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 1A). 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 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 capable of up-regulating p53 activity in vitro or in vivo.[16] The high-resolution structure of the p53AD•hDM2 complex reveals a recognition epitope composed primarily of three p53AD residues (F19, W23 and L26) 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 using constrained, non-natural 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, with IC_{50}'s for inhibiting the p53•hDM2 interaction of 100 - 300 nM;[24] these molecules also resemble p53AD's primary recognition epitope. Since all these inhibitors appear to pre-organize 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, facilitating their use as in vitro and in vivo tools to probe the intricate p53/hDM2 pathway.

Avian pancreatic polypeptide (aPP, Figure 1) 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 1B) positions the three critical hDM2 contact residues (F19, W23 and L26) and five residues important for aPP folding (L14, F17, L21, Y24, L25) on the solvent-exposed and solvent-sequestered faces, respectively, of the aPP α-helix. An M13 pIII-fusion library (Library 1, Figure 1B) was constructed based on this alignment, with five solvent-exposed residues within aPP's α-helix 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 x 10^7 unique transformants, ensuring that it would evaluate DNA sequence space with > 83% confidence.[26] Three rounds of selection for binding to GST-$hDM2_{1-188}$ immobilized on glutathione-coated microtiter plates at 4 °C 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 re-synthesized with a cysteine residue at the C-terminus to facilitate labeling with 5-iodoacetamidofluorescein and subsequent analysis of hDM2 affinity as described.[27]

Fluorescence polarization (FP) analysis indicated that all five miniature proteins identified during this first selection bound $hDM2_{1-188}$ in the micromolar to nanomolar concentration range (Figure 2A and unpublished results). The ligand with highest affinity, **1.1** (Figure 1B), binds $hDM2_{1-188}$ with an equilibrium dissociation constant (K_d) of 120 ± 21 nM, a 2-fold improvement over the unstructured $p53AD_{15-31}$ peptide (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, also an improvement over the unstructured p53AD peptide (IC_{50} = 8.8 ± 0.5 μM under the same conditions). Circular dichroism (CD) 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

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°C, values significantly lower than those of aPP itself.[9] These data indicate 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] demonstrated that both direct target contacts as well as indirect packing interactions contribute to high affinity and specificity.[28] 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 designed to further optimize either protein folding (Library 2) or miniature protein•hDM2 contacts (Library 3) (Figure 1B). Library 2 included **1.1** variants possessing diversity at positions 2, 4, 5 and 7 along the PPII helix. The residues at these positions contribute significantly to both folding and function in the DNA-binding miniature protein p007.[1,28] Library 3 included **1.1** variants with diversity at four positions C-terminal to the hDM2 recognition motif in an attempt to introduce additional contacts within hDM2's p53AD-binding pocket. Each library contained 2 x 10^8 unique transformants, a value sufficient to evaluate DNA sequence space with 100% confidence.[26] The Library 2 and 3 phage pools were each panned for four rounds, two rounds at 4 °C and two rounds at room temperature, to enrich for library members with improved hDM2 affinity relative to **1.1**. 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 re-synthesized for FP analysis as described above. Significantly, the sequence of **1.1** was identified once in the later rounds of the Library 3 selection, implying it may have already been well-optimized for hDM2 binding.

The affinity and inhibitory potency of each second-generation hDM2 ligand was determined by FP analysis (Figures 2A-C), and K_d and IC_{50} values derived from curve fits to the FP data[27] are summarized in Table 1. Interestingly, 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** (Figure 1B) possesses an equilibrium affinity of 1480 nM. 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 represent 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). The most α-helical miniature protein, **3.3**, is approximately 54% α-helical at room temperature and retains its structure up to 50 °C, after which it undergoes α-helix-to-β-sheet conversion. 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).

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 are synthetically accessible, allowing easy access to derivatives with broad functionality 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 applications 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 to modulate protein-protein interactions within the cell.

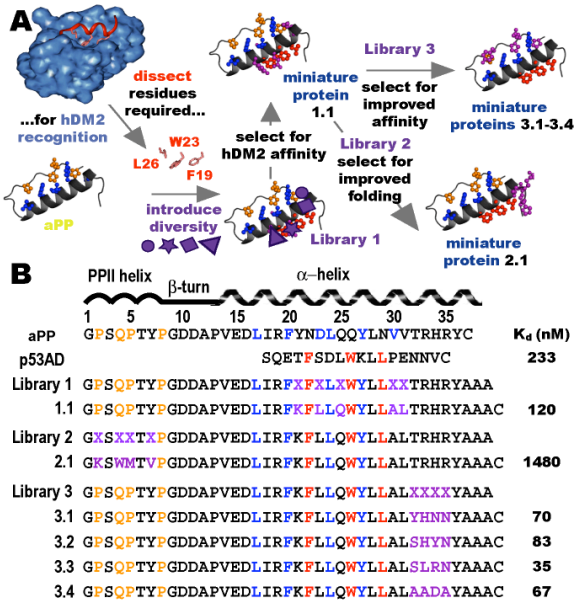

Figure 1. (A) Protein grafting strategy for the design of miniature protein ligands for hDM2. (B) Sequence alignment of aPP, p53AD, and miniature proteins and libraries discussed herein, and equilibrium dissociation constants (K_d) of complexes with $hDM2_{1-188}$. Residues in orange and blue stabilize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

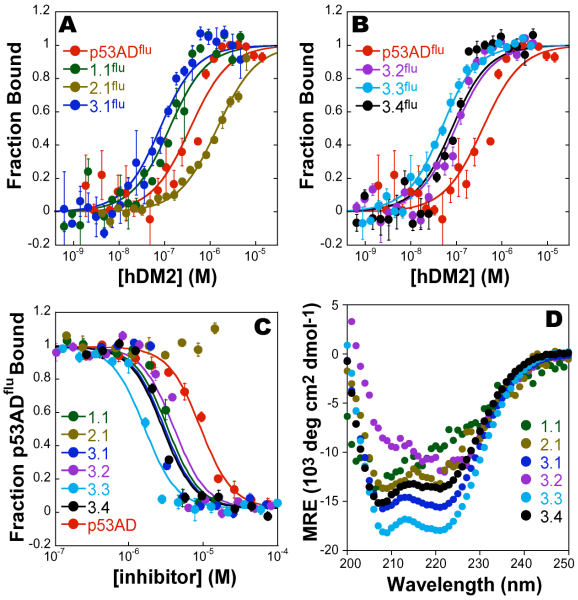

Figure 2. (A-B) Direct binding of fluorescein-labeled polypeptides to $hDM2_{1-188}$ as monitored by fluorescence polarization (FP). 25 nM $peptide^{flu}$ was incubated at room temperature with serial dilutions of $hDM2_{1-188}$ in PBS, pH 7.4 until equilibrium was reached. (C) Inhibition of $p53AD_{15-31}{}^{flu}$•$hDM2_{1-188}$ complexation by unlabeled peptides as monitored by FP. Peptide inhibitors were incubated at various concentrations with 0.5 μM $hDM2_{1-188}$ and 25 nM $p53AD_{15-31}{}^{flu}$ in PBS, pH 7.4 with 1 mM DTT and 2% DMSO until equilibrium was reached. (D) CD spectra of hDM2-binding miniature proteins. Spectra were obtained at 25 °C with 2 - 20 μM miniature protein in 1 mM sodium phosphate/borate/citrate buffer, pH 7.0. Data is the average of three trials, and has been background-corrected and adjusted for concentration and number of residues.

Table 1. Equilibrium dissociation constants of fluorescein-labeled peptides incubated with $hDM2_{1-188}$ (K_d), half-maximal inhibitory concentrations of unlabeled peptides for the $p53AD_{15-31}{}^{flu}$•$hDM2_{1-188}$ interaction (IC_{50}), and CD mean residue ellipticity minima at 222 nM (MRE_{222}) for selected miniature proteins. Data and curve fits are shown in Figures 2A-B, 2C, and 2D, respectively. Values are means and standard errors from three independent FP assays.

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
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

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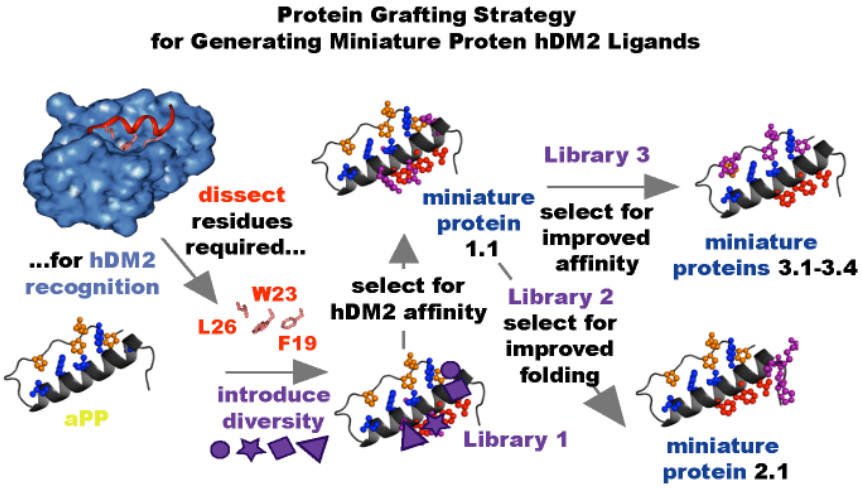

We have previously described a protein grafting strategy for the design of miniature proteins that bind DNA or protein surfaces with high affinity and selectivity. Here we describe the development and characterization of miniature proteins that bind the human double minute 2 oncoprotein (hDM2) and inhibit its interaction with a peptide derived from the activation domain of p53 (p53AD). An initial library of miniature protein variants presenting the minimal hDM2 recognition epitope from p53AD was further optimized to yield several miniature proteins with robust folds and nanomolar affinity for hDM2. The inhibitory activities of these miniature proteins correlate with the stability of the protein fold, emphasizing the benefit of presenting the p53AD epitope on a miniature protein scaffold. Miniature proteins such as those developed herein are both synthetically tractable and encodable, two features that will facilitate further optimization and future applications as in vivo probes of the complex p53/hDM2 pathway.