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Miniature Protein Inhibitors of the p53–hDM2 Interaction

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We have developed a strategy for the design of miniature proteins that bind DNA[1–3] or protein surfaces[4–7] with high affinity and selectivity. This strategy, which is often called protein grafting,[8–11] involves dissecting a functional recognition epitope from its native α-helical or polyproline type II (PPII) helical context and presenting it on a small but structured protein scaffold (Figure 1 A). Here we describe the development and characterization of miniature proteins that bind the human double-minute 2 oncoprotein (hDM2) in the nanomolar concentration range, and inhibit its interaction with a peptide ($p53AD_{15–31}$) derived from the activation domain of p53 (p53AD).[12–14]

hDM2 is the principal cellular antagonist of the tumor-suppressor protein p53.[15] Elevated hDM2 levels are found in many solid tumors that express wild-type p53 and there is considerable interest in hDM2 ligands that are capable of up-regulating p53 activity in vitro or in vivo.[16] The high-resolution structure of the p53AD–hDM2 complex has revealed a recognition epitope that is composed primarily of three p53AD residues (F19, W23, and L26); these are located on one face of a short α-helix.[14] Although the p53AD peptide possesses little α-helical structure in the absence of hDM2,[14,17] augmenting the level of intrinsic α-helix structure in p53AD by using constrained, unnatural amino acids dramatically increases affinity for hDM2 in vitro and activity in vivo.[18,19] In addition, several other scaffolds have been used to display the p53AD epitope, including large proteins,[20] cyclic β-hairpin peptides,[21] retro-inverso peptides,[22] and β-peptides.[13,23] The first highly active small-molecule inhibitors were reported in 2004 and had IC_{50} values for inhibiting the p53–hDM2 interaction in the 100–300 nM range.[24] These molecules also resembled p53AD's primary recognition epitope. Since all these inhibitors appear to preorganize the p53AD epitope to some degree, we reasoned that protein grafting would be a logical route to developing miniature protein hDM2 ligands. In contrast with the previously reported molecules, miniature protein-based inhibitors would be both synthetically tractable and genetically encodable. This would facilitate their use as in vitro and in vivo tools for probing the intricate p53/hDM2 pathway.

Avian pancreatic polypeptide (aPP, Figure 1 B) is a small, well-folded miniature protein that consists of an eight-residue PPII helix linked through a type I β-turn to an eighteen-residue α-helix.[25] Structure-guided alignment of the α-helical segments of p53AD and aPP (Figure 1 B) positions the three critical hDM2 contact residues (F22, W26, and L29 in the aPP-aligned sequence) and five residues important for aPP folding (L17, F20, L24, Y27, V30) on the solvent-exposed and solvent-sequestered faces, respectively, of the aPP α-helix. An M13 pIII-fusion library (library 1, Figure 1 B) based on this alignment was constructed, in which five residues within aPP's α-helix were varied across all twenty amino acids. The variable positions were selected to 1) foster additional interactions with hDM2, 2) sustain the aPP fold, and 3) enable remodeling of the aPP α-helix to better mimic the slightly distorted p53AD α-helix.[14] The phage library contained 6×10^7 unique transformants; this

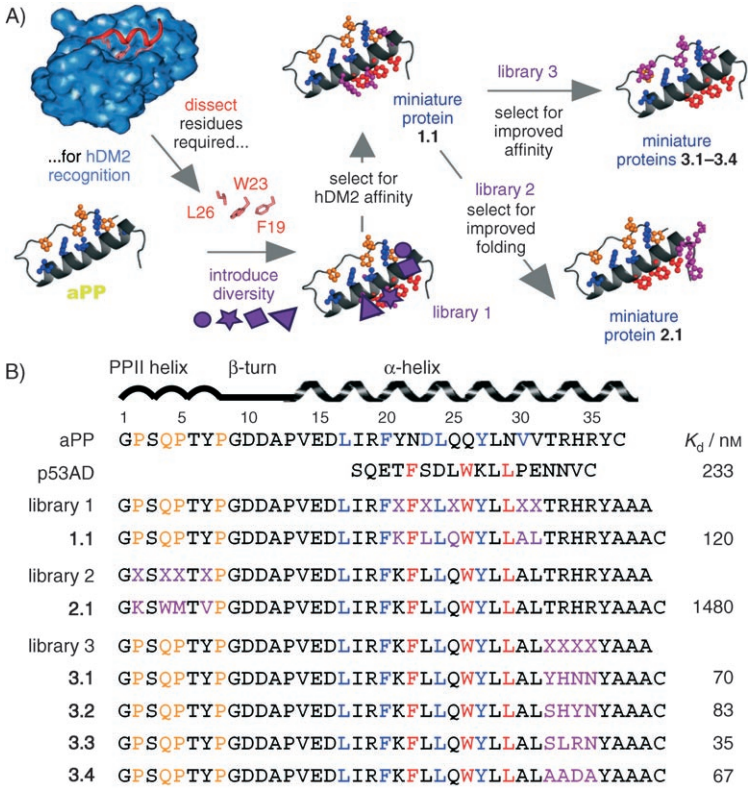

Figure 1. A) Protein-grafting strategy for the design of miniature-protein ligands for hDM2. B) Sequence alignment of aPP, p53AD, miniature proteins, and libraries discussed herein. K_d values of complexes with $hDM2_{1–188}$ are indicated. Residues in orange and blue stabilize the aPP hydrophobic core; those in red contribute to the binding of hDM2. Residues varied in each library are indicated by an X (purple). Peptides have free N termini and amidated C termini.

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ensured that it would evaluate DNA sequence space with >83% confidence.[26] Three rounds of selection (at 4 °C) for binding to GST–hDM2$_{1-188}$, which was immobilized on glutathione-coated microtiter plates, led to a 100-fold enrichment in affinity compared to phage displaying aPP. Phagemid clones from rounds 2 and 3 were sequenced, and five sequences were synthesized with a cysteine residue at the C-terminus to facilitate labeling with 5-iodoacetamidofluorescein and subsequent analysis of hDM2 affinity as described.[13]

Fluorescence polarization (FP) analysis indicated that all five miniature proteins identified during this first selection bound hDM2$_{1-188}$ in the micro- to nanomolar concentration range (Figure 2A and unpublished results). The ligand with highest affinity, **1.1** (Figure 1B), bound hDM2$_{1-188}$ with an equilibrium dissociation constant (K_d) of 120±21 nM, which is a twofold improvement over the unstructured peptide p53AD$_{15-31}$ (K_d = 233±32 nM). This miniature protein inhibits p53AD$_{15-31}^{flu}$–hDM2$_{1-188}$ complexation (Figure 2C) with an IC_{50} of 3.2±0.4 μM, which is also an improvement over the unstructured p53AD peptide (IC_{50} = 8.8±0.5 μM under the same conditions). The CD spectra of **1.1** (Figure 2D) indicates that this miniature protein is roughly 28% α-helical at 25 °C, with a T_m value of 47 °C; these values are significantly lower than those of aPP.[9] These data indicate that the overall secondary and tertiary structures of **1.1** are less stable than those of aPP. Detailed analysis of DNA binding by the aPP-based miniature protein, p007,[1] has demonstrated that direct target contacts as well as indirect packing interactions contribute to high affinity and specificity.[27] Thus we reasoned that the hDM2 affinity of **1.1** might be improved further by optimizing either the stability of the protein fold or the recognition properties of the hDM2-contacting interface.

Two second-generation M13 phagemid libraries of **1.1** variants were therefore designed to further optimize either protein folding (library 2) or miniature-protein·hDM2 contacts (library 3; Figure 1B). Library 2 included **1.1** variants that possessed diversity at positions 2, 4, 5, and 7 along the PPII helix. Residues at these positions contribute significantly to both folding and function in the DNA-binding miniature protein p007.[1,27] In an attempt to introduce additional contacts within hDM2's p53AD-binding pocket, library 3 was constructed to include **1.1** variants with diversity at four positions C-terminal to the hDM2 recognition motif. Each library contained 2×10^8 unique transformants, which is sufficient for evaluating DNA-sequence space with 100% confidence.[26] Library 2 and 3 phage pools were each panned for four rounds to enrich for library members with improved hDM2 affinity relative to **1.1**; two rounds were performed at 4 °C, and two rounds were performed at room temperature. Phagemid clones identified in the third and fourth rounds were sequenced, and one representative molecule from the library 2 selection and four from the library 3 selection were synthesized for FP analysis as described above. Significantly, the sequence of **1.1** was identified once in the later rounds of library 3 selection. This implies that this miniature protein might have already been highly optimized for hDM2 binding.

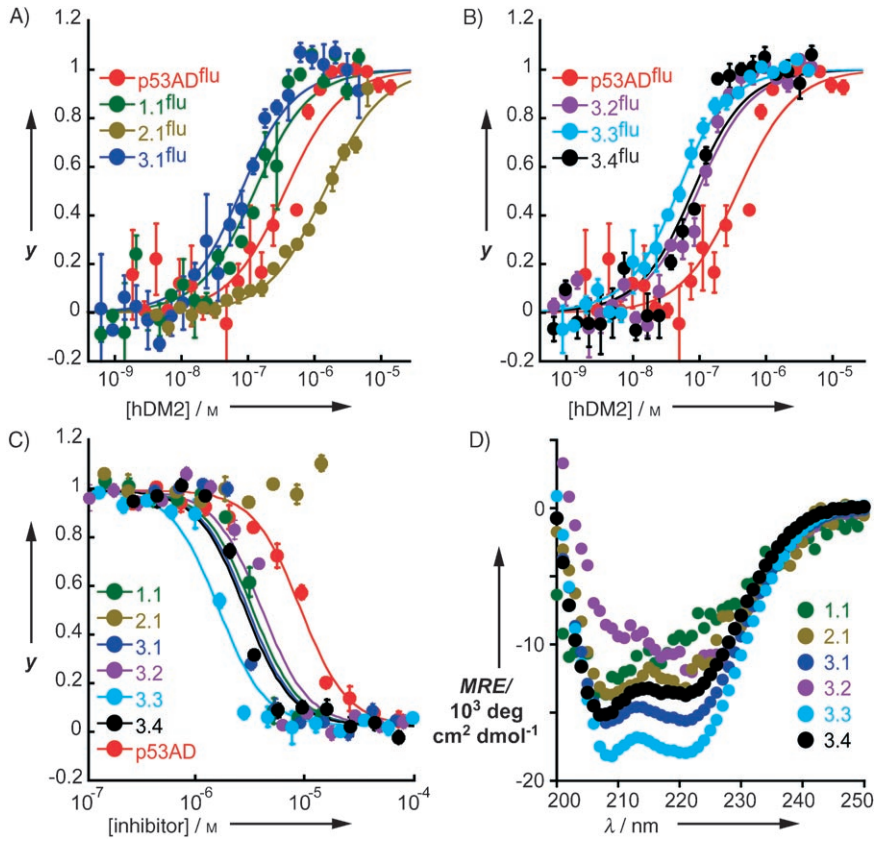

Figure 2. A) and B) Direct binding of fluorescein-labeled (flu) polypeptides to hDM2$_{1-188}$ as monitored by FP. The corresponding peptide sequences are shown in Figure 1. Fluorescently labeled peptide (25 nM) was incubated at room temperature with serial dilutions of hDM2$_{1-188}$ in PBS (pH 7.4) until equilibrium was reached. C) Inhibition of p53AD$_{15-31}^{flu}$–hDM2$_{1-188}$ complexation by unlabeled peptides as monitored by FP. Peptide inhibitors were incubated at various concentrations with hDM2$_{1-188}$ (0.5 μM) and p53AD$_{15-31}^{flu}$ (25 nM) in PBS (pH 7.4) with DTT (1 mM) and DMSO (2%) until equilibrium was reached. D) CD spectra of hDM2-binding miniature proteins. Spectra were obtained at 25 °C with miniature proteins (2–20 μM) in sodium phosphate/borate/citrate buffer (1 mM of each, pH 7.0). Data points are the average of three trials, and have been background-corrected and adjusted for concentration and number of residues.

The affinity and inhibitory potency of each second-generation hDM2 ligand was determined by FP analysis (Figure 2A–C). K_d and IC_{50} values derived from curve fits to the FP data[13] are summarized in Table 1. The single sequence identified from library 2, in which variation was restricted to the PPII helix

region of **1.1**, bound hDM2 poorly—polypeptide **2.1** showed an equilibrium affinity of 1480 nM (Figure 1B). The lack of improved sequences, or even the parent peptide (**1.1**) sequence among those selected from library 2 indicates that peptide **2.1** might have been selected due to some advantage in host growth, phage assembly, or phage infectivity.[28] By contrast, several high-affinity ligands were identified from library 3 in which residues within the α-helix were varied to optimize the miniature-protein·hDM2 interface. The most potent miniature protein (**3.3**) binds hDM2 with a K_d of 35 ± 3 nM and inhibits $p53AD_{15-31}^{flu}$–$hDM2_{1-188}$ complexation with an IC_{50} of 1.6 ± 0.2 μM. These values show marked improvements over the unstructured p53AD peptide ($\Delta\Delta G = -1.1$ kcal mol^{-1}). The CD spectra of the miniature proteins selected from library 3 indicate that they each contain significantly higher levels of α-helix structure than **1.1** or **2.1** (Figure 2D). Miniature protein **3.3** is approximately 54% α-helical at room temperature and retains its structure at higher temperatures up to 50 °C. Overall, there appears to be a direct relationship between the extent of α-helix structure in a miniature protein and equilibrium affinity for the p53AD-binding pocket of hDM2 (Table 1).

Table 1. Properties of the selected miniature proteins and control peptides derived from p53AD.[a]

Peptide	K_d [nM]	IC_{50} [μM]	MRE_{222} [deg cm^2 dmol^{-1} res^{-1}]
$p53AD_{15-31}$	233 ± 32	8.8 ± 0.5	n.d.[b]
1.1	120 ± 21	3.2 ± 0.4	−9340
2.1	1480 ± 91	> 150	−11800
3.1	70 ± 11	2.9 ± 0.5	−15500
3.2	83 ± 13	3.9 ± 0.5	−12020
3.3	35 ± 3	1.6 ± 0.2	−17870
3.4	67 ± 15	2.7 ± 0.2	−13480

[a] Values show equilibrium dissociation constants (K_d) of fluorescein-labeled peptides incubated with $hDM2_{1-188}$, half-maximal inhibitory concentrations (IC_{50}) of unlabeled peptides for the $p53AD_{15-31}^{flu}$–$DM2_{1-188}$ interaction, and CD mean-residue ellipticity minima at 222 nM (MRE_{222}); values are means ± standard errors from three independent FP assays; data and curve fits are shown in Figure 2A–D. [b] n.d. = not determined

In conclusion, we have provided further evidence that protein grafting, in combination with functional selection, provides rapid access to miniature-protein ligands for globular-protein surfaces. The hDM2 ligands we have described herein have high affinities and inhibitory potencies. They can be readily synthesized, which allows easy access to derivatives with broad functionalities, such as fluorescence, cross-linking agents, or protein-transduction domains.[29] These miniature proteins are also genetically encodable—a feature that will facilitate further optimization and future application as in vivo probes of the complex p53/hDM2 pathway. Thus, miniature proteins such as **3.3** have particular promise as novel and facile tools for modulating protein–protein interactions within the cell.

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