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 DPMI-α, β, γ. The prototypic D-peptide inhibitor DPMI-α binds 25−109MDM2 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 superactive D-peptide antagonist of MDM2, termed DPMI-δ, of which the binding affinity for 25−109MDM2 has been improved over DPMI-α by 3 orders of magnitude (Kd = 220 pM). X-ray crystallographic studies validate DPMI-δ as an exceedingly potent inhibitor of the p53−MDM2 interaction, promising to be a highly attractive lead drug candidate for anticancer therapeutic development.

INTRODUCTION

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 overexpressed 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 homologue 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, 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 DPMI-α,β,γ, that are resistant to proteolytic degradation.10 The prototypic D-peptide inhibitor DPMI-α binds 25−109MDM2 at an affinity of 220 nM and kills tumor cells in vitro and inhibits tumor growth in vivo by reactivating the p53 pathway.

We have previously shown that DPMI-α (TNWYANLEKLLLR) adopts a left-handed α-helical conformation, burying several bulky hydrophobic side chains (highlighted in bold typeface) into the p53-binding cavity of 25−109MDM2 (Figure 1A). Among those, Trp3 and Leu7 are the two most critical residues of DPMI-α, contributing a combined free energy of 7.6 kcal/mol to 25−109MDM2 binding, an equivalent Kd value of 10−6 M.10b 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 that of Leu7. These findings largely led to the design of DPMI-β (TAWYANFEKLLR), which contains the N2A/L7F double mutation and binds 25−109MDM2 with a Kd value 35 nM.10c Of note, a separate mirror image phage screening under more stringent conditions identified DPMI-γ (DWWPLAFFEALLR), which contains a Phe residue at position 7 and binds 25−109MDM2 at an affinity of 53 nM.10b Here we report that an ultrahigh affinity (Kd = 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 anticancer therapeutic development.

RESULTS AND DISCUSSION

Structural analysis of DPMI-α(25−109)MDM2 and DPMI-γ(25−109)MDM2 suggested that the aromatic side chain of a Phe residue in DPMIs would not fully occupy its cognate binding site on MDM2. Therefore, we hypothesized that chlorination of the phenyl ring of Phe7 of DPMI-α (chlorination at positions 2, 3, and 4), along with 4-Br−Phe7−DPMI-β, to compete for MDM2 binding with N-acetyl-(15−29)p53 to which carboxylfluorescein (FAM) was conjugated via its Lys24 side chain. The following order of binding activity was obtained on the basis of IC50

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Table 1. Dissociation Equilibrium Constants (Kd, nM) of DPMI-β, 6-F-Trp3−DPMI-β, p-CF3−Phe7−DPMI-β, and DPMI-δ for Synthetic (25−109)MDM2 and (24−108)MDMX

<table>
<thead>
<tr>
<th></th>
<th>DPMI-β</th>
<th>6-F-Trp3−DPMI-β</th>
<th>p-CF3−Phe7−DPMI-β</th>
<th>DPMI-δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDM2</td>
<td>37.8 ± 0.9</td>
<td>140 ± 1.0</td>
<td>0.45 ± 0.41</td>
<td>0.22 ± 0.21</td>
</tr>
<tr>
<td>MDMX</td>
<td>1440 ± 41</td>
<td>1040 ± 59</td>
<td>569 ± 25</td>
<td>200 ± 10</td>
</tr>
</tbody>
</table>

*Each Kd value (mean ± SD) was obtained from three independent measurements*
whereas DPMI-β bound MDM2 at an affinity of 37.8 nM, in good agreement with the published value of 34.5 nM,\textsuperscript{10a} p-CF$_3$-Phe7-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 (25–109)MDM2 in complex with p-CF$_3$-Phe7-PMI-β at 1.8 Å resolution (SI Table S3, Figures S3–S4). As displayed in Figure 1D, the left-handed helix of p-CF$_3$-Phe7-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 DTrp3, p-CF$_3$-Phe7, and DLeu11 as well as the side chains of DTyr4 and DLeu10. Overall, p-CF$_3$-Phe7-PMI-β binding to MDM2 closely resembles its parental peptide DPMI-α as previously reported (SI Figures S5–S6). However, p-trifluoromethylation of Dphe7 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 S61 to 640 Å$^2$). In addition, one more H-bond is formed between DAla2 N of p-CF$_3$-Phe7-PMI-β and Glu72 Oe1 of MDM2. To accommodate the large side chain of p-CF$_3$-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-PMI-β (25–109)MDM2 complex revealed that Trp3 would also be permissible to fluorination at multiple positions of its side chain. We replaced Trp3 in p-CF$_3$-Phe7-PMI-β with 6-F-Trp, and the resultant d-peptide 6-F-Trp3-p-CF$_3$-Phe7-PMI-β bound to (25–109)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 pPMI-β. When 6-F-Trp3 was incorporated into p-CF$_3$-Phe7-PMI-β, the resultant double mutant 6-F-Trp3/p-CF$_3$-Phe7-PMI-β, termed DPMI-δ, bound (25–109)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 (SI 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.\textsuperscript{12} The “lid” has been shown to reduce the binding affinity for MDM2 of 12-mer l-peptide ligands by 5-fold.\textsuperscript{12} It may be anticipated that the $K_d$ value of DPMI-δ reported here for (25–109)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.\textsuperscript{10,11} We quantified the interactions of (24–108)MDMX with pPMI-β, p-CF$_3$-Phe7-PMI-β, 6-F-Trp3-pPMI-β, and DPMI-δ using SPR techniques, and the data are shown in Figure 1C and Table 1. Unexpectedly, p-trifluoromethylation of Phe7 enhanced pPMI-β binding to MDMX by only 2.5-fold, while fluorination of Trp3 slightly improved it. As a result, pPMI-β bound to (24–108)MDMX with a $K_d$ value of 200 nM, 3 orders of magnitude weaker than its binding to MDM2. These SPR results are in accord with FP measurements (SI 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.\textsuperscript{13} In fact, fluorinated aliphatic amino acids have been commonly used in protein design to improve protein stability while having little impact on protein structure.\textsuperscript{14} It has been suggested that fluorination of alkanes enhances hydrophobicity due to an increased molecular size and thus a greater free-energy penalty for hydration.\textsuperscript{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.\textsuperscript{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 pPMI-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.\textsuperscript{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.\textsuperscript{18}

**CONCLUSION**

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 traditionally been 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,\textsuperscript{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.

**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-PMI-β (25–109)MDM2 complex, data collection, structure solution, and refinement. This material is available free of charge via the Internet at http://pubs.acs.org.

Accession Codes

The coordinates and structure factors have been deposited in the PDB with accession code 3TPX.

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**Notes**

The authors declare no competing financial interest.
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