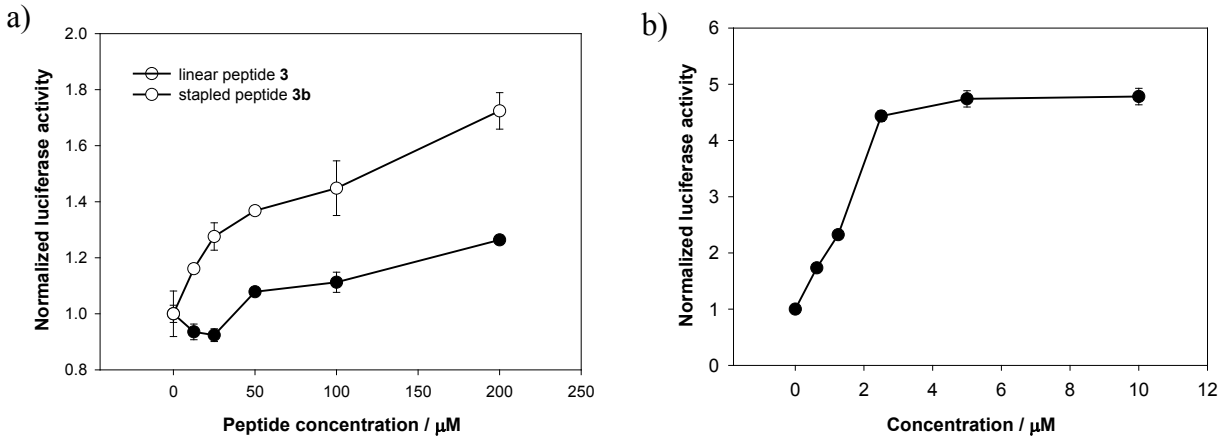


Fig. S6. The p53-dependent transcriptional activation of luciferase in PA1 cells treated with (a) linear peptide **3** or stapled peptide **3b,** (b) nutlin-3. The average transcriptional activities along with standard deviations were plotted.

Reference:

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[S3] Czarna, A.; Popowicz, G. M.; Pecak, A.; Wolf, S.; Dubin, G.; Holak, T. A., *Cell Cycle* **2009**, *8*, 1176-1184.

[S4] Wang, D.; Liao, W.; Arora, P. S. *Angew. Chem. Int. Ed.* **2005**, *44*, 6525-6529.