An Ultrahigh Affinity D-Peptide Antagonist Of MDM2 For P53 Activation

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Synthesis of peptides and proteins

All peptides and proteins used in this work were chemically synthesized as previously described.[1,2] Peptides and proteins were purified to homogeneity by reversed-phase HPLC, and their molecular masses were ascertained by electrospray ionization mass spectrometry. Peptide and protein quantification was performed by UV measurements at 280 nm using molar extinction coefficients calculated according to the published algorithm.[3]

Surface plasmon resonance (SPR)-based competitive binding assay

Quantification of the binding affinity of D-peptides for MDM2 and MDMX was performed at 25 °C on a Biacore T100 SPR instrument using the previously published SPR-based competitive binding method.[1,2] Briefly, a fixed concentration of MDM2 (30 nM) or MDMX (100 nM) was incubated in 10 mM HEPES buffer containing 150 mM NaCl, 0.005% surfactant P20, pH 7.4, with varying concentrations of peptide inhibitor for 30 min before SPR analysis of free protein on a $^{15\text{-}29}$p53 peptide-immobilized CM5 sensor chip (17 RUs). The samples were injected at a flow rate of 20 μL/min for 2 min, followed by 4 min dissociation. The concentration of unbound MDM2/MDMX protein in solution was deduced, based on p53-association RU values, from a calibration curve established by RU measurements of different concentrations of MDM2/MDMX protein injected alone. Non-linear regression analysis was performed using GraphPad Prism 4 to give rise to K_d values based on the equation K_d = [peptide][MDM2/MDMX]/[complex].

Fluorescence polarization (FP) assay

An N-terminally acetylated $^{(15\text{-}29)}$p53 peptide was synthesized by Boc-chemistry SPPS and purified to homogeneity by preparative C18 RP-HPLC. Succinimidyl ester-activated carboxyfluorescein (FAM-NHS) was covalently conjugated to *N*-acetyl-(15-29)p53 via its Lys24 side chain in DMF, and the resultant product *N*-acetyl-(15-29)p53-FAM HPLC-purified and lyophilized. The peptide-MDM2 binding experiments were performed in 96-well plates on a Tecan Infinite M1000 fluorescence plate reader. Serially diluted D-peptides were prepared in Tris-HCl buffered saline (10 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.0) and incubated with 10 nM *N*-acetyl-(15-29)p53-FAM and 250 nM $^{(25\text{-}109)}$MDM2 in a total volume of 150 μl per well. After a 30-min incubation at room temperature, fluorescence polarization was measured at λ_{ex} = 470 nm and λ_{em} = 530 nm. Non-linear regression analyses were performed to give rise to IC_{50} values – concentrations of D-peptide at which 50% of the fluorescent p53 peptide is displaced from its complex with MDM2. For binding studies with $^{(24\text{-}108)}$MDMX, a protein concentration of 1 μM was used under otherwise identical conditions.

Crystallization of the p-CF_3-Phe7-^{D}PMI-β-$^{(25-109)}$MDM2 complex
Crystals were obtained using the hanging-drop vapor diffusion method at room temperature. Each drop contained 1 µl of complex (at 10 mg/ml in 10 mM HEPES, 0.1 mM TCEP, pH 7.5) and 1 µl of reservoir solution consisting of 0.1 M MES, pH 5.5, 25% PEG 4000, and 0.15 M ammonium sulfate. Crystals grew typically in one day as irregular plates measured approximately at 0.10 x 0.15 x 0.05 mm. They belonged to the $C222_1$ space group, and each asymmetric unit contained three structurally independent complexes.

Data Collection, Structure Solution, and Refinement
The crystals were rapidly soaked in the well-solution containing 30% (v/v) glycerol and flash-cooled to 100K in a nitrogen stream. X-ray diffraction data were collected using a BL9-1 at the Stanford Synchrotron Radiation Lightsource (SSRL) on a MAR325 detector and were integrated and scaled with the HKL2000 package.[4] The structure was solved by the molecular replacement method with program Phaser from the CCP4 suite,[5] using the ^{D}PMI-α-$^{(25-109)}$MDM2 structure as a search model (PDB: 3LNJ). Several cycles of model building and TLS refinement were carried out with Phenix and Refmac and coupled with manual refitting with COOT.[6] Data collection and refinement statistics are summarized in **Table S3**. The coordinates and structure factors have been deposited in the PDB with accession code 3TPX. Molecular graphics were generated using Pymol (http://pymol.org).

(1) (a) M. Liu, M. Pazgier, C. Li, W. Yuan, C. Li, W. Lu, *Angew. Chem. Int. Ed.* **2010**, *49*, 3649. (b) M. Liu, C. Li, M. Pazgier, C. Li, Y. Mao, Y. Lv, B. Gu, G. Wei, W. Yuan, C. Zhan, et al., *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 14321.
(2) (a) M. Pazgier, M. Liu, G. Zou, W. Yuan, C. Li, C. Li, J. Li, J. Monbo, D. Zella, S. G. Tarasov, et al., *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 4665. (b) C. Li, M. Pazgier, C. Li, W. Yuan, M. Liu, G. Wei, W.-Y. Lu, W. Lu, *J. Mol. Biol.* **2010**, *398*, 200.
(3) C. N. Pace, F. Vajdos, L. Fee, G. Grimsley, T. Gray, *Protein Sci*. **1995**, *4*, 2411.
(4) Z. Otwinowski, W. Minor, *Methods Enzymol.* **1997**, *276*, 307.
(5) Collaborative Computational Project, Number 4, *Acta Crystallogr. D Biol. Crystallogr*. **1994**, *50*, 760.
(6) P. Emsley, K. Cowtan, *Acta Crystallogr. D Biol. Crystallogr.* **2004**, *60*, 2126.

Table S1. D-peptide binding to MDM2 as quantified by FP using N-acetyl-(15-29)p53-FAM.

Peptide	**IC_{50}, nM (mean ± S.D., n=3)**
^{D}PMI-β	149±12
o-Cl-Phe7-^{D}PMI-β	197±34
m-Cl-Phe7-^{D}PMI-β	3370±1092
p-Cl-Phe7-^{D}PMI-β	80.3±4.0
p-Br-Phe7-^{D}PMI-β	80.7±1.8

Table S2. D-peptide binding to MDM2 as quantified by FP using N-acetyl-*p*-Br-Phe7-^{D}PMI-β-FAM.

Peptide	**IC_{50}, nM (mean ± S.D., n=3)**
^{D}PMI-β	880±11
p-F-Phe7-^{D}PMI-β	258±3
p-Cl-Phe7-^{D}PMI-β	63.0±12.2
p-Br-Phe7-^{D}PMI-β	41.4±19.0
p-I-Phe7-^{D}PMI-β	35.4±15.0
p-CF_3-Phe7-^{D}PMI-β	24.8±7.6
p-CH_3-Phe7-^{D}PMI-β	101±15
p-CN-Phe7-^{D}PMI-β	278±90
6-F-Trp3-^{D}PMI-β	420±28
^{D}PMI-δ	11.0±6.0

Table S3. Data collection and refinement statistics

Data Collection	
Space group	$C222_1$
Cell parameters, Å	a=70.5, b=211.8, c=45.3
Complex/a.u.	3
Resolution [a], Å	50-1.8 (1.85-1.81)
Number of reflections	
Total	65,951
Unique	34,777
R_{merge} [b], %	4.8 (54.2)
Completeness, %	100 (99)
Redundancy	6.5 (6.4)
I/σ, (I)	41.7 (3.3)
Refinement Statistics	
Resolution, Å	20-1.8
R [c], %	19.8
R_{free} [d], %	23.1
No. of protein atoms	2,110
No. of D-peptide atoms	342
No. of water molecules	229
Ligand/Ion	36
Overall B value $(Å)^2$	
Protein	30.7
D-peptide	37.2
Water	39.7
Ligand/Ion	61.5
Root mean square deviation	
Bond lengths, Å	0.02
Bond angles, °	1.69
Ramachandran plot	
Most favored region, %	98.8
Additional allowed region, %	1.2
Generously allowed region, %	0.0
Disallowed region, %	0.0
PDB code	3TPX

[a]All data (outer shell).

[b]$R_{merge} = \sum | I - \langle I \rangle | / \sum I$, where I is the observed intensity and $\langle I \rangle$ is the average intensity obtained from multiple observations of symmetry-related reflections after rejections

[c]$R = \sum \| F_o | - | F_c \| / \sum | F_o |$, where F_o and F_c are the observed and calculated structure factors, respectively

[d]R_{free} = defined by Brunger, A. T. (1992) Free R value: a novel statistical quantity for assessing the accuracy of crystal structures, *Nature 355*, 472-475.

Table S4. D-peptide binding to MDMX as quantified by FP using N-acetyl-(15-29)p53-FAM.

Peptide	**IC_{50}, nM (mean ± S.D., n=3)**
^{D}PMI-β	7603±509
6-F-Trp3-^{D}PMI-β	3516±613
p-CF_3-Phe7-^{D}PMI-β	1824±179
^{D}PMI-δ	1026±158

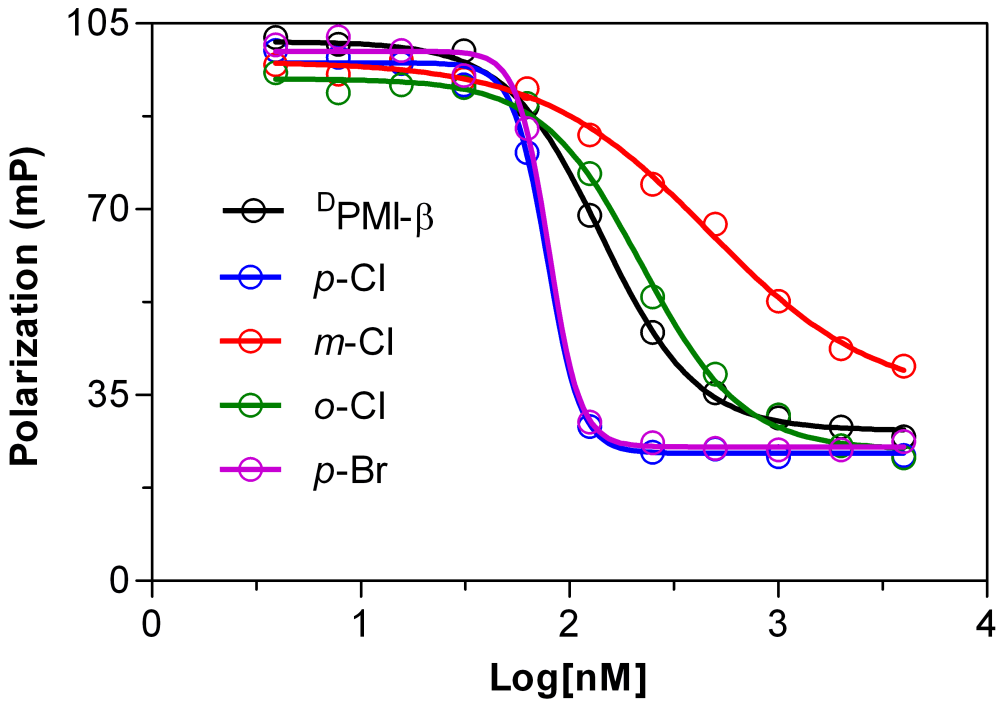

Figure S1. Representative competition of D-peptides for MDM2 binding with a fluorescently labeled p53 peptide as quantified by FP techniques. The measurements were performed in 96-well plates (Corning Life Science) on a Tecan Infinite M1000 fluorescence plate reader. Serially diluted D-peptides were prepared in Tris-HCl buffered saline (10 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.0), to which aliquots of AcO-p53-FAM and $^{25\text{-}109}$MDM2 were sequentially added to respective final concentrations of 10 and 250 nM in a total volume of 150 μl per well. After a 30-min incubation at room temperature, fluorescence polarization was measured at an excitation wavelength of 470 nm and an emission wavelength of 530 nm. IC_{50} values (mean ± S.D.) were calculated from three independent experiments and are tabulated in Table S1.

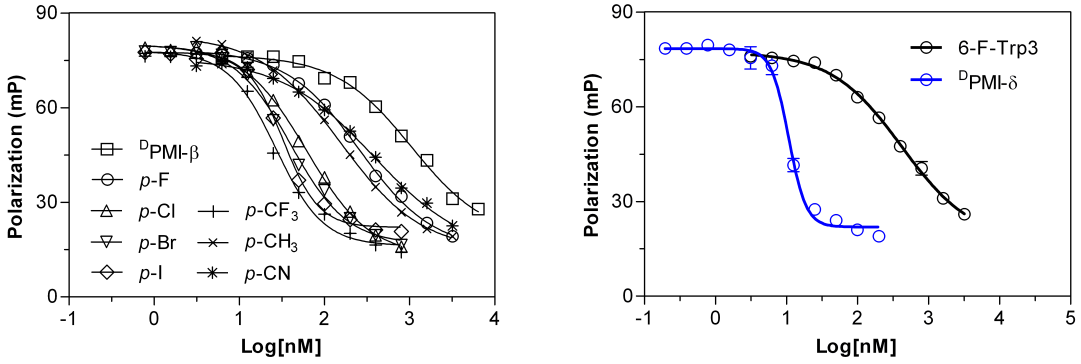

Figure S2. Representative competition of D-peptides for MDM2 binding with a fluorescently labeled ^{D}PMI-β peptide as quantified by FP techniques. The measurements were performed in 96-well plates (Corning Life Science) on a Tecan Infinite M1000 fluorescence plate reader. Serially diluted D-peptides were prepared in Tris-HCl buffered saline (10 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.0), to which aliquots of AcO-*p*-Br-Phe7-^{D}PMI-β-FAM and $^{25\text{-}109}$MDM2 were sequentially added to a final concentration of 10 nM each in a total volume of 150 μl per well. After a 30-min incubation at room temperature, fluorescence polarization was measured at an excitation wavelength of 470 nm and an emission wavelength of 530 nm. IC_{50} values (mean ± S.D.) were calculated from three independent experiments and are tabulated in Table S2.

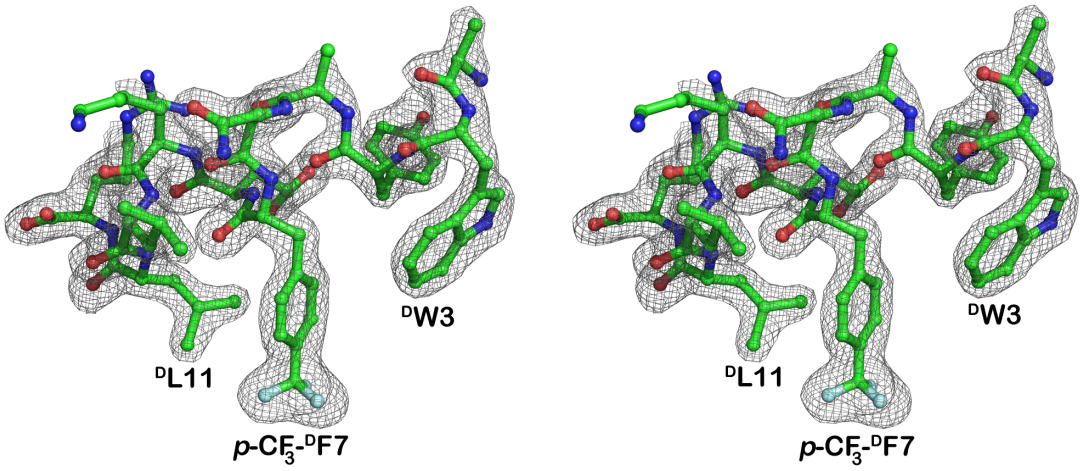

Figure S3. 2Fo-Fc electron density map, contoured at 1.5σ, of *p*-CF_3-Phe7-^{D}PMI-β in complex with $^{(25\text{-}109)}$MDM2. *p*-CF_3-Phe7-^{D}PMI-β is shown in ball-and-sticks, and the side chains of the residues docking directly into the p53-binding cavity of $^{25\text{-}109}$MDM2 are labeled. Whereas residues 2-12 of *p*-CF_3-Phe7-^{D}PMI-β are well-defined in the electron density map, no density was detected for N-terminal ^{D}Thr1 in any of the three structurally independent *p*-CF_3-Phe7-^{D}PMI-β/$^{(25\text{-}109)}$MDM2 complexes present in the asymmetric unit of crystal, suggesting that ^{D}Thr1 is fully disordered in the complex.

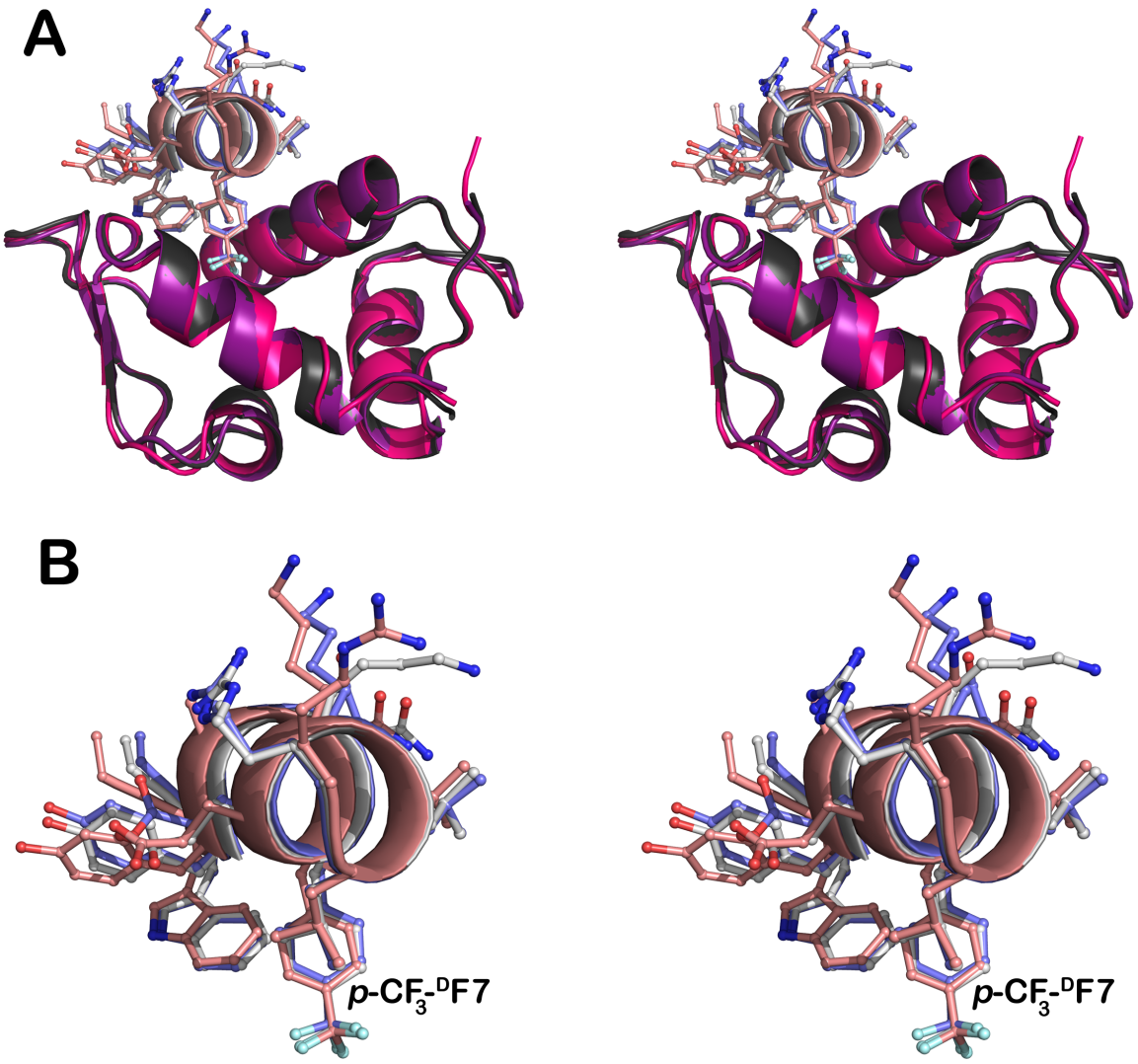

Figure S4. Crystal structure of $^{(25\text{-}109)}$MDM2 in complex with *p*-CF_3-Phe7-^{D}PMI-β. (**A**) Stereo view of three superimposed complexes present in the asymmetric unit of crystal where MDM2 molecules are aligned. They are colored as follows: AB, black/grey; CD, violet/pink; EF, magenta/blue. The Cα- traces of MDM2 and *p*-CF_3-Phe7-^{D}PMI-β are shown as ribbons, whereas the side chains of the D-peptide are also shown as ball-and-sticks. The three *p*-CF_3-Phe7-^{D}PMI-β-$^{(25\text{-}109)}$MDM2 complexes in the symmetric unit are very similar and can be superimposed in pairs with the root mean square deviation (RMSD) values in the range of 0.3 - 0.5 Å for equivalent Cα atoms. (**B**) Structural alignment of three crystallographically independent *p*-CF_3-Phe7-^{D}PMI-β peptides with equivalent Cα atoms superimposed. The RMSDs calculated for 11 Cα atoms vary between 0.2 and 0.3 Å.

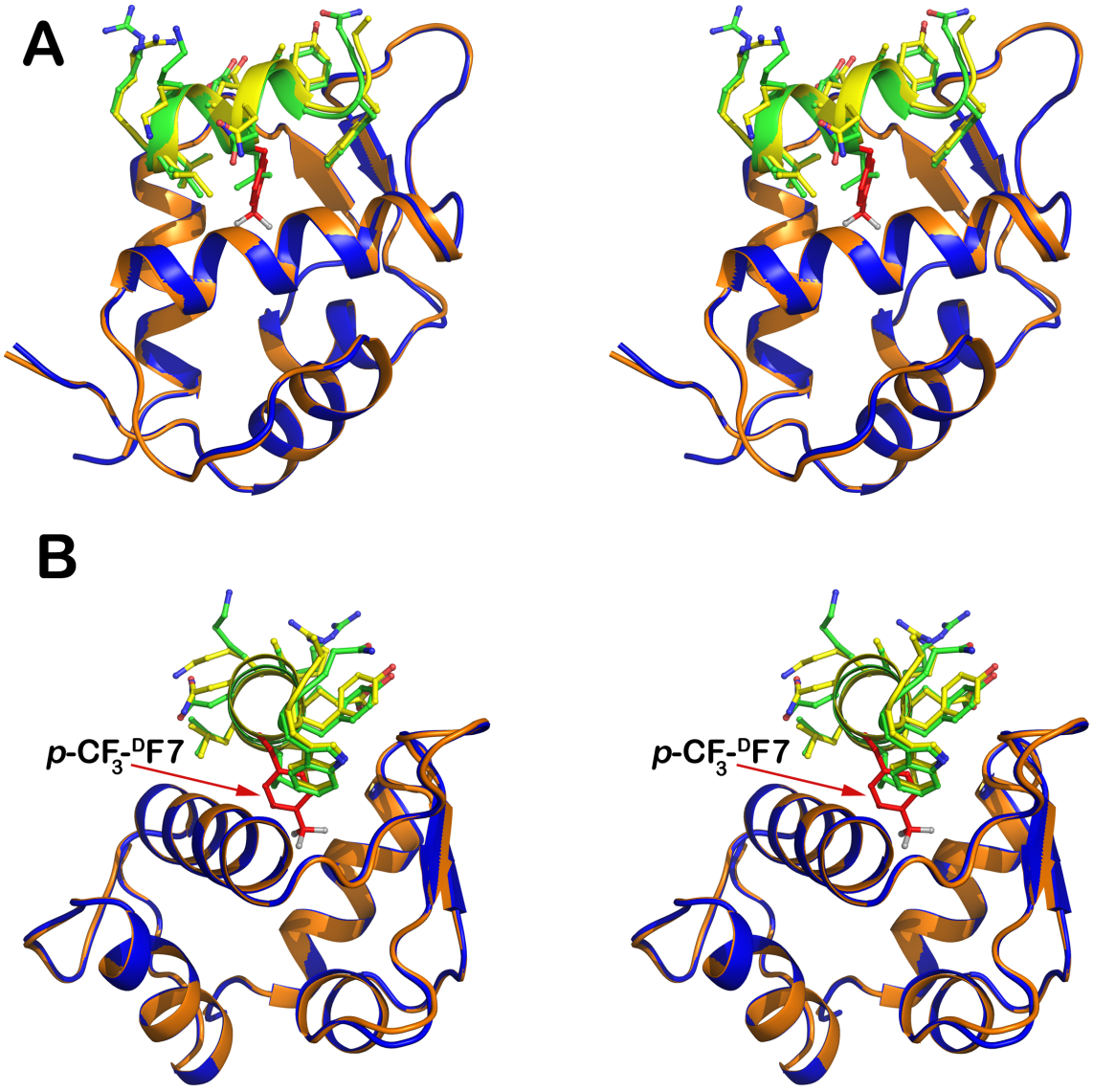

Figure S5. Structural comparison of *p*-CF_3-Phe7-^{D}PMI-β and ^{D}PMI-α in complex with $^{25\text{-}109}$MDM2. (**A**) Stereo view of two superimposed complexes where MDM2 molecules are aligned, and (**B**) its 90° rotation. *p*-CF_3-Phe7-^{D}PMI-β-$^{(25\text{-}109)}$MDM2 (yellow/blue) and ^{D}PMI-α-$^{(25\text{-}109)}$MDM2 (pdb code 3LNJ, green/orange) complexes can be superimposed with the RMSD of equivalent Cα atoms between 0.2 Å to 0.5 Å. *p*-CF_3-Phe7-^{D}PMI-β and ^{D}PMI-α bind to MDM2 similarly in a rigid, left-handed helical conformation. *p*-CF_3-^{D}Phe7 buries the CF_3 group 3.8 Å deeper as compared to Cγ of ^{D}Leu7.

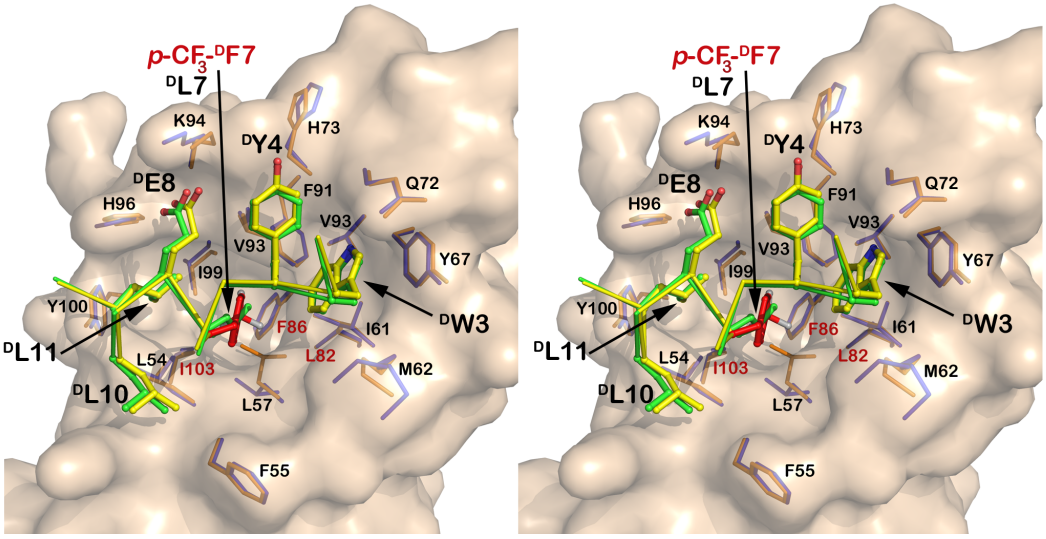

Figure S6. Stereo drawing of a superposition of the binding interfaces of *p*-CF_3-Phe7-^{D}PMI-β-$^{(25\text{-}109)}$MDM2 (yellow/violet) and ^{D}PMI-α-$^{(25\text{-}109)}$MDM2. *p*-CF_3-Phe7-^{D}PMI-β and ^{D}PMI-α are shown in a Cα ribbon diagram together with contact residues in ball-and-sticks. MDM2 contact residues are depicted as sticks over its molecular surface from the *p*-CF_3-Phe7-^{D}PMI-β-$^{(25\text{-}109)}$MDM2 complex. The residues of MDM2 that make hydrophobic contacts exclusively with *p*-CF_3-^{D}Phe7 are colored in red.

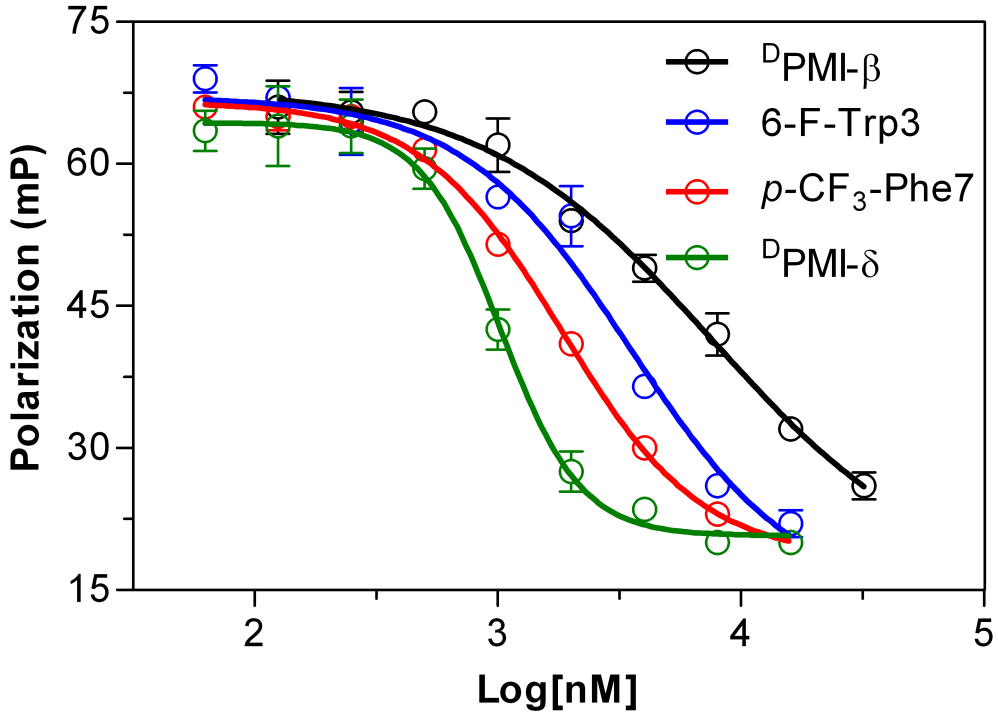

Figure S7. Representative competition of D-peptides for MDMX binding with a fluorescently labeled p53 peptide as quantified by FP techniques. The measurements were performed in 96-well plates (Corning Life Science) on a Tecan Infinite M1000 fluorescence plate reader. Serially diluted D-peptides were prepared in Tris-HCl buffered saline (10 mM Tris, 150 mM NaCl, 1 mM EDTA, pH 7.0), to which aliquots of AcO-p53-FAM and $^{24\text{-}108}$MDMX were sequentially added to respective final concentrations of 10 and 1000 nM in a total volume of 150 μl per well. After a 30-min incubation at room temperature, fluorescence polarization was measured at an excitation wavelength of 470 nm and an emission wavelength of 530 nm. IC_{50} values (mean ± S.D.) were calculated from three independent experiments and are tabulated in Table S4.