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An Ultrahigh Affinity D-Peptide Antagonist Of MDM2

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Abstract

The oncoprotein MDM2 negatively regulates the activity and stability of the p53 tumor suppressor, and is an important molecular target for anticancer therapy. Aided by mirror image phage display and native chemical ligation, we have previously discovered several proteolysis-resistant duodecimal D-peptide antagonists of MDM2, termed ^{D}PMI-α, β, γ. The prototypic D-peptide inhibitor ^{D}PMI-α binds $^{(25\text{-}109)}$MDM2 at an affinity of 220 nM, and kills tumor cells *in vitro* and inhibits tumor growth *in vivo* by reactivating the p53 pathway. Herein, we report the design of a super-active D-peptide antagonist of MDM2, termed ^{D}PMI-δ, of which the binding affinity for $^{(25\text{-}109)}$MDM2 has been improved over ^{D}PMI-α by three orders of magnitude (K_d = 220 pM). X-ray crystallographic studies validate ^{D}PMI-δ as an exceedingly potent inhibitor of the p53-MDM2 interaction, promising to be a highly attractive lead drug candidate for anticancer therapeutic development.

Keywords

p53; MDM2; MDMX; D-peptides; antitumor agents; drug design; mirror image phage display; native chemical ligation

Functional inhibition of the p53 tumor suppressor protein by its negative regulators MDM2 and MDMX, whose genes *MDM2* and *MDMX* are often amplified and/or over-expressed in many tumors harboring wild type *TP53*, directly contributes to tumor development and progression.[1] MDM2 is an E3 ubiquitin ligase that specifically targets p53 for proteosomal degradation[2] – a process potentiated by MDM2 hetero-oligomerization with its homolog MDMX.[3] Both MDM2 and MDMX can also antagonize p53 transcription activity by sequestering p53 transactivation domain via their N-terminal p53-binding domains.[4] Disrupting the p53-MDM2/MDMX inhibitory complex to rescue wild type p53 function has been validated as a viable therapeutic strategy for cancer treatment.[5] Different structural classes of MDM2/MDMX antagonists exist as potential anticancer drug candidates,

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ASSOCIATED CONTENT

Supporting Information

Experimental procedures including synthesis of peptides and proteins, surface plasmon resonance (SPR)-based competitive binding assay, fluorescence polarization assay, crystallization of the p-CF_3-Phe7- ^{D}PMI-β-(25-109)MDM2 complex, data collection, structure solution, and refinement as well as Tables S1-S4 and Figures S1-S7. The coordinates and structure factors have been deposited in the PDB with accession code 3TPX. This information is available free of charge via the Internet at http://pubs.acs.org.

including low molecular weight compounds,[6] small peptides and peptidomimetics,[7] and miniature proteins,[8] among others. Using mirror image phage display coupled with native chemical ligation,[9] we have previously discovered several 12-mer D-peptide antagonists of MDM2, termed ^{D}PMI-α, β, γ, that are resistant to proteolytic degradation.[10] The prototypic D-peptide inhibitor ^{D}PMI-α binds $^{(25\text{-}109)}$MDM2 at an affinity of 220 nM, and kills tumor cells *in vitro* and inhibits tumor growth *in vivo* by reactivating the p53 pathway. An ultrahigh affinity (K_d = 220 pM), protease-resistant D-peptide is designed to antagonize MDM2 by specifically targeting its p53-binding cavity, promising to be a highly attractive lead drug candidate for anti-cancer therapeutic development.

We have previously shown that ^{D}PMI-α (TN**WY**AN**L**EKL**L**R) adopts a left-handed α-helical conformation, burying several bulky hydrophobic side chains (highlighted in bold typeface) into the p53-binding cavity of $^{25\text{-}109}$MDM2 (Figure 1A). Among those, Trp3 and Leu7 are the two most critical residues of ^{D}PMI-α, contributing a combined free energy of 7.6 *k*cal/mol to $^{25\text{-}109}$MDM2 binding – an equivalent K_d value of 10^{-6} M.[10a] Sequence analysis of 18 phage-selected binding clones indicated that while Trp3 was totally conserved, Leu7 was not, as both Phe and Trp residues were also found at position 7. In fact, mutational analysis identified Phe7 as the best residue, registering a 3.5-fold stronger binding to MDM2 than Leu7. These findings largely led to the design of ^{D}PMI-β (TA**WY**AN**F**EKL**L**R), which contains the N2A/L7F double mutation and binds $^{(25\text{-}109)}$MDM2 with a K_d value 35 nM.[10a] Of note, a separate mirror image phage screening under more stringent conditions identified ^{D}PMI-γ (D**WW**PLA**F**EAL**L**R), which contains a Phe residue at position 7 and binds $^{(25\text{-}109)}$MDM2 at an affinity of 53 nM.[10b]

Structural analysis of ^{D}PMI-α-$^{(25\text{-}109)}$MDM2 and ^{D}PMI-γ-$^{(25\text{-}109)}$MDM2 suggested that the aromatic side chain of a Phe7 residue in ^{D}PMI's would not fully occupy its cognate binding site on MDM2. Therefore, we hypothesized that modifications to Phe7 side chain to improve its size and/or hydrophobicity would enhance MDM2 binding by these D-peptide ligands. To test this hypothesis, we used ^{D}PMI-β as our model peptide, and first evaluated the positional effect of chlorination of the phenyl ring of Phe7 of ^{D}PMI-β on MDM2 binding. A fluorescence polarization (FP)-based competition assay was developed to quantify the ability of three Cl-Phe7- ^{D}PMI-β peptides (chlorination at positions 2,3 and 4), along with 4-Br-Phe7- ^{D}PMI-β, to compete for MDM2 binding with *N*-acetyl-(15-29)p53 to which carboxyfluorescein (FAM) was conjugated via its Lys24 side chain. The following order of binding activity was obtained on the basis of IC_{50} values: 4-Cl-Phe ≈ 4-Br-Phe > Phe > 2-Cl-Phe ≫ 3-Cl-Phe (Figure S1 and Table S1). Clearly, chlorination or bromination at the *para* position of Phe7 enhanced ^{D}PMI-β binding to MDM2, while chlorination at the *meta* and *ortho* positions weakened it.

In light of these initial findings, we concentrated on the *para* position of Phe7 and synthesized five additional *p*-X-Phe7-^{D}PMI-β peptides, where X = F, I, CH_3, CF_3, and CN. To improve FP assay sensitivity and dynamic range, a more potent, FAM-labeled *p*-Br-Phe7- ^{D}PMI-β peptide was used under otherwise identical experimental conditions. As shown in Figure S2 and Table S2, the following order of MDM2-binding activity ensued for *p*-X- Phe7-^{D}PMI-β: CF_3 > I > Br > Cl > CH_3 > F > CN > H (Phe). The trifluoromethyl substitution at the *para* position of Phe7 emerged as the best modification to enhance ^{D}PMI-β binding to MDM2. For accurate quantification, we performed a previously established, surface plasmon resonance (SPR)-based competitive binding assay[8b,11] for $^{(25\text{-}109)}$MDM2 interacting with ^{D}PMI-β and *p*-CF_3- Phe7-^{D}PMI-β. As shown in Figure 1B and Table 1, whereas ^{D}PMI-β bound MDM2 at an affinity of 37.8 nM, in good agreement with the published value of 34.5 nM,[10a] *p*-CF_3-Phe7-^{D}PMI-β bound MDM2 with a K_d value of 450 pM – a dramatic increase in binding affinity by 80-fold.

To better understand the structural basis of the enhanced binding of the trifluoromethylated peptide to MDM2, we determined the crystal structure of $^{(25\text{-}109)}$MDM2 in complex with *p*-CF_3-Phe7-^{D}PMI-β at 1.8 Å resolution (Table S3, Figure S3-S4). As displayed in Figure 1D, the left-handed helix of *p*-CF_3-Phe7-^{D}PMI-β anchors deep inside the hydrophobic p53-binding cleft of MDM2 and establishes multiple hydrophobic interactions within the pocket primarily through the bulky side chains of ^{D}Trp2, *p*-CF_3-^{D}Phe7 and ^{D}Leu11 as well as the side chains of ^{D}Tyr4 and ^{D}Leu10. Overall, *p*-CF_3-Phe7-^{D}PMI-β binding to MDM2 closely resembles its parental peptide ^{D}PMI-α as previously reported (Figure S5-S6). However, *p*-trifluoromethylation of ^{D}Phe7 induces new interactions within the pocket with Leu82, Phe86 and Ile103 of MDM2 (Figure 1E), and significantly enlarges the total buried surface area (BSA) of the D-peptide in the complex (from 561 $Å^2$ to 640 $Å^2$). In addition, one more H-bond is formed between ^{D}Ala2 N of *p*-CF_3-Phe7-^{D}PMI-β and Glu72 Oε1 of MDM2. To accommodate the large side chain of *p*-CF_3-^{D}Phe7 two residues of MDM2 (Leu57 and Ile99) reorient in the p53-binding pocket (Figure 1F).

Importantly, structural analysis of the *p*-CF_3-Phe7-^{D}PMI-β-$^{(25\text{-}109)}$MDM2 complex revealed that Trp3 would also be permissible to fluorination at multiple positions of its side chain. We replaced Trp3 in ^{D}PMI-β with 6-F-Trp, and the resultant D-peptide 6-F-Trp3-^{D}PMI-β bound to $^{(25\text{-}109)}$MDM2 with a K_d value of 14 nM as determined by the SPR-based competitive binding assay (Figure 1B and Table 1), representing a 2.5-fold enhancement in binding affinity relative to ^{D}PMI-β. When 6-F-Trp3 was incorporated into *p*-CF_3-Phe7- ^{D}PMI-β, the resultant double mutant 6-F-Trp3/*p*-CF_3-Phe7-^{D}PMI-β, termed ^{D}PMI-δ, bound $^{(25\text{-}109)}$MDM2 at an affinity of 220 pM, suggesting that the energetic effects of Trp3 and Phe7 modifications were additive. These results were confirmed by an independent assay based on FP techniques (Figure S2 and Table S2). It is worth noting that the N-terminal peptide (residues 1-24) of MDM2 is known to form a partially structured "lid" in the apo protein, occluding ligand binding to MDM2 in a ligand size-dependent manner.[12] The "lid" has been shown to reduce the binding affinity for MDM2 of 12-mer L-peptide ligands by five fold.[12c] It may be anticipated that the K_d value of ^{D}PMI-δ reported here for $^{(25\text{-}109)}$MDM2 would be higher than that for full-length MDM2.

D-peptide ligands, unlike their L-peptide counterparts, display a much greater disparity between MDM2 and MDMX binding, with a strong preference for MDM2 over MDMX.[10,11] We quantified the interactions of $^{(24\text{-}108)}$MDMX with ^{D}PMI-β, *p*-CF_3-Phe7-^{D}PMI-β, 6-F-Trp3-^{D}PMI-β and ^{D}PMI-δ using SPR techniques, and the data are shown in Figure 1C and Table 1. Unexpectedly, *p*-trifluoromethylation of Phe7 enhanced ^{D}PMI-β binding to MDMX by only 2.5-fold, while fluorination of Trp3 slightly improved it. As a result, ^{D}PMI-δ bound to $^{(24\text{-}108)}$MDMX with a K_d value of 200 nM - three orders of magnitude weaker than its binding to MDM2. These SPR results are in accord with FP measurements (Figure S7 and Table S4). Obviously, understanding the structural basis of the strong preference of D-peptide ligands for binding to MDM2 over MDMX will provide important insights into designing specific antagonists to target either protein.

Fluorocarbons are known to be substantially more hydrophobic than corresponding hydrocarbons.[13] In fact, fluorinated aliphatic amino acids have been commonly used in protein *de novo* design to improve protein stability while having little impact on protein structure.[14] It has been suggested that fluorination of alkanes enhances hydrophobicity due to an increased molecular size, thus a greater free-energy penalty for hydration.[15] The high electronegativity of fluorine also enables the strongly polar C-F bond to engage in inductive interactions with surrounding polar groups and to alter hydration dynamics at fluorinated molecular surfaces.[16] We have demonstrated that although *p*-trifluoromethylation of Phe7 gave rise to the greatest improvement, iodination, bromination or even chlorination at the *para* position of the phenyl ring was similarly effective in improving ^{D}PMI-β binding to

MDM2. Of note, replacement of a critical Trp residue by 6-Cl-Trp has been shown to dramatically enhance the binding affinity of several peptide and peptidomimetic antagonists for MDM2 due to enhanced van der Waals interactions and polarization effects between the 6-Cl-Trp side chain and its interacting partners of MDM2.[17] Given that the p53-binding cavity of MDM2/MDMX is hydrophobic in nature, halogenation (and fluorination in particular) will likely become a powerful tool for the design of exceedingly potent activators of p53 for therapeutic use.[18]

Different structural classes of drug candidates such as small peptides with unsurpassed affinity and specificity are urgently needed to combat cancer and infectious disease. L-peptides have been traditionally considered to be “undruggable” due primarily to their strong susceptibility to proteolytic degradation *in vivo* and inability to efficiently traverse the cell membrane. Drug discovery based on the scaffold of protease-resistant D-peptides,[19] when coupled with advanced drug delivery technologies, offers a viable and robust solution to the problems both academia and industry are facing today. Our work on the design of ultrahigh affinity D-peptide antagonists of MDM2/MDMX to activate the p53 tumor suppressor may spearhead the development of new classes of anticancer therapeutics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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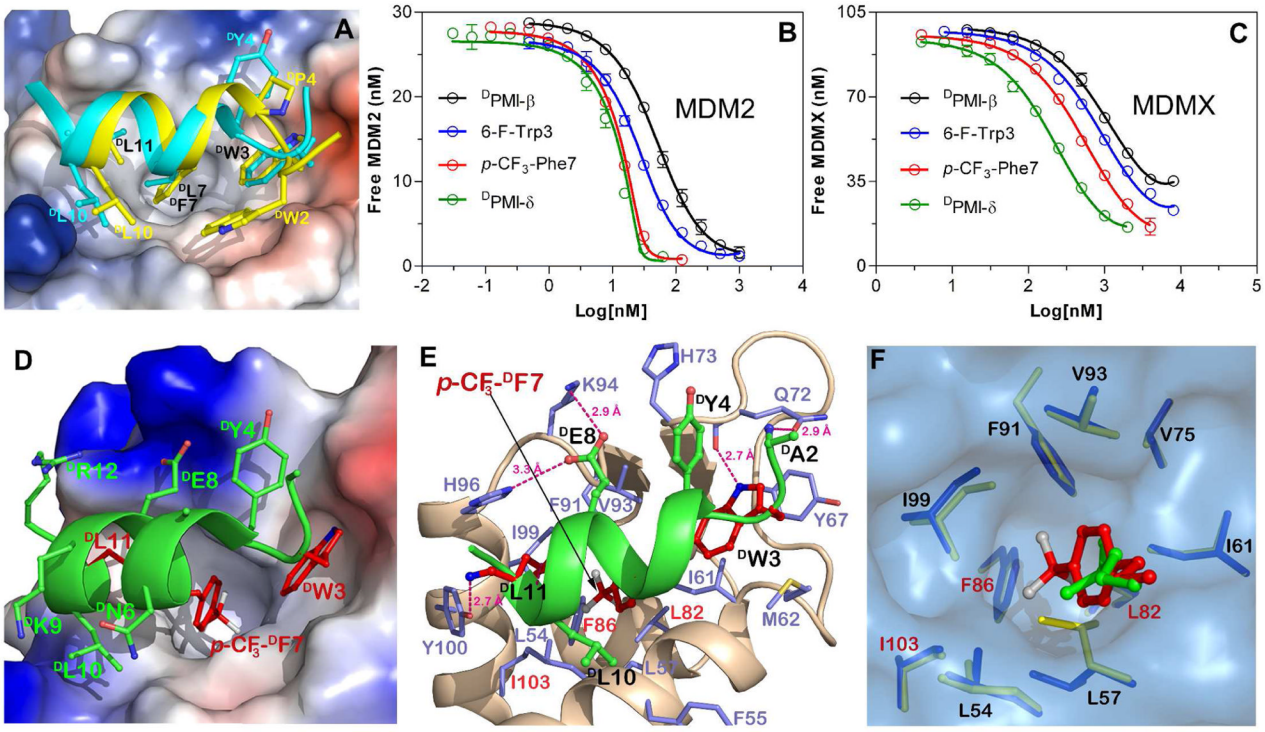

Figure 1.

(**A**) MDM2-binding modes of ^{D}PMI-α and ^{D}PMI-γ peptides. The structures of ^{D}PMI-α-$^{(25-109)}$MDM2 (PDB:3LNJ) and ^{D}PMI-γ-$^{(25-109)}$MDM2 (PDB:3IWY) are superimposed based on MDM2 molecules with ^{D}PMI-α (cyan) and ^{D}PMI-γ (yellow) displayed on the molecular surface of MDM2 complexed with ^{D}PMI-γ. The electrostatic potential displayed on MDM2 surface is colored red for negative, blue for positive, and white for apolar. The D-peptides are depicted in a Cα ribbon diagram where only the side chains of the residues involved in MDM2 binding are shown as ball-and-sticks. Interactions of 30 nM $^{(25-109)}$MDM2 (**B**) or 100 nM $^{(24-108)}$MDMX (**C**) with a two-fold dilution series of ^{D}PMI-β, *p*-CF_3-Phe7- ^{D}PMI-β, 6-F-Trp3-^{D}PMI-β and ^{D}PMI-δ as quantified by SPR-based competitive binding assays. The K_d values obtained from three independent measurements are tabulated in Table 1. (**D**) *p*-CF_3-Phe7- ^{D}PMI-β bound in the hydrophobic pocket of MDM2. The D-peptide is shown as ribbon and its side chains are shown as ball-and-sticks. The three most critical residues for MDM2 binding, ^{D}Trp3, *p*-CF_3-^{D}Phe7 and ^{D}Leu11, are colored in red as in panel **E**. (**E**) The *p*-CF_3-Phe7-^{D}PMI-β-$^{(25-109)}$MDM2 complex interface. Contact residues of MDM2 and *p*-CF_3-Phe7-^{D}PMI-β are shown as sticks and ball-and-sticks, respectively, and hydrogen bonds as red dashes. The *p*-CF_3-Phe7-^{D}PMI-β peptide is anchored in the p53-binding cavity of MDM2 primarily through multiple hydrophobic interactions involving ^{D}Trp3, *p*-CF_3-^{D}Phe7 and ^{D}Leu11 and the side chains of ^{D}Tyr4 and ^{D}Leu10. In addition, five inter-molecular H-bonds are formed, including ^{D}Ala2 N-Glu72 Oε1, ^{D}Trp3 Nε1-Gln72 O, ^{D}Glu8 Oε1-Lys94 Nζ, ^{D}Glu8 Oε2-His96 Nδ1 Nε2, and ^{D}Leu11 O-Ty100 Oη. (**F**) Comparison of the binding pockets of *p*-CF_3-^{D}Phe7 and ^{D}Leu7. The structures of *p*-CF_3-Phe7-^{D}PMI-β-$^{(25-109)}$MDM2 (red/blue) and ^{D}PMI-α-$^{(25-109)}$MDM2 (green/yellow, PDB:3LNJ) are superimposed based on MDM2 molecules. The residues lining the p53-binding pocket are depicted as sticks over the molecular surface of MDM2 complexed with *p*-CF_3-Phe7- ^{D}PMI-β-$^{(25-109)}$MDM2. Leu82, Phe86 and Ile103 of MDM2 make contacts exclusively with *p*-CF_3-^{D}Phe7, which is buried 3.8 Å deeper within the p53-binding pocket than ^{D}Leu7. The side chains of Leu57 and Ile99

of MDM2 shift (from yellow to blue) to accommodate the trifluoromethyl group of *p*-CF_3-^{D}Phe7 in an enlarged binding pocket.

Table 1

Dissociation equilibrium constants (K_d, nM) of ^{D}PMI-β, 6-F-Trp3-^{D}PMI-β, *P*-CF_3-Phe7-^{D}PMI-β, and ^{D}PMI-δ for synthetic $^{(25-109)}$MDM2 and $^{(24-108)}$MDMX.*[a]*

	^{D}PMI-β	6-F-Trp3-^{D}PMI-β	*p*-CF_3-Phe7-^{D}PMI-β	^{D}PMI-δ
MDM2	37.8±0.9	14.0±1.0	0.45±0.41	0.22±0.21
MDMX	1440±41	1040±59	569±25	200±10

*[a]*Each K_d value (mean ± S.D.) was obtained from three independent measurements